Supplementary Methods

Exome sequencing

Exome capture was carried out with the Nextera Rapid Capture Exome Kit (Illumina, Inc., San Diego, CA). The kit covers 214,405 exons with a total size of about 37 Mb. Sequencing was done using HiSeq4000 sequencers (Illumina) to produce 2 × 150-bp reads, and pooling up to nine exomes per lane. The bioinformatics pipeline was based on the 1000 Genomes Project data analysis pipeline (Wang et al., 2013) and on Genome Analysis Toolkits (GATK) best practice recommendations (McKenna et al., 2010). In short, raw sequencing data were first converted to standard fastq format using bcl2fastq (Illumina 2.17.1.14), and then aligned to the GRCh37 (hg19) build of the human reference genome using Burrows–Wheeler Aligner (BWA) software (Li et al., 2009). Alignments were converted to binary bam file format, sorted on the fly and de-duplicated without intermediate input-output-operations to temporary files to achieve maximal performance. Variant calling was performed on using GATK HaplotypeCaller with standard parameters. The quality score emitted from GATK HaplotypeCaller is the phred-scaled posterior probability that all samples in a callset are homozygous reference under the prior of variants occurring in the population. It describes the likelihood of wrong evidence for the variant. Practically few high quality reads with evidence for a variant will cause low quality scores, but more high quality reads supporting the variant will result in higher quality scores. As such the quality score describes genomic site in contrast to GQ or PL that describe the confidence in correct genotype assignment at the given site.

Immunoblotting of human brain tissue lysates

Tissues were lysed at 4^0C in lysis buffer (RIPA buffer, complete protease inhibitor cocktail (Roche) and 1 mM DTT, and centrifuged at 13.200 RPM for 20 min at 4^0C. Protein lysates were collected and total protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher). The samples were loaded (30ug) on a Criterion precast gel 4-12% (#3450125, Bio-Rad Laboratories) and run at 100-120 volt for 1 hour. Proteins were transferred to a Trans-Blot Turbo nitrocellulose membrane (#1704159, Bio-Rad) using the Mini Trans-Blot Electrophoretic Transfer System (Bio-Rad Laboratories). The membrane was then blocked in washing buffer (Phosphate Buffered Saline with Tween 0.1%) containing 5% milk (ELK Milk powder, Campina) for 30 min at room temperature. Primary antibodies, rabbit anti-TUBA4A (1:8000, AP13535b, Abcepta), and rabbit anti-GAPDH (1:1000, GTX100118, Genetex) were incubated overnight at 4^0C in PBS-T with 5% milk. The membrane was washed with PBS-T and incubated with IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody (#926-32211, LI-COR) in PBS-T and finally washed with PBS-T. The membrane was scanned using Odyssey clx (LI-COR) and analysed with Image Studio Lite (LI-COR).

Design and validation of TUBA4A constructs

HA-tagged constructs were made by cloning the HA sequence (YPYDVPDYA; 5'-TACCCATACGATGTTCCAGATTACGCT - 3') and human TUBA4A cDNA sequence into the pCDNA3 expression vector carrying a CMV promoter, using PCR and Gibson Assembly (New England Biolabs). The R105C and R320C mutants were generated using the Quikchange Lightning Site-Directed mutagenesis kit (Agilent), according to the manufacturer's instructions. The specific mutagenic primers used are :

R105C Fwd 5'- CAGCCAACAACTATGCGTGCGGTCATTACACCATT- 3'
Rev 5'- AATGGTGTAATGACCGCACGCATAGTTGTTGGCTG- 3'
R320C: Fwd 5'- GCTGCCTGCTGTACTGTGGAGATGTGGTG - 3'
Rev 5'- CACCACATCTCCACAGTACAGCAGGCAGC - 3'

After cloning, all constructs were verified using Sanger sequencing (Macrogen).

