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Claudia Goettsch, … , Ralf P. Brandes, Katrin Schröder


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NADPH oxidase 4 limits bone mass by promoting osteoclastogenesis

Claudia Goettsch,1,2 Andrea Babelova,3 Olivia Trummer,4 Reinhold G. Erben,5 Martina Rauner,1 Stefan Rammelt,6,7 Norbert Weissmann,8 Valeska Weinberger,3 Sebastian Benkhoff,3 Marian Kampschulte,9 Barbara Obermayer-Pietsch,4 Lorenz C. Hofbauer,1,7 Ralf P. Brandes,3 and Katrin Schröder3

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ROS are implicated in bone diseases. NADPH oxidase 4 (NOX4), a constitutively active enzymatic source of ROS, may contribute to the development of such disorders. Therefore, we studied the role of NOX4 in bone homeostasis. Nox4–/– mice displayed higher bone density and reduced numbers and markers of osteoclasts. Ex vivo, differentiation of monocytes into osteoclasts with RANKL and M-CSF induced Nox4 expression. Loss of NOX4 activity attenuated osteoclastogenesis, which was accompanied by impaired activation of RANKL-induced NFATc1 and c-JUN. In an in vivo model of murine ovariectomy–induced osteoporosis, pharmacological inhibition or acute genetic knockdown of Nox4 mitigated loss of trabecular bone. Human bone obtained from patients with increased osteoclast activity exhibited increased NOX4 expression. Moreover, a SNP of Nox4 was associated with elevated circulating markers of bone turnover and reduced bone density in women. Thus, NOX4 is involved in bone loss and represents a potential therapeutic target for the treatment of osteoporosis.

Introduction
Bone is a dynamic organ that undergoes constant remodeling in response to external stimuli. While bone formation is mediated by osteoblasts, bone resorption is performed by osteoclasts, which derive from the myeloid lineage. In vivo as well as in cell culture models, osteoclastogenesis requires the differentiation factors RANKL and M-CSF (1). M-CSF induces the expression of RANK, the receptor of RANKL on myeloid cells. RANKL binding initiates osteoclast differentiation and activation (2) and is responsible for the induction of osteoclastic genes like tartrate-resistant acid phosphatase (TRAP) and cathepsin K (3). Mature osteoclasts are also activated by RANKL, which maintains their survival (4) and induces cytoskeletal rearrangement, promoting bone resorption (5).

Osteoclastogenesis can also be promoted by cytokines like TGF-β, which act in a biphasic manner on osteoclast formation (6). TGF-β has been linked to oxidative stress, as it increases the formation of ROS and induces the expression of NADPH oxidase 4 (NOX4) (7).

In the NOX family, whose sole function is to produce ROS, NOX4 takes an exceptional position. NOX4 is induced in the course of differentiation in many cells and is constitutively active. Thus, ROS production by NOX4 is largely controlled by the expression level of the enzyme. NOX4 produces H2O2 (8), whereas other NOX proteins generate superoxide anions, require complex activation steps, and show tissue-specific expression. The permanent production of H2O2 by NOX4 potentially adjusts the cellular redox status and interferes with slow processes like differentiation of mesenchymal cells, such as cardiac myocytes, smooth muscle cells, or adipocytes (9).

It has long been suggested that ROS stimulate osteoclasts (10), and impaired antioxidative defense is thought to contribute to increased bone resorption (11). Increased ROS production has also been linked to osteoclastogenesis in cell culture models (12, 13), but the enzymatic source and mechanisms of ROS-induced osteoclastogenesis have not been deciphered.

In the present study, we showed that NOX4 contributes to osteoclastogenesis, that loss of NOX4 activity prevents bone loss in mice, and that NOX4 is associated with increased parameters of bone resorption in humans.

