Supplementary Materials and Methods

In vivo calcein labeling and calculation of bone formation rate (BFR)

Calcein labeling and BFR calculation was previously described (1). Briefly, mice were intraperitoneally injected with 20 mg/kg of calcein (Sigma-Aldrich) or 30 mg/kg of Alizarin (Sigma-Aldrich) in a 2% sodium bicarbonate solution. Mice were labeled 5 days for 2-month-old mice, 8 days for 8-month-old mice and 2 days prior to sacrifice. Tibia, femur and calvarias were fixed in 70% ethanol and embedded in methylmethacrylate and sectioned. Pictures were taken by a fluorescence microscope (Olympus BX51, Center Valley, PA). Analysis of bone formation rate (BFR) per bone surface (BS) was performed by using Bioquant Osteo (Bioquant Image Analysis Corp.). Mineral apposition rate (MAR) is the distance between the midpoints of the two labels divided by the time between the midpoints of the interval. Mineralizing surface per bone surface (MS/BS) represents the percentage of bone surface exhibiting mineralizing activity. Bone formation rate per bone surface (BFR/BS) is the volume of mineralized bone formed per unit time and per unit bone surface. It's calculated as the product of MAR and MS/BS, (BFR=MAR * (MS/BS)).

Reverse Transcription and Real-Time PCR

Total RNA from OCs was isolated with the RNeasy mini kit (Qiagen, Valencia, CA) and digested with DNase to eliminate genomic DNA. Complementary DNA was made using the SuperScript II first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). qPCR was performed using SYBR Advantage mix (Bio-Rad) with

mouse-specific primers as specified in Table I.

Measurement of cAMP levels in bone marrow macrophages

Cells were plated in a 48-well plate ($2x10^5$/ml) and grow over night in growth media (10% FBS-MEM alpha media supplemented with 60ng/ml recombinant MSCF). The concentration of ATP in the extracellular space can reach levels of up to 80μM (2) and the average concentration of ADP in cells is 849±367μM (3). Therefore, we used ADP concentrations in the range of 10μM to100μM. IBMX is a phosphodiesterase inhibitor and 100μM IBMX (3-isobutyl-1-methylxanthine) was used in all experiment to prevent cAMP degradation during the assay. The cells were stimulated with appropriate reagents for 10 minutes at 37°C, in the presence of IBMX. Control wells were treated with vehicle to measure the basal cAMP levels. All experiments were performed in the growth medium. Pertussis toxin (100ng/ml) was added 12 h before experiment. Cyclic AMP levels were determined using a cAMP Chemiluminescent Imunoassay Kit (Invitrogen) according to the manufacturer's instructions.

Reference List

1. Rauch,D.A., Hurchla,M.A., Harding,J.C., Deng,H., Shea,L.K., Eagleton,M.C., Niewiesk,S., Lairmore,M.D., Piwnica-Worms,D., Rosol,T.J. et al 2010. The ARF tumor suppressor regulates bone remodeling and osteosarcoma development in mice. *PLoS. One.* **5**:e15755.

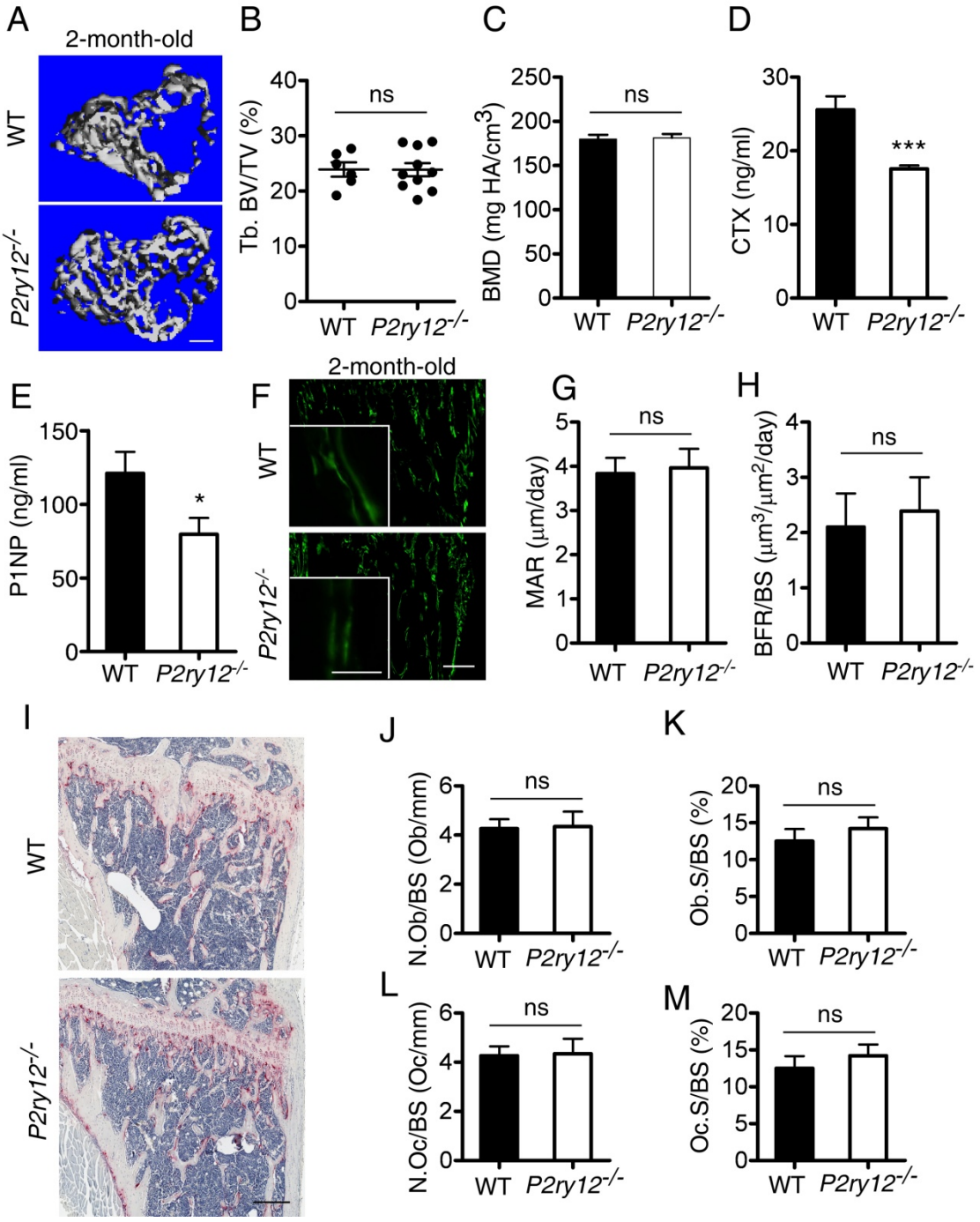
2. Corriden,R., Insel,P.A., and Junger,W.G. 2007. A novel method using fluorescence microscopy for real-time assessment of ATP release from individual cells. *Am. J. Physiol Cell Physiol* **293**:C1420-C1425.

