Stability of mRNA influences osteoporotic bone mass via CNOT3

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Osteoclastogenesis is under the control of posttranscriptional and transcriptional events. However, posttranscriptional regulation of osteoclastogenesis is incompletely understood. CNOT3 is a component of the CCR4 family that regulates mRNA stability, but its function in bone is not known. Here, we show that *Cnot3* deficiency by deletion of a single allele induces osteoporosis. *Cnot3* deficiency causes an enhancement in bone resorption in association with an elevation in bone formation, resulting in high-turnover type bone loss. At the cellular level, *Cnot3* deficiency enhances receptor activator of NF-κB ligand (RANKL) effects on osteoclastogenesis in a cell-autonomous manner. Conversely, *Cnot3* deficiency does not affect osteoblasts directly. *Cnot3* deficiency does not alter RANKL expression but enhances receptor activator of NF-κB (RANK) mRNA expression in bone in vivo. *Cnot3* deficiency promotes RANK mRNA stability about twofold in bone marrow cells of mice. *Cnot3* knockdown also increases RANK mRNA expression in the precursor cell line for osteoclasts. Anti-CNOT3 antibody immunoprecipitates RANK mRNA. *Cnot3* deficiency stabilizes luciferase reporter expression linked to the 3′-UTR fragment of RANK mRNA. In contrast, *Cnot3* overexpression destabilizes the luciferase reporter linked to RANK 3′-UTR. In aged mice that exhibit severe osteoporosis, *Cnot3* expression levels in bone are reduced about threefold in vivo. Surprisingly, *Cnot3* deficiency in these aged mice further exacerbates osteoporosis, which also occurs via enhancement of osteoclastic activity. Our results reveal that CNOT3 is a critical regulator of bone mass acting on bone resorption through posttranscriptional down-regulation of RANK mRNA stability, at least in part, even in aging-induced osteoporosis.

RNA stability | osteopenia

Osteoporosis is a major bone disease affecting over 20 million patients in the United States. The pathophysiology of osteoporosis is based on the imbalance between bone resorption and bone formation (1). These two arms are regulated by the activities of osteoclasts and osteoblasts, respectively (2). Both posttranscriptional and transcriptional events are considered to be involved in the regulation of bone resorption. However, compared with transcriptional regulation of bone metabolism, posttranscriptional regulation is scarcely understood (3).

Posttranscriptional regulation of gene expression coordinates with transcriptional control of cell fate and activity (4). Transcriptional control directs the course of bone resorption by production of mRNAs encoding proteins that are in charge for enhancement or suppression of bone resorption. These mRNAs often exist for a long time; therefore, even the cessation of production of mRNAs would not immediately alter the direction and/or activities of bone resorption. Regulation of mRNA stability can change the levels of gene expression more quickly and flexibly. Because bone resorption has to be kept in balance with respect to the metabolism of calcium, which is changing daily or hourly, rapid and flexible regulation of bone resorption is particularly important. However, whether posttranscriptional regulation is involved in bone resorption and/or the disease state of major bone diseases, such as osteoporosis, is still elusive. CNOT3 is a component of the CCR4-NOT complex and is involved in the regulation of mRNA stability (5, 6). However, whether *Cnot3* has any function in bone has not been known. Therefore, we examined the role of *Cnot3* in the regulation of bone mass.

Results

***Cnot3* Deficiency Causes Osteopenia.** We examined the effects of *Cnot3* deficiency on bone in adult mice (4-mo-old males). Because complete deficiency of *Cnot3* is embryonically lethal, we used *Cnot3* heterodeficient mice (we refer to these mice as *Cnot3*-deficient or KO mice hereafter). First, we examined bone volume in these mice using microcomputed tomography (micro-CT). Three-dimensional micro-CT analysis indicated that *Cnot3* deficiency caused a sparse osteoporotic pattern in the trabecular bone compared with control (Fig. 1 *A* vs. *B*; WT, WT control mice; KO, *Cnot3*-deficient mice). Quantification of trabecular bone mass indicated that *Cnot3* deficiency suppressed the levels of the bone volume per tissue volume (BV/TV) compared with that in WT mice (Fig. 1*C*). Further structural element analysis of micro-CT examination indicated that *Cnot3* deficiency suppressed the levels of the trabecular number (Fig. 1*D*). Conversely, *Cnot3* deficiency increased the levels of trabecular separation (Fig. 1*E*) and trabecular spacing (Fig. 1*F*). Trabecular

Significance

Osteoporosis is a highly prevalent disease affecting nearly 20 million people in the United States and is life-threatening in elderly patients. However, underlying pathophysiology regarding the posttranscriptional control of bone resorption is incompletely understood. CNOT3 is a molecule involved in mRNA stability in yeast to mammalian cells, but its role in bone regulation is not known. We discovered that *Cnot3* deficiency specifically enhances receptor activator of NF-κB (RANK) mRNA stability and leads to osteopenia in healthy young adult animals. Moreover, *Cnot3* levels are reduced in ageing-induced osteoporosis, and *Cnot3* deficiency further exacerbates such osteoporosis significantly. As a mechanism, *Cnot3* binds to RANK mRNA and its 3′-UTR renders *Cnot3*-dependent instability to the reporter gene. Our results reveal *Cnot3* regulation in aging-induced osteoporosis.

