eNeuro

Research Article: New Research | Disorders of the Nervous System

The X-linked autism protein KIAA2022/KIDLIA regulates neurite outgrowth via N-cadherin and δ-catenin signaling

KIDLIA in brain development and dendrite growth

James Gilbert[1] and Heng-Ye Man[1,2]

[1]*Department of Biology, Boston University, 5 Cummington Mall, Boston, MA 02215*

[2]*Department of Pharmacology & Experimental Therapeutics, Boston University School of Medicine, 72 East Concord St., L-603, Boston, MA 02118*

DOI: 10.1523/ENEURO.0238-16.2016

Received: 11 August 2016

Revised: 21 September 2016

Accepted: 14 October 2016

Published: 21 October 2016

Author Contributions: JG and HYM designed research, JG performed research and analyzed the data, and JG and HYM wrote the paper.

Funding: National Institute for Health Mental Health
R01 MH079407

Funding: National Natural Science Foundation of China
81428009

Conflict of Interest: The authors report no conflicts of interest

This work was supported by NIH grant R01 MH079407 and National Natural Science Foundation of China (81428009) (HYM). The authors declare no competing financial interests.

Corresponding Author: Heng-Ye Man, Tel: 617-358-4283. Email: hman@bu.edu

Cite as: eNeuro 2016; 10.1523/ENEURO.0238-16.2016

Title: The X-linked autism protein KIAA2022/KIDLIA regulates neurite outgrowth via N-cadherin and δ-catenin signaling

Running Title: KIDLIA in brain development and dendrite growth

Authors: James Gilbert[1], Heng-Ye Man[1,2]

[1] Department of Biology, Boston University, 5 Cummington Mall, Boston, MA 02215

[2] Department of Pharmacology & Experimental Therapeutics, Boston University School of Medicine, 72 East Concord St., L-603, Boston, MA 02118

Corresponding Author: Heng-Ye Man (617-358-4283, hman@bu.edu)

Author Contributions: JG and HYM designed research, JG performed research and analyzed the data, and JG and HYM wrote the paper

Number of Figures: 7

Number of Tables: 0

Number of Multimedia: 0

Abstract: 250 words

Significance Statement: 103

Introduction: 656 words

Discussion: 1177 words

Acknowledgments: We would like to thank Dr. Greg Dillon and Dr. Angela Ho for technical assistance, and Man Lab members for helpful discussion.

Conflicts of Interest: The authors report no conflicts of interest

Funding Sources: This work was supported by NIH grant R01 MH079407 and National Natural Science Foundation of China (81428009) (HYM). The authors declare no competing financial interests.

Abstract

Our previous work showed that loss of the KIAA2022 gene protein results in intellectual disability with language impairment and autistic behavior (KIDLIA, also referred to as XPN). However, the cellular and molecular alterations resulting from a loss-of-function of KIDLIA and its role in autism with severe intellectual disability remain unknown. Here we show that KIDLIA plays a key role in neuron migration and morphogenesis. We find that KIDLIA is distributed exclusively in the nucleus. In the developing rat brain, it is expressed only in the cortical plate and sublate region but not in the intermediate or ventricular zone. Using *in utero* electroporation, we find that shRNA-mediated knockdown of KIDLIA leads to altered neuron migration and a reduction in dendritic growth and disorganized apical dendrite projections in layer II/III mouse cortical neurons. Consistent with this, in cultured rat neurons, a loss of KIDLIA expression also leads to a suppression in dendritic growth and branching. At the molecular level, we find that KIDLIA suppression leads to an increase in cell-surface N-cadherin and an elevated association of N-cadherin with δ-catenin, resulting in a depletion of free δ-catenin in the cytosolic compartment. The reduced availability of cytosolic δ-catenin leads to elevated RhoA activity and reduced actin dynamics at the dendritic growth cone. Furthermore, in neurons with KIDLIA knockdown, over-expression of δ-catenin or inhibition of RhoA rescues actin dynamics, dendritic growth and branching. These findings provide the first evidence on the role of the novel protein KIDLIA in neurodevelopment and autism with severe intellectual disability.

Significance Statement

Autism spectrum disorder (ASD) is a neurodevelopmental impairment with a strong genetic basis. The cellular and molecular mechanisms linking the autism and intellectual disabillity - related genes to the impairment in brain development remain to be fully elucidated. This is the first study to examine the distribution, expression and neurobiological function of KIAA2022/KIDLIA, a novel gene protein implicated in ASD and intellectual disability. We report that KIDLIA is a neuron-specific nuclear protein expressed in the subplate and cortical plate in

the developing brain. Loss of KIDLIA expression impairs neuron migration, dendritic growth and morphogenesis *via* regulation of the N-cadherin-δ-catenin signaling pathway and actin dynamics.

Introduction

Autism spectrum disorder (ASD) is described on the basis of its three core symptoms of diminished language and communication, impaired social interactions and the tendency for repetitive behaviors (Lord *et al*, 1989). ASD is becoming increasingly devastating due to its high prevalence, impact on families and cost to society. Approximately 1 in 68 individuals in the United States have ASD and roughly 30% of those with ASD have intellectual disability (ID) (Baio, 2014).

The mammalian X chromosome is enriched with genes expressed in the brain, as demonstrated by the high incidence of X-linked ID (Nguyen and Disteche, 2006; Skuse, 2005). Previous work from our group and others identified loss of function of KIDLIA (also referred to as KIAA2022 or Xpn) at chromosome Xq13.2 as the gene responsible for severe ID and autistic behavior in several families (Cantagrel *et al*, 2009; Cantagrel *et al*, 2004; Charzewska *et al*, 2014; Ishikawa *et al*, 2012; Kuroda *et al*, 2015; Van Maldergem *et al*, 2013). We have previously shown that knockdown of KIDLIA in rat hippocampal neurons led to impaired neurite outgrowth (Van Maldergem *et al*, 2013). Patients with a loss of KIDLIA show symptoms typical of ASD including febrile seizures, repetitive behaviors, impaired language, microcephaly and strabismus (Cantagrel *et al*, 2009; Cantagrel *et al*, 2004; Van Maldergem *et al*, 2013), establishing the gene as a causal factor for ASD with ID.

Accumulating evidence has shown that alterations in neurite outgrowth and branching are a common phenotype in neurodevelopmental disorders including ID and ASD (DiCicco-Bloom *et al*, 2006; Zikopoulos and Barbas, 2010). Many autism-related mutations, such as those in phosphatase and tensin homolog on chromosome ten (PTEN), tuberous sclerosis complex 1 (TSC1), or SHANK3, result in an excess of branching (Kwon *et al*, 2006; Peça *et al*, 2011; Weston *et al*, 2014). While others, such as mutations in methyl CpG binding protein 2 (MeCP2), thousand and one kinase 2

(TAOK2) and endosomal Na+/H+ exchanger 6 (NHE6) lead to diminished branching (Belichenko *et al*, 2009; de Anda *et al*, 2012; Ouyang *et al*, 2013). As a novel gene product involved in autism with severe intellectual disability, the role for KIDLIA in brain development including neurogenesis, neuron migration and neuron morphogenesis has not been investigated. Our previous work has suggested an involvement of KIDLIA in neurite outgrowth (Van Maldergem *et al*, 2013), but the molecular mechanisms remain unknown.

N-cadherin-mediated extracellular interactions have been shown to be required for dendrite growth (Tan *et al*, 2010). A major molecule that interacts with the cytoplasmic C-terminal of N-cadherin is δ-catenin. δ-catenin, a member of the p120 catenin family, is neuron specific and binds to the juxtamembrane segment of N-cadherin (Lu *et al*, 1999). δ-catenin is a major candidate gene in autism and part of a protein network that is strongly involved regulating dendrite growth (Turner *et al*, 2015). Overexpression of δ-catenin can induce dendritic protrusions in hippocampal neurons (Martinez *et al*, 2003), while loss of δ-catenin reduces dendritic growth and branching (Arikkath *et al*, 2008; Elia *et al*, 2006).

For the first time we report a role for KIDLIA in neuron migration and dendrite morphological development. We show that KIDLIA is expressed exclusively in the nucleus and is neuron specific. We find that loss of KIDLIA produces aberrant neuronal migration with major defects in apical dendrite growth and orientation *in vivo*. Knockdown of KIDLIA *in vitro* results in a decrease in dendritic growth and actin dynamics. A loss of KIDLIA also leads to increased N-cadherin at the plasma membrane and an elevated interaction between N-cadherin and δ-catenin. This increased association depletes the cytoplasmic pool of δ-catenin causing activation of RhoA-GTP. Consistent with this, we find that after KIDLIA knockdown in neurons, overexpressing δ-catenin or inhibiting RhoA activity rescues the defects in dendritic growth and actin dynamics. These findings strongly support a role for the N-cadherin-δ-catenin-RhoA signaling system in the KIDLIA-dependent dysregulation of dendritic morphogenesis, providing novel insights into the mechanism of KIDLIA-dependent autism and ID.

