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## **De novo and Inherited Monoallelic Variants in Tubulin Alpha-4A (*TUBA4A*) cause Ataxia and Spasticity.**

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### **Abstract:**

Alpha-tubulin 4A encoding gene (*TUBA4A*) has been associated with familial amyotrophic lateral sclerosis (fALS) and fronto-temporal dementia (FTD), based on identification of likely pathogenic variants in patients from distinct ALS and FTD cohorts. By screening a multicentric French cohort of 448 unrelated probands presenting with cerebellar ataxia, we identified ultra-rare *TUBA4A* missense variants, all being absent from public databases and predicted pathogenic by multiple in-silico tools. In addition, gene burden analyses in the 100,000 genomes project (100KGP) showed enrichment of *TUBA4A* rare variants in the inherited ataxia group compared to controls (OR : 57.0847 [10.2- 576.7]; p = 4.02 x10<sup>-07</sup>). Altogether, we report 12 patients presenting with spasticity and/or cerebellar ataxia and harboring a predicted pathogenic *TUBA4A* missense mutation, including 5 confirmed *de novo* cases and a mutation previously reported in a large family presenting with spastic ataxia. Cultured fibroblasts from 3 patients harboring distinct *TUBA4A* missense showed significant alterations in microtubule organisation and dynamics, providing insight of *TUBA4A* variants pathogenicity. Our data confirm the identification of a hereditary spastic ataxia disease gene with variable age of onset, expanding the clinical spectrum of *TUBA4A* associated phenotypes.

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## *TUBA4A* monoallelic variants cause spastic ataxia

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**Running title:** *TUBA4A* monoallelic variants cause spastic ataxia.

**Key words:** Ataxia; *TUBA4A*; Spasticity, De novo; Tubulinopathy, Cerebellar atrophy

**Abbreviation:** ALS: Amyotrophic Lateral Sclerosis; CCAS: Cerebellar Cognitive and Affective Syndrome; FTD: Fronto-Temporal Dementia. HA: Hereditary Ataxia; HSP: Hereditary Spastic Paraplegia; LMN: Lower motor neuron; MAP: Microtubule Associated Protein; MT: Microtubules; MSP: Multiple Systemic Proteinopathy; PTM: Post-Translational Modifications; PLS: Primary Lateral Sclerosis; UMN: Upper motor neuron; WES/WGS: Whole Exome/Genome Sequencing.

## **Introduction:**

Microtubules (MTs) are composed of  $\alpha/\beta$  tubulin heterodimers and play fundamental roles during the development of axons, dendrites, and neuronal synapses. The tubulin gene family contains several isotypes which follow a well-orchestrated spatio-temporal expression pattern.

<sup>1</sup> Pathogenic variants in more than half of the 26 known tubulin encoding genes have been described in recent decades as a cause of tubulinopathies. Tubulinopathies represent a wide range of overlapping neurodevelopmental and neurodegenerative disorders, remarkable by the significant variability of clinical presentations.<sup>2,3,4,5</sup> The Alpha-tubulin 4A isoform encoded by

*TUBA4A* monoallelic variants cause spastic ataxia

*TUBA4A*, has been proposed as the cause of familial Amyotrophic Lateral Sclerosis (fALS) 22 and/or Fronto-Temporal Dementia (FTD), based on identification of likely pathogenic variants in unrelated patients from several fALS/FTD cohorts. <sup>6,7,8</sup> However, other studies state insufficient evidence to support its causal role in fALS and FTD. <sup>9</sup> Very recently, the p.Glu415Lys *TUBA4A* variant has been identified in a large Italian family with spastic cerebellar ataxia and in an unrelated sporadic patient with *de novo* occurrence. <sup>10</sup> Herein we describe 12 patients from 11 unrelated families harboring ultra-rare missense variants in *TUBA4A*, including 5 *de novo* variants. Most patients presented with a neurodegenerative disorder leading to spastic cerebellar ataxia with variable age of onset and disease severity. We carefully characterized the patients' phenotype by providing detailed clinical data and imaging, when available. Functional studies on MT properties were performed on cultured fibroblasts from 3 patients.

## Material and Methods

### Genetics analysis

#### Patient recruitment

**Patients 1, 2 and 5** were part of a larger French cohort of 448 index cases from unrelated families, presenting with unexplored inherited ataxia and/or spastic paraplegia, screened by Clinical exome sequencing using Illumina NextSeq technology ([www.illumina.com/trusightone](http://www.illumina.com/trusightone)), including 366 families previously described. <sup>11</sup> **Patients 3, 4, 7, 8 and 12** were recruited through Genematcher Exchange. <sup>12</sup> Patients 3 and 4 were screened by Whole-Genome Sequencing performed on French sequencing platform using Illumina NovaSeq technology (AURAGEN and SEQOIA platforms). Genetic screening of patients 7, 8 and 12 was performed by WES. **Patients 6, 9, 10, 11** were part of the UK 100,000 Genome Project (100KGP) and were identified through a rare-variant gene-based burden case-control

*TUBA4A* monoallelic variants cause spastic ataxia

analysis (Supplemental Table S1).<sup>13,14,15</sup> Variant calling and clinical sequence interpretation was performed following the Broad Institute's Genome Analysis Toolkit (GATK4) best practices and the guidelines from the American College of Medical Genetics.<sup>16</sup> All the *TUBA4A* variants are given following the canonical MANE select Refseq NM\_006000.3. Probands and available relatives from each family were tested for variant segregation by Sanger sequencing.

## **Functional assays**

### **Fibroblast isolation and culture**

Studies of microtubules organization and dynamics were performed on cultured fibroblasts from 3 patients (patient 1, 2 and 7) and 2 controls (patient 1's father and patient 2's mother) obtained from skin biopsies (Supplemental data).

### **Western immunoblotting and Immunofluorescence**

The antibodies used were the mouse anti- $\alpha$ -tubulin (T6199; Sigma-Aldrich; dilution immunofluorescence (IF): 1:5000, dilution Western Blot (WB): 1:10 000), the rabbit anti-4A $\alpha$ -tubulin (SAB2102603; Sigma-Aldrich; dilution IF: 1:50, dilution WB: 1:1000), the mouse anti-Acetylated  $\alpha$ -tubulin (Acetyl K40) (clone TEU318; Stemcell technologies; dilution IF: 1:2000) and the rat anti-Tyrosinated  $\alpha$ -tubulin (clone YL1/2; dilution IF: 1:1000). DyLight Fluor and Alexa Fluor secondary antibodies were from Thermo Fisher Scientific and LifeTechnologies respectively. Western immunoblotting and immunofluorescence staining and imaging protocols are given in supplemental data.

## **Results**

### **Monoallelic *TUBA4A* missense variants identified in 12 ataxia patients.**

## *TUBA4A* monoallelic variants cause spastic ataxia

WES/WGS allowed identification of 9 pathogenic or possibly pathogenic monoallelic *TUBA4A* missense variants in 12 patients with ataxia, from 11 unrelated families, including 2 previously reported variants (p.Arg105Cys and p.Glu415Lys) and 5 *de novo* (missense) variants identified by trio analysis (Figure 1 and 2). Clinical exome sequencing screening was performed in a multicentric cohort of 448 unrelated probands presenting with inherited cerebellar ataxia. Three *de novo TUBA4A* variants (p.Pro173Ser, p.Pro173Arg, and p.Glu415Lys) were identified in patients 1, 2 and 5, respectively, who presented with early-onset cerebellar ataxia (24y, 22y and 10y, respectively) with or without spasticity. Through GeneMatcher exchange, 5 additional patients (from 4 families) presenting with a similar neurodegenerative disorder and carrying a rare *TUBA4A* missense variant were identified (patients 3, 4, 7, 8 and 12).<sup>12</sup> Interestingly, patient 3 harbors the same *de novo* p.Pro173Arg identified in patient 2 in whom an earlier onset of cerebellar ataxia was noticed (2y), similar to the age of onset of patient 4 (5y) who was found to carry the *de novo* variant p.Cys316Tyr. The two *TUBA4A* variants p.His28Arg (brother patients 7 and 8) and p.Glu423Lys (patient 12) should be considered of unknown significance (VUS) since parental segregation could not be performed due to late onset of the disease (46y, 45y and 62y, respectively).

Subsequently, *TUBA4A* was independently investigated as a candidate gene for the clinical indication of hereditary ataxia (HA) within the latest available rare disease 100KGP dataset, i.e. 35,008 rare disease families, including 1103 hereditary ataxia probands used as cases and 20,904 non-neurologic probands used as controls. According to ACMG guidelines, variants were defined as rare when minor allele frequency was below  $1 \times 10^{-5}$ , which corresponds to a heterozygote carrier frequency of 1/50 000 individuals. The signal was based on 6 *TUBA4A* distinct missense variants within the cases vs 2 in controls, leading to a significant burden of *TUBA4A* rare variants assessed via a two-sided Fisher exact test (i.e., Exomiser variant score of  $\geq 0.80$ , (6/1103 cases vs 2/20,904 controls, OR = 57.0847 [10.2- 576.7],  $p = 4.02 \times 10^{-07}$ ).

## *TUBA4A* monoallelic variants cause spastic ataxia

The p.Arg214Cys and p.Arg264His variants identified in cases were not retained due to the high allele frequency of additional missense variants affecting the same residues in databases (84 and 21 heterozygous carrier on GnomAD v4, respectively). However, pathogenicity of the p.Arg214Cys and p.Arg264His variants could not be fully excluded, and further investigations are needed. Interestingly, a p.Arg215Cys variant has already been identified in a fALS patient.

