

The extended tubulin superfamily

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Summary

Although most eukaryotic cells can express multiple isotypes of $\alpha\beta$ -tubulin, the significance of this diversity has not always been apparent. Recent data indicate that particular $\alpha\beta$ -tubulin isotypes, both genome encoded and those derived by post-translational modification, can directly influence microtubule structure and function – thus validating ideas originally proposed in the multi-tubulin hypothesis over 25 years ago.

It has also become increasingly evident over the past year that some (but intriguingly not all) eukaryotes encode several other tubulin proteins, and to date five further members of the tubulin superfamily, γ , δ , ϵ , ζ and η , have been identified. Although the role of γ -tubulin in the nucleation of microtubule assembly is now well established,

far less is known about the functions of δ -, ϵ -, ζ - and η -tubulin. Recent work has expanded our knowledge of the functions and localisation of these newer members of the tubulin superfamily, and the emerging data suggesting a restricted evolutionary distribution of these ‘new’ tubulin proteins, conforms to established knowledge of microtubule cell biology. On the basis of current evidence, we predict that δ -, ϵ -, ζ - and η -tubulin all have functions associated with the centriole or basal body of eukaryotic cells and organisms.

Key words: Tubulin, Cytoskeleton, Microtubule, Flagellum, FtsZ, Evolution

*‘My soul, sit thou a patient looker on;
Judge not the play before the play is done:
Her plot hath many changes; everyday
Speaks a new scene; the last act crowns the play.’*
Francis Quarles (1592-1644)

Introduction

Microtubules constitute one of the major components of the cytoskeleton of eukaryotic cells and are involved in many essential processes, including cell division, ciliary and flagellar motility and intracellular transport (Hyams and Lloyd, 1993). The basic building block of the microtubule is the heterodimeric protein $\alpha\beta$ -tubulin that assembles in a head-to-tail arrangement to form a linear protofilament. The subsequent formation of lateral interactions between protofilaments results in their assembly into the wall of the cylindrical microtubule. The intrinsic molecular pattern of the $\alpha\beta$ -tubulin repeat defines both the polarity of microtubules and the differing properties of the plus and minus ends. The minus end of the microtubule is usually located at a microtubule-organising centre (MTOC), whose form and architecture varies from cell to cell and organism to organism (Fig. 1). Although MTOCs, which include the centrosome, flagellar basal bodies and spindle pole bodies, have diverse architectures, they exhibit discrete similarities in their biochemistry and molecular components.

The involvement of microtubules in a wide set of cellular structures and processes was widely recognised from early studies of their cell biology. This led to the development of the multi-tubulin hypothesis (Fulton and Simpson, 1976), which acknowledged tubulin diversity and proposed that distinct microtubule structures within a cell comprise different forms of tubulin protein. It is now apparent that most eukaryotic cells can indeed express multiple isotypes of $\alpha\beta$ -tubulin and that this

diversity can be further elaborated by a kaleidoscopic array of post-translational modifications (reviewed by Luduena, 1998). Although the functional significance of this diversity has been, and still is, difficult to weave into a consistent cell biological description, there is increasing evidence that different $\alpha\beta$ -tubulin isotypes and modifications can influence microtubule structure and function.

It is becoming increasingly evident, however, that eukaryotic cells encode other tubulin proteins, a further five members of the tubulin superfamily, γ , δ , ϵ , ζ and η , having been identified to date (Chang and Stearns, 2000; Dutcher and Trabuco, 1998; Oakley, 2000a; Oakley and Oakley, 1989; Ruiz et al., 2000; Vaughan et al., 2000). Since the identification of these tubulins comes from work in organisms whose genomes are not completely sequenced, further members of the tubulin superfamily probably still await discovery. Here, we review recent work on α - and β -tubulin isotypes, both genome encoded and produced by post-translational modification, but also re-examine the multi-tubulin hypothesis in the light of the recent extension of the tubulin superfamily.

Gene-encoded isotype-specific functions for α - and β -tubulin?

The early recognition that eukaryotic organisms express multiple isotypes of α -tubulin and β -tubulin, along with conserved expression of particular isotypes in certain metazoan tissues, suggested that the multi-tubulin hypothesis might have some substance. However, in certain systems experimental evidence did not produce support for the notion of isotype-specific locations or functions. When particular gene-encoded isotypes were expressed in a heterologous environment they appeared to locate and function in the new microtubule context, and some gene-swap experiments indicated isotypes to be

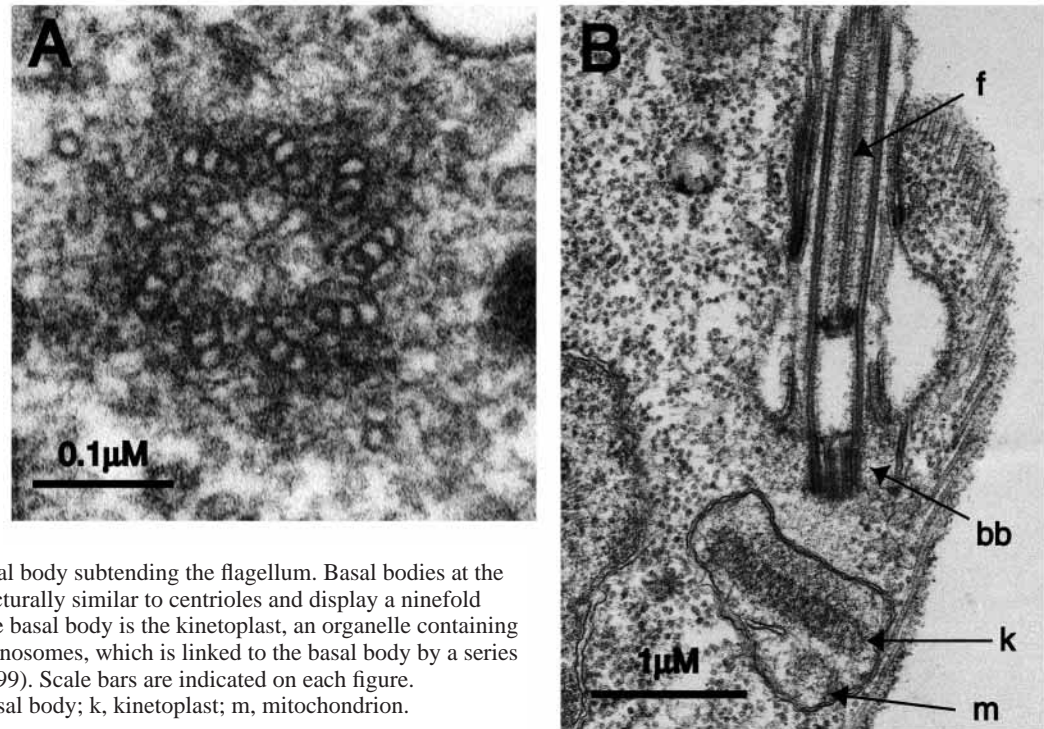


Fig. 1. (A) Electron micrograph of a section through a mammalian centriole showing the typical nine triplet organisation of microtubules (A, B and C tubules).