3. Traut,T.W. 1994. Physiological concentrations of purines and pyrimidines. *Mol. Cell Biochem.* **140**:1-22.

Table 1. Primers for qPCR

Gene	Primer sequence (5'-3')	PCR product size (bp)
P2ry1	Forward primer: CAGGGTTTATGCCACTTATCAGG Reverse primer: AGATCATCTCAGGGATGTCTTGTG	282
P2ry12	Forward primer: GGGCGTACCCTACAGAAACA Reverse primer: TGTTGACACCAGGCACATCC	205
Igtb3	Forward primer: TGGTGCTCAGATGAGACTTTGTC Reverse primer: GACTCTGGAGCACAATTGTCCTT	86
TRAP	Forward primer: CAGCTGTCCTGGCTCAAAA Reverse primer: ACATAGCCCACACCGTTCTC	218
Ctsk	Forward primer: GAGGGCCAACTCAAGAAGAA Reverse primer: GCCGTGGCGTTATACATACA	203
Nfatc1a	Forward primer: GGTAACTCTGTCTTTCTAACCTTAAGCTC Reverse primer: GTGATGACCCCAGCATGCACCAGTCACAG	240
CTR	Forward primer: TGGTTGAGGTTGTGCCCAATGGAGA Reverse primer: CTCGTGGGTTTGCCTCATCTTGGTC	1a: 392 1b: 503
DC-STAMP	Forward primer: ACAAACAGTTCCAAAGCTTGC Reverse primer: TCCTTGGGTTCCTTGCTTC	74
OSCAR	Forward primer: CTGCTGGTAACGGATCAGCTCCCCAGA Reverse primer: CCAAGGAGCCAGAACCTTCGAAACT	310
RANK	Forward primer: CACAGACAAATGCAAACCTTG Reverse primer: GTCTTCTGGAACCATCTTCTCC	400
Csf1r	Forward primer: GCGATGTGTGAGCAATGGCAGT Reverse primer: AGACCGTTTTGCGTAAGACCTG	341
Gapdh	Forward primer: TTCACCACCATGGAGAAGGC Reverse primer: GGCATGGACTGTGGTCATGA	236

S1.

