5 Microtubules and Neurodevelopmental Disease: The Movers and the Makers

Martin Breuss and David A. Keays

Abstract

The development of the mammalian cortex requires the generation, migration and differentiation of neurons. Each of these cellular events requires a dynamic microtubule cytoskeleton. Microtubules are required for interkinetic nuclear migration, the separation of chromatids in mitosis, nuclear translocation during migration and the outgrowth of neurites. Their importance is underlined by the finding that mutations in a host of microtubule associated proteins cause detrimental neurological disorders. More recently, the structural subunits of microtubules, the tubulin proteins, have been implicated in a spectrum of human diseases collectively known as the tubulinopathies. This chapter reviews the discovery of microtubules, the role they play in neurodevelopment, and catalogues the tubulin isoforms associated with neurodevelopmental disease. Our focus is on the molecular and cellular mechanisms that underlie the pathology of tubulin-associated diseases. Finally, we reflect on whether different tubulin genes have distinct intrinsic functions.

Keywords

Microtubule • Cytoskeleton • Neurodevelopment • Neuronal migration • Tubulinopathies

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1 Introduction

Microtubules were discovered in the early 1950s by De Robertis and Franchi, who observed that axons of amphibian sciatic nerves were composed of "large bundle[s] of parallel, tightly packed fibrils" (De Robertis and Franchi 1953). They were similarly described by the neurocytologist Sanford Palay in 1956 as "numerous, long, tubular elements of the endoplasmic reticulum, about 180 Å wide and remarkably straight" (Palay 1956). Soon after Palay's description, various papers reported these structures in different cell types and organisms, such as avian and murine tumours or interstitial cells of hydra (De-The 1964; Slautterback 1963; Ledbetter and Porter 1964). Whereas Slautterback still assumed that they were membranous structures that could be involved in ion transport, De-Thê argued that the "protein nature of these microtubules is very probable" (De-The 1964; Slautterback 1963). In the same year, Ledbetter and Porter described a 13-fold radial symmetry of microtubules in plants and proposed an arrangement of longitudinal subunits that form hollow tubes (Ledbetter and Porter 1964). Shortly after this paper, the protein subunits that form microtubules were isolated, and finally, 1 year later, Mohri published the amino-acid composition of this protein and coined the expression tubulin (Mohri 1968; Shelanski and Taylor 1967). Stephens reported that there are at least two types of microtubule proteins, describing α- and β-tubulins and the tubulin-heterodimer (Stephens 1970). This work culminated in a conference in tubulin biology in 1975 (Taylor 1975) by which time a basic understanding of microtubule structure and function were established (see Fig. 5.1).

2 Tubulin Diversity and Genetics

Shortly after this conference, it became apparent that the α- and β- isoforms were not single entities, but rather existed in multiple flavours. N-terminal sequencing and biochemical separation experiments led Hayashi and colleagues to conclude that both types of tubulins include multiple isotypes consisting of slightly different amino acids (Bryan et al. 1978). The advent of molecular cloning further expanded the tubulin family. cDNA libraries constructed by Cowan and colleagues from chicken brain mRNA resulted in the identification of four α- and four β-tubulin genes; a list which has grown over the years (Krauhs et al. 1981; Lopata et al. 1983; Cleveland et al. 1978, 1980; Cowan et al. 1981; Wilde et al. 1982a, b; Cowan and Dudley 1983; Hall et al. 1983; Little et al. 1981; Ponstingl et al. 1981). With the completion of the human and mouse genome sequences, we now know that there are seven α- and eight β-tubulins in mice; and eight α- and nine β-tubulins in humans (Table 5.1).

With the exception of the carboxy terminus, the α- and β-tubulin isoforms exhibit a high degree of sequence homology; however, their expression pattern varies (Lewis et al. 1985; Villasante et al. 1986; Wang et al. 1986; Burgoyne et al. 1988). For instance, in humans the β-tubulin isoform TUBB1 is specifically found in platelets and megakaryocytes, whereas TUBB3 is expressed in post-mitotic neurons (Wang et al. 1986; Schulze et al. 2004; Liu et al. 2007). Similarly, in *Arabidopsis thaliana*, the *ArathTub9* isoform accumulates specifically in male reproductive tissue, the pollen, whereas *ArathTub1* is preferentially found in roots and leaves (Oakley et al. 2007; Snustad et al. 1992; Cheng et al. 2001).

3 The Multi-tubulin Hypothesis

The existence of this extended gene family with distinct expression patterns led investigators to speculate that the different tubulin isoforms possess unique functional properties, accounting for the extraordinary diverse role microtubules play in eukaryotic cells (Fulton and Simpson 1976). This concept, which is referred to as the multi-tubulin hypothesis, was advanced by Raff and co-workers who employed the