(B) Electron micrograph of a longitudinal section through a trypanosome cell, showing the basal body subtending the flagellum. Basal bodies at the base of flagella (and cilia) are structurally similar to centrioles and display a ninefold symmetry. Also visible beneath the basal body is the kinetoplast, an organelle containing the mitochondrial genome of trypanosomes, which is linked to the basal body by a series of filaments (reviewed by Gull, 1999). Scale bars are indicated on each figure. Abbreviations: f, flagellum; bb, basal body; k, kinetoplast; m, mitochondrion.

functionally interchangeable (Luduena, 1998; Wilson and Borisy, 1997). At a lower level of resolution, however, it was possible to show experimentally that the intrinsic properties of isotypes can influence the function of a new tubulin array when expressed heterologously. For instance, tubulin from the amoeba of *Physarum* is insensitive to the action of colchicine. When introduced into a mammalian cell, it incorporates into microtubules, endowing the cell with colchicine resistance (Prescott et al., 1989).

It is important to recognise that, by necessity, many of the mammalian cell and protist studies used proliferating cells. The recognition that divergent α - and β -tubulin isotypes can participate in different microtubule structures in such cells was entirely consistent with studies in some protists. For example, when the amoeba of *Physarum* differentiate to form plasmodia, the intermediate cells in transition acquire new microtubule arrangements quickly, but the tubulin isotype configuration changes only over a number of cell cycles (Burland et al., 1992). Hence, mixing and co-assembly of tubulin isotypes would be of particular benefit to cells in such differentiation transitions.

Molecular studies in *Caenorhabditis elegans*, however, indicate that particular tubulin isotypes can influence the supramolecular organisation of microtubular structures. For instance, the β -tubulin isotype MEC-7 from *C. elegans* is expressed primarily in microtubules within the axons of touch receptor neurons (Hamelin et al., 1992). Although *C. elegans* microtubules normally consist of 11 protofilaments, these axonal microtubules are structurally distinct and consist of 15 protofilaments. In *mec-7*-null mutants, however, microtubules based on 11 protofilaments are formed, which indicates that the MEC-7 isotype specifically influences the structural organisation of the axonal microtubule (Savage et al., 1994). The α -tubulin MEC-12 is also required for touch sensitivity and 15-protofilament-microtubule assembly (Fukushige et al., 1999).

Raff and colleagues have provided an elegant dissection of the $\alpha\beta$ -tubulin isotype family in *Drosophila*, revealing important roles and attributes of specific isotypes particularly in axonemal morphogenesis (Hoyle and Raff, 1990; Raff et al., 2000; Wilson and Borisy, 1997). Raff et al. noted that the presence of the amino acid sequence EGEFXXX (where X is an acidic residue) close to the C-terminus correlates with the assembly of certain β -tubulin isotypes into axonemes (Raff et al., 1997). Luduena extended this analysis to a large number of tubulin isotypes and confirmed the absence of this sequence in many protists that do not form flagella (yeasts and filamentous fungi) and its presence in many that do (ciliates, flagellates and algae; Luduena, 1998). Some evidence suggested that this type of sequence is unlikely to operate as a simple signal sequence for axonemal microtubule assembly itself (Luduena, 1998). Rather, it may influence the interaction, and therefore function, of these microtubules with accessory proteins and, of course, provide target sites for C-terminal isotype-specific post-translational modifications (see later).

Raff and colleagues have also studied morphogenesis of *Drosophila* basal bodies, which contain only the β 1-tubulin isotype, and the sperm flagellar axoneme, where only the β 2-tubulin isotype is used. They asked whether β 1-tubulin alone can function in axonemes and found that it cannot (Raff et al., 2000). In these males, 9+0 axonemes were initiated at the basal body, and they extended for only a fraction of the normal length. Males possessing equal amounts of the isotypes showed equimolar incorporation of both into axonemes. Finally, increasing the amount of β 1-tubulin produced axonemes that had 10 doublets, and the addition of the extra doublet occurred by a mechanism of lateral insertion rather than templating at the basal body. There was also ectopic occurrence of doublets in the cytoplasm, which Raff and colleagues suggest represents promiscuous initiation, and reflects β 1-tubulin function in assembly of the triplet microtubules of the basal body. These

studies emphasise the fact that tubulin isotype can matter! Small differences between two β -tubulin isotypes influence their ability to form particular microtubule types. Overall, these studies emphasise the fidelity of axonemal doublet assembly from the basal body, which is influenced by direct templating or local concentrations of nucleating molecules. However, the properties of particular tubulin isotypes (e.g. $\beta 2$ but not $\beta 1$ tubulin) have an enormous influence upon assembly of the central pair microtubules and associated structures.

Structure of the $\alpha\beta$ -tubulin dimer

Although many eukaryotes encode multiple α - and β -tubulin genes, sequence analysis reveals that the amino acid sequences of these isotypic variants are generally well conserved, extreme divergence being limited to the C-terminal region. This remarkable conservation in sequence results from constraints imposed by the formation of the tertiary structure of the tubulin heterodimer. Nogales et al. have recently developed an atomic model of $\alpha\beta$ -tubulin, using electron crystallography approaches (Nogales et al., 1998b), and this demonstrates that α - and β -tubulin monomers have nearly identical structures, which can be divided into three domains. The N-terminal domain (residues 1-206), consisting of five α -helices and six parallel β -sheets, forms a classical Rossman fold and creates the GTP-binding domain. The second (intermediate) domain again comprises α -helices and β -sheets and in β -tubulin forms the binding site for the microtubule-stabilising drug taxol. The position of the taxol-binding site suggests mechanisms for its microtubule-stabilising activity (Amos and Lowe, 1999; Downing, 2000). The remaining domain comprises two α -helices, which form a crest on the outside surface of the microtubule protofilament. Although the extreme C-terminal region of $\alpha\beta$ -tubulin cannot be visualised in the atomic model, evidence suggests this is also exposed on the outside surface of the microtubule. Given that these residues represent the major source of variation between $\alpha\beta$ -tubulin isotypes, it has often been proposed that this domain determines the functional specificity of tubulin isotypes.

