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Formin 2 Regulates Lysosomal Degradation of Wnt-Associated β -Catenin in Neural Progenitors

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Abstract

Although neural progenitor proliferation along the ventricular zone is regulated by β -catenin through Wnt signaling, the cytoskeletal mechanisms that regulate expression and localization of these proteins are not well understood. Our prior studies have shown that loss of the actin-binding Filamin A (FlnA) and actin-nucleating protein Formin 2 (Fmn2) impairs endocytosis of low-density-lipoprotein-receptor-related protein 6 (Lrp6), thereby disrupting β -catenin activation, resulting in decreased brain size. Here, we report that activated RhoA-GTPase disengages Fmn2 N- to C-terminal binding to promote Fmn2 activation and redistribution into lysosomal vesicles. Fmn2 colocalizes with β -catenin in lysosomes and promotes its degradation. Further, Fmn2 binds the E3 ligase Smurf2, enhances Smurf2-dependent ubiquitination, and degradation of Dishevelled-2 (Dvl2), thereby initiates β -catenin degradation. Finally, Fmn2 overexpression disrupts neuroepithelial integrity, neuronal migration, and proliferation-phenotypes in E13 mouse embryos, as seen with loss of Fmn2+FlnA function. Conversely, co-expression of Dvl2 with Fmn2 rescues the proliferation defect due to Fmn2 overexpression in mouse embryos. These findings suggest that there is a homeostatic feedback mechanism in the cytoskeletal-dependent regulation of neural proliferation within the cerebral cortex. Upstream, Fmn2 promotes proliferation by stabilizing the Lrp6 receptor, leading to β -catenin activation. Downstream, RhoA-activated Fmn2 promotes lysosomal degradation of Dvl2, leading to β -catenin degradation.

Key words: Actin, cortical development, lysosomal degradation, neural proliferation, vesicle trafficking

Introduction

Corticalogenesis can be viewed through separate distinct stages involving neural proliferation, specification, neuronal migration, and subsequent differentiation (Bystron et al. 2008; Götz and Huttner 2005). With initial proliferation, formation of the rostral neural tube (telencephalon) involves expansion of a single-cell-layered, pseudostratified neuroepithelium. Neuroepithelial cells subsequently extend their processes from the apical side (ventricular zone) to the basal lamina and undergo interkinetic nuclear migration along the apical-basal axis throughout the cell cycle. Specification then involves daughter cells either adopting neural

progenitor, intermediate progenitor, or differentiated neuronal identities.

The process of cerebral cortex development is dependent on precise temporal and spatial expression of various proteins in the Wnt-signaling cascade to allow for both the expansion of the neural progenitor pool (neurogenesis) and the adoption of neural or neuronal cell fates (specification). Wnt signaling plays multifaceted effects on the development of cerebral cortex through noncanonical (PCP) and canonical β -catenin pathways. The PCP pathway is activated via the binding of Wnt to Frizzled (Fz) and its coreceptor. Activation leads to the recruitment of

Dishevelled (Dvl) and formation of a complex with the formin homology protein, Daam1. Daam1 then stimulates the small G-protein Rho through a guanine exchange factor, activating Rho-associated kinase (ROCK) (Habas et al. 2001; Nakagawa et al. 2017). The noncanonical Wnt pathway can also mediate calcium release and effect Cdc42, which has been implicated in neural specification (Cappello et al. 2006). PCP occurs independently of β -catenin, establishes polarity through its regulation of cytoskeletal dynamics, and thereby regulates cell shape, structure, and function, as well as ventral patterning of the neuroepithelium. In the canonical pathway, Wnt binding to Lrp5/6 disrupts the Gsk3 β -Axin destruction complex leading to β -catenin accumulation in the nucleus followed by transcription activation. Changes in transcriptional activation of β -catenin show direct correlation with changes in neural proliferation and brain size (Chenn and Walsh 2003).

Although cytoskeletal changes have long been implicated in PCP, our recent studies implicate a parallel formin-dependent pathway in the canonical Wnt pathway through the Lrp6 receptor and the actin-binding Filamin A (FlnA) and actin-nucleating Formin 2 (Fmn2) proteins (Lian et al. 2016). Loss of FlnA and Fmn2 impairs proliferation, thereby generating multiple embryonic phenotypes, including microcephaly. FlnA interacts with the Wnt coreceptor Lrp6. Loss of FlnA and Fmn2 impairs Lrp6 endocytosis, downstream Gsk3 β activity, and β -catenin accumulation in the nucleus. While these findings implicate an actin-signaling pathway that regulates neural progenitor proliferation in developing cerebral cortex, the downstream effector pathways and their role in the regulation of β -catenin are not known.

Here, we demonstrate that activated RhoA-GTPase disengages Fmn2 auto-inhibition and promotes translocation of the N-cadherin- β -catenin complex to lysosomal circular vesicles. Activated Fmn2 binds the E3 ubiquitin ligase Smurf2 at its proline-rich region and promotes Smurf2-dependent ubiquitination of Dvl2, but not β -catenin. Loss of Dvl2 expression leads to β -catenin degradation. Moreover, the overexpression of Dvl2 rescues Fmn2-induced degradation of β -catenin and neural progenitor proliferation in vivo. The loss of these adherens junction molecules due to Fmn2 overexpression disrupts neuroepidymal integrity, migration, and proliferation. Taken collectively, the actin-nucleating Fmn2 promotes upstream Lrp6 coreceptor-dependent endocytosis and activation of β -catenin to increase proliferation of neural progenitors during corticogenesis (Lian et al. 2016). Downstream, RhoA-activated Fmn2 enhances ubiquitination of Dvl2 through Smurf2 and inhibits β -catenin function through a negative feedback mechanism, thereby impairing neural proliferation during cortical development.

Materials and Methods

In Utero Electroporation of Fmn2 Constructs

All mouse studies were performed with approval from the Institutional Animal Care and Use Committees (IACUC) of Harvard Medical School and Beth Israel Deaconess Medical Center in accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals. GFP and Fmn2-GFP plasmids (2.0 μ g/ μ) were injected into the lateral ventricles of E12 and E13 embryos followed by in utero electroporation as described previously (Zhang et al. 2012). Briefly, timed-pregnant CD1 female mice (Charles River Laboratories) were anesthetized with ketamine and xylazine. The uteri of the mouse were carefully removed from the abdominal cavity, and

the embryos were hydrated with warmed phosphate-buffered saline (PBS at 37 °C). One to two microliters of the plasmid in sterile PBS (with 0.05% Fast Green) was injected into the lateral ventricles. Five electrical pulses (39 V, 50 ms duration with a 1 s interval per pulse) were delivered to the embryos using an Electro Square Porator T820 (BTX, Harvard Apparatus). The embryos were sacrificed 24 h post-electroporation, fixed in 4% paraformaldehyde (PFA), and sectioned on a cryostat at 12–14 μ m thicknesses. More than 3 embryos were analyzed following electroporation per experimental variable.

Plasmids and Antibodies

pCAG-Fmn2-GFP plasmid was constructed by PCR-amplifying the *Fmn2* gene (courtesy Dr. Philip Leder's laboratory) and the PCR product was inserted into the pCAG-GFP vector (Addgene) by restriction enzyme digestion. pcDNA3-Fmn2N-V5, pEGFP-FH1FH2, and pGEX-GST-Fmn2C plasmids were similarly made by PCR amplification and digestion with restriction enzymes. The pCMV5 expression vectors carrying Flag-tagged wild-type (WT), dominant-negative (DN), and constitutively active (CA) RhoA, Cdc42, and Rac1 were gifted from Dr. Takaya Satoh at Kobe University Graduate School of Medicine. For the construction of GST-tagged WT, DN, and CA proteins (RhoA, Cdc42, and Rac1), the pCMV5 expression vectors were cut via restriction enzymes and these cDNAs ligated into a pGEX-6p-3 vector containing a GST tag. The pET21-Fmn2N-His plasmid was prepared by cutting the Fmn2N fragment from pcDNA3-Fmn2N-V5 and inserting it into the pET21-His-tagged vector.

