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Phenotypic spectrum of the tubulin-related disorders and functional implications of disease-causing mutations

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A spectrum of neurological disorders characterized by abnormal neuronal migration, differentiation, and axon guidance and maintenance have recently been attributed to missense and splice-site mutations in the genes that encode α-tubulin and β-tubulin isotypes TUBA1A, TUBA8, TUBB2B, and TUBB3, all of which putatively coassemble into neuronal microtubules. The resulting nervous system malformations can include different types of cortical malformations, defects in commissural fiber tracts, and degeneration of motor and sensory axons. Many clinical phenotypes and brain malformations are shared among the various mutations regardless of structural location and/or isotype, while others segregate with distinct amino acids or functional domains within tubulin. Collectively, these disorders provide novel paradigms for understanding the biological functions of microtubules and their core components in normal health and disease.

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Phenotypic spectrum of the tubulin-related disorders

Twenty-three heterozygous missense mutations in *TUBA1A* have been reported [1–5,6••,7•]. All are sporadic and likely *de novo*, and four have been found in more than one unrelated individual. Most children harboring these mutations have microcephaly, severe motor and intellectual disabilities, and seizures. A subset also has simple strabismus and/or facial weakness, but none has paralytic strabismus or congenital fibrosis of the extraocular muscles (CFEOM). Fetopsy and imaging studies reveal that most *TUBA1A* mutations cause various grades of classic lissencephaly, ranging from the complete loss of gyri and sulci (agyria) to brains with simplified, abnormally thick convolutions (pachygyria). Cortical lamination is significantly disturbed and the six-layer cortex is reduced to four thicker layers, and in some individuals, only to two thin layers. The hippocampus and brainstem are usually malformed and hypoplastic. There is typically partial or complete absence of the corpus callosum and hypoplasia of the internal capsule and corticospinal tract associated with dysmorphic basal ganglia. Cerebellar vermian hypoplasia is also prominent, and *TUBA1A* mutations are estimated to account for approximately 30% of lissencephaly associated with cerebellar malformations [7•] (Table 1).

Five heterozygous missense mutations in *TUBB2B* that are all sporadic and *de novo* have been reported [8••]. Similar to the *TUBA1A* phenotype, children harboring *TUBB2B* mutations are typically microcephalic and have severe motor and intellectual disabilities often accompanied by seizures. Fetopsy and imaging studies reveal perturbed cortical cell migration and abnormal development of radial glia. Instead of classic lissencephaly, however, these children have polymicrogyria, a brain malformation characterized by excessive small gyri that are separated by shallow sulci, giving the brain a cobblestone-like appearance. The polymicrogyria in affected individuals is bilateral, asymmetric, and typically more predominant in the frontal and temporal lobes. Similar to *TUBA1A* mutations, partial or complete agenesis of the corpus callosum, dysmorphisms of the basal ganglia and cerebellum, and brainstem hypoplasia are common radiological findings (Table 1).

TUBB3 mutations cause a more diffuse spectrum of brain malformations and neurological disabilities, and certain phenotypes often segregate with particular amino acid substitutions [9••,10•]. Fourteen heterozygous missense mutations have been reported, which are a mixture of familial and *de novo* mutations, and six have been identified in more than one unrelated individual. The first eight *TUBB3* missense mutations to be reported cause the

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Table 1

Summary of the phenotypic spectrum of the tubulin-related disorders.

Tubulin isotype	TUBA1A	TUBB2B	TUBB3 (a)	TUBB3 (b)
Number of reported mutations	25 Missense	Five missense	Six missense	Eight missense
Phenotypic spectrum				
Clinical disorder	Severe LIS to MCD	PMG	MCD	Axon guidance disorder, CFEOM3
Intellectual and social disability	Generally severe	Generally severe	Mild to severe	Normal to moderate, mutation dependent
Head circumference	Most microcephalic	Microcephalic	<3–50%	Generally normal, mutation dependent
Epilepsy	Can be present	Can be present	Can be present	Absent
Strabismus	Comitant to absent	Not indicated	Comitant to absent	Incomitant (CFEOM3) to rarely absent, mutation dependent
Degenerative peripheral neuropathy	No	No	No	Yes, mutation dependent
Fetopsy and/or MRI neuroimaging data				
Published fetopsy	Yes	Yes	Yes	No
Cortex	Complete agyria to mild gyral malformation	Bilateral to assymetric polymicrogyria	Lissencephaly to gyral disorganization	Generally normal
Major cortical location	Diffuse, posterior, perisylvian, or frontal	Frontal, parietal, and temporal lobes	Perisylvian to frontoparietal	NA
Corpus callosum	Agenesis to mild dysgenesis, probst bundles reported	Agenesis to mild dysgenesis	Agenesis to normal	Agenesis to normal; probst bundles reported
Evidence of primary axon guidance defect	Possibly	No	Yes	Yes
Basal ganglia appearance	Dysmorphic to normal	Dysmorphic to normal	Dysmorphic to normal	Dysmorphic to normal
Cerebellum	Vermis > generalized hypoplasia, occasionally normal	Vermis > generalized hypoplasia	Vermis > generalized hypoplasia	Mild dysplasia to normal
Gross brainstem appearance	Hypoplasia to normal	Hypoplasia to normal	Hypoplasia to normal	Normal

