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LETTERS

Vitamin E decreases bone mass by stimulating osteoclast fusion

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Bone homeostasis is maintained by the balance between osteoblastic bone formation and osteoclastic bone resorption[1–3]. Osteoclasts are multinucleated cells that are formed by mononuclear preosteoclast fusion[1,2,4,5]. Fat-soluble vitamins such as vitamin D are pivotal in maintaining skeletal integrity. However, the role of vitamin E in bone remodeling is unknown. Here, we show that mice deficient in α-tocopherol transfer protein (*Ttpa*$^{-/-}$ mice), a mouse model of genetic vitamin E deficiency[6], have high bone mass as a result of a decrease in bone resorption. Cell-based assays indicated that α-tocopherol stimulated osteoclast fusion, independent of its antioxidant capacity, by inducing the expression of dendritic-cell–specific transmembrane protein, an essential molecule for osteoclast fusion, through activation of mitogen-activated protein kinase 14 (p38) and microphthalmia-associated transcription factor, as well as its direct recruitment to the *Tm7sf4* (a gene encoding DC-STAMP) promoter[7–9]. Indeed, the bone abnormality seen in *Ttpa*$^{-/-}$ mice was rescued by a *Tm7sf4* transgene. Moreover, wild-type mice or rats fed an α-tocopherol–supplemented diet, which contains a comparable amount of α-tocopherol to supplements consumed by many people, lost bone mass. These results show that serum vitamin E is a determinant of bone mass through its regulation of osteoclast fusion.

Bone mass is maintained constant from puberty until menopause by a balance between osteoblastic bone formation and osteoclastic bone resorption, a process called bone remodeling[1–3]. Osteoclasts are multinucleated polykaryons that develop from monocyte-lineage hematopoietic precursors through sequential steps: an initial phase of proliferation and a late phase of differentiation and maturation[1,2,10,11]. Hormones and cytokines have pivotal roles in osteoclast development. Specifically, macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are indispensable for the proliferation of preosteoclasts and the differentiation and maturation of osteoclasts, respectively[1–3,12]. Among the fat-soluble vitamins A, D and K are well known for their ability to affect the skeleton[13,14], however, vitamin E was not examined well in the aspect of bone remodeling.

Vitamin E is a lipid-soluble antioxidant that inhibits lipid peroxidation by scavenging reactive oxygen species and is believed to be protective against arteriosclerotic change and the aging process[15]. Indeed, vitamin E is one of the most popular supplements in the United States; more than 10% of adults in the United States currently take vitamin E daily[16]. Vitamin E, which is a mixture of tocopherols and tocotrienols, is absorbed from food and is transported to the liver, where α-tocopherol transfer protein (α-TTP) mediates the selective transfer of α-tocopherol into lipoproteins[6]. Accordingly, α-tocopherol is the most predominant isoform of vitamin E in the body. Mice deficient in α-TTP (*Ttpa*$^{-/-}$ mice) show ataxia and infertility as a result of reduced serum α-tocopherol concentrations (**Fig. 1a**), which can be rescued by dietary supplementation with α-tocopherol[6,17].

To address the role of vitamin E in bone remodeling, we first studied *Ttpa*$^{-/-}$ mice. The *Ttpa*$^{-/-}$ mice developed a high–bone-mass phenotype in both their vertebrae and long bones as a result of a lower bone resorption compared to wild-type (WT) mice, as evidenced by a lowering of osteoclast surface and deoxypyridinoline, a bone resorption marker (**Fig. 1b–e** and **Supplementary Fig. 1**)[18]. In contrast, the amount of bone formation was unchanged in *Ttpa*$^{-/-}$ mice compared to WT mice. (**Fig. 1d**). This high–bone-mass phenotype was attributed to reduced serum concentrations of vitamin E (**Fig. 1a**) rather than the α-TTP deficiency in the body, as supplementation with α-tocopherol in the diet, which normalized the serum concentrations of α-tocopherol in *Ttpa*$^{-/-}$ mice (**Fig. 1f**), rescued the bone abnormality seen in *Ttpa*$^{-/-}$ mice (**Fig. 1g** and **Supplementary Fig. 2**). In line with this observation, *Ttpa*$^{+/-}$ mice, whose serum concentrations of α-tocopherol are between those of WT and *Ttpa*$^{-/-}$ mice[17], also had an

