Kumar, R., Pilz, D., Babatz, T., Cushion, T., Harvey, K., Topf, M., Yates, L., Robb, S., Uyanik, G., et al. (2010). TUBA1A mutations cause wide spectrum lissencephaly (smooth brain) and suggest that multiple neuronal migration pathways converge on alpha tubulins. *Human Molecular Genetics, 19*(14), 2817-2827. [http://dx.doi.org/10.1093/hmg/ddq182](http://dx.doi.org/10.1093/hmg/ddq182)
**INTRODUCTION**

Lissencephaly (LIS, smooth brain) comprises a group of severe brain malformations associated with deficient neuronal migration that results in mental retardation, epilepsy and when severe a shortened lifespan (1–3). In the most severe form, the

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surface of the cerebral hemispheres is completely smooth (agyria), whereas less severe forms of LIS are characterized by simplified folding patterns with abnormally broad gyri (pachygyria). Classic LIS (agyria–pachygyria spectrum) is typically characterized by a loosely organized and markedly thickened four-layer cortex (compared with the normal six-layered cortical architecture) and apparently normal cerebellum, although some patients have mild vermis hypoplasia (4). However, some rare forms of LIS are associated with a disproportionately small cerebellum, referred to as LIS with cerebellar hypoplasia (LCH). Several subtypes are known including LCH group b associated with Reelin pathway defects and another type characterized by a very poorly organized two-layer cortex (4,5). Classic LIS and LCH most often occur as isolated malformations, but rarely occur as part of malformation syndromes.

LIS has a strong genetic basis. Several genes that underlie LIS are directly or indirectly associated with modulation of microtubules, which are cytoskeletal elements involved in key cellular processes including mitosis, cytokinesis, vesicle transport, and neuronal migration. Heterozygous or hemizygous mutations in the microtubule-associated genes LIS1 on 17p13.3 and DCX on Xq22.3q23 account for ~85% of classic LIS (2,3,6,7). Co-deletion of the nearby LIS1 and YWHAE genes underlies Miller–Dieker syndrome (8–10), which consists of severe classic LIS and characteristic facial dysmorphism. Mutations in other genes including ARX, RELN and VLDLR have been identified in other forms of LIS but collectively these account for only a small proportion of patients with LIS.

The most recent gene implicated in LIS encodes tubulin alpha1A (TUBA1A) and resides on chromosome 12q13.12 (11–14). TUBA1A is a critical structural subunit of microtubules that is transiently expressed during neuronal development (15). Here, we report screening of the largest cohort of LIS patients examined to date to determine the frequency of TUBA1A mutations in patients with LIS, the spectrum of phenotypes associated with TUBA1A mutations, and the functional consequences of different TUBA1A mutations on microtubule function.

We identified novel and recurrent TUBA1A mutations in ~1% of children with classic LIS and surprisingly in ~30% of children with LCH spanning a wide spectrum of severity. We also detected a novel TUBA1A mutation in one child with agenesis of the corpus callosum (ACC) and cerebellar hypoplasia (CBLH) without LIS. We provide structural and functional data suggesting that LIS-associated mutations of TUBA1A operate via diverse mechanisms, perturbing tubulin α–β dimerization and most commonly affecting binding sites for microtubule-associated proteins (MAPs).

**RESULTS**

**TUBA1A mutations identified in 7% of unexplained classic LIS and 32% of LCH**

We screened the complete TUBA1A coding region and flanking 5′- and 3′-untranslated regions using bi-directional Sanger sequencing in 125 patients with LIS, including 72 patients with classic LIS, 22 with subcortical band heterotopia (SBH), 29 with LCH and two with LIS and ACC. Mutations of DCX and LIS1 were excluded by sequencing or by review of brain imaging, so that patients with posterior-predominant LIS (the LIS1 pattern) had normal sequencing results for LIS1, but may or may not have had DCX testing, and vice versa. We identified 5 missense mutations in 72 patients with classic LIS (~7%) and 10 missense mutations in 29 patients with LCH (~34%) (Table 1). We subsequently added mutations in two further patients with TUBA1A mutations found by clinical testing (p.M377V and p.R390C), but did not include them in our cohort analysis. Eight mutations (p.L92V, p.V137D, p.D218Y, p.A270T, p.N329S, p.M377V, p.R390C and p.M425K) were novel, while three (p.R402C, p.R402H and p.R422H) were recurrent mutations previously reported in other patients. We identified p.R402C in five patients, p.R402H in two patients, and p.R422H in two patients.

