CHMP5 controls bone turnover rates by dampening NF-κB activity in osteoclasts

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Physiological bone remodeling requires that bone formation by osteoblasts be tightly coupled to bone resorption by osteoclasts. However, relatively little is understood about how this coupling is regulated. Here, we demonstrate that modulation of NF-κB signaling in osteoclasts via a novel activity of charged multivesicular body protein 5 (CHMP5) is a key determinant of systemic rates of bone turnover. A conditional deletion of CHMP5 in osteoclasts leads to increased bone resorption by osteoclasts coupled with exuberant bone formation by osteoblasts, resembling an early onset, polyostotic form of human Paget's disease of bone (PDB). These phenotypes are reversed by haploinsufficiency for Rank, as well as by neutralizing anti-RANK receptor antibody (Sims and Gooi, 2008). Thus, CHMP5 tunes NF-κB signaling downstream of RANK in osteoclasts to dampen osteoclast differentiation, osteoblast coupling and bone turnover rates, and disruption of CHMP5 activity results in a PDB-like skeletal disorder.

Tight coupling of the activity of osteoblasts to build bone and osteoclasts to resorb bone is necessary to maintain the local biomechanical properties of bone. Moreover, this coupling activity limits the effectiveness of current therapies to treat osteoporosis, as antiresorptives targeting osteoclasts also induce a decrease in osteoblast activity, and the ability of the PTH agonist teriparatide to promote bone formation is partially counterbalanced by increased osteoclast resorptive activity (Sims and Gooi, 2008). Thus, understanding the basis of osteoclast/osteoblast coupling is vital to the development of more effective therapies to treat bone diseases.
coupling is central to designing improved approaches to treat diseases of low bone mass such as osteoporosis.

Paget’s disease of bone (PDB) affects 1–5% of adults over the age of 55 and is characterized by a wave of increased lytic osteoclast activity coupled with exuberant osteoblast activity, ultimately resulting in bone thickening, sclerosis, and expansion (van Staa et al., 2002; Daroszewska and Ralston, 2006; Ralston et al., 2008). These increases in osteoblast function and bone formation are believed to be driven by alterations in osteoclasts by as yet unknown coupling mechanisms (Roodman and Windle, 2005; Gåh松 and Roodman, 2014). Notably, rare forms of PDB-like syndromes such as familial expansile osteolysis, early onset familial PBD, expansile skeletal hyperphosphatasia, juvenile PDB (JPD); familial idiopathic hyperphosphatasia, and inclusion body myopathy associated with Paget’s disease of bone and frontotemporal dementia (IBMPFD) concurrently affect many bones (Daroszewska and Ralston, 2006). Risk factors influencing development of classical and rare forms of PDB include genetic variants in CSF1, TNFRSF11A, TNFRSF11B, SQSTM1/p62, VCP/p97, and OPTN, which are closely connected to the NF-κB pathway downstream of receptor activator of NF-κB (RANK), a cytokine receptor that has an essential function to drive osteoclast differentiation, and the proteasomal degradation pathway (Albagha et al., 2010, 2011). Additionally, environmental stressors such as chronic viruses (MV) infection have been implicated in PDB development (Kurihara et al., 2011). However, despite these advances, the molecular pathobiology of both PDB development and the exaggerated coupling between osteoclast and osteoblast activities is unclear.

Previously, in an attempt to identify novel regulators of the NF-κB pathway, we identified charged multivesicular body protein 5 (CHMP5) as copurifying with the cytosolic NF-κB–IkBα complex from rabbit lung tissue extracts (Shim et al., 2006). CHMP5 is a mammalian orthologue of the yeast VPS60/Ip1, and is essential for late endosomal trafficking and multivesicular body formation as a component of the endosomal sorting complex required for transport (ESCRT) machinery (Babst et al., 2002; Köhler, 2003; Shim et al., 2006). The ESCRT machinery in turn has been demonstrated to be involved in an increasing number of diverse cellular procedures beyond traditional endocytic trafficking such as autophagy, cytokinesis, cell polarity, migration, and viral budding (Raiborg and Stenmark, 2009; Rusten et al., 2012). To study the contribution of CHMP5 to NF-κB signaling in vivo, CHMP5 

Expression of CHMP5 in osteoclasts

To examine the relevance of CHMP5 to osteoclast biology, we examined CHMP5 expression in long bones. Intriguingly, CHMP5 is highly expressed in mature osteoclasts in trabecular and cortical bones, whereas its expression was near the limit of detection in osteoblasts and chondrocytes in the groove of Ranvier and the growth plate (Fig. 1 A and not depicted). Accordingly, protein levels of CHMP5 were significantly increased in osteoclasts in response to RANKL (Fig. 1 B) and CHMP5 deficiency does not affect alkaline phosphatase activity (ALP) or the mineralization capacity of human BM stromal cell (BMSC)–derived osteoclasts (unpublished data). Thus, these findings imply that, among bone lineage cells, CHMP5 only functions in osteoclasts by virtue of its selective expression.

Osteoclast–specific deletion of Chmp5 produces a high turnover, PDB–like bone phenotype

As germline deletion of Chmp5 results in early embryonic lethality in mice (Shim et al., 2006), we generated a Chmp5 floxed allele and bred these mice with Cathepsin K (Ctsk)-Cre mice (Chiu et al., 2004) to generate mice with a deletion of Chmp5 specifically in osteoclasts (referred to herein as Chmp5CKO, CKO). Chmp5CKO mice were born in a Mendelian ratio, but are markedly smaller than littermate controls, though their growth rate and complete blood cell counts are comparable to those of their littersmates (unpublished data). Unexpectedly, Chmp5CKO mice develop early onset, severe, and progressive skeletal deformities with a Pagetoid appearance throughout both the axial and appendicular skeleton, leading to a gradual expansion of affected bones (Fig. 1, C–J). Additionally, trabecular and cortical bone mass in long bones and trabecular bone mass in vertebrae were both markedly reduced in Chmp5CKO mice due to an increase in osteoclast numbers (Fig. 1, E and G). Histological analysis revealed numerous Pagetoid lesions with disorganized architecture and hyperdynamic bone as indicated by an increase in bone resorption surface area and numbers of osteoclasts specifically in these Pagetoid lesions (Fig. 1, H–J). Thus, these results demonstrate that deletion of CHMP5 in osteoclasts increases osteoclast differentiation and bone resorption activity in mice, resulting in PDB–like skeletal deformities.