To evaluate the expression levels of TUBA4A constructs, cells were transfected in 6-well plates with the indicated constructs and 24 hours later, scraped and lysed in SDS sample Buffer (Laemmli, 1970, Nature) containing 2% SDS, 10% glycerol, 5% b-mercaptoethanol, 33 mM Tris pH 6.8, and Protease Inhibitor Cocktail (Roche, according to manufacturers' instructions). Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% non-fat powder milk (Sigma) or 5% bovine serum albumin (BSA, Sigma) in PBS with 0.1% Tween-20 (PBS-T) and incubated with primary antibodies (rabbit anti-TUBA4A, AP13535b, Abcepta; mouse anti-HA antibody, #2367, Cell Signaling Technologies), overnight at 4^0C. After three washes, membranes were incubated with Horseradish Peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit and anti-mouse; GE Heathcare) at 1:10,000 dilution for one hour. Proteins were visualized by enhanced chemiluminescence detection (ECL, GE Healthcare).

Microtubule repolymerization assay and cell viability

COS1 and COS7 cells were cultured at 37°C, and 5% CO_2 in Dulbecco's Modified Eagle's Medium (D-MEM, Gibco) supplemented with 10% Fetal Calf Serum and 1% Penicillin/Streptomycin mix (Gibco). Cells were seeded on 18 mm coverslips and transfected with the indicated plasmids encoding HA-tagged wild type or mutant TUBA4A proteins using Xtremegene HD (Roche) according to the manufacturer's instructions.

Twenty-four hours after transfection, we treated COS1 cells with 10 µM nocodazole (Sigma) for 2 hours at 37°C. Next, we transferred the coverslips into warm medium for the indicated times to wash out the nocodazole and immediately plunged them into cold methanol at -20°C for 10 minutes to fix the cells. Cells were permeabilized in 0.15% Triton X-100 in PBS, and blocked for 1 hour at room temperature using 1% BSA in PBS containing 0.05% Tween-20 (PBS-T). Cells were incubated first with primary antibodies (rabbit anti-β-tubulin antibody; Abcam, ab6026, and

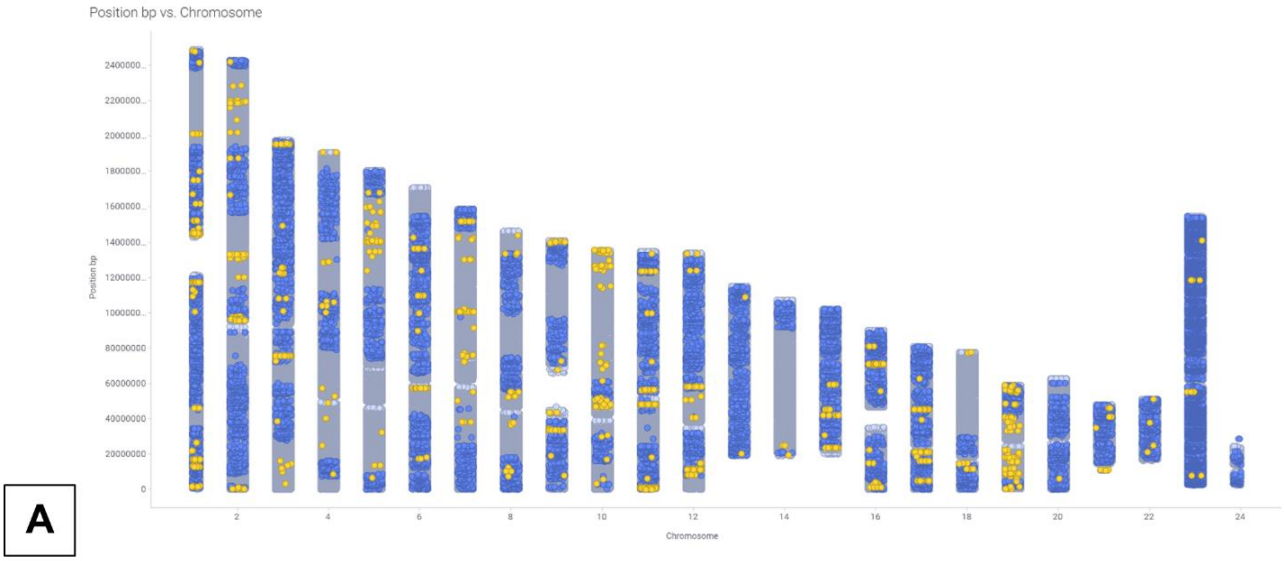
mouse anti-HA antibody, #2367, Cell Signaling Technologies) for 1 hour, washed three times in PBS-T and then with secondary antibodies (goat anti-rabbit Alexa Fluor-488, and goat Anti-mouse Alexa Fluor 594; Invitrogen) for 45 minutes. Coverslips were mounted in Prolong Gold containing DAPI (Invitrogen). Cells were imaged on a Leica SP5 laser scanning confocal microscope using a 63X oil objective (HCX PL APO CS) with a 2X spectral PMT for 488 nm excitation and a 1X spectral hybrid detector for 561 nm excitation. All images were acquired with identical settings. For assessing recovery after nocodazole washout, we acquired 8-10 images for each condition and time point and counted the number of transfected cells positive for a) endogenous asters and/or MT staining as visualized with anti-β-tubulin antibody, and b) HA-TUBA4A WT, R105C and R320C incorporation at asters and MTs visualized with anti-HA antibody. Results were combined from two independent experiments.