Materials and Methods

Antibodies, plasmids and drugs. Primary antibodies to the following proteins were used: rabbit anti-KIAA2022 (1:100 (brain slice), 1:500 (primary culture) for immunohostochemistry (IHC), 1:500 for western blot (WB), Sigma-Aldrich Cat. #: HPA000404, RRID: AB_1079208), mouse anti-NeuN (1:100 for IHC; Millipore), rabbit anti-GFAP (1:100 for IHC; Millipore Cat. #: MAB377, RRID: AB_2298772), mouse anti-Tau1 (1:800 for IHC; EMD Millipore Cat. #: MAB3420, RRID: AB_94855), rabbit anti-MAP2 (1:1000 for ICC; Abcam Cat. #: ab70218, RRID: AB_1269354) mouse anti-N-Cadherin (1:1000 for WB and 5 µg for immunoprecipitation (IP), respectively; BD Transduction Laboratories Cat. #: 610920, RRID: AB_2077527), mouse anti-δ-catenin (1:1000 for WB, 5 µg for IP; BD Transduction Laboratories Cat. #: 611536, RRID: AB_398994), mouse anti-RhoA (1:500 for WB; Cytoskeleton, Inc. Cat. #: ARH03-A, RRID: AB_10708069), mouse HDAC1 (1:1000 for WB; Cell Signaling Cat. #: 5356P, RRID: AB_10858225), mouse anti-α-tubulin (1:5000 for WB; Sigma-Aldrich Cat. #: 00020911 RRID: AB_10013740), mouse anti-GAPDH (1:3000 for WB; Abcam Cat.#: ab8245 RRID: AB_2107448). The following secondary antibodies were used: IgG-HRP for WB (1:5000; BioRad, mouse (Cat. #: 170-6516 RRID: AB_11125547) and rabbit (Cat. #: 170-6515 RRID: AB_11125142)) and Alexa Fluor 488 (1:700, Molecular Probes, mouse: Cat. #: A21121 RRID: AB_141514; rabbit: Cat. #: A11094, RRID: AB_221544) and Alexa Fluor 555 (1:700, Molecular Probes, mouse: Cat. #: A21127 RRID: AB_141596; rabbit: Cat. #: A21428 RRID: AB_141784) for ICC.

GFP–δ-catenin was a kind gift from S. Bamji (University of British Colombia), pEGFP-N1 was obtained from Addgene (Cat. #: 2491, RRID: SCR_005907). For lentiviral shRNA, two KIDLIA shRNA sequences and a scrambled shRNA sequence were designed using the siRNA Wizard v3.1 (Invivogen, http://www.invivogen.com/sirnawizard/) and cloned into the pLKO.1-TRC cloning vector (Addgene, Cat. #: 10878, RRID: SCR_005907) using the AgeI and EcoRI sites. For *in utero* electroporation, the same shRNA sequences were cloned into the pCGLH GFP vector using the BglII and SalI sites. KIDLIA siRNA oligomers for transfection were purchased from Qiagen. The RhoA

inhibitor, CN06, was added directly to the culture media for the times indicated (10 μM, Cytoskeleton, Inc. Cat #: CN06).

Primary neuronal culture. Cortical and hippocampal brain tissue were dissected out from E18 rat fetus brains of either sex and prepared for primary culture. Tissues were first digested with papain (0.5mg/ml in HBSS, Sigma-Aldrich Cat. #: 4762) at 37°C for 15 min, then gently triturated in trituration buffer (0.1% DNase (Cat. #: PA5-22017 RRID: AB_11153259), 1% ovomucoid (Sigma-Aldrich Cat. #: T2011)/1% bovine serum albumin (Sigma-Aldrich Cat. #:05470) in DMEM) until neurons were fully dissociated. Dissociated cortical neurons were then counted and plated into either 6-well plates or 60mm Petri dishes (Greiner Cellstar) for western blot experiments. Hippocampal neurons were plated on 18mm circular coverslips (Carolina Cat. #: 633013, No. 0 in 60mm Petri dishes (5 coverslips/dish) and 6 well glass-bottom imaging dishes (In Vitro Scientific Cat #: P06-20-1-N) for immunocytochemistry and FRAP experiments. Both dishes and coverslips were coated with poly-L-lysine (Sigma-Aldrich Cat #: P2636, 100 μg/ml in Borate buffer) overnight at 37°C then washed three times with sterile DI water and left in plating medium [MEM (500 mL) containing 10% fetal bovine serum (Atlanta Biologicals Cat. #: S11550), 5% horse serum (Atlanta Biologicals Cat. # S12150), 31 mg L-cysteine, and 1% penicillin/streptomycin (Corning Cat. #: 30-002-CI) and L-glutamine (Corning Cat. #: 25-005-CI) before cell plating. Plating medium was replaced by feeding medium (Neurobasal medium supplemented with 1% horse serum, 2% B-27 and 1% Pen/Strep/L-Glu) the day after cell plating. Neurons were maintained in feeding medium with 5'-Fluoro-2'-Deoxyuridine (10 μM, Sigma-Aldrich Cat. #: F0503) supplemented at DIV5 to suppress glial growth until experimental use. Cultures treated with virus were given 250 μl of viral medium added directly to the plating medium at time of plating. The virus was removed 18-24 hr later when feeding medium was added.

Neuronal transfection and viral infection. Hippocampal neurons were transfected at the time of plating in 6 well glass bottom dishes for FRAP experiments using Lipofectamine 2000 (Thermo Fisher

Cat. #: 11668019) and the target plasmid DNA or siRNA per the manufacturer's suggestion. For live imaging experiments, neurons were transfected at DIV5. For one well containing two mL plating medium + cells, 3 µL Lipofectamine 2000 and 1 µg plasmid DNA + 2 µL siRNA (20 µM) were first separately diluted in 50µL MEM then mixed and incubated at room temperature for 20 min to form the transfection complex. The transfection complex was added to the wells and incubated at 37°C for 4 hr before the medium was removed and replaced with feeding medium. Neurons were then cultured for stated times for FRAP or live imaging experiments.

Recombinant lentiviruses were produced by transfecting HEK293T cells with plasmids for the shRNA constructs with viral packaging and envelope proteins (pRSV/REV, pMDLg/RRE, and pVSVG) using polyethylenimine (PEI) reagent (Polysciences, Inc. Cat #: 23966). Conditioned medium containing lentivirus was harvested after 48 h, centrifuged at 1000 × g for 10 min, filtered through a 0.45µm filter and stored at −80°C. Neurons were infected with lentivirus on the day of plating and media was changed 1 d later to feeding medium.

Animals. Timed pregnant CD-1 mice were purchased from Charles River Laboratories (Cambridge, MA, strain code: 022) for *in utero* electroporation experiments. All animals were maintained in accordance with guidelines of the Boston University Institutional Animal Care and Use Committee. Care was taken to minimize suffering of the animals during surgical procedures. The first neonatal day was considered to be postnatal day 0 (P0).

***In utero* electroporation (IUE).** IUE was performed, as described previously (Gal *et al*, 2006), on timed pregnant CD-1 dams purchased from Charles River Laboratories at embryonic day 14.5. Briefly, dams were anesthetized *via* intraperitoneal (IP) injection of a ketamine/xylazine mixture and the uterine horns were exposed *via* midline laparotomy. One to two microliters of plasmid DNA mixed with 0.1% fast green dye (Sigma-Aldrich Cat. #: F7258) was injected intercerebrally through the uterine wall and amniotic sac using a pulled glass micropipette. The plasmid vectors were used at a final

concentration of between 2 and 3 µg/µl. The anode of a tweezertrode (Harvard Apparatus) was placed over the dorsal telencephalon above the uterine muscle and four 35 V pulses (50 ms duration separated by a 950 ms interval) were applied with a BTX ECM830 pulse generator (Harvard Apparatus). Following electroporation, the uterine horns were returned to the abdomen and the cavity was filled with a warm saline solution and the incisions were closed with silk sutures. The dams were then placed in a clean cage and monitored closely during recovery. The pups were allowed to mature with the mother until the times indicated. To collect the electroporated brains, animals were anesthetized with an IP injection of ketamine/xylazine and transcardially perfused with ice-cold PBS. The brains were removed and placed into a 4% paraformaldehyde in PBS solution at 4°C for 6 hrs, followed by overnight incubation in a 30% sucrose PBS solution at 4°C. The brains were placed in trays and submerged in OCT embedding medium (Tissue-Tek Cat. #; 25608-930) and flash frozen by placing the trays in a bath of methanol mixed with dry ice. Frozen brains were cut in 35 µm sections on a Leica CM 1850 cryostat (Leica Biosystems) at -20°C. These procedures were reviewed and approved by the Boston University Institutional Animal Care and Use Committee.

