Cell Reports

Supplemental Information

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

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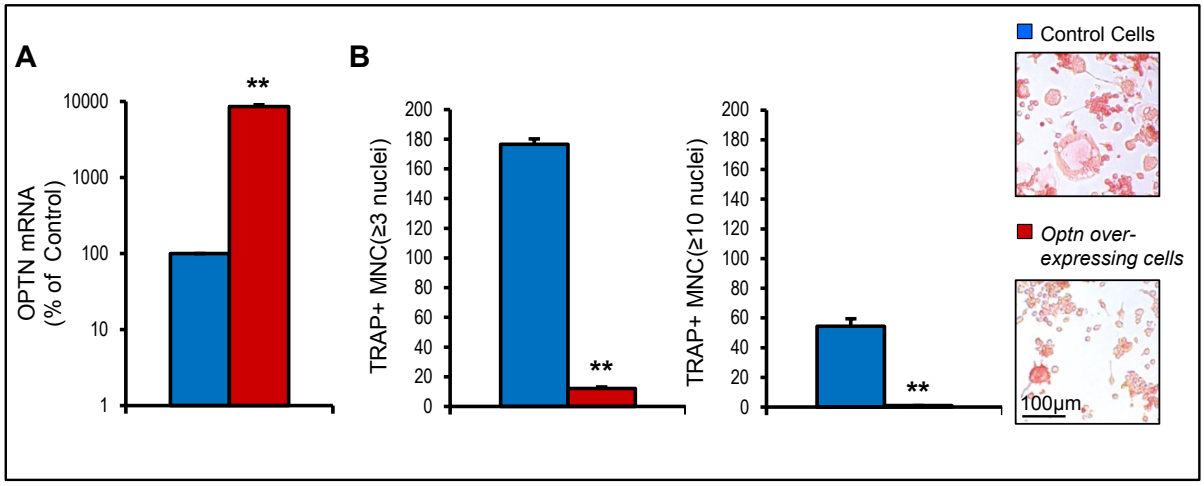

Figure S1. Overexpression of *Optn* in RAW Cells Reduces their Ability to Form TRAP+ Multinucleated (MNC) Cells Upon Stimulation with RANKL, Related to figure 2. (**A**) *Optn* mRNA expression in cells overexpressing *Optn* (red bar) compared to control cells (blue bar). mRNA levels were assayed by qRT-PCR and normalised for 18s rRNA and presented as % of control cells. (**B**) The number of TRAP+ MNC (≥3 nuclei) or hypernucleated MNC (≥10 nuclei) generated from RAW cells overexpressing *Optn* compared to control cells. Values are means ± SEM representative from two-three independent experiments. ** P< 0.01.