**Defining the phenotypic spectrum of TUBA1A mutations**

Detailed brain imaging studies were available for all 17 patients with TUBA1A mutations. We immediately recognized that mutations involving the arginine residue at codon 402 resulted in phenotypes indistinguishable from those associated with mutations of the LIS1 gene, while mutations at other codons produced more complex forms of LIS. From our detailed review of these imaging studies, we were able to delineate five distinct phenotype groups that are most easily appreciated with multiple views in several planes, as shown in Figs 1–4 and Supplementary Material, Figure S1.

The first group consists of five patients with the recurrent p.R402C mutation (Fig. 1). All had classic LIS with frontal pachygyria and posterior agyria corresponding to classic LIS grade 3 with posterior more severe than anterior gradient, rounded hippocampi with a thick outer rim (Ammon’s horn), intact but dysmorphic corpus callosum (missing rostrum plus flat genu and anterior body) and most often normal cerebellar structure. We observed a slightly more severe gyral malformation intermediate between grades 2 and 3, and mild cerebellar vermis hypoplasia in one patient each. This appearance is identical to the most common pattern found with mutations or deletions of the LIS1 gene.

Our second group consists of two patients with the recurrent p.R402H mutation (Fig. 2). Both had severe, classic LIS with nearly complete agyria, the same hippocampal and callosal defects as in the first group and moderate cerebellar vermis hypoplasia. This pattern resembles the brain imaging appearance found in patients with the Miller–Dieker syndrome (MDS) and co-deletion of LIS1 and YWHAE, except the cerebellar vermis hypoplasia is more severe in our two patients.

The third group consists of five patients with mild or moderate LCH and heterogeneous missense mutations (p.V137D, p.A270T, p.M377V, p.R422H) throughout the gene (Fig. 3). The brain imaging appearance is novel, consisting of diffuse pachygyria with no areas of agyria, mild-to-moderate asymmetry of the gyral malformation that could appear most severe over either the central convexity or the posterior pole and a thick cortex that is, however, not as thick as seen in patients with LIS1 (or TUBA1A p.R402C or p.R402H) mutations. The asymmetry is unusual for pachygyria; we considered...
whether this could be polymicrogyria, but the cortex is too thick and cortical infolding was not seen so we think this is a variant of pachgyria and not polymicrogyria. The posterior frontal, perisylvian and parietal regions were more severely affected than the occipital poles, another striking difference from the LIS1 mutation pattern in which the entire posterior brain including the occipital poles is most severely involved. One patient had a prominent deep cellular layer in the subcortical perisylvian region that resembles a short band heterotopia, but the overlying cortex is not indicating pachgyria and not SBH (Fig. 3N). The hippocampus is malformed as in the previous groups. Our three patients with severe pachgyria also had absent or nearly absent corpus callosum, thin brainstem and severe cerebellar hypoplasia. The cerebellar malformation consisted of small and upwardly rotated vermis, cystic dilatation of the 4th ventricle, less severe cerebellar hemisphere hypoplasia and mild to moderately enlarged posterior fossa, thus meeting the criteria for DWM. In the second, the cerebral vermis differed from the typical form of this disorder. The least severely affected subjects had only moderate pachgyria, mildly dysmorphic corpus callosum with absent spenium and mild cerebellar vermis hypoplasia.

