

# LRP6 exerts non-canonical effects on Wnt signaling during neural tube closure

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Low-density lipoprotein receptor related protein 6 (*Lrp6*) mutational effects on neurulation were examined using gain (*Crooked tail*, *Lrp6*<sup>Cd</sup>) and loss (*Lrp6*<sup>−/−</sup>) of function mouse lines. Two features often associated with canonical Wnt signaling, dorsal–ventral patterning and proliferation, were no different from wild-type (WT) in the *Lrp6*<sup>Cd/Cd</sup> neural tube. *Lrp6*<sup>−/−</sup> embryos showed reduced proliferation and subtle patterning changes in the neural folds. Cell polarity defects in both *Lrp6*<sup>Cd/Cd</sup> and *Lrp6*<sup>−/−</sup> cranial folds were indicated by cell shape, centrosome displacement and failure of F-actin and GTP-RhoA accumulation at the apical surface. Mouse embryonic fibroblasts (MEFs) derived from *Lrp6*<sup>Cd/Cd</sup> or *Lrp6*<sup>−/−</sup> embryos exhibited elevated and decreased RhoA basal activity levels, respectively. While ligand-independent activation of canonical Wnt signaling, bypassing Lrp-Frizzled receptors, did not activate RhoA, non-canonical Wnt5a stimulation of RhoA activity was impaired in *Lrp6*<sup>−/−</sup> MEFs. RhoA inhibition exacerbated NTDs in cultured *Lrp6* knockout embryos compared with WT littermates. In contrast, a ROCK inhibitor rescued *Lrp6*<sup>Cd/Cd</sup> embryos from NTDs. Lrp6 co-immunoprecipitated with Disheveled-associated activator of morphogenesis 1 (DAAM1), a formin promoting GEF activity in Wnt signaling. Biochemical and cell biological data revealed intracellular accumulation of Lrp6<sup>Cd</sup> protein where interaction with DAAM1 could account for observed elevated RhoA activity. Conversely, null mutation that eliminates Lrp6 interaction with DAAM1 led to lower basal RhoA activity in *Lrp6*<sup>−/−</sup> embryos. These results indicate that Lrp6 mediates not only canonical Wnt signaling, but can also modulate non-canonical pathways involving RhoA-dependent mechanisms to impact neurulation, possibly through intracellular complexes with DAAM1.

## INTRODUCTION

Low-density lipoprotein receptor related protein 6 (Lrp6), together with Frizzled (Fzd), is an essential component of cell surface receptors for the canonical Wnt/β-catenin signaling pathway (1,2). Wnts are a family of secreted molecules that regulate numerous developmental events through several distinct signal transduction pathways, which are broadly categorized as canonical or non-canonical (3–5). The canonical pathway signals via Wnt ligand binding to Lrp5/6-Fzd1–10 heterodimers to set off a downstream cascade that ultimately results in blocking the ubiquitination and destruction of β-catenin, resulting in cytosolic accumulation and translocation of β-catenin to the

nucleus where it activates TCF/Lef-dependent target gene transcription (6).

Non-canonical Wnt signaling is characterized by Wnt ligand actions transduced through Fzd and/or other receptors, such as Ryk and Ror, that do not result in β-catenin stabilization (reviewed in 5,7,8). Non-canonical effectors have been broadly grouped into either modulators of cytoskeleton remodeling (including JUN N-terminal kinase and the small GTPases, principally RhoA), or Ca<sup>2+</sup>-dependent enzymes (e.g. Ca<sup>2+</sup>/calmodulin-dependent protein kinase and protein kinase C) (5,9). Non-canonical signaling that modulates the cytoskeleton is ascribed to the planar cell polarity (PCP) pathway, because genetic mutations of the relevant genes in *Drosophila*,

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*Xenopus*, *Zebrafish* and mice result in a loss of cell polarity within the plane of an epithelial sheet (10–14). In mice, the Wnt/PCP phenotype has been closely associated with a severe NTD, craniorachischisis, in which the neural tube remains largely or completely open (15–17). These morphogenetic alterations require exquisitely regulated changes in cell shape via cytoskeletal reorganization (18–20). Neurulation in vertebrates requires proper regulation of RhoA signaling (21), which is disrupted in Wnt/PCP mutant mice such as Loop tail (22). Thus, there is an established link between non-canonical Wnt signaling, RhoA regulation, cytoskeletal organization and NTDs.

Historically, Lrp6 has been considered necessary for canonical Wnt signaling only, but studies also implicated Lrp6 in events associated with Wnt/PCP signaling, such as *Xenopus* gastrulation (23), axis elongation in *Xenopus* and neural tube closure in a mouse (24). However, the mechanisms by which Lrp6 achieves these functions during neurulation remain an open question. Two NTD-prone mouse lines, *Lrp6<sup>Cd</sup>* and *Lrp6<sup>-/-</sup>*, provide examples of a gain of function and a loss of function mutation in *Lrp6*, respectively (25,26). *Crooked tail* (Cd) mice carry a single amino acid substitution (G494D) in the extracellular domain of Lrp6 (25), and homozygous *Lrp6<sup>Cd/Cd</sup>* mice display a range of phenotypes, including embryonic lethality, exencephaly (failure of cranial neural tube closure) and runted pups with severe lumbosacral and tail deformities (crooked tails). Exencephaly in *Cd/Cd* embryos can be prevented by prenatal folate supplementation, making it an important model of human NTDs (27). In contrast, *Lrp6* null (*Lrp6<sup>-/-</sup>*) gene-trap mice display an embryonic phenotype primarily characterized by body axis truncation, limb defects, eye and palate defects and a high incidence of exencephaly and/or spina bifida (failure of caudal neural tube closure) (26). Unlike the rescue from NTD in *Lrp6<sup>Cd/Cd</sup>* embryos, prenatal folate supplementation exacerbates NTD in *Lrp6<sup>-/-</sup>* conceptuses and leads to earlier embryonic lethality (28).

The *Lrp6<sup>Cd</sup>* mutation results in a protein that is resistant to Dkk1 antagonism of cytosolic  $\beta$ -catenin stabilization in response to Wnt3a, indicating that *Lrp6<sup>Cd</sup>* is a gain of function mutation (25). This lack of inhibitory function would interfere with the temporal regulation of canonical Wnt signaling and could result in net hyperactivity. However, NTD associated with Wnt pathway genes have so far been ascribed to effects on Wnt/PCP signaling (reviewed in 29). The discovery that mutations in Lrp6 are associated with NTDs suggests either that the canonical pathway also regulates neurulation, or that Lrp6 has a role outside the canonical pathway to affect cranial neurulation in mice. Here, we attempt to resolve these possibilities through comparison of Wnt pathway effects in the neural folds of *Lrp6<sup>Cd</sup>* and *Lrp6<sup>-/-</sup>* embryos. We reason that Wnt pathway functions during neurulation that are altered by both loss and gain of function *Lrp6* alleles are likely to reflect Lrp6-dependent cascades that are critically important for neural tube closure.

## RESULTS

### NTD in Crooked tail is not explained by deregulation of the canonical Wnt pathway

Wnt1 and Wnt3a play a role in the proliferation of progenitor cells in the neural tube, where targeted gene knockout causes

loss of nervous system segments and overexpression results in overgrowth (reviewed in 30). Gain and loss of function mutations in  $\beta$ -catenin also recapitulate these effects (31,32). In addition to cell proliferation, canonical Wnt signaling is essential for dorsal–ventral patterning of the neural tube (reviewed in 33–35). Patterning defects were previously identified in *Lrp6<sup>-/-</sup>* neural tube, which displays expanded Pax3 expression at E9.5 (26). We evaluated canonical Wnt signaling activity in the neural tube of *Lrp6* mutants using a TCF/Lef transcriptional reporter mouse line and also compared cell proliferation and dorsal–ventral patterning in the neural tube of mutant and wild-type (WT) siblings.

Transgenic animals carrying a LacZ cassette expressed from a TCF/Lef-responsive promoter (BatGal (36)) were crossed with the Cd line to assess canonical Wnt/ $\beta$ -catenin-dependent gene transcription. Embryos recovered immediately before or after cranial neurulation displayed  $\beta$ -galactosidase reporter activity indicating that  $\beta$ -catenin-dependent gene transcription in *Lrp6<sup>Cd/Cd</sup>::BatGal* embryos was reduced in crania and unchanged in somites at E9.5, regardless of whether cranial folds were open or closed (Fig. 1A). At E8.5, during completion of neural tube closure, reporter activity was similar in both WT::BatGal and *Lrp6<sup>Cd/Cd</sup>::BatGal* embryos. In addition, LacZ expression detected in tissue sections by immunostaining was similar to whole-mount enzymatic detection with X-Gal (Supplementary Material, Fig. S1). Canonical Wnt signaling in the presence of mutant Lrp6 was further examined in mouse embryonic fibroblast (MEF) cells derived from the *Lrp6<sup>Cd</sup>* and *Lrp6<sup>-/-</sup>* mouse lines (Fig. 1B–D). Cultures were incubated with vehicle or recombinant Wnt3a and collected protein lysates were subjected to western blot analysis, probed with antibodies for total  $\beta$ -catenin and unphosphorylated (activated)  $\beta$ -catenin (37). Compared with littermate controls, basal levels of active  $\beta$ -catenin were lower in mutant MEFs and were induced to a lesser extent by Wnt3a (basal unphosphorylated  $\beta$ -catenin: 33.96 *Lrp6<sup>Cd/Cd</sup>* versus 100 WT; 52.19 *Lrp6<sup>-/-</sup>* versus 100 WT; stimulated: 84.64 *Lrp6<sup>Cd/Cd</sup>* versus 165.51 WT; 84.04 *Lrp6<sup>-/-</sup>* versus 373.97 WT,  $n = 3$ ,  $P < 0.05$ ) (Fig. 1B and C). Similar to the reporter mouse embryos, the difference between mutant and WT activated levels was substantially smaller for *Lrp6<sup>Cd/Cd</sup>* than for *Lrp6<sup>-/-</sup>* MEFs (stimulated values: 84.64 *Lrp6<sup>Cd/Cd</sup>* versus 165.51 WT compared with 84.04 *Lrp6<sup>-/-</sup>* versus 373.97 WT, or 2-fold reduction versus 4.5-fold reduction in active WNT signaling  $P < 0.05$ ). Another readout of canonical signaling, Wnt-dependent transcription of Axin2 mRNA, was compared using quantitative RT-PCR (Fig. 1D). Transcriptional activity paralleled the changes observed for activated  $\beta$ -catenin [basal Axin2 mRNA: 21.32 *Lrp6<sup>Cd/Cd</sup>* versus 100 WT; 9.97 *Lrp6<sup>-/-</sup>* versus 100 WT; stimulated: 65.58 *Lrp6<sup>Cd/Cd</sup>* versus 319.41 WT; 31.81 *Lrp6<sup>-/-</sup>* versus 815.91 WT, or 4.8-fold (*Lrp6<sup>Cd/Cd</sup>*) versus 25.5-fold (*Lrp6<sup>-/-</sup>*) reduction in active Wnt signaling  $n = 3$ ,  $P < 0.05$ ]. Together, these data indicate that *Lrp6<sup>Cd</sup>* is certainly not a hyperactive allele and may be hypomorphic with respect to canonical Wnt signaling through  $\beta$ -catenin in the neural tube.

