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Role of ZEB1 in skeletal muscle: Regulation of cell differentiation, response response to tissue damage and regeneration

Laura Siles Mena

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Regulation of cell differentiation, response to tissue damage and regeneration

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Doctorand
Laura Siles Mena

Director de Tesi
Antonio Postigo

Grup de Regulació Transcripcional de l'Expressió Gènica
Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS)
Barcelona

PREFACE

The experimental study presented in this doctoral thesis was performed at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) in Barcelona in the laboratory of Transcriptional Regulation of gene expression under the supervision of Antonio Postigo. The work was supported by a FPU scolarship from the Spanish Government (Ministerio de Educación, Cultura y Deporte) and a scolarship from the University of Barcelona School of Medicine.

ABSTRACT

ZEB1 is a transcription factor best known for its role in cancer progression and metastasis. It is also expressed during embryonic development of different tissues although its function and mechanism of action have not always been elucidated. In this dissertation I show that ZEB1 is involved in muscle differentiation during embryonic development and it is also required for muscle response after injury and regeneration.

We found that, in the nucleus of myoblasts, ZEB1 represses muscle differentiation genes through direct binding to G/C-centered E-boxes present in the regulatory regions of muscle differentiation genes. Albeit to different degrees depending on the target gene, transcriptional repression of these genes by ZEB1 is mediated by its recruitment of the corepressor CtBP. Binding of ZEB1 to E-boxes in differentiation genes displaces MyoD and prevents their transcriptional activation during the myoblast stage. As myoblasts fuse, MyoD displaces ZEB1 from its DNA binding sites and differentiation proceeds. Knockdown of *Zeb1* induces muscle differentiation genes, thus accelerating the formation of myotubes.

Muscle regeneration after damage depends on a timely regulated transition from pro- to anti-inflammatory signals. Injury of *Zeb1*-deficient mice results in increased recruitment of inflammatory macrophages and expression of pro-inflammatory cytokines, which delays the regenerative process. Adult muscle regeneration relies on a pool of functional SCs and we show that *Zeb1*-deficient SCs undergo premature activation after isolation and culture by downregulating *Pax7* and quiescence-associated genes (*Foxo3, Hes* genes) and upregulating *Myod1*. Moreover, its regenerative potential when transplanted into *mdx* hosts is reduced compared to wild-type SCs and exhibit increased senescence in culture.

These results establish ZEB1 as an important potential regulator of muscle differentiation and regeneration by modulating inflammatory response and SC myogenic progression in response to injury. They also set ZEB1 as a potential therapeutic target in muscle dystrophies or following muscle insult.

RESUM

ZEB1 és un factor de transcripció conegut pel seu paper en progressió tumoral i metàstasi. També s'expressa durant el desenvolupament embrionari de diferents teixits tot i que la seva funció i mecanisme d'acció encara no han estat establerts. En aquesta tesi mostro que ZEB1 està implicat en la diferenciació muscular durant el desenvolupament embrionari i que es necessari en la resposta al dany i regeneració muscular.

Hem trobat que, en els nuclis dels mioblasts, ZEB1 reprimeix gens de diferenciació muscular per unió directa a seqüències "E-box" amb nucleòtids G/C en posició central en les seves regions reguladores. Encara que en diferents graus, depenent del gen diana, la repressió exercida per ZEB1 es fa mitjançant el reclutament del seu corepressor CtBP. La unió de ZEB1 a aquestes "E-boxes" desplaça MyoD evitant la seva activació transcripcional. Un cop els mioblasts es fusionen, MyoD desplaça ZEB1 de la seva unió a l'ADN donant lloc al procés de diferenciació. D'aquesta manera, la inhibició de *Zeb1* indueix els gens de diferenciació muscular accelerant la formació de miotubs.

La regeneració després del dany muscular depèn de la transició de senyals proinflamatoris a antiinflamatoris. La lesió muscular de ratolins deficients per *Zeb1* produeix un elevat nombre de macròfags inflamatoris i l'expressió de citocines pro-inflamatories que retarden el procés regeneratiu. La regeneració del teixit muscular adult requereix la participació d'una població de cèl·lules satèl·lit funcionals. Els nostres resultats demostren que les cèl·lules satèl·lit deficients per *Zeb1* s'activen precoçment un cop aïllades i posades en cultiu. Aquesta activació succeeix per la inhibició de *Pax7* i de gens associats a la quiescència d'aquestes cèl·lules (*Foxo3, Hes*) i la activació de *Myod1*. A més a més, presenten una més alta senescència i la seva capacitat regenerativa és reduïda quan es trasplanten en ratolins *mdx* en comparació a les wild-type.

Aquests resultats situen ZEB1 com un important regulador de la diferenciació i la regeneració muscular per modulació de la resposta inflamatòria i de la progressió de les cèl·lules satèl·lit en la resposta al dany muscular. També suggereixen ZEB1

com una potencial diana terapèutica en distròfies musculars o en resposta a la lesió del múscul esquelètic.

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ABBREVIATIONS

BMD	Becker muscular dystrophy
BMDM	bone marrow-derived macrophages
CFSE	5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
CID	CtBP interaction domain
CK	creatine kinase
CSA	cross sectional area
CSC	cancer stem cell
CTX	cardiotoxin
DM	differentiation medium
DM1	Steinert’s disease
DMD	Duchenne muscular dystrophy
EBD	Evans blue dye
EMT	epithelial-to-mesenchymal transition
GM	growth medium
H&E	haematoxylin & eosin
MHC	myosin heavy chain
MRF	muscle regulatory factors
SC	satellite cell
SGMSC	standard growth medium for satellite cells

INTRODUCTION

INTRODUCTION

The muscular system is one of the most important tissues that accounts for approximately 40% of the total body mass in higher vertebrates. Muscle is a soft tissue composed by muscle itself, tendons and perimysium. It encompasses two large groups: striated (cardiac and skeletal) and smooth muscle. Muscles and fibers therein can be also classified according to other criteria like their fast or slow twitch, their metabolic activity, etc.

The skeletal muscle constitutes the largest group and is under the control of the somatic nervous system through neuromuscular junctions that innervate muscle fibers. Its development in the embryo (myogenesis) is a complex process that results from specific waves of gene expression, which are orchestrated by signalling pathways, transcription factors and epigenetic regulation (Wakelam et al., 1985; Carrió et al., 2015). Adult skeletal muscle is under continuous homeostatic control and adaptation and, compared to other adult tissues, retains significant regenerative capacity in response to injury.

1. Skeletal muscle

In vertebrates, striated muscle is only found in skeletal and heart muscle. Muscle fibers form fascicles surrounded by extracellular matrix proteins. In turn, muscle fibers are formed by myofibers that arise from the fusion of mononucleated myoblasts during myogenesis. Thus, myofibers contain several nuclei, which are positioned along the length of the fiber. The smaller functional unit within the myofiber capable to contract is the sarcomere, which contains actin and myosin filaments that confer to the muscle its striated appearance (Figure 1). Of note, myosin exists in different isoforms depending on the species, age and muscle type that result in highly specialized muscles with different contractile (slow- or fast-twitch) properties (Braun et al., 2011).

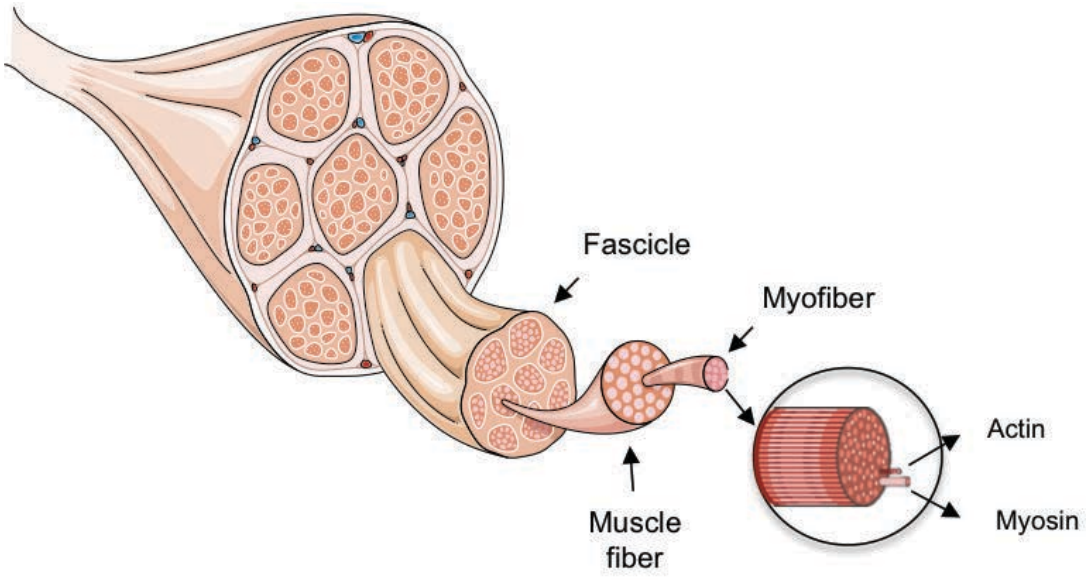


Figure 1. Representation of skeletal muscle organization. Skeletal muscle is composed by compacted fascicles of muscle fibers, which are in turn formed by myofibers. *Adapted with permission from Servier Medical Art.*

Muscle fibers are surrounded by the basal lamina—a protein cover—whose integrity is important for the maintenance of myofibers function and that serves as a scaffold for newly regenerated myofibers (de Palma et al., 2014). Laminins are the most abundant components of skeletal basal lamina (Sanes et al., 2003). Importantly, adult muscle stem cells, named satellite cells (SCs), which are essential for myofiber regeneration upon injury, are located under the basal lamina (Chang et al., 2014) (Figure 2).

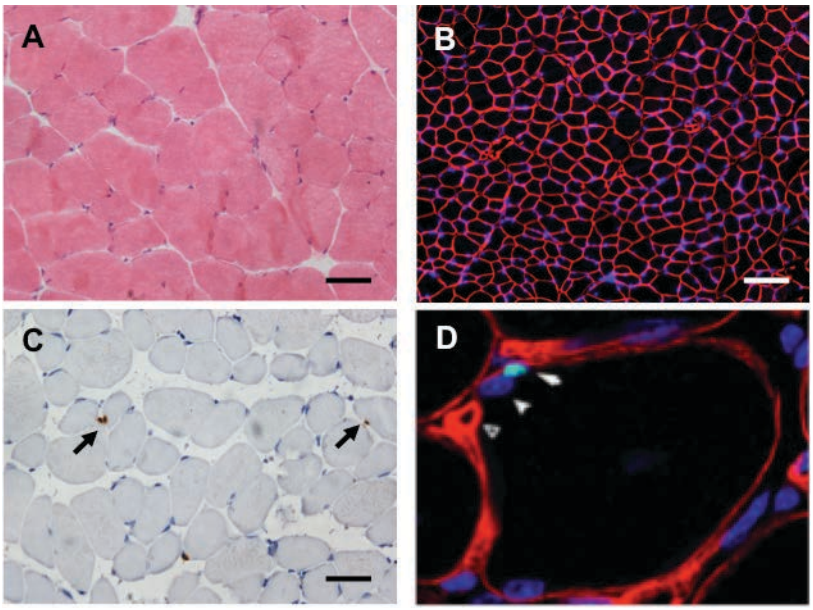


Figure 2. Histological analysis of skeletal muscle and SCs. (A) Haematoxylin and eosin (H&E) staining of skeletal muscle. Scale bar represents 50 μm. **(B)** Laminin (in red) and nuclei (in blue) costaining of skeletal muscle. Scale bar represents 100 μm **(C)** Pax7 staining of skeletal muscle. Arrows show cells expressing Pax7 in the nucleus. Scale bar represents 50 μm. **(D)** SCs (arrow) reside beneath the basal lamina (red) and are marked by Pax7. In mature muscle, they are always associated with a myonucleus (arrowhead) and are in close proximity to local capillaries (empty arrowhead). *Panels A-C, originated from our own results. Panel D, reproduced with permission from Bentzinger, et al., 2012. ©Cold Spring Harbor Laboratory Press.*

1.1. Transcriptional regulation of muscle differentiation

Myogenesis is orchestrated by a specific set of transcription factors, referred as muscle regulatory factors (MRFs) that comprise Myf-5, MyoD, myogenin and MRF4/Myf6 (Tierney et al., 2016). Structurally, MRFs are basic helix-loop-helix (bHLH) proteins that form homodimers with themselves or heterodimers with E proteins to bind to E-box sequences (CANNT) in the regulatory regions of target genes (Braun et al., 2011; Bentzinger et al., 2012). MRFs activate the transcription of muscle genes in a spatiotemporal-defined manner. During myogenesis, there is a division of labour among MRFs, with Myf-5 and MyoD determining lineage commitment and myogenin driving terminal differentiation of myoblasts into myotubes (Braun et al., 2011) (Figure 3).

Overexpression of MRFs in non-muscle cells (e.g., fibroblasts) is sufficient to induce muscle gene expression and, to different degrees, drive a myogenic differentiation (Davis et al., 1987; Weintraub et al., 1989; Braun et al., 1989). Myf5 and MyoD emerge from two independent myogenic lineages and, consequently, there is some redundancy between them (Ott et al., 1991; Rudnicki et al., 1993; Bentzinger et al., 2012). In turn, myogenin and MRF4 are more closely related to terminal differentiation rather than to cellular commitment. Thus, muscle differentiation genes can also be divided according to their temporal pattern of expression on early (e.g., Myf5, MyoD), intermediate (e.g., myogenin) or terminal markers (e.g., MHC, troponins).

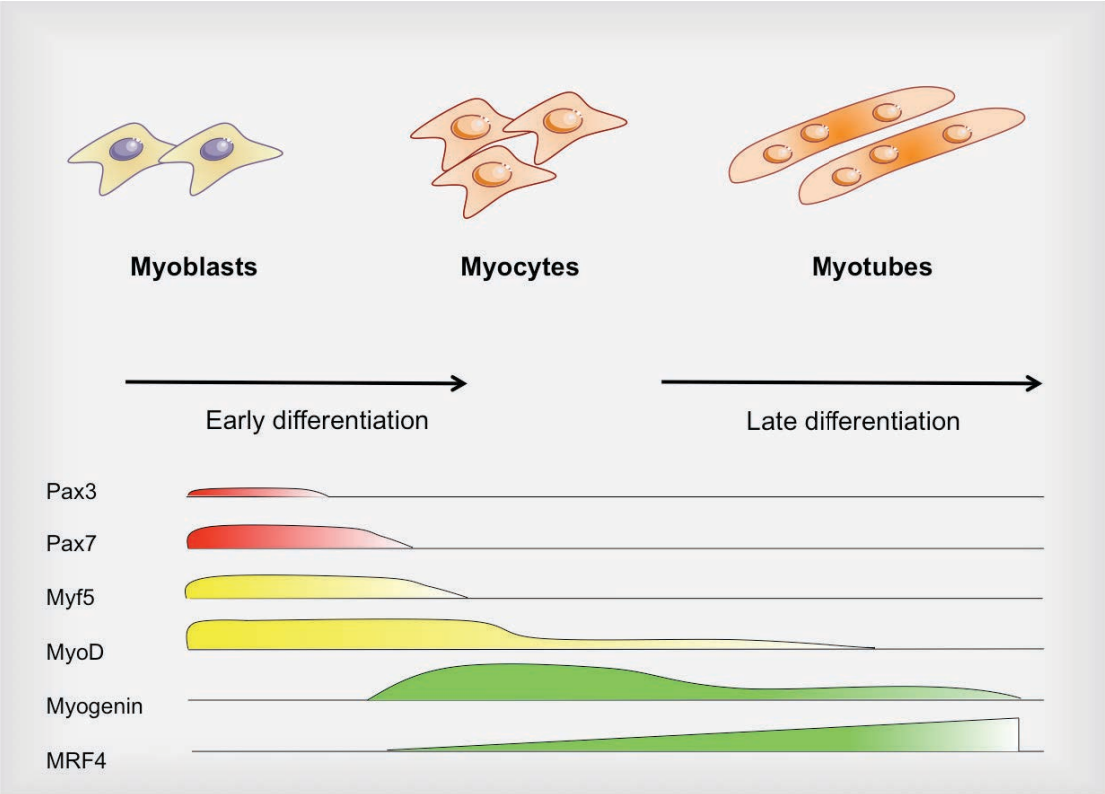


Figure 3. Schematic view of MRFs expression during myogenesis. In early differentiation, Pax7, Myf5 and MyoD are upregulated, whereas Myogenin and MRF4 drive terminal differentiation. *Adapted with permission from Bentzinger et al., 2012. ©Cold Spring Harbor Laboratory Press.*

Another important family of transcription factors involved in myogenesis is paired box proteins Pax3 and Pax7, which are expressed in early differentiation (Figure 3) and during embryonic development (Figure 4). Their functions are partially

overlapping with one compensating for the lack of the other in the mouse embryo (Relaix et l., 2004; Relaix et al., 2005). However, they play different important roles during early myogenesis. For instance, Pax3 is essential in establishing founder and progenitors cells in the limbs formation and Pax7-expressing cells contribute later to the SC pool and the formation of secondary myofibers (Maqbool & Jagla, 2007).

Nevertheless, Pax genes and MRFs cannot explain by themselves the complete pattern of temporal and spatial gene expression during myogenesis. For instance, in myoblasts, MyoD does not induce downstream muscle differentiation target genes, as its function is temporarily blocked through multiple mechanisms. Interaction of MyoD (and/or its E protein partners) with a number of factors prevents MyoD from entering the nucleus (e.g., interaction of MyoD with I-mfa/MDFI), from binding to DNA (e.g., with Twist, Id, and Mist-1), and/or from activating its targets (e.g., with Mist-1 and MyoR) (Benezra et al., 1990; Spicer et al., 1996; Chen et al., 1996; Lu et al., 1999a; Lemercier et al., 1998).

Other transcription factors without specific myogenic activity are important during muscle differentiation. For instance, Six proteins that bind to Eya1 and Eya2 in the nucleus to activate Pax3, MyoD, MRF4 and myogenin (Grifone et al., 2005) or MEF2, which contributes to myogenesis by synergizing with MRFs at the transcriptional level (Molkentin et al., 1995).

1.2. Formation of skeletal muscle in the developing embryo

The first muscle fibers arise from mesoderm-derived cells that generate additional fibers along them (Bentzinger et al., 2012). Muscles in the limbs and trunk derive from cells of the segmented paraxial mesoderm, known as somites (Braun et al., 2011). Somites give rise simultaneously to the mesenchymal sclerotome and the epithelial dermomyotome. Cells of the dermomyotome express Pax3 and Pax7 and, albeit to a lesser extent, Myf5 (Jostes et al., 1990; Goulding et al., 1991; Kiefer & Hauschka, 2001). Then, the dermomyotome develops different myotomal regions important for the generation of myogenic progenitors and the formation of limb buds. Pax3 but not Pax7 is expressed in the migratory cells that will form the limb buds (Bentzinger et al., 2012). The myotome is the most primitive muscle structure that

contains high numbers of MyoD- and Myf5-committed muscle cells (Bentzinger et al., 2012) (Figure 4).

At the latest stage of embryonic development, muscle progenitors delaminate from the myotome and migrate to give rise to the different trunk, limbs and diaphragm muscles (Cinnamon et al., 1999; Vasyutina & Birchmeier, 2006). Once the mature muscle is formed, progenitors enter into quiescence and reside as SCs (Gros et al., 2005; Relaix et al., 2005; Schienda et al., 2006).

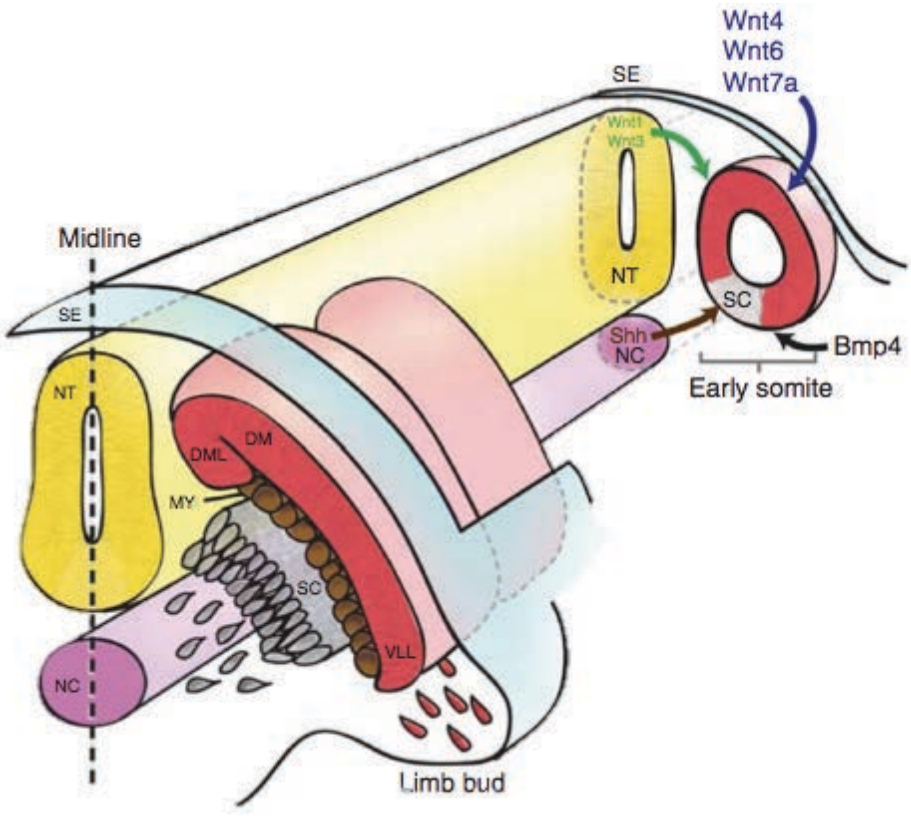


Figure 4. Schematic view of embryo transverse section in somitogenesis. Morphogens secreted from various domains in the embryo specify the early somite to form the sclerotome (SC) and dermomyotome (DM). Wnts secreted from the dorsal neural tube (NT) and surface ectoderm (SE) along with bone morphogenetic protein (BMP) from the lateral plate mesoderm maintain the undifferentiated state of the somite, whereas Shh signals from the neural tube floor plate and notochord (NC) to induce the formation of the sclerotome. As the sclerotome segregates, muscle progenitor cells from the dorsomedial (DML) and ventrolateral (VLL) lips of the dermomyotome mature to give rise to the myotome (MY). In limb buds, Pax3-dependent migrating progenitors delaminate from the ventrolateral lips to later give rise to limb muscles. *Adapted with permission from Bentzinger, et al. 2012. ©Cold Spring Harbor Laboratory Press.*

Of note, epithelial-to-mesenchymal transition (EMT) is a molecular reprograming process by which epithelial cells acquire mesenchymal characteristics (Nieto et al., 2016). EMT is essential during dermomyotomal development

(Bentzinger et al., 2012). EMT factors control the migratory function of myogenic progenitors to reach their target sites, suggesting an important role in muscle stem cells establishment (Jaffredo et al., 1988; Brand-Saberi et al., 1993).

1.3. Signalling pathways and regulation of muscle differentiation

Multiple signalling pathways including IGF, Wnt, Shh, BMP and Notch regulate muscle development (Figure 4). Insulin-like growth factors (e.g., IGF1 and IGF2) regulate myogenesis and muscle repair by increasing MRFs expression in different species (Jiménez-Amilburu et al., 2013; Zanou and Gailly, 2013). They are also involved in muscle hypertrophy and regeneration after damage (Musarò et al., 2001).

Wnt ligands act via activation of either the non-canonical pathway or the canonical β-catenin/TCF transcriptional complex, when binding to their cellular Frizzled (Fzd) receptors (van Amerongen & Nusse, 2009). Among Wnt ligands, Wnt1, Wnt3, Wnt4, Wnt6 and Wnt7a are of particular importance during embryogenesis (Parr et al., 1993). Different Fzd receptors are expressed along the developing somite conferring specificity on the downstream cellular signalling to activate MyoD or Myf5 expression (Borello et al., 2006; Brunelli et al., 2007).

In turn, members of the bone morphogenetic proteins (BMP) family inhibit muscle gene expression. BMP belongs to the TGF-β superfamily and acts through activation of SMAD proteins on target genes (Miyazono et al., 2005). BMP4 maintains the quiescence and the undifferentiated state of muscle progenitors through sustained Pax3 expression (Pourquié et al., 1995).

As in other tissues, Notch signalling regulates cellular differentiation and is also involved in vertebrate myogenesis (Hirsinger et al., 2001; Schuster-Gossler et al., 2007). Notch signalling inhibits MyoD expression through RBP-J as well as indirectly via Notch target genes (e.g., Hes1). Analyses of mice deficient for the Notch ligand *Dll1* or the transcription factor RBP-J revealed that Notch signalling activates myogenic precursors expansion while preventing their differentiation (Schuster-Gossler et al., 2007; Vasyutina et al., 2007).

In addition, muscle development is also regulated by epigenetic events. A specific DNA demethylation signature driven by Pax7 is required to achieve muscle-cell identity and for MyoD activation (Carrió et al., 2016). Thus, mounting evidence indicates that transcriptional programs during myogenesis are modulated by epigenetic modifications.

1.4. Muscle differentiation: from myoblasts to myofibers

Myoblasts are primordial muscle cells that conform muscle tissue. They proliferate and fuse forming multinucleated myofibers. Genetic evidence suggests that myoblasts originate from myogenic precursor cells in somites and prechordal mesoderm. Their differentiation involves multiple signalling pathways, transcription factors and chromatin remodelling proteins. Interestingly, many of them also participate in postnatal myogenesis (Bentzinger et al., 2012).

Myosin heavy chain (MHC) is one of the most contractile proteins in cells, which is largely determined by its ATPase activity (Nguyen et al., 1982; Miller et al., 1990). In murine striated muscle, there are seven MHC isoforms expressed by myofibers (Lyons et al., 1990). Depending on the physiological demand, there is a switch between these isoforms conferring an inherent plasticity to muscle tissue (Agbulut et al., 2003). Myofiber subtype is influenced by several factors, including age, physical exercise or hormonal/metabolic activity (Agbulut et al., 2003).

Skeletal muscles can be classified according to the isoform of MHC expressed (Figure 5). All muscles express more than one isoform, but depending on their relative abundance, they adopt specific contractile or twitching characteristics. Moreover, these isoforms are stage- and muscle-specific, thus generating a high functional heterogeneity (Lyons et al., 1990; Agbulut et al., 2003). Whereas in cardiac muscle only MHCα and MHCβ exist, in skeletal muscle there are adult fast (MHCIIA, IIX/D and IIB) and adult slow (MHCI/β) fibers (Agbulut et al., 2003). For instance, adult mouse gastrocnemius expresses high levels of fast MHCIIB isoform and soleus muscle is predominantly formed by the slow MHCI isoform (Agbulut et al., 2003). In developing skeletal muscle several isoforms have been described: eMHC (embryonic), pnMHC (perinatal) and MHCβ (Lyons et al., 1990). Extraocular muscles express a specific isoform only found there (eoMHC) (Lyons et al., 1990).

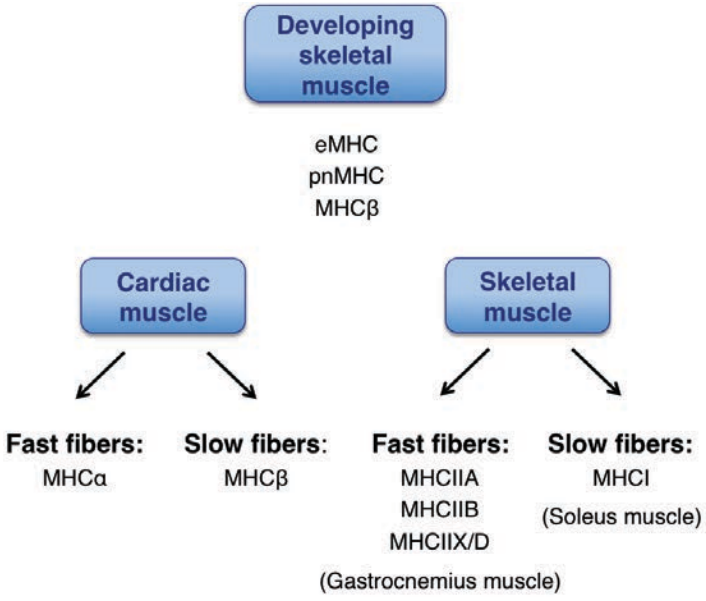


Figure 5. MHC isoforms expression depending on muscle tissue type and on its slow/fast twitch characteristics. Muscles express different isoforms of MHC protein conferring specific metabolic and functional features.

Most of our current understanding of the molecular mechanisms regulating muscle differentiation has been possible by the characterization of transgenic mouse models lacking specific muscle genes. Nevertheless, use of cell culture systems has also provided a powerful tool for the dissection of transcriptional regulation of muscle differentiation. Thus, the mouse myoblast C2C12 cell line is a widely used model to

study muscle differentiation. C2C12 myoblasts proliferate and can be forced to exit cell cycle and differentiate when they are switched from a medium rich in growth factors (referred as growth medium, GM) to another deprived for most of them (differentiation medium, DM). In DM media, myoblasts fuse when high confluence is reached to form multinucleated cells (C2C12 myotubes) that are terminally and irreversibly differentiated (Figure 6).

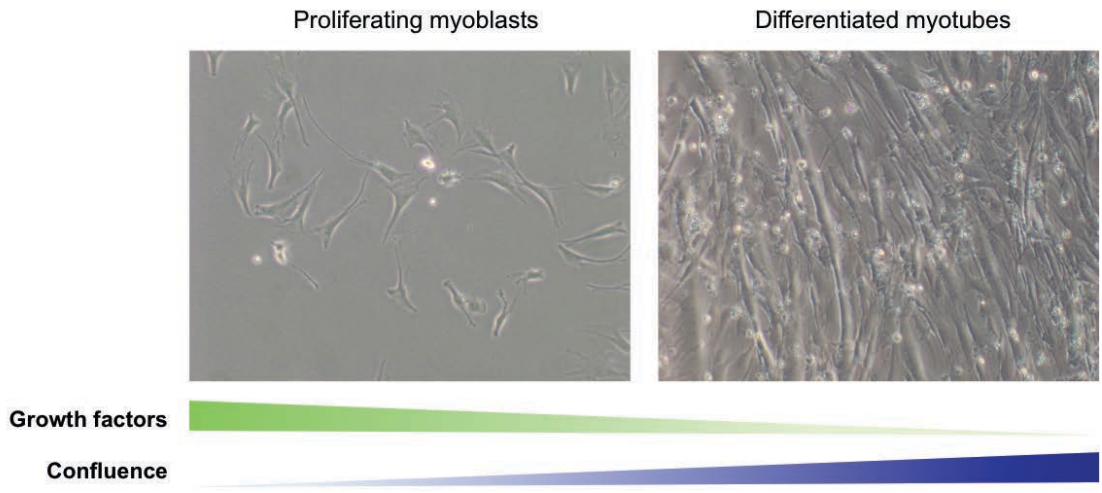


Figure 6. C2C12 *in vitro* model of muscle differentiation. Non-confluent cycling myoblasts differentiate into myotubes when confluence is reached upon changing media from GM to DM. *Pictures originated from our own results.*

1.5. Muscle differentiation in adult muscle: satellite cells

SCs were originally defined as remnants of embryonic development responsible for muscle regeneration after injury (Tierney et al., 2016). However, SCs are a highly heterogeneous population whose origin remains controversial. In fact, SCs are closely related to the myogenic progenitors that arise from the myotomic structures (Relaix et al., 2005; Schienda et al., 2006; Hutcheson et al., 2009; Lepper & Fan, 2010). Many signalling pathways and transcription factors controlling muscle embryonic development also participate during adult muscle regeneration (Tajbakhsh et al., 2009; Bentzinger et al., 2012). Although other adult stem cells, like bone marrow-derived progenitors or pericytes, have myogenic potential (Ferrari et al., 1998; Dellavalle et al., 2007), only SCs can replenish or account for their function in mice where Pax7 has been specifically deleted in SCs (Lepper et al., 2011).

SCs are covered by the basement membrane surrounding myofibers (Mauro et al., 1961). The microenvironment surrounding SCs (the so-called SC niche) is important to maintain their quiescence and to support their self-renewal capacity (Scadden et al., 2006; Jones & Wagers, 2008). The precise molecular mechanisms by which their quiescence and stemness are maintained remains only partly understood.

When SCs are activated, their symmetric or asymmetric divisions controlled by different signalling pathways generate committed myogenic cells or daughter stem cells (Conboy et al., 2002; Dhawan et al., 2005; Kuang et al., 2008; Pallafacchina et al., 2010). Wnt proteins regulate SC commitment and self-renewal upon activation in different directions. For instance, Wnt3a drives SCs toward a myogenic differentiation programme (Brack et al., 2008), whereas Wnt7a, which is released from regenerating myofibers, drives the non-canonical signalling to expand SC pool through symmetric divisions (Le Grand et al., 2009). This phenomenon enhances the muscle regenerative potential after injury. Notch signalling promotes SC self-renewal and regulates SC quiescence through expression of different Notch targets (e.g., *Hes6*, *Heyl*) (Wen et al., 2012). Contrary, p38/MAPK pathway directly regulates MyoD expression, thus promoting SC differentiation (Palacios et al., 2010) (Figure 7). Other factors like myostatin or TGF-β also regulate SC expansion (Bentzinger et al., 2010). Nevertheless, the mechanisms controlling asymmetric cell division are not fully understood (Kuang et al., 2007; Kuang et al., 2008; Almada & Wagers, 2016; Tierney et al., 2016).

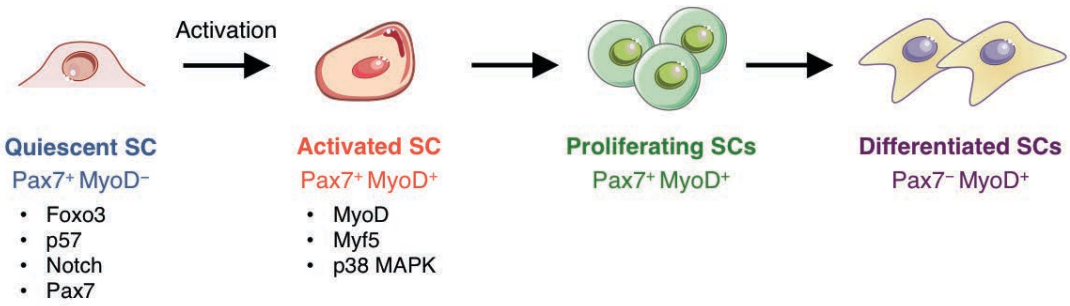


Figure 7. **Myogenic progression of SCs during regeneration.** Quiescent SCs express Pax7 and lack MyoD. Following muscle damage, SCs become activated and proliferate. Proliferating SCs induce MyoD expression and eventually generate committed cells that will generate myoblasts.

2. Muscle damage and regeneration

Skeletal muscle is a highly metabolic tissue with a rapid turnover. Several physiological activities need the continuous participation of muscle contraction, which may cause myofiber damage. Muscle has therefore an effective mechanism to regenerate damaged myofibers (Kumar et al., 2009; Charge et al., 2004). In addition to physiological breakdown, injury can arise in the context of pathological conditions or in the geriatric muscle (Aziz et al., 2012).

Lack of physical activity, inappropriate nutrition or immobilisation can result in muscle wasting and atrophy. Although closely related to atrophy but regulated by different signalling pathways, are a group of muscle conditions referred as myopathies. Myopathies can be chronic or appear in an acutely fashion, like in the context of metabolic syndromes or inflammation. A subgroup of myopathies is the so-called muscular dystrophies, included in the group of neuromuscular disorders. Most of them have a hereditary and genetic origin and they are characterised by progressive muscle wasting and weakness of variable distribution and severity (Marotta et al., 2009; Guiraud et al., 2015).

2.1. Human muscular dystrophies

Human muscular dystrophies comprise several genetic disorders that emerge most often during childhood and have different prognosis. They are characterised by the loss of muscle integrity with the subsequent muscle wasting and a decrease in life span. Diagnosis is based mainly on clinical and analytical features, immunohistochemical analysis of muscle biopsies and/or genetic studies. During their active stages, muscle destruction is accompanied by increased levels of creatine kinase (CK) in serum (Rahimov and Kunkel, 2013). Unfortunately, up to date there are no effective therapies to treat them (Guiraud et al., 2015).

The most common and severe form of muscular dystrophy is the Duchenne muscular dystrophy (DMD), a severe X-linked recessive pathology affecting mainly children and with a poor prognosis. DMD is caused by loss-of-function genetic alteration in the dystrophin gene (Magri et al., 2011). Dystrophin is a protein that

connects myofiber to the surrounding extracellular matrix becoming vital for myofiber integrity and structural stability (Koenig, 1987; Spitali et al., 2013; Tabebordbar et al., 2016). The most affected muscle in DMD patients is the diaphragm, which is the cause of respiratory failure and leads to death in late adolescence (Emery et al., 2002).

A related muscular dystrophy is the Becker muscular dystrophy (BMD), which usually takes a milder phenotype (Takeshima et al., 2010). In BMD, dystrophin is produced in lesser amounts than in healthy individuals. Hence, there is a broad range of phenotypes with different clinical features (de Palma et al., 2014).

Myotonic dystrophy Type I (DM1), also known as Steinert's disease, is another musculoskeletal disease with variable levels of severity. It is the most common autosomal dominant muscular dystrophy (Romeo et al., 2012).

Over their lifespan, dystrophic muscles undergo repeated cycles of myofiber degeneration and subsequent regeneration led by SCs. However, continuous SC activation and regeneration results in a loss of SCs regenerative capacity and replacement of muscle by fat and connective tissue (de Palma et al., 2014). In addition, alterations in SCs divisions-associated genes, also lead to the progressive loss of muscle tissue (Dumont et al., 2015).

2.2. Muscle injury

When injury occurs, immune cells are rapidly recruited into damaged tissue to eliminate dead myofibers and promote muscle regeneration. This cellular infiltration causes an inflammatory response, which is important in the subsequent repair of the injured muscle. It is mainly formed by mononucleated cells among damaged myofibers or found in the interstitial space (Figure 8).

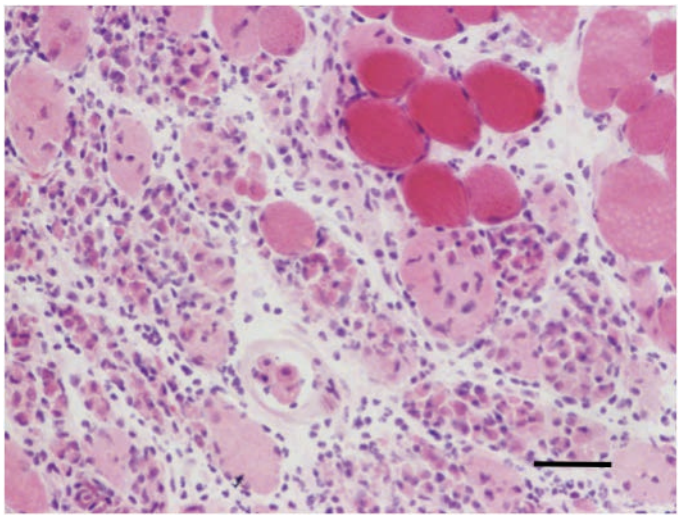


Figure 8. Histological analysis of injured skeletal muscle. Upon muscle injury, immune cells rapidly invade damaged tissue and are observed in high numbers as mononucleated cells infiltrated between muscle fibers. Scale bar represents 50 µm. *Picture originated from our own results.*

Neutrophils (F4/80^{-} Ly6C^{+}) are the first immune cells that extravasate and infiltrate muscle after acute injury. Following neutrophil invasion, there is a second wave of immune cells led by macrophages whose numbers increase progressively to reach a peak between 24h and 48h post-injury (Tidball & Villalta, 2010). Damaged muscle first recruits F4/80^{+} Ly6C^{+} CX3CR1^{low} circulating monocytes with a pro-inflammatory phenotype and expressing high levels of iNOS and other pro-inflammatory markers. Later on, these macrophages switch their phenotype and become anti-inflammatory macrophages, which are CX3CR1^{high} (Arnold et al., 2007) and Ly6C^{low} and that also express high levels of the mannose receptor MRC1 (CD206) and CD163 (Capote et al., 2016). In addition, injured myofibers also secrete chemokines and cytokines that contribute to the inflammatory environment needed for both inflammation and repair. A balanced pro-inflammatory and anti-inflammatory environment is required to resolve injury and to promote muscle regeneration (Ochoa et al., 2007; Tidball, 2017).

The initial response of skeletal muscle to injury is driven by Th1 cytokines, namely IFN-γ (*Ifng*) and TNF-α (*Tnf*) (Figure 9). Other secreted factors by injured muscle and/or invading macrophages, like iNOS (*Nos2*) or IL-6 (*Il6*), further potentiate the inflammatory response. In addition, the C-C motif chemokine ligand 2 (*Ccl2*), helps recruiting pro-inflammatory monocytes expressing the CCL2 high

affinity receptor CCR2 (Lu et al., 2011b) (Figure 9). CCL2/CCR2 signalling is required for a proper regeneration through the mobilization of SCs (Ochoa et al., 2007; Lu et al., 2011a; Lu et al., 2011b). Thus, inflammatory response and regeneration are linked and closely coordinated for muscle homeostasis in the context of muscle damage.

After inflammatory macrophages peak out, injured muscles release anti-inflammatory factors to promote their repair (Tidball & Villalta, 2010). These macrophages express high levels of CD206 and are activated by Th2 cytokines, like IL-4 (*Il4*), IL-10 (*Il10*) and IL-13 (*Il13*). Of note, engagement of CD206 promotes the expression of anti-inflammatory cytokines leading to a positive feedback that reduces muscle inflammation and damage (Tidball & Villalta, 2010; Tidball, 2017) (Figure 9).

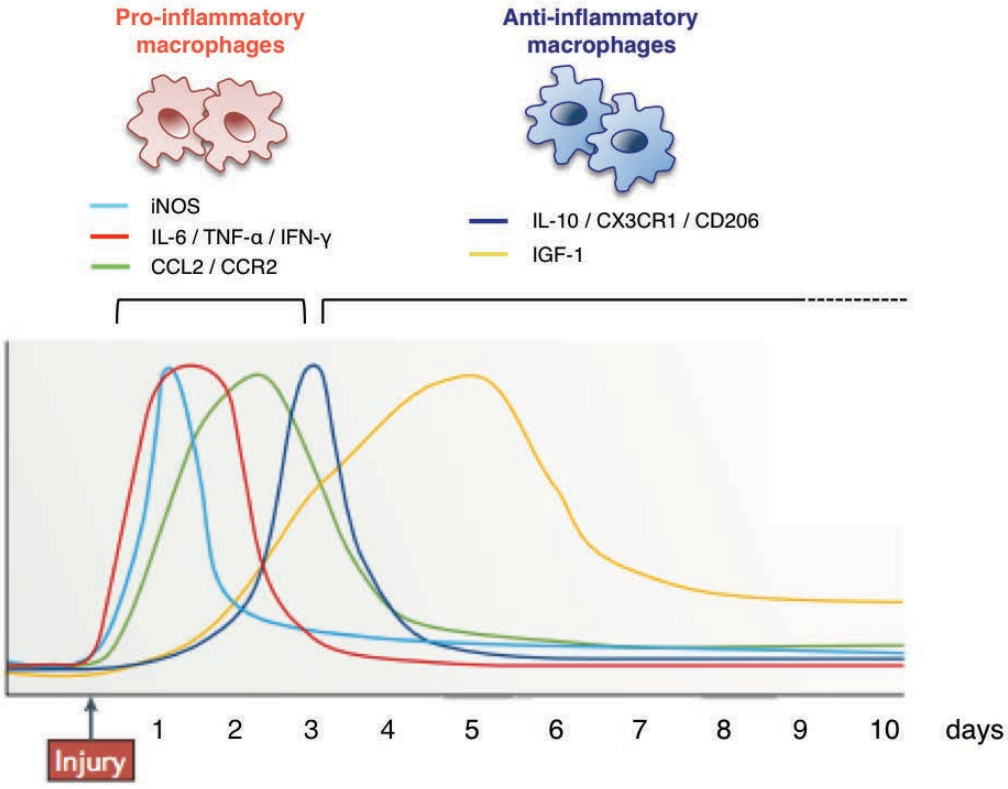


Figure 9. Expression of pro- and anti-inflammatory markers following muscle injury. Within hours of injury, rapid invasion by neutrophils and macrophages occur that promote an inflammatory phenotype with high levels of iNOS, IL-6, CCL2 and others. IL-10 levels begin to increase accompanied by the attenuation of the inflammatory response switching to a pro-regenerative environment. *Adapted with permission from Tidball, 2017.*

2.3. Muscle regeneration

Muscle regeneration is driven by SCs, that once activated proliferate and generate myogenic cells. Under normal physiological conditions SCs remain in a quiescent state. Upon muscle injury, regeneration of the damaged fibers requires the mobilization of SCs to the site of injury, where they become activated. Dissection of SC pool heterogeneity and the mechanisms regulating cell fate determination are critical to understand SCs action and functional exhaustion seen in muscular dystrophies.

In contrast to other adult tissues, skeletal muscle regeneration is highly effective, even after multiple rounds of injury. Consequently, SCs have been the object of study for years as a therapy in muscular diseases. However, SCs cannot be isolated and expanded *in vitro* indefinitely because upon activation they become irreversible differentiated (Montarras et al., 2005; Boonen & Post, 2008). In addition, the number of SCs that can be obtained from muscular biopsies limits their use in clinical practice.

Histologically, muscle regeneration is characterised by the presence of centrally nucleated myofibers. Myonuclei from fibers undergoing regeneration migrate to the centre of the myofiber, which expresses an embryonic MHC isoform named eMHC (encoded by *Myh3* gene) (Wu et al., 2015) (Figure 10).

