SUPPLEMENTAL METHODS

LC-MS/MS analyses

Differentiated mouse calvaria osteoblast cells were treated for 20 hours with vehicle, warfarin (50μM) or Decanoyl-RVKR-CMK (50μM). After treatment, supernatants from control and treated groups were collected and concentrated on a spin column (3 kD MWCO Amicon, EMD Millipore) pre-washed with H_2O. Buffer was then changed to 100 mM ammonium bicarbonate. Concentrated proteins were reduced using 45 mM DTT and digested with Arg-C (Progema). The LC-MS/MS analyses were performed on a LTQ Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany) equipped with a Proxeon nanoelectrospray ion source. The LC column was a C18 reversed phase column packed with a high-pressure packing cell. The buffers used for chromatography were 0.2% formic acid (buffer A) and 100% acetonitrile/0.2% formic acid (buffer B). About 0.6 μg of each sample was loaded on-column at a flowrate of 600 nL/min and eluted with a 2 slope gradient at a flowrate of 250 nL/min. Buffer B first increased from 2 to 40% in 60 min and then from 40 to 80% in 20 min. LC-MS/MS data acquisition was accomplished using an eleven scan event cycle comprised of a full scan MS for scan event 1 acquired in the Orbitrap. The mass resolution for MS was set to 60,000 (at m/z 400) and used to trigger the nine additional MS/MS events acquired in parallel in the linear ion trap for the top nine most intense ions. Standard proteomics parameters were used for the mass spectrometer. Protein database searches were performed with Mascot 2.5 (Matrix Science) against mouse. The mass tolerances for precursor and fragment ions were set to 10 ppm and 0.6 Da, respectively. The enzyme specified was semi-Arg C and two missed cleavages were allowed. Methionine oxidation and glutamic acid carboxylation were specified as variable modifications.

In vitro bone resorption assay

Calvarias from 9-months old *Furin*$^{flox/flox}$ and *Furin*$_{osb}^{-/-}$ mice were collected and devitalized by 3 cycles of sonication of 10 seconds each in 1X sterile PBS at maximum power and by incubation with 70% ethanol for 72h at 4ºC with agitation. Calvarias of each genotype were cut into two equal pieces and cultured in 48-well plates in 100% FBS for 2 hours followed by the addition of DMEM media overnight. At day 0, pre-osteoclastic RAW 264.7 cells

(ATCC) were plated on the calvarias at a density of 5000 cells/well in DMEM media supplemented with 10% FBS. At day 1, media was changed to osteoclast differentiation media, i.e. αMEM containing 10% FBS and supplemented with 10ng/ml of recombinant mouse RANK ligand (R&D). Media was changed at day 3 and 5 of culture. At day 5, media was replaced by 200 μL of fresh media and collected 24 hours later for OCN measurement by ELISA. Calvarias and osteoclastic cells were fixed in 10% formalin, washed with PBS 1X and stained for tartrate resistant acid phosphatase (TRAP) activity to assess osteoclast differentiation. Images of TRAP staining were taken at RT using a dissecting microscope (Discovery.V12; Carl Zeiss) connected to AxioCam ERc5s camera: objectives were 0.63X and 0.8X motorized zoom. Pictures were taken using the Zen 2012 software (Carl Zeiss). The percentage area of TRAP staining on calvaria and the area of each calvaria was quantified using Image J software. OCN concentration was normalized to surface area of corresponding calvaria of each mouse.