This view of the structure of the tubulin dimer has been extended to reveal the arrangements within the microtubule itself. A high-resolution model of the microtubule was recently generated by docking the 3-D crystal structure of tubulin into a 20 Å 3-D reconstruction of a microtubule (Amos, 2000; Nogales et al., 1999). This model predicts the detailed architecture of the microtubule and provides an insight into the molecular

interactions between tubulin molecules (see Fig. 2). These studies have revealed that most of the lateral interactions between tubulin protofilaments occur through the M-loops on one side contacting helix H3 in the adjacent protofilament

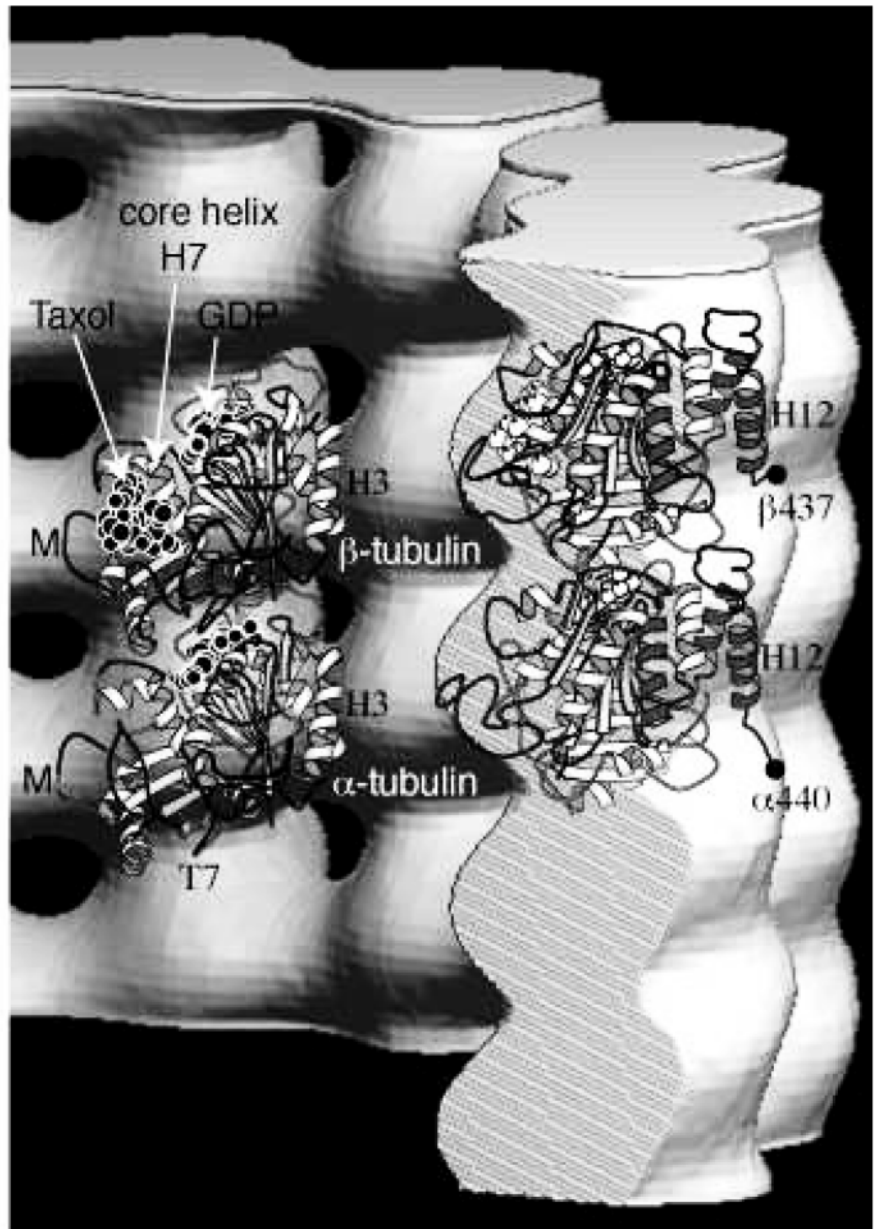


Fig. 2. Current model of microtubule structure at atomic resolution. The crystal structure of tubulin, represented as a ribbon model, is docked onto a low-resolution 3-D image of tubulin protofilaments. The figure shows a section through a microtubule; on the left is a view from inside the microtubule and on the right from one side of a protofilament. Lateral interactions between protofilaments occur through the M-loops on one side contacting the helix H3 in the adjacent protofilament. At the top of each monomer is a guanine-nucleotide-binding site; GTP (bound to α -tubulin) and GDP (bound to β -tubulin) is shown occupying sites at the interface between tubulin subunits. The nucleotide is contacted by loop T7 of the next subunit in the protofilament. Also shown (as a space-filling molecule) in a pocket on the inside surface of the β -tubulin subunit is the microtubule-binding drug taxol. At the tubulin C-terminus, the last residues visible by electron microscopy ($\alpha 440$ and $\beta 437$) are indicated by black circles; the conformations of the last 8-10 residues are unknown. Figure reproduced from Amos, 2000, with permission from Elsevier Science.

(Nogales et al., 1999). Structural interactions between tubulin protofilaments and molecules such as motor proteins and microtubule-associated proteins can also be predicted from this model (Amos, 2000).

Post-translational modifications of α - and β -tubulin

Both α - and β -tubulin undergo numerous post-translational modifications (Ludueno, 1998; McRae, 1997; Rosenbaum, 2000). Indeed in organisms such as the protists that express identical α - and β -tubulins (Silflow, 1991), post-translational modifications provide the only source of variation. Modifications such as acetylation, palmitoylation, phosphorylation and polyglutamylation are post-translational modifications found on other proteins; others such as detyrosination and polyglycylation appear to be tubulin specific. Because most tubulin modifications are reversible, they provide the potential for generating highly dynamic subgroups within the tubulin population. Although these modifications could impart distinct biochemical differences on tubulin molecules, note that they could also conceal isotype-specific differences and consequently reduce tubulin heterogeneity (McRae, 1997). Identification and elucidation of the cellular distribution of modified tubulins has been aided by use of specific monoclonal antibodies and, indeed, some of the modifications were initially identified by means of these reagents.

Tubulin acetylation

Many isotypes of α -tubulin are modified by the addition of an acetyl group to a lysine residue at position 40. Although the precise function of acetylated α -tubulin is uncertain, this modification is widespread, being found on α -tubulin from vertebrates to many protists. Acetylated α -tubulin is associated particularly with stable microtubular structures, such as axonemes, although this is not an invariant rule – for instance, in trypanosomes the ephemeral microtubules of the mitotic spindle are acetylated. Acetylation is a post-assembly phenomenon (Sasse and Gull, 1988), and the general correlation with stable microtubules is really a reflection of the relative length of time that the individual microtubule subunits are presented as a substrate for tubulin acetyltransferase (Maruta et al., 1986). Molecular engineering of protists has shown that organisms that normally express lots of acetylated α -tubulin can use non-acetylated tubulin (Rosenbaum, 2000). Engineering of *Tetrahymena* to express α -tubulin that cannot be acetylated at Lys40 produced mutant cells devoid of acetylated tubulin but expressing no novel phenotype (Xia et al., 2000). *Tetrahymena* is proving to be an excellent organism for such studies, because it constructs its microtubules from single α - and β -tubulin nascent isotypes. These studies suggest that acetylated α -tubulin is not important under the conditions tested or alternatively that other events/proteins can supplant the normal function. However, Stanchi et al. recently described a novel, tissue-specific isoform of α -tubulin in human and mouse (Stanchi et al., 2000) that is highly divergent around the acetylation motif. This isotype does not possess the Lys40 residue necessary for acetylation, and the amino acid sequence from position 32 to 45 is also highly divergent from all other human/mouse isotypes. The intriguing possibility that this isotype has evolved in a cellular environment in which the

acetylation motif is released from normal constraints needs to be addressed.

