Mutations in the murine homologue of TUBB5 cause microcephaly by perturbing cell cycle progression and inducing p53 associated apoptosis

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Summary

Microtubules play a critical role in the generation, migration, and differentiation of nascent neurons in the developing vertebrate brain. Mutations in the constituents of microtubules, the tubulins, are known to cause an array of neurological disorders, including lissencephaly, polymicrogyria, and microcephaly. In this study we explore the genetic and cellular mechanisms that cause TUBB5 associated microcephaly by exploiting two new mouse models: a conditional E401K knockin; and a conditional knockout animal. We report that these mice present with profound microcephaly due to a loss of upper layer neurons that correlates with massive apoptosis and upregulation of p53. This phenotype is associated with a delay in cell cycle progression and ectopic DNA elements in progenitors, which is dependent on the dosage of functional Tubb5. Strikingly, we report ectopic Sox2 positive progenitors and defects in spindle orientation in our knockin mouse line, which are absent in knockout animals. This work sheds light on the functional repertoire of Tubb5, reveals that the E401K mutation acts by a complex mechanism, and demonstrates that the cellular pathology driving TUBB5 associated microcephaly is cell death.
Introduction

The generation of the mammalian neocortex critically relies on a functional microtubule cytoskeleton (Ayala et al., 2007; Kuijpers and Hoogenraad, 2011). Microtubules mediate the separation of sister chromatids in neuronal progenitors, translocate the nucleus in post-mitotic migrating neurons, and enable axonal elongation, connecting disparate brain regions (Dent et al., 2011; Dickson, 2002). Mutations in the tubulin genes themselves (TUBA1A, TUBB2A, TUBB2B, TUBB3, TUBB5, TUBA4A, TUBB4A, TUBG1) have been implicated in a variety of human neurological diseases, now referred to as the tubulinopathies (Bahi-Buisson et al., 2014; Breuss and Keays, 2014; Smith et al., 2014). These disorders include lissencephaly, polymicrogyria, amyotrophic lateral sclerosis, and ocular motility disorders (Jaglin et al., 2012; Smith et al., 2014; Tischfield et al., 2011). We have recently reported that mutations in the beta tubulin isoform TUBB5 can cause microcephaly with structural brain abnormalities (Breuss et al., 2012; Ngo et al., 2014). Tubb5 is widely expressed throughout embryonic development, and is enriched in the developing cortex, where it is found in radial glial progenitors, intermediate progenitors and post-mitotic neurons. As is true for the vast majority of tubulin mutations that cause human disease, those in TUBB5 are heterozygous and de novo. The preponderance of such mutations and absence of disease-causing null alleles has led to the assertion that tubulin mutations act by a gain of function mechanism rather than haploinsufficiency (Hu et al., 2014; Kumar et al., 2010). In this manuscript we investigate this contention by generating an E401K conditional knockin and a conditional knockout line. The E401K mouse line recapitulates a mutation found in a patient who presented with severe microcephaly (-4SD), partial agenesis of the corpus callosum, but with an intact cortical architecture. We show that our Tubb5 mouse lines model the microcephalic phenotypes observed in patients and that this phenotype can be attributed to mitotic defects in neuronal progenitors and massive p53 induced apoptosis. We further demonstrate that loss of function mutations in Tubb5 are capable of causing microcephaly in the mouse, however, the E401K acts by a complex mechanism exhibiting subtle gain of function effects.
Results

