

Supplementary Information

Trio cooperates with Myh9 to regulate neural crest-derived craniofacial development

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Figure S1

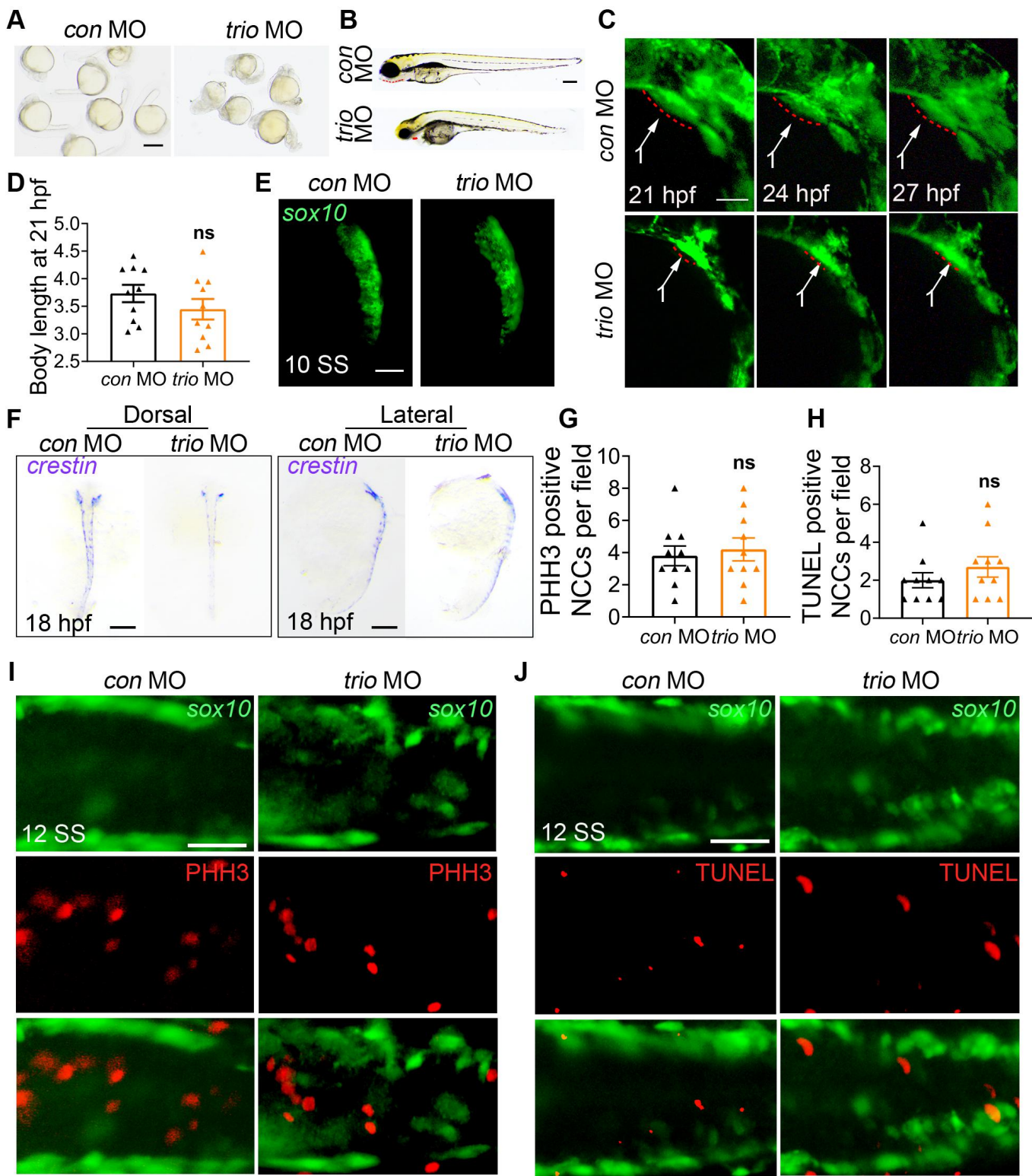


Figure S1: *trio* loss-of-function caused defects in NCC descendant differentiation and migration.

