Mutations in KATNB1 Cause Complex Cerebral Malformations by Disrupting Asymmetrically Dividing Neural Progenitors

Ketu Mishra-Gorur,1,2,3,4,19 Ahmet Okay Çağlayan,1,2,3,4,19 Ashleigh E. Schaffer,5 Chiswili Chabu,2,6 Octavian Henegariu,1,2,3,4 Fernando Vonhoff,7 Gözde Tuğçe Akgümüş,1,2,3,4 Sayoko Nishimura,1,4 Wenqi Han,3,8 Shu Tu,9 Burçin Baran,1,2,3,4 Hakan Gümüş,10 Cengiz Dilber,11 Maha S. Zaki,12 Heba A.A. Hossni,13 Jean-Baptiste Rivière,14 Hülya Kayserili,15 Emily G. Spencer,5 Rasim Ö. Rosti,6 Jana Schroth,8 Hüseyin Per,10 Caner Çağlar,1,2,3,4 Çağrı Çağlar,1,2,3,4 Duygu Dölen,1,2,3,4 Jacob F. Baranoski,1,2,3,4 Sefer Kumandas,10 Frank J. Minja,16 E. Zeynep Ersön-Onay,1,2,3,4 Şhrkant M. Mane,2,17 Richard P. Lifton,2,6 Tian Xu,2,6 Haig Keshishian,7 William B. Dobyns,18 Neil C. Chi,9 Nenad Sestan,3,4,8 Angeliki Louvi,1,3,4 Kaya Bilgüvär,2,17 Katsuhito Yasuno,1,2,3,4 Joseph G. Gleeson,5,* and Murat Günel1,2,3,4,*

1Department of Neurosurgery
2Department of Genetics
3Department of Neurobiology
4Yale Program on Neurogenetics
Yale School of Medicine, New Haven, CT 06510, USA
5Neurogenetics Laboratory, Department of Neurosciences, Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA 92039, USA
6Howard Hughes Medical Institute, Yale School of Medicine, New Haven, CT 06510, USA
7Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06511, USA
8Kavli Institute for Neuroscience, Yale School of Medicine, New Haven, CT 06510, USA
9Department of Medicine, University of California, San Diego, La Jolla, CA 92039, USA
10Division of Pediatric Neurology, Department of Pediatrics, Erciyes University Medical Faculty, Kayseri 38039, Turkey
11Division of Pediatric Neurology, Department of Pediatrics, Sütçü Imam University Medical Faculty, Kahramanmaraş 46100, Turkey
12Clinical Genetics Department, Human Genetics and Genome Research Division, National Research Center, Cairo 12311, Egypt
13Department of Neurology, National Institute of Neuromotor System, Cairo 12311, Egypt
14Equipe Génétique des Anomalies du Développement, EA 4271, Université de Bourgogne, 21078 Dijon, France
15Department of Medical Genetics, Istanbul Medical Faculty, Istanbul University, Istanbul 34093, Turkey
16Department of Radiology
17Yale Center for Genome Analysis
Yale School of Medicine, New Haven, CT 06510, USA
18Departments of Pediatrics and Neurology, University of Washington and Center for Integrative Brain Research, Seattle Children’s Research Institute, Seattle, Washington 98105, USA
19Co-first author
*Correspondence: jogleeson@rockefeller.edu (J.G.G.), murat.gunel@yale.edu (M.G.)
http://dx.doi.org/10.1016/j.neuron.2014.12.014

SUMMARY

Exome sequencing analysis of over 2,000 children with complex malformations of cortical development identified five independent (four homozygous and one compound heterozygous) deleterious mutations in KATNB1, encoding the regulatory subunit of the microtubule-severing enzyme Katanin. Mitotic spindle formation is defective in patient-derived fibroblasts, a consequence of disrupted interactions of mutant KATNB1 with KATNA1, the catalytic subunit of Katanin, and other microtubule-associated proteins. Loss of KATNB1 orthologs in zebrafish (katnb1) and flies (kat80) results in microcephaly, recapitulating the human phenotype. In the developing Drosophila optic lobe, kat80 loss specifically affects the asymmetrically dividing neuroblasts, which display supernumerary centrosomes and spindle abnormalities during mitosis, leading to cell cycle progression delays and reduced cell numbers. Furthermore, kat80 depletion results in dendritic arborization defects in sensory and motor neurons, affecting neural architecture. Taken together, we provide insight into the mechanisms by which KATNB1 mutations cause human cerebral cortical malformations, demonstrating its fundamental role during brain development.

INTRODUCTION

A mechanistic understanding of human brain development has only recently begun to be elaborated at the gene level, with the discovery of disease-causing mutations in monogenic forms of malformations of cerebral cortical development (MCDs). MCD syndromes have traditionally been classified on the basis of...
imaging findings that correlate with disturbances at distinct phases of cortical development, including proliferation of neural progenitors (e.g., leading to genetic forms of microcephaly), neuronal migration (e.g., pachygyria, lissencephaly, subcortical and periventricular heterotopias), and postmigratory development and organization (e.g., schizencephaly, polymicrogyria) (Barkovich et al., 2012). Phenotypic overlap between these MCD disorders is commonly observed, with a single gene mutation leading to multiple cortical abnormalities, suggesting that diverse cerebral malformations can have a unified underlying causation (Bilgüvar et al., 2010).

Genetic studies have also highlighted significant heterogeneity in the molecular pathways underlying MCDs, with the possible exception of autosomal recessive primary microcephaly (MCPH), which is associated with a plethora of genes (e.g., ASPM, CDK5RAP2, CASC5, CENPJ, CEP63, CEP135, CEP152, STIL, and WDR62) that encode proteins involved in cytoskeletal control of the mitotic apparatus, including centrosomes and mitotic spindle poles (Bettencourt-Dias et al., 2011; Bilgüvar et al., 2010; Kaidi et al., 2010; Thornton and Woods, 2009).

Despite dramatic differences in brain size and complexity, animal models have proven invaluable in elucidating the biology of MCDs, for example, by confirming the importance of centrosome in microcephaly (Kaidi et al., 2010). In Drosophila, homozygous loss of either asp (abnormal spindle, ortholog of human ASPM, mutated in MCPH5, OMIM#608716) or cnn (centrosomin, ortholog of human CDK5RAP2, mutated in MCPH3, OMIM#604804) affects asymmetric cell division during development (Bond et al., 2005; Wakefield et al., 2001). Similarly, both mouse and zebrafish models of human SCL/TAL1-interrupting locus gene (STIL), mutated in MCPH7 (OMIM#612703), have shown that STIL plays a role in centrosome duplication and function and mitotic spindle organization and signaling (Izraeli et al., 1999; Pfaff et al., 2007).

The centrosome functions as the primary microtubule-organizing center of the cell, and in humans, mutations in microtubule-associated proteins (DCX, LIS1, NDE1) (Akurraya et al., 2011; Bakircioğlu et al., 2011; Gleeson et al., 1998; Reiner et al., 1993) or tubulin isoforms (TUBA1A, TUBA8, TUBB2B, and TUBB3) (Abdollahi et al., 2009; Jaglin et al., 2009; Kumar et al., 2010; Tischfield et al., 2010) also underlie defects in cellular proliferation, neuronal migration, and cortical organization. Proper functioning of microtubules is in turn dependent on the tight control of their length, number, as well as cargo movement (Shu et al., 2004; Tanaka et al., 2004).

