Mutations in Human Beta-2b Tubulin Result in Asymmetrical Polymicrogyria

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SUPPLEMENTARY INFORMATION:

I/ SUPPLEMENTARY FIGURES

II/ SUPPLEMENTARY NOTE

Supplementary Results:

- Expression data

Sections of embryos taken at embryonic day 14.5 (E14.5) and 16.5 (E16.5) showed strong labeling restricted to encephalic structures (cortex, cerebellum, brainstem), spinal cord, retina and also to the peripheral nervous system (dorsal root ganglia). We also note strong *Tubb2b* expression in cortical plate at both stages (Supplementary Figure 5).

In addition, we assessed the effect of the two mutations (p.L228P, p.S172P) on the level of *TUBB2B* mRNA expression in patient-derived cells and tissues. We found that in both cases, expression in lymphoblastoid cells and lung tissue was respectively decreased 4-fold and 11-fold, respectively, compared with wild type controls (data not shown). Though such results are not usual in the context of missense mutations, we cannot exclude an effect of the mutations on the stability of the mRNA.

- Biochemical investigations

To further characterize the folding of two of the TUBB2B mutants (p.F265L and p.S172P) that displayed impaired heterodimer incorporation upon expression in reticulocyte lysate, we repeated these reactions and analyzed the products kinetically. These experiments revealed other quantitative defects in the heterodimer assembly pathway, including a steady increase in mutant CCT/β-tubulin binary complexes, either a delay (p.F265L) or absence (p.S172P) of TBCD/β-tubulin co-complexes, and a relatively long persistence of prefoldin/β-tubulin co-complexes (Supplementary Fig. 8).

To examine the mechanism of defective heterodimer assembly of the p.F265L and p.S172P mutant polypeptides in detail, we did reconstituted folding reactions *in vitro* using purified components[1,2]. We found no measurable deficiency in the efficiency with which these mutant polypeptides were cycled by CCT (Supplementary Fig. 10).

On the basis of these data, together with of our biochemical investigations showing defects in the folding heterodimer assembly pathway in the case of p.S172P and p.F265L mutant TUBB2B, we conclude that 1) There is a dramatic failure of p.F265L

and p.S172P CCT-generated folding intermediates to stably interact with TBCA and 2) These mutations result in a reduced efficiency of intermediate interaction with TBCD. The latter conclusion is further reinforced by our observation that the p.S172P mutation results in a failure to form a stable "supercomplex"[2] in *in vitro* folding reactions performed with purified components in the presence of the slowly hydrolysable GTP analog, GTP-γ-S (Supplementary Fig. 11).

- Cellular investigations

We further tested whether the five TUBB2B mutations might affect microtubule dynamics by assessing microtubule repolymerization after a cold-induced depolymerization step. In the case of p.S172P, we observed two major defects. First, we noted a conspicuous absence of the mutant protein in the aster at each time point (Supplementary Figure 12). Similar data were obtained in the case of p.F265L. Second, we found a significantly reduced rate of microtubule regrowth from the centrosome (Supplementary Figure 12), suggesting that expression of the mutant protein impaired the ability of endogenously expressed heterodimers to assemble at microtubule plus ends. This experiment, taken together with the overexpression of the same mutant *in vivo* (see main text, Fig. 5b) suggests that a dominant negative effect cannot be excluded for this p.S172P mutation. For the other mutations, subtle effects on tubulin turnover or dynamics cannot be excluded, but were not observed in a regrowth assay (data not shown).

