Human TUBB3 Mutations Perturb Microtubule Dynamics, Kinesin Interactions, and Axon Guidance

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SUMMARY

We report that eight heterozygous missense mutations in TUBB3, encoding the neuron-specific β-tubulin isotype III, result in a spectrum of human nervous system disorders that we now call the TUBB3 syndromes. Each mutation causes the ocular motility disorder CFEOM3, whereas some also result in intellectual and behavioral impairments, facial paralysis, and/or later-onset axonal sensorimotor polyneuropathy. Neuroimaging reveals a spectrum of abnormalities including hypoplasia of oculomotor nerves and dysgenesis of the corpus callosum, anterior commissure, and corticospinal tracts. A knock-in disease mouse model reveals axon guidance defects without evidence of cortical cell migration abnormalities. We show that the disease-associated mutations can impair tubulin heterodimer formation in vitro, although folded mutant heterodimers can still polymerize into microtubules. Modeling each mutation in yeast tubulin demonstrates that all alter dynamic instability whereas a subset disrupts the interaction of microtubules with kinesin motors. These findings demonstrate that normal TUBB3 is required for axon guidance and maintenance in mammals.

INTRODUCTION

Nervous system development is highly dependent upon the microtubule cytoskeleton. Microtubules are copolymers assembled from tubulin heterodimers, which contain several different α- and β-tubulin isotypes encoded by separate genes (Lopata and Cleveland, 1987). Microtubule behavior varies according to isotype composition, suggesting that each isotype may have properties necessary for specific cellular functions (Joshi and Cleveland, 1990; Luduena, 1993); however, precise functions for most tubulin isotypes remain poorly characterized. β-tubulin isotype III (TUBB3), one of at least six β-tubulins found in mammals, is distinct because purified microtubules enriched in TUBB3 are considerably more dynamic than those composed from other β-tubulin isotypes (Panda et al., 1994), and because its expression is primarily limited to neurons (Katsetos et al., 2003). TUBB3 expression is greatest during periods of axon guidance and maturation; levels decrease in the adult central nervous system (CNS) but remain high in the peripheral nervous system (PNS) (Jiang and Oblinger, 1992). Thus, the unique dynamic properties and spatio-temporal expression pattern of TUBB3 suggest that it could have a specific function for nervous system development and axon maintenance.

The development of human brainstem ocular motor neurons is particularly vulnerable to gene mutations that affect cytoskeletal proteins and axon guidance (Miyake et al., 2008; Yamada et al., 2003). Congenital fibrosis of the extraocular muscles type 3 (CFEOM3) is a rare ocular motility disorder in which affected individuals are born with blepharoptosis (drooping eyelids) and restricted eye movements (Doherty et al., 1999; Mackey et al., 2002). Using CFEOM3 as a marker for gene mutations that regulate human nervous system development and function, we now report that eight different heterozygous missense mutations in TUBB3, also known as TuJ1, result in CFEOM3 in isolation or as a component of several previously undefined neurological syndromes. Neuroradiological and pathological findings in humans and a knock-in mouse model, respectively, demonstrate that TUBB3 is necessary for guidance of commissural fibers and cranial nerves. Furthermore, disease-associated mutations can alter microtubule dynamics, and a subset perturbs the interaction of microtubules with kinesin motor proteins.
Thus, our work to define the TUBB3 syndromes establishes the requirement for a neuronal β-tubulin isotype in axon guidance and normal brain development.

RESULTS

Eight Heterozygous TUBB3 Mutations Alter Six Amino Acid Residues

CFEOM3 in the absence of additional neurological signs or symptoms (“isolated CFEOM3”) is an ~90% penetrant autosomal dominant disorder that had previously been mapped to chromosome 16q in pedigrees BN and DP (OMIM#600638, Figure S1A, Tables S1A and S1B) (Doherty et al., 1999; Mackey et al., 2002). The critical region for the CFEOM3 gene was 3.5 Mb and flanked by D16S498-16qter. To identify the CFEOM3 gene, we screened coding exons and intron-exon boundaries of six positional candidates in probands from BN, DP, and additional families with isolated CFEOM3. We identified three heterozygous TUBB3 missense changes in 15 unrelated pedigrees: 784C > T (R262C) in 11 pedigrees, 904G > A (A302T) in 3 pedigrees, and 185G > A (R62Q) in 1 pedigree (Figure S1B, Tables S1B–S1D).

We had ascertained study participants with CFEOM and additional neurological symptoms and, given the pan-neuronal expression of TUBB3 in humans (http://www.hudsen.org, HUDSEN Human Gene Expression Spatial Database, ID: 411), we next sequenced DNA from these probands. We identified 5 additional heterozygous TUBB3 missense changes in 13 unrelated pedigrees. 1249G > C (D417H) and 1249G > A (D417N) alter the same residue and cosegregate in a dominant fashion in 1 and 4 pedigrees, respectively. The remaining mutations, 1138C > T (R380C), 785G > A (R262H), and 1228G > A (E410K), were found in 1, 2, and 6 pedigrees, respectively, and each arose de novo as sporadic disease or from presumed germline mosaicism (Figure S1B, Tables S1A and S1E). Each of the eight mutations segregated with the TUBB3 phenotype, was absent in parents of sporadic individuals, and was not present on over 1,700 control chromosomes. The independent nature of the recurrent mutations is supported by de novo occurrences, ethnic and geographic diversity among probands, and multiple disease-associated haplotypes (Table S1C).

TUBB3 Mutations Can Result in Congenital Oculomotor Nerve Hypoplasia and Later-Onset Peripheral Axon Degeneration

Congenital ocular motility defects resulting from R262C, A302T, R380C, and D417N amino acid substitutions ranged from mild to severe (Figures 1A–1E), as previously described for pedigree BN (Doherty et al., 1999), whereas all participants with R262H, E410K, and D417H severe CFEOM3 and congenital facial weakness (Figures 1G and 1H). Many subjects had aberrant eye movements and several had ptotic eyelid elevation associated with synkinesis of jaw movements (Marcus Gunn phenomenon), clinical manifestations of aberrant innervation of cranial musculature by the trigeminal nerve. We conducted magnetic resonance (MR) imaging of the intracranial motor nerves and orbital contents of affected members of four R262C or D417N pedigrees. Similar to imaging of individuals with KIF21A missense mutations (Demer et al., 2005), which cause the isolated ocular motility disorder CFEOM1 (Yamada et al., 2003), we found hypoplasia of the oculomotor nerve and the muscles innervated by its superior division—the levator palpebrae superiors and superior rectus—as well as the medial rectus muscle innervated by its inferior division (Figures 1J–1L, Figure S1C). The oculomotor nerve also aberrantly innervated the lateral rectus muscle, normally innervated by the abducens nerve. Thus, ocular motility restrictions and/or synkinesis of lid elevation with jaw movements could be explained by axon guidance defects.

All subjects harboring D417H or R262H were born with congenital wrist and finger contractures, suggesting maldevelopment of spinal motor neurons (Figure 1I), and developed lower extremity weakness and sensory loss in the first decade of life. Most subjects harboring D417N and the oldest patient harboring E410K developed lower extremity weakness and sensory loss in the second to third decade, all in the absence of congenital contractures (Figure 1F). Electromyography revealed chronic, generalized sensorimotor polyneuropathy that was predominantly axonal and diagnosed as Charcot-Marie-Tooth Type 2 (CMT2) in some subjects. Several participants harboring D417N without CFEOM3 developed polyneuropathy (Table S1E), suggesting that TUBB3 mutations can cause an isolated CMT2-like disorder.

TUBB3 Mutations Can Result in Commissural Axon and Basal Ganglia Malformations that Segregate with Developmental Disabilities

Brain imaging was reviewed from individuals with each TUBB3 mutation except D417H and appeared normal only in the R62Q subject. Common findings were dysgenesis of the corpus callosum (CC), anterior commissure (AC), and internal capsule; generalized loss of white matter; and basal ganglia dysmorphic abnormalities that correlated with specific mutations (Figure 2). No images showed cortical dysplasia or evidence of cortical migration defects. Intellectual and behavioral impairments generally correlated with the severity of CC dysgenesis. Individuals with A302T, E410K, R262H, and R380C had more severe CC dysgenesis and mild to moderate intellectual, social, and behavioral impairments. By contrast, those with R62Q, R262C, or D417N substitutions had absent or mild CC dysgenesis and most were developmentally normal (Table S1).