A concerted action of polymerizing and severing enzymes regulates microtubule length. Indeed, mutations in SPAST, encoding the microtubule-severing enzyme spastin, result in progressive axonal degeneration and autosomal dominant spastic paraplegia (SPG4, OMIM#182601), thus linking microtubule remodeling to neurodegeneration (Hazan et al., 1999). Katanin, the only other well-characterized microtubule-severing enzyme, composed of a catalytic, p60 (KATNA1), and a regulatory, p80 (KATNB1), subunit, acts by disrupting contacts within the polymer lattice (McNally and Vale, 1993). In developing neurons, Katanin localizes to microtubules and centrosomes and is essential for microtubule shortening and release (Ahmad et al., 1999). Katanin functions in cell division (McNally et al., 2006; Zhang et al., 2007), neuronal morphogenesis (Karabay et al., 2004; Yu et al., 2008), and assembly and disassembly of cilia and flagella (Casanova et al., 2009; Sharma et al., 2007).

p60/KATNA1 is a member of the AAA (ATPases Associated with diverse cellular Activities) domain containing protein family, whereas p80/KATNB1 binds to p60 and targets it to subcellular structures including the centrosome, further mediating its interactions with Dynemin, LIS1, and NDEL1 (Hartman et al., 1998; McNally et al., 2000). A missense mutation in the highly conserved WD40 domain of Katanin has been shown to cause azoospermia and male sterility in mice (O’Donnell et al., 2012).

Here, by studying patients with MCDs, we identify deleterious mutations in KATNB1 that result in a spectrum of MCD disorders, including microcephaly co-occurring with lissencephaly or less severe neuronal migration abnormalities such as periventricular or subcortical heterotopias. Knockdown of KATNB1 orthologs in zebrafish (Danio rerio; katanb1) and Drosophila (kat80) results in a small brain, recapitulating the human phenotype. Further, in Drosophila, kat80 is essential for the formation of the mitotic spindle, and its loss results in supernumerary centrosomes and delayed anaphase onset (AO), preferentially affecting asymmetrically dividing neuroblasts (NBs) in vivo. Lastly, kat80 predominantly regulates neuronal dendritic arborization. Taken together, these findings demonstrate a fundamental role of KATNB1 in human cerebral cortical development and pathology.

RESULTS
Whole-Exome Sequencing Identifies Recessive Mutations in KATNB1 in Patients with Malformations of Cortical Development

We performed whole-exome capture and next-generation sequencing of germline DNA of over 2,000 children, who were mainly products of consanguineous unions. In Family 1 (NG-961), the two affected siblings (kinship coefficient 0.23; Table S1) displayed cognitive delay and seizures (Table S1). Physical exam revealed microcephaly, with MRI demonstrating subcortical heterotopia (Figure 1A; Table S1 available online). Exome sequencing (Table S1) identified two homozygous predicted deleterious missense variants; one was previously reported and affected the glucosaminyl (N-acetyl) transferase 2 gene (encoding a blood group II antigen) (p.Glu298Lys, rs139794913) resulting in cataracts, a phenotype not seen in our patients. The other, a p.Ser535Leu mutation, was novel and affected the KATNB1 gene (Table S1).

Family 2 (NG-LIS-711), with a history of three early-pregnancy losses, also had two affected members (kinship coefficient 0.12; Table S1) that exhibited severe cognitive delay and autistic features (Table S1). MRI scans confirmed microcephaly and revealed severe simplified gyral pattern and corpus callosum abnormality (Figure 1B; Table S1). Exome sequencing (Table S1) detected only two homozygous variants, one affected the metallothionein–4 gene (p.TyrTrp300CysArg), while the other was a KATNB1 predicted deleterious missense mutation (p.Leu540Arg) (Table S1).

In Family 3, a 21-month-old female (NG-MIC-2584) (who also had a sister that died 25 days after birth) presented to medical attention with jaundice, respiratory distress, and severe delay in motor and mental development and was found to be
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Microcephaly (Table S1). MRI demonstrated microlissencephaly and grossly dilated ventricles (Figure 1C). Exome sequencing revealed a homozygous frameshift mutation (p.Val150Cysfs*22) in KATNB1.

In Family 4, the patient (NG-MIC-1218-4) exhibited mild cognitive delay (Table S1). Her MRI was mainly remarkable for microcephaly (Figure 1D). Exome sequencing of the patient and her unaffected sibling (kinship coefficient of 0.23; Table S1) identified 21 homozygous variants observed only in the patient. Twelve of these variants were novel, including a predicted deleterious missense mutation (p.Val45Ile) in KATNB1.

Prompted by the identification of multiple independent homozygous mutations in KATNB1, we then searched our exome sequencing databases for any potential compound heterozygous patients and identified a fifth patient (NG-PNH 226). This was a single affected offspring of a nonconsanguineous union whose clinical and radiologic findings have previously been reported (Wieck et al., 2005). She displayed severe cognitive and motor developmental delay and advanced microcephaly; MRI confirmed the microcephaly and revealed partial microgyria and polymicrogyria (see also Figure S1; Table S1).

**Figure 1. KATNB1 Mutations in MCD Patients**

(A) Kindred NG-961. Pedigree structure depicting a first cousin consanguineous union is shown on the left. Coronal T2-weighted images (left) and axial T1-weighted images (right) show symmetric nodular gray matter heterotopia in the bilateral corona radiata, indicated with white arrows, in both affected siblings. Sanger sequencing confirmation of the p.Ser535Leu mutation in KATNB1 is shown at the bottom.

(B) Kindred NG-LIS-711. Complex pedigree structure is shown at left. Axial (upper) and sagittal (lower) T2-weighted images reveal a microlissencephalic brain with grossly dilated ventricles. Sanger sequencing confirmation of the p.Val150Cysfs*22 homozygous mutation is shown at the bottom.

(C) Kindred NG-MIC-2584. Pedigree structure demonstrating a first cousin consanguineous union with two affected children (one deceased) is shown on the left. Axial (upper) and sagittal (lower) T2-weighted images reveal a microlissencephalic brain with grossly dilated ventricles. Sanger sequencing confirmation of the p.Val150Cysfs*22 homozygous mutation is shown at the bottom.

(D) Kindred NG-MIC-1218. Pedigree structure (top left) and axial T1- (upper) and sagittal T2- (lower) weighted images revealing a microcephalic brain with grossly normal architecture. The patient is homozygous for the p.Val45Ile mutation (bottom).

(E) Exon-intron structure of KATNB1 is shown. Solid bars on top indicate the functional interaction domains and their localization to the KATNB1 protein. The location of each mutation and the associated phenotype are noted. MCP, microcephaly; Het, heterotopia; PaGY, pachygyria; PMG, polymicrogyria (see also Figure S1; Table S1).
classic pachygyria (lissencephaly) and polymicrogyria, but resembles the cortical malformation seen in other severe congenital microcephaly syndromes, especially NDE1 (Alkuraya et al., 2011; Bakircioglu et al., 2011). At the genetic level, exome sequencing of these patients revealed all to harbor rare homozygous or compound heterozygous LOF, splice site, or predicted deleterious missense KATNB1 mutations, including p.Val45Ileu, p.Val150Cysfs*22, p.Ser535Leu, p.Leu540Arg, and IVS9-1G > A;p.Gly578Asp (Figure 1; Table S1). All mutations were confirmed by Sanger sequencing and segregated as expected, with both parents of the affected children being heterozygous for the respective mutations. None of the mutations were observed in the public databases or among the 3,000 exomes sequenced at Yale including 1,460 ethnically matched control chromosomes. These findings provide conclusive evidence that mutations in KATNB1 are the underlying genetic cause of the observed MCD phenotypes.

Mutations in KATNB1 Disrupt Mitotic Spindle Architecture

To study the functional consequences of the KATNB1 mutations at the cellular level, we used dermal fibroblast cultures established from skin biopsies of two patients (siblings NG-961-1 and NG-961-4, carrying the p.Ser535Leu mutation) and an unaffected heterozygous parent (NG-961-2). Western analysis revealed reduced localization of KATNB1 (G–I), NDE1 (J–L), and KATNA1 (M–O) to the mitotic spindle and increased number of centrosomes (arrow) as seen by staining for β-tubulin (P–R). Panels marked with a prime (′) show merged images of primary antigen and DAPI (blue) staining (Q–R′). Consistent with the observations in patient fibroblasts, transfection of HeLa cells with wild-type and mutant forms of KATNB1 results in reduced localization of mutant form of KATNB1 (green) to centrosomes and abnormal spindle formation (tubulin staining; red) in anaphase cells (S–T'). The specific KATNB1 mutation assayed/investigated is indicated at the top of the panel. Coimmunoprecipitation of wild-type and mutant forms of KATNB1 with KATNA1 (U) and NDE1 (V) shows reduced interaction of mutant KATNB1 with both proteins. Scale bars, 5 μm (A–C and P–R), 1 μm (D–O, S, and T). All confocal images were captured using identical settings (see also Figure S2).

