Supporting Information

Supporting Materials and Methods

Generation of the *Dpp3* KO mice and genotyping

We used the *Cre/lox* technology to generate mice in which *Dpp3* gene was ubiquitously inactivated (*Dpp3* KO). Commercially available, murine embryonic stem cell clones carrying a floxed *Dpp3* allele ($Dpp3^{tm1a(EUCOMM)Hmgu}$, International Knockout Mouse Consortium, European Conditional Mouse Mutagenesis Program EUCOMM) were injected into C57BL/6 blastocysts. Dpp3 floxed mice were generated at the Institute for Research in Biomedicine (IRB, Barcelona, Spain). The chimeric mice thus obtained were crossed with wild-type C57BL/6J mice, to confirm germline transmission of the targeted allele. The progeny was bred with FLPo Deleter mice (Wu et al., 2009) to remove the neomycin-resistance cassette carried by the targeted allele (*Dpp3* CKO mice), and afterward crossed with B6.C-Tg(CMV-cre)1Cgn/J (*Cre*) mice to achieve ubiquitous *Dpp3* gene inactivation. Through all the intervened breedings, we generated a colony of $Dpp3^{+/-}$ mice on a pure C57BL/6J background. After we demonstrated that $Dpp3^{-/-}$ (*Dpp3* KO) mice were fertile, the colony was maintained also at the homozygous state. As control mice, we used $Dpp3^{+/+}$-Cre negative littermates (WT) obtained from $Dpp3^{+/-}$ x $Dpp3^{+/-}$ mating and their own progeny.

Litters were genotyped by PCR amplification of genomic DNA (PCR parameters: 94°C for 5 min, then 35 cycles, 94°C for 30 s, 60°C for 40 s and 72°C for 40 s, then 72°C for 5 min). When crossing *Dpp3* CKO mice with *Cre* mice, PCR amplification with primers: 5'Ufrt: 5′-GCGCCGGAACCGAAGTTCCTA-3′, DR: 5′-AGCTCATACTGTCCCCCTCT-3' generated a 1234 bp amplicon for the mutant CKO allele and a 214 bp one for the mutant KO allele. Cre alleles were identified by PCR for Cre sequences in the following conditions: 95°C for 2 min, then 35 cycles, 95°C for 30 s, 57°C for 30 s and 72°C for 1 min, then 72°C for 5 min, using forward primer 5'-

CGACCAGGTTCGTTCACTCA-3', reverse primer 5'-TGATCCTGGCAATTTCGGCT-3'. When crossing *Dpp3*$^{+/-}$ mice, primers used for PCR amplification were 5'Ufrt, DR and DF (5′-GGTTCCTACGTCTCGTCACC-3′), producing a 342 bp product in the presence of the wild-type (WT) allele and a 214 bp product in the presence of the mutated one.

STimulated Emission Depletion (STED) microscopy

BM cell suspension was obtained from long bones of WT and *Dpp3* KO 6 week old male mice, as described previously in the main text. BM cells were seeded on dentin discs in 96-well plate ($4x10^5$ cells/well) and cultured in osteoclastogenic medium, as described in the main text. At day 6, all the samples were fixed in 4% PFA for 10 min at RT, permeabilised with 0.5% Triton™ X-100 (Sigma Aldrich) in PBS and blocked with PBS 5% FBS for 1 hour at RT. Alexa Fluor 488-Phalloidin (Thermo Fisher Scientific) was diluted 1:40 in blocking buffer and incubated for 30 min at RT. Nuclei were visualized with DAPI (Thermo Fisher Scientific). STED xyz images were acquired with a Leica SP8 STED3X laser scanning confocal microscope system (voxel size, 44 x 44 x 83 nm). Alexa Fluor®488-conjugated Phalloidin was excited with a 488 nm argon laser and emission collected from 496 nm to 605 nm. 660 nm CW-depletion laser was used for STED acquisition. DAPI was acquired in standard confocal settings, using a 405 nm laser for excitation and collecting emission from 410 to 464 nm. Stack sequential acquisition was applied. Images were acquired with a Leica HC PL APO 100x/1.40 oil STED White objective.

