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Unravelling the molecular signalling regulating embryonic and feuella gtal myogenesis: the role of Nfix as “master” gene in regulating fetal genetic program

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Contents

PART I

During my PhD I worked on a project, that I started during my Master of Science internship, about the functional cooperation between Sox6 and Nfix during murine prenatal myogenesis. The results of this project have been recently published on Cell Reports where I am first author. This work addressed the role of Sox6, during embryonic myogenesis, in regulating the expression of the slow myosin heavy chain (MyHC-I). We demonstrated that Sox6, which is a well-known inhibitor of MyHC-I during fetal myogenesis, is instead responsible for MyHC-I activation during the embryonic myogenesis via Mef2C-dependent mechanism. Notably, we reported that the switch in Sox6 function at the onset fetal myogenesis occurs through the interaction with the transcription factor Nfix. Finally, we demonstrated that the functional cooperation of Sox6 and Nfix is evolutionary conserved in mouse and zebrafish (Part II). Across this project, I started to work on my PhD project about the identification of the molecular mechanisms involved in the regulation of Nfix expression during fetal myogenesis. The data obtained are now collected in manuscripts one already published (Part II) and one in preparation (Part III).

Abstract

Skeletal muscle development occurs through successive developmental phases, named embryonic and fetal myogenesis, involving the differentiation of distinct myogenic populations: the embryonic and fetal myoblasts. An important work developed in 2007 identified specific features of embryonic and fetal myoblasts, demonstrating that these two populations of muscle progenitors represent intrinsically different myogenic lineages. The identification of the transcriptional factor Nfix was the major step in understanding how muscle progenitor fate decisions are mediated. Nfix, expressed in fetal but not in embryonic muscles, is necessary for the acquisition of fetal myogenic identity, activating fetal-specific genes and repressing the expression of embryonic one, such as slow myosin heavy chain (MyHC-I). Another repressor of MyHC-I, during fetal myogenesis, is the transcription factor Sox6, which inhibits MyHC-I by direct binding to the proximal promoter of MyHC-I gene. Interestingly, Sox6 at variance with Nfix is also expressed during embryonic myogenesis, when embryonic myoblasts form fibers that express high level of MyHC-I. We demonstrated that Sox6 has opposite roles in regulating MyHC-I expression between embryonic and fetal myogenesis. Specifically, during embryonic myogenesis, Sox6 indirectly promotes MyHC-I expression via transcriptional activation of Mef2C. On the contrary, during fetal myogenesis, Nfix allows the proper binding of Sox6 to the MyHC-I promoter with the consequent repression of MyHC-I expression. In addition, we provide evidence that the functional interplay of Nfix and Sox6 is conserved also in zebrafish. Although Nfix functions were partially characterized, nowadays the mechanisms that regulate its expression during fetal myogenesis are still unknown. Another transcription factor more expressed during fetal myogenesis is JunB. We assessed that JunB is required and sufficient to induce the expression of Nfix, acting as direct activator of it. To better elucidate this pathway, we started to study the possible involvement of RhoA and its major kinase ROCK, since growing investigations have shown that RhoA/ROCK regulate skeletal muscle differentiation. We demonstrated that RhoA/ROCK are active only during embryonic myogenesis and their inhibition in embryonic myoblasts increased the expression of both Nfix and JunB. The interference with the RhoA/ROCK signalling led also to the increased activation of the ERK kinases, which we show are necessary for Nfix and JunB up-regulation. In summary, we have identified the RhoA/ROCK axis as an important negative regulator of JunB and Nfix expression during embryonic myogenesis, through the inhibition of ERK activity. Conversely, during fetal myogenesis, the ERK kinases are active and allow JunB and Nfix expression. Finally, Nfix is sufficient to activate the fetal genetic program and to permit the complete maturation of prenatal skeletal muscles.

Introduction

1. Skeletal muscle organization and function

1.1 Cellular physiology and contraction

Skeletal muscle is the most abundant tissue in our body and it is responsible for all the voluntary movements, breathing, the storage of nutrient reserves and for the maintenance of posture and body temperature. Each skeletal muscle is made up of a large number of syncytial cell, known as myofibers. A myofiber is a multinucleated single muscle cell, formed from the fusion of many precursor cells. Each muscle fiber is surrounded by a cell membrane or sarcolemma, composed by a lipid bilayer, that is in contact with numerous thin collage fibrils and specialized proteins such as laminin, which functions as a scaffold for the myofiber. Inside each muscle fibers, the cytoplasm is mainly composed by myofibrils, which constitute the contractile apparatus. The myofibrils are composed by repeated segments, named sarcomers, formed by parallel actin (thin) and myosin (thick) filaments, thus representing the core of muscle contraction. Our current understanding of muscle contraction derives from A.F. Huxley and R. Niedergerke (1954) and H. E. Huxley and J. Hanson (1954), that observed a sarcomere shortening during contraction (Figure 1). The shortening is due to change in length of actin filaments, while the thick filaments of myosin remain constant in length. These observations led them to propose a sliding filament theory, which state that the sliding of actin on myosin generates a shortening of actin filament length, resulting in a shortening of the sarcomere and thus of the muscle. The sliding is due to the capability of myosin to pull upon actin to shorten the sarcomere, thanks to the power stroke of myosin, obtained through the hydrolysis of ATP. Skeletal muscle contractions are neurogenic, as motor neuron input is required to produce muscle contractions. The motor neuron excitation provokes the release from the sarcoplasmatic reticulum of calcium ions, which interact with troponin of the thin filament, causing a movement of the tropomyosin molecules. All these movements allow the binding between actin and myosin, starting the process of contraction.

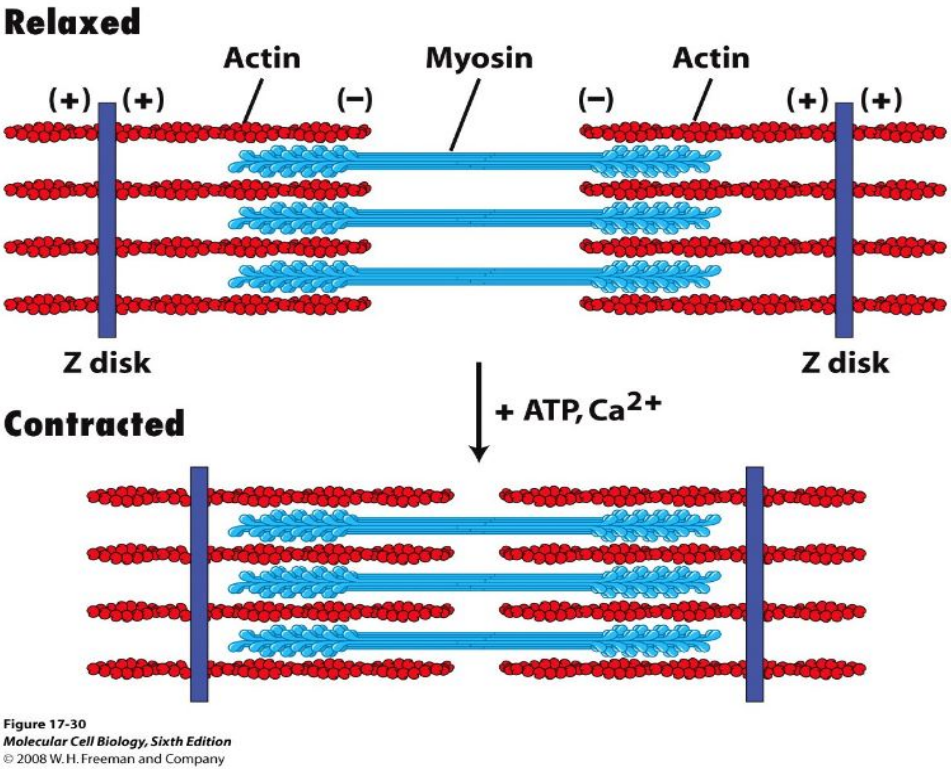

Figure 1: Scheme of a sarcomere. The diagram shows the structure of a relaxed and contracted sarcomere, composed by thick myosin filaments (in light blue) and thin actin filaments (in red). The thin actin filaments are tethered at the Z-discks and interdigitated between myosin filaments. The contracted sarcomere is shorter because of the sliding of actin filaments (Freeman & Company, Molecular Cell Biology, Sixth Edition, 2008).

1.2. The heterogeneity of skeletal muscle

Skeletal muscles are heterogeneous tissues to fulfill different functional demands such as continuous low-intensity activity (posture), repeated submaximal contractions (locomotion) or fast and strong maximal contractions (jumping, kicking). The heterogeneity of skeletal muscles is due to different fiber types that compose each muscle. Nowadays, the fibers are classified into four major types (Table 1), marked by the expression of different isoforms of myosin heavy chain (MyHC): MyHC-I, MyHC-2a, MyHC-2x and MyHC-2b (DeNardi et al. 1993; Chakkalakal et al. 2012). Specific MyHC isoforms are also present in developing muscle: embryonic MyHC (MyHC-emb) and perinatal or neonatal MyHC (MyHC-neo), encoded by *Myh3* and *Myh8* respectively. The developing MyHC isoforms are expressed during prenatal development and in regenerating muscles, which recapitulate the molecular processes involved during embryonic development (Eusebi et al., 1986; Schiaffino et al., 1986; Whalen et al., 1990). The power and the velocity of shortening of each muscle are determined by the type of myosin, that is present in the fiber, and both decrease in the order MyHC-I < MyHC-2a < MyHC-2x < MyHC-2b (Reiser et al., 1985; Bottinelli et al., 1996). These observations lead to subdivide the fibers in slow-twitching fibers or type I fibers, which express MyHC-I, and fast-twitching fibers expressing MyHC-2a, MyHC-2x and MyHC-2b (Schiaffino and Reggiani, 2011). Slow and fast-twitching fibers differ also in metabolic properties. Slow fibers are able to generate ATP by oxidative mitochondrial processes and their ATP consumption during contraction is not high, allowing them to maintain contractile activity for long time without showing fatigue. Conversely, fast fibers generate ATP very rapidly through glycolytic processes, setting a limit to the duration of their contractile activity (Schiaffino and Reggiani, 2011). Based on their metabolic properties, myofibers can be subdivided into slow-oxidative (S), fast-oxidative-glycolitic (FOG) and fast-glycolitic (FG) fibers (Ashmor et al., 1972; Peter et al., 1972). The FOG fibers are the type 2a fibers, expressing MyHC-2a isoform, and they possess both oxidative and glycolytic enzyme complement (Barnard et al., 1971), while type 2x and 2b fibers have only a high content of glycolytic enzymes (Nemeth et al., 1981; Park et al., 1987). The heterogeneity of skeletal muscles is early established during prenatal development and can be regulated by extrinsic signals during adulthood.

	Type I fibers	**Type 2a fibers**	**Type 2x fibers**	**Type 2b fibers**
Contraction time	Slow	Moderately Fast	Fast	Very fast
Resistance to fatigue	Small	Medium	Intermediate	Low

Activity used for	Aerobic	Long-term anaerobic	Short-term anaerobic	Short-term anaerobic
Power produced	Low	Medium	High	Very High
Mitochondrial density	High	Intermediate	Low	Low
Glycolytic capacity	Low	High	High	High
Myosin Heavy Chain	Myh7	Myh2	Myh1	MYH4

Table 1: Characteristics of muscle fiber types

2. Skeletal muscle development

2.1. The onset: somitogenesis

Mesoderm is anatomically divided into paraxial, intermediate, and lateral mesoderm, with respect to position from the midline. During prenatal development, the myogenic progenitors, named myoblasts, derive from presomitic mesoderm (PSM) and they give rise to all the body skeletal muscles (Christ and Ordahl, 1995), while the head and neck muscles derived from anterior paraxial unsegmented mesoderm and from prechordal mesoderm (Buckingham et al., 2003). In the course of development, PSM gives rise to somites through segmentation around embryonic day (E) 8.0 and E13.5 in mouse (Pourquié, 2003). Somites are bilaterally paired cellular spheres, that develop in a rostral-to-caudal direction flanked the neural tube. The segmentation of the paraxial mesoderm is a tight regulated process that depends on clock mechanisms known as clock-and-wave front model. On the basis of this model the segmentation is controlled by an oscillator (clock), which gives the periodicity of the process, and a wave front, that is a gradient of signaling molecules. This model is supported by numerous experimental observations, which show that genes activated by Notch, Wnt and FGF signaling pathways are expressed in a time-fashion way and the crosstalk of these signaling cause the oscillation in the expression of segmentation genes (Hofmann et al., 2004). The manipulation of the wave front, constituted by a gradient of Notch, Wnt and FGF, results in an altered segmentation because of the lack of the oscillatory expression of a network of genes controlling the rhythmic specification of the future segments (Aulehla & Pourquié 2006; Wahl et al., 2007; Goldbeter & Pourquié, 2008; Hubaud & Pourquié, 2014). The somites, formed through segmentation, are initially composed by epithelial cells, which undergo a high number of morphological changes, leading the formation of somatic compartments. The ventral portion of each somite is the sclerotome, which is formed through the epithelial-to-mesenchimal transition and contributes to cartilage and bone (Verbout, 1985; Christ and Wilting 1992). The dorsal part of each somites is the dermamyotome, which maintains the epithelial composition and, during development, yield skeletal and smooth muscles (Huang and Christ 2000; Yusuf and Brand-Saberi 2006; Ben-Yair and Kalcheim 2008), dermis (Kalcheim et al., 1999; Ben-Yair et al., 2003; Ben-Yair and Kalcheim 2005) and nearby cartilage (Huang and Christ 2000). In turn, the dermamyotome can be subdivided into two portions: the dorso-medial or epaxial and the ventro-later or hypaxial part. The dorsal muscles derive from the epaxial domain, while ventral trunk and limbs musculatures originate from hypaxial domain (Christ and Ordahl 1995; Huang and

Christ 2000). The hypaxial muscles are generated through an extensive migration of myogenic precursor cells to enrich their final destination (Vasyutina and Birchmeier, 2006). The delamination from the lips of the dermamyotome is controlled by different key molecules, such as scatter factor/hepatocyte growth factor (SF/HGF) and its receptor c-Met, and by many transcription factors as *Lbx1* and *Six* factors. In mouse mutant for c-Met (Bladt et al., 1995) or HGF (Schmidt et al., 1995) the limb bud and diaphragm are not colonized by myogenic cells, demonstrating that this paracrine signaling is required for the migration of muscle precursors. *Lbx1* is also involved in the regulation of delamination, because the inactivation of *Lbx1* causes the lack of muscles in both forelimbs and hindlimbs (Schafer and Braun, 1999), while Six1 and Six4 control the proliferation and the survival of migrating cells (Li et al., 2003; Laclef et al., 2003). When the precursor cells reach their target sites, they start to differentiate and to fuse into multinucleated myotubes, constituting the embryonic skeletal muscles. Other precursors remain in an undifferentiated state and continue to proliferate, to accomplish differentiation in the late stages of development, allowing the complete maturation of prenatal skeletal muscles. Moreover, shortly after the onset of somitogenesis, some muscle progenitors terminally differentiate in mononucleated muscle cells, named myocytes. The myocytes form the primary myotome, located in the ventral portion of dermamyotome and elongated along the axis of the embryo.

2.2. The patterns of somitogenesis: extrinsic factors

The dermamyotome domains are established by morphogene gradients. The tissues surrounding the somites, such as the dorsal ectoderm, the neural tube, the notochord and the lateral mesoderm, produce different signaling molecules, which control the activation of the myogenic program (Figure 2). Sonic hedgehog (Shh), secreted from the notochord and floor plate of neural tube, is involved in the positive specification of epaxial domain. Shh is essential for the formation of the sclerotome, but also for the maturation of dermomyotomal cells. In the absence of Shh there is somite cell death, most sclerotomal cells are lost (Chiang et al., 1996; Zhang et al., 2001) and myogenic lineage progression is prevented (Feng et al., 2006; Hammond et al., 2007). Wnt factors (Wnt -1, -3a and -4) are expressed in the dorsal half of the neural tube and play a crucial role in the commitment of epaxial dermamyotome, promoting proliferation and maintenance of

myogenic progenitor population (Parr et al., 1993; Hollyday et al., 1995; Munsterberg et al., 1995; Cauthen et al., 2001). Mouse mutants for Wnt-1 do not develop the dermomyotome and lack the expression of the myogenic genes (Ikeya and Takada, 1998), and experiments of explant cultures of mouse mesoderm demonstrate that Wnt-1 strongly induce myogenesis (Tajbakhsh et al., 1998). Conversely, Wnt-7a induces the development of hypaxial dermomyotome in a ventro-later position of each somites and it emerges from the ventral half of the neural tube and from the surface ectoderm (Kenny-Mobbs and Thorogood, 1987; Parr et al., 1993; Cossu et al., 1996; Pourquié et al., 1996). The correct spatio-temporal specification of hypaxial progenitors is also due to BMP4 by the lateral mesoderm. BMP4 retains muscle cells in an undifferentiated state (Pourquié et al., 1995), contributing to the expansion of myogenic pool through the prevention of differentiation. Wnt and BMP proteins are secreted in overlapping gradients (Itasaki and Hoppler, 2010) and it is known that Wnts antagonize BMP signals in the dorsomedial lip of the dermomyotome through the activation of Noggin (Hirsinger et al., 1997; Marcelle et al., 1997). This antagonism controls the timing and pattern of myogenic phenotype (Reshef et al., 1998). Another pathway critical for muscle development is mediated by Notch. Notch is a cell-surface receptor, that may initiate an intracellular signaling cascade, when activated by its ligands: Jagged1, Jagged2, Delta-like 1, Delta-like 3 and Delta-like 4 (Artavanis-Tsakonas et al., 1995). The activation of Notch causes sequential proteolytic cleavage events, which result in the liberation from the plasma membrane of Notch intracellular domain (NICD). Once liberated, NICD enters the nucleus, where it activates transcription of target genes (Kopan and Ilagan, 2009). Activated Notch signaling is fundamental during prenatal myogenesis for the maintenance of the undifferentiated state of muscle progenitors, preserving the pool of myoblasts. On the contrary the cessation of Notch activity promotes muscle differentiation (Schuster-Gossler et al., 2007; Mourikis et al., 2012).

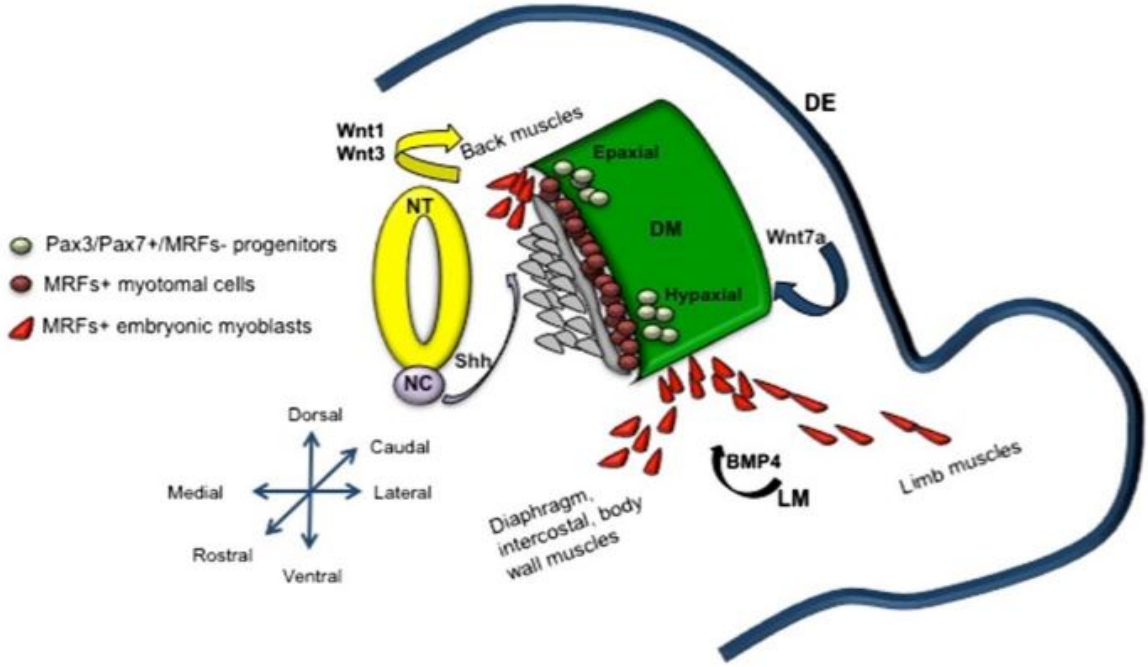

Figure 2: Model of early phases of myogenesis in mouse at E10.5. The model illustrates the morphogenes secreted by the tissus surrounding the somites, which influence the commitment of myogenic precursors. DM dermomyotome, DE dorsal ectoderm, NC notochord, LM lateral mesoderm, MRFs myogenic regulatory factors (Rossi and Messina, 2014).

2.3. The control of myogenic specification, determination and differentiation

Myogenesis in the embryo is initially orchestrated by the myogenic regulatory factors, MRFs, together with the paired-homeobox transcription factors, Pax3 and Pax7. Pax3 is required for myogenic specification (Tajbakhsh et al., 1997), while the MRFs control muscle cell determination and differentiation (Tajbakhsh and Buckingham, 2000). Cells in the mouse dermamyotome express Pax3 and Pax7. However, Pax3, unlike Pax7, is expressed in the hypaxial portion, where it plays an important role in regulating the survival of myogenic progenitor cells (Borycki et al., 1999), the limb musculature development (Franz, 1993; Bober et al., 1994; Goulding et al., 1994; Relaix et al., 2004), and MRFs expression (Maroto et al., 1997; Bajard et al., 2006). Moreover, Pax3 controls the delamination and the migration of muscle precursor cells, because Pax3 activates the expression of c-met, receptor of SF/HGF. SF/HGF are factors that delineate the migratory route of migrating muscle progenitors (Dietrich et al., 1999). On the other hand, Pax7 appears to be dispensable for

embryonic muscle development, but it plays a fundamental role in the maintenance of adult muscle stem cells, the satellite cells (Seale et al., 2000; Relaix et al., 2006). Transgenic mice carrying Pax mutation better elucidate the functions of these transcription factors. In Pax3 mutants the hypaxial domain is lost, leading to the absence of limb and diaphragm muscles (Tajbakhsh & Buckingham 2000), while epaxial-derived muscles are less affected (Bober et al., 1994; Daston et al., 1996; Tremblay et al., 1998). The mice mutant for Pax7 do not exhibit any embryonic muscle phenotype (Mansouri et al., 1996), because of a probable compensation due to Pax3. Indeed, muscle formation is more defective in *Pax3-Pax7* double-knockout embryos, when compared with the single mutant alone (Relaix et al., 2005). Skeletal muscle determination and differentiation depend on MRFs expression. The MRF family is a group of four related basic-helix-loop-helix proteins (bHLH): MyoD (Davis et al., 1987), Myf-5 (Braun et al., 1989), Myogenin (Wright et al., 1989), and MRF4 (Rhodes and Konieczny, 1989). MRFs are master regulators of myogenic lineage, because the expression of any one of them in several non-myogenic cell types converts those cells into skeletal muscle (Weintraub, 1993). The protein structure of MRFs is characterized by a basic-helix-loop-helix domain (bHLH doman), that is high conserved. The main function of bHLH domain is the dimerization and the binding of E-box (enhancer box), a DNA motif with CANNTG as core sequence (Jones, 2004; Berkes and Tapscott, 2005). The crucial role of MRFs in regulating myogenesis was further revealed by models of MRF null mice. Based on knock-out data, it was possible reveal a hierarchy and the functions of MRF network. Mice lacking *MyoD* or *Myf5* have no long-term effects on muscle development, that appears normal but delayed if compared with wild-type embryos. In *Myf5*-null embryos were observed only some epaxial defects, while *MyoD*-null embryos have primary hypaxial defects (Rudnicki et al., 1992; Braun et al., 1992; Tajbakhsh et al., 1997). Knock-out model for both *MyoD* and *Myf5* is devoid of myoblasts and myofibers, demonstrating that both factors are required for the determination of muscle progenitors and that they act upstream of MRF4 and Myogenin (Rudnicki et al., 1993). Mutations in the gene encoding for *Myogenin* cause drastic defects in myoblast differentiation, despite the correct expression of MyoD (Hasty et al., 1993). Moreover, the double mutant for both *Myogenin* and *Myf5* or *Myogenin* and *MyoD* have the same phenotype with the mice lacking only *Myogenin*, confirming that Myf5 and MyoD are upstream of Myogenin (Rawls et al., 1995). *MRF4*-mutants have different phenotypes due to the various functions of MRF4, which is involved both in myogenic determination and also in myofiber differentiation and maturation (Hinterberg et al., 1991; Kassar-Duchossoy et al., 2004). The different but partially overlapping functions of MRFs

reflect also their expression pattern during development. Myf5 and MyoD are the first expressed MRFs for the commitment of myoblasts, followed by the expression of Myogenin and MRF4 in differentiating myoblasts (Hannon et al., 1992; Pownall & Emerson, 1992). In particular, Myf5 appears early in the epaxial domain, while MyoD is expressed later in the hypaxial portion in a Pax3-dependent way. This suggests that axial muscles activate myogenesis through Myf5, while dorso-later myogenesis depends on Pax3 and MyoD activated pathways (Cossu et al., 1996b).

The myocyte enhancer factor 2 (Mef2) family is another family of transcription factors crucial during skeletal muscle myogenesis. Vertebrates have four Mef2 genes (Mef2A, Mef2B, Mef2C and Mef2D), that belong to the evolutionarily ancient MADS family of transcription factors (Shore and Sharrocks, 1995). Mef2 factors have a conserved MAD-box and a Mef2 domain in the N-terminal portion. The Mef2 domain is necessary for the homo- or hetero- dimerization and for the DNA binding to the consensus DNA sequence CAT(A/T)4TAG (Andres et al., 1995; Gossett et al., 1989; Molkentin and Olson, 1996; McKinsey et al., 2002a; Sebastian et al., 2013). Whereas the C-terminus contains the transcriptional activation domain, that is divergent among the different Mef2 members (Potthoff and Olson, 2007). Mef2 factors play a role in the hierarchical regulation of muscle-specific gene expression, although Mef2 do not have myogenic activity. Only the co-expression of Mef2 with MRFs proteins leads to amplification of myogenic differentiation program, increasing the expression of myosin heavy chain isoforms and other muscle structural genes (Molkentin et al., 1995; Wang et al., 2001). The four Mef2 genes are expressed in different tissues as endothelium and bone (Arnold et al., 2007) with higher expression in striated muscles and brain (Edmondson et al., 1994). During prenatal skeletal muscle development, Mef2 mRNAs and proteins were detected in both fetal and embryonic muscles, with the exception of the Mef2D1b isoform, which is restricted to fetal muscle (Ferrari et al., 1997). In particular, *Mef2C* is the first *Mef2* gene expressed around E9 in mouse, while Mef2A and Mef2D are expressed a day later (Edmondson et al., 1994). Moreover, it is known that MRFs activate the expression of *Mef2* genes and in turn Mef2 factors stimulate the expression of MRFs and their own expression (Cheng et al., 1993; Yee and Rigby, 1993; Dodou et al., 2003; Wang et al., 2001), establishing an auto- and cross- regulatory circuits, that maintain muscle phenotype (Penn et al., 2004; Blais et al., 2005). Moreover, it was demonstrated a negative-feedback loop between Mef2C and the histone deacetylase 9 (HDAC9); in fact, Mef2C activates the expression of HDAC9, which, once expressed, restrain Mef2C from excessive activity (Haberland et al., 2007). The understanding of Mef functions in developing muscles was elucidated using mouse mutants. *Mef2A* and *Mef2D* knock-

out mice have no observable phenotype in skeletal muscles (Potthoff et al., 2007a; Potthoff et al., 2007b), while *Mef2C*-null mice exhibit lethality at E9.5 due to cardiovascular defects (Lin et al., 1997). For this reason, there were generated skeletal muscle specific *Mef2C* knock-out under Myogenin and muscle creatin kinase (MCK) promoters. The deletion of Mef2C under Myogenin promoter causes lethality at postnatal day 1 (P1) with myofiber disarray and defects in sarcomere assembly. In contrast, the mice with the deletion on Mef2C under the control of MCK promoter are viable without the disruption of myofiber organization (Potthoff et al., 2007b). In addition, Mef2C regulates fiber type specification, because muscle-specific deletion of Mef2C in a mixed mouse genetic background results in a decrease of slow–twitch fibers, while the over-expression of a super active form of Mef2C (MEF2C-VP16) promotes slow-fiber phenotype (Potthoff et al., 2007). More recently, Anderson et al. confirm that Mef2C is required for normal fiber type composition and discover a new function of Mef2C in regulating glucose uptake (Anderson et al., 2015).

Once committed to myogenic lineage by the expression of MRFs, Pax and Mef factors, myoblasts undergo differentiation. The differentiation requires prior irreversible cell cycle withdrawal. The cell cycle exit is a high regulated process that involves the down-regulation of cell cycle activators, such as cyclins and cyclin-dependent kinases (CDK), and the up-regulation of cell cycle inhibitors, such as Retinoblastoma (Rb), p21 and p27. The induction of growth arrest is mediated by MyoD by activation of the cyclin-dependent kinase inhibitor p21 (Guo et al., 1995; Halevy et al., 1995), which inhibits a wide range of CDKs essential for cell cycle progression (Sherr and Roberts, 1999). MyoD also induces the expression of Retinoblastoma, ensuring the inhibition of cell cycle progression (Martelli et al., 1994). When myoblasts leave the cell cycle, muscle cell fusion begins, allowing the formation of muscle fibers.

2.4. Waves of differentiation: embryonic and fetal myogenesis

During prenatal myogenesis, different types of myofibers develop from different lineage of myogenic progenitors and in a striking time regulated manner (Millet and Stockdale 1986). In vertebrates, skeletal myofibers prenatally develop in two distinct waves, which depend on the differentiation of two populations of myogenic precursors, named embryonic and fetal myoblasts

(Stockdale, 1992; Biressi et al., 2007a; Tajbakhsh, 2009). Embryonic myoblasts are responsible for embryonic or primary myogenesis, which starts in mouse around embryonic day 10.5 (E10.5), giving rise to primary myofibers. Embryonic myogenesis establishes the basic muscle pattern, while fetal myogenesis allow the growth and the complete maturation of prenatal muscles. During fetal myogenesis, which occurs between E14.5 and E17.5, fetal myoblasts fuse to each other or with primary fibers, forming secondary fibers. These two waves of differentiation need the determination of muscle progenitors, through the expression of Myf5, MyoD and MRF4 (MRFs), the commitment to the differentiation and the maturation of myofibers, through the expression of Myogenin and myosin heavy chain (MyHC) isoforms. Embryonic and fetal myoblasts derive from a common progenitor population that co-expresses Pax3 and Pax7 ($Pax3^+/Pax7^+$ cells) during somitogenesis (Kassar-Duchossoy et al., 2005; Relaix et al., 2005). The $Pax3^+/Pax7^+$ population at the beginning of embryonic myogenesis down-regulates Pax7, generating a population of $Pax3^+/Pax7^-$ cells. $Pax3^+/Pax7^-$ cells are bipotent and contribute to the formation of primary fibers and endothelium, while Pax7 is required for fetal myogenesis. The progenitors that terminally differentiate during embryonic myogenesis are only a fraction (Kassar-Duchossoy et al., 2005; Relaix et al., 2005). The remaining committed but undifferentiated progenitors express Pax7 but not Pax3 and are called Pax3-derived $Pax7^+$ cells. Pax3-derived $Pax7^+$ cells are responsible for fetal myogenesis, starting from E14.5 (Figure 3) (Hutcheson et al., 2009; Messina and Cossu, 2009).