The following antibodies with corresponding dilutions were used for the studies: mouse anti-V5 (1:2000, Life Technologies R960-25), mouse anti-His tag (1:2000, Life Technologies R932-25), goat anti-GST tag (1:1000, GE Healthcare 27457701), rabbit anti-Flag (1:1000, Sigma Aldrich F1804 and F7425), mouse anti-E-cadherin and anti-N-cadherin (1:100, BD Biosciences 610181 and 610920), anti-LAMP1 rat and rabbit antibodies (1:200, Abcam ab25245 and ab 24170), mouse anti-TSG101 (1:500, Santa Cruz sc-7964), anti-EEA1 mouse and rabbit antibodies (1:200, Abcam ab70521 and ab2900), mouse anti-RhoA (1:50, Santa Cruz, clone:26C4, sc-418), and rat anti-RhoA (clone: lulu51) were gifted from Dr. Shigenobu Yonemura at RIKEN Center for Developmental Biology, mouse anti-Rac1 (1:1000, Millipore 05-389), rabbit anti-cdc42 (1:1000, Santa Cruz sc-87), and Alexa Fluor 488- or 594-phalloidin (1:50, Invitrogen A12379 and A12381). Rabbit anti-Smurf2 (cat.12024, 1:1000), rabbit anti-Axin1 (cat.2087, 1:1000), rabbit anti-Dvl2 (cat.3224, 1:1000), rabbit anti-Nedd4L (cat.4013, 1:1000), and mouse anti-Nedd4 (cat. 2740, 1:1000) were from Cell Signaling Technology. Mouse anti- β 1-integrin (cat.610467, 1:1000), mouse anti- β -catenin (cat.610153, 1:1000), and mouse anti-Nedd4 (cat.611480, 1:1000) were from BD Biosciences. Rabbit anti-Rab7 (ab77993, 1:100) was from Abcam. Mouse anti- α -tubulin was from Santa Cruz (1:1000, sc-32293).

Protein Expression in *Escherichia coli* and Purification

pET21-Fmn2N-His and pGEX-6p-3 plasmids carrying GST-fused RhoA, Rac1, Cdc42, and Fmn2C were transformed in BL21 cells. Positively transformed clones were inoculated in LB medium with ampicillin and incubated at 37 °C until they reached an OD = 0.7. Protein expression was induced with 0.3 mM IPTG at room temperature overnight. Cells were harvested by centrifugation and suspended in PBS containing 0.1% Triton X-100, PMSF, and protease inhibitor cocktail. Cells were lysed by sonication (Sonicator Ultrasonic Processor W-385, Heat Systems, Inc) and precipitated by centrifugation at 12 000 $\times$ g for 10 min at 4 °C. The

supernatants were incubated with Glutathione Sepharose 4B beads (GE Healthcare) at room temperature for 1 h, and the beads were washed 3 times with PBS containing 0.1% Triton X-100 and protein inhibitor cocktail. GST-fusion proteins bound to beads were stored in 50% glycerol/PBS or were eluted with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The purified proteins were dialyzed, concentrated, and stored in PBS containing 1 mM DTT and protein inhibitor cocktail at -80°C .

GDP and GTP Loading Assays

Purified wild-type RhoGTPases RhoA, Rac1 and Cdc42 (5 μM) were loaded with GTP γS or GDP at 30°C in a reaction solution (40 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM DTT, and 0.2 mM GTP γS or GDP) for 15 min. The reaction was stopped by adding 10 μl 100 mM MgCl_2 (in 50 mM Tris-HCl). GTP γS or GDP-loaded RhoGTPase solution was placed on ice for use.

RhoGTPase Pulldown and Competition Assays

The pcDNA3-Fmn2N-V5 plasmid was transfected into cultured HEK293 cells. After 24 h, 293 cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, proteinase inhibitor cocktail and phosphatase inhibitor cocktail). The cell lysis solution was centrifuged at 13 000 rpm (Eppendorf Centrifuge 5415 D) for 15 min at 4°C and the supernatant was incubated with the purified RhoGTPase beads at 4°C for 3 h. The beads were washed 3 times with the above buffer, and the Fmn2N-V5 pulled down by RhoGTPases was identified by western blot.

GST-Fmn2C beads were incubated with purified Fmn2N-HIS tag in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT at 30°C for 1 h. The beads were washed 3 times with the buffer and then aliquoted into tubes. The competition solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl_2 , and 1 mM DTT) containing varied amounts of GTP-, GDP-RhoA, or CA-RhoA were added to the reaction tubes and incubated at 30°C for 30 min. The beads were washed 3 times with the competition buffer. Amounts of Fmn2N-V5 that remained on the beads after competition with RhoA were measured by western blot.

Ubiquitination Assays

Eighty percent confluent 293 T cells were transfected with His-ubiquitin construct alone, or together with (Flag or myc-tagged) Smurf2, Flag-Dvl2, and Fmn2-GFP for 24 h and then treated with 20 μM MG132 for 5 h. Cells were washed once with PBS and then detached from dish by flushing with PBS. Cells were centrifuged at 800 rpm (Eppendorf Centrifuge 5415 D) for 5 min and then lysed in buffer A (0.1 M phosphate buffer (pH 8.0), 6 M guanidine HCl, and 10 mM imidazole). The lysates were sonicated (Sonicator W-385) 10 times at level 3 and then centrifuged at 13 000 rpm (Eppendorf Centrifuge 5415 D) for 10 min. The supernatants were incubated with 50 μl Ni-NTA resin at room temperature for 3 h. The Ni-NTA resin was centrifuged and washed with buffer A twice and then washed twice with a buffer mixture consisting of buffer A/buffer TI (1:3; buffer TI: 25 mM Tris-HCl (pH 6.8), 20 mM imidazole), and washed once with buffer TI. Ni-NTA resin was suspended in 100 μl SDS sample buffer and 5–10 μl sample supernatants were loaded for western blotting. For β -TrcP-regulated ubiquitination, the procedure was

same as the above, except that the β -TrcP construct (Addgene) was used instead of the Smurf2 construct.

Immunoprecipitation was also used for ubiquitination assays. Eighty percent confluent 293 T cells were transfected with HA-ubiquitin construct alone, or together with (Flag or myc-tagged) Smurf2, Flag-Dvl2, and Fmn2-GFP for 24 h and then treated with 20 μM MG132 for 5 h. Cells were lysed in HEPES buffer (25 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, protease, and phosphatase inhibitor cocktails), and immunoprecipitation was performed with anti-HA antibody as described previously.

Cell Culture and Transfections

Neural progenitor cells were cultured as described previously (Lu et al. 2011). For expansion of cultures, mouse neural progenitors were suspended in neural stem cell medium to generate neurospheres. Neural progenitors were dissociated and cultured on laminin-coated dishes or coverslips for assays of transfection and proliferation.

The Fmn2-GFP construct was transiently transfected into cells for specified times and then fixed for staining. Primary mouse embryonic fibroblasts (MEF) from E13 embryos were cultured in MEF medium (DMEM, 10% FBS plus nonessential amino acids and 2-mercaptoethanol) and were transiently transfected with Fmn2-GFP construct for analysis. Neuro-2 and 293 cells were cultured in 10% FBS/DMEM medium.

Western Blot Analyses

Western blot studies were performed according to previously described methods (Lian et al. 2012).

Immunostaining and Colocalization Analysis

Immunostaining was performed according to previously described procedures in the provided references (Lu et al. 2006; Lian et al. 2012). ImageJ software was used for the measurement of proteins at the membrane or cytoplasmic vesicles.

For colocalization analysis, immunofluorescent images were opened in ImageJ and colored images split into black and white (Dunn et al. 2011). Correlation coefficients were measured using the JACoP procedure in the Plugins window of ImageJ. Pearson's coefficient (r), Manders' coefficients, and Costes' automatic threshold values were obtained in ImageJ. To simplify the colocalization analysis, we adopted Pearson's coefficient (r) values as correlation coefficients. If the r value was more than 50%, the two proteins were defined as being colocalized.

Statistical Analyses

Statistical analyses were performed as previously described (Lu et al. 2006; Lian et al. 2012). Briefly, western blot data (from at least 3 independent experiments) were analyzed by using ImageJ software. Protein distribution and colocalization in cells ($n \geq 5$) were done using ImageJ Plugins software. The numbers of cells stained/costained from serial tissue sections ($n \geq 9$, were performed for each experimental variable $n \geq 3$) were counted manually, analyzed, and quantified using Microsoft Excel. The t-test, standard deviation, and ANOVA and pairwise P -values were calculated to determine significance (Lian et al. 2016).

