B. SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of Kif21a knock-in mice

A 11.2 Kb genomic DNA fragment containing *Kif21a* exons 12 through 22 was subcloned from a 129 BAC (RPCI-22) library using recombineering technology (Copeland et al., 2001; Liu et al., 2003). The 2,827C→T mutation was introduced into the 4th nucleotide of exon 20 by site directed mutagenesis (QuickChange XL, Stratagene). This point mutation created a new *BsrD I* restriction site in the targeting plasmid that was later used to genotype ES cells and mice. In addition, a floxed neomycin (*Neo*) cassette was inserted into intron 19, which conferred resistance to G418 when electroporated into ES cells. Excision of the floxed *Neo* cassette was later obtained in the presence of Cre recombinase protein (pOG231 plasmid, courtesy of S. O'Gorman) leaving behind a single *loxP* site (34 bp) in intron 19. J1 ES cells (Li et al., 1992) were transfected with the targeting vector and subsequently selected with G418. Ninety-six surviving colonies were picked and screened by PCR followed by Southern blot using probes outside of the targeting construct. Ten clones had loxP located in intron 19, of which restriction analysis revealed that 6 carried the R954W mutation. After karyotyping analysis, two R954W positive ES clones were microinjection into C57BL/6J blastocysts that were implanted into pseudo pregnant CD1 female (Robertson, 1987). Chimeric animals were obtained from both independent ES cell clones. Germline transmission was observed in both lines.

The Neo cassette was removed both *in vivo* resulting in progeny on a mixed 129S1/C57BL/6J background, and *in vitro* by transfecting positive ES clones with pOG231 plasmid expressing Cre recombinase resulting in mice on a pure 129S1 background. Penetrance of the CFEOM1 phenotype fell when mice were backcrossed to a C57BL/6 background, was intermediate on a mixed 129S1/C57BL/6J, and was highest on the 129S1 background. Thus, all analyses were performed on the pure 129 genetic background unless otherwise indicated. In addition, one ES cell clone containing the loxP site without the point mutation (*Kif21aloxP*) was used to generate a *Kif21a*^{loxP} control line. *Kif21a*^{loxP} mice were found to be indistinguishable from their WT littermates.

Generation of Kif21a knock-out motor truncated Kif21a$^{KOMT/KOMT}$ *mice*

An 11.0 kb genomic DNA fragment containing *Kif21a* exons 2 through 7 was subcloned from a 129 BAC (RPCI-22) library using recombineering technology (Copeland et al., 2001; Liu et al., 2003). First, a LoxP site and *Neo* gene flanked by *FRT* sites were introduced upstream of exon 6; excision of the *FRT Neo* cassette left behind a *FRT* and a *loxP* site at this locus. Next, a floxed Neo cassette was targeted downstream of exon 2. To determine if *Cre* could successfully excise exons 2-6, the plasmid was electroporated into cell lines expressing *Cre* recombinase and excision of a 7.6 Kb region and Neo cassette was confirmed. This vector was then electroporated into 129/S1 ES cells and correctly targeted ES cells clones were identified by PCR and Southern blot probes. Eleven correctly targeted clones were identified, and clones were transfected with a plasmid expressing Cre recombinase to excise the region flanked by the *loxP* sites. Two mutant ES clones were then microinjected into C57BL/6J blastocysts and implanted into pseudo-pregnant CD1 female. Genotypes were confirmed by Southern blotting, PCR, and DNA sequencing. The *Kif21a* knockout targeting vector excised exons 2-6, resulting in an out-of-frame deletion that removed most of the functional *Kif21a* motor domain (encoded by exons 2-8).

Map1b$^{-/-}$ mice

Experiments were performed using the previously reported *Map1b$^{-/-}$* mice (Meixner et al., 2000). *Map1b$^{+/+}$;Isl^{MN}:GFP* and *Map1b$^{-/-}$Isl^{MN}:GFP* mice were generated by crossing *Map1b$^{-/-}$* mice with C57BL/6J *Isl^{MN}:GFP* transgenic mice (Lewcock et al., 2007).

Visual behavioral test

Behavioral threshold acuity was evaluated using standard methods in 4 *Kif21a$^{KI/KI}$* and 4 WT unrestrained mice (Prusky et al., 2004). Visual tracking was tested and was present in all mice at 0.1 cyc/deg, 0.2 cyc/deg, and 0.4 cyc/deg for each direction of rotation.

Lipophilic dye placement, sample preparation and imaging

Anterograde and retrograde labeling of ocular cranial nerves and EOMs at E14.5 and PO were performed using spectrally distinct lipophilic dyes applied at specific axial levels of the brainstem to label the oculomotor, trochlear, and abducens nuclei bilaterally and/or unilaterally. NeuroVue® Maroon (red) or Jade (green) dye (MTTI, West Chester, Pennsylvania) soaked filter strips were applied subsequent to preparation of tissues of interest (Duncan et al., 2011; Fritzsch et al., 2005; Tonniges et al., 2010). After dye diffusion, brainstems and orbits were observed for successful dye placement and completely labeled samples were prepared for confocal microscopy. Consecutive scans and stacks of images through the nerve and/or each nucleus were collected using a Leica TCS SPE microscope utilizing Leica LAS software. For multi-color labeled oculomotor axons at E12.5, red dye was placed adjacent to contralateral oculomotor nucleus at E12.5 and given twenty-four hours to preferentially label superior division motor neurons and their axons as they crossed the midline. Green dye was then placed on the ipsilateral nucleus, labeling both contralateral superior division axons and ipsilateral inferior division motor neurons and axons.