Immunocytochemistry. Hippocampal neurons were washed twice in ice-cold ACSF and fixed for 10 min in a 4% paraformaldehyde/4% sucrose solution at room temperature. Cell membranes were permeabilized for 10 min in 0.3% Triton-X-100 (Sigma-Aldrich Cat. #: T-9284) in phosphate buffered saline (PBS), rinsed three times in PBS, then subjected to a blocking procedure (1 hr PBS + 5% goat serum). After blocking, cells were incubated with primary antibodies (in 5% goat serum PBS) for 2-hr at room temperature, washed and incubated with Alexa Fluor-conjugated fluorescent secondary antibodies (1:700) for an additional hour. Cells were then mounted to microscopy glass slides with Prolong Gold anti-fade mounting reagent (Thermo Fisher Cat. #: P36930) for subsequent visualization.

Microscopy. For *in vitro* analysis of neuronal morphology, a Carl Zeiss inverted fluorescent microscope was used to collect images with a 40× oil-immersion objective (numerical aperture, 1.3) and collected with AxioVision Release 4.5 software. Neuron images were quantified in Sholl analysis using NIH ImageJ (see below).

Brain sections taken after IUE were imaged using a Zeiss LSM 700 laser scanning confocal microscope. 25 µm, 40× Z-stack images were acquired using the Zen software package. GFP positive cells were traced with the NeuronJ plugin in ImageJ for analysis of dendrite morphology. Sections from at least three embryos were counted and analyzed for each experiment, and surgeries for each combination of plasmids were repeated to confirm the results.

Sholl analysis. MAP2-positive dendrites were traced from images of neurons stained with MAP2 and tau1 using the NeuronJ (RRID:SCR_002074) plugin in ImageJ (RRID: SCR_003070). The Snapshot tool in NeuronJ was used to save the tracings as an image file that was converted to 8-bit and these images were analyzed with the Sholl analysis plugin in ImageJ. The range of measurement was set using the straight line tool traced from the center of the soma to the outermost neurite. Dendrite intersections were analyzed from a starting radius of 10 µm with 10 µm steps to the outer radius. The resulting number of intersections per cell were used to calculate the mean and the standard error mean for each radius interval. Treatments were applied to sister cultures that originated from the same neuron culture preparation.

Western Blot. Cortical neurons cultured in either 60 mm (3×10^6 cells / dish) or 6-well plates (10^6 cells / well) were treated with virus at the time of plating and any other drugs as stated. Neurons were lysed in Laemmli 2X sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl) and boiled for 10-min at 95°C for SDS page electrophoresis. After separation in SDS page, proteins were transferred to PVDF membranes (Bio-Rad) and probed for different targets with the stated antibodies. Immunoblots were visualized using a

chemiluminescence detection system (GE Healthcare) and exposed to Fuji medical X-ray films (Fisher Scientific), scanned and analyzed using ImageJ.

Quantitative Real-Time PCR. Total RNA from rat cortical cultures was purified using TRIzol (Thermo Fisher Cat #: 12183555) and reverse transcribed using the SuperScript III Reverse Transcription System (Thermo Fisher Cat. #: 12574035). The optical density A260/A280 ratio was confirmed to be >1.9 for each sample. For N-cadherin, we used the oligonucleotides: 5′-ATCATTCGCCAAGAGGAAGG-3′ and 5′-GGCTGAAAATAGACCCTGTGA-3′. Quantitative real-time PCR was performed with a 7300 real-time PCR system (Applied Biosystems) using Power SYBYR Green Master Mix (Applied Biosystems Cat. #: 4367659) with the following PCR conditions: initial hold at 95 °C for 10 min, followed by 40 cycles of a 15 s denaturing step at 95 °C and a 60 s annealing and extension step at 60 °C. Transcript levels were normalized to the housekeeping gene GAPDH using the oligonucleotides: 5′-CCATCAACGACCCCTTCATT-3′ and 5′-CTGAGAATGGGAAGCTGGTC-3′.

Cell Surface Protein Biotinylation Assay. Cortical neurons were rinsed with ACSF once then incubated with EZ Link-Sulfo-NHS-LC-Biotin (Thermo Scientific Cat #: 21327) dissolved in 1 ACSF (1 mg/ml) for 10 minutes at room temperature and another 20 minutes at 4°C. Excess biotin reagent was quenched with two washes of ACSF with 20 mM glycine followed by another two washes with ACSF. Neurons were then lysed in lysis buffer 1 (1X PBS with 0.5% SDS, 0.5% SDOC, 1% Triton-X-100) with the addition of a protease inhibitor cocktail (Roche Cat. #: 04693116001), sonicated, then head-to-toe rotated at 4°C for 30 minutes to achieve thorough cell lysis. After spinning down at 13,000 rpm for 15 minutes, 1/10 of the supernatant volume was mixed with an equal volume of Laemmli 2X sample buffer, boiled 10 minutes at 95°C and saved as a total lysate control. The rest of the supernatant was removed to a new Eppendorf 1.5ml tube containing 40 µL pre-equilibrated NeutrAvidin beads (Thermo Fisher Cat. #: 29200), while the pellet was discarded. NeutrAvidin beads, along with the supernatant, were head-to-toe rotated at 4°C for at least two hours. The beads were

rinsed three times in PBS (with 0.5% Triton-X-100), before being mixed with an equal volume of Laemmli 2X sample buffer and boiled 10 minutes at 95°C in preparation for western blot analysis.

Fluorescence after photobleaching (FRAP). Images were taken with a Zeiss LSM 700 laser scanning confocal microscope. The inverted microscope was equipped with an incubation system featuring temperature and CO2 control. All experiments were performed at 37°C and 5% CO2. Live images were acquired using a 63x oil immersion objective lens (NA 1.32).

FRAP experiments were performed using the following protocol: 3 single pre-bleach scans were acquired at 3 s intervals, followed by up to 5 bleach scans at full laser power until the fluorescence reached 25% of the original pre-bleach levels, over a circular area of 4 µm in diameter. During the post-bleach period, scans were acquired at 3 s intervals.

Fluorescence was quantified using the FRAP plugin in ImageJ. Background fluorescence was measured in a random field outside of the region of interest and subtracted from all the measurements. Growth cone fluorescence was determined for each image and normalized to the initial pre-bleach fluorescence to determine the rate of fluorescence recovery.

The net fluorescence recovery (mobile fraction, M_f) measured in the region of interest was determined as $M_f = (F_{pre} – F_{post}) - (F_{pre} – F_{end})$ and the immobile fraction (IM_f) was calculated as $IM_f = (F_{pre} – F_{post}) - (F_{end} – F_{post})$, where F_{end} is the ROI mean intensity at the steady-state, F_{post} represents ROI intensity after photobleaching, and F_{pre} is the mean ROI intensity pre-bleach.

RhoA activation assay.

RhoA activity was assessed using a RhoA Activation Assay Biochem Kit according to the manufacturer's instructions for a (Cytoskeleton Inc. Cat. #: BK036). Briefly, GTP-RhoA was immunoprecipitated from whole cell lysates with glutathione S-transferase-tagged Rhotekin bound to glutathioneagarose beads. The beads were washed and the immunoprecipitates were analyzed by

western blot using a RhoA-specific monoclonal antibody. The lysate was also probed for total RhoA and the GTP-RhoA was normalized to total RhoA levels.

Statistical analyses.

An unpaired Student's t-test or one-way ANOVA with *post-hoc* Tukey's test was used as appropriate.

Results

KIDLIA shows nuclear localization and neuron-specific expression *in vivo*

In situ hybridization studies have shown a lack of KIDLIA mRNA in proliferating, BrdU positive cells, indicating that it may only be expressed in post-mitotic neurons (Cantagrel *et al*, 2009), however, KIDLIA protein expression has not been examined in neurons. By immunostaining KIDLIA in brain slices from postnatal day 0 (P0, day of birth) mouse cortex, we observed an exclusive nuclear localization of KIDLIA that co-localized with the nuclear marker Hoescht (Figure 1A). To further investigate the KDILIA expression pattern in cortical layers *in vivo*, we compared the KIDLIA distribution in embryonic day 17 (E17) and P0 mouse brains. In E17 brain slices, KIDLIA was not expressed in the ventricular zone (VZ) and intermediate zone (IZ). Up from the VZ, only minimal background staining of KIDLIA protein was detected until the subplate (SP) region where KIDLIA expression levels were sharply increased and maintained throughout the entire CP (Figure 1B). Similarly, immunostaining of P0 brains showed that strong KIDLIA expression was restricted to the CP in contrast to the VZ/IZ area (Figure 1C). To determine whether KIDLIA was preferentially expressed between neurons and glia, we co-immunostained KIDLIA with the neuron-specific marker NeuN, or the glial marker GFAP. We found that immunofluorescent signals of KIDLIA completely co-localized with NeuN positive neurons at P0 and P14 (Figure 1D, 1E), but not with GFAP-positive glia (P14) (Figure 1F), indicating that KIDLIA expression was neuron specific.

