MITF and PU.1 Recruit p38 MAPK and NFATc1 to Target Genes during Osteoclast Differentiation

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Transcription factors NFATc1, PU.1, and MITF collaborate to regulate specific genes in response to colony-stimulating factor-1 (CSF-1) and receptor activator of NF-κB ligand (RANKL) signaling during osteoclast differentiation. However, molecular details concerning timing and mechanism of specific events remain ill-defined. In bone marrow-derived precursors, CSF-1 alone promoted assembly of MITF-PU.1 complexes at osteoclast target gene promoters like cathepsin K and acid 5 phosphatase without increasing gene expression. The combination of RANKL and CSF-1 concurrently increased the levels of MAPK-phosphorylated forms of MITF, p38 MAPK, and SWI/SNF chromatin-remodeling complexes bound to these target promoters and markedly increased expression of the genes. NFATc1 was subsequently recruited to complexes at the promoters during terminal stages of osteoclast differentiation. Genetic analysis of Mitf and Pu.1 in mouse models supported the critical interaction of these genes in osteoclast differentiation. The results define MITF and PU.1 as nuclear effectors that integrate CSF-1/RANKL signals during osteoclast differentiation to initiate expression of target genes, whereas a complex that includes NFATc1 may act to maintain target gene expression in differentiated cells.

Bone is a dynamic tissue that maintains both physical integrity and calcium homeostasis in vertebrate species. Consequently, bone is modeled and continuously remodeled beginning in embryogenesis and throughout the lifetime of mammals and other animals. Bone integrity and function are maintained by an exquisite balance between the two major cell types involved in the remodeling process, the osteoblast and the osteoclast (1–3). Osteoblasts, originating from mesenchymal progenitor cells, are responsible for the mineralization of bone matrix. Osteoclasts are of hematopoietic origin and are highly specialized multinuclear cells capable of resorbing bone. It is now well accepted that many human disorders of bone, including common human disorders like osteoporosis, Paget’s disease, rheumatoid arthritis, and cancer bone metastases, reflect an imbalance in the differentiation and function of these two cell types (1, 4).

Two essential cytokines, macrophage colony-stimulating factor-1 (CSF-1) and receptor activator of NF-κB ligand (RANKL), are produced by osteoblasts and are necessary and sufficient for osteoclast differentiation (5–8). Signaling pathways engaged by these cytokines can activate a number of transcription factors required for osteoclast differentiation, including NF-κB, c-Fos, NFATc1, PU.1, and MITF (2, 9–13). Of these, MITF (microphthalmia-associated transcription factor) is a basic helix-loop-helix leucine zipper protein closely related to the transcription factor E (TFE) family, composed of TFE3, TFEB, and TFEC gene products (14, 15). MITF has been implicated in the survival and differentiation of developmentally unrelated cell types, including melanocytes and osteoclasts (15, 16). Strong evidence for a critical role of MITF and TFEB in terminal osteoclast differentiation is provided by the severe osteopetrotic and osteoclast phenotype in mice homozygous for Mitf- (mi) or Mitf- or alleles, or double mutants homozygous for the Mitf-vga hypomorphic allele and a Tfe3 null allele (16–18).

How MITF selectively affects gene expression and differentiation of developmentally unrelated cell types is a question of general biological interest. In osteoclasts MITF has been shown to regulate three definitive osteoclast target genes, tartrate-resistant acid phosphatase/acid phosphatase 5 (TRAP/Acp5) (19), cathepsin K (Ctsk) (20), and Oscar (21). Interactions between MITF and the Erythroblastosis virus E26 oncogene homolog (ETS) family transcription factor PU.1 at least partly account for the ability to regulate these target genes in osteoclasts (21, 22). However, MITF and PU.1 are expressed in macrophages and osteoclasts, as well as the mononuclear precursors for both of these cell types, posing an additional question about how correct gene regulation patterns are maintained in different cell

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types. In recent work, we demonstrated that subcellular localization of MITF was affected by signaling-dependent interactions with 14-3-3 proteins, providing one mechanism that regulates MITF activity in myeloid precursor cells (23). In addition, MITF, PU.1, and NFATc1 can act in combination to synergistically activate reporter genes, and both PU.1 and NFATc1 have been localized to the Ctsk promoter by the chromatin immunoprecipitation technique (24). These data suggest that NFATc1 may be the key factor whose expression and activation distinguish between genes regulated by MITF and PU.1 in macrophages versus osteoclasts (21, 24, 25).