LIS = lissencephaly; MCD = malformations of cortical development; PMG = polymicrogyria; CFEOM3 = congenital fibrosis of the extraocular muscle type 3.

paralytic eye movement disorder CFEOM3, which results from hypoplasia of the oculomotor nerve(s) and secondary atrophy of extraocular muscles. Depending upon the specific amino acid substitution, patients can develop sensorimotor polyneuropathy due to the progressive degeneration of motor and sensory axons in the limbs, and are sometimes born with wrist and finger contractures, facial paralysis, and mild to moderate intellectual and behavioral disabilities. Brain malformations include agenesis or hypoplasia of commissural axon tracts, hypoplasia of the corticospinal tract, and dysmorphic basal ganglia with fusion of the caudate and putamen. Overall, this initial combination of clinical and radiological findings point to a generalized defect in axon guidance and maintenance [9••] (Table 1).

More recent findings have expanded the TUBB3 phenotypic spectrum. Six additional *TUBB3* missense mutations have been identified in individuals with predominant frontal polymicrogyria or simplified and disorganized gyral patterning [10•]. Five of the mutations alter different residues from the initial set of *TUBB3* mutations, while one results in a different substitution at the same residue. Remarkably, none of these patients have CFEOM3, facial paralysis, or signs of sensorimotor polyneuropathy, although most have intermittent or permanent nonparalytic strabismus. Similar to *TUBA1A* and *TUBB2B* mutations, brainstem and cerebellar vermian hypoplasia are also common; however, these are not prominent findings in the initial series of *TUBB3* mutations [9••]. Both sets of *TUBB3* mutations commonly cause corpus callosum dysgenesis and corticospinal tract hypoplasia, and fetopsy and diffusion tensor imaging (DTI) from the second set of *TUBB3* mutations further support a generalized defect in axon guidance [10•]. Basal ganglia dysmorphisms are also present, and thus appears to be a defining phenotype of the dominant tubulin-related disorders (Table 1).

Finally, two consanguineous pedigrees segregating severe developmental delay, seizures, and optic nerve hypoplasia as a recessive trait have been reported to