***In utero* knockdown of KIDLIA regulates neuronal migration without affecting the multipolar to bipolar transition**

To investigate KIDLIA's role in neuronal migration, we utilized *in utero* electroporation (IUE) to knockdown KIDLIA expression during early development. KIDLIA shRNA-GFP, or a scrambled control, was electroporated into mouse embryonic brains at E15 and returned to the pregnant dam to develop (Figure 2A). Brains were first collected at E17 and immunostained for KIDLIA. Indeed, in GFP-positive shRNA electroporated neurons, KIDLIA expression was significantly reduced compared to scrambled controls and nearby non-electroporated neurons (Figure 2B).

Callosally projecting pyramidal neurons are born around E15 in the VZ and migrate to their final location in layers II/III in the CP. At E17, 60% of the neurons electroporated with scrambled shRNA were found in the IZ and only 13% had entered the CP. Strikingly, after electroporation of KIDLIA shRNA, 44% of the neurons were found in the IZ and 28% of the neurons had already migrated into the CP (Figure 2C, 2D). These results strongly indicate that loss of KIDLIA expression altered the migration process in the developing brain.

To investigate the effect of KIDLIA on neuron migration at a later stage, we next collected brains at P0 after IUE at E15. In agreement with our findings at E17, we found that 79% of KIDLIA-shRNA neurons had already reached the upper CP at P0, compared to only 64% in scrambled controls. Additionally, in shRNA electroporated brains, only 5% of neurons were found in the IZ compared to 15% in scrambled control brains (Figure 2E and 2F). Interestingly, although KIDLIA knockdown resulted in changes in the relative layer distribution of the migrating neurons, it did not affect their final laminar destination, with the majority of the electroporated neurons residing in layer II/III (Figure 2E).

After birth in the VZ, multipolar pyramidal neurons move within the IZ/SP where they must adopt a bipolar morphology to enter the CP. This multipolar to bipolar transition (MBT) is key to proper migration and integration of neurons into the cortical plate. KIDLIA protein begins to be expressed in the SP region below the cortical plate at E17 and may be involved in neuronal migration, we therefore

wanted to know whether loss of KIDLIA expression resulted in neurons bypassing the multipolar stage allowing them to move into the CP directly. In mice electroporated with KIDLIA shRNA at E15, we found that in E17 brains both KIDLIA shRNA and scrambled control neurons showed a typical multipolar morphology in the IZ/SP region. Similar to controls, KIDLIA knockdown neurons showed a normal bipolar morphology after entering the CP (Figure 2G and 2H). These findings suggest that KIDLIA doesn't affect the MBT during neuronal migration.

Knockdown of KIDLIA affects apical dendrite orientation *in vivo*

To examine the role of KIDLIA in neuronal development, we analyzed dendritic growth and soma location of neurons following IUE of KIDLIA shRNA at E15. At P4, neurons have reached their proper laminar location at layer II/III but the somas of neurons electroporated with KIDLIA-shRNA were located closer to the pial surface (Figure 3A and 3C), possibly arising from a facilitated migration rate and/or a disruption in the termination of migration. Similar to scrambled controls, neurons with KDILIA shRNA showed a clear apical dendrite growing as single straight process directed orthogonally to the pial surface (Figure 3A and 3B). However, neurons expressing KIDLIA shRNA had a longer apical neurite (Figure 3A, 3D). The percentage of cells whose apical neurites reached the pia was not significantly different from the control at P4 (Figure 3E).

Strikingly, brains analyzed at P14 showed that electroporation of KIDLIA shRNA significantly disrupted the orientation of the apical dendrite. The apical dendrites of neurons after KIDLIA knockdown were largely disorganized and not directed toward the pia, compared to age-matched controls (Figure 3F and 3G). A significant increase and variation in the angle of the apical dendrite to the pia was observed in KIDLIA shRNA electroporated brains compared to scrambled controls (Figure 3H). KIDLIA knockdown also produced a 35% decrease in the length of the major apical dendrite and a significant decrease in the distance of their somas to the pia (Figure 3I and 3J). Additionally, after KIDLIA knockdown, the number of dendrite tips that reached the pia was significantly decreased compared to controls (Figure 3K), presumably due to a disorganization of the apical dendrite

orientation and decreased dendritic growth. These *in vivo* results showed that after knockdown of KIDLIA, neuronal somas were positioned at sites closer to the pial surface at both P4 and P14. A loss of KIDLIA caused aberrant dendrite orientation and decreased apical dendrite growth, indicating that the directed outgrowth and branching of the dendritic tree was significantly impaired, possibly as a consequence of the neurons' early arrival into the CP or a subtle shift in laminar localization.

Knockdown of KIDLIA decreases dendritic outgrowth in primary cultured neurons.

To further investigate the molecular mechanisms underlying the effect of KIDLIA on dendritic growth and arborization, we suppressed KIDLIA expression in primary cultured neurons. We infected cortical neurons at the time of plating (DIV 0) with lentiviral shRNA containing the same targeting sequences as those used for IUE *in vivo*, and found a ~60% reduction in KIDLIA protein expression 6 days after infection (Figure 4A and 4B). Consistent with the *in vivo* observations, we confirmed a similar nuclear localization of KIDLIA in cultured neurons (Figure 4A, 4C).

To investigate the effect of KIDLIA knockdown on dendritic outgrowth, we infected neurons with KIDLIA shRNA virus at DIV0 and immunostained for MAP2 and tau1 at DIV12 to label the dendrite and axon, respectively (Figure 4D). Strikingly, Sholl analysis revealed a large decrease in dendritic branching after KIDLIA knockdown (Figure 4E). Compared to scrambled controls, we observed an ~25% reduction in the length of the longest dendrite, a nearly 50% reduction in the number of dendritic branches, and a 55% reduction in total dendritic length (Figure 4F-H). These results show that loss of KIDLIA expression severely stunted neuronal development *in vitro*.

Knockdown of KIDLIA leads to an increase in surface N-cadherin-δ-catenin association

N-cadherin, the neural member of the cadherin superfamily, has been shown to be involved in neurite outgrowth (Bard *et al*, 2008). N-cadherin is structurally composed of five extracellular cadherin domains, a single-pass transmembrane domain, and an intracellular domain that interacts with catenins which regulate downstream signaling cascades including the Rho GTPases.

A previous study has indicated that in PC12 cells, knockdown of KIDLIA up-regulates the expression of N-cadherin (Magome *et al*, 2013). We therefore sought to investigate whether knockdown of KIDLIA affected N-cadherin expression and localization in neurons. In DIV6 neurons which were infected with KIDLIA shRNA at DIV0, we found that KIDLIA knockdown led to a ~25% increase in N-cadherin protein levels compared to scrambled controls (Figure 5A and 5B). Because KIDLIA is localized in the nucleus, we next examined whether KIDLIA regulates N-cadherin gene transcription. We infected DIV0 cultured cortical neurons with KIDLIA shRNAs for 7 days, and measured N-cadherin mRNA by RT-PCR using the cell lysates. Indeed, after knockdown of KIDLIA we found a 35% increase in N-cadherin mRNA compared to scrambled shRNA and untreated controls (Figure 5C). This data suggests that the KIDLIA-induced increase in N-cadherin protein amount may result from an up-regulation in its gene transcription and translation.

To assess the membrane localization of N-cadherin after KIDLIA knockdown, we isolated cell-surface proteins through biotinylation and immunoprecipitation with Sulfo-NHS-SS-Biotin. Surprisingly, we found a greater than 2-fold increase in surface N-cadherin levels after shRNA-mediated knockdown of KIDLIA compared to scrambled controls (Figure 5D and 5E), indicating that KIDLIA knockdown not only increased overall N-cadherin expression, but also caused a translocation from the cytosolic compartment to the cell surface.

δ-catenin is a critical functional mediator for N-cadherin *via* a direct protein association. δ-catenin expression can induce a dendritic-like morphology in fibroblasts (Yu and Malenka, 2003) and stimulates neurite outgrowth in hippocampal neurons (Martinez *et al*, 2003). Importantly, an increase in membrane-localized N-cadherin suppresses the growth-promoting effects of δ-catenin, suggesting that surface N-cadherin acts as a buffer regulating cytosolic δ-catenin availability (Kim *et al*, 2008). Given that δ-catenin is brain specific (Abu-Elneel *et al*, 2008), and that its localization and ability to induce neurite outgrowth is tightly linked with N-cadherin, we hypothesized that the increased membrane expression of N-cadherin sequesters δ-catenin at the surface and thus depletes its cytosolic pool.