R262C Tubb3 Substitution Results in Impaired Axon Guidance but Normal Cortical Architecture in Mice

To further examine the nature of the nervous system defects in humans, we generated a disease mouse model harboring the most common amino acid substitution (R262C) (Figure S2A). Wild-type (WT) and Tubb3<sup>+/R262C</sup> mice were born at the expected Mendelian frequencies; heterozygous mice appeared healthy, did not display external eye phenotypes, and had histologically normal appearing brains. Similarly, the R262C phenotype in humans can be nonpenetrant and, when penetrant, is limited to CFEOM3. By contrast, Tubb3<sup>R262C/R262C</sup> mice failed to breathe normally and died within hours of birth. Overall brain size was similar between WT and Tubb3<sup>R262C/R262C</sup> littermates, although basal ganglia asymmetries were sometimes present.

Histological analysis at E18.5 revealed normal cortical layer thickness and architecture in both homozygous and
heterozygous mice versus WT littermates, and neocortical layer-specific markers confirmed that layering was preserved (Figures 3A–3H, Figure S2C). The hippocampus and dentate gyrus also appeared normal (Figure S2B). Thus, consistent with human MR findings, heterozygous and homozygous R262C mice do not show evidence of cortical cell migration defects.

Tubb3R262C/R262C mice showed defects in the guidance of commissural axons and cranial nerves. There was significant thinning and/or absent midline crossing of the AC throughout its anterior-posterior axis compared to WT mice; it appeared tortuous and often had aberrant fiber projections at the midline. The AC was also thinner at the midline in Tubb3+/R262C mice, as found in human patients (Figures 3I and 3K, Figure S2D). Agenesis of the CC with bundles of stalled axons (Probst bundles) adjacent to the midline was observed in 2/5 homozygous mutant mice, both of which were on a mixed C57BL/6J:129S6 background (Figures 3J and 3L). By contrast, CC morphology was normal in all 18 mixed background WT mice analyzed. In the three homozygous mice in which the CC crossed the midline, it appeared abnormally thin in two and thick in one (Figure 3K). Whole-mount neurofilament staining at embryonic day (E) 11.5–E12 revealed numerous defects in the guidance and branching of cranial nerves (Figures 3M–3P). The oculomotor nerve failed to reach the correct muscle anlage and instead projected toward the position of the superior oblique muscle that is normally innervated by the trochlear nerve. Trochlear nerve growth was often stalled, and the trigeminal nerve failed to grow and branch properly compared to WT littermates. These data strongly support a primary defect in the guidance of axons.

Tubb3R262C/R262C Mice Have Increased Microtubule Stability and Decreased Kif21a Microtubule Interactions

There was a decrease in the level of TUBB3 protein in Tubb3R262C/R262C and Tubb3+/R262C versus WT mice. Although protein levels in homozygous mutant mice were approximately 30% of WT values, remaining mutant protein incorporated into microtubules that were polymerized in vitro from brain extracts, as well as into microtubules throughout the cytoskeleton of dissociated cortical and hippocampal neurons (Figures 4A, 4B, and 4D). Because Tubb3 is thought to be the most
dynamic β-tubulin isotype, we asked whether protein loss and/or the remaining mutant protein altered microtubule stability in the brain. We detected an approximate 30% increase in the levels of de-tyrosinated α-tubulin (Figure 4C), a posttranslational modification that is indicative of stable microtubules (Webster et al., 1987). We also measured the steady-state level of tubulin polymerization in brain extracts from WT and homozygous mutant littermates. Although overall β-tubulin levels were decreased in Tubb3R262C/R262C brain lysates, there was an increase in the amount of tubulin polymerization as detected by a larger microtubule pellet (Figure 4D). Taken together, these data suggest that microtubule stability is increased in Tubb3R262C/R262C mutants.

We next asked whether Kif21a microtubule interactions are altered in Tubb3R262C/R262C mutants because heterozygous mutations in this kinesin in humans cause isolated ocular motor dysfunction with clinical and neuroradiological signs of cranial nerve axon guidance defects (Demer et al., 2005; Yamada...
et al., 2003). We analyzed the ability of Kif21a to copurify with microtubules isolated from the brains of Tubb3<sup>R262C/R262C</sup> mutant and WT littermates in the presence of adenosine triphosphate (ATP). A significant decrease of Kif21a was detected with microtubules from the brains of Tubb3<sup>R262C/R262C</sup> mice (Figure 4E). Thus, reduced Tubb3 microtubule incorporation and/or the residual mutant protein affect the ability of Kif21a to interact with microtubules and may contribute to oculomotor nerve misguidance in mice and humans. Commissural axon guidance defects and other TUBB3 disease-specific phenotypes not found in CFEOM1 patients suggest that additional motors and/or microtubule-associated proteins (MAPs) beyond Kif21a may also be affected.

**TUBB3 Mutants Show Variable Reductions in Heterodimer Formation but Form Microtubules in Mammalian Cells**

A series of interactions with protein chaperones fold quasi-native tubulin monomers into functional αβ-tubulin heterodimers that can then polymerize into microtubules (Lewis et al., 1996). Point mutations in α- and β-tubulin can impair interactions with chaperone proteins, resulting in reduced heterodimer formation in mammalian cells (Jaglin et al., 2009; Keays et al., 2007) and could explain low protein levels observed in mutant mice. Thus, we asked whether R262C and the other seven substitutions affect the formation of TUBB3 heterodimers.

The coding sequences of WT and all eight TUBB3 mutants were fused to a C-terminal V5 epitope tag (TUBB3-V5) and expressed in rabbit reticulocyte lysate. This lysate is a cell-free system that contains necessary molecular chaperone proteins for de novo tubulin heterodimer formation (Cleveland et al., 1978); resulting heterodimers are comprised of rabbit α-tubulin and WT or mutant TUBB3-V5. WT and mutant proteins were
expressed and translated at equivalent levels. Under native conditions, R62Q, R262C, A302T, and R380C showed significant reductions in heterodimer yield, whereas the remaining four mutants generated moderate yields (Figure 4F). This latter group was mixed with bovine brain tubulin and taken through two successive rounds of microtubule polymerization and

Figure 4. TUBB3R262C/R262C Mice Have Low TUBB3 Protein Levels, Altered Microtubule Stability, and Decreased Kif21a Interactions
(A) TUBB3 protein levels are reduced in TUBB3+/R262C (WT/KI) and TUBB3R262C/R262C (KI/KI) versus WT mice.
(B) TUBB3 R262C heterodimers incorporate into microtubules throughout cell bodies, neurites, and growth cones of dissociated cortical neurons as seen by colocalization with tyrosinated α-tubulin and actin. Variable reductions in TUBB3 staining intensity are noted between WT and mutant neurons.
(C) Levels of de-tyrosinated α-tubulin are increased in brain lysates from mutant versus WT mice.
(D) Brain lysates from TUBB3R262C/R262C mice show increased microtubule polymerization at steady-state levels despite lower levels of β-tubulin. Mutant TUBB3 is detected in the pellets (p).
(E) Levels of Kif21a are reduced on TUBB3R262C/R262C mutant microtubules polymerized in vitro from brain lysates and incubated with ATP, whereas levels of Kif3a remain constant.
(F) In vitro transcription and translation of WT and TUBB3 mutant heterodimers in rabbit reticulocyte lysate. Products analyzed by SDS (top) and nondenaturing (native, bottom) gel electrophoresis and stained with an anti-V5 antibody against the C-terminal tag demonstrate that although transcription and translation are not affected by the mutations, there can be significant and variable decreases in the yield of native heterodimers.
(G) Synthesized WT, R262H, E410K, and D417H/N heterodimers cycle with native bovine tubulin at equivalent efficiency; vertical lines denote removed empty lanes.
*p < 0.05, **p < 0.001. Error bars denote standard error of the mean (SEM). See also Figure S3.
depolymerization. The ability of these mutant heterodimers to co-cycle with native tubulin in vitro was equivalent to WT (Figure 4G).

