The Multifunctional Protein Fused in Sarcoma (FUS) Is a Coactivator of Microphthalmia-associated Transcription Factor (MITF)*

Received for publication, June 19, 2013, and in revised form, November 8, 2013. Published, JBC Papers in Press, November 20, 2013, DOI 10.1074/jbc.M113.493874

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**THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 1, pp. 326 –334, January 3, 2014 © 2014 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

The microphthalmia-associated transcription factor (MITF) is required for terminal osteoclast differentiation and is a signaling effector engaged by macrophage colony-stimulating factor 1 (CSF-1) and receptor activator of nuclear factor-κB ligand (RANKL). MITF exerts its regulatory functions through its association with cofactors. Discovering the identity of its various partners will provide insights into the mechanisms governing gene expression during osteoclastogenesis. Here, we demonstrate that the proto-oncogene fused in sarcoma (FUS), the chromatin remodeling ATPase BRG1, and MITF form a trimeric complex that is regulated by phosphorylation of MITF at Ser-307 by p38 MAPK during osteoclast differentiation. FUS was recruited to MITF target gene promoters Acp5 and Ctsk during osteoclast differentiation, and FUS knockdown abolished efficient transcription of Acp5 and Ctsk. Furthermore, sumoylation of MITF at Lys-316, known to negatively regulate MITF transcriptional activity, inhibited MITF interactions with FUS and BRG1 in a p38 MAPK phosphorylation-dependent manner. These results demonstrate that FUS is a coregulator of MITF activity and provide new insights into how the RANKL/p38 MAPK signaling nexus controls gene expression in osteoclasts.

Differential gene expression is vital for understanding complex cellular differentiation processes. The pathways engaged by these cytokines activate a number of transcription factors required for osteoclast differentiation, including the microphthalmia-associated transcription factor (MITF), a basic helix-loop-helix leucine zipper transcription factor (5–7). MITF partners with the Ets factor PU.1/SPI-1 to regulate the expression of genes such as tartrate-resistant acid phosphatase/acid phosphatase 5 (TRAP/Acp5) and cathepsin K (Ctsk) during osteoclast differentiation (8, 9). In committed osteoclast precursors, MITF and PU.1 recruit corepressors to silence these target genes (10). Upon engagement of CSF-1 and RANKL signaling, MITF is directly phosphorylated by mitogen-activated protein kinases (MAPK) ERK1/2, and p38 MAPK at residues Ser-73 and Ser-307, respectively (5, 6). The presence of the phosphorylated active forms of MITF at target promoters correlates with the recruitment of p38 MAPK, the SWI/SNF chromatin remodeling complex, and RNA polymerase II, initiating target gene transcription (9). MITF is also modified by sumoylation at Lys-182 and Lys-316. These modifications decrease MITF activity without affecting nuclear localization (11).

Fused in sarcoma (FUS) is a member of a multifunctional RNA-binding protein family that includes EWS and TAF15 proteins. FUS is a fusion partner in chromosomal translocations found in patients with myxoid liposarcoma (12). Mutations in the C terminus of FUS have been found in both familial and sporadic amyotrophic lateral sclerosis patients (13, 14). Members of the FUS/EWS family are involved in pre-mRNA splicing and the export of fully processed mRNA to the cytoplasm (15). In addition, FUS and members of this family interact with RNA polymerase II and the TFIIID complex and are implicated in transcriptional initiation and elongation (16, 17). FUS and EWS associate with several gene-specific transcription factors, including nuclear hormone receptors, suggesting a more specialized, gene-specific regulatory role for these proteins in the regulation of gene expression.
The purpose of this study was to identify unique binding partners of MITF to provide additional insight into the mechanisms of MITF function in osteoclasts. We used mass spectrometry to discover proteins that interact with MITF in osteoclast-like cells and identified FUS as one interaction partner. We demonstrate that FUS is part of a trimeric complex with BRG1 and MITF that is required for efficient expression of osteoclast target genes. The formation of this complex is regulated by p38 MAPK-dependent phosphorylation of MITF at Ser-307 and inhibited by sumoylation of MITF at Lys-316. The work reveals FUS as a critical coactivator necessary for MITF function during osteoclast differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Cells (COS-7, RAW264.7 C4, RAW264.7 C4 stably expressing FLAG-MITF and murine bone marrow-derived myeloid precursors (BMMs)) were cultured as described previously (5, 21, 22). Differentiation of RAW264.7 C4 cells and BMMs was optimized using 20 ng/ml soluble recombinant human RANKL (PeproTech) and 50 ng/ml recombinant human colony-stimulating factor-1 (CSF-1, a gift from David Hume). For transient expression of proteins, COS-7 cells were transfected using Lipofectamine (Invitrogen) as detailed in the figure legends, according to the manufacturer’s instructions. For transient expression of proteins, RAW264.7 C4 cells were transfected using the Nucleofector (Lonza) according to the manufacturer’s recommendations.