Tubulin C-terminal modifications

Interestingly, many tubulin modifications occur in the exposed acidic C-terminal domain of $\alpha\beta$ -tubulin, the region that also provides the major source of genetically encoded isotopic α - and β -tubulin variation. These modifications include the tyrosination/detyrosination of α -tubulin and the polyglutamylation and polyglycylation of both α - and β -tubulin. Unfortunately, these regions are not resolved in the crystal structure of tubulin, but their positions in the model strongly suggests that they are exposed on the outer surface of the microtubule (Nogales, 2000).

The tubulin tyrosination cycle involves the enzymatic removal of the C-terminal tyrosine residue present on some α -tubulin isotypes by a specific carboxy-peptidase, and its subsequent restoration by a tubulin-tyrosine ligase (reviewed by Idriss, 2000). Although the functional relevance of this modification is not always clear, highly stable microtubules such as those of the axoneme are detyrosinated, and this appears to reflect the length of time the individual α -tubulin substrate molecule has spent in a microtubule. The trypanosome cytoskeletal microtubules provide an opportunity for visualisation of this process along individual microtubules (Sherwin and Gull, 1989), and there are extensive descriptions of the distribution of detyrosinated α -tubulin in microtubule arrays. The tubulin-tyrosine ligase enzyme, its gene and related genes have been characterised (Ersfeld et al., 1993; Trichet et al., 2000). Although we still do not fully understand the functional implications of the tubulin tyrosination cycle, recent evidence indicates that detyrosination of tubulin can regulate interaction of microtubules with vimentin intermediate filaments by a kinesin-dependent mechanism (Kreitzer et al., 1999).

Removal of the penultimate glutamate residue from the α -tubulin polypeptide produces $\Delta 2$ -tubulin, a derivative that is unable to act as a substrate for tubulin-tyrosine ligase, and this truncated protein is therefore removed from the tyrosination cycle. $\Delta 2$ -tubulin is particularly prevalent on microtubular structures such as the axonemes of flagella and cilia and also in mammalian brain cell microtubules.

The tubulin modifications polyglutamylation and polyglycylation involve the attachment of oligoglutamyl and oligoglycyl side chains of variable length to specific glutamate residues located near the C-terminus of both α - and β -tubulin. These side chains can be of considerable length for instance, axonemal tubulin of *Paramecium* is modified by up to 34 glycyl residues (Bre et al., 1998), and the microtubules of *Trypanosoma brucei* contain 15 glutamyl residues per α -tubulin subunit (Schneider et al., 1997). Both α - and β -tubulin can be simultaneously modified by both polyglycylation and polyglutamylation (Mary et al., 1996), as well as being glutamylated at multiple sites (Schneider et al., 1998).

Polyglutamylation and polyglycylation are again particularly associated with stable microtubule structures such as the axonemes of cilia and flagella. Antibodies specific for these modifications inhibit the beating of flagella and cilia, implicating polyglutamylation and polyglycylation in regulation of axonemal function (Gagnon et al., 1996).

The axonemes of virtually all eukaryotic cilia and flagella

Table 1. A snapshot of the properties of each member of the tubulin family*

| Tubulin | Localisation/function |
|---------------|--|
| $\alpha\beta$ | Heterodimeric protein; basic building block of microtubules |
| γ | Essential role in initiating microtubule assembly at MTOCs, such as spindle pole body, centrosome and basal body |
| δ | Localised to the centrosome or basal body and other regions. δ -tubulin mutants exhibit basal-body defects - doublet rather than triplet arrangement of microtubules owing to specific loss of C tubule |
| ϵ | Localised to the centrosome in cell-cycle-dependent manner |
| ζ | Localised to the basal body area in <i>Trypanosoma brucei</i> |
| η | No localisation data available. <i>Paramecium</i> η -tubulin mutants show rare defects in basal bodies - lacking some microtubules from basal body triplets |

*The size of the data-sets available for each tubulin protein varies considerably - see main text for full details.

are remarkably similar in their organisation, consisting of nine outer doublet microtubules (designated the A and B tubules) surrounding a central pair of singlet microtubules. Studies on the axonemes of sea urchin sperm have also shown that that A and B tubules have differing degrees of tubulin modification (Multigner et al., 1996). The extent of tubulin polyglycylation and polyglutamylolation also varies in a gradient along the flagellum, so that a variety of structurally distinct regions appear to exist within the flagellum/cilium axoneme in different systems (Pechart et al., 1999). Recently, differences have been noted between the modifications present in centriolar microtubules and those of the axoneme. Centriolar microtubules appear to be polyglutamylated but not polyglycylation (Million et al., 1999). Polyglutamylolation appears to be critical for the stability of centriole microtubules, since microinjection of monoclonal antibodies specific for polyglutamylated tubulin isotypes, results in the transient disappearance of centrioles in mammalian cells (Bobbinet et al., 1998).

Polyglutamylolation also represents the major post-translational modification of axonal tubulin in neuronal cells, where it appears to regulate the differential interaction between microtubules and microtubule-associated proteins (MAPs). For instance, MAPs such as Tau and kinesin exhibit optimal binding to tubulin modified by ~3 glutamyl residues, binding affinity decreasing with increased polyglutamyl chain length (Boucher et al., 1994; Larcher et al., 1996). In contrast, increasing polyglutamyl chain length does not appear to affect the binding affinity of MAP1A significantly (Bonnet et al., 2001). Bonnet et al. suggest that the differential binding of MAPs to polyglutamylated tubulin could facilitate their selective recruitment to distinct microtubule populations and thereby modulate the functional properties of microtubules.

The importance of the polyglycylation modification has also been dissected by molecular genetic approaches in *Tetrahymena* (Xia et al., 2000), in which, as we have discussed above in the context of tubulin acetylation, one can engineer modifications to post-translational modification sites in both α - and β -tubulin. Modifying the multiple polyglycylation sites in α -tubulin, such that the modified tubulin isotype was unable to form in the mutant cell, produced no observable phenotype. In contrast, β -tubulin polyglycylation was essential. However, reducing but not eliminating polyglycylation of β -tubulin, by modifying a proportion of the sites, resulted in slow growth, reduced motility and defects in cytokinesis. Interestingly, a double mutant that has a fully non-glycylation β -tubulin and an α -tubulin that has a wild-type C-terminus from β -tubulin is viable. This α - β chimeric tubulin becomes hyperglycylation, which suggests that it is the level of polyglycylation modification rather than the specific isotype that is important.