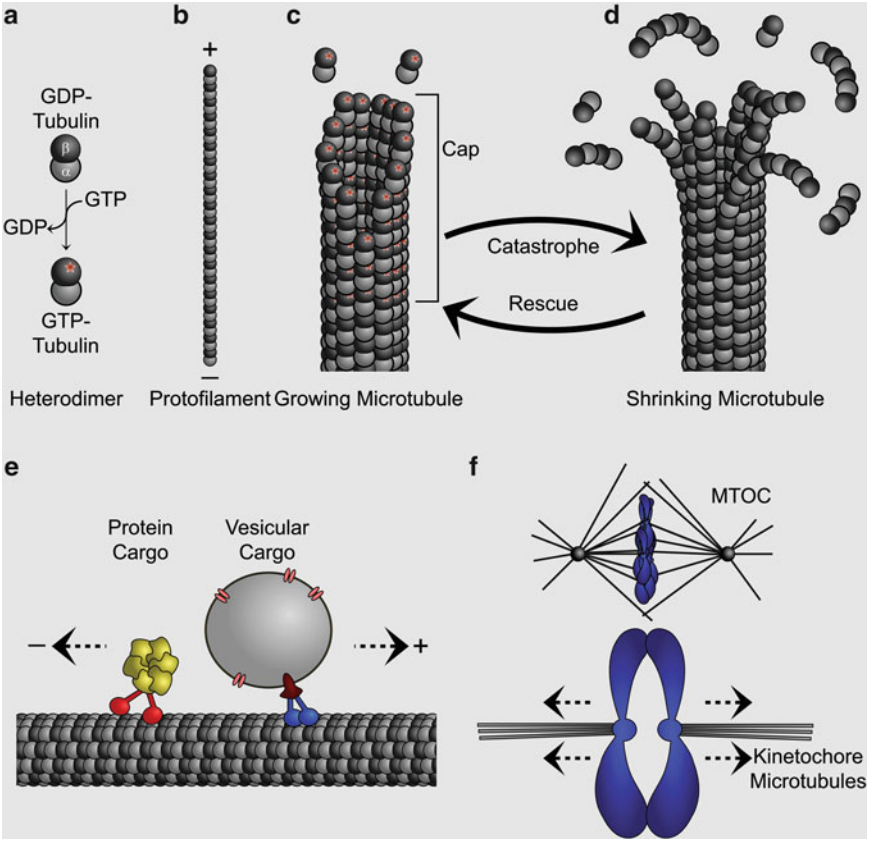


Fig. 5.1 Microtubule biology and cellular function. (**a**) Tubulin heterodimers are the basic building units of microtubules. Both isoforms can bind to a guanidine nucleotide. Whereas α-tubulin cannot hydrolyze and exchange GTP, **β**-tubulin can exist in two states: GTP- or GDP-bound. GTP-tubulin is the active state of the heterodimer with regards to polymerization capability (indicated with a *star*). (**b**) Tubulin heterodimers build up protofilaments by a juxtapositional head-to-tail arrangement. The β-tubulin is oriented towards the so called plus-end, the α-tubulin towards the minus-end. Protofilaments are arranged into a hollow microtubule and have lateral interactions with each other. Note that protofilaments are not formed per se, but are always part of the microtubule complex. (**c**) A polymerizing microtubule will add GTP-bound heterodimers, which will hydrolyze their nucleotide. This results in a GTP-tubulin cap at the plus-end of the microtubule. Due to low concentrations (*in vitro*) or the activity of certain accessory proteins the microtubule can change into a depolymerizing state; this transition is called catastrophe. (**d**) Depolymerizing microtubules are characterized by curved protofilaments (a structural trait of GDP-bound heterodimers) at the plus-tip end that leave the microtubule complex. The reversal of this state into a polymerizing microtubule is called rescue. (**e**) Rigid microtubules are used as transport highways for different cargo, such as macromolecular protein complexes or vesicles. Specialized motor proteins can attach to the microtubule lattice and show processive movement towards the plus- or the minus-end; these motors will bind to cargo and transport it along the microtubule. (**f**) Dynamic microtubules are needed for chromosome alignment in the metaphase plate (*upper panel*) and for sister chromatid separation during anaphase (*lower panel*). *MTOC* Microtubule Organizing Center, centrosome. Modelled on Akhmanova and Steinmetz (2008, 2010), Conde and Caceres (2009), Dogterom et al. (2005), Kuijpers and Hoogenraad (2011) and Schliwa and Woehlke (2003)

genetic tools available in *Drosophila* to replace the testes specific tubulin β-2 with the developmentally expressed β-3 tubulin (Kemphues et al. 1979). The β-3 isoform could support the assembly of a cytoskeletal array, but the substitution nevertheless resulted in defects of axoneme structure, meiosis, and nuclear shaping (Hoyle and Raff 1990; Raff et al. 1997). The Raff group was further able to show that the architecture of microtubules was influenced by the isoform

Table 5.1 List of all human and Murine Tubulin-Isotypes

	Symbol		Name	NCBI Gene ID	
	Mouse	Human	Mouse/Human	Mouse	Human
α-Tubulins	Tuba1a	TUBA1A	Tubulin, α 1A	22142	7846
	Tuba1b	TUBA1B	Tubulin, α 1B	7846	10376
	Tuba1c	TUBA1C	Tubulin, α 1C	22146	84790
	Tuba3a	–	Tubulin, α 3A	22144	–
	Tuba3b	–	Tubulin, α 3B	22147	–
	–	TUBA3C	Tubulin, α 3C	–	7278
	–	TUBA3D	Tubulin, α 3D	–	113457
	–	TUBA3E	Tubulin, α 3E	–	112714
	Tuba4a	TUBA4A	Tubulin, α 4A	22145	7277
	Tuba8	TUBA8	Tubulin, α 8	53857	51807
β-Tubulins	Tubb1	TUBB1	Tubulin, β 1 Class VI	545486	81027
	Tubb2a	TUBB2A	Tubulin, β 2A Class IIA	22151	7280
	Tubb2b	TUBB2B	Tubulin, β 2B Class IIB	73710	347733
	Tubb3	TUBB3	Tubulin, β 3 Class III	22152	10381
	Tubb4a	TUBB4A	Tubulin, β 1 Class VI	22153	10382
	Tubb4b	TUBB4B	Tubulin, β 4B Class IVB	227613	10383
	Tubb5	TUBB5	Tubulin, β 5 Class I	22154	203068
	Tubb6	TUBB6	Tubulin, β 6 Class V	67951	84617
	–	TUBB8	Tubulin, β 8 Class VIII	–	347688

For all isoforms the human and the murine gene symbols are given in addition to a full name. Note that a revised nomenclature for the β-tubulin isoforms is shown and based on the nomenclature of the α-tubulin isoforms (Khodiyar et al. 2007)

composition. Transgenic expression of the moth β-tubulin (Hvβt) alongside the β-2 isoform resulted in a Drosophila germline that was dominated by microtubules with 16 protofilaments, not the usual 13 (Hoyle and Raff 1990; Raff et al. 1997).

Parallel to these reports a mutagenesis screen performed in the group of Martin Chalfie identified a specific β-tubulin isoform (MEC7) that caused loss of touch receptivity in the nematode worm *C. elegans* (Savage et al. 1989, 1994). Similar to Raff and colleagues, they observed that the isoform composition of microtubules could affect the microtubule superstructure. MEC-7 mutants showed a shift from microtubules with 15 protofilaments to microtubules with just 11 protofilaments. This result was mirrored by another *C. elegans* strain harboring mutations in the α-tubulin MEC-12, which is also highly expressed in touch-sensitive neurons and is believed to co-assemble with MEC-7. Mutations in this tubulin again resulted in the loss of microtubules with 15 protofilaments (Fukushige et al. 1999).

These findings suggested that the protofilament number is a fixed inherent property of microtubules which is dependent on the tubulin composition. This is supported by the finding that the predominant configuration of mammalian microtubules in cells is 13 protofilaments (McIntosh et al. 2009; Tilney et al. 1973). However, *in vitro* experiments have shown that vertebrate tubulin-heterodimers by themselves assemble into microtubules ranging from 8 to 17 protofilaments (Chretien et al. 1992; Chretien and Wade 1991). Therefore, preference for a specific number of protofilaments for one isoform can only be determined by interaction with factors present *in vivo*.