Immunoprecipitation, GST Pulldown Assays, and Western Blotting—Whole cell lysates were prepared as described previously (21). For sumoylation experiments, lysis buffer was supplemented with N-ethylmaleimide. Analysis of endogenous sumoylation was performed as described previously (11, 23). Whole cell lysates were immunoprecipitated with anti-FLAG or anti-MITF antibody pre-coupled to protein A/G-agarose beads (Amersham Biosciences) or with GSH-Sepharose beads (Amersham Biosciences). For in vitro binding assays, recombinant GST or GST-MITF fusion bait protein expressed in Escherichia coli from pGEX2T-GST vector was purified with GSH-Sepharose beads and subsequently incubated with cell lysate from RAW264.7 C4 cells. The beads were washed twice with lysis buffer supplemented with 2 mM DTT, twice with lysis buffer containing 0.5 M LiCl, and twice with PBS. The proteins were eluted directly into SDS sample buffer, subjected to SDS-PAGE, and stained with Coomassie Blue for MALDI-TOF analysis. Trypsin peptides were subjected to MALDI-TOF mass spectrometry using a cyano-4-hydroxycinnamic acid matrix and finally analyzed using a Bruker Reflex III (Bruker, Bremen, Germany) mass spectrometer operated in the linear, positive ion mode with an N2 laser.

Plasmids and Mutagenesis—The M-form of MITF in p3×FLAG-Myc-CMVTM-24 expression vector (FLAG-MITF) or pEBG-GST expression vector was described previously (22). Single or double point mutations of MITF were generated by the QuikChange method (Stratagene). FUS full-length, FUS N-terminal fragment (aa 1–259), and FUS C-terminal fragment (aa 260–518) were cloned from RAW264.7 C4 cells as described previously (22) into pcDNA 6/V5-His expression vector using primers with incorporated BamHI and NotI restriction sites. Sequences of PCR primers used for cloning and mutagenesis constructs are available upon request.

shRNA Construct Generation—To effect the silencing of FUS gene, the pSUPER.retro vector was used in concert with a pair of specific oligonucleotides, forward 5′-gatcccccagagttacagtggttatgtctctttgaacataaccactgtaactcttggg-3′ and reverse 5′-agcttttccaaaaacagttacagtgttgctcttgactacgttaactctttggg-3′, that contain a unique 19-nucleotide sequence derived from the mRNA transcript of the gene that corresponds to the sense strand of the pSUPER-generated siRNA, which in turn corresponds to a 19-nucleotide sequence within the mRNA. In the mechanism of RNAi, the antisense strand of the siRNA duplex hybridizes to this region of the mRNA to mediate cleavage of the molecule. These forward and reverse oligonucleotides were annealed and cloned into the vector between the unique BglII and HindIII enzyme sites. This positions the forward oligo at the correct position downstream from the H1 promoter’s TATA box to generate the desired siRNA duplex. Annealing and cloning steps were performed according to the manufacturer’s protocol.

Chromatin Immunoprecipitation (ChIP) and qPCR—ChIP assays were performed as described by Luo et al. (25) with modifications (9) using FUS antibodies. The qPCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems) in the 7500 Real Time PCR System (Applied Biosystems) as described previously (26). RNA expression was calculated as described previously (27).