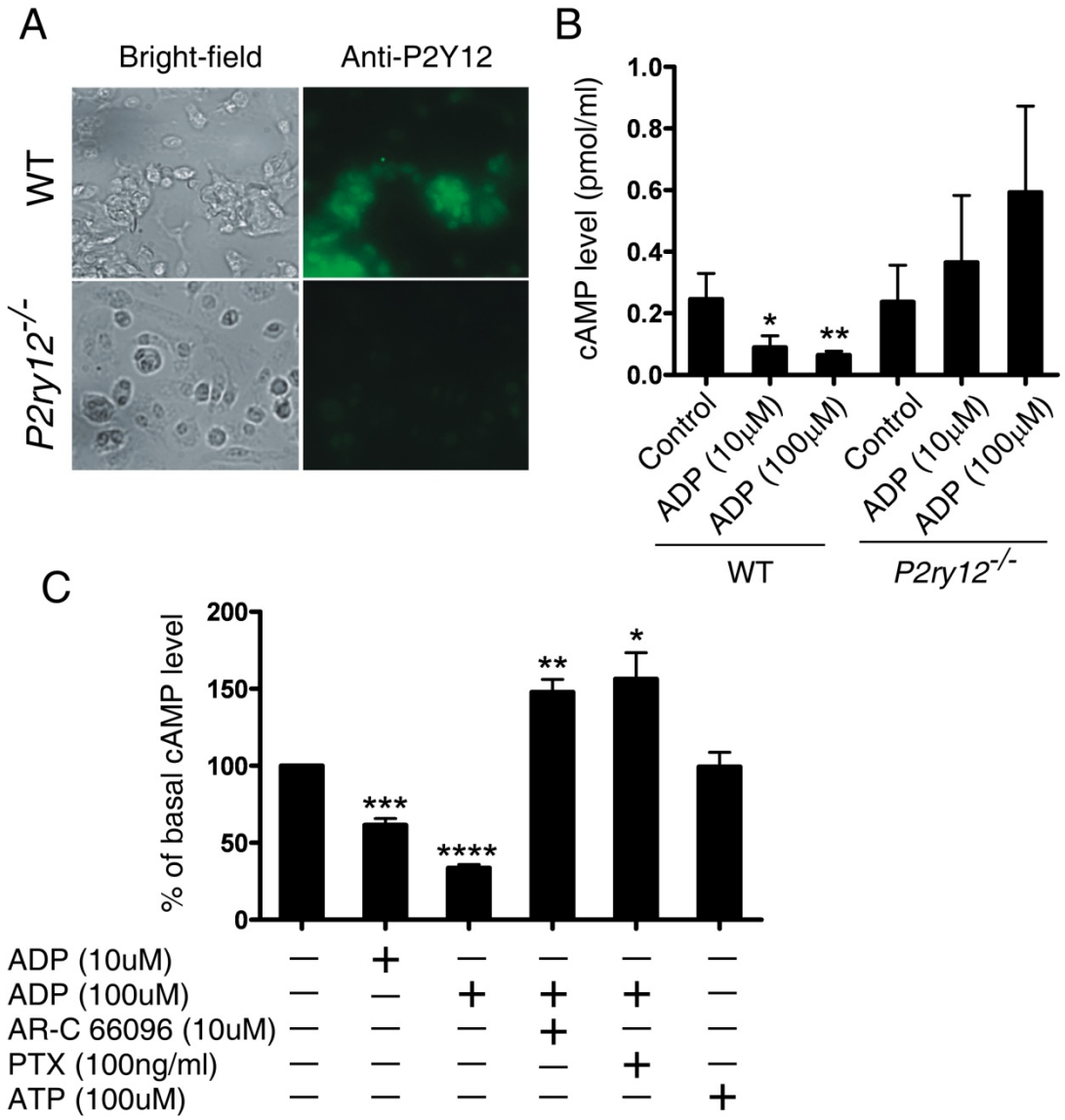

A
2-month-old
WT
P2ry12-/-
B
Tb. BV/TV (%)
ns
WT
P2ry12-/-
C
BMD (mg HA/cm3)
ns
WT
P2ry12-/-
D
CTX (ng/ml)
***
WT
P2ry12-/-
E
P1NP (ng/ml)
*
WT
P2ry12-/-
F
2-month-old
WT
P2ry12-/-
G
MAR (μm/day)
ns
WT
P2ry12-/-
H
BFR/BS (μm3/μm2/day)
ns
WT
P2ry12-/-
I
WT
P2ry12-/-
J
N.Ob/BS (Ob/mm)
ns
WT
P2ry12-/-
K
Ob.S/BS (%)
ns
WT
P2ry12-/-
L
N.Oc/BS (Oc/mm)
ns
WT
P2ry12-/-
M
Oc.S/BS (%)
ns
WT
P2ry12-/-

Supplementary Figure 1

2-month-old *P2yr12*$^{-/-}$ mice showed normal bone phenotype

(A-C) The primary and secondary spongiosa of the tibias from 2-month-old male WT (n=6) and *P2ry12*$^{-/-}$ (n=10) littermate mice were analyzed by µCT. **(A)** Representative 3D reconstructions of trabecular bone. Scale bar: 200µm. **(B)** Calculation of trabecular bone volume to total bone volume (BV/TV) and **(C)** bone mineral density (BMD). **(D, E)** Serum concentration of the C-terminal fragment of collagen I (CTX) and N-terminal propeptide of type I procollagen (P1NP) measured by ELISA. **(F)** Bone formation was visualized by double calcein labeling and visualized in the trabecular bone. Representative pictures: 2.5x, scale bar: 300µm, 20x, scale bar: 50µm. The MAR and BFR are shown **(G, H)**. Bone histology, representative TRAP staining **(I)**, osteoblast number and surface **(J, K)**, osteoclast number and surface **(L, M)** in the primary and secondary spongiosa of the tibia. Scale bar: 300µm. Data represent mean±SD, n=6. *p< 0.05, **p< 0.01, ***p< 0.001.

S2.


A
Bright-field
Anti-P2Y12
WT
P2ry12-/-
B
cAMP level (pmol/ml)
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Control
ADP (10μM)
ADP (100μM)
Control
ADP (10μM)
ADP (100μM)
WT
P2ry12-/-
C
% of basal cAMP level
200
150
100
50
0
***
****
**
*
ADP (10uM)
ADP (100uM)
AR-C 66096 (10uM)
PTX (100ng/ml)
ATP (100uM)

Supplementary Figure 2

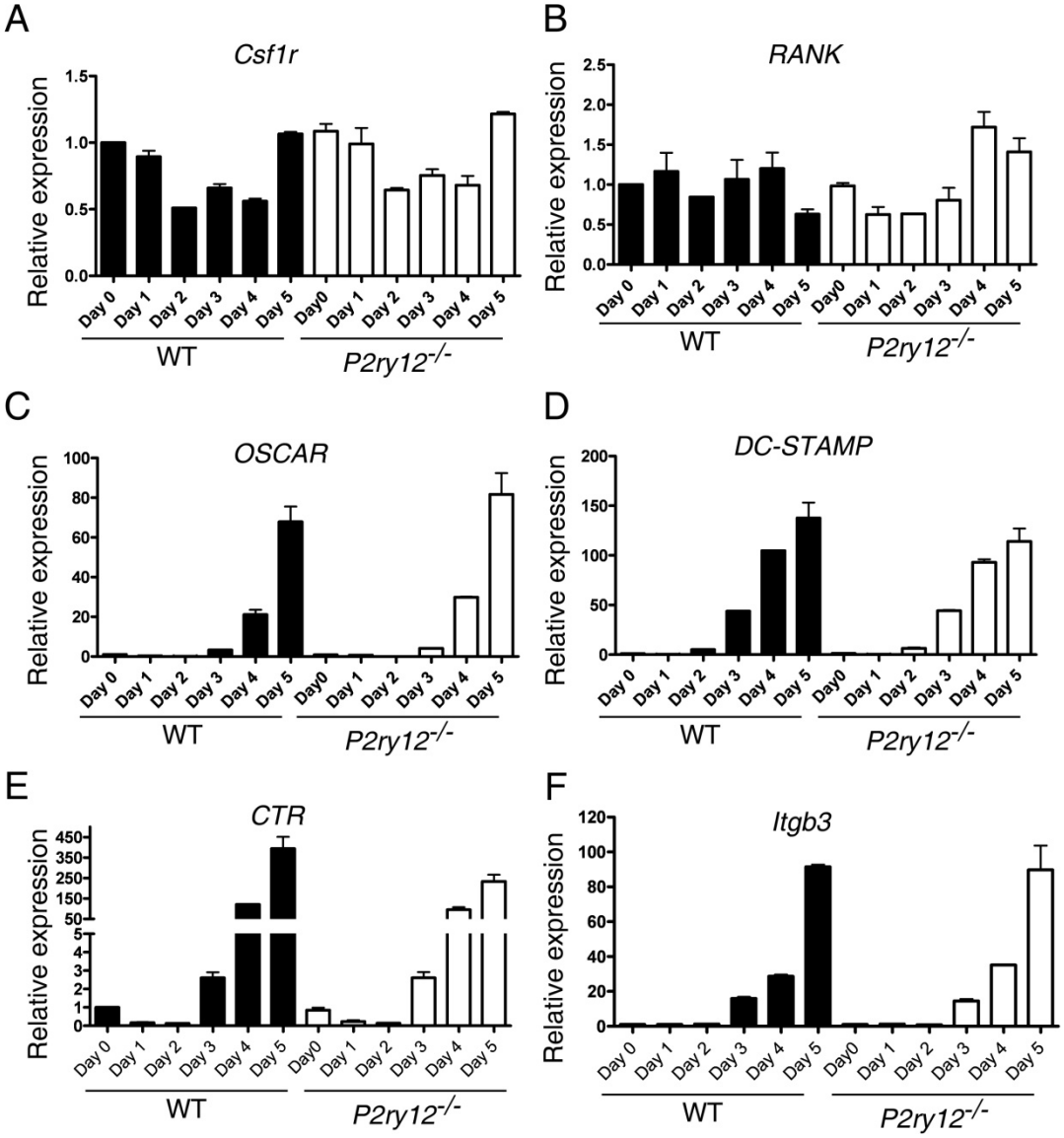
P2Y12 receptor is expressed on macrophages and downregulates intracellular cAMP level following ADP stimulation

