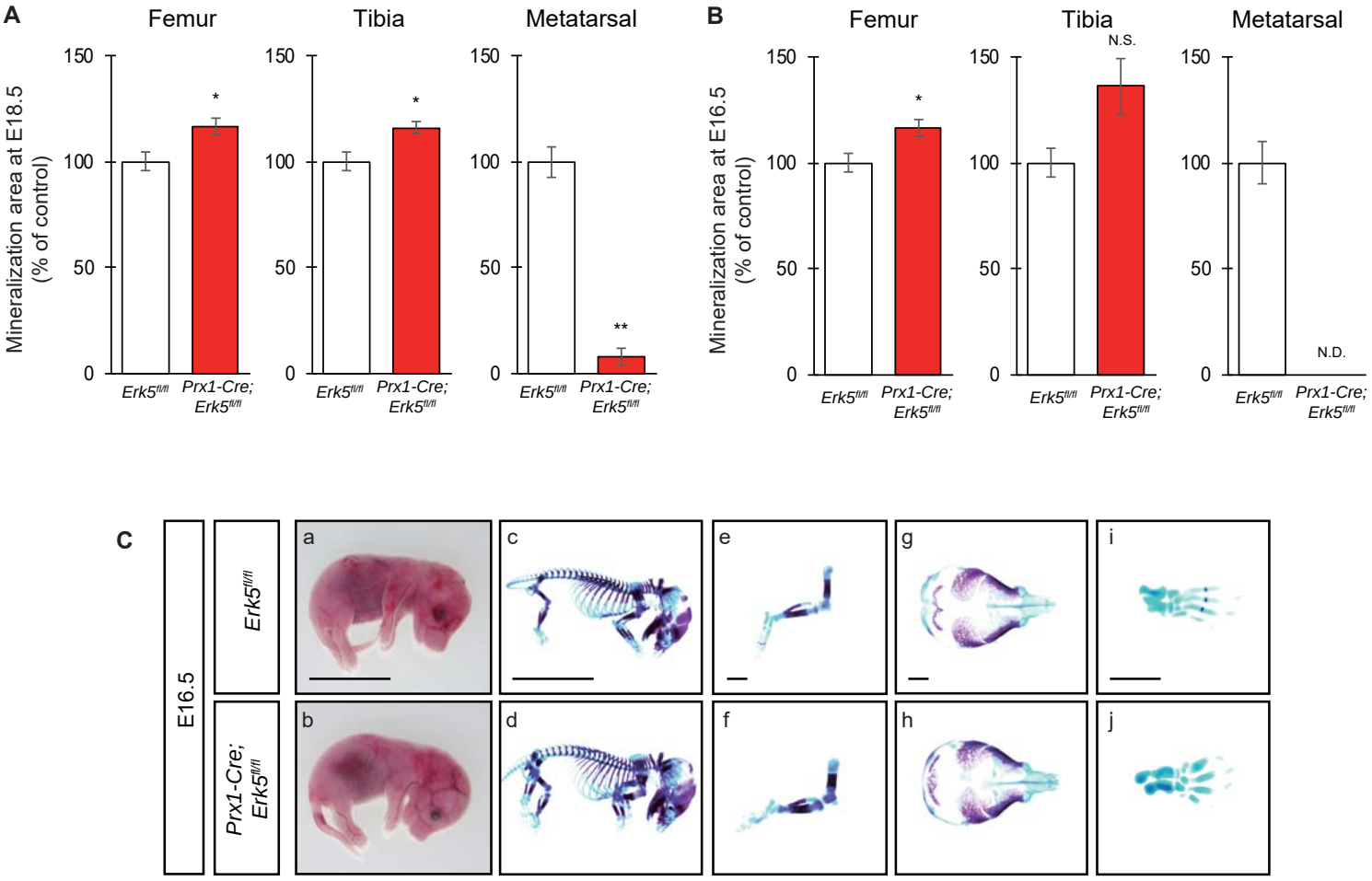


Figure S1. Phenotypes of *Prx1–Cre;Erk5$^{fl/fl}$* embryos. Quantitative data of mineralization area of femur, tibia and metatarsal of *Erk5$^{fl/fl}$* and *Prx1-Cre;Erk5$^{fl/fl}$* embryos at (A) E18.5 and (B) E16.5 (n=3). (C) The whole and parts of skeleton of *Erk5$^{fl/fl}$* and *Prx1-Cre;Erk5$^{fl/fl}$* embryos at E16.5. Embryos were double stained with alizarin red and alcian blue. Bar=10 mm (a–d) and Bar=1 mm (e–j). Representative images of skeletal preparations derived from more than 3 embryos from different litters are shown. *P<0.05, **P<0.01, significantly different from the value obtained in control embryos (A and B). Statistical significance was determined using the two-tailed, unpaired Student's *t*-test (A and B). N.S., not significant. N.D., not detected.

