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# Role of the Srf transcription factor in adult muscle stem cells

Aikaterini Papaefthymiou

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# Université Paris Descartes

## Ecole doctorale Bio Sorbonne Paris Cité

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*Institut Cochin*

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By Aikaterini Papaefthymiou

PhD thesis in Development Discipline

Supervised by Dr. Athanassia Sotiropoulos

Defended on 30th of November 2016

In front of a jury composed of:

Montarras Didier: Research Director - Institut Pasteur, Rapporteur

Duprez Delphine: Research Director - CNRS, Rapporteur

Hénon Sylvie: Professor - University of Paris Diderot, Examineur

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**Résumé:** Le muscle squelettique adulte est un tissu avec une grande plasticité étant donné qu'il adapte sa taille suite à la surcharge fonctionnelle et il régénère suite à une lésion. La base de cette plasticité est la myofibre et les cellules souches associées, les cellules satellites (CS). Suite aux stimuli, les CS sortent de la quiescence, elles s'activent, prolifèrent, s'engagent dans la voie myogénique et fusionnent entre elles ou bien avec la fibre pré-existante. Une partie des CS retourne à la quiescence afin de maintenir le « pool » de progéniteurs. Ce projet a pour objectif de mieux caractériser des voies de signalisation responsables des adaptations des CS au cours de la régénération et de l'hypertrophie compensatoire.

Srf est un facteur de transcription, particulièrement exprimé dans les muscles. Les gènes cibles de Srf sont des gènes qui participent à la régulation de la prolifération cellulaire et des gènes codant des protéines sarcomériques du muscle ou bien des gènes ayant un rôle dans l'adhésion cellulaire, la migration et l'organisation du cytosquelette. Il a été montré que la perte de fonction de Srf dans la lignée de cellules musculaire C2C12 inhibe leur prolifération et leur différenciation et que Srf contrôle l'expression de MyoD qui est un gène de détermination myogénique. Aucune donnée n'est disponible à ce jour concernant la fonction de Srf dans les CS *in vivo*. Nous avons généré des souris dépourvues de Srf spécifiquement dans les CS adultes. Les CS ont été recrutées par l'hypertrophie et la régénération musculaire. En parallèle des études *ex vivo* ont été menées afin de préciser si les phénotypes observés sont cellule-autonomes et afin de disséquer les mécanismes sous-jacents.

Nous montrons que la perte de Srf dans les CS affecte fortement les processus de régénération et d'hypertrophie suggérant un rôle de Srf dans le contrôle du destin cellulaire de CS. Nos études montrent que la perte de Srf dans les SC n'affecte pas leur prolifération et leur engagement dans la différenciation myogénique. Par contre, leur motilité et leur capacité de fusion sont fortement réduites.

Afin d'identifier les effecteurs de Srf impliqués dans la motilité et le défaut de fusion des CS mutantes, nous avons réalisé des études transcriptomiques et identifié le set de gènes dont l'expression est altérée par la perte de Srf dans des conditions de prolifération et de différenciation. L'analyse des fonctions altérées nous a indiqué que la voie de signalisation du cytosquelette d'actine était perturbée. En effet les CS dépourvues de Srf expriment moins d'actine et présentent une organisation du cytosquelette d'actine perturbée. Des expériences de sauvetage utilisant un modèle de souris permettant la surexpression inducible d'actine alpha dans les CS dépourvues de Srf ont montré que la surexpression d'actine chez les mutants Srf était suffisante pour rétablir partiellement l'organisation du cytosquelette et améliorer les capacités de fusion des CS. De manière intéressante, seule la fusion hétérotypique (entre une cellule contrôle et une cellule mutante), et pas la fusion homotypique (entre deux cellules mutantes), est rétablie par l'expression de l'actine. *In vivo*, le rétablissement de la fusion hétérotypique restaure la croissance hypertrophique des muscles alors que l'altération de la régénération chez les mutants Srf n'est que faiblement améliorée par la surexpression de l'actine. Cette étude nous a permis d'avoir une vision d'ensemble et mécanistique de la contribution du facteur de transcription Srf dans la biologie des CS et de mettre en évidence l'importance structurale du maintien du cytosquelette d'actine pour la fusion des cellules musculaires.

**Mots clés:** cellules souches musculaires, serum response factor, hypertrophie musculaire, régénération musculaire

**Title:** Role of the Serum Response Factor (SRF) transcription factor in adult muscle stem cells

**Abstract:** The adult skeletal muscle is a high plastic tissue as it adapts its size upon overload and it is capable of regeneration upon muscle lesion. The skeletal muscle is composed of a specialized syncytium, the myofiber, which is the functional unit of the muscle and a small population of myogenic progenitors, residing adjacent to the myofibers, termed as satellite cells (SCs). SCs are the muscle-specific stem cells which endow the skeletal muscle with its remarkable capacity to repair and to maintain homeostasis during muscle turnover. In resting adult muscles, SCs are quiescent but they activate upon exposure to stimuli. The activated SCs (myoblasts) proliferate extensively and subsequently differentiate and fuse between them or pre-existing myofibers, a series of cellular events called myogenesis. In parallel to the myogenesis, a reserve population of SCs escapes the myogenic program and self-renews to replenish the SC pool. The current project aims to further characterize the signalling pathways involved in SC functions during muscle regeneration and compensatory hypertrophy (CH).

Srf is a muscle-enriched transcription factor with Srf-target genes implicated in cell proliferation, differentiation (sarcomeric proteins), adhesion, migration and cellular cytoskeleton. Studies in C2C12 mouse myogenic cell line showed that Srf loss prevent the myoblast proliferation and differentiation by down-regulating the expression of the myogenic determinant MyoD gene.

We used a genetic murine model for adult SC-specific Srf-loss in order to conduct *in vivo* and *ex vivo* studies for the Srf role in SCs. Compensatory hypertrophy and regeneration are the two means by which SCs were recruited. We show that loss of Srf in SCs affects the regeneration process and the CH suggesting the Srf role in the SC fate. Srf-depleted SCs display probably no defect in their proliferation and differentiation but reduced capacity in motility and fusion. Transcriptomic analysis revealed altered actin cytoskeleton and signalling. Srf-depleted SCs show reduced actin expression and altered actin cytoskeleton. Rescue of actin expression in Srf-depleted SCs partially restored the cytoskeleton organization and the fusion process. Interestingly by actin overexpression only the heterotypic/asymmetric fusion was established but not the homotypic/symmetric fusion. Therefore actin overexpression restored the hypertrophic growth in the CH (*in vivo* model of heterotypic fusion) but failed to do so in the regeneration (*in vivo* model of homotypic fusion).

This study contributed to the *in vivo* investigation of the Srf mechanistic role in adult SCs and underlined the importance of actin cytoskeleton maintenance in the fusion of myogenic cells.

**Keywords:** muscle satellite cells, serum response factor, skeletal muscle hypertrophy, skeletal muscle regeneration, actin cytoskeleton

*L'enfer, c'est les autres....*

*Jean-Paul Sartre*

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Emilie, écoute....keep living for counting! ...mais enfin une Française belle qui pense comme moi !

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Πολύτιμοί μου λίθοι καλά στην καρδιά μου σας έχω φυλαγμένους...



## La ville

Tu t'es dit "J'irai ailleurs, un autre pays,  
un nouveau rivage doivent exister, une ville autre.  
Tous mes efforts ici sont condamnés;  
et mon cœur n'est que mort, enterré.  
Jusqu'à quand ce marasme? Où que je tourne mes yeux,  
où mon regard se pose, je ne vois que ruines  
celles de ma vie gâchée, depuis toutes ces années  
ici, où je ne suis que l'épave de moi-même.

Il n'y aura pas d'autres pays,  
tu chercheras en vain d'autres rivages,  
la ville te poursuivra. Dans ces mêmes  
rues tu iras roder. Et tu vieilliras  
dans ces mêmes quartiers; tes cheveux  
blanchiront dans ces mêmes maisons.  
Toutes les routes te ramèneront ici,  
dans cette même ville.  
Pour ce qui est d'ailleurs - n'espère pas -  
pour toi point de navire, point de chemin.  
De la façon dont ici,  
dans ce petit coin tu as raté ta vie,  
tu l'as ruinée partout, sur toute la terre.