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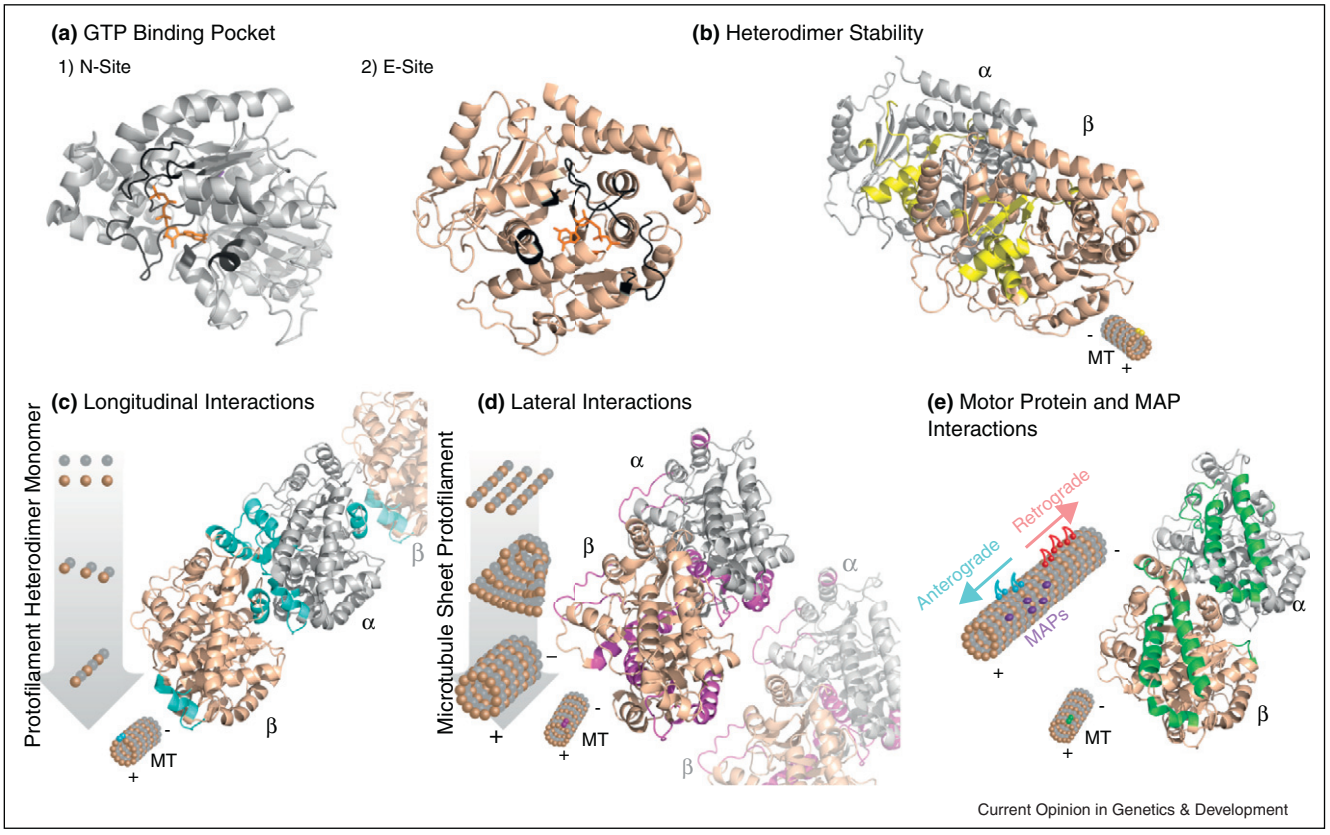
harbor the same homozygous 14 base pair intronic deletion upstream of exon 2 of the *TUBA8* gene, altering transcript splicing and resulting in greatly reduced TUBA8 protein levels in patient derived lymphoblastoid cells. Brain imaging of affected family members revealed extensive bilateral polymicrogyria, dysplastic or absent corpus callosa, and brainstem dysmorphisms [11•]. Although these phenotypes converge with those described above, additional mutations are needed to definitively support *TUBA8* as a polymicrogyria associated gene.

Amino acid substitutions reside in three structural domains of tubulin necessary for microtubule functions

Microtubules are dynamic polymers comprising tandem repeats of αβ tubulin heterodimers, which assemble in a head to tail fashion at the growing ends of microtubules to form a sheet of longitudinal protofilaments. Lateral interactions between neighboring protofilaments cause the sheet to close, thereby forming the hollow, cylindrical microtubule body [12•,13]. The structural conformation of longitudinal protofilaments is tightly regulated and,

Figure 1



Tubulin comprises three separate structural domains that participate in heterodimer stability, longitudinal and lateral protofilament interactions, nucleotide exchange and hydrolysis, and microtubule–protein interactions. **(a)** α-tubulin and β-tubulin each contain a GTP binding pocket (black) at their N-site and E-site (1 and 2, respectively), found within the N-terminal domain of the protein. Nucleotide binding is essential for protein folding, heterodimer stability, and microtubule dynamics. The N-site is formed primarily by α-tubulin residues (1, black) and is stabilized by interactions with some β-tubulin residues (not shown). GTP (1, orange) bound to α-tubulin is nonexchangeable because the N-site is buried at the intraheterodimer interface. The E-site is formed primarily by residues located near the plus-end of β-tubulin (2, black), and also interacts with some α-tubulin residues located at the interheterodimer interface (not shown). GTP bound to β-tubulin is eventually hydrolyzed to GDP (2, orange) once heterodimers have incorporated into microtubules, and the energy released causes microtubule disassembly due to protofilament curling (depicted in Figure 3). This force is countered by lateral protofilament interactions and the abundance of GTP bound β-tubulin heterodimers at the microtubule plus-end. **(b)** Residues found between the N-terminal and intermediate domains of tubulin (yellow) help regulate the stability of the tubulin heterodimer and structural rearrangements coupled to GTP hydrolysis. **(c)** Longitudinal interactions are mediated by a series of highly conserved residues found at the interfaces between the interheterodimer and intraheterodimer (light blue), and facilitate the stability of tubulin heterodimers and the assembly of longitudinal protofilaments (cartoon on left). **(d)** Lateral protofilament interactions are facilitated by residues that flank the sides and inner surface of heterodimers (purple), and are important for 'zipping up' the open sheet of longitudinal protofilaments into a hollow tube (cartoon on left). Lateral protofilament interactions also regulate microtubule dynamics following GTP hydrolysis. **(e)** MAP and motor protein interactions are mediated by external α-helices and adjacent grooves on the outer surface of the microtubule (green). Cartoon on the left depicts motor protein movement and MAP binding along the surface of a microtubule. 3D tubulin schematics were generated using PyMol (PDB: 1JFF). Cartoons labeled 'MT' in panels (b)–(e) depict the orientation of heterodimers relative to the assembled microtubule. Transparent tubulin in panels (c) and (d) are approximations of longitudinal and lateral interactions, respectively, showing adjacent heterodimer subunits within microtubules.