Deletion of CHMP5 augments RANKL sensitivity to osteoclast differentiation

To examine the role of CHMP5 in osteoclast development, BM monocytes (BMMs) isolated from Chmp5CKO and Chmp5CKO mice were treated with M-CSF and RANKL in vitro. During osteoclast differentiation CHMP5 expression was markedly increased in Chmp5CKO osteoclasts, whereas Chmp5CKO osteoclasts fail to induce CHMP5 expression (Fig. 2 A). Osteoclast differentiation and sensitivity to RANKL were

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Figure 1. Chmp5 deletion in osteoclasts results in enhanced osteoclast differentiation and activity. (A) Expression of CHMP5 in long bones. Immunohistochemistry for CHMP5 in the femurs of 4-wk-old male WT mice. TB, trabecular bone. Arrows indicate osteoclasts. (B) Analysis of CHMP5 expression during in vitro osteoclast differentiation of primary BMMs by immunoblotting. (C) Radiography of the hindlimbs (left) and skulls (right) of 6-wk-old male Chmp5fl/fl and Chmp5Ctsk mice. The images represent three mice per group. (D, F, and G) µCT analysis of the femurs, the tibias, and the vertebrae of 12-wk-old male Chmp5fl/fl and Chmp5Ctsk mice. Shown are representative sagittal sections of femurs (D, top), tibias (D, middle), L4 vertebrae (D, bottom) with quantification of maximal diameter of distal femur and total volume of middle femur (M.T.V; F), 3D reconstructions of femurs (G, left), and cross section of L4 vertebrae (G, right). (E) Hematoxylin and eosin staining (top, low power; middle, high power) and TRAP staining (bottom, high power) of 12-wk-old male Chmp5fl/fl and Chmp5Ctsk femurs. (H and I) Von Kossa staining (H), TRAP staining (I, top), and hematoxylin and eosin staining (I, bottom) of 12-wk-old male Chmp5fl/fl and Chmp5Ctsk tibias (H) and Pagetoid lesions (I). (J) Histomorphometric analysis of 12-wk-old male Chmp5fl/fl tibias and Chmp5Ctsk Pagetoid lesions. n > 5 mice per group in D, F, G, and J. All error bars indicate SEM by the Mann–Whitney test. **, P < 0.01; ***, P < 0.001. Bars: (A) 25 µm; (E) 100 µm; (H) 300 µm; (I) 50 µm.
dramatically increased in Chmp5Ctsk BMMs, as indicated by an increase in tetrarate-resistant acid phosphatase (TRAP) staining and activity (Fig. 2, B–D) and osteoclast marker gene expression (Fig. 3, A and B). Likewise, Chmp5Ctsk osteoclasts displaced markedly increased bone resorptive activity with a 2–3-fold increase in the number and area of resorption pits and in the liberation of type I collagen C-terminal telopeptide (CTX; Fig. 2 E). This increase in resorptive activity likely reflected both increased differentiation and proliferation, as carboxyfluorescein succinimidyl ester (CFSE) labeling demonstrated increased proliferation of Chmp5Ctsk BMMs under osteoclast differentiation conditions (unpublished data). Similar to observations made in human PDB patients (Rebel et al., 1974), Chmp5Ctsk osteoclasts were larger and contained more nuclei than Chmp5fl/fl osteoclasts (Fig. 2 F).

Osteoclast differentiation is accompanied by cytoskeletal reorganization necessary to orient the resorptive machinery toward the bone surface and to seal around the resorption pit (Teitelbaum and Ross, 2003) where the Src protooncogenes serve as key regulators of this process (Soriano et al., 1991; Zou et al., 2007; Izawa et al., 2012). Intriguingly, Chmp5Ctsk osteoclasts show elevated levels and activation of Src and increases in the activation of the downstream effectors Syk and active GTP-bound Rac1 (Fig. 2, G–I). In keeping with these observations, actin ring formation was enhanced in Chmp5Ctsk osteoclasts (Fig. 2 J). Thus, CHMPS5 coordinately regulates several aspects of osteoclast differentiation necessary for resorptive activity.

Deletion of CHMPS5 augments production of osteoblast/osteoclast coupling factors in osteoclasts

To determine how CHMPS5 regulates gene expression in osteoclasts, we performed RNA sequencing analysis of Chmp5fl/fl and Chmp5Ctsk osteoclasts. As expected, gene-set enrichment analysis (GSEA) showed an enrichment of genes associated with osteoclast differentiation and fusion in Chmp5Ctsk osteoclasts, and the increase in gene expression was confirmed by RT-PCR analysis (Fig. 3, A and B). Expression of IL–6, a signature gene of Pagetic osteoclasts (Hoyland et al., 1994; Roodman et al., 1992; Roodman and Windle, 2005) was also increased by 3–4-fold in Chmp5Ctsk osteoclasts (Fig. 3 C). Intriguingly, genes associated with Ephrin B-mediated reverse signaling were enriched in Chmp5Ctsk osteoclasts (Fig. 3 D; Zhao et al., 2006). Additionally, Chmp5Ctsk osteoclasts show an increase in expression of several clastokines, secreted osteoclast-derivated coupling factors that promote osteoblast activity, including Efnb2 (Zhao et al., 2006), Sphk1 (Ryu et al., 2006), Cltrc1 (Takeshita et al., 2013), Wnt10b (Ota et al., 2013), and Bmp6 (Teti, 2013; Fig. 3 E). The high expression of several clastokines in Chmp5Ctsk osteoclasts prompted us to examine the ability of Chmp5Ctsk osteoclasts to promote osteoblast differentiation. Co-culture with Chmp5Ctsk osteoclasts increased mineralization and ALP activity to a greater extent than that seen with corresponding WT controls (Fig. 4, A and B). As the same effects were observed with conditioned medium from Chmp5Ctsk osteoclasts, CHMPS5-deficient osteoclasts also promote osteoblast differentiation in vitro via secreted coupling factors (Fig. 4 C).

Signaling between EphB4 on osteoblasts and its primary ligand ephrinB2 on osteoclasts stimulates osteoblast differentiation (Zhao et al., 2006). To determine the contribution of the EphrinB2–EphB4 pathway to the enhanced osteoblast coupling seen in Chmp5Ctsk osteoclasts, EphB4-Fc fusion protein was added to osteoclast/osteoblast co-cultures to suppress EphrinB2–EphB4 signaling (Fig. 4, D and E). Treatment with EphB4-Fc decreased osteoblast mineralization activity, reversing the effect of Chmp5Ctsk osteoclasts to promote osteoblast differentiation. Thus, the EphrinB2–EphB4 pathway is likely to be one important contributor to the enhanced osteoblast coupling activity of Chmp5Ctsk osteoclasts, though other pathways are likely to contribute as well.

Next, we examined the in vivo expression levels of the coupling factors known to be important for bone remodeling by RNA sequencing of marrow-flushed tibias. As shown in Fig. 4 F, Chmp5Ctsk long bones showed elevated levels of both osteogenic factors such as Tgfβ1 (Tang et al., 2009), Igf1 (Xian et al., 2012), and Lf (Cornish et al., 1997) and an osteoclastogenic factor Csf1 (Wei et al., 2006), whereas expression of other genes, including Cxcl12, Fg2, Bmp2, and Bmp6 in Chmp5Ctsk long bones was comparable to levels in Chmp5fl/fl bones. An elevated Rankl/Opg ratio was also observed in Chmp5Ctsk long bones, suggesting an increase in bone remodeling activity (Fig. 4 G). As reported in BMSCs and osteoblasts isolated from PDB patients (Naot et al., 2007), Chmp5Ctsk long bones show distinct patterns of expression of two WNT signaling antagonists, an increased expression of Dickkopf1 (Dkk1) and a decreased expression of Sclerostin (Sost; Fig. 4 H). Collectively, Chmp5 deletion in osteoclasts enhances transcriptional programs corresponding to osteoclast differentiation/activity and also augments production of osteoclast–osteoblast coupling factors.

