

Autoamplification of NFATc1 expression determines its essential role in bone homeostasis

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NFATc1 and NFATc2 are functionally redundant in the immune system, but it was suggested that NFATc1 is required exclusively for differentiation of osteoclasts in the skeletal system. Here we provide genetic evidence that *NFATc1* is essential for osteoclast differentiation in vivo by adoptive transfer of *NFATc1*^{-/-} hematopoietic stem cells to osteoclast-deficient *Fos*^{-/-} mice, and by *Fos*^{-/-} blastocyst complementation, thus avoiding the embryonic lethality of *NFATc1*^{-/-} mice. However, in vitro osteoclastogenesis in *NFATc1*-deficient cells was rescued by ectopic expression of NFATc2. The discrepancy between the in vivo essential role of *NFATc1* and the in vitro effect of NFATc2 was attributed to selective autoregulation of the *NFATc1* gene by NFAT through its promoter region. This suggested that an epigenetic mechanism contributes to the essential function of *NFATc1* in cell lineage commitment. Thus, this study establishes that *NFATc1* represents a potential therapeutic target for bone disease and reveals a mechanism that underlies the essential role of *NFATc1* in bone homeostasis.

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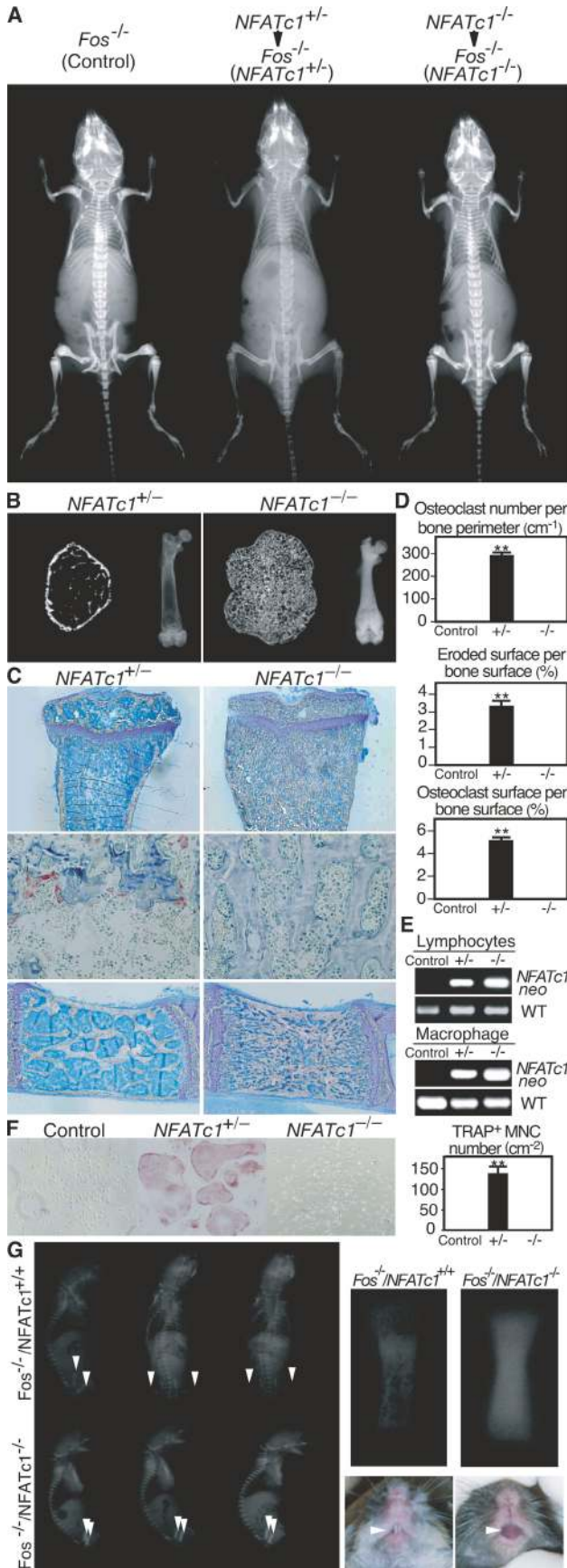
Abbreviations used: AP, activator protein; BMM, bone marrow monocyte/macrophage precursor cell; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; ES, embryonic stem; FLC, fetal liver cell; MeCP2, methyl-CpG binding protein 2; RANKL, receptor activator of NF-κB ligand.

The mechanism underlying irreversible cell fate determination is critical for an understanding of biologic systems in vertebrates, where the NFAT family of transcription factors plays important roles (1, 2). Mice deficient in individual NFAT proteins exhibit a relatively mild phenotype in immune cells or neurons (1, 2), which suggests that the different family members play redundant roles in these systems (1–3). Homeostasis of the skeletal system depends on a balance between bone-forming osteoblasts and bone-resorbing osteoclasts (4). The critical role of *NFATc1* in the skeletal system was suggested by selective and strong induction of *NFATc1* in bone marrow monocyte/macrophage precursor cells (BMMs) that were stimulated with a key cytokine for osteoclastogenesis, receptor activator

of NF-κB ligand (RANKL) (5, 6). Although our previous in vitro analyses indicated that NFATc1 is the essential transcription factor for osteoclast differentiation (5), little is known about its in vivo function in osteoclastogenesis because targeted disruption of the *NFATc1* gene in mice results in embryonic lethality. Furthermore, there is a contrasting report that NFATc2 also activates this process and may have a redundant role (7). Considering the close functional similarity of NFATc1 and NFATc2 (3, 7), it is important to determine whether *NFATc1* is essential for osteoclast differentiation in vivo, and, if so, how it exerts an indispensable function in this cell type.

In this report, we provide in vivo genetic evidence for the essential role of *NFATc1* by two novel techniques: adoptive transfer of hematopoietic stem cells to osteoclast-deficient

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Fos^{-/-} mice and *Fos*-deficient blastocyst complementation. *NFATc1*-deficient osteoclast precursor cells cannot differentiate into osteoclasts. Conversely, osteoclastogenesis of *NFATc1*-deficient cells is rescued by forced expression of *NFATc2*. The discrepancy between the essential role of *NFATc1* and the in vitro effect of *NFATc2* is attributed to selective autoregulation of the *NFATc1* gene by *NFAT* through its promoter region. Our analysis indicates that the essential role of *NFATc1* in bone homeostasis is not determined by its distinct biochemical properties, but is determined by its unique spatiotemporal activation mechanism during osteoclastogenesis.