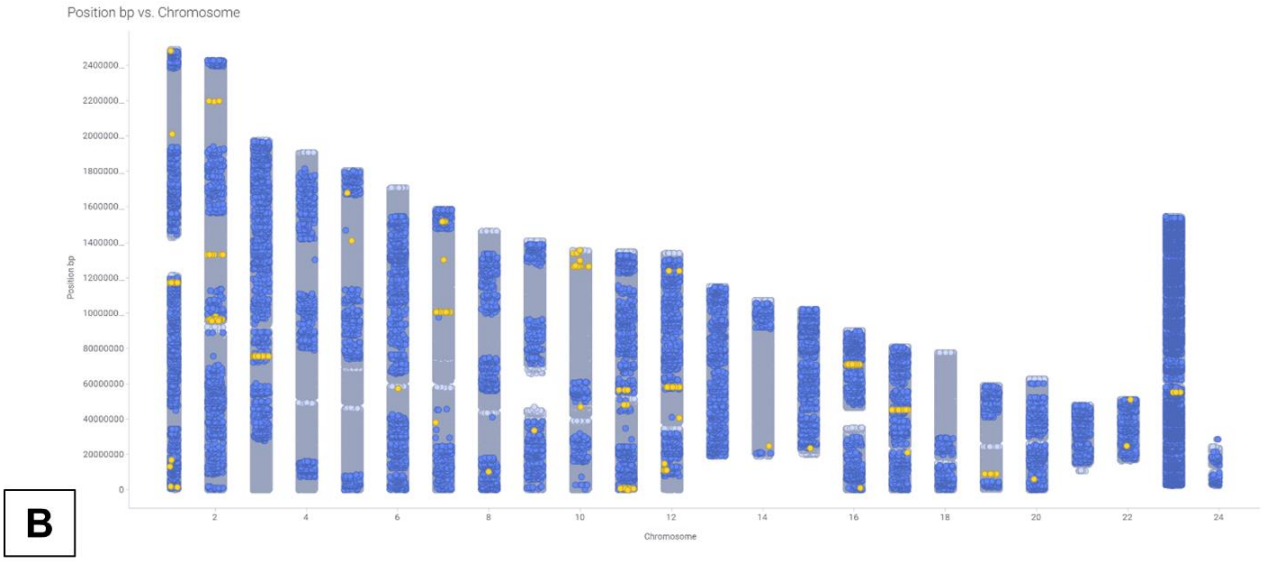
Additionally, we evaluated cell viability of wild type compared to the R105C construct by fixing the cells after 24, 48, and 72 hours, and processed as described above. The percentage of transfected cells was examined with anti-HA and anti-tubulin antibodies using immunofluorescence.

