

Bimodal, Reciprocal Regulation of Fibroblast Growth Factor Receptor 1 Promoter Activity by BTEB1/KLF9 during Myogenesis

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Expression of the gene encoding fibroblast growth factor receptor 1 (FGFR1) and subsequent FGFR1-mediated cell signaling controls numerous developmental and disease-related processes. The transcriptional regulation of the FGFR1 gene is central to these developmental events and serves as a molecular model for understanding transcriptional control of growth factor receptor genes. The FGFR1 promoter is activated in proliferating myoblasts via several Sp1-like binding elements. These elements display varying levels of activation potential, suggesting that unique protein-DNA complexes coordinate FGFR1 gene expression via each of these sites. The Krüppel-like factor, BTEB1/KLF9, was expressed in both proliferating myoblasts and differentiated myotubes *in vitro*. The BTEB1 protein was nuclear-localized in both cell types. BTEB1 activated the FGFR1 promoter via interaction with the Sp1-like binding site located at –59 bp within the FGFR1 promoter. FGFR1 gene expression is down-regulated during myogenic differentiation, and FGFR1 promoter activity is correspondingly reduced. This reduction in FGFR1 promoter activity was attributable to BTEB1 interaction with the same Sp1-like binding site located at –59 bp in the FGFR1 promoter. Therefore, BTEB1 is capable of functioning as a transcriptional activator and repressor of the same promoter via the same DNA-binding element and demonstrates a novel, bimodal role of BTEB1 during myogenesis.

INTRODUCTION

Vertebrate skeletal muscle development is partly characterized by expansive proliferation of myogenic precursor cells or myoblasts during fetal development. This growth of mononucleated myoblast populations in muscle-forming regions throughout the fetus is followed by myoblast fusion and differentiation to form multinucleated muscle fibers. Myoblast departure from the cell cycle during differentiation is permanent under normal developmental conditions, and the resulting muscle fibers are postmitotic. Fetal myoblast growth and differentiation occur at defined times in development. In developing chickens, fetal or secondary myogenesis occurs between embryonic days (ED) 8 and 18 (Stockdale, 1992). Therefore, the developmentally regulated proliferation of myoblasts and subsequent formation of muscle fibers is a tightly controlled process invoking regulators of both proliferation and differentiation.

Fibroblast growth factors (FGFs) comprise a family of more than 20 signaling proteins that regulate a broad range of developmental processes, including cell proliferation and differentiation. These effects are mediated by four high-affinity fibroblast growth factor receptors (FGFRs) in verte-

brates (Coumoul and Deng, 2003; Eswarakumar *et al.*, 2005). FGFR1 is most abundant in mesodermally derived tissues such as skeletal, cardiac, and smooth muscle. In proliferating cells, FGFR activity initiates the mitogen-activated protein kinase (MAPK)/Ras signal transduction cascade (Eswarakumar *et al.*, 2005; Katoh and Katoh, 2006).

FGFR1 function in myogenesis has been investigated both *in vitro* and *in vivo*. Overexpression of wild-type (WT) FGFR1 in Sol8 myogenic cells increased proliferation and delayed differentiation. Conversely, expression of a truncated FGFR1 mutant decreased proliferation and enhanced differentiation (Scata *et al.*, 1999). In addition, expression of a dominant negative FGFR1 variant in chick limbs resulted in a significant loss of muscle mass, decreased muscle fiber density, and a reduction of myoblasts (Flanagan-Steet *et al.*, 2000). In accord with its function *in vitro* and *in vivo*, FGFR1 is expressed in proliferating myoblasts, and expression is down-regulated during myogenic differentiation (Moore *et al.*, 1991; Patel *et al.*, 1999). Therefore, transcriptional regulation of FGFR1 gene expression is critical for normal myogenesis.

Like many growth factor receptor genes, FGFR1 promoter activity is regulated by several “Sp” factor binding sites (DiMario, 2002). The chicken FGFR1 promoter contains three Sp factor binding sites within the 75 bp proximal to the start of transcription (Patel *et al.*, 1999; Parakati and DiMario, 2002). These binding sites are comprised of GC boxes, CA sites, and CT motifs that bind Sp factors. Studies of the Sp1 interaction with the FGFR1 promoter demonstrated that Sp1 interacted directly with the most proximal Sp binding site (Parakati and DiMario, 2002). The most distal of these sites demonstrated the least Sp1-mediated activation of the pro-

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Abbreviations used: BTEB1, basic transcription element binding protein 1; KLF, Krüppel-like factor; FGFR1, fibroblast growth factor receptor 1; ED, embryonic day.

motor. These studies suggested that other Sp1-like proteins regulated FGFR1 promoter activity in myogenic cells.

Basic transcription element binding proteins (BTEBs) are members of the Krüppel-like C2H2 zinc finger family of transcriptional regulators. These factors are characterized by three C-terminal zinc finger domains and highly variable N-terminal regions. Sp1 is also a C2H2 zinc finger transcription factor and recognizes GC-rich binding sites, CA sites, and CT motifs in promoter regulatory regions, although some preferential binding of specific zinc finger proteins for particular binding sites has been suggested (Crossley *et al.*, 1996). The structural and functional similarities of Krüppel-like factor (KLF) family members as well as Sp1 have been previously reviewed (Dang *et al.*, 2000; Pearson *et al.*, 2008). Because of the overlap of binding specificities of the factors, direct competition for binding sites and promoter regulation is possible (Matsumoto *et al.*, 1998; Kaczynski *et al.*, 2001). Because many of these factors display opposite transcriptional regulatory properties, occupancy of promoter elements by specific KLF family members significantly affects promoter activity. There are three known members of the BTEB subfamily. Basic transcription element binding protein 1 (BTEB1), also designated Krüppel-like factor 9 (KLF9), can activate or repress promoter activity depending on the cell type and number of binding sites (Imataka *et al.*, 1992). BTEB3 and BTEB4 function primarily as transcriptional repressors (Kaczynski *et al.*, 2001, 2002) and have not yet been detected as expressed gene products in skeletal muscle cells. Given the complexity of the FGFR1 promoter with respect to the multiple, functional Sp1-like binding sites in the proximal regulatory region and the observed differential regulation of the promoter via these sites, further analysis of promoter regulation was conducted to address the hypothesis that FGFR1 promoter activity is regulated by a member of the KLF family of transcription factors, distinct from Sp1.