Results

Fmn2 Interacts and Overlaps with RhoA on the Plasma Membrane and Circular Vesicles

Several members of the Formin family exhibit auto-inhibition, where the actin-nucleating function that is triggered by the Formin FH2 domain is inhibited. This is due to the binding of a DID/GBD domain (Diaphanous auto-inhibitory/GTPase-binding domain) at the N-terminus of Formin to a DAD (Diaphanous auto-regulatory domain) at the C-terminus of Formin. Competitive binding of RhoGTPases to the GBD is thought to dissociate the binding of GBD to DAD, thereby releasing Formin auto-inhibition and triggering Formin activation and actin nucleation (Higgs 2005). Although other Formin subfamily proteins contain auto-inhibitory domains in their N-terminus (Kobielak et al. 2004; Bor et al. 2012), the Formin subfamily including Fmn1 and Fmn2 do not contain homologous DID and GBD domains. However, the *Drosophila* formin, Cappuccino, which shares homology with the mouse Fmn1/2 subfamily, also lacks these domains but has been shown to exhibit auto-inhibition via activation that is dependent on Rho1-GDP to GTP conversion, analogous to the RhoA-GDP to GTP for the formins (Rosales-Nieves et al. 2006). To test whether Fmn2 exhibits RhoGTPase-dependent auto-inhibition, we first made Fmn2 N-terminal and Fmn2 C-terminal constructs. GST-RhoGTPases are then loaded with GTP γ S and GTP γ S to produce inactive (GDP) and active (GTP) states, respectively. Fmn2N-V5 expressed in 293 cells is then precipitated from cell lysate supernatants using the purified GST-RhoGTPases or GST-Fmn2C. The pull-down assays show that the Fmn2 N-terminus unselectively interacts with Cdc42 and Rac1 in both their active (GTP) and inactive (GDP) states. However, the Fmn2 N-terminus selectively interacted with RhoA-GTP (active) (Fig. 1A). These findings verify the selective association of Fmn2 with RhoA-GTP alone, implying that Fmn2 protein contains a RhoGTPase-binding domain in its N-terminus. Additionally, Fmn2N interacts with Fmn2C (Fig. 1A, last column), consistent with auto-inhibition. To identify whether RhoA-GTP could compete off Fmn2C to Fmn2N binding, we performed competitive binding assays. Purified GST-Fmn2C and GST-Fmn2N were first incubated and pulled down using GSH beads. Equal amounts of GSH beads binding the Fmn2C and Fmn2N complex were incubated with varying amounts of active or inactive RhoA. Results show that although the strength of binding between RhoA and Fmn2N is weaker than that between Fmn2C and Fmn2N (Fig. 1A), active RhoA (GTP γ S-loaded or constitutively active G14V) does compete off the binding of Fmn2N to Fmn2C in a dose-dependent manner (Fig. 1B, top). Inactive GDP γ S-loaded RhoA does not compete off Fmn2N to Fmn2C binding, even under conditions of 5-fold excess GDP-RhoA (Fig. 1B, bottom). Overall, these results suggest that active RhoA might bind the same site in Fmn2N as Fmn2C and competes with Fmn2C at this binding site. Collectively, these studies support the finding that Fmn2, like the *Drosophila* formin Cappuccino (Rosales-Nieves et al. 2006), exhibits RhoGTPase-dependent auto-inhibition through N- and C-terminal binding despite the absence of specific DID and DAD regions.

To further verify this interaction, we transiently transfected a full-length Fmn2-GFP construct into MEF and neural progenitor cells and examined the colocalization of Fmn2 with endogenous RhoA in both cell types. Immunolabeling shows that endogenous RhoA overlaps with Fmn2 at the plasma membrane and also on circular vesicles (Fig. 1C) in both cells (data not shown for neural progenitors). Notably, no circular vesicles appear in nontransfected MEF cells, suggesting that Fmn2

might regulate the formation of circular vesicles together with RhoA. In our prior work, we found that endocytosed lipid rafts (cholera toxin B) are colocalized with caveolin-positive circular vesicles and that FlnA and Fmn2 mediate caveolae endocytosis (Lian et al. 2016). Given that both Fmn2 and RhoA are localized at circular vesicles, we asked whether RhoA could affect Fmn2 localization on the vesicles. We transiently expressed Fmn2-GFP with Flag-tagged dominant-negative (DN)-RhoA and constitutively active (CA)-RhoA in subconfluent MEF cells (Fig. 1D). Similar changes were also seen in neural progenitor cells. Immunostaining of these cells shows that more Fmn2 is localized at cytoplasmic vesicle aggregations in CA-RhoA-expressing cells compared with plasma protrusion and circular vesicles in DN-RhoA-expressing cells by 6–8 h after transfection, indicating that RhoA activation regulated Fmn2 localization (Fig. 1D). Notably, CA-RhoA expression causes contraction and rounding of most cells, implying a decrease in the peripheral cell membrane area likely secondary to endocytosis. Collectively, these results suggest that activated RhoA-GTP would selectively release Fmn2 auto-inhibition and promote endocytosis.

Expression of Fmn2 Causes Aggregation of N-Cadherin and β -Catenin in Lysosomal Vesicles

Our observations indicate that RhoA-GTP releases Fmn2 auto-inhibition, leading to activation of Fmn2 FH1 (aa735 to aa1113) and FH2 (aa1114 to aa1518) domains. These domains are presumed to stimulate the formation of polarized actin fibers and might assist in Fmn2-dependent endocytosis, observed from our prior studies. We therefore examined whether Fmn2 actually enhanced actin cytoskeletal formation. Fmn2 overexpression causes a loss in actin fibers in subconfluent MDCK cells, after prolonged expression (greater than 24 h) leading to blebbing of the cell (Fig. 2A). Similarly, compared with GFP expression, Fmn2 also caused a loss in the cell adherens junction proteins, E-cadherin and β -catenin at this prolonged time point (Fig. 2B and C), suggesting that Fmn2 overexpression did not cause a stabilization of actin fiber and adherens junction proteins, but rather promoted their degradation. Moreover, Fmn2 surprisingly does not demonstrate significant colocalization with actin fibers in MEFs (or in neuroblastoma cells, data not shown), but rather displays localization along actin-positive vesicles within cells after transient expression of Fmn2-GFP (Fig. 3A and left graph in panel 3F) in transient transfections after 8 h. No increased cell death was observed with Fmn2 overexpression by caspase 3 immunostaining (Suppl Fig 1). These results suggest that Fmn2 might not promote the formation of actin fibers, but rather assist in the assembly/disassembly of an actin network for vesicle trafficking.

We have previously shown that FlnA and Fmn2 promote endocytosis of the Wnt coreceptor Lrp6 to activate β -catenin transportation (Lian et al. 2016). N-cadherin at cell adhesion sites is linked to the actin cytoskeleton via the α - and β -catenin complex (Hirano and Takeichi 2012). We therefore examined N-cadherin localization in Fmn2-expressing MEFs. Transient expression of Fmn2 in MEFs shows colocalization of N-cadherin and Fmn2 along the cell membrane, in vesicles adjacent to the cell membrane and in perinuclear circular vesicles (Fig. 3B and middle graph in panel 3F). Immunostaining with the lysosomal marker, LAMP1, shows that the Fmn2-positive perinuclear circular vesicles reside in the lysosomal compartment (Fig. 3C). Less-extensive overlapping staining is seen with the early endosomal marker, EEA1, whereas the late endosomal and lysosomal

Figure 1. Fmn2 physically and functionally interacts with RhoA-GTP. (A) Western blot shows that the N-terminus of Fmn2 (FMN2N, aa1 to aa734 of Fmn2, pcDNA3-Fmn2N-V5) can be pulled down by purified activated RhoA-GTP but not inactivated RhoA-GDP (upper blot). FMN2N also interacts with the C-terminus of Fmn2 (FMN2C, aa1519 to aa1567 of Fmn2, pGEX-GST-Fmn2C) and nonspecifically binds both the active and the inactive forms of other RhoGTPases, Cdc42, and Rac1 (lower blots). Schematic diagram illustrates the various domains of Fmn2 protein (FMN2N, FH1, FH2, and FMN2C domains). (B) Western blot shows that active but not inactive RhoA competitively inhibits binding of FMN2N with FMN2C. The amount of FMN2N bound to GST-Fmn2C-beads gradually decreases with increasing concentrations of active RhoA (RhoA-GTP and RhoA-G14V) (upper panel), whereas no change is seen with inactive GDP-RhoA (lower panel). GST-Fmn2C-bound glutathione beads were incubated with purified FMN2N-V5 and incubated with varied amounts of GTP-loaded, constitutively active (G14V), or GDP-loaded RhoA. (C) Fluorescent photomicrographs demonstrate RhoA and Fmn2 colocalization at the cytoplasmic membrane and vesicles. Fmn2 overlaps with RhoA at the membrane and circular vesicles (see arrowheads) in MEF cells. MEF cells were transiently (8 h) transfected with Fmn2-GFP construct, fixed by 10% trichloroacetic acid (TCA), and stained with anti-RhoA and anti-GFP antibodies. Higher magnification images from the framed sections of the upper panel are represented in the lower panel. (D) Fluorescent photomicrographs show that activated RhoA directs Fmn2 localization. Transfection of a constitutively activated RhoA (CA-RhoA) in MEF cells causes Fmn2 to redistribute into the cytoplasmic particles (arrowhead), whereas inactivated, dominant-negative RhoA (DN-RhoA) promoted Fmn2 expression along the cellular protrusion (arrow). Fmn2 is evenly distributed along the cell membrane and vesicles in control cells. Noticeably, more Fmn2 is localized at circular vesicles in DN-RhoA-transfected cells (arrowhead) but appears at aggregated particles in CA-RhoA-transfected cells (arrowhead). Progenitor cells were transiently transfected (8 h) with Fmn2-GFP together with empty vector (control), Flag-tagged DN-RhoA and CA-RhoA plasmids, fixed by PFA, stained with anti-GFP and anti-Flag antibodies, and visualized by confocal microscopy. Nuclei were counterstained with DAPI. Protein levels localized at the cytosolic and cytoplasmic membrane were measured by ImageJ software. Statistical analyses ($n = 6$) are shown graphically to the right. Scale bars = 10 μ m. Error bar is S.D., ANOVA, $P < 0.05$, and * $P < 0.05$.