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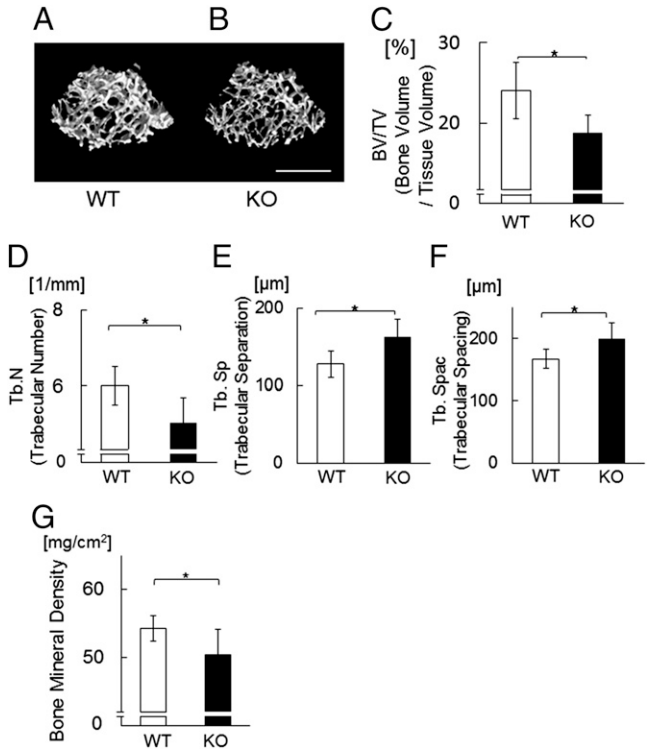

Fig. 1. *Cnot3* deficiency suppresses bone mass. Three-dimensional micro-CT images of distal femora of WT (*A*) and *Cnot3*-deficient (KO; *B*) mice (young adults, 14- to 16-wk-old). (Scale bar, 1 mm.) The trabecular BV/TV (*C*), trabecular number (Tb.N; *D*), trabecular separation (Tb.Sp; *E*), and trabecular spacing (Tb.Spac; *F*) in distal femora were quantified by 3D micro-CT. (*G*) BMD levels of whole femurs of 14- to 16-wk-old WT and *Cnot3*-deficient mice were determined by dual-energy X-ray absorptiometry (n = 5–7 for each group). All data are expressed as mean ± SD. *$P < 0.05$.

thickness was slightly reduced, but the difference was not statistically significant (see Fig. S4*A*). We further determined bone mineral density (BMD) based on dual-energy X-ray absorptiometry. *Cnot3* deficiency suppressed BMD compared with that in WT mice (Fig. 1*G*). These observations indicate that *Cnot3* deficiency suppresses bone mass levels.

***Cnot3* Deficiency Enhances Bone Resorption.** *Cnot3* deficiency suppresses bone mass levels. This could be due to the alteration in bone resorption, the alteration in bone formation, or both. To elucidate this point, we examined the effects of *Cnot3* deficiency on bone resorption. Osteoclasts were examined in the histological sections of bone [Fig. 2 *A* and *B*, cells positive for tartrate-resistant acid phosphatase (TRAP) are indicated by red staining]. *Cnot3* deficiency increased the number of osteoclasts per bone surface (Fig. 2*C*). Furthermore, *Cnot3* deficiency also enhanced the levels of osteoclast surface per bone surface (Oc.S/BS; Fig. 2*D*). These observations indicate that *Cnot3* deficiency increases bone resorption. Therefore, this increase in osteoclastic activity (both number and surface) contributes to the development of osteopenia in *Cnot3*-deficient mice.

***Cnot3* Deficiency Elevates the Levels of Bone Formation.** With respect to the other arm of bone metabolism that may contribute to the induction of osteopenia by *Cnot3* deficiency, we examined whether the decrease in bone mass is influenced by the effects of *Cnot3* deficiency on bone formation activity. To address this aspect, we conducted dynamic bone histomorphometry by injecting calcein to evaluate bone formation. *Cnot3* deficiency increased the bone formation rate (BFR; Fig. 2*E*). *Cnot3* deficiency also increased the mineralizing surface per bone surface (MS/BS), which roughly represents the number of active osteoblasts per bone surface (Fig. 2*F*). These observations indicate that *Cnot3* deficiency causes a high-turnover type bone metabolism that leads to the reduction of bone mass and results in osteopenia.

***Cnot3* Deficiency Increases Osteoclastic Development in Bone Marrow Cells.** Because histomorphometric analyses indicated that *Cnot3* deficiency increases osteoclast number and osteoclast surface in vivo, we addressed whether *Cnot3* regulates osteoclastic development through receptor activator of NF-κB ligand (RANKL)/receptor activator of NF-κB (RANK) signaling. To examine this point, bone marrow cells were prepared from WT and *Cnot3*-deficient mice. These cells were subjected to in vitro osteoclastogenesis by stimulation with RANKL (Fig. 3 *A* and *B*; TRAP is shown as red staining). *Cnot3* deficiency increased the number of TRAP-positive multinucleated cells developed in culture upon the treatment with RANKL (Fig. 3*C*). These observations indicate that *Cnot3* deficiency increases RANKL effects on bone marrow cells, which are induced to differentiate into osteoclasts. Also, these data indicate that the effects of *Cnot3* deficiency are cell-autonomous because they are observed within the bone marrow cells.