Results
NOX4 negatively affects bone density in mice by increasing osteoclast numbers. To identify the effect of NOX4 on bone homeostasis, WT and Nox4–/– mice were studied. As confirmed by Western blot analysis, NOX4 expression was apparent in femur lysates of WT mice, but not Nox4–/– mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI67603S1). Bones of Nox4–/– mice displayed greater trabecular width and thickness than those of WT mice (Figure 1, A–C), whereas trabecular number (WT, 4.3 ± 0.4 per mm; Nox4–/–, 4.4 ± 0.3 per mm) and separation (WT, 217 ± 23 µm; Nox4–/–, 207 ± 14 µm) were similar. Compared with their WT littermates, Nox4–/– mice also exhibited 30% greater trabecular bone mineral density (BMD) of the distal femur (Figure 1D). Importantly, this effect was specific for Nox4, as genetic deletion of Nox2 or Nox1 had no effect on BMD (Nox2–/–, 345.3 ± 12.5 mg/cm²; Nox2–/–, 329.0 ± 18.9 mg/cm²; Nox1–/–, 345.3 ± 12.5 mg/cm²; Nox2–/–, 329.0 ± 18.9 mg/cm²).
As a functional consequence, the biomechanical strength of bones from Nox4–/– mice was significantly higher than that of WT mice (Figure 1E).

Bone resorption, but not bone formation, depends on NOX4. The elevated BMD in Nox4+/- mice suggests that the enzyme either promotes bone resorption or inhibits bone formation. As revealed by calcein labeling, bone formation rate was not altered by genetic deletion of Nox4 (Figure 2A and B); additionally, the mineral apposition rate and double-labeled surface were not different between WT and Nox4+/- animals (Figure 2C and Supplemental Figure 2A). Moreover, plasma levels of procollagen 1 N-terminal peptide, a marker of osteoblast activity, were similar between WT and Nox4-/- mice (Supplemental Figure 2B).

To verify this, the effect of NOX4 on osteoclastogenesis was determined in murine cells in vitro. Bone marrow mononuclear cells (BMNCs) were stimulated with M-CSF and RANKL, and differentiated osteoclasts were identified by their multinucleated morphology and positive TRAP staining. Osteoclast formation was 56% lower in BMNCs of Nox4–/– versus WT mice (Figure 2, H and I). This suggests that the lower number of osteoclasts in Nox4–/– mice is indeed a consequence of impaired differentiation. NOX4 is a mediator of RANKL signaling. To provide functional evidence for a role of NOX4 in the process of osteoclast differentiation, NOX4 expression and ROS formation were measured. During RANKL- and M-CSF-stimulated osteoclastogenesis of WT BMNCs, Nox4 mRNA expression greatly increased (Figure 3A), which was associated with a substantial increase in ROS production. In contrast, ROS formation did not increase in the course of differentiation of cells obtained from Nox4–/– mice (Figure 3B), suggesting a potential role of NOX4-derived ROS for RANKL-induced differentiation.

As RANK expression was not different between M-CSF–primed WT and Nox4–/– BMNCs (data not shown), we determined the role of NOX4 in RANKL signaling. Treatment of M-CSF–primed murine mononuclear cells with RANKL for 30 hours induced a marked increase in basal cytosolic Ca²⁺ in WT, but not Nox4–/–, cells. Moreover, catalase prevented the RANKL-induced increase in cytosolic Ca²⁺ in WT cells, but had no effect on basal Ca²⁺ in Nox4–/– cells (Figure 3C). Recently, a family of active small-molecule NOX4 inhibitors has been developed (14). Similar to the effects of catalase or Nox4 deletion, treatment with a compound of this family, GKT137831 (20 μmol/l), prevented the RANKL-induced increase in cytosolic Ca²⁺ in WT cells, without affecting the Ca²⁺ concentration in cells obtained from Nox4–/– mice (Figure 3D). These data identified NOX4-derived H₂O₂ as an important mediator of RANKL-induced signaling during osteoclastogenesis.