We next evaluated markers for the dorsal (Pax3), middle (Pax6) and ventral (Nkx2.2) portions of the neural tube of *Lrp6* mutant embryos. Compared with WT siblings (Fig. 2A, E, I), the *Lrp6<sup>-/-</sup>* embryos showed an expansion of the Pax3 and Pax6 protein expression domains (Fig. 2C and G) [*Lrp6<sup>-/-</sup>* versus

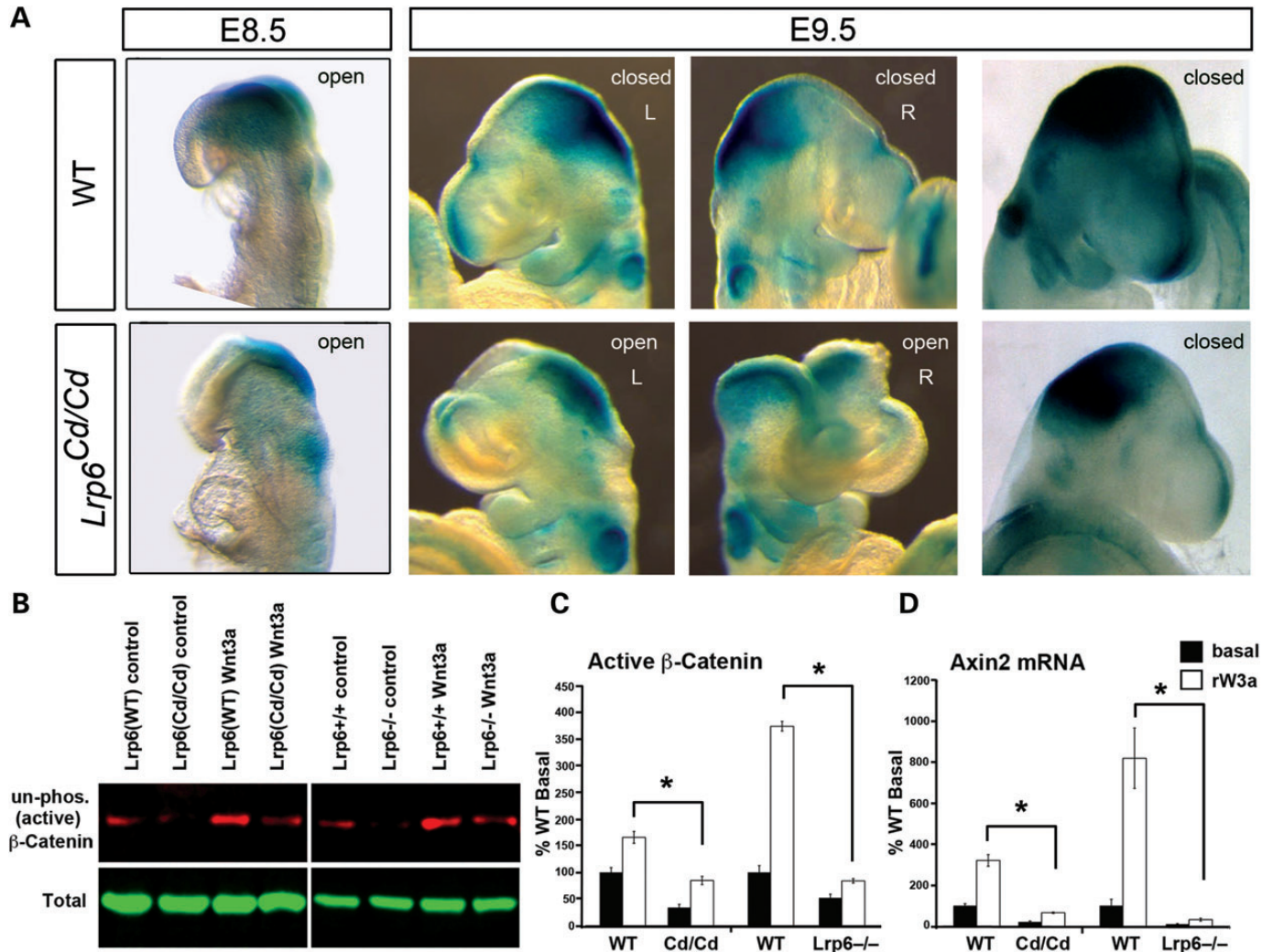


**Figure 1.** *Lrp6<sup>Cd</sup>*-driven TCF/Lef transcription *in situ* and *in vitro*. (A) WT and *Lrp6<sup>Cd/Cd</sup>* embryos carrying the TCF/Lef-reporter transgene, BatGal, were collected at E8.5 and E9.5 and stained with X-gal to visualize canonical Wnt signaling-dependent gene transcription in whole-mount embryos. At E8.5, there is no phenotypic difference between mutant and WT sibling. Two E9.5 *Lrp6<sup>Cd/Cd</sup>* embryos are shown, one with open cranial folds and one that successfully completed cranial neurulation despite being homozygous for the mutation (far right panel), and therefore is phenotypically comparable with the WT sibling. (B) Western blot of Wnt3a responses measuring unphosphorylated (active) β-catenin (red channel) normalized to total β-catenin (green channel) in MEFs derived from the *Lrp6<sup>Cd</sup>* and *Lrp6<sup>-/-</sup>* mouse lines. (C) Quantification of basal and recombinant Wnt3a (rW3a) stimulated levels of activated β-catenin, in mutant compared with littermate-derived WT control MEFs. (D) Quantitative RT-PCR of Axin2 mRNA in control versus rW3a treated MEF cells. \* $P < 0.05$ ,  $n = 3$ .

WT:  $60.4 \pm 0.32\%$  versus  $57.2 \pm 1.01\%$   $\mu\text{m}/\mu\text{m}$  Pax3  $t(4) = 3.19$ ;  $61.6 \pm 3.75\%$  versus  $52.0 \pm 1.54\%$   $\mu\text{m}/\mu\text{m}$  Pax6  $t(5) = 2.69$ ,  $P < 0.05$ , reported as percentages in order to control for size variation among embryos]. However, the extent of patterning gene expression was no different in *Lrp6<sup>Cd/Cd</sup>* embryos (Fig. 2B, F, J), suggesting that altered dorsal–ventral patterning is not the primary cause of NTD in *Lrp6<sup>Cd/Cd</sup>* embryos.

Phospho-histone3 (PH3), an M-phase marker, was used to label dividing neural tube cells at the midbrain/hindbrain junction. In contrast to the reduced divisions in *Lrp6<sup>-/-</sup>* embryos [Fig. 3C and D and (28)],  $(9.92 \pm 0.89$  *Lrp6<sup>-/-</sup>* versus  $11.88 \pm 0.27$  WT, PH3cells/200  $\mu\text{m}$ ,  $t(18) = 2.88$ ,  $P < 0.05$ ), no difference was observed in proliferation between WT and *Lrp6<sup>Cd/Cd</sup>* neural folds (Fig. 3A, B, D).

Apoptosis is also essential to neural tube closure, as disruptions of nearly a dozen genes that are either anti- or pro-apoptotic

have been found to cause NTDs in mice (reviewed in 29). Cell death among genotypes was compared using LysoTracker, a reagent that allows whole-mount visualization of cells with activated lysosomes, a hallmark of apoptosis. No differences in the pattern of cell death in the neural folds were observed between WT, *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* (Fig. 3E–J). Collectively, the above results show that defects in canonical Wnt signaling-dependent functions of proliferation, dorsal–ventral patterning and apoptosis are not sufficient to explain the exencephaly in *Lrp6<sup>Cd/Cd</sup>* mutant embryos.

#### Apical-basal cell polarity is disrupted in crooked tail and *Lrp6<sup>-/-</sup>* neural folds

Cranial neurulation requires formation of a dorsal–lateral hinge point (DLHP) in the neural folds (38). Therefore, the DLHP was

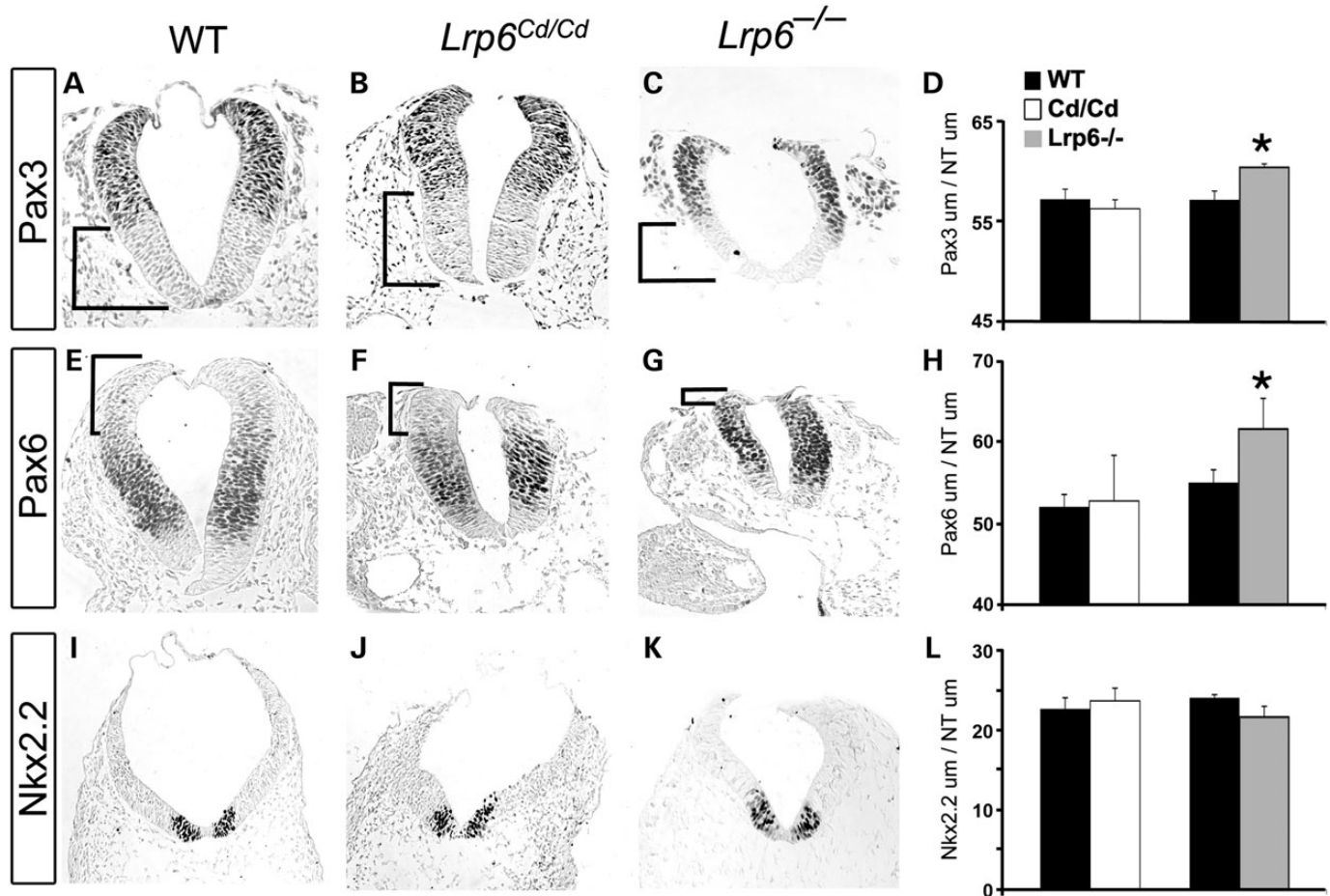


**Figure 2.** Dorsal–ventral patterning of the neural folds is normal in *Lrp6<sup>Cd/Cd</sup>* and modestly altered in *Lrp6<sup>-/-</sup>*. (A–D) The linear extent of Pax3 immunolabeling is slightly expanded ventrally in *Lrp6<sup>-/-</sup>*, but no different in *Lrp6<sup>Cd/Cd</sup>* compared to WT in E9.5 neural folds. (E–H) Pax6 immunolabeling is expanded dorsally in *Lrp6<sup>-/-</sup>*, but remains unchanged in *Lrp6<sup>Cd/Cd</sup>*. (I–L) Localization of the ventral floorplate marker, Nkx2.2, was the same in all genotypes. \* $P < 0.05$ .