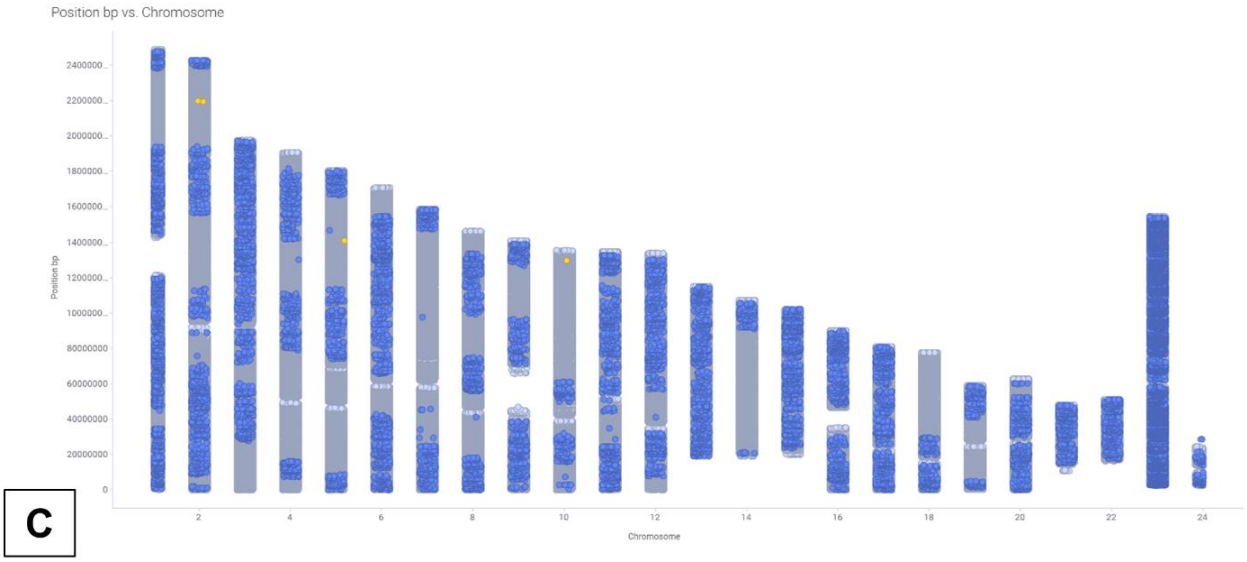
Microtubule integrity assay

To examine the stability of MTs upon expression of the various TUBA4A constructs, we co-transfected COS7 cells with HA-tagged tubulins and EMTB-mCherry, an indirect fluorescent marker of MTs (Bulinski et al., 1999). After transfection, cells were fixed and mCherry signal was imaged to assess the overall integrity of the MT network as previously described (Smith et al., 2014). For each image, a region of interest (ROI) was selected and the signal was thresholded to remove background noise. A circularity filter (circularity index ≥ 0.2) was then applied to the thresholded ROI to remove granular staining. The resulting image represented the filamentous staining. The ratio between the fluorescence intensities of the filtered cytoskeleton versus the thresholded ROI yielded an integrity index ranging from 0 (no integrity) to 1 (perfect integrity).