Cavafis

traduit par François Sommaripas

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## Abbreviations

ABPs: Actin-Binding Proteins  
bHLH: basic Helix-Loop-Helix  
BMP: Bone Morphogenetic Protein  
BrdU: Bromo-deoxy-Uridine  
CH: Compensatory Hypertrophy  
CSA: Cross-Sectional Area  
CTX: Cardiotoxin  
E: Embryonic Day  
ECs: Endothelial Cells  
ECM: Extra Cellular Matrix  
EdU: Ethylo-deoxy-Uridine  
FCM: Fusion-Competent Myoblast  
FGF: Fibroblast Growth Factor  
FC: Founder Cell  
GI: Gastro Intestinal tract  
HGF: Hepatocyte Growth Factor  
IEGs: Immediate-Early Genes  
IGF-1: Insulin-like Growth factor-1  
IP: Intraperitoneal  
KO: Knock-out  
MADS: MCM1-Agamous-Deficiens-SRF  
MAPKs: Mitogen-Activated Protein Kinases  
MEF2: Myocyte Enhancer Factor 2  
MND: Myo-Nuclear Domain  
MRFs: Myogenic Regulatory Factors  
MRTFs: Myocardin Related Transcription Factors  
Mstn: Myostatin  
Mammalian Target of Rapamycin: mTOR  
MyHC: Myosin Heavy Chain

PI: Plantaris muscle

QSCs: Quiescent Satellite Cells

SCs: Satellite Cells

SMCs: Smooth Muscle Cells

SRE: Serum Response Element

SRF: Serum Response Factor

TA: Tibialis Anterior

TCFs: Ternary Complex Factors

TF: Transcription Factor

Tg: Transgene

TGF- $\beta$ : Transforming Growth Factor- $\beta$

TMX: Tamoxifen

TNF- $\alpha$ : Tumor Necrosis Factor- $\alpha$

VEGF: Vascular Endothelial Growth Factor

## Introduction

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### 1. Skeletal Muscle: an organ for locomotion and energy metabolism

**Movement** is a defining feature of all animals and the evolutionary advantages of efficient locomotion led to several solutions for the construction of the motile organs (the muscles) in all animal phyla. **Skeletal muscle**, which constitutes about 40% of the mass of the human body, is the **organ** with the specificity to transform the chemical energy to mechanical energy (potential and kinetic energy) as well as heat energy. Its metabolism affects the metabolic balance of the entire organism and is the major body protein reservoir. Skeletal muscle is the most flexible structure in vertebrate organisms as it exerts diverse functions, enabling both crushing with great force and movement with exquisite precision (Braun and Gautel, 2011). The main muscle activity is the **contraction** of muscle cells, which is under the voluntary control and it is initiated by the nerves impulses.

The functional cellular units responsible for skeletal muscle contraction are cylindrical, multinucleated **muscle fibers** (myofibers). The skeletal myofiber is a specialized syncytium<sup>1</sup>, which contains thousands of myonuclei within a common, undivided cytoplasm, forming an elongated cell under the plasma membrane called sarcolemma.

The myofiber nuclei are postmitotic and under normal conditions cannot re-enter a proliferative state to contribute to additional nuclei. They lose this ability of mitosis once the myogenic progenitors' nuclei have been incorporated into myotubes during the embryonic and postnatal development. In adult life, somatic **stem cell** populations participate in the homeostasis of their host tissues. During postnatal life, myofiber growth and repair of skeletal muscle depend on muscle stem cells, otherwise named **satellite cells** (Mauro, 1961; Moss and Leblond, 1971).

---

<sup>1</sup> from Anc Greek: σύν (syn) = "together" + κύτος (kytos) = "box, i.e. cell"

## Part 1: Origins of Skeletal Muscle and Satellite cells

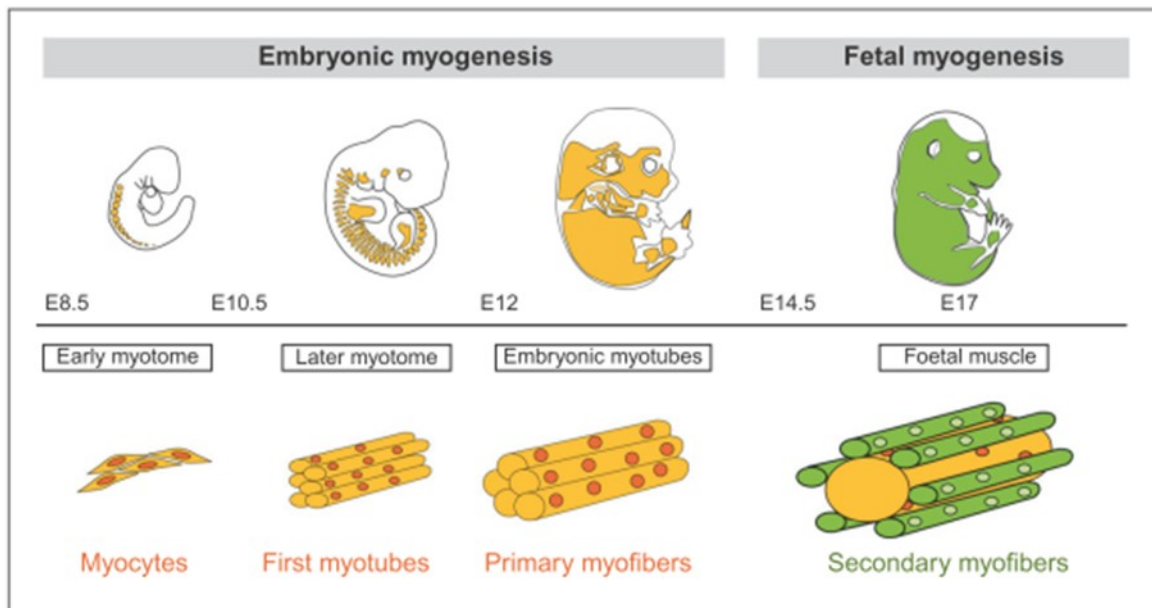
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The three germ layers (endoderm, mesoderm, and ectoderm) are formed during gastrulation. The **mesoderm** is at the origin of muscles, gonads, secretory organs, and connective tissue. Mesoderm is anatomically separated into **paraxial**, **intermediate**, and **lateral** mesoderm, with respect to position from the midline (Bentzinger et al., 2012). Skeletal muscle cells of higher vertebrates arise during midgestation (in mice between embryonic day 9 (E9) and E12) from three different locations within the middle layer of cells in the primitive embryo: the segmented somitic paraxial mesoderm, the unsegmented cranial paraxial mesoderm and the prechordal mesoderm; these represent different parts of the mesoderm along the rostrocaudal axis (Braun and Gautel, 2011).

In mouse, skeletal muscle development occurs in several phases (Figure 1). First, in the E8.5 of gestation, **primary** myofibers develop displaying the earliest expression of myogenin and muscle-specific genes (desmin, titin and  $\alpha$ -actin genes). Around E13 of gestation, **secondary** myofibers form in trunk and limbs, and they develop parallel to the primary myofibers, that serves as a scaffold for the orientation of differentiating myocytes of a second wave of myoblasts (Buckingham and Mayeux, 2012).

Satellite cells (SCs), the stem cells of adult skeletal muscle, arise around E17 of development as a **unique myogenic cell lineage**. They constitute the principal proliferative cell population of developing skeletal muscle. Late in fetal development at around E16.5-18.5, these cells **occupy a satellite cell position** adjacent to the myofibers, a characteristic of progenitor cells in postnatal muscle (Kassar-Duchossoy et al., 2005; Relaix et al., 2005).

Further skeletal muscle maturation occurs during the **postnatal** period for about 2-3 weeks with the SC nuclear accretion to contribute to multinucleated myofibers (Tajbakhsh, 2009). The adult number of myonuclei and satellite cells is established by 3 weeks of postnatal development. Subsequently, the muscle mass undergo extensive hypertrophic **growth** with increased cellular protein content to dominate in order to achieve growth of the musculature to adult size (White et al., 2010).



