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Scuola di Dottorato in Scienze Biologiche e Molecolari

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# Functional cooperation of Sox6 and Nfix regulates fiber type specification during pre-natal muscle development

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*Index*

# ABSTRACT

During muscle development, embryonic and fetal myoblasts in turn differentiate to form slow-twitch and fast-twitch fibers, respectively. While consistent data have been provided on the mechanisms involved in adult fiber remodeling, the molecular regulation of slow/fast specification during embryogenesis is still unclear. Previous work from our laboratory identified nuclear factor I X (Nfix) as a key regulator of fetal myogenesis. Nfix acts as an activator of several fetal-specific genes and at the same time as a repressor of embryonic genes (such as the slow myosin heavy chain isoform MyHC-I). The aim of my project was to better define the possible co-factors involved in Nfix function during muscle pre-natal development. Here we show that Sox6, a direct repressor of the slow-twitch phenotype, cooperates with Nfix in fetal muscle to silence MyHC-I expression at the transcriptional level. Sox6 and Nfix are co-expressed in fetal myoblasts and co-immunoprecipitate in muscle cells, suggesting contribute of both to a repressive complex acting during fetal myogenesis. By ChIP assay, we show that Nfix is required for the binding of Sox6 to the *MyHC-I* proximal promoter during muscle development. Moreover, we show that Sox6 is absolutely necessary for normal MyHC-I regulation. Importantly, Sox6 acts as an activator of MyHC-I rather than a repressor during embryonic myogenesis. Interestingly, we show that the zebrafish orthologs Nfixa and Sox6 cooperate in repressing the slow-twitch phenotype during zebrafish developmental myogenesis. These data demonstrate the evolutionary conserved cooperation of Sox6 and Nfix in the repression of embryonic muscle genes, pointing out to the importance of a synergy among transcriptional factors in the achievement of adult skeletal muscle phenotype.

# STATE OF THE ART

## 1. Skeletal muscle organization and function

### *1.1 Characteristics of skeletal muscle*

Skeletal striated muscle is the most abundant tissue in the body and includes trunk and appendicular muscles, extra-ocular muscles, jaw and tongue muscles, laryngeal muscles and diaphragm. The fundamental functions of skeletal muscle are related to body mobility and posture maintenance, but it also plays an important role in whole body metabolic health.

Skeletal muscle is composed of elongated, multinuclear cells that are called muscle fibers (or myofibers) and represent the contractile cells of the tissue. Myofibers of different muscles have variable diameter (from 10 to 100 μm) and length (from 1 mm to several cm). The myofiber plasma membrane (sarcolemma) entirely surrounds the cytoplasm (sarcoplasm). Most of the sarcoplasm is occupied by myofibrils, which represent the contractile machinery of the fiber. Each myofibril measures 1-2 μm in diameter and spans along the entire length of the fiber. The other cell organelles, such as myonuclei, mitochondria and the sarcoplasmic reticulum (SR) are located in a peripheral position around myofibrils.

Muscle fibers run in parallel within the same muscle and are surrounded by a delicate net of connective tissue (endomysium) that contains blood capillaries, nerve endings and a basal membrane, which is in direct contact with the sarcolemma of each fiber (see Figure 1.1). The connective tissue also forms thicker layers that separate different fiber fascicles (perimysium), and an outer layer covering the muscle surface (epimysium). The connective layers have not just a role of containment, but are fundamental for the transmission of muscular force (Borg and Caulfield, 1980; Purslow and Trotter, 1992). At their extremities, muscle fibers are connected to the tendons by myotendinous junctions that enable a strong connection between the muscle fiber actin filaments and the tendon collagen fibrils (Huijing, 1999). The force generated by muscle is thus transferred via the tendons to bones (trunk, appendicular

and jaw muscles) or to connective tissue (mimic and extra-ocular muscles, diaphragm).

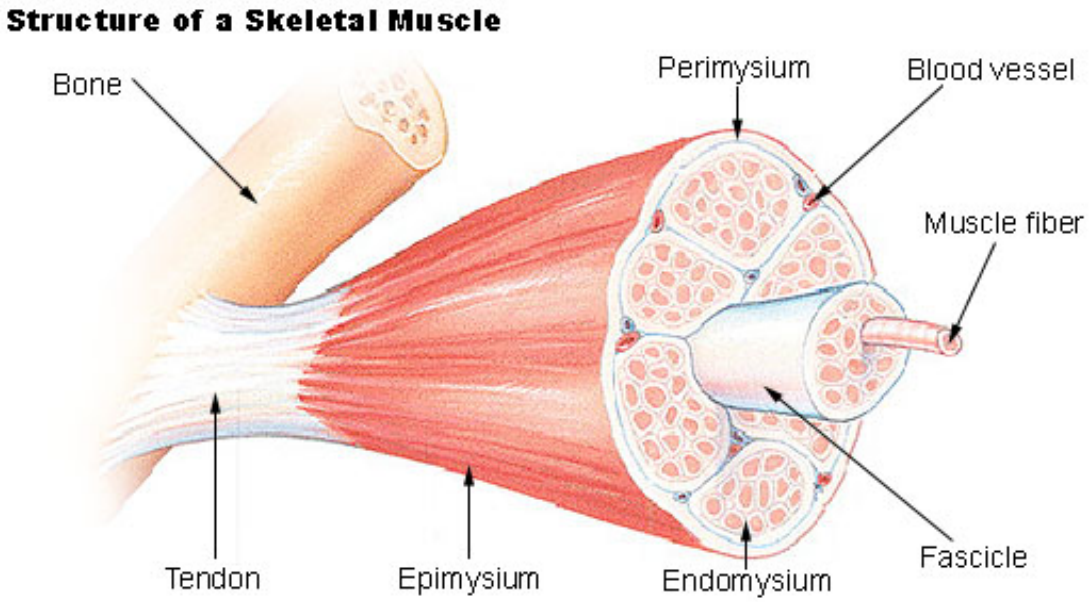


**Figure 1.1. Skeletal muscle structure.** Skeletal muscle is organized in fiber fascicles that are surrounded by septa of connective tissue called perimysium. The connective tissue also forms the endomysium and the epimysium. Skeletal muscle fibers are directly connected to the tendon, which allows transmission of the force generated by muscle to the bone levers. Adapted from: Gray's Anatomy of the Human Body, 2007.

## *1.2 Muscle contraction*

Myofibrils are composed of contractile units called sarcomeres which are connected in series and in parallel to form the typical striated pattern of skeletal muscle with alternating dark and light bands (see Figure 1.2). Sarcomeres are formed by several types of proteins: in particular, the interaction between thick myosin filaments and thin actin filaments is fundamental for muscle contraction. Each myosin molecule is composed of two heavy chains (MyHC) and four light chains (MyLC) arranged to form a long tail and a globular head. The myosin head is the motor domain of the myosin molecule, since it contains both the ATP- and the actin-binding domains that are required for hydrolyzing ATP and generating force. The tail of the molecule is formed by a long α-helix that allows dimerization of the heavy chain. In the thick

filament, each myosin molecule is oriented with the head projecting towards the thin filaments.

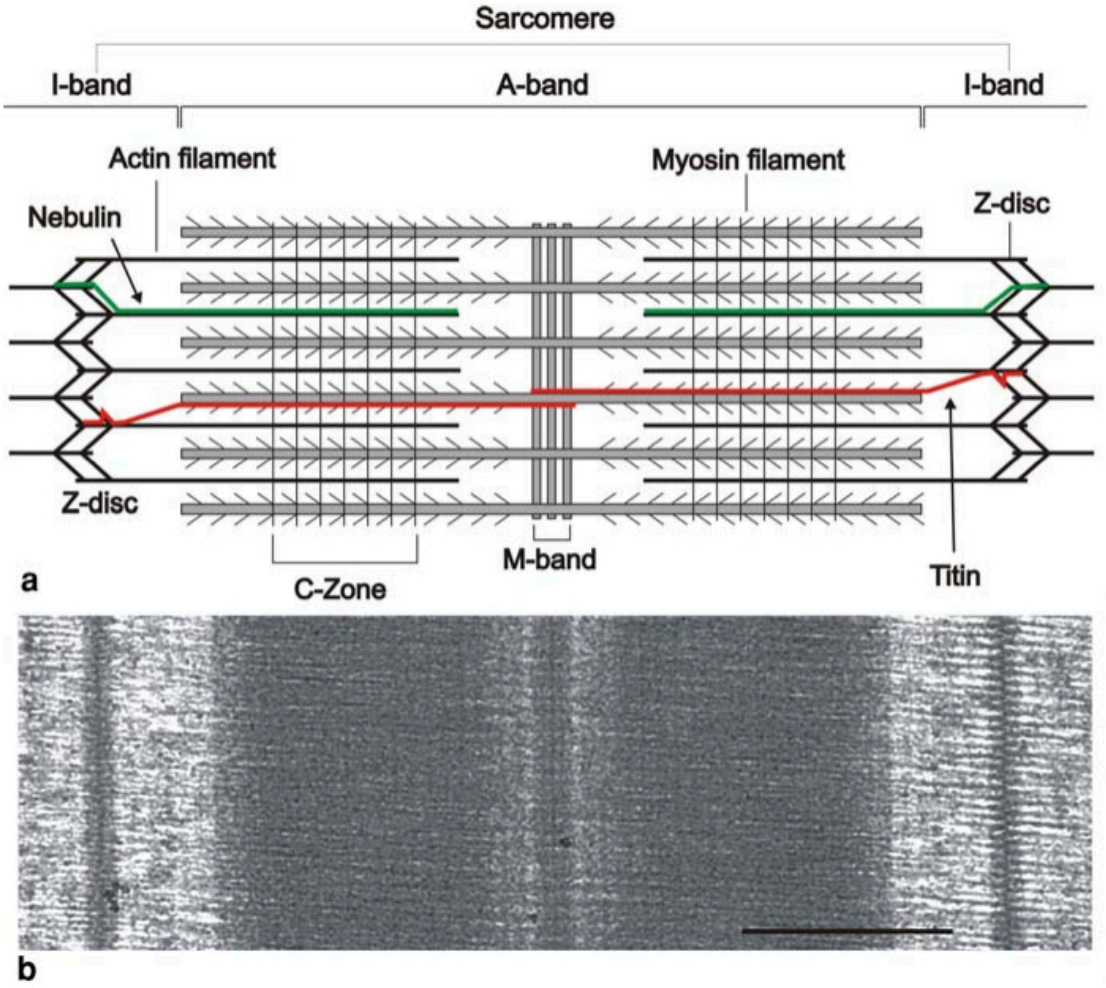


**Figure 1.2. Sarcomere structure.** a) Diagram showing the main components of the sarcomere. The A-band (anisotrope, dark) comprises thick myosin filaments that are linked at the center by the M-band assembly. The I-band (isotrope, light) comprises thin actin filaments that are tethered at the Z-disks and interdigitate with myosin filaments in the A-band. Nebulin and titin are giant proteins that contribute to the structure of the Z-disk. b) Electron micrograph of a longitudinal section of fish white (fast) muscle showing the details of the sarcomere. Scale bar: 500 nm. Adapted from: Luther, 2009.

During contraction, the thick filaments pull the thin filaments towards the center of the sarcomere, thus shortening the length of the entire muscle fiber. At rest, regulatory proteins located on the thin filament prevent the interaction between myosin and actin: tropomyosin is a coiled-coil protein that covers the binding sites for myosin along the actin filament and is associated to a troponin protein complex. Each

troponin molecule is formed by three different subunits: TnC, TnI and TnT. Troponin control of tropomyosin is regulated by sarcoplasmic calcium ($Ca^{2+}$) concentration. During excitation-contraction coupling in skeletal muscle, $Ca^{2+}$ ions are released into the sarcoplasm by the SR in response to depolarization of the sarcolemma. In presence of high $Ca^{2+}$ concentrations, the TnC subunit of troponin binds to a total of four $Ca^{2+}$ ions, thus allowing a conformational change of the tropomyosin molecule that uncovers the myosin binding sites on the actin filament and allows the sliding of the two filaments (Baylor & Hollingworth, 2011). The process of sarcomere contraction is dependent on ATP, which is hydrolyzed by the myosin head and releases the energy necessary for myosin head oscillation and filaments sliding (Huxley, 2004).

Skeletal muscle contracts in response to a voluntary stimulus. Muscle fibers are stimulated to contract by the neurotransmitter acetylcholine (ACh) that is released at the ending of a somatic motor neuron in response to a train of action potentials (Fambrough 1979). Except during development, each muscle fiber is contacted by a single motor neuron: the ensemble of muscle fibers innervated by one neuron is called muscle unit, while that ensemble together with its motor neuron is called motor unit. The neuromuscular junction (NMJ) represents the communicative link between neuron and muscle fiber: here the pre-synaptic axon directly contacts the sarcolemma, which forms invaginations called post-synaptic folds where the nicotinic receptors for ACh are present. After the release of ACh by exocytosis from the pre-synaptic terminal, there is a change in the permeability of the muscle fiber membrane and increased entrance of positive ions that leads to membrane depolarization and generation of the action potential. The wave of depolarization extends to the transverse tubules, deep invaginations of the sarcolemma that contain voltage-sensing $Ca^{2+}$ channels (dihydropyridine receptor, DHPR). DHPRs are functionally coupled to the $Ca^{2+}$ release channels of the SR (ryanodine receptor, RyR) in molecular arrangements called tetrads. Activation of DHPR leads to a large and fast release of $Ca^{2+}$ by the SR that triggers the contractile response of myofibrils. Calcium represents a powerful intracellular messenger in skeletal muscle fibers, being able not only to trigger contraction via binding to troponin, but also to activate protein phosphorylation or de-phosphorylation via activation of calmodulin and calcium-dependent kinases (Wood & Slater, 2001).

## *1.3 Functional heterogeneity of skeletal muscle*

Adult skeletal muscle is composed of different populations of fibers, presenting a wide range of physiological and biochemical differences. This heterogeneity enables the same muscle to respond to different functional demands: from continuous or time-prolonged actions (such as posture and locomotion) to fast and strong maximal contractions (such as swimming, sprinting, etc.). The first classification of muscles diversity was based on organoleptic properties such as color: slow-twitch muscles, characterized by oxidative metabolism, high myoglobin content and continuous activity, were generally defined as red muscle; in contrast fast-twitch muscles, characterized by glycolytic metabolism, low myoglobin content and phasic activity, were defined as white muscles (Needham, 1926).

A further step in the characterization of muscle fiber types took place with the identification and the cloning of different *MYH* genes that specify for distinct MyHC isoforms in mammals (Peter et al. 1972; Schiaffino et al. 1988; Weiss et al. 1999) (see Figure 1.3). The central role of myosin in muscle contraction and the differential expression of several MyHC isoforms in various fibers make MyHC the best marker for fiber typing. It was thus possible to distinguish four different adult fiber types in mouse on the basis of the MyHC isoforms expression: slow-twitch type I fibers, expressing the slow MyHC isoform (MyHC-I or MyHC-β, encoded by *MYH7*), and three distinct fast-twitch type II fibers (IIa, IIx and IIb), each one expressing one of the fast adult MyHC isoforms: MyHC-IIa, MyHC-IIx and MyHC-IIb (encoded by *MYH2*, *MYH1* and *MYH4*, respectively) (DeNardi et al. 1993; Chakkalakal et al. 2012). Different biochemical approaches demonstrated the presence of a spectrum of fibers with pure and hybrid composition in MyHC isoforms, according to the following scheme: I ↔ I/IIA ↔ IIA ↔ IIA/IIX ↔ IIX ↔ IIX/IIB ↔ IIB (Gorza, 1990; Bottinelli et al. 1994). The fiber types present differences in mechanical power (i.e., mechanical energy released per unit of time) and maximum velocity of shortening, which are intrinsically determined by the type of MyHC that is expressed in the fiber (Nyitrai et al. 2006). Both values increase in the following order: I<IIa<IIx<IIb, with IIb fibers exhibiting the highest values in all species and muscles examined up to now.

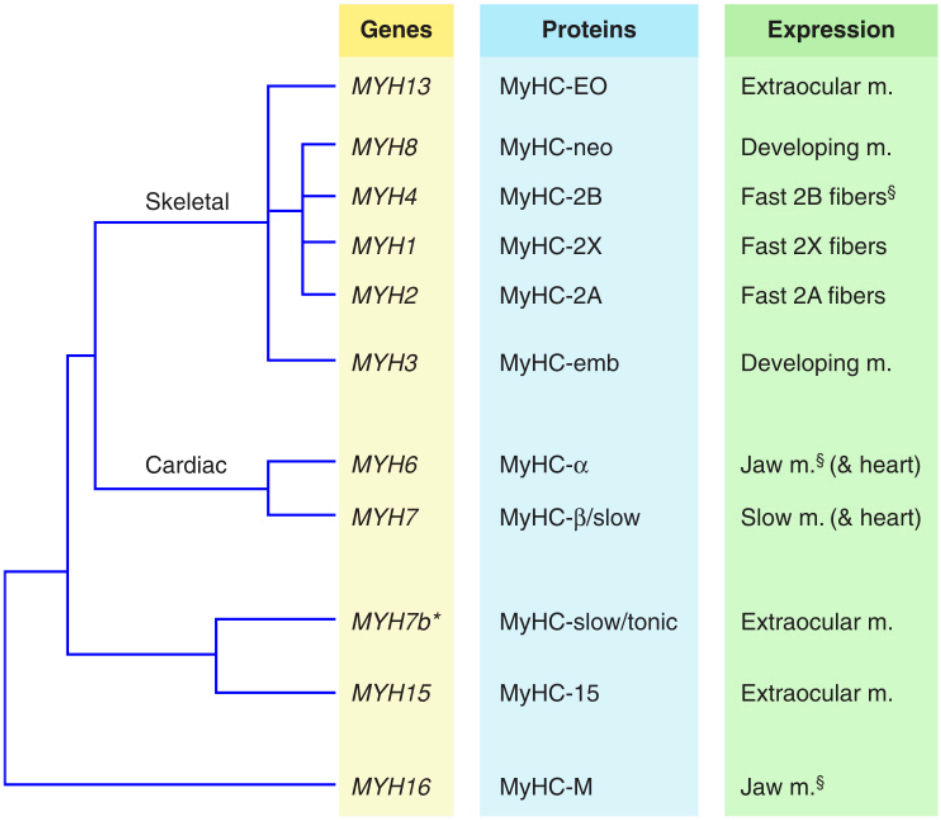


**Figure 1.3. Heterogeneity of mammalian MyHC isoforms.** Diagram showing the different mammalian *MYH* genes with the corresponding proteins and their expression pattern. On the left, a phylogenetic tree shows the relationships between the different *MYH* isoforms. §Expression limited to some mammalian species: in particular, the levels of MyHC-IIb vary between rodent and human muscles, the latter being virtually negative for MyHC-IIb. **MYH7b* is expressed in both cardiac and skeletal muscle at the transcript level, but only in extra-ocular muscles at the protein level. Adapted from: Schiaffino & Reggiani, 2011.

The different fiber types also exhibit distinct metabolic properties: type I fibers generate ATP by oxidative mitochondrial processes and have the ability to maintain contraction for long time without fatigue. Type II fibers have high content of glycolytic enzymes that generate ATP very rapidly but with limited duration of contraction (Nemeth et al. 1981; Park et al. 1987). However, type IIa fibers also possess the oxidative enzyme complement, and are therefore called fast oxidative glycolytic (FOG) fibers (Barnard et al. 1971).

Significant variations in the fiber types exist among different species. For example, in rodent muscles type IIx fibers have intermediate levels of the oxidative enzyme succinate dehydrogenase (SDH) and intermediate shortening velocity between IIa and IIb fibers, while in human muscles, where MyHC-IIb expression is not detectable, IIx

fibers have the lowest content of SDH and the highest shortening velocity of all fiber types (Smerdu et al. 1994). Specific MyHC isoforms are also expressed in developing and regenerating muscle, and in specific anatomical districts, such as extra-ocular and jaw muscles (Wieczorek et al. 1985; Rossi et al. 2010). In particular, MyHC-emb and MyHC-neo (encoded by *MYH3* and *MYH8*, respectively) are the most expressed during embryonic development, with a pattern that is recapitulated during muscle regeneration (Eusebi et al. 1986; Schiaffino et al. 1986; Whalen et al. 1990).

During adult life, the fiber type is strictly regulated by extrinsic signals such as muscle activity and hormones. The program of fiber type specification during development and adulthood will be further discussed in Section 3.

# 2. Skeletal muscle development and growth

## *2.1 Mechanisms of myogenic differentiation*

During embryonic development, myofibers are formed by fusion of myogenic progenitors called myoblasts that arise from pre-somitic mesoderm (PSM) (Christ & Ordahl, 1995). By contrast, some of the head and neck muscles are derived from anterior paraxial unsegmented mesoderm and prechordal mesoderm (Buckingham et al. 2003). Proliferative myoblasts withdraw from the cell cycle at different developmental stages and give rise to differentiated post-mitotic myocytes, which in turn fuse with each other to form multinucleated muscle fibers. The process of differentiation into the skeletal myogenic lineage is tightly regulated at the molecular level. A family of basic helix-loop-helix (bHLH) transcription factors named muscle regulatory factors (MRFs) is able to activate the program of muscle differentiation by initiating the transcription of regulatory and structural muscle specific genes. The MRF family includes *Myf5*, *MyoD*, *MRF4* and *myogenin*. Each of these transcription factors is able to myogenically convert non-muscle cells when ectopically expressed (Pinney et al. 1988; Choi et al. 1990; Liu et al. 2008). MRFs form heterodimers with other non-myogenic bHLH factors (typically the E proteins) and bind specific sequences of DNA called E-boxes (CANNTG) in the enhancers and promoters of muscle specific genes (Beylkin et al. 2006; Cao et al. 2010). In particular, *Myf5* and

*MyoD* are activated during myoblasts proliferation and early differentiation (Emerson 1990), which implies their involvement in maintenance of progenitor muscle cells, while *MRF4* and *myogenin* are expressed in terminally differentiated muscle cells (Edmondson & Olson 1989; Wright et al. 1989). Expression of Myf5 and MyoD is a key step in the commitment into the skeletal muscle lineage, since disruption of both genes results in the complete absence of myogenic cells (Rudnicki et al. 1993), while single mutants display no major defects in myogenesis (Rudnicki et al. 1992). *Myogenin* mutant mice show dramatic reduction in all skeletal muscles, demonstrating that the other MRFs are not able to compensate for myogenin function (Hasty et al. 1993; Nabeshima et al. 1993). Moreover, myogenin is correctly expressed in *MyoD:MRF4* double mutants but is not able to compensate for muscle formation, indicating that myogenin is not sufficient to induce muscle differentiation alone (Rawls et al. 1998). Interestingly, when the Myf5 gene is substituted with myogenin in the MyoD:Myf5 double mutant muscle differentiation takes place, even if with low efficiency, suggesting non-redundant functions for the different MRFs throughout development (Wang & Jaenisch, 1997). MRFs cooperate with different factors in order to activate the expression of muscle-specific genes. An important family of co-factors is the myocyte enhancer factor-2 (MEF2) family which bind to a specific sequence called MADS-box motif that is found in regulative regions of several muscle-specific genes (Olson et al. 1995). MEF2 factors can cooperate with heterodimers of myogenic bHLH proteins and E proteins, and the DNA-binding domains of these factors mediate the physical interaction required for functional cooperation (Molketin et al. 1995; Black & Olson 1998). Several recent studies have demonstrated that MyoD is required for enhancer activity in distal regulative regions of several muscle-specific genes, including *myogenin*, *MyoD*, *Myf5* and *MRF4* (Yee & Rigby, 1993; Cheng et al. 1993; Chang et al. 2004; Carvajal et al. 2008). Notably, MyoD is able to recruit the acetyltransferases p300 and PCAF leading to acetylation of histones H3 and H4 (Yuan et al. 1996; Puri et al. 1997; Rampalli et al. 2007). Further studies have suggested that epigenetic modification and chromatin arrangement might be important in limiting MRF recruitment to muscle enhancers (Cao et al. 2010; Blum et al. 2012), and putative co-factors of the MRFs (including NFATc, c-Jun, Runx1 and Meis) have been validated (Bengal et al. 1992; Knoepfler et al. 1999; Wang et al. 2005; Daou et al. 2013). The process of myogenic differentiation is tightly linked to cell cycle control: in fact, both Myf5 and MyoD

have been shown to be involved in myogenic progenitors expansion (Megeney et al. 1996; Ustanina et al. 2007; Zhang et al. 2010), while myogenin is a marker of terminal differentiation involved in cell cycle exit (Andrès & Walsh, 1996). An extensive literature has supported a role for MyoD in cell cycle exit that is required for differentiation (reviewed in De Falco et al. 2006): in particular it was shown that *MyoD* null myoblasts are not able to withdraw from the cell cycle (Cornelison et al. 2000). Moreover, MyoD has been shown to induce the expression of the Cdk inhibitor p21 that leads to activation of the retinoblastoma tumor suppressor protein (Rb) (Halevy et al. 1995; Zhang et al. 1999). However, it was recently reported that myogenin but not MyoD is sufficient to induce cell cycle exit in proliferating myoblasts by indirect regulation of different cell cycle genes (Liu et al. 2012). Moreover by ChIP analyses for MyoD and myogenin it was shown consistent overlap of MyoD and myogenin transcriptional targets (Blais et al. 2005). On the basis of these observations it was proposed that MyoD might create an open chromatin structure to recruit myogenin for strong transactivation of cell cycle inhibitor genes (Sing & Dilworth, 2013).

Importantly, paired homeobox (*Pax*) genes are also involved in myogenic specification. *Pax3* and its paralog *Pax7* have fundamental and nonredundant functions in the specification of embryonic and post-natal myogenic progenitors. Both Pax3 and Pax7 are expressed in the dermomyotome, with the highest levels of Pax3 in the dorsal and ventral lips and the highest levels of Pax7 in the central domain (Kassar-Duchossoy et al. 2005). In particular, Pax3 is specifically required for proper migration of embryonic myogenic progenitors towards their target regions: in fact Pax3 directly induces expression of *c-met*, which encodes a tyrosine kinase receptor for HGF (hepatocyte growth factor/scatter factor) that is critical for myogenic cells delamination and migration (Epstein et al. 1996; Relaix et al. 2004). Moreover, Pax3 and Myf5 control expression of MyoD which is not able to rescue the complete loss of muscle in *Pax3:Myf5* double mutants (Tajbakhsh et al. 1997), whereas ectopic expression of Pax3 in embryonic tissues is sufficient to induce Myf5, MyoD and myogenin in both paraxial and lateral plate mesoderm (Maroto et al. 1997). Both Pax3 and Pax7 expression are dependent on Six homeoproteins, which are considered to be at the apex of the genetic regulatory cascade that drives myogenic commitment (Rossi & Messina, 2014). At the limb bud level, Six1 and Six4 control migration and delamination of dermomyotomal cells through regulation of Pax3, whereas in the

myotome they positively regulate expression of myogenin, MRF4 and MyoD (Grifone et al. 2005). Notably, Pax3 is expressed at high levels in myogenic progenitors until E13.0 and it is then repressed, whereas Pax7 expression is maintained also in fetal myoblasts (Horst et al. 2006) and post-natal satellite cells (see Section 2.3). Pax3 acts upstream of MyoD during early myogenesis: indeed no MyoD transcript is detected in Splotch (*Pax3* loss-of-function) mice. Moreover, MyoD is not able to rescue muscle loss in *Pax3:Myf5:MRF4* triple mutants, since expression of MyoD is dependent on either Pax3 or Myf5 (Tajbakhsh et al. 1997). On the other hand, Pax7 is dispensable for embryonic muscle development (Seale et al. 2000), while in *Pax3:Pax7* double mutants only the early myotome is formed and subsequent muscle development is interrupted (Relaix et al. 2005). As discussed in the next paragraph, a recent study has elegantly demonstrated a more defined role for Pax3+ and Pax7+ progenitors during development (Hutcheson et al. 2009). Importantly, Pax7 is able to directly activate Myf5 expression by recruiting the histone methyltransferase complex that directs methylation of histone 3 lysine 4 (H3K4) (McKinnel et al. 2008), thus inducing chromatin remodelling and transcriptional activation. Moreover, recent works have discovered genome-wide targets of Pax3 and Pax7 (Soleimani et al. 2012): it was shown in adult myoblasts that Pax3 binds only to a subset (5%) of Pax7 transcriptional targets. Despite the significant overlap in Pax3 and Pax7 functions, Pax7 specifically regulates clusters of genes that are involved in proliferation and inhibition of differentiation by binding to the homeobox motif with higher affinity than Pax3.

## *2.2 Pre-natal development*

All the skeletal muscles of the trunk and the limbs originate from the PSM. Somites are transient metameric structures that are formed by epithelial condensation of the PSM at either side of the neural tube (Nowicki & Burke, 2000; see Figure 1.4). The process of somite formation takes place from the anterior end of the PSM between embryonic day (E) 8.0 and E13.5 in the mouse (Pourquié, 2003). Immediately after epithelialization the ventral part of the somite undergoes an epithelial-to-mesenchymal transition to produce sclerotome progenitors that will form rib and vertebral cartilage, while the dorsolateral domain forms the epithelial dermomyotome, that will give rise to dermis and skeletal muscle progenitors (Brill et al. 1995; Cossu

et al. 1996; Borycki et al. 1997). Cells from the dorsal medial lip of the dermomyotome migrate ventrolaterally to form epaxial dermis and myotome (which will contribute to back muscles), while cells from the ventral lateral lip contribute at the interlimb level to the hypaxial aspect of the myotome (Denetclaw et al. 1997; Olivera-Martinez et al. 2000). The myotome is formed by mononucleated myocytes that span alongside the entire somite and is eventually integrated into the trunk musculature (Tajbakhsh & Buckingham, 2000). Importantly, hypaxial progenitors migrate towards the lateral plate mesoderm to form limb and limb girdle muscles (Cinnamon et al. 1999). The process of delamination and migration of hypaxial progenitors into the limb bud is dependent on positional cues (in particular HGF) provided by mesenchymal non-myogenic cells of the limb bud (Birchmeier & Brohmann, 2000). Pax3+ cells migrating from the somite have not yet activated the myogenic program, and they start expressing Myf5 and MyoD only when they reach the limb (Tajbakhsh & Buckingham, 1994).