The transcription factors nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), and activator protein 1 (AP-1) are involved in RANKL-induced osteoclastogenesis (15). NFATc1 activation depends on calcineurin activation, which is directly activated by Ca²⁺ or cleavage by the calcium-dependent protease μ-calpain. Indeed, in response to RANKL, active μ-calpain and nuclear accumulation of NFATc1 increased in M-CSF–primed murine BMNCs, effects that were absent in cells from Nox4–/– mice (Figure 3, E and F).

262.2 ± 19.7 mg/cm³; Nox1y/–, 268.7 ± 11.0 mg/cm³; n = 8; P = NS). As a functional consequence, the biomechanical strength of bones from Nox4–/– mice was significantly higher than that of WT mice (Figure 1E).

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Calcineurin expression, in contrast, was not modulated by NOX4 (data not shown). Moreover, AP-1 appeared to be controlled by NOX4: RANKL treatment of M-CSF–primed BMNCs increased JNK phosphorylation in cells from WT, but not Nox4–/–, cells (WT, 30% ± 1% relative increase after 15-min RANKL stimulation; Nox4–/–, 1% ± 1% relative increase; n = 3; *P < 0.05). This resulted in a lack of nuclear c-JUN accumulation in Nox4 –/– cells (Figure 3F). In accordance with these observations, RANKL-mediated Oscar induction was attenuated by catalase, by genetic deletion of Nox4, and by the calpain inhibitor calpeptin (Figure 3G). Thus, in the course of osteoclastogenesis, NOX4 is required for RANKL-induced activation of the differentiating transcription factors NFATc1 and AP-1.

A SNP of NOX4 is associated with altered bone density and plasma markers for bone turnover in middle-aged women. We next sought to study the contribution of NOX4 to human bone metabolism and performed SNP analyses. For NOX4 rs11018628, a significant association for altered parameters of bone metabolism was found when the dominant TT allele was compared with the combined CC and CT alleles: levels for serum alkaline phosphatase, CTX, and osteocalcin were significantly different between groups in an existing osteoporosis screening study (Tables 1 and 2 and ref. 16). To determine the biological significance of these findings, bone density measurements were performed. Hip BMD T-scores in females, but not males, were significantly higher in the dominant allele; accordingly, there was a trend toward higher areal hip bone density in the female subgroup (Table 3). Although these observations do not prove a direct role of NOX4 in osteoporosis development, they suggest that NOX4 contributes to bone characteristics in humans. As rs11018628 is localized in an intron of NOX4, a direct effect on enzyme activity is not plausible, suggestive of an effect on NOX4 expression. To address this, immortalized EBV-transduced B-lymphocytes of the CC and TT genotypes were obtained from the Coriell Institute (see Methods). Nox4 mRNA was undetectable in these cells under basal conditions, but increased in the course of differentiation induced by vitamin D3. Importantly, Nox4 mRNA expression after 3 days

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**Figure 2**

Role of NOX4 in bone remodeling. (A) Representative images of calcein-labeled bone surface. (B) Bone formation rate and (C) mineral apposition rate in bones of WT and Nox4–/– mice. (D) Western blot analysis and statistical analyses of densitometry (values shown relative to WT control) from femur lysates for OSCAR, RUNX2, and GAPDH as loading control. (E) Number of TRAP-positive osteoclasts (N.Oc) per bone perimeter (B.Pm), counted in histomorphometric analysis, and (F) representative images of TRAP-stained sections counterstained with Mayer’s hemalaun. (G) Serum levels of CTX. (H and I) Number (H) and representative images (I) of osteoclasts differentiated ex vivo from bone marrow cells of WT and Nox4–/– mice, as identified by TRAP staining and multinucleation. Scale bars: 500 μm (A); 100 μm (F and I). Data are mean ± SEM (n = 3–9). *P < 0.05.
of differentiation was significantly higher in cells obtained from subjects carrying the CC versus the TT allele (Supplemental Figure 3). Collectively, these data indicate that NOX4 expression is higher in subjects carrying the CC allele of NOX4 rs11018628, which is associated with increased markers of bone turnover.