Embryonic and fetal myoblasts are intrinsically distinct populations, which were initially characterized according to their *in vitro* features. They deeply differ in terms of morphology, responses to extracellular signals and myofibers that they generate (Stockdale et al., 1992; Biressi et al., 2007b). The phenotypic diversity is revealed *in vitro,* when embryonic and fetal myoblasts were cultured under differentiating conditions, to allow their fusion and the formation of myotubes. Embryonic myoblasts generate small myotubes with few myonuclei (<20 nuclei per myotube), while fetal myoblasts form large myotube with significantly more nuclei (>20) if compared with the embryonic ones. Moreover, if these two population were cultured under conditions that promote proliferation, embryonic myoblasts are less proliferating, because are more prone to differentiate, if compared with fetal myoblasts (Biressi et al., 2007b). The prenatal muscle progenitors have also different sensitivity to different growth factors, such as TGF-β1 or BMP4 (Cossu et al., 1988; Cusella-DeAngelis et al., 1994). In particular, embryonic myoblast differentiation is unaffected by TGF-β1 or BMP4, that inhibit the differentiation of fetal myoblasts (Biressi et al., 2007b). Once differentiated, embryonic and fetal myoblasts generate respectively

primary and secondary fibers, which have different speed of contraction and metabolisms. Primary fibers are programmed for a mainly oxidative and slow phenotype, because they express high levels of slow myosin heavy chain isoform (MyHC-I) and low level of fast MyHC isoforms. On the contrary, secondary fibers adopt a glycolitic and fast phenotype, being negative for MyHC-I, but expressing perinatal and fast isoforms of myosin heavy chain (Stockdale et al., 1992). The phenotypic diversity of embryonic and fetal myoblasts depends on a different transcriptome, which was revealed by genome-wide gene expression assays, by analyzing freshly purified Myf-5 positive embryonic and fetal myoblasts (Biressi et al., 2007). The array analysis shows that a significant number of genes involved in signaling transduction are differentially expressed. This observation explains the divergent response of embryonic and fetal progenitors to the TGF-β1 and BMP4 treatment. In particular, the inhibitory Smad6 and Smad7, negative regulator of TGF-β signaling, are more expressed in embryonic myoblasts, while genes that are involved in the transduction of TGF-β pathway, such as PKCθ, Decorin and Biglycan are more expressed in fetal myoblasts (Biressi et al., 2007b). Moreover, many cell adhesion molecules, genes encoding metabolic and structural protein and transcription factors are differentially expressed. Interestingly, Nfix and JunB, two crucial transcription factors important for muscle differentiation and maturation, were found to be more highly expressed in fetal myoblasts (Biressi et al., 2007b, Messina et al., 2010). The diverse gene expression profile of embryonic and fetal myoblasts clearly demonstrates that these two populations are intrinsically different.

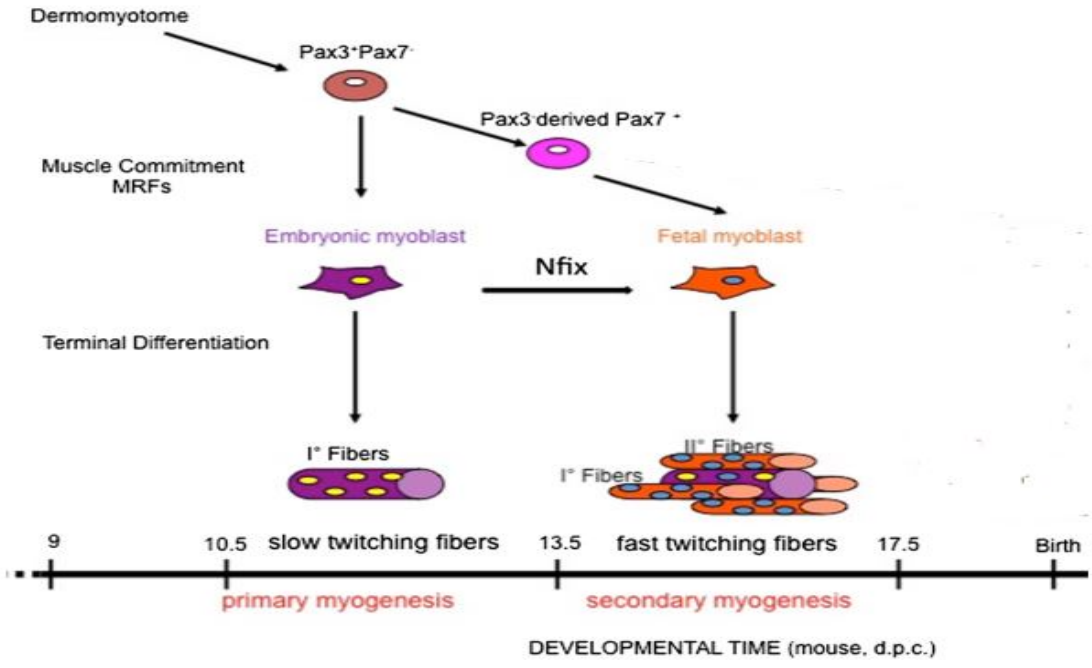

Figure 3: Scheme of prenatal myogenic lineages responsible for embryonic and fetal myogenesis. Embryonic myoblasts ($Pax3^{+}$-$Pax7^{-}$) differentiate around E10.5 in primary slow-twitching fibers, while fetal myoblasts (Pax3-derived $Pax7^{+}$), starting from E14, give rise to secondary fast-twitching fibers. Nfix is the transcription factor, which regulates the transition from embryonic to fetal myogenesis. *d.p.c* days post-coitum (modified from Rossi and Messina, 2014).

2.5. Postnatal development and regeneration

Postnatal muscle growth is achieved by an increase in size of each myofibers by fusion of postnatal myoblasts. The muscle progenitors responsible for the postnatal muscle growth are the Satellite cells (SCs), the resident stem cells of skeletal muscle (Seale et al., 2000). Satellite cells are the third population of muscle progenitors and they are responsible not only for postnatal growth but also for repair and regeneration of skeletal muscle tissue in response to stress, induced by weight bearing or by damage, such as injury or extensive exercise (Grounds and Yablonka-Reuveni, 1993; Charge and Rudnicki, 2004). SCs were first identified by their unique anatomical position, situated underneath the basal membrane of myofibers and outside the sarcolemma (Mauro, 1961). Beside their unique anatomical position, SCs can be identified by expression of several molecular markers, including Pax7 (Seale et al., 2000), M-cadherin (Irintchev et al., 1994), Syndecan-3 and -4 (Cornelison et al., 2001), CD34 (Beauchamp et al., 2000), α7-integrin (Burkin and Kaufman, 1999) and Calcitonin receptor (Fukada et al., 2007). In normal physiological conditions, SCs are mostly quiescent and they can be activated in response to damage, to regenerate muscle tissues. Muscle regeneration occurs in distinct phases: degeneration of myofibers (necrosis), inflammation, regeneration, and functional repair (Musarò, 2014). Myofiber necrosis involves the disruption of sarcolemma, influx of calcium ions and cellular organelles destruction, which stimulates a host inflammatory response. The inflammatory response is a coordinate process, critical for regeneration and for the activation of SCs (Teixeira et al., 2003; Summan et al., 2006; Tidball and Wehling-Henricks, 2007). The first wave of inflammation is mainly composed by neutrophils, then a major role is played by macrophages, which eliminate cellular debris in the necrotic area and sustain SC proliferation and differentiation (Tidball and Villalta, 2010). Once activated upon exposure to signals from a damaged environment, SCs enter the cell cycle, proliferate and differentiate, recapitulating the molecular program, that occurs during prenatal development

(Tajbakhsh & Cossu, 1997). Thus, SCs start to proliferate and activate the expression of Myf5 and MyoD (Cooper et al., 1999; Zammit et al., 2002), generating an amplified population of myoblasts, called myogenic precursors cell (mpc). The myogenic precursors cells undergo cell-to-cell fusion to rapair damaged myofiber or to form de novo multinucleated myofibers, differentiating through the expression of Myogenin and MRF4 (Smith et al., 1994; Yablonka-Reuveni & Rivera, 1994). In addition to generate muscle precursors cells for differentiation, SCs maintain also their own population by self-renewal (Collins et al., 2005). Indeed, they can be classified as myogenic stem cells (Church et al., 1966; Moss & Leblond, 1971). SCs derive from Pax3+/Pax7+ embryonic progenitor cells during prenatal development and they can be visible at the end of fetal myogenesis, when basal lamina is formed and SCs assume their peculiar position (Gros et al., 2005; Relaix et al., 2005; Sambasivan et al., 2013).

2.6. Muscle development in zebrafish

The process of prenatal muscle development in teleosts, in particular in zebrafish, is similar to mammals but the timing and the specification of myogenic progenitors show particular differences. In zebrafish, the first cells committed into myogenic lineage arise before the onset of somitogenesis, which starts immediately after gastrulation (Kimmel et al., 1995). The presomitic population of muscle progenitors is composed by adaxial cells (Stickney et al., 2000). Adaxial cells express Myf5 and MyoD, before the formation of dermamyotome, and migrate radially from the notochord, generating a single layer of superficial slow-twitching fibers, positive for slow MyHC isoform (sMyHC1) and for the transcription factor Prox1a (Figure 4)(Devoto et al., 1996; Stickney et al., 2000). In particular, adaxial cells are specified by notochord-derived Hedgehog (Hh) signals, which lead to the expression of Prdm1, a PR-domain-containing protein (Blagden et al., 1997; Barresi et al., 2000; Roy et al., 2001). Prdm1 is necessary and sufficient to drive slow-twitch differentiation (Roy et al., 2001; Baxendale et al., 2004), by inhibiting the expression of *sox6*, a repressor of slow-specific gene transcription, and by acting as a direct repressor of fast-specific genes (von Hofsten et al., 2008). This mechanism is not conserved in mouse development (Vincent et al., 2012). Fast fibers originate from dermamyotomal progenitors, which form the second component of the primary myotome (Blagden et al., 1997; Stellabotte et al., 2007). Fast fibers are multinucleated and they specifically express the fast MyLC isoform, Mylpfa (Figure 4) (Roy et al., 2001; Liew et al., 2008). After this embryonic period, the second wave of skeletal muscle growth occurs around 48 hour post-fertilization (dpf) by hyperplasia, due to the activity of Pax3+ Pax7+

cells of the dermomyotome (Devoto et al., 1996; Hollway et al., 2007; Elworthy et al., 2008; Stellabotte and Devoto, 2007).

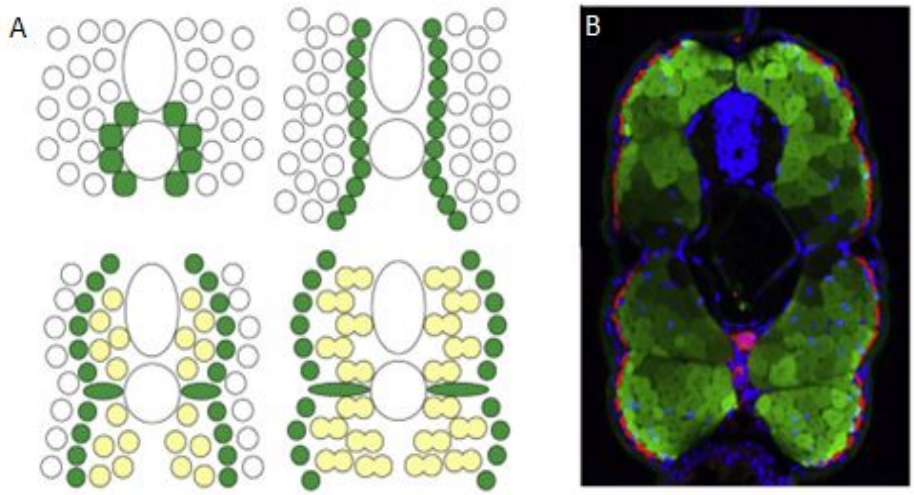

Figure 4: A) Scheme of transverse section of successively older zebrafish embryos. The representation shows the lateral migration of adaxial cells (in green), generating a monolayer of slow-twitching fibers. In yellow is represented the fusion of fast-twitching myoblasts in the central part of embryos. B) Transverse section at the truck level of a 48 hours old zebrafish embryo. The immunofluorescence shows the fiber type composition of myotome. The slow fibers (in red) form a sub-cutaneous layer, while fast-twitching fibers (in green) make up the bulk of the myotome (modified from Jackson and Ingham, 2013).

3. Molecular regulation of myogenic diversity during pre-natal myogenesis

3.1. The role of Nfix

The main regulator of the transition from embryonic to fetal myogenesis is the transcription factor Nfix (Messina et al., 2010). Nfix belongs to the nuclear factor I (NFI) family, which is composed by four closely related genes: *Nfia*, *Nfib*, *Nfic* and *Nfix* (Gronostajski, 2000). The NFI factors have a highly conserved N-terminal domain, which mediates the DNA-binding and the dimerization (Mermod et al., 1989; Gounari et al., 1990). NFI proteins recognize a specific palindromic

consensus binding sequence 5'-PyTGGCA-N3-TGCCAPu-3' as homodimers or heterodimers (Goyal et al., 1990; Kruse & Sippel, 1994b). The C-terminal portion, that exhibits a high variability, is composed by a transcriptional modulation domain for both activation and repression of transcription. Additional variability between NFI factors is due to alternative splicing, encoding more splice isoforms (Kruse and Sippel, 1994a). NFI consensus binding sites were found in promoters and enhancers of genes specific for different tissue, including brain (Martynoga et al., 2013), lung (Bachurski et al., 1997), liver (Jackson et al., 1993), muscle (Spitz et al., 1997) and skeletal elements (Artlett et al., 1998; Gronostajski et al., 2000). Indeed, NFI factors are expressed in different tissue both during development and in adulthood and their expression patterns are restricted in some specific tissue and fine regulated in time (Chaudhry et al., 1997). The analysis of mouse mutant for each of the NFI factors reveals that they have distinct and crucial roles in different organs. *Nfia* $^{-/-}$ mice have severe brain defects with a lack of genesis of corpus callosum and ventricular dilation, causing early hydrocephalus. The neurological deformity causes a high prenatal mortality in *Nfia* $^{-/-}$ embryos (das Neves et al., 1999). *Nfib*-deficient mice possess lung and forebrain defects (Steele-Perkins et al., 2005), whereas *Nfic* mutants have agenesis of tooth roots and aberrant incisor development (Steele-Perkins et al., 2003). Finally, *Nfix*$^{-/-}$ mice have growth retardation and die postnatally between postnatal day 21 (P21) and P28. They present enlargement of brain ventricles and partial agenesis of corpus callosum. Moreover, *Nfix*-mutants show spine deformation with impaired endochondral ossification and skeletal muscle defects (Driller et al., 2007; Messina et al., 2010). The phenotypes of NFI-deficient mice suggest that these factors have both overlapping functions, for example in the regulation of brain development, and tissue specific functions essential for prenatal development. The NFI factor more expressed in skeletal muscle during myogenesis is Nfix (Messina et al., 2010), and its expression is rapidly increased during fetal myogenesis, being virtually absent in embryonic myoblasts (Biressi et al., 2007b). Once expressed, Nfix is able to drive the complete maturation of prenatal muscles, allowing the switch from embryonic to fetal myogenesis. In particular, Nfix represses embryonic specific genes and activate fetal ones (Messina et al., 2010).

Fetal myoblasts silenced for *Nfix* have an impaired fusion and generate small myotubes, acquiring a more embryonic-like phenotype. Moreover, silencing of Nfix in fetal progenitors causes the down-regulation of fetal specific genes, such as *MCK* and *β-enolase*, and the up-regulation of embryonic markers, such as MyHC-I. On the contrary, embryonic myoblasts over-expressing Nfix, form larger myotubes in comparison with control cells and early down-regulate slow MyHC.

Interestingly, Nfix over-expressing cells don't up-regulate MCK, suggesting a requirement of other cofactors. Deeper analysis demonstrates that Nfix is able to induce MCK expression, acting in a complex with Mef2A and PKCθ. In particular, the expression of MCK is dependent on Mef2A, a transcription factor that is equally expressed during all the prenatal development. The capability of Mef2A to bind MCK promoter is dependent on a post-translational phosphorylation, that occurs specifically during fetal myogenesis (Ferrari et al., 1997) and the phosphorylation of Mef2A is due to PKCθ, whose expression is fetal-specific (Biressi et al., 2007b). Nfix acts as a bridge between Mef2A and PKCθ, allowing Mef2A phosphorylation and the consequent activation of MCK. Moreover, Nfix is able to repress MyHC-I expression both indirectly, through the inhibition of Nfatc4 (Messina et al., 2010), and directly, acting in a complex with Sox6 (Taglietti et al., 2016). Interestingly, during fetal myogenesis, Nfix cooperates with Sox6 in the inhibition of slow MyHC and this cooperation is necessary for the switch of Sox6 activity between embryonic and fetal myogenesis (Taglietti et al., 2016). The Nfix negative regulation of slow MyHC is conserved during vertebrate evolution. Indeed, in zebrafish, the ortholog of *Nfix*, *nfixa*, negatively regulate nfatc4, which in turn, is responsible for the physiological expression of slow MyHC, as in mouse (Pistocchi et al., 2013). Also, the functional cooperation between Nfix and Sox6 is conserved during zebrafish myogenesis and is required for the proper regulation of slow MyHC (Taglietti et al., 2016). The role of Nfix in mouse was also characterized in embryos (E12.5) over-expressing Nfix under muscle specific promoter, Tg:*Mlc1f-Nfix2*, and in fetuses (E16.5) with a skeletal muscle-specific deletion of *Nfix* (Messina et al., 2010), demonstrating that Nfix is required and sufficient for the induction of fetal program of muscle gene expression (Figure 5). Moreover, skeletal muscle-specific *Nfix*-deficient muscles (E16.5) have a delayed assembly of sarcomeres and the diameter of myofibers is reduced, suggesting that Nfix may be involved in the regulation of many other genes. Nowadays, the mechanisms that temporally regulate the up-regulation of Nfix specifically at the beginning of fetal myogenesis are not fully known. Indeed, Pax7 is sufficient to activate Nfix, because its over-expression in embryonic myoblasts causes a precocious activation of Nfix, but *Pax7*-null muscles normally express Nfix. These observations corroborate the idea that other genes can be involved in the regulation of Nfix expression (Messina et al., 2010). Finally, it was recently clarified the role of Nfix also during post-natal myogenesis. As observed during prenatal development, adult *Nfix*-null muscles have altered homeostasis and morphology, characterized by a reduced muscle fiber size and over-expression of slow MyHC isoform (Rossi et al., 2016). Moreover, *Nfix*-null mice have a delayed regeneration, which correlates with an increased expression of Myostatin, a potent

inhibitor of myogenesis and satellite cell activation (Taylor et al., 2001; McCroskery et al., 2003). Myostatin expression is directly represses by Nfix, influencing the proper timing of muscle regeneration (Rossi et al., 2016).

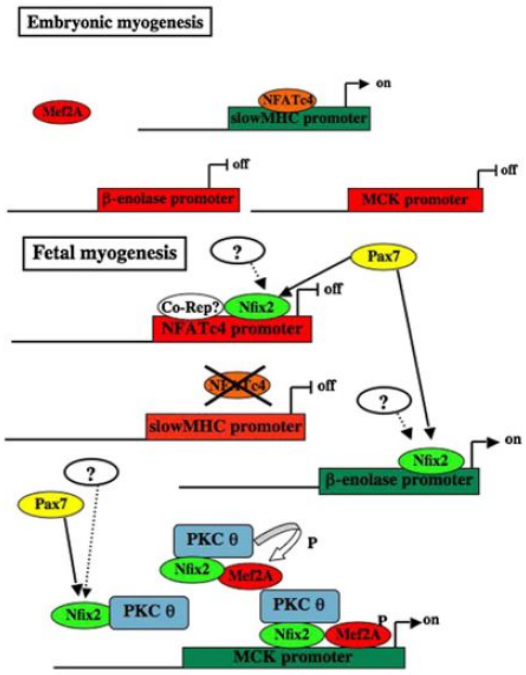

Figure 5: Scheme of Nfix mechanisms of action in regulating the transition from embryonic to fetal myogenesis. During fetal myogenesis, Nfix directly represses Nfatc4, causing the inhibition of slow MyHC expression, and directly promotes β-enolase activation. Nfix acts also as a bridge between PKCθ and Mef2a, allowing MCK up-regulation (Messina et al., 2010).

3.2. The role of Sox6

The SRY-related HMG box (Sox) family in mammals is composed by 22 genes, divided in eight groups (SoxA-SoxH) (Schepers et al., 2002). All the Sox factors have a highly conserved high-mobility group (HMG) domain, which mediates DNA binding to ATTGTT or related sequence motifs (Badis et al., 2009; Kondoh and Kamachi, 2010; Kamachi and Kondoh, 2013). The HMG domain allows also the interaction with cofactors (Yamashita et al., 2000) and it contains two nuclear localization signals (NLS), required for the shuttling between cytoplasm and nucleus (Malki et al., 2010). The shuttling, but also the transcriptional activity of Sox factors, is regulated post-translationally by modifications including phosphorylation (Huang et al., 2000), acetylation (Thevenet et al., 2004) and sumoylation (Gill, 2005; Hattori et al., 2006). SoxD group includes *Sox5*, *Sox6* and *Sox13* genes, whose expression has been demonstrated in different cell lineages as chondrocytes (Lefebvre et al., 1997), vessels (Roose et al., 1998), oligodendrocytes (Stolt et al., 2006), erythrocytes (Dumitriu et al., 2006) and skeletal muscle (Hagiwara et al., 2000). SoxD factors do not possess a transactivation/repression domain, indicating that the recruitment of cofactors is necessary to elicit transcriptional activation or repression activity (Kamachi et al., 2001). Sox6 has important roles in the development of different organ and tissues. *Sox6*-deficient mice die after birth, failing to breathe. Those that survive die in early life, because of the stop to feed (Smits et al., 2001). During skeletal muscle development, Sox6 plays a crucial role in regulating fiber type differentiation in secondary fibers. Fetal *Sox6*-mutant muscles have a significantly higher expression of slow fiber type-specific genes, whereas fast fiber-specific genes are decreased (Hagiwara et al., 2005), suggesting that Sox6 functions as a transcriptional repressor of slow-twitching phenotype. In particular, Sox6 acts as a direct repressor of a specific marker of slow fibers: MyHC-I. Sox6 is able to directly bind the proximal cis-regulatory element of MyHC-I promoter (350-bp upstream of the transcription start site) and a distal enhancer (between -3.5 kb and -2.5 kb from the transcription start site), which are both necessary for the optimal MyHC-I expression in skeletal muscle (Shimizu et al., 1992; Giger et al., 2000; Hagiwara et al., 2005). Furthermore, Sox6 function was elucidated through genome-wide ChIP-seq analysis in fetal (E18.5) myotubes differentiated *in vitro*. Interestingly, the comparison of Sox6 ChIP-seq with the phosphorylated form of Pol II ChIP-seq, which is considered to be a transcriptionally active form of Pol II, associated with highly transcribed gene (Brookes and Pombo, 2009), reveals that the 84% of Sox6 bound genes are transcriptionally inactive or transcribed at a very low level in myotubes (An

et al., 2011). Gene Ontology (GO) analysis reveals that Sox6 target genes show the highest enrichment for genes relevant for myofibril establishment. In particular, Sox6 regulates proteins, which define the fiber types but it is not directly involved in the transcriptional regulation of the fastest MyHC isoforms (An et al., 2011). Sox6 is implicated in fiber type specification also in zebrafish. In zebrafish embryos lacking Prdm1a, adaxial cells differentiate into fast-twitching fibers, because they ectopically express *sox6*. This transformation is suppressed by the transient knock-down of sox6, suggesting that sox6 also during zebrafish development acts to repress slow-twitching genes (von Hofsten et al., 2008; Jackson et al., 2015).

3.3. The role of JunB

Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra1 and Fra2) and ATF proteins are members of AP-1 family. AP-1 is a complex of heterodimers or homodimers, which determine the transcriptional regulation of different target genes (Angel and Karin, 1991; Aronheim et al., 1997; Bakiri et al., 2000). The members of AP-1 family are basic leucine-zipper (bZIP) proteins, because they interact and dimerize through a leucine-zipper motif, and they contain a basic domain for the binding with the DNA in specific DNA response element, named TPA-responsive element (TRE). In particular, Fos proteins can only heterodimerize with Jun factors, while the Jun proteins can both homo- and hetero-dimerize to form transcriptionally active complexes (Nakabeppu et al., 1988; Smeal et al., 1989; Jochum et al., 2001). Moreover, AP-1 members can interact with other factors through the leucine-zipper domain, expanding their regulatory potential (Deng & Karin, 1992). The AP-1 proteins differ considerably in their trans-regulation abilities (Chiu et al., 1989; Bos et al., 1990), but they exhibit similar DNA-binding specificity (Nakabeppu et al., 1988). In spite of the similar DNA-binding specificity, it is known that different dimers preferentially bind to different DNA elements, as the cAMP-response element (CRE), the MAF-recognition elements (MAREs) and the antioxidant-response elements (AREs) (Chinenov and Kerppola, 2001; Eferl and Wagner, 2003). The AP-1 main function is the transduction of extracellular stimuli into the cells, converting growth factor signals into changes in expression of specific target genes (Passegue et al., 2002; Eferl and Wagner 2003). JunB is the member of AP-1 family most characterized in skeletal muscle tissue

and, during myogenesis, it is more highly expressed during fetal myogenesis compared with embryonic myogenesis, as Nfix (Wilkinson et al., 1989; Biressi et al., 2007b). JunB is essential for prenatal development, because the inactivation of JunB results in embryonic lethality around E9.5 because of defects in vasculogenesis in the extra-embryonic tissues (Schorpp-Kistner et al., 1999; Jochum et al., 2001). To rescue the embryonic lethality, it was generated *JunB-/- Ubi-JunB* transgenic mouse, which expresses JunB under the human ubiquitin C promoter, resulting in a reduced expression of JunB (Schorpp-Kistner et al., 1999; Passegue et al., 2001; Hartenstein et al., 2002). *JunB-/- Ubi-JunB* mice have a decrease in body size and reduced length of appendicular bones, due to impaired endochondral bone growth (Hess et al., 2003). The role of JunB in skeletal muscle was characterized only in adult muscles. In normal adult tissue, JunB is required for the maintenance of muscle mass and its down-regulation induces muscle atrophy. Conversely, JunB over-expression causes hypertrophy by enhancing protein synthesis and myosin expression (Raffaello et al., 2010). Moreover, JunB can specifically reduce the stimulation of proteasomal-dependent protein degradation by reducing the activity of FoxO3, a transcription factor that induces atrophy through the activation of atrogenes (Sandri et al., 2004; Raffaello et al., 2010). Thus, it is clear that JunB plays an important role in skeletal muscle maintenance and its strong up-regulation specifically during fetal myogenesis leads to speculate that JunB may be important also for prenatal muscle development.

4. From the extracellular signal to the signaling pathways

4.1. ERK kinases

The extracellular regulated kinase 1 and 2 (ERK1 and ERK2) belong to MAPK family that includes also JNK and p38 MAPKs. The ERKs are known to be one of the principal kinases activated by growth factors and the constitutive activation of ERKs is associated with alterations in cell proliferation, differentiation, and apoptosis (Scholl et al., 2005). ERK kinases can be activated by a high number of ligand- or self-activated tyrosine kinase receptors, such as EGFR, FGFR, IGFR, PDGFR and insulin receptor (Chang et al., 2001; Karnoub and Weinberg, 2008). The ERKs have distinct biological functions *in vivo*. *Erk1*-deficient mice are viable and fertile, with defect in thymocyte maturation (Pages et al., 1999), enhanced long-term memory and it is characterized by

an up-regulation of ERK2 signaling in response to glutamate (Mazzucchelli et al., 2002). *Erk2* mutant mice are embryonic lethal around E6.5 and the embryos present alteration in mesoderm differentiation (Yao et al., 2003). The signaling cascade of ERKs is usually initiated by Ras activation, which transmits the signal by phosphorylating MAPKK proteins. MAPKK, in turn, activate MAPK/ERK kinases, named MEK (Ahn et al., 1991; Gomez and Cohen, 1991), that specifically phosphorylates and activates ERK kinases (Seger and Krebs, 1995). Nevertheless, it was postulated the existence of MEK-independent mechanisms involved in ERKs activation (Johnson et al., 2005; Cuevas et al., 2007). Once activated, the ERKs phosphorylate a large number of substrates (Yoon et al., 2006), that can be localized in the cell cytoplasm or in the nucleus (Seger et al., 1991). Nowadays only some transcription factors, activated by the ERKs, were identified, such as Elk1 (Hipskind et al., 1991; Gille et al., 1992) and JunB (Textor et al., 2006; Cevik et al., 2008). The ERK kinases control the caudo-rostral maturation of PSM, allowing the correct segmentation and somite formation in chick embryos. In particular, ERK activity FGF8-dependent prevents cellular epithelialization and regulates motility along the antero-posterior axis of the PSM (Delfini et al., 2005). Although ERK function is well characterized during early somitogenesis, the role of ERKs in regulating myogenesis is controversial, because the available data are conflicting. It is reported that the ERKs have different activities at early versus late stages of muscle differentiation (Bennett and Tonks, 1997). *In vivo* studies on chick embryos show that the ERK shuttling between nucleus and cytoplasm provide the switch between the two cellular responses: myogenic proliferation and differentiation (Michailovici et al., 2014). In particular, the activation of ERKs, due to FGF activity, causes their translocation into the nuclei, where they promote the proliferation of myogenic progenitors and the inhibition of differentiation. Indeed, the cytoplasmatic localization of ERK correlates with the fusion and differentiation of myoblasts (Michailovici et al., 2014). Also *in vitro* studies show that ERK signaling is crucial for both proliferation and fusion of myoblasts (Jones et al., 2001). Furthermore, Li and Johnson in 2006 report that ERK1 and ERK2 play non redundant functions, using C2C12, mouse myoblast cell line, silenced for ERK1 (C2C12siERK1) or for ERK2 (C2C12siERK2). They demonstrate that C2C12siERK2 cells grow lower and fail to fuse, if compared with control cells or with C2C12siERK1 myoblasts, suggesting that ERK2 is necessary for myofiber formation (Li and Johnson, 2006). Consistently, the treatment of C2C12 with ERK phosphorylation inhibitor, U0126, inhibits myoblast fusion and differentiation (Jiang et al., 2015). On the contrary, it is reported that 23A2 and L6 myoblasts are committed to terminal differentiation upon ERK inhibition, leading to the contrast conclusion that ERK activity is necessary for the inhibition of

terminal differentiation (Coolican et al., 1997; Weyman & Wolfman, 1998). The reasons for these controversial results are unclear but probably the reason depends on the different cell types and conditions used. Moreover, ERK kinases may be only transiently activated and their function may depend on their cellular localization and on the crosstalk with other signaling, complicating the clear identification of their role in myoblasts.