Our fourth group consists of four patients with novel heterozygous missense associations with severe LCH that corresponds to our previous LCH groups c, d and f (5) by brain imaging (p.D218Y, p.N329S; p.M425K; Fig. 4) or fetal neuropathology at 21 weeks gestation (p.L92V). In all, the walls of the cerebral hemispheres are thin, and the cerebral surface completely smooth except for a few abortive sulci at the frontal pole in the third patient. The cortex demonstrates a slightly different pattern in each patient. In the first, the cerebral wall is very thin, but the cortex and white matter can be distinguished (LR07-213; p.D218Y; Fig. 4A–E). In the second, the cortex fills the entire mantle and creates an undulating pattern along the walls of the lateral ventricles, a rare LIS pattern (LR05-388; p.N329S; Fig. 4F–J). The same pattern was seen in an LCH patient with no sample available to test (patient LP99-059 shown in Fig. 6D–F in 5). In the third patient, the cortex and white matter can barely be distinguished. The amygdala and hippocampus are seen as small thickenings of the temporal lobe (LR08-388, p.M425K; Fig. 4K–O). The corpus callosum is absent in all three patients. In the first two, the brainstem is markedly thin and the cerebellum consists of a nubbin with the vermis < 1 cm in size. The posterior fossa is mildly enlarged, but the overall appearance is atypical for DWM because of the minute cerebella. In the third patient, the brainstem is moderately small, and the cerebellar defects meet the criteria for mild DWM.

The remaining subject was identified following fetal terminal angulation for ventriculomegaly and other anomalies at ~21 weeks gestation (CM–66, p.L92V). The brain was small (weight 31 g, expected 45 g) and had a generalized deficiency of neuronal migration with neurons seen diffusely through the mantle (thus no layers), a small amorphous group of neurons in the area of the hippocampus, severe dilatation of the third and lateral ventricles and absent corpus callosum. The brainstem was very small with absent descending corticospinal tracts and inferior olives and small basis pontis. The cerebellum was also very small with absent dentate nuclei and formation of only an indistinct cerebellar cortex.

Our last group consists of a single patient carrying a novel de novo heterozygous missense mutation p.R390C with mildly reduced number of gyri and shallow sulcal depth that we designate as a ‘simplified’ gyral pattern, plus complete ACC and moderate CBLH (Supplementary Material, Fig. S1). The cortical-white matter interface appeared mildly irregular in places, but the cortex was not thick and we found this unconvincing for pachgyria, polymicrogyria or another cortical dysplasia; however, the resolution of the study was low. This phenotype is closest to—but milder than—mild-moderate LCH. Cellular studies of TUBA1A mutations

Since all TUBA1A mutations reported to date are de novo and affect a single allele of an autosomal gene, a dominant-negative mechanism of action is plausible. Consistent with this hypothesis, TUBA1A mutations reported to date do not profoundly affect the ability of mutant TUBA1A to incorporate into the cytoskeleton in recombinant systems (12,16).