Immunoreactivity of NOX4 is increased in human osteoporosis. When human PBMCs were differentiated into osteoclasts in culture, expression of NOX4 increased during the 21-day protocol, as did that of NOX1 (Figure 4A and Supplemental Figure 4). Since NOX1 did not affect bone density in vivo (see above), we focused our analyses on NOX4. One major feature of osteoclasts is the resorption of extracellular matrix. In a PIT formation assay, the NOX4 inhibitor GKT137831 dose-dependently prevented the degradation of the matrix (Figure 4, B and C), which suggests that NOX4 is essentially involved in osteoclastogenesis in human cells as well.

To determine the role of NOX4 in human bone metabolism, we compared the expression of the protein in a set of human bones by immunostaining. Staining intensity of NOX4 was markedly higher in bones obtained from patients with untreated osteoporosis compared with healthy bones (mean age, 51 ± 1.5 years; Figure 4, D and E). Moreover, bone samples from patients with Paget disease, a focal disorder of enhanced bone remodeling, also displayed greater NOX4 immunoreactivity.

NOX4 contributes to bone loss in a mouse model of osteoporosis. To test a direct causal link between NOX4 and osteoporosis, we stimulate bone resorption by estrogen withdrawal in mice. Female mice were subjected to ovariectomy, and bone loss was determined 6 weeks later. As expected, the operation induced a reduction of bone density of approximately 25% and an increase in Oscar expression in bones, whereas Runx2 expression remained unchanged (Supplemental Figure 5). After ovariectomy, NOX4 expression gradually increased at the protein and mRNA levels (Figure 5, A and B), again supporting a potential role of NOX4. Therefore, we next studied the effect of acute genetic deletion of Nox4 by a tamoxifen-activated Cre recombinase (Nox4 fl/fl -ERT-Cre +/0 mice; see Methods) on bone density. In ovariectomized mice, treatment with tamoxifen itself prevented bone loss to some extent, which was most likely a consequence of the partial agonist activity of tamoxifen on the estrogen receptor, which also shortens osteoclast lifespan and prevents osteocyte apoptosis (17). Despite this, acute Nox4 deletion in Nox4 fl/fl -ERT-Cre +/0 animals had an additive inhibitory effect on bone loss (Figure 5C). These data demonstrate that NOX4 is a potential target for the pharmacological prevention of osteoporosis development. In order to verify this aspect, we treated ovariectomized WT mice with the NOX inhibitor GKT137928, administered by
daily oral gavage over 6 weeks after ovariectomy, and compared its effect with the gold standard therapeutic bisphosphonate (pamidronate; 10 mg/kg once per week i.p.). Pamidronate massively increased total and trabecular bone density to an extent exceeding even the bone density of the sham-operated mice by approximately 2-fold. The effect of GKT137928, in contrast, was less pronounced, but still attenuated ovariectomy-induced bone loss more than 60% (Figure 5, D and E). Biomechanical strength analysis of the bones was used to determine the functional consequence of this intervention. The breaking strength of bone from mice treated with GKT137928 was significantly higher than that of mice receiving solvent (solvent, 14.1 ± 0.3; GKT137928, 15.1 ± 0.3; n = 8–15; P < 0.05). Thus, pharmacological inhibition of NOX4 inhibited bone loss in a model of osteoporosis in mice.

## Discussion

Although ROS have been linked to bone loss for many decades (18), our understanding of the mechanisms underlying this phenomenon has changed greatly in recent years. Whereas a concept of redox imbalance and oxidative stress leading to tissue damage was traditionally favored, it is now progressively becoming clear that ROS are pivotal modulators of cell signaling. This concept, however, implies precisely controlled ROS generators, and some of these generators are in fact the NOX proteins (19).