4.2. RhoA

Rho-related small GTPases is a family of low molecular weight GTPases divided into subfamilies: Rho, Rac1, Cdc42 and Rnd. The main function of Rho GTPases is the transduction of extracellular signal into intracellular events, including remodeling of cytoskeleton, control of cell morphology and motility and regulation of gene expression (Narumiya 1996; Bar-Sagi and Hall, 2000; Takai et al., 2001). The subfamily RhoA includes three members named RhoA, RhoB and RhoC, which share high homology. They differ in the C-terminal portion, which influence the interaction with regulators and the subcellular localization (Hori et al., 1991). All the member of the Rho family cycle between an inactive, Guanosine diphosphate (GDP)-bound state and an active, guanosine triphosphate (GTP)-bound state. The oscillation between the GDP-bound and GTP-bound state is regulated by different classes of proteins: GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and GDP-dissociation inhibitors (GDIs)(Figure 6). The activation of Rho effectors depends on GAP proteins, which accelerate the intrinsic GTPase activity of Rho GTPases, promoting the transduction of the signal to their targets (Saras et al., 1997; Taylor et al., 1998). The activation of Rho is regulated by GEFs proteins, which catalyze the exchange of GDP for GTP, thanks to the DH domain with nucleotide exchange activity (Hart et al., 1996; Fukuhara et al., 1999). On the contrary GDIs inhibit Rho activity, blocking the guanine nucleotide exchange (Figure 6) (Sasaki and Takai, 1998; Olofsson, 1999).

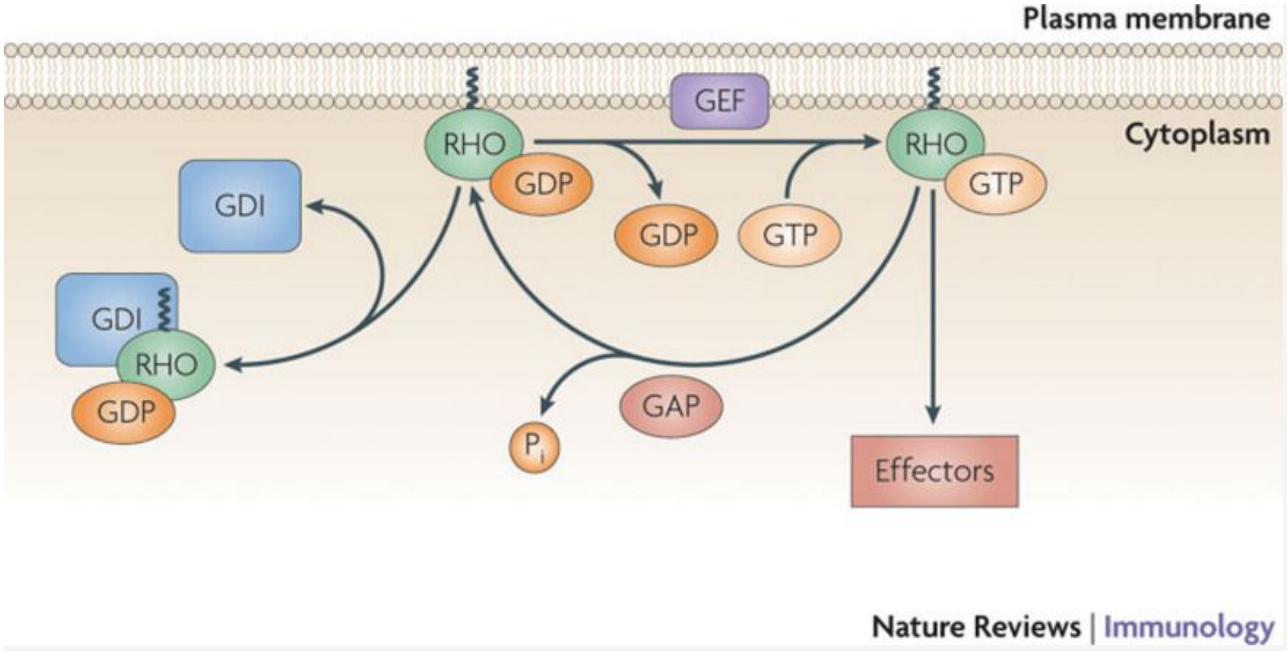

Figure 6: Scheme of regulation of Rho GTPase. The Rho GTPase is anchored to the cell membrane, where the GEF proteins catalyze the release of GDP, allowing the binding to GTP. The GAP proteins increase the GTPase activity of Rho GTPases. The GDIs can sequestrate GDP-bound Rho proteins, inhibiting the binding of Rho GTPases to the cell membrane (Tybulewicz and Henderson, 2009).

The most characterized Rho GTPase is RhoA, which mediates intracellular responses by activating downstream targets. The direct effectors of RhoA are the kinases ROCK and the non-kinase proteins as mDia, rhophilin and rhotekin (Watanabe et al., 1996; Reid et al., 1996; Matsui et al., 1996). ROCK is a serine/threonine kinase, which has a kinase domain in the N-terminal portion, a middle coiled-coil region and a Cys-rich zinc finger-like motif in the C-terminus (Fujisawa et al., 1996; Nakagawa et al., 1996). In mouse, two ROCK kinases were isolated, ROCK I and ROCK II, which may have tissue-specific functions because they are differentially expressed in distinct tissues. In particular, ROCK I is ubiquitously expressed expect in the brain and muscle, while ROCK II is abundant in the brain, muscle, heart, lung and placenta (Fujisawa et al., 1996). The cellular responses of RhoA are studied with different approaches *in vitro*, because *RhoA*-null mice die during early embryogenesis. The role of RhoA in different cell types and processes has been investigated using pharmacological inhibitors, such as C3 exoenzyme, a non specific Rho inhibitor, or Y27632, a competitive inhibitor of ATP binding, selective for ROCK (Uehata et al., 1997; Ishizaki

et al., 2000). Alternatively, there were identified different dominant negative (RhoG17V) and constitutive active forms of RhoA (RhoV14), that are wildly used for *in vitro* studies. RhoA and its kinases ROCK are required for myogenesis and their activity must be fine regulated in time to allow muscle differentiation (Castellani et al., 2006). In skeletal muscle, RhoA is essential for muscle development; indeed, the differentiation of muscle progenitors is blocked if RhoA signaling is inhibited by treatment with ROCK inhibitor or by expressing the dominant negative form of RhoA (Takano et al., 1998). In addition, RhoA activates MyoD and α-actin gene expression (Carnac et al., 1998; Wei et al., 1998) through an indirect mechanism, which involves the redistribution of serum responsive factor (SRF) cofactors from the cytoplasm into the nucleus. In fact, SRF activity correlates with the ration of F-actin (polymerized actin) to G-actin (un-polymerized) in the cytoplasm, because the SRF cofactor MAL in retained in the cytoplasm in presence of high concentration of G-actin. On the contrary, the increased polymerization of actin, promoted by RhoA effectors, leads to the release of MAL from G-actin and the consequent translocation into the nucleus, where it mediates the transcriptional activation of SRF target genes (Miralles et al., 2003). SRF is a MADS-box transcription factor, which control growth factor regulated genes, such as AP-1 factors and different muscle-specific genes (Buckingham, 1994; Soulez et al., 1996). In muscle progenitors, RhoA can be activated by N-cadherin-dependent cell-cell adhesion (Charrasse et al., 2002), providing a link between cell-cell adhesion and downstream signaling of RhoA-SRF, to induce muscle-specific expression of MyoD. Instead, Sordella et al. demonstrate that mice lacking the RhoA inhibitor p190-Rho GAP are defective for adipogenesis but tend to undergo myogenic differentiation (Sordella et al., 2003), suggesting that RhoA and ROCK regulates the adipogenic-myogenic switch during prenatal development (Sordella et al., 2003). Thus, RhoA seems to be involved in the specification of myoblasts but down-regulation of RhoA activity in committed myoblasts is essential for cell cycle withdrawal and myoblasts fusion (Nishiyama et al., 2004), concluding that its activity must be tightly regulated in a finely coordinated in a time-dependent manner to ensure skeletal muscle formation (Castellani et al., 2006; Charrasse et al., 2006).

Aim of the projects

Mammalian skeletal muscle is composed of different fiber types, whose identity is first established during prenatal development (Schiaffino & Reggiani, 2011). This heterogeneity of muscle fibers depends on differentiation of distinct classes of myogenic progenitors, named embryonic and fetal myoblasts. In particular, embryonic myoblasts differentiate into multinucleate muscle fibres, primary fibers, around E11 in the mouse, while the differentiation and fusion of fetal myoblasts takes place between E14.5 and E17.5, giving rise to secondary fibers. In general, it is well established that mammalian primary fibers are programmed for a predominantly slow-twitching phenotype, whereas secondary fibers adopt a fast phenotype (Wigmore & Evans, 2002). It was demonstrated, through genome wide expression analysis carried on purified embryonic and fetal, that the different phenotype of primary and secondary fibers depends on intrinsic factors. This analysis identified many differentially expressed genes and clearly revealed that embryonic and fetal myoblasts have distinct genetic programs, suggesting that the proper transition of skeletal muscle from the embryonic to the fetal phenotype requires a switch in the transcriptional status of differentiating myoblasts (Biressi et al., 2007b). The “master” regulator of embryonic to fetal transition is the transcription factor Nfix (Messina et al., 2010). To drive fetal myogenesis, Nfix is specifically activated around E14.5 and it is virtually absent during the earlier phases of skeletal muscle development. Nowadays, the mechanisms that temporally regulate its expression are still unknown. Nfix, once expressed, allows the acquisition of fetal fiber phenotype, activating fetal specific genes, and repressing embryonic ones, such as the slow myosin heavy chain (MyHC-I) (Messina et al., 2010). Another well-known repressor of MyHC-I during fetal myogenesis is the transcription factor Sox6 (Hagiwara et al., 2007; An et al., 2011). Interestingly, Sox6, at variance of Nfix, is expressed also during embryonic myogenesis, when embryonic myoblasts form primary fibers that express high level of MyHC-I, despite its role of inhibitor of slow twitching program.

Therefore my phD work has been developed by addressing two main aims:

- The identifications of Sox6 functions in regulating MyHC-I during embryonic myogenesis and the characterization of the interplay between Nfix and Sox6 during fetal myogenesis.

- The elucidation of the molecular mechanisms involved in the regulation of Nfix expression during prenatal myogenesis.

Main Results:

1. Nfix induces a switch in Sox6 transcriptional activity to regulate MyHC-I expression in fetal muscle

During my PhD I worked on a project, that I started during my Master of Science internship, about the functional cooperation between Sox6 and Nfix during murine prenatal myogenesis. The results of this project have been recently published on Cell Reports. Here below the main conclusions of this work, whose paper is included in the present Thesis (Part II):

- during embryonic myogenesis, Sox6 acts indirectly as a positive regulator of MyHC-I via a Mef2C-dependent mechanism, in sharp contrast with its well-known and characterized function during fetal myogenesis
- during fetal myogenesis, Sox6 and Nfix phisically and functionally cooperate in the direct inhibition of MyHC-I expression
- the functional cooperation of Sox6 and Nfix is evolutionary conserved also in zebrafish and it is required for proper skeletal muscle development

2. RhoA-ERK axis regulates secondary myogenesis through the control of JunB and Nfix expression

The second project, that I developed during my PhD Thesis, aims to identify and define the molecular signaling responsible for the expression of Nfix at the onset of fetal myogenesis. Here below, the main results achieved, that are now collected in a manuscript in preparation (Part III).

- JunB and Nfix are both specifically expressed at the onset of fetal myogenesis
- JunB is sufficient and necessary for Nfix activation, while Nfix does not control JunB expression but maintains its own expression.

- JunB is not able to promote the switch from embryonic to fetal myogenesis, which is strickly controlled by Nfix

- The expression of JunB and Nfix is negatively regulated by RhoA/ROCK, during embryonic myogenesis, through the inhibition of ERK kinase activity

- ERK kinases, being completely active during fetal myogenesis, are necessary for JunB and Nfix expression

Conclusion and future perspective

Skeletal myogenesis occurs in successive developmental stages, which involve the differentiation of two distinct populations of muscle progenitors: embryonic myoblasts and fetal myoblasts. These two cell populations give rise to heterogeneous muscle tissues, which are composed of different type of fibers in terms of shape, size, contractile activity and metabolism. In particular, embryonic myoblasts fuse into multinucleated primary fibers, starting around E10.5 in mouse. The formation of primary fibers is considered the first wave of muscle differentiation, called embryonic myogenesis. The second wave of myogenesis, named also fetal myogenesis, takes place between E14.5 and E17.5 in mouse, when fetal myoblasts form secondary fibers. This multiphasic process of differentiation gives rise to different fiber types. In particular, primary fibers are slow-twiching fibers, which express high level of the slow and embryonic isoforms of myosin heavy chain (respectively MyHC-I and MyHC-emb); in contrast, secondary fibers are fast-twitching fibers, expressing fast and neonatal isoforms of myosin heavy chain (MyHC-2a, MyHC-2x, MyHC-2b and MyHC-neo). In general, primary fibers are programmed for a mainly slow phenotype and oxidative methabolism, whereas secondary fibers adopt a fast and glycolytic phenotype (Biressi et al., 2007b; Stockdale, 1992). The diversification of muscle fibers during embryonic and fetal stages is independent from neural and hormonal influences. In the past years, some key factors, that may regulate the fiber type specification, has been identified, such as Sox6 (Hagiwara et al., 2005) and Nfix (Messina et al., 2010). Sox6, belonging to group D of Sox factors, is a well known inhibitor of slow-twitching phenotype during fetal myogenesis (Hagiwara et al., 2005; An et al., 2011), whereas Nfix is a transcription factor specifically expressed during fetal myogenesis, that is a crucial activator of the fetal genetic program, allowing both the repression of embryonic genes, such as MyHC-I, and the activation of fetal ones, such as glycolytic enzymes (β-enolase and MCK). We have demonstrated a function switch in Sox6 activity on MyHC-I expression during embryonic

to fetal myogenesis. Our results show that, during fetal myogenesis, Nfix and Sox6 are coexpressed in fetal muscles and cooperate in inhibiting MyHC-I expression. Indeed, we found that Nfix acts as a fundamental co-factor of Sox6, allowing its direct binding to the proximal promoter of *Myh7* gene, critical for Sox6-dependent fetal repression of MyHC-I (Hagiwara et al., 2007). Conversely, during embryonic myogenesis, when Nfix is not yet activated, Sox6 is expressed starting from E12.5, when embryonic progenitors form slow-twitching fibers positive for MyHC-I. In embryonic myogenic progenitors, Sox6, through the direct activation of Mef2C, induce MyHC-I expression, contributing to the specification and maintenance of primary fibers. All together, our data show that the reversal in Sox6 functions from embryonic to fetal myogenesis is due to the interplay with Nfix, that, once expressed, instructs Sox6 for the direct repression of MyHC-I. Moreover, we provide evidence for a conserved cooperation of Sox6 and Nfix orthologs in zebrafish. The fetal-specific expression of Nfix is known to be regulated by Pax7, that is sufficient but not necessary for Nfix activation (Messina et al., 2010), corroborating the idea that additional genes are responsible for Nfix expression. With the aim to identify the molecular and cellular mechanisims by which Nfix expression is achieved during fetal myogenesis, we focused our attention on JunB, the second transcription factor more highly expressed in fetal compared to embryonic myogenesis (Biressi et al., 2007b). We demonstrate that JunB is both sufficient and necessary for the direct induction of Nfix during prenatal skeletal myogenesis, starting from E14.5 in mouse. Therefore, we show that the activation of the fetal genetic program is strikingly dependent on Nfix expression; indeed, JunB alone is not able to drive embryonic to fetal molecular changes. Moreover, we identified the RhoA/ROCK pathway as, at least, one of the main signaling involved in the maintenance of embryonic identity, thanks to their inhibitory effect on ERK kinase activity, which is necessary for JunB and Nfix expression. During fetal myogenesis, we observed a decrease in RhoA/ROCK activity, followed by ERK kinase activation, which allows JunB and Nfix

expression. Nowadays, it remains unknown the upstream inputs, which orchestrate the activity of these signaling pathway and the clarification of them will let us to understand how myogenic progenitors are instructed during skeletal myogenesis. Another crucial point, still unknown, is the knowledge of Nfix functions during fetal myogenesis. We know that Nfix is sufficient to drive fetal myogenesis, repressing directly and indirectly MyHC-I expression and activating fetal- specific genes, such as β-enolase and MCK (Messina et al., 2010), but the skeletal muscle phenotype of *Nfix*-null fetuses is more severe. The morphological changes of fetal muscles lacking Nfix, such as sarcomere disorganization and misexpression of mature isoforms of contractile proteins (Messina et al., 2010), strongly suggest that this transcription factor may regulate the expression of many other genes. To better elucidate Nfix functions, it would be crucial to known all its target genes, which still remain an unsolved issue. The comprehension of the mechanisms involved in the achievement of the respective identities of the two populations of myoblasts and the clarification of Nfix functions during fetal myogenesis will contribute to augment the knowledge of the mechanisms regulating the process of skeletal myogenesis.

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PART II

LIST OF PUBLISHED PAPERS

Taglietti Valentina, Maroli Giovanni, Cermenati Solei, Monteverde Stefania, Ferrante Andrea, Rossi Giuliana, Beltrame Monica and Messina Graziella.

“Nfix induces a switch in Sox6 transcriptional activity to regulate MyHC-I expression in fetal muscle”.

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Article

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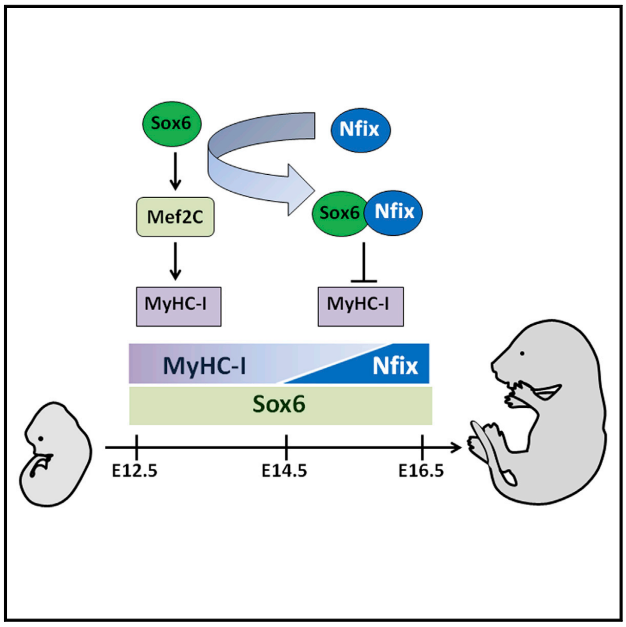
Nfix Induces a Switch in Sox6 Transcriptional Activity to Regulate MyHC-I Expression in Fetal Muscle

Graphical Abstract



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In Brief

Taglietti et al. reveal molecular mechanisms defining muscle fiber specification, driven by the key transcription factors Nfix and Sox6, during mouse and zebrafish development. They show that Nfix reverses Sox6 function between embryonic and fetal phases of myogenesis, allowing the proper expression of slow MyHC.

Highlights

- Sox6 has opposite roles in MyHC-I regulation during embryonic and fetal myogenesis
- In embryonic muscle, Sox6 enhances MyHC-I expression via regulation of Mef2C
- In fetal muscle, Nfix is required for Sox6-mediated repression of MyHC-I
- The Sox6 and Nfixa orthologs cooperate in repressing *smyhc1* in zebrafish

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Nfix Induces a Switch in Sox6 Transcriptional Activity to Regulate MyHC-I Expression in Fetal Muscle

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SUMMARY

Sox6 belongs to the Sox gene family and plays a pivotal role in fiber type differentiation, suppressing transcription of slow-fiber-specific genes during fetal development. Here, we show that Sox6 plays opposite roles in MyHC-I regulation, acting as a positive and negative regulator of MyHC-I expression during embryonic and fetal myogenesis, respectively. During embryonic myogenesis, Sox6 positively regulates MyHC-I via transcriptional activation of Mef2C, whereas during fetal myogenesis, Sox6 requires and cooperates with the transcription factor Nfix in repressing MyHC-I expression. Mechanistically, Nfix is necessary for Sox6 binding to the MyHC-I promoter and thus for Sox6 repressive function, revealing a key role for Nfix in driving Sox6 activity. This feature is evolutionarily conserved, since the orthologs Nfixa and Sox6 contribute to repression of the slow-twitch phenotype in zebrafish embryos. These data demonstrate functional cooperation between Sox6 and Nfix in regulating MyHC-I expression during prenatal muscle development.

INTRODUCTION

In vertebrates, the process of skeletal muscle development occurs in subsequent steps that involve distinct populations of myogenic progenitors, the myoblasts, which arise from the dermomyotomal domain of somitic mesoderm (Christ and Ordahl, 1995). The process of myogenic differentiation is initiated in mesodermal cells by a family of basic-helix-loop-helix (bHLH) transcription factors, named muscle regulatory factors (MRFs), that are able to activate transcription of muscle-specific markers such as the myosin heavy chain (MyHC) isoforms (Pinney et al., 1988; Cao et al., 2010). Embryonic myoblasts sustain a first wave of myogenesis between embryonic day 10 (E10) and E12 in the mouse and give rise to primary myofibers that establish the primitive shape of muscle and express high levels of the slow MyHC isoform (MyHC-I, encoded by the *Myh7* gene) and of the embryonic MyHC isoform (MyHC-emb, encoded by *Myh3*) (Schiaffino et al., 1986; Stockdale, 1992). A second wave of muscle differentiation takes place between E15 and E18, driven by fetal myoblasts that form secondary fibers, characterized by low levels of MyHC-I and high levels of neonatal MyHC (MyHC-neo, encoded by *Myh8*) (Eusebi et al., 1986; Lyons et al., 1990; Daou et al., 2013). Eventually, primary fibers conserve the slow-twitch phenotype typical of embryonic muscle, while secondary fibers lose the expression of several embryonic-specific markers such as MyHC-I and acquire expression of fast-twitch markers (Ferrari et al., 1997; Biressi et al., 2007). Embryonic and fetal myoblasts, once isolated from the embryo, are committed to a specific fiber type, suggesting the involvement of intrinsic factors rather than nerve activity in the establishment of fiber phenotype (Page et al., 1992). These observations suggest that the proper transition of skeletal muscle from the embryonic to the fetal/post-natal phenotype requires a switch in the transcriptional status of differentiating myoblasts.

In our previous study, we have shown that the transcription factor Nfix, a member of the nuclear factor I (Nfi) family, has a key role in the establishment of fetal muscle phenotype and in the downregulation of slow MyHC both in fetal and adult muscles (Messina et al., 2010; Rossi et al., 2016). We have shown that Nfix is strongly expressed in fetal myoblasts and indirectly represses MyHC-I expression via the transcription factor Nfatc4, a positive regulator of *MyHC-I* in skeletal muscle (Calabria et al., 2009; Messina et al., 2010). We have also reported that the zebrafish (*Danio rerio*) Nfix ortholog Nfixa has an evolutionarily conserved role in the transition from slow-twitch to fast-twitch myogenesis (Pistocchi et al., 2013). In the past few years, it has been shown that Sox6, a member of the Sry-related HMG box (Sox) factor family, which is highly conserved in vertebrates, plays a critical role in fetal fiber specification through direct repression of *MyHC-I* by binding to the 5′-upstream region in two different binding sites. The first is located −200 bp from the transcription start site (TSS) in the proximal promoter and is sufficient for Sox6-dependent *MyHC-I* repression in fetal myotubes (Hagiwara et al., 2007; An et al., 2011), and the second

is located −2,900 bp from the TSS in a distal muscle enhancer that is required for full promoter activity (Giger et al., 2000; Blow et al., 2010). As a consequence, *Sox6*-null mouse muscle displays increased levels of MyHC-I and a general switch toward a slower phenotype (Hagiwara et al., 2007; An et al., 2011; Quiat et al., 2011). Of note, studies in zebrafish have shown that Sox6 is restricted to fast-twitch fibers during embryonic muscle development and that ectopic Sox6 expression in adaxial cells (the slow muscle progenitors in zebrafish) leads to the silencing of slow-twitch genes (such as the slow MyHC isoform *smyhc1* and the transcription factor *prox1a*) (von Hofsten et al., 2008; Wang et al., 2011; Jackson et al., 2015).

Here, we observed that Sox6, at variance with Nfix, is expressed at comparable levels in embryonic and fetal myoblasts, despite its role of inhibitor of slow twitching program. Intriguingly, we demonstrated that Sox6 has opposite roles in regulating MyHC-I expression between embryonic and fetal myogenesis in mouse. Specifically, during embryonic myogenesis, Sox6 indirectly promotes *MyHC-I* expression via transcriptional activation of Mef2C. As a consequence, Sox6 deficiency in embryonic muscle leads to a strong downregulation of *MyHC-I*. On the contrary, during fetal myogenesis, Sox6 cooperates with Nfix to repress *MyHC-I* in a complex in which Nfix is necessary for the proper binding of Sox6 to the *MyHC-I* promoter in fetal myotubes. Finally, we show that Nfixa and Sox6 together regulate sMyHC in zebrafish embryos, revealing an evolutionarily conserved mechanism that is required for the acquisition of normal muscle phenotype.

RESULTS

Sox6 Transcriptionally Promotes MyHC-I Expression during Embryonic Myogenesis

Sox6 has been intensively studied as an inhibitor of slow muscle phenotype during the fetal period. However, we observed that Sox6 is also expressed during embryonic myogenesis, which is mainly characterized by the expression of typical slow genes such as the slow MyHC isoform MyHC-I. *Myf5*$^{GFP\text{-}P/+}$ embryos and fetuses were collected at E12.5 or E16.5, and GFP-positive myoblasts were isolated via fluorescence-activated cell sorting (FACS) as previously described (Messina et al., 2010). Using quantitative real-time PCR and western blot, we found that *Sox6* levels do not significantly change, whereas *Nfix*, as known, is drastically upregulated in fetal progenitors (Figures 1A–1C). Importantly, these data show that Sox6 and Nfix proteins are co-expressed only during fetal myogenesis. We also performed extensive immunofluorescence analysis on frozen mouse embryo sections from E10.5 to E18.5 in order to follow Sox6 protein expression throughout development. Notably, Sox6 is first expressed between E11.5 and E12.5 in primary fibers that express high levels of MyHC-I (Figures S1A–S1H), whereas at E17.5, the localization of Sox6 is almost completely associated with secondary fibers that are negative for MyHC-I (Figures S1I–S1L), as previously described (An et al., 2011; Richard et al., 2011). We therefore decided to investigate the possible function of Sox6 during embryonic myogenesis, a function not apparently linked to repression of MyHC-I. To this aim, we performed immunofluorescence analysis on E12.5 muscle sections from homozygous mice carrying the *Sox6*^{lacZ} allele (hence referred to as *Sox6*-null mice) (Smits et al., 2001). Surprisingly, the staining for MyHC-I is strongly decreased in *Sox6* null in comparison to wild-type (WT) muscle (Figures 1D and 1E). Importantly, no differences in total MyHC content were assessed in embryonic muscle groups of *Sox6*-null embryos (Figures 1G and 1H), implying that the decrease in MyHC-I expression is not due to delayed or aberrant muscle differentiation. We also performed western blot and quantitative real-time PCR on embryonic muscle lysates to confirm the immunofluorescence data, and our results showed a decrease of MyHC-I protein in *Sox6*-null samples, without major changes in total MyHC content (Figures 1J and 1K). Interestingly, the phenotype of *Sox6*-null embryonic muscle is reminiscent of the Tg:Mlc1f-*Nfix2* gain-of-function embryo (Figures 1F and 1I), in which the Nfix2 splice variant is ectopically expressed in muscle cells from E11.5, leading to a fetal-like muscle phenotype (Kelly et al., 1997; Messina et al., 2010). Importantly, Nfix expression in embryonic skeletal muscle is not altered in the absence of Sox6 at both the protein and mRNA levels (Figures S2A–S2D). Taken together, these results show that Sox6 is expressed at equal levels in skeletal muscle during embryonic and fetal myogenesis and that deletion of *Sox6* during the embryonic period unexpectedly leads to downregulation of *MyHC-I*.

Sox6 Positively Regulates the Slow-Twitch Phenotype of Embryonic Myoblasts by Binding to *Mef2C* Promoter

In order to define a possible mechanism by which Sox6 regulates the transcription of MyHC-I during embryonic myogenesis, we performed chromatin immunoprecipitation (ChIP) for Sox6 in differentiated embryonic myoblasts. We found that Sox6 does not significantly bind either to the proximal or to the distal regulative regions upstream of *MyHC-I* (Figure 2A), thus suggesting that Sox6 is not able to directly regulate *MyHC-I* transcription in embryonic muscle. To confirm this hypothesis, we performed luciferase assays on WT and *Sox6*-null embryonic differentiated myoblasts with vectors containing the 3,500-bp *MyHC-I* full 5′-upstream region (MyHC-I 3500), the 408-bp *MyHC-I* proximal promoter sequence (MyHC-I 408), or the mutated forms of the distal and proximal canonical Sox6 binding sites, MyHC-I 3500 m and MyHC-I 408 m (Figure S3A) (Hagiwara et al., 2007; An et al., 2011). As expected, in the absence of Sox6, we found a significant reduction of firefly luciferase activity in all the conditions with the only notable exception of the 408 WT construct (Figure 2B), suggesting that Sox6 is promoting MyHC-I expression in embryonic myocytes without direct binding to its canonical binding sites. In order to identify a possible indirect mechanism by which Sox6 enhances MyHC-I expression in embryonic muscle, we focused on the transcription factor Mef2C, a known positive regulator of the slow phenotype (Wu et al., 2000; Potthoff et al., 2007; Anderson et al., 2015). To verify the interaction between Mef2C and the *MyHC-I* promoter in embryonic myoblasts, we performed a ChIP assay, which showed direct binding of Mef2C on the proximal *MyHC-I* promoter (Figure S3B). Interestingly, *Mef2C* mRNA is downregulated in E12.5 *Sox6*-null muscle, at variance with the closely related *Mef2A* (Figure 2C). By ChIP on embryonic differentiated myoblasts, we found that Sox6 directly binds to a region located in

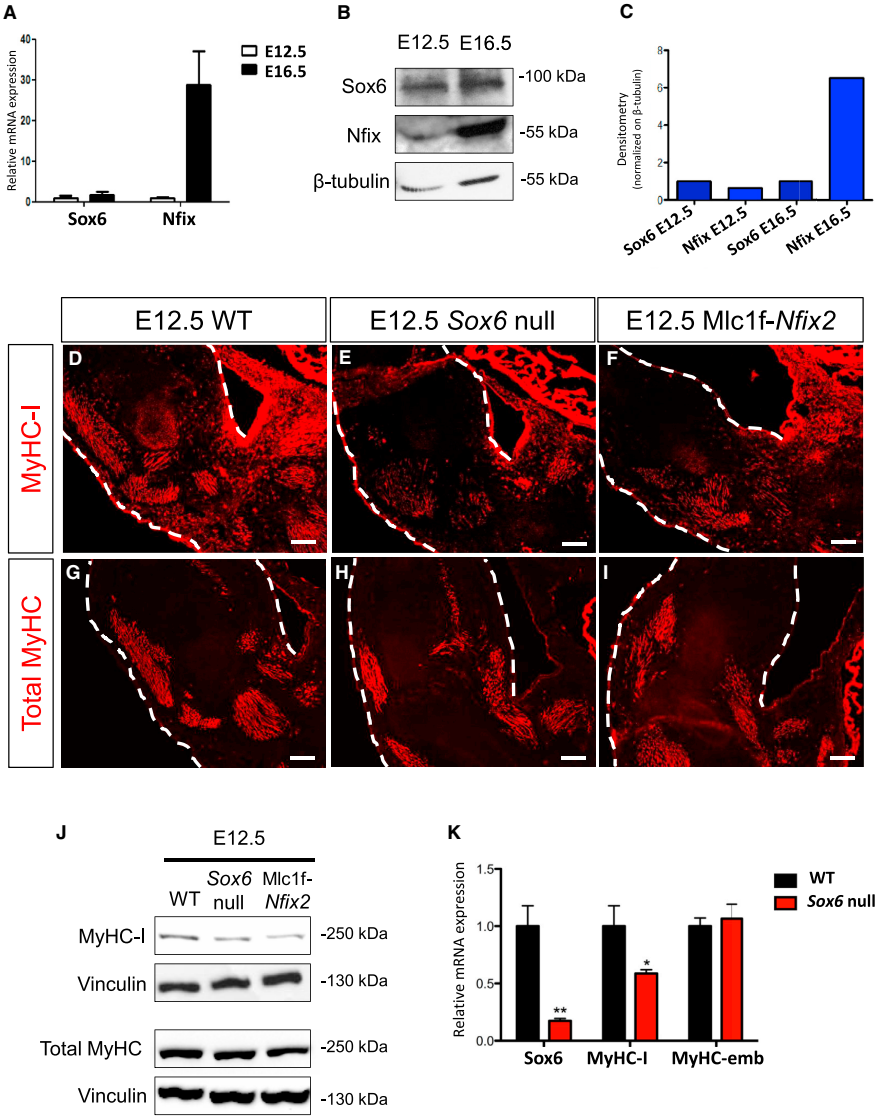

Figure 1. Sox6 Acts a Positive Regulator of the Slow-Twitch Phenotype during Embryonic Myogenesis

(A) Quantitative real-time PCR analysis on freshly isolated *Myf5*$^{GFP-P/+}$ embryonic (E12.5) and fetal (E16.5) myoblasts showing relative expression of *Sox6* and *Nfix* transcripts in the two populations.