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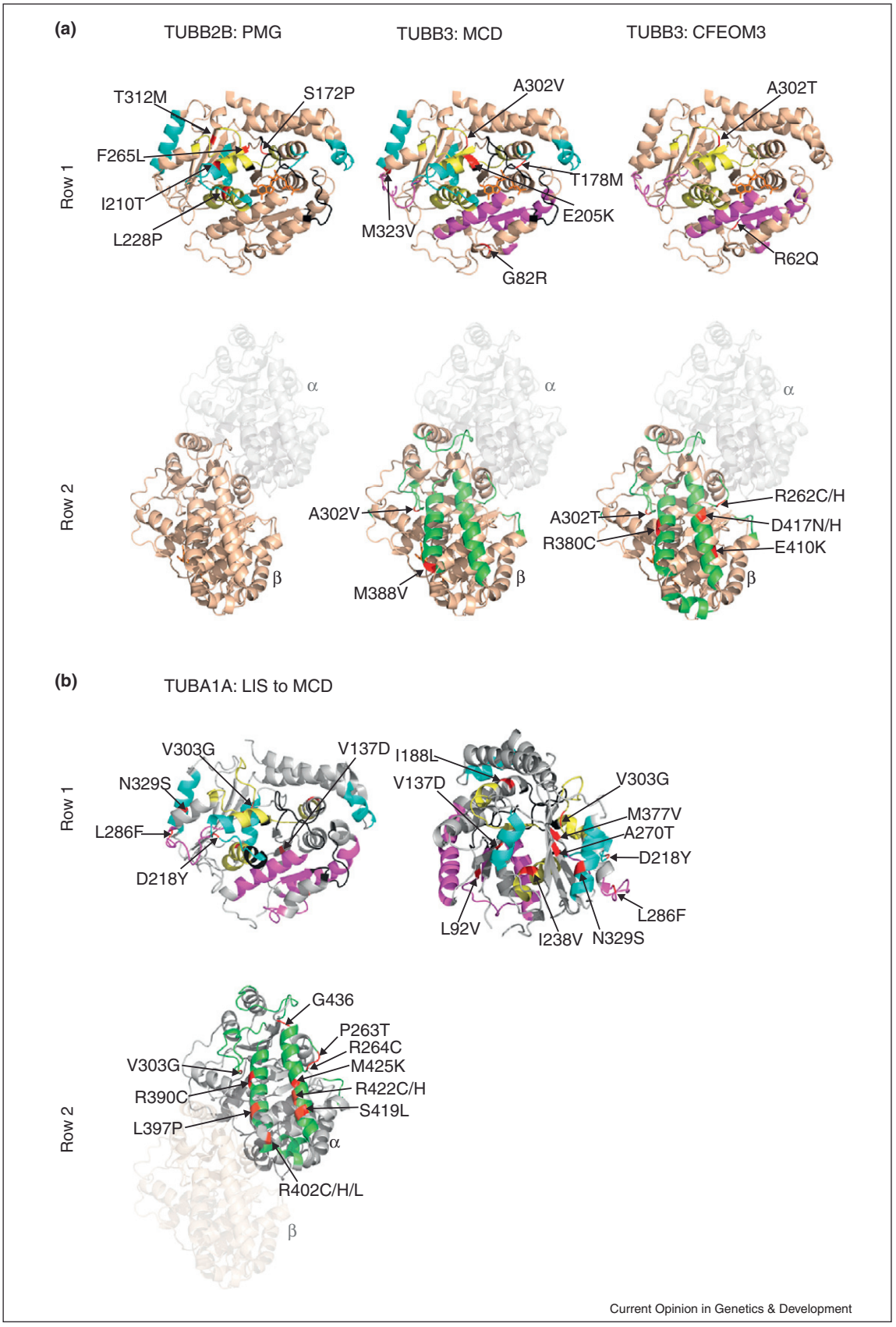
Figure 2