**Figure 2. C-Terminal Mutant Forms of KATNB1 Disrupt the Mitotic Spindle and Display Reduced Interaction with NDEL1 and KATNA1**

(A–C) As evidenced by β-tubulin staining, microtubule architecture of the interphase dermal fibroblasts, derived from patients and their parents, is intact. However, the mitotic spindle is significantly disrupted and malformed in patient-derived cells in anaphase (D–F). Patient fibroblasts also show reduced localization of KATNB1 (G–I), NDE1 (J–L), and KATNA1 (M–O) to the mitotic spindle and increased number of centrosomes (arrow) as seen by staining for β-tubulin (P–R). Panels marked with a prime (′) show merged images of primary antigen and DAPI (blue) staining (Q–R′). Consistent with the observations in patient fibroblasts, transfection of HeLa cells with wild-type and mutant forms of KATNB1 results in reduced localization of mutant form of KATNB1 (green) to centrosomes and abnormal spindle formation (tubulin staining; red) in anaphase cells (S–T'). The specific KATNB1 mutation assayed/investigated is indicated at the top of the panel. Coimmunoprecipitation of wild-type and mutant forms of KATNB1 with KATNA1 (U) and NDE1 (V) shows reduced interaction of mutant KATNB1 with both proteins. Scale bars, 5 μm (A–C and P–R), 1 μm (D–O, S, and T). All confocal images were captured using identical settings (see also Figure S2).
NDEL1 and KATNA1 localization in mitotic cells and found both to be reduced in the mitotic spindle (Figures 2 J–2L and 2M–2O, respectively). Also, patient-derived fibroblasts displayed aberrant number of centrosomes (Figures 2 P–2R). Patient fibroblasts also show reduced localization of KATNB1 (G and H), LIS1 (I and J), and Dynein (K–N) to the mitotic spindle and spindle poles. Panels marked with a prime (’) show merged images of primary antibody and DAPI (blue) staining (A–N). All confocal images were captured using identical settings.

We also assessed the effect of N-terminal domain mutation in dermal fibroblast cultures from the index case of family NG-2584 (carrying the p.Val150Cysfs*22 mutation) and an unaffected parent. As with the p.Ser535Leu mutant fibroblasts, we failed to detect an impact of the mutation on microtubule architecture in interphase cells, but mitotic cells displayed spindle morphology defects (Figures 3C–3D’) and supernumerary centrosomes (Figures 3C–3D’). In mitotic cells, spindle pole localization of KATNB1 was strikingly affected (Figures 3G–3H’), while that of LIS1 was slightly reduced (Figures 3I–3J’). Similarly, Dynein levels at the spindle poles and the spindle proper were dramatically reduced (Figures 3K–3N’).

**KATNB1 Expression in the Developing Brain**

The above findings demonstrate that KATNB1 mutations impact the overall spindle dynamics and integrity by affecting the assembly of the NDEL1/KATNA1/Dynein/LIS1 complex at the spindle poles and microtubules. To understand how these mutations lead to severe cortical abnormalities in humans, we next investigated KATNB1 expression in the developing human brain by interrogating the Human Brain Transcriptome Database (Kang et al., 2011) and found it to be stably expressed throughout fetal development, starting shortly after conception (Figure 4 A). Expression levels remained elevated into infancy particularly in neocortex, hippocampus, and striatum, with high levels still detected in the adult brain. The high levels of expression and localization in neural progenitor cells and postmitotic neurons during early development suggest a continuing role of KATNB1 in neuronal proliferation, migration, and laminar organization of the human cortex.

We then investigated KATNB1 expression in the developing mouse and zebrafish brains. In mouse, Katnb1 was initially expressed in neural progenitors until midneurogenesis, and subsequently in postmitotic neurons in the cortical plate; in postnatal brain, Katnb1 was expressed widely (Figures 4B–4F). Similarly,
Katnb1 was also highly expressed in the developing zebrafish brain. During early developmental stages, katnb1 mRNA expression was ubiquitous throughout the embryo, including the cephalic region. As the embryo develops further, katnb1 mRNA expression profile became more restricted (Figures 4G–4K).

**Loss of KATNB1 Orthologs in Zebrafish and Drosophila Results in Microcephaly**

Based on the finding of diffuse katnb1 expression in the developing zebrafish brain, we initially used this model organism to study KATNB1 function. Knocking down katnb1, the single ortholog, by morpholino injection resulted in a significant reduction of the midbrain size (p = 9.16 x 10-7) (Figures 5A–5E), recapitulating the major phenotypic finding in humans.

Next, to gain a detailed mechanistic insight into the biology of KATNB1, we extended our studies to Drosophila, a model that has been successfully implemented to study human MCD-associated genes. kat80 (the single fly ortholog of human KATNB1; Goldstein and Gunawardena, 2000) has been shown to be ubiquitously expressed in both embryonic and larval stages (Chintapalli et al., 2007; Frise et al., 2010). To examine the potential role of kat80 in regulating brain size, we employed the GAL4/UAS system (Xu and Rubin, 1993). We used Prospero-GAL4 to drive expression of kat80 RNAi (kat80-IR) in neural progenitor cells.
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(NBs) and their newly born progeny (ganglion mother cells), which constitute the majority of cells in the developing larval brain (Figure 5H) (Isshiki et al., 2001). kat80-IR resulted in markedly reduced brain size (microcephaly) of third instar larvae as compared with controls (Figures 5I and 5J) with a concomitant reduction in the number of differentiated cells (1,080 ± 110 versus 673 ± 116 in WT [yw] and kat80-IR flies, respectively; p = 0.034). Approximately 30% of the kat80-IR brains were reduced to one-tenth of normal size (Figure 5J).

**kat80 Loss in Drosophila Results in Mitotic Spindle Abnormalities, Delay in AO, and Mitotic Failure**

The above findings demonstrate that KATNB1 regulates neurogenesis in both vertebrates and invertebrates, but do not reveal the underlying molecular mechanism. As mutations affecting NB numbers are also known to impact brain size (Lee et al., 2006), we next examined whether kat80-IR-expressing larval brains had fewer cells. Larval central brain NBs are specified during embryogenesis, then enter quiescence, progressively exiting during larval life to reach a total number of ~100 per brain lobe at third instar. NBs can be identified by their large size and expression of molecular markers, including the cell polarity protein Miranda (Mir) and transcription factor Worniu (Wor) (Ashraf et al., 2004; Lai et al., 2012). We used Wor-GAL4 to express kat80-IR and GFP:Mira to identify NBs and scored the number of Wor/Mir-positive cells. We found that third instar kat80-IR larval brains had on average ~70 NBs per lobe as compared to ~100 in WT brains (Figures 5G, 5K, and 5L; p < 0.002), indicating that kat80 regulated brain size at least partly by controlling the NB number, which could be due to either excessive cell death or reduced cell proliferation. TUNEL staining showed no apparent ectopic cell death (Figures S3A–S3D) in kat80-IR.
clones, indicating that, at least in the third instar larvae, kat80 knockdown affects brain size independently of cell death.

Given the impact of KATNB1 loss on mitotic spindle (Figures 2D–2F and 3C–3D), we postulated that in kat80-IR brains the remaining ~70 NBs could have cell cycle progression defects, further affecting the production of NB progeny and leading to microcephaly. Hence, we examined cell cycle progression in NBs using time-lapse imaging, and scored the time elapsed between nuclear envelope breakdown (NEBD; determined by the initial detection of microtubules in the center of the cell) and AO (defined by the first sign of separation of sister chromatids), as previously described (Siller et al., 2005) (Figures 6A–6C and 6J). We made use of Worniu-Gal4 to drive GFP::Miranda in order to mark the NBs and the microtubule-binding protein Zeus::mCherry to label the mitotic spindle. The NEBD-AO interval was significantly elongated in kat80-IR NBs as compared with controls (17.86 min ± 3.59 min versus 10.33 min ± 0.82 min, respectively; p = 0.0075), with AO extending over 2 hr in ~13% of NBs.