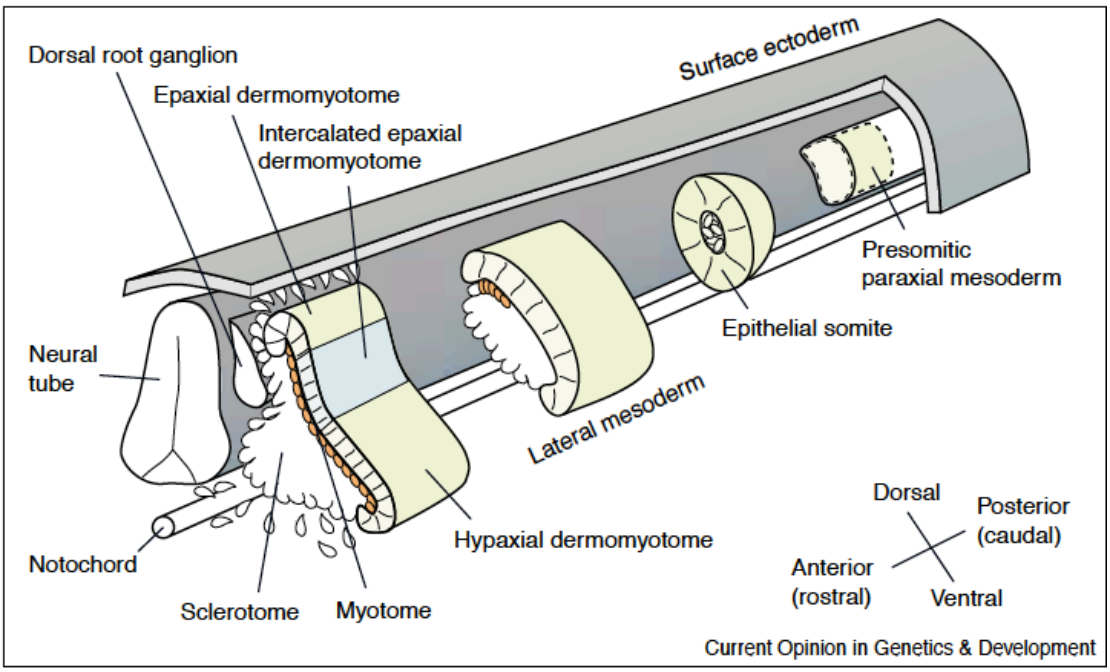


**Figure 1.4. Representation of somite development.** After removal of surface ectoderm, the consecutive steps of somitogenesis are enlighted. Note that the anterior somites are the most differentiated ones, and consist of mesenchymal sclerotome, myotome and epithelial dermomyotome. On the contrary, posterior paraxial mesoderm is still unsegmented. Enlighted in the picture are also the distinct regions of the dermomyotome: epaxial (or dorso-medial), intercalated epaxial (or central) and hypaxial (or ventro-lateral) domains. Adapted from Arnold, 2006.

During the early phases of muscle development in the somite and the limb, commitment into the myogenic lineage is induced by paracrine factors secreted from the surrounding tissues. In particular, Wnt1 and Wnt3a (from the neural tube) and Shh (from the notochord) cooperate for the induction of Myf5 in the epiaxial dermomyotome domain, while Wnt7a (from the dorsal ectoderm) and FGF5 (from lateral mesoderm) induce expression of Pax3 and MyoD in the hypaxial domain (Munsterberg et al. 1995; Tajbakhsh et al. 1998; Borycki et al. 1999; Cossu & Borello 1999). This is consistent with the phenotype observed in *Myf5* and *MyoD* null embryos which display moderate defects in epaxial and hypaxial myogenesis, respectively (Rudnicki et al. 1992; Kablar et al. 1997). Among the different extrinsic signals that positively regulate myogenesis the most studied are Shh and Wnt: Shh is essential for dermomyotomal progenitors commitment into the myogenic lineage (Borycki et al. 1998), while abrogation of the Wnt pathway leads to dermomyotome defects and reduction of Pax3 and Myf5 expression (Borello et al. 2006). Moreover it

was shown that Bmp4 produced in the lateral plate mesoderm is important to inhibit muscle differentiation and to delay Myf5 and MyoD induction in Pax3+ cells (Pourquié et al. 1995).

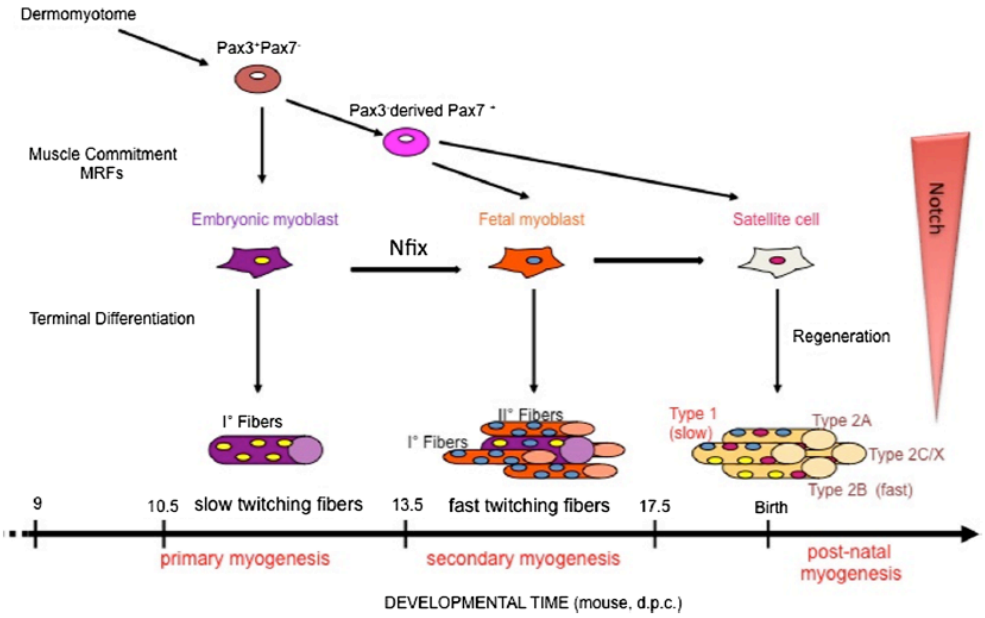


**Figure 1.5. Schematization of the different myogenic progenitors involved in muscle development.** Embryonic myoblasts form slow primary fibers between E11 and E13.5, while fetal myoblasts form fast secondary fibers between E14 and E18. The transcription factor Nfix is a marker of fetal myoblasts/myocytes. The Notch signal is gradually inactivated along the differentiation route. After E18, a third population of post-natal myoblasts called satellite cells is required for muscle growth and regeneration. From Rossi & Messina, 2014.

The process of skeletal muscle development occurs in subsequent steps that involve distinct populations of committed myoblasts (Biressi et al. 2007a, 2007b; Hutcheson et al. 2009) and result in the formation of different developing fibers (Cusella-De Angelis et al. 1994). 'Embryonic' myoblasts sustain a first wave of differentiation between embryonic day (E) 10 and E12 in the mouse and give rise to primary myofibers that establish the primitive shape of muscle and express high levels of MyHC-I and of the embryonic MyHC isoform (MyHC-emb) (Schiaffino et al. 1986; Stockdale et al. 1992). A second wave of muscle differentiation takes place between E15 and E18, driven by fetal myoblasts that form secondary fibers (initially smaller and surrounding primary fibers), which are characterized by low levels of MyHC-I and high levels of the fetal/neonatal isoform MyHC-neo (Eusebi et al. 1986; Lyons et al. 1990; Daou et al. 2013). Starting from E16.0, muscle fibers heterogeneity is

evident in skeletal muscle (Hagiwara et al. 2007). 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 such as muscle creatine kinase (MCK) and the glycolytic enzyme β-enolase (Eno3) (Ferrari et al. 1997; Biressi et al. 2007b). 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). Moreover, embryonic and fetal myoblasts exhibit different requirements for several extrinsic and intrinsic factors, including TGF-β (Biressi et al. 2007b), MRF4 (Kassar-Duchossoy et al. 2005), Pax3/7 and β-catenin (Hutcheson et al. 2009). In a recent report (Hutcheson et al. 2009) genetic ablation of either Pax3+ or Pax7+ cells was conduced to demonstrate the specific roles of these myogenic populations in pre-natal development. Importantly, loss of the Pax3+ lineage leads to embryonic lethality and prevents the emergence of Pax7+ cells, whereas ablation of the Pax7+ lineage only leads to defects in fetal muscle development. These data suggest that the Pax3+ lineage is responsible for primary myogenesis. Interestingly, recent observations have demonstrated that a population of Pax7+ dermomyotomal stem cells is expanded during embryonic myogenesis by activation of the Notch pathway, and that this is critical for muscle progenitors maintenance (Mourikis et al. 2012), suggesting that a single self-renewing population may be responsible for the different myogenic waves.

## *2.3 Post-natal muscle growth and regeneration*

Adult skeletal muscle is a stable tissue endowed with strong regenerative capabilities. After birth the number of myofibers in each muscle remains constant, but they grow in size by fusion of post-natal myoblasts, named satellite cells (SCs). SCs represent a third wave of myogenic progenitors, and in resting conditions (such as in adult uninjured muscle) they occupy a specific anatomical position underneath the basal membrane of skeletal fibers 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), Pax3 (that is expressed exclusively in diaphragm and forelimb muscle SCs) (Relaix et al. 2006), M-cadherin (Irintchev et al.

1994), c-Met (Allen et al. 1995), syndecan-3 and -4 (Cornelison et al. 2001), CD34 (Beauchamp et al. 2000), α7-integrin (Burkin et al. 1999) and calcitonin receptor (Fukada et al. 2007). Among these, Pax7 is probably the most commonly used since it is specifically expressed in SCs during both the quiescient and proliferative state, and across different species such as mouse, human and zebrafish (Seale et al. 2000; McLoon et al. 2003; Hammond et al. 2007). SCs are present in all skeletal muscles but unequally distributed in different muscles and fiber types. In particular, a higher number of SCs is found associated with slow-twitch fibers compared with fast-twitch fibers of the same muscle (Gibson & Schultz, 1982). It was also reported that SCs are more frequently found adjacent to muscle capillaries (Schmalbruch & Hellhammer, 1977) and to the NMJ (Wokke et al. 1989), suggesting that paracrine factors produced by non-muscle cells may have a role in the regulation of SCs activity. SCs number also decreases with age: while at birth SCs nuclei account for the 30% of sublaminar muscle nuclei, this percentage is reduced to <5% in 2 month old adult mice (Bischoff, 1994). This decrease is mainly due to fusion of SCs with the growing fibers during the early post-natal period. In adult uninjured muscle SCs are mostly in G0 (quiescence), but during the first phases of muscle regeneration they start proliferating and activate expression of Myf5 and MyoD (Cooper et al. 1999; Zammit et al. 2002). This amplified population of myoblasts (also called myogenic precursor cells, mpc) is responsible for repair of injured fibers and for the formation of a new pool of sublaminar SCs (Yao et al. 1993; Smith & Schofield, 1997; Heslop et al. 2001). Therefore SCs can be defined as a population of resident adult stem cells, characterized by their ability to differentiate into the myogenic lineage, and to self-renew. However, the embryonic origin of SCs is still unclear. In recent years, many works have demonstrated that SCs arise from the Pax3/Pax7 myogenic progenitors of the dermomyotome, which remain undifferentiated into the late fetal period and are enveloped in the basal lamina of developing fibers (Gros et al. 2005; Relaix et al. 2005; Sambasivan et al. 2013). Myogenic progenitors from the dorsal aorta can also contribute to post-natal muscle growth suggesting a common origin for SCs and endothelial progenitors (De Angelis et al. 1999). In the last years several works have demonstrated that different classes of non-myogenic cells such as hematopoietic stem cells, mesenchymal stem cells and muscle derived stem cells can contribute to regeneration upon transplantation or direct injection into injured skeletal muscle (reviewed in Tedesco et al. 2010). However, the role of these progenitors during

normal muscle development, and their possible contribution to the SCs pool has not yet been elucidated. Moreover, recent works have unambiguously demonstrated that ablation of the Pax7+ SCs pool leads to a complete loss of muscle regeneration (Lepper et al. 2011; Sambasivan et al. 2011). Pax7 has a critical role in SCs development since *Pax7* null mice display normal pre-natal myogenesis but a drastic decrease in adult SCs number and reduced muscle regeneration (Seale et al. 2000; Oustanina et al. 2004). Further investigations have shown that in absence of Pax7 SCs undergo apoptosis instead of entering quiescence during the early post-natal period and Pax3 is not able to rescue for Pax7 in Pax3-expressing SCs (Relaix et al. 2006). Several works have investigated the heterogeneous nature of adult SCs: a first important discovery was achieved by using the *Myf5*-Cre and ROSA-EYFP Cre-reporter alleles (Kuang et al. 2007). The authors have identified a subpopulation of SCs that have never expressed Myf5, and that divide by asymmetric cell division to give rise to a basal Pax7+/Myf5- stem cell and an apical Pax7+/Myf5+ progenitor cell committed to myogenic differentiation. Importantly, a recent report shows that ablation of Pax7 in Myf5+ cells leads to postnatal decline in SCs number and complete loss of muscle regeneration (Gunther et al. 2013). However, the Pax7+ SCs pool is not completely lost even in the absence of both Myf5 and MyoD, suggesting that a small amount of adult SCs might be derived by non-myogenic progenitors during normal muscle development.

## *2.3 Muscle development in zebrafish (Danio rerio)*

Teleosts, and zebrafish in particular, have been widely used as animal models for practical reasons, including ease of genetic manipulation and transparence of embryos/larvae, which allows direct visualization of embryonic development. Myogenesis in teleosts shares several common features with mammals: the presence of different progenitor cells and fiber types, the formation of the embryonic dermomyotome and the presence of extrinsic signals regulating commitment and differentiation into the myogenic lineage. However, there are particular differences in several aspects of muscle development that will be briefly discussed here.

In zebrafish, somitogenesis is immediately initiated after gastrulation. At the end of the first day post-fertilization (dpf), all the somites are formed and divided into

sclerotome and myotome domains (Kimmel et al. 1995). Moreover, a dermomyotome domain was also described in teleosts, in which Pax3 and Pax7 mark myogenic progenitors that are maintained during development, thus leading to a continuous contribution to larval and post-larval muscle growth (Stellabotte & Devoto, 2007). However, unlike in mammals, the first cells to commit into the myogenic lineage arise from the medial region of the PSM, which lies adjacent to the notochord. These mesodermal progenitors, called adaxial cells, start to express Myf5 and Myod before the formation of the dermomyotome, and eventually migrate from the notochord to form the mononuclear superficial slow-twitch fibers that uniquely express the slow MyHC isoform sMyHC1 and the transcription factor Prox1a (Devoto et al. 1996; Stickney et al. 2000). On the other hand, the dermomyotome progenitors will contribute to the medial multinucleated fast-twitch fibers of zebrafish muscle that specifically express the fast MyLC isoform MyLpfa (Liew et al. 2008). Importantly, the molecular mechanisms underlying slow and fast muscle lineage specification have been characterized in zebrafish. It was shown that secretion of Hedgehog (Hh) family members from the notochord and ventral spinal chord is required for the formation of slow-twitch muscle (Lewis et al. 1999; Barresi et al. 2000). Hh acts initially on adaxial cells and induces expression of the zinc finger domain transcription factor Prdm1a that functions as a direct repressor of several fast-twitch specific genes (including *mylpfa*, the fast troponin I *tnni2* and the transcription factor *sox6*) (von Hofsten et al. 2008). This mechanism is not conserved in mouse development (Vincent et al. 2012). Moreover, it was shown that slow and fast muscle differentiation present different requirements for Myod and Myf5: slow adaxial cells differentiation requires either Myf5 or Myod and is disrupted in *myf5:myod* double morphants (Hammond et al. 2007). On the contrary, Myod is specifically required for fast-twitch fibers formation (Groves et al. 2005): it was recently shown that Pbx homeoproteins cooperate with Myod for the induction of the fast-twitch phenotype (Maves et al. 2007).

After the embryonic period, new muscle fibers are formed in different locations by stratified hyperplasia in a way that is independent from myotome formation and Hh signaling (Barresi et al. 2001). This secondary zebrafish myogenesis is driven by Pax3+ Pax7+ cells of the dermomyotome and leads to the formation of both slow-twitch and fast-twitch fibers (Stellabotte et al. 2007).

# 3. Molecular mechanisms of fiber type specification

Here we will discuss in detail the molecular mechanisms involved in adult fiber type plasticity, and those involved in fiber type specification during the pre-natal period, including the Six homeoproteins, Nfix, Sox6 and MyoD/NFATc1.

## *3.1 Adult fiber type plasticity*

It has been known for many years that cross innervation of slow-twitch and fast-twitch muscles lead to a gradual transformation of the fiber characteristics toward the phenotype of the other fiber type (Pette and Vrbova, 1985). Moreover, direct electrical stimulation of the muscle gives rise to adaption of the fiber phenotype to the applied pattern of activity, suggesting that calcium intracellular levels and not trophic factor released by the motorneuron may be involved in fiber type plasticity (Windisch et al. 1998). Interestingly, the modulation of intracellular calcium is able to partly recapitulate the phenotype changes of fiber plasticity (Kubis et al. 1997). In the last years many molecular pathways have been identified which play important roles in adult fiber plasticity. The first to be discovered by in vitro and in vivo experiments was the calcineurin/NFAT pathway (Chin et al. 1998). Calcineurin is a cyclosporin-sensitive, calcium-regulated serine/threonine phosphatase that controls the subcellular localization of the different NFATc factors in skeletal muscle cells and lymphocytes (Timmerman et al. 1996). In the presence of ectopic slow-twitch stimulation or constitutive expression of an activated form of calcineurin, NFATc proteins are shuttled into the fiber nuclei where they positively regulate expression of several slow-twitch markers, such as myoglobin, MyHC-I and the slow troponin C isoform (Chin et al. 1998; Naya et al. 2000; McCullagh et al. 2004; Calabria et al. 2009). Along with NFATc, the transcription factors MEF2 (MEF2A, B, C and D) are also involved in fiber type plasticity since both NFAT and MEF2 consensus binding sites have been identified within the regulative regions of slow fiber specific genes (Chin et al. 1998). MEF2 factors are maintained in an inactive state by members of the family of class II histone deacetylase (HDAC4, 5, 7), which form complexes with MEF2

proteins in the nucleus (Miska et al. 1999). The repression mediated by HDACs is controlled by the phosphorylation state of HDACs, which in turn is regulated by calmodulin dependent kinase (CaMK). In presence of high levels of intracellular calcium, CaMK is activated and phosphorylates HDACs, which are bound by 14-3-3 proteins and exported in the cytoplasm (Lu et al. 2000; McKinsey et al. 2001), thus leading to MEF2 activation. In adult muscle, HDAC4, HDAC5 and HDAC7 are transcriptionally expressed at high levels in slow muscles; however, their protein levels are enriched in fast fibers since class II HDACs are selectively ubiquitinated and degraded via the proteasome in slow-twitch fibers (Potthoff et al. 2007). A cross-talk exists between the calcineurin/NFAT and the MEF2 pathway: in fact, MEF2 activity is strongly enhanced in the presence of activated calcineurin (Wu et al. 2000). Other factors have been shown to play a role in adult fiber plasticity: in particular AMPK (Winder et al. 2001), PPARs (Luquet et al. 2003) and PGC-1α (Lin et al. 2005).

## *3.2 Pre-natal fiber type specification: role of the Six homeoproteins*

The sine oculis homeobox (Six) proteins, a family of highly conserved transcription factors (Six1-Six6 in vertebrates) that play important roles in development, are characterized by the Six domain and the Six-type homeobox, both of which are essential for specific DNA-binding properties and interaction with the Eya/Dach co-factors (Kawakami et al. 2000). Eya factors act as phosphatases that inhibit the activity of the co-repressor Dach; moreover Eya factors recruit components of the Six complex such as p300/CBP (Jemc and Rebay, 2007). Six1, Six4, Eya1 and Eya2 are all expressed in the dermomyotome and mark Pax3+ progenitors, even if their expression is maintained in differentiated muscle. It was reported that ectopic expression of Six1 and Eya2 in pre-somitic mesoderm is sufficient to induce expression of Pax3 and MRFs (Heanue et al. 1999). Analysis of *Six1/Six4* and *Eya1/Eya2* double mutant mice shows that expression of Pax3 is lost in the hypaxial dermomyotome, leading to aberrant delamination and migration of progenitor cells. Moreover, the expression of Myf5, MRF4 and MyoD is compromised with loss of all the muscles derived from the hypaxial dermomyotome, revealing a critical function

for the Six/Eya complex in myogenesis (Grifone et al. 2005; 2007). Recent reports demonstrated that Six proteins are recruited directly on the promoters of *MyoD* and *Myf5* genes (Giordani et al. 2007; Relaix et al. 2013). Moreover, the *myogenin* proximal promoter is also controlled by Six/Eya through a conserved MEF3 binding site (Spitz et al. 1998), even if myogenin expression is partially retained in double mutant mice. Importantly, Six proteins also play a role in pre-natal fiber type specification: in fact it was shown that *Six1/Six4* deficient dorsal muscles (that form normally) display down-regulated levels of fast-twitch markers and up-regulation of several slow-twitch markers, including MyHC-I at the protein level (Richard et al. 2011). Moreover, absence of Six1/Six4 resulted in re-localization of the transcription factor Sox6 from the nucleus into the cytoplasm, suggesting a cross-talk between Six/Eya and Sox6 regulative networks (see Section 3.5). Six1 and Six4 are enriched in fast adult muscle fibers, and they are able to directly bind to the promoters of several fast-twitch genes in embryonic muscle (Niro et al. 2010).

## *3.3 Pre-natal fiber type specification: role of Nfix*

Nuclear factor I x (Nfix) belongs to the Nfi family that in vertebrates comprises four genes: *Nfix*, *Nfia*, *Nfib* and *Nfic* (Gronostajski, 2000). They encode for site-specific DNA binding factors with a conserved N-terminal DNA-binding and dimerization domain and a C-terminal transactivation/repression domain. The homologs of these four *Nfi* genes have been identified in every vertebrate species examined from *Xenopus laevis* to mouse and human (Roulet et al. 1995; Chaudhry et al. 1997; Kulkami & Gronostajski, 1996). In contrast, the cephalocordate *Amphioxus* genome only contains one Nfi gene that has undergone several duplication events during chordate development (Fletcher et al. 1999). The Nfi transcripts are post-transcriptionally modified by alternative splicing of exons in the 3' regions (leading to different C-termini fused to the DNA-binding domain) and alternative promoter usage (Santoro et al. 1988; Inoue et al. 1990; Apt et al. 1994; das Neves et al. 1999). The alternative splicing is evolutionary conserved (Kruse & Sippel, 1994) and differently regulated in distinct cell types (Apt et al. 1994; Chaudhry et al. 1997). The Nfi factors bind as dimers to the palindromic consensus sequence TTGCC(N5)GCCAA with strong affinity (Nowock et al. 1985); however, Nfi factors can also bind to individual

half sites (Meisterernst et al. 1988). The Nfi factors were first identified as crucial regulators of adenovirus DNA replication (Nagata et al. 1983). Eventually, it was demonstrated that Nfi proteins play also fundamental roles in gene expression: Nfi consensus sequences are found in promoters and enhancers of tissue-specific genes that are important for development and differentiation of several organs, including brain (Bedford et al. 1998), kidney (Leahy et al. 1999), cartilage (Szabo et al. 1995), and skeletal muscle (Funk & Wright, 1992; Spitz et al. 1997). In particular, *Nfix* null mice show decreased growth rate from post-natal day (P) 8 to P22 and die at about P22 exhibiting brain malformation, defects in endochondrial ossification and in the gastrointestinal tract (Driller et al. 2007). However, when supplemented with soft dough feed, they increase their weight and can survive to adulthood (Campbell et al. 2008). By the use of these null strains, it has been shown that Nfix plays crucial roles in the development of cerebellar neurons (Piper et al. 2011). Moreover, Nfix is expressed in the adult subependymal zone and in the dentate gyrus of the hyppocampus, the two regions of adult brain that contain proliferative adult stem cells, and here drives direct repression of the *Sox9* gene (Fuentealba et al. 2012; Heng et al. 2014). Importantly, high-throughput ChIP-seq with an antibody recognizing all the Nfi isoforms was performed in neural stem cells (NSCs) (Martynoga et al. 2013), revealing the ability of Nfi factors to bind to the enhancers of several neural-specific genes that are activated during quiescence (along with the transcriptional activator p300/CBP). Moreover, the authors have shown that Nfix is up-regulated when NSCs are treated with BMP4 (leading to an undifferentiated quiescent state), and that Nfix is both sufficient and necessary for the acquisition of quiescence in NSCs.

The role of Nfi factors (and in particular Nfix) in skeletal muscle development has been addressed for the first time in a recent publication by our laboratory (Messina et al. 2010). In this extensive work it was demonstrated that Nfix is a master regulator of fetal-specific muscle transcription, acting both as a repressor of embryonic specific genes (in particular *MyHC-I*) and as an activator of fetal-specific genes (such as *Eno3* and *MCK*). The Nfix transcript and protein are barely expressed in embryonic myoblasts and muscle tissue and start to be expressed at high levels at the beginning of fetal myogenesis. Once activated in fetal myoblasts, Nfix binds to the promoter of the *NFATc4* gene, thus leading to indirect MyHC-I silencing. Moreover, Nfix binds alone to the *Eno3* proximal promoter thus activating Eno3 expression. Finally, Nfix is able to activate expression of MCK by forming a complex with PKCθ and MEF2A at

the 5'-upstream region of *MCK*. In particular, the presence of Nfix allows the binding between PKCθ and MEF2A and PKCθ-dependent phosphorylation of MEF2A, thus leading to binding of the complex at the *MCK* enhancer region (Ferrari et al. 1997). MEF2A is constitutively expressed in skeletal muscle, while PKCθ expression is activated in fetal myoblasts by Nfix and other unknown factors. As a consequence, conditional deletion of *Nfix* in fetal skeletal muscle results in higher levels of NFATc4 and MyHC-I and lower levels of Eno3 and MCK, while ectopic expression of Nfix in embryonic muscle leads to activation of the fetal program and repression of embryonic/slow genes. The factors that are responsible for Nfix induction in fetal myoblasts are until now unknown: it was shown that Pax7 is sufficient to activate Nfix expression in embryonic muscle cells; however, Pax7 is not necessary for Nfix expression in fetal muscle, suggesting a more complicated mechanism. Intriguingly, a recent work has identified Nfix as a DNA-hypomethylated marker in human fetal tissue (compared to adult blood samples), suggesting an epigenetic regulation of the Nfix locus during pre-natal development (Wang et al. 2014).

Importantly, our laboratory has also shown that the zebrafish ortholog nfixa has a conserved role in the repression of the embryonic muscle phenotype (Pistocchi et al. 2013). Nfix has two ortholog genes in zebrafish: Nfixa and Nfixb, but only Nfixa is expressed in skeletal muscle. *nfixa* transcript has a peak at 2 dpf, in the transition period between the initial myotome formation and the stratified hyperplasia phase. As in mouse, Nfixa drives indirect repression of sMyHC1 via repression of *nfatc4*: indeed, knock-down of *nfatc4* partly rescues the slower phenotype of *nfixa* morphants. Moreover, *nfixa* morphants display a strong motility phenotype (with partial or complete immotility) after 3 dpf: this is due to defects in the sarcoplasmic reticulum and loss of the RyR ion channel. This phenotype was never observed in *Nfix* null mice, suggesting that Nfix has both divergent and conserved functions in muscle development throughout vertebrate evolution.

## *3.4 Pre-natal fiber type specification: role of Sox6*

The SRY-related HMG box (Sox) family comprises 20 genes that are divided in eight groups (SoxA-SoxH) in vertebrates (Schepers et al. 2002). The SoxD group has evolved from a single *SoxD* gene (Larroux et al. 2008): in fact invertebrates such as

*C. Elegans* possess only one *SoxD* gene, whereas a single copy of all the three *SoxD* genes (Sox5, Sox6, Sox13) is present in the lower vertebrate classes including gnathostoma (Bowels et al. 2000). Expression of the three SoxD factors has been demonstrated in several cell lineages, including chondrocytes (Lefebvre et al. 1998), oligodendrocytes (Stolt et al. 2006), erythroid cells (Dumitriu et al. 2006), vessels (Roose et al. 1998) and skeletal muscle (Hagiwara et al. 2000). Overlapping functions for Sox5 and Sox6 (which share high homology) have been reported during development of different tissues (Stolt et al. 2006; Lefebvre, 2010). The SoxD proteins differ from the rest of Sox factors for the presence of a N-terminal leucin zipper domain and a glutamine-rich domain that are important for protein-protein interactions (Connor et al. 1995; Iguchi et al. 2007). The family conserved HMG box domain that enables SoxD factors to bind a specific consensus sequence (AACAAT motif) is located in the C-terminal region (Lefebvre et al. 1998). The HMG box also allows interactions with other proteins (Yamashita et al. 2000), and contains the nuclear localization signals (NLS) that are required for nuclear-cytoplasmic shuttling of Sox factors (Malki et al. 2010). Importantly, SoxD factors lack trans-repression or trans-activation domains, but they are able to participate in transcriptional regulation (Lefebvre, 2010). For instance, Sox5 and Sox6 cooperate with Sox9 to activate extracellular matrix genes during chondrocytes development (Lefebvre et al. 1998; Han and Lefebvre, 2008). On the other hand, Sox5 and Sox6 are able to block Sox10-dependent activation of myelin genes in oligodendrocytes (Stolt et al. 2006) by competing with Sox10 for the binding in the 5'-upstream regulative regions. Moreover, Sox6 has been shown to repress the *cyclin D* gene in pancreatic cells by forming a complex with HDAC1 and β-catenin (Iguchi et al. 2007) and to repress the *Fgf-3* gene in association with the transcriptional repressor CtBP2 (Murakami et al. 2001). Sox6 has important roles in the development of different organ and tissues. Therefore, the *Sox6* null mice die at P7-P10 due to breathing failure (Smits et al. 2001); moreover, they present severe dwarfism and abnormal sternum formation. The formation of cartilage is severely impaired in *Sox5/Sox6* double mutants, suggesting functional redundancy for Sox5 and Sox6.