To investigate this hypothesis, we performed co-immunoprecipitation studies using lysates of DIV6 cultured cortical neurons infected with viral KIDLIA shRNAs. We found that knockdown of KIDLIA significantly enhanced the association of N-cadherin with δ-catenin. Meanwhile, we observed a major reduction in the amount of free unbound δ-catenin in the supernatant following immunoprecipitation (Figure 5F). These results indicate that loss of KIDLIA results in an increase in the association of N-cadherin with δ-catenin at the plasma membrane, resulting in a reduction in free cytosolic δ-catenin.

δ-catenin is involved in KIDLIA-dependent changes in neurite growth and arborization

Because free cytosolic δ-catenin is required for its downstream signaling, we next examined whether over-expression of δ-catenin after loss of KIDLIA could rescue the deficits in neurite outgrowth and branching. Neurons were treated with scrambled, or KIDLIA lentiviral shRNA, at DIV0 and transfected with δ-catenin-GFP, or GFP control, one day later (Figure 5G). At DIV6, Sholl analysis revealed a large decrease in dendritic branching in shRNA + GFP cells compared to scrambled + GFP. However, in neurons over-expressing δ-catenin-GFP + shRNA, neurite branching was restored to levels similar to scrambled controls (Figure 5H). These results strongly indicate that the decrease in neurite outgrowth and branching after knockdown of KIDLIA was caused by a reduction in free δ-catenin availability.

Loss of KIDLIA disrupts actin dynamics at the neurite growth cone

Actin is dynamically regulated at the neurite tips, which plays a crucial role in neurite growth (Meberg and Bamburg, 2000; Nicholson-Dykstra *et al*, 2005). To examine whether KIDLIA is implicated in cytoskeletal dynamics, we performed fluorescence after photobleaching (FRAP) experiments with fluorescently labeled actin after knockdown of KIDLIA with siRNA. To test the efficacy of the KIDLIA knockdown, scrambled or KIDLIA siRNA was co-transfected with GFP at DIV0, and immunostainings of KIDLIA at DIV4 showed a ~60% reduction in protein expression (Figure 6A and 6B). Next, GFP-

actin was transfected at DIV0 into hippocampal neurons with scrambled, or KIDLIA siRNA, and FRAP was performed on the growth cones at DIV4 (Figure 6C). In neurites from KIDLIA siRNA transfected neurons, the recovery rate of the actin signal was drastically reduced (Figure 6D). Compared to scrambled siRNA controls, KIDLIA knockdown showed a 70% decrease in the mobile fraction (M_f) of actin (Figure 6G) and a subsequent increase in the immobile fraction (IM_f) of actin (Figure 6H).

Due to the large reduction of free δ-catenin in the cytosol after KIDLIA knockdown, which is known to be involved in actin regulation, we next sought to examine whether over-expression of δ-catenin could rescue actin dynamics with the transfection of δ-catenin-GFP after siRNA-mediated knockdown of KIDLIA (Figure 6E). Indeed, FRAP revealed that in δ-catenin transfected neurons, actin dynamics were restored (Figure 6F) and the mobile and immobile fractions of actin returned to levels similar to controls (Figure 6G and 6H), indicating that the impaired actin dynamics likely resulted from a reduction of free cytoplasmic delta-catenin.

The role for RhoA in the KIDLIA-dependent impairment of actin dynamics

A major downstream effector of δ-catenin is the Rho-GTPase pathway, which links the cadherin/catenin complex to actin dynamics. RhoA GTPase activation leads to an inhibition in neurite growth (Kozma *et al*, 1997; Leeuwen *et al*, 1997). Studies have shown that free cytosolic δ-catenin, but not the membrane associated fractions, inhibit RhoA activity (Kim *et al*, 2008). Because KIDLIA regulates the association of δ-catenin to surface N-cadherin, and thus reduces the availability of cytosolic δ-catenin, we wanted to know whether KIDLIA knockdown affected RhoA activity. The Rho activity status alternates between an active, GTP-bound state and an inactive, GDP-bound state. To perform RhoA activity assays, we infected neurons with lentivirus containing scrambled or KIDLIA shRNA. Neuron lysates were incubated with a GST fusion protein containing the binding domain of the Rho effector protein rhotekin, so that the active form of RhoA was isolated by immunoprecipitation. We then probed for total RhoA in lysates and the immunoprecipitated active RhoA *via* western blot (Figure 7A). Interestingly, we found that KIDLIA knockdown led to a 60%

increase in the amount of active RhoA compared to scrambled controls (Figure 7B). Since RhoA activation prevents neurite initiation and induces neurite retraction, our findings suggest that the inhibitory effect of KIDLIA knockdown on dendrite growth may be mediated by RhoA.

To further determine the role of RhoA activity in the KIDLIA-dependent effects on neuron development, we first performed FRAP experiments to examine the actin dynamics with the application of CN06, which directly inhibits the primary RhoA effector ROCK (Ishizaki *et al*, 2000). In KIDLIA knockdown neurons, application of CN06 1 hr prior to FRAP was sufficient to rescue the actin dynamics to levels similar to scrambled controls (Figure 7C and 7D). Furthermore, we wanted to determine whether the RhoA signaling pathway was responsible for the KIDLIA-dependent impairment in dendritic arborization. We therefore infected neurons with KIDLIA shRNA virus at DIV0 followed by CN06 treatment from DIV4-DIV7. Dendritic structure indicated by MAP2 staining was examined *via* Sholl analysis. Indeed, after KIDLIA knockdown in neurons, we found that CN06 incubation rescued the defects in dendritic growth and branching (Figure 7E and 7F). These findings establish a molecular process in which KIDLIA knockdown induces an increase in membrane-bound N-cadherin and sequestration of cytosolic δ-catenin, leading to activation of the RhoA pathway and a subsequent alteration in actin dynamics, eventually causing suppression in neurite growth and branching (Figure 7G).

Discussion

Our study provides the first evidence showing that KIDLIA, the protein product of the recently identified XLID gene KIAA2022, plays an important role in neuron migration and morphogenesis. In mouse brain, knockdown of KIDLIA results in a redistribution of more neurons in the cortical plate at earlier time points and a subtle, but potentially consequential mis-localization of neurons within their destined cortical layers. In addition, we find that early growth of the leading apical neurite is positively regulated in KIDLIA knockdown neurons, however at a later time point at P14, loss of KIDLIA causes an inhibition in apical dendrite growth and a disruption of dendritic orientation. Given that IUE only

transfects a small portion of neurons derived at a specific time, the majority of the neighboring neurons remain unaffected, the effects of KIDLIA are likely neuron autonomous, rather than being caused by external environmental factors.

How neuron migration is regulated by KIDLIA remains to be investigated. KIDLIA protein expression was mostly restricted to the cortical plate with a distinct band of expression turning on in the subplate region just prior to the CP at E17 in mouse brain. IUE of KIDLIA shRNA led to more neurons migrating to upper cortical layers with a closer positioning of the soma to the pia. After migration, neurons reached their proper laminar layers, however the aberrant positioning of the soma relative to the pia may indicate a disruption in the termination of the migration process, and/or the final somal translocation after migration to layer II/III. However, we observed no change in the multipolar transition phase prior to entry into the CP. Future studies using *in utero* electroporation of KIDLIA shRNA at E12.5 to label layer V/VI neurons would be useful to investigate whether loss of KIDLIA could mis-localize deeper layer neurons to upper cortical layers. Additionally, aberrant soma positioning could be due to premature cell cycle exit during proliferation resulting in an earlier arrival of neurons to their destined cortical layers. The distinct expression pattern of KIDLIA within and just below the CP in embryonic brains suggests that KIDLIA may act as a checkpoint factor for CP entry; a loss of KIDLIA would therefore offer neurons free access to pass the IZ-CP border and enter into the CP.

We have observed similar changes in neurite growth in cultured neurons. KIDLIA knockdown led to a significant reduction in dendrite length and complexity. Using this *in vitro* system, we investigated the cellular mechanisms responsible for the disrupted dendritic development. We found that loss of KIDLIA induced an increase in total N-cadherin levels with a substantial increase in the surfaced-localized fraction, which was accompanied with an elevated association of surface N-cadherin with δ-catenin. The increased δ-catenin/N-cadherin association led to a depletion of the free cytosolic pool of δ-catenin thereby increasing the activation of the downstream RhoA pathway. The RhoA pathway is a major mediator of actin dynamics and neurite outgrowth and complexity.

