**Fig. S1. *Lats1/2* are expressed in the neural tube during early craniofacial development.**
A. RNAscope of *Lats1* (left) and *Lats2* (right). *Lats1* and *Lats2* puntci are found in the cranial neuroepithelium at E10.5. Cellular membranes stained with WGA (white) and nuclei stained with DAPI (blue). Boxed areas are shown at higher magnification in bottom panels. Outline delineates neural tube. Scale bars: 100 µm. B. Expression pattern of *Lats1* (top) and *Lats2* (bottom) during neural tube development. DESeq2 count normalization for *Lats1/2* transcripts in embryonic mouse neural tubes at E8.5, E9.5 and E10.5 (RNAseq data from Yu et al. 2017, NCBI SRA accession number: SRP070626).
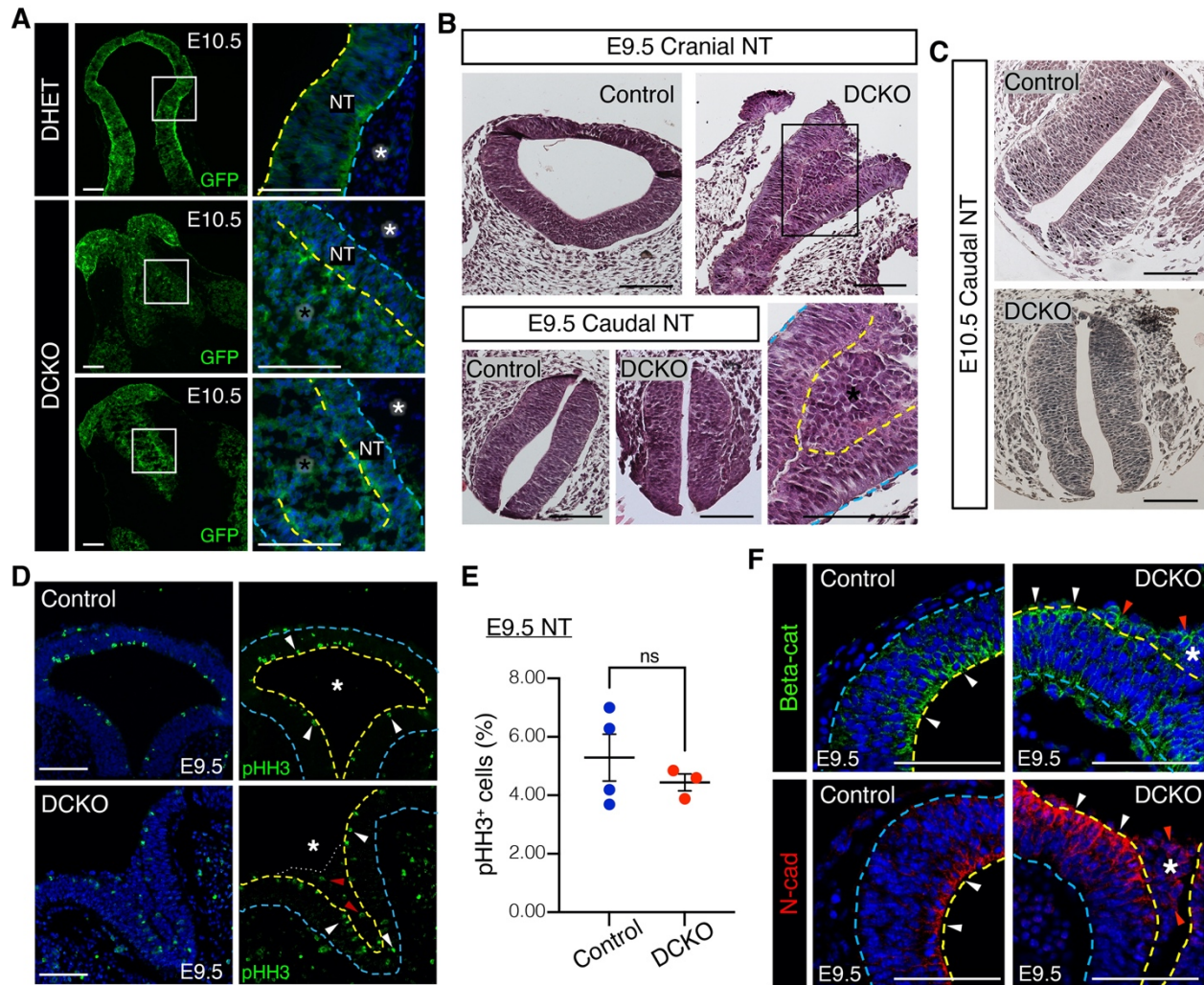
**Fig. S2.** *Lats1/2* **deficiency causes neuroepithelial cell phenotypes in cranial but not caudal neural tube.** A. Immunostaining of membrane-bound GFP reporter. GFP+ cells were found infiltrating the ventricular area in DCKO neural tubes (black asterisk). No recombination was detected in the craniofacial mesenchyme (white asterisk). Nuclei were stained with DAPI (blue). Boxed areas are shown at higher magnification in panels to the right. B-C. Histologic analysis showing H&E staining of coronal sections at E9.5 (B) and E10.5 (C). B. In E9.5 control embryos, neuroepithelial cells have a pseudostratified configuration in cranial and caudal neural tube. In E9.5 *Lats1/2* DCKO embryos, cellular infiltration can be detected inside the closing cranial neural folds (magnification, asterisk) but not in the caudal neural tube.