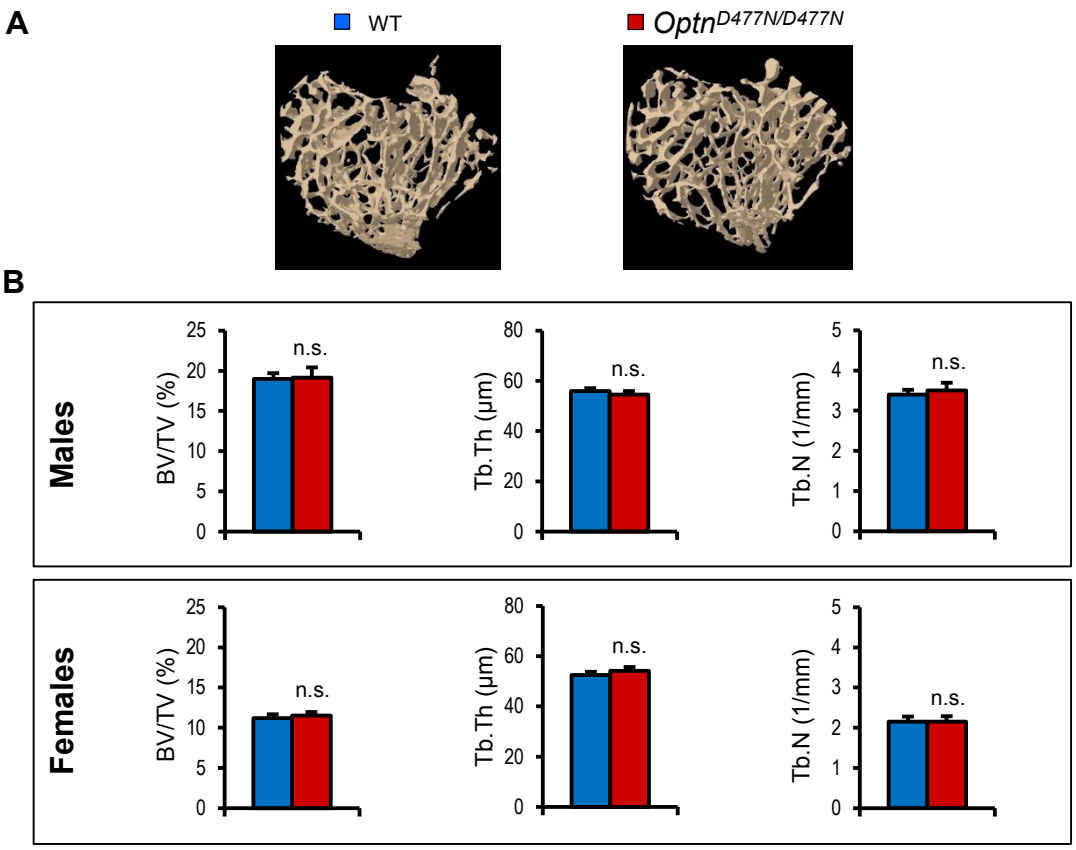

Figure S2. Analysis of Bone Structure in *Optn*$^{D477N/D477N}$ Mice by MicroCT, Related to figure 3. (**A**) Representative MicroCT images of trabecular bone from the tibial metaphysis of *Optn*$^{D477N/D477N}$ and WT mice. (**B**) Comparison of bone volume/total volume (BV/TV); trabecular thickness (Tb.Th) and trabecular number (Tb.N) between WT and mutant male and female mice. Values are means ± SEM from eight mice per group. n.s.; not significant.

Table S1. WT, *Optn*$^{D477N/D477N}$, and *Optn*$^{\Delta Ex12/\Delta Ex12}$ Mice Screened for the Presence of PDB-Like Lesions by MicroCT, Related to figure 3.

Mouse Model	*Optn*$^{D477N/D477N}$									*Optn*$^{\Delta Ex12/\Delta Ex12}$	
Age group	8-9 months			12 months			15-18 months			7-12 months	
Genotype	WT	D477N		WT	D477N		WT	D477N		WT	ΔEx12
Males (*n*)	7	11		8	12		2	6		3	4
Females (*n*)	13	7		12	13		6	3		4	4
Total (*n*)	20	18		20	25		8	9		7	8
Male with lesion (*n*)	0	0		0	0		0	0		0	0
Female with lesion (*n*)	0	0		0	0		0	1		0	0
Total with lesion (*n*)	0	0		0	0		0	1		0	0

Supplemental Experimental Procedures

Reagents:

Cells: RAW 264.7 (ATCC). *Media*: Minimal Essential Medium Eagle alpha modification (α-MEM; Sigma), Fetal Calf Serum (FCS; Hyclone) and L-Glutamine (Invitrogen). The complete α-MEM medium consisted of α-MEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. *Antibodies*: OPTN (1:250, Cayman), c-Fos (1:100, Calbiochem), Actin (1:1000, Sigma), Phospho-IκBα (Ser32; 1:1000 Cell Signalling) and CYLD (1:1000, Cell Signalling). *Cytokines*: Murine M-CSF (Prospec Tech.) and human recombinant RANK-L (gift from Dr. Patrick Mollat-Proskelia SASU). *Other reagents:* Protein G-agarose (Calbiochem), puromycin (GIBCO), penicillin; streptomycin; and Alamar blue (Invitrogen), geneticin (Neomycin analogue-G418; Life Technology), Alizarin red; cetylpyridinium chloride; calcein; vitamin C; and β glycerophosphate (Sigma). *Kits*: Cignal Lenti NF-κB reporter (SA Biosciences), Mission mouse *Optn* shRNA pLKO.1-puro clones (Sigma), Trans-lentiviral packaging kit (Thermo Scientific), GenEZ ORF clone for *Mus musculus* Optineurin (OMu13999; Genscript), GenElute Mammalian Total RNA kit (Sigma), qScript cDNA SuperMix kit (QuantaBioscience), SensiFAST Probe No-ROX kit (Bioline), Steady Glo Luciferase Assay (Promega) and jetPEI-Macrophage transfection reagent (Polyplus Transfection).

