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Figures

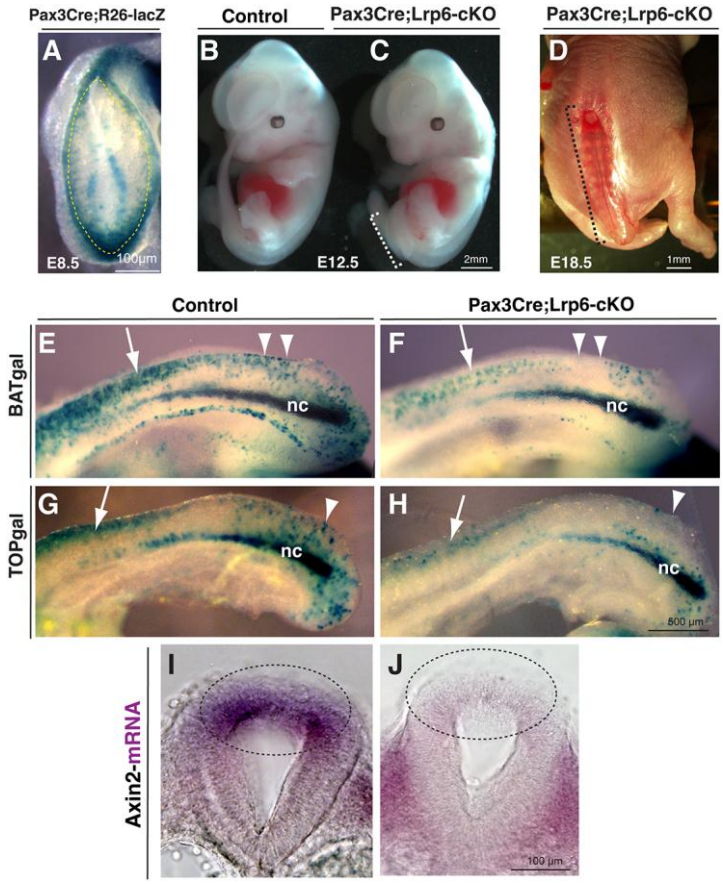

Figure 1. Spinal bifida aperta and diminished canonical Wnt signaling by conditional ablation of *Lrp6* in Pax3-expressing dorsal neural folds. (**A**) Dorsal-posterior view of an X-gal stained (*blue*) E8.5 embryo for genetic fate mapping of *Pax3*$^{Cre/+}$*;Rosa26-lacZ* demonstrates the Cre recombination pattern in the dorsal region of the recently closed and pending-closing posterior neuropore (PNP, dashed curve). (**B-D**) The conditional mutants of *Pax3*$^{Cre/+}$*;Lrp6-cKO* embryos exhibit open spinal NTDs as shown at E12.5 and E18.5. Brackets indicate the open lesion regions. (**E-H**) Sagittal caudal bodies of X-gal stained Wnt/ß-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/ß-catenin target and feedback gene *Axin2* that is high in the dorsal PNP of a littermate control (dashed circle in I) and low in the mutant PNP (J) at E9.5.