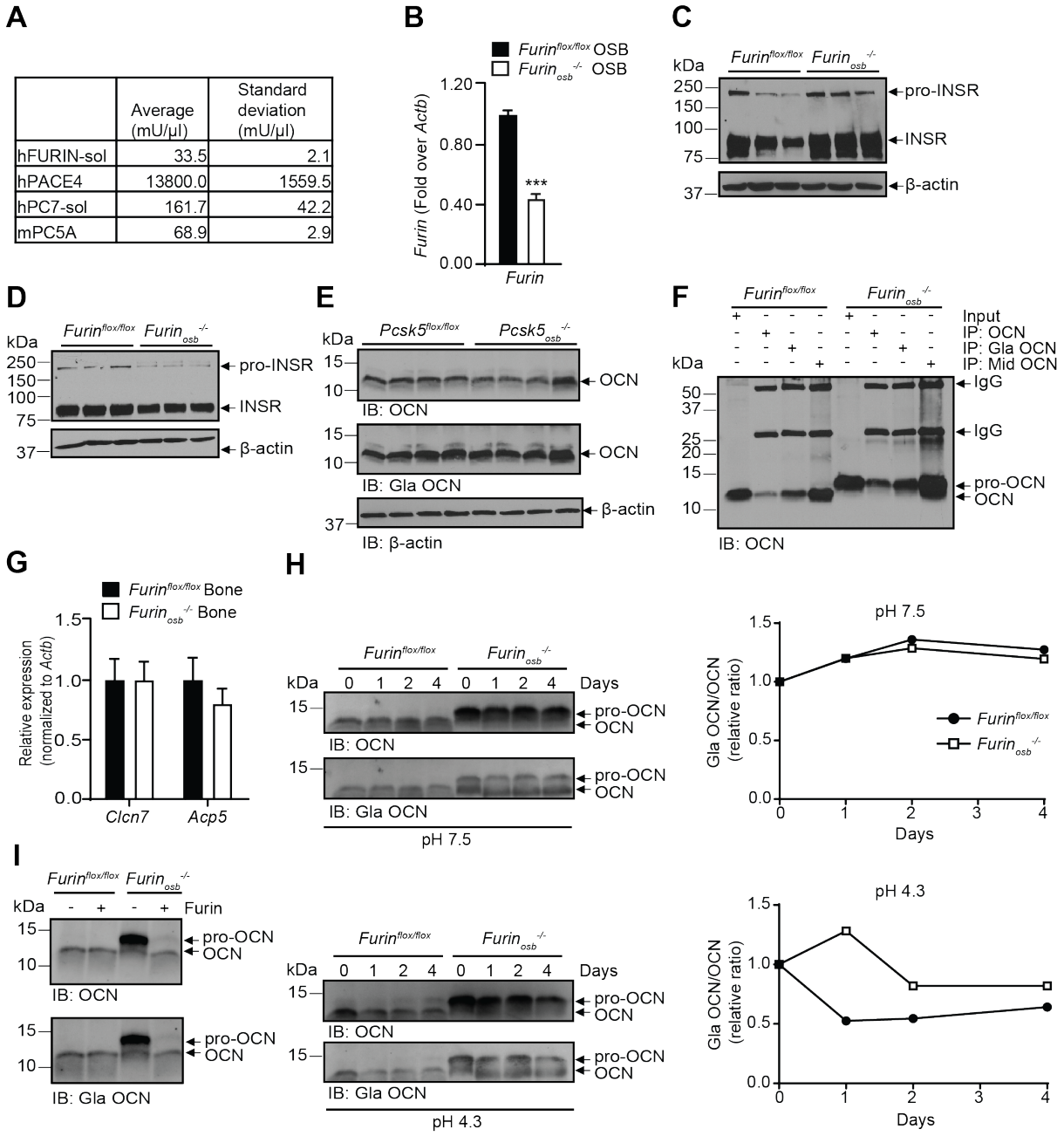
Microscopy

Mouse pro-OCN or R46A/R48A/R49A pro-OCN mutant cDNA was cloned in p3xFLAG-Myc-CMV™-23 in Hind III and BamHI restriction sites to generate N-terminally 3xFLAG tagged pro-OCN. Plasmids were transfected in mouse calvaria osteoblasts plated on glass coverslips and cultured in osteoblast medium. Following 24 hours of transfection, cells were fixed in 4% paraformaldehyde for 15 min and permeabilized using 0.1% triton in 1X PBS. Following blocking for 1 hour in 10% Bovine Serum Albumin (BSA) prepared in 1X PBS, the cells were incubated with rabbit anti-furin and mouse anti-FLAG primary antibodies (F1804; Sigma Aldrich) overnight at 4°C. After three washes with 1X PBS for 5 min each, cells were incubated with Cy3 anti–mouse (1:1,000) (715-165-150; Jackson Immunoresearch) and Alexa Fluor 488 anti–rabbit (1:1,000) (711-545-152; Jackson Immunoresearch) secondary antibodies for 1 h. Cells were then washed 3 times with 1X PBS, stained with DAPI for 2 min to stain DNA, and mounted on slides with FluorSave reagent (EMD Millipore). Cells were imaged at RT on a confocal microscope (Leica TCS SP8) using a 63X 1.40 Oil CS2 objective with oil Immersol 158F and Leica Application Suite X (LAS X) software. Using image J software the area of red signal of FLAG-pro-OCN and blue signal of the nucleus were quantified after setting up an equal threshold for all conditions. Percentage of FLAG-pro-OCN signal was calculated using this formula:

Area of FLAG-pro-OCN (%) = 100 X (Area of red signal / Cytoplasm area)

Cytoplasm area = Total cell area – nucleus area

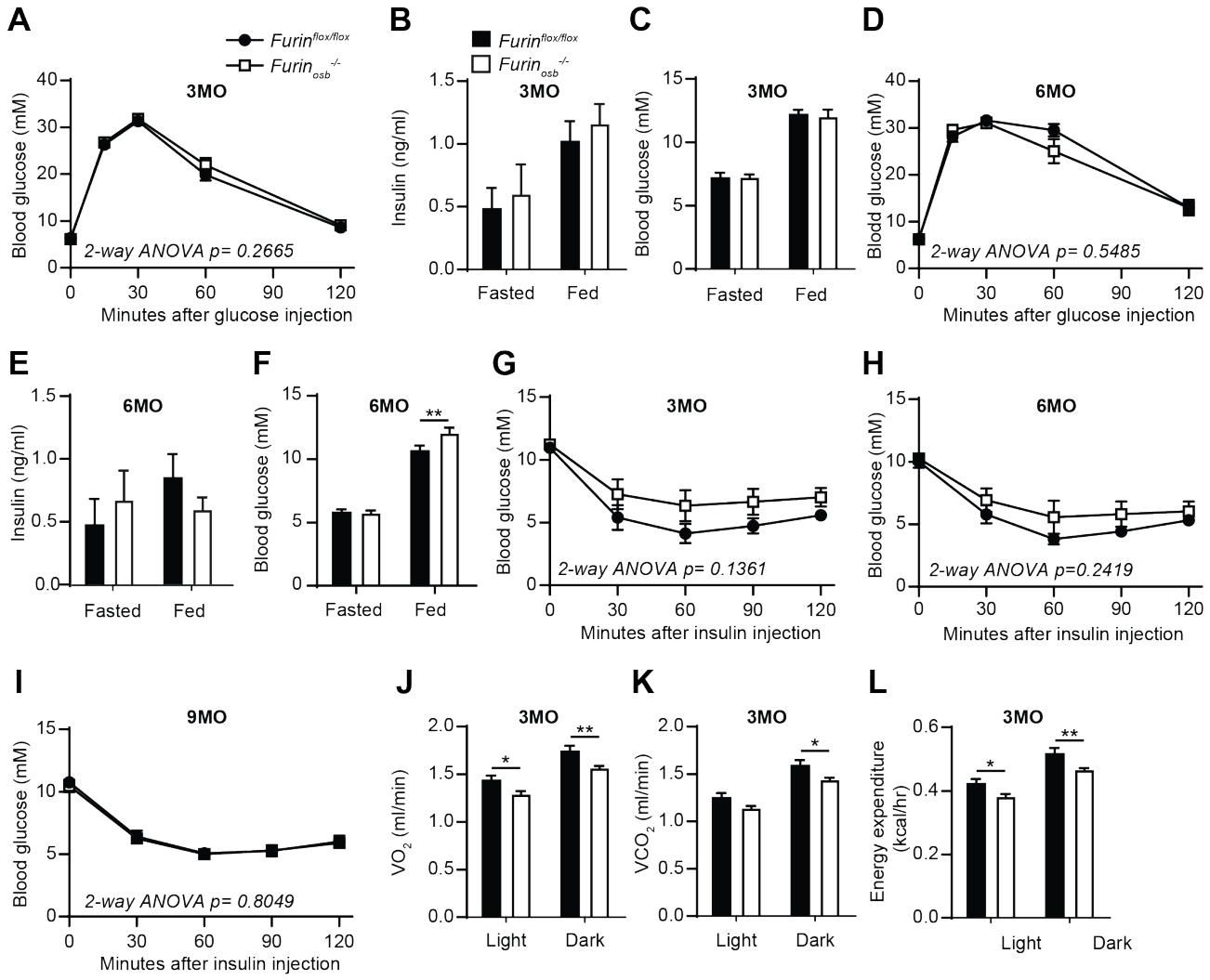
SUPPLEMENTAL FIGURES



