SMURF2 regulates bone homeostasis by disrupting SMAD3 interaction with vitamin D receptor in osteoblasts

Zhan Xu1, Matthew B. Greenblatt2, Guang Yan1, Heng Feng1, Jun Sun1, Sutada Lotinun3, Nicholas Brady4, Roland Baron3, Laurie H. Glimcher5 & Weiguo Zou1

Coordination between osteoblasts and osteoclasts is required for bone health and homeostasis. Here we show that mice deficient in SMURF2 have severe osteoporosis in vivo. This low bone mass phenotype is accompanied by a pronounced increase in osteoclast numbers, although Smurf2-deficient osteoclasts have no intrinsic alterations in activity. Smurf2-deficient osteoblasts display increased expression of RANKL, the central osteoclastogenic cytokine. Mechanistically, SMURF2 regulates RANKL expression by disrupting the interaction between SMAD3 and vitamin D receptor by altering SMAD3 ubiquitination. Selective deletion of Smurf2 in the osteoblast lineage recapitulates the phenotype of germline Smurf2-deficient mice, indicating that SMURF2 regulates osteoblast-dependent osteoclast activity rather than directly affecting the osteoclast. Our results reveal SMURF2 as an important regulator of the critical communication between osteoblasts and osteoclasts. Furthermore, the bone mass phenotype in Smurf2- and Smurf1-deficient mice is opposite, indicating that SMURF2 has a non-overlapping and, in some respects, opposite function to SMURF1.
Bone remodelling is an important physiological process to preserve the structural integrity of the skeleton, and is achieved through the balance between bone formation by osteoblasts and bone resorption by osteoclasts. Osteoblasts, which originate from mesenchymal precursors, secrete the extracellular matrix required for bone mineralization during both embryonic development and the postnatal remodelling that is critical for bone homeostasis in adults. Osteoclasts, which are multinucleated giant cells originating from the myelomonocytic lineage, degrade existing bone through an acidic and enzymatic process. Imbalance between the activity of osteoblasts and osteoclasts during bone remodelling can result in reduced bone mass, which underlies the pathogenesis of osteoporosis, the most common skeletal disease.

To ensure balance between the catabolic and anabolic activities during skeletal remodelling, many factors mediate the coordination between osteoblasts and osteoclasts. For example, during bone resorption, active transforming growth factor-β (TGF-β) is released from bone matrix, recruiting bone marrow (BM) mesenchymal stem cells, which can be further converted to osteoclasts. RANK, secreted by osteoblast lineage cells, including both osteoblasts and osteocytes, binds to its receptor RANK on the cell membrane of osteoclast progenitors, promoting osteoclast formation and function. The expression of RANK is regulated at the cellular level by multiple signalling pathways, including schnurri-3 (ref. 8), EBF2 (ref. 9), parathyroid hormone (PTH), PTH-related protein, TGF-β and vitamin D

Smurf2+/− mice show a reduced bone mass phenotype. Nevertheless, we have noted that many genes identified in in vitro shRNA silencing screens to alter osteoblast differentiation do not do so in vivo (unpublished data). To determine the in vivo relevance of SMURF2 within the skeletal system, we examined the skeleton of 1-week-old Smurf2+/− mice by alcin blue and alizarin red staining. Smurf2+/− mice had no overt developmental defects at weaning, and Smurf2+/− mice were smaller than control littermates in terms of both body size and weight (Fig. 2a,b; Supplementary Fig. 2B). Smurf2+/− mice displayed normal growth plate architecture. However, less trabecular bone was present in femurs of 12-week-old Smurf2+/− mice, compared to wild-type (WT) controls (Fig. 2c). Further analysis by micro quantitative computed tomography (μ-QCT) demonstrated that the distal femurs of Smurf2+/− mice had decreased trabecular bone volume (BV/TV), trabecular number (Tb.N) and cortical thickness (C.Th) with normal trabecular thickness (Tb.Th; Fig. 2d–h). The fifth lumbar vertebra of Smurf2+/− mice similarly showed reduced BV/TV, Tb.N and C.Th, but not Tb.Th compared with WT littermates (Fig. 2i–m). Thus, in contrast to the increased bone density in Smurf1-deficient mice, loss of Smurf2 in mice resulted in reduced bone mass. As seen in older mice, Smurf2+/− mice begin to show low bone mass even at 1 week of age (Supplementary Fig. 2C). In situ hybridization for Col1 expression was also performed. As shown in Supplementary Fig. 2D, the extent of Col1 expression was reduced in Smurf2+/− mice, though there did appear to be some increase in the per-cell intensity of staining within this reduced area. Thus, SMURF2 plays a similar role in regulating bone mass during both the early modelling and later remodelling stages of bone formation.

Smurf2−/− mice have increased bone resorption. To understand the relative contributions of osteoblast and osteoclast activities to the osteopenia seen in Smurf2−/− mice, we next performed histomorphometric analysis of WT and Smurf2−/− mice to evaluate static and dynamic parameters of bone formation and resorption (Fig. 3a–i; Supplementary Table 1). Consistent with the μ-QCT data, histomorphometry also