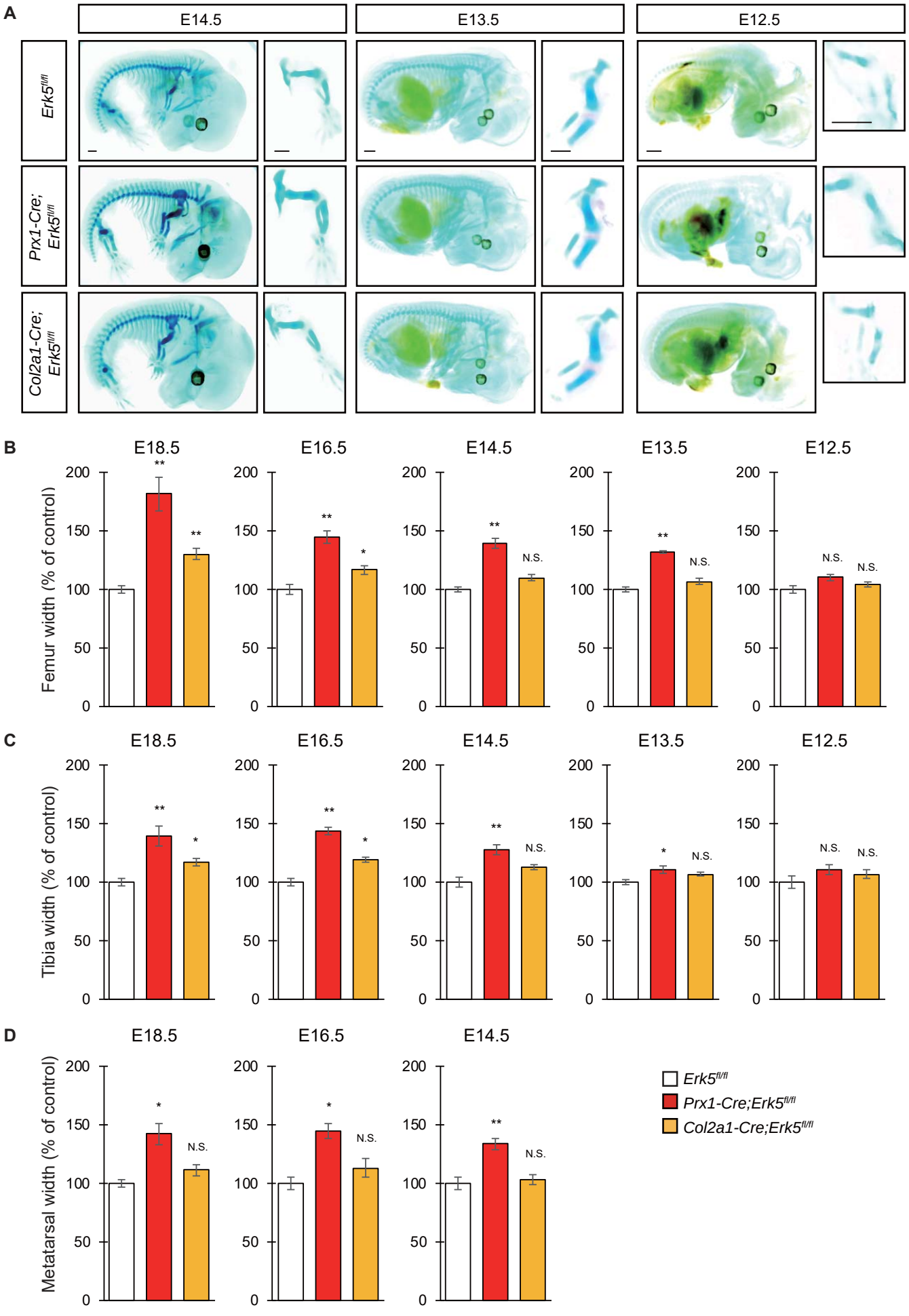


Figure S2. Phenotypes of *Prx1–Cre;Erk5$^{fl/fl}$* and *Col2a1–Cre;Erk5$^{fl/fl}$* embryos. (A) The whole and parts of skeleton of *Erk5$^{fl/fl}$* and *Prx1-Cre;Erk5$^{fl/fl}$* and *Col2a1–Cre;Erk5$^{fl/fl}$* embryos at E14.5, E13.5 and E12.5. Embryos were double stained with alizarin red and alcian blue. Bar=500 μm. Representative images of skeletal preparations derived from more than 3 embryos from different litters are shown. Quantitative data of width of (B) femur, (C) tibia and (D) metatarsal of *Erk5$^{fl/fl}$* and *Prx1-Cre;Erk5$^{fl/fl}$* and *Col2a1–Cre;Erk5$^{fl/fl}$* embryos at E12.5-18.5 (n=3). *P<0.05, **P<0.01, significantly different from the value obtained in control embryos (B-D). Statistical significance was determined using the two-tailed, unpaired Student's *t*-test (B-D). N.S., not significant.

