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Nf1 haploinsufficiency alters myeloid lineage commitment and function, leading to deranged skeletal homeostasis

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Abstract

While nullizygous loss of *NF1* leads to myeloid malignancies, haploinsufficient loss of *NF1 (Nf1)* has been shown to contribute to osteopenia and osteoporosis which occurs in approximately 50 percent of neurofibromatosis type 1 (NF1) patients. Bone marrow mononuclear cells of haploinsufficient NF1 patients and $Nf1^{+/-}$ mice exhibit increased osteoclastogenesis and accelerated bone turnover, however the culprit hematopoietic lineages responsible for perpetuating these osteolytic manifestations have yet to be elucidated. Here we demonstrate that conditional inactivation of a single *Nf1* allele within the myeloid progenitor cell population (*Nf1-LysM*) is necessary and sufficient to promote multiple osteoclast gain-in-functions, resulting in enhanced osteoclastogenesis and accelerated osteoclast bone lytic activity in response to pro-resorptive challenge *in vivo*. Surprisingly, mice conditionally *Nf1* heterozygous in mature, terminally differentiated osteoclasts (*Nf1-Ctsk*) do not exhibit any of these skeletal phenotypes, indicating a critical requirement for *Nf1* haploinsufficiency at a more primitive/progenitor stage of myeloid development in perpetuating osteolytic activity. We further identified p21Ras dependent hyper-phosphorylation of Pu.1 within the nucleus of *Nf1* haploinsufficent myelomoncytic osteoclast

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precursors, providing a novel therapeutic target for the potential treatment of NF1 associated osteolytic manifestations.

Introduction

Myelomonocytic precursor cells give rise to multinucleated, bone resorptive osteoclasts (OCs)[1]. The physiologic lineage commitment of monocyte-macrophage precursor cells is pivotal to maintain skeletal homeostasis. Deregulated OC differentiation, recruitment, and/or function can result in either osteoporosis or osteopetrosis[2,3], affecting not only the skeleton but altering the bone marrow niche as well[4-6]. The cytokines macrophage-colony stimulating factor (M-CSF)[7] and receptor activator of nuclear factor κB ligand (RANKL)[8,9] are indispensable for macrophage and OC development, respectively. O*p/op* mice lacking M-CSF exhibit severe osteopetrosis due to an absence of both OCs and macrophages. Adoptive transfer of wild-type (WT) hematopoietic cells is insufficient to correct osteopetrotic phenotypes in *op/op* recipient mice[10], suggesting that the failure of macrophage and OC differentiation in *op/op* mice is contingent on the extrinsic absence of M-CSF as opposed to intrinsic deficits in either the M-CSF receptor (c-Fms) or intracellular signaling effectors. By contrast, hypersensitivity of macrophages to M-CSF and RANKL in *SHIP* deficient mice has been shown to result in osteoporosis[11].

Mutations in the NF1 tumor suppressor gene lead to malignant and non-malignant disease manifestations of neurofibromatosis type I (NF1), including cutaneous and plexiform neurofibromas, optic nerve gliomas, malignant peripheral nerve sheath tumors (MPNSTs), juvenile myelomoncytic leukemia (JMML), cognitive impairment, cardiovascular disease, and skeletal defects[12]. Neurofibromin, the protein encoded by NF1, functions as a GTPase-activation protein (GAP) for Ras, negatively regulating its functional activity[13]. Experimental data now indicates that *Nf1* gene dose (*Nf1* haploinsufficiency) in hematopoietic derived cells plays a pivotal role in multiple NF1 associated phenotypes including plexiform neurofibromas, neointima formation, and skeletal anomalies osteopenia and osteoporosis[14].

Clinical studies demonstrate that approximately 50 percent of the NF1 patient population suffers from osteopenia or osteoporosis[15-20], resulting in significantly increased rates of long-bone fracture[20,21]. Mononuclear cells cultured from the peripheral blood of NF1 patients and the bone marrow of $Nf1^{+/-}$ mice exhibit increased OC differentiation and bone resorptive capacity *in vitro*[22-24]. However, the functional requirement for *Nf1* haploinsufficiency in perpetuating these osteolytic manifestations has yet to be elucidated in a step-wise and lineage restricted fashion within the hematopoietic compartment. Although $Nf1^{+/-}$ myelomonocytic OC progenitor cells exhibit intrinsic, p21-Ras and PI3K dependent hypersensitivity to M-CSF and RANK-L[22], the putative contribution of extrinsic alterations in cytokine levels within the *Nf1* deficient bone microenvironment is difficult to segregate as a confounding factor. For instance, hypersecretion of osteopontin (OPN)[25], transforming growth factor-beta1 (TGF-β1)[26] and RANKL[27] by *Nf1* null osteoprogenitor cells, together with the decreased expression of the RANKL decoy receptor, osteoprotegerin

(OPG)[27], have each been implicated as potential paracrine factors perpetuating osteolytic activity in murine models of the disease.

To understand the cell autonomous and step-wise role of *Nf1* gene dose in regulating myeloid lineage commitment and OC differentiation, we generated *Nf1-LysM* and *Nf1-Ctsk* mice harboring conditional inactivation of a single *Nf1* allele in myeloid progenitor cells[28] and mature OCs[29], respectively. Here we demonstrate that haploinsufficient loss of *Nf1* within myeloid progenitor cells is necessary and sufficient to perpetuate multiple OC gain-in-functions both *in vitro* and *in vivo*, reminiscent of the phenotype of $Nf1^{+/-}$ mice[22]. We further delineate a mechanism by which p21-Ras hyperactivation results in accumulation of phosphorylated Pu.1 within the nucleus of myelomoncytic osteoclast precursors, which may be associated with enhanced osteoclastogenesis, and provide a novel therapeutic target for the potential treatment of NF1 associated osteolytic manifestations.

Methods

Animals

Nf1-floxed ($Nf1^{flox/flox}$) mice were generated in the laboratory of Dr. Luis Parada (University of Texas Southwestern Medical Center) as described previously[30]. *LysMCre* mice, generated by Dr. Irmgard Forster (University of Duesseldorf)[28], and *CtskCre* mice, generated by Dr. R.A. Davey (University of Malbourne, Australia)[29] were obtained from the Jackson Laboratory. Breeding of $Nf1^{flox/flox}$ mice with *LysMCre* and *CtskCre* mice yielded *LysMCre;*$Nf1^{flox/+}$ and *CtskCre;*$Nf1^{flox/+}$ mice (abbreviated respectively as *Nf1-LysM* and *Nf1-Ctsk* throughout this manuscript) which were maintained at the Indiana University School of Medicine in accordance with the Institutional Animal Care and Use Committee and Institutional Review Board guidelines. Cre mediated recombination of the floxed *Nf1* allele was validated by PCR and western blot (Supplemental Figure 1A-C). The genotype of wild-type (WT) mice were either $Nf1^{flox/flox}$;$Cre^{(-)}$ or $Nf1^{flox/+}$;$Cre^{(-)}$ for each colony. For all experiments, WT mice were obtained from the same colony as the corresponding mutant mice.

Bone marrow isolation

Bone marrow was flushed from the femur, tibia, and iliac crest in a 5 mL volume of Iscove's Modified Dulbecco's Media (IMDM, Gibco/Invitrogen), supplemented with 1% fetal bovine serum (FBS, Hyclone, ThermoScientific) using a 1.5 inch 23-guage needle. Low density bone marrow mononuclear cells (BMMNCs) were isolated by density gradient centrifugation for 30 minutes at 1750 rpm (gh-3.8 rotor, Beckman Coulter) on a 3.5 mL volume of Histopaque (Sigma). The buffy coat layer was collected and washed with IMDM or other media prior to further assays.