(A) Represent bright field images of *con* MO embryos and *trio* MO embryos at 24 hpf. Bar = 200 μ m. hpf: hours post-fertilization; MO: morpholino. (B) Represent bright field images of *con* MO and *trio* MO embryos at 96 hpf. Bar = 100 μ m. Red dotted line: mandible. (C) Lateral view of time-lapse images of *con* MO and *trio* MO Tg(*sox10:egfp*) embryos at 21, 24, 27 hpf. The red dotted lines outline the first NCC

migratory stream. Bar = 500 μ m. **(D)** Quantification of body length of *con* MO and *trio* MO embryos at 21 hpf (n = 10). **(E)** *con* MO and *trio* MO Tg(*sox10:egfp*) zebrafish at 10 SS. SS: somite stage. Bar = 500 μ m. **(F)** WISH performed for the NCC migration marker *crestin* in *con* MO and *trio* MO zebrafish embryos. The dorsal and lateral views of 18 hpf embryos show *crestin* expression in NCC migratory streams. Bar = 200 μ m. WISH: whole-mount in situ hybridization. **(G, I)** Image and quantitative analysis of PHH3 staining of *con* MO and *trio* MO Tg(*sox10:egfp*) embryos indicate NCC proliferation in the dorsal neural tube at 12 SS (n = 10). PHH3: phosphohistone H3. Bar = 500 μ m. **(H, J)** Image and quantitative analysis of TUNEL staining of *con* MO and *trio* MO Tg(*sox10:egfp*) embryos indicate NCC apoptosis in the dorsal neural tube at 12SS (n = 10). Bar = 500 μ m. TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling. All data are represented as mean $\pm$ S.E.M (two-tailed t test ns, not significantly).