Results

Smurf2 deficiency increases osteoblast differentiation. We previously performed an RNA interference-based loss-of-function screen in human mesenchymal stem cells to identify novel regulators of osteoblast differentiation. We specifically screened nine members of the Ned4 E3 ligase family, using robust induction of alkaline phosphatase (ALP), an early marker of osteoblast differentiation, as readout (Supplementary Fig. 1). Short hairpin RNAs (shRNAs) directed against the Ned4 E3 ligase Smurf2 reduced Smurf2 expression and increased ALP level (Fig. 1a,b; Supplementary Fig. 1). Consistently, Smurf2-targeted shRNAs also induced elevated levels of mineral deposition as determined by Von Kossa staining (Fig. 1c,d). To further explore the function of SMURF2 during osteoblastogenesis, we investigated the function of SMURF2 in primary osteoblasts. Mice lacking Smurf2 generated by deletion of exon 9 were previously described to be viable and fertile. Loss of SMURF2 was confirmed by immunoblotting extracts of the lung and liver (Supplementary Fig. 2A). In vitro, primary Smurf2−/− calvarial osteoblasts displayed an increased induction of ALP and enhanced mineralized matrix formation, confirming the results of the shRNA screen (Fig. 1e,f). Consistent with this enhanced differentiation, expression of characteristic osteoblast markers, including tissue-nonspecific Alp, collagen1 alpha 1 (Col1α1), bone sialoprotein (Bsp), osteocalcin (Ocn) and Atf4 were increased in Smurf2−/− osteoblasts (Fig. 1g). Collectively, these data demonstrate that knockdown of Smurf2 in human mesenchymal stem cells and knockout of Smurf2 in primary osteoblasts augment osteoblast differentiation in vitro.

Smurf2−/− mice have increased bone resorption. To understand the relative contributions of osteoblast and osteoclast activities to the osteopenia seen in Smurf2−/− mice, we next performed histomorphometric analysis of WT and Smurf2−/− mice to evaluate static and dynamic parameters of bone formation and resorption (Fig. 3a–i; Supplementary Table 1). Consistent with the μ-QCT data, histomorphometry also
showed that Smurf2−/− mice had a significant decrease in both BV/TV and Tb.N with a concomitant increase in trabecular spacing (281%), but no change in Tb.Th (Fig. 3b–d; Supplementary Table 1), a characteristic phenotype associated with increased bone resorption. Indeed, the decreased bone volume was associated with a significant increase in both the number and bone surface covered by osteoclasts (N.Oc/Bpm and Oc.S/BS; Fig. 3e,f). In addition, the numbers of osteoblasts per bone perimeter (N.Ob/B.Pm) and osteoblast surface per bone surface (Ob.S/BS) were increased in Smurf2−/− mice compared to the WT control mice. However, the bone formation rate normalized to bone volume was not significantly increased (Fig. 3g–i; Supplementary Table 1). Given that the unexpected decrease in bone volume in Smurf2−/− mice was accompanied by increases in osteoclast number, we next examined levels of a serum marker of bone resorption activity, collagen type 1 cross-linked C-telopeptide (CTX). As shown in Fig. 3j, CTX was significantly increased in Smurf2−/− mice, indicating the elevated bone resorption activity in Smurf2−/− mice. We next performed a more extensive histochemical analysis of osteoclasts and observed quantitative increases in osteoclast numbers along the surface of trabecular bone.
indicated by the presence of tartrate-resistant acid phosphatase-positive (TRAP-positive) osteoclasts. Consistent with the increase of osteoclast numbers, expression of characteristic osteoclast marker genes including **Dc-stamp**, **cathepsin K (Ctsk)** and **carbonic anhydrase 2 (Car2)** were increased in the calvaria of Smurf2-/- mice (Fig. 3m), although the expression level of **NFATc1** and integrin beta 3 (**Itgb3**) was not much affected in Smurf2-deficient mice, which could be due to the expression of NFATc1 and Itgb3 in other cell types besides osteoclasts. Collectively, these data indicate that the decrease in bone volume in Smurf2-/- mice is due to increased bone resorption.

Smurf2-/- osteoblasts have increased RANKL expression. To address whether the apparent increase in osteoclast activity seen in Smurf2-/- mice was intrinsic to the osteoclast lineage, BM from Smurf2-/- mice and WT littermates was collected and cultured in the presence of the osteoclastogenic factors RANKL and macrophage colony-stimulating factor (M-CSF).
Osteoclast differentiation was normal in Smurf2−/− male mice, as indicated by a similar number of multinucleated TRAP-positive osteoclasts and supernatant TRAP activity, when compared to BMMs from WT mice (Fig. 4a–c). Further, induction of markers of osteoclast differentiation, including NFATc1, Cathepsin K and Dc-stamp, was comparable in WT and Smurf2−/− osteoclasts (Fig. 4d–f). Thus, osteoclast differentiation is intact in the absence of Smurf2. These results suggest that the elevated bone resorption observed in Smurf2−/− mice is extrinsic to the osteoclast lineage, which is also consistent with the very low level of expression of Smurf2 in osteoclasts.