WT and mutant TUBB3-V5 expression constructs were next transfected into HeLa cells that have well demarcated microtubules. Immunostaining against the C-terminal V5 tag and α-tubulin to detect the overall microtubule network revealed incorporation of WT and mutant heterodimers. Cells expressing R62Q and R262C TUBB3, however, had lower and more punctate microtubule incorporation, whereas A302T, R380C, and the remaining mutants showed robust incorporation that was similar to WT (Figure S3). Interestingly, patients harboring R62Q or R262C have the mildest phenotypes, and this could result from lower amounts of mutant heterodimer incorporation into microtubules compared to the other mutants. Thus, although four disease-associated substitutions show scant heterodimer formation in vitro, all of the mutants can be incorporated into microtubules in mammalian cells, albeit at varying levels.

TUBB3 Amino Acid Substitutions Are Located in Different Structural Domains within β-Tubulin

TUBB3-disease substitutions reside in regions of β-tubulin implicated in the regulation of microtubule dynamics, motor protein trafficking, and interactions with MAPs (Li et al., 2002; Lowe et al., 2001) (Figures 5A–5D, Movie S1). Residues R62
and A302 reside in regions proposed to mediate lateral interactions between longitudinal units of β-tubulin heterodimers, called protofilaments, which assemble to form cylindrical microtubules. Lateral protofilament interactions aid microtubule assembly and regulate dynamics (Nogales and Wang, 2006). By contrast, residues R380, E410, and D417 are found in paired α helices H11 (R380) and H12 (E410, D417) on the external surfaces of microtubules that mediate interactions with numerous motor proteins and MAPs (Al-Bassam et al., 2002; Uchimura et al., 2006). Residue R262 is located in the loop between helix H8 and strand 7 of β-tubulin below helix H12 and forms a putative hydrogen bond with H12 through the carbonyl oxygen of residue D417. Upon mutation, this hydrogen bond is abolished, potentially affecting the tertiary protein structure and motor protein interactions with microtubules (Figure 5E).

The locations of the TUBB3 substitutions suggest that some could directly affect lateral protofilament interactions and microtubule dynamics, whereas others may also perturb motor protein interactions. Notably, these substitutions can result in diagnostically distinct phenotypes (Figure 5F), leading us to ask whether they could alter TUBB3 function and cause dominant effects upon microtubule behavior.

**Insertion of Human TUBB3 Mutations into the Yeast β-Tubulin Locus Affects Cell Viability and Growth**

The sequence and protein structure of β-tubulin is conserved between different isotypes and across species (Luduena, 1993). TUBB3 shares considerable homology with the single β-tubulin isotype in budding yeast (Tub2p), including conservation of all disease-associated residues (Figure S4). Budding yeast provides advantages for studying the behavior of microtubules harboring TUBB3-disease substitutions (Reijo et al., 1994). Mutations can be introduced into Tub2p by homologous recombination, and a single β-tubulin isotype avoids the potential diminution of mutant phenotypes due to an abundance of other tubulin isotypes.

To examine potential dominant effects of disease-associated mutations, we inserted each into the yeast TUB2 locus, and two independent heterozygous diploid strains for each mutation were isolated and grown on nutrient-rich media. Heterozygous diploids were recovered at the expected frequency and did not display growth defects on rich medium, establishing that all of the mutant heterozygous strains were viable. However, only R62Q and R380C haploid spores were viable when present as the sole copy of TUB2, and they grew slowly compared to WT (Table S2).

**TUBB3 Disease Substitutions Result in Benomyl Resistance and Alter Microtubule Dynamics**

To determine if mutant substitutions affect microtubule dynamics, WT, heterozygous, and surviving haploid TUB2 mutant strains were plated on media containing increasing amounts of benomyl, a compound that destabilizes microtubules and consequently inhibits cell division. Control strains grew normally on complete media without benomyl but were modestly or completely inhibited by increasing drug concentrations. By contrast, all mutant diploid strains demonstrated varying degrees of benomyl resistance compared to WT (Figures S5A and S5B); this is in contrast to a previously reported TUB2 mutagenesis screen, in which alterations of conserved residues more often caused benomyl supersensitivity than resistance (Reijo et al., 1994). These results suggest that TUBB3 syndrome mutations in Tub2p increase the stability of microtubules by rendering them resistant to depolymerization. Finally, a heterozygous diploid strain was generated in which one copy of TUB2 was deleted. Unlike the TUBB3 substitutions, TUB2 haploinsufficiency resulted in benomyl supersensitivity (Figures S5A and S5B). Thus, the TUBB3 substitutions do not result in complete loss of TUB2 function and appear to cause dominant effects on microtubule behavior.

Using time-lapse microscopy, we then asked how TUBB3 syndrome mutations alter microtubule dynamics. We monitored astral microtubules in G1 cells given that microtubules are most dynamic during G1 (Carminati and Stearns, 1997). GFP-Tub1p (α-tubulin) labeled microtubules from heterozygous strains R262C, R262H, A302T, and E410K, and haploid strains R62Q and R380C were imaged because these residues reside within different regions of the tubulin dimer.

Substitutions A302T, R62Q, and R380C resulted in similar and significant changes to most measured parameters of microtubule dynamics (Figures 6A–6C). Mutant microtubules were more stable and had longer lifetimes compared to WT and spent the majority of time in prolonged paused states instead of growing and shortening. The frequency of transition to microtubule depolymerization (catastrophe) was reduced, whereas the frequency of transition to polymerization (rescue) was unaffected in A302T heterozygotes and increased in R62Q and R380C haploids. The rates of both polymerization and depolymerization were significantly reduced. R380C microtubules appeared less dynamic than R62Q and A302T microtubules and spent more time in paused states, although WT tubulin in heterozygous A302T cells might dampen stability. Thus, A302T, R62Q, and R380C substitutions appear to increase the stability of microtubules and significantly diminish overall dynamics (Movie S2). A302 and R62 are found in loops within β-tubulin hypothesized to regulate lateral protofilament interactions and thus might be predicted to alter stability when mutated. In contrast, this finding is somewhat unexpected for R380C, given that this residue resides in H11, and to our knowledge this outer helix has not been previously implicated in the regulation of microtubule dynamics.

Diploid R262C, R262H, and E410K substitutions resulted in changes to dynamics distinct from those described above (Figures 6B and 6C). Microtubules in these heterozygous cells spent similar amounts of time growing compared to WT, but the average duration of individual growth events was prolonged and astral microtubules in these strains were longer on average (Figure S5C). The frequency of catastrophe events was only slightly less, whereas the frequency of rescue events was significantly less than WT. Finally, these mutants showed a decreased rate of polymerization and increased rate of depolymerization. Thus, mutant R262C, R262H, and E410K astral microtubules were often long and grew at reduced polymerization rates, followed by a more rapid and complete disassembly to the spindle pole body without recovery (Movie S2).
Mutation at Amino Acid Residues E410, D417, and R262 Result in a Loss of Kinesin Localization on Microtubule Plus Ends

Amino acid residues E410 and D417 of yeast β-tubulin have been identified as microtubule binding sites for conventional kinesin (Kif5) in vitro (Uchimura et al., 2006), and we predict that R262 forms a hydrogen bond with D417, which is abolished by R262C/H substitutions (Figure 5E). Perturbations to microtubule dynamics observed in R262C/H and E410K cells, including the faster rate of depolymerization and reduced rescue frequency, are similar to those found following the deletion of Kip3p (Figures 7A and 7F) (Gupta et al., 2006). Time-lapse video microscopy revealed a strong reduction of Kip3p-3YFP along the lengths and tips of growing microtubules in R262C/H, E410K, and D417H/N cells (Movie S3). Overall, R262C/H, E410K, and D417H/N cells all had a ~70%–80% decrease in signal intensity compared to WT (Figures 7B–7J and 7U). We next examined the plus-end localization of Kip2p-3YFP, as Kip2p is the second major plus-end-directed kinesin motor found in the budding yeast cytoplasm. Kip2p-3YFP localization was normal in WT cells; it was absent from spindle pole bodies and was speckled along the lengths and highly localized to the plus ends of cytoplasmic microtubules (Figures 7K and 7P) (Carvalho et al., 2004). Mutant R262C/H, E410K, and D417H/N cells had a significant, albeit less drastic and more variable, decrease in Kip2p-3YFP plus-end localization compared to Kip3p (Figures 7L–7T and 7V). There were no significant differences in the protein levels of Kip3p-3YFP and Kip2p-3YFP between WT and mutant cells to account for the quantitative differences (Figure S6).

