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Supplementary Information for

Not just for Patterning: *Hox* Genes Function in the Adult Mammalian Skeleton

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Materials and Methods

MicroCT and quantification of images

MicroCT analyses were performed using a lab microCT (Skyscan 1176; Bruker, Billerica, MA, USA) at 9 µm isotropic resolution utilizing a 0.3° rotation step, 0.5 mm aluminum filter, and 2 frame averaging. Data from the microCT scans were processed and analyzed using MicroView (v2.1.2 Advanced Bone Application; GE Healthcare Preclinical Imaging).

Quantification of osteocytes and tdTomato+/SOST+ cells were obtained by taking three 40X images at the distal, medial, and proximal region along the ulna and the cells were manually quantified using ImageJ. An average among the three regions was calculated for each animal. Quantification of the ellipsoid osteocytes were performed by manually quantifying ellipsoid osteocytes and all osteocytes within the normal and abnormal bony matrix in a 40X image of the ulna to calculate the percentage of ellipsoid-shaped osteocytes. Quantification of osteoclasts was performed using the Bioquant Osteo software V17.2.6 (Bioquant Image Analysis Corp., Nashville, TN) according to standard procedures[1].

Skeletal Preparations

E17.5 embryos were skinned and eviscerated, fixed in 100% ethanol overnight then in acetone overnight. Specimens were stained with Alcian blue in a solution containing 15mg Alcian blue (Alcian blue 8GX, Sigma, A5268), 80ml of 95% ethanol and 20ml of glacial acetic acid up to two days. The skeletons were

rinsed in 100% ethanol overnight and cleared in 2% KOH for 3 hrs. The specimens were transferred into alizarin red staining solution with 50mg/L alizarin red (Alizarin Red S, Sigma, A5533) in 2% KOH for 3-5 hrs. The tissue was then cleared in 1% KOH with 20% glycerol and transferred through an increasing glycerol series (20%, 50%, 80%), finally into 100% for long-term storage.

qRT-PCR for *Hoxd11* expression

To analyze deletion of *Hoxd11* following recombination, control and Hox11 conditional mutant were collected after 1-month following the initiation of deletion. The soft tissue from both forelimb zeugopod skeletons were removed and the bone marrow was flushed from both the radius and ulna into 1ml of digestion buffer (2mg/ml of collagenase type IV (Thermo Scientific, 17104-019) and 3mg/ml of dispase II (Thermo Scientific, 17105-041) in 1X PBS) using a 30G needle. The bones were subsequently minced with a razor in a petri dish under a tissue culture hood and the resulting pulp was transferred into digestion buffer with the flushed bone marrow. Three digestion steps were carried out at 37°C with periodic agitation to obtain a single cell suspension. After each period of digestion, cells in suspension were collected into cell culture media containing DMEM with 4.5 g/L D-glucose (Gibco), 1X Glutamax (Gibco), 1mM sodium pyruvate (Gibco), 15% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin. Red blood cells were lysed on ice at a final concentration of 0.5X. The cells were then strained through a 100μM cell strainer and plated. The cells were expanded, passaged twice, then

subsequently sorted by a MA900 (Sony) cell sorter to obtain Hoxa11eGFP-expressing cells. RNA was extracted using the Qiagen RNeasy micro kit (Qiagen, 74004) and cDNA was synthesized using Superscript™ (Thermo Fisher, 11904018). *Hoxd11* expression was measured relative to GAPDH. qPCR was performed with the following primer set using Roche PowerUp™ SYBR™ Green Mastermix: Hoxd11R AGTGAGGTTGAGCATCCGAG, Hoxd11F ACA CCAAGTACCAGATCCGC. ΔCt values were calculated relative to GAPDH.