Osteoblastic lineage cells support osteoclast differentiation in vitro and in vivo by expressing RANKL, a major inducer of osteoclast differentiation and function.2,6,7,19. To investigate the ability of Smurf2−/− osteoblast lineage cells to support osteoclastogenesis, we performed co-culture experiments. Osteoblasts from Smurf2−/− mice showed an enhanced ability to support osteoclastogenesis, as indicated by the number of giant multinucleated cells and the TRAP activity of culture supernatants (Fig. 4g–i). Similarly, RNA obtained from osteoclasts cultured with Smurf2−/− osteoblasts displayed increased expression of terminal markers of osteoclast differentiation, including NFATc1, Cathepsin K, Dc-stamp and Itga5 (Fig. 4j–m). These results demonstrate that SMURF2 acts within osteoblasts to regulate osteoclast differentiation, prompting us to identify the osteoclastogenic factor(s) produced...
**Figure 4 | Smurf2−/− osteoblasts express high levels of RANKL driving osteoclastogenesis.** (a-f) Osteoclastogenesis of bone marrow cells from 4-week-old mice in vitro. (a) TRAP staining of osteoclasts in culture. Scale bar, 0.5 cm. (b) Statistical analysis of TRAP-positive cell numbers in the osteoclast differentiation culture. (c) Culture supernatants were assayed for TRAP activity via colorimetric readout (A405). (d-f) Gene expression analysis of osteoclast cultures was examined by quantitative reverse transcriptase–PCR. (g–m) Osteoclastogenesis of osteoblasts/bone marrow cells by OB-OC co-culture in vitro using neonatal calvarial osteoblasts. (g) TRAP staining of osteoclasts in co-culture, Scale bar, 0.5 mm. (h) Statistical analysis of TRAP-positive cell numbers in the co-culture shown in g. (i) Co-culture supernatants were assayed for TRAP activity via colorimetric readout (A405). Red line, osteoblast cells were from Smurf2−/− mice; black line, osteoblast cells were from WT mice. (j–m) Gene expression analysis of osteoblasts/bone marrow cells co-cultures was examined by quantitative reverse transcriptase–PCR. (n) Gene expression of major osteoclast differentiation regulators in neonatal calvarial osteoblasts. (o) Serum RANKL was determined from 5-week-old male WT and Smurf2−/− mice. Data represent mean ± s.d. (n = 10 for each genotype, ***P < 0.001, **P < 0.01, *P < 0.05, NS, not significant. Student’s t-test). (p) Expression of RANKL, SMURF2 and TUBULIN in primary neonatal calvarial.
by osteoblasts that are regulated by SMURF2. RANKL and OPG, which are expressed in osteoblast cells, are major regulators of osteoclast differentiation\(^{12,20}\). Indeed, Smurf\(_2\)\(^{-/-}\) calvarial osteoblasts had increased RANKL expression but there was no effect on OPG expression (Fig. 4n). The expression of other osteoclast differentiation regulators, including M-CSF, Semaphorin 3A (Sem3a)\(^{21}\) and parathyroid hormone-like peptide (Pthilh or PthrP)\(^{22}\), was not significantly altered in Smurf\(_2\)\(^{-/-}\) osteoblasts. Similar to the data from neonatal calvarial osteoblast cell cultures, osteoblasts from the long bones of 5-week-old Smurf\(_2\)\(^{-/-}\) mice showed increased Rankl expression, but the expression of Opg and other osteoclast differentiation regulators was unchanged (Supplementary Fig. 2E). Concurrently, Smurf\(_2\)\(^{-/-}\) mice displayed higher serum levels of RANKL (Fig. 4o). The higher level of RANKL in Smurf\(_2\)\(^{-/-}\) mice was also confirmed by immunoblotting using cultured primary calvarial osteoblasts (Fig. 4p). Taken together, these data indicate that Smurf2-deficient osteoblast cells augment osteoclastogenesis by increasing the expression of RANKL.

**SMAD3 ubiquitination inhibits RANKL expression.** We next sought to investigate the mechanism in which SMURF2 regulates RANKL expression. The WW domains of SMURF2 interact with the PPXY motif of its substrate and dozens of SMURF2-interacting proteins have been reported. We therefore examined whether proteins known to interact with SMURF2 could affect RANKL expression and found that NEK6, SERTAD2 and SMAD3 could increase RANKL expression when C3H10 T1/2 cells were infected with lentivirus expressing these proteins (Supplementary Fig. 3A–C). Next we determined whether these three proteins are ubiquitination substrates of SMURF2. As shown in Supplementary Fig. 3D–F, SMAD3, but not NEK6 and SERTAD2, could be ubiquitinated by SMURF2, consistent with the previous reports that SMAD3 is a major substrate of SMURF2-mediated ubiquitination\(^{23–26}\). These findings led us to pursue the possibility that SMURF2 regulates RANKL expression by controlling the signalling pathways related to SMAD3, a member of the family of receptor-regulated Smads proteins. To examine whether the higher RANKL levels in Smurf\(_2\)\(^{-/-}\) osteoblasts are related to SMAD3 ubiquitination, we asked whether knockdown of Smad3 interfered with the increased RANKL levels in Smurf\(_2\)\(^{-/-}\) osteoblasts. As shown in Fig. 5a, knockdown of Smad3 decreased RANKL levels in both WT and Smurf\(_2\)\(^{-/-}\) osteoblasts. Moreover, Smad3 deficiency eliminated increased RANKL production by Smurf\(_2\)\(^{-/-}\) osteoblasts, indicating that the regulation of RANKL expression in Smurf\(_2\)\(^{-/-}\) mice is SMAD3 dependent (Fig. 5a). It was reported previously that SMURF2 negatively regulates TGF/Smad signalling either by inducing the degradation of Smad\(^{27–30}\) or by interfering with the formation of homotrimeric or heterotrimeric SMAD3 complexes via the ubiquitination of SMAD3 (ref. 26). We next treated primary calvarial osteoblasts with the TGF-β signalling inhibitor SB525334 and found that the level of RANKL could be reduced by SB525334 in both WT and Smurf\(_2\)\(^{-/-}\) osteoblasts. However, Smurf\(_2\)\(^{-/-}\) osteoblasts still displayed higher RANKL expression than WT cells after SB525334 treatment (Fig. 5b), indicating that elevated TGF-β signalling is not responsible for the higher RANKL expression in Smurf\(_2\)\(^{-/-}\) osteoblasts. This is consistent with the lack of Smad-binding elements in the proximal RANKL promoter\(^{4,31}\). The SMAD3 dependence of increased RANKL expression in Smurf\(_2\)\(^{-/-}\) mice suggested that another Smad3-binding transcription factor might control RANKL expression. RUNX2, vitamin D receptor (VDR) and CCAAT/enhancer-binding protein directly bind to the RANKL promoter and regulate RANKL expression. Among these, VDR is a member of the nuclear hormone receptor superfamily of transcriptional regulators and mediates the diverse biological effects of calcitriol (1,25-(OH)\(_2\))\(_\text{D}_3\) and its analogues\(^{32,33}\). It has also been widely studied for its therapeutic potential in treating various forms of skeletal pathology\(^{34–36}\). Previous studies reported five mouse and human vitamin D response elements (VDRES) in the enhancer regions upstream of the RANKL transcriptional start sites\(^{11,37–39}\). We confirmed the interaction between SMAD3 and VDR, as previously reported\(^{40}\), by immunoprecipitation of FLAG epitope-tagged SMAD3 followed by immunoblotting with an anti-HA antibody to detect HA-tagged VDR (Fig. 5c). The interaction between endogenous SMAD3 and VDR was also confirmed in calvarial osteoblast cells in vivo (Fig. 5d). The interaction between SMAD3 and VDR is direct as it can be recapitulated in a cell-free system using recombinant proteins (Fig. 5e, f). We next examined whether SMAD3 and VDR can function together to induce RANKL expression. As shown in Fig. 5g, h, simultaneous infection of C3H10 T1/2 with SMAD3- and VDR-expressing lentiviruses led to an elevation in RANKL expression. We also examined the activity of Rankl promoter using luciferase reporter assay. Consistently, VDR and SMAD3 could additively increase the activity of Rankl promoter (Fig. 5i). This Rankl promoter contains VDR-binding element but does not contain SMAD3-binding element. These data suggest that SMAD3 could additively increase RANKL expression through directly interacting with VDR, which binds to Rankl promoter and increases RANKL expression.

