

RESEARCH ARTICLE

The role of Lrp6-mediated Wnt/ β -catenin signaling in the development and intervention of spinal neural tube defects in mice

Tianyu Zhao, Moira McMahon, Kurt Reynolds, Subbroto Kumar Saha, Arjun Stokes and Chengji J. Zhou*

ABSTRACT

Neural tube defects (NTDs) are among the common and severe birth defects with poorly understood etiology. Mutations in the Wnt co-receptor *LRP6* are associated with NTDs in humans. Either gain-of-function (GOF) or loss-of-function (LOF) mutations of *Lrp6* can cause NTDs in mice. NTDs in *Lrp6*-GOF mutants may be attributed to altered β -catenin-independent noncanonical Wnt signaling. However, the mechanisms underlying NTDs in *Lrp6*-LOF mutants and the role of Lrp6-mediated canonical Wnt/ β -catenin signaling in neural tube closure remain unresolved. We previously demonstrated that β -catenin signaling is required for posterior neuropore (PNP) closure. In the current study, conditional ablation of *Lrp6* in dorsal PNP caused spinal NTDs with diminished activities of Wnt/ β -catenin signaling and its downstream target gene *Pax3*, which is required for PNP closure. β -catenin-GOF rescued NTDs in *Lrp6*-LOF mutants. Moreover, maternal supplementation of a Wnt/ β -catenin signaling agonist reduced the frequency and severity of spinal NTDs in *Lrp6*-LOF mutants by restoring *Pax3* expression. Together, these results demonstrate the essential role of Lrp6-mediated Wnt/ β -catenin signaling in PNP closure, which could also provide a therapeutic target for NTD intervention through manipulation of canonical Wnt/ β -catenin signaling activities.

KEY WORDS: Lrp6, Wnt/ β -catenin signaling, Spinal neural tube defects, Genetic rescue, Pharmacological intervention

INTRODUCTION

The neural tube is the precursor structure of the brain and spinal cord. Failure of proper neural tube closure at different anatomical regions along the anterior–posterior body axis results in various types of severe neural tube defects (NTDs), such as spina bifida (spinal NTDs) and exencephaly (cranial NTDs), the former of which is the most common type of NTD in humans (Greene and Copp, 2014). Although NTDs affect more than 300,000 newborns worldwide annually, of which ~3000 cases occur in the United States (Centers for Disease and Prevention (CDC), 2004; Zaganjor et al., 2016), the etiology of NTDs remains poorly understood due to its complexity (Copp et al., 2013; Wallingford et al., 2013). Folate

supplementation may prevent a considerable portion of NTDs, but the majority of NTDs that occurred in developed countries with mandatory folic acid fortification are considered unpreventable by folate intake alone (Williams et al., 2015). Therefore, it is imperative to study fundamental mechanisms of normal and defective neural tube closure, which could lead to the development of novel prevention strategies for NTDs.


Mutant mice have been widely used for NTD mechanistic and preventative studies (Greene and Copp, 2005; Zohn, 2020). More than 200 genes have been linked with NTDs in mice (Harris and Juriloff, 2007, 2010), suggesting that the genetic basis of neural tube closure is highly complex. However, only a small number of genes, including several involved in Wnt signaling, have been associated with human NTDs (Allache et al., 2014; Au et al., 2010; De Marco et al., 2014).

Morphogenetic Wnt signaling plays vital roles in early embryonic development, including gastrulation and neurulation. Wnt ligands bind to various types of receptors and co-receptors to transduce signals via β -catenin-dependent (canonical) and β -catenin-independent (noncanonical) pathways. The noncanonical Wnt/planar cell polarity (PCP) signaling pathway regulates cytoskeleton dynamics and collective tissue movements, such as convergent extension, which may drive neural tube closure (Copp et al., 2003; Wallingford, 2006; Wang et al., 2019; Ybot-Gonzalez et al., 2007). The rare but severest type of NTDs, craniorachischisis, is characterized by an entirely open brain and spinal cord (Tobin et al., 2019), and results from failure of the initial neural tube closure at the boundary between the future hindbrain and spinal cord. Craniorachischisis is associated with defective convergent extension and has been mainly found in mice with mutant PCP signaling genes, such as *Ptk7* and the *Vangl*, *Celsr*, *Dvl* and *Fzd* families (De Marco et al., 2011; Juriloff and Harris, 2012). Several of these PCP components, such as *Fzds* and *Dvls*, also play essential roles in the canonical Wnt/ β -catenin signaling pathway (MacDonald et al., 2009).

The Wnt co-receptor *Lrp6* acts upstream of β -catenin in the canonical pathway and is required for a wide range of processes pertaining to embryogenesis and organogenesis, including neural tube closure (Alrefaei and Abu-Elmagd, 2022; Carter et al., 2005; He et al., 2004; Kokubu et al., 2004; Mao et al., 2001; Pinson et al., 2000; Song et al., 2009, 2010; Tamai et al., 2000; Wang et al., 2016; Wehrli et al., 2000; Zhou et al., 2008, 2004, 2010). Multiple studies have identified mutations in human *LRP6* gene from patients with NTDs (Allache et al., 2014; Lei et al., 2015; Shi et al., 2018). In mice, both hypermorphic and hypomorphic mutations in *Lrp6* gene can cause NTDs. The cranial NTDs in the spontaneous mutant *crooked tail* mice (Carter et al., 2005) and spinal NTDs in novel N-ethyl-N-nitrosourea (ENU)-induced *Skax26* mice (in combination with heterozygous *Vangl2^{LP}*) (Allache et al., 2014) are caused by hypermorphic *Lrp6*. In contrast, spinal NTDs in the spontaneous *ringelschwanz* mice are caused by hypomorphic

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Lrp6 (Kokubu et al., 2004), and *Lrp6*-null mutants exhibit partially penetrant cranial NTDs and fully penetrant spinal NTDs (Pinson et al., 2000; Zhou et al., 2010). It has been reported that altered noncanonical Wnt signaling may contribute to NTDs in hypermorphic *Lrp6* mutants (Allache et al., 2014; Gray et al., 2013). However, the causal mechanisms of NTDs in *Lrp6*-deficient mutants and the explicit role of *Lrp6*-mediated canonical Wnt/ β -catenin signaling in neural tube closure are thus far obscure.