Osteoblast activity is highly increased in Chmp5Ctsk mice

To examine whether the elevated levels of the coupling factors observed in Chmp5Ctsk osteoclasts correspond to increased osteoblast activity in vivo, dynamic histomorphometry analysis was conducted. As seen in Fig. 5 (A–B), bone formation rate, mineral apposition rate, and numbers of osteoblasts are markedly increased in periosteal Pagetic lesions. Likewise, in situ hybridization demonstrated increased numbers of mature osteoblasts expressing type 1 collagen α1 (Col1) and osteocalcin (Ocn) in these bone lesions (Fig. 5 C). Consistent with a previous study showing that mature osteoblasts express high levels of vascular endothelial growth factors (VEGFs) and with clinical observations that Pagetoid lesions are hypervascular to the point of occasionally causing high-output heart failure in severe polyostotic cases (Chakravorty, 1978; Zajac and Phillips, 1985; Deckers et al., 2000), these bone lesions are hypervascular and display elevated levels of VEGF expression (Fig. 5, D–F). Additionally, the serum levels of the bone turnover markers ALP, CTX, and N-terminal propeptide of type 1 procollagen (P1NP) were all significantly increased in...
Figure 2.  Chmp5 deletion leads to an increase in osteoclast differentiation and RANKL responsiveness. (A–C) Chmp5fl/fl and Chmp5Cre BMMs were cultured with 40 ng/ml of M-CSF and 20 ng/ml of RANKL for the indicated time periods, and cell lysates were immunoblotted with the indicated antibodies (A). Alternatively, BMMs were cultured with different concentrations of RANKL and multinucleated cells were stained with TRAP (B), and then TRAP activity was measured by colorimetric analysis (C) after 6 d of culture. ***, P < 0.005 by a Bonferroni-corrected two-tailed Student’s t test. (D) Chmp5fl/fl and Chmp5Cre BMMs were cultured with 40 ng/ml of M-CSF and 10 ng/ml of RANKL for the indicated time periods and TRAP activity was measured by colorimetric analysis. ***, P < 0.005 by a Bonferroni-corrected two-tailed Student’s t test. (E) Bone resorptive activity of Chmp5fl/fl and Chmp5Cre osteoclasts. Shown are representative images of in vitro bone resorption assays (right) with quantification of pit number, resorption area, and medium CTX (left, nM). *, P < 0.05; **, P < 0.01 by a two-tailed Student’s t test. Red arrows indicate bone resorption pits. n = 5 fields/slide. (F) Photomicrographs of TRAP-positive osteoclasts in Chmp5fl/fl trabecular bone and Pagetoid lesions in Chmp5Cre mice (CKO; left). Additionally, the number of nuclei in TRAP-positive osteoclasts was counted (n = 30 cells/slide). **, P < 0.01 by a Bonferroni-corrected two-tailed Student’s t test. (G) Elevated levels of Src transcripts in Chmp5Cre osteoclasts. RT-PCR analysis was performed with total RNAs isolated from cultured Chmp5fl/fl and Chmp5Cre osteoclasts after 4 d of culture. ***, P < 0.001 by a Bonferroni-corrected two-tailed Student’s t test. (H and I) After 4 d of culture, Chmp5fl/fl and Chmp5Cre osteoclasts were lysed and immunoblotted with the indicated antibodies (H). Alternatively, GTP-bound Rac1 and total Rac1 were determined by immunoblotting with anti-Rac1 antibody (I). (J) Enlarged actin rings in Chmp5Cre osteoclasts. Chmp5fl/fl and Chmp5Cre osteoclasts were immunostained with FITC-phalloidin. n = 3 independent experiments in A–D and F–J. All error bars indicate SEM. Bars: (B) 60 µm; (E) 100 µm; (F) 25 µm; (J) 100 µm.
Antiresorptive treatments reverse the PDB-like bone phenotype of Chmp5Ctsk mice

To examine if the increased sensitivity of Chmp5Ctsk osteoclasts to RANKL observed in vitro was responsible for the PDB-like bone phenotypes observed in vivo, the genetic interaction between CHMP5 and the RANKL receptor RANK was examined by comparing WT, Rank−/− (Rank-Het), Chmp5Ctsk (CKO), and Chmp5Ctsk;Rank−/− (CKO;Rank-Het) mice (Fig. 6). Though Rank-Het mice do not display detectable bone phenotypes at baseline (Dougall et al., 1999), CKO;Rank-Het mice displayed a substantial reversal of the PDB-like bone phenotypes in Chmp5Ctsk mice, consistent with a high bone turnover state and early onset and polyostotic de novo formation of expansile Pagetoid lesions on periosteal surfaces. In this respect, the phenotype of Chmp5Ctsk mice best resembles the severe polyostotic forms of Paget’s seen in human JPD or other heritable PDB-like disorders such as familial expansile osteolysis (Bakwin and Eiger, 1956) as opposed to typical sporadic PDB that typically manifests as focal lesions in patients over age 50 (Daroszewska and Ralston, 2006; Galson and Roodman, 2014).

Chmp5Ctsk mice, consistent with a high bone turnover state (Fig. 5 G). Given that CHMP5 expression and osteoclast differentiation and activity were all normal in the cultured osteoclasts from Chmp5Ctsk bone marrow stromal cells (BMSCs; unpublished data), this strongly suggests that the high bone turnover state is intrinsic to the function of CHMP5 in osteoclasts. Collectively, Chmp5Ctsk mice demonstrate a high bone turnover state complete with early onset and polyostotic de novo formation of expansile Pagetoid lesions on periosteal surfaces. In this respect, the phenotype of Chmp5Ctsk mice best resembles the severe polyostotic forms of Paget’s seen in human JPD or other heritable PDB-like disorders such as familial expansile osteolysis (Bakwin and Eiger, 1956) as opposed to typical sporadic PDB that typically manifests as focal lesions in patients over age 50 (Daroszewska and Ralston, 2006; Galson and Roodman, 2014).

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can potentially reduce their rate of bone turnover and prevent the development of skeletal deformity (Cundy et al., 2004, 2005; Polyzos et al., 2014). Thus, assessment of the response of Chmp5Ctsk mice to antiresorptives is important to determine the relevance of the model to human PDB-spectrum disorders and also to mechanistically evaluate the contribution of RANKL signaling.

Chmp5fl/fl or Chmp5Ctsk mice were treated with PBS, alendronate, zolendronate, or recombinant OPG-Fc fusion (OPG-Fc) weekly from 2–6 wk of age (Fig. 7). The low bone mass, bone expansion, and elevated by Rank haploinsufficiency (Fig. 6, D–F). Thus, a partial reduction in RANK expression levels imparted by Rank haploinsufficiency can reverse the PDB-like bone phenotype of Chmp5Ctsk mice, arguing that the ability of CHMP5 deletion to increase osteoclast responsiveness to RANK stimulation is central to the pathogenesis of the PDB-like skeletal disorder in Chmp5Ctsk mice.