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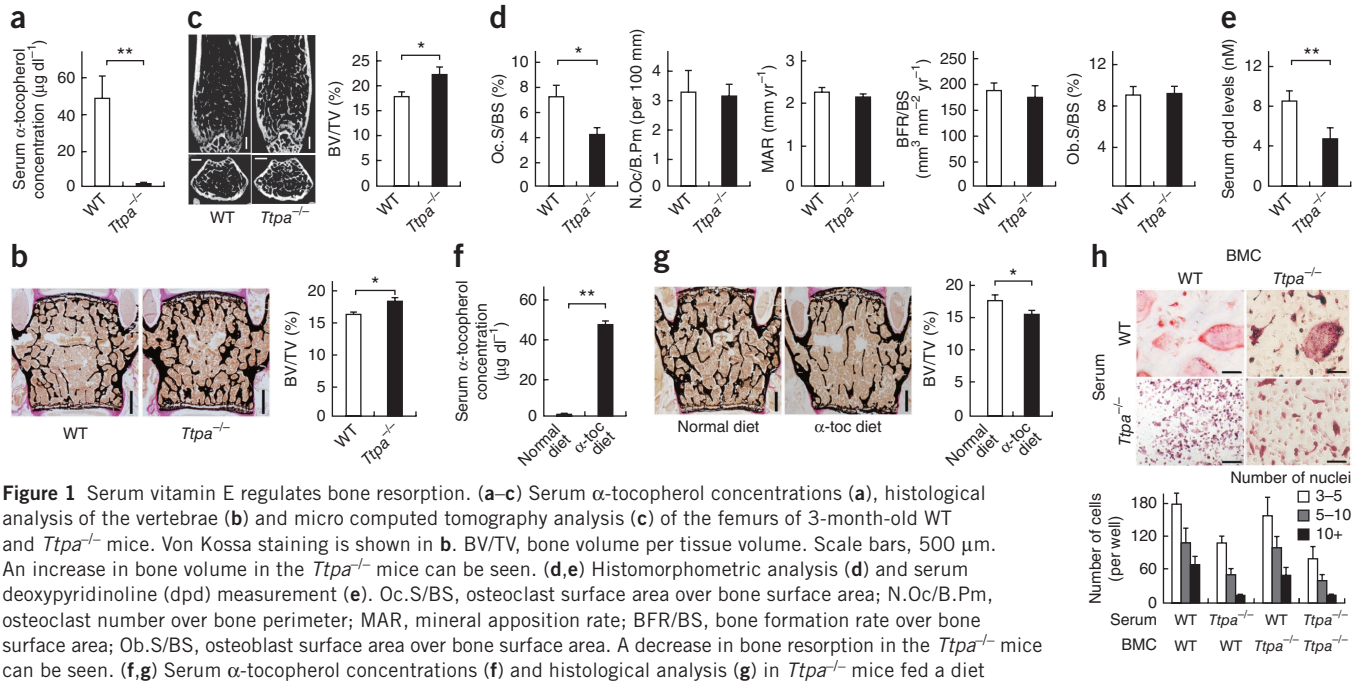

Figure 1 Serum vitamin E regulates bone resorption. (**a**–**c**) Serum α-tocopherol concentrations (**a**), histological analysis of the vertebrae (**b**) and micro computed tomography analysis (**c**) of the femurs of 3-month-old WT and *Ttpa*$^{-/-}$ mice. Von Kossa staining is shown in **b**. BV/TV, bone volume per tissue volume. Scale bars, 500 μm. An increase in bone volume in the *Ttpa*$^{-/-}$ mice can be seen. (**d**,**e**) Histomorphometric analysis (**d**) and serum deoxypyridinoline (dpd) measurement (**e**). Oc.S/BS, osteoclast surface area over bone surface area; N.Oc/B.Pm, osteoclast number over bone perimeter; MAR, mineral apposition rate; BFR/BS, bone formation rate over bone surface area; Ob.S/BS, osteoblast surface area over bone surface area. A decrease in bone resorption in the *Ttpa*$^{-/-}$ mice can be seen. (**f**,**g**) Serum α-tocopherol concentrations (**f**) and histological analysis (**g**) in *Ttpa*$^{-/-}$ mice fed a diet supplemented with α-tocopherol (α-toc diet). Von Kossa staining is shown in **g**. Scale bars, 500 μm. A decrease in bone volume as a result of the α-toc diet can be seen. (**h**) Serum α-tocopherol affects osteoclast differentiation. BMCs from the femurs of WT or *Ttpa*$^{-/-}$ mice were differentiated into osteoclasts in the presence of serum from WT or *Ttpa*$^{-/-}$ mice without addition of FBS. TRAP staining (left) and the number of osteoclasts (right) are shown. Scale bars, 50 μm. A decrease in the number of osteoclasts from WT BMCs with *Ttpa*$^{-/-}$ serum can be seen, whereas *Ttpa*$^{-/-}$ BMCs differentiated into osteoclasts normally with WT serum. $^{*}P < 0.05$, $^{**}P < 0.01$ by Tukey-Kramer testing (**b**) or Student's *t* test (**a**, **c**–**g**). All data are means ± s.e.m.

intermediate bone mass (**Supplementary Fig. 1b**). Moreover, osteoclast development *in vitro* was hampered when WT bone-marrow cells (BMCs) were cultured with the serum from *Ttpa*$^{-/-}$ mice (**Fig. 1h**), and this defect was ameliorated when this serum was supplemented with α-tocopherol or when serum from WT mice was used for the culture (**Fig. 1h** and **Supplementary Fig. 3**). In contrast, *Ttpa*$^{-/-}$ BMCs differentiated into osteoclasts normally when cultured with FBS or serum from WT mice (**Fig. 1h** and **Supplementary Fig. 3**). Thus, serum vitamin E regulates bone mass *in vivo* by affecting bone resorption.

Next, to examine the role of vitamin E in osteoclast development, we treated osteoclasts that were derived from WT BMCs and stimulated by RANKL with α-tocopherol *in vitro*. α-tocopherol stimulated osteoclast differentiation in a dose-dependent manner, as shown by an increase in the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts (**Fig. 2a**), whereas the proliferation of osteoclast precursors and the survival of mature osteoclasts were unchanged by treatment with α-tocopherol (**Fig. 2b**,**c**). Osteoblastic differentiation and proliferation were not altered by α-tocopherol treatment (**Fig. 2d** and **Supplementary Fig. 4**), further indicating that vitamin E affects bone mass through osteoclasts rather than osteoblasts. Notably, α-tocopherol not only increased the generation of TRAP-positive multinucleated osteoclasts but also markedly increased the proportion of larger osteoclasts compared to the total osteoclasts (**Fig. 2e**), which indicated that vitamin E stimulated osteoclast maturation. As a result, α-tocopherol increased bone resorption by inducing the formation of additional mature osteoclasts (**Fig. 2f**).