Boxed area shown at higher magnification in panel to the bottom. C. E10.5 DCKO caudal neural tubes don't have cellular infiltration nor organization phenotypes. D. Immunostaining of proliferation marker pHH3. At E9.5, mitotic cells appear in the apical edge of control neural tubes while they appear distributed along the apicobasal axis of DCKO neural tubes (white arrowheads). Positive cells are also found in the infiltrating cells within the ventricle in the *Lats1/2* DCKO mutants (red arrowheads). Asterisk points the ventricular space/side of the cranial neural tube. E. Quantification of pHH3 positive cells in the neural tube (control, n = 4; DCKO, n = 3). Data were compared using an unpaired t-test. All error bars represent SEM. F. Immunostaining of Beta-catenin and N-cadherin. At E9.5, Beta-catenin and N-cadherin are expressed in the apical edge of the neural tube of control embryos and *Lats1/2* DCKO mutants (white arrowheads). Beta-catenin and N-cadherin expression can also be appreciated in ventricular infiltrating cells (asterisk) in *Lats1/2* DCKO mutants (red arrowheads). Scale bars: 100 µm. Outline delineates neuroepithelium. NT: neural tube, ns: not significant. The yellow line indicates the apical edge; the blue line indicates the basal edge.
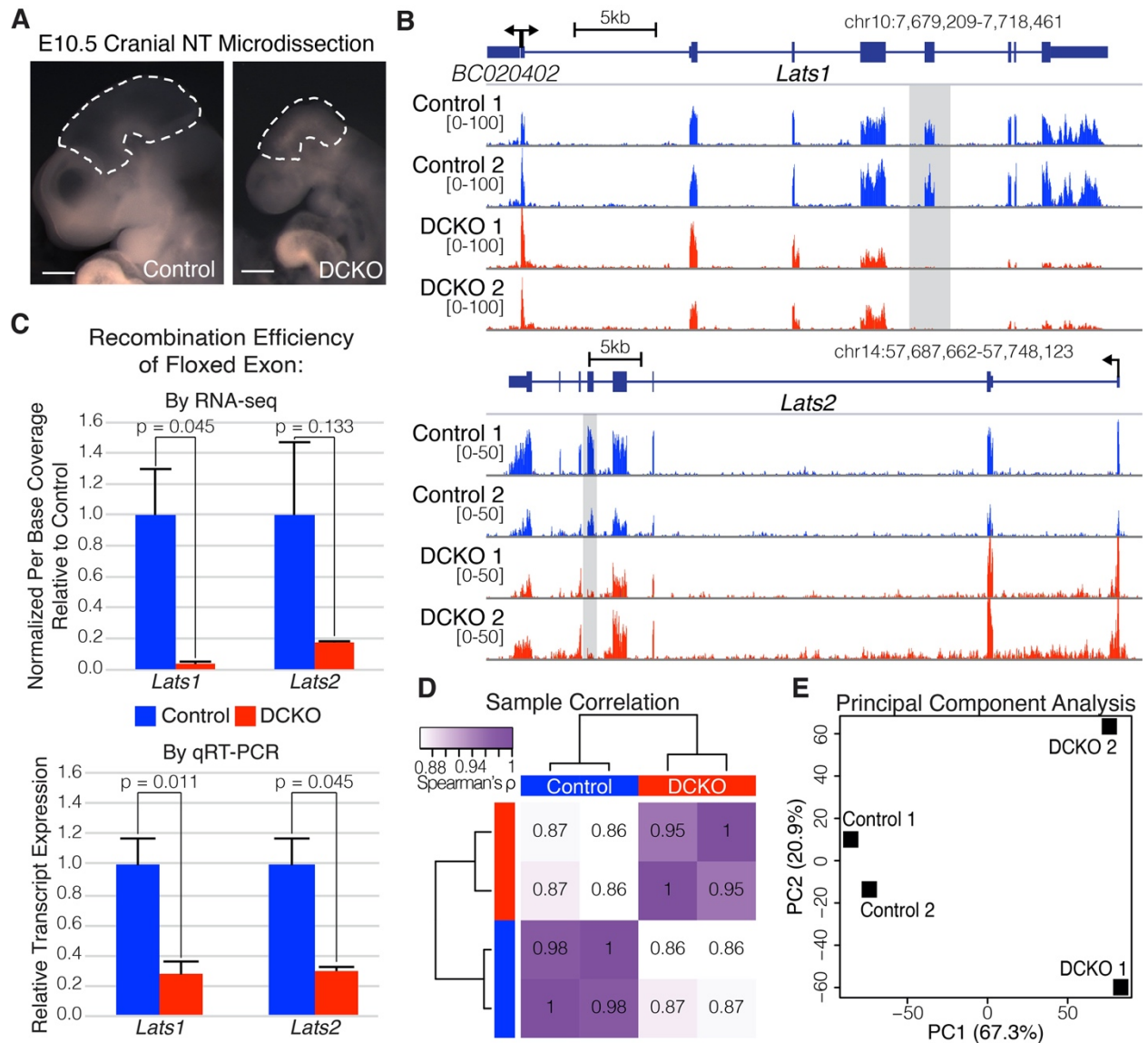
**Fig. S3. Quality control for RNA-Seq Analysis.** A. Cranial neural tubes were microdissected from 2 control (left) and 2 *Lats*1/2 DCKO (right) embryos at E10.5 along the dashed lines for transcriptional profiling. Scale bars: 500µm. B. Genome browser tracks for *Lats1* (mm10 chr10:7,679,209-7,718,461) and *Lats2* (mm10 chr14:57,687,662- 57,748,123) with raw read distribution for each sample (control in blue; DCKO in red). Highlighted in grey are the respective exons deleted upon cre-mediated recombination. C. Quantification of cre-mediated recombination for both *Lats1* and *Lats2* relative to controls using the normalized per base pair