Colonogenic progenitor assays

To determine the frequency of myeloid progenitors in bone marrow, colony-forming unit-macrophage/monocyte (CFU-M) of BMMNCs were performed by seeding 2.5×10^4 BMMNCs into 35-mm gridded dishes containing methylcellulose supplemented with varying doses of murine recombinant M-CSF (0.1, 1, 10, and 50 ng/mL) for 7 days at 37°C

in a 5% CO_2 incubator[22]. Colony type and numbers were counted on an inverted light microscope.

Osteoclast differentiation

Murine osteoclasts were cultured *in vitro* from mouse BMMNCs as described previously[22] using α-MEM medium supplemented with 10% FBS in the presence of murine recombinant macrophage-colony stimulating factor (M-CSF, 30 ng/mL) and murine recombinant receptor activator of nuclear factor kappa-B ligand (RANKL, 20 ng/mL). On day three of culture, the cytokines were changed to M-CSF (30 ng/mL) and RANKL (60 ng/mL) for an additional three days of culture. Adherent cells were then fixed and stained for tartrate resistant acid phosphatase (TRACP) according to the manufacturer's instructions (Sigma-Aldrich, USA). TRACP-positive staining osteoclast cells were visualized and photographed with a Nikon TE2000-S microscope (Nikon Inc., Melville, NY) equipped with a QImaging camera (Fryer Company Inc., Cincinnati, OH). Mature osteoclasts were defined as multinucleated TRACP-positive staining cells containing greater than or equal to three nuclei. Osteoclast nuclear number and area were scored using NIH Image J Software.

Bone resorption assays

BMMNCs were seeded on dentine slices (ALPCO Diagnostic, Windham, NH) and cultured in the presence of M-CSF and RANK-L at 37°C, 5% CO_2 for 7 days. The area of resorptive “pits” was quantified on low power fields using NIH Image J Software.

Flow cytometry

BMMNCs were incubated for 45 minutes at 4°C with saturating concentrations of anti-mouse antibodies in ~100 μL 3% FBS/0.09% NaN_3 in PBS with 0.25 μg anti-mouse CD16/CD32 (“Fc Block”). For the myeloid progenitor analysis, the following antibodies from BD Biosciences were used: FITC-conjugated anti-lineage markers (CD3, CD4, CD8, B220, Mac1, Gr1, Ter119), anti-CD16/32-PE, anti-CD34-PacificBlue, anti-Sca1-APC-Cy7, and anti-c-Kit-PerCP-Cy5.5. For the mature lineage analysis, the following antibodies from BD Biosciences were used: anti-CD3-FITC, anti-CD8-PacBlue, anti-B220-V500, anti-Mac1-PE, anti-Gr1-PECy7, anti-CD4-APC-Cy7, anti-CD45.2-PerCP-Cy5.5. Cells were analyzed on an LSR II 407 flow cytometer, and single color compensation controls were acquired using polystyrene microbeads (BD Biosciences). All post-acquisition analyses were performed with FlowJo 7.6.3 software (TreeStar, WA) with gating parameters determined by fluorescence minus-one controls.

The following gating definitions were used: MPs, myeloid progenitors; GMPs, granulocyte-monocyte progenitors; MEPs, megakaryocyte-erythroid progenitors. MPPs: $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$; MPs: $Lin^{-}Sca1^{-}c\text{-}Kit^{+}$. CMPs: $Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{+}Fc\gamma RII/II^{-/lo}$. GMPs: $Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{+}Fc\gamma RII/\ II^{+}$. MEPs: $Lin^{-}Sca1^{-}c\text{-}Kit^{+}CD34^{-}Fc\gamma RII/II^{-}$. HSPCs: $CD150^{+}Lin^{-}CD48^{-}CD41^{-}Sca1^{+}c\text{-}Kit^{+}$. Myeloid cells: $CD3^{-}B220^{-}$; Monos: $CD3^{-}B220^{-}Gr1^{-}Mac1^{+}$.

Pro-resorptive challenge

12-week old, female WT, *Nf1-LysMCre*, and *Nf1-CtskCre* mice underwent either ovariectomy or sham surgery[22]. Briefly, mice were anesthetized using a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg) administered by intraperitoneal (IP) injection. A 2-cm midline dorsal skin incision was performed, followed by incision of the peritoneal cavity to identify and excise the ovaries. Bone mineral density (BMD) was measured once prior to surgery and again after 6 weeks to track the percent change in BMD over time. The mice were subsequently euthanized and the long bones were dissected for further analysis by micro-computed tomography (μCT) and histological methods described below. The cohort sizes of the experimental (OVX) and sham operated control groups were chosen to achieve sufficient statistical power to detect a significant difference in bone mineral density between genotypes within treatment groups six weeks status-post surgery, according to our previously published work[22].

Peripheral dual-energy X-ray absorptiometry (pDEXA)

Bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DEXA) with a Lunar Piximus densitometer (GE Lunar II, Faxitron Corp., Wheeling, IL)[22]. Mice were anesthetized with a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg) injected IP. Animals were scanned in the prone position with arms and legs extended. A region of interest was defined as the distal femur adjacent to the growth plate (12 × 12 pixels). For ovariectomy experiments, the percent change in BMD was determined by comparing the initial measurement with an endpoint scan, acquired 6 weeks following OVX or sham surgery.

Micro-computed tomography (μCT)

Formalin fixed femora were placed in the gantry of a VivaCT 40 micro-computed tomographer (Scanco Medical AG, Bassersdorf, Switzerland). Images were acquired at 55 kV and 145 mA with a voxel size of 10.5 μm. A region of interest was defined as 100 transverse CT slices beginning 250 μm away from the growth plate and extending proximally. Fractional bone volume (BV/TV, Fraction) and architectural properties of trabecular reconstructions: trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, mm^{-1}), trabecular spacing (Tb.Sp, 1/mm), and connectivity density (Conn.D., mm^{-3}) were calculated as described previously[31]. For quantitative evaluation of tibial fracture calluses, contouring was performed for 100 transverse CT slices extending in both directions from the fracture midline. Callus fractional bone volume (BV/TV) and volumetric bone mineral density (vBMD) were measured within the callus, excluding the cortical bone comprising the original tibial shaft.

Quantitative histomorphometry

Tissues were fixed in 10% formalin for 48 hours, demineralized for 2 weeks in 10% EDTA, and embedded in paraffin. 3.5 μm thick longitudinal sections were cut using a rotary microtome (Leica). Trabecular bone volume fraction (BV/TV) of the secondary spongiosa and osteoblast number (N.Ob/BS) normalized to the bone surface were quantified on H&E stained sections of the distal femur at 200× magnification using BIOQUANT OSTEO v11.2

software (BIOQUANT Image Analysis Inc., Nashville, TN). In a similar fashion, osteoclast number normalized to the bone surface (N.Oc/BS) was quantified on tartrate resistant acid phosphatase (TRACP) stained sections at 200× magnification.

Western blotting

Following stimulation with M-CSF, RANKL, and various inhibitors, nuclear and cytoplasmic protein fractions were harvested using a Nuclear/Cytosol Fractionation kit (Biovision, San Francisco, CA). Isolated proteins were fractionated using NuPAGE 4-12% Bis-Tris Gels (Invitrogen) and electro-transferred to PVDF membranes. Immunoblots were carried out using antibodies specific to Pu.1 (Abcam) and β-actin (Sigma). After incubation with anti-rabbit IgG or anti-mouse IgG (GE Healthcare) antibodies conjugated with HRP, signals were detected using ECL chemoluminescence substrate (ECL Prime, GE Healthcare). Intensity of bands was determined using Image J software.

Phosphatase treatments

Nuclear protein lysates were isolated from *Nf1*$^{+/-}$ osteoclast progenitor cells following 12 hours stimulation with M-CSF (30 ng/mL) and RANKL (30 ng/mL). Potato acid phosphatase (Sigma) was reconstituted at a concentration of 0.05 U/μL. Heat activation was performed by heating the reconstituted phosphatase to 100°C for 15-20 minutes prior to incubation with nuclear protein lysates. Phosphatase treatments were performed by adding 0.05 U of either active or heat inactivated phosphatase to nuclear protein lysates, which were incubated at 37°C for a duration of 30 minutes. SDS was subsequently added to each sample, followed by heating to 100°C for 10 minutes to quench to reaction prior to western blotting.