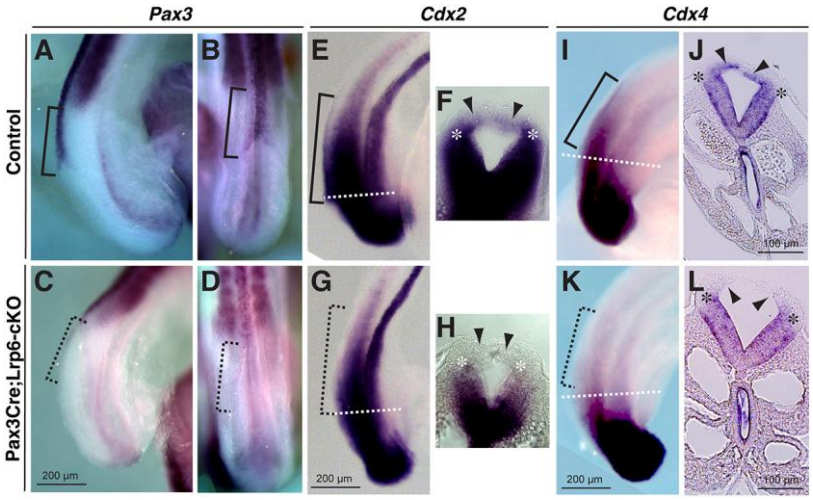

Figure 2. Wholemount in situ hybridization results show diminished gene expression of NTD-associated transcription factors of *Pax3*, *Cdx2*, and *Cdx4* in the dorsal posterior neuropores (PNPs) of the *Pax3-Cre;Lrp6-cKOs* at E9.5. (**A-D**) *Pax3* expression is strong at the PNP closure site as shown in a littermate control embryo (brackets in A, sagittal view and in B, dorsal view), which is diminished specifically at the defective closure site of the mutant PNP (dashed 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 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 bracket in K and arrowheads in L). Asterisks indicate the dorsolateral hinge points.

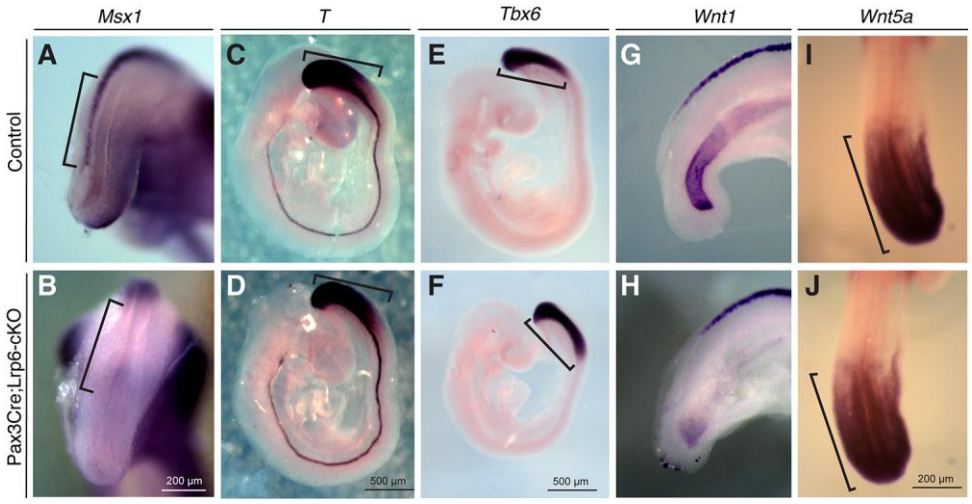

Figure 3. Wholemount in situ hybridization results of *Wnts* and additionally relevant Wnt signaling downstream target genes around the PNP regions of the littermate controls and *Pax3-Cre;Lrp6-cKOs* at E9.5. (**A,B**) *Msx1* is restrictively expressed in the dorsal PNP of the normal control embryo (bracket in A), which is significantly diminished in the mutant PNP (bracket in B). (**C-J**) No obvious changes of *T* (C,D), *Tbx6* (E,F), *Wnt1* (G,H), and *Wnt5a* (I,J) expression patterns around the mutant PNP regions compared to respective expression patterns in the littermate controls.