Supplementary Figures and Tables





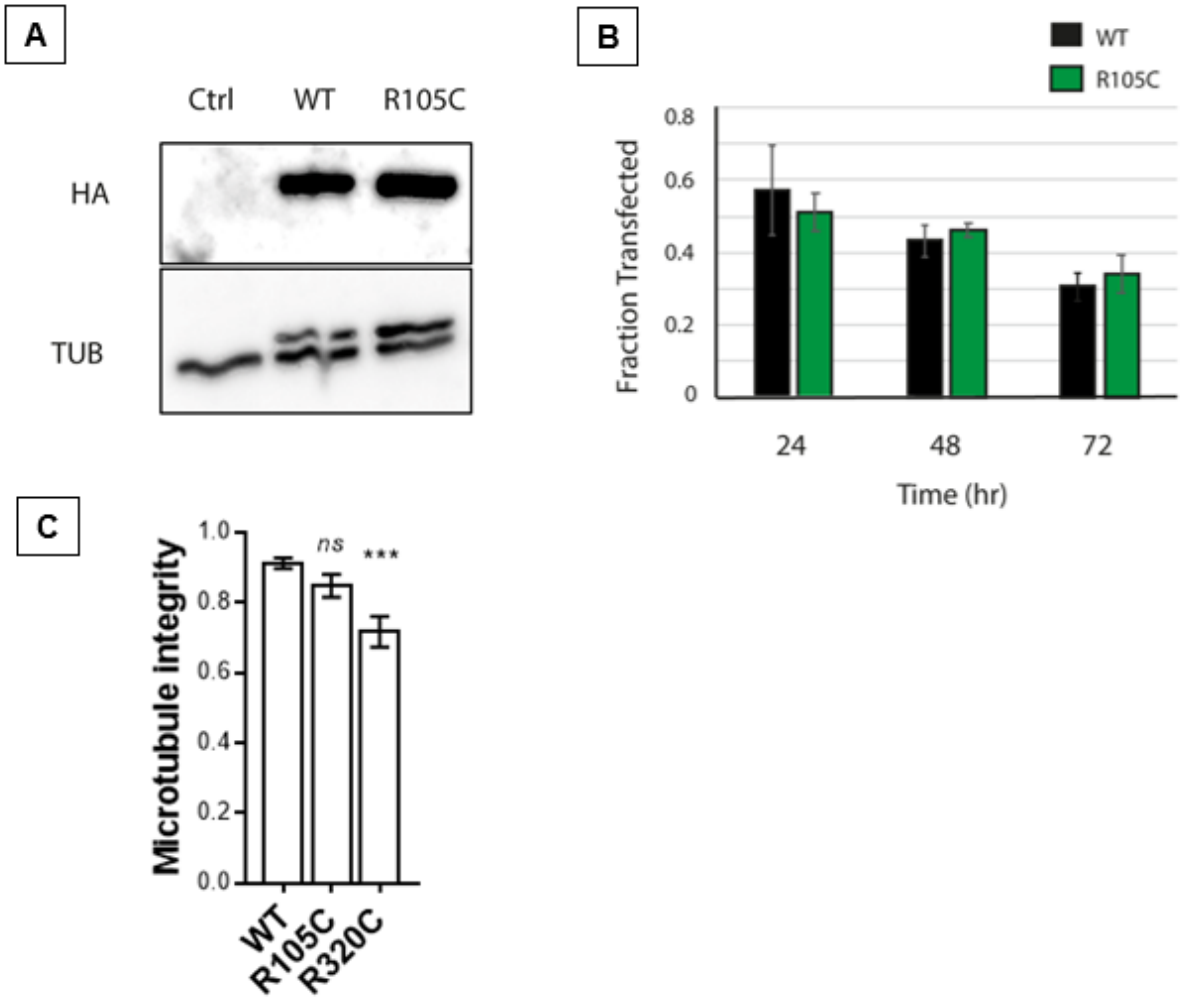


Supplementary Figure 1. Haplotype sharing analysis based on Global Screening Array (GSA) combined with exome sequencing variant filtering.