In contrast to osteoclastogenesis, *Cnot3* deficiency did not significantly affect osteoblastic mineralized nodule formation in the cultures of bone marrow cells (Fig. S1*A*). Further investigation on the role of *Cnot3* in the transcription of the bone morphogenetic protein (BMP) response element-luciferase

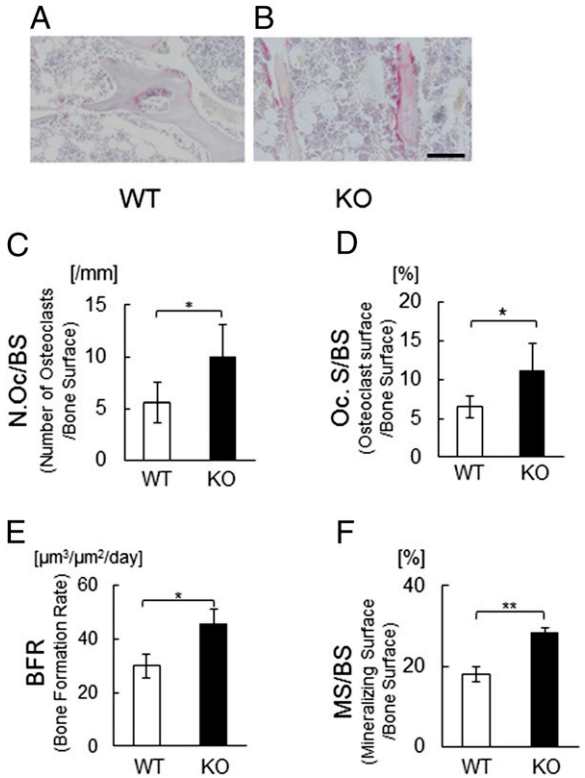

Fig. 2. *Cnot3* deficiency causes high-turnover type osteopenia. TRAP staining of the decalcified sections of the proximal tibiae in WT (*A*) and *Cnot3*-deficient (KO; *B*) mice is shown. (Scale bar, 100 μm.) The number of osteoclasts per bone surface (N.Oc/BS; *C*) and Oc.S/BS (*D*) were quantified based on bone histomorphometric analysis (n = 6–7). Bone formation parameters, including BFR (*E*) and MS/BS (*F*), were determined based on histomorphometric analysis of femora in WT and *Cnot3*-deficient mice (n = 3–4). All data are expressed as mean ± SD. *$P < 0.05$; **$P < 0.01$.

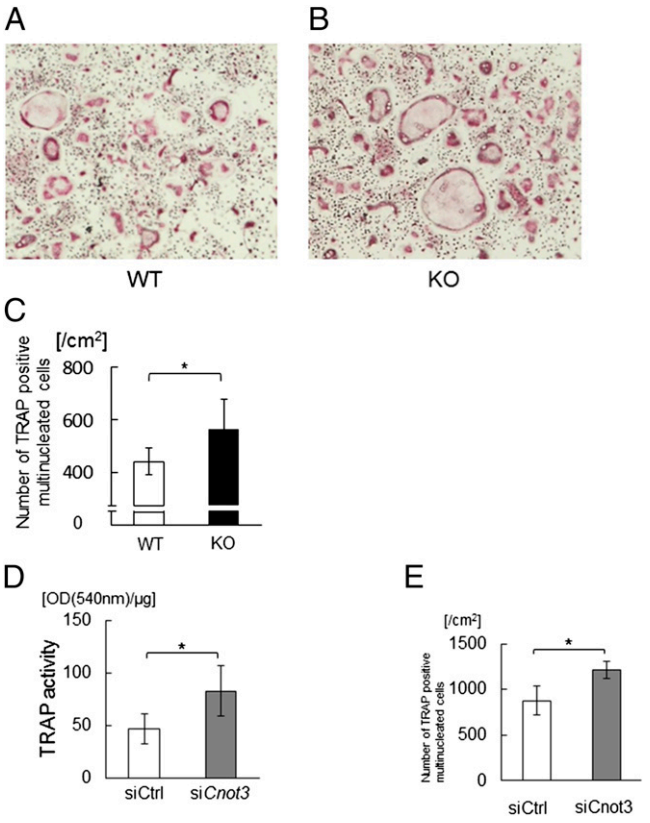

Fig. 3. *Cnot3* deficiency increases osteoclast development cell-autonomously. Osteoclast differentiation in the cultures of the bone marrow cells obtained from WT (*A*) and *Cnot3*-deficient (KO; *B*) mice. (Scale bar, 500 μm.) (*C*) Numbers of TRAP-positive multinuclear cells were counted ($n = 6$ for each group). TRAP activity (*D*) and TRAP-positive multinucleated cell number (*E*) in the cultures of RAW264.7 cells transfected with siRNA for either control (siCtrl) or *Cnot3* (siCnot3) ($n = 5$ for each group) are shown. All data are expressed as mean ± SD. *$P < 0.05$.

reporter did not reveal any effects of *Cnot3* (Fig. S1*B*). Down-regulation of *Cnot3* did not affect osteoblastic proliferation (Fig. S1*C*) or differentiation upon treatment with BMP (Fig. S1*D*) or osteogenic medium (Fig. S1*E*). These data indicate that *Cnot3* suppresses RANKL-induced development of osteoclasts cell-autonomously, although it does not play a major role in bone formation by osteoblasts.