Deconvolution was applied to collected image using Huygens Professional software (SVI, Scientific Volume Imaging). Image processing was performed with Imaris (Bitplane) software.

Colony Forming Unit-Osteoblasts culture

BM cell suspensions were obtained by flushing both hind limbs of 5-week old WT or *Dpp3* KO mice with α-MEM supplemented with 15% FBS, 1% P/S and 1% glutamine (OB medium). Cells were seeded (10^6 cells/cm^2 in 6-well plates) and cultured to induce osteoblast colony (CFU-OBs) differentiation in the same culture medium as above, supplemented with 50 μM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone (osteogenic induction medium, OIM; all reagents from Sigma-Aldrich), for 21 days. Then cells were fixed; calcium mineralization was evaluated by Alizarin Red (Sigma Aldrich) staining and quantified according to standard procedures.

Primary calvarial osteoblast culture

Calvariae were dissected from WT and *Dpp3* KO newborn mice (P3-4), cleaned from adherent soft tissues and then sequentially digested in 1x HBSS (EuroClone SpA, Pero, Italy) containing 1 mg/ml collagenase type IV (Sigma-Aldrich), 0,025% Trypsin (EuroClone SpA) and 1% P/S, at 37°C. The cells from digests 2-4 were collected, pooled, and washed, then plated in 6-well plate in OB medium until cells reached confluence.

For osteogenic differentiation, cells were plated in 24-well plates (1.5×10^5 cells/well) and cultured in OIM for 7, 14 and 21 days. At the different time-points, cells were either fixed for the evaluation of calcium mineralization by Alizarin Red staining, or processed for RNA isolation and gene expression analysis of osteogenic marker genes and antioxidant genes, as described.

Moreover, at baseline and after 7 days of osteogenic induction, phosphatidyl serine externalization in WT and *Dpp3* KO cells was assessed by Annexin V/PI staining and FACS analysis.

References

WU, Y., WANG, C., SUN, H., LEROITH, D. & YAKAR, S. 2009. High-efficient FLPo deleter mice in C57BL/6J background. *PLoS One,* 4**,** e8054.

Supporting Figure Legends

Supporting Fig. 1. *Dpp3* KO mice do not display major bone abnormalities at a young age. MicroCT analysis of lumbar vertebrae in WT and *Dpp3* KO mice at 2 months of age. For each evaluation, n≥7 per genotype.

Supporting Fig. 2. *Dpp3* KO mice display mild cortical bone alterations. **a** MicroCT analysis of the femur in WT and *Dpp3* KO mice at 6 months of age. Closed porosity (Po(cl)%); Volume of closed pores (Po.V(cl)); Number of closed pores (Po.N(cl)); Surface of closed pores (Po.S(cl)); Cortical thickness (Ct.Th); Total cross-sectional area (Tt.Ar); Cortical area (Ct.Ar); Cortical area fraction (Ct.Ar/Tt.Ar); Medullary area (Ma.Ar); Periosteal perimeter (Ps.Pm); Endocortical perimeter (Ec.Pm). **b** Stiffness, deflection and fracture load of WT and *Dpp3* KO tibiae and femurs, as assessed through the 3-point-bending test. For each evaluation, n=6 per genotype.

Supporting Fig. 3. a Time-course analysis of *in vitro* osteoclast differentiation from WT and *Dpp3* KO precursors on plastic, at the indicated time-points (n=3 for each genotype) **b** Time-course analysis of Ctsk and Mmp9 expression during osteoclast differentiation from WT and *Dpp3* KO precursors (n=3 for each genotype). **c** Representative images of 3D-STED super-resolution microscopy in WT and *Dpp3* KO osteoclasts differentiated on dentin discs. Left: nuclei stained with DAPI. Middle: actin stained with Phalloidin. Right: merge. Scale bar: 20 μm. **d** qPCR analysis of genes related to the Nrf2 pathway in *in vitro* differentiated WT and *Dpp3* KO osteoclasts. For each evaluation, n=6 per genotype. **e** qPCR analysis of the *Dpp4* gene in total bone (n=3 for each genotype) and in *in vitro* differentiated osteoclasts (n=6 for each genotype) from WT and *Dpp3* KO mice.

