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CHAPTER ONE

Molecular Insights into Intracellular RNA Localization

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Contents

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Abstract

Localization of mRNAs to specific destinations within a cell or an embryo is important for local control of protein synthesis. mRNA localization is well known to function in very large and polarized cells such as neurons, and to facilitate embryonic patterning during early development. However, recent genome-wide studies have revealed that mRNA localization is more widely utilized than previously thought to control gene expression. Not only can transcripts be localized asymmetrically within the cytoplasm, they are often also localized to symmetrically distributed organelles. Recent genetic, cytological, and biochemical studies have begun to provide molecular insight into how cells select RNAs for transport, move them to specific destinations, and control their translation. This chapter will summarize recent insights into the mechanisms and function of RNA localization with a specific emphasis on molecular insights into each step in the mRNA localization process.

1. INTRODUCTION

RNA localization is the process by which a cell or organism transports and anchors specific RNAs at predetermined locations. Studies in a variety of systems have shown that proper RNA localization is an important feature of early embryonic patterning, neuronal cell function and various aspects of cell motility and polarization (Martin and Ephrussi, 2009; St Johnston, 2005). Localization of mRNA serves several functions, all of which are used by various cells and organisms. First, localization of an mRNA, which can produce many copies of protein through repeated rounds of translation, can be more efficient than translating a protein at a random location and relying of transport or diffusion to localize it to the site of action. This rationale for localizing mRNAs is especially important in very large cells, such as neurons, oocytes, and embryos. Second, mRNA localization can be used to prevent the accumulation of protein in a place that would be harmful to the cell or organism because mRNA localization is tightly coupled to translational repression, such that mRNAs in transit are translationally repressed. Third, mRNA localization can allow a cell to respond quickly to a stimulus by activating translation of a localized transcript. This mode of cellular response is more rapid than relying on transducing a signal to the nucleus to mount a transcriptional response. Finally, an underappreciated function of localized RNAs is that they can serve as a scaffold for assembly of protein complexes and function in a manner independent of simple protein translation.

Classic studies of mRNA localization have highlighted the importance of localized mRNAs in oocytes/early embryos and highly polarized cells

(such as neurons) (Du et al., 2007). In these large cell types, it is hypothesized that diffusion alone would not be sufficient to localize a protein to the site of action in a reasonable amount of time. These studies provide us with the greatest understanding of the function of mRNA localization and the mechanisms that act to target and transport mRNAs to their destinations. However, many recent studies have shown that mRNA localization is not restricted to large, highly specialized cells, but that it is a common feature of nearly all cell types. Furthermore, many genome-wide studies have begun to suggest that the majority of all mRNAs will have specific destinations (Blower et al., 2007; Du et al., 2008; Lecuyer et al., 2007; Sharp et al., 2011; Shepard et al., 2003) so that localization of mRNAs is the rule rather than a specialized process used by a few mRNAs in a few specialized cell types.

In general, all localized mRNAs require a common set of processes to occur. First, the mRNA must contain a *cis*-acting sequence that targets the mRNA to a specific destination; commonly referred to as a "zipcode" (Jambhekar and Derisi, 2007). Second, the zipcode must be recognized by RNA-binding proteins that serve to link the mRNA to the appropriate transport machinery. Third, the complex of RNA and RNA-binding proteins must be localized to the appropriate destination, either by diffusion or more commonly through active transport along the cytoskeleton. Fourth, the RNA must be anchored at the final destination. Finally, an interconnected theme is that localized mRNAs are most often translationally repressed during the process of transport and anchoring, so there must be a mechanism to release the mRNAs from the transport/anchoring complex to allow the mRNA to be translated at the final destination (Oleynikov and Singer, 1998). This chapter will briefly summarize the current state of the field for each of these areas, highlight recent studies that provide exciting new information about each of these topics and point the reader to more comprehensive reviews on each of the individual topics. One large class of specifically localized RNAs that will not be addressed in this chapter is noncoding RNAs that are retained in the nucleus. I point the reader to an excellent recent review on this topic (Wang and Chang, 2011).

2. EXTENT AND LOCATIONS OF mRNA LOCALIZATION

Classic studies of mRNAs localization during oogenesis and embryogenesis have shown that mRNAs localize to specific places in oocytes and early embryos and that the localization of these mRNAs is important for establishing pattern formation during early development

(Bashirullah et al., 1998). Another very prominent cell type that exhibits dramatic RNA localization are neurons, where several well-characterized mRNAs are known to localize to axons and function in neuronal signaling (Holt and Bullock, 2009; Martin and Ephrussi, 2009). These studies have been reviewed in detail elsewhere and will not be discussed in detail here. However, many of these important early studies lead to the view that localization of mRNAs was a niche process used only in very specialized cells. Over the years 2000–2010, the application of genome-wide technologies has changed this view dramatically and suggests that the majority of the transcriptome is localized to a nonrandom position within the cell. Below I highlight some of the novel destinations that serve as sites of mRNA localization (Fig. 1.1).

2.1. Endoplasmic Reticulum

2.1.1. Co-translational Signal Sequence-Dependent mRNA Localization

The ER is a large, membrane-bound organelle that regulates protein secretion, aspects of calcium handling and lipid biosynthesis. The ER is also one of the major cellular sites of protein translation as most transmembrane and secreted proteins are translated on the surface of the ER. One of the best-understood mechanisms of mRNA localization involves the co-translational localization of mRNAs encoding N-terminal signal sequences (SS) to the endoplasmic reticulum (Walter and Blobel, 1981a, 1981b; Walter et al., 1981). Many mRNAs encoding transmembrane, luminal, and secreted proteins contain an N-terminal signal sequence. When these mRNAs are exported from the nucleus to the cytoplasm, translation initiates on cytoplasmic ribosomes. Translation of the signal sequence triggers binding of the signal recognition particle (SRP) to the SS, which stalls translation and triggers localization of the mRNA:nascent peptide:ribosome complex to the signal recognition receptor on the ER surface (Gilmore et al., 1982). Translation then restarts and the newly synthesized peptide is co-translationally inserted into the lumen of the ER through the Sec61 channel. This is a classic case of mRNA localization to the site of action of the coded protein, and provides the paradigm for co-translational mRNA localization.

2.1.2. Translation-Independent mRNA Localization

The signal sequence hypothesis suggested that all mRNAs that are bound to the surface of the ER will contain an N-terminal signal sequence, will be bound to actively translating ribosomes, and localize to the ER in a

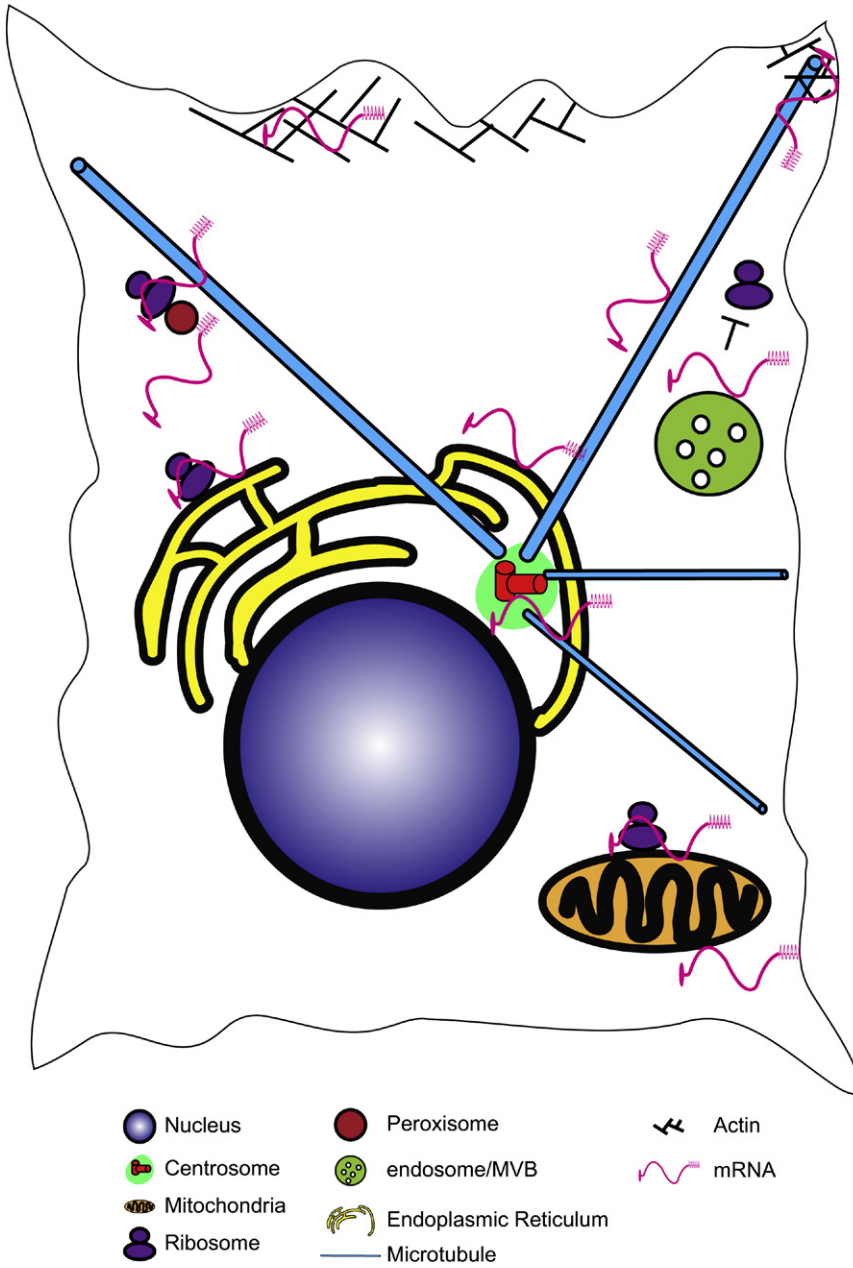

Figure 1.1 ***Sites of intracellular mRNA localization.*** Cartoon of a typical eukaryotic cell depicting sites with identified localized mRNAs. Translation-dependent localization is depicted as an mRNA associated with a ribosome at a destination, while translation-independent localization is depicted as mRNA localization in the absence of a ribosome. (For color version of this figure, the reader is referred to the online version of this book).

translation-dependent manner. However, co-translational localization of mRNA to the ER does not account for all mRNA localization to the ER (Adesnik et al., 1976). Additionally, many mRNAs that do contain signal sequences can localize to the ER in a translation-independent manner (de Jong et al., 2006; Diehn et al., 2000). Furthermore, mutation or RNAi of components of the SRP does not affect the viability or growth rate of any organism where it has been studied, suggesting that there are alternative methods to localize mRNAs to the ER membrane (Hann et al., 1989; Ren et al., 2004). Taken together, these studies have suggested that alternative methods exist to localize mRNAs to the ER. A recent genome-wide study used biochemical fractionation and sequential detergent extraction to demonstrate that there is a wide range of mRNA association with the ER, from mRNAs exclusively localized to the ER to mRNAs equally present in the ER and cytosol to mRNAs exclusively present in the cytosol (Chen et al., 2011). First, many mRNAs encoding cytosolic or nuclear proteins were associated with the ER although the overall distribution of these types of mRNAs was biased toward the cytoplasmic fraction. Second, only mRNAs encoding secreted proteins showed the expected translation-dependent mRNA localization to the ER, while mRNAs encoding resident proteins of the endomembrane system showed a stable and translation-independent association with the ER (Chen et al., 2011), consistent with an FISH-based assay of mRNA localization to the ER (Cui et al., 2012). This study demonstrated that many mRNA-binding proteins are present on the surface of the ER, and that the p180 protein plays a role in translation-independent mRNA localization to the ER (Cui et al., 2012). Taken together, these studies demonstrate that there are multiple modes of mRNA association with the ER. mRNAs encoding selected protein components of the endomembrane system can localize independently of translation (possibly through the p180 protein or other unidentified receptors). mRNAs encoding secreted proteins localize in an SRP-dependent manner, and mRNAs encoding cytosolic proteins can localize in a currently undefined manner.

These studies have revealed an unexpected complexity in mRNA localization to the ER, but many important questions remain unanswered. First, what features in an mRNA direct translation-independent localization? Analysis of an ER-localized mRNA that contained both a signal sequence and a transmembrane domain revealed that mutation of the reading frame preserves localization potential (Cui et al., 2012). These data suggest that mRNA localization information may be encoded in protein coding sequences. This principle would be similar to that found for the RNA

sequence coding or the signal peptide, which directed mRNA export from the nucleus independently of the translated protein (Palazzo et al., 2007). Using the mRNA sequence that codes for a specific protein domain to localize the mRNA to the destination of the protein would be an efficient manner for the cell to encode two functionally coupled pieces of information into the same RNA sequence. While this is an attractive hypothesis with some experimental support, it will need to be tested directly. Another outstanding question is what proteins are involved in targeting mRNAs to the ER in a translation-independent manner. The p180 protein has been implicated in localization of a subset of mRNAs to the ER but is not required for all mRNA localization. Furthermore, the RNA-binding domain in p180 shows no sequence preference (Cui et al., 2012), so it is unclear how specific mRNAs would be targeted to the ER. Elucidation of both the *cis*-acting RNA sequences and *trans*-acting protein factors will allow a greater understanding of translation-independent mRNA localization to the ER.

2.1.3. Localization of Cytosolic mRNAs to the ER

As stated above, several studies have shown that mRNAs encoding cytoplasmic proteins copurify with the ER. There is essentially nothing known about the mechanism that drives these mRNAs to the ER, but a recent study examining the subcellular location of protein translation has provided a rationale for mRNA localization to the ER (Reid and Nicchitta, 2012). The recent development of "ribosome profiling" to measure ribosome-protected fragments on mRNAs as a proxy for translation rate has enabled the rapid, genome-wide analysis of translation in a variety of systems (Ingolia, 2010; Ingolia et al., 2009, 2011). A recent study using ribosome profiling combined with cell fractionation examined the rates of translation in the cytosol to that on the surface of the ER (Reid and Nicchitta, 2012). Consistent with the studies discussed above, this study found that both signal sequence-containing and cytosolic mRNAs were actively translated on the ER. Interestingly, this study found that the density of ribosomes per mRNA was approximately twice as high on the ER as it was in the cytosol, suggesting that the surface of the ER is a more efficient environment for protein translation (Reid and Nicchitta, 2012). More efficient translation could provide a rationale for localization of mRNAs encoding cytoplasmic proteins to the ER. Although this study has provided an intriguing rationale for mRNA localization to the ER, the functional consequences or mechanism of localization are currently

unknown. Important future questions will involve defining how mRNAs are recruited to the ER for translation, and how translation on ER-bound ribosomes impacts protein function.

2.1.4. Links to Asymmetric Cytoplasmic mRNA Localization Pathways

In addition to coupling localization of secreted and endomembrane protein mRNAs to ER-bound ribosomes, localization of mRNAs to the ER has many links to asymmetric cytoplasmic mRNA localization pathways. In budding yeast, localization of the *ASH1* mRNA to the bud tip on actin cables is coincident with movement of the ER (Schmid et al., 2006). In *Xenopus* oocytes, the mRNA-binding protein Vera cofractionates with the ER and is important for the localization of the Vg1 mRNA to the vegetal cortex of the oocyte (Deshler et al., 1997). Furthermore, the Balbiani Body, which is the major site of mRNA localization in *Xenopus*, is rich in mitochondria and ER (Kloc et al., 2004). Finally, localization of seed storage protein mRNAs in rice endosperm cells is coupled to the ER (Choi et al., 2000). Taken together, these studies suggest that the ER may be one of the main sites of intracellular mRNA localization and that this organelle is likely to control many different processes in addition to the production of proteins of the endomembrane and secretory systems. Furthermore, the fact that many asymmetric cytoplasmic mRNA localization pathways are coupled to the ER suggests that understanding how and why particular mRNAs are localized to the ER will be a key to understanding many aspects of mRNA localization in a cell.

2.2. Mitochondria

Mitochondria are the cellular sites of respiration, energy production and some aspects of calcium handling. Mitochondria are unique organelles as they contain a fraction of the genetic information required for their maintenance, and must also import many proteins that are encoded by nuclear DNA (Ernster and Schatz, 1981). Import of proteins into the mitochondria was thought to occur posttranslationally as proteins translated in vitro are capable of import into the mitochondria (Neupert and Herrmann, 2007). However, ribosomes are present on the surface of yeast mitochondria, suggesting that there may be co-translational import of some proteins into the mitochondria (Kellems et al., 1974, 1975). Genome-wide studies of purified yeast mitochondria have demonstrated that several hundred mRNAs copurify with mitochondria-bound ribosomes (Gadir et al., 2011; Saint-Georges et al., 2008). Interestingly, the majority of the mitochondria-localized

mRNAs code for proteins that function in the mitochondria, consistent with a role for co-translational protein import. Localization of a subset of these mitochondria-localized mRNAs has revealed that translation of the N-terminal mitochondrial targeting sequence (MTS), as well as specific RNA elements both in the coding regions and 3′UTRs are important for mRNA targeting to mitochondria (Corral-Debrinski et al., 2000).

Globally, the majority of mitochondria-localized transcripts depend on translation to target to the mitochondria, but as is the case with the ER, there is a class of mRNAs that localize independently of translation (Saint-Georges et al., 2008). Two studies have shown the Puf3 RNA-binding protein is important for localization of a subset of transcripts to the mitochondria: Puf3 associates primarily with transcripts that function at the mitochondria (Gerber et al., 2004) and is required for the localization of these transcripts to the organelle (Gadir et al., 2011; Saint-Georges et al., 2008). Interestingly, deletion of mitochondria-targeting elements within the ATP2 mRNA resulted in defects in mitochondrial respiration and defects in protein import into the mitochondria, demonstrating that mRNA localization to mitochondria is critical for the proper function of the organelle (Margeot et al., 2002). In addition, a recent study of in *Xenopus* cultured neurons identified a nuclear protein, Lamin B2, as being translated in the axon. Surprisingly, the axonal pool of translated LB2 protein localized to mitochondria where it was required for proper mitochondrial function (Yoon et al., 2012). It will be interesting to determine how some messages localize independently of translation and what role those messages play in mitochondrial function.

2.3. Peroxisomes

Peroxisomes are membrane-bound organelles involved in oxygen scavenging and some aspects of lipid metabolism. Peroxisomes derive from budding of ER-derived vesicles and import of peroxisomal proteins (Ma et al., 2011). A recent study examined the localization of many mRNAs encoding peroxisomal proteins in budding yeast and found that a subset of mRNAs is localized to the peroxisome (Zipor et al., 2009). The peroxisome-localized mRNAs included both structural peroxisomal proteins and proteins that undergo posttranslational import into peroxisomes. Interestingly, a subset of peroxisomal mRNAs also localized to the ER, suggesting that the complex biogenesis of peroxisomes may also be reflected in complex patterns of mRNA localization. Similar to the localization of mRNAs to mitochondria, there is a contribution of a *cis*-acting zipcode that functions independently of protein translation to localize mRNAs to the peroxisome.

For example, localization of the PEX14 mRNA was shown to be partially dependent on the RNA-binding protein Puf5. Surprisingly, localization of the PEX14 mRNA to peroxisomes was not required for protein function as a partially localization-deficient construct was able to complement a null mutation of PEX14 (Zipor et al., 2009). Localization of mRNAs to peroxisomes appears to have many features in common with mRNA localization to mitochondria. Future work will be required to determine if there are functional consequences to disruption of peroxisomal mRNA localization and if this phenomenon is conserved in other species.

2.4. Endo/Lysosomes

Endosomes are formed by the invagination of the plasma membrane and are triggered by the activation of cell surface receptors (Hurley, 2008). Endosomes control the sorting of activated cell surface receptors either to the plasma membrane for further use or to the lysosome for degradation. Several studies have found an unexpected link between lysosomes and RNA regulation. For example, two recent studies have linked components of the multivesicular body (MVB) biogenesis pathway and RNAi-mediated gene repression (Gibbings et al., 2009; Lee et al., 2009). Both studies found that components of the RNAi machinery associate with endosomes/MVBs and that depleting components of the endosome machinery impair the ability of the cell to silence transcripts containing miRNA target sequences. Although it was not examined directly, these results strongly suggest that mRNAs targeted for repression will be localized to endosomes/MVBs. Another study found that components of endosomes colocalize with markers of cytoplasmic P-bodies (Gibbings et al., 2009), which are thought to be sites of mRNA storage or degradation. Taken together, these studies demonstrate that endosomal membranes are sites of mRNA storage and processing for degradation.

The link of RNAs to endosomes has several exciting implications. First, mRNAs and miRNAs have recently been found to be excreted from cells in microvesicles/exosomes (Skog et al., 2008; Valadi et al., 2007), and it was recently demonstrated that the core components of the machinery required for MVB formation are also required for the formation of exosomes (Baietti et al., 2012). Therefore, RNA-binding proteins present on endosomes/MVBs could play a role in determining which RNAs are selected for inclusion into exosomes and secretion outside of the cell. Another exciting possibility is that mRNAs linked to endosomes could couple activation of a cell surface receptor to a translational response. One study found that stimulation of neuronal cells with nerve growth factor

triggered axonal translation of the transcriptional activator CREB. Newly synthesized CREB was then transported to the nucleus on endosomes and was important for the transcriptional response to the stimulus (Cox et al., 2008). This suggests that localization of the CREB mRNA to distal axons, perhaps in the context of endosomes, could serve to poise the mRNA to increase axon-localized translation in response to activation of an extracellular receptor.

Another interesting link of endosomes to mRNA transport has come from studies of two more established systems of mRNA localization, localization of the *bicoid* mRNA in *Drosophila* oocytes, and transport of mRNAs in the hyphae of *Ustilago maydis* (Baumann et al., 2012; Irion and St Johnston, 2007). A visual genetic screen for mutants that influence the localization of the *bicoid* mRNA identified a component of the ESCRT–II complex, a complex that is part of the machinery required for MVB formation, as being required for *bicoid* mRNA localization (Irion and St Johnston, 2007). The authors further demonstrated that all members of ESCRT-II are required for *bicoid* localization while components of ESCRT-I and ESCRT-III are not required for this process. This suggests that ESCRT-II has a role in RNA localization that is independent of its role in MVB formation. The filamentous fungus *U. maydis* represents a very different system of RNA transport, where mRNAs are transported bidirectionally in the hyphae through the action of both dynein and kinesin motors, yet endosomes were similarly required for mRNA localization. The majority of mRNA transport is mediated by the Rrm4 protein (Becht et al., 2006). A recent study demonstrated that motile mRNAs colocalize with shuttling endosomes and that endosome biogenesis is required for efficient mRNA movement (Baumann et al., 2012). These results are similar to the observation that the *Drosophila* Rab11 protein, which is a marker for recycling endosomes, colocalizes with the *oskar* RNA at the posterior pole of oocytes (Dollar et al., 2002). Furthermore, the genomic RNAs of retroviruses are localized to endosomes en route to the plasma membrane for viral budding (Basyuk et al., 2003).

Taken together, these studies suggest that endosomes are likely to be the major sites of mRNA localization and control. Linkage of endosomes to the RNAi pathway suggests that endosomes are likely to be sites of storage for translationally repressed mRNAs. Since endosomes have many well-documented connections to cytoskeletal transport pathways (Scita and Di Fiore, 2010), the cell may use the well-established endosome transport pathways to move mRNAs along with endosomes. The fact that endosomes are the site of activated cell surface receptors provides the cell with

a mechanism to link the translation of localized mRNAs to extracellular signals. Finally, the link of endosomes/MVBs with exosomes suggests that the cell may also use endosomes to compartmentalize specific mRNAs into exosomes for the purpose of cell-to-cell communication.

2.5. Actin Cytoskeleton

Although the actin cytoskeleton is not a membrane-bound organelle such as those previously discussed, it is a prominent site of mRNA localization. Indeed, the mRNA encoding chicken β-actin mRNA was shown to localize to the cell protrusions and to the leading edge of motile fibroblasts (Lawrence and Singer, 1986). Furthermore, impairment of β-actin mRNA localization was shown to interfere with the speed and directionality of cell motility (Kislauskis et al., 1994, 1997), which are actin-dependent processes. Several recent studies have augmented our understanding of mRNA localization to the actin cytoskeleton. One hypothesis for the importance of mRNA localization is that co-translation of all members of a multiprotein complex could facilitate the assembly of that complex. This hypothesis was confirmed for the actin-nucleating Arp2/3 complex as the mRNAs for all members of the complex localize to cell protrusions (Mingle et al., 2005). Interestingly, knockdown of one member of the complex resulted in changes in the speed and direction of cell migration. These changes in motility could be rescued by a properly localized mRNA but not completely restored by an mRNA that was targeted to another cellular compartment, demonstrating that the localization of one of the mRNAs of the Arp2/3 complex is important for the function of the complex (Liao et al., 2011). In addition, a recent genome-wide study of mRNAs associated with actin-rich cell protrusions demonstrated that ~50 mRNAs were enriched in these protrusions and that a subset of these mRNAs were localized to the cell protrusions in a microtubule-dependent manner that required the APC protein (Mili et al., 2008). Altogether, studies of actin-localized mRNAs suggest that mRNAs present on the actin cytoskeleton are likely to be involved in control of actin dynamics and other processes related to cell motility.

2.6. Microtubule Cytoskeleton

Microtubules are used for the majority of long-distance transport within cells. Microtubules are important for the localization of the majority of known localized mRNAs (with the notable exception of localized mRNAs in *Saccharomyces cerevisiae*, which require actin-based transport) through the action of various motor proteins. However, several studies suggest that

microtubules are not just a highway for transporting mRNAs but can also serve a destination for some localized transcripts (Martin and Ephrussi, 2009). Purification of microtubules from a variety of organisms (sea urchin, frog, human) has demonstrated that hundreds of mRNAs co-purify with microtubules (Blower et al., 2007; Hamill et al., 1994; Rodriguez et al., 2005; Sharp et al., 2011; Suprenant, 1993; Suprenant et al., 1993, 1989). The majority of microtubule-localized mRNAs code for proteins that appear to function in cell cycle and microtubule-related processes. Our group recently tested this idea directly by knocking down the mRNAs for 10 uncharacterized microtubule-localized transcripts in HeLa cells. We found that the majority of these mRNAs coded for proteins required for proper microtubule organization during both interphase and mitosis (Sharp et al., 2011). Work in *Xenopus* oocytes has also found that both spindle localization and translational control of a subset of mRNAs are important for completion of both meiotic divisions, highlighting the link between mRNA localization and translational control (Eliscovich et al., 2008).

In addition to containing mRNAs that control the microtubule cytoskeleton, microtubules also contain a number of transcripts that appear to be translationally repressed and are likely to be passive cargo on the spindle rather than active participants in controlling microtubule-related events (Blower et al., 2007). This idea is consistent with recent work looking at RNA localization in early cell divisions of the snail *Ilyanassa obsoleta* (Lambert and Nagy, 2002; Rabinowitz and Lambert, 2010). In this work, the authors used RNA FISH to examine several mRNAs important for early development. Some of these transcripts show an unequal distribution during cell division by selectively associating with one of the two centrosomes of a dividing blastomere. This suggests that unequal segregation of important developmental mRNAs by the mitotic spindle could be an important mechanism for the establishment of early patterning asymmetries. While many mRNAs are known to localize to meiotic and mitotic microtubules, little is known about the sequences or proteins involved in this process. There is also nothing known about the functional consequences of disrupting mRNA localization to microtubules. These all will be important future areas of research.

2.7. Summary

Taken together, there is an enormous diversity in the destinations of localized mRNAs. However, several common themes are evident. First, mRNAs tend to localize to the site of action of the coded protein. Second, many types of mRNA localization appear to require a combination of *cis*-acting

RNA sequences and co-translational mRNA targeting. Finally, intracellular membranes are one of the most common destinations for localized mRNAs, suggesting that the cell may use well-established membrane sorting machinery to shuttle mRNAs to different destinations within the cell.

3. MECHANISMS OF mRNA LOCALIZATION

3.1. *Cis*-Acting mRNA Localization Signals

A basic principle of mRNA localization is that some mRNAs contain sequence elements, termed "zipcodes," that are recognized by specific protein factors that link them to the transport machinery. Zipcodes can range from a few nucleotides (5–6) to hundreds of base pairs. With a few notable exceptions, there is no consensus sequence for an RNA zipcode. Further, localization activity of an mRNA can be conferred by many low-specificity sequence elements acting in concert. There are several excellent reviews covering the state of the field of cis-acting sequence elements (Jambhekar and Derisi, 2007) and trans-acting proteins (Martin and Ephrussi, 2009; St Johnston, 2005), so I will not go into any detail on these topics here, but will highlight some recent studies that have revealed unexpected complexity in types of zipcodes.

3.1.1. Splicing-Dependent Localization of Oskar

For the majority of localized mRNAs examined to date, all the information necessary to recapitulate localization is present in the RNA sequence. This is demonstrated by the ability of fluorescently labeled, in vitro synthesized mRNAs to localize to the correct destination when injected into the appropriate system. However, there are several notable exceptions, and one of the best-understood examples is the *oskar* RNA in *Drosophila* oogenesis. Initial work on localization of *oskar* mapped the localization activity of the transcript to the 3′UTR and showed that these sequences were sufficient to localize a transcript when fused to a reporter RNA (Gunkel et al., 1998; Kim-Ha et al., 1993). However, when the *oskar* 3′UTR was assayed for localization activity in a mRNA null mutant, it was not sufficient to confer full localization activity (Hachet and Ephrussi, 2004), which led the authors to propose that the 3′ UTR localized by "hitchhiking" with endogenous transcripts (presumably through 3′UTR-mediated dimerization of *oskar* mRNA molecules). Mutagenesis experiments revealed a requirement for splicing of the first *oskar* intron in concert with the 3′UTR to confer full localization activity, demonstrating that the nuclear history of a transcript

can be important for the localization of the RNA in the cytoplasm. A recent study from the Ephrussi lab has now provided a very detailed mechanistic explanation for the requirement for mRNA splicing (Ghosh et al., 2012). Splicing of the first intron creates a stem–loop structure that is absolutely required for *oskar* localization but is not sufficient to localize intronless versions of the RNA, indicating that other factors are required for localization. Previous genetic evidence had indicated that components of the exon junction complex (EJC) were required for *oskar* localization, and using an in vitro system, it was shown that all the components of the EJC are deposited just upstream of the first exon:exon junction created by splicing. These results suggested that formation of the stem–loop must work in concert with deposition of the EJC to effect localization. Taken together, these studies suggest that some transcripts will require a very complex series of processing events, and that sequential assembly of *trans*-acting factors may be required to achieve proper mRNA localization.

3.1.2. Dimerization-Dependent mRNA Localization

While most mRNA localization signals function entirely in *cis* by recruiting specific proteins to promote localization, another class of zipcodes promotes intermolecular mRNA dimerization to achieve RNA localization. The *Drosophila bicoid* mRNA is one of the best-characterized localized mRNAs and is known to localize to the anterior of the *Drosophila* oocyte in a microtubule-dependent manner. The localization of *bicoid* is dependent on a large section of the 3′UTR that is predicted to form a series of stem–loop structures and long regions of double-stranded RNA (dsRNA; MacDonald, 1990; Macdonald and Kerr, 1998; Macdonald et al., 1993; Macdonald and Struhl, 1988). Localization of *bicoid* is dependent on a number of proteins including the dsRNA-binding protein Staufen (Ferrandon et al., 1994; St Johnston et al., 1991). While studying the interactions of *bicoid* with Staufen, it was observed that unpaired loops on the *bicoid* 3′UTR were important for localization, and that these loops had the potential to pair in *trans* with other *bicoid* molecules (Ferrandon et al., 1997). Mutation of bases in the loops abolished localization but could be restored by complementary mutations in other *bicoid* molecules. *Bicoid* also demonstrated the ability to dimerize in vitro, and dimerization ability correlated with localization activity in vivo. Although the dimerization ability of *bicoid* RNA is important for recruitment of Staufen in embryos and for localization of *biocid* mRNA to the apical side of early embryos and to embryonic mitotic spindles, it is not required for localization to

the anterior of the *Drosophila* oocyte (Snee et al., 2005). Another recent study has also highlighted the use of RNA dimerization motifs to promote RNA localization. *Oskar* RNA (described above) is known to be packaged into large RNA granules containing many molecules of *oskar* RNA (Glotzer et al., 1997), and as described above, the 3′UTR of *oskar* RNA can hitchhike to the posterior of the oocyte using the endogenous transcript. To determine if *oskar* hitchhiking and oligomerization requires RNA:RNA base pairing, the authors investigated the base pairing of *oskar* in vitro (Jambor et al., 2011). They found that *oskar* was able to form oligomers in vitro that depended on unpaired bases present in a loop in the 3′UTR, similar to the mechanism used by *bicoid*. The authors then demonstrated that pairing between different *oskar* molecules is necessary for the hitchhiking behavior of the *oskar* 3′UTR. Interestingly, mutation of bases involved in *trans* pairing did not affect the localization of a full length *oskar* transcript, demonstrating that dimerization is not absolutely necessary for localization in vivo. While the localization of neither *oskar* nor *bicoid* is dependent on dimerization activity, it is possible that dimerization facilitates packaging into higher order particles for more efficient transport or translational control. Another interesting possibility is that pairing of other (as yet unknown) RNAs with the dimerization elements in *bicoid* or *oskar* could facilitate their localization within the oocyte.

3.2. Assembly of Active RNA Localization Complexes

In order for an RNA to localize to the correct destination, it must contain the correct *cis*-acting sequence elements that are processed in a defined temporal and spatial manner as discussed above. In addition to processing of the zipcodes, proteins must be loaded onto the localizing RNA to facilitate assembly of a localization-competent RNP. While studies in a number of genetically tractable model systems, most notably *S. cerevisiae* and *D. melanogaster*, have provided lists of proteins required for the localization of specific mRNAs, a clearly defined understanding of how a localization-competent RNP is formed has been lacking. In addition, when the interaction of genetically identified localization factors with mRNAs is examined in vitro, it has been difficult to reconstitute the exquisite specificity of the interaction (Dienstbier et al., 2009; Du et al., 2008). A biochemical and structural study of the assembly of the *ASH1* localization complex has begun to provide the first clear picture of how a series of modest affinity and specificity interactions can act synergistically to promote the high affinity and high specificity interactions observed in vivo (Muller et al., 2011).

In *S. cerevisiae*, the *ASH1* mRNA is transported to the bud tip of the daughter cell through myosin-driven transport along actin cables. Once in the daughter cell, *ASH1* represses mating-type switching in the daughter cell. Studies from a number of labs have found that *ASH1* contains a dispersed localization sequence consisting of four elements—E1, E2A, E2B and E3—that are implicated in transport of the mRNA. In vivo, the E3 element can mediate efficient transport, suggesting that some of the localization sequences serve redundant functions (Heym and Niessing, 2012). A series of proteins have also been implicated in mRNA transport: She2, an RNA-binding protein that contacts *ASH1* in the nucleus; She3, an adapter for myosin-driven transport; and Myo4, the motor for transport (Heym and Niessing, 2012). Additionally, the RNA-binding proteins, Puf6, Khd1 and Loc1, are part of the localization complex and required for efficient *ASH1* transport but their roles are less well-defined than She2, She3 and Myo4. Although a nearly complete set of players was known for ASH1 mRNA localization, it was not clear until recently how these proteins functioned together to generate a high specificity RNP.

To understand how She2, She3, Puf6 interact with each other and the *ASH1* mRNA localization element to generate a localization complex, the interactions of purified proteins with the *ASH1* E3 RNA sequence were examined in vitro (Muller et al., 2011). Consistent with previous results, She2 interacted directly with the *ASH1* E3 element (Niessing et al., 2004) but only showed a modest preference for *ASH1* E3 compared to nonlocalizing RNAs (~2- to 10-fold higher affinity than for localizing RNAs). In contrast, the Puf6 protein bound RNAs with a modest affinity and specificity, showing little preference for RNAs containing a Puf6 consensus binding sequence. When assayed in tandem, She2 and Puf6 exhibited no synergism for RNA binding but were able to form a trimeric complex mediated by RNA as a scaffold. In contrast, She2 and She3 interact directly with one another and also with RNA to form a stoichiometric complex. Although there was no previous indication that She3 was an RNA-binding protein, it bound RNA with a modest affinity and no specificity in vitro. When the affinity and specificity of She2 and She3 were assayed with *ASH1* localization elements, it revealed that the complex of She2/She3 has a dramatically higher affinity for RNA-containing zipcodes compared to control RNAs This demonstrates conclusively that high affinity and specific recognition of a zipcode requires the concerted action of two low-affinity RNA-binding proteins, which is likely to be a paradigm relevant to many other zipcode-binding proteins. Consistent with the previous results, when

the She3 surfaces that interact with both She2 and *ASH1* RNA were identified and mutated in vivo, they disrupted RNA transport of *ASH1* mRNA. This study leads to the first nearly complete mechanistic model for how a transport-competent RNP is formed. First, *ASH1* is transcribed in the nucleus where it is bound by She2, Puf6, and Loc1. Following export to the cytoplasm, the interaction of She2 and She3 with each other and with the zipcode drives mRNP remodeling to form a very stable, highly specific mRNP that is competent to interact with Myo4 and be transported to the bud neck. Two important ideas emerge from this study. First, it will be important to obtain nearly complete lists of the factors that interact with localizing mRNAs to understand mRNP complex assembly, and it will be important to use in vitro studies to understand how each factor interacts with the others to promote high-affinity and specific-zipcode binding. Second, the identification of She3 as a direct RNA-binding protein suggests that the proteins that interact directly with localizing mRNAs will not be limited to proteins that have well-known and annotated RNA-binding domains, but will likely encompass a much wider range of proteins. This idea has been confirmed by two recent studies using poly-A purification to identify proteins that interact directly with mRNAs. Both studies found that hundreds of proteins that have no known RNA-binding domains directly contact mRNAs (Baltz et al., 2012; Castello et al., 2012). It will be interesting to extend this type of analysis to localized mRNAs.

4. MOTOR-DRIVEN DIRECTED TRANSPORT OF LOCALIZATION-COMPETENT mRNPS

In order to achieve localization of an mRNA to a distinct cytoplasmic location, an assembled RNP is generally transported along the cytoskeleton by molecular motors. While there are some examples of mRNA localization through diffusion and entrapment or selective protection against degradation, these are the exceptions and most examples of mRNA localization are driven by active transport along the cytoskeleton (Martin and Ephrussi, 2009). Several questions about motor-driven mRNA transport have been addressed by recent studies. First, is the direction of transport determined by the polarity of the cytoskeleton? Second, is the directionality of transport determined by the number and type of motor proteins associated with the RNP? What is the fate of a transport particle and associated motor protein once it has reached its destination?

4.1. Role of Cytoskeletal Polarity in mRNA Localization

In many cell types that utilize mRNA localization, there is a well-defined polarity to the cytoskeleton that facilitates transport of mRNPs to one end of the cytoskeletal filaments. For example, in neuronal cells, the centrosome is located in the cell body near the nucleus, and the microtubule plus ends extend into the axons and dendrites. Thus, kinesin motor proteins are used for transport of cargo to the distal portions of the cell (anterograde transport) and dynein motors are used to transport cargo back to the cell body (retrograde transport). Another system with a well-defined cytoskeletal polarity is the early *Drosophila* embryo, where the centrosome is located at the surface of the embryo and the plus ends of microtubules extend into the interior of the embryo. In this system, dynein motors are used to transport mRNP cargo to the surface of the embryo (Gaspar, 2011). However, many systems that utilize mRNA localization do not have a defined polarity of the cytoskeleton, which raises the question as to how directional transport is achieved on a nonpolarized cytoskeleton.

While the *Drosophila* oocyte is a classic system for the study of mRNA localization, it is also a system that has a very poorly defined cytoskeletal polarity (Cooley and Theurkauf, 1994). During stages 8–10 of oogenesis, when many mRNAs are known to localize, the oocyte has a gradient of microtubules with the majority of microtubules nucleated from the anterior of the oocyte. Consistent with this idea, transport of mRNAs from the nurse cells to the oocyte is driven by the dynein motor and associated factors (Gaspar, 2011). An open question in the field of RNA transport has been to determine how transcripts that localize to the posterior of the oocyte achieve this localization. Mutation of the kinesin heavy chain results in a loss of *oskar* mRNA from the posterior (Brendza et al., 2002; Cha et al., 2002), supporting the idea that *oskar* localization is driven by kinesin-mediated transport on a polarized cytoskeleton from the anterior to the posterior of the oocyte. However, direct measurement of *oskar* transport had not been observed until recently.

Movement of *oskar* localization particles tracked by live imaging revealed a small portion (~15%) of *oskar* transcripts moving in a manner consistent with motor-driven transport. Interestingly, *oskar* particles appeared to be moving in all different directions rather than showing a concerted, directed transport to the posterior of the oocyte. However, analysis of hundreds of particles revealed that there was a weak bias in the directionality of transport, with ~57% of all transport particles moving toward the posterior of

the oocyte. Furthermore, these movements were almost entirely toward the plus ends of microtubules and were dependent on the action of kinesin. Various mutants known to affect the localization of *oskar* were found to affect different steps of the localization process, with some proteins involved in establishing the net directionality of transport and others involved in affecting the rate. The conclusion from this study is that over time, an accumulation of mRNA can occur through the use of very small differences in the net direction of transport on a weekly polarized cytoskeleton. The results from this study will likely be applicable to transport of other mRNAs in the *Drosophila* oocyte as well as RNA transport in other oocyte systems. A notable example of another oocyte system that has a very poorly defined cytoskeletal organization is the *Xenopus* oocyte. Although many mRNAs are transported to each hemisphere of these oocytes, two studies using different methodologies have argued for very different polarity of the oocyte cytoskeleton (Messitt et al., 2008; Pfeiffer and Gard, 1999), which will impact models of how directional transport of mRNAs is achieved. Live imaging of specific mRNAs will likely be required to help define how transport operates in this system.

4.2. Influence of Transport Complex Composition on Transport Direction

In the simplest case, a localized mRNA will become incorporated into a transport complex that is linked to one molecular motor and will localize to its destination on a cytoskeleton with a well-defined polarity. While this is the case for some localized mRNAs (such as *ASH1*), localization of an mRNA is usually much more complicated, as illustrated above. A recent study examining the composition and function of the *ASH1* localization complex has discovered some unexpected complexity in the assembly of the complex in vivo (Chung and Takizawa, 2010). As discussed above, localization of *ASH1* depends on She2, She3 and Myo4. However, Myo4 is a nonprocessive motor protein and would not be expected to mediate transport of a cargo as a monomer (Reck-Peterson et al., 2001). Purification of a transport-competent *ASH1* mRNP revealed that the transport complex contained four copies of Myo4 and She2, suggesting a role for protein (or RNA) oligomerization in assembly of a transport-competent mRNP and providing a molecular explanation for the processive movement of the ASH1 mRNP. Interestingly, using an artificial reporter system that could recruit a defined number of Myo4 motors to an RNA demonstrated that recruitment of more motor proteins leads to a greater

efficiency of mRNA transport, a theme that is consistent with the results discussed below.

As mentioned in the preceding section, one of the great challenges to understanding the specificity of mRNA transport is that the exact composition and biochemical activity of most transport complexes is not known. Reconstitution of mRNA-binding complexes has provided a major insight into recognition of mRNA zipcodes. However, assembly of full transport complexes is considerably more complicated as it requires the inclusion of molecular motors, which are technically challenging to work with in vitro. In *Drosophila* embryos, certain transcripts (e.g. *hairy*, *fushi tarazu*, *K10*) localize to the apical centrosome of the embryo, and this behavior can be recapitulated by fluorescently labeled transcripts injected into the embryo (Bullock and Ish-Horowicz, 2001). Analysis of injected wild-type and localization-deficient transcripts in vivo demonstrated that both classes of transcripts exhibit fast bidirectional movement along microtubules, consistent with motor-driven transport (Bullock et al., 2006, 2003). However, localization-competent RNAs exhibited more frequent and longer movements to the minus ends of microtubules, which may result in the net accumulation of the localized mRNA at the minus ends of microtubules (Bullock et al., 2006). In addition, mutation or overexpression of the localization factors Egalitarian and Bicaudal-D led to slower or faster RNA transport respectively, suggesting that recruitment of a different number of motor proteins could be responsible for the different relative transport activities (Bullock et al., 2006). However, in these experiments, it was not possible to directly visualize the motor proteins involved, and as a result, it was not possible to prove that differences in motor number account for differences in motility.

A recent study using partially purified mRNPs from *Drosophila* embryos has begun to provide insight into how directional transport of mRNPs is achieved (Amrute-Nayak and Bullock, 2012). From the above results, it was possible that recruitment of more molecules of dynein or an increased activity of dynein could result in the increased minus end directed processivity observed in vivo. In a clever experiment to study the behavior of localization-competent mRNPs at a single molecule level, mRNAs were incubated with *Drosophila* embryo extract, selected for microtubule binding, and then analyzed at a single molecule level using TIRF microscopy. Remarkably, behavior of single molecules of the localization-competent and incompetent versions of the *K10* RNA in vitro was consistent with movement of the RNAs in vivo. Both RNAs exhibited fast movement to both the plus and minus ends of microtubules, with the localization-competent

RNA exhibiting an increased frequency and longer runs toward the minus ends. Furthermore, GFP-tagged dynein or a subunit of the dynein accessory dynactin complex was covisualized with fluorescent mRNA in vitro. Sequential photobleaching was used to measure the number of motors associated with each transport particle and was found to differ by ~50% between a localization-competent RNA and a localization-incompetent RNA. These data confirm the idea that recruitment of more motor proteins is responsible for increased processivity toward the minus end.

Another open question is to determine how many mRNA molecules are present in each mRNP transport particle. Work on *oskar* localization complexes (Glotzer et al., 1997) and purification of kinesin-associated RNPs from neuronal cells (Kanai et al., 2004) suggests that many molecules of RNA may be present in localization complexes, but this has not been tested rigorously. Using sequential photobleaching, the authors showed that each transport complex contains only one molecule of mRNA (Amrute-Nayak and Bullock, 2012). Furthermore, this was confirmed by FISH in *Drosophila* embryos for two mRNAs that both localize to the apical side of the embryo: *h* and *eve* mRNAs do not colocalize, suggesting that transport particles only contain single mRNA molecules. This result is consistent with a recent FISH study in neurons demonstrating little colocalization between different mRNAs in transport complexes (Mikl et al., 2011). Taken together, the single molecule studies of *K10* mRNA localization complexes demonstrate that localization complexes can consist of a single mRNA that recruits multiple motor proteins. Interestingly, localization-incompetent mRNAs can also recruit motor proteins, and the role of the localization sequence is to increase the recruitment of one particular type of motor protein to achieve a bias in the directionality of transport. This demonstrates that very subtle changes in protein complex composition can result in dramatic localization changes in vivo. It will be important to extend these studies to determine what other motors are recruited to the RNP (kinesin?) and how many copies of each of the accessory proteins are recruited. A long-term goal will be to understand the molecular mechanism by which a localization element results in the recruitment of additional copies of motor proteins to an mRNA.

4.3. RNA Anchoring

Once a transported RNA reaches its destination, the RNA must be anchored in order to retain the localized distribution. In many organisms that localize mRNAs via transport along the microtubule cytoskeleton, the

function of RNA anchoring is served by the actin cytoskeleton. However, we understand very little about the molecular mechanisms that transfer an RNA from the microtubule cytoskeleton to actin cytoskeleton. Another mechanism of RNA anchoring is to change the activity of the motor proteins involved in the transport RNP from active motors to static anchors. Two studies of RNA localization in *Drosophila* have provided clear evidence for this mechanism and begun to shed light on the molecular details of the process (Delanoue and Davis, 2005; Delanoue et al., 2007).

As described above in several previous sections, one of the major types of mRNA transport in *Drosophila* embryos is the movement of mRNAs involved in developmental pattern formation to the apical centrosome. Movement of these mRNAs occurs in a dynein-dependent manner and requires Egl and BicD for transport. However, little was known about the mechanism of RNA anchoring at the centrosome. It was shown that disruption of the actin cytoskeleton had no effect on RNA transport or anchoring, while disruption of microtubules dramatically delocalized the transported RNA (Delanoue and Davis, 2005). Interestingly, dynein was shown to colocalize with localized, anchored RNAs. In addition, interference with dynein function through a blocking antibody completely disrupted RNA that was already anchored at the cortex. Surprisingly, inhibition of dynein motor activity, but not the dynein complex, blocked active transport but retained RNA anchoring. These results show that incorporation into an RNP can change dynein from an active motor protein to a static anchor once it has reached its destination at the microtubule minus ends. A follow-up study examining the role of dynein in localization of the *gurken* RNA in the *Drosophila* oocyte reached a similar conclusion (Delanoue et al., 2007). Interestingly, mutation of the Squid protein, an hnRNP, results in an inability of dynein to change to a static anchor at the destination. However, the molecular details of how mRNPs can change the motor activity of dynein remain unknown. One interesting implication from these studies is that mRNA transport complexes do not necessarily function solely as passive cargo on the cytoskeleton, but have the potential to feedback and change the activity of factors that influence the cytoskeleton, a theme that will be discussed in the last section of this chapter.

5. FORMATION AND STRUCTURE OF RNA GRANULES

One of the most distinctive features of localized RNAs is their ability to form cytologically visible cytoplasmic structures termed *RNA granules*

(Anderson and Kedersha, 2006). RNA granules are most obviously visible in the germ line of most model organisms (P-granules in *C. elegans*, polar granules in *Drosophila*, and germinal granules in *Xenopus*). These granules are composed of RNAs and RNA-binding proteins that are known to control the formation of germ cells in the developing embryos and were one of the first recognized sites of mRNA localization. Germ cell granules (GCG) share many similarities with other types of RNA granules found in somatic cells, such as stress granules and P-granules (Anderson and Kedersha, 2006). Stress granules are large cytoplasmic aggregates of mRNAs and RNA-binding proteins that accumulate upon cellular stress (e.g. heat shock or arsenite treatment) and are thought to function as sites of mRNA storage during cellular response to stress. P-bodies are also visible sites of mRNAs and RNA-binding proteins but because of the protein composition of P-bodies, they are linked to RNA decapping, deadenylation and RNA destruction (Balagopal and Parker, 2009). One common feature of all RNA granules is that they are higher order assemblies of many RNAs and RNA-binding proteins that appear to function in temporary or long-term storage of mRNAs. Although RNA granules are visible cytoplasmic structures, very little was known about the mechanisms of formation or functions of these structures until recently.

5.1. Mechanisms of RNA-Granule Formation

Insight into the physical properties and formation of RNA granules has come from careful analysis of the composition and behavior of *C. elegans* P-granules. In an important study, live cell imaging was used to examine the behavior of the *C. elegans* P-granule component PGL-1 in the gonad and early embryo (Brangwynne et al., 2009). Interestingly, PGL-1 exhibited behavior consistent with a liquid droplet, such as dissolution, condensation and various flow behaviors. Measurement of the viscosity of P-granules suggested that they have a viscosity comparable to glycerol (~1000 times that of water) and colloidal liquids. One of the most interesting properties is regulated dissolution and condensation. At high component concentrations, PGL-1 condensed to form P-granules, but when the effective protein concentration was lowered, the granules dissolved. These results suggested that weak interactions between P-granule components might be important for their formation.

The molecular interactions that lead to the liquid-like behavior of P-granules was addressed by two recent studies that explored the molecular requirements for the formation of P-granules (Updike et al., 2011; Updike

and Strome, 2009; Voronina and Seydoux, 2010). Both studies made the important observation that several proteins present in P-granules (the Vasa-related proteins GLH-1, GLH-2 and GLH-3) contain repeats of the amino acids phenylalanine and glycine, and these were required in GLH-1 for granule formation (Updike et al., 2011). This was informative because FG-repeats are one of the prominent features of the proteins that line the central channel of the nuclear pore (Weis, 2003). It has been hypothesized that weak interactions between the FG-containing nucleoporins are responsible for creating a selective hydrogel-like barrier between the nucleus and the cytoplasm. In fact, it was recently demonstrated that very concentrated solutions of FG-repeats from the yeast nucleoporin Nsp1p form a solid hydrogel through hydrophobic interactions (Frey et al., 2006), which allows only proteins with appropriate characteristics to pass through (Frey and Gorlich, 2007). Although nucleoporins exhibit hydrogel-like properties at high concentrations, while P-granules exhibit liquid-like properties in vivo, the properties of homotypic interaction and selective permeability appeared to suggest a common mechanism. Consistent with a link between the nuclear pore and P-granules, P-granules exhibited a size exclusion of fluorescent dextrans, similar to the nuclear pore (Updike et al., 2011). In addition, RNAi against a subset of nuclear pore proteins disrupted P-granule formation (Voronina and Seydoux, 2010). Furthermore, GFP-fusions of several nuclear pore proteins colocalized with P-granules and mutation of the *C. elegans* Nup98 homolog disrupted P-granule formation. Interestingly, the mouse Nup98 also interacted with the mouse Vasa homolog in mouse testes extract, suggesting that nucleoporins and nucleoporin-like interactions may be important for GCG formation in other systems (Voronina and Seydoux, 2010). Taken together, these results suggest that GCG exhibit many properties of nuclear pores and that regulated, homotypic interactions between FG-repeats are important for granule coalescence.

Biochemical insight into the mechanism of RNA granule formation recently came from two studies analyzing a compound causing cellular differentiation (Han et al., 2012; Kato et al., 2012). While analyzing proteins that bound to a differentiation-inducing drug, the authors noticed that the addition of this compound to cellular extracts caused the aggregation and precipitation of many cellular proteins. Identification of the precipitated proteins revealed that a substantial fraction was known RNA-binding proteins. Furthermore, analysis of the coprecipitated RNAs from cellular extracts identified a large number of mRNAs known to exhibit distinct subcellular

localizations, suggesting that regulated protein aggregation may have a physiological role. Mapping the aggregation-promoting activity of precipitated RNA-binding proteins identified low complexity (LC) sequence domains that were both necessary and sufficient for protein aggregation. The LC regions contained repeats of tyrosine, serine and glycine, similar to the FG repeats found in nuclear pore proteins. Also similar to the FG-repeats found in nucleoporins, the LC domains of RNA-binding proteins were able to form hydrogels in very concentrated solutions in vitro. Interestingly, hydrogels could recruit RNAs as fusion proteins and from cellular extracts, demonstrating that LC-based sequence aggregation can be used to concentrate RNA and RNA-binding proteins into granules. Importantly, mutation of serine residues within the LC sequences blocked hydrogel formation and also blocked localization of the proteins to stress granules formed in tissue culture cells (Kato et al., 2012). Analysis of the interaction of LC regions from various RNA-binding proteins revealed that homotypic interactions were favored but that there were also differing degrees of heterotypic affinities between LC regions from different proteins. This gradient of affinities could suggest a possible mechanism for generating RNA granules of mixed compositions, containing different RNAs and RNA-binding proteins.

While these studies were preformed primarily in vitro, the parallels with P-granule formation suggest a compelling model for RNA granule formation. RNA-binding proteins bound to target RNAs interact with one another via homotypic interactions between repetitive LC sequences. These interactions are promoted by high local protein concentrations that might be facilitated by transport of many copies of RNPs to a specific destination. Once the proteins are aggregated, they are relatively static and likely prevent translation of the RNAs present within the granules. Importantly, RNPs can be released from the granules either by lowering effective protein concentration, a mechanism known to occur in *C. elegans* (Brangwynne et al., 2009), or by phosphorylation of the self-association domains (Han et al., 2012). While this is a compelling model that fits with in vivo evidence from many different model systems, these ideas will all need to be tested in vivo.

5.2. Function of RNA Granules

While the vast majority of evidence from a variety of model systems supports the model that RNA granules are sites of mRNA storage, several studies of the cytoplasmic polyadenylation binding protein (CPEB) from *Aplysia californica* suggest that sites of RNA granules could also be sites of high enzymatic activity that promote protein translation. CPEB proteins are

proteins that recognize the cytoplasmic polyadenylation element (CPE) in the 3′UTR of mRNAs under posttranscriptional control (Richter, 2007). CPEB proteins are implicated in both repression of mRNAs and activation of the mRNA through extension of poly-A tail length in response to a signaling cue (Pique et al., 2008). In *Aplysia*, the CPEB protein contains an unusual N-terminal extension that has some similarity to prion forming domains from yeast (Si et al., 2003). The N-terminus of CPEB behaves as a prion in yeast and appears to form amyloid-like aggregates. Interestingly, aggregation of *Aplysia* CPEB is required for full CPEB activity when expressed in yeast, and blocking prion-like aggregates when expressed in neurons interferes with synaptic function (Si et al., 2010). These results suggest that there is a role for regulated aggregation in promoting the CPEB-mediated cytoplasmic polyadenylation (Si et al., 2003). It will be important to determine if regulated aggregation of other RNA-binding proteins plays a role in the activation of other RNA processing enzymes, or if the majority of RNA granules are sites of RNA storage. Interestingly, amyloid-like aggregation of other RNA-binding proteins has been observed, but in these cases, it has been associated with loss of function of the aggregated proteins. These data suggest that amyloid-like aggregation of RNA-binding proteins could contribute to neurodegenerative diseases (Sun et al., 2011). In the future, it will be important to distinguish between regulated aggregation of RNA-binding proteins, such as that observed in vitro and in *C. elegans* P-granules, and the irreversible, prion-like protein aggregation that could result in deleterious consequences to the organism.

6. REGULATION AND ROLES OF LOCALIZED mRNAS

6.1. Translational Regulation

Once an mRNA has been localized and anchored at a specific destination, it needs to be activated for translation in response to an external, internal or developmental signaling cue. Translational control of localized mRNAs has recently been discussed in an excellent and comprehensive review (Besse and Ephrussi, 2008) and will not be covered here in detail. However, I will summarize one study to illustrate one of the best-understood examples of translational control of a localized mRNA.

One of the first examples of a localized mRNA is the β-actin transcript from chick fibroblasts. The transcript was shown to localize to the leading edge of motile fibroblasts and the localization activity mapped to a small region of the 3′UTR termed the *zipcode*. The zipcode of β-actin

mRNA is bound by the ZBP-1 protein that is required for mRNA localization. Interference with mRNA localization has been shown to affect cell migration but it was not understood how the interaction of the zipcode with ZBP-1 controlled β-actin translation (Condeelis and Singer, 2005). ZBP-1 acts as a zipcode-dependent translational repressor in vitro in rabbit reticulocyte lysate and in vivo in neuronal cells (Huttelmaier et al., 2005).

Interestingly, ZBP-1 contains a tyrosine residue near one of the RNA-binding domains that resides within a consensus sequence for the Src protein kinase, and ZBP-1 is in fact phosphorylated by Src in vitro and in vivo. Phosphorylation of this residue reduced binding of ZBP-1 to the β-actin transcript, demonstrating that phosphorylation by Src can serve as a signal to trigger translational activation. Consistent with this model, knockdown of ZBP-1 in neuronal cell culture resulted in a decrease in neuronal outgrowth, which could be rescued by wild-type ZBP-1, but not by a non-phosphorylatable ZBP-1 variant. Taken together, these results suggest that ZBP-1 associates with β-actin transcripts in the nucleus during transcription, and remains bound to the transcript during transport in the cytoplasm. Once in the cytoplasm, ZBP-1 suppresses the translation of β-actin mRNA until a signal to release the RNA is received in the form of phosphorylation by the Src tyrosine kinase. Importantly, both premature translation induced by ZBP-1 RNAi and failure to relieve translational inhibition result in failures of neuronal outgrowth. This study demonstrates that temporal and spatial coupling of mRNA localization to translational control is critical for the function of the encoded protein (Huttelmaier et al., 2005). The paradigm of signaling-induced mRNA release is likely to be applicable to other RNA-binding proteins, and also fits well with the observation that phosphorylation of serine residues in LC regions can release RNA-binding proteins from RNA granules. It will be important to understand the signaling pathways and proteins that contribute to release of RNA from mRNP transport complexes to fully understand local translational control.

6.2. Noncoding Roles

This chapter has been based on the idea that most localized RNAs are mRNAs and that the primary function of restricting the localization of the mRNA is to control the temporal and spatial expression pattern of the protein. However, there is increasing evidence that some localized mRNAs function as RNA rather than or in addition to serving as a template for protein production. This topic has recently been reviewed (Kloc et al., 2011)

and will be discussed briefly to highlight additional possible roles of localized RNAs.

One of the first hints for non-protein-coding roles for localized RNAs came from the characterization of the Xlsirst (*Xenopus laevis* short interspaced repeats) family of RNAs in *Xenopus.* Xlsirst are a family of ~80 bp repeats that are highly transcribed during oogenesis. Interestingly, sense strand versions of this repeat are localized to the Balbiani Body and vegetal cortex of the oocyte in pattern nearly indistinguishable from other well-characterized localized mRNAs (e.g. Vg1 and Xcat2; Kloc et al., 1993). When the function of the Xlsirts was explored by removal using antisense deoxyoligonucleotides (ODN), it was found that Xlsirts were required for the proper localization of the Vg1 transcript to the vegetal cortex of the oocyte (Kloc and Etkin, 1994). Since Xlsirts are a tandemly repeated RNAs that lack protein-coding capacity, this was a direct demonstration that localized, noncoding RNAs could serve a function, in this case to promote the localization of a protein-coding RNA. The idea that localized RNAs can function as RNA rather than as a protein gained further support from study of the *Xenopus* localized mRNA VegT (Heasman et al., 2001). VegT is another mRNA that is localized to the vegetal cortex of the oocyte and encodes a T-box transcription factor important for development (Zhang et al., 1998). Interestingly, destruction of the VegT RNA by injection of ODN resulted in delocalization of a number of mRNAs from the vegetal cortex (including Vg1), but blocking protein production from the mRNA did not result in the same phenotype. These data demonstrate that in addition to Xlsirts, the VegT mRNA also plays a role in promoting mRNA localization, independently of protein-coding capacity.

Insight into how Xlsirts and VegT could function to promote mRNA localization has come from a careful analysis of the cytoskeleton in oocytes. In *Xenopus*, many mRNAs that are localized to the vegetal cortex are anchored at the cortex by association with either the actin or keratin cytoskeletons. Destruction of Xlsirt and VegT revealed that the cortical cytokeratin cytoskeleton was affected, while actin remained unaffected (Kloc et al., 2005). Consistent with this result, both mRNAs localized to the vicinity of cytokeratin filaments, suggesting that they may act directly to promote proper keratin assembly. Thus, RNAs can act to influence mRNA localization through modulation of cytoskeletal structure.

Additional support for the idea of a structural role for RNA in cytoskeletal assembly has come from study of the mitotic and meiotic spindle. Using *Xenopus laevis* egg extracts to study meiosis II spindles, it was demonstrated

that proper assembly of the spindle was dependent on the presence of RNA (Blower et al., 2005). RNaseA treatment of extracts resulted in dramatic changes to spindle microtubule organization, while inhibition of translation using a variety of translation inhibitors did not change spindle morphology, demonstrating that RNA plays a structural role in microtubule assembly. Interestingly, immunodepletion of Rae1, a protein component of an RNA complex, also resulted in dramatic defects in spindle assembly in vitro, perhaps providing a starting point to understand how RNA can promote spindle assembly. The structural role of RNA in spindles assembly has also been confirmed in a permeabilized cell assay using human cells (Hussain et al., 2009), suggesting that a structural role for RNA in mediating microtubule assembly is likely to be conserved in many organisms.

Although these studies suggest that localized RNAs will play direct, protein coding-independent roles, the molecular details of these processes remain unclear. In the case of Xlsirts and VegT, we know which RNAs act to promote cytokeratin assembly, but we do not know how these RNAs exert their effects, which proteins they bind, or how they affect cytokeratin assembly and maintenance. In the case of the role of RNA in microtubule assembly, we know only that RNA is required for spindle assembly, but we do not know which RNAs promote microtubule assembly or whether proteins other than the Rae1 complex are involved. In both cases, it will be important to understand the RNAs that are required for each of these events, and the RNA-binding proteins that are affected. This knowledge will need to be integrated with an understanding of how the RNA-binding proteins affect cytoskeletal dynamics.

One final example of a protein coding-independent role of a localized RNA comes from study of the *oskar* RNA in *Drosophila*. During *Drosophila* embryogenesis, the oskar protein is required for proper segmentation patterning. However, the classic genetic alleles of *oskar* were loss of protein alleles but did not lead to loss of the mRNA. Analysis of new alleles of *oskar* that completely lacked mRNA production showed stronger phenotypes, with null animals not producing eggs and exhibiting an early oogenesis arrest (Jenny et al., 2006). Interestingly, this oogenesis arrest could be rescued by a protein null allele, or by the *oskar* 3′ UTR, suggesting that the *oskar* mRNA plays a translation-independent role early in oogenesis. There is currently no understanding of how the oskar 3′UTR functions to promote early oogenesis in the absence of protein.

Taken together, these studies support the notion that localization of RNAs (both coding and noncoding) can serve functions in addition to

the well-documented spatial and temporal control of protein synthesis. Given the recent discovery of thousands of noncoding RNAs (Wang and Chang, 2011) and the fact that both mRNAs and ncRNAs can play structural roles, it seems likely that many new functions of localized RNAs that are independent of protein-coding capacity are yet to be discovered.

7. PERSPECTIVE

As detailed above, the localization of an mRNA to a specific destination within a cell or embryo is a widely utilized mechanism for the spatial and temporal control of protein expression. Mechanistically, it is an extremely complicated process involving co-transcriptional processing, linkage to transport complexes, anchoring at a destination and ultimately relief of translational inhibition. While genetics and cytology in several prominent model organisms have provided a basic picture of how mRNA localization is regulated, many open questions remain. Below I have highlighted several interesting questions that will be addressed in the future.

7.1. *cis*-Acting Localization Sequences

As described in the preceding section, many individual *cis*-acting sequences have been described that direct mRNAs to specific destinations. However, many of the localization sequences that have been discovered are large and poorly defined. In addition, there are very few cases where there is obvious homology between defined localization sequences, which has dramatically limited understanding of the logic of zipcodes that sort mRNAs within a cell. A technical aspect that has hampered the field is that mapping of localization elements is a labor intensive process that is generally accomplished one mRNA at a time. In order to achieve better predictive ability for defining zipcodes computationally, we will need much larger datasets of localization elements. Given the recent advances in identification of protein-binding sites within RNAs by high-throughput sequencing (Darnell, 2010), it is possible that these technologies can be applied to localized mRNAs to identify a much larger set of zipcodes. This, coupled with improved methods of RNA structure determination and structural homology prediction (Parisien and Major, 2008), may lead to dramatic increases in our ability to detect and predict mRNA localization elements.

7.2. Biochemical Reconstitution of mRNA Localization Complexes

Genetic and cell biological studies in a wide range of model organisms have provided a list of localized RNAs and proteins that are likely to interact with these RNAs, however, we lack a detailed understanding of the molecular interactions that select certain RNAs from transport and the mechanisms that achieve selective transport. The past year has seen several studies that have begun to provide biochemical insight into both RNA recognition and biased directional transport. Future studies will likely continue to try to understand how combinations of low specificity interactions lead to high specificity RNA binding and how subtle biases in transport complex composition lead to dramatic biases in RNA distribution in a cell or an embryo. Finally, the ability to reconstitute certain aspects of RNA granule formation in vitro should provide the ability to understand the regulated self-association of RNA-binding proteins that will provide a platform for exploring the functional implications of these interactions for RNA transport, translational control and RNA anchoring in vivo.

7.3. Noncoding Functions of Localized RNAs

Recent studies of the transcriptomes of a number of organisms have discovered hundreds to thousands of RNAs with little to no protein-coding capacity (Ulitsky et al., 2011). Current research has focused on the hypothesis that many/most of these noncoding RNAs will regulate nuclear processes (Wang and Chang, 2011) although there has been no systematic study of the localization of these RNAs. Given the studies of RNAs such as Xlsirts, it seems likely that many ncRNAs will have cytoplasmic functions and many may function through localization to specific compartments of the cell. Future studies will likely focus on which ncRNAs are localized to specific compartments within the cell and how they function. It will be important to understand which proteins bind to ncRNAs and how RNA binding changes the activity of the bound protein or complex. Given that some mRNAs also appear to function in a translation-independent manner, it will also be important to differentiate between RNA and protein-coding functions of localized mRNAs.

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CHAPTER TWO

A Hypothesis on the Origin and Evolution of Tubulin

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Contents

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Abstract

Tubulin, the protein subunit of microtubules (MTs), is an α/β heterodimer. In this chapter, a hypothesis on the evolution of the tubulin molecule is proposed, based in part on recent reports on the structures and functions of different forms of tubulin and its relatives. The concentration is on three main areas. 1) Evolution of the vertebrate β-tubulin isotypes. In addition to providing a clear idea about the relationships among these isotypes, recent data suggest that tubulin may have functions that do not involve being in a MT, namely, that it can function as an isolated α/β dimer or as a non-MT polymer. 2) Examination of the entire tubulin superfamily, which includes not only tubulins α, β, γ, δ, ε, η, and others but also a variety of prokaryotic proteins. The hypothesis is presented that the common ancestor of all these proteins formed a filamentous curving polymer that used the energy of GTP hydrolysis to apply force to nucleic acids and/or membranes and that this common ancestor may have been coeval with the first cells. A variety of chaperones, motors and MT-associated proteins may have coevolved with tubulin and their histories illuminate that of tubulin. The

branched, highly negatively charged C-terminal domain present on α- and β-tubulin appears to be a relatively recent addition to tubulin. 3) The hypothesis is presented that the C-terminal domain may have been of prebiotic origin and that it gradually developed into a protein serving particular metabolic functions whose gene eventually became fused with those of α- and β-tubulin. Finally, some experiments are proposed that could illuminate the probability of these hypotheses.

ABBREVIATIONS

Abl Abelson murine leukemia viral oncogene homolog
APC Adenomatous polyposis coli
Arg Abl-related gene
CAP-Gly Cytoskeleton-associated protein, glycine-rich
CCT Chaperonin-containing TCP-1
CENP Centromere protein
CEP Centrosomal protein
CLASP Cytoplasmic linker-associated protein
CLIP Cytoplasmic linker protein
C-Nap1 Centrosomal Nek2-associated protein
CoA, etc. Tubulin-binding cofactor A, etc.
CPEB Cytoplasmic polyadenylation element-binding protein
CRIPT Cysteine-rich interactor of PDZ3
CRMP Collapsin response mediator protein
DISC Disrupted in schizophrenia
EB1 End-binding protein 1
FoxO Forkhead box protein
γ-TuRC γ-tubulin ring complex
γ-TuSC γ-tubulin small complex
GEF Guanine nucleotide exchange factor
GLUT Glucose transporter
HIF Hypoxia-inducible factor
HSP Heat-shock protein
LCFTA Latest common FtsZ/tubulin ancestor
LECA Latest eukaryotic common ancestor
MAP Microtubule-associated protein
NAP Nucleosome assembly protein
ODF2 Outer dense fiber protein 2
PDZ Postsynaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1), and zonula occludens-1 protein
PELP1 Proline-glutamate and leucine-rich protein
RHAMM Hyaluronan-mediated motility receptor
RPAP RNA polymerase II-associated protein
STOP Stable tubule-only polypeptide
TCP-1 T complex protein 1
TRPV1 Transient receptor potential cation channel subfamily V

TTLL Tubulin-tyrosine ligase-like
VDAC Voltage-dependent anion channel
WASH Wiscott–Aldrich syndrome protein
XMAP *Xenopus* microtubule assembly protein

1. INTRODUCTION

Tubulin is the subunit protein of microtubules (MTs), organelles performing a large number of functions within eukaryotic cells; the best known, or canonical, MT-based structures are the mitotic spindle, the interphase MT network, and the axonemes of cilia and flagella (Dustin, 1984; Kreis and Vale, 1999; Cleveland, 1999). In this chapter, where I hope to take the reader toward an understanding of how this complex protein originated and evolved, we shall discuss these and many other functions of tubulin, including some that may have nothing to do with MTs. MTs are formed from heterodimeric tubulin, consisting of α- and β-tubulin polypeptides (Bryan and Wilson, 1971; Ludueña et al., 1977). To add to the complexity, both α and β exist as numerous isotypes, differing in amino acid sequence and encoded by different genes (Sullivan and Cleveland, 1986; Ludueña, 1998). We shall look closely at the individual β-tubulin isotypes in vertebrates because these may be the best understood. This examination will reveal that some of these isotypes have very noncanonical functions and this information will illuminate the evolutionary pathways traced by tubulin and its relatives in the tubulin superfamily, whose structures and functions will give us a sense of what the first tubulin was like in the original eukaryotic cell. Tubulin has relatives among the prokaryotes; these will have their stories to tell that will point to the origin of their common ancestor perhaps as far back as the first living cell. Finally, we will examine the highly unusual C-terminal region of α- and β-tubulin and present the argument that this domain may have a separate story from the rest of the tubulin molecule, and indeed may be of prebiotic origin.

The starting point of this study is to appreciate that the tubulin molecule is subject to tremendous functional constraints. The α and β subunits each have to bind to GTP and join each other to form an α/β heterodimer; these in turn constitute a cylinder, in which the dimers are joined longitudinally into 13 protofilaments that associate laterally into a MT (Li et al., 2002). The MT is asymmetric, with a helical discontinuity or "seam" between two of the protofilaments (Mandelkow et al., 1986). The α/β dimers in MTs generally polymerize onto γ-tubulin (Leguy et al., 2000; Moritz and Agard, 2001). The polymerization process is connected to the hydrolysis of the GTP bound to the β subunit (Carlier et al., 1987; Caplow et al., 1994). This process allows MTs to be dynamic structures: hydrolysis of GTP alters the tubulin conformation so as

to weaken lateral interactions (Wang and Nogales, 2005). When tubulin bound to GDP is exposed at the ends, disassembly is favored (Carlier and Pantaloni, 1981; Desai and Mitchison, 1997; Nogales et al., 1999; Wang and Nogales, 2005; Nogales and Wang, 2006; Buey et al., 2006; Bennett et al., 2009). Such dynamics are important in processes like mitosis. Other MT populations, such as those in axonemes, are very stable (Behnke and Forer, 1967). In addition, MTs do not operate in a vacuum. In order to carry out their numerous and varied functions, the tubulin molecule, either in a MT or as a free dimer, has to interact directly or indirectly with many other proteins. The list of these proteins appears to grow almost monthly (Gache et al., 2005). Table 2.1 shows only a partial list in which the proteins are grouped into categories; these categories are likely to overlap. The list also adds nonprotein factors, such as polysaccharides, RNA, and lipid membranes. In addition, a very large number of proteins are only implied in Table 2.1; these include the various components of MT-based organelles, such as the centrosome and the axoneme. These are likely to include scaffolding proteins and, for any one of these organelles, the total number of component proteins may number in the hundreds (Morris et al., 2003; Li et al., 2004; Nicastro et al., 2006; Pederson et al., 2008; Özlu et al., 2010; Diener et al., 2011). Although much of the data shown in Table 2.1 come from studies done in mammalian cells, similar studies find numerous proteins belonging to the categories listed in Table 2.1 that bind to tubulin or MTs in other animals, plants and fungi (Orosz et al., 1999; Romagnoli et al., 2010; Li et al., 2011; Gusnowski and Srayko, 2011; Panteris et al., 2011). Adding to the complexity is that tubulin may have to discriminate between differentially modified forms of some of these proteins (Kiris et al., 2011; Fauquant et al., 2011). Some of the entities listed in Table 2.1 may bind to tubulin or MTs either directly or indirectly; in some cases, we do not know how they bind.