Here we showed that the constitutively active NOX4 is induced during osteoclast differentiation and that its global knockout leads to increased bone density. Alterations in the bones of Nox4−/− mice did not affect the compacta, but were restricted to trabecular bone, the site of osteoporosis development. Indeed, acute genetic knockdown of Nox4 or its pharmacological inhibition attenuated trabecular bone loss in mice after ovariectomy, and in vitro osteoclastogenesis was attenuated after loss of NOX4 activity. These findings are in line with the more general observations that ROS promote osteoclastogenesis in bone marrow–derived cells (12) and in the RAW264.7 macrophage cell line (13).

Given the multiple targets of ROS and the complex alterations in signaling and gene expression occurring in the course of differentiation, it is challenging to define the exact position of NOX4 in the signaling network. RANKL increases intracellular calcium (11) and activates JNK (20) in M-CSF–primed BMNCs, effects that were both attenuated by loss of NOX4 activity in the present study. The increase in intracellular calcium during osteoclast differentiation has been linked to the induction of transient receptor potential cation channel V2 (TRPV2), TRPV5 (21, 22), and sarco(endo)plasmic reticulum Ca2+ ATPase (SERCA) (23). Indeed, the activities of these proteins (24, 25) and of other regulators of intracellular calcium are known to be redox sensitive (26, 27). Calcium-induced osteoclast differentiation is mediated by the calcium-stimulated phosphatase calcineurin, which activates nuclear translocation of NFATc1, leading to the expression of OSCAR and other proteins. Activation of calcineurin occurs via a calmodulin-dependent conformational change that releases the autoinhibitory loop of the regulatory subunit calcineurin B (28). An alternative mode of activation is the proteolytic cleavage of the autoinhibitory loop of calcineurin A by μ-calpain, leading to constitutive activation of calcineurin (29). Based on our present findings, we conclude that the RANKL-induced increase in cytosolic Ca2+ and μ-calpain activation are NOX4 dependent, and thus calcineurin-mediated nuclear NFATc1 translocation does not occur in Nox4-deficient cells. Cooperation of NFATc1 with the transcription factor AP-1 is required for osteoclastogenesis (30). AP-1 requires active JNK MAP kinase, which phosphorylates the AP-1 component c-JUN. MAP kinase activation is among the best-studied of the redox-sensitive processes, which are mediated by transient ROS-mediated oxidation of cysteines in phosphatases (31). However, the sources of ROS — the MAP kinases and phosphatases involved in this process — vary greatly among cell types. NOX4 overexpression in RAW264.7 cells increased JNK phosphorylation (32), and ROS-dependent inhibition of the phosphatase MKP-1 (33), which dephosphorylates JNK in osteoclasts (34), has been implicated in this process in fibroblasts.

Although eventually, only studies in humans treated with NOX4 inhibitors will unravel the importance of this NOX for bone loss, several lines of evidence in the present study suggest NOX4 as a possible pharmacological target for the treatment of osteoporosis: (a) acute genetic deletion or pharmacological inhibition of NOX4 attenuated bone loss in the murine ovariectomy model, (b) NOX4 immunoreactivity was increased in osteoprotic bones, and (c) SNP analysis suggested that NOX4 expression in humans is associated with altered parameters of bone metabolism. Thus, the present study provided evidence on multiple levels to qualify NOX4 as a novel pharmacological target for the treatment of bone loss. With GKT137831 and GKT137928, the first specific NOX inhibitors without antioxidant properties