Previous case reports have suggested that treatment of patients with JPD with antiresorptives such as bisphosphonates, recombinant OPG, or the anti-RANKL antibody Denosumab...
serum levels of bone turnover markers observed in Chmp5^Ctsk mice were reversed by treatment with antiresorptive agents. Osteoclast differentiation was completely abolished by OPG-Fc treatment, whereas treatment with bisphosphonates blocked bone resorption activity (Fig. 7, D–F). Overall, OPG-Fc elicited a more substantial reversal of the phenotype of Chmp5^Ctsk mice than either alendronate or zolendrate. Coupled with the finding that Rank haploinsufficiency rescues the Chmp5^Ctsk phenotype, this result confirms that the development of the PDB-like phenotype in Chmp5^Ctsk mice is highly dependent on the relative levels of RANK signaling and osteoclast differentiation/activity in vivo.
Figure 6. Rank haploinsufficiency reverses PDB-like bone phenotypes in Chmp5Ctsk mice. (A–C) µCT analysis of the femur and tibia of 12-wk-old male Rank+/+;Chmp5fl/fl (WT), Rank+/-;Chmp5fl/fl (Rank het), Rank+/-;Chmp5Ctsk (CKO), and Rank+/-;Chmp5Ctsk (CKO;Rank het) mice. Shown are representative 3D reconstructions of femurs and tibias (A) with quantification of bone volume/total volume (BV/TV), trabecular separation (Tb.Sp), trabecular number (Tb.N), midshaft volume (M. TV), and maximal femoral diameter. (B and C; n = 7 mice/group). Red arrows indicate maximal femoral diameter. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by a Bonferroni-corrected two-tailed Student’s t test. (D and E) Femurs of the indicated mice were stained for histological analysis, including hematoxylin and eosin and TRAP staining, and the images are compilation/mosaics (E). TRAP-positive osteoclasts were counted in trabecular bones of WT and CKO;Rank het mice and Pagetoid lesions in CKO mice (n = 7 mice/group; D1). ***, P < 0.001 by a Bonferroni-corrected two-tailed Student’s t test. (F) Serum levels of CTX, P1NP, and ALP in 12-wk-old male WT, Rank het, CKO, and CKO;Rank het mice (n = 7 mice/group). *, P < 0.05; **, P < 0.01; ***, P < 0.001 by a Bonferroni-corrected two-tailed Student’s t test. All error bars indicate SEM. Bars: (E, top and middle) 100 µm; (E, bottom) 25 µm.
Figure 7. Antiresorptive treatments reverse PDB-like bone phenotypes in Chmp5CKO mice. (A–C) μCT analysis of the femurs and tibias in 6-wk-old male Chmp5fl/fl and Chmp5CKO mice. Mice were randomized to treatment with PBS, alendronate (Alen), zolendronate (Zolen), or OPG-Fc (OPG) weekly from 2–6-wk-old via IP injection. Shown are representative 3D reconstructions of femurs and tibias (A) with quantification of bone volume/total volume (BV/TV), trabecular separation (Tb.Sp), midshaft volume (M. TV), and maximal femoral diameter (B and C; n = 5 mice/group). Red arrows indicate maximal diameter. **, P < 0.01; ***, P < 0.001 by a Bonferroni-corrected two-tailed Student’s t test. (D and E) Femurs of the indicated mice were stained for histological analysis, including hematoxylin and eosin and TRAP staining. RAP-positive osteoclasts were counted in trabecular bones of Chmp5fl/fl and Chmp5CKO mice treated with PBS, alendronate, or OPG-Fc (n = 5 mice/group; E), *, P < 0.05 by a Bonferroni-corrected two-tailed Student’s t test. (F) Serum levels of CTX, P1NP, and ALP in 6-wk-old male Chmp5fl/fl and Chmp5CKO mice treated with PBS, Alen, Zolen, or OPG (n = 5 mice/group). All error bars indicate SEM. **, P < 0.01 by a Bonferroni-corrected two-tailed Student’s t test. Bars: (D, top and middle) 100 µm; (D, bottom) 25 µm.
CHMP5 suppresses RANK-mediated NF-κB activation in osteoclasts

We previously reported that CHMP5 functions as a negative regulator of NF-κB signaling downstream of proinflammatory cytokines, though the mechanism of this effect was unclear (Shim et al., 2006). Size-exclusion chromatography of the preosteoclast line Raw 264.7 showed that endogenous CHMP5 cofractionated with a subset of NF-κB RelA and IκBα in an ~550-kD complex (Fig. 8 A). Immunoprecipitation analysis confirmed that both endogenous and overexpressed CHMP5 interacts with RelA and IκBα in an osteoclast line and HEK293 cells, respectively (Fig. 8, B and C). However, the interactions with NF-κB p50, NF-κB p100, and IκBβ were not observed, indicating selective interactions between CHMP5 and NF-κB and IκB isoforms (unpublished data).

In this context, luciferase assay with a NF-κB responsive reporter showed that RANKL-induced NF-κB activation was significantly inhibited by CHMP5 overexpression, whereas CHMP5 deficiency enhanced RANKL-induced activation of NF-κB (Fig. 8 D). Accordingly, Chmp5Ctsk osteoclasts displayed an increase in NF-κB DNA-binding activity and a decrease in IκBα levels (Fig. 8, E and F). Blocking this NF-κB activity ablates enhanced activity of TRAP in Chmp5Ctsk osteoclasts, suggesting that aberrant NF-κB signaling is responsible for the phenotypes observed in Chmp5Ctsk osteoclasts (Fig. 8 G). Furthermore, phosphorylation levels of IκKα/β
were relatively normal in Chmp5Ctsk osteoclasts (Fig. 8 F) and CHMP5 overexpression inhibited NF-κB activation by a constitutively active IKKβ, but not by RelA (unpublished data). Thus, these results suggest that CHMP5 functions downstream of the IKK complex and upstream of RelA.

**CHMP5 and USP15 dampen RANK-mediated NF-κB activation and osteoclast differentiation via IkBα stabilization**

To gain insight into the mechanism by which CHMP5 regulates NF-κB signaling, affinity purification–based mass spectrometry was performed to identify CHMP5–binding proteins. Because CHMP5 interacts with other CHMP family proteins as a component of the endosomal sorting complexes required for transport (ESCRT) machinery (Shim et al., 2006; Tsang et al., 2006), Flag-tagged CHMP5-WT and a CHMP5 mutant (D3) that fails to bind to other CHMP proteins were used to identify non-ESCRT related proteins that are associated with the NF-κB pathway (unpublished data). As expected, ESCRT components, including VPS4B and CHMP1B, 2A, 2B, and 4B, were pulled down by CHMP5-WT but not the CHMP5-D3 mutant. Ingenuity pathway analysis of the CHMP5-binding proteins identified enrichment of the protein ubiquitination pathway including the PDB genetic risk factor VCP/p97 (Yamanaka et al., 2012) and the deubiquitinating enzyme USP15 (Schweitzer et al., 2007; unpublished data). Likewise, size exclusion chromatography showed that endogenous VCP/p97 and USP15 cofractionated with a subset of CHMP5, RelA, IkBα, and the E3 ligase β-Trcp, which is consistent with a previous study showing that VCP/p97 binds ubiquitinated IkBα and β-Trcp (Fig. 8 A; Dai et al., 1998; Li et al., 2014). Furthermore, immunoprecipitation analysis confirmed that both endogenous and overexpressed CHMP5 interact with USP15, VCP/p97, and β-Trcp in an osteoclast line and HEK293 cells, respectively (Fig. 8 H and unpublished data). Thus, in osteoclasts, CHMP5 is a component of the 550-kD protein complex containing a subset of NF-κB RelA/IκBα and binds proteins involved in the regulation of ubiquitin–mediated proteasomal degradation, including VCP/p97, USP15, and β-Trcp (Fig. 8 I). Given that CHMP5 binds the RelA–IκBα complex alongside USP15, VCP/p97, and β-Trcp, we hypothesized that CHMP5 may regulate NF-κB signaling via modulating ubiquitination of IκBα. An in vitro ubiquitination assay revealed that IκBα ubiquitination was markedly inhibited by overexpression of CHMP5 or USP15 (Fig. 9 A), which is accompanied with pulse-chase labeling experiments showing that IκBα stability was increased by overexpression of CHMP5 or USP15 (Fig. 9, B and C). Accordingly, the ability of RANKL to induce IκBα degradation was enhanced in the absence of CHMP5 or USP15 (Fig. 9, D and E). Thus, CHMP5 and USP15 appear to stabilize IκBα in osteoclasts via suppression of ubiquitination–mediated proteasomal degradation. Given in vitro ubiquitination and deubiquitination assays that either SCFβ-Trcp-mediated ubiquitination of IκBα or ubiquitinated IκBα levels were not affected by addition of recombinant CHMP5, CHMP5 is unlikely to suppress the IκBα ubiquitination or directly possess deubiquitinating enzyme activity (unpublished data). CHMP5 overexpression did not inhibit RANKL–induced NF-κB activation in the absence of USP15, indicating that CHMP5 requires USP15 to suppress NF-κB signaling downstream of RANKL (Fig. 9 F). In this respect, USP15 overexpression inhibited RANKL–induced NF-κB activation (Fig. 9 G) and USP15 knockdown increased sensitivity of RANKL to osteoclast differentiation (Fig. 9 H). Collectively, these data suggest that the CHMP5–USP15 complex suppresses RANK-mediated NF-κB activation via IκBα stabilization in osteoclasts, in which mechanism it prevents proteasomal degradation of IκBα leading to the retention of NF-κB in an inactive cytosolic complex.