**Figure 1** : Waves of skeletal muscle formation. There is a first wave of skeletal muscle formation, termed embryonic or primary myogenesis. This is followed by a second wave of fetal or secondary myogenesis. The timing of this transition depends on the onset of innervation which varies at different sites in the embryo. E: Embryonic day of mouse development. From (Buckingham and Mayeuf, 2012)

## Chapter 1: Trunk and Limb Myogenesis

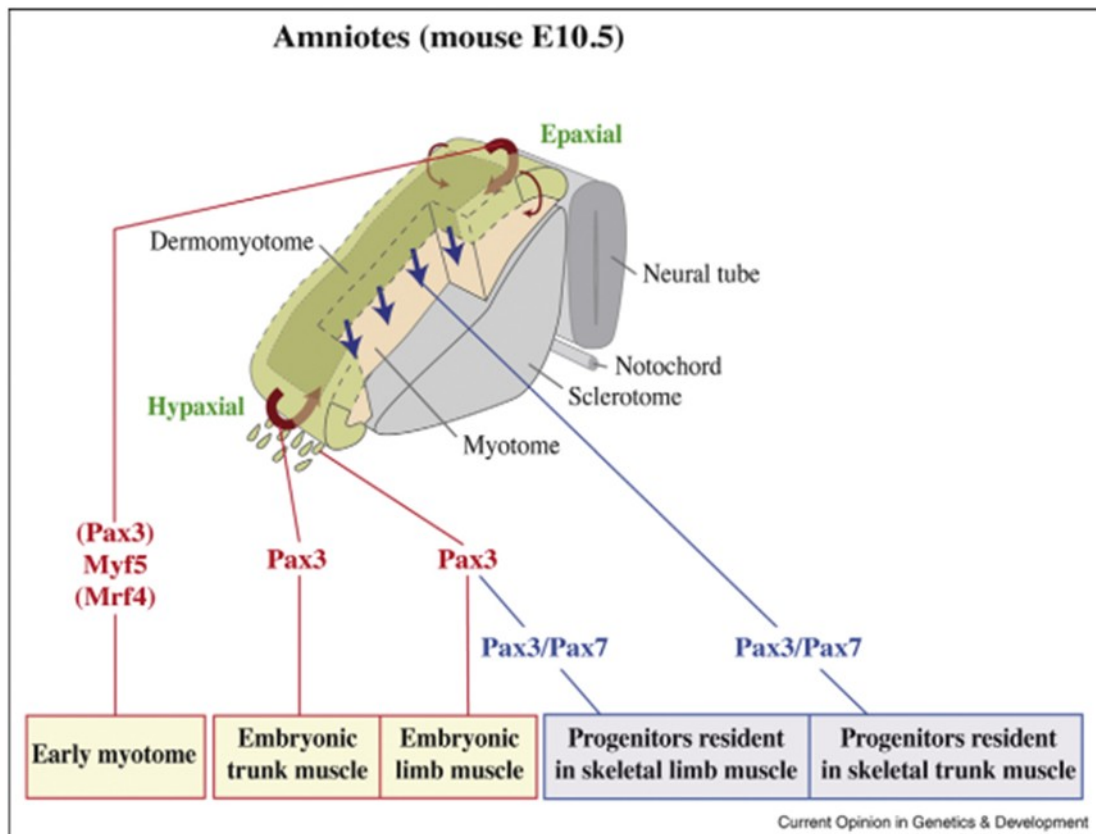
Skeletal muscle in the trunk and limbs derives from **somites** that progressively form by segmentation of paraxial mesoderm on either side of the neural tube and notochord, following an anterior-posterior developmental gradient. Somites are the first **metameric structures** and a characteristic paradigm of segmentation in mammalian embryos (Bentzinger et al., 2012). Segmentation starts from embryonic day 8 (E8.0). The somite is initially an epithelial ball of cells that subsequently distribute into a ventral mesenchymal **sclerotome**, giving rise to the bones of the vertebral column and ribs and an adjacent (dorsal-most part) **syndetome**, a source of muscle tendons in the trunk. The dorsal part of the somite, the **dermomyotome**, retains an epithelial structure for longer and gives rise to dorsal dermis and all the skeletal muscles of the trunk and limbs, as well as endothelial and smooth muscle cells of blood vessels, and brown fat (Buckingham and Rigby, 2014).

### Myotome

The **first muscle mass** to form, under the dermomyotome, is the myotome, which has an epaxial and a hypaxial component (Figure 2). Dorsal muscles are formed from the epaxial part of the dermomyotome and myotome, whereas lateral trunk and limb muscles are



derived from the hypaxial domains (Buckingham et al., 2003). Myogenic precursor cells delaminate from the edges of the dermomyotome and they differentiate into myocytes to form the underlying differentiated muscle of the myotome. Subsequently, these myocytes undergo cell fusion to form multinucleated muscle fibers followed by cleavage and reorganization of the growing and splitting trunk muscle mass. At the level of the fore- and hindlimbs, cells delaminate from the hypaxial dermomyotome and migrate into the early limb bud where they subsequently differentiate into skeletal muscle (Buckingham and Mayeuf, 2012).



**Figure 2 :** Trunk and limb skeletal muscles in amniotes originate from the somites. The dermomyotome contains multipotent progenitor cells of different cell types, including the skeletal muscle progenitors. Even if Pax3 does not directly control early epaxial myogenesis (i.e. formation of the early myotome), all muscle cells derived from the somite have expressed Pax3 in their history. The early myotome and embryonic myofibres originate from cells that have expressed only Pax3 (indicated in red). In the central portion of the dermomyotome (darker green region) and in muscle progenitors once they have migrated to the limb, Pax7 expression is initiated in Pax3 positive cells. Pax3 expression, contrary to Pax7, is downregulated in cells that will contribute to fetal myogenesis (indicated in blue). From (Buckingham and Vincent, 2009)

Both **delamination** and **migration** depend on the presence of c-met, a tyrosine kinase receptor, which interacts with its ligand hepatocyte growth factor HGF, produced by

non-somitic mesodermal cells that thus delineate the migratory route. In mutant mouse embryos, which lack functional c-met or HGF, skeletal muscle is absent from the limbs. Transcription of the c-met gene depends on Pax3 transcription factor (Epstein et al., 1996).

## Cranial Myogenesis

Craniofacial muscles are associated with **head** (including extra-ocular muscles, jaw muscles and facial muscles) and **neck** structures. The formation of head muscles differs significantly (evolutionarily, morphologically, molecularly) from the formation of their counterparts in the trunk and limbs. In the embryo, these structures derive from distinct mesoderm populations. The head musculature originates from the **unsegmented cranial paraxial mesoderm**, which is positioned along both sides of the neural tube and notochord, and it is located anterior to the somites (Sambasivan et al., 2011a).

## Chapter 2: Upstream Regulators of Myogenesis

### 2.1. Paired-Homeobox Transcription Factors

**Pax** genes encode evolutionarily conserved (paired box: a DNA-binding sequence) transcription factors that play critical roles in **organ development** and **tissue specification**. Pax genes are divided into four subfamilies based on sequence similarities depending on the presence of an additional DNA-binding homeodomain and/or an octapeptide region, which serves as a binding motif for protein co-factors (Blake and Ziman, 2014).

**Pax3** and **Pax7** constitute one of the four Pax subfamilies. Pax7 is unique in the family for the presence of a C-terminal 14 amino acids sequence, the OAR (Otp/aristaless/Rax) (Mayran et al., 2015). Similarities in their protein sequence and expression pattern reflect a common origin, as they arose by duplication from a unique ancestral Pax3/7 gene at the onset of vertebrate evolution (Paixão-Côrtés et al., 2015). Pax3 and Pax7 function **upstream** of the myogenic regulatory factors (see the corresponding paragraph) in the trunk and limbs and thus control the **entry** of cells into the **myogenic program** (Relaix et al., 2005). Unlike the myogenic regulatory factors, Pax3 and Pax7 **are not tissue specific**, being also expressed in neuroectoderm, in subdomains of the brain, in the dorsal neural tube, and in neural crest (Buckingham and Rigby, 2014).