We have previously demonstrated that conditional ablation of β -catenin in the dorsal neural folds diminishes expression of the paired-box gene *Pax3*, which encodes a transcription factor critical to neural tube closure, resulting in an open spinal NTD phenotype (Zhao et al., 2014). The β -catenin-Tcf/Lef1 complex modulates activity of the *Pax3* promoter, and ectopic activation of *Pax3* transgene can rescue spinal NTDs in β -catenin mutants. Moreover, β -catenin and *Pax3* cooperate to regulate the caudal type of homeobox gene *Cdx2* for caudal body axis elongation (Zhao et al., 2014). In the current study, we demonstrate that conditional ablation of *Lrp6* in dorsal neural folds causes fully penetrant spinal NTDs, as a potential consequence of diminished activities of canonical Wnt/ β -catenin signaling and its downstream target gene *Pax3*, which are consistent with those occurring in conditional β -catenin mutants. Moreover, our genetic and pharmacological rescue approaches demonstrate the crucial roles of *Lrp6*-mediated Wnt/ β -catenin signaling in the development of spinal NTDs.

RESULTS

Conditional gene targeting of *Lrp6* in *Pax3*-expressing dorsal neural fold causes posterior neuropore (PNP) closure defects and diminished canonical Wnt/ β -catenin signaling

To determine the cell lineage-specific role of *Lrp6* in neural tube closure, we carried out conditional gene-targeting analyses by breeding *Lrp6^{lox}* mice (Zhou et al., 2010) with the *Pax3^{Cre}* knock-in mice (Lang et al., 2005). Genetic fate mapping of *Pax3^{Cre}* mice crossed with the Cre reporter *Rosa26-lacZ* revealed restrictive activities of the Cre recombinase in the dorsal neural folds, including in the recently closed dorsal midline region and the dorsal edge cells along the pending closure sites of the PNP of embryonic day (E)8.5 mouse embryos (Fig. 1A). Genetic fate mapping of *Pax3^{Cre}* at E9.5 PNP has been detailed in our previous study (Zhao et al., 2014). The neural tube closes completely from E10.5 onwards in the non-Cre homozygous *Lrp6^{lox/lox}* or in the double-heterozygous *Pax3^{Cre/+};Lrp6^{lox/+}* littermate control mice (Fig. 1B). However, the conditional knockout *Pax3^{Cre/+};Lrp6^{lox/lox}* (abbreviated as *Pax3Cre;Lrp6-cKO* or *Lrp6-cKO*) mice exhibit fully penetrant tail truncations and spinal bifida, as shown by the consistently open PNP at E12.5 and open caudal spinal cord at E18.5 (Fig. 1C,D). These results indicate that *Lrp6* is required in the *Pax3*-expressing dorsal edge cells for PNP closure, which is consistent with the role of β -catenin in the same lineage for PNP closure, as we previously demonstrated (Zhao et al., 2014).

To validate whether *Lrp6*-mediated Wnt/ β -catenin signaling is disrupted in the mutant PNP, we incorporated two representative Wnt/ β -catenin signaling reporter mouse lines, *BATgal* (Maretto et al., 2003) and *TOPgal* (DasGupta and Fuchs, 1999), to the conditional gene-targeting approaches. X-gal staining for the signaling reporter lacZ of either *BATgal* or *TOPgal* indicated a clear reduction of Wnt/ β -catenin signaling activities in the dorsal PNP of *Lrp6-cKO* embryos at E9.5, compared to that of their triple heterozygous littermate controls (Fig. 1E-H). The signaling reporter shows intensive Wnt/ β -catenin signaling activities in the notochord (Ukita et al., 2009), which was not altered in the mutants as *Pax3^{Cre}*

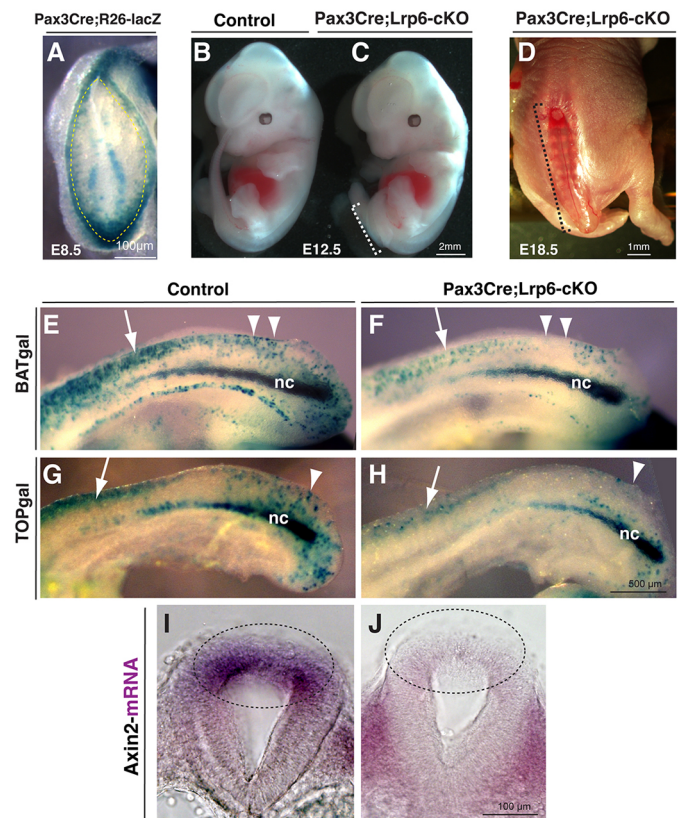


Fig. 1. Spinal bifida aperta and diminished canonical Wnt signaling by conditional ablation of *Lrp6* in *Pax3*-expressing dorsal neural folds. (A) Dorsal–posterior view of an X-gal-stained (blue) E8.5 embryo for genetic fate mapping of *Pax3^{Cre/+};Rosa26-lacZ* demonstrates the Cre recombination pattern in the dorsal region of the recently closed and pending-closing posterior neuropore (PNP; indicated by dashed line). (B–D) The conditional mutants of *Pax3^{Cre/+};Lrp6-cKO* embryos exhibit open spinal neural tube defects (NTDs), as shown at E12.5 and E18.5. Dashed line brackets indicate the open lesion regions. (E–H) Sagittal caudal bodies of X-gal-stained Wnt/ β -catenin signaling reporters *BATgal* or *TOPgal* show higher activities in the littermate control embryos (E,G) and diminished activities in the *Pax3^{Cre/+};Lrp6-cKO* embryos (F,H) at E9.5. Arrows indicate recently closed dorsal neural tube regions. Arrowheads indicate the closing or pending-closing regions. nc, notochord. (I,J) Transverse sections show *in situ* hybridization signal of a Wnt/ β -catenin target and feedback gene *Axin2*, which is high in the dorsal PNP of a littermate control (dashed line oval in I) and low in the mutant PNP (dashed line oval in J) at E9.5.