Tubb5 perturbation causes microcephaly in the mouse

In order to explore the genetic and cellular pathology underlying TUBB5 perturbations we engineered two conditional Tubb5 transgenic mouse lines, a conditional knockin of the E401K patient mutation and a conditional knockout (Fig. S1A). Both, the conditional knockin and the knockout lines rely on loxP sites flanking the terminal exon (exon 4). In the case of the E401K knockin a modified version of exon 4 harboring the E401K amino acid substitution was inserted downstream of this exon, permitting its conditional activation. mRNA analysis showed that in the absence of the Cre recombinase the E401K mutation was not expressed (data not shown). We employed a Nestin promoter to drive Cre-recombinase allowed the specific investigation of the E401K-knockin and the knockout in the developing brain (Tronche et al., 1999). In the presence of this Cre recombinase cDNA sequencing confirmed that the E401K mutation is expressed, and qPCR analysis at E14.5 showed that Tubb5 is expressed at similar levels when compared to controls (Fig. S1D). We analyzed adult mice employing Nissl stains and volumetric magnetic resonance imaging (MRI). Unexpectedly, we found that heterozygous E401K animals expressing the Cre recombinase, showed no significant reduction in brain size in comparison to littermate controls (Fig. 1A-B and E-F). This contrasted with homozygous E401K animals and heterozygous Tubb5 null alleles, both of which presented with large reductions in total brain (E401K/E401K: P<0.0001; KO/+: P<0.0001; n=3) and cortical volume (E401K/E401K: P<0.0001; KO/+: P=0.0025; n=3) (Fig. 1C-D, G-H and I-J). This reduction was also observed in other brain regions, including the corpus callosum, cerebellum, hippocampus and putamen (Fig. S2D-E). Cortical thickness was similar in affected animals and wild-type littermate controls at E12.5, E14.5 and E16.5 (Fig. 1K-L). However, at P0 the cortex was notably thinner in affected lines, and severely reduced in adulthood (E401K/E401K: P<0.01; KO: P<0.001; n=3). This suggests that the underlying defect has its origin in development and is exacerbated postnatally (Fig. 1K-L). Attempts to generate adult homozygous null animals driven by Nestin-Cre were unsuccessful, due to prenatal and early postnatal lethality (no animals could be recovered past weaning age). Taken together these data show that our Tubb5 mouse lines model the microcephaly described in humans with TUBB5 mutations, and
that loss of function mutations can result in phenotypes consistent with the tubulin associated disease states.

**Preservation of cortical architecture but loss of upper neuronal layers in Tubb5 mutants.**

Next we examined the cortical architecture of our Tubb5 mutant lines employing a number of laminar markers. Consistent with the human phenotype we did not observe any gross defects in the laminar structure of the cortex when staining with Foxp2 (layer VI marker), Er81 (layer V marker) or Cux1 (layer II-IV marker) in our homozygous E401K animals or our heterozygous Tubb5 null alleles (Figures 2A-L and S3A-C). However, quantitation of these layers revealed a large reduction in the number of Cux-1 positive superficial neurons in our Nestin-Cre positive homozygous E401K animals and our heterozygous null animals (E401K/E401K: P<0.01; KO/+: P=0.002; n=3) (Fig. 2O). As there was no difference in the number of Er81 and Foxp2 cells between affected animals and littermate controls, the relative contribution of layers II-IV to total cortical thickness was changed dramatically (Figures 2M-N and S3D).

**Neuronal progenitors in Tubb5 mutant mice**

As cortical lamination proceeds in an inside-out pattern with the time of birth correlating with the cellular identity and final position of neurons (Greig et al., 2013), we explored the loss of upper layer neurons by investigating the developing cortex at E14.5. We first asked whether the reduction in neuronal number might be attributed to a reduction in the number of neuronal progenitors (Figures 3A-H and S4A-B). Staining with Tbr2 revealed no significant difference in the number of intermediate progenitors in our Nestin-Cre positive homozygous E401K animals or heterozygous null animals when compared to littermate controls at E14.5 (Fig. S4J). We did observe a small reduction in Sox2 positive apical progenitors in our heterozygous knockout animals that was not apparent in our homozygous E401K knockin animals (KO/+: P=0.0494; n=3; E401K/E401K: P>0.05) (Figures 3A-D and S4A-B and 3I). Strikingly, in these knockin animals we observed an increase in the number of ectopic Sox2 and Tbr2 positive progenitor cells that were not present in the heterozygous knockout (Sox2: E401K/E401K: P<0.01; Tbr2: E401K/E401K: P<0.001; n=3) (Figures 3C, G and M and S4D-
F and K). Staining with the phosphorylated form of the intermediate filament vimentin revealed that a subpopulation of these ectopic cells showed basal processes reminiscent of basal radial glial cells (Fig. S4G-H) (Florio and Huttner, 2014). The presence of ectopic progenitors has been associated with defects in spindle orientation, which can lead to depletion of the progenitor pool and premature production of post-mitotic neurons. This has been proposed to be the underlying cellular pathology responsible for microcephaly (Fish et al., 2006; Thornton and Woods, 2009). To assess spindle orientation in our mouse lines we stained for N-Cadherin, γ-Tubulin, pH3 and DNA at E14.5 permitting the three-dimensional assessment of the spindle angles relative to the ventricular surface (Xie et al., 2013) (Fig. 3P). Comparison of spindle orientation in our heterozygous knockout animals with littermate control animals revealed that the vast majority of divisions were in the horizontal plane. In the case of our E401K homozygous animals a significantly higher percentage of vertical orientations were observed in comparison to controls (E401K/E401K: P<0.01, n=4 animals, n>76 cells) (Figures 3Q and S5A-F). We conclude that the presence of ectopic progenitors in our E401K mutant mice may result from changes in the spindle orientation but this does not have a significant impact on the number of progenitors and is unlikely to account for the severe microcephalic phenotype observed in adulthood.