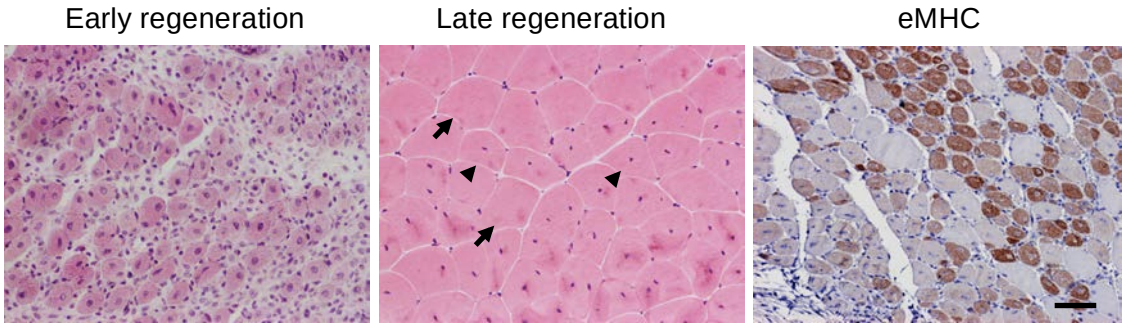


Figure 10. Histological analysis of regenerating muscles. In early regeneration, small myofibers start to form exhibiting centrally located nuclei, along with infiltrated immune cells. In late regeneration, normal muscle structure is almost re-established with centrally nucleated myofibers (arrowheads) coexisting with healthy fibers (arrows). eMHC marker is commonly used for the identification of regenerating myofibers after injury. Scale bar represents 50 µm. *Pictures originated from our own results.*

2.4. Therapeutic strategies in muscular dystrophies

Study of human muscle dystrophies has greatly benefited from the use of genetically modified mice. For instance, the *mdx* mouse, that harbours a premature stop codon in dystrophin gene, is considered the best model of human DMD (Coulton et al., 1988; Willmann et al., 2009). *mdx* mice display both areas of damage and regeneration and have an overall shorter life span compared to wild-type ones (Roig et al., 2004). However, higher regenerative capacity—which relates to the longer telomeres (Sacco et al., 2010)—in *mdx* mice results in milder phenotype vis-à-vis human patients. At later stages, myofibers are replaced by fibroblasts and fat cells that lead to collagen deposition and muscle fibrosis (Willmann et al., 2009). As with muscle degeneration, except for the diaphragm, fibrosis is also less severe in the *mdx* model than in DMD patients (Nakamura et al., 2011).

Due to the wide clinical variability of muscular dystrophies, management and treatment of individuals may vary. However, there is no effective cure for any of the dystrophies and treatment is limited to palliative care. In recent years, newer approaches, such as gene or stem cell therapy, have been explored in as potential treatment. Gene therapy has been directed to replace a functional dystrophin protein or the δ-sarcoglycan gene in mice models with promising results (Wang et al., 2000). The correct delivery into all muscle groups and the host's immunological response are still issues of concern. Other approaches include the use of oligonucleotides to repair point mutations or use of aminoglycoside antibiotics (gentamicin), which causes read-through of stop codons (Barton-Davis et al., 1999).

Despite its potential, stem cell therapy has many limitations. First, mutations account for only a small proportion of the dystrophies. In addition, adult SCs represents a very small population and difficult to maintain in a quiescent state *in vitro* to ensure their myogenic potential (Aziz et al., 2012). Cultured SCs have been reported to exhibit a limited ability to regenerate large areas of damaged muscle (Peault et al., 2007). Signalling at the SC niche is important to maintain SC quiescence and thus enhance their therapeutic ability (Montarras et al., 2005; Aziz et al., 2012). Poor recovery of muscle due to regenerative exhaustion in long-term treatments is also an important problem (Reimann et al., 2000; Luz et al., 2002). Nevertheless, it has been described that a small proportion of bone marrow stem cells

intravenously transplanted into *mdx* mice can restore dystrophin expression (Gussoni et al., 1999). Lastly, the use of embryonic stem cells to treat dystrophic muscle is also being explored (Darabi et al., 2008; Darabi et al., 2011).

3. ZEB1

The ZEB family of transcription factors comprise ZEB1 (also known as, δEF1, Tcf8, among others) and ZEB2 (SIP1) proteins in higher organisms. They are zinc finger/homeodomain proteins (Gheldof et al., 2012) that specifically recognize G/C-centered E-boxes in the regulatory regions of their target genes (Brabletz & Brabletz, 2010; Sanchez-Tilló et al., 2012). ZEB1 has been best characterised during tumour progression and metastasis. Expression of ZEB factors drives an EMT by repressing and activating epithelial and mesenchymal specification genes, respectively (Brabletz & Brabletz, 2010; Sánchez-Tilló et al., 2011a; Nieto et al., 2016). In addition, ZEB inhibits cell differentiation in a number of tissues such as cartilage, bone and the hematopoietic compartment among others (Vandewalle et al., 2009). In lower organisms there is only a single ZEB-related protein. Interestingly, the orthologue of ZEB1/2 in *Drosophila melanogaster*, zfh1, regulates muscle and neural development (Vandewalle et al., 2009).

EMT also occurs during normal development and in the formation of the musculoskeletal system (Vandewalle et al., 2009). Cellular detachment and migration into newly formed tissues are necessary during development, processes also occurring in malignant tumour progression (Gheldof et al., 2012). In fact, most EMT factors were originally identified as regulators of embryogenesis and cell differentiation and only later recognized for their role in cancer progression (Kalluri et al., 2009; Thiery et al., 2009).

3.1. ZEB1 structure and mechanism of action

ZEB factors have a conserved structure across species (Sanchez-Tilló et al., 2012). *ZEB1* and *ZEB2* are two highly homologous genes that encode for proteins of 1124 and 1214 aminoacids, respectively. ZEB proteins contain highly conserved two zinc-finger clusters at their N-terminal and C-terminal ends that mediate their binding to DNA (Sánchez-Tilló et al., 2011b). ZEB1 and ZEB2 are highly modular proteins, with independent domains that interact with other transcriptional factors as well as with non-DNA transcriptional regulators. For instance, ZEB proteins recruit

corepressors (CtBP1/2) and coactivators (p300) to mediate their transcriptional activities (Postigo et al., 1997; Sanchez-Tilló et al., 2011b; Gheldof et al., 2012). A homeodomain of ZEB1 is located at the central region of the protein (Figure 11).

ZEB1 binds to E-box and E-box like sequences (CANNT) on the regulatory regions of genes, with CACCTG as the highest affinity motif (Ikeda and Kawakami, 1995; Remacle et al., 1999; Postigo et al., 1999b). It has been proposed that the presence of two E-box sequences enhances the binding of ZEB1 to DNA (Remacle et al., 1999).

In addition, ZEB1 transcriptional activities are modulated by post-translational modifications. For instance, SUMOylation by Pc2 or acetylation by p300/pCAF disrupts ZEB binding to CtBP (Postigo, 2003a; Postigo et al., 2003b; Long et al., 2005; van Grunsven et al., 2006). Although its transcriptional significance remains unclear, phosphorylation of ZEB1 varies widely among cell types and may contribute to cell type specific activities (Vandewalle et al., 2009; Costantino et al., 2002). As a transcription factor, ZEB1 expression is mainly nuclear, although it has also been noted in the cytoplasm. Different phosphorylation sites on ZEB1's nuclear localization and export sites regulate its cellular location (Isella et al., 2015; Llorens et al., 2016). Post-translational modifications also modulate whether ZEB1 acts as a transcriptional activator or as a repressor (Vandewalle et al., 2009).

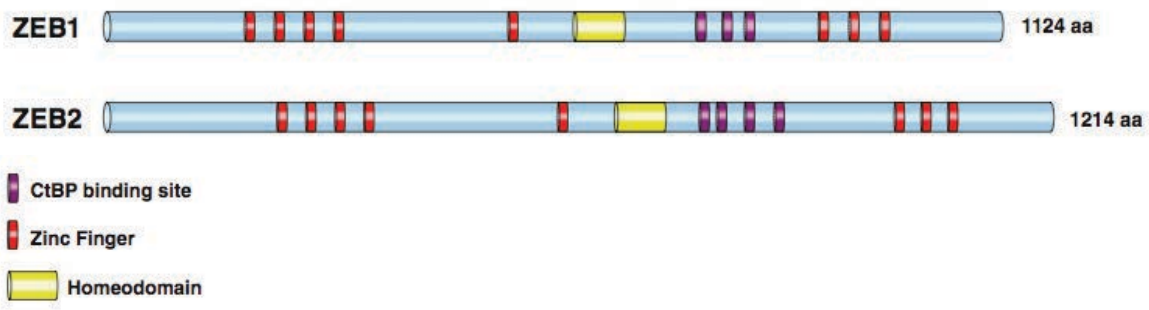


Figure 11. Schematic representation of ZEB1 and ZEB2 structure. Both proteins show conserved structure with similar domains to exert transcriptional regulation. *Reproduced with permission from Sánchez-Tilló et al., 2012.*

ZEB1 contains three binding sites for the CtBP corepressor that form the so-called CtBP interaction domain (CID) (Postigo et al., 1999a). Specifically, CtBP

binds to PLDLS and PLDLS-like sequences near the C-terminal zinc finger clusters (Vandewalle et al., 2009). Many of the genes and functions regulated by ZEB1 depend on CtBP-mediated repression, e.g. IL-2 expression in T-cells or regulation of epithelial genes in human tumour cells (Grooteclaes et al., 2000; Wang et al., 2009). However, evidence indicates that ZEB1 represses transcription through CtBP-independent mechanisms that vary depending on the cell type, target gene, etc. (Sánchez-Tilló et al., 2010; Gheldof et al., 2012).

Epigenetic regulation plays a key role in EMT and cancer metastasis (Wang et al., 2013) and is another mechanism of ZEB1's activity regulation. Cancer stem cells (CSCs) and non-CSCs exhibit a plasticity to switch from one state to the other through ZEB1's promoter chromatin configuration in breast cancer. Thus, high ZEB1 expression is related to CSCs phenotype and to a more aggressive tumour (Chaffer et al., 2013). On the other hand, CtBP forms a complex with histone modificators and other corepressors (e.g. HDAC1/2, HMTs, CoREST) that allow transcriptional repression of targeted genes (Shi et al., 2003; Wang et al., 2013). CoREST repressor complex comprises members of the SWI/SNF chromatin remodelling factors, such as BAF57 and BRG1, the last one also recruited by ZEB1 (Battaglioli et al., 2002; Sánchez-Tilló et al., 2010).

ZEB1 can also inhibit gene expression passively, by displacing other activators of the bHLH family from E-boxes on target genes (Genetta et al., 1994). In line with this observation, ZEB1 was shown to counteract MyoD/Myf5 or MyoD/Myf6-mediated transcriptional activation of p73. p73 is expressed during muscle differentiation and is controlled by MRFs and other transcriptional factors (Fontemaggi et al., 2001). ZEB1 contains several repressor domains with distinct transcriptional specificity. For instance, a repressor domain located close to the C-terminal Zinc finger cluster blocks the MEF2C transcriptional activity (Postigo et al., 1999b).

3.2. Signalling pathways and upstream regulation of ZEB1

ZEB proteins are at the crossroads of several developmental pathways, regulating both proliferation and differentiation programs. Thus, they are involved in

multiple signalling pathways regulating different processes depending on cell type. Of note, signalling pathways that control stem cell homeostasis during embryogenesis and later in adults (e.g., Wnt, Shh, Notch, Hippo, TGFβ) are regulating ZEB1 function (Hill et al., 2013; Nieto et al., 2016).

Notch pathway, which controls myogenic progenitors differentiation, activates ZEB1 expression, while ZEB1 enhances this pathway by indirectly increasing Jag1 and its coactivators (Wang et al., 2009; Brabletz et al., 2011). Moreover, ZEB1 has been described to activate Wnt pathway (Schmalhofer et al., 2009; Sánchez-Tilló et al., 2011a), which is important during muscle differentiation and regeneration after injury. Hippo/YAP signalling pathway is also known to induce and cooperate with ZEB1 (Liu et al., 2010; Lehmann et al., 2016). Lastly, both ZEB proteins are downstream of Snail transcription factor (Guaita et al., 2002) that regulates the timing of MyoD-driven muscle differentiation (Soleimani et al., 2012).

MicroRNAs control gene expression and are important regulators of epigenetic programmes. Several miRs regulate ZEB activity. Among them, miR-200 family are the most characterised ones regulating ZEB in human tumourigenesis (Davalos et al., 2012; Wang et al., 2013). In turn, ZEB also regulates transcription of different miRs, like miR141 and miR200c (Burk et al., 2008).

3.3. ZEB1 expression during embryonic development and in adult tissues

Zeb1-/- mice die close to birth with numerous bone and cartilage defects (Takagi et al., 1998). Thus, the *Zeb1* knockout mouse model can only be used during embryo development. For most tissues, *Zeb1+/-* mice express approximately half of mRNA and protein levels than *Zeb1+/+* counterparts.

Early in embryogenesis, *Zeb1* mRNA is highly expressed in the presomitic and lateral mesoderm. Once somites are formed, *Zeb1* is then moderately expressed and shows a homogenous staining. However, it becomes again upregulated when the limbs start to form. In E9.5 embryos, *Zeb1* is highly expressed in the entire limb bud. Later, when limb buds grow, it adopts a posterior expression pattern. On the other

hand, in the somites of the trunk, *Zeb1* expression is confined to the myotomal regions, coinciding with cells that will migrate to the limb (Figure 12). By E12.0, when limb is formed, *Zeb1* is transiently expressed in the interdigit mesenchyme and finally in the cartilage, when the skeletal pattern is already formed (Takagi et al., 1998).

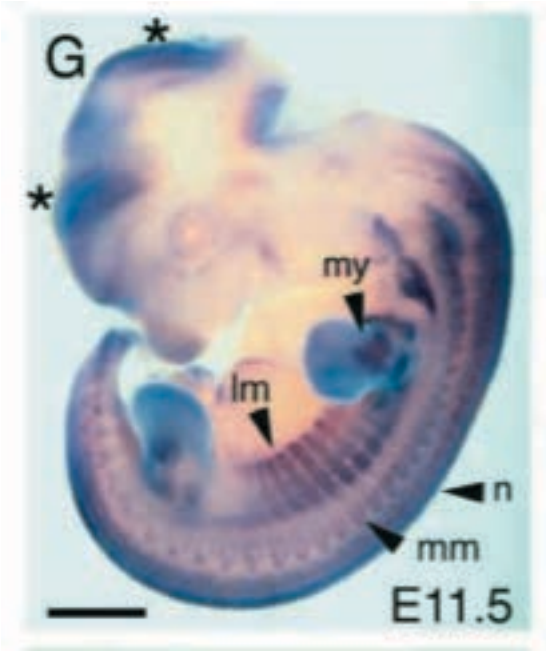


Figure 12. ZEB1 expression in early mouse embryo development analysed by *in situ* hybridization. In the transverse section of the trunk *Zeb1* is expressed in the lateral (lm) and medial (mm) myotome, in the neural tube (n) and in migrated myotome (my) by E11.5. Asterisks indicate non-specific signals produced by probes trapped in the brain ventricles. Scale bar represents 500 μm. *Reproduced with permission from Takagi, et al., 1998.*

Post-natal *Zeb1* mRNA expression is downregulated and its expression is restricted to some human adult tissues. Interestingly, skeletal muscle is one of the tissues expressing highest levels of ZEB1 (Human Atlas database, *www.proteinatlas.org*).

RATIONALE AND OBJECTIVES

RATIONALE AND OBJECTIVES

ZEB1 is best known for its role promoting tumour progression, but it also represses cell differentiation in different tissues, including bone, cartilage and hematopoietic cells.

Muscle differentiation is essential, both during embryonic myogenesis as well as in muscle regeneration. Muscle fibers are subjected to continuous breakdown, which causes myofiber degeneration and require its regeneration by SCs. Thus, due to its regenerative capacity, skeletal muscle represents an ideal model to study ZEB1 function upon tissue injury.

The general aim of this dissertation is to investigate a potential role of ZEB1 in muscle development and dissect the molecular mechanisms by which ZEB1 regulates myogenic differentiation and muscle regeneration in the context of muscle injury.

The specific objectives of this dissertation are to characterise:

1. ZEB1 function in skeletal muscle during development and differentiation using cell line-based and *in vivo* models.
2. ZEB1 expression and function upon muscle injury.
3. ZEB1's role in satellite cells during muscle regeneration after damage.

MATERIALS AND METHODS

MATERIALS & METHODS

Mice models

mdx (Jackson Laboratories, ME, USA) (Bulfield et al., 1984) and *Zeb1+/-* (Takagi et al., 1998) mice strains were used in these studies. All animal handling procedures were approved by the local ethics committee. *mdx* mice strain was crossed with *Zeb1+/-* mice to generate an heterozygous offspring which was again crossed to finally obtain *mdx;Zeb1+/+* and *mdx;Zeb1+/-* offspring. In selected experiments, mice were injected intramuscularly into the gastrocnemius with 10 µM cardiotoxin (CTX) from *Naja mossambica mossambica* (Sigma-Aldrich, St. Louis, MO, USA). When 2 rounds of CTX were administered, mice were allowed to recover for 14 days between both injections. To assess myofiber damage 0,1ml/10g body weight of Evans Blue Dye (EBD, Sigma-Aldrich) was injected intraperitoneally.

Plasmids

Expression vectors used were obtained from the following researchers: full-length mouse *Zeb1* was obtained from M. Saito (Tokyo University, Japan) (Shirakihara et al., 2007), full-length mouse *Zeb1* with mutated CtBP binding sites was obtained from Y. Higashi (Institute for Developmental Research, Kasugai, Japan) (Furusawa et al., 1999) and pEMSV-MyoD was from the late H. Weintraub (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) (Davis et al., 1987). The *Xenopus* MyoD construct, containing the full-length cDNA plus the 5' and 3' untranslated regions and cloned into pSP64T, was obtained from J. Gurdon (Gurdon Institute, Cambridge, United Kingdom). Simian virus 40 (SV40)–β-galactosidase (β-gal) was purchased from Promega, pcDNA3 was purchased from Invitrogen, and the pBluescript SK vector was purchased from Stratagene-Agilent (Santa Clara, CA, USA). Firefly luciferase reporters for the promoters used in this article were provided by the following researchers: 5.5 kb of rat α-*MHC* (*Myh4*) promoter was obtained from E. N. Olson (University of Texas Southwestern, Dallas, TX, USA), (Lu et al., 1999b), 2.3 kb of quail *troponin I* (*Tnnt1*) was obtained from S. Konieczny (Purdue University, West Lafayette, IN, USA) (Johnson, 1996), a bp -1256 mouse *muscle creatine kinase* (*Ckm*) promoter was obtained from S. A. Leibovitch (INRA, Montpellier, France)

(Reynaud, 2000), and a 4-kb enhancer plus a 2.7-kb promoter of the human *Myod1* gene were obtained from J. P. Capone (McMaster University, Hamilton, ON, Canada) (Hunter et al., 2001).

Antibodies

Antibodies used in different experiments are listed below.

Primary Abs	Source	Clone (Catalog Number)
Western Blot		
ZEB1	Santa Cruz Biotechnologies	H-102 (sc-25388)
CtBP1/2	Santa Cruz Biotechnologies	E-12
Myogenin	BD Biosciences	F5D
α-tubulin	Sigma	B5-1-2
Immunohistochemistry		
MHC (Pan MHC)	Developmental Studies Hybridoma Bank	MF20
ZEB1	Santa Cruz Biotechnologies	H-102 (sc-25388)
Laminin	Santa Cruz Biotechnologies	48H-2 (sc-59854)
MyoD	Santa Cruz Biotechnologies	C-20
BrdU-FITC	BD Biosciences	B44
eMHC	Developmental Studies Hybridoma Bank	F1.652
Pax7	Developmental Studies Hybridoma Bank	Pax7
Myh2	Developmental Studies Hybridoma Bank	SC-71
Myh4	Developmental Studies Hybridoma Bank	BF-F3
GFP	Aves Lab	GFP-1020
CD206-Alexa Fluor 488	AbD Serotec	MR5D3
ChIP		
ZEB1	Santa Cruz Biotechnologies	E-20X
MyoD	Santa Cruz Biotechnologies	C-20
***Drosophila* assays**		
MHC	D. Kiehart (Duke University)	
MEF2	B. M. Patterson (National Cancer Institute, NIH)	
FACS analysis		
F4/80-APC	BioLegend	BM8 (123116)
C11b-PE	ImmunoTools	M1/70.15 (22159114)
Ly6C-PerCP-Cy5.5	eBioscence	HK1.4 (45-5932-82)
SCs isolation		
α7 integrin	MBL International	3C12 (K0046-3)
CD34-Biotin	eBioscience	RAM34
CD45-PE	eBioscience	RA3-6B2 (12-0452-81)
C11b-PE	ImmunoTools	M1/70.15 (22159114)
CD31-PE	BD Biosciences	MEC 13.3 (553373)
Sca1-PE	BD Biosciences	D7 (553108)
Streptavidin APC Cy7	BD Biosciences	554063
Alexa Fluor 647 anti-mouse	Life Technologies	A-21240

Secondary Abs	Source	Catalog Number
HRP anti-mouse	Jackson ImmunoResearch	715-035-151
HRP anti-rabbit	Jackson ImmunoResearch	111-035-144
Alexa Fluor 488 anti-mouse	Jackson ImmunoResearch	715-545-150
Alexa Fluor 488 anti-chicken	Jackson ImmunoResearch	103-545-155
Rhodamine RedX anti-rat	Jackson ImmunoResearch	712-295-153
Alexa Fluor488 anti-rabbit	Jackson ImmunoResearch	711-545-152
Rhodamine RedX anti-mouse	Jackson ImmunoResearch	715-295-151

Table 1. List of antibodies used depending on the assay.

RNA interference

The three set of siRNAs used to target mouse *Zeb1* originated as follows.

siRNA	Sequence 5' ⇒ 3'	Ref
si1 ZEB1	GACCAGAACAGUGUUCCAUGUUUAA	MSS210696 from Life Technologies
si2 ZEB1	AACUGAACCUGUGGAUUAU	Lacher et al. 2011
si3 ZEB1	GAAGAACCCUUGAACUUGU	sc-38644 from Santa Cruz Biotechnology
	GAACAGUGUUCCAUGUUUA	
	CAACCAUGAAGGAUCUAUA	
siCtl	UAUAGCUUAGUUCGUAACC	12935-200 from Life Technologies
siCtl-Luc	GAUUAUGUCCGGUUAUGUA	Judge et al. 2005
siCtBP	GAACUGTGUCAACAAGGAC	Wu et al. 2006

Table 2. siRNA sequences used.

As negative controls in interference experiments, siRNA Low-GC (siCtl) and an additional control siRNA targeting firefly luciferase (siCtl-Luc) for Western blot and quantitative real-time PCR (qRT- PCR) studies were used. For C2C12 differentiation and transcriptional assays either 200 nM or 100 nM of siRNA duplexes were used respectively.

Cell culture and transfections

C2C12 and C3H-10T1/2 cells were obtained from the American Tissue Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 12% fetal bovine serum (FBS) (Sigma-Aldrich). Cells were transiently transfected with expression or reporter vectors by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) and/or with small interfering RNA (siRNA) oligonucleotides by using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions.

Transcriptional assays

Transcriptional assays were carried out with firefly luciferase reporter vectors and equal molar amounts of either expression plasmids encoding *Zeb1* (0.4 μg) or MyoD (0.6 μg) or, as controls, the corresponding empty expression vector. For the luciferase-reporter promoters, 0.2 μg were used except for the MHC promoter that 0.4 μg were loaded. As an internal control for transfection efficiency, 1 μg of SV40–β-gal was co-transfected at each point. The total amount of DNA transfected was equalized by adding the promoterless pBluescript SK vector (Stratagene-Agilent) as required. Firefly luciferase activity was assessed with a Luciferase Assay System kit (Promega, Madison, WI, USA), whereas β-galactosidase activity was determined with Luminescent β-Galactosidase Detection Kit II (Clontech, Mountain View, CA, USA). Relative luciferase units (RLU) were assessed with a Modulus II Glomax microplate detection system (Promega). RLU values were expressed as the means of duplicates and are representative of at least four independent experiments.

Western blots

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% SDS, 50 mM Tris pH 8, 2 mM EDTA, plus protease inhibitors) and loaded onto polyacrylamide gels. Gels were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Merck Millipore, Germany). Following blocking for nonspecific antibody binding with 5% non-fat milk, membranes were incubated with the corresponding primary and HRP-conjugated secondary antibodies (see Table 1) before the reaction was developed by using the Pierce ECL Western blotting substrate or SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA). Western blots shown are representative of at least four independent experiments.

Myogenic conversion assays

For differentiation of C2C12 myoblasts into myotubes, cells were seeded into 6-well plates and grown in growth medium (GM) (DMEM plus 20% FBS) until they reached 80 to 90% confluence, at which time the GM was switched to differentiation medium (DM) (DMEM supplemented with 2% horse serum; Sigma-Aldrich) and maintained

in DM for different periods. In selected experiments as specifically indicated, cells were maintained in GM even after reaching full confluence.

In myogenic conversion of cultured SCs, $5x10^3$ freshly isolated SCs were seeded in 24-well-plate and allowed to differentiate in SGMSC. Differentiated cultures were fixed in -20°C-chilled methanol and blocked with 2% gelatin (from cold-water fish skin; Sigma-Aldrich) before being stained with pan-myosin antibody MF-20 for 2 h, washed with phosphate-buffered saline (PBS), incubated either with HRP-conjugated anti-mouse IgG antibody (JIR), counterstained with hematoxylin and developed with a 3,3-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA) or with AlexaFluor 488 anti-mouse (Jackson Immunoresearch, West Grove, PA, USA) and counterstained with DAPI ProLong antifade (Life Technologies). Stained cultures were then examined under a Zeiss Axiovert HXP120V microscope (Zeiss, Oberkochen, Germany).

Chromatin immunoprecipitation assays

ChIP assays were performed using an EpiQuick ChIP kit (Epigentek Group Inc., Farmingdale, NY, USA) according to the manufacturer's instructions. C2C12 cells as either non-confluent cycling myoblasts or terminally differentiated myotubes were incubated during 20 min with a 1% formaldehyde solution (Electron Microscopy Sciences, Hatfield, PA, USA) at room temperature, followed by incubation with 1.25 M glycine. Upon sonication of cell lysates, chromatin was immunoprecipitated with the corresponding specific or control antibodies, and amplification of DNA fragments was assessed by qRT-PCR. As an IgG control for chromatin immunoprecipitation (ChIP) assays, normal rabbit IgG and normal goat IgG-containing serum were purchased from SCBT and JIR, respectively.

Identification of potential DNA binding sequences for ZEB1/MyoD and design of primers for qRT-PCR was conducted by using MacVector 12.5 software (MacVector Inc., Apex, NC, USA). For the ZEB1/MyoD CACCTG site at position -1068 of the mouse *Myh4* promoter, the primers used to amplify the region between bp -1107 and -1014 of this promoter were as follows: forward primer 5'-TATAAAAGATTTTACCTGCCA-3' and reverse primer 5'-ATATTTTCAACCACTGTTCT-3'. For the ZEB1/MyoD CAGGTG site at position -

1046 of the mouse skeletal slow troponin T1 (*Tnnt1*) promoter, the primers used to amplify the region between bp -1105 and -1020 of this promoter were as follows: forward primer 5'-TCTCAAACCAAAGCAAAACCAA-3' and reverse primer 5'-AGTTCCCCGTACCTCATACTCT-3'. A 191-bp region of the mouse *Gapdh* promoter, lacking consensus binding sites for ZEB1/MyoD, was amplified by using forward primer 5'-AGCTACTCGCGGCTTTACG-3' and reverse primer 5'-AAGAAGATGCGGCCGTCTCT-3', modified from those described previously (Noh et al., 2010). In all qRT-PCRs, values shown represent relative binding in relation to input and are the averages of data from two independent ChIP assays, each performed in triplicate.

RNA extraction and quantitative real-time PCR

Total RNA from C2C12 cells was extracted with the SV Total RNA isolation system kit (Promega). RNA was then used to synthesize cDNA by using a reverse transcription kit [random hexamers and GoScript (Promega)]. Total RNA from gastrocnemius muscle or *Xenopus* ectodermal explants (animal caps) was extracted with TRIzol (Life Technologies) and retrotranscribed with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) or with oligo(dT) and SuperScript II reverse transcriptase (Life-Technologies) for *Xenopus* experiments.

Total RNA from SCs was extracted with the Arcturus® PicoPure® RNA Isolation kit (Life Technologies). RNA was retrotranscribed with High-Capacity cDNA Reverse Transcription Kit. mRNA levels were determined by qRT-PCR Chromo4 (Bio-Rad, Hercules, CA, USA) using either SYBR green/ROX (GoTaq; Promega) (for C2C12 cells, gastrocnemius muscle and SCs) or iQSYBRGreen Supermix (Bio-Rad) (for *Xenopus* animal caps). Primers used are listed in Table 3. Relative gene expression was calculated by the ΔΔCT method using Opticon Monitor 3.1.32 software and normalizing values relative to *Gapdh* housekeeping gene or to *eef1a1* in *Xenopus* animal caps experiments.

Target gene	Forward 5' ➔ 3'	Reverse 5' ➔ 3'	Ref
Mouse genes			
Zeb1 (mice)	AACTGCTGGCAAGACAAC	TTGCTGCAGAAATTCTTCCA	Own design
Zeb1 (C2C12)	ACCCCTTCAAGAACCGCTTT	CAATTGGCCACCACTGCTAA	Abe et al. 2008
TnnT1	GATTCTGTATGAGAGGAAAAAG	TCATA TTTCTGTTGCTTCAACTT	Ogilvie et al. 2000
Myog	CACTGGAGTTCGGTCCCAA	TGTGGGC GTCTGTAGGGTC	Caretti et al. 2006
Myod1	TGGGATATGGAGCTTCTATCGC	GGTGAGTCGAAA CACGGATCAT	Dogra et al. 2006
Myf5	TCTGGTCCCGAAAGAACAGC	CTTTTATCTGCAGCACAT GCATT	Tanaka et al. 2003
Il6	AACGATGATGCACTTGCAGA	TGGTACTCCAGAAGACCAGAGG	Nakajima et al. 2014
Ifng	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC	Ydens et al. 2012
Tnf	AGGGTCTGGGCCATAGAACT	CCACCACGCTCTTCTGTCTAC	Chen et al. 2014
Nos2	GACGAGACGGATAGGCAGAG	GCACATGCAAGGAAGGGAAC	Aoshiba et al. 2007
Ccl2	GGGATCATCTTGCTGGTGAA	AGGTCCCTGTCATGCTTCTG	Deguchi et al. 2013
Ccr2	AGCACATGTGGTGAATCCAA	TGCCATCATAAAGGAGCCA	Kitamoto et al. 2013
Il10	ACCAGCTGGACAACATACTGC	TCACTCTTCACCTGCTCCACT	Bencze et al. 2012
Mrc1	CCATTTATCATTCCCTCAGCAAGC	AAATGTCACTGGGGTTCCATCACT	Hayes et al. 2015
Cx3cr1	CTGCTCAGGACCTCACCAT	TTGTGGAGGCCCTCATGGCTGAT	Fang et al. 2005
Myh2	CGATGATCTTGCCAGTAATG	ATAACTGAGATACCAGCG	Abe et al. 2008
Myh4	TAAGCACGAGCGCAGAGTGAAGGAACT	GCTGGATCTTACGGAACTTGGCCAGGT	Caretti et al. 2006
Myh3	CTTCACCTCTAGCCGGATGGT	AATTGTCAGGAGCCACGAAAAT	Zhou et al. 2009
Pax7	GAGAAACCCCGGGATGTTCAG	ATCCAGACGGTTCCCTTTCT	Own design
Cdkn2a	AGACCGACGGGCATAGCTT	TAGCTCTGCTCTTGGGATTGG	Own design
Cdkn1a	CAACCCATCTGCATCCGTTTCACC	GAGTGGGGACCATTCCTGTCTTCA	Ring et al. 2003
Cdkn1c	ACCCCGCGCAAACGT	AGATGCCCAGCAAGTTCTCTCT	Tury et al. 2011
Ccna2	AAGAGAATGTCAACCCCGAAA	ACCCGTCGAGTCTTGAGCTT	Sousa-Victor et al. 2014
Hes1	GCGAAGGGCAAGAATAAATG	TGTCTGCCTTCTCTAGCTTGG	Murta et al. 2015
Hes6	GCCGGATTTGGTGTCTACAT	TCCTGAGCTGTCTCCACCTT	Ogura et al. 2015
Foxo3	GATAAGGGCGACAGCAACAG	CTGTGCAGGGACAGGTTGT	Nowak et al. 2007
Gapdh (C2C12)	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC	Liu et al. 2008
Gapdh (mice)	CGACTTCAACAGCAACTCCCACTCTTCC	TGGGTGGTCCAGGGTTTCTTACTCCTT	Banerjee et al. 2013

***Xenopus* genes**			
ef1α	CACCATGAAGCCCTTACTGA	ACCTGTGCGGTAAAAGAACC	
tnnI1	CAGTAGCATTCCAGGGCAGT	TATGTAGCCCCAATGGGAAA	
tnnI2	CTCTTCAGCGGGGATATTGA	ATTTGAGCCCCTCCTTGAGT	
ckm	ACAAACCAGTGTCCCCTCTG	CCACACCAGGAAGGTCTTGT	
acta1	GCTGACAGAATGCAGAAG	TTGCTTGG AGGAGTGTGT	
myod1	GACCTGCCAATGTTGTGTTG	CAAAAAGTGGTCCGCAAGTT	

Table 3. Primers used for quantitative qRT-PCR.

Xenopus embryo microinjection and *in situ* hybridization

Xenopus laevis embryos were obtained by *in vitro* fertilization as described previously (Kroll et al., 1998) and were staged according to methods described previously (Nieuwkoop et al., 1967). Linearized pcDNA3-*Zeb1*$_{wt}$, pcDNA3-*Zeb1*CID$_{mut}$, and pSP64T-*MyoD* expression vectors were used to produce capped RNA by using the SP6-Message Machine kit (Ambion, Thermo Fisher Scientific). A morpholino oligonucleotide (MO) against *Xenopus zeb1* (van Grunsven et al., 2006) (5'-AGATCTGCCAAAGTTGAGCGTTT-3') was purchased from Gene Tools LLC. Where indicated, 200 pg of *zeb1* RNA or 20ng of *zeb1* MO was injected into one blastomere at the 2-cell stage, along with 20 pg of β-galactosidase RNA. Embryos were further cultured in 0.2X Marc's modified Ringer's (MMR) solution containing 4% Ficoll and 100 µg/ml gentamicin.

For animal cap isolations, both cells of pigmented embryos at the 2-cell stage were injected with 100 pg of *MyoD* plus either 100 pg *Zeb1*$_{wt}$ or *Zeb1*CID$_{mut}$ mRNA, and embryos were raised up to the blastula stage (stages 8 to 9). Twenty ectodermal explants per sample were then isolated and incubated at 25°C in 0.7X MMR supplemented with 1 mg/ml bovine serum albumin (BSA) and 100 µg/ml gentamicin. For *in situ* hybridization assays, embryos were grown to stages 15 to 16, fixed in minimum essential medium with formaldehyde, stained with Red-Gal (Research Organics, Sigma-Aldrich), and processed as described previously (Seo et al., 2005).

Drosophila stocks, crosses, and immunostaining

Full-length cDNA for *zfh-1*, obtained from Z. C. Lai (The Pennsylvania State University, Philadelphia, PA), was inserted into the NotI/XbaI sites of pUAST to

generate independent stocks of upstream activation sequence (UAS)-*zfh-1* flies (genotype, *w118;* P{w^{+mC} = UAS-zfh-1.P}2B). UAS-*zfh-1*-CID_{mut} stocks are identical to UAS-*zfh-1* stocks, except that the CtBP-interacting domain (CID) at ^{790}PLDLS^{796} is mutated to a nonbinding sequence, ^{790}ASASA^{796} (genotype, P{w^{+mC} = UAS-zfh-1-CIDm}2B). UAS-*zfh-1* and UAS-*zfh-1*-CID_{mut} stocks are homozygous, viable, and fertile second-chromosome insertions deposited at the Bloomington Drosophila Stock Center (Bloomington, IN) under identification numbers 6879 and 6880, respectively. *zfh-1* expression in UAS-*zfh-1* and UAS-*zfh-1*-CID_{mut} was induced by crossing them with 24B-Gal4 or MEF2-Gal4 stocks obtained as a kind gift from M. Bates (University of Cambridge, United Kingdom). Embryos were allowed to develop at 25°C before being examined for expression of different proteins. After standard fixation, embryos were blocked with 50% normal goat serum in PBS and incubated with primary and secondary antibodies before the reaction was developed with a DAB substrate kit (Vector Laboratories). Following colour development, embryos were mounted in 80% glycerol and examined on a Zeiss Axioplan-2 microscope.

Immunohistochemistry

FFPE mouse tissue samples corresponded to 4 µm sections from embryonic day 18.5 (E18.5) C57BL/6J wild-type and *Zeb1-/-* embryos (Takagi, 1998). Dissected mouse gastrocnemius were mounted in OCT-corks (Optimal Cutting Temperature, Sakura Finetek, Torrance, CA, USA), frozen in liquid-nitrogen-cooled-isopentane and immediately stored at -80ºC till ready to cut. 7 µm cryosections were performed in a Leica Cryostat (CM 1950) and sections were fixed in ice-cooled acetone prior to staining procedure. Frozen human dystrophies biopsies were obtained from the Spanish National Biobank Network.

Frozen sections were permeabilised in PBS 0,25% Triton X-100 for 30min. FFPE-slides were subjected to deparaffination and hydration by using standard protocols, followed by heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0) for 15 min and then followed by 0,3% H_2O_2 incubation to block endogenous signalling. All tissue sections were then blocked to minimize unspecific IgG binding in PBS, 4%BSA, 5% Normal Donkey or Goat Serum (depending on secondary antibodies) (Jackson Immunoresearch) and 0,5% Tween 20. Detection was developed with the

DAB substrate kit (Vector Laboratories) and counterstained with haematoxylin. H&E staining was performed just after acetone fixation by haematoxylin and eosin incubations. Microscopic examination was carried out in a Nikon Olympus BX41.

For ZEB1 expression analysis in human dystrophies biopsies, the relative number of myofibers (0% to 100%) expressing ZEB1 in the cytoplasm of human muscle dystrophies samples was codified according to the following score (1 to 5, 1: 0-20%; 2: 21-40%; 3: 41-60%; 4: 61-80%; and 81-100%). In case of ZEB1's nuclear staining was scored from 1 to 3 according to the number of positive nuclei of each picture (1: 0-30%; 2: 31-60%; 3: 61-100%) from 1 to 3 according to the number of positive nuclei of each. At least 5 pictures of each biopsy were analysed. Creatine kinase (CKs) levels associated to each patient were provided by the Spanish National Biobank Network.

Immunofluorescence

Dissected mouse gastrocnemius were mounted in OCT-corks (Optimal Cutting Temperature, Sakura Finetek, Torrance, CA), frozen in liquid-nitrogen-cooled-isopentane and immediately stored at -80ºC till ready to cut. 7 µm cryosections were performed in a Leica Cryostat (CM 1950) (Leica, Wetzlar, Germany) and sections were fixed in ice-cooled acetone. Slides were then incubated in 0,1% $NaBH_4$ PBS to block endogenous signalling and blocked to minimize unspecific IgG binding in PBS, 4%BSA, 5% Normal Donkey or Goat Serum (depending on secondary antibodies) (Jackson Immunoresearch) and 0,5% Tween 20. Detection was developed with immunofluorescent-conjugated secondary antibodies. DAPI ProLong antifade (Life Technologies) was used for nucleus counterstaining. Microscopic examination was carried out in a Nikon Eclipse E600 or in the inverted microscope Zeiss Axiovert 200, when required. Images were analysed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) for CSA analysis and for positive stained areas.

Flow cytometry analysis (FACS) of macrophage infiltrate

CTX injection was administered into gastrocnemius muscle 2 days before being euthanized. Gastrocnemius muscle was weighted and prepared for immunostaining. Briefly, muscle was minced for digestion with DMEM (Lonza), 0,05% Pronase

(Merck-Millipore) and 2 U/ml Collagenase Type I (Sigma-Aldrich) at 37ºC. When there were few or no tissue chunks, cell suspension was filtered through a 70 µm cell strainer (Thermo Fisher Scientific), centrifuged and erythrocytes were removed with Red Blood Lysis Buffer (Sigma-Aldrich). Cells were then counted and incubated in a blocking solution for Mouse Gamma Globulin (Jackson Immunoresearch) prior to staining. Cell suspension was analysed with FACS Canto equipment and FlowJo software (Tree Star, San Carlos, CA, USA). Macrophages isolation from injured muscle was performed with FACS AriaII equipment and software.

Isolation and culture of mouse satellite cells

Mouse satellite cells (SCs) were isolated according to a published procedure (Pasut et al., 2012). Briefly, muscles were digested with Collagenase Type I (Sigma-Aldrich) and Dispase II (Sigma-Aldrich). Red Blood Lysis Buffer (Sigma-Aldrich) was used to remove erythrocytes from cellular suspension according to manufacturer's instructions. Sorting was performed with FACS Aria II equipment and software. Sorted SCs were cultured in gelatin-coated dishes with Standard Growth Medium for Satellite Cells (SGMSC: DMEM, 20% FBS (Sigma-Aldrich), 10% HS (Sigma-Aldrich), 1% CEE (SeraLab, UK) and 1%P/S) allowing both proliferation and differentiation.

Generation and transplant of macrophages

Bone marrow total cells (BMTCs) were obtained from 6-8 weeks-old CL57BL/6 wild-type and *Zeb1*+/- mice (Takagi, 1998) and differentiated into macrophages as described (Gonçalves et al., 2008). Briefly, bone marrow from femur and tibia were flushed with PBS and BMTCs collected centrifuged and resuspended in DMEM supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin-streptomycin (Pen/Strep) (Lonza), medium hereafter referred as complete medium. To generate bone marrow–derived macrophages (BMDM), BMTCs were cultivated with 20 ng/ml of recombinant CSF2 (ImmunoTools GmbH, Friesoythe, Germany) during 6 days. Every 2 days, half of the medium was replaced with fresh medium supplemented with CSF2. Macrophages were then incubated with the green fluorescent cell tracer 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Sigma-Aldrich). The following day, $1x10^3$ macrophages were pelleted and resuspended in cold PBS before

being injected intramuscularly in *mdx* mouse gastrocnemius. 2 days after, mice were injected with EBD before being sacrificed and gastrocnemius muscle dissected for immunofluorescence analysis. Left untreated gastrocnemius was used as control. Muscle sections were done for the subsequent ImageJ analysis of CFSE and EBD areas.

BrdU incorporation and satellite cells immunostaining

Isolated SCs were cultured for 24h (before the first division, to assess activation) and labelled with BrdU (1.5 µg/mL, Sigma-Aldrich) for 1h. BrdU-labelled SCs were pelleted, fixed in 4% formaldehyde and resuspended in gelatin. The resulting gelatin block was embedded in paraffin and section. SCs sections were hybridized with the indicated antibodies and detected by immunostaining. Positively stained cells were quantified as the percentage of the total number of analysed cells.

Transplant of satellite cells

Isolated SCs were immediately seeded in 24 well-plates and infected with a CMV-GFP lentiviral supernatant (a kind gift from Jeronimo Blanco, CSIC, Barcelona, Spain) in the presence of 2,5 µg/mL polybrene (Sigma-Aldrich) for 16h. Medium was replaced and living cells were counted. $1.5x10^4$ cells were transplanted into CTX-injured gastrocnemius of *mdx* recipient mice. 1 month later, mice were sacrificed and gastrocnemius muscle was dissected for immunohistochemistry analysis (Ikemoto et al., 2007).

Assessment of satellite cells senescence

SA-β-galactosidase (SA-β-gal) activity was detected in SCs using the senescence β-galactosidase staining kit (Cell Signalling, Danvers, MA, USA), according to the manufacturer's instructions. SA-β-gal-positive cells were quantified from the total cells counted in at least 15 independent pictures.

Statistical analysis

GraphPad Prism software version 5.0a and GraphPad outlier calculator (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses. Quantitative data

are expressed as means ± standard error of the mean (SEM, represented as error bars). A Mann–Whitney test (non-parametric, two-sided) was used for pairwise comparisons among groups at each time point. Statistical significance was set at a $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). Spearman correlation analysis was also performed with GraphPad Prism software.

RESULTS

RESULTS

I. ZEB1 inhibits muscle differentiation *in vitro* and *in vivo*

Knockdown of *Zeb1* induces precocious expression of muscle differentiation genes

Several proteins bind and titrate MyoD, thus preventing MyoD from prematurely activating its target genes in myoblasts (Berkes et al., 2005). MyoD binding to DNA is also temporarily delayed through *cis-level* mechanisms to control the timing of muscle differentiation (Soleimani et al., 2012). In myoblasts, MyoD is excluded from G/C-centered E-boxes at the regulatory regions of muscle differentiation genes. Instead, it binds to the promoters of proliferation-associated genes. MyoD only occupies and activates these E-boxes in muscle differentiation genes as differentiation progresses (Soleimani et al., 2012). Since ZEB1 is expressed early in development and binds exclusively to G/C-centered E-boxes, we questioned whether its knockdown in myoblasts would allow MyoD to bind these E-boxes and trigger a precocious expression of muscle differentiation genes.

To test this hypothesis, we used the C2C12 cell myogenic conversion model (Blau et al., 1983; Bergstrom et al., 2002; Blais et al., 2005). When cultured in GM, C2C12 cells maintain a proliferating myoblast-like phenotype, and despite expression of MyoD, levels of proteins like the intermediate differentiation gene Myogenin or the terminal differentiation markers myosin heavy chain (MHC) and troponin remain low or absent. Only when C2C12 cells exit the cell cycle upon reaching confluence and/or are switched into a DM do they fuse and terminally differentiate to form multinucleated MHC-positive myotubes.

We found that knockdown of endogenous ZEB1 in C2C12 cells resulted in precocious expression of differentiation markers. Upon switching to DM, Myogenin protein became detectable earlier in C2C12 cells knocked down for *Zeb1* with a specific siRNA against *Zeb1* (si1ZEB1) than in cells transfected with a siRNA control (siCtl) (Figure 13A). Upregulation of MHC also occurred earlier and reached higher levels in C2C12 cells knocked down for *Zeb1* with si1ZEB1 than in siCtl cells (Figure

13B). A similar premature expression of MHC occurred when using two additional and independent siRNAs against *Zeb1* (si2ZEB1 and si3ZEB1) (Figure 13C and D).

