**Title**

Tubulin Genes and Malformations of Cortical Development

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**Abstract**

A large number of genes encoding for tubulin proteins are expressed in the developing brain. Each is subject to specific spatial and temporal expression patterns. However, most are highly expressed in post-mitotic neurons during stages of neuronal migration and differentiation. The major tubulin subclasses (alpha- and beta-tubulin) share high sequence and structural homology. These globular proteins form heterodimers and subsequently co-assemble into microtubules. Microtubules are dynamic, cytoskeletal polymers which play key roles in cellular processes crucial for cortical development, including neuronal proliferation, migration and cortical laminar organisation. Mutations in seven genes encoding alpha-tubulin (*TUBA1A*), beta-tubulin (*TUBB2A, TUBB2B, TUBB3, TUBB4A, TUBB*) and gamma-tubulin (*TUBG1*) isoforms have been associated with a wide and overlapping range of brain malformations or "Tubulinopathies". The majority of cortical phenotypes include lissencephaly, polymicrogyria, microlissencephaly and simplified gyration. Well-known hallmarks of the tubulinopathies include dysmorphism of the basal ganglia (fusion of the caudate nucleus and putamen with absence of the anterior limb of the internal capsule), midline commissural structures hypoplasia and/or agenesis (anterior commissure, corpus callosum and fornix), hypoplasia of the oculomotor and optic nerves, cerebellar hypoplasia or dysplasia and dysmorphism of the hind-brain structures. The cortical and extra-cortical brain phenotypes observed are largely dependent on the specific tubulin gene affected. In the present review, all the published data on tubulin family gene mutations and the associated cortical phenotypes are summarized. In addition, the most typical neuroimaging patterns of malformations of cortical development associated with tubulin gene mutations detected on the basis of our own experience are described.

**Keywords**

**Introduction**

The formation of the mammalian cerebral cortex comprises a complex sequence of events, requiring precise regulation at molecular and cellular levels (Kriegstein and Noctor, 2004; Gotz and Huttner, 2005; Ayala et al., 2007). A large number of genes encoding for tubulin proteins are highly expressed during stages of mammalian cerebral cortex development (Leandro-Garcia et al., 2010). The most common isoforms (alpha- and beta-tubulins) are globular subunits that co-assemble into large polymers called microtubules (Figure 1). Microtubule polymers are cylindrical, scaffold-like structures with the ability to steadily grow (by incorporation of alpha/beta-tubulin heterodimers) and rapidly collapse (releasing heterodimers from the polymer). The robust, but dynamic nature of microtubules is harnessed to facilitate cell support, intracellular trafficking and cell division amongst other functions (Mitchison and Kirschner, 1984; Desai and Mitchison, 1997; Feng and Walsh, 2001; Guzik and Goldstein, 2004). The large proportion of tubulin genes expressed during brain development suggests different tubulins isoforms may be required for specific microtubule functions during the three major stages in cerebral cortex formation: neurogenesis, neuronal migration and post-migrational organisation (Figure 2) (Tischfield and Engle, 2010).

During neurogenesis, immature neurons are generated by asymmetrical cell division of radial glial cells within the ventricular and subventricular zones (Rakic, 1982). As a major component of spindle fibres, microtubules provide the mechanical force behind mitosis and, therefore, proliferation of neuronal cells. Immature neurons are subsequently required to migrate increasingly vast distances from proliferative regions to form the neocortex at the brain surface (Rakic, 1974; Angevine and Sidman, 1961). Cell locomotion is achieved by bundles of polarised microtubule polymers that generate leading neurites away from the cell body in the direction of migration. Following neurite outgrowth, a separate population of dynamic microtubules envelope the nucleus and associated organelles and pull them in the direction of migration. Cycles of neurite outgrowth and 'nucleokinesis' generate net movement of immature neurons, which are repeated until specific locations within the developing neocortex are achieved. Following migration, neuronal

communication is facilitated by generation and stabilisation of axonal microtubule fibres to form synaptic connections.

Mutations affecting seven genes encoding for alpha- (*TUBA1A*), beta- (*TUBB2A, TUBB2B, TUBB3, TUBB4A, TUBB*) and gamma-tubulin (*TUBG1*) have been identified in individuals with a range of malformations of cortical development (MCDs) (Keays et al., 2007; Jaglin et al., 2009; Tischfield et al., 2010; Breuss et al., 2012; Hersheson et al., 2013; Lohmann et al., 2013; Poirier et al., 2013; Cushion et al., 2014).

Tubulin gene mutations might alter the dynamics properties of microtubule polymers in several ways. Potential effects include tubulin heterodimer or microtubule dysfunction, alterations to GTP binding and/or disrupted interaction between microtubule polymers and associated proteins (e.g. motor proteins). The overlapping range of MCDs that result from tubulin gene variants have been defined tubulin-related cortical dysgeneses or "Tubulinopathies" (Jaglin and Chelly, 2009; Poirier et al., 2010; Tian et al., 2010; Tischfield and Engle, 2010; Jansen et al., 2011; Romaniello et al., 2014). The tubulin-related cortical phenotypes may include lissencephaly, polymicrogyria, schizencephaly, microlissencephaly and simplified gyration.