(a)
TUBB2B: PMG
TUBB3: MCD
TUBB3: CFEOM3
Row 1
T312M
S172P
F265L
I210T
L228P
A302V
T178M
E205K
M323V
G82R
A302T
R62Q
Row 2
α
β
A302V
M388V
A302T
R380C
R262C/H
D417N/H
E410K
(b)
TUBA1A: LIS to MCD
Row 1
V303G
V137D
N329S
L286F
D218Y
I188L
V137D
V303G
M377V
A270T
D218Y
L286F
L92V
I238V
N329S
Row 2
G436
P263T
R264C
M425K
V303G
R390C
R422C/H
S419L
L397P
α
R402C/H/L
β
Current Opinion in Genetics & Development

therefore, the structures of α-tubulin and β-tubulin are highly conserved throughout eukaryotes.

Tubulin comprises three separate structural domains that are formed by β-sheets that alternate with α-helices; these are the N-terminal, intermediate, and C-terminal domains and, in β-tubulin, correspond to residues 1–229, 230–371, and 372–450, respectively [14••]. These three structural domains serve at least five functions (Figure 1). The GTP binding pocket is formed by residues in the N-terminal structural domain (Figure 1a); GTP binding is important for protein folding, the structure and stability of tubulin heterodimers, and the conformation of longitudinal protofilaments necessary for lateral interactions and microtubule nucleation. Interactions between the N-terminal domain and the adjacent intermediate domain participate in structural rearrangements resulting from the hydrolysis of GTP bound by β-tubulin (Figure 1b). GTP hydrolysis causes straight protofilaments to curl outwards, resulting in microtubule depolymerization [14••,15•]. Other residues within this domain mediate longitudinal (Figure 1c) and lateral (Figure 1d) interactions that are necessary for both heterodimer and microtubule stability. Finally, motor protein and microtubule-associated protein (MAP) interactions occur through residues at the C-terminus that form alpha helices on the external surface of tubulin (Figure 1e). These residues mediate interactions with kinesin and dynein motors that facilitate intracellular transport in the anterograde and retrograde directions, respectively, as well as other MAPs that extrinsically regulate the dynamic properties of microtubules. Certain residues found in this domain are also important for the stability of longitudinal protofilaments [14••,16,17•,18].

Disease-causing amino acid substitutions in TUBA1A, TUBB2B, and TUBB3 are widely distributed among the three domains of tubulin and are therefore predicted to perturb different microtubule functions according to their structural locations (Figure 2). Several mutations alter residues that interact directly with the GTP nucleotide (TUBB2B S172P and TUBB3 T178M) or those located directly adjacent (TUBB2B L228P and TUBB3 E205K). Other mutations are located at contact surfaces between the intraheterodimer and interheterodimer and thus are predicted to alter longitudinal protofilament interactions (Figure 2, row 1). Both of these regions of tubulin are highly complementary and contain stretches of absolutely conserved residues; thus, a single amino acid substitution affecting one of these residues could be expected to significantly impede the formation of heterodimers, the overall ability of microtubules to polymerize, or the conformational changes essential to the dynamic properties of microtubules [14••]. Another subset of mutations affects residues that lie in proximity to the interface between the N-terminal and intermediate domains (Figure 2, row 1) [14••]. Likewise, these amino acid substitutions may also affect heterodimer stability and/or the dynamic properties of microtubules by altering the structure of tubulin during nucleotide exchange and hydrolysis. Overall, most of these types of mutations cause a range of gyral malformations, suggesting that microtubule stability may be diminished during key processes of cell migration, such as the coupling of the nucleus with the centrosome [19,20].

Several mutated residues in TUBA1A and TUBB3 fall within or immediately adjacent to the regions that participate in lateral interactions. Lateral interactions occur primarily between N-terminal loops of one heterodimer with the large loops in the intermediate domains of an adjacent heterodimer in the flanking longitudinal protofilament (Figure 1d, Figure 2, row 1) [21]. These interactions permit the assembly of microtubules, regulate their growth and shortening properties, and also provide the stabilizing force that allows them to curve and bend without breaking [21]. In neurons, this is particularly important because microtubules are arranged in dense networks, and in order to maintain their structural integrity, they must be resistant to forces that cause bending or buckling as is commonly seen *in vivo* [22]. At least one of these residues, R62Q in TUBB3 associated with isolated CFEOM3, was found to diminish the growth and shortening properties of microtubules and made them more resistant to depolymerization when introduced into yeast β-tubulin [9••]. The remaining mutations cause gyral and other brain malformations, but it is not known how they affect the stability or dynamic properties of microtubules.