As SMAD3 can interact with VDR and additively augment the expression of RANKL in osteoblasts, we examined whether SMURF2-mediated SMAD3 monoubiquitination could block the formation of the SMAD3–VDR complex. To test whether ubiquitination imposes a sterically hindrance to the interaction of SMAD3 and VDR, we co-transfected plasmids expressing SMURF2, ubiquitin and SMAD3 into HEK-293T cells to give rise to both an unmodified 55 kDa and an ubiquitin-modified 72 kDa SMAD3 protein product, then extracted SMAD3 proteins to conduct in vitro interaction analysis using purified VDR. This demonstrated that only the unmodified 55 kDa but not the modified 72 kDa SMAD3 was able to interact with recombinant VDR (Fig. 6a). Recombinant SMAD4 was used as a control in this assay. These data indicate that ubiquitination of SMAD3 disrupts the interaction between SMAD3 and VDR. Ubiquitin conjugation occurs at Lys ε-amino groups of targeted proteins. Since our study and others\(^{40,41}\) suggested that the SMAD3 MH1 domain interacts with VDR (Fig. 6b), we next determined whether the MH1 domain can be modified by SMURF2-induced ubiquitination. The linker domain of SMAD3 contains a PY motif, which directly interacts with SMURF2 (Supplementary Fig. 4A, B). While the linker domain fragment of SMAD3 could not be ubiquitinated, SMAD3 1–231, which contains both the MH1 and linker domains, can be ubiquitinated, and this ubiquitination can be augmented by SMURF2 (Supplementary Fig. 4C, D), indicating that the MH1 domain contains ubiquitination sites. Moreover, the interaction between VDR and the SMAD3 MH1 domain was also blocked by SMAD3 ubiquitination (Fig. 6c), indicating that ubiquitination interfered with the binding between SMAD3 and VDR. Previous studies have demonstrated that K33, K53 and K81 within the SMAD3 MH1 domain are important sites for monoubiquitination\(^{42}\). We constructed a SMAD3 mutant (SMAD3-K3R), where K33, K53 and K81 were substituted with arginine and were therefore unsuitable for ubiquitin attachment. As shown in Fig. 6d, SMAD3-K3R mutation led to a marked reduction in the level of SMURF2-mediated ubiquitination, and showed a stronger interaction with VDR when it influence on RANKL expression.
compared to WT SMAD3 (Fig. 6e,f). Consistently, SMAD3-K3R showed increased interaction with exogenous VDR protein in 293T cells compared to the WT SMAD3 (Fig. 6g). Similarly, more endogenous VDR proteins were co-immunoprecipitated by exogenous SMAD3-K3R compared to the WT SMAD3 in calvarial osteoblasts (Fig. 6h). We next treated both WT and Smurf2−/− primary calvarial osteoblasts with 1,25-(OH)_{2}D_{3}. As shown in Fig. 6i, 1,25D treatment could increase levels of VDR as previously reported. Furthermore, we found that RANKL levels were augmented by 1,25D in WT osteoblasts. However, 1,25D treatment failed to further augment RANKL levels in Smurf2−/− osteoblasts (Fig. 6i). The resistance to RANKL induction incurred by 1,25D treatment in Smurf2−/− osteoblasts indicates enhanced SMAD3-related VDR signalling. To test whether ubiquitination influences recruitment of VDR and SMAD3 to the VDREs, we performed chromatin immunoprecipitation (ChIP) assays and found that SMURF2 deficiency increased recruitment of SMAD3 to the portion of the RANKL promoter covering the VDREs (Fig. 6i); however, there was no significant difference in VDR recruitment between WT and Smurf2−/− osteoblasts (Fig. 6i). Hence, we conclude that SMURF2-mediated SMAD3 monoubiquitination interferes with the formation of a SMAD3–VDR complex, hence preventing RANKL induction by this complex.