Notably, α-tocopherol treatment administered only during the osteoclast maturation phase significantly increased osteoclast size and the number of nuclei per osteoclast (**Fig. 2e**), whereas α-tocopherol treatment administered at any other period did not have these effects (**Fig. 2e**), further indicating that vitamin E specifically affects late osteoclast maturation (that is, osteoclast fusion). Moreover, vitamin E also stimulated the generation of foreign-body giant cells, which are developed through macrophage fusion (**Fig. 2g**), further suggesting that vitamin E stimulates cell fusion. Indeed, the sizes of the TRAP-positive osteoclasts were smaller in the *Ttpa*$^{-/-}$ mice, which is in agreement with our *in vitro* observations (**Supplementary Fig. 2**). Taken together, the results suggest that vitamin E stimulates osteoclast fusion.

Because vitamin E is well known as an antioxidant[15], we next studied whether the antioxidant properties of vitamin E were indispensable for its ability to stimulate osteoclast fusion. With the exception of α-tocopherol, none of the isoforms of vitamin E, including α-tocotrienol, which is 100-fold stronger in antioxidant activity than α-tocopherol[15], stimulated osteoclast fusion (**Fig. 2h**). Moreover, except for α-tocopherol, none of the antioxidants tested, including ascorbic acid, which is the primary water-soluble antioxidant[19–22], stimulated osteoclast fusion (**Fig. 2i** and **Supplementary Fig. 5**). In line with these observations, hydrogen peroxide did not affect osteoclast fusion when it was present at a concentration that did not affect cell viability (**Supplementary Fig. 5**). Taken together, these results clearly show that, unlike other vitamin E isoforms and antioxidants, α-tocopherol specifically regulates osteoclast fusion independent of its antioxidant activity.

To address the molecular mechanism of the α-tocopherol–specific ability to stimulate osteoclast fusion, we analyzed the molecular markers of osteoclast differentiation after treatment with α-tocopherol. Among the many genes involved in osteoclast differentiation, only the expression of the differentiation marker genes, such as *Trap* and *Ctsk*[1,2,4,12], was increased, whereas the expression of other genes key for osteoclast differentiation, such as *Nfatc1* (nuclear factor of activated T cells c1)[1,2,4,12], was unchanged (**Fig. 3a**). We focused on