Figure 6. kat80-IR Delays Anaphase Onset in Drosophila Central Brain Neuroblasts, Causing a Reduction in Their Numbers (A–C) kat80-IR was expressed under worniu-Gal4, UAS-mir::GFP, UAS-zeus::mCherry. Thirty NBs from worniu>kat80-IR and six NBs from worniu>gal4 third instar larval brains were used for time-lapse imaging. Wild-type NBs exhibit anaphase onset at ~10.33 ± 0.82 min after nuclear envelope breakdown. kat80-IR leads to increase in anaphase-onset time with an average of about 17.9 ± 3.59 min (error bars indicate SD; two-tailed t test, p = 0.008). In addition, four NBs failed to display anaphase onset even after 2 hr of imaging (C). Snapshots of live imaging of third instar larval brains expressing kat80-IR under worniu > Gal4, mir-GFP, zeus-mcherry. A wild-type (D–F) and a kat80-IR (G–I) NB undergoing division are shown. kat80-IR expression results in multiple centrosomes (asterisks in G and H) and multipolar and barrel-shaped spindles (arrowhead in I). (J) Quantification of time to anaphase onset of 30 kat80-IR NBs compared with wild-type (yw) cells reveals significant delay in mutant NBs.
(n = 30) of kat80-IR NBs, indicating that kat80 knockdown significantly delayed NB cell cycle progression.

Since patient-derived fibroblasts showed spindle and centrosome defects (Figures 2D–2F, 2P–2R, 3C, 3D, 3M, and 3N), we also examined kat80-IR NBs for similar abnormalities. We observed supernumerary centrosomes and multipolar and/or barrel-shaped spindles (Figures 6D–6I), suggesting that kat80 regulates Drosophila brain size in vivo by controlling both number as well as cell cycle progression of NBs.

**Differential Effects of kat80 Loss in the Optic Lobe**

Unlike the embryonically derived central brain NBs, those in the optic lobe are specified from neural epithelium (NE), which expands during larval life by undergoing symmetric divisions in the epithelial plane (Figure 5H). In contrast, optic lobe NB specification is accompanied by a coordinated switch from symmetric to asymmetric division (Figure 5E), a 90° rotation of the division axis, and expression of specific molecular markers (Egger et al., 2007, 2011; Homem and Knoblich, 2012). Thus, the optic lobe is ideal to study symmetrically versus asymmetrically dividing cells. We used GH146-Gal4 to drive kat80-IR expression in the outer and inner proliferation centers of the optic lobe NE (Berdnik et al., 2008). Overall, no defects in NE morphology or spindle orientation were detected (Figures 7A–7E), indicating that kat80 does not regulate spindle integrity or cell cycle progression of symmetrically dividing NE cells. In sharp contrast, but similar to our observations in the central brain, the number of mitotic NBs (Mir-positive) was significantly reduced in the kat80-IR-expressing larval brains compared with controls (Figures 7F–7G’ and 7J–7L). In addition, kat80-IR brains showed supernumerary centrosomes (average number/cell = 3) and twice as many cells in metaphase (57% ± 7% versus 26.9% ± 1.5% in kat80-IR versus WT [yw], respectively) (Figures 7H–7I and 7M). These observations strongly suggested that kat80 loss specifically affects asymmetrically dividing neural progenitor cells, at least in the Drosophila optic lobe.

**Kat80 Loss Impacts on Dendritic Arborization**

Finally, previous reports demonstrated changes in neuronal architecture and dendritic arborization not only in MCD patients (Barak et al., 2011; Kaindl et al., 2010), but also in other intellectual disability and related syndromes, including the Rett syndrome (Armstrong, 2005). Therefore, we studied potential neuronal structural abnormalities of the differentiated neurons in the kat80-IR Drosophila larvae. We examined the dendritic arborization (da) sensory neurons, which innervate the overlying larval epidermis and fall into four categories based on dendritic branching pattern and complexity (Grueber et al., 2007). Due to their accessibility and stereotyped morphology, da neurons serve as a model for dendritic growth, maintenance, and tiling (Jan and Jan, 2010). We used pickpocket-Ga4 (ppk-Ga4) (Grueber et al., 2007) to drive kat80-IR expression in class IV da neurons, which are characterized by highly branched dendritic trees. In kat80-IR Drosophila larvae, dendritic arborization was dramatically reduced (Figures 8A and 8B), and the total number of dendritic termini was significantly diminished (Figure 8E; p < 0.01), suggesting that dendritic extension and number in peripheral neurons were compromised by kat80 loss.

We next extended our observations to the Drosophila CNS by examining dendritic arborization of adult flight motorneurons (MN1–5) that innervate the dorsal longitudinal flight muscle (Consoulas et al., 2002; Ikeda and Koening, 1988). Among all singly identifiable flight motorneurons, MNS serves as a paradigm of dendritic architecture (Consoulas et al., 2002; Vonhoff and Duch, 2010). Using D42-Gal4 to express kat80-IR primarily in adult flight motorneurons (Vonhoff and Duch, 2010) resulted in reduced dendritic arborization of MN5 (Figures 8C and 8D). Because dendritic defects in flight motorneurons alter flight performance (Vonhoff et al., 2012), we tested flight ability and found a significantly reduced flight response in adult D42 > kat80-IR versus WT flies (Figure 8F). Innervation of flight muscles was normal, as confirmed by imaging of MN axons and their targeting into the neuromuscular junction (NMJ) (Figures S5A and S5B). Further, although the number of boutons was not altered in the NMJ of D42 > katp80-IR larvae, their diameter was significantly larger (Figures S5C–S5H), suggesting defective axonal transport. Finally, we did not detect any significant changes in axonal structure (data not shown). Taken together, these observations suggested that kat80 regulates dendritic arborization of sensory and motor neurons in Drosophila.

**DISCUSSION**

Here, we report the identification of four homozygous deleterious mutations and one compound heterozygous deleterious mutation in KATNB1 in multiple independent patients with pleomorphic cerebral cortical phenotypes of varying severity, consisting primarily of microcerebriformies, in which microcephaly co-occurred with neuronal migration abnormalities, ranging from white matter nodular heterotopia to lobar or global pachygyria, as well as cortical organization problems, including polymicrogyria.

KATNB1 encodes the p80 regulatory subunit of the microtubule-severing enzyme Katanin. A subset of the identified mutations (p.Leu540Arg, p.Ser535Leu, and p.Gly578Asp) localize to the N-terminal region, which interacts with the molecular motor protein Dynein and LIS1 (O’Donnell et al., 2012; Toyo-Oka et al., 2005). Patient-derived dermal fibroblasts displayed disorganized mitotic spindles and expressed lower amounts of KATNB1, similar to findings in the Taily mouse, which carries a hypomorphic allele of p80 (O’Donnell et al., 2012). The disease-causing mutations affect the interaction of KATNB1 with NDEL1 and KATNA1, disrupting their efficient localization to the centrosome and to the mitotic spindle during division. This is consistent with previous findings demonstrating that NDEL1 is required for Katanin localization to the centrosome during cell division (Toyo-Oka et al., 2005), suggesting that KATNB1, KATNA1, and NDEL1 are interdependent for their respective localization to the centrosomes. Similarly, fibroblasts harboring a N-terminal mutation in KATNB1 display spindle defects and a significant reduction in the amount of Dynein localizing to the spindle and centrosomes. Thus, KATNB1 mutations result not only in decreased KATNB1 protein levels, but also...
reduced localization of the Katanin complex and other effector molecules to target areas, causing mitotic spindle defects. To gain mechanistic understanding into KATNB1 function, we used *Drosophila*, a model system that has provided invaluable insight into the mechanism of action of genes involved in human MCD syndromes (Gonzalez et al., 1990; Liu et al., 2000; Rujano et al., 2013; Saunders et al., 1997; Siller and Doe, 2008; Yamamoto et al., 2014). Loss of the KATNB1 ortholog results in microcephaly in both *Drosophila* and zebrafish, recapitulating the human phenotype. In addition, in the accompanying paper by Hu et al., *Katnb1* knockout in mice leads to severe cortical abnormalities by affecting centriole and cilia biogenesis during development (Hu et al., 2014 [this issue of Neuron]).
KatNB1 Mutations Cause Cerebral Malformations

KatNB1 is a known component of the Katanin complex, which plays a crucial role in microtubule dynamics. Studies have shown that KatNB1 mutations lead to microcephaly, a condition characterized by an abnormally small head. KatNB1 is involved in the regulation of microtubule severing and transport, processes that are tightly linked to the proper development of the vertebrate cerebral cortex.