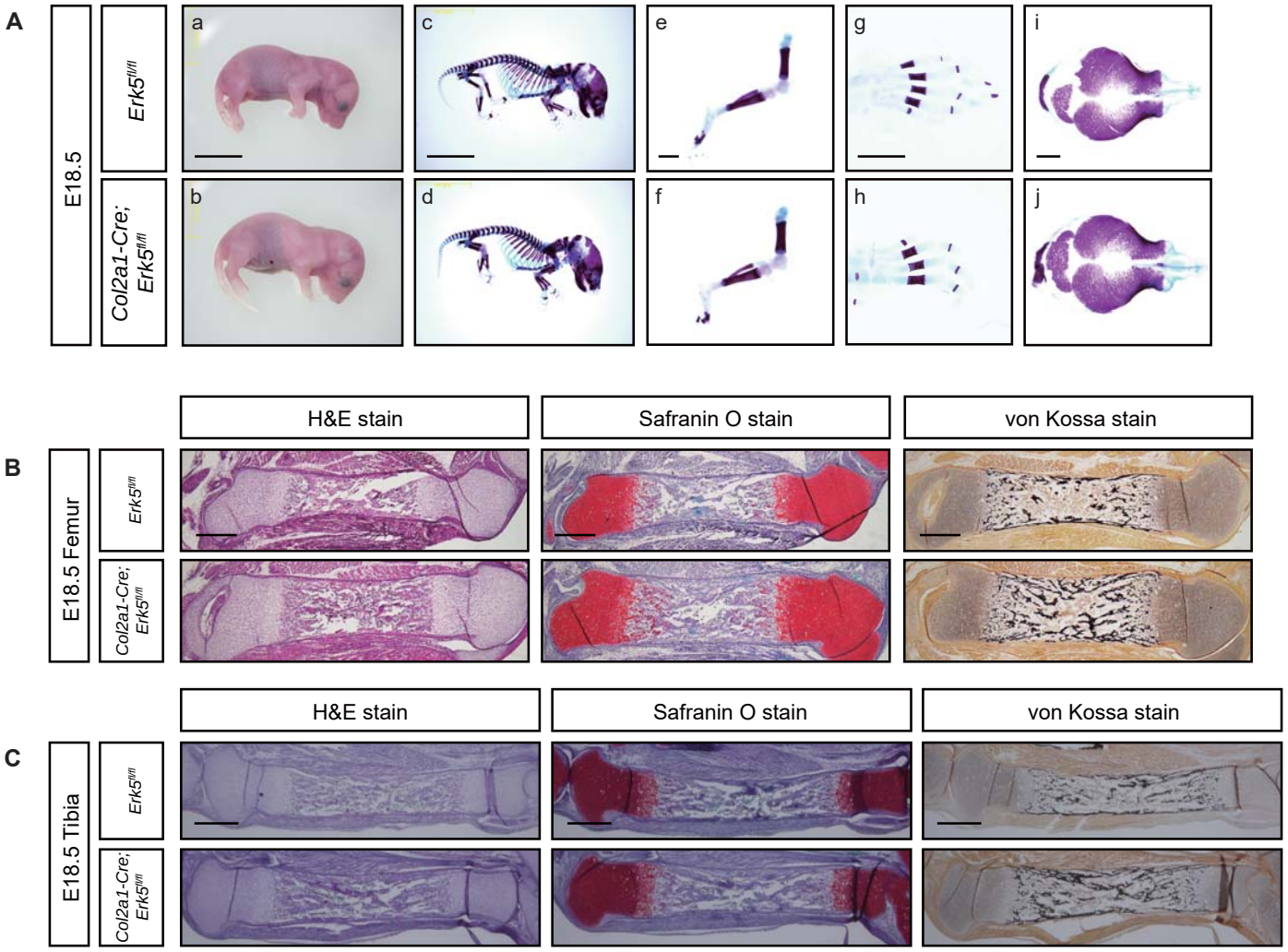


Figure S3. Phenotypes of *Col2a1–Cre;Erk5$^{fl/fl}$* embryos. (A) The whole and parts of skeleton of *Erk5$^{fl/fl}$* and *Col2a1-Cre;Erk5$^{fl/fl}$* embryos at E18.5. Embryos were double stained with alizarin red and alcian blue. Bar=10 mm (a–d) and Bar=1 mm (e–j). Histological analyses of the (B) femur and (C) tibia of *Erk5$^{fl/fl}$* and *Col2a1-Cre;Erk5$^{fl/fl}$* embryos at E18.5. Femur and tibia were stained with H&E, Safranin O and von Kossa. Bar=500 μm. Representative images of skeletal preparations and histological analyses derived from more than 3 embryos from different litters are shown.