To selectively label the oculomotor nerve in the anterograde direction, the dorsal skin and calvaria were removed to visualize the brain. Tissue was removed until the cerebral aqueduct and sulcus isthmi could be seen. NeuroVue ® dye (MTTI, West Chester, Pennsylvania, www.mtti.com) soaked filter strips were placed in the oculomotor nucleus, or just lateral to the nucleus in the midline, depending on the experiment. Anterograde tracing of EOM innervation was then visualized by first removing the cornea, lens, vitreous and pigment from the globe. An incision was made in the sclera and the globe was folded back on itself with EOMs attached. Individual EOMs were then isolated and mounted in glycerol.

For retrograde labeling of oculomotor nuclei, a small wedge of dye soaked filter was positioned in the posterior orbit. The preparation was then placed in 4% PFA at 36°C and allowed to diffuse for 6 days. After lipophilic dye diffusion, successful dye placement was verified by visualizing labeled cranial nerves exiting the brainstem using a Leica M205 microscope with epiflourescent imaging (Leica, Wetzlar, Germany). For visualization of oculomotor nuclei following retrograde labeling, the embryo was fixed, embedded in gelatin, kept at 4°C in PFA/PBS solution for at least 4 days, and then either flat-mounted or sectioned at 100 µm thickness, depending on age. The preparation was then mounted on a glass slide with glycerol. Quality of the preparation was controlled with visualization on a Nikon E800 using Metamorph software.

Histology

For orbital and EOM histology, mice were anesthetized and perfused intracardially with PBS followed by 4% Paraformaldhyde (PFA). Globes with intact EOMs were isolated by orbital dissection. Orbits were fixed for 24 hrs in 4% PFA, washed in PBS and dehydrated through graded alcohol washes and embedded in paraffin. The orbit was then serially cut in 10 μm cross-sections at on a Leica microtome. All sections were collected, mounted on coated slides, and stained with hematoxylin and eosin and examined by light microscopy.

For histological analysis of adult mouse brains, mice were intracardially perfused with 4% PFA or 10% neutral formalin, the skull removed, and the brains carefully dissected. Brains were fixed in 4% PFA or 10% neutral formalin for 24-48 hrs and then processed for embedding in paraffin. Serial sections at 5μm or 10μm thickness were collected and mounted on superfrost-coated slides. Sections were subsequently stained using the Nissl method with or without Luxol fast blue. Results were examined and images were acquired by light microscopy on an Olympus BX51 epifluorescence microscope (Olympus, Center Valley, Pennsylvania) or Nikon Eclipse-80i transmitted light microscope (Nikon, Melville, New York) with Spot Xplorer CCD camera and Spot Version 4.6 acquisition software (Diagnostic Instruments, Sterling Heights, Michigan).

Immunohistochemistry

Embryonic brains were fixed in 4% paraformaldehyde and embedded for cryo- or paraffin sectioning. Frozen sections of 8 or 10μm, depending on embryonic stage, were cut on a Leica 3050S cryostat (Leica, Wetzlar, Germany), and thaw-mounted on superfrost slides. Sections were washed in PBS at RT and antigen unmasking was performed if required. Thereafter, sections were permeabilized and blocked with 1-5% normal goat or donkey serum in 0.1% Triton/PBS at RT for 1 hr. Sections were incubated overnight with primary antibody in blocking buffer at 4°C. After removal of primary antibody and three PBS washes, sections were incubated with corresponding secondary antibodies conjugated with Alexa fluorophores 488 and 594 (Invitrogen, Carlsbad, California). Sections were coverslipped and signal was resolved using an Olympus BX51 epifluorescence microscope (Olympus, Center Valley, Pennsylvania) with Spot Xplorer CCD camera and Spot Version 4.6 acquisition software (Diagnostic Instruments, Sterling Heights, Michigan).

For DAB staining, frozen sections were post fixed with 4% PFA for 30 mins. Endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide solution in methanol for 30 mins, and sections were blocked with 10% goat or fetal calf serum in 0.1% Trition/PBS for 1 hr and incubated overnight with primary antibody at 4°C. Sections were incubated for 1 hr with horse radish peroxidase conjugated secondary antibodies (Jackson Immunoresearch, West Grove, Pennsylvania), drained, washed, and signal was visualized by DAB Fast (Sigma, St. Louis, Missouri) according to manufacturer's recommendations.

Primary antibodies used for immunohistochemistry were rabbit anti-Kif21a (described in (Tischfield et al., 2010)) mouse anti-Islet1 (clone 394D, Developmental Studies Hybridoma Bank, Iowa City, Iowa), rabbit anti-Phox2b (kind gift from J. Burnet), Activated Caspase 3 (Cell Signaling Technology, Inc. Danvers, Massachusetts), mouse anti-Tuj1 (Sigma, St. Louis, Missouri), mouse anti-myogenin F5D (Abcam, Cambridge, Massachusetts), and mouse anti-Neurofilament (clone2H3, Developmental Studies Hybridoma Bank, Iowa City, Iowa). Alexa fluor 594 or Alexa fluor 488 conjugated goat anti-rabbit and goat anti-mouse IgG(H+L) (Invitrogen, Carlsbad, California) secondary antibodies were used for signal detection.