coverage across the affected exons. Bottom: Secondary validation of *Lats1* and *Lats2* exon deletion by qRT-PCR using primers targeting the deleted exons. Data were compared using an unpaired t-test. D. Spearman correlation comparing whole transcriptome following removal of lowly expressed genes for the four samples. E. Plot displaying the principal component analysis (PCA) along PC1 and PC2 for the four samples. Percentages of variance explained per component noted in parentheses.
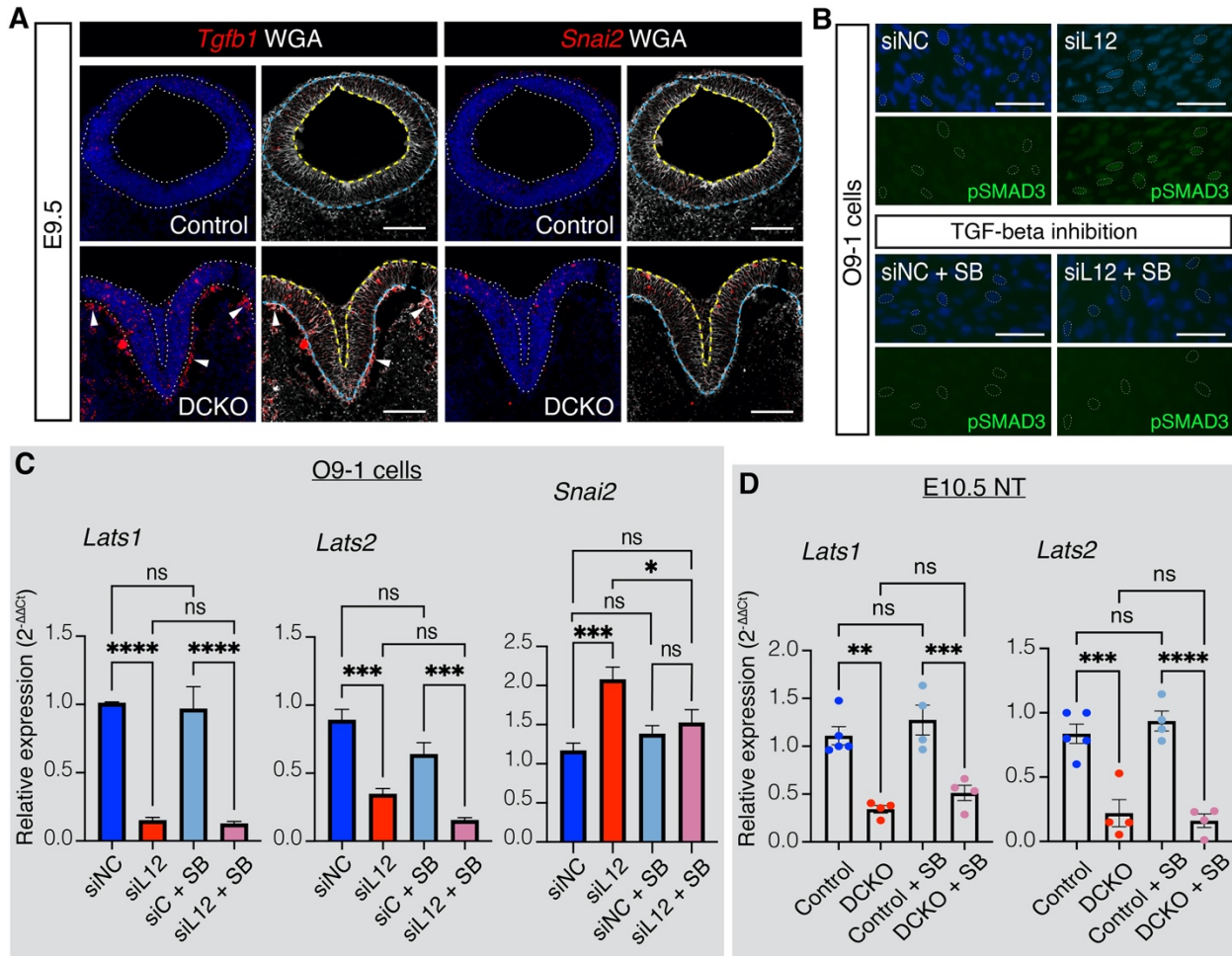
**Fig. S4. Lats1/2 regulates TGF-beta directed EMT in the neural tube and O9-1 cranial neural crest cells.** A. RNAscope for *Tgfb1* and *Snai2.* Left: At E9.5, DCKO neural tubes exhibit an increase in *Tgfb1* within the neuroepithelium and basally migrating cells (arrowheads) compared to control neural tubes. Right: Likewise, *Snai2* transcript levels are upregulated in DCKO neural folds and neuroepithelium compared to control neural tubes. Outline delineates neuroepithelium. The yellow line indicates the apical edge; the blue line indicates the basal edge. Nuclei counterstained with DAPI (blue). Scale bars: 100 μm. B. Immunostaining for TGF-beta downstream effector pSMAD3 in O9-1 cells. *Lats1/2* KD (siL1/2) increased pSMAD3 expression in O9-1 cells, which was inhibited by SB431542 (siL1/2 + SB). Several nuclei outlined for easier examination. Nuclei counterstained with