Figure 2 Legend Three-dimensional mapping of disease-causing amino acid substitutions in TUBB2B, TUBB3, and TUBA1A. Functional domains of tubulin are colored as per Figure 1(a)–(e), and residues altered by mutations are highlighted in red. Row 1: panel depicting mutations altering residues located in the GTP binding pocket, and regions of heterodimer stability, and longitudinal and lateral interactions. 'Side views' are shown for each tubulin, and an additional 'longitudinal' view is shown for TUBA1A. Row 2: panel depicting mutations altering residues located in regions of MAP/motor protein interactions. 'Top-down' view onto the external helices of tubulin is shown. **((a)**, TUBB2B: PMG) Substitutions in TUBB2B that cause polymicrogyria alter amino acids in domains important for GTP binding, heterodimer stability, and longitudinal interactions (row 1), but are not located in regions that mediate lateral interactions (row 1) or MAP/motor protein binding (row 2). ((a), TUBB3: MCD) Substitutions in TUBB3 that cause gyral malformations are located primarily in regions that regulate GTP binding, heterodimer stability, and longitudinal and lateral interactions (row 1). Exceptions are M388 and A302 (row 2). A302 is located within a loop that could be important for both heterodimer stability and MAP/motor protein interactions. Similarly, M388 could also regulate MAP/motor protein interactions, and is in proximity to residues at the plus-end of β-tubulin that mediate interheterodimer contacts. ((a), TUBB3: CFEOM3) Substitutions in TUBB3 that cause axon guidance defects and CFEOM3 are largely found in regions of MAP/motor protein interactions (row 2). The exceptions are R62 and A302, the former of which is located in a loop mediating lateral interactions. **((b)**, TUBA1A: LIS to MCD) Substitutions in TUBA1A are located in all major functional domains. PMG = polymicrogyria; MCD = malformations of cortical development; CFEOM3 = congenital fibrosis of the extraocular muscles type 3; LIS = lissencephaly.