| Subject ID  | DNA ID   | Diagnosis | Status | Amino acid | Inheritance | Group assignment |
|-------------|----------|-----------|--------|------------|-------------|-----------------|
| CM-66       | CDF-0005 | LCH       | Novel  | L92V (c.274C > G) | De novo     | LCH × Sev group 4 |
| CM-107      | CDF-0023 | LCH       | Novel  | V137D      | Unknown     | LCH × mild group 3 |
| LR07-213    | 07.3123  | LCH       | Novel  | D218Y (c.652G > T) | De novo     | LCH × Sev group 4 |
| LR07-244    | 06.1048  | LCH       | Novel  | A270T (c.808G > A) | Unknown     | LCH × Mod group 3 |
| LR05-388    | 06.1242  | LCH       | Novel  | N329S (c.986A > G) | De novo     | LCH × Sev group 4 |
| LP95-073    | 00.0107  | LIS       | Reported (13,14) | R402C (c.1204C > T) | Unknown     | LIS × Mod group 1 |
| LR07-908    | 05.2831  | LIS       | Reported (13,14) | R402C (c.1204C > T) | De novo     | LIS × Mod group 1 |
| LR06-210    | 07.1424  | LIS       | Reported (13,14) | R402C (c.1204C > T) | De novo     | LIS × Mod group 1 |
| LR08-035    | 08.0750  | LIS       | Reported (13,14) | R402C (c.1204C > T) | De novo     | LIS × Mod group 1 |
| LR06-064    | 06.1029  | LIS       | Reported (13,14) | R402C (c.1204C > T) | Unknown     | LCH × Mod group 1 |
| LP97-039    | 99.1608  | LCH       | Reported (12,14) | R402H (c.1204G > A) | De novo     | LIS × Sev group 2 |
| LP97-041    | 01.2278  | LCH       | Reported (12,14) | R402H (c.1204G > A) | De novo     | LIS × Sev group 2 |
| LR05-052    | CDF-0014 | LCH       | Reported (11,13) | R422H (c.1265G > A) | De novo     | LCH × Mod group 3 |
| LR08-340    | CDF-0016 | LCH       | Reported (11,13) | R422H (c.1265G > A) | De novo     | LCH × Mod group 3 |
| LR08-388    | 08.3908  | LCH       | Novel  | M425K      | Unknown     | LCH × Sev group 4 |

Sev, severe; Mod, moderate.

*Parental DNA unavailable for testing.
To confirm this finding for a range of TUBA1A mutants reported here and elsewhere, we introduced the mutations I188L, P263T, R264C, L286F, R402H, R402C and S419L into a construct expressing C-terminally FLAG-tagged TUBA1A and transfected these into P19 cells (Fig. 5). Confocal microscopy revealed that in each case, recombinant TUBA1A is clearly made and the typical tubulin architecture is observed. This suggests that these mutants readily incorporate into the cellular cytoskeletal network. This finding does not support a recent study that reported haploinsufficiency as a cause of the disease phenotype (16) and we chose to undertake detailed structural modeling to predict the functional consequences of existing and novel TUBA1A mutations.

Structural modeling of TUBA1A mutations

The N329S mutation is located on α-helix H10 of α-tubulin (residues 324–337) (17) at the interface between α- and β-tubulin monomers. H10 interacts with α-helix H6 (residues 206–214) and the adjacent loop (the B5–H5 loop, residues 171–183) on β-tubulin, both of which directly interact with GTP (Fig. 6). In the wild-type structure, the interaction of H10 with β-tubulin is stabilized by the side chains of two residues, K326 and N329. The K326 amino group forms an H bond with the backbone oxygen of Y210 and is also involved in a cation–π interaction with the aromatic ring of F214. The N329 carboxamide group forms two H bonds, one with the D179 carboxylate group and the other with the V177 backbone oxygen (on the B5–H5 loop). The N329S mutation will not allow the latter H bond interactions and therefore is likely to destabilize the interaction between α- and β-tubulin monomers around H6 and the B5–H5 loop, possibly affecting the conformation necessary for GTP binding.

Six additional TUBA1A mutations cluster around α-helices H11 and H12 of α-tubulin (R402H, R402C, S419L, R422H, R422C and M425K) and they are likely to affect the...
interaction between these helices. Because H11–H12 lie on the interface between α-tubulin and various MAPs [e.g. kinesin KIF1A (18), DCX (19), MAP2c (20), and Dynein (21)], the mutations are also likely to affect the interaction between tubulin and MAPs. The interaction network between H11 and H12 is complex. First, the guanidinium ion of R402 is involved in a cation–π interaction with the aromatic ring of Y399, which in turn forms an H bond with S419-Oγ. Clearly, both the R402H and the R402C mutations will abolish the cation–π interaction, since neither histidine nor cysteine can be involved in this type of interaction. Furthermore, because a leucine side chain cannot form an H bond, the S419L mutation cannot stabilize Y399 in the ideal position to form a cation–π interaction with R402. Second, the guanidinium ion of R422 forms three H bonds with the carboxylate group of D396 and a salt bridge with the carboxylate group of D392. Neither the R422C nor the R422H mutations can form these interactions (although histidine is able to form an H bond, it is too far from D396). The M425K mutation might also interrupt this network by competing with R422 for the interaction with D392 (due to a change from a neutral residue to a positively charged one). Finally, the L397P mutation is expected to introduce a kink in H11 (since the cyclization of the proline side chain prevents the regular backbone H bond formation), which will affect the position of D396 and D392 relative to R422 and possibly their interaction.