(A) Immunofluorescence analysis of P2Y12 expression in day 3 osteoclast culture.

(B) ADP inhibits cAMP production in WT macrophages but not *P2ry12*$^{-/-}$ macrophages.

(C) ADP inhibits cAMP production in bone marrow macrophages via P2Y12 receptor. Bone marrow macrophages were stimulated with ADP or ATP for 10 minutes. Where indicated, the P2Y12 inhibitor (AR-C 66096) was added 10 minutes prior to stimulation or PTX (100ng/ml) was added 12 h prior. All experiments were performed in the presence of 100 μM IBMX. Data are the mean±S.E.M. from at least four independent experiments. *p< 0.05, **p< 0.01, ***p<0.001

S3.

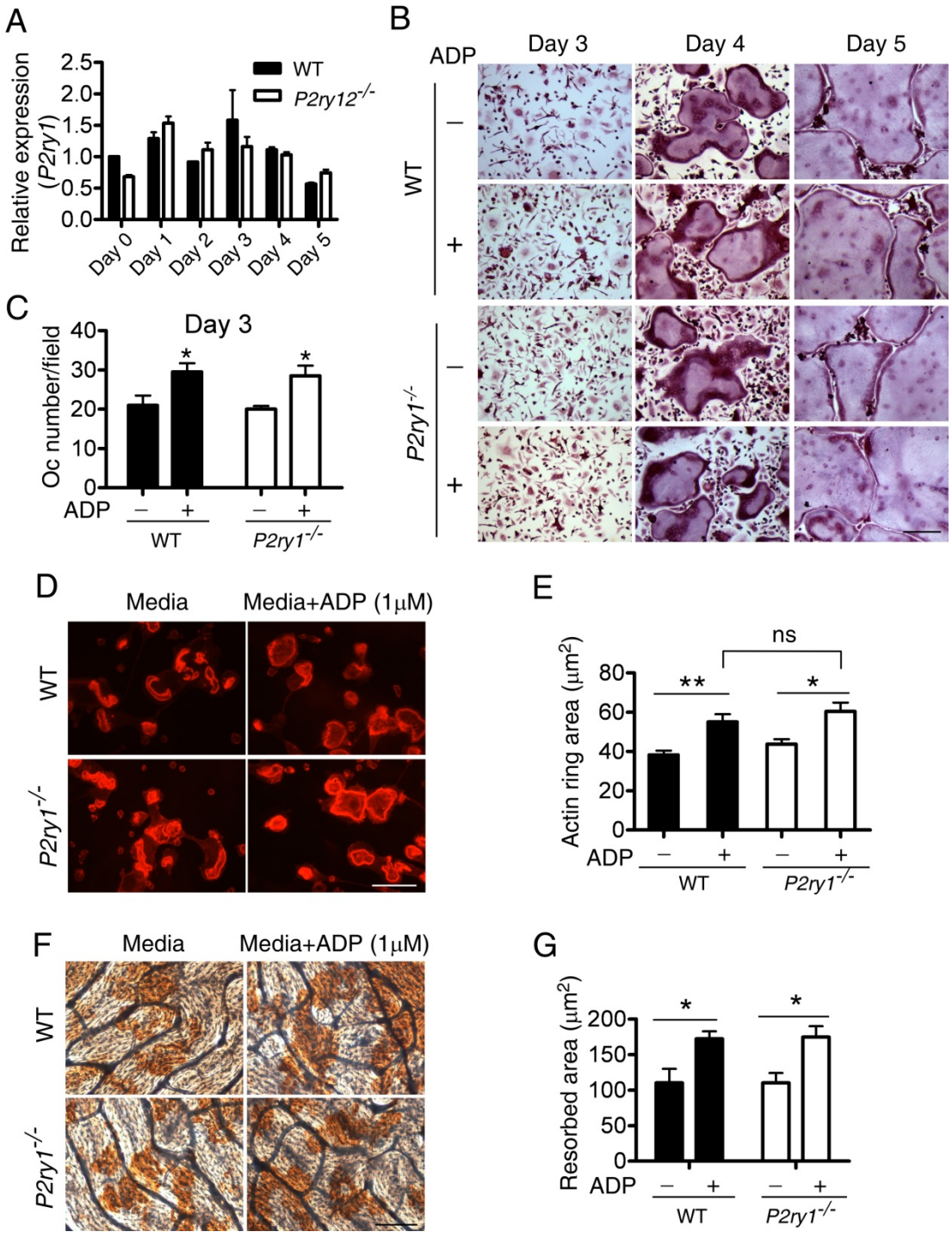

A
Csf1r
Relative expression
1.5
1.0
0.5
0.0
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day0
Day 1
Day 2
Day 3
Day 4
Day 5
WT
P2ry12-/-
B
RANK
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
WT
P2ry12-/-
C
OSCAR
Relative expression
100
80
60
40
20
0
WT
P2ry12-/-
D
DC-STAMP
Relative expression
200
150
100
50
0
WT
P2ry12-/-
E
CTR
Relative expression
450
350
250
150
50
5
4
3
2
1
0
WT
P2ry12-/-
F
Itgb3
Relative expression
120
100
80
60
40
20
0
WT
P2ry12-/-