is not expressed in the notochord. Because these signaling reporters may not visualize some less robust but functional Wnt/ β -catenin signaling *in vivo*, we further examined the expression of *Axin2*, a general downstream target gene and negative feedback regulator of the canonical Wnt/ β -catenin signaling pathway (Jho et al., 2002). Whollemount *in situ* hybridization showed a substantial reduction in *Axin2* mRNA at E9.5 in the dorsal PNP of *Lrp6-cKO* embryos compared to that in the littermate control embryos (Fig. 1I,J). These results demonstrate diminished Wnt/ β -catenin signaling in *Lrp6*-deficient dorsal neural folds.

NTD-associated transcription factors and β -catenin downstream target genes are diminished in the dorsal PNPs of *Pax3Cre;Lrp6-cKO* mutants

To address the molecular mechanisms underlying spinal NTDs in *Lrp6-cKO* mutants, we examined a panel of NTD-associated genes that are specifically expressed in the dorsal neural folds, including

the transcription factor *Pax3* (Epstein et al., 1991; Goulding et al., 1991) and the caudal-type homeobox genes *Cdx2* and *Cdx4* (Young et al., 2009; van Nes et al., 2006). Wholemount *in situ* hybridization showed diminished expression of *Pax3*, *Cdx2* and *Cdx4* in the dorsal neural folds of *Lrp6*-cKO embryos at E9.5 (Fig. 2). We have previously demonstrated that both *Pax3* and *Cdx2* are downstream target genes of β -catenin signaling, and *Cdx2* is additionally regulated by *Pax3* during PNP closure (Zhao et al., 2014). The transcription factor *Msx1* is a regulator of *Pax3* (Monsoro-Burq et al., 2005), and it is also a known β -catenin downstream effector (Foerst-Potts and Sadler, 1997; Song et al., 2009). *Msx1* was restrictively expressed in the dorsal midline during PNP closure, but it was absent in *Lrp6*-cKO PNPs at E9.5 (Fig. 3A,B). These results demonstrate the consistent roles of *Lrp6* and β -catenin in regulation of these key downstream target genes during PNP closure.

We next examined whether conditional ablation of *Lrp6* in the dorsal neural folds affects additional critical genes, such as T-box, *Fgf* and *Wnt* genes in the tailbud signaling center, which are required for caudal body axis formation (Gofflot et al., 1997) and may also play a role in caudal neural tube closure. Wholemount *in situ* hybridization demonstrated that the expression patterns of brachyury (*T*) (Wilkinson et al., 1990), *Tbx6* (Chapman and Papaioannou, 1998), *Wnt1* (Parr et al., 1993) and *Wnt5a* (Yamaguchi et al., 1999) were not apparently altered in *Lrp6*-cKO tailbuds, dorsal neural folds or related caudal tissues at E9.5 (Fig. 3C-J). The expression patterns of *Fgf8*, *Fgf17* and *Fgf18* (Maruoka et al., 1998) were also not altered in the mutant tailbuds or related tissues (Fig. 4A-F). *Mesp2*, modulated by Notch and *Fgf* signaling (Niwa et al., 2011), is restrictively expressed in the presomites (Takahashi et al., 2000) and remained unaffected in E9.5 *Lrp6*-cKOs (Fig. 4G,H). These results suggest that the tailbud signaling center and new somite formation are not altered in the *Lrp6*-cKOs and might not contribute to PNP closure.

Unchanged PCP signaling, proliferation and apoptosis in the dorsal PNPs of *Pax3Cre;Lrp6*-cKO mutants

To examine whether *Lrp6* deficiency in the dorsal neural folds affects noncanonical Wnt/PCP signaling, we first examined two representative genes, *Vangl2* (Kibar et al., 2001) and *Ptk7* (Lu et al., 2004), which are required for PCP signaling and neural tube closure. The expression of these genes was unaltered in the dorsal neural folds of *Lrp6*-cKO mutants at E9.5 (Fig. 5A-D). *Dvl2* is a mediator of canonical and noncanonical Wnt signaling pathways and is

required for neural tube closure (Hamblet et al., 2002). *Dvl2* phosphorylation does not trigger β -catenin signaling and can be detected by band shifts of immunoblots (Gonzalez-Sancho et al., 2004). We detected no changes in *Dvl2* phosphorylation in PNP samples of *Lrp6*-cKO mutants (Fig. 5E). In addition, proliferation, as shown by bromodeoxyuridine (BrdU) incorporation assays, and apoptosis, as shown by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays, were unaffected in the dorsal neural folds of *Lrp6*-cKO mutants (Fig. 6). These results suggest that conditional inactivation of *Lrp6* in dorsal neural folds may not modify PCP signaling, proliferation or apoptosis during caudal neural tube closure.

Genetic activation of β -catenin can rescue closure defects in *Pax3Cre;Lrp6*-cKO mutants

To verify that *Lrp6* acts through β -catenin to regulate PNP closure, we tested genetic rescue experiments by gain-of-function (GOF) of β -catenin in *Lrp6*-cKO mutants. *Ctmb1^{flox(ex3)}* mice were used, in which the conditional removal of the floxed exon 3 (that encodes a phosphorylation site for β -catenin protein degradation) will stabilize β -catenin, leading to constitutive activation of canonical Wnt signaling (Harada et al., 1999). The results indicated rescued PNP closure in the compound mutants of *Pax3Cre;Lrp6*-cKO; β -catenin-GOF mutants (five out five embryos) at E10.5 (Fig. 7). However, the dorsal midline region and roof plate were markedly widened in the mutants, suggesting a side effect of constitutive activation of β -catenin signaling in the dorsal neural folds.