**Smurf2 mesenchymal conditional KO mice have osteopenia.** To further explore the mechanism whereby SMURF2 controls the coupling between bone formation and resorption in vivo, a mice line harbouring a Smurf2 allele in which exon 14 is flanked...
by loxP sites were generated (Supplementary Fig. 5A,B), hereafter called Smurf2\(^{2/2}\) mice. We intercrossed Smurf2\(^{2/2}\) mice with Prx1-Cre expressing mice, which express CRE recombinase in the mesenchyme of the limbs and skull but not in the axial skeleton\(^43\). We collected RNA from femurs and found reduced Smurf2 messenger RNA (mRNA) levels in Prx1-Cre Smurf2\(^{2/2}\) mice as expected (Supplementary Fig. 5C). µ-QCT analysis demonstrated that Prx1-Cre Smurf2\(^{2/2}\) mice showed lower femoral bone mass than Smurf2\(^{2/2}\) controls (Fig. 7a–e). Thus, the bone mass phenotype of Smurf2 mesenchymal conditional knockout (KO) mice was similar to that of germline Smurf2\(^{-/-}\) mice. On the basis of our proposed model that Smurf2 deficiency
in the mesenchymal/osteoblast lineage augments RANKL expression, leading to increased bone resorption, we expected that 
Ppx1-Cre Smurf2<sup>F/F</sup> mice would show increased osteoclast numbers and activity. Indeed, histology demonstrated that 
Ppx1-Cre Smurf2<sup>F/F</sup> mice displayed increased numbers of osteoclasts (Fig. 7i) and increased CTX levels (Fig. 7g), indicating elevated systemic bone resorption activity. We also found increased RANKL expression in the bones of Ppx1-Cre Smurf2<sup>F/F</sup> mice compared with Smurf2<sup>F/F</sup> mice by quantitative PCR (Fig. 7h). RANKL levels are known to change during the course of osteoblast differentiation. To assess the contribution of osteoblast differentiation states to differences in RANKL levels, we cultured calvarial osteoblasts from Smurf2<sup>F/F</sup> mice and then infected the cells with lentivirus-expressing GFP and CRE recombinase. As shown in Fig. 7i, CRE lentivirus led to reduced SMURF2 expression accompanied by higher RANKL levels. When the cells were cultured in non-differentiation media for 48 h after the virus infection, there was no difference in osteoblast differentiation determined by osteoblast gene expression, but CRE virus induced knockout of Smurf2 mRNA accompanied with increased Rankl expression (Supplementary Fig. 5D). These data indicated that the effects of Smurf2 on Rankl expression are not the results of the generalized increase in the differentiation stage. When the cells were cultured in osteoblast differentiation media for 4 days, there were increased osteoblast differentiation in Cre-infected cells and the increase of Rankl expression are kept (Supplementary Fig. 5E), it is hard to distinguish the effects of Smurf2 itself from the effects of generalized increase in differentiation due to the limitations of the experiments. Taken together, these findings support our model that SMURF2 in osteoblast lineage cells directly controls the expression of RANKL through interfering with the Smad3–VDR complex to regulate osteoclastic bone resorption (Fig. 7j).

**Discussion**

SMURF1 and SMURF2 are a C2-WW-HECT class of ubiquitin ligases that play important roles in regulating cell signalling, cell polarity and motility<sup>16,44,45</sup>. Multiple lines of evidence including their similar expression patterns, high degree of amino-acid sequence homology, and the ability to promote ubiquitination of the same targets<sup>14,15,26,27,29,46</sup> suggests that SMURF1 and SMURF2 display partial functional redundancy. The embryonic lethality of Smurf1<sup>/−</sup>Smurf2<sup>/−</sup> double-deficient mice provides definitive genetic evidence of their functional redundancy<sup>16</sup>. Hence, our finding that Smurf2<sup>−/−</sup> animals display decreased bone mass, opposite to the skeletal phenotype of Smurf1-deficient mice, is unexpected and suggests novel SMURF2 functions. We performed osteoblast differentiation assays with primary human mesenchymal stem cells and examined the effects of SMURF2 and SMURF1 on osteoblast differentiation and RANKL expression. As expected, knockdown of either Smurf1 or Smurf2 in human mesenchymal stem cells increased osteoblast differentiation as determined by ALP and alizarin red staining. However, only Smurf2 shRNAs, but not Smurf1 shRNAs, increased Rankl expression. Thus, while SMURF1 and SMURF2 have overlapping functions to inhibit osteoblast differentiation, their effects on RANKL expression diverge (Supplementary Fig. 6).

Our current study suggests that SMURF2 regulates osteoblast-dependent osteoclastogenesis by controlling RANKL expression through SMAD3 ubiquitination-related VDR signalling, a distinct function not likely to be shared by SMURF1, which negatively regulates osteoblast activity through controlling MEKK2 ubiquitination. Huang et al.<sup>42</sup> demonstrated that there are no differences in cortical, subchondral and trabecular bone between WT and homozygous Smurf2 gene-trap mice at 4 or 16–24 months of age. We believe that the divergent results between their study and the current report likely reflect the clear presence of residual levels of SMURF2 in Smurf2 gene-trap mice.<sup>48</sup>

Ubiquitination is a major mechanism for controlling protein functional activity and physiologic responses<sup>19</sup>. Previous studies identified a long list of Smurf ubiquitin substrates, including SMAD1s, 2/3, 5/8, 6/7/1, type 1 TGF-β and BMP receptors, MEKK2 (ref. 15), Par6 (ref. 50), Gsk3 (ref. 51), Connexin 43 (ref. 52), RhoA (ref. 45), KLF5 (ref. 53) and others. Ectopic expression of SMURF1 and/or SMURF2 can degrade and destabilize these substrates. However, conflicting reports have emerged as to whether SMURF1 and/or SMURF2 can induce the ubiquitination and degradation of these proteins under physiological conditions<sup>26</sup>. Two independent studies failed to detect changes in SMAD2, SMAD3 or type I receptor protein levels in either Smurf1 or Smurf2-deficient primary fibroblast cells<sup>15,26</sup>. Instead of polyubiquitination and degradation, SMURF2 induced the multiple-site monoubiquitination of SMAD3 in vivo and inhibited the nuclear accumulation of the SMAD3 complex<sup>26</sup>. Here we confirmed that SMURF2 mediates monoubiquitination of SMAD3. As with the SMAD3/SMAD4 complex, monoubiquitination of SMAD3 impedes the formation of a SMAD3–VDR complex, which promotes the expression of RANKL in osteoblasts. VDR is a member of the nuclear receptor family of transcription factors and induces RANKL upregulation by vitamin D, 1,25-dihydroxyvitamin D3. There are five sites of VDR dimer binding 16, 22, 60, 69 and 76 kb upstream of the RANKL transcriptional start site<sup>11</sup>. Consistent with the existence of VDR-binding sites, monoubiquitination of SMAD3 that disrupts the VDR complex inhibits RANKL signalling. Hence, our study identifies SMURF2 as a regulator of osteoclastogenesis and bone homeostasis that act upstream of RANKL signalling.

