Supplementary Information

Regulation of sclerostin by the SIRT1 stabilization pathway in osteocytes

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Running title: A CK2/USP4/SIRT1 pathway regulates sclerostin expression in osteocytes.

Supplementary Table 1

Construct	Sequence
$(AspSerSer)_6$	GATTCATCAGATTCTTCTGATTCATCCGACTCTTCTGACAGTTCAGACAGC TCT
amiR-33-Ctrl (*amiR-Ctrl*)	TTTGTCTTTTATTTCAGGTCCCAGATCTAGGGCTCTGCGTTTGCTCCAGGTA GTCCGCTGCTCCCTTGGGCCTGGGCCCACTGACAGCCCTGGTGCCTCTGGC CGGCTGCACACCTCCTGGCGGGCAGCTGTGTACAAACTACTTGAGAGCAG GTGTTCTGGCAATACCTGCCTGCTCTGTAATAGTTTGTACACGGAGGCCTG CCCTGACTGCCCACGGTGCCGTGGCCAAAGAGGATCTAAGGGCACCGCTG AGGGCCTACCTAACCATCGTGGGGAATAAGGACAGTGTCACCCCTGCAGG GGATCCGGTGGTGGTGCAAATCA
EGFP-probe	6FAM-CGCGATCACATGGTCCTGCTGG-TAMRA
amiR-33-mSost-1 (*amiR-Sost1*)	tttgtcttttatttcaggtcccAGATCTAGGGCTCTGCGTTTGCTCCAGGTAGTCCGCTGC TCCCTTGGGCCTGGGCCCACTGACAGCCCTGGTGCCTCTGGCCGGCTGCAC ACCTCCTGGCGGGCAGCTGTGAcaagtaggcagatgaggcacTGTTCTGGCAATACC TGGTGCCTCAAGTACCTACTTGTCACGGAGGCCTGCCCTGACTGCCCACG GTGCCGTGGCCAAAGAGGATCTAAGGGCACCGCTGAGGGCCTACCTAACC ATCGTGGGGAATAAGGACAGTG TCACCCCTGCAGgggatccggtggtggtgcaaatca
amiR-33-mSost-2 (*amiR-Sost2*)	tttgtcttttatttcaggtcccAGATCTAGGGCTCTGCGTTTGCTCCAGGTAGTCCGCTGC TCCCTTGGGCCTGGGCCCACTGACAGCCCTGGTGCCTCTGGCCGGCTGCAC ACCTCCTGGCGGGCAGCTGTGtgacctctgtggcatcattccTGTTCTGGCAATACCTG GGAATGATCGCGCAGAGGTCACACGGAGGCCTGCCCTGACTGCCCACGGT GCCGTGGCCAAAGAGGATCTAAGGGCACCGCTGAGGGCCTACCTAACCAT CGTGGGGAATAAGGACAGTGTCACCCCTGCAGgggatccggtggtggtgcaaatca

Supplementary Table 2

Gene	Forward	Reverse
Mouse *Csnk2a1*	TATGTGGAGCTTGGGTTGTATG	CAAGATATCGTTGAAACGTGGA
Mouse *Csnk2a2*	CAACAATGAGAGGGTGGTTGTA	TGACACAGGGTCCTTTACAGTG
Mouse *Csnk2b*	TCTTCTGTGAGGTGGATGAAGA	CTCTTCATCAGGTTCCAGGTCT
Mouse *Dmp1*	GAAAGCTCTGAAGAGAGGACGG	CCTCTCCAGATTCACTGCTGTC
Mouse *Sost*	CTTCAGGAATGATGCCACAGAGGT	ATCTTTGGCGTCATAGGGATGGTG
Mouse *Phex*	CTGGCTGTAAGGGAAGACTCCC	GCTCCTAAAAGCACAGCAGTGTC
Mouse *Tnfsf11* (RANKL)	CAGCATCGCTCTGTTCCTGTA	CTGCGTTTTCATGGAGTCTCA
Mouse *Tnfrsf11b* (OPG)	CGGAAACAGAGAAGCCACGCAA	CTGTCCACCAAAACACTCAGCC
Mouse *Pdpn*	GAGGAACTGTCCACCTCAGC	CGTTTCATCCCCTGCATTAT
Mouse *Mepe*	GGGAAGGAAACCAGGAGAAG	GGTGTGTTTGGTGTGTTTGC
Mouse *Fgf23*	CGTCATAGCCATTCTCCAGCGT	AACAGGAGCCATGACTCGAAGG
Mouse *Ibsp*	CAGGGAGGCAGTGACTCTTC	AGTGTGGAAAGTGTGGCGTT
Mouse *Bglap*	GCAGCACAGGTCCTAAATAG	GGGCAATAAGGTAGTGAACAG
Mouse *Sp7*	ATGGCGTCCTCTCTGCTTGA	GAAGGGTGGGTAGTCATTTG
Mouse *Col1a1*	ACTGTCCCAACCCCCAAAG	ACGTATTCTTCCGGGCAGAA
Mouse *Axin2*	GCAGATGAACCTGAAGGATACC	TTGATGCCATCTCGTATGTAGG
Mouse *Ctsk*	AGCAGAACGGAGGCATTGACTC	CCCTCTGCATTTAGCTGCCTTTG
Mouse *Nfatc1*	GGTGCCTTTTGCGAGCAGTATC	CGTATGGACCAGAATGTGACGG
Mouse *Acp5*	GCGACCATTGTTAGCCACATACG	CGTTGATGTCGCACAGAGGGAT
Mouse *Sirt1*	GTCTCCTGTGGGATTCCTGA	ACACAGAGACGGCTGGAACT
Mouse *Usp4*	AGCACTGCAAAGTGGAAGTGTA	GAGCTTCCTCATCTCCTTCTCA
Mouse *Rplp0*	TGGCCAATAAGGTGCCAGCTGCTG	CTTGTCTCCAGTCTTTATCAGCTGCAC

Supplementary Figure Legends

Supplementary Fig. 1. The expression of CSNK2B in bone-residing osteoblasts and osteocytes.

Cryosectioned femurs of 8-week-old wildtype mice were co-immunostained for CSNK2B (green) and osteocalcin (red) and or sclerostin (red) to detect mature osteoblasts (a) and osteocytes (b), respectively. DAPI was used for nuclear staining. Arrows indicate CSNK2B-expressing osteocalcin^{+} osteoblasts (a) or sclerostin^{+} osteocytes (b). BM, bone marrow; TB, trabecular bone; CB, cortical bone. Scale bar, 25 μm (a, b). Data are representative of two independent experiments.

Supplementary Fig. 2. The expression of CSNK2B in DMP1-expressing osteoblasts and osteocytes.

(a) GFP-expressing DMP1^{+} osteoblasts and osteocytes in the cryosectioned femurs of eight-week-old *Dmp1;Rosa26*$^{mT/mG}$ mice were analyzed by fluorescence microscopy. DAPI was used for nuclear staining. Scale bar, 1 mm (left) and 400 μm (right).

(b) Cryosectioned femurs of 8-week-old *Dmp1;Rosa26*$^{mT/mG}$ mice were stained for CSNK2B (red) and CSNK2B-expressing GFP^{+} osteoblasts and osteocytes were analyzed by fluorescence microscopy. Arrows indicate CSNK2B-expressing DMP1^{+} osteoblasts and osteocytes that reside in the cortical bone (CB). Scale bar, 25 μm. Data are representative of two independent experiments.

Supplementary Fig. 3. Histologic analyses for femoral bones of *Csnk2b*$^{fl/fl}$ and *Csnk2b*^{Dmp1} mice.

(a) H&E staining of femoral sections of 8-week-old *Csnk2b*$^{fl/fl}$ and *Csnk2b*^{Dmp1} male mice. Scale bar, 50 μm.

(b) Immunohistochemistry for cathepsin k in 8-week-old *Csnk2b*$^{fl/fl}$ and *Csnk2b*^{Dmp1} femurs. Arrow heads indicate cathepsin k^{+} mature osteoclasts. M, muscle; CB, cortical bone. Scale bar, 50 μm. Data are representative of two independent experiments.

Supplementary Fig. 4. The expression of osteocyte differentiation genes in *Csnk2b*$^{fl/fl}$ and *Csnk2b*^{Dmp1} bones.

mRNA levels of osteocyte marker genes in 8-week-old *Csnk2b*$^{fl/fl}$ and *Csnk2b*^{Dmp1} tibias. Data are representative of three independent experiments. A two-tailed unpaired Student's t-test for comparing two groups (error bars, SD of biological replicates).

Supplementary Fig. 5. Effect of a CK2 inhibitor on osteocyte differentiation *in vitro*.

(a) Immunoblot for phospho-CK2 substrates in Ocy454 cells treated with vehicle (DMSO) or 0.1 μM of a CK2 inhibitor.

(b) mRNA levels of osteocyte marker genes in Ocy454 cells treated with vehicle (DMSO) or 0.1 μM of a CK2 inhibitor 6 days after osteocyte differentiation culture. Data are representative of two

independent experiments. A two-tailed unpaired Student's t-test for comparing two groups (b; error bars, SD of biological replicates).

Supplementary Fig. 6. Effects of *Csnk2b* deficiency on late stage differentiation of mature osteoblasts.

(a) Diagram of the study and treatment methods.

(b) mRNA levels of osteoblast genes in mature *Csnk2b*$^{fl/fl}$ calvarial osteoblasts (COBs) 6 days after osteogenic differentiation.

(c, d) *Csnk2b*$^{fl/fl}$ COBs were transduced with lentivirus encoding vector control (WT) or CRE recombinase (Δ*Csnk2b*) 6 days after osteogenic culture, and then further differentiated up to 18 days. Mineralization by alizarin red staining with quantification (c) and osteogenic markers expression (d) were determined. Data are representative of two independent experiments. Ordinary one-way ANOVA with Sidak's multiple comparisons test (b) or a two-tailed unpaired Student's t-test for comparing two groups (c, d) (b-d; error bars, SD of biological replicates).

Supplementary Fig. 7. *Csnk2b*^{Dmp1} mice display low bone mass.

MicroCT analysis showing a decrease of femoral bone mass in 8-week-old *Csnk2b*$^{fl/fl}$ and *Csnk2b*^{Dmp1} male (a; n=7) and female (b, c; n=5) mice. 3D-reconstruction images (a, b) and quantification (c) are displayed. Trabecular bone volume/total volume (Tb. BV/TV), trabecular thickness (Tb. Th), and cortical thickness (C. Th) were measured. Scale bar, 500 μm (a, b). A two-tailed unpaired Student's t-test for comparing two groups (c; error bars, SD of biological replicates).

Supplementary Fig. 8. Paracrine effects of *Csnk2b*-deficient osteoblasts on osteoblast and osteoclast differentiation.

The CM harvested from *WT* or and Δ*Csnk2b* mature osteoblasts (described in Supplementary Fig. 6a) was added to osteogenic culture of wildtype bone marrow stromal cells (BMSCs) and 6 days later, ALP activity (a) and osteogenic gene expression (b) were analyzed. Alternatively, the CM was added to osteoclastogenic culture of wildtype bone marrow monocytes (BMMs) and 6 days later, osteoclast differentiation was assessed by TRAP staining (c), TRAP activity (d), and osteoclast gene expression (e). Scale bar, 200 μm (c). Data are representative of two independent experiments. A two-tailed unpaired Student's t-test for comparing two groups (a, b, d, e; error bars, SD of biological replicates).

Supplementary Fig. 9. The expression of osteocyte differentiation genes in *Csnk2b*-deficient osteocytes.

mRNA levels of osteocyte marker genes in *shScr* or *shCsnk2b*-expressing Ocy454 cells 6 (a) or 18 (b) days after osteocyte differentiation culture were assessed by RT-PCR. Data are representative of three independent experiments. A two-tailed unpaired Student's t-test for comparing two groups (a, b; error bars, SD of biological replicates).

Supplementary Fig. 10. OPG-Fc treatment reverses the paracrine effects of *Csnk2b*-deficient osteocytes on osteoclast differentiation.