Complicating the picture still further, recent evidence suggests that tubulin dimers may have functions that do not involve forming part of a MT; these putative functions add further to the list of molecules with which tubulin interacts and we shall discuss them in more detail below. All these factors—geometry, dynamics, and protein–protein interactions—must certainly impose a tremendous number of constraints on tubulin evolution and probably account for the high degree of conservatism in the amino acid sequences of both α and β tubulin.

2. TUBULIN ISOTYPES

It is very likely that tubulin isotypes arose as a way to relieve some of these constraints. MTs composed of different dimers have altered dynamics

Table 2.1 Macromolecules that interact with tubulin and/or microtubules*

Category	Examples	References
Microtubule-associated proteins	Tau, MAP1A, MAP1B, MAP1S, MAP2, MAP4, E-MAP-115 Doublecortin, APC, CRMP-2	(Binder et al.,1985; Munemitsu et al., 1994; Su et al., 1995; Fukata et al., 2002; Kar et al., 2003; Kita et al., 2006; Wakabayashi et al., 2008; Tint et al., 2009; Fourniol et al., 2010; Cheng et al., 2010; Xie et al., 2011)
Motor proteins	Kinesin, flagellar dynein, Cytoplasmic dynein	(Gusnowski and Srayka, 2011)
Plus-end-binding proteins	EB1, CLIP-170, CLASPs, XMAP215, astrin	(Honnappa et al., 2006; Mishima et al., 2007; Weisbrich et al., 2007; Akhmanova and Steinmetz, 2008, 2010; Vitre et al., 2008; van Haren et al., 2009; Dunsch et al., 2011; Li et al., 2011)
Minus-end-binding proteins	Patronin	(O'Rourke and Sharp, 2010)
Microtubule-severing proteins	Katanin, spastin	(Iwaya et al., 2010; Panteris et al., 2011; Dráberova et al., 2011)
Collapsin response mediator proteins		(Lin et al., 2011)
Nuclear import proteins	RPAP4	(Yokoyama et al., 2009; Forget et al., 2010; Roth et al., 2011)
Chaperonins	Prefoldin, CCT, HSP90, HSP60, Tubulin cofactors A, B, C, D, E	(Gao et al., 1994; Tian et al., 1996,1997; Garnier et al., 1998; Mitra et al., 2007; Weis et al., 2010)
Receptors	Adrenergic receptors, RHAMM	(Duvernay et al., 2011; Tolg et al., 2010)
Glucose transporters	GLUT4	(Fletcher et al., 2000; Guilherme et al., 2000; Semiz et al., 2003)

Channel proteins	VDAC, TRPV1	(Carré et al., 2002; Rostovtseva et al., 2008; Rostovtseva and Bezrukov, 2008; Goswami and Hucho, 2008; O'Brien et al., 2012; Storti et al., 2012)
Guanine nucleotide exchange factor		(Varma et al., 2010)
Signal transduction proteins	Gs, Inositol 1,4,5-trisphosphate 3-kinase A, FoxO, CRIPT	(Gundersen and Cook, 1999; Passafaro et al., 1999; Rasenick et al., 2004; Layden et al., 2008; Roychowdhury and Rasenick, 2008; Yu et al., 2009; Elias and Archibald, 2009; Davé et al., 2011; Nechipurenko and Broihier, 2012; Lee et al., 2012)
Dynamic regulatory Factors	STOPs, stathmin	(Margolis et al., 1986; Redeker et al., 2000; Wallon et al., 2000; van der Vaart et al., 2009)
Actin filaments	Dystrophin, actin, ACF7, Arg, GEF-H1	(Karakesisoglou et al., 2000; Krendel et al., 2002; Chuong et al., 2004; Miller et al., 2004; Prins et al., 2009; Okada et al., 2010; Schaumacher et al., 2010; Stroud et al., 2011)
Intermediate filaments	Desmoplakin	(Lechler and Fuchs, 2007)
Tubulin-modifying enzymes	Tubulin–tyrosine ligase, Tubulin carboxypeptidase, TTLL1–TTLL13, HDAC6	(Tuerk et al., 2007; Janke and Bulinski, 2011)
Metabolic enzymes	Most glycolytic enzymes	(Walsh et al., 1989; Somers et al., 1990; Lehotzky et al., 1993; Volker et al., 1995; Vértessy et al., 1997, 1999; Götz et al., 1999; Orosz et al., 2000; Gitlits et al., 2000; Wágner et al., 2001; Kovács et al., 2003; Chuong et al., 2004; Andrade et al., 2004; Keller et al., 2007; Romagnoli et al., 2010; An et al., 2010; Cassimeris et al., 2012)

Continued

Table 2.1 Macromolecules that interact with tubulin and/or microtubules*—cont'd

Category	Examples	References
Centrosomal/basal body components	Hundreds	(Andersen et al., 2003; Guo et al., 2006; Hebbar et al., 2008; Keller et al., 2009; Carvalho-Santos et al., 2010)
Axonemal proteins		
Cytokinesis proteins		(Özlu et al., 2010)
Kinetochore proteins		(Yuan et al., 2009; Espeut et al., 2012; Chan et al., 2012)
Linkers to membranes	CEP290, septin 2, α-synuclein	(Skehel et al., 2000; Alim et al., 2002; Spiliotis et al., 2008; Craige et al., 2010; Hu et al., 2010; Hu and Nelson, 2011; Bowen et al., 2011)
Anchoring proteins†	Dynactin, Nudel, Maskin, Centrobin	(Quintyne et al., 1999; Guo et al., 2006; Albee and Wiese, 2008; Gudi et al., 2011)
RNA-binding proteins	CPEB	(Groisman et al., 2000)
Nuclear envelope proteins	Nesprin-4	(Dahl and Kalinowski, 2011)
Other proteins	DISC1, HIF-1α, Parkin	(Ren et al., 2003; Morris et al., 2003; Carbonaro et al., 2012)
Glycogen		Fridman et al., 2012
Membranes		(Bhattacharyya and Wolff, 1975; Stephens, 1981; Bernier-Valentin et al., 1983; Stephens et al., 1987; Gauthier-Kemper et al., 2011)
Myelin		(Gozes and Richter-Landsberg, 1978; De Nechaud et al., 1983)
Phospholipids		(Klausner et al., 1981)
mRNA		(Carbonaro et al., 2011; Pesiridis et al., 2011)

*This is a partial list of proteins and other macromolecules reported to interact with tubulin, organized into categories, to illustrate the full complexity of the roles of microtubules in our cells. Some of these proteins may bind directly to tubulin, some bind indirectly, and in many cases, it is unclear whether the binding is direct or indirect.
†These proteins could be considered part of the centrosome.

and bind differently to other proteins (Panda et al., 1994; Banerjee et al., 1992). Many functions of MTs could, in principle, be shared among different isotypes. The discussion that follows will describe this in more detail. However, the apparent distributions of functions revealed by experiments in vitro and in vivo are not necessarily the most obvious. In other words, as we will see, the vertebrate βIV isotype is uniquely adapted to form the stable MTs of cilia and flagella, but it can also form the highly dynamic MTs of the mitotic spindle and, conversely, the βI and βV isotypes also form part of the axoneme (Jensen-Smith et al., 2003a; Vent et al., 2005). Similarly, although *C. elegans* uses unique α and β isotypes to form the giant 15-protofilament MTs of the touch-sensitive neurons (Savage et al., 1989; Fukushige et al., 1999), mammals also have giant 15-protofilament MTs in the pressure-sensitive pillar cells of the inner ear, but these do not require special isotypes (Tucker et al., 1992; Hallworth and Ludueña, 2000).

2.1. β-Tubulin Isotypes in Vertebrates

Most of the vertebrate β isotypes fall into six categories: βI, βII, βIII, βIV, βV and βVI (Ludueña, 1998). Several of these—βI, βII, βIII, βIV, and βV—occur in fish, amphibians, birds and mammals. A few reptile sequences are now known: these include βI, βII, βIII, βIV and βVI. There has been further speciation, with βIVa and βIVb occurring in mammals and βIa and βIb in primates. Except for βVI, the isotype differences are highly conserved in evolution (Table 2.2). The differences among the isotypes cluster in the C-terminal region. For mammals, birds, reptiles, and amphibians, these are given in Table 2.3 together with some other key elements of their sequence. The corresponding elements in fish tubulins are given in Table 2.4.

Perhaps the best way to begin a discussion on the evolution of vertebrate β-tubulin isotypes is to summarize what is known or speculated about their

Table 2.2 Relationships among vertebrate β isotypes

Isotype	% Sequence identity to βI	% Divergence between mammals & birds
βI	100.0	0.0
βI	94.6	1.1
βIII	90.2	0.4
βIVa	96.0	n/a
βIVb	97.3	1.7
βV	91.4	1.7
βVI	76.7	19.1

Table 2.3 Key portions of the sequences of β-tubulin isotypes of mammals, birds, reptiles, and amphibians*

Species	124–129	237–240	C-terminal sequence	Accession Number
Class Ia				
Human	AESCDC	TTCL	YQDATAEEEEDFGEEAEEEA	BAB63321
Mouse	AESCDC	TTCL	YQDATAEEEEDFGEEAEEEA	NP_035785
Chicken	AESCDC	TTCL	YQDATAEEEEDFGEEAEEEA	NP_990646
Gekko	AESCDC	TTCL	YQDATAEEEEDFGEEAEEEA	AAW51376
Xenopus	AESCDC	TTCL	YQDATAEEEEDFNEEAEEEA	AAH49004
Class Ib				
Human	AESCDC	TTCL	YQDATAEEEEDFGEEAEEEA	AAD33873
Class II				
Human	SESCDC	TTCL	YQDATADEQGEFEEEEGEDEA	AAN85571
Mouse	SESCDC	TTCL	YQDATADEQGEFEEEEGEDEA	NP_033476
Chicken	SESCDC	TTCL	YQDATADEQGEFEEEGEEDEA	NP_001004400
Anolis	SESCDC	TTCL	YQDATADEQGEFEEEGEEDEA	XP_003224445
Xenopus	SESCDC	TTCL	YQDATADEQGEFEEEEDEA	NP_001079533
Class III				
Human	CENCDC	TTSL	YQDATAEEEGEMYEDDEEESEAQGPK	AAN52035
Mouse	CENCDC	TTSL	YQDATAEEEGEMYEDDDEESEAQGPK	NP_075768
Chicken	CENCDC	TTSL	YQDATAEEEGEMYEDDEEESEQGAK	NP_001026769
Anolis	CENCDC	TTSL	YQDATAEEEGEMYEDDEEESEAGGGK	XP_003229334
Xenopus	CENCDC	TTSL	YQDATAEEEGEMYEDDEEESEAQGK	NP_001088455
Class IVa				
Human	AESCDC	TTCL	YQDATAE**EGEFEEE**AEEEVA	NP_006078
Mouse	AESCDC	TTCL	YQDATAE**EGEFEEE**AEEEVA	NP_033477

Class IVb				
Human	AESCDC	TTCL	YQDATAEE**EGEFEEE**AEEEVA	AAN87335
Mouse	AESCDC	TTCL	YQDATAEE**EGEFEEE**AEEEVA	NP_666228
Chicken	AESCDC	TTCL	YQDATAEE**EGEFEEE**AEEEAE	NP_001074329
Anolis	AESCDC	TTCL	YQDATAE**EGEFEEE**AEEEVA	XP_003216796
Xenopus	AESCDC	TTCL	YQDATAEEEGEFEEGEEEENA	NP_001080566
Class V				
Human	CEHCDC	TTSL	YQDATANDGEEAFEDEEEEIDG	NP_115914
Mouse	CEHCDC	TTSL	YQDATVNDGEEAFEDEDEEEINE	NP_080749
Chicken	CEHCDC	TTSL	YQEATANDGEEAFEDDEEEINE	NP_001026183
Xenopus	CEHCDC	TTSL	YQEATANDEEEAFEEDEEEVNE	NP_00107939
Class VI				
Human	SESCDC	TTSL	FQDAKAVLEEDEEVTEEAEMEPEDKGH	NP_110400
Mouse	SESCDC	TTSL	FQDVRAGLEDSEEDVEEAEVEAEDKDH	XP_619851
Chicken	CESCDC	TTSL	YQDATADVEEYEEAEASPEKET	AAA49120
Anolis	CEGCDC	TTSL	YQDATADVVEYEEVEEEEEVSQEEKEAP	XP_003229040

*The table gives the C-termini and the critical cysteines in β-tubulin isotypes in the four classes of tetrapods. Canonical axonemal sequences are given in boldface and underlined. Corresponding sequences in other isotypes are underlined. *Gekko* = *Gekko japonicas*; *Anolis* = *Anolis carolinensis*, the green anole.
References: (Valenzuela et al., 1981; Sullivan et al., 1986a, 1986b; Wang et al., 1986; Murphy et al., 1987; Monteiro and Cleveland, 1988; Good et al., 1989; Bieker and Yazdani-Buicky, 1992; Ranganathan et al., 1998; Shiina et al., 2001; Crabtree et al., 2001; Banerjee, 2002a, 1986b; Klein et al., 2002; Bhattacharya et al., 2008; Cucchiarelli et al., 2008; Navarro-Nunez et al., 2011; Yang et al., 2011).

Table 2.4 Key portions of the sequences of β-tubulin isotypes of fish*

Species	124–129	237–240	C-Terminal sequence	Accession number
Class I				
Oreochromis	SESCDC	TTCL	YQDATAEE**EGEFEEE**AEEDA	XP_003450643
Oryzias	AESCEC	TTCL	YQDATAEE**EGEFEEE**VEEDA	BAD93273
Danio	SESCDC	TTCL	YQDATAEE**EGEFEEE**AEDDA	AAN33030
Class II				
Danio	SENCDC	TTCL	YQDATADEMGEYEEDDLEDEEDVRH	NP_001019593
Salmo	SENCDC	TTCL	YQDATADEVGEYEEDELEDAD-QDVQQHHDVRH	NP_001167344
Class III				
Salmo	CENCDC	TTCF	YQDATTEEEGEMYEDDEEESESQAR	ACI33858
Class IV				
Oreochromis	AESCDC	TTCL	YQEATAEE**EGEFEEE**GEEDMA	XP_003445489
Salmo	AESCDC	TTCL	YQDATAEE**EGEFEEE**GEEELA	ACN12631
Squalus	AEGCDC	TTCL	YQ----------	ABY48069
Leucoraja	AEGCDC	TTCL	----------	ABY48070
Class V				
Oreochromis	CEHCDC	TTSL	YQEATANDGEENFEDEEDEINE	XP_003452462
Tetraodon†	CEHCDC	TTSL	YQEATANDGEENFQDEEDEI	CAG05304
Class I/IV				
Oreochromis	AESCDC	TTCL	YQDATAE**EGEFEEE**GEEEVA	XP_003450025

*The table gives the C-termini and the critical cysteines in β-tubulin isotypes in fish, both Osteichthyes and Chondrichthyes. Canonical axonemal sequences are given in boldface and underlined. Corresponding sequences in other isotypes are underlined. Dashed line indicates sequence is not known. *Oryzias* = *Oryzias latipes*, the medaka, or ricefish. *Danio* = *Danio rario*, the zebrafish. *Salmo* = *Salmo salar*, the Atlantic salmon. *Oreochromis* = *Oreochromis nilotica*, the Nile tilapia. *Squalus* = *Squalus acanthia*, the spiny dogfish. *Leucoraja* = *Leucoraja erinacaea*, the little skate. *Tetraodon* = *Tetraodon nigroviridis*, the green spotted pufferfish. *Squalus* and *Leucoraja* are Chondrichthyes; the others are Osteichthyes.

†Note: *Tetraodon* has an extra 20 amino acids at its N-terminus.

References: (Oehlmann et al., 2004; Tsukamoto et al., 2005; Leong et al., 2010; Engel and Gallard, 2010; Engel et al., 2010; Huang et al., 2011).

functions. It has long been known that all of the β isotypes are capable of carrying out two of the three canonical functions of MTs, namely, forming the mitotic spindle and the interphase network (Lopata and Cleveland, 1987). However, the strong conservation of the isotype differences implies that they may also have specific individual functions. Several different approaches have been used to elucidate these functions. The development of antibodies specific for the βI, βII, βIII, βIV, and βV isotypes has been very useful (Banerjee et al., 1988, 1990, 1992, 2008; Roach et al., 1998). One limitation is the inability of the βII antibody to distinguish between βIIa and βIIb and of the βIV antibody to discriminate between the βIVa and βIVb isotypes. However, this has been compensated by development of RNA probes capable of distinguishing among all of the β isotypes, which have been used to measure isotype gene expression (Hiser et al., 2006; Lobert et al., 2010). The antibodies serve to measure levels of the isotypes in different cells and localize them in cells and tissues by immunohistochemistry. In addition, the antibodies have been used to purify the αβII, αβIII and αβIV dimers from different sources, mostly bovine brain; these dimers have then been used to measure MT assembly, GTPase activity, conformational stability, MT dynamics, posttranslational modifications, and interactions with MT-associated proteins (MAPs), motor proteins and drugs (Ludueña and Banerjee, 2008b). Finally, the effects of altering the expression of the different isotypes on cell structure and behavior have been examined.

2.1.1. βI Isotype

This isotype has been well-studied in birds and mammals, especially in rodents. βI appears to be found in virtually every tissue, cell and subcellular compartment that has been examined; in other words, if MTs are present, it is almost certain that βI will be part of them (Roach et al., 1998; Leandro-Gárcia et al., 2010). Perhaps the most striking aspect of βI is that there is relatively little evidence for a specialized function. βI occurs in all cilia and flagella that have been examined but it is not the major β-tubulin component of these organelles (Jensen-Smith et al., 2003a). There are, however, three reports that hint at specialized functions for βI. The first is the work of Lezama et al. (2001) who found that MTs enriched for βI are less likely to interact with actin filaments. In this respect, βI differs from the βIV isotype, to be discussed below, which appears to have a particular affinity for actin (Walss-Bass et al., 2001a). The second finding is that βI, together with the α-tubulin isotype, forms a complex with the nucleolar

protein fibrillarin (Yanagida et al., 2004). Fibrillarin is associated with small nucleolar RNAs (Reichow et al., 2007); the significance of its binding to βI is a mystery; it is also not clear whether other β-tubulin isotypes could form this complex as well or whether this is a specific function of the βI isotype. The fact that both fibrillarin and small nucleolar RNAs are found in Archaea (which do not have nucleoli) suggest that the association between fibrillarin and βI may reflect a very ancient linkage with a putative tubulin ancestor (Omer et al., 2000). Finally, Guo et al. (2010) find that silencing βI expression in differentiating human neuroblastoma cells in vitro is lethal. Obviously, this finding speaks to a vital function for βI, such as intracellular transport. However, one could always imagine that the problem is not that βI has a unique function, but that under the specific conditions obtaining in these particular cells during differentiation, the other isotypes present (βII and βIII) could not assume that function at that time. It is significant that knocking down βI expression in undifferentiated cells has no effect on viability, suggesting that under those conditions βII and βIII could pick up the slack, as it were; in short, this experiment does not really reveal a unique function for βI.

2.1.2. βII Isotype

The tissue distribution of the βII isotype is considerably more restricted than that of βI (Tables 2.3 and 2.4); βII is the predominant β isotype in the nervous system, where it is expressed in both neurons and glia (Havercroft and Cleveland, 1984; Burgoyne et al., 1988; Roberson et al., 1992). In glia, βII is particularly abundant in Schwann cells (Roberson et al., 1992). βII is also found in muscles as well as in several other tissues, but at much lower levels (Arai et al., 2002; Dozier et al., 2003; Leandro-García et al., 2010). In comparison with βI, which is found in most cell types in any given tissue, βII may be expressed in fewer cell types. For example, βI is expressed in most layers of the skin but βII only in the stratum granulosum (Roach et al., 1998). In subcellular localization, βII also differs from βI. Where the latter is found in almost every cellular compartment, including axonemes, βII is largely absent from axonemes (except in olfactory cilia) (Woo et al., 2002). In embryos, however, βII is much more widely expressed than in adult tissues (Arai et al., 2002; Nakamura et al., 2003; Jensen-Smith et al., 2003b). In primates, there are two forms of βII: βIIa and βIIb. The latter occurs in the retina, the small intestine, and probably in the brain as well (Crabtree et al., 2001; Leandro-García et al., 2010). At present, there is no hint of a functional difference between βIIa and βIIb.

Further compartmentalization of βII is seen in neurons. Although in many neurons, βII occurs both in the cell bodies and the neurites (Guo et al., 2011), there are clues that point to a specific role for βII in neurite outgrowth. βII expression greatly increases when neurons are stimulated with nerve growth factor (Joshi and Cleveland, 1989), while βII is the major isotype synthesized when nerves are damaged and is the major isotype transported to the site of neuronal regeneration (Hoffman and Cleveland, 1988; Hoffman and Ludueña, 1996). In differentiating human neuroblastoma cells in vitro, βII is apparently localized to the neurites (as opposed to βI and βIII, which occur in both cell bodies and neurites); silencing βII almost totally inhibits differentiation of neurites without significant effects on cell viability (Guo et al., 2010). Finally, when neuronal development is followed in mice, neurons excised and cultured at the time of birth appear to have significant accumulation of βII in the neurites as opposed to the cell bodies (Guo et al., 2011). Not surprisingly, βII appears to be more widespread in developing neurons than in mature neurons (Jiang and Oblinger, 1992). Nor should it be surprising that mutations in βII can cause serious brain malformation (Jaglin et al., 2009; Tischfield et al., 2010).

The mechanism by which βII exercises its probable controlling role in neurite development is unknown. βII most likely is part of a signal transduction pathway whose components are yet to be identified, a pathway which may lead, among other destinations, to the increased synthesis of membrane lipids that necessarily accompanies neurite outgrowth. One possible component of this pathway has been suggested: Kidins/ARMS, a protein that appears to bind to tubulin as well as to various neuronal MAPs (Higuero et al., 2010). Another possible linker is tau, which binds to tubulin and can link to membranes (Abi Farah et al., 2006; Gauthier-Kemper et al., 2011) but the mechanisms by which either protein regulates neurite development are not yet clear.

There is yet more evidence for subcellular compartmentalization of βII. Armas-Portela et al. (1999) found that βII is localized to the perinuclear region, namely, the vicinity of the centrosome, and to the cell cortex in HeLa cells in interphase. Along these lines, in skeletal muscle, which is rich in βII (Yeh and Ludueña, 2004), MTs are nucleated directly on the nuclear envelope rather than on the centrosome (Bugnard et al., 2005). Walss et al. (1999) observed the presence of βII in the nuclei of cultured rat kidney mesangial cells. Many cancer cells also contain βII in their nuclei (Ranganathan et al., 1997; Walss-Bass et al., 2002; Xu and Ludueña, 2002; Yeh and Ludueña, 2004). βII occurs in the nuclei of certain normal cell types including placenta and

pancreatic endocrine cells (Yeh and Ludueña, 2004). Nuclear βII-tubulin has been studied most thoroughly in cultured rat kidney mesangial cells. Nuclear βII is particularly concentrated in the nucleoli and occurs as an αβII dimer, which is capable of binding to colchicine and taxol. Perhaps most significantly, the nuclear αβII dimer does not form MTs but rather a filamentous reticulum (Walss et al., 1999; Walss-Bass et al., 2001; Xu and Ludueña, 2002). There is no nuclear localization sequence in the structure of βII. Microinjected fluorescently labeled αβII dimer has to wait for a cell cycle to enter the nucleus before the nucleus is reassembled after mitosis (Walss-Bass et al., 2001b). It is certainly striking that so many tumor cells contain βII in the nucleus, but it is curious that they express βII at all. Various tumors express βII when the tissues from which they are derived express little or none (Ranganathan et al., 1997; Yeh and Ludueña, 2004; Cucchiarelli et al., 2008).

The findings about nuclear βII are consistent with the observation of Kourmouli et al. (2001) that the nuclear protein heterochromatin protein 1 binds specifically to the αβII dimer. Since heterochromatin protein 1 also binds to the nuclear envelope, Kourmouli et al. (2001) suggest that βII may play a role in the reassembly of the nuclear envelope after mitosis. One could then build on this concept to postulate that if βII indeed plays such a role, then there must be a mechanism in most cells by which βII can exit the nucleus or else be degraded when the nuclear envelope is reassembled after mitosis. This mechanism may be deficient in cancer cells, certain cultured cells and perhaps in placental cells, thereby leaving βII "stuck" in the nucleus during interphase.

Is there a common thread that binds together all these varied observations and speculations about βII—association with neurite outgrowth, transformation, nuclear envelope reformation, centrosomes and the cell cortex? Actually, the common link may not be a thread but a membrane. Every one of these observations places βII close to a membrane (either the nuclear envelope or the cell membrane), even linked indirectly to a membrane (via heterochromatin protein 1) and playing a possible role in major rearrangements of those membranes which certainly accompany neurite outgrowth, transformation and nuclear envelope reassembly. The fact that the nuclear role involves βII in non-MT form raises the possibility that the membrane association is a very ancient one.

2.1.3. βIII Isotype

The β*III isotype* occurs in fish, amphibians, birds and mammals but its tissue distribution is even narrower than that of βII; in fact, it is actually absent

from most tissues. It is abundant in the brain but found only in neurons, unlike βII which occurs in both neurons and glia (Burgoyne et al., 1988). βIII is concentrated in specific regions of the brain in a pattern which varies with development (Liu et al., 2007). βIII is found in neurons of the peripheral nervous system as well (Katsetos et al., 2003a). βIII also occurs in the testis, particularly in Sertoli cells and sperm cells and at lower levels in the vestibular organ, nasal epithelia and the colon (Lewis and Cowan, 1988; Roach et al., 1998; Pětnicová et al., 2001; Perry et al., 2003). βIII differs structurally from the other β isotypes in some potentially significant manners. 1) βIII has a serine at position 239 where the βI, βII and βIV isotypes have a cysteine. Cys239 is a residue that is extremely easy to oxidize (Little and Ludueña, 1985; Bai et al., 1989) and its oxidation blocks MT assembly. 2) βIII has a cysteine at position 124 where the βI, βII and βIV isotypes have a serine. Although the specific properties of cys124 have not been closely examined, it is probably important that it is very close to the highly conserved cys127 and cys129. A cluster of similar topography (i.e. CXXCXC) occurs in von Willebrand's protein and the cysteines in the cluster undergo a disulfide–sulfhydryl interchange when von Willebrand's protein dimerizes during blood coagulation (Mayadas and Wagner, 1992; Dong et al., 1994; Katsumi et al., 2000). Whether such an interchange occurs with βIII tubulin is unknown, but the possibility should be considered. All vertebrate βIIIs that have been sequenced contain cys124 and ser239, so these unusual features are highly conserved. It is particularly striking that outside of the vertebrates, not a single animal β-tubulin has anything other than a cysteine at position 239. Most protists and plants have either cys239 or cys238 or both. Only fungal β-tubulins consistently lack a cysteine residue in this area. Outside of the vertebrates, very few β-tubulins with cys124 have been identified. These comparisons highlight the unusual nature of βIII having both cys124 and ser239. One hypothesis that has been proposed is that the close proximity in the three-dimensional structure of tubulin of cys239 and cys354 (also highly conserved) would allow for formation of a disulfide bridge between these two residues (Bhattacharya and Cabral, 2009). This bridge would be absent in βIII. This could perhaps explain the fact that when assembly occurs in the absence of MAPs, there is a significant delay in nucleation of αβIII that is not observed in αβII or αβIV (Lu and Ludueña, 1994; Banerjee et al., 1992). It is interesting that when the dynamic behavior of MTs formed from any one of these three dimers is measured, MTs made of αβIII are significantly more dynamic, suggesting that αβIII, being more rigid, may be less likely to adapt its conformation to binding adjacent

dimers (Panda et al., 1994). 3) Another unusual feature of βIII occurs at the C-terminus. Normally, this is a highly negatively charged region lacking any basic residues. However, in the case of βIII in amphibians, birds and mammals, the C-terminal residue is actually a lysine. In addition, a few residues in from the lysine is a serine that is phosphorylated (Ludueña et al., 1988). Except for βVI, whose C-terminus is very unusual (Table 2.3), no other vertebrate β-tubulin isotype has a serine in this area. Phosphorylation of this serine in βIII appears to promote binding to MAPs (Khan and Ludueña, 1996). The C-terminal region of βIII in fish lacks both the serine and the lysine residues.

As mentioned above, the αβII, αβIII and αβIV dimers have been purified from bovine cerebra and studied in vitro (Banerjee et al., 1992). In every property examined, the αβIII dimer differs significantly from the other two: αβIII binds less well to colchicine, thiocolchicine, desacetomidocolchicine, 5-(2′,3′,4′-trimethoxyphenyl)-1-methoxytropone, nocodazole, estramustine, and vinblastine (Banerjee and Ludueña, 1992; Banerjee et al., 1994, 1997, 1999; Laing et al., 1997; Xu et al., 2002; Khan and Ludueña, 2003). MTs made from αβIII are most resistant to having their dynamic behavior inhibited by paclitaxel (Derry et al., 1997). The intrinsic (colchicine-induced) GTPase activity of αβIII is the greatest of the three dimers (Banerjee, 1997). Of the three, the conformational stability of αβIII is the greatest. This has been shown directly using differential scanning calorimetry (Schwarz et al., 1998) and indirectly using stop-flow fluorescence to measure the rate at which tubulin conformation shifts when colchicine analogs bind to it (Banerjee et al., 1994, 1997). Another approach used a series of analogs of *N,N′*-polymethylene(bis-iodoacetamide) derivatives of different chain length as molecular calipers; these compounds are able to form two intrachain cross-links in β-tubulin, one between cys239 and cys354 and the other between cys12 and either cys201 or cys211 (Ludueña and Roach, 1981a; Roach and Ludueña, 1984; Little and Ludueña, 1985, 1987). When the different dimers are treated with these derivatives, neither of these cross-links form in αβIII (Sharma and Ludueña, 1994). The lack of the cross-link involving cys239 is not surprising since βIII lacks this residue, but cys12, cys201 and cys211 are all present in βIII; therefore, the fact that the second cross-link does not form indicates that the conformation of αβIII renders at least one of these cysteines inaccessible, or else that at least one of them is engaged in a disulfide bond.

The cellular and tissue distribution of βIII, as well as its structural properties, could give us some clues as to its possible functions. Clearly βIII is

important in the nervous system, where it accounts for 25% of the total β-tubulin in bovine cerebra (Ludueña et al., 1982). This is less than βII, which accounts for 58% of the total β-tubulin (Banerjee et al., 1988); however, since βIII is found only in neurons, as opposed to βII, which occurs in both neurons and glia (Burgoyne et al., 1988), βIII must account for a fraction much >25% of the total β-tubulin in neurons. Estimates in cultured neuroblastoma cells suggest that βII and βIII are expressed in comparable quantities, accounting, respectively, for 41–46% and 26–27% of the total β-tubulin in these cells (Guo et al., 2010).

At first glance, one might deduce that βIII is somewhat redundant with βII in neurons since βIII appears to be regulated similarly to βII. For example, βIII increases after axotomy and, in response to nerve growth factor, can promote neurite outgrowth (Joshi and Cleveland, 1989; Moskowitz et al., 1993; Tucker et al., 2008). However, in almost every case in neuronal cells where βII and βIII are compared, βIII appears to be less important than βII. For instance, the large bolus of βII delivered by slow axonal transport to regenerating sensory axons is not accompanied by a significant increase in βIII (Hoffman and Ludueña, 1996). Similarly, while silencing βII expression can almost totally inhibit neurite outgrowth in differentiating neuroblastoma cells, silencing βIII has only a small inhibitory effect (Guo et al., 2010). Furthermore, although there is evidence suggesting occasional restriction of βII to neurites, such evidence is lacking for βIII with the single apparent exception of βIII but not βII, being seen in the calyx, a specialized nerve ending in the vestibular organ (Perry et al., 2003; Guo et al., 2010, 2011). Finally, the fact that mutations in βIII are less catastrophic in brain development than mutations in βII once again suggests a lesser importance for βIII (Tischfield et al., 2010). In short, in terms of neurite outgrowth, it appears that whatever βIII does, βII does it better, raising the question of what βIII has actually been doing in neurons for over half a billion years.

Certainly, the high dynamic behavior of MTs assembled from the αβIII dimer is potentially functionally significant (Panda et al., 1994). One could argue that this explains why βIII is abundant in a variety of embryonic tissues since cells that are rapidly growing, migrating and changing their shapes may require very dynamic MTs; in subsequent developmental stages, βIII stops being expressed in many tissues (Jiang and Oblinger, 1992; Jensen-Smith et al., 2003b; Dráberová et al., 2008). A similar explanation could account for the abundance of βIII in the invasive margin of colorectal cancer, which must be made of mobile cells containing dynamic MTs (Portyanko et al., 2009). MTs in mature neurons do not seem to be very dynamic, and it is

apparently harmful if they become so (Fanara et al., 2007). In fact, one function of MAPs such as MAP2 and tau, which are very abundant in neurons, is that they inhibit MT dynamics (Gamblin et al., 1996; Panda et al., 2003). Thus, it may be argued that MAP2 and tau evolved to suppress the dynamic behavior of βIII. This argument is supported by the fact that the phosphorylation of the unique serine in the C-terminal region of βIII promotes MAP binding (Khan and Ludueña, 1996). In the brain, this serine is generally fully phosphorylated, so it is not surprising that the dynamic behavior of αβIII is suppressed. This conclusion, however, still begs the question. If αβIII dynamics are necessarily suppressed in neurons, why express βIII at all? Why not replace it with a less dynamic isotype and not worry about phosphorylating it or binding it to MAPs?

Recent work by Jouhilahti et al. (2008) has revealed a potentially novel function for βIII. In certain tumor cell lines and also in normal fibroblasts and keratinocytes, βIII is associated with the mitotic spindle. It appears early in prophase and then disappears at the end of mitosis, about the time that the midbody forms, always associated with α-tubulin. It is likely that the rapid dynamics of MTs made from αβIII could be very useful in mitosis. It would be of great interest if βIII plays such a transient role in many cell types and the subject certainly merits more investigation in normal cell lines.

The evidence is pointing us to a potentially unique function for βIII, based on its lack of cys239, with its easily oxidized sulfhydryl group. The tissues in which βIII is most strongly expressed tend to have high levels of free radicals and reactive oxygen species. For instance, neurons, vestibular cells and Sertoli cells are rich in free radicals such as nitric oxide (NO•) and superoxide anion (O_2^{-}•) (Gally et al., 1990; Bredt and Snyder, 1992; Mruk and Cheng, 2000; Takumida and Anniko, 2000, 2002; Blottner and Luck, 2001; Holstein et al., 2001; Nie and Wang, 2002; Kon et al., 2002; Lee and Cheng, 2003; Mungrue and Bredt, 2004; Cappelletti et al., 2004). Although free radicals and reactive oxygen species are not particularly elevated in the colon and nasal epithelia, one must remember that these two tissues are directly exposed to any free radicals in the food we eat or the air we breathe. Free radicals and reactive oxygen species are very abundant in cancer cells, particularly those that are metastatic and aggressive (Punnonen et al., 1994; Schiff et al., 2000; Portakal et al., 2000; Ray et al., 2000; Brown and Bicknell, 2001). The same is true for βIII, which is especially common in aggressive and drug-resistant tumors with poor prognosis (Matsuzaki et al., 1987; Asai and Remolona, 1989; Scott et al., 1990; Katsetos et al., 1991, 2001, 2002,

2003a, 2003b; Maraziotis et al., 1992; Furuhata et al., 1993; Ranganathan et al., 1996, 1998a,b; Galmarini et al., 2008; Sangrajang et al., 1998; Woulfe, 2000; Dumontet et al., 2002; Hisaoka et al., 2003; Ferrandina et al., 2006; Lee et al., 2007; Sève et al., 2007, 2008; Umezu et al., 2008; Terry et al., 2009; McCarroll et al., 2010).

Most of our cells have MTs in which the βI, βII, or βIV isotypes predominate. Since all these contain the highly reactive cys239, it is reasonable to imagine that even a small amount of oxidative stress could oxidize cys239 and thus interfere with MT assembly and especially with any process requiring rapid MT assembly—mitosis, for example. $O_2^{-}\cdot$ and NO· combine to form peroxynitrite, which reacts with tubulin to form disulfide bridges between the α and β subunits and thus inhibit MT assembly (Beckman et al., 1994; Landino et al., 2002, 2007). Peroxynitrite can also inhibit MT assembly by reacting with tau and MAP2 (Landino et al., 2004c). Cys239 is near the α/β interface (Nogales et al., 1998a) and is quite likely to be part of such a disulfide. Even though many cells use the thioredoxin or glutaredoxin systems, or even ascorbic acid, to reduce these disulfides and restore MT assembly (Landino et al., 2004a, 2004b; 2006), one could argue that the whole problem could be avoided by using β-tubulin that lacks cys239. In short, there is an adaptive reason that could explain why cells with high levels of free radicals and reactive oxygen species also express βIII. In support of this argument, Cumming et al. (2004) showed that stressing a neuronal cell line caused formation of disulfide bridges within the cell, despite the generally accepted rule that cytoplasmic proteins contain few disulfide bonds (Hwang et al., 1992); among the proteins containing these bridges was βII-tubulin. Also, there is considerable evidence that subjecting cells to oxidative stress raises βIII levels by inducing hypoxia-inducible factor, which in turn promotes synthesis of βIII (Moeller and Dewhirst, 2004; Kimbro and Simons, 2006; Griguer et al., 2006; Quintero et al., 2006; Raspaglio et al., 2008).

If βIII protects cells against oxidative stress, then one would expect that decreasing βIII expression would make cells more sensitive to such stress. This appears to be the case. In cultured human neuroblastoma cells, knocking down βIII has only small effects on viability or cell differentiation, but can be lethal when the cells are treated with glutamate and glycine, which promote oxidative stress (Rivot et al., 1999; Guo et al., 2010). Knocking out βIII in tumor cell lines makes them very sensitive to antitumor drugs, not only tubulin-binding drugs such as Vinca alkaloids and paclitaxel—which interact less well with βIII (Derry et al., 1997; Khan and Ludueña, 2003), but also to cisplatin, etoposide and doxorubicin, which are less likely to bind

to tubulin (Gan et al., 2007) (although at high concentrations cisplatin can inhibit MT assembly (Tulub and Stefanov, 2001)). It is probably significant that doxorubicin is a strong promoter of oxidative stress (Eliot et al., 1984; Vásquez-Vivar et al., 1997; Singal and Iliskovic, 1998; Minotti et al., 1999; Tokarska-Schlattner et al., 2006; Cardoso et al., 2008).

In short, a variety of observations suggest that one function of βIII may be to protect cells by forming MTs whose polymerization is less likely to be affected by oxidative stress. Whether βIII can exercise a more general protection against oxidative stress is not clear. The question that naturally arises is whether the cysteine cluster at positions 124, 127 and 129 in βIII could act to reduce adventitious disulfide bonds arising in other proteins. It is conceivable that a disulfide bond in this region might leave MT assembly unaffected, in contrast to a disulfide bond involving cys239 which is incompatible with MT assembly. In this context, it is interesting that Cumming et al. (2004) showed that application of oxidative stress to neuroblastoma cells caused formation of a disulfide bond in βIII. Whether that involved the region of residues 124–129 is unknown.

There is, however, one intriguing finding that may be relevant at this point, a finding that also speaks to a non-MT function for βIII. Mitochondria have long been known to associate closely with MTs (Ball and Singer, 1982; Bernier-Valentin and Rousset, 1982; Stephens, 1986) but it appears that the free tubulin dimer may play a major role in these organelles (Sheldon et al., 2011). The αβIII dimer is enriched about threefold in the outer mitochondrial membranes of certain tumor cells (Carré et al., 2002). The αβII dimer is present there as well, but is only half as enriched. Since mitochondria are major producers of reactive oxygen species and free radicals, it is easy to imagine βIII being the "guardian of the gate" and protecting the cell against these toxic chemicals. However, it is thought that one of the roles of the mitochondrial tubulin dimer is to inhibit the voltage-dependent anion channel (VDAC) (Rostovtseva et al., 2008). In cardiac cells, this role is played by αβII (Guzun et al., 2011). It is probably significant that VDAC is associated with mitochondrial creatine kinase (Saks et al., 1993; Schlattner et al., 2005), an enzyme specifically inhibited by doxorubicin, a drug which, as mentioned above, becomes more lethal to cells when βIII is knocked down (Tokarska-Schlattner et al., 2006). In fact, VDAC, creatine kinase, tubulin and ATP synthase have been proposed to be a "mitochondrial interactosome," playing a major role in regulation of respiration (Saks et al., 1995, 2010; Appaix et al., 2003; Lemasters and Holmuhamedov, 2006; Rostovtseva and Bezrukov, 2008). Cicchillitti et al. (2008) showed that mitochondrial βIII

is bound noncovalently to a large number of proteins, including α-tubulin, and is specifically linked by disulfide bonds to glutathione transferase μ4, but, interestingly, not to α-tubulin. One could perhaps argue that any tubulin dimer in the mitochondrial membrane could inhibit VDAC but that enriching this area with αβIII conveys the additional benefit of protecting the cells from free radicals and reactive oxygen species, and that in order to do this, βIII must interact with proteins involved in repair of damage caused by oxidative stress. This is an argument analogous to the one advanced earlier, namely, that cells could use any tubulin isotype for MT assembly but that cells exposed to oxidative stress would favor βIII because of its protective effect.

2.1.4. βIV Isotype

The β*IV isotype* appears to have a very clear function. Without exception, in every cilium or flagellum that has been examined, the predominant β isotype is βIV; these include retinal rods, sperm flagella and the cilia of trachea, oviduct, vestibular hair cells, olfactory neurons, brain ependyma and the efferent duct of the testis (Renthal et al., 1993; Lu et al., 1998; Woo et al., 2002; Perry et al., 2003; Jensen-Smith et al., 2003a; Daniely et al., 2004). The association of βIV with axonemal MTs is not surprising. Raff et al. (1997), after surveying all the then known β-tubulin sequences, proposed that for a β-tubulin to localize to axonemes, it needed to have near the C-terminus, the sequence EGEFXXX (where X is D or E). Of the vertebrate isotypes, only βIV has such a sequence: EGEFEEE. In mammals, βIV has speciated into βIVa and βIVb (Lewis et al., 1985). Although both these contain EGEFEEE, βIVa is found only in the brain while βIVb is widespread. A survey using a monoclonal antibody that could not distinguish between βIVa and βIVb found βIV expressed in many tissues, being particularly concentrated in ciliated cells (Roach et al., 1998). Another survey using RNA probes that could distinguish between the mRNA's of the two isotypes found βIVa located only in the brain while βIVb was particularly concentrated in the testis, heart and skeletal muscle (Leandro-García et al., 2010). In the brain, βIV is located both in glia and neurons; one study found it in oligodendrocytes (Wu et al., 2009).

Further work by Raff et al. indicated that EGEFXXX was not absolutely required for incorporation into axonemes; however, the "signal sequence" had to be similar and the phenylalanine in the middle was definitely required (Popodi et al., 2008). It is interesting that the "signal sequences" in the βI and βV isotypes are similar to EGEFXXX and are the only ones of the

vertebrate β isotypes to have F in the middle of the sequence; predictably, βI and βV are the only isotypes besides βIV to occur in axonemes.

Is the sequence EGEFEEE just a signal determining axonemal localization or does it somehow contribute to axonemal function? As we shall see later, it is probably both. Does the rest of the βIV molecule play a role in axonemal function? There is no direct answer to this question in vertebrates. However, an elegant experiment performed by Hoyle et al. (1995) in *Drosophila* suggests that the rest of the molecule is important. *Drosophila* has its own set of β isotypes. When the EGEFEEE of the axonemal isotype is placed on another isotype, the latter localizes to the flagella but the outer doublet MTs do not assemble properly. The conclusion from this experiment is that the rest of the β isotype molecule does indeed play a role in axonemal function but the nature of that role is not known.

It is clear that the distinction between βIVa and βIVb has been conserved in mammals at least since the rodent and primate lines diverged about 75–84 million years ago (Murphy et al., 2001; Dawkins, 2004); this could imply some functional difference between the two; the nature of this difference, if any, is unknown. There is one possible clue, however, suggesting that such a difference may exist. Gan and Kavallaris (2008), using lung cancer cell lines, showed that knocking down βIVb had no effect on resistance to taxanes but significantly promoted apoptosis. At first glance, this may seem surprising since taxane resistance is often accompanied by elevated βIV and the αβIV dimer is almost as insensitive to taxol as is αβIII (Derry et al., 1997; Makarovsky et al., 2002; Galmarini et al., 2003). However, when one of the same cell lines was examined by Nicoletti et al. (2001), the mRNA for βIVb accounted for only 0.2% of the total mRNA's for β-tubulin isotypes. A knockdown of βIVb, therefore, should have no effect on taxane resistance; the observation that such a presumably tiny quantity of a single isotype regulates apoptosis suggests a possible unique function for βIVb. In contrast, Yang and Cabral (2007), using CHO cells, showed that transfection of small quantities of βIVa caused increased sensitivity to taxanes. In their experiments, βIVa accounted for 18–89% of the total β-tubulin. Cell morphology and MT organization appeared normal even when βIVa was 71% of the total β-tubulin, but 89% βIVa strongly interfered with mitosis. Yang and Cabral (2007) did not address the possibility of regulation of apoptosis, but they showed that mutagenizing Ala115 in βIVa to Ser115, its equivalent in βIVb, abolished the supersensitivity to taxanes. Analogous mutations at other positions affected sensitivity to colcemid. All these positions are involved in lateral dimer–dimer interactions in MTs. These observations

suggest that βIVa and βIVb could have some functional difference; the most one could speculate at this time is that βIVa might have a greater propensity to form stable MTs, something that could be adaptive in neurons.

The αβIV dimer has been purified from bovine brain and studied in vitro. The study of Leandro-García et al. (2010) suggests that there is much more βIVa in the brain than there is βIVb; therefore, it is likely that the experiments performed in vitro with purified αβIV dimer from the brain were largely done with αβIVa. Its properties are generally different from those of αβIII and very similar to those of αβII with a few possibly significant exceptions. It binds with greater affinity to nocodazole, colchicine and colchicine analogs than do either αβII or αβIII (Banerjee and Ludueña, 1992; Banerjee et al., 1994, 1997; Xu et al., 2002). Detailed analysis of the binding of the colchicine analogs using stop-flow fluorescence indicates that the key difference is that the rate of the conformational change in tubulin that ensues upon binding of the drug is significantly faster for αβIV than for αβII (and far more rapid than for αβIII) (Banerjee et al., 1994, 1997). One implication of these results is that the conformation of αβIV is less rigid than those of the other two dimers.

The cross-linking experiments described above also implied an unusually flexible conformation for αβIV. As mentioned above, a series of analogs of *N,N'*-polymethylene bis(iodoacetamide), of general formula $ICH_2CONH(CH_2)_nNHCOCH_2I$, were used to form an intrachain cross-link in β-tubulin between cys12 and either cys201 or cys211. The amount of the cross-linked product is easily measured by its unique electrophoretic mobility (Laemmli, 1970; Roach and Ludueña, 1984). When αβII is treated with these derivatives, the major yield of cross-linked product is obtained using the $n = 2$ derivative with a smaller yield obtained with the $n = 4$ derivative and even less with the others. However, when αβIV was treated with them, although the major yield was still obtained with the $n = 2$ derivative, substantial yields were obtained with all the other derivatives ($n = 3, 4, 5, 6, 7, 10$). In other words, for αβII, the derivative needed to have a precise geometry to generate a substantial amount of the cross-link, while for αβIV, this was not so important. The simplest explanation for this is that αβIV has a less rigid conformation than does αβII. One could postulate an adaptive advantage for this property of βIV: if it has to form part of a structure (the axoneme) whose MTs frequently have to bend, then it would help to have these MTs made of a more flexible kind of tubulin.

The αβIV dimer differs from the other dimers in its interaction with MAPs. Where both αβII and αβIII assemble very well with either tau or

MAP2, αβIV assembles to only half the extent (Banerjee et al., 1992). One explanation might be a more rapid decay of the tubulin arising from a less rigid conformation. Another may simply be that both αβII and αβIII are largely concentrated in the nervous system, as are tau and MAP2 (Binder et al., 1985; Garner and Matus, 1988); therefore, the βII and βIII isotypes could have coevolved with tau and MAP2 to have a particularly strong interaction with these proteins. In contrast, βIV is a relatively minor component of brain tubulin (accounting for 14% of the total β-tubulin) (Banerjee et al., 1992) and it is widespread in other tissues, so it may not have evolved to bind well to tau and MAP2. Conceivably, it might bind very well to MAPs that are also widespread in tissues, such as MAP4 (Parysek et al., 1984), although this has not been tested.

One final property of αβIV is that in rat kidney mesangial cells that have been treated to depolymerize their MTs, the βIV co-localizes with actin stress filaments (Walss-Bass et al., 2001a). A small amount of co-localization between actin and βIV was seen in these cells as well. No such co-localization occurred with either βI or βII. In short, it appears that βIV may have a unique ability to interact with actin filaments, although whether it binds directly to actin is unknown. The high level of βIV in cardiac muscle cells is consistent with this idea (Guzun et al., 2011). It is interesting that in this respect, βI and βIV appear to have contrasting roles, βIV promoting and βI inhibiting actin binding (Lezama et al., 2001). It is also interesting that the clearest evidence for the interaction between βIV and actin involves the αβIV dimer and not the intact MT. In view of the evidence that αβII and αβIII appear to have functions that involve them acting in non-MT form, it would not be too surprising if αβIV can also function without being part of a MT.

2.1.5. βV Isotype

The last two isotypes in our collection—βV and βVI—are somewhat odd. However, while βVI confines its oddity to only a few cell types, βV is widespread in tissues. βV is found in fish, amphibians, birds and mammals (Ludueña, 1998). Studies have shown that the βV isotype is expressed in most human tissues, including the skin, skeletal, smooth and cardiac muscles, endothelial cells, bile duct, lymph nodes, spleen, breast, islets of Langerhans, testis, tonsil, placenta, prostate, and fetal liver, but is virtually absent from the brain, thymus, bone marrow, and leukocytes (Leandro-García et al., 2010; Chao et al., 2012). In no tissue is βV the major β-tubulin isotype; the expression of βV, relative to the other β isotypes, is greatest in the lung. Similar but not identical results were obtained in an earlier study, in which

βV was found to be expressed in chicken chondrocytes, skeletal muscle, smooth muscle, glia, lymphocytes, testis, intestine, spleen, bursa and thymus, but not in the brain, hepatocytes, or spinal cord neurons (Sullivan et al., 1986). The differences in expression may reflect nothing more than the rodent/primate divergence of 75–84 million years ago or the mammal/bird divergence of 310 million years ago (Kumar and Hedges, 1998; Murphy et al., 2001; Dawkins, 2004), or there could be some functional significance—we do not know. More recently, Guo et al. (2011) found what appears to be a transient expression of βV in the neurons excised from the brains of newborn mice; they found no evidence for βV in neurons from the brains of either embryonic or adult mice. This could conceivably be correlated with an increase in production of reactive oxygen species in the neurons of newborn rats (Tsatmali et al., 2005).

What makes βV unusual is that silencing its expression in CHO cells causes apoptosis or alters mitosis, while overexpressing it in the same cells almost completely disrupts the MT cytoskeleton (Bhattacharya et al., 2008; Bhattacharya and Cabral, 2009). How can these apparently contradictory results be reconciled? There is no doubt that βV can form MTs (Lopata and Cleveland, 1987); perhaps, however, its conformation differs from other tubulin dimers in such a way that having too much βV can disrupt MTs while having a small amount might be acceptable (Bhattacharya and Cabral, 2009). In this regard, we should recall that in no tissue does βV appear to account for more than 12–13% of the β-tubulin expressed (Leandro-García et al., 2010). Thus, we can postulate either that a certain amount of βV, when combined with other β-isotypes in a MT, provides dynamic and other properties that are suited to mitosis or that βV has a specific interaction with some other protein and that this interaction is necessary for mitosis to occur (Bhattacharya et al., 2008).

βV has been found in bovine tracheal cilia and treatment of these cilia with an antibody to the C-terminal region of βV can prevent ciliary beating (Vent et al., 2005). This finding suggests that βV can play a role in axonemal MTs even though it lacks the EGEFXXX signal sequence. Preliminary results indicate that βV is largely in the central pair MTs in the axoneme, while βIV is mostly in the outer doublet MTs (Dossou and Hallworth, 2010).

βV has certain similarities to βIII, which it somewhat resembles in overall sequence. βV lacks the serine present in the C-terminal region of βIII. However, both proteins have ser239 and cys124. It is interesting that overexpressing βIII can also inhibit MT assembly (Hari et al., 2003), but much

less than overexpressing βV. Bhattacharya and Cabral (2009) have found that mutating ser239 in βV to cys239 abolishes the MT-disrupting ability of βV. As mentioned above, βI, βII, βIVa and βIVb have cys239; Bhattacharya and Cabral (2009) hypothesize, based on the proximity of cys239 to cys354 in the three-dimensional structures of these proteins, that the two residues form a disulfide bond and that this bond can stabilize the conformation of the protein and lead to more stable MTs.

One other resemblance between βV and βIII is that both are overexpressed in a variety of tumors; interestingly, these are often not the same tumors (Cucchiarelli et al., 2008; Leandro-García et al., 2010). There is also evidence that while high levels of βIII are associated with poor outcome, the opposite is true for βV (Christoph et al., 2012). Likewise, it is striking that βV may be almost absent from the brain but present in many other tissues, while βIII is abundant in the brain and rare elsewhere. It may very well be that the common thread tying together these disparate observations is that βV and βIII may actually share a function, conceivably one of the protecting cells from oxidative stress, and that, to benefit from that function, a cell only needs to express one of them.

2.1.6. βVI Isotype

The βVI isotype is expressed mainly in hematopoietic cells and has been studied in chicken erythrocytes and mammalian spleen, platelets and megakaryocytes (Wang et al., 1986; Murphy et al., 1987). It is also expressed in leukocytes, bone marrow and fetal liver as well as some tumors, but it is not a major constituent of the latter; in leukocytes, βVI constitutes the major β isotype expressed (Leandro-García et al., 2010). βVI is quite different in sequence from the other isotypes and the differences are not particularly conserved in evolution, to the point where it is not clear that avian and mammalian βVI should really constitute a single β-tubulin isotype class. In chicken, βVI is the major constituent of the marginal band of MTs, a set of MTs forming a circle at the edge of the erythrocyte (Murphy and Wallis, 1983); there is evidence that the number of MTs in the marginal band controls the size of the erythrocyte (Gontakowska-Witalińska and Witaliński, 1976). Mammalian erythrocytes do not have MTs; βVI, however, forms the marginal band in platelets. Although previously thought to consist of a single MT wound around itself several times (Italiano et al., 2003), the marginal band of platelets has recently been shown to consist of several MTs, one of which is a long, stable MT while the others are shorter and very dynamic (Patel-Hett et al., 2008). βVI has a unique propensity to form these circular

MTs; in platelets, 95% of βVI is in the marginal band, while only 58% of βI and 45% of βII are in these structures (Schwer et al., 2001). Furthermore, silencing βVI results in platelets whose marginal bands are formed from other β isotypes; these platelets are spherical and not discoid (Schwer et al., 2001). Such alterations in platelet shape can cause a bleeding disorder (White and De Alarcon, 2002). It is interesting that βVI, when transfected into CHO cells, will form a marginal band-like set of MT fragments (Yang et al., 2011).

βVI has some unusual features in its sequence and also some similarities with βIII and βV. Like βIII and βV, βVI has ser239 instead of the easily oxidized cys239. Also, chicken βVI has the unusual cys124, while mammalian βVI does not. In addition, βVI has two extra cysteines, not present in the other isotypes: cys37 and cys315. It is likely that one of these cysteines forms part of the disulfide bond that has been observed in platelet tubulin (Ikeda and Steiner, 1978). Lastly, the C-terminus of βVI, although overall negatively charged, contains a lysine residue, as is also true for βIII.

βVI transfected into CHO cells incorporates into MTs of the interphase network but once mitosis begins, the presence of βVI disrupts the mitotic spindle (Yang et al., 2011). From this observation, one might speculate that βVI can fit into MT of the interphase network which can be somewhat curved, but has trouble fitting into MTs of the mitotic spindle which tend to be straighter. This speculation is corroborated by the tremendous formation of spiral aggregates that occurs when chicken erythrocyte tubulin is treated with vinblastine, far beyond that which occurs with chicken brain tubulin (Ludueña et al., 1985). Vinblastine is known to cause tubulin to polymerize into spiral protofilaments (Erickson, 1975). βVI could fit much better into these curved protofilaments. Hence, it is not surprising that its normal function is to form circular MTs. In addition, βVI suppresses MT dynamics and nucleation (Yang et al., 2011), all of which is consistent with an isotype destined to form a stable MT wound around itself several times. It is likely that while the stable MT of the marginal band contains largely βVI, the other more dynamic MTs in it may be enriched for other β isotypes.

2.2. Evolution of Vertebrate β-Tubulin Isotypes

Figure 2.1 shows our current understanding of the relationships among the vertebrate β-tubulin isotypes correlated with the different stages of vertebrate evolution. The scheme shown in Fig. 2.1 is actually less complex than the one previously advanced (Ludueña and Banerjee, 2008b). Before there

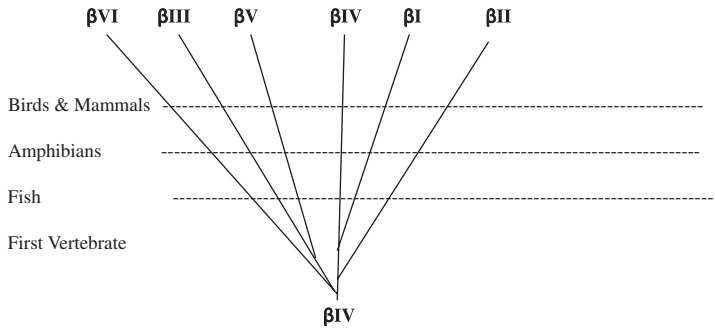


Figure 2.1 ***Relationships among vertebrate β-tubulins.***

was no evidence of βI or βV in fish and hence, it was argued that these isotypes originated later than βII, βIII and βIV; now, we know that both βI and βV occur in fish and hence, we have to postulate that all the isotypes except for βVI appeared at roughly the same time. The possible functions of each isotype are summarized in Table 2.5.

2.2.1. Similarity between βIV and the Ancestral Vertebrate β-Tubulin Isotype

There are four reasons why it is likely that when the first vertebrates appeared, they had at least one β-tubulin isotype, and that this was βIV.

1) βIV is the only vertebrate β-tubulin isotype that contains in its C-terminal region, the sequence EGEFXXX (where X is either D or E). This sequence has been shown to be required for incorporation of a β-tubulin isotype into axonemal MTs, most likely into the unique and complex outer doublet MTs (Raff et al., 1997; Dossou and Hallworth, 2010). Every vertebrate axoneme that has been examined contains βIV. Because axonemes are widespread in animals, protists and some plants, it is very reasonable to propose that the first vertebrate contained cilia or flagella and that the β-tubulin used to form them itself had the signal sequence. To postulate that some other isotype could have been the ancestral vertebrate β-isotype would require the assumption that axonemes temporarily disappeared from the ancestral vertebrate and that both axonemes and the signal sequence then reappeared. This seems unnecessarily cumbersome. It is much simpler to postulate that the earliest vertebrate contained a β-isotype that had the signal sequence and that the other isotypes may have evolved from it. Thus, is it likely that βIV is the closest to the original β isotype in vertebrates.

Table 2.5 Possible functions of vertebrate β-tubulin isotypes

Isotype	State	Interphase	Spindle	Axoneme	Other
βI	Microtubule	√	√	√	Cell viability, inhibits actin binding Forms outer doublet MTs
	Dimer				Binding to fibrillarin?
βII	Microtubule	√	√		Neurite formation
	Dimer/ Network				Nuclear envelope reassembly
βIII	Microtubule	√	√		Very dynamic; protects MTs from ROS
	Dimer				Outer mitochondrial membrane, Protection of cell?
βIV	Microtubule	√	√	√	Forms axonemal microtubules (outer doublets)
	Dimer				Binds to actin
βV	Microtubule	√	√	√	Forms axonemal microtubules (central pair?)
	Dimer				Disrupts microtubules
βVI	Microtubule				Forms curved microtubules in the marginal band of platelets and erythrocytes

2) The animals from which the vertebrates evolved, namely the invertebrate chordates, appear to express multiple β isotypes, almost all of which contain the EGEFXXX sequence (Table 2.6). The hemichordate *Saccoglossus kowalevskii*, from which the vertebrates may have diverged about 530 million years ago (Shu et al., 1999; Takezaki et al., 2003; Aris-Brosou and Yang, 2003; Dawkins, 2004), appears to express up to 10 β-tubulin isotypes, all of which have the EGEFXXX signal sequence in their C-terminal region (Freeman et al., 2008). (Ayala et al. (1998) put the chordate divergence earlier, at 600 million years ago; if this is correct, the other dates that would follow would have to be adjusted accordingly.) Moving further afield, the cephalochordate *Branchiostoma* (formerly called *Amphioxus*) appears to express three β isotypes, and again all of them contain EGEFEEE. The cephalochordates may have diverged about 560 million years ago (Winchell et al., 2002; Dawkins, 2004). The urochordates, possibly diverging about 565 million years ago

Table 2.6 Key portions of the sequences of β-tubulin isotypes of close relatives of the vertebrates*

Phylum/Species	124–129	237–240	C-Terminal sequence	Accession number
Hemichordata				
Saccoglossus	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGEDEEAA	NP_001171814
	AESCDC	TTCL	YQDATAEE**EGEFEEE**EGEDAEEA	NP_001171743
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGEDEEAA	NP_001171819
	SESCDC	TTCL	YQDATAEE**EGEFDEE**EGEAEEEN	XP_002741583
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGDDEEAA	XP_002741272
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGEVEDD	XP_002741274
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGEVEDD	XP_002741273
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EAEEDEA	XP_002741271
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EEGEDEAE	XP_002741270
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGEDDEAA	XP_002735335
Cephalochordata				
Branchiostoma	SESCDC	TTCL	YQDATAEE**EGEFDEE**EGEEDME	XP_002608917
	SESCDC	TTCL	YQDATAEE**EGEFDEE**EGEEDME	EEN64927
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EEEGDELA	XP_002609500
	SESCDS	TTCL	YQDATAEE**EGEFDEE**EGEEDME	XP_002602733
Urochordata				
Halocynthia	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGEDEEV	BAA22382
	AEGCDC	TTCL	YQDATAEE**EGEFDEE**EEDENA	BAA22381
Ciona	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGEDEEA	XP_002130315

Oikopleura	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGEDA	CBY15537
	AESCDC	TTCL	YQEATADDEGEYDEDEEELE	CBY12859
	AESCDC	TTCL	YQEATAED**EGEFDEE**EEEEEA	CBY12609
	AENCDC	TTCL	QQYQEAGIEEEEEGFEEEED	CBY24440
	AESCDC	TTCL	YQEATAED**EGEFDEE**GEEEYEEA	CBY08384
	AESCDC	TTCL	YQEATAED**EGEFDEE**EEEEEA	CBY07085
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EGEDA	CBY40309
Echinodermata				
Strongylocentrotus	AESCDC	TTCL	YQDATAEE**EGEFDEE**EEDGHEQ	XP_001192190
	AEGCDC	TASL	YQDATIDD**EGEFEEE**EGEVDPEEA	XP_001197414
	AESCDC	TTCL	YQDATAEE**EGEFDEE**EEEEEEP	XP_001201898
	SEGCDC	TTCL	YQDATVEDEGDFDEEDPEEEEDEEAA	XP_001197584

*The table gives the C-termini and the critical cysteines in β-tubulin isotypes in the other Chordate sub-phyla, the Hemichordata, Cephalochordata, and Urochordata, as well as the Echinodermata, the major phylum that is most closely related to the Chordates (Dawkins, 2004). Canonical axonemal sequences are given in boldface and underlined. Corresponding sequences in other isotypes are underlined. *Saccoglossus* = *Saccoglossus kowalevskii*, the acorn worm. *Branchiostoma* = *Branchiostoma floridae*, the Florida lancelet. *Halocynthia* = *Halocynthia roretzi*, the Korean common sea squirt. *Ciona* = *Ciona intestinalis*, the transparent sea squirt. *Oikopleura* = *Oikopleura dioica*, an appendicularian, free-living sea squirt. *Strongylocentrotus* = *Strongylocentrotus purpuratus*, the purple sea urchin.
References: (Miya and Satoh, 1997; Freeman et al., 2008; Putnam et al., 2008; Denoeud et al., 2010).

(Winchell et al., 2002; Dawkins, 2004), have been studied a little more thoroughly. *Halocynthia roretzi* and *Ciona intestinalis* each express two β isotypes, all of them containing EGEFXXX (Miya and Satoh, 1997). Six β isotypes have been predicted in *Oikopleura dioica*; of these, four have EGEFEEE and one has the very similar EGEYDED. The sixth is somewhat different: the sequence in the corresponding area is EEGFEEE (Denoeud et al., 2010). The sixth one has substantial differences elsewhere in its sequence, raising the possibility that it could be a pseudogene. Finally, all the known β-tubulin isotype sequences from echinoderms, diverging about 570 million years ago (Wada and Satoh, 1994; Bromham and Degnan, 1999; Furlong and Holland, 2002; Bourlat et al., 2003; Dawkins, 2004), contain the EGEFXXX sequence or something very similar. In contrast, among the protostomes and other animals, from which the deuterostomes diverged about 590 million years ago (Ayala et al., 1998; Giribet, 2002; Valentine, 2002; Aris-Brosou and Yang, 2003; Dawkins, 2004), the EGEFXXX sequence appears to be present in only a minority of β isotypes (Table 2.7). In short, the message is clear: the vertebrates evolved from a set of animals, most of whose β-tubulin isotypes contained the signal sequence that presently is only found in βIV.

3) The axonemal signal sequence EGEFXXX is widespread among fish isotypes. For example, *Oreochromis niloticus*, the Nile tilapia, appears to express seven isotypes, of which six have EGEFEEE. It is interesting that the single exception appears to be similar to βV. The sequence of its signal region is EENFEDE, not very far removed from EGEFXXX. One can readily imagine this proto-βV evolving from βIV.

4) Several fish β-tubulin sequences have been identified as βI and some as roughly equally related to βI and βIV. All these contain the EGEFXXX sequence, making it very plausible that βI at least evolved from βIV.

2.2.2. Evolution of Other β-Tubulin Isotypes in Vertebrates

When and how could the other β-tubulin isotypes have arisen? A large number of tubulins from bony fish (Osteichthyes) have been sequenced. What would be most helpful at this point would be complete sequences from the cartilaginous fish (Chondrichthyes), which diverged from the other vertebrates about 460 million years ago (Dawkins, 2004). However, only two partial sequences are available from the Chondrichthyes; these appear to be related to βIV (Engel and Gallard, 2010; Engel et al., 2010). It is a pity that these fragments did not include the region of the signal sequence. The analysis of these fragments indicates that they are both much more

Table 2.7 Key portions of the sequences of β-tubulin isotypes of other animals*

Phylum/species	124–129	237–240	C-terminal sequence	Accession number
Mollusca				
Octopus	CEGCEC	TTCL	YQEARSTDSDEYDNEEYYNQQEE	AAA116611
Arthropoda				
Drosophila	SEGCDC	TTCL	YQEATADE**EGEFDED**EEGGGDE	AAF54373
	AESCDC	TTCL	YQEATADEDAEFEEEQEAEVDEN	AAN16132
	CENCDC	TTCL	YQEATADDEFDPEVNQEEVEGDCI	AAF47193
	SEGCDC	TTCL	YQEATADDEVEFDDEQAEQEGYESEVLQNGNGE	ACZ95047
Annelida				
Mesenchytraeus	AESCDC	TTCL	YQEATAEE**EGEFEEE**EGEAA	ABO28786
Nematoda				
Caenorhabditis	AESTDC	TTCL	YQEAAADEDAAEAFDGE	CAA33320
Platyhelminthes				
Echinococcus	CEACDC	TTSL	YQEVGIDDDYGEEEAAPEE	CAB91642
	AESCDC	TTCL	YQDATAED**EGEFDED**EEVEEA	CAB91641
	CESCDC	TTCL	YQEAGIGDDEEEDEEGVMGEEIDA	CAB91640
Cnidaria				
Scleronephthya	SEGCDC	TTCL	YQEATAEE**EGEFEEE**EEEEEA	AAU14270
Porifera				
Suberites	AESCDC	TTCL	YQDATAEE**EGEFDEE**EEEEAEA	CAD79598
Amphimedon	AESCDC	TTCL	YQEATADDEAEFDEEEEEEAA	XP_003383793
Mesozoa				
Dicyema	AESCDC	TTCL	YQDAAADEEGEMGEEEDEEAA	BAF46879

*The table gives the C-termini and the critical cysteines in β-tubulin isotypes in selected other animal phyla. Canonical axonemal sequences are given in boldface and underlined. Corresponding sequences in other isotypes are underlined.
References: (Tomarev et al., 1993; Adams et al., 2000; Brehm et al., 2000; Schroeder et al., 2004; Yum and Woo, 2004; Ogino et al., 2007; Tartaglia and Shain, 2008).

similar to βIV than to βII. However, these fragments were both prepared from testis, a tissue one would expect to be rich in βIV and lack βII, so we cannot draw firm conclusions as yet. Fortunately, it is possible to detect the presence of βIII in vertebrates because when tubulin is reduced and carboxymethylated and analyzed by polyacrylamide gel electrophoresis in the system of Laemmli (1970), βIII migrates more slowly than do the other β isotypes (Little, 1979). Using this approach, βIII has been found in brains of various bony and cartilaginous fish, but not in fish eggs (Ludueña et al., 1982; Detrich et al., 1987). There is no sign of any isotype resembling βIII in the hemichordates, cephalochordates or urochordates; hence, we must postulate that βIII appeared sometime between 530 and 460 million years ago.

The recent discovery of βV in fish indicates that both βIII and βV were present at least 460 million years ago. As is the case with avian and mammalian βV, fish βV has cys124, ser239 and a C-terminal region very similar to other βVs. It is also conceivable that βV evolved earlier. Given the resemblance between βV and βIII, it is possible that, when reduced and carboxymethylated, βV might co-migrate with βIII on polyacrylamide gel electrophoresis. In other words, it is conceivable that the band previously identified as βIII in shark brain tubulin (Ludueña et al., 1982) might actually be βV. At present, there is no way of knowing. In short, it is possible that βIII and βV appeared as far back as 530 million years ago. In fact, if they both evolved from βIV, βV may even have evolved first since it contains a sequence very similar to that of the "signal" sequence of βIV. The discussion above at least raises the possibility that βIII may play an important role in protecting MTs and perhaps the whole cell against oxygen toxicity. Given that βV and βIII have the same distribution of cysteines, it is possible that βV could have played a similar role, and perhaps still does. In light of this possibility, it is possible that βIII and/or βV appeared not long after a significant jump in O_2 levels in the Earth's atmosphere to at least 18% of present levels, an event dated by various analyses to 0.54–1.05 billion years ago (Cloud, 1988; Canfield and Teske, 1996; Kah et al., 2004; Holland, 2006; Frei et al., 2009). The increased oxygen levels may be reflected in the appearance in the fossil record of bones, shells and carapaces, all of which are associated with collagen whose biosynthesis requires molecular oxygen (Towe, 1970; Gorres and Raines, 2010).

The βII isotype probably appeared early as well. Not enough is known about the β-tubulins of sharks and their relatives, but it is likely that βII is expressed in these animals and, hence, may have evolved from βIV about 530 million years ago. The putative ability of βII to link to membranes may

have been adaptive to the purpose of generating highly asymmetric cells such as neurons. Of course, many invertebrates have neurons and do not appear to need a particular β isotype for their generation, so this is at best a speculation. There is evidence for βI in fish, so it likely appeared at least 460 million years ago. βVI is a bit of a mystery. Although certain fish sequences have been classified as βVI, it is not clear that they bear a particularly close relationship in sequence to reptilian, avian or mammalian βVI, whose relationship with each other is also unclear. In short, it is possible that the sequences categorized as βVI may have evolved separately from each other or else from an ancestor present in the earliest vertebrates. It is even possible that an ancestral βVI, distinct from βIV, could have been present before the vertebrates appeared. There is no evidence for that, however.

Interestingly, actin has a story analogous to that of the vertebrate β-tubulin isotypes. Like tubulin, actin polymerizes, but into filaments instead of MTs. In birds and mammals, actin exists as six isotypes, whose functions appear to be distinct from each other, and only rarely can one actin isotype replace another (Perrin and Ervasti, 2010). Actin appears to exist in nuclei in monomeric form, where its functions are as yet unclear (Skarp and Vertiainen, 2010). As was suggested for the βIII and βV isotypes of tubulin, actin may also play a role in protecting cells from oxidative stress (Farah et al., 2011).