**Distinct mitotic defects cause p53-mediated apoptosis in the knockin and the knockout**

Next we examined our mutant lines for defects in mitosis by staining with the M-phase marker pH3 at E14.5. A comparison with littermate controls revealed no significant difference in heterozygous knockout animals, but a notable increase in the number of positive cells in our E401K homozygous mutants (E401K/E401K: P<0.05; KO/+: P>0.05; n=3) (Fig. 3I-L and N-O). One explanation for this increase is a lengthening of M-phase, caused by a delay in mitotic progression. To assess this we stained with the chromosomal passenger complex Aurora B, pH3 and Hoechst, permitting identification and scoring of cells in metaphase and ana-/telophase (Figures 4A-F and S6A-B) (Adams et al., 2001). We observed an enrichment of cells in metaphase in our homozygous E401K mutant animals in comparison to littermate controls (Fig. 4O). These data are consistent with the observed increase in pH3 staining, and suggest stalling of the cell cycle during M-phase. This phenotype was
absent in our heterozygous knockout animals, however, in these animals we observed a significant increase in ectopic DNA elements, such as chromosome bridges or globular Hoechst positive structures outside the metaphase plate (Fig. 4G-N and P). As defects in mitotic progression and lagging chromosomes have been associated with aneuploidy induced apoptosis in the embryonic brain, we stained for cleaved Caspase-3 in our mutant lines (Insolera et al., 2014; Marthiens et al., 2013) (Figures 3Q-T and S6C). We observed a significant induction of apoptosis in both our mutant lines that was more severe in our heterozygous null mutant (E401K/E401K: P<0.05; KO/+: P=0.0067; n=3) (Fig. 4Y). Consistent with previous reports we also observed a concomitant increase of the upstream regulator p53 (E401K/E401K: P<0.0001; KO/+: P<0.0001; n=3) (Figures 4U-X and Z and S6D) (Insolera et al., 2014; Marthiens et al., 2013). To confirm the role of p53 as an apoptotic driver we crossed our Tubb5 knockout animals (KO/+) onto a p53 null background (Marino et al., 2000). This resulted in a complete rescue of the prolific apoptosis in our heterozygous knockout animals (P<0.05; n=2) (Fig. S6 E-G). We conclude that distinct defects during mitosis drive p53 dependent apoptosis in our Tubb5 mutant mouse lines leading to a smaller brain size.

Defects in mitotic progression are dependent on the dose of Tubb5

Finally, we investigated the origin of the different phenotypes in our Tubb5 mutant lines. Specifically, we sought to establish whether or not the mitotic progression defect and ectopic progenitors observed in homozygous E401K mutant animals, but absent in our heterozygous knockout animals, could be attributed to the dosage of functional Tubb5. We therefore examined the cortices of homozygous knockout animals, which did not survive into adulthood but are viable at E14.5. Aurora B, pH3 and Hoechst staining revealed a severe mitotic progression defect and abnormal chromosomal elements (Fig. 5A-H). This was accompanied by a massive increase in the number of cleaved Caspase-3 (P=0.0004; n=3) and p53 positive cells (P=0.003; n=3) (Fig. 5I-N). Interestingly, the orientation of the spindle in mitotic progenitors in homozygous knockout animals was indistinguishable from controls (Fig. S7D-G), and Sox2 staining revealed no increase in ectopic progenitors (P>0.05; n=3) (Fig. S7A-C). These data show that Tubb5 has a dose dependent effect on cell cycle progression and apoptosis.
However, the presence of ectopic progenitors in our homozygous E401K mouse indicates that this missense mutation has a more complex mode of action with subtle gain of function effects.