Maternal supplementation of Wnt/ β -catenin signaling agonists reduces the frequency and severity of spinal NTDs in *Pax3Cre;Lrp6*-cKO mutants

The finding that defective Wnt/ β -catenin signaling pathway is essentially related to spinal NTDs provides a significant translational implication for NTD intervention. We therefore attempted to treat spinal NTDs in *Lrp6*-cKOs through maternal supplementation of lithium ion, a commonly used psychotherapeutic medication and well-known Wnt signaling agonist acting through inhibition of intracellular β -catenin degradation (Cohen and Goedert, 2004; Meijer et al., 2004). After intraperitoneal injections of lithium chloride (LiCl) solution to the pregnant females at E7.5-E9.5, ~18% (5/28) of the *Lrp6*-cKO mice had completely closed neural tubes and elongated tails, as determined at E18.5, which were not observed in the control

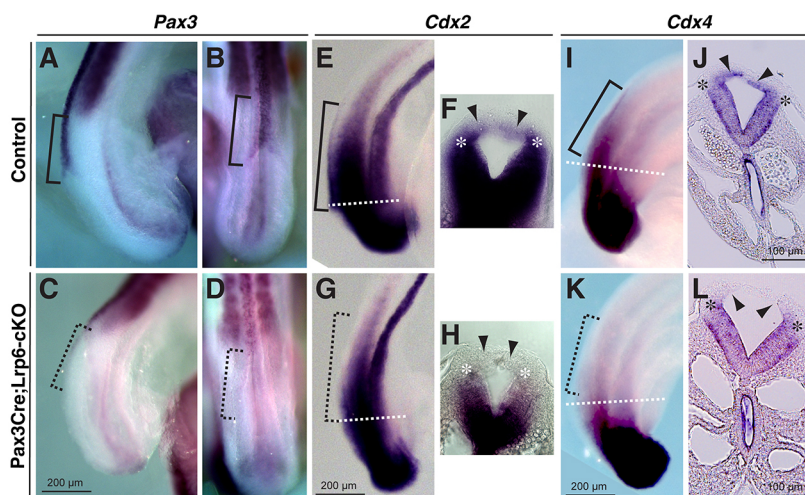


Fig. 2. Wholemount *in situ* hybridization results show diminished gene expression of NTD-associated transcription factors *Pax3*, *Cdx2* and *Cdx4* in the dorsal PNPs of *Pax3-Cre;Lrp6*-cKOs at E9.5. (A-D) *Pax3* expression is strong at the PNP closure site, as shown in a littermate control embryo (brackets in A, sagittal view and in B, dorsal view), whereas it is diminished specifically at the defective closure site of the mutant PNP (dashed line brackets in C, D). (E-H) *Cdx2* is widely expressed in the caudal body of the control embryo, including dorsal PNP (bracket in E, sagittal view; arrowheads in F, transverse section from the region of the dashed line in E), and it is specifically diminished in the dorsal PNP of the mutant embryo (dashed line bracket in G and arrowheads in H). (I-L) *Cdx4* is expressed in the dorsal PNP of the control embryo (bracket in I and arrowheads in J), and it is specifically diminished in the dorsal PNP of the mutant embryo (dashed line bracket in K and arrowheads in L). Asterisks indicate the dorsolateral hinge points.

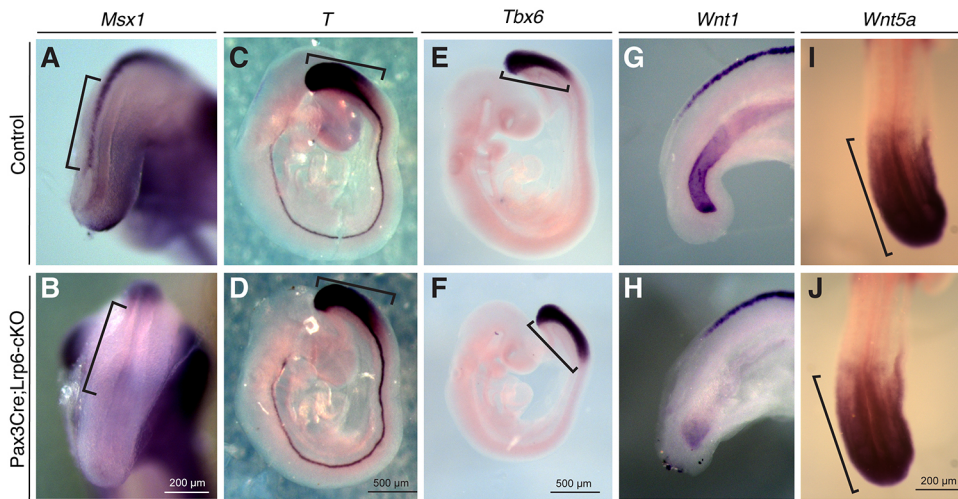


Fig. 3. Wholemount *in situ* hybridization results on Wnt genes and additionally relevant Wnt signaling downstream target genes around the PNP regions of littermate controls and *Pax3Cre;Lrp6-cKO*s at E9.5. (A,B) *Msx1* is restrictively expressed in the dorsal PNP of the normal control embryo (bracket in A), and its expression is significantly diminished in the mutant PNP (bracket in B). (C-J) No obvious changes in *T* (C,D), *Tbx6* (E,F), *Wnt1* (G,H) and *Wnt5a* (I,J) expression patterns around the mutant PNP regions were observed compared to respective expression patterns in the littermate controls.

group of *Lrp6*-cKOs treated with the NaCl placebo (0/30 *Lrp6*-cKOs) (Fig. 8A-C). Conversely, 20% (6/30) of the *Lrp6*-cKOs in the control group exhibited severe closure defects up to the lumbar level, which were not observed in the LiCl-treated *Lrp6*-cKOs (0/28). These results demonstrate effective intervention of spinal NTDs in *Lrp6*-cKOs by lithium treatment. Lesions of variable lengths at the sacrococcygeal levels were also observed in both the LiCl-treated and placebo groups of *Lrp6*-cKOs, suggesting that environmental or other factors may affect canonical Wnt signaling activity and NTD severity. After lithium treatment, *Pax3* expression in the neural folds of *Lrp6*-cKOs was restored (Fig. 8D).

Together, these results elucidate the essential roles of the *Lrp6*-mediated canonical Wnt/ β -catenin signaling pathway in murine caudal neural tube closure, which may provide significant clues to both the etiology and prevention of human NTDs.