In the present work, CSF-1 and RANKL are demonstrated to regulate the nuclear activity of MITF and PU.1, resulting in increased occupancy of osteoclast target promoters by the phosphorylated, activated form of MITF, and in the co-recruitment of the SWI/SNF chromatin-remodeling complex and activated p38 mitogen-activated protein kinase (MAPK). Following these events, NFATc1 is recruited to target promoters. These results indicate that signaling-dependent regulation of MITF and PU.1 is critical in the initiation of transcription of target genes during osteoclast differentiation.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies against MITF and phospho-S307-MITF were described previously (11), anti-phospho-S73 antibody was kindly provided by David E. Fisher (Boston, MA). Anti-Pu.1 antibody was raised in rabbits against the peptide DLV TYDSELYORPMHDYC representing amino acids 18–35 of mouse Pu.1 and affinity-purified using a column containing the target peptide (BIOsource, Hopkinton, MA). Anti-NFATc1 antibody and RNA polymerase antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-p38 MAPk and histone H3 were purchased from Upstate Cell Signaling (Charlottesville, VA). Anti-BRG1, anti-BAF57, and anti-BAF155 antibodies are described elsewhere (26). Monoclonal RNA polymerase II H5 antibody, which recognizes phosphorylated serine 2 in the C-terminal repeat domain, was from Covance (Covance Research Products, Berkeley, CA).

**Culture and Analysis of Osteoclast-like Cells**—Detailed procedures for OCL differentiation have been described previously (27, 28). Briefly, hematopoietic precursors were obtained from spleens of wild type and mutant mice. OCLs were grown in Dulbecco’s modified Eagle’s medium containing 50 ng/ml CSF-1 for 3 days on non-tissue culture plastic dishes; at this point, the non-adherent cell fraction was isolated for further studies. For CSF-1 starvation, non-adherent cells were washed twice, plated on tissue culture dishes, and maintained in medium without CSF-1 for 12 h. For differentiation, non-adherent cells were transferred to tissue culture dishes and treated with 50 ng/ml CSF-1 and 100 ng/ml RANKL for different times indicated in the figures.

**Chromatin Immunoprecipitation, Sequential Chromatin Immunoprecipitation (ReChIP), and qPCR**—Chromatin immunoprecipitation (ChIP) assays were performed as described by Luo et al. (29). Briefly, osteoclast precursors were plated at a density of 3 x 10⁶ cells per 10-cm dish and treated with cytokines for various times as indicated in the figure legends, and cells were cross-linked with 1% final concentration of formaldehyde at 37 °C for 10 min before harvest. Soluble chromatin was prepared following sonication with a Branson 250 digital sonifier (Branson Ultrasonics, Danbury, CT) to an average DNA length of 200–1000 bp. ~5 x 10⁷ cell equivalent (one-sixth) of the sheared soluble chromatin was pre-cleared with tRNA-blocked Protein G-agarose, and 10% of the pre-cleared chromatin was set aside as input control. Immunoprecipitation was carried out with 5 μg of antibodies as indicated in the figures overnight at 4 °C. Immune complexes were pulled down using Protein G-agarose, washed, and eluted twice with 250 μl of elution buffer (0.1 M NaHCO₃, 1% SDS), and cross-linking was reversed in 200 mM NaCl at 65 °C overnight with 20 μg of RNase A (Sigma). DNA was purified following protease K treatment (Invitrogen) with the Qiagen PCR purification kit using the manufacturer’s instructions.

Samples were analyzed by real-time PCR either by SYBR Green super mix (Bio-Rad) for Ctsk promoter or by the Roche universal probe library (Roche Diagnostics, Indianapolis, IN) probe using the Faststart TaqMan master kit (Roche Diagnostics) for Acp5 promoter. The threshold for the promoter being studied was adjusted by that of input values and represented as relative abundance. All qPCR reactions were analyzed by melt curve analysis and agarose gels to confirm the presence of a single specific band.

For ReChIP assays, pre-cleared soluble chromatin from 6 x 10⁶ cells harvested from two 100-mm culture dishes were immunoprecipitated with Pu.1 antibody. After washing, the Pu.1-immune complex was disrupted with 10 mM dithiothreitol at 37 °C for 30 min with shaking and diluted 50-fold with the ChiP buffer. This eluted immune complex was divided and immunoprecipitated with the second specific antibodies as indicated in the figures, and the complexes were analyzed as above.

**Immunoprecipitation, Immunofluorescence, Western Blotting, and GST Pulldown Assays**—Procedures for immunoprecipitation, Western blotting, immunofluorescence, and GST pulldown assays have all been recently described (23).