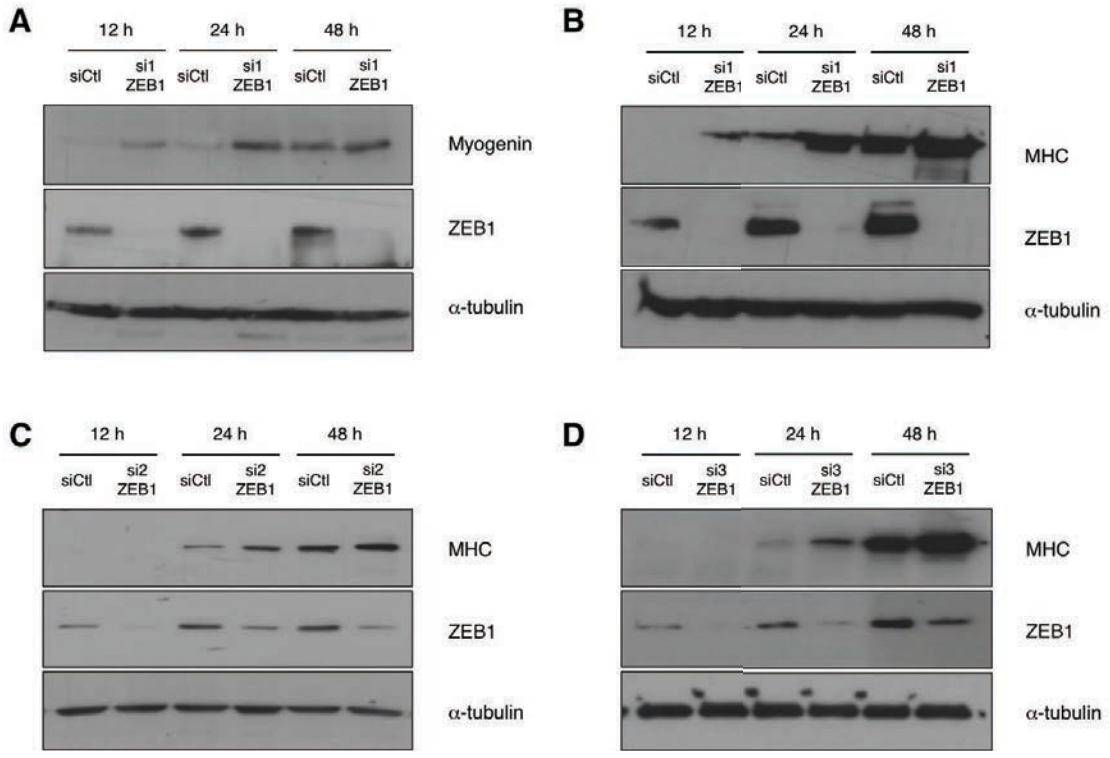


Figure 13. Knockdown of *Zeb1* induces earlier protein expression of muscle differentiation genes. C2C12 cells, transfected with specific siRNAs against *Zeb1* (si1ZEB1, si2ZEB1, or si3ZEB1) or a siRNA control (siCtl), were allowed to differentiate for up to 48 h after being switched to differentiation medium. At the indicated time points, cells were lysed and assessed by Western blotting for ZEB1 and either Myogenin **(A)** or pan-MHC **(B to D)**.

The earlier induction of muscle genes following *Zeb1* knockdown (Figure 14G) was also examined at the mRNA level. Consistent with the above-described results, mRNA for MHC isoforms IIa (*Myh2*) and IIb (*Myh4*) accumulated more rapidly and to higher levels in *Zeb1* knockdown C2C12 cells than in the counterpart siCtl control cells (Figure 14A and B). In addition, mRNA expression levels of the differentiation genes for troponin (*Tnnt1*) and myogenin (*Myog*) and the determination genes for *Myod1* and *Myf5* also displayed an earlier induction profile in *Zeb1*-knocked-down cells than in controls (Figure 14C to F). Together, these results indicate that *Zeb1* knockdown triggers premature induction of muscle differentiation-associated genes and suggest that endogenous ZEB1 may impose a delay on the ability of MRFs to drive muscle differentiation.

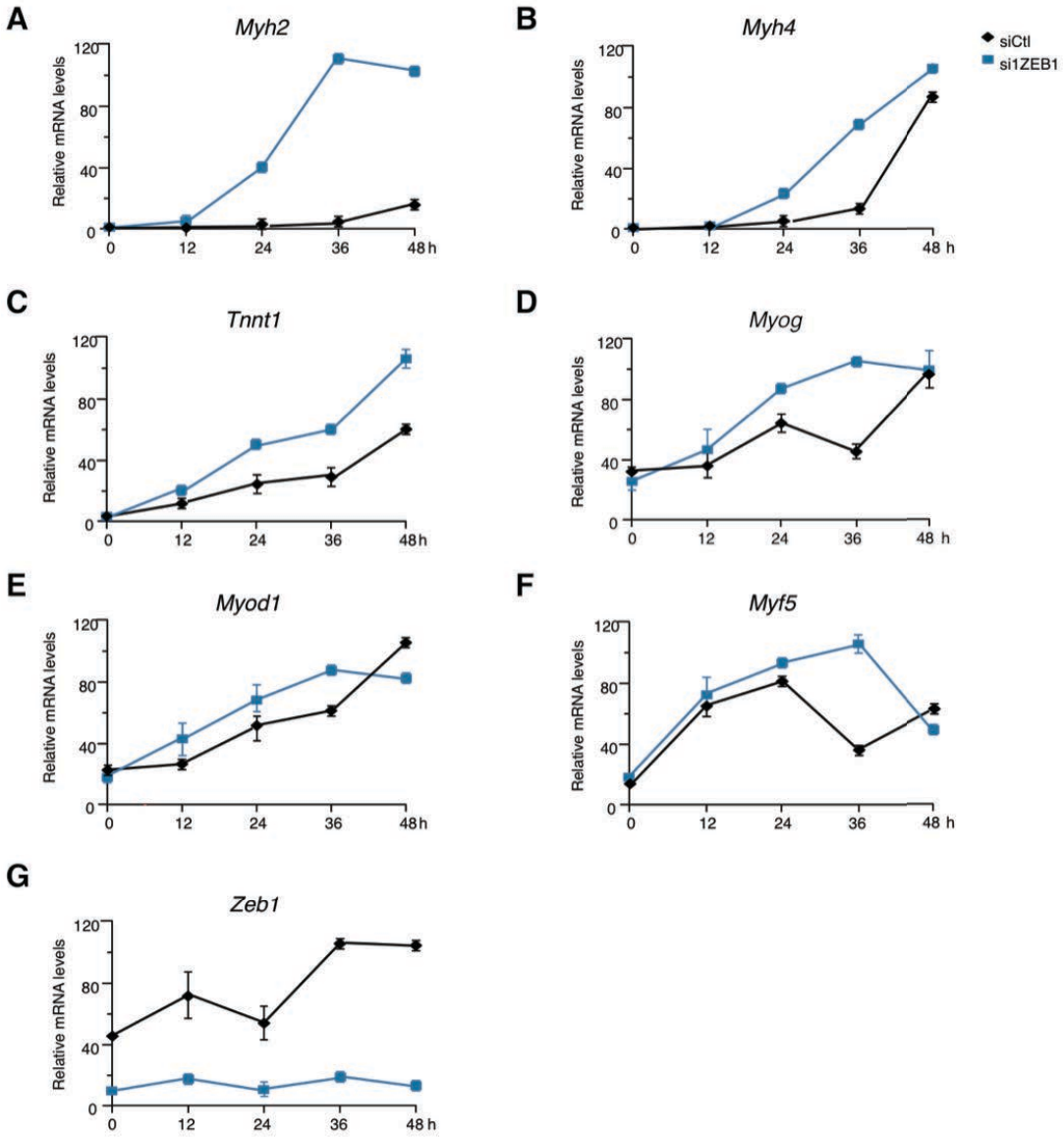


Figure 14. *Zeb1* knockdown induces precocious mRNA expression of several muscle genes. C2C12 cells knocked down for *Zeb1* or transfected with a siCtl were allowed to differentiate for up to 48 h after being switched to differentiation medium. At the indicated time points, mRNA levels for MHC type IIa (*Myh2*) **(A)**, MHC type IIb (*Myh4*) **(B)**, *Tnnt1* **(C)**, *Myog* **(D)**, *Myod1* **(E)**, *Myf5* **(F)**, and *Zeb1* **(G)** were determined by qRT-PCR relative to levels of *Gapdh* as a reference gene. Data shown are a representative case of four independent experiments.

Zeb1 knockdown accelerates myotube formation

Next, we examined whether the induction of these muscle differentiation genes in C2C12 cells upon *Zeb1* knockdown translated into their earlier terminal differentiation into multinucleated myotubes. Compared to control cells, the formation of myotubes in cells knocked down for *Zeb1* was significantly accelerated,

as evidenced morphologically and by quantification of the number of nuclei in MHC-positive cells with respect to the total number of nuclei (Figure 15A to D). At later time points, the difference in the number of nuclei in MHC-positive cells between both experimental conditions was reduced. However, myotube size (number of nuclei per myotube) was still considerably larger in *Zeb1* knockdown cells than in control counterparts (Figure 15C displays the number of MHC-positive myotubes with more than 4 nuclei). Likewise, although by 72 h, the number of MHC-positive myotubes with more than 4 nuclei was similar under both conditions (Figure 15C), *Zeb1* knockdown cells still had more nuclei per myotube than control cells (e.g., more myotubes in siZEB1 than in siCtl exceeded 6 nuclei) (Figure 15A).

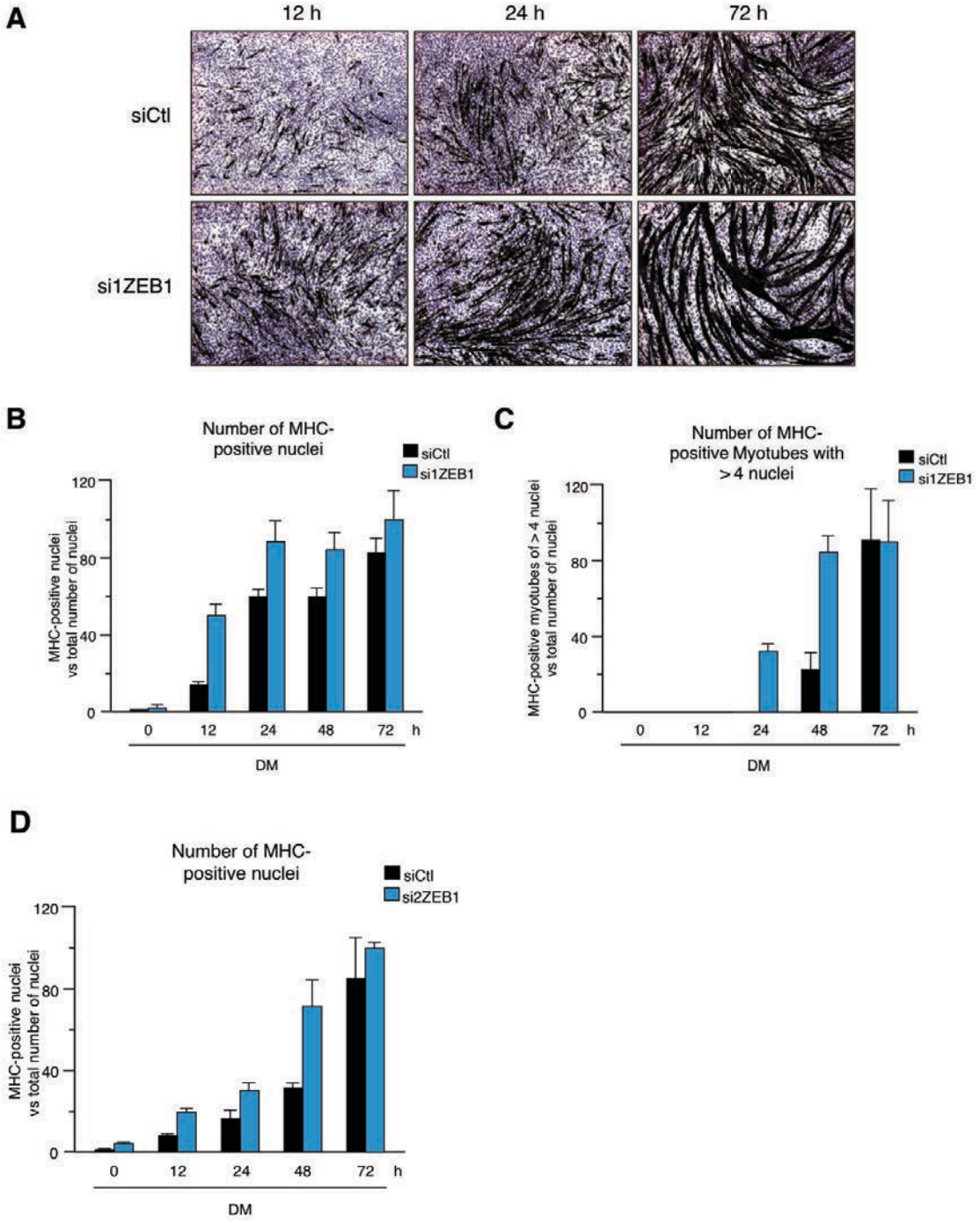


Figure 15. *Zeb1* knockdown accelerates myotube conversion. (A) C2C12 cells transfected with si1ZEB1 or siCtl were allowed to differentiate. At the indicated time points, cells were fixed and immunostained for MHC. Nuclei were counterstained with haematoxylin. Magnification 4x. Captures shown are representative of five independent experiments. **(B)** Quantification of the number of nuclei in MHC-positive cells with respect to the total number of nuclei in the myogenic conversion experiments shown in panel A. **(C)** Quantification of the number of MHC-positive myotubes with more than 4 nuclei with respect to the total number of nuclei. **(D)** Same as panel B but with si2ZEB1. Counts in panels B to D are the averages of four representative fields.

Of note, *Zeb1* knockdown also induced accelerated myotube formation in C2C12 cells that were continuously maintained in GM, even after they reached confluence (Figure 16A and B). This suggests that *Zeb1* knockdown can trigger differentiation once cells have undergone contact-inhibition-mediated cell cycle exit. Together, these results indicate that under normal conditions, endogenous ZEB1 delays myoblast-to-myotube conversion, while *Zeb1* knockdown is sufficient to activate (by relieving repression) a gene signature associated with terminal muscle differentiation.

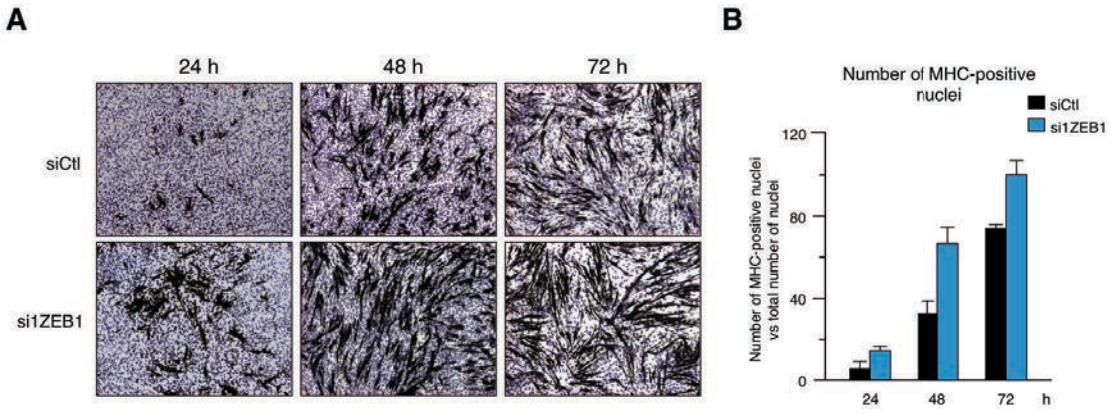


Figure 16. ***Zeb1* knockdown accelerates myotube conversion. (A)** Knockdown of *Zeb1* also accelerates myotube formation in C2C12 cells maintained in GM. C2C12 cells were transfected with siRNAs against ZEB1 or the siRNA control, but upon reaching confluence, cells were maintained for the rest of the experiment in GM. Time zero refers to the time point when cells reached confluence. Magnification 4x. **(B)** Quantification of the number of nuclei in MHC-positive cells with respect to the total number of nuclei in myogenic conversion assays where cells were maintained in GM for the entire experiment, as described above in figure 15.

Stage-dependent differential binding of ZEB1 and MyoD to muscle differentiation genes

For most of its known target genes, ZEB1 inhibits their expression by binding to G/C-centered E-boxes at their regulatory regions and actively repressing transcription (Genetta et al., 1994; Sekido et al., 1994; Postigo et al., 1997a; Postigo et al., 1999b; Brabletz & Brabletz, 2010; Sanchez-Tilló et al., 2011b; Sánchez-Tilló et al., 2012). We therefore questioned whether the observed changes in muscle gene expression upon *Zeb1* knockdown involve binding of ZEB1 to G/C-centered E-boxes in muscle differentiation genes. As MyoD binds G/C-centered E-boxes at muscle differentiation genes in myotubes but not in myoblasts (Soleimani et al., 2012), we also wondered whether ZEB1 could be occupying these E-boxes at the myoblast stage.

The regulatory regions of muscle terminal differentiation genes are particularly enriched for G/C-centered E-boxes (Fong et al., 2012; Soleimani et al., 2012). We therefore decided to test the ability of ZEB1 and MyoD to bind to these E-boxes in the promoters of mouse *Myh4* and *Tnnt1* through chromatin immunoprecipitation (ChIP) assays of C2C12 myoblasts and myotubes (Figure 17). In myoblasts, antibodies against ZEB1, but not its respective control IgG, immunoprecipitated regions of the *Myh4* and *Tnnt1* promoters containing G/C-centered E-boxes (Figure 17). Interestingly, MyoD did not bind to the same region of either promoter in myoblasts. Conversely, when these experiments were performed with DNA from myotubes, the reverse was observed: MyoD, but not ZEB1, was found to bind to G/C-centered E-box-containing regions in both promoters (Figure 17). Lastly, both anti-ZEB1 and anti-MyoD antibodies failed to immunoprecipitate a fragment of the mouse *Gapdh* promoter lacking G/C-centered E-boxes (Figure 17). These results indicate that, in line with our original hypothesis and in an opposite pattern from MyoD, endogenous ZEB1 binds directly to muscle differentiation gene promoters in myoblasts but not in myotubes.

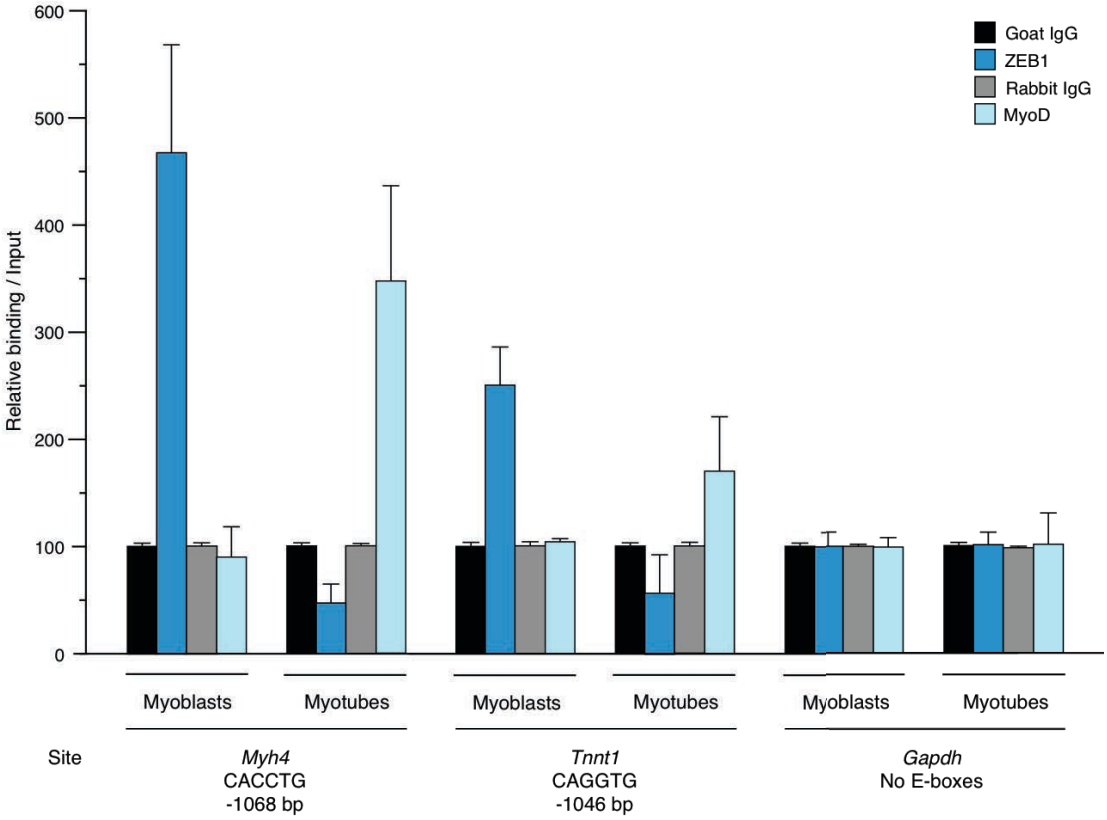


Figure 17. ZEB1 binds to G/C-centered E-boxes in muscle differentiation genes. ZEB1 and MyoD display differential binding to G/C-centered E-boxes in muscle differentiation genes in myoblasts and myotubes. Shown

are data for qRT-PCR of fragments of the mouse *Myh4*, *Tnnt1* and *Gapdh* promoters immunoprecipitated in ChIP assays from C2C12 myoblasts and myotubes with ZEB1 antibody, MyoD antibody or their respective IgG controls. Values represent binding relative to the input averaged from two experiments, each performed in triplicate, and are representative of at least three essays.

Inhibition of muscle differentiation by ZEB1 at the myoblast stage involves CtBP-mediated transcriptional repression

Next, we explored whether negative regulation of muscle differentiation genes by ZEB1 occurs via transcriptional repression. C2C12 cells were co-transfected at the myoblast or myotube stage with either siCtl or specific siRNAs against *Zeb1* and luciferase reporters containing the promoter regions of selected muscle genes. Knockdown of *Zeb1* in non-confluent cycling C2C12 myoblasts upregulated the basal transcriptional activity—that is, relieving ZEB1-mediated repression—of the *Myh4*, *Tnnt1*, muscle creatine kinase (*Ckm*), and *Myod1* gene promoters, the latter to a much lesser extent (Figure 18A to D). Interestingly, when the experiment was carried out with C2C12 cells that had been maintained in DM and differentiated into myotubes, interference of *Zeb1* had no or a very limited effect (Figure 18A to D).

ZEB1 represses transcription through recruitment of different corepressors, some still undetermined, that act in a promoter- and tissue-specific manner (Postigo et al., 1999b; Sánchez-Tilló et al., 2011b). The ZEB1 corepressor CtBP mediates repression of targets of the myogenic MEF2 factor via recruitment of histone deacetylase complex HDAC9/MITR (Zhang et al., 2001). We used a specific siRNA against *Ctbp* (siCtBP) (Figure 19A, left) to investigate a potential contribution of CtBP to ZEB1-mediated transcriptional inhibition of muscle genes. We found that *Ctbp* knockdown in C2C12 myoblasts upregulated, albeit to different degrees, the basal activity of *Myh4*, *Ckm*, and *Myod1* promoters (Figures 18A, C and D). For these three promoters, the effect of concomitant knockdown of *Ctbp* and *Zeb1* on C2C12 myoblasts was similar to the effect of *Zeb1* (or *Ctbp*) single knockdown (Figures 18A, C and D), suggesting that CtBP is the main cofactor in the repression of these muscle genes by ZEB1 at the myoblast stage. Interestingly, relief of repression of the *Tnnt1* promoter by *Ctbp* knockdown in C2C12 myoblasts was below the extent of upregulation following knockdown of *Zeb1* (Figure 18B), indicating that ZEB1-mediated regulation of this gene also involves (and with a greater contribution) other

corepressors (also see below). In contrast, as occurred for *Zeb1* knockdown, interference of *Ctbp* in myotubes had no or only limited effects on the transcription of all four promoters (Figures 18A to D).

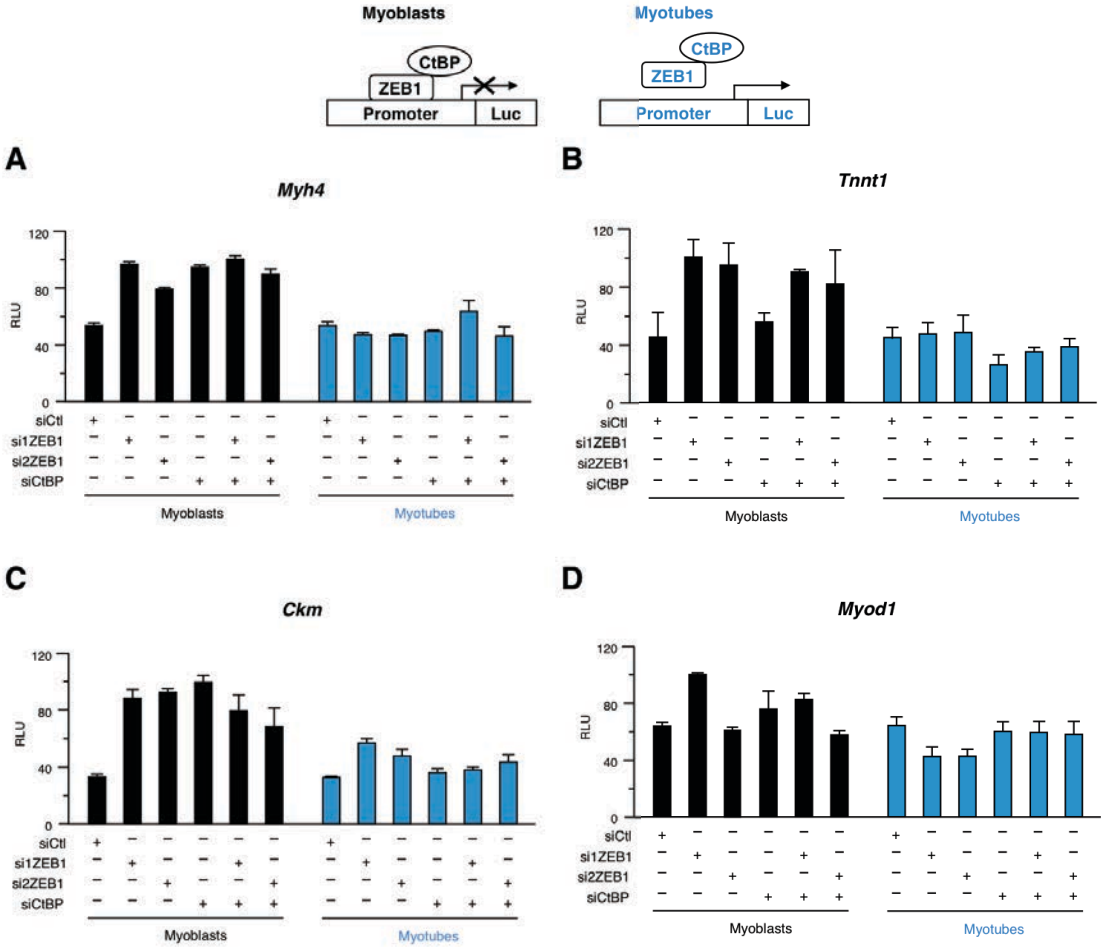


Figure 18. **ZEB1 represses muscle genes largely through a CtBP-dependent mechanism. (A)** Non-confluent cycling C2C12 myoblasts or confluent myotubes allowed to terminally differentiate for 48 h were cotransfected with siCtl, si1ZEB1, si2ZEB1 or *Ctbp* (siCtBP) or equal molar amounts of their different combinations along with luciferase reporter for the *Myh4* promoter. The knockdown efficiency of siCtBP in C2C12 cells is shown in panel in Figure 19A, left. **(B)** Same as panel A but with the *Tnnt1* promoter as a luciferase reporter. **(C)** Same as panel A but with the *Ckm* promoter as a luciferase reporter. **(D)** Same as panel A but the *Myod1* promoter.

Active transcriptional repression of muscle genes by ZEB1 and its dependence on CtBP were then confirmed in C3H-10T1/2 fibroblasts, a well-established cell-based model to study transcriptional regulation by MRFs (Davis et al., 1987; Magli et al., 2010). Constitutively, C3H-10T1/2 fibroblasts do not express muscle genes, but their transfection with *Myod1* is sufficient to induce the formation of myotubes, although C3H-10T1/2 cells never reached the extent of myogenic conversion

observed for C2C12 cells (Davis et al., 1987; Weintraub et al., 1989). C3H-10T1/2 cells were co-transfected with *Myod1* and luciferase reporters for the four promoters described above, and the expression of *Zeb1* was modulated by its overexpression or knockdown. We found that *Zeb1* knockdown in C3H-10T1/2 cells (Figure 19A, middle) upregulated the transcription of all four promoters (Figure 19B), while overexpression of *Zeb1* inhibited their activity (Figure 19C).

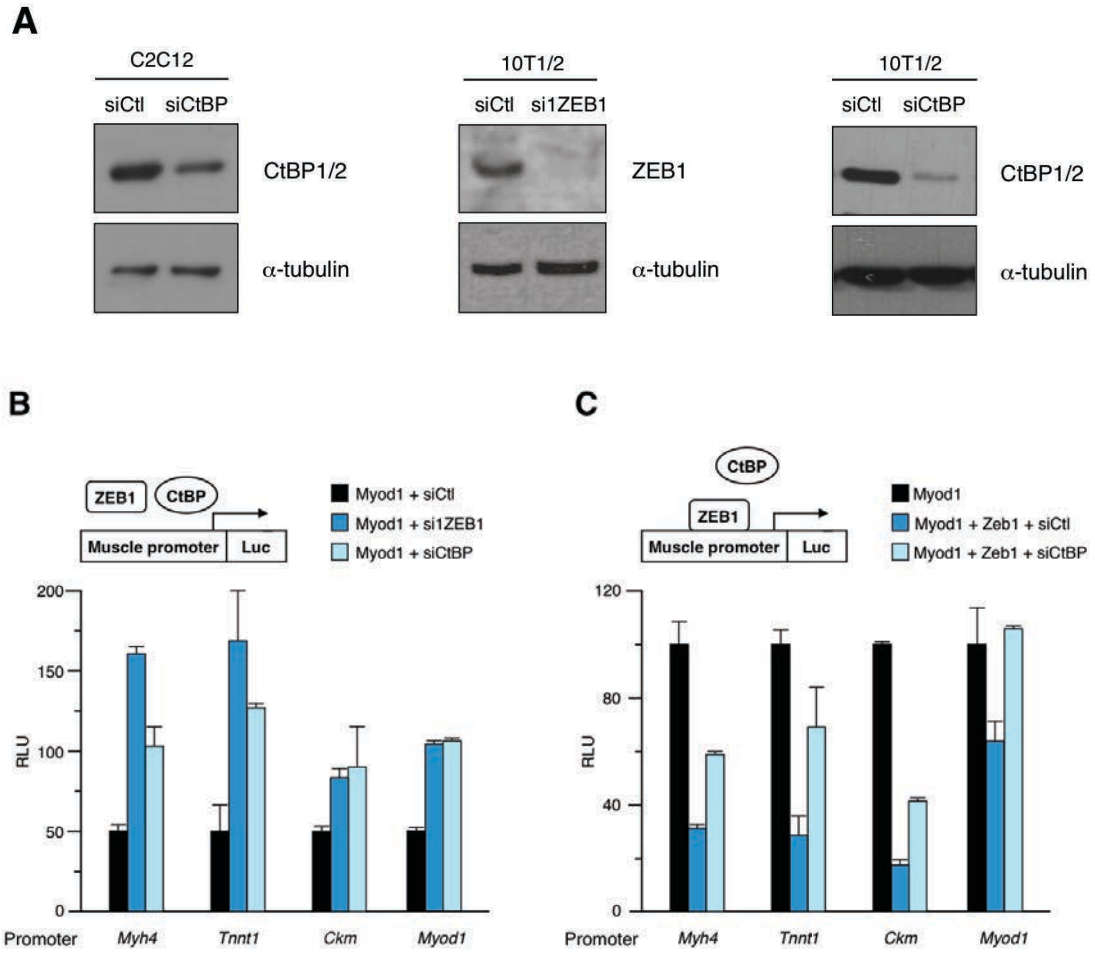


Figure 19. **ZEB1 represses muscle genes largely through a CtBP-dependent mechanism. (A)** Knockdown efficiency of siRNAs against *Ctbp* and *Zeb1* in C2C12 and C3H-10T1/2 cells assessed by Western blotting. (*In the left*) C2C12 cells were transfected with either siCtl or siCtBP. (*Middle*) C3H-10T1/2 cells were transfected with either siCtl or si1ZEB1. (*Right*) Same as the left panel but with C3H-10T1/2 cells. **(B)** C3H-10T1/2 fibroblasts were cotransfected with luciferase reporters for muscle gene promoters, as in Figure 18, plus either siCtl or specific siRNAs against *Zeb1* or *Ctbp1/2*. **(C)** C3H-10T1/2 fibroblasts were cotransfected with luciferase reporters for muscle gene promoters, an expression vector for *Myod1* and combinations of either an expression vector for *Zeb1* or equal molar amounts of its corresponding empty expression vectors plus either siCtl or siCtBP.

Knockdown of *Ctbp* in C3H-10T1/2 cells (Figure 19A, right) augmented the basal activity of these four promoters, including that of troponin (Figure 19B). The relief of repression obtained with siCtBP was similar to or below that observed with interference of *Zeb1* alone. Likewise, elimination of CtBP partially reversed the repressor effect of ZEB1 on all four promoters albeit to different levels (Figure 19C). The differential degree of dependence on CtBP for the basal endogenous repression of the troponin promoter by ZEB1 in C2C12 and C3H-10T1/2 cells supports the above-mentioned evidence that the identity of ZEB1 corepressors varies in a promoter- and tissue-specific manner (Sánchez-Tilló et al., 2011b). Altogether these results with cell-based systems indicate that CtBP mediates a significant share of ZEB1-mediated repression of muscle gene expression.

Dependence of ZEB1 on CtBP for the regulation of muscle gene expression was next examined *in vivo* in *Xenopus* and *Drosophila* embryos. *Xenopus* embryos were microinjected with MyoD and either wild-type ZEB1 ($Zeb1_{wt}$) or a version of ZEB1 where its CtBP-interacting domain (CID) had been mutated ($Zeb1_{CIDmut}$). At blastula stages, ectodermal explants were isolated from these embryos, and the effect of both ZEB1 variants on MyoD-mediated induction of muscle gene expression was examined (Figure 20). ZEB1 inhibited mRNA expression of muscle actin (*act3*), *ckm*, slow skeletal troponin I type 1 (*tnni1*), fast skeletal troponin I type 2 (*tnni2*), tropomyosin 1 α chain (*tpm1*), and *myod1*. Mutation of the CID region in *Zeb1* partially alleviated—and to different degrees—repression of all the genes examined except for *tnni1*, where ZEB1 inhibition seemed to take place independently of CtBP (Figure 20). These results demonstrate that repression of muscle differentiation genes by ZEB1, both in cell-based systems and *in vivo*, occurs to a large extent in a CtBP-dependent manner.

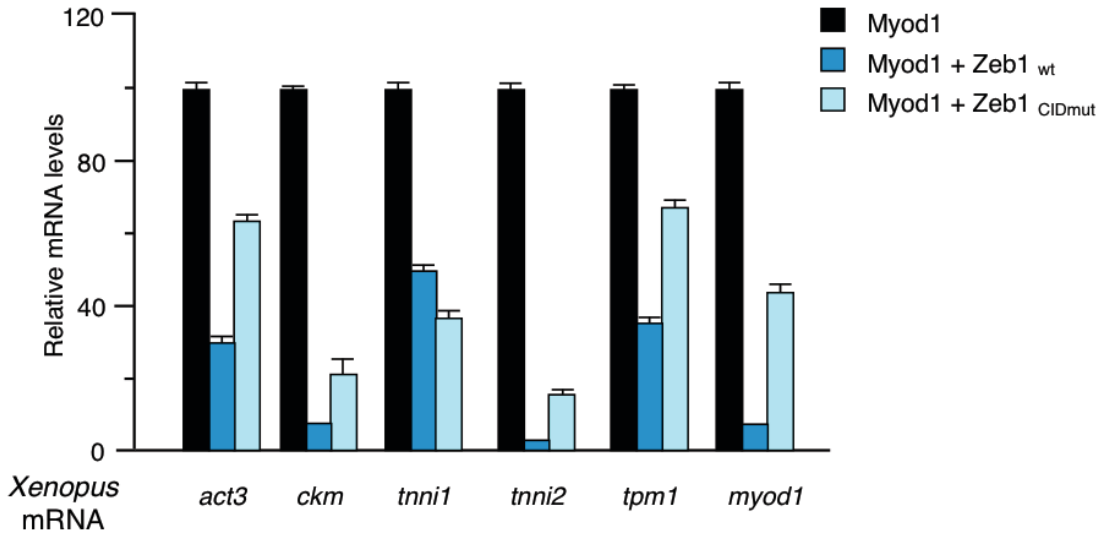


Figure 20. ***In vivo*** **regulation of muscle gene expression by ZEB1 depends on CtBP.** Differential requirement for CtBP in ZEB1-mediated repression of muscle genes in *Xenopus* ectodermal explants. *Xenopus* embryos microinjected with mRNA encoding *Myod1* and either $Zeb1_{wt}$ or $Zeb1_{CIDmut}$ were used to generate ectodermal explants, and qRT-PCR was used to examine MyoD-induced expression of muscle markers. Data shown are a representative case of three independent experiments.

The ZEB1 ortholog in *Drosophila*, zfh-1, is also a transcriptional repressor that binds to G/C-centered E-boxes and interacts with CtBP (Lai et al., 1993; Postigo et al., 1999a; Postigo et al., 1999c). Overexpression of zfh-1 in *Drosophila* embryos under the control of the heat shock protein 70 promoter results in a muscle phenotype that includes, but is not limited to, a downregulation of *Mhc* and *Mef2* expression in the somatic musculature (Postigo et al., 1999c). As in any overexpression experiment, these results cannot rule out that overexpression of *zfh-1* is titrating out other proteins, including CtBP. On the other hand, loss of *zfh-1* does not trigger accelerated myogenic differentiation but rather a complex phenotype with simultaneous loss, gain and mispositioning of muscle precursors (Lai et al., 1993), thus suggesting that the role of zfh-1 during *Drosophila* myogenesis is not fully conserved in vertebrate ZEB1. Despite these caveats, we decided to examine whether the muscle phenotype observed upon *zfh-1* overexpression was dependent on CtBP. *zfh-1* is normally downregulated after gastrulation in most mesodermal derivatives, including muscle (Lai et al., 1991). We used a Gal4-UAS system to maintain the expression of wild-type zfh-1 (UAS-*zfh-1*) or a zfh-1 version unable to interact with CtBP (UAS-$zfh\text{-}1_{CIDmut}$) in the developing muscle under the control of the 24B-Gal4 or MEF2-Gal4 mesoderm-specific driver lines (Figure 21A for 24B-Gal4 and 21B for MEF2-Gal4).

As described previously (Postigo et al., 1999c), maintaining *zfh-1* expression yielded alterations in somatic muscle development and Mhc and Mef2 expression that are more complex than just a block in muscle differentiation (Figure 21A for 24B-Gal4 and 21B for MEF2-Gal4). Nevertheless, the muscle phenotype observed was dependent on CtBP, as no apparent effect on the embryos crossed with UAS-*zfh-1*$_{CIDmut}$ was observed (Figure 21A for 24B-Gal4 and 21B for MEF2-Gal4) even though the zfh-1$_{CIDmut}$ protein is stably expressed (Leatherman et al., 2008). Collectively, these results demonstrate that repression of muscle differentiation genes by ZEB1 (and likely zfh-1), both in cell-based systems and in *in vivo* models, occurs to a large extent in a CtBP-dependent manner.

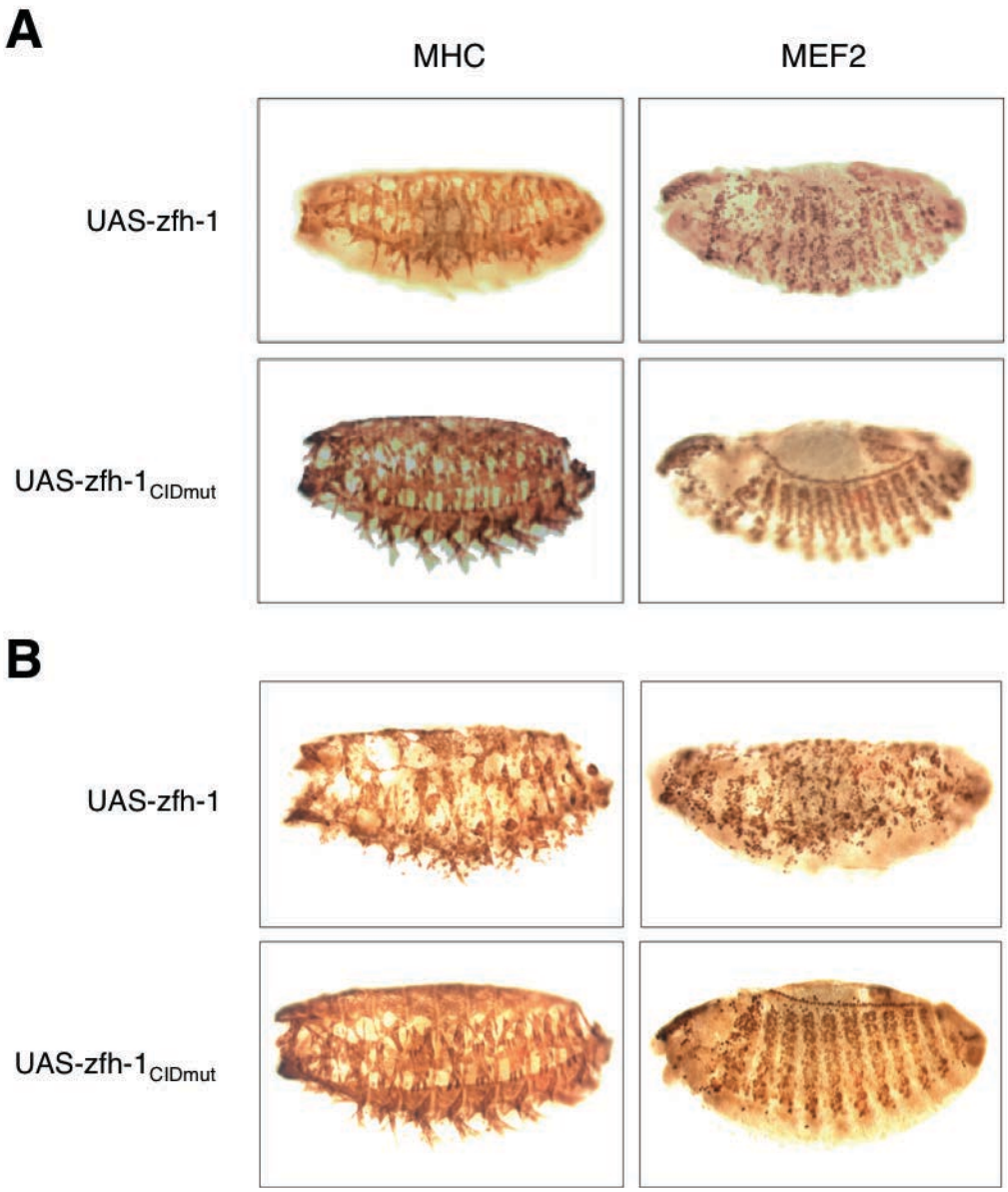


Figure 21. *In vivo* regulation of muscle gene expression by zfh-1 depends on CtBP. Muscle phenotypes of *Drosophila* embryos expressing two copies of UAS-*zfh-1* or UAS-*zfh-1*$_{CIDmut}$ under the control of the 24B-Gal4 **(A)** or the MEF2-Gal4 **(B)** mesodermal gene as a driver. Embryos were fixed and stained for MEF2 (stage 14) and MHC (stage 16).

Loss of *Zeb1* induces muscle differentiation gene expression *in vivo*

We next sought to confirm whether the induction of muscle genes upon *zeb1* knockdown observed by cell-based approaches was recapitulated *in vivo.* To this end, we examined the effect of overexpressing and knocking down *zeb1* during *Xenopus* embryonic development. *Zeb1* mRNA was co-injected along with β-galactosidase mRNA as a lineage tracer into one of two blastomeres at the 2-cell stage. This

introduced the injected mRNA into one bilateral half of the embryo, marked in pink (by staining for the Red-Gal β-galactosidase substrate) and oriented to the right in Figure 22. The contralateral half of the embryo served as a control. Embryos were then raised to neurula stages, and *in situ* hybridization was used to examine the expression of collagen type II (*col2a1*), a marker of differentiated mesoderm, and muscle actin (*act3*), a muscle marker (Figure 22, purple stains). We found that overexpression of ZEB1 strongly repressed the expression of both markers (Figure 22). Conversely, *zeb1* knockdown by microinjection of a specific morpholino oligonucleotide (zeb1 MO) expanded the expression domains of both markers (Figure 22). These results parallel our findings in cell-based systems and support a role for ZEB1 in restraining the expression of muscle differentiation genes *in vivo.*

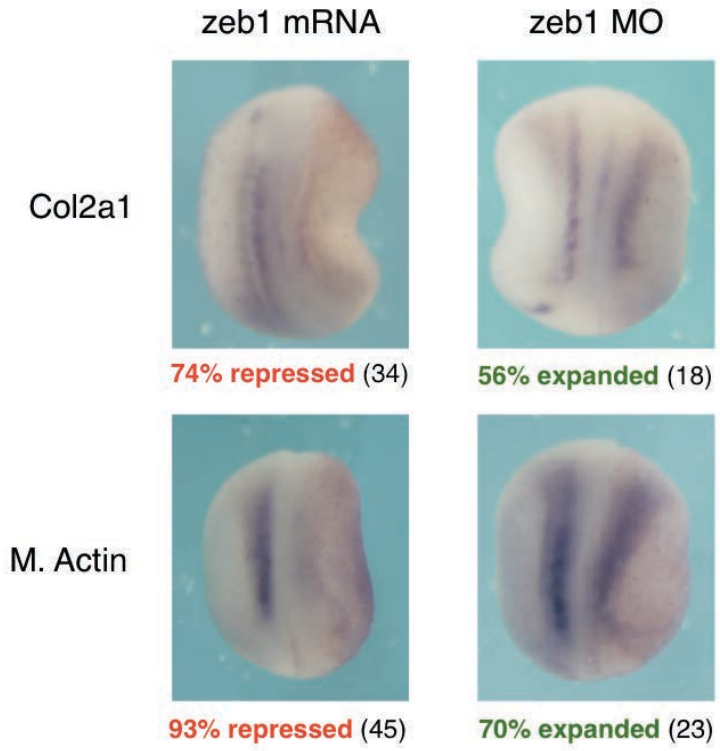


Figure 22. ZEB1 represses muscle gene expression *in vivo*. ZEB1 regulates muscle gene expression in *Xenopus* embryos. *In situ* hybridization for col2a1 and muscle actin (M. Actin) was performed in *Xenopus* embryos injected with mRNA for zeb1 (*zeb1* mRNA) or a specific morpholino oligonucleotide against zeb1 (*zeb1* MO), along with mRNA encoding β-galactosidase to mark the injected side (oriented rightward). Percentages of embryos with each phenotype are indicated, with the number of embryos analysed shown in parentheses. The uninjected contralateral half of the embryo serves as an internal control (oriented leftward).

