Blimp1-mediated repression of negative regulators is required for osteoclast differentiation

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Regulation of irreversible cell lineage commitment depends on a delicate balance between positive and negative regulators, which comprise a sophisticated network of transcription factors. Receptor activator of NF-\(\kappa\)B ligand (RANKL) stimulates the differentiation of bone-resorbing osteoclasts through the induction of nuclear factor of activated \(\kappa\)B cell 1 (NFATc1), the essential transcription factor for osteoclastogenesis. Osteoclast-specific robust induction of NFATc1 is achieved through an autoamplification mechanism, in which NFATc1 is constantly activated by calcium signaling while the negative regulators of NFATc1 are suppressed. However, it has been unclear how such negative regulators are repressed during osteoclastogenesis. Here we show that B lymphocyte-induced maturation protein-1 (Blimp1; encoded by \textit{Prdm1}), which is induced by RANKL through NFATc1 during osteoclastogenesis, functions as a transcriptional repressor of anti-osteoclastogenic genes such as \textit{Irf8} and \textit{Mafb}. Overexpression of Blimp1 leads to an increase in osteoclast formation, and \textit{Prdm1}-deficient osteoclast precursor cells do not undergo osteoclast differentiation efficiently. The importance of Blimp1 in bone homeostasis is underscored by the observation that mice with an osteoclast-specific deficiency in the \textit{Prdm1} gene exhibit a high bone mass phenotype caused by a decreased number of osteoclasts. Thus, NFATc1 choreographs the determination of cell fate in the osteoclast lineage by inducing the repression of negative regulators as well as through its effect on positive regulators.

B lymphocyte induced maturation protein 1 | nuclear factor of activated \(\kappa\)B cell 1 | osteoclastogenesis | transcription | bone

Osteoclasts are multicellular cells of monocyte/macrophage lineage that degrade bone matrix. The maintenance of bone homeostasis is dependent on the balance between bone-resorbing osteoclasts and bone-forming osteoblasts (1, 2). Excessive bone resorption by osteoclasts is often associated with diseases accompanied by pathological bone loss, including osteoporosis and rheumatoid arthritis (3–6); thus research into the mechanisms of osteoclast differentiation is both biologically and clinically important.

Receptor activator of NF-\(\kappa\)B ligand (RANKL) is a key cytokine that stimulates the osteoclast precursor cells to fuse and undergo differentiation into osteoclasts (7, 8). Macrophage colony-stimulating factor (M-CSF) also is an essential cytokine for osteoclastogenesis, which is important for the survival of the osteoclast lineage and the expression of RANK, the receptor for RANKL (9). RANKL binding to RANK results in the recruitment of the TNF receptor-associated factor 6 (TRAF6), leading to the activation of NF-\(\kappa\)B and JNK (10). RANK also stimulates the induction of \textit{Fos} and thus activates the activator protein-1 (AP-1) complex containing c-Fos (11). NF-\(\kappa\)B initiates the initial induction of nuclear factor of activated T cells 1 (NFATc1), the expression of which is autoamplified by NFATc1 binding to its own promoter in cooperation with c-Fos (12). This autoamplification mechanism enables a robust induction of \textit{Nfatc1} specific to osteoclasts (13).

During osteoclastogenesis, NFATc1 is constantly activated by calcium signaling, which is dependent on Ig-like receptors such as osteoclast-associated receptor (OSCAR) and triggering receptor expressed in myeloid cells 2 (TREM2) (1, 2). These receptors are associated with the adaptor molecules, DNAX-activating protein 12 (DAP12) and Fc receptor common \(\gamma\) subunit (FcR\(\gamma\)), both of which contain the immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic domain (14, 15). Spleen tyrosine kinase (Syk) is recruited to the tyrosine-phosphorylated ITAM and makes a complex with the Tec family kinases activated by RANK, which efficiently induces phospholipase \(\gamma\)1 (PLC\(\gamma\)1) phosphorylation leading to the activation of calcium signal through inositol triphosphate (16). NFATc1 functions as the key regulator of osteoclast differentiation (13, 17, 18) by inducing fusogenic genes such as \textit{Tm7sf4} [encoding dendritic cell-specific transmembrane protein (DC-STAMP)] (19, 20) and \textit{Atp6v0d1} (21) in addition to a number of genes (such as \textit{Acp5}, \textit{Calc}er, and \textit{Igb3}) expressed in mature polarized osteoclasts that are involved in the regulation of bone-resorbing activity (1). Recent reports indicate that NFATc1 activity is negatively regulated by other transcription factors such as IFN regulatory factor-8 (IRF-8) and v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MafB) during osteoclastogenesis (22, 23). The expression of such negative regulators was observed to be repressed through osteoclastogenesis. This repression is consistent with the notion that high NFATc1 activity is a prerequisite for efficient osteoclastogenesis (12), but the mechanism by which the expression of these anti-osteoclastogenic regulators is repressed during RANKL-induced osteoclastogenesis has remained obscure.