Figure S2

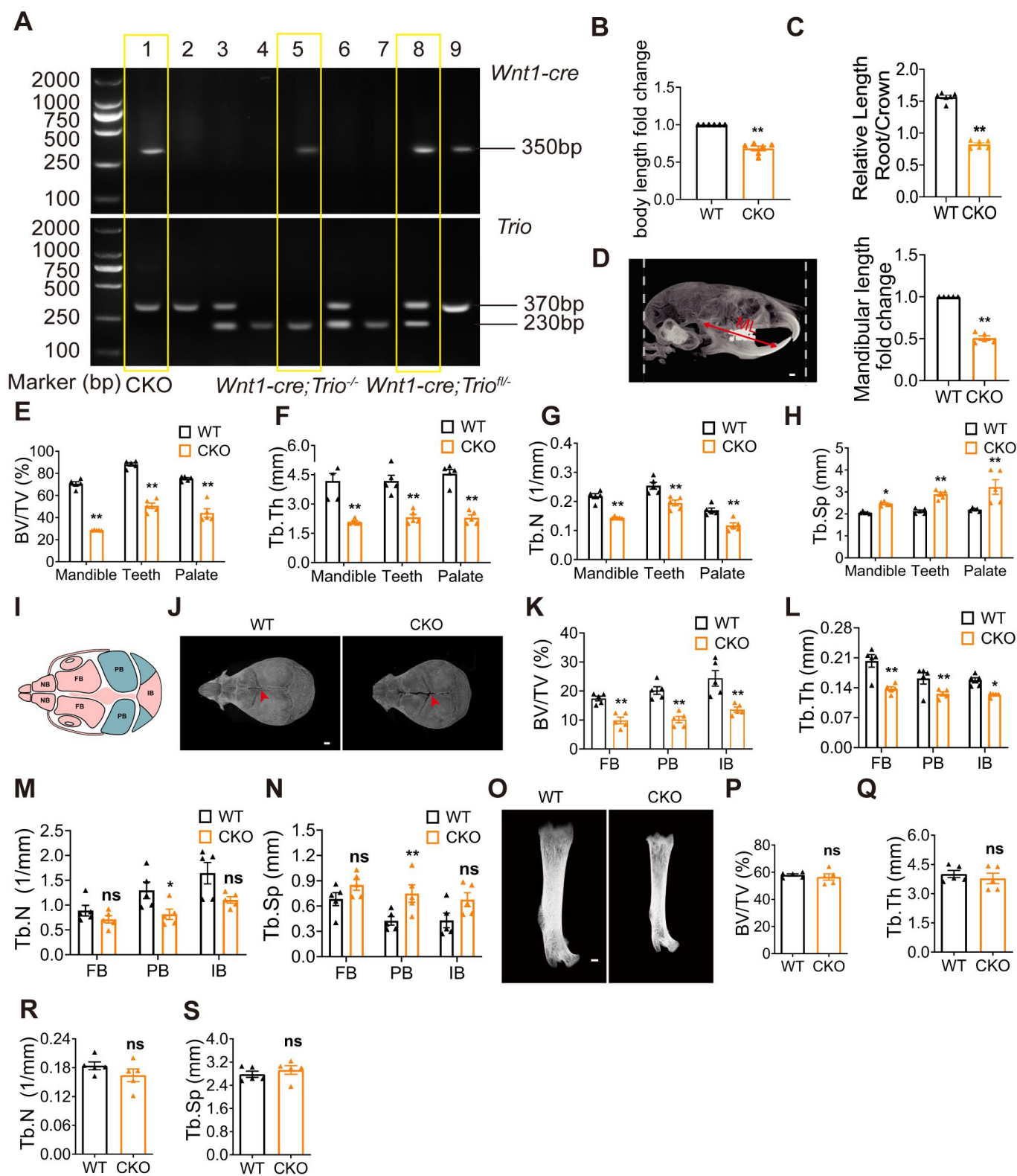


Figure S2: Trio-conditional-knockout mice showed more osteoporotic bone in the skull, but had no change in the femur.

(A) Mice DNA genotypes of *Wnt1-cre* and *Trio-flox* analyzed by PCR. Upper gel represented bands of *Wnt1-cre*. The lower gel contained bands of *Trio*. *Wnt1-cre*: 350-bp, *Trio-flox* homozygote (*Trio^{fl/fl}*): 370-bp, *Trio-flox* heterozygote (*Trio^{fl/-}*): 370 and 230-bp, *Trio* wildtype (*Trio^{-/-}*): 230-bp. Lane 1: *Wnt1-cre*; *Trio^{fl/fl}* = CKO, lane 5: *Wnt1-cre*; *Trio^{-/-}*, lane 8: *Wnt1-cre*; *Trio^{fl/-}*. (B) Quantification of body length fold change of WT and CKO mice (n = 5). (C) Quantification analysis of relative length root/crown of WT and CKO mice at P21 (n = 6). (D) Longitudinal sketch of skull and quantification of mandibular length (ML) (n = 5). Bar = 500 μ m. (E-H) Quantification of Micro-CT images of the mandible, teeth, palate area obtained from WT and CKO mice at P21. BV/TV: bone volume fraction, Tb.Th: trabecular thickness; Tb.N: trabecular number; Tb.Sp: trabecular separation (n = 5). (I) Schematic top view of mice skull. Pink indicated the origin of neural crest and blue indicated the origin of mesoderm. NB: nasal bone; FB: frontal bone; PB: parietal bone; IB: interparietal bone. (J) Top scanning of WT and CKO mice skull at P21. CKO skull displayed incomplete closure of cranial suture (red arrow). (K-N) Quantification of micro-CT images of frontal bone (FB), parietal bone (PB) and interparietal bone (IB) from WT and CKO mice at P21. (O) Longitudinal scanning of femur. Bar = 500 μ m. (P-S) Quantification of BV/TV, Tb.Th, Tb.N, Tb.Sp of femur (n = 5). All data are represented as mean $\pm$ S.E.M (two-tailed t test * p < 0.05, ** p < 0.01, ns, not significantly).