RESULTS

In vivo evidence for the essential role of *NFATc1* in osteoclast differentiation shown by the fetal liver cell (FLC) transfer

Because in vivo analysis of the role of *NFATc1* has been hampered by embryonic lethality (8, 9), one may postulate that a conditional gene targeting strategy should be appropriate to avoid the difficulty. However, there is no desirable promoter to drive *cre* (encoding Cre recombinase) in the very early stage of osteoclast development. Adoptive transfer of hematopoietic stem cells to irradiated lymphocyte-deficient mice (10, 11) is used as an established method to analyze the function of such a gene in the study of lymphocytes (3). Here we applied this method to the skeletal system using mice lacking *c-Fos* (*Fos*^{-/-} mice) (12, 13) as osteoclast-deficient recipients. Hematopoietic stem cells that were derived from *NFATc1*^{+/-} and *NFATc1*^{-/-} FLCs were injected into the liver of busulfan-treated newborn mice of *Fos*^{-/-} background, in which no osteoclasts form because of a cell autonomous defect (13). Osteopetrosis is amelio-

Figure 1. In vivo evidence for the essential role of *NFATc1* in osteoclast differentiation. (A) Radiographic analysis of *Fos*^{-/-} mice transferred with *NFATc1*^{+/-} or *NFATc1*^{-/-} FLCs. Severe osteopetrosis persists after *NFATc1*^{-/-} FLC transfer. (B) Microradiographic analysis of femur (left: microcomputed tomography; right: microradiograph). Bone marrow cavity is filled with unresorbed bone in *NFATc1*^{-/-} FLC chimera. (C) Histologic examination of tibia (top: toluidine blue staining; middle: tartrate-resistant acid phosphatase [TRAP] staining for detection of osteoclasts and lumbar vertebra; bottom: toluidine blue staining). No osteoclasts are observed in *NFATc1*^{-/-} FLC chimera. (D) Histomorphometric evaluation of osteoclasts in *NFATc1*^{+/-} and *NFATc1*^{-/-} FLC chimera. (E) Reconstitution of the hematopoietic system of chimeric mice by the donor cells. PCR primers specific for joint region between the *NFATc1* locus and the neomycin-resistant gene (*NFATc1 neo*) and for wild-type (WT) *NFATc1* gene are used. The similar chimeric ratios were confirmed by quantitative PCR. (F) Complete blockade of in vitro osteoclastogenesis in osteoclast precursor cells from *NFATc1*^{-/-} FLC chimera. Splenocyte-derived osteoclast precursor cells were cultured in RANKL (50 ng ml⁻¹) and M-CSF (10 ng ml⁻¹). We counted multinucleated (>3 nuclei) cells (MNCs) positive for TRAP staining. (G) Radiographic analysis of neonates generated by *Fos*^{-/-} blastocyst complementation. Note the difference in radio-opacity at the femur (arrowheads). Right panels show the magnified view of the femur of neonates and tooth eruption of 6-wk-old mice. All of the *Fos*^{-/-}/*NFATc1*^{-/-} chimeric mice exhibited osteopetrosis, but osteoclastogenesis is restored in *Fos*^{-/-}/*NFATc1*^{+/-} chimeric mice when ES cells are transmitted to the hematopoietic system of recipient mice. **P < 0.001 versus control.

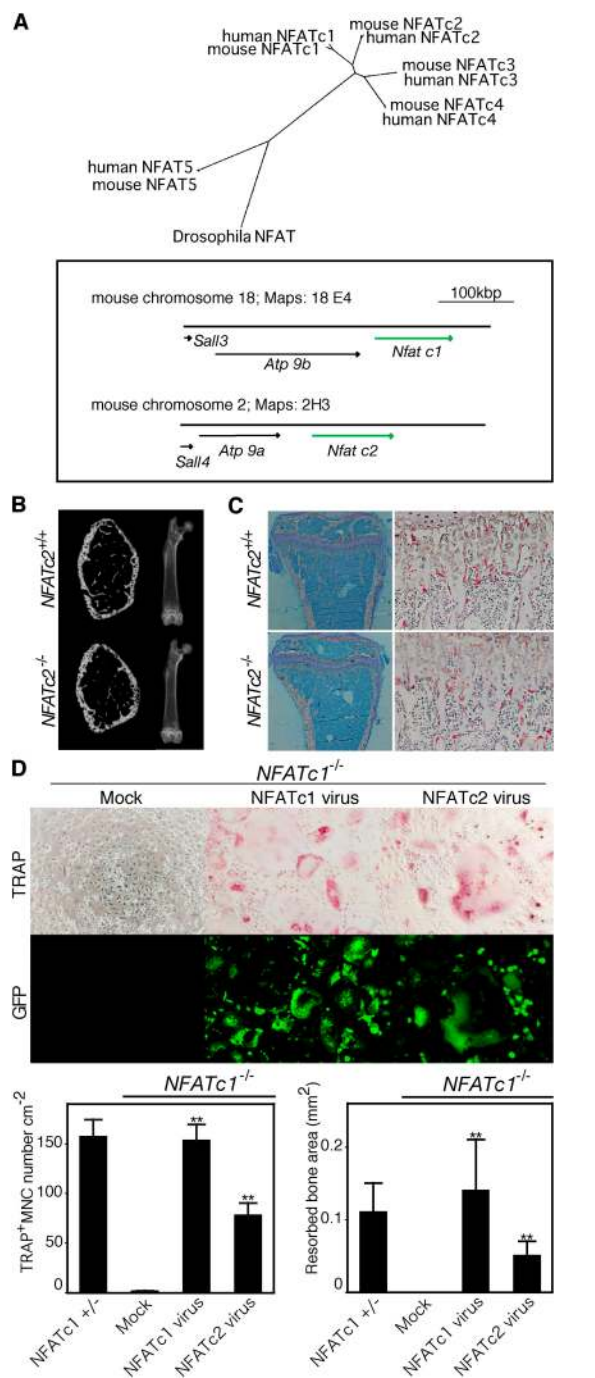


Figure 2. In vitro compensation of *NFATc1* deficiency by forced expression of *NFATc2*. (A) Phylogenetic tree analysis for the DNA binding (Rel homology) region of NFAT family proteins (top). A wide-range genomic view of *NFATc1* and *NFATc2* genes suggests that these genes were generated by chromosomal gene duplication (bottom). (B) Loss-of-function analyses of *NFATc2* in osteoclast differentiation. Microradiographic analysis of femur derived from *NFATc2*^{-/-} mice at 5 wk of age (see Fig. 1 B). (C) Histologic examination of tibia from *NFATc2*^{-/-} mice (left: toluidine blue staining; right: tartrate-resistant acid phosphatase staining [TRAP]). (D) Recovery of osteoclastogenesis in *NFATc1*^{-/-} FLCs by retrovirus-mediated expression of *NFATc1* or *NFATc2*. Forced expression of *NFATc2* as well as that of *NFATc1* induces formation of osteoclasts