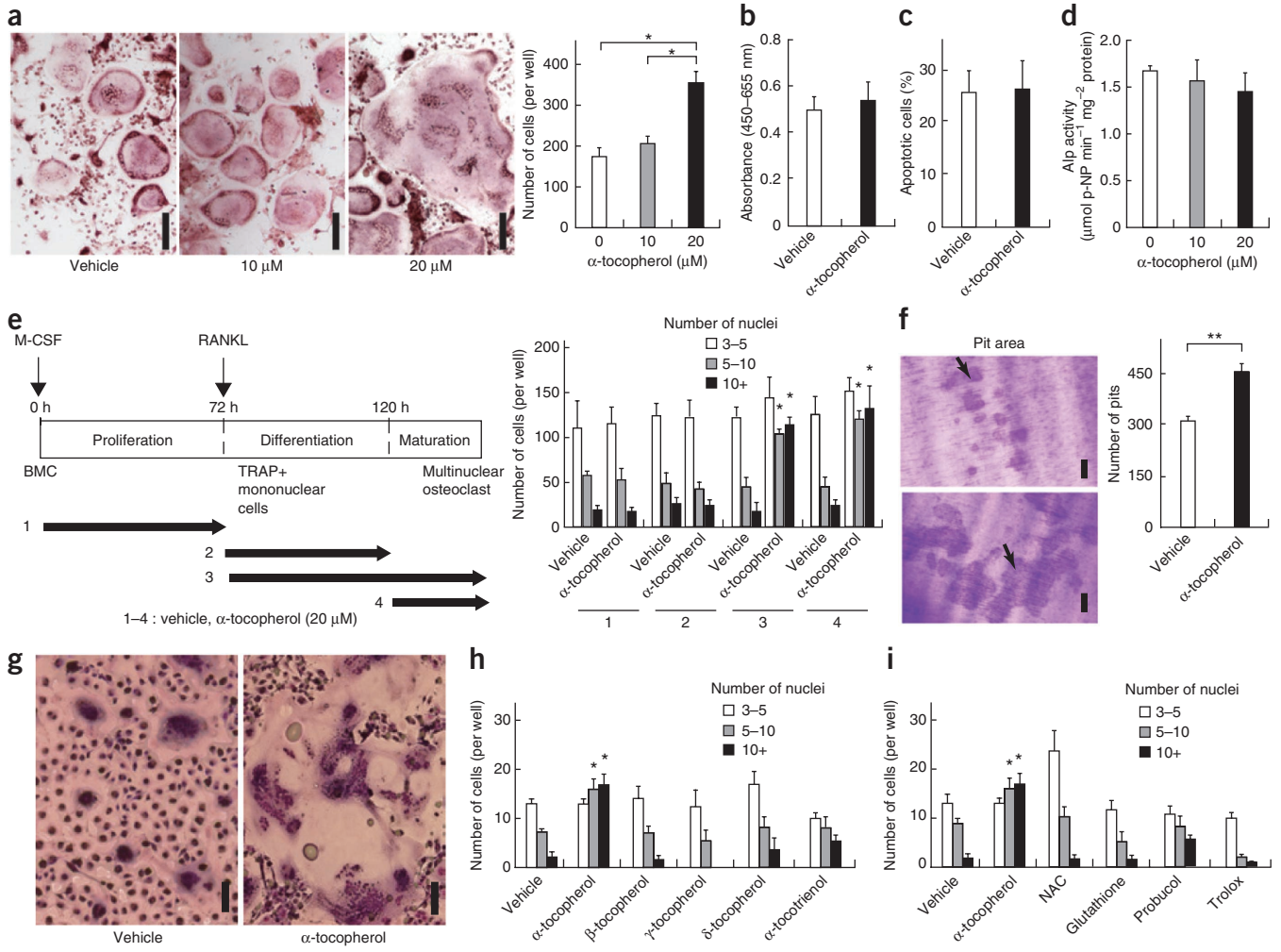

Figure 2 Vitamin E stimulates osteoclast fusion independent of its antioxidant activity. (**a**–**c**) The effect of α-tocopherol on osteoclast differentiation, proliferation and apoptosis. (**a**) BMCs were cultured with M-CSF, RANKL and 10% FBS. TRAP-stained cells (left) and the number of cells with more than three nuclei (right) are shown. An increase in osteoclasts after α-tocopherol treatment can be seen. (**b**) BrdU assay. BMCs were cultured with M-CSF, 10% FBS and α-tocopherol. (**c**) TUNEL assay. BMCs were cultured with M-CSF, RANKL and 10% FBS. (**d**) The effect of α-tocopherol on osteoblasts. An alkaline phosphatase (Alp) assay is shown. p-NP, p-nitrophenol. (**e**) The effect of α-tocopherol on osteoclast fusion. BMCs were cultured with M-CSF and RANKL, and α-tocopherol was added in the proliferation (1), differentiation (2 and 3) or maturation (3 and 4) phase. An increase in the proportion of multinucleated osteoclasts (3 and 4) can be seen. (**f**) The effect of α-tocopherol on bone resorption. A pit formation assay is shown. The eroded area (arrows, left) and the number of pits (right) are shown. BMCs were cultured on dentin with M-CSF and RANKL. α-tocopherol was added later. An increase in bone resorption can be seen. (**g**) Giant-cell progenitors from bone marrow were treated with α-tocopherol. (**h**,**i**) BMCs were cultured with M-CSF and RANKL. Vitamin E isoforms and antioxidants were added later. $*P < 0.05$, $**P < 0.01$ by Tukey-Kramer testing (**a**) or Student's *t* test (**f**,**h**,**i**). Scale bars, 50 μm. All data are means ± s.e.m.

dendritic-cell–specific transmembrane protein (DC-STAMP), a molecule essential for osteoclast fusion, because among the osteoclast-fusion–related genes[4,5], the gene encoding DC-STAMP (*Tmsf4*) was the only one whose expression was induced by α-tocopherol treatment (**Fig. 3a**). Notably, none of the other vitamin E isoforms induced *Tm7sf4* expression (**Fig. 3b**). Conversely, *Tm7sf4* expression was significantly decreased in *Ttpa*$^{-/-}$ mice, whereas expression of other fusion-related genes was unchanged, with the exception of osteoclast stimulatory transmembrane protein, which is another important molecule that is involved in osteoclast fusion (**Fig. 3c**)[23,24]. Next, to clarify the functional role of DC-STAMP in α-tocopherol–induced osteoclast fusion, we performed four sets of gain- and loss-of-function experiments for DC-STAMP. First, overexpression of *Tm7sf4* in RANKL-induced osteoclasts derived from WT mice markedly increased osteoclast fusion even in the absence of α-tocopherol (**Fig. 3d**). Conversely, although knockdown of *Nfatc1* in BMCs derived from WT mice eliminated the appearance of TRAP-positive mononuclear and multinuclear cells, knockdown of *Tm7sf4* only reduced the multinucleation of osteoclasts (that is, osteoclast fusion) (**Fig. 3e** and **Supplementary Fig. 6**), even in the presence of α-tocopherol. Moreover, osteoclast precursors isolated from *Tm7sf4*$^{-/-}$ mice did not differentiate into multinucleated osteoclasts in the presence of α-tocopherol (**Fig. 3f**). Furthermore, *Tm7sf4* transgenic mice[25] rescued the bone abnormality of the *Ttpa*$^{-/-}$ mice, as shown by a decreased bone volume accompanied by an increased bone resorption in the transgenic mice (**Fig. 3g** and **Supplementary Fig. 7**), which is consistent with the hypothesis that vitamin E induces osteoclast fusion through DC-STAMP. Taken together, these results indicate that the induction of DC-STAMP is necessary and sufficient for α-tocopherol to stimulate osteoclast fusion *in vitro* and *in vivo*.

Next, to gain insight into the molecular pathway of the induction of DC-STAMP by α-tocopherol, we examined whether α-tocopherol activates signaling pathways crucial for osteoclast differentiation. Among the pathways we studied, only the p38 pathway was specifically activated by α-tocopherol, as shown by the increase in phosphorylation of p38α, together with mitogen-activated protein kinase kinases 3 and 6 (Mkk3/6), a molecule that is upstream of p38α (**Fig. 4a** and **Supplementary Fig. 8**)[26]. Stimulation of p38α results in the downstream activation of the transcriptional regulator microphthalmia-associated transcription factor (Mitf)[27], an essential molecule for osteoclast maturation and fusion[1], and, indeed, treatment with α-tocopherol increased Mitf phosphorylation (**Fig. 4b**). Moreover, an antibody against Mitf immunoprecipitated the region containing the putative Mitf binding site in the *Tm7sf4* promoter (**Fig. 4c**), showing that Mitf binds to this site *in vivo*. To address the functional role of p38α and Mitf activation in α-tocopherol–induced osteoclast fusion, we knocked down *Tm7sf4* or *Mitf* in osteoclasts derived from WT mice. Knockdown of *Mapk14* (a gene encoding p38) or *Mitf* abolished the stimulatory effect of α-tocopherol on osteoclast fusion (**Fig. 4d**) and *Tm7sf4* induction (**Supplementary Fig. 6**). Conversely, overexpression of *Mapk14* significantly stimulated