DAPI (blue). Scale bars: 100 μm. C. Relative *Lats1*, *Lats2*, and *Snai2* transcript levels in control (siNC) and *Lats1/2* KD (siL12) O9-1 cells in absence and presence of SB431542. TGF-beta inhibition did not alter *Lats1* and *Lats2* expression. *Snai2* transcript upregulation in absence of *Lats1/2* was prevented by TGF-beta inhibition (+ SB). Data from 4 independent experiments with 2 replicates each and were compared using one-way ANOVA followed by Tukey's multiple comparisons test, *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. D. Relative *Lats1* and *Lats2* transcript levels of E10.5 control and DCKO microdissected neural tube tissue in absence and presence of SB431542. TGF- beta inhibition did not alter *Lats1* and *Lats2* expression (n = 4 - 5 neural tubes). Data were compared using one-way ANOVA followed by

Tukey's multiple comparisons test, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. NT: neural tube, ns: not significant. All error bars represent SEM.

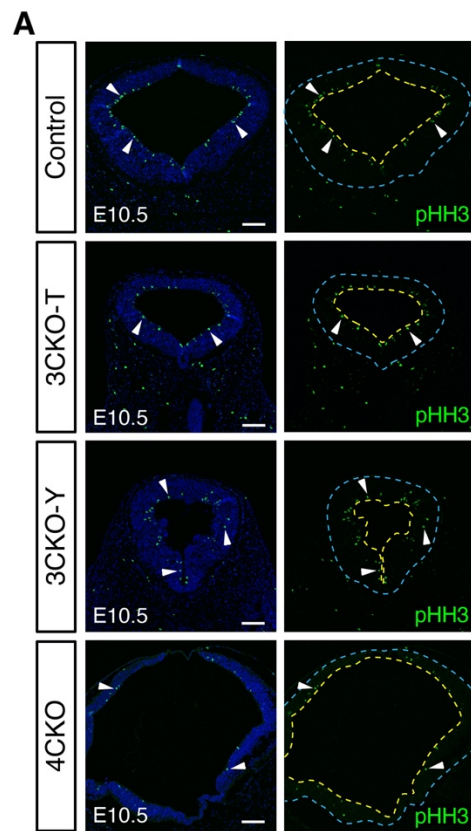**Fig. S5. Hippo signaling maintains neuroepithelial cell behavior in the dorsal neural tube.** A. Immunostaining of proliferation marker pHH3. Mitotic cells appear around the apical edge of the neural tube in control and *Yap/Taz* rescue embryos (arrowheads). Nuclei were stained with DAPI (blue). Scale bars: 100 µm. Outline delineates neuroepithelium. The yellow line indicates the apical edge; the blue line indicates the basal edge.

## Table S1. Mouse genotype nomenclatures.

| Labels | Genotypes |
|---|---|
| Control* | *Wnt1-Cre2* negative |
| DCKO | *Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/\Delta}$ |
| DHET | *Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/+}$ |
| haplo | *Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/\Delta}$, *Yap*$^{F/+}$, *Taz*$^{F/+}$ |
| 4CKO | *Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/\Delta}$, *Yap*$^{F/\Delta}$, *Taz*$^{F/\Delta}$ |
| 3CKO-L1 | *Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/\Delta}$, *Yap*$^{F/\Delta}$, *Taz*$^{F/\Delta}$ |
| 3CKO-L2 | *Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/+}$, *Yap*$^{F/\Delta}$, *Taz*$^{F/\Delta}$ |
| 3CKO-Y | *Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/\Delta}$, *Yap*$^{F/+}$, *Taz*$^{F/\Delta}$ |
| 3CKO-T | *Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/\Delta}$, *Yap*$^{F/\Delta}$, *Taz*$^{F/+}$ |
| 2CKO | *Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/+}$, *Yap*$^{F/\Delta}$, *Taz*$^{F/\Delta}$<br>*Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/\Delta}$, *Yap*$^{F/+}$, *Taz*$^{F/\Delta}$<br>*Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/\Delta}$, *Yap*$^{F/\Delta}$, *Taz*$^{F/+}$<br>*Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/+}$, *Yap*$^{F/+}$, *Taz*$^{F/\Delta}$<br>*Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/+}$, *Yap*$^{F/\Delta}$, *Taz*$^{F/+}$<br>*Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/\Delta}$, *Yap*$^{F/+}$, *Taz*$^{F/+}$ |
| 1CKO | *Wnt1-Cre2*, *Lats1*$^{F/\Delta}$, *Lats2*$^{F/+}$, *Yap*$^{F/+}$, *Taz*$^{F/+}$<br>*Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/\Delta}$, *Yap*$^{F/+}$, *Taz*$^{F/+}$<br>*Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/+}$, *Yap*$^{F/\Delta}$, *Taz*$^{F/+}$<br>*Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/+}$, *Yap*$^{F/+}$, *Taz*$^{F/\Delta}$ |
| 4HET | *Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/+}$, *Yap*$^{F/+}$, *Taz*$^{F/+}$ |

*Cre-negative controls included were from litters with DCKO mutant embryos present

**Table S2. Survival rates of *Lats1/2* mutants at different developmental stages.** *Wnt1-Cre2*, *Lats1*$^{F/+}$, *Lats2*$^{F/+}$ males were crossed with *Lats1*$^{F/F}$, *Lats2*$^{F/F}$ females to generate neural crest-specific conditional *Lats1/2* mutants. *Lats1/2* DCKO mutant embryos were embryonic lethal after E10.5. Control and compound mutant embryos had no obvious developmental problems. Data were compared using a chi-squared test, ***p < 0.001.