In recent years, an important function for Sox6 in skeletal muscle has been identified (Hagiwara et al. 2005; 2007). In *Sox6* null mice the activation of the fetal program is completely lost and by E16 several slow-twitch markers such as MyHC-I and slow TnI are dramatically up-regulated in fetal muscle. Sox6 is able to directly bind to the

proximal promoter of the *MyHC-I* gene at a consensus sequence that is located at -200 bp from the TSS, in a regulative region that is critical for MyHC-I regulation in adult denervated soleus muscle (Huey et al. 2003). Importantly, ChIP-seq for Sox6 was recently performed on fetal differentiated myoblasts (An et al. 2011): the authors have identified 1066 binding sites that are particularly enriched in intronic and intergenic regions, in proximity to E-boxes and Tead/MCAT motifs, suggesting a direct function of Sox6 in the regulation of a wide range of genes. Strikingly, more than 90% of the total binding sites were overlapping with MyoD and myogenin ChIP-seq enriched regions. Most of the Sox6 targets were not associated with active Pol-II binding, suggesting that Sox6 acts prevalently as a transcriptional repressor in fetal muscle. Notably, the ChIP-seq has allowed identification of many Sox6 targets that are involved in skeletal and heart muscle function, including *MyHC-I*, *MyHC-IIa*, *MyHC-IIx*, *α-cardiac MyHC*, *Myh7b*, *Tnnc1*, *Tnni1*, *Prox1*, *Sox6*, *Nfatc3*, and others. In particular, the authors have confirmed the binding of Sox6 to the *MyHC-I* proximal promoter region (-350 bp from the TSS) and also identified a second binding site in a distal muscle enhancer that is required for full gene activation in the adult soleus muscle and in the embryonic heart (Giger et al. 2000; Blow et al. 2010). Moreover, phenotypic characterization of a *Myf5-Cre/Sox6*$^{flox/flox}$ transgenic line has unambiguously demonstrated that Sox6 is required for fetal fiber specification: conditional deletion of *Sox6* results in up-regulation of slow-twitch genes in all the developing fibers starting from the beginning of secondary myogenesis. Sox6 is expressed at the protein level in the nuclei of secondary MyHC-I negative fibers and occasionally in the cytoplasm of fetal and post-natal muscle fibers. It is known that Sox factors can be regulated by subcellular localization via interaction with the HMG box domain with nuclear transport proteins (Argentaro et al. 2003): all the SoxD proteins possess conserved NLS sequences and a nuclear export sequence (NES) (Sim et al. 2008). In particular, the N-terminal NLS binds to calmodulin and the binding allows nuclear translocation of Sox factors by an unknown mechanism (Hanover et al. 2009). The C-terminal NLS binds to β-importin, leading to association with the RanBP2 nucleoporin and transport via the nuclear pore (Forwood et al. 2001). Finally, the NES is bound by the export receptor CRM1/exportin1 and nuclear export of Sox9 and Sox10 in the developing gonad (Gasca et al. 2002). Moreover, Sox factors are targeted by different post-translational modifications, including phosphorylation (Huang et al. 2000), acetylation (Thevenet et al. 2004), sumoylation (Hattori et al.

2006). A recent work has also shown that Sox6 is post-translationally targeted by the E3 ubiquitin-ligase Trip12 for proteasome degradation (An et al. 2013), strongly suggesting that Sox6 may be regulated by sub-cellular localization/protein stabilization in skeletal muscle.

The mechanisms involved in Sox6 activation during mouse development are until now unknown. It has been shown that Sox6 is not expressed in the developing dermomoyotome and myotome between E9.5 and E11.5 (Vincent et al. 2012), suggesting that Sox6 expression might be quickly activated at the beginning of embryonic or fetal myogenesis. Moreover, Six1/Six4 deficient epaxial muscles display relocalization of Sox6 from the nuclei to the cytoplasm and down-regulation of HDAC4, along with consistent up-regulation of embryonic-specific genes (Richard et al. 2011), suggesting that Six factors may be important in the regulation of Sox6 expression. However, due to the severe phenotype of *Six1/Six4* double mutants, it is unclear whether this is a direct transcriptional effect. Sox6 regulation is better defined in zebrafish muscle development: it is known that Hh-dependent Prdm1a expression directly represses Sox6 in adaxial cells and allows normal fast muscle fiber specification (von Hofsten et al. 2008). Sox6 is expressed in the fast domain of developing zebrafish from the 10-somite stage and represses several slow-specific genes such as *smyhc1*, *tnnc1b* and *prox1a* when ectopically expressed in adaxial cells. Reciprocally, knock-down of *sox6* results in ectopic expression of Tnnc1b in fast muscle fibers at the protein level. Interestingly, morpholino (MO) mediated knock down of both *myf5* and *myod* leads to *sox6* down-regulation specifically in somites, suggesting that the MRFs may activate *sox6* expression (Wang et al. 2011). In the same work it has also been shown that *sox6* transcription is de-repressed in slow twitch muscle at 4 dpf, in the absence of Sox6 protein, suggesting a mechanism of post-transcriptional regulation in slow twitch fibers. Importantly, zebrafish *sox6* transcripts contain several recognition sequences for miR-499, a micro-RNA previously identified in mouse, that is located in the nineteenth intron of the *Myh7b* gene and negatively targets *Sox6* mRNA (McCarthy et al. 2009; van Rooij et al. 2009). Intriguingly, *miR-499/miR-208* double null mice display decreased levels of MyHC-I, while forced expression of miR-499 under the *MCK* regulative regions leads to massive up-regulation of MyHC-I (van Rooij et al. 2009). In zebrafish, Hh-dependent Prdm1a expression spatially restricts activation of *Myh7b/miR-499* that is sufficient to repress Sox6 at the post-transcriptional level (Wang et al. 2011).

However, it is unlikely that this mechanism is conserved in mouse development, since Hh signaling and Prdm1 seem not to be involved in fiber type specification in mammals (Vincent et al. 2012).

## *3.5 Pre-natal fiber type specification: role of MyoD/NFATc2*

As already mentioned in Section 3.1, the nuclear factors of activated T-cells (NFAT) play a pivotal role in adult fiber plasticity. Five NFAT genes have been identified: NFATc1-c4 (that are regulated by calcineurin) and NFAT5. The NFATc factors are all expressed in adult skeletal muscle and have different combinatorial effects on distinct MyHC isoform: in particular MyHC-I is actively regulated by all four isoforms, while MyHC-IIb is positively regulated only by NFATc4 (Calabria et al. 2009). Intriguingly, NFATc3 cooperates with MyoD to induce myogenin expression during somite development (Armand et al. 2008). Most importantly, a recent work has demonstrated a critical role for NFATc2 and MyoD in the transcriptional activation of MyHC-neo during the fetal period (Daou et al. 2013). *NFATc2/MyoD* double null mice display loss of MyHC-neo in fetal and post-natal muscle with normal expression of MyHC-I and MyHC-emb. The MyoD/NFATc2 association is mediated by the C-terminal domain and the DNA-binding domain of NFATc2 and by the HLH domain of MyoD. MyoD/NFATc2 bind directly to the *MyHC-neo* regulative regions, that contain several E-boxes and three NFAT binding sites.

## *3.6 Post-natal fiber type specification*

During the early post-natal period the fiber type composition of each skeletal muscle undergo dramatic changes, due to maturation of the neuromuscular system, activation of thyroid hormone, and other unknown factors. For instance, between P0 and P22 in mouse the developmental MyHC isoforms MyHC-emb and MyHC-neo (that represent the majority of total MyHC at birth) are completely substituted by the adult isoforms MyHC-IIa, MyHC-IIx and MyHC-IIb in fast-twitch muscles (for example, the tibialis anterior, the gastrocnemius, the extensor digitorum longus [EDL], the vastus) (Schiaffino et al. 1988). The transcripts of MyHC-IIa, -IIx and -IIb are first detected by P2-5 in rat hindlimb muscles and display a specific spatial pattern of expression in different muscles: for example, in the tibialis anterior MyHC-IIa transcripts and

proteins are mostly expressed in deep regions, whereas MyHC-IIb transcripts are present only in the superficial areas (DeNardi et al. 1993). Additionally, in fast-twitch muscle the small proportion of type I fibers that are present from the fetal period are selectively lost during early post-natal growth (Whalen et al. 1984; Agbulut et al. 2003). In contrast, in slow-twitch muscle (such as the soleus) the period of early post-natal growth is characterized by transformation of type IIa fibers into type I fibers (Kugelberg, 1976). These changes in fiber type composition can be explained by the combined action of intrinsic programs (i.e. activation of transcription factors, epigenetic regulation) and the effect of extrinsic signals (hormones, muscle activity, etc.). A role in post-natal muscle fiber switch has been proposed for different transcription factors, including Six1/Eya1 (Grifone et al. 2004), PGC-1α (Handschin et al. 2007) and notably Sox6 (Quiat et al. 2011). Intriguingly, Sox6 is enriched in fast-twitch adult muscles, and conditional deletion of *Sox6* under control of *MCK-Cre* leads to dramatic increase in MyHC-I, MyHC-IIa, MyHC-IIx and decrease of MyHC-IIb in fast-twitch muscles. However, by using a fetal/post-natal unspecific Cre, the authors are likely overestimating the effect of Sox6 in post-natal fiber type specification. It is known that during this period the primitive polyneuronal innervation is lost and definitive motor units are formed (Jansen & Fladby, 1990), but the relationship with fiber type change has remained elusive. Denervation in the perinatal period significantly alters fiber type composition of slow-twitch muscle, leading to complete loss of MyHC-I expression after 4 weeks (Gambke et al. 1983). On the contrary, the expression of MyHC-IIb in fast-twitch muscle is not altered with denervation (Butler-Browne et al. 1982) or by treating muscles with α–bungarotoxin (Weydert et al. 1987), suggesting an activity independent mechanism for type IIb fibers development, even if the down-regulation of MyHC-neo requires innervation (Russell et al. 1993). The thyroid hormone (that is produced at high levels during early post-natal development) has a positive effect on type IIb fiber type specification, since MyHC-IIb is precociously induced in neonatal muscles by thyroid hormone injection even in the absence of the nerve (Russell et al. 1988). Importantly, SCs (that represent a third wave of myogenic progenitors active during the early post-natal period) may contribute to these changes by continuous incorporation into the growing myofibers during the early post-natal period. However, no formal evidence for SCs contribution to post-natal fiber type specification has been provided until now (Schiaffino & Reggiani, 2011).

# AIM OF THE THESIS

In recent years, several transcription factors involved in muscle fiber type specification have been identified, but the links among these factors have remained elusive. The aim of this work was to verify and characterize a possible cooperation between Nfix and Sox6 in the regulation of skeletal muscle development, focusing on fiber type specification. As reported in the State of the Art section, Nfix and Sox6 share common targets in fetal muscle, and are both required for the achievement of normal muscle phenotype in rodents and teleosts. In this work we provide evidences for functional cooperation between Nfix and Sox6 that is necessary for normal muscle development. We also demonstrated that this cooperation is evolutionary conserved between mouse (*Mus musculus*) and zebrafish (*Danio rerio*).

# MAIN RESULTS

***Pericytes resident in postnatal skeletal muscle differentiate into muscle fibers and generate satellite cells***

As reported in the State of the Art section, the contribution of non-myogenic progenitors to muscle fiber growth and SC pool during development has never been demonstrated. Importantly, we have shown that during the early post-natal period in mouse, perivascular cells (pericytes) positive for TN-AP (tissue non-specific alkaline phosphatase), NG2, PDGFRβ and negative for the endothelial marker VE-Cadherin actively contribute to muscle fibers growth by hyperplasia, and also produce new satellite cells that form a reservoir subsequently activated during muscle regeneration (Dellavalle et al. 2011). Clonally-cultured pericytes are bipotent cells that differentiate into both vascular smooth muscle cells and skeletal muscle cells. These results are not in contrast with the recent finding that ablation of Pax7 positive cells leads to a complete loss of muscle regeneration (Lepper et al. 2011) since we demonstrated that TN-AP positive cells acquire expression of Pax7 before differentiating into the skeletal muscle lineage. To our knowledge, this is the first report proving the contribution of non-myogenic progenitors (here identified as pericytes) to skeletal muscle homeostasis and regeneration.

## *The transcription factors Nfix and Sox6 cooperate in regulating muscle fiber type specification during pre-natal development*

**Sox6 is expressed in both embryonic and fetal muscle and plays different roles in fiber type specification**

Previous analyses conduced in other laboratories (Hagiwara et al. 2007; An et al. 2011) have unambiguously demonstrated that Sox6 is able to directly bind to the regulative regions of different embryonic-specific markers in fetal muscle cells, thus repressing them. However, a detailed characterization of Sox6 expression in the developing muscle has been never addressed. We demonstrated that Sox6, at variance

with Nfix, is expressed in both embryonic (E12.5) and fetal (E16.5) purified myoblasts. By the use of a polyclonal anti-Sox6 antibody we found that Sox6 protein is expressed in skeletal muscle cells starting from E12.5 and at this stage it is mainly detected in the cytoplasm of myogenin/MyHC positive myocytes. Then, starting from E16.5 many of the newly formed skeletal fibers acquire nuclear staining for Sox6, and by E17.5 its expression is completely restricted to secondary fibers that are negative for MyHC-I. By Western Blot on fractionated muscle lysates, we were able to demonstrate that Sox6 protein is present in both nuclear and cytoplasmic fraction of embryonic muscle but only in the nuclear fraction of fetal muscle, suggesting that Sox6 is regulated via sub-cellular localization during pre-natal myogenesis. In order to better elucidate Sox6 functions during muscle development, we analyzed embryonic and fetal skeletal muscle samples from the *Sox6* null mouse strain (Smits et al. 2001) with different biochemical approaches. Unexpectedly, we found that Sox6 has opposite functions in fiber type specification between primary and secondary myogenesis. In particular, Sox6 acts as an activator of MyHC-I in embryonic muscle, whereas in fetal muscle it is critical for MyHC-I transcriptional repression. As a result, *Sox6* null embryos display reduced levels of MyHC-I, while *Sox6* null fetuses show dramatic up-regulation of MyHC-I, which leads to complete disruption of fetal fiber specification. Therefore, we focused on Sox6 function during the embryonic period (E12.5), which was not previously described. By ChIP assay, we identified two direct targets of Sox6 in embryonic muscle: *MyHC-I* and *Eno3*, which are regulated in opposite ways. Interestingly, Eno3 is markedly up-regulated in Sox6 deficient embryonic muscle, which is suggestive of a slow-to-fast conversion that is also observed when Nfix is ectopically expressed during embryonic myogenesis under the control of a muscle-specific enhancer (Messina et al. 2010).

**Nfix and Sox6 non-redundantly and cooperatively regulate fetal fiber specification**

In order to demonstrate a possible functional cooperation between Nfix and Sox6, we analyzed fetal skeletal muscle from the *Nfix* null strain (Campbell et al. 2008) that displays up-regulation of MyHC-I as previously reported (Messina et al. 2010). Notably, we found that Sox6 protein is invariantly expressed in the nuclei of 60% of total muscle fibers, confirming that nuclear/cytoplasmic shuttling of Sox6 in the fetal period is not regulated by Nfix. However, we found a strong increase in the

percentage of Sox6/MyHC-I double positive fibers in *Nfix* null muscle, suggesting that Nfix is required for Sox6-dependent repression of MyHC-I. To elucidate a mechanism of cooperation, we performed co-immunoprecipitation (Co-IP) assay in differentiated C2C12 myoblasts: the results show that Sox6 and Nfix can physically associate with each other, suggesting the formation of a transcriptional complex. To demonstrate the recruitment of the Nfix/Sox6 complex at the *MyHC-I* promoter, we performed chromatin immunoprecipitation (ChIP) for Sox6 in wild type and *Nfix* null fetal myotubes. We found that the binding between Sox6 and the proximal *MyHC-I* promoter is completely abolished in the absence of Nfix. However, Sox6 is still able to bind to a distal muscle-specific enhancer of *MyHC-I*. Both these regions have been previously identified as functional Sox6-binding sites in fetal myotubes, but only the proximal region is required for the repressive activity of Sox6 (An et al. 2011). Moreover, in absence of Nfix, Sox6 is aberrantly present at the *Eno3* promoter and here it is likely repressing Eno3 expression. These data demonstrate that Nfix is required for the binding ability and function of Sox6 in fetal muscle.

In a parallel way, we sought to investigate whether Sox6 is required for normal Nfix function during fetal myogenesis. Hence, we performed different analyses on wild type and *Sox6* null fetal muscle and found that Nfix is normally expressed in the absence of Sox6. In both fetal muscle and cultured fetal myotubes we found a slight decrease in *Nfix* mRNA levels, but no differences in Nfix protein expression. Importantly, NFATc4 – a known negative target of Nfix (Messina et al. 2010) – is correctly down-regulated in *Sox6* null fetal muscle, suggesting that Nfix is still able to regulate some of its target genes in the absence of Sox6. However, NFATc4 down-regulation has no effect on MyHC-I expression that is drastically up-regulated in *Sox6* null fetal muscle. Moreover, Eno3 expression is lost in the absence of Sox6, suggesting that Nfix and Sox6 cooperate for activation of *Eno3*. Taken together, these data demonstrate that functional cooperation between Nfix and Sox6 is necessary for normal fetal fiber specification in skeletal muscle.

**Functional cooperation of Sox6 and Nfixa is evolutionary conserved in zebrafish**

It is known that both the zebrafish Sox6 and Nfixa have an evolutionary conserved role in the repression of slow-twitch genes in zebrafish (von Hofsten et al. 2008; Pistocchi et al. 2013). We thus wondered whether this functional cooperation between Nfixa and Sox6 is also conserved in zebrafish myogenesis. As a preliminary analysis,

we performed immunofluorescence for Sox6 on 48 hours post-fertilization (hpf) embryos: we found that Sox6 is expressed in the majority of muscle fiber nuclei. Intriguingly, we found that the outer superficial fibers positive for sMyHC1 present cytoplasmic staining for Sox6, suggesting that Sox6 subcellular localization may be differentially regulated in slow and fast muscle domains during zebrafish development. 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 qRT-PCR analysis we found that the expression of slow-twitch specific genes such as *smyhc1* and *prox1a* is markedly increased in absence of Sox6, whereas fast-twitch genes such as *mylpfa* (fast myosin light chain isoform) and *tnnc2* (fast troponin C isoform) are expressed at equal or lower levels. Moreover, the *nfixa* transcript is drastically up-regulated since 24 hpf in *sox6* morphants, suggesting that Sox6 might negatively regulate *nfixa*, whereas *nfatc4* is prematurely down-regulated, likely reflecting high Nfixa levels. 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 suboptimal doses of *sox6*-MO and *nfixa*-MO. Strikingly, qRT-PCR results show that *smyhc1* and *prox1a* are significantly up-regulated in the double morphants, whereas *mylpfa* levels do not change. Moreover, our analysis reveals that *sox6* and *nfixa* transcripts are up-regulated in *nfixa* and *sox6* morphants respectively, suggesting a mechanism of compensation that is lost when both factors are targeted for knockdown. Therefore, we conclude that functional cooperation of Sox6 and Nfix is evolutionary conserved in mouse and zebrafish.

# DISCUSSION AND PERSPECTIVES

In the recent years, many works have demonstrated that the intrinsic transcriptional properties of embryonic and fetal muscle progenitors are important to set the fiber type in the absence of nerve activity. Moreover, several transcription factors contributing to whole muscle fiber type 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 the NFAT factors (Daou et al. 2013). However, the network of transcription factors controlling fiber type specification during embryogenesis is still far from being fully characterized, and functional interactions among the different regulators are until now completely unknown. In this work we provided evidence for functional cooperation of Nfix and Sox6 in the repression of the slow-twitch phenotype during fetal muscle development. At variance with 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 at the protein level. Consistently we found that Sox6 protein is expressed at high levels in skeletal muscle *in vivo* starting from 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 the Sox6 transcript is absent in mouse embryonic myotome from E9.5 to E10.75 (Vincent et al. 2012), suggesting that Sox6 might be quickly activated in embryonic myoblasts at the beginning of primary myogenesis. However, we found that Sox6 protein is differentially localized in embryonic and fetal muscle and during the embryonic period is mainly found in the cytoplasm of myocytes and myogenic cells. It is known that Sox factors are regulated by subcellular localization in different tissues (Malki et al. 2010). Sox6 is aberrantly accumulated in the cytoplasm of fetal fibers lacking both Six1 and Six4, thus leading to an up-regulation of slow-twitch genes (including *MyHC-I*) and a consequential decrease in fast-twitch genes expression (Richard et al. 2012). Moreover, Sox6 protein was found in the cytoplasm of muscle fibers in post-natal and adult mouse (An et al. 2011), suggesting that an unknown mechanism controlling Sox6 subcellular localization might be important in regulating Sox6

function in fiber type specification. The recent finding that Sox6 is targeted for proteasomal degradation by the E3 ubiquitin ligase Trip12 in C2C12 myoblasts (An et al. 2013), strongly suggests that Sox6 function may be specifically controlled at the post-translational level. However, Sox6 protein was also found in the nuclei of embryonic myocytes, suggesting that Sox6 may be active as a transcription factor during primary myogenesis. Consistently, we found two downstream targets of Sox6 in embryonic muscle: *MyHC-I* and *Eno3*, which are regulated in opposite ways. To our knowledge, this is the first report of a possible function for Sox6 in the transcriptional regulation of embryonic muscle. Unexpectedly, Sox6 deficiency during primary myogenesis leads to a faster muscle phenotype with low levels of MyHC-I and high levels of Eno3. This is followed in *Sox6* null fetuses by dramatic up-regulation of MyHC-I and loss of Eno3 expression. These data are consistent with previous characterizations of the *Sox6* null phenotype (Hagiwara et al. 2007; An et al. 2011; Quiat et al. 2011) and demonstrate that Sox6 plays opposite roles in fiber type specification during development. It was shown by ChIP and ChIP-seq analyses that the *MyHC-I* regulative regions contain at least two conserved binding sites for Sox6 (promoter and enhancer), while no Sox6 binding sites were found in the *Eno3* promoter (An et al. 2011). We found that Sox6 binds to both *MyHC-I* binding sites in embryonic and fetal myogenic cells but with different affinity (higher in fetal myotubes). Moreover, Sox6 binds to the *Eno3* promoter in embryonic but not in fetal myoblasts. Thus, the binding ability of Sox6 is differentially regulated in discrete myogenic progenitor populations or at different times during development, which contributes to the high versatility of Sox6 functions. In contrast, we have shown that Nfix acts as a repressor of the slow-twitch phenotype in both embryonic and fetal muscle.

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 (Murakami et al. 2001). Therefore, it is likely that the reversal in Sox6 function is due to different factors that are progressively recruited and activated during muscle development. Preferential partners of Sox6 during embryonic myogenesis are likely positive transcriptional regulators, such as the MRFs (MyoD and Myog in particular), which bind to the same regulative regions of Sox6 in muscle cells (An et al. 2011). Then, starting from E16.5, we found that Nfix acts as a fundamental co-factor of Sox6, able to physically associate with Sox6 and to facilitate its binding with the *MyHC-I*

regulative regions, in particular the proximal promoter that was shown to be critical for Sox6-dependent fetal repression (Hagiwara et al. 2007). Strikingly, our ChIP analysis reveals that in absence of Nfix the binding status of Sox6 in fetal myotubes resembles that of embryonic differentiated myoblasts, in particular for *Eno3*. Moreover, our study on *Nfix* null and *Sox6* null fetuses clearly demonstrates that Sox6 and Nfix are independently expressed during secondary myogenesis and that neither Sox6 nor Nfix are able to restore wt MyHC-I expression when the other one is not present. Finally, we demonstrated that Sox6 is required for at least two of the Nfix transcriptional functions in fetal muscle: repression of *MyHC-I* and activation of *Eno3*. The transcriptional regulation of NFATc4 and MyHC-neo was not affected, suggesting that Sox6 is dispensable for the Nfix-induced repression of *NFATc4* and for MyoD/NFATc2-induced activation of *MyHC-neo* (Daou et al. 2013).

In this work we have also provided evidence for a conserved transcriptional cooperation of Sox6 and Nfixa in zebrafish. Sox6 is expressed both at mRNA and protein levels in fast muscle fibers that are negative for sMyHC1, and it is here co-expressed with Nfixa that is required for sMyHC1 down-regulation after 72 hpf (Pistocchi et al. 2013). We show that, as in mouse, Sox6 is crucial for repression of slow-twitch genes, even in presence of elevated Nfixa levels. Moreover, our double MO assay clearly reveals that the two factors can cooperate in repressing the slow muscle phenotype, whereas expression of fast-twitch genes such as *mylpfa* (Liew et al. 2008) was unaffected.

In conclusion we have presented a complex model of regulation of the embryonic/fetal transcriptional switch that not only involves Nfix activation, driven by Pax7 and other unknown factors (Messina et al. 2010), but also functional cooperation between Nfix and Sox6 that is conserved in mammals and teleosts. Future work will be addressed to study the relationship between Sox6 and Nfix in the context of post-natal development and adult skeletal muscle growth.

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# Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells

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Skeletal muscle fibres form by fusion of mesoderm progenitors called myoblasts. After birth, muscle fibres do not increase in number but continue to grow in size because of fusion of satellite cells, the postnatal myogenic cells, responsible for muscle growth and regeneration. Numerous studies suggest that, on transplantation, non-myogenic cells also may contribute to muscle regeneration. However, there is currently no evidence that such a contribution represents a natural developmental option of these non-myogenic cells, rather than a consequence of experimental manipulation resulting in cell fusion. Here we show that pericytes, transgenically labelled with an inducible Alkaline Phosphatase CreERT2, but not endothelial cells, fuse with developing myofibres and enter the satellite cell compartment during unperturbed postnatal development. This contribution increases significantly during acute injury or in chronically regenerating dystrophic muscle. These data show that pericytes, resident in small vessels of skeletal muscle, contribute to its growth and regeneration during postnatal life.

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Satellite cells are myogenic stem/progenitor cells of postnatal skeletal muscle and have a prominent role in tissue growth and regeneration. Recent work has demonstrated that, like embryonic myoblasts, most, if not all, satellite cells in the body originate from the Pax3+/Pax7+ cells in the somites and become detectable in their specific niche, underneath the basement membrane of skeletal muscle fibres, from approximately E16.5 in the mouse[1–4]. However, numerous reports indicate that, on transplantation, cells from tissues other than muscle (for example, bone marrow, brain, adipose tissue and blood vessels) can fuse with regenerating muscle fibres and also contribute to the satellite cell pool[5–8]. It is therefore important to understand whether contribution to skeletal muscle by non-myogenic progenitors is restricted to artificial conditions created by transplantation, or whether it occurs during unperturbed pre- and post-natal development. To address this question, we used a Cre-loxP lineage tracing strategy to follow the *in vivo* fate of progenitors associated with the vasculature. Recently, we reported that pericytes associated with the micro-vasculature of human muscle are able to differentiate into skeletal muscle *in vitro* and *in vivo*, on transplantation into dystrophic, immune-deficient mice[9]. A major problem in pericyte biology is the lack of specific markers[10]. Thus, we chose alkaline phosphatase (AP) that, in contrast to other pericyte markers such as the neuro-glial 2 proteoglycan (NG2)[11] or platelet-derived growth factor receptor beta (PDGFRβ)[12], is not expressed in skeletal muscle fibres, nor in myogenic progenitors, but is restricted to the micro-vasculature of striated muscle in postnatal life. This strategy allowed us to label a subset of pericytes and endothelial cells, as in the mouse both of these cell types express AP. A second reporter mouse *Tg:VE-Cadherin(PAC)-CreERT2* (ref. 13) allowed us to selectively follow endothelial cell fate inside skeletal muscle. Our data show that during postnatal growth, pericytes, but not endothelial cells, contribute to fibre development and to the satellite cell pool and, that this contribution is further enhanced by acute or chronic tissue regeneration.

## Results

**Alkaline phosphatase is expressed in muscle microvessels**. In preliminary studies, we examined in great detail the expression of AP in postnatal and adult skeletal muscle. AP expression is robust and widespread in tissues such as intestine, bone and liver, whereas it is restricted to the micro-vasculature of other tissues, such as striated muscles, in many species[14–16] including human[9]. We performed extensive immunofluorescence, confocal analysis on adult and juvenile skeletal muscle to confirm AP expression in vessel-associated cells. Both AP+ endothelial cells and pericytes were unequivocally identified by their anatomical niche and by labelling with different antibodies that recognize either one or the other cell population (Fig. 1a–l; Supplementary Fig. S1a–c).

We never detected AP+ cells that are not associated with small vessels; on the other hand, less than 20% of total vessels express AP. The *in vivo* expression pattern of AP in postnatal skeletal muscle was also confirmed by analysing mice carrying one copy of the *TN-AP*$^{\beta geo}$ allele[17]. In this mouse strain, β-gal+ cells are only associated with vessels, whereas myofibres and satellite cells are always unlabelled (Supplementary Fig. S1d).