RESULTS

FUS Is a p38 MAPK-dependent Coactivator of MITF—To identify new MITF binding partners, we employed the previously described RAW264.7 C4 cell line that stably expresses FLAG-MITF at levels comparable with endogenous MITF (22). Cell lysates from either mock-infected control (RAW264.7 C4, expression vector control) or FLAG-MITF-expressing cells (RAW264.7 C4 F-M) were prepared and purified by immunoprecipitation with anti-FLAG antibody coupled to protein A/G-agarose beads. A number of polypeptides copurified specifically with
the epitope-tagged FLAG-MITF (Fig. 1A, compare lanes 1 and 2). Protein bands that were communoprecipitated with MITF were excised from the gels and digested with trypsin, and the identity of peptides was determined by mass spectrometry. In total, peptides representing 11 different proteins were identified with varying levels of confidence, including MITF itself. Two peptides in the C terminus of the proto-oncogene FUS (Fusion Involved in t(12;16) In Malignant Liposarcoma) were identified with a relatively high degree of confidence (see Table 1). To confirm that FUS interacts with MITF, recombinant GST-MITF protein purified from bacteria was used in binding assays with RAW264.7 C4 cell extracts followed by immunoblotting with FUS antibody. GST-MITF, but not GST, specifically pulled down endogenous FUS (Fig. 1B, compare lanes 1 and 2).

To map the interaction sites between FUS and MITF, full-length V5-tagged FUS, the V5-FUS-N-terminal domain (aa 1–259), or the V5-FUS-C-terminal domain (aa 260–518) (Fig. 1C, top panel) was coexpressed with FLAG-MITF in COS-7 cells. MITF complexes were communoprecipitated with FLAG antibody, and Western blots of the captured material were probed with V5 antibody. Both full-length FUS and the C-terminal domain of FUS were communoprecipitated with FLAG-MITF (Fig. 1C, lanes 2 and 4, respectively). These results indicate that FUS interacts with MITF through its C-terminal RNA binding domain. In complementary experiments, full-length V5-tagged FUS was coexpressed with full-length MITF, the MITF N-terminal region (aa 1–178), or the MITF C-terminal region that includes the basic helix-loop-helix zipper domains (aa 178–419) (Fig. 1D, top panel). The results indicated that FUS interacts with the C-terminal region of MITF (Fig. 1D), which includes the leucine zipper and the Ser-307 p38 MAPK phosphorylation site (5).

MITF-FUS Interaction Is Increased by CSF-1/RANKL Signaling—We next investigated whether CSF-1/RANKL signaling could affect the interaction of endogenous FUS with MITF during osteoclast differentiation. MITF was communoprecipitated from cell extracts prepared from primary murine bone marrow derived myeloid precursors that were treated with CSF-1/RANKL for 24 and 72 h. The results demonstrated that although a complex containing MITF and FUS could not be detected in bone marrow-derived myeloid precursors, treatment with CSF-1/RANKL stimulated formation of an MITF-FUS complex by 24 h (Fig. 2A). The level of FUS in the MITF complex increased following 72 h of cytokine treatment, a time point at which MITF target genes are maximally induced (Fig. 2A) (5, 9, 22).

We confirmed the results obtained in primary cells in the RAW264.7 C4 F-M system. In this system, MITF tagged with the FLAG epitope could be coprecipitated with endogenous FUS (Fig. 2B). Association of endogenous FUS with FLAG-MITF was observed within 6 h of CSF-1/RANKL treatment, and the amount of complex formation was maximal by 24 h. Previous work from our group demonstrated that BRG1 can also form a complex with MITF. We detected MITF-BRG1 interactions in these same experiments with kinetics similar to the MITF-FUS complex (Fig. 2B). Our previous work has shown that osteoclast differentiation is accelerated in the RAW264.7 C4 F-M system relative to BMMs, with cell fusion and maximal gene expression occurring at 24–48 h, compared with 72 h in primary BMMs, findings consistent with the differing kinetics of FUS-MITF and BRG1-MITF interactions seen in experiments here (9, 22).

