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Optineurin Negatively Regulates Osteoclast Differentiation by Modulating NF-κB and Interferon Signaling: Implications for Paget’s Disease

**Highlights**
- Susceptibility to Paget’s disease is associated with reduced optineurin expression
- Optineurin knockdown enhances osteoclast differentiation
- Loss of optineurin function increases bone turnover in vivo
- Optineurin inhibits osteoclast formation by modulating NF-κB and IFN-β signaling

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**In Brief**
Using mouse models, Obaid et al. identify a role of optineurin in bone metabolism as a negative regulator of osteoclast differentiation. Loss of optineurin function leads to increased bone turnover in mice, suggesting a mechanism by which genetic variants in optineurin predispose to Paget’s disease of bone.

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Optineurin Negatively Regulates Osteoclast Differentiation by Modulating NF-κB and Interferon Signaling: Implications for Paget’s Disease

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SUMMARY
Paget’s disease of bone (PDB) is a common disease characterized by osteoclast activation that leads to various skeletal complications. Susceptibility to PDB is mediated by a common variant at the optineurin (OPTN) locus, which is associated with reduced levels of mRNA. However, it is unclear how this leads to the development of PDB. Here, we show that OPTN acts as a negative regulator of osteoclast differentiation in vitro and that mice with a loss-of-function mutation in Optn have increased osteoclast activity and bone turnover. Osteoclasts derived from Optn mutant mice have an increase in NF-κB activation and a reduction in interferon beta expression in response to RANKL when compared to wild-type mice. These studies identify OPTN as a regulator of bone resorption and are consistent with a model whereby genetically determined reductions in OPTN expression predispose to PDB by enhancing osteoclast differentiation.

INTRODUCTION
Paget’s disease of bone (PDB) is a common skeletal disorder characterized by osteoclast activation, which provokes increased but disorganized bone turnover, leading to pathological fractures, bone deformity, and bone pain. The disease has a strong genetic component, but the genes responsible have not been fully characterized. The most important predisposing gene is SQSTM1, which is mutated in ~10% of patients with the disease. The SQSTM1 causal mutations increase osteoclastogenesis by enhancing receptor activator of nuclear factor kappa B (NF-κB) (RANK) signaling in osteoclasts and their precursors (Cavey et al., 2006; Daroszewska et al., 2011; Hiruma et al., 2008). Linkage studies in families (Lucas et al., 2008) coupled with genome-wide association studies (GWAS) (Albagha et al., 2010, 2011) have identified a strong susceptibility locus for PDB at the OPTN locus on chromosome 10p13. The OPTN gene encodes optineurin, a ubiquitously expressed cytoplasmic protein involved in many cellular processes including regulation of NF-κB signaling (Zhu et al., 2007), autophagy, and innate immunity (Wild et al., 2011), but the role of optineurin in bone metabolism is unknown. Here, we investigated the role of OPTN in regulating bone turnover and evaluated the molecular mechanisms by which variants at the OPTN locus predispose to PDB.

RESULTS AND DISCUSSION
Reduced Expression of OPTN Predisposes to PDB
In order to identify disease-causing mutations in the OPTN locus, we conducted mutation screening of the coding exons of OPTN in 200 PDB patients, but results showed no mutations in the protein-coding region (data not shown). However, the top GWAS hit (rs1561570) was found to be a strong expression quantitative trait locus (eQTL) in human monocytes (Zeller et al., 2010) and in peripheral blood mononuclear cells (Westra et al., 2013) with substantially reduced levels of OPTN mRNA expression in carriers of the PDB-predisposing “T” allele with an evidence of an allele-dose effect (Figure 1). These observations indicate that susceptibility to PDB is associated with reduced expression of OPTN and raise the possibility that OPTN might act as a negative regulator of osteoclast function.

Optn Knockdown Enhances Osteoclast Differentiation
Here, we studied the expression of optineurin during osteoclast differentiation and investigated the effects of Opn knockdown in primary bone-marrow-derived macrophage (BMDM) cultures. Expression of optineurin increased considerably during osteoclast differentiation, following stimulation of the cultures with macrophage colony-stimulating factor (M-CSF) and receptor...
However, osteoblast-osteoclast co-culture assays showed that of the optineurin protein (homozygous for a D-to-N amino acid substitution at codon 477), remodelling in vivo, we conducted skeletal phenotyping of mice.