**DISCUSSION**

The tubulin gene family in humans is extensive, comprising at least nine α, nine β, two γ and single δ and ε subunits, all expressed in different temporal, spatial and subcellular locations. *TUBA1A* is one of three α-tubulin genes that cluster on chromosome 12q13.12. The encoded protein is formed by a core of two β-sheets surrounded by α-helices and can be divided into three functional domains: (i) an N-terminal domain (residues 1–205) that contains the guanine nucleotide-binding region; (ii) an intermediate domain (residues 206–381) containing the Taxol drug-binding site and (iii) a C-terminal domain (resides 382–451) that likely constitutes the binding surface for MAPs and molecular motors such as kinesins and dyneins (17).

**Mutation detection rates and testing recommendations in LIS-SBH subtypes**

We identified *TUBA1A* mutations in 17 patients, most (67%) residing in the C-terminal domain. Five were detected in patients with unexplained classic LIS (~7%), all at codon R402. Another 10 mutations were found in patients with LCH (~34%) scattered through most of the coding region of the gene. In children with classic LIS resembling the LIS1 (posterior predominate) pattern, the expected mutation detection rate is ~85% (7). Our data predict that another ~1% of patients with classic LIS will have mutations of *TUBA1A* (i.e. 7% of the remaining 15% of LIS yet unexplained).

In children with LCH—a rare subtype of LIS—these results increase the mutation detection rate from essentially nil up to ~30%, making *TUBA1A* the first gene to explain a significant proportion of this rare phenotype. A few patients with another LCH variant [LCH group b (5)] have mutations of *RELN* or *VLDLR* (22–24).

Although published reports (14,25) suggest that *TUBA1A* mutations are found in some patients with SBH, we found no mutations in a series of 22 patients with unexplained...
SBH. We did see small areas partly resembling SBH in two patients with the mild–moderate form of LCH (as shown in Fig. 3N), but the brain imaging phenotype in these individuals was clearly dominated by pachygyria, not SBH.

Importantly, we found one mutation in a girl with ACC and CBLH but without LIS. This observation suggests that mutations of \textit{TUBA1A} may be responsible for a subset of children with isolated ACC, isolated CBLH or—as in our patient—combined ACC and CBLH without obvious LIS. Further studies in these latter groups are clearly needed.

These results have significant implications for clinical molecular testing for LIS. We conclude that sequencing of \textit{TUBA1A} is so far indicated in two groups of patients. First are those with classic LIS and negative deletion and sequencing analysis for \textit{LIS1} and \textit{DCX}. On brain imaging, we recognize classic LIS based on the presence of extensive agyria–pachygyria, 12–20 mm thick cortex and no more than mild CBLH. Second are those with LIS and moderate-to-severe CBLH, the so-called LCH, whether or not the corpus callosum is small. Experts in brain imaging in this group of disorders could exclude patients with a frontal gradient (LIS most severe in the anterior frontal lobes) and patients with the rare pattern associated with \textit{RELN} or \textit{VLDLR} mutations (mild frontal pachygyria, hippocampal hypoplasia and very small and near-afoliar cerebellum), but we do not recommend excluding these patterns generally. Although we did find one subject with combined ACC and CBLH without LIS, we do not have sufficient data to justify clinical testing of \textit{TUBA1A} in children without LIS at this time.