Discussion

In this paper we have explored the genetic and cellular mechanisms that cause TUBB5 associated microcephaly by exploiting two new mouse models: a conditional E401K knockin; and a conditional knockout animal. We report that both lines present with severe microcephaly, recapitulating the primary pathological feature observed in affected patients. Comparison of these lines with each other and littermate controls has revealed that the reduction in brain size is due to distinct mitotic defects in apical progenitors which drive p53 associated apoptosis. Our results demonstrate that the severity of phenotypes represent a continuous spectrum dependent on the dosage of functional Tubb5 (Fig. 6).

This work and a number of recent studies have contributed to our growing understanding of the cellular etiology that is responsible for microcephaly. Those genes that are known to cause this disease, which include ASPM, CDK5RAP2, WDR62, MCPH1, CEP152 and STIL, are intimately associated with the mitotic apparatus and centrosomes (Alcantara and O'Driscoll, 2014). While mouse models for these genes have not always resulted in strong phenotypes, some of these have revealed defects in asymmetric cell division (Fish et al., 2006; Gruber et al., 2011; Pulvers et al., 2010). These defects, which prematurely shift the output of progenitors to neurons, have been proposed to be a common functional link between different microcephaly causing genes (Thornton and Woods, 2009; Woods et al., 2005). This model, however, has increasing been challenged. Niswander and colleagues have recently shown that a mouse model of Wdr62 does not present with abnormal spindle orientation, but rather defects in spindle stability promote mitotic arrest and consequently cell death (Chen et al., 2014). Our conditional knockout mouse likewise presents with normal spindle orientation, but severe microcephaly due to massive apoptosis which is associated with p53 upregulation. The involvement of p53 in the pathogenesis of microcephaly has been further
highlighted by recent studies that have employed mouse models to alter the number of centrioles in radial glial progenitors (Insolera et al., 2014; Marthiens et al., 2013). These studies, along with ours, suggest that microcephaly is caused by a variety of defects during mitosis including temporal perturbation of the cell cycle, impaired spindle stability, multi-polar spindles, and aneuploidy that then engage apoptotic mechanisms through p53 induction.

In this manuscript we report the specific loss of upper layer neurons in our mouse models, whereas those in the deeper layers are largely preserved. What might be the explanation for this? This could reflect the onset of the Nestin-Cre induced recombination and the half-life of the Tubb5 protein. This Cre-line reaches its peak of recombination at E10.5 to E11.5, showing efficient recombination by 12.5 (Dubois et al., 2006; Insolera et al., 2014). However, the stability of existing Tubb5 transcript and protein may prevent any defects prior to E14.5, when deep layer neurons are generated (Greig et al., 2013). Alternatively, the preservation of deep layer neurons may reflect functional complementation by another beta tubulin isoform. The tubulin gene family contains multiple isoforms, with dynamic expression patterns (Breuss et al., 2015). It is conceivable that one of these isoforms (e.g. Tubb2b) are able to compensate for the lack of Tubb5 at earlier time points, but not at later stages. Finally, it is possible that progenitor cells exhibit distinct sensitivities to spindle perturbations during neuronal development. A specific loss of upper layer neurons following depletion of Nde1 in a full-body homozygous knockout supports this idea (Feng and Walsh, 2004).

This study has also provided insight into the genetic mechanisms by which tubulin mutations cause human disease. It is known that some tubulin mutations, including the E401K mutation, cause defects in the chaperone mediated folding pathway, impairing tubulin heterodimer formation. These data alone would suggest the mutations act by loss of function, but the absence of disease causing null alleles has led weight to the argument that all tubulin mutations act by gain of function (Hu et al., 2014; Kumar et al., 2010). This contention has been further supported by overexpression of these folding mutations in vivo by in utero electroporation which resulted in severe phenotypes (Breuss et al., 2012; Ngo et al., 2014). The characterization of our Tubb5 conditional knockout mouse has
demonstrated that null alleles can result in severe phenotypes consistent with the tubulinopathies. However, detailed analysis of the E401K mutant mouse has shown that the mode of action in the case of this missense mutations is complex. While we cannot exclude the possibility that the ectopic Sox2 positive progenitors in the E401K mouse line are a consequence of dose dependency, their absence in the knockout line suggests that this missense mutation has a subtle hypermorphic or neomorphic effect. This, however, does not seem to be the mechanism that drives microcephaly, which we attribute to apoptosis resulting from mitotic defects associated with a reduction in the pool of functional heterodimers.