Figure 6. TUBB3 Disease Amino Acid Substitutions Result in Changes to Microtubule Dynamic Instability

(A and B) Life-time history plots depicting the lengths of microtubules (y axis) over time (x axis) in G1 cells from (A) haploid WT and TUB2 mutants and (B) heterozygous diploid WT and TUB2 mutants demonstrate that Tub2p substitutions perturb microtubule dynamic instability. For WT and each mutation, one microtubule representing data from the collective analysis has been selected and plotted.

(C) Summary table of individual dynamic instability parameters. Number of events is listed in parentheses. *p < 0.05, **p < 0.001, ***p < 0.0001.

See also Figure S5, Table S2, and Movie S2.

long microtubules that result in mispositioning of the mitotic spindle away from the bud neck (Gupta et al., 2006).
accumulation on cytoplasmic microtubule plus ends, suggesting that kinesin interactions on microtubules are reduced in humans harboring these amino acid substitutions. These data provide in vivo evidence to support previous in vitro studies (Uchimura et al., 2006) implicating E410 and D417, as well as identifying an additional residue, R262, as important amino acids for proper microtubule-kinesin interactions. These findings also support diminished KIF21A microtubule interactions found in TUBB3R262C/R262C mice (Figure 4E).

The localization of Kip3p-3YFP and Kip2p-3YFP on the plus ends of cytoplasmic microtubules was not significantly altered between WT, R62Q and A302T heterozygous mutant cells (Figures 7U and 7V), and these residues are distal from known kinesin-microtubule interaction sites. Cytoplasmic microtubules in heterozygous R380C cells had an approximately 50% increase in the amount of Kip3p-3YFP at the plus ends but a nonsignificant increase in the amount of Kip2p-3YFP (Figures 7U and 7V). Thus, residues R62Q, A302T, and R380C all diminish microtubule dynamics in a similar fashion and do not significantly reduce the levels of kinesin on the plus ends of microtubules.

**DISCUSSION**

**Phenotype-Genotype Correlations in the TUBB3 Syndromes Support a Dominant Etiology**

We have identified TUBB3 as the mutated gene underlying a series of autosomal dominant disorders of axon guidance that we collectively call the TUBB3 syndromes. In 29 unrelated families, we identified eight unique heterozygous missense mutations that alter six amino acid residues. Phenotype-genotype correlations are emerging for these syndromes, and although the TUBB3 phenotypes limited to CFEOM3 and polyneuropathy often segregate in autosomal dominant families, those with more severe clinical findings typically arise de novo. Overall, most adults with isolated CFEOM3 have R262C or, less commonly, R62Q or A302T substitutions. Some children with isolated CFEOM3, however, harbor the D417N substitution and are at risk of developing a polyneuropathy in their teens or twenties. CFEOM3 with developmental delay, CC agenesis, and basal ganglia dysmorphisms in the absence of facial weakness may be predictive of the R380C substitution. Association of severe CFEOM3 with facial weakness,
developmental delay, moderate to severe CC dysgenesis, and likely a late-onset polyneuropathy predicts the E410K substitution, while the addition of finger contractures, basal ganglia dysmorphisms, and early onset polyneuropathy would suggest R262H or D417H substitutions.

Multiple findings suggest that the mutations underlying the TUBB3 syndromes primarily alter microtubule function in a dominant fashion, although we cannot rule out partial loss of function for some. First, recurrent missense mutations in the absence of truncating mutations are most consistent with altered rather than loss of protein function as a primary genetic etiology. Second, facial paralysis and progressive sensorimotor polyneuropathy occur only in those individuals harboring R262H, E410K, D417H/N, the four mutations that permit heterodimer formation and efficient microtubule incorporation, and have dominant effects upon microtubule function by perturbing kinesin interactions in yeast. Third, among the four mutations that result in scant heterodimer formation, A302T and R380C have considerably more microtubule incorporation in mammalian cells and cause more severe phenotypes than R62Q and R262C. Finally, R262C and R262H result in relatively isolated CFEOM3 and a severe TUBB3 phenotype, respectively; these two substitutions are distinguished because R262H permits much more efficient heterodimer formation and microtubule incorporation than R262C both in vitro and in mammalian cells. Thus, more severe phenotypes and developmental disabilities that correlate with particular mutations might reflect a greater extent of mutant heterodimer incorporation and the specific nature of the dominant effect (i.e., dynamics, protein interactions).

**Motor Protein Trafficking Defects Result in Progressive Axonal Neuropathy**

Progressive axonal neuropathies can result from inactivating mutations in kinesin and dynein accessory proteins, underscoring the vulnerability of motor neurons and peripheral axons to protein trafficking defects (Chevalier-Larsen and Holzbaur, 2006). The TUBB3 syndromes now demonstrate that tubulin mutations resulting in secondary motor protein transport defects can also cause axonal neuropathies. TUBB3 expression is maintained at high levels only in the adult PNS (Jiang and Oblinger, 1992), supporting a role for TUBB3 in health and maintenance of peripheral motor and sensory axons. This role is in addition to the function of TUBB3 in cranial nerve development, and perturbations in protein trafficking caused by a subset of TUBB3 substitutions may also explain, in part, the cellular etiology of CFEOM3. Notably, recurrent dominant mutations in KIF21A result in CFEOM1 and, in rare families, CFEOM3 (Yamada et al., 2004). Our findings in mice suggest that mutations in TUBB3 can diminish KIF21A-microtubule interactions, possibly accounting for oculomotor nerve axon guidance defects.

**TUBB3 Is Required for Proper Axon Guidance**

Neuroimaging, clinical manifestations of cranial motor nerve misrouting, and the phenotypic analysis of a TUBB3 disease mouse model elucidate a critical role for TUBB3 in proper axon guidance. Throughout nervous system development, differentiating neurons require dynamic populations of microtubules in order to appropriately respond to growth and guidance cues (Gordon-Weeks, 2004; Kalil and Dent, 2005). Because TUBB3 is the most dynamic β-tubulin isotype and the only one with expression primarily restricted to the CNS and PNS (Katsetos et al., 2003), it has been hypothesized that the dynamic properties of TUBB3 could be required for specific developmental processes (Panda et al., 1994). We expand upon these earlier observations by demonstrating that microtubule stability is increased in homozygous R262C mice, a finding that could result from the combined effects of Tubb3 heterodimer loss and the remaining mutant heterodimers. Dynamic changes could be exacerbated further by mutant heterodimers that diminish microtubule dynamics and incorporate at higher levels (R380C), resulting in more severe axon guidance phenotypes.

**Tubulin Isotypes Have Divergent Cellular Functions**

Certain aspects of the TUBB3 syndromes, including CC dysgenesis and basal ganglia dysmorphisms, converge with those resulting from heterozygous missense mutations in TUBA1A and TUBB2B in humans (Jaglin et al., 2009; Keays et al., 2007). Each of these three tubulin isotypes are highly expressed in post-mitotic differentiating neurons and the phenotypic similarities suggest that they have important overlapping functions (Coksaygan et al., 2006; Jaglin et al., 2009; Liu et al., 2007). However, the primary brain malformations resulting from mutations in TUBA1A and TUBB2B are lissencephaly, pachygyria, and/or gray matter heterotopias that result from cell migration defects. By contrast, humans and mice with TUBB3 substitutions do not show signs of cortical cell migration defects, and a recent study of TUBA1A mutation-negative subjects ascertained on the basis of agryria and pachygyria failed to identify mutations in TUBB3 (Poirier et al., 2007) and instead identified TUBB2B (Jaglin et al., 2009).