Supporting Fig. 4. a Amount of ROS production expressed as MFI of ROS^+ WT and *Dpp3* KO osteoclast precursor cells (OCPs), as assessed by FACS analysis in the presence of the indicated stimuli. **b** Amount of ROS production evaluated as MFI of ROS^+ Ly-$6G^+$ WT and *Dpp3* KO cells as assessed by FACS analysis in the presence of the indicated stimuli; percentage of WT and *Dpp3* KO early apoptotic PMN cells and measurements of ROS production in this cell population, assessed as above. For each evaluation, n=3 per genotype per group. **p*<0.05, ***p*<0.01, ****p*<0.001

Supporting Fig. 5. *Dpp3* KO osteoblasts show increased osteogenesis and altered expression of antioxidant molecules. **a** Representative images of Alizarin Red stained CFU-OBs from WT and *Dpp3* KO mice and corresponding quantification. **b** and **c** qPCR analysis of osteogenic marker genes and antioxidant genes (respectively) in *in vitro* differentiated WT and *Dpp3* KO osteoblasts. **d** Time-course analysis of mineralization by WT and *Dpp3* KO primary osteoblast cultures, as assessed by Alizarin Red staining (representative images for each time-point) and quantification. **e** Time-course analysis of the expression of osteogenic marker genes in WT and *Dpp3* KO primary osteoblast cultures upon osteogenic induction. **f** FACS analysis of Annexin V/PI staining of WT and *Dpp3* KO primary osteoblast cultures in basal condition (OB medium) or after osteogenic induction (OI medium). **g** qPCR analysis of antioxidant genes in WT and *Dpp3* KO primary osteoblast cultures. n=6 per genotype in panels **a**-**c**, n=3 per genotype in panels **d**-**g**. **p*<0.05, ***p*<0.01.

Supporting Fig. 6. a OPG level in the supernatant of co-cultures. **b** Gene expression analysis of selected genes relevant for osteoclast/osteoblast crosstalk. For each experimental condition, n=3.