MATERIALS AND METHODS

Cell Culture

Fetal myoblasts were isolated from ED13 chicken leg muscles as previously described (O'Neill and Stockdale, 1972; Crow and Stockdale, 1986). Myoblasts were seeded into collagen-coated plates at a density of $1 \times 10^6/35$ -mm plate. Growth medium consisted of 10% horse serum (Hyclone, Logan, UT), 5% chick embryo extract, 1.32 mM CaCl_2 , 2 mM glutamine, and antibiotic/antimycotic (Invitrogen, Carlsbad, CA) in Ham's F-10 medium (Sigma, St. Louis, MO). Myotube cultures were incubated in medium supplemented with 10 μM cytosine arabinoside (AraC). Medium was replaced every other day. *Drosophila* SL2 cells (ATCC, Manassas, VA) were cultured in 90% Schneider's *Drosophila* medium/10% fetal bovine serum (Hyclone) supplemented with 2 mM glutamine and antibiotic/antimycotic (Invitrogen) as previously described (Parakati and DiMario, 2002).

DNA Constructs, Transfection, and Promoter Analysis

The chicken FGFR1 promoter-reporter construct, 3284FGFR1Luc, contains 3284 bp of FGFR1 DNA sequence upstream from the start of transcription. This FGFR1 sequence was isolated by restriction digests using SacI and BglII and was ligated upstream to the firefly luciferase gene in pGL3Basic (Promega, Madison, WI). Mutations were introduced into specific sites of the FGFR1 promoter using QuickChange PCR-based site-directed mutagenesis (Stratagene, La Jolla, CA) and sequence specific mutagenic oligonucleotides. The following forward primers and their anti-sense complementary primers were used to generate the respective DNA constructs: mu29FGFR1Luc: CTCTCTTCTCCTATCCACAGCTCAC; mu48FGFR1Luc: CCCTGCACCT-TATCCTGACTCTCTTTC; and mu59FGFR1Luc: CAGCAGCGGGACAGGG-GGTTAACTGCCTCTGCCCTGAC. Nucleotide substitutions introduced into the FGFR1 promoter are indicated by the bold, underlined sequence. The double mutant m29m48FGFR1Luc was generated using the mu48 mutagenic oligonucleotides and mu29FGFR1Luc as the DNA template. Mutations were confirmed by DNA sequencing.

The pPacBTEB1 expression plasmid (kindly provided by T. Elton, Brigham Young University) contains BTEB1 cDNA transcriptionally regulated by the *Drosophila* actin promoter. The pCMVBTEB1 contains the BTEB1 cDNA cloned into pCDNA3.1 (Invitrogen).

Myoblasts were transfected 24 h after isolation with 1 μg WT or mutant FGFR1Luc constructs and varying amounts of pCMVBTEB1. For promoter analyses, pSV-RL (Promega) was cotransfected for expression of Renilla luciferase to normalize for variation in transfection efficiencies. Equal amounts of total DNA were transfected, and pBluescript vector DNA (Stratagene) was used to equalize DNA content for transfection. DNAs were transfected using Lipofectamine 2000 (Invitrogen). For transfection of SL2 cells, medium was replaced with 100% Schneider's *Drosophila* medium overnight. Cells were transfected with 2 μg WT or mutant FGFR1Luc constructs and varying amounts of pPacBTEB1 using Cellfectin transfection reagent (Invitrogen). pBluescript was used to equalize DNA content for transfection, and pSV-RL was used to normalize transfection efficiencies.

FGFR1 promoter activities were measured using the Dual-Glo Luciferase Assay (Promega). Activities were determined 24 h and 9 d after transfection for myoblasts and myotubes, respectively. Firefly luciferase activities were normalized to Renilla luciferase activities to control for any differences in transfection efficiencies. Promoter activities in SL2 cells were measured 48 h after transfection.

Chromatin Immunoprecipitation

Chromatin immunoprecipitations from ED13 chicken myoblasts and myotubes were performed as previously described (Mitchell and DiMario, 2010) with the following modifications. Chromatin was immunoprecipitated with 16 μg BTEB1 antibody, and endogenous precipitated FGFR1 DNA was amplified using the following forward and reverse primers: CTGTTTTTCAGT-GCCAACT and CATGGGGCCCCGTCGGCCCGTG. Exogenous transfected FGFR1Luc DNA was amplified using the following primers: CTGTTT-TCAGTGCCAACT and CAGCGGTCCATCATCCAGCGGATAGAA.

RT-PCR

RNA from myoblasts and myotubes was extracted using RNA STAT-60 (Tel-Test B). RNA was then reverse transcribed and amplified using Access RT-PCR reagents (Promega) and the following forward and reverse BTEB1-specific primers: CACGCTACTGTGGTGTCACTGGC and GTTCCACCCC-ACCATGATCAAGCG. Identification of the amplified DNA as BTEB1 cDNA was verified by DNA sequencing.