A) The SNPs that mark opposite homozygosity and therefore indicate the borders of a common haplotype are shown in blue. The areas in between represent shared haplotype blocks among four affected individuals. All shared heterozygous coding or splicing variants (missense/nonsense/frameshift) identified by exome sequencing in five affected individuals are shown in yellow.

B) Variants left depicted in yellow after filtering for minor allele frequency <0.01%

C) Four variants are left depicted in yellow after additional filtering (segregation with unaffected relative and quality control). Only the variant in *TUBA4A* is highly expressed in brain.

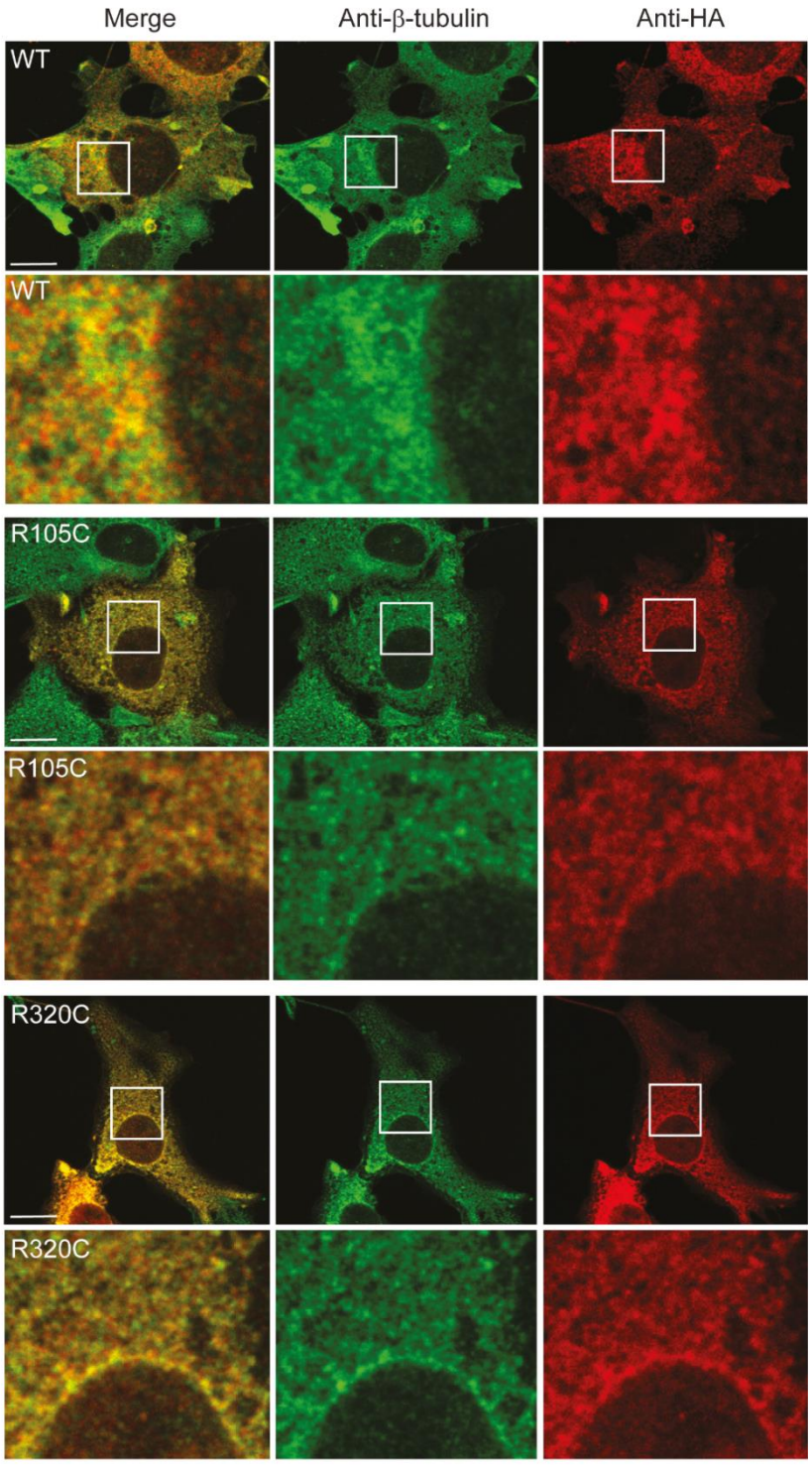


Supplementary Figure 2. Verification of tubulin constructs, cell viability, and microtubule integrity experiments.