(B) Western blot on lysates from freshly isolated *Myf5*$^{GFP-P/+}$ embryonic and fetal myoblasts. β-Tubulin was used to normalize the amount of protein loaded.

(C) Quantitative densitometry of the protein expression levels of Sox6 and Nfix at E12.5 and E16.5.

(D–I) Immunofluorescence on E12.5 muscle sections from WT (D and G), *Sox6* null (E and H), and Tg:Mlc1f-*Nfix2* (F and I) mice stained with anti-MyHC-I (D–F) or anti-MyHCs (MF20) antibodies (G-I). Dashed lines highlight the forelimb anlagen contour. Scale bars, 100 μm.

(J) Western blot on E12.5 muscle samples from WT, *Sox6* null, and Tg:Mlc1f-*Nfix2* mice. Vinculin was used to normalize the amount of loaded proteins.

(K) Quantitative real-time PCR on E12.5 muscle tissue from WT and *Sox6*-null mice (*$p < 0.05$; **$p < 0.01$; $n = 3$).

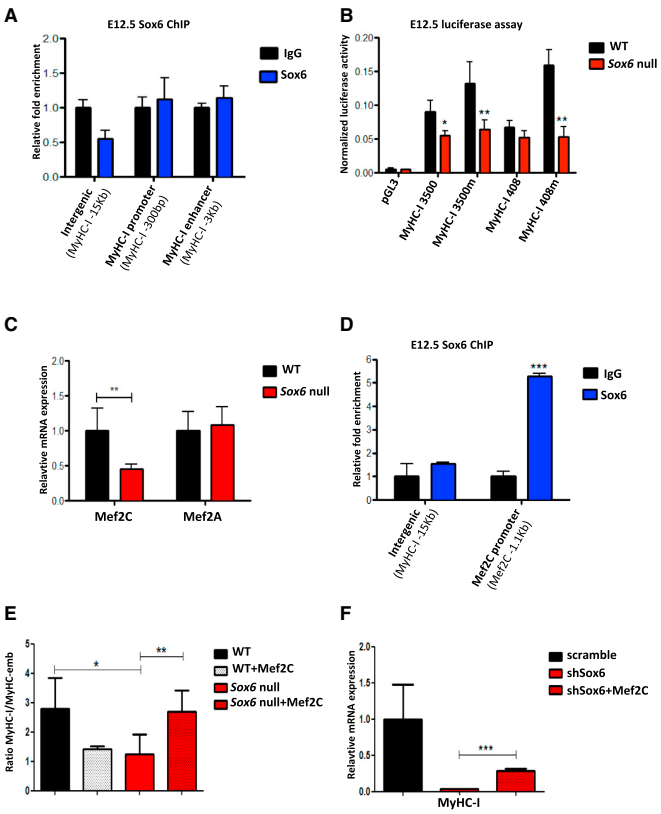

Figure 2. Sox6 Indirectly Activates MyHC-I Expression in Embryonic Myoblasts via a Mef2C-Dependent Mechanism

(A) ChIP assay with anti-Sox6 antibody on E12.5 differentiated myoblasts. Three different chromatin regions were tested: a negative control region (intergenic) located 15 kb upstream of *MyHC-I* TSS, the *MyHC-I* proximal promoter (−375 bp; MyHC-I promoter), and the *MyHC-I* distal enhancer (−2,900 bp; MyHC-I enhancer).

(B) Luciferase report assay on WT and *Sox6*-null E12.5 differentiated myoblasts transfected with control pGL3-basic, MyHC-I 408, MyHC-I 408 m, MyHC-I 3,500, and MyHC-I 3,500 m constructs (*$p < 0.05$; **$p < 0.01$; $n = 2$).

(C) Quantitative real-time PCR for *Mef2C* and *Mef2A* on E12.5 muscle tissue from WT and *Sox6*-null mice (**$p < 0.01$; $n = 2$).

(D) ChIP assay with anti-Sox6 antibody on E12.5 differentiated primary myoblasts showing binding of Sox6 to a region located −1.1 kb upstream of *Mef2C* TSS (***$p < 0.001$; $n = 3$). As negative control, we used the *MyHC-I* −15 kb intergenic region.

(E) Quantitative real-time PCR for *MyHC-I* and MyHC-emb (MyHC-I/MyHC-emb ratio) on WT and *Sox6*-null differentiated embryonic myoblasts transfected with a Mef2C-overexpressing vector (*$p < 0.05$; **$p < 0.01$; $n = 2$).

(F) Quantitative real-time PCR for MyHC-I on embryonic myoblasts purified from *Myf5*$^{GFP-P/+}$ mice, infected with scramble or shSox6 virus. Myf5$^{GFP-P/+}$ purified embryonic myoblasts infected by lentiviruses expressing the shRNA for Sox6 were transfected with Mef2C-overespressing vector (***$p < 0.001$; $n = 2$).

the *Mef2C* promoter (Figure 2D), indicating that Sox6 is a direct activator of Mef2C during embryonic myogenesis. In order to validate a possible Mef2C-mediated mechanism of MyHC-I regulation, we transfected WT and *Sox6*-null unpurified embryonic myoblasts with a vector overexpressing Mef2C. Mef2C overexpression in Sox6-deficient cells (Figure S3C) leads to an increase of *MyHC-I* expression when normalized on the levels of *MyHC-emb* to account for the total number of myogenic cells (Figure 2E). To validate these data, we also transduced purified embryonic myoblasts with a lentiviral vector expressing small hairpin RNA (shRNA) against Sox6 to achieve in vitro Sox6 knockdown (Figure S3D). We then transfected cells with Mef2C-overexpression vector (Figure S3D) and assessed the levels of *MyHC-I* mRNA by quantitative real-time PCR (Figure 2F). Strikingly, overexpression of Mef2C was able to partially rescue MyHC-I expression in shSox6 cells (up to 30% of the levels of scramble-transduced cells). These results show that during the embryonic period, Sox6 acts indirectly as a positive regulator of MyHC-I via a Mef2C-dependent mechanism, in sharp contrast with its well-known and characterized function during fetal myogenesis (An et al., 2011; Quiat et al., 2011; see below).

Sox6 Is Necessary for Nfix Binding to the *MyHC-I* Promoter during the Fetal Period

Since Sox6 has opposite roles in MyHC-I regulation in embryonic and fetal muscle, and since Nfix is only expressed during fetal myogenesis (Messina et al., 2010), we decided to investigate the possible cooperation between Nfix and Sox6 during fetal myogenesis. We first investigated Nfix expression and function in *Sox6*-null fetuses (E16.5). As previously described in other *Sox6*-null mouse models (Hagiwara et al., 2007; An et al., 2011; Quiat et al., 2011), fetal fiber specification is completely disrupted in the absence of Sox6. Indeed, in contrast to WT, *Sox6*-null fetal muscle displays very high levels of MyHC-I by immunofluorescence, quantitative real-time PCR, and western blot (Figures S4A–S4D). We also looked at Nfix on sections from the *Sox6*-null mice and found that despite the dramatic increase in MyHC-I expression, Nfix is correctly expressed in the nuclei of muscle fibers in the absence of Sox6 (Figures 3A–3D), and western blot analysis did not reveal differences in Nfix protein content (Figure S4C). These data suggest that Nfix is normally expressed in fetal muscles lacking Sox6 but unable to properly repress *MyHC-I*. To verify whether Sox6 is required for Nfix function, we performed ChIP for Nfix in WT fetal myotubes, which revealed binding of Nfix to *MyHC-I* promoter in the same region of the proximal Sox6 binding site (−200 bp) (Figure 3E). Interestingly, we

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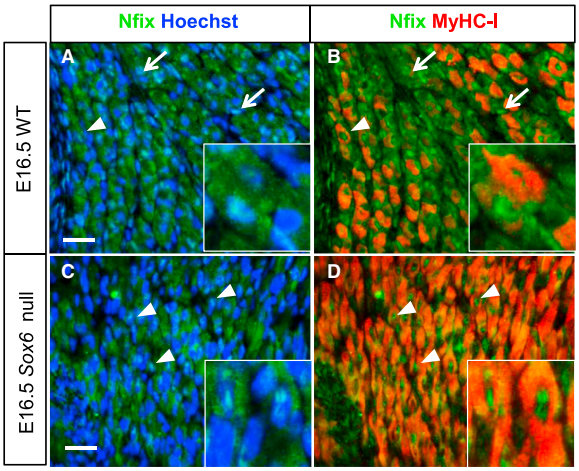

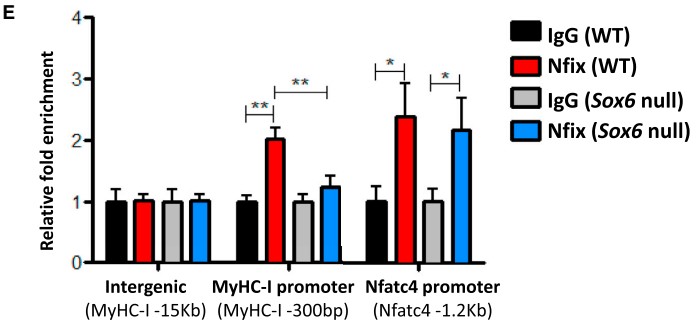

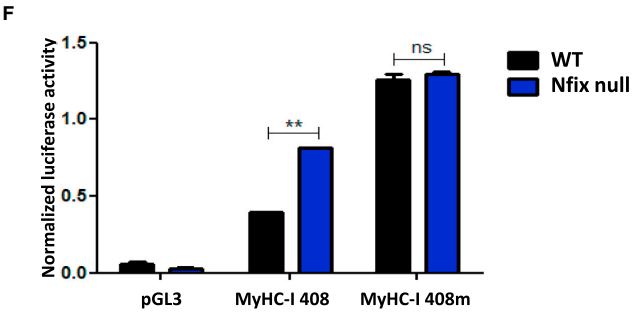

Figure 3. Sox6 Is Required for Normal Nfix Function in Fetal Muscle

(A–D) Immunofluorescence with anti-Nfix (green) and anti-MyHC-I (red) antibodies on E16.5 muscle sections from WT (A and B) or *Sox6*-null (C and D) mice. Arrows indicate Nfix-positive nuclei in secondary (MyHC-I negative) fibers. Arrowheads indicate Nfix-positive nuclei in MyHC-I-positive fibers. Nuclei are counterstained with Hoechst. Scale bars, 25 μm. Higher magnifications of Nfix-positive fibers are shown in the insets.

(E) ChIP assay with anti-Nfix antibody on WT and *Sox6*-null E16.5 myotubes on negative control region (intergenic) located 15 kb upstream of *MyHC-I* TSS, the *MyHC-I* proximal promoter (−375 bp; MyHC-I promoter), and the *Nfatc4* promoter region (−1.2 kb upstream of the Nfatc4 TSS) (*$p < 0.05$; **$p < 0.01$; $n = 2$).

(F) Luciferase report assay on WT and *Nfix*-null differentiated fetal myoblasts (E16.5) transfected with control pGL3-basic, MyHC-I 408, and MyHC-I 408 m vectors (**$p < 0.01$; $n = 2$).

observed that in *Sox6*-null fetal myotubes, the binding of Nfix to the *MyHC-I* promoter is significantly reduced. Importantly, Nfix binding to *Nfatc4* promoter is not impaired in the absence of Sox6 (Figure 3E), suggesting that Nfix is still able to bind to other transcriptional targets in the absence of Sox6. We further validated the repressive role of Nfix on the *MyHC-I* proximal promoter by performing a luciferase assay with MyHC-I 408 and MyHC-I 408 m vectors in WT and *Nfix*-null fetal myotubes (Figure 3F). Our results showed increased luciferase expression in the absence of Nfix with the vector carrying the WT sequence, suggesting that indeed Nfix represses MyHC-I by acting on the proximal promoter regulative region, in spite of the absence of any Nfix consensus sequences (data not shown). Importantly, Nfix-dependent negative regulation of MyHC-I is lost when the proximal Sox6 binding sequence is mutated, demonstrating that Sox6 is required for Nfix binding to the proximal MyHC-I promoter (Figure 3F). Overall, these results suggest a possible crosstalk between Nfix and Sox6 in regulating MyHC-I expression at the proximal promoter region.

Nfix Is Necessary and Sufficient for Sox6 Regulation of MyHC-I Expression

To better investigate a possible reciprocal interplay between Nfix and Sox6, we performed immunofluorescence for Sox6 and total MyHC on frozen muscle sections from E16.5 WT and *Nfix*-null mice (Campbell et al., 2008). We found that Sox6 is expressed in both WT and *Nfix*-null muscles (Figures S4E and S4F), implying that Nfix is dispensable for normal Sox6 expression in fetal muscle. Interestingly, immunofluorescence for Sox6 and MyHC-I on sections from the same mice revealed that in the absence of Nfix, there is a marked increase in the number of Sox6-positive fibers that co-express MyHC-I, in contrast to WT muscles (Figures 4A–4D). We further

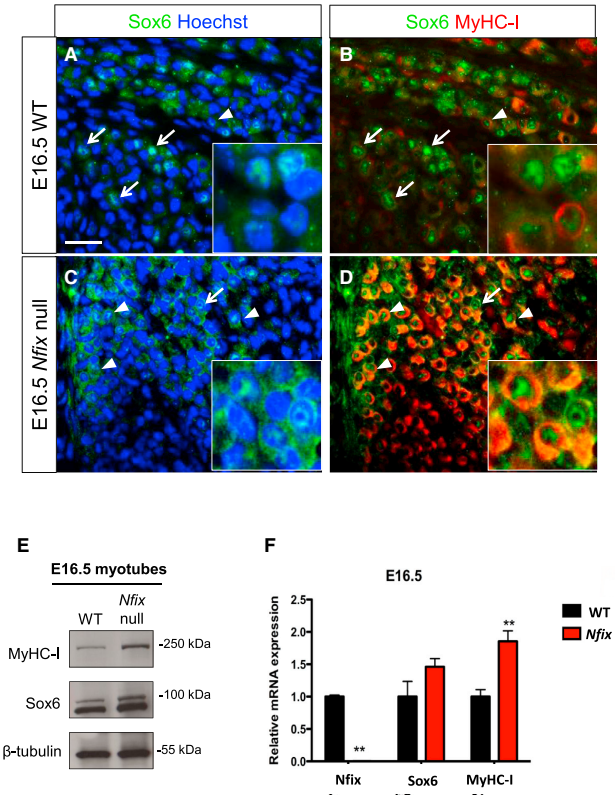

Figure 4. Nfix Is Necessary for the Correct Function of Sox6 in Fetal Muscle

(A–D) Immunofluorescence with anti-Sox6 (green) and anti-MyHC-I (red) antibodies on fetal (E16.5) muscle sections from WT (A and B) and *Nfix*-null (C and D) mice. Arrows indicate secondary fibers, which present nuclear Sox6 expression and low or absent staining for MyHC-I. Arrowheads indicate fibers co-expressing nuclear Sox6 and MyHC-I. Nuclei are counterstained with Hoechst. Scale bars, 25 μm. Higher magnifications are shown in the insets.

(E) Western blot on lysates from WT and *Nfix*-null E16.5 myotubes. β-Tubulin was used to normalize the amount of proteins loaded.

(F) Quantitative real-time PCR on WT and *Nfix*-null E16.5 myotubes (**$p < 0.01$; $n = 3$).

validated these data by western blot and quantitative real-time PCR on differentiated fetal myotubes and observed that *Nfix*-null myotubes express higher levels of MyHC-I than WT cells (Figures 4E and 4F). These data show that in the absence of Nfix, the repressive activity of Sox6 on MyHC-I is partially impaired, even if the normal expression pattern of Sox6 is maintained. The results obtained in both *Sox6* and *Nfix*-null fetuses led us to hypothesize a functional cooperation between Nfix and Sox6 in MyHC-I regulation. Therefore, we tested the ability of Nfix and Sox6 to bind to each other in a multi-protein complex. To this aim, we used fetal myotubes transiently transfected with an Nfix2 hemagglutinin (HA) vector (see Experimental Procedures) and performed a co-immunoprecipitation (coIP) assay from nuclear extracts for HA and Sox6. CoIP revealed the presence of a complex containing both Sox6 and Nfix2-HA, as shown in Figure 5A. Therefore, we wondered whether, as observed for Nfix fetuses in absence of Sox6, Nfix might be required for the binding of Sox6 to the *MyHC-I* promoter as well. Hence, we performed ChIP for Sox6 in WT and *Nfix*-null fetal myotubes. Our results showed a decrease in Sox6 binding to the two different sites in the *MyHC-I* 5′-upstream region (Figure 5B). Notably, the binding with the proximal promoter was completely lost in the absence of Nfix, whereas the binding with the distal enhancer was reduced by 50%. Interestingly, ChIP for Sox6 on the *Mef2C* promoter reveals that in WT fetal myotubes, Sox6 is not able to bind to the *Mef2C* promoter, at variance with what happens during the embryonic stage. On the contrary, Sox6 binding on the *Mef2C* promoter still occurs in *Nfix*-null fetal myotubes. These data indicate that Nfix is required for the proper binding of Sox6 to the *MyHC-I* promoter during fetal myogenesis. Finally, we performed ChIP for Sox6 on WT and Tg:Mlc1f-*Nfix2* embryonic myoblasts (Figure 5C). Strikingly, Nfix overexpression leads to a switch in the binding properties of Sox6; indeed, we found that Sox6 is bound to the *MyHC-I* promoter, but not to the *Mef2C* promoter, in Tg:Mlc1f-*Nfix2* embryonic myoblasts. These data demonstrate that Nfix is necessary and sufficient for the binding of Sox6 to the MyHC-I proximal promoter and therefore for Sox6 repressive activity on MyHC-I.

Functional Cooperation of Sox6 and Nfixa Is Evolutionarily Conserved in Zebrafish

It was previously demonstrated that both Sox6 and Nfixa have an evolutionarily conserved role in the repression of slow-twitch genes in zebrafish (von Hofsten et al., 2008; Pistocchi et al., 2013; Jackson et al., 2015). We thus wondered whether a functional interplay between Nfixa and Sox6 is conserved in zebrafish myogenesis. As a preliminary analysis, we performed quantitative real-time PCR on trunk and tail regions isolated from zebrafish embryos at 1, 2, and 3 days post-fertilization (dpf) and found that the *sox6* transcript is expressed at high levels at 1 dpf and is steadily downregulated up to 3 dpf (Figure S5A), whereas the *nfixa* transcript peaks at 2 dpf (Figure S5B), as shown previously (Pistocchi et al., 2013). Additionally, we performed immunofluorescence for Sox6 and total MyHC (MF20 antibody) or the slow MyHC isoform sMyHC (F59

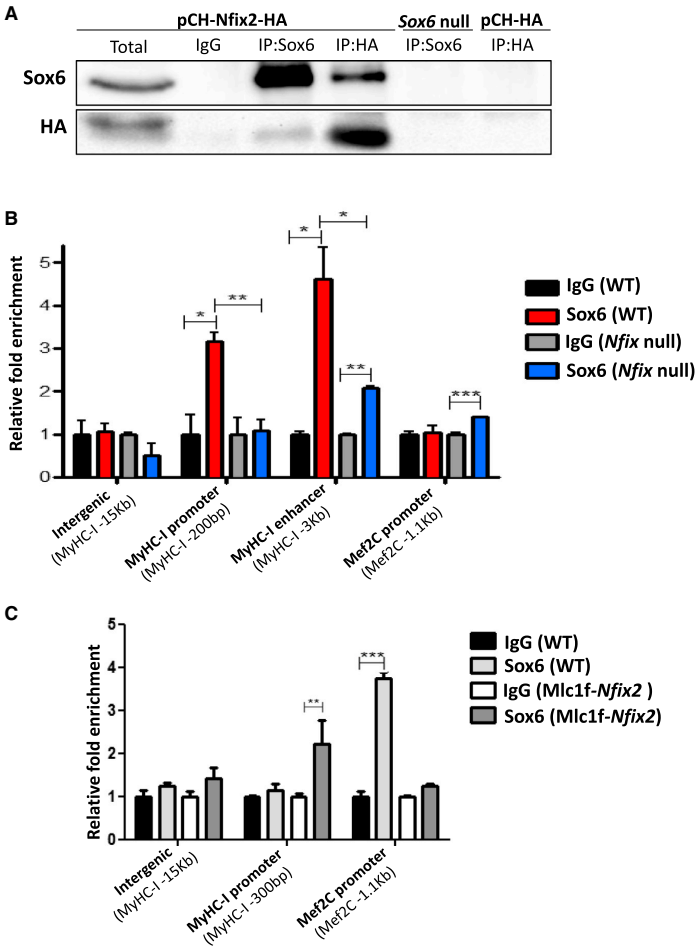

Figure 5. Nfix Is Required for Fetal-Specific Binding of Sox6 to *MyHC-I* Regulative Regions

(A) Immunoprecipitation assay, from nuclear extracts, on fetal myoblasts transfected with Nfix2-HA vector, showing the immunoprecipitation of Sox6 (IP Sox6) and HA (IP HA). T, total lysate; IgG, negative control; IP, immunoprecipitated. The coIPs for Sox6 on *Sox6*-null myotubes and for HA on fetal myoblasts transfected with the HA-only expressing vector (pCH-HA) were used as negative controls.

(B) ChIP assay with anti-Sox6 antibody on WT and *Nfix*-null E16.5 myotubes on the same chromatin regions described in Figures 2A and 2D (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 2$). As a negative control, we used only the *MyHC-I* −15 kb region.

(C) ChIP assay on unpurified embryonic myoblasts from WT or Tg:Mlc1f-*Nfix2* to test Sox6 binding on MyHC-I and the Mef2C promoter. The intergenic region was used as a negative control and IgG as an unrelated antibody (**$p < 0.01$; ***$p < 0.001$; $n = 2$).

antibody) on 2 dpf embryos and found that Sox6 protein is specifically expressed in the nuclei of fast muscle fibers that are negative for sMyHC (Figures 6A and 6B), whereas the outer superficial fibers positive for sMyHC are mostly negative for Sox6 (data not shown). Interestingly, we observed that a minority of slow superficial fibers show cytoplasmic staining for Sox6 (Figures 6C–6J), suggesting that Sox6 subcellular localization might be spatially regulated in the different muscle domains. In order to elucidate the role of Sox6 in slow-twitch genes regulation, we performed morpholino (MO)-mediated knockdown of *sox6* (von Hofsten et al., 2008). By quantitative real-time PCR analysis (Figures S5C and S5D), we found that the expression of *smyhc1* is markedly increased in *sox6* morphants at 2 dpf, whereas the fast-twitch gene *mylpfa* (fast myosin light chain isoform) is expressed at equal levels. Moreover, the *nfixa* transcript is drastically upregulated in *sox6* morphants, suggesting that Sox6 might negatively regulate *nfixa* (Figure S5E). We conclude that Sox6 is a critical repressor of the slow-twitch phenotype in zebrafish and that Nfixa is not able to compensate for *sox6* knockdown. To verify a possible cooperation between Nfixa and Sox6, we performed co-injections of morpholinos against *sox6* and *nfixa* at lower doses with respect to those previously described (von Hofsten et al., 2008; Pistocchi et al., 2013) in order to minimize their effect when injected separately (see Experimental Procedures). Co-injection of these doses of MOs resulted in synergistic defects in motility in touch-response assays (Figure S6). Control embryos and the vast majority of *sox6* or *nfixa* morphants readily swam away when touch-stimulated. On the contrary, 66% of double partial morphants were either shivering or bending their tails before eventually moving away but within a shorter distance (Figures S6A and S6C; Movie S1). The synergistic effect is more evident when lowering the doses (see Figures S6B and S6D). Strikingly, quantitative real-time PCR results show that *smyhc1* is significantly upregulated only in the double morphants at 48 hpf, whereas the level of *myl*1, an early marker for differentiating fast muscle cells (Burguière et al., 2011), does not change (Figure 6K). Moreover, we validated the increased expression of sMyHC (F59 antibody) in *sox6*/*nfixa* double morphants by western blot (Figure 6L). We thus conclude that functional cooperation of Sox6 and Nfix is required for proper skeletal muscle development and that this cooperation is evolutionarily conserved in mouse and zebrafish.

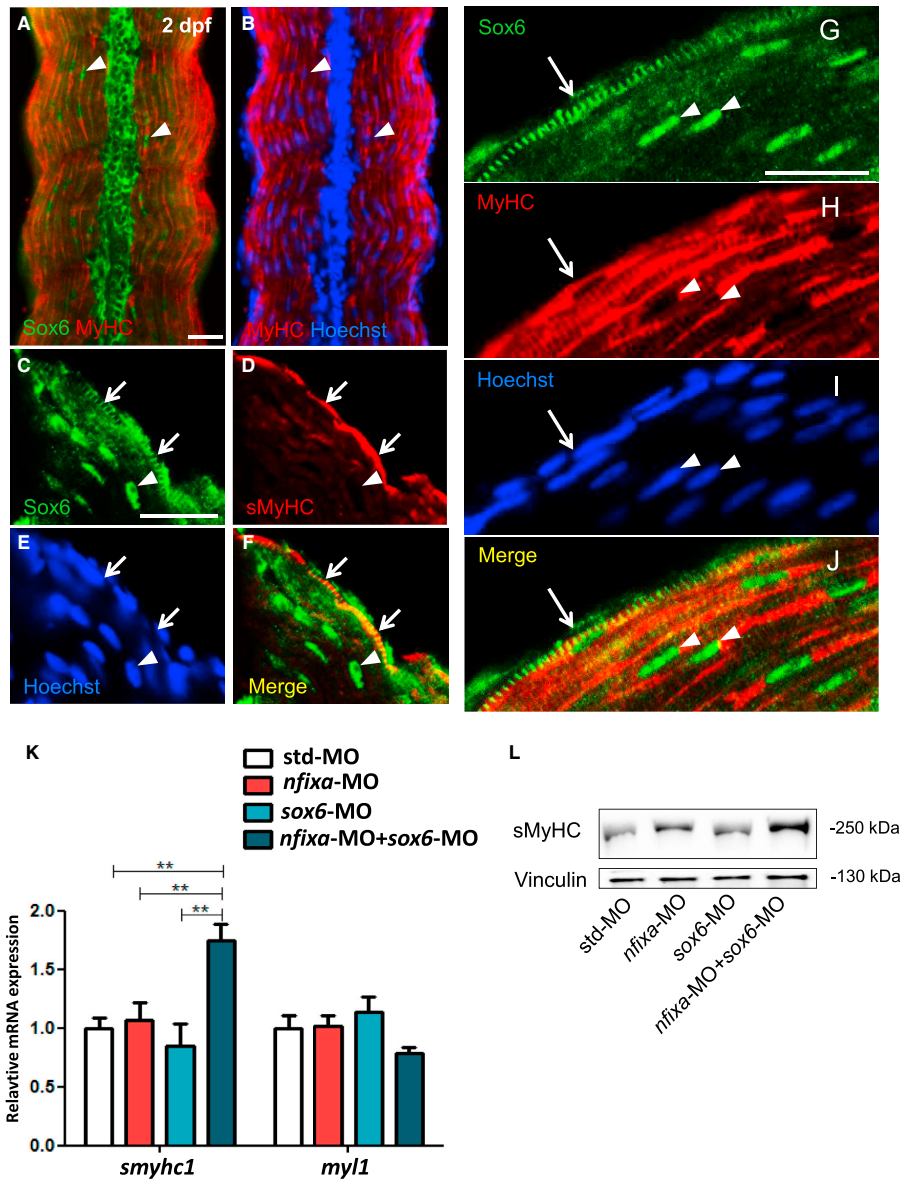

Figure 6. Functional Cooperation of Sox6 and Nfixa Is Conserved in Zebrafish

(A–J) Immunofluorescence with anti-Sox6 antibody (green) and anti-MyHC antibody (red) (A, B, and G–J) or with anti-Sox6 (green) and anti-sMyHC (F59, red) (C–F) on 2 dpf zebrafish muscle longitudinal sections. Arrowheads indicate Sox-positive nuclei in fast-twitch muscle fibers. Arrows indicate Sox6 staining in the cytoplasm of superficial slow fibers. Approximately one-fifth of the sMyHC-positive superficial cells displayed cytoplasmic Sox6 staining, whereas fast fibers negative for sMyHC only displayed nuclear Sox6 staining. Nuclei are counterstained with Hoechst. Scale bars, 25 μm.