The associated phenotypes may be an expression of decreased proliferation due to impaired mitosis during neurogenic stages, axon guidance disturbance (abnormal fascicles and axon tracts, abnormalities of internal capsule and corpus callosum, hypoplasia of brainstem and corticospinal tracts) and/or of migration or post-migration defects (abnormal cortex and hippocampal lamination, cerebellar dysplasia) (Tischfield et al., 2011; Cushion et al., 2013; Bahi-Buisson et al., 2014; Kato, 2015; Oegema et al., 2015; Breuss et al., 2016; Romaniello et al., 2015 & 2017). Characteristic hallmarks of tubulin gene mutations include dysmorphic basal ganglia (fusion of the caudate nucleus and putamen with absence of the anterior limb of the internal capsule), rounded thalami, midline commissural structures hypoplasia, agenesis of the corpus callosum and anterior commissure, and cerebellar hypoplasia/dysplasia (Amrom et al., 2014; Romaniello et al., 2015 & 2017; Breuss et al., 2017; Patel et al., 2017; Whitman and Engle, 2017). Frequently associated

defects include hypoplasia of the oculomotor and optic nerves, dysmorphic hind-brain structures, and a wide spectrum of MCDs. Each tubulin gene is associated with a predominant phenotype (Bahi-Buisson et al., 2014). MCDs result from a dysregulation in one of the intricate mechanisms underlying cerebral cortex formation: cell proliferation (e.g. microcephaly), neuronal migration (e.g. lissencephaly-pachygyria spectrum) and post-migrational organization (focal or diffuse polymicrogyria and schizencephaly) (Bahi-Buisson and Cavallin, 2016; Barkovich et al., 2012). In addition to MCDs, other diseases have been associated with tubulin genes mutations. These include "dystonia Type 4 (DYT4)" and "hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC)" caused by mutations in *TUBB4A* gene (Hersheson et al., 2013; Lohmann et al., 2013; Blumkin et al., 2014), female infertility associated with mutations in *TUBB8* gene (Feng et al., 2016), and congenital macrothrombocytopenia caused by *TUBB1* gene mutations (Kunishima et al., 2009).

In this review, all published data on tubulin gene mutations and resultant cortical phenotypes are summarized in order to further delineate the predominant MCDs associated with each gene. In addition, the most typical neuroimaging patterns of MCDs associated with tubulin gene mutations detected on the basis of our own experience are described.

**Methods**

A MEDLINE search on PubMed and OMIM Programs of the published literature yielded 62 studies that met inclusion criteria [Malformations of cortical development] and [Tubulin gene mutations in human brain]. The period of the literature search runs from 2007 to 2017. As the number of reported mutations in *TUBB2A* and *TUBG1* are few, we have also included two further studies published in 2018 (Sferra et al., 2018; Brock et al., 2018).

**Results**

**Tubulinopathies and MCDs:**

***TUBA1A* [MIM602526] 12q13.12**

The first tubulin gene associated with impaired cerebral cortical development was *TUBA1A*, encoding for Tubulin Alpha 1A (Keays et al., 2007). *TUBA1A* is predominantly expressed in post-mitotic neurons, notably in the cortex, hippocampus, cerebellum and brainstem, with expression reducing soon after birth (Hall and Cowan, 1985; Poirier et al., 2007). *TUBA1A* was initially screened in humans with lissencephaly after a murine model carrying an ENU-induced mutation in the orthologous gene, *Tuba1*, produced a phenotype characteristic of mouse lissencephaly. Keays and colleagues subsequently identified two *TUBA1A* mutations, p.Arg246Cys & p.Arg402His, associated with an agyric and pachygyric brain surface respectively (Keays et al., 2007). Following this initial discovery, subsequent publications identified a number of novel and recurrent *TUBA1A* mutations, and a predominant *TUBA1A*-specific phenotype began to emerge of lissencephaly with cerebellar hypoplasia (LCH), plus abnormalities of the corpus callosum and basal ganglia/internal capsule (Poirier et al., 2007; Bahi-Buisson et al., 2008; Fallet-Bianco et al., 2008; Morris-Rosendahl et al., 2008; Kumar et al., 2010). The 'classical' lissencephaly phenotype (similar to that associated with *LIS1* mutations) is limited to substitutions affecting the Arg402 residue (Kumar et al., 2010). At present, 85 *TUBA1A* variants have been identified in individuals with MCDs (see Table 1). Resultant phenotypes range from lissencephaly, without or with congenital microcephaly (microlissencephaly), through polymicrogyria-like cortical malformations (either diffuse, predominantly perisylvian or multifocal), to mildly simplified or unaffected cerebro-cortical surface (see Table 1) (Keays et al., 2007; Poirier et al., 2007; Bahi-Buisson et al., 2008; Fallet-Bianco et al., 2008; Morris-Rosendahl et al., 2008; Kumar et al., 2010; Jansen et al., 2011; Sohal et al., 2012; Cushion et al., 2013; Okumura et al., 2013; Poirier et al., 2013; Zanni et al., 2013; Amrom et al. 2014, Bahi-Buisson et al., 2014; Kamiya et al., 2014; Romaniello et al., 2014 & 2017; Shimojima et al., 2014; Kato, 2015; Oegema et al., 2015; Mutch et al., 2016). Microlissencephaly has only been observed in foetal cases. It represents the most severe grade of cortical dysplasia combining extreme microcephaly and lissencephaly with hemispheres lacking

primary fissures and olfactory sulci (Fallet-Bianco et al., 2008 & 2014; Lecourtois et al., 2010; Bahi-Buisson et al., 2014). Subcortical band heterotopia (SBH) has also been reported (Morris-Rosendahl et al., 2008; Mozanski et al., 2012). One isolated instance of Hirschsprung disease and Syndrome of Inappropriate Antidiuretic Hormone secretion (SIADH), in addition to agyria with microcephaly, has been described in a girl harbouring a novel *TUBA1A* variant predicted to cause a p.Cys200Tyr substitution (reported as p.Cys402Tyr) (Hikita et al., 2013). Two individuals with hydranencephaly-like cortical dysgeneses were identified as having p.Cys25Phe and p.Arg64Trp substitutions (Yokoi et al., 2015), and a p.Ala270Ser variant in a boy presenting with microphthalmia and congenital cataracts in addition to microcephaly and mildly simplified cerebral gyral patterning (Myers et al., 2015). At present, the vast majority of *TUBA1A* mutations described are *de novo,* heterozygous missense variations. Two siblings with a polymicrogyric cortex and an inherited p.Ile5Leu mutation from an asymptomatic mother mosaic for the corresponding variation have been reported (Jansen et al., 2011). The bulk (~68%) of *TUBA1A* mutations have been associated with the most severe cortical phenotypes (e.g. microlissencephaly, lissencephaly, LCH, and diffuse polymicrogyria-like malformations), with a smaller proportion (~28%) attributed to "central" or perisylvian pachygyric or polymicrogyric cortices, and around 4% in individuals presenting with mildly simplified or unaffected brain surface patterning (Bahi-Buisson et al., 2014).