examined using the mutant lines intercrossed with mice that ubiquitously express myristoylated Venus fluorescent protein (39) to allow visualization of cell shape *in vivo*. *Lrp6<sup>Cd/Cd</sup>::myr-Venus* or *Lrp6<sup>-/-</sup>::myrVenus* embryos often fail to form a DLHP (Fig. 4B and C). DLHP formation requires cells to lengthen in the apical–basal axis and narrow along the apical surface of cells in the medial–lateral axis (apical constriction), thus creating a bend in the tissue (40,41). Both *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* embryos showed a significant increase in the circularity of cells along the apical surface (Fig. 4D), consistent with a cell shape defect that could impair DLHP formation (WT  $0.57 \pm 0.003$  versus *Lrp6<sup>Cd/Cd</sup>*  $0.72 \pm 0.018$   $t(4) = 4.83$ ,  $P < 0.01$  or WT  $0.61 \pm 0.018$  versus *Lrp6<sup>-/-</sup>*  $0.70 \pm 0.005$   $t(4) = 6.72$   $P < 0.01$ ).

Despite the prior association of Lrp6 with canonical signaling, the defects in cell shape observed in *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* suggest that the neural folds have a polarity defect that prevents cells from properly elongating in the apical–basal axis. Furthermore, evidence in *Xenopus* indicates that both gain and loss of Lrp6 function impairs PCP-associated convergent extension cell movements during gastrulation (23). We therefore reasoned that NTDs in *Lrp6* mutants may result from polarity defects often attributed to Wnt/PCP signaling. Centrosome alignment, an

important marker of cell polarity during neural tube closure, was examined along the apical surface of the neural folds through  $\gamma$ -tubulin immunostaining. Unlike their WT siblings (Fig. 4E and E'), both *Lrp6<sup>Cd/Cd</sup>* (Fig. 4F and F') and *Lrp6<sup>-/-</sup>* (Fig. 4G and G') embryos had centrosomes located closer to the center of the cells rather than at the apical pole.

This failure of centrosome localization to the neural fold apical surface may result from altered small GTPase activity. Cdc42 disruption during *Drosophila* wing formation resulted in failure of cells to elongate in the apical–basal direction (42). This failure was partly due to disorganization of the actin cytoskeleton (42). Subsequently, numerous studies from *Drosophila* to mice have linked Cdc42 with both the establishment of polarity and non-canonical Wnt signaling (reviewed in 12,43,44). Levels of activated Cdc42 were reduced in both *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* compared with WT MEFs (Fig. 4H) (*Lrp6<sup>Cd/Cd</sup>*:  $29.7 \pm 12.6\%$  of WT  $t(3) = -6.12$ ,  $P < 0.01$ ; *Lrp6<sup>-/-</sup>*:  $46.2 \pm 16.0\%$  of WT  $t(3) = -3.37$ ,  $P < 0.05$ ). Together with their enhanced circularity, this mislocalization of  $\gamma$ -tubulin in neural fold cells indicates a significant polarity defect in both the *Lrp6* mutant lines and an important role for Lrp6 in maintaining apical–basal cell polarity during neurulation.

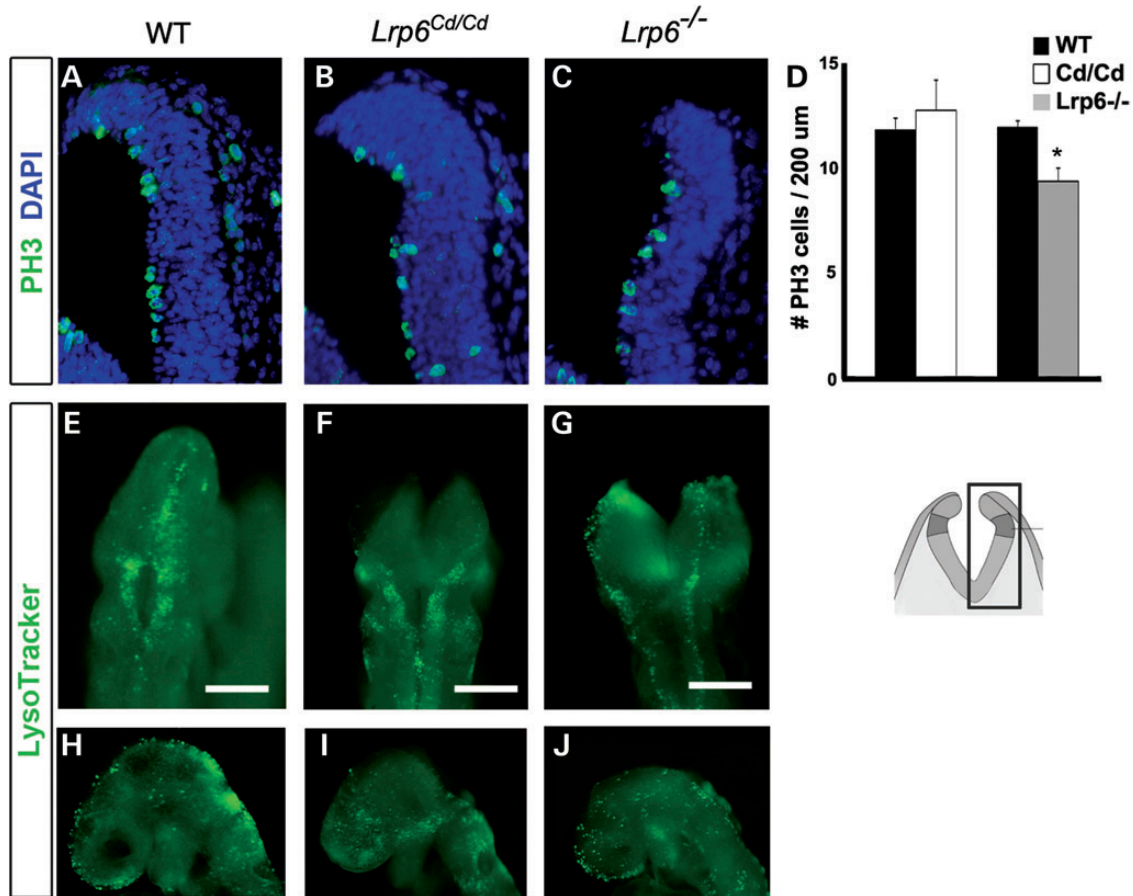


**Figure 3.** Disruption of cell division and apoptosis is not apparent in *Lrp6<sup>Cd/Cd</sup>*, and *Lrp6<sup>-/-</sup>* embryos show expected decreases in cell division. (A–D) No difference in phospho-histone 3 (PH3, green) labeling was observed throughout the neural folds of E9 *Lrp6<sup>Cd/Cd</sup>* embryos compared with WT littermates, whereas *Lrp6<sup>-/-</sup>* nulls showed a significant reduction in labeling (\* $P < 0.01$ ). (E–J) LysoTracker (green) showed similar amounts and distribution of dying cells in *Lrp6<sup>Cd/Cd</sup>*, *Lrp6<sup>-/-</sup>* and WT embryos.

### F-Actin and RhoA are deregulated in *Lrp6* mutants *in vivo* and *in vitro*

During neurulation, F-actin accumulates at the apical surface (Fig. 5A, reviewed in 45) accompanied by apical accumulation of GTP-bound RhoA, which serves as a molecular switch in actin polymerization [Fig. 5D, and (46)]. Histological sections from *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* embryos showed a failure of apical F-actin accumulation in the neural folds (Fig. 5B and C). A GST-tagged Rhotekin-binding domain (RBD) peptide was applied to tissue to bind active RhoA *in vivo*. Compared with WT, active RhoA was generally increased in *Lrp6<sup>Cd/Cd</sup>* and diminished in *Lrp6<sup>-/-</sup>* neural folds with no accumulation at the apical surface (Fig. 5D, E, F).

RhoA activity was next measured *in vitro* using RBD fused to agarose beads to pull down GTP-bound RhoA (Fig. 5G and H). *Lrp6<sup>Cd/Cd</sup>* MEFs contained elevated GTP-RhoA levels compared with WT MEFs, whereas there was significantly less active RhoA in *Lrp6<sup>-/-</sup>* MEFs than WT controls ( $257 \pm 56.5\%$  of WT in *Lrp6<sup>Cd/Cd</sup>*,  $t(5) = 2.76$ ,  $P < 0.05$ ;  $62 \pm 10.8\%$  of WT in *Lrp6<sup>-/-</sup>*,  $t(7) = -3.50$ ,  $P < 0.05$ ). Stimulation of mutant MEFs with recombinant Wnt3a, sufficient to increase GTP-RhoA 2-fold in WT cells, was ineffective in either *Lrp6<sup>-/-</sup>* or *Lrp6<sup>Cd/Cd</sup>* cells. RhoA is a known downstream target of the Wnt/PCP

pathway (47,48). Myosin light chain (MLC) is a prominent target downstream of RhoA and its effector kinase, ROCK. Phospho-MLC2 is required for vertebrate neurulation (21) and is a key element of PCP signaling in the embryonic node (49) and for apical constriction during neurulation. Levels of p-MLC2 were therefore examined in NIH3T3 cells transfected with pLrp6-GFP or pLrp6<sup>Cd</sup> (Fig. 5I and J) and in lysates of WT, *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* MEFs (Fig. 5K and L). In both settings, p-MLC2 levels were elevated in Lrp6<sup>Cd</sup>-expressing cells, and the phosphoprotein was reduced in Lrp6 null cells, providing strong evidence for a role of Lrp6 in RhoA-dependent, non-canonical Wnt signaling.

To further test the participation of Lrp6 in non-canonical Wnt signaling, we examined RhoA activity levels in the presence of recombinant Wnt5a, a ligand considered to act exclusively in the non-canonical Wnt pathway, likely through the Ror tyrosine kinase receptor family (8). Again, basal levels of active RhoA were elevated in *Lrp6<sup>Cd/Cd</sup>* and reduced in *Lrp6<sup>-/-</sup>* MEFs (Fig. 6A). Interestingly, the Wnt5a ligand failed to further stimulate RhoA activity in MEF cultures from either mutant line (Fig. 6A). Moreover, when the GSK3 $\beta$  inhibitor CHIR99021 was used to activate canonical Wnt signaling downstream of the Lrp-Fzd receptors (50), there was no activation of basal RhoA activity (Fig. 6B, Supplementary Material, Fig. S2).