Although this series of elegant experiments goes a long way to providing explanations for tubulin modifications (Rosenbaum, 2000), we probably still have some way to go to fit the explanation into a global context. The evidence regarding essential functions of polyglycylation revealed in *Tetrahymena* can be considered in evolutionary terms. Polyglycylation does not appear to be a universal modification of tubulin. The general observation is that most of the modifications appear to have developed early in the evolution of eukaryotic cells and are present in many protists. A comparison of representatives of three ancient groups – the trichomonads (*Tritrichomonas mobilensis*), the trypanosomatids (*T. brucei*) and the diplomonads (*Giardia lamblia*; Schneider et al., 1998; Schneider et al., 1997; Weber et al., 1997) – is informative in this respect. Tubulin acetylation and tubulin polyglutamylolation are present in all three, but tyrosination has been detected only in trypanosomes and may therefore have appeared after the trichomonads diverged from the rest of the eukaryotes. Conversely, polyglycylation has been detected in the diplomonads and not in the other two groups, which suggests it is an ancient modification that was subsequently lost in trichomonads and trypanosomatids. So, although polyglycylation appears to be an essential function in the ciliate *Tetrahymena*, the ancient flagellate *T. brucei* appears to be able to operate without it.

The extended tubulin superfamily

In addition to α - and β -tubulin, several other tubulins have now been identified, the number of distinct tubulin classes identified to date standing at seven (Chang and Stearns, 2000; Dutcher and Traubco, 1998; Oakley, 2000a; Oakley and Oakley, 1989; Ruiz et al., 2000; Vaughan et al., 2000). Most of these tubulins have now been shown to have distinct subcellular localisations and an emerging, diverse set of functions (see Table 1).

γ -tubulin

γ -tubulin was first identified in the filamentous fungus *Aspergillus nidulans* as a result of a genetic screen designed to identify proteins that interact with β -tubulin (Oakley and Oakley, 1989). *mipA* encodes a protein that was clearly a new member of the tubulin family and was termed γ -tubulin. It is approximately 30% identical to α - and β -tubulin, has subsequently been described in a wide variety of eukaryotic organisms and is likely to be present in all eukaryotes. γ -tubulin is located in centrosomes and other MTOCs, such as the spindle pole body (SPB) of fungi, where it plays an essential role in initiation of microtubule assembly. Eukaryotic cells, however, contain a large amount of soluble γ -tubulin. Some is

associated with the TCP-1 chaperonin complex, but much is associated with evolutionary conserved protein complexes (γ -TuRC and γ -TuSC) that play a central role in nucleation of assembly at the minus end of microtubules (Oakley, 2000a).

δ -tubulin

The gene encoding δ -tubulin was also first identified by genetic means, as a mutation resulting in defective basal body function in the green alga *Chlamydomonas* (Dutcher and Trabuco, 1998). The mutation results in elevated frequencies of formation of unflagellate cells, and their flagellar basal bodies possess doublet rather than the typical triplet arrangement of microtubules, owing to the specific loss of the C-tubule. Recently Garreau de Loubresse et al. have shown that inactivation of δ -tubulin in *Paramecium* results not only in the loss of the C-tubule but also in the loss of the B- and A-tubules at one or more triplet sites within the basal body (Garreau de Loubresse et al., 2001).

Database searches have also now identified δ -tubulin in humans, mice, rats and trypanosomes (Chang and Stearns, 2000; Vaughan et al., 2000), and we have observed that it is also present in other protists, such as the malarial parasite *Plasmodium*. Immunofluorescence detection of δ -tubulin in *Chlamydomonas* suggests that it is localised to the basal body area (Dutcher, 2001). Chang and Stearns immunolocalised human δ -tubulin to a centrosomal region between centrioles and also to an intercentriolar location between duplicated centrosomes (Chang and Stearns, 2000). However immunolocalisation of mouse δ -tubulin in somatic cells (Smrzka et al., 2000) suggested that this protein has a cytoplasmic and nuclear location, being only particularly enriched at spindle poles during mitosis. In contrast, in mouse sperm cells, δ -tubulin is associated with the perinuclear ring of the manchette, centriolar vaults and the flagellum.

Understanding δ -tubulin function in flagellated and non-flagellated cells will require ultrastructural localisation of the protein. This fine level of structural information will also probably be required for the other members of the extended tubulin superfamily that seem to have restricted distributions. Obviously, as more tubulins come to light, it makes the production of specific antibodies all the more necessary and all the more difficult!

ϵ -tubulin

ϵ -tubulin was discovered independently in mammalian cells by genomic approaches (Chang and Stearns, 2000) and in trypanosomes by a combination of genomic and cloning survey approaches (Vaughan et al., 2000). Database searching shows that this new tubulin is present in many but not all eukaryotes (see below). Immunolocalisation of ϵ -tubulin and GFP-tagged ϵ -tubulin in mammalian cells shows it to be located to the centriolar area, and this localisation shows cell-cycle-dependent modulation. During the early phase of the cell cycle, ϵ -tubulin associates predominantly with the old centrosome and only later in the cell cycle becomes associated with both the old and new centrosome (sometime after centrosome segregation). Mammalian cell centrosomes clearly exhibit cell-cycle-dependent protein patterns that reflect the structurally complex nature of replication and maturation of centrioles within the centrosome (Lange et al., 2000; Lange and Gull, 1995; Lange and Gull, 1996; Tassin and Bornens, 1999).

Immunolocalisation at higher resolution will be required if we are to unravel how the intricacies of ϵ -tubulin positioning at the centrosome relate to the replication and maturation of the centrioles at the centrosome core. Although the role of ϵ -tubulin is at present unresolved, Chang and Stearns have observed that centrosomes can nucleate microtubule assembly irrespective of ϵ -tubulin content, which indicates that ϵ -tubulin is not involved in microtubule nucleation (Chang and Stearns, 2000).

ζ -tubulin

ζ -tubulin was discovered in trypanosomes, and the only full-length sequences available so far are from *T. brucei* and *Leishmania major* (Vaughan et al., 2000). However, there is evidence (see below) from database searches that this branch of the tubulin superfamily also exists in other organisms (Dutcher, 2001). Immunofluorescence and immunoelectron microscopy has revealed that ζ -tubulin localises to the basal body region in trypanosomes and at the centriolar region in some animal cells (S.V., A. Baines, P.G.M. and K.G., unpublished).

η -tubulin

η -tubulin was discovered by genetic means in *Paramecium* and is encoded by the *SM19* gene (Ruiz et al., 2000). The *sm 19-1* mutation in *Paramecium* produces a cell phenotype in which basal body duplication is inhibited, the oral apparatus is reduced and γ -tubulin becomes mislocalised. Although we have no precise localisation of η -tubulin so far, the mislocalisation of γ -tubulin in *sm 19-1* mutants may indicate that η -tubulin tethers γ -tubulin (or γ -tubulin complexes) to the basal body. Ultrastructural studies of *sm 19-1* mutants reveal a rare, but perhaps important, defect in some basal bodies that lack microtubules from their triplets (3% of cross sections of basal bodies). Interestingly, the *Paramecium* η -tubulin sequence shows an extreme C-terminus that resembles that of α -tubulin (it contains a C-terminal EY dipeptide; see below).