**Analysis of RNA Expression**—RNA was extracted using TRIzol (Invitrogen). Residual contaminating genomic DNA was removed using 2 units of DNase 1 (Roche Applied Science) for 20 min at room temperature, and RNA was purified with the RNeasy kit (Qiagen). 2 μg of purified total RNA was reverse transcribed by Superscript III reverse transcriptase (Invitrogen) with random hexamer primers. Primers used for real-time PCR were picked by Oligo v4.0 software, and sequences used are available upon request. The real-time PCR was conducted using SYBR Green super mix (Bio-Rad) in an iCycler real-time detection system (Bio-Rad). The PCR threshold was determined by using the iCycler PCR baseline-subtracted curve fit method. The threshold for the gene being studied was adjusted by that of a reference gene (ribosomal protein L4). All reactions were examined by agarose gels to confirm the presence of a single specific band.

**Mouse Mutants and Bone Histomorphometry**—All mice were maintained on a C57B/6J background. Wild-type mice were used to make bone marrow precursors for the in vitro experiments. To generate mice for the genetic experiments mice het-
ereosynovial osteoclasts. Images of stained sections were acquired using a Spot 2 digital charge-coupled device camera on a Nikon E-1000 microscope, and ImagePro Plus was used to quantify the association of MITF and PU.1 with regulatory regions and 3′-regions. Analysis of regions corresponding to internal exons/introns (also depicted in Fig. 1B) were used as negative controls. MITF and PU.1 could not be detected at either of these promoters (Fig. 1C). In addition, PU.1, despite the nuclear localization of this factor, could not be detected at either of these promoters (Fig. 1C). After addition of CSF-1, MITF and PU.1 were enriched at both promoters (Fig. 1C), indicating that MITF plays a crucial role in the initial recruitment of PU.1 to these promoters. Quantitative real-time PCR (qPCR) was used to confirm the results (supplemental Fig. S1B).

In addition to the 5′-region of the Ctsk and Acp5 genes, 3′-regions corresponding to internal exons/introns (also depicted in Fig. 1B) were used as negative controls. MITF and PU.1 could not be detected at this portion of the Ctsk or Acp5 genes (Fig. 1C). Additional negative controls performed were a reaction in which preimmune IgG (no antibody) was included, and analysis of the 5′-regulatory region of the Hprt gene, a gene that is not regulated by MITF or PU.1 (Fig. 1C). Histone H3 was routinely used as a positive control for all ChIP experiments, and signals

FIGURE 1. CSF1 promotes nuclear localization of MITF and recruitment of MITF-PU.1 complex to Ctsk and Acp5 promoters. A, immunofluorescence analysis of MITF and PU.1 subcellular localization with or without CSF-1. Murine bone marrow cell culture from which CSF-1 was withdrawn for 12 h (−) and supplemented for 12 h (+) as indicated were fixed and analyzed for subcellular localization of MITF and PU.1 using specific antibodies. Scale bar, 5 μm. The cells were scored for cytoplasmic or nucleocytoplasmic staining from five experiments, and results are available in supplementary Fig. S1. B, graphical representation of regions analyzed for Ctsk and Acp5 promoters by ChIP, including both 5′ regulatory regions and 3′ regions. C, analysis for the association of MITF and PU.1 with Ctsk and Acp5 promoters in the absence or presence of CSF-1 by qPCR; shown are PCR products that are present after 35 cycles. P, PCR with primers flanking MITF and PU.1 sites in promoter as depicted in B; E, PCR with primers that flank the intron/exon regions. For CSF-1: −, cell culture from which CSF-1 was withdrawn for 12 h; +, cells that were grown with CSF-1 alone.

The affect of CSF-1 on MITF and PU.1 nuclear function was studied using the technique of chromatin immunoprecipitation (ChIP). Fig. 1B represents the regions of Ctsk and Acp5 genes that were analyzed following the ChIP technique, regions previously demonstrated to contain binding sites for these transcription factors (19, 20) (see supplemental Fig. S2A for sequences of the promoter regions containing binding sites for these factors). Upon withdrawal of CSF-1, as expected from previous results, MITF was not found at Ctsk and Acp5 promoters (Fig. 1C). In addition, PU.1, despite the nuclear localization of this factor, could not be detected at either of these promoters (Fig. 1C). After addition of CSF-1, MITF and PU.1 were enriched at both promoters (Fig. 1C), indicating that MITF plays a crucial role in the initial recruitment of PU.1 to these promoters. Quantitative real-time PCR (qPCR) was used to confirm the results (supplemental Fig. S1B).