Osteoblast-Osteoclast Co-culture Assay

Osteoblastic cells (5 x 10^4 cells per well) isolated from the calvariae of 7-10 week old mice by sequential collagenase/EDTA digestion were co-cultured with bone marrow cells (5 X 10^5 cells per well) in a 24-well plate in α-MEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% Heat inactivated FCS and 10 nM 1α,25$(OH)_2$ D3. The culture medium was replaced every 2-3 days and cells were fixed after 8 days, TRAP stained and TRAP positive multinucleated osteoclasts were counted.

Immunoblotting and Immunoprecipitation

Cells were lysed using RIPA buffer and cell lysates were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto Hybond-P (Amersham) membranes. Membranes were blocked with 5% (w/v) non-fat milk in Tris buffered saline with TBS-tween (50 mM Tris, 150 mM NaCl, 0.1%

[v/v] Tween-20) and probed with rabbit primary antibodies. After washing with TBST, membranes were incubated with anti-rabbit horseradish peroxidise conjugated secondary antibody (1:5000) washed and visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) on a SynGene GeneGnome/ Licor imager. Intensities of bands were quantified using SynGene Gene Tools software/ Image Studio Lite Ver 3.1 software.

To immunoprecipitate OPTN, 50 µg of cell extract protein was incubated overnight at 4°C with 4 µg of sheep anti-mouse Optn antibody (s308c). After rotation overnight at 4°C with 15 µl protein G-agarose, the beads were collected by centrifugation, washed three times with modified RIPA buffer, denatured and subjected to SDS-PAGE, and immunoblotted as mentioned above.

Optn Overexpression

RAW cells were seeded at a density of 1.5 million cells per T25 and transfected with GenEZ *Optn* ORF expression plasmid using jetPEI according to the manufacturer protocol. A stable line of *Optn* overexpressing cells was generated following 3 weeks of selection using 400 ug/ml neomycin. Overexpression was confirmed by qRT-PCR. Cells overexpressing *Optn* or control cells were seeded in 96-well plate (5000/well) in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) and stimulated with RANKL (100 ng/ml) for 4 days. Cells were then fixed, TRAP stained and TRAP positive multinucleated cells were counted.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated using GenElute Mammalian Total RNA Kit and RNA was quantified using the Nanodrop 1000 Spectrophotometer. Complementary DNA was generated by RT-PCR using the qScript cDNA SuperMix kit following the manufacturer's instructions. Primers and labelled probes were designed using the Primer 3 and the Roche Diagnostics website (Roche). The primer sequences were as follows: IFNB1-F, 5'-cacagccctctccatcaacta-3', IFNB1-R, 5'-catttccgaatgttcgtcct-3', TNFSF11-F, 5'-tgaagacacactacctgactcctg-3', TNFSF11-R, 5'-ccacaatgtgttgcagttcc-3', IL6-F, 5'-gctaccaaactggatataatcagga-3', IL6-R, 5'-ccaggtagctatggtactccagaa-3', OPTN-F, 5'-gctccgaaatcaagatggag-3', and OPTN-R, 5'-gcagagtggctaacctggac-3'. Real-time PCR was performed using SensiFAST Probe No-ROX kit on a Chromo 4TM Detector and quantified using the Opticon MonitorTM software version 3.1.

Samples were normalized to 18s rRNA expression. 18s cDNA was amplified with the VIC-labelled predesigned probe-primer combination from Applied Biosystems (4319413E) allowing two channel detection of one cDNA.