### *TUBB2B* [MIM612850] 6p25.2

*TUBB2B* mutations were initially described in four individuals and one foetus with bilateral, asymmetric and predominantly posterior polymicrogyria (Jaglin et al., 2009). The authors provided evidence of high expression of murine *Tubb2b* in post-mitotic neurons and, to a lesser extent, radial glia progenitors. As with *TUBA1A* mutations, abnormal cerebellar/vermis, basal ganglia, and corpus callosum were also described. Histological examination of the foetus showed disorganisation of cortical neurons and radial glial fibres, and neuronal over-migration beyond the pial basement membrane. To date, 40 *TUBB2B* mutations have been described, the majority of which associated

with cortical abnormalities closely resembling polymicrogyria, especially in perisylvian or "central" regions (see Table 1) (Jaglin et al., 2009; Cederquist et al., 2012; Guerrini et al., 2012; Romaniello et al., 2012 & 2014; Cushion et al., 2013; Amrom et al., 2014; Bahi-Buisson et al., 2014; Jamuar et al., 2014; Oegema et al., 2015). The histological examination of a foetus and neuronal migration deficits observed in *Tubb2b* knockdown rats (Jaglin et al., 2009) have suggested that *TUBB2B*-related malformations are distinct from classic 'post-migration' polymicrogyria (as classified in Judkins et al., 2011), and should rather be considered "polymicrogyria-like" cortical malformations (Cushion et al., 2013). This pattern (either predominantly central or generalized, with a thick cortex and irregular surfaces on both the pial and grey-white junction sides) described in fact an unlayered polymicrogyria combined with neuronal overmigration and neuronal heterotopias more reminiscent of cobblestone lissencephaly than classic polymicrogyria. An identical pattern was subsequently observed by other authors (Bahi-Buisson et al., 2014; Fallet-Bianco et al., 2014; Romaniello et al., 2017). In addition, one of the *TUBB2B* mutated cases (p.Leu207Pro) described by Cushion et al., (2013) showed a lissencephaly pattern with an agyric cortex, and bilateral band of heterotopic grey matter suggesting an overlap between *TUBA1A*- and *TUBB2B*-related lissencephalies. As with *TUBA1A* mutations, genetic variations in *TUBB2B* are now associated with phenotypes ranging from lissencephalic brain surfaces to a mildly simplified or unaffected gyral patterning (Guerrini et al., 2012; Cushion et al., 2013; Bahi-Buisson et al., 2014; Oegema et al., 2015). One occurrence of fetal akinesia deformation sequence and microlissencephaly has also been reported in a foetus with a *TUBB2B* p.Cys239Phe mutation (Laquerriere et al., 2016). A distinctive genotype-specific phenotype comprising a polymicrogyria-like cortical surface with congenital fibrosis of the extra-ocular muscle (CFEOM) has been reported in a family with a *TUBB2B* p.Glu421Lys variant, segregating in an affected mother and two daughters (Cederquist et al., 2012). CFEOM is a recognised feature in *TUBB3*-associated tubulinopathies, also discussed in this review. Two isolated instances of polymicrogyric cortex plus open-lip schizencephaly have been described in patients harbouring novel variants (p.Gly140Ala & p.Cys354Arg) (Romaniello et al., 2012 & 2014). As

with most tubulinopathies, reported *TUBB2B* variants are predominantly *de novo,* missense mutations. However, one complex insertion/deletion c.1080_1084delCCTGAinsACATCTTC [p.Leu361_Lys362delinsHisLeuGln] has been described in an individual with severe microcephaly and simplified gyral patterning (Romaniello et al., 2014). Inherited mutations have been described however: these include the CFEOM-associated p.Glu421Lys variant, two siblings who inherited a p.Gly13Ala mutation from their asymptomatic father who was mosaic for the variant (Cederquist et al., 2012; Oegema et al., 2015). A somatic, mosaic p.Arg380Pro *TUBB2B* mutation was identified in an individual with pachygyria (Jamuar et al., 2014). More recently, a recessive p.Arg390Gln mutation in *TUBB2B* was detected in a consanguineous family affected by Uner Tan syndrome (Breuss et al., 2017). In addition to the quadrupedal locomotion, all the three patients showed severe cerebellar hypoplasia but no detectable MCD or typical basal ganglia malformation was reported.

### *TUBB3* [MIM602661] 16q24.3

*TUBB3* has a unique function in nervous system development and axon generation and maintenance due to its dynamic properties and neuron-specific expression patterns (Tischfield et al., 2010). *TUBB3* mutations were first identified in the ocular motility disorder, congenital fibrosis of the extra ocular muscles type 3 (CFEOM3). CFEOM3 is a highly penetrant autosomal dominant disorder, and missense mutations are found in affected families and in sporadic individuals (Tischfield et al., 2010). Almost all mutation carriers had an ocular motility disorder, and several also had ptosis with synkenetic jaw movements (Marcus Gunn phenomenon). Some had associated facial weakness, congenital wrist and finger contractures, and/or subsequent development of weakness and sensory loss in the lower extremities in the first to third decade of life. The latter was consistent with a predominantly axonal polyneuropathy and those without CFEOM3 presented as a Charcot-Marie-Tooth hereditary neuropathy type 2 (CMT2)-like disorder. Most patients had anomalies on brain imaging, including defects of the corpus callosum and internal capsule, dysmorphic basal ganglia and reduction in periventricular white matter. No detectable MCDs were

reported in this cohort however. Mild to moderate intellectual disability appeared to correlate with the degree of callosal abnormalities.