To investigate the effects of Optn on osteoclast activity and bone activations in WT cultures subjected to shRNA knockdown cultures. We found no difference between OptnD477N/D477N and WT mice in the ability of calvarial osteoblasts to form bone nodules in vitro (Figure 3B). However, osteoblast-osteoclast co-culture assays showed that osteoblasts from OptnD477N/D477N mice had reduced ability to support osteoblast differentiation when compared to WT osteoblast cultures (Figure 3C). In contrast, and consistent with bone marrow culture data presented in Figure 3A, osteoclast precursor cells derived from OptnD477N/D477N mice showed enhanced sensitivity to osteostimulation that appears to compensate for the reduction in the ability of mutant osteoblast to support osteoclast differentiation (Figure 3C).

Quantitative bone histomorphometry showed that OptnD477N/D477N mice had significantly increased number of osteoclasts per bone surface (Oc.N/BS) and resorption surfaces (Oc.S/BS) as compared with WT (Figure 3D). Indices of bone formation (osteoid surface per bone surface [OS/BS], osteoid volume per bone volume [OV/BV], and mineral apposition rate [MAR]) were also significantly higher in OptnD477N/D477N mice (Figures 3E and 3F), and there was a non-significant trend (p = 0.08) for an increase in bone formation rate (BFR/BS). These observations illustrate that optineurin negatively regulates osteoclast activity both in vitro and in vivo. The lack of an effect on osteoblast differentiation in vitro suggests that the increased bone formation we observed in OptnD477N/D477N in vivo was most probably secondary to the increase in bone resorption, as occurs in humans with PDB (Ralston et al., 2008). Since PDB is also characterized by the development of focal osteolytic lesions that predominantly affect older people, we performed further skeletal phenotyping of OptnD477N/D477N mice to investigate bone mass and bone structure and to look for evidence of focal osteolytic lesions in aged mice using micro-computed tomography (micro-CT) scanning of lower limbs. There was no difference between genotypes in BV/TV, trabecular number, or structure in young mice (Figures S2A and S2B). However, analysis of older mice aged between 8 and 18 months revealed evidence of a focal osteolytic lesion in the left femur in one OptnD477N/D477N mutant mouse aged 15 months (Figure 3G; Table S1). We then investigated the presence of bone lesions in another loss-of-function mouse model in which the C-terminal polyubiquitin-binding domain of Optn has been deleted, resulting in a truncated protein with low expression (OptnΔEx12/ΔEx12). Analysis of the hindlimbs of OptnΔEx12/ΔEx12 mice (n = 8) by micro-CT showed no Paget’s-disease-like lesions (Table S1). These observations illustrate that while the D477N loss-of-function mutation in Optn increases bone turnover, this does not result in net bone loss, presumably because the increase in bone resorption is coupled with that of bone formation. The development of a focal osteolytic lesion in one OptnD477N/D477N mutant mouse (~10% of mice aged ≥15 months), but not in OptnΔEx12/ΔEx12, supports the hypothesis that loss of function in optineurin can lead to a PDB-like phenotype while illustrating that additional factors must also be present for focal osteolytic lesions to become fully penetrant.

Optn Regulates Osteoclast Differentiation by Modulating NF-κB and Interferon Signaling

Controlled RANKL-induced NF-κB activation is essential for osteoclast differentiation and function and for the maintenance of normal bone turnover. Previous studies have suggested OPTN as a negative regulator of TNF-α-induced NF-κB activation in immune cells (Maruyama et al., 2010; Nagabhushana et al., 2011; Sudhakar et al., 2009; Zhu et al., 2007), but its role in RANKL-induced NF-κB activation is yet unknown. In order to investigate the effects of Optn on intracellular signaling in osteoclasts, we studied RANKL-induced NF-κB activation during osteoclast differentiation in cultures from OptnD477N/D477N mice as well as in Optn knockdown cultures. We found no difference in RANKL-induced NFκB activation, as measured by phosphorylation of IκBα, in BMDMs from D477N mutant mice compared with WT (Figure 4A). However, following RANKL stimulation, there was a progressive increase in NF-κB activation that was...
significantly greater in cultures from OptnD477N/D477N as compared with WT mice, with a maximal effect at 3 days (Figure 4B). Similar findings were observed in Optn knockdown cultures in which reduced expression of Optn by knockdown resulted in enhanced NF-κB activity after RANKL stimulation (Figure 2D). These findings indicate that the inhibitory effects of Optn become most apparent as osteoclast differentiation proceeds and is consistent with the observation that Optn levels increase substantially during osteoclast differentiation (Figure 2A). A similar mechanism has previously been reported in Cyld null mice, which show no abnormalities of RANKL-induced NF-κB activation in BMDMs but show enhanced NF-κB activation as osteoclast differentiation proceeds (Jin et al., 2008).