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DISCUSSION

Adult skeletal muscle is composed of two major fiber types presenting a wide range of physiological and biochemical differences. Slow-twitch type I fibers use oxidative metabolism and express the slow MyHC isoform MyHC-I; in contrast, fast-twitch type II fibers present glycolytic or mixed metabolism and express three fast MyHC isoforms (MyHC-IIa, IIx/d, and IIb) (Peter et al., 1972; Schiaffino et al., 1988; Chakkalakal et al., 2012). The phenotype of post-natal muscle fibers is strictly regulated by extrinsic signals such as muscle activity and hormones (Butler-Browne et al., 1982; Gambke et al., 1983; Russell et al., 1988). Additionally, different factors controlling adult muscle plasticity have been identified, including the Nfatc (Calabria et al., 2009) and Mef2 (Wu et al., 2000; Potthoff et al., 2007; Anderson et al., 2015) transcription factor families and PGC-1α (Li et al., 2002). On the contrary, the molecular mechanisms by which muscle fiber diversity is achieved during pre-natal development are still poorly understood. It was shown that the intrinsic transcriptional properties of embryonic and fetal myogenic progenitors are important to set the fiber type in the absence of nerve activity (Cho et al., 1993; Cusella-De Angelis et al., 1994). Moreover, in recent years, several transcription factors contributing to developmental muscle fiber specification have been identified, including Sox6 (Hagiwara et al., 2007; An et al., 2011), Nfix (Messina et al., 2010), Six1/Six4 (Richard et al., 2011), and Nfatc2 (Daou et al., 2013). However, the network of transcription factors controlling fiber specification during embryogenesis is still far from being fully characterized, and until now, functional interactions among the different regulators were completely unknown.

In this work, we provided evidence for functional interplay between Nfix and Sox6 in controlling expression of the slow MyHC isoform during mouse pre-natal muscle development.

In contrast to Nfix, which is a specific marker of fetal myogenesis (Messina et al., 2010; Mourikis et al., 2012), Sox6 is expressed in both embryonic and fetal purified myoblasts at the mRNA and protein levels. Consistently, we found that Sox6 protein is expressed in skeletal muscle in vivo starting between E11.5 and E12.5. This was unexpected, since Sox6 is known to be a repressor of MyHC-I, which along with MyHC-emb is expressed in all embryonic fibers (Hagiwara et al., 2007; Hutcheson et al., 2009). It is known that *Sox6* transcript is absent in mouse primary myotome between E9.5 and E10.75 (Vincent et al., 2012), suggesting that Sox6 is quickly activated in embryonic myoblasts at the beginning of primary myogenesis. Unexpectedly, Sox6 deficiency during primary myogenesis leads to a transient faster muscle phenotype with low levels of MyHC-I. This is followed in *Sox6*-null fetuses by dramatic upregulation of MyHC-I at the transcription level, consistently with previous characterizations of the *Sox6*-null phenotype (Hagiwara et al., 2007; An et al., 2011; Quiat et al., 2011). Our results demonstrate that Sox6 plays opposite roles in MyHC-I expression during development. Importantly, in embryonic myogenic cells, Sox6 does not bind to the two canonical *MyHC-I* binding sites, which are both dispensable for Sox6-dependent embryonic regulation of MyHC-I, at least in our culturing conditions. These data strongly suggest an indirect mechanism of regulation. We found that Sox6 directly binds to the promoter of *Mef2C* and activates its expression in primary myofibers. Mef2C is part of a transcription factor family that is constitutively expressed in muscle cells since early embryogenesis (Edmondson et al., 1994) and plays an important role in the specification and maintenance of type I fibers (Chin et al., 1998; Wu et al., 2000; Anderson et al., 2015). Importantly, conditional deletion of *Mef2C* in skeletal muscle leads to a drastic reduction in slow-twitch fibers (Potthoff et al., 2007). Our rescue experiment, although partial, clearly shows that one of the Sox6-dependent effects on MyHC-I transcription in embryonic myofibers is mediated by Mef2C, which is directly targeted by Sox6 in embryonic, but not fetal, muscle. This function led us to hypothesize that the binding ability of Sox6 is differentially regulated in discrete myogenic progenitor populations or at different times during development according to different co-factors, which contribute to the high versatility of Sox6 functions. It is known that SoxD factors, lacking *trans*-acting functional domains, have a critical requirement for co-factors in order to regulate transcription of target genes (reviewed in Hagiwara, 2011). Therefore, it is likely that the reversal in Sox6 function is due to different factors that are progressively recruited and activated during muscle development. Indeed, we found that during fetal myogenesis, Nfix acts as a fundamental co-factor of Sox6 and is able to form a complex with Sox6, which is no longer able to bind to the *Mef2C* promoter but can bind to *MyHC-I* regulative regions, in particular the proximal promoter that was shown to be critical for Sox6-dependent fetal repression of MyHC-I (Hagiwara et al., 2007). In our previous study, we showed that Nfix negatively regulates MyHC-I by repressing Nfatc4, a positive regulator of MyHC-I (Messina et al., 2010). Interestingly, we have now found that during fetal myogenesis, Nfix is also present at the *MyHC-I* proximal promoter along with Sox6, suggesting that a physical association between these two proteins may be required for proper MyHC-I downregulation. Since Nfix presents both a *trans*-repression domain and a *trans*-activation domain, we hypothesized that formation of a complex with Sox6 might provide the basis for the transcriptional repression at the *MyHC-I* locus (Figure 7). Indeed, Nfix and Sox6 were found to be part of the same complex in fetal myotubes and are both present at the *MyHC-I* proximal promoter. Moreover, our study on *Nfix*-null and *Sox6*-null fetuses clearly shows that Sox6 and Nfix are independently expressed during secondary myogenesis and that neither Sox6 nor Nfix is able to correctly downregulate MyHC-I expression when the other one is not present. A scheme of the scenario in which Sox6 and Nfix behave

(K) Quantitative real-time PCR analysis on trunk and tail regions at 48 hpf from embryos injected with std-MO or suboptimal doses of *nfixa*-MO (0.25 pmol), *sox6*-MO (0.1 pmol), or *nfixa*-MO + *sox6*-MO (**$p < 0.01$; N = 2).
(L) Western blot for sMyHC at 52 hpf on trunk and tail regions of embryos injected with std-MO or suboptimal doses of *nfixa*-MO, *sox6*-MO, or *nfixa*-MO + *sox6*-MO. Vinculin was used to normalize the amount of loaded proteins.

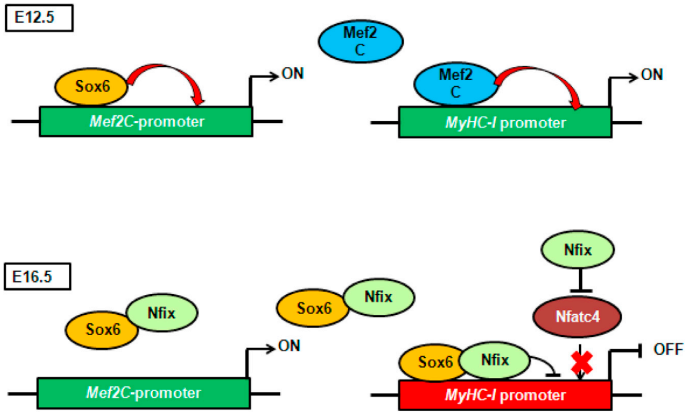

Figure 7. Model of Sox6 and Nfix Interplay during Pre-natal Myogenesis

Scheme illustrating the functions of Sox6 and Nfix during embryonic (E12.5) and fetal (E16.5) myogenesis. Transcription factors are represented by circles, whereas the regulative region of *MyHC-I* is indicated by rectangles that are green when transcription is activated and red when transcription is repressed.

and cooperate during pre-natal muscle development is shown in Figure 7.

In this work, we have also provided evidence for a conserved transcriptional cooperation of Sox6 and Nfixa in zebrafish. We have shown that Sox6 is crucial for the regulation of *smyhc1*, in line with the findings of Jackson et al. (2015). It was shown that Sox6 expression is silenced in zebrafish slow fibers by the combined effects of Prdm1a and miR-499 (von Hofsten et al., 2008; Wang et al., 2011). We found that a minority of sMyHC-positive cells display cytoplasmic staining for Sox6, suggesting that Sox6 might also be regulated through subcellular localization in different fiber types. Notably, our double partial knockdown performed with suboptimal doses of *sox6*-MO (von Hofsten et al., 2008) and *nfixa*-MO (Pistocchi et al., 2013) caused a severe impairment in the touch-evoked escape response, suggesting that Sox6 and Nfixa cooperate in proper muscle function. Molecularly, our data suggest that the two transcription factors can act together to repress *smyhc1* expression in zebrafish embryos, whereas the expression of a typical fast-twitch gene, such as *myl1* (Burguière et al., 2011), was unaffected in double partial morphants.

In conclusion, we have presented a complex model of regulation of embryonic to fetal MyHC-I regulation that involves a functional interplay between Nfix and Sox6 that is conserved in mammals and teleosts.

EXPERIMENTAL PROCEDURES

Mouse Strains and Fish Lines

The following murine lines were used: *Myf5*$^{GFP\text{-}P/+}$ (Kassar-Duchossoy et al., 2004), *Nfix* null (Campbell et al., 2008), *Sox6*$^{lacZ/+}$ (Smits et al., 2001), and Tg:Mlc1f-*Nfix2* (Messina et al., 2010). For each of these lines, the genotyping strategy has been described in the references. CD1 WT mice (Jackson Laboratory) were used as well. Mice were kept in pathogen-free and controlled conditions, and all procedures conformed to Italian law (D. Lgs n° 2014/26, implementation of the 2010/63/UE) and were approved by the Animal Welfare Body of the University of Milan and the Italian Minister of Health (1212/2015PR). Zebrafish were raised and maintained according to established techniques. The following line was used: AB (from Carole Wilson, UCL, London, UK).

Immunofluorescence on Sections

Mouse or zebrafish embryos were fixed in 4% paraformaldehyde, extensively washed in PBS, and incubated overnight in PBS containing 15% sucrose. Samples were then frozen in nitrogen-chilled isopentane and kept at −80°C until use. Cryostat sections (8 μm thick) were permeabilized in 1% BSA, 0.2% Triton X-100 in PBS for 30 min at room temperature and then incubated for 1 hr in blocking solution (10% goat serum in PBS) and overnight with the primary antibody or with mock PBS. After incubation for 45 min with the fluorescent-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), sections were washed in PBS 0.2% Triton X-100 and mounted, and fluorescent immunolabeling was recorded with a DM6000 Leica microscope. The following primary antibodies were used: rabbit anti-Sox6 (Abcam, 1:300), rabbit anti-Nfix (Novus Biological, 1:200), mouse anti-MyHC-I (Sigma, 1:200), BAD5 (monoclonal, 1:2), MF20 (monoclonal, 1:2), and F59 (monoclonal, 1:10). Nuclei were stained with Hoechst (1:1,000).

Cell Sorting and Culturing

Dissected *Myf5*$^{GFP\text{-}P/+}$ embryonic or fetal muscles were digested by 0.15 mg/mL Collagenase (Sigma), 1.5 mg/mL Dispase (GIBCO), and 0.1 mg/ml Dnase I (Sigma) for 30 min at 37°C in agitation as described in Biressi et al. (2007). After centrifugation and filtration, cells were collected in DMEM, 20% fetal bovine serum (FBS), 2 mM EDTA, and 20 mM HEPES. For cell sorting, we used the Sorter BD FACSAria. GFP-positive cells were collected for mRNA and protein extraction. For the preparation of unpurified fetal myoblasts after digestion of tissue, cells were pre-plated for 30 min on a plastic dish to lose fibroblasts, which normally adhere to plastic. Unpurified cells were kept in incubation at 37°C in 20% HS (20% horse serum in DMEM) and 24 hr later allowed to differentiate in DM (2% horse serum in DMEM) for 48 hr.

Lentivirus Production, Transduction, and Transfection

Preparation of viral particles were performed by co-transfecting pLKO.1-shSox6 vectors (Thermo Fisher Scientific) or non-targeting shRNA vectors (30 μg), together with the packaging plasmids pMDLg/p (16.25 μg), pCMV-VSVG (9 μg), and pRSV-REV (6.25 μg), in HEK293T cells. Transfection was performed using the calcium phosphate transfection method. Viral particles were collected 40 hr after transfection and subjected to ultracentrifugation at 20,000 rpm for 2 hr at 20°C (Beckman Coulter, Optima L-100 XP). The concentrated viral particles were re-suspended in PBS and stored in aliquots at −80°C until further use. Embryonic myoblasts were transduced by overnight incubation with viral preparation. These preparations were used to transduce embryonic myoblasts at an MOI of 10. The day after transduction, embryonic myoblasts were transfected with Mef2C (pCDNAI/A-Mef2C) or the empty vector as a negative control using Lipofectamine LTX (Invitrogen).

Protein Extraction and Western Blot

Cultured cells were washed twice in ice-cold PBS and then lysed (30 min in ice) with RIPA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton-X, 0.1% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl in deionized water) plus protease inhibitors (1 mM PMSF). For zebrafish, only trunk and tail regions were used, cutting out the head, and protein was extracted in Laemmli buffer

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(3 μL per embryo). Protein was harvested after centrifugation, quantified by absorbance reading at 750 nm, and stored at −80°C. 30 μg protein was resolved on 8%–12% polyacrylamide gels or on MiniProtean TDX Gels (Bio-Rad) after denaturation at 95°C for 5 min with SDS-PAGE Loading sample buffer 2X (100 mM Tris [pH 6.8], 4% SDS, 0.2% Bromophenol blue, 20% glycerol, and 10 mM dithiothreitol). For western blot analysis, proteins were transferred onto nitrocellulose with the iBlot Dry Blotting System (Invitrogen). Following transfer, membranes were blocked in 5% milk in Tris-buffered saline (TBS)-T (TBS plus 0.02% Tween20) for 1 hr at room temperature. The primary antibodies used were rabbit anti-Sox6 (Abcam; 1:1,000), mouse anti-MyHC-I (Sigma; 1:5,000), mouse anti-β-tubulin (Santa Cruz Biotechnology; 1:5,000), rabbit anti-Nfix (Novus Biologicals; 1:500), mouse anti-HA (Covance; 1:500), mouse anti-vinculin (Sigma; 1:1,000), and mouse anti-sMyHC (F59, Hybridoma Bank) anti-mouse sarcomeric MyHC (MF20, Hybridoma Bank). Horseradish peroxidase (HRP)-conjugated antibodies (Bio-Rad) were used as secondary antibodies, and the signal was revealed with the ChemiDoc MP System (Bio-Rad).

Transfection and Co-immunoprecipitation Assays

For transfection of fetal myoblasts, WT or Sox6-null cells were plated on 90-mm dishes and allowed to reach 80% confluence in proliferating conditions. Cells were transfected with pCH-Nfix2-HA or pCH-HA plasmids with Lipofectamine LTX (Invitrogen) overnight at 37°C. Cells were kept in DM for 36 hr after transfection, and then nuclear extracts were prepared by collecting 5×10^6 cells in 400 μL ice-cold hypotonic buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, protease inhibitors, 1 mM PMSF, and phosphatase inhibitors) for 15 min in ice. Then, NP40 was added to a final concentration of 0.625%, and cells were centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant was discarded as cytoplasmatic extract, while the pelleted nuclei were resuspended in 100 μL immunoprecipitation lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% NP40, 1 mM EDTA, 100 mM NaCl, and protease and phosphatase inhibitors. Immunoprecipitation was performed using the immunoprecipitation kit Dynabeads Protein A (Life Technologies). Dynabeads Protein A (50 μL) was incubated for 2 hr with gentle rotation at room temperature with 5 μg rabbit anti-Sox6 antibody (Abcam), rabbit anti-HA antibody (Santa Cruz), or non-related rabbit immunoglobulin G (IgG) (Santa Cruz). Then, the bead-antibody complex was incubated with gentle rotation for 2 hr at 4°C with 1.5 mg total protein lysates per condition. The eluted proteins were denatured with non-reducing SDS-PAGE loading sample buffer (100 mM Tris [pH 6.8], 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and loaded onto a gel.

Chromatin Immunoprecipitation and Reporter Assay

ChIP for Sox6 and luciferase assays were performed as previously published (An et al., 2011). For the detailed protocol, see Supplemental Experimental Procedures.

RNA Extraction and Analysis

RNA from homogenized mouse or zebrafish embryos and isolated cells was extracted with NucleoSpin RNA kits (Macherey-Nagel) following the manufacturer's instructions. Eluted RNA was checked on agarose gels, quantified with a Nanodrop spectrophotomer, and stored at −80°C. Approximately 0.5 μg RNA was used with the ImProm-II Reverse Transcriptase kit (Promega). Real-time PCR was performed on cDNA using SYBR Green Supermix (Bio-Rad) and the CFX Connect Real Time System (Bio-Rad). After amplification, relative mRNA expression levels were calculated using standard curves from cDNA dilutions and normalized on the *Gapdh* expression levels. For quantitative real-time PCR in zebrafish, we used *rpl8* to normalize the mRNA levels. The primers used are listed in Tables S1 and S2.

MO Microinjections

The antisense MOs (Gene Tools) used in this study, *sox6*-MO1 (von Hofsten et al., 2008) and *nfixa*-MO (Pistocchi et al., 2013), were previously described. MOs, diluted in Danieau buffer (Nasevicius and Ekker, 2000), were injected at the one- to two-cell stage. Escalating doses of each MO were tested for phenotypic effects; as control for unspecific effects, each experiment was performed in parallel with an std-MO (standard control oligo) with no target in zebrafish embryos. We usually injected 0.25 pmol *sox6*-MO per embryo. For combined knockdown experiments, we injected *sox6*-MO and *nfixa*-MO at 0.1 and 0.25 or 0.08 and 0.125 pmol per embryo, respectively.

Statistical Analysis

Values were expressed as means ± SD. Statistical significance was assessed by unpaired Student's t test with Prism 5 software. Statistical significance with a probability of less than 5%, 1%, or 0.1% is indicated in each graph with a single, double, or triple asterisk, respectively, followed by the number of independent experiments (n).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.082.

AUTHOR CONTRIBUTIONS

Conceptualization, G. Maroli, V.T., and G. Messina; Methodology, S.C., A.F., and S.M.; Investigation, V.T., G. Maroli, S.M., S.C., A.F., and G.R.; Writing – Original Draft, G. Maroli and V.T.; Writing – Review & Editing, V.T., S.C., G.R., G.C., M.B., and G. Messina; Supervision, M.B. and G. Messina; Funding Acquisition, G. Messina.

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Supplemental Information

Nfix Induces a Switch in Sox6 Transcriptional Activity to Regulate MyHC-I Expression in Fetal Muscle

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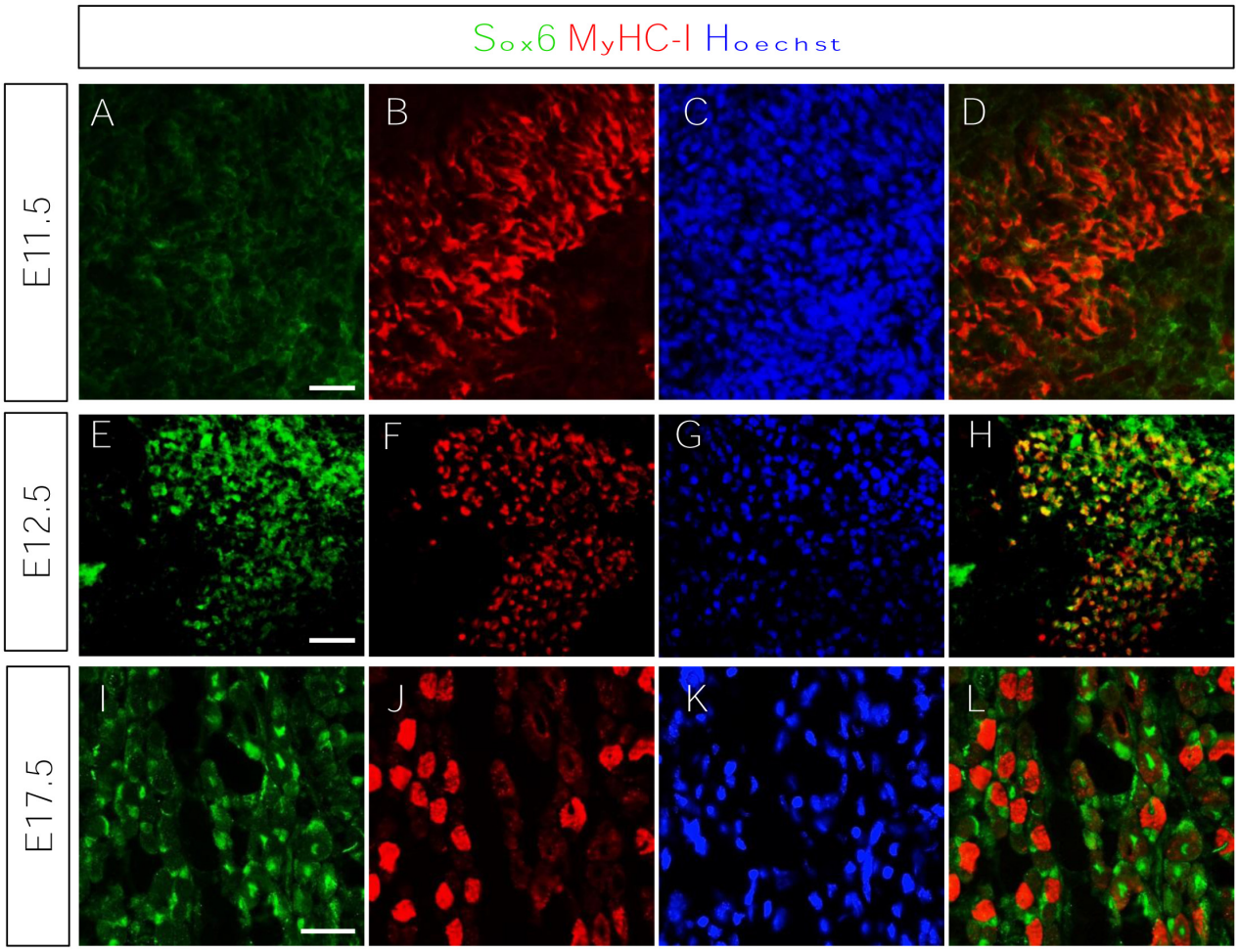
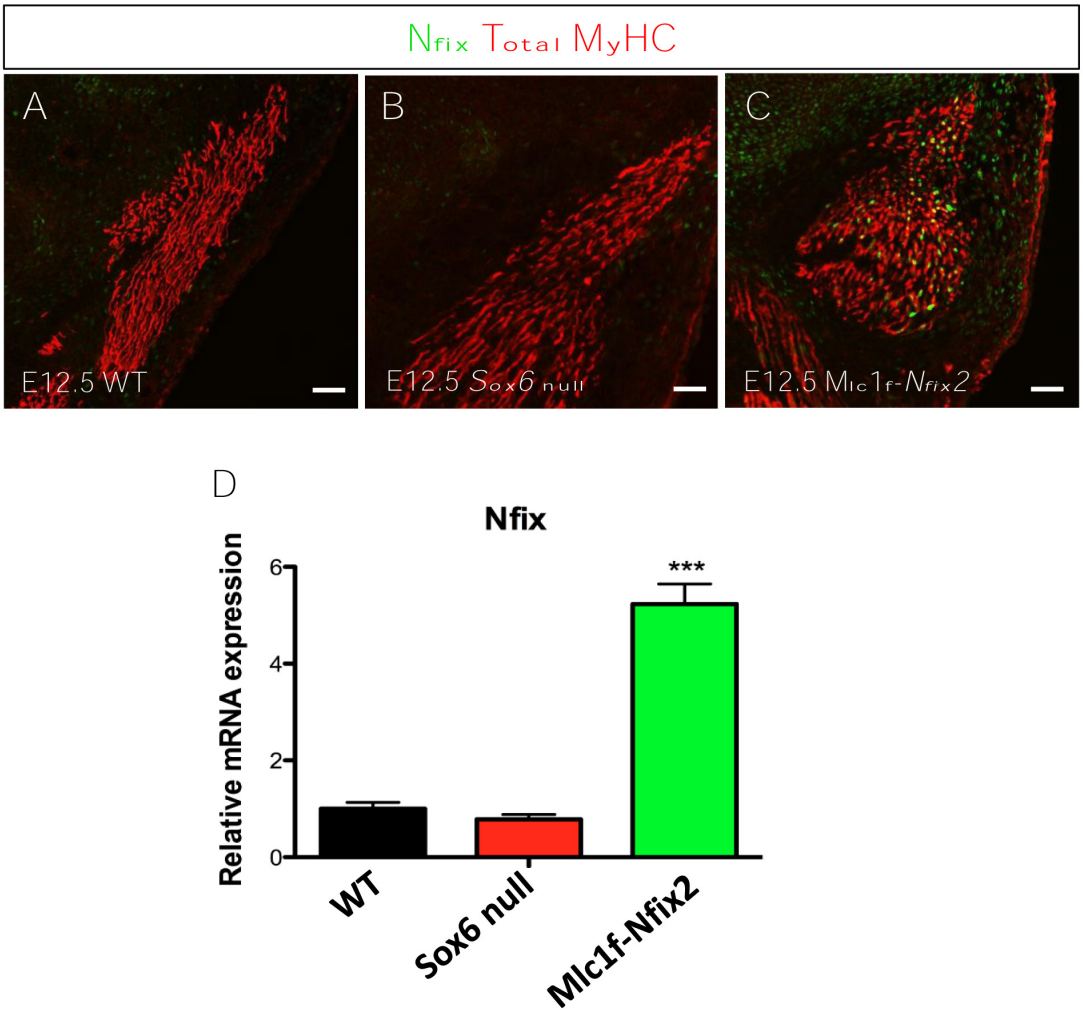
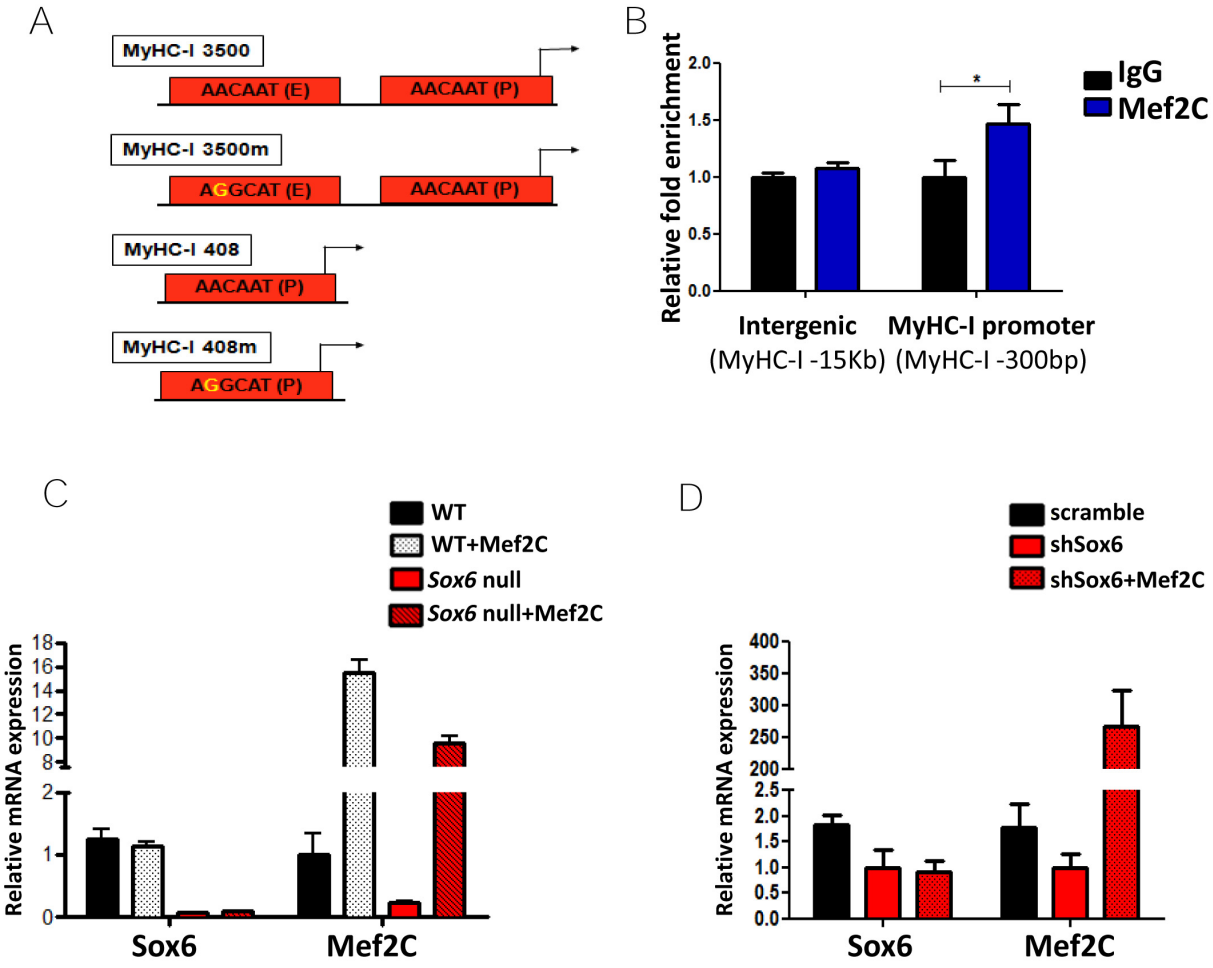


Supplemental Figure S1. Related to Figure 1. Sox6 is expressed in both embryonic and fetal muscle cells.

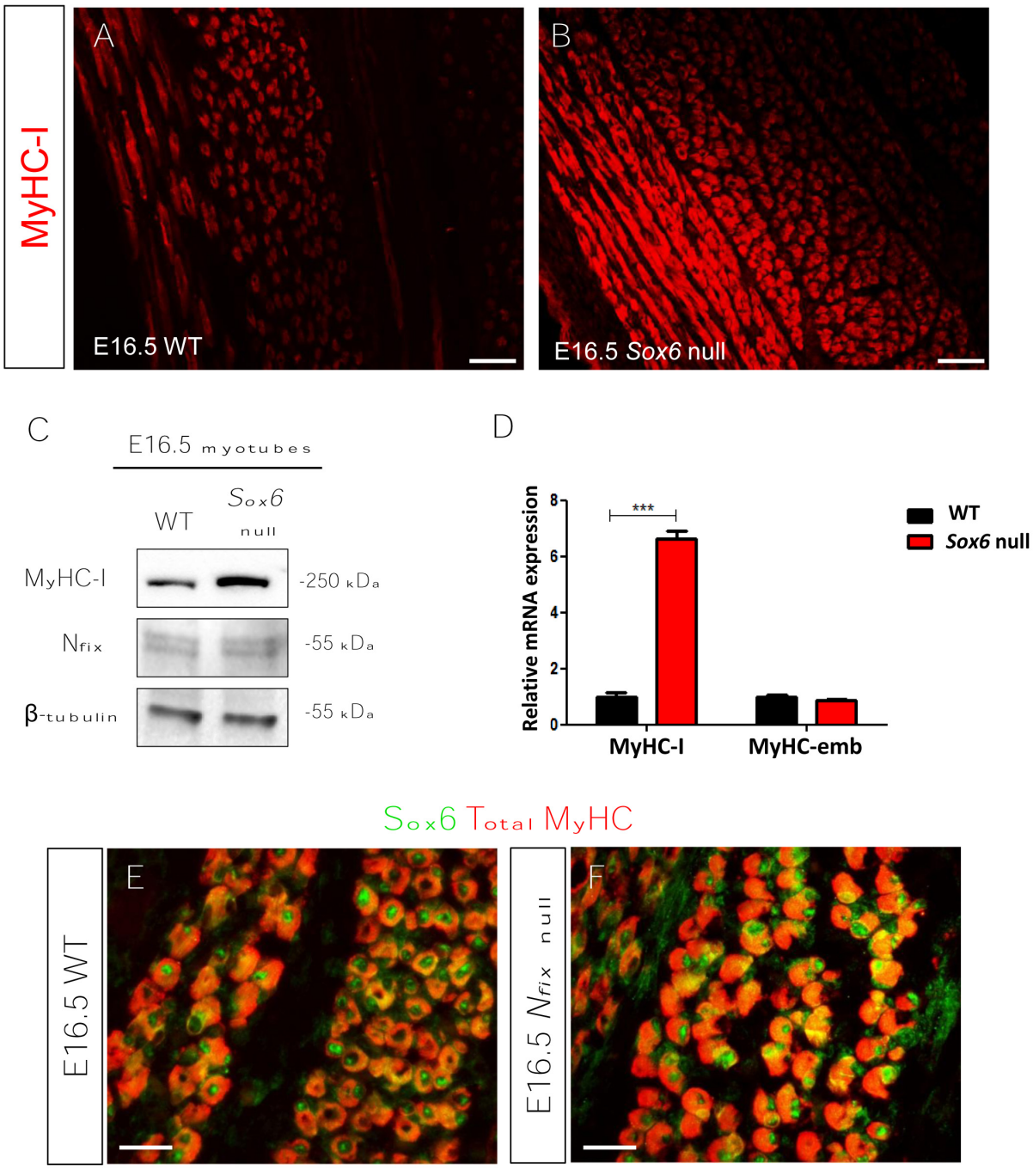
A-L) Immunofluorescence with anti-Sox6 (green) and anti-MyHC-I (red) antibodies on E11.5 (**A-D**), E12.5 (**E-H**) and E17.5 **(I-L)** muscle sections. Nuclei are counterstained with Hoechst. Scale bars: 25 µm.