**Figure 8. kat80-IR Results in Reduced Dendritic Arborization in Central and Peripheral Nervous System**

(A and B) Larval class IV sensory neurons in the peripheral nervous system (PNS) were visualized using UAS>CD8-GFP expressed under the control of the PPK-GAL4 driver. Morphological analysis of dendrites of class IV neurons, which display distinct morphology, was only performed in segments A3 and A4. We observed a significant reduction in dendrite extension in kat80-IR larvae (B) as compared to the wild-type, shown in (A). (C and D) kat80-IR reduced dendritic arbor of motoneuron 5 in the CNS (D) as compared to wild-type flies (C). Dendrites of adult flight motoneurons in the CNS were visualized by expressing UAS>CD8-GFP under the control of the D42-GAL4 or C380-GAL4 driver. Asterisk marks the motoneuron 5 cell body.

(E) The total number of terminal dendrites is statistically significantly reduced in kat80-IR versus wild-type larvae as counted manually on z-projections (mean ± SEM; WT, 420 ± 15.67; kat80-IR, 369.6 ± 7.8; p = 0.01 [n = 10 cells, 7 larvae for WT, and n = 10 cells, 5 larvae for kat80-IR larvae]).

(F) The effect of reduced dendritic arborization of flight motoneurons was assessed in a flight assay. Expression of kat80-IR using the D42 driver (expressed in adult motoneurons controlling wing muscles) resulted in severely impaired flight response, as assessed by the landing distance in the cylinder (left two columns in black, landing distance in millimeters; mean ± SEM; D42 driver; WT [yw], 42.4 ± 8.7; kat80-IR, 411.7 ± 30.7 [p = 0.0001]). In contrast, kat80-IR expression in PNS sensory neurons under the ppk driver did not affect the flight response, as expected (right two columns in gray, landing distance in millimeters; mean ± SEM; ppk driver; mean ± SEM; WT, 122.6 ± 21.8; kat80-IR, 69 ± 19.9), n = 20 adult males for each genotype (see also Figure S5).

KatNB1 mutations could be due to deregulation of signaling mechanisms that control NB specification, e.g., the Notch and JAK/STAT pathways. However, we found no effect of kat80 loss on either pathway in the optic lobe (Figure S4), suggesting that kat80 regulates NB numbers independent of these cues. Indeed, it is known that in the optic lobe, intracellular signaling events, and not spindle orientation, regulate NB specification and hence cell fate (Egger et al., 2007). Thus, in kat80-IR larvae, the deficit in optic lobe NBs is also likely due to a cell fate specification defect, a notion also supported by our observation that, at some frequency, we did indeed observe kat80-IR larvae with normal-size brains containing supernumerary NBs (K.M.-G., unpublished observations). This would suggest a role for Katanin in cell fate specification, which was previously shown to be independent of spindle orientation. Therefore, in the Drosophila larval brain, kat80 plays a dual role such that its loss not only compromises the initial pool of cells per se (exit from quiescence or cell fate specification), but also their proliferative capability (delayed AO, spindle, and centrosomal defects), resulting in severe microcephaly.

Finally, microtubule severing and transport are known to play a central role in neurogenesis (Franker and Hoogenraad, 2013). We observed a striking reduction of dendritic arborization of both central and peripheral neurons in kat80-IR flies, a finding that is consistent with the reported significant reduction of the dendritic field area and the number of the dendritic numbers could be due to deregulation of signaling mechanisms that control NB specification, e.g., the Notch and JAK/STAT pathways. However, we found no effect of kat80 loss on either pathway in the optic lobe (Figure S4), suggesting that kat80 regulates NB numbers independent of these cues. Indeed, it is known that in the optic lobe, intracellular signaling events, and not spindle orientation, regulate NB specification and hence cell fate (Egger et al., 2007). Thus, in kat80-IR larvae, the deficit in optic lobe NBs is also likely due to a cell fate specification defect, a notion also supported by our observation that, at some frequency, we did indeed observe kat80-IR larvae with normal-size brains containing supernumerary NBs (K.M.-G., unpublished observations). This would suggest a role for Katanin in cell fate specification, which was previously shown to be independent of spindle orientation. Therefore, in the Drosophila larval brain, kat80 plays a dual role such that its loss not only compromises the initial pool of cells per se (exit from quiescence or cell fate specification), but also their proliferative capability (delayed AO, spindle, and centrosomal defects), resulting in severe microcephaly.
KATNB1 Mutations Cause Cerebral Malformations

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.12.014.

AUTHOR CONTRIBUTIONS

K.M.-G. designed, performed, and analyzed in vitro and in vivo (Drosophila) experiments to characterize the KATNB1 mutations and wrote the manuscript. A.O.C. and A.E.S. performed genetic analysis, identified KATNB1 mutations, and summarized the genetic, clinical, and radiological findings. C. Chabu helped design Drosophila experiments and write the manuscript and C. Chabu and T.X. helped analyze the data. O.H. generated and validated all constructs used in the study. F.V. worked on the dendritic arborization and bouton analysis studies and F.V. and H. Keshishian analyzed the data. G.T.A. experimentally verified human mutations. S.N. and W.H. performed expression analyses in mouse and human tissue and S.N., W.H., A.L., and N.S. analyzed the data. S.T. performed the zebrafish experiments and S.T. and N.C.C. analyzed the data. A.O.C., C.D., M.S.Z., H.A.A.H., J.-B.R., H.G., H. Kayserili, E.G.S., R.O.R., H.P., S.K., and W.B.D. ascertained and recruited patients, diagnosed and clinically evaluated patients, and collected samples. J.S. performed genetic investigation in patients. B.B., Caner Caglar, Cagri Caglar, D.D., and J.F.B. assisted in experimental work. F.J.M. performed radiological analysis. E.Z.E.-O. and K.Y. performed bioinformatic analysis. S.M.M. and R.P.L. oversaw exome sequencing of the patient samples. A.L. wrote the manuscript. K.B. analyzed the genetic data. J.G.G. analyzed the genetic data and led the research. M.G. analyzed the genetic data, wrote the manuscript, and led the research.

ACKNOWLEDGMENTS

We thank the patients and families who contributed to this study. This work was supported by the Yale Program on Neurogenetics and National Institutes of Health (NIH) Grants U54HG006504 (Yale Center for Mendelian Disorders, to R.P.L., M.G., M. Gerstein, and S.M.M.), U01MH081896 (to N.S.), and R01MH103616 (to M.G. and K.B.). This work was also supported by R01NS041537 and P01HD070494 (to J.G.G. and N.C.C.). R.P.L. and J.G.G. are Investigators of the Howard Hughes Medical Institute. We are grateful to the Gregory M. Kiez and Mehmet Kutman Foundation for continuing support.

Accepted: December 3, 2014
Published: December 17, 2014

REFERENCES


Supplemental Information

Mutations in *KATNB1* Cause Complex Cerebral Malformations by Disrupting Asymmetrically Dividing Neural Progenitors

A. g.57787300G>A

Proband

Father

Mother

B. g.57790282G>A

Proband

Father

Mother

Figure S1
(Related to Figure 1)
Figure S2
(Related to Figure 3)
Figure S3
(Related to Figure 5)
Figure S4
(Related to Figure 7)
Figure S5
(Related to Figure 8)
Supplemental Data

Supplemental Figure S1. Electropherograms obtained via Sanger sequencing analysis of patient NG-PNH-226 and her parents. Whole-exome sequencing identified two compound heterozygous mutations in this patient (marked in red, wild-type (WT) marked in green). (A) The G>A transition at the splice acceptor site variant; (B) shows the G>A transition leading to the missense p.Gly578Asp variant. Co-segregation analysis revealed that the patient inherited the splice acceptor site variant from her mother and the missense variant from her father.