To identify the negative transcriptional regulators in osteoclast differentiation, we performed a genome-wide screening of RANKL-inducible transcription factors and found that B lymphocyte-induced maturation protein-1 (Blimp1) (encoded by \textit{Prdm1}) is crucial for the repression of anti-osteoclastogenic genes such as \textit{Irf8} and \textit{Mafb}. Blimp1 is a transcriptional repressor that plays crucial roles in the differentiation and/or function of osteoclasts.

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Data deposition: GeneChip data are available for download from the Genome Network Platform (http://genomenetwork.nig.ac.jp/).

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various kinds of cells such as macrophages and lymphocytes (24). In particular, Blimp1 is necessary for B cells to undergo terminal differentiation into plasma cells through direct repression of transcription factors such as Pax5, Bcl6, and Myc, which are specifically important in the lineage commitment and proliferation of B cells (24, 25).

Here we show that Blimp1 is induced by RANKL through NFATc1 and functions as a global repressor of negative regulators of NFATc1 during osteoclastogenesis. We also provide genetic evidence that Blimp1 is important for bone homeostasis by its promotion of osteoclastogenesis. These results reveal a previously undescribed aspect of the NFATc1-mediated transcriptional program of osteoclastogenesis, the repression of negative regulators by an NFATc1 target gene.

Results and Discussion

Genome-Wide Screening of Transcription Factors Involved in Osteoclastogenesis. To explore the transcription factors involved in osteoclast differentiation, we performed a genome-wide screening of mRNAs expressed in bone marrow-derived monocyte/macrophage precursor cells (BMMs) stimulated by RANKL. We clustered the data of 1,675 transcription factors (26) using k-means algorithm into 22 groups (27). Four clusters of genes exhibited a common expression pattern characterized by a steady increase (by more than 2-fold at each time point after RANKL treatment) (Fig. 1A, Upper). Clusters I, II, III, and IV contained 72, 54, 48, and 7 transcription factors, respectively (Fig. 1A, Upper). From these four clusters, we finally selected 13 genes that are highly expressed in mature osteoclasts (an average difference >500 at 3 days) (Fig. 1A, Lower). Among the 13 genes selected were Nfatc1, Ppargc1b, and Fosl2, which are known to function as transcriptional activators in osteoclast differentiation (13, 28, 29). Blimp1 also has been classified with this group of transcription factors but is known to be a transcriptional repressor required for plasma cell function (24).

We confirmed that both the protein and mRNA expression of Prdm1 increased markedly during osteoclastogenesis (Fig. 1 B and C). Prdm1 was dramatically induced in BMMs stimulated by RANKL in the presence of M-CSF (Fig. 1C). It has been reported that Prdm1 expression increases with the macrophage differentiation induced by phorbol-12-myristate 13-acetate (30), but M-CSF treatment alone did not induce the Prdm1 expression in this course. To test whether Prdm1 induction by RANKL is dependent on NFATc1, we investigated the Prdm1 expression in the presence of a calcineurin inhibitor, cyclosporin A. As expected, Prdm1 induction was greatly suppressed by the treatment with cyclosporin A, suggesting that Prdm1 expression is regulated by NFATc1 (Fig. 1D). We performed a computational search on the sequence from 5 kb upstream to 5 kb downstream of the transcription start site of the Prdm1 gene and found an NFATc1-binding site in a region conserved in the human and mouse sequences (Fig. 1E). Indeed, ChIP analysis showed that NFATc1 binds directly to the site in BMMs stimulated with RANKL for 2 days but not in unstimulated BMMs (Fig. 1E). These results suggest that Prdm1 is a direct transcriptional target of NFATc1 during osteoclastogenesis.