<sup>6</sup> The four other *TUBA4A* missense variants identified in patients 6, 9, 10 and 11 were absent from control cohorts. Interestingly, patient 6 was found to have the same variant identified in patient 5 (p.Glu415Lys). The p.Arg105Cys variant was identified in patient 10 who presented with very late onset (60y) pure cerebellar ataxia associated with severe cerebellar atrophy and CCAS. This variant was previously reported in a large Dutch family exhibiting an heterogeneous clinical presentation related to FTD.<sup>8</sup> Two other missense variants, p.Asp33Val and p.Leu259Val, were identified in a proband from a family of 4 members affected with ataxia (patient 9), and in a sporadic case presenting with early onset spastic ataxia (patient 11), respectively. These two variants remain of unknown significance since parents or relatives were unavailable for segregation analyses. All identified variants affect evolutionarily highly conserved residues (in most Eukaryota, Figure 2) and were predicted as pathogenic across a series of *in silico* prediction tools. All variants were absent from several databases including GnomAD v.4.0 and Regeneron database (Supplemental Table S2 )

## **Clinical findings**

Our multicentric cohort consists of 12 patients from 11 unrelated families, 5 females and 7 males, whose age at last evaluation ranged from 20 to 67 years. Table 1 provides an overview of the main clinical findings. All patients presented with gait disturbance/ ataxia with a highly variable age of onset ranging from 2y to 60y (mean age of onset: 28y). Spasticity and spastic gait were reported in more than half of the patients (7/12, 58.3%). Neurological examination also revealed increased deep-tendon reflexes in two thirds of the cohort (8/12, 66,6%), while



## *TUBA4A* monoallelic variants cause spastic ataxia

dysarthria, ankle clonus and bladder dysfunction were reported in less than half of the patients (5/12, 41.6 %). Ophthalmological evaluation revealed nystagmus or abnormal eye movements in half of the patients (6/12, 50%) and cognitive decline was noted in one third of the cases (4/12, 33,3%). Interestingly, a neurodevelopmental disorder was noticed only for patients 2 and 3 who carried the same p.Pro173Arg variant and presented with learning disabilities. Apart from 2 cases who showed amyotrophy or upper limb muscular weakness (2/12, 16,6%), there was no evidence of lower motor neuron (LMN) involvement in the other patients. Brain imaging was performed for 11 cases and showed cerebellar atrophy in seven cases ranging from mild to severe (7/11, 63%) (Supplemental Figure S1). No abnormal signal at the level of cerebellar nuclei was noticed on MRI images of these 7 patients. Most identified patients (8/12, 66,6%) presented with a slowly or moderately progressive disease (data not available for 4 patients). (Supplemental Table S3).

## **Protein modelling**

The effect of missense variants on alpha tubulin protein structure was investigated using homology modeling of TUBA1A experimentally validated models. Steric clashes were predicted for most reported variants and were particularly relevant for p.Asp33Val, p.Pro173Arg, and p.Cys316Tyr. The Proline impacted by p.Pro173Arg/Ser variant interacts with the two C-terminal alpha helices, thereby conferring an important role in protein tertiary structure. Interestingly, p.Pro173Arg/Ser and p.Arg105Cys are located close to residues involved in GTP binding domain and could lead to defects in tubulin GTP interaction (Supplemental Figure S2). The C-terminal p.Glu415Lys and p.Glu423Lys variants which lie outside the globular structure led to introduction of positively charged Lysine in an enriched polar negatively charged residue region. Most of the reported variants were predicted by DynaMut software to destabilize the protein, and lead to differential vibrational entropy energy

*TUBA4A* monoallelic variants cause spastic ataxia

between wild-type and mutant ( $\Delta\Delta S_{\text{Vib}} \text{ ENCoM}$ ) as recently predicted for ALS related *TUBA4A* variants.<sup>17</sup> (Supplemental Table S3)

## **MTs functional studies**

MTs studies were performed on cultured fibroblasts from patients 1, 2 and 7 (carrying the p.Pro173Ser, p.Pro173Arg, and p.His28Arg variants, respectively). Fibroblasts from patients's 1 father and patients's 2 mother were used as controls (n=2). By an immunostaining approach, we first investigated aspects of the endogenous form of alpha4A-tubulin protein in fibroblasts of patients and did not observe any abnormal distribution such as granules or modifications in soluble levels of these isoforms (Figure 3C). Protein expression level of total alpha-tubulin and alpha4A tubulin isoform quantified by Western Blotting on fibroblast lysates from all tested patients were comparable to controls (Figures 3A & B). By contrast, the ratio of alpha4A-tubulin isoform versus total alpha-tubulin in MTs assessed by an immunostaining approach, was significantly increased in fibroblasts from patients 2 (p.Pro173Arg) and 7 (p.His28Arg), while it was significantly decreased in patient 1 (p.Pro173Ser) (Figure 3C & D). These differences in the incorporation rate of the alpha4A-tubulin isoform in tested patients may therefore result from defects of alpha4A-tubulin protein incorporation in MTs rather than lower *TUBA4A* protein expression.

Acetylated and tyrosinated tubulin levels were quantified in patients' fibroblasts by immunostaining, as these Post Translation Modifications (PTMs) play a crucial role in regulating microtubule stability.<sup>18,19,20</sup>

We noticed a significant increment of MTs tyrosination level in all three patients' fibroblasts when compared to controls (Figure 3E & F) as well as a significantly decreased MTs acetylation level in fibroblast from patients 2 (p.Pro173Arg) and 7 (His28Arg) (Figure 3G & H). Surprisingly, no difference in MTs acetylation level was observed in fibroblast from patient 1 (p.Pro173Ser) (Figure 3G & H).

## Discussion

### ***TUBA4A* variants are associated with inherited ataxia phenotype.**

We report a cohort of unrelated patients harboring heterozygous pathogenic (1), likely pathogenic (4) or possibly pathogenic (4) variants in *TUBA4A* presenting with inherited cerebellar ataxia with or without spasticity. The clinical presentations were distinct from the fALS22 and FTD presentations previously associated with heterozygous *TUBA4A* variants.<sup>6</sup>

Association between cerebellar ataxia and a *TUBA4A* variant was very recently suggested, based on p.Glu415Lys variant segregation in a large family presenting with spastic ataxia (LOD score = 3.6) as well *de novo* occurrence in a sporadic patient with ataxia.<sup>10</sup>

Identification of additional 5 unrelated patients carrying *de novo* missense mutations, including 3 impacting the same residue (Pro173) clearly indicates *TUBA4A* causal role in the disease. In our cohort, the age of onset of neurological signs was highly variable, ranging from 2y to 60y (mean age: 25y). According to our data, the estimated frequency of *TUBA4A* pathogenic variants among patients with inherited ataxia from the French (3/448), and UK 100KGP (4/1103) cohorts is 0.45% (7/1551).

### **A dominant negative effect of *TUBA4A* variants ?**

Functional studies performed on cultured fibroblasts from 3 patients showed significant differences in the incorporation level of alpha4A tubulin isoforms in MTs. Increased MTs tyrosination level and decreased acetylation level noticed in patients 2 and 3 support impact on MTs stability (Figure 3).<sup>20,18</sup> These result could be consistent with an already suggested dominant negative mechanism of *TUBA4A* mutants.<sup>10,6,17</sup> However, contribution of a partial loss of function or a gain of function mechanism cannot be formally excluded.

### **Alpha 4A tubulin, a particular isoform.**

*TUBA4A* monoallelic variants cause spastic ataxia

*De novo* variants impacting proline 173 in *TUBA1A* or *TUBB2B* (p.Pro173Leu) have already been associated with global neuro-developmental disorders with seizure and multiple brain malformations, distinct from the isolated spastic ataxia found in patient 1, 2 and 3.<sup>2,21</sup> Expression pattern of *TUBA4A* isoform has been shown to exhibit age related increment in fetal human and in adult mice brains.<sup>6,1</sup> In contrast with most tubulin isoforms, a minor involvement of *TUBA4A* during neurogenesis may allow a normal brain development as it was already suggested for *TUBB4A* associated with adult onset dystonia 4 (DYT4).<sup>3</sup>

Alpha4A tubulin contain the longest C-terminal polyglutamic side chain across the tubulin family, that is highly involved in Microtubules associated proteins (MAPs) binding and in tubulin PTMs.<sup>18,19,22</sup> The p.Glu423Lys and p.Glu415Lys identified variants could disrupt MAP association or PTMs, as suggested for a *de novo* C-terminal variant in *TUBB2A* identified in early onset spastic ataxia families.<sup>23</sup>

### **Is ALS a clinical continuum with Spastic Paraplegia and Ataxia ?**

Our patients carrying monoallelic *TUBA4A* variants presented with progressive neurological disorder associating cerebellar degeneration and predominant upper motor neuron (UMN) lesions leading to spasticity and are distinct from classical ALS patients who present simultaneous UMN and Lower motor neuron (LMN) impairment. Hereditary spastic paraplegia (HSP) and Primary lateral sclerosis (PLS) are characterized by pure UMN damage leading to spasticity, and are difficult to distinguish from atypical forms of ALS due to important overlaps.<sup>24</sup> It is not clear whether these atypical forms are distinct types of disease or are milder ends of an ALS phenotypic spectrum.<sup>25</sup> However, it is interesting to notice that pathogenic variants in several genes (e.g *ALS2*; *SPG11*; *KIF5A*) have been associated with either ALS, PLS or HSP phenotypes.<sup>26,27,28</sup> Additionally, other genes such as *SOD1* have been associated with either ALS by a dominant gain of function mechanism or HSP by a recessive loss of function, supporting the hypothesis of a clinical continuum.<sup>29</sup>

*TUBA4A* monoallelic variants cause spastic ataxia

## Conclusion

Our study confirms the identification of an inherited ataxia phenotype associated with *TUBA4A* rare variants. Disease onset and severity prediction remain unclear, and no obvious genotype/phenotype correlation was identified. The highly heterogeneous phenotype associated with *TUBA4A* variants is reminiscent of variability seen in other neurodegenerative disorders, such as Multiple System Proteinopathy (MSP) disorders that include ALS and FTD in the clinical spectra.<sup>30</sup> Drawing a distinctive line between *TUBA4A* patients with ALS-FTD or ataxia can be challenging based on the limited number of cases and functional studies reported so far. Further studies are needed for a better understanding of *TUBA4A* pathophysiology.