Subsequently, the *TUBB3* phenotypic spectrum was broadened following the description of frontal polymicrogyria, simplified and disorganized gyral patterning, asymmetric cortical dysplasia, polymicrogyria-like cortical dysplasia and gyral disorganization with multifocal dysplasia, with or without extra-cortical features (Poirier et al., 2010; Singh and Tsai, 2010; Tischfield et al., 2010; Chew et al., 2013; Bahi-Bouisson et al., 2014; Oegema et al., 2015; Whitman et al., 2015 & 2017; Fukumura et al., 2016; Shimojima et al., 2016; Patel et al., 2017; Romaniello et al., 2017; ). One foetal case was reported with microlissencephaly (Fallet-Bianco et al., 2014) and Oegema et al., (2015) reported a diffuse irregular gyration and sulcation in two cases, and an irregular gyral pattern in one case. Within the subset of individuals with MCD, most *TUBB3* mutations were *de novo* (Poirier et al., 2010; Bahi-Buisson et al., 2014; Oegema et al., 2015). Inherited variants have been described. These include p.Glu205Lys and pMet323Val, which were both consistent with familial, dominant transmission, and a homozygous *TUBB3* p.Ala302Val variant (Poirier et al., 2010). This variant was heterozygous in the 'affected' mother and, despite paternal DNA being unavailable, a high level of homozygosity upon SNP array analysis suggested consanguinity.

It had been hypothesised that the two *TUBB3*-related phenotypes were due to different microtubule functions affected by the corresponding subset of *TUBB3* mutations. However, two *de novo* variants (p.Gly71Arg & p.Gly98Ser) have recently been identified in individuals with both MCDs and syndromic CFEOM3, suggesting a common microtubule dysfunction shared by both phenotypes (Whitman et al., 2015).


### *TUBB* (*TUBB5*) [MIM191130] 6p21.33

*TUBB* (also referred to as *TUBB*5) *de novo* missense mutations have been reported in three unrelated patients aging between 2 years 6 months to 4 years 10 months (Breuss et al., 2012) (see Table 1). They were microcephalic (OFC -2.5SD to -4SD) and had mild to severe global

developmental delay, but no seizure disorder. Neuroimaging showed focal polymicrogyria, localized subcortical band heterotopia a hypoplastic cerebellar vermis in one individual, who also had retinal dysplasia and microphthalmia. The other two patients were not reported to have a cortical dysplasia. However, neuroimaging showed dysmorphic basal ganglia and hypoplasia or partial agenesis of the corpus callosum. In embryonic mice, *Tubb5* is the most highly expressed tubulin isotype in the brain. It is expressed predominantly in radial glia, intermediate progenitors and post-mitotic neurons, and its depletion affects cell-cycle dependent proliferation as well as neuronal positioning (Breuss et al., 2012; Ngo et al, 2014). Isrie et al. described additional *TUBB* patients with circumferential skin creases that also present with microcephaly, suggesting a role for this tubulin isoform beyond correct brain development (Isrie et al., 2015).

## *TUBA8* [MIM605742] 22q11.21

Mutations in Tubulin alpha 8 (*TUBA8*) were previously reported to cause cortical malformation with an autosomal recessive inheritance pattern (Abdollahi et al., 2009). Four individuals from two consanguineous families of Pakistani origin were found to harbour a homozygous *TUBA8* 14bp deletion in *TUBA8* intron 1. This led to abnormal splicing and a shortened transcript lacking exon 2. Neuroimaging showed extensive bilateral polymicrogyria, a thin or absent corpus callosum and a hypoplastic brain stem (Abdollahi et al., 2009). All presented with severe infant-onset epilepsy, severe developmental delay, hypotonia and optic nerve hypoplasia.

Following this initial report however, Braun et al. (2010) demonstrated low levels of *Tuba8* in the developing brain of mice and humans, bringing into question the pathogenicity of genetic variations described. In addition, Diggle et al., (2017) demonstrated a role of *Tuba8* in spermatogenesis but not in brain development using a mouse knockout model. Moreover, the authors highlighted a homozygous, loss-of-function *SNAP29* mutation in the individuals described in the original study by Abdollahi et al., and postulate that SNAP29 deficiency, rather than TUBA8 deficiency, caused

the brain phenotypes observed (Diggle et al., 2017). There is currently no evidence to support *TUBA8* as a candidate gene for MCDs.

### *TUBB2A* [MIM615101] 6p25.2

Recent studies have described six individuals harbouring *de novo* missense mutations in *TUBB2A* (Cushion et al., 2014; Lee et al., 2014; Rodan et al., 2017; Ejaz et al., 2017). Cushion et al., (2014) reported two mutations (p.Asn247Lys & p.Ala248Val) affecting adjacent amino acids within a highly-conserved loop of encoded beta-tubulin protein. One individual presented with infantile spasms at 5 months and brain MRI revealed a diffuse, simplified gyral pattern, reduced white matter, globular basal ganglia, mildly hypoplastic cerebellar vermis, thin and dysmorphic corpus callosum, mild brainstem hypoplasia and moderately enlarged third and lateral ventricles. The second child presented with hypotonia at 4 months and multifocal epileptiform discharges at 11 months. Neuroimaging revealed corpus callosum dysmorphisms, but normal cortex, basal ganglia and thalami.