Repression Activity of Blimp1 Is Important for Osteoclast Differentiation. Although it has been reported previously that Blimp1 functions as a transcriptional repressor in other cell types (24, 25), transcription factors can function as either a positive or a negative transcriptional regulator in a context-dependent manner (31). To investigate whether Blimp1 acts as a transcriptional activator or repressor during osteoclast differentiation, we constructed Blimp1 variants that constitutively function as a transcriptional activator or repressor (32). We fused either the transactivation domain of herpes simplex virus VP16 or the repressor domain of Drosophila engraved to Blimp1 (AD-Blimp1 and RD-Blimp1, respectively) (Fig. 2A) and overexpressed these variants in BMMs using the retrovirus vector (Fig. 2B) to test their effects on osteoclast formation. The introduction of RD-Blimp1 into BMMs by retroviral transfer led to increased osteoclast differentiation, whereas the overexpression of AD-Blimp1 inhibited osteoclastogenesis. Overexpression of AD-Blimp1 was confirmed by reverse transcription-PCR (Fig. 2B, Left). Results in the presence of M-CSF and RANKL are shown. **, P < 0.01. (E) Recruitment of NFATc1 to the Prdm1 promoter region. Schematic view of the NFATc1-binding site in the regulatory region of Prdm1 (expanded, Lower Left). DNA sequences conserved between the mouse and human sequences were identified using VISTA Genome Browser (http://genome.ucsc.edu/vista/index.shtml). Arrowheads indicate the primer set used for ChIP. The sites colored red represent the NFATc1-binding site. ChIP assay and representative results (Lower Right).

Fig. 1. Increased expression of Prdm1 during osteoclastogenesis. (A) GenecChip analysis of transcription factor expression during osteoclast differentiation. Among the 22 clusters, clusters I to IV were characterized by a steady increase. The gene expression increased mainly in the early (I), intermediate (II), or late (III) phase, or continuously (IV). The log-transformed value of the mRNA expression of each gene is depicted by a black line, and the average of the black lines is shown in red. (B) Immunoblot analysis to detect the expression of Blimp1 protein in BMMs stimulated with RANKL in the presence of M-CSF. (C) mRNA expression of Prdm1 induction by RANKL treatment alone did not induce the Prdm1 expression in this course. To test whether Prdm1 induction by RANKL is dependent on NFATc1, we investigated the Prdm1 expression in the presence of a calcineurin inhibitor, cyclosporin A. As expected, Prdm1 induction was greatly suppressed by the treatment with cyclosporin A, suggesting that Prdm1 expression is regulated by NFATc1 (Fig. 1D). We performed a computational search on the sequence from 5 kb upstream to 5 kb downstream of the transcription start site of the Prdm1 gene and found an NFATc1-binding site in a region conserved in the human and mouse sequences (Fig. 1E). Indeed, ChIP analysis showed that NFATc1 binds directly to the site in BMMs stimulated with RANKL for 2 days but not in unstimulated BMMs (Fig. 1E). These results suggest that Prdm1 is a direct transcriptional target of NFATc1 during osteoclastogenesis.

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osteoclast formation (Fig. 2 C and D). These results suggest that Blimp1 functions mainly as a transcriptional repressor in the promotion of osteoclast differentiation.