FtsZ

FtsZ was originally defined as a bacterial cell division protein that can form protofilament structures and has a weak sequence homology to tubulin. The 3-D structure of the protein is remarkably similar to that of tubulin (Erickson, 1998; Lowe and Amos, 1998; Nogales et al., 1998a). The FtsZ family of proteins is represented in Bacteria, some of the Archaea, chloroplasts and some mitochondria (Margolin, 2000). Our recent bioinformatics analysis using PRINTS, and other approaches, has led to the discovery of a new group of FtsZ proteins encoded in the genomes of some of the Archaea. There are therefore sets of major subtypes of FtsZ, and the relationship of these to the new tubulins may provide some insight to common ancestors and/or evolutionary routes.

Sequence gazing

Post-translational modifications

Aligning the amino acid sequences of the new tubulins with α and β can be informative in assessing their potential post-translational modifications. It is of course impossible to be categorical. An initial analysis suggests the following.

Acetylation is unlikely, because the lysine residue at position 40 that becomes acetylated in α -tubulin is absent from all other tubulin sequences. Similarly, human and trypanosome γ -, δ - and ϵ -tubulin, as well as trypanosome ζ -tubulin, each lack a C-terminal tyrosine residue, which indicates that these new tubulins are unlikely to be modified by the tyrosination cycle to which α -tubulin is subjected. The C-termini of γ -, δ -, ϵ -, ζ - and η -tubulin generally lack the glutamate motifs that appear to be important for polyglutamylolation and polyglycylation (see Fig. 3). Hence, although we must await biochemical assessment, it seems unlikely that these members of the tubulin superfamily are subjected to these modifications. One exception is perhaps δ -tubulin, which has a small group of glutamates.

The monoclonal antibody YL1/2 is widely used to detect the presence of tyrosinated α -tubulin in microtubule structures, because it recognises an epitope minimally dependent on a C-terminally located tyrosine (or phenylalanine) residue preceded by aspartate or glutamate residue (Wehland et al., 1984). The existence of such a putative C-terminal epitope (-EY) in *Paramecium* η -tubulin is intriguing, particularly given that the α -tubulins of *Paramecium* lack the C-terminal epitope necessary for recognition by YL1/2. It has often been observed that this antibody stains basal bodies or centrioles in some organisms. This monoclonal might be detecting η -tubulin alone and/or tyrosinated α -tubulin at these sites in such organisms. As stated earlier, however, the discovery of these new tubulins indicates that, until the reactivities of antibody probes towards all tubulins are established, care must be taken in interpretation of such studies.

Structural comparisons

We have manually aligned the six full-length members of the tubulin superfamily identified in the African trypanosome, *T. brucei* (Fig. 3). Our alignment indicates that there are a number of insertions and deletions in the δ -, ϵ - and ζ -tubulin sequences when compared with $\alpha\beta$ -tubulin. Insertions and deletions that are >5 residues in length are highlighted in Fig. 3 and shown schematically in Fig. 4. The secondary structure of pig α -tubulin has also been added to indicate where these insertions and deletions might be located. Although this alignment takes into account this structure, so that insertions and deletions are restricted to loop regions wherever possible, some deletions appear to result in a disruption of the predicted secondary structure. This is most clearly exemplified in the ζ -tubulin sequence, in which a predicted deletion towards the C-terminus could result in the loss of H10. In the $\alpha\beta$ -tubulin sequence, H10 is predicted to be one of the regions that makes lateral contacts between protofilaments and longitudinal dimer contacts at the minus end surface of tubulin (Inclan and Nogales, 2001). However, given the current dearth of knowledge of ζ -tubulin function, the significance of this potential deletion is unclear.

Although it is evident from the alignment shown in Fig. 3 that the δ -, ϵ - and ζ -tubulin sequences contain a significant number of insertions and deletions, our analysis suggests that key regions involved in nucleotide binding (loops T1-T7) are conserved in all of the tubulin sequences. However, from this sequence gazing, we predict that while δ - and ϵ -tubulin could hydrolyse GTP, ζ -tubulin does not. We base this prediction on the observation that, from our alignment, Glu254 in α -tubulin

is conserved in the *T. brucei* δ - and ϵ -tubulin. This residue, which is conserved in all α -tubulins, is essential for GTP-hydrolytic activity. These results are in accord with the analysis of the human δ - and ϵ -tubulin sequence by Inclan and Nogales, who also recently proposed that δ - and ϵ -tubulin could probably hydrolyse GTP (Inclan and Nogales, 2001). We would point out that Inclan and Nogales suggested that the *T. brucei* δ -tubulin was divergent at this key residue (since it possesses an alanine instead of a glutamate residue); however, our alignment indicates that this glutamate residue is also conserved in *T. brucei*.

We have also noted that the δ -, ϵ - and ζ -tubulins show more variation in sequence than $\alpha\beta$ -tubulin. When the first protistan β -tubulins were sequenced in the 1980s, much discussion initially concentrated on the observation that β -tubulins participating in the construction of the complex axoneme/basal bodies of flagella and cilia appeared to be less divergent than those that do not participate (see, for instance, Singhofer-Wowra et al., 1986). This theme can be considered again in terms of these new tubulins, in the rather different context of participation in the construction of the main wall of a microtubule or particular MTOC. Naturally, there are other explanations possible and caveats even to this one. However, the finding that at least six diverse members of the tubulin superfamily are present in one trypanosome cell suggests cell biological distinctions in their functions. Whatever the precise mechanism of microtubule nucleation (Erickson, 2000), γ -tubulin is acknowledged to be a universal component of MTOCs (Oakley, 2000b). A distinct possibility is that these new tubulins are components of MTOCs or particular subsets of microtubules.

Using the structure of $\alpha\beta$ -tubulin as a model, Inclan and Nogales analysed potential interactions of γ -, δ - and ϵ -tubulin with the $\alpha\beta$ -tubulin microtubule (Inclan and Nogales, 2001). Sequence comparisons of α -, β -, γ -, δ - and ϵ -tubulin showed clear conservation of sequence in certain regions, leading to the speculation that functionally important contacts were being maintained. From this sequence/structure analysis, Inclan and Nogales suggested that δ -tubulin interacts longitudinally with α -tubulin at the minus end of the microtubule whereas ϵ -tubulin binds to the plus end of β -tubulin and acts as a microtubule-plus-end-capping protein. Similar analysis of the ζ - and η -tubulin sequences has not yet been undertaken. These *in silico* studies are extremely useful in predicting potential interactions of these new tubulins with the $\alpha\beta$ -tubulin microtubule, although possible interactions with γ -tubulin or other protein components of the centrosome or basal body also need to be addressed.