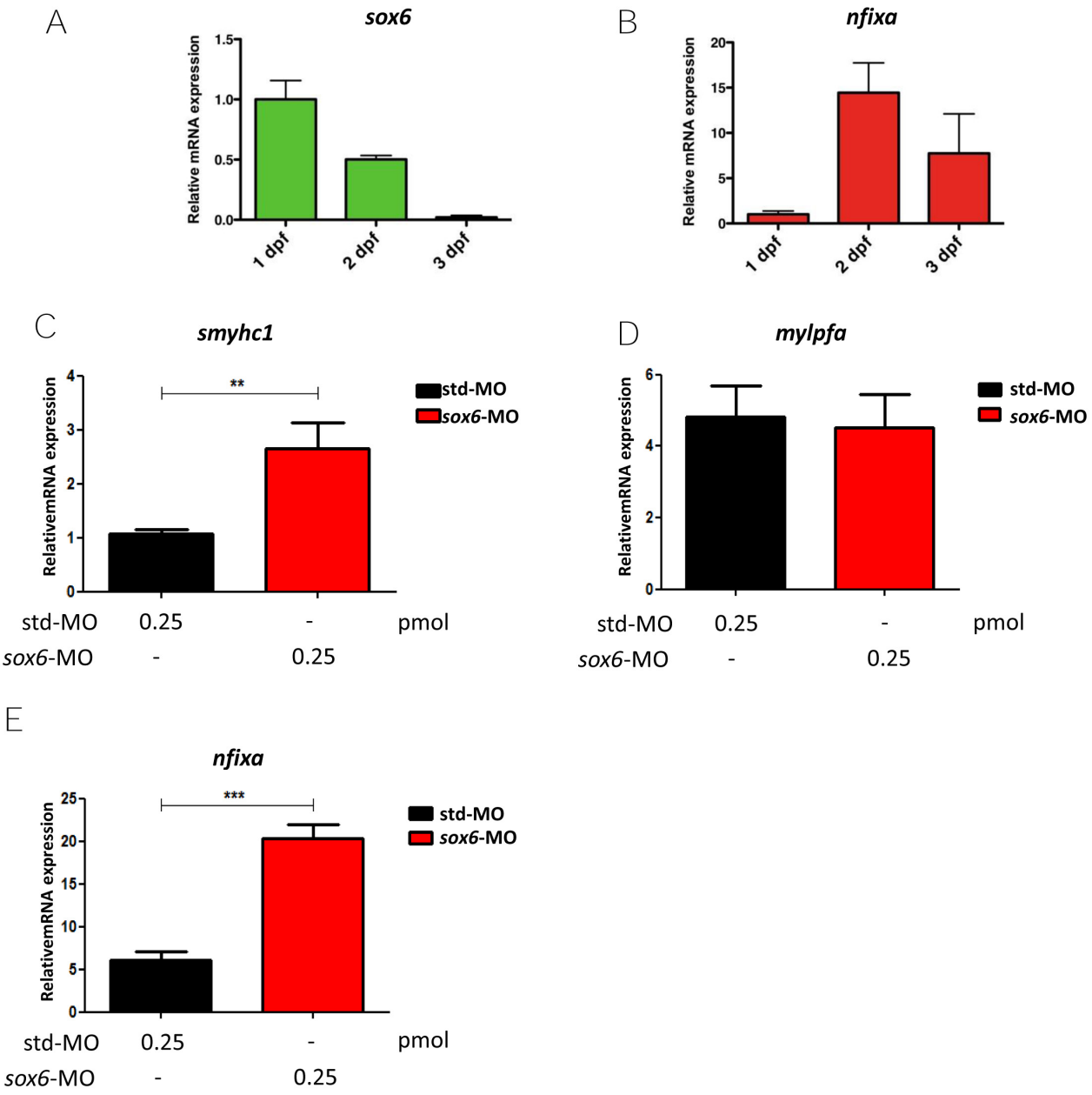
Supplemental Figure S2. Related to Figure 1. Nfix is not up-regulated in *Sox6*-null embryonic muscle.
A-C) Immunofluorescence with anti-Nfix antibody (green) and MF20 (red) on E12.5 muscle sections from WT (**A**), *Sox6* null (**B**) and Tg:Mlc1f-*Nfix2* (**C**) embryos. Scale bar: 50 µm. **D)** qRT-PCR for *Nfix* on WT, *Sox6* null and Tg:Mlc1f-*Nfix2* E12.5 muscle tissue (***p<0.001; N=3).



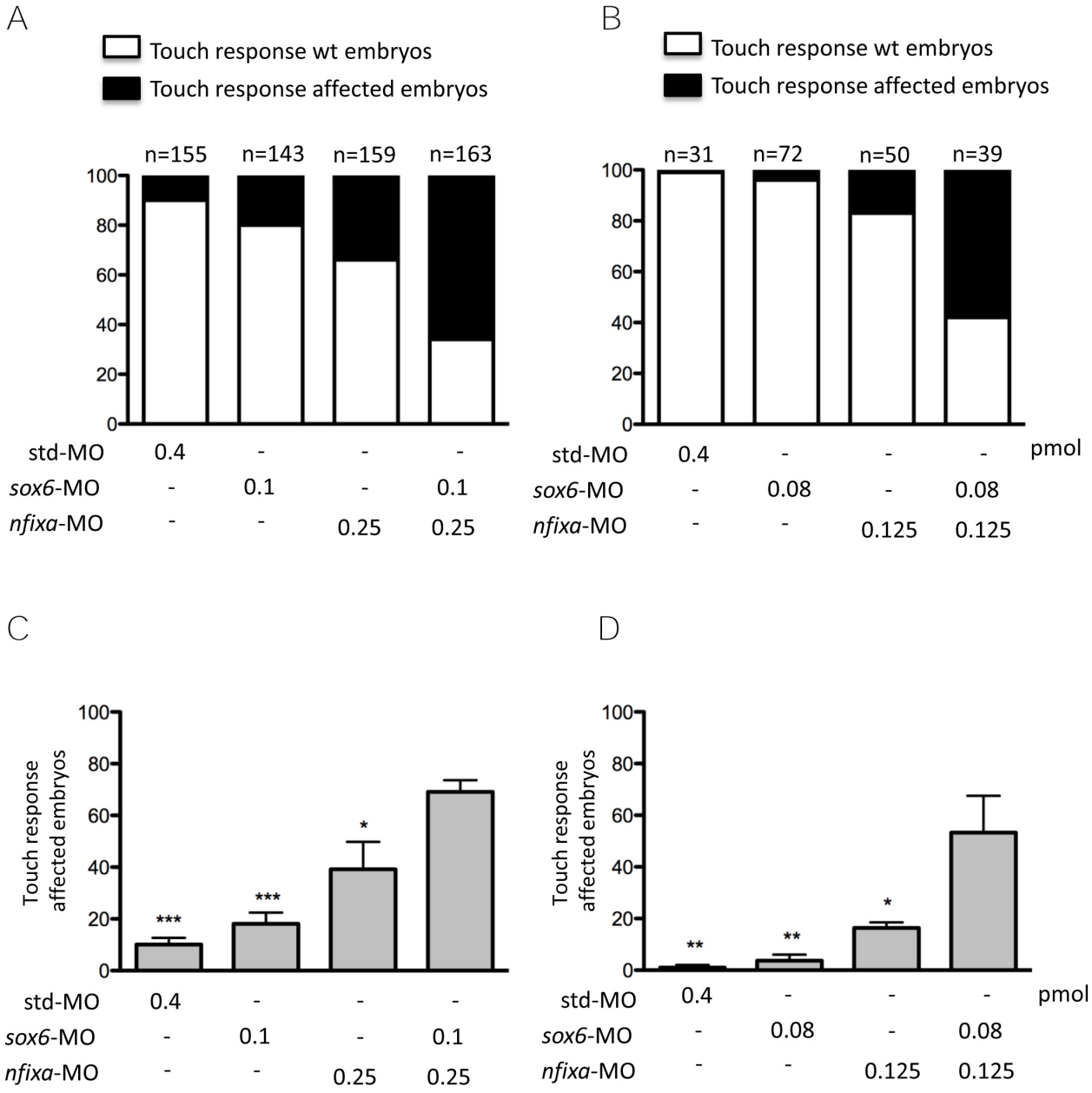
Supplemental Figure S3. Related to Figure 2. Sox6, through Mef2C, positively regulates MyHC-I in embryonic myotubes.
A) Scheme of the luciferase vectors used in Fig. 2B and Fig. 3F. See also Hagiwara et al. 2007 and An et al. 2011 for a complete description of the vectors. **B)** Chromatin immunoprecipitation (ChIP) for Mef2C on an intergenic region located at -15 Kb upstream of the MyHC-I TSS and on the MyHC-I proximal promoter. (*p<0.05; N=2). **C)** qRT-PCR for Sox6 and Mef2C on WT and *Sox6* null differentiated embryonic myoblasts transfected with a Mef2C-overexpressing vector. Related to Fig. 2E. **D)** qRT-PCR for Sox6 and Mef2C on purified embryonic myoblasts treated with control shRNA lentivirus (scramble), with anti-Sox6 shRNA lentivirus (shSox6) and with both shSox6 lentivirus and Mef2C overexpressing vectors (shSox6+Mef2C). Related to Fig. 2F.



Supplemental Figure S4. Related to Figure 3 and Figure 4. Sox6 and Nfix are independently expressed in fetal muscle.
A, B) Immunofluorescence with anti-MyHC-I on E16.5 muscle sections from WT (**A**) and *Sox6* null **(B)** mice. Scale bar: 50 µm. **C)** Western blot on E16.5 WT and *Sox6* null myotubes. β-tubulin was used to normalize the amount of proteins loaded. **D)** qRT-PCR analysis on E16.5 WT and *Sox6* null muscle tissue (***p<0.001; N=3). **E, F)** Immunofluorescence with anti-Sox6 antibody (green) and anti-MyHC antibody (red) on fetal (E16.5) muscle sections from WT and *Nfix* null mice. Scale bars: 25 µm.



Supplemental Figure S5. Related to Figure 6. *sox6* and *nfixa* expression levels during wt embryo development and gene expression analysis in *sox6* morphants.
A, B) qRT-PCR for *sox6* **(A)** and *nfixa* **(B)** on trunk/tail regions collected from 1, 2 and 3 dpf zebrafish embryos. **C-E)** qRT-PCR for *smyhc1, mylpfa, and nfixa* on trunk/tail regions at 2 dpf collected from embryos injected with std-MO or *sox6*-MO (**p<0.01; ***p<0.001; N=2).



Supplemental Figure S6. Related to Figure 6. Touch-evoked escape response assay on std-MO, *sox6*-MO, *nfixa*-MO and *nfixa*-MO+*sox6*-MO injected embryos.
A, B) Touch-evoked escape response assay was performed on *sox6* and *nfixa* single morphants and on the combined double partial morphants, injected using slightly higher **(A)** or lower **(B)** doses. Graphs show the percentage of embryos with or without defects (white and black bars, respectively) in touch-evoked escape response assay. The co-injection of both lower and higher doses of MOs resulted in synergic defects in motility in touch-response assays. **C, D)** Statistical analysis of touch-evoked escape response assay shown in A and B, respectively (*p<0.05; **p<0.01; ***p<0.001).

Supplemental Movie S1. Related to Figure 6. Impaired escape response in double partial *sox6/nfixa* morphants.
The movie shows, sequentially, touch-evoked escape responses of 2 pdf control embryos (std-MO 0.4 pmol), single partial *sox6* or *nfixa* knocked down embryos (*sox6*-MO or *nfixa*-MO, 0.1 pmol and 0.25 pmol, respectively), and double partial morphants (*sox6*-MO+*nfixa*-MO). The most representative embryos are shown for each condition (see Supplemental Fig. S6A). In particular, the *sox6/nfixa* double partial morphant corresponds to the most drastic and representative phenotype, within the range of escape-response impairments observed.

SUPPLEMENTAL TABLES

Supplemental Table S1. Primers used for mRNA expression analysis with quantitative Real Time PCR (*Mus musculus*)

Gene	Primers sequence	Reference
Sox6	F: 5'-AATGCACAACAAACCTCACTCT-3' R: 5'-AGGTAGACGTATTTCGGAAGGA-3'	
MyHC-I (Myh7)	F: 5'-AGGGCGACCTCAACGAGAT-3' R: 5'-CAGCAGACTCTGGAGGCTCTT-3'	Mathew et al. 2011
MyHC-emb (Myh3)	F: 5'-GCAAAGACCCGTGACTTCACCTCTAG 3' R: 5'-GCATGTGGAAAAGTGATACGTGG-3'	Mathew et al. 2011
Nfix	F: 5'-CACTGGGGCGACTTGTAGAG-3' R: 5'-AGGCTGACAAGGTGTGGC-3'	Mourikis et al. 2012
Mef2c	F: 5'-AGTACACCGAGTACAACGAGC-3' R: 5'-GCCTGTGTTACCTGCACTTGG-3'	
Mef2a	F: 5'-TTGATGGGGGAATGACAACT-3' R: 5'-TAGTCCTGTGGGGAATGGAT-3'	
Gapdh	F: 5'-GGCATGGACTGTGGTCATGA-3' R: 5'-TTCACCACCATGGAGAAGGC-3'	

Supplemental Table S2. Primers used for mRNA expression analysis with quantitative Real Time PCR (*Danio rerio*)

Gene	Primers sequence
nfixa	F: 5'-ACCGAAGAAACCGACTGGTG-3' R: 5'-TCTGTGGCCATTGTAGTTCAGG-3'
sox6	F: 5'-GCGCATGGAATCGGACAG-3' R: 5'-GGCTTGTGTGGAGAGGTAGAG -3'
smyhc1	F: 5'-GCTAACAGGCAGGCATCAGA-3' R: 5'-GTTGCATTTGGGAATCCTTGACA- '
mylpfa	F: 5'-GCGGCTTCAGACTTCTCTTCTTG-3' R: 5'- CTTCTTGGGTGCCATGTCGAG-3'
myl1	F: 5'-GGAACAGTGATGGGTGCTGA-3' R: 5'-CGTTTTCATCCTCCTGGCCT-3'
rpl8	F: 5'-GCAAACAGAGCCGTTGTTG-3' R: 5'-CCTTCAGGATGGGTTTGTCA-3'

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reporter assay

Luciferase expression vectors driven by the *MyHC-I* promoter were kindly provided by Dr. K.M. Baldwin (University of California, Irvine) and Dr. N. Hagiwara (University of California, Davis). The MyHC-I 408 and MyHC-I 3500 constructs contain 408 bp and 3500 bp of the 5'-upstream sequence of the rat *MyHC-I* gene, respectively (Fig. S2A; Huey et al. 2002). The MyHC-I 408m and MyHC-I 3500m constructs were generated by introducing ACA to CAG substitutions to disrupt the proximal and the distal Sox6 binding sites, respectively (Fig. S2A; Hagiwara et al. 2007; An et al. 2011). These lucifearse vectors were co-transfected with the *Renilla* luciferase vector (pcDNA-Rluc) into embryonic or fetal primary myoblasts using Lipofectamine 2000 (Invitrogen). The day after transfection, cells were washed in PBS and allowed differentiation in DM. After 2 more days, firefly and *Renilla* luciferase activities were measured using Dual-Glo Luciferase Assay System (Promega) and a luminometer according to the manufacturer's instructions. pGL3-basic vector was used as a negative control.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously published (An et al. 2011). Briefly, primary myoblasts were crosslinked for 10 min at room temperature in 1% formaldehyde (Sigma) in PBS, then the crosslinking was quenched with 0,25M glycine in PBS (10 min) and the cells were pelleted and lysed in Sonication Buffer containing 10mM Tris-HCl pH 8.0, 1mM EDTA, 1% SDS in deionized water. Sonication was performed in a Bioruptor Diagenode with three pulses of 5 min at maximum intensity. Chromatin was de-crosslinked and checked for 200-500bp DNA fragments enrichment. Immunoprecipitation was performed with 3-5 µg of the following antibodies: rabbit anti-Sox6 (Abcam), rabbit anti-Nfix (Novus Biologicals), rabbit anti-Mef2C (Cell Signaling) and normal goat IgG (Santa Cruz). After elution, de-crosslinking, DNA extraction and precipitation, the samples were analyzed by qRT-PCR.

The following primers were used for amplification of the *MyHC-I* proximal promoter region (promoter): 5'-CCCCACCCCCTGGAACT-3' (fw), 5'-CCAGCTAGGAAACAATTGGAAGTG-3' (rev); for the *MyHC-I* distal promoter region (enhancer): 5'-ACACCGCCCACTCAATACAC-3' (fw), 5'-GCCCTCTCCAAACACTCTTG-3' (rev); for a negative control region located 15 Kb upstream of

the *MyHC-I* gene (intergenic): 5'-TCGGACCGGAGTGTTAGGAA-3' (fw), 5'-ACCCTGGAGTCTCAGCATCG-3' (rev) (An et al. 2011); *Nfatc4* promoter (-1.2Kb): 5'–GGCGCTTAACCCTTTAGGTG-3' (fw). 5'-CAAGACAGGGGAGCAGTCAC-3' (rev). We also used the following primers for the *Mef2c* promoter (-1.1Kb): 5´-AACCTAAGGGTTTTGTTATGACGC-3' (fw), 5´-ACGGGTGGGACTTTTTAGGAG-3´(rev). The fold enrichment of each sample was calculated as percentage of input for internal control and then normalized on the IgG value.

Touch-evoked escape response assay

The screening for embryonic motility was performed as follows: embryos at 2 dpf were subjected to a tactile stimulus. Using a needle, a gentle stimulus was applied to the tail of the larvae and their reaction observed. Wild-type embryos at this stage of development have a normal activity. Upon application of the tactile stimulus they swim away from the source of the stimulus (Granato et al. 1996).

Movies were recorded with a Leica MZFLIII stereomicroscope equipped with a DFC 480-R2 digital camera and the LAS imaging software. Movies were edited using the Adobe Premiere program.

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PART III

Manuscript in preparation

RhoA-ERK axis regulates secondary myogenesis through the control of JunB and Nfix expression

Summary

The transition from embryonic to fetal myogenesis is a crucial switch, required for the complete maturity of skeletal muscles. Mechanistically, the transcription factor Nfix, specifically expressed during fetal myogenesis, is needed for the beginning of fetal myogenesis. Here we investigate the mechanisms regulating Nfix expression, showing that JunB is both necessary and sufficient for Nfix activation. Moreover, we show that the temporal progression of prenatal muscle development is timed by the RhoA/ROCK, which maintains the embryonic myogenesis, suppressing JunB and Nfix expression. RhoA/ROCK elicit their effects repressing ERK kinases activity, which promote the fetal genetic program. In conclusion, RhoA/ROCK/ERK axis constitutes one of the major pathway that regulates the progression of prenatal myogenesis through the control of JunB and Nfix expression.

Introduction

Prenatal skeletal muscle development is a biphasic process, which involves the differentiation of two distinct populations of muscle progenitors, known as embryonic and fetal myoblasts (Biressi et al., 2007b; Hutcheson et al., 2009). The first wave of muscle differentiation, named embryonic myogenesis, takes place around embryonic day (E) 10.5-12.5 in mouse. During this phase embryonic myoblasts are committed to differentiate in primary fibers, establishing the primitive architecture of prenatal muscles. Primary fibers are mainly slow-twitching fibers expressing high levels of slow Myosin heavy chain (MyHC) isoform (MyHC-I, encoded by the *Myh7* gene) and embryonic MyHC isoform (MyHC-emb, encoded by *Myh3* gene) (Schiaffino et al., 1986; Stockdale et al., 1992). Then, the second wave of differentiation is called fetal myogenesis and occurs between E14.5 and E17.5. During this latter phase, fetal myoblasts give rise to fast-twitching secondary fibers. Secondary fibers express high level of neonatal MyHC (MyHC-neo, encoded by *Myh8*)

and glycolytic enzymes, and they are characterized by low levels of slow MyHC (Eusebi et al., 1986; Lyons et al., 1990; Daou et al., 2013). The formation of secondary fibers allows the complete maturation of prenatal muscles and confers the fiber type diversification, to fulfil different functional demands of adult skeletal muscles (Schiaffino and Raggiani, 2011).

Embryonic and fetal myoblasts are two intrinsically different population of muscle progenitors that differs in terms of morphology, extracellular signal response and gene expression profile (Biressi et al., 2007b), indicating that a transcriptional change is needed to switch from embryonic to fetal myogenesis. The major regulator of the transition from embryonic to fetal gene transcription is Nfix, a transcription factor mainly expressed during fetal myogenesis (Biressi et al., 2007b; Messina et al., 2010). The key role of Nfix is due to its capability to activate fetal specific markers, such as the muscle creatine kinase (MCK) and β-enolase (eno3), and to repress embryonic-specific genes, as *Myh7* (Messina et al., 2010; Taglietti et al., 2016). Moreover, it has been reported that Nfix regulates also postnatal muscle homeostasis and the proper timing of regeneration (Rossi et al., 2016). Interestingly, the second transcription factor more highly expressed during fetal myogenesis is JunB (Biressi et al., 2007b). JunB is a member of activator protein 1 (AP-1) family, which maintains muscle mass and prevents atrophy in adult muscles (Raffaello et al., 2010), whereas its role during prenatal development is still unknown. Cellular identity during development is known to be defined by intrinsic factors but how the temporal progression is achieved is still a matter of research. Moreover, the integration of environmental signals is fundamental for the orchestration of prenatal development but, although different pathways activated by extracellular stimuli have been described, the molecular mechanisms that translate them into gene expression program are not known.

The Rho GTPase RhoA plays important roles in many intracellular signaling (Kimura et al., 1996; Amano et al., 1996) through the activation of its major effector, the Rho-kinase ROCK. The interplay of the RhoA/ROCK pathway with various signaling molecules, such as the ERK kinases (Zuckerbraun et al., 2003; Li et al., 2013), is known to affect the proper transduction of extracellular signals, conditioning the gene expression network. Here we report that the RhoA/ROCK axis confers the identity of embryonic myoblasts, repressing the activation of the ERK kinases and, as a consequence, of JunB. Conversely, during fetal myogenesis, the ERK activity is necessary for the expression of JunB, which directly activate Nfix, promoting the beginning of fetal myogenesis program and the complete maturation of prenatal muscles.

Results

Analysis of temporal gene expression of Nfix and JunB

Although it was demonstrated that Nfix and JunB are highly expressed specifically during fetal myogenesis (Biressi et al., 2007b; Taglietti et al., 2016), the temporal profiles of their expression are not known. We first analyse the transcript levels of Nfix and JunB on Myf5$^{GFP-P/+}$ myoblasts, freshly isolated by FACS from cellular suspensions obtained from Myf5$^{GFP-P/+}$ embryonic muscles, at E11.5, E12.5 and E13.5 and from fetal muscles at E14.5, E15.5, E16.5 and E17.5. Both Nfix and JunB start to be expressed around E14.5 and their expression drastically increases at E15.5 to remain still high until E17.5 (Figure 1 A-B). Western blot analysis on total skeletal muscle lysates at different stages showed a similar profile of Nfix and JunB expression seen by qRT-PCR (Figure 1 C). These data confirm that Nfix and JunB expression occurred only during fetal stages and demonstrate that their activation starts at E14.5.

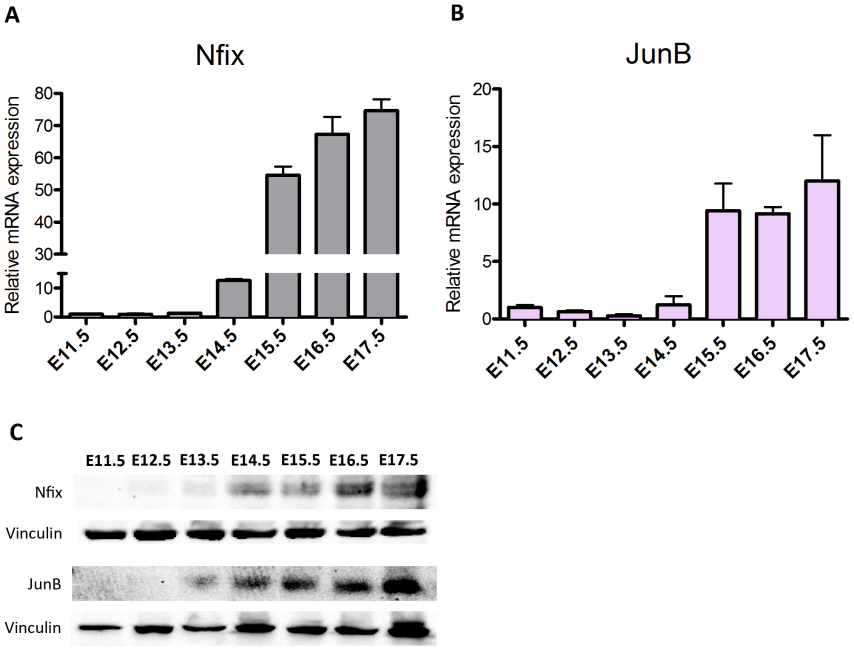


Figure 1. Developmental timing of Nfix and JunB expression. **A-B)** qRT-PCR analysis on purified Myf5$^{GFP-P/+}$ myoblasts dissected from E11.5 up to E17.5 muscles, showing the expression profile of Nfix (A) and JunB (B) at different developmental stages. **C)** Western Blot on purified Myf5$^{GFP-P/+}$ myoblasts isolated from E11.5 up to E17.5 muscles. Vinculin was used to normalize the total amount of loaded proteins.

JunB is sufficient for the induction of Nfix

To better characterize the patterns of expression of Nfix and JunB in fetal muscle progenitors, we performed immunostaining on Myf5$^{GFP-P/+}$-purified myoblasts obtained from fetuses at E14.5, E15.5 and E16.5. Freshly isolated myoblasts were maintained in culture for 2 hours to allow their adhesion and then were stained for Nfix and JunB (Supplementary Figure S1). In all the time points analysed, a high percentage of fetal myoblasts co-expressed Nfix and JunB (E14.5 = 77.2%, E15.5 = 85%, E16.5 = 82 %), but at E14.5 and E15.5 there were some myoblasts which were positive only for JunB (E14.5 = 10.3%, E15.5 = 10.2%). Conversely, at E16.5 few myoblasts were positive for Nfix but not for JunB (13.9%) (Figure 2A-C). Since JunB seems be expressed earlier than Nfix, we decided to investigate the possible interplay between Nfix and JunB. With this aim, unpurified embryonic myoblasts were transfected with pcDNA3.1X-JunB expressing vector and the expression of Nfix was analysed by Western Blot. The analysis revealed that Nfix is precociously activated in embryonic myoblasts overexpressing JunB, if compared with the embryonic myoblasts expressing a control vector (pcDNA3.1x) (Figure 2D). To further confirm this result, Myf5$^{GFP-P/+}$-purified embryonic myoblasts were induced to express JunB upon pcDNA3.1X-JunB transfection and the transcript levels of Nfix were examined by qRT-PCR. The pure population of embryonic myoblasts over-expressing JunB expressed also Nfix, whereas it was virtually absent in the control myoblasts, suggesting the requirement of JunB for Nfix induction (Figure 2E). Interestingly, the embryonic myoblasts, which overexpress JunB and Nfix, precociously down-regulate the typical embryonic marker MyHC-I, and up-regulate the fetal marker, β-enolase, described to be normally respectively inhibited (Taglietti et al., 2016) and activated by Nfix (Messina et al., 2010). These results suggest that the induction of JunB in embryonic myoblasts is sufficient for the expression of Nfix and the activation of the fetal genetic program. To evaluate whether JunB acts as a direct activator of Nfix, we performed *in silico* sequence analysis of Nfix promoter, founding two consensus activator protein-1 (AP-1) sites, 5'-TGA[G/C]TCA-3'

(Chinenov and Kerppola, 2001; Eferl and Wagner, 2003), located about 200 base pairs (bp) and 1400bp upstream of the transcription start site (TSS). To address whether JunB is responsible for the direct transcriptional activation of Nfix on those two sites, we performed a chromatin immunoprecipitation assay (ChIP) for JunB on fetal differentiated myoblasts. As shown in Figure 2F, JunB is able to directly bind Nfix promoter in the region proximal to the TSS (-200bp) but not the distal one (-1400bp). MyHC-2b promoter was used as positive control sequence for the ChIP of JunB (Raffaello et al., 2010). Taken together, these results show that JunB is sufficient to directly promote the transcriptional activation of Nfix.