### Pax3

In the mouse, Pax3 is expressed in presomitic mesoderm and then throughout the somite, before becoming restricted to the dermomyotome as somites mature (Buckingham

and Rigby, 2014). The mutation of Pax3 in **Splotch** mice leads to defects in neural tube closure (spina bifida and exencephaly), in the migration of the neural crest cells and in limb muscle formation. Pax3-mutant embryos show somite defects and abnormalities in segmentation. In consequence, the myotome fails to form correctly leading to trunk muscle defects and perinatal death (Tremblay et al., 1998).

Cells in the central dermomyotome are marked by the expression of both Pax3 and Pax7 proteins however, only Pax3 is expressed in long-range migrating cells (see Figure 2). Pax3-mutant mice have no limb muscles that depend on the delamination and migration of muscle progenitor cells from the hypaxial dermomyotome (Tremblay et al., 1998). Despite Pax3 and Pax7 have partially **overlapping** functions during muscle development; Pax7 can substitute for Pax3 function in somite and trunk muscle development, but only partially in the formation of limb muscles involving long-range migration of muscle progenitor cells (Relaix et al., 2004).

#### Pax3 Expression in Postnatal Muscles

Analysis of adult mice in which the Pax3 gene is targeted with nLacZ reporters revealed the presence of  $\beta$ -galactosidase–positive satellite cells in adult skeletal muscle. The number of such cells varies between muscles. They are particularly **abundant in the diaphragm** but are much less frequent in hindlimb muscles, with the exception of the gracilis muscle. In contrast, 50% of forelimb muscles express Pax3. As in the embryo, expression of Pax3 is not detectable in head muscles. Most ventral trunk muscles are positive, with a striking juxtaposition in the rib area, where intercostal muscles are mainly negative, whereas body wall muscles such as the serratus caudalis dorsalis are positive (Relaix et al., 2006).

#### Pax7

Although the Pax3 function is revealed by the Splotch mutation, no spontaneous mutant is available which indicates the role of Pax7. Pax7 has been originally described as a member of the paired-homeobox gene family that is specifically expressed during **somitogenesis** and **neurogenesis** in the embryo. Pax7 transcripts are present in the myotome of the somites and persist during differentiation into skeletal muscles of the trunk and limbs (Jostes et al., 1990). Its expression pattern both in ectoderm- and mesoderm-derived tissues, suggests a function in central nervous system development and skeletal muscle.

In parallel to myofiber formation, a subpopulation of myogenic precursor cells that do not express the myogenic regulatory factors and maintain Pax3/Pax7 expression is observed in the myotome at E10.5 (see Figure 2). In the absence of both Pax3 and Pax7, further

muscle development is arrested and only the early embryonic muscle of the myotome forms. Cells not expressing Pax3 or Pax7 die or assume a non-myogenic fate (Kassar-Duchossoy et al., 2005; Relaix et al., 2005).

#### Pax7 Germline Mutant Phenotype

Pax7 germline mutants are born but they have increased lethality (die within 2–3 weeks) and **growth retardation** with reduced body weight although the muscle formation is not affected. The cause of death is probably because of dysgenesis of neural crest derivatives (Mansouri et al., 1996).

The markedly decreased muscle mass, the reduced fiber caliber and the reduced number of myonuclei of Pax7 mutant muscle suggested that the postnatal growth phase of skeletal muscle, normally mediated by satellite cells, was deficient in the absence of Pax7 and is attributable to an absence of functional satellite cells (Kuang, 2006). Deletion of Pax7 in mice leads to normal numbers of stem cells at birth followed by excessive **wasting** of stem cells during the first weeks of postnatal development (Oustanina et al., 2004; Relaix et al., 2006).

Satellite cells (SCs), the skeletal muscle stem cells, also express the paired box transcription factor Pax7 (Seale et al., 2000). Pax7 is an essential transcriptional factor (TF) for the **survival** (Relaix et al., 2006), **proliferation** (Oustanina et al., 2004) and **self renewal** (Olguin and Olwin, 2004) of SCs and for an efficient regeneration mediated by Pax7-expressing cells (Günther et al., 2013; Lepper et al., 2011; Sambasivan et al., 2011b).

## 2.2. Sine Oculis–Related Homeobox Transcription Factors

In addition to Pax3 and Pax7, the homeodomain proteins **Six1** and **Six4** are important upstream regulators of myogenesis that directs dermomyotomal multipotent progenitors toward the myogenic lineage (Figure 3). Unlike Pax3 and Pax7, these factors are also **present** in differentiated skeletal muscle (Buckingham and Rigby, 2014). Six family proteins are transcription factors characterized by the presence of two conserved domains, a Six-type homeodomain that binds to DNA and an amino-terminal Six domain that interacts with coactivators or corepressors of transcription (Bentzinger et al., 2012). Six proteins bind to and translocate the eyes-absent homologs **Eya1** and **Eya2** to the nucleus, where they act as **cofactors** to activate Six target genes, such as Pax3, MyoD, MRF4 and myogenin (Grifone, 2005).

Six4 germline mutant mice do not present major developmental defects (Ozaki et al., 2001), while Six1 null mice do not survive and have defects in many organs, including **rib**, **craniofacial** and **muscle** deficiencies (Laclef et al., 2003). Six1 and Six4 double germline

mutant mice develop a more severe phenotype than in the single *Six1* mutant with loss of all muscles derived from the **hypaxial** dermomyotome (limb and many trunk muscles). At the limb bud level, *Six1* and *Six4* homeogenes control early steps of myogenic cell delamination and migration from the somite through the control of *Pax3* gene expression (Grifone, 2005). *Myf5* expression within the epaxial dermomyotome, which is independent of *Pax3*, is unaffected in *Six1* and *Six4* double mutants, and dorsal muscles arising from this structure are the only remaining axial muscles in these mice (Giordani et al., 2007). *Six* orchestration together with *Pax*, at the **onset of myogenesis** is illustrated by regulation of the *Myf5* and *MyoD* gene (Relaix et al., 2013).

In adult muscle ***Six1*** acts as a main determinant of **fast-fiber type acquisition** and maintenance (Sakakibara et al., 2014, 2016) and as a critical regulator of SC self-renewal to ensure a proper muscle regeneration (Le Grand et al., 2012).

### Chapter 3: Myogenic Regulatory Factors

The process of skeletal myogenesis during embryonic development is regulated by a family of **muscle-specific** transcription factors that are expressed in a **spatially** and **temporally** ordered manner (Figure 3). The family of basic helix-loop-helix (bHLH) transcription factors consisted of *Myf5*, *MyoD*, *Myogenin* (*MyoG*), and *MRF4*, form the myogenic regulatory factors (MRFs). The ancestral MRF gene is assumed to have given rise to the four family members in vertebrates, shown by evolutionary analyses of amino acid sequences of this family members (Atchley et al., 1994).