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RESULTS

CSF-1 Promotes Recruitment of MITF-PU.1 Complexes to Cathepsin K and Acid Phosphatase Promoters—

We recently demonstrated that the combined action of CSF-1 and RANKL regulated the subcellular localization of MITF (23). To extend these observations and dissect the effect of CSF-1 alone on MITF function, the subcellular localization of MITF and its association with target promoters was analyzed as a function of CSF-1 stimulation. When CSF-1 was withdrawn from bone marrow-derived macrophage cultures for 12 h, indirect immunofluorescence demonstrated that MITF was found predominantly in the cytoplasm in 80% of cells studied (Fig. 1A and supplementary Fig. S1A). Addition of CSF-1 to these cytokine-deprived cells for 12 h resulted in the partial nuclear localization of MITF, with >80% of cells analyzed having both nuclear and cytoplasmic staining for MITF (Fig. 1A and supplementary Fig. 1A). In contrast, CSF-1 withdrawal and restimulation didn’t significantly affect the nuclear localization of PU.1 (Fig. 1A).
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![Diagram](image)

**FIGURE 2.** RANKL signaling is required for the induction of MITF target genes. A, analysis of osteoclast differentiation by TRAP staining. Cells that were treated with CSF-1 alone or CSF-1 and RANKL for 3 and 5 days were stained for TRAP activity as explained under “Experimental Procedures.” B, relative expression of Ctsk and Acp5 genes from osteoclast precursors treated with CSF-1 alone or CSF-1 and RANKL for 0.5, 3, and 5 days were measured by qRT-PCR. Average of five experiments with RNA isolated in parallel from cells used for ChIP experiments presented in Figs. 2–5 are presented. C, ChIP of osteoclast precursors treated with CSF-1 alone or CSF-1 and RANKL for 0.5, 3, and 5 days with MITF and PU.1 antibodies. Immunoprecipitated chromatin from two independent experiments was analyzed by qPCR for the presence of Ctsk and Acp5 promoter levels that were normalized to the respective input levels. Error bars in all panels represent ±S.D.

were observed for specific antibodies with all primer sets used (Fig. 1C). The same set of negative controls was used for all subsequent ChIP experiments presented for both Ctsk and Acp5 genes (see supplemental Figs. S2–S5 for data).

Although CSF-1 promoted nuclear localization of MITF and recruitment to osteoclast target promoters, CSF-1 alone was unable to promote differentiation of osteoclast-like cells (OCLs) in vitro (Fig 2A) or to activate expression of the Ctsk and Acp5 genes (Fig. 2B). In contrast, the combination of CSF-1 and RANKL induced visible OCL formation after 3 days of treatment and resulted in robust expression of Ctsk and Acp5 genes. After 5 days of cytokine treatment, OCL differentiation was complete, and expression of target genes was at maximal levels (Fig. 2, A and B). ChIP assays were used over this time course of differentiation to determine if MITF-PU.1 complexes were further enriched at target promoters by the action of both cytokines versus CSF-1 alone (Fig. 2C; supplemental Fig. S2B for controls). The combination of CSF-1 and RANKL together for either 0.5 or 3 days resulted in only a marginal increase of <2-fold in occupancy of these two promoters by MITF or PU.1, a change that was not significantly different from the CSF-1 treatment (Fig. 2C, second and third bar graphs in each panel). After addition of both cytokines for 5 days, when OCL differen-

tiation is completed, occupancy of Ctsk and Acp5 promoters by both MITF and PU.1 were maximal but only 2- to 3.5-fold higher than the CSF-1-only treatment (Fig. 2C, fourth bar graph). These data indicate that there are only small changes in the occupancy of these target promoters by MITF and PU.1 over the time course of OCL differentiation, which correlate well with the robust increases in gene expression observed after 3 days of CSF-1/RANKL treatment.

Phosphorylated, Activated Forms of MITF and p38 MAPK Are Present at Target Promoters following CSF-1/RANKL Stimulation—MITF is targeted by both Erk and p38 MAPK pathways in a CSF-1/RANKL-dependent fashion, at residues Ser-73 and Ser-307, respectively, and both phosphorylation events increase MITF activity (11, 12). Using phospho-specific MITF antibodies available for each of these residues, we analyzed whether chromatin-associated MITF located at the Ctsk promoter was phosphorylated at these positions (Fig. 3, A and B; supplemental Fig. S3A for controls). In bone marrow-derived cells that were grown with CSF-1 alone an enrichment of pSer73-MITF at the Ctsk promoter was observed, but pSer307-MITF was not detected. CSF-1 and RANKL treatment for 0.5 days had no additional effect on the level of pSer73-MITF at the Ctsk promoter, but the abundance of pSer307-MITF was substantially increased (Fig. 3B). After 3 days of combined CSF-1/RANKL stimulation, the levels of both pSer73 and pSer307 forms of MITF at the Ctsk promoter were maximal. The pSer73 form of MITF increased ~10-fold at this time, whereas the pSer307 form increased an additional 3-fold above levels detected following 0.5 day of treatment. After 5 days of cytokine treatment, the chromatin-associated levels of both phosphorylated forms of MITF remained elevated and not significantly different from the levels seen at the 3-day time point.