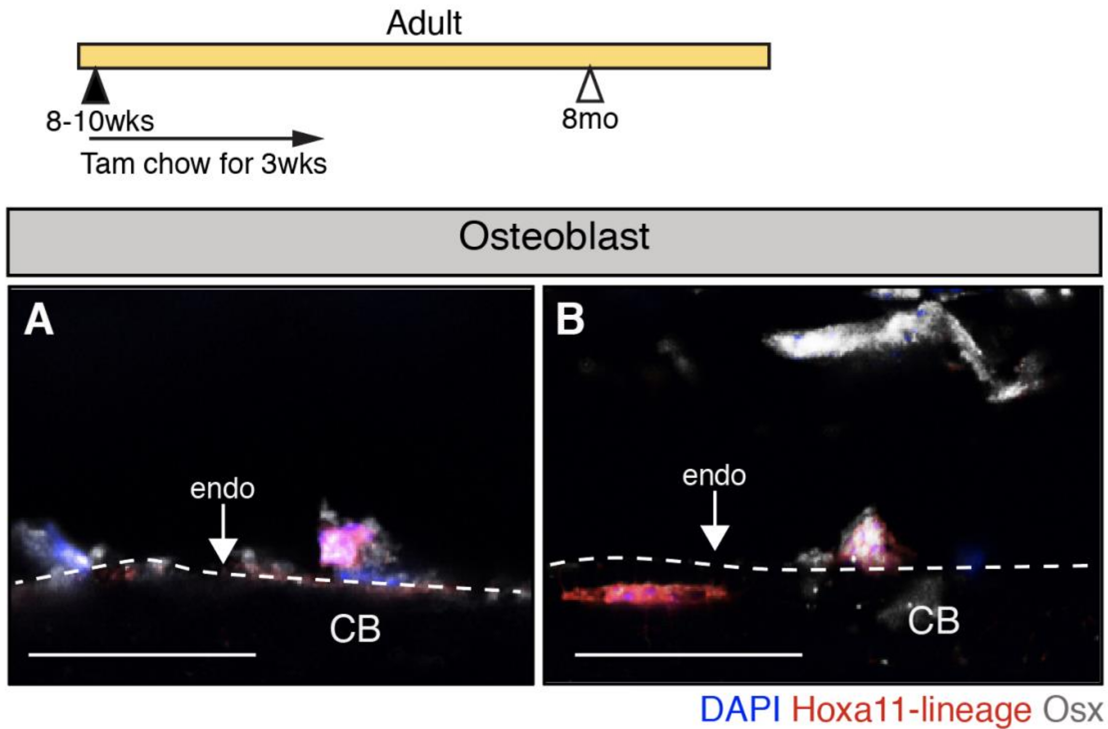


Fig. S1. Adult Hox11-expressing skeletal stem cells continuously contribute to endosteal osteoblasts. Animals harboring the *Hoxa11-CreER$_{T2}$* allele (genotype indicated) were fed on tamoxifen chow for 3 weeks starting at 8-10 weeks of age and collected at 8-months of age. **(A, B)** High magnification images of Hoxa11-lineage marked osteoblasts (red) co-expressing Osx (white) on the endosteal surface. White dashed line demarcates the endosteal surface. DAPI: blue. All images are from the ulna, endo = endosteum, CB = cortical bone. Scale bar, 25μm**.**