marker, Rab7, similarly shows more significant colocalization with Fmn2 (Fig. 3D and right graph in panel 3F). These observations are consistent with the extensive overlapping expression of N-cadherin, Fmn2, and LAMP1 in perinuclear lysosomes after

triple immunolabeling in neural progenitors (Fig. 3E, left panel). As Fmn2 expression promotes translocation of N-cadherin and actin into circular vesicles, we examined whether β -catenin is similarly localized to Fmn2-positive circular vesicles after

Figure 2. Fmn2 expression causes loss of actin fiber and adherence junction proteins including β -catenin (Ctnnb1) and E-cadherin in MDCK epithelial cells. (A) Fluorescent photomicrographs show that overexpression of Fmn2 (fluorescein) causes virtually complete loss of actin filaments (rhodamine, phalloidin staining), leading to cell expansion beyond the normal cell-cell contacts at 24–48 h after Fmn2 transfection, presumably due to effects from Fmn2 activation. The left panels show normal distribution of actin filament in GFP-expressing MDCK cells and the right panels show loss of actin filament in Fmn2-GFP-expressing cell (see arrow). No increased cell death by caspase 3 staining was observed (Suppl Fig. 1). Subconfluent MDCK cells were transfected with GFP or Fmn2-GFP construct for 24–48 h and then fixed with 4% PFA. The cells were stained with anti-GFP and then counterstained with Alexa Fluor 594-phalloidin and DAPI (blue). (B) Fluorescent photomicrographs show that Fmn2-GFP expression causes a decrease in β -catenin expression and localization along the cell membrane compared with GFP control. Noticeably, Fmn2-expressing cells appear to expand with presumptive loss in cell-cell adherence junctions (see arrow). (C) Fluorescent photomicrographs show that Fmn2-GFP expression also causes similar changes to E-cadherin (see arrow-indicated cell). Subconfluent MDCK cells were transfected with GFP or Fmn2-GFP construct for 24 h and then fixed with 4% PFA and costained with anti-GFP and anti- β -catenin or anti-E-cadherin antibodies. Cultures are evaluated by immunofluorescence using an Olympus fluorescent microscope. Scale bar = 10 μ m.

transient Fmn2 expression. β -Catenin immunostaining resides in both Fmn2- and LAMP1-positive circular vesicles in Fmn2-expressing neural progenitor cells (Fig. 3E, right panel). This localization is not appreciated in non-Fmn2-expressing neural progenitor cells (data not shown), suggesting that Fmn2 might mediate lysosomal degradation of the N-cadherin and β -catenin complex following upstream FlnA and RhoA activation.

Fmn2 Directs Lysosomal Degradation of N-Cadherin and β -Catenin Complex

Given that Fmn2 regulates endocytosis of Lrp6 and presumably lysosomal degradation, we first asked whether Fmn2 affects Lrp6 expression. Western blot analyses suggest that total and phosphorylated Lrp6 levels are actually increased with Fmn2 expression (Fig. 4A, data for phospho-Lrp6 not shown). These findings would suggest that upstream activation of Fmn2 would not only promote proliferation through β -catenin transport (Lian et al. 2016) but also by enhancing Lrp6 expression. Given that Fmn2 also promotes redistribution of N-cadherin and β -catenin into lysosomes, we then examined whether Fmn2 affects the expression of these proteins. Although Fmn2 is involved in Lrp6 activation, loss of Fmn2 alone in Fmn2-knockout (null) neural progenitor cells did not significantly decrease the levels of both β -catenin and N-cadherin proteins, as compared with WT cells (Fig. 4B), suggesting the possibility that another downstream Fmn2 function could offset upstream Fmn2/Lrp6 effects on β -catenin levels. Next, we overexpressed Fmn2 in neural cells to determine the fate of the cadherin-catenin complex. We generated several stable Fmn2-expressing Neuro-2 cell clones (Fmn2c1, c2 and c3) by a limiting dilution method. Stable expression of Fmn2 significantly reduces both β -catenin and N-cadherin expression (Fig. 4C). As a comparison, β 1-integrin expression is not diminished and actually increases, suggesting that Fmn2 specifically leads to degradation of the cadherin and

catenin complex, possibly through a lysosomal pathway. The specificity of Fmn2 effects on the proteins' degradation was further confirmed by the unchanged expression levels of other proteins like NeuN, Runx2, and doublecortin (data not shown). To implicate a lysosomal pathway, we used several inhibitors of protein degradation to treat the Fmn2-expressing clones. MG132 blocks protein degradation via both proteasome and, to a lesser extent, lysosomes. Exposure to MG132 leads to a significant accumulation in β -catenin expression in both control cells and in clones of cells expressing Fmn2-GFP (Fig. 4D). Noticeably, multiple bands for β -catenin are apparent in MG132-treated cell samples, suggesting that the degradation of phosphorylated and/or ubiquitinated β -catenin in the cells is inhibited. Treatment with Bafilomycin A1 (BFA1) or chloroquine, both of which are inhibitors for lysosomal degradation, also causes a progressive β -catenin accumulation in Fmn2-expressing cells (Fig. 4E). Finally, treatment with BFA1 plus MG132 produces an added effect on the inhibition of β -catenin degradation compared with that seen only with MG132 (Fig. 4F). Together with the observed localization of these proteins to lysosomes, the results suggest that downstream Fmn2 promotes degradation of the N-cadherin- β -catenin complex through the lysosomal pathway, thereby offsetting upstream Fmn2-dependent β -catenin activation through Lrp6.

Fmn2 Promotes β -Catenin Degradation via Regulating Smurf2-Dependent Ubiquitination of Dishevelled-2

Prior studies showed that degradation of β -catenin is dependent on ubiquitination through the β -TrcP E3 ligase (Winston et al. 1999). Therefore, we first examined whether Fmn2 regulates β -TrcP-triggered ubiquitination of β -catenin. Fmn2 alone or together with β -TrcP did not increase ubiquitination of β -catenin (data not shown), suggesting that Fmn2-regulated degradation of β -catenin does not occur through this pathway. Given these findings, we next asked whether the WW

Figure 3. Fmn2 expression causes redistribution of N-cadherin and β -catenin into the lysosomal compartment. (A) Fluorescent photomicrographs indicate that while Fmn2 (green) nucleates actin, it does not localize along actin filaments (red, arrows, upper panel) but rather shows significant overlap with vesicle-associated actin (arrows, lower panel). MEF cells were transfected with Fmn2-GFP construct for 8 h, fixed, and stained with anti-GFP antibody, Alexa fluor 594-phalloidin, and DAPI. Immunofluorescent images were taken by confocal microscopy. (B) Fluorescent photomicrographs demonstrate colocalization of Fmn2 (green) with N-cadherin (red) in various cellular compartments. Colabeling is seen along the cytoplasmic membrane (arrowhead, upper panel), just under the membrane (arrows, upper panel), and is followed by staining in circular vesicles (see arrows, lower panel). MEF cells were transfected with Fmn2-GFP construct for 6–8 h, fixed, and stained with anti-GFP and anti-N-cadherin antibodies and DAPI. (C) Fluorescent photomicrographs show that Fmn2-associated circular vesicles are associated with the lysosomal compartment. Both panels indicate that Fmn2-positive circular vesicles situated near the nucleus were LAMP1-positive (see arrows), whereas a few Fmn2-positive vesicles adjacent to the peripheral cell membrane were LAMP1-negative (arrowhead). These findings might suggest recent endocytosis followed by trafficking to the lysosomal compartment. MEF cells were transfected with Fmn2-GFP construct for 6–8 h, fixed, and stained with anti-GFP and anti-LAMP1 antibodies. (D) Fluorescent photomicrographs demonstrate overlapping expression of Fmn2 (fluorescein) with the early endosomal antigen marker (EEA1) and late endosomal/lysosomal marker (Rab7). EEA1 expression shows less co-expression with Fmn2 (arrowhead), consistent with a primary role for Fmn2 in lysosomal processing. MEF cells cultured in DMEM/10% FBS were transfected with Fmn2-GFP construct for 8 h, then fixed with 4% PFA, and stained with anti-EEA1 and anti-Rab7 antibodies. (E) Fluorescent photomicrographs demonstrate overlapping expression of N-cadherin or β -catenin (Ctnnb1) with Fmn2 and LAMP1 by triple labeling. The left panel shows that N-cadherin and Fmn2 are localized in LAMP1-positive lysosomes (arrows) and the right panel shows that β -catenin and Fmn2 are also colocalized in LAMP1-positive lysosomes near the nucleus (arrow). Neural progenitor cells transiently expressing Fmn2-GFP were fixed and stained with anti-GFP, anti-LAMP1, and anti-N-cadherin or anti- β -catenin antibodies. (F) Statistical significance for colocalization of the respective proteins is shown graphically. The relative amounts of these proteins were measured using ImageJ software, and the correlation coefficients (Pearson's coefficients, r) was measured by using the ImageJ plugins colocalization analysis procedure. A correlation coefficient greater than 0.5 suggests that there is a significant degree of overlap between actin and Fmn2 at the vesicles and cell membrane, whereas no significant colocalization is seen with F-actin fibers and Fmn2 (left panel). Similarly, significant overlap is seen in the localization of N-cadherin, β -catenin, Fmn2, and lysosomes (right panels). Sample number = 5–10 cells per variable. Scale bar = 10 μ m. * P < 0.05, ** P < 0.01.