DISCUSSION

Lrp6-mediated Wnt/ β -catenin signaling during neural tube closure

This study reveals the essential role of *Lrp6*-mediated Wnt/ β -catenin signaling in caudal neural tube closure. *Lrp6* is a Wnt co-receptor acting upstream of β -catenin in the canonical pathway. We found

fully penetrant spinal NTDs in *Pax3Cre;Lrp6*-cKO mutants, which is phenotypically consistent with *Pax3Cre;* β -catenin-cKO mutants, as demonstrated in our previous study (Zhao et al., 2014), suggesting a consistent role of *Lrp6* and β -catenin in caudal neural tube closure. At the molecular signaling level, we observed diminished expression of several genes encoding transcription factors, including *Pax3*, *Cdx2*, *Cdx4* and *Msx1*, in the dorsal neural folds of *Lrp6*-cKO mutants, which were identically downregulated in the β -catenin-cKO neural folds, further suggesting that *Lrp6* and β -catenin act in the same signaling cascade to regulate critical downstream target genes during caudal neural tube closure. Our genetic rescue experiments revealed that constitutively active β -catenin in the dorsal neural folds can rescue closure defects in *Lrp6*-cKO embryos, which firmly validates that *Lrp6* acts through β -catenin to promote caudal neural tube closure. Moreover, maternal supplementation of an agonist of the Wnt/ β -catenin signaling could reduce the frequency and severity of NTDs in *Lrp6*-cKO mutants, further substantiating the essential roles of *Lrp6*-mediated Wnt/ β -catenin signaling in caudal neural tube closure.

We employed *Pax3^{Cre}* knock-in mice for conditional gene-targeting analyses of both *Lrp6* and β -catenin, which generated consistent phenotypic and mechanistic results. Our genetic fate-mapping experiments demonstrated that *Pax3^{Cre}* became activated

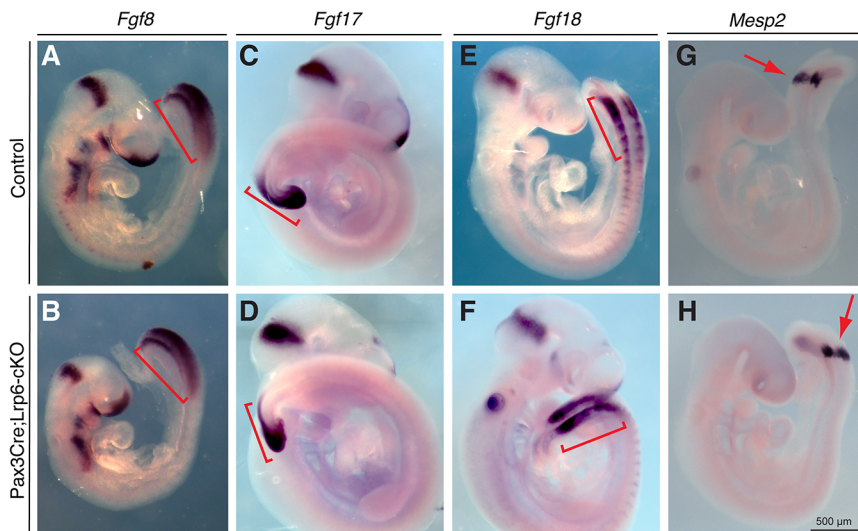


Fig. 4. Wholemount *in situ* hybridization results on Fgf genes and related *Mesp2* expression around PNP regions of the littermate controls and *Pax3Cre;Lrp6-cKO*s at E9.5. (A-F) No obvious differences in *Fgf8* (A,B), *Fgf17* (C,D) and *Fgf18* (E,F) expression around PNP regions (brackets) between the control and mutant embryos were observed. (G,H) No obvious differences in Fgf-regulated *Mesp2* expression in the presomites between the littermate control and mutant embryos were observed.

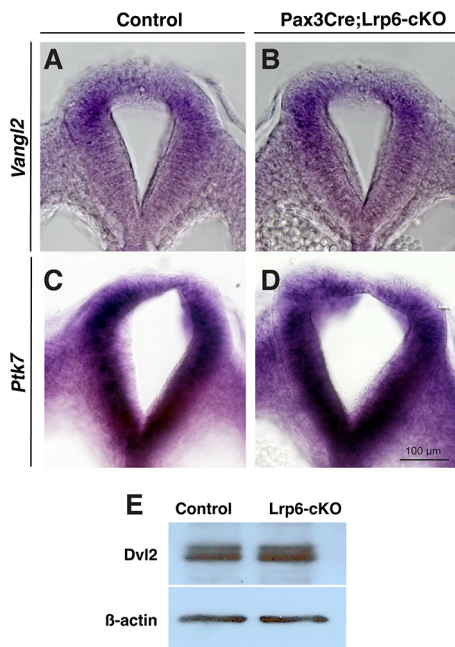


Fig. 5. Noncanonical Wnt/PCP signaling activities in littermate controls and Pax3-Cre;Lrp6-cKOs at E9.5. (A–D) Transverse sections after wholemount *in situ* hybridization show no obvious changes in *Vangl2* (A,B) and *Ptk7* (C,D) expression patterns at the PNP closure sites in normal control and mutant embryos. (E) Immunoblots show no differences in phosphorylated (higher band that is linked with PCP signaling) and nonphosphorylated (lower band) Dvl2 proteins between the control and mutant PNP samples.

in the border region between the non-neural surface ectoderm and neural plate in the pending closure sites of dorsal neural folds at E8.5. It has been demonstrated that conditional removal of β -catenin using *Grhl3^{Cre}* in these border cells also causes spinal NTDs (Kimura-Yoshida et al., 2015). However, it remains unclear whether *Pax3^{Cre}* and *Grhl3^{Cre}* activities overlap in the same border cells during primary neurulation. We recently demonstrated that *Grhl3*