(a-c) The conditioned medium (CM) was harvested from *shScr*- or *shCsnk2b*-expressing Ocy454 cells and added to osteoclastogenic culture of wildtype BMMs in the absence or presence of OPG-Fc. 6 days later, osteoclast differentiation was assessed by TRAP staining (a), TRAP activity (b), and osteoclast gene expression (c). Scale bar, 200 μm (a). Blue arrows, $TRAP^+$ multinucleated osteoclasts.

(d, e) Alternatively, the CM was added to osteogenic culture of wildtype BMSCs in the absence or the presence of OPG-Fc. 6 days later, ALP activity (d) and osteogenic gene expression (e) were analyzed. Data are representative of two independent experiments. A two-tailed unpaired Student's t-test for comparing two groups (b, c, d [*shScr* vs. s*hCsnk2b*], e) or ordinary one-way ANOVA with Sidak's multiple comparisons test (d [vehicle vs. OPG-Fc]) (b-e; error bars, SD of biological replicates).

Supplementary Fig. 11. Effects of a CK2 or USP4 inhibitor on SIRT1 ubiquitination.

HEK293T cells expressing Flag-*SIRT1* and His-ubiquitin were treated with vehicle (DMSO), 0.1 μM CK2 inhibitor (a) or 100 μM USP4 inhibitor (b). 2 days later, cells were treated with 10 μM MG132 for 6 hours and then lysed, pull-downed with Ni-NTA agarose, and immunoblotted with anti-SIRT1 antibody. Alternatively, Ni-NTA fractions were immunoblotted with an anti-ubiquitin antibody as a loading control.

Supplementary Fig. 12. Identification of SIRT1-binding deubiquitinating enzymes.

HEK293T cells expressing were transiently transfected with Flag-tagged DUBs and HA-ubiquitin and 2 days later, cells were treated with 10 μM MG132 for 6 hours. Cells were lysed, pull-downed with Flag-conjugated agarose, and immunoblotted with SIRT1. Input controls were immunoblotted with FLAG or SIRT1.

Supplementary Fig. 13. Loading control for total ubiquitinated proteins in *USP4*- or *USP22*-overexpressing cells.

The Ni-NTA fractions used for Fig. 6b, c, and f were immunoblotted with anti-ubiquitin antibody as loading controls.

a

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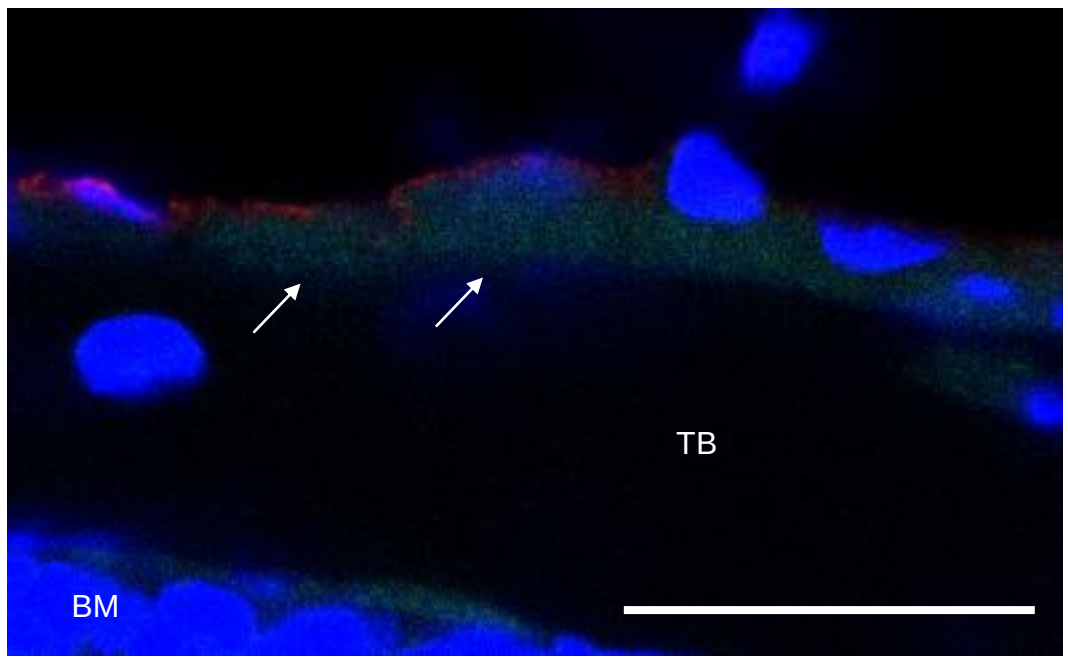

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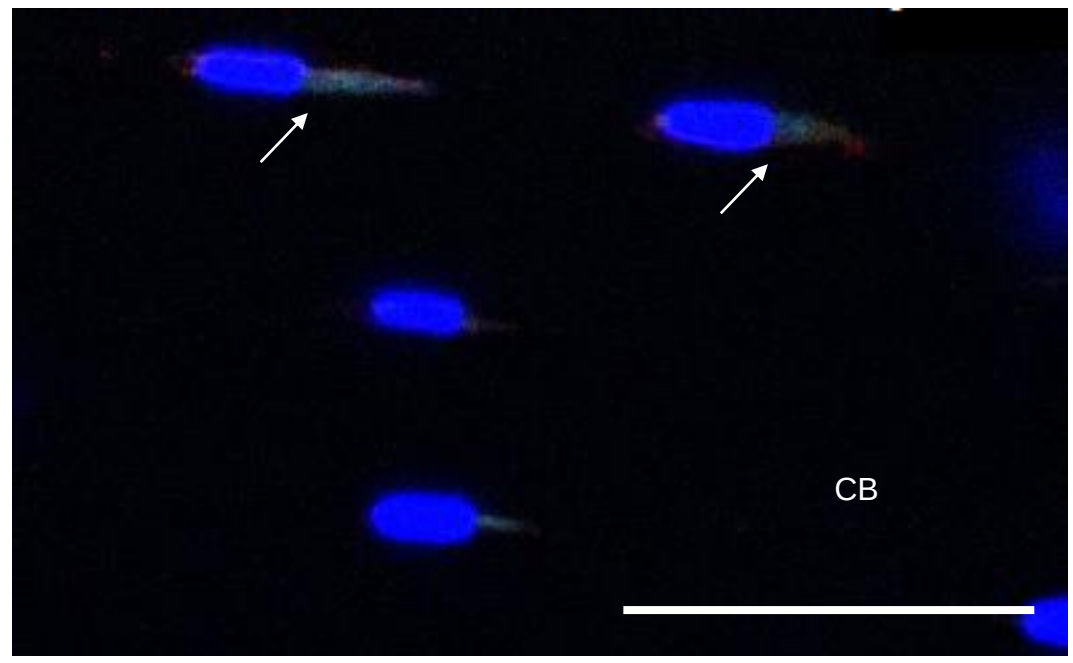
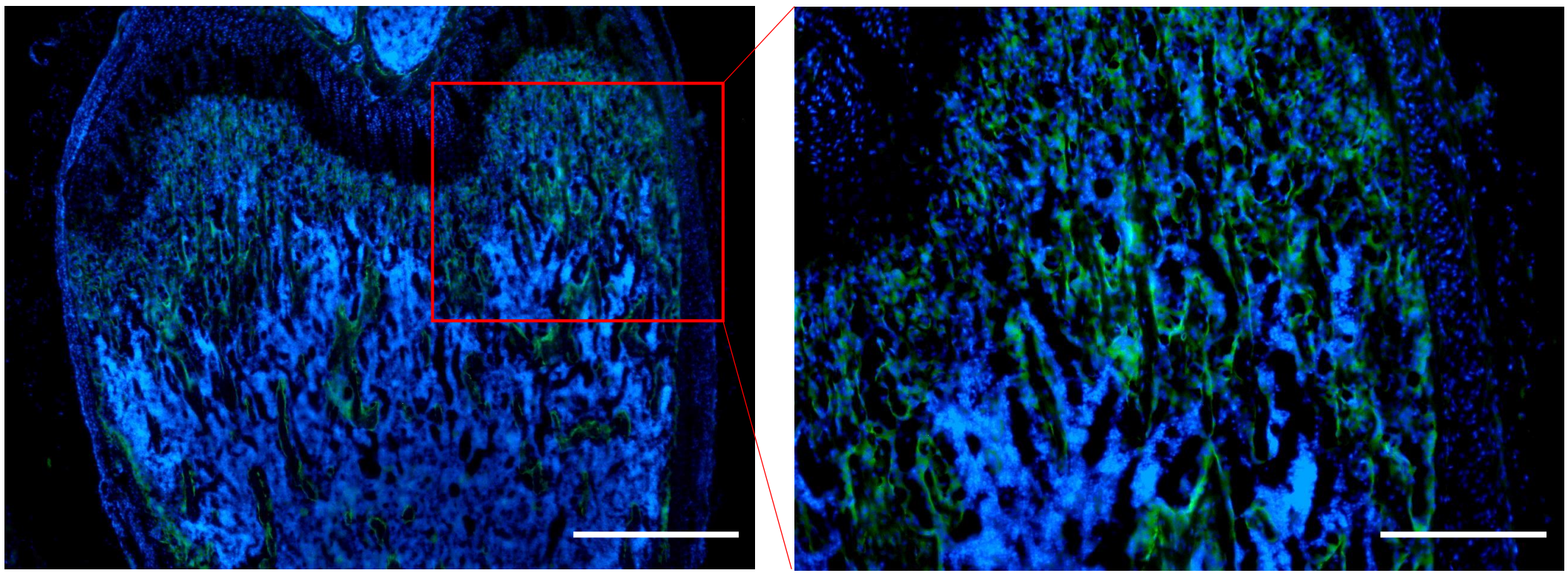
IF:CSNK2B/Sclerostin



Supplementary Fig. 1

a

Dmp1;Rosa26$^{mT/mG}$



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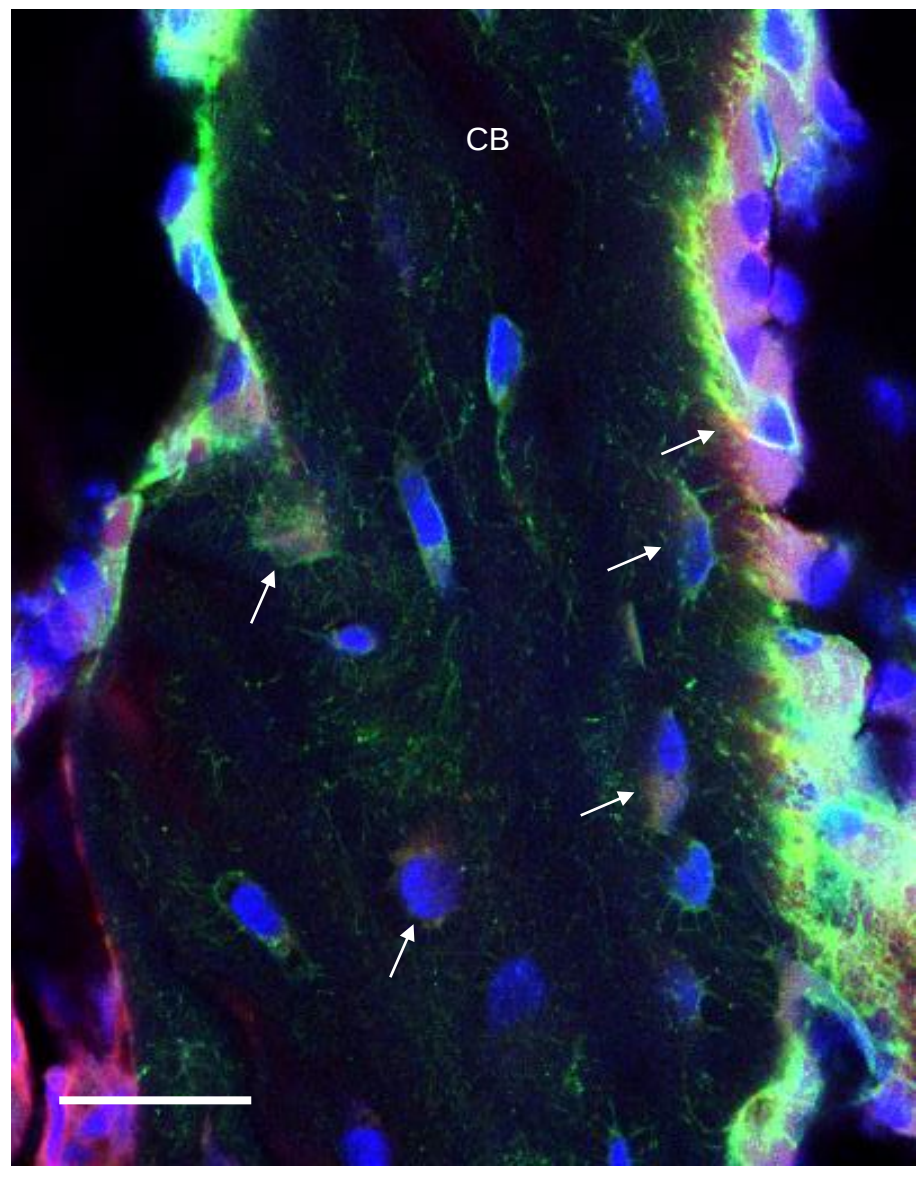
IF: CSNK2B