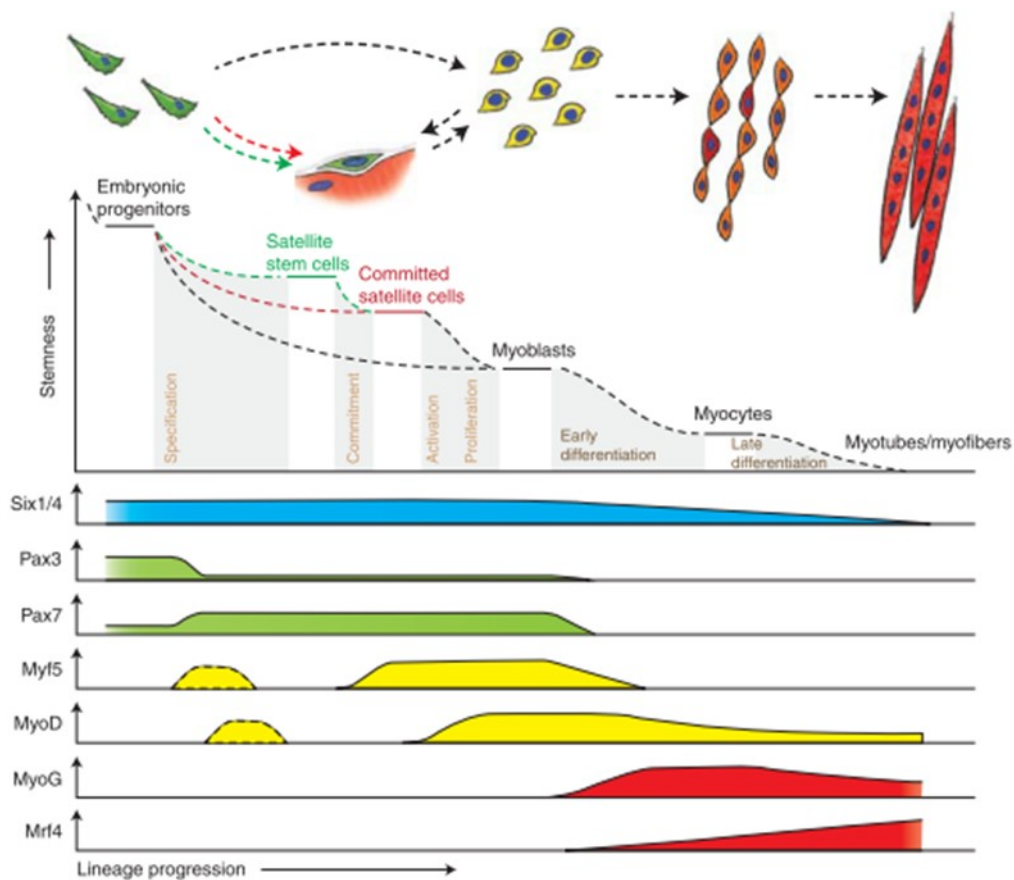
The MRFs **cooperatively** activate transcription and myogenesis through protein-protein interactions with members of the myocyte enhancer factor 2 (**MEF2**) family of MADS domain transcription factors (Molkentin et al., 1995) and the ubiquitously-expressed **E proteins** (Londhe and Davie, 2011). They have potential **target genes with E boxes** (cis-acting DNA control elements which are present in the promoters and enhancers of muscle-specific genes) (Arnold and Braun, 1996).

The MRFs have differential patterns of expression in the developing somites and are exclusively expressed in skeletal muscle<sup>2</sup>. In the mouse embryo, *Myf5* is the earliest MRF to be expressed in the dorsomedial lip of dermomyotome at E8.0. *Myogenin* is expressed after *Myf5* at E8.5 and *MRF4* at E9.0. *MyoD* is the last to be expressed in the somite at E10.5. Cells migrating toward the limb do not express MRFs until they have reached the limb bud (Francetic and Li, 2011). The MRFs null mutations analyses also suggest **overlapping** functions between them.

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<sup>2</sup> *Myf5* expression is also found in non-muscle tissues, such as preadipocytes and neurons

The existence of **dominant-acting** regulators in muscle cell differentiation has been predicted from early cell fusion experiments which demonstrated that muscle-specific genes can be transactivated in non-muscle nuclei of heterocaryons (the parental nuclei remain intact) between differentiated skeletal muscle cells and various non-muscle cell types (Blau et al., 1983). When they are overexpressed in nonmuscle cells, they will activate the myogenic program, with suppression of other cell fates and formation of differentiated muscle (Weintraub et al., 1991). Since then the possibility of converting one cell type to another by transdifferentiation has become a major issue in the stem cell field.



**Figure 3 :** Hierarchy of transcription factors regulating progression through the myogenic lineage. Muscle progenitors that are involved in embryonic muscle differentiation skip the quiescent satellite cell stage and directly become myoblasts. Some progenitors remain as satellite cells in postnatal muscle and form a heterogeneous population of stem and committed cells. Activated committed satellite cells (myoblasts) can eventually return to the quiescent state. Six1/4<sup>3</sup> and Pax3/7 are master regulators of early lineage specification, whereas Myf5 and MyoD commit cells to the myogenic program. Expression of the terminal differentiation genes, required for the fusion of myocytes and the formation of myotubes, are

<sup>3</sup> They are also expressed in adult mature myofibers

performed by both myogenin (MyoG) and MRF4<sup>4</sup>. From (Bentzinger et al., 2012). This figure refers the requirement of each TF on myogenic cellular level and not in developmental time.

## MyoD

Lineage-specific markers are expressed in proliferating myoblasts prior to terminal differentiation and the myoblast determination gene number 1 or MyoD is one of them. MyoD was isolated as the cDNA clone hybridized to a myoblast/myotube cDNA library, as the key regulator that can change the cell fate in transfected fibroblast-like 10T1/2 cells by inducing the myogenic phenotype (Davis et al., 1987). The phenomenon of myogenic conversion remains remarkable in that a single transcription factor can exert this overriding effect and this set of experiments establish the conceptual framework for the discovery of induced pluripotent stem cells from somatic cells (Hochedlinger, 2010).

MyoD transcripts are detectable at **E10.5** in the somites and myotomal muscles that had already expressed MyoG two days earlier. MyoD expression pattern in somites, after the MyoG expression, suggest that early myogenesis in the myotome is not dependent on this TF (Sassoon et al., 1989). MyoD is the last of the four myogenic HLH transcription factors to be activated in development and thus, may be essential for the SC function in postnatal growth and/or regeneration of skeletal muscle rather than in the regulation of *de novo* differentiation of skeletal myocytes (Megeney et al., 1996).

Mice carrying a null MyoD mutation are **viable** and **fertile**, and they do not exhibit any morphological or physiological abnormalities in skeletal muscle (Rudnicki et al., 1992). Mutant animals reveal an abundant expression of Myf5 in their muscle indicating that functional redundancy is a feature of the MRF regulatory network and that Myf5 may partially substitute for the function of MyoD in the control of the skeletal myogenic developmental program.

MyoD is **dispensable** for the development of skeletal muscle however, in its absence the postnatal growth is attenuated and muscle regeneration is less efficient confirming its important role in SC function. MyoD-mutant animals show increased number of MyoD-deficient satellite cells but they exhibit decreased rates of proliferation (Megeney et al., 1996). Thus, the lack of MyoD promotes the satellite cell self-renewal rather than progression through the developmental program as the MyoD mutant myoblasts are **differentiation defective** (Cornelison et al., 2000; Sabourin et al., 1999). In this case, MyoD plays a cell autonomous and non-redundant role in regulating the dynamic balance between proliferation, differentiation and renewal that normally establishes an appropriate satellite cell pool size.

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<sup>4</sup> Mrf4 also directs embryonic but not foetal muscle progenitor cell commitment

In addition to its importance in the myogenic determination and differentiation, MyoD has a role in the proliferation and growth of myoblasts as it was shown by the *ex vivo* culture of MyoD-null myoblasts and their sequential passages. Primary cultures of MyoD<sup>-/-</sup> mice were poorly growing and displayed signs of senescence a phenotype more accentuated over the four subsequential passages accompanied with the progressive loss of Myf5 expression (Montarras et al., 2000). Further study confirmed the MyoD capacity to interact with and to affect components of the cell cycle machinery by initiating the expression of Cdc6 gene after myoblasts transition out of quiescence in order to progress through the cell cycle (Zhang et al., 2010). This could justify the early requirement for MyoD expression within hours after the SC activation and before SC duplication (Jones et al., 2005).

In the adult, MyoD is not expressed by quiescent satellite cells but in activated, proliferating myoblasts (Yablonka-Reuveni and Rivera, 1994). Nevertheless, adult satellite cells have passed through a developmental stage where the MyoD locus was active (Kanisicak et al., 2009).

## Myf5

Among the four MRFs, Myf5 is the earliest to be expressed at **E8** in the somites, in the epaxial domain, adjacent to the neural tube, subsequently is activated in the opposing hypaxial domain and is markedly reduced after E14 (Francetic and Li, 2011). Newborn Myf5-deficient animals were **viable** and **fertile** (Kaul et al., 2000). In addition, Myf5 mutants lack epaxial (dorsal) muscles (Kassar-Duchossoy et al., 2004).