Supporting Table 1. Primers used for RT-PCR and qPCR analysis

RT-PCR	
Mouse gene	**Primers**
Dpp3	F: 5'-CTGAAGAAGAGTACCAGGCA-3' R: 5'-GCACTGAGGTTCTGAGAGT-3'
Gapdh	F: 5'-TGTCAGCAATGCATCCTGCA-3' R: 5'-TGGATGCAGGGATGATGTTC-3'
qPCR	
Mouse gene	**Primers**
Dpp3	F: 5'-GGCTTCAGTCCTCAACACAGA-3' R: 5'-TCTCTTCAGCTTGGAGGTCAA-3'
Opg	F: 5'-GTTTCCCGAGGACCACAAT-3' R: 5'-CCATTCAATGATGTCCAGGAG-3'
Rankl	F: 5'-GAAGGCTCATGGTTGGATGT-3' R: 5'-GTAGCCCAAGGGTATTTCAG-3'
Ctsk	F: 5'-GAACAGATTCTCAACAGCAGGA-3' R: 5'-GAGACAGAGCAAAGCTCACCA-3'
Mmp9	F: 5'-ACGACATAGACGGCATCCA-3' R: 5'-GCTGTGGTTCAGTTGTGGTG-3'
Nrf2	F: 5'-CATGATGGACTTGGAGTTGC-3' R: 5'-CCTCCAAAGGATGTCAATCAA-3'
Ho-1	F: 5'-AGGCTAAGACCGCCTTCCT-3' R: 5'-TGTGTTCCTCTGTCAGCATCA-3'
Sqstm1	F: 5'-AAAGAAGTGGACCCATCTACAGAG-3' R: 5'-CGCCTTCATCCGAGAAAC-3'
Bach1	F: 5'-TCTGAGACGGACACGGAAG-3' R: 5'-ATTGAATGGCAGCTTCACCT-3'
Sod1	F: 5'-CCATCAGTATGGGGACAATACA-3' R: 5'-GGTCTCCAACATGCCTCTCT-3'
Runx2	F: 5'-CTTCACAAATCCTCCCCAAG-3' R: 5'-GGCGGGACACCTACTCTCAT-3'
Alp	F: 5'-AAGGCTTCTTCTTGCTGGTG-3' R: 5'-GGTGTATCCACCGAATGTGA-3'
Col1α1	F: 5'-CTCCTGGTATTGCTGGTGCT-3' R: 5'-TTCACCAGGAGAACCTTTGG-3'
Spp1	F: 5'-TTTACAGCCTGCACCCAGA-3' R: 5'-CAGATTCATCCGAGTCCACA-3'
Foxo-1	F: 5'-CTTCAAGGATAAGGGCGACA-3' R: 5'-GACAGATTGTGGCGAATTGA-3'
Efnb2	F: 5'-TCTGTGTCATCGGTTGGCTACGTT-3' R: 5'-ACAGACGCACAGGACACTTCTCAA-3'
Ephb4	F: 5'-TGACTGTAAACTCGGTACTCAAGAA-3' R: 5'-GGATGCCACTGATCTTCTAACC-3'
Wnt10b	F: 5'-TTCACGAGTGTCAGCACCA -3' R: 5'-AAAGCACTCTCACGGAAACC-3'
Lrp5	F: 5'-CATGGACATCCAAGTGCTGA-3' R: 5'-TTGTCCTCCTCGCATGGT-3'

Bmp6	F: 5’-GTGACACCGCAGCACAAC-3’ R: 5’-TCGTAAGGGCCGTCTCTG-3’
Sema4D	F: 5’-AAGTGGGTGCGCTACAATG-3’ R: 5’-TCAAGGAGCTGGTGTAGTTGG-3’
Plxnb1	F: 5’-GGTTGGAGACCAGCCTTG-3’ R: 5’-CCGGTCTCACAGTGTAGCTG-3’
Wnt5a	F: 5’-CAAATAGGCAGCCGAGAGAC-3’ R: 5’-CTCTAGCGTCCACGAACTCC-3’
Ror2	F: 5’-CCCAACTTCTACCCAGTCCA-3’ R: 5’-TGTCCGCCACAGATGTATTG-3’
Gapdh	F: 5’- AGGTCGGTGAACGGATTTG-3’ R: 5’-TGTAGACCATGTAGTTGAGGTCA-3’