2.2.3. Gene Duplication Event

Obviously, the tubulin isotypes of the higher vertebrates are very different from their ancestor, with only the βIV isotype containing the signal sequence. The complexity of the isotypes in amphibians, birds and mammals likely arose early in vertebrate history and reflects the well-known genomic complexity that separates vertebrates from invertebrates (Dehal and Boore, 2005). If the whole genome duplicated up to two times in the first vertebrate, there was certainly an opportunity for the tubulin genes to duplicate. If the ancestral vertebrate β-tubulins were multifunctional, and if further functions were acquired, then the selective pressure to separate some of the functions by evolving even more isotypes may have been enormous. A fundamental feature of evolution is the concept that gene duplication followed by mutation of the resulting genes could lead to the appearance of novel functions (Ohno, 1973). A potential problem with that idea, however, is that selection might disfavor any mutation that compromises the function of the original protein. However, more recent thinking postulates that first, a single gene encodes a protein to perform a function; then, the protein begins to perform multiple functions. If that gene then duplicates, the

resulting genes might diverge by acquiring mutations, where mutations in one gene might favor one function and mutations in the other would favor the second function (Piatigorsky and Wistow, 1991; Hughes and Hughes, 1993; Hughes, 1994). This process very likely explains the divergence of β-tubulin isotypes in vertebrates. In other words, the admittedly speculative functions involving tubulin in non-MT form shown in Table 2.5 may not be recently acquired functions but very ancient ones distributed among more recently evolved isotypes. We shall apply this reasoning later to the evolution of the tubulin superfamily.

2.3. Vertebrate α-Tubulin Isotypes

Although many α-tubulin isotypes have been identified and sequenced in vertebrates, there is much less to say about them here. Altogether, seven classes of α isotypes have been proposed. In terms of sequence, Classes I, II, III and IV are very similar to each other. Their distributions have only been described in broad strokes. The isotypes in Class I are widespread in distribution although one is found largely in the brain. The isotypes of Class II are found mostly in the testis, while Class III is found in both brain and muscle and Class IV occurs in blood cells. Class V will be discussed in a moment. The highly divergent class VI (known as αTT1) has been reported only as a minor component of mouse testis (Hecht et al., 1988), while Class VII was described in *Xenopus* ovaries (Wu and Morgan, 1994). There is also an unclassified α isotype in the zebrafish (Ludueña and Banerjee, 2008b). When a given isotype is reported only in a single vertebrate, it is difficult to speculate about its evolutionary significance.

The α isotypes of Classes I, II, III, IV, and VII have, in common, a lysine at position 40. This residue is found in most known α-tubulins. Lys40 is often acetylated, a modification that conveys a great deal of stability to MTs (LeDizet and Piperno, 1986). The α isotypes of Classes I, II, and IV have EEY at the C-terminus. The C-terminal tyrosine can be removed by a tubulin carboxypeptidase and added back by a tubulin–tyrosine ligase. The significance of these reactions in the very early evolution of tubulin will be discussed later. The α isotypes of Class II have EE at the C-terminus to which the ligase can add the tyrosine to create EEY (Gu et al., 1988). One major feature of α-tubulin is that it binds to GTP; this GTP is neither hydrolyzed nor exchanged (Bai et al., 1998). Its precise function is unknown although mutations that alter the GTP-binding site on α can be highly deleterious (Keays et al., 2007).

The most intriguing α isotypes are those of Class V, referred to as α8, found in cardiac and skeletal muscle and the testis. These have EEF at the C-terminus; the phenylalanine cannot be removed by tubulin carboxypeptidase. In short, these isotypes do not participate in the tyrosination/detyrosination cycle. Furthermore, they lack lys40, so they cannot be acetylated. In addition, they have an unusual sequence at positions 35–45, which is very different from that of the other αs. This region is thought to be a loop on the inner MT wall that can make contact with adjacent protofilaments (Stanchi et al., 2000). Interestingly, these three unique features have been highly conserved in the evolution of Class V among vertebrates. It is likely, therefore, that Class V α has some particular functional role, but what it is we cannot say. In short, class V α could be the one exception to the hypothesis that β is more "important" than α; to summarize, for the different βs, we have at least a glimmer of specific functions; the βs have more complex distributions than the αs; the GTP bound to α appears totally passive and most of the drugs that target tubulin bind to β.

2.4. Evolution of α- and β-Tubulin in Other Eukaryotes

Although many sequences of both α- and β-tubulin are available from the entire eukaryotic world, few isotypes have been studied in as much detail as have the ones from vertebrates. Instead of evolution of the eukaryotes illuminating the functions of the different tubulins, it appears to be the other way around, in that tubulin sequences are being used to solve the puzzle of the relationships among the various eukaryotic phyla (Keeling and Doolittle, 1996; Keeling et al., 1999; Schütze et al., 1999; Baldauf et al., 2000; Moriya et al., 2001; Edgcomb et al., 2001; Nishi et al., 2005; Sakaguchi et al., 2005; Tyler et al., 2010). An interesting question is whether the various functions that have been hypothesized for specific tubulin isotypes in vertebrates can also be carried out by tubulin in other eukaryotes. Very little evidence is available at this time although recent work suggests that in plants, alterations of reactive oxygen species concentrations can affect tubulin polymerization (Livanos et al., 2011). It is likely that tubulin isotypes started to appear about the time that animals originated between 660 and 720 million years ago (Valentine, 2002). Our survey of the vertebrate tubulins suggests that they do not always need to form MTs. Outside of the vertebrates, there is one piece of evidence consistent with this: in *Toxoplasma*, there is a conoid structure consisting of a bundle of about nine tubulin protofilaments that are not in MT form (Hu et al., 2002).

3. EVOLUTION OF THE TUBULIN/FtsZ SUPERFAMILY

The α- and β-tubulin polypeptides are 36–42% identical and 63% homologous in sequence and very close in their higher order structures (Ponstingl et al., 1981; Krauhs et al., 1981; Little and Seehaus, 1988; Burns, 1991; Nogales et al., 1998a). Other tubulins have been found, namely, γ-, δ-, ε-, η-, ζ-, ι-, θ- and κ-tubulins, bearing more distant relationships to α and β; together these constitute the *tubulin superfamily* (Fig. 2.2) (McKean et al., 2001; Dutcher, 2001, 2003a; Ludueña and Banerjee, 2008c). In addition, there are several prokaryotic proteins that are related as well, although less closely, and together with the tubulins, they constitute part of a larger superfamily: the *tubulin/FtsZ superfamily*. The distributions and properties of these proteins are given in Table 2.8. After describing the individual members of this superfamily, we will speculate as to how this superfamily originated and evolved. We will begin by examining the common features that these proteins share.

3.1. The Players: Eukaryotes

Figure 2.2 shows the relationships among the members of the tubulin superfamily, based on their amino acid sequences. It is reasonable to speculate that the degree of relatedness in sequence corresponds to the order in

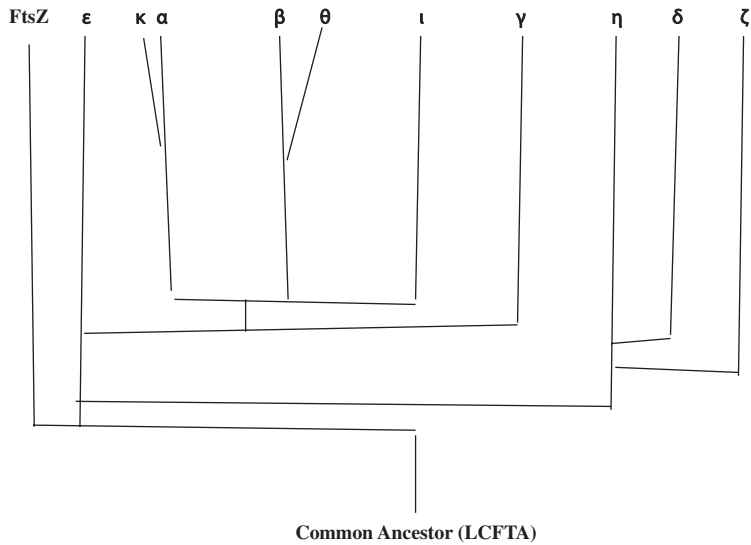


Figure 2.2 ***Hypothesis for the evolution of the tubulin superfamily.*** *(Based on Dutcher (2003a)).*

Table 2.8 The tubulin/FtsZ superfamily: distribution, subcellular localization and polymer structure*

A. Eukaryotes

					Subcellular Localization			Nature of Polymer	GTPase
Tubulin	**Animals**	**Plants**	**Fungi**	**Protists**	**Basal Body**	**Centrosome**	**Other**		
α,β	+	+	+	+	+	+	Ubiquitous	Microtubule, ring, reticulum, filament	Yes
γ	+	+	+	+	+	+	Nucleus	Ring	Probably
δ	+	+		+	+	+	Perinuclear ring	Ring, filament	
ε	+	+		+	+	+		Filament?	Probably
η	+			+	+				
ζ	+			+	+				
θ				+	+				
ι				+					
κ				+					

B. Prokaryotes

Protein	**Eubacteria**	**Eury**	**Cren**	**Nano**	**Kor**	**Thaum**	**Nature of Polymer**	**GTPase**
FtsZ	+	+	+	+	+	+	Filament, ring	Yes
RepX	+						Filament	Probably
TubZ	+						Filament	Yes
BtubA	+						Filament	Yes
BtubB	+						Filament	Yes
α-Tubulin†	+							
Tubulin‡				+				

*Abbreviations: Eury = Euryarchaeota; Cren = Crenarchaeota; Nano = Nanoarchaeota; Kor = Korarchaeota, Thaum = Thamarchaeota.
†Beggiatoa protein resembling α-tubulin.
‡Thaumarchaeota protein resembling tubulin.
References: (Banks et al., 2011; Yutin and Koonin, 2012).

which they evolved. Since most of the members of the tubulin superfamily occur in each of the eukaryotic kingdoms, it is likely that the last eukaryotic common ancestor (LECA) expressed these proteins. Also, since the ability to form centrioles, basal bodies and axonemes can occur in any of these kingdoms, it is possible that the LECA also possessed these organelles. The fact that only α and β can form MTs and that the others cannot suggests that the ancestor of these proteins did not form MTs. The argument shall be presented that it is likely to have formed a filament instead.

3.1.1. α- and β-Tubulin

We have already discussed the MT-forming properties of α- and β-tubulin. The propensity of tubulin to form rings and that these rings can nucleate MT assembly in vitro has long been known (Pantaloni et al., 1981). However, there is considerable evidence that the α/β heterodimer can also form a filament and that this filament exerts force on DNA. The ends of MTs depolymerize into curved protofilaments (Mandelkow et al., 1991; Arnal et al., 2000; Höög et al., 2010). Small fibrils have been seen connecting the curved protofilaments to the inner kinetochore in the chromosome (McIntosh et al., 2008) (Fig. 2.3). Although the composition of these fibrils is still unknown, McIntosh et al. (2010) calculated that a set of curving protofilaments arising from a single MT could exert a force of 0.2–0.6 pN, presumably arising from GTP hydrolysis; they compared this model to the way that FtsZ in bacteria mediates cytokinesis (see Section 3.2.1). McIntosh et al. (2010) also proposed that tubulin protofilaments could have been the original mechanism by which tubulin separated DNA molecules in mitosis; presumably, the complex mitotic spindle arose later. In this model, the MT itself becomes irrelevant. There is thus evidence that tubulin in the mitotic spindle is connected indirectly to the DNA. The fact that GTP hydrolysis increases the curvature of these protofilaments is consistent with these hypotheses (Müller-Reichert et al., 1998).

3.1.2. γ-Tubulin

γ-Tubulin has long been known as a nucleator of MTs, playing that role for the centrosome, the basal body and the central pair MTs of axonemes (Fuller et al., 1995; Kierszenbaum, 2002; McKean et al., 2003). γ-Tubulin molecules associate laterally to form a ring in a process that requires the participation of other proteins (Oegema et al., 1999; Aldaz et al., 2005; Cuschieri et al., 2007; Kollman et al., 2008) (Fig. 2.4). The α/β dimer then adds longitudinally to this ring to form a MT; the geometric properties of the γ-tubulin ring determine the number of protofilaments in the MT as well

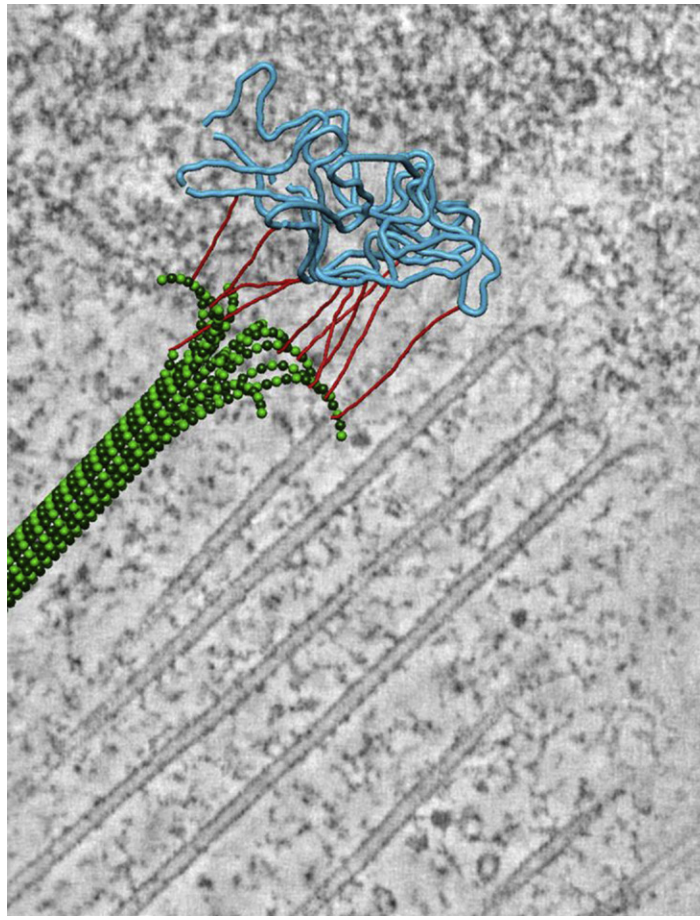


Figure 2.3 ***Kinetochore microtubules***. Note curved protofilaments at the ends of the microtubules. These connect to the DNA. Courtesy of Mary Morphew, Fazly Ataullakhanov, Ekaterina Grishchuk, and Richard McIntosh, Dept. M.C.D. Biology, Boulder, CO. (For color version of this figure the reader is referred to the online version of this book).

as the pitch (Moritz et al., 2000). This has been studied most closely in the yeast *Saccharomyces cerevisiae*, in which two molecules of γ-tubulin and one molecule of each of the proteins Spc97 and Spc98 form the γ-tubulin small complex (γ-TuSC). Seven γ-TuSC, together with about three molecules of γ-tubulin, form a ring onto which 13 protofilaments of α/β dimers can polymerize; the extra three molecules of γ-tubulin are thought to stabilize the ring (Erlemann et al., 2012). The possibility exists that some kind of chaperonin may be required for γ-tubulin to fold properly (Geissler et al., 1998). γ-Tubulin can bind to either GDP or GTP, making no difference to

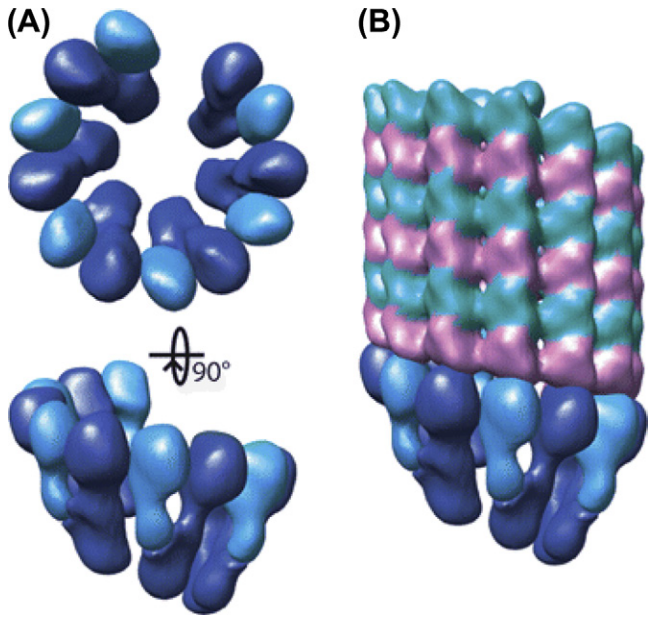


Figure 2.4 ***Model for the oligomerization of γ-TuSC***. (A) Multiple γ-TuSCs with γ- and α/β-tubulin were aligned with the minus end of a microtubule to indicate a possible arrangement of a higher order, γ-TuRC-like structure that would be competent for microtubule nucleation. (B) The γ-TuSC assembly from A shown making longitudinal contacts with the minus end of a microtubule. (*Figure 9 in Kollman, J.M., Zelter, A., Muller, E.G.D., Fox, B., Rice, L.M., Davis, T.N., Agard, D.A., 2008. The structure of the γ-tubulin small complex: implications of its architecture and flexibility for microtubule nucleation. Mol. Biol. Cell 19, 207–215. Reprinted with permission).* (For color version of this figure the reader is referred to the online version of this book).

the γ-tubulin conformation (Rice et al., 2008). Whether GTP hydrolysis is necessary for γ-tubulin polymerization into a ring is not clear; however, binding of GTP to γ-tubulin appears to be required for nucleation of basal bodies (Shang et al., 2005). Besides the centrosome and basal body, γ-tubulin is often found at other sites of MT nucleation: the cortex of plant cells, bicoid RNA structures in *Drosophila*, membranes of embryonal carcinoma cells, and in the macronucleus of *Tetrahymena* (Schnorrer et al., 2002; Murata et al., 2005; Macurek et al., 2008; Kushida et al., 2011). In the centrosome, γ-tubulin binds to ninein, thereby anchoring MTs to the centrosome, and also binds to pericentrin and centrosomin (Dictenberg et al., 1998; Mogensen et al., 2000; Delgehyr et al., 2005; Nigg, 2007; Zhang and Megraw, 2007).

In addition to nucleating MTs, γ-tubulin appears to have other functions. In the nucleus, it interacts with E2F proteins that in turn bind to

promoter regions of DNA; it also binds to the promoter for retinoblastoma protein 1 (Höög et al., 2011; Ehlén et al., 2012). In short, γ-tubulin interacts both directly and indirectly with DNA, a function not too different from the way the α/β dimer interacts indirectly with the DNA in the kinetochore (Fig. 2.3). The nuclear γ-tubulin is specifically associated with the nucleoli, and with ribosomal DNA as well as with the tumor suppressor protein C53 that regulates the cell cycle (Hořejši et al., 2012). Just as was the case with nuclear βII described above (Walss et al., 1999), nuclear γ-tubulin does not enter the nucleus through nuclear pores but during the process of reformation of the nucleus during mitosis (Hořejši et al., 2012). γ-Tubulin can bind to the βγ subunit of G-proteins and may help to activate Gβγ (Montoya et al., 2007). In *Aspergillus*, γ-tubulin regulates the anaphase-promoting complex (Nayak et al., 2010). In mammalian cells, γ-tubulin binds to the Golgi protein p115, thus interacting indirectly with a membrane and localizing the Golgi near the centrosome (Radulescu et al., 2011). γ-Tubulin also binds to WASH, a protein involved in actin filament nucleation (Monfregola et al., 2010). One as yet poorly understood behavior of γ-tubulin is that it can form a complex with β-tubulin in glioma cells (Katsetos et al., 2007); the functional significance of this complex formation and whether it also occurs in normal glial cells is unknown. To summarize, γ-tubulin, in addition to nucleating tubulin, can interact with DNA and a variety of proteins. If we imagine a proto-centrosome without α- and β-tubulin, and without MTs, perhaps what we are seeing in these alternative functions of γ-tubulin are relicts of a major role once played by γ or its ancestor in the proto-centrosome.

Just as is apparently the case with α- and β-tubulin, the pressure of performing multiple functions may have caused the appearance of isotypes of γ-tubulin. In mammals, the γ1 isotype is widespread and its absence is lethal; the γ2 isotype is present largely in the brain and its absence is not serious, but it nucleates MTs in the centrosome (Yuba-Kobo et al., 2005; Vinopal et al., 2012). Whether the two forms of γ-tubulin actually have specific functions is not clear. In the ciliate *Euplotes*, there are two forms of γ-tubulin: one is associated with centrosomes and one with basal bodies (Marziale et al., 2008). Although this suggests a functional difference between the two, the specific nature of this difference is unknown.

3.1.3. δ-Tubulin

δ-Tubulin occurs in basal bodies and centrioles; it has been observed in humans and other vertebrates, tunicates (*Ciona*), and a variety of protists (Dutcher and Trabuco, 1998; Chang and Stearns, 2000; Smrzka et al., 2000;

Piard-Ruster and Stearns, 2001; Inaba and Satouch, 2002; Sidjanin et al., 2002; Kato et al., 2004). δ-Tubulin seems to be localized in filaments that join adjacent centrioles to each other within the centrosome (Chang and Stearns, 2000). Deletion of δ-tubulin causes formation of basal bodies with nine doublet MTs instead of the canonical nine triplets (O'Toole et al., 2003). In mice, there are two forms of δ; one is widespread in the tissues; a larger form occurs in the testis (Smrzka et al., 2000). The latter forms two kinds of polymer, both involving spermiogenesis; one constitutes the pre-perinuclear and perinuclear rings of the spermatids; the second forms an intercellular bridge between spermatocytes (Kato et al., 2004). The rings are substantially larger than those formed by γ-tubulin and their substructure has not been analyzed. These structures have also not been observed outside of mice. Regardless, all these findings suggest that δ-tubulin can participate in formation of some kind of filamentous polymer and plays an important role in the development of the centriole/basal body.

3.1.4. ε-Tubulin

ε-Tubulin has been observed in humans and other vertebrates as well as in a variety of protists (Vaughan et al., 2000; Chang and Stearns, 2001; Dupuis-Williams et al., 2002; Dutcher et al., 2002; Morrison et al., 2003). ε-Tubulin localizes to basal bodies and centrosomes. In basal bodies of *Paramecium*, ε-tubulin is located very close to the triplet MTs and at each end of the structure (Ross et al., 2011). Within the centrosome, ε-tubulin is preferentially located in the older centriole; it could conceivably be connected to a dense fiber that is associated with the older centriole (Nakagawa et al., 2001). Deletion of ε causes the triplet MTs in centrioles or basal bodies to become doublets or singlets and also disrupts centriole duplication, mitosis and meiosis (Dupuis-Williams et al., 2002; Dutcher et al., 2002; Chang et al., 2003; Ross et al., 2011). Interestingly, mutations in the putative nucleotide-binding domain of ε are lethal (Ross et al., 2011), suggesting that ε may not only bind to guanine nucleotides but that this binding is essential to its function.

If δ- and ε-tubulin play important roles in the formation of the centrosome or basal body, it is not surprising that they have not been found in fungi, which lack these structures. They also appear to be generally missing from plants, which also lack basal bodies and centrioles. However, sequencing of the full genome of the spike moss *Selaginella moellendorfii* suggests the presence of both δ- and ε-tubulin in these plants (Banks et al., 2011). It is interesting that the phylogenetic position of *Selaginella*, which is an ancient genus with 350 million years in the fossil record (Banks, 2009), has

been somewhat controversial (Lyon, 1901). Nevertheless, it appears to be a lycopsid, not quite a fern but close. Unlike many other vascular plants, ferns produce spermatozoid cells with complex cilia that show the typical 9 + 2 pattern of eukaryotic cilia as well as centrioles and basal bodies with nine triplet MTs (Renzaglia et al., 1998, 1999; Sakaushi et al., 2003). It is interesting also that, again unlike other vascular plants, the β-tubulin isotypes of *S. moellendorfii* include some with the EGEFXXX signal sequence, suggesting that they form part of these flagella (Banks et al., 2011) (Table 2.9). In short, it appears that δ- and ε-tubulin are indeed present in plants, but only in those plants that have centrioles and basal bodies.

3.1.5. η-Tubulin

η-Tubulin has been observed in the protists *Chlamydomonas* and *Paramecium* and the animals *Tribolium*, *Ciona*, and *Xenopus* (Libusová and Dráber, 2006; Siebert et al., 2008); it is required for basal body duplication (Ruiz et al., 2000). Mutations in η cause delocalization of both β- and γ-tubulin in the basal bodies (Ruiz et al., 2000, 2004). These findings suggest that η is capable of binding either directly or indirectly to other forms of tubulin, and, hence, that it can participate in formation of some kind of polymer.

3.1.6. Other Tubulins

In the past few years, several new tubulins have been discovered; their functions are unknown. ζ-Tubulin has been identified in basal bodies of the protists *Trypanosoma* and *Leishmania* and, interestingly, *Xenopus* (Dutcher, 2003a; El-Sayed et al., 2005; Peacock et al., 2007). It is absent in yeast and *Arabidopsis*. Although one cannot generalize from these few examples and argue that ζ is present only in protists and animals and not in plants or fungi, nevertheless, so far, ζ has appeared only in organisms that have centrioles and/or basal bodies and one might speculate that ζ may play some kind of role in these organelles (Vaughan et al., 2000; Dutcher, 2001; McKean et al., 2001).

The genome of *Paramecium* contains θ-, ι-, and κ-tubulins (Ruiz, 2003). Little is known of their functions. κ and θ are 56% identical in sequence, respectively, to α and β in *Paramecium*, allowing one to conclude that they are, in effect, isotypes of α and β. Even ι is not so different, being 29% identical to yeast β.

3.1.7. The Ancestral Tubulin in LECA

Based on the common features of the proteins in the tubulin superfamily, can we deduce what the ancestral tubulin in LECA did? As argued above, it is unlikely to have formed MTs. However, as we have seen, the αβ dimer can form filaments as can δ, while γ can form rings. Modeling and genetic

Table 2.9 Key portions of the sequences of β-tubulin isotypes of certain plants*

Phylum/species	124–129	237–240	C-Terminal sequence	Accession Number
Plants				
Anemia	AENCDC	TCCL	YQDATAERE**GEYEED**YDEA	CAA48929
	AENCDC	TCCL	YQDATAEPE**GXYEED**EDEA	CAA48930
Selaginella	AESCDC	TCCL	YQDASA**DDEYEEE**PEEELQS	EFJ38783
	AESCDC	TCCL	YQDASA**EEEYDEE**AEELEQ	EFJ38610
	AESCDC	TCCL	YQDASADD**EGEFEEE**A	EFJ38329
	AEGCDC	TCCL	YQDASADE**EGEFEEE**GAEA	EFJ29447
	AESCDC	TCCL	YQDASADE**EGEFEEE**EGEG	EFJ36383
	AEACDC	TCCL	YQDARPDD**EGDYGEEEE**QEGEYSQMS	EFJ36035
	AENCDC	TCCL	YQDAAVE**ADYEDD**DEEQAA	EFJ35470
	CEGADC	TCCL	YQDASID**DDDFGED**PTCSSCQSTA	EFJ29026
	AESCDC	TCCL	YQDATADE**EGEFEEE**E	EFJ28795

*The table gives the C-termini and the critical cysteines in β-tubulin isotypes in two plants. Canonical axonemal sequences are given in boldface and underlined. Corresponding sequences in other isotypes are underlined.
References: (Moepps et al., 2005).

studies suggest that ε and η can associate with other tubulins (Inclán and Nogales, 2000; Ruiz et al., 2004), while the fact that δ, ε, η, and ζ are probably localized to either the centrosome or the basal body or both implies that they are part of a structure. Otherwise, since these organelles are not bounded by a membrane, these tubulins would be able to drift throughout the cell and would not be localized anywhere. Besides MTs, these organelles contain filaments made of the protein rootletin which, aided by the protein C-Nap1, connects the centrioles to each other and to the basal body (Mayor et al., 2000; Bahe et al., 2005; Yang et al., 2006). The centrosomal protein ODF2 also forms filaments (Donkor et al., 2004). It is possible, therefore, that δ, ε, η, ζ, and θ could be part of these filaments, since they do not appear to form MTs. Perhaps they form a scaffold that organizes the nine triplet MTs. Even if they are parts of filaments, we cannot necessarily conclude that any one of these tubulins forms a homogeneous polymer. Nevertheless, it is not unreasonable to suggest that the ancestral tubulin formed filaments having the diameter of a single molecule. Since the GTP-binding site is conserved in all these tubulins, it is very probable that the ancestral tubulin bound to GTP and, as we shall argue later, hydrolyzed it, utilizing the energy thus produced in either the assembly or the function of the filament. It is perhaps not coincidental that the most stable part of the tubulin molecule is the GTP-binding site (Keskin et al., 2002). Furthermore, it is striking that every member of the tubulin superfamily whose subcellular location is known is found in the basal body and α, β, γ, δ, and ε occur in the centrosome as well. The two organelles undoubtedly have a common origin, consistent with the observation that basal bodies can form from centrioles, just as centrioles form from each other, although the molecular mechanism by which this occurs is a mystery (Kobayashi and Dynlacht, 2011). As we have seen, the αβ dimer as well as γ can interact indirectly with DNA. It is conceivable that the ancestral tubulin also interacted indirectly with DNA and perhaps constituted an organelle that we could call a *proto-centrosome.* It is also possible that the proto-centrosome connected to an area of the cell membrane that acted as a sensory membrane patch, perhaps being involved in phagocytosis and mating (Jékely and Arendt, 2006). The proteins currently involved in intraflagellar transport bear an ancestral relationship to vesicle coats and nuclear pore complexes (Jékely and Arendt, 2006). It is conceivable that the connection of the proto-centrosome to a receptor-rich region of the cell membrane is extremely ancient, possibly even preceding the appearance of MTs. Locating the proto-centrosome close to this patch makes sense. Absorption of nutrients could take place very close to the

chromosome. Perhaps the proto-mitochondrion was taken up at this time, thereby establishing a source of energy in the vicinity. Perhaps it is relevant to point out that the interaction of tubulin dimers with VDAC is preceded by binding of the tubulin dimer to the mitochondrial membrane (Rostovtseva et al., 2012). Jékely and Arendt (2006) propose that the first MTs arising in the vicinity of the proto-centrosome could have caused protrusion of the sensory membrane patch, which would have allowed it to function more efficiently. The appearance of the centriole and its duplication could have allowed one daughter to serve as the root for axonemal tubulins and the other as an originator for MTs of the mitotic spindle.

When we examine the prokaryotic members of the tubulin superfamily, the probable functions of the ancestral tubulin in LECA will become much clearer.

3.2. The Players: Prokaryotes

The discovery that certain prokaryotic proteins are related to tubulin transformed the field of tubulin evolution. These proteins can be joined with the tubulin superfamily to constitute the larger tubulin/FtsZ superfamily (Fig. 2.5). Here, we shall survey the relevant structural and functional properties of FtsZ and its relatives.

3.2.1. FtsZ

When bacteria divide, the protein FtsZ forms filaments that tug on the cell membrane and perform cytokinesis (Bi and Lutkenhaus, 1991). FtsZ

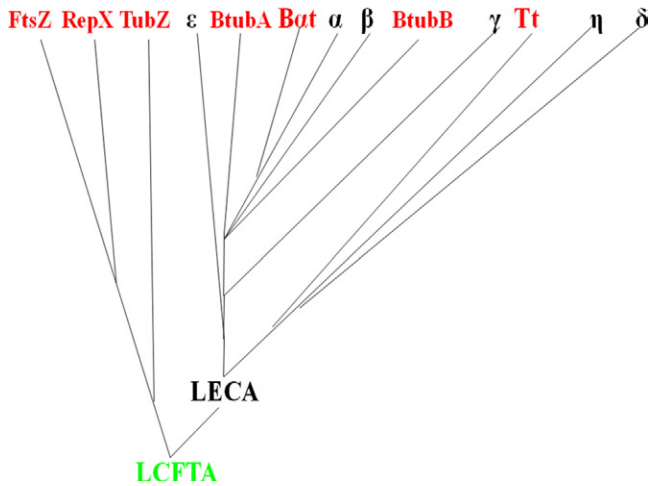


Figure 2.5 ***The tubulin/FtsZ superfamily.*** Proteins found in prokaryotes are in red, eukaryotes in black and LCFTA in green. Bαt = *Beggiatoa* α-tubulin; Tt = Thaumarchaeota tubulin. (For color version of this figure the reader is referred to the online version of this book).

has a sequence 7% identical to that of α- and β-tubulin and the three-dimensional structures of the three proteins are very similar (Fig. 2.6); the sequence identity increases to 10% at the nucleotide-binding site (Nogales et al., 1998a, 1998b; Löwe and Amos, 1998). FtsZ and tubulin share an overall general similarity in function: they both play a role in cell division. However, FtsZ is involved in cytokinesis and does not directly act on the bacterial DNA. In eukaryotes, cytokinesis is mediated by actin filaments, while tubulin forms the mitotic spindle which moves the DNA (Löwe and Amos, 2009). In prokaryotes, tugging on DNA is carried out by actin-like filaments; in short, comparing prokaryotes and eukaryotes, one could imagine that actin-like and tubulin-like proteins have switched roles (Löwe and Amos, 2009). FtsZ is widespread, being found in most eubacteria and, of the presumptive archaeal kingdoms, FtsZ commonly occurs among the Euryarchaeota and has recently been observed in at least some members of the Crenarchaeota, Korarchaeota, Nanoarchaeota and Thaumarchaeota (Margolin et al., 1996; Baumann and Jackson, 1996; Lu et al., 1998; Gribaldo and Brochier-Armanet, 2006; Cann, 2008; Elkins et al., 2008; Makarova and Koonin, 2010; Makarova et al., 2010; Yutin and Koonin, 2012). Interestingly, archaeal FtsZ is slightly more similar to tubulin than is eubacterial FtsZ (Margolin et al., 1996). FtsZ is also expressed in some mitochondria and chloroplasts, where it plays a role in the fission and reproduction of these

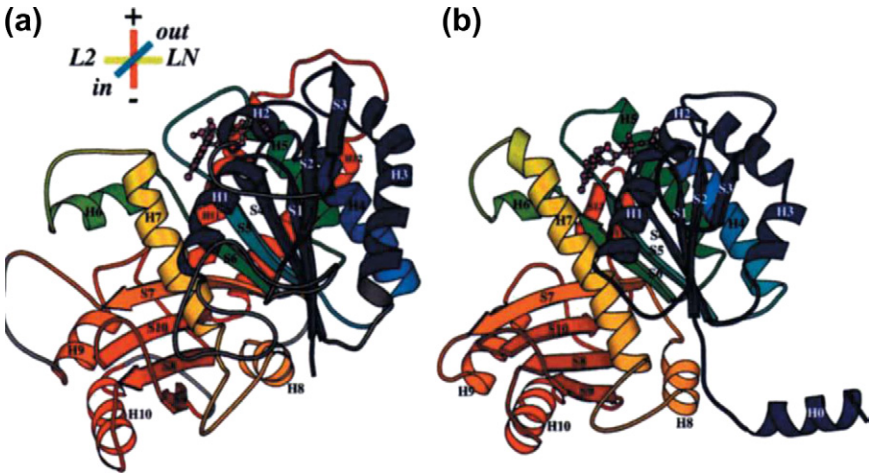


Figure 2.6 ***Ribbon diagrams of β-tubulin (a) and FtsZ (b)*** *(From Nogales et al. (1998b). Reprinted by permission from Macmillan Publishers Ltd: Nature Structural Biology Volume 5, pp. 451–458, Tubulin and FtsZ form a distinct family of GTPases (Fig. 1) copyright 1998).* (For color version of this figure the reader is referred to the online version of this book).

organelles (Beech et al., 2000; Vaughan et al., 2004; Glynn et al., 2007; Olson et al., 2010). Nevertheless, it is important to recall that not all cells use systems based on FtsZ or tubulin to divide; many Archaea, Eubacteria and even mitochondria and chloroplasts use filaments made of dynamin, ESCRT III or other proteins (Arimura and Sutsumi, 2002; Gao et al., 2003; Osteryoung and Nunnari, 2003; Praefcke and McMahon, 2004; Low and Löwe, 2006; Samson et al., 2008; Lindås et al., 2008). One can readily imagine that the earliest cells could have had a variety of generally similar filament-forming proteins—FtsZ, ESCRT-like, dynamin, intermediate filament-like, and actin-like—that formed some kind of cytoskeleton that aided in cell division and other functions and that their many varied descendants may have retained certain sets of these proteins for that or other purposes (Møller-Jensen and Löwe, 2005; Derman et al., 2009; Cabeen et al., 2011).

Returning to FtsZ, the resemblance to tubulin was noted early, especially in the key role of GTPase in both proteins (Bramhill and Thompson, 1994; Mukherjee and Lutkenhaus, 1994; Erickson, 1995). In the phosphate-binding portion of the GTP-binding site, where tubulins have the sequence GGGTGSG, FtsZ has GGGTGTG (Lutkenhaus, 1993) (Table 2.10). FtsZ polymerizes in vitro into curved dynamic filaments (Erickson et al., 1996; Stricker et al., 2002; Oliva et al., 2003; Graumann, 2009). In the cell, these filaments can aggregate or even polymerize further to create the Z-ring whose constrictions pinch the cell in two (Anderson et al., 2004). Although GTP binding is required for FtsZ filament formation (Adams and Errington, 2009), the precise role of GTP hydrolysis in FtsZ is not exactly the same as with MTs. First, unlike tubulin, hydrolysis of GTP does not alter FtsZ conformation (Wang and Nogales, 2005; Erickson, 2009). Second, instead of being internal to the molecule, as is the case with tubulin, GTP is hydrolyzed at the interface where FtsZ molecules meet in the filament (Scheffers et al., 2002). In short, GTPase appears to be required for cytokinesis itself, i.e. constriction of the Z-ring, rather than for FtsZ polymerization or dynamics (Lu et al., 2001; Chen and Erickson, 2009; Jaiswal et al., 2010), although a role for GTPase in filament nucleation has been proposed (Mukherjee et al., 2001). Resemblances to tubulin do not end here. The overall negative charge of both FtsZ and tubulin allow for cation-induced bundling of FtsZ filaments and of MTs, thus facilitating, respectively, formation of the Z-ring and the mitotic spindle (Rieder and Bajer, 1977; Needleman et al., 2004; Miraldi et al., 2008; Kuchibhatla et al., 2011; Popp and Robinson, 2012). FtsZ resembles tubulin in that its functions are mediated through

Table 2.10 Nucleotide binding sites in the tubulinFtsZ superfamily*

Protein	Phosphate-binding site	Species
Eukaryotes		
α-tubulin	SFGGGTGSG	Mouse
β-tubulin	SLGGGTGSG	Mouse
γ-tubulin	SIAGGTGSG	Mouse
δ-tubulin	SMAGGTGSG	Mouse
ε-tubulin	SMGGGTGSG	Mouse
ζ-tubulin	SLVGGTGSG	*Trypanosoma cruzi*
η-tubulin	SLCGGTGSG	*Xenopus laevis*
θ-tubulin	SLGGGTGSG	*Paramecium tetraurelia*
ι-tubulin	SITGGTGSG	*Paramecium*
κ-tubulin	SVGGGTGSG	*Paramecium*
FtsZ (chloroplasts)	GMGGGTGTG	*Chlamydomonas reinhardtii*
FtsZ (mitochondria)	GLGGGTGTG	*Cyanidioschyzon merolae* (Rhodophyta)
Eubacteria		
FtsZ	GMGGGTGTG	*Bacillus subtilis*
RepX	GLGGGTGTG	*Bacillus cereus*
TubZ	GAGGGVGTG	*Bacillus thuringiensis*
BtubA	AIGGGTGSG	*Prosthecobacter dejongeii*
BtubB	SIGGGSGSG	*Prosthecobacter dejongeii*
α-tubulin-like	GVDGGTGSG	*Beggiatoa*
Archaea		
Euryarchaeota FtsZ	GLGGGTGTG	*Methanosphaera stadtmanae*
Crenarchaeota FtsZ	GLGGGTGTG	Uncultured Crenarchaeote
Korarchaeota FtsZ	GMGGGTGTG	*Korarchaeum cryptofilum*
Nanoarchaeota FtsZ	GMGGGTGTG	*Nanoarchaeum equitans*
Thaumarchaeota FtsZ	GLGGGTGTG	*Nitrosoarchaeum koreensis*
Thaumarchaeota tubulin	AMGGGTGSS	*Nitrosoarchaeum koreensis*

*There are other parts to the nucleotide-binding site, but we are focusing on the part that binds to the phosphate.
References: (Inclán and Nogales, 2000; McKean et al., 2001; Dupuis-Williams, 2002a,b; Jenkins et al., 2002; Ruiz, 2003; Wang et al., 2003; Ruiz et al., 2004; Miyagishima et al., 2004; El-Sayed et al., 2005; Tinsley and Khan, 2006; Fricke et al., 2006; Larsen et al., 2007; Elkins et al., 2008; Dodson et al., 2009; Aylett et al., 2010; Takaki et al., 2010; Yutin and Koonin, 2012).

association with a variety of other proteins, although FtsZ has nowhere nearly as many companions as tubulin has (Table 2.1) (Adams and Errington, 2009). Among these proteins are FtsA and ZipA which connect FtsZ to the cell membrane, EzrA, which regulates FtsZ polymerization and dynamics, and SepF, which bundles FtsZ filaments (Addinall and

Lutkenhaus, 1996; Hale and de Boer, 1999; Chung et al., 2007; Singh et al., 2007, 2008; Adams and Errington, 2009). Also like tubulin, FtsZ from certain species, but not all, has a highly charged C-terminal domain which plays a role in binding of associated proteins (Singh et al., 2007). However, unlike tubulin, the C-terminal domain of FtsZ, when present, is rich in both acidic and basic residues (in *B. subtilis*, for instance, its sequence is DDTLDIPTFLRNRNKRG) (Vaughan et al., 2004; Singh et al., 2007); there is no sign that the C-terminal domains of the two proteins are related. Finally, although FtsZ's main role is in cytokinesis, the polymerization of FtsZ is closely coupled to the replication of DNA; in fact, inhibition of DNA replication inhibits Z-ring formation (Inoue et al., 2009). Thus, there is at least a functional link between FtsZ and DNA even if there is no chain of proteins that can be traced as is the case with tubulin (McIntosh et al., 2008, 2010).

3.2.2. TubZ

FtsZ is not the only prokaryotic tubulin-like protein. TubZ from *Bacillus anthracis* and *Bacillus thuringiensis* assembles into a force-generating polymer, which can be either a double- or a quadruple-helical array of TubZ molecules (Srinivasan et al., 2011). GTP binding is necessary for filament formation and GTP hydrolysis alters the conformation of the molecule (Aylett et al., 2010). In these organisms, the TubZ filament aids in segregation of a plasmid that encodes a toxin (Aylett et al., 2010; Srinivasan et al., 2011). TubZ filaments are dynamic structures, undergoing treadmilling, and their dynamic behavior is inhibited when GTPase activity is blocked (Larsen et al., 2007; Chen and Erickson, 2008). TubZ interacts with DNA via a protein TubR; it is proposed that TubZ uses treadmilling to bring the TubR–DNA plasmid to the edge of the cell, where it can then be easily released (Ni et al., 2010).

3.2.3. RepX

In addition to TubZ, *B. anthracis* encodes RepX which is also involved in replication of the toxin-encoding plasmid (Tinsley and Khan, 2006). RepX requires GTP to polymerize and is 20–22% identical in sequence to FtsZ (Tinsley and Khan, 2006; Akhtar et al., 2009).

3.2.4. BtubA and BtubB

The eubacterium *Prosthecobacter* expresses two proteins BtubA and BtubB, whose sequence places them in the tubulin superfamily. In fact, BtubA is closer to α, and BtubB closer to β, than even γ-tubulin (Jenkins et al., 2002). BtubA and BtubB can form heterodimers and polymerize into bundles of

filaments, in which BtubA and BtubB alternate (Sontag et al., 2009). Their three-dimensional structures are very close to those of α- and β-tubulin (Schlieper et al., 2005). Although both BtubA and BtubB bind and hydrolyze GTP, hydrolysis by BtubB appears to play a more important role in polymerization (Sontag et al., 2005). The actual function in *Prosthecobacter* of the filaments that they form is unknown, although it is possible that they play a role in the growth of the stalks characterizing this group of bacteria (Staley et al., 1976). The following evidence suggests that BtubA and BtubB arose in *Prosthecobacter* by horizontal gene transfer from an early eukaryote. 1) There is no evidence for any other eukaryotic protein in *Prosthecobacter* (Schlieper et al., 2005). 2) Some species of *Prosthecobacter* have FtsZ as well as BtubA and BtubB, implying that BtubA and BtubB were never required for cell division (Pilhofer et al., 2007). 3) As mentioned above, BtubA and BtubB are far more closely related to α- and β-tubulin than they are to FtsZ (Jenkins et al., 2002).

Unlike FtsZ, TubZ and RepX, the *Prosthecobacter* tubulins are not outliers in the tubulin superfamily, but rather right in the middle of it. They have an important story to tell and we shall return to them later.

3.2.5. Other Prokaryotic Proteins

One genus of the Thaumarchaeota contains in its genome a protein with a distant relationship to the tubulins; conceivably, it could have arisen by horizontal gene transfer or alternatively, it could point to a tubulin ancestor in the Archaea (Yutin and Koonin, 2012). Also, the giant bacterium *Beggiatoa* contains a protein similar to α-tubulin but half its size (Yutin and Koonin, 2012). There is as yet no evidence about the properties of these proteins or if they polymerize.

3.3. Evolution of Structure and Function in the Tubulin/FtsZ Superfamily

3.3.1. The Common Ancestor of Tubulin and FtsZ

The branches of the tubulin/FtsZ superfamily are arranged according to sequence differences (Fig. 2.5). Our working assumption is that the further apart in sequence two proteins are the earlier they separated from a common ancestor. In order to turn these diagrams into an evolutionary story, however, we need to survey the properties of their members and see what they do and do not have in common. Having already discussed the likely properties of the ancestral tubulin that lived in LECA, we shall extend this analysis to the protein that would have been the latest common

FtsZ-tubulin ancestor (LCFTA). Several of its likely characteristics are easy to predict. First, every member of this superfamily has a highly conserved site for binding guanine nucleotides (Table 2.10). This implies that LCFTA bound to GTP or GDP. Second, FtsZ, TubZ, RepX, BtubA and BtubB as well as β-tubulin are able to hydrolyze GTP. Structural modeling predicts the same for γ-tubulin as well. Thus, it is likely that LCFTA had GTPase activity.

A second question is if LCFTA polymerized and what kind of polymer it formed. We know that FtsZ, RepX, TubZ, BtubA, and BtuB form filaments in a process coupled to their GTPase activities (Sontag et al., 2005; Larsen et al., 2007; Akhtar et al., 2009). We have argued that the ancestral tubulin in LECA also formed filaments. Thus, the preponderance of the evidence suggests that LCFTA also formed filaments, which certainly are much simpler structures than MTs and hence, more likely to have been ancestral. GTP hydrolysis was probably required for this process.

The final question is that of function. The centrosome, with which α, β, γ, δ, and ε are associated, plays a major role in cell division, being the anchor of the mitotic spindle, which separates the chromosomes during mitosis. FtsZ plays a critical role in cell division, but is connected with cytokinesis rather than separation of DNA. TubZ and RepX are not associated with cell division, but with the related process of DNA replication in plasmids. BtubA and BtubB are speculated to play a role in budding, perhaps analogous to cell division (Staley et al., 1976). In short, LCFTA was probably involved in cell division. There is a strong degree of mechanistic unity as well. It is particularly striking that various members of the FtsZ-tubulin superfamily bind indirectly to either DNA or a membrane via a structure formed by some other protein. The α/β dimer is connected to the chromosomal DNA by a filament whose nature is as yet undetermined (McIntosh et al., 2008). The MAP tau, which promotes MT assembly (Weingarten et al., 1975), can bind directly to double-stranded DNA in vitro, although whether tau actually serves as a link between tubulin and DNA is not clear (Hua et al., 2003). γ-Tubulin binds to E2F which in turn binds to DNA (Höög et al., 2011). TubZ binds to TubR, which binds to the plasmid DNA (Ni et al., 2010). In short, it is likely that LCFTA interacted with nucleic acids or membranes via some other kind of protein. Putting this all together, we can speculate that LCFTA formed filamentous curving polymers that used the energy of GTP hydrolysis to accomplish work, tugging either on a membrane or on a nucleic acid. The prokaryotic descendants of LCFTA diverged according to whether they acted on membranes (FtsZ) or on nucleic acids (TubZ, RepX), while its eukaryotic descendant, the ancestral tubulin of LECA,

specialized in pulling on DNA and, in some of its descendants, lost its ability to connect to membranes. However, this ability was not necessarily lost entirely. We have already mentioned possible interactions between tubulins and membranes (Kourmouli et al., 2001; Carré et al., 2002) (Table 2.5).

3.3.2. Back to the Beginning: the Origin of LCFTA

Now that we have made a tentative reconstruction of the properties of LCFTA, we may speculate as to its birth. A recent hypothesis about the origin of the genetic code, would, if true, strongly support a tremendous antiquity for LCFTA. Davis (1999) presents a compelling hypothesis that amino acids acquired their codons in an order corresponding to the length of the biosynthetic pathways by which the amino acids were formed from arguably the first metabolic system, carbon-fixing cycles acting jointly with mineral assemblies, preceding not only the formation of the first cell but also the genome itself. By this argument, the first amino acids to form would be aspartate and glutamate followed very closely by asparagine and glutamine, and then alanine. The analysis proceeds from there to compare the amino acid sequences of proteins that occur in all three kingdoms of life. Then, amino acid residues that are conserved within a given protein family are weighted according to the assignment order of their codons to predict which proteins arose the earliest (Davis, 2002). The results suggest that ferredoxin was the first protein to appear, long before the first cell; this is consistent with the observation, based on its sequence, that ferredoxin could have originated by repeated duplication of a tetrapeptide (Eck and Dayhoff, 1966). (We shall return to ferredoxin later). Glutamine synthetase is postulated to be the second oldest protein (Kumada et al., 1993). By this type of analysis, based on the nature of the amino acids conserved in its sequence, FtsZ, and by extension, the common FtsZ-tubulin ancestor, would be one of the 9–10 most ancient proteins (Davis, 2002; Erickson, 2007; Wickstead and Gull, 2011). LCFTA would have appeared when the ancestral cell was enclosed by a membrane (Davis, 2002), consistent with the membrane connection of FtsZ. (It should be noted that this hypothesis about the origin of the genetic code is not universally accepted (Amirnovin, 1997)).

Unlike the case with ferredoxin, however, no simple repeating peptide has been identified in FtsZ. Nevertheless, in the structure of FtsZ, there are two domains, one that binds to GTP and one that has residues that can hydrolyze GTP when FtsZ polymerizes. The GTPase site is formed when the catalytic domain from one FtsZ molecule interacts with the GTP-binding domain of another FtsZ molecule as would happen in a filament. Oliva

et al. (2004) hypothesize that the two domains were once separate proteins encoded by different genes that eventually fused (Fig. 2.7). It is not unreasonable to speculate that LCFTA would have had the same structure. In other words, the most distant ancestor of tubulin could have arisen from the fusion of two genes. In addition to being able to bind or interact with GTP, we could further speculate that this most distant ancestor could have had a propensity to polymerize. In this context, it is interesting to note that both FtsZ and tubulin can be induced to polymerize into straight or curved filaments by the presence of mica (Hamon et al., 2009); mica is still one of the most abundant minerals on the Earth's surface and it has also been found in meteorites, making its presence on the early Earth a virtual certainty (Hazen, 2012). Perhaps the most distant FtsZ-tubulin ancestor was unable

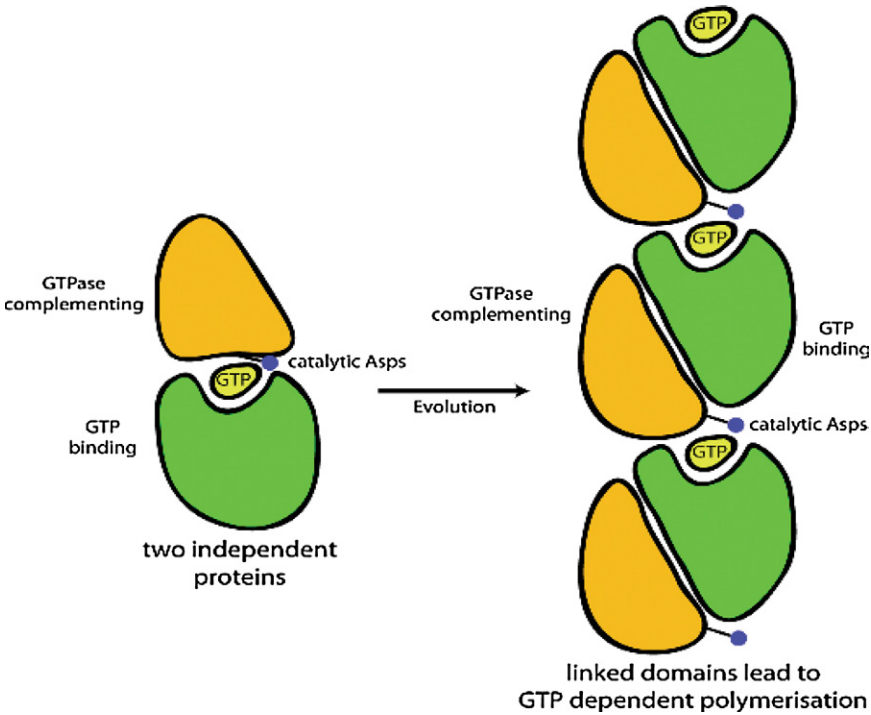


Figure 2.7 ***Hypothetical scheme from Oliva et al. (2004) about the origin of FtsZ.*** The authors speculate that FtsZ arose from a fusion of two ancestors, a GTP-binding protein and a GTPase complementing protein that had two aspartates able to catalyze the hydrolysis of GTP, provided that the two proteins bind to each other. *(Reprinted by permission from Macmillan Publishers Ltd: Nature Structural and Molecular Biology Volume 11, pp. 1243–1250, Structural insights into FtsZ protofilament formation (Supplementary Fig. 3) copyright 2004).* (For color version of this figure the reader is referred to the online version of this book).

to polymerize on its own but required the appropriate mineral environment, and its first evolutionary step was to acquire the ability to polymerize without minerals. The ability to polymerize may have been common among the first proteins. A large number of enzymes, both in prokaryotes and eukaryotes, polymerize; sometimes, these polymers have cytoskeletal functions (Ingerson-Mahar et al., 2010; Barry and Gitai, 2011). One could argue that if metabolizing systems appeared before cell membranes, then it was adaptive for enzymes to form polymers so as not to drift away. Eventually, divergent evolution might have given rise to an active monomeric enzyme while its polymerizing sibling might have had a cytoskeletal role (Barry and Gitai, 2011). The FtsZ/tubulin ancestor may originally have been adaptive because it hydrolyzed GTP for energy; its ability to form filaments may have been only a bonus. I am not proposing that LCFTA was the ancestor of all the GTPases; not all GTPases are related to the tubulin/FtsZ superfamily (Sage et al., 1995; Leipe et al., 2002).

3.3.3. The Possible Host of LCFTA

If FtsZ and tubulin had a common ancestor, it is likely to be very ancient. The presence of FtsZ in Eubacteria and the Archaea argues for the presence of the FtsZ/tubulin ancestor in the earliest cells. This brings us to the current models for the origin of cells. Essentially, there is agreement that there are three super-kingdoms of life: Eukaryotes, Eubacteria and Archaea, with Archaea having extremely deep divisions among Euryarchaeota, Crenarchaeota, Korarchaeota, Nanoarchaeota and Thaumarchaeota—it is hard to culture many Archaea and hence, very difficult indeed to sequence their genomes (Gribaldo and Brochier-Armanet, 2006). There are many hypotheses as to the relationships among these kingdoms, and the question of the origin of eukaryotes has been especially controversial. Some have proposed that all three kingdoms are coeval (Carlile, 1982), others that the prokaryotes were first, the nucleus arising by endosymbiosis of an Archaebacterium (Gupta and Goldring, 1996; Embley and Martin, 2006). Others have speculated that the eukaryotes were the first to appear (Reanney, 1974; Forterre and Philippe, 1999; Kurland et al., 2006). A common theme is that an archaeal cell acted as host to other archaeal or eubacterial symbionts and thus gave rise to the eukaryotic cell (Ribeiro and Golding, 1998; Rivera et al., 1998; Forterre, 2011); alternatively, an eubacterium could have arisen from the fusion of an archaeal and an eukaryotic cell (Devos and Reynaud, 2010). It has even been proposed that before the first cell actually formed and acquired a limiting membrane, some kind of reproducing

metabolic-mineral cluster—a "pre-cell"—may have already diverged into the three kingdoms (Wächstershäuser, 2006). Another idea is that the original archaeal host is represented in today's eukaryotes by the "cell body," namely, the nucleus and MT-organizing centers, sometimes extrapolated to include the proteins and other structures involved in information storage, packaging, replication and expression (Baluška et al., 1997, 2004; Horiike et al., 2001; Embley and Martin, 2006). By this approach, metabolic enzymes and cortical structures such as actin would have appeared later in a series of endosymbiotic events (Embley and Martin, 2006). Another hypothesis is that the ancestral first cell is of a type that no longer exists, a cell with RNA in its genome—a cell named a chronocyte; from this chronocyte would have evolved the other kingdoms (Poole et al., 1998; Hartman and Fedorov, 2002; Hartman et al., 2006).

It is not my aim to choose among these various hypotheses, but rather to see how the evolution of tubulin and FtsZ from LCFTA fits into them. Much hinges on whether the similarities between FtsZ and tubulin reflect a common ancestry or convergent evolution. If the former is the case, then one would speculate that LCFTA originated in one of the first cells. If the latter is true, then there was no LCFTA and it is more difficult to speculate as to how tubulin could have arisen. The suggestion has been made that tubulin arose as a cortical protein in an early eukaryotic cell (Cavalier-Smith, 1992). Convergent evolution is perhaps a less likely explanation for the following reasons. First, convergence would attest that FtsZ and tubulin developed common three-dimensional structure, filamentous polymerization and GTPase activity—all three. However, FtsZ and tubulin have commonalities in their GTP-binding site that are uniquely common to both of them and which distinguish them from other GTPases (Nogales et al., 1998b; Colicelli, 2004; Erickson, 2007).

Reviewing the various models, it is difficult to decide if LCFTA would more likely have arisen in the Eubacteria or the Archaea. However, if LCFTA arose in a chronocyte, this would imply that the occasional association of RNA with MTs, already discussed, may be a vestige of that chronocyte. The existence of long noncoding RNA's, of still mysterious function, as well as the long list of ribonucleoproteins containing possibly catalytic RNA's, suggests that perhaps the RNA world is more than vestigial (Jeffares et al., 1998; Mercer et al., 2009); one proposed function for this RNA has been to act as scaffold for histone modification complexes (Tsai et al., 2010); since a similar function has been proposed for nucleosome assembly proteins that undergo the same unusual

polyglutamylation as does tubulin (Regnard et al., 2000), it is conceivable that there is more tubulin–RNA interaction than we have yet discovered. By comparing sequences of different α- and β-tubulins, one finds that they are 75–85% conserved and one can estimate an evolutionary rate (Erickson, 2007). However, the sequence differences between tubulin and FtsZ are of such a magnitude that extrapolation would predict that their common ancestor would have lived before the Earth was created 4.5 billion years ago (Doolittle, 1995; Hartman and Fedorov, 2002; Erickson, 2007). The way out of this conundrum is to propose that the common ancestor evolved much more rapidly, which is easy to imagine in organisms whose genome is made of RNA instead of DNA (Hartman and Fedorov, 2002).

A subsidiary question is that of when all this evolution took place. Based on isotopic evidence, the first cell may have lived as many as 3.5–3.8 billion years ago (Mojzsis et al., 1996; Ueno et al., 2006) and the oldest microfossils may have been present 3.4 billion years ago (Schopf and Packer, 1987; Wacey et al., 2011) although the interpretation of these microfossils has been questioned (Brasier et al., 2002). The first possible eukaryotic cells, known as acritarchs, lived about 1.7–1.9 billion years ago (Knoll, 1994; Javaux, 2007); the observation of numerous cytoplasmic processes in these cells strongly suggests the presence of a cytoskeleton (Javaux et al., 2001); however, it should be noted that only 1.5–1.1 billion years ago did unambiguously eukaryotic cells appear (Berney and Pawlowski, 2006; Knoll et al., 2006). Furthermore, it has been proposed that these acritarchs were really prokaryotes and that the eukaryotes *per se* may not have appeared until 850 million years ago (Cavalier-Smith, 2002a, 2002b, 2006, 2010). It is, at any rate, given the large number of competing hypotheses about the origin of eukaryotes, difficult to estimate when they might have appeared (Roger and Hug, 2006). An interesting question is to ask how the transition to an oxidizing atmosphere would have affected the FtsZ/tubulin ancestral protein. This event, probably caused by the appearance of photosynthesis about 2.7 billion years ago (Brocks et al., 1999; Summons et al., 1999), has been dated to about 2.1–2.5 billion years ago by measuring sulfur-containing minerals and isotopes of sulfur, chromium and other elements (Canfield, 1998; Farquhar and Wing, 2003; Bekker et al., 2004; Frei et al., 2009). Granted that modern tubulin is extremely sensitive to oxidation (Mellon and Rebhun, 1976; Little and Ludueña, 1985; Bai et al., 1989), nevertheless, the levels of oxygen 2 billion years ago were probably only a fraction of the present levels and perhaps may not have affected tubulin.

3.3.4. *Lessons of* Prosthecobacter

As we have discussed, BtubA and BtubB of *Prosthecobacter* appear to have arisen by horizontal gene transfer (Schlieper et al., 2005). Close analysis of the sequences of BtubA and BtubB indicates that each one is a mosaic of α- and β-tubulin sequences (Martin-Galiano et al., 2011), suggesting that, rather than BtubA evolving from α and Btub B from β, a single tubulin was transferred to *Prosthecobacter* just prior to the divergence of α and β. In a sense, the *Prosthecobacter* tubulins give us a last look into the nature of tubulin at this critical time in its evolution just before MTs appeared. *Prosthecobacter* has much to teach us, but its lessons are not about the properties it has but about the ones it does not have: 1) *Prosthecobacter* tubulins do not form MTs; 2) they do not need to be dimers in order to polymerize; 3) they lack the highly negatively charged C-terminal domains present in α- and β-tubulin; and 4) perhaps most importantly, they can dimerize and polymerize without intervention by any kind of chaperone (Martin-Galiano et al., 2011), in contrast to tubulin, which requires no less than seven other proteins just to form the dimer, without which MT assembly will not proceed (Tian et al., 1996; Lopez-Fanarraga et al., 2001). *Prosthecobacter* is suggesting to us that the tubulin from which α- and β-tubulin arose lacked these properties as well. How do we go from this filament-forming monomer to an MT-forming dimer?

3.3.5. *Origin of Microtubules*

Any hypothetical scenario needs to have each step be adaptive. At first glance, this seems to be a tall order; however, one reasonable speculation may be as follows. We begin with the αβ common ancestor, very likely able to add longitudinally or laterally to γ-tubulin or δ-tubulin, and perhaps longitudinally to ε and η. It is not an unreasonable assumption that this filament-forming protein would have interacted with other proteins in order to carry out its various functions; these proteins could be the ancestors of the chaperones and tubulin-binding cofactors. At this stage, tubulin could have formed a curving protofilament that was linked via a fibril to DNA as McIntosh et al. (2008, 2010) have postulated. From here, the evolution to MTs could have proceeded in four steps.

3.3.5.1. Fusion of the Gene for the C-terminus with that of the Ancestral αβ-Tubulin

As will be argued below, the C-terminus could be of separate origin. The fusion of its gene with that of the αβ ancestor may have conveyed to the tubulin molecule the ability to bind to glycolytic enzymes and

mitochondria. The C-terminal domains of tubulin, to this day, bind to mitochondria and to the glycolytic enzymes aldolase, glyceraldehyde 3-phosphate dehydrogenase, and pyruvate kinase (Volker and Knoll, 1993; Carré et al., 2002; Kovacs et al., 2003). The fact that some of these interactions are inhibitory may be irrelevant; the reversibility of the binding of the C-terminus of tubulin to mitochondria and glycolytic enzymes could allow any small inhibition that may have been caused by the binding to be significantly compensated by having these enzymes localized to the vicinity of the tubulin filament. In other words, by including the C-terminus, the tubulin filament could gather energy sources closer to the proto-centrosome. This would definitely be adaptive since it would allow for faster replication.

There was a price to pay for this benefit, however, the C-terminus would have destabilized the tubulin molecule. One way to imagine the effect of adding the C-terminal domain to tubulin is to look at experimental data on the effect of removing it. Treatment with subtilisin cleaves both α- and β-tubulin so as to remove their C-terminal domains (Serrano et al., 1984b; Maccioni et al., 1986); the cleavage is heterogeneous and may remove more than just the negative C-terminal domain (Maccioni et al., 1986; Redeker et al., 1992b; Lobert et al., 1993). Nevertheless, the effects of the removal of the C-terminus are striking. Without the C-terminal domains, tubulin's conformation changes, losing a great deal of its α-helical content and becoming more compact and stable (Maccioni et al., 1986). The tubulin molecule has long been known to decay, namely, that it loses the ability to bind to drugs and to assemble into MTs (Wilson, 1970). Decay is not a matter of proteolytic digestion but simply that the molecule denatures over time (Schwarz et al., 1998). This can be demonstrated by measuring the time-dependent increase in exposure of sulfhydryl groups and hydrophobic areas (Ludueña and Roach, 1981a; Prasad et al., 1986). Both these effects are greatly decreased by subtilisin treatment; in fact, removal of the C-terminus appears to hide a substantial hydrophobic domain (Sarkar et al., 1995; Chaudhuri and Ludueña, 1997, 2001). Conceivably, this may be a domain involved in MT formation. Modeling studies are consistent with this hypothesis, indicating that one effect of the C-terminal domains is to increase the flexibility of other parts of the tubulin molecule (Freedman et al., 2011). Removal of the C-termini also favors tubulin's polymerization into vinblastine-induced protofilaments but inhibits formation of MTs; instead, under assembly conditions, subtilisin-treated tubulin forms a bundle of filaments that is

C-shaped in cross-section (Serrano et al., 1984b, 1986; Sackett et al., 1985). A later study involved deleting portions of the C-terminus of the βI- and βIII-tubulin isotypes and then transfecting them into HeLa cells and examining their incorporation into the cell's MTs; essentially, the results showed that removal of the C-terminal domain of β blocked incorporation into MTs, although formation of α/β dimers was not affected (Joe et al., 2009) (Table 2.11); removal of the C-terminus of α has the same effect (Lefèvre et al., 2011). Although the precise sites where subtilisin cleaves in βI and βIII are not known, if these sites correspond to the site on βII, then there is an additional complexity because truncated forms of βI and βIII shorter than if cleaved by subtilisin are still able to incorporate into preformed MTs. It may be that the last few residues of the C-terminal region are required for formation of MTs *de novo*. Extrapolating from these results, one could argue that the appearance of the C-terminal domain would have made the tubulin molecule less stable and less likely to form filaments but more likely to form MTs.

3.3.5.2. Divergence of α and β

The next step may have been the divergence of α and β. Using the same arguments advanced above regarding the tubulin genes in the early vertebrates, we can imagine the αβ ancestor gradually acquiring more functions, each mediated by binding to a different protein. Duplication of the gene for the αβ ancestor would have led to daughter genes which would have eventually diverged into α and β, each one retaining binding to one set of proteins, not necessarily the same. It is not difficult to imagine that the filaments thus formed contained alternating α and β molecules. Interestingly, modeling studies have raised the possibility that the interactions between α and β could actually increase each other's flexibility (Freedman et al., 2011). In other words, putting α and β in the same filament could have increased the need for chaperones.

3.3.5.3. Appearance of the Chaperones

At some point, there would be an adaptive advantage to stabilizing tubulin's conformation. If we imagine that the unstabilized tubulin, after addition of the C-terminus, only found the "right" conformation a small fraction of the time, then sequential intervention of the chaperones and cofactors would have increased that fraction and ultimately permitted more efficient formation of filaments. This would have been adaptive by allowing for more rapid replication and larger chromosomes. This is where the chaperones and

Table 2.11 Introns and the C-Termini of α- and β-tubulin

A. Introns near the C-terminus

Species	Before the intron	After the intron
α-tubulin		
Chlorella vulgaris	EKDYEE	VGAESAEADGEDEGEEY
β-tubulin		
Eimeria tenella	EYQQYQ	DATAEEEGEFDEEEGVMDAEGAA
Toxoplasma gondii	EYQQYQ	DATAEEEGEFDEEEGEMEAEEEA
Cylicocyclus nassatus	EYQQYQ	EATADDMGDLDAEGAETAYPEE

B. Effect of truncating the C-terminus on incorporation of human α, βI and βIII isotypes into microtubules (note: although the human sequences do not have an intron at this point, the space is indicated to allow for comparison with part A of this table).

Construct	Sequence	Incorporation into Microtubules (%)*
α wild-type	AALEKDYEE VGVDSVEGEGEEEGEEY	100†
α Δ13	AALEKDYEE VGVD	100†
α Δ23	AAL	0†
βI wild-type	EYQQYQ DATAEEEEDFGEEAEEEA	85
βI Δ16	EYQQYQ DA	97
βI Δ17	EYQQYQ D	0
βIII wild-type	EYQQYQ DATAEEEGEMYEDDEEESEAQGPK	95
βIII Δ5	EYQQYQ DATAEEEGEMYEDDEEESE	91
βIII Δ10	EYQQYQ DATAEEEGEMYEDD	87

Continued

Table 2.11 Introns and the C-Termini of α- and β-tubulin—cont'd

B. Effect of truncating the C-terminus on incorporation of human α, βI and βIII isotypes into microtubules (note: although the human sequences do not have an intron at this point, the space is indicated to allow for comparison with part A of this table).		
βIII Δ20	EYQQYQ DATA	80
βIII Δ21	EYQQYQ DAT	82
βIII Δ22	EYQQYQ DA	23
βIII Δ23	EYQQYQ D	0
βIII Δ24	EYQQYQ	0
βIII Δ25	EYQQY	0

C. Subtilisin-generated C-terminal peptides in pig brain tubulin.	
	Sequence
αI	SVEGEGEEEGEEY
βII	GEFEEEEGEDEA

*The constructs, labeled with green fluorescent protein (GFP), were transfected into HeLa cells. The number indicates the percentage of HeLa cells in which the constructs were incorporated into microtubules.
†In the study by Lefèvre et al. (2011), the percentage of cells was not formally counted, but it appeared from the text that all of the GFP-labeled α wild-type and αΔ13 were incorporated into microtubules while little or none of the αΔ23 was incorporated.
References: Intron locations (Perumal et al., 2005); *Chlorella* (Yamada et al., 1993); *Eimeria* (Zhu and Keithly, 1996); *Toxoplasma* (Nagel and Boothroyd, 1988); *Cylicocyclus* (von Samson-Himmelstjerna et al., 2002); α truncations (Lefèvre et al., 2011); βI and βIII truncations (Joe et al., 2009); subtilisin peptides (Redeker et al., 1992b).

tubulin-binding cofactors could have entered the picture. We are talking about no less than seven proteins that are required to allow the α/β dimer to form. These proteins include prefoldin, CCT, and tubulin-binding cofactors A–E (Table 2.12) (Yaffe et al., 1992). Where did these factors come from? What were they before they chaperoned tubulin? We will examine them in turn; it is possible that each one was a kind of primitive MAP, mediating the functions of the ancestral tubulin.

3.3.5.3.1. CCT. CCT is thought to have coevolved with its favorite substrates, actin and tubulin (Llorca et al., 2000; Archbald et al., 2001; Dunn et al., 2001; Li and Wu, 2003). Unlike the other associated proteins, it hydrolyzes ATP to fold its substrates (Melki, 2001). It is interesting that tubulin interacts with at least three of the subunits of CCT (Llorca et al., 2000; Muñoz et al., 2011). This suggests a very long-standing connection between CCT and tubulin. Perhaps the ancestor of CCT bound to those regions of tubulin to which CCT binds at the present day (Ritko-Vonsovici and Willison, 2000; Llorca et al., 2001). It may very well be that CCT arose as an associated ATPase that helped to mediate the functions of both actin and tubulin. One could further postulate that actin and tubulin, in switching from monomeric to polymerized states, were unique in undergoing conformational changes as part of normal function. Conceivably, CCT may have bound to the proteins in either or both conformational states and could have, without changing its function too much, accelerated the conformational change or helped ensure that temporary exposure of hydrophobic areas did not lead to nonspecific aggregation. Since we are postulating that the ancestral tubulin operated in the proto-centrosome, it is likely that these factors were in the centrosome as well. It is of great interest, therefore, that several of the subunits of CCT, specifically CCT1, CCT2, CCT4, CCT5, and CCT8, are located in the centrosome to this day; CCT1 is localized specifically to the "mother" centriole, just as is ε-tubulin (Seo et al., 2010). Another chaperonin, derived from CCT, is located in the centrosome as well, as is the chaperonin Hsp90 (Lange, 2002; Kim et al., 2005). It is intriguing that these chaperonins form a complex with some of the Bardet–Biedl complex proteins (Seo et al., 2010). Lesions in these proteins cause the disorder called Bardet–Biedl syndrome, and it is thought that these proteins form a structure, the BBSome, which is transported along the axonemes of primary cilia in early development (Jin and Nachury, 2009). Essentially, what Seo et al. (2010) have shown is that chaperonins can mediate the interaction between MTs of the axoneme and the BBSome proteins, acting, in a sense, like MAPs.

Table 2.12 Stages in the folding of α- and β-Tubulin to form the α/β dimer*

α-Tubulin	β-Tubulin
Step 1. α + prefoldin → α-prefoldin	β + prefoldin → β-prefoldin
Step 2. α-prefoldin + CCT + ATP → α-CCT + prefoldin + ADP + P_i	β-prefoldin + CCT + ATP → β-CCT + prefoldin + ADP +P_i
Step 3. α-CCT + CoB → CoB-α + CCT	β-CCT + CoA → CoA-β + CCT
Step 4. CoB-α + CoE → CoE-α + CoB	CoA-β + CoD → CoD-β + CoA
Step 5. CoE-α + CoD-β + CoC → CoE-CoD-CoC-αβ	
Step 6. CoE-CoD-CoC-αβ + GTP → CoE + CoD + CoC + αβ + GDP + P_i	

*This starts with the α- and β-tubulin chains as they come off the ribosome.
References: (Melki, 2001; Llorca et al., 2001; Grynberg et al., 2003; Simons et al., 2004).

Chaperonins of the CCT family occur in Archaea, both Euryarchaea and Crenarchaea (Archibald et al., 2000, 2001; Archibald and Roger, 2002). The association of CCT with tubulin raises the possibility that the tubulin–CCT association arose after the Eubacteria diverged from the Archaea and the Eukaryotes, but it is clearly very ancient and is likely to have occurred in LECA.