***Cnot3* Knockdown Promotes Osteoclast Development in Precursor Cells.** Because *Cnot3* deficiency enhances RANKL-induced osteoclastogenesis in bone marrow cells, we further examined whether *Cnot3* knockdown affects RANKL effects on osteoclastic differentiation of their precursor cell line, RAW264.7. To do this, siRNA against *Cnot3* or control siRNA was transfected to knock down *Cnot3* in RAW264.7 cells. These cells were then treated with RANKL, and we examined the effects of *Cnot3* deficiency on the expression of the osteoclastic differentiation marker. In control cells, RANKL treatment increased TRAP activity (Fig. 3*D*). In contrast, *Cnot3* knockdown superenhanced the RANKL effects on the RAW264.7 cell differentiation into osteoclasts, as monitored by the levels of TRAP activity (Fig. 3*D*). In addition to such biochemical features, morphological examination on the osteoclastogenesis indicated that *Cnot3* knockdown in RAW264.7 cells increased the number of RANKL-induced TRAP-positive multinucleated cells (Fig. 3*E*). These data again indicate that *Cnot3* deficiency enhances RANKL effects on osteoclastic differentiation of the precursor cell line. Thus, *Cnot3* deficiency enhances RANKL-induced osteoclastogenesis in primary cultures of bone marrow cells and osteoclast precursor cell lines in a cell-autonomous manner.

***Cnot3* Localizes in the Cytoplasm of Osteoclast Precursor Cells.** To address how *Cnot3* regulates bone mass through the control of RANKL-induced differentiation of osteoclasts cell-autonomously, we examined the intracellular localization of immunofluorescent signals of *Cnot3* in the precursor cells for osteoclasts. In RAW264.7 cells, *Cnot3* was localized within the cytoplasm before induction of differentiation (Fig. 4 *A–C*; *Cnot3*, green; DAPI, blue; several mononuclear cells are shown). After treatment of the cells with RANKL for 3 d, these cells differentiated into osteoclasts and fused to exhibit multinucleation (Fig. 4 *D–F*; *Cnot3* and DAPI in a single multinucleated osteoclast). After osteoclastic differentiation, *Cnot3* remained localized within the cytoplasm (Fig. 4 *D* and *F*; *Cnot3*, green; DAPI, blue). These observations reveal that *Cnot3* protein is expressed in the cells of osteoclast lineages and that it localizes, at least mainly, within the cytoplasm during osteoclastogenesis.

***Cnot3* Deficiency Enhances RANK Expression.** To explore the target of *Cnot3*, we examined the expression of mRNAs in the bone of *Cnot3*-deficient mice in comparison to WT mice. Because *Cnot3* deficiency enhanced RANKL effects in osteoclasts, we examined RANKL expression in bone. However, we found that *Cnot3* deficiency did not increase RANKL expression (Fig. S2*A*). In contrast, *Cnot3* deficiency enhanced the expression of RANK gene compared with WT in vivo (Fig. 4*G*). This mRNA regulation by *Cnot3* deficiency was specific to RANK mRNA, because mRNA levels of Mitf and Opg were not altered (Fig. S2 *B* and *C*). To test whether this effect of *Cnot3* deficiency on the levels of RANK mRNA is cell-autonomous, RANK mRNA levels were examined in RAW264.7 cells that were treated with either siRNA for *Cnot3* or control siRNA. We observed that *Cnot3* knockdown up-regulated RANK mRNA levels in these osteoclast precursor cells (Fig. 4*H*).

We further examined whether the increase in ex vivo osteoclastogenesis in *Cnot3*-deficient cells is due to increased progenitor number. *Cnot3* deficiency did not affect the levels of osteoclast progenitor cells (c-kit$^-$, CD11b$^+$, and c-fms$^+$ cells) estimated based on fluorescence-activated cell sorting (Fig. S3*A*, WT; Fig. S3*B*, *Cnot3* KO; and Fig. S3*C*, quantification). As another angle to estimate the levels of osteoclast progenitor cells, we examined the number of bone marrow-derived macrophages (BMMs) based on F4/80 antibody staining. *Cnot3* deficiency did not increase the number of F4/80-positive cells compared with WT (Fig. S3*D*). We also examined whether the increase in ex vivo osteoclastogenesis in *Cnot3*-deficient cells is due to increased RANKL expression. We found that *Cnot3* deficiency did increase RANK mRNA expression in the bone marrow cells (Fig. S3*E*) but that it did not increase RANKL, Opg, Mitf, or c-Fos mRNA expression in the cells used for osteoclastogenesis experiments in cultures (Fig. S3 *F–I*). These results indicate that the increase in ex vivo osteoclastogenesis in *Cnot3*-deficient cells is not due to increased progenitor number or to increased RANKL expression in these cells.