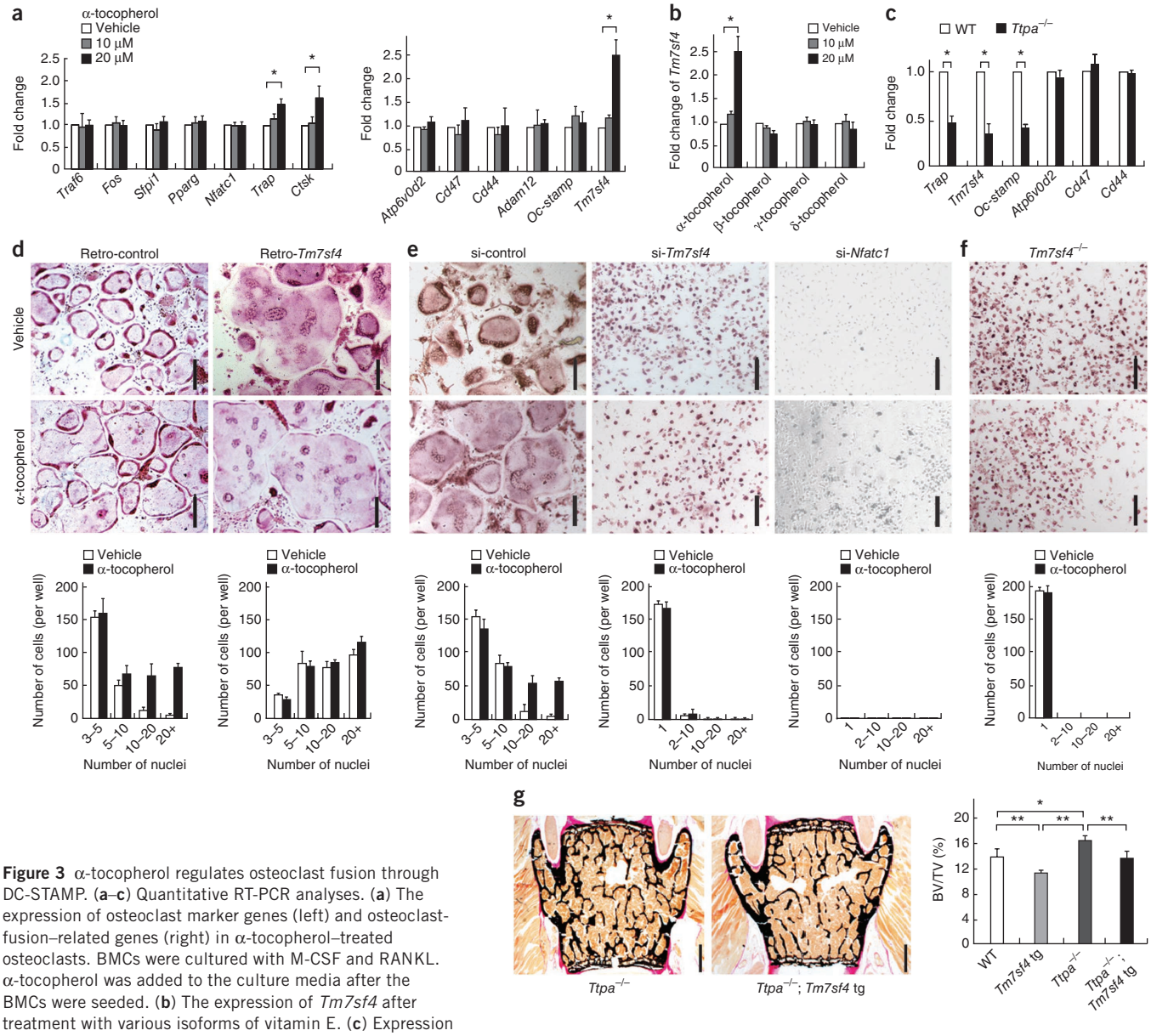

Figure 3 α-tocopherol regulates osteoclast fusion through DC-STAMP. (**a**–**c**) Quantitative RT-PCR analyses. (**a**) The expression of osteoclast marker genes (left) and osteoclast-fusion–related genes (right) in α-tocopherol–treated osteoclasts. BMCs were cultured with M-CSF and RANKL. α-tocopherol was added to the culture media after the BMCs were seeded. (**b**) The expression of *Tm7sf4* after treatment with various isoforms of vitamin E. (**c**) Expression of osteoclast-marker genes in WT and *Ttpa*$^{-/-}$ femurs. A decrease of the expression of *Tm7sf4* among the osteoclast-fusion–related genes can be seen. (**d**–**f**) DC-STAMP is essential for α-tocopherol–induced osteoclast fusion. (**d**) Retroviral overexpression of DC-STAMP. An increase in osteoclast fusion by DC-STAMP in the absence of α-tocopherol can be seen. Retro-*Tm7sf4*, retroviral overexpression of *Tm7sf4*; retro-control, retroviral overexpression of control vector. (**e**,**f**) The effect of α-tocopherol on siRNA-treated BMCs (**e**) and *Tm7sf4*$^{-/-}$ BMCs (**f**). si-control, non-targeting siRNA; si-*Tm7sf4*, siRNA to *Tm7sf4*; si-*Nfatc1*, siRNA to *Nfatc1*. A decrease in osteoclast fusion even in the presence of α-tocopherol can be seen. BMCs from WT (**d**,**e**) and *Tm7sf4*$^{-/-}$ (**f**) mice were cultured with M-CSF and RANKL. α-tocopherol was added to culture media after the BMCs were seeded. Scale bars, 50 μm. (**g**) Histological analysis of the vertebrae from WT, *Tm7sf4* transgenic (*Tm7sf4* tg), *Ttpa*$^{-/-}$ and *Ttpa*$^{-/-}$; *Tm7sf4* tg mice. Scale bars, 500 μm. $^{*}P < 0.05$, $^{**}P < 0.01$ by Tukey-Kramer testing (**a**,**b**,**g**) or Student's *t* test (**c**). All data are means ± s.e.m.