Another instance of the *TUBB2A* p.Ala248Val variant has since been described in a 4 year-old boy with developmental delay, spastic diplegia and exaggerated startle, but absence of epilepsy (Rodan et al., 2017). Contrary to the individual harbouring this variant in the Cushion et al. study, neuroimaging showed focal anterior temporal pachygyria. An individual with developmental delay, seizures, perisylvian polymicrogyria and microcephaly has also been described, harbouring a *TUBB2A* p.Gln291Pro mutation affecting a distinct region of the protein subunit (Lee et al., 2014). An individual with a *TUBB2A* p.Arg262His mutation was described with bilateral posterior sylvian polymicrogyria, asymmetric ventricles, abnormal frontal gyration and severe neurological phenotype, characterized by arthrogriposis multiplex congenital and dysmorphic facial features (Ejaz et al., 2017). More recently, a *TUBB2A* p.Asp417Asn variant was identified in a patient with a sacsinopathy-like progressive spastic ataxia syndrome, cerebellar atrophy but with no reported cortical malformation (Sferra et al., 2018).

To date, six missense mutations have been described (see Table 1). Whilst this number is too small to delineate any robust genotype-phenotype correlations, Ejaz et al. (2017) suggested that the differing severities of phenotypes observed so far may be due to residue-specific effects. The role of *TUBB2A* in nervous system development is unclear. In both humans and mice, expression is notably less than beta-tubulins *TUBB2B*, *TUBB* and *TUBB3* during gestation (Breuss et al., 2012), perhaps corresponding to the relatively mild cortical phenotypes observed in some affected individuals thus far.

### *TUBB4A* [MIM602662] 19p13.3

The disease phenotypes associated with mutations in *TUBB4A* are the most distinct of the tubulinopathy genes. Genetic variations in *TUBB4A* were originally associated with dystonia Type 4 (DYT4) (Hersheson et al., 2013; Lohmann et al., 2013). However, a number of *TUBB4A* mutations were described in individuals with hypomyelination, most commonly with atrophy of the basal ganglia and cerebellum (H-ABC), but also with isolated and 'unclassified' hypomyelination, 'hypomyelination mimicking Pelizaeus-Merzbacher disease', and an isolated instance of hereditary spastic paraplegia (Simons et al., 2013; Blumkin et al., 2014; Ferriera et al., 2014). It is postulated that mutations in *TUBB4A* are associated with a disease presentation continuum, with DYT4 and H-ABC at the mild and severe of the spectrum respectively. No detectable cortical phenotypes are described. For a comprehensive review of *TUBB4A*-related disorders, see Nahhas et al., (2016).

### *TUBG1* [MIM191135] 17q21.2

Gamma-tubulin 1 (*TUBG1*) is one of two human γ-tubulin genes. It associates with several other proteins to form the γ-tubulin ring complex (γTuRC). This complex functions as a scaffold for the formation of microtubules, a process called "microtubule nucleation". It also forms a cap at the minus-end of the microtubule to stabilise the growth of the microtubule at the plus-end. (Kollman et al., 2011; Poirier et al., 2013). Eleven patients from ten families with missense mutations in *TUBG1*

have been described to date (Poirier et al., 2013; Brock et al., 2018) (see Table 1). The core features of *TUBG1*-related malformations resemble those of *PAFAH1B1* (encoding LIS1) mutations: these include pachygyria with a posterior to anterior gradient, lateral ventricle enlargement and reduction in white matter but without basal ganglia, corpus callosum, brainstem or cerebellar abnormalities characteristic of many tubulinopathies (Brock et al., 2018). The authors suggest this may be due to the distinct role of gamma-tubulin from both alpha- and beta-tubulins.

**MCD Tubulinopathy spectrum in our series:**

The MCD pattern of twenty-eight patients carrying tubulin gene mutations (*TUBA1A* n=14; *TUBB2B* n= 8; *TUBB3* n= 6) and previously described (Romaniello et al., 2012; Romaniello et al., 2014; Romaniello et al., 2017) has been revised and details reported (Figure 3).

*TUBA1A-related MCD spectrum*: 8/14 patients of our *TUBA1A* gene mutated patients had a detectable MCD. Polymicrogyria was observed in seven cases with different patterns: four patients had isolated perisylvian polymicrogyria, two patients had perisylvian polymicrogyria-associated with a thick and pachygyric cortex in the occipital lobe, and one had polymicrogyria and diffuse pachygyria. The only patient without polymicrogyria, showed a diffuse pachygyria with subcortical band heterotopia (Figure 3).

*TUBB2B-related MCD spectrum*: 7/8 patients (87.5%) of our patients carrying mutation in the *TUBB2B* gene presented a MCD upon neuroimaging. Polymicrogyria was observed in 6 cases: 3 cases showed diffuse polymicrogyria (with associated SCH in 2) and 3 showed perisylvian polymicrogyria (associated with thick and pachygyric occipital cortex in 1). One patient had no polymicrogyria but a simplified gyral pattern associated with small heterotopic nodules (Figure 3).

*TUBB3-related MCD spectrum*: none of our *TUBB3* gene mutated patients (n=6) presented a detectable MCD.

**Conclusions**

The development of the central nervous requires precise regulation of a massive number of cell division and migration events within a specific spatial and temporal window. Neurons and glial cells must proliferate, migrate to appropriate areas in the cerebral cortex and project multiple cellular extensions (axons and dendrites) to form synapses, thereby allowing communication with other neurons and the creation of networks (Barkovich, 2013). Microtubules play key roles during stages of cerebral cortex development: they provide cell structure and generate the intracellular forces required by neurons to migrate and develop axonal and dendritic processes, whilst providing organized scaffolds for intracellular transport by motor proteins (e.g. kinesins and dyneins). The importance of precisely regulated microtubule function during cerebrocortical formation is highlighted by both the number of tubulin genes expressed during brain development, as well as the ever-growing number of pathogenic tubulin mutations.