Supplemental Figure S2. Reduced protein levels in patient-derived dermal fibroblasts and possible aneuploidy upon expression of mutant forms of KATNB1: (A) Western analysis indicates reduced levels of KATNB1 in patient- as compared with parent-derived fibroblasts. (B-C”) HeLa cells transfected with WT and mutant forms of KATNB1. Reduced localization to the kinetochores and lagging chromosomes during metaphase (arrow) upon expression of mutant KATNB1.

Supplemental Figure S3. kat80-IR does not lead to increased apoptosis. TUNEL staining of Drosophila 3rd instar larval brains with kat80-IR clones does not show increased apoptosis within the clones.

Supplemental Figure S4. kat80-IR does not affect Notch or JAK/STAT signaling in the optic lobe. The GH146 driver was used to express kat80-IR in Drosophila larval brains. 3rd instar larval brains were stained for Notch and STAT. Images are 3D projections of equivalent Z stacks and were captured using identical confocal settings.

Supplemental Figure S5. kat80-IR does not affect muscle innervation. (A,B). Adult flies expressing kat80-IR under C380-GAL4 driver, which directs expression in adult flight motoneurons (MN1-MN5), show normal innervation of the dorsal longitudinal flight muscle (DLM) via the PDMN nerve. (C-F).

Bouton size but not numbers were affected by kat80-IR: NMJ at muscle 4 (C,D) and muscle 13 (E,F) were imaged and quantified for size (G) and number (H). Bouton size was significantly increased in kat80-IR 3rd instar larvae: muscle 4: mean± SEM: yw: 2.71±0.098; kat80-IR:3.276 ±0.118; P = 0.0023; muscle 13: mean± SEM: yw: 2.755±0.0853; kat80-IR:3.306 ±0.088; P = 0.0004

Supplemental Table S1A: Clinical Characteristics of patients with KATNB1 mutations.

Supplemental Table S1B: Additional physical exam findings of patients.

Supplemental Table S1C: Radiological features of patients.

Supplemental Table S1D: Calculated relationships.
**Supplemental Table S1E:** Overlapping HBD segments between relatives.

**Supplemental Table S1F:** HBD segments of patients.

**Supplemental Table S1G:** KATNB1 mutations identified by whole-exome sequencing.

**Supplemental Table S1H:** HBD variants of patients.
Experimental Procedures

Human subjects. The study protocol was approved by the Yale Human Investigation Committee (HIC) (protocol number 0908005592). Institutional review board approvals for genetic and MRI studies, along with written consent from all study subjects, were obtained by the referring physicians at the participating institutions. All fetal human tissues were collected under guidelines approved by the Yale HIC (protocol number 0605001466). Human fetal brains at 20 and 22 weeks of gestation were obtained from the Human Fetal Tissue Repository at the Albert Einstein College of Medicine (CCI number 1993-042).

Whole-exome capture and sequencing. NimbleGen 2.1M human exome array (Roche Nimblegen, Inc.) was used to capture the exomes of all samples according to the manufacturer’s protocol with modifications, described previously. Sequencing of the library was performed on HiSeq2000 using a barcoding technology 74 base paired end, 6 samples per lane. The Illumina pipeline version 1.8 was used for image analysis and base calling.

Genome-wide genotyping. Selected samples were analyzed using 610K Quad Bead Chips according to the manufacturer’s protocol (Illumina).

Sanger sequencing. Coding regions and exon-intron boundaries of KATNB1 were evaluated by Sanger sequencing using standard protocols. Amplicons were cycle sequenced on ABI 9800 Fast Thermocyclers, and post cycle sequencing clean-up was carried out with CleanSEQ System (Beckman Coulter Genomics). The amplicons were analyzed on 3730×L DNA Analyzer (Applied Biosystems Inc.).

Identifying expression levels in the human fetal brain. The Human Brain Transcriptome database was used to identify the levels of KATNB1 expression at selected time points throughout human development. Procedure for interrogation and procurement of results were per their previously established protocols.

Exome sequence data analysis

Data preprocessing: Sequence reads were obtained through Illumina CASAVA pipeline. To achieve high quality alignment result, each read was subjected to trimming and filtering. We used cutadapt to trim PCR primer sequences from 3’-end. We also trimmed 3’-end of the read, starting from an ambiguous base call and continuing to trim systematically low quality 3’-end reads using an algorithm implemented in BWA. We filtered reads if the resulting read length is shorter than 35 bp after
trimming. We also filtered reads with overall low quality (> 80% of the bases were < Q14) or low entropy/complexity (all but 3 or less bases are identical). A read pair was filtered if one of the ends was filtered. These trimming and filtering operations were carried out using an in-house perl script and the results were stored in FASTQ format files. We then aligned remaining sequence reads to a human genome reference (GRCh37) using a 1000 genomes project FASTA file (human_g1k_v37_decoy.fasta). Reads were initially aligned by BWA \(^5\) (v0.5.9-r16) and then unaligned reads and subset of the aligned reads were processed by Stampy \(^6\) (v1.0.21). Results were stored in BAM files. To exclude the contribution of PCR duplicates in the variant calling, we inferred duplicated DNA fragments and marked to be excluded from the downstream analysis using MarkDuplicates algorithm implemented in Picard-tools (http://picard.sourceforge.net).

**Variant calling and filtering:** We called variants for all 9 samples reported here together using freebayes (v9.9.2-36-gff98393) across the union of target and tiled intervals of two platforms (Agilent and Nimblegen) padded for 50 base pairs at each end. Variant (or allele) types detected were SNP, INDEL, MNP or other complex change (COMPLEX). We filtered the following SNPs: multi-allelic sites, QUAL < 20, QUAL / AO < 4, FS > 100, EPP > 300, or RPP > 200, where QUAL is the phred-scaled probability that the detected variant is false, AO is the depth of alternate-supporting allele, FS is the phred-scaled P-value of Fisher’s exact test to detect strand bias in the reads, EPP and RPP are the end and read placement probability calculated by freebayes. For other variant types, the following variants were filtered: multi-allelic sites, QUAL < 10, QUAL / AO < 5, MQ < 20, MQM < 20, or ABP > 300, where MQ is the mean mapping quality, MQM is the mean mapping quality of alternate-supporting reads, ABP is the allele balance probability calculated by freebayes. To exclude low-confident genotype calls, we used the likelihood ratio between the maximum and the second highest phred-scaled genotype likelihoods, which we call GQ. We filtered heterozygous or homozygous genotypes if the GQ were less than 20 or 10, respectively

**Variant annotation:** We annotated functional consequence of the variants using variant effect predictor (v73). Using this information, we extracted variants that were located in exons or in splice regions up to 8 base pairs out from the exon-intron boundary. We annotated variants known to present in 1000 genomes project phase 1 (v3) and ESP6500SI (v2). Conservation scores inferred by GERP++ \cite and phastCons \cite, repeat masked elements, and regions of known segmental duplications were also annotated. We used vcfintersect program of vcflib to intersect our callset with known mutations in dbSNP (dbsnp_137.b37.excluding_sites_after_129.vcf) and a “gold-standard” INDEL sites (Mills_and_1000G_gold_standard.indels.b37.vcf).
**Homozygosity by descent segment detection:** To identify segments homozygous by descent (HBD), we used germline (v1-5-1). We included only sites without missing genotype calls as required by the program. We ran the program with the options “-err_hom 0 -err_het 0 -bits 32 -min_m 1 -homoz -homoz-only.” The identified segments were incorporated into the whole variant call set, and extended until we observed a heterozygous genotype. For relative pairs NG961 and LIS-711, we identified overlapping HBD segments and calculated the proportion of variant sites that coincide with each other. NG961 siblings shared 6 homozygous segments that were identical by descent, whereas only 1 of shared segments were IBD for LIS-711, consistent with the fact that LIS-711 patients were double first cousins.