Remarkably, TUBB2B and TUBB3 are the major β-tubulin isotypes expressed in the nervous system and share 90% protein sequence homology, including all disease-associated amino acids. These isotypes differ, however, in the sequence of their C-terminal tails, dynamic behavior in vitro, and unique posttranslational modifications (Banerjee et al., 1990; Khan and Luduena, 1996), suggesting that functional differences between these isotypes may account for phenotypic distinctions in humans and mice. Our results now greatly expand upon previous observations that different β-tubulin isotypes may have evolved in higher vertebrates to serve specific cellular functions (Luduena, 1993) and support a critical role for TUBB3 in axon guidance.

**EXPERIMENTAL PROCEDURES**

Detailed methods can be found in the Extended Experimental Procedures available online.

**Clinical Genetic Studies**

Probands were ascertained based on affection with CFEOM. Participants were enrolled by the Engle Laboratory at Children's Hospital Boston or by collaborating laboratories following appropriate Institutional Review Board approval and informed consent. Clinical data were obtained from participants and medical providers. Genetic linkage and mutation detection by direct sequencing and DHPLC were performed as previously described (Doherty et al., 1999; Miyake et al., 2008) and using NCBI reference sequences NM_006086 and NT_010542.
Magnetic Resonance Imaging

Diagnostic MRI scans were reviewed from participants with each TUBB3 substitution except D417H. Images are T1 (B–E, G, B’, F’), T2 (A, F, H, J–N, A’–D’, G’, H’), Flair (I). Orbital MRI was performed as described previously (Demer et al., 2005).

Mouse Histology, Whole-Mount Neurofilament Staining, and Dissociated Neuronal Cultures

E18.5 embryonic brains on mixed 129/B6 and pure 129S6 backgrounds were fixed in 4% paraformaldehyde and embedded for cryo- or paraffin sectioning. Ten to twelve micrometer sections were blocked with 5% normal goat/donkey serum and 0.1% Triton in PBS at room temperature for 1 hr and incubated with primary antibody in 1% serum and 0.01% Triton at 4°C overnight. Secondary antibody was added at room temperature for 1 hr.

Microtubule Repolymerization Assay

E18 whole brains from WT, heterozygous, and homozygous mutant mice were homogenized in BRB80 buffer and centrifuged at 50,000g at 4°C for 30 min. One milliliter of GTP was added to supernatant and microtubules were polymerized for 30 min at 37°C. Samples were layered on a 30% sucrose cushion and centrifuged for 20 min at 37°C at 100,000 g to pellet the microtubules.

Copolyurification of Kinesin with Microtubules

E18 brains from WT and homozygous mutant mice were homogenized with a glass homogenizer in BRB80 buffer containing protease inhibitors and PMSF and incubated on ice for 20 min. A tubulin-rich fraction was obtained by centrifuging the lysates at 4°C for 30 min at 50,000 g. One millimolar ATP, 1 mM GTP, and 10 μM taxol were added to the supernatants and incubated at 37°C for 30 min. Samples were then layered on top of a 30% sucrose cushion and centrifuged for 20 min at 37°C at 100,000 g. Pellets were resuspended in SDS buffer and run on SDS NuPAGE 8%–12% Bis-Tris gels (Invitrogen).

In Vitro Heterodimer Formation and Cycling

One microgram of plasmid DNA was added to 50 μl of reticulocyte cocktail according to manufacturer’s instructions (Promega TNT T7 Coupled Reticulocyte Lysate), and the reactions were incubated at 30°C for 90 min. Following incubation, the products were chased with 300 ng bovine brain tubulin and 1 mM MgCl2 and GTP for 30 min at 37°C, and 2 μl and 10 μl from the overall reactions were run on SDS NuPAGE 8%–12% Bis-Tris (Invitrogen) and Native-PAGE 4%–16% Bis-Tris Gels (Invitrogen), respectively. WT and mutant TUBB3 heterodimers were detected with a monoclonal V5 antibody (Invitrogen) against the C-terminal tag. Remaining products were cycled with bovine brain tubulin.

Mutation Modeling

Each TUBB3 substitution was plotted on the solved protein structure for the αβ-tubulin heterodimer (Protein Data Bank [PDB]: 1JFP) using PyMOL software (1.1r1, http://www.pymol.org/). Hydrogen bonds were predicted using the bond distance measurement function and were also modeled using the Swiss-Pdb Viewer (DeepView). The protein model movie was created using PyMOL.

Yeast In Vivo Microtubule Dynamics

Cells were seeded overnight in SD-complete media, imaged at room temperature (-26°C) on an Axio Image MI (Zeiss) scope with a 63× Plan Fluar 1.4 N.A. objective, and captured using a CoolSnap HQ camera (Photometrics). The typical acquisition protocol acquired five z-series fluorescent images at 0.75 μm axial steps and one differential interference contrast (DIC) image corresponding to the central fluorescent image. Time-lapse image series were acquired at 8 s intervals.

Kip3p-3YFP and Kip2p-3YFP Microtubule Plus-End Localization

Mutant and WT CFP-Tub1p-expressing cells containing either Kip3p-3YFP or Kip2p-3YFP were grown and imaged as described above in the in vivo microtubule dynamics section. Seven z-series images were merged into a single projection image (maximum) using deconvolution microscopy and the nearest neighbor algorithm function in Slidebook software (Intelligent Imaging Innovations, CO). In randomly selected fields, the plus ends of all identifiable microtubules in multiple cells in all phases of the cell cycle were marked with a circle of equal radius, and the average plus-end intensity was then calculated using quantification software (Slidebook). At least two clones from WT and mutant strains were analyzed on at least two separate days, and net signal intensities from microtubule plus ends and cell backgrounds were determined by averaging the values obtained from the total population on each day.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, three tables, three movies, and references and can be found with the article online at doi:10.1016/j.cell.2009.12.011.

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EXTENDED EXPERIMENTAL PROCEDURES

Clinical and Genetic Studies
Genomic DNA was extracted from saliva using Oragene-DNA Self-Collection Kits (DNA Genotek) or from blood using Gentra Puregene Blood Kits (QIAGEN). When a sequence variant was found in the proband, co-segregation within the family was confirmed. To determine if variants are common polymorphisms, SNP databases were interrogated and the presence of each variant was assessed in at least 850 DNA control samples (1700 chromosomes) of mixed ethnicity, including Caucasian (American, European, Australian), African American, and Middle Eastern. PCR amplicons that included the variation were analyzed by either denaturing high-performance liquid chromatography (DHPLC, Transgenomic, Inc., Omaha, NE) with appropriate denaturing temperatures and acetonitrile gradients, or by fragment size analysis following restriction digestion by XhoI (New England Biolabs, Ipswich, MA). TUBB3 sequencing and DHPLC primers are provided in Table S3. Sequencing primers, DHPLC and PCR conditions are available upon request. For pedigrees in which a single individual was affected and harbored a de novo mutation, paternity and maternity were confirmed by the appropriate inheritance of at least ten informative polymorphic markers.

Pedigree Genotype and Phenotype Drawings
Patient pedigrees, phenotypes, and genotypes were created using Progeny software and the combined lab/clinical/LIMS software package (http://www.progenygenetics.com).

Mouse TUBB3 Targeting Construct and Generation of Mutant F1 Mice
inGenious targeting laboratory (iTL, Stony Brook, NY) designed the targeting construct, performed injections into 129/SvEv cells, and generated mutant F1 mice. Detailed construct information is available upon request.