Supplementary Fig. 2

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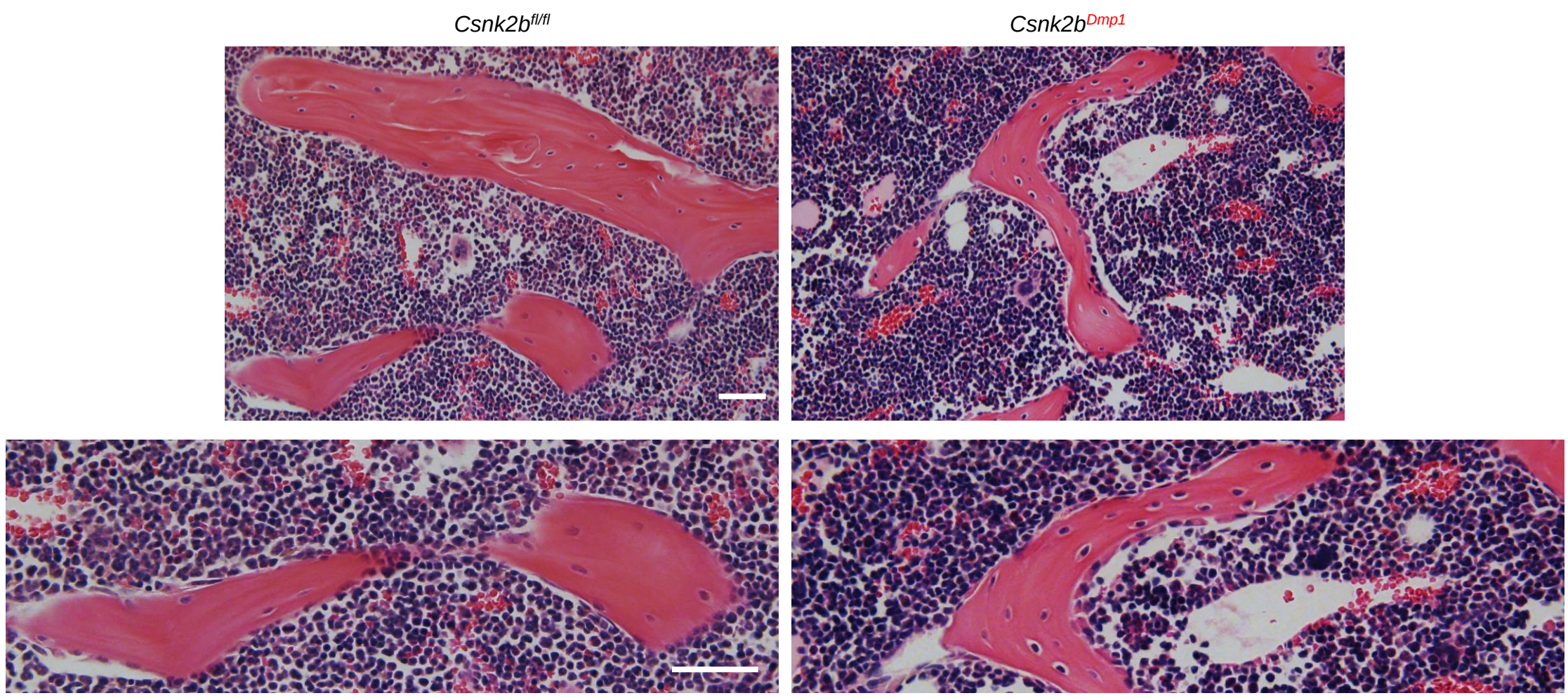

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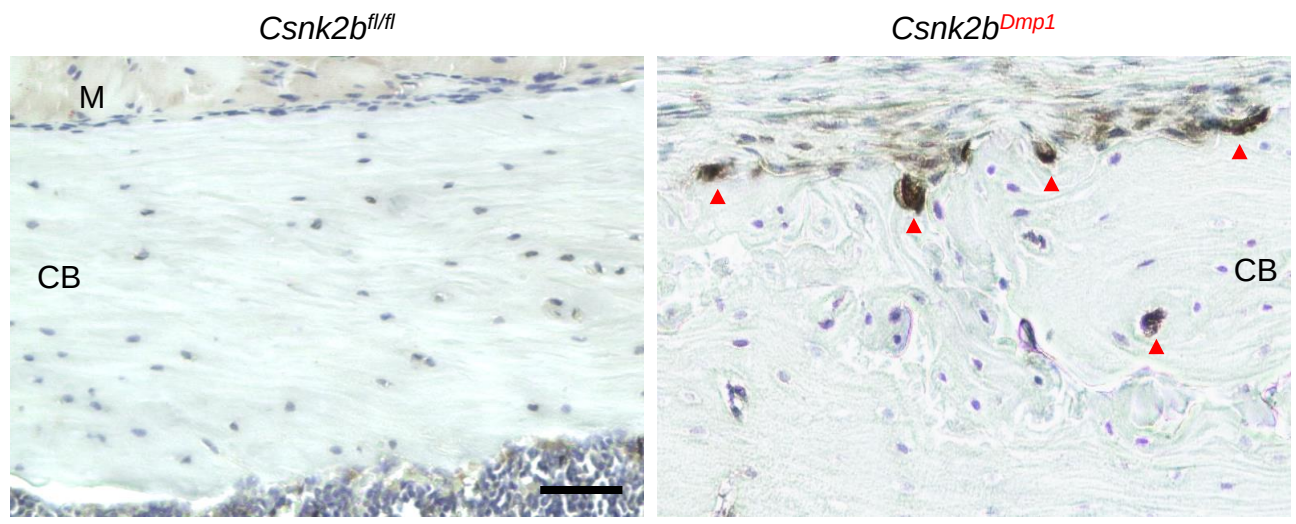

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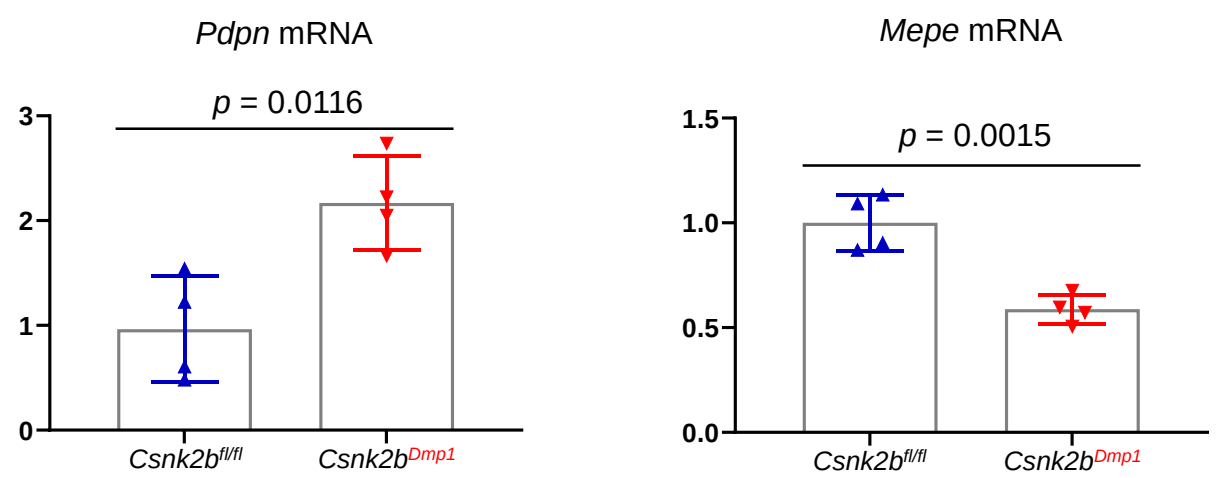
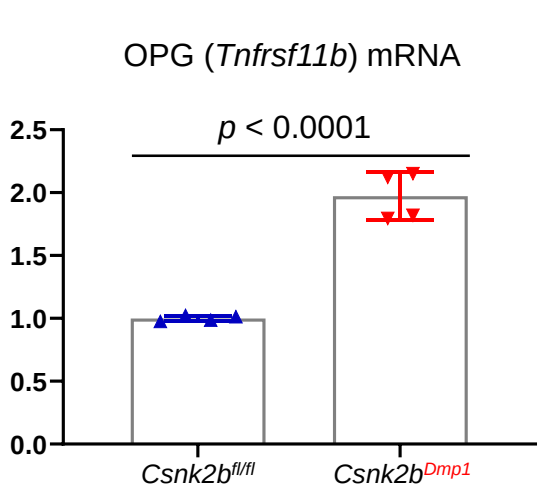
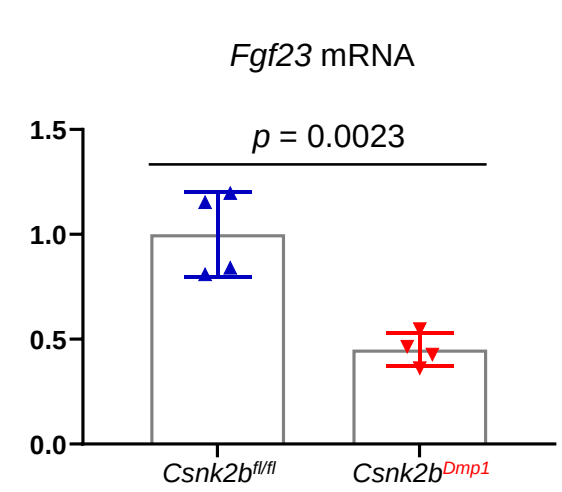
Supplementary Fig. 3