**Candidate mutations:** To narrow down the candidate mutations, we filtered a mutation if one of the following conditions were met:

1. Any of the European American (EA) or African American (AA) cohorts of ESP6500SI had non-reference allele frequency > 0.005;
2. Any of EA or AA cohorts of ESP6500SI had one or more individuals with non-reference homozygous genotype;
3. Any of the 1000 genomes subpopulations had non-reference allele frequency > 0.005; or
4. It is a SNP for which the consequence is missence and both 2 programs (SIFT and PolyPhen) did not predict deleterious or damaging effect.

We searched for homozygous mutations shared by sibs (NG961 and LIS-711) within shared HBD segments.

**Cell culture and Transfection**

**Skin Biopsy and Dermal Fibroblast Culture:** A 4mm skin punch biopsies were taken under local anesthesia from the umbilical area of NG961-1, NG961-4, and their father (NG961-2) using a standard procedure\(^7,8\). Samples were stored in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, cat. no. 11965-084) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, cat. no. 10438-026), 1% (1x) L-glutamine (Gibco, cat. no. 25030-081), 2% (1x) Penicillin-Streptomycin (Gibco, cat. No. 15140-122) and processed immediately on arrival. Following wash with PBS (Sigma Chemical Co., Saint Louis, USA), the samples were cut into small fragments, which were laid onto the surface of 100 mm\(^2\) Petri dishes, in square areas marked by perpendicular lines made with scalpel blades. The fragments were allowed to air dry so as to adhere the dermis side of specimens to the culture dish. The fragments were then
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cultured in DMEM with 10% heat-inactivated fetal bovine serum, 1% (1x) L-glutamine, 1% (1x) Penicillin-Streptomycin at 37°C. The fibroblasts typically grew within 7-9 days. HeLa cells were cultured in RPMI with 10% FCS and transfected using lipofectamine (Life technologies) and standard protocols.

Cloning and mutagenesis: Cloning of all genes in expression vectors was performed using the Gateway system (Life Sciences). Gateway BP and LR reactions were performed using BP and LR clonase, respectively, according to the vendor instructions. PCR reactions to amplify genes and/or gene fragments were performed using the high-fidelity Accuprime Pfx DNA polymerase (Life Sciences) while all mutagenesis reactions were performed using the high-fidelity Phusion DNA polymerase (New England Biolabs). Original full length cDNAs of various genes were purchased from the following vendors: KATNB1 from Openbiosystems/ Thermo Scientific (catalog MHS1011-58773); NDEL1 from Addgene (plasmid 12572); KATNA isoform 1 (catalog IHS1380-97652414; GenBank NM_007044) from Thermo Scientific; KATNA isoform 2 (catalog HsCD00510530) and PAFAH1/LIS1 (catalog HsCD00515632) from ASU (Arizona State University) DNA repository DNAs. B1/B2 PCR reactions were performed using primers below, in order to amplify the cDNA of all genes. For KATNA1, the B1 primer was combined with either one of the B2KATNA primers to amplify the two isoforms of KATNA1. B1/B2 PCR products containing the cDNA of the desired gene were purified using the PCR purification kit (Qiagen) and were recombined using BP clonase into the shuttle plasmid pDONR-zeo (Life Sciences). This yielded so-called cDNA ENTRY clones. All genes were fully sequenced at this stage. When needed, site-directed PCR mutagenesis was performed on these cDNAs ENTRY clone and each mutagenesis was followed by full sequencing of the respective cDNA to confirm the presence of the desired mutation and the absence of any novel PCR-caused mutation in the ORF of that gene. All mutagenesis primers were designed using the QuickChange Primer Design website (Agilent Technologies). Once the sequence of any ENTRY clone was confirmed, the respective plasmid DNA was recombined using LR clonase into a common, CMV promoter expression vector, pCDNA-DEST40 (Life Sciences), which carries a V5-HIS tandem tag at the 5” end of the Gateway cassette. All B2 primers depicted below have no STOP codon. This allowed tagging of all genes with the V5-HIS tandem tags present in pCDNA-DEST40.

For co-IP experiments, it was necessary to tag some of the genes with different peptide tags. Via a similar approach, we tagged LIS1, NDEL1 and KATNA1 with 6-Myc, Myc and HA in separate constructs. In addition, the commercially obtained cDNA of LIS1 had a missense mutation (A instead of G in position 982 which was repaired via PCR mutagenesis, using primers listed below).
The following KATNB1 mutations were targeted using site directed PCR mutagenesis: C>G P440R; C>T R459W; C>T S535L; G>A V45I; T>G L540R and G>A G578D. In each mutagenesis primer pair, the bold/underscored codon in the forward primer is the one targeted by mutagenesis. In each B1 primer, the bold/underscored GCCACC is the Kozak sequence, immediately in front of the start ATG codon. In each B2 primer, the bold/underscored codon is the last (non-STOP) codon of the ORF of the respective gene.

S535LF: GTGGCCATCAACGACCTGTGGTGGTG
S535LR: CACCACCACCAACAGGTGTTGGATGCGCAC

B1KATNB1: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGCCACCCCTGTGGTCACCAAGAC
B2KATNB1ns: GGGGACCACTTTGTACAAGAAAGCTGGGTC CCAGTCCACACTGGGCATTGAGCGGTGCA

B1NDEL: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGATGGTGAAGATATACCAGATT
B2NDELns: GGGGACCACTTTGTACAAGAAAGCTGGGTC CACACACACACTGAGAGGCAGCATACC

B1-KATNA: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGATCTTCTTATGATTAGTGA
B2KATNA1ns: GGGGACCACTTTGTACAAGAAAGCTGGGTC ATAGCATGATCCAAACTCAAATATCCA
B2KATNA2ns: GGGGACCACTTTGTACAAGAAAGCTGGGTC AGGACGCATCCCTGACGGCAAAGGAATATAGA

**Antibodies and Immunostaining**

For immunocytochemistry cells were fixed in 4% paraformaldehyde (4% PFA) for 10 min or 4% PFA (10 min) followed by chilled methanol for 5 min at -20°C. Cells were pre-blocked in blocking solution containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories), 1% bovine serum albumin, 0.1% glycine, 0.1% lysine and 0.1% Triton X-100 in PBS for one hour at room temperature. After pre-blocking, sections were incubated in primary antibodies diluted in blocking solution on a horizontal shaker at 4°C for 2 hours, and washed in PBS at room temperature for three times. Sections were then incubated with secondary antibodies in blocking solution for 2 hours and washed in PBS for three times at room temperature. All samples slices were mounted with VECTASHIELD containing DAPI (Vector Laborotaries), and imaged with Zeiss LSM or Leica TCS SP2 laser scanning confocal microscopy system. Primary antibodies used for immunocytochemistry are: anti-KATNB1 (Rabbit, 1:200; Sigma-Aldrich), Katanin A1 (R&D Systems, 1:100 dilution); NDEL1 (Origene, 1:50 dilution); V5 (Rockland Immuno); myc (Origene); HA (1:100 dilution); Dynein (Sigma, 1:100); Lis1 (Thermo-Fisher, 1:100). Alexa Fluor 488-
conjucated anti-rabbit IgG and Cy3 conjugated anti-mouse IgG raised in donkey (1:400; Life Technolgies, NY) were used as secondary antibodies.