osteoclast fusion in the absence of α-tocopherol (**Fig. 4e**). These results clearly show that α-tocopherol regulates osteoclast fusion through p38α, Mitf and DC-STAMP. Currently, the molecular mechanism by which α-tocopherol induces p38α activation is unknown. A report showing that α-tocopherol succinate, which is a redox-silent analog of α-tocopherol, induces the activation of mitogen-activated protein kinase kinase kinase 5 (Ask-1)[28], which is an upstream protein kinase in the Mkk3/6-p38α pathway, suggests that α-tocopherol may use this same pathway in an antioxidant-independent manner.

Finally, to address the clinical relevance of our observations, we fed WT mice for 8 weeks with a diet containing an amount of α-tocopherol that is comparable to that found in supplements consumed by many people (**Fig. 4f**)[29]. WT mice fed a α-tocopherol–supplemented diet showed a 20% decreased bone mass after 8 weeks, with a concomitant increase in bone resorption and osteoclast size (**Fig. 4g**,**h** and **Supplementary Figs. 2** and **9**). Moreover, WT rats fed the same α-tocopherol–supplemented diet also had a 20% loss of bone mass after 8 weeks (**Fig. 4i** and **Supplementary Fig. 10**), showing that excessive intake of vitamin E is deleterious to maintaining bone mass in rodents. Notably, when WT mice were fed a diet supplemented with δ-tocopherol or antioxidants, we observed no bone loss (**Supplementary Fig. 9**), further indicating that α-tocopherol decreases bone mass independent of its antioxidant activity.