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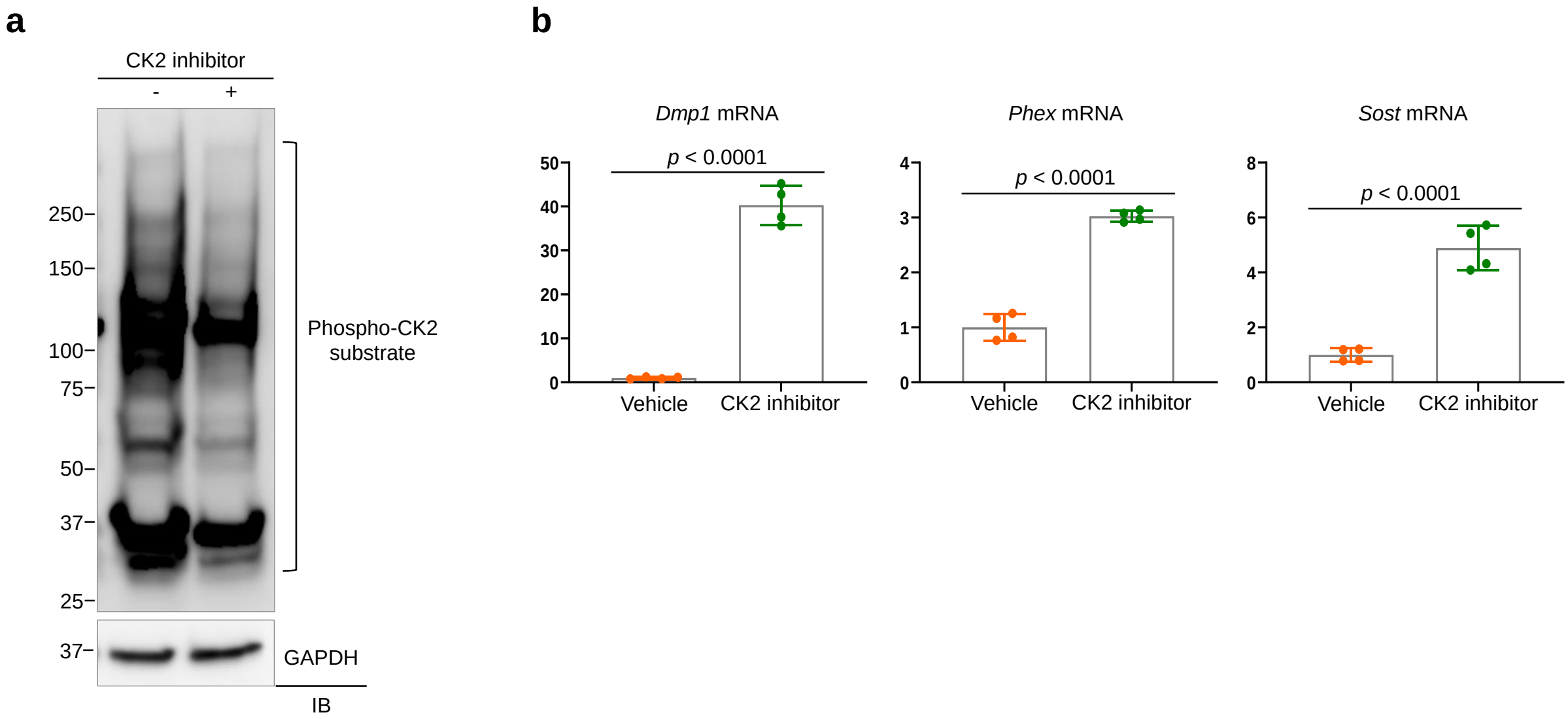

Supplementary Fig. 5

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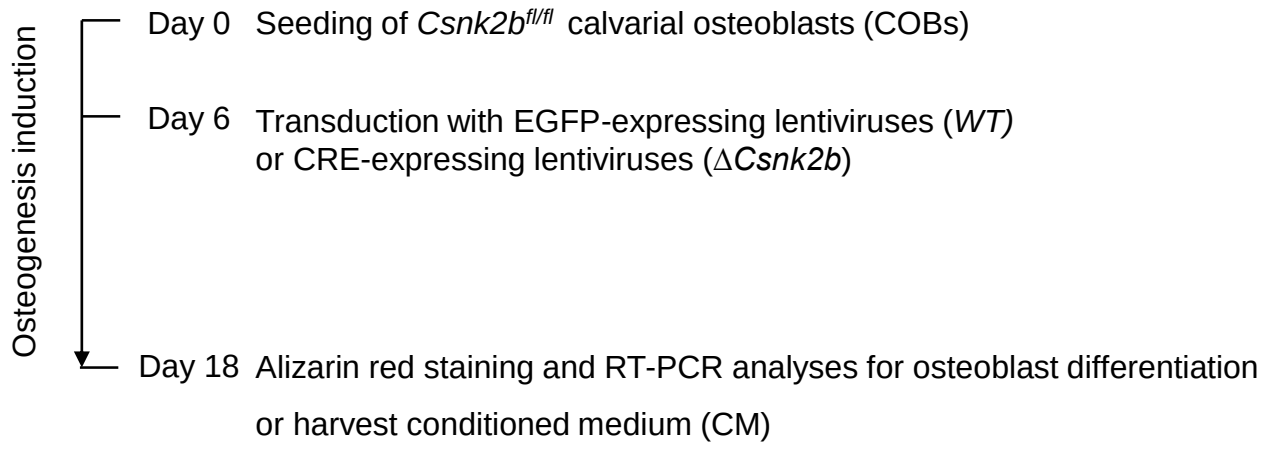

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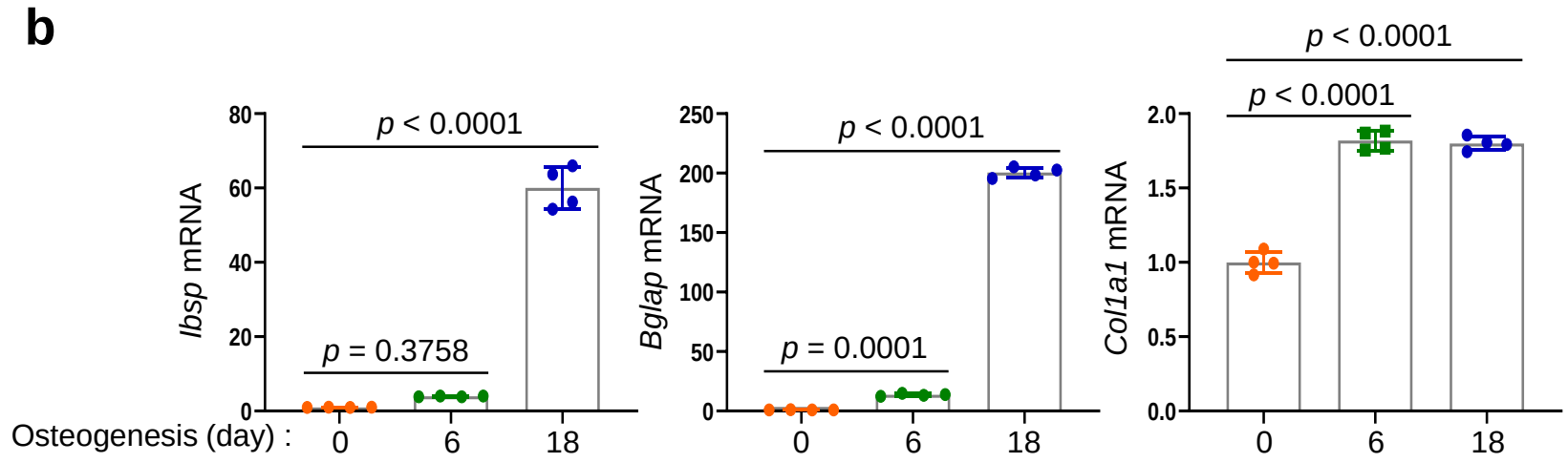

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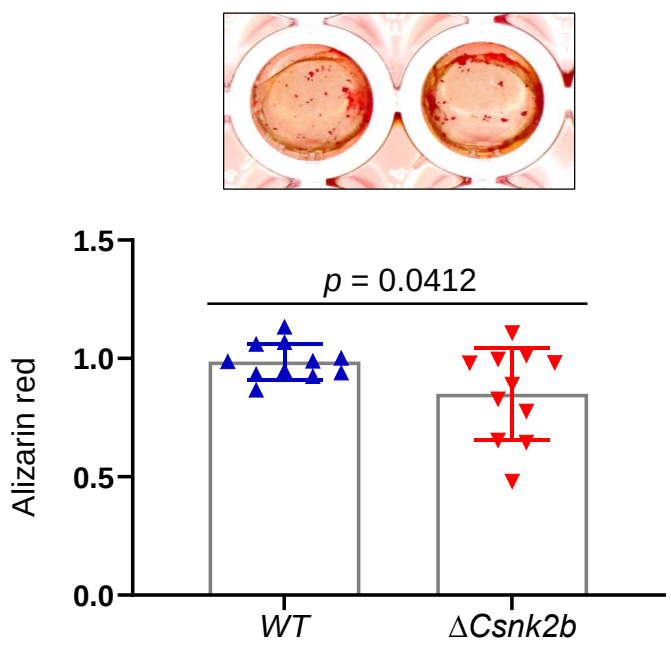

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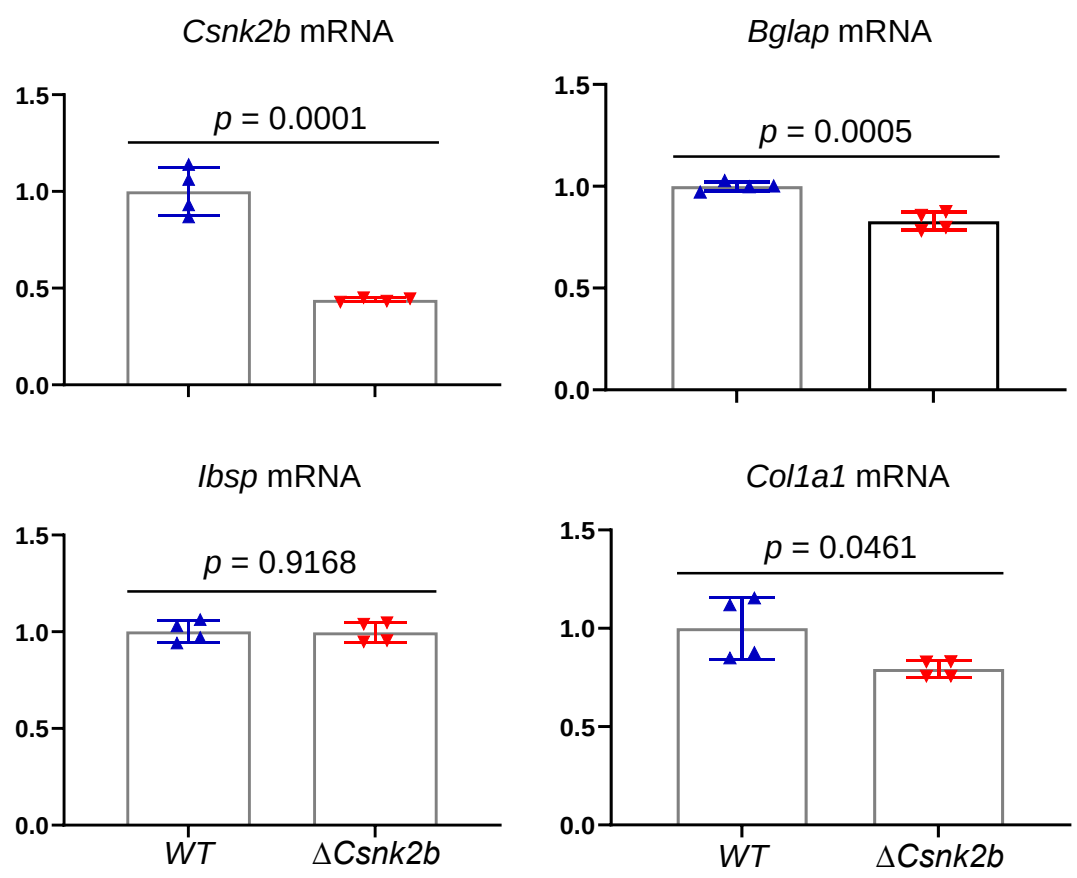

Supplementary Fig. 6

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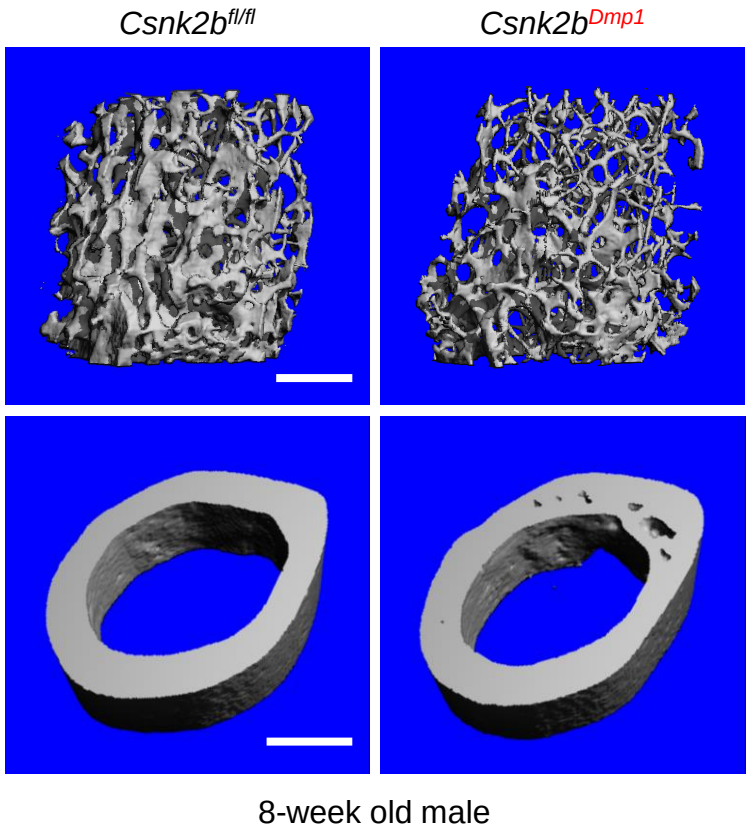