Figure S3

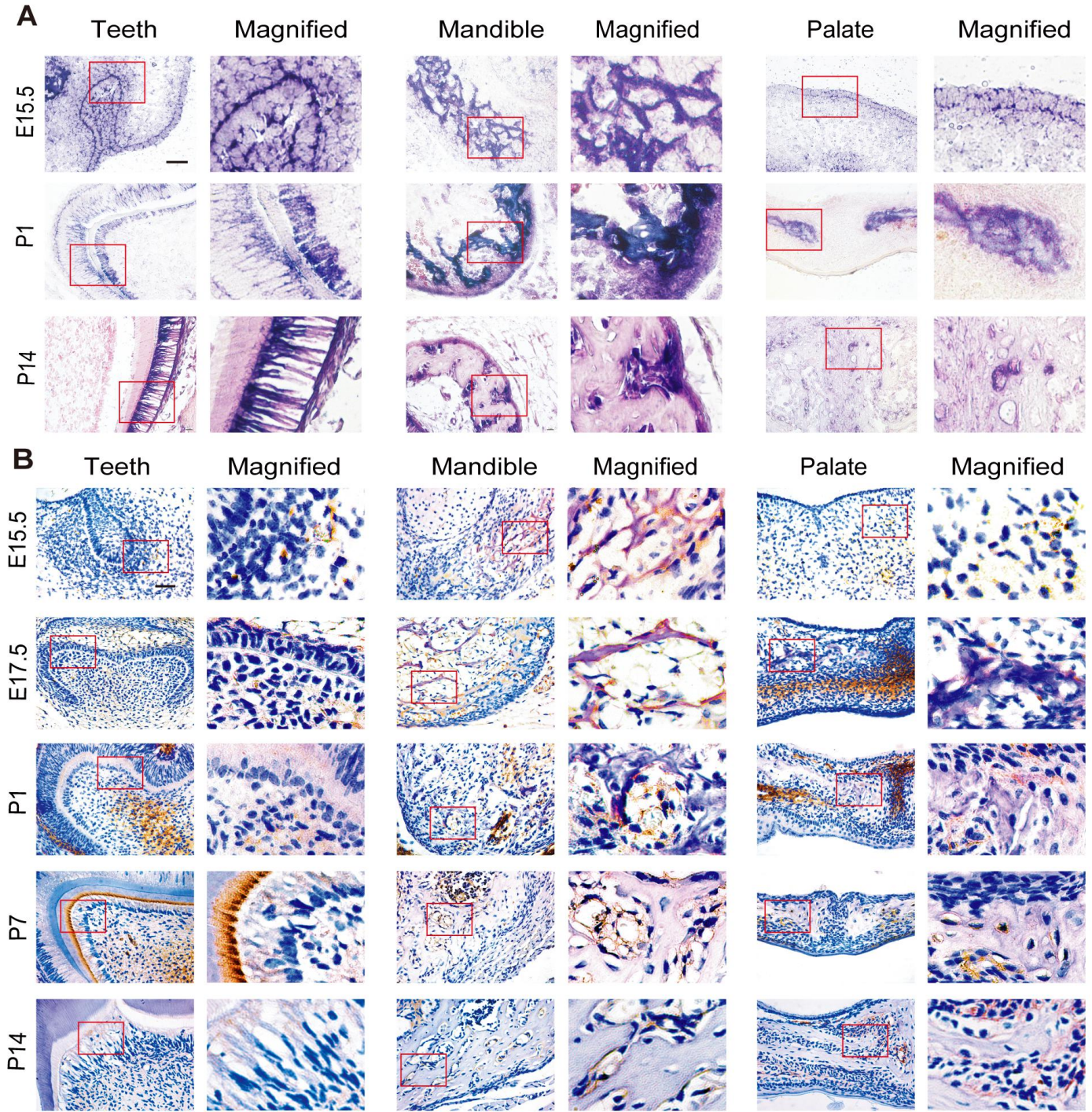


Figure S3: Trio showed expression in teeth, mandible and palate of mice.

(A) WISH of Trio expression of mice at E15.5, P1 and P14. Bar = 100 μ m. Following panels showed higher magnifications of boxed areas. (B) Immunohistochemical staining for Trio expression of mice at E15.5, E17.5, P1, P7 and P14. Bar = 100 μ m. Following panels showed higher magnifications of boxed areas.