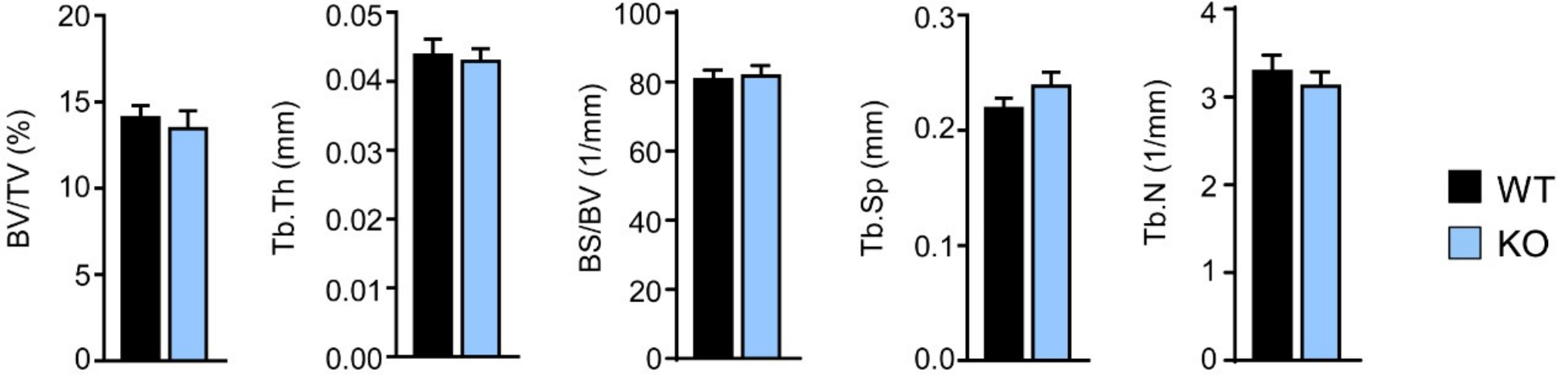
Supporting Table 2. MicroCT analysis and data comparison in SHAM and OVX WT and *Dpp3* KO mice.

	SHAM		OVX	
Parameters	WT	KO	WT	KO
BV/TV (%)	11.87±2.01	9.40±2.51	9.73±1.31	7.33±0.73
Tb.Th (mm)	0.046±0.004	0.043±0.005	0.042±0.003	0.039±0.001
BS/BV (1/mm)	76.17±6.89	82.01±9.67	80.95±5.76	87.79±2.26
Tb.Sp (1/mm)	0.30±0.04	0.36±0.06	0.33±0.06	0.40±0.03
Tb.N (1/mm)	2.59±0.39	2.15±0.43	2.32±0.25	1.87±0.15