Statistical analysis

Differences between experimental groups were interrogated using the Student's t-test or either one- or two-factor analysis of variance (ANOVA) statistical tests as appropriate. In the instances where the ANOVA was significant, post-hoc testing was performed between individual groups using the Newman-Keuls multiple comparison test. An alpha level of 5% was set as the type I error rate for all studies, with p values < 0.05 required to reject the null-hypothesis.

Results

Conditional *Nf1* haploinsuffiency in myeloid progenitor cells promotes expansion of the OC precursor pool, enhancing OC maturation and bone lytic activity

Osteoclastogenesis is a dynamic process requiring the commitment and proliferation of early myeloid progenitors, followed by terminal myelomonocytic differentiation, and ultimately fusion to multinucleated OCs with bone resorptive activity[1]. Mononuclear cells harvested from the bone marrow of *Nf1*$^{+/-}$ mice and peripheral blood of human NF1 patients exhibit an increased propensity for OC differentiation and bone resorption *ex vivo* due to p21-Ras mediated hypersensitivity to M-CSF and RANK-L[22-24]. Yet no genetic study has directly assessed the stage of myeloid or OC differentiation at which *Nf1* haploinsufficiency is permissive of these gain-in-functions. To investigate the temporal role of *Nf1* gene dose in

regulating early versus late stage osteoclast development, we began by comparing myeloid lineage commitment and proliferative capacity of BMMNCs harvested from *Nf1-LysM*, *Nf1-Ctsk*, and wild-type (WT) mice. When BMMNCs isolated from these mice were cultured in semisolid methycellulose media supplemented with varying concentrations of M-CSF, we found that the number of colony forming unit-macrophage (CFU-M) per femur were significantly increased in *Nf1-LysM* BMMNCs versus WT with increasing doses of M-CSF (Figure 1A). In a complementary approach, flow cytometric analysis of the bone marrow revealed a significant increase in the frequency of myeloid progenitors (Figure 1B). Thus, conditional *Nf1* haploinsufficiency in early stage myeloid progenitor cells is sufficient to promote expansion of the OC precursor pool as previously observed in $Nf1^{+/-}$ mice[22]. By contrast, however, when we compared the frequency of CFU-M generated from *Nf1-Ctsk* BMMNCs in M-CSF supplemented methycellulose culture, we observed no significant difference as compared to the WT control (Supplemental Figure 2A), indicating that conditional *Nf1* haploinsufficiency restricted to the terminal stages of OC differentiation does not alter the lineage commitment or frequency of more primitive myeloid progenitor cells.

We next sought to assess the capacity of *Nf1-LysM*, *Nf1-Ctsk*, and WT BMMNCs to undergo terminal OC differentiation in response to M-CSF and RANK-L. Given the increased frequency of myelomonocytic OC progenitor cells within the bone marrow of *Nf1-LysM* mice, we hypothesized that BMMNCs harvested from these animals would concordantly exhibit enhanced osteoclastogensis. Indeed, the number of tartrate resistant acid phosphatase (TRACP) positive staining multinucleated OCs (Figure 1C), number of nuclei per osteoclast, and mean osteoclast size (Figure 1D) were significantly increased in *Nf1-LysM* BMMNC cultures as compared to WT controls suggesting increased osteoclast progenitor fusion and recapitulating the hallmark features of osteoclasts cultures derived from the bone marrow of $Nf1^{+/-}$ mice [22] and the peripheral blood of human NF1 patients[23,24]. Although *Nf1-Ctsk* mice do not exhibit any apparent increase in the frequency of OC progenitors within the bone marrow, we nonetheless reasoned that *Nf1* haploinsufficiency, even restricted to late stage osteoclastogenesis, might still be sufficient to potentiate osteoclast maturation. As expected, we observed no significant difference in the osteoclastogenic capacity of *Nf1-Ctsk* BMMNCs as compared to WT cultures (Supplemental Figure 2B), suggesting that *Nf1* haploinsufficiency in the terminal stages of OC development alone is insufficient to potentiate osteoclast differentiation as compared to genetic disruption of a single *Nf1* allele in more primitive myeloid progenitor cells. These data also serve as a negative control to exclude the putative contribution of paracrine factors from *Nf1* heterozygous mature osteoclasts that could act on immature cells of the myeloid lineage to promote terminal differentiation.

A defining morphological feature of mature osteoclasts is the organization of the actin cytoskeleton to form a specialized cell–extracellular matrix that provides the appropriate microenvironment for bone matrix degradation. This complex structure is formed by the coalescence of actin cytoskeletal structures termed podosomes that arrange into identifiable patterns such as clusters, rings and ultimately belts that constitute a functional sealing zone[32]. Here, we show that compared to WT controls, *Nf1-LysM* osteoclast cultures

exhibit significantly higher levels of belt formation (Figure 1E,F), another characteristic cellular feature observed in *Nf1*$^{+/-}$ osteoclasts with increased bone resorptive activity[33].

To directly assess the functional capacity of OCs to degrade bone matrix, OCs were cultured on dentine slices in the presence of M-CSF and RANK-L to quantify the area of "pits" generated by osteoclast mediated erosion. Consistent with previous data demonstrating increased bone resorptive capacity of OCs cultured from the *Nf1*$^{+/-}$ mice[22], we observed a significant increase in "pit" area on dentine slices seeded with *Nf1-LysM* OCs as compared to WT controls (Figure 1G). Consistent with previous negative findings, *Nf1-Ctsk* OC cultures did not exhibit a significant difference in "pit" resorptive capacity as compared to WT (Supplemental Figure 2C).

Nf1 haploinsufficiency in myeloid progenitor cells is necessary and sufficient to accentuate osteolytic activity *in vivo*

Intriguingly, although *Nf1*$^{+/-}$ OCs exhibit multiple gain-in-functions[22], *Nf1*$^{+/-}$ mice do not spontaneously exhibit commensurate reductions in bone mass or quality as compared to WT mice[34]. By contrast, we previously demonstrated that the consequences of *Nf1* haploinsufficiency can be unmasked *in vivo* following ovariectomy (OVX) induced pro-resorptive challenge, whereby *Nf1*$^{+/-}$ mice lose bone mass at approximately double the rate of WT littermates perpetuated by excess osteolytic activity[22]. To characterize the functional consequences of cell-autonomous *Nf1* haploinsufficiency in myeloid progenitor cells versus terminally differentiated osteoclasts in response to pro-resorptive stress, we ovariectomized *Nf1-LysM* and *Nf1-Ctsk* mice and monitored changes in their bone mass and bone mineral density as compared to WT controls.

Consistent with previous findings, we observed no significant baseline differences in bone mineral density, bone mass, or trabecular architecture between either *Nf1-LysM* (Supplemental Figure 3A, B) or *Nf1-Ctsk* mice (Supplemental Figure 4A, B) versus WT controls. However, following OVX induced pro-resorptive challenge, the rate of bone loss was significantly increased in *Nf1-LysM*-OVX mice as compared to WT controls. *Nf1-LysM*-OVX mice exhibited a 10.8 ± 1.5 (SEM) percent reduction in distal femoral bone mineral density (BMD) during the 6 weeks following OVX surgery versus WT animals which lost only 6.8 ± 0.7 (SEM) percent of distal femoral BMD (Figure 2B). As an additional control group, sham-operated animals gained approximately 1-3% of femoral BMD over the 6-week time course. Representative micro-computed tomography (μCT) reconstructions illustrate the marked loss of trabecular bone in OVX *Nf1-LysM* mice as compared to OVX WT controls (Figure 2A). Corroborating these data, quantitative μCT evaluation revealed significant deficits in femoral bone volume fraction (BV/TV) (Figure 2C), with commensurate changes in multiple bone microarchitecture parameters, including trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular spacing (Tb.Sp) between OVX *Nf1-LysM* and OVX WT mice (Figure 2D-F, respectively). Finally, histomorphometric enumeration of TRACP positive multinucleated OCs lining the trabecular bone surface revealed significantly increased osteoclast numbers in the femora of OVX *Nf1-LysM* mice (Figure 2G,H), implicating enhanced osteoclastogenesis as the pivotal factor promoting excess bone catabolic activity in *Nf1-LysM* animals.