A) COS1 cells were transfected with HA-tagged WT tubulin or TUBA4A-R105C construct. Cell lysates were collected after transfection and analyzed by western blot using anti-HA or anti-α-tubulin (TUB) antibodies. This reveals specific detection by the HA antibody of the transfected proteins, and indicates that both HA-tagged proteins are expressed at similar levels.

B) Cells were fixed 24, 48, or 72 hours after transfection and the percentage of transfected cells was examined using anti-HA and anti-tubulin antibodies in an immunofluorescence approach. We did not observe a difference in the fractions of transfected cells at any time point, indicating that the TUBA4A-R105C construct is not toxic to the cells. WT 24h, n=109 cells; WT 48h, n=258 cells; WT 72H, n=163 cells; R105C 24h, n=121 cells; R105C 48h, n=131 cells; R105C 72h, n=219 cells.

C) COS7 cells were co-transfected with HA-tagged tubulins and EMTB-mCherry. After transfection, cells were fixed and mCherry signal was imaged to assess the overall integrity of the microtubule network. The R320C mutation, used as positive control, has a significant effect on the integrity of the network, while the R105C mutant has not. ***p<0.001, n=16, 24, 23 for WT, R105C, and R320C, respectively (one way-ANOVA and Dunnet's post hoc test).



Supplementary Figure 3. Nocodazole washout figures at 0 minutes.

HA-tagged wild type (WT) and TUBA4A-R105C and -R320C constructs were transfected into COS1 cells. We studied the repolymerization potential of microtubules one day after transfection, at 0, 5, 10, and 30 minutes following nocodazole wash-out. Cells were stained with anti-β-tubulin (green) to visualize all microtubules and anti-HA antibody (red) for the transfected constructs (wild type, R105, and R320C). Insets are magnified below the overview images, which show the cellular area where the centrosome is normally located. At the 0 minute time point, the centrosome is not visible in any of the cells. Scale bar: 10 µm.

Gene	Transcript	Nucleotide change	Protein change	SNP	MAF GnomAD	CADD score (v1.3)	Brain expression (TPM)*	LOF Z-score**	pLI**	Annotation
TUBA4A	NM_006000.2	c.313C>T	p.Arg105Cys	Novel	0	32	76	1,82	2,96E-02	Microtubule cytoskeleton
ZNF142	NM_001105537.2	c.1318G>T	p.Asp440Tyr	rs1490267320	4.0^{E}-06	28.5	9	1,48	1,88E-15	Transcriptional regulation
PTPRE	NM_001323355.1	c.1369C>T	p.Arg457Trp	rs777749200	1.2^{E}-05	34	7	4,29	1,00E-02	Cell differentiation, signal transduction
ARAP3	NM_022481.5	c.1312G>A	p.Glu438Lys	rs536292464	8.1^{E}-05	27.8	5	5,97	9,68E-01	Cytoskeleton, signal transduction

Supplementary Table 1. Candidate variants identified after filtering of exome sequencing data.

*Average brain expression across all regions in TPM (read counts divided by the length of the gene in kilobases); derived from the Genotype-Tissue Expression (GTEx) Project.

** Higher LOF Z-score indicates that the transcript is more intolerant of variation (more constrained). This Z-score is highly correlated with gene length (r = 0.57), with longer genes having higher scores (more intolerant) due to higher confidence in their predictions. Therefore, the probability of being loss-of-function intolerant (pLI) is the suggested metric for evaluating a gene's intolerance (constraint) of loss-of-function variation.