**High Bone Mass Phenotype Induced by Osteoclast-Specific Deficiency of Prdm1.** Because Prdm1-deficient mice are embryonically lethal (25), to investigate the role of Blimp1 in osteoclasts in vivo, we crossed Prdm1lox/– mice (25) with Csklox/+ mice (33) to disrupt the Prdm1 gene specifically in the osteoclast lineage (Prdm1OC–). Microcomputed tomography clearly indicated that the bone volume was greatly enhanced in Prdm1OC– mice at the age of 12 and 32 weeks (Fig. 3A and C). The osteoclast number was significantly reduced in the metaphyseal region, and the bone marrow was abnormally filled with trabecular bone in Prdm1OC– mice (Fig. 3B and C). Bone morphometric analysis indicated an increase in bone volume associated with a reduced osteoclast number and a decrease in the indicators of osteoclastic bone resorption (Fig. 3C). Osteoblastic parameters were slightly decreased in Prdm1OC– mice (Fig. 3C), possibly because of an osteoclast-osteoblast coupling mechanism. These results collectively suggest that the increase in bone mass in Prdm1OC– mice was caused by impaired osteoclastic bone resorption owing to a defect in osteoclast differentiation. Having normal tooth eruption, the mice exhibit a high bone mass phenotype. (A) Histological analysis of the proximal tibia of Prdm1lox/lox and Prdm1OC– mice (Left, toluidine blue staining, purple marks cartilage; Center, von Kossa staining, black indicates mineralized bone; Right, TRAP staining, red marks osteoclasts). (C) Parameters for osteoblastic bone resorption and osteoblastic bone formation in the bone morphometric analysis of Prdm1lox/lox and Prdm1OC– mice at the age of 32 weeks. * P < 0.05; **, P < 0.01; N.S., not significant. (B) Histological analysis of the proximal tibia of Prdm1lox/lox and Prdm1OC– mice (Left, toluidine blue staining, purple marks cartilage; Center, von Kossa staining, black indicates mineralized bone; Right, TRAP staining, red marks osteoclasts). (C) Parameters for osteoblastic bone resorption and osteoblastic bone formation in the bone morphometric analysis of Prdm1lox/lox and Prdm1OC– mice at the age of 32 weeks. * P < 0.05; **, P < 0.01; N.S., not significant.

**Impaired Osteoclastogenesis in Prdm1-Deficient Cells.** In vitro osteoclast differentiation was evaluated by counting the multinucleated cells (MNCs) positive for the osteoclast marker tartrate-resistant acid phosphatase (TRAP) after stimulation of BMMs with RANKL in the presence of M-CSF. The number of TRAP-positive MNCs was markedly reduced in the Prdm1OC– cells compared with the Prdm1lox/lox cells (Fig. 4A). Quantitative genomic PCR analysis indicates that the Prdm1 gene was excised efficiently 2 days after RANKL treatment (Fig. 4B). Consistently, the protein and mRNA expression of Prdm1 was diminished in Prdm1OC– cells 2 days after RANKL stimulation (Fig. 4C and D). These results indicate that the Prdm1 gene was disrupted in the middle stage of osteoclast differentiation (24–48 h after RANKL stimulation in the 72-h period of differentiation). There was no significant difference in the number of CD11b+ cells in the BMMs (Fig. 4E), suggesting that Prdm1OC– cells contain a normal number of osteoclast precursor cells. RANKL-stimulated induction of the osteoclast-specific genes Nfatc1, Acp5, and Ctsk was severely reduced in Prdm1OC– cells (Fig. 4D).
To evaluate the effect of Blimp1 overexpression, we constructed a retroviral vector carrying the HA-tagged Blimp1 gene. We transduced the Prdm1ΔOC/– cells with the viral vector and evaluated the TRAP-positive MNC formation. Impaired osteoclast formation in Prdm1ΔOC/– cells was significantly restored by the overexpression of Blimp1 (Fig. 4F), demonstrating that Blimp1 has an ability to promote osteoclastogenesis.