In recent years, mutations in the tubulin isotypes have been identified in a wide range of complex disorders of brain development involving MCDs (Abdollahi et al., 2008; Jaglin et al., 2009; Kumar et al., 2010; Poirier et al., 2010; Breuss et al., 2012; Cushion et al., 2014).

Alpha- and beta-tubulins form heterodimers that incorporate into microtubules. The structure of tubulin protein subunits can be grossly divided into three separate structural domains: the N-terminal, intermediate, and C-terminal domains (Singh and Tsai, 2010; Tischfield and Engle, 2010). The N-terminal structural domain forms the GTP-binding pocket required for protein folding and stability, heterodimer formation and regulation of microtubule dynamics via changes in protofilament conformation (Singh and Tsai 2010). Residues predominantly within the intermediate domain mediate longitudinal and lateral interactions with adjacent tubulin subunits within the microtubule, necessary for polymer stability. Interactions between motor proteins and microtubule-associated proteins (MAPs) occur principally through residues at the C-terminus, that form helices on the external surface of tubulin and a 'tail' of mostly acidic residues that extends away from the microtubule polymer. These residues are predicted to mediate interactions with kinesin and dynein

to regulate the dynamic behaviour of microtubules and are necessary for neuronal migration, differentiation and axon guidance (Singh and Tsai, 2010; Tischfield and Engle, 2010).

Mutated residues are widely distributed among the three protein domains, and are therefore predicted to perturb varying microtubule functions according to their structural locations. Several mutations alter residues that interact directly with the GTP nucleotide or those located directly adjacent to it (e.g. *TUBB2B* p.Gly140Ala; Romaniello et al., 2012). Some mutations are predicted to affect lateral interactions between tubulin subunits of adjacent protofilaments (e.g. Asn256Ser; Guerrini et al., 2012).

Other mutations affect residues at contact surfaces between the intra- and interdimer interfaces (e.g. *TUBB2B* p.Gly98Arg; Cushion et al., 2013) and thus are predicted to alter longitudinal protofilament interactions and microtubule stability (Nogales et al., 1999).

Correct microtubule function is critical for successful neuronal proliferation, migration and cortical maturation. Mutations affecting genes encoding for MAPs (e.g. DCX and LIS1) are associated with neuronal migration disorders (Reiner et al., 1993; Gleeson et al., 1998). Mutation affecting residues on the surface of microtubules that mediate protein interactions with kinesin, dynein and other MAPs are associated with axon guidance and maintenance defects and oculomotor disorders (Tischfield et al., 2010).

Despite the overlapping phenotype, mutation-specific phenotypes in tubulin isotypes exist, due both to the specific function of the related isotype and to the different functional areas of the protein involved. This leads to a genotype/phenotype correlation based not only on the primary sequence, but also on the tertiary structure and the three-dimensional arrangement of the protein (Tischfield et al., 2010). In a recent study, Bahi-Buisson et al., (2014) analysed the correlation between alpha- and beta- tubulin gene mutation location and the associated broad phenotype, showing how variability in tubulin-related disorders depends on microtubule functional defects predicted (i.e. mutations that predominantly interfere with heterodimerisation may not affect cortical development) (Bahi-Buisson et al., 2014).

A number of recurrent tubulin gene mutations have been described in unrelated patients who share strikingly similar brain phenotypes. For example, *TUBA1A* p.Arg264Cys has been identified in a number of individuals with characteristic central pachygyria (Poirier et al., 2007; Bahi-Buisson et a., 2008; Bahi-Buisson et al., 2014). Additionally, different amino acid substitutions affecting the same residue have also been shown to cause comparable cortical malformations, such as the recurrent TUBA1A p.Arg402Cys and p.Arg402His variants (Kumar et al., 2010; Bahi-Buisson et al., 2014). However, a number of identical mutations have been found in patients demonstrating dissimilar brain abnormalities. Examples include *TUBA1A* p.Arg390Cys, associated with both asymmetrical perisylvian polymicrogyria and mildly simplified gyration (Kumar et al., 2010; Poirier et al., 2013), and *TUBB2A* p.Ala248Val identified in one individual with seemingly normal cortical but in another with pachygyria (Cushion et al., 2014; Rodan et al., 2016). Reasons for such phenotypic variabilities are unclear but may be explained by different genetic background and/or environmental factors which may contribute, or even due to inconsistencies in the diagnosis or interpretation of neuroimaging.

The broad spectrum of tubulinopathy MCDs described to date ranges from severe agyria (with or without microcephaly), through pachygyria and polymicrogyria-like malformations, to mildly or unaffected cerebrocortical patterning at the less severe end. In addition to the cortical phenotype, extra-cortical brain features including abnormalities of the corpus callosum, basal ganglia and cerebellar hemispheres or vermis are almost consistently present (Abdollahi et al., 2008; Jaglin et al., 2009; Kumar et al., 2010; Poirier et al., 2010; Breuss et al., 2012; Cushion et al., 2014; Romaniello et al., 2015 & 2017). Recently, patients with highly-characteristic cerebellar, brainstem and basal ganglia abnormalities, but without striking cortical involvement, revealed a number of mutations in either *TUBA1A*, *TUBB2B* and *TUBB3* genes, suggesting that brain surface malformations may be less of an indication towards tubulin gene involvement than originally perceived (Oegema et al., 2015; Romaniello et al., 2017). We now observe a range of malformations resulting from variations within the same gene, whilst it was originally thought that

mutations affecting different tubulin genes gave rise to exclusively distinct phenotypes. Despite this, affected genes are associated with predominant cortical phenotypes (Bahi-Buisson et al., 2014). Currently, these include *TUBA1A* and *TUBG1* with lissencephalic brain surfaces, *TUBB2B* with polymicrogyria-like malformations, subsets of *TUBB3* mutations associated with either polymicrogyria-like malformations or unaffected cortex, but CFEOM3 ocular deficits, *TUBB* with microcephalic brains with or without apparent cortical involvement, *TUBB4A* with seemingly unaffected brain surface but hypomyelination with atrophy of the basal ganglia and cerebellum, and *TUBB2A* with mildly simplified cortical patterning.