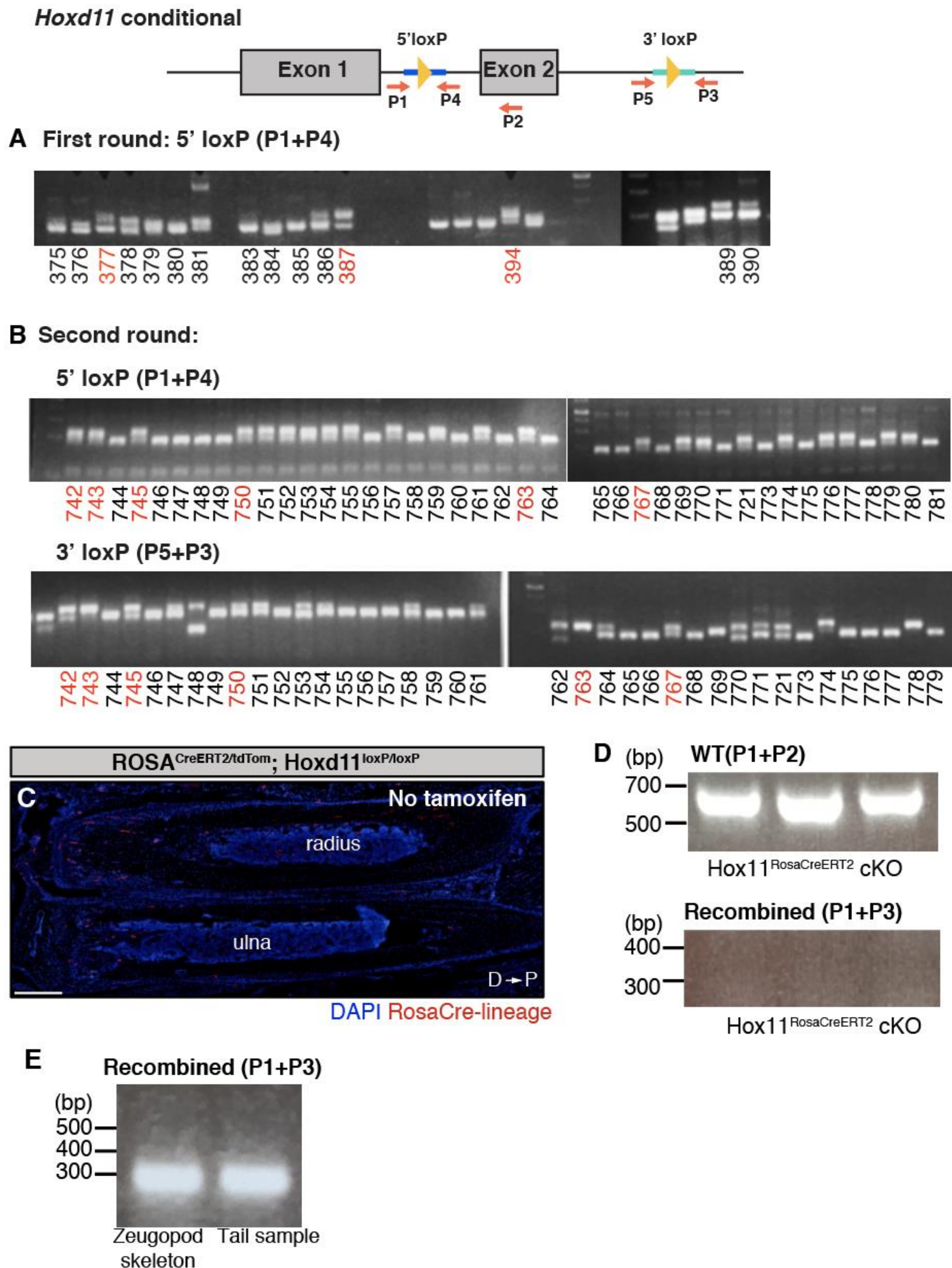


Fig. S2. CRISPR/Cas9 generation of the *Hoxd11* conditional allele. LoxP sites were sequentially targeted both 5' and 3' of *Hoxd11* exon2. Homology sequence used in the donor sequences are highlighted with thick dark blue line (5' loxP) and thick light blue line (3' loxP). Orange arrows mark the approximate location of the PCR primers used to confirm correct targeting. **(A)** PCR

genotyping of founder (F0) animals for insertion of the 5' loxP site. Animals #377, 387, and 394 were sequence verified and male F1 animals were used as stud males for second round of targeting. **(B)** PCR genotyping of F0 animals for insertion at the 5' loxP (top panel) and 3' loxP (bottom panel). Animals # 742, 743, 745, 750, 763 and 767 were heterozygous for 5' loxP and sequence verified to harbor correctly targeted 3' loxP. Animals #742 and 745 were found to be chimeric. Animal #763 contained the loxP sites in *trans*. Animals # 743, 750 and 767 contained the loxP sites in *cis* and #743 was selected to be the final founder. **(C)** 8-week old adult with the genotype $ROSA^{CreERT2/LSL\text{-}tdTomato}$; $Hoxd11^{loxP/loxP}$ without tamoxifen administration shows minimal recombination visualized by ROSA-lineage marked cells (red). DAPI: blue. Scale bar: 200µm. **(D)** PCR analysis shows that the minimal recombination seen in the zeugopod bones do not result in detectable recombined bands (bottom panel) and robust control band (top panel). Three samples are biological replicates of the same genotype. **(E)** Equally strong detection of the recombined PCR band between zeugopod skeleton and tail sample taken from $Hox11^{ROSACreERT2}$ conditional mutants collected at 6 months of age.