Whether the kinases responsible for phosphorylation of these sites were also enriched at the Ctsk promoter was examined (Fig. 3C and supplemental Fig. S3B for controls). This analysis demonstrated that the activated, phosphorylated form of p38 MAPK was not detected in cells treated with CSF-1 alone, but chromatin-associated phospho-p38 MAPK could be observed in cells treated with both CSF-1 and RANKL (Fig. 3C, gray bar graphs). The levels of phospho-p38 were also maximal after 3–5 days of treatment, increasing ~3-fold over the level seen at 0.5 day of cytokine treatment. In contrast, Erk1 and -2 were not detected at the Ctsk promoter under the same conditions (Fig. 3C, black bar graphs).

To more directly address whether phosphorylation of MITF and recruitment of p38 MAPK correlates with transcriptional activation of the target genes, ChIP was used to determine occupancy of the target promoters by RNA polymerase II (pol II) (Fig. 3D and supplemental Fig. S3B). An antibody that recognizes total RNA pol II, as well as one that recognizes pol II phosphorylated on the C-terminal tail, were used. The experiments demonstrated that levels of RNA pol II at the promoters correlate well with osteoclast differentiation, target gene expression, and the presence of phosphorylated MITF phospho-p38 MAPK than with the presence of the MITF-PU.1 complex alone.
Enrichment of NFATc1-associated Ctsk and Acp5 Promoters Is Observed in Activated Osteoclasts—NFATc1 plays a central role in osteoclast differentiation and has been shown to collaborate with MITF and PU.1 in activation of Ctsk and Acp5 promoters (13, 24, 25) (see supplemental Fig. S2A for sequences of the promoter regions). The kinetics of NFATc1 recruitment to the Ctsk and Acp5 promoter was examined in the in vitro differentiation system (Fig. 4A and supplemental Fig. S4). Unexpectedly, qPCR analysis following ChIP demonstrated that NFATc1 was enriched at both Ctsk and Acp5 promoters only after 5 days of CSF-1/RANKL treatment (Fig. 4A). As expected, treatment of cells with CSF-1 alone for 5 days did not result in NFATc1 recruitment to the target promoters (supplemental Fig. S4B). Kinetically, recruitment of NFATc1 followed robust expression of these genes, recruitment of RNA pol II (Figs. 2B and 3D), and enrichment of the activated, phosphorylated forms of MITF and of activated p38 MAPK (Fig. 3).

To verify that the three transcription factors were present at these promoters in the same cells, at day 5 of differentiation, sequential ChIP, or “ReChIP,” experiments were performed using the PU.1 antibody (Fig. 4B). In preliminary experiments the PU.1 antibody was found to be more avid and thus better suited to this approach than either MITF or NFATc1 antibodies, i.e. more DNA was recovered with the PU.1 antibody allowing several antibodies to be used simultaneously in the ReChIP. Cross-linked chromatin was first immunoprecipitated with PU.1 antibody, and the antibody complex was dissociated (see “Experimental Procedures”). Subsequently, equal portions of the material recovered from the PU.1 ChIP were simultaneously re-precipitated with MITF, NFATc1, or PU.1 antibodies. The amount of DNA present in the second PU.1 precipitation determined by qPCR was assumed to be 100%, and relative MITF and NFATc1 levels were calculated as % co-occupancy (gray bars). Supernatants after the second immunoprecipitation were examined by qPCR (dark bars), demonstrating that little Ctsk promoter DNA remained after the second immunoprecipitation. All data presented in A and B are the mean of two independent experiments done in duplicate. Error bars indicate ± S.D.

Enrichment of NFATc1-associated Ctsk and Acp5 Promoters Is Observed in Activated Osteoclasts—NFATc1 plays a central role in osteoclast differentiation and has been shown to collaborate with MITF and PU.1 in activation of Ctsk and Acp5 promoters (13, 24, 25) (see supplemental Fig. S2A for sequences of the promoter regions). The kinetics of NFATc1 recruitment to the Ctsk and Acp5 promoter was examined in the in vitro differentiation system (Fig. 4A and supplemental Fig. S4). Unexpectedly, qPCR analysis following ChIP demonstrated that NFATc1 was enriched at both Ctsk and Acp5 promoters only after 5 days of CSF-1/RANKL treatment (Fig. 4A). As expected, treatment of cells with CSF-1 alone for 5 days did not result in NFATc1 recruitment to the target promoters (supplemental Fig. S4B). Kinetically, recruitment of NFATc1 followed robust expression of these genes, recruitment of RNA pol II (Figs. 2B and 3D), and enrichment of the activated, phosphorylated forms of MITF and of activated p38 MAPK (Fig. 3).
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FIGURE 5. BRG1/mSWI/SNF recruitment to Ctsk and Acp5 promoters during OCL differentiation. A, ChIP analysis using antibodies specific for BRG1, BAF57, and BAF155, respectively, for the presence of Ctsk promoter at indicated cytokine treatments and time points. B, ChIP analysis for the Acp5 promoter, the same as described in A for Ctsk. C, sequential ChIP, using PU.1 as the first antibody followed by PU.1, Brg1, Baf57, and Baf155 antibodies for the second immunoprecipitation. PU.1 levels were set to 100% as in Fig. 3 (gray bars). Supernatants after the second immunoprecipitation were examined by qPCR ([open bars]). All data presented in A–C are the mean of two independent experiments done in duplicate. Error bars indicate ± S.D.