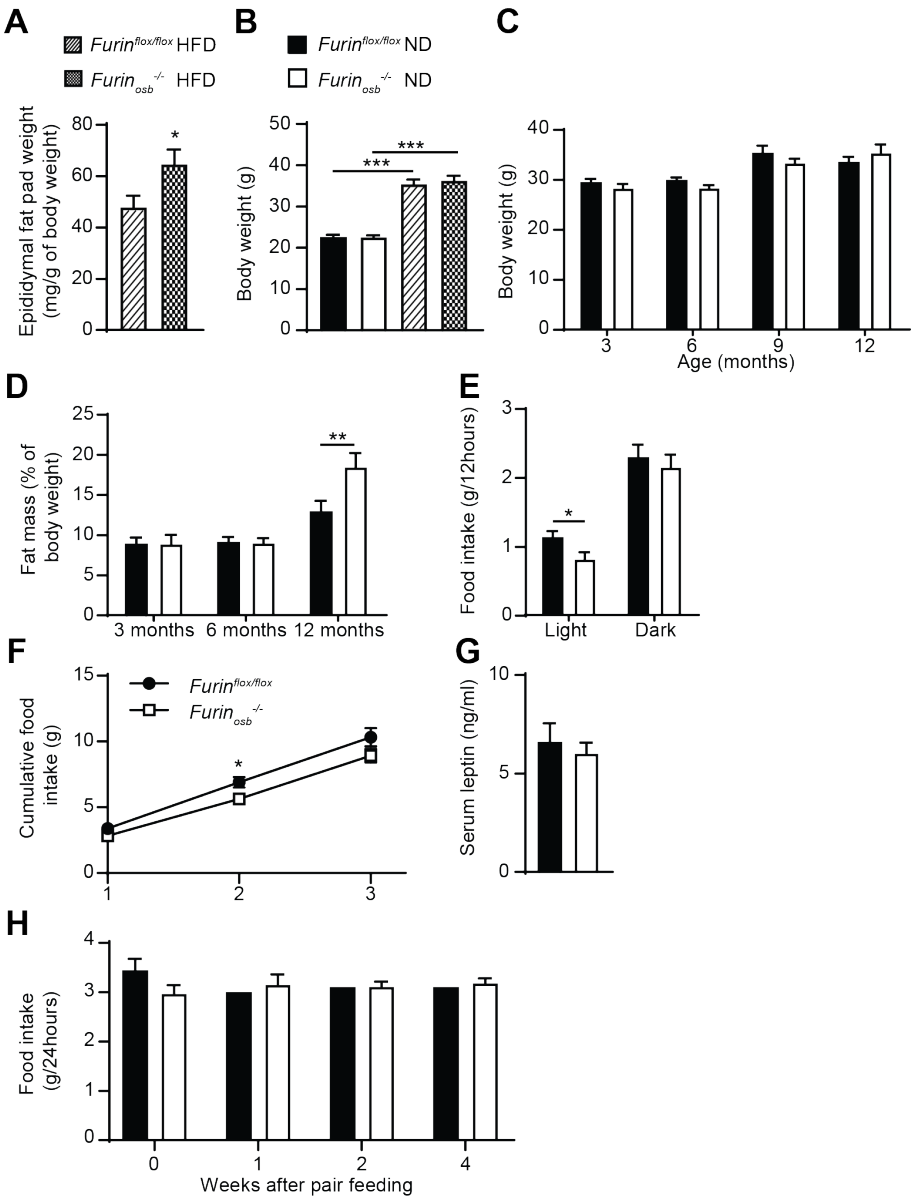
	Average (mU/µl)	Standard deviation (mU/µl)
hFURIN-sol	33.5	2.1
hPACE4	13800.0	1559.5
hPC7-sol	161.7	42.2
mPC5A	68.9	2.9