So, what factors determine the protofilament number? Brouhard and colleagues have demonstrated that the microtubule associated protein

DCX (Doublecortin) stabilizes the 13 protofilament configuration *in vitro* and they argue that this might be one of the main mechanisms that ensure correct protofilament numbers in neurons (Bechstedt and Brouhard 2012). Likewise, the nucleation by a γ-tubulin ring complex contributes to a consistent width of cellular microtubules (Moritz et al. 2000). Posttranslational modifications may also play a role. Goodman and colleagues have shown that acetylation of the α-tubulin MEC-12 stabilizes the 15 protofilament configuration found in *C. elegans* touch receptive cells (Cueva et al. 2012). The deletion of the responsible acetylase, ATAT-2, results in highly variable protofilaments numbers. This observation has led Goodman and colleagues to the proposition that acetylation promotes the formation of salt bridges that mediate lateral interactions between protofilaments (Cueva et al. 2012). In their model an interaction between glutamate at position 55 and lysine 40 exists within the α-tubulin (αE55-αK40). This salt bridge is disrupted by acetylation of the K40 residue, favoring an interaction between adjacent heterodimers (αE55-α′H283), the angle of which is consistent with 15 protofilament microtubules (Cueva et al. 2012).

4 Posttranslational Modifications

Acetylation is but one of a myriad of different posttranslational modifications associated with the tubulins. Others include detyrosination, polyglutamylation, polyglycylation, palmitolyation and phosphorylation (Janke and Bulinski 2011; Westermann and Weber 2003). These modifications affect tubulin dynamics and stability, the interaction with motor proteins and also non-motor microtubule associated proteins. The amino acid sequence of individual tubulin isoforms influences their respective posttranslational modifications. For instance, in mice and humans TUBA8 lacks a lysine at the critical residue 40, and consequently cannot be acetylated. This contrasts with the remaining members of the α-tubulin family, all of which have this residue, and therefore can be subject to this modification (Fukushige et al. 1999; Stanchi et al. 2000). Likewise, some tubulin isoforms, such as the testis specific cα2 in chicken, lack a carboxy-terminal tyrosine residue and are therefore not subject to detyrosination (Pratt et al. 1987).

5 Tubulin proteins in Neurodevelopmental Disease – The Makers

Since their discovery in the 1950s it is has been clear that microtubule function is essential for the formation and function of the nervous system in a broad range of animal species, whether it be a nematode, a fruit fly, a frog or a rodent (Goldstein and Yang 2000; Gerson et al. 1976; Gray 1975, 1976; Ward et al. 1975; Poulain and Sobel 2010). It is no surprise that the same holds true for the development of the human brain. Microtubules facilitate neurogenic division, they drive neuronal migration, and they are required for neuronal differentiation and circuit formation (Ayala et al. 2007; Kuijpers and Hoogenraad 2011) (Fig. 5.2). Here we discuss the role of the different tubulins in these processes with a focus on human diseases caused by mutations in these genes (Fig. 5.3).

6 *TUBA1A* – The First

The tubulin gene family was first implicated in neurodevelopmental disease following the cloning of an N-ethyl-N-nitrosourea (ENU) induced *Tuba1a* mutation in the *Jenna* mouse mutant (Keays et al. 2007). It was identified in a screen for hyperactive behavior, but also showed defects in working memory and presented with an exaggerated acoustic startle response (Edwards et al. 2011; Keays et al. 2007, 2010). Histological examination revealed wave-like perturbations of the adult cortex, a fractured pyramidal layer of the hippocampus and structural abnormalities in the superior colliculus; defects which were

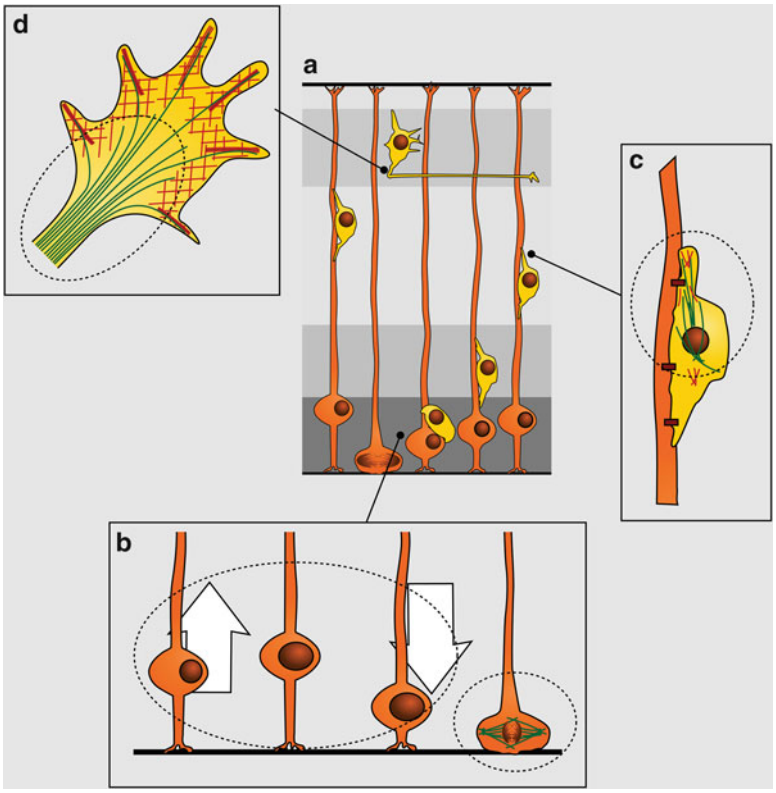


Fig. 5.2 Examples for microtubule functions in neurodevelopment. (**a**) Schematic of the developing cortex. Radial glial progenitors span the entirety of the cortex from the ventricular to the pial surface (shown in *orange*). They undergo mitosis to generate other types of progenitors (not shown) and neurons (shown in *yellow*). Postmitotic neurons migrate along their radial glial mother cell to their final destination in the cortical plate where they will differentiate and extend their axons. (**b**) Detailed view of the interkinetic nuclear migration of a radial glial cell. The cell nuclei migrate basally (*upwards*) during G1 phase, undergo S phase and migrate apically (*downwards*) during G2. Finally, cells will undergo mitosis at the ventricular surface. Microtubules are required for interkinetic nuclear migration and spindle formation (shown in *green*) which mediates sister chromatid separation in M phase. (**c**) Neuronal migration requires nuclear translocation. Nuclei are surrounded by a microtubule cage (nuclear cage; shown in *green*) that connects with the actomyosin network (shown in *red*) via the centrosome. (**d**) Differentiating neurons extend their neurites to form connections. Axonal projections have to cover large distances within the brain and the correct pathfinding requires the establishment of a growth cone. This specialized structure consists of microtubules (shown in *green*) that provide a rigid platform which interacts with the actin cytoskeleton (shown in *red*), facilitating the extension of the lamellipodia

attributed to impaired neuronal migration. These phenotypes were reminiscent of mouse models of lissencephaly (*Lis1*, *Dcx*, and the *Reeler* mouse), a disease which is characterized by a cortex with a smooth surface (Gleeson and Walsh 2000; Guerrini and Parrini 2010) (see also Chap. 1). Speculating that mutations in *TUBA1A* might cause neurodevelopmental disease in humans, a genetic screen identified two *de novo* mutations in this gene (R264C and R402H) in patients with lissencephaly (Keays et al. 2007).