The *Cnot3* deficiency-induced increase in RANK mRNA expression could be due to an increase in the transcriptional activity of RANK gene, an increase in mRNA stability, or both. Because RANK mRNA expression was reported to be transcriptionally enhanced by a Wnt5a and Ror2 system (7), we examined the expression of these genes in bone. However, the levels of Wnt5a and Ror2 mRNAs were not altered in the bone of *Cnot3*-deficient mice (Fig. S2 *D* and *E*). Moreover, the localization of *Cnot3* was cytoplasmic and was hardly detectable within the nuclei of the RAW264.7 cells throughout the differentiation of these cells into osteoclasts (Fig. 4 *A–F*). Furthermore, we

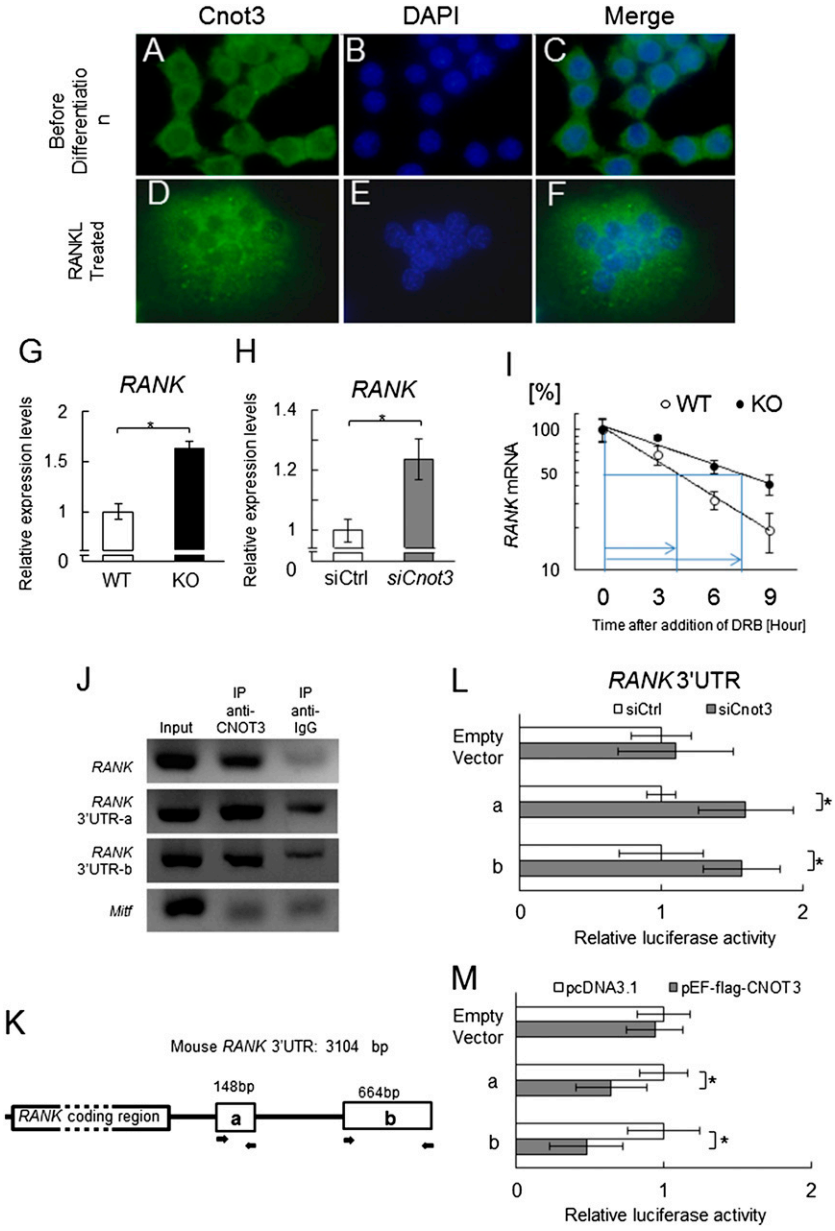

Fig. 4. *Cnot3* deficiency stabilizes RANK mRNA. Images of RAW264.7 cells as osteoclast precursor cells (before differentiation on day 1; *A*–*C*) and those after treatment with RANKL (day 4; *D*–*F*). (Scale bar, 50 μm.) Staining for DAPI (blue; *B* and *E*) and *Cnot3* (green; *A* and *D*) was examined by fluorescence microscopy during osteoclast development. RAW264.7 cells were cultured in the presence of RANKL (50 ng/mL) for 4 d. (*G*) Real-time PCR analysis of RANK mRNA in the femora in WT and *Cnot3*-deficient (KO) mice. (*H*) RAW264.7 cells were transfected with siRNA for *Cnot3* or control siRNA, and RANK mRNA levels were examined ($n = 4$ for each group). Gapdh mRNA levels were used as a control. (*I*) Time course analysis of RANK mRNA decay after treatment with a transcription inhibitor, 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB), in the cultures of BMMs obtained from *Cnot3*-deficient or WT mice. Hypoxanthine-guanine phosphoribosyltransferase mRNA levels were used as a control ($n = 4$ for each point). (*J*) RNA immunoprecipitation followed by RT-PCR analysis of *Cnot3* and RANK mRNA. Cell lysates from RAW264.7 cells were subjected to immunoprecipitation using anti-CNOT3 and control IgG antibodies. The immunoprecipitates were analyzed based on RT-PCR. (*K*) Map of RANK mRNA 3′-UTR. A luciferase assay was conducted using reporter plasmids containing each of the conserved fragments (a and b, as shown in *K*) of RANK 3′-UTR and siRNA (*L*) or an expression vector (*M*) for *Cnot3* in the cultures of RAW264.7 cells ($n = 6$ for each group). All data are expressed as mean ± SD. $^*P < 0.05$.

examined the effects of *Cnot3* knockdown on the transcriptional activities of Tnfrsf11a (RANK) promoter. *Cnot3* knockdown did not affect the transcriptional activities of 2.0-kb Tnfrsf11a promoter (Fig. S2*F*). These observations suggest that *Cnot3* deficiency enhances RANK mRNA levels without affecting transcriptional events of the Tnfrsf11a promoter.