To address how SMURF2 expression is regulated in osteoblasts, we treated preosteoblast cells with TGF-β,
BMP2 and oestrogen. As shown in Supplementary Fig. 7, TGF-β, but not BMP2 or oestrogen, regulates Smurf2 expression. This regulation of Smurf2 expression by TGF-β can increase Smurf2 expression in a dose- and time-dependent manner. TGF-β is widely known to play critical roles in cellular proliferation and differentiation through receptor-regulated SMAD proteins such as SMAD2 and SMAD3, which are phosphorylated upon type I and type II cell surface receptor activation and in turn bind to SMAD4 and translocate into the nucleus, thus propagating the signal. The TGF-β-activated transcriptional programme is involved in various pathological and physiological processes. In particular, TGF-β is abundant in bone matrix and its pivotal role in bone development and remodelling has been addressed. A rich reservoir of TGF-β in the bone milieu can enhance the expression of multiple factors such as PTH-like hormone and JAG1 (Jagged 1) that tip the balance of bone remodelling in favour of osteolysis.

In the setting of osteolytic bone metastases, this process may generate a vicious cycle promoting feed-forward destruction of bone. Although several prominent pathways such as PI3K, MAPK, Rho/RAC1 (ref. 60) and Notch have been shown to regulate bone homeostasis by TGF-β, the exact function of TGF-β during OB differentiation and many other developmental aspects remains unresolved. The regulation of Smurf2

Figure 7 | Deletion of Smurf2 in mesenchymal cells leads to increased bone resorption. (a–e) μ-QCT analysis of proximal femur from 5-week-old Smurf2fl/fl and Smurf2prx1 male mice for bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and cortical thickness (C.Th). Scale bar, 500 μm. (f) Histological sections of tibias from 5-week-old Smurf2fl/fl and Smurf2prx1 male mice stained for tartrate-resistant acid phosphatase (TRAP). Scale bar, 100 μm. (g) Serum CTX levels in 5-week-old Smurf2fl/fl and Smurf2prx1 male mice. Value represent means ± s.d. (n = 6 for each genotype, **P < 0.01, *P < 0.05, NS, not significant. Student’s t-test). (h) Quantitative PCR analysis of Rankl expression in the bone of Smurf2prx1 compared to the Smurf2fl/fl male mice. Values represent means ± s.d. (n = 3 for each genotype, **P < 0.01, *P < 0.05, Student’s t-test). (i) Expression of RANKL, SMURF2, SMAD3 and TUBULIN in Smurf2fl/fl calvarial osteoblast cells infected with the lentivirus-expressing GFP and CRE recombinase. (j) Model depicting the mechanism through which SMURF2-mediated monoubiquitination of SMAD3 leads to decreased RANKL expression.
expression by TGF-β is consistent with the previous work, showing that TGF-β stimulates Smurf2 expression by Smad-independent pathways such as the PI3K/Akt pathway via the TGF-β receptor61. This regulation of SMURF2 expression by TGF-β suggests that the role of SMURF2 in preventing excessive OC formation may be also regulated by TGF-β.

Lipophilic hormone vitamin D exerts important biological functions on the maintenance of calcium and bone homeostasis through activating VDR, which is a ligand-dependent transcription factor belonging to the nuclear receptor superfamily12,62. VDR mediates 1,25(OH)2D function via heterodimerizing with the retinoid X receptor and consequently interacting with response elements in the promoter regions of target genes, including Ca metabolism mediators such as TRPV6 (ref. 64).

Mechanistically, VDR ligands could facilitate VDR binding to response elements in the promoter regions of target genes, the retinoid X receptor and consequently interacting with VDR/SMAD coregulated genes and reduce SMAD3 occupancy at these sites, leading to the inhibition of fibrosis67. Overall, VDR-induced RANKL expression. However, the dependence of vitamin D in the function of VDR in our model needs further investigation to determine.

Crosstalk between TGF-β and vitamin D signalling pathways has been suggested by their cooperative actions on osteoblasts66. Explaining these observations, TGF-β treatment can enhance the transactivation activity of VDR through the SMAD3/VDR interaction40. Synergy between SMAD3 and VDR was also observed to regulate the human osteoclast promoter in transient transfection experiments. In contrast to these synergistically cooperative actions, some reports reveal antagonistic actions between vitamin D and TGF-β67. Recent studies claim that VDR ligands inhibit TGF-β1/SMAD-induced activation of hepatic stellate cells and improve liver fibrosis in mice67. Mechanistically, VDR ligands could facilitate VDR binding to VDR/SMAD coregulated genes and reduce SMAD3 occupancy at these sites, leading to the inhibition of fibrosis67. Overall, these synergistic or antagonistic actions between vitamin D and TGF-β could occur in a tissue-specific manner. The tissue-specific gene expression pattern and amounts of Smad(s), VDR and their receptors may be responsible for tissue-specific differences in cooperative actions between SMADs and VDR.

Bone mass is under sophisticated control of bone formation by endocrine and paracrine signals, and bone resorption through osteoblast-dependent or hematopoietic lineage-autonomous mechanisms. Our finding shows that deletion of Smurf2 in osteoblast affects both aspects of bone homeostasis. Thus, SMURF2 in osteoblasts may serve as a rheostat for bone homeostasis by coordinating the activities of osteoblasts and osteoclasts to regulate bone mass. The role of Smurf2 in osteoblasts is similar to that observed in Smurf1 loss-of-function studies in terms of increased osteoblast differentiation35. However, the in vivo phenotype of Smurf1−/− and Smurf2−/− mice is non-overlapping and in some respects diametrically opposite functions.