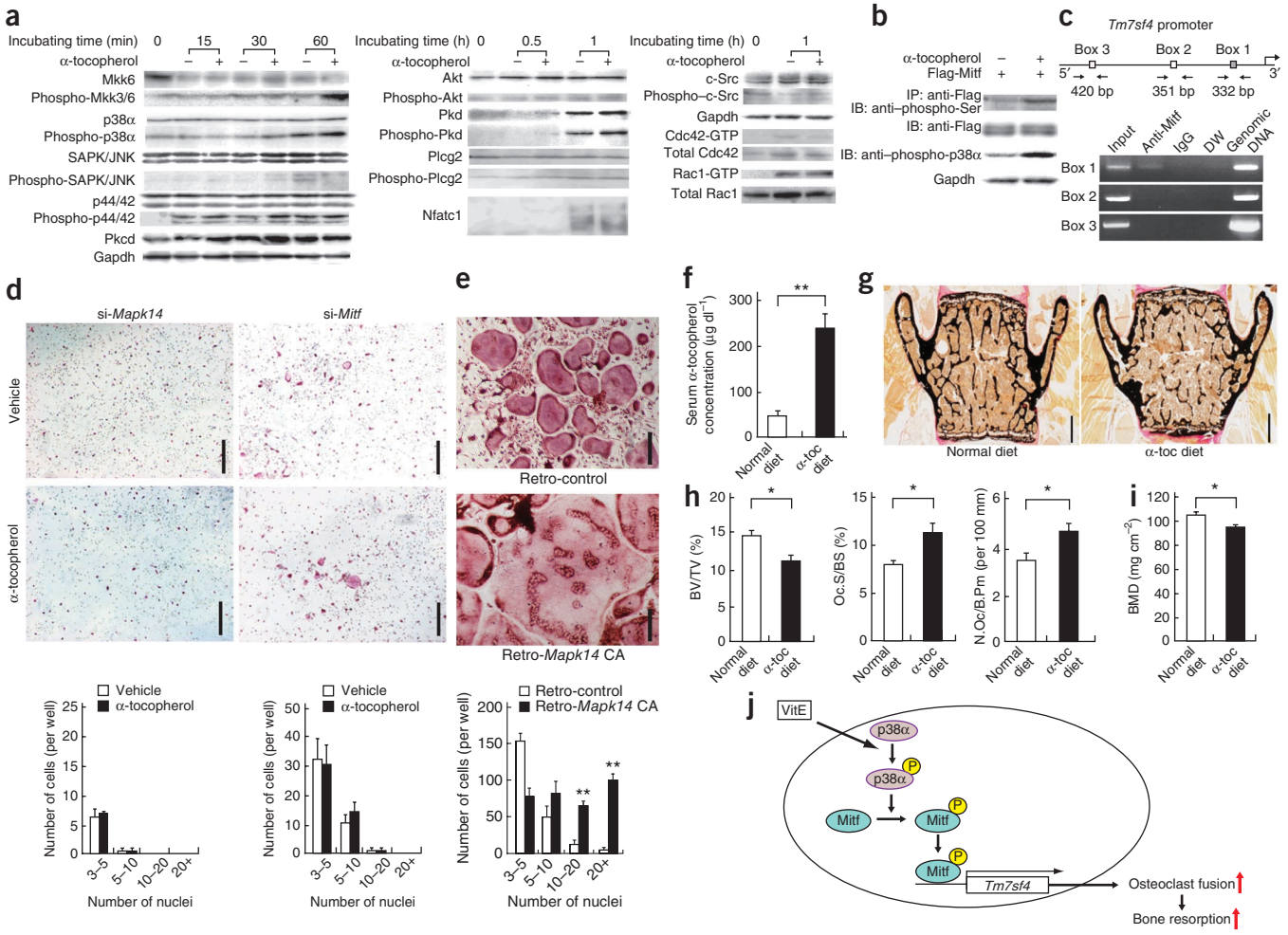

Figure 4 α-tocopherol decreases bone mass through p38α and Mitf. (**a**) Protein analysis of the α-tocopherol–treated osteoclasts. BMCs cultured with M-CSF only (left, middle) or mature osteoclasts that were induced by M-CSF and RANKL (right) were stimulated with α-tocopherol (20 μM) (– or +) and RANKL (middle right). Phospho-, phosphorylated; SAPK/JNK, mitogen-activated protein kinase 9 or mitogen-activated protein kinase 8; p44/42, mitogen-activated protein kinase 3 or cyclin-dependent kinase 20; Pkcd, protein kinase C, δ; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Akt, thymoma viral proto-oncogene 1; Pkd, protein kinase D; Plcg2, phospholipase C, γ 2; Nfatc1, nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1; c-Src, Rous sarcoma oncogene. (**b**) Immunoprecipitation analysis. An increase in the phosphorylation of p38α after treatment with α-tocopherol in p38α-expressing HEK293 cells can be seen. (**c**) Chromatin immunoprecipitation assay. Three potential binding sites (boxes 1–3) in the *Tm7sf4* promoter are shown (above). An antibody against Mitf (anti-Mitf) specifically immunoprecipitated the region containing the box 1 site of the *Tm7sf4* promoter. IgG, immunoglobulin G; DW, distilled water. (**d**,**e**) Gene knockdown (**d**) and retroviral overexpression (**e**) in osteoclasts. BMCs derived from WT mice were cultured with M-CSF and RANKL. α-tocopherol was added later. Scale bars, 50 μm. A decrease in osteoclast fusion even in the presence of α-tocopherol in BMCs treated with siRNA to *Mapk14* (si-*Mapk14*) (encoding p38α) or siRNA to *Mitf* (si-*Mitf*) (**d**) and an increase in osteoclast fusion in the absence of α-tocopherol in constitutively active p38α (*Mapk14*CA)-expressing BMCs (**e**) can be seen. (**f**–**h**) Analyses of WT mice and rats fed an α-tocopherol–supplemented diet. Serum α-tocopherol concentrations in these animals (**f**) and histological (**g**) and histomorphometric analyses (**h**). Scale bars, 500 μm. AA decrease in bone mass and an increase in bone resorption after α-tocopherol treatment can be seen. (**i**) Dual-energy X-ray absorptiometry analysis. A decrease in bone mineral density resulting from a α-tocopherol–supplemented diet. (**j**) The proposed mechanism of vitamin E (VitE)-induced osteoclastic fusion. P, phosphorylated. $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t* test. All data are means ± s.e.m.

In summary, we show that vitamin E stimulates bone resorption and decreases bone mass by inducing osteoclast fusion (**Fig. 4j**). Moreover, we provide evidence that serum vitamin E is a determinant of bone mass. In contrast with our results, several reports have indicated that vitamin E participates in bone anabolism and that 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), which is a vitamin E analog, inhibits inflammation-induced osteoclast differentiation[30–33]. In our experiments, trolox mildly inhibited osteoclast differentiation when we added it during the early osteoclast differentiation period (**Supplementary Fig. 11**), but it did not induce osteoclast fusion when we added it during the maturation phase (**Fig. 2i**). Alternatively, differences in the methodologies or the ages of the animals that were used in the previous compared to the present analyses may explain the discrepant results. In addition, several reports have shown the beneficial effects of α-tocopherol on human bone, which probably occur by reducing oxidative stress[21,34,35]. Nevertheless, most of these studies used a small sample size and were not well controlled. Given the widespread use of vitamin E, and especially α-tocopherol, as a supplement in humans, a larger, controlled study that addresses its effects on human bone is warranted.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

K.F. conducted most of the experiments. M.I., H.O. and C.M. conducted mice analyses. T.F. and S.S. conducted *in vitro* experiments. T.M. provided DC-STAMP–related mice. K.T. and H. Tamai conducted the analyses of vitamin E serum concentrations. T.N.-K. performed western blots. H.A. provided *Ttpa*$^{-/-}$ mice. T.K. and H. Takayanagi conducted gene expression analyses. S.T., K.S., A.O. and H.I. designed the project. S.T. supervised the project and wrote most of the manuscript. S.K. designed the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. We purchased the C57BL/6J mice from the Charles River Laboratory and Oriental Yeast, and we purchased the Wister rats from CLEA Japan. *Ttpa*$^{-/-}$, *Tm7sf4*$^{-/-}$ and *Tm7sf4* transgenic mice were previously described[6,9,25]. We crossed *Ttpa*$^{-/-}$ and *Tm7sf4* transgenic mice to obtain *Ttpa*$^{-/-}$; *Tm7sf4* transgenic mice. We fed the mice a diet supplemented with α-tocopherol (600 mg per kg of food; Sigma) from 4–12 weeks of age, and we fed the rats the same diet from 6–14 weeks of age. The α-tocopherol–supplemented diet was made by CLEA Japan. We analyzed seven or eight mice in each group. We maintained all animals under a 12-h light-dark cycle with *ad libitum* access to food and water. All animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conformed to the relevant guidelines and laws.