**CHMP5 mediates deubiquitination of the VCP/p97 complex via USP15**

VCP/p97 is a type II AAA-ATPase that regulates unfolding of proteins as a ubiquitin–selective chaperone, and genetic variants in VCP cause inclusion body myopathy associated with Paget’s disease of bone and frontotemporal dementia (IBMPFD; Rape et al., 2001; Watts et al., 2004). As it has also been suggested that VCP/p97 regulates ubiquitin–mediated proteasomal degradation of IκBα (Dai et al., 1998), we speculated that VCP/p97 may cooperate with CHMP5 and USP15 to regulate IκBα degradation. To confirm the observation that the CHMP5 complex in osteoclasts contains the deubiquitinating enzyme USP15 and VCP/p97 (Fig. 8 A and I), the physical interactions between CHMP5, USP15, and VCP/p97 were studied using cell-free immunoprecipitation assay (Fig. 10 A). Intriguingly, CHMP5 interacts directly with both USP15 and VCP/p97, whereas there was no direct interaction between USP15 and VCP/p97. Likewise, in osteoclasts the interaction of USP15 with VCP/p97 was markedly decreased in the absence of CHMP5 (Fig. 10 B), suggesting that CHMP5 mediates the interaction between USP15 and VCP/p97. Previous studies have shown that VCP/p97 and its cofactors interact with ubiquitinated client proteins and facilitate their degradation in proteasome (Koegel et al., 1999; Dai and Li, 2001). We therefore examined the effect of CHMP5 and USP15 on ubiquitination levels of VCP/p97 client proteins using a ubiquitination assay (Fig. 10 C). Similar to our observation that IκBα ubiquitination was suppressed by CHMP5 working together with USP15 (Fig. 9 A), overexpression of CHMP5 or USP15 down-regulated the ubiquitination of proteins physically associated with Myc-VCP/p97. Thus, CHMP5 is likely to dampen ubiquitination of VCP/p97–client proteins via recruitment of USP15 to VCP/p97.

IBMPFD is associated with missense mutations in VCP/p97, the majority of which are located in the N-terminal and the D1 ATPase domains (Nalbandian et al., 2011). Among these, R155H has been reported as the most common VCP mutation causing IBMPFD, and A232E is associated with severe clinical manifestations such as early onset of PDB and aggressive myopathy (Watts et al., 2004). To examine if IBMPFD–associated
Figure 9. CHMP5 and USP15 suppress RANKL-induced NF-κB activation and osteoclast differentiation via stabilization of IκBα. (A) HEK293 cells were transfected with Chmp5 or Usp15 along with Flag-IκBα, His-ubiquitin, and Myc-β-Trcp, treated with 10 µM MG132, and subjected to IκBα ubiquitination (top) and protein expression assays (bottom). (B and C) HEK293 cells were transfected with control vector, Chmp5, or Usp15 along with Flag-IκBα, and IκBα stability was determined by pulse–chase labeling with [35S]-methionine followed by autoradiography and quantified by ImageJ (C). (D and E) RAW264.7 cells expressing control vector, Chmp5, or Usp15 shRNA were stimulated with 25 ng/ml of RANKL at different time points, and immunoblotted with the indicated antibodies (D). IκBα degradation was quantified using ImageJ and normalized to Hsp90 expression (E). (F and G) RAW264.7 cells expressing control vector or Usp15 shRNA were transfected with Chmp5 along with PBII-luc and Renilla (F). Alternatively, RAW264.7 cells were transfected with Usp15 in a dose-dependent manner along with PBII-luc and Renilla (G). Luciferase activity was measured and normalized to Renilla *, P < 0.05; **, P < 0.01; ***, P < 0.001 by a Bonferroni-corrected two-tailed Student’s t test. (H) RAW264.7 cells expressing control vector or Usp15 shRNA were cultured in the presence of M-CSF and different concentrations of RANKL TRAP staining was performed after 6 d. (I) WT BMMs were treated with vehicle or NF-κB inhibitor for 1 h before RANKL stimulation. After 48 h of stimulation with RANKL, Chmp5 and Usp15 expression was analyzed by RT-PCR. ***, P < 0.0001 by a Bonferroni-corrected two-tailed Student’s t test. Data in A–I represent n = 3 independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Bar: (H) 60 µm.