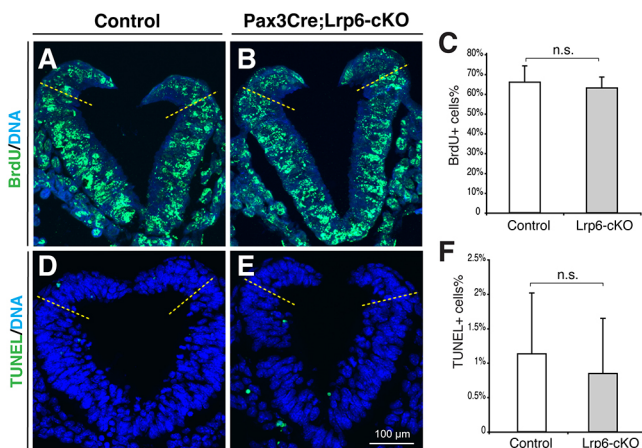


Fig. 6. Proliferation and apoptosis at the PNP closure sites of the littermate controls and Pax3-Cre;Lrp6-cKOs at E9.5. (A–C) BrdU incorporation and detection experiments show no significant differences in proliferating cells in the dorsal PNPs above the dorsolateral hinge points (dashed lines in A,B, transverse PNP sections) between the control and mutant embryos. (D–F) TUNEL assays demonstrate no significant differences in apoptotic cells (green in D,E) in the dorsal PNPs between the control and mutant embryos. n.s., no statistical significance ($P > 0.05$; unpaired, two-tailed Student's *t*-test).

regulates dynamic movements of the non-neural surface ectodermal border cells for multicellular rosette formation and convergent cellular protrusions to promote caudal neural tube closure (Zhou et al., 2020). Conditional gene-targeting analyses of *Lrp6* using *Grhl3^{Cre}* may clarify whether Lrp6-mediated Wnt/ β -catenin signaling is also activated in these border cells as a modulator of their molecular properties and cellular dynamics for neural tube closure.

Lrp6 and noncanonical Wnt signaling during neurulation

Lrp6 may exert a key role in the signaling interactions between canonical and noncanonical Wnt pathways. Loss-of-function (LOF) of Lrp6-mediated Wnt/ β -catenin signaling may affect noncanonical Wnt/PCP signaling directly or indirectly. Our results showed that *Lrp6* deficiency did not change the expression patterns of two representative NTD-associated PCP signaling genes, *Vangl2* and *Ptk7*, in the mutant PNP, suggesting that Lrp6-mediated Wnt/ β -catenin signaling does not directly regulate transcriptional activation of these PCP signaling genes. We also showed that *Lrp6* deficiency did not change the Dvl2 phosphorylation level. Because Dvl2 phosphorylation is independent of Lrp5/Lrp6 co-receptors and does not stabilize β -catenin (Gonzalez-Sancho et al., 2004), it is an excellent indicator of altered noncanonical Wnt signaling. It has been reported that different Wnts may signal through different co-receptors, Lrp6 for canonical signaling and Ror1/Ror2 for noncanonical signaling, and that canonical and noncanonical Wnts may exert reciprocal inhibition by competition for Fzd binding (Grumolato et al., 2010). Interestingly, an early study demonstrated that either LOF or GOF of *Lrp6* disrupted the convergent extension that is a hallmark of PCP signaling during *Xenopus* gastrulation (Tahinci et al., 2007). The authors showed that an intracellular domain of Lrp6 can inhibit Wnt/PCP signaling by unknown mechanisms, while potentiating Wnt/ β -catenin signaling. Thus, Lrp6 may serve as a molecular switch from noncanonical to canonical Wnt signaling (Tahinci et al., 2007). Nevertheless, it remains unknown whether or how the noncanonical inhibitory function of this intracellular domain of Lrp6 contributes to neural tube closure. Another study reported that the extracellular domains of Lrp6 (or its homolog Lrp5) can also inhibit noncanonical Wnt signaling *in vitro* (Bryja et al., 2009). These authors further showed that *Wnt5a* deficiencies partially rescued heart defects and fully rescued the partially penetrant cranial NTD exencephaly in *Lrp6*-null mutants, suggesting that GOF of noncanonical Wnt signaling is the cause of these defects in *Lrp6*-null mutants (Bryja et al., 2009). However, *Wnt5a* deficiencies had no rescue effects on the fully penetrant spinal NTDs of *Lrp6*-null mutants, suggesting distinctly different mechanisms underlying cranial and spinal NTDs in *Lrp6*-deficient mutants. Above all, it will be important to determine whether upregulation of the Wnt/PCP signaling activities can indeed cause NTDs.

In contrast, the hypermorphic *Lrp6^{crooked tail}* homozygous embryos exhibit cranial NTDs with defective RhoA signaling and apical–basal cell polarity, suggesting a defective noncanonical Wnt signaling mechanism (Carter et al., 2005). The compound mutants of another hypermorphic *Lrp6^{Skax2}* homozygous combined with heterozygous *Vangl2^{Lp}* exhibit spinal NTDs (Allache et al., 2014), reiterating a defective noncanonical Wnt signaling mechanism in these hypermorphic Lrp6 mutants. Nevertheless, it remains unknown whether or how noncanonical Wnt signaling is altered and may contribute to NTDs in *Lrp6*-deficient mutants and its contribution to NTD incidence. Mutants deficient in the PCP signaling gene *Ptk7* exhibit the severest NTD, craniorachischisis

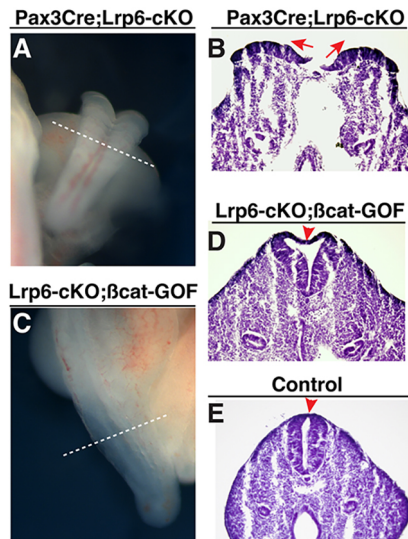


Fig. 7. Genetic rescue of PNP closure defects in the *Pax3-Cre;Lrp6-cKOs* by β -catenin gain-of-function (GOF). (A,B) Failed PNP closure as shown in the dorsal–posterior view of an *Lrp6-cKO* embryo (A) and in a transverse PNP section (arrows in B, cut through the dashed line in A) at E10.5. (C,D) Rescued PNP closure as shown in the dorsal–posterior view of an *Lrp6-cKO;β-catenin-GOF* embryo (A) and in a transverse PNP section that shows abnormally widened but closed dorsal PNP (arrowhead in D, cut through the dashed line in C) at E10.5. (E) A transverse PNP section of a littermate control embryo shows normally closed PNP at the dorsal midline (arrowhead in E) at E10.5.