Supplementary Discussion:

Our data are consistent with the view that *TUBB2B*-related forms of PMG are primarily due to haploinsufficiency. The cellular consequences of *TUBB2B* haploinsufficiency, and the mechanisms by which this results in polymicrogyria, an unlayered cortex, and heterotopic neuronal cells remain unknown. We propose that the neuropathophysiology of the *TUBB2B*-related PMG might result from a combination of both neuronal migration impairment and radial glia dysfunction that lead respectively to ectopic neurons in the white matter and cerebellum, and to pial membrane breaches. On the one hand, our study provides evidence for the involvement of migration disruption in the pathology, as RNAi and fetal brain

histopathological analyzes showed a migration arrest of neurons within the IZ/SZV**.** On the other hand, with respect to the overmigration of neurons and the presence of excessive gyration, we propose that these neuropathological features are the result of microtubule dysfunctions in radial glia. Previous studies on the G-protein-coupled adhesion receptor 56 (*Gpr56*) animal model of PMG have shown that disturbances in the structure of the pial basal membrane result in overmigration of neurons in mice[3]. *TUBB2B* haploinsufficiency in radial glia could impair microtubule dependent processes that include recycling or intracellular trafficking of transmembrane receptors such as GPR56, which in turn could affect the integrity of the basement membrane through disruptions of cell-cell (neuron-radial glia) and/or cell-matrix (radial glial endfeet-pial BM) interactions.

III/ SUPPLEMENTARY METHODS

Patients and Analysis of *TUBB2B*

Prior to *TUBB2B* analysis, and in accordance with the diagnosis proposed by the referring physician, mutations in the following genes were previously excluded by the diagnostic center : *DCX* (RefSeq NM_181807), *LIS1* (RefSeq NM_000430), *ARX* (RefSeq NM_139058), *TUBA1A* (RefSeq NM_006009), and *GPR56* (RefSeq NM_201524). For patients with PMG syndromes, DNA was also analyzed by CGH-microarray using the NimbleGen chip 385,000-probes (385 K) array (Roche-NimbleGen, Madison, WI) to exclude potential pathogenic CNVs.

For the fetuses, a diagnosis of severe brain developmental delay with gyral abnormalities had been established by ultrasound and/or MRI.

To better define clinical and imaging phenotypes associated with *TUBB2B* mutations, all patients found to be carrying mutations in *TUBB2B* were re-examined and their clinical and imaging features compared.

For each patient, the complete *TUBB2B* coding sequences (RefSeq NM_178012) and splice sites were amplified in five independent PCR reactions from genomic DNA. Primer sequences and positions, PCR conditions and product sizes are available under request. PCR products were checked by 2% agarose gel electrophoresis, and analyzed by direct sequencing using the BigDye dideoxyterminator chemistry (Applied Biosystems, Foster City, CA), and ABI3700 DNA analyzer (Applied Biosystems). For the nomenclature of *TUBB2B* mutations, nucleotide numbering is designated according to the cDNA reference sequence in GenBank Accession number NM_178012, in which the “A” of the ATG translation initiation codon is nucleotide #1 and the initiation codon is codon #1.

Expression studies

To define the spatio-temporal profile of *Tubb2b* expression (in the absence of specific antibodies), we used RNA ISH. We did not attempt to produce antibodies and study TUBB2B protein expression because the chances to get a specific antibody for *Tubb2b* are really thin. The extreme homology of *TUBB2B* with the next-door *TUBB2A,* which differs only by 2 amino acids out of 445 (See Supplementary Figure 1) did not favor the option of protein expression analysis.

For quantitative RT-PCR expression studies, total RNA samples were prepared from mouse brains from E8.5 to E16.5 embryos, newborn and adult mice, and from primary cultured cortical neurons, primary cultured astro-glial cells and the C6 glial cell line. Primary cultures of cortical neuronal cells were established from cell suspensions of fetal mouse brain at embryonic day 15.5. Dissociated cells were plated and cultured for 2 to 28 days on microwell poly-D-lysine/laminin-coated coverslips (Sigma-Aldrich Inc., St. Louis, MO).

Quantitative RT-PCR was performed using oligonucleotides F2 5'-AACAGCAAGAAGCTAACCAGG and R1 5'-AGGCACATATTTATTACCAGTTGC for mouse *Tubb2b* RNA analysis. Oligonucleotides specific for *cyclophilinA* (NM_008907) were used as a control. Amplification was done using SYBR Green chemistry (LightCycler FastStart DNA Master, Roche diagnostics, Switzerland) and Roche amplification technology. All experiments were reproduced three times in duplicate.