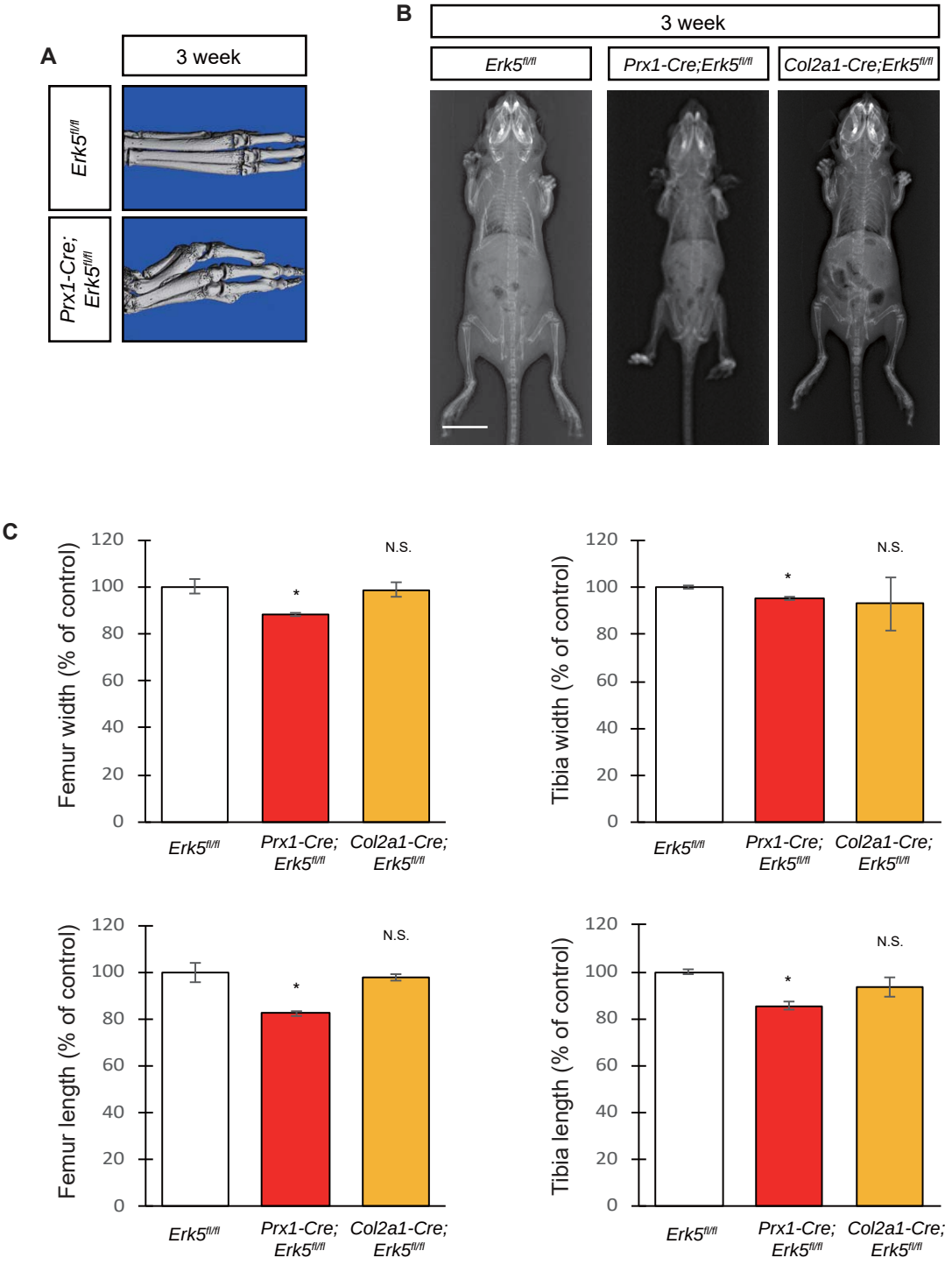


Figure S4. Phenotypes of *Prx1–Cre;Erk5$^{fl/fl}$* and *Col2a1–Cre;Erk5$^{fl/fl}$* mice and *Osx-Cre;Erk5$^{fl/fl}$* embryos. (A) μCT 3D images of the foot of *Erk5$^{fl/fl}$* and *Prx1-Cre;Erk5$^{fl/fl}$* mice at 3 week-old. (B) CT images of *Erk5$^{fl/fl}$*, *Prx1-Cre;Erk5$^{fl/fl}$* and *Col2a1-Cre;Erk5$^{fl/fl}$* mice at 3 week-old. Bar=10 mm. Representative images of μCT and CT derived from more than 3 mice are shown. (C) Quantitative data of width and length of femur and tibia of *Erk5$^{fl/fl}$* and *Prx1-Cre;Erk5$^{fl/fl}$* and *Col2a1–Cre;Erk5$^{fl/fl}$* mice at 3 week-old (n=3). (D) The whole and parts of skeleton of *Osx-Cre* and *Osx-Cre;Erk5$^{fl/fl}$* embryos at E18.5. Embryos were double stained with alizarin red and alcian blue. Bar=10 mm (a–b) and Bar=1 mm (c–h). Representative images of skeletal preparations derived from more than 3 embryos from different litters are shown. Statistical significance was determined using the two-tailed, unpaired Student's *t*-test (C). N.S., not significant.