rated and bone marrow cavities are observed when *NFATc1*^{+/-} FLCs are transferred to *Fos*^{-/-} mice (Fig. 1, A and B). Tooth eruption, which is not seen in *Fos*^{-/-} mice (13), is observed in these mice (unpublished data). In contrast, when *NFATc1*^{-/-} FLCs are transferred to *Fos*^{-/-} mice, they exhibit severe osteopetrosis and bone marrow cavities remain occupied with unresorbed bone (Fig. 1, A and B). Histologic analyses show that osteoclast number and parameters of bone resorption are normalized only when *NFATc1*^{+/-} cells are transferred (Fig. 1, C and D). Repopulation of hematopoietic cells was confirmed by PCR in splenic lymphocytes and monocyte/macrophage precursor cells (Fig. 1 E). Consistently, osteoclast precursor cells that were derived from the *NFATc1*^{-/-} FLC chimera could not generate osteoclasts in culture (Fig. 1 F). These results indicate that *NFATc1* is indispensable for osteoclast formation in vivo, and that FLC transfer to osteoclast-deficient mice is a beneficial tool for in vivo analysis of the osteoclast lineage.

Fos^{-/-} blastocyst complementation

Rag-2-deficient blastocyst complementation is an alternative method for analyzing the lymphocyte-specific functions of gene, loss of which results in embryonic lethality (14). To provide additional genetic evidence for the essential role of *NFATc1* in osteoclastogenesis, we applied this method to an analysis of the osteoclast lineage using *Fos*^{-/-} blastocysts (referred to as *Fos*-deficient blastocyst complementation). We analyzed chimeric neonates that were generated by injection of wild-type or *NFATc1*^{-/-} embryonic stem (ES) cells into *Fos*^{-/-} blastocysts. The chimeric mice that result from injection of normal ES cells into *Fos*^{-/-} blastocysts (*Fos*^{-/-}/*NFATc1*^{+/+} chimera) should develop mature osteoclasts, which are derived from the injected ES cells. In fact, all of the chimeric mice recovered from osteopetrosis, and tooth eruption was observed when the injected ES cells were transmitted into the hematopoietic lineage (Fig. 1 G). In contrast, chimeric mice that result from injection of *NFATc1*^{-/-} ES cells into *Fos*^{-/-} blastocysts (*Fos*^{-/-}/*NFATc1*^{-/-} chimera) remain severely osteopetrotic, and have a defect in tooth eruption that is due to a lack of osteoclasts (Fig. 1 G and not depicted). Taken together with the results of FLC transfer, these observations provide clear evidence that *NFATc1* is essential for osteoclast differentiation in vivo.

NFATc2 is dispensable for osteoclastogenesis in vivo and in vitro

NFATc2 is another NFAT protein that is expressed at a low level in osteoclast precursor cells (5, 7). In addition to the similarity of the DNA binding (Rel homology) domain, the genomic view of the *NFATc1* and *NFATc2* loci suggest that *NFATc2* is the evolutionally closest relative of *NFATc1* among the NFAT proteins (1, 2, 15, 16) (Fig. 2 A). In addition

with pit-forming activity on dentin slices (RANKL, 50 ng ml⁻¹). Infection efficiency was monitored by GFP, which is expressed bicistronically. **P < 0.001 versus mock.