Cell counts, quantification, and statistical analyses

Absolute motor neuron cell counts were obtained by counting each Nissl stained neuron with a distinctive nucleolus and cytoplasm in the oculomotor, trochlear, abducens, and facial nucleus in each serial section through the brainstem. Quantification of Phox2b positive cell in the oculomotor nucleus was performed by counting each Phox2b positive cell with a discernable portion of the nucleus in every serial section of the midbrain at E11.5. For oculomotor midline cell counts, within each serial section all Islet1 positive cells with a discernable portion of their nucleus visible located on the midline, floor plate, and adjacent to the oculomotor nucleus were scored using the brush function in Photoshop.

Unless mentioned in the text, results are presented as mean with standard deviation or standard error of the mean, as indicated. All differences of the means between two sample sets were assessed by two-tailed Student t-test or Wilcoxon test and data sets were plotted using GraphPad Prism software. At least three animals in each group were analyzed and significance was set at $p < 0.01$.

Flat-mount retina immunofluorescent staining

Retinas were carefully dissected from adult mice and fixed in 4%PFA, flat mounted and blocked in 10% normal goat serum with 0.2%Triton in 1xPBS. Samples were incubated in primary mouse anti-neurofilament antibody (1:1000; clone2H3, Developmental Studies Hybridoma Bank, Iowa City, Iowa) and secondary Alexa-Fluor 594 goat anti-mouse antibody (1:1000; Invitrogen, Carlsbad, California) at 4°C overnight. All antibodies were diluted in blocking solution. Images were acquired using an Olympus BX51 epifluorescence microscope (Olympus, Center Valley, Pennsylvania) with Spot Xplorer CCD camera and Spot Version 4.6 acquisition software (Diagnostic Instruments, Sterling Heights, Michigan).

Whole mount embryo immunohistochemistry

Embryos were fixed in 4% PFA at 4°C for 2 hrs, washed in PBT and dehydrated with successive 15 min washes in 25, 50, 75 and 100% MeOH/PBT and at –20°C overnight. Embryos were bleached in 4:1:1 MeOH/DMSO/H2O2 at RT for 3-5 hrs. After bleaching, embryos were rehydrated for 15 min each in 75, 50, and 25% MeOH/PBT, then washed twice with PBT at RT. Embryos were incubated twice in PBSMT for 1 hr each before adding anti-neurofilament antibody (clone2H3, Developmental Studies Hybridoma Bank, Iowa City, Iowa) diluted in PBSMT (1:1000) and incubated overnight at 4°C with gentle rocking. Following overnight incubation embryos were wash twice in PBSMT at 4°C and 3 times in PBSMT at RT for 1 hr. Thereafter, Peroxidase-conjugated Goat anti-Mouse IgG secondary antibody (Jackson Immunoresearch, West Grove, Pennsylvania) diluted 1:500 in PBSMT (2µl into 1ml of PBSMT) was added and embryos were incubated with gentle rocking at 4°C overnight. The following day, embryos were washed twice in PBSMT at 4°C, and 3 times at RT for 1 hr, and washed once in PBT at RT for 20 min. Embryos were then incubated in 0.3mg/ml DAB (Sigma) in 0.5% NiCl2 (10mg tablet in 34ml of 0.5% in NiCl2) PBT at RT for 30 min or more. H2O2 at 0.0003% (1/100 30% dilution and 1/1000 dilution) was then added and embryos were incubated at RT until desired color density was reached (usually ~10 min). Embryos were then rinsed in PBT and dehydrated through a series of 30%, 50%, 80%, 100%, and 100% MeOH for additional 30 min. Finally, embryos were cleared in benzyl alcohol:benzyl benzoate (1:2) (BABB) in glass containers and visualized by on a Nikon dissecting scope with Spot camera and software.

Fluorescent whole mount embryo immunohistochemistry

Whole mount E10.5-E12.5 embryos were prepared as previously described (Huber et al., 2005). Mouse anti-neurofilament (1:1000; clone2H3, Developmental Studies Hybridoma Bank, Iowa City, Iowa) and rabbit anti-Tuj1 (1:500; Abcam, Cambridge, Massachusetts) primary antibodies, and Alexa-Fluor 594 goat anti-mouse (1:1000; Invitrogen, Carlsbad, California) and Alexa-Fluor donkey anti-rabbit (1:1000; Invitrogen, Carlsbad, California) secondary antibodies were used to stain prepared embryos. Once stained, embryos were carefully dissected down the midline sagittal plane and flat mounted on coverslips with BABB. Samples were imaged on a Zeiss LSM 700 series laser scanning confocal microscope and images were acquired using Zen Software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and uniformly adjusted for contrast and brightness using ImageJ software (NIH).

Oculomotor nerve diameters were measured blind to genotype in ImageJ by drawing a line that perpendicularly bisected the diameter of the nerve, obtaining the number of pixels comprising the line, and converting the pixels to microns. This was done at the proximal exit point after all nerve branches fasciculated into one bundle, at the widest part of the nerve, and at the most distal part of the nerve prior to branching at the orbit. Both left and right oculomotor nerves were analyzed for at least 3 embryos for each time point. Statistical significances were determined by one-way ANOVA between ages E10.5, E11.5, and E12.5, and two-tailed independent t-tests for proximal and distal nerve diameters within and between genotypes. Significant p-value < 0.0036 using the Bonferroni correction for multiple comparisons.