Supplemental Figure 1. Biochemical characterization of the *Furin*$_{osb}^{-/-}$ mice. (A) Enzymatic unit activity of different PCs. **(B)** Relative *Furin* expression in bone marrow osteoblasts derived from *Furin*$^{flox/flox}$ and *Furin*$_{osb}^{-/-}$ mice assessed by QPCR (n=3). **(C-D)** Western blot analysis of insulin receptor on bone extracts from *Furin*$^{flox/flox}$ or *Furin*$_{osb}^{-/-}$ mice **(C)** and on cell extracts from bone marrow derived osteoblasts from *Furin*$^{flox/flox}$ or *Furin*$_{osb}^{-/-}$ mice **(D). (E)** Western blot analysis of total (OCN) and γ-carboxylated OCN (Gla OCN) on bone extracts from *Pcsk5*$^{flox/flox}$ or *Pcsk5*$_{osb}^{-/-}$ mice. **(F)** OCN immunoprecipitation by the different anti-OCN antibodies used in OCN ELISAs and detection of OCN by Western

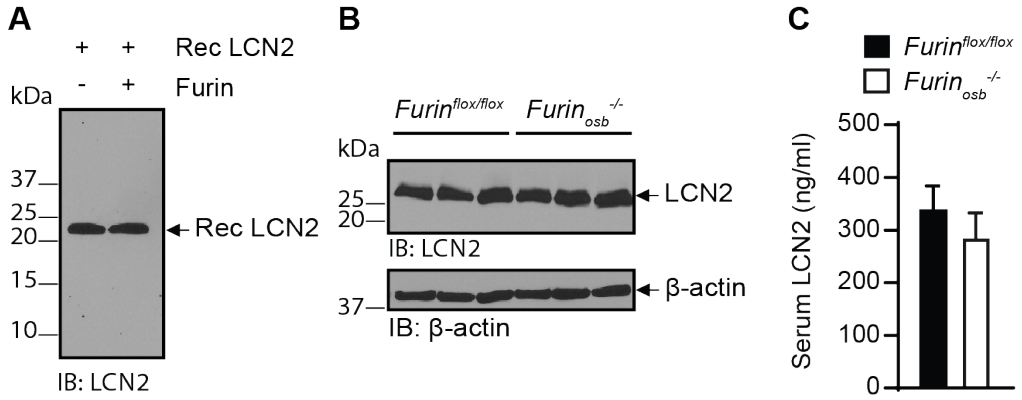
blotting. Immunoprecipitation was performed on bone extracts from *Furin*$^{flox/flox}$ or *Furin*$_{osb}^{-/-}$ mice. **(G)** QPCR analysis of *Clcn7* and *Acp5* gene expression in calvaria bone from *Furin*$^{flox/flox}$ or *Furin*$_{osb}^{-/-}$ mice. **(H)** Western blot analyses of in vitro decarboxylation of OCN over 4 days (left panels). Bone extracts from *Furin*$^{flox/flox}$ or *Furin*$_{osb}^{-/-}$ mice were incubated in phosphate buffered solution at pH 7.5 or pH 4.3 during 0-4 days at 37°C. Right panel: Quantification of Gla OCN/OCN ratio. **(I)** In vitro digestion of OCN in bone extract from *Furin*$^{flox/flox}$ and *Furin*$_{osb}^{-/-}$ mice incubated for 1 hour with 1U of furin. Released OCN was assessed by Western blot using total OCN and Gla OCN antibodies. Results are given as means ± SEM. ***, $p<0.001$ using unpaired two-tailed Student's *t* tests.



Supplemental Figure 2. Metabolic phenotype of *Furin*$^{flox/flox}$ and *Furin*$_{osb}^{-/-}$ mice at 3, 6 and 9 months. Glucose metabolism phenotyping of *Furin*$^{flox/flox}$ (n=7-11) and *Furin*$_{osb}^{-/-}$ (n=7-10) mice fed a normal diet at 3 months **(A-C)** and 6 months **(D-F)** of age. **(A, D)** Glucose tolerance test. Mice were fasted for 16 h and injected I.P. with 2g/kg glucose. **(B, E)** Fasting and fed serum insulin measurements. **(C, F)** Fasting and fed blood glucose measurements. **(G-I)** Insulin tolerance test on *Furin*$^{flox/flox}$ and *Furin*$_{osb}^{-/-}$ mice fed a normal diet at 3 **(G)**, 6 **(H)** and 9 **(I)** months of age**.** Mice were fasted for 5h and injected I.P. with 0.75U/kg of insulin. **(J-L)** Metabolic parameters of 3-month-old *Furin*$^{flox/flox}$ (n=9) and *Furin*$_{osb}^{-/-}$ (n=7) mice. Oxygen consumption **(J)**, carbon dioxide release **(K)** and heat production (energy expenditure) **(L)**. 3MO: 3-month-old; 6MO: 6-month-old; 9MO: 9-month-old. Results are given as mean ± SEM. *, $p<0.05$; **, $p<0.01$ using 2-way ANOVA for repeated measurements with Bonferroni multiple comparisons testing.