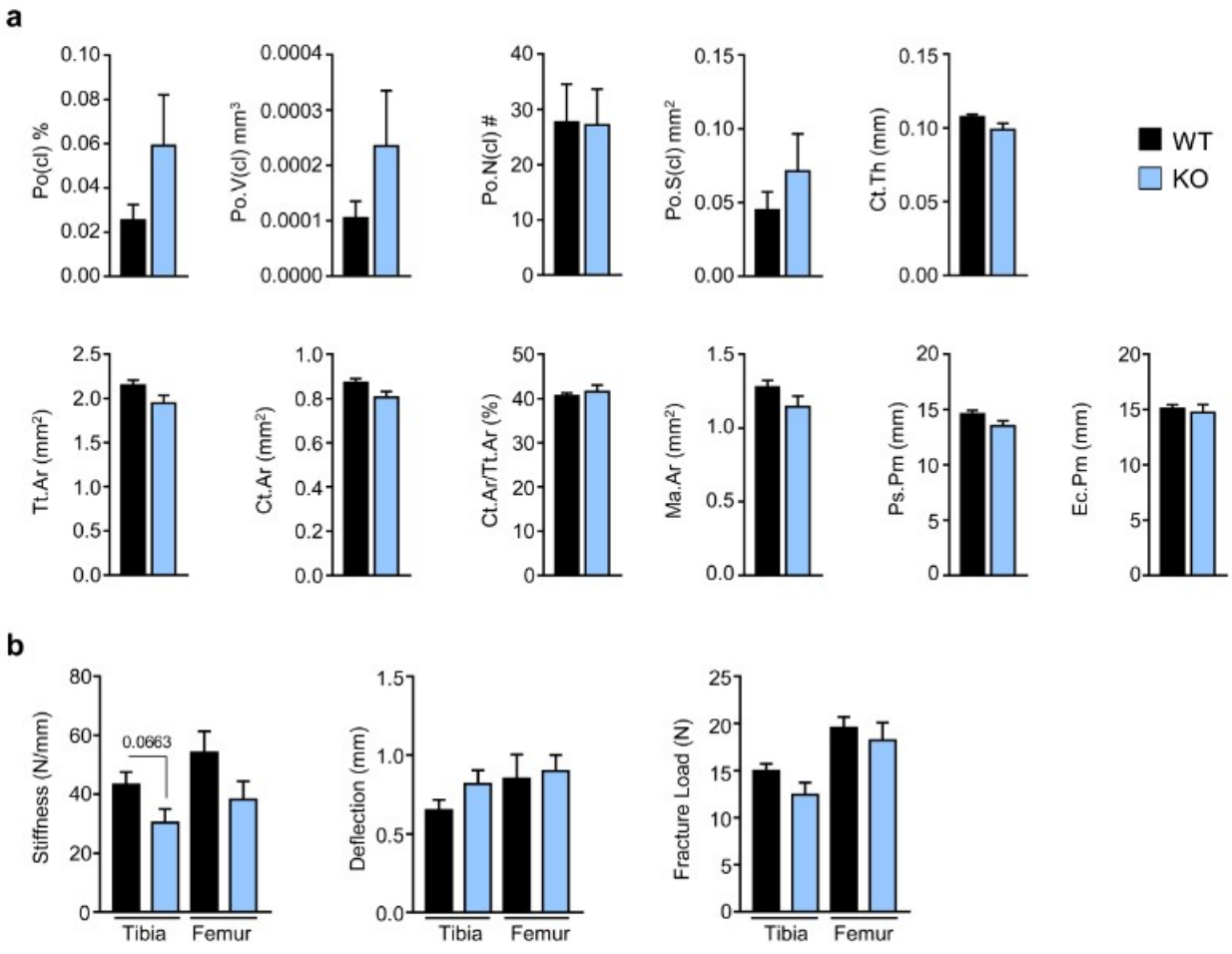
Comparisons	***p* value**
WT SHAM vs KO SHAM	0.1
WT OVX vs KO OVX	0.0022**
WT SHAM vs WT OVX	0.0487*
KO SHAM vs KO OVX	0.0772
WT SHAM vs KO SHAM	0.3495
WT OVX vs KO OVX	0.039*
WT SHAM vs WT OVX	0.0823
KO SHAM vs KO OVX	0.0568
WT SHAM vs KO SHAM	0.2763
WT OVX vs KO OVX	0.0199*
WT SHAM vs WT OVX	0.2194
KO SHAM vs KO OVX	0.1824
WT SHAM vs KO SHAM	0.0801
WT OVX vs KO OVX	0.0276*
WT SHAM vs WT OVX	0.3348
KO SHAM vs KO OVX	0.1723
WT SHAM vs KO SHAM	0.0998
WT OVX vs KO OVX	0.0024**
WT SHAM vs WT OVX	0.1658
KO SHAM vs KO OVX	0.1538

All data are expressed as mean ± SD; *p<0.05; **p<0.01.