We have noted previously the unusual distribution of the new tubulins in eukaryotic genomes and a general correlation with triplet basal bodies or axonemes (Vaughan et al., 2000). Certainly, evidence now indicates that δ -, ϵ -, ζ - and η -tubulin all localise to, or affect functions at, the centriole or basal body area of cells (Chang and Stearns, 2000; Dutcher, 2001; Ruiz et al., 2000). We discuss below how the evolutionary distribution of the tubulin superfamily members fits with known microtubule cell biology. In the context of tubulin sequence gazing, we note that some of those organisms that do not possess δ -, ϵ -, ζ - and η -tubulins, such as yeast and *C. elegans*, are organisms whose γ -tubulins are rather divergent (Burns, 1995; Keeling and Logsdon, 1996; Spang et al., 1996). One constraint on the γ -

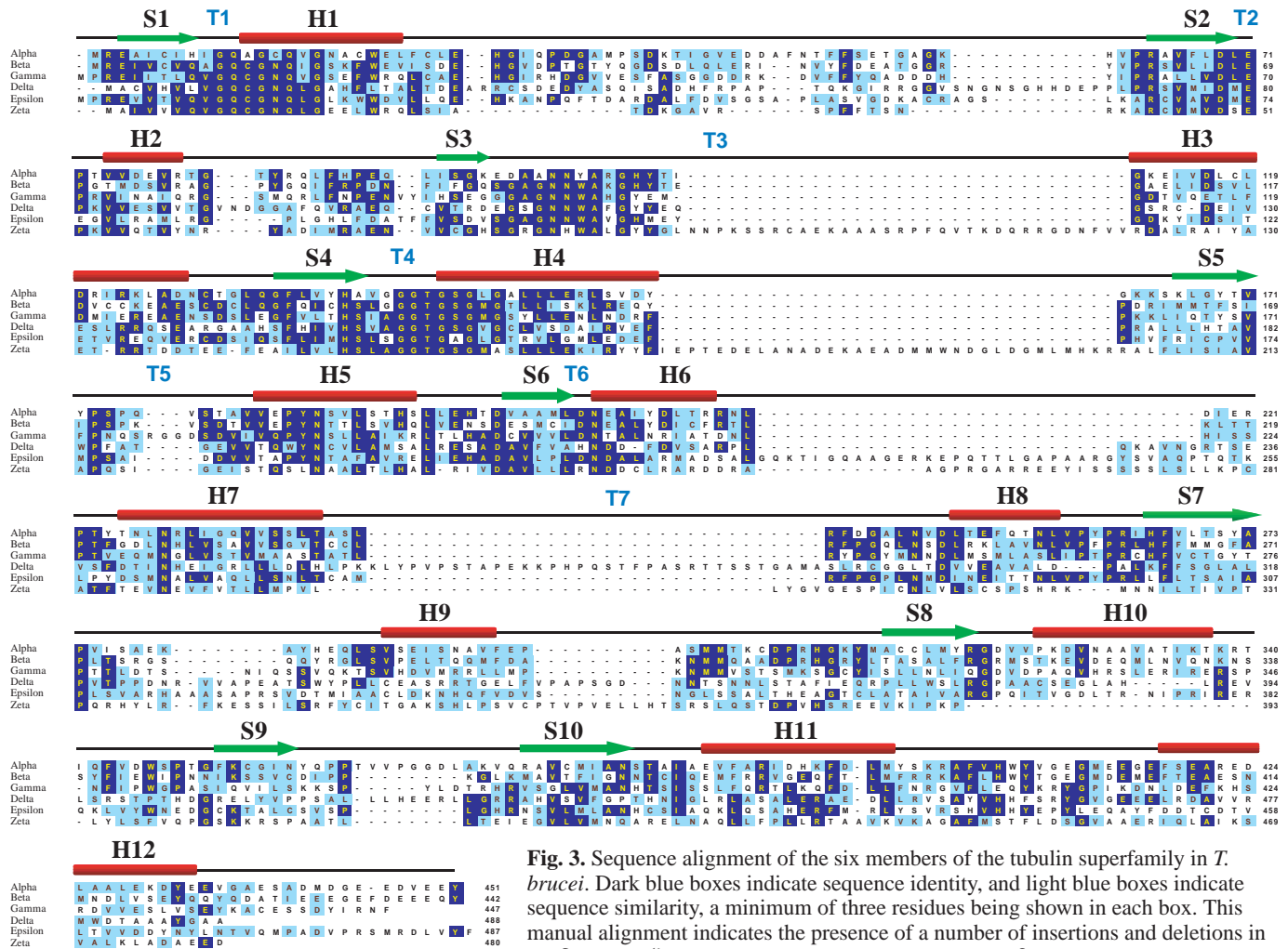


Fig. 3. Sequence alignment of the six members of the tubulin superfamily in *T. brucei*. Dark blue boxes indicate sequence identity, and light blue boxes indicate sequence similarity, a minimum of three residues being shown in each box. This manual alignment indicates the presence of a number of insertions and deletions in the δ -, ϵ - and ζ -tubulin sequence in comparison with $\alpha\beta$ -tubulin. Secondary structural elements of pig α -tubulin are indicated (PDB number 1TUB) to suggest where these insertions and deletions may be located. T1-T7 indicate loops that contain amino acid residues directly involved in nucleotide contacts (Nogales et al., 1998b).

structural elements of pig α -tubulin are indicated (PDB number 1TUB) to suggest where these insertions and deletions may be located. T1-T7 indicate loops that contain amino acid residues directly involved in nucleotide contacts (Nogales et al., 1998b).

tubulin sequence might be the need to interact with δ -, ϵ -, ζ - and η -tubulin in particular MTOCs. Organisms such as yeast would be released from such constraints, and their γ -tubulin sequences could have diverged as a consequence.