Intriguingly, when analogous studies were performed in *Nf1-Ctsk* mice, whereby *Nf1* haploinsufficiency is restricted to mature, terminally differentiated OCs, we observed no significant differences in bone mass or microarchitecture parameters when comparing WT- and *Nf1-Ctsk*-OVX mice. Although bone loss occurred in both the mutant and control animals, the percentage reduction in femoral BMD was equivalent between WT- and *Nf1-Ctsk*-OVX mice (Figure 3A). Representative μCT reconstructions illustrate comparable bone loss in the distal femur between ovariectomized WT and *Nf1-Ctsk* mice (Figure 3B). Quantitatively, we detected no significant difference in femoral BV/TV (Figure 3C) or trabecular architecture parameters (Figure 3D-I.) when comparing *Nf1-Ctsk* and WT animals following OVX. Collectively, these data imply that conditional *Nf1* haploinsufficiency in mature OCs is insufficient to perpetuate increased osteolytic lytic activity in response pro-resporptive challenge; but rather, genetic ablation of *Nf1* in more primitive myeloid progenitor cells appears to be required to permit expansion of the OC precursor pool within the bone marrow, thereby promoting accelerated bone resorption *in vivo*.

Previous studies have demonstrated the *LysM*-Cre driver to be relatively specific to primitive myeloid lineages within the hematopoietic compartment[28]. Nonetheless, to further confirm that the observed phenotype in *Nf1-LysM* mice was unrelated to possible ectopic recombination or “leakage” of the *LysM*-Cre driver in the osteoblast lineage, osteoblasts were enumerated on the bone surface in WT and *Nf1-LysM* mice (Supplemental Figure 3C). We observed no significant difference in the number of osteoblasts per mm bone surface (Ob.N/BS, mm^{-1}) within the distal femur when comparing *Nf1-LysM* mice to the WT control, suggesting that the enhanced bone loss in *Nf1-LysM* mice in response to pro-resorptive stress is primarily related to increased osteoclast bone lytic activity.

Nf1 haploinsufficiency in OC progenitors results in Ras-dependent accumulation of phosphorylated Pu.1 in the nucleus

Given that haploinsufficient loss of *Nf1* in myeloid progenitor cells is critical to promoting increased osteolytic activity *in vivo*, we therefore reasoned that neurofibromin may play a pivotal role in regulating the expression and/or activity level of myeloid dependent transcription factors. Pu.1 transcriptional activity is required for myelomonocytic and osteoclast differentiation, whereby mice lacking Pu.1 exhibit severe osteopetrosis and are deficient in both macrophages and OCs secondary to impaired expression of the M-CSF receptor, c-Fms[35]. Here, we show that the expression level of Pu.1 is increased in the nucleus of *Nf1* haploinsufficient OC progenitors relative to WT controls stained with FITC-labeled anti-Pu.1 (green) and Hoescht (blue) (Figure 4A). We next performed western blot analysis to examine Pu.1 protein levels in OC progenitors treated with M-CSF and RANK-L. When we probed with Pu.1-specific antiserum (Santa Cruz, CA), the predominant protein detected in WT nuclear lysates migrated at an apparent molecular weight of approximately 35 kD. Intriguingly, the expression of two additional bands in the 40-55 kD range was markedly enriched in the $Nf1^{+/-}$ nuclear lysates across multiple timepoints (Figure 4B).

Phosphorylation of Pu.1 is known to retard its migration in SDS-PAGE electrophoresis[36,37]. To confirm that these band shifts represent phosphorylated forms of

Pu.1, nuclear lysates were treated with potato acid phosphatase prior to electrophoresis. Phosphatase treatment resulted in complete conversion of the shifted bands to the lower molecular weight, unphosphorylated Pu.1 species (Figure 4C). By contrast, treatment of the lysates with heat denatured potato acid phosphatase did not alter the migration or intensity of the shifted bands, thus validating that these bands correspond specifically to phosphorylated Pu.1 species (Figure 4C).

Given neurofibromin's function as a GTPase activating protein (GAP) for p21-Ras, we therefore reasoned that p21-Ras hyperactivation in *Nf1* haploinsufficent osteoclasts may underlie the accumulation of the phosphorylation form of Pu.1 in the nucleus. To test this hypothesis, we transduced primary *Nf1*$^{+/-}$ c-kit^{+} hematopoietic cells with a recombinant retrovirus encoding the full length *NF1* GAP-related domain (GRD) and a selectable marker, *pac*, which confers resistance to puromycin[38]. As compared to *Nf1*$^{+/-}$ c-kit^{+} cells expressing MSCV-*pac* alone, reconstitution of the full length *NF1-GRD* in *Nf1* haploinsufficient c-kit^{+} cells significantly attenuated the phosphorylation of nuclear Pu.1 (Figure 4D). Given recent findings implicating Pu.1 phosphorylation in the transcriptional activation of terminal myeloid specific gene programs[39,40], we postulate this to be a previously unrecognized mechanism by which *Nf1* haploinsufficiency engenders myeloid lineage commitment, thereby promoting osteoclastogenesis and hyper-resorptive activity.

Discussion

OCs cultured *ex vivo* from the bone marrow of *Nf1*$^{+/-}$ mice and the peripheral blood of human NF1 patients exhibit multiple gain-in-functions[22-24], consistent with clinical observations that NF1 patients have reduced bone mineral density[15-19,41] and are predisposed to increased fracture risk[20,21]. However, the functional requirement for *Nf1* haploinsufficiency within hematopoietic cells to perpetuate these osteolytic manifestations has yet to be elucidated in a step-wise, lineage restricted fashion. Here we demonstrate that conditional inactivation of a single *Nf1* allele within the myeloid progenitor cell population (*Nf1-LysM*) is necessary and sufficient to promote multiple osteoclast gain-in-functions, resulting in increased osteoclast progenitor fusion, enhanced osteoclastogenesis, and accelerated osteoclast bone lytic activity in response to pro-resorptive challenge *in vivo*. Intriguingly, mice conditionally *Nf1* heterozygous in mature, terminally differentiated osteoclasts (*Nf1-Ctsk*) do not recapitulate these phenotypes, indicating a critical requirement for *Nf1* haploinsufficiency at a more primitive stage of myeloid development in perpetuating enhanced osteolytic activity. Given that genetic ablation of a single *Nf1* allele in terminally differentiated osteoclasts alone is insufficient to augment the rate of bone lytic activity, we thus postulate that expansion of the osteoclast precursor pool and an overall increase in osteoclast numbers are the preponderant factors driving bone resorption in the context of *Nf1* haploinsufficiency (Figure 5).