The introduction of a *TUBA1A* genetic test into clinical practice has resulted in the identification of a host of disease causing mutations in this gene (Poirier et al. 2007, 2012; Fallet-Bianco et al. 2008; Bahi-Buisson et al. 2008; Morris-Rosendahl et al. 2008; Kumar et al. 2010; Lecourtois et al. 2010; Jansen et al. 2011; Mokanszki et al. 2012; Sohal et al. 2012; Hikita et al. 2013). Most patients identified have *de novo* mutations and present with a spectrum of phenotypes that extends from an absence (agyria), to a

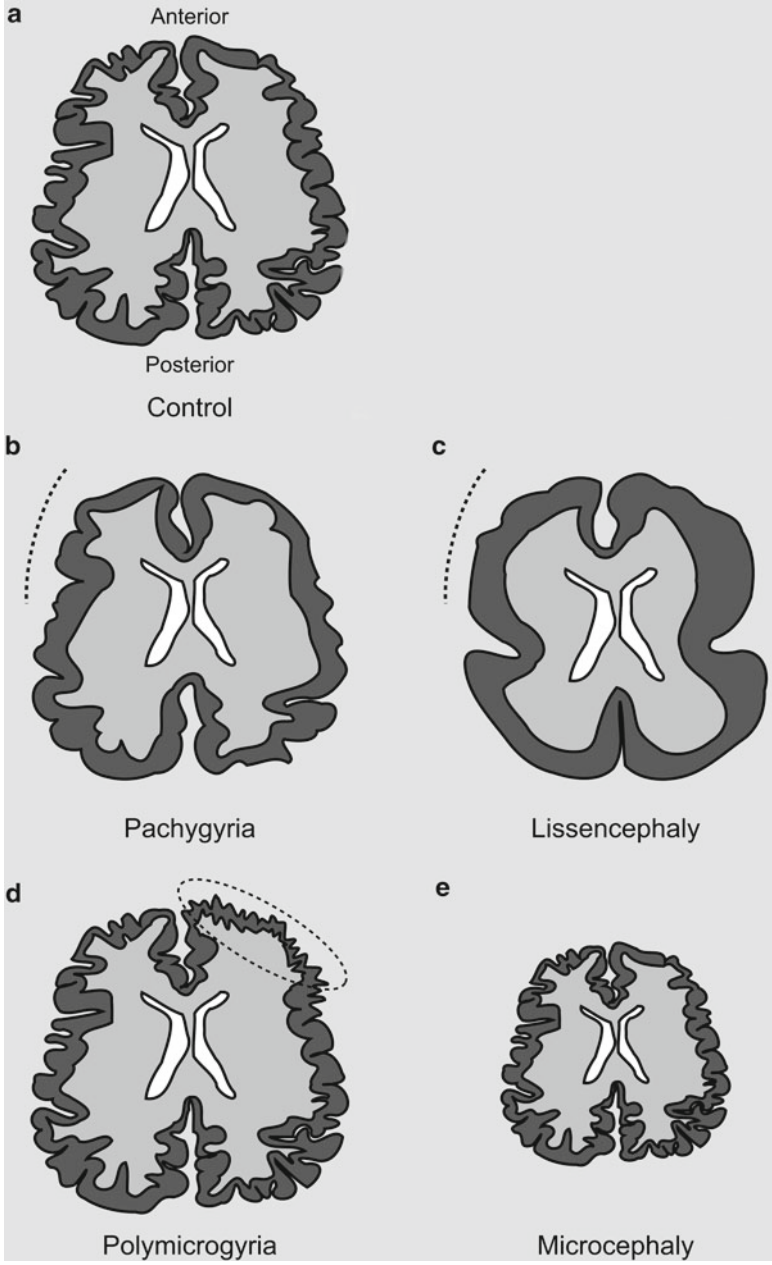


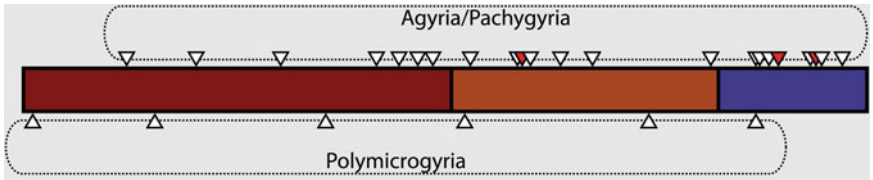
Fig. 5.3 Spectrum of tubulinopathies. (**a**) Depiction of an axial section of a control brain with regular distribution and number of sulci and gyri. (**b**) Pachygyric (meaning thick gyri) patients show a reduction in the number and increase in the size of their gyri (indicated with a *dotted line*). (**c**) Lissencephalic (meaning smooth brain) patients show a complete absence of sulci and gyri. Both pachygyria and lissencephaly can be present as a gradient from anterior to posterior. (**d**) Polymicrogyric (meaning many small gyri) patients show an increase in the number of gyri with a decreased size. This is often focally localized and asymmetric. (**e**) Microcephalic (meaning small head) patients show a reduction in overall brain size (−2SD from the mean). Microcephaly vera (or primary microcephaly) occurs in the absence of other cortical malformations

reduction (pachygyria) or even an increased number of gyri (polymicrogyria) (Poirier et al. 2007, 2012; Kumar et al. 2010). These cortical phenotypes are frequently accompanied by hypoplasia or agenesis of the corpus callosum, hypoplasia of the brain stem, dysgenesis of the basal ganglia, ventricular dilation, and hypoplasia of the cerebellum (Sohal et al. 2012; Kumar et al. 2010). In addition, almost all patients with *TUBA1A* mutations present with a reduction in brain size (−1 S.D. to −7 S.D. from mean), most classifying as microcephalic (less than −2 S.D. below mean; more than 90 %) (Poirier et al. 2007, 2012; Sohal et al. 2012; Kumar et al. 2010).

7 Molecular and Cellular Mechanisms of *TUBA1A* Mutations

What is the underlying molecular defect that results in the disease state in patients with *TUBA1A* mutations? TUBA1A, like all tubulins protein, has three major domains; an N-terminal domain (1–229), an intermediate domain (230–371), and a C-terminal domain (372–450). The N-terminal domain harbors a GTP binding pocket that, in the case of α-tubulins, is non-exchangeable and is thought to act as a structural co-factor (Nogales et al. 1997; Spiegelman et al. 1977). In the case of the *Jenna* mouse it was shown that the S140G mutation caused impairment in GTP binding, and, consequently, a dramatic reduction in heterodimer formation. The mutant heterodimers, however, were able to incorporate into the microtubule cytoskeleton, suggesting that the mutation acted by haploinsufficiency. Similarly, the human mutations V303G, L397P, and R402C all result in a reduction in heterodimer levels, which have been attributed to molecular defects in the tubulin folding pathway (Tian et al. 2008, 2010). It is apparent, however, that some disease causing tubulin mutations have no effect on the efficiency of chaperon mediated tubulin folding whatsoever. For instance, *in vitro* analysis of the P263T, L286F, R402H, and S419L mutations has shown that they do not cause impaired heterodimer folding. In the case of the P263T mutation the incorporation of mutant heterodimers into the microtubule lattice has a deleterious effect of microtubule dynamics and growth, lending itself to the conclusion that some tubulin mutations act by a dominant negative mechanism (Tian et al. 2010). Mutations that fall within this class may influence the binding of microtubule associated proteins such as DCX or the kinesins (Amos and Schlieper 2005). Tubulins might also interact with unknown microtubule associated proteins that are vital for the formation of the developing brain.