Oculomotor nerve immunofluorescence

Embryos from *Kif21a*$^{+/+}$*;Isl*^{MN}*:GFP* and *Kif21a*$^{KI/KI}$*;Isl*^{MN}*:GFP* were dissected out at E11.5, E12.5, E13.5 and E15.5 in HBSS buffer, the forebrain removed, and the remaining embryos fixed in 4% PFA/1X PBS overnight. Oculomotor nerves together with the surrounding tissue were then carefully dissected out in 0.01M PBS and were flat mounted in 1XPBS containing 70% glycerol and 0.01M KOH for direct imaging or for immunofluorescence staining. For immunofluorescence, tissue was washed x3 with 0.01M PBS, and blocked with 10% normal goat serum and 1% TritonX-100 in 1X PBS overnight at 4^{o}C. The next day, the tissue was incubated with primary antibody mouse anti-neurofilament (clone2H3, Developmental Studies Hybridoma Bank, Iowa City, Iowa) for 36 hrs at 4^{o}C. After 6 washes with 1XPBST (0.1% TritonX-100), tissue was incubated with Alexa Fluor 594 conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibodies (1:1000, Invitrogen, Carlsbad, California) overnight at 4^{o}C. Tissues were then flat mounted on 1XPBS plus 70% glycerol and 0.01M KOH. Z-stack images were acquired using a Zeiss LSM 700 series laser scanning confocal microscope and images were acquired using Zen Software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and reconstructed using ImageJ (National Institutes of Health).

Two-photon imaging

E12.5-E14.5 *Kif21a*$^{+/+}$*;Isl*^{MN}*:GFP* and *Kif21a*$^{+/KI}$*;Isl*^{MN}*:GFP* embryo orbits were prepared for imaging by removing the forebrain and fixing the embryo overnight in 4% PFA. Orbits were carefully microdissected from embryos in PBS and placed on a glass coverslip in 70% glycerol in 0.01M potassium hydroxide to clear surrounding tissue. Samples were imaged on a Zeiss inverted Axio Observer LSM 710 NLO 2-photon microscope equipped with a Mai Tai DeepSee Laser (Spectraphysics) using a LD C-Apochromat 40x/1.1 water immersion objective. Images

were uniformly adjusted for contrast and brightness using Imaris x64 version 7.2.3 software (Bitplane), then labeled and stitched into a Quicktime movie using iMovie (Apple).

Western blotting

Denatured protein samples in LDS Sample Buffer (Invitrogen) resulting from pertinent experiments were loaded on NuPAGE 4-12% Bis-Tris gels (Invitrogen, Carlsbad, California), and subjected to SDS-PAGE electrophoresis. Proteins were then transferred from gels to nitrocellulose membranes (Invitrogen, Carlsbad, California) and membranes were blocked with 5% non-fat-milk in 0.01M PBST (0.1%Tween20) at RT for 1 hr. Membranes were then incubated overnight at 4^{o}C with primary antibodies diluted in 0.01M PBST (0.1%Tween20) as follows: rabbit anti-Kif21a (1:5000, generated by our lab as described (Tischfield et al., 2010)) mouse anti-Kif1a (1:1000, BD Biosciences, San Jose, California), mouse anti-Kif3a (1:1000, BD Biosciences, San Jose, California), mouse-anti kinesin heavy chain (KHC) (1:3000, EMD Millipore Corporation, Billerica, Massachusetts), mouse anti-α-tubulin (1:10,000, Sigma, St. Louis, Missouri), mouse-anti β-actin (1:10,000, Abcam, Cambridge, Massachusetts), mouse anti-Myc (1:1000, Santa Cruz Biotechnology, Inc, Santa Cruz, California), mouse-anti GFP (1:1000, Santa Cruz Biotechnology, Inc, Santa Cruz, California), and rabbit-anti RFP (1:1000, Abcam, Cambridge, Massachusetts). Following several washes with PBST, membranes were incubated with goat anti-rabbit or goat anti-mouse secondary antibodies (1:10,000, Jackson Immunoresearch, West Grove, Pennsylvania) at RT for 1 hr. Following several washes with PBST, Chemiluminescent substrate (Amersham ECL Plus, GE Healthcare Life Sciences, Pittsburgh, PA) was added, and images were captured real-time at 10-20 sec intervals using a FujiFilm LAS-4000 with CCD camera (GE Healthcare Life Sciences, Pittsburgh, PA). Protein measurement and quantification were performed using MultiGauge software (GE Healthcare Life Sciences, Pittsburgh, PA).

Microtubule co-sedimentation assay

Tissue lysates were made from E18.5 *Kif21a*$^{+/+}$ and *Kif21a*$^{KI/KI}$ mouse brains, or cell lysates were made from transfected HEK293 cells by 1ml BRB80 buffer (80 mM PIPES / KOH, 1mM EGTA, 1mM $MgCl_2$, pH 6.8) supplemented with Halt^{TM} protease inhibitor and phosphatase inhibitor (single-use cocktail 100x, Thermo Fisher Scientific, Inc, Rockford, Illinois). Crude tissue/cell were centrifuged at 55,000 rpm for 35 mins at 4^{o}C, 400µl supernatants (S1 fraction) with equal protein concentrations were added at final concentration with 2mM AMP-PNP (5'-adenylylimidodiphosphate, an nonhydrolyzable ATP analog inhibiting detachment of kinesin from microtubules) or ATP (Sigma, St. Louis, Missouri), 1 mM GTP, 50 µg/ml palitaxol-stabilized microtubules (cytoskeleton, Inc, Denver, Colorado), 20 µM palitaxol (Sigma, St. Louis, Missouri), and 5% glycerol and were incubated for 35 mins at 37^{o}C. For microtubule co-sedimentation assay of *Map1b* mice, microtubules were polymerized with endogenous brain tubulin to ensure that microtubule polymerization were not generally altered by loss of Map1b. The reactions were overlaid on a 500µl 30% sucrose cushion and centrifuged at 55,000rpm for 35 mins at 25^{o}C, resulting in a pellet containing microtubules and associated proteins (P2 fraction) and soluble supernatants (S2 fraction). Soluble supernatants (S2 fraction) were kept and microtubule pellets (P2 fraction) were rinsed twice by lysate buffer and re-suspended and de-polymerized completely by 1x loading buffer. All fractions were denatured with 1x loading buffer and were analyzed by SDS-PAGE and western blot. Three independent experiments were performed for both the *in vivo* and *in vitro* studies. The ability of kinesin to bind to microtubules was represented as the ratio of the amount of kinesin protein presented in P2 fraction to the total amount of kinesin protein presented in both P2 and S2 fraction. Statistical analysis was