Previous studies have shown that the deubiquitinase enzyme CYLD plays an important negative regulatory role in NF-κB signaling in osteoclasts by interacting with p62 and TRAF6 (Jin et al., 2008). It has also been reported that optineurin is required for CYLD-dependent inhibition of NF-κB activation in immune cells (Nagabhushana et al., 2011). Since the D477N mutation is located in the region that binds to CYLD (Nagabhushana et al., 2011), we studied the effects of the mutant protein on CYLD binding by immunoprecipitation in osteoclasts. This confirmed that the D477N Optn variant had an impaired ability to bind CYLD compared with WT Optn (Figure 4C), indicating that the inhibitory effect of Optn on osteoclast is mediated, in part, by a CYLD-dependent pathway. It has previously been shown that the expression of mutant OptnD477N protein was higher than that of WT in multiple tissues including BMDMs (Gleason et al., 2011). In line with these findings, we also observed that the expression of the mutant OptnD477N protein during osteoclast differentiation was higher than that of the WT (Figures 4C and 4E). This difference was more noticeable from day 3 post-RANKL stimulation (Figure 4E), possibly due to increased NF-κB activity, since a putative NF-κB binding site has been reported in Optn promoter (Sudhakar et al., 2009). However, the increased levels of expression of the mutant OptnD477N protein were clearly not sufficient to compensate for its reduced ability to suppress NF-κB activity and osteoclast differentiation.

Studies have shown that while RANKL stimulates osteoclast activity, it also initiates a negative auto-regulatory effect on osteoclasts through induction of interferon beta (IFN-β) expression (Takayanagi et al., 2002; Hayashi et al., 2002), which has an inhibitory effect on osteoclast by interfering with RANKL-induced expression of c-Fos (Takayanagi et al., 2002). Since previous studies have shown that optineurin is involved in IFN-β signaling in immune cells (Gleason et al., 2011; Munitic et al., 2013), we investigated if Optn plays a role in this negative regulatory loop in osteoclast by studying the expression of IFN-β and c-Fos in both WT and OptnD477N/D477N mutant cells. We found

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**Figure 2. Optn Knockdown in Mouse Bone-Marrow-Derived Macrophages Enhances Osteoclast Differentiation**

(A) Immunoblot showing the expression of Optn during osteoclast differentiation. BMDMs were stimulated with M-CSF (25 ng/ml) and RANKL (100 ng/ml), and Optn expression was examined in cell lysate at the indicated time points. Anti-β-actin was used as loading control.

(B) Immunoblot showing Optn knockdown in BMDMs. Cells were transduced with lentiviral particles expressing shRNA targeting the Optn gene or non-targeting control (NTC).

(C) Enhanced osteoclast differentiation in Optn knockdown BMDM. Optn-depleted or NTC cells were stimulated with M-CSF (25 ng/ml) and RANKL (indicated concentrations), and TRAP+ multinucleated cells (MNC) were counted and shown as mean ± SEM from three independent experiments.