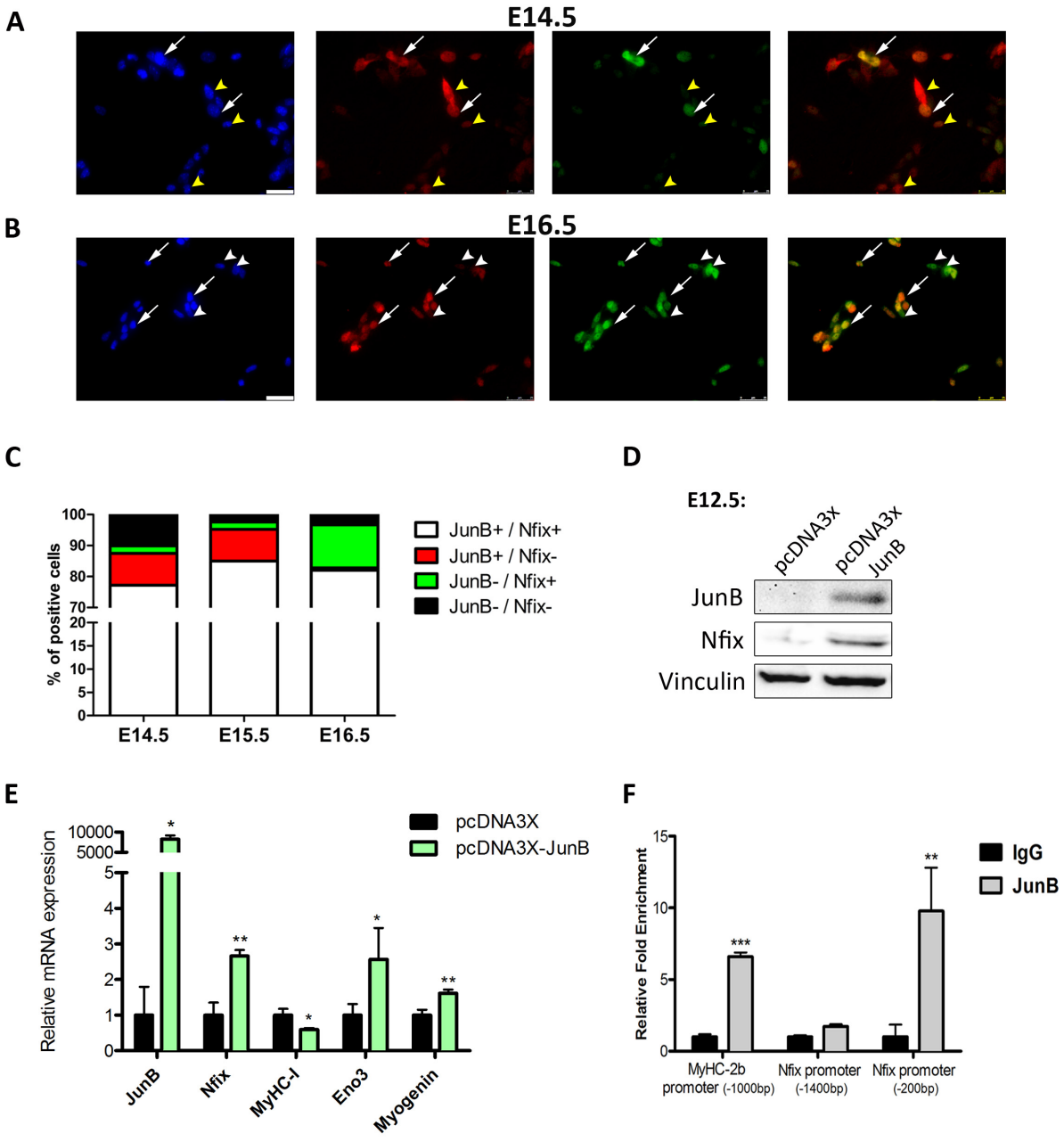


Figure 2. JunB acts as a direct activator of Nfix A-B) Immunofluorescence for JunB (red) and Nfix (green) on freshly isolated Myf5$^{GFP-P/+}$-purified myoblasts at E14.5 (A) and E16.5 (B). Nuclei were counterstained with Hoechst. White arrows indicate myoblasts coexpressing JunB and Nfix. White head-arrows, in Figure A, mark nuclei positive for JunB but negative for Nfix, while yellow head-arrows, in Figure B, indicate myoblasts expressing Nfix but not JunB. Scale bars: 25 µm. **C)** Graph showing the percentage of JunB+/Nfix+ (white bar), JunB+/Nfix- (red), JunB-/Nfix+ (green) and Junb-/Nfix- (black). Percentages are expressed in respect to the total number of nuclei. **D)** Western Blot on lysates from unpurified embryonic (E12.5) myoblasts over-expressing JunB (pcDNA3x-JunB) compared with control myoblasts (pcDNA3x). The Western Blot shows that embryonic myoblasts transfected with pcDNA3x-JunB precociously expressed Nfix. Vinculin was used to normalize the total amount of loaded proteins. **E)** qRT-PCR on Myf5$^{GFP-P/+}$-purified embryonic myoblasts (E12.5) transfected with pcDNA3x-JunB over-expressing vector or with pcDNA3x, as control. The JunB over-expressing embryonic myoblasts have an increased expression of Nfix, that correlates with a decrease of MyHC-I expression and an up-regulation of Eno3 (*p<0.05, **p<0.01; N=3). **F)** ChIP assay with anti-JunB on fetal myotubes (E16.5) on positive control region (MyHC-2B promoter), Nfix promoter distal region located 1400bp upstream of Nfix TSS and Nfix proximal promoter (-200bp). IgG were used as an unrelated antibody (**p<0.01; N=3).

Nfix does not control JunB expression but directly activate its own expression

We then wondered whether the overexpression of Nfix in embryonic muscles would induce the expression of JunB. For this reason we analyzed the mRNA levels of JunB in embryonic myoblasts transfected with pCH-Nfix2 vector, which enables the over-expression of Nfix. Interestingly, the over-expression of Nfix does not induce the expression of JunB in embryonic myoblasts (Fig. 3A). To confirm this result, we evaluated the protein levels of JunB in embryonic myoblasts purified from a transgenic mice overexpressing Nfix, Tg:*Mlc1f*-Nfix2 mice, under the transcriptional control of the myosin light chain1F promoter and enhancer (Jiang et al., 2002; Messina et al., 2010). Western blot analysis shows that JunB was virtually absent at E12.5 in Tg:*Mlc1f*-Nfix2 embryonic myoblasts as in wild type (WT) littermates (Figure 3B). As expected JunB was also correctly expressed in *Nfix*-null fetal myoblasts (Fiure 3C-D), indicating that Nfix does not control JunB expression. Moreover, to evaluate if Nfix, once expressed, is able to maintain its own expression we transduced fetal unpurified myoblasts with a lentiviral vector expressing the dominant-negative Nfi-engrailed (NFI-ENG), a fusion protein composed by the Drosophila ENG transcriptional repression domain fused with Nfia DNA binding and

dimerization domain (Bachurski et al., 2003). The over-expression of NFI-ENG causes the inhibition of Nfi factor transactivation activity. The NFI-ENG fetal myoblasts show a strong down-regulation of Nfix, if compared with fetal myoblasts expressing only the engrailed domain (ENG), as control (Figure 3E), suggesting that Nfi factors activate the transcription of Nfix. To confirm this data we, then, performed a ChIP assay for Nfix in fetal differentiated myoblasts, founding a direct bind of Nfix on its own promoter, in a NFI consensus binding site located 1000bp upstream from the TSS (Figure 3F). Overall, these results demonstrate that Nfix is not able to control JunB expression, but it is able to promote and maintain its own expression.

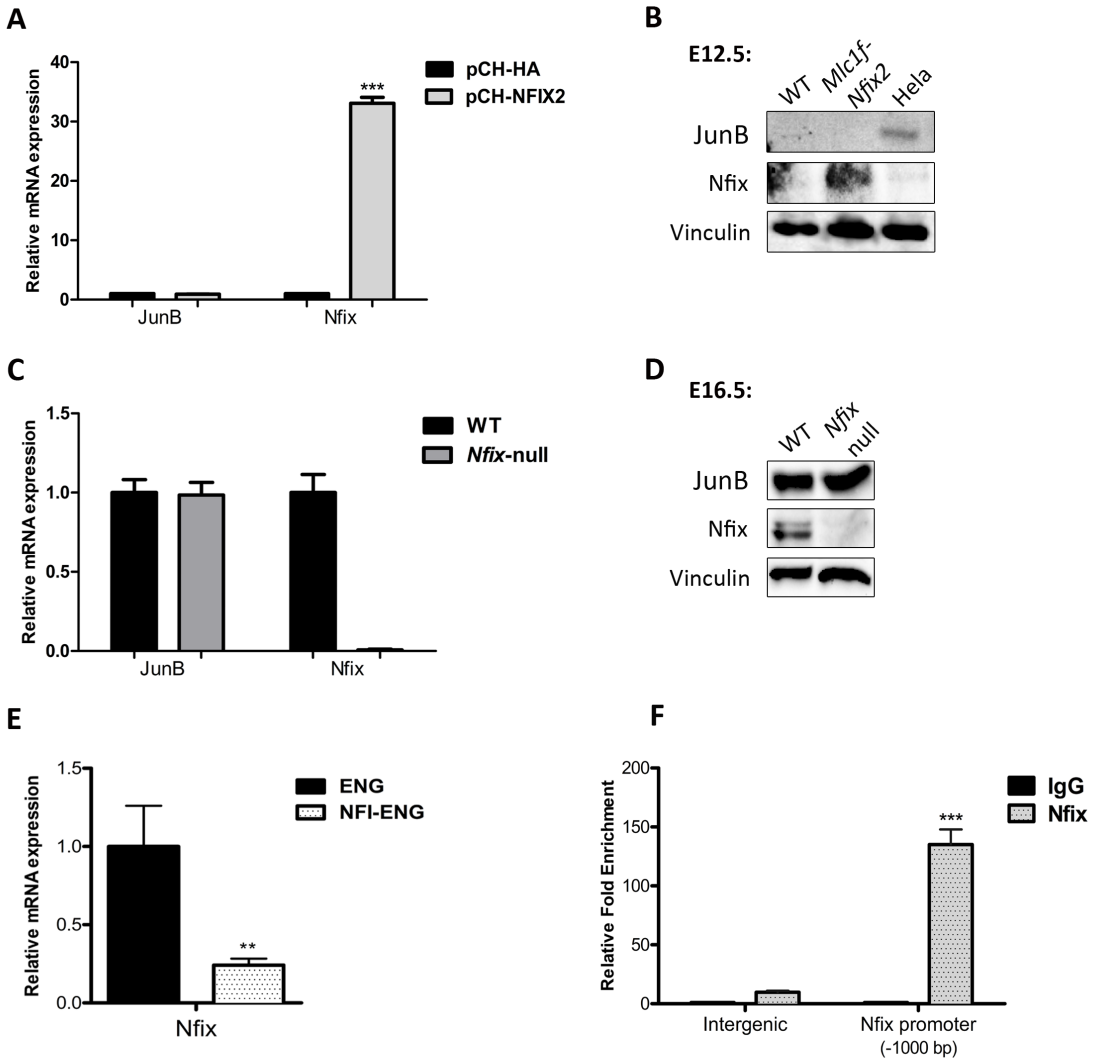


Figure 3. Nfix does not regulate JunB but promotes its own expression A) qRT-PCR for JunB and Nfix on embryonic (E12.5) myoblasts transfected with Nfix over-expressing vector (pCH-Nfix2) or a control vector (pCH-HA) (***p<0.01; N=3). **B)** Western Blot on WT and Mlc1f-Nfix2 embryonic muscles (E12.5) and Hela protein extracts, as positive control for JunB expression. The Western Blot show Nfix and JunB protein levels and Vinculin was used to normalize the total amount of loaded proteins. **C)** qRT-PCR for JunB and Nfix on WT and Nfix-null fetal muscles (E16.5), showing the normal expression of JunB in the absence of Nfix. **D)** Western Blot on lysates from WT and Nfix-null fetal muscles (E16.5). Vinculin was used to normalize the total amount of loaded proteins. **E)** qRT-PCR for Nfix on unpurified fetal myoblasts transfected with the dominant-negative NFI-engrailed (NFI-ENG) compared with fetal myoblasts expressing only the engrailed domain (ENG) (**p<0.01; N=3). **F)** Chip assay on unpurified fetal myotubes using anti-Nfix antibody, to test Nfix binding on its own promoter (-1000bp; Nfix promoter). Intergenic region has been used as a negative control and IgG as an unrelated antibody.

JunB is necessary for the induction of Nfix but not for the direct activation of fetal myogenic program

Since we observe that JunB is sufficient to promote the expression of Nfix, we decided to further investigate whether JunB was also necessary to activate Nfix and thus the fetal program (Messina et al., 2010). With this aim, we isolated by cell sorting purified fetal myoblasts from $Myf5^{GFP\text{-}P/+}$ muscles and JunB silencing by small hairpin RNA (shRNA) was performed. As control $Myf5^{GFP\text{-}P/+}$-purified fetal myoblasts were transduced with a scrambled targeting sequence vector. When cultured under conditions that promote differentiation, purified fetal myoblasts silenced for JunB display the typical embryonic phenotype, characterized by mononucleated differentiated myocytes and multinucleated myotubes containing only few nuclei (Supplemental Figure S2A) (Biressi et al., 2007b). The selective inhibition of JunB impairs fetal myoblasts differentiation and fusion as evidenced by decreased number of nuclei per myotube, decreased fusion index and decreased area of each myotubes (Supplemental Figure S2B-D). Most importantly, silencing of JunB abolishes the expression of Nfix, as shown in Figure 4A and in Figure 4B, whereas the typical embryonic marker slow MyHC was robustly induced (Figure 4B). As the fetal program was affected, we were wondered whether the effect on fetal myogenesis was specifically due to the silencing of JunB or to the consequent down-

regulation of Nfix in shJunB fetal myoblasts. For this reason, we transduced shJunB purified fetal myoblasts with HA-tagged Nfix2 expression vector (shJunb+Nfix2) (Supplemental Figure S2E) and we cultured them under differentiating conditions. After 3 days *in vitro*, fetal shJunB+Nfix2 cultures contained larger myotubes than fetal shJunB cultures with more nuclei in clusters in the center of the myotubes (Figure 4C). Furthermore, the morphology of shJunb+Nfix2 myotubes was similar to what observed in both scramble and Nfix2-transduced cultures (Figure 4C), indicating that the over-expression of Nfix in shJunB myoblasts is sufficient to restore the fetal program. To assess whether the fetal morphological restoring is associated with a transcriptional change, we examined by Western Blot the expression of the typical embryonic marker MyHC-I. As show in Figure 4D, fetal myoblasts selectively inhibited for JunB expressed high level of slow MyHC-I after differentiation, but the up-regulation of MyHC-I is lost in shJunb+Nfix2 fetal differentiated myoblasts, which correctly down-regulate it. Moreover, while wild-type (WT) embryonic myoblasts over-expressing JunB down-regulate MyHC-I and activate β-enolase, as a consequence of Nfix up-regulation, in Nfix-null embryonic myoblasts the over-expression of JunB does not leads to any change in MyHC-I and β-enolase, used respectively as marker of embryonic and fetal myogenesis (Supplemental Figure S2F and Figure 4E). These data demonstrate that JunB is required for Nfix induction and Nfix, acting downstream JunB, is uniquely required for the activation of the fetal program. Thus, JunB alone is not able to activate the fetal genetic program.

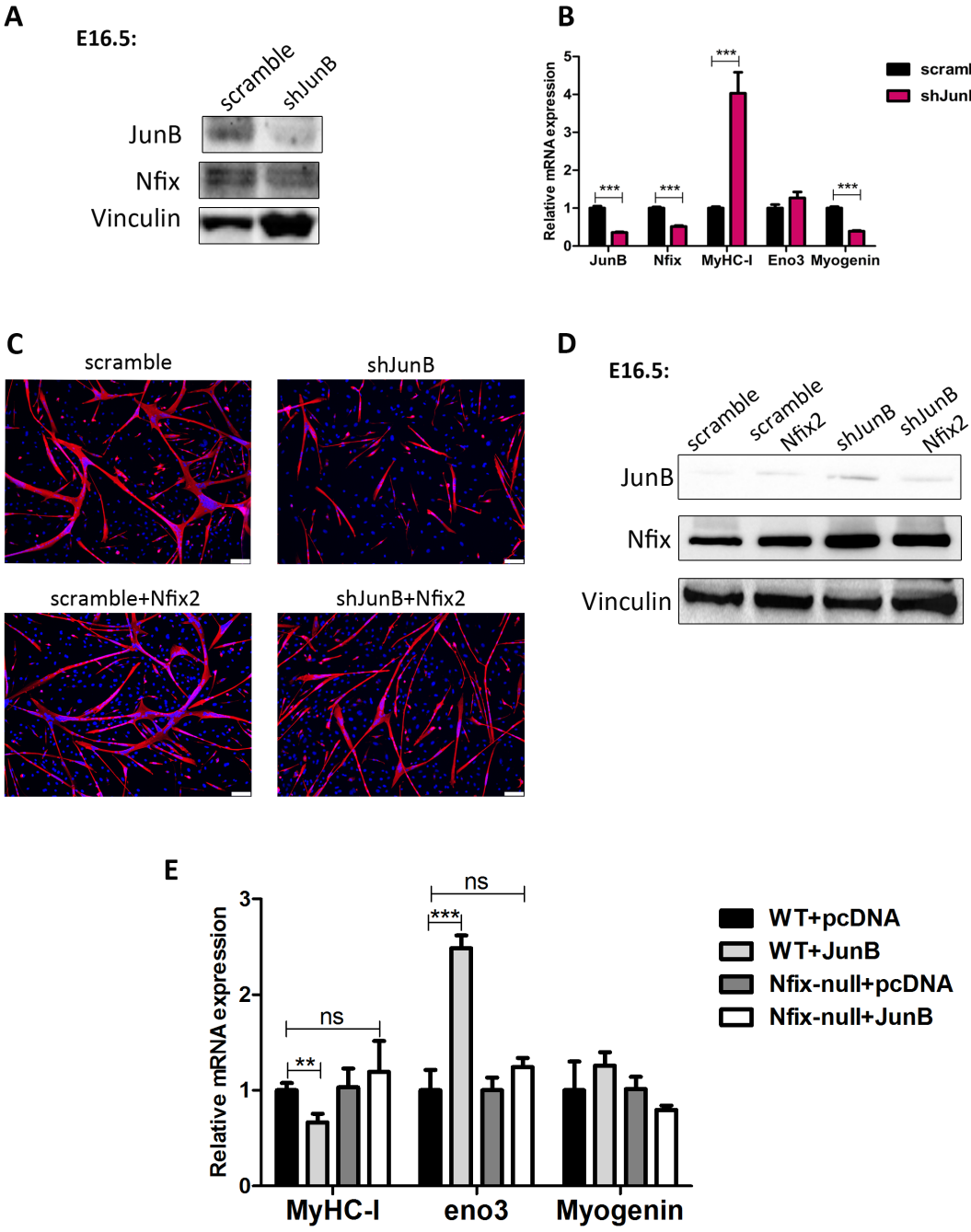


Figure 4. Silencing of JunB leads to the acquisition of embryonic features through the down-regulation of Nfix. A) Western Blot on shJunB or control (scramble) fetal differentiated myoblasts, showing a decrease in Nfix activation in the absence of JunB. Vinculin was chosen to normalize the total amount of loaded proteins. **B)** qRT-PCR on Myf5$^{GFP-P/+}$-purified fetal myoblasts infected by lenti-shJunB or scramble vector, analyzing the expression of Nfix and MyHC-I. **C)** Immunofluorescence on differentiated Myf5$^{GFP-P/+}$-purified fetal myoblasts co-transduced with shJunB and HA-Nfix2 lentivectors (shJunB+Nfix2) or the single and the non-targeting (scramble) vectors as controls, showing MyHCs (MF20) in red and Hoechst in blue. The scale bar represents 100 µm. **D)** Western Blot on differentiated fetal myoblasts (scamble+pCH-HA,

scramble+Nfix2, shJunB+pCH-HA, shJunB+Nfix2), showing MyHC-I expression. MyHCs (MF20) and Vinculin were used to normalize the total amount of loaded proteins. **E)** qRT-PCR on WT and Nfix-null embryonic myoblasts trasfected with JunB overexpressing vector or with a control vector (pcDNA). The analysis shows a statistical significant down-regulation of MyHC-I and an up-regulation of Eno3 only in WT embryonic cells that overexpress JunB. The regulation of MyHC-I and Eno3 is lost in Nfix-null myoblasts over-expressing JunB (**p<0.01; ***p<0.01; N=3).

The axis RhoA/ROCK negatively regulates ERK activity

It has been described that the Rho GTPases RhoA is required for the myogenic process and their activity must be fine regulated in time to allow a proper muscle differentiation (Castellani et al., 1996). To understand if the activity of RhoA is timely regulated during prenatal muscle development, GST-Rhotekin pulldown assay was performed on lysates of E12.5, E14.5 and E16.5 myoblasts and the amount of active Rho GTPases was determined by Western Blot. As shown in Figure 5A-B, the amounts of GTP-bound activated Rho was higher at E12.5 and E14.5, whereas at E16.5 there is a strong decrease of their active state, indicating that Rho GTPases are selectively activated during embryonic myogenesis and shuttled down during fetal stage. RhoA is an upstream activator of ROCK kinases and requires ROCK activity for its effects, which impinge myogenesis (Nishiyama et al., 2004; Pelosi et al., 2007). For this reason, to confirm the activation of RhoA signaling, we examined the phosphorylation of MYPT1 in Thr 696, specifically due to ROCK (Seko et al., 2003; Muranyi et al., 2005) during prenatal skeletal muscle development. The phosphorylation of ROCK substrate, MYPT1, was detectable only during early phase of myogenesis between E11.5 and E13.5, confirming that RhoA and ROCK are both active during primary myogenesis. The RhoA/ROCK axis is known to regulate many intracellular substrates to orchestrate their signaling, such as the ERK kinases (Zuckerbraun et al., 2003; Li et al., 2013). We therefore checked the activity of ERK kinesis during prenatal development. The phosphorylation of the ERK kinases, which is associated with their activity, is present only during fetal myogenesis, being completely activated starting from E14.5 until E17.5 (Figure 5D). Given that RhoA/ROCK signaling is engaged in the regulation of ERK activity, we tested whether the RhoA/ROCK axis regulates ERK activity. To this aim, unpurified embryonic myoblasts were treated with a well known ROCK inhibitor, Y27632 (Uehata et al., 1997). Immunoblot analysis

demonstrates that embryonic myoblasts treated with Y27632 show a strong increase in ERK activity, suggesting that ROCK negatively affects the activation of ERKs. Conversely, the amount of phosphorylated ERK in fetal myoblasts expressing the constitutively-activated form of RhoA (RHOV14) was decreased compared with control cells (Figure 5E-F). Taken together, these results indicate that ROCK mediates the negative regulation of RhoA signaling on the activity of ERK kinases.

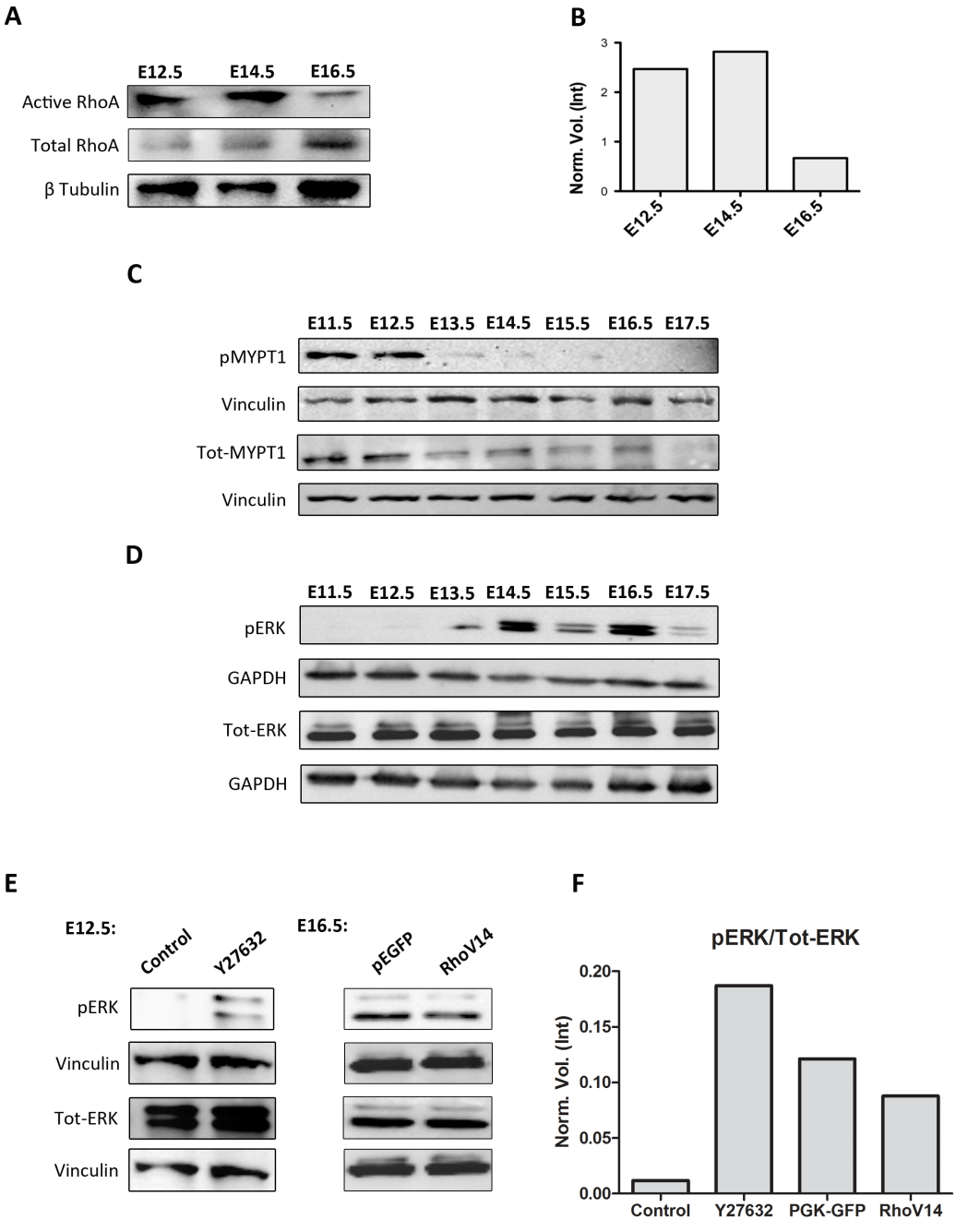
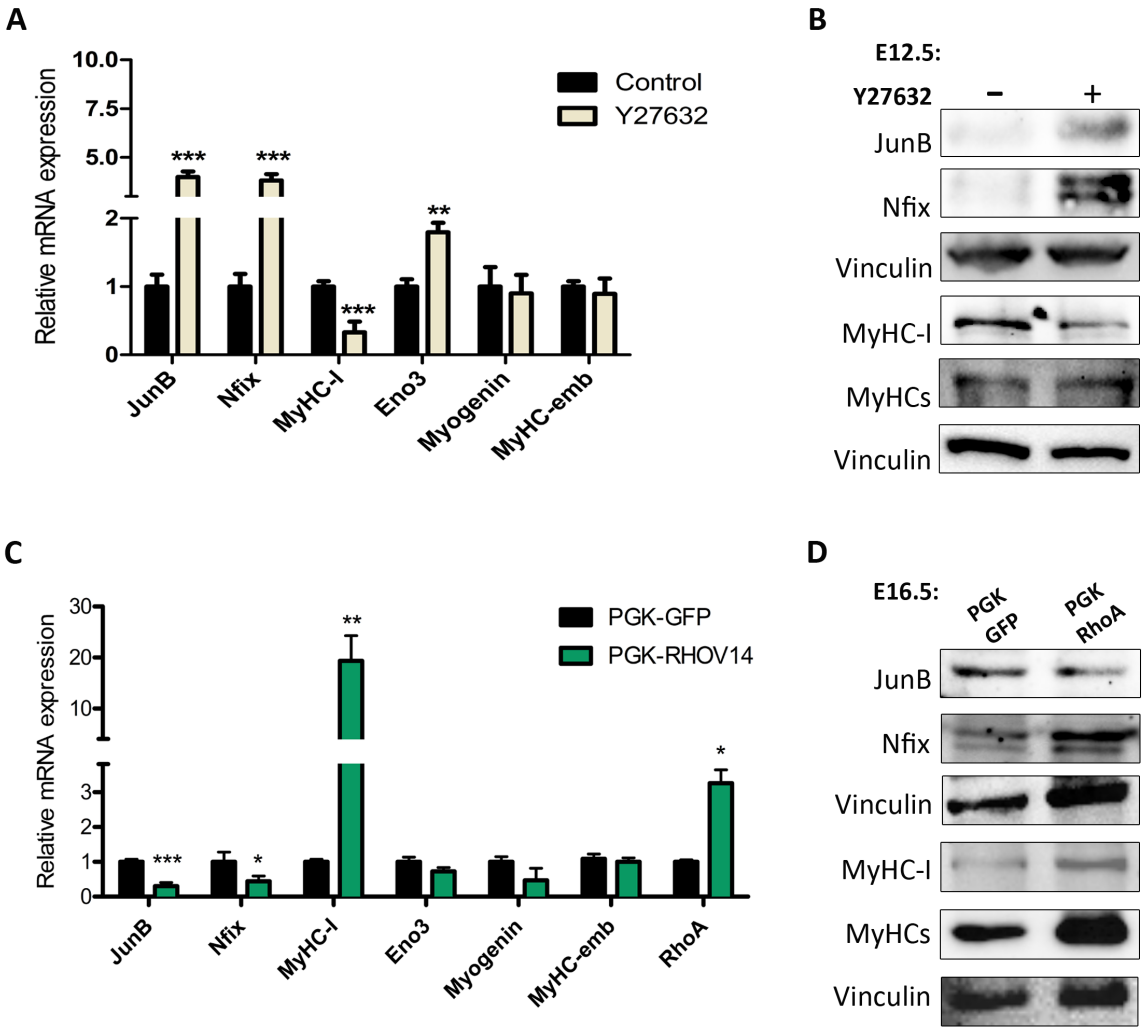


Figure 5. The axis RhoA/ROCK inhibits ERK kinases activity during embryonic myogenesis A) Pull-down assay on lysates of freshly isolated myoblasts at E12.5, E14.5 and E16.5. The precipitated active Rho GTPases were detected by Western Blot (upper panel) and the amount of input was shown in the lower panel. ß-tubulin was used to normalize the total amount of loaded input. **B)** Quantitative densitometry of active Rho GTPases normalized on the ratio between the total amount of Rho GTPases and ß-tubulin. **C)** Western Blot analysis of proteins isolated from E11.5 to E17.5 of prenatal muscles, showing ROCK activity through the detection of MYPT1 known to be phosphorylated at Thr696 by ROCK. The total MYPT1 (Tot-MYPT1) and Vinculin were chosen to normalize the amount of loaded proteins. **D)** Western Blot analysis of proteins isolated from E11.5 to E17.5 of prenatal muscles for phosphorylated ERK (pERK) and total ERK (Tot-ERK). GAPDH was used to normalize the total amount of loaded proteins. **E)** Western Blot analysis on unpurified embryonic (E12.5) myoblasts treated daily with ROCK inhibitor (Y27632) or vehicle (control), showing an increased phosphorylation of ERK kinases upon ROCK inhibition (left panel). In the right panel the Western Blot was performed on unpurified fetal myoblasts transduced with a lentiviral vector expressing the constitutively activated form of RhoA (RhoV14) or a control vector (PGK-GFP), showing a decrease in ERK phosphorylation in RhoV14 fetal differentiated myoblasts compared with the control cells. **F)** Quantitative densitometry of phosphorylated ERK kinases normalized on the ratio between the total amount of ERK and Vinculin. Vinculin was used to normalize the total amount of loaded proteins.