3.3.5.3.2. Prefoldin. Prefoldin may have a similar story. Its substrates seem to be limited to actin and tubulin in eukaryotes and to FtsZ, MreB (prokaryotic analog of actin), and perhaps other proteins in Archaea (Hansen et al., 1999; Leroux et al., 1999; Siegert et al., 2000; Li and Wu, 2003). Prefoldin, which is shaped like a jellyfish, appears to connect with its substrates by its tentacles and hand them off to CCT (Siegert et al., 2000; Martín-Benito et al., 2002; Simons et al., 2004). Prefoldin interacts with tubulin at two sites, that are homologous in α-, β-, and γ-tubulin (Rommelaere et al., 2001). This observation raises the possibility that prefoldin interacted with the common ancestor of α-, β-, and γ-tubulin, perhaps connecting it with other filaments. As we have argued for CCT, prefoldin could have been a filament-associated protein that mediated the functions of actin and tubulin.

3.3.5.3.3. Tubulin Cofactors A–E. Based on their current association with β-tubulin in the folding process, it is possible that tubulin cofactors A and D were associated with β, perhaps mediating the function of the αβ ancestor (Weisbrich et al., 2007). Parts of tubulin cofactor A bear a distant resemblance to spectrin, a cytoskeletal protein (Guasch et al., 2002; Djinovic-Carugo et al., 2002). Also, cofactor A binds to native rather than unfolded tubulin, raising the possibility that it could have bound to an ancestral

tubulin filament rather than starting out as a chaperone (Steinbacher, 1999). Tubulin cofactors B and E interact with α and, interestingly, with its C-terminal domain (Weisbrich et al., 2007). In other words, addition of the C-terminal domain could have introduced these two cofactors into the picture. Finally, tubulin cofactor C interacts with the α/β dimer; it is possible that this, too, once bound to the αβ ancestor. The N-terminal domain of cofactor C has a distant connection to spectrin, while its C-terminal domain resembles an adenylyl cyclase-associated protein that can bind to actin (Grynberg et al., 2003). Cofactor D has an armadillo domain characteristic of proteins that interact with the cytoskeleton (Peifer et al., 1994; Grynberg et al., 2003). The fact that the domain structures of the tubulin cofactors are very different is consistent with a complex descent (Grynberg et al., 2003). These observations support the hypothesis that the tubulin cofactors evolved from proteins involved with an ancestral tubulin that participated in formation of a filamentous structure.

My hypothesis is that the tubulins arose as filamentous polymers constituting the proto-centrosome of the first eukaryotes. If these hypotheses are true, then one would expect that some of the proteins with which the first tubulins interacted in the proto-centrosome could still be present in the descendants of this organelle today. It is of great interest, therefore, to find that proteomic analyses of basal bodies and centrosomes reveal the presence of a large number of such proteins, including chaperonins, dynein, 14-3-3, and even fibrillarin, which has been reported to bind to the βI isotype in mammals (Yanagida et al., 2004; Keller et al., 2005; Kilburn et al., 2007). Several forms of thioredoxin, which may interact with tubulin, are present as well (Kilburn et al., 2007). Interestingly, human and protist cilia contain some of these proteins also, including chaperones and 14-3-3 (Ostrowski et al., 2002; Pazour et al., 2005).

The concept of chaperones being related to MAPs is not totally unprecedented. It is interesting that dyneins, which have long been associated with MTs, are related to some prokaryotic proteins, the MoxR family; these are ATPases that function as chaperones (Mitchell, 2007). The likelihood that an MT-associated motor and a chaperone protein can have a common ancestor is consistent with the hypothesis that the tubulin-binding cofactors could be derived from tubulin-binding proteins.

3.3.5.4. Microtubule Formation

We are postulating that αβ dimers formed filaments that tugged on the DNA as has been proposed by McIntosh et al. (2010). If γ-tubulin rings

were in existence, one could imagine these filaments being anchored to them; this would permit several filaments to pull on the DNA at once. Under these conditions, DNA molecules could get larger and carry more genes; hence, evolution would favor these arrangements. Further strength would be contributed by mutations that allowed for bundling of the filaments. It is possible that the negatively charged C-termini would drive the filaments to join with each other via hydrophobic interactions. The presence of the γ-tubulin ring at the base of this structure would have determined that MTs contain 13 protofilaments. When MTs are formed in the absence of a γ-tubulin ring in vitro, they often have different numbers of protofilaments (McEwen and Edelstein, 1977; Evans et al., 1985; Chrétien et al., 1992). In short, formation of MT could be inevitable given the binding to the γ-tubulin ring.

3.3.5.5. Origin of γ-Tubulin

Prosthecobacter may have one further lesson to teach us, namely, about the origin of γ-tubulin. Currently, γ-tubulin has to interact with several other proteins, including perhaps a chaperonin, to form a ring (Geissler et al., 1998). It is likely that the acquisition of this entourage was something that happened to γ-tubulin when it diverged from the common ancestor of α, β, γ, and the *Prosthecobacter* tubulins. Conceivably, forming a ring could have aided γ-tubulin in its role in the proto-centrosome and then, fortuitously, the γ-tubulin ring served to nucleate MTs.

3.3.6. Kinesins

Somewhere during this development, kinesins would have become associated with tubulin. In the curving protofilament model, GTP hydrolysis could initially have provided all the energy needed to separate DNA molecules during mitosis. Eventually, however, DNA molecules grew larger and more force was required to separate them. Such force could have been provided by kinesin-13, a form of kinesin that does not mediate transport but rather depolymerizes MTs, and uses ATP to power bending of tubulin protofilaments (Moores et al., 2002; Mennella et al., 2009; Domnitz et al., 2012). Kinesin would have added the energy of ATP hydrolysis to that of GTP hydrolysis generated by the protofilament itself. Kinesin-13 found in kinetochores binds to curved protofilaments (Mulder et al., 2009; Endow et al., 2010); it may have been able to help separate DNA molecules even in the absence of MTs. Later, as MTs appeared, other kinesins, such as kinesins-5, -8, and -14, joined the process and helped to establish the complex

mitotic spindle of the present day (Ferenz et al., 2010; Woodruff et al., 2010; Civelekoglu-Scholey et al., 2010; Hentrich and Surry, 2010). In some organisms, such as yeast, the coordination among the kinesins led to replacement of kinesin-13 by kinesins-8 and -14 (EmsMcClung and Walczak, 2010; Chen et al., 2011).

If MTs were preceded by filaments aided by kinesin-13 to separate DNA molecules, then kinesin-13 must have existed in the earliest eukaryotes as well. However, family trees of kinesins do not put kinesin-13 at the root: kinesin-13 is a minor branch of a very robust tree (Lawrence et al., 2002; Dagenbach and Endow, 2004; Miki et al., 2005). Most of the kinesins are present among all the eukaryotes, hence, the evolution of the kinesin family probably took place in LECA long before the appearance of MTs (Iwaba and Miyata, 2002; Wickstead et al., 2010). Based on the family tree, the ancestral kinesin is unlikely to have been an MT depolymerizer. It may have been an ATPase that "walked" on some kind of filamentous structure, perhaps carrying vesicles. The kinesins at the root of the kinesin family at the present day can cross-link MTs, thus acting like MAPs (Lawrence et al., 2002; Seeger and Rice, 2010; Rank et al., 2012) raising the possibility that kinesin could have accelerated the protofilament bundling that led to MT formation. If the earliest kinesins arose in the absence of MTs, it is possible that they used the energy of ATP hydrolysis to cross-link filaments made from the tubulin ancestor to build the proto-centrosome. Some of the kinesins perhaps bound DNA directly as well. Kinesin itself may have evolved from a G-protein, with ATP replacing GTP as the bound nucleotide (Kull et al., 1998). Similarities in mechanism between kinesin and small GTPases have been noted (Dietrich et al., 2008).

An interesting question is the nature of the binding of kinesin to tubulin. Kinesin-1 appears to bind largely at the C-terminus and this binding is required for it to induce MT depolymerization (Niederstrasser et al., 2002; Seeger and Rice, 2010). Since, as will be argued below, the C-terminus is probably a late addition to tubulin, if the same is true for kinesin-13, then the participation of this or any other kinesin in MT function would likely have occurred at the time the MT appeared, rather than earlier as was argued above. However, there is also evidence that kinesin binds elsewhere than at the C-terminus (Rodionov et al., 1990; Marya et al., 1994; Skiniotis et al., 2004). In fact, binding at the C-terminus activates the ATPase activity of kinesin, while binding elsewhere does not; this is particularly true of the C-terminus of β-tubulin (Skiniotis et al., 2004). This implies that kinesin

could have acted as a cross-linking element in the proto-centrosome, only acquiring its ability to depolymerize MTs when tubulin picked up its C-terminus. Kinesin appears to bind to both α and β; raising the possibility that it bound to the αβ ancestor (Larcher et al., 1996). On the other hand, other evidence suggests that kinesin binds to β significantly better than it does to α, so it is possible that kinesin originally bound to β (Song and Mandelkow, 1993; Uchimura et al., 2006).

Less is known about the binding of dynein to tubulin. Its binding site overlaps with that of kinesin and it is known to include the C-terminus (Mizuno et al., 2004; Vent et al., 2005). It may be that dynein, like kinesin, binds both at the C-terminus and elsewhere and that its interaction with tubulin could have begun at the same time as kinesins. Alternatively, if dynein only binds at the C-terminus, then the dynein–tubulin interaction would have begun later.

3.3.7. Origin of Centrioles, Basal Bodies and Axonemes

Once MTs appeared, it was not a quick step to appearance of the highly organized centrioles and basal bodies. This subject has been reviewed in recent years (Hartman, 1993; Azimzadeh and Bornens, 2004; Martinez-Campos et al., 2004; Mitchell, 2004, 2007; Richards and Cavalier-Smith, 2005; Satir et al., 2007; Bornens and Azimzadeh, 2007; Marshall, 2009; Azimzadeh and Marshall, 2010; Carvalho-Santos, 2011). Centrioles and basal bodies are almost interchangeably similar to each other (Dirksen and Crocker, 1965; Dirksen, 1991; Dutcher, 2003b). The basal body is essential to forming the axoneme; the centriole is at the core of the mitotic spindle but is not absolutely necessary for its formation, although its presence is thought to increase the fidelity of chromosome separation (Marshall, 2007). Of the two centrioles in a centrosome, one, the "mother" centriole, nucleates MTs of the mitotic spindle and the interphase network; the "mother" can also move to the cell periphery and act as a basal body (Lange, 2002; Rebollo et al., 2007; Rusan and Peifer, 2007; Strnad and Gönczy, 2008; Lonczak and Khodjakov, 2009; Kobayashi and Dynlacht, 2011).

It may be illuminating to see how the centriole/basal body develops at the present time. The centriole appears to start with a hub, around which grow nine spokes. The ends of the spokes are connected to form a cartwheel of ninefold symmetry. At the end of each spoke, singlet MTs appear, which turn into doublets and then into triplets (Allen, 1969; Anderson and Brenner, 1971; Pearson and Winey, 2009). ε-Tubulin is necessary for the doublets and δ-tubulin for the triplets to form (Pearson and Winey, 2009).

It is likely that the original MT could have helped organize filaments in its vicinity to bind to the DNA or RNA and thus helped to organize cell division. Using proteomics approaches, looking through many taxa to see which proteins are most commonly associated with centrioles, a set of about 14 "core proteins" has been identified that are very likely to have coevolved with MTs; these may originally have been proteins binding to LCFTA and mediating its functions (Lange, 2002; Andersen et al., 2003; Dobbelaere et al., 2008; Hodges et al., 2010). One of these proteins is tektin, a member of the intermediate filament protein class, which is thought to form a filament running parallel to the MT. Tektin is found in axonemes, basal bodies, centrioles and the midbody (Setter et al., 2006; Duncan et al., 2008). One of the repeating units within tektin is the length of the tubulin dimer, suggesting that tektin could regulate the length of the MT (Amos, 2008). This might have helped shape the centriole, whose triplet MTs are all the same length (Dirksen, 1991). The intermediate filaments have equivalents among the prokaryotes (Ausmees et al., 2003), so it is quite possible that tektin was associated with the ancestral tubulins, including the proto-centrosome, from the very beginning. Based on an extensive survey, Carvalho-Santos et al. (2010) propose that three other proteins would have appeared very early: these are SAS6, which nucleates the cartwheel and is essential for the ninefold symmetry, BLD10/CEP135, which is also necessary for cartwheel formation, and SAS4/CPAP (Loncarek and Khodjakov, 2009; Pearson et al., 2009; Jerka-Dziadosz et al., 2010; Van Breugel et al., 2011). Later, depending on the taxon, other proteins would have been recruited; for example, in animals and fungi, SAK/PLK4, CP110 and SPD/CEP192 might have appeared, the latter perhaps involved in organizing the pericentriolar matrix (Carvalho-Santos et al., 2010). Centrin and centrobin, which have roles in centriole duplication, would have appeared early as well (Pearson et al., 2009; Gudi et al., 2011). CEP110 and ninein promote centrosome maturation (Ou et al., 2002). Other than tektins, none of these proteins appear to have prokaryotic homologs, so the earliest centriole proteins may have arisen in the first eukaryote. A variety of specific proteins are involved in transforming a centriole into the very similar basal body (Hoyer-Fener, 2010). Given the ninefold symmetry of the cartwheel, MTs could have formed at the end of the spokes. Tektin filaments lying alongside the singlets could have allowed for doublet and triplet formation, with the tektin filaments perhaps forming some of the protofilaments in the region where the incomplete B-tubule touches the A-tubule, or where the C-tubule touches the B-tubule (Setter et al., 2006). Pericentrin, which binds to γ-tubulin, is another component

that forms the pericentriolar matrix and could have existed long before the centriole appeared (Dictenberg et al., 1998; Bornens, 2002; Dammermann and Merdes, 2002; Nigg, 2007). Once MTs appeared, it is possible that the inherent flexibility of the tubulin molecule could have contributed to a gradient of conformational effects that may have influenced the binding of various associated proteins and created the apparently complex geometry of MT-based structures (Roth and Pihlaja, 1977). Modeling based on measurements of the dynamic behavior of MTs made from specific isotypes suggests that the isotypic composition can affect the nature of tubulin isotypes that add on to growing MTs (Rezania et al., 2008); in short, forming MTs allows for a wide range of functional complexity.

Once the centriole formed, one model proposes that its location near the membrane could have allowed formation of the axonemal MTs (Jékely and Arendt, 2006). Another model proposes that the centriole nucleated MTs that grew into a Golgi vesicle and that this assemblage then moved toward the membrane where the vesicle fused with the cellular membrane and MTs in it became the axonemal MTs (Molla-Herman et al., 2010). The latter model could easily explain why the ciliary membrane differs in its lipid content from the other cellular membranes, being enriched for sterols, glycolipids and sphingolipids (Satir and Christensen, 2007; Tyler et al., 2008; Emmer et al., 2010); this would imply a higher degree of order in ciliary membranes. One can imagine the appearance of a set of proteins which would connect the base of the axoneme with the membrane, and inhibit exchange of lipids between the axonemal membrane and the cell membrane (Craige et al., 2010). The first cilia could have been primary cilia with nine outer double MTs and no central pair (9 + 0). These would have functioned largely as sensory receptors (Davis et al., 2006). Appearance of motor proteins such as dynein could have led to gliding motility (Mitchell, 2004). The central pair MTs and their associated radial spoke proteins would have allowed for and regulated the beating of the cilia, leading to the current 9 + 2 structure.

Other hypotheses for the origin of cilia have been put forward. One is that the evolution of the cilium drove that of the centriole/basal body. In other words, a group of MTs in the cilium would have allowed for gliding motility. Doublet MTs would have been favored because they were stronger (Mitchell, 2004). Another idea is that the cilium was a 3 + 0 structure, then 6 + 0 and then 9 + 0, each one providing greater motile power than the one before (Hartman, 1993). Implicit in these ideas is that the proto-centrosome

was an amorphous entity that developed MTs as a response to help anchor the axonemal MTs, with the centriolar MTs becoming doublets as the axonemal MTs became doublets and acquiring ninefold symmetry as the number of doublets in the axoneme rose to nine. However, this does not explain why the centriole/basal body requires triplet MTs, nor is it consistent with the work of Van Breugel et al. (2011) who find strong evidence for ninefold symmetry in the core of the centriole.

Another hypothesis is that the centriole/basal body originated as a virus particle, which was incorporated into a host cell; by this hypothesis, the viral genome would have encoded γ-, δ-, ε-, η-, and ζ-tubulins as well as tektins and other proteins specific to the centrosome/basal body and required for its assembly (Satir et al., 2007). The idea is that the virus would have been able to direct formation of a cartwheel structure and that the host would have contributed α- and β-tubulins as well as the intraflagellar transport and motor proteins. The central pair MTs could then have arisen as misplaced cytoplasmic MTs that ended up anchored in the axoneme. This hypothesis, which makes a valuable contribution, is consistent with the idea that viruses are coeval with life and played a role in major events in the history of life (Forterre, 2006) and also has the advantage of accounting for the occasional RNA molecules that seem to be associated with the centriole (Hartman, 1975) and addresses the central pair MTs, whose origin is somewhat difficult to explain. However, it does not explain what forces would have caused the evolution of the γ-, δ-, ε-, η-, and ζ-tubulins in the virus. It also implies that α- and β-tubulins would have evolved in a host cell separate from γ-, δ-, ε-, η-, and ζ-tubulins. Although this is not impossible, it may be simpler to try to explain the evolution of the whole tubulin superfamily in one coherent story. By the story advanced here, the presence of RNA associated with the centriole may be evidence not that the centriole was once a separate organism, but rather that the proto-centrosome was connected to the genome, perhaps when it was still made from RNA.

A close look at the centrosomal-associated RNA (cnRNA) could perhaps illuminate this issue; so far cnRNA has only been observed in animals, specifically, humans, *Xenopus*, *Drosophila*, and the mollusks *Ilyanassa* and *Spisula* (Groisman et al., 2000; Lambert and Nagy, 2002; Alliegro et al., 2006; Lécuyer et al., 2007; Kingsley et al., 2007; Blower et al., 2007). Some RNA binds to MTs of the mitotic spindle and is apparently actively engaged in translation while so doing (Blower et al., 2007). In *Spisula*, there are five species of cnRNA, one of them apparently encoding an RNA-dependent

nucleotide polymerase; some of these cnRNAs may not be encoded in the nuclear DNA, raising the possibility that the cnRNA is an independent entity, rather than an RNA that happens to bind to the centrosome (Alliegro et al., 2006; Alliegro, 2008; Alliegro and Alliegro, 2008). In *Ilyanassa*, about 3–4% of the cellular RNA is cnRNA (Kingsley et al., 2001). The 16 identified cnRNAs encode three zinc finger proteins (which bind to DNA), ankyrin (a protein linking the membrane to the cytoskeleton (Bennett and Stenbuck, 1979)), and an RNA-binding protein (Kingsley et al., 2001). Some of the cnRNA is poor in introns, in that respect, but no other, resembling prokaryotic genomes (Alliegro and Alliegro, 2008). Some of these findings are consistent with the hypothesis of an independent origin for the centrosome (Alliegro, 2008). On the other hand, if we hypothesize, as we have here, that tubulin arose in an early eukaryotic cell where it may have played a role in binding to membranes or to the nucleic acid genome, it is striking that cnRNA encodes proteins that do exactly these things. By this argument, cnRNA in the organisms where it appears to occur could be a vestige of a very ancient function of the primitive tubulin ancestor. This line of reasoning is only strengthened by the possibility that the earliest cells could have had a genome made of RNA (Gilbert, 1986). In evaluating these arguments, however, we should recall that cnRNA has only been seen in animals and in a relatively small number of animals at that. cnRNA could be a separate entity that arose multiple times to serve particular functions in different lines of animals; the fact that, in cell division in *Ilyanassa*, cnRNA localizes with only one of the daughter centrosomes does suggest some specificity of function (Kingsley et al., 2001).

3.3.8. Kinetochores

Although much speculation has gone into the evolution of the centriole, the evolutionary history of the organelle at the other end of MTs, namely, the kinetochore, is more obscure. The kinetochore, which in essence connects the MT plus end with the DNA, consists of an inner and an outer plate surrounded by a fibrous corona (Rieder, 1982; Rieder and Salmon, 1998; Maney et al., 1999; Maiato et al., 2004); the kinetochore varies in composition among organisms. A variety of approaches have been used to analyze the structure of the components of the kinetochore; it is likely that as many as 40–50 proteins constitute the kinetochore (Okai et al., 2004; Wang et al., 2007; Dunsch et al., 2011; Maskell et al., 2010; Petrovic et al., 2010; Hua et al., 2011; Suzuki et al., 2011; Espeut et al., 2012). Some of the components of the kinetochore are apparently highly conserved in evolution

(Hua et al., 2011). A broader analysis of the distribution of the kinetochore components may generate a clearer idea of how the kinetochore evolved. Current knowledge may offer us clues. First, as already mentioned, the kinetochore is the site of the as-yet-uncharacterized fibrils that link the kinetochore to the curved protofilaments at the end of the MT (McIntosh et al., 2008). These fibrils point to a very ancient mechanism by which tubulin filaments could help to separate chromosomes, a mechanism that could antedate MTs themselves (McIntosh et al., 2010). Second, farnesylation of CENP-E and CENP-F occurs in the kinetochore and is required for binding to MTs and for cell cycle progression (Ashar et al., 2000; Hussein and Taylor, 2002). Normally, attachment to a protein of the highly hydrophobic farnesyl group, an isoprenoid derivative, allows the protein to be anchored to a membrane (Kitten and Nigg, 1991; Zhang and Casey, 1996; Dong et al., 2002). CENP-E is a kinesin-like motor protein, classified as kinesin-7, which helps separate the chromosomes during mitosis (Yen et al., 1992). CENP-F plays a role in regulation of the cell cycle (Rattner et al., 1994). With the possible exception of the nuclear envelope, it is not clear that the kinetochore interacts with a membrane, and in the case of CENP-E, one would imagine that anchoring it to a membrane would impede its motor function. In short, it is possible that farnesylation of these two proteins is a relict of a time when the tubulin filaments and their apparatus separating the DNA may have been connected to a membrane. Since then, the membrane association may have been lost but some of the other components may have bound to the farnesyl group and thus retention of this modification was favored.

3.3.9. Did Microtubules Originate in Spirochetes

The proposal has been made that tubulin and MTs originated in spirochetes which then became ectosymbionts in early eukaryotes to which they conveyed these organelles (Szathmary, 1987; Margulis et al., 2006). Three spirochetes have had their genomes sequenced: *Borrelia*, *Leptospira*, and *Treponema*; each of these encodes FtsZ but no tubulin (Picardeau et al., 2008; Han et al., 2011; Porcella et al., 2011). A spirochete protein that reacts with antibodies to tubulin is related to the chaperone Hsp65 (Munson et al., 1993). It appears likely that the spirochetes separated from other bacteria after the FtsZ/tubulin divergence. However, MT-like structures have been seen in a variety of prokaryotes although the proteins that compose them have not been identified (Bermudes et al., 1994); should these proteins turn out

eventually to form part of the FtsZ-tubulin superfamily, then the arguments above may need to be modified.

4. THE ORIGIN OF THE C-TERMINAL TAIL OF TUBULIN: BACK BEFORE THE BEGINNING?

The C-terminal domains of α- and β-tubulin have a unique combination of properties. First, they are highly negatively charged. Second, they are the sites where many MAPs, motors and other proteins bind to tubulin; as such, they play major roles in regulation of MT assembly, dynamics and function. Third, they are the sites of a variety of posttranslational modifications, some of which, namely, polyglutamylation, polyglycylation, and detyrosination/tyrosination, are highly unusual. Finally, as mentioned above, the presence of the C-terminal domain both destabilizes tubulin's conformation and determines that tubulin will form MTs and not some other kind of polymer.

In this section of this chapter, the argument will be made that the C-terminal domain of α- and β-tubulin may have evolved as a separate entity. There are four reasons underlying this argument. First, none of the other members of the tubulin superfamily or their more distant prokaryotic relatives have a domain like this. Even *Prosthecobacter* BtubA and BtubB, which we have argued, are the closest descendants of the common αβ ancestor, lack the C-terminal domain, suggesting that the C-terminal domain was added after BtubA and BtubB diverged from the common αβ ancestor. Second, although absent in many eukaryotes, certain organisms still retain an intron between the exon encoding the C-terminal domain and the rest of the tubulin gene. The actual presence or absence of introns in αβ can by itself be used to construct a family tree; it is thought that the original α and β had multiple introns, which have gradually disappeared, over the course of evolution (Perumal et al., 2005). There is one intron, in particular, found in the β-tubulin genes of the protists *Eimeria* and *Toxoplasma* and the nematode *Cylicocyclus* that is placed such that the C-terminal domain is precisely that which we have found retains the few residues required to determine MT assembly as well as the highly negative residues (Table 2.12) (Perumal et al., 2005; Joe et al., 2009). In addition, there are similarly placed introns in many of the other β-tubulins as well as in α-tubulin (Perumal et al., 2005). The third reason arises from an analysis of tubulin's posttranslational modifications and will be discussed in more detail below. Fourth, analysis of the C-terminal domain as an independent entity suggests that it could be even more ancient than the tubulin/FtsZ ancestor; this will also be discussed below.

4.1. Evolutionary Significance of Posttranslational Modifications of Tubulin

Tubulin undergoes a wide variety of posttranslational modifications (Table 2.13). The mechanisms and functions of these modifications have been reviewed very well recently (Janke and Bulinski, 2011; Garnham and Roll-Mecak, 2012) and need not be repeated here, where the main emphasis is on the relevance of these modifications to the evolution of the tubulin molecule and of its C-terminal region in particular. In assessing the experiments that address functions of posttranslational modifications, it is important to keep in mind that biochemical experiments are generally set up to obtain answers to yes/no questions, such as "Does blocking this modification affect this process?" It is always possible that the various posttranslational modifications may have functions that are as yet totally unknown.

As Table 2.13 shows, the number of different kinds of posttranslational modifications of tubulin is impressively large, perhaps more than that of any other protein. Focusing on the likely evolutionary history of the modifications, however, it is clear that some are rather narrowly distributed in the eukaryotic world. For example, even though phosphorylation of tubulin is widespread, the specific residues that are phosphorylated can be very variable and there is no strong indication of conservation of this modification outside of a particular phylum, let alone a kingdom. Thus, it is likely that many of these modifications arose during the evolution of a particular class or phylum of organisms and cannot speak to the status of posttranslational modification of the ancestral tubulin. That said, however, there are four modifications that appear in all the kingdoms except the fungi. Since fungi appear to lack all of the modifications of tubulin and since they are probably an offshoot of the animals (Baldauf, 2003), the most probable explanation is that the LECA had these four modifications and that the fungi lost them. Three of these modifications occur exclusively in the C-terminal domain and they shall be discussed in detail; the exception is acetylation, which merits a brief discussion of its own.

4.1.1. Acetylation

α-Tubulin is acetylated at lysine 40; except in fungi, this residue is highly conserved in evolution, even in the primitive protest *Giardia* (Campanati et al., 1999; Ludueña and Banerjee, 2008a). The enzymes that add and remove this acetyl group are themselves highly conserved in evolution, suggesting that this is an important modification; interestingly, the acetyltransferase involved appears to be specific for tubulin (North et al., 2003; Akella

Table 2.13 Posttranslational Modifications of Tubulin

Modification	Subunit/site	Animals	Plants	Fungi	Protists
C-terminal					
Polyglutamylation	α	Yes	Yes	No	Yes
Polyglutamylation	β	Yes	No	No	Yes
Polyglycylation	α	Yes	No	No	Yes
Polyglycylation	β	Yes	No	No	Yes
Detyrosination/ tyrosination	α	Yes	Yes	No	Yes
Deglutamylation	α (penultimate glu)	Yes	No	No	Yes
Phosphorylation	β/S441, S444	Yes	No	No	No
Phosphorylation	α/C-term Tyr	Yes	No	No	No
VA removal★	βIVb	Yes	No	No	No
Methylation†	α/E434	No	No	No	Yes
Methylation†	β/D or E	No	No	No	Yes
Δ5‡	α	No	No	No	Yes
Elsewhere					
Acetylation	α/K40	Yes	Yes	No	Yes
Palmitoylation	α/C376	Yes	No	No	Yes
Phosphorylation	α or β/S,T,Y§	Yes	Yes	No	Yes
SUMOylation	α, β	Yes	No	No	Yes
Ubiquitinylation	α, γ	Yes	No	No	No
Arginylation	α8/S114	Yes	No	No	No
Arginylation	β6/A64, E125	Yes	No	No	No
Glycosylation	α, β	Yes	No	No	No
Methylation	α/K				
Lysinoalanine cross-linking	α, β	Yes	No	No	No
Isoaspartylation	α, β	Yes	No	No	No
Polyamination	α, β	Yes	Yes	No	No
Nitration	α, β	Yes	No	No	No
ADP-ribosylation	α, β	Yes	No	No	No

★The removal of the last two residues (VA) in the βIVb isotype of tubulin was prominent in liver cancer in rats (Miller et al., 2008). There is no evidence for any other occurrence.
†So far, this modification has been seen only in *Toxoplasma gondii* (Xiao et al., 2010). Its functional significance is unknown.
‡So far, this modification, removal of the last 5 residues of α, has been seen only in *Toxoplasma gondii* (Xiao et al., 2010). Its functional significance is unknown.
§Human α-tubulin can be phosphorylated at S165 and pig brain β-tubulin at T409 and S420 (Yoshida et al., 2003; Abeyweera et al., 2009).
References: (Amir-Zaltsman et al., 1982; Wandosell et al., 1987; Correia et al., 1993; Najbauer et al., 1996; Prasad and Dey, 2000; Hino et al., 2003; Yoshida et al., 2003; Plessmann et al., 2004; Starita et al., 2004; Rosas-Acosta et al., 2005; Iwabata et al., 2005; Dremina et al., 2005; Verhey and Gaertig, 2007; Wong et al., 2007; Ludueña and Banerjee, 2008a; Cicchillitti et al., 2008; Miller et al., 2008; Abeyweera et al., 2009; Del Duca et al., 2009; Song et al., 2009,2010; Wloga and Gaertig, 2010; Xiao et al., 2010).

et al., 2010; Shida et al., 2010). Acetylation is apparently always associated with stable subsets of MTs, such as those of axonemes, centrioles and basal bodies (LeDizet and Piperno, 1986; Fouquet et al., 1994; Boggild et al., 2002; Bane et al., 2002; Matsuyama et al., 2002). One can readily imagine that a modification that promotes MT stability would be important in the formation of centrioles and basal bodes and that since, as has been argued above, these structures were present in LECA, it is likely that acetylation was as well. It is perhaps significant that the set of other proteins that are modified by acetylation of lysines is enriched for proteins that interact with nucleic acids, such as histones, transcription factors and ribosomal proteins and even occurs in an archaeal DNA-binding protein (Polevoda and Sherman, 2002; Wardleworth et al., 2002; Naryzhny and Lee, 2004; Odintsova et al., 2003). This observation is consistent with our hypothesis that the ancestral tubulin interacted directly or indirectly with nucleic acids. Given the arguments made above that the αβ ancestor was unstable, it is reasonable to speculate that tubulin acetylation could have arisen to counter this. In addition to promoting MT stability, tubulin acetylation is required for inhibition of MT-associated membrane Na^+, K^+-ATPase as well as promoting sliding of the endoplasmic reticulum and interaction of the latter with mitochondria (Casale et al., 2001; Santander et al., 2006; Arce et al., 2008; Friedman et al., 2010). Whether these functions also played a role in LECA is not clear.

4.1.2. Detyrosinolation/Tyrosinolation

Most α-tubulins in animals, plants and protists—but not fungi—are synthesized with a C-terminal tyrosine, preceded by a glutamate; in most of these organisms, the C-terminal tyrosine is removed by a carboxypeptidase (Kumar and Flavin, 1981). What is unusual is that the tyrosine can then be added back by the action of a tubulin–tyrosine ligase with the concomitant hydrolysis of ATP (Barra et al., 1974). Synthesis of this peptide bond (α/α linkage) involves neither ribosomes nor tRNA. In short, α-tubulin molecules are able to cycle by addition or removal of the C-terminal tyrosine; tyrosinolation generally occurs on the free tubulin dimer, while detyrosinolation happens to polymerized tubulin (Wehland and Weber, 1987; Webster et al., 1987). There are some exceptions to this story. Certain α isotypes (α4 in vertebrates) are synthesized with a C-terminal glutamate, but they can be tyrosinolated by the ligase (Gu et al., 1988). The unusual α8 isotype in vertebrates is synthesized with a C-terminal phenylalanine and is subject to neither the carboxypeptidase nor the ligase, hence removing itself from this cycle (Stanchi et al.,

2000). Finally, it is worth mentioning that in some animals, the penultimate glutamate is subject to removal also, creating Δ2-tubulin which, since the glutamate is never restored, is permanently removed from the cycle (Paturle-Lafanechère et al., 1991; Mary et al., 1996).

This leads to two questions: 1) what is the function of the C-terminal tyrosine and 2) what is the purpose of removing it? One answer to the first question is that the presence of the C-terminal tyrosine permits interaction with important CAP-Gly proteins such as CLIP-115 and $p150^{glued}$ (Peris et al., 2006). We have already noted that the tubulin-binding cofactors B and E require the presence of the C-terminal tyrosine in order to bind to tubulin and we have speculated that when the C-terminal region was added to the rest of the tubulin molecule, it could have brought these chaperones with it (Weisbrich et al., 2007). The fact that α-tubulin in the primitive protist *Giardia* has a C-terminal tyrosine that does not get removed (Weber et al., 1997) is consistent with the idea that there was a particular early function for this residue. Obviously, the detyrosinolation/tyrosinolation cycle allows for regulation of the binding of CAP-Gly proteins and this could certainly be one of its purposes. In addition, there is considerable evidence that detyrosinolation and tyrosinolation of tubulin are associated, respectively, with increased and decreased MT stability (Kreis, 1987; Khawaja et al., 1988), so it is possible that this cycle is one of the several mechanisms regulating MT dynamics. The effect is indirect, however: rather than the presence or absence of the C-terminal tyrosine directly affecting MT stability, its absence promotes the interaction of MTs with certain stabilizing proteins (Infante et al., 2000; Peris et al., 2009). It is therefore not surprising that tubulin is detyrosinolated in the centriole, which is a very stable structure (Fouquet et al., 1994). The asymmetric distribution of tyrosinolated and detyrosinolated tubulin in the outer doublet MTs of the axoneme suggests that this modification could regulate motility (Mary et al., 1996; Multigner et al., 1996; Péchart et al., 1999). These three possible functions (binding of CAP-Gly proteins, regulation of MT dynamics and regulation of axonemal motility) are all likely to be very ancient. However, other functions, such as the apparent critical role of tubulin–tyrosine ligase in neuronal function, specifically neurite morphogenesis, where different α isotypes differ in their tyrosinolation status, are probably more recent developments (Redeker et al., 1998; Erck et al., 2005).

4.1.3. Polyglutamylation

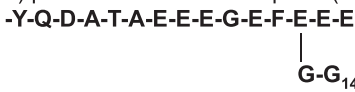
Polyglutamylation, first reported in 1990 (Eddé et al., 1990), involves addition of a glutamate to the γ-carboxyl group of a glutamate residue in the

C-terminal region of either α or β, via an α/γ linkage, followed by addition of several other glutamates to the first one via α/α linkages, although further glutamates can be added by α/γ linkages, resulting in branched side chains (Wolff et al., 1994). This modification is found in tubulins of animals, plants and protists including *Giardia*, *Plasmodium* and *Toxoplasma* (Rüdiger et al., 1992; Rüdiger and Weber, 1993; Bré et al., 1994; Mary et al., 1994a, 1994b, 1996; Weber et al., 1997; Péchart et al., 1999; Fennell et al., 2008; Hoyle et al., 2008; Xiao et al., 2010; Pathak et al., 2011) (Fig. 2.8). The precise residues that are glutamylated vary depending on the organism and the isotype; the number of glutamates added is also variable. In mouse brain α, up to six glutamates are added, in *Tetrahymena* α, up to 11 (Redeker et al., 1991; Wloga et al., 2008). Up to 16 glutamates can be added to mouse brain β in samples enriched for the glutamylating enzyme (Mukai et al., 2009), but whether this number is physiologically significant is not clear. Normally, in adult mammalian brains, the α1/2 isotype has four glutamates, α4 has five, βI has one, βII has five, βIII has six, and βIV has four (Alexander et al., 1991; Mary et al., 1996; Redeker et al., 1998, 2010). Neonatal brains have a lesser degree of polyglutamylation (Redeker, 2010). The degree of polyglutamylation varies greatly among different MT organelles. In general, stable MTs have more glutamates added than do less stable ones (Wolff et al., 1992; Audebert et al., 1993; Rao et al., 2001; Kann et al., 2003; Verdier-Pinard et al., 2003). Axonemal tubulin is always polyglutamylated (except in the morphologically unusual flagella of the sperm cells of certain insects), although the degree varies between α and β and with specific location within the axoneme (Plessmann and Weber, 1997; Pechart et al., 1999; Bobinnec et al., 1999; Mencarelli et al., 2005; Hoyle et al., 2008). Nonetheless, the highest degree of polyglutamylation occurs in centrioles and basal bodies where up to 17 glutamates can be added (Fouquet et al., 1994; Geimer et al., 1997; Bobinnec et al., 1998; Lechtreck and Geimer, 2000). Polyglutamylation is catalyzed by a family of tubulin–tyrosine-ligase-like (TTLL) proteins, one of whose members is the tubulin–tyrosine ligase that adds a tyrosine to the C-terminal end of α (Janke et al., 2005; Van Dijk et al., 2007; Janke and Bulinski, 2011). There are also less well-known deglutamylases that remove the glutamates (Kimura et al., 2010).

The functions of polyglutamylation are apparently complex. The degree of polyglutamylation has been shown to influence the binding of MAPs to tubulin; it could also allow for fine-tuning of functions (Boucher et al., 1994; Larcher et al., 1996; Bonnet et al., 2001; Janke et al., 2008). It has been shown to promote MT severing, a process whereby a severing protein

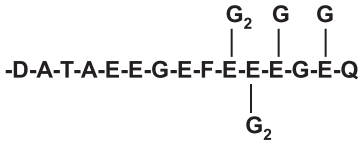
a) α-Tubulin from bovine sperm (Plessmann and Weber, 1997):

b) β-Tubulin from bovine sperm (Plessmann and Weber, 1997):



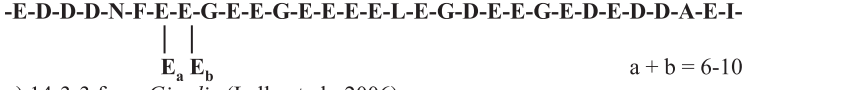
c) α-Tubulin from mouse brain (Redeker et al., 1991):

d) β-Tubulin from *Paramecium* (Vinh et al., 1999):



e) NAP-1 from HeLa cells (Regnard et al., 2000):

f) NAP-2 from HeLa cells (Regnard et al., 2000):



g) 14-3-3 from *Giardia* (Lalle et al., 2006):

Figure 2.8 ***Polyglutamylated and polyglycylated regions of α- and β-tubulins and NAP-1 and NAP-2.*** The number of glutamates or glycines in a chain is shown by the subscript. For the NAPs and 14-3-3, the specific number of glutamates or glycines attached at each of the modified residues is not known, but the total for each protein is known and the range is given.

such as spastin binds to the C-terminal end to reshape the tubulin molecule and cause the MT to fall apart (White et al., 2007; Lacroix et al., 2010). Both these roles suggest that polyglutamylation may have a role in regulating MT dynamics; a role in cell morphogenesis has also been proposed (Spiliotis et al., 2008). Furthermore, as is the case with detyrosinolation, polyglutamylation is unevenly distributed in axonemal MTs and may thus modulate axonemal motility (Péchart et al., 1999).

Polyglutamylation is an unusual modification, but it is not restricted to tubulin. Nucleosome assembly proteins (NAPs) 1 and 2 are also polyglutamylated. These proteins have glutamates added to the γ-carboxyl groups of glutamates 356 and 357 of NAP1 and to glutamates 347 and 348 of NAP2; up to 9 and 10 glutamates are added, respectively to NAP1 and NAP2 (Regnard et al., 2001) (Fig. 2.8). The glutamylated sequences, although not exactly at the C-terminus, are not far away from it; they are similar to the glutamylated segments of α- and β-tubulin in that they are rich in glutamates and contain aspartates and glycines (Regnard et al., 2001). NAP1 and NAP2 are thought to be chaperones for particular histones (Rodriguez et al., 1997; Adams and Kamakaka, 1999; Park and Luger, 2006). The protein PELP1 (proline–glutamate and leucine-rich protein) is also polyglutamylated, once again in a region rich in glutamates, although this phenomenon has been observed largely in pancreatic cancer cells; PELP1 is involved in chromatin remodeling and its polyglutamylation promotes cell growth (Kashiwaya et al., 2010). In addition to these two proteins, there is evidence that a large number of other proteins can be polyglutamylated (Van Dijk et al., 2008). This is based on two approaches: one is to overexpress the glutamylating enzyme in HeLa cells and observe which proteins have radioactive glutamate added; the second is to use a monoclonal antibody specific for polyglutamylated tubulin. In these experiments, the authors identified proteins, many of which are involved in chromatin shaping, nuclear-cytoplasmic trafficking, and transcription regulation. It is striking that most of the proteins subject to polyglutamylation interact directly or indirectly with DNA or RNA. Nevertheless, two points to remember are 1) if the modifying enzyme is present at an abnormally high concentration, it could conceivably interact with a protein that it does not normally modify, and 2) that an antibody that recognizes a certain modification in one protein, namely tubulin, may, in a different protein, recognize an epitope that does not have this modification. Further analysis using mass spectrometry with proteins purified from wild-type cells should make it definitively clear exactly which proteins are naturally polyglutamylated.

4.1.4. Polyglycylation

Polyglycylation is similar to polyglutamylation; glycines are added to the γ-carboxyl groups of glutamates in the C-terminal region via α/γ linkages; further glycines can then be added to these via α/α linkages (Redeker et al., 1992a) (Fig. 2.8). So far, polyglycylation has only been seen in tubulin from protists and animals, however, its presence in *Giardia* suggests it is likely to

be very ancient (Bressac et al., 1995; Bré et al., 1996; Mary et al., 1994b, 1996, 1997; Multigner et al., 1996; Weber et al., 1997; Péchart et al., 1999; Xia et al., 2000; Hoyle et al., 2008; Wloga et al., 2008). Unlike the case with polyglutamylation, polyglycylation is concentrated in axonemal MTs where, in the case of *Paramecium*, up to 20 glycines are added to α and up to 18 to β; in the case of β, the polyglycylation sites are within the axonemal signal sequence EGEFXXX (Rüdiger et al., 1995; Xie et al., 2000; Redeker et al., 2005, 2010). Lesser amounts of polyglycylation have been observed in MTs in neurons and the cochlea (Bré et al., 1998; Saha and Slepecky, 2000; Bane et al., 2002; Banerjee, 2002c). Generally, polyglycylation occurs in more stable MTs like those of the basal body and the axonemes; interestingly, centriolar MTs do not appear to be polyglycylated (Million et al., 1999). Unlike polyglutamylation, where the glutamates are added to only a single glutamate, the glycines are added to four or five glutamates (Xia et al., 2000). In general, it appears that polyglycylation of tubulin is a requirement for proper axonemal formation although its precise role is uncertain (Thazhath et al., 2002; Wloga et al., 2009). In fact, in the gerbil, polyglycylation may not be necessary for ciliary beating (Dossou et al., 2007). The mechanism by which polyglycylation exerts its effect on axonemal formation is not clear. Xia et al. (2000) have mutated all five of the glutamate polyglycylation sites in *Tetrahymena* β-tubulin; they have shown that only two of the sites have to be present for appropriate axonemal formation, but it does not matter which ones: any two will do. Also, mutating sufficient glutamates in β to preclude axonemal formation can be corrected by adding the wild-type C-terminal region from β onto α (Xia et al., 2000). In other words, some of the polyglycylation sites have to be present but it does not matter which ones they are nor even whether they are on α or β. This raises an interesting question: if the C-terminal region carries out its function independently of whether it is on α or β, could it have once been able to do so if it was attached to neither, namely, as a free polyglycylated polypeptide?

As is the case with polyglutamylation, more proteins than tubulin are polyglycylated. In *Giardia*, the protein 14-3-3 is polyglycylated at two glutamates close to the C-terminus (Lalle et al., 2006); 14-3-3 is a strongly conserved and very ancient group of eukaryotic proteins serving a variety of functions all of which appear to involve interacting with other proteins, generally phosphorylated proteins (Yaffe et al., 1997; Petosa et al., 1998; Rosenquist et al., 2000). The sequence containing the polyglycylated glutamates is not similar to the C-termini of tubulin (Lalle et al., 2006) (Fig. 2.8).

Another polyglycylated protein is NAP1, which is also polyglutamylated (Ikegami et al., 2008). In *Tetrahymena*, Pgp1p, a protein related to chaperones, is polyglycylated in a process that is thought to be important for viability of the organism (Xie et al., 2007). The polyglycylation site resembles tubulin's C-terminus in being rich in glutamates and aspartates but differs strikingly in that the basic residues lysine and arginine account for half of the residues in this region (Xie et al., 2007). It has been proposed that polyglycylating or monoglycylating enzymes may also have further protein substrates but these have not yet been identified (Rogowski et al., 2009). It is hard to find common threads connecting these proteins and the other polyglutamylated proteins to tubulin, other than 14-3-3 acting as a linker and NAP1 and Pgp1p perhaps as chaperones.

4.1.5. Evolutionary Aspects

As mentioned above, all four of these posttranslational modifications are widely distributed phylogenetically, indicating that they are likely to be very ancient and may have occurred in LECA. Except for acetylation, all of them involve addition of amino acids. Not surprisingly, the enzymes that catalyze these reactions appear to be related and generally share a core domain of very similar architecture (Janke et al., 2005; Szyk et al., 2011); they constitute a group called the TTLL family (Table 2.14). One might imagine that by fitting the sequences into a family tree, one could then deduce which came first. These family trees suggest that the polyglutamylases constitute one group and the polyglycylases, another with the tubulin–tyrosine ligase almost sandwiched in between (Janke et al., 2005; Wloga et al., 2008). Perhaps it is meaningful that the phylogeny is based on the nature of the amino acid added rather than whether the preferred substrate is α-tubulin or β-tubulin or whether the enzyme adds the amino acid to an α- or a γ-carboxyl group. One could speculate that the ligase family evolved before α- and β-tubulin diverged, or that it evolved in tandem with the C-terminal domain prior to this domain joining tubulin. Conceivably, the ancestral enzymes originated as amino acid-binding enzymes that then acquired the ability to add the amino acid to a substrate. The fact that only a few of these enzymes have been identified as adding glutamates or glycines to nontubulin substrates may only reflect our ignorance but could also indicate that proteins such as NAPs 1 and 2 and the rest may only have appeared as substrates much later.

Table 2.14 The TTLL enzyme family

Enzyme	Preferred substrate	Amino Acid added	Initiation	Elongation	Other substrates
TTL	α	Tyr	√		
TTLL1	α	Glu		√	
TTLL2		Glu			
TTLL3	α	Gly	√		β
TTLL4	β	Glu	√		α, PELP1
TTLL5	α	Glu	√		
TTLL6	α	Glu		√	
TTLL7	β	Glu	√		
TTLL8	α	Gly	√		
TTLL9		Glu			
TTLL10	α	Gly		√	β, NAP1
TTLL11	α	Glu		√	
TTLL12		Gly			
TTLL13	α	Glu		√	

References: (Ikegami et al., 2006, 2008; Kashiwaya et al., 2010).

4.2. Origin and Evolution of the C-terminal Domain of Tubulin

4.2.1. Possible Prebiotic Origin of the C-terminal Domain of Tubulin

The argument has been made above that the C-terminal domain may have once been a separate polypeptide and not part of tubulin. With that in mind, let us look at the sequences of some C-termini with a new eye (Fig. 2.8). It is natural to want to put facts into hierarchical categories as when we give prominence to the amino acid linkages that are made on the ribosomes and call the others "posttranslational modifications." If we step away from the hierarchy, we will see that the amino acid linkages shown in Fig. 2.8 are largely created by enzymes with no ribosomal involvement. Even the C-terminal tyrosine of α, although often encoded in the genome, can be removed and then added back enzymatically. Could such structures as these have originated without involvement of ribosomes or genomes or even cells?

Apparently, it is possible. Fox and Harada (1958) showed that if a mixture of amino acids was heated anhydrously at 170 °C, the amino acids would polymerize into "proteinoids" that possessed some limited enzymatic activity and primitive cell-like morphology (Fox et al., 1974, 1987; Nakashima and Fox, 1987; Fox, 1991). The proteinoids were apparently branched but their structures were never rigorously determined; however, glutamate had to be present for their formation, presumably because glutamate's γ-carboxyl group allowed for α/γ as well as α/α linkages (Mark et al., 1964; Fox and

Dose, 1972). The resemblance of these proteinoids to the C-termini shown in Fig. 2.8 is obvious, particularly since the branching in both proteinoids and C-termini could be complex (Wolff et al., 1994). Nevertheless, before arguing that the C-terminal region originated prebiotically, we should ask whether there is some likelihood that their component amino acids could have been present on a lifeless Earth. This is indeed likely. First, the most abundant amino acid in these C-termini is glycine which is also far and away the most abundant amino acid synthesized in prebiotic experiments as well as being present in meteorites and even moon dust (Murphy et al., 1970; Ring et al., 1972; Schlesinger and Miller, 1983; Kminek et al., 2002). Other amino acids also prominent in the C-termini are glutamate, aspartate, alanine and valine, which are also common in prebiotic experiments and in meteorites (Schlesinger and Miller, 1983; Kminek et al., 2002). Except for glycine, which exists only as a single enantiomer, chirality is a potential problem, with living systems using only L-amino acids and prebiotic experiments generating only racemic mixtures of amino acids; meteorites also contain mixtures of L- and D-amino acids, although they are not always perfectly racemic (Pizzarello and Cooper, 2001; Kminek et al., 2002). In other words, it may be difficult to generalize from a proteinoid made from a racemic mixture of amino acids to a polyglutamylated polypeptide such as the tubulin C-terminus, containing only L-amino acids. However, there may be a way out of this conundrum.

Recent thinking in the field of life's origins has suggested that the first self-replicating system could have arisen underwater from alkaline hydrothermal vents, which naturally are full of minerals and contain tiny compartments which could have mimicked cells and allowed for synthesis and concentration of biopolymers (Martin and Russell, 2007). One of these, the Lost City serpentinite formation under the Atlantic Ocean, has been extensively studied (Kelley et al., 2005; Proskurowski et al., 2008; Martin et al., 2008). Minerals of various kinds can catalyze synthesis of oligopeptides and oligonucleotides, some of them quite long (Ferris et al., 1996; Ferris, 2006; Ertem et al., 2007). Among the minerals, quartz and calcite are thought to have been quite abundant; these exist in chiral forms which selectively adsorb particular stereoisomers of the amino acids; for example, left-handed calcite selectively adsorbs L-aspartate, while the titanium mineral rutile binds selectively to L-glutamate and L-aspartate (Hazen et al., 2001; Hazen and Sholl, 2003; Hazen, 2006; Jonsson et al., 2009; Parikh et al., 2011). Adsorption of glutamate to rutile or hydrous ferric oxide could have led to formation of chiral polymers joined by peptide bonds (Sverjensky et al., 2008). The amino acid tyrosine, which is the

most abundant C-terminal residue of α-tubulin, may seem an exception to this story of simplicity, but even tyrosine and phenylalanine can form peptide bonds abiotically in the presence of iron and nickel (Huber and Wächtershäuser, 1998). In short, the hypothesis that the C-terminal region of tubulin could have arisen prebiotically is not an unreasonable one. Analogous evolutionary schemes have been proposed for at least one other case. The iron–sulfur mineral greigite, which arguably could have been present on the early Earth, has a three-dimensional structure very similar to that of the iron–sulfur cluster in ferredoxin and acetyl-CoA dehydrogenase. It has been suggested that greigite or something like it could have had a catalytic function at the time of the first life and that later a peptide was associated with the mineral and eventually, the iron–sulfur cluster became part of these proteins (Milner-White and Russell, 2005; Russell, 2006).

4.2.2. Ancient Features of the C-Terminal Domain

Besides their architecture and the abundance of amino acids that could have been easily synthesized abiotically, there are other primitive features in the structure and function of the C-terminus that are consistent with a very ancient origin even if not necessarily a prebiotic one. First, as already mentioned, many of the amino acid linkages are not made on the ribosome. The TTLL enzymes that make them could be descended from an ancestor more ancient than the ribosome, an amino acid polymerase that drew on the energy stored in ATP (Janke et al., 2005; Wloga et al., 2008). Second, the majority of amino acids that are encoded in the C-terminal domains (glutamate, aspartate, glycine, alanine, glutamine, asparagines, and serine) are among the earliest to have acquired codons according to the hypothesis of Davis (1999, 2002) about the origin of the genetic code. In fact, a typical C-terminal domain could be, by this analysis, even older than Davis' proposed earliest protein, namely, ferredoxin. Third, there is a certain lack of specificity about at least one of the enzymes involved in modifying tubulin, namely the tubulin–tyrosine ligase. Although the enzyme requires tubulin in its native state as one of its substrate, it can readily add phenylalanine, L-3,4-dihydroxyphenlyalanine, nitrotyrosine, or azatyrosine instead of tyrosine (Arce et al., 1975; Raybin and Flavin, 1977; Kalisz et al., 2000; Purro et al., 2003). Such lack of specificity is what one would expect from a primitive enzyme. Fourth, a similar lack of specificity characterizes the role of polyglycylation. As mentioned above, although polyglycylation of β-tubulin is required for proper axoneme formation, neither the location nor the extent of the modification matters (Vinh et al., 1999; Xia et al., 2000; Duan and Gorovsky, 2002; Redeker et al., 2005; Dossou et al., 2007).

4.2.3. Evolution of the C-Terminal Domain

We postulate here that the C-terminal domain, including its polyglycyl and polyglutamyl side chains, may have originated prebiotically, and then that part of it became encoded in the genome, existing as a separate polypeptide modified by enzymes of the TTLL family; only later on would the gene for the C-terminal domain have fused with the gene for the αβ ancestor (Table 2.15). If the C-terminal domain of tubulin evolved on its own, regardless of whether it arose prebiotically, it must have had some function in order for it to have survived prior to fusion with the αβ tubulin ancestor. Modeling studies have suggested that the C-terminal domain could have some secondary structure (Luchko et al., 2008); in the putative ancient and separate C-terminal domain, that could have given it some stability as well as increased the specificity of its interactions with other molecules. Some of the current properties of the C-terminal domain could give us a clue about these interactions. First, the C-terminal domain is the binding site for MAPs, specifically tau and MAP2 (Mukrasch et al., 2007); however, it is not the only site: tau also binds to the N-terminal region of α-tubulin (Serrano et al., 1984a; Littauer et al., 1986; Devred et al., 2004). Similarly, the MT-severing protein katanin binds to both the C-terminal and to another region on tubulin (White et al., 2007). It is easy to imagine that the C-terminal domain could have bound to an ancestor of tau or katanin, which in turn bound to a member of the tubulin superfamily. In other words, the C-terminal domain could have helped to anchor the filaments which we have postulated were the product of the tubulin superfamily ancestor and which we propose to have been the proto-centrosome, a structure involved in genome replication. The anchoring could originally have been to a mineral surface onto which a glutamate polymer was adsorbed (Jonsson et al., 2009; Parikh et al., 2011). Later, if the C-terminal domain became encoded as a separate polypeptide, then this anchoring/organizing function would have continued. From there to actually fusing with tubulin appears to be a small step. Among the MAPs binding to the C-terminal domain could have been the ancestors of tubulin-binding cofactors B and E, as we have already proposed.

Studies have shown that tubulin can act as a chaperone in that it protects the activity of enzymes, even to restoring the activities of unfolded enzymes (Manna et al., 2001). This activity of tubulin requires the C-termini of α and β (Sarkar et al., 2001). In short, it is possible that the putative free C-terminal might have had chaperone activity that would have been very valuable in an era where enzymes were likely to have been very unstable and temperatures were highly variable. Perhaps along the same lines, it is possible that the C-terminal domain stabilized filaments formed by the

Table 2.15 Hypothetical evolution of C-Terminus

1. Branched polymers enriched for glycine and/or glutamate arise abiotically.
2. These polymers aid the first cell in regulating metabolism, filament formation and nucleic acid condensation.
3. TTLL family originated as ATPases which then acquired the ability to put amino acids together. They could have developed into polyglutamylases and polyglycylases. The C-terminus could have been a substrate.
4. Part of the branched polymer was encoded as a short peptide.
5. The gene for the polymer fused with the genes of α- and β-tubulin, NAP1, NAP2, 14-3-3, and other proteins.

ancestral tubulin; one piece of suggestive evidence is that the presence of a high concentration of glutamate protects tubulin against decay and also promotes its polymerization into bundles of filaments (Hamel, 1981a,b; Hamel et al., 1982). It is only a small further step to postulate that one function of the C-terminal domain and its putative polyglutamate side chain was to stabilize filaments formed by the ancestral tubulin.

The C-terminal domain could have had several other functions. Currently, this domain binds to the glycolytic enzymes aldolase, glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase (Volker and Knull, 1993; Kovacs et al., 2003). The interactions with the first two of these are inhibitory but that does not appear to be the case with pyruvate kinase; one could argue that the C-terminal domain could have localized this ATP-producing enzyme close to the proto-centrosome and, hence, generated the energy required for cell division.

The C-terminal domain also binds to the VDAC in the outer mitochondrial membrane (Carré et al., 2002). VDAC is a protein that regulates ADP/ATP exchange; tubulin causes closure of the channel (Rostovtseva and Bezrukov, 2008) (Fig. 2.9). This appears to be a very ancient function of the C-terminal domain; organisms that lack mitochondria also have C-terminal domains that are missing or shorter (*Giardia*, *Enterocytozoon*, *Encephalitozoon*) (Rostovtseva et al., 2008). The evidence that VDAC inhibition involves the C-terminal domain is that tubulin lacking the C-terminal domain is not inhibitory; it is thought that the negative charges in the C-terminal domains of α- and β-tubulin bind to positively charged residues in the VDAC channel (Rostovtseva and Bezrukov, 2008); phosphorylation of the outer surface of VDAC increases inhibition probably by promoting binding to the body of the tubulin molecule (Sheldon et al., 2011). Interestingly, synthetic peptides corresponding to the C-terminal domains are not inhibitory—they are thought to simply slide in and out of the channel without

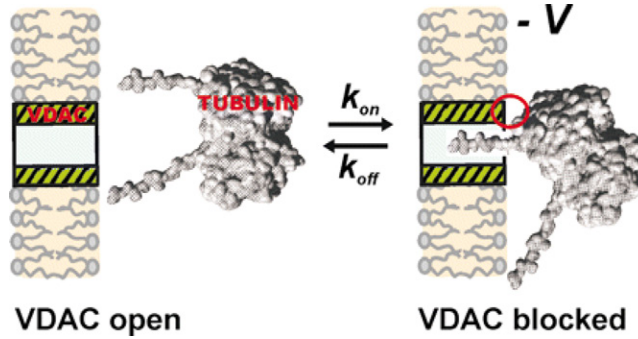


Figure 2.9 ***Model of VDAC–tubulin interaction.*** From Rostovtseva et al. (2008). Note that when VDAC is blocked the C-terminal domain of one of either α- or β-tubulin is in the VDAC channel. There may be an additional interaction between VDAC and the main body of the tubulin molecule (red ellipse). *(From Rostovtseva et al. (2008) Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. Proc. Natl. Acad. Sci. U.S.A. 105, 18746–18751, Fig. 4. Reprinted with permission).* (For color version of this figure the reader is referred to the online version of this book).

affecting it; presumably, the bulk of the tubulin molecule settles in outside the VDAC and keeps the C-terminal domain stuck inside the channel. This is consistent with models that indicate that the diameter of the C-terminal domain is 0.9 nm while that of the channel is 1.2 nm (Gurnev et al., 2012). It has been proposed that there is an "interactosome" consisting of VDAC, tubulin, creatine kinase and ATP synthase and that the presence of tubulin in this complex and its interaction with glycolytic enzymes allows tubulin to play a major role in regulation of cellular metabolism (Saks et al., 1993; Lemasters and Holmuhamedov, 2006; Gonçalves et al., 2007; Timohhina et al., 2009; Maldonado et al., 2010; Cassimeris et al., 2012).

How would a free C-terminal domain fit into these schemes, especially if experiments show that this domain has no effect? These experiments were done with synthetic peptides lacking polyglutamylation or polyglycylation. It is possible that either a branched C-terminal domain could fit better into the VDAC channel or the polyglutamyl or polyglycyl branches could bind to the surface of the outer mitochondrial membrane and that the encoded portion of the C-terminus could then be held in the VDAC channel. Alternatively, perhaps the polyglutamyl side chain could do the inhibiting while the encoded part binds to the outside of VDAC. If either of these is true and if the C-terminal domain is also binding to the proto-centrosome, then mitochondria could end up localized in that area; given that mitochondria have a very high concentration of VDAC, then it is easy to imagine that even if a few VDAC channels are closed because of binding to the C-terminal

domains, then overall, there would be a large increase in energy being provided by the mitochondria for cell division. One needs also to remember that if two branches of a structure each fit into a VDAC channel, then two mitochondria could be linked and even more energy would be provided for the cell's purposes. If these arguments are correct, then the C-terminal domain could have been involved in mitochondrial function when the mitochondrial ancestor entered the cell prior to the origin of MTs.

It is also possible that the C-terminal domain could have been associated with mitochondrial ancestors. The evolution of VDAC is complex and multiple forms exist at the present time (De Pinto et al., 2010). There are similarities in the structures of VDAC and bacterial porins, channel proteins found in Eubacteria and Archaea (Besnard et al., 1997; Makarova and Koonin, 2010); it is not yet clear whether these reflect a common ancestor or convergent evolution (Young et al., 2007; Zeth and Thein, 2010). In either case, it is possible that the C-terminal domain regulated the activity of these channel proteins.

It is also conceivable that the polyglutamate or polyglycine side chains could have had functions on the separate C-terminal domain. For instance, polyglycine could have formed a "nest" for binding phosphates in nucleotides (Milner-White and Russell, 2005); even a polypeptide that is enriched for glycine could have bound to phosphates or sulfates (Dreusicke and Schulz, 1986).

At some point, the gene for the C-terminal domain could have fused with that of the αβ ancestor. If the C-terminal domain bound to the tubulin-binding cofactors B and E, as we have proposed, bringing them to the table, as it were, would have partly ameliorated the destabilization of tubulin induced by attachment of the C-terminal domain. This domain could also have fused with an ancestor of NAP1 and NAP2. Variation among the C-terminal domains of tubulin is quite large, making it difficult to reconstruct their evolution even while bound to tubulin; of the polyglutamylation or polyglycylation sites that have been identified in other proteins, it is difficult to be discern whether these represent a separate gene fusion event or whether they arose independently.

5. EXPERIMENTAL TESTS OF THESE HYPOTHESES

5.1. Evolution of Vertebrate β Isotypes and Other α- and β-Tubulins

Although this is a relatively straightforward story, due to the large number of α and β sequences that are available, a closer study of the functions of

individual isotypes could be very useful. Elucidation of the functions of the βV and α8 isotypes in vertebrates could be particularly illuminating. The interaction of the isotypes with the chaperones would be of interest. For example, given the higher rigidity of βIII, does it need all seven of the chaperones and cofactors to form a dimer? If disulfide linkages form in tubulin, it would be helpful to know which cysteines are involved. Correlation of the entities listed in Table 2.1 with specific tubulin isotypes and further studies with the isotypes acting as dimers or non-MT polymers could generate a robust mechanism of how the different isotypes could have evolved, including the pressures to which they responded, especially in vertebrates.

5.2. Evolution of the Tubulin/FtsZ Superfamily

Obviously, many of the steps in the development of the tubulin/FtsZ superfamily are just hypotheses, but many of these hypotheses could be tested and stronger hypotheses constructed, in essentially five ways. First, sequencing more proteins and more genomes and evaluating their relatedness could yield results that modify the hypotheses advanced above. For example, a recent study raises the possibility that the common ancestor of FtsZ and tubulin may also have been ancestral to histone 2A and actin (Gardiner et al., 2008). The observation that actin may occur in eukaryotic nuclei and interact with DNA, as it does in prokaryotes, is consistent with this hypothesis (Skarp and Vartiainen, 2010). Sequencing of genomes from more members of the various archaeal kingdoms would provide a clearer context for those tubulin or FtsZ relatives found among them and may reveal new members of the superfamily (Makarova and Koonin, 2010; Yutin and Koonin, 2012). We would have a clearer idea of whether these proteins arose by horizontal gene transfer or represent pathways of evolution different from the one described here. The proteins composing the fibrils connecting the kinetochores to MTs (McIntosh et al., 2008) have not yet been identified; it would be of great interest if these proteins were somehow related in sequence to proteins connecting FtsZ with the membrane in bacteria or indeed with any other tubulin- or FtsZ-binding protein. We should probably not be too hopeful, however. Filamentous tubulin-binding proteins such as tau and MAP2 have very little secondary structure and their activity even survives boiling (Fellous et al., 1977). Such proteins, most of whose sequence is in essence a spacer, are unlikely to be as conserved in evolution as proteins of very complex higher order structure such as tubulin.

Second, few, if any, studies have been done on purified δ-, ε-, η-, ζ-, θ-, ι-, and κ-tubulins. Other than their amino acid sequences and, in a few

cases, the effects of mutations, we know very little about their properties. Studies on their stability, which we could predict should be high, would be a useful place to start. Their ability to polymerize with themselves or each other, and their GTPase activities could all be measured. Co-localization and co-immunoprecipitation studies might allow identification of the proteins with which these other tubulins interact and could give us a far clearer idea of their functions than we presently have. Entering functions on the superfamily tree shown in Fig. 2.5 would certainly be a useful exercise.

Third, much of the hypothesizing done here involves tubulin filaments plus or minus the C-terminus. There are various ways to produce and study tubulin filaments. We can presently generate single protofilaments of tubulin using vinblastine (Erickson, 1975); it is easy to subject these filaments to protein chemical and colchicine-binding assays (Palanivelu and Ludueña, 1982). Certainly, the binding of MAPs, chaperones, kinesins, and other proteins to these filaments could be measured and the results could be illuminating. However, we must keep in mind that the presence of vinblastine could alter the tubulin conformation and block some putative binding sites, thus potentially limiting the utility of these filaments to explore these questions (Ludueña and Roach, 1981b, 1981c; Ludueña et al., 1986). Tubulin filaments or ribbons can also be formed by treatment with glutamate, paclitaxel, colchicine or mica; some of these filaments even have GTPase activity (Andreu and Timasheff, 1982; Hamel et al., 1982; Andreu et al., 1983; Elie-Caille et al., 2007; Hamon et al., 2009). Alternatively, we could prepare α- and β-tubulin genetically modified to lack the C-terminus, or treated with subtilisin to remove it. These could conceivably form filaments, single or bundled. We could use these systems to examine interactions with the proteins listed in Table 2.1 and/or described in the text above. It would be interesting, for example, if these truncated tubulins did not need the full set of chaperones or cofactors to polymerize. If only kinesin-13 and no other kinesin bound to these filaments or filament bundles, then that would support the concept that the original function of this kinesin-13 was to promote curving of tubulin protofilaments.

Fourth, more experiments should be done in which the interactions between individual kinesins and tubulin are studied in detail; it may be that if the specific location of the kinesin-binding sites on tubulin were to be superimposed on the kinesin family tree that the origin and evolution of the tubulin–kinesin interaction would become much clearer. Similar experiments could be done with dynein.

Fifth, our understanding of the centriole, basal body and axoneme is still very primitive. It is not even entirely clear exactly what the composition of

these structures is. Therefore, the constituent proteins have to be identified and their three-dimensional structures determined and possible prokaryotic homologs identified. Then their arrangements within the centriole, basal body and axoneme have to be worked out. Recent evidence indicates that the arrangement of even apparently simple structures such as the nexin link in the axoneme is very complex (Heuser et al., 2009). The work of Kohno et al. (2011) who used a variety of protein chemical approaches to determine the arrangement of proteins in the radial spoke could serve as a model.

Sixth, given the known sequences of *Prosthecobacter* BtubA and BtubB, it may be possible to deduce the sequence of the αβ ancestor and re-construct it so that its polymerization, GTPase, chaperone-dependence and stability could be measured. These experiments could make the hypotheses advanced in this review more robust or lead us to discard them altogether.

5.3. Origin of the C-Terminal Domain

The hypotheses proposed here about the prebiotic origin and evolution of the C-terminal domain of α- and β-tubulin may seem highly improbable but they could be evaluated by experimentation. Thus, we could easily synthesize peptides corresponding to the C-termini of α- and β-tubulin. We could also mix appropriate amino acids in the proportions corresponding to current C-termini with high levels of glutamate or glycine or both and heat them under anhydrous conditions (Fox and Harada, 1958) to generate proteinoids, presumably highly branched. These proteinoids could be made of a racemic mixture of amino acids or else constructed using only L- or D-amino acids. Alternatively, one could chemically construct a C-terminal peptide that is polyglutamylated or polyglycylated at the appropriate sites. With greater difficulty, it may be possible to specify exactly how many glutamates or glycines there could be in a side chain although this may not be necessary since such a degree of homogeneity does not appear to exist in vivo. Alternatively, one could culture cells in the presence of overexpressed members of the TTLL family and then purify the tubulin, cleave off the C-termini with subtilisin and then purify the C-terminal domains. The small amount of heterogeneity introduced by subtilisin may actually be only a minor problem (Lobert et al., 1993).

Once C-terminal domains, modified and unmodified, have been prepared, there are a variety of questions that could be asked. Their ability to act as chaperones for enzymes or to cause tubulin to polymerize into filaments

or to bind to these filaments could be measured quite easily. Binding to the long list of proteins with which tubulin is known to interact could also be assessed (Table 2.1). We already know that unmodified C-termini do not interact with VDAC (Rostovtseva et al., 2008); however, it is certainly possible that a modified C-terminal domain could do so. The results of these experiments could give a much clearer picture of the possible functions of the postulated independent C-terminal domain.

Learning more about the enzymes that modify tubulin could also be a fruitful area of investigation. There are several blank spaces in Table 2.14. Extending our knowledge of the substrate specificities of the TTLL family of enzymes as well as the acetylating and deacetylating enzymes could give a clearer picture of how these enzymes evolved. It may also be of interest to explore the connection between the TTLL family and the enzymes that add glutamates to folate (Young et al., 2008).

6. CONCLUDING REMARKS

Our story began with a detailed analysis of the structures and evolution of vertebrate β-tubulin isotypes that suggested that tubulin could have functions that do not involve being part of MT. This conclusion was then applied to the evolution of the superfamily that includes all the different forms of tubulin and its prokaryotic relatives. It is much easier to envisage these proteins forming a filament than a complex cylinder like MT. The theme widespread in the tubulin/FtsZ superfamily is of a polymerizing GTPase that accomplishes work using the energy of GTP hydrolysis. It is proposed that about the time of the first cell, two proteins came together to form the LCFTA, one a hydrolase and the other a GTP-binding protein, and that the structure of the connection directly led to filaments which used the energy of GTP hydrolysis to promote cell division by acting either on the DNA or on the cell membrane. When the first eukaryotic cells appeared, there was a major parting of the ways with the prokaryotes using FtsZ to perform cytokinesis by acting on the cell membrane and the eukaryotes using tubulin to act on the DNA. These tubulin filaments may have joined with other polymers to form a proto-centrosome to aid in accomplishing this task. At the same time, the ancestral tubulin diverged into γ-, δ-, ε-, and η-tubulins and the αβ-tubulin ancestor, each of which probably had specific tasks to perform in the proto-centrosome. Eventually, still at the stage of the LECA, the ancestral αβ tubulin acquired a third component, a C-terminus, likely

Table 2.16 Hypothetical evolution of Tubulin

First cells
1. A GTP-binding protein associates with a catalytic protein to form a GTPase.
2. GTPase forms filament that tugs on nucleic acid or membrane.
3. Divergence of FtsZ, which forms polymers that act on membrane, from ancestral tubulin whose polymers act on nucleic acids.
Early eukaryotic cells
4. Appearance, in order, of ε, η, δ, γ, and β, which associate to form a filamentous proto-centrosome, which helps to separate DNA.
5. Kinesin and other proteins bind to filament to help it do its job.
6. Lateral gene transfer to *Prosthecobacter*.
7. Acquisition of C-terminus, causing destabilization of the molecule.
8. Evolution of binding proteins into chaperones to stabilize tubulin.
9. Divergence of α and β that form the α/β dimer, which forms polymers that pull on DNA.
10. Bundling of filaments aids that process.
11. Microtubule appears.
12. Association with kinesin-13.
13. Formation of centriole and basal bodies.
14. Formation of cilia and flagella.
15. Divergence of eukaryotes.
Later eukaryotes
16. Origins of protists, animals, plants, and fungi. Centrioles lost from fungi and most plants.
17. Appearance of vertebrates.
18. Doubling of genome in vertebrates allows for appearance of multiple isotypes of α-, β-, and γ-tubulin.
Tubulin isotypes appear in other taxa but are most abundant in animals and plants.

to be even more ancient and perhaps of prebiotic origin, and already performing various useful functions in the cell. The C-terminal domain would have contributed these functions while at the same time destabilizing the tubulin molecule, leading to the appearance of stabilizing proteins, such as the chaperones and the tubulin-binding cofactors. The pressure of multiple proteins binding to the αβ ancestor led to divergence of α and β and appearance of the α/β dimer. Eventually, the C-terminus promoted the formation of MTs, which formed organelles such as basal bodies, centrioles and axonemes, all prior to the divergence of the eukaryotes. These hypotheses are summarized in Table 2.16. Once the eukaryotes diverged into the four great kingdoms of animals, plants, fungi and protists, further

developments occurred. Especially in the animals and plants, the appearance of multicellularity coupled with cell differentiation caused the evolution of isotypes of α-, β-, and γ- to the point where higher animals and plants often have about six to eight isotypes each of α and β and two of γ. The plethora of isotypes may in part explain the long list of tubulin-binding factors shown in Table 2.1. It is perhaps an ironic indication of how far tubulin has evolved from its putative ancestral three genes that the tissue with the greatest complexity of tubulin isotypes, namely the neuron, is also the one whose tubulins are speculated to participate in a quantum mechanical fashion in our thought processes (Hameroff and Watt, 1982; Hameroff, 1998; Craddock and Tuszynski, 2010). These speculations are based on the fundamental characteristics of the tubulin molecule, namely, its flexibility, GTPase activity, and highly negative C-terminal domains (Trpišová and Tuszynski, 1997; Tuszynski et al., 1997; Hameroff et al., 2002; Rezania and Tuszynski, 2009). These are the ancient features whose origins have been woven into the story told in this chapter.