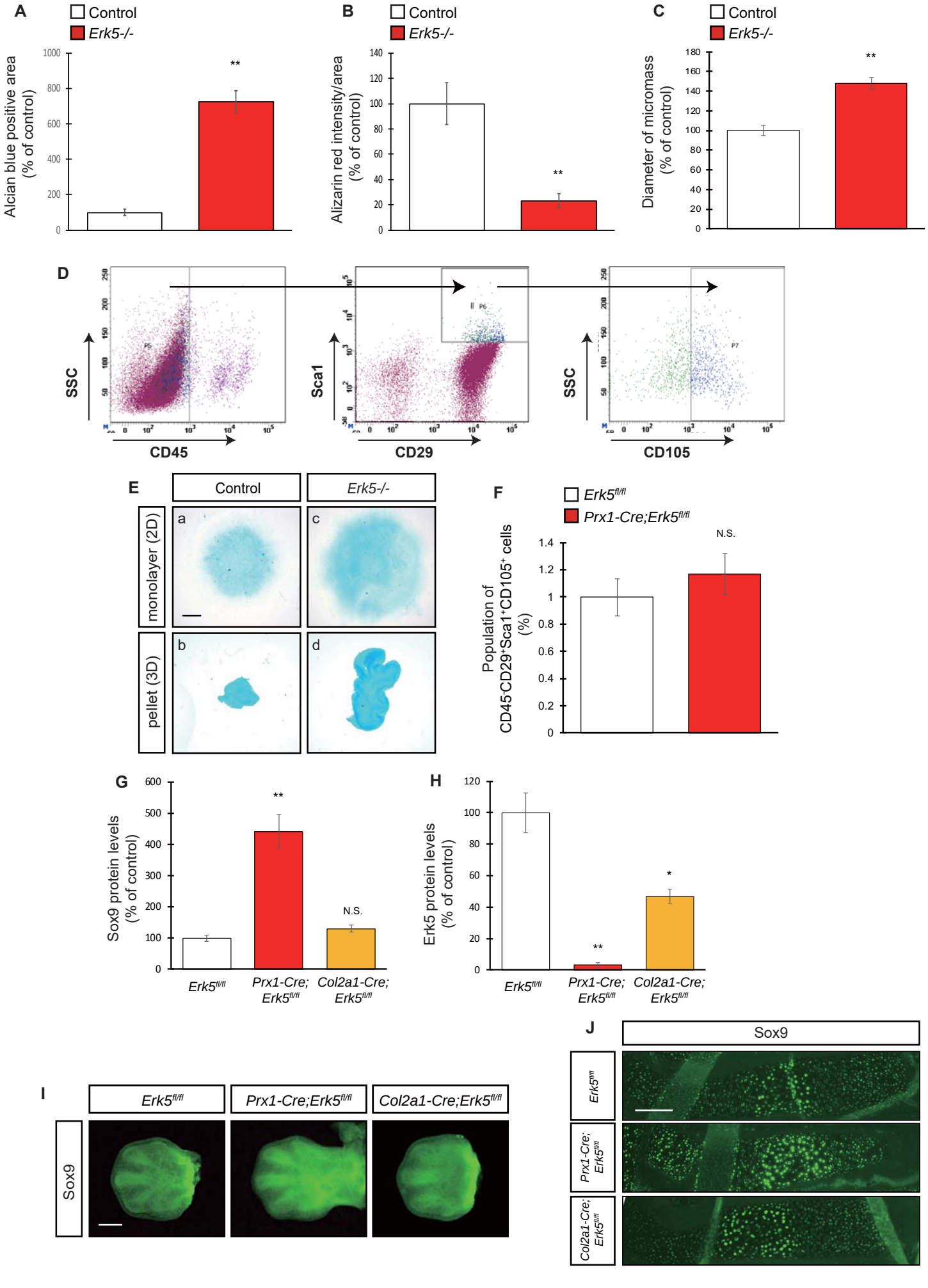
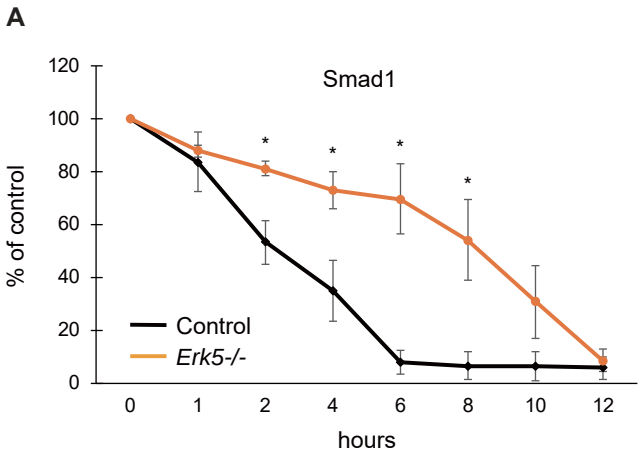
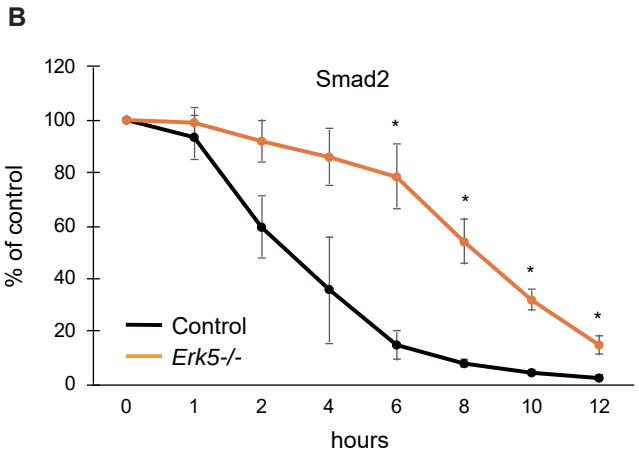
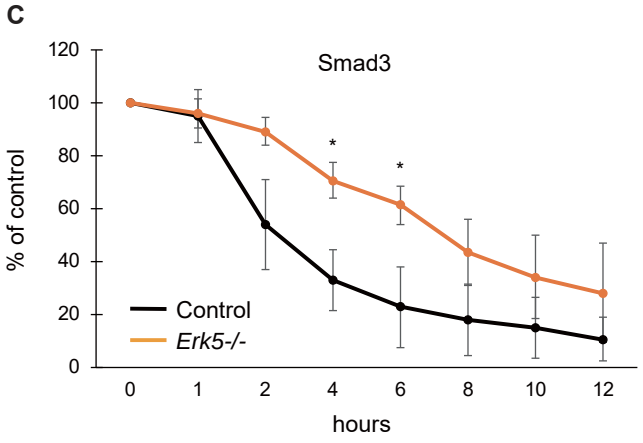
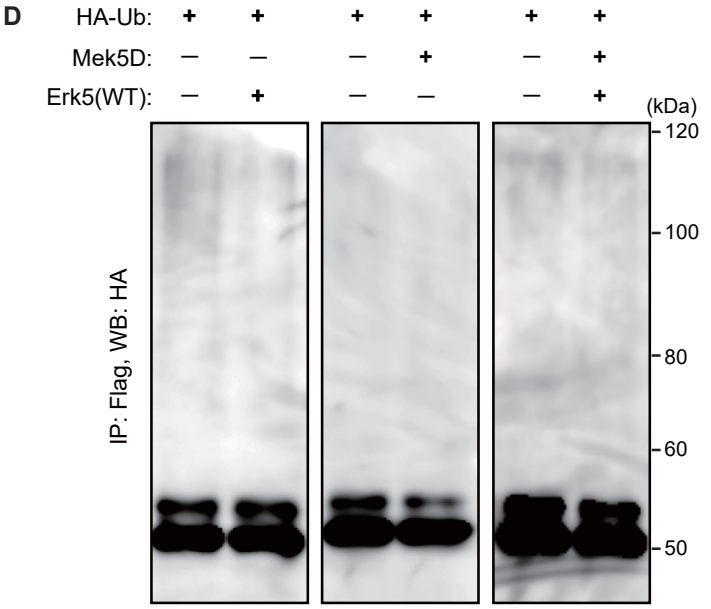