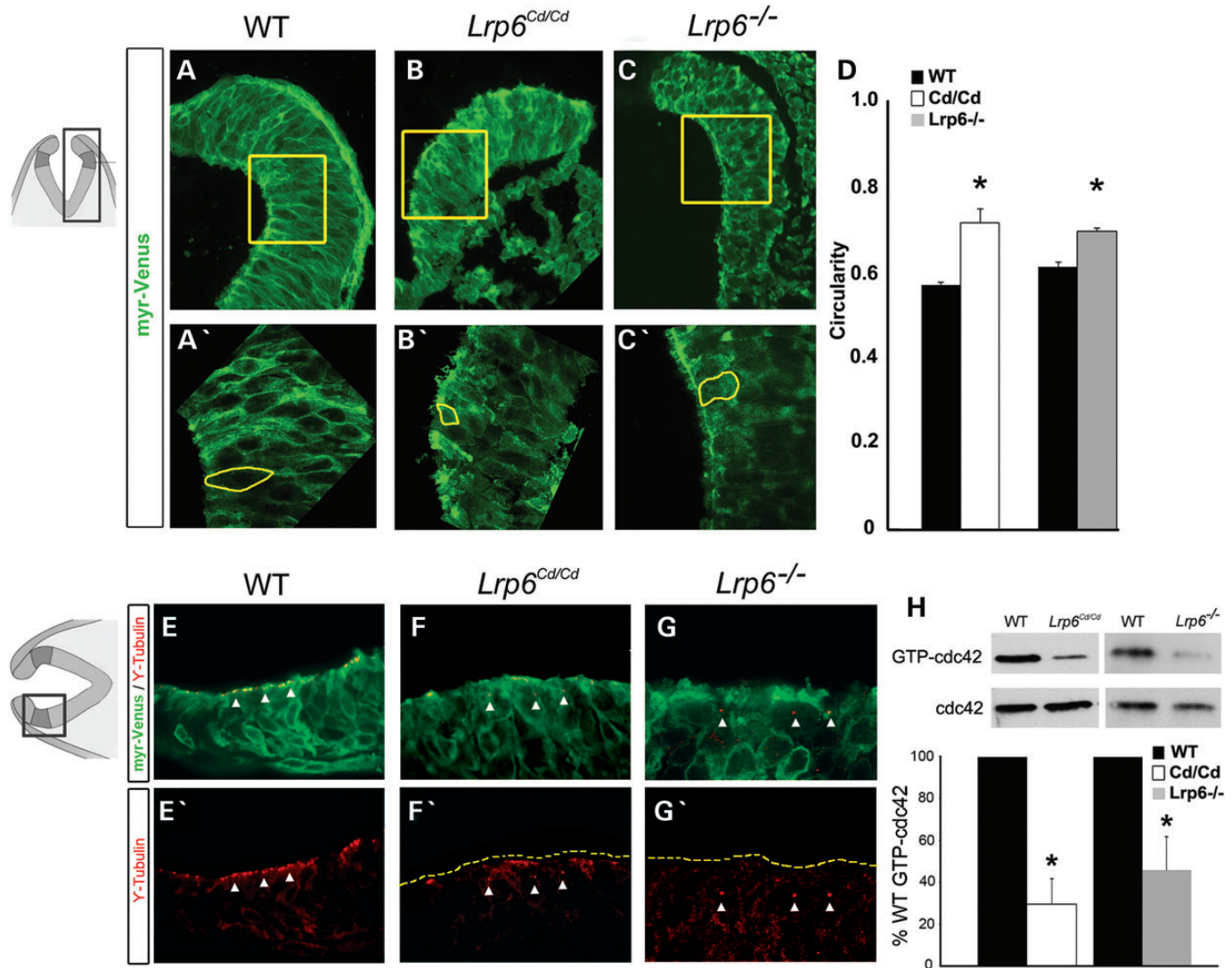


**Figure 4.** Defects in cell shape and polarity in the cranial neural folds of both *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* embryos. (A–C) Sections through the cranial folds of E9 embryos carrying the myr-Venus reporter immunolabeled for GFP reveal cell shape and show that compared with WT there is a failure of cell elongation throughout the *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* neural folds, particularly at the DLHP. (A'–C') Higher magnification images ( $\times 63$ ) in which individual cells have been traced (yellow) illustrate the changes in cell shape. (D) The average shape of cells along the apical surface is measured as circularity, where 1.0 reflects an exactly round measurement and numbers closer to zero reflect an increasing deviation from a perfect circle. Cells are significantly more rounded in both *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* neural folds ( $n = 3$  animals per genotype, 100–150 cells per animal,  $*P < 0.01$ ). (E–H) Cell polarity is disrupted in *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* neural folds and Cdc42 is deregulated in mutant MEFs. (E–G) Merged immunofluorescence images show cell shape (myr-Venus GFP, green) and centrosomes ( $\gamma$ -tubulin, red). In contrast to WT sections, centrosomes fail to accumulate  $\gamma$ -tubulin or position themselves at the apical surface in *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* neural folds. (H) Western blots from PAK-bead pulldowns of the GTP-Cdc42 from MEF cells show a decrease in the active form compared with total cdc42 levels.  $n = 4$ ,  $*P < 0.05$ .

Thus, whether directly or indirectly, *Lrp6* has an impact on the ability of Wnt5a to regulate non-canonical signaling, while the canonical pathway by itself is insufficient to activate RhoA.

#### Proper RhoA regulation is required for neural tube closure in *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* embryos

We next sought to determine whether the deregulation of RhoA observed in the neural folds is an essential mechanism underlying the NTDs in both *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* mutants. Embryos ( $n = 156$  *Lrp6<sup>-/-</sup>* line;  $n = 272$  *Lrp6<sup>Cd/Cd</sup>* line) were collected at E8.5 and were subjected to *ex vivo* roller culture in

media containing a ROCK inhibitor to antagonize the major downstream effector of RhoA during cranial neurulation. Previous reports demonstrated a differential effect of ROCK inhibitor on convergent extension and neurulation in other mutant mice with defective PCP signaling (22). In the present study, heterozygous *Lrp6<sup>+/-</sup>* embryos were more susceptible to NTD in the presence of ROCK inhibitor compared with their WT littermates (Fig. 6C). In contrast, NTD in *Lrp6<sup>Cd/Cd</sup>* embryos, in which basal RhoA activity is elevated, were rescued by increasing doses of ROCK inhibitor (Fig. 6D). The 33% rate of neural tube closure observed for *Lrp6<sup>Cd/Cd</sup>* embryos in the control culture condition is consistent with previous reports of neurulation rates in *Cd/Cd*

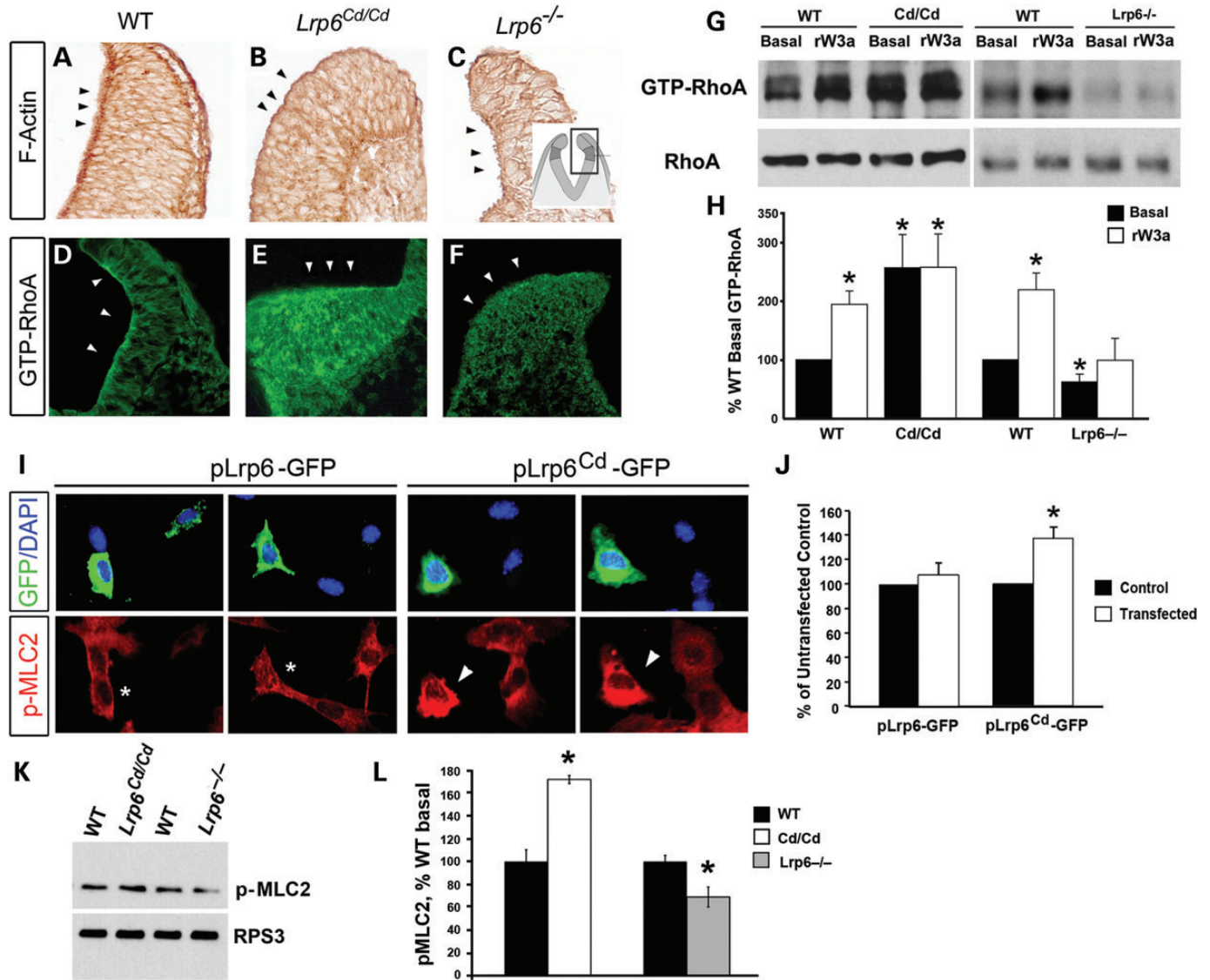


**Figure 5.** Defects in cytoskeletal RhoA activity regulation in *Lrp6* mutants. (A–C) Both *Lrp6*<sup>Cd/Cd</sup> and *Lrp6*<sup>-/-</sup> embryos fail to accumulate F-actin at the apical surface of the neural folds. (D–F) GTP-RhoA is elevated throughout the neural folds in *Lrp6*<sup>Cd/Cd</sup>. In contrast, RhoA activity in *Lrp6*<sup>-/-</sup> is generally reduced and GTP-RhoA fails to accumulate at the apical surface. (G, H) MEF cells isolated from Cd/Cd embryos show elevated basal levels of GTP-RhoA that cannot be further stimulated by recombinant Wnt3a (rW3a), whereas *Lrp6*<sup>-/-</sup> MEFs show diminished basal levels that are unresponsive to Wnt stimulation.  $n = 6$  \* $P < 0.05$ . (I) NIH 3T3 cells transfected with pLrp6-GFP or pLrp6<sup>Cd</sup>-GFP plasmids immunostained for GFP and p-MLC2, phosphorylated on threonine residue 19. In contrast to cells expressing Lrp6-GFP (white \*), those with Lrp6<sup>Cd</sup>-GFP (white arrowhead) showed visibly more p-MLC2 labeling than untransfected cells, quantified in the bargraph (J;  $n = 12$  cells each condition \* $P < 0.02$ ). (K, L). Western blots of MEF cell lysates from mutants and their WT siblings showed p-MLC2 was elevated to 175% (*Lrp6*<sup>Cd/Cd</sup> versus WT) and reduced to 70% (*Lrp6*<sup>-/-</sup> versus WT) of controls (loading normalized either to ribosomal protein S3, or to total MLC2 \* $P < 0.01$ ,  $n = 6$  each genotype).

mice (27), while at 5  $\mu$ M ROCK inhibitor 82% of *Lrp6*<sup>Cd/Cd</sup> embryos closed their neural tubes, a significant rescue ( $z = 2.13$ ,  $P < 0.05$ ). Furthermore, the rescued *Lrp6*<sup>Cd/Cd</sup> embryos show a restoration of centrosomes and their positioning at the apical pole of neural fold cells (Fig. 6E). Thus ROCK inhibition interferes with neurulation in *Lrp6*<sup>-/-</sup> through a mechanism that further decreases the already reduced levels of GTP-RhoA. In contrast, the *Lrp6*<sup>Cd/Cd</sup> mutation, associated with elevated RhoA activity, renders embryos resistant to deleterious effects of ROCK inhibitor, which instead rescues *Lrp6*<sup>Cd/Cd</sup> mutants from NTD and restores centrosomes to the apical pole of the neural tube.