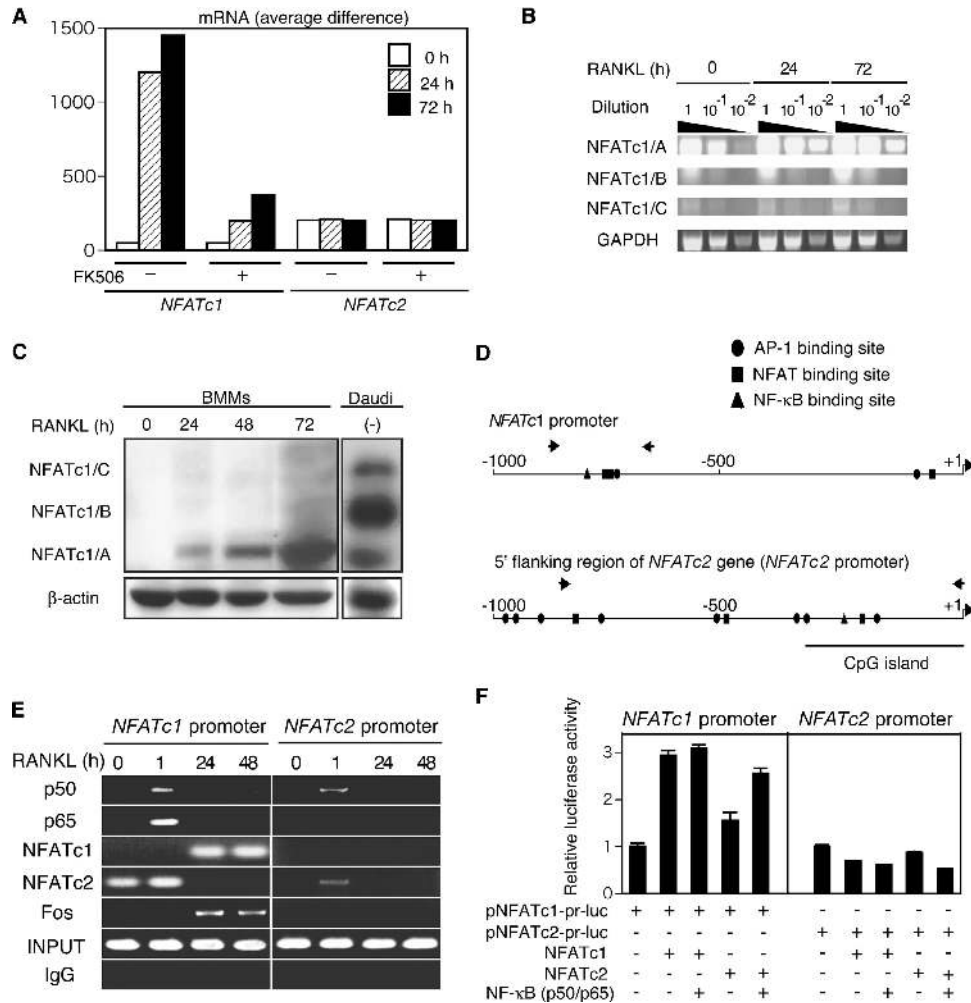


Figure 3. Autoamplification of *NFATc1* during osteoclastogenesis. (A) GeneChip analysis of mRNA expression of *NFATc1* and *NFATc2* in RANKL-stimulated BMMs. Strong induction of *NFATc1* by RANKL is inhibited by FK506 (2.5 μg ml⁻¹), which inhibits NFAT activity. *NFATc2* expression is constitutive and is not affected by FK506. (B) Semiquantitative RT-PCR analysis of the mRNA of *NFATc1* isoforms in BMMs. *NFATc1/A* is induced ~10-fold by RANKL. (C) Immunoblot analysis of *NFATc1* isoforms during RANKL-induced osteoclastogenesis in BMMs. A Daudi lymphoma cell line

is shown as a positive control for all three isoforms (A, B, and C) of *NFATc1* (22). (D) A schematic illustration of putative transcription factor binding sites in the 5' flanking region of the *NFATc1* (20) and *NFATc2* genes. Binding sites in the 5' flanking region of the *NFATc2* gene were determined by the TRANSFAC retrieval program. Arrows indicate primers for ChIP experiments. (E) ChIP assay of *NFATc1* and *NFATc2* promoters in RANKL-stimulated BMMs. (F) Luciferase assay of *NFATc1* and *NFATc2* promoters in HEK293T cells.

tion, overexpression of *NFATc2* facilitates the differentiation of osteoclasts (7) (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>). These results prompted us to investigate osteoclast differentiation in *NFATc2*-deficient (*NFATc2*^{-/-}) mice (17). Although *NFATc2* is involved in the regulation of chondrocytes at advanced age, bone development of *NFATc2*^{-/-} mice has never been well defined (18). Skeletal development occurs normally, and we observed no increase in bone mass in *NFATc2*^{-/-} mice as shown in Fig. 2 B [see also reference 19]. We found no abnormality in the number of osteoclasts, or in the parameters of osteoclastic bone resorption (Fig. 2 C and Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>) in *NFATc2*^{-/-} mice. Consistently, *NFATc2*^{-/-} BMMs that are stimulated by

RANKL normally differentiate into osteoclasts with bone-resorbing activity (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1> and not depicted). In addition, RANKL induction of *NFATc1* in *NFATc2*^{-/-} cells is comparable to that in *NFATc2*^{+/+} cells (Fig. S3). Thus, *NFATc2* is dispensable for RANKL-induced *NFATc1* expression and osteoclastogenesis.

Osteoclast differentiation of *NFATc1*-deficient cells is rescued by forced expression of *NFATc2*

Because *NFATc1* and *NFATc2* have a similar ability to activate gene expression in T cells (1–3), it has been unexpected that *NFATc1* plays a nonredundant role in the skeletal system. *NFATc1*^{+/-} cells differentiate into osteoclasts with bone-

resorbing activity when they are stimulated by RANKL in the presence of M-CSF, whereas *NFATc1*^{-/-} cells are unable to generate osteoclasts (Fig. 2 D), despite normal development into the monocyte/macrophage lineage (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>). To investigate the function of NFATc1 and NFATc2 further, we ectopically expressed NFATc1 and NFATc2 under the control of the *LTR* promoter using retrovirus-mediated gene transfer (20) in *NFATc1*^{-/-} osteoclast precursor cells. As expected, the formation of bone-resorbing osteoclasts was rescued by retroviral expression of NFATc1. Surprisingly, osteoclast formation recovered, albeit at a reduced efficiency, as the result of forced expression of NFATc2. This demonstrated that forced expression of NFATc2 compensates for the loss of the *NFATc1* gene (Fig. 2 D).

Selective autoamplification of *NFATc1* during osteoclastogenesis

How can we reconcile the *in vivo* essential role of *NFATc1* in osteoclastogenesis with the observation that *NFATc1* deficiency is compensated for by forced expression of NFATc2? Whereas NFATc2 is expressed constitutively in BMMs at a low level, mRNA of *NFATc1* is induced selectively and potently by RANKL (5) (Fig. 3 A). The induction of *NFATc1*, but not *NFATc2*, is down-regulated by the calcineurin inhibitor, FK506, which suppresses the activity of NFAT (Fig. 3 A). This suggests that *NFATc1* is autoregulated selectively by NFAT when BMMs are stimulated with RANKL, and that the resulting expression pattern may explain the specificity of *NFATc1*. Although *NFATc1* also is autoregulated in T cells (20, 21), the expression pattern during osteoclastogenesis (autoamplification) is characterized by a monotone increase and magnitude of amplification. Three isoforms (A, B, and C) of NFATc1 are well documented in T cells (20, 22), but it remains to be clarified which isoform is involved in osteoclastogenesis. Using specific PCR primers, we found that mRNA expression of the shortest isoform, NFATc1/A, is induced selectively in RANKL-stimulated BMMs (Fig. 3 B); immunoblot analysis yielded consistent results (Fig. 3 C). Therefore, we investigated the regulatory mechanism of the P1 promoter of the *NFATc1* gene, which regulates NFATc1/A induction (20), in comparison with the *NFATc2* promoter. To determine the transcriptional start site of mouse *NFATc2* gene, a 5' rapid amplification of cDNA ends experiment was performed. The putative transcription factor binding sites in the 5' flanking region of the *NFATc1* and *NFATc2* gene are shown in Fig. 3 D. Unexpectedly, these results suggest that not only the *NFATc1* promoter but also the *NFATc2* promoter contain multiple NFAT binding sites.

