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, Lrp6Cd) and loss (Lrp62/) 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 Lrp6Cd/Cd neural tube. Lrp62/ embryos showed reduced proliferation and subtle patterning changes in the neural folds. Cell polarity defects in both Lrp6Cd/Cd and Lrp62/ 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 Lrp6Cd/Cd or Lrp62/ 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 Lrp62/ MEFs. RhoA inhibition exacerbated NTDs in cultured Lrp6 knockout embryos compared with WT littermates. In contrast, a ROCK inhibitor rescued Lrp6Cd/Cd 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 Lrp6Cd 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 Lrp62/ 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/b-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 b-catenin, resulting in cytosolic accumulation and translocation of b-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 b-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++-dependent enzymes (e.g. Ca++/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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**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 β-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−/− 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/β-catenin-dependent gene expression. Embryos recovered immediately before or after cranial neurulation displayed β-galactosidase reporter activity indicating that β-catenin-dependent gene transcription in Lrp6Cd/Cd::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 Lrp6Cd/Cd::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 Lrp6Cd and Lrp6−/− 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 β-catenin and unphosphorylated (activated) β-catenin (37). Compared with littermate controls, basal levels of active β-catenin were lower in mutant MEFs and were induced to a lesser extent by Wnt3a (basal unphosphorylated β-catenin: 33.96 Lrp6Cd/Cd versus 100 WT; 52.19 Lrp6−/− versus 100 WT; stimulated: 84.64 Lrp6Cd/Cd versus 165.51 WT; 84.04 Lrp6−/− versus 373.97 WT, n = 3, P < 0.05) (Fig. 1B and C). Similar to the reporter embryos, the difference between mutant and WT activated levels was substantially smaller for Lrp6Cd/Cd than for Lrp6−/− MEFs (stimulated values: 84.64 Lrp6Cd/Cd versus 165.51 WT compared with 84.04 Lrp6−/− 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 β-catenin [basal Axin2 mRNA: 21.32 Lrp6Cd/Cd versus 100 WT; 9.97 Lrp6−/− versus 100 WT; stimulated: 65.58 Lrp6Cd/Cd versus 319.41 WT; 31.81 Lrp6−/− versus 815.91 WT, or 4.8-fold (Lrp6Cd/Cd) versus 25.5-fold (Lrp6−/−) reduction in active Wnt signaling n = 3, P < 0.05]. Together, these data indicate that Lrp6Cd is certainly not a hyperactive allele and may be hypomorphic with respect to canonical Wnt signaling through β-catenin in the neural tube.

We next evaluated markers for the dorsals (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−/− embryos showed an expansion of the Pax3 and Pax6 protein expression domains (Fig. 2C and G) [Lrp6−/− versus
WT: $60.4 \pm 0.32\%$ versus $57.2 \pm 1.01\% \mu m / \mu m$ Pax3 $t(4) = 3.19; 61.6 \pm 3.75\%$ versus $52.0 \pm 1.54\% \mu m / \mu m$ 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 \( \text{Lrp6}^{Cd/Cd} \) embryos (Fig. 2B, F, J), suggesting that altered dorsal–ventral patterning is not the primary cause of NTD in \( \text{Lrp6}^{Cd/Cd} \) mutant 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 \( \text{Lrp6}^{-/-} \) embryos [Fig. 2B, F, J], (9.92 \pm 0.89 \( \text{Lrp6}^{-/-} \) versus 11.88 \pm 0.27 WT, PH3 cells/200 \( \mu m \), $t(18) = 2.88, P < 0.05$), no difference was observed in proliferation between WT and \( \text{Lrp6}^{Cd/Cd} \) 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, \( \text{Lrp6}^{Cd/Cd} \) and \( \text{Lrp6}^{-/-} \) (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 \( \text{Lrp6}^{Cd/Cd} \) mutant embryos.