Supplementary Figure 3

***P2yr12*$^{-/-}$ osteoclasts display normal regulation of differentiation markers by qPCR.**

(A) Macrophage Colony Stimulating Factor Receptor (MCSFR, *Csf1r*), **(B)** Receptor Activator of Nuclear Factor κ B (*RANK*), **(C)** osteoclast-associated receptor (*OSCAR*), **(D)** dendritic cell-specific transmembrane protein (*DC-STAMP*), **(E)** Calcitonin receptor (*CTR*), **(F)** Integrin β3 (*Itgb3*). All data were normalized to levels of *gapdh*, comparing WT (left) and *P2ry12*$^{-/-}$ (right) cells on days 0-5 of OC differentiation. Data represent mean±SD from three independent experiments.

S4

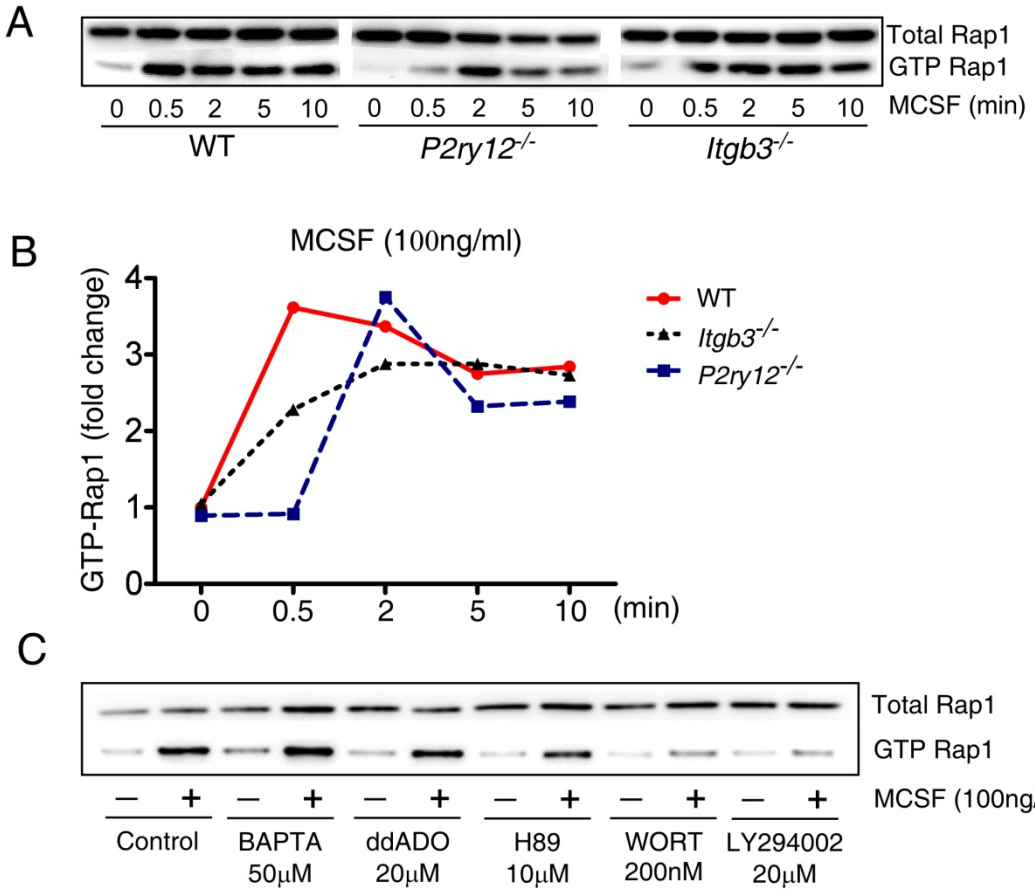

A
Relative expression (P2ry1)
2.5
2.0
1.5
1.0
0.5
0.0
WT
P2ry12-/-
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
B
ADP
Day 3
Day 4
Day 5
WT
P2ry1-/-
–
+
C
Day 3
Oc number/field
40
30
20
10
0
ADP
–
+
WT
P2ry1-/-
D
Media
Media+ADP (1μM)
WT
P2ry1-/-
E
Actin ring area (μm2)
80
60
40
20
0
ns
**
*
ADP
WT
P2ry1-/-
F
Media
Media+ADP (1μM)
WT
P2ry1-/-
G
Resorbed area (μm2)
200
150
100
50
0
*
*
ADP
WT
P2ry1-/-

Supplementary Figure 4

***P2ry1*$^{-/-}$ osteoclasts have intact responses to ADP**

(A) *P2ry1* gene expression in WT and *P2ry12*$^{-/-}$ during OC differentiation, analyzed by quantitative real time PCR. **(B)** WT and *P2ry1*$^{-/-}$ OCs stained for TRAP on days 3, 4 and 5 of OC differentiation with M-CSF and RANKL, in the presence or absence of ADP**.** Scale bar: 200μm. **(C)** Quantitation of OC (≥ 3 nuclei) number at day 3 of osteoclast culture. **(D-G)** BMMs derived from 2 month old WT or *P2ry12*$^{-/-}$ mice were cultured with RANKL and MCSF on bone slices for 5 days. ADP was added at a final concentration of 1μM where indicated. **(D)** Actin ring formation was determined by immunofluorescence following Rhodamine phalloidin staining. Scale bar: 100μm. **(E)** Quantification of actin ring area. **(F, G)** Quantification of pits area. Scale bar: 100μm. n = 3 per condition. Data represent mean±SD, *p< 0.05, **p< 0.01, ***p<0.001.