performed by using a two-tailed paired Student's t test for in vivo experiments and by one-way ANOVA with post-hoc Tukey t test for multiple comparisons for in vitro experiments.

Differential centrifugation and cell fractionation

Brains were dissected from *Kif21a*$^{+/+}$ and *Kif21a*$^{KI/KI}$ mice at E18.5 and were homogenized in 1 ml Buffer A (20mM HEPES, 100mM K-aspartate, 40mM KCl, 5mM EGTA, 5mM $MgCl_2$, 2mM Mg-ATP, 1mM DTT, pH 7.2) supplemented Halt^{TM} protease inhibitor and phosphatase inhibitor (single-use cocktail 100x, Thermo Fisher Scientific, Inc, Rockford, Illinois). Crude brain tissue lysates were incubated on ice for 30 mins and were pre-cleared by centrifugation at 1,000 g for 5 mins. The supernatants (S0 fraction) were kept and protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc, Rockford, Illinois). After being adjusted to the same concentrations, equal volumes of WT and KI supernatants were centrifuged at 3,000 g, and both pellets (P1 fraction, representing primarily mitochondria) and supernatants (S1 fraction) were kept. The supernatants were centrifuged further at 10,000 g and both pellets (P2 fraction, representing primarily large organelles such as lysosomes) and supernatants (S2 fraction) were kept. The supernatants were centrifuged further at 100,000 g and both pellets (P3 fraction, representing primarily small organelles such as ER and Golgi as well as the insoluble cytoskeleton) and supernatants (S3 fraction) were kept. The pellets were resuspended and homogenized further with Buffer A containing 1% TritonX-100, and then were centrifuged again at 100,000 g. Both pellets (P4 fraction, representing the detergent-insoluble cytoskeleton-enriched fraction) and supernatants (S4 fraction, representing the detergent-soluble membrane-enriched fraction) (Marszalek et al., 1999; Okada et al., 1995) were kept. All centrifugations were performed at 4^{o}C. Each pellet from each fraction was re-suspended in the same volume of Buffer A. Loading buffer was added, and the sample was denatured and analyzed by SDS-PAGE and western blot. Three independent experiments were performed. Relative levels of proteins in each fraction were represented as the ratio of protein intensity in each fraction to summed intensity in fractions. Statistical analysis was performed using a two-tailed paired Student's t test.

DNA constructs and mutagenesis

The cDNA of human FL *KIF21A* (1-1674) (NCBI accession No. NM_001173464.1, NP_001166935) was amplified by PCR from human fetal brain cDNA and subcloned into pcDNA3.1/myc-his (B) vector (Invitrogen). *KIF21A* mutations were introduced into the construct by using QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, California). Constructs of pBA-GFP-*Kif21a* and pBA-*Kif21a* (1-417)-GFP expressing mouse FL Kif21a (1-1573) (NCBI accession No. NM_016705.3, NP_057914) and truncated Kif21a (1-417) with GFP fused at the N-terminus and C-terminus, respectively (kind gifts from G Banker). *Kif21a* mutations were introduced into the pBA-GFP-*Kif21a* by using QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, California). Mouse N-terminal GFP-fused FL and truncated *Kif21a* amplified by PCR using WT or mutant pBA-GFP-Kif21a as template were subcloned into pCDNA3.1(+) vector (Invitrogen, Carlsbad, California). Mouse FL and truncated *Kif21a* amplified by PCR using WT or mutant pBA-GFP-*Kif21a* as template were subcloned in pCDNA3.1 (+)/mCherry vector. A 12-amino acid-linker (DRRWIRRIWRRP) was inserted between GFP and Kif21a, and an 11-amino acid-linker (GGDYKDDDKGG) was inserted between Kif21a and mCherry. Human motor, 3rd coiled-coil and WD40 domain of KIF21A and mouse WT and mutant 3rd coiled-coil domains of Kif21a were subcloned in pGEX4T2 vector (GE Healthcare Life Sciences, Pittsburgh, PA). All constructs were verified by DNA sequencing.

Cell culture and transfection

HEK293 cells, HeLa cells, and COS7 cells were cultured in DMEM medium (Invitrogen, Carlsbad, California) supplemented with 10% FBS and 1% penicillin-streptomycin. Transfections were performed on the day after cells were seeded. HEK293 cell were transfected using Calphos^{TM} mammalian transfection kit (Clontech, Mountain View, California), and HeLa and COS7 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, California). Cells were fixed for immunofluorescence or were lysed for biochemistry assays 24 to 36 hrs after transfection.