\textbf{Genotype–phenotype analysis}

Our genotype–phenotype analysis revealed two major phenotype subsets, each with varying degrees of severity. The first subset (groups 1 and 2) involves all seven patients with mutations of codon R402, where the brain phenotype matches the well-known phenotype associated with mutations in \textit{LIS1} or combined \textit{LIS1} and \textit{YWHAE} mutations. Specifically, the recurrent p.R402C phenotype resembles the \textit{LIS1} deletion or intragenic mutation phenotype, whereas the p.R402H phenotype resembles MDS caused by the co-deletion of \textit{LIS1} and \textit{YWHAE}. The second subset (groups 3 and 4)
involves patients with mutations of any other codon that differ dramatically from the LIS1-associated pattern. Mutations of other codons have much more variable phenotypes with more variable severity and (anterior–posterior) gradient of LIS and more consistent hypoplasia of the corpus callosum and cerebellum.

The second subset includes children with the rare LCH phenotype. In group 3, the gyral pattern often appeared most severe in the middle (posterior frontal or perisylvian-parietal regions), rather than at the frontal or occipital pole. Also, the callosal and cerebellar defects varied substantially in severity. Finally, the LCH seen in our group 4 is far more severe than ever seen in patients with LIS1 or codon R402 mutations. Taken together, our genotype–phenotype analysis suggests that mutations involving R402 specifically disrupt the LIS1-associated neuronal migration pathway, whereas other mutations are likely to disrupt multiple pathways and functions.

We reviewed the descriptions and available brain images for other published patients with TUBA1A mutations and found them generally compatible with our analysis of the phenotype spectrum (11–14,25). This was particularly true for the high-resolution images provided in Fig. 1 in the most recent paper (13).

Mechanisms of action for TUBA1A mutations

The mechanism(s) leading to the LIS or LCH phenotypes with heterozygous mutations is not clear, and we considered both haploinsufficiency and a dominant-negative effect. Several lines of evidence may support a dominant-negative mechanism of action. First, our data and those of others (12,16) show that mutant TUBA1A protein readily incorporates into the cellular cytoskeletal network, rather than failing to incorporate as might be expected with loss of function. Second, the observation of a nonsense mutation, frameshift mutation or gene deletion in even a single patient would support haploinsufficiency, but only missense mutations of TUBA1A have been identified to date. To extend this observation, we
searched several large cytogenetic databases including Deci-
pher (https://decipher.sanger.ac.uk/), DGAP (http://www.
bwhpathology.org/dgap/) and ECARUCA (http://agserver01.
azn.nl:8080/ecaruca/ecaruca.jsp), as well as PubMed, but
found no reports of individuals with TUBA1A deletions.
Further, no 12q13.12 deletions that contain TUBA1A were
found in approximately 15 000 subjects with developmental dis-
orders screened by microarrays (Swaroop Aradhya, GeneDx;
Lisa Schaffer and Jill Rosenfeld, Signature Genomics, personal
communications, 2009). We cannot exclude the possibility that
heterozygous null mutations of TUBA1A cause early embryonic
lethality, which would uphold haplinsufficiency. However, we
think that the pathogenic mechanisms are more complex than
simple haplinsufficiency given that LIS-associated TUBA1A
mutations incorporate and maintain typical microtubule struc-
ture. These mutations could disrupt the binding sites for
MAPs, leading to altered function or possibly dominant-
negative effects.