Supplemental Figure 3. Reduced energy expenditure and food intake in *Furin*$_{osb}^{-/-}$ mice at 3 months of age. (A) Epididymal fat pad weight normalized to the body weight of *Furin*$^{flox/flox}$ and *Furin*$_{osb}^{-/-}$ mice fed a high fat high sucrose diet (HFD) for 10 weeks**. (B)** Body weight of *Furin*$^{flox/flox}$ and *Furin*$_{osb}^{-/-}$ mice fed a normal chow diet (ND) or a HFD at 3 months of age. **(C)** Body weight of *Furin*$^{flox/flox}$ and *Furin*$_{osb}^{-/-}$ mice fed a normal diet at 3, 6, 9 and 12 months of age. **(D)** Fat mass % to body weight in *Furin*$^{flox/flox}$ and *Furin*$_{osb}^{-/-}$ at 3, 6, 9 and 12 months of age. In panels **(A)** through **(D)**, n=7-12 mice per group were analyzed. **(E and F)** Food intake in the light and dark cycle **(E)** and cumulative food intake over 3 days **(F)** in *Furin*$^{flox/flox}$ (n=9) and *Furin*$_{osb}^{-/-}$ (n=7) at 3 months of age. **(G)** Serum leptin levels in 6-month-old *Furin*$^{flox/flox}$ (n=11) and *Furin*$_{osb}^{-/-}$ (n=9) mice fed a normal diet. **(H)** Average daily food intake in *Furin*$^{flox/flox}$ (n=9) and *Furin*$_{osb}^{-/-}$ (n=7) before initiation of pair feeding (week 0) and after 1, 2 and 4 weeks of pair feeding. Results are shown as mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001 using unpaired two-tailed Student's t tests (**A**), 1-way ANOVA with Bonferroni multiple comparisons testing (**B**), 2-way ANOVA for non- with Bonferroni multiple comparisons testing (**D-F**).



Supplemental Figure 4. LCN2 is not processed by furin in osteoblasts. (A) *In vitro* processing assay of recombinant LCN2 incubated for 1h with furin. Released LCN2 was assessed by Western blot. **(B)** Western blot analysis of LCN2 on bone extracts from *Furin*$^{flox/flox}$ or *Furin*$_{osb}^{-/-}$ mice. **(C)** Serum LCN2 level in *Furin*$^{flox/flox}$ (n=6) or *Furin*$_{osb}^{-/-}$ (n=6) mice after 16h fasting followed by 2 hours of re-feeding.