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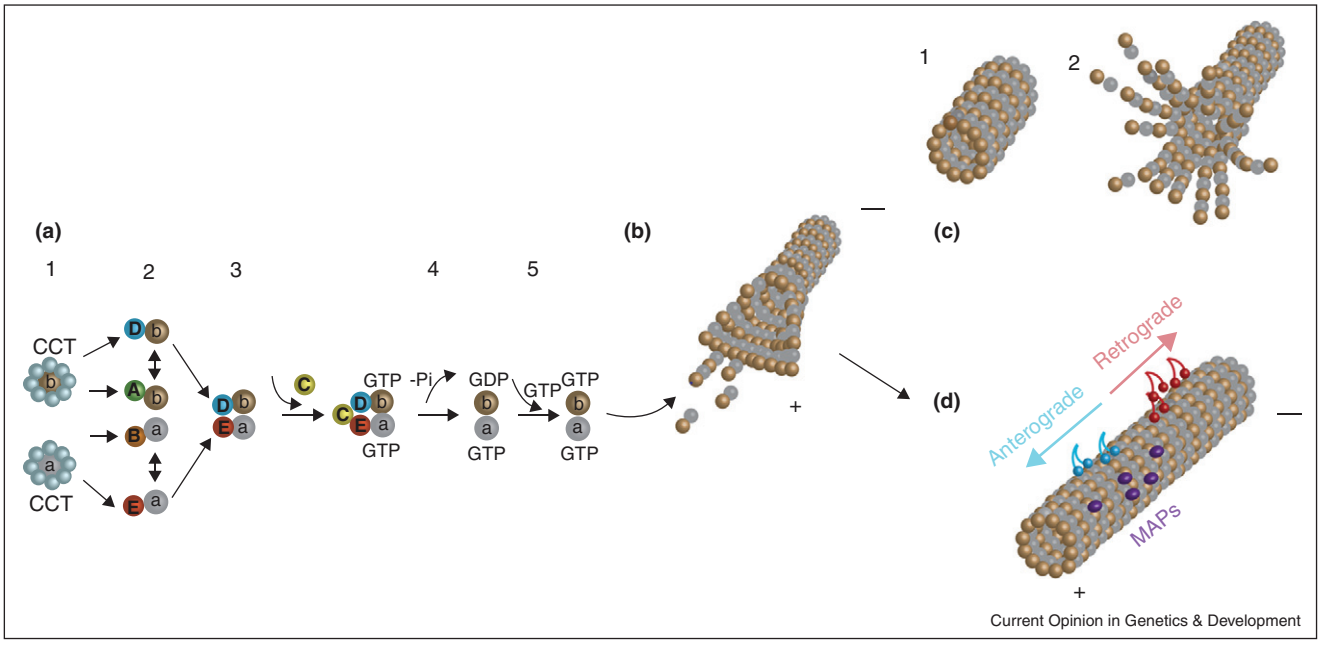
Finally, many substitutions mutate residues on the surface of microtubules that mediate protein interactions with kinesin, dynein, and other MAPs. For example, DCX (*doublecortin*) is a MAP that causes X-linked lissencephaly, and it has recently been shown to interact with α-tubulin R264, a recurrent mutation found in TUBA1A (Figure 2b, row 2) [23,24]. Intriguingly, several of these types of mutations are associated with distinct neurological impairments and/or brain malformations that suggest specific perturbations to developmental pathways involved in cell migration or axon guidance and maintenance. For example, recurrent R402C and R402H substitutions found on the external surface of TUBA1A (Figure 2b, row 2) cause a phenotype indistinguishable from the classical form of lissencephaly resulting from *LIS1* mutations or *LIS1* and *YWHAE* codeletions (Miller–Dieker syndrome), respectively [7•,25]. LIS1 forms a large protein complex with dynein that is essential for proper neuronal migration and leading neurite extension, suggesting R402 is particularly important for some aspect of LIS1/dynein function [7•,26,27]. TUBB3 residues associated with CFEOM3 predominately affect amino acids that are important for kinesin–microtubule interactions (Figure 2a, row 2) [9••,17•]. Many of these mutations are associated with sensorimotor polyneuropathy, underscoring the association between neurodegeneration and motor trafficking defects, as well as CFEOM3 [28]. CFEOM1, a nearly indistinguishable eye movement disorder, results from heterozygous mutations in the motor and stalk regions of the kinesin KIF21A, suggesting its involvement in the disease [9••,29,30]. Considering that phenotypic distinctions can occur between those mutations that affect kinesin and/or MAP interactions, this provides an opportunity to study how specific residues on the surface of microtubules differentially regulate the vast number of protein interactions [9••].

Mutations can alter both the overall abundance and functions of tubulin heterodimers

All mutations reported thus far in *TUBA1A*, *TUBB2B*, and *TUBB3* have been heterozygous missense mutations.

Figure 3



Summary depicting the proposed functional effects of disease-causing mutations in α-tubulin and β-tubulin. **(a)** Overview of the tubulin heterodimer folding pathway. (1) Nascent tubulin polypeptides are delivered to the cytosolic chaperone (CCT) in order to generate folding intermediates with GTP binding pockets. (2) α-tubulin and β-tubulin folding intermediates are then released, bound, and stabilized by a second set of chaperones, TBCA and TBCD (β-tubulin, b) and TBCB and TBCE (α-tubulin, a). (3) TBCD and TBCE form a complex to coassemble the tubulin heterodimer, and bind TBCC. (4) This triggers the hydrolysis of GTP bound to β-tubulin and releases the tubulin heterodimer from the folding complex. (5) Following the exchange of GDP for GTP in β-tubulin, the heterodimers are capable of incorporating into microtubules. Mutations in tubulin are predicted to diminish the levels of functional tubulin heterodimers by disrupting the formation of the GTP binding pocket and/or interactions with protein chaperones. **(b)** Tubulin heterodimers assemble in a head to tail fashion to form a sheet of longitudinal protofilaments at the growing plus-end of a microtubule. Lateral interactions between adjacent protofilaments cause the open sheet to close and assemble into a hollow tube. **(c)** Mutations in α-tubulin and β-tubulin found at interheterodimer interfaces and/or regions of lateral protofilament interactions are predicted to impede the polymerization and dynamic properties of microtubules, resulting in microtubules that may be (1) relatively nondynamic with reduced frequencies of growth and shortening, or (2) unstable and more likely to depolymerize. **(d)** The external microtubule surface interacts with kinesin and dynein motors, allowing the anterograde and retrograde transport of proteins and organelles along microtubules. MAPs also bind to the external surface and extrinsically regulate microtubule stability and dynamics. Mutations found in the external helices of tubulin are demonstrated or predicted to alter these types of protein interactions.