For the preparation of probes for RNA *in situ* hybridization, *in vitro* transcription was performed using a specific 168pb probe of *Tubb2b* mouse mRNA subcloned into pCR4-topoTA cloning vector (Invitrogen, Carlsbad, CA). The probe corresponds to 121 bases upstream and 45 bases downstream of the A of the ATG translation initiation codon according to the *Mus musculus* GenBank cDNA reference sequence (NM_023716). For all experiments, sense and antisense probes were generated in parallel in order to assess the specificity of the signal. Briefly, RNA *in situ* hybridization using ^{35}S-labelled probes was carried out on frozen mouse sections in a 50% (v/v) formamide solution at 60°C. Sections were washed in 50% (v/v) formamide, then in successively stringent SSC solutions, with a final wash in 0.1x SSC at 60°C. Slides were autoradiographed (using BioMAX XAR, Kodak, Pathé, Paris, France) then dipped in diluted Kodak NTB2 emulsion (Kodak, Pathé, Paris, France) and exposed for 7-15 days. Emulsions were developed and sections counterstained with hemalun, mounted in VectaMount (Vector laboratories, Peterborough, UK) and examined by light microscopy.

Magnetic activated cell sorting and quantitative RT-PCR

Rat C6 cells cultured following standard procedures were co-transfected with a mU6-pro vector (containing CDS-sh, 3'UTR-sh, scrambled CDS-sh, scrambled 3'UTR-sh or an unrelated scrambled sequence as a control) and a plasmid carrying a CMV

promoted gene encoding the surface marker *CD20* (molar ratio 10:1) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Recovery of transfected cells was performed as described[4]. Briefly, cells were collected 48 hours after transfection, and CD20 positive cells were selectively sorted using a mouse anti-human CD20 primary antibody (Southern Biotech, Birmingham, AL) and goat anti-mouse magnetic microbeads (Miltenyi Biotech, Auburn, CA). CD20 labeled cells were positively selected on MS magnetic columns (Miltenyi Biotech, Auburn, CA).

The efficiency of RNAi on *Tubb2b* mRNA stability was assessed on CD20 positive purified populations by qRT-PCR, using the following forward (5'-CCCAGACCCAAGGCAGCAAG) and reverse (5'-CCATGCTCATCACTGATGACC) primers on a LightCycler 480 Real-Time PCR System (Roche Applied Science, Basel, Switzerland). Quantifications were obtained using the advanced relative quantification mode (Light Cycler 480 software v1.5.0). *Tubb2b* expression was normalized to the expression of *cyclophilinA* in the same sample. The efficiencies of our shRNAs in repressing *Tubb2b* mRNA abundance were represented as a percentage of the effect induced by the control unrelated mismatch shRNA.

Histology and analysis of rat brain sections

Embryonic brains were removed five days later (E20) and fixed using Antigenfix (Diapath, Martinengo, Italy) overnight at 4˚C. They were further embedded in 4% (w/v) agar (Sigma Aldrich, St Louis, MO) and coronally sectioned at 100 µm thickness on a vibratome HM650V (Microm, Walldorf, Germany). *NeuroTrace* 500/525 green-fluorescent Nissl staining (1:500, Molecular Probes-Invitrogen, Carlsbad, CA) was performed on free-floating sections of RFP/shRNA transfected brains to observe the position of RNAi electroporated cells within overall cortical structures. Sections were mounted in Fluoromount-G (Southern-Biotech, Birmingham, AL) and analyzed with a confocal imaging system (FluoView-Olympus, Center Valley, PA). Plot profiles of fluorescence across cortical thickness were generated on sections of independent brains using ImageJ (NIH, Bethesda, MD).