The large majority of tubulinopathy mutations are heterozygous, missense variants of autosomal dominant inheritance. They frequently result in amino acid substitutions that affect otherwise extremely conserved residues within protein primary sequences. Consistent with the devastating syndromes that result from these mutations, they are primarily of *de novo* inheritance. A number of recurrent *de novo* variations have been reported. Neuroimaging of unrelated individuals harbouring the identical mutations frequently share similar brain dysgeneses. In addition, mutations affecting equivalent residues of different tubulin isoforms have also been identified (e.g. *TUBA1A* p.Arg390Cys & *TUBB2B* p.Arg380Cys) highlighting critical residues to tubulin protein function during brain development. Despite occurring on equivalent residues, the resultant phenotype will often depend on the affected gene, supporting the notion that tubulinopathy genes are associated with predominant malformations. There may also be issues over the mutation-origin timing of the *de novo* mutations leading to heterogeneous developmental outcomes and with late-stage mutations not being detected in lymphocyte DNA (Pouduri et al., 2012; Jamuar et al., 2014; Lee et al., 2014).

Three hot spot mutations were found representing 40% of all known *TUBA1A* mutations (Arg264, Arg422 and Arg402). Based on clinical and neuroimaging findings, Bahi-Buisson & Cavallin, (2016), reported *TUBA1A* pathogenic variants p.Arg402Cys or p.Arg402His, preferentially related to lissencephalies and microlissencephalies phenotype (p.Thr56Met, p.Asn101Ser, p.Arg264His,

p.Leu286Phe, p.Val303Gly, p.Arg320His, p.Lys326Asn, p.Val371Glu, and p.Glu429Gln the variants associated with microlissencephaly), while p.Arg264Cys to polymicrogyria-like cortical dysplasia. Histological examinations in a *Tuba1a* mutant mouse revealed defects attributed to impairment of neuronal migration as mechanisms structural abnormalities of cortex and fractured pyramidal layer of the hippocampus (Keays et al., 2007). The *TUBA1A* gene is highly expressed in post-mitotic neurons, but not in glia in the human and mouse brain (Gloster et al., 1994). Murine expression studies showed that the *Tuba1a* gene is largely absent from the proliferative ventricular zone and its expression peaks at 16.5 embryonic days when neuronal migration is underway (Bamji and Miller, 1996; Gloster et al., 1994). Successful migration requires cycles of leading process extension in the direction of locomotion, translocation of the nucleus and retraction of trailing processes (Tsai and Gleeson, 2005; Ayala et al., 2007). All of these processes are reliant on a dynamic network of microtubules polymers which, if impaired by *TUBA1A* gene mutations, primarily lead to disorders of migration (Breuss and Keays, 2014).

In our experience, 57 % of our *TUBA1A* gene mutated patients showed an MCD pattern. Contrary to the literature however, none showed classical lissencephaly or microlissencephaly, perhaps since no foetus had been studied. Conversely, most patients showed a polymicrogyria pattern, mainly perisylvian (75%), so that it seems to be a prevalent *TUBA1A* associated pattern. In accordance with literature, it is possible to assume that the perisylvian regions is the main brain location associated in *TUBA1A* mutated patients. With regards to *TUBB2B*, 87.5% of patients from our cohort carrying mutation in the *TUBB2B* gene present a MCD. In line with the literature, polymicrogyria is most prevalent, without a specific pattern. Polymicrogyria was generalized in one case (p.Ile210Thr), associated with SCH in two (p.Gly140Ala, p.Cys354Arg), and perisylvian polymicrogyria was observed in 3/8 cases (37.5%) (p.Asp327Gly, p.Arg380Cys). Therefore, the involvement of perisylvian regions, although present, is not as prevalent as in *TUBA1A* mutations.

Intriguingly, polymicrogyria is also the predominant phenotype in individuals harbouring *TUBB3* mutations (when an MCD is reported). Polymicrogyria and simplified and disorganized gyral

patterning, asymmetric cortical dysplasia, polymicrogyria-like cortical dysplasia and gyral disorganization with multifocal dysplasia were, in fact, the most commonly described *TUBB3*-associated MCD patterns (Poirier et al., 2010; Singh and Tsai, 2010; Tischfield et al., 2010; Bahi-Buisson et al., 2014; Whitman et al., 2015; Fukumura et al., 2016; Shimojima et al., 2016; Patel et al., 2017). Nevertheless, in our cohort, none of the patients carrying a *TUBB3* gene mutation had a detectable MCD.

Evidence to support altered protein function in tubulinopathies is provided by a recent description of two monozygotic twins harbouring a *de novo* 0.32Mb deletion of chromosome 16q24.3, encompassing *TUBB3* amongst other genes (Grønborg et al., 2015). Neuroimaging did not detect any cerebral cortex, basal ganglia, corpus callosum or optic nerve abnormalities, despite both cases presenting with global development delay, secondary microcephaly, mild facial dysmorphism and mild spastic diplegia. This suggests that congenital brain malformations are not secondary to haploinsufficiency of *TUBB3* (Grønborg et al., 2015). The same cannot necessarily be said for the other tubulin isoforms, especially as Jaglin et al. (2009) and Breuss et al. (2012) have both demonstrated brain abnormalities following knockdown of *Tubb2b* and *Tubb5* in mice, respectively (Jaglin et al., 2009; Breuss et al., 2012 & 2016).