S 5

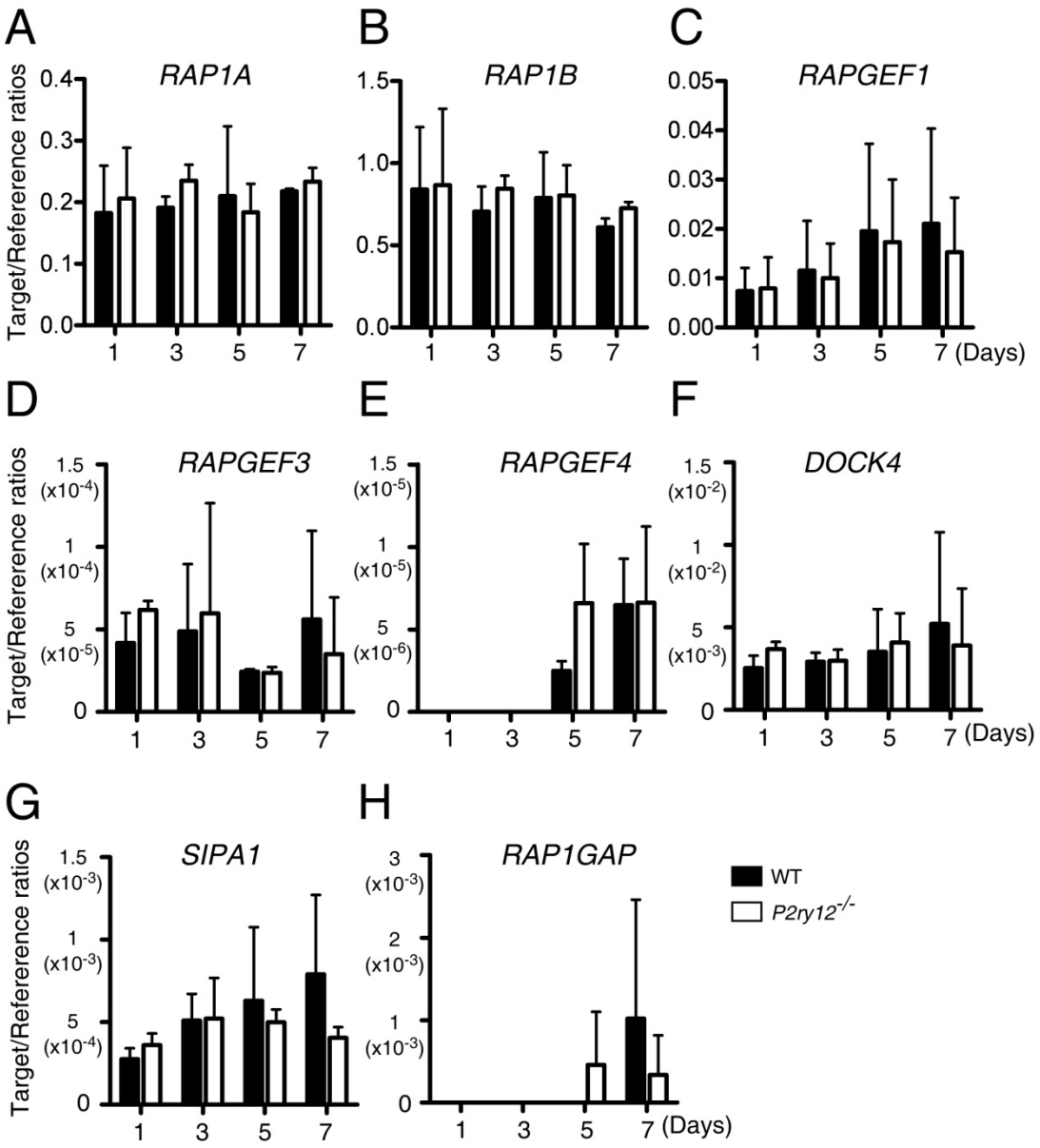

A
Total Rap1
GTP Rap1
0 0.5 2 5 10 0 0.5 2 5 10 0 0.5 2 5 10
MCSF (min)
WT
P2ry12-/-
Itgb3-/-
B
MCSF (100ng/ml)
GTP-Rap1 (fold change)
4
3
2
1
0
0 0.5 2 5 10 (min)
WT
Itgb3-/-
P2ry12-/-
C
Total Rap1
GTP Rap1
– + – + – + – + – + – +
MCSF (100ng/ml)
Control
BAPTA 50μM
ddADO 20μM
H89 10μM
WORT 200nM
LY294002 20μM

Supplementary Figure 5

***P2ry12*$^{-/-}$ OCs showed impaired MCSF-induced Rap1 activation**

(A) Rap1 and Rap1-GTP from WT and *P2ry12*$^{-/-}$ pre-OCs (day 3 OC culture) stimulated with 100ng/ml MCSF for 0-10 minutes. Total cell lysate was used for detection of Rap-1. Activated Rap1 (Rap1-GTP) was pulled-down by using Ral GDS-RBD labeled agarose beads. **(B)** Densiometry of GTP-Rap1 induction versus WT time 0**. (C)** WT pre-OCs was treated with or without 100ng/ml MCSF and chelation of calcium (BAPTA), adenylate cyclase inhibitors (ddADO), PKA (H89) or inhibitors of PI3K (wortmannin or LY294002) as indicated. Total Rap1 and activated Rap1 were detected as above.

S6.