Recurrent R402 mutations
Mutations involving the R402 residue have been reported in a
total of eleven patients to date [seven described here and four
elsewhere (13,14)], demonstrating that this codon is a muta-
tional hotspot in TUBA1A. Our modeling data show that the
R402 residue is located on α-helix H11, which lies at the
outer surface of microtubules at the interface between
α-tubulin and various MAPs and molecular motor proteins,
including kinesin KIF1A (18). Our cellular assays clearly
demonstrated that mutant R402 proteins are translated, loca-
lize to the cytoplasm and incorporate properly into the cyto-
skeletal network. Collectively, our data indicate that
mutations involving the R402 residue are more likely to
affect the interaction between tubulin and tubulin-binding
proteins than to affect overall tubulin architecture. However,
further biochemical experiments would be needed to substanti-
ate these predictions. Given that R402 mutations result in a LIS1-like phenotype, we hypothesize that mutations involving
this residue specifically disrupt the LIS1 signaling pathway.
We propose that this may occur in a kinesin-dependent
manner that involves a LIS1-dynein-transportable microtubule
(tMT) complex, based on a recent observation that LIS1 and
cyttoplasmic dynein co-migrate towards the plus end of micro-
tubules in a complex with tMT (26). In this model, LIS1 fixes
dynein on tMT (freighter tubulins), which are then transported
to the plus end of cytoskeletal MTs in a kinesin-dependent
manner. Thus, we hypothesize that the R402 mutation specific-
ally interferes with kinesin-mediated anterograde transport of
LIS1-coupled dynein, without disrupting other functions of the
TUBA1A protein.

We further hypothesize that the LCH-associated mutations
of TUBA1A disrupt two or more distinct neuronal migration
pathways that converge on TUBA1A, such as LIS1-related
dCX-related pathways, resulting in the chaotic LIS gradi-
ent and severe callosal and cerebellar phenotypes. It follows
that mutations at less critical residues might cause corpus cal-
losum and cerebellar malformations without obvious LIS, as
we now document in one patient.

In conclusion, we have identified numerous novel mutations
in TUBA1A, including several recurrent mutations at two
codons that result in homogenous phenotypes. We provide
mutation frequency data that will contribute to clinical diagnos-
tics and demonstrate a wider spectrum of phenotypes than pre-
viously reported that now includes severe and moderate
classic LIS (groups 1 and 2), severe and moderate LCH
(groups 3 and 4) and ACC and CBLH not associated with LIS
(group 5). We also show that LIS-associated mutations are
likely to act in a dominant-negative manner by incorporating

Figure 5. Recombinant wild-type and mutant FLAG-tagged TUBA1A incorporate into the normal interphase microtubule network. After transfection into P19
cells, wild-type and mutant recombinant TUBA1A were visualized using methanol fixation and immunostaining using anti-FLAG antibodies.
into microtubules and disrupting tubulin-MAP interactions. Certainly, tubulin genes are emerging as strong candidates for analysis in neuronal migration disorders, given recent additional reports of mutations in TUBB2B in asymmetrical polymicrogyria (27) and TUBA8 in polymicrogyria with optic nerve hypoplasia (28).

MATERIALS AND METHODS

Patients

The Institutional Review Boards at the University of Chicago and the University Hospital of Wales approved this study. From our large cohort of subjects with unexplained LIS documented by review of brain imaging (available for all subjects and reviewed by W.B.D. or D.T.P.) and DNA samples available (n = 125), we selected 72 patients with classic LIS (29 females and 43 males), 22 with SBH (13 females and 9 males), 29 with LCH (17 females and 12 males) most of whom also had partial or complete ACC and two females with LIS and ACC but normal cerebellum on imaging. We combined the latter two groups for further analysis. All LIS subgroups were defined years before this study was conducted (5,29,30). Testing for nonsense mutations, missense mutations, deletions or duplications in LIS1 and DCX was negative in most patients (7). In the remainder, the most appropriate gene was tested with negative results. Specifically, we tested only LIS1 in some
patients with posterior-predominant LIS and tested DCX only in some with anterior-predominant LIS. We subsequently added two patients in whom TUBA1A mutations were found in clinical laboratories (one unexpectedly given the lack of LIS) and referred to us.