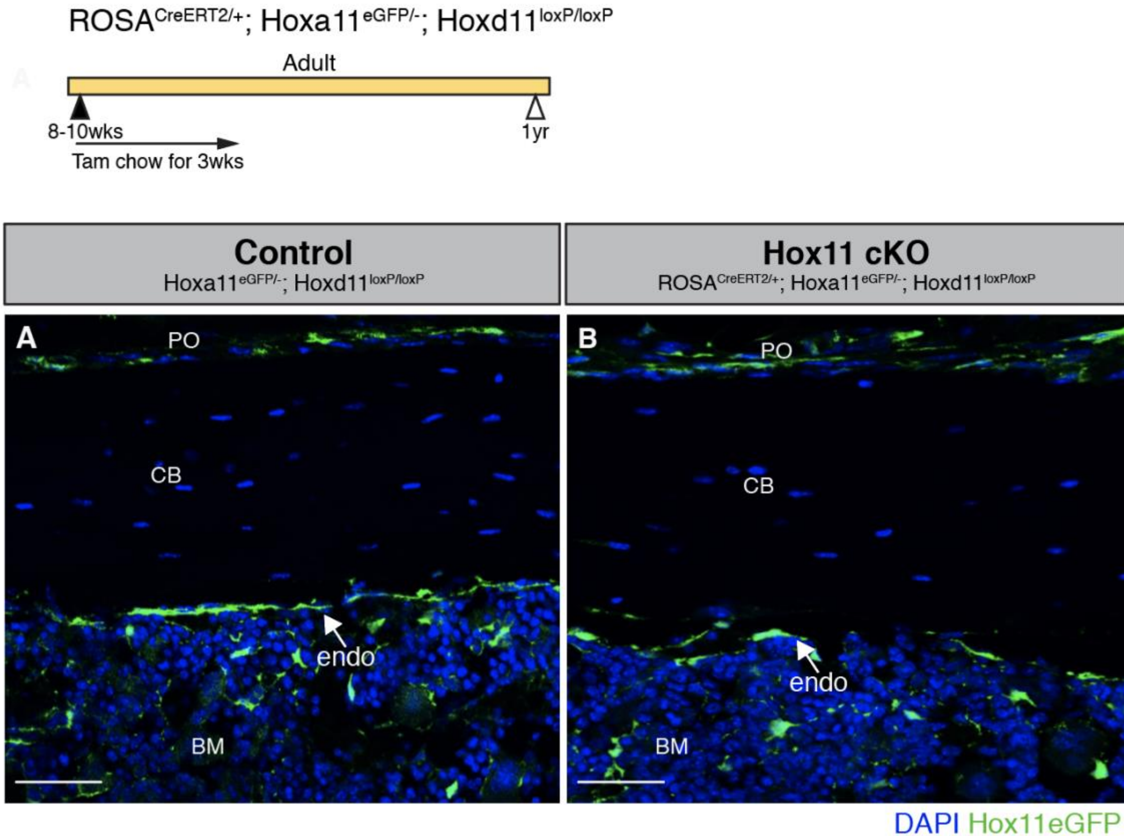
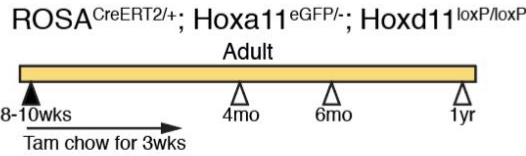


Fig. S3. Hox11-expressing skeletal stem cells are maintained in the Hox11 conditional mutants. Hox11ROSACreERT2 conditional mutants were fed on tamoxifen chow for 3 weeks starting at 8-10 weeks of age and collected at 1-year of age. **(A, B)** Hox11eGFP-expressing skeletal stem cells (green) are present in the expected locations at 1-year of age in both the control (A) and Hox11 conditional mutants (B). All images are from the ulna, PO = periosteum, CB = cortical bone, endo = endosteum, BM = bone marrow. DAPI: blue. Scale bar, 75μm.