(Lu et al., 2004). Unexpectedly, a later study demonstrated that *Ptk7* modulates both canonical and noncanonical Wnt signaling through physical interaction and stabilization of *Lrp6* proteins (Bin-Nun et al., 2014). Therefore, *Ptk7* or related PCP signaling may actually act upstream of *Lrp6*-mediated Wnt/ β -catenin signaling, suggesting

complex signaling crosstalk between the canonical and noncanonical Wnt pathways during neural tube closure.

NTD intervention by targeting *Lrp6*-mediated Wnt/ β -catenin signaling

The current study showed that maternal supplementation of LiCl during a relatively short period (from E7.5 to E9.5) can reduce the frequency and severity of spinal NTDs, with ~18% of *Lrp6-cKO* mutants exhibiting completely closed neural tubes and an additional 20% with reduced NTD lesion sizes in the rescue group. Lithium inhibits *Gsk3* enzymic activities (Klein and Melton, 1996) to stabilize β -catenin in the canonical Wnt signaling pathway (Liu et al., 2002). Thus, the results of our NTD rescue experiments by lithium support the role of *Lrp6*-mediated Wnt/ β -catenin signaling in PNP closure and may also provide a translational implication for NTD intervention using small-molecule agonists of Wnt/ β -catenin signaling (Fig. 8E). There are several other *Gsk3* inhibitors that can be used for therapeutic treatments (Cohen and Goedert, 2004; Meijer et al., 2004), including the small-molecule CHIR99021 and 6-bromindirubin-3'-oxime (BIO), which have been used widely as potent Wnt agonists (Sato et al., 2004; Silva et al., 2008). It is important to note that *Gsk3* inhibitors are not specific to Wnt signaling and have broad functions, such as in insulin signaling, NFAT signaling and Hedgehog signaling (Cline et al., 2002; Crabtree and Olson, 2002; Price and Kalderon, 2002; Ring et al., 2003). Regardless of these broad effects, our results showed that lithium supplementation reduced the frequency and severity of NTDs in *Lrp6*-deficient mutants by restoring the expression of *Pax3*, which is a key downstream target gene of Wnt/ β -catenin signaling. As demonstrated in our previous work, genetic activation of *Pax3* can rescue spinal NTDs in the conditional β -catenin mutants (Zhao et al., 2014). Intriguingly, *p53* (also known as *Trp53*) LOF by genetic or pharmacological approaches can rescue NTDs in

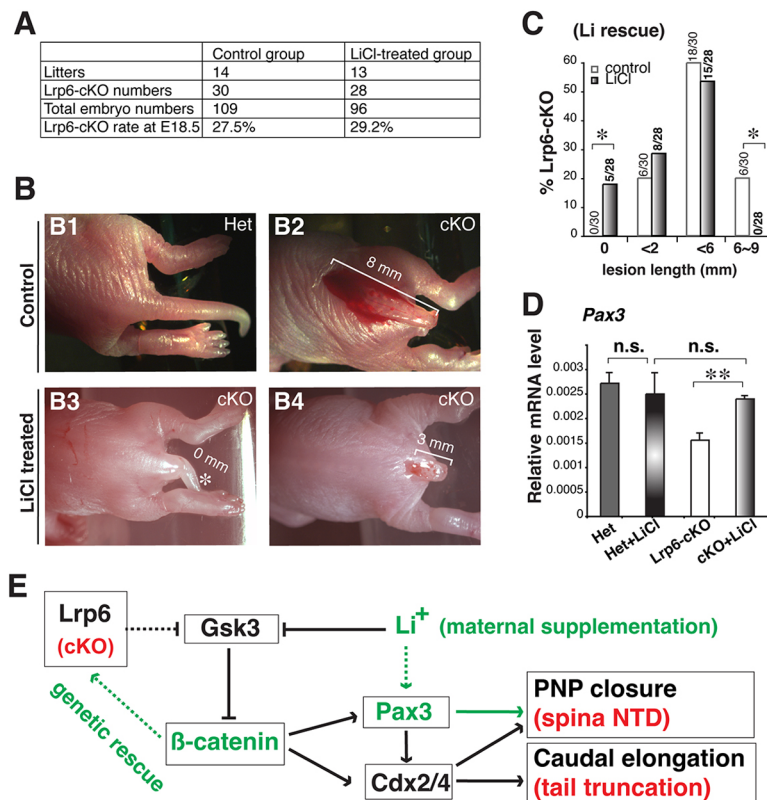


Fig. 8. Pharmaceutical intervention of spinal NTDs in *Pax3-Cre;Lrp6-cKOs* by maternal supplementation of a Wnt/ β -catenin signaling agonist. (A) The embryo numbers and *Lrp6-cKO* ratios are not significantly different between the control and lithium-treated groups at E18.5 as compared with the expected Mendelian ratio (25% cKOs) ($P > 0.05$, chi-square test). (B) Dorsal–caudal body views of a double heterozygous (Het) *Pax3^{Cre};Lrp6^{fllox/+}* embryo that shows no NTD and with normal tail (B1), an *Lrp6-cKO* embryo in the control group that shows the severest lumbosacral NTD (with ~8 mm lesion length, bracket in B2), an *Lrp6-cKO* embryo treated with lithium that shows a fully closed or rescued spinal cord (0 mm lesion length in B3, asterisk shows partially rescued tail growth), and a mutant embryo treated with lithium that shows milder NTD (with 3 mm lesion length in B4) at E18.5. (C) Rescue effects of spinal NTDs in *Lrp6-cKOs* examined at E18.5 after maternal supplementation of lithium chloride (LiCl) from E7.5 to E9.5. The lesion lengths (mm) were measured under a microscope. * $P = 0.02$ (Fisher exact test); after all samples combined and averaged in each group, $P = 0.01$ (unpaired, two-tailed Student's *t*-test). (D) RT-qPCR results demonstrate significant restoration of *Pax3* mRNA in the lithium-treated *Lrp6-cKO* PNPs at E9.5. n.s., no statistical significance ($P > 0.05$); ** $P < 0.01$ (unpaired, two-tailed Student's *t*-test). (E) Illustrative summary of *Lrp6*-mediated β -catenin–*Pax3/Cdx2/Cdx4* signaling underlying PNP closure/elongation and spinal NTD; the latter can be rescued by either genetic activation of β -catenin or maternal supplementation of lithium ion, which stabilizes intracellular β -catenin by inhibiting *Gsk3* in the canonical Wnt signaling pathway, thus restoring a key downstream transcription factor *Pax3* in *Lrp6*-deficient PNPs. Red font, mutants and phenotypes; green font and arrows, genetic or pharmacological rescues demonstrated in the current study.