Mice carrying a homozygous targeted deletion of *Zeb1* die close to birth with cleft palate and a number of bone and cartilage abnormalities (Takagi et al., 1998). Although at E10 to E11 levels of MyoD, Myogenin, and Ckm are not significantly

affected in these mice (Takagi et al., 1998), a molecular analysis of muscle differentiation at later stages has not been carried out. From the results shown up to here, one might expect that the temporal pattern of expression of muscle differentiation gene expression would be accelerated in *Zeb1-/-* mouse embryos.

Of all adult MHC isoforms, IIb/MYH4 is induced not only the earliest (around E14.5) but also at much higher levels than the rest, while mRNA for isoform IIa/MYH2 starts to be detected only after E17.5 (Lu et al., 1999b). We decided to examine by immunohistochemistry the expression of both adult MHC isoforms during late development in sibling mouse embryos that were either wild-type or null for *Zeb1*. We found that by E18.5, expression of MYH4 was only slightly higher in *Zeb1-/-* embryos than in the normal *Zeb1*+/+ sibling counterparts (Figure 23A). Meanwhile, MYH2, which was barely detectable in *Zeb1*+/+ mouse embryos, displayed earlier (higher) expression in the *Zeb1-/-* sibling counterpart embryos (Figure 23B). These results, consistent with the cell-based and *in vivo* data described above, indicate that loss of *Zeb1* results in an acceleration of the temporal pattern of the muscle differentiation gene signature.

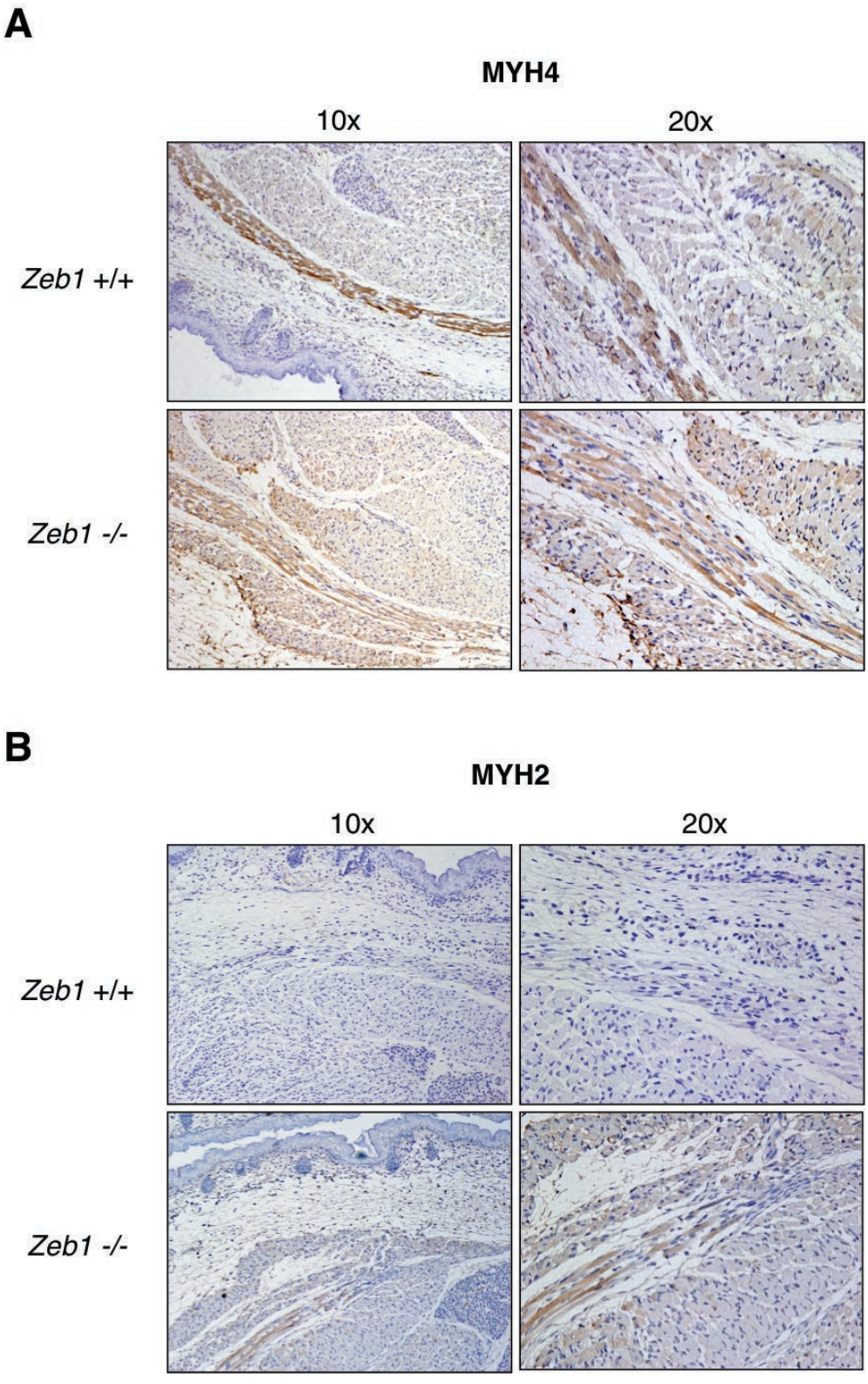


Figure 23. **ZEB1 represses MYH2 expression in the developing mouse embryo.** Muscle differentiation gene expression is accelerated in embryos from mice with targeted deletion of *Zeb1*. **(A)** Shown is immunohistochemistry for MHC IIb (MYH4) in the developing muscle of *Zeb1*+/+ and *Zeb1*-/- E18.5 sibling mouse embryos. Magnifications of 20x and 10x are shown. **(B)** Same as panel B but with immunostaining for MHC isoform IIa (MYH2).

Altogether, the above data set ZEB1 as an important regulator of muscle differentiation in a temporal-specific manner. MRFs drive skeletal myogenesis but the precise temporal and spatial pattern of muscle development involves a wide range of myogenic regulators. Experiments in cell line-based systems and *in vivo* models revealed that ZEB1 imposes a temporary delay in muscle gene expression and differentiation. In contrast to MyoD, ZEB1 binds to G/C-centered E-boxes in muscle differentiation genes at the myoblast stage but not in myotubes.

These results demonstrate a repressor activity of muscle differentiation exerted by ZEB1 during embryonic development. Next, we questioned whether ZEB1 participates in adult muscle normal homeostasis and disease, and focused in muscle regeneration after injury. Adult muscle regeneration is believed to recapitulate the differentiation programme seen during skeletal muscle development. We first analysed ZEB1 in biopsies of several muscle damage models to evaluate its expression in damaged and regenerating myofibers.

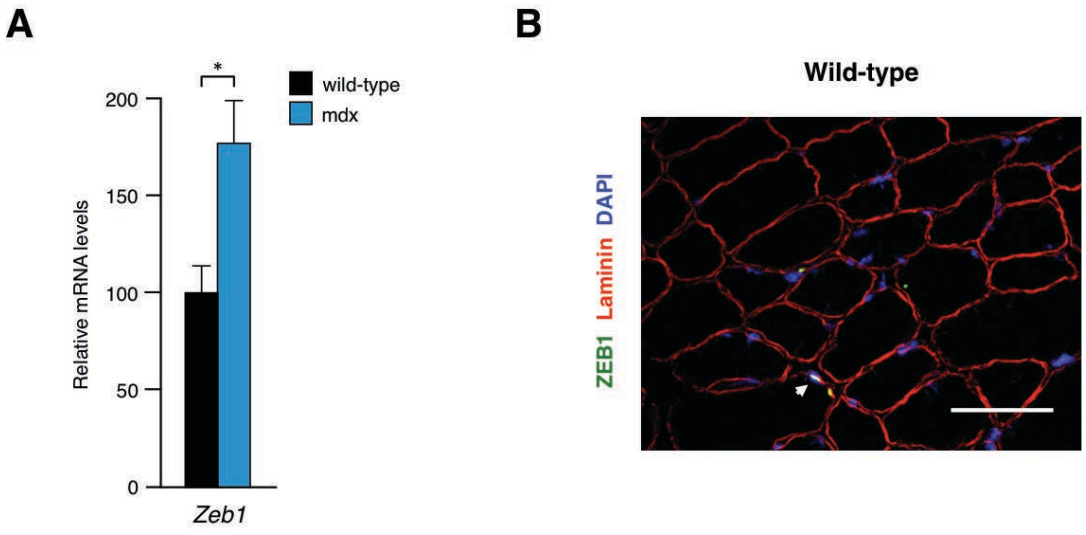
→ The results showed in this chapter have been published in:

Siles, L., et al. (2013). ZEB1 imposes a temporary stage-dependent inhibition of muscle gene expression and differentiation via CtBP-mediated transcriptional repression. *Molecular and cellular biology*, 33(7), 1368-1382.

II. ZEB1 protects muscle from damage

ZEB1 is upregulated and ectopically expressed in undamaged fibers of dystrophic muscles

To investigate a potential role for ZEB1 in normal and dystrophic adult muscle, we first examined its expression in the gastrocnemius muscle of wild-type and *mdx* mice and found that *Zeb1* mRNA levels were upregulated in the latter (Figure 24A). Muscle samples were then immunostained for ZEB1 along with laminin to assess fiber integrity. In wild-type muscle, ZEB1 was restricted to a subset of peripheral nuclei (white arrow in Figure 24B). In contrast, in the *mdx* muscle, within areas with signs of damage ZEB1 was found not only in peripheral nuclei but, surprisingly, also in the cytoplasm of some myofibers. (Figure 24C). Compared to human dystrophic muscles, muscles in *mdx* young mice retain stronger regenerative capacity and display numerous central nuclei, a marker of regeneration (Sacco et al., 2010). Interestingly, many of these central nuclei were positive for ZEB1 (yellow arrowheads in Figure 24C).



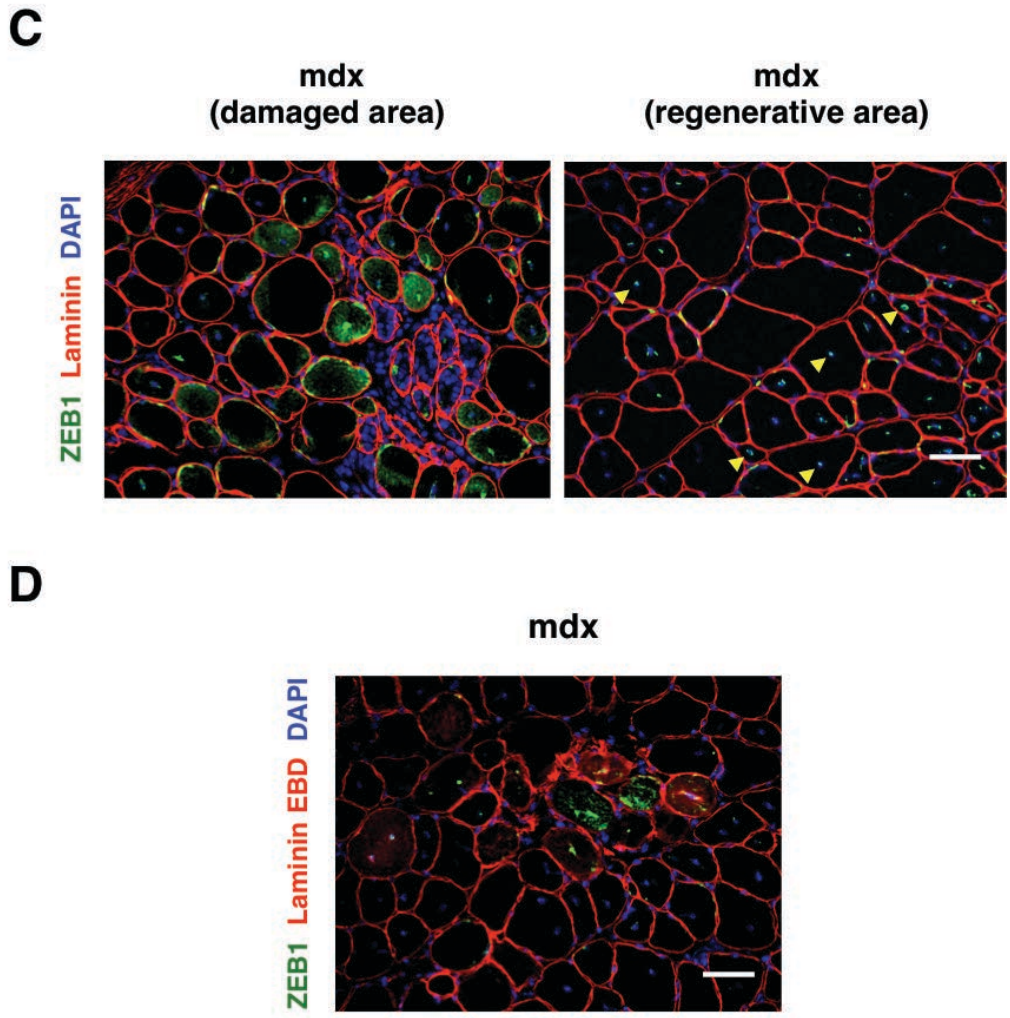


Figure 24. ZEB1 is upregulated and ectopically expressed in undamaged fibers of dystrophic muscles. (A) Expression of *Zeb1* mRNA in the gastrocnemius muscle of 2-month old wild-type and *mdx* mice. Data shown is representative of at least 5 mice for each genotype. **(B)** Gastrocnemius muscle was assessed for ZEB1 and laminin expression along with DAPI counterstaining. In wild-type muscles, ZEB1 is restricted to some peripheral nuclei (white arrowh). **(C)** As in B but in dystrophic *mdx* muscle. ZEB1 is also in the cytoplasm of some fibers within areas with signs of degeneration and in central nuclei of fibers with signs of regeneration (yellow arrowheads). **(D)** As in B but before being sacrificed mice were injected i.p. with EBD. Scale bar in all pictures represents 50 µm.

ZEB1 expression was also examined in mice that, before being sacrificed, had been injected intraperitoneally (i.p.) with Evans blue dye (EBD), a red fluorescent dye that incorporates into damaged myofibers *in vivo* (Matsuda et al., 1995). Notably, cytoplasmic expression of ZEB1 was related to undamaged myofibers that failed to capture EBD (Figure 24D). These results indicate that ZEB1 is upregulated in dystrophic muscle where its ectopic cytoplasmic expression occurs only in non-damaged fibers.

We next sought to test these results in normal and dystrophic human muscle. As in wild-type mice, ZEB1 was expressed in a subset of peripheral nuclei in healthy human muscle (arrowheads in Figure 25) and, like in *mdx* mice, in samples from a series of DMD, BMD and DM1 muscle dystrophies, ZEB1 was found not only in some peripheral nuclei (arrowheads, Figure 25) but also in the cytoplasm of some fibers. ZEB1 was also expressed in the few central nuclei existing in human dystrophic muscles (arrows in Figure 25).

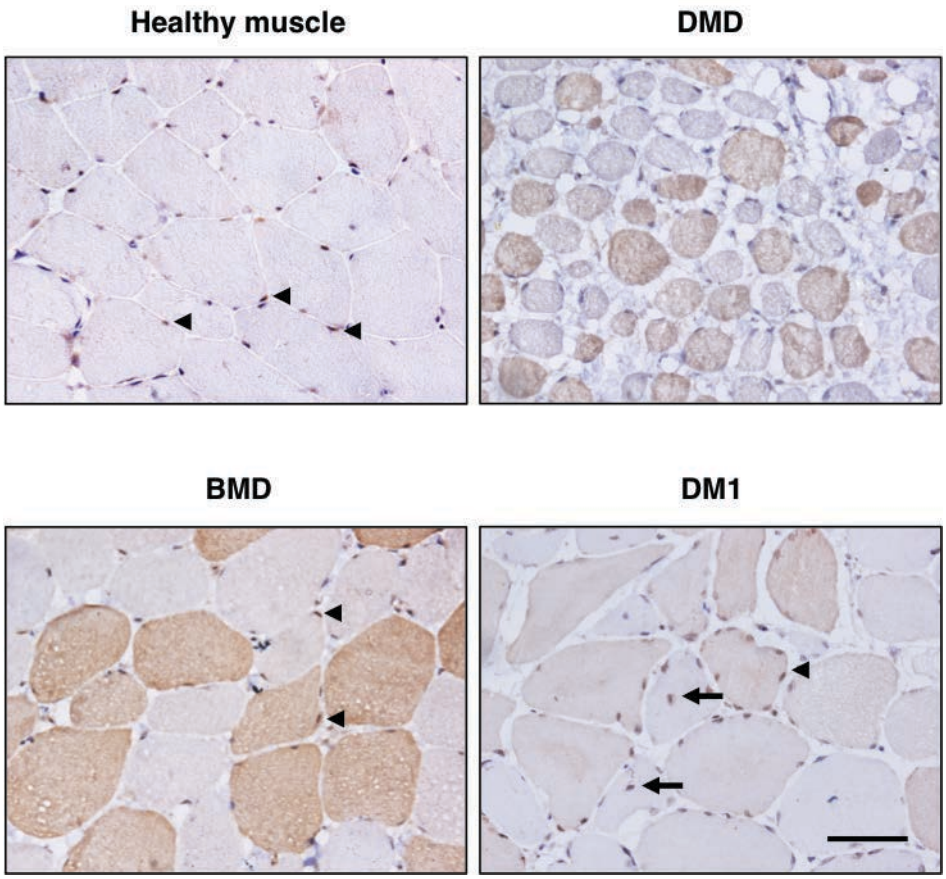


Figure 25. ZEB1 is expressed in human dystrophic muscle. ZEB1 expression in healthy and dystrophic human muscles. Arrowheads indicate ZEB1 positive peripheral nuclei and arrows centrally-located ones. Scale bar represents 50 μm.

We examined whether creatine kinase levels (CK) in our series of human muscle dystrophies associated to the cellular distribution of ZEB1. Interestingly, CK levels maintained a strong negative correlation with cytoplasmic ZEB1 expression (Spearman's ρ: -0.80) (Figure 26A). The relative number of fibers displaying

cytoplasmic ZEB1 expression was also higher among those patients with lower CKs (Figure 26B). Together with the results in EBD-treated *mdx* mice, these data indicate that cytoplasmic ZEB1 expression in dystrophic muscle associates to undamaged fibers.

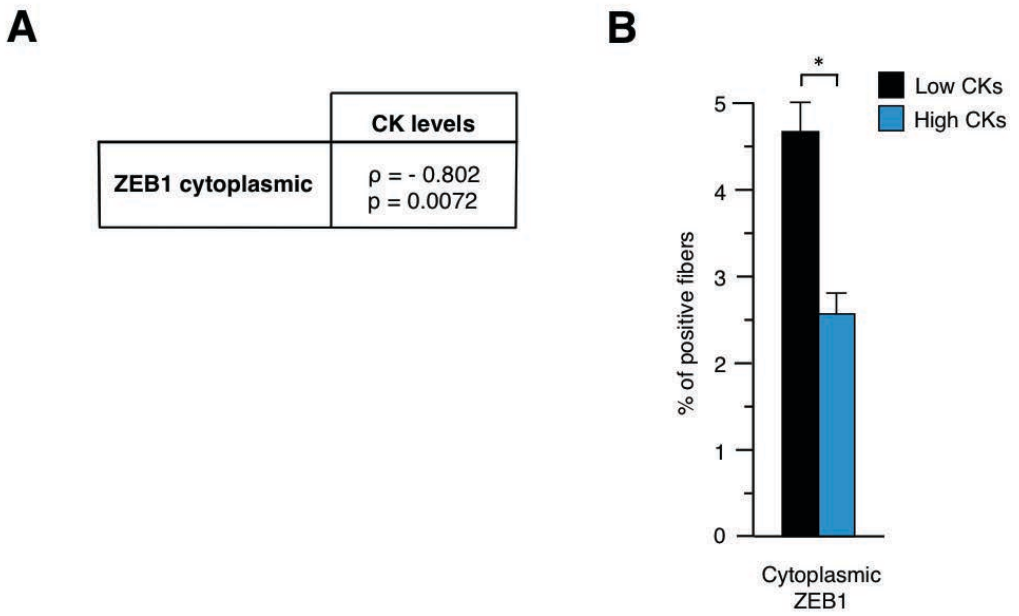


Figure 26. CK levels in human muscle dystrophies are positively correlated with nuclear ZEB1 and negatively correlated with cytoplasmic ZEB1. (A) Spearman correlation analysis of human dystrophies according to ZEB1 nuclear and cytoplasmic expression. **(B)** The score representing the relative number of fibers displaying cytoplasmic ZEB1 is higher among human muscle dystrophy patients with lower CKs.

ZEB1 protects dystrophic muscles from damage

Zeb1-/- mice die around birth (Takagi et al., 1998). Nevertheless, partial downregulation of *Zeb1* blocks ZEB1 functions that depend on a fine threshold of its expression. Thus, deletion of one *Zeb1* allele in cancer cells is sufficient to block tumour progression in *Zeb1*+/- mice (Liu et al., 2014). The gastrocnemius muscle in *Zeb1*+/- mice —which expresses about half of *Zeb1* mRNA levels than in wild-type mice (Figure 27A)— displayed overall macroscopic and histological structure (Figure 27B).

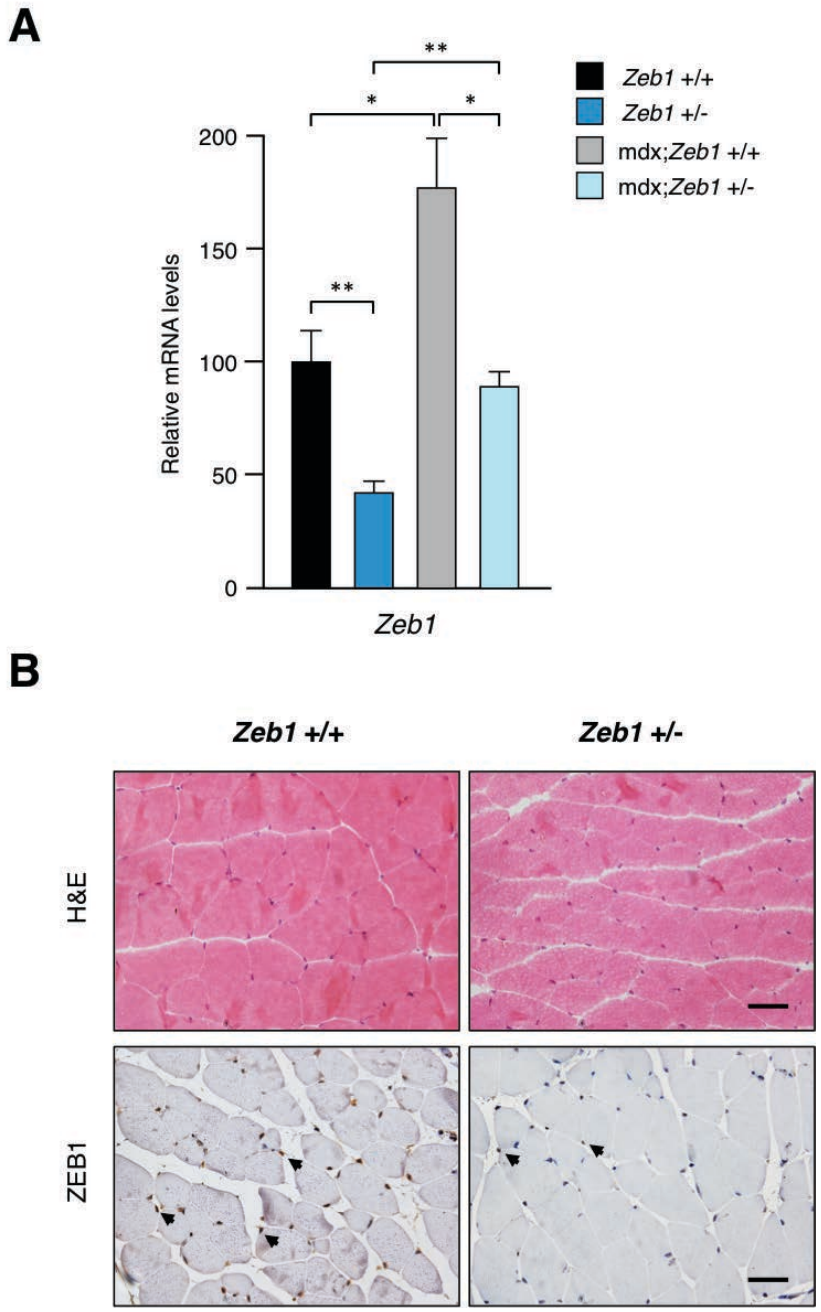


Figure 27. Healthy *Zeb1*+/- gastrocnemius display normal histological structure. (A) Relative *Zeb1* mRNA levels in the gastrocnemius muscle of wild-type and *mdx* mice (*Zeb1*+/+, *Zeb1*+/-, *mdx*;*Zeb1*+/+ and *mdx*;*Zeb1*+/-) was determined by qRT-PCR with respect to *Gapdh*. n=6. **(B)** Immunohistochemical characterization of *Zeb1*+/+ and *Zeb1*+/- gastrocnemius muscle by H&E and ZEB1 staining. Arrowheads indicate ZEB1 positive nucleus. Scale bar represents 50 µm.

Under basal condition, *Zeb1*+/- mice have similar gastrocnemius weight compared to *Zeb1*+/+ counterparts (Figure 28A). However, cross-sectional area (CSA) analysis revealed that fibers in the *Zeb1*+/- gastrocnemius muscle have a larger average size with fewer smaller size fibers and more larger size ones than in wild-type

mice (Figures 28B to D). Of note, dystrophic muscles display greater level of fiber size variability than healthy muscles with smaller-size fibers being more resistant to necrosis (Karpati et al., 1988). Therefore, the myofiber hypertrophia in *Zeb1*-deficient muscles can potentially make them more susceptible to muscle injury.

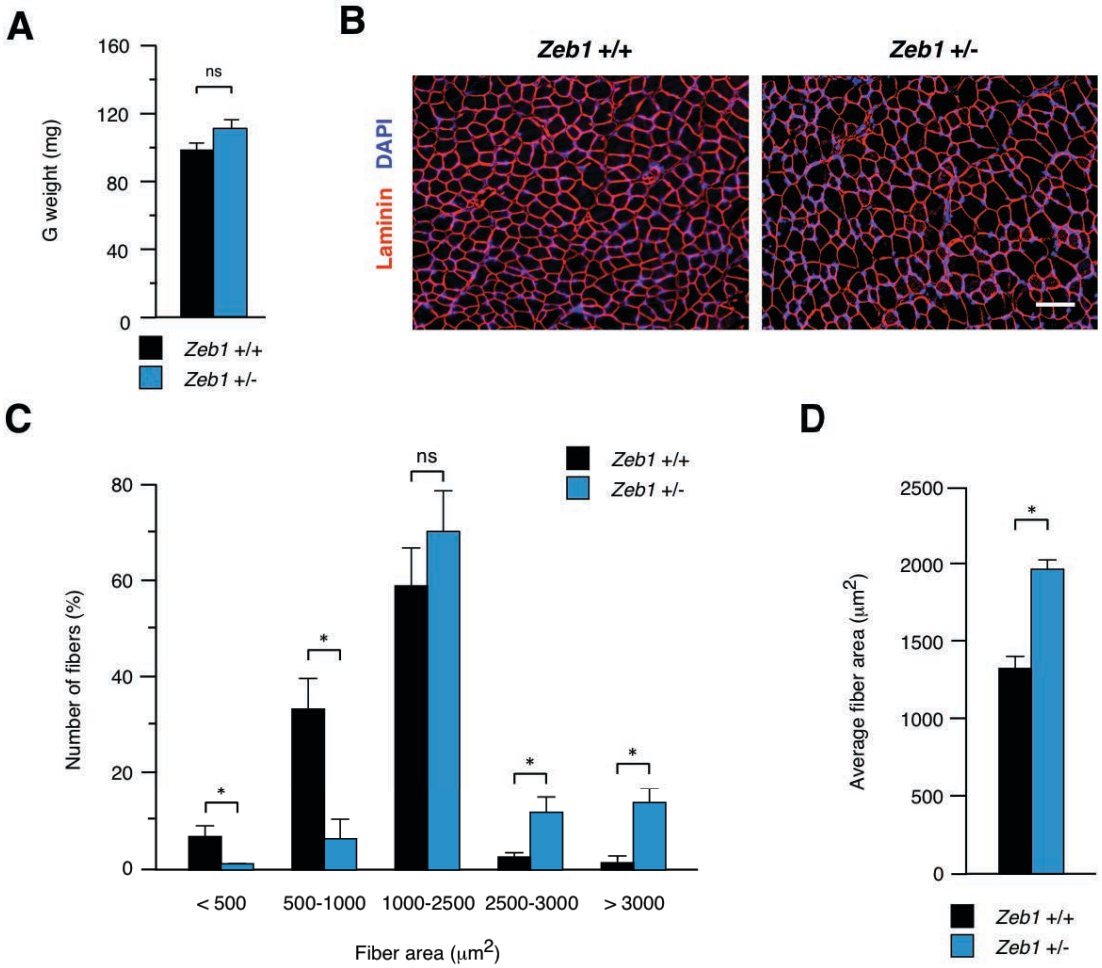


Figure 28. Untreated *Zeb1*+/- gastrocnemius has normal muscle structure but increased CSA than *Zeb1*+/+. **(A)** Gastrocnemius weight of uninjured *Zeb1*+/+ and *Zeb1*+/- mice. n=6. **(B)** Representative images of laminin staining of *Zeb1*+/+ and *Zeb1*+/- muscle. Scale bar represents 100 μm. **(C)** CSA analysis of *Zeb1*+/+ and *Zeb1*+/- muscle. n=4. **(D)** Mean fiber diameter of *Zeb1*+/+ and *Zeb1*+/- gastrocnemius muscle calculated from CSA analysis. n=4.

The data above suggest that ZEB1 may have a protective role in muscle damage. To test this hypothesis, *Zeb1* expression in the *mdx* mouse was downregulated by crossing it with *Zeb1*+/- mice to generate *mdx*;*Zeb1*+/- mice (Figure 27A), whose phenotype was then examined at young (2-month-old) and older (10-15-month-old) age. At 2-moths of age, total body and gastrocnemius

weight in *mdx;Zeb1*+/- mice were reduced compared to that of *mdx* mice with full levels of *Zeb1* [hereafter referred as *mdx;Zeb1*+/+] (Figures 29A and 29B) as also did the total body weight. In contrast, at 10-15-month age, *mdx;Zeb1*+/- mice were heavier, which might be related with persistence of inflammation in their muscles (see below, Figure 32).

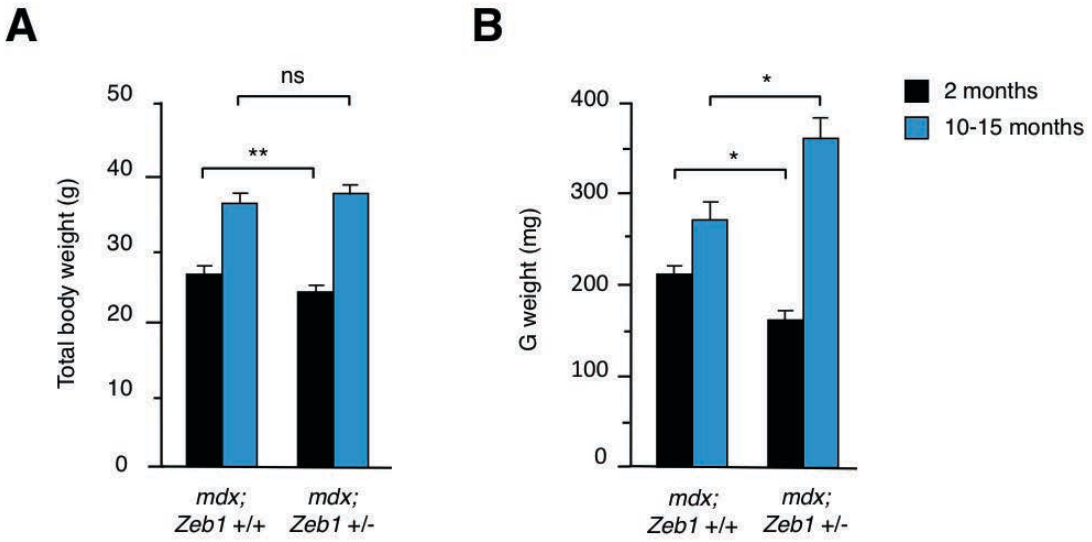


Figure 29. **Total body and gastrocnemius weight of *mdx;Zeb1*+/+ and *mdx;Zeb1*+/- mice. (A)** Total body weight of *mdx;Zeb1*+/+ and *mdx;Zeb1*+/- mice at 2 and 10-15-months-old. n=6. **(B)** Gastrocnemius weight of *mdx;Zeb1*+/+ and *mdx;Zeb1*+/- mice at 2 and 10-15-months-old. n=6.

Muscles in mice from both genotypes exhibited fiber size variability and areas of degeneration, inflammation and regeneration but in 2-months-old *mdx;Zeb1*+/- muscle there was a higher degree of fiber abnormalities (Figure 30A). In addition, fiber damage and inflammatory infiltration was more extensive in *mdx;Zeb1*+/- mice at both ages analysed (Figure 30B).

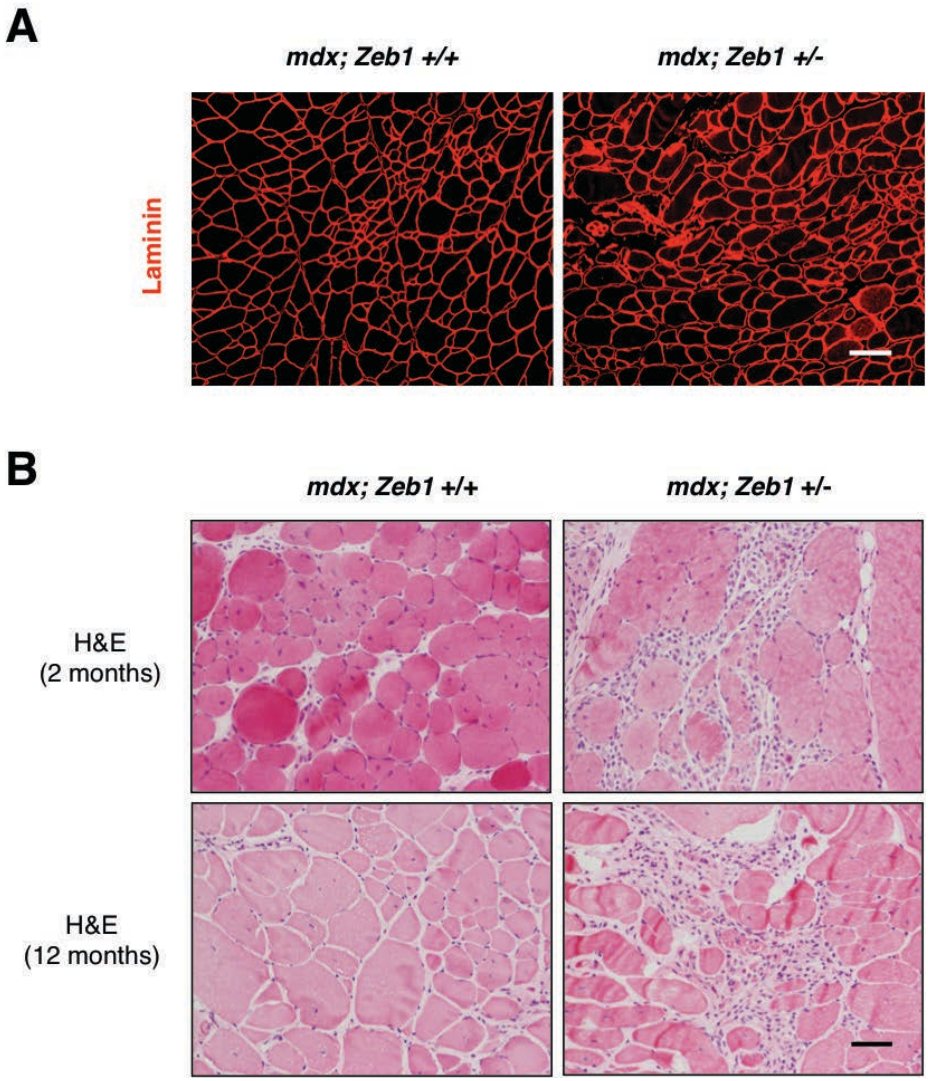


Figure 30. Gastrocnemius immunohistochemical analysis of *mdx;Zeb1*+/+ and *mdx;Zeb1*+/- mice. (A) Laminin staining of 2-months-old *mdx;Zeb1*+/+ and *mdx;Zeb1*+/- mice. Scale bar represents 100 µm. **(B)** Gastrocnemius structure was analysed by H&E staining. Scale bar represents 50 µm.

Ectopic cytoplasmic expression of ZEB1 was lower in *mdx;Zeb1*+/- muscles than in *mdx;Zeb1*+/+ counterparts and cytoplasmic ZEB1^{+} fibers correspond to non-infiltrated ones (Figure 31), further supporting our results above that ZEB1 cytoplasmic expression associates to undamaged fibers.

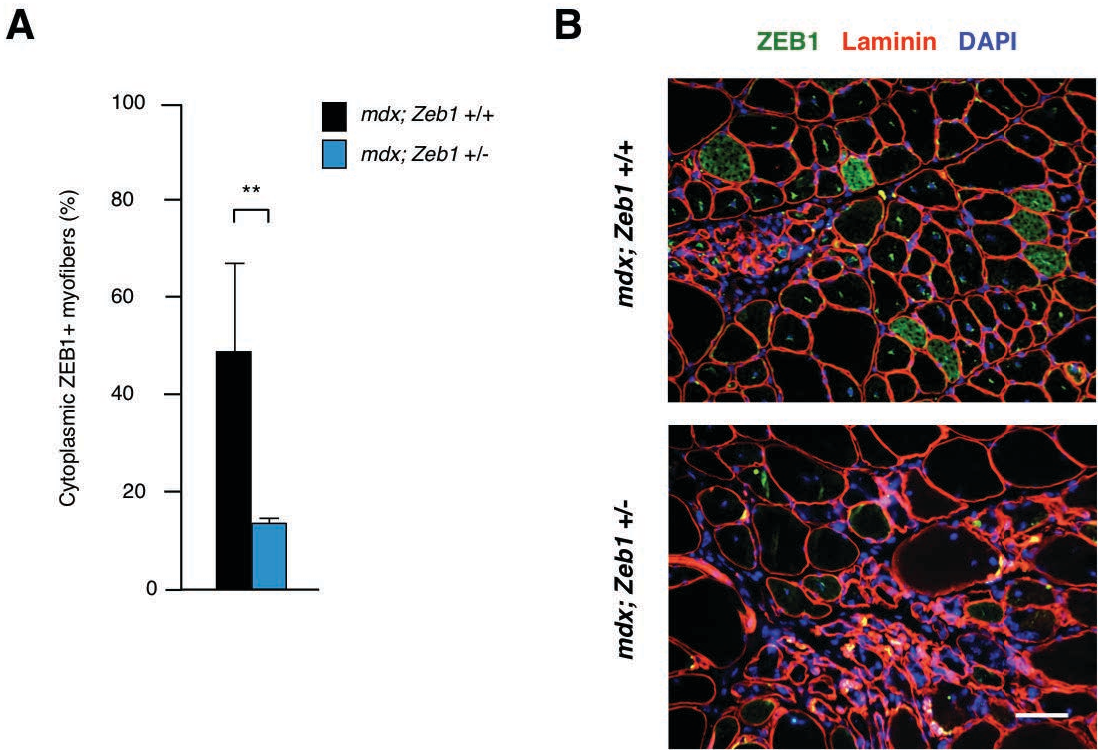


Figure 31. ZEB1 is expressed in the cytoplasm of *mdx* gastrocnemius muscle. (A) The percentage of myofibers expressing cytoplasmic ZEB1 is lower in *mdx*;*Zeb1*+/- muscles than in *mdx*;*Zeb1*+/+ counterparts. **(B)** As in (A) but the cellular distribution of ZEB1 was assessed by immunostaining. Scale bar represents 50 µm.

Lastly, EBD staining corroborated that the damaged area in the *mdx;Zeb1*+/- gastrocnemius, from both young and older mice, more than doubled that in *mdx;Zeb1*+/+ counterparts (Figure 32). These data indicate that expression of full levels of *Zeb1* has a protective role in the dystrophic muscle and that its downregulation in *mdx* mice resulted in lower number of fibers with cytoplasmic ZEB1, more inflammatory infiltration and greater fiber damage.

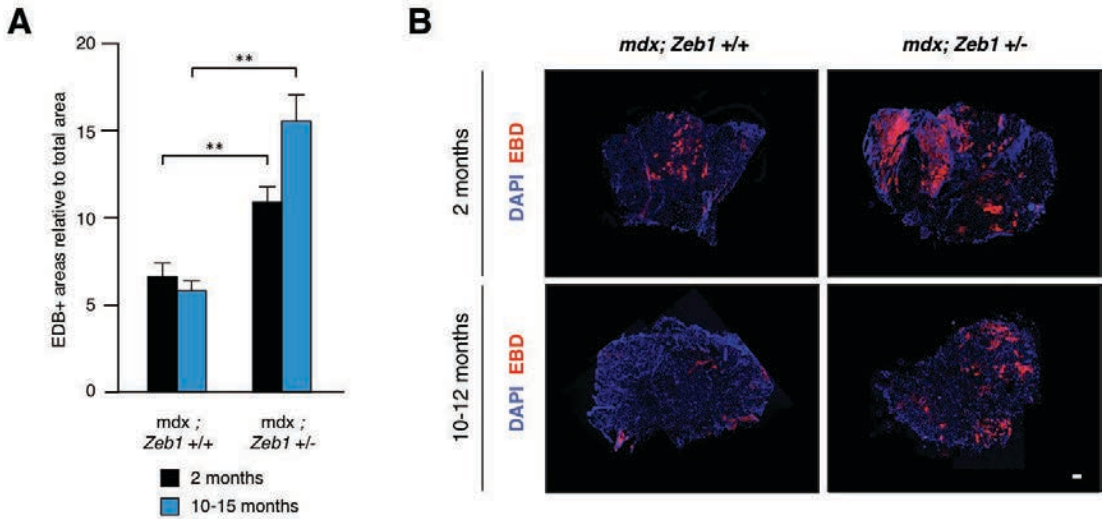


Figure 32. ***mdx;Zeb1+/-* gastrocnemius exhibits increased muscle damage. (A)** Quantification of EBD^{+} areas relative to total area of the cut for each animal. n=5. **(B)** Immunohistochemical analysis of gastrocnemius muscle with EBD staining. Scale bar represents 200 µm.

ZEB1 protects muscle against acute injury

To further investigate the protective role of ZEB1 in muscle damage, we evaluated the response to intramuscular injection of the snake venom cardiotoxin (CTX), a well-established model of acute muscle injury (Garry et al., 2016). CTX induces local necrosis with inflammatory infiltration, mostly by macrophages, which is eventually followed by muscle regeneration.

The gastrocnemius muscle of *Zeb1*+/+ and *Zeb1*+/- mice was injected with CTX and subsequent muscle injury and regeneration were assessed at different time points (Figure 33). During the first two days following CTX injection muscles from both genotypes display local necrosis accompanied by abundant inflammatory infiltrate, processes were more intense and extensive in *Zeb1*+/- muscles. By day 4 post-injection, gastrocnemius muscle started to show signs of regeneration but necrosis and inflammation continued to be higher in *Zeb1*-deficient mice. By day 7 post-injection, wild-type muscles had an almost normal histological architecture with most of the inflammation already resolved and numerous centrally nucleated myofibers indicative of regeneration. In contrast, muscles in *Zeb1*-deficient mice still

showed zones of necrosis, inflammatory infiltrate and areas of abnormal regeneration with myofibers of different sizes. Lastly, at 14 days post-injection, while wild-type muscles were totally reconstituted, muscles in *Zeb1*+/- mice still showed scattered regions of necrosis and infiltrate along with regenerated areas. These results indicate that, *Zeb1*+/- muscles undergo enhanced damage with extensive tissue degeneration and higher inflammation than *Zeb1*+/+ ones.