### Improper intracellular regulation of Lrp6<sup>Cd</sup>

We previously showed that the *Cd* point mutation impairs the interaction of Lrp6<sup>Cd</sup> with its known chaperone protein MESD, reducing levels of biotinylated Lrp6 at the cell surface (25). Subsequent studies identified Lrp6 as the target of glycosylation that changes the electrophoretic mobility of the protein so that it appears as a doublet on western blots at around 220 and 240 kDa, well above its predicted molecular weight of 185 kDa (51,52). Here, the *Cd* mutation is shown to produce a 220 kDa immature form of the protein, as well as a novel fragment of ~150 kDa (Fig. 7A). The latter fragment is consistent

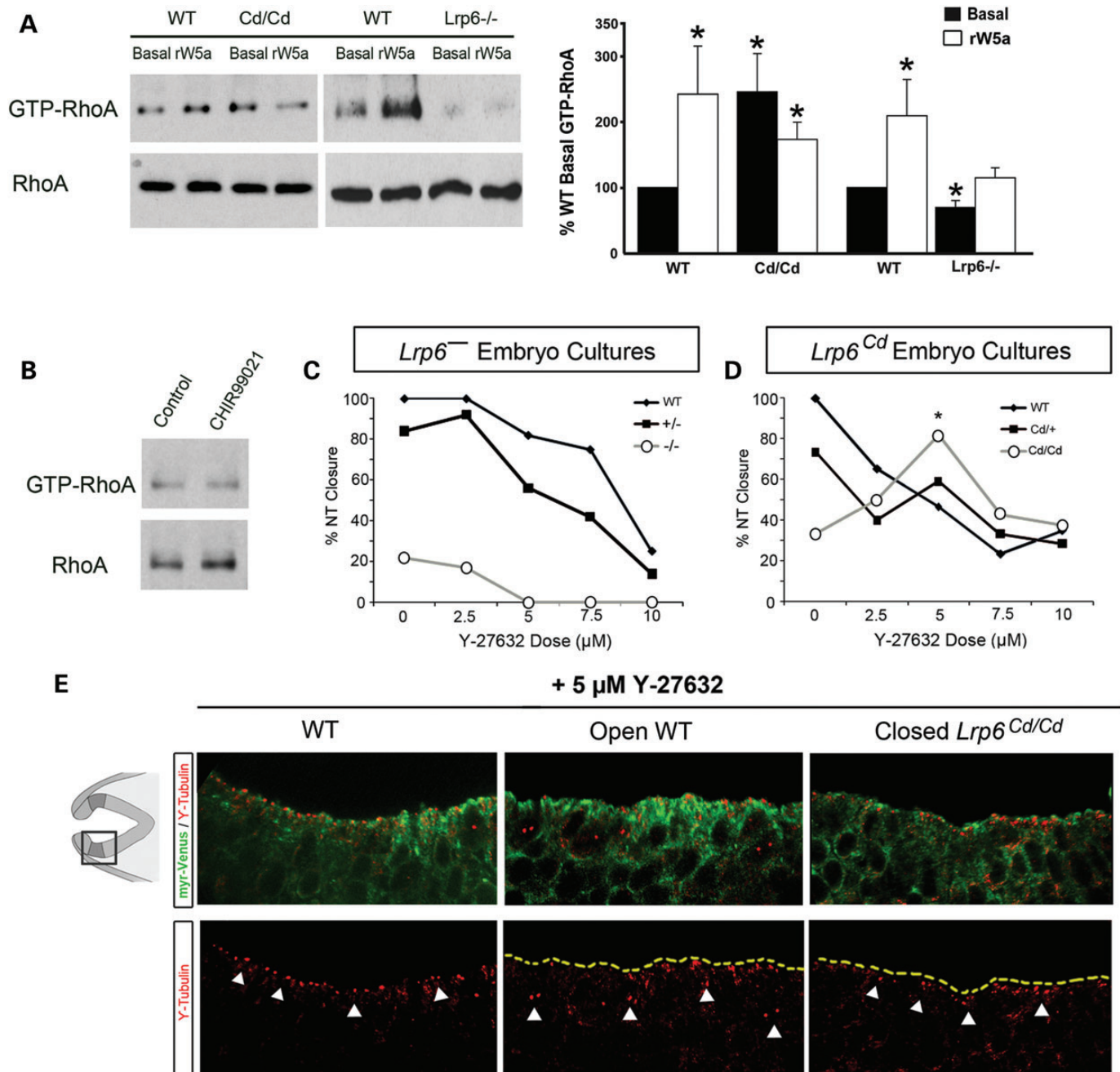


**Figure 6.** *Lrp6* genotype and non-canonical stimulation of RhoA signaling in NTDs. (A) RhoA activity in MEF cells derived from *Lrp6<sup>Cd/Cd</sup>* or *Lrp6<sup>-/-</sup>* and their WT littermate embryos. Cultures were stimulated for 10 min in recombinant Wnt5a. (B) WT MEFs. Basal RhoA activity levels were examined after exposing cells for 4 h to the GSK3β inhibitor, CHIR99021 (10 μM). Canonical Wnt activation independent of Lrp-Fzd does not elevate RhoA activity levels. (C and D) Whole embryo cultures of animals harvested at E8.5 and maintained *ex vivo* for 24 h before phenotypic scoring and genotyping. Graphs show the effects of ROCK inhibitor, Y-27632, on neural tube closure in the *Lrp6<sup>-/-</sup>* (C) and *Lrp6<sup>Cd</sup>* (D) mouse lines. (C) Heterozygous, *Lrp6<sup>+/-</sup>* embryos failed neurulation more frequently at lower doses of Y-27632 when compared with WT littermates, producing a leftward shift in their dose response curve. (D) The 5 μM dose of Y-27632 rescued *Lrp6<sup>Cd/Cd</sup>* embryos from NTD, decreasing the incidence of NTD compared with those cultured with no inhibitor. \**P* < 0.05. (E) Whole embryos cultured in the presence of 5 μM Y-27632. In contrast to a WT with closed cranial folds, the open cranial folds in a WT embryo show displaced γ-tubulin-labeled centrosomes, while the closed cranial folds of the *Lrp6<sup>Cd/Cd</sup>* embryo show more centrosomes are aligned at the apical surface.

with improper cleavage of Lrp6 occurring at the site of the Cd mutation, which would be detectable with the antibody directed against the C-terminal portion of Lrp6. Treatment of WT and *Cd/Cd* MEF lysates with the deglycosylating agent PNGase F

caused a shift in Lrp6 protein to an ~200 kDa species (Fig. 7B, Lane 3) consistent with the removal of the post-translational glycosylation of Lrp6 reported previously (51,52). Treatment of cells with Tunicamycin to prevent glycosylation *in*