Dual X-ray absorptiometry analyses. We measured the bone mineral density of the femurs of all animals by DCS-600 (ALOKA), as previously described[18]. We examined at least eight mice for each group.

Histological and histomorphometric analyses. We injected mice with calcein (25 mg per kg of body weight; Sigma) and stained the undecalcified sections of the lumbar vertebrae using von Kossa and TRAP, as previously described[18,36]. We performed static and dynamic histomorphometric analyses using the OsteoMeasure Analysis System (OsteoMetrics); the Oc.S/BS and N.Oc/B.pm values were calculated for the slices that stained positive for TRAP. We analyzed seven or eight mice in each group.

Cell culture. *In vitro* osteoclast differentiation was accomplished as previously described[18]. Briefly, BMCs of 6–8-week-old mouse femurs were cultured in minimum essential medium α supplemented with FBS in the presence of human M-CSF (10 ng ml^{-1}; R&D Systems) for 3 d and then differentiated into osteoclasts using human RANKL (50 ng ml^{-1}; PeproTech) and M-CSF for 3 d. The osteoclast culture using mouse serum is decribed in detail in the **Supplementary Methods**. The pit formation assay, BrdU assay and TUNEL assay were performed as previously described[18], and the details are described in the **Supplementary Methods**. The foreign-body giant-cell culture was established as previously described[37]. Briefly, BMCs were collected in DMEM (Sigma) with 10% FBS. Cells were stimulated with interleukin-4 (IL-4) (10 ng ml^{-1}) for 48 h, fixed and stained with May-Grünwald-Giemsa for evaluation. The *in vitro* primary osteoblast culture was established as previously described[38,39] (**Supplemntary Methods**). α-tocopherol was added to the culture media at 20 μM or at the indicated concentrations. Other vitamin E isoforms were added at 20 μM, N-acetylcysteine was added at 2 mM, glutathione was added at 5 mM, probucol was added at 5 μM and trolox was added at 200 μM. We cultured all cells in triplicate or quadruplicate wells and repeated each experiment more than three times. Additional details are given in the **Supplementary Methods**.

Transfection and retroviral infection. A total of 20 nM siRNA (Invitrogen) was transfected into BMCs derived from the femurs of WT mice using HiPerFect (QIAGEN). After transfection, cells were cultured using the same methods as those used for the osteoclast differentiation. Complementary DNA (cDNA) of *Tm7sf4* was cloned from osteoclasts using PCR. The constitutively active form of p38α was obtained from Addgene. Retrovirus was produced by the retroviral vector pMXs-IRES-GFP system, which was based on Moloney murine leukemia virus, as previously described[40]. Briefly, we collected the virus produced by packaging cells after 2 d of transfection. BMCs were infected with the retrovirus for 2 d in the presence of M-CSF. After 2 d of infection, cells were stimulated by RANKL to differentiate into osteoclasts. The details are described in the **Supplementary Methods**.

Quantitative RT-PCR analyses. To acquire RNA from mouse bones, we flushed bone marrow out of the femurs with PBS and used the bones as previously described[18]. RNA was extracted using TRIzol (Invitrogen), and reverse transcription was performed for cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). We performed quantitative analyses of gene expression using the Mx3000P Real-Time PCR System (Stratagene). We examined the gene expressions in triplicate or quadruplicate individually and repeated each experiment more than three times.

Protein analyses. We collected cell lysate protein using radioimmunoprecipitation assay buffer with a phosphatase inhibitor cocktail (Nacalai) and the Complete Mini protease inhibitor cocktail (Roche). To detect RAS-related C3 botulinum substrate 1 (Rac1) and cell division cycle 42 (Cdc42) expression, we used Active GTPase Pull-Down and Detection kits (Thermo Scientific). For detecting the phosphorylation of Mitf, we used Anti-Flag M2 Affinity beads (Sigma), horseradish peroxidase–conjugated M2 antibody to Flag (1:1,000; Sigma) and antibody to phopshorylated serine (1:1,000; Millipore). Further details are given in the **Supplementary Methods**. We examined the expressions individually and repeated each experiment more than three times.

Chromatin immunoprecipitation (ChIP). We used ChIP-IT Express Chromatin Immunoprecipitation Kits (Active Motif), following the manufacturer's instructions. Briefly, we cultured BMCs from WT mice for 2 d. Cells were crosslinked with 0.4% formaldehyde, and the reaction was stopped by adding glycine. Fixed cells were resuspended in lysis buffer and sonicated for 5 min (with cycles of 30 s on and 30 s off). The supernatant was used immediately for ChIP experiments.

Statistical analyses. We performed statistical analyses using Tukey-Kramer testing for multiple comparisons and Student's *t* tests for two-group comparisons. Values were considered statistically significant at $P < 0.05$. All data are means ± s.e.m. Results are representative of more than four individual experiments.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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Erratum: Vitamin E decreases bone mass by stimulating osteoclast fusion

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In the version of this article initially published, it was incorrectly stated that the mice were fed a diet supplemented with α-tocopherol at 600 mg per kg of body weight. Instead, the food itself contained 600 mg of α-tocopherol per kg. The error has been corrected in the HTML and PDF versions of the article.