Pax3 mutants, suggesting a role of apoptosis in the development of NTDs (Pani et al., 2002). We did not detect significantly altered apoptosis in *Lrp6*-deficient mutants, but it would be important to test whether *p53* LOF can rescue NTDs in *Lrp6*-deficient mutants as well. Conversely, severe midline defects, including cranial and spinal NTDs and midline facial clefts, occurred in mice with triple knockout of the intrinsic apoptotic genes *Bax*, *Bak* (also known as *Bak1*) and *Bok* (Ke et al., 2018), demonstrating that developmental apoptosis is required for normal neural tube closure and related midline fusion processes. Thus, completely inhibiting apoptosis seems inappropriate for preventing NTDs.

In summary, this study demonstrated that conditional ablation of *Lrp6* in dorsal neural folds resulted in spinal NTDs due to diminished canonical Wnt/ β -catenin signaling and downstream target genes. This could be rescued either by genetic activation or pharmacological stabilization of β -catenin *in vivo* (Fig. 8E), indicating LRP6-mediated Wnt/ β -catenin signaling as a novel target for intervention for NTDs in humans.

MATERIALS AND METHODS

Animals

The *Lrp6*^{fllox} mice for conditional gene-targeting analyses have previously been described (Zhou et al., 2010). The *Pax3*^{Cre} knock-in mice (Engleka et al., 2005), *Rosa26-lacZ* mice (Soriano, 1999), and canonical Wnt/ β -catenin signaling reporter lines of *BATgal* (Maretto et al., 2003) and *TOPgal* (DasGupta and Fuchs, 1999) mice donated by different investigators were obtained through The Jackson Laboratory. *Ctnnb1*^{fllox(ex3)} mice (MGI:1858008, gift from M. Taketo, Kyoto University, Kyoto, Japan) (Harada et al., 1999) were used for genetic rescue by conditional activation of β -catenin. These mouse strains were maintained on a C57BL/6J or a mixed B6;129 background. All mice were housed in the vivarium at the University of California, Davis (Davis, CA, USA). Pregnant, timed-mated mice were euthanized prior to cesarean section. Noon of the conception day was designated as E0.5. All research procedures using mice were approved by the University of California, Davis Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

X-gal staining and wholemount *in situ* hybridization

Mouse embryos were fixed in 1% paraformaldehyde (PFA) for ~30 min on ice and processed for X-gal staining as previously described (Song et al., 2009; Wang et al., 2011; Zhao et al., 2014). Embryos fixed in 4% PFA overnight at 4°C were processed for wholemount *in situ* hybridization using digoxigenin-labeled antisense RNA probes as previously described (Zhao et al., 2014). Antisense RNA probes were synthesized based on sequence information provided by the Allen Brain Atlas (<https://portal.brain-map.org/>). At least three mutants and three littermate control (Cre-lacking or double heterozygous) embryos were used for each *in situ* experiment, which showed consistent results.

Maternal administration of LiCl

In vivo stimulation of the Wnt/ β -catenin signaling pathway by LiCl was carried out as previously described with minor modifications (Song et al., 2009; Tian et al., 2010). Pregnant females were injected intraperitoneally with 200 mg/kg LiCl or an equivalent dose of a NaCl control solution on E7.5, E8.5 and E9.5. Embryos were collected at E18.5 for phenotype analysis or at E9.5 (1 h after the third-day injection) for real-time reverse transcription quantitative PCR (RT-qPCR) analyses.

RNA isolation and real-time RT-qPCR

Total RNAs were isolated from the caudal neural folds and pooled from five E9.5 embryos of the mutant or control groups. Heterozygous *Pax3*^{Cre/+} embryos were used as the control. After reverse-transcription, real-time PCR was carried as described in our previous publications (Song et al., 2009; Zhao et al., 2014). The mRNA level of *Pax3* was normalized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) to allow for

relative comparisons among different experimental groups using the $\Delta\Delta C_t$ method.

BrdU labeling, immunohistochemistry and TUNEL assays

Acute BrdU labeling was performed by intraperitoneal injection of BrdU at 100 mg/kg body weight to the pregnant mice 1 h prior to sampling. Immunohistochemistry was carried out on paraffin or frozen sections using primary antibodies against BrdU (1:100; M0744, Dako) and Alexa Fluor-conjugated secondary antibodies (1:400; Molecular Probes). TUNEL assays were performed using the Dead End Fluorometric TUNEL System (Promega) as detailed by the manufacturer. The total numbers of BrdU- or TUNEL-positive cells were counted in the dorsal PNP above the dorsolateral hinge points on each confocal micrograph (Fig. 6) then divided by the total cell numbers on the same areas to obtain the percentages of the positive cells. In each experiment, at least three sections from three mutants or three littermate controls at the same age were counted and averaged for statistical analyses.

Immunoblotting

Immunoblotting was carried out according to the standard protocol (Wang et al., 2016). Protein samples were pooled from the caudal part of five embryos of E9.5 control or *Pax3Cre;Lrp6*-cKO embryos. Primary antibodies against Dvl2 (1:200; sc-13974, Santa Cruz Biotechnology) and β -actin (1:5000; sc-1616, Santa Cruz Biotechnology) were used.

Statistical analyses

At least three littermate controls and three mutant embryos were used for each statistical evaluation. Significances were assessed by unpaired, two-tailed Student's *t*-test or pairwise comparison (one-way ANOVA) when appropriate. In all cases, $P \leq 0.05$ was considered statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.Z., C.J.Z.; Methodology: T.Z., C.J.Z.; Validation: T.Z., M.M., K.R., S.K.S., A.S., C.J.Z.; Formal analysis: T.Z., M.M., K.R., S.K.S., C.J.Z.; Investigation: T.Z., M.M., K.R., S.K.S., C.J.Z.; Resources: C.J.Z.; Data curation: T.Z., A.S., C.J.Z.; Writing - original draft: C.J.Z.; Writing - review & editing: M.M., K.R., S.K.S., C.J.Z.; Visualization: T.Z., C.J.Z.; Supervision: C.J.Z.; Project administration: C.J.Z.; Funding acquisition: C.J.Z.

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