Cortical Layering, Whole-Mount Neurofilament Staining, Antibodies, and Preparation of Dissociated Neuronal Cultures
Cortical architecture and organization was examined at E18.5 by Nissl staining in five KI/KI and five wild-type embryos. Cortical layering was further assessed at E18.5 in five KI/KI and five WT/WT embryos using the following antibodies: rabbit polyclonal anti-Cux-1 for cortical layers II, III, and IV (CDP, Santa Cruz); rat monoclonal anti-Ctip2 (Abcam) for cortical layers V and VI; rabbit polyclonal anti-Tbr1 (Abcam) for cortical layer VI; mouse monoclonal anti-Reelin (Millipore) for cortical layer I. White matter tracts (CC and AC) were examined using rat monoclonal anti-L1 (Chemicon) to stain axons. All secondary antibodies were purchased from Jackson ImmunoResearch. Pictures were taken using an Olympus BX51 scope equipped with a Spot Xplorer camera and either 4x UPlanFL 0.13 NA or 10x UplanFL 0.3 NA objectives. Brains serially sectioned in the coronal plane through the anterior commissure were reviewed and the anterior commissure and cortex measurements were taken from the slide in which the anterior commissure was thickest at the midline. Anterior commissure measurements were obtained from Nissl-stained sections (KI/KI n = 4, WT/KI n = 3, WT/WT n = 3). Cortical thickness measurements were obtained from Tbr1-immunostained sections, and by determining the distance between the subcortical boundary of layer six and the pial boundary of layer one (KI/KI n = 5, WT/WT n = 5). Statistical analysis was performed using an unpaired Student’s t test between WT versus WT/KI and WT versus KI/KI. * = p < 0.05.

Whole-mount neurofilament staining was performed as described previously (Mar et al., 2005) using a monoclonal anti-neurofilament antibody (2H3, Developmental Study Hybridoma Bank, Univ. of Iowa). A total of six KI/KI and six wild-type E11.5–E12.5 embryos were analyzed. Pictures were taken at 5x magnification using a Nikon SMZ 1500 dissection scope equipped with a Spot Xplorer camera.

Primary dissociated cortical cultures were prepared from E18.5 WT and mutant embryos. Brains were incubated briefly in Papain and triturated using a 1 ml pipette. Cellular debris was removed by centrifugation, and 50,000 cells were plated on poly-D-lysine at 8 µg/cm² on glass coverslips in a 24 well dish. Neurons were plated in Neurobasal media (Invitrogen) supplemented with 2% B27, 200 mM glutamine, 25 mM glutamate, and 2 mM Pen/Strep and then incubated at 37°C with 5% CO₂. Three days after plating, cells were fixed in 4% paraformaldehyde containing 8% sucrose and processed for immunocytochemistry. Staining was performed using the TuJ1 monoclonal antibody (Covance), rat monoclonal Ty-tubulin (YL1/2, Abcam), and rhodamine labeled phalloidin (Molecular Probes, Invitrogen). Z-plane images were captured using a Nikon TE2000 inverted scope equipped with a Photometrics CoolSnap HQ² camera and a 60x Plan Apo 1.4 NA objective. Images were processed using deconvolution software from the NIS-Elements advanced research software package (Nikon) at 20 iterations.

Tubb3 Protein Levels
Levels of TUBB3 protein were determined using whole-brain lysates from E18.5 WT and mutant embryos and the mouse monoclonal TuJ1 antibody (Covance). Images were captured using a LAS-4000 epiluminator (FujiFilm), and protein quantification was performed using MultiGauge software (FujiFilm) and Adobe Photoshop (CS3).
Microtubule Repolymerization Assay

Microtubule pellets were washed and resuspended in SDS loading buffer and run on SDS NuPAGE 8%–12% Bis-Tris gels (Invitrogen). Primary and secondary antibody staining was performed as described in the following section entitled coprecipitation of kinesin with microtubules. The following antibodies were used: monoclonal antibody against alpha-tubulin (DM1a, Sigma), a monoclonal antibody against beta-tubulin (D66, Sigma), monoclonal antibody against TUBB3 (TuJ1, Covance), and a monoclonal antibody against actin (ab8226, Abcam). Levels of de-tyrosinated tubulin were determined from E18.5 whole brain lysates from seven different WT and mutant mice prior to centrifugation using a mouse monoclonal antibody (aa12 Abcam). Images were captured using a LAS-4000 epi-illuminator (FujiFilm), and protein quantification was performed using MultiGauge software (FujiFilm) and Adobe Photoshop (CS3). Mutant values were normalized against WT on each separate experimental day, and values were scaled according to total loading volume as estimated by the amount of actin loading control (Abcam #8226). Statistical analysis was performed using a two-tailed paired Student’s t test.

Copurification of Kinesin with Microtubules

Kif21a immunoblotting at 1:5000 dilution was performed using a polyclonal antibody generated from a synthetic peptide corresponding to mouse Kif21a amino acids (aa) 1657–1676 (NH2-[C}NLQDGQLSDTGDLGEDIAASN-COOH)/human KIF21A aa 1655-1674 with an L/I mismatch at aa 1651. The peptide was purified, conjugated to KLH, and injected into rabbits. The antiserum was affinity purified and ELISA testing was performed (Bethyl, Inc.). By Western blot, anti-Kif21a antibody recognizes a band of ~180 kDa and 185 kDa in mouse and human, respectively. Specificity of anti-Kif21a antibody was tested by absence of a band with pre-immune absorption and confirmed by antigen (peptide) competition, and staining was completely blocked using 50 folds molar excess of the peptide. Kif3a immunoblotting was performed using a monoclonal antibody (611508, BD Biosciences) at 1:1000 dilution. Both were applied overnight at 4°C in solution containing 0.1% Triton in PBS and 5% non-fat dried milk. The following morning, gels were washed and secondary antibodies were added at concentrations of 1:10,000 (goat anti-rabbit and goat anti-mouse, Jackson Immunoresearch) for 1 hr at room temperature. Chemiolluminescence (Western Lightning Plus-ECL) agent was added, and images were captured using a LAS-4000 epi-illuminator (FujiFilm). Protein quantification was performed using MultiGauge software (FujiFilm) and Adobe Photoshop (CS3). Mutant values were normalized against WT on each separate experimental day (n = 6), and values were scaled to the total loading volume as estimated by the amount of alpha-tubulin in the microtubule pellet (DM1a, Sigma). Statistical analysis was performed using a two-tailed paired Student’s t test.

Construction of TUBB3-V5 Expression Plasmids

The ORF of TUBB3 was amplified from a human fetal brain cDNA library (Marathon-ready cDNA, Clontech) without a stop-codon and recombined into the Gateway pcDNA3.2 V5 destination vector (Invitrogen) according to manufacturer’s instructions. Site-directed mutagenesis (Stratagene, La Jolla, CA) was performed according to manufacturer’s instructions for insertion of each nucleotide base-pair change, and mutagenesis primer sequences are provided in Table S3.

TUBB3 Heterodimer Synthesis and Cycling

Ninety microliters of synthesized reticulocyte products were mixed with depolymerized bovine brain tubulin purchased from Cytoskeleton (Denver, CO) at a total concentration of 5 mg/ml, supplemented with GTP at a final concentration of 1mM, and taken through two cycles of polymerization and depolymerization as previously described (Zabala and Cowan, 1992), n = 3. Images were captured using a LAS-4000 epi-illuminator (FujiFilm).

HeLa Cell Transfection, Fixation, and Immunohistochemistry

Cells were plated at a density of 20,000 cells/well in a 24 well plate on glass coverslips and cultured in media containing DMEM with sodium pyruvate, 10% horse serum, 5% FBS, 4 mM glutamine, and 2 mM Pen/Strep. Twenty four hours after plating, cells were transfected (Lipofectamine, Invitrogen) with WT and mutant TUBB3-V5 expression constructs. The following day, cells were fixed in ice-cold methanol (to extract soluble tubulin) at −20°C for 10 min. Following two additional PBS washes, the cells were incubated in blocking buffer (PBS, 5% Goat Serum, 1% BSA, 0.1% Triton-X) for 30 min. The cells were colabeled with antibodies against the V5 tag (Invitrogen) and FITC-conjugated α-tubulin (DM1A, Sigma) at dilutions of 1/5000, and 1/400, respectively. A Cy3 mouse fluorescently conjugated secondary antibody (Molecular Probes) was applied at 1 μg/ml and glass coverslips were mounted on slides using VectaShield HardSet mounting media (Vector labs). Microscopy was performed using an Axio Image MI (Zeiss) scope and a 63x Plan Fluor 1.4 N.A. objective, and images were captured using a CoolSnap HQ camera (photometrics). Exposure times were held constant for WT and each mutant, and histograms are adjusted equally for both channels.

