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TRANSCRIPTIONAL ENHANCER FACTOR 1 (TEF-1/ TEAD1) MEDIATES ACTIVATION OF *IFITM3* GENE BY BRG1

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Abstract

The interferon inducible transmembrane (IFITM) proteins mediate several cellular processes such as homotypic cell adhesion functions of interferons (IFNs) and cellular anti-proliferative activities. We show that the BAF complex mediated induction of *IFITM3* is dependent on binding of the transcriptional enhancer factor 1 (TEF-1/TEAD1) to the M-CAT like elements of its promoter. TEF-1 knock-down reduced the BAF complex mediated activation of *IFITM3* promoter. In the absence of the BAF complex, TEF-1 is repressive to *IFITM3* expression. The regulation of *IFITM3* by TEF-1 demonstrates that TEF-1 dependent regulation is more widespread than its previously established role in the expression of muscle specific genes.

1. INTRODUCTION

Interferons (IFNs) play critical roles in tumor surveillance by controlling apoptosis and through cellular anti-proliferative and differentiating activities. They also play major roles in cellular defense against viral and parasitic infection [1,2]. The expression of several interferon inducible genes require the chromatin remodeling SWI/SNF-like BAF complexes for their basal as well as the IFN inducible expression [3-6]. Among these are the interferon inducible transmembrane protein (IFITM) family genes, which comprise of *IFITM1* (9−27), *IFITM2* (1−8U) and *IFITM3* (1−8D) [7,8]. These genes have been implicated in several cellular processes such as homotypic cell adhesion functions of IFN and cellular anti-proliferative activities [7, 9,10]. The expression level of IFITM genes have also been found to be up-regulated in a number of cancer cells [1,10].

We previously found that the BAF complex is constitutively associated with the promoters of the IFITM genes and maintains an open chromatin structure at the promoter for rapid induction by IFNs or viral infection [3]. We had also shown that the BAF complex-mediated induction of the *IFITM3* is dependent on two critical DNA elements in its promoter [5]. The first element located between the positions −152 and −138, contains an Sp1 binding motif. Sp1 recruited BAF complex to the promoter and a mutation in this site reduced BAF complex-mediated activation significantly. The second element was identified upstream of the Sp1 binding motif, between the positions −173 and −152. Deletion of this region resulted in a significant reduction in the BAF complex-mediated activation as well. Though the sequence analysis of this region did not reveal any consensus protein binding motifs [5], a sequence, 5’-AGGAATTTGT-3’, resembling an M-CAT motif was identified. In this study, we present evidence that the second critical element in the *IFITM3* promoter is bound by the transcriptional enhancer factor 1

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(TEF-1/TEAD1). TEF-1 regulates the BRG1-dependent activation and IFN-induction of the *IFITM3* gene.

2. MATERIALS AND METHODS

2.1. Constructs and antibodies

pREP4-Luc, pREP7-BRG1 and pREP4-puro were described earlier [3,6]. pREP4-TM3-Luc was constructed as reported earlier, by PCR amplifying the *IFITM3* promoter from position −238 to −25 [5]. Point mutations of the *IFITM3* promoter were performed using the QuickChange kit (Stratagene). TEF-1 antibodies were obtained from BD transduction laboratories (610923).

2.2. Cell culture, transfection and luciferase assay

SW-13 and Hela cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1 mM glutamine. For reporter assays, the reporter constructs were transfected into SW-13 or HeLa cells and luciferase activity was measured 72 hrs after transfection using the dual luciferase assay kit (Promega). Transfections were carried out using superfect transfection reagent (Qiagen).

2.3. Electrophoretic mobility shift assay (EMSA)

For EMSA, the nuclear extracts from SW-13 and HeLa cells or the *in vitro* translated TEF-1 were used. Detailed nuclear extracts isolation and EMSA procedures are provided in the supplemental information.

2.4. DNA affinity protein purification and mass spectrometry

For DNA affinity purification, biotinylated oligonucleotides containing the 7-bp protein-binding site were concatamerized using the self primed PCR technique [11,12]. Complete details of the DNA affinity protein purification are provided in the supplemental information. MALDI-TOF mass spectrometry was carried out at the Lerner Research Institute, Mass Spectrometry Laboratory for Protein Sequencing, the Cleveland clinic foundation, Cleveland, Ohio.

2.5. RNA interference, RNA isolation, cDNA synthesis and quantitative PCR

TEF-1 siRNAs were obtained from Qiagen. Total RNAs were isolated from HeLa or SW-13 cells as described earlier [6]. cDNA was synthesized using SuperScript III RNase H- reverse transcriptase (Invitrogen). TaqMan probes and universal RT-PCR master mix were obtained from Applied Biosystems Inc.

2.6. In vitro translation

HeLa cell mRNAs were used to synthesize the cDNA encoding TEF-1. The cDNA was cloned into the vector, pBluescript KS (Stratagene). TNT Quick Coupled Transcription/Translation system was used for in vitro translation (Promega).

3. RESULTS

3.1. Identification of the sites essential for IFITM3 promoter activation

SW-13 cells do not express detectible levels of either BRG1 or hBRM, the essential ATPase subunits of the BAF complexes. Reconstitution of active BAF complex by the transient expression of BRG1 activates a number of genes including the IFN-inducible *IFITM3* [6]. We previously found that a 213 bp promoter fragment of *IFITM3* contains sequences responsive

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to BRG1 activation in SW-13 cells [5]. Though the Sp1 binding site between −144 and −139 plays a critical role, the region between −173 and −152 also contribute significantly to the BRG1-mediated activation of the *IFITM3* promoter [5], suggesting that more elements may mediate the BRG1 activation. To identify the BRG1 response sequences between the positions −173 and −152 of the *IFITM3* promoter, we generated luciferase constructs containing the sequences from −238 to −25 in pREP4 episomal vector, which forms a regular chromatin structure. Mutants were constructed with mutations spanning the promoter region between −175 and −138. Activities of the constructs were analyzed by transient transfection assays (Fig. 1A). The wild-type promoter construct showed an activation of over 10-fold when BRG1 was transiently transfected into the cells. Mutation C resulted in the most significant decrease in promoter activity, whereas the mutations A and B modestly reduced the promoter activity (Fig. 1A). Mutation E, which overlaps the mutation C also showed a modest reduction in the promoter activity. Thus the mutations C and E together are a 7-bp region that is important in mediating the BRG1 activity.

To analyze the protein-DNA interactions in this region we carried out electrophoretic mobility shift assays (EMSA) using a 21-bp [γ^{-32}P]ATP labeled oligonucleotide probe spanning the positions from −162 to −142 (Fig. 1B). When incubated with the nuclear extracts of SW-13 cells, retardation in the migration of the wild-type probe due to the formation of a protein/DNA complex was observed (Fig. 1C, lane 1). Competition with a 100-fold excess of the unlabelled probe diminished the signal intensity of the complex (Fig. 1C, lane 2). Furthermore, an excess of the unlabelled mutant probe with mutations in the 7-bp sequence (Fig. 1B, highlighted in bold), did not compete with the complex (Fig. 1C, lane 3), showing that the protein-DNA interaction was specific.

3.2. Transcriptional enhancer factor 1 binds to IFITM3 promoter

To identify the proteins that bind to the probe, we used DNA affinity chromatography. In this procedure, biotinylated oligonucleotides containing the 7-bp protein-binding site (Fig. 1B, WT) were concatamerized by PCR amplification [11,12]. The PCR products were bound to streptavidin coated magnetic beads and were incubated with SW-13 nuclear extracts. The bound factors were resolved on an SDS-PAGE gel and stained with silver nitrate (Fig. 2A, WT). In parallel, control purification was carried out with mutant oligonucleotides (Fig. 1B, M). A comparison of the protein bands from the two samples revealed a 50-kDa band in the wild-type eluate, which was absent in the mutant eluate (Fig. 2A, compare lanes WT and M). The protein band was excised from the gel and analyzed by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry. Six peptides were identified from this sample, which were identical to the sequence of the protein, transcriptional enhancer factor 1 (TEF-1) (Fig. 2B). TEF-1 is a member of the eukaryotic TEA/ATTS family of transcription factors, which contains the highly conserved DNA binding domain that recognize the canonical M-CAT sequence motif, 5'-AGGAATG-3' [13,14].

To investigate whether the probe shift in the EMSA assays (Fig. 1C) were caused by the binding of TEF-1, we carried out EMSA supershift assays using anti TEF-1 antibodies. TEF-1 antibodies supershifted the protein/DNA complex formed with both SW-13 (Fig. 3A, lane 4) and HeLa nuclear extracts (Fig. 3B, lane 4). Addition of pre-immune serum did not result in any supershifted complexes (Fig. 3A and B, lanes 3). We then used the *in vitro* translated TEF-1 to confirm its binding to the probe. As shown in figure 3C, the *in vitro* translated TEF-1 formed a complex with the probe, with migration similar to that observed with the endogenous TEF-1 in SW-13 (Fig. 1C and 3A) and HeLa nuclear extracts (Fig. 3B). While 100-fold excess of wild-type unlabelled probe competed with the complex formation, the mutant probe did not (Fig. 3C, compare lanes 2 and 3). In addition, the *in vitro* translated TEF-1/probe complex was supershifted when incubated with TEF-1 antibodies (Fig. 3C, lane 5). Thus the properties of

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the *in vitro* translated TEF-1 were similar to that of the endogenous protein, confirming that the protein bound to the probe was indeed TEF-1.

The *IFITM3* promoter contains another sequence, between the positions −170 and −164 (5′-AGTAATT-3′), that resembles the M-CAT sequence. Mutation of this sequence also caused a modest decrease in BRG1-induced activation (Fig. 1A, Mut B). We used EMSA to examine whether TEF-1 can bind to this sequence. For this, we used a 36-bp probe (Fig 3D, probe WT1), encompassing both the −170 5′-AGTAATT-3′ −164 region (Fig. 3D, site B, in bold) and the identified TEF-1 binding site (Fig. 3D, site A, in bold) in the *IFITM3* promoter. When incubated with SW-13 nuclear extracts, the probe formed a protein/DNA complex, which was supershifted by TEF-1 antibodies (Fig. 3E, lanes 1 and 2). Mutation in site B (Fig 3D, probe M2) did not alter the gel mobility shift or the supershift with TEF-1 antibodies (Fig 3E, lanes 3 and 4). There was no significant variation in the signal intensity of the shifted complexes formed with the wild-type and mutant probe, M2 (Fig 3E, compare lanes 1,2 with 3,4) showing that the TEF-1 binding is largely unaffected. When site A in the probe was mutated (Fig 3D, probe M1) the protein/DNA complex signal intensity was greatly reduced (Fig. 3E, lanes 5 and 6), showing that TEF-1 bound the probe much less efficiently. Interestingly, the migration pattern in the EMSA and EMSA supershift assays was similar to that of the wild-type probe showing that indeed TEF-1 has a secondary binding site between the positions −170 and −164 of the *IFITM3* promoter. Thus the reduction in the BRG1 mediated activation in the *IFITM3* promoter mutant B (Fig. 1A, Mut B) could be attributed to the loss of a TEF-1 binding site. To confirm that the bands observed in figure 3E, lanes 5 and 6, were due to the binding of TEF-1 to site B and not due to some residual binding to the mutated site A, we carried out EMSA with a shorter probe which lacked site A (Fig. 3D, probe WT2). The migration of the wild-type probe was retarded by the *in vitro* translated TEF-1 and the complex was supershifted by TEF-1 antibodies (Fig. 3E, lanes 7 and 8). When site B in the probe was mutated (Fig. 3D, M3), no retardation of the probe migration was seen (Fig. 3E, lanes 9 and 10), showing clearly that the TEF-1 bound to a secondary site in the *IFITM3* promoter, though much less efficiently.

Next, we tried to demonstrate binding of TEF-1 to the *IFITM3* promoter *in vivo*, using chromatin immunoprecipitation (ChIP) assays. Unfortunately, we could not detect enrichment with the TEF-1 antibodies compared to pre-immune controls (data not shown), which can be explained by inaccessible epitopes or antibodies not being suitable for ChIP analysis. Therefore, we used the following methods to confirm the involvement of TEF-1 in *IFITM3* regulation.

3.3. IFITM3 promoter activity is dependent on TEF-1

In order to determine whether TEF-1 is involved in the regulation of *IFITM3* we knocked-down TEF-1 in cultured cells. As shown in figure 4A, knock-down of TEF-1 in SW-13 cells by transfection with siRNAs targeting TEF-1 mRNA resulted in its reduction by over 70% and a similar reduction in the protein levels was also observed (Fig. 4B). Neither BRG1 expression nor IFN-α treatment affected the TEF-1 expression level (Fig. 4A). The basal expression of *IFITM3* was low in SW-13 cells (Fig. 4C). Induction with IFN-α resulted in a slight increase in the expression. Transient transfection of BRG1 and thereby reconstitution of the active BAF complex resulted in a significant increase in the basal and the IFN-α induced *IFITM3* expression levels (Fig. 4C). When TEF-1 levels in the cells were reduced by siTEF-1, a two-fold decrease of the BRG1-mediated induction of the basal *IFITM3* expression was observed (Fig. 4C). A corresponding decrease in the IFN-α induced expression level also was also observed. Interestingly, the basal and the IFN-α induced levels of IFITM3 expression were higher in TEF-1 knock-down SW-13 cells in the absence of the active BAF complex, when compared to the cells without TEF-1 knock-down (Fig. 4C). This suggests a repression activity caused or mediated by TEF-1 in the absence of the BAF complex on the *IFITM3* promoter.

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TEF-1 regulation of *IFITM3* promoter was also tested in HeLa cells, which has an active BAF complex. As shown in figure 4D, IFN-α treatment induced the promoter activity significantly. Transfection of the siTEF-1 into HeLa cells, which caused 70% reduction in the TEF-1 expression (data not shown), resulted in a significant decrease in both the basal and IFN-α induced promoter activity (Fig. 4D).

4. DISCUSSION

TEF-1 belongs to a family of four transcription factors that also include TEF-3, TEF-4 and TEF-5. They contain the highly conserved N-terminal TEA-DNA binding domain (TEAD) [13-16] recognizing the canonical M-CAT motif in several muscle-specific gene promoters [17-19]. While TEF-1 is known to regulate several M-CAT dependent promoters that are muscle specific, TEF-1 mRNA has been detected in several other tissues, suggesting that the M-CAT binding activity could be involved in the regulation of non-muscle specific genes as well [20]. In this study, we have found that TEF-1 regulates the interferon-inducible *IFITM3* gene in non-muscle cells.

EMSA assays showed that the TEF-1 protein binds specifically to the 5′-AGGAATT-3′ sequences between the positions −155 and −149 in the *IFITM3* promoter. This is a one base pair variation from the canonical M-CAT motif, 5′-AGGAATG-3′. Mutation in the TEF-1 binding site resulted in significant reduction in the promoter activity (Fig. 1A). Knock-down of the TEF-1 by siRNAs resulted in decreased *IFITM3* mRNA levels (Fig. 4C) and promoter activity in reporter assays (Fig. 4D), thereby clearly showing the involvement of TEF-1 in *IFITM3* gene regulation.

In addition to the sequences between −155 and −149 (Fig. 3D, site A) in the *IFITM3* promoter, TEF-1 also exhibits a weak binding to a secondary site, 5′-AGTAATT-3′ (Fig 3D, site B) between positions −170 and −164, which varies from the canonical M-CAT motif in the first and fifth positions. A systematic study to identify nucleotides in the M-CAT motif, most important for the activation of the c-TNT promoter in the chick embryo primary muscle cells has shown that a shift in the fifth position of the TEF-1 binding site 5′-CATTCCT-3′ from C to A resulted in a significant decrease in the promoter activity [21]. This could explain the weaker binding of TEF-1 to site B and stronger binding to site A (Fig. 3E). However, even though the majority of the activation potential seems to arise from site A, site B also contributes to the activity of the *IFITM3* promoter as its mutation resulted in a modest decrease in BRG1-mediated activation (Fig 1A, mutants A and B). Both the TEF-1 binding sites A and B are completely conserved between *IFITM3, IFITM2* and *IFITM1* [7], suggesting a similar mechanism in the regulation of all the IFITM genes.

Several cofactors that are required for TEF-1 activity have been identified [13,22,23]. TONDU, the mammalian homolog of *Drosophila vestigial* gene, binds to all the four TEAD proteins [24]. YAP65, that interacts with all the four TEAD proteins has been shown to be a general transcriptional co-activator in mammalian cells [15]. TAZ also has been shown to be a co-activator of TEF-1 [13]. This study showed that TEF-1 and the BAF complex cooperatively activate the *IFITM3* gene, which suggests that the BAF complex could be a co-activator of TEF-1 during the regulation of *IFITM3*.

In this study, we found that TEF-1 knock-down resulted in an increase in the basal expression levels of the *IFITM3* gene in SW-13 cells, suggesting that TEF-1 could play a repressive role in the absence of the co-activating BAF complex. TEF-1 has been suggested to act as a repressor of the *α*-tropomyosin transgene in the early embryo. The lack of the co-repressors or the presence of co-activators has been speculated as the reason for the loss of repressive activity of TEF-1 during the later stages of development [25]. Over-expression of TEF-1 resulting in

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the repression activity of TEF-1 is also well known. It has been suggested that this phenomenon could be due the squelching, where the co-activator could be titrated out by the binding of the over-expressed TEF-1 [15,26-28]. Thus the repressive effect of TEF-1 could be due to a co-repressor activity, which may be recruited by TEF-1 in the absence of the co-activator, the BAF complex being such a co-activator in our studies.

We previously found the involvement of Sp1 in the BRG1 mediated activation of the *IFITM3* gene. In this study, we have identified a novel player, TEF-1 in its regulation. Our data indicate that the expression of the IFITM genes is regulated by multiple transcription factors through interaction with the chromatin remodeling complexes and suggests a role for TEF-1 in the IFN signaling pathway

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Supplementary Material

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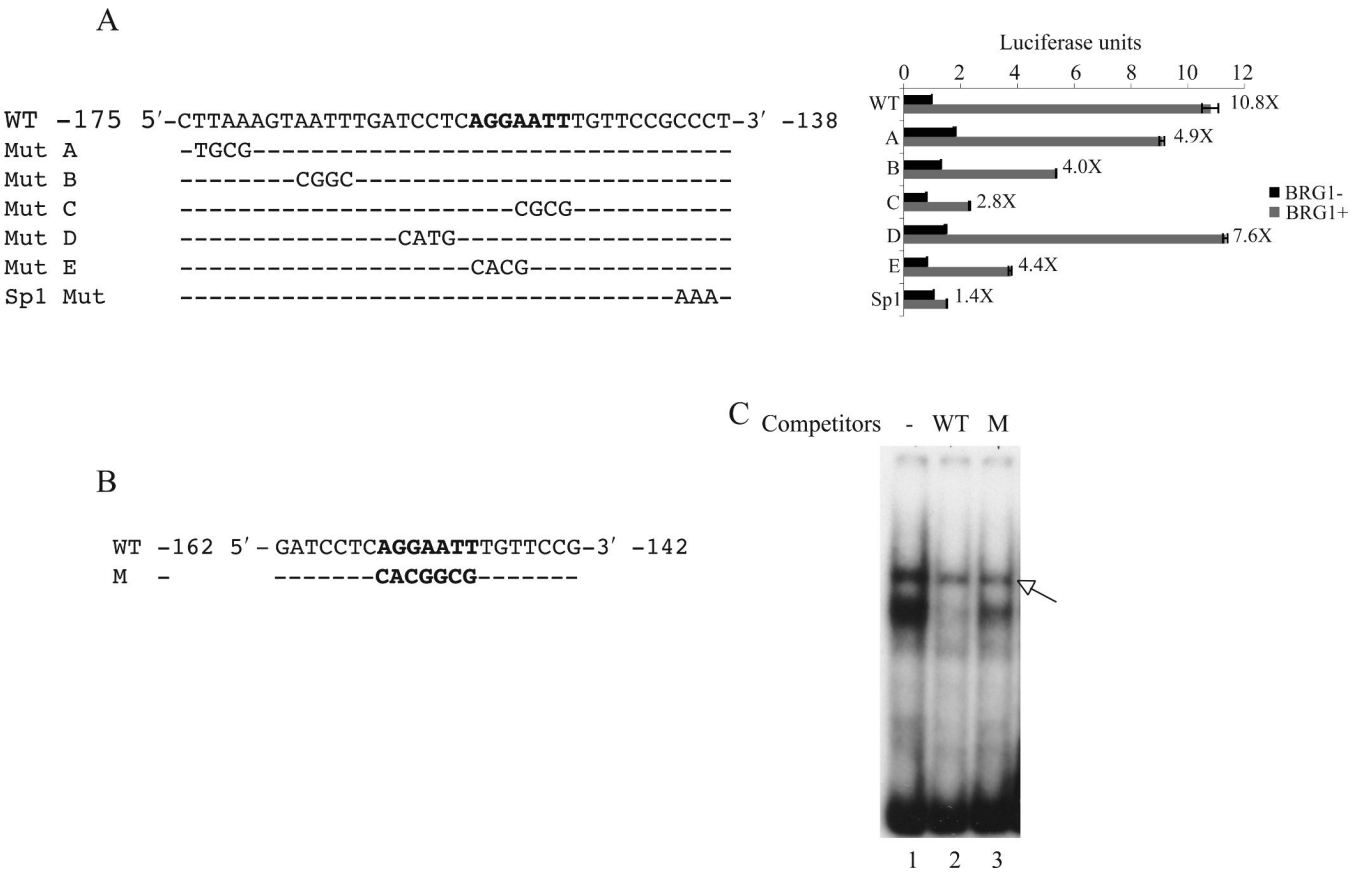

Fig.1.

Identification of the sites critical for the BAF complex dependent regulation of *IFITM3* promoter. (A) Left panel: Sequence between the positions −175 and −138 of the *IFITM3* promoter in the luciferase reporter constructs (pREP4-TM3-Luc). WT:wild type promoter. The 7-bp site important for the *IFITM3* promoter activation is shown in bold. Mut A to Mut E : promoter mutants, mutated sites are indicated; Sp1 Mut: Sp1 binding site mutant. Right panel: luciferase activities of the corresponding constructs. (B) Sequence of the oligonucleotide probes used for EMSA in Figures 1C and 3A, B and C. WT: wild-type probe; M: mutant probe, mutated site shown in bold. (C) EMSA with SW-13 nuclear extracts. A 100-fold excess of the wild-type or mutant competitors were used. WT: wild-type competitor (Lane 2), M: mutant competitor (Lane 3), Lane 1: no competitor. Arrow - non-specific band

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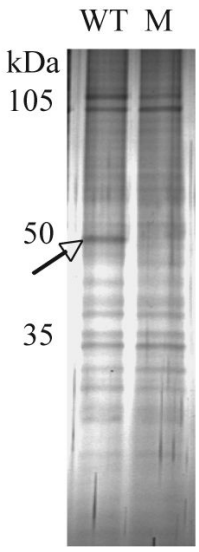
A



B

Peptides Identified	Position in TEF-1
KIILSDEGK	57-65
NELIAR	70-75
KQVSSHIQVLAR	88-99
ALQHMAAMSSAQIVSATAIHNK	118-139
LVEFSAFLEQQR	218-229
VCSFGK	326-331

Fig.2.

Purification and identification of the protein binding to the 7-bp region of the *IFITM3* promoter. (A) Proteins purified from the SW-13 nuclear extracts by DNA affinity chromatography using the wild-type (Fig. 1B, WT) and mutant probes (Fig. 1B, M) were resolved by SDS-PAGE and stained with silver nitrate. The 50-kDa band (arrow) was excised from the gel and analyzed by MALDI-TOF mass spectrometry. (B) The six peptides that were identified by MALDI-TOF MS and their positions in the TEF-1 protein.

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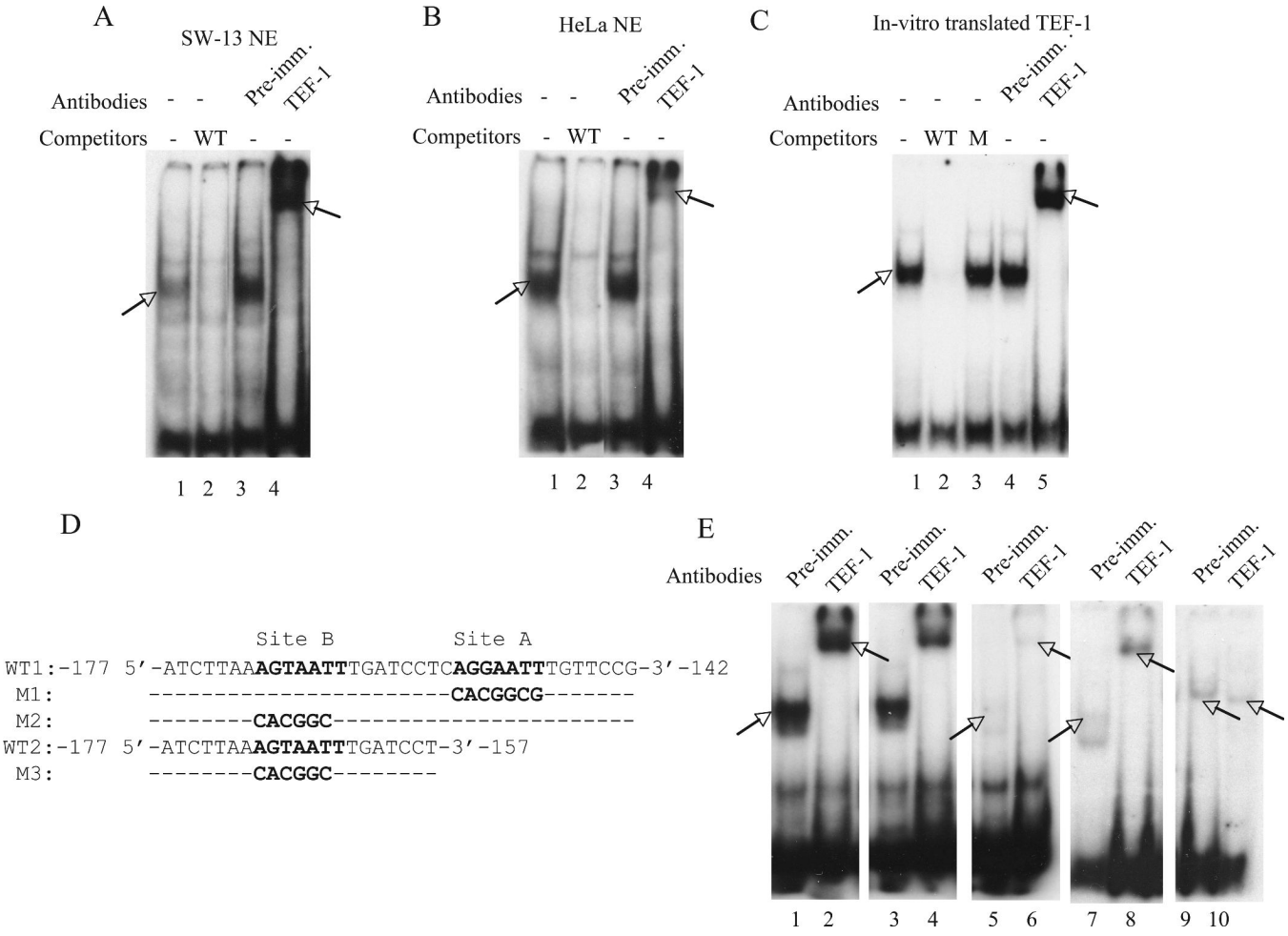

Fig.3.

TEF-1 binds to the *IFITM3* promoter. (A) EMSA of the wild-type probe (Fig. 1B) with SW-13 nuclear extracts. A protein/DNA complex was formed (lane 1) which was competed by a 100-fold excess of the unlabeled wild-type probe (WT). TEF-1 antibody super-shifted the complex (lane 4), while the pre-immune serum did not (lane 3). The shifted and supershifted bands are indicated by arrows. (B) EMSA was performed as above, with HeLa nuclear extracts. (C) EMSA was performed as above, with *in vitro* translated TEF-1. M: 100-fold excess of the mutant probe (Fig. 1B, M). (D) WT1, M1 and M2: probes used in the EMSA shown in figure 3E, lanes 1−6 (WT1: lanes 1 and 2; M1: lanes 5 and 6; M2: lanes 3 and 4). Sites A and B: primary and secondary TEF-1 binding sites, respectively, in the wild-type probe (WT). M1 and M2: site A and site B mutant probes, respectively. Mutated sites are in bold. WT2 and M3: probes used in the EMSA shown in Fig. 3E, lanes 7−10 (WT2:lanes 7 and 8; M3: lanes 9 and 10). WT2 :wild-type probe, M3: mutant probe. Mutated sites are in bold. (E) EMSA with the SW-13 nuclear extracts and *in vitro* translated TEF-1.The protein/DNA complex formed between the SW-13 nuclear extracts and the wild-type probe (WT1, lane 1) is supershifted by TEF-1 antibodies (lane 2). A protein/DNA complex is formed with the site B mutant probe (M2, lane 3) and is supershifted by the TEF-1 antibodies (lane 4). The complex formation is significantly reduced when the site A mutant (M1) probe was used. A very faint complex formation is observed (lane 5, arrow) that is supershifted by TEF-1 antibodies (lane 6, arrow). The protein/DNA complex formed between the *in vitro* translated TEF-1 and the wild-type probe (WT2, lane 7) is supershifted by TEF-1 antibodies (lane 8). The mutant probe (M3, lanes

9 and 10) did not form a specific complex. The non-specific bands in lane 9 and 10 are marked with arrows.

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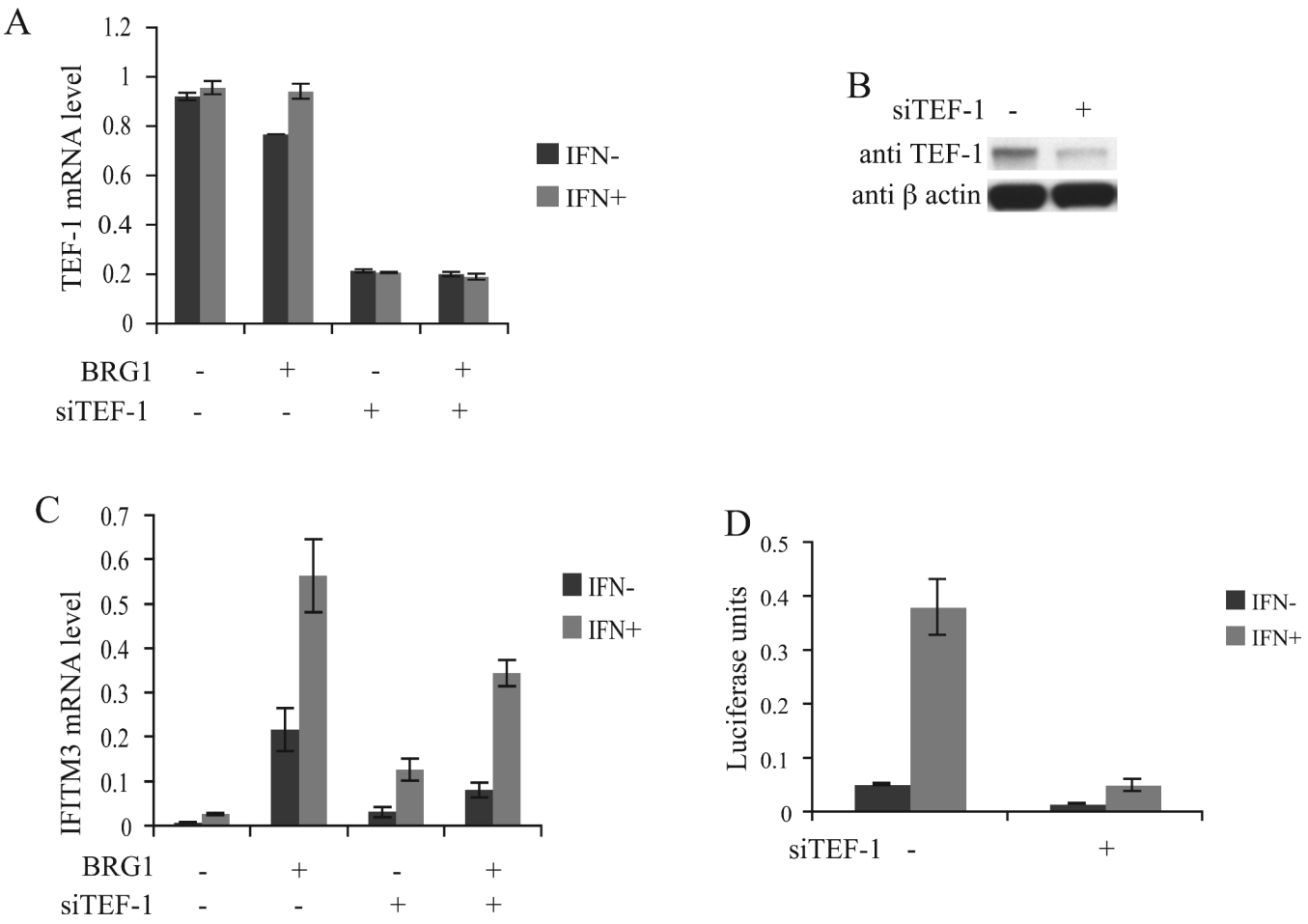

Fig.4.

TEF-1 knock-down results in the reduction of *IFITM3* promoter activity and expression levels. (A) SW-13 cells were transfected with siTEF-1 RNA oligonucleotides or the control RNA oligonucleotides and co-transfected with either pREP7-BRG1-Hygromycin or pREP7-Hygromycin control vector. Total RNAs were isolated from the cells selected with hygromycin for 48 h and TEF-1 mRNA levels were determined using quantitative real-time PCR. The cells were induced with 1000 u/ml α-interferon (IFN-α) for 12 h. (B) Western blot showing the TEF-1 levels in SW-13 cells transfected with siTEF-1 RNA oligonucleotides or the control RNA oligonucleotides and co-transfected with pREP7-Hygromycin. Protein was isolated from the cells selected with hygromycin for 48 h. β actin was used as control (C) *IFITM3* mRNA levels in SW-13 cells. Conditions same as in figure 4A. (D) *IFITM3* promoter activity in the wild-type and TEF-1 knock-down HeLa cells. pREP4-TM3-Luc was co-transfected into HeLa cells with siTEF-1 RNA oligonucleotides or the control RNA oligonucleotides. The cells were induced with 1000 u/ml IFN-α for 12 h. Luciferase activity was measured 48 h after transfection.