Figure S4

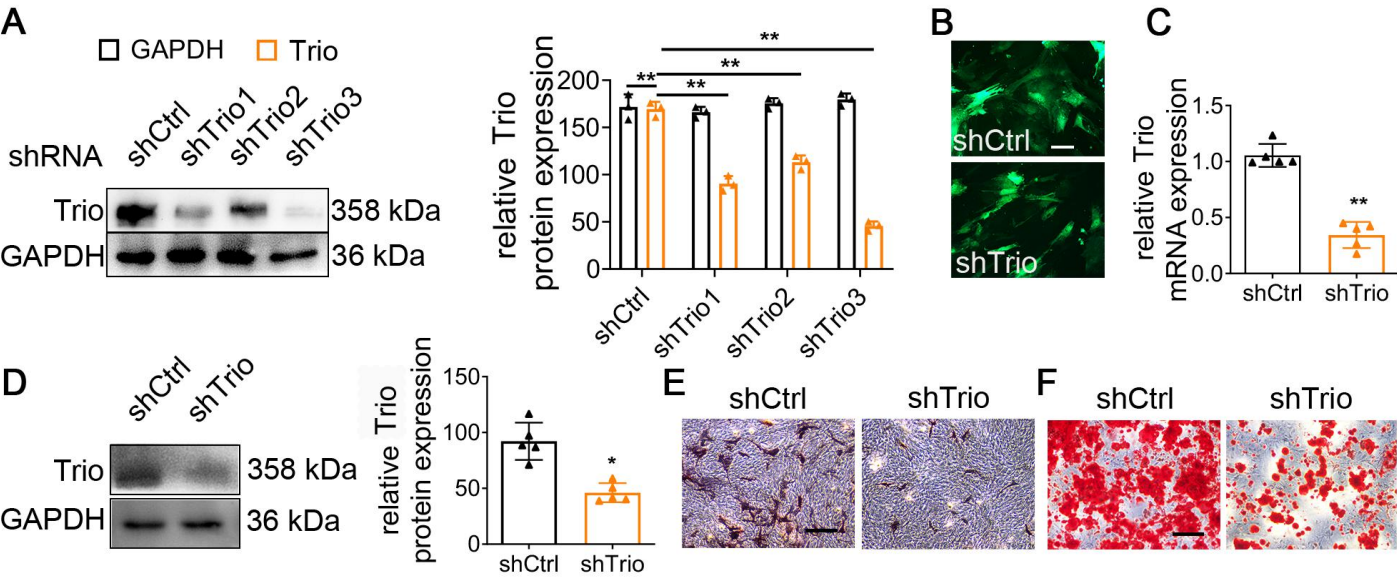


Figure S4: Knockdown of Trio in NCCs influenced cell proliferation, apoptosis and osteogenesis differentiation *in vitro*.

(A) Lentiviral particles encoding shRNA-3 targeting the Trio gene (shTrio3) was selected for its high Trio knockdown efficiency by western blot analysis. Quantitative analysis of western blot bands followed (n = 3). (B) Fluorescence microscopy images proved shTrio lentivirus transfection efficiency of NCCs. Bar = 100 μ m. (C) qRT-PCR analysis performed to detect Trio mRNA expression in shCtrl and shTrio NCCs (n = 5). (D) Western blot conducted to test the Trio knockdown efficiency after shTrio transfection. Quantitative analysis of western blot bands followed (n = 5). (E) Alkaline phosphatase (ALP) staining of NCCs. Bar = 100 μ m. (F) Alizarin red staining (ARS) of NCCs. Bar = 100 μ m. All data represent mean $\pm$ S.E.M (two-tailed t test * p < 0.05, ** p < 0.01, ns, not significantly).