Statistical testing

Fluorescence intensities were recovered in a table format and further processed for statistical analysis using InStat (GraphPad, La Jolla, CA). First, normal distributions of data sets were assessed using the two samples Kolmogorov-Smirnov test. In each

group, the distribution did not differ significantly from that of a normal (Gaussian) distribution as indicated by *P* values >0.10. Thus, one-way Analysis Of Variances (ANOVA) (alpha=0.05) were used on unmatched groups, on the basis of the null hypothesis stating that there are no differences between means of different classes, suggesting that the variance of the within-class samples should be identical to that of the between-class samples. In cases where *P* values <0.05 reject the null hypothesis, a post-hoc test was performed (Tukey-Kramer multiple comparisons test, alpha=0.05) to discriminate pairs of populations that significantly differ.

Cloning

A full-length cDNA encoding the human TUBB2B sequence was generated by PCR using a human brain cDNA library (Clontech, Mountain View, CA) as template. The PCR product was cloned into the pRK5 vector (Addgene, Cambridge, MA) and checked by DNA sequencing. Various mutations corresponding to those associated with cortical dysgenesis (see Results) were introduced by site directed mutagenesis using a Quikchange II kit (Stratagene, La Jolla, CA). A tag encoding the FLAG epitope (DYKDDDDK) was incorporated by PCR in-frame with the C-terminus of the TUBB2B wild type sequence to allow the distinction of the transgene from other highly homologous endogenously expressed β-tubulin polypeptides. These products were also cloned into the pET-23b(+) vector (Novagen, Madison, WI). All constructs were checked by DNA sequencing

Kinetic *in vitro* translation experiments

In kinetic experiments, aliquots (1.5 μl) were withdrawn from the reaction at various times, diluted into 5 μl of gel loading buffer (gel running buffer supplemented with 10% glycerol and 0.1% bromophenol blue), and stored on ice prior to resolution on a non-denaturing gel[1,2].

***In vitro* folding reactions**

To investigate the ability of CCT to cycle various target proteins, these were presented by sudden dilution from urea to CCT[5] at 0˚C in order to allow the formation of the CCT/β-tubulin binary complexes. These complexes were then separated from unincorporated probe by passage over a microcolumn of Sephadex G25, and

incubated with a large molar excess of the mitochondrial chaperonin Hsp60. This chaperonin is incapable of generating productive tubulin folding intermediates, and therefore acts as a trap that captures intermediates discharged from CCT[6]. In experiments performed to assess the GTP hydrolysis-dependent release of *de novo* assembled tubulin heterodimers from the TBC-containing supercomplex, GTP-γ-S was substituted in place of GTP at a concentration of 1 mM.

Microtubule repolymerization assay

In regrowth experiments to determine the dynamic behavior of microtubules, cells were incubated at 4˚C for 30 min 24 hours post-transfection, restored to 37˚C, and fixed at various brief intervals thereafter with -20˚C methanol. Cells were stained with a polyclonal anti-FLAG antibody (to visualize protein expressed from the transgene; green) and a monoclonal anti-α-tubulin antibody (to visualize the overall microtubule network; red) (DM1A, Sigma-Aldrich Inc., St. Louis, MO).

Sequence alignments

Alignments were performed using ClustalW algorithm in Bioedit sequences alignment editor (Ibis Biosciences, Carlsbad, CA). Protein sequences of β-tubulin 2B of different species were obtained from USCS Genome Browser (http://genome.ucsc.edu/); human TUBB2B (Q9BVA1); *Mus musculus* Tubb2b (AAI38937); *Rattus norvegicus* NC_005116; *Bos Taurus* NP_001003900; *Xenopus tropicalis* NP_989275; *Gallus gallus* NP_001004400; *Danio rerio* NP_942104; *Drosophila melanogaster* NP_523795; *Caenorhabditis elegans* NP_509585; *Saccharomyces cerevisiae* YFL037W; human TUBB2A (Q13885); human TUBB2C (P68371).

IV/ SUPPLEMENTARY REFERENCES

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