### Table 1

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<th>Men</th>
<th>Women</th>
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<tr>
<td>Age (yr)</td>
<td>51.08 ± 12.15</td>
<td>61.80 ± 10.70</td>
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<td>Weight (kg)</td>
<td>83.3 ± 12.1</td>
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<td>Height (cm)</td>
<td>176.8 ± 7.0</td>
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<td>Calcium (mmol/l)</td>
<td>2.48 ± 0.12</td>
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<td>Phosphate (mg/dl)</td>
<td>2.94 ± 0.58</td>
<td>3.71 ± 0.57</td>
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<td>25(OH) vitamin D (ng/ml)</td>
<td>33.22 ± 11.52</td>
<td>32.65 ± 15.07</td>
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<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>69.15 ± 17.50</td>
<td>94.50 ± 35.80</td>
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<tr>
<td>CTX (ng/ml)</td>
<td>0.239 ± 0.134</td>
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<tr>
<td>Osteocalcin (ng/ml)</td>
<td>17.7 ± 5.8</td>
<td>7.0 ± 6.7</td>
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<tr>
<td>Parathyroid hormone (pg/ml)</td>
<td>40.7 ± 12.8</td>
<td>28.1 ± 7.7</td>
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<td>Lumbar BMD (g/cm²)</td>
<td>1.016 ± 0.147</td>
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<td>Lumbar BMD T-score</td>
<td>−0.655 ± 1.329</td>
<td>−1.594 ± 1.436</td>
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<td>Lumbar BMD Z-score</td>
<td>−0.150 ± 1.380</td>
<td>−0.075 ± 1.387</td>
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<td>Femoral neck BMD (g/cm²)</td>
<td>0.855 ± 0.121</td>
<td>0.694 ± 0.123</td>
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<td>Femoral neck BMD T-score</td>
<td>−0.543 ± 0.893</td>
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<td>0.292 ± 0.900</td>
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<tr>
<td>Total hip BMD (g/cm²)</td>
<td>1.017 ± 0.123</td>
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<td>−1.176 ± 1.198</td>
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<tr>
<td>Total hip BMD Z-score</td>
<td>0.304 ± 0.826</td>
<td>0.031 ± 1.085</td>
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Data are mean ± SD. n = 228 (men); 180 (women).

### Table 2

<table>
<thead>
<tr>
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<th>CC+CT</th>
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<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>86.8 ± 28.1</td>
<td>78.7 ± 29.7</td>
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<tr>
<td>CTX (ng/ml)</td>
<td>0.285 ± 0.125</td>
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<tr>
<td>Osteocalcin (ng/ml)</td>
<td>10.07 ± 6.79</td>
<td>9.33 ± 11.07</td>
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Data are mean ± SD. n = 1 (CC); 56 (CT); 351 (TT).
and good biocompatibility have become available (35) and are currently in preclinical testing. Although the isoform specificity of the compounds is limited, our experiments performed in Nox1- and Nox2-deficient mice as well as previous work (13) excluded NOX1 and NOX2 as mediators of osteoclast function under physiological conditions. Nevertheless, under different circumstances, such as inactivation, atrophy, or inflammation, other sources of ROS, like NOX2 or mitochondria, might be relevant for bone loss (36).

In conclusion, we provide a direct link between NOX4 and bone loss: NOX4 expression was increased in osteoporotic bone in humans, and SNP analyses linked NOX4 to bone metabolism. In mice, in vivo inhibition or knockdown of NOX4 prevented bone loss secondary to estrogen withdrawal, and global Nox4-knockout mice exhibited increased bone density. These results suggest that bone loss disorders could be attenuated by inhibition of NOX4.

### Methods

For details of BMD measurement, bone histology and histomorphometry, bone biomechanics analysis, cell culture and ex vivo cell differentiation, pit formation, amplex red assay for H2O2 production, intracellular calcium measurement, immunoblotting, quantitative PCR, surgical procedures, and statistical analyses, see Supplemental Methods.

**Studies using human cohorts and cells.** The cohorts at Graz University were studied (16, 37), and genotyping for NOX4 SNPs was carried out by Taq-Man fluorogenic 5′ exonuclease assay. Immortalized B lymphocytes carrying the alleles TT or CC were obtained from the Coriell Institute. The following cell lines were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research: GM12864, GM12874, GM18571, GM18573, GM18576, GM18577, GM18972, GM18976, GM18978, GM18969, GM19138, GM19145, GM19222, GM19223, GM19210, and GM19238.