## Data availability

Data are available upon reasonable request.

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*TUBA4A* monoallelic variants cause spastic ataxia

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## **Ethics declaration**

All procedures were conducted according to the guidelines of the Declaration of Helsinki and all patients/their guardians included in the study consented to participation according to ethical approval of the recruiting institutions.

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## **Competing interest**

The authors report no competing interests.

## **Author Contributions**

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Writing-review & editing: M. Be, M.K, H.Mo, M.Bo, A.D

## **Supplementary material**

Supp Methods

Table S1, S2, S3 and S4

Figure S1, S2 and S3

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*TUBA4A* monoallelic variants cause spastic ataxia

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## TUBA4A monoallelic variants cause spastic ataxia

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## Figure and Table legends

### Table 1 – Clinical and genetic findings of 12 patients reported in this study.

All variants are reported based on RefSeq NM\_006000.3. AD = Autosomal dominant; CCAS:

*TUBA4A* monoallelic variants cause spastic ataxia

Cerebellar Cognitive and Affective Syndrome. HSP= Hereditary Spastic paraplegia; ID = Intellectual Disability; DD = Developmental delay; LL = Lower Limbs; UL = Upper Limbs; NA = Not available.

**Figure 1 – Schematic drawing of the TUBA4A protein.** Missense mutations reported

below the figure are those identified in this study in patient presenting with hereditary ataxia (HA) while variants reported on top are those already identified in ALS-FTD patients.

Confirmed *de novo* variants are indicated in red. Variants indicated in green with dotted line arrows (p.Arg214Cys and p.Arg264His) were identified during the 100KGP burden analysis in HA patients who were not included in the cohort due to high frequency of missense affecting the same residue in databases.

**Figure 2 – Pedigrees of the reported families and protein conservation study.**

A) Pedigrees of reported families. Affected individuals are represented by black symbols.

Index cases are indicated by an arrow. individuals tested for *TUBA4A* variant are indicated by an asterisk (\*). Apart from the younger unaffected sister of patient 7 and 8 who was found to carry the p.His28Arg (in grey) , all tested unaffected individuals were negative for the variant.

Confirmed *de novo* variants are indicated in red B) Protein sequence conservation throughout Eukaryotic kingdoms. Nematostella v., Acropora m., Hydra v., Trichoplax a. are distant metazoa. Tetrahymena t.; Plasmodium g.; Toxoplasma g. are protists (alveolata). Mutant residues are indicated on top.

**Figure 3 –MT functional studies on cultured fibroblasts from 3 patients.**

Microtubules organization was analyzed on cultured fibroblasts from 3 patients (carrying p.Pro173Arg, p.Pro173Ser and p.His28Arg, respectively) and 2 controls. For immunostaining panel, all images were processed and analysed using Fiji software (National Institutes of

## *TUBA4A* monoallelic variants cause spastic ataxia

Health). For each condition,  $n=30$  fibroblasts per patient and control were analyzed.

Microtubules were delineated using the segmented line tool on the total alpha-tubulin channel.

For all fluorescent intensity measurements, the background signal was measured in an adjacent area of microtubules and subtracted. For MTs' acetylation and tyrosination level assessment, individualized microtubules chosen in peripheral regions of fibroblasts were quantified. Statistical analyses were done with Prism 6, GraphPad Software. Data distribution was assumed to be normal, but this was not formally tested. P values are represented as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ . **A)** alpha4A-tubulin immunoblots of lysates from cultured fibroblasts. Total alpha-tubulin was used as a loading control. **B)** alpha4A-tubulin expression level quantification from blots shown in A. **C)** Representative immunostaining of total alpha-tubulin and alpha4A-tubulin isoform in cultured patient fibroblasts. **D)** Quantification of alpha4A-tubulin incorporation level in microtubules from patient fibroblasts shown in C. **E)** Representative immunostaining of tyrosinated tubulin in cultured patient fibroblasts. **F)** Quantification of tyrosinated tubulin in microtubules shown in E. **G)** Representative immunostaining of acetylated tubulin in cultured patient fibroblasts. **H)** Quantification of acetylated tubulin in microtubules shown in G.

**Table 1 Clinical and genetic findings of 12 patients reported in this study**

General	Family 1	Family 2	Family 3	Family 4	Family 5	Family 6	Family 7		Family 8	Family 9	Family 10	Family 11
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12
Gender/Ethnicity	M/Caucasian	M/Caucasian	F/Caucasian	F/Caucasian	F/Caucasian	F/Caucasian	M/Caucasian	M/Caucasian	M/Caucasian	M/Caucasian	F/India	M/Caucasian
Age at last examination	36y	47y	20y	32y	32y	36y	60 y	51 y	42y	65y	20y	62 y
<b>Genetics findings</b>												
DNA	c.517C>T	c.518C>G	c.518C>G	c.947G>A	c.1243G>A	c.1243G>A	c.83A>G	c.83A>G	c.98A>T	c.313C>T	c.775C>G	c.1267G>A
Protein	p.Pro173Ser	p.Pro173Arg	p.Pro173Arg	p.Cys316Tyr	p.Glu415Lys	p.Glu415Lys	p.His28Arg	p.His28Arg	p.Asp33Val	p.Arg105Cys	p.Leu259Val	p.Glu423Lys
Inheritance	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	Unknown	Not maternal	Not maternal	Unknown	Dominant	Unknown	Unknown
<b>Clinical findings</b>												
Initial symptoms	Ataxia	Spastic ataxia	Ataxia	Ataxia	Spastic ataxia	Ataxia	Spastic gait	Spastic gait	Ataxia	Ataxia	Ataxia	Spastic ataxia
Age of onset	24 y	22 y	2 y	5 y	10 y	17 y	46 y	45 y	20 y	60 y	10 y	54 y
Developmental delay	-	LD	LD	-	-	-	-	-	-	-	-	-
Ataxia (SARA score)	+ (16.5)	+ (21)	+ (NA)	+ (11)	+ (17)	+ (NA)	-	-	+ (NA)	+ (23)	+ (12)	+ (NA)
Dysarthria	+	+	+	+	Mild	-	?	?	-	+	+	?
Eyes movements	Saccadic pursuit	Nystagmus	?	+	nystagmus	Nystagmus	N	N	N	N	Nystagmus	N
UL weakness	-	+	-	-	-	?	-	-	-	-	-	+
LL weakness	-	+	++	-	+	?	+	+	-	-	-	+
Spasticity	-	+	+	-	+	-	+	+	-	-	++	+
Extensor planter reflex	-	+	++	-	+	-	+	+	-	-	+	-
Increased deep tendon reflex	-	+	+	+	+	-	+	+	-	-	+	+
Ankle clonus	-	+	+	+	-	-	+	+	-	-	+	-
Cognitive decline	-	-	NA	+	-	-	+	+	-	CCAS	-	-
Bladder dysfunction	-	-	-	-	+	-	+	+	-	-	+	-
Amyotrophy	-	-	-	-	-	-	-	-	-	-	-	+
EMG	Myopathic signs	Normal	UL elongated motor potential	normal	Elongated motor potential	NA	Perturbed	Perturbed	NA	NA	Normal	NA
Brain MRI	CA+	CA+	CA+	CA++	N	NA	N	vascular insult	CA	CA++	CA++	N
Other features	Increased CPK	-	scoliosis	-	-	-	Cirrhosis	Cirrhosis	Hepatic cyst	-	-	-
Disease progression	Moderate	Moderate	Slow	Slow	slow	NA	Moderate	Fast	NA	Slow	Slow	Slow

All variants are reported based on RefSeq NM\_006000.3. + = present; - = absent; AD = autosomal dominant; CA = cerebellar atrophy; CCAS = cerebellar cognitive and affective syndrome; CPK = creatin phosphokinase; DD = developmental delay; LD = learning disability; LL = lower limbs; N = normal UL = upper limbs; NA = not available.