Recruitment of NFATc1 and other transcription factors to the *NFAT* promoters

Chromatin immunoprecipitation (ChIP) analysis shows that NFATc2 is recruited to the *NFATc1* promoter at the earliest phase of osteoclast differentiation (Fig. 3 E). 24 h after RANKL stimulation, NFATc1 mainly occupies the promoter instead of NFATc2, and c-Fos is concomitantly recruited to

the promoter (Fig. 3 E); this is consistent with the strong induction of *NFATc1* at this time point. This occupancy persists during terminal differentiation of osteoclasts, and indicates that autoamplification of *NFATc1* occurs during this process and that c-Fos may contribute to it. This is congruent with the critical role of c-Fos in RANKL-mediated induction of *NFATc1*, which was reported previously (5, 23). NF- κ B is activated rapidly by RANKL (24), and a recent study suggests that NF- κ B activity is important for RANKL-mediated induction of *NFATc1* at the early phase of osteoclastogenesis (25). Consistent with this, NF- κ B components p50 and p65 are recruited to the *NFATc1* promoter 1 h after RANKL stimulation. However, this recruitment is not observed after 24 h (Fig. 3 E), which suggests that NF- κ B is important for the initial induction of *NFATc1* in cooperation with NFATc2. Although NFATc2 is detected on the *NFATc1* promoter without RANKL stimulation, *NFATc1* is not induced under this condition, which suggests that additional stimulation, such as NF- κ B activation, is important for the efficient triggering of this promoter. This is consistent with the observation in the luciferase assay that the *NFATc1* promoter is not activated fully by NFATc2 alone, but is activated markedly by the coexpression of NFATc2 and NF- κ B (Fig. 3 F). The basal calcineurin activity in osteoclast precursor cells may contribute to the nuclear localization of NFATc2 without RANKL addition (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>). Because osteoclast differentiation was not affected in *NFATc2*^{-/-} mice, the function of NFATc2 evidently can be compensated by other factors, although it may contribute to physiologic regulation of the initial induction of *NFATc1*. A reporter plasmid that is driven by the *NFATc1*-P1 promoter, but not by the *NFATc2* promoter, is activated markedly by NFAT in cooperation with NF- κ B (Fig. 3 F). These results indicate that the *NFATc1* promoter is distinct from the *NFATc2* promoter in that the former is exclusively under autoregulation by NFAT; this further suggests that an autoregulatory mechanism contributes to the essential role of *NFATc1*.

Epigenetic regulation of *NFATc1* gene determines its unique spatiotemporal induction pattern during osteoclastogenesis

To gain insight into the selective recruitment of NFATc1 to the *NFATc1* promoter, we examined the association of transcriptional cofactors with these promoters. *NFATc1* promoter is associated increasingly with transcriptional coactivators with histone acetylase activity, such as CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF), but is dissociated with histone deacetylase 1 (HDAC1; Fig. 4 A). It is notable that histone acetylation in the *NFATc1* promoter increased gradually after RANKL stimulation and that the high acetylation status was sustained, but this was not observed in the *NFATc2* promoter (Fig. 4 B). Methylation of histone H3 lysine 4, which is characteristic of the transcriptionally active locus, is up-regulated exclusively in the *NFATc1* promoter (Fig. 4 B). Conversely, the *NFATc2* promoter is associated con-

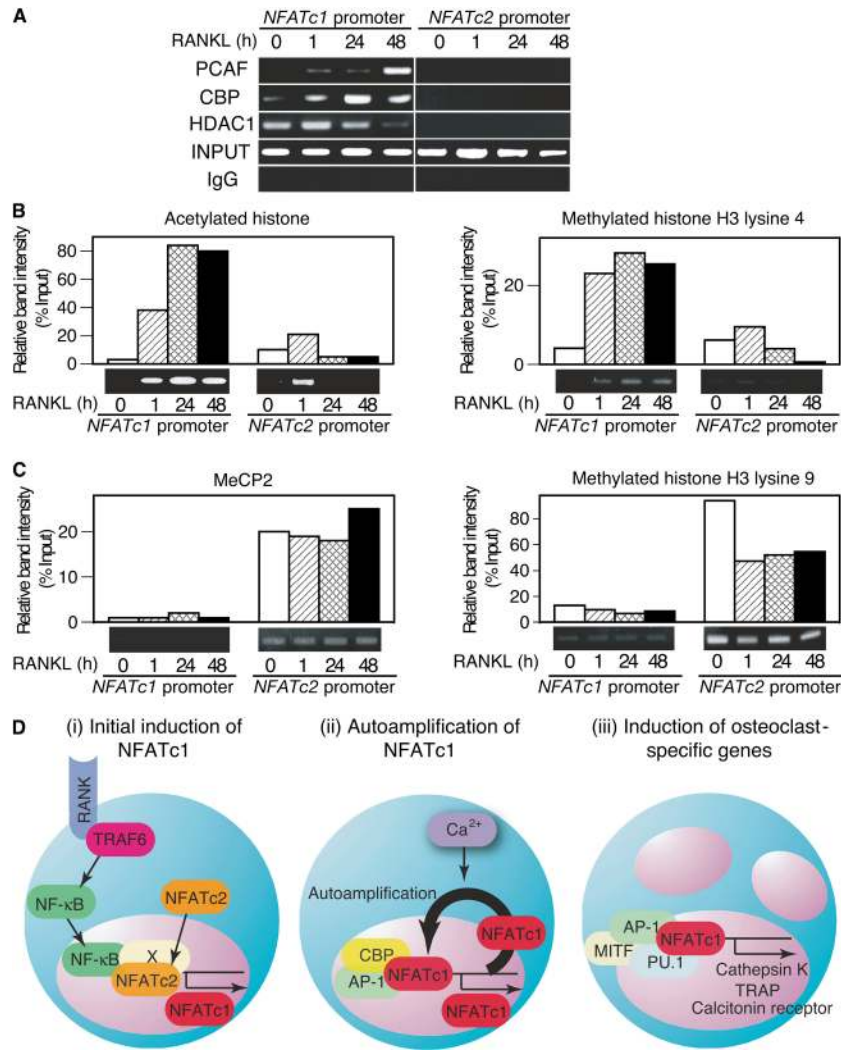


Figure 4. Epigenetic regulation of *NFATc1* promoter underlies the selective autoamplification of *NFATc1*. (A) Analysis of chromatin modification–related factors in *NFATc1* and *NFATc2* promoters by ChIP in RANKL-stimulated BMMs during osteoclastogenesis. (B) ChIP assay for acetylated histone and methylated histone H3 lysine 4. (C) ChIP assay for MeCP2 and methylated histone H3 lysine 9. (D) A schematic diagram of three stages of osteoclast differentiation that are governed by NFATc1. (i) NFATc2 is recruited to the *NFATc1* promoter at the very early phase, but this is not enough to activate the *NFATc1* promoter. The binding of RANKL to receptor activator of NF-κB (RANK) results in the recruitment of TNF receptor–associated factor (TRAF) 6, and leads to the activation of downstream molecules, such as NF-κB (25, 34). Cooperation of NFATc2 and NF-κB activates the initial induction of