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Missense mutations in the absence of nonsense, frameshift, or genomic deletions support altered protein function rather than haploinsufficiency as a primary genetic etiology of these tubulin-related disorders, and this is reinforced by phenotype–genotype correlations associated with recurrent mutations [9••,31]. By contrast, homozygous splice-site mutations in *TUBA8* delete amino acids that are necessary for the structural integrity of tubulin and likely result in loss of protein function; however, heterozygous carriers do not have reported phenotypes, suggesting nervous system development is presumably more or less normal in the context of *TUBA8* haploinsufficiency [11•]. Thus, with the exception of *TUBA8*, several lines of evidence support dominant-negative effects rather than tubulin haploinsufficiency as sufficient to cause the various types of neurological and structural brain impairments.

Other findings, however, suggest that some mutations cause a reduction in the overall amount of tubulin heterodimers containing the mutant isotype, adding an important caveat to this argument. *In vitro* assays have revealed that approximately half of the amino acid substitutions in TUBA1A, TUBB2B, and TUBB3 that have been tested result in a significant decrease in the production of functional tubulin heterodimers due to the failure to properly interact with one or more chaperone proteins that capture and fold nascent tubulin polypeptides [8••,9••,10•,32,33•,34] (Figure 3). Upon overexpression in mammalian cells, some of these epitope tagged tubulin mutants demonstrate a moderate to severe decrease in microtubule incorporation as would be expected from compromised folding. These results are confounded by the ability of many of these poorly folded mutant heterodimers to still incorporate into interphase microtubules at levels equivalent to wild-type, although this could be an artifact from overexpression. Nonetheless, knockdown of *Tubb2b* in the embryonic rat cortex by *in utero* RNAi has reduced protein levels by 60%, causing arrested cell migration in a manner reminiscent of the human TUBB2B phenotype [8••].

There are not always clear phenotypic differences between those tubulin mutations that significantly diminish heterodimer synthesis and microtubule incorporation versus those that permit levels equivalent to wild-type. However, in some instances, there appears to be a trend among recurrent mutations such that increased levels of microtubule incorporation correlate with more widespread and often severe nervous system malformations. This is best demonstrated by different recurrent mutations altering the same residue. For example, the most common TUBB3 substitution, R262C, predominately results in isolated eye movement restrictions, whereas R262H causes severe eye movement restrictions in addition to other neurological impairments and brain malformations. Introducing both of these mutations into yeast β-tubulin similarly altered the growth and shortening properties of microtubules and diminished kinesin–microtubule interactions; however, *in vitro* folding assays and overexpression in mammalian cells demonstrated that the R262H substitution resulted in the generation of more tubulin heterodimers with higher levels of microtubule incorporation than R262C [9••]. Perhaps in a similar manner, recurrent TUBA1A substitutions R402H and R402C are distinguishable because the former usually cause a more severe lissencephaly phenotype with complete agyria versus R402C, and also permit higher levels of heterodimer formation and microtubule incorporation [7•,33•]. Thus, in some circumstances, the manifestation and/or severity of nervous system impairments may depend on the relative abundance of mutant heterodimers compared to wild-type, combined with their ability to incorporate into the microtubule cytoskeleton such that dynamics, motor protein, and/or MAP interactions are affected in different dominant-negative fashions.

Conclusion

Many questions still remain with regard to how the different mutations in α-tubulin and β-tubulin cause the range of reported neurological impairments and structural brain malformations. At present, much of the evidence supports that the dynamic properties and functions of microtubules are altered in several fashions. These include diminishing the overall abundance of functional tubulin heterodimers, altering GTP binding, altering longitudinal and lateral protofilament interactions, and impairing microtubule interactions with kinesin, dynein, and other MAPs (Figure 3). It is conceivable that each mutation disturbs one or more of these processes to different extents, and therefore, it is often difficult to predict phenotypic outcomes based solely upon structural locations and putative functional effects. Nonetheless, certain phenotypes including classic lissencephaly, CFEOM3, and polyneuropathy are unique or more common to mutations in one particular isotype or in different functional domains within tubulin. This is intriguing because expression levels are known to vary across isotypes according to cell type and developmental stage, and there are reported differences in their subcellular localizations and ability to influence microtubule dynamics [35–37]. Therefore, all of these factors should be considered when designing experiments to examine the function of different tubulin isotypes and their disease-related amino acid substitutions in the developing nervous system.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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