Figure 4. N-cadherin and β-catenin (Ctnnb1) undergo degradation through a Fmn2-dependent lysosomal and proteasomal pathway. (A) Western blot analyses show that Fmn2 expression enhances Lrp6 expression, consistent with enhanced upstream activation of β-catenin pathway and enhanced proliferation. Neuro-2 cells were transfected with Fmn2-GFP or GFP construct and then Fmn2-expressing single-cell clones were selected by limiting dilution method. Lrp6 protein levels in GFP control Neuro-2 cells and Fmn2-GFP-expressing clones (Fmn2c1 and c2) were examined by western blot (upper panel) and the statistical analyses from 3 independent assays are graphically shown in the lower panel. (B) Western blot analyses, however, also show that loss of Fmn2 does not lead to a decrease in β-catenin expression compared with WT neural progenitors. Neural progenitor cells were isolated from E13 littermate embryos and cultured in neural stem culture medium. Western blot was performed by using total cell lysates. The upper panel shows a representative result, and the graphics in the lower panel shows quantification of western blot data from 3 pairs of neural progenitor samples. (C) Constitutive expression of Fmn2 significantly reduced not only β-catenin expression (top band) but also N-cadherin expression (the middle bands, at both short and long exposure (Long Expor) times). As a representative control, expression of β1-integrin was significantly increased in Fmn2-expressing Neuro-2 cells. Alpha-tubulin served as a loading control (bottom band). Statistical analysis (see quantitation graphically in lower panel) from 3 assays was done by using ImageJ. (D) Western blot analyses show that Fmn2-dependent degradation of β-catenin occurs via the proteasomal and lysosomal pathways. β-Catenin expression in Fmn2-expressing clonal lines is significantly increased after MG132 treatment (upper band). Tubulin was used as a loading control. Statistical findings are summarized below graphically. Fmn2-expressing cell clones were treated with 10μM MG132 (an inhibitor for proteasome and lysosomal pathway) for 3 h and then lysed in sample buffer. (E) Fmn2-stimulated degradation of β-catenin is inhibited by inhibitors of lysosomal degradation. Western blot shows that treatment of Neuro-2 cells constitutively expressing Fmn2 (Fmn2c1) with either bafilomycin or chloroquine inhibits β-catenin within 1–3 h. The Fmn2-expressing Fmn2c1 cell clone was treated with 100 nM bafilomycin A1 (BFA1, an inhibitor of vacuolar-type H (+)-ATPase in lysosomes) or 50 μM chloroquine (another inhibitor for lysosomal degradation) for varied times and then lysed in sample buffer for western blot. (F) Western blot shows that β-catenin levels in MG132+BFA1-treated samples are increased compared with MG132-only treated samples. Statistical analyses ($n \geq 3$) are shown graphically at the bottom. Fmn2c1 cells were treated by 10μM MG132 alone or together with 100 nM BFA1 for 1 and 3 h, and the samples were then lysed in sample buffer for western blot analyses. The numbers (1) and (3) refer to treatment duration of 1 and 3 h, respectively. Similar results were obtained for the other Fmn2-expressing cell lines. All statistics were performed from $n \geq 3$ replications. * $P < 0.05$; *** $P < 0.001$.

interacting proline-rich domains of Fmn2 could interact with another E3 ubiquitin ligase. Several E3 ligases in the Nedd4 family contain tryptophan-rich WW domains, which specifically bind to proline-rich domains, as found in Formin proteins. We therefore tested whether Fmn2 interacts with these proteins. Fmn2-only weakly immunoprecipitates Smurf1, does not interact with Nedd4, but exhibits much stronger binding to Smurf2, suggesting some degree of specificity of the interaction (Fig. 5A). Immunostaining further shows that Fmn2 expression highly overlaps with endogenous and exogenous Smurf2 but not Nedd4 at the cytoplasmic membrane and circular vesicles in neural progenitor cells (Fig. 5B, C, Suppl Fig 2). Lastly, Smurf2

co-immunoprecipitates with the proline-rich Fmn2 C-terminus (FH1FH2C) but not the N-terminus (Fmn2N) (Fig. 5D, E), suggesting that Smurf2 might bind to the proline-rich domain, as identified in other proline-rich proteins. Given that Fmn2 expression causes β-catenin degradation, we therefore asked whether Smurf2 could enhance β-catenin ubiquitination. Overexpressing Smurf2 alone or together with Fmn2, however, did not increase ubiquitination of β-catenin (Fig. 5F). Additionally, we did not find any effect of Smurf2 on GSK3b ubiquitination as previously reported (data not shown).

Given our recent findings of Fmn2 regulation of upstream Wnt coreceptor Lrp6 turnover (Lian et al. 2016), we addressed

Figure 5. Smurf2 interacts with Fmn2 but does not directly mediate ubiquitination of β -catenin. (A) Western blot demonstrates specific Fmn2 interactions with both Smurf1 and Smurf2 by immunoprecipitation. Stronger binding is seen with Smurf2 compared with Smurf1 (upper and middle panels), whereas no binding is observed between Fmn2 and Nedd4 under the same conditions (lower panel). 293 T cells were transfected with Flag-tagged Smurf1 or Smurf2 and Fmn2-GFP constructs for 24 h and then lysed in co-immunoprecipitation buffer. The lysates were incubated with normal IgG or anti-GFP antibody, Flag-tagged Smurf1 or Smurf2 or endogenous Nedd4 was co-immunoprecipitated using anti-GFP antibodies. (B) Fluorescent photomicrographs show that Fmn2 colocalizes with Smurf2. Neural progenitor cells were transfected with Fmn2-GFP alone or together with Flag-Smurf2 for 6–8 h, fixed, and costained for endogenous Smurf2 (upper panels) or Flag-Smurf2 (lower panels) and Fmn2-GFP, using anti-Smurf2, anti-Flag, and anti-GFP antibodies. (C) Correlation coefficient analyses ($n \geq 5$) show that Fmn2 is colocalized with Smurf2 but not Nedd4 (Suppl Fig 2). Correlation coefficient was measured with NIH ImageJ software using the plugins colocalization analysis procedure. The Pearson's coefficient (r) above 50% indicates a significant colocalization relationship. (D) Smurf2 binds to Fmn2 C-terminal (FH1FH2C domain). 293 T cells were transfected with only Flag-tagged Smurf2 or cotransfected with GFP-FH1FH2C constructs for 24 h, and then the Flag-tagged Smurf2 was co-immunoprecipitated using anti-GFP antibody. The upper panel shows that Smurf2 was pulled down only in GFP-FH1FH2C-cotransfected cells. (E) Smurf2 does not bind to Fmn2 N-terminal (aa1-aa734). 293 T cells were transfected with only Fmn2N-v5 or cotransfected with Flag-tagged Smurf2 constructs for 24 h and then Fmn2N-v5 was co-immunoprecipitated using anti-Flag antibody. (F) Western blots show that Smurf2 alone or together with Fmn2 do not alter β -catenin ubiquitination. No significant change in the intensity of the upper bands (see arrow) is seen following transfection of Fmn2 and Smurf2 (lanes 1 and 2), and His-Ub pulldown, as compared with control cells (lane 4). The lower panel indicates the amounts of all ubiquitinated proteins pulled down by Ni-NTA resin. 293 T cells were transfected with His-tagged ubiquitin (His-Ub), Myc-Smurf2, and/or Fmn2-GFP constructs for 24 h and then treated with 20 μ M MG132 for 5 h. The cells were lysed in guanidine-denaturing buffer and the lysates were centrifuged at 13 000 $\times$ g for 10 min. Ubiquitinated β -catenin was pulled down from cell supernatants with Ni-NTA resin. Scale bar = 10 μ m.