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I had planned to send this chapter for comments and suggestions to the late Dr. Lynn Margulis who pioneered work in this area, but sadly she passed away before this work was finished. In honor of her enormous contributions to our understanding of the early evolution of life, I would like to dedicate this review to her memory.

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CHAPTER THREE

Dynamin: Expanding Its Scope to the Cytoskeleton

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Contents

Abstract

The large GTPase dynamin is well known for its actions on budded cellular membranes to generate vesicles, most often, clathrin-coated endocytic vesicles. The scope of cellular processes in which dynamin-mediated vesicle formation occurs, has expanded to include secretory vesicle formation at the Golgi, from other

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endosomes and nonclathrin structures, such as caveolae, as well as membrane remodeling during exocytosis and vesicle fusion. An intriguing new facet of dynamin's sphere of influence is the cytoskeleton. Cytoskeletal filament networks maintain cell shape, provide cell movement, execute cell division and orchestrate vesicle trafficking. Recent evidence supports the hypothesis that dynamin influences actin filaments and microtubules via mechanisms that are independent of its membrane-remodeling activities. This chapter discusses this emerging evidence and considers possible mechanisms of action.

1. INTRODUCTION

The cytoskeleton forms a dynamic structural framework that works in concert with cellular membranes and the extracellular matrix (ECM) to maintain cell shape and tissue integrity, execute migration, morphogenesis and cell division, and orchestrate protein and membrane trafficking. Several hundred proteins regulate the dynamic interactions of cytoskeletal filaments and membranes during fundamental cellular processes. The large GTPase dynamin, which is best known for its activities that remodel membranes, is emerging as a regulator of cytoskeletal filaments. Whereas dynamin is soundly established to generate vesicles during endocytosis (Doherty and McMahon, 2009; Ferguson and De Camilli, 2012; Praefcke and McMahon, 2004; Ramachandran, 2011; Schmid and Frolov, 2011), its original discovery as a microtubule-binding protein (Shpetner and Vallee, 1989), together with early observations of defective neurite extension and growth cone migration by cultured neurons from *Drosophila* carrying a defective dynamin gene (Kim and Wu, 1987), provided early hints that dynamins may also influence processes that depend on cytoskeletal filaments. Over subsequent decades, insights into the abilities of dynamin to influence the dynamics of select actin filament networks and microtubules, and of its possible molecular mechanisms of action, have emerged. In this chapter, we highlight those actin- and microtubule-dependent processes in which a regulatory role for dynamin is supported. An outstanding challenge is to demonstrate unequivocally that dynamin influences cytoskeletal networks independently from its well-characterized functions in endocytosis. To provide a contextual framework, we begin with a succinct review of the membrane-remodeling activities of dynamin during endocytosis and exocytosis.

2. DYNAMIN—A MEMBRANE REMODELING GTPase

Endocytosis is the process whereby contents of the plasma membrane and extracellular fluids are delivered to the intracellular milieu via vesicles

formed at the plasma membrane (Conner and Schmid, 2003; Doherty and McMahon, 2009). Endocytosis occurs by many different mechanisms, including clathrin-mediated endocytosis (CME), clathrin-independent endocytosis, macropinocytosis, phagocytosis and caveolin-dependent endocytosis. Of these many different routes, the mechanisms by which clathrin mediates endocytosis is best understood. During CME, adapter proteins bind and cluster membrane receptors and, together with assembled clathrin lattices, initiate the inward budding of the plasma membrane; as the membrane invaginates more deeply, it remains tethered via a thin, tubular membrane neck. Finally, dynamin-mediated scission of the thin, tubular tether releases a vesicle containing the internalized membrane and fluid components to the cytoplasm (Doherty and McMahon, 2009; Ferguson and De Camilli, 2012; McMahon and Boucrot, 2011; Schmid and Frolov, 2011).

Several nonmutually exclusive models for the mechanisms by which dynamin catalyzes scission have been proposed. A common feature of all models is the assembly of dynamin as a helical polymer around the thin, tubular necks that tether deeply invaginated clathrin-coated pits to the plasma membrane. Some models invoke GTP hydrolysis-dependent conformational changes within the assembled dynamin polymer that mechanically constricts, extends or twists the membrane necks, leading to scission and vesicle formation (Danino et al., 2004; Roux et al., 2006; Stowell et al., 1999; Sweitzer and Hinshaw, 1998). Recent evidence from structural studies supports the idea that a mechanochemical powerstroke may be involved (Chappie et al., 2011). Another model suggests that GTP hydrolysis-dependent cycles of assembly and disassembly of dynamin oligomers on thin necks subsequently lead to membrane scission, perhaps by locally destabilizing structure at membrane interfaces of the neck (Bashkirov et al., 2008; Pucadyil and Schmid, 2008). An outstanding question is how GTP hydrolysis, the associated conformational changes within the dynamin collar or its cycles of assembly and disassembly are linked to membrane scission.

More recently, dynamin was implicated in membrane remodeling during exocytosis (Anantharam et al., 2011; Fulop et al., 2008; Gonzalez-Jamett et al., 2010; Holroyd et al., 2002; Jaiswal et al., 2009). During chromaffin granule secretion, the rate of dynamin GTPase activity controlled the rate of fusion pore expansion and thereby, regulated the kinetics and amount of released secretory vesicle content (Anantharam et al., 2011). Mutant dynamin with an accelerated rate of GTP hydrolysis increased the rate of fusion pore expansion and the frequency of full fusion with the plasma membrane, whereas mutant dynamin with decreased rates of GTP hydrolysis stabilized

fusion pores and delayed expansion to full fusion. How the GTPase cycle modulates fusion pore expansion is not known. The GTPase cycle may control interactions of dynamin with other components of the fusion machinery, such as synaptophysin (Gonzalez-Jamett et al., 2010). Alternatively, assembled dynamin oligomers may restrict fusion pore expansion with cycles of GTP hydrolysis regulating the half-life of assembled dynamin at the fusion pore and, thereby, fusion pore dynamics (Anantharam et al., 2011). The cortical actin network also modulates fusion pore dynamics (Jaiswal et al., 2009) and a function for dynamin in regulating actin filaments at sites of exocytosis has not been ruled out. The ability of dynamin to regulate fusion pores during exocytosis expands the repertoire of cellular processes influenced by this versatile, membrane-remodeling GTPase.

2.1. Dynamin Structure and Biochemistry

Dynamin is a large GTPase composed of 100 kDa subunits that associate as tetramers. Tetrameric dynamin further oligomerizes to form a helical polymer around tubular templates, such as the necks of deeply invaginated clathrin-coated pits. Interestingly, dynamin also forms helical polymers around other structures with the appropriate electrostatic charge and diameter, such as microtubules and bundles of actin filaments (Gu et al., 2010; Shpetner and Vallee, 1989). Dynamin also assembles as stable ring-like structures in the absence of tubular templates (Hinshaw and Schmid, 1995). Recent X-ray crystallographic studies of nearly full-length dynamin1 revealed a dimeric structure composed of two extended, antiparallel dynamin polypeptides with a subunit–subunit interface situated near the base of the central "stalk" domain and the globular, N-terminal GTP-binding G-domains oriented at opposite ends of the extended dimer (Faelber et al., 2011; Ford et al., 2011). A pleckstrin homology (PH) domain was situated in a foot-like globular structure at the base of each stalk, creating the membrane-binding face of the extended dimer. Not yet examined is the structure of the C-terminal proline-rich domain (PRD) of dynamin, however, it is expected to project toward the G-domain surface, opposite the membrane-binding surface. Through these major domains (G-domain, stalk, PH domain and PRD), dynamin executes several activities, including hydrolysis of GTP, self-assembly via interactions between dynamin stalk regions, binding to anionic lipids via the PH domain, and interactions with a growing list of proteins that bind primarily via the PRD. Each of these domains and activities is required for vesicle scission by dynamin.

2.1.1. Dynamin GTPase Cycle

Unassembled dynamin binds GTP with low affinity and exhibits a low basal rate of GTP hydrolysis that is stimulated upon self-assembly as oligomers (Stowell et al., 1999; Warnock and Schmid, 1996). Recent biochemical and structural studies indicated that assembly-stimulated GTP hydrolysis results from interactions of G-domains between adjacent rungs of the helical dynamin polymer assembled around a tubular template (Chappie et al., 2010, 2011). Several PRD-interacting proteins, including Grb2 (Barylko et al., 1998), cortactin (Mooren et al., 2009) and SNX9 (Soulet et al., 2005) stimulate the basal rate of GTP hydrolysis by dynamin.

The importance of GTP hydrolysis by dynamin for membrane remodeling was first highlighted by electron microscopic images of synaptic membrane preparations incubated in the presence of GTPγS, a nonhydrolyzable GTP analog (Takei et al., 1995). Elongated membrane tubules decorated by electron-dense ring-like structures formed in such preparations; the tubules also labeled with anti-dynamin antibodies. Subsequent characterization of mutant dynamin proteins defective in GTP binding, the GTP hydrolysis catalytic rate or assembly-stimulated GTPase activity supported the hypothesis that GTP hydrolysis by dynamin powered membrane scission during CME (Marks et al., 2001; Song et al., 2004a, 2004b). Cryo-electron microscopy of dynamin assembled on lipid tubules suggested that GTP hydrolysis-dependent conformational changes within the assembled dynamin polymer were coupled to constriction of membrane tubules, leading ultimately to membrane scission (Hinshaw and Schmid, 1995; Sweitzer and Hinshaw, 1998). Indeed, recent structures of a dynamin1 fusion protein obtained in the presence of either nonhydrolyzable or transition-state guanine nucleotides provides evidence of a GTPase-dependent powerstroke (Chappie et al., 2009, 2011). Whether the putative powerstroke exerts forces that drive scission is not yet known.

2.1.2. Dynamin's Pleckstrin Homology (PH) Domain Mediates Interactions with Phospholipids

Positively charged amino acids within the dynamin PH domain mediate binding to acidic phospholipids, particularly phosphoinositides (Ferguson et al., 1994; Zheng et al., 1996). While the affinity of a single PH domain for anionic lipids is low, cooperative binding by oligomeric dynamin increases the avidity (Klein et al., 1998). Additionally, the PH domain appears to play an active role in vesicle scission. Dynamin binding to liposomes was coupled to partial insertion into the membrane of a hydrophobic variable loop,

VL1, of the PH domain (Ramachandran et al., 2009; Ramachandran and Schmid, 2008). Membrane insertion by VL1 may sense or generate membrane curvature along tubular membranes or destabilize bilayer structure to promote membrane scission (Bethoney et al., 2009; Ramachandran et al., 2009). Whereas the primary structure of the PH domains of dynamin1 and dynamin2 are quite similar, these dynamin isoforms exhibit distinct abilities to assemble and generate curvature on lipid templates (Liu et al., 2011b). The differential activities of dynamin1 and dynamin2 were attributed to a single amino acid residue in a different variable loop,VL3, of the PH domain. Switching one amino acid in dynamin2 VL3 to tyrosine, which exists at that position in dynamin1, was sufficient to confer dynamin1-like membrane scission and curvature-generating properties to dynamin2 (Liu et al., 2011b). In neurons, dynamin1 drives rapid recycling of synaptic vesicle membranes, whereas dynamin2 is the workhorse for slow endocytic events that require clustering of membrane receptors, adapter proteins and the assembly of clathrin lattices. Thus, distinct modes of membrane remodeling may be governed by differential interactions of dynamin isoforms with membranes.

2.1.3. C-terminal, Proline-Rich Domain

More than 30 proteins interact directly with dynamin, the majority of which bind the C-terminal PRD (Ferguson and De Camilli, 2012; Schafer, 2004). The PRD contains several PXXP motifs that interact with SH3-domain-containing proteins, but the specific binding sites within the PRD for most dynamin-binding partners have not been determined. During endocytosis, the PRD targets dynamin to clathrin-coated structures (CCS) through interactions with amphiphysin and endophilin (David et al., 1996; Ringstad et al., 1999; Sundborger et al., 2011; Takei et al., 1999). Dynamin is targeted to select F-actin networks via PRD-mediated interactions with cortactin or Abp1 (Kessels et al., 2001; McNiven et al., 2000). Interestingly, a region of the PRD restricts dynamin interactions with microtubules during interphase but phosphorylation within the PRD by mitotic kinase activity permits dynamin interactions with microtubules during mitosis (Morita et al., 2010; Shpetner et al., 1996). Thus, given such a diverse array of PRD-interacting partners and coupled with GTPase-regulated self-assembly, dynamin is positioned to function as a multidimensional scaffold capable of acting in different cellular processes through a complex array of dynamic protein–protein interactions. Identifying how these interactions are regulated in time and space to govern different dynamin-dependent functions remains an important goal.

2.2. Experimental Approaches to Explore Novel Dynamin Functions

Inhibiting dynamin has proven a useful strategy to explore its contributions and mechanisms of action during cellular processes, particularly endocytosis. Several mutant dynamin proteins that negatively impact membrane receptor internalization have been characterized, including those that alter GTP binding and/or hydrolysis, block self-assembly as oligomers and decrease interactions with phospholipids or interacting proteins. A common tool used to disrupt dynamin-dependent endocytosis is dynamin-K44A, a mutant defective in binding GTP (Damke et al., 1994). Since dynamin-K44A potently blocks endocytosis, identifying cytoskeletal-related functions for dynamin is challenged if decreased endocytic activity impacts surface receptors or signaling pathways that impinge on the cytoskeleton. Moreover, gain-of-function effects of dynamin-K44A on actin filaments, such as actin filament cross-linking (Mooren et al., 2009), further confound interpretations of dynamin actions on cytoskeletal filaments. Overexpressed dynamin proteins may indirectly perturb cellular processes by sequestering dynamin-binding partners or inducing cytotoxic effects (Liu et al., 2011a). Thus, strategies that monitor endocytosis activity and control the level of dynamin protein expression must be employed when using exogenously expressed dynamin proteins.

Genetic ablation or "knockout" of dynamin genes in mice or approaches that conditionally deplete dynamin genes within specific cells or tissues can reveal roles for dynamin in cellular processes (Ferguson et al., 2009; Liu et al., 2008). Transient, RNAi-mediated knockdown typically achieves up to 90% depletion of dynamin proteins (Ezratty et al., 2005; Mooren et al., 2009). As with some mutant dynamin proteins, discerning novel functions for dynamin using gene knockout or siRNA-mediated depletion is complicated if endocytosis is grossly perturbed. Thus, quantitative assessment of endocytic activity and of potential downstream signals should be carried out in concert with studies of novel functions for dynamin. However, when coupled with experiments in which expressed mutant dynamin proteins selectively restore endocytosis but not other processes, knockdown or genetic ablation approaches may reveal novel functions for dynamin.

New pharmacological agents that acutely block GTP hydrolysis by dynamin are frequently used to disrupt endocytosis (Macia et al., 2006; Yamada et al., 2009). However, the specificity, mechanism of action and selectivity of the available dynamin-blocking drugs are not sufficiently characterized to

be useful probes of novel, endocytosis-independent activities for dynamins. Finally, injected dynamin antibodies were used to inhibit dynamin function in some studies (Henley et al., 1998; Jones et al., 1998; Thompson et al., 2004).

3. ACTIONS OF DYNAMIN AND ACTIN FILAMENTS DURING ENDOCYTOSIS

Both dynamin and actin filaments support CME and other forms of endocytosis in mammalian cells, including phagocytosis, macropinocytosis and caveolae-mediated endocytosis (Di et al., 2003; Galletta et al., 2010; Gold et al., 1999; Henley et al., 1998; Kaksonen et al., 2006; Lamaze et al., 1997; Lanzetti, 2007; Liu et al., 2008; Merrifield et al., 1999; Mooren et al., 2012; Schlunck et al., 2004; Shajahan et al., 2004; Tse et al., 2003). In contrast in yeast, actin filaments together with other factors that induce membrane curvature, execute vesicle scission for endocytosis (Aghamohammadzadeh and Ayscough, 2009; Kaksonen et al., 2003; Mooren et al., 2012; Weinberg and Drubin, 2012). In mammalian cells, actin filaments facilitate vesicle scission but are not absolutely required (Ferguson et al., 2009; Itoh et al., 2005; Taylor et al., 2012). Actin filaments were also implicated in supporting other steps of the endocytic process, including membrane invagination, formation or stabilization of deeply invaginated CCS or movement of newly formed vesicles away from the plasma membrane (Girao et al., 2008; Liu et al., 2010; Mooren et al., 2012; Qualmann et al., 2000). Before considering how dynamin regulates cytoskeletal filaments outside the realm of endocytosis, we discuss emerging evidence supporting interdependent actions of dynamin and actin filaments during CME.

Both actin filaments and dynamin are recruited to CCS at the plasma membrane. Low amounts of dynamin, but little actin, associated with nascent CCS when they first formed (Merrifield et al., 2002). Just prior to scission, dynamin accumulated rapidly at CCS; recruitment of actin, presumably from de novo assembly, preceded the rapid burst of dynamin recruitment and scission by ~20 s (Taylor et al., 2012). Factors that promote actin assembly (Arp2/3 complex, N-WASP and cortactin) were recruited to CCS prior to the scission event, whereas factors that promote F-actin disassembly (cofilin and coronin) appeared later, suggesting that F-actin turnover at CCS is temporally coupled to vesicle scission (Taylor et al., 2012).

A role for dynamin in F-actin turnover at CCS was also supported from studies of fibroblasts from conditional dynamin1/dynamin2 double-knockout mice. F-actin was highly enriched at deeply invaginated CCS in

fibroblasts lacking both dynamin isoforms (Ferguson et al., 2009). Arp2/3 complex, an actin-nucleating factor that induces actin assembly (Pollard, 2007), was also enriched at CCS, indicating that dynamin was not required to recruit or activate the machinery for F-actin assembly. In contrast, dynamin likely functions to limit actin assembly or promote its turnover at CCS (Fig. 3.1). The transient appearance of small patches of dendritic filament networks closely associated with invaginated CCS is consistent with this idea (Collins et al., 2011).

Dynamin and actin appear to function interdependently at CCS to power vesicle scission. Disrupting the actin cytoskeleton in cells lacking dynamin with pharmacologic agents increased the number of shallow, wide-necked, CCS at the expense of deeply invaginated CCS, supporting a role for actin filaments in membrane invagination and in stabilizing the thin necks that link CCS to membranes (Ferguson et al., 2009). Moreover, an intact actin cytoskeleton greatly enhanced dynamin recruitment and the efficiency of scission (Taylor et al., 2012). Thus, actin filaments facilitate endocytosis by stabilizing invaginated membranes, promoting the formation of deeply invaginated CCS with thin tubular necks and reinforcing recruitment of dynamin. The thin tubular necks may be ideal templates for assembly of dynamin collars. Actin filaments also could provide additional binding sites for dynamin at CCS via direct interactions (Gu et al., 2010) or indirect interactions with the F-actin- and dynamin-binding proteins, cortactin or Abp1 (Kaksonen et al., 2000; Merrifield et al., 2005; Zhu et al., 2005). Whether or not dynamin influences actin filament turnover or filament organization for efficient vesicle formation remains to be explored.

Whereas actin filaments facilitated dynamin recruitment at CCS, the dynamin GTPase cycle also influenced F-actin assembly and disassembly at CCS, lending additional support for interdependent roles for dynamin and actin filaments during endocytosis (Taylor et al., 2012). Mutant dynamin proteins with impaired rates of GTP hydrolysis decreased the rate of actin recruitment and increased the rate of F-actin disassembly at CCS (Taylor et al., 2012). However, mutant dynamin proteins with increased rates of GTP hydrolysis had no effect on actin dynamics at CCS, suggesting that a threshold level of GTP hydrolysis by dynamin was sufficient to influence actin filaments.

Dynamin also may catalyze scission in coordination with actin filaments during some clathrin-independent processes. The cytokine receptor γc is internalized via a clathrin-independent pathway that requires

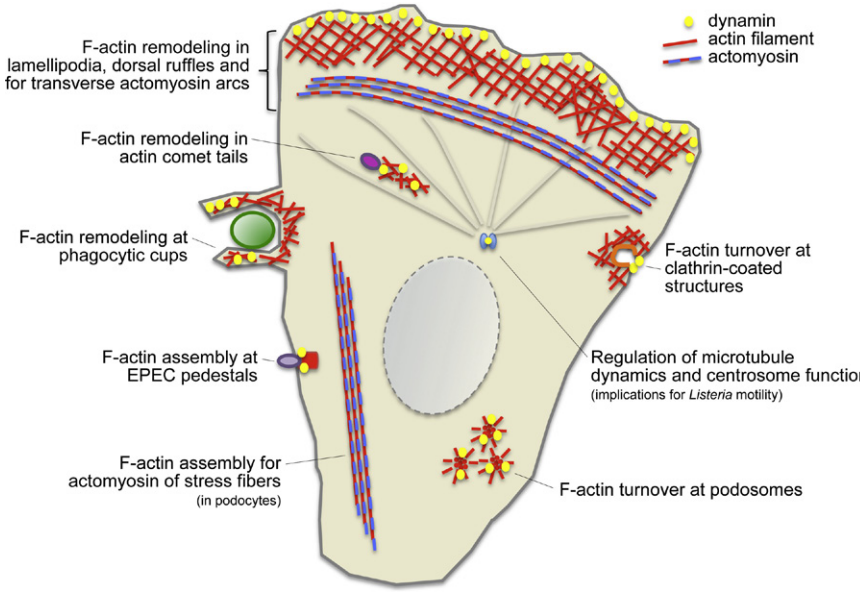

Figure 3.1 Dynamin influences cytoskeletal networks via a variety of mechanisms, including recruiting actin assembly factors, bundling and cross-linking actin filaments, remodeling actin filaments, promoting actin filament turnover and influencing microtubule dynamics and centrosome cohesion. During endocytosis, dynamin modulates actin filament turnover as clathrin-coated structures separate from the plasma membrane (Ferguson et al., 2009). Dynamin contributes to phagosome formation for phagocytosis (Di et al., 2003; Gold et al., 1999) and influences microtubule dynamics and centrosome cohesion via unknown mechanisms (Tanabe and Takei, 2009; Thompson et al., 2004). Dynamin is involved in formation and stability of podosomes and invadopodia, actin-rich structures that promote migration and ECM degradation in some cells (Buccione et al., 2004; Ochoa et al., 2000; Oser et al., 2009). Dynamin is enriched on the F-actin comet tails elaborated by intracellular bacterial pathogens (Lee and De Camilli, 2002; Orth et al., 2002); the function of dynamin for bacterial motility is not known, but indirect effects arising from the influence of dynamin on microtubule stability are not ruled out (Henmi et al., 2011). F-actin assembly at pedestal structures elaborated by pathogenic *E. coli* depends on dynamin recruitment to bacterial attachment sites (Unsworth et al., 2007). As a component of the dendritic actin networks of lamellipodia and ruffles, dynamin remodels actin filaments to support filament turnover and actomyosin formation (Chua et al., 2009; Krueger et al., 2003; McNiven et al., 2000; Mooren et al., 2009). Actin filament uncapping by dynamin is implicated in maintaining the actomyosin-containing stress fibers in podocytes (Gu et al., 2010). (For color version of this figure, the reader is referred to the online version of this book).

dynamin2 and its binding partner, cortactin, which also binds actin filaments (Sauvonnet et al., 2005). As will be discussed in detail below, cortactin may be an important biochemical link between dynamin and actin filament networks for endocytosis and other cellular processes.

Phagocytosis, an endocytic process in which cells engulf particles, depends on F-actin-rich protrusions of the plasma membrane. In cells in which dynamin2 was perturbed either by injecting anti-dynamin antibodies or by expressing mutant dynamin2-K44A, actin-rich protrusions extended around internalizing particles but the protrusions arrested before engulfment was complete (Di et al., 2003; Gold et al., 1999; Tse et al., 2003). Thus, dynamin2 may remodel actin filaments within protrusive structures to complete phagosome formation (Fig. 3.1). Similar to phagocytosis, internalization of extracellular fluid via macropinocytosis depends on dynamic actin filaments that support sheet-like ruffles that collapse rearward to encapsulate extracellular fluid (Kerr and Teasdale, 2009; Lim and Gleeson, 2011) and on dynamin (Liu et al., 2008; Schlunck et al., 2004). Whether or not dynamin remodels membranes, modulates actin filaments, or both during macropinocytosis and the late stages of phagocytosis has not been examined.

In summary, considerable evidence supports the view that dynamin and actin filaments play interdependent roles during clathrin-dependent and clathrin-independent endocytic processes in mammalian cells. Although not universally required for CME, a functional actin cytoskeleton promotes formation and stability of deeply invaginated clathrin-coated pits and facilitates efficient vesicle scission. Cortactin, which interacts with actin filaments and with dynamin, is likely to link dynamin and actin filaments during many forms of endocytosis. However, other dynamin-interacting proteins may coordinate the concerted actions of dynamin and actin filaments in a site-specific manner.

4. BEYOND ENDOCYTOSIS: DYNAMIN AS A CYTOSKELETAL REGULATOR

Dynamin was first discovered as a nucleotide-sensitive microtubule-binding and -bundling protein (Shpetner and Vallee, 1989). Dynamin (or in Greek, force or power) was so named based on speculations that it generated nucleotide hydrolysis-dependent force-producing interactions between bundled microtubules. Subsequent work showed dynamin hydrolyzed GTP, an activity that was stimulated by microtubules (Maeda et al., 1992; Shpetner and Vallee, 1992). When dynamin was identified as the product of the *shibire* gene in *Drosophila melanogaster* (Chen et al., 1991; van der Bliek and Meyerowitz, 1991), together with observations that dynamin decorated thin membrane tubules with clathrin-capped tips in GTPγS-treated

synaptic membrane preparations (Takei et al., 1995), its destiny as a key regulator of vesicle scission was solidified. Understanding alternate functions and mechanisms of action of dynamin, particularly those that involve cytoskeletal dynamics, only recently returned to the forefront. In the sections that follow, we discuss evidence that implicates dynamin in regulating the actin and microtubule cytoskeletons.

4.1. Dynamin and Microtubules

Since its discovery as a microtubule-binding protein, dynamin was implicated in several microtubule-dependent processes in animal cells, including efficient mitosis, maintenance of centrosome structure and for microtubule dynamic instability (Tanabe and Takei, 2009; Thompson et al., 2004, 2002). Dynamin did not localize with microtubules in interphase cells but was localized with γ-tubulin of interphase and mitotic centrosomes (Chircop et al., 2011; Thompson et al., 2004). Dynamin was strikingly enriched along the bundled microtubules of the mid-zone spindle and mid-body during late stages of mitosis and cytokinesis (Hamao et al., 2009; Thompson et al., 2002). Although early events in mitosis and mid-zone microtubules appeared normal in mitotic fibroblastoid cells lacking dynamin2, the time required to complete cytokinesis was longer compared with control cells (Liu et al., 2008). Similarly, decreasing the expression of the single dynamin protein expressed in *Caenorhabditis elegans* or activating a temperature-sensitive mutant dynamin blocked cytokinesis at a late stage (Thompson et al., 2002). Whether or not increased genomic instability results from decreased levels of endogenous dynamin2 remains to be explored. Taken together, these findings indicate that dynamin acts directly on bundled microtubules of the mid-body to promote cell division. Depleting dynamin2 also caused individual centrosomes to separate by as much as 3 μm, without apparent gross perturbations to overall microtubule organization (Thompson et al., 2004). Thus, dynamin2 maintains centrosome cohesion in interphase cells but how this activity contributes to microtubule organization, polarized morphology or cell migration is not known.

The dynamin PRD negatively regulates interactions of dynamin with microtubules. A mutant form of dynamin lacking the C-terminal portion of the PRD strongly decorated microtubules of interphase cells and conferred resistance to cold-induced microtubule disassembly (Hamao et al., 2009). During mitosis, association of dynamin with mid-zone microtubules required phosphorylation within the PRD by cyclin-dependent kinase1,

which presumably overrides the intrinsic block for binding microtubules (Chircop et al., 2011; Morita et al., 2010). The actions of dynamin on interphase centrosomes and on mitotic microtubules provide evidence supporting a *bona fide* physiologic function for dynamin in regulating select microtubule-dependent processes (Ishida et al., 2011; Thompson et al., 2004).

Investigations of interphase cells depleted of dynamin2 revealed increased numbers of stable microtubules containing high levels of acetylated tubulin, a marker of stable microtubules (Fig. 3.1) (Henmi et al., 2011; Tanabe and Takei, 2009). The increased stable microtubules were coupled to defects in transport of Golgi-derived vesicles, perhaps as a result of inefficient vesicle capture by stable, as opposed to dynamic, microtubules (Tanabe and Takei, 2009). Thus, through its effects on microtubule dynamics, dynamin2 may indirectly influence vesicle transport at the Golgi and mid-body. Stable microtubules in dynamin2-depleted cells may also negatively impact cell migration through effects on focal adhesion disassembly (Kaverina et al., 1999).

4.2. Links between Dynamin and F-actin Networks

Identifying functional links between dynamin and the actin cytoskeleton is an area of intense investigation. Early evidence implicating dynamin in actin-related functions came from studies of neurons from *D. melanogaster* carrying temperature-sensitive mutations in the *shibire* gene, which encodes fly dynamin. *Shibire* flies become paralyzed at restrictive temperatures due to defects in synaptic transmission (Kim and Wu, 1987; Poodry and Edgar, 1979; van der Bliek and Meyerowitz, 1991). Cultured neurons from *shibire* flies also exhibit defective neuritogenesis at restrictive temperatures, including stunted neurite extension and decreased growth cone motility (Kim and Wu, 1987). Subsequent studies implicated dynamin in other actin-dependent processes, including cell migration (Kruchten and McNiven, 2006), invasion through ECM (Baldassarre et al., 2003), apical constriction in epithelial cells (Chua et al., 2009) and select forms of actin-dependent bacterial pathogenesis (Lee and De Camilli, 2002; Orth et al., 2002; Unsworth et al., 2007). Because dynamin action in endocytosis may influence signaling pathways that impinge on actin filaments, clarifying the relationship between dynamin regulation of actin filament networks and its endocytic functions is the primary challenge toward gaining a full understanding of the scope of dynamin activities. In the following section, we discuss emerging evidence supporting the hypothesis that dynamin is a

multifunctional protein that directly influences actin filament networks in addition to its membrane-remodeling activities.

Dynamin, particularly the ubiquitously expressed dynamin2, was found associated with several actin filament networks, including the F-actin networks of protrusive structures, such as lamellipodia, dorsal ruffles and phagocytic cups, the actin networks of podosomes and invadopodia, *Listeria* comet tails and with actomyosin "belts" at the apical surface of epithelial cells (Chua et al., 2009; Gold et al., 1999; Gray et al., 2005; Krueger et al., 2003; Kurklinsky et al., 2011; Lee and De Camilli, 2002; Orth et al., 2002). One feature shared by most of these actin networks is that they exist as dendritic networks of branched actin filaments formed through the action of the actin nucleator, Arp2/3 complex. Many dynamin-interacting partners link dynamin to nucleation-promoting factors for Arp2/3 complex, such as N-WASP (Salazar et al., 2003; Schmitz et al., 2004; Soulet et al., 2005), WAVE (Cestra et al., 2005) and cortactin (McNiven et al., 2000; Weed et al., 2000). Thus, dynamin2 associates primarily with dendritic actin filament networks that typically undergo rapid turnover.

4.2.1. Podosomes and Invadopodia

Podosomes are dynamic cell–ECM adhesive structures formed at the ventral surface of macrophages, osteoclasts and in Rous sarcoma virus-infected cells (Buccione et al., 2004; Ochoa et al., 2000). Actin filaments of podosomes are highly dynamic and arranged in a radial array surrounding a core of bundled filaments (Luxenburg et al., 2007). ECM degradation occurs at the ventral cell surface in close association with podosomes. Invadopodia are longer lived than podosomes, possess robust ECM degradation activity and are typically elaborated by invasive tumor cells to enhance metastatic migration of through tissues (Artym et al., 2011; Baldassarre et al., 2003; Linder, 2009; Linder et al., 2011).

Dynamin2 was implicated in regulating actin dynamics and/or ECM-degrading activity at podosomes and invadopodia via a mechanism that depends on GTP binding and hydrolysis (Baldassarre et al., 2003; Bruzzaniti et al., 2005; Ochoa et al., 2000). Whereas podosomes formed in cells expressing the dominant mutant protein, dynamin2-K44A, F-actin turnover at podosomes was decreased, suggesting that dynamin2 GTPase activity regulated F-actin turnover at podosomes (Fig. 3.1) (Ochoa et al., 2000). Exogenous dynamin2-K44A could act in a dominant manner to stabilize filaments by cross-linking (Gu et al., 2010). On the other hand, podosomes and invadopodia failed to form in

cells expressing a truncated mutant form of dynamin2 lacking the C-terminal PRD, suggesting a role for full-length dynamin2 in formation or stabilization of these structures (Lee and De Camilli, 2002; Baldassarre et al., 2003). In osteoclasts, a complex of dynamin2 and the adapter protein, Cbl, which binds the dynamin2 PRD, may coordinate recruitment of F-actin regulators or activators at podosomes to control osteoclast migration on bone. Phosphorylation by c-Src kinase destabilized the dynamin2–Cbl complex, suggesting a link between adhesion signaling, migration and dynamin2-mediated regulation of actin dynamics at podosomes (Bruzzaniti et al., 2005).

Dynamin2 may stabilize F-actin networks of invadopodia in concert with its interacting partner, cortactin, which also binds F-actin (Oser et al., 2009); however, the actions of dynamin2 and cortactin for efficient vesicle trafficking or secretion of ECM-degrading metalloproteases have not been ruled out. In this regard, dynamin2 and cortactin were implicated in formation of secretory vesicles at the trans-Golgi (Cao et al., 2005; Salvarezza et al., 2009; Weller et al., 2010) and cortactin-promoted secretion of metalloproteases and ECM degradation at invadopodia (Clark and Weaver, 2008).

4.2.2. F-actin Comets and Pedestals

Many bacterial pathogens of mammalian cells commandeer the host-cell actin filaments and their regulatory machinery to promote infection (Haglund and Welch, 2011). Dynamin2 is associated with F-actin structures elaborated by two such bacterial pathogens, the F-actin tails of *Listeria monocytogenes* and the F-actin pedestals elaborated by enteropathogenic *Escherichia coli* (EPEC) (Fig. 3.1). Actin tails induced by *Listeria* propel the bacteria through the cytoplasm, eventually guiding them into protrusions that become engulfed by adjacent neighbor cells. Using this mechanism, *Listeria* spreads from cell to cell, evading immune detection. Since motility of *Listeria* via actin tails does not involve vesicle budding or endocytosis, the presence of dynamin2 along the F-actin tails provided compelling evidence for an actin-dependent and endocytosis-independent function for dynamin2 (Henmi et al., 2011; Lee and De Camilli, 2002; Orth et al., 2002). Although dynamin2 was not required for *Listeria* motility in reconstituted systems (Loisel et al., 1999), dynamin2 may organize actin filaments of comet tails for optimal bacterial movement in the cellular milieu or efficient cell-to-cell spread of *Listeria* via F-actin-rich projections that drill into neighboring cells. The PRD was critical for targeting dynamin2 to F-actin tails (Lee and De Camilli, 2002; Orth et al., 2002). Experimentally induced F-actin tails generated by expression of phosphatidylinositol-4-phosphate,

5-kinase (PIP5K) were similarly decorated by dynamin (Orth et al., 2002). Whereas F-actin tails formed in *Listeria*-infected cells depleted of dynamin2, the tails were shorter and *Listeria* moved more slowly compared to bacteria in infected control cells (Henmi et al., 2011). Expression of dynamin mutant proteins defective in GTPase activity also decreased F-actin tail number, tail length and rate of movement of *Listeria* and PI5K-induced comet tails (Henmi et al., 2011; Lee and De Camilli, 2002; Orth et al., 2002). Exogenously expressed mutant dynamin proteins might indirectly influence motility of PIP5K-induced vesicular compartments if, as a result of decreased membrane scission activity in cells expressing mutant dynamins, PI5K-generated vesicles formed less frequently or were larger in size, creating different templates for F-actin tail formation (Cameron et al., 2004).

An intriguing link between dynamin2, *Listeria* motility and microtubules was recently uncovered. As discussed above, microtubule stability, the content of acetylated tubulin in microtubules and the rate of *Listeria* motility all depended on the cellular concentration of dynamin2 (Henmi et al., 2011; Tanabe and Takei, 2009). These apparently unrelated findings prompted the suggestion that increased stable microtubules in dynamin2-depleted cells might hinder bacterial movement (Fig. 3.1) (Henmi et al., 2011). In support of this idea, addition of agents that alter the global organization or abundance of cellular microtubules restored normal *Listeria* movement to dynamin2-depleted cells. These agents may create a cellular milieu that allows maximal propulsion.

In contrast with *Listeria* invasion of the cytoplasm in infected cells, EPEC remain bound to the outside of infected cells, anchored at the plasma membrane via an F-actin structure called a pedestal (Goosney et al., 2001). EPEC attach to cells via the bacterial protein, Tir, which then recruits the adapter protein Nck to the intracellular membrane face. Subsequent recruitment of actin polymerizing factors lead to formation of F-actin pedestals (Wunderlich et al., 1999). Depletion of dynamin2 or expression of mutant dynamin proteins decreased the accumulation of F-actin at EPEC pedestals, implicating dynamin2 in pedestal formation and/or maintenance (Fig. 3.1) (Unsworth et al., 2007). Since dynamin2 appeared at sites of EPEC binding in concert with N-WASP but before Arp2/3 complex, dynamin2 may create a multiprotein scaffold that recruits factors required for de novo F-actin assembly at pedestals. Alternatively, or in addition, dynamin2 may orchestrate local rearrangements in cortical F-actin at bacterial

attachment sites to establish a filament architecture conducive for pedestal formation.

4.2.3. Dynamin and Other Dendritic Actin Networks: Lamellipodia, Cortical Ruffles and Phagocytic Cups

Dynamin was found associated with dendritic actin networks of lamellipodia (McNiven et al., 2000), transient cortical ruffles elaborated on the dorsal surface of PDGF-stimulated and -unstimulated cells (Krueger et al., 2003; Schafer et al., 2002) and protrusions extended by macrophages during phagocytosis (Gold et al., 1999). Recruitment of dynamin to some of these actin networks depended on the PRD, most likely via interactions with the PRD- and F-actin-interacting protein, cortactin, which is also enriched in these networks (Gray et al., 2005; Krueger et al., 2003; McNiven et al., 2000; Mooren et al., 2009). Expression of mutant dynamin proteins lacking the PRD or having defective GTPase activity, or microinjection of anti-dynamin antibodies, perturbed dynamics of many of these networks (Fig. 3.1). For example, in macrophage-like cells in which dynamin function was perturbed with injected antibodies or by expressing dynamin2-K44A, protrusions still extended around incoming IgG-coated particles, but arrested before engulfment was complete (Di et al., 2003; Gold et al., 1999; Tse et al., 2003). Thus, dynamin2 may remodel actin filament networks within phagocytic cups to complete phagosome closure. However, a role for dynamin2 in modulating exocytic vesicles and their fusion at nascent phagosomes has not been ruled out (Di et al., 2003).

4.2.4. Dynamin and Actomyosin Networks

The participation of dynamin2 in regulating actomyosin networks was recently uncovered (Fig. 3.1) (Chua et al., 2009; Mooren et al., 2009). Contractile actomyosin networks at the apical junctions maintain the organization of epithelial sheets and drive morphogenesis that shapes developing tissues and organisms (Hartsock and Nelson, 2008; Lecuit and Wieschaus, 2002). Actomyosin networks also comprise a key component of the cell migration machinery (Vicente-Manzanares et al., 2009). Expressed mutant dynamin2 -K44A proteins having decreased GTP hydrolysis activity induced constriction at apical junctions of epithelial cells; importantly, perturbing endocytic activity via alternate mechanisms did not induce apical constriction (Chua et al., 2009). Thus, the effects of dynamin2-K44A on contractility did not result solely from decreased endocytosis. Apical constriction by dynamin2-K44A depended on Rho-ROCK signaling and the dynamin2-interacting

protein, cortactin. Additionally, depleting dynamin2 in epithelial cells disrupted cell–cell adherens junctions (Chua et al., 2009). Loss of adherens junction integrity in dynamin-depleted epithelial cells may result from altered recycling of E-cadherin, which initiates and maintains dynamic junctions (Ivanov et al., 2005). Dynamin2 may have dual roles at adherens junctions: recycling cell–cell adhesion molecules and modulating contractility via pathways that influence actomyosin function. Although dynamin2 was not associated with actomyosin networks in nonepithelial cells, it might influence contractility through effects on the dendritic actin networks that form and maintain actomyosin networks (Ehrlich et al., 2002; Kovacs et al., 2002; Verma et al., 2004). Consistent with this idea, the organization of select actomyosin networks comprising transverse arcs was perturbed in dynamin2-depleted U2-OS cells (Fig. 3.1) (Mooren et al., 2009).

4.2.5. Neurites, Growth Cones and Dendritic Spines

Paralysis of the *Drosophila* mutant *shibire*^{ts1} led investigators to ultimately establish a role for dynamin in recycling synaptic membrane that controls synaptic transmission (van der Bliek and Meyerowitz, 1991). However, cultured neurons from *shibire*^{ts1} flies also exhibited defects in neurite extension, suggesting that dynamin also influenced actin-dependent processes (Kim and Wu, 1987; Torre et al., 1994). At that time, little was known about the mechanisms by which neuronal growth cones migrated to extend neurites, aside from the general participation of actin filaments and microtubules (Letourneau et al., 1986; Marsh and Letourneau, 1984). Renewed interest in investigating a role for dynamin in regulating actin-dependent processes in neurons came with the identification of alternatively spliced isoforms of dynamin3, a dynamin family member that, with dynamin1, was enriched in neuronal tissue. Whereas some splice variants of dynamin3 worked cooperatively with dynamin1 to support synaptic transmission (Raimondi et al., 2011), other dynamin3 isoforms were highly enriched at the tips of dendritic spines of cultured hippocampal neurons (Gray et al., 2005). When expressed in neurons, one splice variant of dynamin3 (dynamin3-baa) altered the morphology of spine-like protrusions along neurites from short, mushroom-shaped structures to elongated filopodia characteristic of immature dendritic spines (Gray et al., 2003, 2005). The ability of dynamin3-baa to influence spine morphology depended on its interaction with cortactin, implicating dynamin3-baa and cortactin in regulating F-actin-based structures in developing neurons (Gray et al., 2005).

A complex of dynamin2 and cortactin was also implicated in regulating another neuronal appendage, the growth cone (Kurklinsky et al., 2011). Growth cones are dynamic structures at the tips of axons that guide elongating axons during neuronal development. Lamellipodia and filopodia, composed of branched actin filaments and actin filament bundles, respectively, comprise the growth cone periphery. These F-actin networks, together with microtubules in the central body of growth cones, respond to guidance cues and orchestrate the direction and rate of growth cone advance. Cortactin and dynamin2, but not dynamin1 or dynamin3, were localized with adhesion proteins at the transition zone between the peripheral and central regions of growth cones. When overexpressed, cortactin or dynamin2 enhanced growth cone spreading and the recruitment of the F-actin cross-linking protein, α-actinin, to the transition zone (Kurklinsky et al., 2011). Thus, dynamin2 and cortactin appear to influence the architecture of F-actin networks of growth cones, which in turn, may regulate adhesion to the ECM and the rate of neurite outgrowth.

4.2.6. Dynamin Action during Whole-Cell Migration and Tumor-Cell Invasion

Cell migration requires the concerted actions of many cellular processes: actin filament and microtubule dynamics, formation of adhesive structures, integrin receptor cycling, contractility, and controlled directionality. Dynamin may contribute to cell migration via several nonmutually exclusive mechanisms: by recycling integrin receptors to influence cell-substrate adhesion (Chao and Kunz, 2009; Ezratty et al., 2005), by regulating secretion of degradative enzymes involved in ECM invasion (Cao et al., 2005), by recruiting actin assembly factors through its PRD-interacting partners to drive protrusive behavior (Kruchten and McNiven, 2006), by generating signaling molecules associated with internalized receptors that regulate the cytoskeleton downstream of endocytic activity (Feng et al., 2012), or by maintaining microtubule organization and dynamics required to maintain cell polarity and focal adhesion disassembly (Ezratty et al., 2005; Kaverina et al., 1999; Tanabe and Takei, 2009; Thompson et al., 2004). As discussed above, dynamin2 is a component of lamellipodia and of growth factor-induced circular ruffles on the plasma membrane, both of which are associated with advancing the leading edge of migrating cells (Krueger et al., 2003; McNiven et al., 2000; Schafer et al., 2002). Through dynamin's direct interactions with signaling enzymes, such as the p85 subunit of phosphatidylinositol 3-phosphate kinase (PI3K) (Feng et al., 2012), Src (Cao et al., 2010; Foster-Barber and Bishop, 1998), or

FAK (Ezratty et al., 2005; Wang et al., 2011), dynamin could organize signaling complexes that fine-tune the migration machinery for efficient motility. A future challenge is to elucidate the molecular mechanisms by which dynamin regulates these many different elements that, together, move a cell.

The contributions of dynamin to metastatic tumor progression may be tumor-specific. Dynamin2 expression was elevated in tissues of patients with aggressive, metastatic pancreatic cancer (Eppinga et al., 2012). In cultured pancreatic cancer cells, cellular levels of dynamin2 correlated directly with whole-cell migration rate, ECM invasion and metastatic potential in vivo. The ability of dynamin2 to promote migration of pancreatic cancer cells depended on phosphorylation by c-Src kinase, which together with interactions of dynamin and FAK, may contribute to focal adhesion disassembly and migration (Ahn et al., 2002; Cao et al., 2010; Ezratty et al., 2005; Wang et al., 2011; Weller et al., 2010). Cellular levels of dynamin2 similarly correlated directly with cell growth and invasion in an experimental model of glioblastoma, however, in this system, interactions of dynamin2 with PI3K and the phosphatase, SHP-2, were involved (Feng et al., 2012). Thus, in two different model systems, dynamin2 supported metastatic behavior via mechanisms that involve complexes with signaling proteins. Whereas the studies cited above point to a promigratory function for dynamin2 in tumor-cell invasion, metastatic progression of cervical squamous cell carcinoma and expression of the ECM-degrading metalloprotease, MMP-2, were inversely correlated with dynamin2 expression (Lee et al., 2010).

4.3. Molecular Mechanisms of Actin Filament Regulation by Dynamin

Understanding the molecular mechanisms by which dynamin regulates cytoskeletal filaments is an active goal. Initial clues that dynamin might regulate cytoskeletal filaments arose from identifying its direct interactions with microtubules (Shpetner and Vallee, 1989) and actin regulatory proteins (Ferguson and De Camilli, 2012; Kruchten and McNiven, 2006; McNiven et al., 2000; Schafer, 2004). The list of dynamin-interacting proteins continues to expand, with dynamin now recognized as a versatile molecular scaffold that could assemble a myriad of different protein complexes, including actin assembly factors (Krueger et al., 2003; McNiven et al., 2000; Weed et al., 2000), scaffolds and enzymes involved in signal transduction (Cao et al., 2010; Lai et al., 1999; Okamoto et al., 1999; Salazar et al., 2003) and membrane curvature sensing and generating proteins (Anggono and

Robinson, 2007; David et al., 1996; Grabs et al., 1997). Most often, interactions with binding partners occur between the dynamin PRD and SH3 domain(s) of the partner protein. Indeed, a mutant form of dynamin lacking the PRD rarely functions in cells. Among dynamin isoforms, the PRD varies most in primary amino acid sequence (Urrutia et al., 1997). The potential for dynamin family members to participate in a complex array of activities as specified by the diverse interactions of its PRD is vast. In this section, we review recent research that provides mechanistic insight for how dynamin regulates actin filaments.

4.3.1. Dynamin and Cortactin

The F-actin-binding protein cortactin appears to mediate many actin-dependent processes in which dynamin is implicated to participate. Cortactin binds the dynamin PRD and, like dynamin, is enriched in lamellipodia (McNiven et al., 2000; Weed et al., 2000), dorsal ruffles (Krueger et al., 2003), invadopodia (Clark et al., 2007), podosomes (Tehrani et al., 2006), actin comets (Barroso et al., 2006) and at the trans-Golgi network (Cao et al., 2005). Other activities of cortactin, specifically its ability to bind and activate the actin-nucleating activity of Arp2/3 complex and to stabilize branched F-actin junctions (Weaver et al., 2001), highlight cortactin as a key bridge between dynamin and dynamic actin filament networks. Although dynamin was recently found to directly bind actin filaments with sub-micromolar affinity (Gu et al., 2010), cortactin greatly potentiates the association of dynamin with actin filaments in vitro (Gray et al., 2005; Mooren et al., 2009). Several different complexes of dynamin and cortactin, together with other actin regulators or signaling molecules, have been identified (Cao et al., 2005; Chua et al., 2009; Gray et al., 2005; Krueger et al., 2003; Kurklinsky et al., 2011; McNiven et al., 2000). Cortactin also stimulated the basal GTPase activity of dynamin2 in vitro, but the biological significance of this activation is unknown (Mooren et al., 2009).

Biochemical experiments performed with purified proteins in vitro have provided intriguing clues of molecular mechanisms by which dynamin might influence actin filament dynamics and organization. An ongoing challenge will be to validate these mechanisms in living cells and identify how each may be executed in space and time to influence actin-dependent processes. It is likely that dynamin exploits many of the intrinsic activities required for membrane remodeling and scission in its actions on actin filaments. Dynamin2 and cortactin, which together potentiate

multivalent actin filament cross-linking, tightly bundled actin filaments *in vitro;* importantly, GTP hydrolysis by dynamin2 remodeled the bundled filaments from tight to loosely associated (Mooren et al., 2009). When viewed in real time using total internal reflection fluorescence microscopy, filaments in bundles actively unraveled upon addition of GTP (Mooren et al., 2009). GTP hydrolysis by dynamin2 also influenced the organization of actin filaments associated with PI-4,5-P_2-containing liposomes (Schafer et al., 2002). GTPase-dependent changes in the relative orientations of filaments within bundled networks could influence access and binding to filaments of other actin regulatory factors, such as cofilin (Mooren et al., 2009). Moreover, actin filament severing was observed as a result of dynamin2-mediated GTP hydrolysis (Mooren et al., 2009); filament severing could influence filament dynamics either by promoting F-actin turnover or by promoting *de novo* actin assembly, depending on the cellular context. Thus, GTP hydrolysis by dynamin2 and cortactin may influence actin filaments via a broad range of mechanisms, including promoting actin assembly, promoting actin filament turnover or remodeling filament architecture.

4.3.2. Direct Binding of Actin Filaments by Dynamin

The recent discovery that dynamin directly binds actin filaments brought a novel twist to the scope of mechanisms by which dynamin might regulate actin filaments. Dynamin bundled actin filaments *in vitro* via interactions of filaments with conserved basic residues within an α-helix of the dynamin stalk (Gu et al., 2010). Interestingly, ring-shaped dynamin oligomers preferentially associated with short actin filaments, which also maximally stimulated GTP hydrolysis by dynamin. Unexpectedly, dynamin rings promoted "uncapping" of filaments capped at their barbed end by gelsolin (Gu et al., 2010). GTP hydrolysis was not required to uncap filaments, which occurred in the presence of the nonhydrolyzable GTP analog, GTPγS (Gu et al., 2010). Like filament severing, dynamin-mediated filament uncapping could promote either actin assembly or filament disassembly, depending on the local conditions. Support for the hypothesis that dynamin-mediated filament uncapping promoted actin assembly came from studies of cultured podocytes depleted of dynamin2, in which actomyosin-containing stress fibers were disrupted and the accessibility of actin filament barbed ends was reduced (Fig. 3.1) (Gu et al., 2010).

In summary, a growing body of evidence supports the hypothesis that proteins of the dynamin family act directly, or indirectly via PRD-binding

partners, on actin filaments. The GTP hydrolysis-dependent activities of dynamin on actin filaments identified in in vitro experiments could modulate cellular actin networks via several different mechanisms, including stimulating local F-actin assembly, promoting F-actin turnover and remodeling actin filament architecture. Additionally, dynamin may modulate actin networks by acting as a dynamic molecular scaffold that recruits factors for de novo actin assembly (Cestra et al., 2005; Feng et al., 2012; Gomez et al., 2005; Salazar et al., 2003; Soulet et al., 2005; Wang et al., 2011; Xin et al., 2009).

5. DYNAMIN MUTATIONS, CYTOSKELETON AND HUMAN DISEASE

Mutations in the gene encoding dynamin2 have been linked to two diseases that affect muscle and peripheral nerve function: centronuclear myopathy (CNM), a disorder resulting in muscle degeneration (Bohm et al., 2012; Durieux et al., 2010), and Charcot–Marie–Tooth (CMT) disease, a neuropathy of peripheral nerves (Zuchner et al., 2005). Clustered mutations in the dynamin2 stalk and PH domain, along with a few others, have been linked to CNM and CMT (Bitoun et al., 2005; Zuchner et al., 2005). Some disease-associated mutant dynamin proteins are associated with microtubules. For example, exogenously expressed dynamin2-Δ3, a CMT-related dynamin with a small deletion within the PH domain, prominently decorated microtubules and induced increased levels of acetylated tubulin along microtubules of cultured COS-1 cells (Tanabe and Takei, 2009). In other studies, however, cellular levels of acetylated tubulin appeared normal when dynamin2-Δ3 was expressed at near endogenous levels in fibroblastoid cells lacking wild-type dynamin2 (Liu et al., 2011a). Surprisingly, CME was not perturbed in fibroblastoid cells expressing some common mutant dynamin2 proteins associated with CNM or CMT; rather, slight defects in selected secretory pathways and in non-clathrin-dependent endocytic pathways were noted (Liu et al., 2011a). To the extent that disease-associated mutant dynamin2 proteins have been studied, none perturbed PDGF-stimulated macropinocytosis, an actin-dependent endocytic process (Liu et al., 2011a). A recently developed mouse model of CNM in which a common human mutation, dynamin2-R465W, was expressed in skeletal muscle highlights a role for dynamin2 in several aspects of muscle tissue homeostasis, including sarcomere assembly, contractility and myofiber integrity.

6. CONCLUDING REMARKS

The spectrum of cellular activities, in which dynamin is implicated to participate, is expanding rapidly. Evidence collected in the years 2000–2010 bolsters hypotheses that dynamin may broadly influences cytoskeletal actin and microtubule networks via a diverse repertoire of molecular mechanisms. Future work will focus on clarifying the relationship between dynamin's actions during endocytosis or other membrane-remodeling events and its actions on cytoskeletal filaments. During endocytosis and exocytosis, dynamin may execute membrane remodeling in concert with actin filament remodeling to maximize efficiency. New mutant dynamin proteins that allow investigators to separate dynamin's activities on membranes and cytoskeletal filaments will be needed to clarify this relationship. Moreover, because the scope of cellular processes in which dynamin modulates the cytoskeleton is broad, it will be necessary to investigate the physiological significance of its actions in different cellular contexts.

Clearly, the focus of most investigations to date has centered on the actin-related functions of dynamin. In this regard, biochemical experiments have offered intriguing clues of potential molecular mechanisms that must now be examined in living cells. Despite its discovery as a microtubule-bundling protein, very little is known about the molecular mechanisms by which dynamin influences microtubules. This aspect of dynamin action is particularly mysterious because dynamin does not associate prominently with microtubules in interphase cells yet depleting dynamin impacts microtubule dynamics globally. Identifying how dynamin influences microtubule dynamic instability remains an important focus for future investigations. With respect to actin filaments, GTP hydrolysis by dynamin, in combination with its myriad of direct and indirect interactions with actin filaments and their regulators, provides a diverse array of mechanisms by which dynamin could modulate actin filament networks. In keeping with the restricted distribution of dynamin within most networks, dynamin's actions are likely to be local, rather than global. Site-specific actions of dynamin may serve to coordinate local reorganization of the actin cytoskeleton at other cellular structures, such as at clathrin-coated pits, EPEC pedestals, actin comets and podosomes. However, local actin filament remodeling by dynamin within lamellipodia or adherens junctions may ultimately influence actin networks required for contractility and whole-cell migration.

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CHAPTER FOUR

Beta-Barrel Scaffold of Fluorescent Proteins: Folding, Stability and Role in Chromophore Formation

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Contents

Abstract

This review focuses on the current view of the interaction between the β-barrel scaffold of fluorescent proteins and their unique chromophore located in the internal helix. The chromophore originates from the polypeptide chain and its properties are

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influenced by the surrounding protein matrix of the β-barrel. On the other hand, it appears that a chromophore tightens the β-barrel scaffold and plays a crucial role in its stability. Furthermore, the presence of a mature chromophore causes hysteresis of protein unfolding and refolding. We survey studies measuring protein unfolding and refolding using traditional methods as well as new approaches, such as mechanical unfolding and reassembly of truncated fluorescent proteins. We also analyze models of fluorescent protein unfolding and refolding obtained through different approaches, and compare the results of protein folding *in vitro* to co-translational folding of a newly synthesized polypeptide chain.

1. INTRODUCTION

Fluorescent proteins (FPs) are a powerful tool for the bioimaging of single molecules, intact organelles, live cells, and whole organisms. Fluorescence microscopy has become an invaluable approach in the fields of biochemistry, biotechnology, and cell and developmental biology. The great advantage of FPs in comparison with synthetic dyes and quantum dots is that they can be genetically introduced into cells, tissues, or whole organisms. FPs can be used to mark whole cells, to label and visualize single protein molecules, and to monitor their dynamics and interactions with other proteins.

Being enclosed in a β-barrel scaffold, the chromophore of FPs represents a unique fluorescent probe that is introduced into a target object within its own microenvironment (Fig. 4.1). The fluorescent properties of such a label are not sensitive to the environment but genetic engineering can be used to construct the microenvironment artificially. The construction of the first mutant variant of the wild-type green fluorescence protein (GFP) from the jellyfish *Aequorea victoria* with improved properties was the beginning of the continued development of FP variants. The new variants allow the use of advanced techniques, and development of novel methods requires the design of new FP variants. A pallet of FPs ranging from blue to far-red with different quantum yields, fluorescence lifetimes and photochemical characteristics has been developed. Remarkably, though some of these variants have <25% amino acid identity with the wild-type protein, all of them have a β-barrel fold with the chromophore inside.

It is generally established that the existence of a unique FP chromophore relies on the barrel and that its properties depend on the protein matrix vicinity. Much less is known about the influence of the chromophore on the β-barrel. This chapter summarizes what is known about the relationship between the β-barrel scaffold and the unique chromophore that it contains. The first two parts provide an overview of the current knowledge

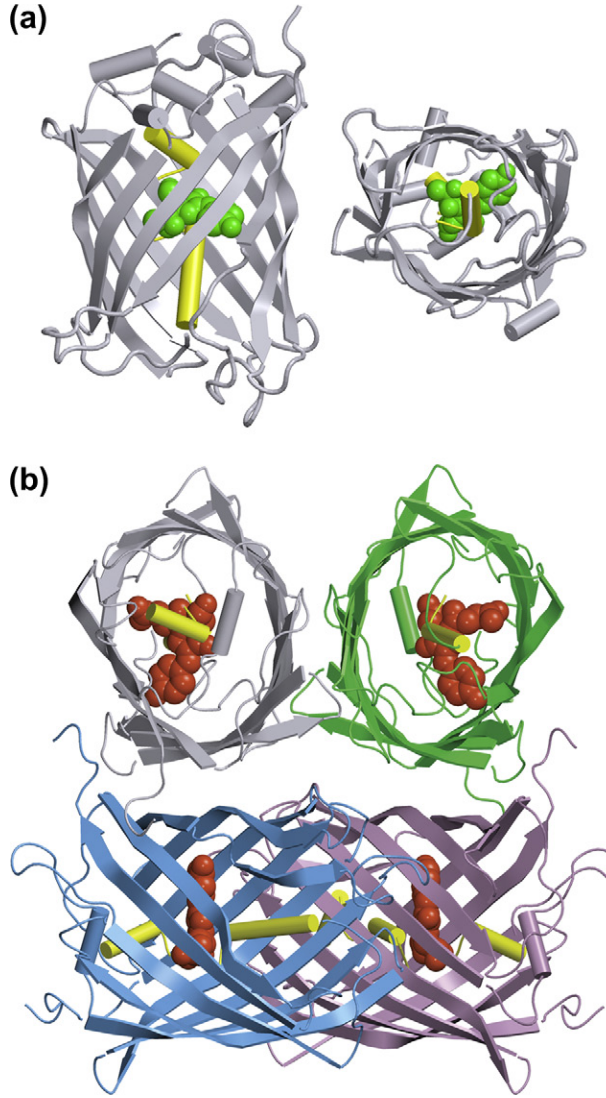

Figure 4.1 ***Three-dimensional structure of sfGFP (PDB code 2B3P, Pedelacq et al., 2006) in two projections*** (*a*) and of DsRed1 from *Discosoma* sp. (PDB code 1G7K, Yarbrough et al., 2001) (*b*). The chromophores of sfGFP and DsRed1 are shown as *green* and *red space-filling unions*, respectively. A central α-helix bearing the chromophore is shown in *yellow*. Monomers of DsRed1 are displayed in different colors. The drawing was generated by the graphic programs VMD (Humphrey et al., 1996) and Raster3D (Merritt and Bacon, 1997). (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book).

about the effect of the β-barrel scaffold on the chromophore properties. First, we summarize a variety of chromophore structures found in FPs, and we describe the autocatalytic and light-induced chromophore formation and transformations and discuss the interaction of the chromophore with protein matrix of the β-barrel. Then, we describe the structure and fluorescent properties of the chromophore from *A. victoria* GFP, its genetically engineered variants, and FPs from other organisms.

The last two parts are devoted to the problems of FP folding and stability, and how they are influenced by the chromophore. Internal interactions play a crucial role in all β-barrel proteins and in FPs, in particular, identifying them as globular proteins. For this reason, we begin with a brief reminder of the fundamental principles of globular protein folding and a description of the first examinations of the stability of different FPs. These studies were complicated by FP high stability, as quasi-stationary dependences were obtained only after several days of protein incubation in a denaturant. Furthermore, some FPs are prone to aggregation. The development of cycle3-GFP and superfolder-GFP (sfGFP), which do not aggregate and for which the refolded protein has the same properties as the native protein, allowed the possibility of a careful systematic study of unfolding–refolding processes. In this work, we attempt to analyze the reasons for the discrepancies in the description of these processes presented in different papers. We also analyze what is known from the literature on hysteresis during FP unfolding and refolding processes and the role that chromophore plays in it.

We present studies performed both using traditional methods to examine protein unfolding–refolding as well as new approaches, such as mechanical unfolding and reassembly of truncated FPs. We analyze the comparability of the results obtained using the different methods. We also compare the results of FP folding in vitro with co-translational folding of a newly synthesized polypeptide chain.

2. CHROMOPHORE FORMATION AND TRANSFORMATIONS IN FLUORESCENT PROTEINS

The fluorescence of FPs and their engineered variants spans the spectral range from blue to far-red. The main determinant of the emission hue is the chemical nature of the chromophore housed within the β-barrel. The amino acids in the chromophore environment fulfill multiple functions. Some of them contribute to chromophore synthesis; the contacts of other

amino acids with the chromophore are related to the further adjustment of FP spectroscopic features and structural stability.

2.1. Chromophore Structures Found in Fluorescent Proteins

The ability of FPs to fluoresce in the visible spectral region descends from the chromophore hidden in the β-barrel scaffold. The position of the FP on spectral scale is mostly determined by the chemical structure of the chromophore, i.e. the more extended the system of π-conjugated electrons, the more red-shifted emission. Chromophore maturation does not require the involvement of any cofactors or enzymatic components except for molecular oxygen. The only prerequisite for the initiation of chromophore maturation is correct protein folding, which results in the bending of the central α-helix, exposing the chromophore-forming tripeptide and arranging the catalytic amino acids in a position that is favorable for chromophore synthesis. The chromophore is self-generated from an internal tripeptide, X65Tyr66Gly67, through a multistep reaction that includes cyclization, dehydration, oxidation and, in some cases, hydrolysis. The first step of this complex reaction, tripeptide cyclization and the subsequent proton abstraction from the α-carbon of Tyr66, is supposed to be promoted by Arg96 and Glu222 with Arg96 playing the role of an electrostatic catalyst and Glu222 acting as base catalyst (Sniegowski et al., 2005; Wood et al., 2005). These amino acids are highly conserved among FPs. In addition to the catalytic function of Glu222, inspection of the changes in the crystallographic structure of EGFP induced by excessive X-ray irradiation revealed a stabilizing role of Glu222 (Royant and Noirclerc-Savoye, 2011). X-ray-induced EGFP bleaching was shown to be related to Glu222 decarboxylation and the associated rearrangement of the hydrogen bond network. On the basis of these observations, it was proposed that Glu222 contributes the rigidity of the chromophore cavity, thus restricting chromophore flexibility and preventing it from nonradiative deactivation of the excited state. The other absolutely conserved amino acid is the chromophore-forming glycine residue located at position 67. Substitution of Gly67 with any other residue impairs chromophore synthesis. It is believed that glycine is the only residue at position 67 that allows the formation of a central α-helix with the required kinked conformation. In that conformation of the α-helix, the amide nitrogen of Gly67 is in close proximity to the carbonyl carbon of the residue at position 65 and can perform a nucleophilic attack. In all natural FPs, position 66 is occupied by a Tyr residue but it can be replaced with any aromatic amino acid, as has been shown for artificial FP variants. Indeed,

a cyan-emitting variant of GFP contains Trp at position 66, and a blue-emitting variant of GFP has a His residue instead of Tyr at position 66 (Tsien, 1998). It was shown that chromophore formation takes place in FP variants bearing Ser, Leu or Gly at position 66 but the resulting structures do not fluoresce and they instead undergo further reactions, such as hydrolysis (Barondeau et al., 2006, 2007). These data indicate that Tyr66 provides the proper oxidative chemistry during chromophore maturation and prevents undesirable side reactions, such as backbone fragmentation and hydrolysis. Analysis of more than 200 FP structures available in the Protein Data Bank revealed three highly conserved glycine residues located at positions 31, 33 and 35 (Ong et al., 2011). Interestingly, these internal amino acids are not involved in chromophore maturation, and their functions remain unclear.

A variety of chromophore structures is found in FPs (Fig. 4.2). The GFP-like green emitting chromophore that was the first to be discovered consists of two aromatic rings, including a phenolic ring from Tyr66 and a five-membered heterocyclic structure (Fig. 4.2*a*, (Cody et al., 1993; Ormo et al., 1996)). These two aromatic cycles are incorporated in the chromophore system through the bridge between them. The bridge is oxidized to have a double bond and to accomplish the conjugation of π-electrons from both aromatic rings. Such an expanded system of π-conjugated electrons is able to absorb and emit visible light.

The red emitting DsRed-like (according to DsRed from *Discosoma* species where it was found for the first time) chromophore contains an additional desaturated Cα–N bond at the Gln65 residue that further extends the system of π-conjugated electrons and results in a red shift of the absorption and emission (Fig. 4.2*b*; Gross et al., 2000). An entirely different type of red emitting chromophore is presented by Kaede-like chromophore (from the Kaede derived from the stony coral *Trachyphyllia geoffroyi*). Kaede-like chromophore is observed in a set of FPs, including Kaede, EosFP, dendFP and others (Matz et al., 2006). These proteins bear chromophores composed of three aromatic rings where a GFP-like chromophore core is supplemented by an indole ring from the His65 residue (Fig. 4.2*c*; Mizuno et al., 2003).

In contrast to the DsRed-like chromophores, the blue-emitting chromophore of mTagBFP (Subach et al., 2008) and mTagBFP2 (Subach et al., 2011a) has a shorter π-conjugated system of a five-membered heterocyclic structure and an N-acylimine double bond between the Cα and N atoms of the Leu65 residue, while the phenolic ring of the Tyr66 residue is out of conjugation being nearly perpendicular to the rest of the chromophore (Fig. 4.2*d*; Subach et al., 2010c).

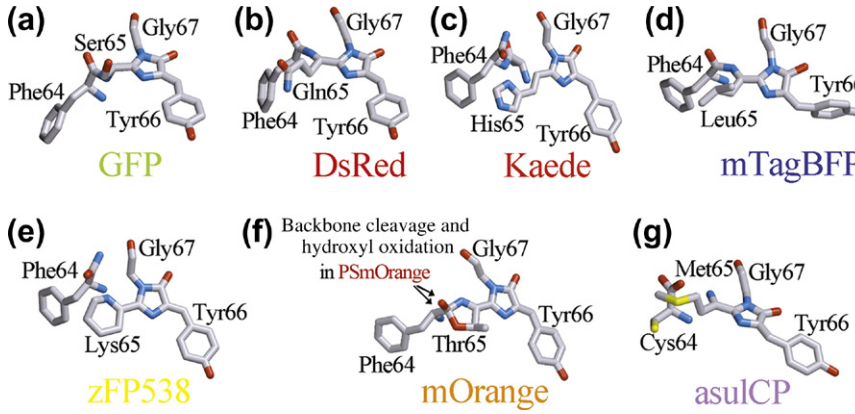

Figure 4.2 ***A variety of chromophore structures in FPs.*** *a* – green chromophore of GFP (PDB code 1W7S; van Thor et al., 2005); *b* and *c* – red chromophores of DsRed (PDB code 1G7K; Yarbrough et al., 2001) and Kaede (PDB code 2GW4; Hayashi et al., 2007); *d* – blue chromophore of mTagBFP (PDB code 3M24; Subach et al., 2010c); *e*–*g* – derivatives of the DsRed-like red chromophore of zFP538 (PDB code 1XAE; Remington et al., 2005), mOrange (PDB code 2H5O; Shu et al., 2006), PSmOrange and asulCP (PDB code 2A50; Andresen et al., 2005). Carbon, nitrogen, oxygen and sulfur are colored in gray, blue, red and yellow, respectively. The drawing was generated based on the Protein Data Bank (Dutta et al., 2009) by the graphic programs VMD (Humphrey et al., 1996) and Raster3D (Merritt and Bacon, 1997). (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book).

There are at least four derivatives of the DsRed-like chromophore. Three of them are three-ring π-systems. The yellow chromophore of zFP538 from *Zoanthus* species has an additional tetrahydropyridine ring derived from the Lys65 residue (Fig. 4.2*e*; Remington et al., 2005). The third dihydrooxazole ring of the orange chromophore found in mOrange, KO and its mutant variants is generated from the Thr65 residue (Fig. 4.2*f*; Shu et al., 2006). It is believed that less effective conjugation of the π-electrons is responsible for the spectroscopic features of yellow/orange FPs. The chromophore in the far-red photoswitched form of PSmOrange (Subach et al., 2011b), a mutant variant of mOrange, is also a three-ring system, in which the third dihydrooxazole ring is further oxidized to have a C═O double bond instead of a hydroxyl group (Fig. 4.2*f*). The far-red emission of light-induced PSmOrange and its enhanced version PSmOrange2 (Subach et al., 2012) is attributed to a more efficient π-conjugation of the GFP-like core with alylimine and carbonyl groups that are involved in the dihydrooxazole ring. Acylimine functionality of the DsRed-like chromophore in the chromoprotein asCP from sea anemone *Anemonia sulcata* and its derivative

KFP undergoes hydrolysis between the carbon atom of the residue at position 64 and the N1 atom of the Met65 residue, leading to chromophore fragmentation (Fig. 4.2*g*; Quillin et al., 2005; Yampolsky et al., 2005). As we can see, position 65 of the chromogenic tripeptide can be occupied by any amino acid, which affects the chromophore chemistry and results in diverse chromophore structures.

Chromophores can adopt both *cis*- and *trans*-configurations with nonplanar *trans*-chromophores seen mostly in nonfluorescent CPs while the nearly planar *cis*- and *trans*-chromophores are characteristic of proteins with a high quantum yield of fluorescence. An analysis of FP structures available in the Protein Data Bank revealed that there is a low frequency of perfectly planar chromophores in FPs (Maddalo and Zimmer, 2006). It was proposed that the chromophore cavity of FPs is not complementary to a planar chromophore, and thus the protein matrix induces chromophore deformation, twisting the phenolic ring of the Tyr66 residue slightly around Cα═Cβ double bond of the bridge. The ethylenic bridge is supposed to prevent the chromophore from undergoing a more prominent deformation. Upon excitation of the chromophore, the π-conjugation of the bridge is reduced and the phenolic ring of the chromophore can rotate freely. In this case, the protein matrix does not allow the chromophore to gain the perpendicularly twisted conformation that is postulated to be the main pathway of nonradiative energy dissipation (Megley et al., 2009). Thus, the microenvironment of the chromophore should be rigid enough in FPs with high quantum yield. Some of the recently developed FPs with far-red emission have a slightly relaxed chromophore microenvironment (Section 2.3). As a result, the quantum yield of those proteins suffers, but their chromophores gain the ability to equilibrate with the polar environment of the protein matrix. Thus, the more pronounced Stokes shift in those proteins is achieved at the expense of their quantum yield (Abbyad et al., 2007).

2.2. Autocatalytic and Light-Induced Chromophore Formation and Transformations

The general scheme of the autocatalytic chromophore maturation is presented in Fig. 4.3. The chromophore posttranslational chemistry is triggered by protein folding that brings the nitrogen of Gly67 and the carbonyl carbon of the residue at position 65 in close proximity to each other (Fig. 4.3*a*). The distance between the amide nitrogen of Gly67 and the carbonyl carbon of X65 at the deformed path of the α-helix inside the β-barrel was calculated to be less than the sum of their covalent radii (Lemay et al., 2008).