3rd instar wandering larvae were dissected in Schneider’s medium (Sigma, St. Louis, MO), fixed in 100 mM Pipes (pH 6.9), 1 mM EGTA, and 1 mM MgCl2 for 25 min and blocked for 1 h in 1X phosphate-buffered saline (PBS) containing 1% goat serum, 5% normal goat serum, 1% normal donkey serum and 0.3 % Triton X-100 (PBT-Blocking). After blocking, specimen were extensively washed in PBT (PBS+0.3% Triton X-100) for 1 h and incubated with primary antibodies in PBT-Blocking overnight at 4°C. Primary antibodies were: rat anti-Miranda (1:100; Chris Doe); mouse anti-αtubulin (BS12, Sigma, 1:1500); mouse anti-γtubulin (GTU88, Sigma, 1:100); rabbit anti-Scrib, 1:1000 (Doe lab); anti-E-cadherin (DSHB); anti-Notch-intra (DSHB); anti-STAT92E (Steve Hou); rabbit anti-phospho-histone H3 (Upstate Biotechnology, Lake Placid, NY; 1:1000); katanin B1 (Sigma, 1:100). Primary antibodies were extensively rinsed off with PBS-BT for 1 h at room temperature, and specimens were incubated with fluorescently conjugated secondary antibodies (Life Technologies, NY) diluted in PBS-BT, followed by extensive rinsing with PBS-BT. For DNA labeling, specimens were mounted in Vectashield mounting medium with DAPI (Vector Labs). Brains were imaged using a Zeiss LSM 510 or Leica TCS SP2 laser scanning confocal microscope (Deerfield, IL) equipped with a 40X NA or 63X NA oil immersion objective, respectively. Figures were assembled in Adobe Photoshop (San Jose, CA). 3D projections and quantifications were performed using IMARIS.

Immunoprecipitation

HeLa cells were transfected with either V5 tagged WT or mutant forms of KATNB1 along with 6XMyckatna1 or HA-NDEL1 using Lipofectamine (Life Technologies, NY). 48 hours after transfection, cells were lysed using a triton-X100 based buffer containing protease inhibitor cocktail (Calbiochem). Clarified supernatants were used for immunoprecipitation using Dynabeads (Life Technologies) according to the Suppliers protocol. Immunoprecipitates were analyzed by Western analysis using standard protocols. The samples were run on 10% SDS-PAGE gels (BioRad) and Western blots were performed according to standard methods. In brief, lysates were separated on gradient gels (4-16 %, BioRad) SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking with 5% milk, the membranes were incubated first with rabbit anti-tag antibodies and then with a secondary HRP conjugated anti-rabbit IgG (Jackson Immunoresearch Labs). Signals were detected with chemiluminescence reagent (Biorad).

Fly Genetics
Oregon R or yw flies were used as wild-type controls. Other fly strains used include: \textit{w;worniu-GAL4, UAS-Miranda::GFP, UAS-Zeus::mCHERRY/ Cyo;Dr/TM6b} (Chris Doe); \textit{w,UAS-CD8-GFP;D42-gal4,cha-gal80; c380-Gal4, UAS-CD8-GFP;cha-gal80; hsFLP;Act-FRT-CD2-FRTgal4,UAS-CD8(n); y[1] w[1118]; P[w[+m*]=GawB]GH146} (Bloomington Stock Center); \textit{kat80} RNAi lines were obtained from VDRC. Three independent \textit{kat80} RNAi lines showed similar results.

\textbf{Time-lapse Analysis of Neuroblast Cell Division in Larval Brain Explants}

Wandering 3rd instar larvae of \textit{worniu-Gal4} crossed to either \textit{yw} or \textit{kat80-IR} flies were dissected in Schneider’s insect medium (Life Technologies) supplemented with 7.5% fetal bovine serum (FBS). Four to five larval brains were immediately transferred into 200 µl of Schneider’s medium supplemented with 7.5% FBS, 0.5 mM ascorbic acid, and fatbodies obtained from 10 wild-type wandering 3rd instar larvae. Brains were mounted with fatbody tissue on a standard membrane (Yellow Springs Instruments, Yellow Springs, OH) and placed on a stainless steel slide as previously described \footnote{9}. Brains were imaged using a Zeiss laser scanning confocal microscope equipped with a 63X NA oil immersion objective. For the mitotic analysis of neuroblasts, stacks containing four focal planes spaced by 1.5 µm were acquired at intervals of 1 minute 30 seconds. Time-lapse image series were converted into movies using IMARIS. All movie frames are maximum intensity projections.

\textbf{Neuron visualization and dendritic analysis}

For visualization of larval sensory cells and to study the effect of kat80 loss, \textit{UAS-CD8-GFP} or \textit{UAS-CD-GFP}, \textit{kat80-IR} were expressed under the control of the \textit{ppk-GAL4} driver that expresses strongly in all class IV sensory neurons and weakly in class III neurons \footnote{10,11}. Analysis was focused on class IV dendrites, which are morphologically distinguishable from class III branches \footnote{10}. Morphological analysis of dendrites was exclusively restricted to the dorsally located ddaC neuron in segments A3 and A4 \footnote{10}. Images were taken using a Bio-rad 1024 confocal microscope. The total number of terminal dendrites was counted manually on z-projections using the cell count function of ImageJ software. Quantification of dendritic coverage was performed in ImageJ software by individually overlaying a grid of squares of 0.1% of the total pixel size of each z-projection image and calculating the number of boxes containing dendrites as a percentage of total box number within the dendritic field.

For visualization of dendrites of adult flight motoneurons and to study the effect of kat80 loss, \textit{UAS-CD8-GFP} or \textit{UAS-CD-GFP}, \textit{kat80-IR} were expressed under the control of the D42-GAL4 or C380-GAL4 driver that drive expression mainly in motoneurons \footnote{12,13}. Additionally, GAL80 was expressed under the choline-
acetyl transferase promoter (Cha-GAL80) to suppress GAL4 activity in all cholinergic neurons\textsuperscript{14} some of which are presynaptic partners of the flight motoneurons\textsuperscript{13,15}. Mouse anti-GFP primary antibody (Rockland Immunochemicals) with fluorescently conjugated secondary antibodies were used as described previously\textsuperscript{16,17} for better visualization of GFP labeled dendrites.

To visualize NMJ arbors third-instar larvae were dissected and fixed as described\textsuperscript{18}. To visualize NMJ arbors, anti-HRP immunocytochemistry was performed as described\textsuperscript{19} with 0.05% diaminobenzidine (DAB; Polysciences, Inc., Warrington, PA) in the presence of 0.003% H\textsubscript{2}O\textsubscript{2}. Larval fillets were mounted in glycerol and examined using bright field imaging. All data were taken from muscles in segment A3 and restricted to type Ib boutons.

\textbf{Behavioral experiments}

Dendritic defects after targeted manipulation of adult flight motoneurons was performed as previously described\textsuperscript{17}. The flight assay was performed according to the method of Drummond et al.\textsuperscript{20,21} and as modified by Nelson et al.

\textbf{Mouse experiments:}

\textit{In situ hybridization:} Embryonic and postnatal brains were fixed, respectively, by immersion in or intracardial perfusion with 4\% paraformaldehyde (PFA), post-fixed in 30\% sucrose in 4\% PFA and sectioned on a cryomicrotome (Leica Microsystems, Wetzlar, Germany). Sections were processed for in situ hybridization as described previously\textsuperscript{22}. RNA probes complementary to mouse \textit{katanin b1} (bases 105 to 908 of the mouse \textit{katanin b1} cDNA) were labeled with digoxigenin-11-UTP. Sections were analyzed using a Stemi stereomicroscope or AxioImager (Zeiss, Oberkochen, Germany) fitted with an AxioCam MRc5 digital camera. Images were captured using AxioVision software (Zeiss) and assembled in Adobe Photoshop.

\textbf{Zebrafish studies:}

\textit{Katnb1 expression pattern analysis by whole-mount in situ hybridization:} Whole-mount \textit{in situ} hybridization was performed as described\textsuperscript{23}. DNA template for anti-sense \textit{katnb1} (accession number: NM\_213018) riboprobe was synthesized by PCR amplification from 24 hpf (hours post fertilization)
zebrafish cDNA with the following primer set: 5’-TGGTGGACGTCCTCAACATA-3’ and 5’-AGTCCAGAGGGCCATAAGT-3’.

**katnb1 morpholino injection and image analysis:** 9 ng of katnb1 morpholino was injected into 1-cell stage wild type embryos as described23. Dorsal view images of 72 hpf control and morphant embryos were taken by a Leica M205 FA dissecting microscope with LAS AF software. Brain size was subsequently measured in ImageJ. Statistical analysis was carried out in Microsoft Excel. katnb1 ATG morpholino: 5’-TTGTGTGGACTGGGTCAAATCACTC-3’ (Genetools).

**References:**