A

4 month		6 month		1 year	
Control	Hox11 cKO	Control	Hox11 cKO	Control	Hox11 cKO

B

Parameter measured		4 month Control n=6	4 month Hox11 cKO n=6	6 month Control n=5	6 month Hox11 cKO n=6	1 year Control n=6	1 year Hox11 cKO n=6
Periosteal perimeter (mm)	radius	2.39±0.2	2.47±0.3	2.42±0.07	2.49±0.1	2.46±0.1	2.50±0.09
	ulna	2.9±0.1	2.88±0.1	3.0±0.04	2.87±0.4	3.02±0.1	2.89±0.2
Endosteal perimeter (mm)	radius	1.13±0.1	1.13±0.13	1.15±0.1	1.15±0.2	1.2±0.2	1.12±0.4
	ulna	1.3±0.2	1.27±0.3	1.32±0.1	1.25±0.3	1.30±0.5	1.21±0.07
Marrow area (mm^2)	radius	0.17±0.06	0.18±0.07	0.19±0.02	0.18±0.01	0.21±0.06	0.19±0.08
	ulna	0.15±0.01	0.17±0.01	0.16±0.03	0.15±0.01	0.20±0.05	0.15±0.02
Cortical thickness (mm)	radius	0.29±0.03	0.29±0.1	0.28±0.02	0.29±0.02	0.27±0.02	0.30±0.3
	ulna	0.32±0.05	0.31±0.03	0.30±0.04	0.31±0.06	0.29±0.02	0.32±0.1

Fig. S4. Long-term deletion of Hox11 function at adult stages do not lead to noticeable gross morphological differences. Hox11 conditional mutants with the *ROSA-CreER$_{T2}$* (genotype indicated) allele along with control animals were fed on tamoxifen chow for 3 weeks beginning at 8-10 weeks of age to delete *Hox11* function and evaluated at 4-months, 6-months, and 1 year of age. **(A)** Top panel: 3D rendering from microCT scans of the zeugopod skeleton (radius/ulna) from control and Hox11$_{ROSACreERT2}$ conditional mutants. Bottom panel: Cross

section of the zeugopod skeleton through the distal end at 10% of the entire length of the ulna. **(B)** Table outlining the morphological measurements generated from microCT scans. Statistics by Student’s t test; $*p < 0.05$.