In skeletal muscle, pericytes and satellite cells are often adjacent to each other[18] (arrows in the Fig. 1m); however, AP was never detected in Pax7+ or MyoD+ satellite cells at any time examined; an example at P7 is shown in Supplementary Figure S1e,f, respectively. These results were confirmed by enzymatic staining for AP on muscles sections from the *Myf5*$^{nlacZ/+}$ mouse (expressing nuclear β-gal only in Myf5+ cells[19]) (Fig. 1n), even after cardiotoxin-induced regeneration (Supplementary Fig. S1g–j). Notably, AP activity was invariably detected outside the basement membrane encircling skeletal muscle fibres and co-localized with platelet endothelial cell adhesion molecule (Pecam)-positive vessels (Fig. 1o). Moreover, in single myofibres isolated from *EDL* or *Tibialis anterior* (*TA*) of P30 *Myf5*$^{nlacZ/+}$ mice, satellite cells were found not to express AP during activation, proliferation or differentiation (Supplementary Fig. S1k–m). In contrast, nonpurified primary cultures of skeletal muscle contained both AP+ and Myf5+ cells, but double positive cells were never detected (Supplementary Fig. S1n). Satellite cells-derived clones did not express AP during proliferation (5 days *in vitro*) or after differentiation (10 days *in vitro*) (Supplementary Fig. S1o,p). Taken together, these results show that, in mouse postnatal skeletal muscle, AP is only expressed by a subset of vessel-associated cells, whereas myogenic cells and myofibres never express AP.

**Tissue nonspecific alkaline phosphatase CreERT2 mice**. Three isoforms of AP have been identified in mouse: the placental (P-AP) and the intestinal (I-AP) isoforms on chromosome 1, and tissue nonspecific AP (TN-AP) on chromosome 4. By RT–PCR with primers specific for the different isoforms[20], we found that adult skeletal muscle express exclusively TN-AP (Supplementary Fig. S2a). To follow the fate of AP+ cells in postnatal skeletal muscle, we generated three transgenic *Tg:TN-AP-CreERT2* mouse lines (#3; #6; #12) (Supplementary Fig. S2b,c). After crossing with *R26R* reporter, the three transgenic lines express similar efficiency of Cre recombination, with little variability among different animals. No signal outside AP+ cells was detected in any of the mouse lines. We chose, for the majority of subsequent experiments line #12, whose founder was more fertile. However, several crucial experiments, such as contribution to muscle fibres, were repeated in the different lines, again with similar results.

To examine the efficiency of recombination during postnatal skeletal muscle development, we subcutaneously injected *Tg:TN-AP-CreERT2:R26R* mice with tamoxifen for 3 consecutive days at P6, P7 and P8, and skeletal muscles were analysed 3 days after the last injection. Approximately 70% of AP+ vessels also express β-gal, indicating efficient recombination, and no β-gal+/AP-negative cells were detected (Fig. 2a). Whole-muscle X-gal staining revealed β-gal+ vessels as expected (Diaphragm and *Quadriceps* muscles are shown in Fig. 2b,c, respectively). Moreover we demonstrated, on skeletal muscle sections from *Tg:TN-AP-CreERT2* mice that also carry the *R26*$^{NZG}$ reporter[21], that β-gal is expressed by both Pecam+ endothelial cells (Fig. 2d,e) and PDGFRβ+ (Fig. 2f,g) or NG2+ (Fig. 2h–k) pericytes at a ratio of about 1:1 (quantification in Fig. 2l: 500 β-gal+ cells were analysed.). The same results were confirmed also with *Tg:TN-AP-CreERT2:R26R* mice. (Supplementary Fig. S2d,e). After control oil injections no β-gal signal was detected (Supplementary Fig. S2f,g).

To further validate this transgenic mouse model and to unequivocally rule out that the Cre recombinase might be activated by satellite cells, we prepared single fibres and nonpurified cells suspension from skeletal muscle of *Tg:TN-AP-CreERT2:R26R-EYFP* mice. Both preparations were cultured for 5 days with 4-OH tamoxifen: no yellow fluorescent protein (YFP) positive cells were found in purified, cultured muscle fibres (Fig. 2m,n); whereas in nonpurified primary cultures both YFP and MyoD were expressed, but never in the same cell (Fig. 2o,p).

By RT–PCR, we showed that freshly isolated YFP+ cells from *Tg:TN-AP-CreERT2:R26R-EYFP* mice (killed 2 days after the last tamoxifen injection), express *TN-AP* but not *Pax7* and *Myf5* messenger RNAs, as opposed to freshly isolated SM/C2.6+ satellite cells from the same mice (Supplementary Fig. S2h). Moreover, quantitative RT–PCR showed that YFP+ cells express high levels of *Cre*, while satellite cells isolated from the same mice do not (Supplementary Fig. S2i; as positive control, we used C2C12 cells infected with recombinant AAV-*Cre*). These results indicate that satellite cells and

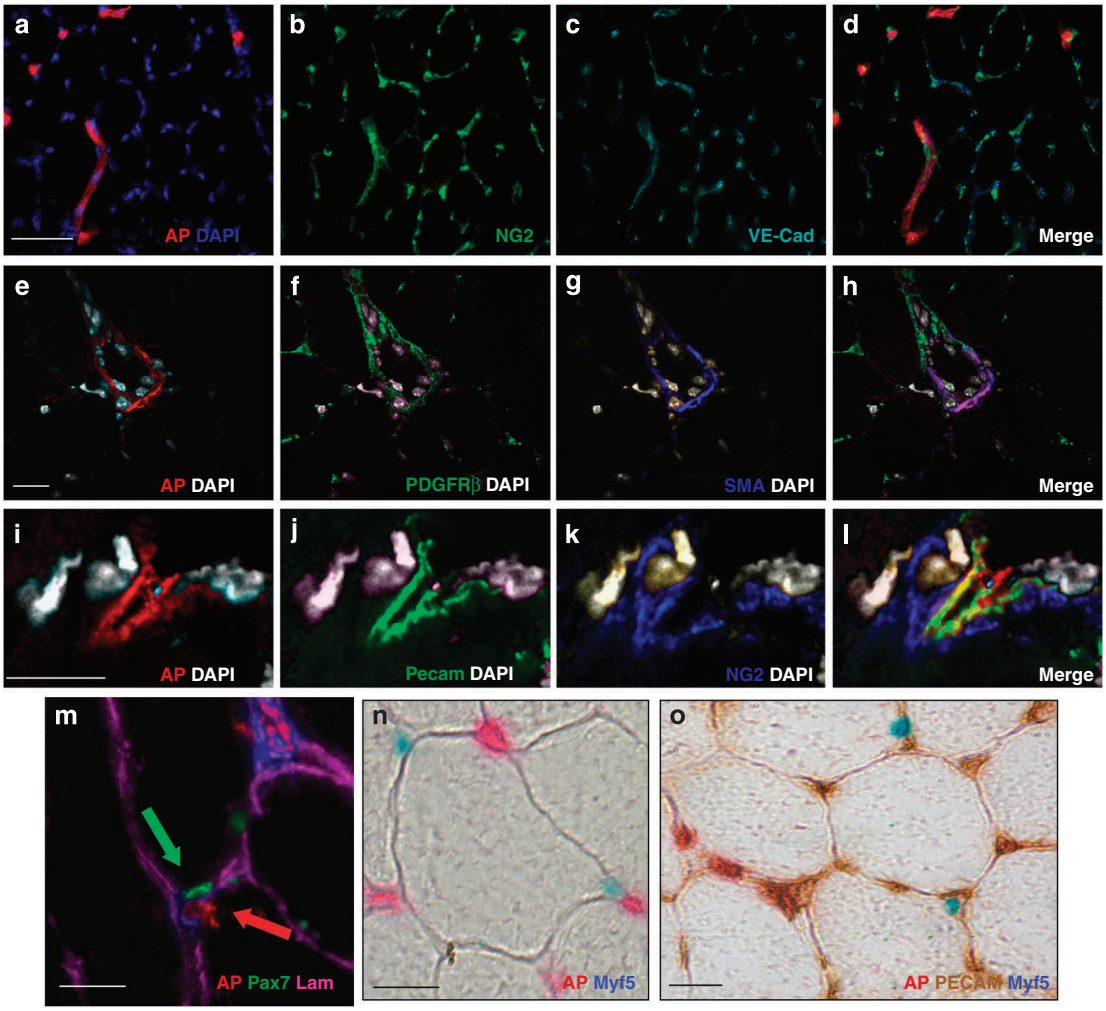


**Figure 1 | AP expression in mouse postnatal skeletal muscle.** (**a**–**d**) Immunofluorescence analysis on 1-month-old mouse muscle sections shows localization of AP within vessel-associated cells. (**a**) AP+ cells are stained in red, nuclei are stained in blue by DAPI (**b**) NG2+ cells are stained in green, (**c**) Ve-Cadherin+ cells are stained in cyan, (**d**) merged image. (**e**–**h**) AP is expressed by PDGFRβ+/SMA+ pericytes. (**e**) AP+ cells are stained in red, nuclei are stained in grey by DAPI, (**f**) PDGFRβ+ cells are stained in green, nuclei are stained in grey by DAPI (**g**) SMA+ cells are stained in blue and nuclei in grey by DAPI, (**h**) merged image. (**i**–**l**) AP is expressed by Pecam+ endothelial cells, surrounded by an AP-negative pericyte. (**i**) AP+ cells are stained in red, nuclei are stained in grey by DAPI, (**j**) Pecam+ cells are stained in green, nuclei are stained in grey by DAPI, (**k**) NG2+ cells are stained in blue, nuclei are stained in grey by DAPI, (**l**) merged image. (**m**) Immunostained *TA* muscle (15-days-old) shows a Pax7+ satellite cell (green arrow), and an AP+ vessel-associated cell (red arrow); Laminin in magenta. We counted 170 AP+/Pax7− cells and 102 AP−/Pax7+ cells in *TA* muscle and 438 AP+/Pax7− cells and 138 AP−/Pax7+ cells in *Soleus* muscle: no co-localization is detected. (**n**,**o**) $Myf5^{nlacZ/+}$ *TA* muscle sections stained for X-gal (blue) and AP (red); Pecam is stained brown in (**o**). (**a**–**d**) Scale bar, 100 μm. (**e**–**o**) Scale bar, 20 μm.

their derivatives never express the transgenic *TN-AP-CreERT2* construct, both *in vivo* and *in vitro*.

**AP-derived cells contribute to postnatal skeletal muscle**. To analyse the fate of AP+/β-gal+ cells, *Tg:TN-AP-CreERT2:R26R* mice were genetically labelled as described above and were killed 8 weeks later, at the end of their growth period. We detected several areas with β-gal+ fibres in all muscles analysed (*Quadriceps*, *Gastrocnemius* and *Biceps femoris* muscles are shown in Fig. 3a–c). Skeletal muscle sections showed many β-gal+ muscle fibres together with β-gal+ vessels (Fig. 3d,e) and several β-gal+/MyHC+ fibres (Fig. 3f). β-gal expression was randomly distributed between fast and slow fibres. The highest percentage of β-gal+ myofibres (7.3±1.2%) was observed in the Diaphragm whereas the lowest was in the *TA* muscle (0.6±0.2%) (Fig. 3g). We also confirmed these results by assaying β-gal activity in homogenates from different muscles (Methods) (Fig. 3h). Similar results were obtained using *Tg:TN-AP-CreERT2:*$R26^{NZG}$ mice (Fig. 3i, quantification in Fig. 3g), where we could clearly detect blue nuclei inside skeletal muscle fibres (Fig. 3j,k), ruling out the possibility of nonspecific staining due to β-gal diffusion inside the tissue. Tamoxifen injections at P1, P2 and P3 resulted in high pup lethality and did not significantly increase the percentage of labelled myofibres, suggesting that the contribution of AP-expressing cells to skeletal muscle remains constant in the first week of postnatal life. On the other hand, later tamoxifen injections, from P8 to 1 month of age, resulted in a progressively lower amount of β-gal+ fibres in adult life, indicating that the myogenic potential of AP+ cells decreases with subsequent mouse growth.

Next, we investigated whether AP+ cells might contribute to the satellite cell pool. To this end, muscles of *Tg:TN-AP-CreERT2:R26R* mice were collected at different time points after tamoxifen induction. No β-gal+/Pax7+ satellite cells were observed *in vivo* 3 days after the last tamoxifen injection; moreover, freshly isolated

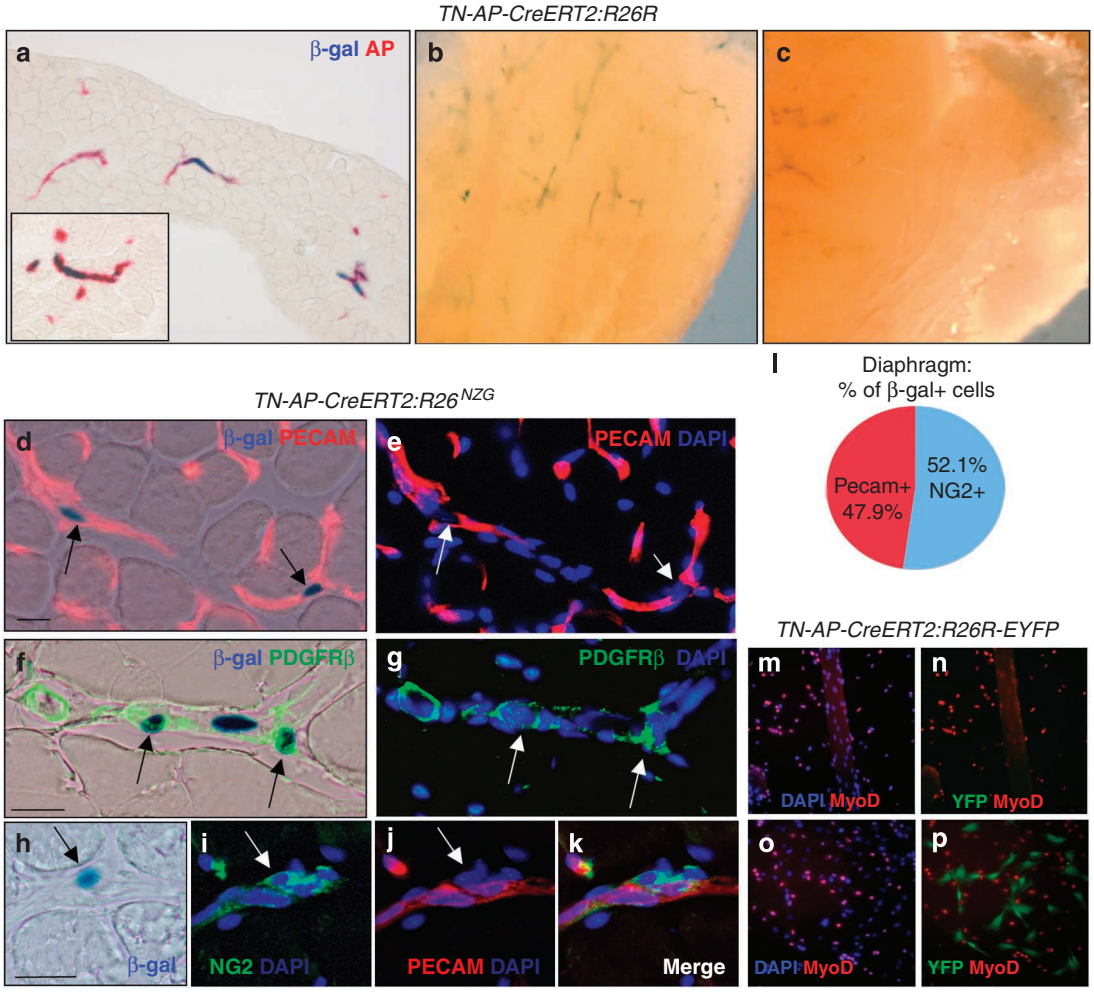


**Figure 2 | *Tg:TN-AP-CreERT2* mouse generation.** (**a**) Section of Diaphragm muscle from double heterozygous *Tg:TN-AP-CreERT2:R26R* mice stained for β-gal (blue) and grey (red) 72 h after tamoxifen injection. Double-positive β-gal+/AP+ vessels are clearly visible. High magnification in the inset. Scale bar, 100 μm. (**b**,**c**) X-gal whole-mount staining of Diaphragm (**b**) and *Quadriceps* (**c**) from *Tg:TN-AP-CreERT2:R26R* mice, 72 h after last tamoxifen injection. (**d**–**k**) *Tg:TN-AP-CreERT2:R26*$^{NZG}$ *Triceps* muscle sections, 72 h after tamoxifen injection, stained with X-Gal and antibodies specific for the endothelial or pericyte lineages. (**d**,**e**) Two β-gal+/Pecam+ endothelial cells are visible (arrows). Pecam is stained in red, nuclei are counterstained with DAPI. Scale bar, 20 μm. (**f**,**g**) Two β-gal+/PDGFRβ+ pericytes surrounding a small vessel. PDGFRβ is stained in green, nuclei are counterstained with DAPI. Scale bar, 20 μm. (**h**–**k**) A β-gal+/NG2+ pericyte surrounding a small vessel. Note that the pericyte is negative for the endothelial marker Pecam. NG2 is stained in green; Pecam in red; nuclei are counterstained with DAPI. Scale bar: 20 μm. (**l**) Distribution of β-gal+ cells within the endothelial and pericyte populations for the Diaphragm muscle; 500 β-gal+ cells were analysed. (**m**,**n**) Single fibre preparation from *Tg:TN-AP-CreERT2:R26R-EYFP TA* muscle cultured for 5 days with 4-OH tamoxifen and stained with anti-MyoD (red) and anti-GFP antibodies (green); DAPI in blue. No YFP+ cells were found in purified fibre preparations. Scale bars, 50 μm (**o**,**p**) Nonpurified cell suspension of *TA* muscle from *Tg:TN-AP-CreERT2:R26R-EYFP* mice, cultured for 5 days with 4-OH tamoxifen and stained for MyoD (red) and GFP (green); DAPI in blue. Scale bars, 50 μm.

satellite cells did not express β-gal activity. However, 1 week after tamoxifen injection, few double-positive satellite cells were identified *in vivo* (Fig. 4a) (0.19% of the total Pax7+ cells in the *TA* and 2.5% in the *Pectoralis*). At later stages, the percentage of double-positive Pax7+/β-gal+ satellite cells progressively increased in all muscles analysed, reaching, for example, 25% of total Pax7+ cells at 1 month after tamoxifen injection in the *Pectoralis* muscle (Fig. 4b). An example of Pax7+/β-gal+ satellite cells underneath the basal lamina of a β-gal-negative fibre is shown in Figure 4c–f. Quantifications of Pax7+/β-gal+ satellite cells detected with time are shown in Figure 4g. Although the total number of Pax7+ satellite cells decreases with time, the number of double-positive Pax7+/β-gal+ progressively increases, reaching a plateau at 3 weeks after tamoxifen induction; a detailed quantification for the *Pectoralis* muscle is shown in Figure 4h. Interestingly, we found a higher number of double-positive Pax7+/β-gal+ cells in the same muscles where we detected more β-gal+ fibres, suggesting that AP-derived cells contribute to skeletal muscle formation mainly by entering the satellite cell compartment, although direct fusion with developing muscle fibres may occur as well.

**Muscle regeneration enhances AP+ pericyte myogenesis**. *Tg:TN-AP-CreERT2:R26R* mice were treated with tamoxifen at P6–P8 and then injected with cardiotoxin in *TA* and *Gastrocnemius* muscles at P30; three weeks after injury all treated muscles showed a strong increase in the percentage of β-gal+ fibres in comparison with the contralateral, uninjured muscle (Fig. 5a,b). A section at high magnification, showing centrally located nuclei, typical of regenerating fibres, is shown in Figure 5c; quantification is shown in Figure 5d. These experiments were confirmed with *Tg:TN-AP-CreERT2:R26R* line #3 and using *Tg:TN-AP-CreERT2:R26*$^{NZG}$ mice (Fig. 5e).

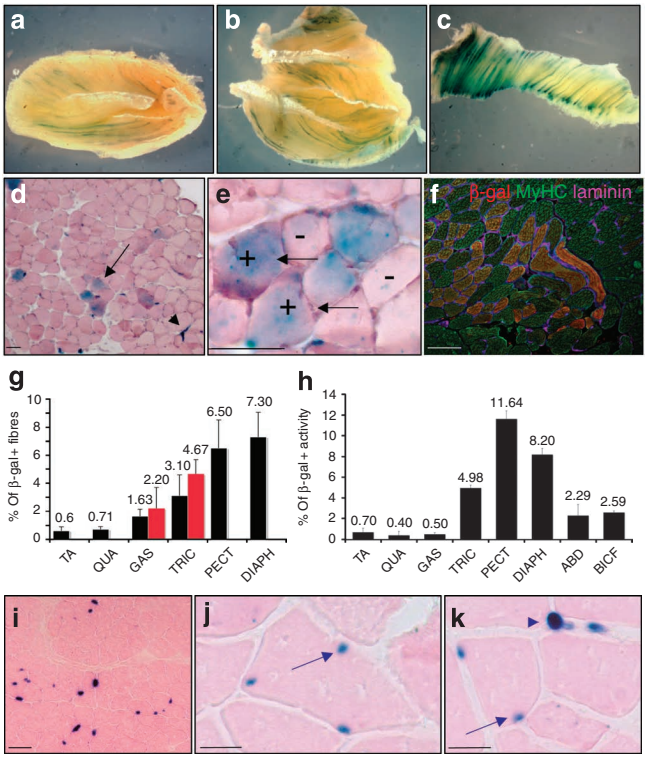


**Figure 3 | Cells derived from TN-AP+ pool contribute to skeletal muscle fibre development.** (**a**–**c**) β-gal staining of three different muscles, 2 months after tamoxifen injection: *Quadriceps* (**a**) *Gastrocnemius* (**b**), *Biceps femoris* (**c**). (**d**,**e**) *Quadriceps* muscle sections stained for β-gal; blue fibres (arrow) along with blue vessels (arrowhead) are visible. The sign (+) marks β-gal+ fibres, whereas the sign (−) marks fibres with very low or no β-gal expression. (**f**) Immunofluorescence on sections from the same muscles shown in (**d**) and in (**e**) stained with anti-β-gal (red), anti-MyHC (green) and anti-Laminin (magenta) antibodies confirms the presence of β-gal positive fibres. Scale bar, 100 µm. (**g**) Quantification of β-gal+ fibres counted on the total fibre number. Different muscles were analysed. Number of experimental animals: $n=15$ for Tg:*TN-AP-CreERT2:R26R* mice, black bars; $n=5$ for *Tg:TN-AP-CreERT2:R26*$^{NZG}$, red bars. Error bars represent standard deviation. (**h**) β-gal activity measurement on muscle homogenates. Standard curve was obtained by different dilutions of *CMV-Cre-R26* and wt muscles. β-gal activity obtained 1 week after the last tamoxifen injection in *TN-AP-CreERT2:R26R* mice (representing labelling of the vessels) was subtracted from the obtained values. QUA, *Quadriceps*; GAS, *Gastrocnemius*; TRIC, *Triceps*; PECT, *Pectoralis*; DIAPH, Diaphragm; ABD, *Abdominal*; BICF, *Biceps femoris*. Number of experimental animals: $n=5$. Error bars represent standard deviation. (**i**–**k**) Muscle sections of *Tg:TN-AP-CreERT2:R26*$^{NZG}$ injected with tamoxifen at P6–P8 and killed 2 months later, stained with X-gal and Eosin. Arrows show β-gal+ nuclei inside skeletal muscle fibres; arrowhead shows β-gal+ nuclei of vessel-associated cells. Scale bar, 100 µm (**i**) or 20 µm (**j**,**k**).

To investigate whether AP+ pericyte-derived satellite cells were able to support a second round of regeneration, six *Tg:TN-AP-CreERT2:R26*$^{NZG}$ mice at 1 month of age were injected with cardiotoxin in both *TA*, as described above. After three weeks, a second cardiotoxin injection (at the same dose) was performed in the right *TA* only. Mice killed 24 h after the second injection showed complete destruction of previously regenerated fibres (Fig. 5f), as compared with contralateral muscles that had received only one injection and appeared similar to what shown in Figure 5e. Mice killed after three extra weeks showed the presence of new β-gal+, centrally nucleated fibres (Fig. 5g).

**Muscular dystrophy also enhances AP+ pericyte myogenesis.** To test whether AP+ pericytes increase their contribution to skeletal muscle during the degeneration/regeneration cycles that characterize the progression of muscular dystrophy, *Tg:TN-AP-CreERT2:R26R:αSG* −/− dystrophic pups and their heterozygous littermates *Tg:TN-AP-CreERT2:R26R:αSG* +/− were injected with tamoxifen at P6–P8 and killed 2 months later. In dystrophic skeletal muscle, we observed a very high percentage of β-gal+ fibres, up to three times more than control mice (Fig. 6a,b). Skeletal muscle sections showed that the regenerated fibres robustly express β-gal (Fig. 6c; quantification in Fig. 6d).

**AP+ pericyte myogenesis is minimal in adult muscle.** Two- and three-month-old male *Tg:TN-AP-CreERT2:R26R* mice were treated intraperitoneally with tamoxifen for 3 consecutive days and, 1 week after tamoxifen injection, almost 70% of AP+ cells were β-gal+. β-gal expression always co-localized with Pecam+ or NG2+ vessel-associated cells (Supplementary Fig. S3a,b).

Two months after tamoxifen injection, all muscles examined showed many β-gal+ vessels; in ~30% of treated mice, we detected very few β-gal+ fibres, only short fragments of which were labelled, suggesting focal contribution of few AP-derived cells during adult life (Supplementary Fig. S3c). Moreover, this minimal contribution did not significantly increase after cardiotoxin-induced regeneration (0.15±0.1 in control *TA* and 0.93±0.4 in cardiotoxin *TA*; $n=4$) (Supplementary Fig. S3e–h; quantification in Supplementary Fig. S3d). The contribution did not increase even when mice were injected with tamoxifen for 5 consecutive days.

**Endothelial cells do not contribute to postnatal myogenesis.** As AP is expressed in both pericytes and endothelial cells, we investigated whether one or both cell types contribute to skeletal muscle formation *in vivo*. *Tg:VE-cadherin-(PAC)CreERT2:R26R* mice[13] were injected at P6–P8 with tamoxifen. Because VE-Cadherin is expressed only in endothelial cells[22], we could selectively follow the fate of these cells. Three days after induction, 87±5% of total vessels were β-gal+ (Fig. 7a) and remained β-gal+ even in the mouse adult life (Fig. 7b,c), indicating a very efficient recombination in this transgenic mouse strain. Neither satellite cells nor myofibres were labelled 3 days or 1 week after tamoxifen injection (Fig. 7d) or, most importantly, 1 or 2 months after tamoxifen injection, in any of the muscles analysed (Fig. 7e). This was also the case after muscle regeneration induced by cardiotoxin injection (Fig. 7f,g). Taken together, these results indicate that in postnatal life, endothelial cells do not contribute to skeletal muscle growth or regeneration, nor to the satellite cell pool.

**AP+ cells generate smooth or skeletal myogenic clones *in vitro*.** To confirm a possible progressive recruitment of AP+ cells into the satellite cell compartment, YFP+ cells, isolated from *Tg:TN-AP-CreERT2:R26R-EYFP* mice (Fig. 8a) at different time points (3 days, 1 week, 1 and 2 months) after tamoxifen injection (P6–P8), were stained for Pax7 (Fig. 8c–e; quantification Fig. 8b). The percentage of double-positive Pax7+/YFP+ cells progressively increased during the first month of life from 0%, 3 days after tamoxifen injection, to 3.7±1.4% after 1 week, and 25.7±2.9% after 1 month, remaining at a similar level at 2 months (30.3±5.2%). One month after cardiotoxin muscle injury, tamoxifen-treated mice showed a similar percentage of YFP+ cells co-expressing Pax7 (29.5±5.4%) (Fig. 8b), suggesting that AP-derived satellite cells can self-renew and maintain a constant contribution to the satellite cell pool. These results were confirmed by quantitative RT–PCR for *Pax7* and *Myf5* (Supplementary Fig. S4a). Surprisingly, AP-derived satellite cells did not express

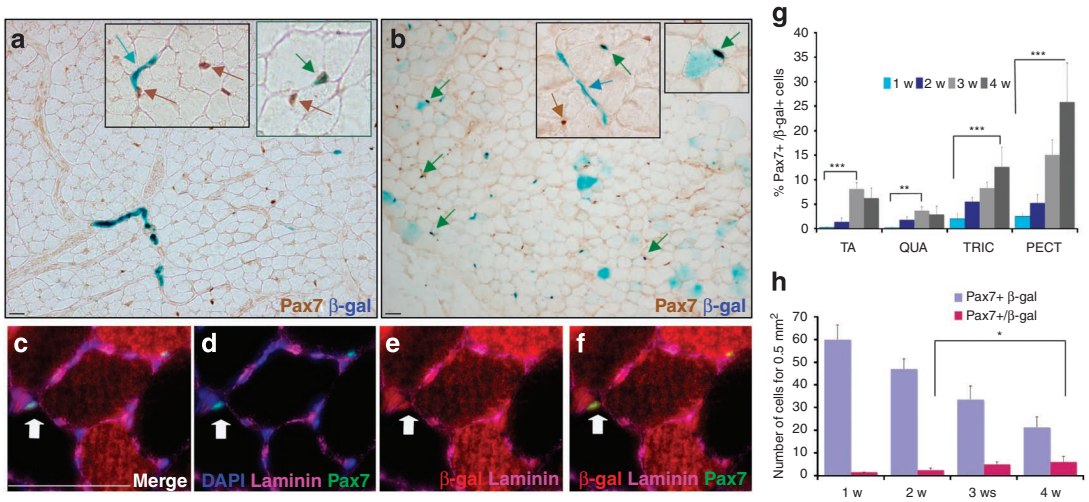


**Figure 4 | Generation of satellite cells from AP+-derived cells during mouse growth.** (**a**) Immunohistochemistry on *Tg:TN-AP-CreERT2:R26R Pectoralis* muscle sections, 1 week after tamoxifen injection, stained for β-gal (blue) and anti-Pax7 (brown). The majority of Pax7+ cells are β-gal negative (brown arrows), whereas vessels are blue (blue arrows). Only few double-positive cells are detectable (green arrow). Insets show high magnification. (**b**) Immunohistochemistry on *Tg:TN-AP-CreERT2:R26R Pectoralis* muscle sections 1 month after last tamoxifen injection stained for β-gal (blue) and Pax7 (brown). Several Pax7+/β-gal+ satellite cells were detected (green arrows) together with β-gal+ fibres. Insets show high magnification. (**c**–**f**) *TA* muscle section of *Tg:TN-AP-CreERT2:R26R* mouse, 2 months after tamoxifen injection. A Pax7+ (green) satellite cell also expresses β-gal (red) (arrows); the satellite cell is associated to a β-gal-negative fibre. Laminin is stained in magenta. Scale bar, 100 μm. (**g**) Pax7+/β-gal+ satellite cells counted on the total number of Pax7+ satellite cells, in different muscles and at different time points after tamoxifen induction: 1 week (pale blue), 2 weeks (dark blue), 3 weeks (pale grey), 4 weeks (dark grey). Number of experimental animals: $n = 5$. Error bars indicate standard deviation. Results were assessed for statistical significance by Student's *t*-test (unpaired, two-tailed) and differences were considered statistically significant at $P < 0.05$. Asterisks in the histogram indicate the different *P* values: $^{**}P < 0.01$ and $^{***}P < 0.001$. (**h**) Total number of satellite cells for microscopic field (~0.5 $mm^2$) at different time points after tamoxifen induction in *Pectoralis* muscle. Blue bars indicate the number of Pax7+/β-gal− satellite cells; red bars indicate the number of Pax7+/β-gal+ satellite cells. The total number of Pax7+ cells decreases with time, whereas the number of double-positive Pax7+/β-gal+ cells increases until 3 weeks after tamoxifen induction, remaining constant afterwards. Number of experimental animals: $n = 5$. Error bars indicate standard deviation. Results were assessed for statistical significance by Student's *t*-test (unpaired, two-tailed) and differences were considered statistically significant at $P < 0.05$. Asterisk in the histogram indicates *P* value: $^{*}P < 0.05$.