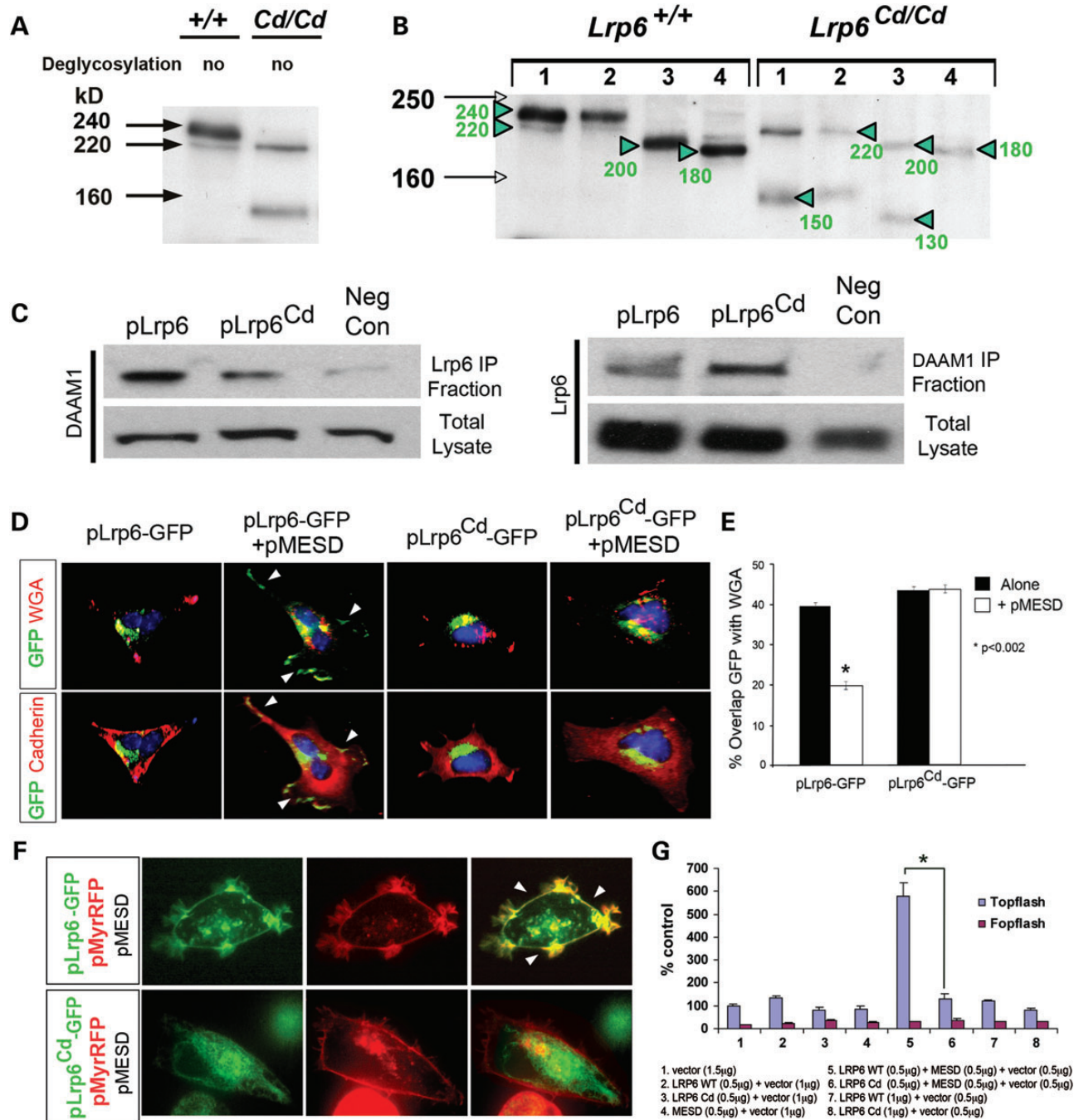


**Figure 7.** Lrp6<sup>Cd</sup> is improperly processed within the cell. (A) Western analysis of Lrp6 in MEFs shows that Lrp6<sup>Cd</sup> fails to form the typical doublet seen in WT and instead produces only the lower isoform and a novel 150 kDa fragment. (B) Treatment of MEF cells with deglycosylating agents shifts Lrp6 bands to smaller apparent molecular weights. Lanes 1 and 2: lysates from untreated cultures prepared with or without glycosylation denaturing buffer; lane 3: lysates treated with PNGase; lane 4: lysates from cells cultured in the presence of Tunicamycin, an inhibitor of protein glycosylation. (C) Lrp6 and DAAM1 are reciprocally co-immunoprecipitated (IP) from cells overexpressing Lrp6 or Lrp6<sup>Cd</sup> protein. Left panels are immunolabeled for DAAM1, right panels for Lrp6. (D) Transfections of NIH3T3 cells with LRP6-GFP fusion protein with equivalent transfer efficiencies for the WT and mutant plasmids confirmed by equal total pixels immunolabeled GFP/cell in the transfected cultures show intracellular GFP fluorescence (green) with WGA-labeled Golgi apparatus (red in upper panel). Dual labeling with anti-pancadherin antibody (red in lower panel) shows the whole cytoplasm and edge of the plasma membrane. When pLrp6-GFP is cotransfected with pMESD, more of the fluorescently labeled receptor gets out of the Golgi (decreased GFP/WGA overlap) and more reaches the plasma membrane (green fluorescence at the cell periphery, arrowheads). In contrast, cells expressing Lrp6<sup>Cd</sup>-GFP display substantial overlap with labeled Golgi, even in the presence of exogenous MESD, with almost no overlap with the plasma membrane. (Blue = DAPI stained nuclei). (E) Quantified overlap of GFP-fluorescence with WGA-labeled Golgi apparatus in cells expressing either Lrp6-GFP or Lrp6<sup>Cd</sup>-GFP with or without added MESD. \*P < 0.0015 compared with WT. n = 12–18 cells per condition. (F) HEK293 cells (live, unfixed) show less mutant Lrp6<sup>Cd</sup>-GFP (green) colocalization (yellow) with the plasma membrane (red, arrowheads) compared with Lrp6-GFP, when cotransfected with myristoylated-red fluorescent protein (pMyrRFP) and pMESD. (G) Transcriptional reporter activity in HEK293 cells transfected with pLrp6<sup>WT</sup> or pLrp6<sup>Cd</sup> with or without pMESD. MESD enhances transcriptional activity of Lrp6<sup>WT</sup>, but has no effect on Lrp6<sup>Cd</sup>-dependent transcription.

*vivo* resulted in a single band in both WT and *Lrp6<sup>Cd/Cd</sup>* cells, each migrating at the predicted molecular weight of Lrp6 of 185 kDa (Fig. 7B, lane 4). Absence of the putative cleavage product in Tunicamycin treated *Lrp6<sup>Cd/Cd</sup>* MEFs suggests that degradation of Lrp6<sup>Cd</sup> requires that the protein is glycosylated and that the aberrant cleavage occurs during or after post-translational processing.

A previous report provided genetic evidence for a role of Lrp6 in the regulation of small GTPases (24). However, the cellular consequences during neurulation and molecular mechanism(s) underlying its effects have not been characterized. Disheveled-associated activator of morphogenesis 1 (DAAM1) is a requisite formin protein linking PCP signaling with changes in GTP-bound RhoA (53–56). Recent reports have also linked DAAM1 with polarity and centrosome reorientation by showing that it co-localizes with both  $\gamma$ -tubulin and myosin IIB (57). We show here that DAAM1 reciprocally co-immunoprecipitates with Lrp6 in NIH3T3 cells transfected with *Lrp6* or *Lrp6<sup>Cd</sup>* cDNA (Fig. 7C), indicating that Lrp6 participates in a protein complex with DAAM1. We therefore reasoned that the implied trafficking defects associated with *Lrp6<sup>Cd</sup>* mutation could result in more Lrp6<sup>Cd</sup> intracellular protein in complex with DAAM1 and so increase basal levels of active RhoA. To test this hypothesis, the subcellular distribution of Lrp6–GFP<sup>Cd</sup> was compared with Lrp6–GFP in transfected NIH3T3 cells (Fig. 7D and E). The plasmids expressing the Lrp6–GFP fusion protein were confirmed by DNA sequencing to differ only in the single nucleotide substitution in the pLrp6<sup>Cd</sup>–GFP mutant and transfections of equal amounts of WT or mutant plasmid resulted in equivalent total GFP fluorescence per cell area. When transfected singly, both the Lrp6–GFP and Lrp6<sup>Cd</sup>–GFP fusion proteins (green) co-localized (yellow) to a similar extent with the intracellular Golgi apparatus (WGA-labeled, red) (Fig. 7D, upper panel). This was quantified in Figure 7E, where similar fractions of the total GFP fluorescence per cell co-localized with the Golgi [ $39.5 \pm 4.6\%$  (Lrp6–GFP) versus  $43.4 \pm 6.3\%$  (Lrp6<sup>Cd</sup>–GFP)]. In contrast, when these plasmids were cotransfected with a construct encoding the Lrp6 chaperone (pMESD) (58), the co-localization of the WT fusion protein to the Golgi complex was sharply reduced while the mutant Lrp6<sup>Cd</sup>–GFP co-localization was unchanged [ $19.8 \pm 4.2\%$  (Lrp6–GFP) versus  $44.0 \pm 7.1\%$  (Lrp6<sup>Cd</sup>–GFP) GFP/Golgi overlap, Fig. 7E]. Qualitatively, more Lrp6–GFP was seen at the cell periphery overlapping the plasma membrane when in the presence of added MESD, indicating that more of the WT protein moved out of the Golgi apparatus to be inserted into the plasma membrane. Moreover, when Lrp6–GFP or Lrp6<sup>Cd</sup>–GFP was cotransfected with plasmids expressing MESD and myristoylated-RFP (to label the plasma membrane), the WT Lrp6–GFP readily colocalized while, in contrast, the Lrp6<sup>Cd</sup>–GFP fusion protein did not colocalize with the membrane, even in the presence of exogenous MESD (Fig. 7F). This failure of membrane insertion of the Lrp6<sup>Cd</sup> protein is consistent with a failure of interaction between the mutant Lrp6 protein and its chaperone, MESD, and is corroborated by a transcriptional reporter assays in transfected HEK293T cells (Fig. 7G), where the unstimulated levels of TCF/Lef transcription were comparable in the WT or mutant constructs. However, when cotransfected with the chaperone MESD, basal activity of the Lrp6<sup>WT</sup> vector was increased nearly 6-fold, while the transcriptional signal of

the mutant Lrp6<sup>Cd</sup> protein, which is unable to interact with MESD, was unchanged by the addition of MESD, presumably due to inefficient plasma membrane insertion of the receptor (25,58). Together the immuno-coprecipitation and subcellular localization data indicate that Lrp6 participates in a complex that includes DAAM1 and that the Cd mutation in Lrp6 impairs its ability to localize to the plasma membrane. Thus, the Lrp6<sup>Cd</sup> protein would accumulate intracellularly where it could promote DAAM1 complex enhancement of RhoA activity (model in Fig. 8).

## DISCUSSION

This study examined the potential mechanisms underlying NTDs in two different mouse *Lrp6* mutants. Numerous essential processes for neural tube closure that are regulated by Wnt signaling were evaluated, including  $\beta$ -catenin-TCF/Lef-dependent gene transcription, cell proliferation and apoptosis, dorsal–ventral patterning, establishment of apical–basal cell polarity and organization of the cytoskeleton. Changes in these features in the Lrp6 mutants are associated with altered RhoA activity levels. Moreover, RhoA activation in the presence of non-canonical Wnt pathway ligand Wnt5a is impaired in the absence of Lrp6. The data suggest that disruption of apical–basal cell polarity and cytoskeleton organization are a primary mechanism underlying NTDs in the *Lrp6* mutants and support a role for Lrp6 in the regulation of cell functions beyond  $\beta$ -catenin-dependent signaling.

Looking for evidence of canonical Wnt effects on neurulation, we found impaired proliferation and expansion of Pax3 and Pax6 expression domains in *Lrp6<sup>-/-</sup>*, but these were unchanged in the *Lrp6<sup>Cd/Cd</sup>* embryonic neural tube. Neither mutation visibly increased apoptosis during advanced stages of neurulation. Nevertheless, the LacZ reporter mouse indicated that canonical Wnt/TCF/Lef-dependent transcription was not elevated in *Lrp6<sup>Cd/Cd</sup>* embryos. This ran counter to the expected outcome given the elevated levels of cytosolic, total  $\beta$ -catenin in *Lrp6<sup>Cd/Cd</sup>* MEF cells, increased nuclear  $\beta$ -catenin in *Lrp6<sup>Cd/Cd</sup>* somites and lack of antagonism by Dkk1 in MEFs and transfected cells previously observed (25). Clearly, the *Lrp6<sup>Cd</sup>* mutation alters the temporal aspect of Wnt signaling as, once activated, Dkk1 does not antagonize the canonical pathway (25). Attenuated elevations of activated  $\beta$ -catenin and Axin-2 mRNA levels in response to Wnt3a in mutant compared with WT cells, together with the inability of cotransfected MESD to enhance Lrp6<sup>Cd</sup>-dependent transcriptional reporter activity, or to increase transit of Lrp6<sup>Cd</sup> out of the Golgi, all suggest that Lrp6<sup>Cd</sup> transcriptional responses in the neural tube may be blunted. Nevertheless, the present study shows that *Lrp6<sup>Cd</sup>* is a gain of function mutation as it is associated with elevated RhoA activity, while *Lrp6<sup>-/-</sup>* embryos display reduced RhoA activity levels and pharmacological inhibition of ROCK, the major effector downstream of RhoA, can rescue NTD and cell polarity defects in *Lrp6<sup>Cd/Cd</sup>* embryos. Our *in vivo* data lend strong support to a growing literature demonstrating context-dependent outcomes of Wnt signaling (reviewed in 5). The present data suggest that canonical Wnt signaling defects do not readily account for NTD in *Lrp6<sup>Cd/Cd</sup>* and that Lrp6 also affects non-canonical functions that, in view of the rather modest patterning changes in the *Lrp6<sup>-/-</sup>* embryos, may be more crucial to advancing neurulation.