Myf5-mutant mice show a **delay** in the myotome development although they expressed the normal set of MRFs (Braun et al., 1992). Similarly, inactivation of the Myf5 gene also allows apparently normal muscle development in the newborn mice, supporting the notion of an extraordinary plasticity in the formation of the myogenic lineage, with the myogenic factors Mrf4 and MyoD expressed in the somites at later developmental stages substituting for the absence of Myf5 (Kassar-Duchossoy et al., 2004).

Skeletal muscles of **adult** Myf5 null mice exhibit a subtle progressive **myopathy**. Adult Myf5 null mice exhibit perturbed muscle regeneration with a significant increase in muscle fiber hypertrophy, delayed differentiation, adipocyte accumulation, and fibrosis after freeze-injury. Satellite cell numbers are not significantly altered in Myf5 null animals and they show a modest **impaired proliferation** under some conditions *in vitro* (Gayraud-Morel et al., 2007).

### *Myf5 and MyoD germline mutant phenotype*



Mice deficient for both MyoD and Myf5 were **immobile** and died soon after birth. Newborn mice are totally devoid of skeletal muscle fibers and myoblasts (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). Fetal myogenesis was severely compromised whereas Mrf4 partially rescues embryonic myogenesis in Myf5/MyoD double-knockout mice (Kassar-Duchossoy et al., 2004). Mice **triple mutant** for Myf5, MyoD and Mrf4 totally lack skeletal muscle and myoblasts (Kassar-Duchossoy et al., 2004). These experiments suggest that formation of skeletal muscle depends on **determination factors** Myf5, MyoD and Mrf4 which direct multipotent progenitors into the myogenic lineage (Sambasivan and Tajbakhsh, 2007).

## MyoG

Myogenin (MyoG) is another member of the MRF which was isolated for inducing the myogenic fate of non myogenic cell line and it shares a very high homology with MyoD in its amino-terminal region (Wright et al., 1989). MyoG binds to the regulatory regions of muscle-specific genes and activates their regulation. Thus, whereas MyoD and Myf5 are active in the lineage specification of muscle cells, myogenin appears to mediate muscle **cell differentiation**. During the embryonic myogenesis, the MyoG is expressed earlier than the MyoD in the myotomal cells of the first somites whereas they are expressed synchronously in the limb buds (Sassoon et al., 1989).

Mice lacking myogenin are born immobile and die at birth because of a virtual absence of myofibers. MyoG is **indispensable** for the skeletal muscle formation during embryogenesis contrary to the MyoD and Myf5-mutants. MyoG-mutant mice show severe reduction of skeletal muscle mass, drastically reduced fiber density with mononucleated cells replacing most of the mature muscle cells (Hasty et al., 1993; Nabeshima et al., 1993).

The initial phase of myotomal differentiation occurred normally in the myogenin-mutant embryos. Primary myogenesis was delayed whereas **secondary** myogenesis was dramatically **affected** indicating that myogenin is not required for the initial aspects of myogenesis, including myotome formation and the appearance of MyoD-expressing myoblasts (Venuti et al., 1995). Myogenin is not essential for commitment of cells to the myogenic lineage, but is important for terminal differentiation. Lack of myosin heavy chain and actin (markers for differentiated muscle cells) expression was diminished in MyoG-mutant mice demonstrating its importance for biochemical and morphological differentiation of skeletal muscle cells (Venuti et al., 1995).

**Postnatal deletion** of MyoG leads to 50% lethality before P10. The survived mice were observably **smaller** in size, not accompanied however with reduced myofiber caliber or compensatory upregulation of any of the myogenic bHLH TF expression. The absence of MyoG did not alter postnatal skeletal muscle growth or function and reveal “an unsuspected

non-cell autonomous role for myogenin in the regulation of tissue growth” (Knapp, 2006). Deletion of MyoG in adult mice renders the skeletal muscle resistant to neurogenic atrophy by diminishing the expression of MuRF1 and Atrogin1 (E3 ubiquitin ligases) and thus preventing the proteasome-mediated protein degradation. Denervation-induced upregulation of MyoG triggers MyoG-dependent transcriptional cascade, involving MuRF1 and Atrogin1, leading to muscle atrophy (Moresi et al., 2010).

## Mrf4

Mrf4 or Myf6 represents the fourth member of MRFs that is capable of converting fibroblasts to stable determined myogenic cells at a very high frequency (Rhodes and Konieczny, 1989). Mrf4 is expressed transiently in the myotome at the same time as Myf5 at the onset of myogenesis and then becomes up-regulated during **late fetal** development to eventually become the predominant myogenic bHLH factor expressed in **adult** skeletal muscle (Hinterberger et al., 1991). Based on its expression pattern, it has been proposed that Mrf4 regulate skeletal muscle maturation and aspects of adult myogenesis (Le Grand and Rudnicki, 2007).

A recent study confirmed the function of Mrf4 in adult muscle fibers as Mrf4 knockdown in adult skeletal muscle induces hypertrophy and prevents denervation-induced atrophy. This effect is dependent on an increase in Mef2<sup>5</sup> transcriptional activity and the consequent upregulation of Mef2 target genes, suggesting that these two TFs act together to regulate growth in adult muscle. Mrf4 is a **negative regulator** of muscle growth by **repressing** Mef2 activity (Moretti et al., 2016).

Three Mrf4 knock-out mice have a range of phenotype from viable with no muscle defects, to lethal phenotype with some muscle defects. Since Myf5 and Mrf4 are genetically linked on the same chromosome, knock-out in one gene results in a *cis* effect by which the expression of the other is also decreased or lost (Olson et al., 1996). In the viable knock-out, Mrf4-mutant mice showed only a slight reduction in expression of a subset of muscle-specific genes but showed a dramatic increase in expression of myogenin, suggesting that it may compensate for the absence of Mrf4 and demonstrating that Mrf4 is required for the down-regulation of myogenin expression that normally occurs in postnatal skeletal muscle (Zhang et al., 1995).

In the Mrf4 and MyoD double mutant the myogenin null phenotype is phenocopied suggesting therefore that Mrf4 or MyoD is necessary to activate the myogenin gene (Kassar-Duchossoy et al., 2004)

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<sup>5</sup> Mef2 transcription factors are co-regulators with MRFs to activate myogenesis

## Part 2: Description of Myofibers

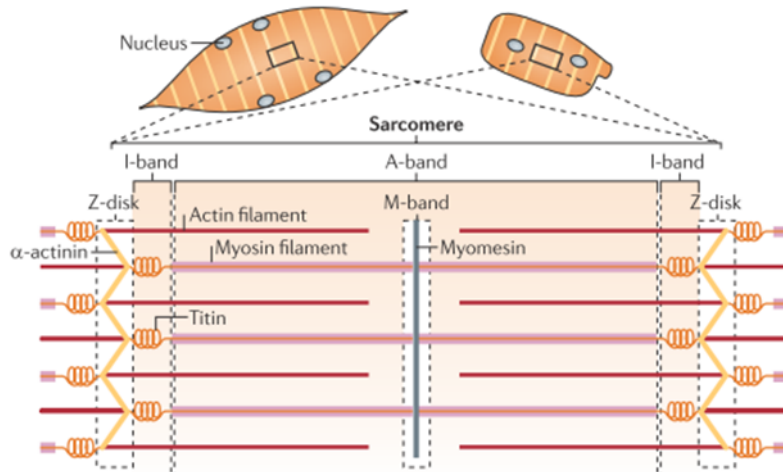
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Myofibers are composed of myosin II **motor proteins** and **actin filaments** that generate force and movement. In the striated muscles that are used for locomotion, actomyosin contraction is amplified in serial and parallel arrangements of numerous contractile units, called **sarcomeres**, which is the basic **functional unit** of skeletal muscle (Figure 4). These are made up of actin and myosin filaments arranged in highly ordered, almost crystalline arrangements, as well as hundreds of regulatory proteins such as the troponin–tropomyosin complex, scaffolding and cytoskeletal crosslinking proteins such as  $\alpha$ -actinin, myomesin and the kinase titin (Braun and Gautel, 2011).