What are the underlying cellular mechanisms that give rise to *TUBA1A*-related disease? In addressing this question it is important to appreciate that *TUBA1A* is highly expressed in post-mitotic neurons, but not glia, in the human and mouse brain (Gloster et al. 1999; Bamji and Miller 1996). Murine expression studies have shown that TUBA1A is largely absent from the proliferative ventricular zone (VZ), and its expression peaks at embryonic day (E) 16.5 (Braun et al. 2010). The migration of neurons requires the extension of the leading process, the translocation of the nucleus and the retraction of the trailing process (Trivedi and Solecki 2011) (see also Chaps. 1, 2, 4 and 7). All of these processes are heavily reliant on a dynamic microtubule network, and could potentially be impaired by mutations in *TUBA1A*. Similarly, neurite outgrowth requires the stable support and dynamic force generated by microtubules (Dent and Gertler 2003). Defects in this process can cause inadequate crossing of the midline, resulting in an abnormal corpus callosum and neurological defects (Engle 2010). Disorders of axon guidance or migration, however, fail to account for the reduction in brain size that is observed in almost all patients with mutations in *TUBA1A*. This is particularly curious, given its post-mitotic expression. One explanation that might account for this phenotype is an increase in neuronal apoptosis, which has been observed in the adult superior colliculus in the *Jenna* mouse (Edwards et al. 2011). This explanation is consistent with the observation that *TUBA1A* associated microcephaly can increase in severity postnatally (Cushion et al. 2013).



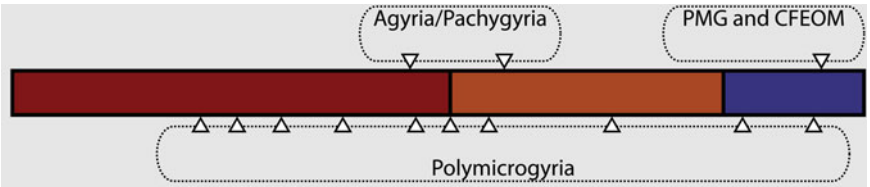
Gene Name:	*TUBA1A* (NCBI Gene ID 7846)
Genomic Location:	Chromosome 12
Size:	451 amino acids
Expression:	Predominantly post-mitotic neurons
Malformations:	Lissencephaly (Agyria) to Pachygyria; or Polymicrogyria; Microcephaly Hypoplasia or agenesis of the corpus callosum Hypoplasia of the brain stem Dysgenesis of the basal ganglia Ventricular dilation Hypoplasia of the cerebellum
Mutations	
Agyria/Pachygyria:	E55K; *L70S*; L92V; V137D; I188L; C200Y; Y210C; D218Y; I238V; P263T; R264C (Hotspot); A270T; L286F; V303G; G366R; R390C; N392S; L397P; R402H; R402C; R402L; S419L; R422C; R422H; M425K; G436R
Polymicrogyria:	I5L; Y161H; V235L; A333V; R390C

Fig. 5.4 Mutations associated with *TUBA1A*

Why do mutations in *TUBA1A* cause a spectrum of distinct neurological disorders? Initially this gene was strongly associated with lissencephaly/pachygyria, but it is now clear that *de novo* mutations can also cause polymicrogyria. For instance, a mutation in valine 235 (V235L) results in bilateral and asymmetric polymicrogyria, whereas mutations in arginine 402 (R402C, R402H) cause classic lissencephaly (Mokanszki et al. 2012; Kumar et al. 2010; Poirier et al. 2007). An analysis of the position of polymicrogyria and lissencephaly causing mutations reveals no obvious pattern (Fig. 5.4). It is conceivable that different diseases are a consequence of defects in different cellular processes associated with microtubule based neuronal migration. However, this would not account for the interesting case of the R390C mutation. This very same mutation has been reported to cause polymicrogyria in a 1-year-old boy and mild gyral simplification and total agenesis of the corpus callosum in another child (Poirier et al. 2012; Kumar et al. 2010). How does the same mutation cause two distinct migration phenotypes? One possibility could be the exposure to different environmental conditions *in utero*; or additional genetic factors that contribute to one or the other phenotype.

8 *TUBB2B* – Expanding the Spectrum

Given that mutations in *TUBA1A* cause neurodevelopmental disease, it was reasonable to speculate that mutations in the β-tubulins might also be pathogenic. Following a genetic screen of



Gene Name:	*TUBB2B* (NCBI Gene ID 347733)
Genomic Location:	Chromosome 6
Size:	445 amino acids
Expression:	Post-mitotic neurons, but also at lower levels in progenitors
Malformations	Polymicrogyria with or without CFEOM; or Agyria/Pachygyria Microcephaly Dysmorphic basal ganglia Cerebellar dys- or hypoplasia Abnormal corpus callosum Brain stem hypoplasia
Mutations	
Polymicrogyria:	G98R; L117P; G140A; S172P; I210T; L228P; A248V; F265L; T312M; R380L; R380S; R380C; D417N
Agyria/Pachygyria:	L207P; N256S
PMG and CFEOM:	E421K

Fig. 5.5 Mutations associated with *TUBB2B*

TUBB2A, *TUBB2B*, and *TUBB2C*, the Chelly group reported the identification of five cases of asymmetrical polymicrogyria (four patients, one aborted fetus) caused by mutations in *TUBB2B* (Jaglin et al. 2009). Besides asymmetrical polymicrogyria, each patient presented with additional features, such as microcephaly, dysmorphic basal ganglia, cerebellar dys- or hypoplasia, abnormal corpus callosum and brain stem hypoplasia. Similar to *TUBA1A*, the spectrum of *TUBB2B* related diseases has expanded rapidly. Engle and colleagues recently reported the occurrence of a mutation (E421K) that causes congenital fibrosis of the extraocular muscles (CFEOM), a specific defect of axon guidance, accompanied by polymicrogyria (Cederquist et al. 2012); axon guidance defects accompanied by polymicrogyria and schizenecephaly have also been reported for a G140A mutation (Romaniello et al. 2012). Pilz and colleagues have described a lissencephalic patient with a *TUBB2B* mutation (L207P), and Guerrini and colleagues have reported an individual with pachygyria and microcephaly with an N256S mutation (Cushion et al. 2013; Guerrini et al. 2012) (Fig. 5.5). To date biochemical analysis has been conducted on five *TUBB2B* mutations (F265L, I210T, L228P, S172P and T312M) and, similar to *TUBA1A* mutations, they influence tubulin heterodimer folding and their incorporation into microtubules in different ways. For instance, the S172P mutation results in arrested tubulin heterodimer folding, whereas the I210T is indistinguishable from the wild-type in biochemical and cellular assays.