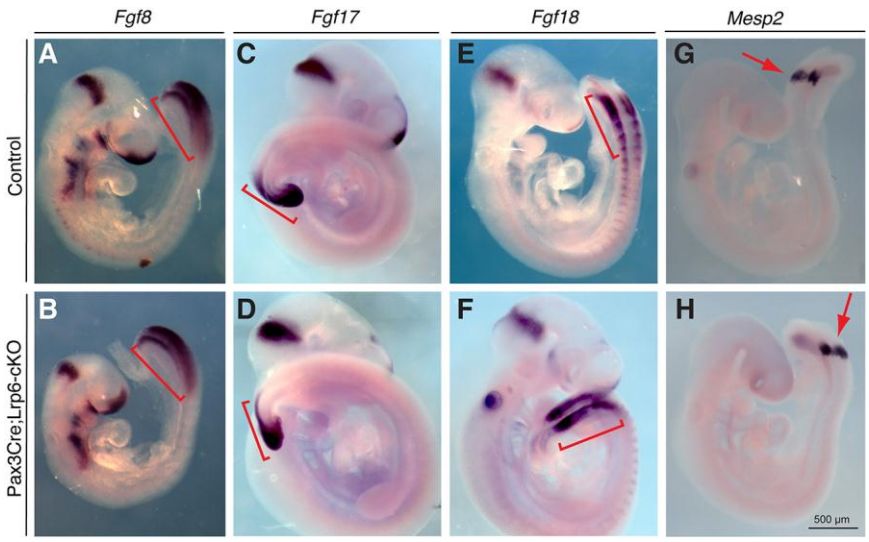

Figure 4. Wholemount in situ hybridization results of *Fgfs* and related *Mesp2* expression around PNP regions of the littermate controls and *Pax3-Cre;Lrp6-cKOs* at E9.5. (**A-F**) No obvious changes of *Fgf8* (A,B), *Fgf17* (C,D), and *Fgf18* (E,F) expression around PNP regions (brackets) between the control and mutant embryos. (**G,H**) No obvious changes of the Fgf-regulated *Mesp2* expression in the presomites of the littermate control and mutant embryos.

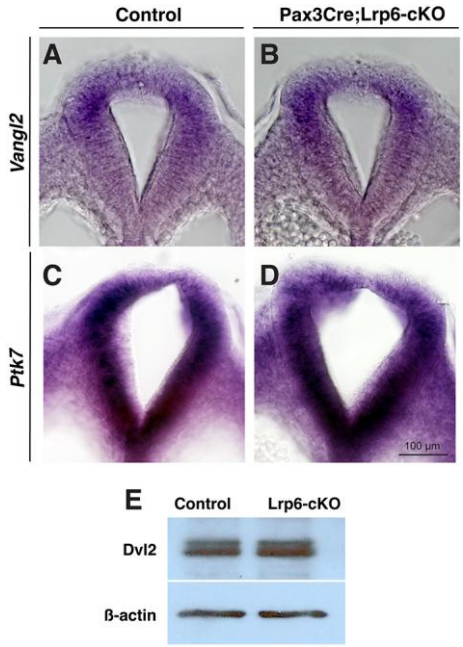

Figure 5. Noncanonical Wnt/PCP signaling activities the littermate controls and *Pax3-Cre;Lrp6-cKOs* at E9.5. (**A-D**) Transverse sections after wholemount in situ hybridization show no obvious changes of *Vangl2* (A,B) and *Ptk7* (C,D) expression patterns at the PNP closure sites of the normal control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

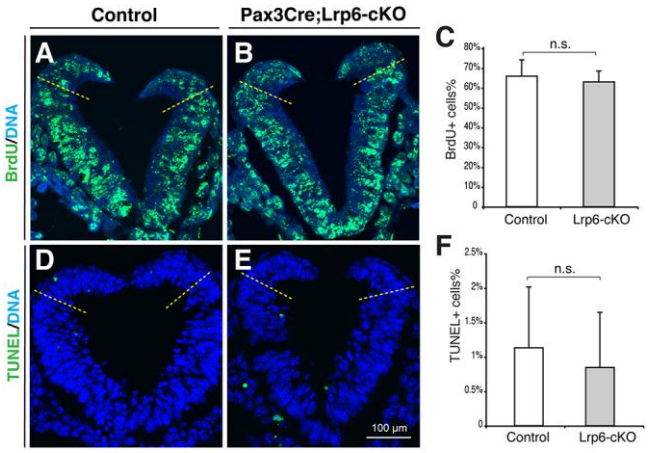

Figure 6. Proliferation and apoptosis at the PNP closure sites of the littermate controls and *Pax3-Cre;Lrp6-cKOs* at E9.5. (**A-C**) BrdU incorporation and detection experiments show no significant changes of the proliferating cells in the dorsal PNPs above the dorsolateral hinge points (dashed lines in A,B, transverse PNP sections) between the control and mutant embryos. (**D-F**) TUNEL assays demonstrate no significant changes of the apoptotic cells (green in D,E) in the dorsal PNPs between the control and mutant embryos. n.s., no statistical significance ($P > 0.05$).