The ERK kinases act downstream the RhoA/ROCK pathway in regulating the fetal myogenesis

To understand if the RhoA/ROCK axis plays a role in the regulation of JunB and Nfix, we analysed the effects of the ROCK inhibitor Y27632 on Myf5$^{GFP\text{-}P/+}$ -purified embryonic myoblasts. The ROCK inhibition led to a statistically significant increase of both JunB and Nfix expression, while did not affect Myogenin and MyHC-emb expression (Figure 6A). As expected, purified embryonic myoblasts treated with Y27632 showed also a strong decrease in the expression of MyHC-I, whereas the fetal marker β-enolase (Eno3) was precociously expressed in embryonic treated myoblasts (Figure 6A). The precious expression of JunB and Nfix and the down-regulation of slow MyHC upon ROCK inhibition were evaluated also by Western Blot (Figure 6B). Conversely, Myf5$^{GFP\text{-}P/+}$-purified fetal myoblasts transduced with a lentiviral vector expressing the constitutively-activated form of RhoA (RHOV14) had a dramatic decrease of JunB and Nfix mRNA levels, while MyHC-I was highly expressed, instead of being repressed. Moreover, purified over-expressing RHOV14 fetal myoblasts were characterized by a decrease in the fetal marker β-enolase,

indicating that RHOV14-expressing fetal myoblasts acquire a more embryonic-like gene transcription profile (Figure 6C). Western Blot analysis confirmed that the two fetal transcription factors analyzed, JunB and Nfix, were strongly down-regulated in RHOV14 fetal myoblasts, whereas slow MyHC was robustly induced (Figure 6D). Considering that RhoA and ROCK negatively affect the activation of ERK, we wondered whether the ERK kinases would regulate JunB and Nfix expression. For this reason, we treated unpurifed fetal myoblasts with ERK antagonist, PD98059, and we examine by Western Blot both JunB and Nfix protein levels. The immunoblot in Figure 6E reveals that the expression of JunB and Nfix were substantially reduced in PD98059-treated fetal myoblasts, indicating that ERK kinases positively regulate fetal myogenesis, through the activation of JunB and Nfix. Thereafter, we examined whether ERK are the downstream module of RhoA/ROCK signaling during myogenesis. As shown in Figure 6F, ROCK inhibition in embryonic myoblasts enhanced ERK phosphoylation and activation leading to the consequent up-regulation of JunB and Nfix, but the co-incubation of Y27632 and PD98059 led to a strong reduction of JunB and Nfix, that were not expressed as in control embryonic myoblasts. This result indicates that ERK kinases are downstream effectors of RhoA/ROCK during prenatal myogenesis and that ERK activity is strikingly necessary for the activation of JunB and Nfix.


A
Relative mRNA expression
10.0
7.5
5.0
2
1
0
Control
Y27632
***
***
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**
JunB
Nfix
MyHC-I
Eno3
Myogenin
MyHC-emb
B
E12.5:
Y27632 – +
JunB
Nfix
Vinculin
MyHC-I
MyHCs
Vinculin
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Relative mRNA expression
30
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PGK-GFP
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E12.5:
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PD98059 – + – +
JunB
Nfix
pERK
Tot-ERK
Vinculin

Figure 6. RhoA and ROCK negatively regulate fetal myogenesis by inhibiting JunB and Nfix while ERK activity promotes their expression A) qRT-PCR on Myf5$^{GFP-P/+}$-purified embryonic myoblasts treated with ROCK inhibitor (Y2763) or vehicle, analyzing the expression of embryonic and fetal markers following ROCK inhibition. **B)** Western Blot analysis on unpurified embryonic myoblasts upon ROCK inhibition or in control myoblasts, showing an increased expression of both JunB and Nfix and a consequent down-regulaiton of MyHC-I. MyHCs (MF20) and Vinculin were used to normalize the total amount of loaded proteins. **C)** qRT-PCR on Myf5$^{GFP-P/+}$-purified fetal myoblasts transduced with the constitutively activated RhoA (PGK-RhoV14) or control (PGK-GFP) lentivectors to check the expression of embryonic and fetal markers. **D)** Western Blot analysis on unpurified fetal myoblasts over-expressing the constitutively-activated form of RhoA (PGK-RhoV14) or control (PGK-GFP) to analyze the protein levels of JunB, Nfix and MyHC-I. MyHCs (MF20) and Vinculin were used to normalize the total amount of loaded proteins. **E)** Western Blot analysis on fetal myoblasts treated with ERK inhibitor (PD98059) or vehicle, showing the inhibition of JunB and Nfix expression after treatment with PD98059. Vinculin was used to normalize the total amount of loaded proteins. **F)** Western Blot analysis on unpurified embryonic myoblasts co-treated with ROCK inhibitor (Y27632) and ERK inhibitor (PD98059) using as a control not treated myoblasts and cells treated with the singular inhibitor. The co-treatment blocks the up-regulation of JunB and Nfix, observed in the myoblasts treated only with ROCK inhibitor.

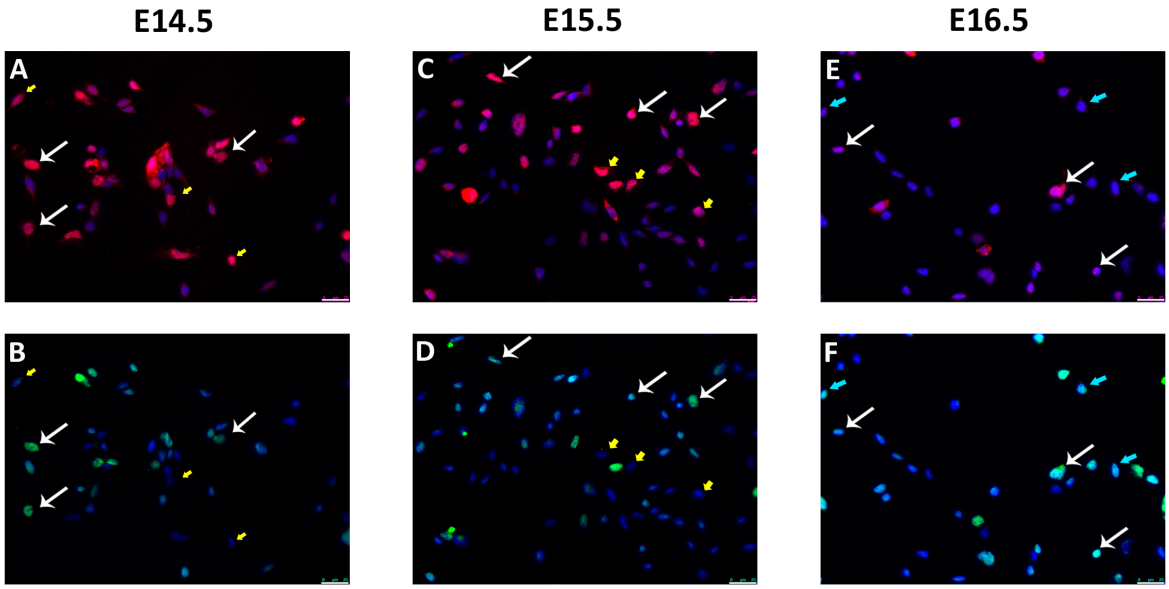
Discussion

Prenatal skeletal muscle development is achieved in two distinct phases to fulfil the different needs of a developing embryo (Biressi et al., 2007a; Stockdale, 1992; Tajbakhsh and Buckingham, 2000). During the embryonic stage, embryonic myoblasts participate to the formation of primary slow-twitching fibers, which form the first architecture of skeletal muscles, while, during the fetal stage, fetal myoblasts differentiate in secondary fast-twitching fibers. Embryonic and fetal myoblasts are heterogeneous populations of muscle progenitors, which differ in term of morphology, response to growth factors and gene expression, being they intrinsically different (Biressi et al., 2007b). To allow the complete maturation of prenatal skeletal muscles a temporal switch from embryonic to fetal myogenesis is needed. This transition is accompanied by changes in gene expression; indeed embryonic-derived fibers express high level of MyHC-I and MyHC-emb, while fetal-derived fibers are negative for MyHC-I and MyHC-emb but express glycolytic enzymes, such as β-enolase, and fast-twitching isoforms of MyHC, as the neonatal MyHC (Schiaffino et al., 1986). Nowadays, there have been some advances in understanding the regulation of embryonic to fetal transition, and we know that the gene expression program, that establishes fetal myogenesis, is mainly controlled by Nfix (Messina et al., 2010). It has been demonstrated that Nfix, is specifically expressed during fetal myogenesis, when it drives the activation of the fetal genetic program (Messina et al., 2010; Taglietti et al., 2016). Intriguingly, the second transcription factor specifically expressed during fetal development is JunB (Biressi et al., 2007b), which plays a crucial role in the maintenance of postnatal muscle physiology (Raffaello et al., 2010). Our finding that JunB and Nfix are co-expressed in fetal progenitors and that JunB target Nfix expression indicate that JunB is a direct activator of Nfix at the beginning of fetal myogenesis. Indeed, the silencing of JunB in fetal myoblasts is associated with the acquisition of an embryonic phenotype, which is completely rescued with the over-expression of Nfix. Our data demonstrate that the fetal genetic program is fully governed by Nfix, being the switch of the two phases of prenatal muscle development strikingly dependent on it. Thus, we demonstrate that JunB alone is not able to regulate the transition from embryonic to fetal myogenesis. Interestingly, in adult muscle, the lack of JunB results in atrophic myofibers, because of its inhibitory effect on *myostatin* expression (Raffaello et al., 2010), and the same phenotype were observed modulating Nfix expression (Rossi et al., 2016). Indeed, in postnatal skeletal muscles, Nfix-deficiency causes a reduction in the mean cross-sectional area of myofibers and in a reduction of Myostatin expression (Rossi et al., 2016). All these

observations suggest that JunB may exert its functions in adult skeletal muscles through the activation of Nfix. Starting from the consideration that both JunB and Nfix are necessary for the maintenance of adult skeletal muscle mass, to deeper elucidate the signalling signalling involved in the temporal regulation of muscle development progression, we focused our attention on RhoA GTPases and on ERK kinases. Indeed, both RhoA GTPases and ERK kinases have been suggested to impact on myofiber size, but, while the inhibition of RhoA signalling leads to increased fiber size (Coque et al., 2014), the inhibition of ERK cascade elicits in muscle atrophy, with a reduction in fiber calibre (Haddad & Adams, 2004; Shi et al., 2009). Interestingly, it was also reported that RhoA exerts its cellular effects through the activation of Rho-Kinases, ROCK (Schofield and Bernard, 2013), which in turn negatively regulates ERK activity (Li et al., 2013; Khatiwala et al., 2009). Although the relationship between the activity of RhoA and ERK kinases was not characterized in prenatal skeletal muscle development, we speculate that their activity may be involved in the control of JunB and Nfix expression. Time course experiments reveal a pronounced enrichment of RhoA and ROCK activity specifically during embryonic myogenesis, while ERK kinases are activated only during fetal myogenesis. Interestingly, we demonstrate that RhoA/ROCK pathway negatively controls the ERK function, which is suppressed when the RhoA/ROCK signaling is activated. Our data highlight the importance of RhoA/ROCK/ERK axis during skeletal myogenesis, and this let us to study whether JunB and Nfix are affected by RhoA/ROCK/ERK signaling. We show that the RhoA/ROCK pathway represses JunB and Nfix expression, leading to a change in the transcriptional profile of muscle progenitors. In particular, the constitutive activation of RhoA in fetal myoblasts causes a persistence of the embryonic marker MyHC-I, due to the down-regulation of JunB and Nfix. Conversely, the inhibition of ROCK in embryonic myoblasts provokes an up-regulation of JunB and Nfix, which correlates with a down-regulation of MyHC-I and a precocious expression of β-enolase. Based on these finding, we decide to deeper investigate also the role of ERK kinases during fetal myogenesis, revealing their crucial role in promoting the fetal program through the positive regulation of JunB and Nfix. These results display that the RhoA/ROCK/ERK axis constitutes, at least, one of the major signaling pathway that regulates the temporal progression of prenatal myogenesis. However, it remains unknown the upstream inputs, which orchestrate the activation or inhibition of these signaling pathway. Understanding how myogenic progenitors are instructed during skeletal myogenesis will be crucial for the comprehension of the mechanisms involved in the initial specification of the two

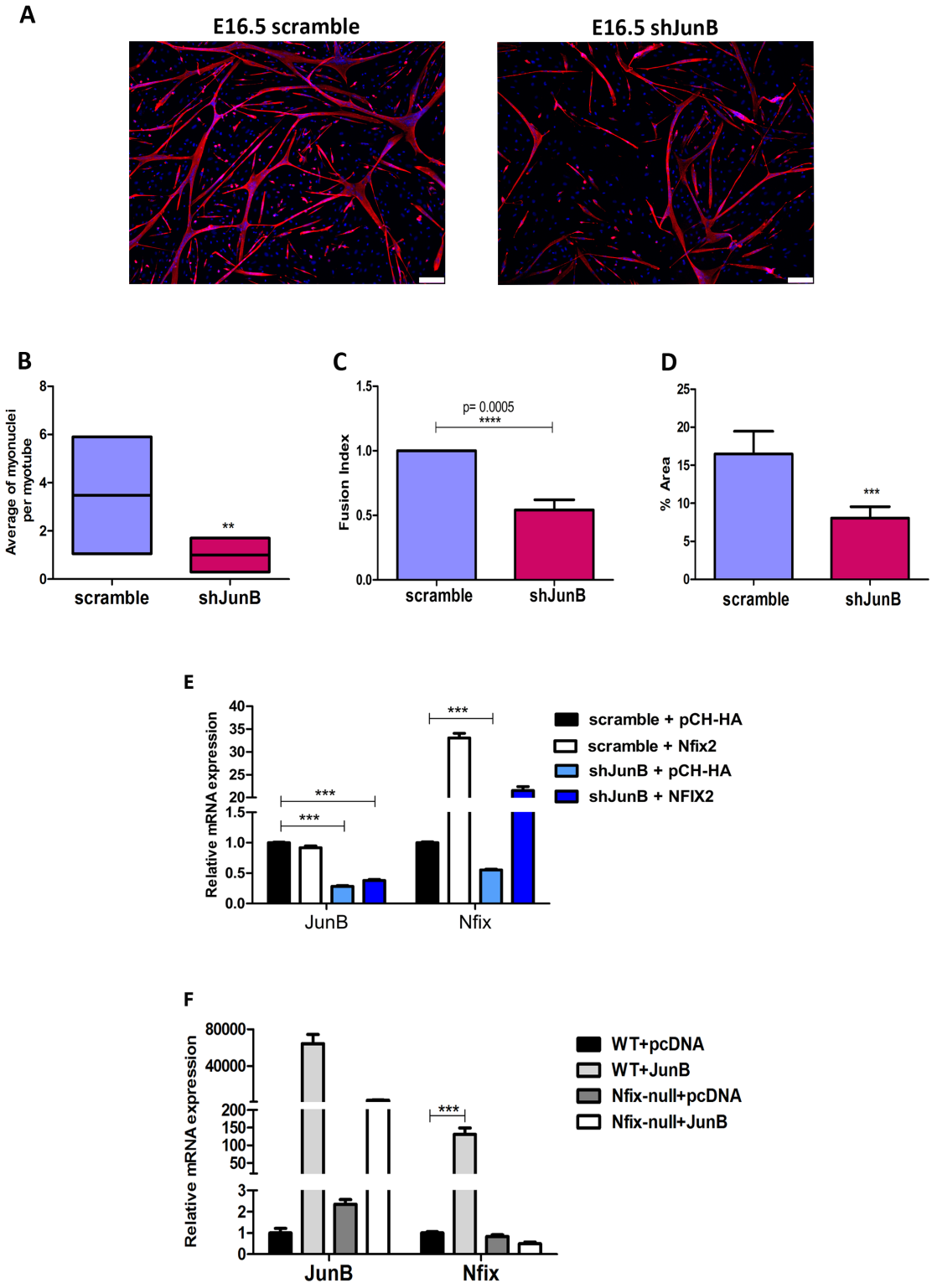
populations of myoblasts and in the achievement of their respective identities. In conclusion, we have identified the RhoA/ROCK pathway as an important regulator of embryonic myogenesis, that maintains JunB and Nfix repressed through the inhibition of ERK activity. With the progression of prenatal development, the RhoA/ROCK signaling is progressively decreased, allowing the activation of ERK kinases, which are necessary for JunB and Nfix expression. Finally, we demonstrate that the transition from embryonic to fetal is strikingly dependent on Nfix, whose expression is directly regulated by JunB. Identification of these signaling pathways may contribute to augment the knowledge of the mechanisms regulating the process of skeletal myogenesis.

Supplemental Figure



Supplemental Figure S1. JunB and Nfix are co-expressed in fetal myoblasts, with JunB earlier expressed than Nfix

A-D) Immunofluorescence for JunB (red) and Nfix (green) on freshly isolated Myf5$^{GFP-P/+}$-purified myoblasts at E14.5. Nuclei were counterstained with Hoechst. White arrows indicate myoblasts coexpressing JunB and Nfix, while yellow arrows indicate myoblasts expressing JunB but not Nfix at E14.5 **(A-B)** and at E15.5 **(C-D)**. Scale bars: 25 µm. **E-F)** Immunofluorescence for JunB (red) and Nfix (green) on freshly isolated Myf5$^{GFP-P/+}$-purified myoblasts at E16.5. Nuclei were counterstained with Hoechst. White arrows indicate myoblasts coexpressing JunB and Nfix, while light blue arrows indicate myoblasts expressing Nfix but not JunB. Scale bars: 25 µm.



Supplemental Figure S2. Silencing of JunB impairs the differentiation of fetal myotubes

A) Immunofluorescence for total MyHCs (MF20) on differentiated Myf5$^{GFP-P/+}$-purified fetal myoblasts transduced with a lentiviral vector expressing small hairpin RNA for the silencing of JunB (shJunB) or with

the non-targeting control vector (scramble). The shJunB-expressing fetal myotubes acquire the typical aspect of embryonic myotubes. **B)** The fusion ability was quantified by counting the number of nuclei for each terminally differentiated myotubes. **p<0.01. **C)** Calculation of the fusion index as the ratio of the number of nuclei inside myotubes to the number of total nuclei. ****p<0.0005. **D)** Quantification of MyHCs positive area (MF20) using image analysis software. ***p<0.005. **E)** qRT-PCR for JunB and Nfix on fetal myoblasts (E16.5) following the transduction with scramble or shJunB vector and with control or Nfix2-over-expressing vector. ***p<0.005; N=3. **F)** qRT-PCR on wild-type (WT) and *Nfix*-null embryonic myoblats (E12.5), transfected with JunB-overexpressing vector or with a control vector (pcDNA). ***p<0.005; N=3.

Material and methods

Mutant Animals

Myf5$^{GFP-P/+}$ (Kassar-Duchossoy et al., 2004), Tg:MLC1f-*Nfix*2 (Messina et al., 2010), *Nfix*-null, obtained from Prof. Richard M. Gronostajski (University of Buffalo) (Driller et al., 2007), and wild-type CD1 mice (Charles River) were used and their genotyping strategies have been published in the references. Female mice were mated with males (2:1) and examined every morning for copulatory plugs. The day on which a vaginal plug was found was designated as 0.5 gestation day. Mice were kept in pathogen-free conditions and all procedures were conformed to Italian law (D. Lgs n 2014/26, implementation of the 2010/63/UE) and approved by the University of Milan Animal Welfare Body and by the Italian Ministry of Health.

Cell isolation and Cell Culture

Myf5$^{GFP-P/+}$ embryonic muscles, isolated at E12.5, or fetal muscles, isolated at E16.5, were mechanically and enzimatically digested for 30 min at 37°C in agitation with 1.5 mg/ml of Dispase (Gibco), 0.15 mg/ml of Collagenase (Sigma), and 0.1 mg/ml Dnase I (Sigma) as previously described (Biressi et al., 2007b). Dissociated cells were filtrated and collected in DMEM high-glucose (EuroClone), 20% fetal bovine serum (FBS, EuroClone), 2mM EDTA and 20mM HEPES. The GFP-positive myoblasts were sorted using Sorted BD FACSAria and cultured in 20% horse serum (HS) medium composed by DMEM high-glucose (EuroClone), 20% HS (EuroClone), 2mM L-Glutamine (Sigma-Aldrich) and 1% Penicillin/Streptomycin (Euroclone). Unpurifed embryonic and fetal myoblasts were obtained using the same enzymatic and mechanic procedure utilized for Myf5$^{GFP-P/+}$-sorted myoblasts, but after the digestions the obtained cells were pre-plated on plastic dish to allow the attachment of fibroblasts. The non-adherent cells were collected and incubated at 37°C in 20% HS. The differentiation was induced decreasing the serum from 20% to 2% and maintaining the cells in 2% HS for 48h or 72h after the medium changing. Embryonic myoblasts were treated daily with 10 μg/μL of ROCK inhibitor Y27632 (Calbiochem). Fetal myoblasts were treated over night (O/N) with 50 mM of ERK antagonist PD98059 (Cell Signaling).

Plasmids and lentivirus production

The following plasmids were used: pCH-Nfix2, pCH-HA (Messina et al., 2010), pLentiHA-NfiEngr, pLentiHA-Engr (Messina et al., 2010), scramble (Sigma-Aldrich) and shJunB plasmids

(SHCLNG-NM_008416, Sigma-Aldrich), PGK-RHOV14 and pcDNA3X-JunB or pcDNA3X, as control. pcDNA3X-JunB plasmid was obtained by subcloning the JunB cDNA (kindly provided by Milena Grossi, University of Rome) in the pcDNA3X vector (ClonTech), whereas PGK-RHOV14 has been produced by cloning the cDNA of RhoA with a single point replacement of glycine with valine at positions 14, RHOV14 (kindly provided by Germana Falcone, CNR Rome), in PGK-GFP vector.

Viral particles were prepared through the co-trasfection of the packaging plasmids pMDLg/p (16.25µg), pCMV-VSVG (9µg) and pRSV-REV (6.25µg) together with each of the following lentiplasmids: shJunB, pLentiHA-Nfix2, PGK-RHOV14 and the respective controls, as scramble, pLentiHA and PGK. Transfection was performed in HEK293T cells by using Calcium Phosphate Transfection method. Viral particles were collected 40h after transfection and concentrated with Lenti-X Concentrator (CloneTech) in PBS 1X. The concentrated viral particles were stored at -80°C until use.

Cell transfection and transduction

For transfection experiments, embryonic or fetal myoblasts were cultures at a confluency of 70%-80% and transfected following the Lipofectamine™ LTX (Invitrogen) transfection protocol. Myoblasts were harvested 48 h after transfection. Transduction of fetal myoblasts was performed by addition of the viral preparation to the cultured cells at the multiplicity of infection (MOI) of 10. After O/N incubation, the medium was changed and the cells were maintained in culture for 72 h after the medium changing, to allow their differentiation.

Immunofluorescence on cultured cells

Cell cultures were fixed for 10 min at 4°C with 4% paraformaldehyde in PBS 1X and then were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich), 1% BSA (Sigma-Aldrich) in PBS 1X for 30 min at room temperature (RT). After the permeabilization, the samples were treated with a blocking solution composed by 10% goat serum (Sigma-Aldrich) for 45 min at RT and incubated O/N at 4°C with primary antibody diluted in PBS. The primary antibodies used are: rabbit anti-Nfix (1:200, Novus Biologicals) and mouse anti-JunB (1:100, SantaCruz Biotechnology). After two washes with PBS, 1% BSA and 0.2%Triton the samples were incubated for 45 min at RT with secondary antibodies (1:250, Jackson Laboratory) and Hoechst (1:500, Sigma-Aldrich). Finally the

cells were washed twice with 0.2% Triton in PBS and mounted with Fluorescence Mounting Medium (Dako). Images were acquired with Leica-DMI6000B fluorescence microscope equipped with Leica DFC365FX camera. And images were merges with Photoshop.

RNA extraction, Retrotranscripion and Real-Time qPCR

NucleoSpin kits RNA XS (Macherey-Nagel) was used for the extraction of total RNA from cultured cells or freshly isolated myoblasts. After quantification of RNA with a NanoPhotometer (Implen), 0.5 µg of total RNA was retrotranscribed with iScript Reverse Transcription Supermix (Bio-Rad). The obtained cDNA was diluted 1:10 in S.A.L.F. water and 5 µl of diluted cDNA was used for the Real-Time qPCR. Real-Time qPCR was performed using SYBR Green Supermix (Biorad) and the primers are listed in Table S1. Relative mRNA expression levels were normalized on GAPDH expression levels.

Protein extraction and Wester Blot

Protein extracts were obtained from cultured myoblasts lysed with RIPA buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 1% Triton-X, 0.1% sodium deoxycholate, 0.1% SDS, 150mM NaCl in deionized water) for 30 min on ice, while total protein extracts from embryonic and fetal muscles were obtained from homogenized tissues in Tissue extraction Buffer (50mM Tris-HCl, 1mM EDTA, 1% Triton-X, 150mM NaCl). Both RIPA and Tissue extraction Buffer were supplemented with protease and phosphatase inhibitors. After lysis, samples were centrifugated at 4°C for 10 min at 10000 rpm and the supernatants were collected and quantified using the DC Protein Assay (Bio-Rad). For the Western Blot assay 30µg of protein extract were denaturated at 95°C for 5 min with SDS Page Loading samble buffer (100mM Tris pH 6.8, 4% SDS, 0.2% Bromophenol blue, 20% Glycerol and 10mM dithiothreitol) and loaded on 8% SDS acrylamide gel. After electrophoresis running, proteins were blotted to a nitrocellulose membrane (Whatman, Protran Nitrocellulose Transfer Membrane), which was blocked for 1 h with 5% milk in Tris-buffered saline solution plus 0.02% Tween20 (Sigma-Aldrich). The primary antibodies were incubated O/N at 4°C in agitation using the following conditions: rabbit anti-Nfix (1:1000, Novus Biologicals), rabbit anti-JunB (1:500, SantaCruz Biotechnology), mouse anti-Vinculin (1:2500, Sigma-Aldrich), mouse anti-slow MyHC (hybridoma Bad5, 1:2, DSHB); mouse anti-total MyHC (hybridoma MF20, 1:5, DSHB), rabbit anti-MYPT1 phosphorylated in thr696 (1:500, SantaCruz), rabbit anti-Tot MYPT1 (1:500, SantaCruz Biotechnology), rabbit anti-pERK (1:1000, SantaCruz Biotechnology), mouse anti-Tot

ERK (1:500, SantaCruz Biotechnology) and mouse anti-GAPDH (1:5000, Sigma-Aldrich). Blots after primary antibody incubation were washed and incubated with secondary antibodies (1:10000, IgG-HRP, Bio-Rad) for 40 min at RT and washed again. The bands were revealed using ECL detection reagent (ThermoFisher) and images were acquired using the ChemiDoc MP System (Biorad).

Chormatin Immunoprecipitation (ChIP) assay

The ChIP protocol was performed on unpurified fetal differentiated myoblasts (E16.5) using $5x10^6$ cells for each immunoprecipitation. Fetal myotubes were fixed with 1% formaldehyde (Sigma-Aldrich) in DMEM high-glucose for 10 min at RT. The fixation was quenched with 125mM Glycine (Sigma-Aldrich) in PBS 1X for 10 min at RT. The cells were rinsed with cold PBS, harvested and centrifugated for 10 min at 2500 rpm at 4°. The cell pellet was lysated and sonicated with a Bioruptor Sonicator (Diagenode) for 15 min, with repeated cycles of 30 sec of sonication and 30 sec of resting. The sonicated suspension was centrifugated for 10 min at 14000rmp at 4°C and the supernatant was stored in aliquots at -80°C. Chromatin was precleared with Protein G Sapharose (Amersham) and Rabbit Serum for 2 h at 4°C on a rotating platform, while Protein G Sapharose was blocked O/N with BSA (10mg/ml) and Salmon Sperm (1 mg/ml) (Sigma-Aldrich). After preclearance, chromantin was incubated O/N at 4°C with 5 μg of antibody: rabbit anti-Nfix (Novus Biologicals), mouse anti-JunB (SantaCruz Biotechnology) and normal rabbit IgG (SantaCruz Biotechnology). The day after blocked Protein G Sapharose was washed and added to the chromatin incubated with the antibodies for 3 h in rotation at 4°C. After incubation, the Protein G Sapharose was spun down and repeatedly washed. The elution was performed O/N at 65°C with 10 mg RNase (Sigma-Aldrich) and 200 mM NaCl (Sigma-Aldrich) to reverse crosslink. The DNA was purified with phenol/chloroform, after treatment with 20 μg of Proteinase K (Sigma-Aldrich). Obtained DNA was analyzed by Real-time qPCR and results were plotted as fold enrichment with respect to the IgG sample. Primers used are listed in Table S2.

Pull-Down assay

Active Rho Pull-Down and Detection kit (ThermoScientific) was performed on 600 μg of cell lysate obtained from unpurified myoblasts (E12.5, E14.5, E16.5), following the manufacturer's instructions.

Table S1:

Gene	Primers sequence	Reference
Nfix	F: 5'-CACTGGGGCGACTTGTAGAG-3' R: 5'-AGGCTGACAAGGTGTGGC-3'	Mourikis et al. 2012
JunB	F: 5'- CCTGTGTCCCCCATCAACAT-3' R: 5'- CAGCCTTGAGTGTCTTCACCT-3'	Mathew et al. 2011
MyHC-I (Myh7)	F: 5'-AGGGCGACCTCAACGAGAT-3' R: 5'-CAGCAGACTCTGGAGGCTCTT-3'	Mathew et al. 2011
MyHC-emb (Myh3)	F: 5'-GCAAAGACCCGTGACTTCACCTCTAG-3' R: 5'-GCATGTGGAAAAGTGATACGTGG-3'	Mathew et al. 2011
Eno3	F: 5'-TTCTACCGCAACGGCAAGTA -3' R: 5'-GACCTTCAGGAGCAGGCAAT-3'	
Myogenin	F: 5'-CTGGGGACCCCTGAGCATTG-3' R: 5'-ATCGCGCTCCTCCTGGTTGA -3'	
RhoA	F: 5'- AGCTTGTGGTAAGACATGCTTG-3' R: 5'- GTGTCCCATAAAGCCAACTCTAC-3'	
Gapdh	F: 5'-GGCATGGACTGTGGTCATGA-3' R: 5'-TTCACCACCATGGAGAAGGC-3'	

Table S2:

Gene	Primers sequence	Reference
MyHC-2b promoter (-1000bp)	F: 5'-GAGGTCCGTAGTCAGTCTCTTTT-3' R: 5'-TACCCCAAGTGTTAGGCTCA-3'	Raffaello et al., 2010

Nfix promoter (-1400bp)	F: 5'- ACACTAGGATTGAGGAAGACTTAGA-3' R: 5'- CAAGGCCTTCTGGGGCTC-3'	
Nfix promoter (-200bp)	F: 5'-TTGAGAATCCACCCAAGCCC-3' R: 5'-AAGCCAACGCCTGATTCTGA-3'	
Nfix promoter (-1000bp)	F: 5'-CAAAGAGGCATCCACTTGCAG-3' R: 5'-GTTTTTGGTVTCCAACTGCCG -3'	
Intergenic	F: 5'- TCGGACCGGAGTGTTAGGAA -3' R: 5'- ACCCTGGAGTCTCAGCATCG -3'	An et al. 2011

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