Apical–basal cell polarity is disrupted in crooked tail and \( \text{Lrp6}^{-/-} \) neural folds

Cranial neurulation requires formation of a dorsal—lateral hinge point (DLHP) in the neural folds (38). Therefore, the DLHP was
examined using the mutant lines intercrossed with mice that ubiquitously express myristoylated Venus fluorescent protein (39) to allow visualization of cell shape in vivo. Lrp6Cd/Cd::myr-Venus or Lrp62/2::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 Lrp6Cd/Cd and Lrp62/2 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 ± 0.003 versus Lrp6Cd/Cd 0.72 ± 0.018 t(4) = 4.83, P < 0.01 or WT 0.61 ± 0.018 versus Lrp62/2 0.70 ± 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 Lrp6Cd/Cd and Lrp62/2 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 ± 0.003 versus Lrp6Cd/Cd 0.72 ± 0.018 t(4) = 4.83, P < 0.01 or WT 0.61 ± 0.018 versus Lrp62/2 0.70 ± 0.005 t(4) = 6.72, P < 0.01).

Together with their enhanced circularity, this mislocalization of γ-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 2. Dorsal–ventral patterning of the neural folds is normal in Lrp6Cd/Cd and modestly altered in Lrp62/2. (A–D) The linear extent of Pax3 immunolabeling is slightly expanded ventrally in Lrp6−/−, but no different in Lrp6Cd/Cd compared to WT in E9.5 neural folds. (E–H) Pax6 immunolabeling is expanded dorsally in Lrp6−/− (Fig. 4G and G′) embryos had centrosomes located closer to the center of the cells rather than at the apical pole.
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). Histological sections from Lrp6Cd/Cd and Lrp62/2 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 Lrp6Cd/Cd and diminished in Lrp62/2 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). Lrp6Cd/Cd MEFs contained elevated GTP-RhoA levels compared with WT MEFs, whereas there was significantly less active RhoA in Lrp6Cd/Cd MEFs than WT controls (257 ± 56.5% of WT in Lrp6Cd/Cd, t(5) = 2.76, P < 0.05; 62 ± 10.8% of WT in Lrp62/2, 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 Lrp62/2 or Lrp6Cd/Cd 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 pLrp6Cd (Fig. 5I and J) and in lysates of WT, Lrp6Cd/Cd and Lrp62/2 MEFs (Fig. 5K and L). In both settings, p-MLC2 levels were elevated in Lrp6Cd-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 Lrp6Cd/Cd and reduced in Lrp62/2 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 GSK3β 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).
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 (<i>n = 156 Lrp6<sup>−/−</sup> line; n = 272 Lrp6<sup>Cd/Cd</sup> line</i>) 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.
mice (27), while at 5 μM 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
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\textsuperscript{Cd} is improperly processed within the cell. (A) Western analysis of Lrp6 in MEFs shows that Lrp6\textsuperscript{Cd} 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\textsuperscript{Cd} 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) and substantial overlap (yellow) 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\textsuperscript{Cd}–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\textsuperscript{Cd}–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\textsuperscript{Cd}–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\textsuperscript{WT} or pLrp6\textsuperscript{Cd} with or without pMESD. MESD enhances transcriptional activity of Lrp6\textsuperscript{WT}, but has no effect on Lrp6\textsuperscript{Cd}-dependent transcription.
vivo resulted in a single band in both WT and Lrp6Cd/Cd 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 Lrp6Cd/Cd MEFs suggests that degradation of Lrp6Cd 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 γ-tubulin and myosin IIB (57). We show here that DAAM1 reciprocally co-immunoprecipitates with Lrp6 in NIH3T3 cells transfected with Lrp6 or Lrp6Cd CDNA (Fig. 7C), indicating that Lrp6 participates in a protein complex with DAAM1. We therefore reasoned that the implied trafficking defects associated with Lrp6Cd mutation could result in more Lrp6Cd intracellular protein in complex with DAAM1 and so increase basal levels of active RhoA. To test this hypothesis, the subcellular distribution of Lrp6–GFP 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 plrp6Cd–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 Lrp6Cd–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 ± 4.6% (Lrp6–GFP) versus 43.4 ± 6.3% (Lrp6Cd–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 Lrp6Cd–GFP co-localization was unchanged [19.8 ± 4.2% (Lrp6–GFP) versus 44.0 ± 7.1% (Lrp6Cd–GFP)] (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 Lrp6Cd–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 Lrp6Cd–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 Lrp6Cd 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 Lrp6WT vector was increased nearly 6-fold, while the transcriptional signal of the mutant Lrp6Cd 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 Lrp6Cd 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 