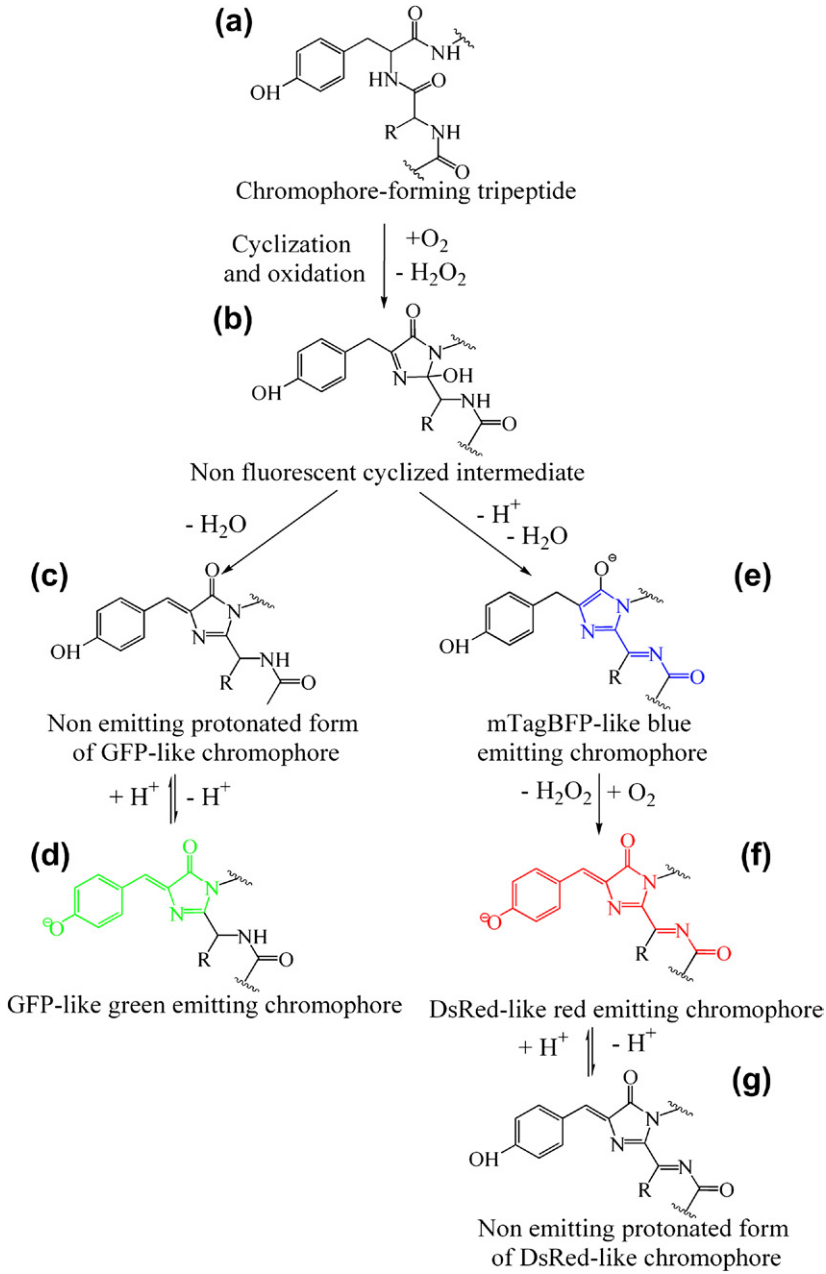

Figure 4.3 ***General scheme of the autocatalytic synthesis of blue, green and red chromophores.*** (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book).

Following the folding, cyclization and oxidation events lead to the formation of the imidazolone-containing product that absorbs at approximately 350 nm and does not emit (Fig. 4.3*b*). Dehydration and oxidation of the Cα–Cβ bond of the Tyr66 residue result in green GFP-like chromophore formation (Fig. 4.3*c*). In this proton abstraction, the catalytic role is played by Arg96 and Glu222. The formation of the red DsRed-like chromophore is more complex (Miyawaki et al., 2012; Subach and Verkhusha, 2012). It includes the accumulation of TagBFP-like blue intermediate first (Pletnev et al., 2010; Subach et al., 2009a, 2009c) (Fig. 4.3*e*). The mechanism of N-acylimine formation of the TagBFP-like chromophore involves cyclization followed by oxidation/dehydration or dehydration/oxidation steps with Glu222 as a base catalyst in the proton abstraction. The red chromophore synthesis requires the final oxidation of the Cα–Cβ bond of the Tyr66 residue, and catalytic functions in this process are suggested for the pairs of Glu222/Lys69, Glu222/Arg69 and Glu222/Arg203 in the case of mCherry (Subach et al., 2009a), FTs (Pletnev et al., 2010) and PAmCherrys (Subach et al., 2009a). The hydroxyl group of the phenolic ring in the chromophores of GFPs and red FPs (RFPs) can be in a protonated or deprotonated state depending on the features of the chromophore microenvironment (Fig. 4.3*c,d* and *f,g*). For example, hydrogen bond formation between Tyr66 and Thr203 stabilizes the anionic form of the green chromophore in GFP and its derivatives (Ehrig et al., 1995; Heim et al., 1994). Substitution of Thr203 with Ile in GFP derivatives, such as sapphire-GFP, results in a predominantly neutral form of the green chromophore because its anionic form cannot be solvated in the absence of the hydroxyl group of Thr203. The introduction of the carboxyl groups in the vicinity of the chromophore is a way to shift the equilibrium to the neutral form of the chromophore (Shi et al., 2007; Shu et al., 2007). The carboxyl groups have pK_a values that are lower than the pK_a of the Tyr66 side chain hydroxyl of the chromophore in the ground state, which encourages the carboxyl ionization and stabilization of the chromophore in the neutral form. The anionic form of the red and green chromophores usually has a high quantum yield when buried inside the protein globule, while their neutral forms emit virtually none.

The photophysics of the green and red chromophores in some GFPs and RFPs is strongly affected by excited state proton transfer (ESPT). This reaction was revealed for the first time for wild-type GFP (Brejc et al., 1997). The protein has an absorption spectrum with two peaks at 395 and 475 nm attributed to the neutral and deprotonated forms of the green chromophore. The light absorption by the neutral form of the green chromophore

enforces the proton abstraction from the hydroxyl of the Tyr66 residue, resulting in an intermediate excited state that differs from the excited state of the anionic chromophore by the conformation of the nearest amino acids to the chromophore. Both the intermediate excited state and the anionic chromophore emit a green light with maxima at 508 and 504 nm. Proton transfer takes place via a proton wire that is composed in GFP of the hydrogen bonded Ser65 and Tyr66 residues of the chromophore and a water molecule and the Ser205 and Glu222 residues. Recently, the second excited state intermediate preceding the fully deprotonated intermediate was observed by combined time resolved mid-infrared and visible pump-dump-probe spectroscopy experiments (Di Donato et al., 2011). The intermediate is characterized by a partial protonation of Glu222 and a shift of the protons involved in a hydrogen bond network. As a result of proton transfer events, the excitation of GFP at the absorption band of the neutral chromophore leads to a large Stokes shift of more than a 100 nm.

It was demonstrated that the same mechanism underlies the large Stokes shift of yellow/red FPs but their proton wires differ from those of GFP. For example, the high-resolution structure of red mKeima indicates that its chromophore is hydrogen bonded to the Asp165 residue via Ser148 (Henderson et al., 2009b). In contrast to mKeima, the recently developed LSSmKate1 and LSSmKate2 (Piatkevich et al., 2010a) and LSSmOrange (Shcherbakova et al., JACS, 2012) proteins do not exhibit an additional absorption from the anionic form of the chromophore. In LSSmKate1 and LSSmKate2, the proton is accepted by carboxyl group of Glu167 directly or by carboxyl group of Ser165 via Asp167. On the basis of these data, it was demonstrated that incorporation of the carboxyl groups in position 165 or 167 creates an ESPT pathway and thus induces a large Stokes shift in several conventional RFPs, including mNeptune, mCherry, mStrawberry, mOrange, and mKO (Piatkevich et al., 2010b). LSSmKates are good probes for two-photon microscopy as they can be efficiently excited with standard two-photon light sources. Their far-red emission in combination with two-photon excitation allows for deep-tissue intravital imaging (Piatkevich et al., 2010a).

In addition to general autocatalytic reactions where the choice between the competitive green and red branches of synthesis and its endpoint is determined by the peculiarities of the chromophore microenvironment, almost all of the transitions of the scheme shown in Fig. 4.3 can be engineered to be time-depended or light-inducible. For example, fluorescent timers developed on the basis of mCherry, such as fast-FT, medium-FT, and slow-FT (Subach et al., 2009c), have time-delays that vary in their extent

in the blue-to-red conversion (E-F transition in Fig. 4.3). The analysis of the crystal structures of the conventional blue-emitting Blue102 mutant of medium-FT and fast-FT combined with site-directed mutagenesis revealed the amino acids accounting for the timing properties of FTs (Pletnev et al., 2010). Amino acids at positions 69 and 82 affect the oxidation speed of Cα–Cβ of Tyr66, with Arg69 and Tyr82 delaying the oxidation and with Lys69 and Leu82 accelerating it. The rate of blue intermediate formation is determined by the amino acid at position 216: Ala and Cys residues at this position slow the acylimine formation, while Ser216 makes it faster. The blue-to-red conversion in Blue102 is blocked by the *trans* chromophore conformation, which is unfavorable for further oxidation. This is due to the presence of a bulky Ile at position 145 instead of Ser145 in fast-FT. The time-delayed blue-to-red conversion of FTs can be used for the temporal resolution of different cellular events.

The irreversible dark-to-red photoconversion of PATagRFP under UV light results in the bright red fluorescent form of the protein, which absorbs at 562 nm and emits at 595 nm (Subach et al., 2010a). Light-induced red fluorescence acquisition is supposed to be a two-step process involving the absorption of two photons by different chromophore intermediates. Presumably, the steps correspond to the B-to-E and E-to-F transitions shown in Fig. 4.3. The PATagRFP demonstrated a good performance in the multicolor single particle tracking photoactivated localization microscopy (spt-PALM) imagining of living cells (Subach et al., 2010a).

The irreversible light-induced E-to-F transition (Fig. 4.3) is realized in the photoactivatable FPs, such as the PAmCherries (Subach et al., 2009b). According to the crystallographic and mass spectrometric data, PAmCherries in the "dark-state" contain a nonplanar chromophore that is identical to the blue chromophore of mTagBFP. The oxidation of the Tyr66 Cα–Cβ bond in PAmCherries following UV light illumination is accompanied by the decarboxylation of the Glu222 and release of a CO_2 molecule via a Kolbe-like radical reaction. Photoactivated PAmCherries exhibit a bright red fluorescence with absorbance and emission maxima at approximately 570 and 595 nm, respectively. These proteins, as well as the other photoactivatable FPs, are intended for application in super-resolution techniques based on PALM (Subach et al., 2009b).

A number of irreversible and reversible photo-induced reactions that are not shown in Fig. 4.3 occur in FPs. We would like to briefly consider these reactions. A group composed of PAGFP, PSCFP and PSCFP2 is able to undergo irreversible phototransformation to a green emitting form

(Chudakov et al., 2004). These proteins before illumination with UV light have the GFP-like green chromophore stabilized in a neutral form. Dark-to-green photoactivation of PAGFP as well as cyan-to-green photoconversion of PSCFP and PSCFP2, similar to PAmCherries, is concomitant with decarboxylation of the Glu222 carboxyl group, which enforces a rearrangement of the chromophore environment and the successive chromophore ionization (Henderson et al., 2009a).

The Kaede subfamily of FPs, which includes Kaede (Ando et al., 2002), EosFP and its variant mEos2 (Wiedenmann et al., 2004), DendFP (Pakhomov et al., 2004) and its engineered monomeric versions Dendra and Dendra2 (Gurskaya et al., 2006), mcavRFP (Kelmanson and Matz, 2003), rfloRFP (Labas et al., 2002), mIrisFP (Adam et al., 2008), and mKikGR (Tsutsui et al., 2005), undergoes green-to-red photoconversion. The proteins in the Kaede subfamily hold the GFP-like two-ring chromophore in the green form and the three-ring Kaede-like chromophore in the red form. The His65 providing the third ring to the chromophore is supposed to be indispensable for Kaede-like chromophore synthesis. The green-like chromophore intermediate in the Kaede subfamily under light absorption is subjected to a series of photochemical reactions leading to the conjugation of the His65 indole ring with the GFP-like core through the Cα–Cβ double bond in His65 (Wiedenmann et al., 2011). The final red-emitting Kaede proteins contain a fluorescent anionic chromophore equilibrated with the nonfluorescent neutral form.

A set of FPs including cyan mTFP0.7 (Ai et al., 2006), green Dronpa (Ando et al., 2004), red asFP595 and its KFP derivative (Chudakov et al., 2003), rsCherry and rsCherryRev (Stiel et al., 2008), the green and red forms of IrisFP (Adam et al., 2008), and red rsTagRFP (Subach et al., 2010b) are capable of reversible photoconversion between fluorescent and nonfluorescent states under light illumination at a wavelength specific for the forward and backward transitions. The fluorescent state is supposed to contain an anionic chromophore, while the nonfluorescent state bears its neutral form, which is flexible enough to dissipate the excitation energy. Following the light absorption, a series of events occur involving a light-induced *cis–trans* isomerization of the chromophore along with the associated structural rearrangements within the chromophore's pocket and alterations of the hydrogen-bond network that change the protonation status of the chromophore (Henderson et al., 2007; Schafer et al., 2008).

An unusual mechanism that has not been previously described underlies a light-driven reversible photoconversion of Dreiklang, a mutant variant

of yellow Citrine (Brakemann et al., 2011). The protein maintains a GFP-like two-ring chromophore in the protonation–deprotonation equilibrium of the Tyr66 hydroxyl. Excitation at the absorption band of the anionic chromophore of Dreiklang with the maximum at 515 nm results in bright yellow fluorescence that peaks at 529 nm. Intense illumination at the absorption band of the neutral chromophore of Dreiklang with the maximum at 405 nm induces a switching to a nonfluorescent state that absorbs at approximately 340 nm. The reverse kindling of the protein is achieved by the illumination of the protein with light at 340 nm. An inspection of the structure of Dreiklang in its on- and off-states by X-ray diffraction analysis and electrospray ionization mass spectrometry experiments revealed the addition/elimination of a hydroxyl group donated by the water in the chromophore vicinity to its five-membered imidazolinone ring at the C═N bond. This chemical modification disrupts the conjugation of the imidazolinone ring with the phenolic ring of the chromophore, and the final structure absorbs in the UV region and does not fluoresce. Remarkably, the wavelength of florescence excitation of Dreiklang (approximately 515 nm) is decouple from that used for switching it off and on (405 and 365 nm), which allows for avoidance of the interlocking of a switching and fluorescence readout in microscopic experiments.

2.3. Interaction of Chromophore with Protein Matrix of β-Barrel

The interactions of the chromophore with amino acids of its microenvironment can have an additional impact on the excitation and/or emission spectra position. The well-known example of the batochromic shift induced by substitution outside the chromophore is the red shift by 20 nm of the yellow fluorescence of YFP, a yellow enhanced version of GFP, with respect to its precursor wild-type GFP (Ormo et al., 1996). The chemical structure of the YFP chromophore remains the same as that of GFP. A 20-nm shift of emission is generated by a Tyr residue introduced instead of Thr203 in the position located above the chromophore. The Tyr is supposed to be involved in the π–π stacking interaction with the phenolic ring of Tyr66 from the chromophore (Wachter et al., 1998).

This approach is realized in the natural yellow-emitting phiYFP from sea medusa *Phialidium* species (Pakhomov and Martynov, 2011). PhiYFP has the most red-shifted spectrum of fluorescence among the proteins bearing the GFP-like chromophore. The fluorescence of phiYFP is red shifted by 10 nm in comparison with YFP derived from GFP, which is indicative of additional

contacts that govern the spectroscopic properties of phiYFP in addition to π–π stacking. Two complementary structural factors that contribute to the yellow fluorescence of phiYFP have been proposed. They are an excitation-induced protonation of the N_2 nitrogen in the imidazolinone ring of the chromophore that takes place through the hydrogen bond network connecting the N_2 nitrogen with a proton donor Glu222 via the Thr65 and the destabilization of the negative charge at the phenolic ring of Tyr66 due to the absence of hydrogen bond donors in the vicinity of the phenol hydroxyl.

The opposite behavior has been observed in cyan amFP486 from *Anemonia majano* (Henderson and Remington, 2005) and mTFP1, a monomeric version of FP from *Clavularia* (Ai et al., 2008), where a stabilization of the negative charge of the phenol in the chromophore results in the blue shift of both absorbance and emission relative to that of GFP. The stabilization is achieved through the interaction of the Tyr66 phenolic ring with the positively charged His199, which is properly oriented against the chromophore by a well-organized hydrogen bond wire.

A batochromic shift of the spectra in the far-red FPs, mNeptune and mPlum, is also not coherent with covalent modification of a DsRed-like chromophore. It is attributed to the interaction of acylimine oxygen of the chromophore with the hydrogen bond donor in the chromophore-bearing pocket. In mPlum, the carboxyl group of the Glu16 residue donates the hydrogen bond that is weak in the ground state, but the light absorption stimulates the reorientation of the Glu16 side chain with subsequent strengthening of the hydrogen bond (Abbyad et al., 2007; Shu et al., 2009). That is, the proposed mechanism for the red shift in the emission with no effect on the absorption spectrum position of mPlum. In mNeptune, a water molecule that occupies a free space created by substitution of Met41 with Gly forms a strong hydrogen bond in both the ground- and excited-states leading to the red shift in the excitation and emission spectra (Lin et al., 2009). Similar to mNeptune, this mechanism causes the red shift in the recently developed far-red proteins eqFP650 and eqFP670 (Shcherbo et al., 2010). These proteins also contain a water-filled cavity due to the substitution of Met44 with less bulky amino acids.

As we can see, the chromophore makes numerous contacts with the protein matrix which affect the photophysics of the chromophore and tune the color of the fluorescence. We suppose that noncovalent interactions of the chromophore with the β-barrel do influence the protein stability. It has been shown that FP mutants that are unable to form chromophores have decreased stability with respect to their chromophore-bearing counterparts.

Indeed, a mutant form of GFP-S65T with the substitution of Gly67, a highly conserved amino acid that is strongly required for chromophore formation, to Ala is two times less stable against guanidine hydrochloride denaturation (Kutrowska et al., 2007) compared with EGFP (Stepanenko et al., 2004), with the mid-point of the transition being approximately 1.2 and 2.3 M for GFP-S65T/Gly67Ala and EGFP, respectively. An analysis of GdnHCl-induced denaturation of two mutant forms of sfGFP defective for chromophore synthesis has been performed (Andrews et al., 2007). The first mutant contains the substitution of catalytic Arg96 to Ala. The second has the substitutions Met88Tyr and Tyr74Met in positions preceding Pro75 and Pro89, precluding the correct conformation of α-helix. Both mutants of sfGFP exhibit significantly lower resistance to GdnHCl compared to sfGFP with the midpoint of transition being approximately 1.3, 0.8 and 4.2 M for sfGFP/Arg96Ala, sfGFP/Met88Tyr/Tyr74Met and sfGFP, respectively (Andrews et al., 2007).

3. STRUCTURE OF FLUORESCENT PROTEINS AND THEIR UNIQUE PROPERTIES

All FPs share the same β-barrel fold that in addition to being vital for fluorescence acquisition, donates them formidable stability. Together with the structural similarity, all FPs have the same drawbacks, mainly a propensity for oligomerization and aggregation. Here, we consider the peculiarities of structural and supramolecular organization of FPs and the ways to overcome their limitations. We start with wild-type GFP from the jellyfish *A. victoria* whose main disadvantage is poor folding at temperatures exceeding the temperature of its natural environment of cold boreal waters.

3.1. *Aequorea victoria* GFP and its Genetically Engineered Variants

Wild-type GFP, discovered first by Shimomura, is a small 25-kDa protein of 238 amino acids (Shimomura, 2006). Its polypeptide chain adopts a β-barrel scaffold that is vital for green fluorescence acquisition (Fig. 4.1). The eleven-stranded β-barrel of GFP is flanked by lids on both sides (Ormo et al., 1996). The barrel encloses an α-helix that runs up the barrel axis. This central α-helix is deformed substantially by the chromophore in the middle section. The chromophore inside the protein globule is protected from the bulk solvent by the barrel and lids. This spatial pattern is highly conserved among all GFP-like proteins.

Interestingly, careful examination of the 266 structures of GFP-like proteins available in the Protein Data Bank showed low variability of the lid segments of the β-barrel, especially of a lid that is opposite to the N- and C-termini (Ong et al., 2011). The amino acids in the next positions are highly conserved, corresponding to residues 89, 91 and 196 on the side where the N- and C-termini are situated and residues 20, 23, 27, 53, 101, 102, 104, 127, 130, 134 and 136 of the other lid of the barrel. In GFP, positions 89 and 196 are occupied by proline amino acids. The peptide bond preceding Pro196 is a typical *trans*-bond, while the side chain of the residue preceding Pro89 is in the *cis*-conformation. This behavior is characteristic of all GFP-like proteins with few exceptions (Ong et al., 2011). Moreover, the mutation of residues preceding Pro89 and Pro196 in GFP results in ineffective chromophore maturation (Andrews et al., 2007). Apparently, the X88-Pro89 and X195-Pro196 patches are important for maintaining of the kink in the α-helix backbone that is required for chromophore synthesis. The function of the highly conserved lid, which is curiously disordered, remains to be discovered. There is an assumption that the lid could be involved in some protein–protein interaction. This makes sense if we take into account that disordered protein segments or completely disordered proteins often have numerous partners and that they gain more structure during interaction with these partners (Turoverov et al., 2010).

GFP has a complex two-peaked excitation spectrum with a major peak at 395 nm and a minor one at 475 nm (Tsien, 1998). These two bands have been attributed to absorption by two chemically distinct chromophore species, i.e. the neutral and anionic form of the green chromophore. Excitation of both the neutral and the anionic chromophore results in green fluorescence with the maximum at 508 nm as the neutral chromophore tends to ionize in the excited state. Direct emission from a neutral chromophore occurs with low probability giving a shoulder in the emission spectra at 475 nm. Excited-state reactions of the chromophore are mediated by a proton wire that is composed of Ser65, Tyr66, Ser205 and Glu222 residues and a bound water molecule in GFP. It should be noted that many amino acids with side chains placed inside the barrel of GFP are charged or polar. Additionally, there are numerous bound water molecules inside the barrel cage. These residues and water molecules connected through hydrogen bonds are involved in chromophore synthesis and determine the photophysical behavior of the chromophore.

The excitation spectrum of GFP is strongly influenced by environmental factors, such as pH, temperature and small concentrations of denaturants

and ions. For example, solution alkalization up to pH values of 10–11 leads to a decrease of the excitation band at 395 nm with a concomitant increase of the excitation band at 475 nm (Tsien, 1998). At elevated temperatures, similar changes are observed. The influence of small concentrations of denaturants and ions will be considered thoroughly in the next section. Wild-type GFP tends to form dimers at increased concentrations, and this aggregation also results in excitation spectrum deviations. In this case, the excitation band at 395 nm increases at the expense of the excitation band at 475 nm (Tsien, 1998).

GFP folds properly at temperature lower the 25 °C, and it accumulates in insoluble aggregates when expressed at 37 °C (Tsien, 1998). This drawback stimulated the creation of mutant variants of GFP with enhanced folding properties. One of these optimized variants is GFPmut1, which is identical to the commercially available and widely used EGFP (enhanced GFP) (Cormack et al., 1996). The GFPmut1/EGFP protein contains a substitution of Phe64 to Leu and Ser65 to Thr. A single mutation, Phe-64Leu, improves protein folding at 37 °C and the mutation Ser65Thr makes GFPmut1/EGFP manifold brighter compared to the wild-type protein. Still, the folding efficiency of GFPmut1/EGFP is quite low. Only 20% of GFPmut1/EGFP is present in the soluble form at 37 °C. The other variant of GFP with a reduced propensity for aggregation is cycle3-GFP or GFPuv (Patterson et al., 1997). It has three amino acid substitutions: Phe99Ser, Met153Thr and Val163Ala. The mutations such as Phe64Leu and Val163Ala are the most often used "folding enhancing" mutations in GFP mutants.

Provided by Invitrogen, a mutant form of Emerald is considered to be one of the best GFP variants based on its brightness and folding features (Shaner et al., 2005). The total list of substitutions in Emerald includes Phe64Leu, Ser65Thr, Ser72Thr, Asn149Lys, Met153Thr, Ile167Thr, and His231Leu. Careful examination of the impact of these substitutions on the brightness of bacterial colonies expressing the mutant form of GFP revealed that the most important substitutions for acquisition of green fluorescence are Phe64Leu, Ser65Thr, Ser72Thr, Asn149Lys, and Ile167Thr (Teerawanichpan et al., 2007). The substitutions Met153Thr and His231Leu were neutral in this regard. The mutation Met153Thr found in cycle3-GFP is believed to diminish protein aggregation at elevated temperatures. On the other hand, in a yellow variant of GFP, the mutation Met153Thr together with Val163Ala and Ser175Gly substitutions have been shown to improve the kinetics of chromophore maturation (Rekas et al., 2002). According to these observations, a VisGreen variant has been created bearing the minimal

set of mutations that are indispensable for achieving the maximal visible fluorescence (Teerawanichpan et al., 2007). They are the aforementioned Phe64Leu, Ser65Thr, Ser72Thr, Asn149Lys, and Ile167Thr. This version performed well in plant and animal cells.

The combination of the "enhanced GFP" mutations and the "cycle3" mutations yielded a GFP+ variant, a protein that possesses bright fluorescence and is able to fold correctly at 37 °C (Scholz et al., 2000; Waldo et al., 1999). It should be noted that GFP+ is also known as "folding-reporter-GFP." Indeed, the folding efficiency, and hence the fluorescence, of GFP+ when it is fused downstream to a cellular protein is strongly correlated with the folding status of the fusion partner (Waldo et al., 1999). GFP+ has been widely used for screening soluble proteins (Pedelacq et al., 2002; van den Berg et al., 2006; Yokoyama, 2003), discovering drugs for degenerative diseases, such as Alzheimer (Kim et al., 2006), and identifying chaperones.

The last set of improved proteins includes super-proteins, such as sfGFP and supercharged-GFP. In contrast to the folding-reporter, the fluorescence of sfGFP is insensitive to misfolding of the fusion partner (Pedelacq et al., 2006). This robustly folded version of GFP has been generated from folding-reporter-GFP by fusing it downstream to poorly folded ferritin and screening libraries for fluorescent colonies. The additional destabilizing burden in the form of insoluble protein has resulted in obtaining sfGFP that possesses an improved tolerance for circular permutation, increased resistance against chemical denaturants and improved folding kinetics. In addition to the folding-reporter mutations, sfGFP bears six extra mutations. They are Ser30Arg, Tyr39Asn, Asn105Thr, Tyr145Phe, Ile171Val and Ala206Val. The greatest impact on the folding features of sfGFP was attributed to Ser30Arg substitution. The arginine residue in this position was shown by crystallography to induce the formation of a five-membered intramolecular ionic network connecting the first, second, fifth and sixth β-strands of the barrel. The ionic network consists of Glu32 (second β-strand), Arg30 (second β-strand), Glu17 (first β-strand), Arg122 (sixth β-strand), and Glu115 (fifth β-strand) residues. Substitution of Tyr39 with Asn was shown to initiate the formation of an α-helix at the loop between the second and third β-strands of the barrel, and it may contribute to the stability of this region. Substitutions of Tyr145 to Phe and Ile171 to Val are supposed to reduce the formation of off-pathway intermediates that are prone to aggregate, while substitutions of Asn105 to Thr and Ala206 to Val presumably increase the solubility of native protein.

It is worth discussing the eCGP123 GFP, although it is not a mutant of GFP, but rather it is derived from Azami-GFP from the stony coral (Kiss et al., 2009). eCGP123 tolerates overnight incubation at 80 °C without considerable loss of green fluorescence. To produce this extremely stable FP, the introduction of "folding interference" domains was applied, as it was performed in the development of sfGFP. Here, heterologous loops were used as destabilizing insertions. The loops were sequentially placed into three β-turns of GFP; the construct was subjected to evolution and selection steps after each insertion. This allows a gradual increase of protein stability, while simultaneous insertion of all three loops would destroy the FP completely.

On the basis of sfGFP with an initial charge of −7, a set of proteins with the charge ranging from −25 to +48 has been created by substituting highly solvent-exposed amino acids with negatively (Glu or Asp) or positively (Lys or Arg) charged residues (Lawrence et al., 2007). These proteins, termed as supercharged GFPs, preserve fluorescence and structure that are identical to those of sfGFP. The key feature of supercharged GFPs is their dramatically enhanced solubility. They remain soluble even in conditions strongly favoring aggregation, i.e. under heating at 100 °C for 1 min or in the presence of 40% of 2,2,2-trifluoroethanol, a chemical that stimulates protein aggregation. Their precursor sfGFP precipitated substantially under the conditions tested.

3.2. Fluorescent Proteins from Other Organisms

GFP-like proteins are found in numerous marine organisms. Together with GFP, 11 GFP-like proteins have been identified in the class Hydrozoa, the phylum Cnidaria until recently. Most of the Hydrozoan proteins are green FPs; the exceptions are a yellow FP from hydromedusa *Phialidium* sp. (phiYFP) and a chromoprotein from an unidentified anthomedusa (Shagin et al., 2004). PhiYFP contains the typical green GFP-like chromophore and, to achieve yellow fluorescence, it utilizes the same structural approach, as it was realized in artificial EYFP derived from GFP, i.e. π–π stacking of the phenolic ring of the chromophore and Tyr203 placed above it. Recent findings imply that the Hydrozoan FP family is more abundant than previously believed. The first example of a multicolored FP in a single Hydrozoan species has been described recently (Aglyamova et al., 2011). GFP homologs of three colors, cyan, green and yellow, have been found in the medusa life cycle stage of the *Obelia* bioluminescent system, while previous attempts to clone FPs from the colonial polyp stage of *Obelia* have failed.

The spectral variability of *Obelia* FPs most likely arises from the microenvironment of the green GFP-like chromophore. All three proteins tend to form stable aggregates composed of up to 128 monomers. In *Obelia*, these proteins localize in subcellular granules containing the photoprotein and FP. This allows for speculation that *Obelia* FPs could be involved in the regeneration of the photoprotein following its oxidation in a bioluminescence reaction (Aglyamova et al., 2011). Recently, two amazing representatives of GFP homologs have been identified in the class Hydrozoa. They are multidomain proteins, namely two-domain green abeGFP from the siphonophore *Abylopsis eschscholtzii* and the four-domain orange-fluorescent Ember from an unidentified jellyfish (Hunt et al., 2012). The analysis of the spectroscopic features of Ember revealed that only one domain contains an orange-emitting chromophore, which is likely a DsRed-like red chromophore; the other three domains bear green-emitting chromophores. Still, the final fluorescence of Ember is orange-red as a result of effective resonance energy transfer from the green subunits to the red one. The exact functions of the multidomain arrangement of these proteins are obscure, though the authors suggest that it contributes to overall protein stability (Hunt et al., 2012).

FPs are also present in bilateral animals of phyla Arthropoda and Chordata. All the nine endogenous FPs discovered in copepods of two families, Pontellidae and Aetedae (phylum Arthropoda, subphylum Crustacea), are green FPs (Hunt et al., 2010; Masuda et al., 2006; Shagin et al., 2004). Copepod GFPs exhibit extremely high brightness and exceedingly fast chromophore formation. The highest quantum yield of 0.92 that is close to the theoretical maximum has been reported for GFPs from copepod *Pontella mimocerami* (Shagin et al., 2004). Genome analysis revealed the occurrence of GFP genes in three amphioxus species of the *Branchiostoma* genus (phylum Chordata, subphylum Chepalochordata (Li et al., 2009)), while mRNA encoding GFPs was isolated only from *Branchiostoma floridae* species (Deheyn et al., 2007). Lancelet *B. floridae* harbors as many as 16 GFP-like proteins (Bomati et al., 2009). Two of the GFP-like proteins from *B. floridae* are likely not to carry a mature chromophore as they contain a 65Gly-Tyr-Ala67 tripeptide instead of a 65Gly-Tyr-Gly67, which is found in the rest of *B. floridae* GFP-like proteins. The only one of the chromophore-forming *B. floridae* GFP-like proteins is a brightly fluorescent green FP; the others are either weakly fluorescent or nonfluorescent. In addition to the diverse spectral features, these GFP-like proteins have distinct expression patterns that suggest that they perform different functions in the animal (Bomati et al., 2009).

The most abundant and color diverse is the family of GFP-like proteins found in sea anemones and corals (phylum Cnidaria, class Anthozoa). Their spectra span the range from cyan to red and purple-blue, and nonfluorescent colors are found as well (Verkhusha and Lukyanov, 2004). This class of GFP homologs suggests two options for red color that are realized through DsRed-like and Kaede-like chromophores.

Virtually all GFP-like proteins are oligomeric (Fig. 4.1). Being monomeric at low protein concentration, GFP tends to form dimers at increased protein concentration; under physiological conditions, it exists as a hetero-tetramer with aequorin, whose excitation energy it accepts and re-emits in the green range of the spectrum (Tsien, 1998). The weak dimerization of GFP and related proteins is easily alleviated by mutation of one of the three hydrophobic residues on the protein surface to charged residues, resulting in charge repulsion (Ala206Lys, Leu221Lys, or Phe223Arg (Zacharias et al., 2002)). Substitution of Ala206 to a bulky Val residue in sfGFP also favors the monomeric form, sterically hindering dimerization (Pedelacq et al., 2006). The other Hydrozoa FPs are obligate dimers, as well as Chordata FPs (Bomati et al., 2009; Shagin et al., 2004). Nearly all Anthozoa and Arthropoda FPs generate tetrameric complexes even at nanomolar concentrations; they are also inclined to form aggregates (Hunt et al., 2010; Yanushevich et al., 2002). The strong tendency of Arthropoda FPs to aggregation is observed as visible protein precipitation during storage. The nature of aggregation is the same for both groups of proteins. It was shown that aggregation is facilitated by electrostatic interaction between positively charged N-terminal patches and negatively charged patches on the FP surface. The introduction of neutral or negatively charged residues instead of positively charged ones increases protein solubility significantly (Yanushevich et al., 2002).

The tetrameric molecule of GFP-like proteins looks like dimer of dimers, where each monomer interacts with two adjacent monomers through two interfaces that differ significantly both in their chemical composition and in the strength of their interactions (Evdokimov et al., 2006; Remington et al., 2005; Wiedenmann et al., 2005; Yarbrough et al., 2001). The weaker one is composed of a central hydrophobic cluster with a few polar amino acids around it. The disruption of this interface is easily achieved by mutating the hydrophobic residue at the dimeric interface to a positively charged arginine or lysine (Campbell et al., 2002). This junction is weakened substantially or completely in naturally occurring dimeric GFP homologs (Loening et al., 2007; Wilmann et al., 2005). The second interface that is present in both dimeric and tetrameric GFP homologs is more

extended; it involves numerous hydrogen bonds and salt bridges between polar residues and buried water molecules. The further stabilization of the region arises from the clasp between the C-termini of neighboring subunits (Yarbrough et al., 2001). Likewise, for the hydrophobic interface, the disruption usually starts with the introduction of charge disturbance, but the additional elimination of existing contacts between the polar groups and the modification of C-terminal patches of protein are required (Campbell et al., 2002). We should note that the interface-forming amino acids of both junctions are highly specific, even for GFP-like proteins from the same origin, which prevents them from heterooligomerization (Stepanenko et al., 2008). The tetrameric organization of Anthozoa and Arthropoda FPs results in a slight deformation of protein subunits, although their total tertiary structure remains conserved (Yarbrough et al., 2001). Thus, the β-barrel of GFP-derived proteins is near perfectly circular in cross-section (Ormo et al., 1996), while the β-barrel of Anthozoa and Arthropoda FPs has an elliptical shape (Yarbrough et al., 2001). This deformation is deemed to be pertinent to chromophore maturation. This is demonstrated by the fact that the disruption of tetramers is often impossible without compromising the fluorescence (Campbell et al., 2002). More specifically, the disruption of the stronger hydrophilic interface always eliminates the fluorescence, while the disruption of the hydrophobic interface usually gives a slightly less or poorly fluorescent mutant form. Therefore, the easy task of engineering the monomeric versions of proteins for which crystallographic data are available is heavily complicated by the need to subject the proteins to further optimization procedures for recovery of the fluorescence. For example, to create a monomeric and fluorescent version of red FP from *Discosoma* coral, as many as 33 amino acid substitutions were introduced (Campbell et al., 2002). That notwithstanding, there are currently monomeric FPs of various colors from violet-blue to far-red (Chudakov et al., 2010).

4. PIONEERING STUDIES OF FLUORESCENT PROTEIN STABILITY

FPs belong to the proteins with β-barrel topology (Tsien, 1998). Crystallographic structures revealed that these proteins resemble an 11-stranded β-can wrapped around a single central helix, in the middle of which is the chromophore (Ormo et al., 1996; Wachter et al., 1998; Yang et al., 1996). The cylinder has a diameter of ~30 Å and a length of ~40 Å (Yang et al., 1996). In the Protein Data Bank, there are more than 1000 structures of

proteins with β-barrel topology. Among the proteins with this structure, 68% are various types of enzymes, and the remainder is binding proteins and transport membrane proteins. Most of them have six or eight β-strands. Membrane proteins have 14–20 β-strands. Among the other proteins with β-barrel topology, only FPs have an 11 β-stranded barrel (Nagano et al., 1999). The extreme resistance to a variety of denaturing effects is a special feature of FPs. For example, they are approximately 1000 times more stable than proteins of the lipocalin superfamily (Stepanenko et al., 2012a). The structure of odorant-binding proteins (pOBP), which represents a β-barrel composed of eight β-strands with a central nonpolar cavity for the binding of hydrophobic odorant molecules, possesses stability that is close to that of the GFP-like proteins (the difference in the free energy of pOBP in the native and unfolded states in the absence of a denaturant is −25 kJ/mol). At the same time, the rate of unfolding of pOBP is 1000 times faster than that of the GFP-like proteins under the same conditions (Staiano et al., 2007). Interestingly, azurin, which has a single tryptophan residue (Trp48) deeply buried in the hydrophobic central cavity of the β-barrel and thus resembles the FPs, is also much less stable to denaturant action (Gabellieri et al., 2008).

Despite an increasing number of studies on the stability and unfolding–refolding process of proteins with β-barrel topology in recent years, these processes have been much less understood than similar processes in α- and α/β-proteins. The β-barrel proteins are typical globular proteins. Before discussing the features of green FP folding, we will briefly discuss the fundamental principles of globular protein folding, which have been developed based on more than half a century of intensive investigation of this problem (see, e.g. Finkelstein and Ptitsyn, 2002; Nolting, 1999).

4.1. Fundamental Principles of Globular Protein Folding

The work of Anfinsen represents the earliest investigation of globular protein folding (Anfinsen, 1973). This work showed that the three-dimensional native structure of each protein is determined by its amino acid sequence. The polypeptide chain apparently adopts the structure corresponding to the minimum of free energy. Somewhat later, it was shown that the primary structure defines not only the three-dimensional structure of the protein in its native state but also a way of achieving it and the existence of a sufficiently high energy barrier between the native and unfolded states. The last point is extremely important because the existence of a high energy barrier between the native and fully or partially unfolded states means the impossibility of the existence of partially folded native states of proteins. Globular

protein can be either native or denatured, partially or fully unfolded. This determines the reliability of performance by the globular proteins of their functions. It is this fact that allows for the possibility of obtaining crystals of proteins and, consequently, the ability to determine their structure by X-ray analysis. In this way, globular proteins drastically differ from synthetic polymer molecules. At the end of the twentieth century, it became apparent that many proteins have an amino acid sequence that, in principle, does not allow them to fold up in a three-dimensional structure; these are the so-called "intrinsically disordered proteins" (IDPs). This term underlines the intrinsic amino acid sequence-determined inability of these proteins to form ordered structures. It is commonly thought that the smaller the content of hydrophobic amino acid residues and the higher the net charge of a polypeptide chain, the smaller the probability that this chain will fold into a compact globule, and, contrary, the greater the propensity of the IDPs to form complexes with other proteins and self-aggregate if, for some reasons, they fail to interact with their partners. Due to these structural peculiarities, IDPs on the one hand play key roles in signaling, recognition, and regulation systems, but on the other hand, their aggregates are strongly related to many of the so-called conformational diseases and amyloidoses in particular. Studies of IDPs comprise a separate and intensively developing research field (see, e.g. Dunker et al., 2008; He et al., 2009; Turoverov et al., 2010; Uversky and Dunker, 2010). We will not stop here on these studies, especially because FPs represent a very different type of proteins, namely, they are globular proteins with many intramolecular contacts.

The main methodological approach for studying the folding and stability of globular proteins is the examination of their in vitro unfolding and refolding induced by external factors, such as changes in the denaturant concentration, pH and temperature of the solution. To this aim, one can record stationary and (or) kinetic dependencies of protein characteristics, the values of which differ in native and denatured states of protein.

If such dependences are determined for the parameters that are linearly related to the concentration of the protein (such as fluorescence intensity at a fixed wavelength of registration), then the difference in the free energies between the native and denaturated states can be evaluated on the basis of the stationary dependence of the fraction of protein in the native and unfolded states on the denaturant concentration, and the free energy barrier between the native and denaturated states can be characterized by the rate constant determined from measurement of the kinetic dependence of the change in this parameter. The use of intensive parameters (fluorescence

spectrum position, the relation of fluorescence intensities at two wavelengths, or fluorescence polarization) is possible only for the qualitative characterization of conformational transitions. Some of these parameters, e.g. parameter that characterizes the fluorescence spectrum position, can also be used for determination of protein fractions in native and denaturated states after specially elaborated correction (Staiano et al., 2007).

According to the current view, protein folding is determined by the protein's energy landscape (Jahn and Radford, 2005; Radford, 2000). This landscape describes the dependence of the free energy on all the coordinates determining the protein conformation. The number of conformational states accessible by a polypeptide chain is reduced while approaching the native state. Therefore, this energetic surface is often called the "energy funnel." Under the influence of external denaturating factors, the energy landscape varies. Under native conditions, the native state of the protein, corresponding to a deep minimum of the free energy, is energetically favorable and, conversely, the unfolded state of the protein is energetically unfavorable, but it corresponds to the minimum of free energy at a high concentration of denaturant (Fig. 4.4). For a long time, it was thought that protein folding is similar to the crystallization process and that a protein can only exist in two states: native and unfolded, with nucleus formation being the limiting step in the folding process. This model, known as the "nucleation and growth" model, well describes the folding of small single-domain proteins that follow the "all-or-none" principle. At intermediate concentrations of denaturant, the free energy of the molecules in the native and unfolded state can be comparable. This means that under these conditions, the concentration of molecules in the native and unfolded states is slightly different. The change in the energy landscape with the increase in the denaturant concentration is shown in Fig. 4.4*a*.

To describe the folding of large proteins, the "sequential protein folding" model, also known as the "framework" or "hierarchic" model, was proposed (Ptitsyn, 1973). It suggests that folding starts with the backbone forming secondary structure elements, which then interact to form a more advanced folding intermediate; the specific packing of the side chains concludes the process. Each stage of the folding process stabilizes the major structural elements formed in the previous state, suggesting the existence of several folding intermediates. In this case, the stationary curves can determine the population of the native, intermediate and unfolded states at each denaturant concentration, while several rate constants determined from the kinetic curves will characterize the existing energy barriers between the existing states.

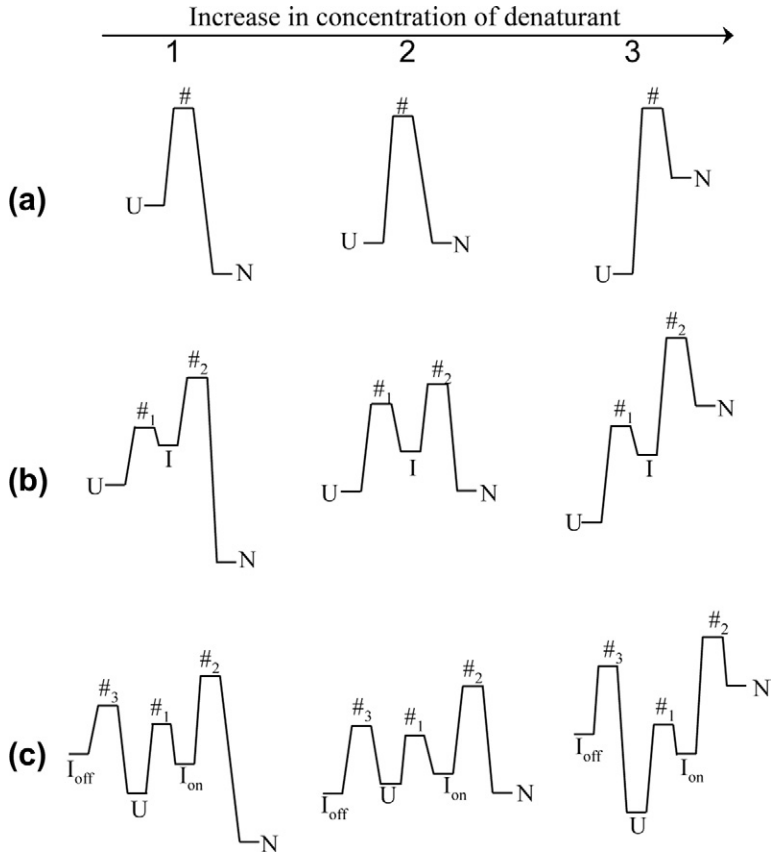

Figure 4.4 ***Change in the energy landscape induced by a denaturant.*** 1 and 3 are the concentrations of denaturant at which the protein is in its native and unfolded states. U, N, I and # are the unfolded, native, intermediate and transition states of protein. I_{on} and I_{off} are the on- and off-pathway intermediate states. *a*. Two-state unfolding–refolding model of protein. *b*. Three-state unfolding–refolding model of protein. *c*. Protein unfolding–refolding via on- and off-pathway intermediates.

The intermediate states, which have the structural elements of the native state, are called on-pathway states. For a long time, it was believed that there is one universal intermediate state referred to as the "molten globule state" (Ohgushi and Wada, 1983). Other partially folded intermediates (e.g. premolten globule and highly ordered molten globule) were later found (Uversky and Ptitsyn, 1996). Figure 4.4*b* shows the change in the energy landscape with the increase in the denaturant concentration for proteins with one on-pathway intermediate state.

Along with the on-pathway states, there can be off-pathway states (traps). These states have structural elements that are not met in the native state. Figure 4.4*c* shows that at reducing the denaturant concentration, both the on-pathway (I_{on}) and off-pathway (I_{off}) states can appear. Molecules that were caught in the "traps" can be folded only after a further decrease of denaturant concentration via unfolding the off-pathway elements.

In living cells, a newly synthesized protein finds itself in the "overcrowded" physiological cell medium, where the concentration of proteins, nucleic acids, and polysaccharides is as high as 400 mg/ml, and where macromolecules occupy up to 40% of the medium volume (Ellis, 2001; Zimmerman and Minton, 1993). Such conditions can greatly affect all biological processes, including protein folding, misfolding, and aggregation (Chebotareva et al., 2004; Minton, 2000; Uversky et al., 2002; van den Berg et al., 2000). The folding of proteins in the living cell is complicated by at least two factors: the existence of unfavorable contacts with "neighbors," and the appearance of the incorrect intramolecular contacts during a co-translational folding (Turoverov et al., 2010). Therefore, in order for the correct folding to occur, a set of special protein-helpers provides assistance. These are the chaperones and the enzymes that regulate the *cis–trans* isomerization of proline and the formation of the disulfide bridges. They prevent protein aggregation and misfolding, accelerate folding, and participate in protein transport (e.g. protein translocation through the membranes) (Bader and Bardwell, 2001; Fink, 1999; Gilbert, 1994; Schmid, 2001).

4.2. Comparative Studies of Green and Red Fluorescent Proteins

Studies of the stability and folding of FPs began almost simultaneously with the intensive studies aimed to create new mutants with improved properties. In the early studies of FPs, their reactions to different treatments were examined. In particular, the effects on wild-type *Aequorea* GFP of treatment with detergents (Bokman and Ward, 1981; Ward and Bokman, 1982), heating (Ward et al., 1982) and proteases (Chalfie et al., 1994) were studied.

The important breakthrough in the construction of new variants of GFP was the construction of EGFP, which has two amino acid replacements in comparison with wild type (Cormack et al., 1996). In particular, one of the most important substitutions was S65T, which shifted the equilibrium constant between the neutral and anionic forms of the chromophore toward the anionic form and, thus, significantly increased the fluorescence quantum

yield. Surprisingly, despite the existence of numerous EGFP applications, its spatial structure was determined only recently (Royant and Noirclerc-Savoye, 2011).

The discovery of a distant homolog of GFP cloned from *Discosoma* coral (Matz et al., 1999), called DsRed for its significantly red-shifted excitation and emission maxima (558 and 583 nm, respectively), has attracted great interest and stimulated the appearance of several papers on its structure, its stability to different denaturants and its processes of folding and unfolding (Verkhusha et al., 2003; Vrzheshch et al., 2000). The structure and stability of DsRed were studied in comparison with EGFP. Both proteins have similar β-barrel folds but possess different oligomeric organization and chromophore structures. These works represent a thorough examination of DsRed treated with different denaturing actions, including heating, GdnHCl, and changes in pH. A large variety of spectroscopic and fluorescence methods was used. The main conclusion was that DsRed is much more stable than EGFP and that the processes of unfolding are highly complex. The authors proposed a kinetic mechanism for DsRed denaturation that includes consecutive conversion of the initial state of the protein to the denatured state through three intermediates. The first intermediate still emits fluorescence, and the last one is subjected to irreversible inactivation. Because of tight DsRed tetramerization, it was suggested that the obligatory protonation of each monomer results in the fluorescence inactivation of the whole tetramer. The remarkable fluorescence stability of DsRed under all conditions that have been studied was attributed to a significant extent to its tetrameric organization.

This conclusion stimulated a systematic analysis of five FPs with different degrees of oligomerization (Stepanenko et al., 2004). For these proteins, the fluorescent and absorbance parameters, the near-UV and visible CD spectra, the accessibility of the chromophores and the tryptophans to acrylamide quenching, and the resistance of these proteins to the guanidine hydrochloride unfolding and the kinetics of the approaching the unfolding equilibrium were compared. In this paper, tetrameric zFP506 was shown to be dramatically more stable than the EGFP monomer, assuming that the association might contribute to the protein conformational stability. RFPs were shown to possess comparable conformational stabilities regardless of oligomerization: monomeric mRFP1 is the most stable species under the equilibrium conditions, and tetrameric DsRed1 possesses the slowest unfolding kinetics. EGFP was shown to be considerably less stable than mRFP1, whereas tetrameric zFP506 was the most stable species

analyzed in this study. It was concluded that the quaternary structure, which is an important stabilizing factor, does not represent the only circumstance dictating the dramatic variations between the FPs in their conformational stabilities.

However, all these studies were complicated by the tendency of FPs to aggregate and by the extremely slow process of their unfolding. The study of FP unfolding–refolding became much more effective with the use of new FP variants with greatly reduced tendencies for aggregation.

In the course of searching for brighter GFP variants using a DNA shuffling approach, a mutant that was 42 times more fluorescent than the wild type was identified (Crameri et al., 1996). This variant has three mutations (Phe99Ser, Met53Thr, Val163Ala) and was named cycle3-GFP, also referred to as GFPuv. It was suggested that more efficient folding and the higher yields of cycle3-GFP were the results of significantly reduced aggregation. The DNA shuffling method was used to improve cycle3-GFP folding. For this purpose, the protein was fused to a very poorly folding protein (H-subunit of ferritin) (Pedelacq et al., 2006). After four rounds of selection, sfGFP was identified. These two variants, cycle3-GFP and sfGFP, became the favorite subjects of FP stability and folding investigations.

5. UNFOLDING–REFOLDING OF FLUORESCENT PROTEINS

Virtually all recent work on the processes of FP unfolding–refolding has been performed with the cycle 3 (Enoki et al., 2006, 2004; Fukuda et al., 2000; Huang et al., 2007, 2008; Melnik et al., 2011a) or sfGFP (Andrews et al., 2009, 2007; Stepanenko et al., 2012b) proteins being subjected to different denaturing effects, including the chemical denaturants GdnHCl (Andrews et al., 2007; Fukuda et al., 2000; Huang et al., 2007) and GTC (Stepanenko et al., 2012b), changes in the pH of the solution (Enoki et al., 2006, 2004), and changes in the ionic strength (Hsu et al., 2010).

Since FPs have a unique chromophore and a single tryptophan residue, Trp57, the fluorescence characteristics of which are sensitive to the structure of the protein, and nonradiative energy transfer from Trp to the chromophore in the neutral state exists in the native state of the protein, fluorescent methods have been used in practically all papers to monitor changes in the structure of the protein under different treatment conditions (Andrews et al., 2007; Enoki et al., 2006, 2004; Fukuda et al., 2000; Huang et al., 2008; Melnik et al., 2011a; Orte et al., 2008; Stepanenko et al.,

2012b). In addition, to characterize the processes of unfolding-refolding, CD (Enoki et al., 2004; Huang et al., 2007), SAX (Enoki et al., 2006), single-molecule fluorescence (Orte et al., 2008), and single-molecule mechanical unfolding (Bornschlogl and Rief, 2011; Dietz et al., 2006; Dietz and Rief, 2004; Mickler et al., 2007) as well as theoretical approaches (Andrews et al., 2008; Reddy et al., 2012) were used.

One would think that such intense study of the same subject by different groups using different experimental approaches would provide a comprehensive view of the processes of the folding–unfolding of proteins. However, the investigators agree only on the fact that these proteins have a very high resistance to denaturing effects. Most believe that the protein has a complex energy landscape with a number of different intermediate states, high energy barriers, and multiple pathways (Andrews et al., 2008, 2007; Chirico et al., 2006; Enoki et al., 2006, 2004; Fukuda et al., 2000; Huang et al., 2007; Jackson et al., 2006; Mickler et al., 2007). In nearly every work, intermediate states in the pathway of FP unfolding–refolding were detected, though the number of intermediates differs from one to four, depending on the use of a denaturant action and registered parameters.

5.1. Intermediate States on Pathway of Fluorescent Protein Unfolding

Practically all developments related to the folding and stability of FPs are discussed in the thorough review by Hsu et al. (2009) with more or less details. The authors presented an unbiased survey of the papers on the theme and discussed a variety of the conclusions on the pathways of FP unfolding–refolding, but they did not demonstrate a preference for any one point of view. It would be of interesting to understand why the results obtained using different methods do not coincide and what is the real pathway of FP unfolding–refolding.

The most traditional approach of investigation of protein folding is the study of its unfolding–refolding processes induced by different concentrations of chemical denaturants. As FPs are highly stable against chemical denaturation, the use of urea is absolutely unacceptable, and in the majority of papers GdnHCl (Andrews et al., 2007; Fukuda et al., 2000; Hsu et al., 2010; Huang et al., 2007; Orte et al., 2008) was used as a denaturating agent. Recently, one study used an even stronger denaturant, namely GTC (Stepanenko et al., 2012b).

In the studies of protein unfolding–refolding processes, it is used to recording the denaturant-induced changes of the parameters which are

sensitive to the changes of protein structure, such as the intensity of the intrinsic fluorescence, the CD signal and others, and the conclusions about the existence of intermediate states are drawn on the basis of the form of the curve. For GFP, it was shown that the dependence of the steady-state fluorescence intensity of the green chromophore correlates with the CD signal change, and can therefore be used to study the processes of FP unfolding–refolding (Fukuda et al., 2000). Unfortunately, in most of the papers, no experimental curves are available, and only calculated dependences of the fraction of molecules in different structural states on the denaturant concentration are given. In all the papers using GdnHCl as denaturant, it was noted that the dependences of the data on the concentration of the denaturant are quasi-stationary and change over time (Andrews et al., 2007; Fukuda et al., 2000; Huang et al., 2007). More clearly, this was shown in the work carried out by the group of Jenning (Andrews et al., 2007). All these dependences (Andrews et al., 2007; Fukuda et al., 2000; Huang et al., 2007) have sigmoidal character and are well described by a two-state transition (Andrews et al., 2007; Fukuda et al., 2000; Huang et al., 2007). However, it was shown that a better approximation is obtained using a three-state approximation (see Fig. 3 in Andrews et al., 2007 and Fig. 7e in Huang et al., 2007). Although the differences between these two approximations are subtle, the three-state approximation was favored, and the conclusion on the existence of a native-like intermediate state was performed.

At the same time, the absorption spectrum of the FP was not recorded in any of the above papers, although it is known that FP absorption spectra are complex. For YFP, a significant sensitivity of the absorption spectra to anions, such as halides, nitrates and thiosulfate, was shown (Wachter and Remington, 1999). The existence of two forms of a yellow chromophore was shown with the change of pH (Hsu et al., 2010; Seward and Bagshaw, 2009). Nonetheless, even in the study of YFP unfolding–refolding, experimental data on chromophore fluorescence intensity were not corrected for the change of absorption at the wavelength of excitation, though such correction must be performed not only for YFP but also in the study of any FP. It was shown that the change in the chromophore absorption spectrum with the change of denaturant has a general character (Stepanenko et al., 2012b). This is caused not only by the change of pH but also by denaturing agents, such as GdnHCl and GTC. Hence, if we are to judge the conformational changes of the protein, we have to eliminate the fluorescence changes induced by changes in the absorption of the chromophore.

The visible absorption spectrum of sfGFP changes dramatically over all ranges of GTC concentrations (Fig. 4.5*a*). However, the most pronounced alterations in the visible absorption spectrum of sfGFP are recorded at concentrations of GTC up to approximately 0.7 M (Fig. 4.5*a*). Here, the visible absorption spectra of sfGFP demonstrated a pronounced drop in the absorption band at 485 nm, which corresponds to the anionic form of the chromophore, with a concomitant rise in the absorption band at 390 nm, which corresponds to the neutral chromophore. Further changes in the intensity of both the absorption maxima can be described as sigmoid curves. Some blue shift of the absorption band at 485 nm is observed at a GTC concentration exceeding 1.3 M. The optical density at approximately 425 nm remains unaltered in all range of GTC concentrations (Fig. 4.5*a*). The presence of such an isosbestic point in the visible absorption spectra of sfGFP indicates that only two types of molecules exist, namely sfGFP molecules with neutral and anionic chromophore forms, and the observed alterations of the visible absorption spectra are caused by changes in the ratio between them. The chromophore fluorescence intensity was corrected on the spectral effect related to the change of absorption at the excited wavelength (Fig. 4.5*b*).

A joint analysis of the changes in the fluorescence intensity of chromophore and tryptophan residue, the parameter A, and the change of the elution volume in response to changes in the denaturant concentration suggested that the structure of the protein varies slightly in the range of 0–0.1 M GTC, it then remains constant up to 0.9 M, and the unfolding of the protein occurs in the range of 0.9–1.7 M GTC. Therefore, the complex nature of the change of fluorescence intensity up to 0.9 M GTC is of purely spectroscopic character, while in the range from 0.9 to 1.7 M GTC, the dependence of the fluorescence intensity is determined by two factors: the continuing change in the optical density and the process of protein unfolding. The characteristics, such as the dependence of the corrected fluorescence intensity of the chromophore, fluorescence anisotropy and the parameter A, on the denaturant concentration provide no evidence about the intermediate states in the denaturating pathway.

Nevertheless, in the literature, there are a number of papers reporting the existence of intermediate states in the way of unfolding. Many studies indirectly indicate the possibility of the existence of intermediate states in the process of protein denaturation. Recent studies show that the stability of FPs is not uniform through the scaffold cylinder (Melnik et al., 2011b, 2011c; Orte et al., 2008). In particular, this was shown in the work by Orte et al. (2008). In this work, cycle3-GFP was probed by

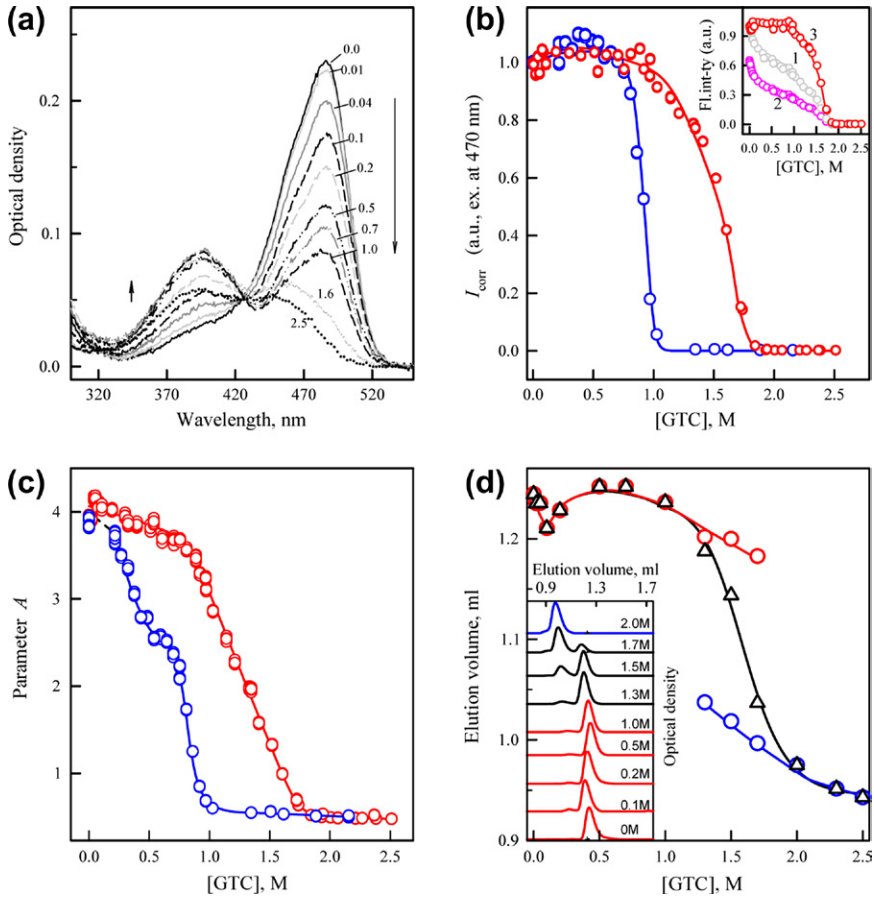

Figure 4.5 ***sfGFP unfolding–refolding induced by GTC (Stepanenko et al., 2012b).*** *a*, change in the absorption spectrum; *b*, chromophore fluorescence intensity corrected to the change of the chromophore absorption spectra and *c*, parameter $A = I_{320}/I_{356}$ of tryptophan fluorescence on GTC concentration. Inset to panel *b*: experimentally recorded chromophore fluorescence intensity (curve 1, gray), corrected to a total density of solution as follows I/W, where $W = (1 - 10^{-D_\Sigma})/D_\Sigma$ (see Kuznetsova et al., 2012; Sulatskaya et al., 2011; Sulatskaya et al., 2012) (curve 2, pink), and corrected to the change of the chromophore absorption spectra (panel a) with the GTC concentration (curve 3, red). *d*, changes of the position of elution peaks of compact and denatured molecules (red and blue circles, respectively) and the change of the averaged elution volume of sfGFP (black triangles). Inset: Changes of the elution profile of sfGFP at increasing denaturant concentrations. The values of the curves specify applied denaturant concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book).

measuring the hydrogen/deuterium (H/D) NMR exchange rates of more than 157 assigned amid protons that contain nearly two-thirds of the GFP amid protons. It was found that amide protons in β-strands 7, 8, 9 and 10 have, on average, higher exchange rates than the others in the β-barrel. Approximately 40 amide protons were found that do not undergo significant exchange, even after several months. It was concluded that most of these residues play an important role in stabilizing the structure of the protein and that they are clustered into a core region encompassing most of the β-strands, at least at one end of the barrel. It was shown that the majority of them are located in β-strands 1–6.

The existence of two regions with different structural stability was confirmed by differential scanning microcalorimetry (Melnik et al., 2011b, 2011c). The microcalorimetric analysis of the nonequilibrium melting of cycle3-GFP and its two mutants, Ile14Ala and Ile161Ala, revealed that the temperature-induced denaturation of this protein most likely occurs in three stages. The first and second stages involve melting of a smaller hydrophobic cluster formed around the residue Ile161, whereas a larger hydrophobic cluster (formed around the residues I14) is melted only at the last cycle3-GFP denaturation step or remains rather structured, even in the denatured state (Melnik et al., 2011b).

A complex energy landscape with at least two intermediate states was suggested in theoretical work (Reddy et al., 2012), though it is not clear what conditions (what denaturant concentration) were used for the calculation and how the results will depend on the denaturant concentration. The shape of the energy landscape depends on the concentration of the denaturant (Section 4.2). Consequently, the rate of unfolding will depend on the denaturant concentration. The kinetic experiments performed using manual mixing and stopped flow exhibit two relaxation phases at 7.0 M GdnHCl and higher and three phases at 6.5 M GdnHCl and lower (Andrews et al., 2007).

Several papers have described five intermediate states of FP unfolding (Enoki et al., 2006). Several approaches were used in these examinations of cycle3-GFP unfolding–refolding, but the main conclusion that there are several intermediate states was made on the basis of intrinsic tryptophan fluorescence (Enoki et al., 2006). In these papers, protein denaturation was induced by changing the pH of the solutions. The recorded complex dependence of fluorescence intensity on the pH was interpreted as the

existence of several intermediate states. At the same time, it was not taken into account that energy transfer from the tryptophan residue to the chromophore depends not only on the distance between them but also on the chromophore ionization state, which changes with pH. Furthermore, the change in the ionization of groups in the microenvironment of the tryptophan residues that are known (White, 1959) to induce fluorescence quenching was not taken into account. In cycle3-GFP, there is carboxyl group from Asp 216 in the vicinity of the indole ring of the tryptophan residue. Its ionization can be changed by reducing the solution pH, even before any structural change occurs. In total, GFP contains 18 Asp and 16 Glu residues whose ionization and remoteness from the indole ring could be changed by changing the solution pH. Additionally, the fluorescence of a tryptophan residue exposed to solution is quenched by water molecules. None of these factors were discussed, and apparently, they were not taken into account in the papers by Enoki et al. (2006, 2004). By the way, the dependence of indole fluorescence on pH below 2.9 is quite well known (White, 1959).

Finally, single-molecule methods based on nonradiative energy transfer (Orte et al., 2008) and the mechanical unfolding (Bornschlogl and Rief, 2011; Dietz et al., 2006; Dietz and Rief, 2004; Mickler et al., 2007) were used to investigate FPs. The advantage of these methods is that they do not need ensemble averaging, and that is why they enable the identification of the existence of parallel unfolding pathways and intermediate states that may not be highly populated (Haustein and Schwille, 2004; Tinnefeld and Sauer, 2005). The unfolding of individual molecules by chemical denaturants is monitored by the changes in the Forster resonance energy transfer (FRET). Usually, two fluorescent dyes (donor and acceptor) must be attached to the target protein, but for FP, it was necessary to bind only one chromophore (Alexa647) because the yellow chromophore of Citrine works as a donor. It is noteworthy that, except for the use of traditional FRET, the authors used a more sophisticated methodology, two-color coincidence detection (TCCD), which makes use of simultaneous excitation of the donor and acceptor by two overlapped lasers (Li et al., 2003; Orte et al., 2006). This method has the advantage of being sensitive to FRET changes, and it allows the additional detection of fully unfolded proteins, in which the fluorescence of the intrinsic fluorophore is quenched but where there is still a signal from the attached reference dye. Unexpectedly, for Citrine-Alexa-647

in native conditions, the presence of two structured states was detected, one of which has a high-FRET efficiency and the other has a lower FRET signal. The authors suggested that the low-FRET species is a partially structured state. At the same time, it is not clear why this state was referred to as a "partially structured intermediate state." By the conventional meaning, the intermediate state is a state that appears on the pathway of protein denaturation, and the population of molecules in this state strongly depends on the concentration of the denaturing agent: this state does not exist in native or completely unfolded conditions. However, a "low-FRET" state was found in native conditions, and its population was practically independent of the GdnHCl concentration. Therefore, the scheme of Citrine unfolding given in the work of Orte et al. (2008) was scarcely supported by the experimental evidence. Furthermore, in this work, the authors also did not take into account the change in the absorption spectrum of the chromophore that is of great importance in the analysis of FRET.

A new powerful technique has become popular for the investigation of the structural stability of molecules, namely single-molecule force experiments, however, it is not evident whether the comparison of the results obtained by this method and those gained from the experiments with chemical denaturants or heating is reasonable. In reality, chemical denaturants and heating influence the protein as a whole; the action is applied simultaneously to all parts of protein, while in the mechanical experiments, the force is applied to local points of the protein.

Nonetheless, a series of single-molecule force experiments was successfully performed in the Rief laboratory on GFP (Dietz et al., 2006; Dietz and Rief, 2004; Mickler et al., 2007), and these studies even elaborate protocol-like instructions for investigators who are beginning to use atomic force microscopy to study mechanical protein unfolding or refolding (Bornschlogl and Rief, 2011). In the first work of this series, it was shown that the mechanical unfolding of GFP proceeds via two metastable intermediate states, which were connected with the detachment of a seven-residue N-terminal α-helix from the β-barrel. It was shown that detachment of this small α-helix completely destabilizes GFP thermodynamically even though the β-barrel is still intact and can bear a load. The second intermediate state was found to be a molecule lacking a full β-strand from the N- or C-terminus (Dietz and Rief, 2004). In the following work, it was shown that the GFP structural response depends upon the direction of the strain

application. It was shown that the energy landscape and the three-dimensional deformation response of functional protein structures can be systematically explored by changing the direction of force application (Mickler et al., 2007).

5.2. Hysteresis in Unfolding and Refolding of Fluorescent Proteins

The unique structure of FPs is their chromophore on the kinked α-helix running through the center of the β-can. The chromophore forms from an autocatalytic reaction of the backbone, involving cyclization, oxidation, and dehydration reactions (Cody et al., 1993; Ormo et al., 1996). Chromophore formation follows the construction of the β-barrel and helix kinking (Barondeau et al., 2003). Despite being surrounded by an 11-stranded β-barrel, the chromophore may isomerize in a hula-twist motion (Andrews et al., 2009). Furthermore, it turns out that the chromophore can be reached by the molecules of the solvent. The latter is proved by the change of its absorption spectrum by low concentrations of chelates just after their addition to the solution (Stepanenko et al., 2012b). This, in turn, causes the change of chromophore fluorescence and can be considered as the appearance of an intermediate state, though it is not a structural, but a spectroscopic effect, and it must be taken into account in all structural examinations of FPs, though it has not been done anywhere.

The formation and maturation of the chromophore in FP is a long-continued process because it requires the overcoming of a high energetic barrier. However, after its formation, the chromophore in its turn plays a crucial role in protein stability. This was most convincingly shown for sfGFP (Andrews et al., 2008, 2009, 2007). It was found that sfGFP unfolding is fully reversible as 100% of the chromophore signal of the native protein was recovered under strong refolding conditions. At the same time, the curve of the fraction of unfolded protein as a function of the final denaturant concentration for the unfolding transition shifts to the lower concentration of denaturant during the time of equilibration. The unfolding transition was found to be practically unchanged only between 96 and 192 h, indicating that a quasi-equilibrium has been achieved. During this period of time, only the unfolding transition curve moved to the smaller concentration of denaturant, while the position of the refolding transition curve did not change. Even after 192 h when the quasi-equilibrium was achieved, the unfolding

and refolding transition curves did not coincide, indicating the existing of hysteresis.

It was suggested that extremely slow processes of unfolding and refolding of FP are related to the isomerization of proline residues (Andrews et al., 2009, 2007; Enoki et al., 2004), which most certainly play an important role in refolding, although the main role in the unfolding process belongs to the chromophore. This was first shown clearly and convincingly in the work by Andrews et al. (2007). In this work, the unfolding–refolding processes of FPs without a chromophore were investigated. For this purpose, the sfGFP/Arg96Ala mutant was chosen because this FP variant was discovered to slow chromophore formation from minutes to months (Wood et al., 2005). The equilibrium unfolding and refolding of sfGFP/Arg96Ala was monitored by the change in tryptophan fluorescence as a function of increased denaturant concentration. The equilibrium unfolding and refolding transitions of this protein were found to be superimposed and showed no evidence of hysteresis. The midpoint of the transition corresponds to 1.3 M GdnHCl, which is significantly lower than the midpoint of the sfGFP quasi-equilibrium unfolding transition (approximately 4.2 M GdnHCl) and even the sfGFP equilibrium refolding transition (approximately 1.8 M GdnHCl) (Andrews et al., 2007). This result was confirmed by the examination of another FP mutant without a chromophore. In the laboratory of prof. Jennings, it was revealed that mutation of the N-terminal residues in two X-Pro peptide sequences Tyr74Pro75 and Met88Pro89 to Tyr74Met/Met88Tyr also hindered chromophore formation. These two mutations map to the helical cap of the barrel, which is tightly pinned to the barrel in the sfGFP. It was shown that the equilibrium refolding and unfolding transitions for this variant also eliminated the hysteresis. These data support the hypothesis that the hysteresis observed in unfolding–refolding is related to chromophore. Interestingly, the cooperativity of the folding–unfolding transitions for the double mutant has decreased from that observed for Arg96Ala with the midpoint at approximately 0.8 M GdnHCl (Andrews et al., 2007). Unfortunately, it was not proven that the β-barrel scaffold of the protein was not disturbed by these mutations.

In many papers, the slowness of the FP's processes of unfolding and refolding is associated with its proline residue isomerization (Andrews et al., 2008, 2009, 2007; Enoki et al., 2006, 2004; Hsu et al., 2009; Jackson et al., 2006). It is well known that proline *cis*−*trans* isomerization plays a key role in the rate-determining steps of protein folding (see, e.g. Levitt, 1981).

Proline residues are unique among natural amino acids because the cyclic side chain of proline prevents the rotation of an N–Cα bond, and the peptide backbone has no amide hydrogen for hydrogen bonding. This amino acid residue has a relatively high intrinsic probability of existing as the *cis* rather than the *trans* peptide isomer (Brandts et al., 1975; Grathwohl and Wuthrich, 1976), whereas for other amino acids, the probability is much smaller (less than 10^{-3}, see Ramachandran and Mitra, 1976).