| Stage | Total # | Control | | Wnt1-Cre2, Lats1$^{F/+}$, Lats2$^{F/+}$ | | Wnt1-Cre2, Lats1$^{F/\Delta}$, Lats2$^{F/+}$ | | Wnt1-Cre2, Lats1$^{F/+}$, Lats2$^{F/\Delta}$ | | Wnt1-Cre2, Lats1$^{F/\Delta}$, Lats2$^{F/\Delta}$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Expected (50%) | Actual | Expected (12.5%) | Actual | Expected (12.5%) | Actual | Expected (12.5%) | Actual | Expected (12.5%) | Actual |
| E9.5 | 125 | 62.5 | 64 | 15.625 | 19 | 15.625 | 15 | 15.625 | 12 | 15.625 | 15 |
| E10.5 | 173 | 86.5 | 85 | 21.625 | 24 | 21.625 | 22 | 21.625 | 17 | 21.625 | 25 |
| P0*** | 151 | 75.5 | 90 | 18.875 | 27 | 18.875 | 18 | 18.875 | 16 | 18.875 | 0 |

**Table S3. Somite staging at different developmental timepoints.** Somite pairs were counted during embryo dissection. Significant developmental delay was detected in the *Lats1/2* DCKO embryos compared to no-cre controls at the E10.5 timepoint but not at E9.5. Nevertheless, all *Lats1/2* DCKO embryos had an open neural tube at E9.5, while *Lats1/2* DCKO embryos had variable neural tube closure by E10.5 (E9.5 control, n = 10; E9.5 DCKO, n = 6; E10.5 control, n = 20; E10.5 DCKO, n = 13). Somite number data were compared using an unpaired t-test and open neural tube proportion data were compared using a two-sample Z-test, ***p < 0.001. ONT: open neural tube, CNT: closed neural tube.

| Stage | Somite pairs | Control | | DCKO | |
|---|---|---|---|---|---|
| | | Somite Average | ONT (%)/ CNT (%) | Somite Average | ONT (%)/ CNT (%) |
| E9.5 | 21-29 | 25.0 ± 1.8 | 0/100 | 23.8 ± 1.9 | 100/0*** |
| E10.5 | 35-39 | 36.8 ± 2.2 | 0/100 | 29.8 ± 3.9*** | 53.8/46.2*** |

**Table S4. Survival rates and craniofacial phenotypes of rescue mutants.** *Wnt1*-Cre2, *Lats1*$^{F/+}$, *Lats2*$^{F/+}$, *Yap*$^{F/+}$, *Taz*$^{F/+}$ males were crossed with *Lats1*$^{F/F}$, *Lats2*$^{F/F}$, *Yap*$^{F/F}$, *Taz*$^{F/F}$ females to generate neural crest-specific *Lats1/2* mutants with varying alleles of *Yap* and *Taz*. Embryos were collected at E10.5. Rescue embryos had variable craniofacial phenotypes. Bolded genotypes were imaged and studied further. Data were compared using a chi-squared test, not significant. NT: neural tube, Fb: forebrain, PA: pharyngeal arches.

| Genotype | # Embryos | Expected (%) | Craniofacial Phenotypes | | |
|---|---|---|---|---|---|
| | | | NT | Fb | PA |
| Control | 66 | 67.50 (50%) | 0/66 | 0/66 | 0/66 |
| **4CKO** | 4 | 4.22 (3.125%) | 3/4 | 4/4 | 2/4 |
| 3CKO-L1 | 5 | 4.22 (3.125%) | 1/5 | 4/5 | 1/5 |
| 3CKO-L2 | 6 | 4.22 (3.125%) | 3/6 | 5/6 | 0/6 |
| **3CKO-Y** | 1 | 4.22 (3.125%) | 0/1 | 0/1 | 0/1 |
| **3CKO-T** | 6 | 4.22 (3.125%) | 0/6 | 4/6 | 1/6 |
| 2CKO | 22 | 25.31 (18.75%) | 4/22 | 6/22 | 5/22 |
| 1CKO | 21 | 16.88 (12.5%) | 0/21 | 1/21 | 1/21 |
| 4HET | 4 | 4.22 (3.125%) | 0/4 | 0/4 | 0/4 |
| TOTAL | 135 | | | | |