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**Abstract**

Osteoclasts are multinucleated giant cells derived from myeloid progenitors. Excessive bone resorption by osteoclasts can result in serious clinical outcomes for which better treatment options are needed. Here, we identified fibronectin leucine-rich transmembrane protein 2 (Flrt2), a ligand of the Unc5 receptor family for neurons, as a novel target associated with the late/maturation stage of osteoclast differentiation. Flrt2 expression is induced by stimulation with receptor activator of nuclear factor-kB ligand (RANKL). Flrt2 deficiency in osteoclasts results in reduced hyper-multinucleation, which could be restored by RNAi-mediated knockdown of Unc5b. Treatment with Netrin1, another ligand of Unc5b which negatively controls osteoclast multinucleation through down regulation of RANKL-induced Rac1 activation, showed no inhibitory effects on Flrt2-deficient cells. In addition, RANKL-induced Rac1 activation was attenuated in Flrt2-deficient cells. Taken together, these results suggest that Flrt2 regulates osteoclast multinucleation by interfering with Netrin 1-Unc5b interaction and may be a suitable therapeutic target for diseases associated with bone remodeling.

## Introduction

Skeletal homeostasis is maintained by balancing the bone forming function of osteoblasts and bone resorbing function of osteoclasts. Functional imbalance of osteoblasts and osteoclasts results in abnormal bone homeostasis (1). Osteoclasts are specialized multinucleated giant cells derived from monocyte/macrophage lineage bone marrow precursors (2). Osteoclast differentiation is initiated and sustained primarily by the osteoclast differentiation factor RANKL, which is mainly produced by osteoblasts and osteocytes (3, 4). Under pathogenic inflammatory conditions, excessive bone resorption by osteoclasts is often observed (5, 6). Currently, bisphosphonate and anti-RANKL antibody (Denosumab), which target early osteoclast commitment and/or osteoclast viability, are used for treatment (7). Although these treatments are efficiently anti-resorptive, long-term use compromises bone strength due to unintended inhibition of coupled bone formation. A better treatment strategy may be to target only late-stage osteoclast biological processes (i.e., osteoclast "maturation"), while preserving early osteoclast differentiation necessary for coupled bone formation (8). To do so in a manner that could lead to novel effective therapeutics to target bone remodeling diseases will require further investigation of the molecular mechanisms that control osteoclast maturation.

A hallmark of osteoclast maturation is multinucleation (9). Multinucleation is required for physiological bone resorbing activities (10), but hyper-multinucleation (defined by osteoclasts with >100 micron diameter) often results in pathological bone loss (5, 6, 11). To identify gene targets associated with the late/maturation stage of osteoclast differentiation, we have previously performed comparative gene expression profiling by using multinucleation as a functional readout (8). As a result, we identified the gene *Flrt2* which encodes the protein fibronectin leucine-rich transmembrane protein 2 (Flrt2) (Supplementary Fig 1). Flrt2 is a member of the fibronectin leucine-rich transmembrane family of proteins (Flrts: Flrt1-3) (12), which are classified as cell adhesion molecules (CAMs) (13), and are also reported to interact with heterophilic receptors,

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functioning as guidance factors in vascular, neural, and early embryonic development (14-21). Flrt2 has been reported to function as a chemorepellent in axon guidance and cell migration through interactions with uncoordinated-5 (Unc5) receptors (19), and also to function in cell adhesion through an interaction with Latrophilins (22).

In this study, we identified Flrt2, which has no previously reported function in bone, as a regulator of osteoclast multinucleation. We report here that gene deletion of *Flrt2* in osteoclasts resulted in reduced osteoclast hyper-multinucleation. RNAi-mediated knockdown of Unc5b in Flrt2-deficient cells restored osteoclast hyper-multinucleation. Treatment with Netrin1, an alternative ligand of Unc5b which has been shown to inhibit osteoclast multinucleation through negative regulation of RANKL-induced Rac1 activation (23), showed no inhibitory effects on Flrt2-deficient cultures. Additionally, RANKL-induced Rac1 activation was impaired in Flrt2-deficient osteoclasts. These results suggest the possibility that Flrt2 and Netrin1 compete for Unc5b binding during osteoclast multinucleation, as osteoclast hyper-multinucleation is inhibited in Flrt2-deficient cells. Collectively, our results reveal a novel role for Flrt2 in fine-tuning of osteoclast multinucleation.

**Results**

**Flrt2 is involved in *in vitro* osteoclast differentiation**

In order to understand the role of Flrt2 in osteoclasts, we first examined *Flrt2* gene expression dynamics during osteoclast differentiation. We generated osteoclasts *in vitro* from mouse bone marrow-derived monocytes (BMMs) treated with M-CSF + RANKL for up to three days, and performed temporal qPCR expression analysis and western blots using whole cell lysates pulled down with a lectin that recognize glycoproteins including Flrt2. Expression of Flrt2 was induced, and peaked on day one after RANKL stimulation (Fig 1A). We next generated Flrt2 conditional knockout mice by crossing floxed Flrt2 mice (Flrt2$^{fl/fl}$) with Mx1Cre mice, which express inducible