Subcellular Fractionation, Western Blotting, and Immunohistochemistry

Cytoplasmic and nuclear protein extracts from myoblasts and myotubes were prepared as previously described (Parakati and DiMario, 2005; Mitchell and DiMario, 2010). Proteins were electrophoresed in a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Blots were placed in blocking solution (2% nonfat dry milk, 0.05% Tween-20 in PBS) overnight. BTEB1, E47 (Santa Cruz Biotechnology, Santa Cruz, CA), and β -actin (Upstate Biotechnology, Lake Placid, NY) antibodies were diluted 1:1000 in blocking solution and incubated with the blots for 1 h at room temperature. Blots were washed with 0.05% Tween-20 in PBS and then incubated in horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) diluted 1:1000 in blocking solution. Blots were washed as before and developed using Lumi-Light Chemiluminescent Substrate (Roche, Indianapolis, IN). The BTEB1 rabbit polyclonal antibody was generated commercially (Cambridge Research Biochemicals, Wilmington, DE) by immunization with the following chicken BTEB1 amino acid sequence conjugated to KLH (keyhole limpet hemocyanin): CSDSLESPEDEDAGSDSD.

BTEB1 was detected by immunohistochemistry in myoblasts and myotubes. Cells were fixed with methanol for 5 min at room temperature and then washed with PBS. Blocking solution (2% bovine serum albumin, 5% horse serum in PBS) was added for 1 h, followed by primary antibody diluted 1:1000 in blocking solution for 1 h. Cells were washed as before and then incubated in fluorescein-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:100 in blocking solution for 1 h. Cells were then incubated in 1.2 μM DAPI in PBS for 10 min and washed as before.

RESULTS

BTEB1 Is Expressed in Proliferating Myoblasts and Differentiated Myotubes

Several experimental approaches were taken to determine the expression of BTEB1 in myoblasts and myotubes. BTEB1 mRNA was identified in extracts of both myoblasts and myotubes by RT-PCR (Figure 1A). BTEB1 mRNA has previously been identified without detection of the BTEB1 protein (Imataka *et al.*, 1992). Therefore, the presence of BTEB1 protein was detected. Western blot analysis of protein extracts from myoblasts and myotubes using a custom antibody directed against chicken BTEB1 detected a single protein of approximately 200 kDa (Figure 1B). E47 and β -actin

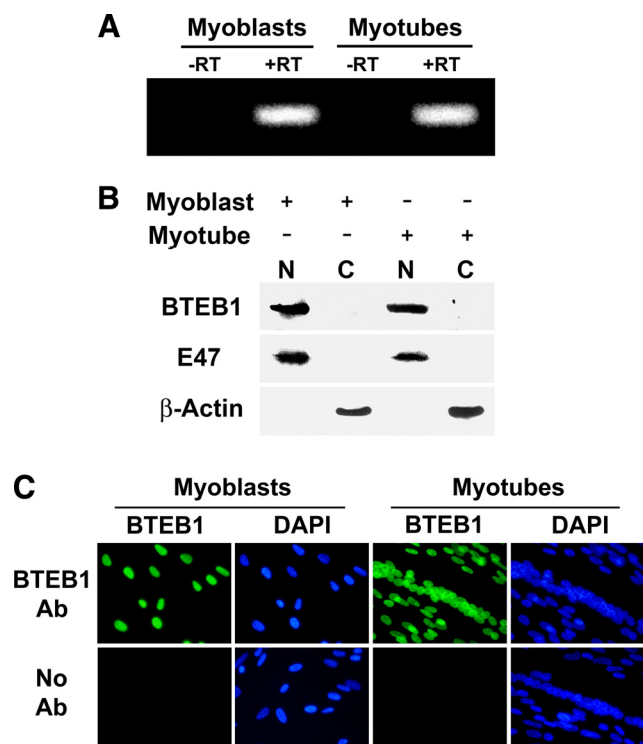


Figure 1. BTEB1 is expressed in myoblasts and myotubes. (A) BTEB1 gene expression in myoblasts and myotubes was assessed by RT-PCR. A 330-bp fragment was detected after amplification of myoblast and myotube cDNA generated from reverse transcriptase (+RT). The same product was not detected from reactions without reverse transcriptase (-RT). (B) BTEB1 protein was detected by Western blot analysis. Nuclear (N) and cytoplasmic (C) protein extracts from myoblast and myotube cultures were western blotted. BTEB1, E47, and β -actin were detected with their respective specific antibodies. E47 and β -actin detection in nuclear and cytoplasmic extracts, respectively, confirmed the separation of these extracts. BTEB1 was detected in nuclear extracts of both myoblasts and myotubes. (C) BTEB1 was immunolocalized to nuclei of myoblasts and myotubes. Myoblast and myotube cultures were immunostained with the BTEB1 antibody (BTEB1 Ab) and fluorescein-conjugated secondary antibody. Control cultures were processed without BTEB1 antibody (No Ab). Nuclei were visualized by DAPI staining.

were detected in nuclear and cytoplasmic extracts, respectively, to confirm the selective preparation of these extracts. Immunostaining of myoblasts and myotubes was also conducted. BTEB1 was detected in nuclei of both myoblasts and myotubes (Figure 1C).

BTEB1 Regulates FGFR1 Promoter Activity Positively in Proliferating Myoblasts

To determine the role of BTEB1 in the regulation of FGFR1 gene expression, myoblasts were transiently transfected with the WT FGFR1 promoter-luciferase construct 3284FGFR1Luc and the BTEB1 expression construct, pCMVBTEB1. FGFR1 promoter activity in myoblasts was increased by BTEB1 in a dose-dependent manner (Figure 2A). Transfection of 20 and 200 ng pCMVBTEB1 into myoblasts in 35-mm tissue culture plates resulted in FGFR1 promoter activities increased by ~2.5- and 6-fold, respectively. Increased FGFR1 promoter activity in the presence of increased pCMVBTEB1 was not a result of increased expression vector (pcDNA3.1) DNA. Transfection of 200 ng pcDNA3.1 empty vector and

3284FGFR1Luc into myoblasts did not significantly affect FGFR1 promoter activity relative to promoter activity in the absence of pcDNA3.1 (Figure 2B).