*Myf5*, suggesting that they may belong to the Pax7+/Myf5−, self-renewing satellite cell population[23].

To test the skeletal myogenic potential of AP-expressing vascular progenitors, we analysed the ability of AP-derived cells to give rise to myogenic clones. YFP+ cells were isolated 1 week after tamoxifen induction (P6–P8) and plated at clonal density in 96 multiwells (1 cell per well). On average, 10% of plated cells gave rise to clones and 30% of these clones contained MyHC-expressing myocytes and myotubes (Fig. 8f), indicating that AP-Cre+, vessel-associated cells have a significant spontaneous myogenic potential after isolation. Most (>90%) of the remaining MyHC-negative clones expressed smooth muscle actin (SMA) (Fig. 8g), in agreement with their pericyte origin and smooth muscle fate. This was confirmed by staining with an antibody recognizing the cytoskeletal protein SM22α, a smooth muscle-specific marker[24] (Fig. 8h); very few clones (less than 1%) contained both MyHC+ and SM22α+ cells (Fig. 8i), indicating that very few AP+ cells maintain a double potency, once isolated and cloned *in vitro*. Freshly isolated YFP+ cells express pericyte and endothelial genes such as Tie2, (endothelial-specific receptor tyrosine kinase 2), VEGFR2 (vascular endothelial growth factor receptor 2), AnnexinV, SMA and PDGFRβ (Supplementary Fig. S4b) and, on *in vitro* culture, YFP+ cells maintain pericyte markers such as NG2, SMA and desmin (Supplementary Fig. S4c–e).

To further investigate the myogenic differentiation process of AP-derived pericytes and its relation with muscles expressing Pax3 *in vivo*, we sorted YFP+ cells from forelimb muscles (whose satellite cells are known to express Pax3) and hindlimb muscles of *Tg:TN-AP-CreERT2:R26R-EYFP* mice, either 1 and 8 weeks after last tamoxifen injection. At different times in culture, we followed Pax7, Pax3 and Myf5 expression by immuno-fluorescence. A high percentage of YFP+ cells, sorted 1 week after last tamoxifen injection, was found to express Pax3; 60% of the total YFP+ cells from the forelimb, and 25% from hindlimb (Supplementary Fig. 5a–c). Pax3 expression rapidly decreases after 48 h in culture, when Myf5 expression first appears. Pax7 is never expressed in culture, indicating that the myogenic differentiation of AP-derived pericytes, in culture, is Pax7-independent (Supplementary Fig. S5d–f). On the other hand, the protein expression profile of YFP+ cells sorted from *Tg:TN-AP-CreERT2:R26R-EYFP*, 8 weeks after last tamoxifen injection, is different and comparable to the typical satellite cells profile. In fact, Pax7 is expressed in many cells (50%) until 72 h in culture and decreases thereafter with a concomitant increase of Myf5 expression (Supplementary Fig. S5g–i) At this stage Pax3 is present at a low frequency (below 10%) only in cells obtained from forelimb muscles. Quantification of myogenic gene expression in YFP+ cells during time in culture, is shown in Supplementary Figure S5j,k, respectively, 1 week or 8 weeks after tamoxifen injection. Direct quantitative comparisons between Pax3 and Pax7 is not possible since Pax3 is also expressed in some smooth muscle cells[25] whereas Pax7 is not. In both cases, YFP+ cells, obtained 1 week or 8 weeks after tamoxifen injection, begin to form myotubes after 5 days in culture. Each experiment was repeated three times with six mice each; polyclonal populations were analysed after 24 and 48 h in culture, whereas single cell-derived clones were analysed at later time points, when clones became clearly identifiable.

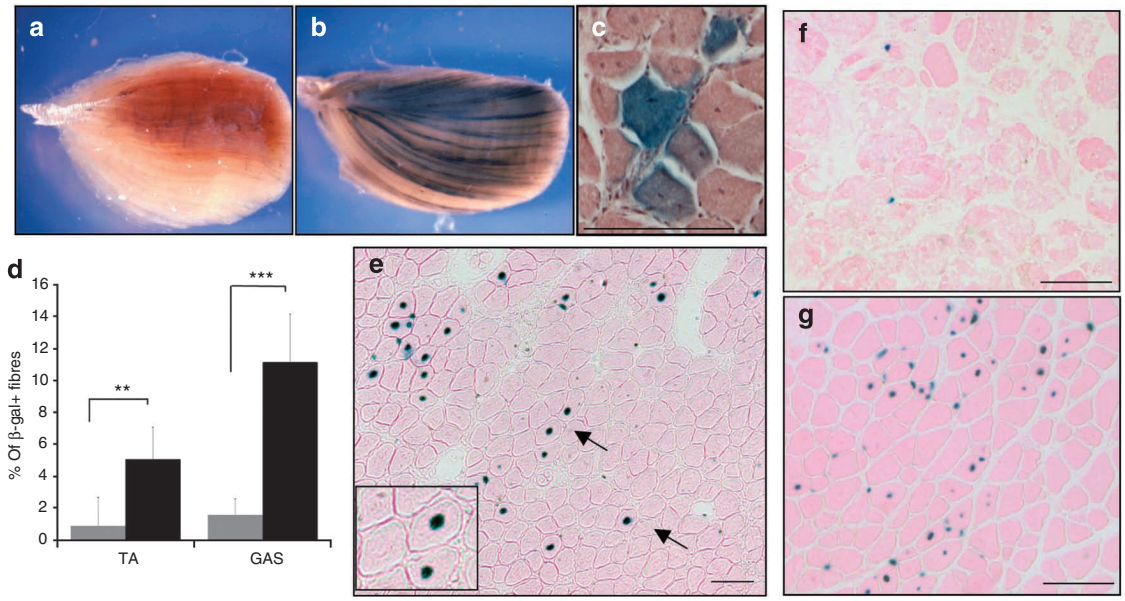


**Figure 5 | AP+ pericytes contribution to skeletal muscle after acute injury.** (**a**,**b**) Whole-mount X-Gal staining of *Tg:TN-AP-CreERT2:R26R* untreated (**a**) or cardiotoxin-treated (**b**) *TA* muscle showing a dramatic increase in the number of β-gal+, regenerated fibres. (**c**) Section of muscles shown in (**b**), stained for β-gal and H&E; β-gal+ centrally nucleated fibres are visible. (**d**) The percentage of β-gal+ fibres is increased from 0.9% in untreated *TA* muscle (grey histogram) to 5.07% in cardiotoxin-treated *TA* muscle (black histogram). In *Gastrocnemius* (GAS), the percentage of β-gal+ fibres is increased from 1.6% in untreated leg (grey histogram) to 11.15% in cardiotoxin-treated (black histogram). Number of experimental animals: $n=5$; error bars indicate standard deviation. Results were assessed for statistical significance by Student's *t*-test (unpaired, two-tailed) and differences were considered statistically significant at $P<0.05$. Asterisks in the histogram indicate the different *P* values: $^{**}P<0.01$ and $^{***}P<0.001$. (**e**) *TA* section of *Tg:TN-AP-CreERT2:R*$^{NZG}$ mice injected with tamoxifen at P6–P8, treated with cardiotoxin at 3 weeks and killed 3 weeks after damage. Many regenerating, centrally nucleated fibres are present (arrows); high magnification is shown in the inset. (**f**,**g**) *TA* sections of *Tg:TN-AP-CreERT2:R*$^{NZG}$ mice injected with two rounds of cardiotoxin and killed 24 h (**f**) or 3 weeks (**g**) after the second injury (details in the text). Scale bar, 100 μm.

## Discussion

Postnatal myogenesis is mainly due to proliferation of juvenile satellite cells that contribute to the development of skeletal muscle by fusing with growing myofibres, and, to its regeneration, by repairing or replacing damaged or degenerated myofibres[26]. Nevertheless, in the last ten years, evidence has accumulated that other progenitors can contribute to skeletal muscle regeneration either by direct fusion or by entering the satellite cell pool[6–8,27–36]. When identified, the anatomical niche of these cells has been often associated with blood vessels (endothelial cells, pericytes and also haematopoietic cells). However, the embryonic origin of these unorthodox myogenic cells and their lineage relationship with satellite cells remain unknown. It was similarly unclear whether skeletal myogenesis is one possible developmental option of these non-myogenic progenitors, or whether it represents the artificial consequence of cell transplantation that, through occasional fusion with regenerating fibres, exposes the donor cell nucleus to the dominant effect of MyoD.

In this work, we used *Tg:TN-AP-CreERT2* mice to follow the fate of vessel-associated cells during postnatal muscle growth. AP is broadly expressed in many pre- and postnatal tissues, and in different cell types such as osteoblasts[37], embryonic stem cells[38], bone marrow stroma cells[39], liver and kidney cells[40], granulocytes[41] and B cells[42]; however, in postnatal mouse striated muscles, AP is expressed only by vessel-associated cells, in both endothelial cells and pericytes, as previously reported[9,14–16], and exhaustively confirmed in this work through a variety of histochemical and immunochemical methods. Most importantly, by using virtually all possible experimental approaches, we demonstrated that both AP and Cre expression are invariably absent in quiescent satellite cells, as well as in proliferating and differentiating myogenic cells *in vitro* or *in vivo*, even after cardiotoxin-induced damage. These preliminary results rule out the possibility of inappropriate lineage tracing, because of undetected expression in different cell types resident in skeletal muscle, and allowed us to investigate the fate of AP-derived vascular cells in growing postnatal skeletal muscle. A time course of reporter gene expression showed that, beside their expected contribution to the micro-vascular wall, pericytes, but not endothelial cells (as revealed by an endothelial-specific CreERT2 mouse), contribute to the growth of a minor but significant percentage of muscle fibres. Moreover, many AP-derived cells enter the satellite cell pool, with a relatively fast kinetics during the first month of postnatal life, suggesting that they also contribute to subsequent muscle regeneration. Indeed, conditions that trigger acute or chronic skeletal muscle regeneration, such as cardiotoxin injection or muscular dystrophy, increase dramatically AP+ cell contribution to skeletal myogenesis (up to five times in the area of acute regeneration and three times in dystrophic muscle). These results show that AP+ pericytes respond to pathological conditions, and, thus, they may serve as a tool for the cell therapy of dystrophic patients and justify the rationale for a clinical trial, with human AP+ pericytes, that is currently running in our Institution.

We found the highest number of β-gal+ fibres and β-gal+ satellite cells in muscles where Pax3 is expressed postnatally, such as forelimb muscles and Diaphragm[43]. Furthermore, we observed that these AP+ vessel-associated cells express Pax3 in young animals (D.A., C.D. and C.G.; unpublished data) and that YFP+ cells, isolated 1 week after tamoxifen injection, express Pax3, and not Pax7, in culture. On the other hand, we have clearly shown that, during mouse growth, AP-derived pericytes become real satellite cells and acquire Pax7+ expression *in vivo*, under the basal lamina and when isolated *in vitro*, two months after tamoxifen induction. It was previ-

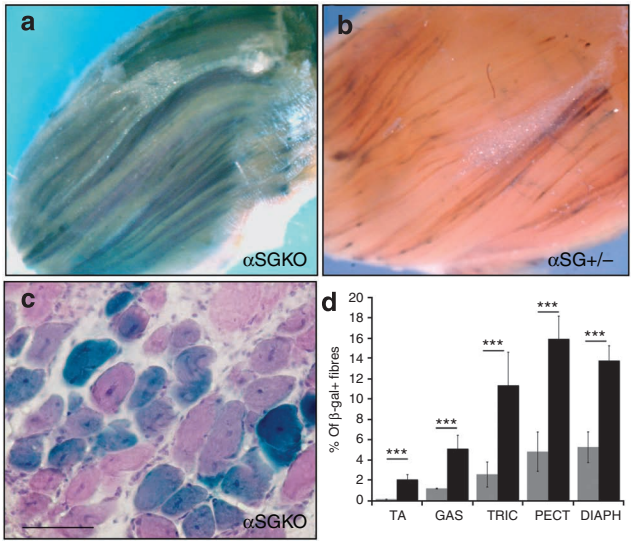


**Figure 6 | AP+ pericytes contribution to skeletal muscle during chronic regeneration.** (**a**,**b**) *Triceps* muscles of *Tg:TN-AP-CreERT2:R26R:αSGKO* (**a**) or *Tg:TN-AP-CreERT2:R26R:αSG+/−* (**b**) mice, injected with tamoxifen at P6–P8, killed two months later and stained for β-gal activity. Many β-gal+ fibres can be detected in the dystrophic muscle. (**c**) Muscle sections of *Tg:TN-AP-CreERT2:R26R:αSGKO* shown in (**a**) stained for β-gal and H&E. Scale bar, 100 μm. (**d**) Percentage of β-gal+ fibres detected in the dystrophic muscles. Grey bars indicate *αSG+/−* background; black bars indicate *αSGKO* background. Number of experimental animals: $n = 5$; error bars indicate standard deviation. Results were assessed for statistical significance by Student's *t*-test (unpaired, two-tailed) and differences were considered statistically significant at $P < 0.05$. Asterisks in the histogram indicate the *P* value: ***$P < 0.001$. GAS, *Gastrocnemius*; TRIC, *Triceps*; PECT, *Pectoralis*; DIAPH, Diaphragm.

ously demonstrated that during embryogenesis Pax3+ progenitors of the dermomyotome give rise not only to myogenic cells, but also to several other mesoderm cell types, including dermal fibroblasts, endothelial cells[44,45], and, notably, to pericytes and smooth muscle of the dorsal aorta[25]. Thus, whereas all satellite cells appear to originate from somites[2], the data presented here are not in contrast with this evidence, because other mesoderm progenitors, such as Pax3-expressing pericytes, also originate from somites. These progenitors may directly differentiate into skeletal muscle while expressing Pax3 (at least *in vitro*), and this occurs more frequently in postnatal forelimb muscles where Pax3 is more abundant; alternatively, they may enter the satellite cells pool and activate Pax7 expression.

AP+ cell contribution to skeletal muscle mainly occurs during the first month of postnatal growth and becomes sporadic in adult mice even in regenerating myofibres after cardiotoxin-induced muscle damage. Probably only during perinatal growth AP-derived pericytes can contribute to skeletal myogenesis and enter the satellite cells pool, in response to a major recruitment of myogenic cells to the maturing muscle fibres. Thus, it is conceivable that this contribution may be higher during prenatal development but, unfortunately, widespread expression of AP at this stage precludes this analysis.

Very recently, three reports[46–48] showed that selective ablation of Pax7-expressing satellite cells during adult life permanently hampers subsequent attempts to regenerate skeletal muscles, leading to the conclusion that no other endogenous cell types may substitute for satellite cells. These results are not in contradiction with those reported here for the following reasons: the ablation of Pax7+ satellite cells was carried out in adult mice, at an age when the contribution of AP+ pericyets to muscle regeneration becomes negligible. It is also possible that satellite cells are required to induce other cell types to adopt a myogenic fate, as suggested by Sambasivan *et al.*[48]

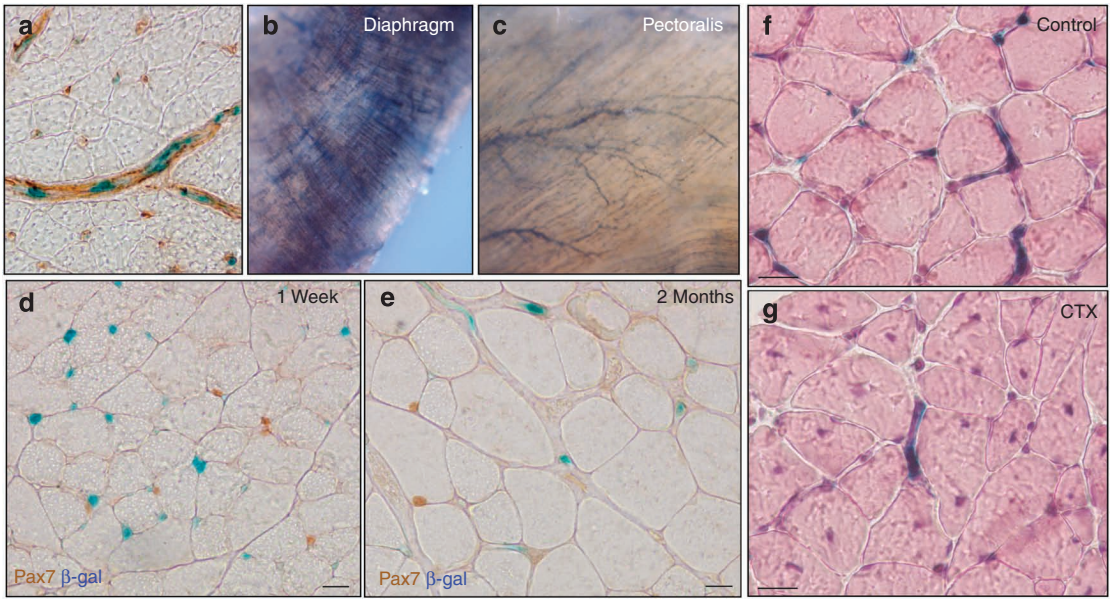


**Figure 7 | Endothelial cells do not contribute to growing or regenerating muscle.** (**a**) *Ve-cadherin(PAC)-CreRT2:R26R Pectoralis* muscle stained for β-gal (blue) and Pecam (brown) 3 days after tamoxifen injections (P6–P8). (**b**,**c**) Whole mount X-Gal staining of *Ve-cadherin(PAC)-CreRT2:R26R* Diaphragm (**b**) and *Pectoralis* (**c**) 2 months after tamoxifen injections (P6–P8). β-gal+ vessels are clearly visible. (**d**,**e**) *TA* muscle sections of *Ve-cadherin(PAC)-CreERT2:R26R* mouse 1 week (**d**) and 2 months (**e**) after tamoxifen injection, stained for β-gal (blue) and anti-Pax7 (brown). No double-positive cells are present. (**f**,**g**) *TA* muscle sections of *Ve-cadherin(PAC)-CreERT2:R26R* mice 1 month after cardiotoxin injection (CTX) (**g**) and contralateral muscle (**f**), stained for β-gal and H&E. No β-gal+ fibres are detected. Scale bar, 20 μm.

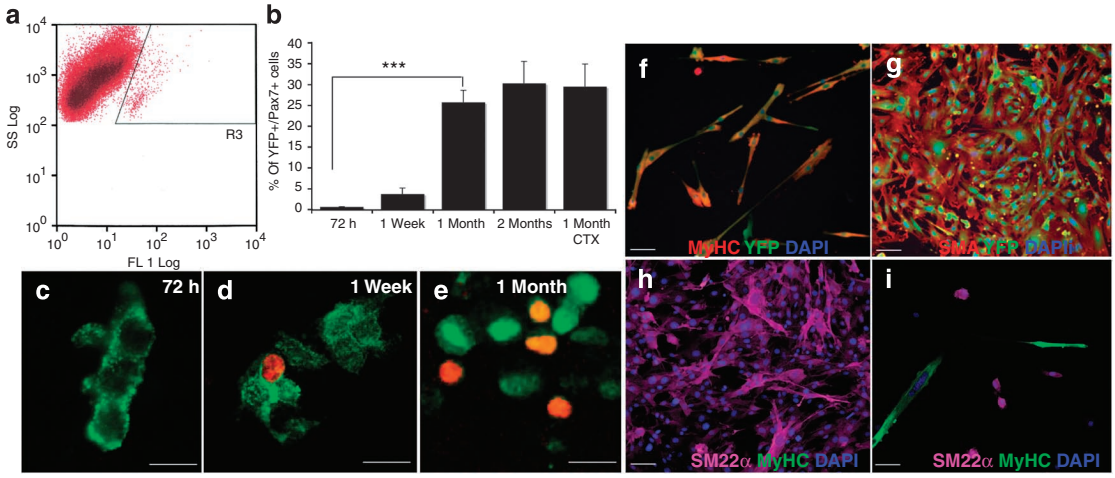


**Figure 8 | Myogenic potential of isolated AP-derived YFP+ cells.** (**a**) FACS analysis with a gate selected to sort YFP+ cells. (**b**) Relative frequency of double-positive YFP+/Pax7+ cells as % of the total YFP+ cells, obtained immediately after sorting, at different time points after tamoxifen induction and after cardiotoxin injection (CTX). Error bars indicate standard deviation. Results were assessed for statistical significance by Student's *t*-test (unpaired, two-tailed), and differences were considered statistically significant at $P<0.05$. Asterisks in the histogram indicate the different *P* values: ***$P<0.001$. (**c**–**e**) Freshly isolated YFP+ cells were stained with anti-GFP antibody (green) and anti-Pax7 (red). Scale bar, 20 μm (**f**) Skeletal myogenic YFP+ clone, stained with anti-MyHC (red), anti-GFP (green) and DAPI (blue). (**g**) Smooth myogenic YFP+ clone, stained with anti-SMA (red), anti-GFP (green) and DAPI (blue). (**h**,**i**) Smooth myogenic (**h**) and mixed (**i**) YFP+ clones stained for anti-MyHC (green), anti-SM22α (magenta) and DAPI (blue). We counted 2 double-positive clones on a total number of 114 clones scored. Scale bars, 40 μm.

In conclusion, we have demonstrated that vascular pericytes, resident in skeletal muscle, are bi-potent (as confirmed by clonal analysis), as they contribute to both the smooth muscle layer of blood vessels and skeletal muscle fibre development, including the satellite cell pool, during unperturbed, postnatal growth of the mouse. It is still unknown how this choice is regulated *in vivo*, although it is plausible that signals emanating from neighbour muscle fibres or endothelium may direct the cells to a skeletal or to a smooth muscle fate, respectively.

## Methods

**Mice generation and genotyping**. To generate *Tg:TN-AP-CreERT2* mice the Cre recombinase fused with estrogen receptor (CreERT2) was cloned in a pGEM-T Easy Vector, between two fragments homologous to *TN-AP* gene in the starting coding region ATG upstream region. The construct was inserted into a bacterial artificial chromosome (BAC) 240 kbp clone (RP23 RP23-400F8 (Start: 137,302,861 and End: 137,529,460)) by homologous recombination in bacteria (spanning from 177 kbp upstream of the ATG, and covering most of the coding region). Molecular analysis demonstrated the correct insert of the construct into the BAC (Supplementary Fig. S2b,c). Digestion by BamHI shows the generation of an extra 2.8 kbp band and the absence of an 8 kbp band in the electroporated BAC compared with the unelectroporated one. Injection of the BAC into fertilized eggs generated a total of seven independent transgenic lines that were tested by crossing with the *R26R* reporter mouse[49]. Three lines were found to express beta-galactosidase and were used for subsequent experiments.

Mice were kept heterozygous and genotyped by FW primer mapping on *TN-AP* sequence (FWTN-AP cgtctagatgagcctagggctggctggagcacag) and RW primer mapping on *Cre* sequence (RWCRE cagccaccagcttgcatga), generating a 1,400 bp band.

Genotyping strategies of other mice, including *Pax7*−/− mutant mice[50], *Myf5*$^{nlacZ/+}$ mice[19], *Ve-Cadherin-(PAC)CreERT2* (ref. 12), *TN-AP*$^{\beta geo/+}$ (ref. 16) have been published. To test our transgenic model, we used three different reporter mice: *R26R*[49] that express cytoplasmic β-gal; *R26*$^{NZG21}$ that express nuclear β-gal and *R26R-EYFP*[51] that express cytoplasmic YFP. Control mice C57/BLJ6 from Jackson laboratory were used. All experiments were performed under internal regulations for animal care and handlings (IACUC 355). Both males and females were used for experiments and they were equally distributed between control and treated groups to avoid gender variations in muscle mass.

**Tamoxifen injection and cardiotoxin treatment**. Young animals were injected subcutaneously with 0.25 mg of tamoxifen, diluted in corn oil, (SIGMA T5648-1G) for 3 consecutive days at 6, 7 and 8 days after birth. Adult animals were treated for 3 consecutive days with 2 mg of tamoxifen by intraperitoneal injection. For the injury protocol, 25 μl of 100 μM cardiotoxin from *Naja mossambica mossambica* (SIGMA C9759) were injected directly into skeletal muscle.

**X-gal staining and immunohistochemistry**. Dissected muscles were fixed with paraformaldehyde (PFA) 4% and 0.25% glutaraldehyde for 30 min at 4 °C. After incubation in X-gal solution O/N at 37 °C, they were washed and dehydrated with increasing sucrose concentration (10–20 and 30%) for 20 min and then frozen within OCT. Eight μm cryostats sections were used for immunohistochemistry and stained with H&E when necessary. The endogenous peroxidases were blocked by $H_2O_2$ 30% in methanol for 30 min. The sections were then incubated with PBS 0.2% Triton 1% BSA for 30 min and with 10% donkey serum for 30 min. Primary antibody incubation was followed by secondary biotinilated antibody (Dako) and ABC kit (Vector Laboratories, PK-6100), enzymatic reaction with DAB (Sigma) was followed under microscope and stopped after the appropriated time.

**β-gal activity measurement**. Muscles were homogenized in buffer (0.25 M Tris pH 7.8, PMSF 1 mM), centrifuged at 15,000 *g*×10 min at 4 °C, and extracted proteins were subsequently quantified by spectrophotometer with DC protein Assay (Biorad, 500-0114). 100 μg of protein were incubated with 500 μl of Z Buffer pH7 (60 mM $Na_2HPO_4$ 40 mM $NaH_2PO_4$ 10 mM KCl, 1 mM $MgSO_4$, 50 mM b-Mercaptoethanol) and 100 μl of ONPG (o-nitrophenyl-b-D-galactoside) 4 mg ml$^{-1}$ at 37 °C for 2.5 h. The reaction was blocked with 250 μl of $Na_2CO_3$ and the level of β-gal activity was evaluated by reading the $OD_{420}$ at the spectrophotometer. To quantify the proportion of β-gal+ fibres in different muscles of *Tg:TN-AP-CreERT2:R26R* mice, we created a standard curve by mixing different proportions (from 0.1 to 50%) of muscle homogenates derived from wild-type and *CMV-Cre-R26* mice. The proportion of β-gal activity only due to β-gal-positive fibres was calculated by subtracting the activity measured in muscles from *Tg:TN-AP-CreERT2:R26R* mice injected with tamoxifen and killed 1 week later, when the β-gal signal is present only in vessel-associated cells.

**Alkaline phosphatase reaction**. Different kits for AP enzymatic detection were used (see below) to exploit different reaction colours. Muscle sections were fixed with PFA 4% at 4 °C for 10 min and, after 3 washes, incubated with BCIP-NBT kit (Roche, 12296226 and 12329020) in an alkaline buffer (NaCl 100 mM, Tris 100 mM PH 9.5, $MgCl_2$ 50 mM, Tween 0.1%) for 20 min in the dark, or with PermaRed/AP (Histo-Line laboratories, K049) for 20 min. Moreover, AP substrate kit from Vector (SK-5100) were used and visualized by confocal microscopy (Leica) at 456 nm, along with other immuofluorescent antibodies.

**Immunofluorescence and antibodies**. Frozen sections and cultured cells were fixed with PFA 4% at 4 °C for 10 min, washed 3× with Triton 0.2% BSA 1% in PBS, and incubated with the same buffer for 30 min, followed by 10% Donkey serum for 30 extra min. Primary antibody was incubated for 1 h at room temperature or O/N at 4 °C in the same buffer. Samples were washed 3 times and then incubated with secondary antibodies (Alexa Fluor 488-594-546 or 647, Invitrogen) for 1 h at RM, washed 3 times and then mounted and examined under epifluorescence or confocal Leica microscope.