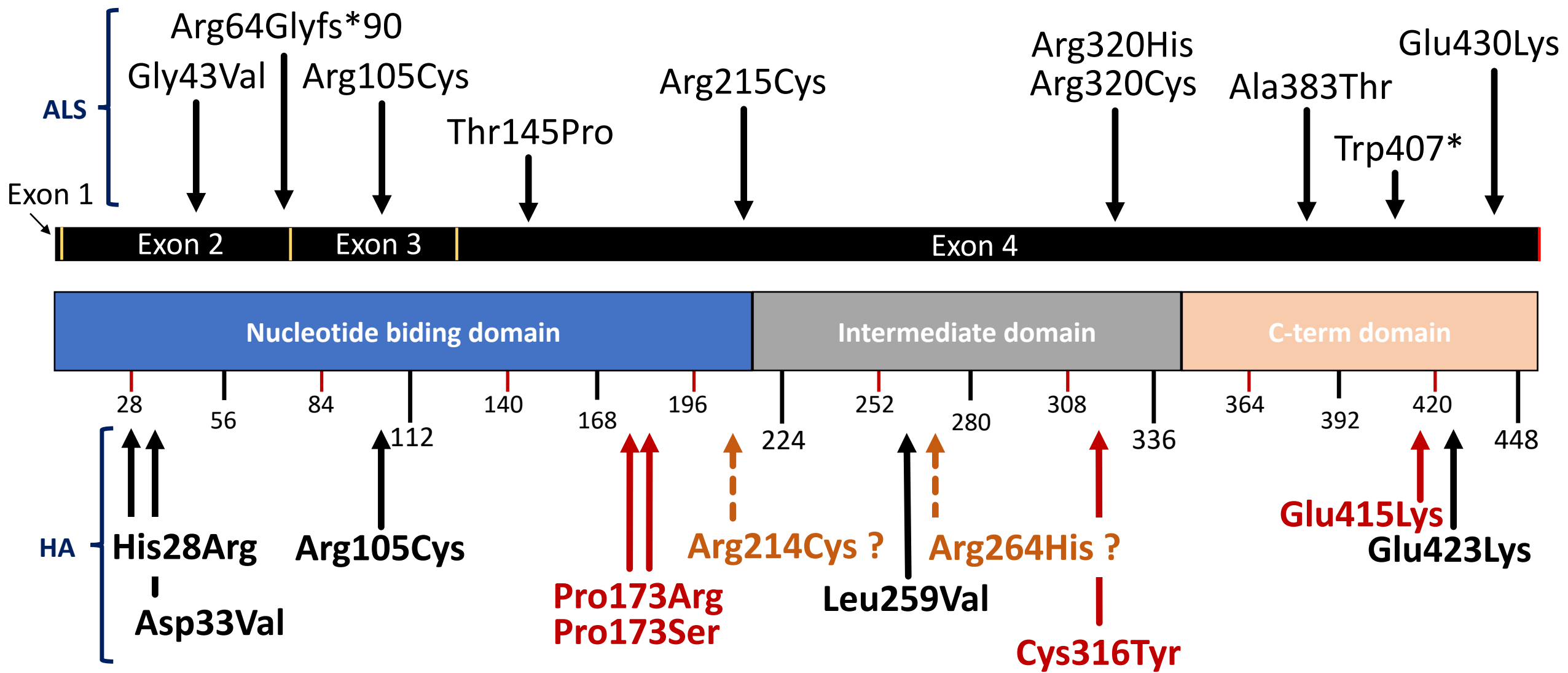
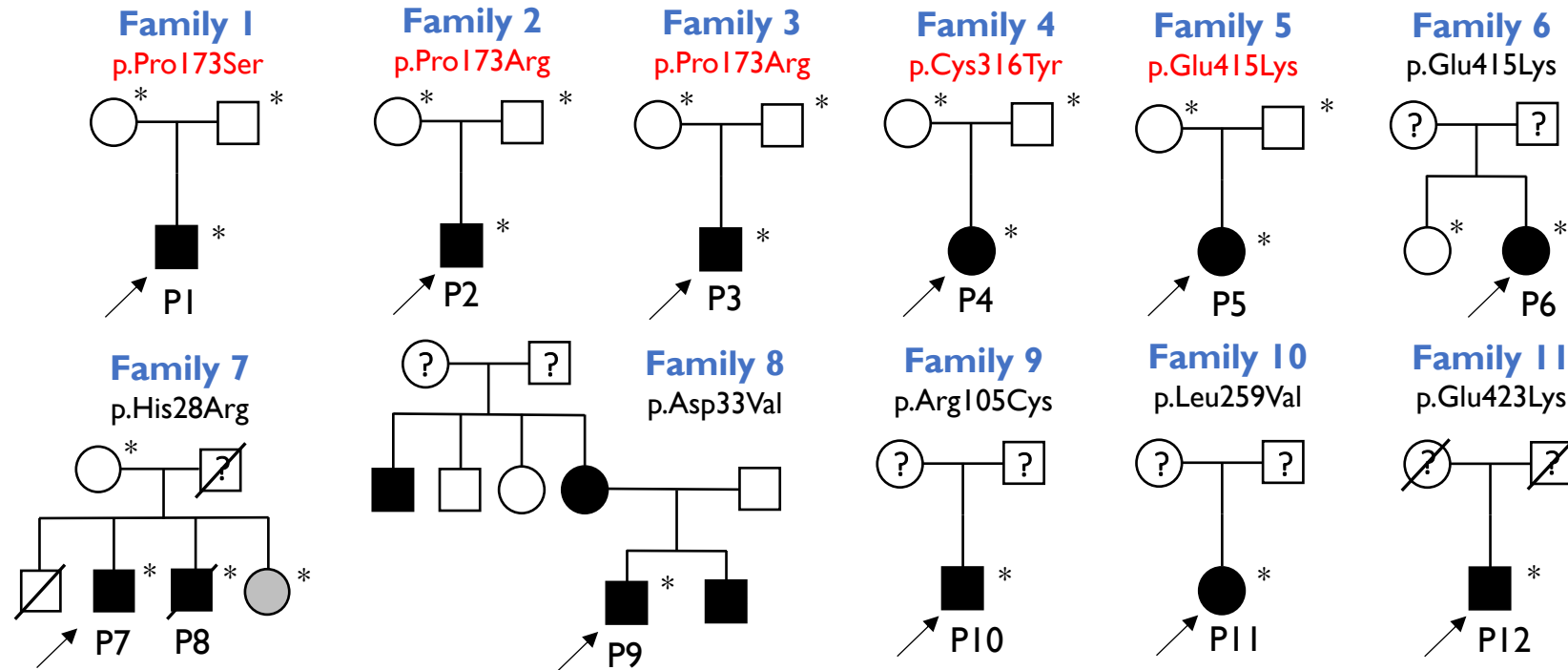