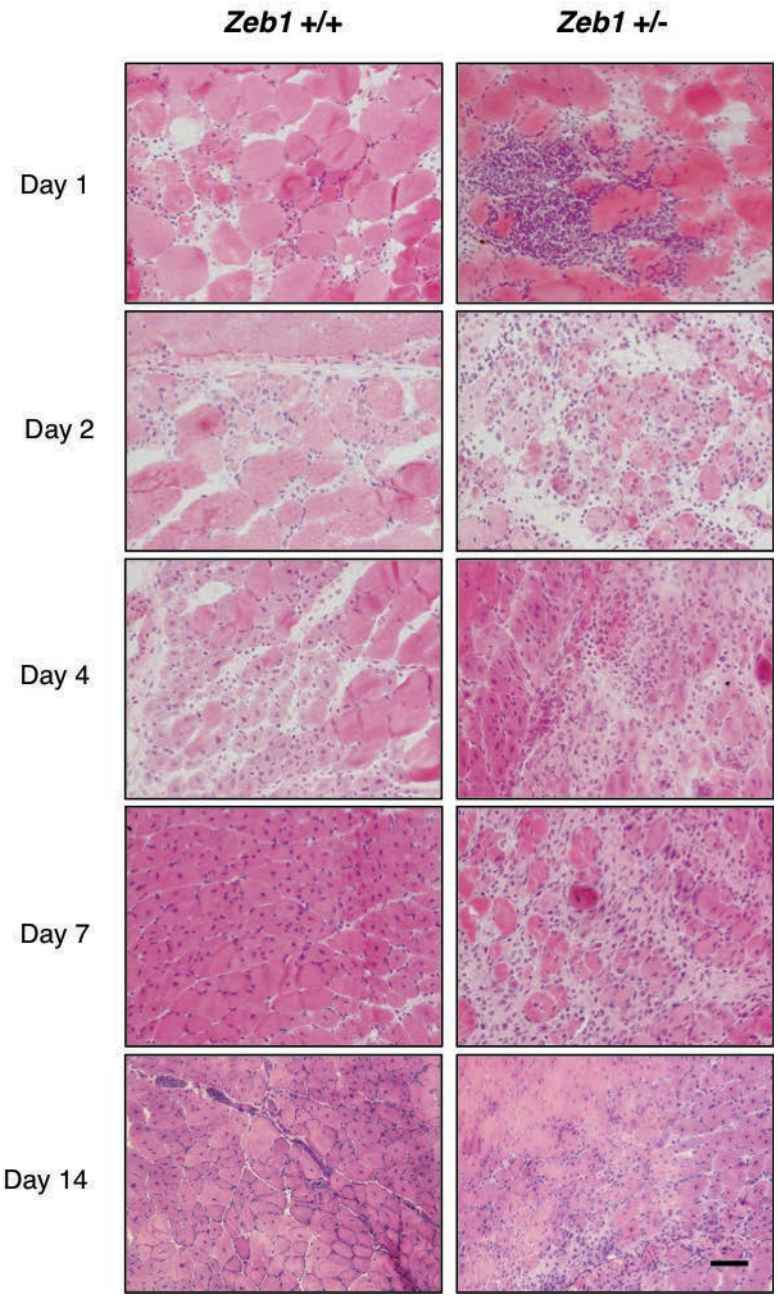


Figure 33. ***Zeb1*-deficient mice exhibit enhanced muscle injury.** *Zeb1*+/+ and *Zeb1*+/- mice were injected with CTX and analysed for H&E at different days p.i. Representative images are shown. Scale bar represents 50 µm.

Levels of *Zeb1* mRNA increased sharply following CTX injection in wild-type mice (Figure 34A) and ectopic cytoplasmic expression of ZEB1 was lower in the more damaged muscles of *Zeb1*-deficient mice (Figure 34B). Altogether, the above results indicate that ZEB1 inhibits muscular inflammatory infiltration in the context of muscular dystrophies and acute injury insults, and suggest an acceleration in the resolution of inflammation and the start of regeneration.

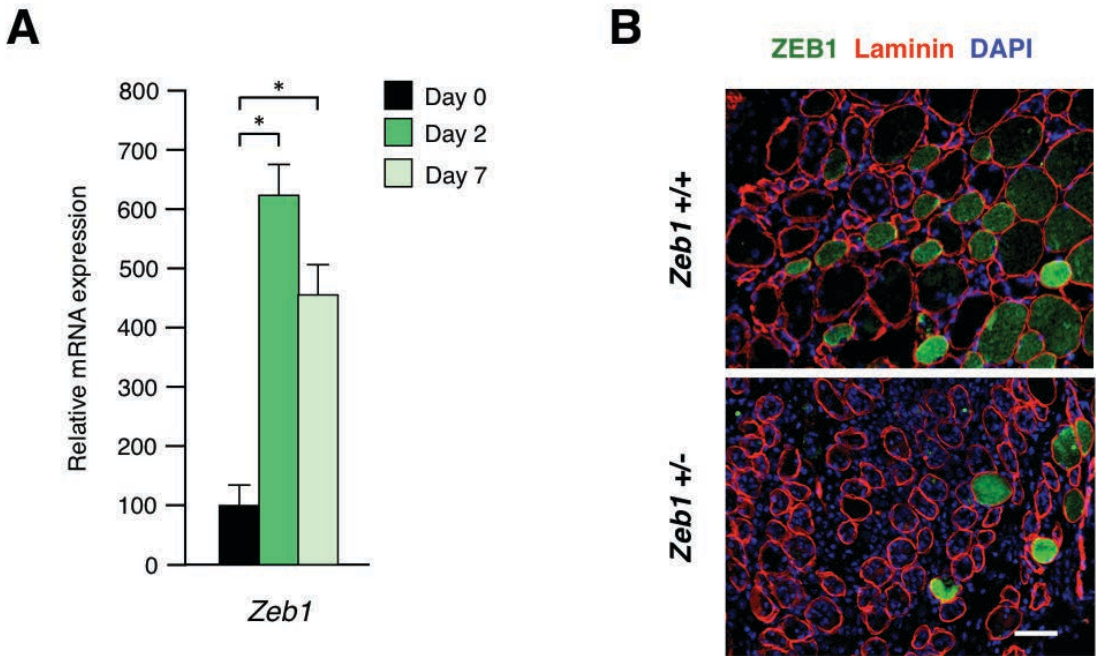


Figure 34. ZEB1 is upregulated upon CTX-mediated injury and is seen in the cytoplasm of undamaged fibers. (A) 2-month-old *Zeb1*+/+ mice were injected with CTX and relative *Zeb1* mRNA levels were assessed. **(B)** ZEB1 and Laminin co-staining of CTX-injected *Zeb1*+/+ and *Zeb1*+/- gastrocnemius showing reduced cytoplasmic ZEB1^{+} myofibers in *Zeb1*-deficient mice. Representative images are shown. Scale bar represents 50 μm.

ZEB1 accelerates the switch to an anti-inflammatory phenotype in infiltrating macrophages upon injury

Since the histological analysis revealed an increase in the infiltrated area in *Zeb1*+/- mice upon CTX injection, we wanted to characterise the role of ZEB1 modulating the inflammatory response to injury. We performed FACS analysis of the gastrocnemius muscle of 2-month-old *Zeb1*+/+ and *Zeb1*+/- mice after 2 days of CTX injection for

the cell surface marker F4/80 and for Ly6C. In line with our results above, the inflammatory infiltrate of *Zeb1*+/- muscles contained more F4/80^{+} and Ly6C^{+} cells than their *Zeb1*+/+ counterparts when injured (Figure 35).

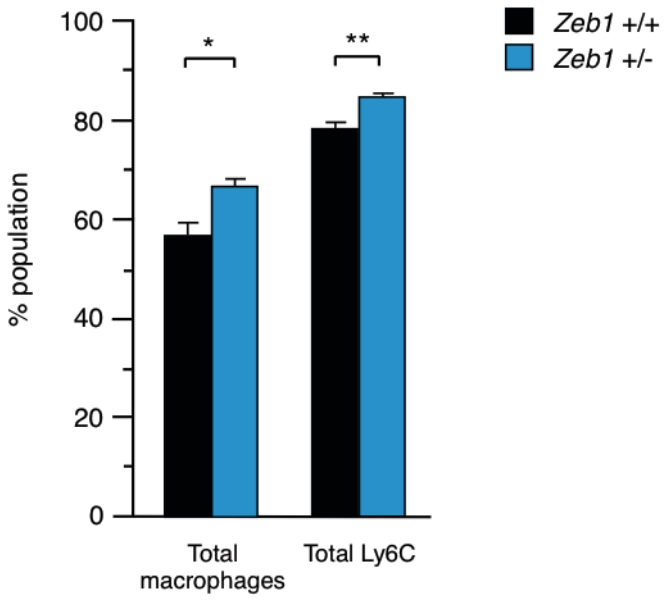


Figure 35. *Zeb1*+/- mice recruit more macrophages upon CTX injection. Total macrophages and Ly6C^{+} cells in 2 days p.i. *Zeb1*+/+ and *Zeb1*+/- gastrocnemius. n=7.

Mounting evidence indicates that the archetypal classification of macrophages as either pro-inflammatory or anti-inflammatory does not reflect the continuum of phenotypes occurring during macrophage activation and that transitional phenotypes are more common *in vivo* (reviewed in Murray et al., 2014). Nevertheless, pro-inflammatory macrophages express high levels of Ly6C (Ly6C^{high}) and the expression of this antigen declines (Ly6C^{low}) as macrophages transition towards an anti-inflammatory and pro-regenerative phenotype (Arnold et al., 2007; Capote et al., 2016). Analysis of the macrophage populations (CD11b^{+} F4/80^{+}) infiltrating the gastrocnemius of *Zeb1*+/+ and *Zeb1*+/- mice two days after CTX injection indicated that the number of Ly6C^{high} macrophages was higher in the latter (Figure 36), thus

indicating that *Zeb1*-deficient gastrocnemius recruits higher levels of pro-inflammatory macrophages during muscle damage.

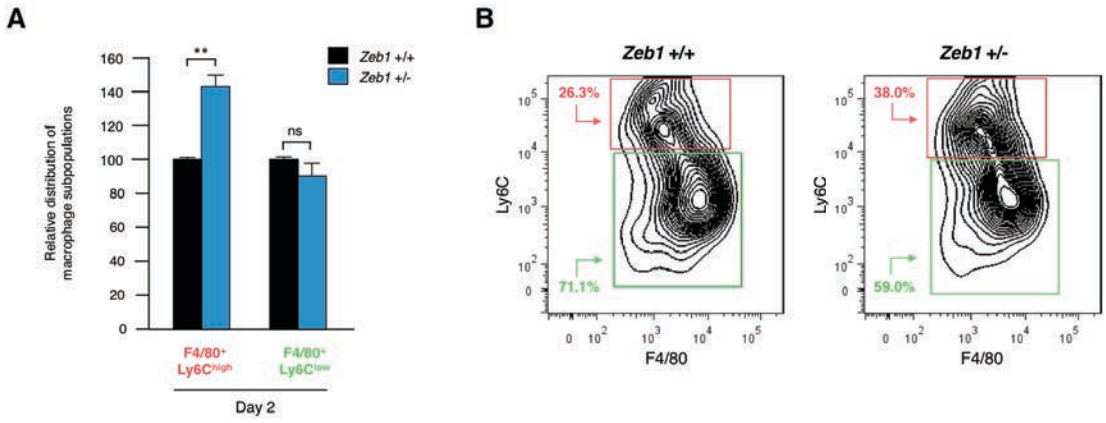


Figure 36. *Zeb1*+/- mice recruit more inflammatory macrophages upon CTX injection. (A) FACS analysis of Ly6Chigh and Ly6Clow macrophages subsets in 2-days p.i. *Zeb1*+/+ and *Zeb1*+/- injured gastrocnemius. n=6. **(B)** Representative FACS plots of macrophages populations assessed in (A).

In the course of muscle injury, damaged myofibers and infiltrating immune cells secrete cytokines and chemokines that create either pro-inflammatory or anti-inflammatory environment (Philippou et al., 2012). We therefore analysed the expression profile of different pro-inflammatory (*Tnf, Il6, Ifng, Nos2, Ccl2, Ccr2*) and anti-inflammatory markers (*Il10, Mrc1, Cx3cr1*) in injured muscles from both genotypes. *Il6, Tnf* and *Nos2* pro-inflammatory markers were upregulated in *Zeb1*+/- CTX-injected muscle at day 2 p.i. *Ccl2* and *Ccr2* mediators of macrophages recruitment were also increased in *Zeb1*+/- injured muscle. Moreover, anti-inflammatory *Mrc1, Cx3cr1* and *Il10* were downregulated in *Zeb1*+/- gastrocnemius at day 2 p.i (Figure 37A). However, no differences were found at day 7 p.i in anti-inflammatory cytokines analysed (Figure 37B). In contrast, *Il6, Ifng* and *Tnf* expression were still higher in *Zeb1*+/- mice 7 days p.i. (Figure 37B). Of note, several studies have shown a role of *Tnf* and *Il6* also in the regulation of muscle differentiation after injury (Chen et al., 2007; Serrano, et al., 2008; Bencze et al., 2012; Kharraz et al., 2013; White et al., 2016). *Ifng* is also considered as an important coordinator of inflammation and the early stages of muscle regeneration (Tidball, 2017). Thus, this upregulation of pro-inflammatory cytokines 7 days p.i. and the subsequent higher inflammation in *Zeb1*+/- mice suggest a possible delay in the regeneration process.

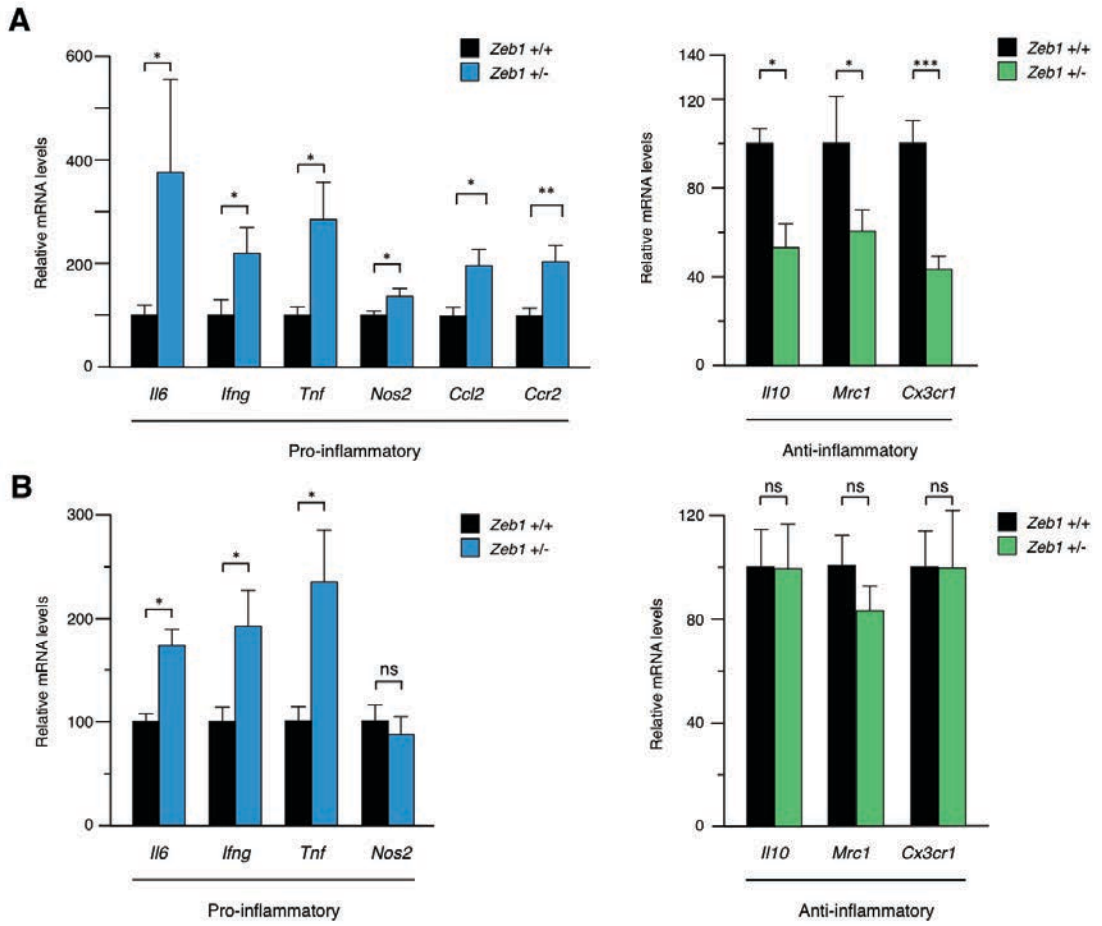


Figure 37. ***Zeb1*+/- muscle exhibits an increased inflammatory response upon CTX injection. (A)** Day 2 p.i. expression levels profile of pro-inflammatory (in blue) and anti-inflammatory (in green) cytokines. The average of at least 5 mice is shown. **(B)** As in (A) but at day 7 p.i. The average of at least 5 mice is shown.

These results were also confirmed by immunostaining of CTX-injured muscles as in with anti-MRC1/CD206. As shown in Figure 38, upregulation of CD206 in response to acute injury was delayed in *Zeb1*+/- mice. It can be therefore concluded that following injury *Zeb1*-deficient muscles show persistence of high levels of pro-inflammatory cytokines and a delay in their switch to an anti-inflammatory phenotype and pro-regenerative state.

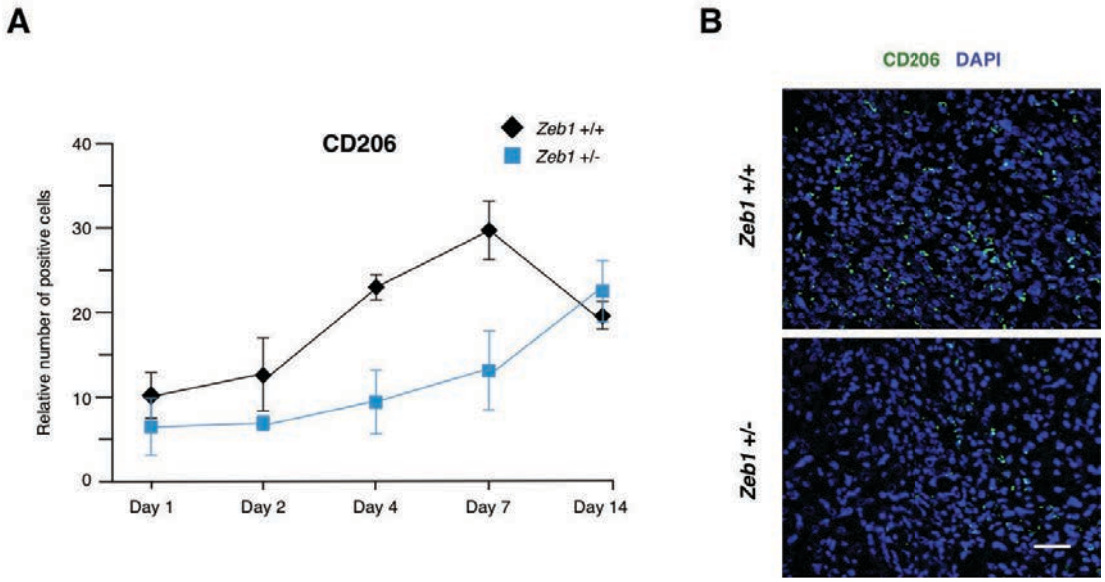


Figure 38. Anti-inflammatory marker CD206 expression is delayed in *Zeb1*+/- injured muscle. (A) CD206 marker was immunohistochemically analysed at different days post-CTX in *Zeb1*+/+ and *Zeb1*+/- gastrocnemius. Representative quantification is shown. **(B)** Representative image of $CD206^{+}$ cells at day 7 p.i. in both genotypes. Scale bar represents 50 µm.

Transplant of *Zeb1*-deficient macrophages induces greater muscle damage than wild-type macrophages

In order to assess whether *Zeb1*+/- macrophages are more capable to induce muscular atrophy, bone marrow-derived macrophages (BMDM) were obtained from bone marrow cells of *Zeb1*+/+ and *Zeb1*+/- mice and stained for 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) cell tracer. Twenty-four hours later, macrophages were injected intramuscularly into 6-months-old *mdx* mice gastrocnemius. After 2 days p.i. EBD-injected *mdx* mice were sacrificed and gastrocnemius muscle was analysed by immunohistochemistry to evaluate damage. We could observe that CFSE positive cells were associated with bigger EBD positive areas when *Zeb1*+/- macrophages were injected. Moreover, *mdx* gastrocnemius receiving *Zeb1*+/- macrophages had more EBD^{+} myofibers related to the total area of the transversal cut (Figure 39). This result indicates that *Zeb1*+/- macrophages are intrinsically able to induce greater damage suggesting that they are more inflammatory than *Zeb1*+/+ macrophages.

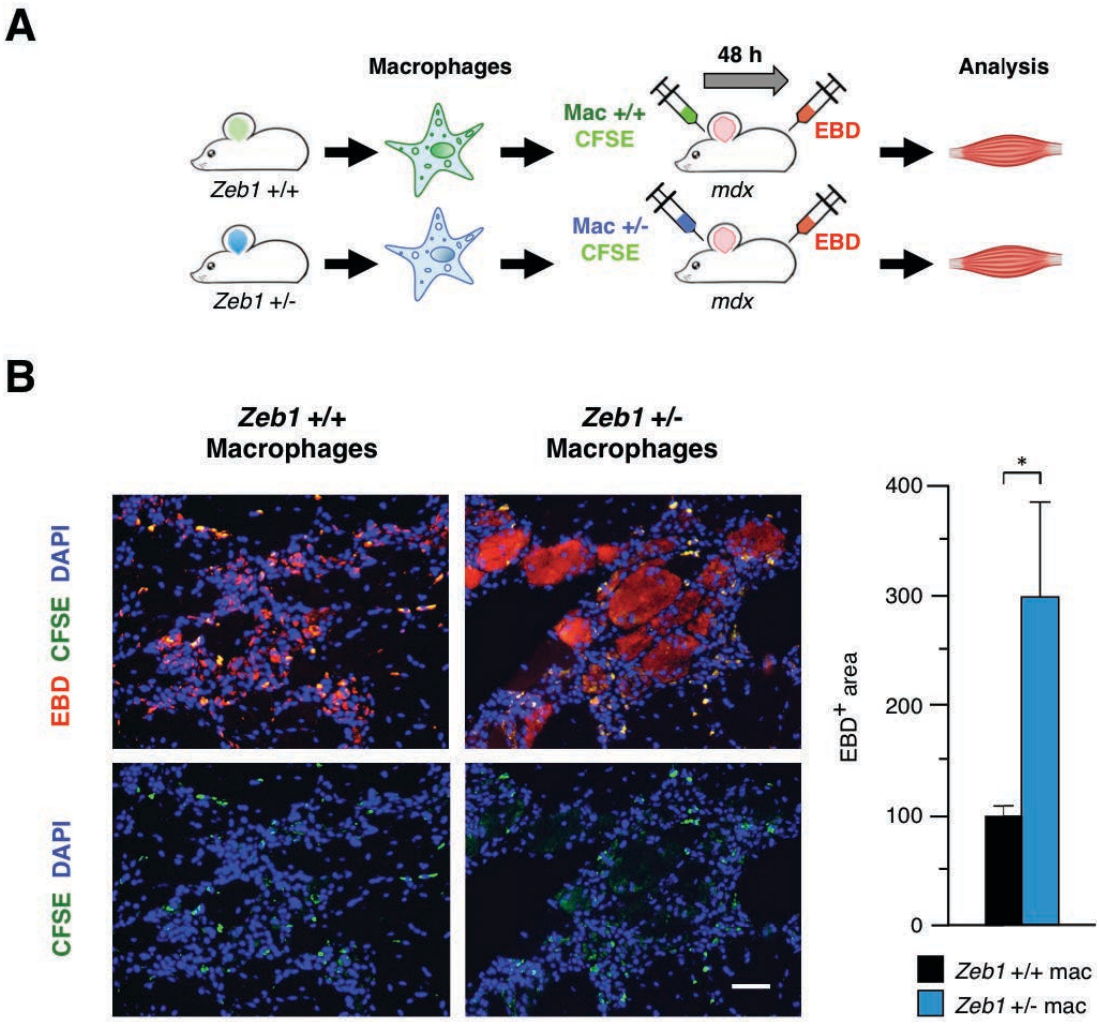


Figure 39. **Transplanted *Zeb1*+/- macrophages induced more muscle damage than *Zeb1*+/+ ones. (A)** Experimental design of bone marrow derived macrophages transplantation into *mdx* hosts. **(B)** Pictures show positive EBD and CFSE areas of dissected muscles injected with *Zeb1*+/+ or *Zeb1*+/- macrophages. The histogram on the right shows the quantification of EBD^+ areas from at least 5 independent pictures for each mouse. Results are represented as the percentage of EBD^+ areas in muscle transplanted with *Zeb1*+/- macrophages relative to muscle transplanted with *Zeb1*+/+ macrophages. n=5. Scale bar represents 50 µm.

III. Full levels of ZEB1 are required for efficient muscle regeneration and satellite cell function

Regeneration is impaired in *Zeb1*-deficient mice upon injury

The expression of ZEB1 in central nuclei of *mdx* muscles (Figure 24C), prompted us to investigate a potential role of ZEB1 in muscle repair. In 2-month-old *mdx*;*Zeb1*+/- mice the number of central nuclei was reduced by 30% compared to same age *mdx*;*Zeb1*+/+ mice (Figure 40A). The gastrocnemius of these mice was also stained for embryonic Myosin Heavy Chain (eMHC), whose upregulation in adult muscle is considered a marker of muscle regeneration. At 2-months of age, the area stained for eMHC in *mdx*;*Zeb1*+/+ mice exceeded by almost twice that in *mdx*;*Zeb1*+/- counterparts (Figure 40B). In contrast, in 10-15-months-old mice, when the regeneration capacity is already significantly reduced even in *mdx;Zeb1*+/+ mice, there was no difference in eMHC expression between both genotypes despite the larger EBD^+ area observed in *mdx;Zeb1*+/- mice (Figure 40B). These results indicate that downregulation of *Zeb1* results in deficient muscle regeneration.

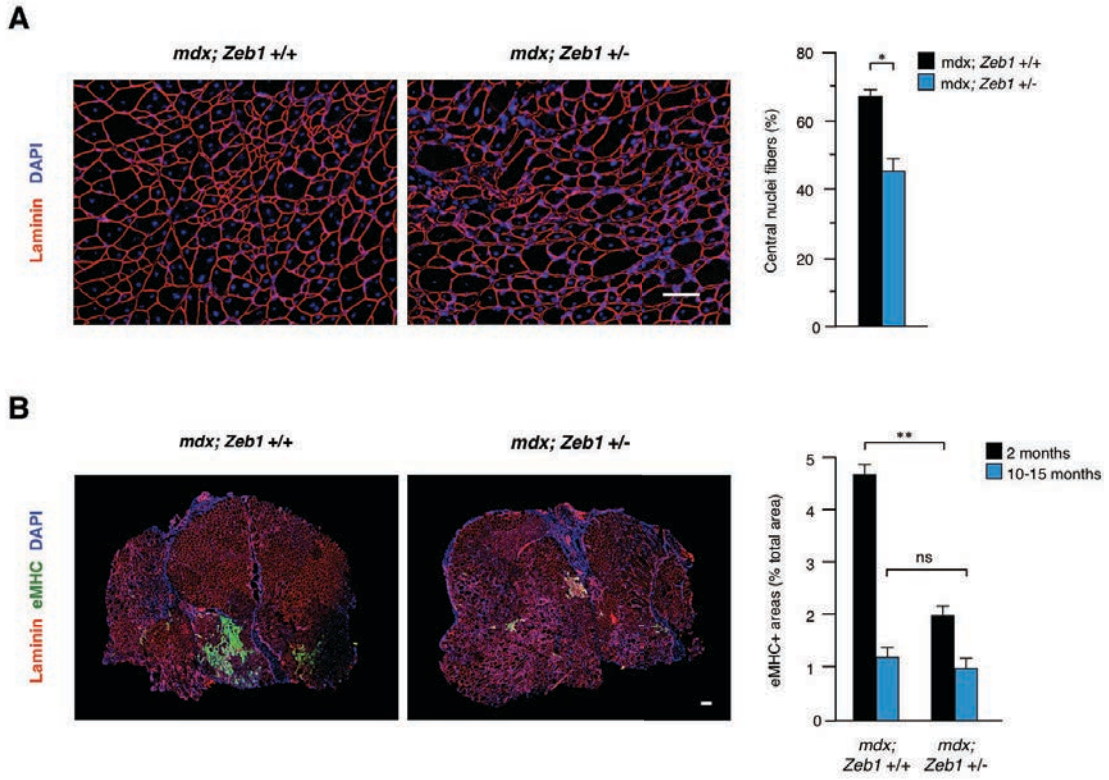


Figure 40. Muscle regeneration is impaired after muscle injury in *mdx;Zeb1*+/- mice. (A) Representative pictures of Laminin and DAPI staining of 2-months-old *mdx;Zeb1*+/+ and *mdx;Zeb1*+/- mice. Chart in the right shows central nuclei areas quantification of 2 independent pictures from each mouse. n=4. Scale bar represents 100 µm. **(B)** Representative pictures of eMHC and Laminin staining of *mdx;Zeb1*+/+ and *mdx;Zeb1*+/- gastrocnemius muscle transversal sections. Chart in the right shows quantification of eMHC^{+} areas in 2- or 10-15-months-old *mdx;Zeb1*+/+ and *mdx;Zeb1*+/- mice relative to total area of the cut. n= 6. Scale bar represents 200 µm.

Regulation of eMHC by ZEB1 was also examined in the context of acute injury. In line with the delay in repair of *Zeb1*+/- muscles following acute injury in Figure 33, we also found that mRNA levels for *Myh3* (the gene encoding eMHC) were lower in *Zeb1*+/- muscles at day 2 p.i. (Figure 41A). In contrast, 7 days after injury, eMHC^{+} areas and *Myh3* expression were higher in *Zeb1*-deficient muscles (Figures 41A and 41B) in concordance with the increased damaged area resulting from the CTX injection. Of note, muscle regeneration in *Zeb1*+/- mice was accompanied by greater fiber size heterogeneity and some abnormalities, which may be related to a deficient maturation of the emerging myofibers in these mice compared to wild-type counterparts (Figure 41C).

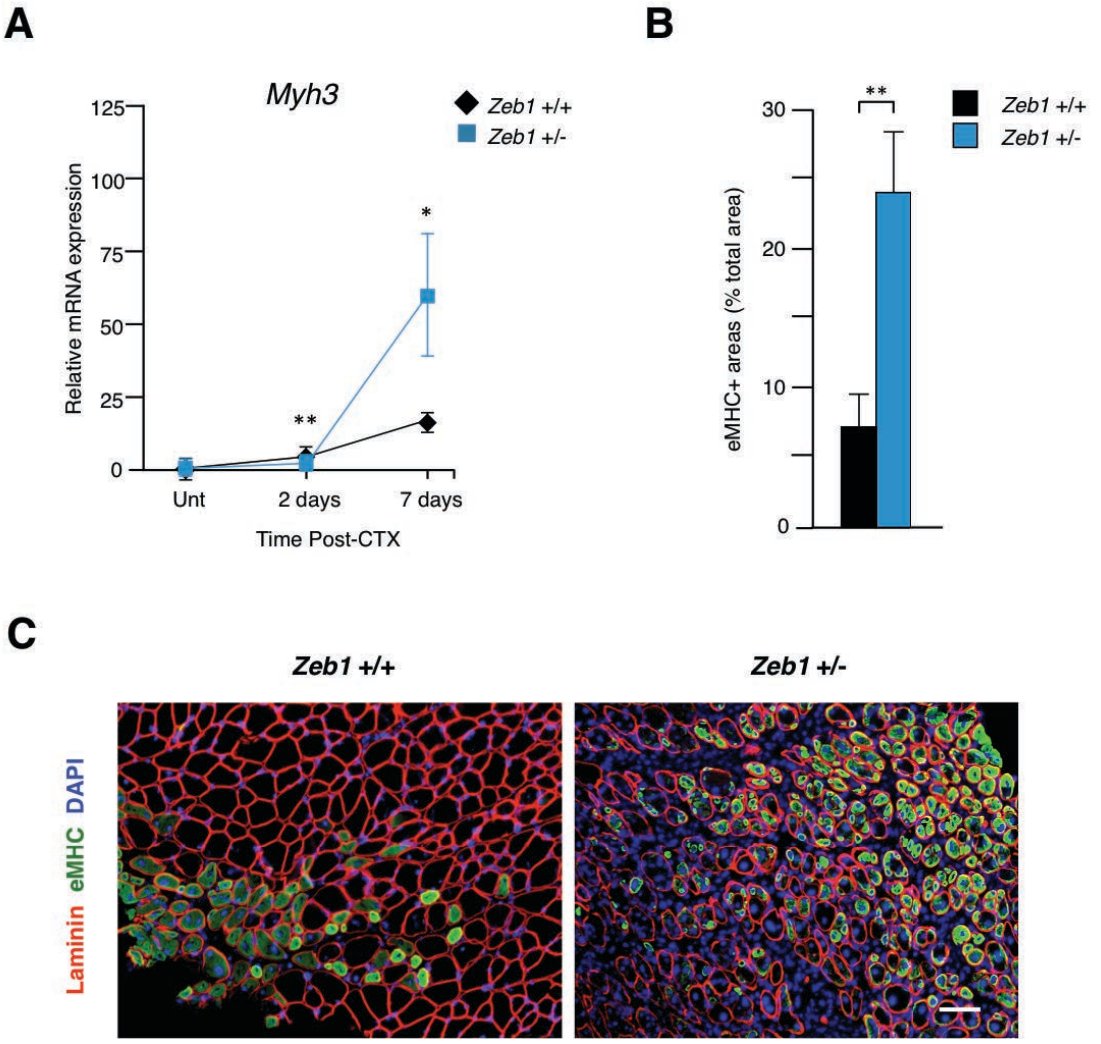


Figure 41. **Muscle regeneration is delayed after muscle injury in *Zeb1*+/- mice. (A)** Relative mRNA levels of *Myh3* gene at day 2 or 7 p.i. in *Zeb1*+/+ and *Zeb1*+/- muscle related to untreated mice. n=6. **(B)** Quantification of eMHC^{+} areas 7 days p.i. relative to total area of the cut of each animal. n= 5. **(C)** Representative pictures of eMHC and laminin staining of CTX injected gastrocnemius at day 7 p.i. are shown. Scale bar represents 100 µm.

Next, we investigated whether regeneration in the gastrocnemius muscle of both genotypes could be affected by exhaustion of muscle regeneration upon successive rounds of CTX. Firstly, we injected CTX and allowed muscle to regenerate for 14 days when the second CTX injection was administered (as in Lepper et al., 2009). At day 28 after the last CTX injection, gastrocnemius muscle was dissected. Morphological analysis of muscles from both genotypes with laminin and DAPI showed structural defects in *Zeb1*+/- gastrocnemius, whereas in *Zeb1*+/+ counterparts the overall muscle morphology was already restored. In addition, in *Zeb1*-deficient muscles, myofiber size was decreased and many areas displayed defective regeneration (Figure 42).

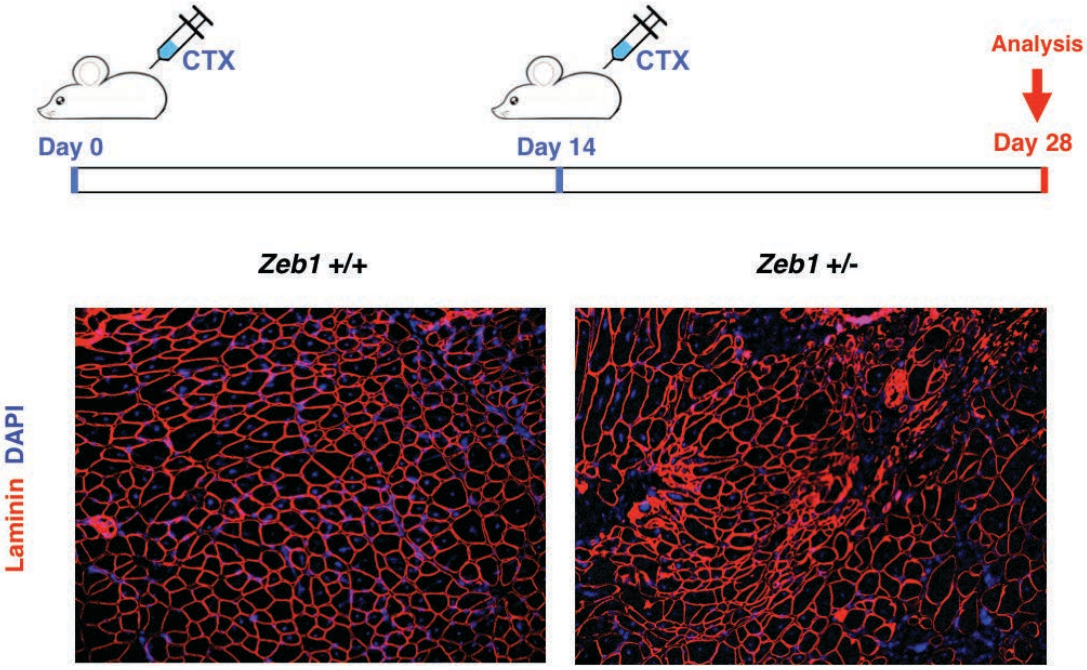


Figure 42. **Exhaustion of muscle regeneration leads to abnormal myofiber formation in *Zeb1*+/- muscle.** Experimental design for two rounds of CTX injury and immunohistochemical analysis of laminin counterstained with DAPI. Representative images from 4 mice for each genotype are shown. Scale bar represents 100 µm.

H&E staining also revealed the existence of infiltrated areas in *Zeb1*+/- gastrocnemius along with small centrally nucleated myofibers at day 28 (Figures 42 and 43). In fact, some necrotic myofibers could still be observed in *Zeb1*+/- muscle (Figure 43, upper panel). In addition, *Zeb1*+/- muscles still contained abundant infiltrate of $CD206^{+}$ anti-inflammatory cells that was not found in wild-type muscles (Figure 43, bottom panel). Meanwhile *Zeb1*+/+ mice appear to have almost completely regenerated muscle.

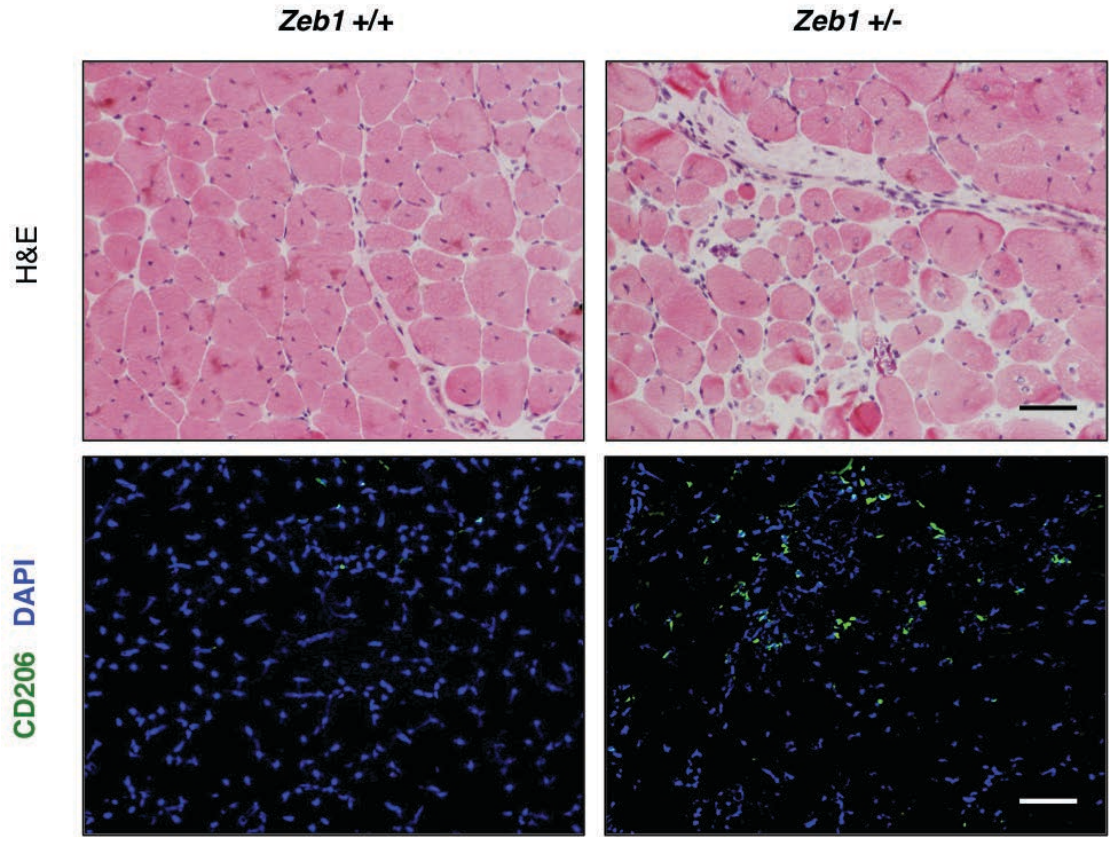


Figure 43. **Exhaustion of muscle regenerative capacity in *Zeb1*+/- gastrocnemius.** H&E and CD206 staining of *Zeb1*+/+ and *Zeb1*+/- mice 14 days after the second CTX injection. Representative images from 4 mice for each genotype are shown. Scale bar represents 50 µm.

Impaired muscle regeneration in *Zeb1*+/- muscles after two rounds of CTX was reflected in lower expression of eMHC (Figure 44A). Although *Zeb1*+/- muscles displayed delayed muscle repair after a single injury insult —as evidenced by the histological progression and pattern of eMHC and *Myh3* expression (Figure 41)— they were eventually able to fully regenerate their damaged areas. However, a second injury further challenged and exhausted the regenerative capacity of *Zeb1*+/- muscles (Figure 44).

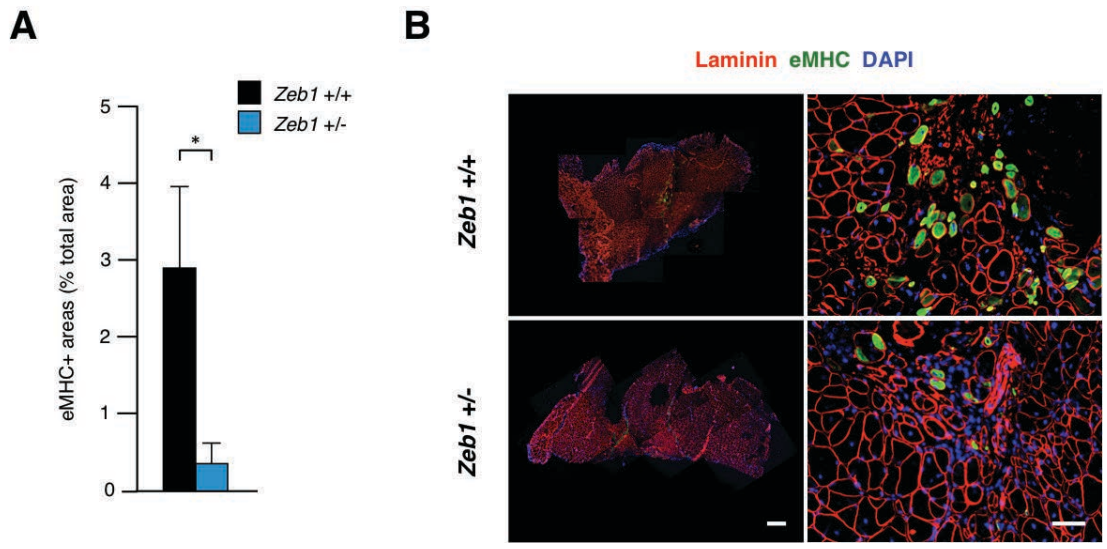


Figure 44. **Exhaustion of regeneration in *Zeb1*+/- gastrocnemius resulted in lower eMHC expression. (A)** Quantification of eMHC$^+$ areas relative to the total section area in each animal. n=4. **(B)** Representative pictures of eMHC and laminin costaining. Pictures in the left show total transversal cut. Scale bar represents 200 µm. Pictures on the right are from a higher magnification of eMHC$^+$ areas of each genotype. Scale bar represents 50 µm.

Altogether, it can be concluded that muscle regeneration is delayed and compromised in *Zeb1*-deficient mice suggesting poorer regenerative capacity of their SCs.

Satellite cells require full levels of ZEB1 to maintain their quiescence-associated gene signature

A pool of functional SCs is essential for muscle regeneration after injury. SCs are activated and become proliferating myoblasts that eventually differentiate to repair muscle damage. *Ex vivo* culture of SCs also leads to their activation and myogenic progression although alters their gene expression profile and reduces their regenerative capacity vis-à-vis freshly isolated SCs (Montarras et al., 2005; Pallafacchina et al., 2010).

The defective and delayed regenerative capacity of *Zeb1*-deficient muscles prompted us to characterise the expression and role of ZEB1 in SCs freshly isolated by FACS —using the sorting protocol described in Pasut et al., (2012) as well as during their myogenic conversion (Figure 45).

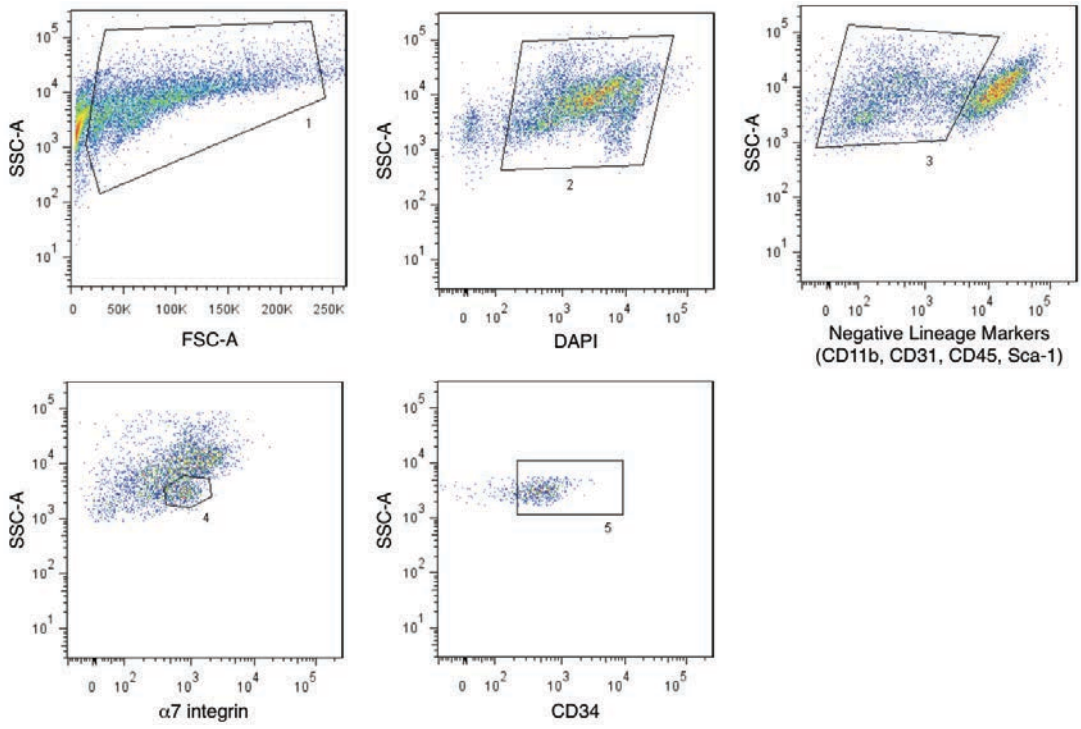


Figure 45. SCs isolation protocol. Shown is the sorting strategy followed for mouse SCs isolation.