Finally the data presented in the manuscript highlights the limitation of studying tubulin mutations by overexpressing disease causing mutations in cell culture and *in vivo* by electroporation. FLAG tagged variants of Tubb5 with the E401K mutation completely fail to incorporate into the microtubule cytoskeleton in Neuro2A cells, but some mutant heterodimers must fold correctly and incorporate *in vivo* in our E401K mouse. Similarly, overexpression of the E401K mutation by *in utero* electroporation revealed no induction of apoptosis, whereas the data presented in this manuscript show that cell death underpins the microcephalic phenotype in our mouse lines. It is therefore evident that if we are to understand the pathology that underlies the expanding spectrum of tubulin associated disease states *in vivo* mouse models will be vital.
Materials and Methods

Generation and maintenance of transgenic mouse lines.

Targeting constructs were generated by modifying a BAC containing the Tubb5 gene (RP24-330C1) with the Red/ET system (Gene Bridges). These were linearized and electroporated into A9 embryonic stem cells (ESCs) derived from 129/Ola and C57BL/6J hybrids (Neely et al., 2010). ESC clones resistant to G418 (Geneticin) were picked and grown before DNA was extracted by isopropanol precipitation. For Southern Blot analysis DNA was digested with EcoRI (Roche, 10200310001), run on a 0.7% agarose gel (TAE), denatured (40%w/v NaOH, 1.5 M NaCl), neutralized (0.05 M NaP₄, pH 7.2), transferred to a Porablot NY plus membrane with 20xSSC overnight (Porablot, 741240) and cross-linked the next day. The hybridization probe (Tubb5_SB_F and Tubb5_SB_R PCR product; 365bp) was radioactively labelled and purified according to the manufacturer’s protocols before incubation with hybridization solution over night at 65°C in a rolling shaker before washing and development (Rediprime II DNA Labelling System, GE, RPN1633; Illustra ProbeQuant G-50 Micro Columns, GE, 28-9034-08; Hybridization Buffer, Sigma, H7033-125ML). Successful recombination of the upstream LoxP site was confirmed by PCR analysis (Tubb5_LoxP_F and Tubb5_LoxP_R). Positive ES cell clones were injected into C57BL/6J blastocysts to generate chimeras (Gossler et al., 1986). Mice were maintained at the animal facilities at the Institute of Molecular Pathology (IMP), Vienna, under specific pathogen free (SPF) conditions on a 14:10 light:dark cycle. Mice were genotyped for presence of the transgene by PCR analysis of genomic DNA extracted from ear clips. Mice were kept in a mixed background and all experiments presented use littermate controls. Prior to experiments mice were crossed to a mouse line expressing the Flp recombinase under the control of a beta-actin promoter to remove the Neomycin resistance cassette (in house bred derivative of the B6.Cg-Tg(ACTFLPe)9205Dym/J line, Jackson laboratories). The p53 conditional knockout mice used in this study, were modified such that Exons 2-10 were flanked by LoxP sites. These mice were obtained from the Jackson Labs (Trp53tm1Brn) and maintained on a C57BL/6J background. qPCR was performed as previously described (Braun et al., 2010; Breuss et al., 2012). All procedures were
carried out accordingly to legal requirements and covered by an approved license (M58/006093/2011/14).