This group of proteins, which is essential for the ordered assembly of actin and myosin filaments into sarcomeres, combines architectural, mechanical and signaling functions in muscle and summarized as the **sarcomeric cytoskeleton** (Gautel and Djinočić-Carugo, 2016). Such signaling functions that are interpreted in gene expression, protein synthesis and protein degradation are the translation of mechanical stimuli to biochemical signals (muscle mechanotransduction).

Responding to the signals from motor neurons, myofibers depolarize and release calcium from the sarcoplasmic reticulum. This drives the movement of actin and myosin filaments relative to one another and leads to sarcomere shortening and muscle contraction (Yin et al., 2013a).

Each myofiber is anchored at its extremities to tendons at the myotendinous junctions. **Myotendinous junctions** are anatomical regions which connects the skeletal muscle to the bone for transmitting the contractile force between the two tissues.



**Figure 4** : Striated muscle structure. The contractile machinery of skeletal muscle syncytial myotubes (left) and single cardiomyocytes (right) is formed from long arrays of sarcomere units, which are joined into myofibrils. The sarcomere (bottom) is constructed from interdigitating, antiparallel filaments of actin and myosin, the elastic titin filaments and the crosslinker proteins for actin —  $\alpha$ -actinin, myosin and myomesin. Sarcomeres contain many other accessory components, including proteins involved in transcriptional regulation and turnover control. Adapted from (Braun and Gautel, 2011)

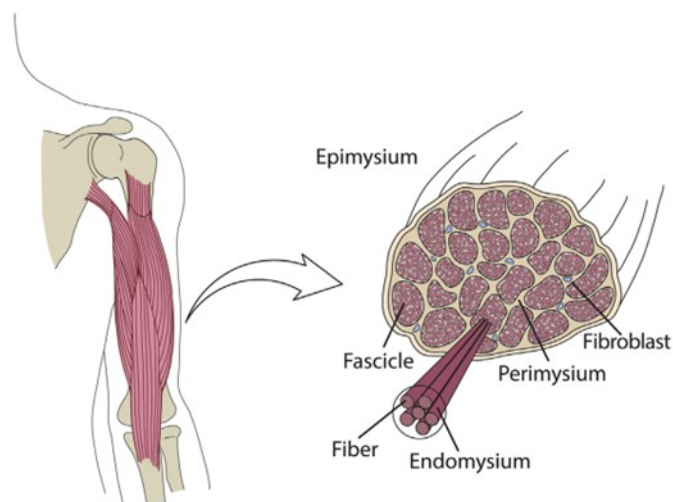
Skeletal muscle comprises different fiber types based on their physiological properties. Skeletal muscle fibers can be grouped into a **slow-contracting**/fatigue-resistant type and a **fast-contracting**/fatigue-susceptible type. Slow and fast muscles differ in the metabolism type (**oxidative or glycolytic**). Myofibers also vary in terms of their **myosin heavy chain (MyHC) isoforms** (I, IIA, IIB, IIX). The fiber type profile can change, in response to hormonal, neural influences and mechanical load, rendering the muscle a **plastic** tissue (Schiaffino and Reggiani, 2011).

## Part 3: Extracellular Matrix

Skeletal muscles are composed primarily of contractile material. However, muscle is a composite tissue of connective tissue, blood vessels and nerves, these “minor” tissues (in terms of relative mass) may strongly influence muscle function. Normal muscle function is also influenced by the skeletal muscle extracellular matrix (**ECM**) or basement membrane that coats the skeletal myofibers. The primary function of extracellular matrix is to **endow** tissues with their specific **mechanical** and **biochemical** properties. ECM bears the majority of muscle passive load as shown by biomechanical studies and alterations in ECM properties are associated to muscle pathology with ECM fibrosis (Gillies and Lieber, 2011). Also, acellular basement membrane provide a scaffold to orient and constrain cells during regeneration (Vracko and Benditt, 1972).

Each myofiber is surrounded by the **endomysium** (also called the basement membrane or basal lamina). Bundles of myofibers are surrounded by the **perimysium**, while the entire muscle is contained within the **epimysium** (Figure 2). The epimysium layer is continuous with the tendons that attach the muscles to the bones (Lund and Cornelison, 2013). Additionally, ECM in skeletal muscle is critical for both longitudinal and lateral force transmission from muscle fibers to tendons (Purslow, 2010).

The basal lamina of skeletal muscle is composed of type IV **collagen**, **proteoglycans**, **glycoproteins** and matrix remodeling enzymes. These ECM molecules are mainly synthesized and excreted by **interstitial muscular fibroblasts** but can also be produced and remodeled by myoblasts during muscle development and regeneration (Chapman et al., 2016).

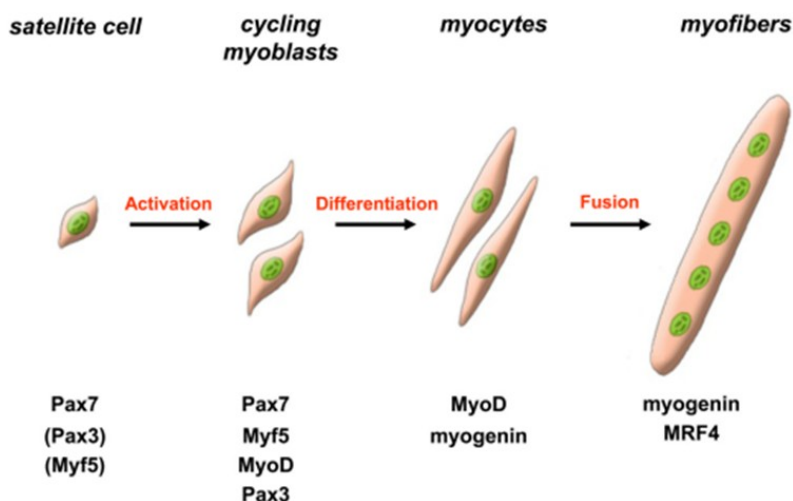


**Figure 5** : Skeletal muscle extracellular matrix structure. Skeletal muscles are composite and hierarchical tissues with three layers of ECM, the epi-, peri- and endomysium. Skeletal muscle fibroblasts reside in the extracellular space between muscle fibers and fascicles, where they secrete ECM proteins to be incorporated into skeletal muscle ECM. From (Chapman et al., 2016)

## Part 4: Satellite Cells

Satellite cells are skeletal muscle-specific **adult stem cells** and their presence seems to be a common feature of all adult skeletal muscles. They account for 2-7% of the nuclei associated with a particular myofiber and the proportion varies with age and muscle group (Gibson and Schultz, 1983). Satellite cells contribute to increase the diameter and length of the existing fibers, as they are the main source of myogenic cells after birth and during regeneration. The satellite cells were initially described in the course of an electron microscopic study in the peripheral region of the skeletal muscle fiber of the frog and were named based on their anatomic location on the surface of muscle fibers, between the myofiber plasmalemma and the basal lamina (Mauro, 1961).

In **resting** muscle, satellite cells remain in a non-proliferative, **quiescent** state. Upon injury or growth **stimulus**, satellite cell become **activated**, enter the cell-cycle and turn into proliferating myoblasts, which differentiate and eventually fuse (Figure 6) to pre-existing myotubes or to each other to form new myotubes (Le Grand and Rudnicki, 2007). Terminal differentiation leads to the activation of many muscle-specific genes, including those encoding the sarcomeric proteins (such as myosin light and heavy chains,  $\alpha$ -actin, troponin I, and troponin T) muscle enzymes, structural proteins of the contractile apparatus and specialized membrane receptors.