VCP/p97 mutations alter the binding affinity of VCP/p97 to CHMP5 or USP15, immunoprecipitation analysis was performed with Myc-tagged VCP/p97 R155H and A232E constructs (Fig. 10, D and E). Both VCP/p97 R155H and A232E displayed markedly decreased binding to CHMP5 and USP15. Similarly, whereas CHMP5 or USP15 can suppress the ubiquitination of the client proteins associated with VCP/p97-WT, VCP/p97-R155H, and VCP/p97-A232E were refractory
Figure 10. CHMP5 mediates deubiquitination of the VCP/p97 complex via USP15. (A) GST or GST-Chmp5 was incubated with the purified proteins, immunoprecipitated with glutathione-agarose, and immunoblotted with the indicated antibodies (top). Alternatively, GST or GST-VCP was incubated with purified CHMP5 protein (bottom). Input indicates loading controls for USP15, VCP, and CHMP5. (B) RAW264.7 cells expressing a control vector or Chmp5 shRNA were cultured with 5 µg of RANKL for 2 d before treatment with 10 µM MG132. Cell lysates were immunoprecipitated with anti-Vcp antibody and protein G–conjugated dynabeads and immunoblotted with the indicated antibodies. (C) HEK293 cells were transfected with Myc-VCP and HA-ubiquitin in the absence or presence of CHMP5 or USP15. 24 h later, cells were treated with 10 µM MG132 and subjected to ubiquitination of Myc-VCP. (D and E) HEK293 cells were transfected with HA-CHMP5 (D) or USP15 (E) and His-ubiquitin along with vector, Myc-VCP (WT), or Myc-VCP mutants (R155H, A232E). 24 h later, cells were treated with 10 µM MG132, immunoprecipitated with anti-Myc antibody–conjugated agarose, and immunoblotted with the indicated antibodies. (F) HEK293 cells were transfected with Myc-VCP (WT) or Myc-VCP mutants (R155H, A232E) and HA-ubiquitin in the absence or presence of CHMP5 or USP15. 24 h later, cells were treated with 10 µM MG132 and subjected to ubiquitination of Myc-VCP. (G) HEK293 cells were transfected with vector, Myc-VCP (WT), or Myc-VCP mutants (R155H, A232E) along with Flag–RANK, PBlu-Iuc and Renilla in the absence or presence of USP15, and 24 h later, luciferase activity was measured and normalized to Renilla. **, P < 0.01 by a Bonferroni-corrected two-tailed Student’s t test. Data in A–G represent n = 2 independent experiments. **, P < 0.01.
to this effect (Fig. 10 F). Thus, the R155H and A232E mutations disrupt the physical and functional engagement of VCP/p97 with CHMP5 and USP15. Consistent with this engagement with CHMP5 and USP15, expression of VCP/p97-WT but not VCP/p97-R155H or VCP/p97-A232E inhibited RANK-mediated NF-κB activation (Fig. 10 G), and addition of USP15 promoted a further decrease in the presence of VCP/p97-WT but not VCP/p97-R155H or VCP/p97-A232E (Fig. 10 G). Thus, the IBMPFD-associated VCP/p97 mutations that disrupt engagement with CHMP5 and USP15 also influence the ability of VCP/p97 to regulate the activation of NF-κB by RANK.

**DISCUSSION**

Our data reveal that CHMP5 is a key dampener of bone remodeling, preventing the development of a high turnover PDB-like state. CHMP5 mobilizes USP15 to deubiquitinate and stabilize IkBα, inhibiting ubiquitin-dependent proteasomal degradation. This dampens RANK-mediated activation of NF-κB in osteoclasts, in turn increasing the threshold of stimulation required for osteoclast differentiation and osteoclast-mediated bone resorption. Intriguingly, CHMP5 and USP15 expression are both up-regulated by RANKL, suggesting that they may function in concert as a negative feedback mechanism to suppress NF-κB activity (Fig. 9 I).

The rescue of the Chmp5 Ctsk bone phenotype by Rank haploinsufficiency demonstrates that exaggerated bone remodeling observed in vivo is directly attributable to the enhanced sensitivity to RANK stimulation observed in osteoclasts in vitro, though this result does not exclude that alterations in other pathways may also contribute to the phenotype observed. Notably, Rank heterozygous mice do not display detectable alterations in basal osteoclast numbers or activity (Dougall et al., 1999). Thus, the ability of CHMP5 deletion to increase the sensitivity of osteoclasts to RANK stimulation in vitro is supported by the observation that Chmp5 Ctsk mice also display an increased sensitivity to reductions in Rank gene dosage in vivo. This conclusion is also reinforced by the observation that OPG-Fc, which directly inhibits RANK activation, is highly effective in rescue of the Chmp5 Ctsk bone phenotype. Unlike OPG-Fc treatment, Chmp5 Ctsk mice treated with bisphosphonates, which suppress osteoclast resorptive activity without directly affecting activation of RANK, still display bone expansion as a result of incomplete blockade of osteoblast activity. Through comparison of these attempts to reverse the Chmp5 Ctsk bone phenotype, we conclude that the osteoclast/osteoblast coupling in Chmp5 Ctsk mice is RANK-dependent but is largely independent of osteoclast resorptive activity. Thus, coupling factors relevant to this model are likely to be directly produced by osteoclasts as opposed to being growth factors liberated from the bone matrix during osteoclast-mediated bone resorption. Consistent with this model, Chmp5 Ctsk osteoclasts display an enhanced ability to promote osteoblast activity in a co-culture system in vitro. Likewise, transcriptome analysis of Chmp5 Ctsk osteoclasts demonstrates that CHMP5 not only suppresses osteoclast differentiation, but it also suppresses a broad transcriptional program driving the expression of several osteoclast/osteoblast coupling factors. Among these coupling factors whose expression is regulated by CHMP5, the EphrinB2–EphB4 pathway is particularly important, as it is necessary for the enhanced coupling activity of CHMP5-deficient osteoclasts. However, the observation that several known osteoclast/osteoblast coupling factors are regulated by CHMP5 indicates that additional pathways also contribute to this phenotype. Thus, by acting as a rheostat to tune RANK signaling in osteoclasts, CHMP5 in turn regulates systemic coupling of osteoclast and osteoblast activity.

Our previous study of mice with germline deletion of Chmp5 indicated the importance of CHMP5 function in late endosomal trafficking to lysosomes and in TGFβ signaling via regulating the lysosomal degradation of TGFβ receptors (Shim et al., 2006). However, lysosomal biogenesis and endosomal structures are largely intact in Chmp5 Ctsk osteoclasts and TGFβ receptor levels and TGFβ-induced SMAD activation were unaltered in the absence of CHMP5 (unpublished data). This may be due to Csk–cre deleting Chmp5 after lysosomal biogenesis is complete in osteoclasts, or the specialization of endosomal/lysosomal structures in osteoclasts may necessitate the use of different pathways for their biogenesis. Additionally, we found that distinct domains mediate the interactions of CHMP5 with the endocytic and NF-κB pathways, implying that functions of CHMP5 in regulating NF-κB activity are biochemically distinct from its role in lysosomal biogenesis in osteoclasts. However, further studies will be required to determine if the functions of CHMP5 to regulate endocytosis and NF-κB pathway are linked.

The spectrum of human PDB and related monogenic forms of PDB encompass a wide range of phenotypic variation (Daroszewskia and Ralston, 2006). In this respect, the phenotype of Chmp5 Ctsk mice fits clearly within this spectrum, as the combination of periosteal hyperdynamic bone deposition, exaggerated bone remodeling coupled with increased activities of osteoclasts and osteoblasts, bone expansion, and osteoclasts displaying increased numbers of nuclei are pathognomonic for Paget’s-spectrum disorders. Although sporadic PDB displays a greatly increased relative risk of osteosarcoma formation (Hansen et al., 2006), no osteosarcomas were observed over the course of this study, and the early onset and polyostotic nature of the Chmp5 Ctsk bone phenotype is markedly different from classical PDB. Some PBD patients have been observed to display narrow fibrosis (Murrin and Harrison, 2004), and this is not observed in Chmp5 Ctsk mice. Similarly, the vascular calcifications associated with JPD are not observed in Chmp5 Ctsk mice (Saki et al., 2013; Whyte et al., 2014). However, further studies will be required to determine the reasons for these differences. Lastly, it is possible that environmental factors are needed for full penetrance of all aspects of PDB phenotypes, as environmental factors which are acknowledged to be especially important for the pathogenesis of PDB (Roodman, 2010).
Given that variants in CHMP5 gene have yet to be identified in human patients with a Paget’s disorder spectrum, we instead considered whether CHMP5 displays a functional interaction with other known genes implicated in a Paget’s spectrum disorder. As variants in VCP/p97 gene cause the Paget’s-related disorder IBMPFD, and VCP/p97 has been implicated in regulation of IkBα, the relationship between CHMP5 and VCP/p97 was studied, demonstrating that CHMP5 serves as an adaptor to recruit the deubiquitinating enzyme USP15 to the VCP/p97 complex. This recruitment then leads to USP15-mediated deubiquitination of VCP/p97 client proteins, likely salvaging them from proteosomal degradation. Accordingly, mutations in VCP/p97 that cause IBMPFD (R155H and A232E) disrupt both the physical and functional interaction with CHMP5 and USP15. Collectively with the PDB-like skeletal phenotype of Chmp5<sup>−/−</sup> mice, this indicates that CHMP5 cooperates with VCP/p97 to regulate RANK signaling and that disruption of this functional interaction may be a key factor in the development of some Paget’s spectrum disorders. This also raises the possibility that VCP/p97 serves an important function to select the substrates targeted by the CHMP5–USP15 complex for deubiquitination.