The immunofluorescence assay was carried out with the following antibodies: rabbit anti-β-galactosidase at 1:100 dilution (Chemicon, AB986), rabbit anti-β-gal at 1:1,000 (MPCappel 559762) and mouse anti-β-gal at 1:200 (Promega Z3783); rabbit anti-laminin at 1:300 (Sigma, L9393) or chicken anti-laminin at 1:500 (Abcam, Ab14055); anti- NG2 1:200 (Chemicon, AB5320) or donated from W. Stallcup, at 1:100; anti-Pecam at 1:2 (gift from Elisabetta Dejana); anti-Pax7 at 1:2 (DHSB); anti-smooth muscle actin at 1:200 (Sigma, A2547); anti-SM22α at 1:100 (Abcam); anti-MyHC at 1:2 (MF20 DHSB); anti-GFP 1:300 (Chemicon, AB3080); anti-Ki67 at 1:500 (Novo Castra, NCL-Ki67p), anti-PDGFRβ at 1:100 (from W. Stallcup), anti-AP at 1:100 (R&D); anti Pax3 at 1:2 (DHSB); anti MyoD at 1:100 (Dako).

**Cell sorting**. Dissected muscles were cut in small pieces and digested by collagenase D 0.1% (Roche, 1088866) and Trypsin 0.25% (Gibco, 15090-046) for 20 min at 37 °C, 2 or 3 cycles. After spin and filtration, the cells were collected in DMEM, 20%FBS, 2 mM EDTA, 20 mM HEPES or PBS 2% FBS, 2 mM EDTA when staining with SM/C2.6 (a gift from S. Takeda).

Cell Sorting was carried out in MoFLo System (DAKO); sorted cells were either cloned in 96 wells or collected for RNA extraction. FACS analysis was performed by Canto (BD); data were analysed by FACSDiva software (BD).

Sorted cells were cultured with DMEM (Sigma) 20%FBS on (low factor) Matrigel (BD, 354248) coated wells, in low (3%) oxygen incubator.

**RT-PCR and quantitative real-time PCR**. The RNA from sorted cells was collected by RNeasy mini (or micro) kit (Qiagen, 74104 and 74004) and converted into double-strand complementary DNA with the Superscript VILO cDNA systhesis kit (Invitrogen, 11754-050), according to the manufacturer's instructions. Real-time quantitative PCR analysis was carried out on cDNA from isolated cells by using an Mx3000P real-time PCR detection system (Stratagene). Each cDNA sample was amplified in duplicate by using the Express SYBR GreenER qPCR Supermix (Invitrogen, 11784-200).

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## Author contribution

A.D. planned and performed many experiments and wrote the manuscript; G.M. performed IF and clonal analysis; DC performed the RT–PCR experiments, E.A. helped with the experiments with VE-Cadherin cre mice, A.I. performed some IF experiments, L.P. prepared constructs, S.A. did all the animal work, R.S. helped with the transgenic construct, S.B. contributed to planning and interpretation of the experiments, S.T. supervised the cloning strategy and corrected the manuscript; G.C. planned the experiments, analysed the results and wrote the manuscript.

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# Functional cooperation of Sox6 and Nfix regulates fiber type specification during pre-natal muscle development

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# SUMMARY

Nuclear factor I X (Nfix) is a key regulator of fetal myogenesis acting both as an activator of fetal-specific genes and as a repressor of embryonic muscle genes, such as *MyHC-I*. Here we show that Sox6, belonging to the Sox factors family, cooperates with Nfix in fetal muscle to repress MyHC-I at the transcriptional level. Sox6 and Nfix are co-expressed in fetal myoblasts and co-immunoprecipitate in muscle cells. By ChIP assay, we show that Nfix is required for Sox6 binding to the *MyHC-I* promoter in fetal myoblasts and thus for Sox6 repressive activity. Moreover, we show that Sox6 is a direct activator of MyHC-I in embryonic muscle and is required for proper Nfix function in fetal muscle. These data demonstrate functional cooperation of Sox6 and Nfix in the regulation of embryonic muscle genes, that is evolutionary conserved in mouse and zebrafish.

# INTRODUCTION

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 myosin heavy chain (MyHC) isoform (MyHC-I, encoded by the *Myh7* gene); in contrast, fast-twitch type II fibers present glycolytic or mixed metabolism and express three fast MyHC isoforms (MyHC-IIa, IIx/d, and IIb, encoded by *Myh2*, *Myh1* and *Myh4* respectively) (Peter et al. 1972; Schiaffino et al. 1988; Chakkalakal et al. 2012). The phenotype of adult muscle fibers is strictly regulated by extrinsic signals such as muscle activity and hormones (Ogata et al. 2005; Mendler et al. 2008). In recent years, many key factors controlling adult muscle diversity and plasticity have been identified, including the NFATc transcription factor family (Calabria et al. 2009), Wnt3a and BMP4 (Kuroda et al. 2013) and class II HDACs (Potthoff et al. 2007). However, the molecular mechanisms by which muscle fiber diversity is achieved during development are still unclear.

In vertebrates the process of skeletal muscle development occurs in subsequent steps that involve distinct populations of myogenic progenitors, named myoblasts, which arise from the dermomyotomal domain of somitic mesoderm (Christ & Ordahl, 1995). The process of myogenic differentiation is initiated in mesodermal cells by a family of bHLH transcription factors named Muscle Regulatory Factors (MRFs) able to activate transcription of muscle-specific markers such as the MyHC isoforms (Pinney et al. 1988; Cao et al. 2010). 'Embryonic' myoblasts sustain a first wave of differentiation between embryonic day (E) 10 and E12 in the mouse and give rise to primary myofibers that establish the primitive shape of muscle and express high levels of MyHC-I and of the embryonic MyHC isoform (MyHC-emb, encoded by

*Myh3*) (Schiaffino et al. 1986; Stockdale et al. 1992). A second wave of muscle differentiation takes place between E15 and E18, driven by fetal myoblasts that form secondary fibers, which are 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 such as muscle creatine kinase (MCK) and the glycolytic enzyme β-enolase (Eno3) (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 a previous study (Messina et al. 2010) we have shown that the transcription factor Nfix, member of the nuclear factor I family, has a key role in the establishment of fetal muscle phenotype. Nfix is drastically up-regulated in fetal compared to embryonic myoblasts and it acts as an indirect repressor of MyHC-I by transcriptional inhibition of NFATc4. Moreover, Nfix activates the expression of the fetal-specific markers Eno3 and MCK by direct regulation of their promoters. Notably, a recent study focused on the zebrafish (*Danio rerio*) ortholog *nfixa* (Pistocchi et al. 2013) suggests an evolutionary conserved role for Nfix in the transition from slow-twitch to fast-twitch myogenesis.

In the last years it was reported that Sox6, a member of the Sry-related HMG box (Sox) factors family highly conserved in vertebrates, plays a critical role in normal

fetal fiber specification. In particular *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). Moreover, several studies have shown that the zebrafish ortholog *sox6* has a conserved function in repressing slow-twitch genes (such as the slow MyHC isoform *smyhc1* and the transcription factor *prox1a*) during embryonic muscle development (von Hofsten et al. 2008; Wang et al. 2011). Here we show that Sox6 forms a complex with Nfix in fetal muscle cells to cooperatively repress MyHC-I. Nfix is required for the proper binding of Sox6 to the *MyHC-I* promoter in fetal myotubes. Notably, during embryonic myogenesis Sox6 acts as an activator rather than a repressor of MyHC-I. On the other hand, Nfix transcriptional activity during fetal myogenesis is severely altered in the absence of Sox6. Finally, we demonstrate that Nfixa and Sox6 functionally cooperate in repressing slow-twitch genes in zebrafish embryos, revealing an evolutionary conserved mechanism that is required for the acquisition of normal muscle phenotype.

# RESULTS

**Sox6 is equally expressed but differentially localized during pre-natal muscle development**

As previously reported (Hagiwara et al. 2007; An et al. 2011; Quiat et al. 2011) Sox6 is a key repressor of the slow/embryonic muscle phenotype. However, a detailed characterization of Sox6 expression during the different myogenic waves has never been conduced. We first investigated whether Sox6 was differentially expressed in muscle progenitors during primary and secondary myogenesis: to this aim *Myf5*$^{GFP\text{-}P/+}$ embryos 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). We interestingly found by quantitative Real Time PCR (qRT-PCR) that *Sox6* mRNA levels do not significantly change between the two populations, whereas *Nfix* is drastically up-regulated in fetal progenitors (Fig. 1A). Moreover, we found no differences in Sox6 protein content between freshly isolated embryonic and fetal myoblasts (Fig. 1B), confirming that Sox6 is equally expressed in these two populations of muscle progenitors. Importantly, these data also show that Sox6 and Nfix proteins are co-expressed in fetal myoblasts. We then performed extensive immunofluorescence analysis on frozen mouse embryo sections from E11.5 to E18.5 in order to follow Sox6 protein expression in muscle cells and muscle fibers throughout development. We found that Sox6 is expressed at high levels in skeletal muscle starting from E12.5: at this stage we found Sox6 positive cells in all the embryonic muscle groups, including thoracic, appendicular and dorsal muscles (data not shown). Importantly, the subcellular localization of Sox6 protein at this stage is mostly cytoplasmic, as revealed by co-localization between Sox6 and total MyHC

stained with the MF20 antibody (Fig. 1C-F). We also performed immunofluorescence for Sox6 and MyoD or for Sox6 and myogenin (Myog) on E12.5 frozen muscle sections (Supplemental Fig. S1A-H): in both cases we found the same cytoplasmic staining observed in the majority of MyHC positive cells, suggesting that Sox6 is preferentially localized in the cytoplasm of embryonic myoblasts and myocytes *in vivo*. This expression pattern is maintained in all muscle groups until E15.5 (data not shown). Then, starting from E16.5, when the initial diversification between primary and secondary fibers occurs, the staining for Sox6 is mainly found in the nuclei of MyHC positive fibers (Fig. 1G-J), even if a lower staining can still be detected in the cytoplasm. We quantified the results obtained by immunofluorescence counting the number of nuclear or cytoplasmic Sox6 expression events on the total number of Sox6/MyHC double positive cells. As shown in Fig. 1K, the percentage of nuclear Sox6 positive cells dramatically increases from 19±8% in the embryonic period to 69±3% in the fetal period. Conversely, the percentage of cytoplasmic Sox6 positive cells decreases from 81±3% to 31±4% in the fetal period. In order to exclude the possibility of unspecific staining, we isolated nuclear and cytoplasmic protein fractions directly from embryonic and fetal muscle tissue and revealed Sox6 localization by Western Blot (Fig. 1L). Results show a marked increase of nuclear Sox6 and a consequential decrease of cytoplasmic Sox6 in fetal compared to embryonic muscle. Interestingly we observed an upper band (marked by the asterisk in Fig. 1L) that is highly enriched in the E16.5 nuclear extract, suggesting that Sox6 may be regulated by post-translational modifications during muscle development. Importantly, our Western Blot analysis clearly indicates that Sox6 protein is also present in the nuclei of embryonic muscle tissue, even if at a lower level compared to fetal muscle. Thus, low levels of Sox6 in the majority of embryonic muscle nuclei are

likely undetectable by immunofluorescence. Taken together, these results demonstrate that Sox6 is localized in both the cytoplasm and the nuclei of myogenic cells during the embryonic period, and it is then rapidly accumulated into the muscle fiber nuclei during fetal myogenesis. Strikingly, starting from E17.5 the nuclear expression of Sox6 becomes restricted to secondary fibers that are negative for MyHC-I (Supplemental Fig. S1I-L), as previously described (An et al. 2011; Richard et al. 2011).

**Sox6 is an activator of MyHC-I during embryonic myogenesis**

Since Sox6 protein is expressed in myocytes from the beginning of embryonic myogenesis, we decided to investigate on its possible function during the embryonic period, when Nfix is normally expressed at very low levels (Messina et al. 2010). 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 (Fig. 2A,B), in a way that is reminiscent of the Tg:Mlc1f-*Nfix2* Nfix gain-of-function embryo, in which the Nfix2 isoform is ectopically expressed in muscle cells from E11.5 recapitulating the expression pattern of the *Myl1* enhancer Mlc1f (Kelly et al. 1997; Messina et al. 2010) (Fig. 2C). Notably, MyHC-I expression in the embryonic heart is not affected, suggesting that MyHC-I down-regulation is restricted to skeletal muscle. Moreover, no differences in total MyHC content were assessed in embryonic muscle groups of both *Sox6* null and Tg:Mlc1f-*Nfix2* embryos (Fig. 2D-F), suggesting that the decrease in MyHC-I expression is not due to delayed or aberrant muscle differentiation. We also performed Western Blot on embryonic muscle lysates to confirm the

immunofluorescence data (Fig. 2G): results show a strong decrease of MyHC-I protein in both *Sox6* null and Tg:Mlc1f-*Nfix2* samples, without major changes in total MyHC content. Interestingly, in *Sox6* null muscles we found a drastic up-regulation of Eno3, a marker of fetal myogenesis (Messina et al. 2010), suggesting that Sox6 may negatively regulate Eno3 expression. These results have been also confirmed by qRT-PCR on wt and *Sox6* null embryonic muscle tissue showing significant *MyHC-I* decrease and *Eno3* increase in absence of Sox6, while the levels of *MyHC-emb* mRNA do not change (Fig. 2H). It was previously reported that Sox6 directly regulates *MyHC-I* by binding to the 5'-upstream region in two different binding sites: the first is located at -375 base pairs (bp) from the transcription start site (TSS) in the proximal promoter and is critical for Sox6-dependent *MyHC-I* repression in fetal myotubes (Hagiwara et al. 2007); the second is located at -2900 bp from the TSS, in a distal muscle enhancer that is required for full promoter activity (Giger et al. 2000; Blow et al. 2010), but its function is unknown (An et al. 2011). In order to determine whether Sox6 acts directly on the *MyHC-I* regulative regions during embryonic myogenesis, we performed chromatin immunoprecipitation (ChIP) for Sox6 in differentiated embryonic myoblasts (Fig. 2I). Interestingly, we found that Sox6 binds to both *MyHC-I* regulative regions, suggesting that Sox6 directly activates MyHC-I expression during embryonic myogenesis. Moreover, Sox6 directly binds to the *Eno3* proximal promoter region in close proximity of the TSS and of a conserved consensus sequence for Nfix (Messina et al. 2010), thus suggesting a direct mechanism of repression mediated by Sox6 in embryonic muscle. Taken together, these data demonstrate that Sox6 acts as a positive regulator of the slow-twitch phenotype during the embryonic period, in sharp contrast with its function in fetal muscle (An et al. 2011; Quiat et al. 2011). Thus, Sox6 has opposite roles in fiber type specification

between embryonic and fetal myogenesis. On the contrary, Nfix is both sufficient and necessary to initiate the fetal-specific program. In fact, ectopic Nfix expression in embryonic muscle leads to transcriptional down-regulation of *MyHC-I* and up-regulation of *Eno3* (Fig. 2J), while Nfix deficiency in fetal muscle results in higher levels of MyHC-I and lower levels of Eno3 (Messina et al. 2010; and see below).

**Sox6 activity is impaired in fetal muscle in the absence of Nfix**

In order to study the possible cooperation of Sox6 and Nfix in fetal myogenesis, we performed immunofluorescence for Sox6 and total MyHC on frozen muscle sections from E16.5 wt and *Nfix* null mice (Campbell et al. 2008): results show that Sox6 is localized in the nuclei of about 60% of total fibers in both wt and *Nfix* null muscles (Supplemental Fig. S2A,B and yellow bars in Fig. 3E). We then performed immunofluorescence for Sox6 and MyHC-I on sections from the same mice. Interestingly, in contrast to wt muscle, where a large proportion of the nuclear Sox6 positive fibers express low or null levels of MyHC-I, in the absence of Nfix we observed a marked increase in the number of nuclear Sox6 positive fibers that co-express MyHC-I (Fig. 3A-D). Quantification of the immunofluorescence data reveals a two-fold increase in the percentage of nuclear Sox6/MyHC-I double positive fibers (from 12±3% to 28±7%; red bars in Fig. 3E). The Western Blot on isolated nuclear extracts from wt and *Nfix* null fetal muscle did not reveal significant differences in nuclear Sox6 protein content (Fig. 3F,G). We further validated these data by qRT-PCR and Western Blot on differentiated fetal myotubes. *Nfix* null myotubes express higher levels of MyHC-I mRNA and protein compared to wt cells, but only a slight increase (non-significant) in Sox6 expression was detected (Supplemental Fig. S2C,D). These data demonstrate that, in the absence of Nfix, Sox6 repressive activity

on MyHC-I is partially impaired, even if the normal expression pattern of Sox6 is maintained. This suggests that cooperation between Nfix and Sox6 is required for normal fetal fiber type specification.

**Nfix is necessary for the binding of Sox6 to the MyHC-I proximal promoter**

On the basis of the results obtained and since Sox6 and Nfix are co-expressed starting from fetal myogenesis, we wondered whether they can bind to each other in a multi-protein complex. To this aim we used C2C12 myoblasts transiently transfected with both Nfix2-HA- and Sox6-expressing plasmids (see Experimental Procedures), and we performed co-immunoprecipitation (Co-IP) assay for HA and Sox6. The Co-IP revealed a binding between Sox6 and Nfix-HA, as shown in Fig. 4A. Since Nfix presents both a trans-repression and a trans-activation domain, but it is unable to directly bind to the *MyHC-I* promoter (Messina et al. 2010), we hypothesized that physical association with Sox6 might provide the basis for transcriptional repression at the *MyHC-I* locus. In order to unravel a possible mechanism of cooperation, we performed ChIP for Sox6 in wt and *Nfix* null fetal myotubes (Fig. 4B). Results show a decrease in the normal binding of Sox6 to the two different sites in the *MyHC-I* 5'-upstream region. Importantly, the binding with the proximal promoter was completely lost in the absence of Nfix, while the binding with the distal enhancer was reduced by 50%. These data demonstrate that Nfix is required for the proper binding of Sox6 to the *MyHC-I* proximal promoter, and thus for the repressive activity of Sox6. Since we demonstrated that Sox6 directly represses *Eno3* in embryonic muscle, we sought to determine whether Nfix might regulate the binding between Sox6 and the *Eno3* promoter as well. Importantly, Sox6 does not bind to the *Eno3* promoter in fetal wt myotubes (An et al. 2011). However, in *Nfix* null myotubes we found the presence of

a binding, implying that Sox6 is incorrectly bound to *Eno3* promoter in absence of Nfix. This further suggests that Nfix is required for the global function of Sox6 in fetal muscle.

**Sox6 is required for normal Nfix function in fetal muscle**

In the light of the observed cooperation between Nfix and Sox6, we also investigated Nfix function in *Sox6* null fetuses (E17.5). As previously described in other mouse models (Hagiwara et al. 2007; An et al. 2011), fetal fiber specification is completely disrupted in the absence of Sox6. Indeed, in contrast to wt muscle, in *Sox6* null fetuses the totality of muscle fibers present high levels of MyHC-I by immunofluorescence (Fig. 5A-B). We also performed immunofluorescence for Nfix on the same sections (Fig. 5C-F) and found that, despite the dramatic increase in MyHC-I expression, Nfix is correctly expressed in the nuclei of muscle fibers in absence of Sox6. Additionally, we performed qRT-PCR on wt and *Sox6* null fetal myotubes. We found a slight but significant decrease of *Nfix* mRNA in the absence of Sox6 (Fig. 5G), suggesting that Sox6 may positively regulate Nfix expression. Importantly, *MyHC-I* mRNA levels are drastically increased (up to 6-fold) in the absence of Sox6. However, by performing Western Blot (Fig. 5H), we found no differences in Nfix protein content. These data demonstrate that Nfix is normally expressed in the absence of Sox6, but unable to properly repress MyHC-I. Since we demonstrated a critical requirement for Nfix in Sox6 muscle-specific activity, we sought to investigate whether Sox6 deficiency might lead to impairments in Nfix function as well. Therefore, we performed qRT-PCR on E12.5 and E16.5 wt and *Sox6* null muscle tissue. We observed that *Nfix* is normally up-regulated during fetal myogenesis in absence of Sox6, even if its levels in the E16.5 *Sox6* null sample are

slightly decreased (consistently with the *in vitro* mRNA analysis; Fig. 6A). As expected, *Sox6* null embryonic and fetal muscles display abnormal *MyHC-I/MyHC-emb* ratios that reflect the down-regulation of *MyHC-I* during primary myogenesis and its subsequent up-regulation in the fetal period (Fig. 6B). Intriguingly, the *MyHC-neo/MyHC-emb* ratio is not affected (Fig. 6C). Moreover, *NFATc4* is markedly up-regulated in *Sox6* null embryonic muscle, but is normally repressed in the fetal period in absence of Sox6 (Fig. 6D), whereas *Eno3* mRNA is barely expressed in *Sox6* null fetuses (Fig. 6E), suggesting that Nfix was able to correctly regulate *NFATc4* but not *MyHC-I* and *Eno3* in absence of Sox6. Taken together, these date indicate that Sox6 is required for the proper transcriptional function of Nfix during fetal development, thus demonstrating a non-redundant and cooperative function for Sox6 and Nfix in the achievement of fetal muscle phenotype (see the model in Fig. 6F).

**Functional cooperation of Sox6 and Nfixa is evolutionary conserved in zebrafish**

It is known that both Sox6 and Nfixa have an evolutionary conserved role in the repression of slow-twitch genes in zebrafish (von Hofsten et al. 2008; Pistocchi et al. 2013). We thus wondered whether functional cooperation between Nfixa and Sox6 is conserved in zebrafish myogenesis. As a preliminary analysis, we performed immunofluorescence for Sox6 and total MyHC (MF20 antibody) or sMyHC1 (F59 antibody) on 48 hours post-fertilization (hpf) embryos: we found that Sox6 is expressed in the majority of muscle fiber nuclei (Fig. 7A,B). Intriguingly, we found that the outer superficial fibers positive for sMyHC1 present cytoplasmic staining for Sox6 (Fig. 7C-J), suggesting that Sox6 subcellular localization may be differentially regulated in slow and fast muscle domains during zebrafish development. Additionally we performed qRT-PCR on tails isolated from zebrafish embryos at 24,

48 and 72 hpf: *sox6* transcript is expressed at high levels at 24 hpf and is steadily down-regulated at 72 hpf (Supplemental Fig. S3A), whereas *nfixa* transcript peaks at 48 hpf (Supplemental Fig. S3B) as previously shown (Pistocchi et al. 2013). 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). *sox6* morphants display a complex phenotype characterized by cardiac edema, vascular defects and abnormal motility that is not observed in control std-MO injected embryos (data not shown). By qRT-PCR analysis (Supplemental Fig. S3C-H) we found that the expression of slow-twitch specific genes such as *smyhc1* and *prox1a* is markedly increased in absence of Sox6, whereas fast-twitch genes such as *mylpfa* (fast myosin light chain isoform) and *tnnc2* (fast troponin C isoform) are expressed at equal or lower levels. Moreover, the *nfixa* transcript is drastically up-regulated since 24 hpf in *sox6* morphants, suggesting that Sox6 might negatively regulate *nfixa*, whereas *nfatc4* is prematurely down-regulated, likely reflecting high Nfixa levels. 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 suboptimal doses of *sox6*-MO and *nfixa*-MO (see Supplemental Experimental Procedures). Strikingly, qRT-PCR results show that *smyhc1* and *prox1a* are significantly up-regulated in the double morphants, whereas *mylpfa* levels do not change (Fig. 7K). Moreover our analysis reveals that *sox6* and *nfixa* transcripts are up-regulated in *nfixa* and *sox6* morphants respectively, suggesting a mechanism of compensation that is lost when both factors are targeted for knockdown. Therefore, we conclude that functional cooperation of Sox6 and Nfix is evolutionary conserved in mouse and zebrafish.

# DISCUSSION

In recent years, many works have demonstrated that the intrinsic transcriptional properties of embryonic and fetal muscle progenitors are important to set the fiber type in the absence of nerve activity. Moreover, several transcription factors contributing to whole muscle fiber type 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 the NFAT factors (Daou et al. 2013). However, the network of transcription factors controlling fiber type specification during embryogenesis is still far from being fully characterized, and functional interactions among the different regulators are until now completely unknown. In this work we provided evidence for functional cooperation of Nfix and Sox6 in the repression of the slow-twitch phenotype during fetal 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 at the protein level. Consistently we found that Sox6 protein is expressed at high levels in skeletal muscle *in vivo* starting from 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 the *Sox6* transcript is absent in mouse embryonic myotome from E9.5 to E10.75 (Vincent et al. 2012), suggesting that Sox6 might be quickly activated in embryonic myoblasts at the beginning of primary myogenesis. However, we found that Sox6 protein is differentially localized in embryonic and fetal muscle and during the embryonic period is mainly found in the cytoplasm of myocytes and myogenic cells. It is known that Sox factors are regulated by subcellular localization in different tissues

(Argentaro et al. 2003; Avilion et al. 2003). Sox6 is aberrantly accumulated in the cytoplasm of fetal fibers lacking both Six1 and Six4, thus leading to an up-regulation of slow-twitch genes (including *MyHC-I*) and a consequential decrease in fast-twitch genes expression (Richard et al. 2012). Moreover, Sox6 protein was found in the cytoplasm of muscle fibers in post-natal and adult mouse (An et al. 2011), suggesting that an unknown mechanism controlling Sox6 subcellular localization might be important in regulating Sox6 function in fiber type specification. The recent finding that Sox6 is targeted for proteasomal degradation by the E3 ubiquitin ligase Trip12 in C2C12 myoblasts (An et al. 2013), strongly suggests that Sox6 function may be specifically controlled at post-translational level. However, Sox6 protein was also found in the nuclei of embryonic myocytes, suggesting that Sox6 may be active as a transcription factor during primary myogenesis. Consistently, we found two downstream targets of Sox6 in embryonic muscle, *MyHC-I* and *Eno3*, which are regulated in opposite ways. To our knowledge, this is the first report of a possible function for Sox6 in the transcriptional regulation of embryonic muscle. Unexpectedly, Sox6 deficiency during primary myogenesis leads to a faster muscle phenotype with low levels of MyHC-I and high levels of Eno3. This is followed in *Sox6* null fetuses by dramatic up-regulation of MyHC-I and loss of Eno3 expression. These data are consistent with previous characterizations of the *Sox6* null phenotype (Hagiwara et al. 2007; An et al. 2011; Quiat et al. 2011) and demonstrate that Sox6 plays opposite roles in fiber type specification during development. It was shown by ChIP and ChIP-seq analyses that the *MyHC-I* regulative regions contain at least two conserved binding sites for Sox6 (promoter and enhancer), while no Sox6 binding sites were found in the *Eno3* promoter (An et al. 2011). We found that Sox6 binds to both *MyHC-I* binding sites in embryonic and fetal myogenic cells but with different

affinity (higher in fetal myotubes). Moreover, Sox6 binds to the *Eno3* promoter in embryonic but not in fetal myoblasts. Thus, the binding ability of Sox6 is differentially regulated in discrete myogenic progenitor populations or at different times during development, which contributes to the high versatility of Sox6 functions. In contrast, we have shown that Nfix acts as a repressor of the slow-twitch phenotype in both embryonic and fetal muscle.

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 (Murakami et al. 2001). Therefore, it is likely that the reversal in Sox6 function is due to different factors that are progressively recruited and activated during muscle development. Preferential partners of Sox6 during embryonic myogenesis are likely positive transcriptional regulators, such as the MRFs (MyoD and Myog in particular), which bind to the same regulative regions of Sox6 in muscle cells (An et al. 2011). Then, starting from E16.5, we found that Nfix acts as a fundamental co-factor of Sox6, able to physically associate with Sox6 and to facilitate its binding with the *MyHC-I* regulative regions, in particular the proximal promoter that was shown to be critical for Sox6-dependent fetal repression (Hagiwara et al. 2007). Strikingly, our ChIP analysis reveals that in absence of Nfix the binding status of Sox6 in fetal myotubes resembles that of embryonic differentiated myoblasts, in particular for *Eno3*. Moreover, our study on *Nfix* null and *Sox6* null fetuses clearly demonstrates that Sox6 and Nfix are independently expressed during secondary myogenesis and that neither Sox6 nor Nfix are able to restore wt MyHC-I expression when the other one is not present. Finally, we demonstrated that Sox6 is required for at least two of the Nfix transcriptional functions in fetal muscle: repression of *MyHC-I* and activation of *Eno3*. The transcriptional regulation of MyHC-neo was not affected, suggesting that

Sox6 is dispensable for the MyoD/NFATc2-induced activation of *MyHC-neo* (Daou et al. 2013).

In this work we have also provided evidence for a conserved transcriptional cooperation of Sox6 and Nfixa in zebrafish. Sox6 is expressed both at mRNA and protein levels in fast muscle fibers that are negative for sMyHC1, and it is here co-expressed with Nfixa that is required for sMyHC1 down-regulation after 72 hpf (Pistocchi et al. 2013). We show that, as in mouse, Sox6 is crucial for repression of slow-twitch genes, even in presence of elevated Nfixa levels. Moreover, our double MO assay clearly reveals that the two factors can cooperate in repressing the slow muscle phenotype, whereas expression of fast-twitch genes such as *mylpfa* (Liew et al. 2008) was unaffected.

In conclusion we have presented a complex model of regulation of the embryonic/fetal transcriptional switch that not only involves Nfix activation, driven by Pax7 and other unknown factors (Messina et al. 2010), but also functional cooperation between Nfix and Sox6 that is conserved in mammals and teleosts. Future work will be addressed to study the relationship between Sox6 and Nfix in the context of post-natal development and adult skeletal muscle growth.

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# EXPERIMENTAL PROCEDURES

### Mouse and fish strains and injection of embryos

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); 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 Lab) were used as well. All animal studies were housed at the Departmental animal house. Mice were kept in pathogen-free conditions and all procedures were conformed to Italian law and approved by the University of Milan and Use Committee.