the presence of these proteins in a single complex (Fig. 4B, black bar graphs). Combined, these results strongly indicated that the majority of all three proteins are present at the target promoters within the same cells.

Recruitment of BRG1 SWI/SNF Complexes to Target Promoters Correlates with Enrichment of Phosphorylated Forms of MITF—To begin addressing the potential mechanism of MITF and PU.1 action, the recruitment of SWI/SNF chromatin-remodeling complexes was studied using ChIP (Fig. 5 and supplemental Fig. S5A). This analysis demonstrated that BRG1, the ATPase-dependent chromatin-remodeling subunit, was not recruited to the Ctsk and Acp5 promoters following CSF-1 treatment alone for 12 h (Fig. 5A), or even for 5 days (supplemental Fig. S5B), or by both CSF-1 and RANKL after 0.5 day of treatment. In contrast, BRG1 could be detected following 3 and 5 days of RANKL/CSF-1 stimulation (Fig. 5A). Similarly, two subunits of mSWI/SNF, BAF57 and BAF155, were also recruited to the Ctsk and Acp5 promoters, strongly suggesting that the entire mSWI/SNF complex was likely present.

ReChIP of PU.1 chromatin complexes again demonstrated that ~40–60% of both Ctsk and Acp5 promoters demonstrated co-occupancy of BRG1, BAF57, and BAF155. Analysis of the supernatant after the second immunoprecipitation detected little promoter DNA signal remaining (Fig. 5B).

MITF can physically interact with factor BRG1 in overexpression studies in fibroblasts (30), but whether physical interactions between BRG1 and MITF could be detected in osteoclasts has not been established. Mammalian expression vectors containing GST-MITF and BRG1 expression were co-transfected into COS-7 cells, and GST pulldown assays were performed on cell lysates (Fig. 6A). These experiments showed that MITF and BRG1 could be found in the same complex (Fig. 6A). Co-immunoprecipitation assays were performed on an RAW264.7 cell line engineered to stably express a FLAG-tagged version of MITF at levels similar to that of endogenous MITF (23). Consistent with the ChIP results, a complex containing both FLAG-MITF and BRG1 could be detected only after treatment of these cells with the combination of CSF-1 and RANKL (Fig. 6B).

Similarly, transiently expressed FLAG-tagged MITF could be co-immunoprecipitated with endogenous BRG1 only after RANKL treatment of RAW264 cells (Fig. 6C). In contrast, FLAG-MITF with a S307A mutation interacted with endogenous BRG1 significantly less efficiently than wild-type in the transient assays (Fig. 6C). Finally, a complex containing both MITF and BRG1 could be detected in primary bone marrow-derived cells treated with the combination of RANKL and CSF-1 for 3–5 days (Fig. 6D, lanes 2 and 3) but not in cells treated with CSF-1 alone (Fig. 6D, lane 1). These results suggest that BRG1 chromatin-remodeling complexes to osteoclast target promoters could be mediated through signal-dependent physical interactions between MITF and BRG1. However, these results do not rule out the possibility that interactions with PU.1 and/or NFATcC1 could also be involved in recruitment of BRG1 to target promoters.

Genetic Interactions between MITF and PU.1 Mutant Alleles Result in Impaired Osteoclast Function and Osteopetrosis—The data obtained during in vitro differentiation of OCLs implicates
TABLE 1

| Pu.1 genotype | Mitf-genotype | No. mice analyzed | No. Op* mice | Op* mice |
|---------------|---------------|-------------------|--------------|----------|
| +/+           | +/+           | 5                 | 0            | 0        |
| +/−           | +/+           | 10                | 0            | 0        |
| +/−           | mi+/+         | 10                | 0            | 0        |
| +/−           | mi/vga        | 18                | 7            | 38.8     |
| +/−           | mi/vga        | 12                | 7            | 100      |

*Op indicates severe osteopetrosis as determined by radiography and failure of tooth eruption.