Extensively branched processes can be induced when RhoA is inhibited *via* δ-catenin. Conversely, when RhoA is activated, dendrite length and the dendritic field is decreased (Li *et al*, 2000; Nakayama *et al*, 2000; Wong *et al*, 2000). The reduction in δ-catenin availability is responsible for the KIDLIA-dependent effect on dendrite morphogenesis because overexpression of δ-catenin rescued neurite growth and dendritic branching as well as actin dynamics in neurons after KIDLIA knockdown. In line with our findings that δ-catenin is a key mediator for the neuronal effects of KIDLIA, δ-catenin was identified as a major genetic target in autism population (Turner *et al*, 2015). Interestingly, of the autism genes positively correlated with δ-catenin, there is a significant enrichment in genes involved in dendrite morphogenesis including PDLIM5, SHANK1, CDKL5, DLG4 (Turner *et al*, 2015). In addition, loss of δ-catenin is implicated in impaired cognitive function and intellectual disabilities (Belcaro *et al*, 2015; Hofmeister *et al*, 2015; Medina *et al*, 2000), indicating a crucial role for δ-catenin in brain development and function.

Under the condition of KIDLIA knockdown, we find an increase in N-cadherin protein levels and an elevated association between N-cadherin and δ-catenin, but the underlying mechanism remains unclear. It is possible that a lack of KIDLIA expression in the nucleus leads to an up-regulation in N-cadherin gene transcription and subsequent protein translation. Regarding KIDLIA-induced changes in protein interaction, it has been shown that δ-catenin is subject to protein palmitoylation (Kang *et al*, 2008), a modification that causes enhanced association of δ-catenin with N-cadherin (Brigidi *et al*, 2014). Also, by binding to the juxtamembrane domain of cadherin, δ-catenin is known to stabilize and increase the amount of N-cadherin at the surface (Reynolds and Carnahan, 2004). It is thus conceivable that a loss of KIDLIA may cause δ-catenin palmitoylation, possibly *via* enhancing the expression of the palmitoyl-acyl transferase DHHC5 (Brigidi *et al*, 2014), leading to higher levels of association between δ-catenin and N-cadherin.

We found that the N-cadherin/δ-catenin effect is mediated by RhoA. The Rho GTPases regulate dendrite growth and branching *via* modulation of cytoskeleton components (Newey *et al*, 2005). The role for RhoA in dendritic branching has recently been established as RhoA activation

leads to a reduction in dendritic branching (Nakayama *et al*, 2000), while RhoA inhibition enhances branching (Neumann and Schweigreiter, 2002). Cytoplasmic δ-catenin has previously been shown to inhibit RhoA by keeping it in the inactive RhoA-GDP state, resulting in an increase in dendritic branching (Martinez *et al*, 2003). Constitutively active RhoA expression has been shown to reduce dendrite length and the volume of the dendritic field (Nakayama *et al*, 2000; Wong *et al*, 2000), while RhoA loss-of-function mutations has also been shown to cause abnormal dendritic arborization (Lee *et al*, 2000).

RT-PCR of mRNA prepared from prenatal and postnatal mouse brain shows a sevenfold increase in KIDLIA expression between embryonic day 10.5 (E10.5) and E18.5, indicating a role for KIDLIA in neural development. KIDLIA mRNA expression reaches a maximum at postnatal day 3 (P3) and is maintained at a low level into adulthood (Cantagrel *et al*, 2004; Ishikawa *et al*, 2012). Thus, KIDLIA expression peaks during key developmental time periods for neuronal growth and maturation. Both *in vivo* in mouse brain and in cultured neurons, immunostainings reveal a co-localization of KIDLIA with the nuclear marker Hoescht. KIDLIA is a large 170 kDa protein with monopartite and bipartite nuclear localization sequences. Given the specific nuclear localization, we suspect a major role for KIDLIA in gene regulation. It is possible that KIDLIA knockdown causes an up-regulation in N-cadherin gene transcription and thus an elevated amount of N-cadherin.

Attenuated growth and structural abnormalities in developing neurons lead to deficiencies in neuronal wiring and synapse formation during brain maturation. Aberrant neuron morphogenesis and synaptogenesis are associated with a number of brain disorders, including Fragile-X mental retardation, Rett syndrome, Down syndrome and CDKL5-related encephalopathy (Belmonte and Allen, 2004; Garner and Wetmore, 2012; Ricciardi *et al*, 2012; Shepherd and Katz, 2011). Given that KIDLIA is a novel gene with largely unknown functions, more studies are needed to elucidate the role of KIDLIA in multiple steps of neuron development including neurogenesis, morphogenesis, synapse maturation, synaptic plasticity and behavior.

Acknowledgments

We would like to thank Dr. Greg Dillon and Dr. Angela Ho for technical assistance, and Man Lab members for helpful discussion. This work was supported by NIH grant R01 MH079407 and National Natural Science Foundation of China (81428009) (HYM). The authors declare no competing financial interests.

Figure Legends

Figure 1. KIDLIA is expressed in neurons and is localized in the nucleus

(A) Immunohistochemistry in brain slices of P0 mouse cortex. KIDLIA was co-localized with the nuclear marker Hoescht. Scale bar = 10 μm. **(B,C)** Immunohistochemistry of KIDLIA at E17. KIDLIA expression began in the SP region of the upper IZ and throughout the cortical plate and was restricted to the cortical plate at P0. Scale bars = 20 μm. **(D, E)** KIDLIA was expressed only in cells positive for the neuronal marker NeuN at P0 (left and P14 (right). **(F)** KIDLIA expression was not observed in cells expressing the glia marker GFAP at P14. Scale bars = 20 μm (full picture); 10 μm (enlarged area). UpCP = upper cortical plate; LoCP = lower cortical plate; SP = subplate; IZ = intermediate zone; VZ = ventricular zone.

Figure 2. *In utero* electroporation of KIDLIA shRNA disrupts neuronal migration but does not affect the multipolar to bipolar transition

(A) Schematic of the *in utero* electroporation (IUE) procedure. Pups were injected with shRNA-GFP DNA into the lateral ventricle (LV) at E15 and the anode of the tweezertrode was placed above the dorsal telencephalon. Pups were the returned to the mother to mature until the times indicated. **(B)** Representative images of scrambled and KIDLIA shRNA electroporated neurons at E17. Immunostaining of KIDLIA shows a clear reduction of KIDLIA expression compared to scrambled controls and nearby non-electroporated neurons. Scale bare = 10 μm. **(C)** Brain slices taken at E17 after IUE of KIDLIA shRNA-GFP, or scrambled shRNA-GFP, at E15. Scale bare = 50 μm. **(D)** KIDLIA

knockdown caused a greater percentage of neurons in the upCP and a smaller fraction in the IZ. More than 1,500 GFP+ neurons from five brains were analyzed in each group. **(E)** Brain slices taken at P0 after IUE of KIDLIA shRNA-GFP, or scrambled shRNA-GFP, at E15. Scale bare = 50 μm. **(F)** Analysis of neuronal migration at P0 showed that more neurons were in the upCP and less in the IZ compared to scrambled controls. More than 1,200 GFP+ neurons from four brains were analyzed in each group. **(G and H)** Analysis of the multipolar to bipolar transition. In E17 brain, multipolar neurons were observed in the upper IZ (magnified lower right) prior to CP entry and neurons showed a normal transition back to a bipolar morphology after CP entry (magnified upper right) in both KIDLIA and scrambled shRNA electroporated neurons. Scale bars = 10 μm. Statistics: ** $p<0.01$, *** $p<0.001$. Error bars, SEM. Yellow dashed line indicates the pia. UpCP = upper cortical plate; LoCP = lower cortical plate; IZ = intermediate zone; VZ = ventricular zone.

Figure 3. Knockdown of KIDLIA affects apical dendrite growth and orientation *in vivo*

(A) Images (left) and tracings (right) of P4 layer II/III cortical neurons after electroporation of GFP labeled shRNA constructs at E15. Yellow dashed lines indicate the pia; scale bar = 10 μm. **(B)** No significant change was observed in the apical dendrite angle to the pia after knockdown of KIDLIA. **(C)** An increase was observed in the length of the major apical dendrite (n = 6) and **(D)** a decrease in the distance of the soma to the pia (n = 6). **(E)** No significant difference was observed in the number of dendrite tips reaching the pia. **(F)** Images (left) and tracings (right) of P14 layer II/III cortical neurons after electroporation of GFP labeled shRNA constructs at E15.5. Dashed lines indicate the pia; scale bar = 10 μm. **(G) Merged** tracings of the major apical dendrites of P14 neurons after *in utero* electroporation of scrambled and KIDLIA shRNA showed that knockdown of KIDLIA disrupted the orientation of the apical dendrites toward the pia. Red bars show the average angle of the dendrites in relation to the pial surface (dashed line). **(H)** Quantification of the angle of the major apical dendrite toward the pia showed a significant increase after loss of KIDLIA expression (n = 5). An angle of 0° indicates a dendrite is growing directly toward the pia. **(I)** A significant decrease in the length of the

major apical dendrite, **(J)** a decrease in the distance of the soma to pia and **(K)** a significant decrease in the percentage of dendrite tips that reached the pia were observed after knockdown of KIDLIA *in vivo*. Statistics: * p<0.05, ** p<0.01, *** p<0.001. Error bars, SEM.