There are 10 proline residues in FPs. The first of them, Pro13, is located in the beginning of the first β-strand, four proline residues (Pro187, Pro192, Pro196, Pro211) are located between β-strands, and another four are in the central α-helix (Pro54, Pro56, Pro58, Pro75). Due to these proline residues, this α-helix is rather kinked and in reality, represents several pieces of α-helix (Fig. 4.6). All these Pro residues are in the *trans* form, while one, Pro89, is in the *cis* form. This residue is located between the fourth β-strand and the central α-helix, dramatically changing the direction of the polypeptide chain. Evidently, it plays a crucial role in the packing of the α-helix inside the β-barrel. Importantly, this is provided by the unfavorable *cis* isomer. Surely, we cannot exclude the role of other proline residues in the cap of the barrel (Pro187, Pro192, Pro196) or proline residues that surely play important roles in kinking the α-helix backbone (Pro54, Pro56, Pro58, Pro75).

The retarding action of proline isomerization on the rate of FP protein refolding was shown in the unfolding–refolding experiment of cycle3-GFP in the presence of cyclophilin A (CycPA), a protein with peptidyl-proline isomerase activity (Andrews et al., 2007; Enoki et al., 2004). Later, this was proved in experiments on cycle3-GFP unfolding–refolding in the presence of *Escherichia coli* trigger factor (TF) (Xie and Zhou, 2008). TF is an efficient molecular chaperone in the catalysis of protein folding reactions that are rate-limited by the isomerization of prolyl bonds (Scholz et al., 1997). It forms a protective shield for nascent polypeptides at the ribosome exit tunnel and assists in the folding of most newly synthesized polypeptide chains (Hoffmann et al., 2006). The spontaneous and TF-assisted folding of GdnHCl denatured cycle3-GFP was probed by tryptophan fluorescence and chromophore fluorescence (Xie and Zhou, 2008). It was shown that in the presence of low concentrations of TF, the fast recovery of Trp57 fluorescence and the fast recovery and slow adjustment phases of the chromophore fluorescence are accelerated, suggesting that those processes are all coupled to proline isomerization. However, with increasing TF concentration, the rate constants for the rapid burial of Trp57 and for the acquisition

and adjustment of chromophore fluorescence were decreased. These results were explained by competition between catalysis and binding by TF. Previously it was shown that TF-assisted protein folding requires repeated binding and release cycles between TF and the folding intermediates (Huang et al., 2000). The higher the concentration of TF, the greater the chance of recapture of substrate intermediates by TF. It was suggested that this binding effect can lead to the arrest of folding (Huang et al., 2002, 2000), so that GFPuv folding was no longer limited by proline isomerization. Thus, it can be concluded that though the limiting-rate role of all the proline residues together in FPs is demonstrated, the role played by each of them is the task of future investigations.

5.3. Circular Permutation and Reassembly of Split-GFP

Many proteins being cleaved into two parts can noncovalently reassemble into a stable and a functional state (Carey et al., 2007). In some cases, fragments can reassemble spontaneously; in others, it occurred after association with other proteins to which fragments of target protein were attached (Kerppola, 2006; Kim et al., 2007; Michnick et al., 2007). The results obtained in the Boxer laboratory testify to the unique ability of fragments of GFP molecules to reassemble spontaneously, which seems to be due to a highly developed system of intramolecular interactions in this protein, which also lead to its high stability (Kent et al., 2008). These authors found an association between recombinant sfGFP (with replacement Thr65Ser), consisting of the first 10 β-strands (GFP 1–10), and a synthetic peptide with the same amino acid sequence as the last β-strand of GFP (GFP 11).

GFP 1–10 isolated from inclusion bodies in denaturing solution does not initially exhibit the absorption or fluorescence characteristics of the GFP chromophore. After the addition of fully synthetic GFP 11, chromophore maturation was observed. As a result, a protein with properties indistinguishable from the intact protein was reconstituted. The lack of structure in GFP 1–10 and the observed formation of the chromophore only after the addition of GFP 11 suggest that GFP 11 induces the precyclization structural constraints necessary for chromophore formation. Thus, the reconstitution of native absorption, fluorescence, and excited-state dynamics, including the deuterium isotope effect, suggested that strand 11 was in the correct orientation, allowing for the reassembly of the fully functional protein. Both the protonated (A) and deprotonated (B) forms of the chromophore were present in whole GFP because Ser at position 65 was introduced. These results

demonstrate that the GFP barrel can be reassembled from pieces where one piece is readily prepared on a peptide synthesizer, thus any natural or unnatural amino acid can be introduced. This system is ideally suited for studying the reassembly of β-barrel structures with a built-in fluorescence reporter, and, by using circular permutation, it may prove possible to apply the same strategy to any strand of the β-barrel.

In subsequent papers (Do and Boxer, 2011; Kent and Boxer, 2011; Kent et al., 2009), a technique to obtain a fragment consisting of the first 10 β-strands was improved. Fragment GFP 1–10 was obtained by recombination: a trypsin cleavage site was specifically engineered into the normally trypsin-resistant GFP loop between the β-strands 10 and 11. After digestion of the loop, the GFP remains intact and spectrally indistinguishable from the uncut protein. The GFP 11 strand was removed by denaturation and then replaced by a fully synthetic strand with any desired sequence containing natural or unnatural amino acids. It occurs that only if GFP 1-10 was refolded from the denaturing solution into a solution containing an excess of GFP 11, the newly associated (GFP 1–10–GFP 11) protein has properties indistinguishable from native GFP.

It was found that if GFP 1–10 was refolded without GFP 11, the properties of GFP 1–10 were quite different from those of the intact and reassembled GFP (Fig. 4.7). Additionally, the fluorescence quantum yield of this refolded form was surprisingly only approximately five times less than the native, fully folded protein (Fig. 4.7). This was unexpected as the fluorescence from denatured GFP 1–10 (or denatured native GFP) is particularly low because the chromophore is in an unstructured environment, and nonradiative decay pathways lead rapidly to the ground state. The high fluorescence quantum yield of the chromophore and the decreased yet still existent secondary structure of GFP 1–10 observed by circular dichroism suggest that GFP 1–10 has some residual structure. Furthermore, it was shown that refolded GFP 1–10 does not bind to GFP 11.

However, it was observed that refolded GFP 1–10 does rebind GFP 11 in the presence of light (initially observed in room light). It was proposed that refolded GFP 1–10 has the chromophore in the *trans* configuration (denoted *trans*-GFP 1–10), but light activation creates a photostationary state with the *cis* configuration of the chromophore (*cis*-GFP 1–10), and only *cis*-GFP 1–10 can bind GFP 11. It was noted that *cis* and *trans* are applied to the chromophores in simple solvent, while these may be twisted somewhat from their ideal geometry by constraints in the protein.

The strategy of creating any structures that do not have one of the β-strands or α-helix begins with the synthesis of full-length GFP with an introduction to the relevant segments of the polypeptide chain loops with sites that can be selectively digested with proteases and circular permutations of the C- and N-termini. Then, the specially introduced fragments of polypeptides are selectively cleaved by proteases, and the split-off fragment is removed under denaturing conditions using chromatography. The deletion is replaced with its synthetic analog with the desired amino acid substitutions. Finally, after transfer to native conditions, one will obtain the protein with the desired properties. Using this approach, the proteins with synthetic β-strand 10 (Do and Boxer, 2011), β-strand 11 (Kent and Boxer, 2011), and the central α-helix with the chromophore (Kent et al., 2009) were created. This method offers a new approach to the construction of fluorescent markers and is a perfect illustration of a crucial role of intermolecular interactions in the formation of FPs.

5.4. Co-translational Folding of Fluorescent Proteins

The material presented above indicates that significant progress in understanding FP unfolding–refolding processes in vitro has been achieved. However, until recently, nothing was known about the processes of these proteins folding in the cell. Meanwhile, this question is of particular interest in the case of FPs because the folding of newly synthesized chains must be substantially different from the refolding of the protein in vitro, as in the latter case, it occurs in the presence of an already formed chromophore. In addition, in the native FPs, as in any other protein and, especially, in proteins with β-barrel topology, the role of the contacts of residues that are significantly remote along the polypeptide chain is important (Fig. 4.6). In vivo, the polypeptide chain of a protein is synthesized in the peptidyl transferase center of the large ribosomal subunit and proceeds vectorially from the N- to the C-terminus. The nascent chain elongates within a narrow tunnel of the ribosome, where the folding into a native structure is not possible, and at the output of the channel, it emerges into a crowded environment of cell cytoplasm. Therefore, it is usually assumed that after synthesis on the ribosome, the nascent polypeptide chain of the protein folds with the help of chaperones and chaperonins.

The co-translational folding of FPs was studied by examining the de novo folding yield of cycle3-GFP expressed by polyribosomes from *E. coli* cells (Ugrinov and Clark, 2010). It was shown that even the longest GFP nascent chain cannot fold to a native conformation, while the C-terminal

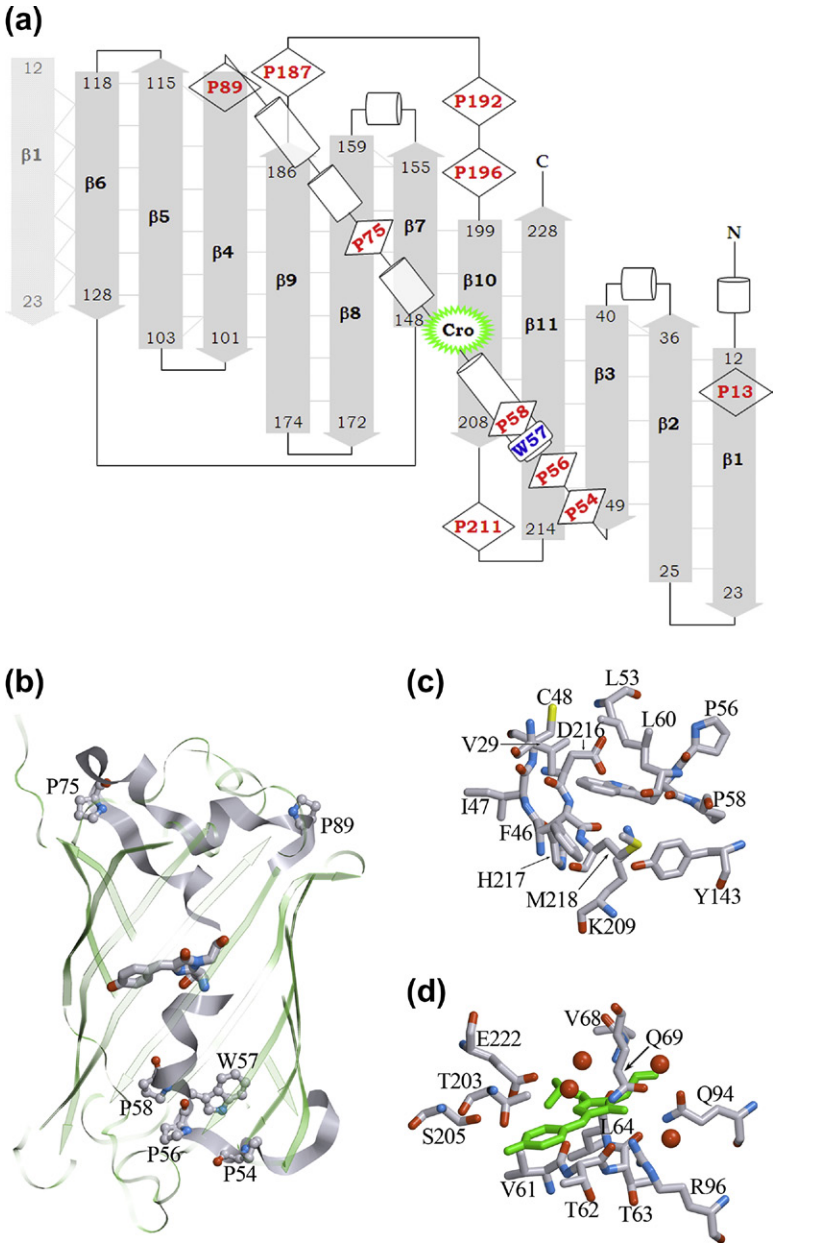

Figure 4.6 ***Structure of sfGFP.*** Localization and microenvironment of the chromophore and the tryptophan residue. *a*, diagram illustrating the formation of the β-barrel of 11 β-strands and the internal helix. The localization of the chromophore (Cro), tryptophan residue W57 and proline residues, including Pro89, the only proline that has the *cis*-conformation is shown; *b*, localization of α-helix in β-barrel. The β-barrel strand in the

residues are conformationally constrained within the ribosomal exit tunnel. At the same time, a GFP variant with a C-terminal extension (CFPex) to span the ribosome exit tunnel, placing all GFP residues outside the tunnel, exhibited measurable quantities of GFP fluorescence tethered to the ribosome (Ugrinov and Clark, 2010).

This result of the in vivo experiment was confirmed and extended by the examination of the folding of ribosome-attached nascent FPs that were synthesized in vitro from truncated RNA transcripts (Kelkar et al., 2012). To generate ribosome-bound FP intermediates of GFP (CFP, EGFP, Venus, and Citrine) and RFP (mStrawberry, mCherry, DsRed, and mTangerine), the variants were fused to a C-terminal reporter protein (CFTR). The RNA transcripts were truncated downstream of the last FP codon and translated in RRL to capture different lengths of the FP C-terminus within the ribosome exit tunnel (Fig. 4.8). In contrast to in vivo expression where nascent chains of different lengths were isolated on polysomes (Ugrinov and Clark, 2010), the in vitro system contains transcripts of a given tether length (Fig. 4.8).

It was shown that the formation of the characteristic FP β-barrel is prevented by sequestration of only a few C-terminal residues within the

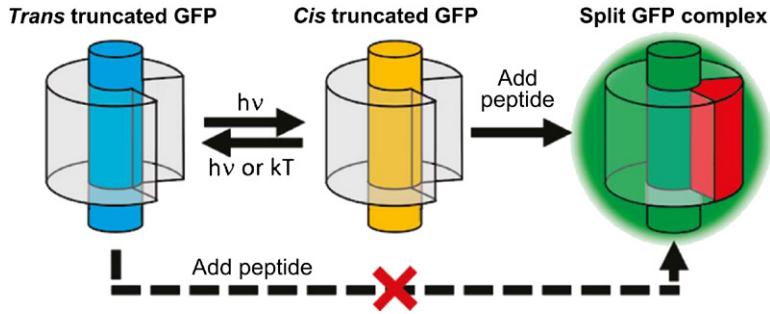

Figure 4.7 ***Reassembling sfGFP 1–10 and a synthetic 11-th β-strand.*** The truncated sfGFP 1–10 after refolding in native conditions does not reassemble with a synthetic peptide corresponding to 11-th β-strand, but it reassembles after light activation. The reassembled structure is identical to the native protein *(Reprinted with permission from (Kent and Boxer, 2011) Copyright 2011 American Chemical Society).* (For color version of this figure, the reader is referred to the online version of this book).

foreground is made transparent. The proline residues that are part of the α-helix, and Pro89, which is localized between the α-helix and the fourth β-strand, are shown; *c*, the microenvironment of W57; *d*, the chromophore microenvironment. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book).

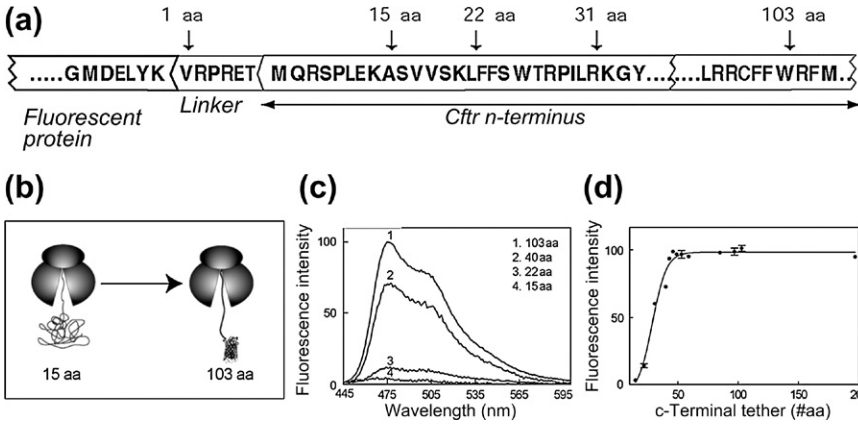

Figure 4.8 ***Co-translational folding of FPs.*** *a*, a diagram of the FP fusion protein showing the C-terminal of the FP, the 6-amino acids linker and the N-terminal residues of the cystic fibrosis transmembrane conductance regulator (CFTR). The truncation sites are indicated by arrows. *b*, a diagram showing co-translational folding of the FP with different C-terminal tether lengths. *c*, the emission fluorescence spectra of the FP with different C-terminal tether lengths. *d*, the dependence of the intensity fluorescence of FP with different C-terminal tether lengths. *(Adapted from figure 1 and figure 2 originally published in Journal of Biological Chemistry (Kelkar et al., 2012) Copyright 2012 the American Society for Biochemistry and Molecular Biology).*

ribosome exit tunnel. In contrast, folding proceeds unimpeded when the last C-terminus residue is extended at least 31 amino acids beyond the ribosome peptidyl transferase center. Thus, the ribosome constrains tertiary folding as expected, but it has no detectable influence on either the kinetics or yield once the C-terminus has exited from the tunnel. It was found that co-translational folding intermediates with 10 β-strands outside the exit tunnel remain kinetically trapped in a non-native, on-pathway intermediate structure that retains folding competence for prolonged periods of time. It was also shown that the final step in FP folding is relatively unaffected by the cellular folding environment. Kinetic analysis revealed that co-translational FP folding involves at least two steps: the formation of a partially folded intermediate and the slow incorporation of the eleventh β-strand (and possibly others) into the final barrel structure (Kelkar et al., 2012). Kelkar et al. (2012) proposed the scheme of co-translational folding of FPs. They proposed that folding occurs through a landscape characterized by rapid formation of a stable N-terminal folding intermediate that likely occurs coincidently with, and may be facilitated by vectorial elongation of the nascent chain. These events are followed by a slow, rate-limiting step after ribosome release that requires the eleventh β-strand to

form the final barrel structure necessary for chromophore catalysis. In cells, these events would normally be coupled when synthesis is completed and the nascent chain is released from the ribosome. Interestingly, despite their conserved architecture, the folding rates for FPs are faster for genetically selected "superfolder" proteins. Future examination of these proteins could clarify the rate-limiting step and the co-translational folding intermediates.

6. CONCLUDING REMARKS

The β-barrel scaffold plays a crucial role in chromophore formation. A properly folded β-barrel is the essential prerequisite for the initiation of chromophore maturation. The most important features for chromophore synthesis are Arg96, which plays the role of an electrostatic catalyst, and Glu222, which acts as a base catalyst. These amino acids are absolutely conserved among FPs. The residue of Gly67 from the chromophore-forming tripeptide is also strongly required for the chromophore cyclization through nucleophilic attack of amide nitrogen of Gly67 to the carbonyl carbon of the residue at position 65. The residue of Tyr66 that is conserved in all natural FPs is believed to provide the proper oxidative chemistry during chromophore maturation and prevent undesirable side reactions, such as backbone fragmentation and hydrolysis. The most variable position is position 65 of the chromogenic tripeptide; the amino acid at this position affects chromophore chemistry and results in diverse chromophore structures. The extent of π-conjugation within the chromophore is the main determinant of the emission hue. However, numerous contacts of the chromophore with the protein matrix further affect the photophysics of FPs, including color adjustment and excited-state proton transfer reactions. Moreover, through these internal interactions, the chromophore tightens the protein structure. Indeed, FP proteins are extremely stable. The quasi-equilibrium dependence of protein unfolding is achieved only on the fourth day of its incubation in solutions of GdnHCl. The high stability of these proteins is clearly manifested when truncated GFP generated by removing some β-strand or even an internal helix is reassembled with a synthetic peptide corresponding to the truncated fragment. Thus, the reassembled protein exhibits properties that are indistinguishable from the native one. The formidable stability that is characteristic to FPs greatly complicates the study of the processes of their unfolding–refolding. Nevertheless, due to the creation of improved GFP variants such as cycle3-GFP and sfGFP, these studies are now possible. As these proteins are not subjected to aggregation, their unfolding is

reversible. However, renaturation occurs at much lower denaturant concentration than unfolding, so that unfolding and refolding dependences do not coincide. The main reason for the existence of hysteresis is the fact that the chromophore is already synthesized and must be correctly positioned when forming the β-barrel scaffold of protein. Obviously, an important role in this process is played by the prolines and in particular, by Pro89, which is the *cis* isoform. Despite the large number of studies on the folding process of FPs that have appeared recently, there is no consensus in the literature on the availability and number of intermediate states. The study of these processes is complicated by the heterogeneity of the protein in its native state due to the presence of protonated and anionic forms of the chromophore and the change in the relative content of these forms depending on the concentration of the denaturant. Despite the fact that the existence of two forms of the chromophore has been known for a long time, until recently, this effect has not been taken into account in the study of FP folding.

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CHAPTER FIVE

Cytoskeletal Proteins: Shaping Progression of Hepatitis C Virus-Induced Liver Disease

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Abstract

Hepatitis C virus (HCV) infection, which results in chronic hepatitis C (CHC) in most patients (70–85%), is a major cause of liver disease and remains a major therapeutic challenge. The mechanisms determining liver damage and the key factors that lead to a high rate of CHC remain imperfectly understood. The precise role of cytoskeletal (CS) proteins in HCV infection remains to be determined. Some studies including our recent study have demonstrated that changes occur in the expression of CS proteins in HCV-infected hepatocytes. A variety of host proteins interact with HCV proteins. Association between CS and HCV proteins may have implications in future design of CS protein-targeted therapy for the treatment for HCV infection. This chapter will focus on the interaction between host CS and viral proteins to signify the importance of this event in HCV entry, replication and transportation.

1. INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of liver disease worldwide. The development of much needed specific antiviral therapies and an effective vaccine have been hampered by the lack of a convenient small animal model. Studies have established the involvement of host-cell cytoskeletal (CS) proteins, including microtubules (MT) and actin filaments in HCV replication, as formation and intracellular transport of HCV replication complexes (RCs). Recently we showed upregulation and interaction of filamin (fila) with HCV proteins in HCV expressing human hepatoma cells compared with parental cells with similar results in liver biopsies from patients with chronic hepatitis C (CHC) vs controls (Ghosh et al., 2011). Recent advances in microarray and proteomics methods have permitted more comprehensive analysis of alterations in gene expression induced by HCV replication and reveal modulation of new genes potentially useful for selection of antiviral targets. Applications of new image-analysis technology to study virus internalization are providing clearer insights into mechanisms used by HCV to enter host cells. The use of dominant negative constructs, specific inhibitory drugs and RNAi to prevent entry through particular pathways selectively has provided evidence for the clathrin-mediated entry of HCV. This chapter will cover host CS and viral protein interactions and their roles in replication, transportation and well-documented entry

mechanisms as well as more recent discoveries in the light of emerging relations between host CS and HCV proteins.

2. OVERVIEW OF HCV

2.1. Virology and Taxonomy

The HCV was cloned and characterized for the first time in 1989 (Choo et al., 1989). HCV is a small, positive-strand, enveloped RNA virus member of the family Flaviviridae (genus *Hepacivirus*) (Alter et al., 1999). It is primarily transmitted parenterally and to a lesser extent through vertical and sexual routes (Gentzsch et al., 2011). Acute HCV infection is typically mild in severity, with only about one-third of the subjects developing jaundice, which is usually mild. Most persons with acute HCV infection do not reach the minimal threshold of clinical disease and are not diagnosed. However, of those infected, 70–85% will develop chronic infection (Milward et al., 2010). HCV is estimated to infect approximately 170–180 million people worldwide (Strnad et al., 2006; Tsao et al., 2006; Milward et al., 2010), including an estimated 2.7 million people in the USA who have evidence of HCV infection and about 4 million who have evidence of prior infection (Alter et al., 1999; Lau et al., 2005). However, mechanisms determining liver damage and key factors leading to high rate of chronic hepatitis remain unclear. Hepatic histo-pathology detected by light microscopy is characteristic but not specific and includes inflammatory lesions of low or moderate intensity and hepatic fibrosis. The common and most characteristic trait of chronic HCV infection involves lesions in hepatocyte nuclei. These changes include swelling, altered shape, hyperchromasia, disturbed nuclear chromatin structure, enlarged and frequently multiple nucleoli and lesions of nuclear envelope. Cellular localization of HCV proteins using immunohistochemical techniques remains difficult and underdeveloped. In most studies, HCV proteins have been detected in the cytoplasm although some reports indicate nuclear localization, especially core protein.

2.2. Genome

The complete virion of HCV contains a positive stranded RNA genome of about 9.6 kb in length, which encodes a polyprotein of about 3000 amino acids. This polyprotein, which is synthesized on host ribosomes, is cleaved by host and viral proteases to generate at least 10 functional proteins, namely, core, two envelope proteins (E1 & E2), p7, and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Watashi

and Shimotohno, 2007; Gentzsch et al., 2011) The extracellular virion is mainly composed of a triad of core and the two envelope proteins. Both NS2 and p7 are thought to be essential cofactors for the assembly and release of the virion. More specifically, NS2 is a protease that mediates the cleavage of the polyprotein at the NS2–3 junction, while p7 forms cation-selective channels. The other NS proteins (from NS3 to NS5B) are required for viral replication. Indeed, NS3-4A (a serine protease) and NS5B (an RNA-dependent RNA polymerase) are thought to play central roles in viral genome replication and assembly (Watashi and Shimotohno, 2007; Ghany et al., 2011).

2.3. Genotypes

HCV has six known major genotypes (1–6), which are further divided into 50 subtypes (1a, 1b, etc.). Variations in relative frequencies of viral genotypes exist in different geographic regions of the world. Genotypes 1a and 1b account for most infections in the USA (70%), with genotype 1a predominating. The remaining 30% of infected individuals in the USA are due mostly to genotypes 2 or 3, with a smaller proportion accounted for by genotypes 4–6 (Boyer et al., 2006). In contrast, 80% of HCV infections in Europe are due to subtype 1b. Genotype 2a is prevalent in Japan, Taiwan, and China; genotype 3 in the UK (Bostan and Mahmood, 2010); genotype 4 in the Middle East and Northern and Central Africa; genotype 5 in South Africa; and genotype 6 in South East Asia (Boyer et al., 2006).

2.4. Progression and Complications

The natural history of acute HCV viral infection is summarized in Fig. 5.1. As shown in the figure, those with chronic infection are unlikely to achieve a spontaneous clearance. Rather, the great majority develop chronic hepatitis, which is usually gradual, albeit progressive. The risk of developing liver cirrhosis is highly variable among different cohorts, but is about 20–30% over the ensuing 25 years. Major protective factors include consumption of coffee on a regular basis and absence of smoking. In addition, absence of iron overload, as is more common in women than men, likely has a protective effect (Scobey et al., 2009). There is also evidence that iron reduction improves the response to interferon-based therapy of CHC (Desai et al., 2008; Caballes et al., 2012) and reduces the risk and rate of progression and/or development of hepatocellular carcinoma (HCC) (Caballes et al., 2012).

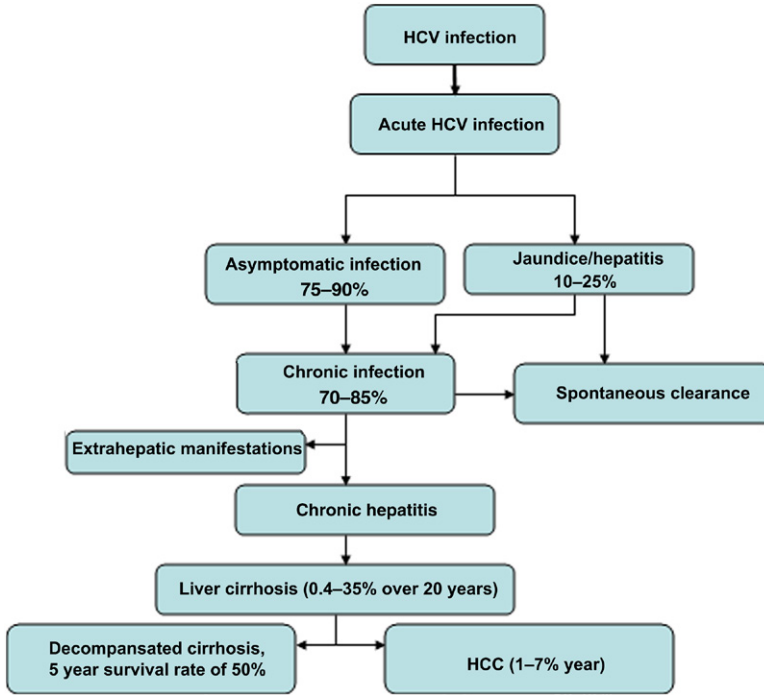

Figure 5.1 Natural history of progression of HCV infection with the estimated percentages of the different outcomes. (For color version of this figure, the reader is referred to the online version of this book).

2.5. Current Strategy for HCV Treatment

For several years, the treatment of CHC has relied on dual therapy with both pegylated interferon α (PEG-IFN-α) and ribavirin (RBV) as standard of care. It is clear that sustained viral response (SVR) rates with PegIFN-A + RBV therapy leave much room for improvement. Furthermore, because approximately 10% of HCV infected patients are eventually found to be null responders to such therapy (Heathcote, 2009; Bailly et al., 2010), an alternative strategy for treatment is necessary. With increased knowledge of the viral life cycle and characterization of key peptides of HCV, a number of compounds of at least five different classes are under development. Inhibitors of the viral NS3/4A protease (boceprevir, telaprevir), however, are the only stat-C drugs currently approved by the Food and Drug Administration (FDA) (Ghany et al., 2011). Although there are some preliminary data that suggest that these new drugs may be active against other genotypes, future studies are needed to confirm these promising results.

2.6. Prognosis

Among those who have developed cirrhosis or advanced hepatic fibrosis with CHC, the risk of hepatic decompensation and death due to cirrhosis and its complications, particularly those associated with portal hypertension (bleeding from gastroesophageal varices, spontaneous bacterial peritonitis, refractory ascites, hepatic encephalopathy) or the development of HCC are major factors that presage premature mortality. Nevertheless, because of the slowly progressive nature of CHC, premature death attributable to CHC occurs relatively late in life. This burden of chronic disease, and the fact that CHC is now the single most common indication for liver transplantation worldwide, highlight the desirability of more thorough screening of at risk populations and more assiduous therapy of persons found to have CHC.

2.7. Progression of Chronic Hepatitis C to HCC

There is strong evidence demonstrating the association of chronic HCV infection to cirrhosis and HCC. CHC leads to liver cirrhosis in approximately 20% of patients within 20 years that follow the infection (Strnad, Lienau, et al., 2006). There are several factors including external and host factors, that can increase the risk of progression of liver disease. Virtually all HCV-related HCC occurs among patients with cirrhosis. A meta-analysis study by Donato et al. (1998) with 21 case–control studies showed the risk for HCC was increased 17-fold in HCV-infected patients compared to HCV-negative controls. Frequently, the initial time of infection is not known, and therefore must be estimated. The mean time of development of cirrhosis was 21 ± 10 years in patients with CHC attributed to blood transfusion as reported by Tong et al. (1995) in a longitudinal U.S. study. According to Castells et al. (1995), the time to development of cirrhosis and HCC was 24 years and 27 years, respectively, in a European study. After 20 years of CHC and development of liver cirrhosis, risk of HCC occurrence is about 1–5% per year. (Gordon et al., 1998; Degos et al. 2000; Chiba et al. 1996; Bruno et al. 1997; NIH Consensus Statement, 1997; Di Bisceglie et al., 1994). Higher estimates in the range of 5–7% per year have been reported from Japan. Because of the increased risk of development of HCC in patients with CHC who have cirrhosis or advanced hepatic fibrosis, it is recommended that such patients receive ongoing screening for development of HCC with abdominal ultrasound every six months.

3. PATHOLOGY OF ACUTE TO CHC WITH EMPHASIS ON CS CHANGES

HCV infects mainly hepatocytes, and the mechanisms of cell entry remain incompletely understood. HCV can replicate and persist also in other cells of the human body, e.g., in peripheral blood mononuclear cells (NIH Consensus Statement, 1997; Di Bisceglie et al., 1994) and pluripotent hematopoietic CD34+ cells (Sansonno et al., 1998). HCV can induce both acute and chronic lesions in hepatocytes. HCV is a typical RNA virus, and thus there is no integration of the viral genome or a piece of genome into host chromosomes. Pathogenesis of persistent HCV infection is dominated by specific defects of cell-mediated immune responses (Koziel et al., 1993). At the acute stage of the infection, CD8+ lymphocytes recognize epitopes of structural and NS HCV proteins, including mainly NS3, NS4 and, to a lesser extent, NS5, but the numbers of such lymphocytes in peripheral blood are low, which indicates that the cells accumulate first and foremost in the liver (Cerny et al., 1995) The cytotoxic lymphocytes, which infiltrate the liver, recognize HCV antigens in the context of MHC class I antigens (Weiner et al., 1995). A preferential presentation of selected HCV peptides may take place with disturbed presentation of the other peptides.

3.1. Overview of CS Proteins

Cytoskeleton represents the basic structural framework of the cell. Three major types of filaments make up the cytoskeleton: microfilaments or actin filaments, MTs, and intermediate filaments. The presence of this system in all cells, as well as their structural diversity and cytoplasmic distribution, has been recognized relatively in recent years. Actins and tubulins are the most abundant CS proteins that support and participate in diverse cellular functions. Recent studies have begun to unveil the pathways that regulate degradation of actin (via TRIM32) (Insall and Machesky, 2009; Saccone et al., 2008) and tubulin (via parkin or cofactor E-like) (Tian et al., 1997). These pathways participate in maintaining appropriate function and quality control of CS proteins through cellular proteostasis. Recent advancement in cell biology and microbiology signifies the involvement of host-cell cytoskeleton in the life cycle of pathogens, such as viruses. The cellular cytoskeleton provides the basis for intra- and intercellular movements. This trafficking is actively exploited by many pathogens and is likely to depend on a combined track of actin filament and MT. Regulation and/or complete

inhibition of intracellular movements through host-cell motors and tracks, as well as the factors involved in host–pathogen interaction, offers a potential strategy and targets for development of more effective antimicrobial therapy. In this chapter, we discussed about the association and emerging link between CS proteins and HCV infection.

3.2. Morphological Lesions Detected by Different Types of Microscopy

3.2.1. Lesions Detected by Light Microscopy in CHC

Histopathological pattern of CHC exhibits no unique characteristic traits. The respective lesions resemble those noted in other chronic viral hepatitis types, e.g. chronic type B hepatitis. Inflammatory infiltrates are present in portal spaces, less frequently within the lobules, the latter usually accompanied by interface hepatitis, formerly called *piecemeal necrosis of hepatocytes* (Fig. 5.2A and B). Occasionally, the inflammatory infiltrate contacts the sites of piecemeal necrosis at the interface of lobules and portal spaces (Fig. 5.2B)

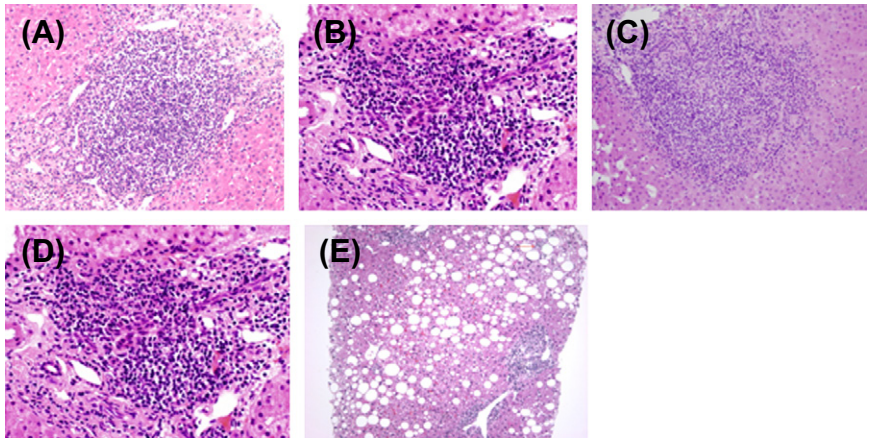

Figure 5.2 Typical hepatic histo-pathology of CHC prominent portal inflammation without bile duct involvement expanding a portal area with interface hepatitis into the surrounding lobule (H & E; 200×) (A). Higher power view of a portal area with numerous lymphocytes involving and expanding a portal area with interface activity (H & E; 400×) (B). Medium-power view of lymphoid aggregate with piecemeal necrosis of surrounding hepatocytes and sinusoidal lymphocytes (H & E; 200×) (C). Low-power view of lobule with prominent steatosis in the lobule. Portal areas with mild inflammation. Focus of lobular inflammation (arrow) also present (H & E; 100×) (D). Low-power view of example of CHC with periportal fibrosis. Note the presence of inflammation around portal area and surrounding fibrosis (Masson trichrome; 100×) (E). (For color version of this figure, the reader is referred to the online version of this book).

(Barwick and Rosai, 1996; Dumoulin et al., 1999). The typical although not pathognomic traits of the morphological pattern in CHC include presence of lymphoid follicles (Fig. 5.2C) and/or accumulation of lymphoid cells in portal spaces or in lobules, damage to cells of bile duct epithelium, fatty degeneration of hepatocytes (Fig. 5.2D), presence of Mallory-Denk bodies in the cytoplasm of hepatocytes (Barwick and Rosai, 1996; Cathomas et al., 1996; Faa et al., 1994; Fischer et al., 1996). In most cases, fibrosis progresses gradually from portal triads (Fig. 5.2E).

3.2.2. Pattern of CHC as Detected by Electron Microscopy

Most viruses are small enough to be at the limit of resolution of even the best light microscopes, and can be visualized in liquid samples or infected cells only by electron microscopy (EM). However, there has been debate about the value of EM for medical virology (Curry et al., 2000; Curry et al., 2006; Madeley et al., 2000; McCaughey et al., 2000). The HCV complete virion consists of a core of genetic material (RNA), surrounded by an icosahedral protective shell of protein, which is further encased in a lipid (fatty) envelope of cellular origin. Two viral envelope glycoproteins, E1 and E2, are embedded in the lipid envelope (Figs 5.3 and 5.4). Even today, little is known about the morphogenesis of this virus. TEM of a virus-like particle (VLP) model obtained by expressing genes encoding the HCV structural proteins has demonstrated that viral budding occurs at the ER membrane and that the HCV core protein drives this process (Blanchard et al., 2003). Fluorescence microscopy has shown that most of the HCV core protein is associated with the surface of lipid droplets (McLauchlan et al., 2002), and TEM has shown that viral budding occurs at the ER membrane in the close vicinity of these lipid droplets (Fig. 5.4) (Ait-Goughoulte et al., 2006; Hourioux et al., 2007).

The ultrastructural patterns in CHC pertain to both cell nucleus and cytoplasm. A detailed analysis of literature on ultrastructural lesions in HCV infections has shown that the pattern remains to be a matter of debate. The other cellular lesions involve nuclear lobulization and the presence of cytoplasmic inclusions. Normal cell nucleus structure was sometimes noted despite the presence of cytoplasmic lesions (Faa et al., 1994; Jonas MM, 1999). Disintegration of nuclear envelope was accompanied by passage of nuclear content to the cytoplasm (Biczysko et al., 2002). Cytoplasmic changes observed in EM in HCV infection are more uniform in many types of cell lines infected in vitro and in human or chimpanzee hepatocytes infected in vivo (Gastaldi et al., 1995; Iacovacci

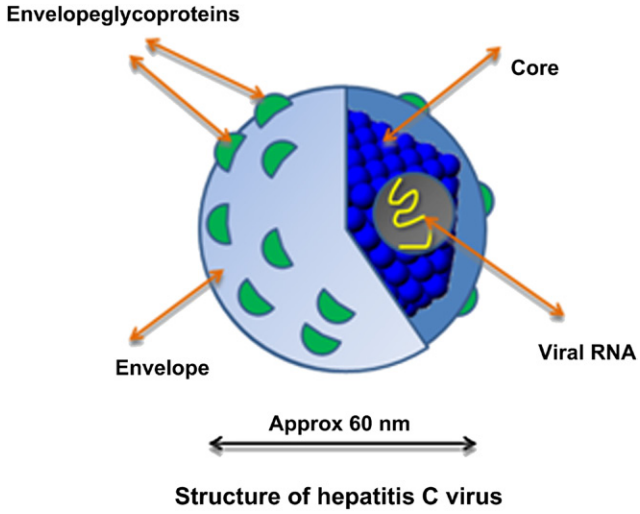

Figure 5.3 Simplified diagram of structure of HCV. *(Adapted from http://en.wikipedia.org/wiki/File:HCV_structure.png. Licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license).* (For color version of this figure, the reader is referred to the online version of this book).

et al., 1997; Jonas MM, 1999; ; Kao et al., 1997). The most common cytoplasmic lesions detected by EM in chronic HCV infection are listed in Table 5.1 and common characteristics of HCV are in Table 5.2.

4. ROLE IN HCV INFECTION AND PATHOGENESIS

A signature of intracellular positive-strand RNA viruses is RCs, which are associated with membranes and composed of viral proteins, replicating RNA, altered cellular membranes and other host factors (Salonen et al., 2005). Studies have established the involvement of the host-cell CS in HCV replication, as formation and intracellular transport of HCV RCs are closely linked to the dynamic organization of the endoplasmic reticulum (ER) and CS network (Presley et al., 1997). The interactions between CS components and cellular membranes are intimate and extensive (Doherty and McMahon, 2008). Both MT and actin filament polymerization are essential for HCV RNA synthesis (Bost et al., 2003). Recently, it was proposed that actin filaments and MT play a role in the migration of HCV RCs to other regions in the cell (Lai et al., 2008). However, the roles of CS proteins at the molecular level in HCV infection and pathogenesis still remain poorly understood. A recent study by Hudacko et al. suggested that

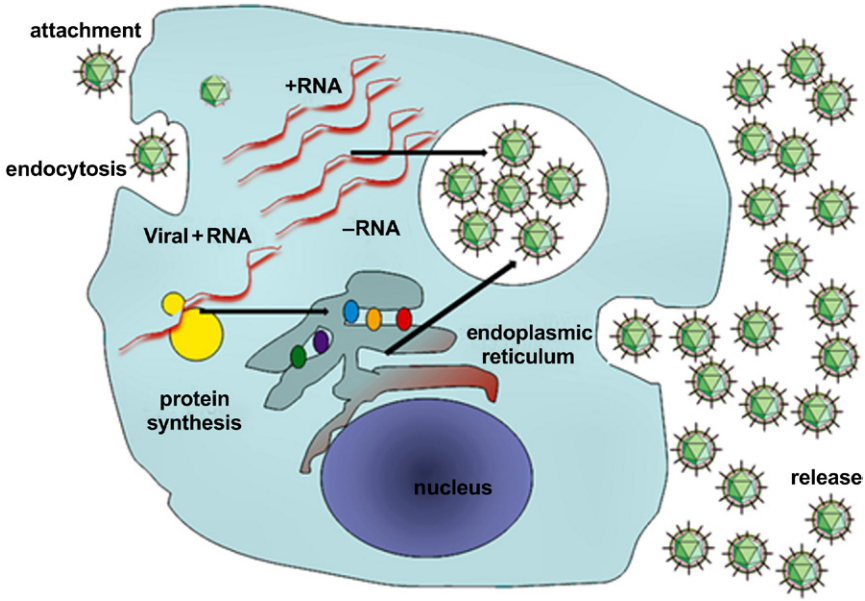

Figure 5.4 ***Simplified diagram of the HCV replication.*** *(Adapted from http://en.wikipedia.org/wiki/File:HepC_replication.png).* (For color version of this figure, the reader is referred to the online version of this book).

elevated serum F-actin antibody titers are commonly encountered in HCV-infected patients and may reflect more active inflammation in liver biopsy samples, similar to autoimmune hepatitis. Fang et al. utilized a proteomic approach to analyze the global protein expression profiles of host cells in absence and presence of HCV replication. So far, detailed characterization of the host response to HCV replication and the cellular factors needed for viral infection and/or replication have not been fully delineated, due to the lack until recently of a robust HCV cell culture system and the lack of powerful biological tools. Recent successful establishment of an HCV sub-genomic replicon cell systems and complete virion infectious systems have provided a useful source. Like other members of Flaviviridae, HCV viral NS proteins as well as host-cell factors are believed to function in the initiation and regulation of HCV viral RNA replication, by formation of an RC. Consistent with previous reports, cellular proteins, such as HSP27 (Choi et al., 2004), α-actinin (Lan et al., 2003), nucleolin (Hirano et al., 2003) and eukaryotic initiation factor 4A-I (Kyono et al., 2002), which are reported to comprise the replication machinery of HCV (Ishido et al., 1998), were found to be elevated in HCV replicon cells. In addition, changes in the

Table 5.1 Most common cytoplasmic ultrastructural alterations in HCV-infected cells

Electron microscope observations	Types of infected cells
Dilated vesicular endoplasmic reticulum (ER)	• Human hepatocytes • TOFE cells
Dilated, degranulated ER with double membranes of semicircular shape ("undulating")	• Human hepatocytes • Human and chimpanzee hepatocytes
Cytoplasmic vesicles with virus-like particles: of 50 nm in diameter, of 50–60 nm in diameter, of 40–45 nm in diameter	• Daudi cells and chimpanzee hepatocytes HepG2 cell line Human fetal hepatocytes • TOFE cells • Human hepatocytes
Virus-like particles within ER cisternae, 45 nm in diameter of 20 nm in diameter	• HeLaG cells • Human hepatocytes
Tubular structures in ER cytoplasm	• TOFE cells • Human hepatocytes • Chimpanzee hepatocytes
Regular mitochondria with dense granules in matrix	• Human hepatocytes
Densely packed fibrils in ER	• Human hepatocytes
Large lipid droplets	• Human hepatocytes • HepG2 and CHO cell lines • Transgenic mice hepatocytes
Increased number of lysosomes	• Human hepatocytes • TOFE cells
Irregular mitochondria with reduced number or absence of cristae	• Human hepatocytes
Paracrystalline inclusions in mitochondria	• Human hepatocytes • Transgenic mice hepatocytes

expression of other CS dynamic regulator proteins in HCV replicon cells were also revealed. Rho GDP-dissociation inhibitor 2, known to regulate Rho and the reorganization of the actin CS (Hall, A, 1998; Sasaki and Takai, 1998), was overexpressed in the presence of HCV replication. It has been speculated that the host-cell CS proteins might be the most likely component of HCV RC. Assembling the RNA polymerase on the cytoskeleton or on cell membranes might ensure appropriate concentrations of replication components, and hence control the rates or efficiencies of replication reactions. Protein–protein interactions among the various HCV NS proteins and hVAP-33 are important for the formation of HCV RC (Lu et al., 2004).Viral proteins have been shown to affect the biology of cellular

Table 5.2 Characteristics of HCV

Family	Flaviviridae
Virion characteristics	Spherical particle of 50 nm (36–65 nm) in diameter with core (30 nm) and envelope extensions
Genome structure	Single-stranded, linear RNA
Presence of viral components in cell nucleus	Around 30 nm spheric or tubular structures • Core protein • HCV RNA plus and minus strands
Presence of viral components in cell cytoplasm	• 45–60 nm virus-like particles • 22–40-nm-thick tubular structures • Core protein (30 nm particles), E2, NS3, NS4, NS5a, NS5b • HCV RNA plus and minus strands

systems, including cell cycles and signaling pathways, contributing to viral pathogenesis (Waris et al., 2001; Flores et al., 1993). HCV proteins disturb the host mitogenic signaling (Macdonald et al., 2003; He et al., 2002; Tan et al., 1999) and apoptosis pathway (Chung et al., 2003; Lan et al., 2002).

4.1. Role in HCV Entry

Recent advancement in HCV cell culture models has significantly improved our understanding and knowledge about molecular virology of HCV infection, including the entry steps. The most important components of this process rely on specific interactions between virus components, mainly envelope proteins and several cellular factors. According to recent reports, HCV entry into host cells is a multistep event and involves steps from particle binding to host cell up to delivery of viral genome to its replication site within the target cells, which are chiefly human hepatocytes. Several cell surface molecules, such as tetraspanin [CD81] (Pileri et al., 1998), SR-BI/Cla1 (Scarselli et al., 2002), Claudin-1 (CLDN-1) (Evans et al., 2007) and Occludin (OCLN) (Liu et al., 2009), are considered as essential receptors/co-receptors for HCV cell entry. In addition, other molecules like glycosaminoglycans, such as heparin sulfate, the lectins DC-specific intracellular adhesion molecule-3-grabbing nonintegrin (SIGN), liver-specific (L)-SIGN (Cormier et al., 2004a), and low-density lipoprotein (LDL)-receptor (LDL-R) (Agnello et al., 1999) have been implicated in HCV cell attachment and entry.

Research on HCV replication and pathogenesis has been hampered by the lack of reproducible in vitro methods of HCV infection. To

Table 5.3 Summary of HCV cell entry in vitro model systems

Model system	Basis of system	Effectivity
HCV-like particles (HCV-LPs)	Baculovirus expression systems (Baumert et al., 1998).	• Particles are not secreted but rather retained in intracellular vesicles. • Not clear how well particles reflect earliest stages of infection by authentic HCV.
HCV pseudoparticles (HCVpp)	Based on the production of lentiviral particles (Bartosch et al., 2003a; Hsu et al., 2003).	• Particles are infectious and show tropism for human liver cells. • HCVpp are not associated with lipoproteins since they are produced in 293T kidney cells that do not synthesize lipoproteins.
Cell culture-produced HCV (HCVcc)	Based on JFH-1, a particular genotype 2a virus strain, or chimeras of this, cloned from the serum of a patient with fulminant HCV.	• This model mimics a natural HCV infection. • Some limitations exist as this system is restricted to two cell lines, Huh-7 and LH86, which have abnormal lipoprotein metabolism, and essentially to the JFH-1 strain.

overcome these limitations, selectable HCV replicons were developed in human hepatoma-derived Huh7 cells, which contain self-replicating HCV RNA and express all viral proteins (Hunt, 2002; Wei et al., 2004; Choi et al., 2004). These systems have been successfully used to study HCV translation and RNA replication revealing important processes of virus–host interactions (Lan et al., 2003; Hirano et al., 2003; Kyono et al., 2002; Shevchenko et al., 1996; Lee et al., 2004). The involvement of HCV proteins has been suggested in a wide range of activities, including cell signaling, transcriptional modulation, transformation, apoptosis, oxidative stress, membrane rearrangement, vesicular trafficking and immune response (Jensen et al., 1999; Hideki et al., 2004; Alan, 1998; Sasaki and Takai, 1998; Ishido et al., 1998; Waris et al., 2001; Flores et al., 1993). Recent establishment of HCV cell culture system, cell culture models that release HCV viral particles, are providing new model systems for molecular studies of the HCV life cycle and virus–host

interactions (Bardag et al., 2004; Keller et al., 2000; Lopez et al., 2000; Wakita et al., 2005; Lindenbach et al., 2005). A recent study using an siRNA approach has identified a set of 26 human genes that modulate virus production and suggested novel for antiviral therapy (Gao et al., 2003; Fang et al., 2006). The main in vitro model systems used to study HCV cell entry are summarized in Table 5.3.

4.1.1. CD81

CD81 is a ubiquitously expressed, unglycosylated membrane protein and an integral member of the tetraspanin family. Pileri et al., 1998 has been proposed CD81 as an HCV receptor molecule and demonstrated that it is sufficient for binding not only the envelope protein (E2) but also HCV particles in humans. Considering the wide distribution of CD81, these results imply that HCV can bind to a variety of cells other than hepatocytes. Activation of Rho GTPases mediates actin-dependent relocalization of the HCV E2/CD81 complex to allow CD81 to be associated with tight junctions (TJ) proteins, ZO-1, OCLN and CLDN-1 (Brazzoli et al., 2008), molecules recently described as HCV co-receptors, which are described later. Finally, CD81 engagement activates the Raf/MEK/ERK signaling cascade, which affects post-entry steps of the virus life cycle (Brazzoli et al., 2008). Therefore, CD81 not only serves as a mere attachment factor, but also actively promotes infection by triggering signaling cascades important for virus entry and more downstream events.

4.1.2. SR-BI/Cla1

SR-BI/Cla1 is a "multiligand" receptor for various types of lipoproteins and also for chemically modified lipoproteins, e.g. oxidized and acetylated LDL (Rhainds and Brissette, 2004), expressed in various mammalian cells but is expressed mostly in the liver and steroidogenic tissues. The antiviral action of interferon recently has been linked to a decrease in the expression levels of SR-BI on the cell surface, thereby restricting virus attachment and entry into hepatocytes (Murao et al., 2008). These data show the crucial role of SR-BI in cell infection by HCV.

4.1.3. Tight Junction Proteins

Tight Junction (TJ) proteins are major components of cell–cell adhesion complexes that differentiate apical from basolateral membrane domains and maintain cell polarity by forming an intramembrane; regulating diffusion of certain molecules (Shin et al., 2006). The TJ complex is composed of

four major types of transmembrane proteins: OCLNs, CLDNs, junction-associated molecules (JAMs) and coxsackie virus B adenovirus receptors (CARs) (Greber and Gastaldelli, 2007). TJs comprise a network of cytoplasmic scaffolding proteins and provide a platform to links to the CS and to intracellular signaling molecules (Balda and Matter, 2008; Tsukita et al., 2008). Evans et al. (2007) identified CLDN1, which is involved in HCV entry. CLDN1 forms networks at TJ and is expressed in all epithelial tissues but predominantly in the liver (Furuse et al., 1998). By contrast, functional studies indicate that CLDN1 plays a role in the postbinding phase of infection, subsequent to HCV binding to CD81 and probably subsequent binding among HCV and SR-BI (Evans et al., 2007). CLDN6 and CLDN9 are two other members of the CLDN-1 family, also mediate HCV entry (Zheng et al., 2007; Meertens et al., 2008). These molecules are expressed in the liver along with CLDN1, however, unlike CLDN1; they are also present in peripheral blood mononuclear cells, another possible HCV replication site beside human hepatocytes. According to recent data, distribution of CLDN1 in TJ correlates with permissiveness to HCV infection (Liu et al., 2009; Yang et al., 2008), thereby confirming that localization of CLDN1 to TJs is critical for viral entry and cellular tropism of HCV. Nevertheless, there is still no evidence for a direct interaction between CLDN1 and the virus.

Recent reports have showed the involvement of another transmembrane component of the TJ, namely, OCLN (60 kD) what is structurally related to CLDN-1s, plays an important role in HCV cell entry and initiation of a productive HCV infection (Liu et al., 2009). OCLN participates in both cell–cell adhesion in the paracellular space and anchoring of the junctional complex to the cytoskeleton. The latter function is accomplished through binding of the C-terminal cytoplasmic region of OCLN to scaffolding zonula occludens proteins (ZO-1, -2 and -3) which mediate binding to actin cytoskeleton (Peng et al., 2003). OCLN polypeptide is delivered to the plasma membrane in an MT- and temperature-dependent manner, whereas its steady-state localization at the cell surface depends on intact microfilaments (Subramanian et al., 2007). Liu et al. demonstrated that reduction of expression of CLDN1 and OCLN by siRNA and shRNA interference inhibited HCVpp and HCVcc cell entry (Liu et al., 2009). Application of confocal microcopy-based image analysis showed that OCLN accumulates in the ER and co-localizes with the E2 HCV protein (Benedicto et al., 2008). Altogether, these data suggest that direct interaction between OCLN and E2, facilitates viral entry through hepatocyte

TJs, and this process may also require a delicate molecular architecture of TJ proteins.

A significant observation was that HCV infection may alter localization of TJ proteins (Benedicto et al., 2008; Liu et al., 2009). CLDN1 and OCLN expression levels were down-regulated following infection, rendering the infected cells refractory to HCVpp superinfection (Liu et al., 2009). Exactly how CLDN1 and OCLN are involved in this stepwise entry process is not completely understood, despite some evidence pointing to a late step (Benedicto et al., 2009; Evans et al., 2007). Recent report proposes that CLDN1 association with CD81 subserves HCV entry (Harris et al., 2010, 2012). Previously, it was thought that OCLN is necessary but later it was found to be dispensable for formation of TJ (Furuse et al., 1993). The observation of involvement and internalization of OCLN is required in HBV entry hints that OCLN may be a critical protein linking virions to endocytic pathways (Coyne et al., 2007). OCLN forms a complex with dynamin, a well-known regulator of endocytosis and actin cytoskeleton (Praefcke and McMahon, 2004), and HCV infection is sensitive to dynamin inhibition. Depletion of dynamin II, the only dynamin in epithelial cells, prevents junctional formation and induces abnormalities in actin assembly (Chua et al., 2009). Dynamin II is known to play an important role in orchestrating epithelial cell polarity by maintaining a balance of membrane protein endocytosis and intracellular membrane traffic (Chua et al., 2009). This report suggests that OCLN could play an important role by forming bridge between surface protein complex and endocytic machinery through its association with dynamin. The necessity of the association between OCLN and dynamin for HCV entry is yet to be established. Confirmatory studies are needed to delineate further the role of OCLN with incoming HCV particles during the sequential entry process and/or facilitate virus internalization.

4.2. Known Alterations of CS Proteins in HCV Infected Cells

A better understanding about host and viral cell interaction, host-cell responses to HCV infection and replication may provide new biomarkers of HCV infection and/or suggest new therapeutic approaches for CHC. The objective of our recent study (Ghosh et al., 2011) was to determine the expression profile of selected differentially regulated and expressed CS proteins and their interaction with HCV proteins and to gain a better understanding about the involvement of host CS and its association with viral proteins in HCV infection. Our study showed that filamin A (fila–A),

an actin binding, and vimentin (vim), an IF family member, were prominently altered in HCV-expressing cells compared with those in parental cells (Huh7) and in liver biopsies of patients with CHC vs controls (Figs. 5.6 and 5.7). Fila is a central player in signal transduction and links the actin CS to various transmembrane proteins and is known to interact with at least 30 other proteins, facilitating intracellular communication (Feng and Walsh, 2004). Vim provides cellular integrity under mechanical stress in vivo with a resilience not related to MT or actin filament networks (Goldman et al., 1996).

We have studied the interactions between fila, vim and HCV proteins and the effect of IFN-α and cytochalasin B (CytoB), an actin inhibitor, on those proteins. Our results suggest that inhibiting up-regulation of these CS proteins and/or interrupting their interaction with HCV proteins may facilitate the development of much needed, novel therapeutic approaches designed to inhibit HCV infection. The major findings in this study are as follows: (i) among several differentially expressed proteins identified, the most notable differences were in the expression of fila and vim in HCV-expressing cells compared to the parental hepatoma cell line (Huh7); (ii) fila co-localized with core, NS3 and NS5A proteins, while vim partially co-localized with core protein; (iii) fila directly interacts with core and NS proteins, while no interaction was observed between vim and NS proteins but partial interaction was observed with core protein; (iv) liver biopsies from patients with CHC showed higher expression of fila and vim compared with noninfected controls; (v) IFN-α led to a dose-dependent decrease in the expression of fila and HCV proteins; (vi) inhibition of actin polymerization by CytoB treatment showed restricted perinuclear localization of HCV proteins and reduced expression of fila. We hypothesized that upregulation of CS proteins following HCV infection can contribute to disease through interaction with HCV proteins. Our results showed strong interaction of fila with NS3 and NS5A, but not with vim and decrease in both HCV protein syntheses and the upregulated expression of host CS protein after IFN-α treatment, further suggesting that overexpression of fila and vim is a key adaptive response of human hepatocytes to HCV infection.

Our results indicate a novel property of fila and vim and suggest that HCV might exploit the actin and microfilament network for the transport of the virus by polymerization-related mechanisms. Such interactions could also play a role at later steps of the viral cycle, for example, during viral morphogenesis and secretion. An interaction between

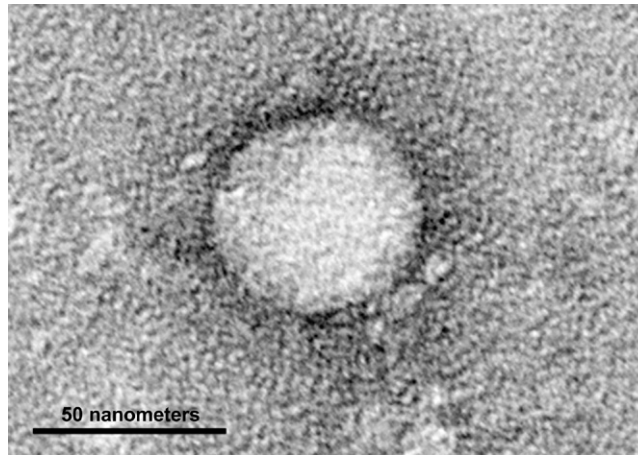

Figure 5.5 Electron micrograph of HCV purified from cell culture. *(Adapted from http://en.wikipedia.org/wiki/File:HCV_EM_picture_2.png).*

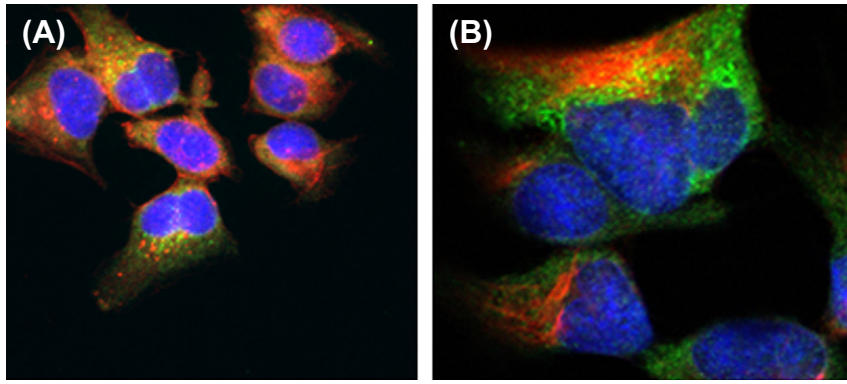

Figure 5.6 ***Expression and co-localization of host CS and HCV proteins in CON1 cells.*** Expression of fila (red, A) and vim (red, B) with HCV core (green in A, B) in CON1 cells. Yellow color represents co-localization of fila/vim with core. (*Adapted from Ghosh et al., 2011 with permission*). (For color version of this figure, the reader is referred to the online version of this book.)

viral proteins of HCV and CS-associated proteins has been reported previously (Bost et al., 2003; Lai et al., 2008). Association of fila and other CS proteins with HIV virions also occurs, suggesting their probable involvement in viral assembly and budding through interaction with viral proteins (Jimenez-Baranda et al., 2007). The precise roles of CS proteins in HCV infection remain to be fully delineated. In our recent study, we are investigating whether increased liver angiogenesis could be a predictor of HCC occurrence in patients with HCV-related

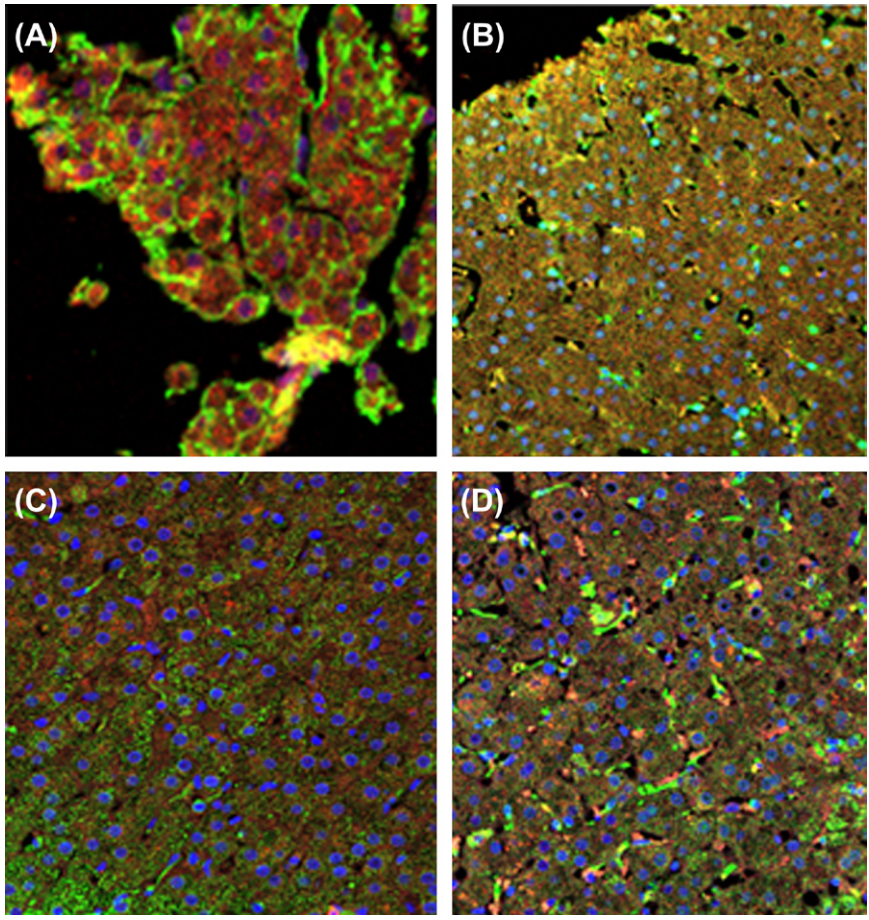

Figure 5.7 ***Association of host CS and HCV proteins in liver biopsies from patients with CHC.*** Expression of host CS proteins (fila and vim in red) with HCV proteins (NS3/NS5A in green) in liver biopsies from patients with CHC. (For color version of this figure, the reader is referred to the online version of this book.)

cirrhosis. A positive association and co-localization was found between higher expression of fila and angiogenic markers with progression of HCV infection to HCC in both human subjects and cell lines. This study suggests that the interaction between fila and angiogenic markers in hepatocytes may contribute to the progression of the HCV to HCC and could be used as a potential target for development of new therapy in the treatment of HCV with HCC. Furthermore, we determined the association between fila and angiogenic markers in HCV-induced cirrhosis and progression to HCC.

In addition, changes in the expression of other CS dynamic regulator proteins in HCV replicon cells were also revealed. Such as Rho GDP-dissociation inhibitor 2, regulator of Rho and involve in reorganization of the actin cytoskeleton (Alan, H 1998; Sasaki and Takai, 1998), was over-expressed in the presence of HCV replication. It has been speculated that the host-cell CS proteins might be the most likely component of HCV RC. Assembling RNA polymerase on cytoskeleton or on cell membranes might ensure appropriate concentrations of replication components, and hence, control rates or efficiencies of replication reactions. Further analyses of functional interactions of fila and vim with structural and NS proteins of HCV will refine roles and significance of these interactions, thereby improving our understanding of mechanisms underlying the progression and pathogenesis of HCV infection. A deeper understanding of the consequences of HCV–CS proteins interactions will facilitate the development of much needed, novel therapeutic approaches targeted to inhibit HCV infection.

4.3. Role in Other Human Liver Diseases

4.3.1. Role in Development of Liver Diseases

As already described, MT, actin microfilaments and IFs are the three main family members of CS proteins comprising the cytoskeleton of eukaryotic cells. Cytoskeleton plays a role in diverse cellular functions including maintenance of cell shape, cell movement, cell division and intracellular organization and trafficking. Therefore, disruption of CS proteins affects cell function and subsequently, the development and progression of several liver diseases including fibrosis, cirrhosis, alcoholic and nonalcoholic liver disease and HCC (Omary et al., 2009; Shepard and Truma et al., 2010). Delineating the regulation of CS protein expression and determining expression profile/pattern is critical for diagnosis and therapeutic intervention for a variety of hepatic diseases.

MTs are composed of α- and β-tubulin heterodimers, which form protofilaments which in turn form hollow tubes. Specifically, in hepatocytes, asymmetric organization of MTs determines surface polarity. Alcohol consumption has been shown to disrupt liver MTs in human alcoholic liver disease showing decreased polymerization and increased free tubulin (Shepard and Truma, 2010). Additionally, colchicine, an alkaloid derived from the plant *Colchicum autumnale*, has been shown to improve liver cirrhosis in experimental animal models mediated by disruption of MTs, interference with transcellular movement of collagen and increased collagenase

production (Tanner et al., 1981; Wardas et al., 1992; Lee et al., 2004). Furthermore, colchicine has been shown to stabilize the plasma membrane and prevent acetaminophen-induced liver injury (Muriel et al., 1993). Colchicine has been used for treatment of primary biliary cirrhosis and other liver diseases with inconsistent results (Gong and Gluud, 2005).

Actin exists as a monomer (G-protein) or a filamentous polymer (F-actin). These dynamic filaments are major components of stress fibers and are located in the leading edge of lamellipodia of migrating cells. Acetaldehyde, a toxic metabolic by-product of ethanol metabolism, forms stable adducts with many proteins including G-actin. However, acetaldehyde does not interfere with actin polymerization (Shepard and Truma, 2010). Although, ethanol does impair actin-related processes including hepatocyte attachment to extracellular matrix (ECM) and cell spreading which requires CS rearrangement. Studies by Schaffert et al. (2006) demonstrated that decreased hepatocyte attachment and spreading is due to diminished Rac and Cdc42 GTPase activity in ethanol-fed animals, while the GTP-bound form of RhoA was not significantly altered. Similarly, in HCV-infected hepatocytes, blocking Rho GTPase family members Rac, Rho and Cdc42 reduces HCV infectivity (Brazzoli et al., 2008). CD81 was found to be a central regulator of these GTPases. Actin was also shown to be cysteine-oxidized by ethanol possibly leading to altered actin dynamics and thereby impairing hepatocyte function. In addition to oxidize protein modifications, α-actin has been identified as being hyperacetylated by ethanol affecting actin-dependent cellular processes.

IFs are the largest cytoskeleton family members composed of approximately 70 different genes, which are regulated in a tissue-specific manner. Mutations in these genes have been shown to be the direct or indirect cause of more than 80 human diseases (Omary et al., 2009). Of the six major IF subgroups, keratins (Type I and II) represent the largest. Mutations in genes encoding keratins, K8, K18 and K19, predispose individuals to liver disease (Omary et al., 2009). K8 and K18 are predominantly expressed in hepatocytes, while desmin and vimentin, examples of subgroup type III, are generally expressed in nonparenchymal liver cell populations, Kupffer, Stellate, and endothelial cells. Cholangiocytes express K7, K8, K18 and K19 (Strnad et al., 2008; Omary et al., 2002). During embryogenesis, hepatocytes also express varying amounts of K19. Keratin expression profiles and distribution depend on cell differentiation and cell type. In addition to changes in keratin expression profiles in response to liver injury, expression levels and posttranslational modifications such as phosphorylation (Ku et al., 2007)

and sumoylation (Snider et al., 2011) are observed. Site-specific phosphorylation of keratin confers hepatoprotection, while hypersumoylation leads to insolubility of keratins limiting their cytoprotective effects. Therefore, expression levels and posttranslational modifications affect keratin function, and ultimately, predispose individuals to a variety of liver diseases.

K8 and K18 knockout mice exhibit mild chronic hepatitis and hepatocytes were sensitized to different stress conditions. Similarly, K18 R89C variant mice were predisposed to thioacetamide-induced liver fibrosis (Strnad et al., 2008). Mutating keratin phosphorylation sites also led to increased sensitivity to liver injury, albeit a milder phenotype was observed. K19 expression is seen in human cholestatic liver disorders as well as alcohol-induced liver damage (Ku et al., 2007; Van Eyken, 1998). HCC generally express K8 and K18 and are commonly used as tumor markers (Omary et al., 2009; Moll et al., 2008). Furthermore, characterization of keratin expression profiles is used as diagnostic measurement for primary tumor origin for metastasis. Additionally, keratin expression patterns can be used to predict tumor prognosis (Omary et al., 2009).

Nonalcoholic fatty liver disease includes two histological types of liver damage: nonalcoholic fatty liver (NAFL; fatty liver without significant inflammation) and nonalcoholic steatohepatitis (NASH; fatty liver and inflammation) (Jou et al., 2008; Syn et al., 2009). Obesity leads to the development of fatty liver, which predisposes individuals to more severe liver injury (fibrosis, cirrhosis and HCC), and studies have shown that mice fed a high-fat diet for 24 weeks had increased expression of vimentin, K8 and K18 (Park et al., 2011). These studies suggest that IF proteins may correlate with progression of hepatic steatosis and inflammation and could prove to be a potential diagnostic biomarker for liver disease in obese humans. Since NAFL and NASH have different prognosis, accurate diagnosis is required for proper treatment.

4.3.2. Hepatic Stellate Cells and Key CS Changes Promoting Fibrosis

Hepatic Stellate cells (HSCs) are the main effector cells of liver fibrosis. Following a fibrogenic stimulus, these cells transdifferentiate from a quiescent to a myofibroblast-like phenotype leading to increased proliferation, migration and contractility (Friedman, 2008). Additionally, activated HSCs express and secrete excessive amounts of ECM proteins, particularly type I collagen resulting in hepatic fibrosis/cirrhosis. HSCs express a number of CS proteins dependent on cell differentiation (Jiroutova et al., 2005). In the quiescent state, HSCs express glial fibrillar acidic protein, while activated

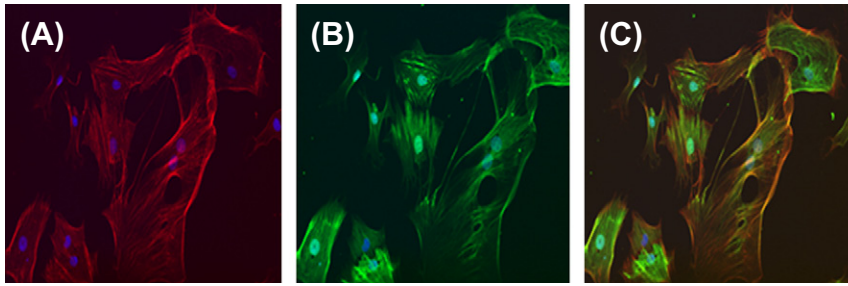

Figure 5.8 ***CS actin (A) co-localizes with NMM II-B (B).*** CS actin (red) and NMM II-B (green) clearly co-localize (C) in culture-activated primary rat HSCs. Nuclei are stained with DAPI (blue) (10×). (For color version of this figure, the reader is referred to the online version of this book.)