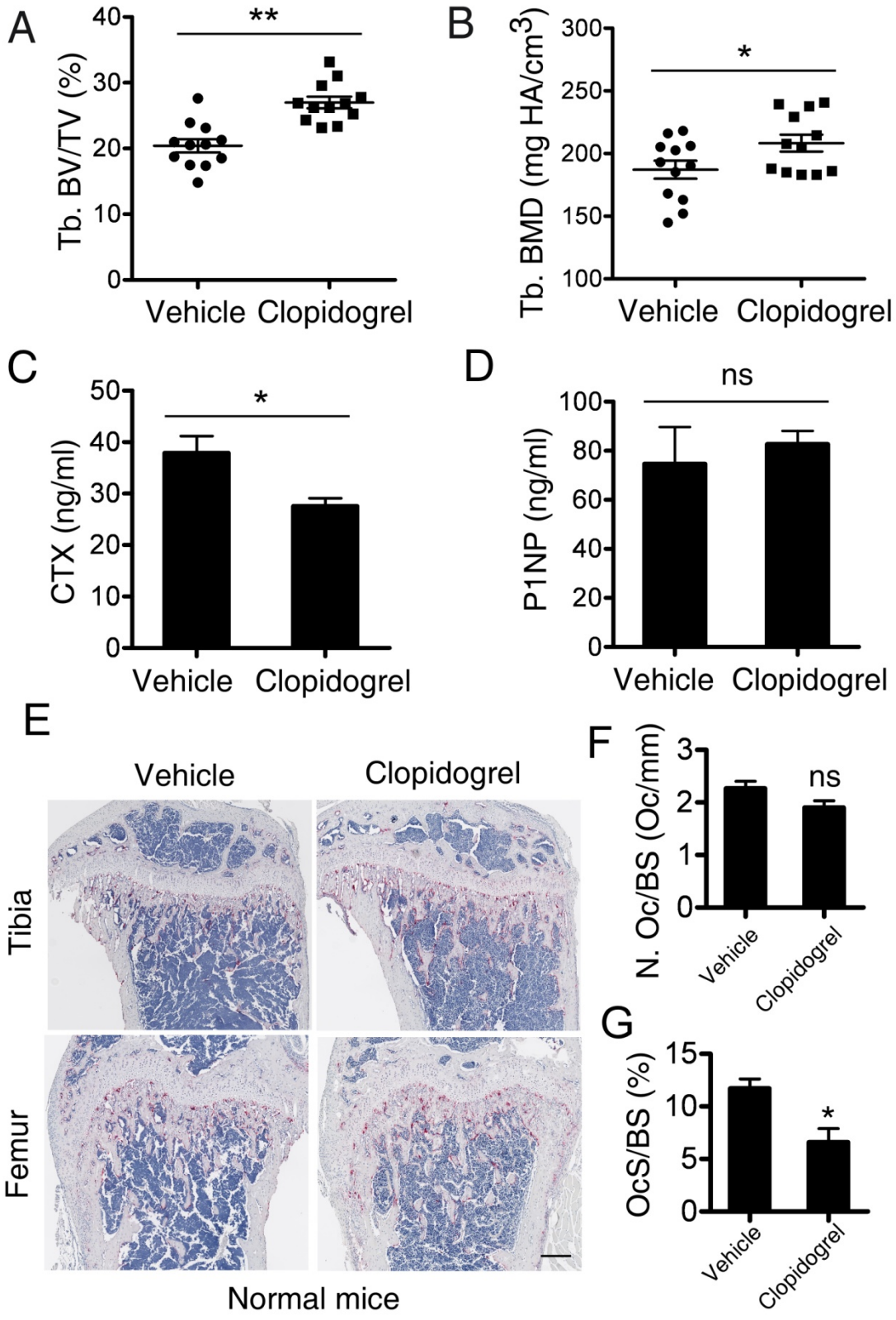

A
RAP1A
B
RAP1B
C
RAPGEF1
D
RAPGEF3
E
RAPGEF4
F
DOCK4
G
SIPA1
H
RAP1GAP
Target/Refererence ratios
(Days)
WT
P2ry12-/-

Supplementary Figure 6

***P2ry12*$^{-/-}$ OCs showed normal mRNA expression of Rap1 regulatory genes during OCs formation**

Expression of Rap1 regulatory genes determined by quantitative real time RT-PCR in developing OCs. **(A)** Ras-related protein Rap-1A (*RAP1A*), **(B)** Ras-related protein Rap-1b (*RAP1B*), **(C)** Rap guanine nucleotide exchange factor 1 (*RAPGEF1*), **(D)** Rap guanine nucleotide exchange factor 3 (*RAPGEF3*), **(E)** Rap guanine nucleotide exchange factor 4 (*RAPGEF4*), **(F)** Dedicator of cytokinesis 4 (*DOCK4*), **(G)** Signal-induced proliferation-associated protein 1 (*SIPA1*), **(H)** Rap1 GTPase-activating protein 1 (*RAP1GAP*). Data represent mean±SD.

S7.

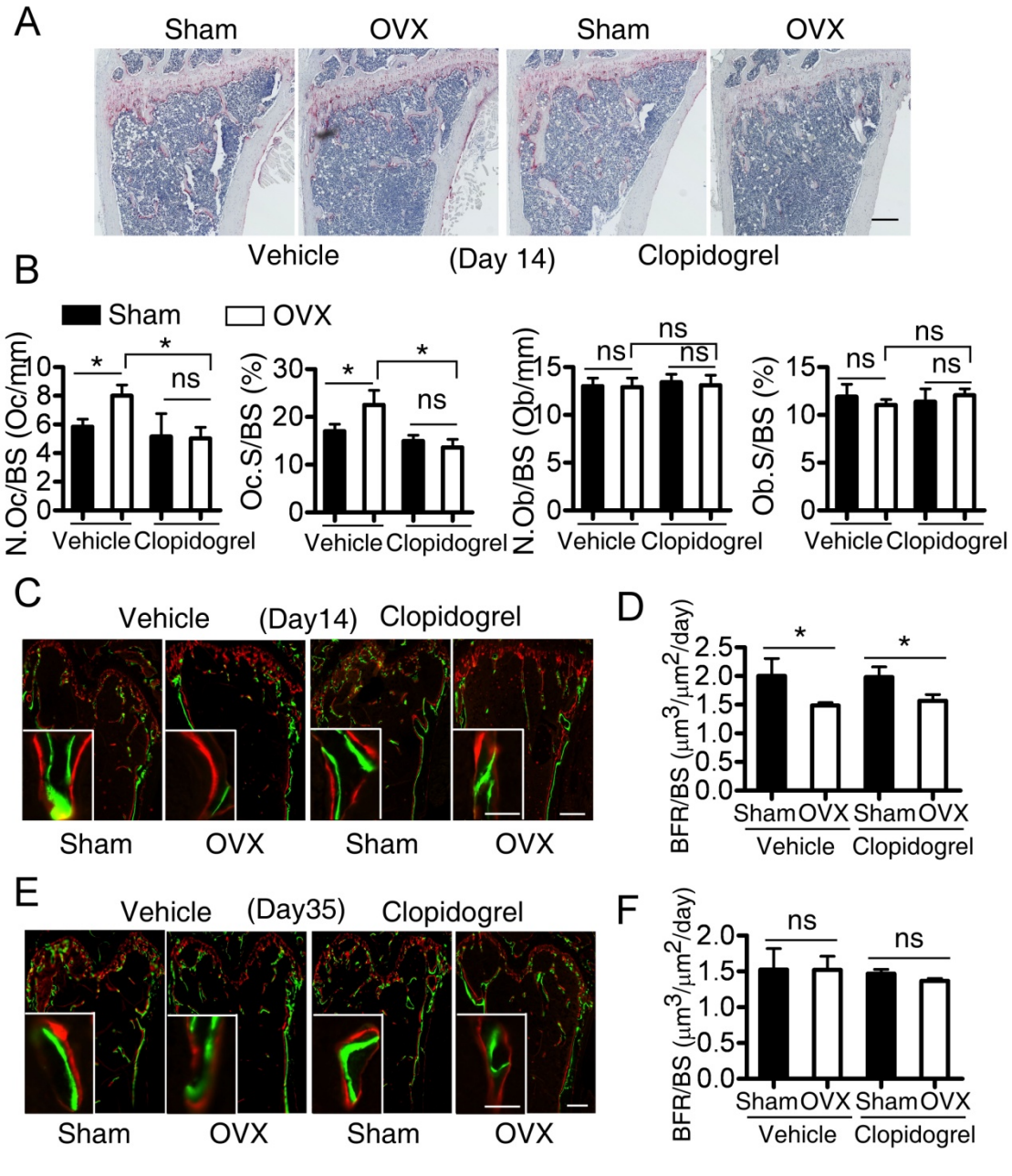

A
Tb. BV/TV (%)
40
30
20
10
0
**
Vehicle
Clopidogrel
B
Tb. BMD (mg HA/cm3)
300
250
200
150
100
*
Vehicle
Clopidogrel
C
CTX (ng/ml)
50
40
30
20
10
0
*
Vehicle
Clopidogrel
D
P1NP (ng/ml)
100
80
60
40
20
0
ns
Vehicle
Clopidogrel
E
Vehicle
Clopidogrel
Tibia
Femur
Normal mice
F
N. Oc/BS (Oc/mm)
3
2
1
0
ns
Vehicle
Clopidogrel
G
OcS/BS (%)
15
10
5
0
*
Vehicle
Clopidogrel

Supplementary Figure 7

Pharmacologic inhibition of P2Y12 receptor in normal mice increased trabecular bone volume.

Clopidogrel treatment (30mg/kg/day in drinking water) of 6 week old WT Balb/c mice for 9 days. *n*=12 per group. **(A, B)** Trabecular bone volume (BV/TV) and bone mineral density of tibiae were analyzed by μCT in the primary and secondary spongiosa of the tibia. **(C, D)** Serum concentration of the C-terminal fragment of collagen I (CTX) and N-terminal propeptide of type I procollagen (P1NP) were measured by ELISA after 9 days treatment with clopidogrel. **(E)** TRAP-stained hindl imbs sections at day 9 after vehicle or clopidogrel treatment. TRAP+ staining (dark red areas) indicate OC presence. Scale bar: 300μm. Osteoclast number and surface in the primary and secondary spongiosa of the tibia are shown **(F, G).** Data represent mean±SD, *$p< 0.05$, **$p< 0.01$, ***$p<0.001$.

S8.


A
Sham
OVX
Sham
OVX
Vehicle
(Day 14)
Clopidogrel
B
Sham
OVX
N.Oc/BS (Oc/mm)
Oc.S/BS (%)
N.Ob/BS (Ob/mm)
Ob.S/BS (%)
ns
Vehicle
Clopidogrel
C
Vehicle
(Day14)
Clopidogrel
Sham
OVX
Sham
OVX
D
BFR/BS (μm3/μm2/day)
Sham
OVX
Sham
OVX
Vehicle
Clopidogrel
E
Vehicle
(Day35)
Clopidogrel
Sham
OVX
Sham
OVX
F
BFR/BS (μm3/μm2/day)
ns
Sham
OVX
Sham
OVX
Vehicle
Clopidogrel

Supplementary Figure 8

P2Y12 antagonism partially protected mice from ovariectomy-induced bone loss

14 week old WT C57BL/6 mice underwent OVX and sham-operation (Sham) were treated with clopidogrel (30mg/kg/day) or vehicle-control in drinking water for indicated time. *n*=8 per group. Tibia from sham-operated (sham) and OVX mice was analyzed by histomorphometry. (**A**) TRAP-stained tibias sections after ovariectomy. Scale bar: 300µm. **(B)** Quantification of osteoclast and osteoblast cells 14 days after OVX**.** OC number (N.Oc) per bone surface (N.Oc/BS) and OB number per bone surface (N.Ob/BS) in the primary and secondary spongiosa of the tibia. **(C, E)** Bone formation was visualized in the trabecular bone by calcein (first) and alizarin red (second) double labeling. Representative pictures: 2.5x, scale bar: 300µm, 20x, scale bar: 50µm. **(D, F)** The quantification of MAR and bone formation rate. Data represent mean±SD, *n*=5. **p*< 0.05, ***p*< 0.01, ****p*< 0.001