8-week old male

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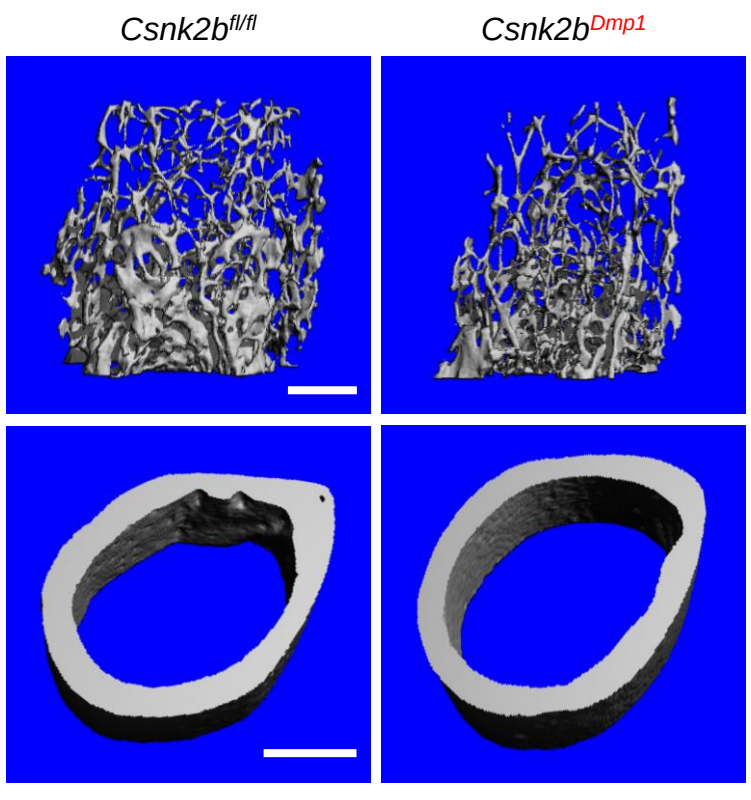

8-week old female

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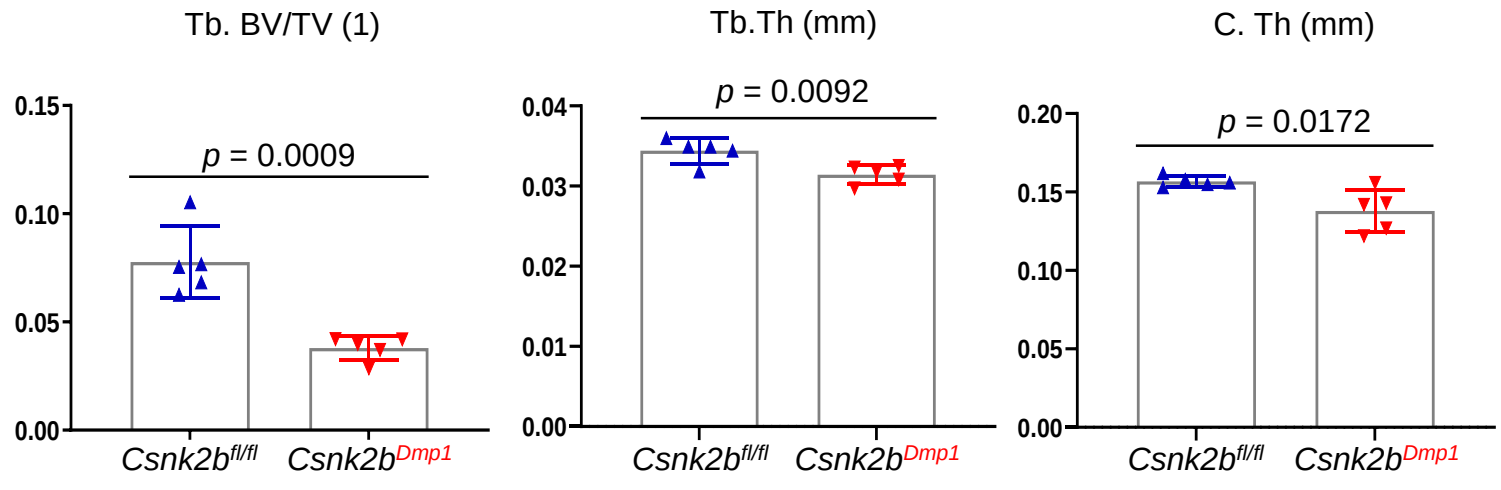

Supplementary Fig. 7

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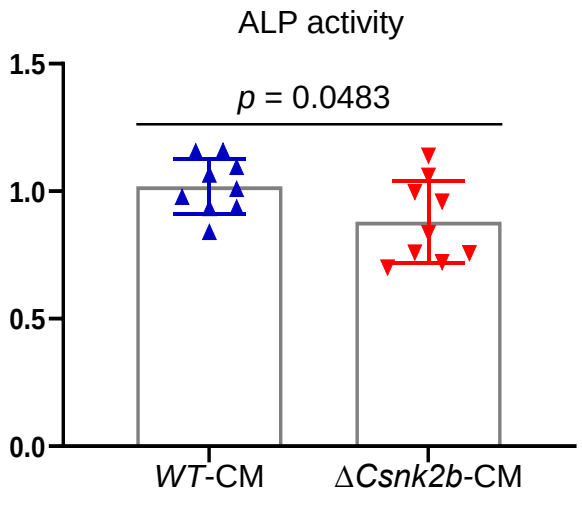

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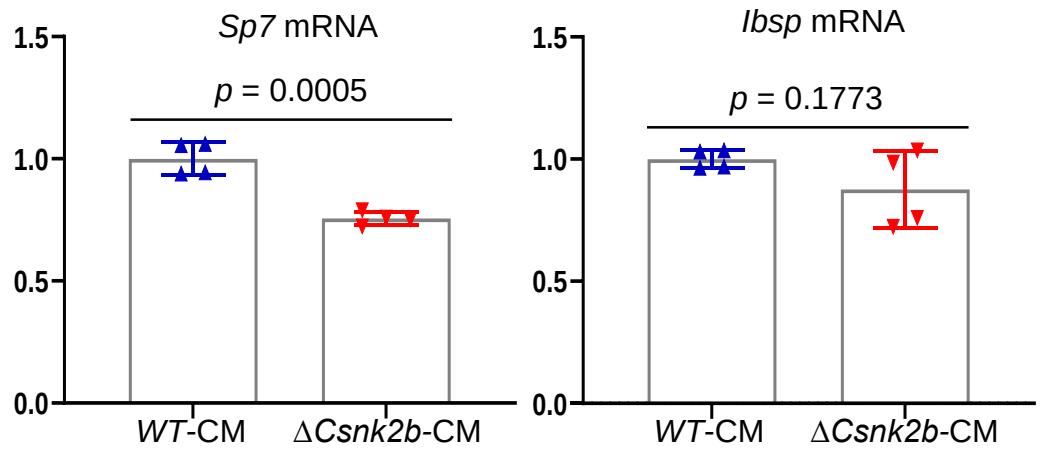

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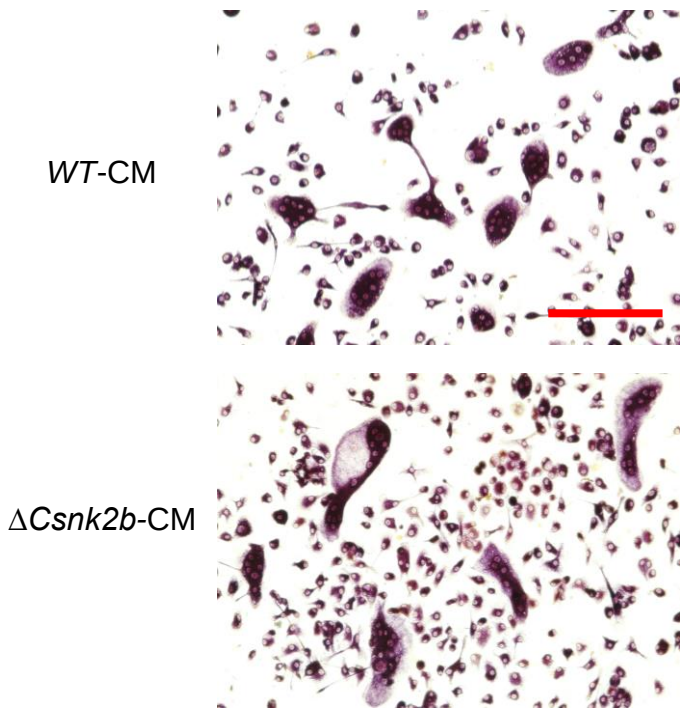

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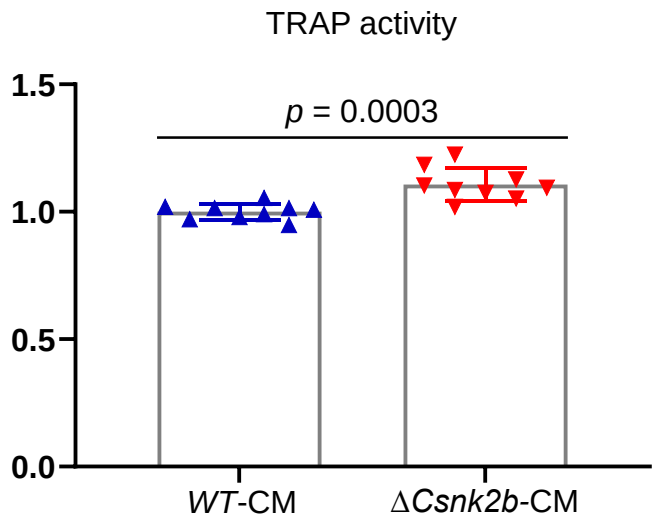

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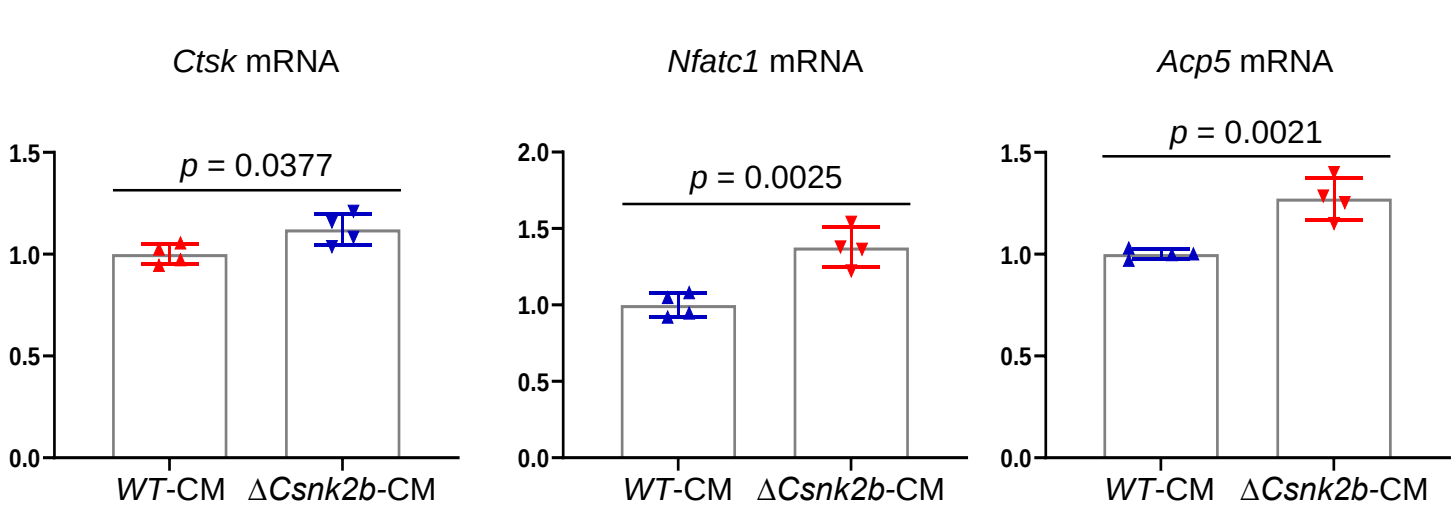