CSF-1/RANKL signaling leads to phosphorylation of MITF at Ser-307 via p38 MAPK, an event critical for MITF action at target genes (5, 9). We utilized RAW264.7 C4 cells to determine
whether phosphorylation of Ser-307 was required for formation of the MITF-FUS complex. For these experiments, FLAG-MITF (WT) or phosphorylation-defective FLAG-MITF S307A was transiently expressed in RAW264.7 C4 cells, and the cells were subsequently treated with CSF-1 and RANKL for different time periods. As in the previous analysis, endogenous FUS
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FUS Is Recruited to MITF Target Genes and Is Required for Their Cytokine-dependent Expression—ChIP assays were used to examine whether endogenous FUS could be recruited to MITF target promoters for the Acp5 and Ctsk genes during osteoclast differentiation (9). Soluble chromatin from BMMS was isolated at various times following cytokine treatment and then immunoprecipitated with FUS antibody (Fig. 3A). In cells treated with CSF-1 alone, both Ctsk and Acp5 promoters showed a negligible enrichment of FUS. In contrast, after treatment with CSF-1/RANKL for 72 h, FUS was enriched by 6-fold at the Acp5 promoter and 15-fold at the Ctsk promoter (Fig. 3A). The kinetics of FUS recruitment to the MITF target promoters was analogous to both FUS-MITF complex formation in BMMs following cytokine treatment (see Fig. 2A) and to the recruitment of BRG1 and polymerase II to these promoters (9).

To demonstrate that FUS functions as a coactivator of MITF, we used short hairpin (sh) RNA to knock down FUS levels in RAW264.7 C4 F-M cells. Delivery of the FUS shRNA expression vector lowered FUS protein and RNA levels ~2.5–3-fold compared with a scrambled shRNA control (Fig. 3B). To evaluate the role of FUS knockdown in the regulation of MITF target genes, transfected cells were cultured without or in the presence of CSF-1 and RANKL for 24 h, and the expression of MITF osteoclast target genes Acp5 and Ctsk was measured by qRT-PCR (Fig. 3C). The results demonstrated a significant reduction of expression of both Acp5 and Ctsk, indicating that FUS interaction is required for MITF function during osteoclast differentiation.

FUS, MITF, and BRG1 Form a Complex Dependent on Ser(P)-307 of MITF—We have previously reported that MITF phosphorylation at Ser-307 also enhances interaction of MITF with BRG1, the ATPase subunit of the mSWI/SNF chromatin remodeling complex (9). To examine the relationship of interactions between MITF, BRG1, and FUS, we overexpressedFLAG-MITF, V5-FUS, and BRG1 in COS-7 cells, cells that do not express endogenous MITF (Fig. 4A). When V5 antibody was used for immunoprecipitation, a complex between BRG1 and V5-FUS could not be detected; however, when all three factors were coexpressed, strong complex formation was detected (Fig. 4A, compare lanes 2 and 3). In a parallel experiment, when the FLAG antibody was used for the immunoprecipitation, the trimeric complex was more abundant when all three factors were overexpressed (data not shown).

To evaluate whether the phosphorylation of MITF by p38 MAPK has any role in the formation of this trimeric complex, we performed a similar series of experiments in COS-7 cells. Following transfection with combinations of V5-FUS, FLAG-MITF WT, or FLAG-MITF S307A and BRG1, cell extracts were prepared, and FLAG antibody was used to precipitate MITF complexes. Complex formation was again enhanced only when MITF, FUS, and BRG1 were coexpressed (Fig. 4B, lane 2 versus lane 4). Interestingly, substitution of p38 MAPK phosphorylation-defective FLAG-MITF S307A resulted in a significant reduction in complex formation, indicating that phosphorylation at S307A is important for efficient complex formation (Fig. 4B, lane 4 versus lane 5, quantified in Fig. 4C). Consistent with these binding studies, overexpression of either V5-FUS or BRG1 in RAW264.7 C4 cells treated with CSF-1 and RANKL led to a significant 2–3-fold increase in expression of MITF target genes Acp5 and Ctsk (Fig. 4D). However, the combination of both was not significantly different from either cofactor alone, likely because MITF protein levels are limiting. BRG1 overexpression did not increase the level of Fus mRNA (Fig.

Could be coprecipitated with FLAG-MITF in a signaling-dependent fashion (Fig. 2C, 1st panel). In contrast, MITF-FUS complex formation with the FLAG-MITF S307A variant was significantly attenuated, a result supporting a function for p38 MAPK signaling in promoting complex formation. Consistent with this result, overexpression of MKK6 led to increased p38 MAPK activation and formation of the FUS-MITF complex, although overexpression of RAF kinase and activation of the ERK pathway did not affect the FUS-MITF complex (Fig. 2D, compare lanes 3 and 4).

**FIGURE 3.** FUS is a coactivator of MITF in CSF-1/RANKL-mediated induction of MITF target genes. A, soluble chromatin from BMMs treated either with CSF-1 alone or CSF-1 and RANKL for the indicated time points were immunoprecipitated with FUS antibody, and enrichment of Acp5 and Ctsk promoters was analyzed by qPCR. The top panel shows the relative positions of the oligonucleotides used with respect to the TSS of each gene. B, evaluation of FUS knockdown by shRNA FUS constructs. Left panel, either scrambled or shFUS retroviral constructs were nucleofected into RAW264.7 C4 F-M cells, and 24 h post-infection, cell lysates were probed for FUS protein and TUBULIN protein (loading control). Right panel, mRNA levels of Fus in presence and absence of CSF-1 and RANKL 24 h post-infection with either scrambled (Scr) or shRNA FUS by qRT-PCR. C, RAW264.7 C4 F-M cells infected and treated as in B were analyzed for mRNA expression of Acp5 and Ctsk in the presence or absence of CSF-1 and RANKL by qRT-PCR. All experiments were the average of at least two independent biological sets done in duplicate, and the error bars indicate S.D.
and FUS and BRG1 overexpression did not increase expression of the MITF target gene Bcl2 (data not shown), which we have previously demonstrated is a gene target that does not respond to CSF-1/RANKL signaling in osteoclasts (10). Furthermore, knockdown of FUS by shRNA in RAW264.7 C4 cells treated with CSF1/RANKL abolished recruitment of FUS on the Ctsk promoter (Fig. 4E, left panel). Importantly, FUS knockdown also abrogated BRG1 enrichment on the Ctsk promoter (Fig. 4E, right panel), further supporting the need for FUS to be present for efficient complex formation in response to signaling.

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4D), and FUS and BRG1 overexpression did not increase expression of the MITF target gene Bcl2 (data not shown), which we have previously demonstrated is a gene target that does not respond to CSF-1/RANKL signaling in osteoclasts (10). Furthermore, knockdown of FUS by shRNA in RAW264.7 C4 cells treated with CSF1/RANKL abolished recruitment of FUS on the Ctsk promoter (Fig. 4E, left panel). Importantly, FUS knockdown also abrogated BRG1 enrichment on the Ctsk promoter (Fig. 4E, right panel), further supporting the need for FUS to be present for efficient complex formation in response to signaling.