FIGURE 1

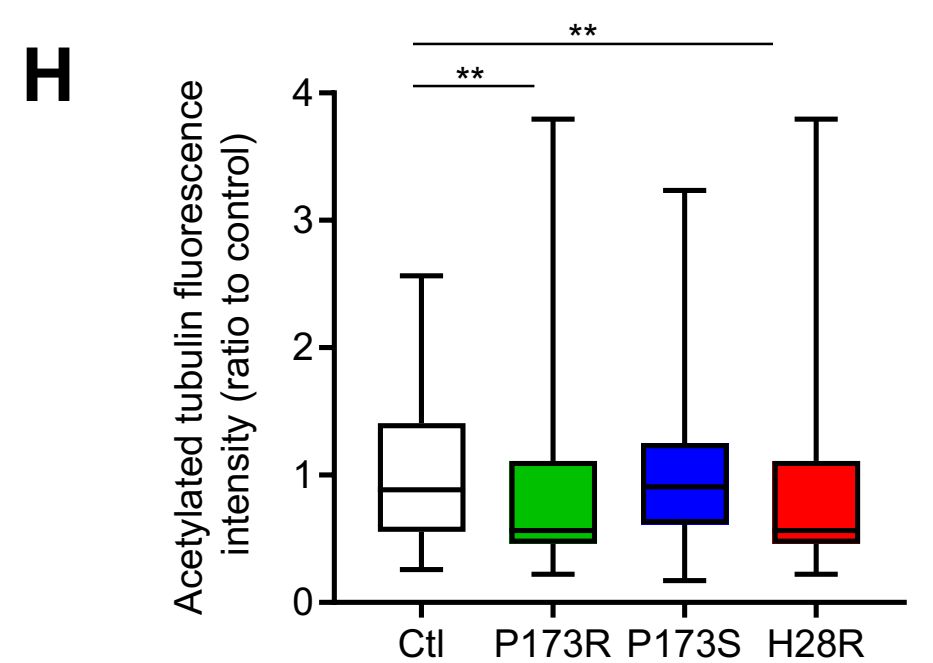
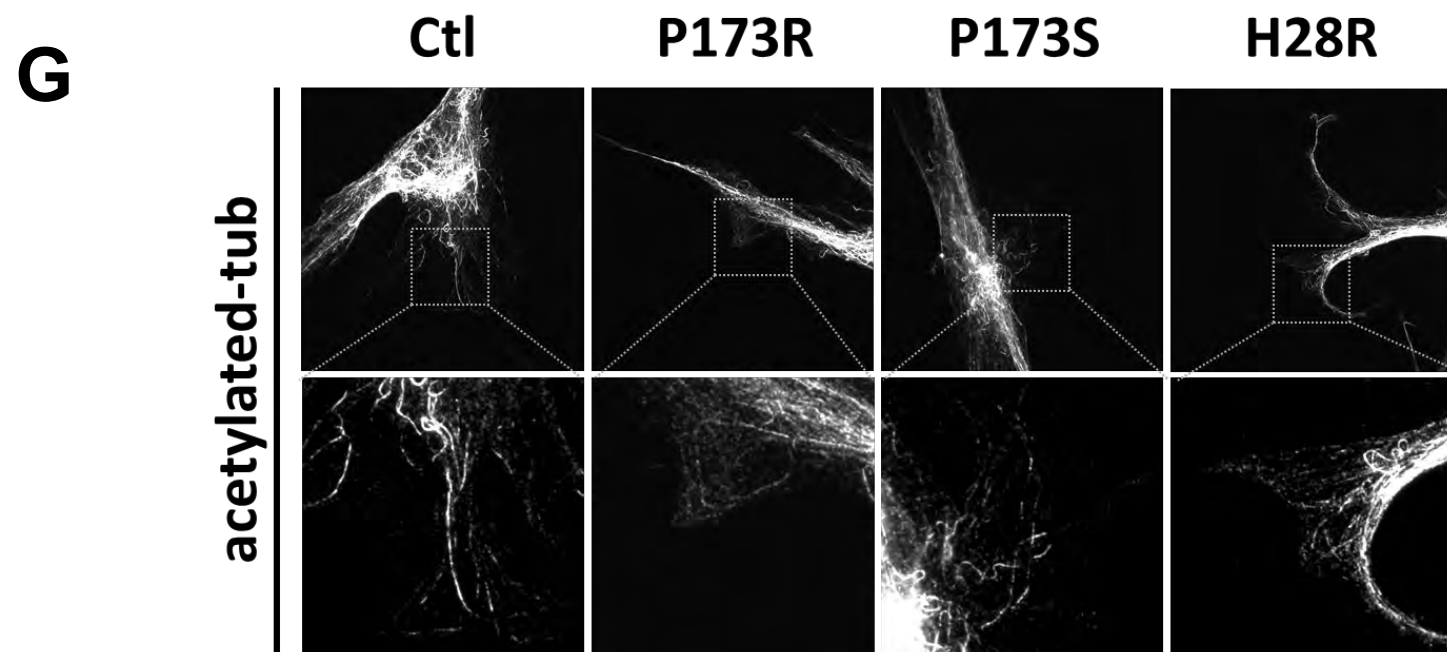
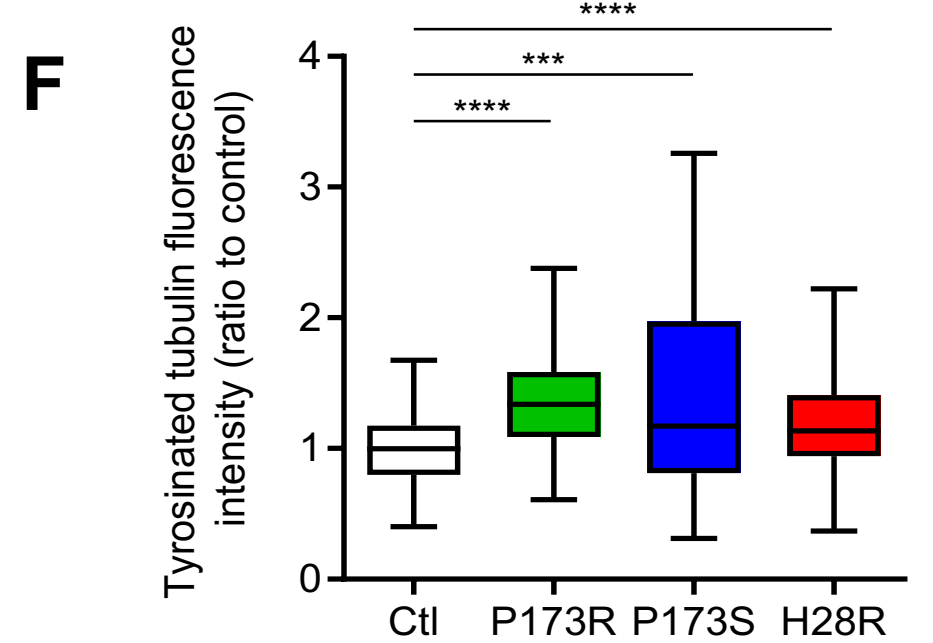
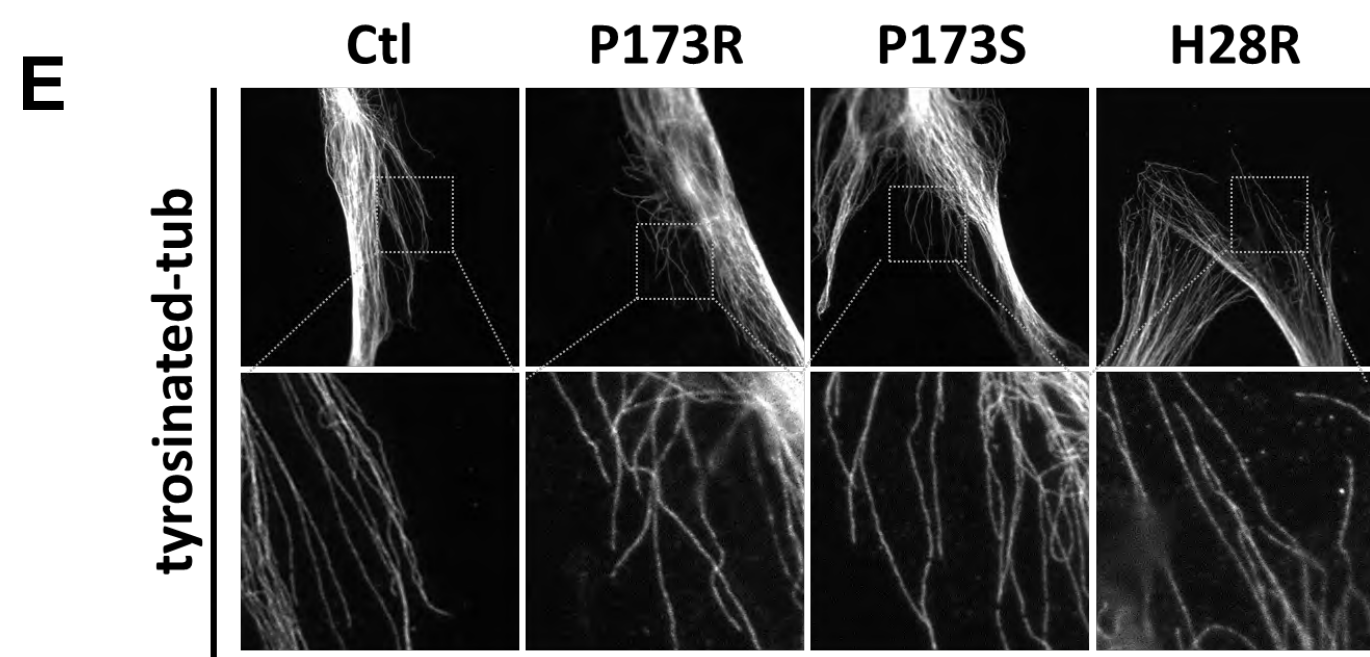