Supplementary Fig. 8

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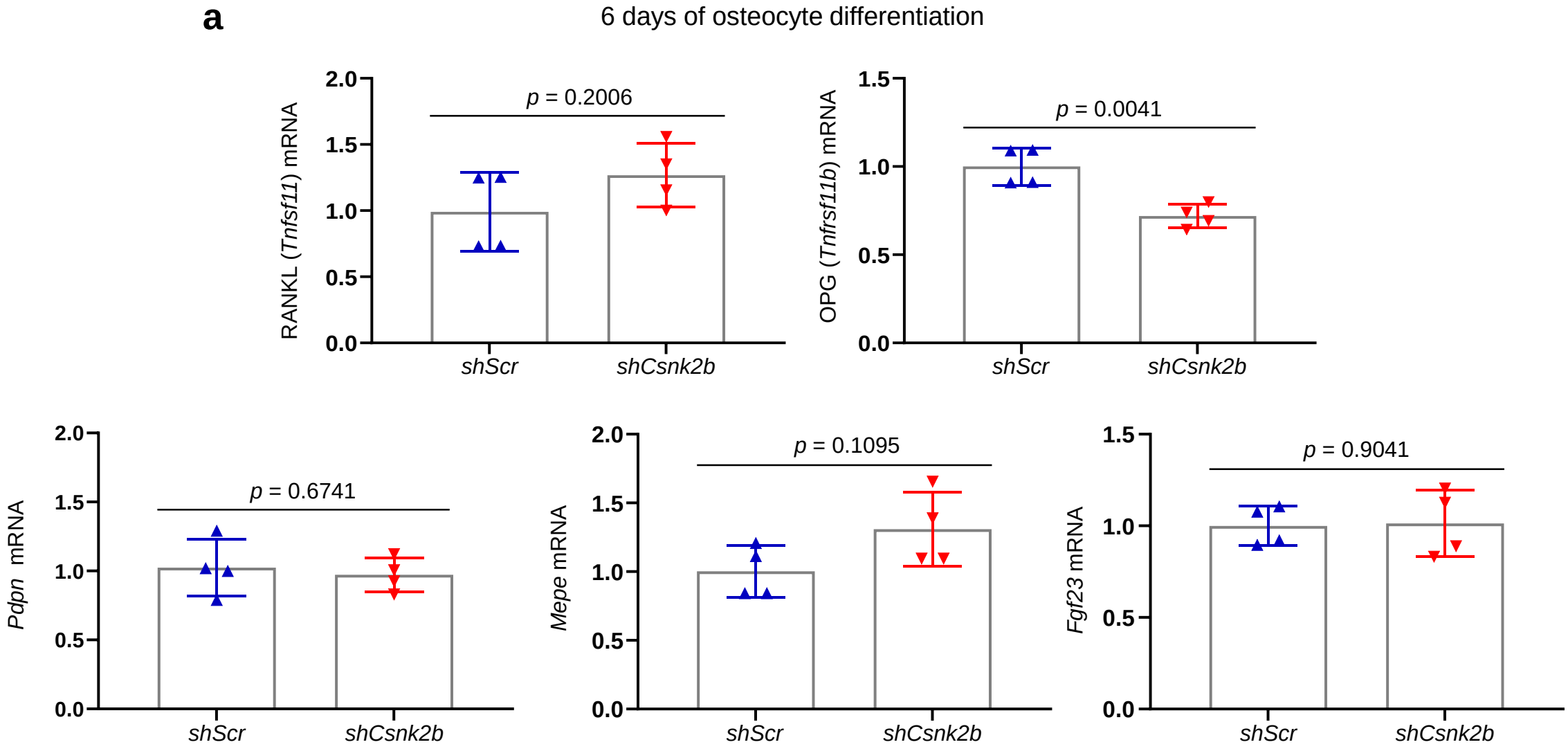

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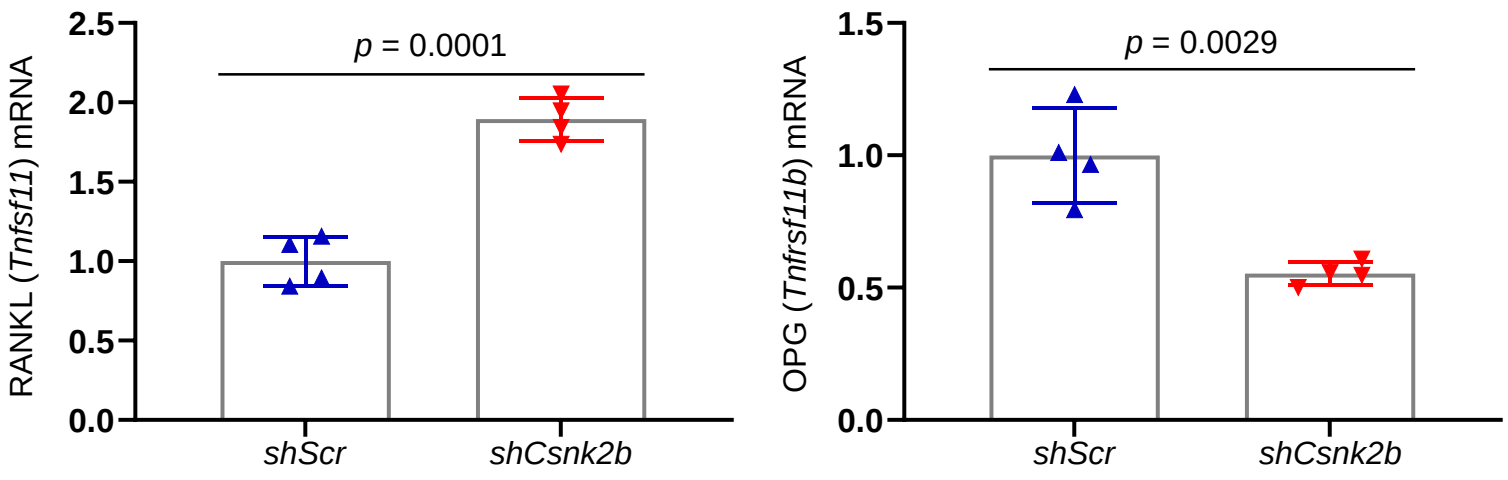
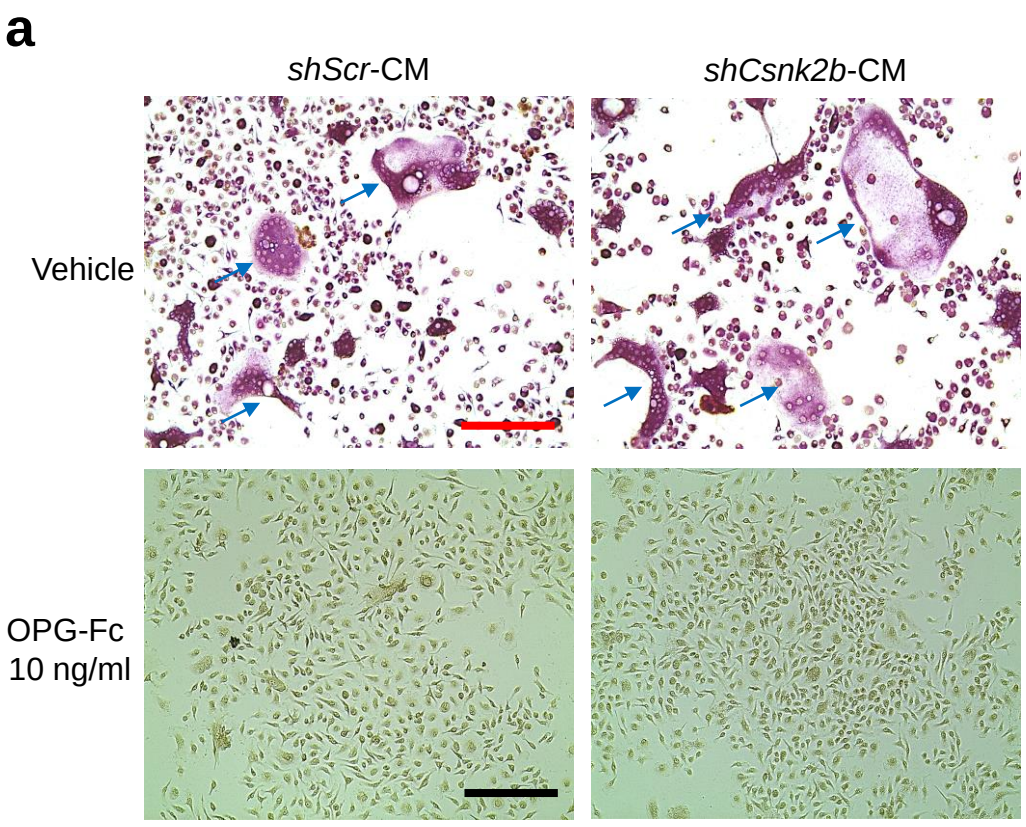
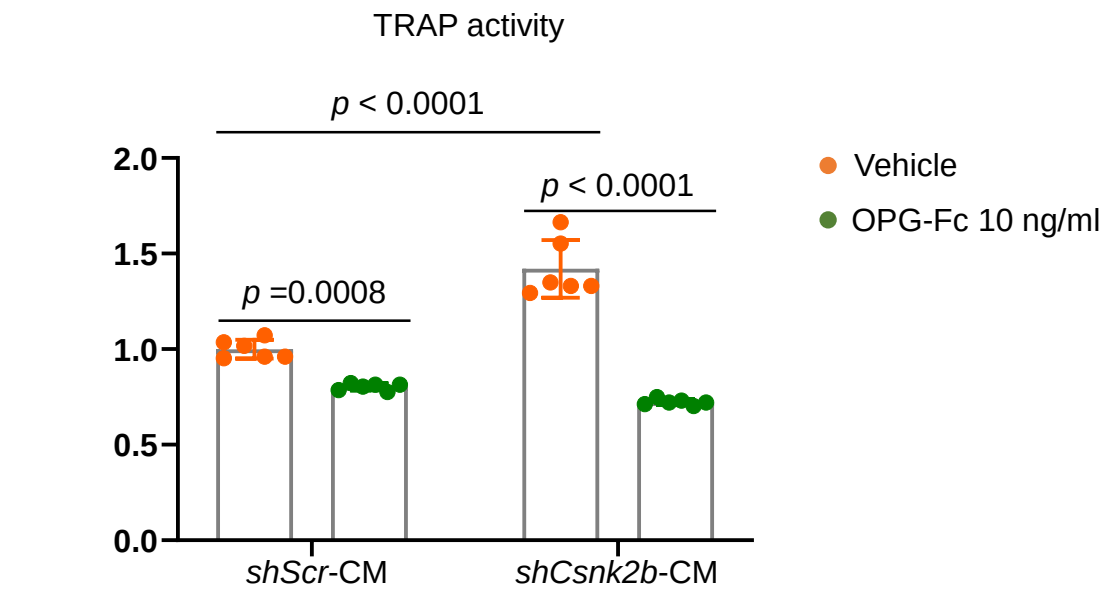
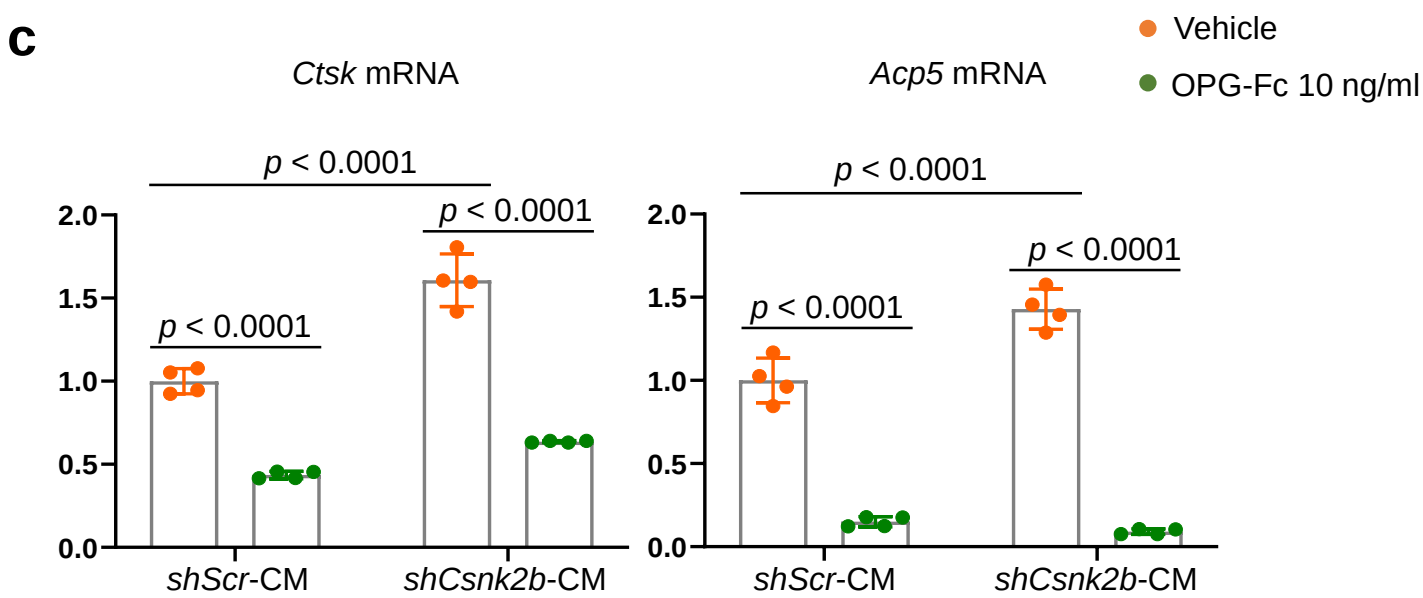
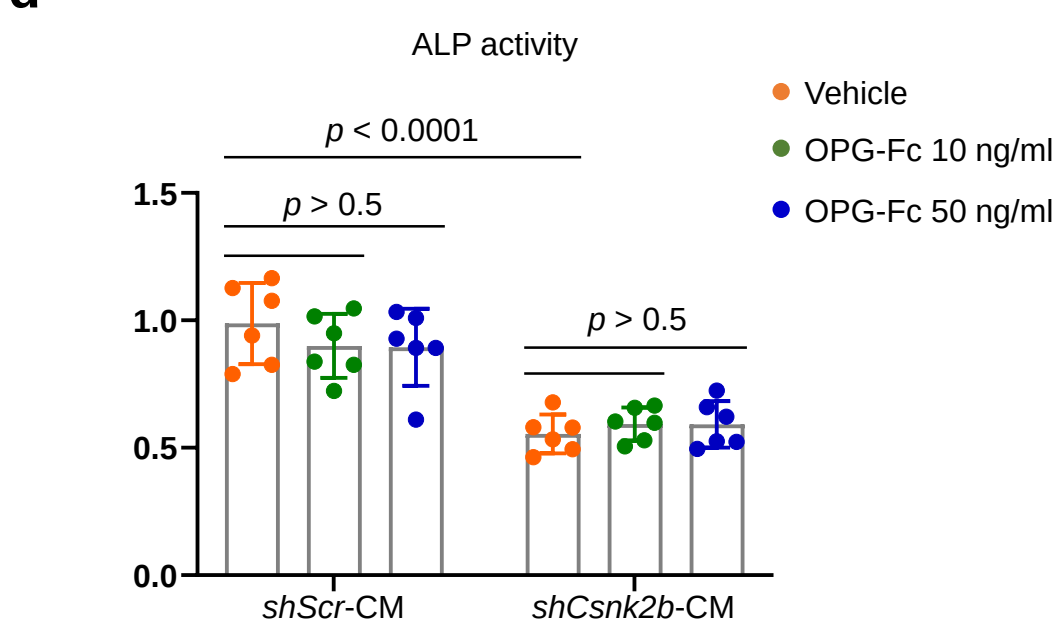