It is still unclear which pathogenic mechanisms are exclusive to each gene (thereby causing predominant gene phenotypes) and which are shared, thus giving rise to the extra-cortical anomalies characteristic of tubulin gene involvement. One potential reason for the overlapping phenotypes arising from tubulin gene mutations may be that, within alpha- and beta-tubulin gene families, expression patterns often intersect with one another (Leandro-Garcia et al., 2010). Therefore, at any one point of time in a neuronal cell, microtubule polymers can be composed of varying proportions of a number of tubulin isoforms (Breuss et al., 2017). Isoforms share extremely high levels of sequence homology, making it extremely difficult to elucidate specific roles of isoforms within cell. It nevertheless appears to support the multi-tubulin hypothesis, which postulates that tubulin isoforms play subtly distinct roles, especially during cerebrocortical formation (Tischfield and

Engle, 2010). Further support of this notion has been recently provided by experiments in mice, demonstrating that overexpression of a number of beta-tubulin genes cannot compensate for the knock-down in *Tubb3* during embryonic brain development (Saillour et al., 2014). A recent observation is that tubulin gene variants can be attributed to a wide range of brain phenotypes, as microtubules are involved in all major stages of cortical development (i.e. neuronal proliferation, migration and organisation). Conversely, mutations affecting MAPs (e.g. *DCX* and *LIS1*) tend to be limited to agyria and pachygyria, suggesting they play more specified roles in neuronal migration rather than cell proliferation and axon generation (Mutch et al., 201). The establishment of predominant phenotypes associated with each gene has been facilitated by the increase in number of mutations identified during recent years. However, are we still missing certain variants responsible for more devastating developmental deficits, and potentially early embryonic lethality? Recent findings have suggested that somatic mosaic mutations, presumably lethal when present in the germline, account for more cases of cerebrocortical malformations than originally predicted (Jamuar et al., 2014). This is a distinct possibility, especially as somatic variations are often undetectable by currently available sequencing techniques. Therefore, not ruling out mosaicism in parents when classifying seemingly *de novo* variants in probands is very important.

The next challenge is to continue the gene-discovery programmes in cortical malformations given the complex genetic architecture and causality, as well as investigating tubulin genes in other disease phenotypes. Model systems may answer some of the questions about timing and origin of *de novo* mutations, spatial and temporal developmental checkpoints, and potential pharmacological or cellular biology intervention platforms.

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**Conflict of interest**

The authors declare that they have no conflict of interests.

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**Figure Titles and Legends**

**Figure 1. Structure and dynamic behaviour of the microtubule**. **A)** Alpha- (pink) & beta-tubulin (grey) protein subunits form heterodimers containing two motifs that bind guanine nucleotides (GTP/GDP); the non-exchangeable site on the a-tubulin, which holds a GDP molecule within the dimer, and the exchangeable site on the b-tubulin which permits the reversible hydrolysis of GTP to GDP and back. **B)** Heterodimers containing GTP-bound beta-tubulin (grey outline) incorporate into the plus-end of polymerising microtubules (beta-tubulin towards the plus-end) to form a GTP-rich cap. Following incorporation, GTP hydrolysis occurs at the beta-tubulin (red outline). An altered conformation of GDP-bound heterodimer creates stress on the straight walls of the polymer. Microtubule-associated proteins (MAPs; e.g. DCX, MAP1B, MAP2 & Tau) decorate the microtubule lattice and provide structural support. Molecular motors (e.g. kinesin & dynein) transport intracellular cargo in predominantly anterograde and retrograde directions, respectively. **C)** If GTP-hydrolysis rate becomes greater than addition of GTP-bound heterodimers, the strain caused by GDP-bound heterodimers results in microtubule catastrophe, whereby tubulin protofilaments peel away from the walls of the polymer and the microtubule depolymerises. Microtubule depolymerisation is also promoted by microtubule-severing enzymes (MSEs; e.g. Katanin & Spastin) and certain kinesins (e.g. Kinesin type 8 and 13).

**Figure 2. The role of microtubules in cerebral cortex development.** Microtubule function is critical to the three major stages of cortical development: **A)** During neurogenesis, immature neurons (IN) arise from radial glial cell proliferation (RGC) in the ventricular and subventricular zones within the developing neocortex. Microtubules (pink) are the major component of spindle fibres, which provide the mechanical forces behind mitosis and enable division of genetic material (blue). **B)** Neuronal migration from ventricular zones to the cortical plate, guided by radial glial processes spanning the neocortex, is achieved by push-and-pull forces of microtubules. Bundles of stable microtubules, with their plus-ends facing away from the cell body, generate a leading neurite in the direction of migration. Subsequently, more dynamic microtubule polymers envelop the

nucleus and associated organelles e.g. centrosome (green), pulling it in the direction of migration to result in net cell movement. **C)** Upon achieving a final destination in the cortical plate, microtubule polymers generate extensive axonal processes to facilitate synaptic connections with other neurons in often-distant brain locations.

**Figure 3: MCDs in tubulinopathies.** T2-weighted MRI sections of different MCDs in patients with mutations in *TUBA1A* (**A, B**) and *TUBB2B* (**C, D**) genes are shown. In **A**, a 3 day old female has a bilateral perysilvian polymicrogyria, while in **B** diffuse pachygyria with subcortical band heterotopia is present. Diffuse bilateral polymicrogyria with right schizencephaly and simplified gyral pattern with small nodules of heterotopias (arrow in **D**) are shown respectively in panel **C** and **D**. All patients were part of the cohort described in Romaniello et al, 2017. MR images of patients B and D were also reported in Romaniello et al., (2014).

**Table 1. MCDs-related-Tubulin genes reported mutations to date**