The myogenic progression of SCs is defined by a highly specific transcriptome (Yin et al., 2013; Almada & Wagers, 2016). Thus, quiescent SCs —unable to incorporate BrdU— express Pax7 but not MyoD. Once SCs are activated and began to proliferate they incorporate BrdU and express MyoD. Lastly, only after SCs exit the cell cycle and differentiate they lose Pax7 expression.

At the time of isolation (0 h), wild-type SCs express more than twice the levels of *Zeb1* found in *Zeb1*+/- SCs (Figure 46). Interestingly, activation of SCs in culture reduced *Zeb1* expression in wild-type SCs at 24 h and 72 h to the levels found in freshly isolated *Zeb1*+/- SCs. Only after 10 days, expression of *Zeb1* further declined in *Zeb1*+/- SCs (Figure 46).

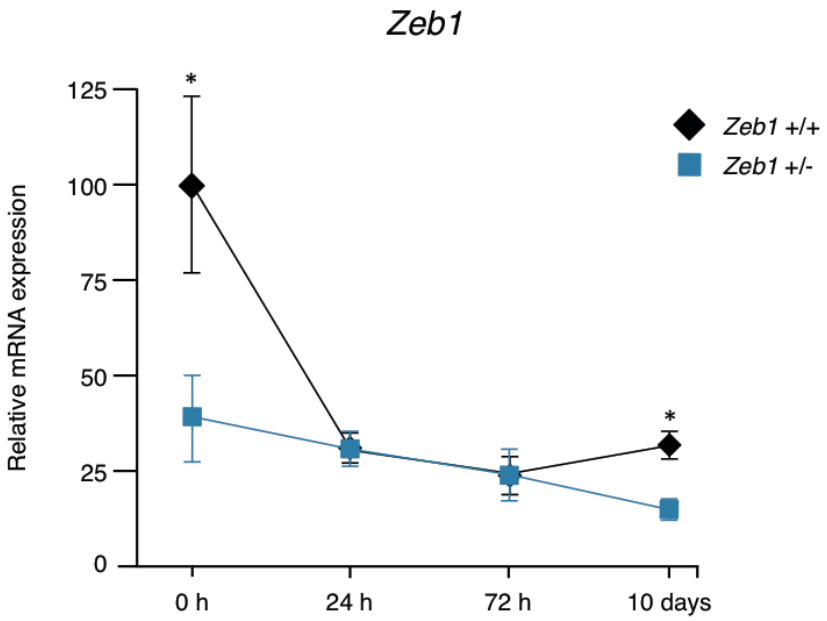


Figure 46. ***Zeb1* is downregulated upon SCs activation in culture.** SCs isolated from *Zeb1*+/+ and *Zeb1*+/- gastrocnemius muscles were analysed for *Zeb1* mRNA levels by qRT-PCR at the time of isolation (0 h) as well as during their activation in culture. n= 4.

Next, we examined whether ZEB1 modulates the myogenic transition of SCs through MyoD expression. SCs were costained for Pax7 and MyoD to characterise quiescent ($Pax7^+$ $MyoD^-$), proliferating ($Pax7^+$ $MyoD^+$) and differentiating ($Pax7^-$ $MyoD^+$) populations (Wu et al., 2015) (Figure 47A). Notably, freshly sorted *Zeb1*+/- SCs exhibited a small population expressing MyoD, higher than ones from *Zeb1*+/+ sorted SCs, which was almost negative. Instead, the population of $Pax7^+/MyoD^-$ cells was reduced in *Zeb1*-deficient cells (Figure 47B). A similar pattern was observed once SC became activated upon 24 h in culture with *Zeb1*+/- SCs harbouring a smaller proportion of cells in quiescence and a larger share of differentiating cells. The share of differentiating cells among in *Zeb1*+/- cultures was even larger after 3 days (Figure 47B).

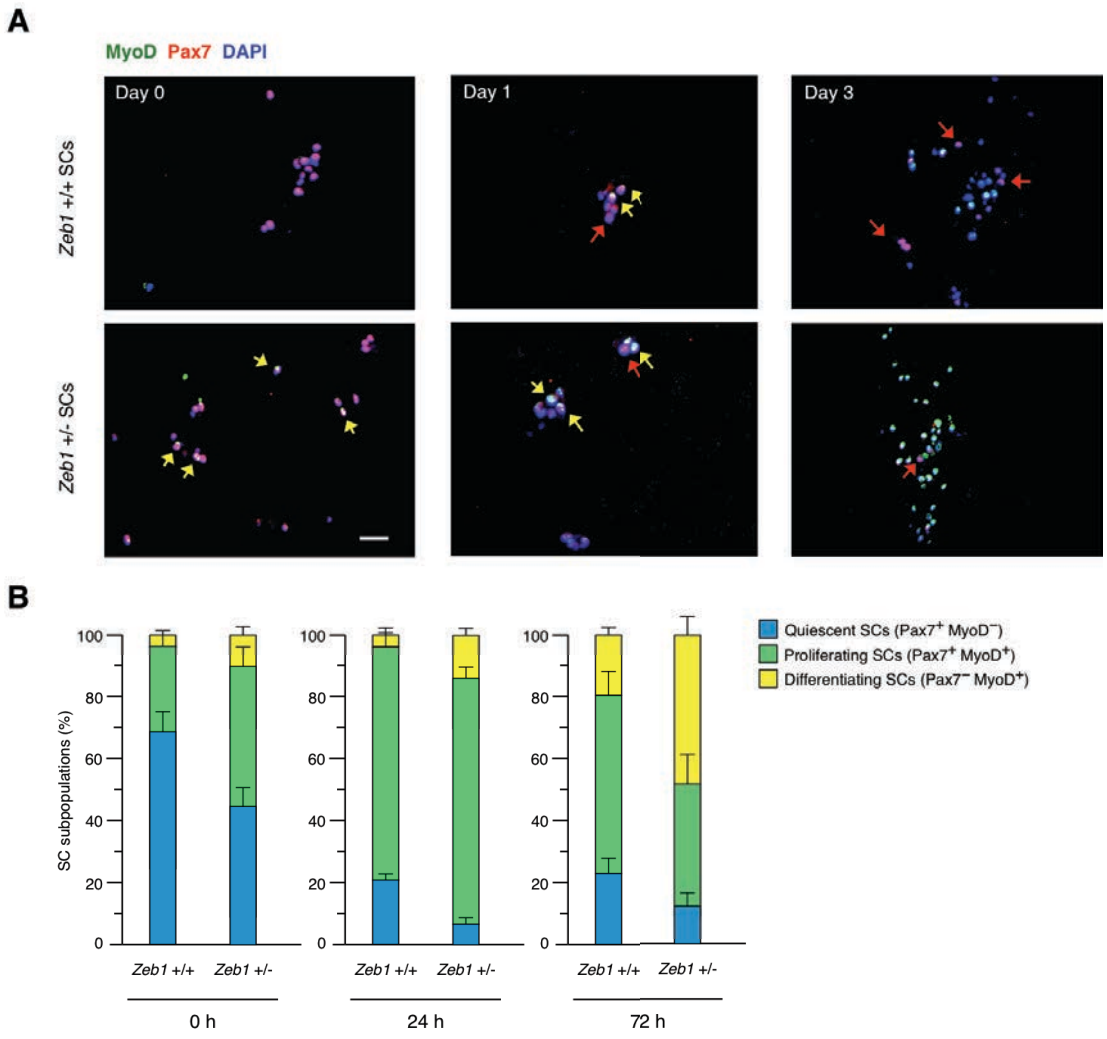


Figure 47. **Downregulation of *Zeb1* in SCs triggers their premature activation and myogenic progression. (A)** SCs isolated from *Zeb1*+/+ and *Zeb1*+/- mice were characterised by immunostaining for their stage based on their expression of Pax7/MyoD, both at the time of isolation and upon activation in culture for 24 h and 72 h. (Yellow arrows show $Pax7^{+}$ $MyoD^{+}$ cells and red arrows $Pax7^{+}$ $MyoD^{-}$ cells). Scale bar represents 50 μm. **(B)** Chart shows quantifications from pictures in (A). Data shown are the average of 4 mice from each genotype.

Pax7 and MyoD expression, whose regulation mediates SC activation, were also analysed at the mRNA level (Megeney et al., 1996). At the time of SC isolation (0 h), *Zeb1*+/- SCs expressed less than half of the levels found in wild-type SCs for *Pax7* and about twice of those for *Myod1* (Figures 48A and 48B). Expression of MyoD increased at 24 h in SCs from both genotypes and decreased afterwards (Figure 48B).

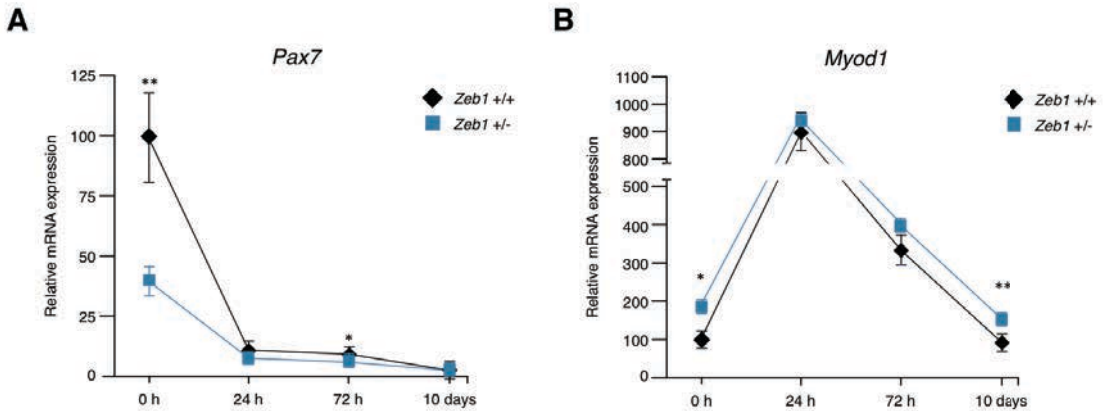


Figure 48. **Downregulation of *Pax7* and upregulation of *Myod1* in *Zeb1*-deficient SCs.** Freshly isolated and cultured SCs from both genotypes were assessed by qRT-PCR for **(A)** *Pax7* and **(B)** *Myod1*. At least 4 mice were analysed for each genotype.

The quiescence or activated status of *Zeb1*+/+ and *Zeb1*+/- SCs was assessed by staining for Pax7 and BrdU to determine the activated population incorporating BrdU ($Pax7^+$ $BrdU^+$) and the quiescent ones ($Pax7^+$ $BrdU^-$) (Sousa-Victor et al., 2014) (Figure 49A). Upon 24h in culture, *Zeb1*+/- SCs displayed increased number of activated $Pax7^+$ $BrdU^+$ cells and consequently a lower share of the quiescent SC population ($Pax7^+$ $BrdU^-$) (Figure 49B). These observations indicate that downregulation of *Zeb1* in SCs triggers their premature activation in culture.

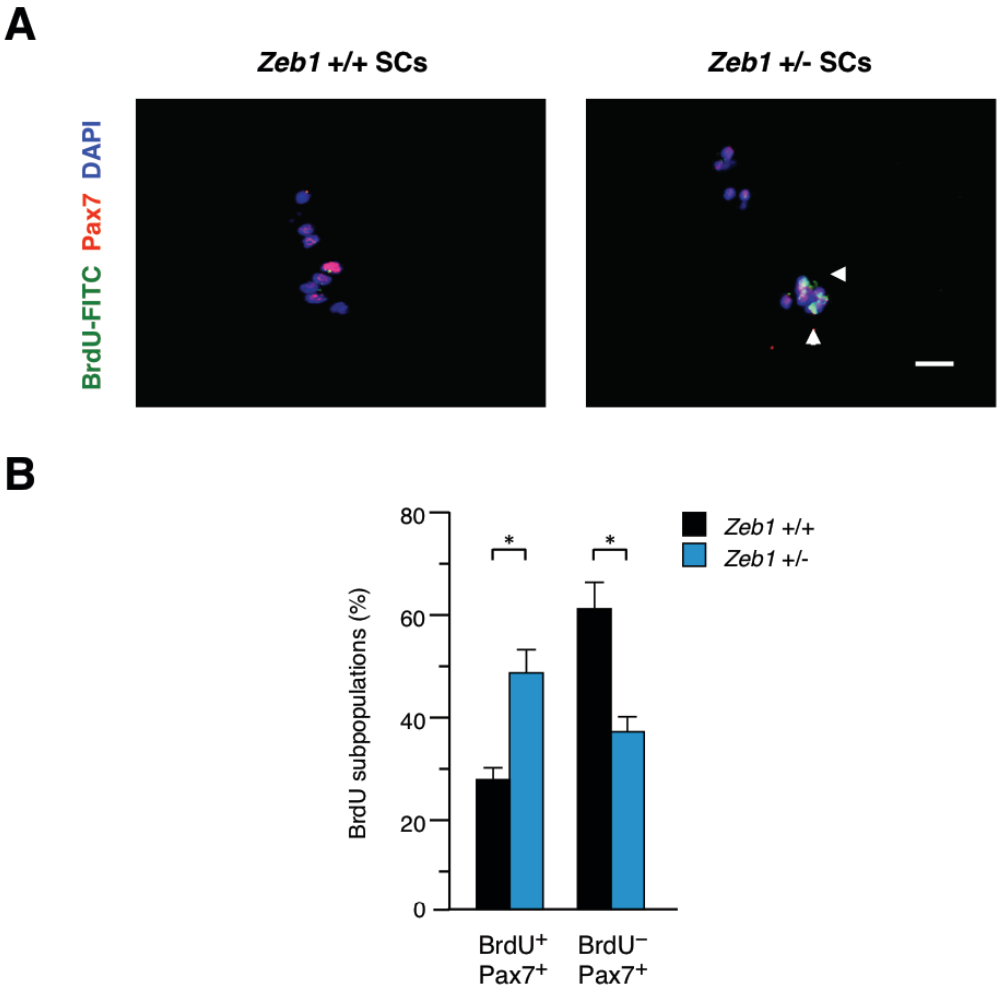


Figure 49. ***Zeb1*+/- SCs are precociously activated when cultured *in vitro*. (A)** BrdU and Pax7 costaining of 24h cultured SCs to assess first activation. **(B)** Quantification of the different SCs populations by their Pax7 and BrdU costaining (arrowheads indicate $Pax7^{+}$ $BrdU^{+}$ cells). n=4. Scale bar represents 20 µm.

To investigate the mechanisms by which ZEB1 inhibits SCs' premature activation we explored whether ZEB1 regulates genes that form part of quiescent SCs' signature both in freshly isolated SCs (0 h) and following culture for up to 10 days. Namely, we examined the mRNA expression levels of: the cyclin-dependent kinase inhibitor $p57^{KIP2}$/*Cdkn1c* involved in quiescence maintenance in a number of stem cells, including SCs (Fukada et al., 2007) and *Foxo3*, which activates Notch1 and Notch3 expression (Gopinath et al., 2014). Both markers of SC quiescence were downregulated in freshly isolated *Zeb1*+/- SCs (Figure 50). Of note, following SC activation and differentiation (24 h, 72 h and 10 days), expression of *Pax7*, *Cdkn1c* and *Foxo3* declined in wild-type SCs to the levels of *Zeb1*+/- ones (Figures 48 and 50).

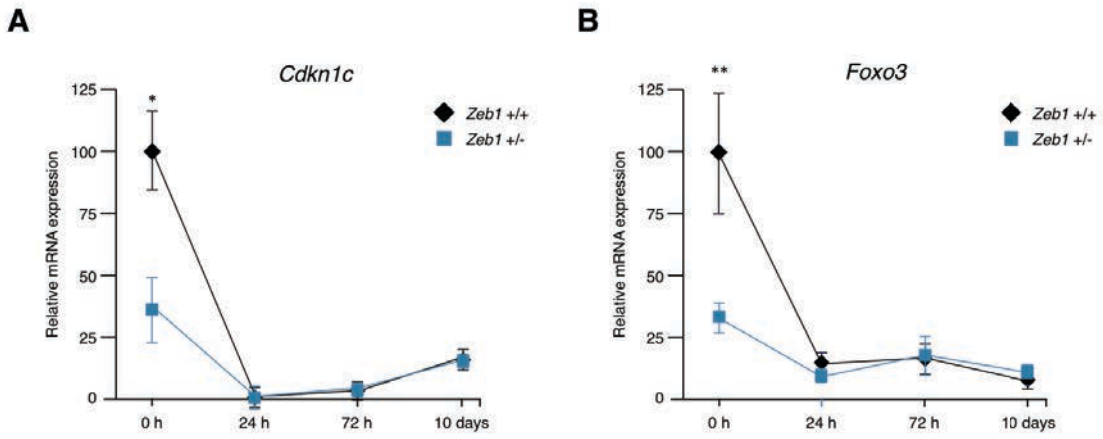


Figure 50. ***Zeb1*-deficient SCs downregulate SCs quiescence-associated genes.** Freshly isolated and cultured SCs from both genotypes were assessed by qRT-PCR for **(A)** *Cdkn1c* and **(B)** *Foxo3*. At least 3 mice were used for each genotype.

Activation of the Notch pathway maintains SC quiescence indirectly through activation of Pax7 (which in turn downregulates MyoD) or via direct repression of MyoD by Notch target genes of the HES and HEY families (Bjornson et al., 2012; Mourikis et al., 2012). In line with their premature activation, *Zeb1*+/- SCs expressed lower levels of *Hes1* and *Hes6* (Figure 51) in a pattern that resembles what occurs in *Foxo3*-deficient cells (Gopinath et al., 2014). Altogether, the above data indicate that freshly isolated *Zeb1*+/- SCs displayed lower levels of quiescence-associated genes and higher *Myod1* expression, which leads to their premature activation and differentiation. It can also be concluded that full levels of ZEB1 are required for SCs to maintain their quiescence.

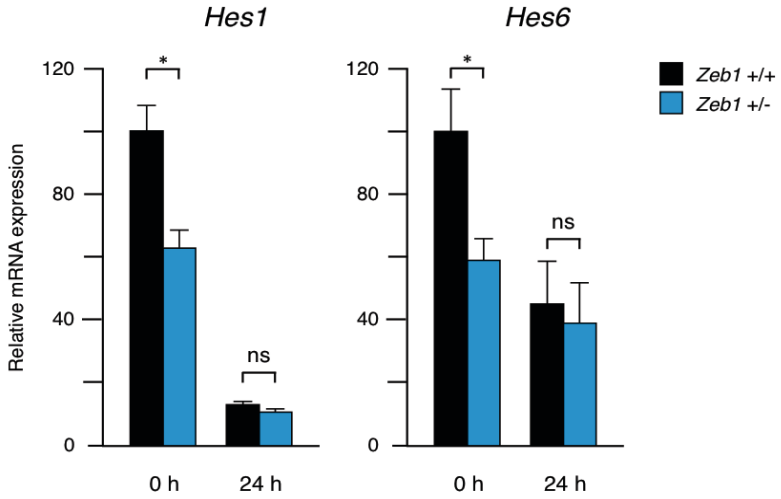


Figure 51. ***Zeb1*+/- SCs are precociously activated through downregulation of Notch signaling.** Relative mRNA levels of *Hes1* and *Hes6* in SCs that were analysed after isolation (0h) or cultured for 24h. At least 4 mice were used for each genotype.

Isolated mouse SCs are capable to differentiate and form myotubes when cultured *ex vivo* as the C2C12 cell line does. *Zeb1*+/+ and *Zeb1*+/- SCs were allowed to differentiate and stained with a pan-MHC antibody (MF-20) counterstained with DAPI to evaluate the number of nuclei per myotube. *Zeb1*+/- SC cultures were able to differentiate (Figure 52A). Interestingly, we observed increased number of single nuclei myoblasts expressing terminal differentiation marker MHC (Figure 52C). These results are in line with our previous analysis showing precocious early differentiation in *Zeb1*+/- cultures by *Myod1* upregulation. On the other hand, the number of larger myotubes (containing more than 10 nuclei per myotube) was dramatically reduced in *Zeb1*+/- SCs cultures (Figure 52C). The total number of MF-20-positive cells was the same in both cultures (Figure 52B). These observations showed that *Zeb1*+/- SCs differentiate rapidly when cultured but suggest an impairment in the formation of larger myotubes (myoblasts fusion).

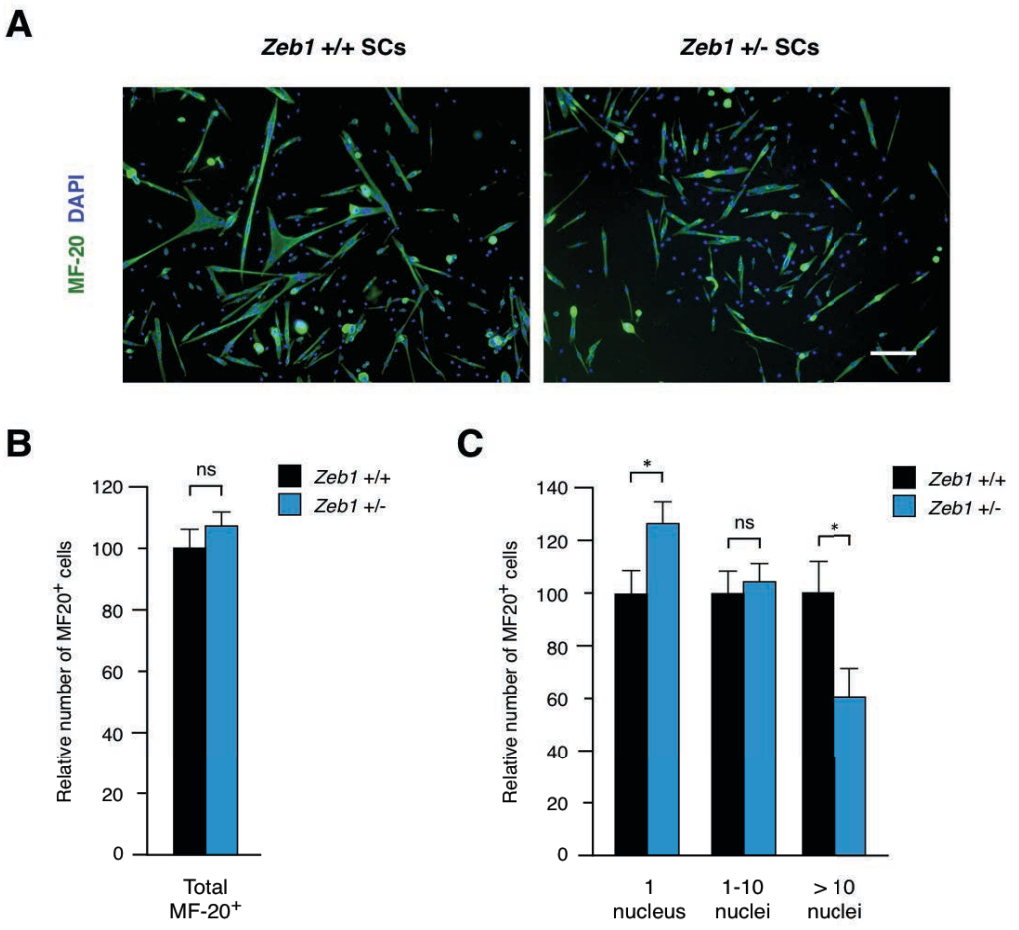


Figure 52. ***Zeb1*+/- SC cultures generate a larger share of MHC$^+$ mononucleated cells and a lower one of MHC$^+$ larger myotubes. (A)** Cultured SCs were allowed to differentiate and stained with MF-20 antibody. Representative pictures are shown. Scale bar represents 100 µm. **(B)** Quantification of total MF-20 positive cells or myotubes. **(C)** As in (B) but MHC$^+$ cells were assessed for the number of nuclei. At least 10 independent pictures of each mouse were quantified. n=5.

ZEB1 inhibits senescence of satellite cells

Inhibition of senescence is required for muscles to maintain their pool of SCs and to mount an efficient regeneration in response to damage. For instance, SCs in young 2-month-old mice with genetic ablation of *Prmt7* displayed premature senescence and deficient repair capacity after acute injury (Blanc et al., 2016). Premature senescence of SCs is also involved in the pathogenesis of muscle dystrophies (Bigot et al., 2008). Lastly, senescence is also responsible of the decline in the pool and functionality of SCs during aging (Musaro et al., 2001; Sousa-Victor et al., 2014). SCs injury and aging induced-senescence, results in the secretion of a specific set of soluble factors —that form the so-called senescence associated secretory phenotype (SASP), chiefly of IL6— that promote plasticity and reprogramming of neighbouring non-senescent cells (Chiche et al., 2017).

ZEB1 inhibits senescence in terminally differentiated fibroblasts and in cancer cells (Liu et al., 2014; de Barrios et al., 2017). Therefore, we questioned whether deficient regeneration of *Zeb1*+/- muscles relates to premature senescence of their SCs. Senescence of SCs from wild-type and *Zeb1*+/- SCs was assessed by staining for β-galactosidase activity, commonly referred as senescence-associated β-galactosidase (SA-β-gal). We found that *Zeb1*-deficient SCs displayed a higher number of senescent cells than wild-type counterparts (Figure 53).

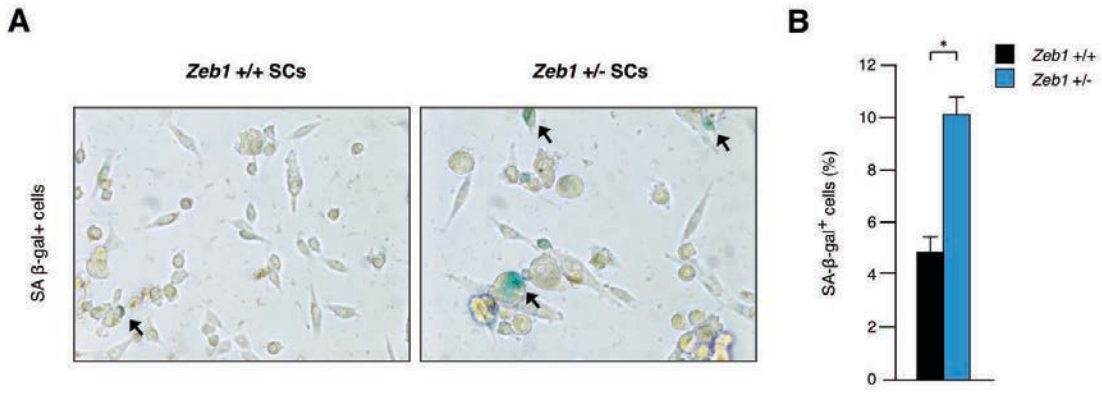


Figure 53. Cultured *Zeb1*+/- SCs are more senescent than *Zeb1*+/+ ones. (A) SA-β-gal^{+} cells in 10 days cultured SCs. Representative images are shown. Black arrows show positive cells. n=4. **(B)** At least 10 independent pictures were counted and are represented as the percentage of the total counted cells.

As in other cell types, premature senescence in SCs form both young or geriatric mice represents an irreversible cell cycle arrest, which is mediated by cell cycle inhibitors p16 (*Cdkn2a*) and p21 (*Cdkn1a*) (Bigot et al., 2008; Sousa-Victor et al., 2014; Blanc et al., 2016). Notably, higher rate of senescence among *Zeb1*-deficient SCs was accompanied by upregulation of *Cdkn2a* and *Cdkn1a* compared to wild-type SCs (Figure 54). Levels of *Ccna2* (cyclin A2) and *Cdkn1c* ($p57^{KIP2}$), which are involved in cell cycle progression of myogenic progenitor cells (Fukada et al., 2007), were also higher in *Zeb1*-deficient SCs respect to wild-type ones (Figure 54). Altogether these data indicate that *Zeb1*+/- SCs exhibit precocious activation and differentiation as well as a premature senescence.

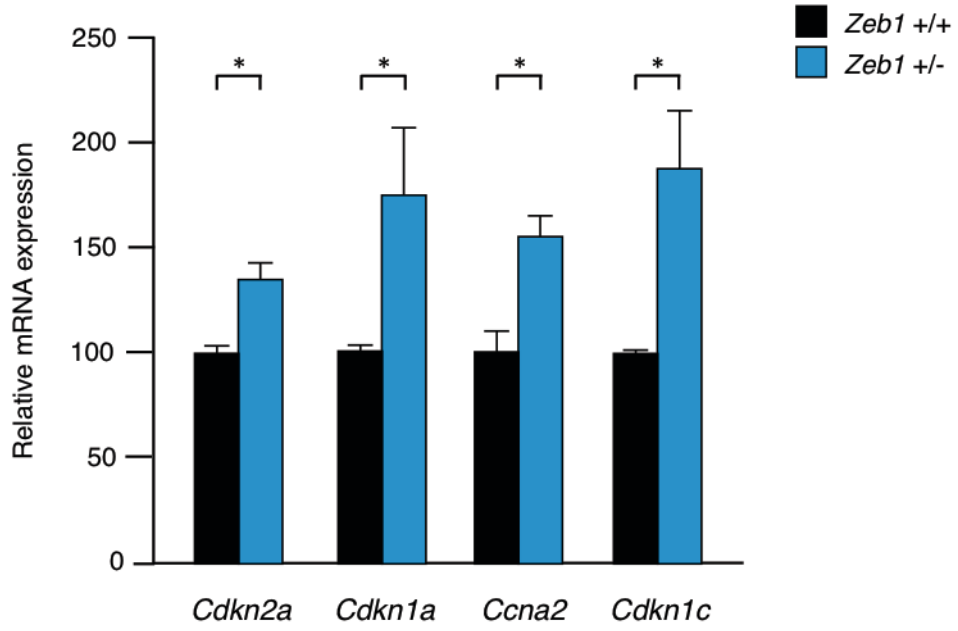


Figure 54. **Cell cycle-associated genes are upregulated in 10-days cultures of *Zeb1*-deficient SCs.** Relative mRNA levels of *Cdkn2a, Cdkn1a, Ccna2* and *Cdkn1c* genes in 10 days-cultured *Zeb1*+/+ and *Zeb1*+/- SCs. n= 5.

Satellite cells depend on ZEB1 expression for efficient muscle regeneration of dystrophic muscle

To study whether the deficient regenerative capacity of *Zeb1*+/- muscles is intrinsic to the SCs themselves, we transplanted SCs from both genotypes into damaged muscles. *Zeb1*+/+ and *Zeb1*+/- SCs were sorted, immediately transduced with a lentivirus encoding for GFP and injected into CTX-treated *mdx* hosts to enhance the

engraftment and muscle regenerative response (Cerletti et al., 2008) (Figure 55A). A month later, the gastrocnemius muscle was analysed for GFP^+ areas that correspond to transplanted SC-derived myofibers by immunohistochemistry (Figure 55B). Quantification of these GFP^+ areas showed that engraftment of *Zeb1*-deficient SCs was about 50% less efficient in regenerating muscle tissue than wild-type SCs (Figure 55C). Importantly, when SCs are cultured, there is a decrease in their regenerative potential due to the loss of $Pax7^+$ population and an increase in $MyoD^+$ committed myoblasts (Montarras et al., 2005). Thus, premature upregulation of MyoD in cultured *Zeb1*-deficient SCs hinders their regenerative capacity when transplanted.

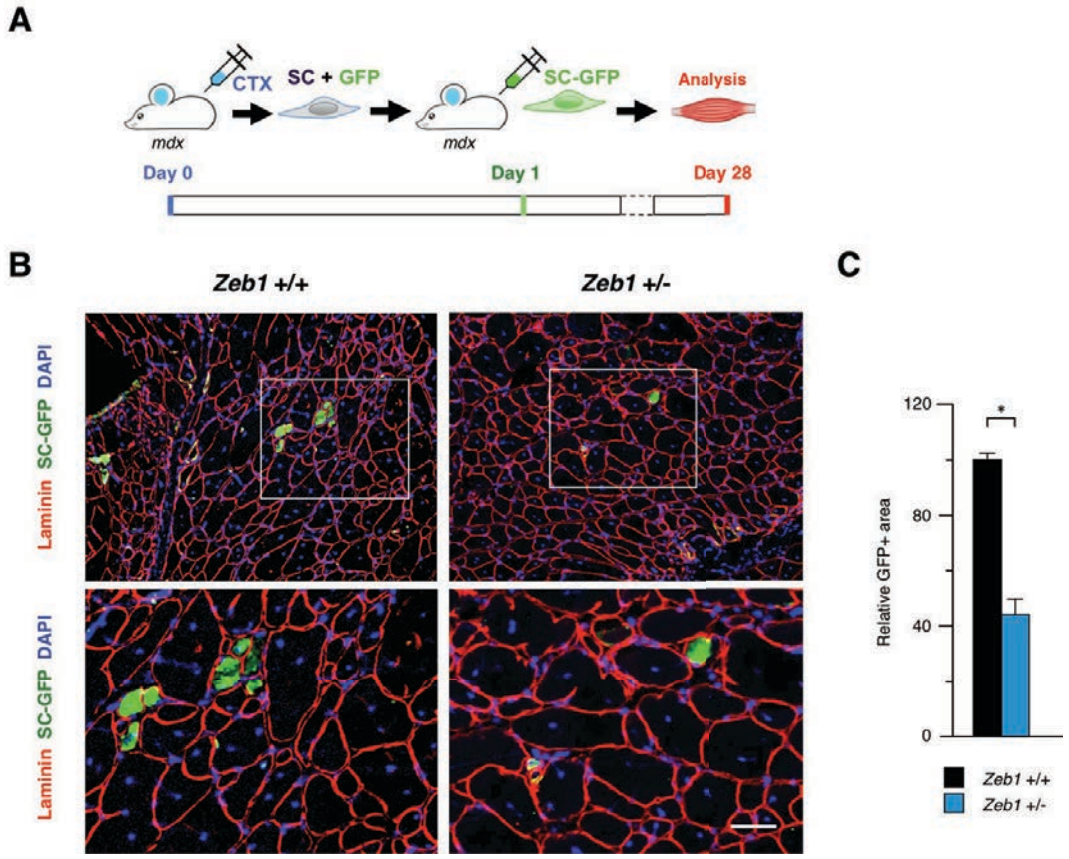


Figure 55. SCs depend on their expression of full levels of *Zeb1* to drive efficient muscle regeneration. (A) GFP-labeled SCs isolated from *Zeb1*+/+ and *Zeb1*+/- mice were transplanted into the gastrocnemius of *mdx* mice that have been previously injected with CTX. **(B)** Four weeks later mice were euthanized and the presence of GFP^+ myofibers was assessed by immunostaining. Representative pictures are shown. Scale bar represents 50 μm. **(C)** Quantification of areas stained for GFP in (B). n= 4.

These data demonstrate that, the deficient repair of *Zeb1*+/- muscles upon injury lies on an intrinsic deficiency of their SCs and that SCs depend on their expression of full levels of *Zeb1* to drive efficient muscle regeneration.

DISCUSSION

DISCUSSION

Skeletal muscle controls its homeostasis and adapts to daily physical activity and tissue insult through different molecular mechanisms. Accordingly, compared to other adult tissues, muscles maintain the capacity to regenerate upon damage. Many of the genes and signalling pathways that participate in myogenesis during embryonic development are also responsible for this regenerative response. However, the molecular cues that coordinate muscle injury during the activation, proliferation and differentiation of SCs are still only partially understood. Using cell line-based systems and different *in vivo* models, we have found that ZEB1 represses muscle gene expression and differentiation by displacing MyoD from its binding sites on the regulatory regions of muscle differentiation genes. In addition, we showed that ZEB1 protects adult muscle from injury and its required for a correct regeneration.

ZEB1 regulates muscle differentiation in a stage-dependent manner. In contrast to MyoD, ZEB1 binds to G/C-centered E-boxes in muscle-specific genes at the myoblast stage but not in myotubes. We also found that, knockdown of endogenous *Zeb1* in non-confluent cycling myoblasts, but not in differentiated myotubes, upregulates the basal transcriptional activity of several differentiation gene promoters resulting in their premature expression and an acceleration of myotube formation. These results demonstrate that these genes are normally under negative regulation by ZEB1 in myoblasts but not in myotubes (Figure 56).

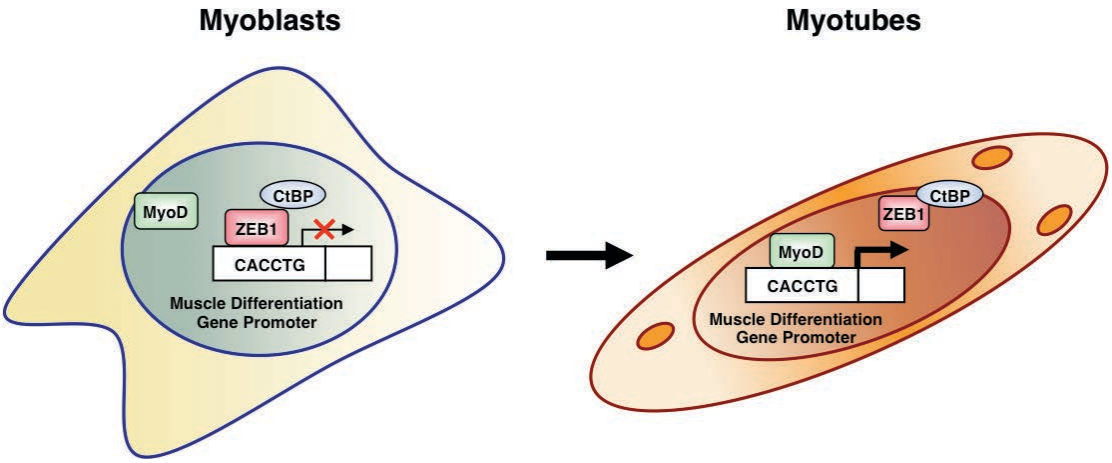


Figure 56. ZEB1 regulates muscle differentiation in a stage-dependent manner. In myoblasts, ZEB1 binds to G/C-centered E-boxes in the regulatory regions of muscle differentiation genes whose transcription would be repressed at least in part via recruitment of CtBP. In myotubes, MyoD displaces ZEB1 from these G/C-centered E-boxes and activates the expression of muscle genes as differentiation progresses.

As ZEB1, Snail also displaces MyoD from the E-boxes of muscle differentiation genes during the myoblast stage (Soleimani et al., 2012). Interestingly, ZEB1 is a downstream target of both Snail1 and Snail2 (Taube et al., 2010; Dave et al., 2011; Wels et al., 2011). Both Snail1 and ZEB1 are expressed in invading cancer cells in carcinomas where they repress an overlapping set of epithelial specification markers. However, they participate at different stages, with Snail1 driving the EMT process and ZEB1 maintaining it (Shirakihara et al., 2007; Taube et al., 2010; Dave et al., 2011). Cells in the dermomyotome also undergo an EMT to delaminate and migrate into the primary myotome (Braun et al., 2011). In fact, Snail1 triggers an EMT in the dermomyotome and regulates the timing of its expression (Delfini et al., 2009). Our data support a similar temporal division of labour between Snail1 and ZEB1 during muscle differentiation.

Transcriptional repression by ZEB1 is mediated by the tethering of different corepressors whose identity varies in a tissue- and target gene-specific manner (reviewed in Sanchez-Tilló et al., 2011b). In the context of muscle gene expression, CtBP mediates repression of MEF2 targets by recruitment of HDAC9/MITR (Zhang et al., 2001). Our results both, in cell line-based systems and in *in vivo* mouse models demonstrated that CtBP mediates ZEB1 repression of muscle genes although to different degrees, being dispensable for the regulation of Tnni1 in *Xenopus* but largely

required in the case of MyoD in C3H10T1/2 fibroblasts. Similarly, lower dependence on CtBP for the repression of TnnT1 in C2C12 cells relative to C3H-10T1/2 fibroblasts corroborated the promoter and tissue specificity of the cofactors involved in ZEB1-mediated repression. Nevertheless, while CtBP accounts for the largest share of ZEB1 repression of muscle differentiation genes, ZEB1-mediated repression also involved CtBP-independent mechanisms that remain to be elucidated for each of these genes. In line with this, repression of E-cadherin by ZEB1 involves other cofactors such as BRG1 although it still remains unclear whether recruitment of BRG1 is direct or is rather mediated through BRG1-associated factors (BAF170, BAF57) known to interact with the coREST complex that includes CtBP itself (Wang et al., 2007; Sanchez-Tilló et al., 2010). In fact, BRG1, a member of the SWI/SNF family of chromatin remodelling factors, facilitates incorporation of the MyoD-BAF60c complex on the regulatory elements of MyoD-target genes in myoblasts prior to activation of transcription (Forcales et al., 2012).

CtBP also mediates other ZEB1 functions. For instance, CtBP mediates ZEB1 inhibition of senescence in cancer cells, an important tumour suppressor mechanism (De Barrios et al., 2017). We have found that, SCs from *Zeb1*+/- muscles exhibit increased senescence when they become activated in culture. In line with the results observed in C2C12 cell line, these *Zeb1*+/- SCs cultures also exhibit a premature activation and differentiation by increasing the population of $MyoD^{+}$ cells. Muscle regeneration and SCs senescence are closely linked. For instance, PRMT7 methyltransferase is required for muscle stem cell self-renewal and regeneration in vivo and in addition, its deletion causes senescence of activated SCs in young mice (Blanc et al., 2016). Of note, cellular reprogramming to pluripotency is currently being explored as a potential strategy for in vivo tissue regeneration of different tissues (Ocampo et al., 2016; Garreta et al., 2017). Controlled reprogramming of somatic cells into defined induced pluripotent cells (iPSCs) for their use in stem cell therapy of muscle dystrophies requires that iPSCs overcome senescence to maintain their stemness (Banito et al., 2009). Furthermore, SCs have arisen as a major cell for in vivo reprogramming in muscle through different senescence-associated factors (e.g. IL-6) secreted by senescent cells after muscle injury (Chiche et al., 2016). Small molecules such as cellular senescence attenuators or senescence/apoptosis regulators are widely used to generate iPSCs (Garreta et al., 2017).

Activation and cell fate commitment of SCs in culture has been associated to a poor regenerative potential (Montarras et al., 2005; Ikemoto et al., 2007). Importantly, *Zeb1*+/- SCs exhibit a defective muscle regeneration in *mdx* and CTX injury mouse models that is accompanied by the formation of abnormal myofibers. *Zeb1*+/- injured gastrocnemius undergoes delayed regeneration at early stages of CTX treatment. However, it expressed higher levels of *Myh3* by day 7 p.i. due to the greater damaged area in comparison to wild-type mice. These data are also in line with the poorer engraftment and myofiber formation by *Zeb1*+/- SC transplant into *mdx* hosts.

In healthy young muscles, SCs maintain a balance between their self-renewal and differentiation that is altered during ageing and in certain pathological conditions. How far stem cells can regulate their quiescence remains unknown. Many proteins and microRNAs participate in the maintenance of quiescence in SCs, e.g., FOXO3, mTORC1, miR-431 or PTEN among others (Rodgers et al., 2014; Gopinath et al., 2014; Consalvi et al., 2015; Wu et al., 2015; Yue et al., 2017). Interestingly, ZEB1 activates mTOR expression in mouse embryo fibroblasts (Liu et al., 2013). Alternatively, ZEB1 may also activate Notch signalling through induction FOXO3. At the time of isolation, *Zeb1*+/- SCs expressed lower levels of *Foxo3*. *Foxo3*-deficient SCs express lower levels of Notch1 and Notch3 receptors and, like *Zeb1*+/- SCs, are unable to maintain their quiescence and prematurely differentiate in culture and express lower levels of Notch target genes *Hes1* and *Hes6.*

Notably, while full levels of *Zeb1* expression maintain quiescence in SCs, just a partial downregulation of *Zeb1* in SCs to half the levels in the wild-type ones was sufficient to drive their activation and differentiation. Likewise, activation of wild-type SCs in culture was accompanied by the downregulation of *Zeb1* to the same levels than those found in *Zeb1*+/- SCs. In that line, in a transgenic mouse model of lung cancer, partial downregulation of ZEB1 in cancer cells —from the deletion of one *Zeb1* allele— was sufficient to block the transition from lung adenoma to adenocarcinoma, indicating that ZEB1's pro-tumour role in cancer cells depends on a fine threshold of its expression (Liu et al., 2014). Our data in muscle showed that the function of ZEB1 in SCs also depends on a similarly narrow threshold of expression. *Zeb1*+/- SCs still express about half of *Zeb1* levels found in wild-type counterparts but, importantly, this downregulation was enough to render *Zeb1*+/- SCs unable to

maintain their quiescence. Likewise, at the time of isolation (0 h), *Zeb1*+/- SCs express about half the levels of *Pax7*, *Foxo3a*, *Hes* genes, and *p57/Cdkn1c* (Figure 57).