whether Smurf2 could interact with other key proteins in the Wnt-signaling cascade, thereby indirectly affecting β -catenin degradation. We systematically tested several molecules in the Wnt-signaling pathway (Lrp6, β -catenin, Gsk3b, and Daam1) and found that Smurf2 not only specifically binds to Dvl2 (lane 2 in Fig. 6A) but also enhances Dvl2 ubiquitination (Fig. 6B). With respect to the role of Fmn2 in this process, we further found that the Smurf2-Dvl2 interaction is significantly enhanced by co-expressing Smurf2 with Fmn2 or by stimulating 293 T cells with Wnt3a, a ligand for Lrp6 (lanes 3 and 4 in Fig. 6A). The proline-rich Fmn2 C-terminus promotes the Smurf2-Dvl2 interaction, whereas the Fmn2 N-terminus weakens this binding (Fig. 6C), suggesting that Fmn2 activity state might be required for binding enhancement. Fmn2, however, does not appear to directly interact with Dvl2 (Fig. 6D and E), suggesting some unrecognized mechanism whereby Fmn2 promotes Dvl2-Smurf2 interactions. Nevertheless, these studies show that Fmn2 enhances the

ubiquitin ligase Smurf2 interaction with Dvl2, a key component of the Wnt pathway that regulates β -catenin stability.

As ubiquitinated proteins are often targeted for degradation, we asked whether Fmn2 might regulate Smurf2-dependent ubiquitination of Dvl2. We found that Fmn2 significantly enhances Smurf2-triggered ubiquitination of Dvl2 (lane 3 in Fig. 6B and lane 6 in Fig. 6F). As a control and measure of specificity for this function, ubiquitination of axin1 is not increased by co-expression of Smurf2 with Fmn2, although axin1 strongly interacts with Dvl2 (Fig. 6G and H). To clarify the functional consequence of Fmn2-enhanced Dvl2 ubiquitination, we next examined the expression of endogenous β -catenin and Dvl2 following transient expression of Fmn2 and Smurf2. Co-expression of Fmn2 and Smurf2 proteins causes a significant decrease in expression of β -catenin and Dvl2. Expression of axin1, as a control, in these cells is not reduced, but rather increased (Fig. 6I), indicating a specificity of function for Fmn2 and Smurf2 in degrading Dvl2 and indirectly, β -catenin. In

Figure 6. Fmn2 regulates β -catenin degradation by promoting Smurf2-mediated ubiquitination and degradation of Disheveled-2 (Dvl2). (A) Fmn2 significantly increases the binding of Smurf2 to Dvl2. Western blot demonstrates increasing amounts of Flag-Dvl2 precipitated via Myc-Smurf2 when Fmn2 is co-expressed or Wnt3a is added (upper panel). Total protein concentrations in cell supernatants for Flag-Dvl2, Myc-smurf2, and Fmn2-GFP are shown in the lower panels. 293 T cells were transfected with the various constructs for 24 h and then treated with or without 100ng/ml Wnt3a for 3 h. Co-immunoprecipitation was performed with anti-Myc antibody. (B) Western blot for immunoprecipitation of HA-tagged ubiquitin (HA-Ub) and Dvl2 indicates that expression of Fmn2 promotes Smurf2-dependent Dvl2 ubiquitination. The amount of ubiquitinated Dvl2 (ub-Dvl2) increased when Fmn2-GFP was expressed with Smurf2 (last lane). 293 T cells were transfected with HA-ubiquitin, Flag-Smurf2, and Fmn2-GFP constructs for 24 h and then treated with 20 μ M MG132 for 5 h. The cells were lysed and immunoprecipitation was performed with anti-HA antibody. (C) The Fmn2 C-terminal enhances the interaction between Dvl2 and Smurf2, whereas the Fmn2 N-terminal weakens this binding. 293 T cells were transfected with Flag-Dvl2, Myc-Smurf2, and HA-FH1FH2C or together with Fmn2N-v5 constructs and then Myc-Smurf2 was co-immunoprecipitated with anti-Flag antibody. The upper panels show increased Myc-Smurf2 pull-down in HA-FH1FH2C-expressing cells relative to control. Conversely, Fmn2N-v5-cotransfected cells show decreased Myc-Smurf2 pull-down compared with control. The lower panels indicate input protein levels for the various constructs. (D and E). Fmn2 does not physically interact with Dvl2. Dvl2 could not be immunoprecipitated by Fmn2-GFP by western blot analyses. Similarly, Fmn2-GFP could not be pulled down by Dvl2. The input levels represent the expression levels of Fmn2 and Dvl2 proteins in the supernatants. F Fmn2 promotes Dvl2 ubiquitination by Smurf2. Levels of ubiquitinated Dvl2 are significantly increased when Fmn2 is co-expressed with Dvl2 (upper panel). Protein concentration in cell supernatants for Flag-Dvl2, Fmn2-GFP, and Myc-Smurf2 are shown in the lower panels. 293 T cells were transfected with His-ubiquitin, Myc-Smurf2, Flag-Dvl2, or Fmn2-GFP constructs for 24 h and then treated with 20 μ M MG132 for 5 h. Ubiquitinated Dvl2 was pulled down from supernatants with Ni-NTA resin. (G) Dvl2 interacts with axin1. Endogenous axin1 was immunoprecipitated by Dvl2. 293 T cells were transfected with Flag-Dvl2 construct and empty vector and immunoprecipitation was performed with anti-Flag antibody. (H) Fmn2 does not promote axin1 ubiquitination. No increase in axin ubiquitination is observed following Smurf2 and Fmn2 expression, at either short or long exposure times. The procedure is same as the above in F. I. Fmn2 together with Smurf2 decreases expression of Dvl2 and β -catenin. Endogenous β -catenin and Dvl2 levels were decreased following transfection of Fmn2 or Fmn2 and Smurf2. To demonstrate the specificity of this degradation pathway, axin levels were not reduced but actually increased when Fmn2 and Smurf2 were expressed. 293 T cells were transfected with Fmn2-GFP and Flag-Smurf2 constructs for 24 h and then lysed. The cell supernatants were normalized for western blot analyses. Statistical analyses for the respective panels (a), (f), and (i) in Fig. 4 are shown graphically. All experiment variables reflect an $n \geq 3$ samples. Signal intensities of the bands were quantified by using NIH ImageJ software. ANOVA, $P < 0.001$ for panel A, C, and I, and pairwise P values are * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

total, Fmn2 enhances Dvl2 ubiquitination, which, in turn, promotes Dvl2 degradation and subsequent degradation of β -catenin.

Incongruent with the potential role for Fmn2 in promoting Dvl2, β -catenin degradation was the observation that null Fmn2 resting cells do not show significantly higher levels of β -catenin expression than that seen in WT cells (see Fig. 4B). One potential explanation would be due to changes in β -catenin protein synthesis to offset the decreased degradation. In fact, the degradation rates for β -catenin and Dvl2 in null Fmn2 cells are significantly

slowed after cultures were exposed to cycloheximide, a protein synthesis inhibitor (Fig. 7A–D). Noticeably, the loss of Fmn2 also impairs β -catenin activity on Axin1 mRNA transcription (Fig. 7E), suggesting an impairment in β -catenin signaling to the nucleus. Lastly, to test whether overexpression of Dvl2 could rescue β -catenin expression, we transiently expressed Flag-Dvl2 in stable Fmn2-expressing cell clones. Overexpression of Dvl2 rescues the decrease in β -catenin expression in these Fmn2-expressing clones (Fig. 7F). As a representative control, expression of FlnA is not

