Supplementary Materials

Deubiquitinating Enzyme USP8 is Essential for Skeletogenesis by Regulating Wnt Signaling

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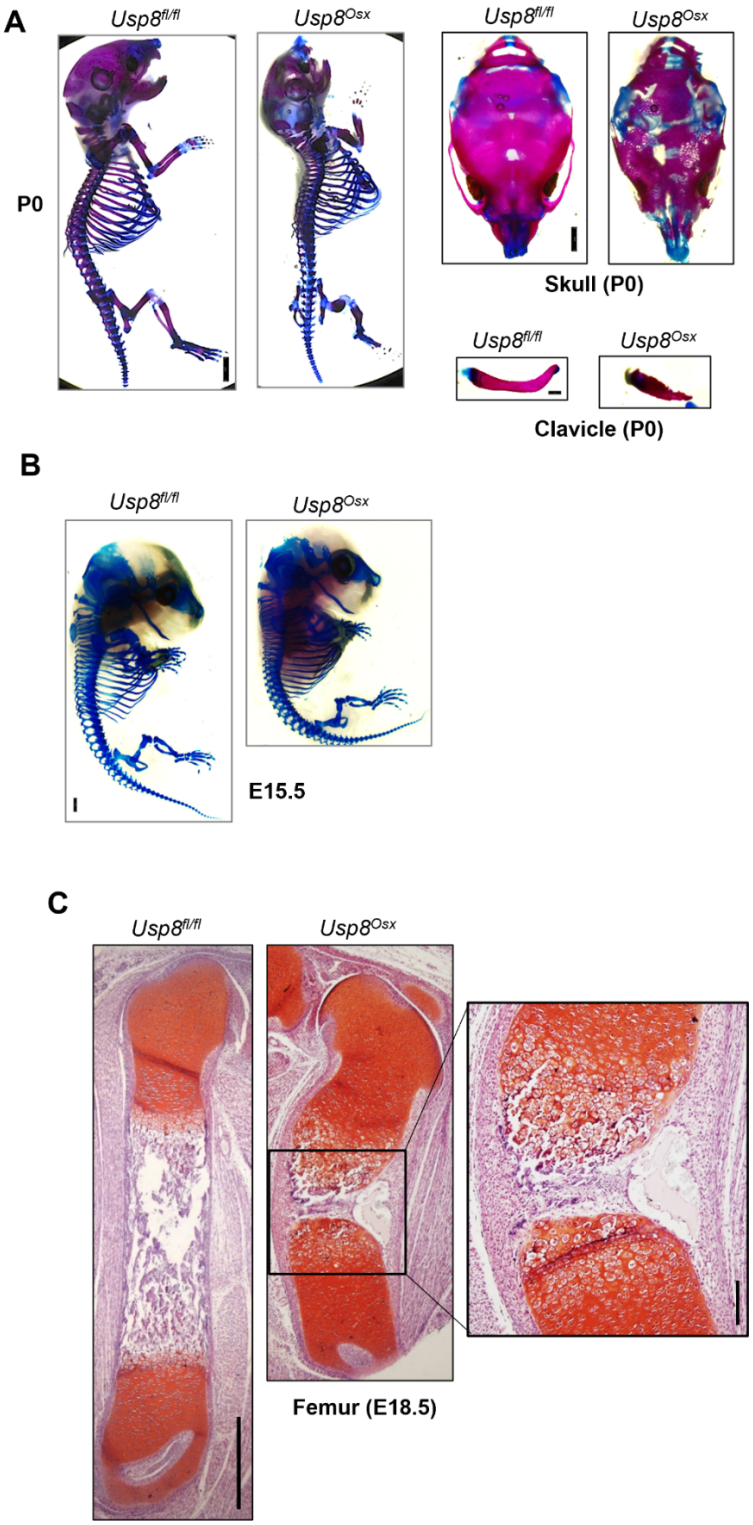
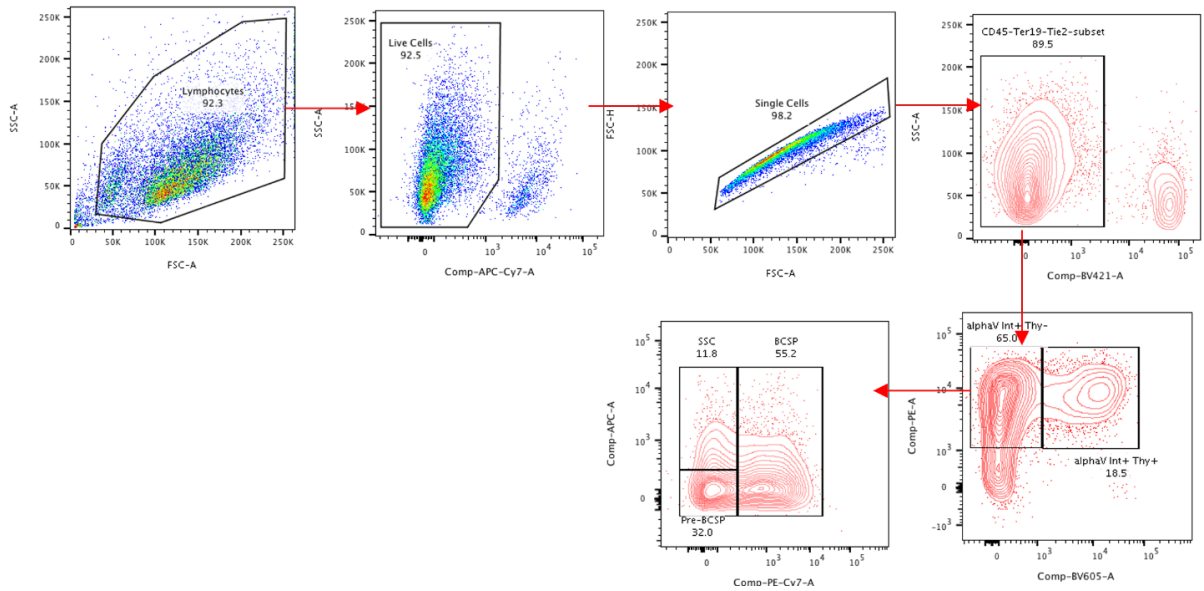
Supplementary Figure S1: *Usp8*^{Osx} mice display impaired ossification during skeletogenesis