MITF and PU.1 as key regulators of osteoclast-specific gene expression during differentiation. To confirm these in vitro observations in vivo, the interaction of two MITF alleles, Mitf<sup>mi</sup> and Mitf<sup>vga</sup>, and a Pu.1 knock-out allele were studied. Mitf<sup>mi</sup> is the result of an in-frame deletion of one of four Arg residues in the basic domain that are required for efficient binding to target promoter (15, 16), whereas the hypomorphic MITF<sup>hma</sup> allele is due to insertion of a transgene in the region 5’ to the structural MITF gene and results in a 10-fold reduction in the level of Mitf RNA produced. Previous work by our group demonstrated that ~25% of mice with the genotype Mitf<sup>mi/+;Pu.1<sup>+/−</sup> display a weak osteopetrotic phenotype, whereas 100% of either MITF<sup>mi/mi</sup> or Pu.1<sup>−/−</sup> mice develop severe osteopetrosis (19).

Although Mitf<sup>mi/+</sup> heterozygous mice have no bone phenotype, lowering the dose of wild-type MITF in Mitf<sup>mi/vga</sup> mice (with wild-type PU.1) resulted in severe osteopetrosis in 38% of mice (7/18) throughout their lifetime, including failure of tooth eruption (Table 1 and Fig. 7A). To confirm that the defects in osteoclast differentiation and function were cell autonomous, differentiation of spleen precursors derived from 4-day-old mice in response to CSF-1 and RANKL was studied (Fig. 7, C and D). Following differentiation in response to CSF-1/RANKL, cells from the Mitf<sup>mi/vga</sup> mice exhibited a few di- and tri-nuclear cells that were Acp5-positive, but differentiation was clearly greatly reduced compared with control (Fig. 7C, middle panel). Cells from the Mitf<sup>mi/vga</sup>, Pu.1<sup>−/−</sup> mice failed to visibly differentiate (Fig. 7C, right panel). RNA was isolated from these cells after 5 days of treatment with CSF-1/RANKL and analyzed by qPCR (Fig. 7D). This analysis demonstrated that the Mitf<sup>mi/vga</sup>;Pu.1<sup>−/−</sup> cells did not express either Acp5 or Ctsk genes in response to CSF-1/RANKL stimulation. Ctsk RNA was not induced in cells from Mitf<sup>mi/vga</sup> mice, whereas Acp5 RNA expression was ~3-fold lower than controls, consistent with the less severe osteoclast phenotype of these mice.

DISCUSSION

Osteoclast differentiation is regulated by a group of ubiquitous transcription factors instead of by cell type-specific transcription factors. These factors, including MITF, PU.1, NFATc1, NF-kB, and c-Fos, individually are expressed in many different cell types and cell lineages but collectively orchestrate a program of gene expression that leads to osteoclast differentiation (2). How the actions of these factors are coordinated, especially at distinct stages of osteoclast differentiation, remain largely unknown. A more detailed understanding of how these factors act during osteoclast differentiation will likely provide insights into how osteoclast dysfunction contributes to common human bone disorders, as well as defining molecular targets that might have translational potential in diagnosis and treatment of bone diseases.

Our results demonstrate a molecular sequence for a subset of genes expressed during osteoclast differentiation. CSF-1 alone can regulate nuclear localization and recruitment of MITF to select target promoters in osteoclast precursors, and MITF recruitment to these promoters resulted in the co-recruitment of PU.1. However, gene expression was not activated by the MITF-Pu.1 complex when CSF-1 alone was present. The combination of CSF-1 and RANKL signaling led to increased levels of phosphorylated, activated MITF that correlated well with recruitment of RNA pol II and robust target gene expression. CSF-1/RANKL likely affect MITF phosphorylation by increasing the level of activated Erk and p38 MAPK in the nucleus, in fact, p38 MAPK was directly recruited to the target promoters at the same time the phosphorylated forms of MITF appear. This results in the recruitment of co-activators that remodel chromatin at target promoters, and subsequently to the recruitment of NFATc1 (see model, Fig. 8). Thus, the MITF-Pu.1 complex acts to integrate CSF-1/RANKL signals directly at target promoters, resulting in downstream events that lead to target gene activation.