Supplemental table 1: List of oligonucleotides used in this study

qPCR primers for gene expression	**Sequence (5'-3')**	**Gene**
Pcsk1-Fw	CCATGCTGCGACTCCTACAA	*Pcsk1*
Pcsk1-Rv	TGGAGGGCTTGTTGAGCTTT	*Pcsk1*
Pcsk2-Fw	TCTAGCAAGCACCCCAAAGG	*Pcsk2*
Pcsk2-Rv	CTCTGCACATGCTTCAGGGA	*Pcsk2*
Furin-Fw	TGTGACGGCTACACCAACAG	*Furin*
Furin-Rv	GCTTCTCATTCTGGTTGCCG	*Furin*
Pcsk4-Fw	CCCTCCTGTTACACCTGCTG	*Pcsk4*
Pcsk4-Rv	TGTGCATGCATGGTTTTGGG	*Pcsk4*
Pcsk5A-Fw	AAACCTGGCCGTCGTGTATT	*Pcsk5 isoforme A*
Pcsk5A-Rv	TGTGCACCTGCATGGTTAGT	*Pcsk5 isoforme A*
Pcsk5B-Fw	GGCAGAACCTACCGTGTCAT	*Pcsk5 isoforme B*
Pcsk5B-Rv	CGATGTCGTCCTCATCGTCC	*Pcsk5 isoforme B*
Pcsk6-Fw	GGCTGTTCTACCTGTGTCCC	*Pcsk6*
Pcsk6-RV	GGCGACTAGTGATTCAGGGG	*Pcsk6*
Pcsk7-Fw	AGATTTGCTGACCCCAGAGC	*Pcsk7*
Pcsk7-RV	GAGGAAATCAAGGGCTGCCT	*Pcsk7*
Actb-Fw	GACCTCTATGCCAACACAGT	*Actb*
Actb-Rv	AGTACTTGCGCTCAGGAGGA	*Actb*
Acp5-Fw	AGTCCTGCTTGTCCGCTAAC	*Acp5*
Acp5-Rv	CCTAAAAGGGGTGAGCCTGG	*Acp5*
Clcn7-Fw	GACTGGCTGTGGGAAAGGAA	*Clcn7*
Clcn7-Rv	TCTCGCTTGAGTGATGTTGACC	*Clcn7*
Primers for genotyping	**Sequence (5'-3')**	**Genotype**
Furin flox-Fw	ATGCTCAAGGCCAGAAGATC	*Furin +/flox*
Furin delta-Fw	GCTGTATTTATTCCGGAGAC	*Furin delta*
Furin flox-Rv	AATCTGTTCCCTGCTGAGGA	*Furin +/flox & Furin delta*
Ggcx flox-Fw	CCATGTGTCCAAAGCATTTCT	*Ggcx +/flox*
Ggcx flox-Rv	TCATTGAGTCCTTCCCGAAC	*Ggcx +/flox*
Bglap1-Fw	TGGAGTGGTCTCTATGACCT	*Ocn +/-*
Neo poly A-Fw	TTCCTTGACCCTGGAAGGTG	*Ocn +/-*
Bglap2-Fw	TTGTGCTGGGGTGGTTTCTG	*Ocn +/-*
Bglap2-Rv	AGCCTTCCCCAACCCCTATT	*Ocn +/-*
Pcsk5 flox-Fw	CAGAATTGCTGTGCTCTGGA	*Pcsk5 +/flox*
Pcsk5 flox-Rv	GTATTGGCATTTCCCTCAGC	*Pcsk5 +/flox*
Cre-Fw	GCGGTCTGGCAGTAAAAACTATC	*OC-Cre*
Cre-Rv	GTGAAACAGCATTGCTGTCACTT	*OC-Cre*
Il-2-Fw	CTAGGCCACAGAATTGAAAGATCT	*OC-Cre*
Il-2-Rv	GTAGGTGGAAATTCTAGCATCATCC	*OC-Cre*
Primers for mutagenesis	**Sequence (5'-3')**	**Mutation**
mOcn R48A/R49A-Fw	gtgaacagactcGCgGCctaccttggagc	R48A/R49A
mOcn R48A/R49A-Rv	gctccaaggtagGCcGCgagtctgttcac	R48A/R49A
mOcn R46A/R48A/R49A-Fw	aggtagtgaacGCactcgcggccta	R46A/R48A/R49A
mOcn R46A/R48A/R49A-Rv	taggccgcgagtgcgttcactacct	R46A/R48A/R49A

Primers for cloning	**Sequence (5'-3')**	**Cloning vector**	**Restriction site included**
Hind III pro Fw	attaaaagcttaagcccagcggccctgagtct	in p3xFLAG-Myc-CMV™-23	Hind III
mOcn BamHI Rv	attaaggatccaatagtgataccgtagatgcg	in p3xFLAG-Myc-CMV™-23	BamHI
EcoRI pro Fw	aattgaattcgccaccatgaggaccctctctc	pIRES2-EGFP-V5	EcoRI
mOcn AgeI Rv	aattaccggtaatagtgataccgtagatgcg	pIRES2-EGFP-V5	AgeI
OCN aa 24 BamHI F	ttaaGGATCCaagcccagcggccctgagtc	pGEX4T3	BamHI
OCN aa 95 EcoRI R	ttaaGAATTCctaaatagtgataccatagatg	pGEX4T3	EcoRI