Therefore, we investigated whether *Cnot3* regulates RANK mRNA stability. To address this point, we examined the stability of the RANK mRNA in BMMs. The mRNA stability levels were quantified after treatment with 5,6-dichloro-1-β-ribofuranosylbenzimidazole, which blocks transcription. In the BMMs obtained from WT mice, RANK mRNA decayed as a function of time (Fig. 4*I*, open circles). In contrast, *Cnot3* deficiency in BMMs stabilized the RANK mRNA about twofold to increase its expression (Fig. 4*I*, closed circles). This effect of *Cnot3* deficiency was specific to RANK mRNA, because *Cnot3* deficiency did not affect Mitf mRNA stability (Fig. S2*G*). These data indicated that *Cnot3* deficiency enhances the stability of the mRNA of RANK, a receptor for RANKL and the key stimulator of osteoclastic differentiation.

***Cnot3* Binds to RANK mRNA.** Because *Cnot3* deficiency enhances mRNA stability, the next question was how *Cnot3* is involved in the regulation of RANK mRNA stability. To examine the possibility of interaction between *Cnot3* and RANK mRNA, anti-CNOT3 (human) antibody that cross-reacts with *Cnot3* (mouse) was used to immunoprecipitate RNA in RAW264.7 cells. We asked whether RANK mRNA is associated with *Cnot3*. Analysis of the precipitates by RT-PCR, obtained by using anti-CNOT3 antibody, indicated that this antibody pulled down RANK mRNA (Fig. 4*J*, RANK). In contrast, anti-CNOT3 antibody did not immunoprecipitate Mitf mRNA more than the background (Fig. 4*J*). As a control, anti-IgG antibody did not pull down RANK mRNA (Fig. 4*J*). Thus, *Cnot3* associates with RANK mRNA.

We analyzed the sequences in the 3-kb 3′-UTR of RANK mRNA and found that there were two regions whose sequences were closely reminiscent between human and mouse RANK

genes. We designated these two 148-bp and 664-bp regions as region a and region b, respectively (Fig. 4*K*). Homologies between human and mouse *Cnot3* within regions a and b are 68% and 69%, respectively. Both of these two conserved 3′-UTR regions were linked to luciferase reporter and then transfected into RAW 264.7 cells. When these cells were treated with siRNA for *Cnot3*, such knockdown increased luciferase activity (Fig. 4*L*), indicating that *Cnot3* knockdown stabilized luciferase via the 3′-UTR of RANK mRNA. Conversely, *Cnot3* overexpression suppressed the levels of luciferase reporter (Fig. 4*M*), indicating that *Cnot3* destabilized the reporter via the RANK 3′-UTR fragments. These RANK 3′-UTR fragments (a and b) were also detected within the precipitates pulled down by anti-CNOT3 antibody (Fig. 4*J*). These observations reveal that *Cnot3* associates with RANK mRNA and regulates its stability via the 3′-UTR.

***Cnot3* Deficiency Exacerbates Osteoporosis Induced by Aging.** Aging causes severe osteoporosis in humans as well as other animals, including mice. If *Cnot3* is regulating bone mass levels, this raises a question as to whether the endogenous expression levels of *Cnot3* may change with aging. To address this point, *Cnot3* expression in bone of aged WT mice at 2 y of age was compared with that of young WT adult mice at 4 mo of age. Strikingly, the data indicated that aging reduced *Cnot3* expression levels in bone about threefold in aged mice compared with young mice (Fig. 5*A*). To examine the pathophysiological relevance of our observations on *Cnot3* further, we examined the effects of *Cnot3* deficiency on this osteoporosis model in the aged mice (2 y of age). We asked whether *Cnot3* is still an endogenous suppressor for bone resorption even at such a nadir of bone mass in these aged mice and even when the levels of *Cnot3* are threefold less compared with those of young adult mice (Fig. 5*A*). In these mice, micro-CT scans indicated that aging-induced osteoporosis was represented by an increase in the sparsity of trabecular bone in aged WT mice compared with young adult WT mice (Fig. 5*B*, WT vs. Fig. 1*A*, WT). Baseline levels of 3D BV/TV in the WT aged mice was about 8% (Fig. 5*D*, WT, open column), and this was about one-third compared with the levels in young adult mice (about 24%; Fig. 1*C*, WT, open column). Surprisingly, in these aged mice, *Cnot3* deficiency even further exacerbated the sparsity of trabecular bone (Fig. 5*C*, KO). Quantification of the 3D trabecular bone structure in aged osteoporotic mice indicated that even in the background of such low levels of bone mass in WT aged osteoporotic mice, *Cnot3* deficiency further exacerbated osteoporosis, because bone volume was further reduced by about 50% (Fig. 5*D*, closed column; BV/TV was about 4%; $P < 0.01$). Elemental analysis of the 3D micro-CT–based structure indicated that aging-induced osteoporosis resulted in a threefold reduction in the baseline levels of trabecular number compared with those in young adult mice [six per millimeter (Fig. 1*D*, open column) vs. two per millimeter (Fig. 5*E*, open column)]. Even so, *Cnot3* deficiency further suppressed trabecular bone number by about 40% [1.2 per millimeter (Fig. 5*E*, closed column); $P < 0.01$]. Baseline trabecular thickness was similar between aged and young adult mice. However, *Cnot3* deficiency reduced the thickness of trabecular bone in aged mice (Fig. 5*F*). Furthermore, *Cnot3* deficiency increased the levels of the trabecular separation (Fig. 5*G*) and trabecular spacing (Fig. 5*H*). With respect to cortical bone thickness, although *Cnot3* deficiency slightly reduced the level without statistical significance in young adult mice (Fig. S4*B*), in aged osteoporotic mice, *Cnot3* deficiency suppressed the cortical bone thickness (Fig. S4*C*). Thus, *Cnot3* deficiency exacerbated aging-induced bone loss. These data indicate that deficiency of CNOT3 exacerbates deterioration of bone mass even in aged osteoporotic mice at their nadir of bone volume. Thus, CNOT3 is a critical molecule endogenously present in the aged osteoporotic mice.