Blimp1 Represses Expression of the Irf8 and Mafb Genes in Osteoclasts. What are the target(s) of Blimp1-mediated repression in the promotion of osteoclastogenesis? To identify the putative Blimp1-regulated genes, we performed a genome-wide screening of mRNAs during osteoclastogenesis using WT and Prdm1ΔOC/ΔOC BMMs (Fig. 5A, Left and Center). We first comprehensive search for the Blimp1-regulated genes during osteoclastogenesis. The expression of 365 genes decreased more than 2-fold every 24 h during osteoclast differentiation (Left). Of these genes, we identified the expression of 116 genes to be sufficiently recovered (>120%) in Prdm1-deficient cells (Center). Prdm1-deficient cells were generated by retroviral introduction of Cre recombinase into Prdm1fl/fox BMMs. A computational search for the Blimp1-binding site in the region conserved between the human and mouse sequences within 500 kb upstream of the transcriptional start site of 116 genes using the ECR Browser (http://ecrbrowser.dcode.org/) showed that 60 genes, including eight transcription factors, have a putative Blimp1-binding site (Right). The number in parentheses indicates the number of Blimp1-binding sites. (B) Recruitment of Blimp1 to the Irf8 and Mafb regulatory regions. Schematic view of the Blimp1-binding site in the regulatory region of Irf8 and Mafb (Upper). BMMs cultured with RANKL and M-CSF for 2 days were used. Arrowheads indicate the primer sets used for ChIP. Sites colored red represent Blimp1-binding sites. ChIP assay and representative results (Lower). (C) Real-time RT-PCR analysis of Irf8 and Mafb mRNAs in Prdm1ΔOC/ΔOC BMMs stimulated with RANKL for 2 days. *, P < 0.05; **, P < 0.01. (D) Real-time RT-PCR analysis of Irf8 and Mafb mRNAs in HA-tagged Blimp1-expressed BMMs stimulated with RANKL for 2 days. **, P < 0.01. (E) A model of Blimp1-mediated regulation of osteoclast differentiation.
selected genes the expression of which was decreased more than 2-fold every 24 h during osteoclast differentiation in WT cells (Fig. 5A, Left). We further selected genes in which the reduced expression was clearly normalized by the Prdm1 deficiency (Fig. 5A, Center). We computationally searched the 5′-flanking region of these genes for the Blimp1-binding site to identify 60 genes having a putative Blimp1-binding site (Fig. 5A, Right). The eight transcription factors were included in these genes (Fig. 5A, Right). In this study, we focused on Irf8 and Mafb because these genes are reported to be negative regulators of osteoclast differentiation (22, 23). We identified nine Blimp1-binding sites in the 5′-flanking sequence of Irf8 and three Blimp1-binding sites in the 5′-flanking sequence of Mafb. A cluster of Blimp1-binding sites is located ≈5.5 kb upstream from the transcription start site of the Irf8 gene. Indeed, ChIP analysis showed that Blimp1 bound to the sequence containing the cluster in the 5′-flanking sequence of Irf8 (Fig. 5B). Blimp1 also bound to one of the three Blimp1-binding sites located ≈16 kb upstream from the transcription start site of the Mafb gene (Fig. 5B). These results suggest that Blimp1 represses the expression of Irf8 and Mafb by directly binding to their regulatory regions. Consistent with this notion, the expression of Irf8 and Mafb was increased in Prdm1−/−OC−/− cells (Fig. 5C), and the forced expression of Blimp1 decreased the expression of Irf8 and Mafb significantly (Fig. 5D). These results suggest that the Irf8 and Mafb genes are among the direct repressive targets of Blimp1 during osteoclastogenesis.

To investigate whether Blimp1 promotes osteoclastogenesis by the down-regulation of Irf8 and Mafb, we generated retroviral vectors carrying Irf8 or Mafb shRNA. However, the introduction of Irf8 and/or Mafb shRNAs into Prdm1−/−OC−/− BMMs had only a minimal rescue effect (Fig. S1), suggesting that Blimp1 exerts its function through the regulation of multiple genes, including Irf8 and Mafb. Pax5 expression is known to be repressed by Blimp1 in plasma cells (24), and Pax5+ mice exhibit an osteocrogenic phenotype because of an increased number of osteoclasts (34), suggesting that Pax5 is a candidate target of Blimp1 in osteoclasts. However, the contribution of Pax5 to the Blimp1-mediated regulation of osteoclastogenesis may be minimal, because the expression of Pax5 is negligible during osteoclastogenesis based on the GeneChip data. Bcl6 also is repressed by Blimp1, and Blimp1 is negatively regulated by BCL6 in plasma cells (24). Although the shRNA-mediated knockdown of Bcl6 did not normalize the decreased osteoclastogenesis in Prdm1−/−OC−/− cells (Fig. S1), we cannot rule out the possibility that Bcl6 may also contribute to the Blimp1-mediated regulation of osteoclastogenesis in cooperation with Irf8 and Mafb.