Figure S5. Erk5 is essential for chondrogenesis through its expression in mesenchymal cells. Quantification of (A) alcian blue stain, (B) alizarin red stain, and (C) diameter of micromass of Figure 2A. (D) Isolation strategy of $CD45^{-}CD29^{+}Sca1^{+}CD105^{+}$ cells. (E) $CD45^{-}CD29^{+}Sca1^{+}CD105^{+}$ cells were isolated from forelimb buds of *Erk5*$^{fl/fl}$ and *Prx1-Cre;Erk5*$^{fl/fl}$ embryos at E12.5, and subsequent 2D monolayer culture and 3D pellet culture, followed by determination of alcian blue staining (n=3). Bar=500 μm. (F) Quantification of population of $CD45^{-}CD29^{+}Sca1^{+}CD105^{+}$ cells in limb buds of *Erk5*$^{fl/fl}$ and *Prx1-Cre;Erk5*$^{fl/fl}$ embryos at E12.5 (n=3). (G and H) Quantification of immunoblot data of Figure 2H. Detection of Sox9 proteins in *Erk5*$^{fl/fl}$, *Prx1-Cre;Erk5*$^{fl/fl}$ and *Col2a1-Cre;Erk5*$^{fl/fl}$ embryos at (I) E12.5 and (J) E16.5 by immunohistochemistry. Bar=100 μm (I) and 500 μm (J). Representative images of histological analyses derived from more than 3 embryos from different litters are shown. *P<0.05, **P <0.01, significantly different from the value obtained in control cells (A-C and F) or control embryos (G and H). Statistical significance was determined using the two-tailed, unpaired Student's *t*-test (A-C and F-H). N.S., not significant.









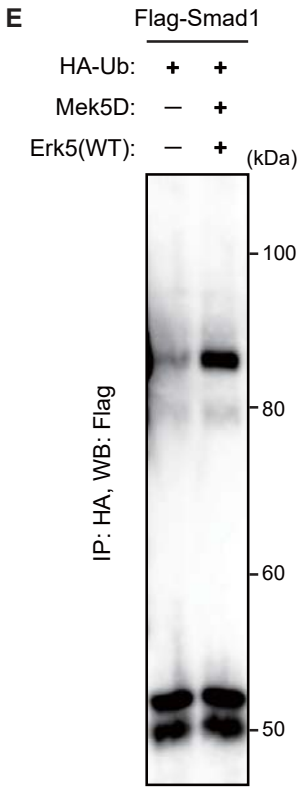
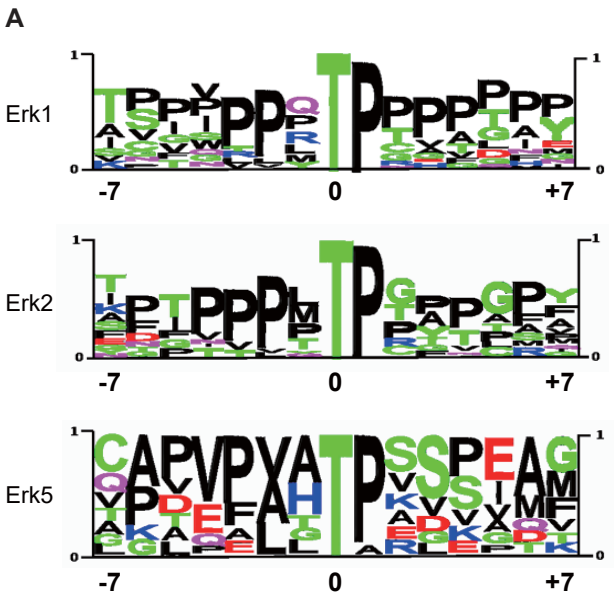
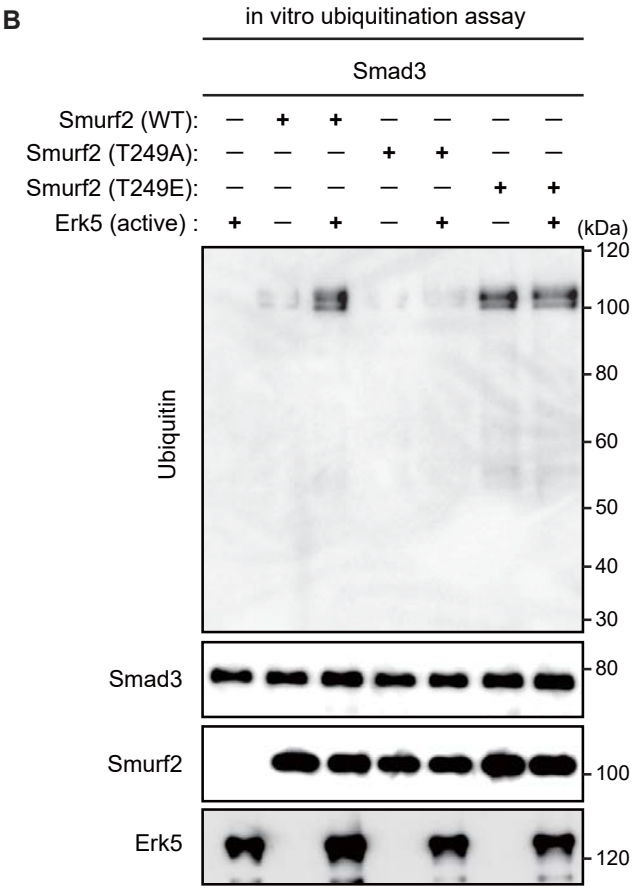