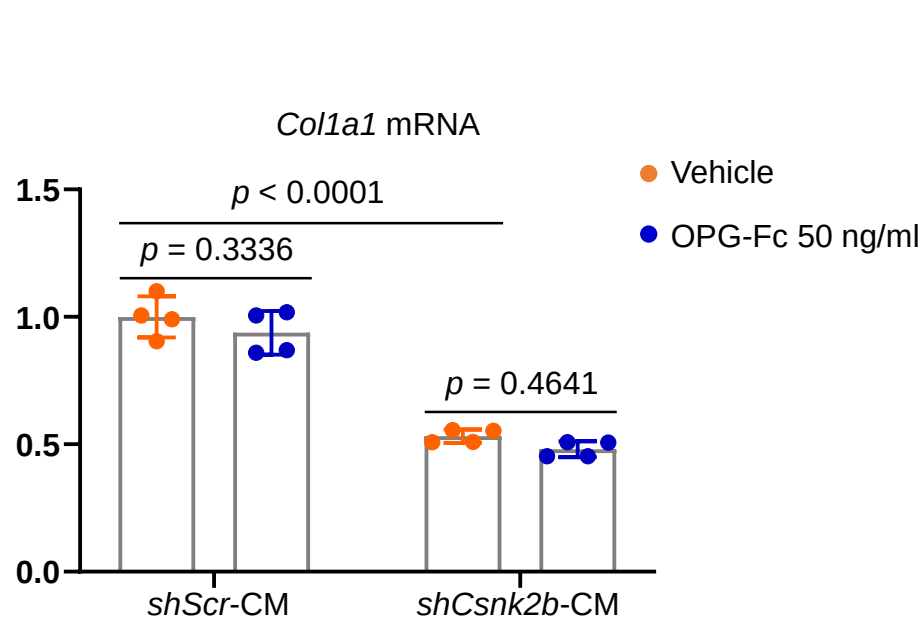
Supplementary Fig. 9



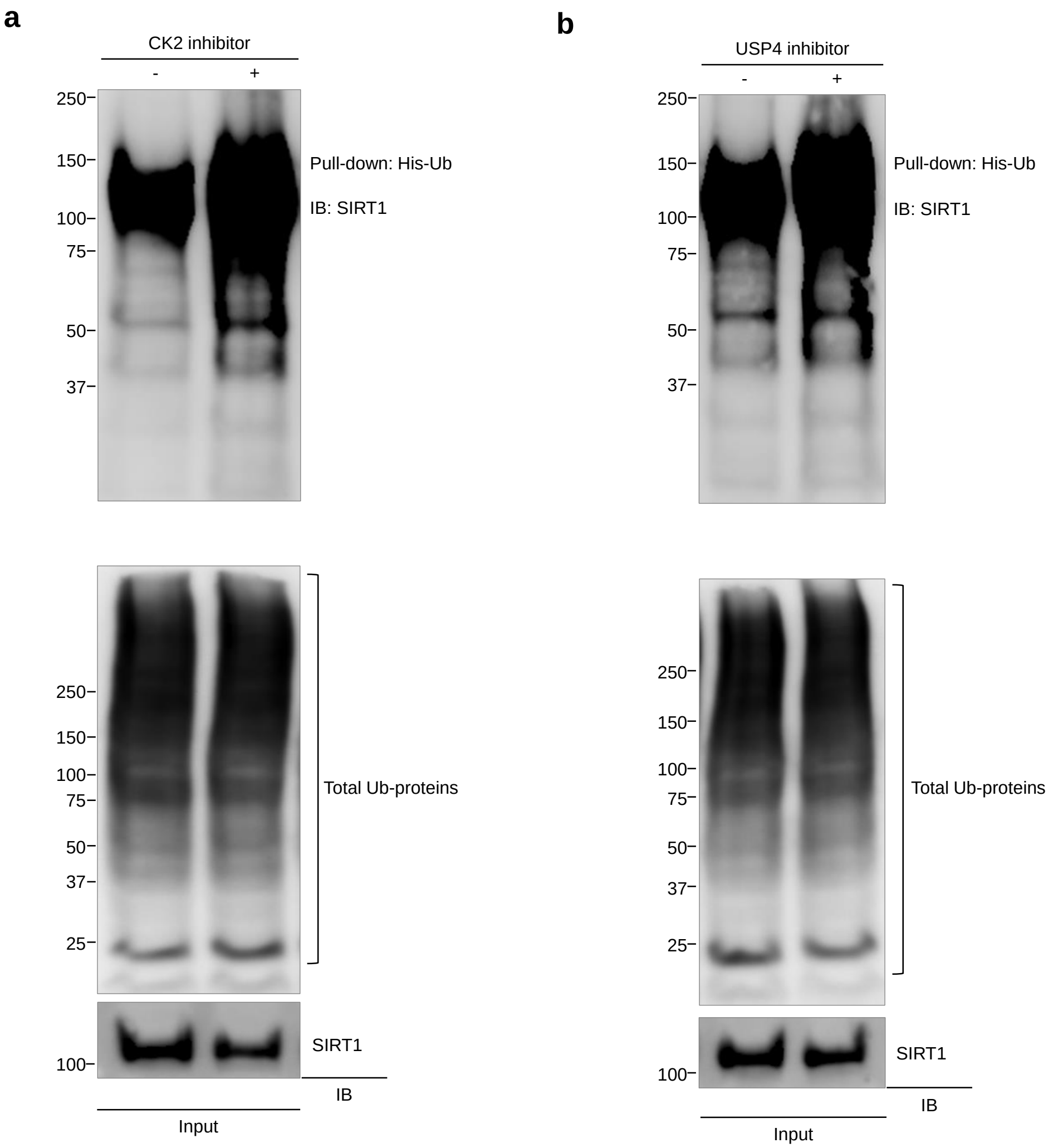




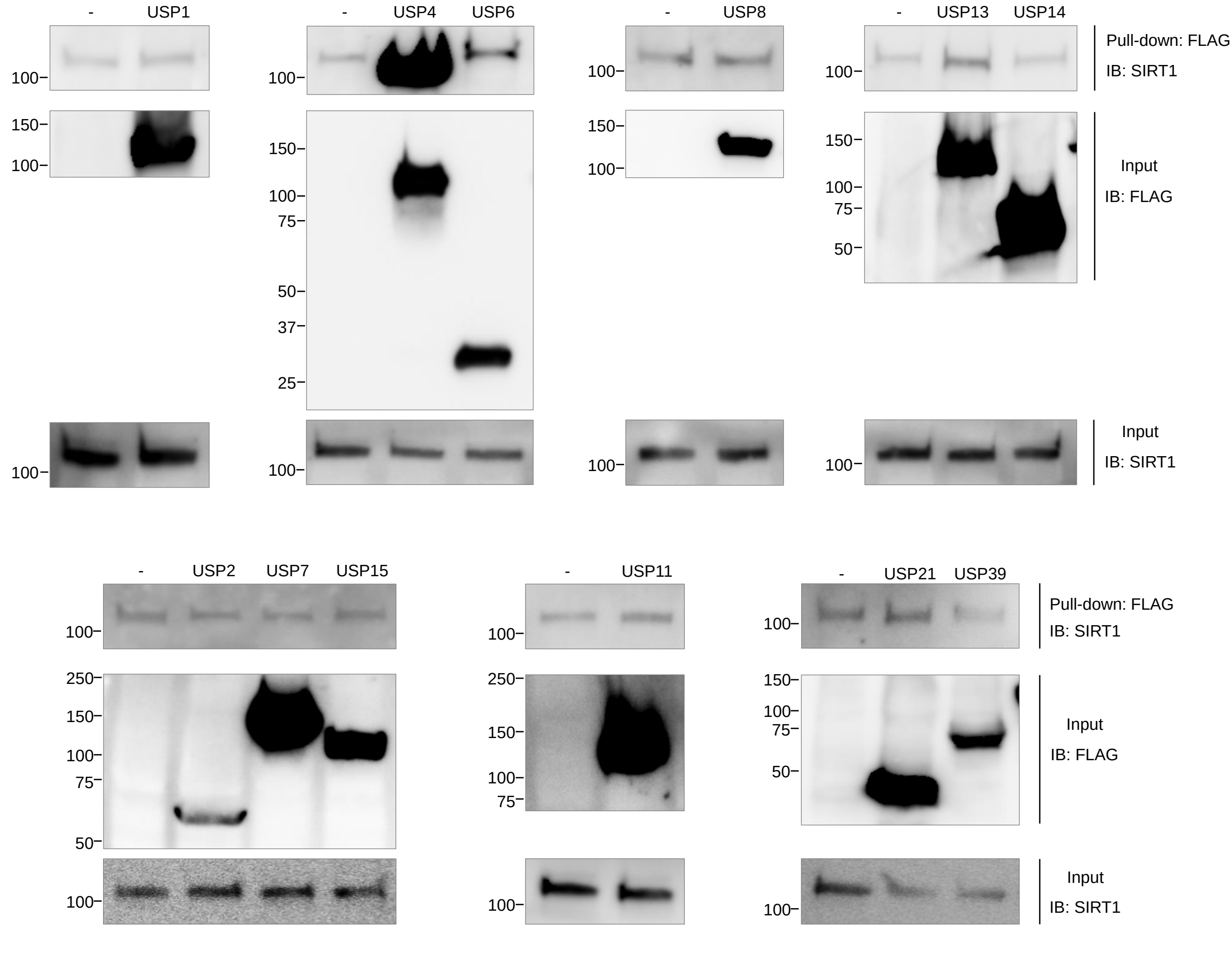




Supplementary Fig. 10



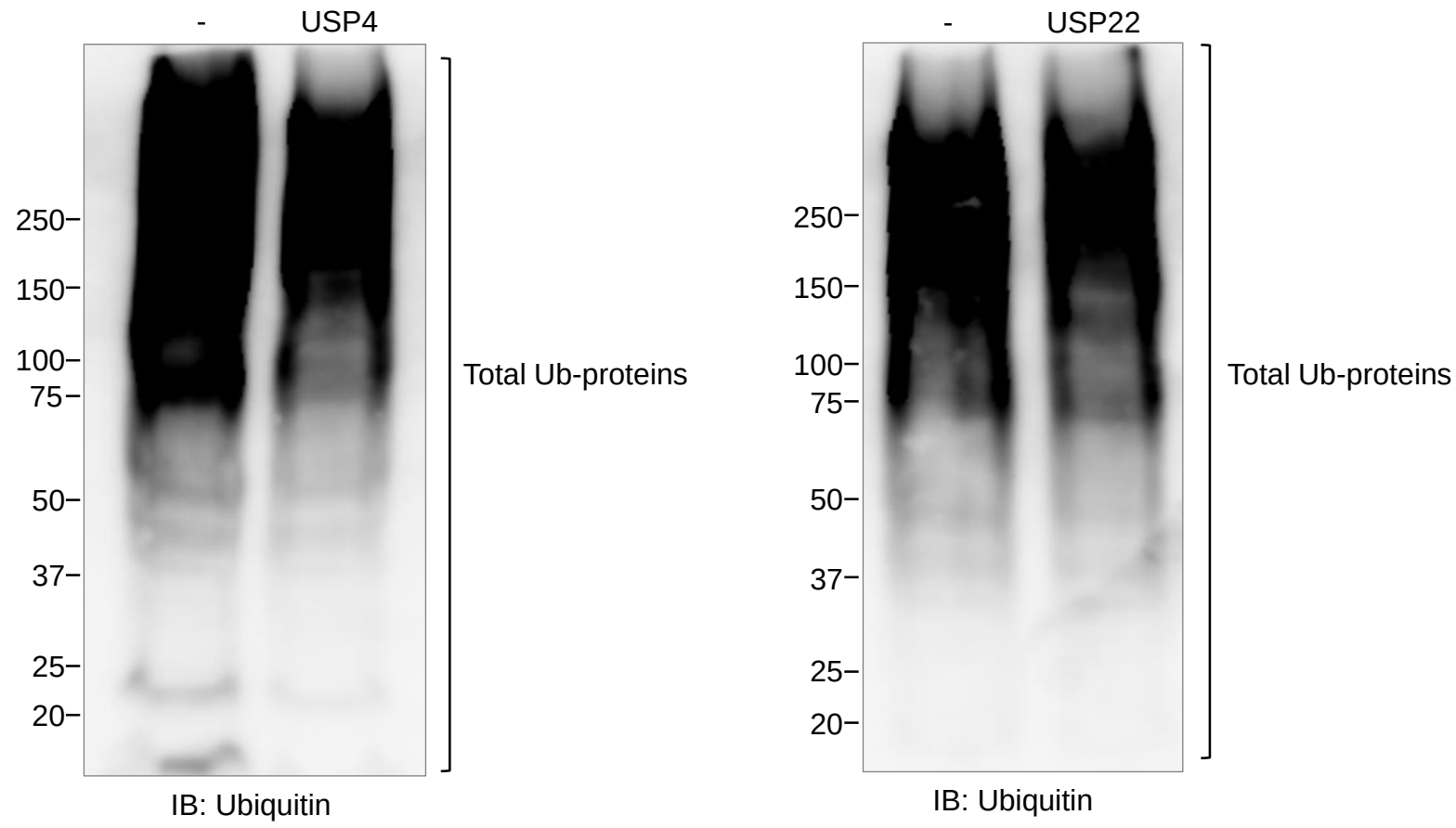
Supplementary Fig. 11



Supplementary Fig. 12