Immunoprecipitation assay

For *in vitro* immunoprecipitation assays, 36 hrs after transfection, HEK293 cells cultured in 100 mm dish were rinsed with PBS and lysed with 1ml ice-cold cell lysate buffer (50mM TrisHCl, 150mM NaCl, 1Mm EDTA, 1% NP40, pH 6.8) supplemented with Halt^{TM} protease inhibitor and phosphatase inhibitor (single-use cocktail 100x, Thermo Fisher Scientific, Inc, Rockford, Illinois). After incubation on ice for 30 mins, cell lysates were clarified by centrifugation at 14,000rpm for 15 mins at 4^{o}C and supernatants were incubated with 2µg primary antibody, mouse anti-Myc (Santa Cruz Biotechnology, Inc, Santa Cruz, California), mouse anti-GFP (Abcam, Cambridge, Massachusetts), and mouse anti-RFP (Abcam, Cambridge, Massachusetts) respectively for 2 to 4 hrs at 4^{o}C with gentle rotation, then with 40µl protein-G agarose beads (Invitrogen, Carlsbad, California) for an additional 2 hrs or overnight. After 5 washes with cell lysate buffer, beads were re-suspended by 40µl 2x loading buffer. The elutions were denatured and analyzed by SDS-PAGE and western blot. Three independent experiments were performed. Immunoprecipitation efficiencies were represented by the relative intensity of co-immunoprecipitated protein/ immunoprecipitated protein. Statistical significance was determined by one-way ANOVA with post-hoc Tukey t test for multiple comparisons.

For endogenous immunoprecipitation assays, mouse brains were dissected at E18.5 and homogenized in ice-cold tissue lysate buffer (50mM TrisHCl, 150mM NaCl, 1Mm EDTA, 1% NP40, pH 6.8) supplemented with Halt^{TM} protease inhibitor and phosphatase inhibitor (single-use cocktail 100x, Thermo Fisher Scientific, Inc, Rockford, Illinois). After incubation on ice for 30 mins, tissue lysates were clarified by centrifugation at 14,000 rpm for 15 mins at 4^{o}C. 1ml supernatants at 1mg/ml protein concentration were incubated with 2µg rabbit anti-Kif21a (generated by our lab as described (Tischfield et al., 2010)) or mouse ati-Map1b (BD Biosciences, San Jose, California) antibody and 40µl protein-G agarose beads (Invitrogen, Carlsbad, California). Immunoprecipitation and western blot were performed as described above.

GST-fused protein purification

Expression of GST-fused WT and mutant 3rd coiled-coil domains in BL21 cells were induced by 0.1 mM IPTG overnight at 16 ^{o}C. Cells were harvested by centrifugation at 7700g at 4^{o}C and sonicated in 1xPBS supplemented with 1% triton and protease inhibitors. Cell lysates were centrifuged at 18,000g for 20 min at 4^{o}C, and supernatants incubated with glutathione sepharose beads (GE Healthcare Life Sciences, Pittsburgh, PA) for 2 hours at 4^{o}C followed by washing in 1XPBS. The bound GST-fused proteins were incubated with Thrombin (80Units/ml, GE Healthcare Life Sciences, Pittsburgh, PA) in 1XPBS to cleave off the GST tag and quenched by Benzamidine sepharose beads (GE Healthcare Life Sciences, Pittsburgh, PA) in 1XPBS containing 0.5MNaCl. The purified proteins were concentrated in BRB80 buffer using a

Microcon Centrifugal Filter (EMD Millipore Corporation, Billerica, Massachusetts) and their final concentrations were determined by SDS-PAGE and coomassie-blue staining using BSA as standard.

In vitro single molecule imaging and measurements

HEK293 cells transfected with GFP-fused WT or mutant Kif21a constructs for 24-36 hrs were lysed in BRB80 buffer (80 mM PIPES, 2 mM MgCl2, 1 mM EGTA[pH 6.8]) supplemented with 2 mM Mg-ATP (Sigma, St. Louis, Missouri), 1% Triton, Halt™ protease and phosphatase inhibitor (single-use cocktail 100x, Thermo Fisher Scientific, Inc, Rockford, Illinois). Clarified lysates were diluted in motility buffer (12 or 30 mM PIPES, 1 mM $MgCl_2$, 2 mM EGTA [pH 6.8], 1 mM DTT, 2mM Mg-ATP, 1.25 mg/ml casein, 2 mM Trolox and 20 µM taxol (Sigma, St. Louis, Missouri)) supplemented with an oxygen scavenger system (Qiu et al., 2012) and added to motility chambers containing taxol-stabilized Cy5-microtubules. Equal protein loading was confirmed by western blot analysis.

Images were recorded using an Olympus IX-81 Total Internal Refection Fluorescence (TIRF) microscope with a 100X 1.45 N.A. oil immersion TIRF objective (Olympus, Center Valley, Pennsylvania). Time-lapsed single molecule images generated by TIRF microscopy were captured using a back-thinned electron multiplier CCD camera (Hamamatsu). An active landing event was defined as a new Kif21a/microtubule association event that occurred after the start of data acquisition and moved for at least 4 pixels in distance (0.6um). An inactive landing event (dead motor) was defined as a new Kif21a/microtubule association event that occurred after the start of data acquisition, lasted at least 4 pixels in time (8 sec), and did not move. Events with durations of less than 4 pixels on both the X- and Y-axes were discarded. Active and inactive landing frequencies were normalized by microtubule length and duration of data acquisition.

For assays measuring the frequency of landing of truncated KIF21A constructs with or without introduction of purified 3rd coiled-coil protein, the motility buffer containing 80 mM PIPES was used. For each construct, data were acquired from two independent experiments, and from three time-lapsed images (acquired every 2 sec for 5 min) for each experiment.