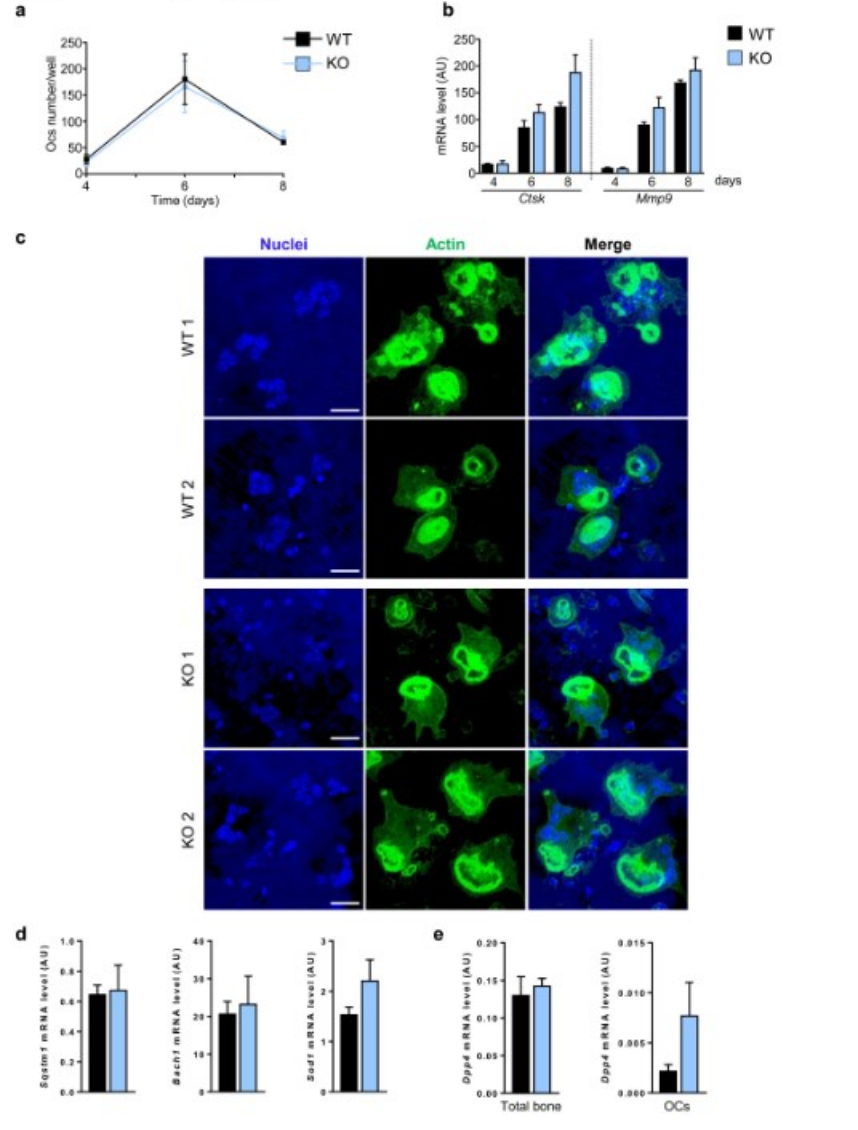
Supplementary Fig 1_R



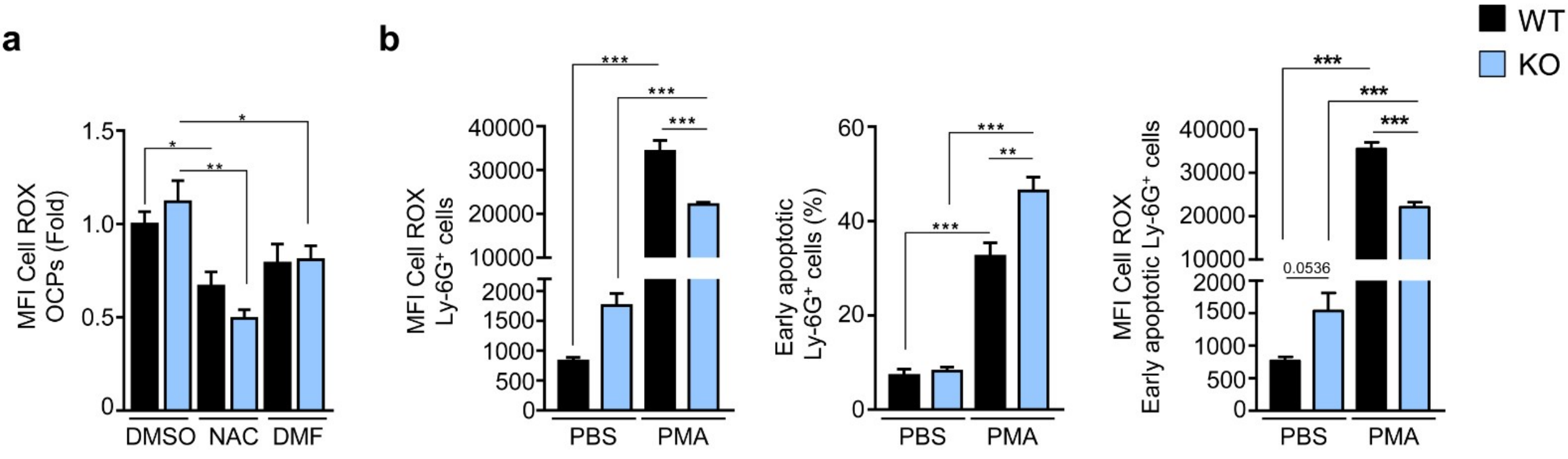
Supplementary Fig 2_R