Cell lineage commitment is strictly regulated to maintain the homeostasis of the biological systems of the multicellular organisms. To this end, cell differentiation signals usually stimulate the negative-feedback regulatory pathways to correct any excess. During osteoclastogenesis, however, NFATc1 activity and expression are maintained at an extremely high level compared with other cell types (12). Therefore, the expression of molecules that inhibit NFATc1 activity needs to be repressed. Blimp1, which is induced by the RANKL–NFATc1 axis, represents the repressor of anti-osteocrogenic genes (Fig. 5E). It is likely that NFATc1 functions as the crucial regulator of osteoclastogenesis both by inducing osteoclastogenic genes and by acting as a repressor of negative regulatory genes.

Accumulating evidence indicates that immune and bone cells share important regulatory molecules, including receptors, signaling molecules, enzymes, and transcription factors. These findings have shed light on the new interdisciplinary research field of osteoimmunology (1, 2). In particular, an unexpectedly high number of factors that regulate osteoclast differentiation were identified originally in immune cells (1, 2). Blimp1 also has been well characterized as a repressor of genes involved in early B-cell differentiation that enable B cells to undergo terminal differentiation into plasma cells (24). The function of Blimp1 in osteoclasts is analogous to its role in plasma cells, in that Blimp1 represses the genes involved in macrophage development such as Irf8 and Mafb (35, 36), thus converting monocyte/macrophage precursor cells into terminally differentiated osteoclasts.

Because Blimp1 acts as a global repressor of multiple anti-osteocrogenic genes, it is possible that inhibitors of Blimp1 function may serve as antiresorptive agents by inducing various factors affecting osteoclastogenesis. In depth understanding of the gene regulatory programs mediated by Blimp1 should help provide a molecular basis for future therapeutic strategies in bone disease.

Materials and Methods

Mice and Analysis of Bone Phenotype. Prdm1fllox/fl and CtskCremt mice were generated previously and have been described elsewhere (25, 33). Both strains were backcrossed with C57BL6 mice for more than six generations. All mice were born and maintained under specific-pathogen–free conditions. All animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conform to relevant guidelines and laws. CT scanning was performed using a SkyScan Xmate A1000 scanner (Cantatecno). Three-dimensional microstructural image data were reconstructed and structural indices were calculated using TRI/3D-BON software (RATOC Systems). Bone morphometric analysis was performed as described (14, 16).

Cell Cultures. The method for in vitro osteoclast differentiation was described previously (13, 14, 16). In brief, bone marrow cells cultured with 10 ng/mL M-CSF (R&D Systems) for 2 days were used as BMMs, which were further cultured with 50 ng/mL RANKL (Peprotech) in the presence of M-CSF (10 ng/mL) for 3 days.

Flow Cytometric Analysis. Two days after stimulation with RANKL, cells were stained with the PE-conjugated anti-CDC11 antibody (BD Biosciences) or control rat IgG. Stained cells were analyzed with a flow cytometer (FACS-Canto II; BD Biosciences).

Quantitative PCR Analysis. Total RNA and cDNA were prepared by SOGEN (Wako Chemicals) and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Genomic DNA was obtained by phenol/chloroform extraction and ethanol precipitation. Quantitative PCR analysis was performed with a LightCycler (Roche) using SYBR Green (Toyobo). The primer sequences used in real-time RT-PCR analysis were Actb, 5′-CTTCTACAATT-AAGCTGTCCTGGTCGTTG-3′ and 5′-TCATGAGGTCTGCTCTGAGCAG-3′; Prdm1, 5′-GGAAGAATG-GCCAGATGCCAAGAGAAG-3′ and 5′-TCCGACAGGAGGATCTGAGGTT-3′; CtskCre/+, 5′-AGGGACCTAATGCGAGGAGATACTA-3′ and 5′-ATGCCAGAGGCGTGTTCTCTATT-3′; Nfatc1, 5′-GGTTACTCTGTCTTCTCCTATCTGAGCTG-3′ and 5′-GGTATGACCCCAAGTGCAACACAGCA-3′; Prdm1, 5′-TGCTATCAAGGCCACCCC-3′ and 5′-CTTCTGGTGGAGACTGGTGAGAC-3′; Marf, 5′-AACGGTACTGTTGAGGAC-3′ and 5′-TACAGAAAAACTCAAGGAAGAG-3′; Irf8, 5′-AAGGCTACCGTGTCTTTCAG-3′ and 5′-GGAAAGGTCTACTGCTGACGACG-3′; engrailed, 5′-TCCGAAATTTGC-3′ and 5′-GGAAGCTTGAGGCAGGACACGAGCA-3′; and 5′-GGGAATTCTTACAGGCGACCACACCG-3′.