**Nissl staining and Immunohistochemistry**

Embryos were harvested at embryonic day (E) 14.5 and E16.5, postfixed in 4% PFA overnight, dehydrated in 30% sucrose, embedded in Neg-50 Frozen Section Medium (Richard-Allan Scientific 6502-APD), and sectioned on a cryostat (12 µm). Brains of adult animals (12-16 weeks of age) were dissected following perfusion with 0.9% NaCl followed by 4% PFA. Sections for Nissl staining were dried, washed in PBS and incubated in cresyl violet for 3 minutes (0.25% cresyl violet acetate (Sigma, C5042) in dH2O with 10 drops of glacial acetic acid added per 100 ml of solution). After a short wash in dH2O, sections were dehydrated in an alcohol series (30%, 70%, 96% and 100% ethanol; 2 minutes each) and xylol (twice; 2 minutes), where they were left until mounting with DPX mountant (Fluka, 44581). Images were acquired on a Mirax slide scanner (Zeiss). For immunohistochemistry sections were incubated with the primary antibody overnight in blocking solution supplemented with TritonX-100 and donkey serum or BSA (see Table S2). For all stainings antigen retrieval was performed with antigen unmasking solution (Vector Laboratories, H-3301). Slides were heated from room temperature to 90°C in a water bath and then placed at room temperature for 30 minutes prior to incubation with the primary antibody overnight (14-16 hours) at 4°C. The next day, sections were washed 3 times in PBS (5 minutes each), before application of a species specific secondary antibody (Molecular Probes) for 1 hour in blocking solution at 4°C. Following staining for 5 minutes with Hoechst 33342 Fluorescent Stain in PBS (1:2000) slides were mounted with Dako Fluorescent Mounting Medium (Dako, Art. Nr.: S302380).

**Magnetic Resonance Imaging and area segmentation.**

For MR analysis adult mice were perfused with 0.9% NaCl and 4% PFA supplemented with 10% ProHance Solution (Bracco Imaging Group, 4002750). MR images were acquired with a 15.2 T Biospec horizontal bore scanner (Bruker BioSpin, Ettlingen, Germany), and BFG6S-100 actively shielded gradient system (750 mT/m maximum gradient strength). A quadrature transmit/receive
volume coil (23 mm inner diameter, Bruker BioSpin) was used. A T1-weighted multi-slice multi-echo (MSME) 3D sequence was used with TR/TE 20/3.7 ms, 1.8 X 1.6 X 1.6 cm$^3$ field of view, 50 X 50 X 100 µm$^3$ spatial resolution, 4 averages. Total imaging time was 1 h 21 min. Each 3D image set was manually segmented using Amira 5.6 (Visualization Science Group). The delineation of different brain structures was performed in axial plane and subsequently controlled in the two other planes. A mouse brain atlas was used as a reference (Paxinos and Franklin, 2004). The brain surface and structures were delineated based on the MRI signal intensity differences. The Amira software package was used for volume calculation of different brain regions and a two-way ANOVA with a Bonferroni correction for multiple comparisons was employed to test significance.

**Quantifications.**

To determine the cortical thickness during development embryonic Nissl sections (E12.5, E14.5, E16.5, P0) were scanned and matched cortical regions of all genotypes were measured. For Sox2, Tbr2, pH3, p53 and cleaved Caspase 3 quantifications images were captured with an LSM710 (Zeiss) and the number or positive cells in the cortex in a field of view measuring 340x340 µm were counted. For each animal four images were quantitated and an average was obtained. In the case of pH3 positive cells this count was normalised to the length of the ventricle. For the quantification of mitotic progression and the occurrence of ectopic DNA elements images were captured with an LSM710 and cells were counted in a field of view measuring 236x236 µm covering the thickness of the entire section in z. Spindle orientation measurements were performed as described previously (Juschke et al., 2014; Postiglione et al., 2011; Xie et al., 2013). All cells assessed for our spindle orientation experiments were directly adjacent to the ventricular surface. For adult layer marker quantifications (Foxp2, Cux1, Er81) confocal images of coronal sections were acquired on an LSM710 (Zeiss) and a 200 µm wide box was positioned across the cortex. For each animal positive cells were counted on 4 images and the number averaged. To determine the relative thickness layers were measured on the same sections. All cell counting was performed blind to genotype. Statistical significance was determined by application of a two-tailed Student’s t-test or a one-way ANOVA with a Bonferroni post test for multiple comparisons with GraphPad Prism. For the cortical thickness during
Development and the relative layer thickness a two-way ANOVA with a Bonferroni post test for multiple comparisons was employed.