As might be expected *TUBB2B* is highly expressed in post-mitotic neurons at key develop-

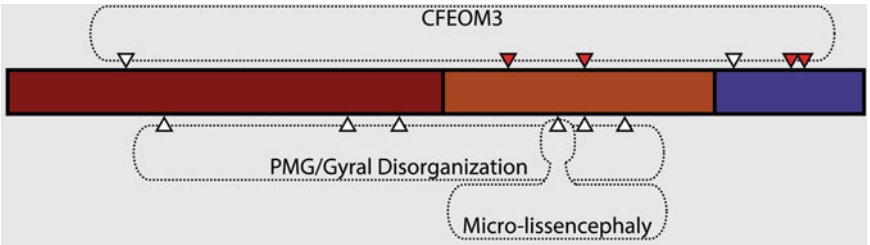
mental time-points, but is also found in progenitor cells at lower levels (Jaglin et al. 2009). *In vivo* knockdown experiments in the rat have shown that *Tubb2b* is required for radial migration. These data have led to the hypothesis that *TUBB2B*-related cortical malformations are due to a combination of impairment in neuronal migration and radial glial dysfunction (Jaglin et al. 2009).

9 *TUBB3* – The Janus Tubulin

The list of tubulinopathy causing genes expanded in 2010 with the addition of *TUBB3* by two independent studies. Engle and colleagues showed that six different heterozygous mutations in this gene caused congenital fibrosis of the extraocular muscles type 3 (CFEOM3), either in isolation or as a component of a syndrome (Tischfield et al. 2010). Interestingly, and in marked contrast to the previously described *TUBB2B* and *TUBA1A* mutations, no neuronal migration deficits or microcephaly could be observed in these patients. The pathogenicity of one mutation (R262C) was explored further by the creation of a transgenic mouse line that replicated various aspects of the human disease. The R262C mutation increased microtubule stability and impaired their interaction with the motor protein Kif21a. Ultimately, this resulted in defects of axon guidance and cranial nerve extension, but not cortical architecture (Tischfield et al. 2010). In the same year, the Chelly group reported six different *TUBB3* mutations (five heterozygous, one homozygous) in nine patients with malformations of cortical development associated with neuronal migration defects (Poirier et al. 2010). All patients suffered from polymicrogyria or gyral disorganization with microcephaly and cerebellar dys- or hypoplasia. With the exception of one individual, these patients presented with brainstem hypoplasia, an abnormal corpus callosum and dysmorphic basal ganglia, but not the CFEOM3 phenotypes described by Engle and colleagues (Fig. 5.6). None of the mutations identified by the Chelly group were the same as those that cause CFEOM3; curiously, however, both studies described different mutations in the same residue, A302. Its substitution with a threonine caused CFEOM3, whereas the A302V mutation was pathogenic in a patient with gyral disorganization (Tischfield et al. 2010; Poirier et al. 2010). These data imply that the mutations probably act by a dominant mechanism and not by haploinsufficiency. This idea is supported by the observation that mutations that cause cortical malformations, in contrast to the ones causing CFEOM3, reduce microtubule stability (Tischfield et al. 2010; Poirier et al. 2010). Finally, it should be noted that the phenotypes associated with *TUBB3* have recently been expanded to include peripheral neuropathy, defective olfactory function, photophobia, cyclic vomiting and hypogonadotropic hypogonadism with analogies to Kallmann syndrome (Chew et al. 2013).

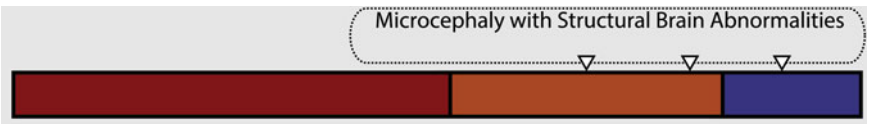
10 *TUBB5* – The Mitotic Tubulin

We have added another tubulin isoform to the list of disease-causing genes: *TUBB5* (Breuss et al. 2012). In contrast to the other tubulinopathies, the primary defect associated with *TUBB5* is microcephaly. We reported three unrelated individuals with *de novo TUBB5* mutations (M299V, V353I, E401K) with only one patient presenting with a notable migration phenotype (M299V) (Fig. 5.7). Similar to other tubulin isotype-dependent disorders, affected individuals presented with dysmorphic basal ganglia and corpus callosum abnormalities. Employing a transgenic mouse line that expresses GFP under the endogenous *Tubb5* promoter, we have shown that *TUBB5* is expressed in radial glial progenitors, intermediate progenitors, and post-mitotic neurons. Depletion of *TUBB5 in utero* by shRNA knockdown perturbed the cell cycle of progenitors and resulted in neuronal migration defects. Similarly, we have found that overexpression of two of the three *TUBB5* mutations (E401K and V353I) increased the percentage of progenitors in M-phase and altered neuronal positioning. Intriguingly, these two mutations affected the tubulin folding pathway in different ways. The behavior of the



Gene Name:	*TUBB3* (NCBI Gene ID 10381)
Genomic Location:	Chromosome 16
Size:	450 amino acids
Expression:	Exclusively post-mitotic neurons
Malformations:	CFEOM3; or PMG/Gyral disorganization Dysmorphic basal ganglia Cerebellar hypoplasia
Mutations	
CFEOM3:	R62Q; R262H; R262C; A302T; R380C; E410K; D417H; D417N
PMG/ Gyral disorganization:	G82R; T178M; E205K; A302V; M323V
Micro-lissencephaly:	M288V

Fig. 5.6 Mutations associated with *TUBB3*



Gene Name:	*TUBB5* (NCBI Gene ID 203068)
Genomic Location:	Chromosome 6
Size:	444 amino acids
Expression:	Progenitors and postmitotic neurons
Malformations:	Microcephaly Dysmorphic basal ganglia Abnormal corpus callosum Abnormal brain stem
Mutations	
Microcephaly with structural brain abnormalities:	M299V, V353I, E401K