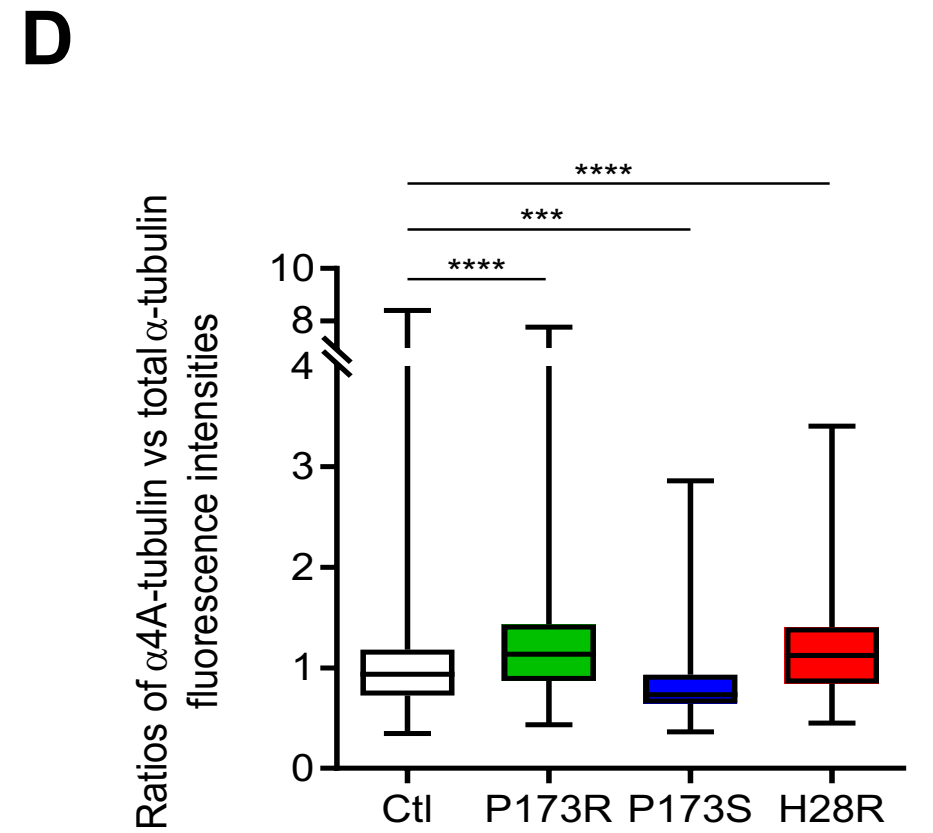
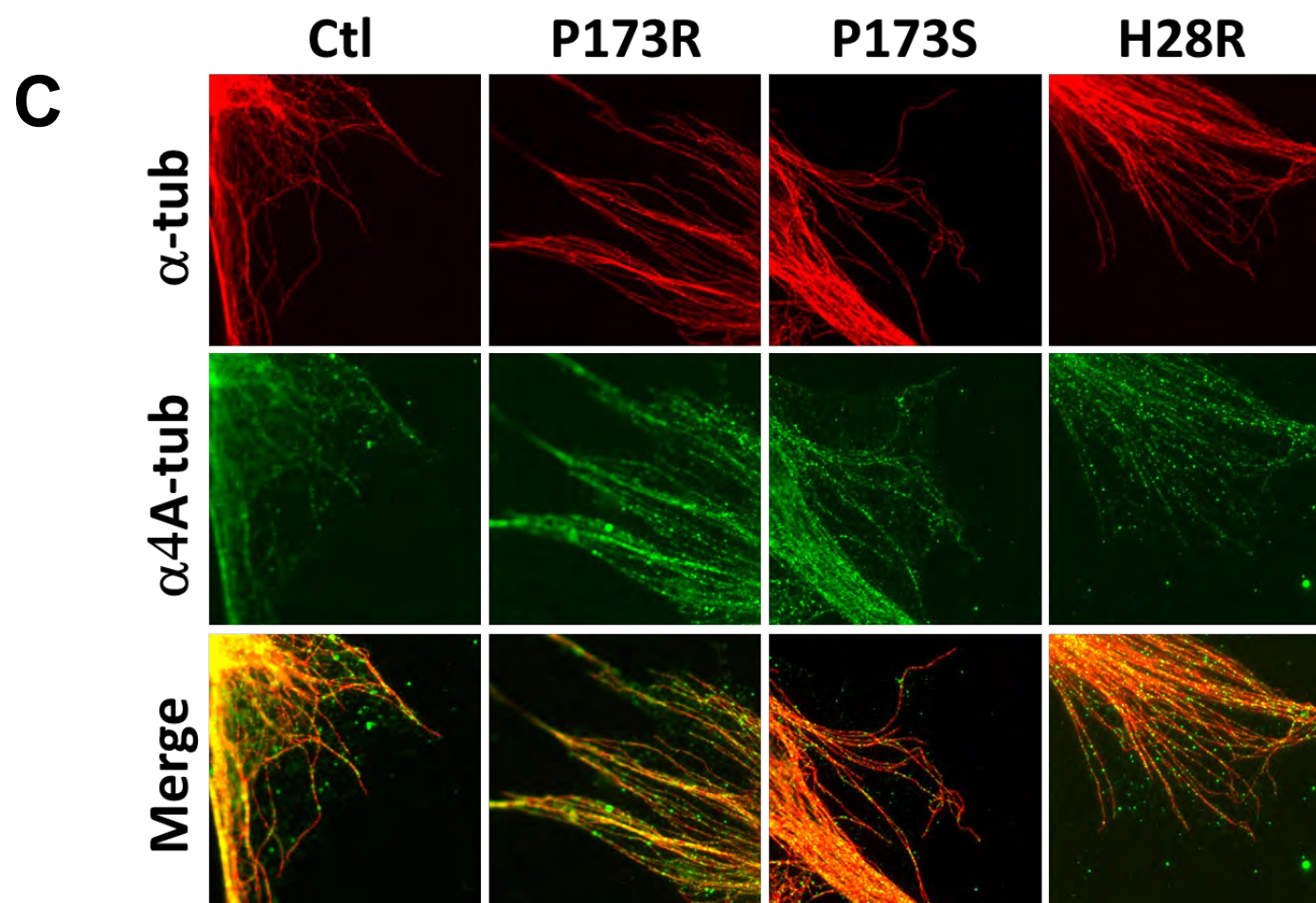
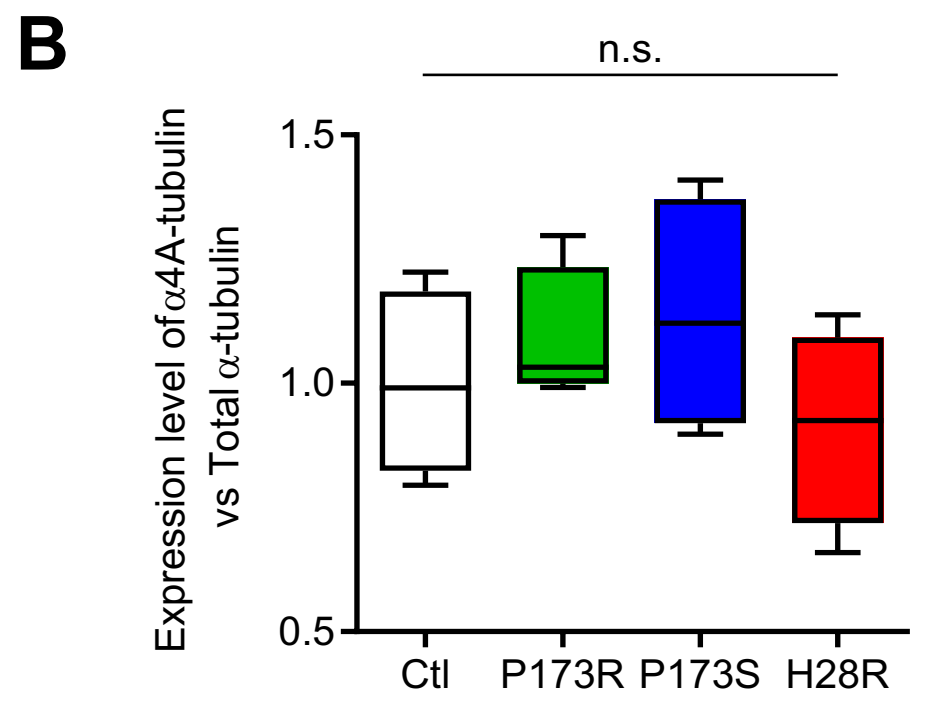
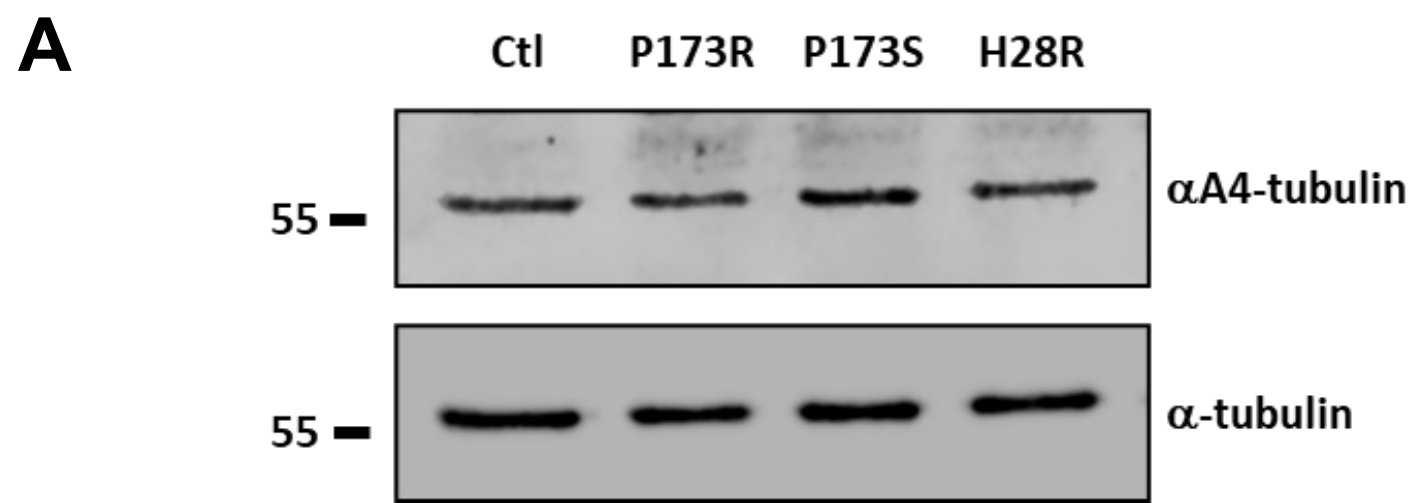
**A**