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Author contributions

Mice were generated by M.B., U.E., D.A.K., F.V and B.W. Experiments were performed by M.B. with help from T.F., K.C., T.G., L.U., N.Y. and M.C.S. Data analysis was done by M.B. The project was designed by M.B. and D.A.K. The manuscript was prepared by M.B. and D.A.K.
References


Fig. 1. Tubb5 perturbation causes microcephaly in the mouse. (A-D) Nissl-stained, coronal sections of the adult brain for (A) Tubb5(E401K/+), (B) Tubb5(E401K/+); Nestin-Cre, (C) Tubb5(E401K/E401K); Nestin-Cre and (D) Tubb5(fl/+); Nestin-Cre. (E-H) Axial magnetic resonance...
images (MRIs) of these genotypes. (I-J) Quantifications of total brain (I) and cortical (J) volume including a heterozygously floxed line without Cre as an additional control. (K-L) Quantifications of the cortical thickness of all five conditions during development and in adulthood for the E401K knockin (K) and the knockout (L) lines. Shown are embryonic day (E) 12.5, E14.5, E16.5, postnatal day (P) 0 and adult. Error bars in I, J, K and L show the mean ± SEM. **/##P<0.01, ***P<0.001, ****P<0.0001 (* and # in K indicate significance relative to Tubb5(E401K/+); Nestin-Cre, respectively); for the statistical analysis shown in I-J we employed a one-way ANOVA with a Bonferroni post test for multiple comparisons for the knockin and a two-tailed Student’s t-test for the knockout; for the statistical analysis shown in K-L we employed a two-way ANOVA with a Bonferroni post-test for multiple comparisons. Scale bars in D and H show 1000 µm and 2000 µm, respectively. n=3 for all conditions.
Fig. 2. Tubb5 mediated microcephaly is caused by a specific loss of upper layer neurons. (A-L) Immunostainings for cortical layers on adult (A, E, and I) Tubb5(E401K/+), (B, F, and J) Tubb5(E401K/+); Nestin-Cre, (C, G, and K) Tubb5(E401K/E401K); Nestin-Cre and (D, H, and L) Tubb5(fl/+); Nestin-Cre. Shown are the layer VI marker FoxP2 (A-D), the layer V marker Er81 (E-H) and the layers II-IV marker Cux1 (I-L). (M-O) Quantifications of the number of FoxP2 (M), Er81 (N) and Cux1 (O) positive cells in a cortical area with a 200 µm width. The laminar structure of the cortex is preserved in all genotypes, however, there is a large reduction in the number of superficial Cux1 positive neurons in homozygous E401K mutants and heterozygous knockout animals. Error bars show
the mean ± SEM. **P<0.01; for the statistical analysis shown in M-O we employed a one-way ANOVA with a Bonferroni post test for multiple comparisons for the knockin and a two-tailed Student’s t-test for the knockout. Scale bar in L shows 100 µm. n=3 for all conditions.
Fig. 3. Ectopic progenitors and an increased mitotic index in the homozygous E401K mutant mouse. (A-P) Immunostainings of the embryonic cortex (E14.5) for (A-D) Sox2 positive radial glial cells, (E-H) Tbr2 positive intermediate progenitors and (I-L) pH3 positive mitotic cells for (A, E, and I) Tubb5(E401K/+), (B, F, and J) Tubb5(E401K/+); Nestin-Cre, (C, G, and K) Tubb5(E401K/E401K); Nestin-Cre and (D, H, and L) Tubb5(fl/+); Nestin-Cre mice. Arrowheads in C
and G indicate ectopic cells positive for the respective markers. (M-O) Quantifications of the number of ectopic Sox2 positive cells (M), the number of pH3 positive cells (N) and the number of abventricular pH3 positive cells (O) for each genotype. The number of pH3 positive was normalized to the length of the ventricular surface. (P) Representative 3D reconstruction of a mitotic cell stained for pH3 (red), γ-tubulin (green), N-Cadherin (green) and DNA (Hoechst, blue). Measurement of the spindle orientation employed a line drawn between the centrosomes marked by γ-tubulin and a plane defined by 5 points on the N-Cadherin marked surface. (Q) Quantification of spindle angle measurements for all five genotypes. Note the significant increase of the mean spindle angle in the homozygous knockin. Error bars in M-O and Q show the mean ± SEM. *P<0.05, **P<0.01; for the statistical analysis shown in M-O and Q we employed a one-way ANOVA with a Bonferroni post test for multiple comparisons for the knockin and a two-tailed Student’s t-test for the knockout. Scale bars in L and P show 50 µm and 5 µm, respectively. n=3 for all conditions except for Q (n=4 animals, n≥76 mitotic cells per condition).