Fig. 5.7 Mutations associated with *TUBB5*

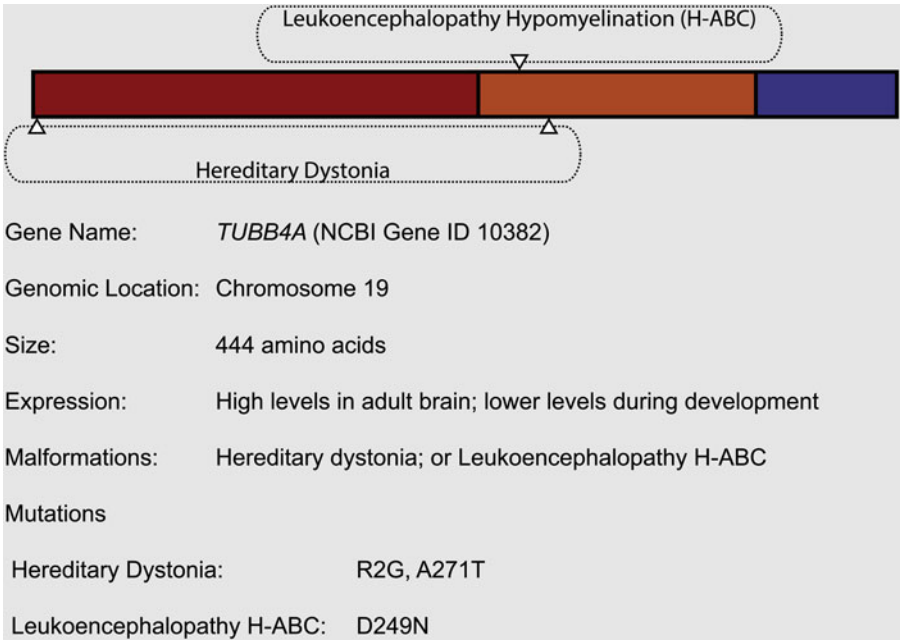


Fig. 5.8 Mutations associated with *TUBB4A*

V353I mutation was indistinguishable from wild-type tubulin, whereas the E401K mutation disrupted the chaperone-mediated folding with a consequent dearth of α/ß heterodimers that failed to incorporate into the cytoskeletal network. This result highlights that tubulin mutations that operate by different mechanisms can still result in similar phenotypes.

11 *TUBB4A* – Postnatal and Motor-Related

There is emerging evidence that the tubulinopathies are not limited to developmental phenotypes. In 2013 two independent groups reported the cloning of an R2G mutation in *TUBB4A* in a multigeneration Australian family that suffered from Whispering Dysphonia. Affected individuals in this family presented with a characteristic "hoppy horse" gait, laryngeal dysphonia, and a thin face (Hersheson et al. 2012; Lohmann et al. 2012). Klein and colleagues additionally described an A271T mutation in an unrelated familial case of segmental dystonia with spasmodic dysphonia (Lohmann et al. 2012). Complementing this finding, Vanderver and colleagues have reported that D249N mutations in *TUBB4A* cause a rare form of hereditary leukoencephalopathy, characterised by hypomyelination with atrophy of the basal ganglia and the cerebellum (H-ABC) (Simons et al. 2013) (Fig. 5.8). Most of the affected individuals, which originated from seven independent families, presented in infancy with motor dysfunction, but with normal cognitive and language development. While the underlying cellular and molecular mechanisms responsible for these phenotypes remain to be defined, it is known that the Asp249 residue forms a salt bridge with Arg2. This is important for the correct positioning of the T7 loop that interacts with the α-tubulin bound GTP. It is therefore a tenable hypothesis that disruption of this bridge impairs heterodimer stability or microtubule dynamics. Given the postnatal motor-deficits in those individuals with *TUBB4A* mutations, it is an unsurprising fact that this gene is expressed at low levels in the developing CNS, but is highly transcribed in adult cerebellum, brainstem and striatum (Breuss et al. 2012; Leandro-Garcia et al. 2010). It remains to be determined which cell types in the adult brain express this gene.

12 *TUBA8* – (Un)Related

TUBA8 was first cloned from a human adult skeletal muscle cDNA library, and was shown to be enriched in heart, skeletal muscle and testis (Stanchi et al. 2000). Sheridan and colleagues implicated this gene in polymicrogyria by undertaking genetic mapping of two consanguineous families (Abdollahi et al. 2009). They found linkage to a 7.42 Mb region that contained 230 genes, one of which was *TUBA8*. Candidate gene sequencing revealed a 14 base pair deletion in intron 1 of *TUBA8* that altered splicing. Despite assertions that this gene is widely expressed in developing neuronal structures, careful analysis in mice and humans has revealed that (unlike other disease-causing tubulins) *TUBA8* is expressed at extremely low levels in the developing brain (Braun et al. 2010). An alternative explanation for the reported polymicrogyria is that an unidentified mutation lies in another gene in the candidate interval. In the absence of additional unrelated patients with mutations in this gene the association of *TUBA8* with neurodevelopment disease should be considered tenuous at best. It may transpire that *TUBA8* is an innocent gene.

13 *TUBG1* – The Third Family Implicated

The tubulin superfamily is not limited to the α- and β-tubulins, but includes the γ-, δ-, ε-, ζ- and η-tubulins (McKean et al. 2001; Dutcher 2001; Oakley 2000; Oakley and Oakley 1989). Chelly and colleagues have recently shown that mutations in the γ-tubulin *TUBG1* cause complex cortical malformations (Poirier et al. 2013). The γ-tubulins are highly conserved in eukaryotes, forming a structural component of the centrosome known as the γ-tubulin ring complex (Oakley 2000; McKean et al. 2001). This complex is known to play a role in the nucleation of microtubules and regulation of the spindle during mitosis (Edgerton-Morgan and Oakley 2012). Chelly and colleagues reported three patients harboring missense *de novo* mutations in *TUBG1* (L387P, Y92C, T331P), one of the two isoforms in humans. Functional analysis revealed that the L387P mutation impairs chaperone mediated folding of TUBG1, whereas the W92C mutation results in decreased frequency of microtubule nucleation from the spindle body (Poirier et al. 2013).

TUBG1 is constitutively expressed throughout the body and its homozygous deletion results in an arrest of development at the morula/blastocysts stage due to mitotic spindle disorganization (Yuba-Kubo et al. 2005). Surprisingly, given the function of γ-tubulin in centrosome regulation, only two of these patients suffered from microcephaly. All patients showed agyria and/or pachygyria with abnormalities of the corpus callosum, highlighting the vanishing boundaries between disorders characterised by defects in proliferation, migration and differentiation. Consistent with this observation, *TUBG1* knockdown by *in utero* electroporation resulted in a drastic impairment in neuronal migration (Poirier et al. 2013). The coupling of the centrosome to the actin cytoskeleton is a critical requirement for the saltatory nuclear translocation in migrating neurons (Tsai and Gleeson 2005) (see also Chaps. 1, 2, 4 and 7).