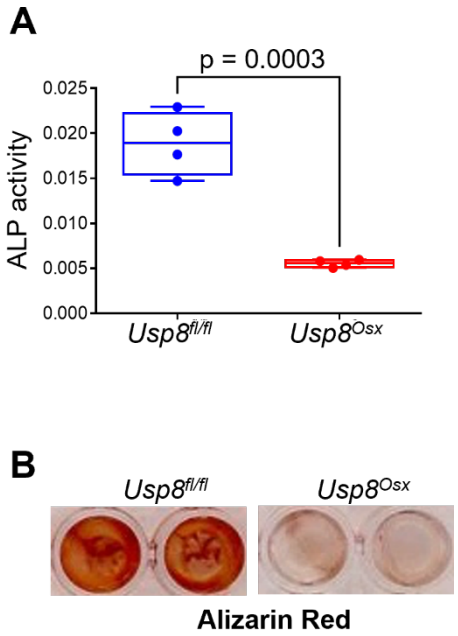
(A) Alizarin red/alcian blue staining of skeletal preparations of the whole body, skulls, and clavicles of P0 *Usp8*$^{fl/fl}$ and *Usp8*^{Osx} embryos. Scale bar, 1 mm. (B) Alizarin red/alcian blue staining of skeletal preparations of the whole body of E15.5 *Usp8*$^{fl/fl}$ and *Usp8*^{Osx} embryos. Scale bar, 1 mm. (C) Safranin O-stained longitudinal sections of E18.5 *Usp8*$^{fl/fl}$ and *Usp8*^{Osx} femurs. Scale bar, 250 µm (left) and 100 µm (right, enlarged inset image).

Supplementary Figure S2: Isolation of mouse SSCs using flow cytometry



(A) Representative flow cytometry dot plots showing the gating strategy to isolate mouse skeletal stem cells (SSCs) from E18.5 *Usp8*$^{fl/fl}$ embryonic limbs. Dot plots showing the different populations while applying the gating strategy, such as BCSP (bone, cartilage, and stromal progenitor, CD45$^-$Ter119$^-$Tie2$^-$αVInt$^+$Thy$^+$CD105$^+$); pre-BCSP (pre-bone cartilage and stromal progenitor, CD45$^-$Ter119$^-$Tie2$^-$αVInt$^+$Thy$^-$CD105$^-$CD200$^-$) and SSC (skeletal stem cell, CD45$^-$Ter119$^-$Tie2$^-$αVInt$^+$Thy$^-$CD105$^-$CD200$^+$). A similar strategy was used to isolate SSCs from E18.5 *Usp8*^{Osx} embryonic limbs.

Supplementary Figure S3: The impaired osteogenic potential of *Usp8*^{Osx} calvarial osteoblasts.



(A, B) Primary calvarial osteoblasts (COBs) isolated from *Usp8*$^{fl/fl}$ and *Usp8*^{Osx} neonates at postnatal day 0 were cultured under osteogenic conditions for 6 days, and ALP activity (A) and alizarin red staining (B) were performed to assess early and late osteoblast differentiation, respectively. Data are shown as a box-and-whisker plot (with median and interquartile ranges) from min to max, with all data points shown. Analysis was performed using the unpaired Student's t-test within Prism.

Supplementary Table S1: Number of offspring derived from the breeding of *Usp8*$^{fl/lf}$ and *Usp8*$^{fl/+}$*;Osx-cre* mice

Age	*Usp8*$^{fl/fl}$	*Usp8*$^{fl/+}$*;Osx*	*Usp8*$^{fl/fl}$*;Osx*	Total
P0	70	23	23	116
P21	124	46	0	170

Supplementary Table S2: Primer Sequences

Gene	Forward	Reverse
Mouse *Usp8*	CGCAATCATCTCCTTCCATT	GATTTGGGAGAAGTAGCCCC
Mouse *Col1a1*	ACTGTCCCAACCCCCAAAG	ACGTATTCTTCCGGGCAGAA
Mouse *Opn*	TGCACCCAGATCCTATAGCC	CTCCATCGTCATCATCATCG
Mouse *Osx*	ATGGCGTCCTCTCTGCTTGA	GAAGGGTGGGTAGTCATTTG
Mouse *Runx2*	TACAAACCATACCCAGTCCCTGTTT	AGTGCTCTAACCACAGTCCATGCA