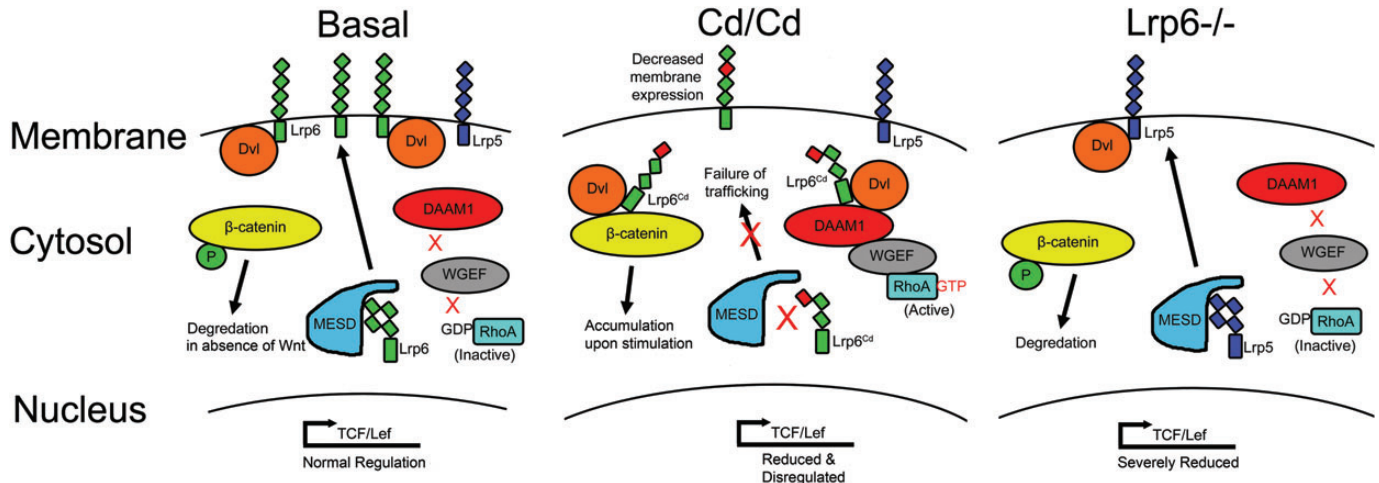


**Figure 8.** Model of  $Lrp6^{Cd}$  deregulation within the cell. Under basal conditions, Lrp6 is efficiently trafficked and inserted into the cell membrane facilitated by MESD. The Cd mutation prevents Lrp6 interaction with MESD and leads to cleavage and defective processing of  $Lrp6^{Cd}$ , all of which reduces localization of  $Lrp6^{Cd}$  on the surface of the cell. The mutant  $Lrp6^{Cd}$  accumulates within the cell to alter levels of  $\beta$ -catenin and increase GTP-RhoA levels through complexation with DAAM1. In the  $Lrp6^{-/-}$  cells, only Lrp5 remains available for signal transduction, and it is insufficient to activate either the canonical or non-canonical pathways to the level necessary for proper neural tube closure.

In contrast to the limited effects of the mutations on canonical signaling parameters in the neural tube, a number of apical–basal cell polarity deficits associated with non-canonical Wnt signaling were found in the neural tube of both the  $Lrp6^{Cd/Cd}$  and  $Lrp6^{-/-}$  embryos. The failure of cells to elongate in the neural folds could account for the improper DLHP formation in both mutants. Unlike mouse mutants closely associated with PCP defects [*Lp*: (59), *Dvl*: (17,60), *Fzd*: (15)], no differences were observed in the width of the floor plate in either  $Lrp6$  mutant line. We did not specifically examine convergence extension in these animals. Nevertheless, cell shape defects in both  $Lrp6^{Cd/Cd}$  and  $Lrp6^{-/-}$  mice coincided with disruption of  $\gamma$ -tubulin localization in the neural folds and deficient Cdc42 activation *in vitro* in both cases, indicating defects in establishing apical–basal cell polarity, a prerequisite for PCP-driven events. This failed polarity was associated with disrupted protein networks implicated in apical constriction (reviewed in 46,61,62), including altered localization of apical F-actin, and GTP-RhoA in the neural folds of both  $Lrp6$  mutants.

GTP-RhoA levels were elevated in  $Lrp6^{Cd/Cd}$  and decreased in  $Lrp6^{-/-}$  MEFs and embryonic neural tube compared with WT. Disruption of RhoA signaling exacerbates NTDs in other mouse models associated with PCP defects (22). Here, ROCK inhibition prevented NTDs in cultured  $Lrp6^{Cd/Cd}$  embryos while exacerbating neurulation defects in  $Lrp6^{+/+}$  animals. Recent reports indicate that phosphorylation of MLC2, a target of ROCK, is controlled by the PCP pathway and that phospho-MLC2 is required for neural tube closure and planar polarization of the embryonic node (21,49). We further demonstrate that, compared with WT,  $Lrp6^{Cd}$  expression in  $Lrp6^{Cd/Cd}$  MEFs or transfected cells produces significant elevation of intracellular phospho-MLC2. Thus a connection among Lrp6 mutants, disregulated RhoA activity, altered phosphorylation of its downstream (MLC2) target through ROCK and, therefore, integrity of non-canonical Wnt signaling pathway elements is compelling. Pharmacological activation of canonical,  $\beta$ -catenin-dependent Wnt signaling using a compound that

bypasses Lrp6/Fzd receptors did not increase RhoA activity, suggesting that changes in RhoA activity in the Lrp6 mutants are not directly related to changes in transcription. Importantly, the degree of RhoA activation in response to Wnt5a, a Wnt ligand acting independently of Lrp6 and instead dependent upon other receptors, activates RhoA to the same levels as Wnt3a, which has mixed canonical and non-canonical signaling potential (63,64). Moreover, Wnt5a treatment does not further increase the already elevated RhoA activity in  $Lrp6^{Cd/Cd}$  MEFs, while RhoA activation by Wnt5a is impaired in  $Lrp6^{-/-}$  MEFs. These results strongly implicate non-canonical Wnt pathway involvement in Lrp6 function with regard to neurulation.

The point mutation in  $Lrp6^{Cd}$  changes an amino acid in the second extracellular  $\beta$ -propeller domain of Lrp6 (25), a region implicated in ligand binding (65). Although Dkk1 still binds  $Lrp6^{Cd}$  protein, the mutation renders Dkk1 unable to antagonize  $\beta$ -catenin stabilization in  $Lrp6^{Cd/Cd}$  MEFs (25). The mutation also reduces cell surface localization most likely because  $Lrp6^{Cd}$  cannot bind its chaperone protein, MESD (Fig. 7) (25,58). Data presented here expand upon these findings to show that  $Lrp6^{Cd}$  fails to produce the mature Lrp6 species and forms a truncated C-terminal fragment, which appears to result from proteolysis dependent upon the Cd mutation. The immature and truncated  $Lrp6^{Cd}$  products were susceptible to the effects of PNGase and Tunicamycin, suggesting that the altered species are generated due to a defect in LRP6 post-translational glycosylation. Importantly, unlike WT fusion protein,  $Lrp6^{Cd}$ –GFP trafficking was much less efficient, regardless of whether MESD was co-expressed. We propose a model in which  $Lrp6^{Cd}$  abnormally accumulates intracellularly because it cannot be properly processed and inserted into the membrane (Fig. 8).

Assessing previous characterization of Lrp6 function in both the canonical and non-canonical Wnt pathways in light of our model, several possible interpretations of our data are evident. Recent work reevaluating the canonical Wnt signaling pathway implicated the Lrp6 cytoplasmic tail as essential for efficient

Dvl signaling and stabilization of  $\beta$ -catenin in the cytosol (66). Intracellular accumulation of C-terminal Lrp6<sup>Cd</sup> fragments in Lrp6<sup>Cd/Cd</sup> cells could facilitate this interaction and contribute to cytosolic  $\beta$ -catenin levels observed in Lrp6<sup>Cd/Cd</sup> embryos (25). Increased cytoplasmic Lrp6<sup>Cd</sup> may also result in abnormal Dvl activation of DAAM1, a formin required for Wnt-dependent changes in RhoA activity (53,54). Here, immunoprecipitation showed that Lrp6 can interact with DAAM1, likely through the complex including Dvl. An intracellular accumulation of mutant protein or peptide that does not reach the plasma membrane could account for the elevated levels of RhoA observed in Lrp6<sup>Cd/Cd</sup> MEFs.

Together, these results indicate that both Lrp6 loss and gain of function disrupt apical–basal cell polarity in the neural folds, and this is associated with suppressed Cdc42 activity and especially with suppressed (in Lrp6<sup>-/-</sup> embryos) or elevated (in Lrp6<sup>Cd/Cd</sup> embryos) RhoA activity. Furthermore, inhibition of RhoA activity (i.e. ROCK inhibition) exacerbates (Lrp6<sup>-/-</sup>) or rescues (Lrp6<sup>Cd/Cd</sup>) mutant embryos from NTD in a genotype-dependent manner. This suggests that Lrp6 functions in neural tube closure primarily as a modulator of a RhoA-dependent, non-canonical Wnt pathway. The present evidence in a mouse is further supported by reports in *Xenopus* that Lrp6 impacts convergent extension during frog neurulation (23). These new data refine our model of defective Lrp6<sup>Cd</sup> action, implicate Lrp6 interaction with non-canonical signaling through a DAAM1-dependent complex, further illuminate the role of Lrp6 in neural tube closure and underscore the importance of further study of Lrp6 point mutants toward understanding the context-dependent functions of this versatile receptor.

## MATERIALS AND METHODS

### Animals

Mice were housed in climate-controlled Thoren units with a 12 h light-dark cycle. All procedures were in accordance with NIH Guidelines and were approved by the Institutional Animal Care and Use Committee at Weill Cornell Medical College. Cd mice were maintained on an A-strain background. Gene-trap mice in which the Lrp6 locus was inactivated (26) were on a stable C3H/HeJ background. TCF/Lef reporter mice (BatGal) were originally generated by Dufort and colleagues (36). Myristoylated Venus GFP (myr-Venus) mice express fluorescent protein that localizes to the inner leaflet of the cellular plasma membrane (39).

### In situ assessment of canonical Wnt activity and cell death

BatGal mice (36) were crossed with Lrp6<sup>Cd/+</sup> mice and offspring of Lrp6<sup>Cd/+</sup> × TCF-LacZ, double heterozygous pairs were evaluated for canonical Wnt reporter activity in the cranial folds at E8.5 and E9.5. Embryos were fixed with 4% paraformaldehyde (PFA) at 4°C, washed in 0.1 M PBS at 4°C, incubated in room temperature buffer (0.02% Igepal, 0.01% Na-deoxycholate, 2 mM MgCl<sub>2</sub>, 100 mM Na-phosphate) for 5 min, transferred to staining solution [1 mg/ml X-Galactosidase, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub> in X-Gal wash buffer] and incubated at 37°C overnight before rinsing in 0.1 M PBS. Embryos were post-fixed with 4% PFA overnight at 4°C.

E9.5 embryos were labeled with LysoTracker (Invitrogen, L-7526), per manufacturer's instructions, to evaluate cell death. Whole mount images were collected on a fluorescent stereomicroscope (Leica M165 FC) with a DFC310 FX camera.