**Mice and animal procedures.** Mice were bred in our animal facility. The Nox2<sup>−/−</sup> and Nox1<sup>−/−</sup> founders of our colonies were obtained from Charles River and provided by K.H. Krause (University of Geneva, Geneva, Switzerland), respectively. Nox4<sup>−/−</sup> mice were generated by targeted deletion of the translation initiation site and of exons 1 and 2 of Nox4 (Genoway) (38). Tamoxifen-inducible Nox4-knockout mice (Nox4<sup>fl/fl-Cre-ERT2+/0</sup>) were produced by crossing homozygous Nox4<sup>fl/fl</sup> mice with tamoxifen-inducible Cre-ERT2<sup>+/0</sup> mice (39). In all experiments, littermate WT and knockout animals were used.

Osteoporosis was induced by ovariectomy, and bone composition was studied 6 weeks after surgery (40). Pharmacological inhibition of NOX4 was achieved by treating mice with the NOX4 inhibitor GKT137831 (41, 42).

### Table 3

**Effect of the SNP rs11018628 in the female subgroup**

<table>
<thead>
<tr>
<th>Bone density parameters&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CC+CT</th>
<th>TT</th>
<th>P</th>
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<tbody>
<tr>
<td>Hip T-score</td>
<td>−1.57 ± 1.550</td>
<td>−1.09 ± 1.102</td>
<td>0.048</td>
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<tr>
<td>Hip Z-score</td>
<td>−0.258 ± 1.384</td>
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<tr>
<td>Hip density (g/cm²)</td>
<td>0.78 ± 0.186</td>
<td>0.841 ± 0.134</td>
<td>0.057</td>
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<th>Bone metabolism markers</th>
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<tbody>
<tr>
<td>CTX (ng/ml)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.261 ± 0.097</td>
<td>0.241 ± 0.204</td>
<td>0.033</td>
</tr>
<tr>
<td>Osteocalcin (ng/ml)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.17 ± 1.90</td>
<td>6.93 ± 7.33</td>
<td>0.002</td>
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Data are mean ± SD. <sup>a</sup>n = 29 (CC+CT); 143 (TT); <sup>b</sup>n = 27 (CC+CT); 141 (TT); <sup>c</sup>n = 29 (CC+CT); 147 (TT).

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**Figure 4**

Role of NOX4 in human bone loss. (A) Nox4 mRNA expression in the course of differentiation of human precursor cells into osteoclasts. (B) Statistical analysis and (C) representative images of PIT formation assays of human PBMCs treated with RANKL with or without GKT137831. (D) Intensity of NOX4 staining in human bone material from healthy subjects and patients with osteoporosis or Paget disease. (E) Representative images of bone slides from a healthy subject and an osteoporotic patient stained for NOX4 (brown) and counterstained with hemalaun (violet). Scale bars: 1 mm (C); 50 μm (E). Data are mean ± SEM (n = 3–6). *P < 0.05.
achieved by administration of the compound GKT137928 (20 mg/kg/d; provided by Genkyotex) by daily gavage. Genetic deletion of Nox4 in Nox4fl/fl-Cre-ERT2+/0 mice was induced by injection of tamoxifen (40 mg/kg i.p. on 3 consecutive days). Interventions were started 2 days after ovariectomy.

**Statistics.** All values are expressed as mean ± SEM, with the exception of SNP data, which are expressed as mean ± SD. Normal distribution of data was analyzed by Kolmogorov-Smirnov test. Categorical variables were compared by χ² test, numeric values were compared by 2-tailed Student’s t test, and multiple-group comparisons were made by ANOVA. Data showing unequal variance or lacking normal distribution were analyzed by Wilcoxon rank-sum test or Kruskal Wallis test. A P value less than 0.05 was considered statistically significant.

**Study approval.** Animal experiments in Frankfurt, Germany, were approved by local governmental authorities (approval no. F28/20) and performed in accordance with animal protection guidelines. Human bone samples were obtained following approval by the Institutional Review Board of the Dresden Medical Faculty (EK245082010). Protocol procedures for cross-sectional study for SNP analyses were approved by the local Ethics Committee of the Medical University of Graz. Written informed consent was obtained from all study participants.

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