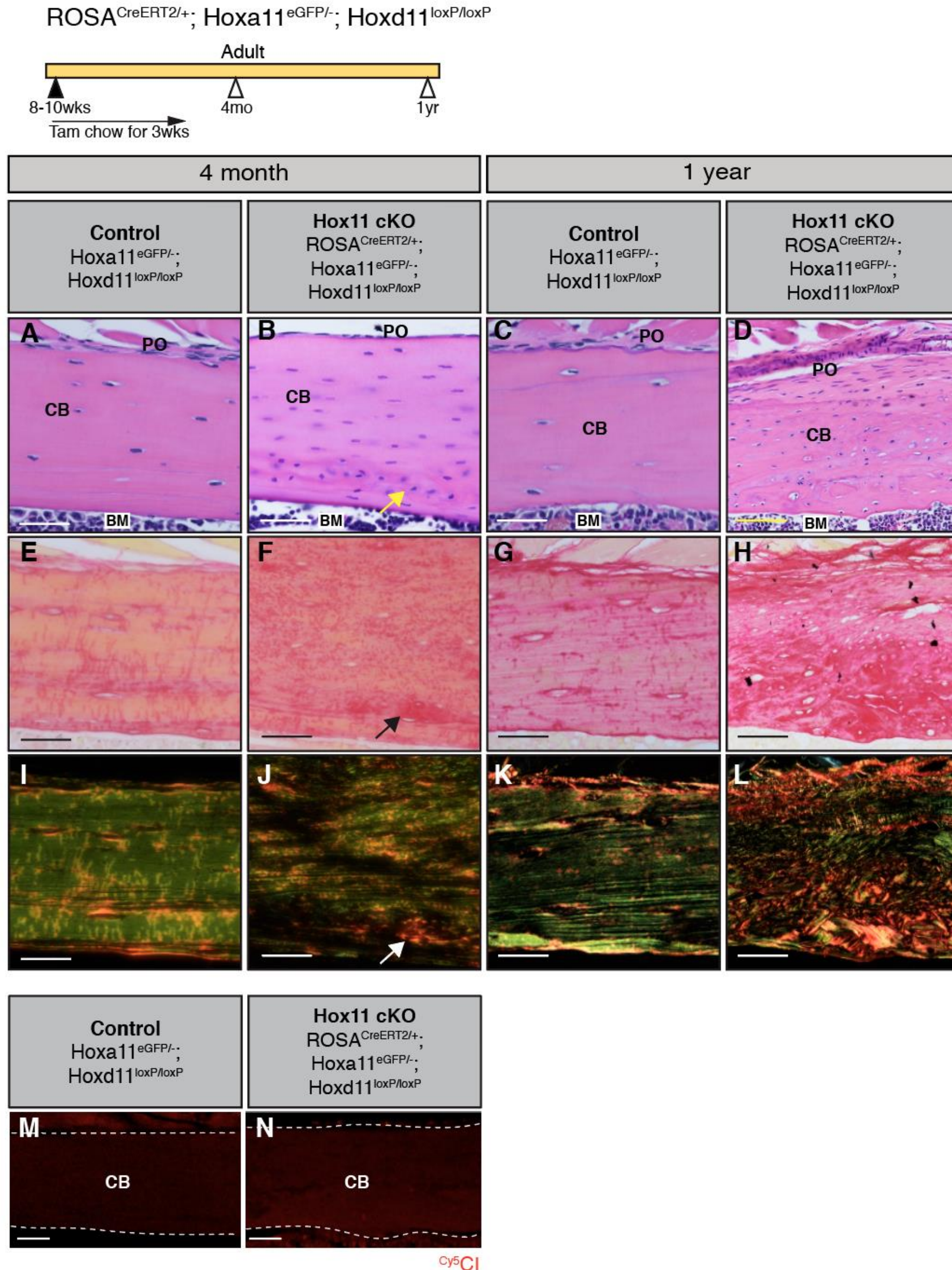


Fig. S5. Adult conditional deletion of Hox11 function lead abnormal matrix at all times points examined. Hox11 conditional mutants with the *ROSA-CreERT2* (genotype indicated) allele along with control animals were fed on tamoxifen chow at 8-10 weeks of age for 3 weeks to delete *Hox11* function and evaluated at 4-months of age, 6-months of age, and 1 year of age. **(A-D)** H&E

stains of control (A, C) and $Hox11_{ROSACreERT2}$ conditional mutant (B, D) at 4-months and 1-year of age. **(E-H)** Brightfield images of picrosirius red stain of consecutive bone sections from A-D. (**I-L)** Polarized light images of picrosirius red stain of bone sections from E-H. Yellow (B), black (F) white (J) arrows highlight the abnormal matrix in the 4-month old conditional mutant bone. **(M, N)** Bone sections from control (M) and $Hox11_{ROSACreERT2}$ conditional mutant (N, 4-months following deletion) were stained with $_{Cy5}$CI. Both bone sections do not show staining. White dashed line marks border of cortical bone. All images are from the ulna, PO = periosteum, CB = cortical bone, BM = bone marrow. Scale bar in all images: 100μm.