The primer sequences used in quantitative genomic PCR analysis were Prdm1 third exon, 5′-gtcgggcaactgcctgc-3′ and 5′-ctcaaatgctgctgctgc-3′; Prdm1 sixth intron/seventh exon, 5′-caaggtagctgcctgtc-3′ and 5′-tgggctttccgagttggt-3′.

GeneChip Analysis. GeneChip analysis was performed as described (13, 14, 16). Briefly, the total RNAs were used for cDNA synthesis by reverse transcription followed by synthesis of biotinylated cRNA through in vitro transcription. After cRNA fragmentation, hybridization with mouse genome 430 2.0 array
ChIP Assay. ChIP assay was performed by the ChIP Assay Kit (Upstate) according to the manufacturer’s instructions with minor modifications. In brief, BMMs were stimulated with RANKL for 2 days with formaldehyde. After quenching with glycine, cells were lysed and sonicated. Sonicated chromatin was incubated with either rabbit anti-Blimp1 polyclonal antibody (32) or anti-NFATc1 antibody (Santa Cruz). We used rabbit serum or rabbit IgG (Santa Cruz) as a control. Immunoprecipitation was performed using protein A Dynabeads (Invitrogen). After extensive washing, protein-DNA crosslinks were reversed, and the precipitated DNA was treated with proteinase K before phenol chloroform extraction and ethanol precipitation. The primer sequences were Prdm1, 5'-AACAGTGGTGGTACGACCTT-3' and 5'-AGGGCTGTTCCTACTGAGG-3'; Irf8, 5'-TCTCCCTCTCTCCTCCTTA-3' and 5'-AAGGCGGCTGACGACAG-3'; Mafb sense, 5'-CACAGGGAAGAAGCAGCTC-3' and 5'-GCACCTGGACGTGACAG-3'; Mafb site 2, 5'-CCTGGTTCTGTCCTGGAAG-3' and 5'-GGAGGGCTATGAAAGGAGAGG-3'; Mafb site 3, 5'-GCCTGACCTCCTGGATAGAAGA-3' and 5'-CGACTGGGAGCGCTTGATAG-3'.

**Plasmid Construction and Retroviral Gene Transfer.** A retroviral vector, pMX-HA-Blimp1, was constructed by inserting DNA fragments encoding HA and Blimp1 into a retroviral expression vector, pMX-ires-GFP (38). The retroviral vectors pMX-HA-Blimp1 and pMX-AD-HA-Blimp1 were constructed by inserting DNA fragments encoding enveloped or VP16 (32) into pMX-HA-Blimp1. A retroviral vector, pMX-Cre, has been described elsewhere (39). Retroviral packaging was performed by transfecting the plasmids into Plat-E cells using FuGENE 6 (Roche) as described (38). Retroviral particles were used for infection after concentration by centrifugation at 8,000 × g for 16 h. After 12-h inoculation, BMMs were stimulated with RANKL.

**Knockdown Analysis.** We used the RNAi system (BD Biosciences) and constructed the Irf8 and Mafb RNAi-Ready pSIREN vector according to the manufacturer's protocol. Briefly, BMMs stimulated with RANKL for 2 days were transfected with Mafb or control RNAi-Ready pSIREN vector. After 12-h incubation with retroviruses carrying the pSIREN vector, BMMs were stimulated with RANKL followed by selection with 2.5 μg/mL puromycin (Invitrogen).

**Immunoblot Analysis.** Immunoblot analysis was performed using antibodies against Blimp1 (NOVUS), HA (Abgent), and Lammin1 (Santa Cruz) as previously described (40). Anti-Lamin1 antibody was used as an internal control.

**Statistical Analysis.** Statistical analysis was performed using the unpaired two-tailed Student’s t test. All data are expressed as the mean ± SEM.

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Supporting Information

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Fig. S1. Effects of retroviral expression of Irf8, Mafb, and Bcl6 shRNA on osteoclastogenesis on Prdm1^{flox/flox} and Prdm1^{ΔOc/–} bone marrow-derived monocyte/macrophage precursor cells stimulated with RANKL.