**B**

Patients	28 R	33 V	105 C	173 R/S	259 V	316 R	415 K	423 K
Homo S.	{...}YCLE <b>H</b> G <b>I</b> Q <b>P</b> D <b>G</b> {...}	{...}NY <b>A</b> R <b>G</b> H <b>Y</b> {...}	{...}F <b>S</b> I <b>Y</b> P <b>A</b> P <b>Q</b> V <b>S</b> T{...}	{...}N <b>L</b> V <b>P</b> Y <b>P</b> R <b>I</b> {...}	{...}K <b>Y</b> M <b>A</b> C <b>C</b> L{...}	{...}E <b>G</b> E <b>F</b> S <b>E</b> A <b>R</b> E <b>D</b> M <b>A</b> A <b>L</b> E <b>K</b>		
Nematostella v.	{...}YCLE <b>H</b> G <b>I</b> Q <b>P</b> D <b>G</b> {...}	{...}NY <b>A</b> R <b>G</b> H <b>Y</b> {...}	{...}F <b>A</b> I <b>Y</b> P <b>A</b> P <b>Q</b> V <b>S</b> T{...}	{...}N <b>L</b> V <b>P</b> Y <b>P</b> R <b>I</b> {...}	{...}K <b>Y</b> M <b>A</b> C <b>C</b> M{...}	{...}E <b>G</b> E <b>F</b> S <b>E</b> A <b>R</b> E <b>D</b> L <b>A</b> A <b>L</b> E <b>K</b>		
Acropora m.	{...}YCLE <b>H</b> G <b>I</b> Q <b>P</b> D <b>G</b> {...}	{...}NY <b>A</b> R <b>G</b> H <b>Y</b> {...}	{...}F <b>S</b> I <b>Y</b> P <b>A</b> P <b>Q</b> I <b>A</b> T{...}	{...}N <b>L</b> V <b>P</b> Y <b>P</b> R <b>I</b> {...}	{...}K <b>Y</b> M <b>A</b> C <b>C</b> L{...}	{...}E <b>G</b> E <b>F</b> S <b>E</b> A <b>R</b> E <b>D</b> L <b>A</b> A <b>L</b> E <b>K</b>		
Trichoplax a.	{...}YCLE <b>H</b> G <b>I</b> Q <b>P</b> D <b>G</b> {...}	{...}NY <b>A</b> R <b>G</b> H <b>Y</b> {...}	{...}F <b>A</b> V <b>Y</b> P <b>A</b> P <b>Q</b> I <b>A</b> T{...}	{...}N <b>L</b> V <b>P</b> Y <b>P</b> R <b>I</b> {...}	{...}K <b>Y</b> M <b>A</b> C <b>C</b> L{...}	{...}E <b>G</b> E <b>F</b> S <b>E</b> A <b>R</b> E <b>D</b> L <b>A</b> A <b>L</b> E <b>K</b>		
Hydra v.	{...}YCLE <b>H</b> G <b>I</b> Q <b>P</b> D <b>G</b> {...}	{...}NY <b>A</b> R <b>G</b> H <b>Y</b> {...}	{...}F <b>A</b> V <b>Y</b> P <b>A</b> P <b>Q</b> V <b>S</b> T{...}	{...}N <b>L</b> V <b>P</b> Y <b>P</b> R <b>I</b> {...}	{...}K <b>Y</b> M <b>S</b> C <b>C</b> L{...}	{...}E <b>G</b> E <b>F</b> S <b>E</b> A <b>R</b> E <b>D</b> M <b>A</b> A <b>L</b> E <b>K</b>		
Tetrahymena t.	{...}F <b>C</b> L <b>E</b> <b>H</b> G <b>I</b> Q <b>P</b> D <b>G</b> {...}	{...}N <b>F</b> A <b>R</b> G <b>H</b> Y{...}	{...}F <b>T</b> I <b>Y</b> P <b>S</b> P <b>Q</b> V <b>S</b> T{...}	{...}N <b>L</b> V <b>P</b> Y <b>P</b> R <b>I</b> {...}	{...}K <b>Y</b> M <b>A</b> C <b>S</b> M{...}	{...}E <b>G</b> E <b>F</b> S <b>E</b> A <b>R</b> E <b>D</b> L <b>A</b> A <b>L</b> E <b>K</b>		
Plasmodium g.	{...}F <b>C</b> L <b>E</b> <b>H</b> G <b>I</b> Q <b>P</b> D <b>G</b> {...}	{...}N <b>F</b> A <b>R</b> G <b>H</b> Y{...}	{...}F <b>C</b> C <b>W</b> P <b>S</b> P <b>Q</b> V <b>S</b> T{...}	{...}N <b>L</b> V <b>P</b> Y <b>P</b> R <b>I</b> {...}	{...}K <b>Y</b> M <b>A</b> C <b>C</b> L{...}	{...}E <b>G</b> E <b>F</b> S <b>E</b> A <b>R</b> E <b>D</b> L <b>A</b> A <b>L</b> E <b>K</b>		
Toxoplasma g.	{...}F <b>C</b> L <b>E</b> <b>H</b> G <b>I</b> Q <b>P</b> D <b>G</b> {...}	{...}N <b>F</b> A <b>R</b> G <b>H</b> Y{...}	{...}F <b>C</b> S <b>W</b> P <b>S</b> P <b>Q</b> V <b>S</b> T{...}	{...}N <b>L</b> V <b>P</b> Y <b>P</b> R <b>I</b> {...}	{...}K <b>Y</b> M <b>A</b> C <b>C</b> L{...}	{...}E <b>G</b> E <b>F</b> S <b>E</b> A <b>R</b> E <b>D</b> L <b>A</b> A <b>L</b> E <b>K</b>		

**FIGURE 2**



**FIGURE 3**



## **Supplemental materials**

### **Supp methods.**

#### **100,000 UK Genome project (100K GP) protocol**

DNA extracted from blood was prepared for GS using TruSeq DNA PCR-Free library preparation. GS was performed on either HiSeq 2000 (Illumina) or HiSeq X (Illumina) platforms as 150 or 125 bp paired-end reads to an average minimum depth of 35× (31×-37×). Sequenced data were aligned to the human genome reference build 37 or build 38 using Isaac Genome Alignment Software. Using the Platypus variant caller, family-based variant calling of insertions, deletions (indels), or single-nucleotide variants was performed. were screened for single nucleotide variants, small insertions/deletions, structural variants (SVs) or copy number variants (CNVs), and short tandem repeats in relevant genes from the PanelApp virtual gene panels (Genomics England) (Martin AR et al. Nat. Genet 2019).

#### **Fibroblast isolation and culture**

Skin biopsies were collected by using a biopsy punch tool and tissue samples were diced into small sections that were placed on the adherent wall of 25 cm<sup>2</sup> flask horizontally positioned. Flasks were filled with 5 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) foetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. They were maintained horizontal at 37 °C under humidified conditions with 5% CO<sub>2</sub>. Then 12 to 24 hours later (depending on the adherence of skin sections toward the flask) they were gently

reversed to cover skin sections with medium. Finally, 10 to 15 days later (depending on the number of fibroblasts growing outside the tissue), skin sections were carefully removed, and fibroblasts were cultured in the supplemented DMEM medium described above.

### **Cell lysis and Western immunoblotting**

Fibroblasts were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 10% sucrose, 0.1% [vol/vol] Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate. Lysates were clarified by centrifugation at 20 000 g for 10 min at 4°C, diluted in Laemmli buffer, electrophoresed on a 10% one-dimensional sodium dodecyl sulfate-polyacrylamide gel and trans-blotted on a nitrocellulose membrane. Membranes were blocked for 30 min in TBS-Tween buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.5% [vol/vol] Tween-20) containing 3% BSA for 30 min and then incubated overnight with primary antibodies in the same buffer. Blots were washed with TBS-Tween buffer and incubated for 1 h with the appropriate DyLight Fluor-conjugated secondary antibodies (680 or 800 conjugate) and quantified with a LI-COR Odyssey fluorescent imaging and associated Image Studio analysis software.

### **Immunofluorescence staining and imaging.**

Cultured fibroblasts were fixed with Methanol/1mM EGTA for 5 min at -20°C. Fixed cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature, blocked with 4% BSA in PBS followed by overnight primary antibody incubation in 2% BSA in PBS. Alexa Fluor 488– or 546–coupled secondary fluorescent antibodies were used at dilution of 1:2000 for 1 h at room temperature. Glass coverslips were mounted in Mowiol mounting medium and cells were imaged with a widefield upright microscope (100×/1.46). All images were processed and analysed

using Fiji software (National Institutes of Health). Microtubules were delineated using the segmented line tool on the total alpha-tubulin channel. For all fluorescent intensity measurements, the background signal was measured in an adjacent area of microtubules and subtracted.

**Table S1: TUBA4A Gene burden analysis in 100K GP.**

A rare-variant gene-based burden case-control analysis was performed within the rare disease component of the 100,000 Genomes Project (100KGP) using an already described analytical framework.<sup>1,2</sup> Cases were defined as all probands recruited under hereditary ataxia ( $n = 1103$ ) whereas corresponding controls were all remaining recruited 100KGP probands minus those recruited under the broad neurological and neurodevelopmental disease group disorder ( $n = 20,904$ ). Variants were defined as rare when minor allele frequency was strictly below  $1 \times 10^{-5}$ . Exomiser was then run on genome data from all probands and available family members, in order to filter for rare coding variants that segregated with the disease status per each possible mode of inheritance. None of the variants in cases were found in control group.

Rare <i>TUB4A</i> variants	Cases (HSP/HA)	Controls
Yes	6	2
No	1,097	20,902
Total	1,103	20,904

**Table S2: *TUBA4A* missense reported in the study and in silico prediction score.**

Positions, exons, and domains localization are given according to hg38 and RefSeq NM\_006000.3 nomenclature. *In silico* prediction score SIFT<sup>3</sup>(0: damaging,1:benign), Alpha missense<sup>4</sup> (1:damaging,0:benign), CADD<sup>5</sup> and REVEL<sup>6</sup> were used. Variants classification was performed according to the guidelines of the American College of Medical Genetics (ACMG)<sup>7</sup>. Frequency includes minor allele frequency found in **GnomAD v1,v2 and v3** (250,000 Exomes and 75,000 Genomes), **Ukbiobank** (150,119 Genomes) and **Queen Square in-house database** (23,000 Exomes). AA: Amino Acid; AlphaM: Alphamissense; LP: Likely Pathogenic; NA: None available; P: Pathogenic; VUS: Variant of unknown significance.