Figure S5

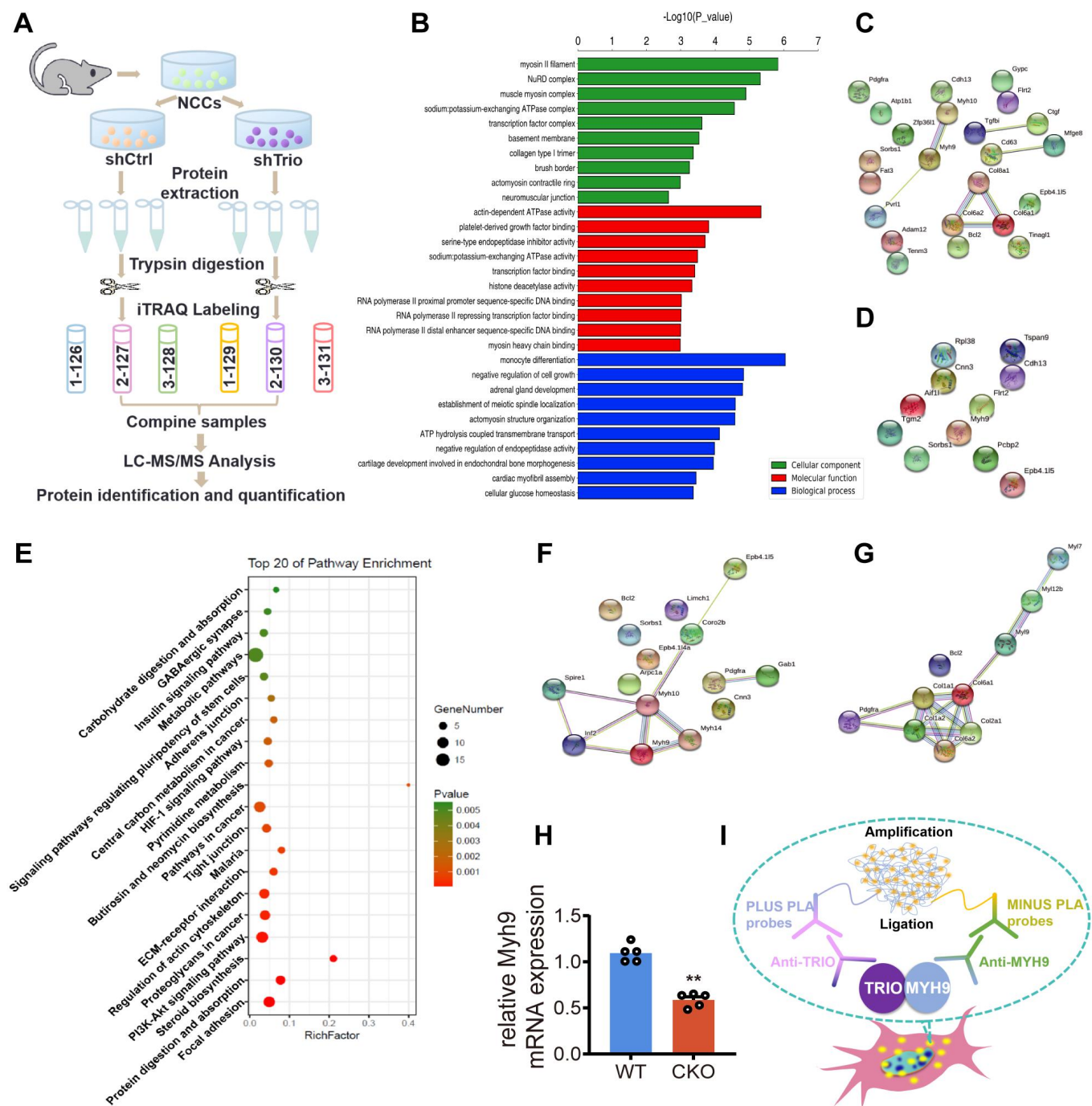


Figure S5: Selection for Myh9 from 297 DEPs in iTRAQ by bioinformatic analysis.