Methods

Mice. Smurf2−/− mice were from Dr Jeffery Wrana’s lab and were previously described16. Conditional Smurf2+/− mice, in which exon 14 of Smurf2 allele is flanked by loxP sites, were generated at Merck and on a C57BL6 background. Pex-1−/− mice were purchased from the Jackson Laboratory. The mutant mice were genotyped by PCR-based genotyping analysis. The DNA was amplified using SMURF2 protein expression. All animals were housed under specific pathogen-free conditions. All experiments using mice were approved by the Institutional Animal Care and Research Advisory Committee of the Shanghai Institute of Biochemistry and Cell Biology.

Cell culture. 293T cells (American Type Culture Collection) were maintained in DMEM containing 10% fetal bovine serum (FBS). CH110 T1/2 cells (American Type Culture Collection) were maintained in α-MEM culture medium (containing 10% FBS, 2 mM l-glutamine (Corning), 1 mM sodium pyruvate (Corning), 10 mM Hepes buffer (Corning), 1% MEM nonessential amino acids (Corning) and 1% penicillin/streptomycin (Gibco)). Primary murine calvarial osteoblasts were isolated from 5-day-old neonates by collagenase/disase II digestion. Calvarial bones were incubated in α-MEM containing 1 mg ml−1 collagenase (Sigma), 2 mg ml−1 dispase II (Roche) and 2% penicillin/streptomycin. The bones were placed in a shaker at 37 °C. Cells were released by 10 min of sequential digestions. The first digestions were discarded, and subsequent three digestions were collected. Cells released from digestion were plated in 5-MEM culture medium and differentiated with 50 μg ml−1 ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma). For in vitro gene inactivation, floxed osteoblasts were infected with either GFP- or CRE-expressing lentivirus. For Von Kossa staining, cells were fixed at day 21 of culture with 10% neutral formalin buffer and stained with 5% silver nitrate for 30 min. For ALP assay, cells were fixed with 10% neutral buffered formalin and subjected to ALP staining on day 7.

In vitro osteoclast differentiation. Mouse BM cells isolated from the femur and tibia were seeded (1 × 106 cells per well in a 24-well plate) and cultured in 5-MEM with 10% FBS containing 10 ng ml−1 M-CSF (PeproTech) for 3 days. Subsequently, nonadherent cells were discarded and adherent BMMs were further cultured in the presence of 20 ng ml−1 M-CSF and 150 ng ml−1 soluble RANKL. Cells were then fixed and stained for the presence of TRAP using a kit according to the manufacturer’s instructions (Sigma-Aldrich).

Co-culture experiments. Mouse calvarial osteoblast cells from WT and Smurf2−/− mice were seeded equally into a 24-well plate (8 × 104 cells per well) and cultured in 5-MEM with 10% FBS, osteoclast precursors (monocytes) were isolated as mentioned above and cultured for 24 h. The nonadherent BM cells (1 × 106 cells per well in a 24-well plate) were collected and cultured with calvarial osteoblasts in the presence of 1,25-dihydroxyvitamin D3 (10 nM; Sigma-Aldrich) and PGE2 (1 μM; Sigma-Aldrich) and the culture medium was half-changed every 2 days. Osteoclasts were identified by TRAP staining68,69.

Antibodies and reagents. Anti-RANKL antibody (ab5039; 1:1,000) was obtained from Abcam. Anti-SMAD3 (C67H9) antibody (9523; 1:1,000) and anti-P-SMAD3 (S423/425) antibody (9520; 1:1,000) were purchased from Cell Signaling Technology. Monoclonal anti-HA antibody (SC-7392; 1:2,000), anti-VDR rabbit polyclonal antibody (SC-1008; 1:1,000), anti-VDR mouse monoclonal antibody (SC-13133; 1:1,000) and anti-TUBULIN antibody (SC-23948; 1:1,000) was from Santa Cruz Biotechnology. Anti-SMURF2 antibody (ABS39; 1:500) was obtained from Millipore. Monoclonal anti-FLAG antibody (F3165; 1:5,000) was obtained from Sigma-Aldrich. Monoclonal anti-MYC antibody (AE010; 1:2,000) was from Abclonal Technology. Monoclonal anti-GFP antibody (66002-1; 1:10,000) was purchased from Proteintech. Rabbit IgG (SC-2027) was purchased from Santa Cruz Biotechnology and Mouse IgG (I5381) was from Sigma-Aldrich.

Recombinant mouse TGF-β1 (catalogue no.5231) was purchased from Cell Signaling Technology; recombinant human BMP-2 (120-02) was from Proteintech; β-estradiol (oestrogen; 02194565.2) was from MP; SB525334 was purchased from Selleckchem.

Skeletal preparation and staining. Mice were exsanguinated and the resulting samples were transferred into acetic for 48 h after overnight fixation in 95% ethanol. Skeletons were then stained in Acan blue and Alizarin red S solution and sequentially cleared in 1% KOH/20% glycerol.

Histology and immunostaining. Limb tissues were skinned and fixed in 4% paraformaldehyde in PBS. Then, specimens were decalcified until soft and pliable. Dehydration of tissues was done by passage through graded ethanol, cleared twice in xylene and embedded in paraffin. Tissue sections (6 μm) were used for haematoyxin–eosin staining or TRAP staining according to the standard protocol. Digoxigenin (DIG)-labelled RNA probes were generated according to the manufacturer’s protocol. DIG-labelled antisense probes were generated to detect Col1a1 mRNA expression. Probes were then hybridized with paraffin sections and visualized using an anti-DIG horseradish peroxidase conjugate system.
µ-CT analysis. The mouse femurs were collected, soft tissues were removed and the remaining tissues were fixed in 70% ethanol and µ-CT analysis scanned a region of 28 mm proximal to the distal growth plate using a Scanco Medical µ-CT 35 system, with a spatial resolution of 7 µm. The region selected for analysis was the region of 2.1 mm in length of distal metaphysis. The interesting region segmented by a fixed threshold was reconstructed into three-dimensional images. The related properties of trabecular bone, such as Tb.Th, Tb.N, BV/TV, C.Th, were then calculated according to distance transformation of the binarized images.[70]

Whole-mount TRAP histochemistry. Calvariae were dissected from 4-week-old mice and fixed in 100% methanol for 5 min (ref. 8). Calvariae were washed in PBS and then TRAP staining was performed according to the instructions of the manufacturer (Sigma).