Zebrafish were raised and maintained according to established techniques. The following strains were used: AB (from the Wilson lab, 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 isopentane/liquid nitrogen. Cryostat sections (8 μm thickness) were permeabilized in 1% bovine serum albumin, 0,2% Triton-X 100 in PBS for 30 min at RT, incubated 1 h in blocking solution (10% goat serum in PBS) and overnight with the primary antibody or with mock PBS. After incubation for 30 min with the fluorescent-conjugated secondary antibody (Jackson ImmunoResearch), sections were again washed in PBS 0,2% Triton-X 100, mounted and examined under epifluorescence microscope (Leica). The following primary antibodies were used: rabbit anti-Sox6 (Abcam ab30455, 1:300), rabbit anti-Nfix (Novus Biological, 1:200), mouse anti-MyHC-I (Sigma, 1:200), MF20 (monoclonal, 1:2), F59 (monoclonal,

1:10), anti-Myog (monoclonal, 1:2), mouse anti-MyoD (BD Biosciences, 1:200 ). Nuclei were stained with Hoechst (1:1000).

**Cell sorting and culturing**

Dissected *Myf5*$^{GFP\text{-}P/+}$ embryos or fetuses were digested by 0,1% collagenase D (Roche) and 0,25% Trypsin (Gibco) for 20 min at 37°C in agitation as described in Biressi et al. 2007. After centrifugation and filtration, the cells were collected in DMEM, 20% FBS, 2 mM EDTA, 20 mM HEPES. Cell sorting was performed in MoFlo System (DAKO). GFP+ cells were collected for mRNA and protein extraction. For the preparation of unpurified myoblasts, after digestion of the tissue we performed a pre-plating of 30 min to separate fibroblasts. Unpurified cells were kept for 12 h in a low oxygen (3%) incubator at 37°C in DF20 (20% FBS in DMEM). Then the plates were checked for fibroblasts contamination and allowed to differentiate in DM (2% horse serum in DMEM) for 48 h.

**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, 1mM EDTA, 1% Triton-X, 0,1% sodium deoxycholate, 0,1% SDS, 150 mM NaCl in deionized water) plus protease inhibitors (1 mM PMSF). Proteins were harvested after centrifugation, quantified by absorbance reading at 750 nm and stored at -80°C. Proteins were then separated on 8%-12% polyacrylamide gels or on MiniProtean TDX Gels (Biorad) migrated for 2-3 h at 100 V (constant voltage). For Western Blot analysis the proteins were transferred onto nitrocellulose with the iBlot Dry Blotting System (Invitrogen). Following transfer, the membranes were blocked in 5% milk in phosphate buffer to eliminate non-specific

binding. The primary antibodies used are: rabbit anti-Sox6 (Abcam ab30455), mouse anti-slow MyHC (Sigma), mouse anti-tubulin (Santa Cruz), rabbit anti-Nfix (Novus Biological), goat anti-Lamin B (Santa Cruz), mouse anti-HA (Covance), mouse anti-vinculin (Sigma), mouse anti-β-enolase (BD Biosciences). HRP-conjugated antibodies (Biorad) were used as secondary antibodies, and the signal was revealed with the ChemiDoc MP System (Biorad). For the nucleus/cytoplasm fractionation protocol, see Supplemental Experimental Procedures.

**Transfection and co-immunoprecipitation assays**

C2C12 myoblasts were plated on 90 mm dishes and allowed to reach 90% confluence in proliferating conditions. Cells were co-transfected with pCHX-Nfix2-HA and ZS-Green IRES Sox6 plasmids with Lipofectamine LTX (Invitrogen) over night at 37°C. Cells were kept in DM for 24 h, then proteins were extracted in IP Lysis Buffer, containing 50 mM Tris-HCl pH 8.0, 1% NP40, 0,5% sodium dehoxycholate, 150 mM NaCl, protease and phosphatase inhibitors. Immunobinding was performed over night at 4°C with 5 µg of rabbit anti-Sox6 antibody (Abcam), rabbit anti-HA antibody (Santa Cruz) or non-related rabbit IgG (Santa Cruz) and 1 mg of total proteins per condition. Sepharose-conjugated Protein A (GE Healthcare) was used for immunoprecipitation of the antibody-antigen complex.

**Chromatin immunoprecipitation (ChIP)**

The ChIP for Sox6 was performed as published (An et al. 2011). For the detailed protocol see the Supplemental Experimental Procedures.

**RNA extraction and analysis**

RNA from homogenized embryos or from pelleted cells was extracted with NucleoSpin RNA kits (Macherey-Nagel) following manufactures instructions. Eluted RNA was checked on 1.6% agarose gels, quantified with a Nanodrop spectrophotomer and stored at -80°C. Approximately 1 µg of RNA was used with the ImProm-II Reverse Transcriptase kit (Promega). Real Time PCR was performed on cDNA using SYBR Green Supermix (Biorad) and the CFX Connect Real Time System (Biorad). After amplification, relative mRNA expression levels were calculated using standard curves from cDNA dilutions, and normalized on the *Gapdh* expression levels. For qRT-PCR in zebrafish we used two different normalizers: *ef1a* and *rpl8*. The primers used are listed in Supplemental Tables S1 and S2.

**MO microinjections**

Antisense morpholinos (MOs; Gene Tools, Philomath, OR) used in this study were already described: *sox6*-MO1 (von Hofsten et al. 2008), *nfixa*-MO (Pistocchi et al. 2013). MOs, diluted in Danieau buffer (Nasevicius and Ekker, 2000), were injected at 1- to 2-cell stage. Escalating doses of each MO were tested for phenotypic effects; as control for unspecific effects, each experiment was performed in parallel with a std-MO (standard control oligo) with no target in zebrafish embryos. We usually injected 0.8-1.0 pmol/embryo of *nfixa*-MO, 0.5 pmol/embryo of *sox6*-MO1. For combined knockdown experiments, we injected *sox6*-MO1 and *nfixa*-MO at 0.1 and 0.25 pmol/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 0,5% was indicated in each graph with *, **, ***

respectively, followed by the number (N) of independent experiments.

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# FIGURE LEGENDS

**Figure 1. Sox6 is equally expressed but differentially localized during primary and secondary myogenesis. A)** qRT-PCR analysis on *Myf5*$^{GFP\text{-}P/+}$ embryonic (E12.5) and fetal (E16.5) myoblasts showing unaltered expression of *Sox6* transcript in the two populations. **B)** Western Blot on lysates from freshly isolated *Myf5*$^{GFP\text{-}P/+}$ embryonic and fetal myoblasts. β-tubulin was used to normalized the amount of protein loaded. **C-F)** Immunofluorescence with antibodies anti-Sox6 (green) and MF20 (recognizing all MyHC isoforms, red) on frozen sections of embryonic (E12.5) skeletal muscle. Arrowheads show co-localization of Sox6 and MyHC in the cytoplasm of myogenic cells. Nuclei are counterstained with Hoechst. Scale bars: 25 μm. **G-J)** Immunofluorescence with antibodies anti-Sox6 (green) and MF20 (red) on frozen sections of fetal (E16.5) skeletal muscle, showing nuclear localization of Sox6 in the developing fibers (arrows). Nuclei are counterstained with Hoechst. Scale bars: 25 μm. **K)** Quantification of the immunofluorescence data (the percentage of nuclear or cytoplasmic Sox6 events was calculated on the total number of Sox6/MyHC double positive cells; N=3). **L)** Western blot with nuclear (N) and cytoplasmic (C) fractions extracted from E12.5 and E16.5 muscle tissue. β-tubulin and Lamin B are shown for normalization of the cytoplasmic and nuclear extracts, respectively. The arrow marks the 90 kDa Sox6 band; the asterisk marks the modified band for Sox6.

**Figure 2. Sox6 acts as a positive regulator of MyHC-I during embryonic myogenesis. A-F)** Immunofluorescence on E12.5 muscle sections from wt (**A,D**), *Sox6* null (**B,E**) and Tg:Mlc1f-*Nfix2* (**C,F**) mice stained with antibodies anti-MyHC-I (**A-C**) or MF20 (**C-F**). Scale bars: 100 μm. **G)** 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 protein. **H)** qRT-PCR on E12.5 muscle tissue from wt and *Sox6* null mice (*p<0,05; **p<0,01; ***p<0,005; N=3). **I)** ChIP assay with anti-Sox6 on E12.5 differentiated myoblasts. Four different chromatin regions were tested: a negative control region (intergenic), the *MyHC-I* proximal promoter (-375 bp; MyHC-I promoter), the *MyHC-I* distal enhancer (-2900 bp; MyHC-I enhancer) and the *Eno3* proximal promoter (*p<0,05; N=2). **J)** qRT-PCR on E12.5 muscle tissue from wt and Tg:Mlc1f-*Nfix2* embryos (*p<0,05; **p<0,01; ***p<0,005; N=3).

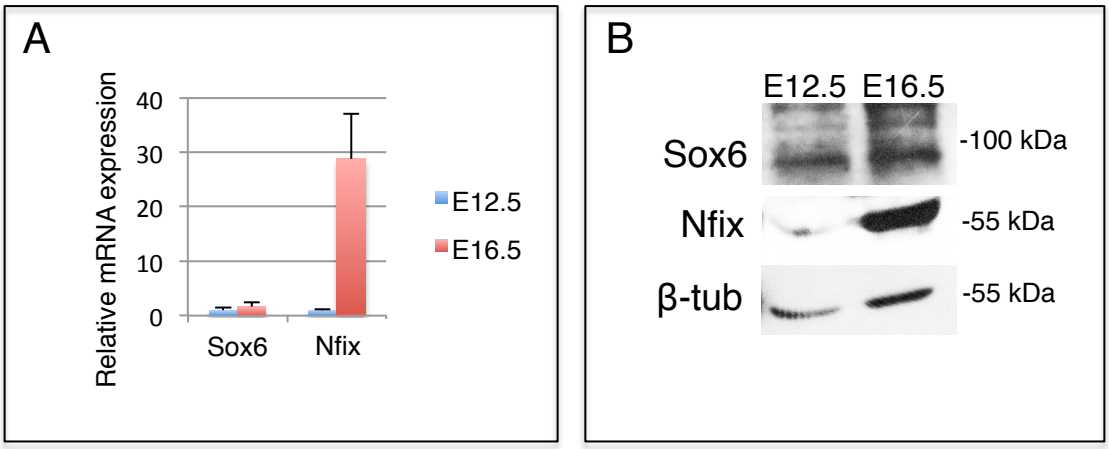
**Figure 3. Nfix is necessary for the correct function of Sox6 in fetal muscle. A-D)** Immunofluorescence with anti-Sox6 (green) and anti-MyHC-I (red) antibodies on fetal (E16.5) muscle sections from wt (**A,B**) and *Nfix* null (**C,D**) mice. Arrows indicate secondary fibers which present nuclear Sox6 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. **E)** Quantification of the immunofluorescence data showing the percentage of nuclear Sox6 positive/MyHC positive fibers (yellow bars) and the percentage of nuclear Sox6 positive/MyHC-I positive fibers in wt and *Nfix* null fetal muscle (red bars; ***p<0,005; N=3). **F)** Western Blot for Sox6 on nuclear (N) protein extracts from wt and *Nfix* null fetal muscle. Lamin B was used to normalize the amount of proteins loaded. Black squares mark lanes run on a separate gel but using the same samples and amounts of input protein. **G)** Quantification of the pixel intensity for Sox6 on two independent Western Blot experiments, one of which is depicted in Fig. 3F.

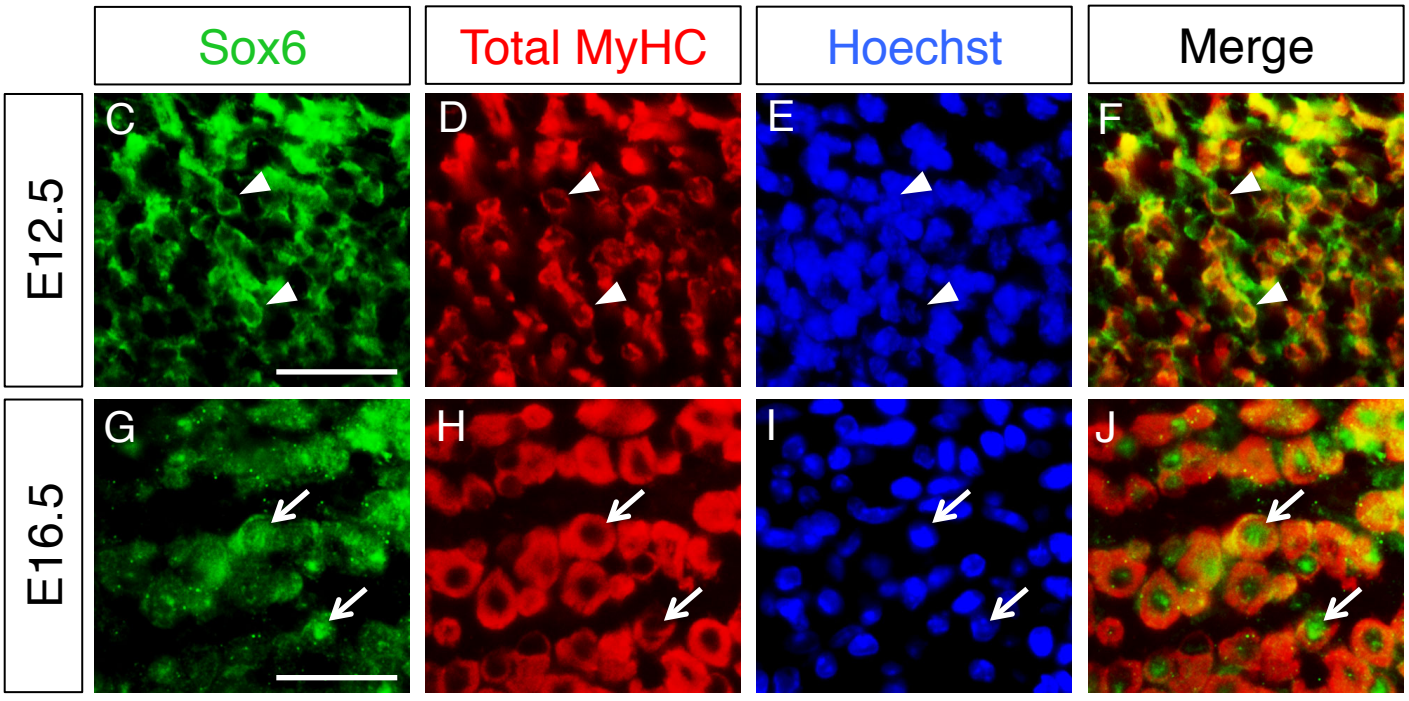
**Figure 4. Nfix is required for the correct binding of Sox6 to the MyHC-I promoter. A)** Immunoprecipitation assay on C2C12 myoblasts transfected with Nfix2-HA- and Sox6 vectors, showing co-immunoprecipitation of HA and Sox6 (T= total lysate; IgG= negative control). **B)** ChIP assay with anti-Sox6 on wt and *Nfix* null E17.5 myotubes on the same chromatin regions descripted in Fig. 2I (*p<0,05; **p<0,01; N=2).

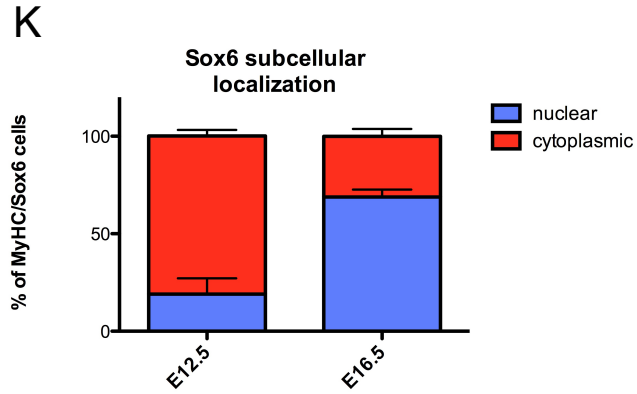
**Figure 5. Sox6 is critical for MyHC-I transcriptional repression in fetal muscle. A,B)** Immunofluorescence with anti-MyHC-I antibody on E17.5 muscle sections from wt (**A**) and *Sox6* null (**B**) mice. Scale bar: 50 μm. **C-F)** Immunofluorescence with antibodies anti-Nfix (green) and anti-MyHC-I (red) on E17.5 muscle sections from wt (**C,D**) or *Sox6* null (**E,F**) 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. **E)** qRT-PCR on wt and *Sox6* null E17.5 myotubes (*p<0,05; **p<0,01; ***p<0,005; N=3). **F)** Western Blot on wt and *Sox6* null E17.5 myotubes. β-tubulin was used to normalize the amount of proteins loaded.

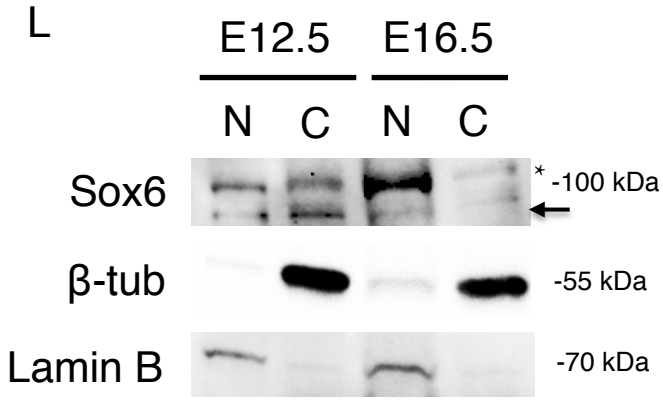
**Figure 6. Sox6 is required for normal Nfix function in fetal muscle. A-E)** qRT-PCR analysis on E12.5 and E16.5 wt (white bars) and *Sox6* null (black bars) muscle tissue. In the case of *MyHC-I/MyHC-emb* ratio (B) and of *MyHC-neo/MyHC-emb* ratio (C) the normalized values of *MyHC-I* and *MyHC-neo* (on *Gapdh*) were divided for the normalized value of *MyHC-emb* (on *Gapdh*) (*p<0,05; **p<0,01; ***p<0,005; N=2). **F)** Model proposed for cooperation between Sox6 and Nfix during primary (E12.5) and secondary (E16.5) myogenesis.

**Figure 7. Functional cooperation of sox6 and Nfixa is conserved in zebrafish. A-J)** Immunofluorescence with anti-Sox6 antibody (green) and MF20 (red) (**A-B,G-J**) or with anti-Sox6 (green) and anti-sMyHC1 (F59, red) (**C-F**) on 48 hpf zebrafish muscle longitudinal sections. Arrowheads indicate Sox6 positive nuclei in fast-twitch muscle fibers. Arrows indicate Sox6 staining in the cytoplasm of superficial slow fibers. Nuclei are counterstained with Hoechst. Scale bars: 25 μm. **K)** qRT-PCR analysis on tails from std-MO, *sox6*-MO, *nfixa*-MO and *sox6*-MO:*nfixa*-MO 48 hpf embryos (*p<0,05; **p<0,01; ***p<0,005; N=2).

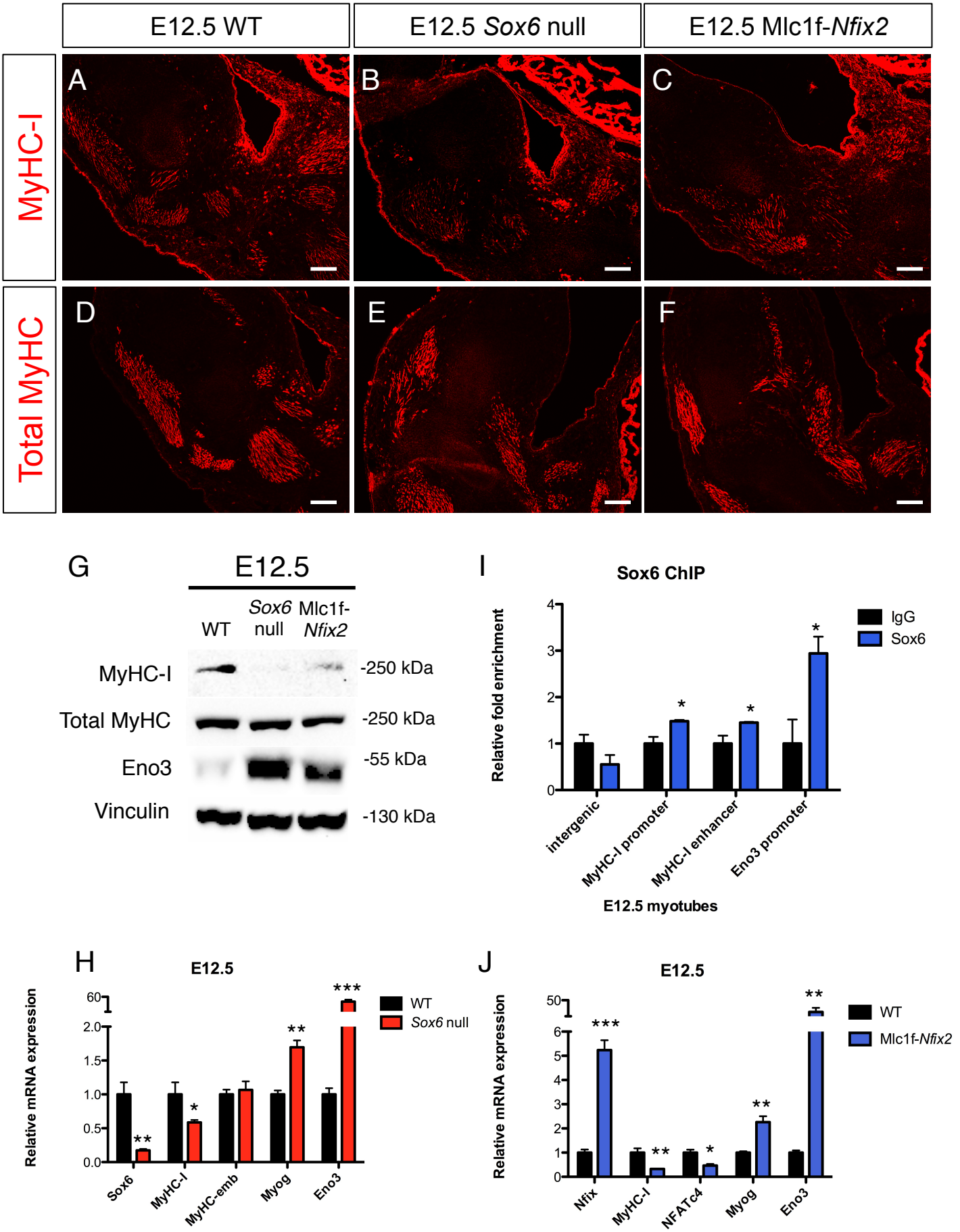


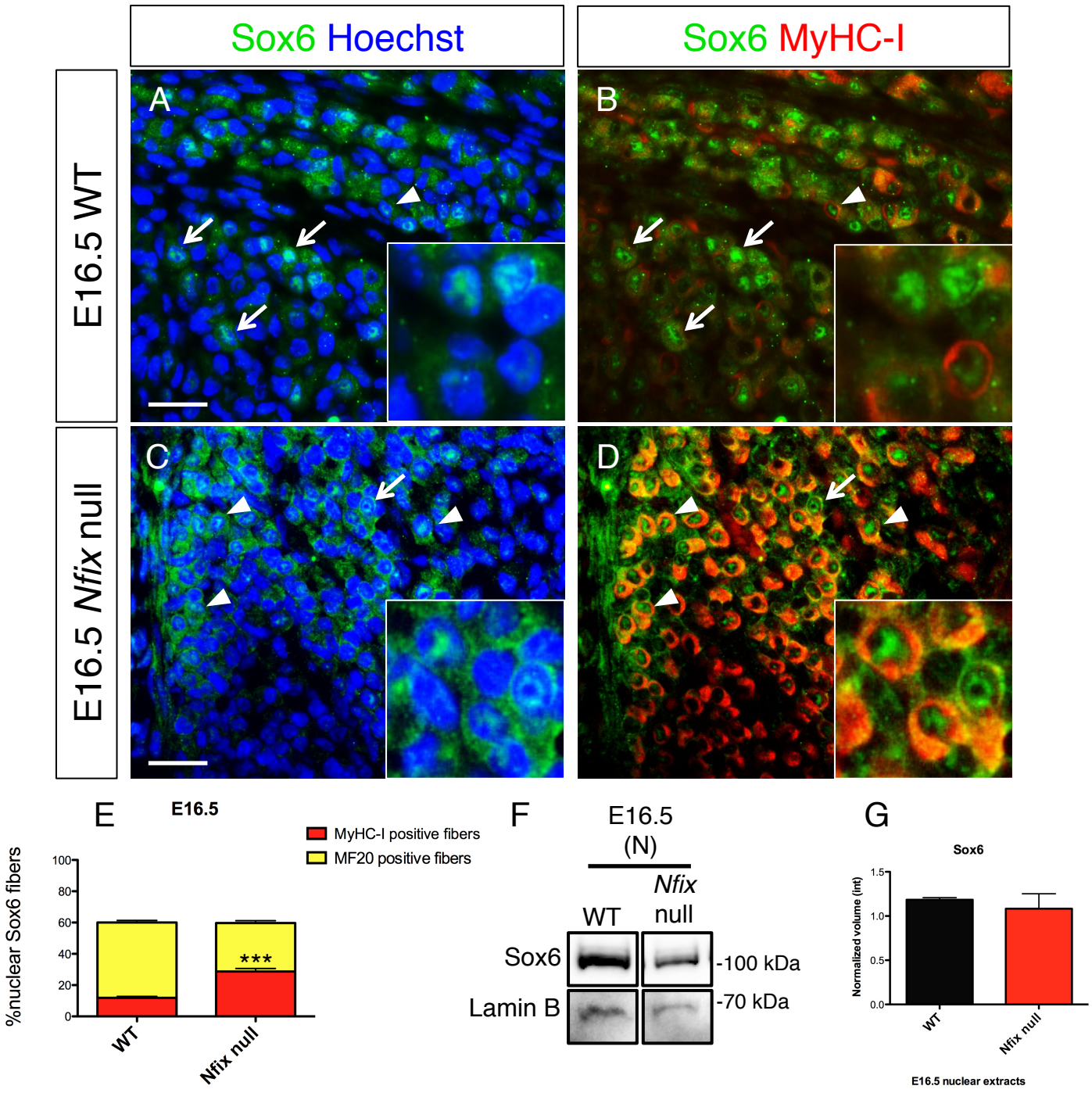




Maroli et al., Fig. 1




Maroli et al., Fig. 2

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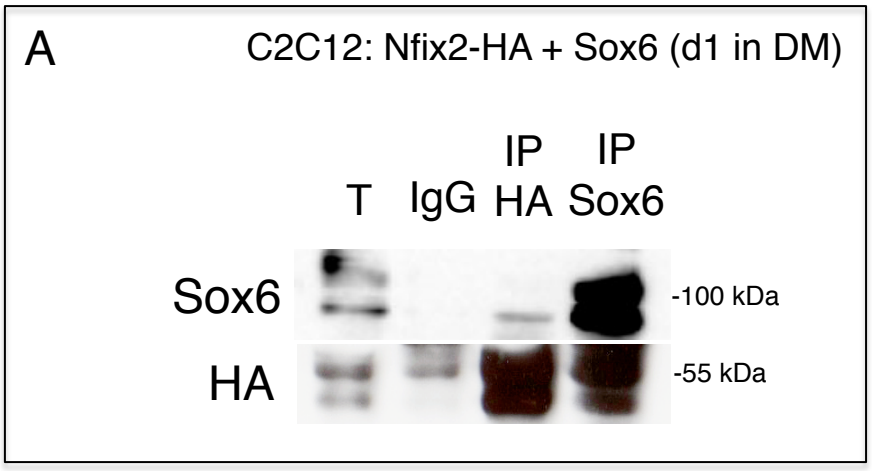