(A) Schematic outline of the sample preparation and iTRAQ protein analysis in shCtrl and shTrio NCCs. iTRAQ, isobaric tags for relative and absolute quantitation. (B) GO enrichment analysis of common DEPs between shCtrl and shTrio group. The vertical coordinate represented categories sorted according to p-value from top to bottom. The more significant items were to the top. (C) main GO clusters within the biological process (BP) involving in cell. adhesion. (D) Main GO clusters within the cell component (CC) involving in focal adhesion. (E) Top 20 enrichment KEGG pathways associated with DEPs. The pathway ranking in this diagram is in order of smaller P -value and bigger

gene number from top to bottom. **(F)** Main GO clusters within the cell component (CC) involving in actin-cytoskeleton. **(G)** Main KEGG clusters involving in focal adhesion. **(H)** qRT-PCR analysis performed to detect Myh9 mRNA expression in the first branchial arch of WT and CKO embryos at E10.5 (n = 5). **(I)** Schematic of the proximity ligation assay (PLA) in NCCs. All data represent mean $\pm$ S.E.M (two-tailed t test $**p < 0.01$).

Figure S6

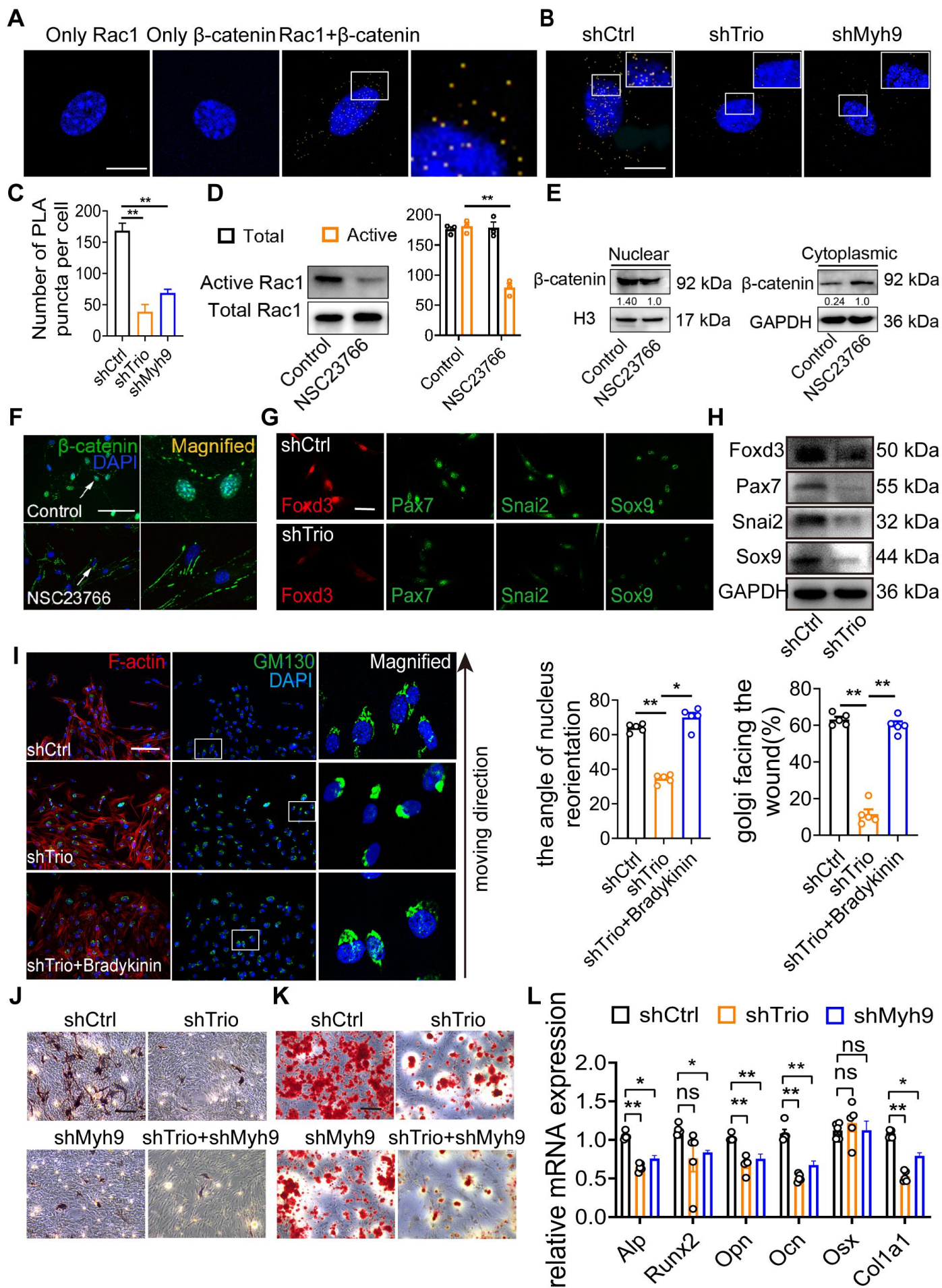


Figure S6: Myh9 assists Trio in regulation of NCC migration via Rac1 and Cdc42 GTPase activation.