14 Microtubule Associated Proteins – The Movers

Microtubules do not act alone, but rather in concert with an orchestra of microtubule associated proteins (MAPs) (Amos and Schlieper 2005) (see also Chaps. 4 and 6). The multitude of tubulin mutations that do not affect folding, and are able to incorporate into a functional cytoskeleton strongly suggest that they act by impairing the interaction with MAPs. There are a multitude of MAPs that could potentially be involved, including the microtubule stabilizer DCX (see also above) which is a key player in the pathogenesis of lissencephaly (Reiner 2013; Caspi et al. 2000; Gleeson et al. 1998). Here, we focus on the movers; dynein and kinesin.

These two classes of proteins are molecular motors that employ microtubules as intracellular

highways to delivery their molecular cargo (Vale and Milligan 2000). In addition they can also act as force generators or influence microtubule stability (Moore and Wordeman 2004; Mitchison and Mitchison 2010). While it is unclear whether isoform composition directly influences the interaction between microtubules and motor proteins, it has been shown that posttranslational modifications are important (Janke and Bulinski 2011). For instance, kinesin family motors increase their microtubule-binding upon detyrosination (Konishi and Setou 2009; Dunn et al. 2008). Similarly, for dynein motors, it has been shown that polyglutamylation directly regulates their interactions with microtubules (Suryavanshi et al. 2010; Kubo et al. 2010).

15 Dynein

Cytoplasmic dynein is a minus-end directed motor protein that consists of two heavy chains and a complex of associated light chains (Vallee et al. 2012; Rodriguez-Crespo 2011). The major cytoplasmic form, dynein 1 (DYNC1H1), is ubiquitously expressed and important for various functions ranging from vesicular transport to nuclear envelope breakdown (Vallee et al. 2012). The minor form, dynein 2 (DYNC2H1), is responsible for transport within cilia and flagella; their beating behavior in turn, is driven by the axonemal class of dyneins (Vallee et al. 2012). Chelly and colleagues reported *de novo* mutations in DYNC1H1 in nine independent cases of pachygryria and/or polymicrogyria (Poirier et al. 2013). Consistent with earlier findings that implicated dynein in peripheral neuropathy and an axonal (type 2) form of Charcot-Marie-Tooth disease, a subset of these patients also showed defects in the peripheral nervous system (Harms et al. 2012; Weedon et al. 2011; Poirier et al. 2013). Disease-causing missense mutations causing malformations of cortical development occurred throughout the protein; however, the mutations affecting the peripheral nervous system seem to cluster in the tail domain.

Although dynein has a multitude of cellular functions, the observed cortical malformations are most likely the result of deficient nuclear translocation in migrating neurons. The critical role dynein plays in this process has been revealed by experiments in the fungus *Aspergillus nidulans*, a eukaryotic model for nuclear migration (Willins et al. 1997). Morris and colleagues reported that mutations in the fungal homolog *NudA* block nuclear migration (Xiang et al. 1994). They further showed genetic interaction of this gene with the *LIS1* homolog, *NudF* (Willins et al. 1997). Subsequent functional characterization of this interaction revealed that dynein and Lis1 are acting in concert with Nde1/Nudel to couple the centrosome and the nucleus to the actin cytoskeleton (Tsai et al. 2007; Sasaki et al. 2000). The importance of this interaction is underlined by the finding that mutations in *LIS1* and *NDE1* cause neurodevelopmental disease (Reiner et al. 1993; Alkuraya et al. 2011; Bakircioglu et al. 2011) (see also Chap. 1).

16 Kinesins

The kinesin superfamily consists of 45 genes (also known as KIFs), classified into 15 families (Hirokawa et al. 2009). The progressive movement of most KIFs is directed toward the microtubule minus-end, although there are some family members that move toward the plus-end. Most are dimeric in structure, which enables them to "walk" along the surface of microtubules, driven by the hydrolysis of ATP. Their preferred substrates are 13 protofilament microtubules, underlining the importance of protofilaments number (Moores et al. 2006). Their main function is to transport of cellular cargo (Hirokawa et al. 2009), however, they also play an important role in the depolymerization of microtubules and force generation during mitosis (Moore and Wordeman 2004). These "movers" have also been implicated in neurological disease: Marchuk and colleagues identified a *KIF5A* mutation (N256S) as causative in hereditary spastic paraplegia, a neurodegenerative disorder (Reid et al. 2002); Engle and colleagues showed that a host of missense mutations in *KIF21A* cause the congenital axon guidance defects CFEOM1 and

CFEOM3 (Yamada et al. 2003, 2004); and Chelly and colleagues identified several mutations in both *KIF5C* and *KIF2A* that cause microcephaly with epilepsy and severe cortical phenotypes, such as polymicrogyria and agyria/pachygryria (Poirier et al. 2013).

17 Reflections and Directions

This review has catalogued those tubulin genes, the "makers", and those microtubule associated motors, "the movers", that cause neurodevelopmental disease. It is apparent that mutations in the neurodevelopmentally expressed "makers" (*TUBA1A*, *TUBB2B*, *TUBB3*, and *TUBB5*) cause a spectrum of diseases with overlapping phenotypes. At this juncture it is not possible to predict a disease phenotype given the residue or isoform mutated. This is because different tubulin mutations act by distinct mechanisms, some by haploinsuffiency, others by dominant means. Dominant mutations, in turn, have different effects on the stability and dynamic properties of microtubules, which is likely to be associated with the binding affinities of various MAPs. The question that arises is whether different tubulin proteins have intrinsic properties that make them distinct? Alternatively, could their unique expression patterns simply provide spatio-temporally critical concentrations? While the classic experiments in invertebrate systems strongly pointed towards tubulin specific function(s), the same cannot be said for the tubulinopathies, which have muddied the scientific waters. One way to address this issue would be to create a series of transgenic mouse models whereby the coding region of one gene of interest (e.g. *Tubb5*) is replaced by each of the seven other β-tubulin isoforms. Driven by the endogenous *Tubb5* promoter, this experiment would reveal, whether Tubb5 for instance, has a specific function in the developing telencephalon.

In the future, we expect that an understanding of tubulin gene function and the underlying molecular mechanisms that give rise to the tubulinopathies will play an important role in the development of novel therapeutics and diagnostic tools. There is growing evidence that neurodevelopmental disorders, once thought to be irreversible, may be treated effectively postnatally (Ehninger and Silva 2011). In the case of loss of function mutations in *TUBA1A* it is conceivable that a small molecule that increased the transcriptional activity at the *TUBA1A* genomic locus might be of utility (Kern et al. 2013). Finally, we expect that in the coming years the tubulinopathies will expand further, encompassing additional genes and disease states. To date genetic screening has primarily been biased by pre-conceived notions of the role of a particular isoform, and the availability of specific patient cohorts. There is already some evidence implicating *de novo TUBA1A* and *TUBB2B* mutations in autism spectrum disorders (Neale et al. 2012; Pinto et al. 2010). With the extension of exome, and eventually, whole genome sequencing into the clinic we expect that many more *de novo* mutations will be found.

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