For run length and velocity assays of FL WT and mutant Kif21a constructs, the motility buffer containing 30 mM PIPES was used. For each construct, data were acquired from two independent experiments, and from three time-lapsed images (acquired every 2 sec for 10 min) for each experiment.

Run lengths and velocities were determined by building and analyzing kymographs using ImageJ (National Institutes of Health). Statistical significance was determined by one-way ANOVA with post-hoc Tukey t test for multiple comparisons.

HeLa cells extraction, immunofluorescence cell staining, and microtubule association assays

HeLa cells were seeded and transfected as described above. 24 hrs after transfection, non-extracted cells were washed with 37°C pre-warmed PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9), and then fixed with 37°C pre-warmed 0.25% glutaraldehyde/PHEM for 10 min. Extracted cells were treated with 37°C pre-warmed extraction buffer (PHEM buffer, 5 µM paclitaxel (Sigma, St. Louis, Missouri), 0.1% Triton-X100) for 2 mins and then fixed with 37°C pre-warmed 0.25% glutaraldehyde/PHEM for 10 mins. All cells were

then washed twice with 0.01M PBS and incubated in 1 mg/ml sodium borohydride for 30 mins to quench the glutaraldehyde. Following two washes with 0.01 M PBS, cells were permeabilized with 0.1% Triton-X100/0.01 M PBS and incubated in blocking buffer (5% normal goat serum, 1% BSA, 0.1% Triton-X100 in 0.01 M PBS) for 1 hr at RT and were then incubated with primary antibodies, rabbit anti-Myc (1:200, Santa Cruz Biotechnology, Inc, Santa Cruz, California) and/or mouse anti-α-tubulin (1:1000, Sigma, St. Louis, Missouri) overnight at 4^{o}C. The following day, following 3 washes with 0.01 M PBS, cells were incubated with Alexa Fluor 594 or Alexa Fluor 488 conjugated goat anti-rabbit and/or goat anti-mouse IgG secondary antibodies (1:1000, Invitrogen, Carlsbad, California) for 1 hr at RT. Both primary and secondary antibodies were diluted in blocking solution. After several washes with 0.01M PBS, cells were mounted on slides by Fluoromount-G reagent (SouthernBiotech, Birmingham, Alabama). Images acquired by a laser scanning confocal microscope (LSM 510 META/NLO; Carl Zeiss MicroImaging, Inc., Thornwood, NY) with LSM software 3.2 (Carl Zeiss MicroImaging, Inc.). 50 transfected cells were counted for microtubule association for each construct for each experiment (n=3). Statistical significance was determined by one-way ANOVA with post-hoc Tukey t test for multiple comparisons

EB3 microtubule dynamics and polymerization assay

An EB3-mCherry construct was co-transfected with GFP-, GFP-WT FL Kif21a, or GFP- mutant FL Kif21a constructs in COS7 cells. 24 hours after transfection, 20-sec time-lapse images were acquired on an inverted Nikon Eclipse Ti epifluorescence microscope described above. Analyses of microtubule growth and dynamics were performed using Nikon Elements software as described in (Hammond et al., 2010). Background subtraction was performed using the mean pixel value of a non-cell region of each image and maximum projections were assembled from sequential images recorded at 2-sec intervals with Nikon Elements software. For ease of visualization, the first frame was depicted in blue, the last frame in red, and intermediate frames in green. EB3-mCherry labeled microtubules that had both blue and red frames visible during the 20s recording were defined as having persistent growth and their velocities were measured as an indication of microtubule polymerization rate. EB3-mCherry labeled microtubules that lacked a red frame or a blue frame or both were defined as having non-persistent growth. The experiment was repeated three times and, for each experiment, at least 3 cells from each condition and for each construct were quantified. Statistical significance was determined by one-way ANOVA with post-hoc Tukey t test for multiple comparisons.

Oculomotor explant culture and analysis

Midbrains were dissected from E11.5 *Isl^{MN}:GFP Kif21a$^{KI/KI}$* and *Kif21a$^{+/+}$* embryos. GFP-positive oculomotor neurons were then visualized using a fluorescence dissecting microscope (Nikon) and the nucleus dissected out by tungsten needles (Fine Science Tools, Foster City, California) in 1X HBSS buffer (Invitrogen). Oculomotor explants were plated on PDL/ laminin (Invitrogen, Carlsbad, California) coated coverslips and cultured in neural basal medium supplemented with B27 (Invitrogen, Carlsbad, California), L-glutamine, streptavidin/ampicillin and 25 ng/ml of BDNF, GDNF, and CNTF (ProSpec, East Brunswick, New Jersey). Following 17-20 hours of culture, oculomotor explants were fixed or used for live imaging.

For oculomotor growth cone behavior and growth assays, images were acquired at 1 min intervals during 30 minutes of live imaging on an inverted Nikon Eclipse Ti epifluorescence microscope with Coolsnap HQ2 camera (Photometrics, Tucson, Arizona) and Nikon Elements

software (Nikon, Melville, New York) with a 10X Plan Apo objective. Data were analyzed using ImageJ software (National Institutes of Health).