DNA isolation, amplification and sequencing
DNA was extracted from leukocytes from EDTA-treated blood using either the Puregene kit (Genta Systems, Inc., Minneapolis, MN, USA) or the MagNAPure Total Nucleic Acid Extraction system (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer’s protocols. PCR-amplification primers were designed using Primer3 (http://frodo.wi.mit.edu/) with M13 forward and reverse tails added to each primer to facilitate high-throughput DNA sequencing (Table 1). DNA was amplified in a reaction comprising: 20 ng genomic DNA, 1× buffer I (1.5 mM MgCl2, Applied Biosystems, Foster City, CA, USA), 1 mM dNTPs (Applied Biosystems), 0.4 μM primer (each of forward and reverse; IDT, Coralville, IA, USA) and 0.25 U AmpliTaq Gold (Applied Biosystems) in a total volume of 10 μl. Thermocycling conditions were as follows: 94°C for 10 min; 35 cycles of 94°C for 30 s, annealing temperature (53–60°C) for 30 s and 72°C for 30 s and final extension of 72°C for 10 min. Variations in reaction composition and cycling conditions were required for a small number of amplicons. PCR products were purified in a 10 μl reaction comprising 6.6 U Exonuclease I and 0.66 U shrimp alkaline phosphatase that were incubated at 37°C for 30 min followed by 80°C for 15 min. Sequencing reactions were performed using BigDye terminators on an ABI 3730XL 96-capillary automated DNA sequencer (Applied Biosystems) at The University of Chicago DNA Sequencing and Genotyping Core Facility. Sequence data were imported as ABI files into Mutation Surveyor v3.10 (SoftGenetics, State College, PA, USA). Sequence contigs were assembled by aligning ABI files against GenBank reference sequence files obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Genbank/). To screen for putative mutations, the entire length of the sample trace was manually inspected for quality and variation from the reference trace. All detected variants were visually reviewed by two trained individuals and were confirmed using bi-directional sequencing.

Transfection experiments
Mutations were introduced into the pRKSTUBA1A-CFLAG expression construct using the Quickchange site-directed mutagenesis kit (Stratagene). This construct expresses C-terminally FLAG-tagged TUBA1A under the control of the CMV promoter. Wild-type and mutant constructs were transfected into P19 cells (ECACC, 95102107) using Lipofectamine LTX reagent (Invitrogen). After 24 h, cells were fixed in ice-cold methanol, permeabilized with 0.1% (v/v) Triton X-100 and stained with antibodies recognizing FLAG (Sigma) and secondary anti-mouse antibodies conjugated to Alexa 488 (Molecular Probes). Structural modeling of TUBA1A mutations
Two atomic models of tubulin complexes were used for the analysis of the mutations: (a) protofilaments of α- and β-tubulin heterodimers and (b) the kinesin KIF1A-microtubule complex. The former model was based on docking of the α- and β-tubulin heterodimer crystal structure (PDB id: 1JFF) (31) into the cryo electron microscopy (cryoEM) map of the intact microtubules at 8 Å resolution (32) (coordinates kindly provided by Ken Downing, Life Sciences Division, Berkeley Lab), while the latter was based on docking of the same structure (PDB id: 1JFF) in addition to the kinesin KIF1A motor domain crystal structure (PDB id: 1VFV) into the cryoEM map of the complex in an AMPNNP (5′-adenyl-β,γ-imidodiphosphate) form at 10 Å resolution (PDB id: 2HXF) (33). In the original models, the interfaces between the domains suffer from minor clashes, most likely due to conformational differences between the structures of the domains in their isolated form and in the context of their assemblies. To remove these clashes, we first added hydrogens using VMD (34) and then applied conjugate gradient energy minimization to the interface between two heterodimers of α- and β-tubulin in the first model and to the interface between KIF1A and α-tubulin in the second model. The minimization was performed using NAMD (35) with the CHARMM27 forcefield (36). The final models were visualized and analysed with chimera (37); mutations were introduced based on the Dunbrack backbone-dependent rotamer library (38) and H bonds were identified with the FindHBond method (39). Cation–π interactions were identified using the program CaPTURE (40).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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