The exuberant bone formation and expansion seen in Chmp5<sup>−/−</sup> mice demonstrates the importance of CHMP5 for the regulation of bone turnover rates. As, to our knowledge, Chmp5<sup>−/−</sup> mice represent one of the most severe mouse models of high turnover metabolic bone disease yet reported, we believe that they may offer an opportunity to identify osteoclast/osteoblast coupling factors with anabolic activity, which may ultimately have utility for the treatment of osteoporosis. Additionally, the early onset, high severity, and complete penetrance displayed by this model may make this an attractive preclinical model to validate treatment for Paget’s-spectrum disorders.

MATERIALS AND METHODS

Materials. Mouse M-CSF and mouse RANKL were purchased from R&D Systems. Antibodies to Chmp5 (Santa Cruz Biotechnology, Inc.), Hsp90 (Cell Signaling Technology, Inc.), ubiquitin (Santa Cruz Biotechnology, Inc.), IkBα (Santa Cruz Biotechnology, Inc.), β-Trcp/HOS (Santa Cruz Biotechnology, Inc.), NF-κB p50 (Santa Cruz Biotechnology, Inc.), anti-HA HRP conjugated antibody (Santa Cruz Biotechnology, Inc.), p65/RelA (Enzo Life Sciences), NF-κB p100 (Cell Signaling Technology), Vcp/p97 (Cell Signaling Technology), Flag (Sigma-Aldrich), GFP (Clontec), GAPDH (Affinity BioReagents), Usp15 (Bethyl Laboratories), Nfatc1 (BioLegend), phospho-IKKα/β (Cell Signaling Technology), Bi-phospho-Syk (Y325/526; Abgent), TGF-β receptor, type 1 (EMD Millipore), phospho-Src Family (Tyr416; Cell Signaling Technology), and Cathepsin K (Biossion) were used for immunoblotting. Anti-HA agarose-conjugated antibody (Santa Cruz Biotechnology, Inc.) and anti-MyHC HRP-conjugated antibody (Santa Cruz Biotechnology, Inc.), and anti-HA HRP-conjugated antibody were used for immunoprecipitation. NF-κB inhibitor Bay 11–7085 and active Rac1 detection kit were purchased from Santa Cruz Biotechnology, Inc. and Cell Signaling Technology, respectively. For recombinant proteins, GST-NF-κB p65 and GST-IkBα were purchased from Enzo Life Sciences, and GST-CHMP5 and GST-p97/VCP were purchased from Novus Biologicals. The recombinant active SCD-Skp2 protein complex and activated IkBα were purchased from EMD Millipore.

Mice. To generate Chmp5<sup>−/−</sup> mice, exon 4 and 5 flanked by a 5’ loxp site and a 3’ loxp-PGKNEO-loxP cassette was cloned into the pEasy-flox vector and the ES clones with homologous recombination were selected by neomycin and validated by Southern blotting. Subsequently, Cre recombinase-expressing construct was transfected into the validated neomycin-resistant clones and further selected to delete the PGK-NEO cassette. Mice bearing the targeted allele were generated by blastocyst injection and backcrossed with C57BL/6 mice up to F8 generation.

Chmp5<sup>−/−</sup> mice were generated by intercrossing Chmp5<sup>+/−</sup> and Cathepsin K-Cre breeders with Chmp5<sup>+/−</sup> mice. Cathepsin K-Cre mice were a gift from S. Kato (University of Tokyo, Tokyo, Japan). Chmp5<sup>−/−</sup>Rank<sup>−/−</sup> mice were generated by intercrossing Chmp5<sup>+/−</sup>Rank<sup>−/−</sup>; Cathepsin K-Cre mice with Chmp5<sup>−/−</sup>Rank<sup>−/−</sup> breeders. All mice were on a C57BL/6 background. All animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the Weil Cornell Medical College subcommittee on animal care (IACUC).

Cell culture, osteoclast, and osteoblast differentiation. Primary BMMs and RAW264.7 cells (American Type Culture Collection) were cultured in α-minimum essential medium (Cellgro) supplemented with 10% FBS (HyClone), 1% penicillin/streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen) in 5% CO₂ at 37°C. Primary calvarial osteoblasts (COBs) and BM-derived stromal cell (BMSCs) were cultured in α-MEM (α-MEM; Invitrogen) containing 10% FBS, 1% penicillin/streptomycin, 2mM L-glutamine, 1% Hepes, and 1% nonessential amino acid. For osteoblast differentiation, the cells were cultured under basal medium containing β-glycerophosphate and ascorbic acid.

For osteoclast differentiation analysis, the femur and tibia were carefully dissected from 6–wk-old male Chmp5<sup>+/−</sup> and Chmp5<sup>−/−</sup> mice, and BM cells collected by flushing were plated overnight in α-MEM plus 10% FBS. Nonadherent cells were collected and seeded on a 100-mm dish with mM-CSF (30 ng/ml, R&D Systems). After 48 h, nonadherent cells were discarded and adherent cells were used as BMMs. BMMs were detached from the 100-mm dish using Detachin (Genlanics). For the osteoclastogenesis, RAW264.7 (2.6 × 10<sup>5</sup> cells/cm<sup>2</sup>) were cultured for 4d in α-MEM containing 5 ng/ml mRANKL (R&D Systems) or 20 ng/ml mM-RANKL and 40 ng/ml mM-CSF, respectively. Alternatively, cells were plated on the bovine cortical bone slices (bonelices.com) and cultured with 20 ng/ml mM-RANKL and 40 ng/ml mM-CSF. The number and area of the resorptive pits on the bone slice were counted and the amount of CTX liberated from the bone slice to medium was measured by an ELISA kit (IDS) to assess bone resorption activity.

Osteoblast culture with osteoclast-conditioned medium. The conditioned medium of Chmp5<sup>+/−</sup> and Chmp5<sup>−/−</sup> osteoclasts was harvested as described previously. (Matsuoka et al., 2014) In brief, after 1 or 2 d culture with α-MEM medium containing 10% FBS, mM-RANKL (20 ng/ml), and mM-CSF (40 ng/ml), Chmp5<sup>+/−</sup> and Chmp5<sup>−/−</sup> BM-BMMs were cultured in the conditioned medium (CM) for 1 or 2 d and CM was harvested. Each CM was concentrated using the Amicon Ultra-15 Filter Unit (10K; EMD Millipore). Co-culture of osteoblasts with Chmp5<sup>+/−</sup> and Chmp5<sup>−/−</sup> osteoclasts was performed in the presence of various concentrations of mouse-Fc or mouse EphB4-Fc (R&D Systems). After 12–18 d of culture, osteoblasts were stained with alizarin red and mineralization activity was measured by colorimetric analysis.