For oculomotor growth cone morphology and protein accumulation assays, explants were fixed in 1XPBS with 4% PFA and 10% sucrose at RT for 15 mins. Immunofluorescence staining was performed as described above using rabbit anti-Kif21a (1:2000), rabbit anti-KANK (1:300,Bethyl Laboratories, Montgomery, Texas), or phalloidin (1:100, Invitrogen, Carlsbad, California). Oculomotor explants were photographed at a fixed exposure time on the inverted Nikon Eclipse Ti epifluorescence microscope described above. GFP-positive growth cones were outlined and the mean fluorescence intensities of each protein measured within the oculomotor nuclei in the midbrain explant and in the GFP-positive growth cones using NIS Elements software. Mean intensity ratios were calculated by dividing the fluorescence intensity in the oculomotor nuclei by the fluorescence intensity in the growth cone.

For the axon outgrowth assay, we performed oculomotor explant cultures embedded in collagen gel (BD Biosciences, San Jose, California) from E11.5 *Thy1*^{MN}*:GFP Kif21a*$^{KI/KI}$ and *Kif21a*$^{+/+}$ embryos as described above. *Thy1:GFP* was chosen for these studies because we find the GFP signal remains stronger for longer periods of time in culture than *Isl*^{MN}*:GFP*. Explants were cultured for 3 days, fixed, and photographed using a laser scanning confocal microscope as described above. Maximum projections were assembled from Z-stacked images and axon outgrowth indexes were defined by the number of pixels of axon GFP fluorescence above background by ImageJ software (National Institutes of Health) as described in (Hansen et al., 2004). At least 3 WT/WT and 3 KI/KI explants were analyzed for each experiment. Statistical significance was determined using the two-tailed paired Student's t test.

Electron microscopy and morphometric analysis

Embryos from E12.5 *Kif21a*$^{+/+}$*;Isl*^{MN}*:GFP* and *Kif21a*$^{KI/KI}$*;Isl*^{MN}*:GFP* mice were dissected out in HBSS buffer. For each embryo, the forebrain was removed and the remaining embryo was immediately fixed in fixation solution (2% paraformaldehyde, 2.5% glutaraldehyde, 0.02% $CaCl_2$, and 2% tannic acid in 0.1M cacodylate buffer) for 4 hrs at RT. Embryos were then moved to fixation solution without tannic acid and fixed overnight at RT. The oculomotor nerve, which was visualized by GFP fluorescence, together with surrounding tissue was carefully dissected out in 0.02% $CaCl_2$/0.1M cacodylate buffer and processed for plastic embedding and electron microscopy. Three cross-sectional levels located proximal to, within, and distal to the bulb in two mutant mice were analyzed, and compared to the equivalent proximal and distal sections from two WT mice. For each of the ten cross-sections, the circumference of each object within the oculomotor nerve was traced by hand using Fiji (National Institutes of Health) and then identified as a cross-sectional axon, longitudinal axon, central growth cone, lamellipodia/filopodia, or degenerating axon by the following criteria (Brunso-Bechtold and Vinsant, 1988; Williams et al., 1986; Williams et al., 1991). Cross-sectional axons were defined as axons with regular circular shapes and similar diameters in X and Y axes, containing an even distribution of at least 3 or 4 microtubules visualized as circles of 25nm diameter, with or without membranes, vesicles and mitochondria. Longitudinal axons were defined as axons having a dominant diameter in one axis with most or all microtubules appearing as longitudinal tubes with 25nm width. Central growth cones were defined as objects, often with an irregular shape, containing only one microtubule in total, or within the portion of the object deemed the growth cone, with or without membranes, vesicles and mitochondria. Lamellipodia and filopodia were defined as objects containing no microtubules, neurofilaments, membranes or mitochondria. Degenerating axons

were defined as objects containing aggregated membranes, vesicles and mitochondria and often containing multiple layer inclusion bodies.

Graphs of the numbers and average areas of objects were generated by Microsoft Excel. In addition, a standard width (1.3μm) grid was placed over the four proximal nerve sections, and the cross-sectional axons that fell completely or partially within each grid were scored by hand using Fiji for number of mitochondria, vesicles, and membranes contained within each. The graph of these numbers was generated by SPSS.

Mass spectrometry

E18.5 wildtype mouse brain lysates were immunoprecipitated using three different anti-Kif21a antibodies recognizing (1) residues C-terminal to the WD40 domain (Tischfield et al., 2010), (2) residues in the1st coiled-coil stalk domain, and (3) residues in the linker between the 2nd and 3rd coiled-coil stalk domains of Kif21. Immunoprecipitated proteins were separated by SDS-PAGE followed by in gel trypsin digestion and mass spectrometry analysis.

GST Pull-down assay

GST-fused motor, 3rd coiled-coil stalk and WD40 domains were purified as described above and bound to glutathione sepharose beads (GE Healthcare Life Sciences, Pittsburgh, PA). Wildtype mouse brains were dissected at E18.5 and homogenized in ice-cold tissue lysate buffer (50mM TrisHCl, 150mM NaCl, 1Mm EDTA, 1% NP40, pH 6.8) supplemented with Halt^{TM} protease inhibitor and phosphatase inhibitor (single-use cocktail 100x, Thermo Fisher Scientific, Inc, Rockford, Illinois). After incubation on ice for 30 mins, tissue lysates were clarified by centrifugation at 14,000 rpm for 15 mins at 4^{o}C. 1ml supernatants at 1mg/ml protein concentration were incubated with 50 μl of the glutathione sepharose beads bound to the GST-fused proteins or to GST alone for 4 hours with gentle rotation at 4^{o}C. After 5 washes with tissue lysate buffer, beads were re-suspended by 50μl 2x loading buffer. The elutions were denatured and analyzed by SDS-PAGE and western blot or coomassie-blue staining.

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