Figure S6. Erk5 modulates stability of Smad proteins. (A-C) Quantification of immunoblot data of Figure 2E. (D) HEK293 cells were transfected with HA-Ub in the presence of Mek5D and/or Erk5(WT) expression vectors without Flag-Smads, and subsequent IP with anti-Flag antibody, followed by immunoblotting with anti-HA antibody (n=3). (E) HEK293 cells were transfected with HA-Ub and Flag-Smads in either the presence or absence of Mek5D and Erk5(WT) expression vectors, and subsequent IP with anti-HA antibody, followed by immunoblotting with anti-Flag antibody. *P<0.05, significantly different from the value obtained in control cells (A-C). Statistical significance was determined using the two-tailed, unpaired Student's *t*-test (A-C).





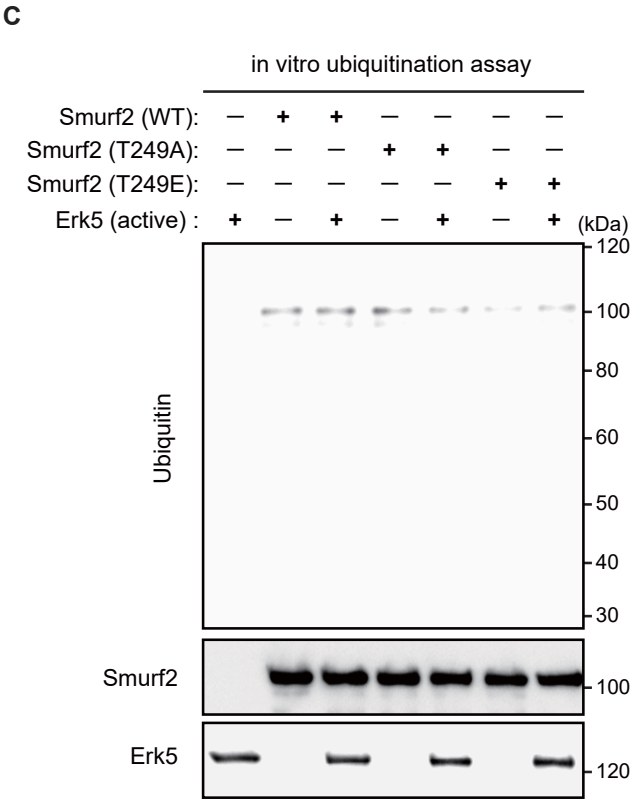


Figure S7. Erk5 directly phosphorylates Smurf2 leading to ubiquitination of Smad proteins. (A) Representative substrate sequence logo of Erk1, Erk2 and Erk5 generated by application from PhosphoSitePlus (www.phosphosite.org/) according to morphology pattern of modification site, which is a spatial combination of specific amino acids. A modification site is defined as the modified residue at 0 position, along with seven flanking amino acids N-terminal (from position -7 to -1) and C-terminal (from position +1 to +7). (B) In vitro ubiquitination assay. Recombinant Smuf2 proteins and Smad3 protein were incubated with active Erk5 in the presence of E1 and UbcH5c, followed by SDS-PAGE (n=4). (C) In vitro ubiquitination assay. Recombinant Smuf2 proteins were incubated with active Erk5 without Smad proteins in the presence of E1 and UbcH5c, followed by SDS-PAGE (n=3).