Figure 4. Knockdown of KIDLIA decreases dendritic growth and branching *in vitro*

(A) Western blot after nuclear/cytoplasmic fractionation of primary rat hippocampal neurons shows clear nuclear expression of KIDLIA with no expression in cytoplasm. Neurons were treated with lentiviral KIDLIA shRNA, or scrambled shRNA, at DIV0 and collected at DIV6. **(B)** Analysis of the western blot data showed that the shRNA significantly reduced KIDLIA expression (n=5); Nuclear loading control: HDAC1; cytoplasmic loading control = GAPDH. **(C)** Immunostaining of KIDLIA in primary rat hippocampal neurons shows co-localization with the nuclear marker Hoescht. **(D)** Immunostaining of dendrites (MAP2) and axons (tau1) at DIV12; scale bar = 10 μm. **(E)** Sholl analysis of dendrite growth at DIV12 showed a significant change in branching (n=14). **(F)** Analysis of MAP2-positive dendrites showed a decrease in the longest dendrite segment (n = 14), **(G)** a decrease in the number of dendrite branches (n= 14) and **(H)** a significant decrease in the sum dendritic length (n=14). Statistics: * p<0.05, ** p<0.01, *** p<0.001. Error bars, SEM.

Figure 5. Knockdown of KIDLIA leads to an increase in surface N-cadherin and the N-cadherin/δ-catenin association

(A) Western blot from DIV6 neuronal lysates after treatment with KIDLIA shRNA virus (DIV0) showing an increase in total N-cadherin levels. Loading control = GAPDH **(B)** Quantification of the western blot data represented in panel A (n = 3, each sample performed in duplicate and averaged). **(C)** Knockdown of KIDLIA expression by shRNA lentivirus caused an increase in N-Cadherin mRNA expression. Gene expression was normalized to the housekeeping gene, GAPDH (n=3, each sample performed in triplicate and averaged). **(D)** Surface biotinlyation of neuronal lysates after treatment with scrambled or KIDLIA shRNA virus showed an increase in surface N-cadherin levels. **(E)** Quantification

of the western blot data shown in panel C (n = 4). **(F)** N-cadherin was co-immunoprecipitated with a dramatically larger fraction of δ-catenin after lentiviral shRNA knockdown of KIDLIA in neuronal lysates. The increased binding of δ-catenin to N-cadherin subsequently depleted the unbound, cytosolic fraction of δ-catenin. **(G)** Images of neurons transfected with scrambled or KIDLIA shRNA either with GFP or δ-catenin-GFP; scale bar = 10 μm. **(H)** Sholl analysis of the transfected neurons from panel E showed that δ-catenin overexpression could rescue the decreased dendrite growth and branching observed after knockdown of KIDLIA (n = 10). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ Error bars, SEM.

Figure 6. Loss of KIDLIA disrupts actin dynamics at the neurite growth cone *via* δ-catenin

(A) Immunocytochemistry of primary rat hippocampal neurons after transfection of scrambled, or KIDLIA siRNA, with GFP. Neurons were transfected at DIV0 and immunostained for KIDLIA (red) at DIV4. **(B)** Analysis of the immunocytochemistry images showed that the siRNA significantly reduced KIDLIA expression (n=10 for both groups). **(C)** FRAP experiments after co-transfection of KIDLIA siRNA, or scrambled siRNA, with actin-GFP. Regions at the growing neurite tip were selected for photobleaching at 488 nm and imaged every 3 seconds. **(D)** Analysis of the FRAP data showed that knockdown of KIDLIA produced a dramatic decrease in actin dynamics after photobleaching n = 9). **(E)** Neurons were co-transfected with KIDLIA siRNA, or scrambled siRNA, with actin-mCherry and δ-catenin-GFP, or GFP alone, and were photobleached at 555 nm and imaged every 3 seconds. **(F)** Analysis of the FRAP data. Overexpression of δ-catenin rescued the actin dynamics after knockdown of KIDLIA (n = 7). **(G)** The mobile fraction, calculated as the difference between the average level of bleaching to the level of recovery, was significantly decreased after knockdown of KIDLIA (n = 7). **(H)** The immobile fraction of actin, calculated as the difference between the initial fluorescence and the level of recovery was significantly decreased (n = 7). Statistics: ** $p<0.01$, **** $p<0.0001$. Error bars, SEM.

Figure 7. Increased RhoA activation leads to impaired actin dynamics

(A) Western blot at DIV6 after treatment with KIDLIA shRNA, or scrambled shRNA virus, at DIV0. RhoA stimulation was performed with treatment of nocodazole (10 μM) for 30 min prior to collection of neuronal lysates. Activated RhoA-GTP was selectively immunoprecipated using beads conjugated to the Rho binding domain of the Rho effector protein, rhotekin, and whole cell lysates were probed for total RhoA levels. Loading control = GAPDH. **(B)** Quantification of the RhoA assay showed a significant increase in the levels of activated RhoA-GTP after KIDLIA knockdown (n = 4). **(C)** Images of neurons transfected with KIDLIA siRNA, or scrambled siRNA, with actin-mCherry and treated with CN06, or vehicle control, 1 hr prior to FRAP experiments. **(D)** Analysis of the FRAP data showed that inhibition of the RhoA pathway could rescue the actin dynamics after knockdown of KIDLIA (n = 6). **(E)** Images of neurons treated from DIV4-7 with CN06, or vehicle control, after treatment with KIDLIA shRNA or scrambled control virus at DIV0; scale bar = 10 μm. **(F)** Sholl analysis of the images in panel C showed that chronic inhibition of the RhoA pathway was sufficient to rescue dendrite outgrowth and branching (n = 11). **(G)** Diagram depicting changes in the N-cadherin-δ-catenin signaling cascade after loss of KIDLIA expression. Increased surface N-cadherin sequesters δ-catenin after KIDLIA knockdown thereby depleting the cytosolic free fraction of δ-catenin. Less inhibition on RhoA increases its activation and subsequent inhibition on neurite outgrowth *via* changes in actin dynamics. Statistics: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error bars, SEM.

References

Abu-Elneel K, Ochiishi T, Medina M, Remedi M, Gastaldi L, Caceres A*, et al* (2008). A delta-catenin signaling pathway leading to dendritic protrusions. *J Biol Chem* **283**: 32781-32791.

Arikkath J, Israely I, Tao Y, Mei L, Liu X, Reichardt LF (2008). Erbin controls dendritic morphogenesis by regulating localization of delta-catenin. *J Neurosci* **28**: 7047-7056.

Baio J (2014). Prevalence of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2010. *Morbidity and mortality weekly report Surveillance summaries (Washington, DC : 2002)* **63**: 1-21.

Bard L, Boscher C, Lambert M, Mege RM, Choquet D, Thoumine O (2008). A molecular clutch between the actin flow and N-cadherin adhesions drives growth cone migration. *J Neurosci* **28**: 5879-5890.

Belcaro C, Dipresa S, Morini G, Pecile V, Skabar A, Fabretto A (2015). CTNND2 deletion and intellectual disability. *Gene* **565**: 146-149.

Belichenko P, Wright E, Belichenko N, Masliah E, Li H, Mobley W*, et al* (2009). Widespread changes in dendritic and axonal morphology in Mecp2-mutant mouse models of Rett syndrome: evidence for disruption of neuronal networks. *J Comp Neurol* **514**: 240-258.

Belmonte MK, Allen G (2004). Autism and abnormal development of brain connectivity. *J Neurosci* **24**: 9228-9231.

Brigidi GS, Sun Y, Beccano-Kelly D, Pitman K, Mobasser M, Borgland SL*, et al* (2014). Palmitoylation of delta-catenin by DHHC5 mediates activity-induced synapse plasticity. *Nat Neurosci* **17**: 522-532.

Cantagrel V, Haddad M-R, Ciofi P, Andrieu D, Lossi A-M, Maldergem L*, et al* (2009). Spatiotemporal expression in mouse brain of Kiaa2022, a gene disrupted in two patients with severe mental retardation. *Gene expression patterns : GEP* **9**: 423-429.