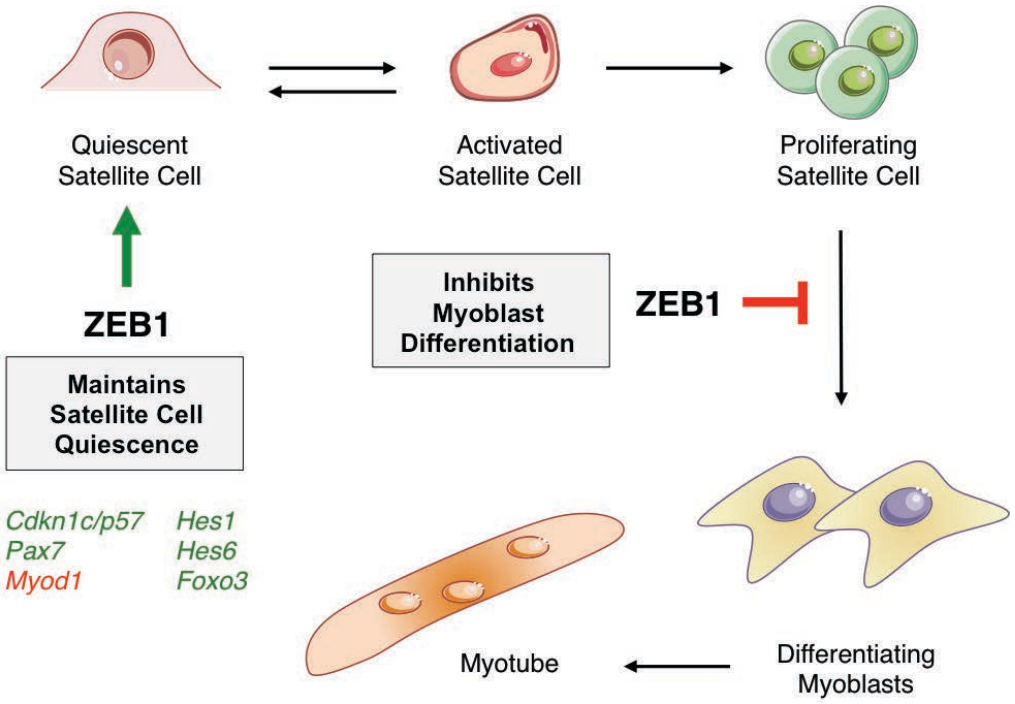


Figure 57. ZEB1 promotes SCs quiescence and inhibits myoblast differentiation. *Zeb1*+/- SCs become rapidly activated when cultured. ZEB1 activates quiescence-related genes in SCs (green) and represses *Myod1* (red).

Muscle injury prompts the activation of SCs that eventually differentiate and regenerate the damaged area. The myogenic progression of SCs depends on a timely balance between the inflammatory reaction produced by muscle damage that activates SCs and the subsequent anti-inflammatory response that promotes SC proliferation and differentiation (Kharraz et al., 2013). We found that ZEB1 was expressed in the centrally-located nuclei of regenerating myofibers. Its expression is upregulated in human and mouse dystrophic muscles as well as in response to acute injury. Interestingly, in both types of muscle damage used in this study, ZEB1 became translocated to the cytoplasm in some undamaged myofibers. Thus, increased ZEB1 in dystrophic human and *mdx* muscles is likely to correspond to its expression in both centrally nucleated and ZEB1^{+} cytoplasmic myofibers. Although ZEB1 has also been noted in the cytoplasm of cancer cells, the significance of ZEB1's ectopic expression remains elusive (Graham et al., 2010; Lehmann et al., 2016). Once at the cytoplasm, ZEB1 may no longer plays its function in the nucleus as a transcription factor (loss-

of-function) and/or it may gain a new and still undetermined function (gain-of-function) unrelated to its traditional role as a DNA binding protein. Interestingly, despite the upregulation of ZEB1 in dystrophic muscles, low levels of cytoplasmic ZEB1 correlate with an increase in myofibers damage and in CKs serum levels, an indicator of myofiber degeneration. Given that ZEB1 protects from muscle damage, modulation of ZEB1 expression and/or cellular distribution could represent a strategy to regulate and ameliorate muscle dystrophy.

The plasminogen activation system is a mechanism that promotes tissue remodelling in homeostasis and in many pathological conditions including skeletal muscle regeneration (Suelves et al., 2005). The protease urokinase plasminogen activator (uPA), is induced in *mdx* muscles and its ablation exacerbates the dystrophic phenotype (Suelves et al., 2007). uPA and plasmin promote cellular infiltration and enhance muscle repair when injured (Suelves et al., 2001), but also can improve the invasive capacity of carcinoma cells. Interestingly, ZEB1 upregulates uPA expression and inhibits that of its inhibitor PAI-I in the murine intestine and in cancer cells by direct binding onto its promoter (Sánchez-Tilló et al., 2013). Other extracellular matrix remodelling proteins are strongly upregulated during *mdx* disease showing its importance on the pathological progression (Marotta et al., 2009).

CCL2-mediated signalling is important not only during injury but also in repair by recruiting $CCR2^{+}$ macrophages, which contribute to muscle regeneration (Lu et al., 2011a; Lu et al., 2011b; Lesault et al., 2012; Tidball, 2017). We found that, compared to *Zeb1*+/+ counterparts, *Zeb1*+/- mice displayed enhanced damaged when crossed with *mdx* mice or in acute injury models. Furthermore, *Zeb1*-deficient muscles triggered a more intense and prolonged inflammatory reaction ($F4/80^{+}/Ly6C^{high}$) and an upregulation of the CCL2/CCR2 axis upon injury. However, although *Zeb1*-deficient muscles upregulate *Ccr2*, their regeneration was impaired likely due to the high levels of inflammation originated in *Zeb1*+/- injured muscle and the inefficiency of *Zeb1*+/- SCs to regenerate after multiple rounds of muscle damage. These data support a role for ZEB1 regulating the timely balance and promoting the transition between the inflammatory response and its resolution (Figure 58).

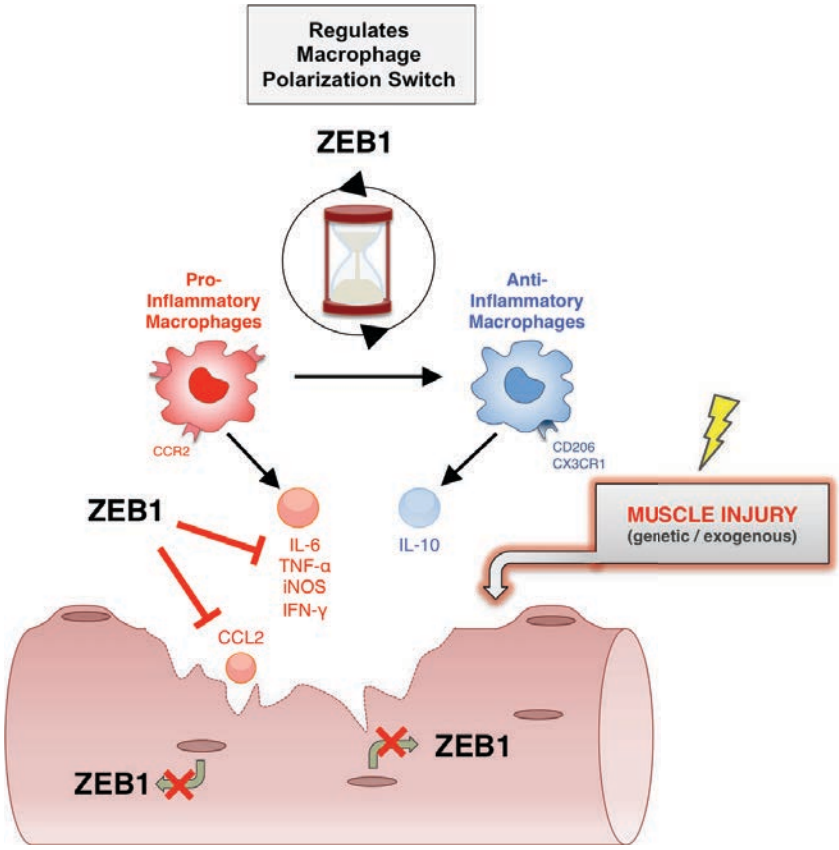


Figure 58. ZEB1 is required for the balance and transition from pro-inflammatory to anti-inflammatory macrophages in response to muscle injury. Injured *Zeb1+/-* muscle exhibits an increased inflammatory response and secretion of pro-inflammatory cytokines. The switch to an anti-inflammatory environment is delayed resulting in defective muscle regeneration.

$CCR2^{+}$ infiltrating macrophages at the sites of injury contribute to phagocytosis and produce high levels of IGF-1 that promotes muscle regeneration (Lu et al., 2011b). IGF has powerful and conserved mitogenic effects on muscle cells across species (Castillo et al., 2004) and it is important both during proliferation and during differentiation (Azizi et al., 2016). In mammals and fishes, IGF-1 is a more potent stimulator of proliferation than insulin (Montserrat et al., 2012). *Ccr2-/-* mice express low levels of IGF-1 due to the deficiency in macrophages recruitment and hence account for a poor regenerative capacity (Lu et al., 2011b).

The inflammatory environment during the first hours after injury is critical to trigger SC-driven myogenesis. Therefore, the regulation of inflammation without affecting muscle regenerative capacity is an important aim in the therapy of muscle dystrophies. However, the coregulation between these two steps has only recently

emerged (Tidball, 2017). Several cytokines during muscle injury are de-regulated in *Zeb1+/-* gastrocnemius (Figure 58). IFN-γ has been postulated as an important coordinator in the first stages of muscle regeneration. Blockade of IFN-γ signalling in injured muscle reduces the activation of the pro-inflammatory macrophages and shifts to an anti-inflammatory phenotype (Villalta et al., 2011). *Zeb1+/-* injured muscle exhibit elevated levels of *Ifng* 7-days post-CTX injection. Also IL-10, which promotes an anti-inflammatory phenotype, is reduced in the early stages of *Zeb1*-deficient CTX-injured muscle. In addition, IL-6 and TNF-α have been described to act promoting muscle damage but also indispensable when regeneration occurs for the transition to the early differentiation stage (Chen et al., 2007; Serrano, et al., 2008; Bencze et al., 2012; Kharraz et al., 2013; White et al., 2016). Serum Il-6 increases in ageing animals, suggesting a role in the immunomodulation of developmental fate of SCs (Tierney et al., 2014). Moreover, IL-6 increases proliferation of myoblasts but not their fusion in culture conditions (Tidball & Villalta, 2010). Consistently with the above-described results, *Zeb1*-deficient mice display elevated levels of *Il6* and *Tnf* during muscle injury and therefore decoupling muscle damage and inflammation from regeneration. This phenotype can also be involved in the possible impairment of myofibers formation. In addition, our results indicate that the role of ZEB1 in the regulation of inflammation and regeneration are intrinsic to the specific phenotypes that ZEB1 drives in macrophages and SCs, respectively, as the independent transplant of these two cell types into wild-type mice demonstrated.

Given the complexity of the interactions between damaged muscle and myeloid cells, treatment of chronic muscle diseases need to preserve the correct balance between pro- and anti-inflammatory signals. DMD is commonly treated with anti-inflammatory corticosteroids (e.g., prednisone) that reduce muscle damage and weakness by inhibiting macrophage and other myeloid cells recruitment (Villalta et al., 2009; Pichavant et al., 2011). Glucocorticoids can also affect macrophage phenotype by promoting a shift to an anti-inflammatory state (Villalta et al., 2009). Transplantation of macrophages into muscle is also being considered as a potential therapy to improve muscle regeneration and function in chronic disease (Lesault et al., 2012). Macrophage polarization towards an anti-inflammatory phenotype *in vivo* may help the engraftment of transplanted stem cells ($Pax7^+$ cells) into dystrophic muscle. Therefore, therapeutic advances rely on an improved understanding of

macrophage function and on the molecular mechanisms regulating the switch between them favouring muscle regenerative potential.

In sum, our data set ZEB1 as an important regulator of muscle integrity and homeostasis both during embryonic myogenesis as well as in muscle injury and repair. They also establish ZEB1 as a potential therapeutic target in human dystrophies and other conditions where muscle regeneration is compromised.

CONCLUSIONS

CONCLUSIONS

The results obtained in this dissertation show that ZEB1 regulates muscle differentiation and participates in muscle homeostasis by controlling the response after damage. Knockdown of *Zeb1* in C2C12 cell line accelerates myotube formation and *Zeb1*-deficient SCs activate prematurely in culture loosing its regenerative potential due to precocious differentiation. This precocious activation occurs through down-regulation of SCs quiescence-associated genes and expression of MyoD. Furthermore, enhanced inflammation in *Zeb1*-deficient injured gastrocnemius increases muscle damage and delays their regeneration. This phenotype contribute to an exhaustion of the regenerative capacity of SCs and hence the impairment of myofiber formation.

From the results presented in this dissertation, I can conclude that:

1. ZEB1 inhibits muscle differentiation in cell line-based systems and during embryonic development in a stage-dependent manner by recruitment of CtBP and displacement of MyoD from the regulatory regions of muscle differentiation genes.
2. ZEB1 is upregulated in injured muscle (genetic or exogenous) and is expressed in centrally-located nuclei of regenerating myofibers and in the cytoplasm of undamaged myofibers.
3. ZEB1 protects muscles from infiltration by inflammatory macrophages, prevents myofiber damage and favours the transition towards an anti-inflammatory response.
4. Full levels of ZEB1 are required for SCs to maintain their quiescence and for efficient muscle regeneration upon injury or when transplanted.

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APPENDIX I

The data presented in chapter I from results of this dissertation resulted in the publication of the following article:

- **Siles, L.,** Sánchez-Tilló, E., Lim, J. W., Darling, D. S., Kroll, K. L., & Postigo, A. (2013). ZEB1 imposes a temporary stage-dependent inhibition of muscle gene expression and differentiation via CtBP-mediated transcriptional repression. *Molecular and cellular biology*, *33*(7), 1368-1382.

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ZEB1 Imposes a Temporary Stage-Dependent Inhibition of Muscle Gene Expression and Differentiation via CtBP-Mediated Transcriptional Repression

Laura Siles,[a] Ester Sánchez-Tilló,[a] Jong-Won Lim,[b] Douglas S. Darling,[c] Kristen L. Kroll,[b] Antonio Postigo[a,d,e]

Group of Transcriptional Regulation of Gene Expression, IDIBAPS, Barcelona, Spain[a]; Department of Developmental Biology, Washington University School of Medicine, St. Louis, Missouri, USA[b]; Department of Oral Health and Center for Genetics and Molecular Medicine, University of Louisville, Louisville, Kentucky, USA[c]; James Graham Brown Cancer Center, Louisville Health Care Center, Louisville, Kentucky, USA[d]; ICREA, Barcelona, Spain[e]

Skeletal muscle development is orchestrated by the myogenic regulatory factor MyoD, whose activity is blocked in myoblasts by proteins preventing its nuclear translocation and/or binding to G/C-centered E-boxes in target genes. Recent evidence indicates that muscle gene expression is also regulated at the *cis* level by differential affinity for DNA between MyoD and other E-box binding proteins during myogenesis. MyoD binds to G/C-centered E-boxes, enriched in muscle differentiation genes, in myotubes but not in myoblasts. Here, we used cell-based and *in vivo Drosophila*, *Xenopus laevis*, and mouse models to show that ZEB1, a G/C-centered E-box binding transcriptional repressor, imposes a temporary stage-dependent inhibition of muscle gene expression and differentiation via CtBP-mediated transcriptional repression. We found that, contrary to MyoD, ZEB1 binds to G/C-centered E-boxes in muscle differentiation genes at the myoblast stage but not in myotubes. Its knockdown results in precocious expression of muscle differentiation genes and acceleration of myotube formation. Inhibition of muscle genes by ZEB1 occurs via transcriptional repression and involves recruitment of the CtBP corepressor. Lastly, we show that the pattern of gene expression associated with muscle differentiation is accelerated in *ZEB1*$^{-/-}$ mouse embryos. These results set ZEB1 as an important regulator of the temporal pattern of gene expression controlling muscle differentiation.

Muscle gene expression is orchestrated by a small set of myogenic regulatory factors (MRFs), namely, Myf-5, MyoD, myogenin, and MRF4, that bind to E-box sequences (CANNTG) in the regulatory regions of target genes by forming homodimers with themselves or heterodimers with E proteins (reviewed in references 1 and 2). During myogenesis, there is a division of labor among MRFs, with Myf-5 and MyoD determining lineage commitment and myogenin driving terminal differentiation of myoblasts into myotubes (1). In addition, there is also a regulatory interplay among MRFs, as Myf5 and MyoD induce myogenin, which in turn regulates MRF4, while MyoD and myogenin can activate their own expression. Overexpression of MRFs in nonmuscle cells (e.g., fibroblasts) is sufficient to induce a number of muscle markers and, to different degrees, drive a myogenic differentiation program (3–5).

However, MRFs cannot explain by themselves the sophisticated pattern of temporal and spatial gene expression during myogenesis. Determination and terminal differentiation of skeletal muscle precursors require the concerted action of MRFs with many other proteins, which modulate the expression, intracellular localization, and/or transcriptional activity of MRFs (1, 6). For instance, in myoblasts, MyoD does not induce downstream muscle differentiation target genes, as its function is temporarily blocked through multiple mechanisms. *Inter alia*, the interaction of MyoD (and/or its E protein partners) with a number of factors prevents MyoD from entering the nucleus (e.g., interaction of MyoD with I-mfa/MDFI), from binding to DNA (e.g., with Twist, Id, and Mist-1), and/or from activating its targets (e.g., with Mist-1 and MyoR) (7–11). However, these negative regulatory factors are still not sufficient to account for the differential gene expression signature in myoblasts and myotubes (1, 2, 6).

Recent works have revealed that, independently of their squelching by proteins like Twist or Id, binding of MRFs to DNA during muscle differentiation is also controlled at the *cis* level. Differential affinity of MyoD vis-à-vis other E-box binding proteins for E-box sequences dictates their occupancy in myoblasts and myotubes (12, 13). Binding of MyoD to MyoD-specific E-boxes promotes transcriptional activation of muscle differentiation genes much more strongly than does MyoD binding to E-box sequences that are also shared with NeuroD2, another basic helix-loop-helix (bHLH) protein that regulates neuronal differentiation (12). In addition, E-box sequence specificity, e.g., G/C versus A/T as central nucleotides, determines DNA occupancy during muscle differentiation (13). In myoblasts, MyoD binds to A/T-centered E-boxes in genes associated with cell proliferation but does not interact with G/C-centered E-boxes that are enriched in the regulatory regions of muscle differentiation genes (13). The latter E-boxes are occupied during the myoblast stage by transcriptional repressors of the Snail family (13). As differentiation progresses, Snail factors are displaced from these E-boxes by MyoD, thus inducing the expression of a muscle differentiation gene signature (13).

Another transcriptional repressor that specifically recognizes G/C-centered E-boxes is ZEB1 (also known as δEF1) (reviewed in references 14 and 15). Like Snail factors, ZEB1 is best known for its

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Address correspondence Antonio Postigo, idib412@clinic.ub.es.

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role in cancer progression, where it triggers an epithelial-to-mesenchymal transition (EMT), endowing cancer cells with a proinvasive phenotype (14, 15). ZEB1 is expressed in the epithelium of the undifferentiated somite and in the dermomyotome during mouse and chick embryogenesis (16, 17). Mutation and overexpression of its ortholog in *Drosophila melanogaster*, zfh-1, leads to altered somatic musculature formation (18, 19). Interestingly, ZEB1 is a downstream target of both MyoD and Snail factors (20–23). In *in vitro* overexpression experiments, ZEB1 competes with bHLH and Snail factors for binding to E-boxes in different genes (e.g., those for immunoglobulin heavy chain enhancer, α4 integrin promoter, CD4 proximal enhancer/promoter, and p73 promoter/intron 1) (24–28).

This pattern of ZEB1 expression during development and its exclusive affinity for G/C-centered E-boxes prompted us to question whether endogenous ZEB1 is regulating muscle gene expression. Using a number of cell-based and *in vivo* models, we show here that ZEB1 imposes a temporary stage-dependent inhibition of muscle gene expression and differentiation via CtBP-mediated transcriptional repression. ZEB1 knockdown triggers early protein and mRNA expression of muscle determination and differentiation genes and, accordingly, precocious myotube conversion. In a reverse pattern with respect to MyoD, ZEB1 binds to G/C-centered E-boxes in muscle differentiation genes at the myoblast stage but not in myotubes. Negative regulation of muscle genes by ZEB1 in myoblasts occurs via transcriptional repression. In both cell-based systems and *Xenopus laevis* and *Drosophila* embryos, we show that this repression involves an interaction of ZEB1 with the CtBP corepressor. Finally, we found that the temporal pattern of muscle differentiation gene expression is accelerated in *ZEB1*$^{-/-}$ mouse embryos. Together, these results indicate that endogenous ZEB1 plays an important role regulating the temporal pattern of gene expression during muscle differentiation.

MATERIALS AND METHODS

Cells, cell culture, and transfections. C2C12 and C3H-10T1/2 cells were obtained from the American Tissue Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza) supplemented with 12% fetal bovine serum (FBS) (Sigma). Cells were transiently transfected with expression or reporter vectors by using Lipofectamine 2000 (Life Technologies) and/or with small interfering RNA (siRNA) oligonucleotides by using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions.

Antibodies. The following commercial antibodies were used in this work: ZEB1 (H-102; Santa Cruz Biotechnology [SCBT]), sarcomeric pan-myosin (MF-20; Development Studies Hybridoma Bank [DSHB]), myosin heavy chain IIa (MyH2) (SC-71; DSHB), myosin heavy chain IIb (MyH4) (BF-F3; DSHB), MyoD (C-20; SCBT), myogenin (F5D; BD Pharmingen), CtBP1/2 (E-12; SCBT), and α-tubulin (B5-1-2; Sigma) antibodies. Antibodies against *Drosophila* muscle markers were obtained as follows: mouse anti-myosin heavy chain (anti-MHC) antibody was a gift from D. Kiehart (Duke University), and rabbit anti-MEF2 antibody was a gift from B. M. Patterson (National Cancer Institute, NIH). Secondary horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG and goat anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch (JIR). For blocking in immunostaining assays, normal serum from the host species of the secondary antibody was purchased from JIR. As an IgG control for chromatin immunoprecipitation (ChIP) assays, normal rabbit IgG and normal goat IgG-containing serum were purchased from SCBT and JIR, respectively.

RNA interference. The three set of siRNAs used in this study to target mouse ZEB1 originated as follows. The siRNA duplex referred to as si1ZEB1, with the sense sequence 5′-GACCAGAACAGUGUUCCAUGUUUAA-3′, was purchased from Life Technologies as a Select RNAi siRNA (MSS210696) guaranteed to have no off-target effects. si2ZEB1 (sense sequence 5′-AACUGAACCUGUGGAUUAU-3′) was described previously (29). Finally, the siRNA referred to as si3ZEB1 was purchased from SCBT (catalog number sc-38644) and consisted of a pool of three different siRNA duplexes of the following sense sequences: 5′-GAAGAACCCUUGAACUUGU-3′, 5′-GAACAGUGUUCCAUGUUUA-3′, and 5′-CAACCAUGAAGGAUCUAUA-3′. As negative controls in interference experiments, the following siRNAs were used: 5′-UAUAGCUUAGUUCGUAACC-3′ and Select RNAi siRNA LO GC (catalog no. 12935-200; Life Technologies). In Western blot and quantitative real-time PCR (qRT-PCR) studies, an additional control siRNA targeting firefly luciferase (5′-GAUUAUGUCCGGUUAUGUA-3′) (30) was also used. The siRNA duplex against CtBP1/2 with the sense sequence 5′-GAACUGTGUCAACAAGGAC-3′ was previously described (31).

Plasmids. Expression vectors used in the study were obtained from the following researchers: full-length mouse ZEB1 was obtained from M. Saito (Tokyo University, Japan) (32), full-length mouse ZEB1 with mutated CtBP binding sites was obtained from Y. Higashi (Institute for Developmental Research, Kasugai, Japan) (33), and pEMSV-MyoD was from the late H. Weintraub (Fred Hutchinson Cancer Research Center, Seattle, WA) (3). The *Xenopus* MyoD construct, containing the full-length cDNA plus the 5′ and 3′ untranslated regions and cloned into pSP64T, was obtained from J. Gurdon (Gurdon Institute, Cambridge, United Kingdom). Simian virus 40 (SV40)–β-galactosidase (β-gal) was purchased from Promega, pcDNA3 was purchased from Invitrogen, and the pBluescript SK vector was purchased from Stratagene-Agilent. Firefly luciferase reporters for the promoters used in this article were provided by the following researchers: 5.5 kb of rat α-MHC promoter was obtained from E. N. Olson (University of Texas Southwestern, Dallas, TX), (34), 2.3 kb of quail troponin I was obtained from S. Konieczny (Purdue University, West Lafayette, IN) (35), a bp −1256 mouse muscle creatine kinase (MCK) promoter was obtained from S. A. Leibovitch (INRA, Montpellier, France) (36), and a 4-kb enhancer plus a 2.7-kb promoter of the human MyoD gene were obtained from J. P. Capone (McMaster University, Hamilton, ON, Canada) (37).

Western blots. Western blot assays were performed as described previously (38). Briefly, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% SDS, 50 mM Tris [pH 8], 2 mM EDTA, plus protease inhibitors) and loaded onto polyacrylamide gels. Gels were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore). Following blocking for nonspecific antibody binding with 5% nonfat milk, membranes were incubated with the corresponding primary and HRP-conjugated secondary antibodies before the reaction was developed by using the Pierce ECL Western blotting substrate or SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific). Western blots shown in this study are representative of at least four independent experiments.

Myogenic conversion assays. For differentiation of C2C12 myoblasts into myotubes, cells were seeded into 6-well plates and grown in growth medium (GM) (DMEM plus 20% FBS) until they reached 80 to 90% confluence, at which time the GM was switched to differentiation medium (DM) (DMEM supplemented with 2% horse serum; Sigma) and maintained in DM for different periods. In selected experiments as specifically indicated, cells were maintained in GM even after reaching full confluence. Cells were fixed in −20°C-chilled methanol and blocked with 2% gelatin (from cold-water fish skin; Sigma) before being stained with pan-myosin antibody MF-20 for 2 h, washed with phosphate-buffered saline (PBS), incubated with HRP-conjugated anti-mouse IgG antibody (JIR), and developed with a 3,3′-diaminobenzidine (DAB) substrate kit (Vector Laboratories). Finally, cells were incu-

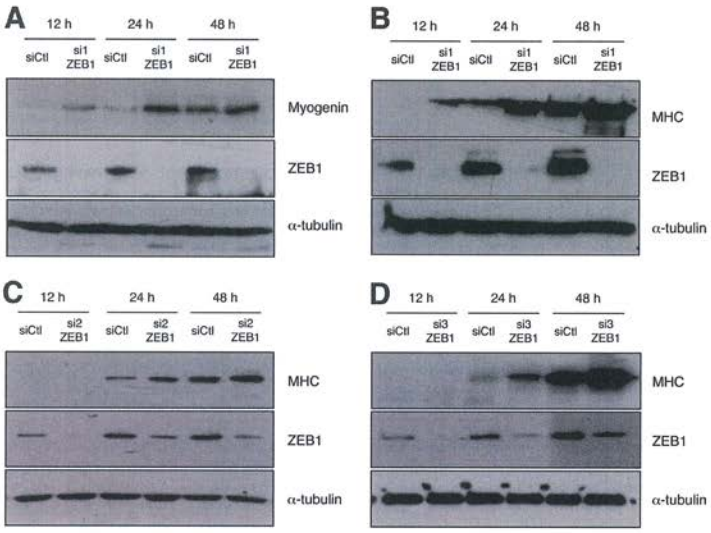


FIG 1 Knockdown of ZEB1 induces earlier protein expression of muscle differentiation genes. C2C12 cells, transfected with either 200 nM specific siRNAs against ZEB1 (si1ZEB1, si2ZEB1, or si3ZEB1) or a siRNA control (siCtl), were allowed to differentiate for up to 48 h after being switched to differentiation medium. At the indicated time points, cells were lysed and assessed by Western blotting for ZEB1 (H-102) and either myogenin (F5D) (A) or MHC (MF-20) (B to D) along with α-tubulin (B5-1-2) as a loading control.

bated for 30 s with hematoxylin for nucleus counterstaining and washed with PBS.

RNA extraction and quantitative real-time PCR. Total RNA from C2C12 cells and *Xenopus* ectodermal explants (animal caps) (see below) was extracted with the SV Total RNA isolation system kit (Promega) and TRIzol (Life Technologies), respectively. RNA was then used to synthesize cDNA by using a reverse transcription kit [random hexamers and GoScript (Promega) for C2C12 cell experiments and oligo(dT) and SuperScript II reverse transcriptase (Invitrogen) for *Xenopus* experiments]. mRNA levels were determined by qRT-PCR using either SYBR green/ROX (GoTaq; Promega) (for C2C12 cells) or iQSYBRGreen Supermix (Bio-Rad) (for *Xenopus* animal caps). Primers used to determine gene expression in C2C12 cells by qRT-PCR were as follows: mouse ZEB1 forward primer 5′-ACCCCTTCAAGAACCGCTTT-3′ and reverse primer 5′-CAATTGGCCACCACTGCTAA-3′, mouse myosin heavy chain IIa (MyH2) forward primer 5′-CGATGATCTTGCCAGTAATG-3′ and reverse primer 5′-ATAACTGAGATACCAGCG-3′ (39), mouse myosin heavy chain IIb (MyH4) forward primer 5′-TAAGCACGAGCGCAGAGTGAAGGAACT-3′ and reverse primer 5′-GCTGGATCTTACGGAACTTGGCCAGGT-3′ (40), mouse troponin T1 (TnnT1) forward primer 5′-GATTCTGTATGAGAGGAAAAAG-3′ and reverse primer 5′-TCATATTTCTGTTGCTTCAACTT-3′ (40), mouse myogenin forward primer 5′-CACTGGAGTTCGGTCCCAA-3′ and reverse primer 5′-TGTGGGCGTCTGTAGGGTC-3′ (41), mouse MyoD forward primer 5′-TGGGATATGGAGCTTCTATCGC-3′ and reverse primer 5′-GGTGAGTCGAAACACGGATCAT-3′ (42), mouse Myf-5 forward primer 5′-TCTGGTCCCGAAAGAACAGC-3′ and reverse primer 5′-CTTTTATCTGCAGCACATGCATT-3′ (43), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5′-AACGACCCCTTCATTGAC-3′ and reverse primer 5′-TCCACGACATACTCAGCAC-3′ (44). Primers used for qRT-PCR of *Xenopus* animal cap cells were as follows: EF1α forward primer 5′-CACCATGAAGCCCTTACTGA-3′ and reverse primer 5′-ACCTGTGCGGTAAAAGAACC-3′, TnnI1 (slow skeletal troponin I type 1) forward primer 5′-CAGTAGCATTCCAGGGCAGT-3′ and reverse primer 5′-TATGTAGCCCCAATGGGAAA-3′, TnnI2 (fast skeletal troponin I type 2) forward primer 5′-CTCTTCAGCGGGGATATTGA-3′ and reverse primer 5′-ATTTGAGCCCCTCCTTGAGT-3′, MCK forward primer 5′-ACAAACCAGTGTCCCCTCTG-3′ and reverse primer 5′-CCACACCAGGAAGGTCTTGT-3′, mActin (muscle actin) forward primer 5′-GCTGACAGAATGCAGAAG-3′ and reverse primer 5′-TTGCTTGGAGGAGTGTGT-3′, and MyoD forward primer 5′-GACCTGCCAATGTTGTGTTG-3′ and reverse primer 5′-CAAAAAGTGGTCCGCAAGTT-3′. Relative gene expression was calculated by the $\Delta\Delta C_T$ method, normalizing values relative to either GAPDH (C2C12 cells) or EF1α (*Xenopus* animal caps) as a reference gene. qRT-PCR data shown are representative of at least three independent experiments, with each point performed in triplicate, and are represented as fold change plus/minus standard deviation.

Chromatin immunoprecipitation assays. ChIP assays were performed as described previously (38), using an EpiQuick ChIP kit (Epigentek) according to the manufacturer's instructions. Briefly, C2C12 cells as either nonconfluent cycling myoblasts or terminally differentiated myotubes were incubated during 20 min with a 1% formaldehyde solution (Electron Microscopy) at room temperature, followed by incubation with 1.25 M glycine. Upon sonication of cell lysates, chromatin was immunoprecipitated with the corresponding specific or control antibodies, and amplification of DNA fragments was assessed by qRT-PCR. Identification of potential DNA binding sequences for ZEB1/MyoD and design of primers for qRT-PCR were conducted by using MacVector 12.5 software (MacVector Inc.). For the ZEB1/MyoD CACCTG site at position −1068 of the mouse MyH4 promoter, the primers used to amplify the region between bp −1107 and −1014 of this promoter were as follows: forward primer 5′-TATAAAAGATTTTACCTGCCA-3′ and reverse primer 5′-ATATTTTCAACCACTGTTCT-3′. For the ZEB/MyoD CAGGTG site at po-

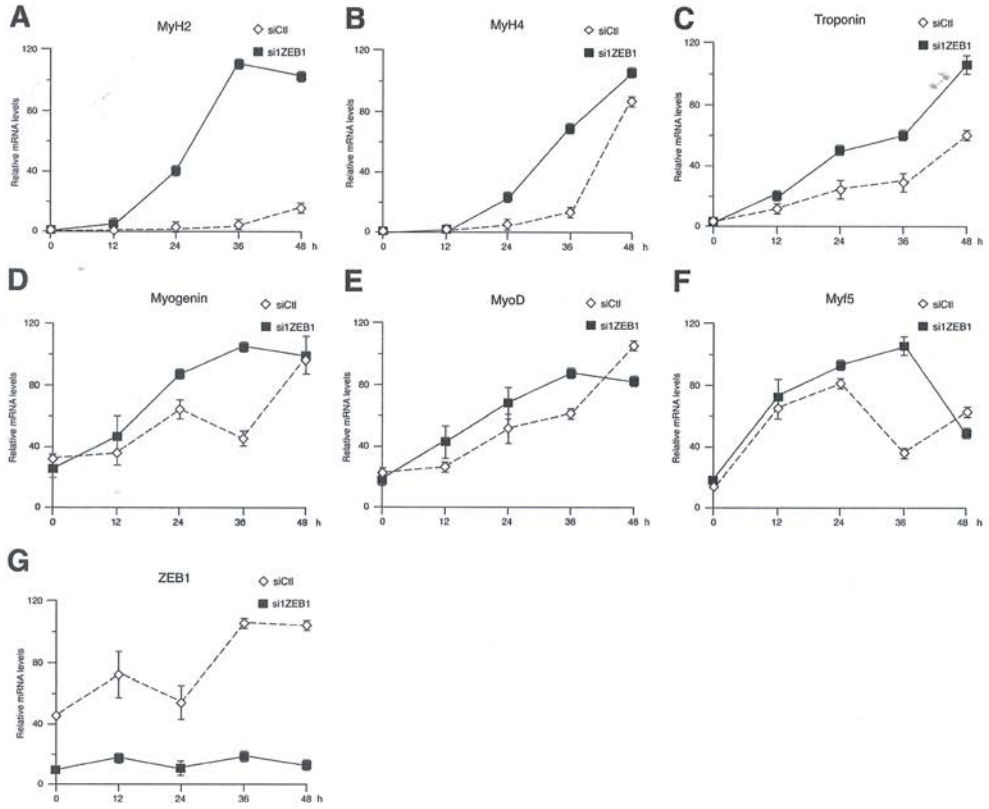
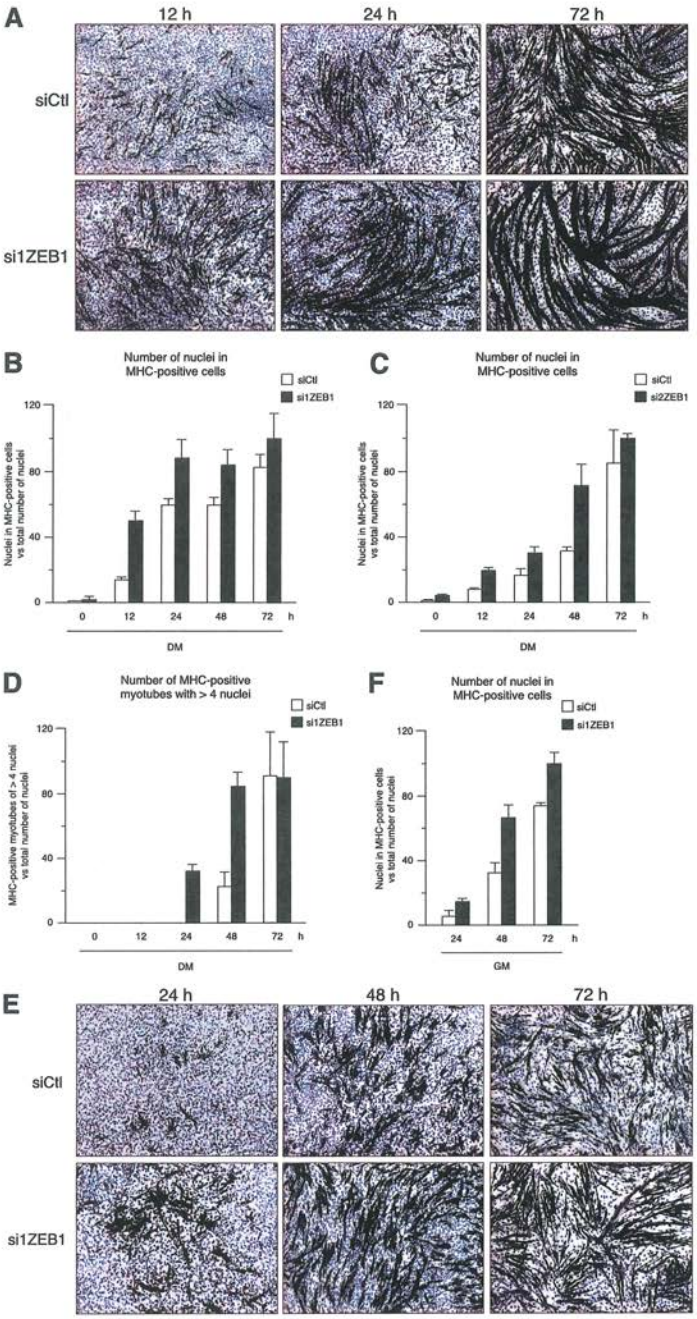


FIG 2 ZEB1 knockdown induces precocious mRNA expression of several muscle genes. As in Fig. 1, C2C12 cells knocked down for ZEB1 (si1ZEB1) or transfected with a siRNA control (siCtl) were allowed to differentiate for up to 48 h after being switched to differentiation medium. At the indicated time points, mRNA levels for MHC type IIa (MyH2) (A), MHC type IIb (MyH4) (B), troponin T1 (C), myogenin (D), MyoD (E), Myf5 (F), and ZEB1 (G) were determined by quantitative real-time PCR (qRT-PCR) relative to levels of GAPDH as a reference gene. qRT-PCR was conducted as detailed in Materials and Methods. Data shown are a representative case of four independent experiments.

sition −1046 of the mouse skeletal slow troponin T1 (TnnT1) promoter, the primers used to amplify the region between bp −1105 and −1020 of this promoter were as follows: forward primer 5′-TCTCAAACCAAAGC AAAACCAA-3′ and reverse primer 5′-AGTTCCCCGTACCTCATACTC T-3′. A 191-bp region of the mouse GAPDH promoter, lacking consensus binding sites for ZEB1/MyoD, was amplified by using forward primer 5′-AGCTACTCGCGGCTTTACG-3′ and reverse primer 5′-AAGAAGAT GCGGCCGTCTCT-3′, modified from those described previously (45). In all qRT-PCRs, values shown represent relative binding in relation to input and are the averages of data from two independent ChIP assays, each performed in triplicate, and representative of at least three experiments.

Transcriptional assays. Transcriptional assays were carried out as described previously (38). Briefly, cells were transfected with firefly luciferase reporter vectors and equal molar amounts of either expression plasmids encoding ZEB1 or MyoD or, as controls, the corresponding empty expression vector. As an internal control for transfection efficiency, 1 μg of SV40–β-gal was cotransfected at each point. Total DNA used in each cotransfection was equalized by adding the promoterless pBluescript SK vector as required. Firefly luciferase activity was assessed with a Luciferase Assay System kit (Promega Corporation), whereas β-galactosidase activity was determined with Luminescent β-Galactosidase Detection Kit II (Clontech). Relative luciferase units (RLU) were assessed with a Modulus II Glomax microplate detection system (Promega). RLU values were expressed as the means of duplicates and are representative of at least four independent experiments.

***Xenopus* embryo microinjection and *in situ* hybridization.** *Xenopus laevis* embryos were obtained by *in vitro* fertilization as described previously (46) and were staged according to methods described previously (47). Linearized pcDNA3-ZEB1$_{wt}$, pcDNA3-ZEB1$_{CtDmut}$, and pSP64T-MyoD expression vectors were used to produce capped RNA by using the SP6-Message Machine kit (Ambion). A morpholino oligonucleotide (MO) against *Xenopus* ZEB1 (48) (5′-AGATCTGCCAAAGTTGAGCGT TT-3′) was purchased from Gene Tools LLC. Where indicated, 200 pg of


A
12 h
24 h
72 h
siCtl
si1ZEB1
B
Number of nuclei in MHC-positive cells
siCtl
si1ZEB1
Nuclei in MHC-positive cells vs total number of nuclei
120
80
40
0
0
12
24
48
72
h
DM
C
Number of nuclei in MHC-positive cells
siCtl
si2ZEB1
Nuclei in MHC-positive cells vs total number of nuclei
120
80
40
0
0
12
24
48
72
h
DM
D
Number of MHC-positive myotubes with > 4 nuclei
siCtl
si1ZEB1
MHC-positive myotubes of > 4 nuclei vs total number of nuclei
120
80
40
0
0
12
24
48
72
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DM
F
Number of nuclei in MHC-positive cells
siCtl
si1ZEB1
Nuclei in MHC-positive cells vs total number of nuclei
120
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72
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GM
E
24 h
48 h
72 h
siCtl
si1ZEB1

ZEB1 RNA or 20 ng of ZEB1 MO was injected into one blastomere at the 2-cell stage, along with 20 pg of β-galactosidase RNA. Embryos were further cultured in 0.2× Marc's modified Ringer's (MMR) solution containing 4% Ficoll and 100 μg/ml gentamicin. For animal cap isolations, both cells of pigmented embryos at the 2-cell stage were injected with 100 pg of MyoD plus either 100 pg $ZEB1_{wt}$ or $ZEB1_{CIDmut}$ mRNA, and embryos were raised up to the blastula stage (stages 8 to 9). Twenty ectodermal explants per sample were then isolated and incubated at 25°C in 0.7× MMR supplemented with 1 mg/ml bovine serum albumin (BSA) and 100 μg/ml gentamicin. For *in situ* hybridization assays, embryos were grown to stages 15 to 16, fixed in minimum essential medium with formaldehyde, stained with Red-Gal (Research Organics), and processed as described previously (49).

***Drosophila* stocks, crosses, and immunostaining.** Full-length cDNA for zfh-1, obtained from Z. C. Lai (The Pennsylvania State University, Philadelphia, PA), was inserted into the NotI/XbaI sites of pUAST to generate independent stocks of upstream activation sequence (UAS)-zfh-1 flies (genotype, w^{118}; P{w^{+mC} = UAS-zfh-1.P}2B). UAS-zfh-1-CID_{mut} stocks are identical to UAS-zfh-1 stocks, except that the CtBP-interacting domain (CID) at ^{790}PLDLS^{796} is mutated to a nonbinding sequence, ^{790}ASASA^{796} (genotype, P{w^{+mC} = UAS-zfh-1-CIDm}2B). UAS-zfh-1 and UAS-zfh-1-CID_{mut} stocks are homozygous, viable, and fertile second-chromosome insertions deposited at the Bloomington Drosophila Stock Center (Bloomington, IN) under identification numbers 6879 and 6880, respectively. zfh-1 expression in UAS-zfh-1 and UAS-zfh-1-CID_{mut} was induced by crossing them with 24B-Gal4 or MEF2-Gal4 stocks obtained as a kind gift from M. Bates (University of Cambridge, United Kingdom). Embryos were allowed to develop at 25°C before being examined for expression of different proteins. After standard fixation, embryos were blocked with 50% normal goat serum in PBS and incubated with primary and secondary antibodies before the reaction was developed with a DAB substrate kit (Vector Laboratories), as described previously (19). Following color development, embryos were mounted in 80% glycerol and examined on a Zeiss Axioplan-2 microscope.

Mouse tissue immunohistochemistry. Mouse tissue samples corresponded to 4-μm sections from embryonic day 18.5 (E18.5) C57BL/6J wild-type (+/+) and δEF1 (ZEB1) null (−/−) embryos (17). Slides were subjected to deparaffination and hydration by using standard protocols, followed by heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0) for 15 min. Slides were then treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase before being incubated with a nonspecific binding blocking solution (5% normal goat or donkey serum depending on the host of the secondary antibody, 4% BSA, and 0.5% Tween 20 in PBS). Next, slides were incubated with the corresponding primary (overnight at 4°C) and HRP-conjugated (1 h at 37°C) antibodies diluted in blocking solution. The immunohistochemistry reaction was developed with the DAB substrate kit (Vector Laboratories), followed by counterstaining with hematoxylin, before being mounted in di-*N*-butylphthalate in xylene solution (DPX; Sigma) for microscopic examination.

RESULTS

Knockdown of ZEB1 induces precocious expression of muscle differentiation genes. The existence of proteins that bind and titrate out MyoD, thus preventing MyoD from prematurely activating its target genes in myoblasts, is well established (reviewed in reference 6). Recent evidence indicates that MyoD binding to DNA is also temporarily delayed through *cis*-level mechanisms to control the timing of muscle differentiation (13). In myoblasts, MyoD is excluded from G/C-centered E-boxes at muscle differentiation genes. MyoD only begins to occupy G/C-centered E-boxes and activate these genes as muscle differentiation progresses (13). Since ZEB1 is expressed early in development and binds exclusively to G/C-centered E-boxes, we questioned whether its knockdown in myoblasts would allow MyoD to bind these E-boxes and trigger a precocious expression of muscle differentiation genes.

To test this hypothesis, we made use of the C2C12 cell myogenic conversion model, widely employed in muscle gene expression studies (20, 50, 51). When grown in high serum (commonly referred as growth medium [GM]), C2C12 cells maintain a proliferating myoblast-like phenotype, and despite expression of MyoD, levels of proteins like the intermediate differentiation gene myogenin or the terminal differentiation markers myosin heavy chain (MHC) and troponin remain low or absent. Only when C2C12 cells exit the cell cycle upon reaching confluence and/or are switched into a low-serum medium (differentiation medium [DM]) do they fuse and terminally differentiate to form multinucleated MHC-positive myotubes.