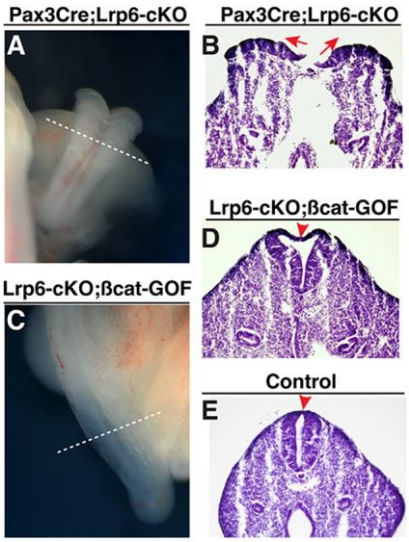

Figure 7. Genetic rescue of PNP closure defects in the *Pax3-Cre;Lrp6-cKOs* by ß-catenin gain-of-function (GOF). (**A,B**) Failed PNP closure as shown in the dorsal-posterior view of an *Lrp6-cKO* embryo (A) and in a transverse PNP section (arrows in B, cut through the dashed line in A) at E10.5. (**C,D**) Rescued PNP closure as shown in the dorsal-posterior view of an *Lrp6-cKO;ß-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.

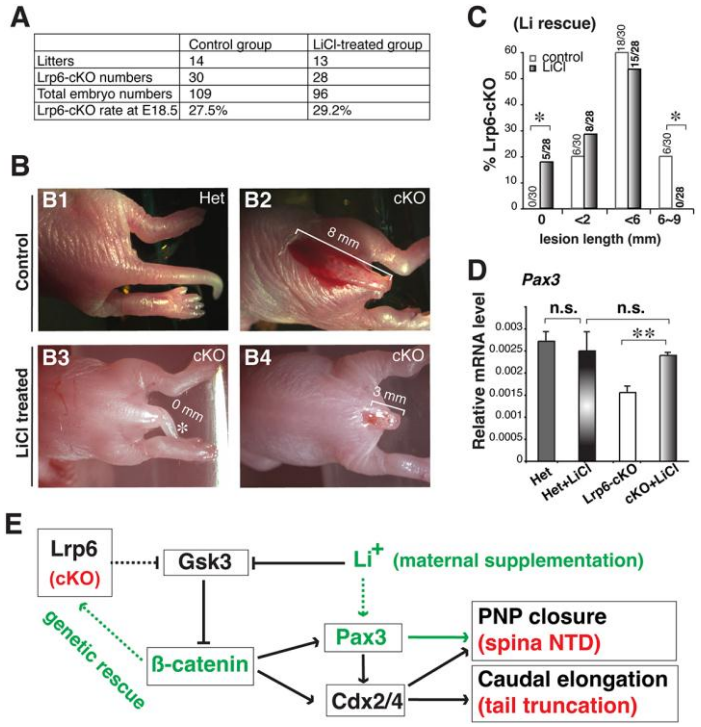

Figure 8. Pharmaceutical intervention of spinal NTDs in the *Pax3-Cre;Lrp6-cKOs* by maternal supplementation of a Wnt/ß-catenin signaling agonist. (**A**) The embryo numbers and *Lrp6-cKO* ratios are not significantly changed between the control and lithium-treated groups at E18.5 as compared with the expected Mendelian ratio (25% cKOs) ($P > 0.05$, Chi-Square test). (**B**) Dorsal-caudal body views of a double heterozygous (Het) of $Pax3^{Cre};Lrp6^{flox/+}$ 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 about 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 from E7.5 to E9.5. The lesion lengths (mm) were measured under the microscope. *, $P = 0.02$ (Fisher exact test); After all samples combined and averaged in each group, $P = 0.01$ (Student's t test). (**D**) RT-qPCR results demonstrate significant restoration of *Pax3* mRNAs in the lithium-treated *Lrp6-cKO* PNPs at E9.5. n.s., no statistical significance ($P > 0.05$); **, $P < 0.01$. (**E**) Illustrative summary of Lrp6-mediated ß-catenin-Pax3/Cdx2/Cdx4 signaling underlying posterior neuropore (PNP) closure/elongation and spinal NTD, the latter can be rescued by either genetic activation of *ß-catenin* or maternal supplementation of lithium ion that stabilizes intracellular ß-catenin by inhibiting Gsk3 in the canonical Wnt signaling pathway, and thus restoring a key downstream transcription factor *Pax3* in Lrp6-deficient PNPs. Red fonts, mutants and phenotypes; green fonts and arrows, genetic or pharmacological rescues demonstrated in the current study.