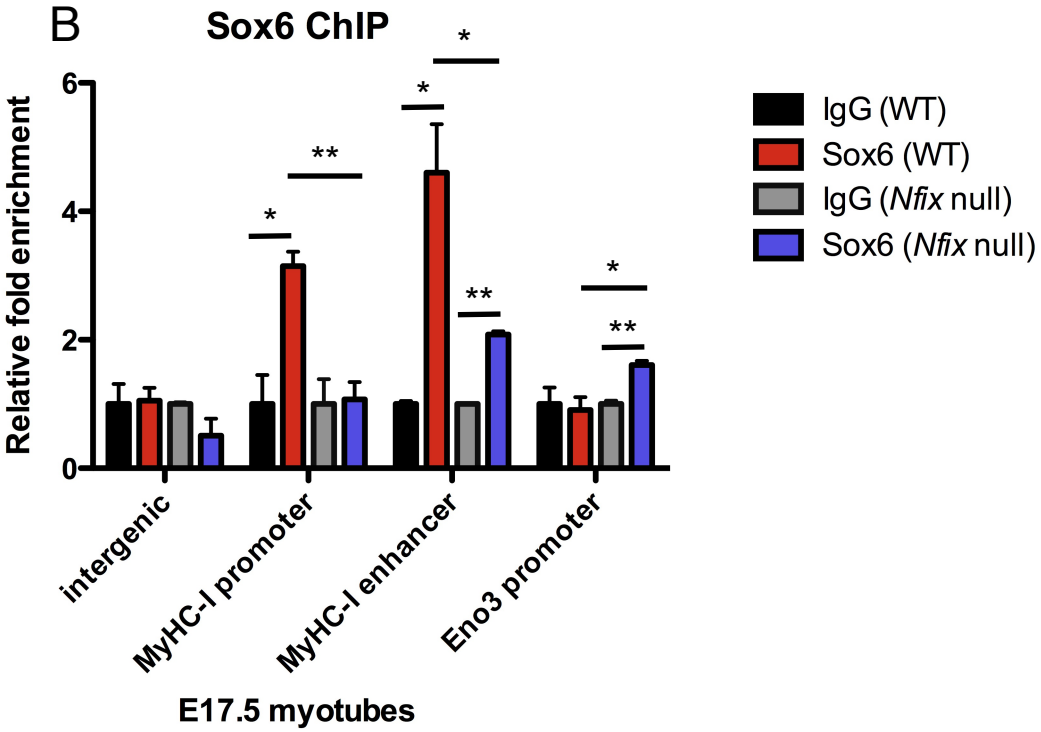


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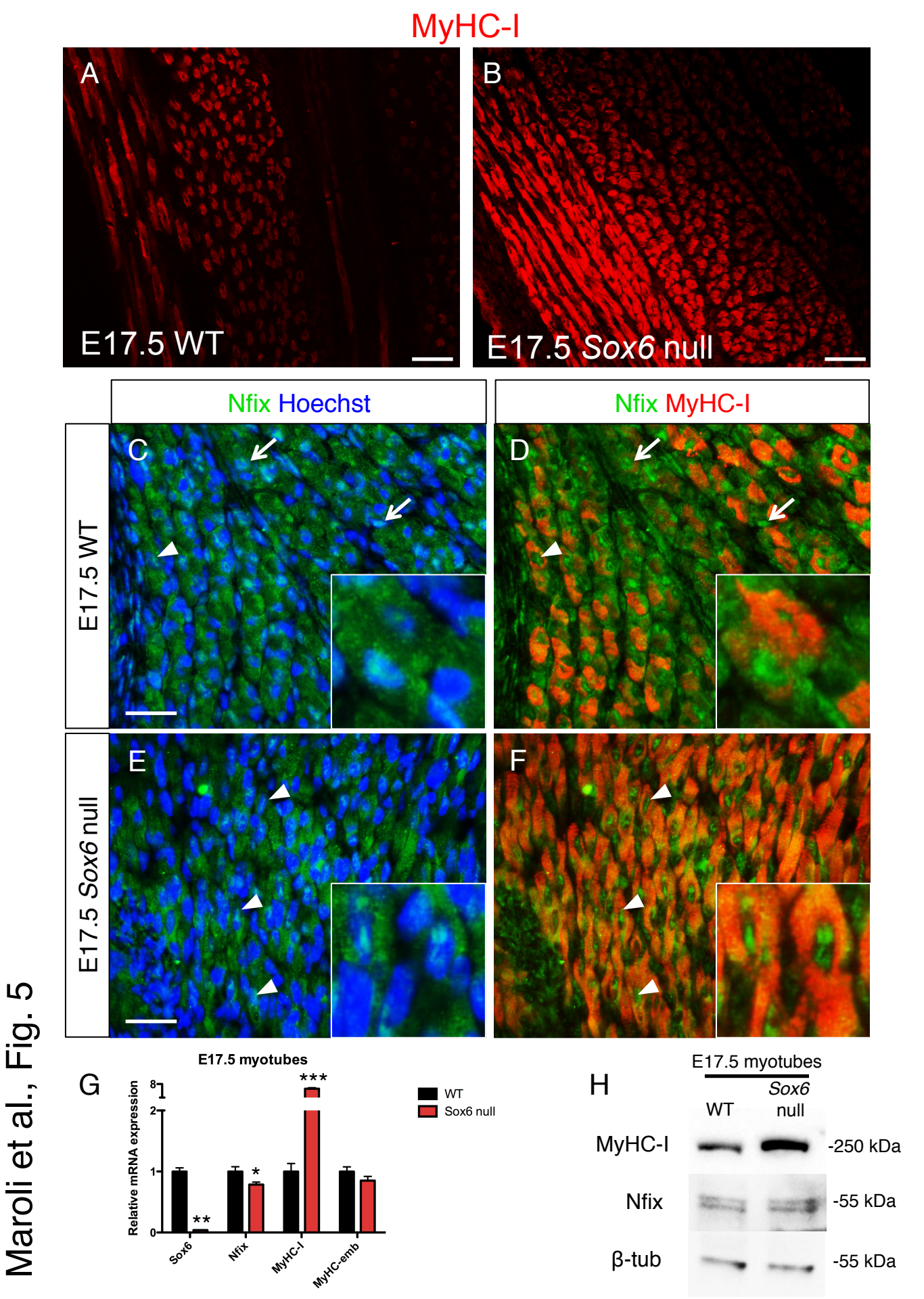
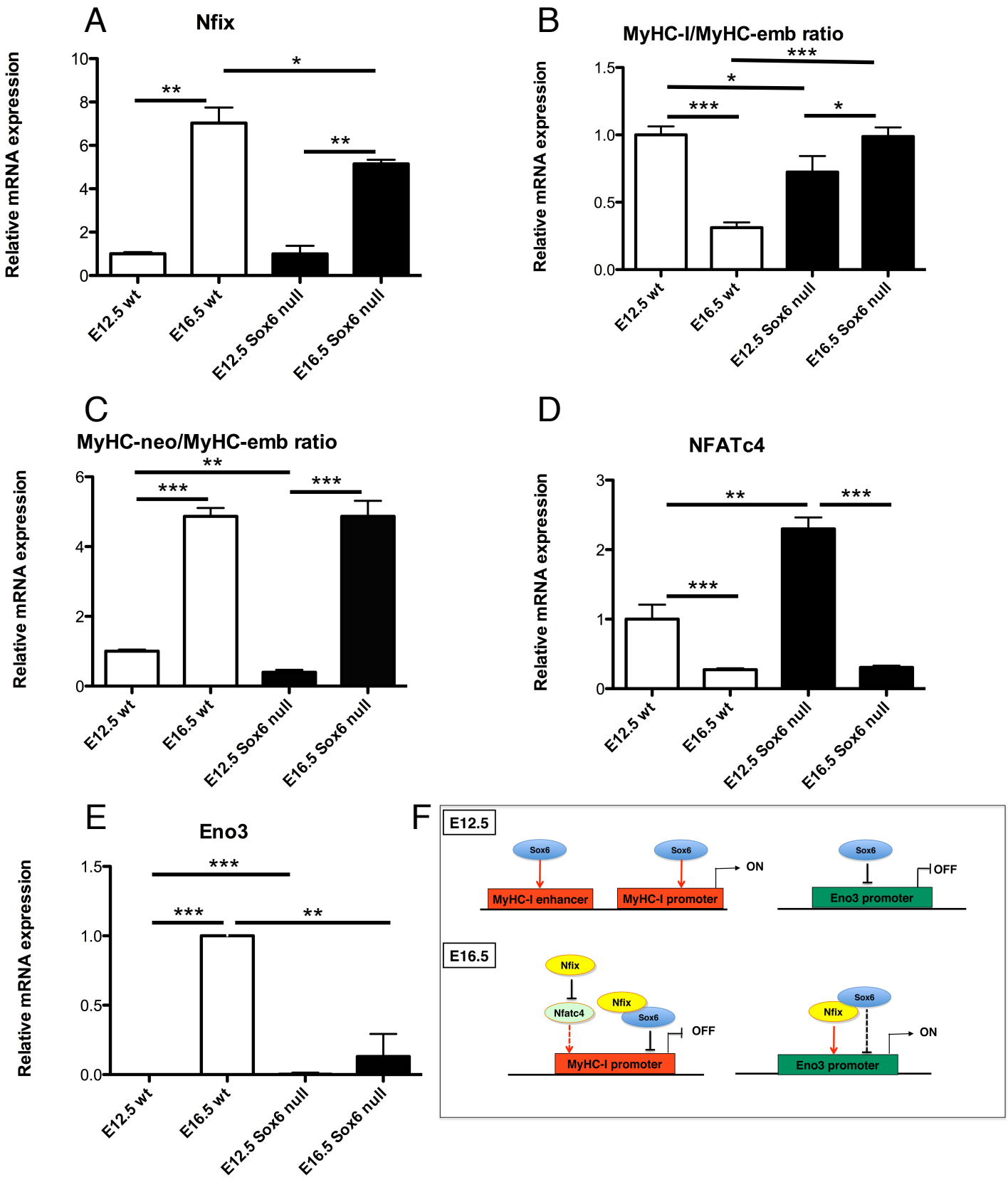
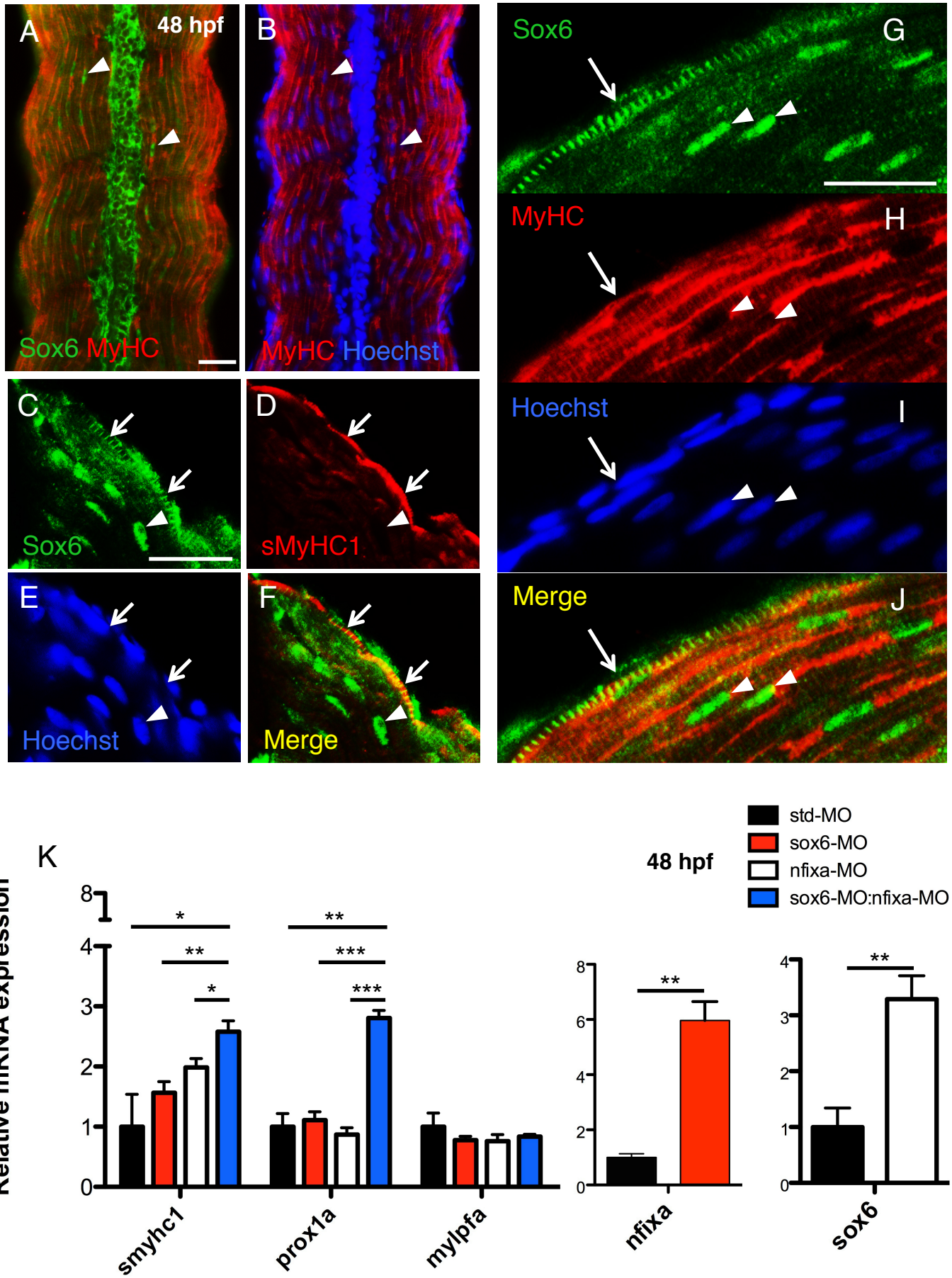



Maroli et al., Fig. 5




Maroli et al., Fig. 6




Maroli et al., Fig. 7

# SUPPLEMENTAL INFORMATION

## SUPPLEMENTAL DATA

**Supplemental Figure S1. Sox6 is expressed in both embryonic and fetal muscle cells. A-H)** Immunofluorescence with antibodies anti-Sox6 (green) and anti-MyoD (red) (**A-D**) or anti-myogenin (red) (**E-H**) on E12.5 muscle sections showing cytoplasmic localization of Sox6 in myoblasts and myocytes (arrows in the panels). **I-L)** Immunofluorescence with antibodies anti-Sox6 (green) and anti-MyHC-I (red) on E17.5 muscle sections, showing nuclear expression of Sox6 in MyHC-I negative (secondary) fibers (arrows). Nuclei are counterstained with Hoechst. Scale bars: 25 μm.

**Supplemental Figure S2. Sox6 is normally expressed in fetal muscle in the absence of Nfix. A,B)** Immunofluorescence with anti-Sox6 antibody (green) and MF20 (red) on fetal (E16.5) muscle sections from wt and *Nfix* null mice. Scale bars: 25 μm. **C)** qRT-PCR on wt and *Nfix* null E17.5 myotubes (** $p<0,01$; N=3). The increase in *Sox6* mRNA expression is not statistically significant. **D)** Western blot on lysates from wt and *Nfix* null E17.5 myotubes showing increased MyHC-I expression and equal Sox6 expression in the absence of Nfix. β-tubulin was used to normalize the amount of proteins loaded.

**Supplemental Figure S3. Knock-down of *sox6* in zebrafish embryos impairs the Nfixa/Nfatc4 axis function. A,B)** qRT-PCR for *sox6* (**A**) and *nfixa* (**B**) on tails

collected from 24, 48 and 72 hpf zebrafish embryos. **C-H)** qRT-PCR for *nfixa*, *nfatc4*, *smyhc1*, *prox1a*, *mylpfa*, *tnnc2* on tails collected from std-MO and *sox6*-MO injected embryos at 24 and 48 hpf (*p<0,05; **p<0,01; ***p<0,005; N=2).

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

| Gene | Primers sequence | Notes |
|---|---|---|
| *Sox6* | F:AATGCACAACAAACCTCACTCT<br>R:AGGTAGACGTATTTCGGAAGGA | |
| *MyHC-I (Myh7)* | F:AGGGCGACCTCAACGAGAT<br>R:CAGCAGACTCTGGAGGCTCTT | Mathew et al. 2011 |
| *MyHC-emb (Myh3)* | F:GCAAAGACCCGTGACTTCACCTCTAG<br>R:GCATGTGGAAAAGTGATACGTGG | Mathew et al. 2011 |
| *MyHC-neo (Myh8)* | F: GTCACGCAATGCAGAAGAGA<br>R: CAGGTCCTTCACCGTCTGTT | Mathew et al. 2011 |
| *Nfix* | F:CACTGGGGCGACTTGTAGAG<br>R:AGGCTGACAAGGTGTGGC | Mourikis et al. 2012 |
| *NFATc4* | F:TACAGCAACAAGCGGGTGTC<br>R:CGGAGAGATGAGTCTGGTAGGG | |
| *Myog* | F:GACATCCCCCTATTTCTACCA<br>R:GTCCCCAGTCCCTTTTCTTC | |
| *Eno3* | F:CAGCTTGTTTCCAGCATGAG<br>R:CTCTACCGACACATCGCAGA | Mourikis et al. 2012 |
| *Gapdh* | F:GGCATGGACTGTGGTCATGA<br>R:TTCACCACCATGGAGAAGGC | |

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

| Gene | Primers sequence | Notes |
|---|---|---|
| *nfixa* | F:ACCGAAGAAACCGACTGGTG | |
| | R:TCTGTGGCCATTGTAGTTCAGG | |
| *sox6* | F:GCGCATGGAATCGGACAG<br>R:GGCTTGTGTGGAGAGGTAGAG | |
| *smyhc1* | F:GCTAACAGGCAGGCATCAGA<br>R:GTTGCATTTGGGAATCCTTGACA | |
| *mylpfa* | F: GCGGCTTCAGACTTCTCTTCTTG<br>R: CTTCTTGGGTGCCATGTCGAG | |
| *tnnc2* | F:CAATCATGACTGACGCGCAA<br>R:GCCACCGTCAGTATCGAACA | |
| *nfatc4* | F:GCGCATAGAAGTACAGCCCA<br>R:TAGCCCACAAGCTTCACCAC | |
| *prox1a* | F: CCAACCACCTCAGTCACCAT<br>R: CCCCTCTTGGATTGTACTTCCT | |
| *ef1a* | F:CTGGAGGCCAGCTCAAACAT<br>R:ATCAAGAAGAGTAGTACCGCTAGCATTAC | |
| *rpl8* | F:GCAAACAGAGCCGTTGTTG<br>R:CCTTCAGGATGGGTTTGTCA | |

# SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Preparation of nuclear and cytoplasmic protein extracts**

Eviscerated embryos and fetuses were homogenized for 1 min in ice with 300-500 µl of STM buffer comprising 250 mM sucrose, 50 mM Tris–HCl pH 7.4, 5 mM MgCl2, protease and phosphatase inhibitor cocktails (Dimauro et al. 2012). After centrifugation, the supernatant was kept as cytoplasmic extract, while the pellet was washed twice in hypotonic buffer without detergents, resuspended in NE buffer (containing 20 mM HEPES pH 7.9, 1.5 mM MgCl2, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton-X-100, protease and phosphatase inhibitors) and centrifuged 30 min at 43000 rpm at 4°C. The supernatant was kept as nuclear extract. Cytoplasmic and nuclear extracts were then quantified and loaded on polyacrylamide gels for Western Blot analysis.

**Chromatin immunoprecipitation (ChIP)**

ChIP was performed as previously published (An et al. 2011). Briefly, primary myoblasts were crosslinked for 10 min at RT in 1% formaldehyde (Sigma) in PBS, then the crosslinking was quenched with 0,25 M glycine in PBS (10 min) and the cells were pelleted and lysed in Sonication Buffer containing 10 mM TRIS-HCl pH 8.0, 1 mM 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 200-500 bp DNA fragments enrichment. Immunoprecipitation was performed with 3-5 µg of the following antibodies: goat anti-Sox6 (Santa Cruz), normal goat IgG (Santa Cruz). After elution, de-crosslinking and 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): CCCCACCCCCTGGAACT (fw), CCAGCTAGGAAACAATTGGAAGTG (rev); for the *MyHC-I* distal promoter region (enhancer): ACACCGCCCACTCAATACAC (fw), GCCCTCTCCAAACACTCTTG (rev); for a negative control region located 15 Kb upstream of the *MyHC-I* gene (intergenic): TCGGACCGGAGTGTTAGGAA (fw), ACCCTGGAGTCTCAGCATCG (rev) (An et al. 2011); and for the proximal *Eno3* promoter: CCAGGTCAGGAGCTTTTGCT (fw), CCGCCTCTCTCATTCCATCC (rev). The fold enrichment of each sample was calculated as percentage of input for internal control and then normalized on the IgG value.

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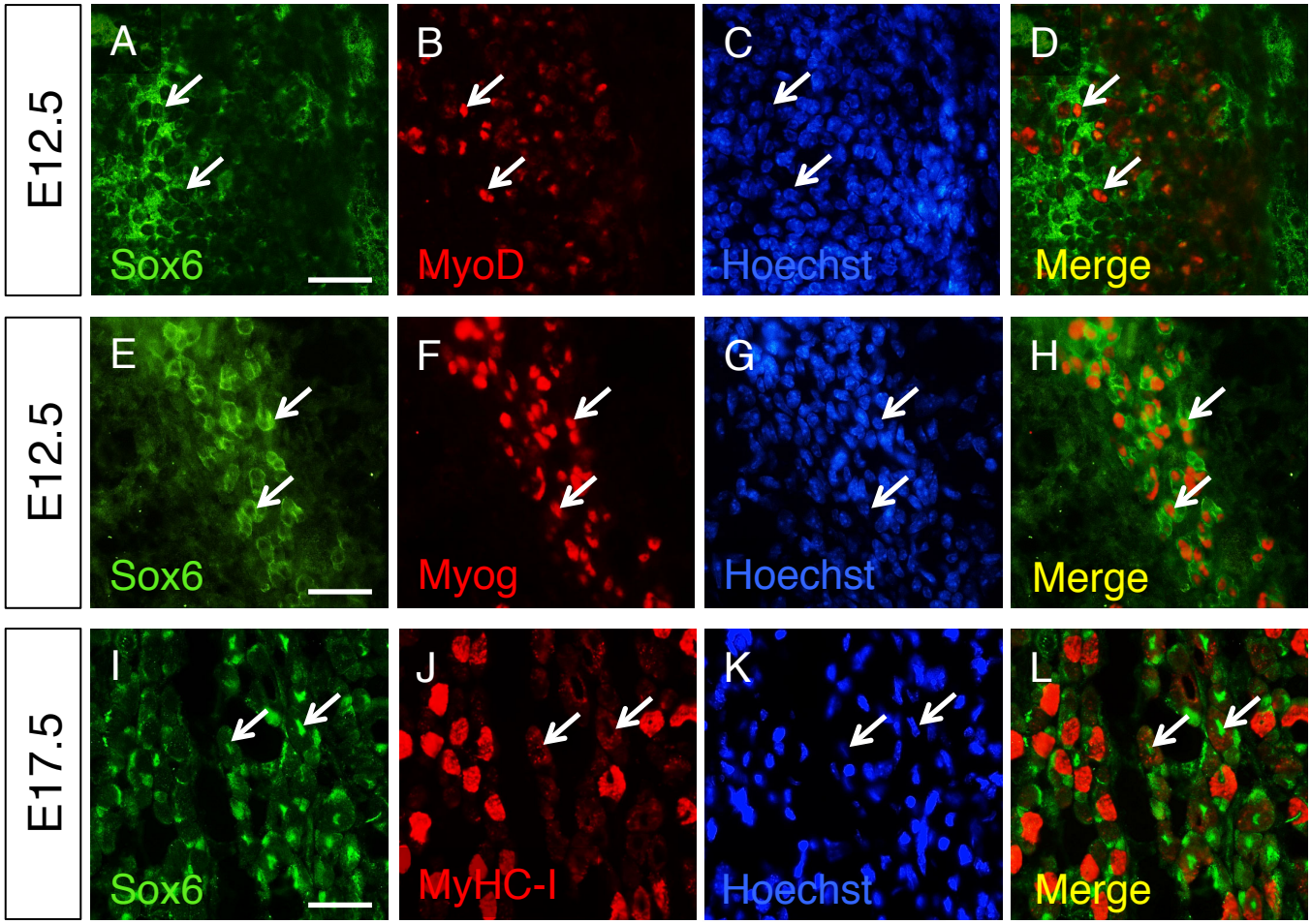
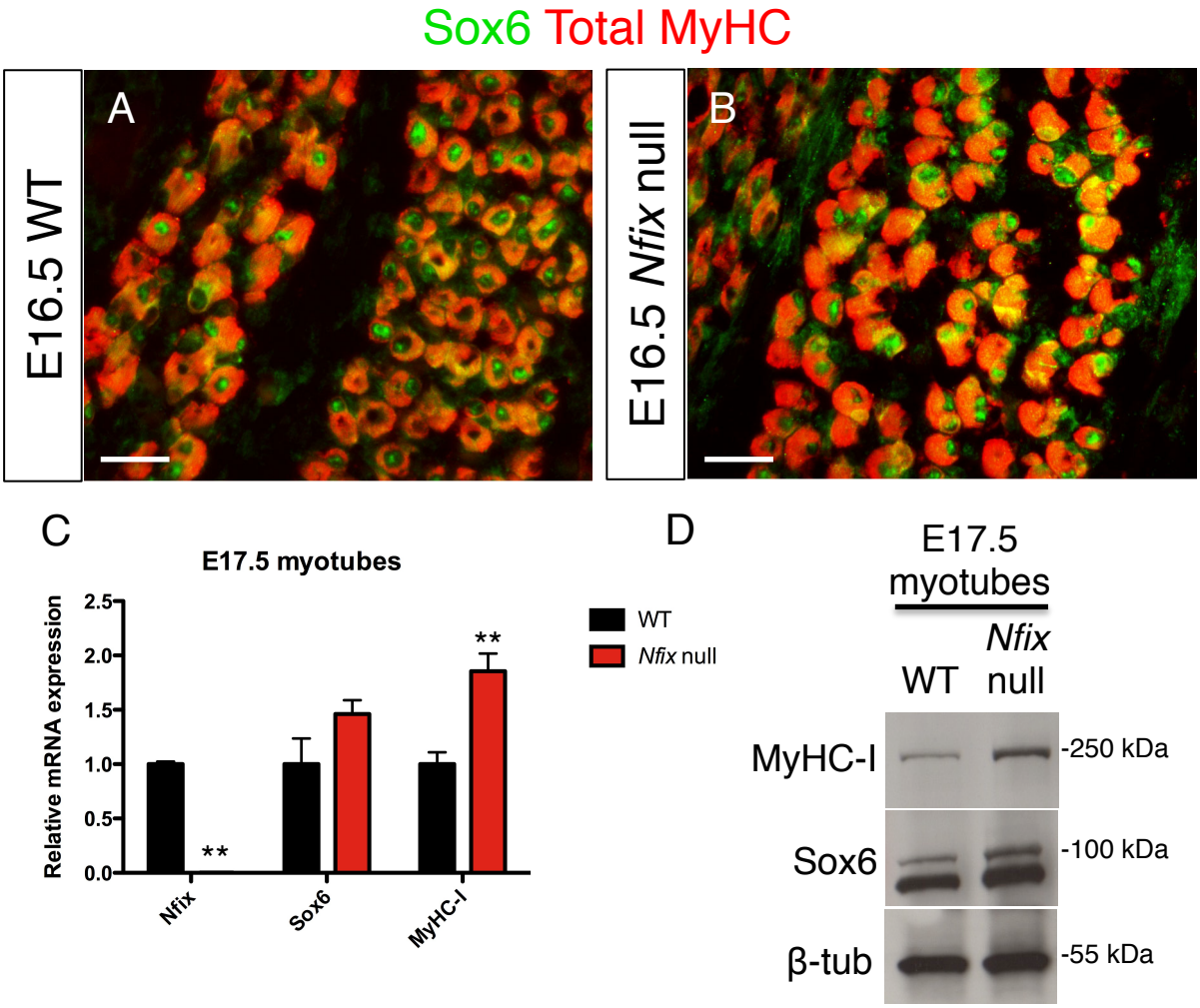



Maroli et al., Fig. S1

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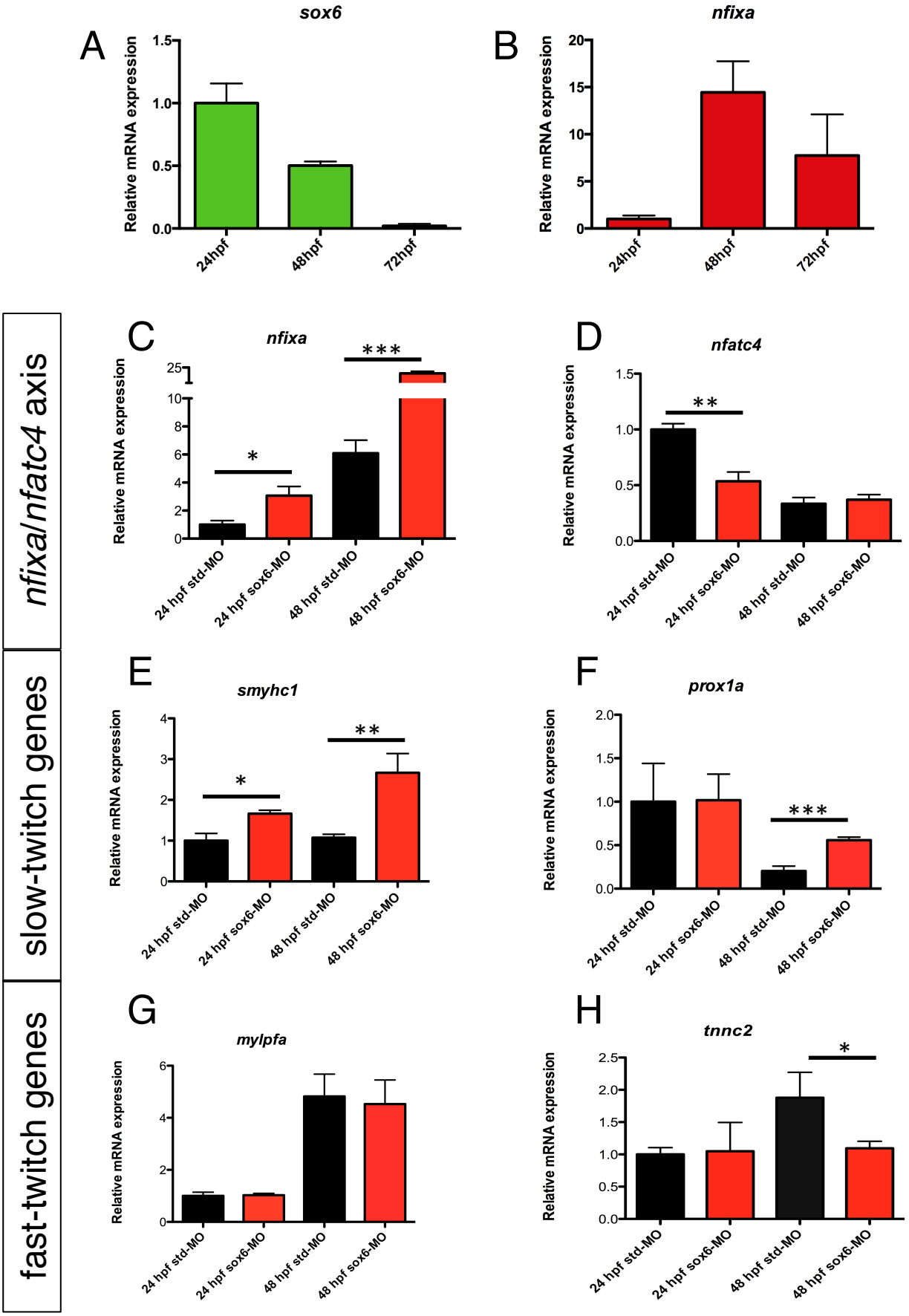


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