In contrast to the *Nf1-Ctsk* model which does not display evidence of osteoclast hyperactivity as observed in *Nf1-LysM* and *Nf1*$^{+/-}$ animals[22], Alanne and colleagues recently reported that transgenic mice harboring conditional *Nf1* nullizygous (*Nf1*$^{-/-}$) osteoclasts driven by *TRAPCre*, exhibit increased bone resorptive capacity and aberrant actin ring formation *in vitro*[42]. Despite differences in *Nf1* gene dose (*Nf1* heterozygosity in

Nf1-Ctsk mice versus biallelic *Nf1* inactivation in the *TRACPCre;Nf1*$^{flox/flox}$ model), we postulate that the apparent osteoclast gain-in-functions in this mouse model are likely due to the expression of *TRAPCre* in more primitive stages of osteoclast/myeloid development as compared to *CtskCre*, which is restricted to mature, terminally differentiated osteoclasts[29]. Supporting this concept, the authors report that *TRACPCre;Nf1*$^{flox/flox}$ mice exhibit a number of extra-osseous features including splenomegaly and megakaryocytosis[42]. These findings are reminiscent of the juvenile myelomonocytic leukemia (JMML)-like myeloproliferative disease (MPD) phenotypes seen following *NF1(Nf1)* loss of heterozygosity (LOH) in the hematopoietic compartment[43,44], and are suggestive of *TRAPCre* recombination of *Nf1* in relatively primitive myeloid OC precursors as previously reported[29]. While the physiological relevance of the *Nf1* nullizygosity within the osteoclast lineage remains questionable given that NF1 patients suffering from osteopenia/osteoporosis typically retain one functional *NF1* allele within the hematopoietic compartment, these data nonetheless provide critical insight regarding the stage of myeloid/OC differentiation at which loss of loss of *Nf1* is permissive of OC gain-in-functions.

Nf1-LysM bone marrow mononuclear cells (BMMNCs) exhibit increased colony forming unit-macrophage (CFU-M) in methylcellulose culture, enhanced osteoclastogenic capacity in response to M-CSF and RANKL, thereby resulting in accelerated osteolytic activity *in vivo* following OVX-mediate pro-resorptive challenge. By contrast, *Nf1-Ctsk* mice did not recapitulate any of these phenotypes in analogous experiments, indicating a critical requirement for *Nf1* haploinsufficiency at a more primitive stage of myeloid development in perpetuating osteolytic activity.

Pu.1 transcriptional activity is indispensable for myelomonocytic and osteoclast differentiation, whereby the absence of Pu.1 in mice leads to severe osteopetrosis and combined deficiency of both macrophages and OCs secondary to impaired expression of the M-CSF receptor, c-Fms [35,45]. Intriguingly, however, supraphysiologic levels of Pu.1 expression do not necessarily correlate with increased myelomoncytic lineage commitment. In fact, recent studies suggest that phosphorylation of Pu.1 plays a central role in modulating its DNA binding affinity[37,46], and is thereby critical to its ability to activate myeloid specific gene programs that promote terminal differentiation[39,40]. For instance, although human myeloid leukemic cell lines have been shown to overexpress Pu.1[47], the transcript levels of Pu.1-dependent myeloid genes, such as *CD11b* and *c-fms*, are relatively low by comparison[48,49]. Carey and colleagues demonstrated that this discrepancy between Pu.1 expression level and transcriptional activity depends on the phosphorylation status of Pu.1[37], whereby Pu.1 phosphorylation induced by tetradecanoylphorbol-13 acetate (TPA) led to growth arrest in leukemic cells[37]. A recent study by Seshire et al. further demonstrated that Pu.1 dephosphorylation at serine residues by the leukemia-associated fusion protein pro-myelocytic leukemia/retinoic acid receptor alpha (X-RARα) inhibits Pu.1 promoter binding and transcription of *c-fms* among other myelomoncytic genes[40].

In the present study, we demonstrate that the *Nf1* tumor suppressor gene modulates nuclear levels of phosphorylated Pu.1 in myeloid OC progenitors, whereby M-CSF/RANKL induced p21Ras hyperactivation (in the context of *Nf1* haploinsufficieny) leads to increased levels of phospho-Pu.1 in the nucleus. Re-expression of the human full-length GAP related domain

(GRD) of *NF1* in primary *Nf1*$^{+/-}$ c-kit^{+} hematopoietic cells significantly attenuated the phosphorylation of nuclear Pu.1, demonstrating the Ras dependent nature of this process. The apparent association between increased phosphorylation of nuclear Pu.1 and enhanced osteoclastogenesis in the context of *Nf1* haploinsufficiency warrants further investigation in future studies.

Collectively, the results of this study suggest that the low bone mass phenotype observed in *Nf1*$^{+/-}$ and *Nf1- LysM* mice (and possibly the phenotype of osteopenia/osteoporosis in NF1 patients) may be due to enhanced osteoclast differentiation and recruitment of osteoclast progenitors which occurs at a relatively primitive stage of myeloid differentiation, driven by Ras mediated hypersensitivity to M-CSF and RANKL. Thus, circumventing the early stage recruitment and expansion of the osteoclast progenitor pool by targeting the molecular pathways driving this process may represent a rational therapeutic strategy in the treatment of NF1-associated osteoporosis. By contrast, bisphosphonates, which have long since been the clinical mainstay of in the treatment of osteoporosis, function to induce apoptosis of mature, terminally differentiated osteoclasts as they begin to resorb the bone matrix. As they accumulate at high concentrations in the bone matrix, bisphosphonates have been associated with a number of deleterious effects over time including the accumulation of microfractures and osteonecrosis among other complications[50]. These pitfalls have led to the advent of other novel anti-resorptive therapies such as denosumab[51], which acts at more primitive stage of osteoclast development as compared to bisphosphonates by blocking the RANKL-dependent terminal differentiation of osteoclast progenitor cells.

Evidence regarding the efficacy of bisphosphonates in the treatment of NF1-associated osteoporosis are inconclusive. A recent clinical study limited to six patients with quite profound osteoporosis did demonstrate a trend toward increased BMD in five out of the six patients in the study, although the effect did not reach statistical significance after 23 months of treatment[52]. By contrast, recent work by Heervä and colleagues has shown that osteoclasts derived from human NF1 patients were resistant to bisphosphonate induced apoptosis versus healthy controls[53]. As such, the application of targeted anti-resorptive agents that act at a more primitive stage of osteoclast differentiation may hold therapeutic promise in the treatment of NF1 associated osteopenia/osteoporosis and warrants further exploration in preclinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Points

- *Nf1* haploinsufficiency in myelomonocytic precursors alters lineage commitment, potentiating osteoclast differentiation and lytic activity.
- *Nf1* haploinsufficient osteoclast gain-in-functions are associated with Ras-dependent increases in nuclear phosphorylated Pu.1.