Fig. 4. Defects in mitotic progression and p53 associated massive apoptosis in Tubb5 mutant mice. (A-C) Immunostainings for AuroraB and pH3 for (A) Tubb5(E401K/+), (B) Tubb5(E401K/+);
Nestin-Cre, (C) Tubb5(E401K/E401K); Nestin-Cre. Arrowheads indicate cells that were scored to be in metaphase. (D-F) Representative images for cells in pro-metaphase, metaphase and ana-/telophase. (G-N) Immunostainings for pH3 and Hoechst nuclear staining for the heterozygous knockout line. H, J, L and N show grayscale images of the Hoechst staining for G, I, K and M, respectively. Arrowheads in H, J, L and N show ectopic DNA elements, a mitotic chromosome bridge in the case of H. The arrowhead in M indicates a pH3 positive bridge connecting the ectopic DNA element to the nucleus. (O) Quantification of the ratio of metaphase and ana-/telophase cells for all genotypes for all mitotic cells in the cortex. (P) Quantification of the appearance of ectopic DNA elements at the ventricular surface. (Q-X) Immunostainings of the embryonic cortex (E14.5) for the apoptotic marker cleaved Caspase-3 (Q-T) and the apoptosis regulator p53 (U-X). (Y-Z) Quantification of the number of cleaved Caspase-3 positive cells (Y) and the number of p53 positive cells (Z) per cortical image. Both, the homozygous knockin and the heterozygous knockout, show a significant increase in the number of cleaved Caspase-3 and p53 positive cells. Error bars in O, P, Y and Z show the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; for the statistical analysis shown in O-P and Y-Z we employed a one-way ANOVA with a Bonferroni post test for multiple comparisons for the knockin and a two-tailed Student’s t-test for the knockout. Scale bars show 20 µm in C, 5 µm in F and G and 50 µm in X. n=3 for all conditions.
Fig. 5. Homozygous knockout of Tubb5 induces mitotic progression defects, ectopic DNA elements and more severe cell death. (A-B) Immunostainings for AuroraB and pH3 for the
homozygous knockout (Tubb5(fl/fl); Nestin-Cre) and a littermate control (Tubb5(fl/fl)). Arrowheads indicate cells that were scored to be in metaphase. (C-F) Immunostainings for pH3 and Hoechst nuclear staining for the homozygous knockout line. D and F show grayscale images of the Hoechst staining for C and E, respectively. The arrowheads in D and F show ectopic DNA elements (mitotic chromosome bridge in the case of F). Arrowhead in C indicates pH3 signal on the ectopic DNA element. (G-H) Quantification of the ratio of metaphase and ana-/telophase cells (G) and the appearance of ectopic DNA elements at the ventricular surface (H) for the homozygous knockout line. (I-L) Immunostainings for the apoptotic marker cleaved Caspase3 (I-J) and the apoptosis regulator p53 (K-L) for the homozygous knockout (Tubb5(fl/fl); Nestin-Cre) and littermate controls (Tubb5(fl/fl)). (M-N) Quantification of the number of cleaved Caspase3 positive cells (M) and the number of p53 positive cells (N) found per cortical image. The homozygous knockout shows a significant increase in the number of Casp3 and p53 positive cells. Error bars in (m-o) show ± SEM. **P<0.01, ***P<0.001; for the statistical analysis shown in G-H and M-N we employed a two-tailed Student’s t-test. Scale bars show 20 µm in B and L, 5 µm in C and 50 µm in J. n=3 for all conditions.
Fig. 6. A model for Tubb5 perturbation in murine cortical progenitors. (A) Upon depletion of functional Tubb5 by 50% ectopic DNA elements appear that are not sufficient to arrest the cell cycle.
but nonetheless activate the p53 pathway and induce apoptosis. If functional levels of Tubb5 are below 50% this results in an outright mitotic progression defect, upregulation of p53 and apoptosis. The schematic also depicts some of the known microcephaly genes associated with the centrosome (Aspm, Cdk5Rap2, Wdr62, Cep152 and Stil). (B) Our data suggest that the E401K mutation also has a gain of function or neomorphic affect on the mitotic spindle. Whereas heterozygous and homozygous Tubb5 knockout animals exhibit no defects in spindle orientation, in our homozygous E401K animals we observed a higher percent of vertical orientations in comparison to control mice. We hypothesise that this defect is responsible for the increase in ectopic progenitors we observe in this mouse line.