Yeast Plasmids

To create mutant TUB2 heterozygous strains, site directed mutagenesis (Stratagene) was used to insert each TUBB3 point mutation into the PCS3 plasmid (Gupta et al., 2001; Sage et al., 1995). Mutagenesis primer sequences are indicated in Table S2. Kip3p-3YFP and Kip2p-3YFP strains with expression controlled by the endogenous promoters were created using plasmids as previously described (Carvalho et al., 2004; Gupta et al., 2006). GFP-Tub1 plasmid was obtained from Aaron Straight (Stanford University School of Medicine) and constructed as previously described (Gupta et al., 2002).
Benomyl Assay
A stock solution of benomyl (10 mg/ml in DMSO, Sigma) was slowly added to hot 1x YPD-agarose media to obtain final concentrations of 4, 6, 9, 12, 15, 18, and 21 μg/ml in 100 mm/20 mm plates. Plates were dried overnight in a room temperature incubator. Yeast strains were seeded overnight in YPD media (1% yeast extract, 2% peptone, 2% glucose) and grown at 24 °C. The next morning, cell density was determined by spectrophotometry at optical density (OD) 600, and equal concentrations of WT and mutant clones were plated at logarithmic 3-fold dilutions and allowed to grow to mid-log phase at 24 °C. Haploid R380C was an exception and required two days of growth prior to reseeding. Colony growth was monitored for up to 4 days at 24 °C. Benomyl resistance was determined at day 4 using the following scale: mild resistance was scored if colony growth did not exceed one logarithmic dilution, moderate resistance if colony growth did not exceed two logarithmic dilutions, and strong resistance if colony growth exceeded three or more logarithmic dilutions compared to wild-type.

Yeast In Vivo Microtubule Dynamics, Image Analysis, and Quantification
Microtubule dynamics were assessed in unbudded cells with no bud growth visible by DIC microscopy. Total number of individual cells analyzed for heterozygous WT, R262C, R262H, A302T, E410K is 16, 19, 18, 19, and 20, respectively. Total number of individual cells analyzed for haploid WT, R62Q, and R380C is 13, 19, and 14, respectively. Microtubules were only scored if the entire length fell within the series of z-focal plane images used for analysis. If possible, multiple cells from the same time-lapse series or multiple microtubules from a single cell were analyzed. The three-dimensional lengths of microtubules at each time point were determined using the ruler function in Slidebook, and calculated by identifying the tip and the base of the microtubules in their respective z-plane images, taking into account the z-plane separation distance. Each series of time-lapse images was analyzed three separate times as independent sets of measurements, which were used to construct microtubule lifetime history plots using the averages of the three length measurements. Microtubule dynamics rates were calculated by linear regression analysis of the lifetime history plots. Growth and shortening events were defined as a set of at least three consecutive time-points with an R² value ≥ 0.8 and a length excursion of ≥ 0.45 μm. Pause events are defined as at least three consecutive time-points during which length change was < ± 0.225 μm. All growth, shortening, and pause events lasted at least 24 s. Catastrophe was defined as a transition to shortening after a period of growth or pause, and rescue was defined as a transition to growth following a period of pause or depolymerization. Brief time periods over which microtubules did not meet the above criteria were ignored and remain unclassified. The total duration spent in growth, shortening, or pause was calculated by dividing the sum of the time spent in each phase by the total evaluated time for all microtubules observed. The mean duration of growth, shortening, and pausing events reflects the average of each individual event. To determine catastrophe frequency, only the time spent in growth and pause were considered. For rescue frequency, only time shrinking was considered. Statistics were calculated with GraphPad PRISM software and using one-way ANOVA and the Dunnett’s post-test with wild-type as the control. Error bars represent standard error of the mean (SEM).

Kip3p-3YFP and Kip2p-3YFP Microtubule Plus-End Localization
In some instances, when the actual locations of microtubule plus ends were in question, a circle was placed at the best approximation of a microtubule plus end or terminal location of a plus-end kinesin signal. Cell background was determined in cells in which plus-end Kip3 and Kip2 localization intensities were obtained using a separate mask function in Slidebook. Average cell background intensity was variable among cells, but excluded any signal that might derive from spindle pole bodies and microtubules. Individual cells with background intensities not representative of the whole were excluded from analysis. The overall mean signal intensities for cell backgrounds and microtubule plus ends were determined once the mean slide background intensity had been subtracted from each. Imaging parameters were held constant across each analysis day, and included exposure times of 800 ms for CFP-Tub1p fusion proteins, 900 ms for kinesin-3YFP fusion kinesin proteins, and a neutral density setting of 50% to minimize bleaching. When instances of significant clonal variability were encountered, a third clone was analyzed. Statistics were calculated with GraphPad PRISM software and using one-way ANOVA and the Dunnett’s post-test with wild-type set as the control.

Kip3p-3YFP Live-Video Microscopy

TUBB3 mutant and WT cells expressing both CFP-Tub1p and Kip3p-3YFP were seeded overnight in SD-complete media at 24 °C. The following morning, the cells were reseeded and grown at 24 °C to reach mid-log phase, and cells were maintained at room temperature during image acquisition. Cells were observed with a fully automated Zeiss 200 M inverted microscope (Carl Zeiss, NY) equipped with a MS-200 stage (Applied Scientific Instrumentation, OR), a Lambda LS 175 W xenon light source (Sutter, CA), and a 100x 1.45 N.A. Plan Fluor objective. Images were acquired using a Hamamatsu EM-CCD photomultiplying camera. The microscope, camera, and shutters were controlled by Slidebook software (Intelligent Imaging Innovations, CO). In randomly selected fields, three Z series fluorescent images were captured at 0.50 μm axial steps at 3 s intervals. Exposure times of 100 ms were captured for CFP and YFP channels. Typically, Kip3p-3YFP signal at the plus ends of wild-type microtubules was consistently detected after 40 image acquisitions (~2 min). Four-dimensional projection images were created using Slidebook and exported for viewing in Quicktime.

Kip2p-3YFP and Kip3p-3YFP Protein Quantification
Mutant and wild-type cells were seeded overnight in SD-complete media at 24 °C. The following morning, cells were reseeded and grown at 24 °C to reach mid-log phase, and cell density was determined by spectrophotometry at OD₆₀₀ prior to cell lysis.
Gel electrophoresis was performed using NuPAGE 8%–12% Bis-Tris gels (Invitrogen). A monoclonal GFP antibody (Roche) was used to immunoblot against Kip2p-3YFP and Kip3p-3YFP, and equivalent total protein loading was confirmed using a rabbit polyclonal antibody against MAP Kinase (Mpk1, Santa Cruz sc-20168). Images were captured using a LAS-4000 epi-illuminator (FujiFilm), and protein quantification was determined using MultiGauge software (FujiFilm).

**Statistics**

Statistics were calculated using GraphPad PRISM software and the Student’s t test or one-way ANOVA with Dunnett’s post-test. Error bars represent standard error of the mean (SEM).

**SUPPLEMENTAL REFERENCES**


Figure S1. CFEOM3 Results from Heterozygous Mutations in TUBB3 and Perturbs Normal Extraocular Muscle Innervation, Relates to Figure 1

(A) Cytogenetic representation and schematic physical map of chromosome 16, indicating the position of the polymorphic markers used in the linkage analyses, and the 3.5 Mb FEOM3 critical region defined by a centromeric recombination event at D16S498 and 16qter (Doherty et al., 1999; Mackey et al., 2002). Approximately 50 genes fall within the critical region, and the full coding regions of MAP1LC3B and PRDM7 (italics) were sequenced prior to the detection of disease mutations in TUBB3 (bold italics).