Figure 7. Fmn2 loss causes impairment in degradation rates of β -catenin and Dvl2 and in β -catenin activity. (A) Fmn2 loss inhibits the β -catenin degradation rate. WT and null Fmn2 neural progenitor cells cultured in neural stem cell culture medium were treated with 200 μ g/ml cycloheximide (CHX) for 0, 1.5, and 3 h, respectively, and then the total cell lysates were prepared for western blot. β -Catenin amounts were measured by using ImageJ software and the degradation curve plot drawn from 3 separate samples is shown graphically in (B). α -Tubulin amounts were used as control. β -Catenin levels at each time point ($P = 0.0098$) were significantly associated with genotype (WT and null Fmn2) but the rate of degradation of β -catenin was not significantly different (ANOVA, $P = 0.3686$) over this time frame given that β -catenin levels were already elevated from impaired degradation with loss of Fmn2. (C) Fmn2 loss slows down Dvl2 degradation rate. WT and null Fmn2 neural progenitor cells cultured in neural stem cell medium were treated with 200 μ g/ml cycloheximide (CHX) for 0, 1, 2, and 4 h, respectively, and then the total cell lysates were prepared for western blot. Dvl2 amounts were measured by using ImageJ software and the plot of Dvl2 degradation rate was drawn from 3 separate samples and shown graphically in (D). α -Tubulin amounts were used as control. Dvl2 levels at each time point ($P = 0.017$) was significantly associated with genotype (WT and null Fmn2) and the rate of degradation over time of Dvl2 showed borderline significance between genotypes ($P = 0.065$). (E) Fmn2 loss impairs β -catenin activation on axin1 transcription. Total mRNAs were prepared from WT and null Fmn2 neural progenitor cells cultured in neural stem cell culture medium and cDNAs were obtained by reverse transcription. Axin1 transcript amounts in WT and null Fmn2 cells ($n = 3$) were quantified by real-time PCR. The relative amount of axin1 RNA in null Fmn2 cells is significantly lower ($<30\%$) than that in WT cells. (F) Dvl2 overexpression rescues β -catenin expression in the Fmn2-constitutively expressing Neuro-2 cell lines. Fmn2-constitutively expressing cell clones (Fmn2c1, c2 and c3) were transfected with Flag-Dvl2 construct overnight. The cell supernatants were prepared and normalized for western blot analyses. Western blot demonstrates increased amounts of β -catenin protein in Dvl2-transfected Fmn2 clones. No change is seen in levels of FlnA, suggesting a fairly specific effect of Dvl2 function on β -catenin expression. Quantification and statistics ($n = 3$) are shown graphically to the right. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

rescued in these cells, suggesting that Fmn2-regulated ubiquitination and degradation of Dvl2 is responsible for the stabilization of β -catenin. Collectively, these findings suggest that Fmn2 promotes Smurf2-dependent ubiquitination and degradation of Dvl2, thereby enhancing β -catenin degradation.

Overexpression of Fmn2 Causes Defects in Neuroepithelial Lining, Neural Cell Migration and Proliferation, and Dvl2 Expression Rescues the Proliferation Defect

To determine the functional role of Fmn2 overexpression in vivo, we performed in utero electroporation of Fmn2-GFP and control GFP constructs into the lateral ventricles of embryonic day 13 murine cerebral cortices and assessed the neuroepithelium and migration. Immunostaining for actin and the adheren junction-associated protein, β -catenin, showed that the apical lining along the lateral ventricular of Fmn2-expressing brain ventricles becomes thinner and at times discontinuous compared with control (Fig. 8A upper and lower panels). By 24 h post-electroporation, most GFP-positive

control neural cells migrate into the upper cortical layers, especially near the outermost molecular layer, whereas the majority of the Fmn2 GFP-expressing cells reside in the intermediate zone (Fig. 8B). No increase in cell death was seen with Fmn2 electroporation (Suppl Fig 1). These results showed that ectopic expression of Fmn2 causes a defect in neuroepithelial integrity and impaired neural cell migration.

Our associated studies show that loss of Fmn2 with FlnA leads to a reduction in neural proliferation and a smaller brain. We therefore examined the effects of Fmn2 overexpression on neural cell proliferation by flow cytometry. Neuro-2 cells, expressing GFP or Fmn2-GFP, were pulse-labeled with BrdU for 40 min, fixed with PFA, and immunolabeled with anti-BrdU antibody. Results from data histograms revealed that the fraction of both BrdU- and GFP-positive cells ($Q2$ = cells that were in the cell cycle) in Fmn2 GFP-expressing cells is lower than that in control GFP-expressing cells (Fig. 8C). Overexpression of Fmn2 significantly decreases BrdU incorporation (17% vs. 28% in control), indicative of reduced proliferation (Fig. 8C). Thus, both loss and overexpression of Fmn2 impairs neural proliferation, suggesting a homeostatic process where Fmn2 overactivation promotes

Figure 8. Fmn2-dependent disruption of neural cell proliferation is rescued by Dvl2. Fmn2 overexpression also impairs the subventricular lining and cell migration. (A) Fluorescent photomicrographs of the lateral ventricles of E13 mouse cortex show that in utero electroporation of Fmn2 (green) causes disruption of the neuroependymal lining. F-actin staining shows actin fibers along the apical lining of Fmn2-GFP-expressing cortex are thinned and discontinuous (see arrows in the left-sided upper panels) compared with that in GFP-alone expressing cortex. Expression of β -catenin (Ctnnb1) along the apical lining of Fmn2-GFP-expressing cortex is also impaired (see arrows in left-sided lower panels). Higher magnification images of the boxed regions are shown to the right. E12 embryos were in utero electroporated with pCAG-GFP or pCAG-Fmn2-GFP constructs, sacrificed at 24 h post-electroporation, and fixed with 4% PFA. Frozen sections (14 μ m) were cut and stained with anti-GFP and anti-Ctnnb1 antibodies. F-actin and nuclei were stained with Alexa Fluor 594-phalloidin and DAPI, respectively. (B) Fluorescent photomicrographs of E13 mouse cortex shows that overexpression of Fmn2 (green) impairs neural cell migration. Most of GFP-alone neural cells have migrated into the upper cortical layers (left image), while most of the GFP-Fmn2-expressing cells only reach into the intermediate zone (right image). Higher magnification of the boxed images is shown below (compare the slower migration seen in the Fmn2-expressing neural cells as compared with GFP-expressing cells). Quantitative analyses (from $n = 8$ pairs of embryos per control and experimental variable) indicate that fewer Fmn2-expressing cells had reached the cortical plate, relative to GFP-expressing cells, consistent with a migration defect. E12 embryos were in utero electroporated with pCAG-GFP or pCAG-Fmn2-GFP constructs, sacrificed at 24 h post-electroporation, and then fixed with 4% PFA. Frozen sections (14 μ m) were stained with anti-GFP antibody and counterstained with DAPI. (C) Flow cytometric analyses show that overexpression of Fmn2 impairs neural cell proliferation. Neuro-2 cells were transfected with GFP and Fmn2-GFP constructs for 24–48 h and then labeled with BrdU for 40 min. The cells were fixed with cold 70% ethanol and stained with anti-GFP and anti-BrdU antibodies. The number of BrdU-positive cells was quantified by flow cytometry. An approximate 40% reduction is seen in the number of BrdU-labeled cells in Fmn2-expressing neural cells relative to GFP-expressing neural cells. (D) Fluorescent photomicrographs of E14 mouse cortex show co-expression of Dvl2 with Fmn2 rescues Fmn2-induced proliferation defect. E13 embryos were in utero electroporated with pCAG-Fmn2-GFP construct alone or together with pCAG-Flag-Dvl2 construct, sacrificed at 24 h post-electroporation, and then fixed with 4% PFA. Frozen sections (14 μ m) were costained with anti-GFP, anti-Ki67 (a proliferation marker) and anti-Flag antibodies, and counterstained with DAPI. The image to the right shows that almost all Fmn2-expressing cells in Fmn2+Dvl2-co-electroporated embryos are Flag-Dvl2-positive. Quantitative analyses are shown graphically to the right (from 18 sections of 3 Fmn2-GFP embryos and 24 sections of 4 Fmn2-GFP+Flag-Dvl2 embryos), confirming that more (77% vs. 64%) Fmn2+Dvl2-co-expressing neural progenitor cells stay in the proliferative status (GFP+ and Ki67+), relative to Fmn2-only expressing cells. No increased cell death was observed with Fmn2 in utero electroporation (Suppl Fig 2). Scale bars = 20 μ m (for right panel of Fig. 8D) and = 50 μ m (for all other panels); ** $P < 0.01$.

downstream Smurf2–Dvl2-dependent β -catenin degradation, whereas Fmn2 inactivation inhibits upstream Wnt-dependent activation of β -catenin through FlnA–Lrp6.

Given that Dvl2 rescues the level of β -catenin protein in Fmn2-expressing cells, we examined if co-expression of Dvl2 with Fmn2 could rescue the Fmn2-induced proliferation defect

to further verify the physiological relevance of their functions. We performed in utero electroporation on E13 embryos with pCAG-Fmn2-GFP construct alone or together with pCAG-Flag-Dvl2 construct. At 24 h post-electroporation, the embryos were harvested, sectioned, and costained with anti-GFP and anti-Ki67 (a proliferation marker) antibodies. Co-staining of GFP with Ki67 indicated that the electroporated neural progenitors remained in a proliferative status, whereas GFP+, Ki67-electroporated neural cells would have exited the cell cycle and proceeded to undergo differentiation (Fig. 8D). Statistical analyses indicate that the ratio of Fmn2+Dvl2-co-expressing proliferating neural progenitor cells is significantly higher (77% vs. 64%) than that of Fmn2-expressing proliferating cells (GFP+ and Ki67+), suggesting that Dvl2 expression can reverse, at least in part, the effect due to Fmn2-impaired proliferation.