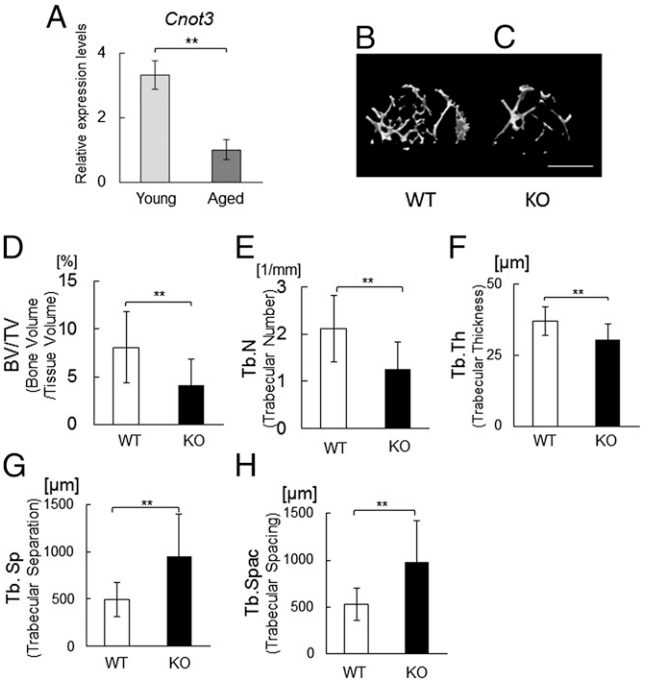

Fig. 5. *Cnot3* deficiency exacerbates aging-induced osteoporosis. (*A*) Real-time PCR analysis of *Cnot3* in whole humeri of young (14- to 16-wk-old) and aged (2-y-old) WT mice. Three-dimensional micro-CT images of distal femora of 2-y-old WT (*B*) and *Cnot3*-deficient (KO; *C*) mice. (Scale bar, 1 mm.) The trabecular BV/TV (*D*), Tb.N (*E*), trabecular bone thickness (Tb.Th; *F*), Tb.Sp (*G*), and Tb.Spac (*H*) of distal femora in 2-y-old WT and *Cnot3*-deficient mice (n = 9–10) were quantified based on 3D micro-CT analysis. All data are expressed as mean ± SD. $^{**}P < 0.01$.

***Cnot3* Deficiency Exacerbates Bone Resorption in the Aged Osteoporotic Mice.** We further examined the pathophysiological relevance of the effects of *Cnot3* deficiency on osteoclasts based on histomorphometry in the osteoporosis model of aged mice in vivo (Fig. S5 *A* and *B*). *Cnot3* deficiency increased osteoclast number per bone surface in the osteoporotic mice almost twofold compared with WT osteoporotic mice (Fig. S5*C*). In these osteoporotic mice, *Cnot3* deficiency increased Oc.S/BS, representing the area covered by osteoclasts, almost threefold (Fig. S5*D*). At the cellular level, we found that *Cnot3* deficiency enhanced twofold the number of TRAP-positive multinucleated cells developed from BMMs in vitro (Fig. S5 *E*–*G*). These observations indicated that *Cnot3* deficiency enhances osteoclastogenesis cell-autonomously even in aged osteoporotic mice.

With regard to bone formation, *Cnot3* deficiency in aged osteoporotic mice slightly enhanced MS/BS (Fig. S5*H*), but not for BFR and mineral apposition rate (Fig. S6 *A* and *B*). In vitro experiments also showed that *Cnot3* deficiency did not affect mineralized nodule formation in the culture of the bone marrow cells obtained from these aged mice (Fig. S6*C*). These observations indicate that *Cnot3* deficiency exacerbates osteoporosis by the enhancement of bone resorption as a major arm in association with an elevation in MS/BS, revealing a further increase in the high-turnover type of bone loss even in the osteoporotic state in the aged mice. Therefore, *Cnot3* deficiency exacerbates osteoporosis even at the nadir of bone mass via further enhancement of bone resorption.

Discussion

We have identified *Cnot3* as a critical regulator of osteoporosis pathophysiology. Because *Cnot3*-null mice are lethal, we examined the effects of *Cnot3* deficiency in heterozygous mice. The deletion of even a single allele of *Cnot3* reduces bone mass in

young adult mice. These mice lacking a single allele of *Cnot3* survive until 2 y of age, which is a normal aging range in these animals. Importantly, although baseline levels of bone mass are significantly reduced in the WT aged osteoporotic mice down to one-third of those in WT young adult mice, single-allele *Cnot3* deletion in these aged osteoporotic mice exacerbated the disease state further down to about 50% that of the WT osteoporotic mice. Therefore, even in the osteoporotic state of the aged WT mice, a single allele of *Cnot3* is playing a protective role in the maintenance of bone mass.