(B) Chromatograms from automated genomic sequence analysis of one individual from a representative pedigree showing each of the eight heterozygous mutations in TUBB3. The nucleotide substitution in each chromatogram is indicated by the double peak and denoted by a black arrow. The corresponding normal and mutated amino acid residues are indicated with brackets under each codon triplet.

(C) Schematic lateral view of the brainstem and left orbit in an individual with normal eye movements (top) and an individual with CFEOM3 (bottom). The extraocular muscles are innervated by brainstem motor neurons with cell bodies that reside in the pons (abducens nucleus, green) and midbrain (trochlear nucleus, brown; oculomotor nucleus, blue). In the normal state (top), abducens motor neurons innervate the ipsilateral lateral rectus (LR) muscle (which is cut in the diagram) while trochlear motor neurons innervate the contralateral superior oblique (SO) muscle. Oculomotor neurons innervate the ipsilateral medial rectus (MR), inferior rectus (IR), and inferior oblique (IO) muscles through branches of its inferior division, and the contralateral superior rectus (SR) and both ipsilateral and contralateral levator palpebrae superioris (LPS) muscles through branches of the superior division. In CFEOM3 (bottom), the most consistent findings are hypoplasia of the LPS and SR muscles and hypoplasia of the superior division of the oculomotor nerve (depicted by pale muscle mass and dotted nerve).
Figure S2. TUBB3 Mouse Targeting Construct and Supplemental Histology, Relates to Figure 3
(A) The R262C substitution is located in exon four of TUBB3. LoxP sites are located in intron 3, and well as downstream from the 3’UTR.
(B) E18.5 sagittal sections demonstrating that the hippocampus and dentate gyrus have developed normally in homozygous mutant mice.
(C) Graph depicting cortical thickness of E18.5 WT (n = 5) and TUBB3R262C/R262C (n = 5) embryos. No differences are detected between WT and homozygous mutants.
(D) Graph depicting maximum midline anterior commissure thickness in E18.5 WT (n = 3), TUBB3WT/R262C (n = 3), and TUBB3R262C/R262C (n = 4) mice showing a linear decrease in the thickness of the AC in mutant embryos. Measurements in TUBB3R262C/R262C embryos were obtained at the thickest crossing point, even if embryos lacked crossing on adjacent sections. *p < 0.05. Error bars denote standard error of the mean (SEM).

Commonly, there is also hypoplasia of the MR muscle (depicted as opaque muscle mass). The levator palpebrae superioris muscle elevates the upper eyelid, and the superior and medial recti supraduct (elevate) and adduct (rotate toward the midline) the globe, respectively. Thus, lack of appropriate innervation with secondary atrophy of these specific extraocular muscles would account for the ptosis and infraducted and abducted (rotated away from the midline) globe position in severe CFEOM3. Individuals with mild and moderate CFEOM3 may have less severe hypoplasia of these muscles.
Figure S3. Mutant TUBB3 Heterodimers Can Incorporate into Mammalian HeLa Cell Microtubules, Relates to Figure 4

(A) HeLa cells were transfected with wild-type-V5 and mutant TUBB3-V5 expression constructs and fixed in methanol to extract soluble, nonincorporated tubulin heterodimers. The left panel shows the endogenous microtubule cytoskeleton in green stained with alpha tubulin, and the middle panel shows the V5-tagged heterodimers in red. The right column shows merged images, and each horizontal row depicts individual mutants. R262C and R62Q heterodimers sometimes incorporate at levels similar to WT but generally are found at lower levels in microtubules. The remaining mutants incorporate at levels similar to WT. Interestingly, although A302T and R380C substitutions show reduced heterodimers synthesis in vitro, levels of incorporation in HeLa microtubules can exceed that of WT. Also note that significantly more R262H heterodimers are found in the microtubule cytoskeleton versus R262C.

(B) Magnified images corresponding to the white boxes in the middle panel. Note that R62Q and R262C microtubules show more punctuate staining versus WT and the remaining mutants. Brightness intensity has been increased in these two mutants for visualization purposes. The remaining mutants show similar microtubule morphology compared to WT.
Figure S4. TUBB3 Disease-Associated Amino Acids Are Highly Conserved in β-Tubulin, Relates to Figure 5

The protein sequence of human TUBB3 aligned with the human β-tubulin isotype TUBB2, mouse and chick TUBB3, and the yeast β-tubulin ortholog, TUB2. The amino acid positions are numbered above each boxed alignment, and the mutated residues found in the TUBB3 syndromes are boxed in red. Note that all mutated amino acid residues are conserved throughout evolution in different species (mouse, chick, yeast) and among different isotypes (TUBB2). Matching residues are shaded in dark gray, and consensus match and non-consensus residues are shaded in light gray and white, respectively. The human TUBB3 protein consists of 450 amino acids and shares 99% overall sequence identity with mouse and chick TUBB3, ~90% with mouse TUBB2, and ~75% with yeast TUB2.
Figure S5. TUBB3 Disease Amino Acid Substitutions Result in Benomyl Resistance and Increased Microtubule Stability, Relates to Figure 6

(A) Diploid strains heterozygous for each CFEM3 Tub2p amino acid substitution were plated at logarithmic dilutions (indicated on left) and on complete media containing increasing dosages of benomyl (indicated on bottom). When compared to WT, all mutant strains demonstrate some degree of benomyl resistance. In contrast, a heterozygous diploid strain haplo-insufficient for TUB2 (TUB2+/−) is benomyl supersensitive.

(B) Chart depicting the extent of benomyl resistance for heterozygous diploid and haploid mutants over drug concentrations ranging from 4 μg/ml to 21 μg/ml. Shaded boxes on the right reflect the degree of benomyl resistance scored for wild-type and each mutant strain. Note that surviving haploid spores from mutant strains R62Q and R380C, homozygous for mutant TUB2, were strongly resistant, exceeding the drug resistance of their heterozygous diploid parents.

(C) Average astral microtubule length in WT and R262C, R262H, and E410K cells. Astral microtubules in R262C, R262H, and E410K mutants were longer on average than wild-type astral microtubules, and these differences were statistically significant for R262H and E410K. Also, D417H and D417N astral microtubules were also longer than average (data not shown). Statistics were calculated using one-way ANOVA, and the Dunnett’s post-test with wild-type set as the control. ***p < 0.0001. Error bars denote SEM.
Figure S6. Protein Levels of Kip3p-3YFP and Kip2p-3YFP in Yeast Cell Lysates, Relates to Figure 7
Levels of Kip3p-3YFP (A) and Kip2p-3YFP (B) fusion proteins (~180 kDa) in two independent mutant TUB2 strains harboring a specific TUBB3 syndrome mutation are equivalent to WT.
**Table S1A. CFEOM3 pedigrees and TUBB3 mutation analysis**

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*Nucleotide numbering starts from the A of the initiation codon (ATG) in coding sequence.*
Table S1B: Pedigrees harboring TUBB3 R262C substitution (784C>T)

Pedigree Phenotype Key S1A, S1C, S1D
- CFEOM3
- facial weakness
- learning disability
- developmental delay
- peripheral neuropathy
- wrist and finger contractures
- peripheral neuropathy and wrist and figure contractures
- spastic diplegia and wrist and finger contractures
- spastic diplegia
- hemiplegia
- non-penetrant carrier
- questionably affected
- non-penetrant carrier

BN

DP

AT

BT
Table S1B (continued): Pedigrees harboring TUBB3 R262C substitution (784C>T)

Table S1C: SNP haplotypes of R262C pedigrees
Table S1D: Pedigrees harboring R62Q and A302T TUBB3 substitutions

Table S1E: Pedigrees harboring R262H, R380C, E410K, D417N and D417H TUBB3 substitutions
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<th>Additional linkage primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>D16STACA F 5'-AGCAGGAGGAGGGATG - 3'</td>
</tr>
<tr>
<td>D16STACA R 5'-AGGTTGGTTGGAGAGC - 3'</td>
</tr>
</tbody>
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