Histology, immunohistochemistry, and in situ hybridization. For histological analysis, hindlimbs were dissected from 6- or 12-wk-old male mice, fixed in 10% neutral buffered formalin for 24–48 h, and decalified by daily changes of 15% tetrasodium EDTA for 2 wk. Tissues were dehydrated through an ethanol series, embedded in paraffin, and sectioned at 7 μm thickness along the coronal plate from anterior to posterior. Decalified femoral sections were stained with hematoxylin and eosin (H&E), Safranin O, and tartrate-resistant acid phosphatase (TRAP).

For in situ hybridization, DIG-labeled probes were prepared to detect type I collagen (Col1) and osteocalcin (Ocn) mRNA expression using the DIG-labeled kit (Roche) according to the manufacturer’s instructions. Paraffin sections were dewaxed and quenched with endogenous peroxidase, and...
then hybridized with the probes. DIG-labeled probe was then detected by immunostaining with anti-DIG-POD and streptavidin-HRP.

For immunohistochemistry, paraffin tissue sections were dewaxed and blocked with 3% goat serum, 1% BSA, 0.1% Triton X-100 in PBS for 1 h at room temperature. Sections were incubated with antibodies specific to CHMP5 (Santa Cruz Biotechnology, Inc.; clone H7), PECAM-1 (CD31, Santa Cruz Biotechnology, Inc.; clone M6), VEGF (EMD Millipore, 07-1420), and collagen type I (Rockland) at 4°C overnight, treated with TSA-biotin (Perkin Elmer), and streptavidin-HRP, as per manufacturer’s instructions, and then visualized with 2.2’-diaminobenzidine tetrahydrochloride.

**µCT analysis, bone histomorphometry, and skeletal preparation.** For µCT analysis, a Scanco Medical µCT 35 system with an isotropic voxel size of 7 µm was used to image the distal femur. Scans were conducted in 70% ethanol and used an x-ray tube potential of 55 kVp, an x-ray intensity of 0.145 mA, and an integration time of 600 ms. For trabecular bone analysis of the distal femur, a upper 2.1-mm region beginning 280 µm proximal to the growth plate was contoured. For cortical bone analysis of femur and tibia, a 30-µm-thick en bloc sample was micromachined, frozen in liquid nitrogen and standard µCT scans were conducted. Cortical and cancellous bone sections were thresholded at 211 and 350 µg HA/cm³, respectively. µCT CT scans of skulls, vertebrae and feet were performed using isotropic voxel sizes of 1.2 and 20 µm. 3D images were obtained from contoured 2D images by methods based on distance transformation of the binarized images. All images presented are representative of the respective genotypes.

For the bone histomorphometry, 20 mg/kg calcine (Sigma-Aldrich) and 30 mg/kg demeclocycline (Sigma-Aldrich) dissolved in 2% sodium bicarbonate solution were injected subcutaneously with 4-d interval at day 0 and day 2 before the sacrifice. Undecalcified tibia samples were fixed in 4% PFA and processed as described. After glutaraldehyde fixation, the samples were dehydrated in a series of graded ethanol concentrations and then embedded in plastic resin for bone sections. The sections were stained with von Kossa and TRAP, and numbers and activities of osteoblasts and osteocytes were quantified using the Bioquant Osteo Image Analysis System.

*Complete blood count test.* Blood was collected from 12-week-old male *Chmp5*fl/fl and *Chmp5*Ctsk mice (n = 5 mice/group) by cardiac puncture immediately after sacrifice. All parameters for complete blood counts were calculated by a Hemavet 950 analyzer (Drew Scientific) according to the manufacturer’s instructions.

**Antiresorptive treatment.** PBS, 2.5 mg/kg alendronate sodium trihydrate (Sigma-Aldrich), 100 µg/kg zolendronic acid monohydrate (Sigma-Aldrich), or 3 mg/kg OPG–Fc (Amgen Inc.) was intraperitoneally injected weekly to *Chmp5*fl/fl and *Chmp5*Ctsk male mice from 2–5 wk of age, and the mice were all sacrificed at 6 wk of age and used for skeletal analysis.

**Bone RNA isolation and RT-PCR analysis.** Bone RNA was prepared as previously described. In brief, tibias were isolated from 6–8-week-old male *Chmp5*fl/fl and *Chmp5*Ctsk mice, and cleaned of surrounding soft tissue, and BM cells were removed by flushing with PBS. Immediately, bones were transferred to the prechilled tubes containing QIAzol lysis reagent (QIAGEN) and homogenized using a Polytron (Kinematic PT 2100, 7 mm tip) under a rack surrounded by liquid nitrogen. cDNA was reverse transcribed using random primers and MultiScribe reverse transcription (Applied Biosystems) and used in real-time PCR with a SYBR Green PCR Master Mix kit (Applied Biosystems). The specific primer pairs are shown in [Table S1](#).
with anti-iIsBta antibody. Alternatively, RAW264.7 cells were infected with lentiviruses expressing control (GFP and Luciferase genes; Broad Institute, Boston, MA), mouse Chmp5 (Sigma-Aldrich), or mouse Usp15 (Sigma-Aldrich) shRNAs, and the cells were treated with 10 mM MG132 for 8 h before 1-h RANKL stimulation. The cells were lysed, immunoprecipitated with anti-iIsBta and protein G–agarose, subjected to SDS-PAGE, and immunoblotted with anti-ubiquitin antibody.

**Biomarkers of bone turnover.** Blood was collected from 6-wk-old male Chmp5flox/flox and Chmp5Cre mice (n = 5/group) by cardiac puncture and divided into two groups. Serum was separated using serum separator tubes and serum levels of ALP (Cloud–Clone), PINP (IDS), and CTX (IDS) were measured using each ELISA kits as per the manufacturer’s instructions.

**Statistical analysis.** Data were shown as the mean ± SEM. Sample sizes were calculated on the assumption that a 30% difference in the parameters measured would be considered biologically significant with an estimate of sigma of 10–20% of the expected mean. α and β were set to the standard values of 0.05 and 0.8, respectively. No animals or samples were excluded from analysis. In Fig. 4 (f and g), mice were randomized to treatment groups (aldendronate, zoledronate, OPG–Fc, or PBS). pCT analysis was performed in a blinded fashion. We first performed the Shapiro–Wilk normality test for checking normal distributions of the groups. If normality tests passed, two-tailed, unpaired Student’s t tests were used for the comparisons between two groups; if normality tests failed, Mann–Whitney tests were used for the comparisons between two groups. For the comparisons of three or four groups, we used one-way ANOVA if normality tests passed, followed by Tukey’s multiple comparison test for all pairs of groups. If normality tests failed, Kruskal–Wallis test was performed and followed by Dunn’s multiple comparison test. The GraphPad Prism software (v6.0a) was used for statistical analysis. P < 0.05 was considered statistically significant. * P < 0.05; ** P < 0.01; *** P < 0.001.

**Online supplemental material.** Table S1 shows the specific primer pairs used in RT–PCR analysis of bone and cultured osteoclast RNAs. Table S2 shows a gene set related to the osteoclast differentiation and fusion modified from Biomarkers of bone turnover.

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