NFATc1, but because *NFATc2*-deficient mice have no obvious defect in osteoclast differentiation, unknown factor(s) (shown as X) may compensate for the loss of *NFATc2* in these mice. (ii) RANKL–RANK interaction cooperates with immunoreceptors to activate the calcium signals (28), which stimulate the NFATc1 activation by way of calcineurin (5). NFATc1 binds to its own promoter, which leads to the robust induction of *NFATc1*; AP-1 (containing c-Fos) is critical for this autoamplification. Selective recruitment of NFATc1 to the promoter of *NFATc1*, but not *NFATc2*, is explained in part, by epigenetic regulation. (iii) Several osteoclast-specific genes, such as *cathepsin K*, *TRAP*, and *calcitonin receptor*, are activated by a transcriptional complex that contains NFATc1 and other cooperators, such as AP-1, PU.1, and microphthalmia-associated transcription factor (MITF).

stantly with methylated DNA-binding proteins, such as the methyl-CpG binding protein 2 (MeCP2) (Fig. 4 C), which suggests that the *NFATc2* promoter specifically is silenced during osteoclast differentiation. Consistent with this notion, we detected high levels of methylated histone H3 lysine 9 only in the *NFATc2* promoter (Fig. 4 C). These results suggest that epigenetic regulation underlies the specific autoamplification of *NFATc1*.

DISCUSSION

Our results illustrate that the essential importance of a gene is not only attributable to the specific biochemical function of the coded protein, but also is determined epigenetically by the distinct regulatory mechanism of gene expression. The role of *GATA-1* and *GATA-3* in erythropoiesis and Th2 cell differentiation are examples in which the essential importance of one member of a group of structurally related pro-

teins of interchangeable function is based on an autoregulatory mechanism (26, 27). These studies suggest that autoregulation of transcription factors is one of the critical mechanisms for cell fate determination (2). In a future study, generation of mice with an *NFATc2* knock-in into the *NFATc1* locus will be an intriguing strategy to provide the conclusive *in vivo* evidence.

As depicted in Fig. 4 D, in the differentiation of osteoclasts, preexisting NFATc2 cooperates with other RANKL-stimulated transcription factors, such as NF- κ B, to activate the initial induction of *NFATc1*, followed by an autoamplification phase of *NFATc1*. It was reported that the activator protein (AP)-1 component, c-Fos, is critical for RANKL-mediated induction of *NFATc1* (5, 23). Consistent with this, c-Fos is recruited selectively to the *NFATc1* promoter at the autoamplification phase (Fig. 3 E). The composite NFAT/AP-1 site is observed exclusively in the *NFATc1* promoter (Fig. 3 D) (20). This lends further support to the notion that the cooperation of NFAT and AP-1 is responsible for the specific activation of the *NFATc1* promoter and its autoamplification.

Calcium signal-mediated activation of NFATc1 also triggers the autoamplification loop of *NFATc1* and ensures a sustained *NFATc1*-dependent transcriptional program (5, 28) in which osteoclast-specific genes are activated by a transcriptional complex that involves NFATc1, AP-1, and other cooperators (5, 29). Accumulating evidence indicates that NFATc1 regulates many osteoclast-specific genes, such as *cathepsin K* (29), *TRAP* (5, 30), *calcitonin receptor* (5, 30), and *osteoclast-associated receptor* (30), in cooperation with other transcription factors (e.g., PU.1 and microphthalmia-associated transcription factor), although the components of the transcriptional complex are not always the same (30) (Fig. 4 D, iii). A distinct pattern of calcium oscillation, which efficiently keeps NFATc1 transcriptionally active in the nucleus (31), may explain the specific spatiotemporal expression of NFATc1 in the osteoclast lineage (5), in contrast to T cells. FK506 inhibited osteoclast formation, even when it was added at the late phase of osteoclastogenesis (48–72 h after RANKL stimulation, unpublished data); this suggests that calcium signaling is also critical for the autoamplification of *NFATc1*.

Successful application of FLC transfer and blastocyst complementation, first established in the immune system, provided genetic evidence that *NFATc1* is essential for osteoclast differentiation and maintenance of the skeletal system. Modulation of the *NFATc1* autoamplification pathway has promise as a strategy for suppressing the excessive osteoclast formation that is characteristic of a variety of bone diseases.

MATERIALS AND METHODS

Mice and analysis of bone phenotype. Generation of *Fos*^{-/-} and *NFATc1*^{-/-} mice has been described previously (8, 13). *NFATc2* mice (17) were provided by L.H. Glimcher (Harvard Medical School, Boston, MA). Histologic, histomorphometric, and microradiographic examinations were performed using essentially the same methods as described (32). Statistical analysis was performed using Student's *t* test. All mice were kept in a specific pathogen-

free environment, and all animal experiments were performed with the approval of the institutional committee of Tokyo Medical and Dental University.

In vitro osteoclastogenesis and retroviral gene transfer. Bone marrow cells or FLCs were cultured in α -MEM (GIBCO BRL) containing 10% FBS (Sigma-Aldrich) and 10 ng ml⁻¹ M-CSF (R&D Systems). After 2 d, adherent cells were used as osteoclast precursor cells. In osteopetrotic mice, osteoclast precursor cells were obtained similarly from splenocytes. We described the method of RANKL-induced *in vitro* osteoclastogenesis, retroviral gene transfer, characterization of osteoclasts, and immunostaining (5, 24, 32). All data are expressed as mean \pm SEM (*n* = 6). Retroviral vectors, pEGZ-NFATc1/A (NFATc1 virus) and pEGZ-NFATc2 (NFATc2 virus), were described previously (20), and infection efficiencies of both retrovirus vectors into osteoclast precursor cells were \sim 40%, as described previously (24).