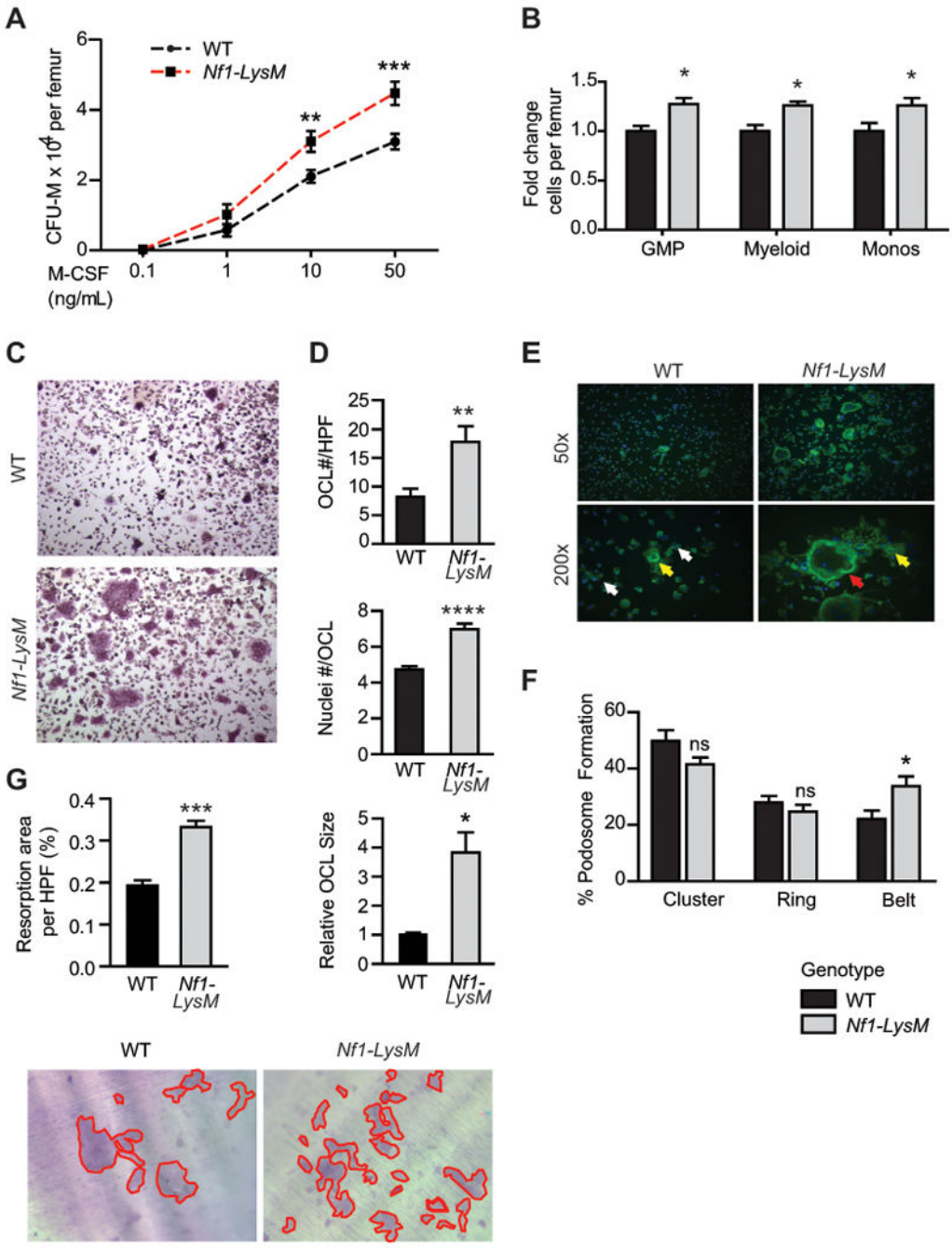

Figure 1. *Nf1* haploinsufficiency in myeloid progenitor cells promotes expansion of the OC progenitor population and potentates OC differentiation

(**A**) The number of colony forming unit monocyte/macrophage (CFU-M) per femur were enumerated following 7 days culture in semisolid methycellulose media. $^{**}P < 0.01$, $^{***}P < 0.001$, *Nf1-LysM* vs WT. $n = 8$ mice per genotype. (**B**) Populations of GMP, myeloid cells, and monocytes in the bone marrow were identified by flow cytometry. Fold changes in the number of cells per femur are reported. $^{*}P < 0.05$, *Nf1-LysM* vs WT. $n = 4$ mice per genotype. (**C**) Representative photomicrographs show TRACP staining of bone marrow

derived osteoclasts cultured in the presence of M-CSF and RANKL for 6 days. (**D**) The number of osteoclasts per high power field (HPF),the number of nuclei per osteoclast, and the mean osteoclast size were quantitated as shown. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *Nf1-LysM* vs WT. *n* = 4 biological replicates per genotype. (**E**) Representative photomicrographs show actin ring formation in bone marrow derived osteoclast cultures at 50× (top) and 200× (bottom) magnification. Cells were stained with Alexa Fluor® 488 Phalloidin (green) and Hoechst (blue). (**F**) Podosome formation was evaluated by determining the percent distribution of actin organization into clusters, rings, and belts. **P* < 0.05, *Nf1-LysM* vs WT. *n* = 4 biological replicates per genotype. (**G**) Osteoclasts were incubated on dentine slices with representative photomicrographs showing resorptive “pits” generated by osteoclast bone lytic activity. Resorptive “pit” area was quantified as shown in the bar graph. ****P* < 0.001, *Nf1-LysM* vs WT.

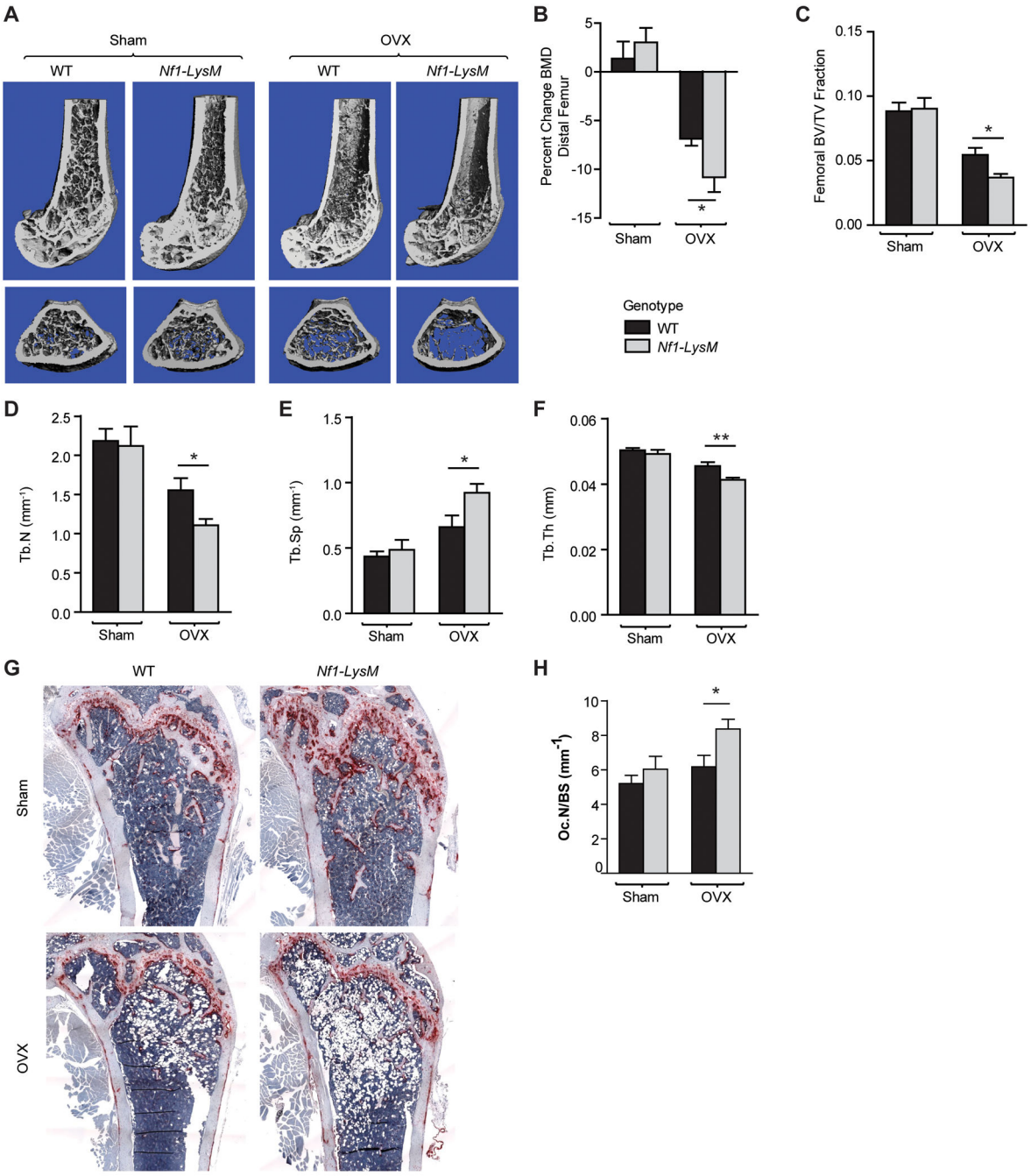

Figure 2. Conditional *Nf1* haploinsufficiency in myeloid progenitor cells potentiates osteolytic activity *in vivo* in response to OVX mediated pro-resorptive challenge