(A) PLA detection and visualization of Rac1 and β -catenin in NCCs. Blue: DAPI nuclear staining. Yellow spots: PLA signals. Bar = 400 μ m. (B) PLA detection and visualization of Rac1 and β -catenin in the shTrio and shMyh9 NCC groups. Blue: DAPI nuclear staining. Yellow spots: PLA signals. Bar = 400 μ m. (C) Quantification of PLA in (B) (n = 3). (D) Image and quantification of Rac1 pull-down activation in the Control and NSC23766 NCC groups (n = 3). (E) Western blot of β -catenin in the nucleus and cytoplasm in the Control and NSC23766 NCC groups. (F) Immunostaining of β -catenin (green) and DAPI (blue) for nuclear β -catenin expression indicated by a white arrow in the Control and NSC23766 NCCs. Bar = 100 μ m. (G) Immunofluorescence staining for markers of migratory NCCs (Foxd3, Pax7, Snai2 and Sox9). Bar = 200 μ m. (H) Western blot of markers of migratory NCCs (Foxd3, Pax7, Snai2 and Sox9) in shCtrl and shTrio cells. (I) Immunofluorescence staining of the Golgi body marker GM130 and F-actin for cell polarization assay. Nuclei were counterstained with DAPI. Quantification analysis of percentage of the angle of nucleus reorientation and Golgi facing the wound (n = 5). Bar = 100 μ m. (J, K) ALP and ARS staining of shCtrl, shTrio, shMyh9 and shTrio + shMyh9 NCCs. Bar = 100 μ m. (L) qRT-PCR of marker genes in osteogenic differentiation (Alp, Runx2, Opn, Ocn, Osx, Colla1) expression (n = 5). All data represent mean $\pm$ S.E.M (two-tailed t test $*p < 0.05$, $**p < 0.01$, ns, not significantly).

Figure S7

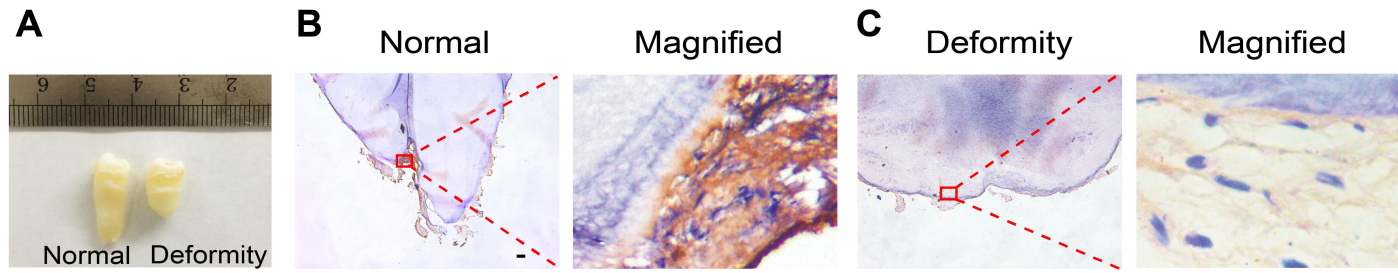


Figure S7: SCAPs in teeth with short roots showed less Trio expression.

(A) General view of normal and deformed teeth of human. (B, C) Immunohistochemistry for Trio for human stem cells of dental papilla (SCAPs) of normal and deformed teeth. Brown staining indicated anti-Trio immunoreactivity. Teeth with short roots expressed decreased Trio. Bar = 100 μ m.