Fetal liver cell transfer. *NFATc1*^{-/-} and *NFATc1*^{+/-} embryos were obtained by crossing *NFATc1*^{+/-} parental mice. Total liver cells (10⁷ cells) from embryonic day (E) 13.5 embryos were injected into the liver of recipient *Fos*^{-/-} newborn (2 d) mice, which were analyzed at 6 wk of age. The recipient mice were treated with busulfan (Sigma-Aldrich) in utero by i.p. administration to pregnant mice (12.5 mg kg⁻¹) twice at 17.5 and 18.5 d postcoitum.

***Fos*^{-/-} blastocyst complementation.** Establishment of *NFATc1*^{-/-} ES cell lines has been described (33). *NFATc1*^{-/-} ES cells or wild-type ES cells (E14K) were injected into E3.5 *Fos*^{-/-} blastocysts that were obtained by fertilizing *Fos*^{-/-} gametes from *Fos*^{-/-} paternal and maternal mice using *in vitro* fertilization technique. Resultant blastocysts were transferred to pseudopregnant foster mothers to generate chimeric mice.

RT-PCR and GeneChip analysis. RNA extraction, semiquantitative RT-PCR, and GeneChip analysis were performed as described previously (5, 32). The following PCR primers were used to detect NFAT isoforms. NFATc1/A: 5'-GGTAACTCTGCTTTCTAACCTTAAGCTC-3' (sense) and 5'-GTGATGACCCAGCATGCACCAGTCACAG-3' (antisense); NFATc1/B: 5'-CCCATCCGCCAGGCTACAGCCGCAGTAA-3' (sense) and 5'-TTCGGTAAGTTGGGATTTCTGAGTGGTACC-3' (antisense); NFATc1/C: 5'-CCCATCCGCCAGGCTACAGCCGCAGTAA-3' (sense) and 5'-TGAGTGGTACCAGATGTGGGTCCAGTTTAT-3' (antisense).

Chromatin immunoprecipitation. ChIP was performed with the ChIP Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions, using antibodies against NFATc1, NFATc2, p300/CBP-associated factor, histone deacetylase 1, p50, CBP, MeCP2 (Santa Cruz Biotechnology, Inc.), p65, acetylated histone, mono/di/trimethyl-histone H3 lysine 4, dimethyl-histone H3 lysine 9 (Upstate Biotechnology), c-Fos (Calbiochem), and normal IgG (Santa Cruz Biotechnology, Inc.). The purified DNA was analyzed by PCR using primers that detect sequences containing the *NFATc1*-P1 promoter: 5'-CCGGGACGCCCATGCAATCTGT-TAGTAATT-3' (sense) and 5'-GCGGGTGCCTGAGAAAGC-TACTCTCCCTT-3' (antisense) and the *NFATc2* promoter: 5'-TTAT-CAGGGAGCACTGCCCATCTCCGCTTT-3' (sense) and 5'-CGGTCTGGCCTGAGCGACAGGCCAGACAA-3' (antisense).

Luciferase reporter gene assay. The reporter plasmid pNFATc1P1-0.8 kb-luc (pNFATc1-pr-luc) was described previously (20). pNFATc2-0.8 kb-luc (pNFATc2-pr-luc) was constructed by inserting a 0.8-kb NheI-Xho fragment of the mouse *NFATc2* promoter into the same sites of the pGL3 basic promoter vector (Promega). The mouse *NFATc2* promoter region was amplified by PCR using following primers: 5'-GCTAGCTGTTTTGTGACTGTTATCATGCTGGG-3' (sense) and 5'-CTCGAGCTTCCTGCTCAAGGCACCTGTTGCAG-3' (antisense). Transfection into HEK293T cells and measurement of luciferase activity were performed as described (5) using the Dual-luciferase reporter assay system (Promega).

Online supplemental material. Fig. S1 shows the gain-of-function analyses of *NFATc1* and *NFATc2* in osteoclasts. Fig. S2 shows the histomorphometric evaluation of osteoclasts in *NFATc2*^{-/-} mice. Fig. S3 shows the normal osteoclast differentiation and expression of NFATc1 in *NFATc2*^{-/-} monocyte/macrophage precursor cells that were stimulated with RANKL/M-CSF. Fig. S4 shows the development into the monocyte/macrophage lineage from *NFATc1*^{-/-} FLCs. Fig. S5 shows the effect of FK506 on the recruitment of NFATc2 to the *NFATc1* promoter without RANKL stimulation. Also, details of the methods for phylogenetic tree analysis and 5' rapid amplification of cDNA ends analysis are available. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>.