(**A**) Representative micro-computed tomography (μCT) shows reconstructed femora in longitudinal (top) and transverse (bottom) cross-sections 6-weeks post-surgery (OVX vs sham-operated controls). (**B**) The OVX-induced reduction (percentage change) in bone mineral density (BMD) of the distal femur was determined by pDEXA measurements acquired before and 6-weeks after ovariectomy vs sham surgery. **P* < 0.05, OVX *Nf1-LysM* vs OVX WT. WT Sham (*n* = 5), *Nf1-LysM* Sham (*n* = 5), WT OVX (*n* = 8), *Nf1-LysM*

Sham ($n = 10$) mice per genotype. (**C**) Trabecular bone volume fraction (BV/TV) was quantified by μCT. WT Sham ($n = 6$), *Nf1-LysM* Sham ($n = 6$), WT OVX ($n = 7$), *Nf1-LysM* Sham ($n = 12$) mice per genotype. *$P < 0.05$, OVX *Nf1-LysM* vs OVX WT. Trabecular microarchitecture parameters including trabecular number (Tb.N) (**D**), trabecular spacing (Tb.Sp) (**E**), and trabecular thickness (Tb.Th) (**F**) were quantified by μCT. WT Sham ($n = 6$), *Nf1-LysM* Sham ($n = 6$), WT OVX ($n = 7$), *Nf1-LysM* Sham ($n = 12$) mice per genotype. *$P < 0.05$, OVX *Nf1-LysM* vs OVX WT**. (G)** Representative photomicrographs of TRACP stained distal femora of WT and *Nf1-LysM* mice 6 weeks following either sham or OVX surgery. (**H**) Osteoclast numbers per mm bone surface (Oc.N/BS) were manually enumerated at 100× magnification on TRACP stained sections of the distal femur. WT Sham ($n = 5$), *Nf1-LysM* Sham ($n = 5$), WT OVX ($n = 3$), *Nf1-LysM* Sham ($n = 5$) mice per genotype. *$P < 0.05$, OVX *Nf1-LysM* vs OVX WT.

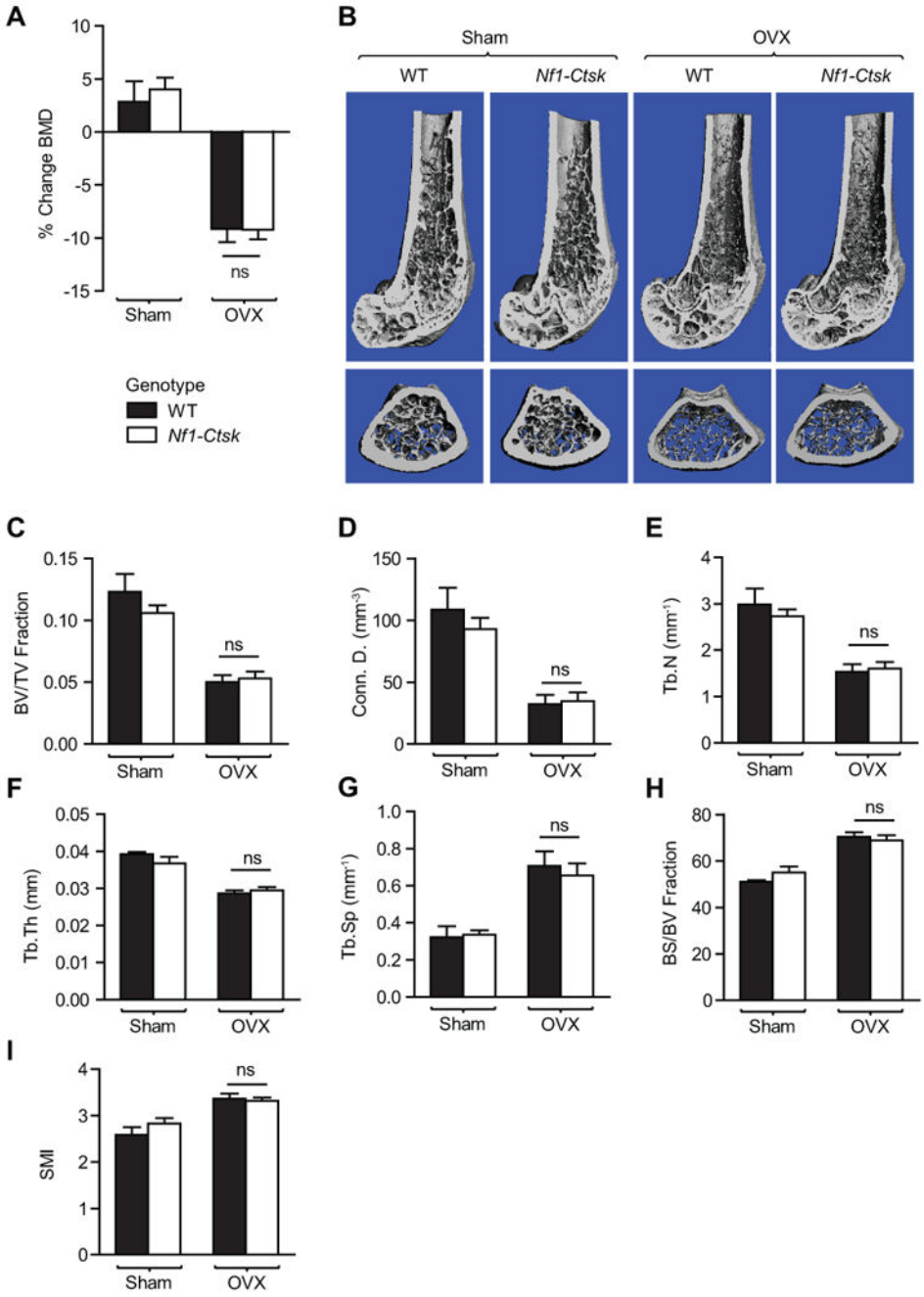

Figure 3. Conditional *Nf1* heterozygosity in terminally differentiated OCs is not sufficient to potentiate bone catabolic activity in ovariectomized *Nf1-Ctsk* mice

(**A**) The percentage change in bone mineral density (BMD) 6 weeks status-post OVX vs sham surgery was compared between WT and *Nf1-Ctsk* mice. WT Sham ($n = 7$), *Nf1-Ctsk* Sham ($n = 6$), WT OVX ($n = 15$), *Nf1-Ctsk* OVX ($n = 10$) mice per genotype. ns = no significant difference. (**B**) Representative μCT reconstructed femora in longitudinal (top) and transverse (bottom) cross-sections for WT versus *Nf1-Ctsk* mice 6 weeks following either sham or OVX surgery. (**C**) Femoral BV/TV was quantified by μCT as shown and

revealed no significant difference between WT and *Nf1-Ctsk* mice. WT Sham ($n = 5$), *Nf1-Ctsk* Sham ($n = 5$), WT OVX ($n = 12$), *Nf1-Ctsk* OVX ($n = 12$) mice per genotype. No significant differences in trabecular microarchitecture parameters were found when comparing connectivity density (Conn.D.) (**D**), trabecular number (Tb.N) (**E**), trabecular thickness (Tb.Th) (**F**), trabecular spacing (Tb.Sp) (**G**), bone surface to bone volume ratio (BS/BV) (**H**), and structure model index (SMI) (**I**) between WT and *Nf1-Ctsk* mice undergoing OVX surgery. WT Sham ($n = 5$), *Nf1-Ctsk* Sham ($n = 5$), WT OVX ($n = 12$), *Nf1-Ctsk* OVX ($n = 12$) mice per genotype.