HSCs express smooth muscle α-actin. Vimentin is detected in HSCs from normal and cirrhotic livers, and although desmin is expressed in quiescent cells, desmin expression increases throughout the transdifferentiation process. Therefore, HSCs have a distinct pattern of IF expression depending on quiescent or activated phenotype contributing to its function. Mutations in IFs K8 and K18 associate with progression of HCV-induced liver fibrosis (Strnad et al., 2006). Quiescent HSCs can respond to physiologic stimuli to alter IF expression needed for proper CS structure, metabolic function and tissue environment interaction to promote wound healing (Guma et al., 2001).

HSC contraction and migration are necessary for the wound-healing process and influence both development and severity of hepatic fibrosis. Myosin proteins act as molecular motors and contribute to cellular contraction, cytokinesis and migration. Myosins bind actin filaments and generate force, using energy from ATP hydrolysis. Specifically, class II myosins are associated with generation of contractile forces (Bresnick, 1999). HSCs display a distinct expression profile of nonmuscle myosin II (NMM II-A, II-B and II-C) isoforms. Upregulation of NMM II-A and II-B expression was associated with F-actin stress fibers in the cellular periphery and throughout the cytoplasm of rat HSCs, respectively (Fig. 5.8). These results suggest that NMM II-A activation facilitates rearrangement of actin bundles into cellular protrusions and NMM II-B incorporates into cytoplasmic stress fibers. Increased expression of NMM II-A and II-B regulates HSC migration, while other myosin II classes likely modulate contraction contributing to development and severity of liver fibrosis (Moore et al., 2011).

One of the earliest events in liver fibrosis is the transdifferentiation/activation of HSCs, and during this process, the HSC undergoes morphological changes (i.e. Stellate cell to a stretched polygon morphology), becomes hypercontractile and increases expression of fibrillar collagens. In general, myofibroblasts have a distinct morphology with the presence of large bundles of microfilaments running along the cell axis (Sandbo and Dulin, 2011). Change in HSC morphology is also accompanied by altered gene expression affecting myofibroblast function to promote the fibrogenic response (Sandbo and Dulin, 2011). Smooth muscle α-actin, a CS stress-fiber component, is upregulated in activated HSCs and is routinely used as a marker of the transdifferentiated phenotype. Changes in actin cytoskeleton modulate several key functions of the myofibroblast namely focal adhesion assembly, ECM reorganization, cell contraction and migration, localization of translational machinery (such as de novo gene expression needed in the formation of lamellipodia in migratory cells) and transcriptional regulation (such as genes encoding CS and contractile components) (Sandbo and Dulin, 2011).

In addition to actin and stress fiber formation, MTs play an important role in cell migration by repeated polymerization and depolymerization events. Delineating the mechanisms regulating MT rearrangement and CS reorganization during HSC activation may uncover new therapeutic targets for the liver fibrosis. MT stability is decreased by destabilizing proteins. superior cervical ganglia 10 (SCG10) sequesters free tubulin leading to MT destabilization and subsequent changes in cell shape and motility (Charbaut et al., 2001; Gavet et al., 1998; Poulain et al., 2007). HSCs express SCG10 and induction is observed during the activation process (Paradis et al., 2010). SCG10 colocalizes with tubulin at the distal end of MT in culture-activated HSCs promoting cell growth and motility. Inhibition of SCG10 by siRNA resulted in decreased HSC proliferation and migration. Additionally, SCG10 expression increases and correlates with fibrotic stages in CHC infected livers. Therefore, these data suggest that SCG10 plays a role in cellular mechanisms associated with HSC activation through MT interference suggesting SCG10 as a potential therapeutic target.

4.3.3. Effects on Endothelial and Kupffer Cells in Liver Injury

Sinusoidal endothelial cells (SECs) play an important role in hepatic microcirculation. SECs separate the sinusoidal blood from hepatocytes; however, the SECs are unique in that they possess fenestrae, open pores, which allows for exchange of fluids, solutes and metabolites between hepatocytes and sinusoidal blood (Yokomori, 2008). Disruption of endothelial fenestrae

impairs substrate exchange and is a major contributor to hepatocyte dysfunction and development of liver cirrhosis (Babbs et al., 1990). Diameter and number of fenestrae is dynamic and can be affected by a variety of compounds (Braet et al., 2004). Studies have demonstrated that actin, myosin and calmodulin modulate contraction and dilation of endothelial fenestrae (Oda et al., 1983; Oda et al. 1986; Van Der Smissen et al., 1986). Manipulating the CS by using actin-disrupting latrunculin A increased the number of fenestrae (Braet et al., 2003). Similarly, as Rho initiates the assembly of cytoplasmic stress fibers, comprised of actin and focal adhesions, treatment of SECs with lysophosphatidic acid, a Rho promoter, contracts fenestrae. In contrast, treatment with an inhibitor of Rho leads to dilation and fusing of fenestrae. Overall, these results imply that Rho changes in fenestrae are mediated through regulation of actin cytoskeleton (Yokomori et al., 2004). SECs that have lost fenestration after culturing express CD31, which becomes detectable in cirrhosis, and CD31 expression inversely correlates with fenestration (DeLeve et al., 2004).

Kupffer cells (KCs) are resident macrophages of the liver and are necessary for the clearance of substances from systemic circulation including bacteria, endotoxins, viruses, cellular debris and other specific macromolecules mainly through phagocytosis (Kolios et al., 2006). The CS system is essential for normal phagocytic activity of KCs (Watanabe et al., 1990). The cytoskeleton plays an important role in cell membrane ruffling and formation of pseudopodia, which are both critical for phagocytic activity. Actin–myosin interaction through calcium–calmodulin systems plays a major role in phagocytic activity (Bretscher, 1991). The active calcium–calmodulin complex activates myosin light chain kinase leading to phosphorylation of myosin. Once myosin is phosphorylated, it can interact with actin inducing cell membrane activity and pseudopodia and subsequently phagocytosis. Additionally, vimentin is also an important CS component for phagocytosis. Studies have shown that age-related damage to the cytoskeleton in KCs decreases phagocytic activity leading to increased severity to endotoxemia (Knook et al., 1989) and other noxious challenges (Videla et al., 2001).

4.3.4. Autophagy and Liver Disease

Autophagy plays a role in numerous cellular processes including homeostasis, development and differentiation, and innate and adaptive immunity (Monastyrska et al., 2009). The autophagic process involves specific membrane rearrangements culminating in the development of cytosolic

double-membrane autophagosomes, which sequester cytoplasmic material marked for degradation. Since cellular cytoskeleton is critical during membrane rearrangements and vesicle-mediated processes, actin microfilaments and MTs have been shown to be important in autophagy (Monastyrska et al., 2009). Recently, autophagy has been shown to play a protective role against the development of liver disease by removing misfolded proteins accumulated in the ER. However, some studies have also demonstrated dysregulated autophagy (i.e. increased or decreased activity) as a critical event in the development and progression of numerous liver diseases (i.e. HCV/HBV infection, alcohol-induced liver injury and HCC) (Rautou et al., 2010). Several recently published reports suggest a role for autophagic proteins in HCV replication and/or secretion (Ait-Goughoulte et al., 2008; Dreux and Chisari, 2009; Dreux et al., 2009; Tanida et al., 2009). However, a recent report by Guevin et al. (2010) provided for the first time a link between an HCV protein, NS5B, and the autophagy machinery using Huh7 cells harboring HCV replicon and with HCVcc. They showed that ATG5 is associated with the membrane and colocalizes with the membranous web constituent, NS4B. Interestingly, strong co-localization between the two proteins was only seen early in infection and was completely absent late in infection. This result may imply that the ATG5–NS5B interaction is required for the onset of the viral replication. Furthermore, autophagic flux is increased during HSC activation, and therefore, may prove to be a new therapeutic strategy for liver fibrosis.

4.4. Roles in Progression of HCV to HCC

HCC is one of the most common fatal cancers worldwide. HBV and HCV infections, exposure to aflatoxin, and excessive intake of alcohol have been identified as major risk factors. However, the molecular mechanisms underlying the development of HCC are still poorly understood. Recently, β-catenin, one of the key components of the Wnt signaling pathway, has been found to be mutated in about 20% of HCCs, suggesting a role of the Wnt pathway in their development. β-catenin is part of a complex of proteins that constitute adherens junctions (AJs). AJs are necessary for the creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells. β-catenin also anchors the actin cytoskeleton and has been reported to be responsible for transmitting the contact inhibition signal that causes cells to stop dividing once the epithelial sheet is complete (Entrez gene: Catenin). Recent reports suggest involvement of β-catenin in various aspects of liver biology including liver development, regeneration

including partial hepatectomy, HGF-induced hepatomegaly, liver zonation, and HCC (Thompson et al., 2007).

Huang et al. (1999) showed that β-catenin and adenomatous polyposis coli (APC) mutations in 22 HCCs associated with HCV infection, using single-strand conformation polymorphism (SSCP) followed by direct DNA sequencing. β-catenin mutations were found in nine (41%) cases, but no APC mutations were found. β-catenin immunohistochemistry revealed nuclear accumulation of β-catenin protein in all nine tumors with a β-catenin mutation and two additional tumors without a mutation. These results suggest that activation of the Wnt signaling pathway by β-catenin mutation contributes significantly to the HCC associated with HCV infection. Recent evidence shows that focal adhesion kinase (FAK) is upregulated in HCV core expressing cells, and mediates, via NFkB (Balsano et al., 2007). Both cell migration and protein expression of several adhesion and CS proteins, such as paxillin and beta1 integrin, are influenced by HCV core protein. Proteomic analysis by Kanamori et al. (2011) led to identification of talin-1, ubiquitous cytosolic protein and promising prognostic marker for HCC. Talin is concentrated at regions of cell–substratum contact in focal adhesions and, in lymphocytes, at cell–cell contacts (Burridge et al., 1983; Burn et al., 1988). It is capable of linking integrins to the actin cytoskeleton either directly or indirectly by interacting with vinculin and alpha-actinin (Alan, 1998). Talin-1 upregulation is associated with HCC progression and may serve as a prognostic marker. Kanamori et al. (2011) and Pan et al. (2012) have proposed an integrated approach to represents a modality to explore novel relationships in a proteome complex and highlights the functional roles of Vim in HCC metastasis. Further detailed analyses will assess the expression profile and function of all the adhesion molecules in HCV-related HCC and are needed to clarify the role of HCV in HCC progression and development of intrahepatic metastases.

4.5. CS Proteins as Potential Therapeutic Target

The current standard of care is based on the addition of protease inhibitors (PIs) to a regimen of PEG–IFN-α and RBV. While addition of protease inhibitors is a useful advance, limitations persist, as for dual therapy. Indeed, similar adverse events are not only seen but also occur more frequently in patients treated with triple therapy than in those treated with dual therapy alone (Ghany et al., 2011). Most of the therapies have common adverse

effects. Thus, the inability of PIs to be used as monotherapy, the lack of alternatives for those who may have poorly tolerated dual therapy, and increased risk of adverse events make it clear that other avenues, expanding on previous regimens, are the future of anti-HCV research. Recapitulating the success of PIs with current knowledge of HCV life cycle, several other drugs could be conceivably used against HCV.

HCV has been shown to require intact dynamic MTs for successful entry and post-fusion steps in its host cell (Roohvand et al., 2009). Furthermore, both MTs and actin are essential in HCV RNA synthesis (Lan et al., 2003; Tsao et al., 2006; Ghosh et al., 2011), such that MT disruption decreases HCV viral titers (Boulant et al., 2008). Although the exact mechanism is still being elucidated, NS3 and NS5A have been shown to be essential elements of the viral RC that interacts with both tubulin and actin (Lai et al., 2008). Other interactions include cytokeratin 8 and Vim with the core protein (Kang et al., 2005); vesicle-associated membrane protein-associated protein (VAP-A &B) binding to NS5A and NS5B (Lai et al., 2008); NS5A interaction with β-catenin (an essential protein that binds type1 cadherins and links them to actin) (Milward et al., 2010); and the possible regulatory role of HCV p16 (F-protein) on the MTs itself (Tsao et al., 2006). Disrupting the interaction of various viral components with the CS, therefore, is a promising target of future pharmacotherapy.

The development of the HCVcc system is a major accomplishment, permitting important insights into virus–host cell interaction. However, this system also has some limitations, including genetic defects of the cell lines used, which do not permit normal production of lipoproteins compared with primary human hepatocytes. Finally, molecules targeting the MT network may prevent initiation of productive HCV infection and drugs down-regulating hepatic VLDL production limit virus propagation by affecting morphogenesis and release of progeny virus from infected cells. In light of the currently limited therapeutic options, the need for more efficacious therapies is obvious.

5. SUMMARY AND KEY QUESTION FOR FUTURE RESEARCH

Future work will be necessary to confirm that both MTs and actin filaments provide intracellular tracks for the transport of HCV RCs to

reach the lipid droplets, where virus assembly occurs. Due to many factors that affect SVR and inherent limitations of current therapy, development of individualized treatment regimens is likely to play a more important role in the future. Although a dedicated folding system for actin and tubulin was discovered nearly two decades ago, our understanding about complex role of CS quality control pathway continues to expand. Many questions in this field remain incompletely understood, particularly about degradation of CS proteins. Elucidating the details of these pathways will continue to shed light on importance of CS proteins in biology. It has also become clear that understanding the quality control of CS proteins will enhance our knowledge of debilitating human diseases, including HCV, cancer, developmental diseases, and neurodegenerative diseases. Although many studies have examined the clinical course of HCV infection, long-term cohort studies are still needed to characterize the risk factor profiles that may predict the eventual outcome in chronic HCV infection, and to further define the outcome of patients with greater than 30 years of HCV infection. Since the response rates to the current HCV therapies are still variable, there is a necessity for innovative new therapies and vaccines. Importantly, the present HCC screening and surveillance methods need to be evaluated to determine if they are effective in reducing morbidity and mortality in patients with cirrhosis.

In **Summary**, the chronic nature of HCV infection influences the clinical approach and management of this disease. Prevention of HCV infection is possible by understanding various mechanisms of viral transmission. Acute HCV infection usually avoids diagnosis because 70–80% of infected individuals are asymptomatic. Most infected persons are unaware of their exposure to HCV, and are not diagnosed until many years later. The rate of chronic HCV infection is affected by the person's age at time of infection, gender, race, and viral immune response. A large proportion of HCV-infected persons, ranging from 70 to 85%, develop chronic HCV infection, and are at risk for advanced liver fibrosis, HCV-related extrahepatic complications, cirrhosis and HCC (Fig. 5.1). An estimated 20–30% of chronic HCV infections advance to end-stage liver disease over three or four decades. Extrahepatic manifestations can occur during chronic HCV infection or cirrhosis. Research is ongoing to determine the histological, biochemical, genetic and demographic markers that may further predict the outcome of HCV infections. Our recent study systematically demonstrated in vitro and in vivo changes that occur in the expression profile of fila and vim in hepatocytes, in response to HCV protein expression associated with

infection by HCV (Ghosh et al, 2011). This study showed association and interaction of fila with viral proteins in HCV infection. It also characterized the effect of IFN-α and CytoB in regulating expression of fila associated with HCV infection, which may contribute to identification of possible therapeutic targets. Like other members of Flaviviridae family, HCV viral NS proteins, as well as host cell factors, are believed to function in the initiation and regulation of HCV viral RNA replication, by formation of an RC. Jimenez-Baranda et al. (2007) described an association of fila and other CS proteins with HIV virions, suggesting their probable involvement in viral assembly and budding through interaction with viral proteins (Jimenez-Baranda et al., 2007). Interactions among viral proteins of other viruses and CS-associated proteins have been reported previously (Bost et al., 2003; Lai et al., 2008). Our results indicate a novel and potentially important property of fila and vim and suggest that HCV might exploit the actin and microfilament network for the transport of the virus by polymerization-related mechanisms.

Altogether, the results from our and other studies reviewed in this report demonstrated a CS requirement for HCV entry, RNA synthesis and viral transport, although the definitive roles of CS proteins in HCV infection remain to be fully delineated. Among several potential mechanisms, we prefer a model that involves actin and MT-mediated formation and/or maintenance of the vesicles, which serve as the membrane-anchoring sites for the HCV RC. This model is especially attractive in light of the discovery of the proximity of ER membrane-associated HCV NS proteins with paracrystalline structures reminiscent of the MT aggregates in HCV-infected chimpanzee hepatocytes or near sites of viral RNA synthesis in Kunjin virus-infected cells (Mottola et al., 2002; Schaff et al., 1990, 1992; Westaway et al., 1997). These CS networks may be used by unenveloped HCV capsid particles for the transportation to perinuclear ER regions in the cytoplasm. There is an essential need for further research to develop a clear understanding about the role of host CS elements and their interactions with HCV and the risk factors that lead to progression of fibrosis, cirrhosis, and HCC. Further analyses of functional interactions of host CS with structural and NS proteins of HCV will refine roles and significance of these interactions, thereby improving our understanding of mechanisms underlying the progression and pathogenesis of HCV infection. Identification of additional viral, host, and external factors will undoubtedly aid in developing means of prevention, early detection, and treatment.

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CHAPTER SIX

Integrins and Small GTPases as Modulators of Phagocytosis

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Contents

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Abstract

Phagocytosis is the mechanism whereby cells engulf large particles. This process has long been recognized as a critical component of the innate immune response, which constitutes the organism's defense against microorganisms. In addition, phagocytic internalization of apoptotic cells or cell fragments plays important roles in tissue homeostasis and remodeling. Phagocytosis requires target interactions with receptors on the plasma membrane of the phagocytic cell. Integrins have been identified as important mediators of particle clearance, in addition to their well-established roles in cell adhesion, migration and mechanotransduction. Indeed, these ubiquitously expressed proteins impart phagocytic capacity to epithelial, endothelial and mesenchymal cell types. The importance of integrins in particle internalization is emphasized by the ability of microbial and viral pathogens to exploit their signaling pathways to invade host cells, and by the wide variety of disorders that arise from abnormalities in integrin-dependent phagocytic uptake.

ABBREVIATIONS

CFTR Cystic fibrosis transmembrane conductance regulator
EHV1 Equine herpes virus type 1
FAK Focal adhesion kinase
FAT Focal adhesion targeting
FERM Band 4.1, ezrin, radixin, moesin
FnBP Fibronectin-binding protein
HCMV Human cytomegalovirus
HSV-1 Herpes simplex virus type 1
ICAM3 Intracellular adhesion molecule 3
ILK Integrin-linked kinase
KGF Keratinocyte growth factor
KSHV Kaposi sarcoma-associated herpes virus
LPS Lipopolysaccharide
MFG-E8 Milk fat globule-EGF factor 8
ORP1L Oxysterol-binding protein-related protein
PAR-2 Protease-activated receptor-2
PI3K Phosphatidylinositol-3-kinase
RILP Rab-interacting lysosomal protein
ROCK Rho-associated coiled coil-containing kinase
RPE Retinal pigment epithelium
UV Ultraviolet

1. INTRODUCTION

1.1. Phagocytosis: Definition and Stages

The cellular uptake of particles with ≥0.5-μm diameter is termed *phagocytosis* (Flannagan et al., 2012; Vicente-Manzanares et al., 2009). Although

a large body of research has focused on how immune cells phagocytose pathogens, many other cell types are capable of phagocytic particle engulfment. They include endothelial, epithelial, neuronal and mesenchymal cells. These cell types are termed "nonprofessional" phagocytes to denote their somewhat lesser ability to take up particles, relative to "professional" phagocytic immune cells, such as macrophages.

The process of phagocytosis is an essential component of the innate and the adaptive immune responses. On the other hand, phagocytosis by nonprofessional phagocytes plays major roles in tissue maintenance, regeneration and remodeling. Irrespective of the cell type and nature of the particle involved, phagocytosis occurs through a series of discrete, sequential steps that include particle recognition and binding to the cell surface, engulfment, and disposition (Flannagan et al., 2012).

1.2. Receptors Involved in Particle Recognition and Binding

Particle recognition and binding occur when receptors on the plasma membrane of the phagocytic cell recognize specific ligands or components on the surface of the particle to be engulfed. In most cases, particle recognition is followed by receptor clustering, forming an arrangement reminiscent of a zipper. This causes the cell membrane to extend and wrap itself around the particle, forming a structure termed "phagocytic cup," which seals and leads to internalization (Caron and Hall, 1998; Griffin et al., 1975; Swanson, 2008). If the particle is a foreign body, such as a pathogen, recognition can occur through the interaction between particle-specific proteins and receptors on the plasma membrane of the phagocyte. For example, lipopolysaccharide (LPS) found on the surface of bacteria is bound by several proteins expressed in immune cells, including the CD14 pattern recognition receptor, as well as the CD204 scavenger receptor (Peiser et al., 2000; Zanoni et al., 2011). Similarly, the pattern recognition receptor dectin-1 binds a variety of lectins and plays key roles in the phagocytosis of fungal pathogens in pulmonary and other tissues (Drummond and Brown, 2011).

Particles can also become coated by factors found in the circulation or in the extracellular matrix, such as immunoglobulin G or fibronectin, respectively. Particles opsonized in this manner can then bind to a variety of cellular receptors, including Fcγ proteins in immune cells, and integrins in both professional and nonprofessional phagocytes (Blystone et al., 1994; Bunting et al., 2002; Swanson, 2008).

During tissue remodeling and maintenance, different types of particles are engulfed, including apoptotic corpses and cell fragments, such as photoreceptor outer segments. A key recognition signal that triggers binding

of these particles to both professional and nonprofessional phagocytes is phosphatidylserine, which is bound by several surface receptors, including TIM and BAI1 (Kobayashi et al., 2007; Park and Ravichandran, 2010). Phosphatidylserine can also be bound by secreted proteins, such as milk fat globule-EGF factor 8 (MFG-E8), which in turn binds to αvβ5 integrins on the surface of retinal pigmented epithelial (RPE) cells, triggering phagocytosis (Law and Nandrot, 2012).

Once a particle has been recognized and bound by phagocytic cell surface receptors, the latter cluster at the attachment site, stimulating activation of a wide array of phagocytic signaling cascades, the exact nature of which depends on the characteristics of the particle and the specific receptors involved. Irrespective of the nature of these signals, they all culminate in local actin polymerization and membrane remodeling, which precede particle ingestion. Notably, many aspects of phagocytosis share similarities with cell migration on two-dimensional substrates, and often involve activation of integrins, especially in nonprofessional phagocytes.

1.3. Phagosome Closure and Particle Engulfment

Internalization occurs after the tips of pseudopods surrounding the particle meet and fuse, through poorly understood mechanisms that are thought to involve contractile events modulated by nonmuscle myosins (Botelho and Grinstein, 2011). In preparation of phagosome separation from the plasma membrane, actin polymerization halts. In the case of particle internalization triggered by αMβ2 integrin stimulation, production of phosphatidylinositol-3,4,5-*tris*phosphate at the phagocytic cup is required, which may form through stimulation of phosphatidylinositol-4-phosphate 5-kinase α. In this manner, a sequence of F-actin assembly and disassembly, necessary for particle binding and engulfment, occurs. Shortly after sealing of the phagocytic vesicle and detachment from the plasma membrane have occurred, localized actin polymerization is triggered once again. This newly formed actin filaments participate in mediating the movement of phagosomes to the cell interior (Bohdanowicz et al., 2010; Dewitt et al., 2006). Once this process has taken place, a series of phagosomal maturation events ensue.

1.4. Phagosome Maturation and Particle Disposition

Following internalization, phagosomes containing particles undergo a series of transition steps collectively termed "maturation." Maturation is accompanied by gradual decreases in intravesicular pH, and culminates in phagosome fusion with acidic lysosomes and degradation of cargo (Kinchen and

Ravichandran, 2008). An exception to this pathway appears to be the engulfment of melanosome-containing vesicles in epidermal keratinocytes. In these cells, the plasma membrane surrounding the phagocytosed melanosomes is removed in the cytoplasm, and these pigment-containing vesicles are then targeted to perinuclear regions, rather than to lysosomes, where they shield nuclear DNA from UV radiation damage (Ebanks et al., 2011).

Early during phagosome maturation, the small GTPase Rab5 is recruited to the phagosome in a dynamin-dependent fashion (Kinchen et al., 2008). At this stage, Rab5 is activated and is able to recruit a variety of other proteins, which mediate the subsequent association of Rab7 with the maturing phagosome (Botelho and Grinstein, 2011; Kinchen and Ravichandran, 2008). Recruitment of Rab7 appears to occur coordinately with dissociation of Rab5, and is essential to prepare the phagosome for lysosomal fusion. Although the precise mechanisms are yet to be elucidated, active Rab7 may mediate recruitment of Rab-interacting lysosomal protein (RILP) and oxysterol-binding protein-related protein (ORP1L). These two factors, in turn, serve as adapters to bind dynein and regulate microtubule minus end-directed transport of the phagosome to the microtubule-organizing center, where lysosomes are found (Wang et al., 2011). The fusion of phagosomes to lysosomes containing multiple degradative enzymes is associated with pronounced intravesicular acidification, resulting in fragmentation and digestion of the phagosomal contents (Botelho and Grinstein, 2011; Wang et al., 2011).

2. INTEGRINS AS PHAGOCYTIC RECEPTORS

Integrins are a multigene family of transmembrane proteins that mediate cell adhesion and signaling. Their biological importance is emphasized by their presence in all multicellular animals, and by the key roles they play in multiple cellular functions. Integrins lack catalytic activity, but are able to transmit signals to and from the extracellular milieu through their interactions with myriad cytoplasmic proteins. In this manner, they are key participants not only in phagocytosis but also in cell survival, adhesion, motility, and mechanotransduction. Interference with integrin binding or activation of cytoplasmic effectors inhibits particle engulfment (Albert et al., 2000).

2.1. Structure and Functions of Integrins

Integrins exist as heterodimeric complexes composed of one α and one β subunit. Although there are 18 α- and eight β-subunits, only 24 heterodimeric combinations have been identified to-date (Hynes, 2002).

A major function of integrins is to bind extracellular matrix substrates, as well as transmembrane proteins of the Ig superfamily expressed on adjacent cells. On this basis, integrins can be broadly divided into three subfamilies, depending on whether they bind to collagen, fibronectin/vitronectin or laminin. In spite of the ability of several integrin heterodimers to bind similar extracellular substrates, they exhibit unique functions, as evidenced by the wide variety of phenotypical alterations in mice in which integrin subunit-encoding genes have been inactivated. Generally, integrins are associated with cell adhesion and migration during embryonic development, tissue maintenance or regeneration, host immune functions and hemostasis (Harburger and Calderwood, 2009). However, αvβ5, αMβ2 and β1 integrins have also been implicated in phagocytosis (Bhalla et al., 2009; Deuretzbacher et al., 2009; Law and Nandrot, 2012; Ross et al., 1992).

Integrins can be found in an inactive, low-affinity state. Binding to ligands or mechanical cues can trigger a series of events, which include conformational changes in the integrin cytoplasmic domain (Humphries, 2002), clustering and interactions with a vast number of cytoplasmic proteins with catalytic and/or scaffold properties. The association of integrins with these proteins results in activation of downstream signaling cascades (Zaidel-Bar et al., 2007). In this manner, integrins orchestrate cellular responses to environmental signals received through chemical and/or mechanical stimuli.

2.2. Integrin-Modulated Signaling

Integrins can be activated bidirectionally, through processes initiated by extracellular factors (termed "outside-in" signaling) or cytoplasmic proteins ("inside-out" activation). The former frequently involves subsequent formation of integrin complexes with various cellular proteins, which link and allow integrins to modulate the cytoskeleton, cellular spreading and migration, as well as survival and proliferation (Margadant et al., 2011). Outside-in signaling results in the formation of cytoplasmic multiprotein complexes that vary in their molecular composition, depending on the cell type and/or stimulus. Proteins such as focal adhesion kinase (FAK) or Src family kinases become activated as a result of outside-in signaling, and trigger a variety of cellular responses that can lead to rearrangements in the actin cytoskeleton. Other important proteins involved in integrin signaling include Rho GTP-binding proteins, paxillin, integrin-linked kinase (ILK), and vinculin (Harburger and Calderwood, 2009). In contrast to outside-in processes, inside-out activation occurs when intracellular stimulators, such as talin or kindlin, bind to the integrin β subunit tail, changing the conformation and

ability of the integrin heterodimers to bind extracellular substrates, and thus regulating adhesion strength (Shattil et al., 2010).

2.3. Phagocytic Integrins and Proteins Associated with Integrin-Mediated Phagocytosis

Although phagocytic particle uptake results in the same outcome, irrespective of the recognition receptor involved, it has recently been recognized that the mechanisms that mediate engulfment can vary greatly, depending on the recognition receptor, as well as the cell type involved. In mammals, phagocytosis during physiological or homeostatic processes is mediated by αMβ2, αVβ3, αVβ5 and/or α6β1 integrins in immune cells (Beller et al., 1982; Hanayama et al., 2002; Savill et al., 1990). In nonprofessional phagocytes, αVβ5 and α2β1 integrins have been associated with particle engulfment (Arora et al., 2000; Nandrot et al., 2007). To-date, all phagocytic processes have been linked to several common signaling pathways, including activation of Rho GTPases and actin polymerization. An important role for integrins in phagocytosis is also underlined by the ability of phagocytic cells to adjust membrane protrusions according to the size, shape and rigidity of the particle to be engulfed. These responses involve mechanosensing by the phagocyte, which is mediated through integrins, and requires Rac1 and downstream target activation. Integrin-mediated phagocytosis occurs with the contribution of several proteins, some of which can directly associate with these transmembrane receptors. Some of the proteins that function downstream from integrin activation during phagocytosis include FAK, ILK, galectin-3, nonmuscle myosin II, and MFG-E8 (Fig. 6.1).

2.3.1. Focal Adhesion Kinase

FAK is a highly conserved 125-kDa protein that shares >95% homology across species (Arold, 2011). Three principal domains have been defined in this protein: an N-terminal band 4.1, ezrin, radixin, moesin (FERM) domain, a central tyrosine kinase domain, and a C-terminus that contains sequences that target its localization to focal adhesions (FAT domain). The FERM and FAT domains mediate multiple protein–protein interactions important for FAK function (Zhao and Guan, 2009). FAK is an unusual kinase, in that it acts mostly as a scaffold protein. Activation occurs as a result of integrin ligation, which causes FAK autophosphorylation at Y397. Autophosphorylation results in a conformational change, which in turn allows Src or Fyn kinase to bind FAK. This sequence of events leads to mutual phosphorylation and activation of FAK and Src or Fyn. The active FAK/Src

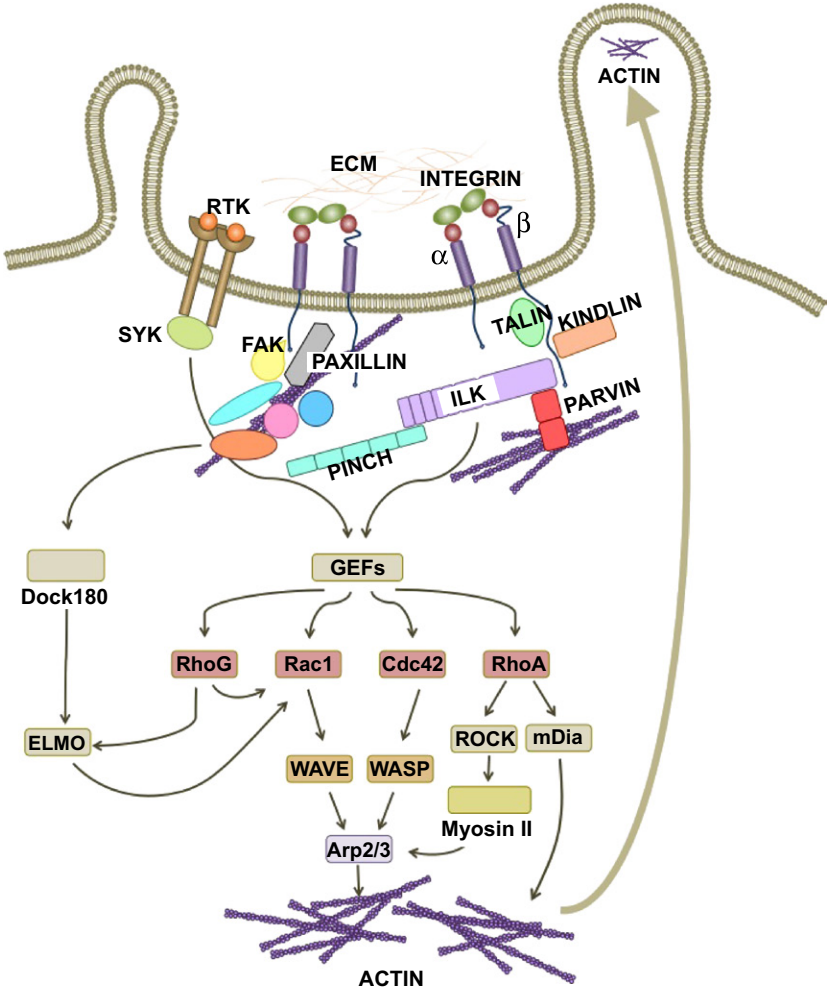

Figure 6.1 ***Signaling pathways through integrins involved in actin polymerization and phagocytosis.*** The interaction between integrins and their extracellular matrix (ECM) ligands induces "outside-in" signaling. During this process, recruitment of signaling factors, such as focal adhesion kinase (FAK), together with scaffold proteins, including paxillin, ILK, PINCH, kindlin, and parvin, links integrin activation with actin polymerization. Under some circumstances, signaling resulting from activation of receptor tyrosine kinases (RTK) modulates and contributes to integrin-promoted phagocytosis. The resulting downstream effects involve activation of guanine nucleotide exchange factors (GEFs) that stimulate RhoG, Rac1, Cdc42, and/or RhoA. Subsequently, the nucleation-promoting factors WAVE and WASP activate the actin nucleation complex Arp2/3, leading to actin polymerization and formation of pseudopods around the particle to be engulfed. During internalization of the particle, nonmuscle myosin proteins play key roles in the separation of the phagosome from the plasma membrane. (For color version of this figure, the reader is referred to the online version of this book).

species then catalyzes phosphorylation of multiple substrates, thus initiating a signaling cascade that regulates a large variety of cellular functions in addition to phagocytosis, including proliferation, migration, and survival (Qin and Rodrigues, 2012; Zhao and Guan, 2009).

FAK regulates formation of lamellipodia, cell protrusions and migration through its ability to modulate F-actin dynamics and Rho GTPase activity (Zhao and Guan, 2009). FAK can either increase or limit RhoA activity, depending on cell type and context. FAK can also mediate activation of Rac1 during cell migration, an effect that may be involved in engulfment of enterobacteria by macrophages (Hudson et al., 2005).

2.3.2. Integrin-Linked Kinase

Integrins associate with various other adapter proteins, forming complexes that link the extracellular matrix with the actin cytoskeleton (Brakebusch and Fassler, 2003; Hannigan et al., 1996; Hynes, 2002). ILK is a scaffold that interacts not only with the cytoplasmic tail of β1 and β3 integrins, but also with a multitude of other factors, including ELMO2 (Ho et al., 2009), PINCH (Braun et al., 2003; Tu et al., 1999; Zhang et al., 2002), α- and β-parvin (Nikolopoulos and Turner, 2002; Olski et al., 2001; Tu et al., 2001; Yamaji et al., 2001), as well as paxillin (Nikolopoulos and Turner, 2001). Although discrepant observations still exist regarding its kinase activity (Dagnino, 2011; Maydan et al., 2010), recent work has shown compelling evidence that ILK is a pseudokinase with no intrinsic phosphorylation capacity (Fukuda et al., 2009, 2011; Lange et al., 2009). ILK is essential for embryo implantation (Sakai et al., 2003) and cell migration (Grashoff et al., 2003; Terpstra et al., 2003), and constantly shuttles into and out of the nucleus (Acconcia et al., 2007; Nakrieko et al., 2008). Significantly, ILK is central in many processes that require actin cytoskeletal remodeling, such as development of front-rear polarity, forward movement and cell migration during tissue repair in vivo (Ho and Dagnino, 2012; Ho et al., 2009; Nakrieko et al., 2011, 2008). Recently, evidence in support of a role for ILK in phagocytosis has begun to emerge. For example, *Streptococcus pyogenes* is able to attach to and invade various mammalian cell types through integrin-mediated processes. Loss of ILK in epithelial cell lines established from the epidermis, the larynx and the cervix results in decreased bacterial adherence and internalization (Siemens et al., 2011; Wang et al., 2006). Similarly, fibroblast cell lines engineered from ILK-deficient tissues fail to support attachment and invasion of *Streptococcus pneumoniae* (Bergmann et al., 2009). The events downstream of ILK involved in these effects are yet to be defined.

2.3.3. Galectin-3

Galectin-3 belongs to a multigene family of β-galactoside-binding proteins widely expressed in mammalian cells (Newlaczyl and Yu, 2011). This lectin is synthesized in the cytoplasm and can either be secreted or shuttle between the cytoplasm and the nucleus. Nuclear galectin-3 participates in regulation of gene transcription and RNA splicing, whereas in the cytoplasm, it is involved in cell survival (Newlaczyl and Yu, 2011). Extracellular galectin-3 binds various membrane proteins, including α3β1 and αMβ2 integrins (Hughes, 2001), thus jointly modulating cell adhesion to the extracellular matrix and phagocytosis.

Some functions of hepatic stellate cells are modulated by extracellular galectin-3. These cells are central in the fibrinogenic responses of the liver, and acquire a myofibroblast phenotype following their phagocytosis of apoptotic hepatocytes (Jiang et al., 2012). Galectin-3 in stellate cells is essential for normal phagocytic capacity, and for expression of transforming growth factor-β and procollagen α1(I). Hepatic galectin-3 binds to and acts as a cross-linking moiety for αVβ3 integrins, which in turn increases the ability of stellate cells to tether apoptotic hepatocytes (Jiang et al., 2012). The interaction of galectin-3 with integrins also triggers autocrine and paracrine signaling events that further enhance the phagocytic capacity of hepatic stellate cells.

2.3.4. Nonmuscle Myosin II

Actin filaments interact with molecular motors to generate contractile forces necessary for phagosome formation, which occurs following the generation of membrane extensions that surround the particle to be engulfed. Myosins are motor proteins indispensable for cellular processes that involve generation of contractile forces. A subset of myosin isoforms generally termed nonmuscle myosins is expressed in all mammalian cells, where they function together with F-actin to induce changes in cell shape and motility (Vicente-Manzanares et al., 2009). Nonmuscle myosins accomplish these functions through their contractile properties, and their ability to form filaments with and cross-link actin. All myosins are composed of a light and a heavy chain. Their ATPase activity and ability to form filaments is regulated by phosphorylation of specific residues in the heavy chain (Vicente-Manzanares et al., 2009). Many kinases can phosphorylate these residues, including Rho-associated coiled coil-containing kinase (ROCK), which functions directly downstream of RhoA activation.

Multiple nonmuscle myosin isoforms have been implicated in phagocytosis. In general, they appear to participate in one of the two main stages. Specifically, some are necessary for extension of pseudopodia following particle binding to the phagocyte plasma membrane, whereas others are necessary for phagosome closure, phagocytic cup squeezing and/or movement to the interior of the cell (Groves et al., 2008). In particular, myosin II is necessary in macrophages for actin assembly during αMβ5-mediated phagocytosis (Olazabal et al., 2002). During this process, αMβ5 stimulation results in activation of RhoA, which in turn activates ROCK. The latter phosphorylates myosin II, which is recruited to sites bound by the particle at a time when the latter is still partially external or has just been engulfed (Olazabal et al., 2002). Under these circumstances, myosin II participates in recruitment of Arp2/3 and actin to the phagosome, and it is required for F-actin remodeling around the particle and engulfment.

Nonmuscle myosin II is also involved in the phagocytic activity of RPE cells. In these cells, phagocytosis results from the coordinated activation of the Mer receptor tyrosine kinase and αvβ5 integrins. During engulfment, Mer mediates myosin II recruitment to phagosomal sites (Strick et al., 2009). Pharmacological myosin inhibition or knockdown impairs the ability of RPE cells to engulf particles, emphasizing the important role of this contractile protein in particle uptake.

2.3.5. Milk Fat Globule-EGF Factor 8 (MFG-E8)

During lactation, several proteins and triglycerides are produced by the mammary epithelium. MFG-E8 was first described as one such factor (Raymond et al., 2009). This 53-kDa glycoprotein is widely expressed in various cell types, and contains an N-terminus cleavable signal peptide, followed by two epidermal growth factor-like repeats. The second repeat contains an RGD integrin-binding domain, which allows MFG-E8 interaction with and activation of αVβ3 and αVβ5 integrins. MFG-E8 also contains two C-terminal Discoidin/F5/8C domains. The second of these domains exhibits high affinity for binding to phosphatidylserine present in cell membranes (Raymond et al., 2009). MFG-E8 is implicated in a variety of physiological functions. It is able to bind apoptotic cells via its Discoidin/F5/8C domains, and its RGD motif is essential for cell engulfment by macrophages. In the mammary gland, MFG-E8 is indispensable for epithelial cell clearance of apoptotic cells during mammary gland involution at the end of lactation, and its absence causes accumulation of apoptotic cell

remnants, duct distension and mastitis (Hanayama and Nagata, 2005). The role of MFG-E8 in mammary gland function extends beyond lactation, as absence of this protein during development results in impaired mammary gland branching morphogenesis, through mechanisms that involve impaired integrin activation (Ensslin and Shur, 2007).

MFG-E8 has high affinity for glycoproteins on the egg surface, and contributes to the ability of sperm to adhere to the egg in an integrin-independent manner. Although MFG-E8 is not indispensable for fertilization, its absence is associated with reduced fertility in mice (Ensslin and Shur, 2007).

2.4. Integrin Modulation of Small GTPases during Phagocytosis

Although multiple types of particles and receptors participate in phagocytosis, the mechanisms associated with engulfment converge on the activation of small GTPases of the Rho family, which direct F-actin remodeling beneath particles bound to the cell surface, for subsequent internalization (Niedergang and Chavrier, 2005).

The most widely studied events in integrin-mediated phagocytosis have focused on the immune cell complement receptor integrin αMβ2, also termed CR3 (Dupuy and Caron, 2008; Flannagan et al., 2012). Early research suggested that αMβ2- and Fcγ-mediated phagocytosis in immune cells differed from each other, in that the former proceeded through activation of RhoA, whereas the latter involved activation of Cdc42 and Rac1 (Caron and Hall, 1998). Further, morphologically, these two processes were reportedly different, as αMβ2-mediated phagocytosis did not appear to involve formation of pseudopods, but rather particle sinking into the plasma membrane (Allen and Aderem, 1996; Kaplan, 1977). More recent studies have challenged this notion, and reported the formation of membrane protrusions around particles being engulfed in response to αMβ2 stimulation. These observations suggest the existence of common elements between those two phagocytic pathways (Bohdanowicz et al., 2010; Patel and Harrison, 2008). Further, comprehensive analyses of Rho GTPase involvement in αMβ2- and Fcγ-mediated phagocytosis in macrophages have established that the former requires Vav3, RhoA and RhoG, whereas the latter is dependent on Cdc42, Rac2 (but not Rac1), and RhoG (Sindrilaru et al., 2009; Tzircotis et al., 2011). Thus, common elements involved in particle internalization appear to exist, irrespective of the initial trigger, although subsequent aspects of phagocytosis, including F-actin remodeling and activation of diverse signaling cascades, may diverge depending on the phagocytic

stimulus. In addition, activation of the small GTPase Rap1 in response to stimulation of various growth factor receptors or bacterial factors is a potent inside-out activating signal for αMβ2 integrins, and enhances phagocytic uptake (Caron et al., 2000).

Given that Rho GTPases are essential regulators of actin reorganization, and that cortical actin polymerization beneath the sites of particle attachment to the plasma membrane is a key driving force for ingestion, it is not surprising that engulfment mediated by integrins in nonimmune phagocytic cells is also critically dependent on Rho GTPase activity. For example, shed photoreceptor outer segments bind to integrin αVβ5 in RPE cells, activating Rac1, but not RhoA or Cdc42, and triggering their phagocytic internalization (Mao and Finnemann, 2012). Similarly, phagocytosis of collagen fibers by fibroblasts proceeds through β1 integrin activation. The latter occurs through both inside-out and outside-in mechanisms mediated, respectively, by Rap1 and Vav2-dependent activation of Rac1 (Arora et al., 2008a, 2008b).

3. BIOLOGICAL IMPLICATIONS: PHAGOCYTIC INTEGRINS AND APOPTOTIC CELL CLEARANCE

Superfluous, senescent or abnormal cells are constantly generated during embryonic development and throughout postnatal life. These cells arise during physiological tissue remodeling and renewal or during regeneration after injury. They are generally eliminated through apoptosis, and are quickly removed by adjacent cells of immune or nonimmune origin, to avoid necrosis and inflammation. This process also mediates clearance from the circulation of aged erythrocytes (Boas et al., 1998), apoptotic germ cells (Elliott et al., 2010), and cells damaged by a variety of insults. Apoptotic corpse removal occurs through processes that involve integrin binding to the cells to be removed, followed by phagocytosis (Dupuy and Caron, 2008). Apoptotic cells can be removed by either immune or nonimmune cells, following sequential steps of recognition, engulfment, and degradation. Cells that are damaged first release chemotactic factors that both attract phagocytes and enhance their capacity to internalize particles. These factors include lysophosphatidylcholine, ATP, UTP, transforming growth factor-β, and CX3CL1 (Ravichandran, 2011). Different cell types release distinct chemoattractant "find-me" signals, and it is not clear whether the latter are tissue-, cell type- or context-specific.

Apoptotic cells also present on their surface molecules that are specifically recognized as engulfment ("eat me") signals by phagocytes. These

signals include modified sugar chains, annexin 1, phosphatidylserine on the outer leaflet of the plasma membrane, intracellular adhesion molecule 3 (ICAM3), thrombospondin 1, and modified CD31 (Ravichandran, 2010). In particular, the presence of phosphatidylserine on the outer leaflet of the plasma membrane appears to universally signal the presence of an "altered self" (as opposed to a foreign, non-self-entity) that must be removed. Phosphatidylserine serves as a ligand, through either direct binding or indirect interactions via bridging molecules, to multiple types of receptors (Ravichandran, 2010; Savill and Fadok, 2000). This ligand on apoptotic cells not only mediates recognition by phagocytes but also triggers internalization.

Both integrins αVβ3 and αVβ5 are involved in engulfment of apoptotic cells, although they appear to do so in a cell-type-specific manner. For example, αVβ3 mediates clearance of apoptotic neutrophils by macrophages, whereas αVβ5 is involved in engulfment by dendritic cells, HEK293 and RPE cells (Albert et al., 2000; Nandrot et al., 2007; Savill et al., 1990). These two integrins bind phosphatidylserine indirectly by interacting with MFG-E8, which serves as a required bridge (Akakura et al., 2004; Hanayama et al., 2004). Significantly, although phagocytosis mediated by integrin αMβ2 occurs through RhoA activation, engulfment through αVβ5 integrins requires Rac1 activation (Hanayama et al., 2004). In addition, apoptotic cell phagocytosis appears to require cooperative interactions between αVβ5 integrins and other receptors, including the Mer tyrosine kinase receptor and CD81 (Chang and Finnemann, 2007; Wu et al., 2005). CD31 activation also enhances surface levels of αVβ5 integrins, which results in further increments in phagocytic ability.

In testes, Sertoli cells play key roles in removal of apoptotic germ cells. In this tissue, several transmembrane receptors, including BAI-1 and Mer, as well as the bridging factor MFG-E8, are necessary for efficient apoptotic cell clearance, through mechanisms that include activation of Rac1 by ELMO1/Dock complexes (Elliott et al., 2010). Although the role of integrins in this process has yet to be explored, the requirement of MFG-E8 suggests their likely involvement.

In general, engulfment of apoptotic cells occurs through two conserved pathways. One involves stimulation of either BAI-1 or integrins, which is followed by ELMO/Dock180 activation of Rac1. The other pathway relies on CED-1 or stabilin-2 stimulation by phosphatidylserine, which also results in Rac1 activation (Ravichandran, 2010). Recently, stabilin-2 was demonstrated to bind integrin αVβ5 during apoptotic erythrocyte engulfment,

indicating that diverse phagocytic pathways can interact with and modulate each other to maximize apoptotic cell clearance (Kim et al., 2012).

4. INTEGRIN-DEPENDENT PHAGOCYTIC TISSUE REMODELING

The soft connective tissue provides support to organs and other tissues in the body, and is mainly composed of fibroblasts. These cells constantly remodel their surrounding environment through the synthesis, deposition and degradation of extracellular matrix proteins, such as collagens (Everts et al., 1996). Matrix remodeling is necessary for tissue homeostasis and regeneration after injury (McGaw and Ten Cate, 1983). Importantly, a balance between extracellular matrix synthesis and degradation is critical for proper tissue function, and defects in degradation are associated with fibrotic disorders. Although extracellular metalloproteases contribute to the breakdown of collagen and other extracellular matrix substrates, engulfment and subsequent lysosomal degradation of collagen fibrils by fibroblasts is also a key component of this process (Birkedal-Hansen et al., 2003; Everts et al., 1996). Collagen is both a ligand and a phagocytic substrate for α2β1 integrins, abundantly expressed in mesenchymal cells (Knowles et al., 1991). A rate-limiting step in collagen degradation via the phagocytic route is its binding to these integrins (Lee et al., 2006). Similar to phagocytosis in other cells, the mechanisms involved in collagen phagocytosis include integrin ligation and clustering, generation of membrane protrusions, Src-dependent phosphorylation and activation of Vav2, which in turn activates Rac1, inducing F-actin remodeling (Arora et al., 2000, 2008b). Collagen interactions with α2β1 integrins in fibroblasts also activate a positive feedback loop that results in enhanced binding to each other. Two additional factors required for collagen binding and internalization are gelsolin and nonmuscle myosin IIa. Significantly, binding of collagen to integrins increases myosin IIa phosphorylation in a Ca^{2+}-dependent manner, indicating that multiple signaling events are involved in collagen uptake by fibroblasts (Arora et al., 2011). In addition, phagocytosis in fibroblasts is stimulated by pressure, via integrin β1-dependent pathways (Bhalla et al., 2009). This process may be activated during tissue repair and remodeling, given that mechanical stimuli, such as changes in extracellular pressure, occur during infection and inflammation. Notably, in spite of the wide expression of α2β1 integrins in multiple mammalian cell types, phagocytosis of collagen fibrils has only been observed in fibroblasts. This suggests the presence of additional co-receptors

or intracellular factors specifically expressed in this cell type. The possibility of engulfment of other extracellular matrix ligands, such as fibronectin, remains unexplored.

5. ROLE OF PHAGOCYTOSIS IN TISSUE MAINTENANCE AND HOMEOSTASIS

5.1. Retina

Retinal pigmented epithelial cells are components of a unique physiological system in which phagocytosis is triggered in a periodic and rhythmic manner. Specifically, the regulation of retinal cell function and behavior is strongly influenced by circadian rhythms, which modulate a renewal program of photoreceptor rod and cone outer segments (LaVail, 1976).

The interaction of retinal neurons with light produces photo-oxidative compounds that accumulate and must be constantly eliminated to maintain proper photoreceptor function (Law and Nandrot, 2012). In this manner, membranous disks in the distal portions of photoreceptor outer segments are constantly produced and shed once a day. Shed outer segments do not accumulate in the retina. Rather, they are phagocytosed by adjacent RPE cells (Young and Bok, 1969), which perform specialized forms of clearance phagocytosis (Fig. 6.2). The importance of this process in the homeostasis of retinal photoreceptors is emphasized by the fact that impairment of RPE phagocytosis results in accumulation of outer segment debris and, eventually, blindness.

As occurs with apoptotic cells, phosphatidylserine located in the outer aspect of the plasma membrane is the recognition signal on shed photoreceptor outer segments that targets them for engulfment (Boesze-Battaglia and Albert, 1992). Integrins also play key roles in shed outer segment recognition. The mechanism involves secretion by RPE cells of MFG-E8, which serves as a bridge between αVβ5 integrins and the shed outer segments (Nandrot et al., 2007). Ligation of αVβ5 integrins results in sequential FAK and Mer tyrosine kinase recruitment and activation at the apical surface of the RPE cell, which are necessary for engulfment (Finnemann, 2003; Nandrot et al., 2007). Activation of αVβ5 integrins also stimulates a parallel pathway that results in MFG-E8-dependent activation of Rac1 (but not of Cdc42 or RhoA), and its recruitment to the plasma membrane (Mao and Finnemann, 2012). Rac1 activation in response to ligation of αVβ5 integrin is required for actin cytoskeleton remodeling at the phagocytic cup, a process that is essential for phagocytosis. In contrast, Rac1 activation

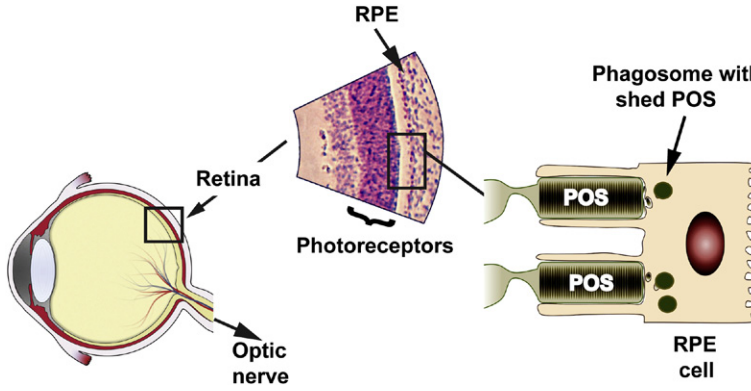

Figure 6.2 ***Phagocytosis of shed photoreceptor outer segments by retinal pigmented epithelium cells.*** In the retina, photoreceptor outer segments (POS) on distal regions of rods and cones are shed daily. Adjacent retinal pigmented epithelium (RPE) cells engulf the shed POS by phagocytosis. This process is mediated via αVβ5 integrins, and is essential for long-term maintenance of retinal function and vision. (For color version of this figure, the reader is referred to the online version of this book).

is dispensable to maintain F-actin in resting RPE cells, indicating a high degree of specificity in these responses.

Other components of the phagocytic pathway in RPE cells are annexin 2 and nonmuscle myosin VIIa. Annexin 2 can regulate F-actin dynamics, is recruited to early phagosomes, and appears to be required for the timely activation of FAK, actin nucleation and polymerization, as well as internalization of the newly formed phagosome. Indeed, mouse *AnxA*2-null RPE cells accumulate phagosomes containing outer segments in apical processes (Law, 2009).

Activation of the Mer receptor tyrosine kinase in RPE cells in response to binding of outer segments is also accompanied by myosin II concentration at phagosome sites (Gibbs et al., 2003). Myosin motor proteins are involved in the generation of the contractile forces required to achieve pseudopod constriction and phagosome closure. Further, mutations in the gene that encodes myosin VIIa result in a variety of disorders, including absence of melanosomes and accumulation of phagosomes in the apical aspect in RPE cells, abnormal retinal electrical properties, and lower rates of disk renewal in photoreceptors (Gibbs et al., 2003).

5.2. Epidermis

The epidermis of the skin serves as a protective barrier against pathogens, chemicals and physical insults, such as UV irradiation. Pigmentation and

photoprotection of the skin are determined by the presence of melanin, which is synthesized in melanocytes and packaged into membrane-bound organelles termed melanosomes. Melanosomes are taken up by epidermal keratinocytes via phagocytosis (Van Den Bossche et al., 2006). Once melanosomes are internalized, they do not follow a typical degradative pathway to the lysosomes. Rather, they are transported into the cell and arranged in a radial microtubule array that surrounds and covers the nucleus, forming a microparasol that protects DNA from UV damage (Byers et al., 2007). UVB irradiation results in the upregulation of melanogenic enzymes in melanocytes, concomitantly with increased expression and activation of protease-activated receptor 2 (PAR-2) and keratinocyte growth factor (KGF) receptor in keratinocytes. The latter are physiological routes that stimulate melanosome uptake in epidermal cells (Cardinali et al., 2005; Sharlow et al., 2000;Virador et al., 2002).

Present in melanosomes is the integral protein Gpnmb, which mediates adhesion to keratinocytes via an RGD motif known to bind integrins, although the precise identity of those integrins remains to be determined. Importantly, Gpnmb is essential for melanosome uptake by keratinocytes. In addition, targeted inactivation in mouse epidermal keratinocytes of the *Intb1* gene, which encodes the β1 integrin subunit, has been shown to impair the phagocytic capacity of these cells, underlining its importance for particle engulfment in this tissue (Sayedyahossein et al., 2012).

The G-protein-coupled receptor PAR-2 is activated by proteolytic cleavage of its extracellular domain, which generates a new N-terminus that functions as a tethered ligand (Dery and Bunnett, 1999). Activation of PAR-2 in keratinocytes induces cyclic AMP production, actin polymerization and α-actinin reorganization. The small GTPase RhoA is also activated by PAR-2 stimulation, and is required for melanosome uptake (Scott et al., 2003; Sharlow et al., 2000). This sequence of signaling events is likely an important contributor to the formation in keratinocytes of long filopodia that wrap around melanosomes, followed by the formation of membrane extensions that mediate engulfment and culminate in particle deposition around the nucleus (Ando et al., 2012).

In the skin, UVB irradiation also causes activation of growth factor receptors and rapid expression of early growth response genes and growth factors. In particular, mesenchymal cells in the underlying dermis produce KGF, which acts in a paracrine manner on keratinocytes to stimulate melanosome internalization, through mechanisms that also involve formation of pseudopodia, engulfment and transport of engulfed particles to perinuclear

regions (Fig. 6.3) (Belleudi et al., 2011; Cardinali et al., 2008, 2005). KGF induces activation of Src and phospholipase C-γ, which is required for phagocytosis. This growth factor also promotes activation of Rac1, which is essential for particle engulfment, as is F-actin remodeling (Sayedyahossein et al., 2012). Significantly, ILK is required as a downstream effector for phagocytosis consequent to KGF receptor stimulation. Although early signaling events downstream from KGF receptor stimulation are present in ILK-deficient epidermal keratinocytes, Rac1 activation and particle engulfment are severely impaired in these cells. In an analogous manner, ILK is also essential for PAR-2 stimulation of phagocytosis (Sayedyahossein et al., 2012).

Cross-modulation between several different phagocytic pathways in keratinocytes appears to exist as inhibition of PAR-2 interferes with KGF-induced particle engulfment (Cardinali et al., 2005). The concept of cross talk between various pathways for efficient melanosome engulfment is further reinforced by the observation that targeted inactivation of *Intb1* also abolishes KGF-induced particle uptake in cultured mouse epidermal keratinocytes (Sayedyahossein et al., 2012).

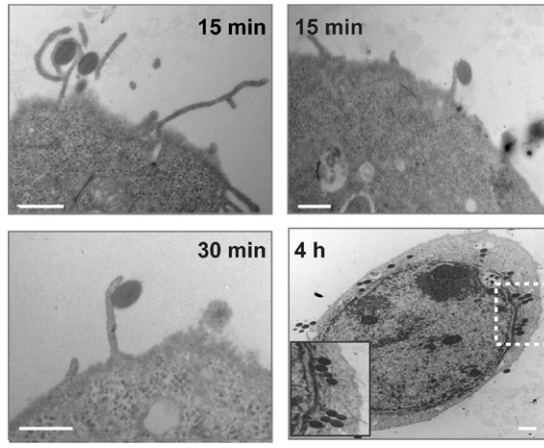

Figure 6.3 ***Formation of pseudopods and particle engulfment by keratinocytes.*** Primary mouse keratinocytes were cultured in serum- and growth additive-free medium for 4 h prior to the addition of KGF (20 ng/ml) and 0.5-μm latex microspheres, which mimic melanosomes. The cells were cultured at 37 °C for the times indicated, and processed for transmission electron microscopy. Note the formation of long, fine pseudopodia around the particles, which precedes their engulfment. After 4 h, a substantial fraction of engulfed particles exhibit a perinuclear distribution. Inset in the 4-h micrograph shows a higher-magnification image of the boxed area. Bar, 1 μm.

6. INTEGRIN-DEPENDENT PHAGOCYTOSIS AND PATHOGENESIS

6.1. Virus and Bacterial Cell Invasion

Phagocytosis of bacteria and viruses by immune cells and their subsequent destruction within cytoplasmic vesicles play key roles in an organism's defense against pathogens (Kaufmann and Walker, 2006). However, many bacteria have evolved mechanisms that allow them to adhere and invade host cells, in which they survive not only escaping antibody-mediated immune responses but also allowing their intracellular persistence and replication (Sinha and Fraunholz, 2010). Pathogenic bacteria express proteins termed invasins, which mediate both adhesion to and internalization by the host cell. Notably, fibronectin-binding integrins are prime targets of bacterial invasins (Hoffmann et al., 2011).

Integrins can be bound and activated by bacteria through two mechanisms. The first involves expression on the bacterium of proteins that directly bind integrins, such as invasin (expressed by *Yersinia enterocolitica*) or the CagL protein of *Helicobacter pylori*, which allows it to colonize the gastric epithelium in the absence of ligand-bound integrins (Isberg and Leong, 1990; Kwok et al., 2007). Most often, bacterial microorganisms express surface proteins that bind extracellular matrix substrates, which, in turn, coat the bacteria and serve as a link with cellular β1 integrins. For example, *Staphylococcus aureus* and *S. pyogenes* express fibronectin-binding proteins (FnBP), which allow their interaction with α5β1 integrins in host cells of immune or epithelial origin (Hauck and Ohlsen, 2006; Nobbs et al., 2009). Indeed, the ability of FnBP to associate with fibronectin appears to be sufficient and critical for bacterial adhesion to and engulfment by host cells, for tissue colonization and development of *S. aureus*-induced diseases, including mastitis, endocarditis and wound infections (Menzies et al., 2002; Que et al., 2005; Sinha and Fraunholz, 2010).

The molecular mechanisms involved in integrin-mediated bacterial uptake have been extensively characterized for *S. aureus* infections. Fibronectin-coated *S. aureus* associates with β1 integrins in host cells, inducing their clustering and triggering outside-in signaling. The signaling pathways activated include accumulation of focal adhesion proteins, such as tensin and zyxin, adjacent to the bound *S. aureus*, as well as activation of Src and FAK (Agerer et al., 2005, 2003). FAK is necessary for internalization, but not for binding of *S. aureus* to the cell surface. This kinase facilitates bacterial

uptake by phosphorylating cortactin, which results in reorganization of the actin cytoskeleton. Significantly, bacterial internalization appears to occur through formation of plasma membrane invaginations, rather than formation of pseudopodia surrounding the bacteria, in a process that may involve formation of lipid rafts (Hoffmann et al., 2011).

In contrast, *S. pyogenes* produces a different type of FnBP that mediates binding to endothelial cells, and induces Rac1 activation and phagocytosis-like uptake (Amelung et al., 2011). Another pathogen, *S. pneumoniae*, colonizes cells in the upper respiratory tract, and is an etiological agent of severe infections, such as sinusitis, otitis media, pneumonia, septicemia and meningitis (Cartwright, 2002). *Streptococcus pneumoniae* produces several factors that interact with surface proteins in epithelial and endothelial host cells, including integrins (Bergmann et al., 2009). Specifically, these microorganisms are capable of binding to vitronectin, which in turn mediates interactions with αVβ3 integrins in nasopharyngeal epithelial cells and in microvascular endothelial cells (Bergmann et al., 2009). This interaction causes formation of cell membrane protrusions reminiscent of filopodia, followed by engulfment. Bacterial invasion of pulmonary host cells requires F-actin remodeling, in a manner dependent on ILK, phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt). Similarly, in HaCaT cells, a human keratinocyte line, and in HEp2 human larynx epithelial cells, *S. pyogenes* bound to plasminogen/plasmin can interact with α5β1 and α1β1 integrins, respectively. This interaction triggers bacterial internalization and cell invasion, through pathways that also require actin polymerization, as well as expression of ILK, AKT and PI3K. Under these circumstances, internalization actually protects the bacteria against killing by macrophages, and is associated with development of various human diseases, from mild superficial infections of the skin and nasopharynx mucous membranes to rare but severely invasive diseases (Siemens et al., 2011; Wang et al., 2006).

Bacteria that belong to the genus *Shigella* cause bacillary dysentery, and act by first invading the colonic mucosal epithelial cell which, upon death, release these microorganisms, allowing them to spread to adjacent, uninfected tissue (Demali et al., 2006). *Shigella* produce a large number of proteins that facilitate their engulfment. Among them, IpaB and IpaC proteins bind to α5β1 integrins, and promote transient adhesion of this microorganism to the host cell (Watarai et al., 1996). In addition, the bacterial protein OspE binds to ILK, increasing surface levels of β1 integrins, inhibiting phosphorylation of paxillin and FAK, and stabilizing focal adhesions (Kim et al., 2009). In this manner, the bacterium ensures that infected host cells

remain attached to the basement membrane, which enhances bacterial ability to invade neighboring cells. Thus, through a unique combination of targeting integrins from the extracellular milieu and modulating their ability to form stable focal adhesions via cytoplasmic proteins, these bacteria ensure not only efficient cellular uptake but also continued tissue colonization.

Recently, the mechanisms of invasion of lung epithelial cells by *Legionella pneumophila* have been investigated (Prashar et al., 2012). Humans become infected by inhalation of aerosols containing these bacteria. Both β1 integrin and E-cadherin attach to and coordinately mediate cellular uptake of *L. pneumophila*. Bacterial attachment to the cell membrane induces formation of filopodia-like protrusions that trap the bacterium at the cell surface. Attachment also promotes formation of pseudopodia that mimic zipper formation with subsequent bacterial internalization. The formation of these membrane extensions is mediated by F-actin and nonmuscle myosin II. Further, efficient uptake partially depends on the ability of the attached bacteria to promote focal adhesion turnover, and to recruit β1 integrins to membrane protrusions (Prashar et al., 2012).

In a manner analogous to that used by bacteria, viruses frequently target integrins to facilitate cell invasion, and in many cases, they use processes akin to phagocytosis, in spite of having a much smaller size of that generally accepted for this process (Stewart and Nemerow, 2007; Tiwari and Shukla, 2012). Herpes family viruses, including Herpes simplex type 1 (HSV-1), equine herpes virus type 1 (EHV-1), human cytomegalovirus (HCMV) and Kaposi sarcoma-associated herpes virus (KSHV), enter immune and nonimmune phagocytic cells through steps that involve formation of lamellipodia and Rho GTPase activation (Dupuy and Caron, 2008; Tiwari and Shukla, 2012). Further, some envelope proteins of HSV-1, EHV-1 and HCMV bind αVβ3 integrin to trigger internalization (Parry et al., 2005; Wang et al., 2005), whereas others promote β1 integrin-dependent uptake (Feire et al., 2004).

Entry into cells of human adenoviruses types 2 and 5 results from cooperative effects of binding to specific membrane receptors and to integrins αVβ3 or αVβ5 (Bergelson et al., 1997; Wickham et al., 1993). Virus entry also requires activation of Rac1 and Cdc42, as well as F-actin remodeling (Li et al., 1998a, 1998b). Similarly, rotaviruses, major etiological agents of human and animal gastroenteritis, penetrate intestinal epithelial cells following binding to α2β1 integrins (Fleming et al., 2011).

6.2. Deficient Apoptotic Cell Clearance

The efficient clearance of apoptotic cells is necessary for homeostasis and maintenance of healthy tissues. This process ensures that toxic or

immunogenic intracellular contents from the dying cells do not reach the local environment, producing a harmful strong inflammatory response. In this scenario, intense immune responses can occur against intracellular antigens or DNA released from the dying cells. As a consequence of inefficient apoptotic corpse clearance, severe autoimmune disorders may develop in humans, such as systemic lupus erythematosus and rheumatoid arthritis (Gaipl et al., 2004, 2005). This process has been elegantly modeled in genetically modified mice that exhibit defects in phosphatidylserine-mediated target recognition and which develop a variety of autoimmune conditions (Lacy-Hulbert et al., 2007; Rodriguez-Manzanet et al., 2010).

In the lung, inefficient apoptotic cell removal is associated with respiratory disease, which, in turn, reinforces abnormal phagocytosis. For example, elevated numbers of apoptotic cells are found in lung secretions and tissues of individuals with chronic obstructive pulmonary disease or asthma, and are accompanied by a reduced phagocytic capacity of alveolar macrophages in culture (Hodge et al., 2003; Janssen et al., 2008). Similarly, lung epithelial cells with mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) exhibit impaired apoptotic cell phagocytosis (Vandivier et al., 2009). This defect is associated with abnormally elevated basal levels of active RhoA, which appear to counter the positive modulation by Rac1 of phagocytosis, thus playing an important role in deficient apoptotic cell clearance (Kreiselmeier et al., 2003; Vandivier et al., 2009). Similarly, cigarette smoking reduces the ability of alveolar macrophages to engulf apoptotic cells, although inhibition of RhoA can ameliorate this deficit (Richens et al., 2009).

Defective clearance of apoptotic cells in atherosclerotic plaques also plays a key role in the pathogenesis of atherosclerosis. In a variety of mouse strain backgrounds, inactivation of the gene encoding MFG-E8 or transglutaminase-2 (an enzyme associated with efficient phagocytosis mediated via αvβ integrins) results in accelerated atherogenesis (Ait-Oufella et al., 2008; Lorand and Graham, 2003). In humans, apoptotic cells are abundant in atherosclerotic plaques, especially as lesions progress and plaques enlarge (Bennett et al., 2012). In these damaged areas, vascular smooth muscle cell apoptosis can be extensive, and delayed clearance of these cells by macrophages causes inflammation due to secondary necrosis. This inflammatory response appears to be a key component of plaque rupture and subsequent adverse cardiac events (Bennett et al., 2012).

The role of phagocytic cell removal signals during carcinogenesis is complex. Tumor cells can evolve a variety of mechanisms to evade removal by phagocytes. For example, in various types of cancers, such as head and

neck, prostate, esophageal carcinomas and non-Hodgkin's lymphoma, tumor cells downregulate the pro-phagocytic signal annexin A1 (Chao et al., 2012). Conversely, the upregulation of "don't eat me" signals has also been reported. Specifically, increased expression of CD47, which allows cells to evade phagocytic uptake by macrophages, is associated with poor prognosis and high relapse rates of a variety of leukemias and solid tumors (Chao et al., 2012). In stark contrast, some apoptotic tumor cells can generate ATP and other factors that stimulate immune responses and phagocytic engulfment by macrophages (Aymeric et al., 2010). Hence, modulation of apoptotic cell clearance can significantly impact tumor progression and the outcome of anticancer therapies, depending on the nature of factors released by apoptotic tumor cells.

Phagocytosis and digestion of photoreceptor outer segment tips shed in a circadian rhythm occurs within hours of release, and is mediated by the RPE cells. Outer segment tips are not apoptotic cell remnants. However, the mechanism of their recognition by the RPE through binding of phosphatidylserine is analogous to that of apoptotic cell recognition. Significantly, in *Intb*5-null mice, which lack β5 integrin, the daily diurnal phagocytosis of outer segments does not occur, and mutant animals lose vision as they age (Nandrot et al., 2004), indicating a key role for β5-mediated phagocytosis in maintenance of retinal function. Impaired phagocytosis resulting from degeneration of RPE cells is also at the root of human age-related macular degeneration (Nandrot et al., 2004).

6.3. Phagocytosis as a Contributor to Neurodegenerative Diseases

In the brain, microglial cells function as resident macrophages to clear pathogens, apoptotic cells or debris, thus maintaining a healthy tissue. These cells produce MFG-E8, which, similar to RPE cells, serves as a bridge between phosphatidylserine on apoptotic neurons, and αvβ integrins on microglia, resulting in Rac1 activation and particle engulfment.

Neurological disorders, such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis, are characterized by generation of strong inflammatory responses, which contribute to increased neuronal loss through apoptosis (Block et al., 2007). Apoptotic neurons are removed through phagocytosis by microglial cells. Defective phagocytosis can be associated with aggravation of these disorders (Fuller and Van Eldik, 2008). Paradoxically, and to further exacerbate this situation, the increased

inflammation that often occurs in chronic neurodegenerative diseases can also trigger microglial phagocytosis of viable and healthy neurons (Fricker et al., 2012).

In some other circumstances in which inflammation occurs, for example, in the presence of the bacterial ligands lipotheicoic acid or LPS, microglia become activated and secrete peroxynitrite. The latter causes phosphatidylserine to become exposed on the outer leaflet of the plasmalemma of otherwise healthy neurons, converting them into targets for phagocytosis by adjacent microglial cells. In this manner, microglia actually cause neuronal death and exacerbate neurodegeneration (Neher et al., 2011). This type of neuronal damage can be prevented with agents that interfere with phagocytosis, even in the presence of inflammatory mediators. Similarly, low concentrations of amyloid β, an etiological agent in the development of Alzheimer's disease, can induce neuronal loss without first causing neuronal apoptosis (Neniskyte et al., 2011). This mechanism of cell loss involves increased microglial phagocytic capacity, together with exposure of phosphatidylserine on the outer aspect of the neuronal plasma membrane. As a result, microglial cells engulf neurites from healthy cells, as well as neuronal cell bodies, in the absence of amyloid β-induced neuronal apoptosis. Under these conditions, impairing phagocytosis also prevents neuronal loss and preserves the neuronal cell population. Thus, abnormal phagocytic activity, irrespective of whether it is impaired or enhanced, appears to be involved in altered neuronal tissue homeostasis and function.

7. CONCLUSIONS AND PERSPECTIVES

Integrins are well-established mediators of cell adhesion and migration, and understanding of the complexity of the pathways that these proteins regulate continues to grow (Margadant et al., 2011). It is becoming increasingly clear that integrins are also central for phagocytosis, and that many pathways activated during forward cell movement are also involved in particle engulfment. Significantly, phagocytosis occurs in many, if not all, cell types and can be mediated by a wide variety of integrins, all of which have a common characteristic of signaling via Rho GTPases to modulate actin cytoskeleton dynamics. Whether integrins linked to intermediate filaments, such as β4, participate in particle engulfment remains to be determined.

In contrast to cell adhesion and mechanotransduction, integrins often appear to require cross modulation with other membrane receptors to elicit a full phagocytic response, indicating that efficient phagocytosis results from coordinate responses to several stimuli. This mode of action is reminiscent of other responses orchestrated by integrins, such as cell migration and proliferation, which require cross talk between these adhesion proteins and a variety of growth factor receptors. Future research focused on understanding the processes that link integrin activation and receptor stimulation will likely shed light into multiple aspects of phagocytic cell function, tissue homeostasis and pathogenesis.

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