Discussion

Although many of the proteins involved in neural progenitor proliferation and specification have been well described, the mechanisms by which cells regulate the spatial and temporal expression of these molecules in the cerebral cortex is not known. We provide the first evidence supporting a role for formin actin-nucleating proteins in regulating neural proliferation through trafficking and degradation of adherens junction proteins. We report that Fmn2 interacts selectively with RhoA to direct actin-dependent vesicle trafficking of catenins and cadherins to the lysosomal compartment. Activated RhoA disengages Fmn2 auto-inhibition, allowing the proline-rich N-terminus of Fmn2 to bind the E3 ubiquitin ligase Smurf2. Smurf2 selectively binds Dvl2, and this binding is enhanced through Fmn2. Smurf2 also promotes ubiquitination of Dvl2, and Dvl2 degradation leads to β -catenin degradation. These observations reveal a cytoskeletal-dependent homeostatic mechanism for regulating neural proliferation (Fig. 9). As shown from our prior study (Lian et al. 2016), upstream Fmn2 promotes Wnt3a-dependent Lrp6 endocytosis, enhances Lrp6 expression, and Gsk3b inactivation, thereby increasing β -catenin nuclear transcription and brain size. Downstream, persistent activation of this pathway, however, leads to Fmn2-induced degradation of β -catenin via Smurf2 and Dvl2 to curtail proliferation via negative feedback.

Disruption of cytoskeletal proteins would be expected to alter various aspects of cortical development, including migration, proliferation, and polarity. For instance, the actin-binding protein FlnA is highly expressed in the ventricular zone of the developing cortex, with loss-of-function mutations causing periventricular heterotopia (PH) in humans. PH is characterized as neuronal nodules in periventricular zone, which was initially thought to originate from a failure in neuronal migration since FLNA has been shown to be essential for migration in many types of non-neuronal cells (Fox et al. 1998; Sheen et al. 2005; Shao et al. 2016). Furthermore, other studies have shown that cytoskeletal proteins are localized to neuronal leading process and indispensable for neuronal migration along glial fibers (Maninova et al. 2017; Mazel 2017). Finally, our recent studies in mouse model shows that FlnA is required for spreading of neural progenitors and migration of neural cells in cerebral cortex (Zhang et al. 2012, 2013). All these findings seem to support the above assumption that PH originates, at least in part, from the failure in FLNA-required migration of neurons. However, the core pathogenesis underlying the PH might be more complex than merely changes in actin effecting cell shape and migration. While FlnA loss in mice impairs neural migration, it does not lead to PH formation, suggesting that FlnA loss might be

Figure 9. Schematic diagram of Fmn2 functions in affecting the Wnt-signaling pathway. Upstream, Fmn2 promotes Wnt3a-dependent Lrp6 activation (phosphorylation) and endocytosis through FlnA binding to Lrp6, and Gsk3b inactivation presumably through endocytosomal formation of phospho-Lrp6-binding Gsk3b or through inhibitory Gsk3b phosphorylation, thereby increasing nuclear β -catenin protein level and brain size. Fmn2 also promotes Lrp6 expression that further enhances this pathway. Downstream, Fmn2 activation by RhoA promotes Smurf2 association, and Smurf2-catalyzed ubiquitination of Wnt downstream molecule Dvl2, thereby causing lysosomal degradation of Dvl2 and β -catenin and inhibiting β -catenin function and brain size.

essential for cell motility but not sufficient for PH formation in mice. Additive loss of FlnB with FlnA, however, does lead to PH in mice but has been proposed to be due to changes in proliferation from interactions between progenitors and the vasculature (Houlihan et al. 2016). Our studies have also suggested that loss of FlnA alone or in combination with Fmn2 leads to microcephaly in mice, with the impairment in progenitor proliferation due to vesicle trafficking defects (Lian et al. 2012, 2016). The current studies further suggest that Fmn2-dependent degradation of adherens junction proteins can regulate cerebral cortical proliferation. Additionally, PH formation has been proposed to be due to breakdown of the neuroepithelial lining and has been observed in multiple human or mouse genes that are known to cause PH (FLNA, BIG2, or alpha-Snap) (Ferland et al. 2009). Trafficking and lysosomal degradation defects would lead to changes in clearance of adherens junction proteins seen in the current study. Further, formin proteins mDia1 and mDia3 regulate neuroepithelial stabilization through apical actin belts, and their loss similarly causes PH in mice (Thumke et al. 2011). Finally, loss of cadherin-related proteins such as FAT, Celsr1, and Vangl also causes formation of periventricular heterotopia (Hirano and Takeichi 2012). Collectively, these recent findings implicate a common molecular mechanism underlying PH pathogenesis and its associated abnormalities whereby changes in the cytoskeletal dynamics (and trafficking as well as degradation) give rise to a more complex neurological phenotype with changes in neural proliferation, migration, and stability of ventricular lining.

Several actin-dependent regulatory processes have been implicated in development of the cerebral cortex. Loss of

Formin 2 with FlnA leads to a reduction in mouse brain size (Lian et al. 2016) with the thought that Formins provide a means of fine-tuning endocytosis (Ryu et al. 2009; Levayer et al. 2011). We now show that Fmn2 activation is tightly regulated by RhoA activation. RhoA is involved in destabilization of actin and microtubules in radial glial scaffolding, and loss of its function has previously been shown to impair neural migration (Cappello et al. 2012). Only RhoA-GTP (and not RhoA-GDP) can selectively disengage Fmn2 auto-inhibition. Moreover, the Fmn2 GBD shows no differential binding between activated and inactivated Cdc42 or Rac, suggesting that these RhoGTPases may be involved in different processes. Another means of regulation extends from RhoA binding to both Fmn2 and FlnA (Ohta et al. 1999). While FlnA likely serves as a scaffold for RhoA to activate Fmn2, we also find that it regulates RhoA activation through binding of various receptors (unpublished observations, Lian and Sheen). In analogous manner, other studies have shown that loss of function of other formins mDia1 and mDia3 leads to disruption of the neuroependymal lining and PH formation in mice (Thumke et al. 2011), whereas changes in other RhoGTPases such as Cdc42 deletion in mouse disrupt the neuroependymal lining, local adherens junctions, and proliferation of basal progenitors, which may lead to neuronal heterotopia (Cappello et al. 2006; Chen et al. 2006). Taken together, the current studies provide a potential mechanistic link whereby different filamin-binding receptors influence the actin cytoskeleton, leading to variable activation of RhoGTPases, consequent formin activation, and initial of different ubiquitin ligases.

Homeostasis may reflect a fundamental characteristic of cytoskeletal proteins that influence neural cell polarity, as excess or insufficient expression of actin cytoskeletal-associated proteins would disrupt progenitor development. For example, Fmn2 is essential for oocyte meiosis via its actin-nucleating function and Fmn2 loss impairs cell division, generating abnormal multichromosomal oocytes (Leader et al. 2002). However, excess Fmn2 also affects actin fiber stabilization and causes multinuclear cell formation through vesicle trafficking (Fig. 2). Likewise, loss of MEKK4 (a MAP kinase and FlnA-binding partner) causes severe heterotopia but leads to increased rather than decreased FlnA expression in developing mouse cerebral cortex. These observations suggest that the dynamics of cytoskeletal proteins and not the amount might be critical for PH formation (Sarkisian et al. 2006) – also consistent with a trafficking role in the maintenance of cell polarity. Thus, insufficient or excessive expression of these cytoskeletal proteins would ultimately affect cell polarity, thereby generating similar phenotypes.

Progression through the cell cycle influences both neural proliferation and cell type specification. We have previously shown that FlnA regulates neural progenitor transition through G2-M phase of the cell cycle by influencing the degradation of cyclinB/Cdk1-associated proteins (Lian et al. 2012). The FlnA-binding RhoA activity decreases rapidly on entry into M phase and then increases after anaphase around the cleavage furrow with FlnA (Nunnally et al. 1980; Yoshizaki et al. 2003). Recent work has implicated aurora kinases as a target of the Wnt/ β -catenin pathway (Dutta-Simmons et al. 2009). Expression of Aurora B reaches a maximum at the G2-M phase transition (Bischoff et al. 1998) and the kinase is necessary for cytokinesis during mitosis. Mislocalization of the protein can disrupt cytokinesis, as it targets a number of proteins to localize to the cleavage furrow including intermediate filaments (Goto et al. 2003). Collectively, these interactions provide a potential mechanism for not only regulation of cell proliferation but also cell

specification. Further studies to clarify how upstream changes in FlnA and RhoA modify catenin-dependent changes in Aurora kinases may provide some insight into how actin-associated proteins direct cell fate specification.

Supplementary Material

Supplementary material is available at *Cerebral Cortex* online.

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