**CSF-1/RANKL Signaling and p38 MAPK Activation Decrease MITF Sumoylation**—MITF is known to be sumoylated at Lys-182 and Lys-316, a modification that decreases the transcriptional activity of MITF but does not appear to influence dimerization, DNA binding, stability, or nuclear localization (11). To test whether CSF-1/RANKL signaling could affect MITF sumoylation in osteoclasts, we tested bone marrow-derived myeloid precursors that were treated with CSF1 alone or CSF1 and RANKL for 3 days (differentiating osteoclasts). Similar to the observations of Fisher and co-workers (11), we observed an endogenous sumoylated form of MITF in myeloid precursors that disappeared after CSF-1/RANKL treatment (Fig. 5A, compare lanes 1 and 2). This endogenous sumoylated form migrated in a similar fashion as sumoylated MITF in COS-7 cells that overexpressed FLAG-MITF and HA-SUMO1 (Fig. 5A, compare lanes 1 and 3). To further evaluate whether this higher molecular weight species is sumoylated MITF and to confirm whether CSF-1/RANKL treatment could be responsible for the desumoylation of MITF, we employed an assay in which GST-MITF and HA-SUMO1 were cotransfected into RAW264.7 C4 cells. Following transfection, cells were treated with CSF-1/RANKL and harvested at two different time points. GST-pulldown followed by Western blotting demonstrated that MITF was modified by SUMO1 (Fig. 5B). Three bands of sumoylated MITF could be detected with antibodies against SUMO1, GST, or MITF consistent with the published data (Fig. 5B) (11). Importantly, the sumoylated species were significantly decreased following 30 h of CSF-1/RANKL treatment (Fig. 5B).
To determine signaling pathways that affect MITF sumoylation, we used a COS-7 cell overexpression system to study the effects of either ERK or p38 MAPK pathways. Overexpression of MKK6 to activate p38 MAPK, but not RAF to activate ERK, resulted in a marked decrease of the sumoylated forms of MITF in a GST-pulldown assay (Fig. 5C, compare lanes 2–4). These results suggest that activation of MITF by the RANKL/p38 MAPK pathway is partially achieved by inhibition of sumoylation.

Sumoylation of MITF at Lys-316 Inhibits Interaction with FUS—Because sumoylation of MITF can be regulated by CSF-1/RANKL signaling, we hypothesized that it might affect formation of the MITF-FUS complex. As a preliminary step, we first tested which of the two MITF sumoylation sites were targeted by p38 MAPK signaling. FLAG-MITF was engineered to alter Lys-182 and Lys-316 separately and together. The MITF vectors encoding these altered proteins were transfected into COS-7 cells along with a vector encoding HA-SUMO1. Analysis of the MITF proteins produced demonstrated that the MITF-K316R protein contained significantly lower amounts of SUMO1 modification than MITF-K182R (Fig. 6A, compare lanes 3 and 4); mutation of both sites leads to a total loss of SUMO1 modification (Fig. 6A). Furthermore, the pattern of sumoylation remaining in the MITF-K316R was identical to wild type FLAG-MITF cotransfected with HA-SUMO1 and MKK6 (Fig. 6A, lanes 4 and 6). These results suggest that sumoylation of MITF at Lys-316 is the target of cytokine and p38 MAPK signaling.

Similar coimmunoprecipitation experiments in COS-7 cells expressing FLAG-MITF wild type and sumoylation mutant proteins, HA-SUMO1 and V5-FUS, were next performed. These experiments demonstrated that sumoylation of MITF decreased the FUS/MITF-WT complex (Fig. 6B, lane 2 versus lane 7). Sumoylated MITF-K182R behaved similarly to wild type (Fig. 6B). In contrast, MITF-K316R formed the FUS complex as efficiently as wild type MITF (Fig. 6B, compare lanes 2 and 9) and independent of sumoylation for residue Lys-182 (compare lanes 2, 7, and 10). Endogenous BRG1 binding to the
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The DISCUSSION section of the text discusses the role of FUS as a coactivator of MITF, highlighting the importance of signaling pathways in regulating osteoclast differentiation. It mentions that phosphorylation of MITF by p38 MAPK increases its transcriptional activity and that FUS may act as a classic coactivator for MITF, recruiting RNA polymerase II and increasing transcription of osteoclast target genes.

The DISCUSSION section also notes the importance of sumoylation of MITF in regulation of its activity. It is stated that sumoylation of MITF at Lys-316 is decreased by CSF-1/RANKL signaling, and SUMO1 modification of this residue inhibits the interaction of MITF with FUS. This results in decreased interaction between MITF and BRG1, and increased interaction between MITF and FUS.

In conclusion, the authors demonstrate a new role for FUS as a coactivator of MITF in osteoclast differentiation, highlighting the importance of signaling pathways in regulating osteoclastogenesis.

Acknowledgments—We are grateful to Dr. David Hume for cytokine CSF-1 and Michael De Lay for critical reading of the manuscript.

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