### Immunohistochemistry

Heterozygous breeding pairs were placed together one evening and separated the following morning, which was designated E0.5. Embryos from intercrosses were harvested on E9.5 and yolk sacs were collected for genotyping. Embryos were fixed in 4% PFA overnight at 4°C prior to paraffin processing (Tissue Tek 2000, Miles Laboratories) or cryoembedding in OCT. Paraffin-embedded tissues were sectioned coronally at 6  $\mu$ m, as before (67). Primary antibodies included: anti-PH3 (Upstate Biotechnology, 16-189, 1:1000), anti-Pax3 (Developmental Studies Hybridoma Bank (DSHB), 1:1000), anti-Pax6 (DSHB, 1:1000), anti-Nkx2.2 (DSHB, 1:500), anti-GFP (Santa Cruz, sc-9996, 1:1000), anti- $\gamma$ -tubulin (Santa Cruz, sc-10732, 1:1000), anti-F-Actin (DSHB, 1:5000), anti-phospho-myosin light chain 2 (p-MLC2), phosphorylated on threonine 19 (Cell Signaling 3675, 1:1000 on Western and 1:200 on ICC) and anti pan-cadherin (Abcam 16505, 1:2000). Immunolabeling was visualized using the species appropriate secondary antibody conjugated to a fluorophore [Alexa Fluor 488, Molecular Probes, A-11070 (rb) & A-11001 (ms), 1:500; Alexa Fluor 568, Molecular Probes, A-10042 (rb), 1:500] or conjugated to HRP (Signet Streptavidin detection system, #2246) for detection with DAB. In fixed cells, Golgi apparatus was labeled with Wheat Germ Agglutinin AlexaFluor®594 (WGA594, Life Technologies W32466) at 10 mg/ml. Cryosections for *in situ* GTP-RhoA labeling were post-fixed in ice cold 4% PFA for 5 min at room temp, washed in H<sub>2</sub>O, quenched with H<sub>2</sub>O<sub>2</sub> for 5 min, washed 3 × 5 min in PBS, blocked with SNIPER for 1 h at room temp and incubated in 10  $\mu$ g/ml RBD-GST fusion protein (Cytoskeleton Inc., RT01) overnight at 4°C. After fusion protein incubation, sections were washed then fixed for 10 min with 4% PFA. Sections were incubated with primary rabbit anti-GST antibody (Calbiochem, PC53, 1:200) for 2 h at 37°C. Immunolabeling of fusion protein was visualized using a secondary antibody conjugated to a fluorophore (Alexa Fluor 488, Molecular Probes A-21206, 1:500).

### Assessment of dorsal ventral patterning

Paraffin embedded tissues were sectioned at 6  $\mu$ m and every 10th section was taken for labeling with each of the antibodies. Sections extended from the midbrain to just below the level of the otic vesicle, or ~10–15 sections per marker per animal, times 4 embryos (40–50 sections per marker per genotype). The measures were simple ratios of traces in NIH ImageJ of the length of labeled neural fold divided by the total length of the fold. Measurements were taken bilaterally to avoid any bias due to asymmetrical embedding and values were averaged over the 10–15 sections for each embryo to avoid sampling error. Statistics were generated on the average ratio for each embryo examined (four embryos per each genotype).

### Estimation of cell circularity

Apical basal polarity has been linked to cell shape changes in the neural folds of vertebrates (68). One measure of cell polarity is their circularity, which measures the degree to which the length and width of the cell approximates a circle (e.g. approaches a ratio of 1) (69). Using ImageJ software (NIH), the perimeter of cells at the apical surface nearest to the location of the DLHP was traced individually on sections of *Lrp6<sup>Cd/Cd</sup>::myrVenus* and *Lrp6<sup>-/-</sup>::myrVenus* embryos and their WT siblings. The circularity of cells was determined by the ImageJ software using the equation: circularity =  $4 \pi$  (area/perimeter<sup>2</sup>).

### Plasmids and transfections

pLrp6 and pLrp6<sup>Cd</sup> plasmids were used in transfections as previously described (25). The DAAM1 coding region including a mycDKK tag at the C-terminus was cloned into the pCMV6 mammalian expression plasmid under the control of a CMV promoter (Origene, RC217675). Human pLrp6–GFP and pLrp6<sup>Cd</sup>–GFP contain a monomeric eGFP sequence at the carboxyterminus cloned into pCMV6-AC (Origene, RG218918). The Cd point mutation was introduced into the human pLrp6–GFP construct by site-directed mutagenesis (QuikChange, Stratagene) per manufacturer's instructions. MESD plasmid (pMESD) was a gift of Dr B.C. Holdner, SUNY Stony Brook. Co-localization of Lrp6–GFP with cell compartments was assessed using WGA594 to label the Golgi apparatus or cadherin to label plasma membrane. Co-transfections with myristoylated-RFP (Myr-RFP) plasmid (pCS2-myr-mCherry, a kind gift from Dr Sean Megason, Harvard Medical School) assessed co-localization of the mutant and WT Lrp6–GFP fusion proteins with the plasma membrane. Plasmid transfers were carried out in NIH3T3 or HEK293 cells in 60 mm dishes and transfected with either single plasmids or in combination using Transfectin (BioRad) per the manufacturer. Transfection efficiencies were normalized either to total GFP/cell area or to Fop luciferase. Top/Fop luciferase reporter assays were carried out as previously described (70).

### GTPase assay, western blotting and immunoprecipitation

MEFs were prepared from E14.5 *Lrp6<sup>+/+</sup>*, *Lrp6<sup>Cd/Cd</sup>* and *Lrp6<sup>-/-</sup>* mouse embryos (71). Active GTP-bound forms of RhoA and Cdc42 were measured using a pull-down assay according to the manufacturer's instructions (Cytoskeleton, PAK02). Before cell lysis, cultures were treated for 10 min with 50 ng/ml recombinant Wnt3a or 100 ng/ml recombinant Wnt5a. To activate canonical Wnt signaling independently of Lrp-Fzd binding, *Lrp6<sup>+/+</sup>* cultures were treated with a highly selective GSK3 $\beta$  inhibitor CHIR99021 (50) at 10  $\mu$ M for 4 h—a dose and incubation time selected from our experimental time course at which we found increases in nuclear  $\beta$ -catenin and cyclin D1 protein (Supplementary Material, Fig. S2). Cells were then harvested for detection of GTP-RhoA or GTP-Cdc42. Rhotekin-PBD or WBD agarose-bound proteins (Cytoskeleton, RT02) were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose. Blots were blocked in 3% BSA and incubated in mouse anti-RhoA (Santa Cruz, sc-418, 1:500) or mouse anti-Cdc42

antibody (BD, 610929 1:500). Labeling was detected using HRP-conjugated anti-mouse secondary antibodies and chemiluminescent reagents (Pierce, pico-34078 or femto-34096). X-ray films were scanned on an optical densitometer and relative protein concentrations were determined using Quantity One software (Bio-Rad). Optical densities of bands corresponding to GTP-bound GTPases were normalized to the amount of total RhoA or Cdc42, measured in western blot lanes loaded with equal amounts of lysate total protein. Alternatively, blots assessing protein expression were probed using rabbit anti-dephosphorylated  $\beta$ -catenin (Cell Signaling 8814, 1:1,000) and mouse anti- $\beta$ -catenin (BD 610153 1:4,000), detected using fluorescent antibodies and quantified on an Odyssey IR imager (LiCor) per manufacturer's protocol.

NIH3T3 cells were transfected with pLrp6 or pLrp6<sup>Cd</sup>, with or without cotransfected pDAAM1-mycDKK constructs. After 36 h recovery, replated cells were lysed in 350  $\mu$ l of RIPA buffer [150 mM NaCl, 1.0% IGEPAL<sup>®</sup> CA-630, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 7.4; 1 mM EDTA with protease inhibitor cocktail (Sigma)]. Lysates were clarified at 10 000 rpm for 5 min, pre-cleared with 25  $\mu$ l of Protein A/G beads (Santa Cruz, sc-2003) at 4°C for 30 min and then incubated with 5  $\mu$ l of Lrp6 antibody (Santa Cruz, sc-25317) or mycDKK antibody (Origene, TA50011) at 4°C with shaking overnight. Lrp6 and mycDKK antibodies were precipitated using 25  $\mu$ l of Protein A/G beads overnight. IP fractions and whole lysates were separated by SDS–PAGE and transferred to nitrocellulose as described above. Blots were blocked with either 1% milk or 3% BSA and probed with Lrp6 or mycDKK antibody.

### q-PCR

Total RNA was extracted from 70 to 80% confluent cultures of MEF cells grown in 12-well plates with TRI reagent (Sigma T9424). Template cDNA was prepared using an iScript first-strand cDNA synthesis kit (Bio-Rad 170-8891). Real-time PCR reactions were set up with a GoTaq SYBR green qPCR master mix (Promega) and run on Applied Biosystems 7500 Fast Real-Time PCR system with the following primers: Axin2-F' TGCATCTCTCTCTGGAGCTG; Axin2-R' ACAGCGAGTTATCCAGCGAC; Gapdh-F' TTGATGGCAACAATCTCCAC; Gapdh-R' CGTCCCGTAGACAAAATGGT.

### Lrp6 glycosylation studies

**Cell Culture:** WT (*Lrp6<sup>+/+</sup>*) and Cd/Cd (*Lrp6<sup>Cd/Cd</sup>*) MEF cells were maintained in DMEM (Invitrogen Corp) containing high glucose with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin (1 U/ml)/streptomycin (1  $\mu$ g/ml) (Invitrogen) as supplements. **RIPA lysis:** Cells were washed with cold PBS and lysed with RIPA buffer, on ice. Lysed cells were centrifuged at 12 000g for 15 min at 4°C and supernatants were collected as cell lysates. **Deglycosylation:** PNGase F (New England BioLabs Inc.) removed carbohydrate residues from proteins according to the company's protocol. Tunicamycin (Sigma-Aldrich) was prepared as a 5 mg/ml stock solution in DMSO. In order to prevent post-translational protein glycosylation, cell cultures were treated with Tunicamycin (5  $\mu$ g/ml, Sigma-Aldrich) for 24 h, and lysates prepared as above. Lrp6 proteins were detected on

western blot using anti-Lrp6 antibody (C-10, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### Embryo culture and inhibitor treatment

Embryos from timed pregnancies of either *Lrp6*<sup>Cd/+</sup> or *Lrp6*<sup>+/-</sup> mating pairs were collected when recovered embryos displayed between 10 and 16 somites and primary neural tube closure was underway, but cranial neurulation was not yet complete. Embryos were prepared for whole embryo culture as detailed elsewhere (72). Embryos were incubated in the roller apparatus (BTC Engineering, Cambridge, UK) at 37°C for 2 h prior to addition of a Rho kinase (ROCK) inhibitor, Y-27632 (Enzo Life Sciences, ALX-270-333) or vehicle control (1 µl/ml DMSO). Embryos were allowed to develop in culture for 24 h from harvest to a time at which cranial neurulation should be complete and WT embryos without inhibitor displayed an average of 23–26 somites. Yolk sacs were removed for genotyping and embryos were photographed on a stereomicroscope with CCD camera (Leica) and scored for somite number.

### Statistical treatment of data

Continuous variable measurements, including the extent of immunohistochemical labeling (Fig. 2), number of PH3 positive cells per µm (Fig. 3), cell circularity (Fig. 4), optical density (Figs 4 and 5) and percent fluorescence overlap with Golgi (Fig. 7) were statistically compared using a two-tailed Student's *t*-test. In order to avoid sampling error from single sections, an average value was generated for each individual embryo that was then used for statistical comparison. Multiple measurements were taken from a series of histological sections (minimum of four sections per immune- or fluorescent protein label, serially sampling the caudal midbrain to otic vesicle) (Figs 2–4). Error bars in all figures represent the standard error of the mean. The frequency of neural tube closure (Fig. 6) was statistically compared using a *z*-test calculated using Microsoft Excel software. The exact values for each graph are reported in the results section using the following format:  $t(n-1) = X$  or  $z = X$ ,  $P < 0.05$ , where  $n$  = sample size (e.g. embryos or cultures),  $X$  is the computed *t* or *z* value and the significance threshold is a *P*-value  $< 0.05$ .

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

*Conflict of Interest statement.* None declared.

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