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REFERENCES

- Crabtree, G.R., and E.N. Olson. 2002. NFAT signaling: choreographing the social lives of cells. *Cell*. 109(Suppl):S67–79.
- Hogan, P.G., L. Chen, J. Nardone, and A. Rao. 2003. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* 17: 2205–2232.
- Peng, S.L., A.J. Gerth, A.M. Ranger, and L.H. Glimcher. 2001. NFATc1 and NFATc2 together control both T and B cell activation and differentiation. *Immunity*. 14:13–20.
- Teitelbaum, S.L., and F.P. Ross. 2003. Genetic regulation of osteoclast development and function. *Nat. Rev. Genet.* 4:638–649.
- Takayanagi, H., S. Kim, T. Koga, H. Nishina, M. Ishiki, H. Yoshida, A. Saiura, M. Isobe, T. Yokochi, J. Inoue, et al. 2002. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev. Cell*. 3:889–901.
- Theill, L.E., W.J. Boyle, and J.M. Penninger. 2002. RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu. Rev. Immunol.* 20:795–823.
- Ikeda, F., R. Nishimura, T. Matsubara, S. Tanaka, J. Inoue, S.V. Reddy, K. Hata, K. Yamashita, T. Hiraga, T. Watanabe, et al. 2004. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. *J. Clin. Invest.* 114:475–484.
- de la Pompa, J.L., L.A. Timmerman, H. Takimoto, H. Yoshida, A.J. Elia, E. Samper, J. Potter, A. Wakeham, L. Marengere, B.L. Langille, et al. 1998. Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature*. 392:182–186.
- Ranger, A.M., M.J. Grusby, M.R. Hodge, E.M. Gravalles, F.C. de la Brousse, T. Hoey, C. Mickanin, H.S. Baldwin, and L.H. Glimcher. 1998. The transcription factor NF-ATc is essential for cardiac valve formation. *Nature*. 392:186–190.
- Shinkai, Y., G. Rathbun, K.P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall, and F.W. Alt. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. 68:855–867.
- Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 68:869–877.
- Wang, Z.Q., C. Ovitt, A.E. Grigoriadis, U. Mohle-Steinlein, U. Ruther, and E.F. Wagner. 1992. Bone and haematopoietic defects in mice lacking c-fos. *Nature*. 360:741–745.
- Grigoriadis, A.E., Z.Q. Wang, M.G. Cecchini, W. Hofstetter, R. Felix, H.A. Fleisch, and E.F. Wagner. 1994. c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science*. 266:443–448.
- Chen, J., R. Lansford, V. Stewart, F. Young, and F.W. Alt. 1993. RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development. *Proc. Natl. Acad. Sci. USA*. 90:4528–4532.
- Ho, S.N., D.J. Thomas, L.A. Timmerman, X. Li, U. Francke, and G.R. Crabtree. 1995. NFATc3, a lymphoid-specific NFATc family member that is calcium-regulated and exhibits distinct DNA binding specificity. *J. Biol. Chem.* 270:19898–19907.
- Graef, I.A., J.M. Gastier, U. Francke, and G.R. Crabtree. 2001. Evolutionary relationships among Rel domains indicate functional diversification by recombination. *Proc. Natl. Acad. Sci. USA*. 98:5740–5745.
- Hodge, M.R., A.M. Ranger, F. Charles de la Brousse, T. Hoey, M.J. Grusby, and L.H. Glimcher. 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity*. 4:397–405.
- Ranger, A.M., L.C. Gerstenfeld, J. Wang, T. Kon, H. Bae, E.M. Gravalles, M.J. Glimcher, and L.H. Glimcher. 2000. The nuclear factor of activated T cells (NFAT) transcription factor NFATp (NFATc2) is a repressor of chondrogenesis. *J. Exp. Med.* 191:9–22.
- Koga, T., Y. Matsui, M. Asagiri, T. Kodama, B. de Crombrughe, K. Nakashima, and H. Takayanagi. 2005. NFAT and Osterix cooperatively regulate bone formation. *Nat. Med.* 11:880–885.
- Chuvpilo, S., E. Jankevics, D. Tyrins, A. Akimzhanov, D. Moroz, M.K. Jha, J. Schulze-Luehrmann, B. Santner-Nanan, E. Feoktistova, T. Konig, et al. 2002. Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. *Immunity*. 16:881–895.
- Zhou, B., R.Q. Cron, B. Wu, A. Genin, Z. Wang, S. Liu, P. Robson, and H.S. Baldwin. 2002. Regulation of the murine *Nfatc1* gene by NFATc2. *J. Biol. Chem.* 277:10704–10711.
- Chuvpilo, S., M. Zimmer, A. Kerstan, J. Glockner, A. Avots, C. Escher, C. Fischer, I. Inashkina, E. Jankevics, F. Berberich-Siebelt, et al. 1999. Alternative polyadenylation events contribute to the induction of NF-ATc in effector T cells. *Immunity*. 10:261–269.
- Matsuo, K., D.L. Galson, C. Zhao, L. Peng, C. Laplace, K.Z. Wang, M.A. Bachler, H. Amano, H. Aburatani, H. Ishikawa, and E.F. Wagner. 2004. Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. *J. Biol. Chem.* 279:26475–26480.
- Takayanagi, H., K. Ogasawara, S. Hida, T. Chiba, S. Murata, K. Sato, A. Takaoka, T. Yokochi, H. Oda, K. Tanaka, et al. 2000. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN- γ . *Nature*. 408:600–605.
- Takatsuna, H., M. Asagiri, T. Kubota, K. Oka, T. Osada, C. Sugiyama, H. Saito, K. Aoki, K. Ohya, H. Takayanagi, and K. Umezawa. 2005. Inhibition of RANKL-induced osteoclastogenesis by (-)-DH-MEQ, a novel NF- κ B inhibitor, through downregulation of NFATc1. *J. Bone Miner. Res.* 20:653–662.
- Takahashi, S., R. Shimizu, N. Suwabe, T. Kuroha, K. Yoh, J. Ohta, S. Nishimura, K.C. Lim, J.D. Engel, and M. Yamamoto. 2000. GATA factor transgenes under GATA-1 locus control rescue germline GATA-1 mutant deficiencies. *Blood*. 96:910–916.
- Ouyang, W., M. Lohning, Z. Gao, M. Assenmacher, S. Ranganath, A. Radbruch, and K.M. Murphy. 2000. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity*. 12:27–37.
- Koga, T., M. Inui, K. Inoue, S. Kim, A. Suematsu, E. Kobayashi, T.

- Iwata, H. Ohnishi, T. Matozaki, T. Kodama, et al. 2004. Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. *Nature*. 428:758–763.
29. Matsumoto, M., M. Kogawa, S. Wada, H. Takayanagi, M. Tsujimoto, S. Katayama, K. Hisatake, and Y. Nogi. 2004. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. *J. Biol. Chem.* 279:45969–45979.
30. Kim, Y., K. Sato, M. Asagiri, I. Morita, K. Soma, and H. Takayanagi. 2005. Contribution of NFATc1 to the transcriptional control of immunoreceptor OSCAR but not TREM-2 during osteoclastogenesis. *J. Biol. Chem.* 280:32905–32913.
31. Tomida, T., K. Hirose, A. Takizawa, F. Shibasaki, and M. Iino. 2003. NFAT functions as a working memory of Ca^{2+} signals in decoding Ca^{2+} oscillation. *EMBO J.* 22:3825–3832.
32. Takayanagi, H., S. Kim, K. Matsuo, H. Suzuki, T. Suzuki, K. Sato, T. Yokochi, H. Oda, K. Nakamura, N. Ida, et al. 2002. RANKL maintains bone homeostasis through c-Fos-dependent induction of *interferon- β* . *Nature*. 416:744–749.
33. Yoshida, H., H. Nishina, H. Takimoto, L.E. Marengere, A.C. Wakeham, D. Bouchard, Y.Y. Kong, T. Ohteki, A. Shahinian, M. Bachmann, et al. 1998. The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity*. 8:115–124.
34. Takayanagi, H. 2005. Mechanistic insight into osteoclast differentiation in osteoimmunology. *J. Mol. Med.* 83:170–179.