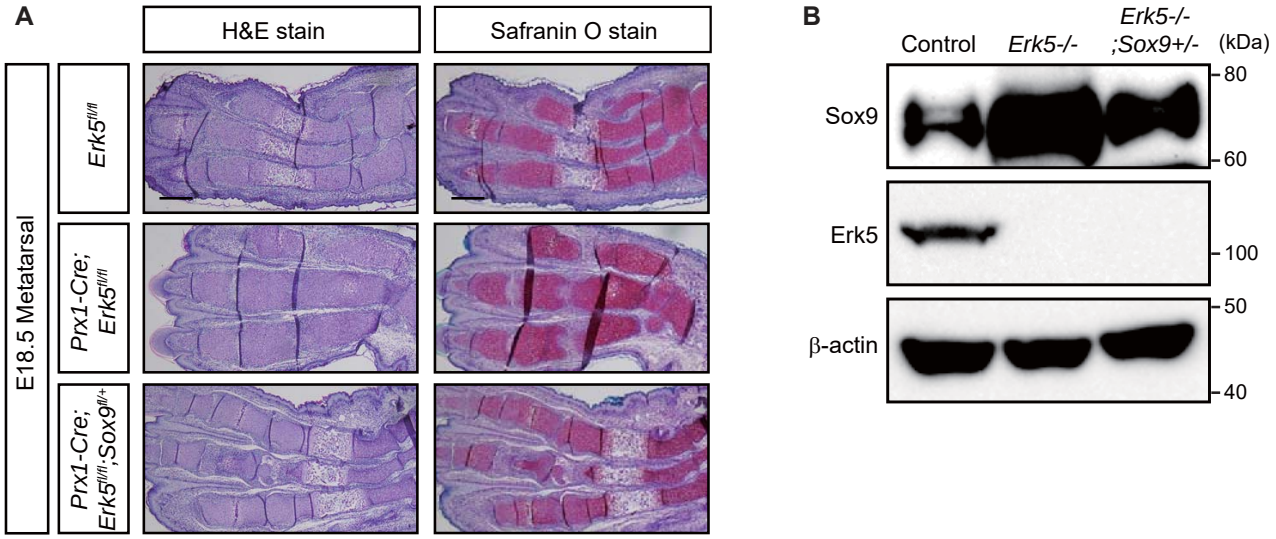


Figure S8. Phenotypes of *Prx1–Cre;Erk5$^{fl/fl}$* and *Col2a1–Cre;Erk5$^{fl/fl}$* embryos and Sox9 protein level in mesenchymal cells. (A) Histological analyses of the metatarsal of *Erk5$^{fl/fl}$*, *Prx1-Cre;Erk5$^{fl/fl}$*, and *Prx1-Cre;Erk5$^{fl/fl}$;Sox9$^{fl/+}$* embryos at E18.5. Metatarsal was stained with H&E and Safranin O. Bar=500 μm. Representative images of histological analyses derived from more than 3 embryos from different litters are shown. (B) Primary mesenchymal cells of *Erk5$^{fl/fl}$*, *Prx1-Cre;Erk5$^{fl/fl}$*, and *Prx1-Cre;Erk5$^{fl/fl}$;Sox9$^{fl/+}$* embryos at E12.5 were isolated, followed by determination of Sox9 protein level (n=3).

Table S1. List of primers used for generating Smurf2 mutant constructs.

Genes	F (5'-3')	R (5'-3')
Smurf2 T249A	TTACATGCTCCTCCAGACCTACCAGAA	TGGAGGAGCATGTAAATGTGTTCTGCT
Smurf2 T249E	TTACATGAACCTCCAGACCTACCAGAA	TGGAGGTTCATGTAAATGTGTTCTGCT

Table S2. List of primers used for real-time PCR.

Genes	Upstream (5'-3')	Downstream (5'-3')
Acan	GAGGAGCTCCAGCACAATATCGA	GGTAGATCTGCAGGGTCGAT
Col2a1	TGGTGGAGCAGCAAGAGCAA	CAGTGGACAGTAGACGGAGGAAA
Col10a1	TGCCCGTGTCTGCTTTTACTGTCA	TCAAATGGGATGGGGGCACCTACT
Mmp13	AGGCCTTCAGAAAAGCCTTC	TCCTTGGAGTGATCCAGACC
Runx2	CCTAGTTAGAGTGGTAGCAGAAGC	ACAGACAACGAAGAAAGTTCCCAC
Smad1	GCTGCCTTAAACAGACAAGCTGG	CCGTGGAGCGGATAAGACAGAAG
Smad2	TGCAACAGTGTGTAAGATCCCACC	GGTTGACAGACTGAGCCAGAAG
Smad3	CACGCAGAACGTGAACACC	GGCAGTAGATAACGTGAGGGA
Smad4	ACACCAACAAGTAACGATGCC	GCAAAGGTTTCACTTTCCCCA
Smad5	ATGAGCTTTGTCAAGGGCTGG	GGAGAGCCCATCTGAGTAAGGAC
Smad6	ATCACCTCCTGCCCCTGT	CTGGGGTGGTGTCTCTGG
Smad7	AAGATCGGCTGTGGCATC	CCAACAGCGTCCTGGAGT
Smad8	ACCAGGACACACAACTCAAACC	GTTCCTTGATGGACGTGGCTG
Sox9	TTTGGGTCTGCCTGGACTGTATGTG	AAGGTCTGTCCGATGTCTCTCTGC

Table S3. List of primers used for ChIP assay.

Fragments	Upstream (5'-3')	Downstream (5'-3')
***Sox9 promoter* BRE (a-b)**	CCAGCTCCGCTTTGACGAGC	CACTTTTCGATGCTGTCTCCGTGG
***Sox9 promoter* SBE (c-d)**	ACCACGGAGACAGCATCGAAAAGT	TTCACACGGAGACCGTTCCAAAACTG

Table S4. List of primers used for generating Smad1 mutant constructs.

Genes	F (5'-3')	R (5'-3')
Smad1 S11A	TTTACAGCTCCAGCTGTGAAGAGACTT	AGCTGGAGCTGTAAAGGAAAATAAACT
Smad1 S132A	GTAGAAGCTCCTGTACTTCCTCCTGTG	TACAGGAGCTTCTACTCTCTTATAGTG
Smad1 S187A	CCTCACGCTCCCAATAGCAGTTACCCA	ATTGGGAGCGTGAGGAAACGGGTGGCT
Smad1 S195A	CCAAACGCTCCTGGGAGCAGCAGCAGC	CCCAGGAGCGTTTGGGTAACTGCTATT
Smad1 S206A	CCTCACGCTCCCACCAGCTCAGACCCA	GGTGGGAGCGTGAGGGTAGGTGCTGCT
Smad1 S214A	CCAGGAGCTCCTTTCCAGATGCCAGCT	ACCAGCTCAGACCCAGGAGCTCCTTTC
Smad1 S456A	ATGGGTGCTCCTCATAATCCTATTTCA	ATGAGGAGCACCCATTTGAGTAAGAAC