Cre recombinase, since global deletion of Flrt2 expression in mice leads to embryonic lethality (17). In Flrt2^{fl/fl}Mx1Cre mice, the *Flrt2* gene is deleted upon polyinosinic-polycytidylic acid (poly I:C) treatment in osteoclast precursors, enabling us to examine the effect of Flrt2 deletion on osteoclast differentiation. We generated BMMs from Flrt2^{fl/fl}, Flrt2^{fl/+}Mx1Cre, and Flrt2^{fl/fl}Mx1Cre mice, and confirmed *Flrt2* gene deletion in genomic DNA from Flrt2^{fl/fl}Mx1Cre BMMs using PCR (Fig 1B). Consistent with this, *Flrt2* gene expression was completely abolished in Flrt2^{fl/fl}Mx1Cre BMMs (Fig 1B). We then examined whether *Flrt2* gene deletion affects osteoclast formation. We prepared Flrt2^{fl/fl} and Flrt2^{fl/fl}Mx1Cre BMMs and cultured with M-CSF + RANKL to generate osteoclasts. We found reductions in total tartrate-resistant acidic phosphatase (TRAP) activity and in frequency of multinucleated TRAP^{+} cells (i.e., mature osteoclasts) in Flrt2^{fl/fl}Mx1Cre cultures compared to Flrt2^{fl/fl} cultures (Fig 1C). Reductions in hyper-multinucleated osteoclasts (>100 $\mu$m) were particularly dramatic (Fig 1C). Message levels for the osteoclast differentiation and maturation markers *Nfatc1*, *CtsK*, and *Atp6v0d2* showed small but still significant differences in Flrt2^{fl/fl}Mx1Cre cultures on Day 3, while *Dcstamp* showed no differences (Fig 1D). Retroviral transduction of Flrt2^{fl/fl}Mx1Cre BMMs with full length Flrt2 completely restored TRAP[+] hyper-multinucleated cells in Flrt2^{fl/fl}Mx1Cre cultures (Fig 1E), suggesting that the phenotypes observed in Flrt2^{fl/fl}Mx1Cre cultures can be attributed to deletion of the Flrt2 gene. These results suggest a role for osteoclast-derived Flrt2 in osteoclast differentiation, and more specifically in osteoclast hyper-multinucleation.

**Osteoclast-derived Flrt2 is required for bone homeostasis**.

To examine whether osteoclast-derived Flrt2 is required for bone development and homeostasis, we performed representative 3D reconstructions of trabecular bone from the femurs of gender- and age-matched Flrt2^{fl/fl} and Flrt2^{fl/fl}Mx1Cre mice. Bone microstructure imaging by high resolution microcomputed tomography ($\mu$CT) of Flrt2^{fl/fl}Mx1Cre mice revealed slight but

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significant increases in bone mass, characterized by augmented bone indices including trabecular bone volume per tissue volume (BV/TV), trabecular number (Tb.N), and concomitant decreases in trabecular spacing (Tb.Sp) (Fig 2). No significant differences in trabecular thickness or cortical bone thickness (Ct.Th) were detected between Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre mice (Fig 2). These results suggest that osteoclast-expressed Flrt2 is involved in bone homeostasis.

**Flrt2, Netrin 1 and Unc5b regulation of osteoclast multinucleation**

To understand the molecular mechanism that regulates Flrt2-mediated osteoclast hyper-multinucleation, we examined RANKL-dependent signaling pathways, including ERK, p38, JNK, and NF-kB, which are activated via the RANK-TRAF6 axis. We found no significant differences in activation of these signaling molecules between Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre cultures (data not shown), suggesting that osteoclast-expressing Flrt2 is not involved in regulation of the RANK-TRAF6 signaling axis.

Flrts have been reported to act as CAMs and also as ligands of repulsive receptors (21, 24). Flrts have also been reported to be proteolytically cleaved near the plasma membrane in neurons (19). To determine whether Flrt2 functions as a CAM or as a ligand in osteoclasts, we examined cleavage of Flrt2 during osteoclast differentiation. We prepared whole cell lysates and supernatants from Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre cultures, pulled down with a lectin, and performed western blotting using Flrt2-specific antibody. Membrane-bound Flrt2, which ranges in size from 80 to 100 kDa, was detected in whole cell lysate of Flrt2$^{fl/fl}$ cultures one day after RANKL stimulation (Fig 3A). Expression of membrane-bound Flrt2 gradually decreased during osteoclast differentiation, and disappeared almost completely in mature osteoclast cultures. By contrast, the cleaved form of Flrt2, which ranges in size from 60 to 80 kDa, was detected in supernatants from two days after RANKL stimulation (Fig 3A). The cleaved form of Flrt2 was decreased after treatment with Brefeldin A, a trafficking inhibitor, suggesting that trafficking of membrane-bound

Flrt2 was inhibited, and consequently, that presence of the cleaved form was decreased (Supplementary Fig 2). Neither membrane-bound nor cleaved forms of Flrt2 were detected in Flrt2^{fl/fl}Mx1Cre cultures (Fig 3A). The cleaved form of Flrt2 was detected in Flrt2^{fl/fl}Mx1Cre cultures after retroviral transduction of Flrt2 (Fig 1E). These results suggest that osteoclast-expressed Flrt2 is cleaved during osteoclast differentiation, and further suggest that Flrt2 might predominantly function as a soluble ligand that interacts with heterophilic receptors, but that does not function as a CAM.

Flrt2 has been shown to interact with Unc5b, Unc5d and/or Latrophilin3 (18-20, 22). Unc5b has been demonstrated as the predominant receptor for Flrt2 in endothelial cells during vascular development (20). To gain insight into the predominant receptor for Flrt2 in osteoclast formation, we performed qPCR expression analysis. We detected expression of *Unc5b* (Fig 3B) but not *Unc5d* or *Latrophilin3* (data not shown). The expression levels of *Unc5b* were comparable between Flrt2^{fl/fl}Mx1Cre and Flrt2^{fl/fl} cultures, and decreased in osteoclasts (Fig 3B). To identify the involvement of Unc5b in Flrt2-mediated regulation of osteoclast multinucleation, we performed RNAi experiments using retrovirus encoding shRNA for Unc5b. RNAi-mediated knockdown of Unc5b in Flrt2^{fl/fl}Mx1Cre BMMs restored osteoclast hyper-multinucleation, and furthermore, knockdown of Unc5b in Flrt2^{fl/fl} BMMs resulted in slightly increased numbers of TRAP[+] hyper-multinucleated osteoclasts (Fig 3B).