BTEB1 Activates the FGFR1 Promoter in the Absence of Sp1

Previous studies have shown that Sp1 positively regulates FGFR1 promoter activity in myoblasts (Parakati and DiMario, 2002). Because BTEB1 also increased FGFR1 promoter activity, the ability of BTEB1 to activate the promoter independently of Sp1 was tested. *Drosophila* SL2 cells lack endogenous Sp1 (Courey and Tjian, 1988) and therefore is an appropriate cell type to assess Sp1-independent transcriptional regulation. The 3284FGFR1Luc reporter construct was transfected alone and in combination with the BTEB1 expression construct, pPacBTEB1. Cotransfection of 0.75 and 3 μ g pPacBTEB1 increased FGFR1 promoter activity about four- and 10-fold, respectively (Figure 3). These results indicate that BTEB1 activates the FGFR1 promoter in proliferating myoblasts and that FGFR1 promoter activation in SL2 cells is independent of Sp1.

Mutation Analysis of the FGFR1 Promoter Regulatory Elements in Myoblasts and SL2 Cells

The chicken FGFR1 proximal promoter region contains three candidate Sp binding sites located at -29, -48, and -59 bp. To determine whether any of these sites mediates BTEB1 activation of the FGFR1 promoter, each site was mutated yielding mu29FGFR1Luc, mu48FGFR1Luc, and mu59FGFR1Luc. The function of these sites was tested by cotransfection of WT or mutant promoter-luciferase constructs along with pCMVBTEB1 into myoblasts. Mutation of the individual binding sites at -29 and -48 bp resulted in 30–40% reduction of promoter activity (Figure 4A). Notably, mutation of the -59-bp binding site resulted in an 85% reduction of promoter activity.

To further characterize the binding site(s) for BTEB1 activation of the FGFR1 promoter, SL2 cells were transfected with mu59FGFR1Luc and increasing amounts (10–200 ng) of pPacBTEB1. These amounts of transfected pPacBTEB1 significantly activated the WT FGFR1 promoter in SL2 cells (Figure 2). BTEB1-mediated activation of the promoter was abrogated by mutation of the Sp site at -59 bp at all amounts of pPacBTEB1 tested (Figure 4B). Importantly, transfection of the amounts of pPacBTEB1 along with the FGFR1 promoter-reporter construct harboring mutations of both the Sp sites at -29 and -48 bp (mu29mu48FGFR1Luc) significantly increased FGFR1 activity (Figure 4C). These results indicate that the binding site located at -59 bp mediates BTEB1 activation of the FGFR1 promoter.

Endogenous BTEB1 Binds to the FGFR1 Promoter in Myoblasts

Chromatin immunoprecipitation analysis was conducted to determine whether endogenous BTEB1 interacts with the FGFR1 promoter in myoblasts. Chromatin isolated from proliferating myoblasts was immunoprecipitated with the BTEB1 antibody, and precipitated DNA was amplified using primers that flanked the three proximal Sp binding sites. Amplification of DNA precipitated by the BTEB1 antibody yielded the expected product size of 330 bp (Figure 5). Positive controls included amplification of input chromatin before immunoprecipitation and amplification of 3284FGFR1Luc plasmid DNA. Negative controls included absence of antibody and use of nonspecific IgG.

To determine the site of BTEB1 interaction with the FGFR1 promoter via chromatin immunoprecipitation, WT

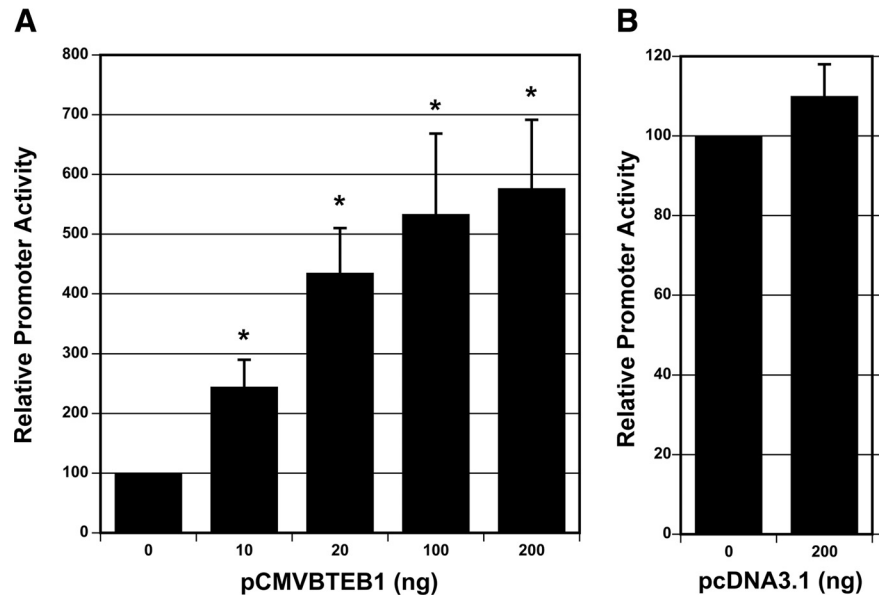


Figure 2. Positive regulation of FGFR1 promoter activity by BTEB1 in myoblasts. (A) ED13 chicken myoblasts were transfected with WT 3284FGFR1Luc and increasing amounts of pCMVBTEB1. Transfection of pCMVBTEB1 significantly (asterisk) increased FGFR1 promoter activity. Bars, means \pm SD ($p < 0.01$; $n = 4$). (B) Myoblasts were transfected with wild-type 3284FGFR1Luc and pcDNA3.1 empty vector DNA. No significant difference was detected. Bars, means \pm SD ($n = 3$).

(3284FGFR1Luc) and mutated (mu59FGFR1Luc) DNA constructs were transfected into myoblasts. Chromatin was then immunoprecipitated and amplified as above (Figure 5). WT 3284FGFR1Luc DNA was amplified, yielding the 330-bp product, indicating that BTEB1 interacted with the exogenous, transfected FGFR1 promoter. However, the FGFR1 promoter harboring a mutation of the binding site at -59 bp (mu59FGFR1Luc) was not amplified after immunoprecipitation with the BTEB1 antibody. Endogenous FGFR1 promoter sequence was not amplified in the transient chromatin immunoprecipitation experiments using WT 3284FGFR1Luc and mu59FGFR1Luc because a firefly luciferase specific

primer was used to distinguish endogenous FGFR1 sequence from FGFR1Luc sequence in the PCR reactions. These results further establish that BTEB1 interacts specifically with the FGFR1 promoter at the -59 -bp binding site.

BTEB1 Represses FGFR1 Promoter Activity in Differentiated Myotubes

FGFR1 gene expression is activated in proliferating myoblasts and repressed in differentiated myotubes (Scata *et al.*, 1999; Patel *et al.*, 1999). Furthermore, this developmentally regulated expression of FGFR1 is required for normal myogenesis (Flanagan-Steet *et al.*, 2000). To determine whether BTEB1 affects FGFR1 promoter activity in differentiated myotubes, WT FGFR1 promoter activity was measured in myotubes after transfection of 3284FGFR1Luc and increasing amounts of pCMVBTEB1 (Figure 6A). Cotransfection of pCMVBTEB1 significantly repressed FGFR1 promoter activity in myotubes in a dose-dependent manner. Greater than 95% promoter repression was observed by transfection of 200 ng pCMVBTEB1.

BTEB1 Interacts with the -59 -bp binding Site in Myotubes

Mutagenesis and transfection of the FGFR1 promoter-reporter constructs as well as chromatin immunoprecipitation were carried out to determine the site of interaction of BTEB1 with the FGFR1 promoter in differentiated myotubes. WT 3284FGFR1Luc, mu29FGFR1Luc, mu48FGFR1Luc, and mu59FGFR1Luc were individually cotransfected with 200 ng pCMVBTEB1. Mutation of the binding sites located at -29 and -48 bp significantly reduced FGFR1 promoter activity (Figure 7). Although FGFR1 gene expression is normally reduced in myotubes compared with myoblasts, mutation of these sites further reduced FGFR1 promoter activity. Conversely, mutation of the binding site located at -59 bp significantly increased FGFR1 promoter activity in myotubes. These cotransfection studies (Figures 6 and 7) indicate that BTEB1 functions as a transcriptional repressor of FGFR1 promoter activity in differentiated myotubes and suggest that the site of this repression is mediated by the binding site located at -59 bp.

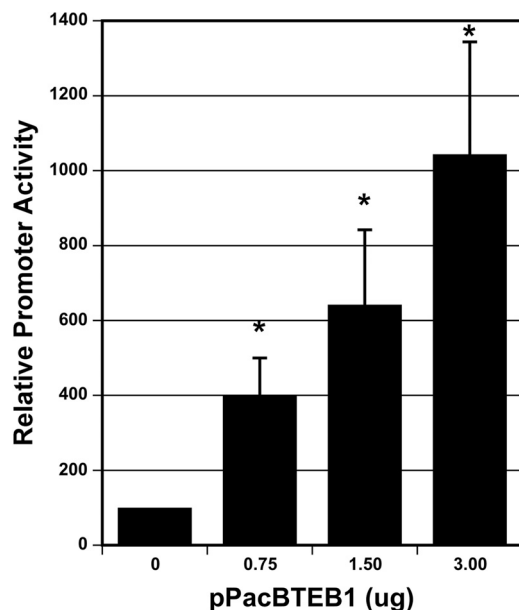


Figure 3. Activation of the FGFR1 promoter by BTEB1 in *Drosophila* SL2 cells. Cells were transfected with WT 3284FGFR1Luc and increasing amounts of the pPacBTEB1 expression construct. Transfection of pPacBTEB1 significantly (asterisk) increased FGFR1 promoter activity. Bars, means \pm SD ($p < 0.01$; $n = 4$).

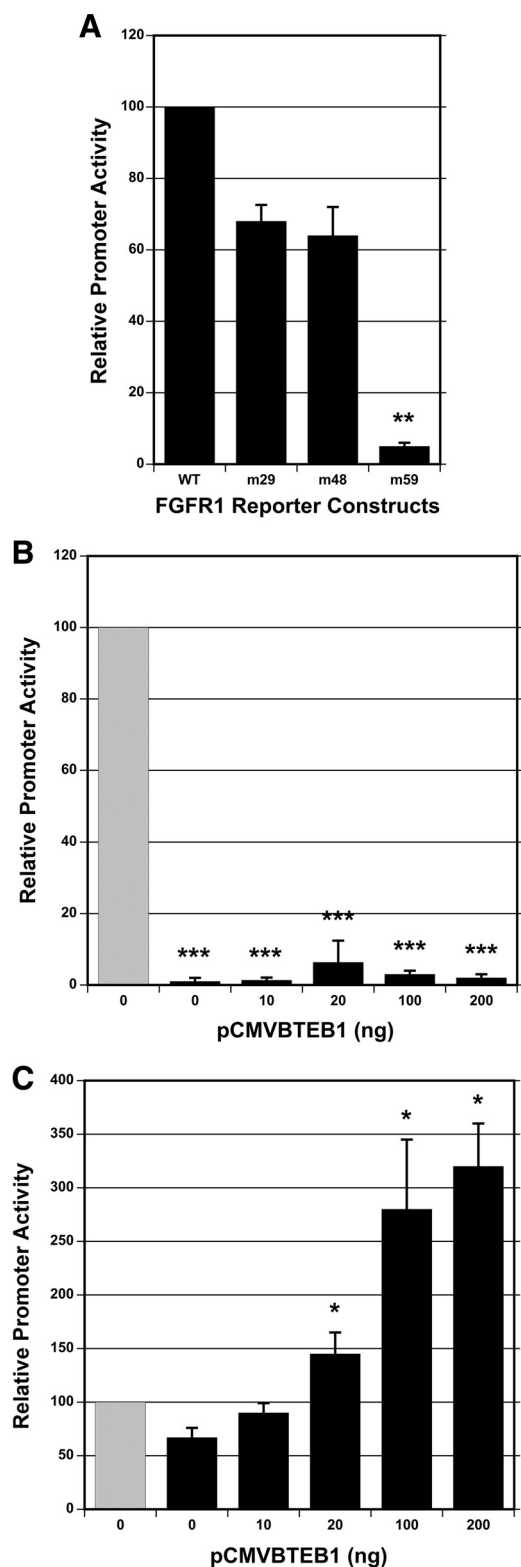


Figure 4. Effects of mutagenesis on FGFR1 promoter activity. (A) *Drosophila* SL2 cells were separately transfected with 2 μ g wild-type (WT) 3284FGFR1Luc, mu29FGFR1Luc (m29), mu48FGFR1Luc (m48), and mu59FGFR1Luc (m59) reporter constructs. Cells were cotransfected with 3 μ g pPacBTEB1. (B) Myoblasts were transfected with WT 3284FGFR1Luc (□) and mu59FGFR1Luc (■) along with increasing amounts of pCMVBTEB1. (C) Myoblasts were transfected with WT 3284FGFR1Luc (□) and mu29mu48FGFR1Luc (■)

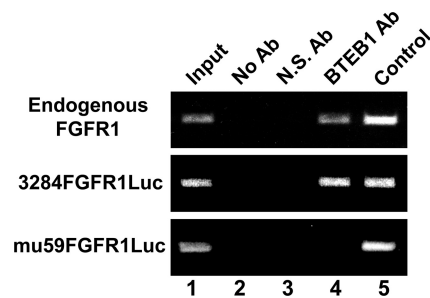


Figure 5. Chromatin immunoprecipitation analyses of BTEB1 interaction with the FGFR1 promoter in myoblasts. Chromatin from proliferating myoblasts was isolated and amplified as described in *Materials and Methods*. Lane 1, input chromatin before immunoprecipitation; lane 2, DNA product from chromatin immunoprecipitated without antibody; lane 3, DNA product from chromatin immunoprecipitated with nonspecific (N.S.) antibody; lane 4, DNA product from chromatin immunoprecipitated with BTEB1 antibody; lane 5, DNA product from amplification of 3284FGFR1Luc plasmid DNA (Control).

To further determine the site of interaction between BTEB1 and the FGFR1 promoter in myotubes, chromatin from myotubes was immunoprecipitated with the BTEB1 antibody, followed by amplification of DNA using FGFR1 gene specific primers that flanked the three Sp binding sites (Figure 8). Endogenous FGFR1 promoter sequence was specifically immunoprecipitated with the BTEB1 antibody. Transient chromatin immunoprecipitation of the WT 3284FGFR1Luc and mu59FGFR1Luc DNAs using the BTEB1 antibody resulted in amplification of the WT FGFR1 promoter sequence, but not the mu59FGFR1Luc promoter sequence. These results demonstrate that BTEB1 interacts with and represses FGFR1 promoter activity in myotubes via the -59-bp binding site.

DISCUSSION

Cell type-specific, developmentally regulated expression of the FGFR1 gene controls a wide array of developmental processes, including myoblast proliferation and differentiation. Studies of FGFR1 gene overexpression correlate increased FGFR1 activity with prolonged myoblast proliferation and reduced or delayed differentiation (Itoh *et al.*, 1996). Conversely, reduced FGFR1 activity resulted in reduced muscle mass, presumably due to reduced myoblast proliferation and premature differentiation (Itoh *et al.*, 1996; Flanagan-Steet *et al.*, 2000). In addition, to its role in normal myogenesis, FGF signaling has been causally linked to progression of prostatic cells toward a metastatic state (Murphy *et al.*, 2010). Altered FGFR1 gene expression is also associated with myeloproliferative neoplasms (Wadleigh and Tefteri, 2010) and a significant proportion of breast cancers (Turner *et al.*, 2010). Therefore, elucidation of the mechanisms that control FGFR1 gene expression have broad implications for understanding normal and diseased cellular processes.

Our previous studies have partially defined the mechanism of transcriptional regulation of the FGFR1 gene during

along with increasing amounts of pCMVBTEB1. Activities of mutant FGFR1 promoters were compared with activity of the WT 3284FGFR1 promoter. Bars, means \pm SD (* p < 0.05; ** p < 0.01; *** p < 0.001; n = 4).

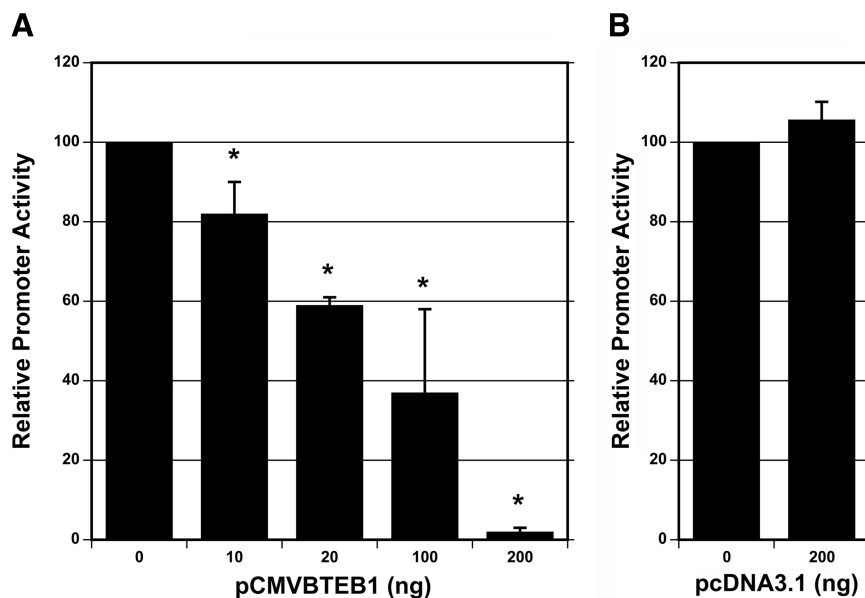


Figure 6. Repression of FGFR1 promoter activity by BTEB1 in myotubes. (A) FGFR1 promoter activities in myotubes transfected with WT 3284FGFR1Luc and increasing amounts of pCMVBTEB1 were measured. Transfection of pCMVBTEB1 significantly reduced FGFR1 promoter activity (* $p < 0.02$, $n = 4$). (B) FGFR1 promoter activities in myotubes transfected with WT 3284FGFR1Luc and pcDNA3.1 empty vector were measured. No significant differences were detected ($n = 4$). Bars, means \pm SD.

myogenesis. The avian FGFR1 promoter contains at least five functional Sp1-like binding elements. Two sites are located more than 1 kb upstream from the start of transcription. These sites bind the Sp1 transcription factor (Patel and DiMario, 2001). The remaining three sites are located in the proximal 70 bp from the start of transcription (Patel *et al.*, 1999; Parakati and DiMario, 2002). These numerous functional Sp1-like binding sites are characteristic of transcrip-

tional regulatory regions that lack overt TATA box sequences (DiMario, 2002). Consequently, the numerous sites may coordinate the location of transcription initiation (Näär *et al.*, 1998). In addition, the numerous functional sites provide the opportunity for complex, finely controlled transcriptional regulation via multiple transcriptional regulatory proteins and interactions among them. Indeed, this was detected in further studies of the FGFR1 promoter focusing on a neighboring E2F-binding site. Here, E2F4 functionally interacted with p107, p130, and Sp1 in a developmentally regulated, dynamic manner (Parakati and DiMario, 2005).

The current study revisited the three proximal Sp1-like binding sites of the FGFR1 promoter. Earlier work had shown that the most proximal site located at -29 bp bound Sp1 in proliferating myoblasts and that mutagenesis of this site significantly reduced FGFR1 promoter activity in myoblasts (Parakati and DiMario, 2002). Interestingly, Sp1-mediated activation of the FGFR1 promoter via the -59 -bp site was reduced relative to activation via the -29 -bp site. This

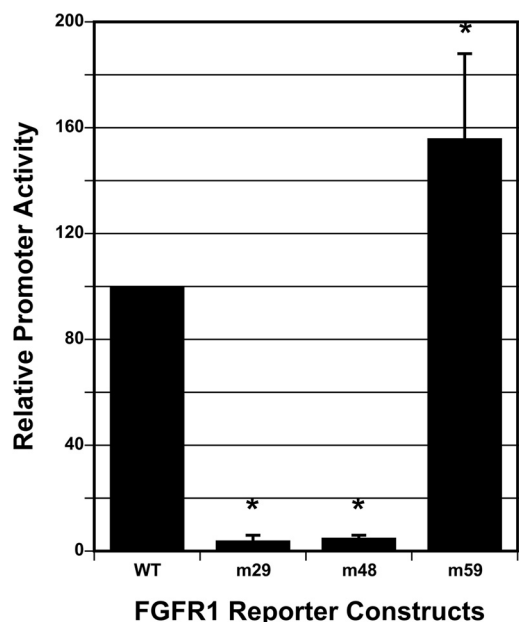


Figure 7. Effects of mutagenesis on FGFR1 promoter activity in myotubes. FGFR1 promoter activities were measured in myotubes transfected with the wild-type (WT) 3284FGFR1Luc, mu29FGFR1Luc (m29), mu48FGFR1Luc (m48), and mu59FGFR1Luc (m59) and cotransfected with 200 ng pCMVBTEB1. mu29FGFR1Luc and mu48FGFR1Luc yielded significantly reduced FGFR1 promoter activity relative to 3284FGFR1Luc. mu59FGFR1Luc yielded significantly increased promoter activity relative to 3284FGFR1Luc (* $p < 0.05$, $n = 4$).