We investigated metabolic bases for the bone loss due to *Cnot3* deficiency. We found that *Cnot3* deficiency increased osteoclast number as well as osteoclast surface in vivo even in the "aged" osteoporotic mice. In vitro experiments indicated that bone marrow cells obtained from *Cnot3*-deficient young adult mice showed an increase in osteoclast development. Importantly, *Cnot3* deficiency in aged mice also showed an increase in osteoclast development cell-autonomously in vitro. Furthermore, RAW264.7 cells, a cell line used as osteoclast progenitors, express *Cnot3*, and *Cnot3* knockdown in these cells cell-autonomously increased the levels of osteoclast development. Thus, *Cnot3* is a cell-autonomous suppressor of osteoclastic differentiation and bone resorption.

In contrast to osteoclasts, although osteoblasts expressed *Cnot3*, *Cnot3* deficiency does not affect the expression of genes encoding proteins related to osteoblastic phenotypes, such as collagen, alkaline phosphatase, Runx2, and Osx, in vivo in bone. Furthermore, *Cnot3* deficiency does not affect in vitro mineralization assay based on calcified nodule formation in isolated bone marrow cells. *Cnot3* down-regulation in osteoblasts did not affect the activity of BMP response element-luciferase reporter. Therefore, *Cnot3* deficiency-induced osteoporosis is due to the enhancement of osteoclasts and bone resorption but is at least not largely dependent on its regulation of osteoblasts or bone formation. These observations would correspond to the effects of *Cnot3* deficiency on a larger increase in the activity of the bone resorption than on an increase in the levels of the osteoblastic activity leading to high-turnover osteoporosis. High turnover alone should not lead to bone loss unless penetration of trabecular elements prevents the usual coupling of bone formation to bone resorption because of the lack of a surface upon which osteoblasts can deposit new bone. Thus, such penetration of trabecular elements and the resulting lack of surface may have occurred in the *Cnot3*-deficient mice.

We examined the bases for the effects of *Cnot3* deficiency on the enhancement of bone resorption and osteoclastic activity. *Cnot3* deficiency enhanced RANKL effects on osteoclastogenesis in BMMs as well as in RAW246.7 cells. However, *Cnot3* deficiency does not affect expression of RANKL in bone or bone marrow cells per se. In contrast, *Cnot3* deficiency enhanced RANK gene expression in bone or bone marrow cells. The presence of *Cnot3* as a factor that regulates RANK expression not only in young adult mice but also in highly osteoporotic aged mice indicates that *Cnot3* is an intrinsic suppressor of bone loss even in the aged osteoporotic mice. Importantly, *Cnot3* expression was reduced threefold in WT aged osteoporotic mice compared with WT young adult mice. Bone resorption is controlled in several ways. RANKL/RANK, Nfatc, OPG, macrophage colony-stimulating factor, the Wnt5a-Ror2 system, sphingosin-1–phosphate, ephrins, semaphorins, estrogen deficiency, disuse/unloading, and sympathetic tone (1, 7–15) have been described. *Cnot3* is unique because it is a previously undescribed molecule regulating osteoclast development via control of mRNA stability and is endogenously opposing against osteopenia even in the aged osteoporotic stage.

Cnot3 deficiency stabilizes mRNA of RANK in the cells of osteoclast lineage. Because regulation of gene expression is managed largely based on transcription, transcriptional events have been studied in detail in bone homeostasis. In contrast to the transcription side, posttranscriptional regulation has not been well understood in terms of its relevance to the pathophysiology of osteoporosis. Our data indicate that *Cnot3* deficiency stabilizes RANK mRNA and exacerbates aging-induced osteoporosis. Hence, *Cnot3* is playing a role in the prevention of excessive bone resorption. Posttranscriptional regulation of gene expression is a more rapid, fine, and flexible type of tuning compared with robust transcriptional regulation. The bone remodeling is based on continuous turnover of bone, with rapid bone resorption followed by bone formation to fill the preceding resorption site. Thus, bone resorption is the first key and fast event. The rapid control of bone resorption by posttranscriptional control may fit such a nature of speed of the bone turnover, especially with respect to the bone resorption arm. Our observation indicates that *Cnot3* regulates bone mass via controlling the mRNA stability of important gene, RANK.

Cnot3 deficiency results in bone loss due to the high-turnover state of bone metabolism. The possibility of developing drug candidates to increase the expression of the *Cnot3* gene could be of interest with respect to unique treatment for osteoporosis.

In conclusion, we have identified a unique molecule, *Cnot3*, that is involved in regulation of bone mass in aged osteoporotic mice.

Experimental Procedures

Because *Cnot3*-null mice are embryonically lethal, we used heterozygous mice in which one allele of the *Cnot3* locus is deleted, and these mice are referred to as *Cnot3*-deficient or KO mice. *Cnot3*-deficient mice in a C57B6/J background were produced by Morita et al. (6) as reported previously. All experiments were performed according to the guidelines for animal welfare at our institution and were approved by our institutional review board at Tokyo Medical and Dental University.

Other information for the materials and methods are described in *SI Experimental Procedures*.

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