**Figure 6** : Schematic representation of adult myogenesis. Quiescent skeletal muscle satellite cell can become activated following stimuli originating from their associated fiber or from the micro-environment. Their proliferating progeny, the skeletal myoblasts, express the paired-box transcriptions factors Pax7 and Pax3, as well as the myogenic regulatory factors Myf5 and MyoD. Once committed to differentiation, myoblasts stop cycling and loose expression of

Pax7, Pax3 and Myf5. Differentiating myogenin+ve myocytes will then align and fuse to form multinucleated myofibers. MRF4 is further required for hypertrophy of the new fibers. From (Le Grand and Rudnicki, 2007)

The descendents of activated satellite cells called **myoblasts** or **myogenic precursor cells**: they are the transient amplifying population, undergoing multiple rounds of division and express MyoD. Satellite cells appear to form a population of stem cells that are **distinct** from their daughter myoblasts as defined by biological and biochemical criteria (Yablonka-Reuveni et al., 2008). A **reserve** population escapes from the differentiation fate and return back to quiescence that assures the replenishment of satellite cell population.

The **replenishment** of the satellite cell population is a critical aspect of muscle tissue regeneration. Repeated injury experiments have shown that satellite cell numbers remain constant even after multiple traumas. The satellite cell pool is constantly replenished during lifetime, although there is a decline in satellite cell numbers and a reduced proliferative capacity in aged individuals (Dumont et al., 2015a). Thus, satellite cells display two hallmarks of stem cells: **lineage-specific differentiation** and **self-renewal** (Buckingham and Montarras, 2008).

## Chapter 1: Transcriptional Control of Adult Myogenesis

Adult myogenesis takes place during muscle **regeneration** and SCs undergoing myogenesis to form **de novo** muscle fibers. During adult adaptation of muscle, aspects of early developmental programs can be reactivated and can cooperate with specific factors to determine fiber-type characteristics. This process, in many but not all respects, **recapitulates** embryonic myogenesis (Yin et al., 2013a). The myogenic potential of satellite cells is under the molecular control of specific Paired-box and bHLH transcription factors which tightly orchestrate the balance of myogenic progression during muscle regeneration (Le Grand and Rudnicki, 2007).

SCs are identified by the expression of Pax7 and Pax7-expressing SCs are required for muscle regeneration as it has been shown by genetic ablation of such cells (Lepper et al., 2011; Sambasivan et al., 2011b). Pax7 is able to drive transcription in quiescent satellite cells, activated and proliferating satellite cells. Expression is then downregulated in cells that initiate terminal differentiation, but is maintained (and transcriptionally active) in those that withdraw from immediate differentiation (Zammit, 2006).

Satellite cells and primary myoblasts lacking Pax7 undergo cell cycle arrest and **precocious differentiation**. *Ex vivo* Pax7 deletion in wild type myoblasts resulted in a striking reduction in the levels of Myf5 mRNA, a reduction in MyoD expression and an



increase in the numbers of satellite cells expressing myogenin, suggesting cell cycle arrest and precocious differentiation (von Maltzahn et al., 2013). In contrast, overexpression of Pax7 caused an increase in the number of EdU-incorporating Pax7-positive SCs and a reduction of nonproliferating cells, as well as an impairment of SC differentiation (Günther et al., 2013). Conditional inactivation of the Pax7 expression in adult satellite cells leads to **progressive loss** of SCs and to differential changes of the SC signature. SCs after loss of Pax7 expression maintain some SC features for several weeks, but not indefinitely. Inactivation of Pax7 gives rise to rare **atypical** SCs with reduced heterochromatin condensation. Pax7-deficient, hypoproliferative SCs are mainly removed by differentiation into myocytes (Günther et al., 2013). Pax7 induces **chromatin modifications** that stimulate transcriptional activation of target genes to regulate entry into the myogenic developmental program (McKinnell et al., 2008).

The myogenic identity is established by the presence and function of specific transcription factors, the MRFs. The Myf5 locus is active in 90% of quiescent satellite cells, which suggests most satellite cells are **committed** to the myogenic lineage (Kuang et al., 2007). MyoD and Myf5 are crucial to drive the gene expression program of activated SCs. **MyoD and Myf5** are induced in satellite cells *in vivo* within 3 hours of cardiotoxin-mediated muscle injury, implicating them in the **early** stages of satellite cell activation (Cooper et al., 1999). The differentiation factors **myogenin and Mrf4** are involved in **later** phases of myogenesis. Induction of myogenin is necessary and sufficient for the formation of myofibers (Le Grand and Rudnicki, 2007). However the specific role of MyoG in adult myogenesis has not been described since the MyoG inactivation in adult satellite cells has not been yet studied. Mrf4 is further required to prevent hypertrophy of the myofibers (Moretti et al., 2016).

MyoD expression is restricted to cells exhibiting myogenic capacity and is a marker of myogenic commitment that directs the upregulation of differentiation-linked genes (Tapscott, 2005). Myogenin **acts downstream** of MyoD and Myf5 since most myoblasts in culture express MyoD or Myf5, but turn on myogenin only when induced to differentiate. During muscle regeneration myogenin mRNA sequences in mononuclear cells were detected as early as 6 h after injury, peaked between 24 and 48 h, and thereafter declined to pre-injury levels at about 8 days. The presence of myogenin mRNA at 6 to 48 h indicates that transcription of this gene is occurring at the same time as replication of muscle precursor cells *in vivo* (Grounds et al., 1992).

**Translational control** of MRF expression also accounts for the transition through the sequential myogenic stages. In quiescent SCs the expression of the Myf5 protein is avoided by sequestration of the Myf5 mRNA in messenger ribonucleoprotein granules and by the action of the microRNA-31, which blocks Myf5 translation (Crist et al., 2012). MyoD protein

expression is also prevented in quiescent SCs by the action of tristetraprolin, a protein that promotes the decay of MyoD mRNA (Hausburg et al., 2015). Moreover, a general repression of translation, mediated by the phosphorylation of the eukaryotic initiation factor eIF2 $\alpha$ , preserves the quiescent state of satellite cells, as cells unable to phosphorylate eIF2 $\alpha$  exit quiescence and activate the myogenic program (Zismanov et al., 2016).

Myogenic differentiation is blocked by the **inhibitor of differentiation** (Id), a bHLH protein that lacks the basic DNA-binding domain and interacts with either MyoD or E proteins. When myoblasts exit the cell cycle Id expression is downregulated, allowing functional heterodimers to be formed and promoting muscle differentiation-specific gene expression (Puri and Sartorelli, 2000).

## Chapter 2: Quiescence

The stable quiescence of adult stem cells in their niche is best demonstrated by measuring the frequency at which they undergo DNA synthesis (Fuchs et al., 2004). In resting adult muscles, satellite cells exist in a **non-cycling**, quiescent state. In 2014, it was shown that the quiescence is not really a dormant state but is composed of two distinct functional phases, **G0** and an 'alert' phase termed **G(Alert)**. Stem cells actively and **reversibly** shift between these phases in response to injury-induced systemic signals (Rodgers et al., 2014). Thereby, the quiescent state is distinct from the cell cycle exit observed prior to differentiation, the most notable difference being its reversibility, which allows cells to return to a proliferative state in response to injury (Dumont et al., 2015a). The rapid cell cycle re-entry of satellite cells after injury suggests that the quiescent state is highly regulated and represents a “ready” state that is primed for activation.

Microarray analyses revealed that quiescent SCs (QSCs) possess a **unique transcriptional profile** that distinguishes them from their more activated progeny. QSCs express approximately 500 uniquely enriched genes (Fukada et al., 2007; Liu et al., 2013; Pallafacchina et al., 2010). Although the functional importance of many of these genes is yet to be understood, it seems likely that this transcriptional program inhibits QSC proliferation, anchors satellite cells in their anatomical niche and provides mechanisms for the efficient transport and processing of lipids that are required for metabolic reactions characteristic of quiescent cells (Almada and Wagers, 2016).

QSCs have a different **epigenetic signature** that has to be preserved through self renewing divisions to assure the original transcriptional state (epigenetic memory). The histone code has been shown to act as a source of inheritable epigenetic information as it can be transmitted from one cell generation to the next. The chromatin of QSCs is maintained at a **permissive state** in which few genes are epigenetically **repressed**

(H3K27me3). SC activation is accompanied by the acquisition of H3K27me3 inactive histone mark. By accumulating H3K27me3 upon SC activation, the chromatin is converted to a more repressed state to restrict thus alternative cell fates (Liu et al., 2013). Histone modifications enzymes (ex. EZH2-PRC2, