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Zeb1 and Tle3 are *trans*-factors that differentially regulate the expression of myosin heavy chain-embryonic and skeletal muscle differentiation

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Abstract

Myosin heavy chain-embryonic encoded by the *Myh3* gene is a skeletal muscle-specific contractile protein expressed during mammalian development and regeneration, essential for proper myogenic differentiation and function. It is likely that multiple *trans*-factors are involved in this precise temporal regulation of *Myh3* expression. We identify a 4230 bp promoter-enhancer region that drives *Myh3* transcription in vitro during C2C12 myogenic differentiation and in vivo during muscle regeneration, including sequences both upstream and downstream of the *Myh3* TATA-box that are necessary for complete *Myh3* promoter activity. Using C2C12 mouse myogenic cells, we find that Zinc-finger E-box binding homeobox 1 (Zeb1) and Transducin-like Enhancer of Split 3 (Tle3) proteins are crucial *trans*-factors that interact and differentially regulate *Myh3* expression. Loss of Zeb1 function results in earlier expression of myogenic differentiation genes and accelerated differentiation, whereas Tle3 depletion leads to reduced expression of myogenic differentiation genes and impaired differentiation. Tle3 knockdown resulted in downregulation of Zeb1, which could be mediated by increased expression of *miR-200c*, a microRNA that binds to *Zeb1* transcript and degrades it. Tle3 functions upstream of Zeb1 in regulating myogenic differentiation since double knockdown of *Zeb1* and *Tle3* resulted in effects seen upon *Tle3* depletion. We identify a novel E-box in the *Myh3* distal promoter-enhancer region, where Zeb1 binds to repress *Myh3* expression. In addition to regulation of myogenic differentiation at the transcriptional level, we uncover post-transcriptional regulation by Tle3 to regulate *MyoG* expression, mediated by the mRNA stabilizing Human antigen R (HuR) protein.

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Author Contributions

Pankaj Kumar, Masum Saini, and Sam J. Mathew were involved in experimental design; Pankaj Kumar, Aatifa Zehra, and Masum Saini carried out the experiments; Pankaj Kumar, Aatifa Zehra, Masum Saini, and Sam J. Mathew analyzed and interpreted the results; and Sam J. Mathew wrote the final manuscript based on the draft written by Pankaj Kumar.

Disclosures

The authors declare no conflicts of interest.

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Thus, Tle3 and Zeb1 are essential *trans*-factors that differentially regulate *Myh3* expression and C2C12 cell myogenic differentiation in vitro.

Keywords

C2C12 cells; mouse; myogenin; myosin heavy chain-embryonic; regeneration; skeletal muscle; Tle3; Zeb11

1 Introduction

The mammalian adult skeletal muscle is composed of diverse cell types including muscle fibers, muscle stem cells, fibroblasts, macrophages, pericytes, etc. Muscle fibers are long, multinucleate, contractile cells that arise by a process of fusion and differentiation of myogenic progenitors during development. The muscle fibers contain sarcomeres which are the functional contractile units, composed of thin and thick filaments. Myosins are the primary contractile proteins that constitute the thick filaments, which are heterohexamers made up of a pair of heavy chains and two distinct pairs of light chains. Myosin heavy chain (MyHC) is the largest subunit of the myosin molecule and is crucial for muscle contractile function. Several MyHC isoforms with varying contractile velocities exist, which are expressed in a temporally and spatially restricted manner. While most MyHC isoforms are expressed in the mature, adult muscle, two isoforms, referred to as the developmental MyHCs, are expressed primarily during developmental stages. These are MyHC-embryonic encoded by the *Myh3* gene and MyHC-perinatal encoded by the *Myh8* gene. These two MyHCs are normally not expressed in most adult muscles except during muscle regeneration following injury or disease. Interestingly, not much was known about these developmental MyHCs, although mutations in them lead to congenital musculoskeletal abnormalities such as Freeman-Sheldon and Trsimus-pseudocamptodactyly Syndromes.[1] Recently, we characterized the role of MyHC-embryonic during embryonic and perinatal stages, where we reported its importance in regulating proper myogenic differentiation.[2]

The highly dynamic expression of MyHC-embryonic and -perinatal, high during developmental stages but absent in the normal adult muscle, and a similar transient increase during muscle regeneration following injury, indicates that the expression of developmental MyHCs is tightly regulated. To attain strict temporal regulation, it is likely that developmental MyHCs are regulated in multiple ways, possibly at the transcriptional, post-transcriptional, translational, and post-translational levels. Several studies have been previously carried out to understand the transcriptional regulation of developmental MyHCs, with respect to *cis*-regulatory elements and *trans*-factors.[3–6] The myogenic regulatory factor (MRF) MyoD has been reported to be a crucial *trans*-factor that binds to consensus E-boxes in the promoter-enhancers of *Myh3* and *Myh8* to regulate their expression.[3] Nuclear factor of activated T-cells (NFAT) isoforms have been reported to bind to the *Myh3* and *Myh8* promoter-enhancer, and cooperate with MyoD to regulate the expression, especially in the case of *Myh8*.[5] Thus, although a few studies have sought to understand the transcriptional regulation of *Myh3*, they have mostly focused on activators, with very little known about repressors. This is significant because *Myh3* expression is downregulated sharply at specific

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time points, including postnatal stages and post-muscle regeneration. It is thus likely that additional *cis*-elements and *trans*-factors are essential for proper regulation of expression of *Myh3*.

Zinc finger E-box binding homeobox 1 protein (Zeb1) is a repressor which negatively regulates myogenic differentiation and the expression of several genes associated with myogenic differentiation such as MyHC-IIb, troponin, muscle creatine kinase and MyoD.[7] The mechanism by which Zeb1 represses its target genes was proposed to be mediated by the C-terminal binding factor (CtBP) corepressors, which recruit histone modifiers such as histone deacetylases (HDACs) to repress transcription.[7] Loss of Zeb1 function has also been reported to lead to defective skeletal muscle regeneration in mice.[8] ZEB1 has been shown to interact with the corepressor Transducin-like Enhancer of Split 1 (TLE1) to transcriptionally repress E-Cadherin in lung adenocarcinoma cells.[9] Transducin-like Enhancer of Split (TLE) proteins are a family of corepressors with vital functions during embryonic development, adult homeostasis and regeneration.[10] Recently, we found that Tle4 prevents Pax7-mediated activation of myogenic factor 5 (*Myf5*) in quiescent muscle stem cells.[11] Tle3 has been shown to be important in promoting white adipose tissue and mitochondrial gene expression.[12–14] Tle3 has also been reported to regulate myogenic differentiation by interacting with MyoD.[15] Thus, Zeb1 and Tle3 are attractive *trans*-factor candidates, which might have roles in regulating *Myh3* expression and myogenic differentiation.

We have identified a 4230 bp promoter-enhancer region of *Myh3* spanning sequences upstream and downstream of the TATA-box, which are both necessary for complete promoter activity in vitro during C2C12 myogenic differentiation and in vivo during mouse skeletal muscle regeneration. Using C2C12 mouse myogenic cell differentiation as model, we find that the MRF MyoG activates *Myh3* promoter activity, whereas Zeb1 represses it. Loss of Zeb1 function results in earlier expression of myogenic differentiation factors and accelerated differentiation. Zeb1 was found to interact with the corepressor Tle3, and Tle3 knockdown led to decreased expression of myogenic differentiation factors and impaired differentiation. Tle3 depletion also resulted in downregulation of Zeb1 levels, possibly due to elevated levels of *miR-200c*, a microRNA that degrades *Zeb1* via a feedback loop mechanism. Double knockdown of *Zeb1* and *Tle3* recapitulated the phenotypes seen upon *Tle3* depletion, indicating that Tle3 functions upstream of Zeb1 in regulating myogenic differentiation. Our study also uncovers a novel Zeb1 binding site in the *Myh3* distal promoter-enhancer region, which represses *Myh3* expression. Tle3 was found to regulate the expression of *MyoG* at the post-transcriptional level, mediated by the Human antigen R (HuR) protein. Thus, we identify Tle3 and Zeb1 as crucial *trans*-factors that differentially regulate *Myh3* expression and C2C12 cell myogenic differentiation.

2 Methods and Materials

2.1 Animal experiments

Wild-type C57BL/6 mice aged 6–8 weeks were used for the in vivo experiments in the present study. All animals were maintained and experimental procedures performed as per the protocols approved by the Institutional Animal Ethics Committee (RCB/IAEC/2017/021).

2.2 Plasmid and DNA constructs

For generating the mouse *Myh3* promoter-enhancer-GFP fusion construct, the 4230 bp mouse *Myh3* promoterenhancer DNA sequence was amplified from the bacterial artificial chromosome RP23-67L23, using specific primers and incorporated into the *pEGFP-C2* vector at the *AseI* and *NheI* restriction sites. Similarly, for cloning the full length and deletion *Myh3* promoter-enhancer constructs into the luciferase reporter *pGL-3 control* vector, *BglII* and *HindIII* restriction sites were used. The *Myh3-Z1-Mut* promoter-enhancer construct was generated by mutating the consensus Zeb1-binding CACCTG sequence to GGAACT in the *Myh3* full length promoter-enhancer construct and cloning into the *pGL-3 control* vector, using the *BglII* and *HindIII* restriction sites. For cloning of *myogenin* 3'untranslated region (UTR), the *myogenin* 3'UTR was PCR amplified using specific primers and inserted into the *pmirGLO* dual-luciferase vector at the *SacI* and *SalI* restriction sites. *Zeb1-Mut* construct was generated by PCR amplification of multiple fragments of Zeb1 coding sequence (excluding amino acids 150–272 of N-terminal and 882–959 of C-terminal zinc finger clusters) having overlapping sequences to each other and *pcDNA3.1/HisC* vector, using *BamHI* and *XbaI* restriction sites. The primers used for cloning are listed in Table 1.

2.3 Cell culture, transfections and nucleofection

C2C12 mouse myoblast cells (ATCC; Cat# CRL-1722) were cultured and maintained as per ATCC guidelines in complete media (DMEM media supplemented with 10% FBS and 1% Penicillin–Streptomycin). For differentiation of C2C12 cells, after plating the cells at day 0 in complete media, it was replaced with differentiation media (DMEM media supplemented with 2% horse serum and 1% Penicillin–Streptomycin) after 48 h (day 2). After 72 h of plating (day 3 onwards), 50% of the media was replaced daily with fresh differentiation media. For knockdown experiments, control, *Tle3*, or *Zeb1* sequence-specific small interfering RNA (siRNA) were reverse transfected into C2C12 myoblasts. Approximately 30 000 C2C12 cells were seeded per well of a 24 well plate containing the transfection mix. The transfection mixture per well comprised 100 μL Opti-MEM (Gibco; Cat# 31985070), 100 nM of *Tle3* (Ambion; Cat# 4390771, ID: S75196 and Ambion; Cat# 1320001, ID: MSS238514) or 100 nM of *Zeb1* (Ambion; Cat# 1320001, ID: MSS210696 and Ambion; Cat# 1320001, ID: MSS210697) or control siRNA (Ambion; Cat# 4390816, ID: 4390847) and 2 μL Lipofectamine RNAiMAX (Invitrogen; Cat# 13778150). For plasmid co-transfections in C2C12 myoblasts, forward transfection was performed. Twenty-four hours prior to plasmid transfection, C2C12 cells were seeded at a density of 2×10^5 cells per well in a 6-well plate, so as to obtain 70%–80% confluency on the day of transfection. 2 μg of *pCMV-3Tag3b-FLAG-Tle3* or control *pCMV-3Tag3b-FLAG* plasmid were transfected with Lipofectamine 2000 (Invitrogen; Cat# 11668-019) as per recommendations of the manufacturer. In a similar manner, *pcDNA3.1/HisC-Zeb1* plasmid was forward transfected in C2C12 cells, after 24 h of *Tle3* siRNA treatment. Cells were harvested for RNA and protein isolation using standard protocols on days 3, 5, and 7 of C2C12 differentiation. Where specified, C2C12 cells were incubated with either the vehicle DMSO or the proteasomal inhibitor MG132 (Sigma; 4747901MG) at 10 μM concentration for 6 h at days 3 and 5 of differentiation post siRNA transfection. Similarly, for *MyoG* mRNA stability, C2C12 cells were incubated with the transcription inhibitor Actinomycin

D (CST; 15021S) at 5 μM concentration for 4- and 8-h time points at days 3 and 5 of differentiation.

For measuring in vitro *Myh3* promoter activity, C2C12 cell nucleofection with full-length and truncated *Myh3* promoter-enhancer plasmid DNA constructs were done using the Lonza Nucleofector 2b (AAB1001, Kit V), program B032, as per the guidelines of the manufacturer. Cells were seeded at a density of 50 000 cells/well of a 24 well dish. For expression of *Myh3* promoter-enhancer fused to GFP, nucleofection was carried out on C2C12 cells cultured on coverslips, and stained for MyHC-embryonic as well as GFP.

2.4 In vivo electroporation and tissue harvesting

For measuring the activity of the *Myh3* promoter constructs in vivo during regeneration post injury, the tibialis anterior (TA) muscle of C57BL/6 mice were given pre-treatment with Hyaluronidase (0.5 U/μL) subcutaneously for efficient delivery of DNA. The mice were anesthetized using appropriate dose of xylazine/ketamine, and luciferase plasmid constructs of the required concentration in sterile saline (as mentioned in Section 2.5) were injected into the TA muscle intramuscularly with a 22-gauge syringe after 2 h of the hyaluronidase treatment. This was followed by transcutaneous standard square wave pulses delivered using stainless steel electrodes according to the manufacturer's guidelines (ECM830 Square Wave Electroporation System, BTX). A total of ten pulses of 175 V/cm were delivered to the muscle, with each pulse of 20 μs duration, at an interval of 100 ms.[16] Electroporated muscles were injured after 72 h of electroporation by injecting 25 μL of 1.2% Barium chloride. The TA muscles were then harvested 5 days post injury (dpi) for performing luciferase assay.

2.5 Luciferase assay

Luciferase assay was carried out using a *firefly* luciferase reporter vector, along with equi-molar amounts of expression vectors containing *MyoG, Zeb1, MyoD, Tle3, Mitf and Zeb1-Mut* genes, as per the experiment. For the *myogenin* 3'UTR expression analysis, *pmirGLO* dual-luciferase vector was used. For the *Myh3* promoter-enhancer full-length and deletion luciferase constructs, transfection was carried out by nucleofection in C2C12 cells, with 250 ng of the *firefly* luciferase construct and 0.5 ng of the *pHRL-CMV renilla* luciferase (Promega; Cat# E6271) as an internal control (for transfection efficiency). For forward transfection of luciferase constructs in C2C12 cells, 600 ng of *firefly* and 0.5 ng of *renilla* luciferase were transfected alone or in combination with expression vectors for *MyoG, Zeb1, MyoD, Tle3, Mitf and Zeb1-Mut* into 50 000 cells/well of a 24 well plate. Likewise, for the 3'UTR expression analysis, 600 ng of the *myogenin* 3'UTR in the *pmirGLO* dual luciferase construct was transfected. The total quantity of plasmid DNA used for transfection was kept constant between control and sample, by equalizing with *pET-28a* (+) vector. After 120 h, cells were lysed and luciferase assay was performed using a luminometer (Promega) with the Dual-Glo Luciferase Assay system (Promega, Cat# E2920).

For measuring *Myh3* promoter activity in vivo during regeneration, 30 μg of *firefly* luciferase construct and 60 ng of *renilla* luciferase construct were electroporated into the TA, harvested (as explained in Section 2.4) and luciferase assay performed using the Dual-

Glo Luciferase Assay system. The data of luciferase activity are represented as relative luciferase units (RLU), comparing the ratio of firefly luciferase activity normalized to renilla luciferase. The percent reduction in *Myh3* promoter activity is equal to 100 minus percent promoter activity of the deletion construct, normalized to the *Myh3* FL promoter activity. As an example, for calculating the percent loss in activity, if promoter activity of DC2 is 170 and that of FL is 2100, then the percent loss or reduction in promoter activity is 100–170/2100 × 100 equals to 92%.

2.6 Total RNA isolation, cDNA synthesis, and qPCR

Total RNA was isolated from cultured C2C12 cells at days 3, 5, and 7 of differentiation using the RNeasy Plus Mini Kit (Qiagen), as per the manufacturer's protocols, followed by cDNA synthesis using 1 μg RNA template, oligo $(dT)_{12–18}$ primer, and superscript III reverse transcriptase (Invitrogen). For miRNA quantification, cDNA was synthesized (miScript II RT kit, Qiagen) from 1 μg RNA template isolated using miRNeasy mini kit (Qiagen) as per manufacturer's instructions. The cDNA samples were used as template for quantitative PCR (qPCR) on an ABI 7500 Fast Real-Time PCR instrument (Applied Biosystems), using gene-specific primers (Table 2). Expression levels of all the mouse genes examined were normalized to *Gapdh* expression. Taqman probes were used for quantification of *Tle3* expression (Applied Biosystems; Cat# Mm00437097_m1) with *Gapdh* used as normalizing control (Applied Biosystems; Cat# Mm99999915_g1). For *miR-200c* expression, Mm_miR-200c_1 miScript primers (Qiagen; miS-cript assay ID# MS00001827) were used along with small non-coding *RNU6* RNA-specific Mm_RNU6-2_11 miS-cript primers (Qiagen; miScript assay ID# MS00033740) as normalizing control.

2.7 Immunoblotting and co-immunoprecipitation

C2C12 cell pellets were lysed in RIPA buffer (Sigma; Cat# R0278-500 mL) containing 1% protease inhibitor (Sigma, P8340-5 mL), to obtain protein lysates. Protein quantification was done using the bicinchoninic acid protein Assay Kit (Pierce; Cat#23225). Protein samples were then subjected to 10% SDS-PAGE, followed by transfer onto a PVDF membrane (Millipore; Cat# iPVH00010). The PVDF membrane was incubated with specific primary antibodies at 4°C overnight, followed by incubation with appropriate secondary antibodies for two hours at room temperature, immunoblotted, developed by chemiluminescence, and imaged using a digital imaging system (ImageQuant LAS4000). Immunoblots were quantified using Image J and represented in the form of fold intensity with respect to beta-actin, in densitometry graphs. For immunoprecipitation, C2C12 cells were crushed to a fine powder in IP lysis buffer (Thermo Scientific; Cat# 87787), followed by centrifugation to obtain clear protein lysates. The protein lysates were pre-cleared by incubating with Protein G-Agarose beads (GE healthcare; Cat# 17-0618-01) for 3 h at 4°C on an end-to-end rotator. Pre-cleared lysates were incubated with the Zeb1 antibody for immunoprecipitation at 4°C overnight. IgG (Millipore; Cat#12-370) was used as the negative control for immunoprecipitation. The antibody bound proteins were captured on IP lysis buffer equilibrated Protein G Agarose beads, washed five times with IP lysis buffer containing 1% protease inhibitor, boiled in 2× Laemmli buffer, and analyzed by immunoblotting. Antibodies used were rabbit anti-TLE3 (Proteintech; 11 372-1-AP) at 4

μg/mL, rabbit anti-Zeb1 (ThermoFisher; PA5-28221) at 0.1 μg/ mL, mouse anti-MyoD (Santa Cruz; sc-32758) at 2 μg/ mL, mouse anti-MyoG (Santa Cruz; sc-12732) at 2 μg/ mL, mouse anti-H3K9me2/3 (CST; 5327) at 0.01 μg/mL, mouse anti-β-actin (CST; 3700) at 1.25 μg/mL, rabbit anti-KMT1A/SUV39H1 (CST; 8729S) at 1 μg/mL, rabbit anti-GFP (Sigma; A11122) at 2 μg/mL, mouse anti-MyHC-embryonic (DSHB; F1.652) at 3 μg/mL, HRP Goat anti-rabbit (Jackson Immuno Research; 111-035-144), and HRP Goat anti-mouse (Jackson Immuno Research; 111-035-003) at 0.08 μg/mL concentrations.

2.8 Chromatin immunoprecipitation (ChIP)

Undifferentiated C2C12 cells were cross-linked in 1% formaldehyde solution and quenched by adding 0.125M glycine. Fixed cells were resuspended in nuclear lysis buffer (supplemented with 1× protease inhibitor) at a density of 1×10^6 cells/100 μL and sonicated to shear the chromatin to an average fragment size of 200–600 bp using Bioruptor Pico (Diagenode). Sheared chromatin was immunoprecipitated using 2 μg of rabbit anti-Zeb1 polyclonal antibody (ThermoFisher; PA5-28221) as well as 3 μg of mouse anti-Zeb1 monoclonal antibody (Novus; 24BA6, NBP2-23484) in separate independent experiments. Antibody-chromatin complexes were immobilized on Protein G magnetic beads (Dynabeads Protein G, Cat#10003D), washed thrice and eluted in elution buffer. The supernatant was collected, reverse crosslinked at 65°C overnight using 5M NaCl, and DNA isolated using phenol-chloroform with chilled ethanol precipitation. The isolated DNA fragments were used to set qPCR using *Myh3* promoter-enhancer-specific primers and control primers for a non-specific region on Chromosome 11 (Primer sequences given in Table 2). Rabbit anti-IgG and mouse anti-IgG incubated beads only negative controls were used and a minimum of three independent experiments were carried out.

2.9 Immunofluorescence staining, imaging and processing

For quantification of C2C12 cell fusion and differentiation index, either *Zeb1* or *Tle3* and control siRNA-treated cells were cultured on gelatin coated coverslips, fixed in 4% paraformaldehyde followed by blocking in 5% goat serum (BioAbChem; Cat# 72-0480) in PBS containing 0.1% Triton-X-100 (MP Biochemicals; Cat# 194854). The coverslips were incubated overnight at 4°C with appropriate concentration of myosin heavy-chain primary antibodies (mixture of MyHC-embryonic, MyHC-slow, and MY-32 antibodies), followed by incubation with fluorophore-conjugated secondary antibodies as well as phalloidin, and mounted using DAPI Fluoromount-G (Southern Biotech; Cat# 0100-20). For fusion and differentiation index, the total number of $DAPI^+$nuclei was counted using the spot and annotation function in Imaris software (http://www.bitplane.com/). The number of $DAPI^+$nuclei within the myotubes and the total number of myotubes were counted using Image J. Fusion and differentiation index were calculated as the percentage of nuclei within myotubes compared to the total number of nuclei. For fusion index, only myotubes with two or more nuclei were counted, whereas for differentiation index, myotubes with a single nucleus expressing MyHC were also counted. Similarly, fusion and differentiation indices were quantified with *pCMV-3Tag3b-FLAG-Tle3* or control *pCMV-3Tag3b-FLAG* transfected C2C12 cells. *Zeb1* and control siRNA-treated cells were stained with phalloidin for reserve cell numbers and normalized to unit area (mm^2). Antibodies and fluorophores used are rabbit anti-TLE3 (Proteintech; 11 372-1-AP) at 4 μg/mL, rabbit anti-Zeb1

(ThermoFisher; PA5-28221) at 0.3 μg/mL, mouse anti-MyHC-embryonic (DSHB; F1.652) at 3 μg/mL, mouse anti-MyHC-slow (Sigma; M8421) at 1.5 μg/mL, mouse anti-myosin (Sigma; M4276) at 1 μg/mL, Goat anti-rabbit Cy2 conjugated (Jackson ImmunoResearch; 111-225-144) at 7.5 μg/mL, Goat anti-rabbit Cy3 conjugated (Jackson ImmunoResearch; 111-165-144) at 7.5 μg/mL, Goat anti-rabbit Biotin conjugated (Jackson ImmunoResearch; 115-065-020) at 2.8 μg/mL, Goat anti-rabbit Biotin conjugated (Jackson ImmunoResearch; 115-065-144) at 2.8 μg/mL, Cy3 conjugated streptavidin (Jackson ImmunoResearch; 016-160-084) at 3.6 μg/mL, Cy2 conjugated streptavidin (Jackson ImmunoResearch; 016-220-084) at 3.6 μg/mL, and Oregon Green 488 Phalloidin (Life Technologies; O7466) at 0.6 U/mL concentrations. Immunofluorescence images were acquired using a Leica TCS SP8 confocal microscope.

2.10 Statistical analysis

For all the experiments, a minimum of three biological replicates were used, analyzed, and represented as mean ±standard error of mean. The data from the experiments were analyzed using parametric, unpaired *t*-test using the GraphPad Prism software. For mRNA stability, mRNA half-life/decay rate was calculated by non-linear regression curve fitting (One phase decay) using the GraphPad Prism software. The p-value ≤.05 was considered significant; p-values are indicated on the graphs along with asterisks denoting significance.

3 Results

3.1 A 4.2 kb genomic region regulates the expression of *Myh3*

To characterize the *cis*-regulatory region regulating *Myh3* expression, we identified a 4230 bp genomic region upstream of the ATG start codon in the third exon of *Myh3*.[2] This 4230 bp *Myh3* promoter-enhancer was cloned upstream of a GFP reporter (*pEGFP-C2-Myh3-FL*), which was transfected into C2C12 mouse myoblasts, where we observed that GFP recapitulated the expression of MyHC-embryonic protein detected by a monoclonal antibody (Figure 1A). This suggests that the 4230 bp genomic sequence upstream of the ATG start codon of *Myh3* contains all the cis-regulatory elements necessary for *Myh3* expression. To further characterize the 4230 bp *Myh3* promoter-enhancer, we generated full length and truncation constructs fused to a firefly luciferase reporter gene. A full-length construct (*Myh3-FL* containing the –1 to –4230 bp sequence), a construct truncated for 1906 bp sequence distal to the promoter (*Myh3-DC2* lacking –2324 to –4230 bp), a construct truncated for the 2502 bp sequence upstream of the TATA box (*Myh3-DC3* lacking –1729 to –4230 bp), and a construct truncated for the 1684 bp sequence downstream of the ATG start codon in the first exon (*Myh3-DC8* lacking –1 to –1684 bp) were fused to the firefly luciferase reporter gene (Figure 1B). These constructs were transfected into C2C12 cells and luciferase activity measured 6 days post transfection. We observed 92%, 95%, and 33% reduction in luciferase activity, respectively, in *Myh3-DC2, Myh3-DC3*, and *Myh3-DC8* constructs compared to *Myh3-FL*, indicating that the genomic region upstream and downstream of the TATA box are required for complete *Myh3* promoter activity in C2C12 cells (Figure 1C). Similarly, these constructs were tested for promoter activity in vivo during regeneration post injury, where we observed a similar trend of promoter activity

as seen in vitro (Figure 1D). This suggests that conserved mechanisms operate to regulate MyHC-embryonic expression during in vitro and in vivo myogenic differentiation.

The *Myh3* promoter-enhancer includes conserved *cis*-elements such as consensus E-box sequences. These E-boxes are the binding sites for basic Helix–loop Helix (bHLH) trans-factors such as MyoD.[3,5] We tested whether the bHLH trans-factor MyoG, which is known to promote muscle differentiation, has any effect on *Myh3* promoter activity. Co-transfection of the *Myh3-FL* promoter along with a *MyoG* plasmid in C2C12 cells, led to a significant increase in *Myh3-FL* promoter activity compared to the control *Myh3-FL* promoter alone, suggesting that MyoG activates *Myh3* expression (Figure 1E). Previous reports suggest that the repressor Zinc finger E-box binding homeobox 1 protein (Zeb1) negatively regulates the expression of the *Myh4* gene (encoding the adult fast MyHC-IIb isoform), by binding to E-box sequences upstream of the promoter.[7] To test whether Zeb1 has any role in regulating MyHC-embryonic, the *Myh3-FL* promoter was co-transfected along with a *Zeb1* plasmid in C2C12 cells, which led to a significant reduction in *Myh3-FL* promoter activity, indicating that Zeb1 represses *Myh3* promoter activity (Figure 1F). As reported previously, co-transfection of a *MyoD* plasmid led to increased *Myh3-FL* promoter activity (Figure 1F).[3] Co-transfection of a non-myogenic control bHLH transcription factor, *melanocyte inducing transcription factor* (*Mitf*) had no discernable effect on *Myh3-FL* promoter activity (Figure 1F). This indicates that the bHLH transcription factors MyoG, MyoD, and Zeb1 are specifically involved in regulating *Myh3* promoter activity.

3.2 Zeb1 inhibits MyHC-embryonic expression and muscle differentiation

Since Zeb1 represses *Myh3* promoter activity, we next tested its role in myogenic differentiation in C2C12 myoblasts. Transfection of a *Zeb1*-specific siRNA led to a significant reduction in Zeb1 transcript (Figure 2A) and protein (Figure 2E,F) expression, at days 3, 5 and 7 of C2C12 myogenic differentiation. *Zeb1* knockdown had little effect on the transcript or protein expression of MyoD, a regulator of early stages of myogenic differentiation, except at day 7 where the transcript levels were significantly decreased (Figure 2B,E,G). However, the transcript and protein expression of MyoG, a regulator of later stages of myogenic differentiation, exhibited a distinct pattern, wherein the levels increased significantly at day 3, was unchanged at day 5, and decreased significantly at day 7 of differentiation following *Zeb1* knockdown (Figure 2C,E,H). Intriguingly, we observed a similar pattern of transcript and protein expression of MyHC-embryonic, a terminal myogenic differentiation marker, with increase at day 3, no change at day 5 and decrease at day 7 of differentiation upon *Zeb1* knockdown (Figure 2D,E,I). This effect was especially apparent at the protein level, where *Zeb1* knockdown led to an ~5-fold increase at day 3 and an ~5-fold decrease at day 7 of differentiation, in MyHC-embryonic levels (Figure 2E,I). A second siRNA targeting *Zeb1* was used, where we observe minor changes in Zeb1 levels in the early-mid stages of differentiation in the control siRNA-treated samples; however, the trends in protein expression of MyoG, MyHC-embryonic and differentiation index were similar to that seen with the first siRNA, thus validating our results (Figure S1).

Since *Zeb1* knockdown led to significant changes in the expression of MyoG and MyHC-embryonic, we next tested whether it had any effect on myogenic differentiation. We found

that the number of reserve cells, the cycling, undifferentiated cells in differentiated C2C12 culture, per unit area were reduced to approximately a third upon *Zeb1* knockdown at day 5 of differentiation, suggesting an increased rate of differentiation (Figure 2J). We observed a sharp increase in the number of differentiated myofibers upon *Zeb1* knockdown at day 5 of differentiation (Figure 2K). Upon quantification, an ~3-fold increase in fusion index as well as differentiation index was observed following *Zeb1* knockdown (Figure 2K,L and S1E). Thus, these results clearly demonstrate that *Zeb1* inhibits myogenic differentiation, since loss of *Zeb1* results in earlier expression of myogenic differentiation markers and enhanced differentiation.

The repressive effect of repressors such as Zeb1 have been reported to be mediated by corepressors such as CtBP.[7] TLEs are a family of corepressors with essential functions in development, differentiation and homeostasis.[10] TLEs have been shown to play important roles in myogenesis.[11,15] Numerous studies suggest that TLEs interact with homeodomain proteins to repress target genes.[10] Since Zeb1 is a homeodomain protein, we hypothesized that it may interact with TLEs. Therefore, we carried out a co-immunoprecipitation experiment by immunoprecipitating Zeb1 and found that it interacts with Tle3 (Figure 2M). Interestingly, we also observed that Zeb1 interacts with MyoD in co-immunoprecipitation experiments, suggesting that Zeb1, Tle3 and MyoD might be part of a protein complex involved in regulating myogenic differentiation (Figure 2M). A previous study reported interaction between Tle3 and MyoD in myogenic differentiation.[15] Both Zeb1 and Tle3 exhibited nuclear localization in immunofluorescence experiments (Figure S2A).

3.3 *Tle3* regulates myogenic differentiation

Since Tle3 interacts with Zeb1, we wished to characterize the role of Tle3 during myogenic differentiation. Transfection of a *Tle3*-specific siRNA led to a significant reduction in Tle3 transcript (Figure 3A) and protein (Figure 3E,F) expression at days 3 and 5 of C2C12 differentiation. As with *Zeb1* knockdown, *Tle3* knockdown had little effect on the transcript or protein expression of MyoD (Figure 3B,E,H). However, unlike with *Zeb1* knockdown, *Tle3* knockdown led to a consistent reduction in MyoG and MyHC-embryonic at the transcript and protein levels at days 3 and 5 of differentiation, except that MyHC-embryonic protein shows very little expression at day 3 of differentiation (Figure 3C–E,H,I).

Consistent with the reduced levels of the late differentiation markers, we observed fewer myofibers at day 5 of C2C12 differentiation upon *Tle3* depletion, which upon quantification exhibited significantly reduced fusion index and differentiation index (Figure 3M–O).
To test whether overexpression of Tle3 has any effect on myogenic differentiation, we transfected the *pCMV-3Tag3b-FLAG-Tle3* or control *pCMV-3Tag3b-FLAG* plasmids into C2C12 cells and allowed them to differentiate until day 5. Overexpression of Tle3 was clearly observed in western blots where the 3xFLAG-tagged Tle3 protein was visible at a slightly higher molecular weight than the endogenous Tle3 protein and elevated expression confirmed by densitometry (Figure 3J, Figure S2B). This led to increased levels of MyHC-embryonic and MyoG in western blots, which were found to be significant upon densitometry (Figure 3J–L). Overexpression of Tle3 also resulted in elevated fusion and differentiation indices (Figure S2D–F). To validate this further, the *Myh3-FL* promoter

activity was quantified following co-transfection of MyoD, MyoD+Tle3 and Tle3 alone (Figure S2I). We found that co-transfection of MyoD could activate the *Myh3-FL* promoter activity as previously reported,[3] while MyoD+Tle3 led to further activation of the *Myh3-FL* promoter, which was not seen with Tle3 alone (Figure S2I). Thus, loss of Tle3 function led to a significant reduction in the levels of MyoG and MyHC-embryonic with consequent reduction in fusion and differentiation indices, whereas overexpression of Tle3 led to elevated levels of MyoG and MyHC-embryonic, increased fusion index and MyoD-mediated activation of *Myh3* promoter, indicating that Tle3 is crucial for normal myogenic differentiation.

3.4 Tle3 functions upstream of Zeb1 and regulates its expression

Since Zeb1 and Tle3 proteins interact and both are involved in myogenic differentiation, we next tested their effect on each other. We observed a significant downregulation by ~50% of Zeb1 protein levels at days 3 and 5 of C2C12 differentiation, following *Tle3* knockdown (Figure 4A,B). To test whether this effect was transcriptional or related to protein stability, we quantified *Zeb1* transcript levels by qPCR at days 3 and 5 of C2C12 differentiation following *Tle3* knockdown and observed a significant, >50% reduction in *Zeb1* transcript levels (Figure 4C). Treatment with the proteasomal inhibitor MG132 failed to rescue the reduction in MyHC-embryonic, Zeb1 and MyoG upon Tle3 depletion, suggesting that Tle3 mediates its effect on myogenic differentiation primarily at the transcriptional or post-transcriptional level (Figure S3F–J). We also quantified the effect of *Zeb1* knockdown on Tle3 expression and observed no change in Tle3 protein or transcript expression at days 3, 5 and 7 of C2C12 differentiation (Figure 4F,G, Figure S2C). This indicates that Tle3 functions upstream of Zeb1 to regulate Zeb1 expression.

Next, we overexpressed Zeb1 in the background of *Tle3* knockdown, since depletion of Tle3 led to reduced Zeb1 levels (Figure S3A–C). This did not rescue the reduction in MyHC-embryonic levels upon Tle3 depletion; on the contrary, this led to a further reduction in MyHC-embryonic levels (Figure S3A–D), which fits well with the increase in MyHC-embryonic levels seen upon Zeb1 depletion (Figure 2E,I).

One of the key regulators of *Zeb1* expression is the microRNA *miR-200c*, reported to regulate *Zeb1* levels in pancreatic, prostrate, colon and breast tumors and during epithelial to mesenchymal transition.[17–19] These studies indicate that Zeb1 and *miR-200c* operate via a negative feedback loop mechanism: Zeb1 represses *miR-200c* expression by binding to three sites on its promoter, whereas *miR-200c* binds to five binding sites in the 3'UTR of *Zeb1* mRNA to downregulate its expression.[20] Thus, when Zeb1 levels are low, *miR-200c* expression increases and vice versa, which could be one mechanism by which *Zeb1* expression is regulated during myogenic differentiation. To test this, we quantified *miR-200c* levels by qPCR following *Tle3* knockdown, and found it to be significantly upregulated at days 3 and 5 of C2C12 differentiation (Figure 4D). The levels of *miR-200c* were also significantly upregulated following *Zeb1* knockdown at days 3, 5 and 7 of differentiation, validating the negative feedback loop between Zeb1 and *miR-200c* (Figure 4H). These results indicate that the downregulation of *Zeb1* upon *Tle3* knockdown, could be mediated by *miR-200c*.

Along with Zeb1, the histone methyltransferase KMT1A is known to regulate the expression of *miR-200c* by repressing the *miR-200c* promoter.[21] KMT1A trimethylates lysine 9 of histone H3 (H3K9), a repressive mark, which prevents premature MyoG expression in the myoblast stage, and inhibits MyoD mediated muscle differentiation.[22] KMT1A protein levels were found to be significantly reduced at all time points of differentiation, in both *Tle3* knockdown as well as *Zeb1* knockdown (Figure 4A,E,F,I). To test whether the decreased KMT1A levels led to a reduction in its function, overall H3K9-trimethylated protein levels were quantified, which was significantly decreased upon *Tle3* knockdown (Figure S3A,E). These results further confirm and explain the upregulation of *miR-200c* levels in both *Tle3* and *Zeb1* individual knockdowns.

3.5 Combined knockdown of *Tle3* and *Zeb1* resembles *Tle3*-only knockdown phenotype

Although Tle3 and Zeb1 proteins interact, individual *Tle3* or *Zeb1* knockdown have contrasting effects on myogenic differentiation. Therefore, we next tested them for epistasis by carrying out double knockdown of *Tle3* and *Zeb1* in C2C12 cells. The knockdown efficiency at day 5 of differentiation was validated by western blotting, which indicated >75% reduction in the protein levels of both Tle3 and Zeb1 (Figure 5A–C). Double knockdown of *Tle3* and *Zeb1* resulted in significantly reduced protein levels of MyoG and MyHC-embryonic at day 5 of differentiation (Figure 5A,D,E), similar to *Tle3*-only knockdown (Figure 3E,H,I), unlike *Zeb1*-only knockdown (Figure 2E,H,I). This indicates that Tle3 functions upstream of Zeb1, since the combined depletion of *Tle3* and *Zeb1* resembles the knockdown phenotype of *Tle3*-only knockdown, with respect to the protein expression of late myogenic differentiation genes.

3.6 Zeb1 represses *Myh3* promoter activity via a novel binding site

Since Zeb1 overexpression led to a reduction in *Myh3* promoter activity (Figure 1F), we wished to test whether Zeb1 has binding sites in the 4230 bp *Myh3* promoter-enhancer. Zeb1 has been shown to bind the E-box sequence CACCTG in the *Myh4* gene promoter and repress its expression.[7] Therefore, we analyzed the *Myh3* promoter-enhancer for consensus CACCTG sites, which led to the identification of two sites; the first is in the distal promoter-enhancer region (-3436 to –3430 bp) and the second in intron 2 (-580 to –574 bp) (Figure 6A). Chromatin immunoprecipitation followed by qPCR for each of the two potential Zeb1 binding E-boxes revealed that Zeb1 occupies only the site in the distal promoter (-3436 to –3430 bp) and not the other site in intron 2 (-580 to –574 bp), compared to control primers for a non-specific region on the same chromosome (Figure 6B,C). These results were validated using a second Zeb1 antibody (Figure S2G–H). We generated an E-box mutant for the –3436 to –3430 bp Zeb1 binding region in the *Myh3* promoter-enhancer luciferase construct (referred to as *Myh3-Z1-Mut*), which exhibited significantly increased luciferase activity compared to the *Myh3-FL* construct (Figure 6E). This confirms that this novel Zeb1 binding site in the *Myh3* promoter-enhancer is required to repress *Myh3* expression. We also generated a mutant *Zeb1* construct lacking the N- and C-terminal zinc finger clusters (amino acids 150–272 and 882–959, respectively) referred to as *Zeb1-Mut* (Figure 6D). Co-transfection of *Zeb1 -Mut* had no effect on *Myh3-FL* promoter activity, indicating that the zinc finger clusters in Zeb1 are required to mediate its regulatory effect on *Myh3*,possibly by facilitating binding (Figure 6E). *Zeb1-Mut* had no additional effect

on *Myh3-Z1-Mut*, suggesting that the zinc finger clusters of Zeb1 and the predicted Zeb1 binding site in the *Myh3* promoter-enhancer are both required for Zeb1-mediated regulation of *Myh3* (Figure 6E). Interestingly, MyoD was able to activate *Myh3-Z1-Mut*, indicating that its effect is at least partially independent of the Zeb1 binding E-box (Figure 6E). Co-transfection of control, non-myogenic bHLH transcription factor Mitf had no effect on *Myh3-Z1-Mut* activity (Figure 6E).

To validate this further, *Myh3-FL* promoter was transfected into C2C12 cells where Zeb1 was silenced by siRNA, which resulted in a significant increase in *Myh3* promoter activity (Figure 6F). Interestingly, depletion of *Tle3* also resulted in a similar effect (Figure 6G), which is most likely due to the reduction in Zeb1 levels upon *Tle3* knockdown (Figure 4A–C).

3.7 Tle3 regulates myogenic differentiation via post-transcriptional mechanisms

It is intriguing that depletion of *Tle3* led to increased *Myh3-FL* promoter activity (Figure 6G) but reduced transcript and protein levels of both MyoG and MyHC-embryonic (Figure 3C–E,H,I). We hypothesized that this can only result from post-transcriptional mechanisms that regulate the expression of the late myogenic differentiation genes such as *MyoG* and *Myh3*, upon *Tle3* knockdown. Therefore, we cloned the *MyoG* 3'UTR into the *pmirGLO* dual luciferase vector, transfected it into C2C12 cells and quantified the luciferase activity. Interestingly, a significant reduction in luciferase activity was observed upon treatment with *Tle3* siRNA (Figure 6H), which was not seen with *Zeb1* siRNA (Figure 6I). This suggests that the stability of the *MyoG* mRNA is decreased upon *Tle3* knockdown. A well-known regulator of the mRNA stability of several myogenic differentiation genes including MyoG is the RNA-binding Human antigen R (HuR) protein, which stabilizes mRNAs by binding to AU-rich regions in the 3'UTR.[23] We found that *HuR* transcript levels were significantly reduced by about half upon *Tle3* knockdown at day 5 of differentiation, indicating a possible mechanism by which Tle3 regulates myogenic differentiation at the post-transcriptional level (Figure 6J). Treatment with the transcription inhibitor Actinomycin D indicated that depletion of *Tle3* leads to a reduction in half-life of *MyoG* transcript levels at days 3 and 5 ofdifferentiation, confirming its effect on mRNA stability (Figure S3K).

4 Discussion

The *Myh3* and *Myh8* minimal promoter-enhancer regions sufficient to drive muscle-specific reporter expression were identified previously and found to respond to MyoD and calcineurin.[3] Beylkin et al. also identified three E-boxes which were necessary for *Myh3* promoter activity, of which the proximal two were shown to be bound by MyoD.[3] The *Myh3* promoter-enhancer region used by Beylkin et al included 14 bp downstream and 791 bp upstream of the TATA box.[3] Another study where intron 1 of *Myh3* along with the proximal promoter was included in the promoter-enhancer construct reported the binding of NFATc1 and NFATc3 to sequences in intron 1, close to which MyoD was shown to bind, to regulate *Myh3* expression.[5] In the current study, the promoter-enhancer region taken spans well beyond the segment used in the previous studies, in both directions; we have used 1728 bp downstream and 2502 bp upstream of the *Myh3* TATA box (Figure 1B). Our results indicate

that *cis*-elements both upstream and downstream of the *Myh3* TATA box are required for complete *Myh3* promoter activity, suggesting that the transcriptional regulation of *Myh3* is more complex than previously thought (Figure 1B–D).

Previous studies report that the MRF MyoD is a crucial *trans*-factor which binds to E-boxes in the *Myh3* promoter-enhancer, to regulate *Myh3* transcription.[3,5] Another MRF, Myf5 was also shown to activate *Myh3* promoter activity, although to a lesser extent than MyoD.[3] Interestingly, not much is known about the later expressed MRF MyoG, with respect to regulation of *Myh3* expression. *Myog* knockdown in primary bovine muscle satellite cells allowed to differentiate caused reduced *Myh3* expression, whereas *MyoG* knockout in C2C12 cells led to loss of myogenic differentiation capability.[24,25] Our results clearly indicate that MyoG is crucial for *Myh3* transcriptional regulation (Figure 1E).

Zeb1 and its *Drosophila* ortholog zfh-1 have been shown to inhibit myogenic differentiation.[26] Consensus G/C-centered E-boxes are bound by Zeb1 specifically in myoblasts and not differentiated myofibers, where it represses transcription of target genes by recruiting the corepressor CtBP.[7] A recent study characterized the role of Zeb1 in muscle regeneration using *Zeb1* heterozygous mice, where it was found to be necessary for the maintenance of muscle stem cell quiescence and macrophage phenotype, leading to impaired muscle regeneration in *Zeb1* heterozygotes.[8] Intriguingly, *Zeb1* heterozygotes following muscle injury exhibited reduced expression of *Myh3* transcript and MyHC-embryonic protein levels at early time points, and elevated levels at later time points, indicating that muscle regeneration is delayed upon loss of Zeb1 function.[8] However, whether Zeb1 has any direct role in regulating *Myh3* expression has not been previously addressed. Our results now identify a novel E-box in the *Myh3* promoter-enhancer, which is bound by Zeb1 to repress *Myh3* expression, indicating a direct role of Zeb1 in regulating *Myh3* transcription (Figure 6A–E). Using a Zeb1 mutant form lacking zinc-finger clusters, we confirm that Zeb1 binding is required to regulate *Myh3* promoter activity (Figure 6D,E). Unlike the reduction in early *Myh3* expression followed by elevated expression at later time points during muscle regeneration following injury reported in *Zeb1* heterozygotes,[8]we observe increased expression of *Myh3* and *Myog* at early time points followed by reduced levels at later time points of C2C12 differentiation (Figure 2C–E,H–I). This could be because the in vivo experiments make use of a germline loss of Zeb1 function in all cell types or because Zeb1 function is not completely abrogated in the *Zeb1* heterozygotes.[8]

TLEs are thought to mediate repression by recruiting histone modifiers such as histone deacetylases (HDACs).[10] Since Tle3 has been reported to be important in myogenic differentiation and interacts with MyoD, we tested whether Zeb1 and Tle3 might have shared functions in myogenic differentiation.[15] Although our results demonstrate that Tle3 and Zeb1 interact with each other and with MyoD during myogenic differentiation (Figure 2M), it is apparent that they also have independent roles, evident by their contrasting effects on differentiation (Figures 2 and 3). Similar results have been reported in adipogenesis, where Tle3 functions as a dual-function transcriptional coregulator working with PPARγto activate pro-adipogenic target promoters while also inhibiting Wnt signaling by preventing Tcf4 activation by β-catenin to reverse β-catenin-mediated repression of adipocyte gene expression.[14] In a follow-up study, Tle3 was found to promote the white-adipocyte-specific

gene expression program and antagonize the Prdm16-mediated brown-adipocyte selective program.[13] This was facilitated by direct interaction between Prdm16 and Tle3, which disrupted the Prdm16-PPARγ interaction.[13] These results suggest that Tle3 can form both activating and repressive transcriptional complexes which are transient in nature to regulate differentiation. Our results indicate that Zeb1 functions to inhibit myogenic differentiation whereas Tle3 promotes differentiation. This suggests that the Zeb1-Tle3 interaction during myogenesis is most likely an antagonistic one, similar to that between Prdm16 and Tle3 discussed above in adipogenesis, to fine tune the rate of differentiation, where Tle3 prevents the inhibition of differentiation by Zeb1. Another Tle family member, Tle1, has been shown to regulate Zeb1 expression in the opposite direction, further supporting our observations with Tle3.[27]

We find that Tle3 functions upstream of Zeb1, since depletion of *Tle3* led to a reduction in Zeb1 levels possibly mediated by *miR-200C*, whereas *Zeb1* knockdown had no effect on Tle3 levels (Figure 4A–C,F–G). This was further validated when we found that double knockdown of *Tle3* and *Zeb1* leads to reduced MyoG and MyHC-embryonic protein levels as seen in *Tle3* knockdown (Figures 3E–I and 5). Interestingly, this bears striking parallels to a study where *miR-200c* when overexpressed during myogenic differentiation led to reduced Zeb1, MyoG, and MyHC levels.[28] Reduction in levels of the histone methyltransferase KMT1A and H3K9-trimethylation seen upon Tle3 depletion suggest that these could play a role in regulating *miR-200c* levels (Figure 4A,E, Figure S3A,E).

Our findings indicate that Tle3 functions at the transcriptional and post-transcriptional levels to regulate *Myh3* expression and myogenic differentiation, which was validated by inhibiting proteasome-mediated degradation (Figure S3F–J). Since Tle3 interacts with MyoD, a direct role in regulating the transcriptional output by occupying E-boxes in the *Myh3* promoter-enhancer is conceivable; accordingly, we find that *Myh3* promoter activity increases upon *Tle3* knockdown, indicating that it functions as predicted as a transcriptional repressor (Figure 6G). On the other hand, Tle3 also functions at the post-transcriptional level to regulate the expression of late myogenic differentiation genes such as *MyoG* and *Myh3* (Figure 6H). This post-transcriptional effect of Tle3 is mediated at least in part by HuR, which is known to stabilize mRNAs.[23] Actinomycin-D mediated inhibition of transcription confirmed that Tle3 is required to stabilize *MyoG* mRNA (Figure S3K).

In conclusion, we have identified that the promoter-enhancer of *Myh3* extends further upstream and downstream of the TATA-box than previously described. We find that Zeb1 and Tle3 are crucial *trans*-factors which interact with each other and differentially regulate the expression of *Myh3* during myogenic differentiation. Tle3 not only functions upstream of Zeb1 but also has post-transcriptional regulatory roles in myogenic differentiation mediated by mRNA stability. Some of the mechanisms underlying this may be regulated by the microRNA *miR-200c* and the RNA-binding protein HuR. Interestingly, how Tle3 regulates the expression of *miR-200c* and HuR is unknown and will be of interest in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during this study.

Abbreviations

CtBP	C-terminal binding protein
HDAC	histone deacetylase
HuR	human antigen R protein
Mitf	melanocyte inducing transcription factor
MRF	myogenic regulatory factor
Myf5	myogenic factor 5
MyHC	myosin heavy chain
MyoD	myoblast determination protein
MyoG	myogenin
NFAT	nuclear factor of activated T-cells
Tle3	transducin-like Enhancer of Split 3
UTR	untranslated region

Zeb1 zinc-finger E-box binding homeobox 1.

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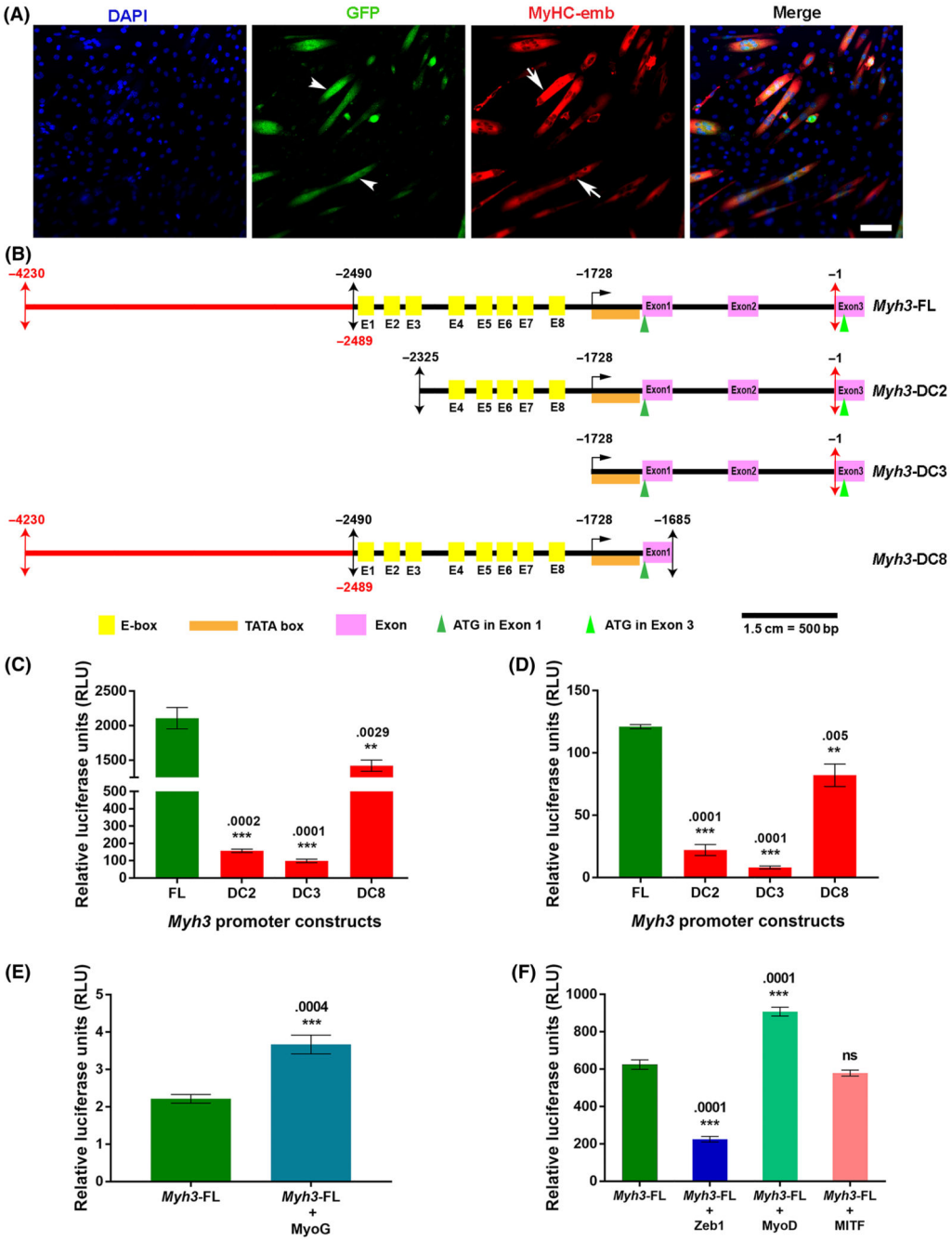

Figure 1. A 4.2 kb genomic region regulates *Myh3* expression.

(A) Immunofluorescence staining for DAPI (blue), GFP (green; arrow heads), and MyHC-embryonic (red; arrows) in C2C12 cells at day 5 of myogenic differentiation; the rightmost panel is a merge of other channels; scale bar is 10 μm. (B) Schematic of the *Myh3* FL, DC2, DC3, and DC8 promoter constructs; scale bar is 1.5 cm =500 bp. (C) In vitro promoter activity of constructs in (B) at day 5 of C2C12 myogenic differentiation. (D) In vivo promoter activity of constructs in (B) during mouse TA muscle regeneration post injury (*n* = 4 per construct). (E, F) Graphs showing the effect of MyoG (E), Zeb1, MyoD and Mitf

(F) on *Myh3-FL* promoter activity in C2C12 cells. Graphs are presented as mean ±SEM of a minimum of three independent experiments; *p-value ≤.05 is considered significant.

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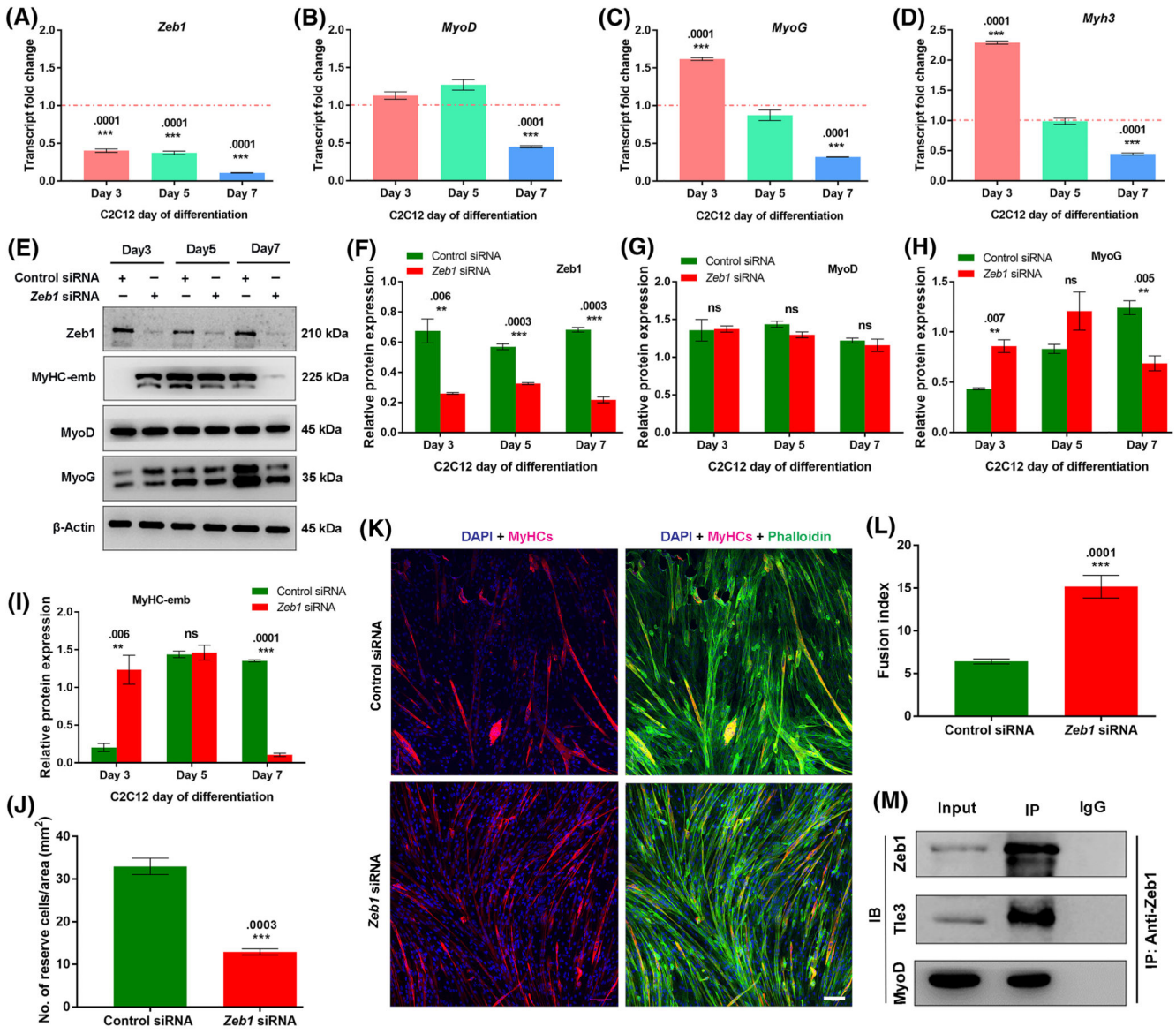

Figure 2. *Zeb1* knockdown leads to accelerated differentiation.

(A–D) Graphs showing transcript expression of *Zeb1, MyoD, MyoG,*and *Myh3*, quantified by qPCR in control and *Zeb1* siRNA-treated cells at days 3, 5 and 7 of C2C12 differentiation. Control transcript levels are set to one (red dotted line in A–D). (E) Western blots for Zeb1, MyoD, MyoG, MyHC-embryonic, and β-actin protein levels and (F–I) their densitometric quantification normalized to β-actin (loading control), on protein lysates of control and *Zeb1* siRNA-treated C2C12 cells at days 3, 5, and 7 of differentiation, respectively. (J) Graph quantifying the number of reserve cells per mm^2 in control siRNA and *Zeb1* siRNA-treated C2C12 cells at day 5 of differentiation. (K) Immunofluorescence images of C2C12 cells treated with control (upper panel) or *Zeb1* siRNA (lower panel), labeled for MyHCs (red), phalloidin (green), and DAPI (blue), at day 5 of differentiation; scale bar is 100 μm. (L) Graph showing fusion index quantifying the number of nuclei (two or more nuclei) per fiber as a fraction of the total number of nuclei in control

and *Zeb1* siRNA-treated cells, at day 5 of differentiation. (M) Immunoblots showing co-immunoprecipitation of Zeb1, Tle3 and MyoD in undifferentiated C2C12 cells with the Rabbit IgG used as control. Graphs are presented as mean ±SEM of a minimum of three independent experiments; **p*-value ≤.05 is considered significant.

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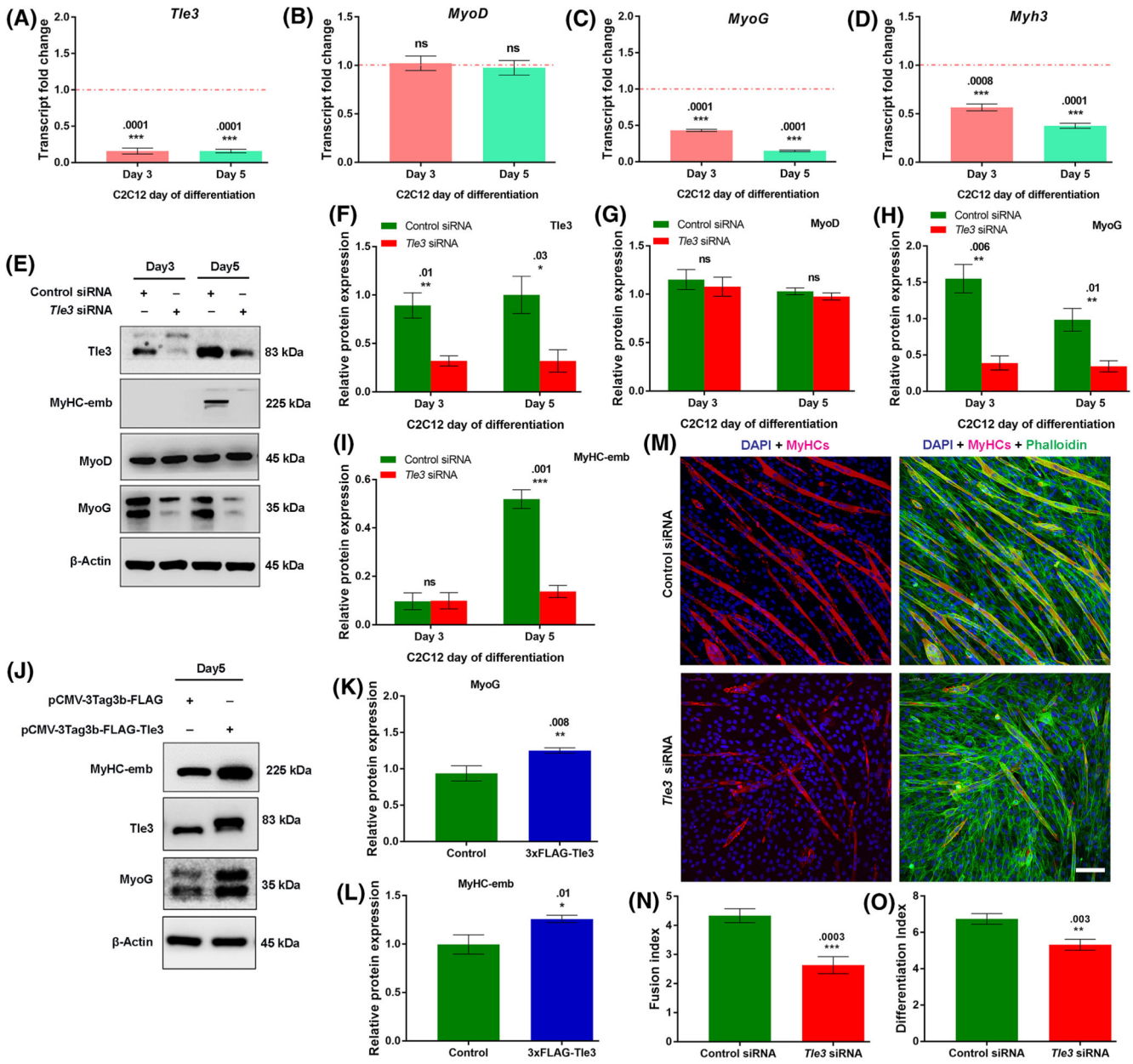

Figure 3. *Tle3* knockdown leads to impaired differentiation.

(A–D) Graphs showing transcript expression of *Tle3, MyoD, MyoG*, and *Myh3*, quantified by qPCR in control and *Tle3* siRNA-treated cells at days 3 and 5 of C2C12 myogenic differentiation. Control transcript levels are set to one (red dotted line in A–D). (E) Western blots for Tle3, MyoD, MyoG, MyHC-embryonic, and β-actin protein levels and (F–I) their densitometric quantification normalized to β-actin (loading control), on protein lysates of control and *Tle3* siRNA-treated C2C12 cells at days 3, and 5 of differentiation, respectively. (J) Western blots for Tle3, MyoG, MyHC-embryonic and β-actin (loading control) on C2C12 protein lysates overexpressing *pCMV-3Tag3b-FLAG-Tle3* or control *pCMV-3Tag3b-FLAG* plasmids, and (K, L) the densitometric quantification of MyoG and MyHC-embryonic at day 5 of differentiation. (M) Immunofluorescence images of C2C12

cells treated with control (upper panel) or *Tle3* siRNA (lower panel), labeled for MyHCs (red), phalloidin (green), and DAPI (blue), at day 5 of differentiation; scale bar is 100 μm. (N, O) Graphs showing fusion and differentiation indices, respectively, quantifying the number of nuclei (two or more nuclei for fusion index) per fiber as a fraction of the total number of nuclei in control and *Tle3* siRNA-treated cells, at day 5 of differentiation. Graphs are presented as mean ±SEM of a minimum of three independent experiments; **p*-value ≤.05 is considered significant.

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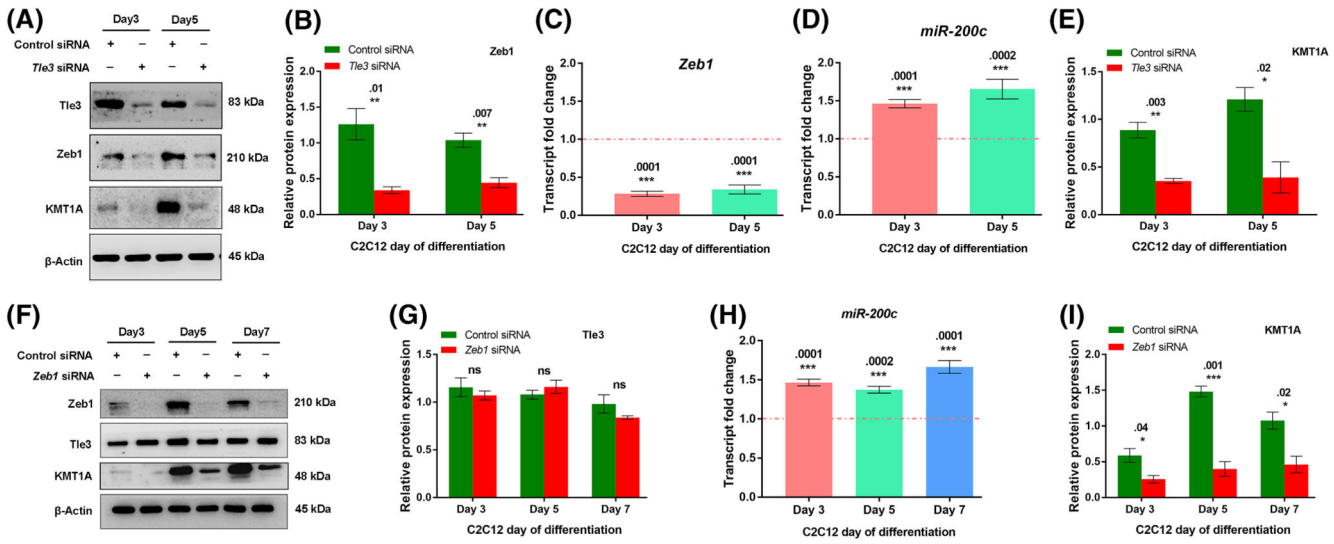

Figure 4. *Tle3* knockdown leads to downregulation of Zeb1 expression.

(A) Western blots for Tle3, Zeb1, KMT1A and β-actin (loading control), on protein lysates of control and *Tle3* siRNA-treated C2C12 cells at days 3 and 5 of differentiation, respectively. (B, E) Graphs showing densitometric quantification of Zeb1 and KMT1A protein levels normalized to β-actin following *Tle3* knockdown at days 3 and 5 of differentiation, respectively. (C, D) Graphs showing transcript levels of *Zeb1* and *miR-200c* following *Tle3* knockdown at days 3 and 5 of differentiation. (F) Western blots for Zeb1, Tle3, KMT1A and β-actin (loading control), on protein lysates of control and *Zeb1* siRNA-treated C2C12 cells at days 3, 5, and 7 of differentiation, respectively, and (G, I) densitometric quantification of Tle3 and KMT1A protein levels normalized to β-actin. (H) Graph showing transcript levels of *miR-200c* following *Zeb1* knockdown at days 3, 5, and 7 of differentiation. Control transcript levels are set to one (red dotted line in C, D and H). Graphs are presented as mean ±SEM of a minimum of three independent experiments; *p-value ≤.05 is considered significant.

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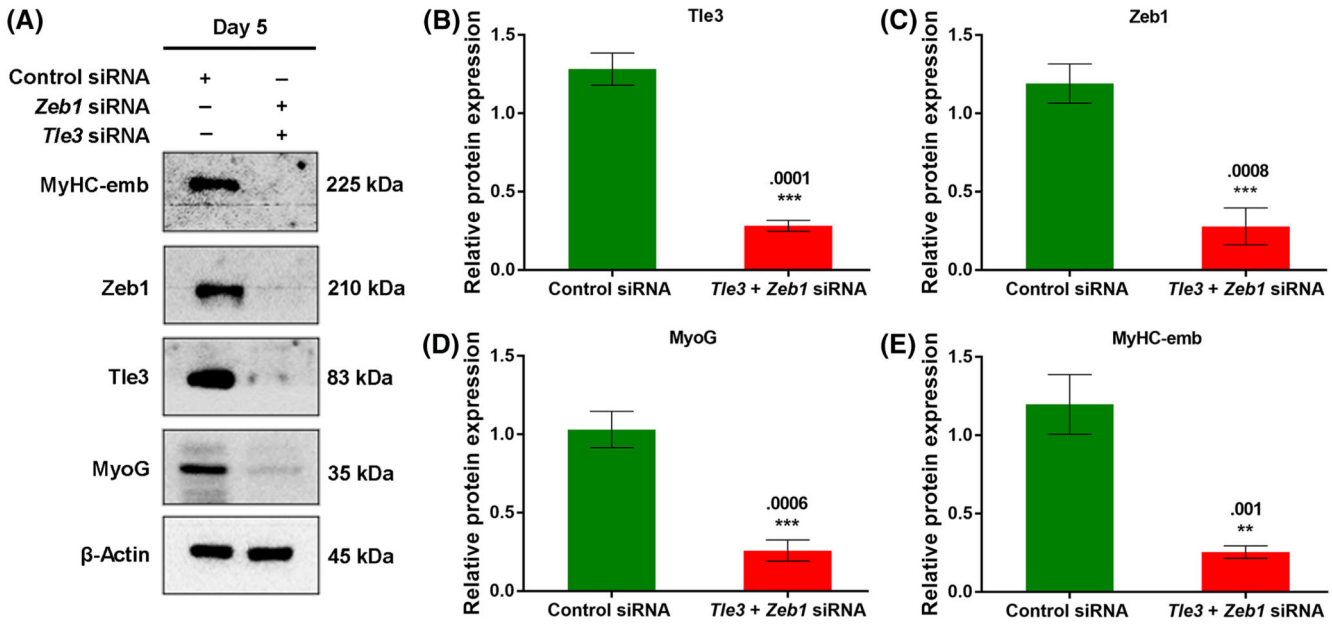

Figure 5. Double knockdown of *Tle3* and *Zeb1* leads to inhibition of muscle differentiation.

(A) Western blots for Tle3, Zeb1, MyoG, MyHC-embryonic, and β-actin (loading control), respectively, on protein lysates of control, and *Tle3+Zeb1* siRNA-treated C2C12 cells at day 5 of differentiation. (B-E) Graphs showing densitometric quantification of Tle3, Zeb1, MyoG, and MyHC-embryonic protein levels normalized to β-actin following the combined knockdown of *Tle3* and *Zeb1* at day 5 of differentiation. Graphs are presented as mean ±SEM of a minimum of three independent experiments; **p*-value ≤.05 is considered significant.

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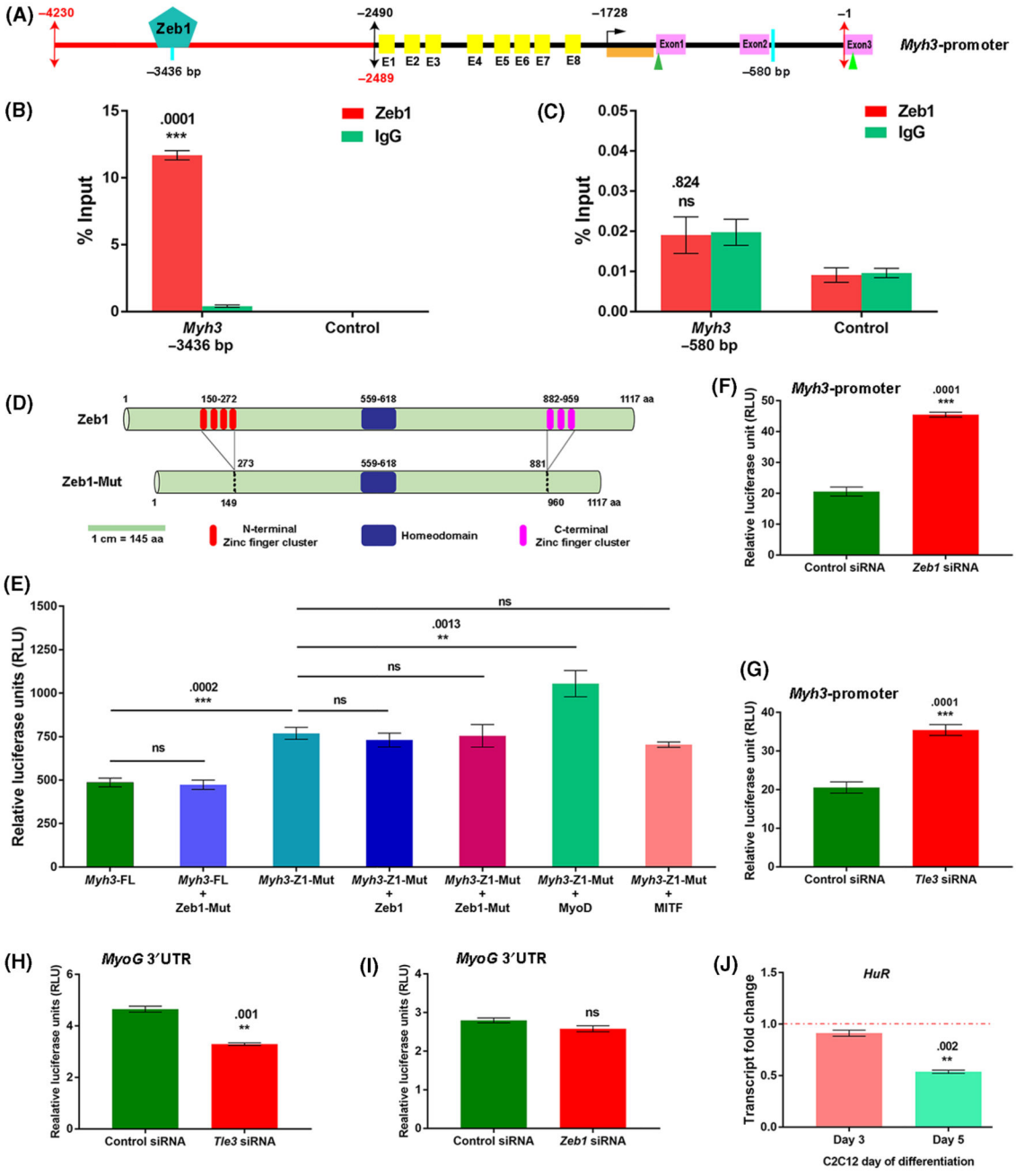

Figure 6. Zeb1 represses *Myh3* promoter activity via a novel binding site, whereas Tle3 regulates muscle differentiation post-transcriptionally.

(A) Schematic representation of the *Myh3* promoter-enhancer depicting the predicted Zeb1 binding E-boxes at –3436 bp (distal region) and at –580 bp (intron 2, upstream of the ATG start site in exon 3), respectively. (B, C) Chromatin immunoprecipitation using Zeb1 and control IgG antibodies on C2C12 myoblasts for the E-boxes starting at –3436 and –580 bp in the promoter-enhancer of *Myh3* and a control region on the same chromosome. (D) Schematic depicting the Zeb1 full length (top) or N- and C-terminal zinc-finger domain deleted Zeb1-Mut (bottom) protein. (E) Graph showing relative luciferase activity

of *Myh3-FL* promoter construct alone, *Myh3-FL* with Zeb1-Mut, *Myh3-Z1-Mut* alone, *Myh3-Z1-Mut* with Zeb1, *Myh3-Z1-Mut* with Zeb1-Mut, *Myh3-Z1-Mut* with MyoD, and *Myh3-Z1-Mut* with Mitf constructs, respectively (left to right), following transfection into C2C12 cells in vitro, at day 5 of differentiation. (F, G) Graphs showing luciferase activity of *Myh3-FL* promoter construct transfected into C2C12 cells in vitro following *Zeb1* and *Tle3* knockdown respectively, at day 5 of differentiation. (H, I) Graphs showing luciferase activity of *MyoG* 3'UTR luciferase construct in vitro, following *Tle3* and *Zeb1* knockdown, respectively, at day 5 of C2C12 differentiation. (J) Graph showing transcript expression of *HuR* quantified by qPCR in control and *Tle3* siRNA-treated cells at days 3 and 5 of C2C12 differentiation; control transcript levels are set to one (red dotted line in J). Graphs are presented as mean ±SEM of a minimum of three independent experiments; **p*-value ≤.05 is considered significant.

Table 1

Oligonucleotides used for cloning.

Gene/vector backbone		Direction	Primer sequence 5' to 3'
MyH3-FL/pEGFP-C2		Forward	TGCATTAGTTATTAATAATCCACTTAAGCTTACCGCT
		Reverse	GACCGGTAGCGCTAGCGGTGTCGGCTGCAAAACAAA
MyH3-FL/pGL-3 Control		Forward	CCCGGGCTCGAGATCTAATCCACTTAAGCTTACCGCT
		Reverse	CCGGAATGCCAAGCTTGGTGTCGGCTGCAAAACAAA
MyH3-DC2/pGL-3 Control		Forward	CTAGCCCGGGCTCGAGTCACTGTGAACATGGGCT
		Reverse	CCGGAATGCCAAGCTTGGTGTCGGCTGCAAAACAAA
MyH3-DC3/*pGL-3 Control*		Forward	CTAGCCCGGGCTCGAGTATAAAAGCCCAGGGGGC
		Reverse	CCGGAATGCCAAGCTTGGTGTCGGCTGCAAAACAAA
MyH3-DC8/*pGL-3 Control*		Forward	CCCGGGCTCGAGATCTAATCCACTTAAGCTTACCGCT
		Reverse	CCGGAATGCCAAGCTTACAGAGAGAAGCATAGAGGA
MyoG3'UTR/*pmirGlo*		Forward	TTGTTTAAACGAGCTCGATTGTCTGTCAGGCTGGGT
		Reverse	ATAAAGACTGGTTGCTATCAGTCGACCTGCAGGCAT
Myh3-Z1-Mut/pGL-3 Control	PCR1	Forward	CCCGGGCTCGAGATCTAATCCACTTAAGCTTACCGCT
		Reverse	TGACATTAAGCATACA**AGTTCC**GAGAGATGGGTGCCTCCAGC
	PCR2	Forward	GAGGCACCCATCTCTC**GGAACT**TGTATGCTTAATGTCAAAGA
		Reverse	CCGGAATGCCAAGCTTGGTGTCGGCTGCAAAACAAA
	PCR3	Forward	CCCGGGCTCGAGATCTAATCCACTTAAGCTTACCGCT
		Reverse	CCGGAATGCCAAGCTTGGTGTCGGCTGCAAAACAAA
pcDNA3.1 /HisC-Zeb1 -Mut	PCR1	Forward	TAAGGTACCAGGATCCATGGCGGATGGCCCCAGGTGTAAGC
		Reverse	CAGGCATCAAGCTAATCAACTGGGAAAATGCATCTGGTGTT
	PCR2	Forward	TGCATTTTCCCAGTTGATTAGCTTGATGCCTGTGAATGGCA
		Reverse	TGCAGTAGGAGTAGCGCATTCCATTCTCTGTCTTCCGAGTT
	PCR3	Forward	GACAGAGAATGGAATGCGCTACTCCTACTGCAAGAGAGGAG
		Reverse	AAACGGGCCCTCTAGACTAAGCTTCATTTGTCTTCTCTTCA

Table 2

Primers used for qPCR and ChIP analysis.

Gene name	Direction	Primer sequence 5' to 3'
Myh3 (MyHC-emb)	Forward	ATGAGTAGCGACACCGAGATG
	Reverse	AAAGCAGTAGGTTTTGGCAT
MyoD	Forward	GCTGCCTTCTACGCACCTG
	Reverse	GCCGCTGTAATCCATCATGC
Myogenin	Forward	CAGTACATTGAGCGCCTACAG
	Reverse	GGACCGAACTCCAGTGCAT
Zeb1	Forward	AACTGCTGGCAAGACAACG
	Reverse	ATGACTGCTGGCTTCTGGT
HuR	Forward	ATGAAGACCACATGGCCGAA
	Reverse	TTCACAAAGCCATAGCCCAA
GAPDH	Forward	GACTTCAACAGCAACTCCCACT
	Reverse	GGTCCAGGGTTTCTTACTCC
Primers used for ChIP analysis		
Myh3 (–3436 bp)	Forward	GGCATGTGAATGGCTCCT
	Reverse	GAACAGATGTCGACAGACCC
Myh3 (–580 bp)	Forward	GATCTCACTGTGTAGCCCCA
	Reverse	TCTGCCTCCCATGCTAAGAA
Control	Forward	GCTTGAAAACGAGGTGGAAA
	Reverse	CCTCCTCAGCCTGTCTCTTG