Expression of NFATc1 is induced by CSF-1/RANKL during osteoclast differentiation, and this factor has been implicated as a “master regulator” of osteoclast differentiation (13, 31). In addition, previous results have shown that the combination of MITF, PU.1, and NFATc1 acts synergistically to activate osteoclast target promoters (24, 25). These findings were interpreted to indicate that osteoclast gene expression initiates with the accumulation of NFATc1, because MITF and PU.1 are expressed in both bone marrow precursors and macrophages (24, 25). In contrast, our results demonstrate that the occupancy of at least some target promoters by an active MITF-Pu.1 complex that includes p38 MAPK and BRG1 correlated well with the initiation of osteoclast differentiation and robust target gene expression following 3 days of cytokine treatment (Fig. 8). The detection of NFATc1 in these promoter complexes instead correlated with the maintenance of expression of these genes in multinuclear osteoclasts capable of bone resorption that are present following 5 days of cytokine treatment (Fig. 8).
Our results do not rule out that NFATc1 can regulate other genes important for osteoclast differentiation and function, for example the calcitonin receptor gene and the β3 integrin gene, in an MITF-PU.1-independent fashion (32, 33). However, the results presented support the conclusion that MITF-PU.1 complex integrates signals necessary for the appropriate temporal regulation of select osteoclast genes like Ctsk and Acp5 during terminal differentiation.

The mechanism underlying MITF-PU.1 action involves recruitment of BRG1-SWI/SNF complexes that can remodel chromatin structure. Such changes in chromatin structure likely facilitate the subsequent recruitment of NFATc1 to these regulatory regions. Direct interactions of NFATc1 with PU.1 have also been reported (24), and such direct interactions could provide an additional level of molecular specificity. The combination of all three transcription factors may also lead to increased recruitment of co-activators like BRG1, facilitating additional modification of chromatin structure and recruitment of RNA polymerase II.

The results of genetic studies with Mitf⁻/⁻, Mitfvga⁻/⁻, and a PU.1 null allele confirm a central role for interaction between these two factors during terminal osteoclast differentiation in vivo, extending our previous studies that identified a partially penetrant bone phenotype in 25% of Mitf⁻/⁻; Pu.1⁻/⁻ mice (19). A strong osteopetrotic phenotype was readily detected in 38% of mice with genotype Mitf⁻/⁻;Pu.1⁻/⁻, whereas lowering Pu.1 gene dosage by one-half increased the bone and osteoclast phenotype of Mitf⁻/⁻ mice to 100%. Taken together with the lack of an osteoclast phenotype in Mitf⁻/⁻ mice, these results are

**FIGURE 7.** Genetic interactions between Mitf and Pu.1 alleles result in impaired osteoclast function and osteopetrosis. A, radiographic analyses of long bones of 30-day-old wild-type, Mitf⁻/⁻;Pu.1⁻/⁻, and Mitf⁻/⁻;Pu.1⁻/⁻ mice showing severe osteopetrosis. B, histomorphometric analysis was performed on TRAP-stained long bone sections of wild-type, Mitf⁻/⁻;Pu.1⁻/⁻ and Mitf⁻/⁻;Pu.1⁻/⁻ for the indicated static bone and osteoclast parameters. C, cell autonomous defects in multinuclear osteoclast formation in Mitf⁻/⁻;Pu.1⁻/⁻ and Mitf⁻/⁻;Pu.1⁻/⁻ mice compared with wild-type mice. In vitro differentiated osteoclasts from 4- to 5-day-old mouse splenocytes were fixed and stained for TRAP activity. D, analysis of expression by qPCR of osteoclast specific genes from wild-type and Mitf⁻/⁻;Pu.1⁻/⁻ and Mitf⁻/⁻;Pu.1⁻/⁻ mutant mice as indicated. Average expression of Ctsk and Acp5 genes from differentiating osteoclasts were normalized against ribosomal protein L4 levels.

**FIGURE 8.** Schematic representation of events during osteoclast differentiation. Bone marrow hematopoietic precursors treated with CSF-1 alone neither differentiate into osteoclasts nor exhibit any osteoclast markers (top panel). However, the MITF complex is recruited to the target genes (bottom panel) with primed histone acetylation levels. By 3 days of combined CSF-1 and RANKL treatments multinuclear TRAP-positive osteoclasts could be observed with robust expression of MITF target genes, also an active phospho-Ser-307 form of MITF is enriched along with recruitment of phospho-p38 MAPK, co-activators, and chromatin-remodeling complexes. By day 5 the gene expression is enhanced, and recruitment of NFATc1 could be observed (bottom panel) with peak in differentiation (top panel).
not consistent with the MITF-mi product acting strictly in a dominant negative fashion to block the function of the related factor TFE-3, a mechanism that has been suggested to account for the bone phenotype of the Mitfmi/mi mutant (18). An alternative hypothesis might be that this mutation also interferes with functional interactions between MITF and PU.1 in response to CSF-1/RANKL signaling. Future experiments using the combination of ChIP with cells derived from Mitf mutant mice will provide additional insights into this problem.

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