(D) RANKL-induced NF-κB activation during osteoclast differentiation. Optn-depleted or NTC cells were transduced with lentiviral particles expressing an NF-κB luciferase reporter followed by stimulation with M-CSF and RANKL, and reporter activity was measured at the indicated time points. Values are mean ± SEM from two independent experiments presented as % of NTC-shRNA. Blots and pictures are representative of three independent experiments. *p < 0.05; **p < 0.01 compared to NTC. See also Figure S1.
that RANKL induced the expression of IFNβ in both WT and OptnD477N/D477N mutant cells, but the expression levels were significantly lower in mutant cells compared to those observed in WT (Figure 4D). Additionally, c-Fos expression during later stages of osteoclast differentiation (3 days post-RANKL stimulation onward) from OptnD477N/D477N mice was higher than that observed in WT. Values are mean ± SEM from two independent experiments presented as percentage of WT/WT combination. (D–F) Histomorphometrical analysis of trabecular bone showing enhanced bone turnover in OptnD477N/D477N mice. Representative images of proximal tibia metaphysis stained with TRAP (D), Von Kossa (E), or calcein double labeling (F). Graphs on the right represent comparison of bone resorption indices (osteoclast number per bone surface [Oc:N/BS] and osteoclast surface per bone surface [Oc:S/BS]), bone formation indices (osteoid surface per bone surface [OS/BS] and osteoid volume per bone volume [OV/ BV]), or dynamic bone formation indices (mineral apposition rate [MAR] and bone formation rate per bone surface [BFR/BS]) between WT and mutant mice. Data are shown as mean ± SEM from seven to eight animals per group. (G) PDB-like lesions observed in the left femur of a 15-month-old OptnD477N/D477N mouse. Micro-CT images showing osteolytic bone lesion within the cortex and histological analysis showing enhanced osteoclastogenesis in the affected region. *p < 0.05, **p < 0.01; n.s., not significant compared to WT. See also Figure S2 and Table S1.

Conclusions

In conclusion, we have demonstrated that reduced expression or loss of Optn function in mice leads to enhanced osteoclast differentiation, identifying Optn as a negative regulator of osteoclast differentiation. The underlying mechanisms are complex but involve RANKL-induced NF-κB activation, an interaction with CYLD, and regulation of IFN-β signaling with regulatory effects that are cell-type specific, dependent on its expression level and on its ability to bind polyubiquitin. Our data
suggest that the common genetic variant rs1561570 at the OPTN locus increases susceptibility to PDB by reducing levels of OPTN expression, probably leading to enhanced osteoclast differentiation.

**EXPERIMENTAL PROCEDURES**

**Reagents**
Details of the materials and reagents can be found in Supplemental Experimental Procedures.

**Mice**
The generation of Optn<sup>Ex12flox/lox</sup> knockin mice was described previously (Gleason et al., 2011). To generate Optn<sup>Ex12flox/lox</sup> mice, the Optn<sup>Ex12flox/lox</sup> mice were first crossed to Fipe<sup>−/−</sup> mice to remove the Fipe transgene, and these mice were then crossed to Bai-1 Cre mice to delete exon 12. All experiments were performed according to institutional, national, and European animal regulations.

**Micro-Computed Tomography Analysis**
Micro-CT analysis was performed using an in vivo Skyscan 1076 or an ex vivo Skyscan 1172 system. Live animals were scanned on the in vivo scanner.
looking for the development of PDB-like lesions as previously described (Daroszewska et al., 2011), and those showing evidence of lesions were further analyzed on the ex vivo scanner and then subjected to histological analysis as described below. Image reconstruction was performed using the Skyscan NRecon package and trabecular bone parameters measured using Skyscan CTAn software.

**Histomorphometrical Analysis**

Animals received two intraperitoneal injections of calcine 3 days apart before being culled. The hindlimbs were fixed for 24 hr in 4% formalin-buffered saline and stored in 70% ethanol prior to embedding in methyl methacrylate. The bones were processed for static and dynamic histomorphometry according to standard techniques. Sections were stained for tartrate-resistant acid phosphatase (TRAP) with aniline blue counterstain to visualize osteoclasts and osteoid analysis was done after Von Kossa staining with Van Gieson counterstaining according to standard protocols as previously described (Erben and Glössmann, 2012). Histomorphometry was performed using custom-built software and followed standardized nomenclature and recommendations (Dempster et al., 2013).

**Osteoblast Culture and Bone Nodule Assay**

Osteoblasts were isolated from the calvarial bones of 2-day-old mice by sequential collagenase/EDTA digestion and cultured in complete αMEM medium. On reaching confluence, cells were detached with trypsin, re-plated in 12-well plates at a density of 1 × 10⁵ cells/well, and cultured in osteogenic medium (complete αMEM supplemented with 50 μg/ml vitamin C and 3 mM β-glycerophosphate). The medium was replaced three times per week, and cultures were continued for up to 21 days. Mineralized nodules were detected using alizarin red staining, and bone nodule formation was quantified by de-calcified bone. Nat. Genet. 42, 520–524.

**SUPPLEMENTAL INFORMATION**

Supplemental information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.071.

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