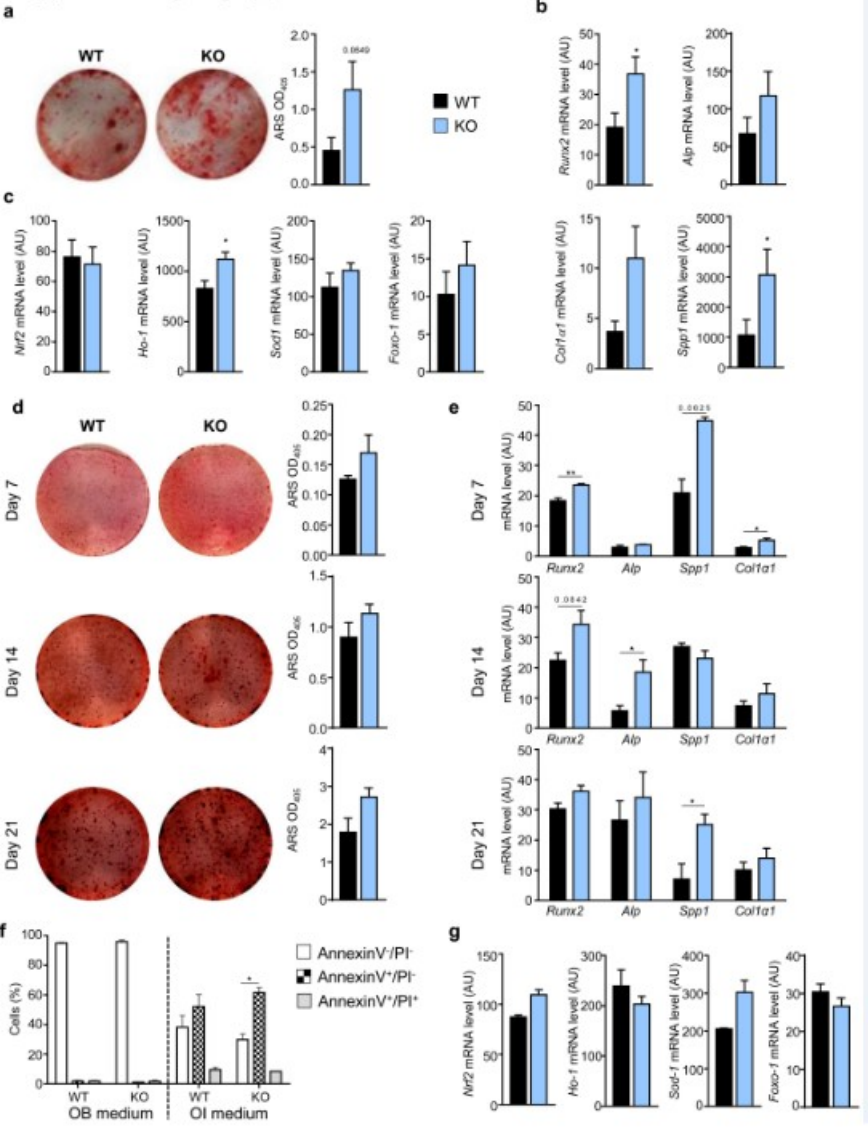
Supplementary Fig 3_R



Supplementary Fig 4_R



Supplementary Fig 5_R



Supplementary Fig 6_R