Abbreviations: SNP, single nucleotide polymorphism. MAF, minor allele frequency in Genome Aggregation Database (GnomAD). CADD, Combined Annotation Dependent Depletion score; TPM, transcripts per million; LOF, loss of function.

Variant	Exon	Domain	Sex	Diagnosis	Age at onset	Disease duration	Family history	Segregational evidence	Functional evidence?	Notes	Reference
V7I	1	N-terminal	F	ND	ND	ND	ND	Absent	Absent		Pensato et al.
G43V	2	N-terminal	M	ALS-FTD	52	4	Dementia	Absent	Absent	Detected in 1 control	Smith et al.
R64Gfs*90	2	GTPase		FTD [SD]	49	ND	PD+MCI	Absent	Absent		Perrone et al.
IVS2+4A>G	2	N/A	M	ALS	57	2	Negative	Absent	Absent	exon 2 skipping	Pensato et al.
R105C	**3**	**GTPase**	**Multiple affected relatives with FTD**					**Yes**	**Absent**		**Current study**
T145P	4	GTPase	M	ALS	48	6	Dementia	Affected father carrier	Absent		Smith et al.
R215C	4	GTPase	F	ALS-FTD	78	ND	Dementia	Absent	Yes	Affects MT stability	Smith et al.
I234T	4	GTPase	ND	ALS	51	ND	Negative	Absent	Absent		Li et al.
R320C	4	C-terminal	M	ALS	64	2	Negative	Absent	Yes	Affects MT polymerization, dynamics, and stability	Smith et al.
R320H	4	C-terminal	F	ALS	41	3	ALS	Absent	Yes	Affects MT polymerization and stability	Smith et al.
T349S	4	C-terminal	F	ALS	62	1	Negative	Absent	Absent		Pensato et al.
T381M	4	C-termina	F	ALS	47	10	ALS+MCI	Affected sibling carrier	Absent	C9orf72 RE present	Perrone et al.
A383T	4	C-terminal	M	ALS	71	2	ALS	Absent	Yes	Affects MT polymerization and stability Detected in 1 control	Smith et al., Li et al.
H393Pfs*19	4	C-terminal	Multiple affected relatives with ALS					No cosegregation with affected relatives	Absent	SOD1 variant with cosegregation	Li et al.
W407*	4	C-terminal	F	ALS	66	3	ALS	Absent	Yes	Affects MT polymerization, dynamics, and stability	Smith et al.
K430N	4	C-terminal	F	ALS	64	4	ALS	not detected in an affected first cousin	Absent		Smith et al.
D438N	4	C-terminal	M	ALS+MCI	59	2	ALS	Absent	Absent		Pensato et al.

Supplementary Table 2. Detailed information of identified and reported variants in *TUBA4A*.

Reported variants from four previous studies are presented with their location in the gene (exon) and protein (domain). The variant shown in red is the variant identified in the current study. The degree of evidence supporting pathogenicity varies, with most variants lacking familial segregation. The two variants G43V and A383T were detected once in a set of 13,023 control samples. The variant T381M was identified in two siblings who also carried a pathogenic repeat expansion (RE) in *C9orf72*, and H393Pfs*19 was found in a family with a pathogenic variant in *SOD1*, segregating with disease. Therefore, these variants are less likely to be the single cause of disease. The variant IVS2+4A>G was proven to abolish the original donor splice site resulting in exon 2 skipping using a minigene splicing assay. Functional experiments have been performed by Smith et al. with ALS-associated variants, showing a disruption of microtubule repolymerization, dynamics, and/or stability. The variant W407* showed the most dramatic effect with deficient formation of heterodimers and incorporation into the MT network.

Abbreviations: ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; MCI, mild cognitive impairment; ND, not described, MT, microtubule.