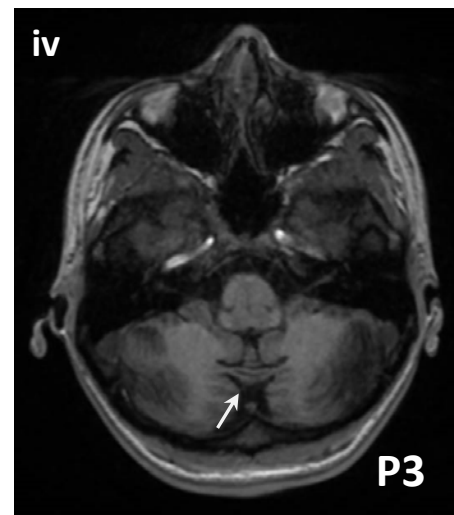
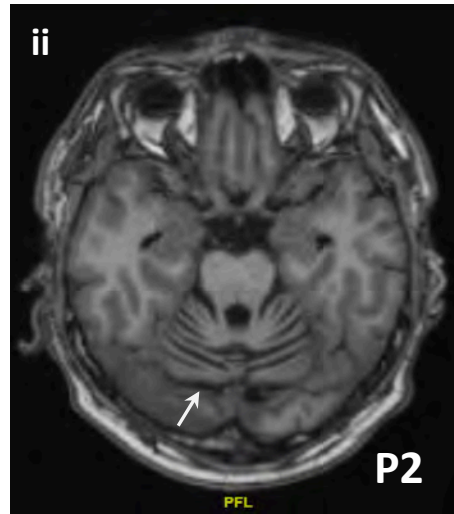
Family	Inheritance	Position (hg38;chr2)	Exon	Codon change	AA change	Frequency	CADD	SIFT	AlphaM	REVEL	ACMG criteria	ACMG
3	unknown	2-219252151-T-C	2	c.83A>G	<b>p.His28Arg</b>	0	25.7	0.0	0.995	0.868	PM2,PP3	<b>3</b>
10	unknown	2-219252136-T-A	2	c.98A>T	<b>p.Asp33Val</b>	0	29.7	0.0	0.784	0,853	PM2,PP3	<b>3</b>
8	unknown	2-219251627-G-A	3	c.313C>T	<b>p.Arg105Cys</b>	0	30	0.0	0.994	0,848	PS1,PM2,PP3	<b>4</b>
1;14	<i>denovo</i>	2-219251181-G-C	4	c.518C>G	<b>p.Pro173Arg</b>	0	27.8	0.0	0.998	0,891	PS2,PM2,PP3	<b>4</b>
2	<i>denovo</i>	2-219251182-G-A	4	c.517C>T	<b>p. Prol73Ser</b>	0	27	0.0	0.986	0,935	PS2,PM2,PP3	<b>4</b>
9	unknown	2-219250924-G-C	4	c.775C>G	<b>p.Leu259Val</b>	0	23.2	N.A	0.977	0,890	PM2,PP3	<b>3</b>
7	<i>denovo</i>	2-219250752-C-T	4	c.947G>A	<b>p.Cys316Tyr</b>	0	26.3	N.A	0.999	0,931	PS2,PM2,PP3	<b>4</b>
5; 6	<i>denovo</i>	2-219250456-C-T	4	c.1243G>A	<b>p.Glu415Lys</b>	0	24.4	N.A	0.998	0,824	PS1,PS2,PS4,PM2,PP3	<b>5</b>
4	unknown	2-219250432-C-T	4	c.1267G>A	<b>p.Glu423Lys</b>	0	28.1	N.A	0.997	0,824	PM2,PP3	<b>3</b>

**Table S3: DynaMut prediction outcomes for *TUBA4A* missense variants identified in the study.**

DynaMut software was used to predict variant effect and vibrational entropy energy differences between wild-type and mutant. <sup>8</sup>

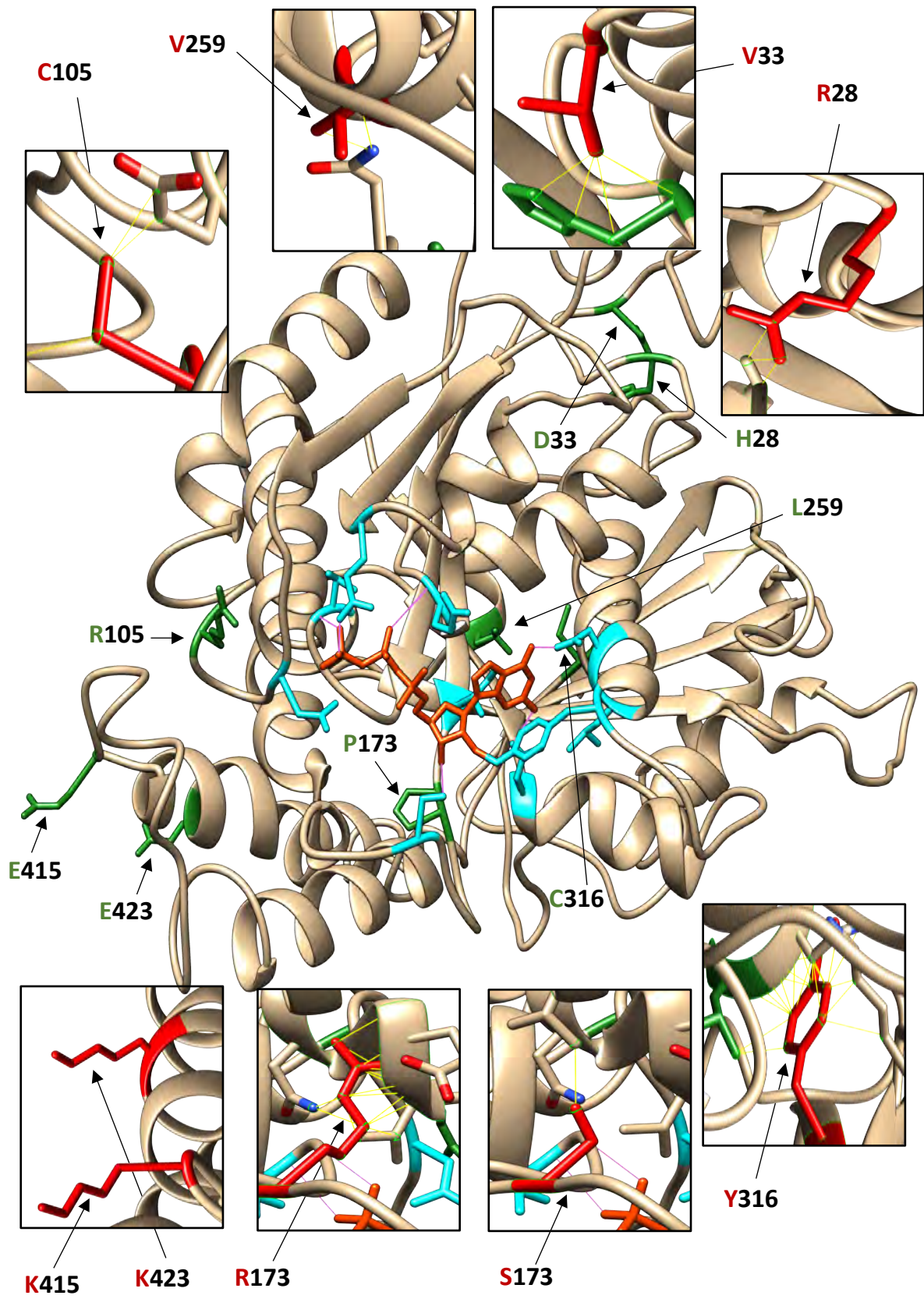
<b>AA change</b>	<b><math>\Delta\Delta G</math> prediction outcome</b>	<b><math>\Delta</math> Vibrational Entropy Energy Between Wild-Type and Mutant(<math>\Delta\Delta S_{\text{vib}}</math> ENCoM)</b>
<b>p.His28Arg</b>	-0.708 kcal/mol (Destabilizing)	0.283 kcal.mol <sup>-1</sup> .K <sup>-1</sup> (Increase of molecule flexibility)
<b>p.Asp33Val</b>	0.947 kcal/mol (Stabilizing)	-0.439 kcal.mol <sup>-1</sup> .K <sup>-1</sup> (Decrease of molecule flexibility)
<b>p.Arg105Cys</b>	-2.113 kcal/mol (Destabilizing)	0.737 kcal.mol <sup>-1</sup> .K <sup>-1</sup> (Increase of molecule flexibility)
<b>p.Pro173Arg</b>	0.519 kcal/mol (Stabilizing)	-0.569 kcal.mol <sup>-1</sup> .K <sup>-1</sup> (Decrease of molecule flexibility)
<b>p. Pro173Ser</b>	-0.707 kcal/mol (Destabilizing)	0.046 kcal.mol <sup>-1</sup> .K <sup>-1</sup> (Increase of molecule flexibility)
<b>p.Leu259Val</b>	-1.195 kcal/mol (Destabilizing)	0.399 kcal.mol <sup>-1</sup> .K <sup>-1</sup> (Increase of molecule flexibility)
<b>p.Cys316Tyr</b>	-0.839 kcal/mol (Destabilizing)	-0.830 kcal.mol <sup>-1</sup> .K <sup>-1</sup> (Decrease of molecule flexibility)
<b>p.Glu415Lys</b>	0.616 kcal/mol (Stabilizing)	-0.009 kcal.mol <sup>-1</sup> .K <sup>-1</sup> (Decrease of molecule flexibility)
<b>p.Glu423Lys</b>	0.271 kcal/mol (Stabilizing)	0.055 kcal.mol <sup>-1</sup> .K <sup>-1</sup> (Increase of molecule flexibility)

**Supp Figure 1** – Brain MRI. Sagittal and axial brain MRI image from patient 2 (i;ii), patient 3 (iii,iv) and patient 4 (v;vi) showing hemispheric and/or vermian cerebellar atrophy (arrows).



**Supp Figure 2 – Protein modelling.** The three-dimensional structure of alpha tubulin was built on the crystallography model from the protein databank (PDB) cryo-electron microscopy structural entry “1TUB”, containing TUBA1A which share 98% of homology with TUBA4A.<sup>9</sup> The protein structure illustration and amino acids were generated by CHIMERA v1.16 (build 42360) and mutation modeling was performed using Dunbrack rotamer 2010.<sup>10</sup> The GTP molecule is shown in orange and residues involved in GTP binding are shown in blue. WT and mutated residues are shown in green and red, respectively. The effect of the mutation was predicted by considering the properties of the substituted amino acids including changes in size, shape, polarity, charge, and rigidity. H-bonds are represented by purple lines. Yellow lines represent steric clashes identified when the distance between residues was one or more Angstroms less than the sum of the van der Waals radii.





## Supplemental references

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