β-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 β-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 Lrp6Cd/Cd, but these were unchanged in the Lrp6Cd/Cd 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 Lrp6Cd/Cd embryos. This ran counter to the expected outcome given the elevated levels of cytosolic, total β-catenin in Lrp6Cd/Cd MEF cells, increased nuclear β-catenin in Lrp6Cd/Cd somites and lack of antagonism by Dkk1 in MEFs and transfected cells previously observed (25). Clearly, the Lrp6Cd mutation alters the temporal aspect of Wnt signaling as, once activated, Dkk1 does not antagonize the canonical pathway (25). Attenuated elevations of activated β-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 Lrp6Cd-dependent transcriptional reporter activity, or to increase transit of Lrp6Cd out of the Golgi, all suggest that Lrp6Cd transcriptional responses in the neural tube may be blunted. Nevertheless, the present study shows that Lrp6Cd is a gain of function mutation as it is associated with elevated RhoA activity, while Lrp6Cd/Cd 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 Lrp6Cd/Cd 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 Lrp6Cd/Cd and that Lrp6 also affects non-canonical functions that, in view of the rather modest patterning changes in the Lrp6Cd/Cd embryos, may be more crucial to advancing neurulation.
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 network signaling 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 significantly elevated levels of intracellular phospho-MLC2. Thus a connection among \( Lrp6 \) mutants, deregulated 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 β-catenin in the cytosol (66). Intracellular accumulation of C-terminal Lrp6Cd fragments in Lrp6CdCd cells could facilitate this interaction and contribute to cytosolic β-catenin levels observed in Lrp6CdCd embryos (25). Increased cytoplasmic Lrp6Cd may also result in abnormal Dvl activation during frog neurulation (23). These new data refine our understanding supported by reports in canonical Wnt pathway. The present evidence in a mouse is further illuminated by the role of Lrp6 in neural tube closure primarily as a modulator of a RhoA-dependent, non-canonical signaling through a DAAM1-dependent 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 Lrp6CdCd 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−/− embryos) or elevated (in Lrp6CdCd embryos) RhoA activity. Furthermore, inhibition of RhoA activity (i.e. ROCK inhibition) exacerbates (Lrp6−/−) or rescues (Lrp6CdCd) 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 Lrp6CdCd action, implicating Lrp6 interaction with non-canonical signaling through a DAAM1-dependent complex, further illuminating 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. Genetrap 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 Lrp6CdCd mice and offspring of Lrp6CdCd::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 MgCl2, 100 mM Na-phosphate) for 5 min, transferred to staining solution [1 mg/ml X-Galactosidase, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 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 μm, 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-γ-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 A-11070 (rb), A-11001 (ms), 1:500). Paraformaldehyde was used for canonical Wnt reporter activity in the cranial folds at E8.5 and E9.5. Embryos from intercrosses were harvested on E9.5 and incubated in 10 μg/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 μm 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</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 = 4π (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 (QuickChange, Stratagene) per manufacturer’s instructions. M Shed 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 Transfectant (BioRad) per the manufacturer. Transfection efficiencies were normalized either to total protein 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 (Cytokeleton, PAK02). Before cell lysis, cultures were treated for 10 min according to the manufacturer’s instructions (Cytoskeleton, RT02). Lysates were clarified at 10 000 rpm for 5 min, pre-cleared with 25 μl of Protein A/G beads (Santa Cruz, sc-2003) at 4°C for 30 min and then incubated with 5 μl 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 μl 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′ TGCATCTCTTCTTGAGCTG; Axin2-R′ TTGATGGCAACAAT. 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′ TGCATCTCTTCTTGAGCTG; Axin2-R′ TTGATGGCAACAAT.

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 μg/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 μg/ml stock solution in DMSO. In order to prevent post-translational protein glycosylation, cell cultures were treated with Tunicamycin (5 μg/ml, Sigma-Aldrich) for 24 h, and lysates prepared as above. Lrp6 proteins were detected on
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). Xi 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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