We found here that knockdown of endogenous ZEB1 in C2C12 cells resulted in precocious expression of differentiation markers. Upon switching to DM, myogenin protein became detectable earlier in C2C12 cells knocked down for ZEB1 with a specific siRNA against ZEB1 (si1ZEB1) than in cells transfected with a siRNA control (siCtl) (Fig. 1A). Upregulation of MHC also occurred earlier and reached higher levels in C2C12 cells knocked down for ZEB1 with si1ZEB1 than in siCtl cells (Fig. 1B). A similar premature expression of MHC occurred when using two additional and independent siRNAs against ZEB1, here referred to as si2ZEB1 and si3ZEB1 (Fig. 1C and D).

The earlier induction of muscle genes following ZEB1 knockdown was also examined at the mRNA level (Fig. 2). Consistent with the above-described results, mRNA for MHC isoforms IIa (MyH2) and IIb (MyH4) accumulated more rapidly and to higher levels in ZEB1 knockdown C2C12 cells than in the counterpart siCtl control cells (Fig. 2A and B). In addition, mRNA expression

FIG 3 ZEB1 knockdown accelerates myotube conversion. (A) C2C12 cells transfected with 200 nM of a specific siRNA against ZEB1 (si1ZEB1) or a siRNA control (siCtl) were grown to confluence in growth medium (GM) (denoted time zero) before being switched to differentiation medium (DM) and allowed to differentiate for up to 72 h in DM. At the indicated time points, cells were fixed and immunostained for MHC (MF-20). Nuclei were counterstained with hematoxylin. Magnification, ×4. Captures shown are representative of five independent experiments with si1ZEB1 and siCtl. For all panels, the number of C2C12 cells throughout the myogenic conversion assay was the same under the ZEB1 knockdown (si1ZEB1, si2ZEB1, and si3ZEB1) and control (siCtl) conditions, as assessed by trypan blue staining (at the beginning of the assays) and by scoring the number of nuclei counterstained with hematoxylin (at the different endpoints) (total cell counts are not shown). (B) Quantification of the number of nuclei in MHC-positive cells with respect to the total number of nuclei in the myogenic conversion experiments shown in panel A. A value of 100 was arbitrarily set for comparison. Counts in panels B to D and F are the averages of four representative fields. (C) Same as panel B but with si2ZEB1. Similar results were obtained with si3ZEB1 (not shown). (D) Quantification of the number of MHC-positive myotubes with more than 4 nuclei with respect to the total number of nuclei. Similar results were obtained for si2ZEB1 and si3ZEB1 (data not shown). (E) Knockdown of ZEB1 also accelerates myotube formation in C2C12 cells maintained in GM. As for panel A, C2C12 cells were transfected with siRNAs against ZEB1 or the siRNA control, but upon reaching confluence, cells were maintained for the rest of the experiment in GM. Time zero refers to the time point when cells reached confluence and corresponds to the time point in panels A to D when cells were switched from GM to DM. (F) Quantification of the number of nuclei in MHC-positive cells with respect to the total number of nuclei in myogenic conversion assays where cells were maintained in GM for the entire experiment, as described above for panel E.

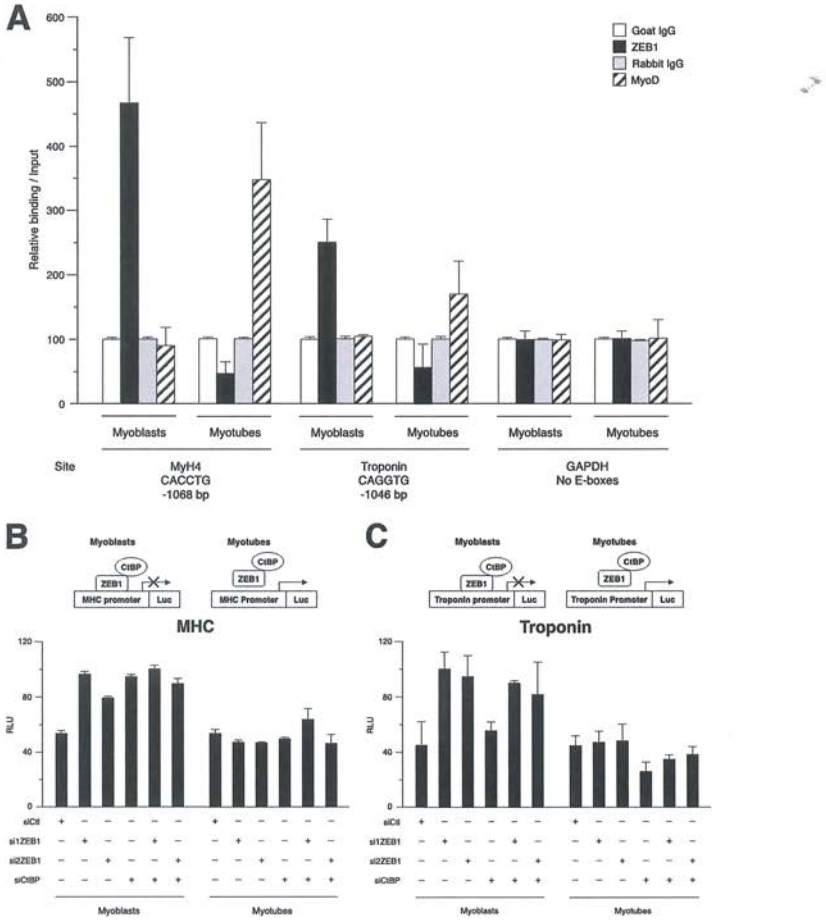

FIG 4 ZEB1 binds to G/C-centered E-boxes in muscle differentiation genes whose transcription is repressed largely through a CtBP-dependent mechanism. (A) ZEB1 and MyoD display differential binding to G/C-centered E-boxes in muscle differentiation genes in myoblasts and myotubes. Shown are data for qRT-PCR of fragments of the mouse Myh4, troponin T1, and GAPDH promoters immunoprecipitated in ChIP assays from C2C12 myoblasts and myotubes with ZEB1 antibody (E-20X), MyoD antibody (C-20), or their respective IgG controls. ChIP assays were performed as described in Materials and Methods. Values represent binding relative to the input averaged from two experiments, each performed in triplicate, and are representative of at least three essays. (B) The basal transcriptional activity of the MHC promoter is under negative regulation by ZEB1 and CtBP in myoblasts but not in myotubes. Nonconfluent cycling C2C12 myoblasts or confluent myotubes allowed to terminally differentiate for 48 h were cotransfected with 100 nM control siRNA (siCtl) or siRNAs against ZEB1 (si1ZEB1 and si2ZEB1) or CtBP (siCtBP) or equal molar amounts of their different combinations along with 0.4 μg of a luciferase reporter for the MHC promoter. Transcriptional assays and assessment of relative luciferase units (RLU) in panels B to E, G, and H were performed as described in Materials and Methods and are representative of at least four independent experiments. The knockdown efficiencies of si1ZEB1, si2ZEB1, and siCtBP in C2C12 cells are shown in panel F and in Fig. 1A to C. (C) Same as panel B but with 0.2 μg of the troponin I promoter as a luciferase reporter. (D) Same as panel B but with 0.2 μg of the MCK promoter as a luciferase reporter. (E) Same as panel B but with 0.2 μg of the MyoD enhancer/promoter. (F) Knockdown efficiency of siRNAs against CtBP and ZEB1 in C2C12 and C3H-10T1/2 cells. (Left) C2C12 cells were transfected with 100 nM either siCtl or siCtBP. CtBP protein levels were assessed by Western blotting using an antibody against CtBP1/2 (E-12) along with α-tubulin (B5-1-2) as a loading control. (Middle) C3H-10T1/2 cells were transfected with 200 nM either siCtl or si1ZEB1. ZEB1 protein levels were assessed by Western blotting using an antibody against ZEB1 (H-102) along with α-tubulin (B5-1-2) as a loading control. (Right) Same as the left panel but with C3H-10T1/2 cells. (G) Muscle genes are also under negative regulation by ZEB1 and CtBP in the C3H-10T1/2 myogenic conversion model. C3H-10T1/2 fibroblasts were cotransfected with luciferase reporters for muscle gene promoters, as in panels B to E, plus either 100 nM siRNA control (siCtl) or specific siRNAs against ZEB1 (siZEB1) or CtBP1/2 (siCtBP). The knockdown efficiencies of si1ZEB1 and siCtBP in C3H-10T1/2 fibroblasts are shown in panel F. (H) Transcriptional repression of muscle genes by ZEB1 depends on CtBP. C3H-10T1/2 fibroblasts were cotransfected with luciferase reporters for muscle gene promoters as in panel G, 0.6 μg of an expression vector for MyoD, and combinations of either 0.4 μg of an expression vector for ZEB1 or equal molar amounts of its corresponding empty expression vectors plus either 100 nM siRNA control (siCtl) or a specific siRNA against CtBP1/2 (siCtBP).

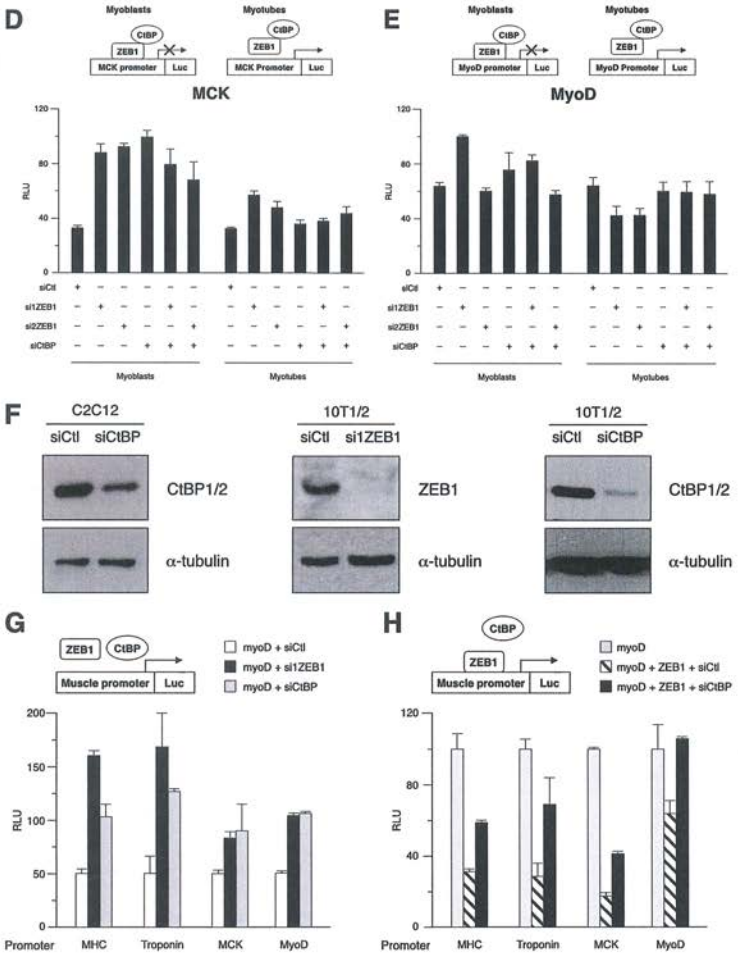

FIG 4 continued

levels of the differentiation genes for troponin and myogenin and the determination genes for MyoD and Myf-5 also displayed an earlier induction profile in ZEB1-knocked-down cells than in controls (Fig. 2C to G). Together, these results indicate that ZEB1 knockdown induces premature induction of muscle genes and suggest that endogenous ZEB1 may impose a delay on the ability of MRFs to drive muscle differentiation.

ZEB1 knockdown accelerates myotube formation. Next, we examined whether this induction of muscle differentiation genes in C2C12 cells upon ZEB1 knockdown translated into their earlier terminal differentiation into multinucleated myotubes. Compared to control cells, the formation of myotubes in cells knocked down for ZEB1 was significantly accelerated, as evidenced morphologically and by quantification of the number of nuclei in MHC-positive cells with respect to the total number of nuclei (Fig. 3A to C; also data not shown for si3ZEB1). At late time points, the difference in the number of nuclei in MHC-positive cells between both experimental conditions was reduced. However, myotube size (number of nuclei per myotube) was still considerably larger in ZEB1 knockdown cells than in control cells (Fig. 3D displays the number of MHC-positive myotubes with more than 4 nuclei). Likewise, although by 72 h, the number of MHC-positive myotubes with more than 4 nuclei was similar under both conditions (Fig. 3D), ZEB1 knockdown cells still had more nuclei per myotube than control cells (e.g., more myotubes in siZEB1 than in siCtl exceeded 6 nuclei) (Fig. 3A).

Of note, ZEB1 knockdown also induced accelerated myotube formation in C2C12 cells that were continuously maintained in

GM, even after they reached confluence (Fig. 3E and F). This suggests that ZEB1 knockdown can trigger differentiation once cells have undergone contact-inhibition-mediated cell cycle exit. Together, these results suggest that under normal conditions, endogenous ZEB1 delays myoblast-to-myotube conversion, while ZEB1 knockdown is sufficient to activate (by relieving repression) a gene signature associated with terminal muscle differentiation.

Stage-dependent differential binding of ZEB1 and MyoD to muscle differentiation genes. For most of its known target genes, ZEB1 inhibits their expression by binding to G/C-centered E-boxes at their regulatory regions and actively repressing transcription (14, 15, 24–26, 52, 53). We therefore questioned whether the observed changes in muscle gene expression upon ZEB1 knockdown involve binding of ZEB1 to G/C-centered E-boxes in muscle differentiation genes. As MyoD binds G/C-centered E-boxes at muscle differentiation genes in myotubes but not in myoblasts (13), we also wondered whether ZEB1 could be occupying these E-boxes at the myoblast stage.

The regulatory regions of muscle terminal differentiation genes are particularly enriched for G/C-centered E-boxes (12, 13). We therefore decided to test the ability of ZEB1 and MyoD to bind to these E-boxes in the promoters of mouse MyH4 and troponin through chromatin immunoprecipitation (ChIP) assays of C2C12 myoblasts and myotubes (Fig. 4A). In myoblasts, antibodies against ZEB1, but not its respective control IgG, immunoprecipitated regions of the MyH4 and troponin promoters containing G/C-centered E-boxes (Fig. 4A). Interestingly, MyoD did not bind to the same region of either promoter in myoblasts. Conversely, when these experiments were performed with myotubes, the reverse was observed: MyoD, but not ZEB1, was found to bind to G/C-centered E-box-containing regions in both promoters (Fig. 4A). Lastly, both ZEB1 and MyoD antibodies failed to immunoprecipitate a fragment of the mouse GAPDH promoter lacking G/C-centered E-boxes (Fig. 4A). These results indicate that, in line with our original hypothesis and in an opposite pattern from MyoD, endogenous ZEB1 binds directly to muscle differentiation gene promoters in myoblasts but not in myotubes.

Inhibition of muscle differentiation by ZEB1 at the myoblast stage involves CtBP-mediated transcriptional repression. Next, we explored whether negative regulation of muscle differentiation genes by ZEB1 occurs via transcriptional repression. C2C12 cells were cotransfected at the myoblast or myotube stage with either siCtl or specific siRNAs against ZEB1 and luciferase reporters containing the promoter regions of selected muscle genes. Knockdown of ZEB1 in nonconfluent cycling C2C12 myoblasts upregulated the basal transcriptional activity—that is, relieving ZEB1-mediated repression—of the MHC, troponin, muscle creatine kinase (MCK), and MyoD gene promoters, the latter to a much lesser extent (Fig. 4B to E). Interestingly, when the experiment was carried out with C2C12 cells that had been maintained in DM and differentiated into myotubes, interference of ZEB1 had no or a very limited effect (Fig. 4B to E).

ZEB1 represses transcription through recruitment of different corepressors, some still undetermined, that seem to act in a promoter- and tissue-specific manner (52, 53). A known ZEB1 corepressor is CtBP (CtBP1 and CtBP2), a non-DNA-binding protein that in turn tethers histone-modifying enzymes (33, 54, 55). CtBP mediates repression of targets of the myogenic MEF2 factor via recruitment of HDAC9/MITR (56). Here, we used a specific siRNA against CtBP (siCtBP) (Fig. 4F, left) to investigate a potential contribution of CtBP to ZEB1-mediated transcriptional inhibition of muscle genes. We found that CtBP knockdown in C2C12 myoblasts upregulated, albeit to different degrees, the basal activity of MHC, MCK, and MyoD promoters (Fig. 4B, D, and E). For these three promoters, the effect of concomitant knockdown of CtBP and ZEB1 on C2C12 myoblasts was similar to the effect of ZEB1 (or CtBP) single knockdown (Fig. 4B, D, and E), suggesting that CtBP is the main cofactor in the repression of these muscle genes by ZEB1 at the myoblast stage. Interestingly, relief of repression of the troponin promoter by CtBP knockdown in C2C12 myoblasts was below the extent of upregulation following knockdown of ZEB1 (Fig. 4C), indicating that ZEB1-mediated regulation of this gene also involves (and with a greater contribution) other corepressors (also see below). In contrast, as occurred for ZEB1 knockdown, interference of CtBP in myotubes had no or only limited effects on the transcription of all four promoters (Fig. 4B to E).

Active transcriptional repression of muscle genes by ZEB1 and its dependence on CtBP were then confirmed in C3H-10T1/2 fibroblasts, a well-established cell-based model to study transcriptional regulation by MRFs (3–5, 8). Constitutively, C3H-10T1/2 fibroblasts do not express muscle genes, but their transfection with MyoD is sufficient to induce the formation of myotubes, although C3H-10T1/2 cells never reached the extent of myogenic conversion observed for C2C12 cells (3, 4). Here, C3H-10T1/2 cells were cotransfected with MyoD and luciferase reporters for the four promoters described above, and the expression of ZEB1 was modulated by its overexpression or knockdown. We found that ZEB1 knockdown in C3H-10T1/2 cells (Fig. 4F, middle) upregulated the transcription of all four promoters (Fig. 4G), while overexpression of ZEB1 inhibited their activity (Fig. 4H).

Knockdown of CtBP in C3H-10T1/2 cells (Fig. 4F, right) augmented the basal activity of these four promoters, including that of troponin (Fig. 4G). The relief of repression obtained with siCtBP was similar to or below that observed with interference of ZEB1 alone. Likewise, elimination of CtBP partially reversed the repressor effect of ZEB1 on all four promoters albeit to different levels (Fig. 4H). The differential degree of dependence on CtBP for the basal endogenous repression of the troponin promoter by ZEB1 in C2C12 and C3H-10T1/2 cells supports the above-mentioned evidence that the identity of ZEB1 corepressors varies in a promoter- and tissue-specific manner (52). In any case, altogether, these results with cell-based systems indicate that CtBP mediates a significant share of ZEB1-mediated repression of muscle gene expression.

ZEB1 dependence on CtBP for the regulation of muscle gene expression was next examined *in vivo* in *Xenopus* and *Drosophila* embryos. *Xenopus* embryos were microinjected with MyoD and either wild-type ZEB1 ($ZEB1_{wt}$) or a version of ZEB1 where its CtBP-interacting domain (CID) had been mutated ($ZEB1_{CIDmut}$). At blastula stages, ectodermal explants were isolated from these embryos, and the effect of both ZEB1 variants on MyoD-mediated induction of muscle gene expression was examined (Fig. 5A). ZEB1 inhibited mRNA expression of muscle actin, MCK, slow skeletal troponin I type 1 (TnnI1), fast skeletal troponin I type 2, tropomyosin 1 α chain, and MyoD. Mutation of the CID region in ZEB1 partially alleviated—and to different degrees—repression of all the genes examined except for TnnI1, where ZEB1 inhibition seemed to take place independently of CtBP (Fig. 5A). These results demonstrate that repression of muscle differentiation genes

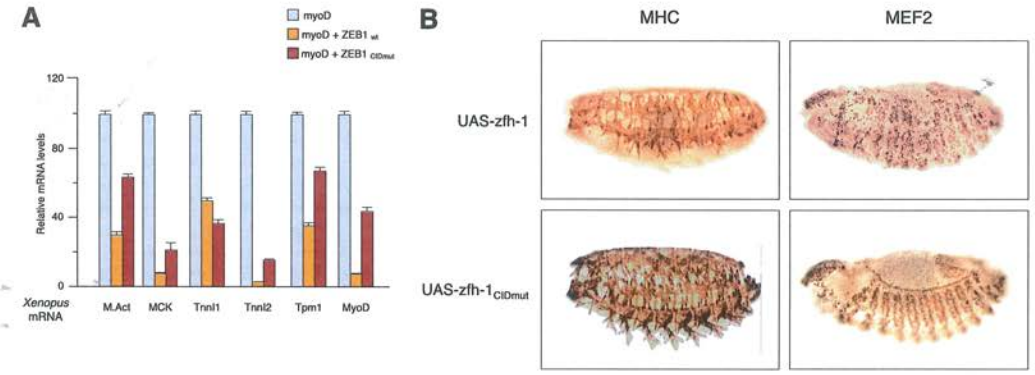

FIG 5 *In vivo* regulation of muscle gene expression by ZEB1/zfh-1 depends on CtBP. (A) Differential requirement for CtBP in ZEB1-mediated repression of muscle genes in *Xenopus* ectodermal explants. *Xenopus* embryos microinjected with mRNA encoding MyoD and either $ZEB1_{wt}$ or $ZEB1_{CIDmut}$ were used to generate ectodermal explants, and qRT-PCR was used to examine MyoD-induced expression of muscle actin (M.Act), MCK, slow skeletal troponin I type 1 (Tnni1), fast skeletal troponin I type 2 (Tnni2), tropomyosin 1 α chain (Tpm1), and MyoD. Isolation of mRNA from ectodermal explants and assessment of relative mRNA levels with respect to the reference gene EF1α were performed as described in Materials and Methods. Data shown are a representative case of three independent experiments. (B) Muscle phenotypes of *Drosophila* embryos expressing two copies of UAS-zfh-1 or UAS-zfh-1_{CIDmut} under the control of the 24B-Gal4 mesodermal gene as a driver. Embryos were fixed and stained for MEF2 (stage 14) and MHC (stage 16). Note that some myofibers were missing from muscle groups in UAS-zfh-1 embryos compared to UAS-zfh-1_{CIDmut} embryos.

by ZEB1, both in cell-based systems and *in vivo*, occurs to a large extent in a CtBP-dependent manner.

The ZEB1 ortholog in *Drosophila*, zfh-1, is also a transcriptional repressor that binds to G/C-centered E-boxes and interacts with CtBP (18, 19, 54). Overexpression of zfh-1 in *Drosophila* embryos under the control of the heat shock protein 70 promoter results in a muscle phenotype that includes, but is not limited to, a downregulation of MHC and MEF2 expression in the somatic musculature (19). As in any overexpression experiment, these results cannot rule out that overexpression of zfh-1 is titrating out other proteins, including CtBP. On the other hand, loss of zfh-1 does not trigger accelerated myogenic differentiation but rather a complex phenotype with simultaneous loss, gain, and mispositioning of muscle precursors (18), thus suggesting that the role of zfh-1 during *Drosophila* myogenesis is not fully conserved in vertebrate ZEB1. Despite these caveats, we decided to examine whether the muscle phenotype observed upon zfh-1 overexpression was dependent on CtBP. zfh-1 is normally downregulated after gastrulation in most mesodermal derivatives, including muscle (57). We used a Gal4-UAS system to maintain the expression of wild-type zfh-1 (UAS-zfh-1) or a zfh-1 version unable to interact with CtBP (UAS-zfh-1_{CIDmut}) in the developing muscle under the control of the 24B-Gal4 or MEF2-Gal4 mesoderm-specific driver lines (Fig. 5B and data not shown for MEF2-Gal4). As described previously (19), maintaining zfh-1 expression yielded alterations in somatic muscle development and MHC and MEF2 expression that are more complex than just a block in muscle differentiation (Fig. 5B for 24B-Gal4; also data not shown for MEF2-Gal4). Nevertheless, the muscle phenotype observed was dependent on CtBP, as no apparent effect on the embryos crossed with UAS-zfh-1_{CIDmut} was observed (Fig. 5B for 24B-Gal4; also data not shown for MEF2-Gal4), even though the zfh-1_{CIDmut} protein is stably expressed (58). Together, these results demonstrate that repression of muscle differentiation genes by ZEB1 (and likely zfh-1), both in cell-based systems and in *in vivo* models, occurs to a large extent in a CtBP-dependent manner.

Loss of ZEB1 induces muscle differentiation gene expression *in vivo*. We next sought to confirm whether the induction of muscle genes upon ZEB1 knockdown observed by cell-based approaches was recapitulated *in vivo*. To this end, we examined the effect of overexpressing and knocking down ZEB1 during *Xenopus* embryonic development. ZEB1 mRNA was coinjected along with β-galactosidase mRNA as a lineage tracer into one of two blastomeres at the 2-cell stage. This introduced the injected mRNA into one bilateral half of the embryo, marked in pink (by staining for the Red-Gal β-galactosidase substrate) and oriented to the right in Fig. 6A. The contralateral half of the embryo served as a control. Embryos were then raised to neurula stages, and *in situ* hybridization was used to examine the expression of collagen type II (Col II), a marker of differentiated mesoderm, and muscle actin, a muscle marker (Fig. 6A, purple stains). We found that overexpression of ZEB1 strongly repressed the expression of both markers (Fig. 6A). Conversely, ZEB1 knockdown by microinjection of a specific morpholino oligonucleotide (ZEB1 MO) expanded the expression domains of both markers (Fig. 6A). These results parallel our findings in cell-based systems and support a role for ZEB1 in restraining the expression of muscle differentiation genes *in vivo*.

Mice carrying a homozygous targeted deletion of *ZEB1* die close to birth with cleft palate and a number of bone and cartilage abnormalities (17). Although at embryonic days 10 to 11, levels of MyoD, myogenin, and MCK are not significantly affected in these mice (17), a molecular analysis of muscle differentiation at later stages has not been carried out. From the results shown up to here, one might expect that the temporal pattern of expression of muscle differentiation gene expression would be accelerated in $ZEB1^{-/-}$ mouse embryos.

Of all adult MHC isoforms, IIb/MyH4 is induced not only the

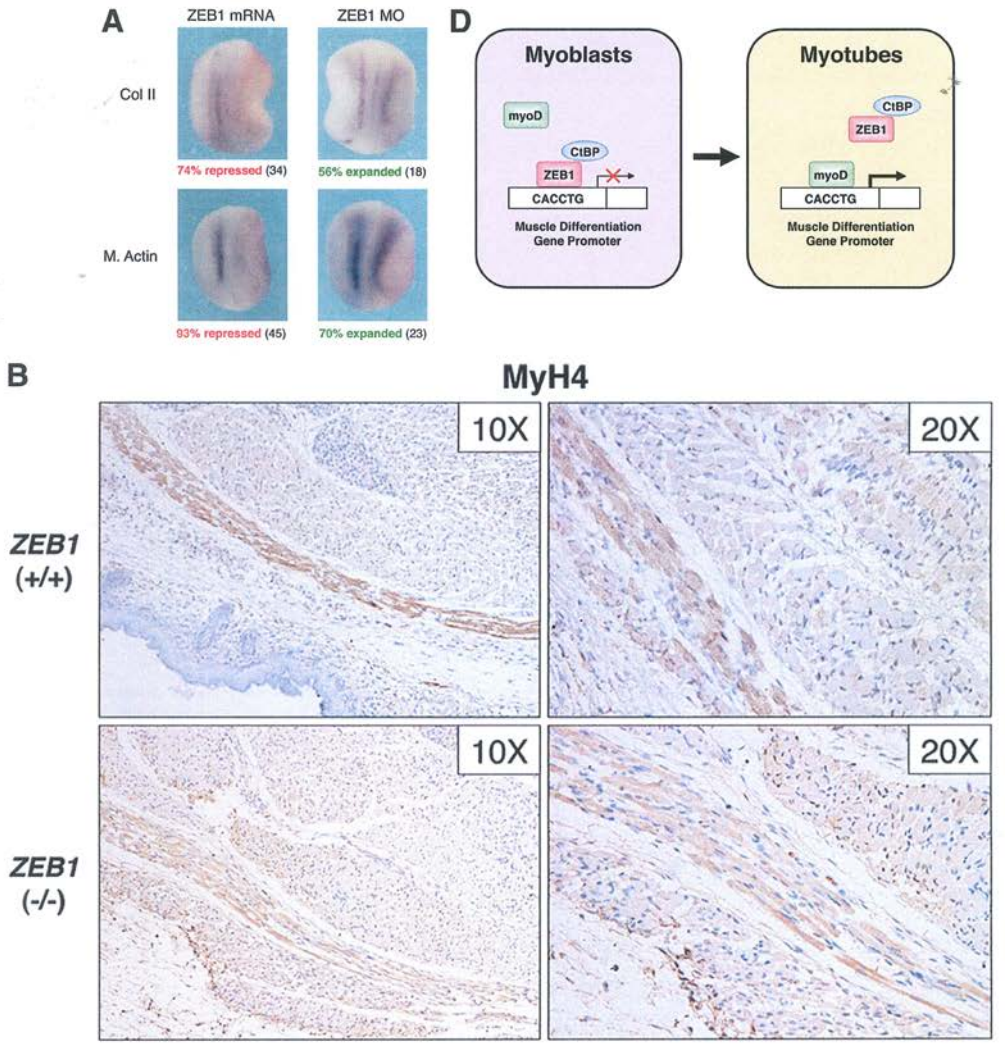

FIG 6 ZEB1 represses muscle gene expression *in vivo*. (A) ZEB1 regulates muscle gene expression in *Xenopus* embryos. *In situ* hybridization for collagen II (Col II) and muscle actin (M. Actin) was performed in *Xenopus* embryos injected with mRNA for ZEB1 (ZEB1 mRNA) or a specific morpholino oligonucleotide against ZEB1 (ZEB1 MO), along with mRNA encoding β-galactosidase to mark the injected side (oriented rightward), as described in Materials and Methods. Embryos were then allowed to develop up to stages 15 to 16. Percentages of embryos with each phenotype are indicated, with the number of embryos analyzed shown in parentheses. The uninjected contralateral half of the embryo serves as an internal control (oriented leftward). (B) Muscle differentiation gene expression is accelerated in embryos from mice with targeted deletion of *ZEB1*. Shown is immunohistochemistry for MHC IIb (MyH4; BF-F3) in the developing muscle of $ZEB1^{+/+}$ and $ZEB1^{-/-}$ E18.5 sibling mouse embryos. MyH4 displayed slightly higher expression levels in $ZEB1^{-/-}$ embryos than in the $ZEB1^{+/+}$ counterparts. Magnifications of ×20 and ×10 are shown. Tissue immunostaining was performed as described in Materials and Methods. (C) Same as panel B but with immunostaining for MHC isoform IIa (MyH2; SC-71). MyH2 was expressed earlier in $ZEB1^{-/-}$ embryos than in the $ZEB1^{+/+}$ sibling counterparts, where it was barely detectable. (D) Schematic model of the role of endogenous ZEB1 regulating the temporal pattern of gene expression during muscle differentiation. In myoblasts, ZEB1 binds to G/C-centered E-boxes in the regulatory regions of muscle differentiation genes whose transcription would be repressed at least in part via recruitment of CtBP. In myotubes, MyoD would displace ZEB1 from these G/C-centered E-boxes, thus activating the expression of muscle genes as differentiation progresses.

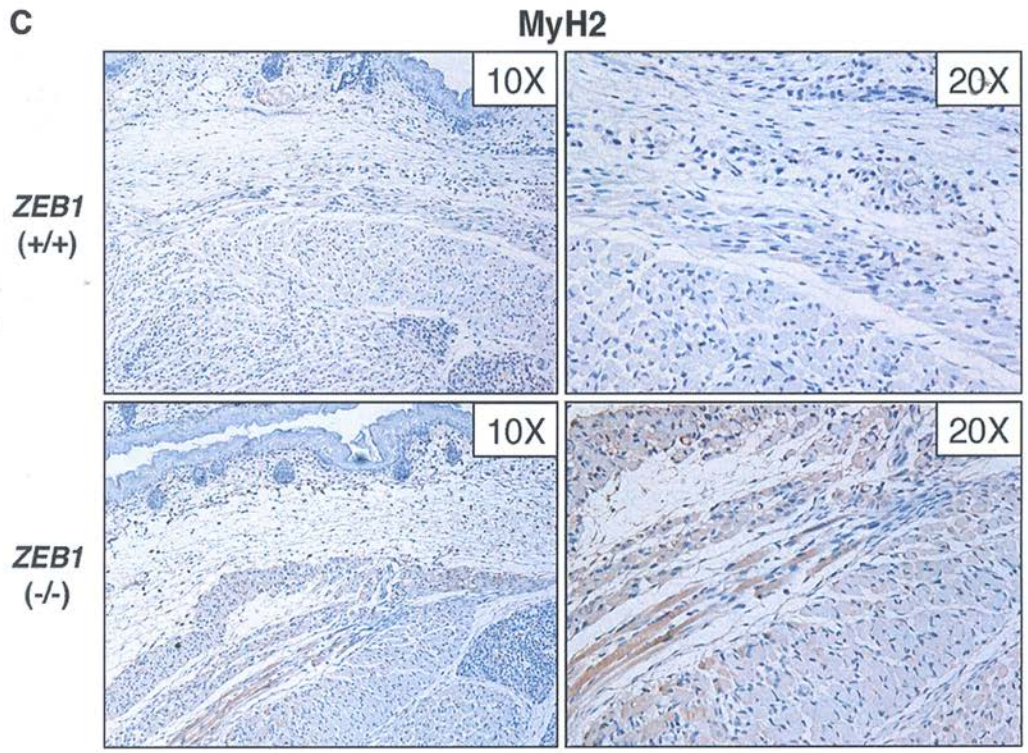

FIG 6 continued

earliest (around E14.5) but also at much higher levels than the rest, while mRNA for isoform IIa/MyH2 starts to be detected only after E17.5 (59). We decided to examine by immunohistochemistry the expression of both adult MHC isoforms during late development in sibling mouse embryos that were either wild type (+/+) or null (−/−) for *ZEB1*. We found that by E18.5, expression of MyH4 was only slightly higher in $ZEB1^{-/-}$ embryos than in the normal $ZEB1^{+/+}$ sibling counterparts (Fig. 6B). Meanwhile, MyH2, which was barely detectable in $ZEB1^{+/+}$ mouse embryos, displayed earlier (higher) expression in the $ZEB1^{-/-}$ sibling counterpart embryos (Fig. 6C). These results, consistent with the cell-based and *in vivo* data described above, indicate that loss of *ZEB1* results in an acceleration of the temporal pattern of the muscle differentiation gene signature.

DISCUSSION

MRFs drive skeletal myogenesis, but the precise temporal and spatial pattern of muscle development involves regulation of the expression and activity of MRFs by a wide range of myogenic agonists and inhibitors. Using cell-based systems and several *in vivo* models, this study demonstrates that the transcriptional repressor ZEB1, which binds only to G/C-centered E-boxes, imposes a temporary delay in muscle gene expression and differentiation. ZEB1 knockdown resulted in premature expression of muscle differentiation genes and an acceleration of myotube formation. Contrary to MyoD, ZEB1 binds to G/C-centered E-boxes in muscle differentiation genes at the myoblast stage but not in myotubes. Inhibition of muscle differentiation by ZEB1 involves transcriptional repression of its targets at the myoblast stage, largely via recruitment of the CtBP corepressor. Finally, we show that the pattern of gene expression associated with muscle differentiation is accelerated in $ZEB1^{-/-}$ mouse embryos.

Binding of MyoD to the regulatory regions of muscle genes and triggering of myogenesis are temporarily blocked in myoblasts by the action of factors that sequester E-proteins and/or MyoD itself or that bind to the same E-box sequences (1, 6). Accordingly, exogenous overexpression of either type of factor (squelching or E-box binding factors) can inhibit MyoD-induced myotube formation in fibroblasts (7–9, 11, 13, 26, 60). However, in addition to the caveat of overexpression experiments mentioned above, displacement of MyoD from E-boxes when these inhibitory factors are overexpressed occurs independently of their DNA binding affinity relative to MyoD. Recently, it was shown that induction of myogenesis by MyoD is also regulated by its affinity for G/C-centered E-boxes with respect to other endogenous E-box binding proteins like Snail factors (13).

In myoblasts, MyoD binds to A/T-centered E-boxes but not to G/C-centered E-boxes, which are predominant in muscle differentiation genes and can be bound by Snail factors. It is only as differentiation progresses that MyoD occupies these E-boxes and activates muscle differentiation (13). ZEB1 binds G/C-centered E-boxes (mostly of the CACCTG/CAGGTG type also preferred by MyoD) in the promoters of a wide range of genes, where it can act in competition with other bHLH and zinc finger factors (24–28). Upon binding to these E-boxes, ZEB1 regulates gene expression mostly by active transcriptional repression (26, 52–54). Here, we found that knockdown of endogenous ZEB1 in nonconfluent cycling myoblasts, but not in differentiated myotubes, upregulates the basal transcriptional activity of several muscle gene promoters. This result suggests that these genes are normally under negative regulation by ZEB1 at the myoblast stage but not in myotubes (Fig. 6D).

Interestingly, ZEB1 is a downstream target of both Snail1 and Snail2 (21–23). Snail1/2 and ZEB1 have been extensively characterized for their role as inducers of an epithelial-to-mesenchymal transition (EMT) in the context of cancer cell invasion and tumor metastasis (14, 15). In triggering an EMT at the invasive front of carcinomas, both Snail1 and ZEB1 repress an overlapping set of epithelial specification markers, but they participate at different stages, with Snail1 triggering the EMT process and ZEB1 maintaining it (21, 22, 32). Cells in the dermomyotome also undergo an EMT to delaminate and migrate into the primary myotome (1). Conceivably, a similar temporal division of labor between Snail1 and ZEB1 could occur during muscle differentiation, as evidence already exists that Snail1 triggers an EMT in the dermomyotome and regulates its timing (61).

Transcriptional repression by ZEB1 is mediated by the tethering of different corepressors whose identity seems to vary in a tissue- and promoter-specific manner (reviewed in reference 52). Thus, repression of CD4 in lymphocytes is dependent on recruitment of the Tip60 histone acetyltransferase, while CtBP mediates ZEB1 repression of the growth hormone gene in the pituitary gland or interleukin-2 and Bcl-6 in lymphocytes. In heterologous Gal4/LexA reporter systems, ZEB1 antagonizes transcriptional activation by most transcription factors tested, including that induced by the myogenic factor MEF2C (53). ZEB1 repression of MEF2-mediated transcription involves the central region of ZEB1, encompassing the interacting domain for CtBP (33, 53, 54). In the context of muscle gene expression, CtBP is known to mediate repression of MEF2 targets by recruitment of HDAC9/MITR (56). Our results for cell-based systems and *in vivo* models demonstrated that CtBP participates in ZEB1 repression of muscle genes although to different degrees, being dispensable for the regulation of Tnni1 in *Xenopus* but largely required in the case of MyoD in C3H-10T1/2 fibroblasts. Similarly, the small dependence on CtBP for the repression of troponin in C2C12 cells relative to C3H-10T1/2 fibroblasts corroborated the promoter and tissue specificity of the cofactors involved in ZEB1-mediated repression. Nevertheless, while CtBP accounts for a large share of ZEB1 repression of muscle differentiation genes, our data also indicate that their inhibition by ZEB1 implicates additional CtBP-independent mechanisms that remain to be elucidated. In line with this, repression of E-cadherin by ZEB1 requires the summative action of several cofactors (62).

MyoD function, rather than its expression, is temporarily blocked in myoblasts (6). Likewise, ZEB1's antimyogenic activity seems to also be regulated during muscle differentiation. Thus, ZEB1 is expressed in the epithelium of the undifferentiated somite and the dermomyotome and continues to be expressed as muscle starts to form (16, 17). The required functional inactivation of ZEB1 during muscle differentiation may take place through several mechanisms, but our results here indicate that, at least in part, this might occur via displacement of ZEB1 from G/C-centered E-boxes by MyoD as muscle differentiation progresses. In keeping with this model, ZEB1 is also in equilibrium with the bHLH E2A protein for binding to the immunoglobulin heavy chain enhancer during B-cell differentiation (24).

In summary, this work has shown that muscle differentiation genes are under transcriptional repression by ZEB1/CtBP in myoblasts. ZEB1 imposes a temporal delay in muscle differentiation, and its regulatory and functional interplay with MyoD appears to play an essential role in controlling the temporal pattern of gene expression during muscle differentiation.

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APPENDIX II

I also coauthored two review articles on ZEB and EMT-related factors:

- Sánchez-Tilló E, **Siles L**, De Barrios O, Cuatrecasas M, Vaquero EC, Castells A, Postigo A. Expanding roles of ZEB factors in tumorigenesis and tumor progression. *Am J Cancer Res*. 2011 1(7):897-912.

- Sánchez-Tilló*, E, Liu*, Y, de Barrios* O, **Siles* L**, Fanlo L, Cuatrecasas M, Darling DS, Dean DC, Castells A, Postigo A. EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness. *Cell Mol Life Sci.* 2012 69(20):3429-56.
(* Equal first contribution)

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Cellular and Molecular Life Sciences

REVIEW

EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness

Ester Sánchez-Tilló · Yongqing Liu · Oriol de Barrios · Laura Siles · Lucia Fanlo · Miriam Cuatrecasas · Douglas S. Darling · Douglas C. Dean · Antoni Castells · Antonio Postigo

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Abstract Cancer is a complex multistep process involving genetic and epigenetic changes that eventually result in the activation of oncogenic pathways and/or inactivation of tumor suppressor signals. During cancer progression, cancer cells acquire a number of hallmarks that promote tumor growth and invasion. A crucial mechanism by which carcinoma cells enhance their invasive capacity is the dissolution of intercellular adhesions and the acquisition of a more motile mesenchymal phenotype as part of an epithelial-to-mesenchymal transition (EMT). Although many transcription factors can trigger it, the full molecular reprogramming occurring during an EMT is mainly orchestrated by three major groups of transcription factors: the ZEB, Snail and Twist families. Upregulated expression of these EMT-activating transcription factors (EMT-ATFs) promotes tumor invasiveness in cell lines and xenograft mice models and has been associated with poor clinical prognosis in human cancers. Evidence accumulated in the last few years indicates that EMT-ATFs also regulate an expanding set of cancer cell capabilities beyond tumor invasion. Thus, EMT-ATFs have been shown to cooperate in oncogenic transformation, regulate cancer cell stemness, override safeguard programs against cancer like apoptosis and senescence, determine resistance to chemotherapy and promote tumor angiogenesis. This article reviews the expanding portfolio of functions played by EMT-ATFs in cancer progression.

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Review Article

Expanding roles of ZEB factors in tumorigenesis and tumor progression

Ester Sánchez-Tilló[1], Laura Siles[2], Oriol de Barrios[3], Miriam Cuatrecasas[4], Eva C. Vaquero[5], Antoni Castells[5], Antonio Postigo[1,6,7]

[1]Group of Transcriptional Regulation of Gene Expression, Dept. of Oncology and Hematology, IDIBAPS, Barcelona, Spain; [2]Master Program in Molecular Biotechnology, University of Barcelona, Spain; [3]Master Program in Biomedicine. University of Barcelona, Spain; [4]Dept. of Pathology (CDB), Hospital Clinic of Barcelona, Spain; [5]Dept. of Gastroenterology, Hospital Clinic of Barcelona, CIBERehd, IDIBAPS, Barcelona, Spain; [6]ICREA, Barcelona, Spain; [7]James Graham Brown Cancer Center, University of Louisville, KY, USA.

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Abstract: The ZEB family of transcription factors regulates key factors during embryonic development and cell differentiation but their role in cancer biology has only more recently begun to be recognized. Early evidence showed that ZEB proteins induce an epithelial-to-mesenchymal transition linking their expression with increased aggressiveness and metastasis in mice models and a wide range of primary human carcinomas. Reports over the last few years have found that ZEB proteins also play critical roles in the maintenance of cancer cell stemness, control of replicative senescence, tumor angiogenesis, overcoming of oncogenic addiction and resistance to chemotherapy. These expanding roles in tumorigenesis and tumor progression set ZEB proteins as potential diagnostic, prognostic and therapeutic targets.

APPENDIX III

I also participated in several projects on the role of ZEB1 driving an EMT and promoting tumor progression in carcinomas and lymphomas.

- Sánchez-Tilló E, de Barrios O, **Siles L**, Cuatrecasas M, Castells A, Postigo A. β-catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness. *Proc Natl Acad Sci USA*. 2011 08(48):19204-9.

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(* Equal contribution)

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β-catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness

Ester Sánchez-Tilló[a], Oriol de Barrios[b], Laura Siles[c], Miriam Cuatrecasas[d], Antoni Castells[e], and Antonio Postigo[a,f,g,1]

Clinical Cancer Research

Human Cancer Biology

ZEB1 Promotes Invasiveness of Colorectal Carcinoma Cells through the Opposing Regulation of uPA and PAI-1

Ester Sánchez-Tilló[1,2], Oriol de Barrios[1], Laura Siles[1], Pier G. Amendola[1], Douglas S. Darling[6], Miriam Cuatrecasas[3], Antoni Castells[2,4], and Antonio Postigo[1,2,5,7]

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The EMT activator ZEB1 promotes tumor growth and determines differential response to chemotherapy in mantle cell lymphoma

E Sánchez-Tilló[1], L Fanlo[1,2,11], L Siles[1,11], S Montes-Moreno[3,11], A Moros[4], G Chiva-Blanch[5,6], R Estruch[5,6], A Martinez[7], D Colomer[4], B Győrffy[8], G Roué[4] and A Postigo*[,1,9,10]

ORIGINAL ARTICLE

ZEB1-induced tumourigenesis requires senescence inhibition via activation of DKK1/mutant p53/Mdm2/CtBP and repression of macroH2A1

Oriol de Barrios,[1] Balázs Győrffy,[2] María Jesús Fernández-Aceñero,[3,4] Ester Sánchez-Tilló,[1] Lidia Sánchez-Moral,[1] Laura Siles,[1] Anna Esteve-Arenys,[5] Gaël Roué,[5] José I Casal,[6] Douglas S Darling,[7] Antoni Castells,[8,9] Antonio Postigo[1,9,10,11]