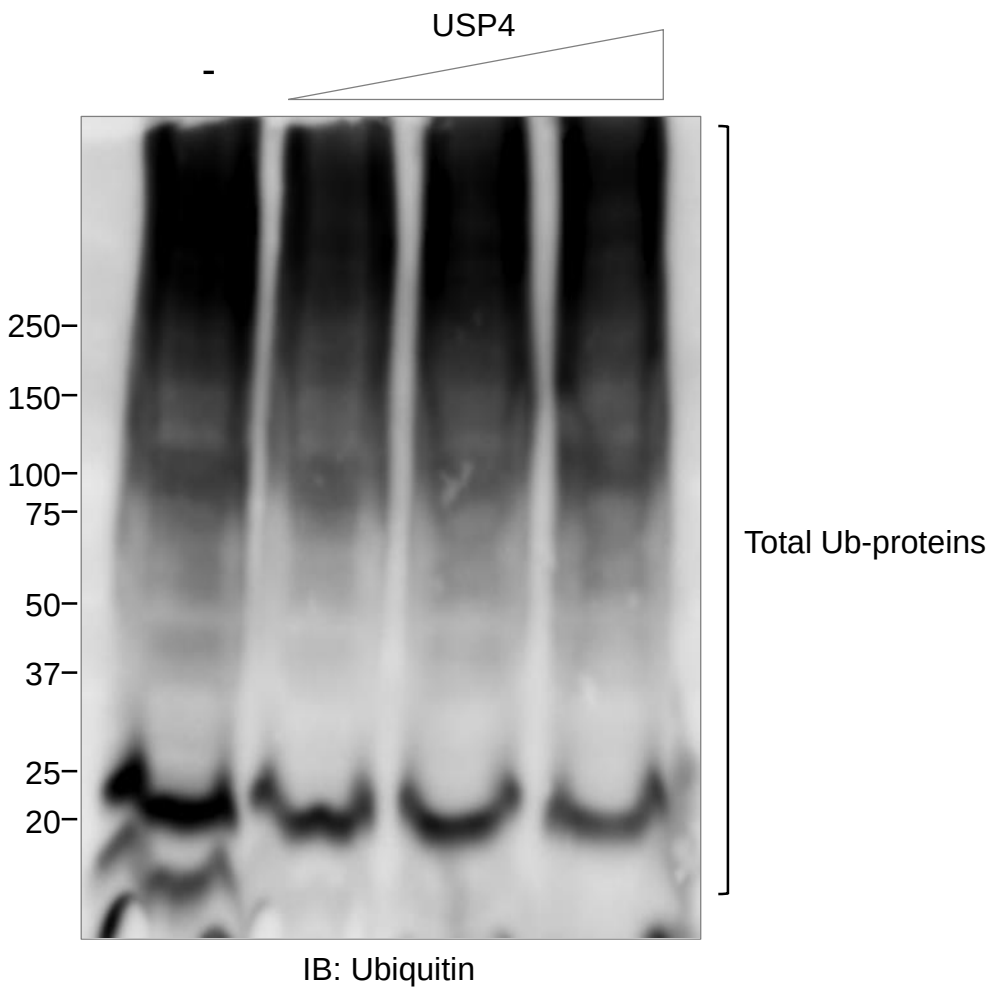
a

Fig. 6b, loading controls for Ni-NTA fractions



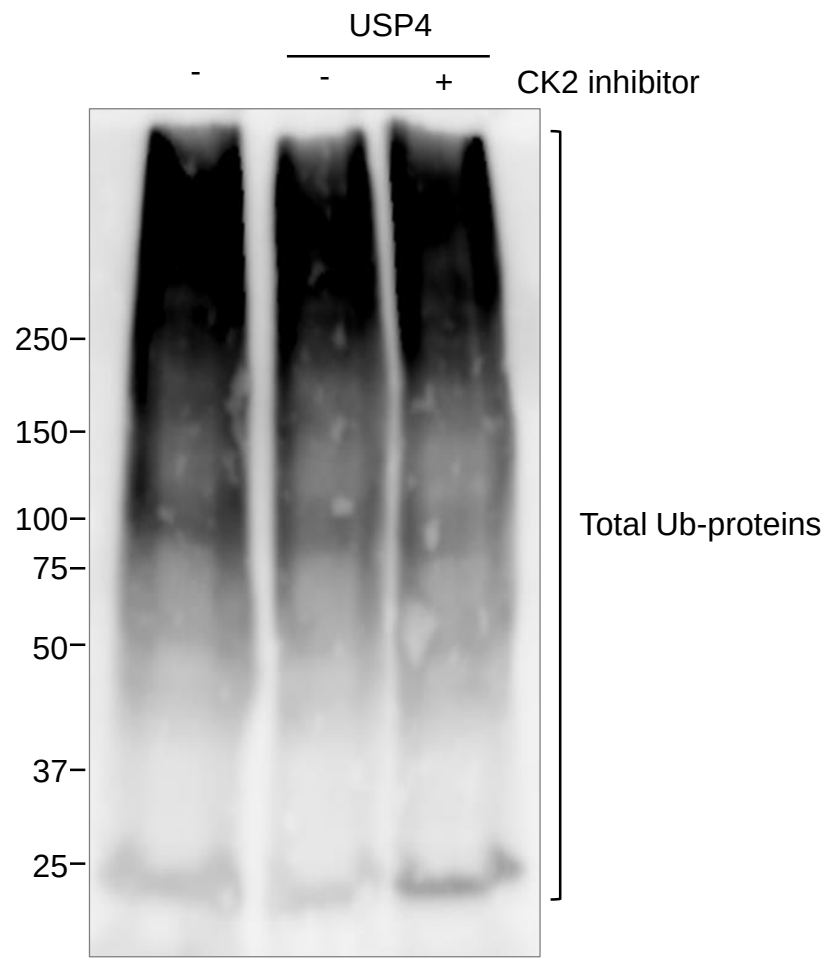
b

Fig. 6c, loading controls for Ni-NTA fractions



c

Fig. 6f, loading controls for Ni-NTA fractions



Supplementary Fig. 13