Immunoblots and immunoprecipitation. Tissues were washed with chilled PBS and lysed in tissue lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA and 0.1% SDS) supplemented with protease inhibitors cocktail (Sigma) by automated tissue homogenate instrument (JXFSTPRP-48; Shanghai Jinxing Industrial Development Co., Ltd.). Cells were washed with chilled PBS and lysed in EBC buffer (50 mM Tris, pH 7.5, 120 mM NaCl and 0.5% NP-40) supplemented with protease inhibitors cocktail (Sigma). Lysates were then separated by SDS–polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies. For co-immunoprecipitation, lysates were incubated with Anti-FLAG M2 Affinity Gel (Sigma F2426) or Anti-HA Agarose Fast Flow (AOGMA, AGM90054) at 4 °C overnight. Precipitates were washed three times with lysis buffer before being resolved by SDS–polyacrylamide gel electrophoresis and were immunoblotted with indicated antibodies. Uncropped scans of all blots are provided as Supplementary Fig. 8.

Ni-NTA agarose purification. Cells were collected at 48 h after plasmid transfection and were lysed in urea lysis buffer (8 M urea containing 0.5% Triton x-100, 100 mM imidazole and 34 mM β-mercaptoethanol). Ni-NTA agarose beads (Qiagen) were then incubated with cell extracts and rotated at room temperature for 4 h or overnight. Precipitates were washed five times with urea lysis buffer and then boiled in 1 × sample buffer.

Real-time reverse transcriptase–PCR analysis. Total RNA was prepared using TRIzol (Sigma) and was reverse-transcribed into cDNA using the PrimeScript RT Reagent kit (TakaRa). The real-time reverse transcriptase–PCR reaction was performed using the Bio-Rad CFX96 system.

Transient transfections and reporter gene assays. For transient transfections, CHH10 T1/2 cells were seeded overnight in a 12-well dish at a concentration of 8 × 10^4 cells per well. Cells were then transfected with a Rankl luciferase reporter gene plasmid and varied combinations of VDR and Smad3 expression constructs, as indicated, using Effectene transfection reagent (Qiagen). pRL–TK (Promega) was co-transfected as a normalization control for transfection efficiency. Forty-eight hours after transfection, luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). pRankl-Luc plasmid was made by inserting Mlu1/Bgl2-digested PCR products, which was amplified using former primer (5'-TTATTGGTACTGTCGAGTGAATGACAATA-3') and reverse primer (5'-TTATTAGttGATCTcGAGGCGGCCTGCA-3'), into Mlu1/Bgl2-digested luciferase plasmid pGL3-Basic.

Serum measurements. Blood samples were collected from WT, Smurf2 germline KO and Smurf2 conditional KO mice. Serum RANKL was measured by using a RANKL Elisa kit (Sangon Biotech) according to the instructions of manufacturer.

ChIP assays. ChIP was performed using the EZ ChIP kit (Millipore) according to the instructions of manufacturer. Recovered DNA samples were subjected to PCR amplification with forward primer (5’-AGGcAGAGGCGGTAGGTGAT-3’) and reverse primer (5’-GTGCCGTGGTTTATTGTTTG-3’) to amplify the vitamin D responsive element binding site on the RANKL promoter region (–937–922).

Statistics. Statistical significance was determined by unpaired, two-tailed Student’s t-test for comparison between two groups with a P value of <0.05 considered statistically significant.

Data analysis. Experimental sample sizes required for adequate power were determined by the expected CVs we have previously observed with each assay, using the standard β value of 0.8 and α value of 0.05, and our sample sizes are similar to those generally employed in the field. All n values defined in the legends refer to biological replicates unless otherwise indicated. Studies were not blinded to investigators or formally randomized. No inclusion or exclusion criteria were used. Data distribution was assumed to be normal, but this was not formally tested[68–70].

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or are available from the authors upon reasonable request.

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Acknowledgements

We thank Dr Jeffrey L. Wrana (University of Toronto, Canada) for providing Smurf2−/− mice line and Dr Marc Wein (Harvard Medical School) for critical reading of the manuscript. We also thank members of the Zou and Glimcher labs for useful discussion. This work was supported in part by grants from the National Natural Science Foundation of China (NSFC; 13171463) and 973 Program from the Chinese Ministry of Science and Technology (MOST; 2015CB945303 and 2014CB946704). Supported by the Strategic Priority Research Program of the Chinese Academy of Sciences, Grant No. XDB19000000. W.Z. is a scholar of the ‘National 1000 Young Talents Program of China’ and the ‘National Science Fund for Excellent Young Scholars’ (NSFC; 81322027). L.H.G. received support from NIH grant HD055601. M.B.G. is supported by a Career Award for Medical Scientists from the Burroughs Wellcome Foundation and NIH grant OD021351.

Author contributions

W.Z. and L.H.G. conceived and supervised the project. Z.X., W.Z. and M.B.G. performed most of the experiments and data analysis. G.Y. performed the immunohistochemistry and biochemical experiments. Z.X. and W.Z. wrote the paper with help from L.H.G. and R.B. helped with animal experiments and most of the experiments and data analysis. G.Y. performed the immunohistochemistry and biochemical experiments. Z.X. and W.Z. wrote the paper with help from L.H.G. and M.B.G.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Xu, Z. et al. SMURF2 regulates bone homeostasis by disrupting SMAD3 interaction with vitamin D receptor in osteoblasts. Nat. Commun. 8, 14570 doi: 10.1038/ncomms14570 (2017).

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