An evolutionary and functional survey of the extended tubulin superfamily

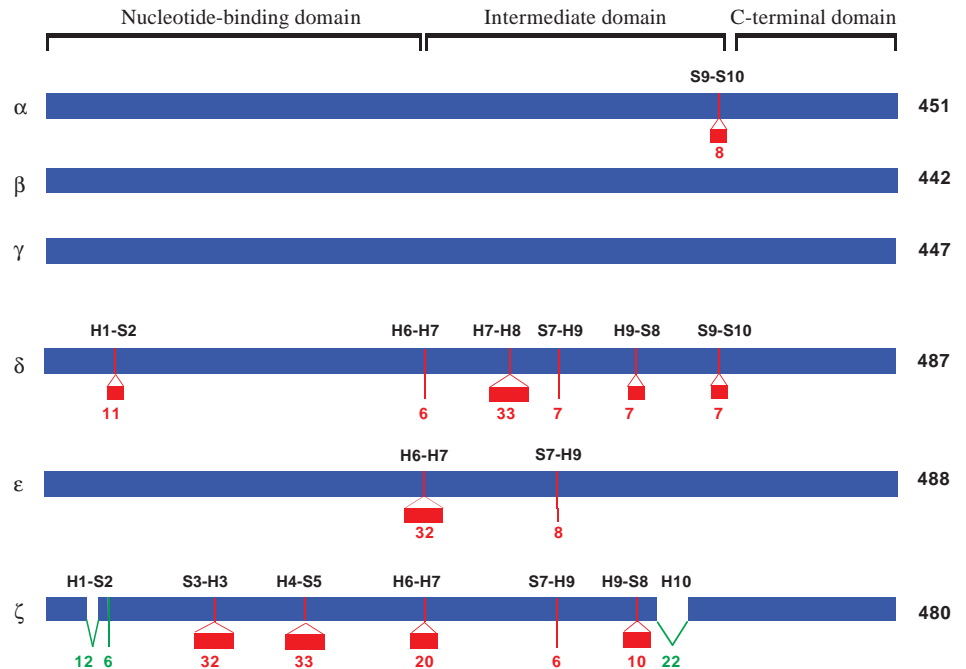
α -, β - and γ -tubulin appear to be present in every eukaryote, and in some cases are the only tubulins present; this is thus the minimal set of tubulins required to define microtubule function in certain eukaryotic cells. In contrast, the new tubulins have a restricted evolutionary distribution (Vaughan et al., 2000). The evolutionarily ancient protozoan *T. brucei*, for instance, exhibits at least six tubulin superfamily members (α , β , γ , δ , ϵ and ζ), whereas several other organisms, whose genomes are either complete or nearly complete, such as *S. cerevisiae*, *Arabidopsis thaliana*, *C. elegans* and *Drosophila melanogaster*, possess only the minimal set of α -, β - and γ -tubulin.

As we discussed above, there is a reasonable correlation between the occurrence of the new tubulins (δ , ϵ , ζ and η) and the appearance of a motile axoneme and triplet microtubule

basal body or centriole. The yeasts and plants are well known for their lack of such structures, whereas *C. elegans* has amoeboid sperm, and the sensory cilia of nematodes lack normal triplet microtubule basal bodies or centrioles (Albertson and Thomson, 1993; Perkins et al., 1986; Wolf et al., 1968). Although centrioles that have nine triplets have been reported in *Drosophila* (Gonzalez et al., 1998; Mahowald and Strassheim, 1970), variations in which the centrioles of *Drosophila* embryos have only doublet microtubules are known to occur. Callaini has debated these structures in insects and points out the possibility that ‘despite the vast literature on insect sperm structure, it is unclear whether these cells have a true centriole’ (Callaini et al., 1999). Microtubule doublets rather than triplets are commonly found in spermatocytes, and there have been erroneous interpretations of centriole structures in insects, since accessory microtubules around the axoneme often extend close to and even beyond the doublets. Hence, even if *Drosophila* has triplet microtubules in its centriole, they might not share all the attributes of more usual triplet microtubule centrioles or basal bodies.

An important question to be addressed, therefore, is why the centrosomes of *Drosophila* can dispense with these new

Fig. 4. Schematic representation of the alignment shown in Fig. 3, indicating the location of insertions and deletions in the *T. brucei* δ -, ϵ - and ζ -tubulin sequence. Red boxes indicate insertions and green chevrons indicate deletions of >5 amino acids; the actual length of each insertion/deletion is indicated. The proposed positions of these insertions/deletions with reference to the secondary structural elements of pig α -tubulin shown in Fig. 3 are denoted; for instance, S9-H10 indicates an insertion present on the loop between strand 9 and helix 10. The exact length of each tubulin sequence is shown at the right-hand side of the figure. Also indicated are the limits of the tubulin regions identified as the nucleotide-binding domain, the intermediate domain and the C-terminal domain.



tubulins. δ - and ϵ -tubulin cannot be the result of recent divergence in the tubulin superfamily, because these tubulins are present in the more evolutionarily ancient protozoan *T. brucei*. Greater knowledge of the molecular functions of δ - and ϵ -tubulin, as well as more detailed studies on the *Drosophila* centrosome, is undoubtedly required if we are to understand why these proteins are absent from the *Drosophila* genome.

The new tubulins are certainly not restricted to the protists, because full length δ - and ϵ -tubulin have been recognised in mammals (Chang and Stearns, 2000; Smrzka et al., 2000). It is perhaps slightly dangerous to identify certain tubulin sequences in genomes represented only by GSS or EST markers. However, both ζ - and η -tubulin appear to be represented in *Xenopus* (Dutcher, 2001), and there are good markers for δ -tubulin in these and other metazoan databases. Such EST databases provide us with intriguing glimpses of the evolutionary landscape, but full characterisation awaits the appearance of complete sequences and their analysis by a variety of bioinformatics approaches (Attwood et al., 2000; Vaughan et al., 2000).

Does this glimpse of the extended tubulin superfamily help our conjectures on function? We have rehearsed above our views on the correlation between possession of 9+0 triplet basal bodies or centrioles and these new tubulins. Thus, possession of these new tubulins (δ , ϵ , ζ , η) may indeed relate to construction of complex centrioles or basal bodies. However, it is important to note other attributes of such organelles apart from their intrinsic organisation. Defined patterns of centriole/basal body duplication and maturation are exemplified in protists such as trypanosomes, *Paramecium* and *Chlamydomonas* but are also seen in the centrioles of mammalian cells. Moreover, some eukaryotic cells are able to divide while expressing flagella/cilia, whereas others only exhibit cilia/flagella in differentiated, non-dividing cells such as sperm or ciliated epithelia. An important function of the new tubulins might be to endow the basal bodies and centrioles of dividing organisms with properties that facilitate their dynamic duplication, maturation and inheritance in certain cells.

Finally, many centrioles, basal bodies and axonemes exhibit very particular appendages and a circumferential polarity or anisotropy. Although there is little direct proof of biochemical non-equivalence of triplets (Beisson and Jerka-Dziadosz, 1999), there are very good structural examples that suggest non-equivalence of triplets in centrioles or basal bodies and doublets in the axoneme. Many of these structural anisotropies of the basal bodies have consequences for the precise positioning of accessory microtubule arrays emanating from these basal body MTOCs. We have long recognised the structural complexity of the basal body region (often most clearly seen in protists) as an integrator of cytoplasmic form through its nucleation of microtubule arrays. This area of eukaryotic cells may now be revealing its biochemical complexity.

The tubulin superfamily saga

The tubulin superfamily appears to be healthy, and our appreciation of its extent is growing. New members and new modifications and relationships observed. There is much still to be done if we are to understand the role of tubulin diversity in cell function and evolution. The quotation from Francis Quarles at the start of this essay seems rather appropriate when one looks back at the past thirty years of tubulin biology and biochemistry. Given the discoveries of the past few years, however, we might still be surprised at how this superfamily is arranged in the years to come. Since the nature of family life in the 21st century often seems to involve extended membership, divorce, renamings, adoptions, unclear parentage and other such events, we should not be too surprised at future events in the tubulin family saga!

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