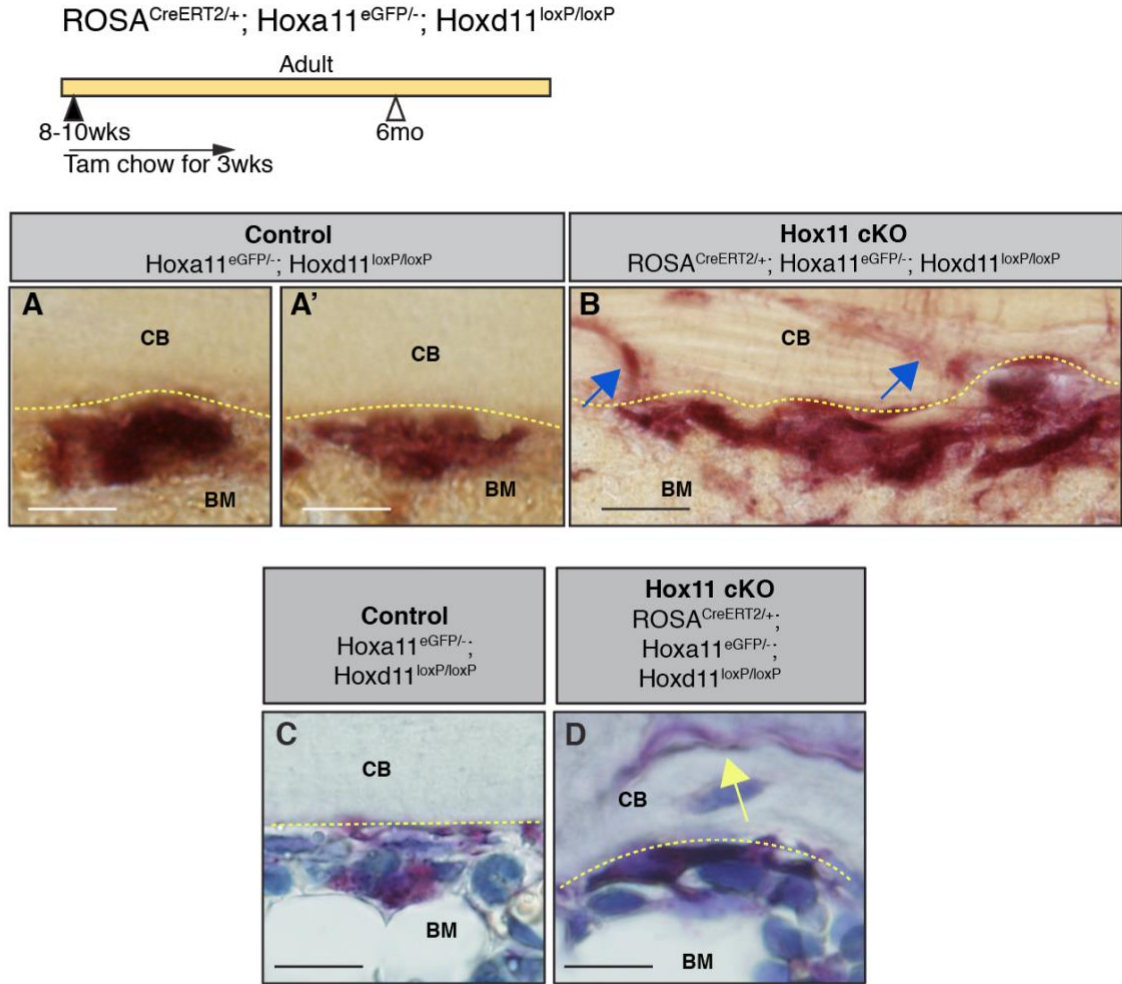


Fig. S6. Distribution of TRAP staining, but not number of osteoclasts is distinct in Hox11 conditional mutant bones. Hox11 conditional mutants with the *ROSA-CreER$_{T2}$* (genotype indicated) allele along with control animals were fed on tamoxifen chow at 8-10 weeks of age for 3 weeks to induce deletion of *Hox11* function and collected at 6-months of age. **(A-B)** Higher magnification of osteoclasts to show apparent TRAP stain leakage into cortical bone matrix. Yellow dashed line demarcates the endosteal bone surface. TRAP enzyme leaked into the bone matrix in mutants (blue arrow). Scale bar: 25μm **(C, D)** TRAP stain with hematoxylin for visualization of nuclei. No cells were associated with TRAP stain in cortical bone (yellow arrow). Yellow dashed line demarcates the endosteal bone surface. Scale bar: 25μm. All images are from the ulna, PO = periosteum, CB = cortical bone, BM = bone marrow.

References

1. Dempster, D. W. *et al.* Histomorphometry Nomenclature: A 2012 Update of the Report of the ASBMR Histomorphometry Nomenclature David. *J. Bone Miner. Res.* **28**, 2–17 (2014).