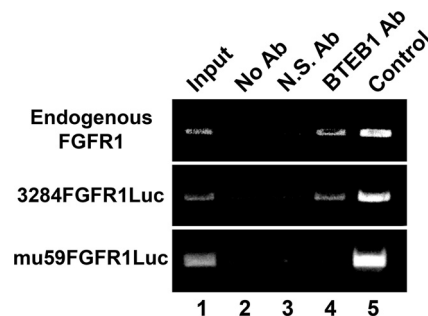


Figure 8. Chromatin immunoprecipitation analyses of BTEB1 interaction with the FGFR1 promoter in myotubes. Chromatin from differentiated myotubes was isolated and amplified as described in *Materials and Methods*. Lane 1, input chromatin before immunoprecipitation; lane 2, DNA product from chromatin immunoprecipitated without antibody; lane 3, DNA product from chromatin immunoprecipitated with nonspecific (N.S.) antibody; lane 4, DNA product from chromatin immunoprecipitated with BTEB1 antibody; lane 5, DNA product from amplification of 3284FGFR1Luc plasmid DNA (Control).

knowledge led to the hypothesis that a transcription factor similar to, but distinct from, Sp1 interacts with the binding site located at -59 bp of the FGFR1 promoter.

BTEB1/KLF9 is a member of the Krüppel-like transcription factor family. It can bind to Sp1-like *cis*-elements such as GC boxes and CT motifs (Imataka *et al.*, 1992) located in the proximal FGFR1 promoter. Because BTEB1 has typically been considered to be an Sp1-like transcriptional activator and mutation of the -59 -bp Sp1-like binding site reduced promoter activity, the role of BTEB1 regulation of the FGFR1 promoter via the -59 -bp binding site was investigated. Endogenous BTEB1 was expressed in both proliferating myoblasts and differentiated myotubes. Furthermore, BTEB1 protein was nuclear localized in both cell types, establishing it as a candidate transcriptional regulator. BTEB1 expression and cellular localization was distinct from that of Sp1, which was detected in myoblasts, but not in myotubes (Parakati and DiMario, 2002).

Similar to Sp1 interaction with the -29 -bp site, BTEB1 activated the FGFR1 promoter in myoblasts via the -59 -bp binding site. This interaction was confirmed through mutagenesis, BTEB1 overexpression studies, chromatin immunoprecipitation, and combinatorial usage of these approaches. Transcriptional activation by BTEB1 has been previously described for the SV40 (Imataka *et al.*, 1992), γ -globin (Asano *et al.*, 2000), and RANTES (Song *et al.*, 1999) promoters. Therefore, BTEB1 activation of the FGFR1 promoter in myoblasts supports its role as a transcriptional activator of diverse promoters in numerous cell types. Importantly, this is the first report of a KLF family member other than Sp1 involved in transcriptional regulation of genes that control skeletal myoblast proliferation and differentiation.

The multiple Sp1-like binding sites within the proximal FGFR1 promoter offer potential strong transcriptional activation. They also provide the opportunity for finely tuned transcriptional control. Although the most proximal -29 -bp site interacted with Sp1 (Parakati and DiMario, 2002), the -59 bp site demonstrated preferential activation of the promoter by BTEB1 (Figures 4 and 5). This distinction of specific Sp1-like *cis*-element interactions with Sp1 versus BTEB1 provides perhaps subtle, but important transcriptional regulation. KLF family members share high sequence homologies within the carboxy-terminal regions that contain the zinc finger DNA-binding domains. However, their amino-terminal regions are highly variable, suggesting KLF-specific interactions with other transcriptional regulators. Such KLF-specific interactions with multiple KLF regulators on the same promoter may provide more complex, dynamic transcriptional control.

The presence of BTEB1, in contrast to the absence of Sp1, in differentiated myotubes encouraged further investigation of its function relative to FGFR1 gene expression. Interestingly, BTEB1 maintained interaction with the -59 -bp binding site of the FGFR1 promoter in myotubes. Furthermore, it mediated transcriptional repression of the FGFR1 promoter. Many KLF family members such as Sp1 function as transcriptional activators. Other KLFs such as KLF10 function as transcriptional repressors (Cook *et al.*, 1998; Zhang *et al.*, 2001). BTEB1's bimodal, reciprocal function of transcriptional activation in myoblasts and transcriptional repression in myotubes via the same DNA-binding site is a novel finding for BTEB1.

The mechanism by which BTEB1 switches its function during myogenesis is not known. It is possible that BTEB1 functions as a transcriptional repressor in myotubes by blocking transcriptional activators. However, to date, we have no data to support this model. Rather, data support a

model in which transcriptional repressor complexes exert their repressor activity on the FGFR1 promoter via dynamic protein-protein interactions among transcriptional regulators and that the structural basis for the interaction between repressor complexes and the FGFR1 promoter are present in both myoblasts with activated FGFR1 gene expression and in myotubes with FGFR1 gene repression. This is evident by the occupancy of both the -59 -bp binding site and the E2F-binding site by BTEB1 and E2F4, respectively, in both myoblasts and myotubes (Figures 5 and 8; Parakati and DiMario, 2005). In the case of the E2F4-binding site, the transcriptional repressor complex is activated by exchange of p107 for p130 and loss of Sp1. It is reasonable to hypothesize that the bimodal, reciprocal function of BTEB1 is also mediated by cell type specific, developmentally regulated interactions of BTEB1 with specific transcriptional coregulators. For example, TEIG1 can function as a transcriptional repressor by interaction with mSin3A and HDAC1 (histone deacetylase 1) via its SID (Sin-interacting domain; Zhang *et al.*, 2001). The potential role of mSin3A in the BTEB1-mediated transcriptional repression of the FGFR1 promoter in myotubes is unclear because forced expression of mSin3A in myogenic cells did not significantly affect FGFR1 promoter activity (unpublished results). Alternatively, BTEB1 may functionally interact with transcriptional regulators resident at the other proximal Sp1-like binding sites of the FGFR1 promoter in differentiated myotubes.

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