Unc5 receptors are known as classical signaling receptors for secreted Netrin ligands in the nervous system (24). In the bone system, Netrin 1 is produced by various bone cells, including osteoclasts (23, 25), and Netrin 1-Unc5b interactions negatively regulate osteoclast multinucleation (23). Interestingly, Netrin 1 has been shown to compete with Flrt2 for Unc5d (19). We found comparable message levels of *Netrin 1* in both Flrt2^{fl/fl} and Flrt2^{fl/fl}Mx1Cre BMMs and osteoclasts (Fig 3C). We examined whether Netrin 1-Unc5b interactions mediated inhibitory effects on Flrt2-deficient osteoclast maturation. We prepared BMMs from Flrt2^{fl/fl} and

Flrt2$^{fl/fl}$Mx1Cre mice and treated them with M-CSF and RANKL in the presence or absence recombinant Netrin 1. Formation of hyper-multinucleated osteoclasts was significantly inhibited in Flrt2$^{fl/fl}$ cultures, consistent with a previous report (Fig 3C) (23). By contrast, an inhibitory effect of recombinant Netrin 1 was not observed in Flrt2$^{fl/fl}$Mx1Cre cultures (Fig 3C). These results suggested that Flrt2 might compete with Netrin 1 for Unc5b, such that Unc5b might be saturated with osteoclast-derived Netrin 1 in the absence of Flrt2, and consequently, no additional inhibition was observed in Flrt2$^{fl/fl}$Mx1Cre cultures by adding recombinant Netrin 1.

Netrin 1-Unc5b interactions have been shown to inhibit RANKL-induced Rac1 activation (23). We therefore examined RANKL-induced Rac1 activation in Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre cultures. We found that RANKL-induced Rac1 activation was diminished in Flrt2$^{fl/fl}$Mx1Cre cultures compared to Flrt2$^{fl/fl}$ cultures (Fig 3D). Rac1 protein levels were normal after RNAi-mediated knockdown of Unc5b and treatment with Netrin 1 in Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre cultures (Fig 3D). These results suggest that Flrt2 is required for proper RANKL-induced Rac1 activation, and further support, to some degree, the idea that osteoclast-derived Flrt2 might compete with Netrin 1 for Unc5b to attenuate the inhibitory effect of Netrin 1 on Rac1 activation. Collectively, our results suggest that osteoclast-derived Flrt2 competes with osteoclast-derived Netrin 1 for Unc5b binding, and contributes to fine-tuning of osteoclast multinucleation.

**Discussion**

Osteoclast maturation involves multinucleation, which occurs via incomplete cytokinesis and/or cell fusion (26). The importance of multinucleation in osteoclast formation is demonstrated by the impaired bone-resorbing activity of osteoclasts that cannot become multinuclear cells (10). Although multinucleation is important for osteoclast function, hyper-multinucleation is often observed in pathogenic inflammatory conditions where it contributes to excessive bone resorption. However, the molecular mechanisms underlying regulation of osteoclast hyper-

multinucleation are not well understood. In this study, we identified Flrt2, which has previously been functionally characterized in systems other than bone, as a regulator for osteoclast multinucleation. Using conditional knockout mice, we revealed an osteoclast-intrinsic role of Flrt2 in multinucleation. Additionally, we showed involvement of Unc5b in Flrt2-mediated regulation of osteoclast multinucleation. Our results reveal Flrt2 as a novel regulator of osteoclast multinucleation.

We showed that expression of Flrt2 message peaked on Day 1 and gradually decreased (Fig 1A). Retroviral transduction of Flrt2, which causes stable expression of Flrt2 restored hyper-multinucleation in Flrt2$^{f/f}$Mx1Cre cultures (Fig 1E), suggesting that reduction of Flrt2 message during the maturation phase (Day 2 to 3 after RANKL stimulation) is not required for osteoclast maturation.

We here showed that osteoclast-expressed Flrt2 is cleaved during osteoclast differentiation, suggesting that osteoclast-derived Flrt2 might function as a ligand, but not as a CAM. A previous report showed that Flrt2 cleavage requires metalloproteinase (MMP) activity (19). In osteoclasts, MMP-9 is the most abundant MMP (27), suggesting the possibility that MMP-9 is involved in Flrt2 cleavage in osteoclasts. Although we showed here that most osteoclast-expressed Flrt2 was cleaved in mature osteoclasts, we cannot conclude that membrane-bound Flrt2 does not play a role in osteoclasts, and further studies will be required to address this issue.

We sought to identify the molecular mechanisms by which Flrt2 regulates osteoclast multinucleation, and found that RNAi-mediated knockdown of Unc5b, the predominant receptor for Flrt2 in endothelial cells and also a receptor for Netrin 1 in osteoclasts, restored hyper-multinucleation in Flrt2$^{fl/fl}$Mx1Cre cultures. These findings suggest that Unc5b is involved in Flrt2-mediated regulation of osteoclast hyper-multinucleation. Given that Netrin 1 competes with Flrt2 for Unc5d (19), we hypothesized that Flrt2 could also compete with Netrin 1 for Unc5b, thus interfering with the Netrin 1-Unc5b-mediated negative regulation on osteoclast multinucleation

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(Fig 3E). Indeed, we showed that 1) the inhibitory effect of Netrin 1 was not observed in Flrt2<sup>fl/fl</sup>Mx1Cre cultures, and 2) RANKL-induced Rac1 activation was inhibited in Flrt2<sup>fl/fl</sup>Mx1Cre cultures. Since message levels of Netrin 1 were not affected by the absence of Flrt2, it is plausible that reduced hyper-multinucleation in the absence of Flt2 is due in some part to negative regulation of Rac1 activation by Netrin 1-Unc5b interactions. However, there is a difference between Flrt2<sup>fl/fl</sup> and Flrt2fl<sup>/fl</sup>Mx1Cre cultures in terms of frequency of hyper-multinucleated osteoclasts after RNAi-mediated knockdown of Unc5b (Fig 3B), suggesting the involvement of a Unc5b-independent mechanism. Further studies will be required to determine the molecular mechanism underlying regulation of osteoclast multinucleation through Flrt2.

Considering the severity of the *in vitro* phenotype of Flrt2<sup>fl/fl</sup>Mx1Cre osteoclast differentiation, the Flrt2<sup>fl/fl</sup>Mx1Cre bone phenotype seems modest. We found expression of another Flrt family member, Flrt3 (but not Flrt1), was induced in both Flrt2<sup>fl/fl</sup> and Flrt2<sup>fl/fl</sup>Mx1Cre cultures (Supplementary Fig 3, and data not shown). Flrt3 has been demonstrated to act as a ligand of Unc5b in the nervous system. Indeed, RNAi-mediated knockdown of Flrt3 in Flrt2<sup>fl/fl</sup>Mx1Cre BMMs showed an additive inhibitory effect on osteoclast hyper-multinucleation (Supplementary Fig 3), raising the possibility of involvement of Flrt3 in osteoclast differentiation and bone homeostasis *in vivo*. Future characterization of Flrt2 and Flrt3 double conditional knockout mice may be useful for addressing this issue. In addition, Flrts are broadly expressed proteins. Given that Flrts are cleaved, cleaved Flrts derived from other tissues might compensate the lack of osteoclast-derived Flrt2 *in vivo*. Further studies will be required to clarify the impact of Flrts in bone homeostasis.

Taken together, although a full understanding of how Fltr2 contributes to osteoclast multinucleation and its impact on bone homeostasis remain unclear, we provide evidence of a fine-tuning function of the chemorepellent Flrt2 in osteoclast multinucleation. With additional characterization, Flrt2 may be a good candidate for a therapeutic target for bone diseases that

are accompanied by bone destruction.

## Materials and Methods

### Mice

Flrt2$^{fl/fl}$ mice were generated as described previously (20). To obtain Flrt2 conditional knock-out mice (Flrt2$^{fl/fl}$Mx1Cre) in hematopoietic cells, homozygous Flrt2$^{fl/fl}$ mice were crossed with Mx1Cre transgenic mice [Tg(Mx1-cre)1Cgn] purchased from Jackson Laboratory. For induction of Cre recombinase expression in Mx1Cre mouse, mice were injected intraperitoneally with 250 $\mu$g Poly(I:C) (SIGMA) 10 days after birth three times every other day. Mice were used for in vitro and in vivo experiments 6 weeks after the first injection. All mice were maintained and used in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

### Reverse transcription and real-time PCR (qPCR)

Total RNAs were extracted from cells using Trizol reagent (Invitrogen), and reverse transcription was performed with High capacity cDNA kit (Thermo Fisher Scientific). qPCR was performed using Quant Studio3 (Applied Biosystems) with the following TaqMan probes: Flrt2 (Mm03809571_m1), Flrt3 (Mm01328142_m1), Unc5b (Mm0050454_m1), DCstamp (Mm04209236_m1), CtsK (Mm00484039_m1), Nfatc1 (Mm00479445_m1), ATP6v0d2 (Mm01222963_m1), 18s (Hs99999901_s1).

### In vitro osteoclast differentiation and tartrate-resistant acid phosphatase (TRAP) staining

BMMs and osteoclasts were prepared as described previously (8). Cells were fixed with 3.7% formalin and stained using the Acid, Phosphatase, Leukocyte (tartrate-resistant acid phosphatase kit) (387A-1KT, Sigma) according to the manufacturer's instructions. TRAP positive

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multi-nuclear cells were counted. Recombinant mouse Netrin1 (1109-N1) was purchased from R&D. Brefeldin A was purchased from Thermo Fisher Scientific.

**Retrovirus preparation and transduction**

To prepare retroviral particles, Plat-E packaging cells were transfected with pMX vectors encoding C-terminally FLAG-tagged Flrt2, pSuper vectors encoding siRNA targets for Unc5b (5'-GCCACGCAGATCTACTTCAAGTGTA -3'), and Flrt3 (5'- GAGCACCATTCAGATAACCACTGCA -3') using PEImax (Polysciences). Empty pMX vector and pSuper vector were used as negative controls. After 3 days, medium containing retrovirus was harvested and passed through a syringe filter (0.45 μm pore diameter). BMMs were transduced with retroviruses for 16 h with hexadimethrine bromide (8 μg/ml) in the presence of M-CSF (60 ng/ml). Infected cells were selected by culturing for 2 days in the presence of puromycin (2 μg/ml) with M-CSF (60 ng/ml). Puromycin-resistant BMMs were used for experiments.

**Immunoblot analysis**

Cells were lysed with sample buffer (0.5% Triton-X 100, 20nM Tris-HCl, pH7.5, 150mM NaCl). Glycoprotein were pulled down from total cell lysates or culture supernatants using lectin (SIGMA: L1882) to enrich Flrt2. Protein concentrations were determined using the Bradford assay. Equal amounts of lysates were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-12% gradient gel and transferred onto polyvinyl difluoride (PVDF) membranes. Western blotting was performed with the following antibodies: anti-Flrt2: AF2877 (R&D), and anti-Actin: sc-47778 (Santacruz). Rac1 activity was measured using Rac1 activation detection kit (Thermo scientific).

**Micro-computed tomography**

Femurs from Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre mice were harvested and scanned by $\mu$CT ($\mu$CT35, SCANCO Medical AG, Brüttisellen, Switzerland). For trabecular bone analysis, scans were performed at the distal femoral metaphysis 0.4 mm proximal to the growth plate. For cortical bone, the mid-diaphysis of femurs was scanned. All scans were performed at a resolution of 6 $\mu$m per slice using an X-ray energy of 55 kvp and an integration time of 300 ms. A total of 200 slices for trabecular bone and 50 slices for cortical bone were analyzed using the instrument's software.

**Statistical Analysis**

All experiments were analyzed using two-way ANOVA or unpaired Student's t-test by Prism 6.0 (GraphPad). $P < 0.05$ was considered statistically significant.

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**Conflict of interest**

The authors declare that they have no competing interests.

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**Figure legends**

**Figure 1. Flrt2 is involved in *in vitro* osteoclast differentiation**

(A) Expression of Flrt2 was quantified by Q-PCR (top) and western blot with glycoprotein-enriched whole cell lysates (lec-WCL) (bottom). (B) Scheme of the Flrt2 knock-out allele (left). PCR genotyping of $Flrt2^{fl/fl}$, $Flrt2^{fl/+}Mx1Cre$, and $Flrt2^{fl/fl}Mx1Cre$ BMMs. Flox allele and targeted allele were detected by genomic PCR with Flox primers (green arrows in left) and Del primers (orange arrows in left), respectively. Cre recombinase was detected by genomic PCR. Asterisk indicates internal controls (middle). Flrt2 expression in $Flrt2^{fl/fl}$ and $Flrt2^{fl/fl}Mx1Cre$ BMMs (right). (C) $Flrt2^{fl/fl}$ and $Flrt2^{fl/fl}Mx1Cre$ BMMs were cultured with M-CSF + RANKL for three days. TRAP activity, frequency of TRAP+ multinucleated cells (3 nuclei or more per cell), and frequency of TRAP+ hyper-multinucleated cells ($>100\ \mu m^2$) are shown. (D) Expression of indicated genes was quantified by Q-PCR. (E) $Flrt2^{fl/fl}$ and $Flrt2^{fl/fl}Mx1Cre$ BMMs were retrovirally transduced with the indicated vectors followed by culture with M-CSF + RANKL for three days. Overexpression of Flrt2 was confirmed by Q-PCR (Day 3 after RANKL) and western blot with lec-WCL and glycoprotein-enriched culture supernatants (lec-SUP) (Day 2 after RANKL). Frequency of TRAP+ hyper-multinucleated cells ($>100\ \mu m^2$) is shown. Scale bars represents 100 $\mu m$. Data are shown as mean ± S.D. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$.

**Figure 2. Osteoclast-derived Flrt2 is required for bone homeostasis**

$\mu CT$ images of femurs from $Flrt2^{fl/fl}$ and $Flrt2^{fl/fl}Mx1Cre$ mice. BV/TV, Tb.Th, Tb.N, Tb.Sp, and Ct.Th are shown. Scale bars represent 0.5 mm. Data are shown as mean ± S.D. *; $p<0.05$, **; $p<0.01$.

**Figure 3. Flrt2, Netrin 1 and Unc5b regulation of osteoclast multinucleation**

(A) Western blot analysis of lec-WCL and lec-SUP was performed using anti-Flrt2 antibody. (B)

Expression of Unc5b message was quantified by Q-PCR (left). Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre BMMs retrovirally transduced with shUnc5b or control vector were cultured with M-CSF + RANKL for three days, and knockdown of Unc5b was determined by Q-PCR (middle). Frequency of TRAP+ hyper-multinucleated cells is shown (right). (C) Expression of Netrin 1 message was quantified by Q-PCR (left). Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre BMMs were cultured with M-CSF + RANKL for three days in the presence or absence of recombinant Netrin 1 (200 ng/ml). Frequency of TRAP+ hyper-multinucleated cells is shown (right). (D) Pre-osteoclasts from Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre mice were stimulated with RANKL for the indicated times, and the activated form of Rac1 was detected (top). Rac1 protein levels in Unc5b-knockdown or Netrin 1-treated cultures were shown (bottom). (E) Schematic model of regulation of osteoclast multinucleation by Flrt2. Osteoclast-derived Flrt2 competes with osteoclast-derived Netrin 1 for a receptor Unc5b, which attenuates negative regulation of Netrin 1-Unc5b-mediated Rac1 activation. In the absence of osteoclast-derived Flrt2, Netrin 1-Unc5b interaction inhibited Rac1 activation, leading to impaired osteoclast multinucleation. Scale bars represents 100 $\mu$m. Data are shown as mean ± S.D. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$.
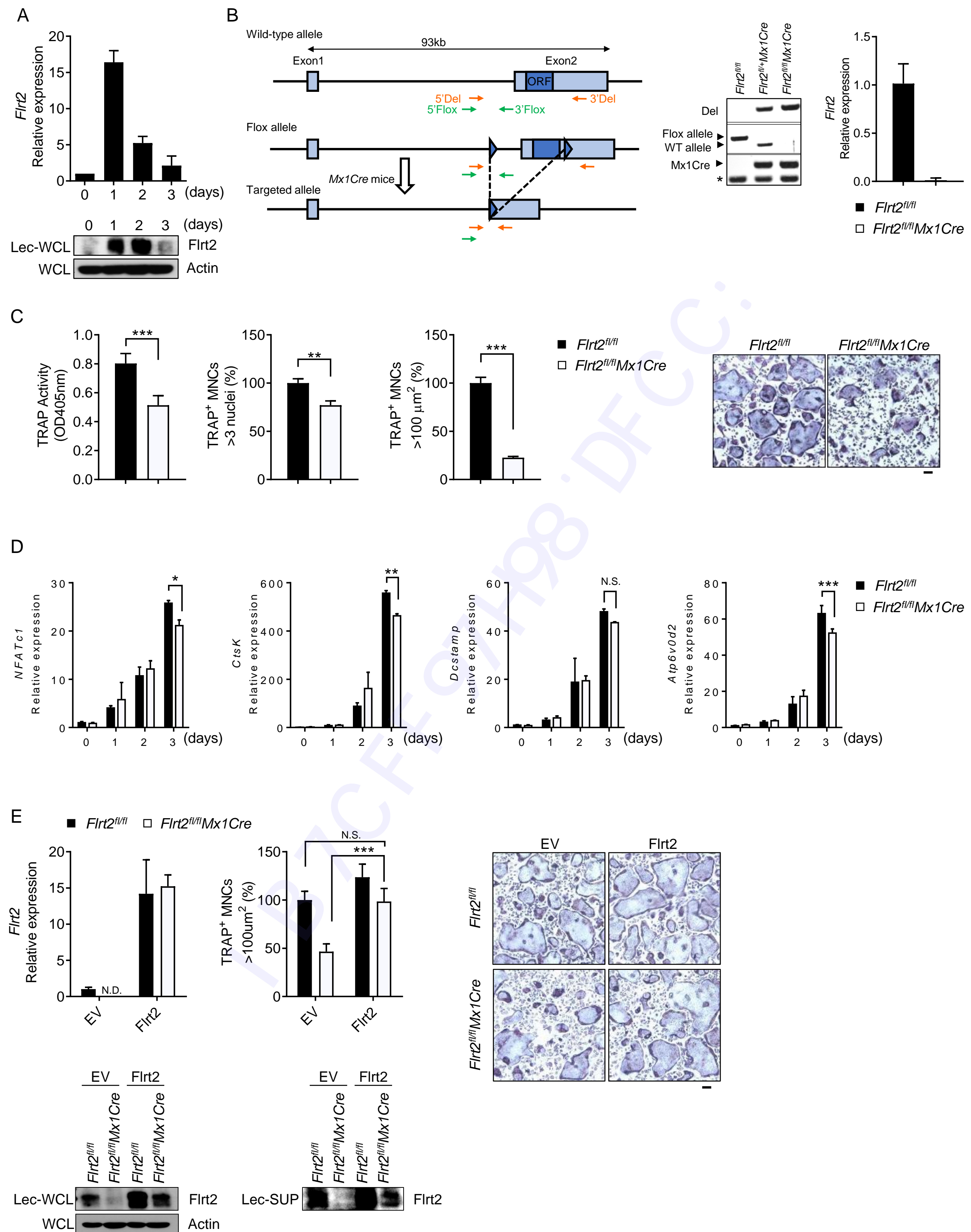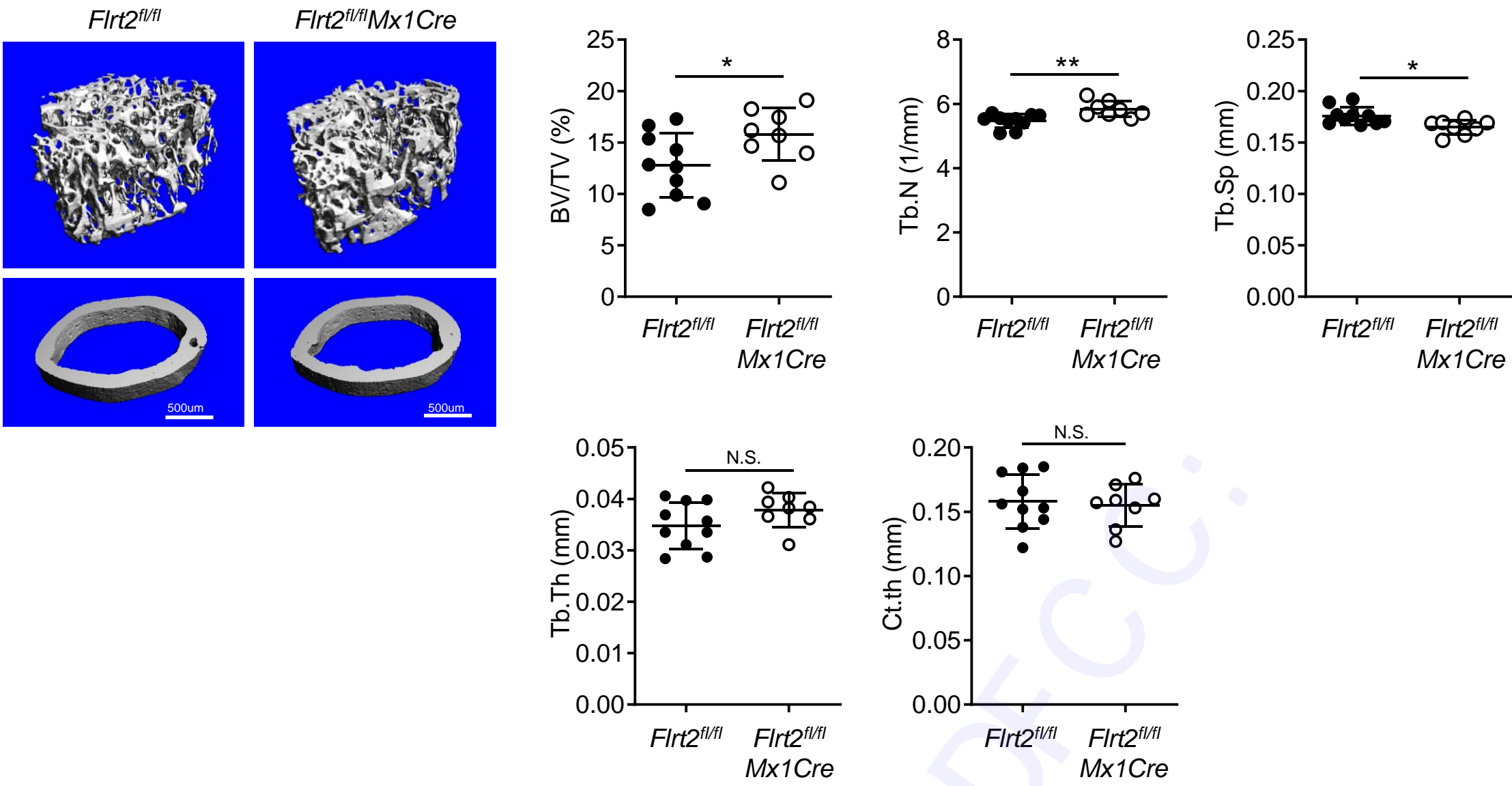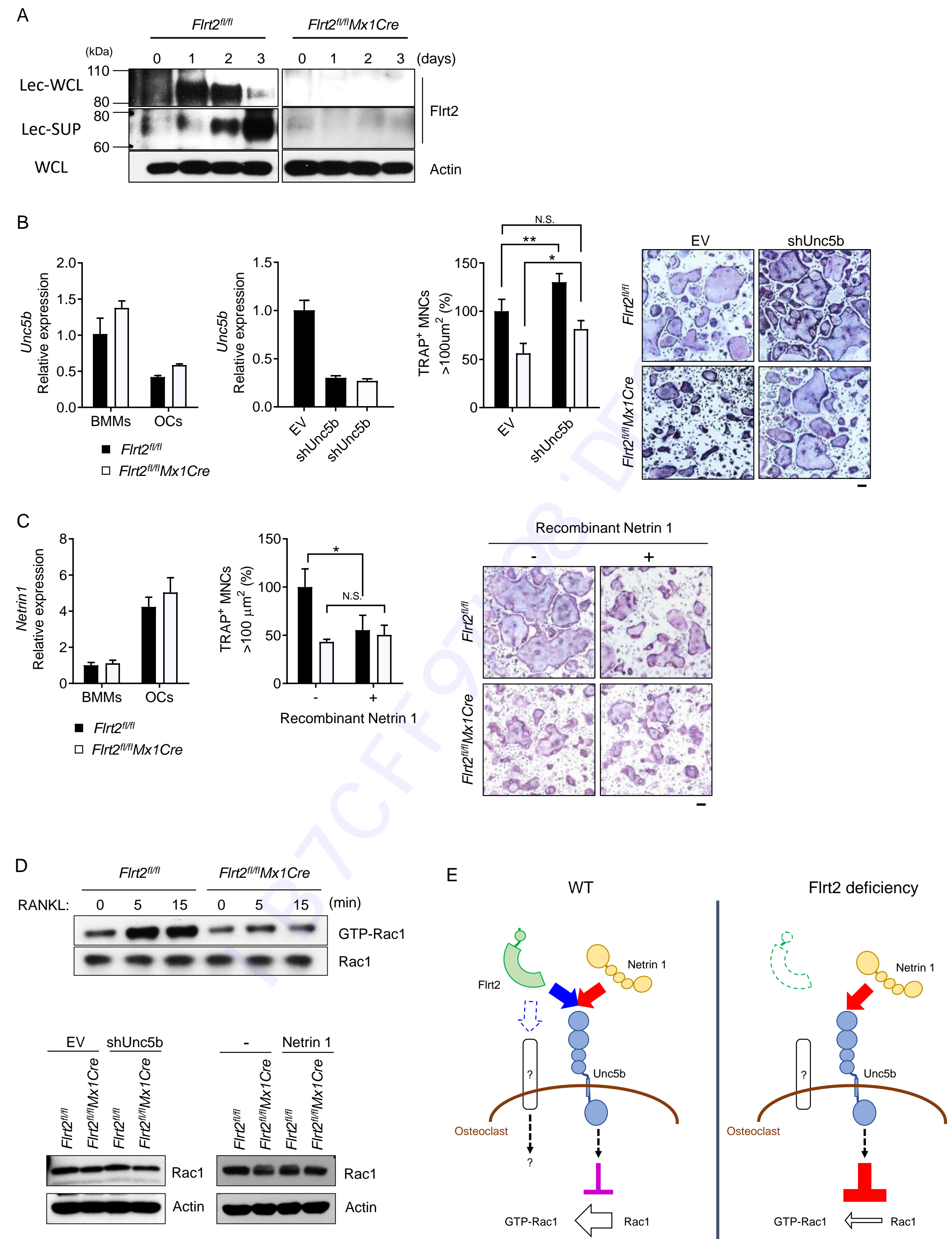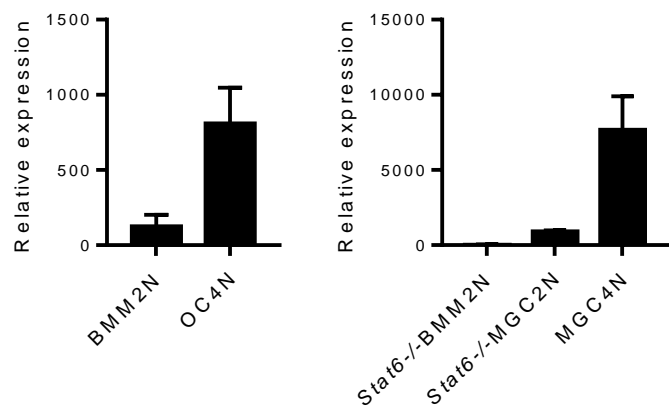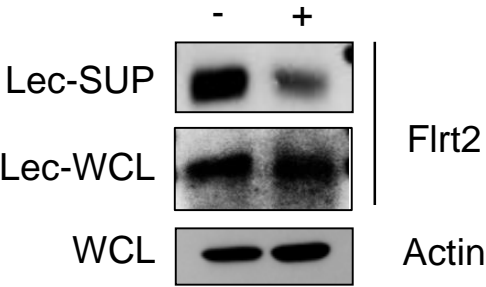
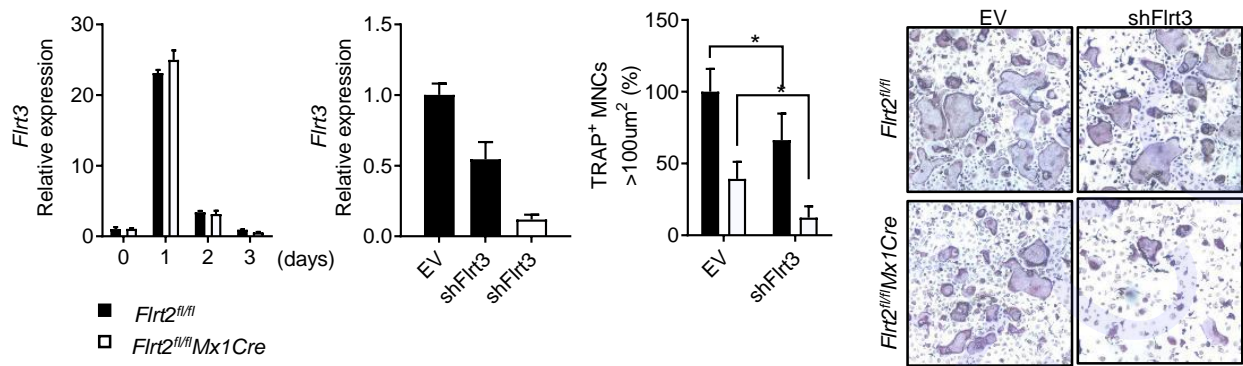# Figure 1

# Figure 2

# Figure 3

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

**Supplementary figure 1**

Cloning of Flrt2 as a novel gene involved in late-stage osteoclast differentiation. Mouse bone marrow-derived monocytes were cultured with either MCSF + RANKL to differentiate into osteoclasts, or IL-4 + IL-3 to generate multinuclear giant cells (MGCs) which also undergo cell fusion, and gene expression was assessed in mononuclear macrophages (BMM2N) versus multinucleation-committed tetraploid osteoclasts (OC4N), or tetraploid MGCs (MGC4N). Because MGC fusion is dependent on STAT6-mediated IL-4 signaling, we also prepared $Stat6^{-/-}$ monocytes ($Stat6^{-/-}$BMM2N) and cells cultured with IL-3 + IL-4 ($Stat6^{-/-}$MGC2N) to filter out potentially cell fusion-independent MGC-associated genes. As a result, we identified the gene *Flrt2*. Monocytes (2N) and multinucleation-committed cells (4N) were sorted and total RNAs were isolated. Relative expression levels of Flrt2 in monocytes and multinucleation-committed cells are shown as mean ± SD of three independent experiments.

**Supplementary figure 2**

Western blot analysis of Flrt2 in lec-SUP and lec-WCL after treatment with Brefeldin A (BFA). Flrt2$^{fl/fl}$ BMMs cultured with M-CSF + RANKL for two days were treated with or without BFA (1 μg/ml) for 8 hrs, WCL and SUP were harvested, and pulled-down with Lectin. Western blot was performed using anti-Flrt2 antibody.

**Supplementary figure 3**

Total RNAs were isolated from Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre BMMs cultured with M-CSF + RANKL for the indicated days, and expression of Flrt3 message was quantified by Q-PCR (left). Flrt2$^{fl/fl}$ and Flrt2$^{fl/fl}$Mx1Cre BMMs retrovirally transduced with shFlrt3 or control vector were cultured with M-CSF + RANKL for three days, and knockdown of Flrt3 was determined by Q-PCR (middle). Cells were stained for TRAP. Frequency of TRAP+ hyper-multinucleated cells is shown (right).

Scale bars represents 100 $\mu$m. Data are shown as mean $\pm$ S.D. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$.