Cantagrel V, Lossi AM, Boulanger S, Depetris D, Mattei MG, Gecz J*, et al* (2004). Disruption of a new X linked gene highly expressed in brain in a family with two mentally retarded males. *J Med Genet* **41**: 736-742.

Charzewska A, Rzońca S, Janeczko M, Nawara M, Smyk M, Bal J*, et al* (2014). A duplication of the whole KIAA2022 gene validates the gene role in the pathogenesis of intellectual disability and autism. *Clin Genet* **88**: 297-299.

de Anda F, Rosario A, Durak O, Tran T, Gräff J, Meletis K*, et al* (2012). Autism spectrum disorder susceptibility gene TAOK2 affects basal dendrite formation in the neocortex. *Nat Neurosci* **15**: 1022-1031.

DiCicco-Bloom E, Lord C, Zwaigenbaum L, Courchesne E, Dager S, Schmitz C*, et al* (2006). The developmental neurobiology of autism spectrum disorder. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26**: 6897-6906.

Elia L, Yamamoto M, Zang K, Reichardt L (2006). p120 catenin regulates dendritic spine and synapse development through Rho-family GTPases and cadherins. *Neuron* **51**: 43-56.

Gal JS, Morozov YM, Ayoub AE, Chatterjee M, Rakic P, Haydar TF (2006). Molecular and morphological heterogeneity of neural precursors in the mouse neocortical proliferative zones. *J Neurosci* **26**: 1045-1056.

Garner CC, Wetmore DZ (2012). Synaptic pathology of Down syndrome. *Adv Exp Med Biol* **970**: 451-468.

Hofmeister W, Nilsson D, Topa A, Anderlid BM, Darki F, Matsson H*, et al* (2015). CTNND2-a candidate gene for reading problems and mild intellectual disability. *J Med Genet* **52**: 111-122.

Ishikawa T, Miyata S, Koyama Y, Yoshikawa K, Hattori T, Kumamoto N*, et al* (2012). Transient expression of Xpn, an XLMR protein related to neurite extension, during brain development and participation in neurite outgrowth. *Neurosci* **214**: 181-191.

Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M*, et al* (2000). Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol Pharmacol* **57**: 976-983.

Kang R, Wan J, Arstikaitis P, Takahashi H, Huang K, Bailey AO*, et al* (2008). Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. *Nature* **456**: 904-909.

Kim H, Oh M, Lu Q, Kim K (2008). E-Cadherin negatively modulates delta-catenin-induced morphological changes and RhoA activity reduction by competing with p190RhoGEF for delta-catenin. *Biochem Biophys Res Commun* **377**: 636-641.

Kozma R, Sarner S, Ahmed S, Lim L (1997). Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol Cell Biol* **17**: 1201-1211.

Kuroda Y, Ohashi I, Naruto T, Ida K, Enomoto Y, Saito T*, et al* (2015). Delineation of the KIAA2022 mutation phenotype: Two patients with X linked intellectual disability and distinctive features. *Am J Med Genet A* **167**: 1349-1353.

Kwon C-H, Luikart B, Powell C, Zhou J, Matheny S, Zhang W*, et al* (2006). Pten regulates neuronal arborization and social interaction in mice. *Neuron* **50**: 377-388.

Lee T, Winter C, Marticke SS, Lee A, Luo L (2000). Essential roles of Drosophila RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. *Neuron* **25**: 307-316.

Leeuwen FN, Kain HE, Kammen RA, Michiels F, Kranenburg OW, Collard JG (1997). The guanine nucleotide exchange factor Tiam1 affects neuronal morphology; opposing roles for the small GTPases Rac and Rho. *J Cell Biol* **139**: 797-807.

Li Z, Aelst VL, Cline HT (2000). Rho GTPases regulate distinct aspects of dendritic arbor growth in Xenopus central neurons in vivo. *Nat Neurosci* **3**: 217-225.

Lord C, Rutter M, Goode S, Heemsbergen J, Jordan H, Mawhood L*, et al* (1989). Autism diagnostic observation schedule: a standardized observation of communicative and social behavior. *J Autism Dev Disord* **19**: 185-212.

Lu Q, Paredes M, Medina M, Zhou J (1999). Δ-Catenin, an adhesive junction–associated protein which promotes cell scattering. *The Journal of cell …*.

Magome T, Hattori T, Taniguchi M, Ishikawa T, Miyata S, Yamada K*, et al* (2013). XLMR protein related to neurite extension (Xpn/KIAA2022) regulates cell-cell and cell-matrix adhesion and migration. *Neurochem Int* **63**: 561-569.

Martinez M, Ochiishi T, Majewski M, Kosik KS (2003). Dual regulation of neuronal morphogenesis by a delta-catenin–-cortactin complex and Rho. *J Cell Biol* **162**: 99-111.

Meberg PJ, Bamburg JR (2000). Increase in neurite outgrowth mediated by overexpression of actin depolymerizing factor. *J Neurosci* **20**: 2459-2469.

Medina M, Marinescu RC, Overhauser J, Kosik KS (2000). Hemizygosity of δ-catenin (CTNND2) is associated with severe mental retardation in cri-du-chat syndrome. *Genomics* **63**: 157-164.

Nakayama AY, Harms MB, Luo L (2000). Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *J Neurosci* **20**: 5329-5338.

Neumann H, Schweigreiter R (2002). Tumor necrosis factor inhibits neurite outgrowth and branching of hippocampal neurons by a rho-dependent mechanism. *J Neurosci* **22**: 854-862.

Newey SE, Velamoor V, Govek EE (2005). Rho GTPases, dendritic structure, and mental retardation. *Dev Neurobiol* **64**: 58-74.

Nguyen D, Disteche C (2006). High expression of the mammalian X chromosome in brain. *Brain Res* **1126**: 46-49.

Nicholson-Dykstra S, Higgs HN, Harris ES (2005). Actin dynamics: growth from dendritic branches. *Curr Biol* **15**: R346-357.

Ouyang Q, Lizarraga SB, Schmidt M, Yang U, Gong J, Ellisor D*, et al* (2013). Christianson syndrome protein NHE6 modulates TrkB endosomal signaling required for neuronal circuit development. *Neuron* **80**: 97-112.

Peça J, Feliciano C, Ting JT, Wang W, Wells MF, Venkatraman TN*, et al* (2011). Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. *Nature* **472**: 437-442.

Reynolds AB, Carnahan RH (2004). Regulation of cadherin stability and turnover by p120ctn: implications in disease and cancer. *Semin Cell Dev Biol* **15**: 657-663.

Ricciardi S, Ungaro F, Hambrock M, Rademacher N, Stefanelli G, Brambilla D*, et al* (2012). CDKL5 ensures excitatory synapse stability by reinforcing NGL-1-PSD95 interaction in the postsynaptic compartment and is impaired in patient iPSC-derived neurons. *Nat Cell Biol* **14**: 911-923.

Shepherd GM, Katz DM (2011). Synaptic microcircuit dysfunction in genetic models of neurodevelopmental disorders: focus on Mecp2 and Met. *Curr Opin Neurobiol* **21**: 827-833.

Skuse D (2005). X-linked genes and mental functioning. *Hum Mol Genet* **14 Spec No 1**: 32.

Tan Z-JJ, Peng Y, Song H-LL, Zheng J-JJ, Yu X (2010). N-cadherin-dependent neuron-neuron interaction is required for the maintenance of activity-induced dendrite growth. *Proc Natl Acad Sci U S A* **107**: 9873-9878.

Turner TN, Sharma K, Oh EC, Liu YP, Collins RL, Sosa MX*, et al* (2015). Loss of delta-catenin function in severe autism. *Nature* **520**: 51-56.

Van Maldergem L, Hou Q, Kalscheuer V, Rio M, Doco-Fenzy M, Medeira A*, et al* (2013). Loss of function of KIAA2022 causes mild to severe intellectual disability with an autism spectrum disorder and impairs neurite outgrowth. *Hum Mol Genet* **22**: 3306-3314.

Weston MC, Chen H, Swann JW (2014). Loss of mTOR repressors Tsc1 or Pten has divergent effects on excitatory and inhibitory synaptic transmission in single hippocampal neuron cultures. *Front Mol Neurosci* **7**: 1.

Wong WT, Faulkner-Jones BE, Sanes JR (2000). Rapid dendritic remodeling in the developing retina: dependence on neurotransmission and reciprocal regulation by Rac and Rho. *J Neurosci* **20**: 5024-5036.

Yu X, Malenka RC (2003). Beta-catenin is critical for dendritic morphogenesis. *Nat Neurosci* **6**: 1169-1177.

Zikopoulos B, Barbas H (2010). Changes in prefrontal axons may disrupt the network in autism. *J Neurosci* **30**: 14595-14609.