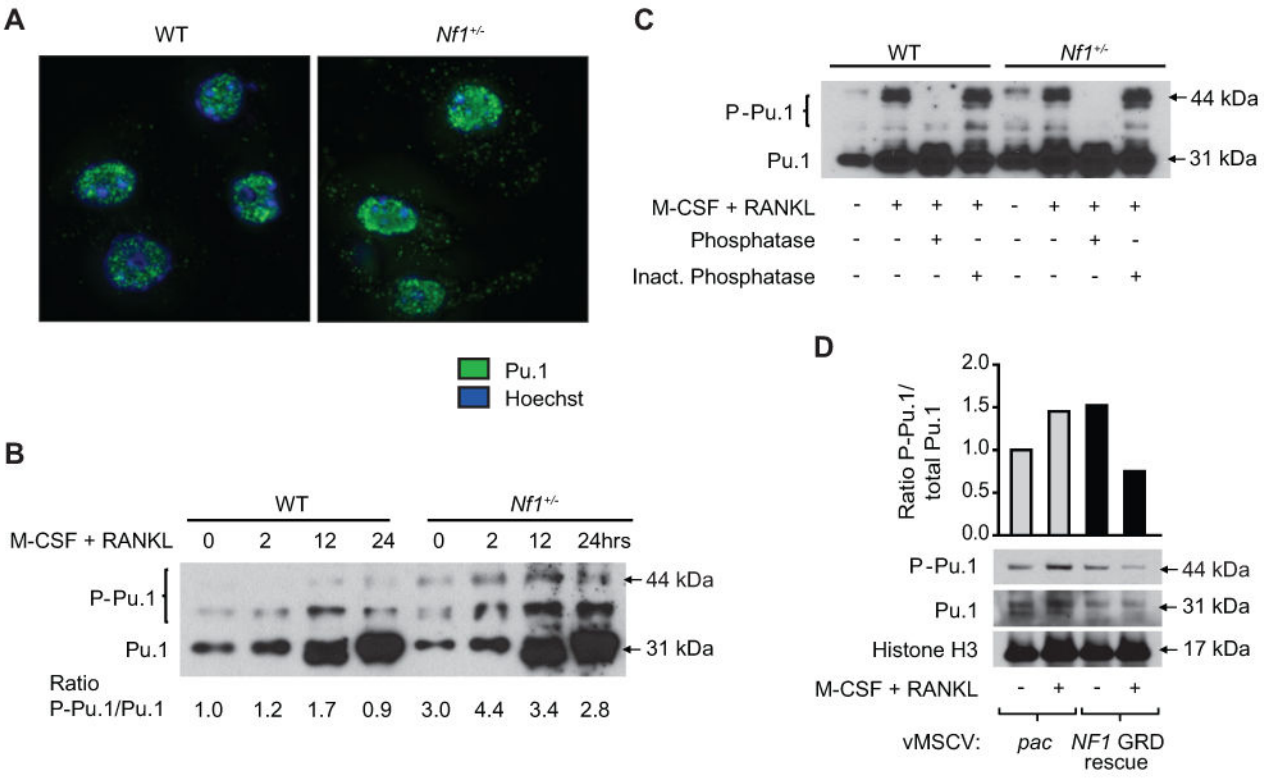

Figure 4. *Nf1* haploinsufficiency drives p21-Ras dependent phosphorylation of Pu.1 in the nucleus of myelomonocytic osteoclast precursors

Primary osteoclast progenitors were isolated from WT and *Nf1*$^{+/-}$ mice according to the same methodology as in previous experiments. (**A**) Representative photomicrographs of WT and *Nf1*$^{+/-}$ primary OC progenitors stained with FITC-labeled anti-Pu.1 (green) and Hoescht (blue). The assay was performed on two independent occasions with similar results. (**B**) Phosphorylated and nonphosphorylated Pu.1 species were detected in OC progenitor nuclear lysates by western blot following stimulation with M-CSF and RANKL for 0, 2, 12, and 24 hours. The expression level of the phosphorylated Pu.1 species was determined by densitometry as a ratio to the predominant non-phosphorylated Pu.1 band as in internal nuclear loading control. This experiment was repeated three times, with similar results. (**C**) Nuclear protein lysates were prepared from OC progenitors following 12 hours stimulation with M-CSF and RANKL. Nuclear lysates were subsequently incubated at 37°C with either phosphatase or heat inactivated phosphatase for a duration of 30 minutes prior to immunoblotting with anti-Pu.1 antibody. This experiment performed two times, with similar results. (**D**) The human full-length GAP related domain (GRD) of *NF1* was re-expressed by retroviral transduction of primary c-kit^{+} hematopoietic cells isolated from the bone marrow of WT and *Nf1*$^{+/-}$ mice. Following puromycin selection, cells were stimulated with M-CSF and RANK-L and phosphorylated and non-phosphorylated Pu.1 species were detected in nuclear lysates by western blot. Histone H3 was detected as an additional nuclear loading control. The ratio of phosphorylated to total Pu.1 was determined by densitometry. This experiment was performed twice, with similar results.

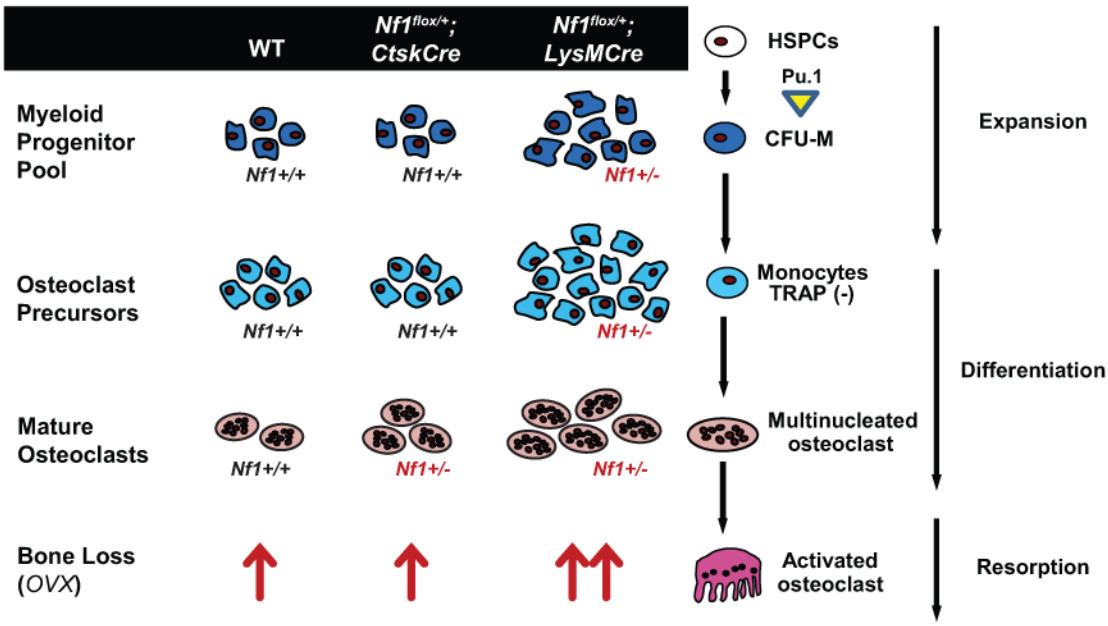

Figure 5. Haploinsufficient loss of *Nf1* in myeloid progenitor cells is required to potentiate osteolytic activity in a mouse model of NF1 associated osteoporosis

Given that genetic ablation of a single *Nf1* allele in terminally differentiated osteoclasts alone (*Nf1-Ctsk*) is insufficient to augment the rate of bone lytic activity, we postulate that expansion of the OC precursor pool (*Nf1-LysM*) and an overall increase in OC numbers are the preponderant factors perpetuating excess osteolytic activity *in vivo* following OVX mediated pro-resorptive challenge. These phenotypes are associated with p21-Ras dependent hyper-phosphorylation of the myeloid transcription factor Pu.1 in the nucleus of *Nf1* haploinsufficent myelomonocytic OC precursors.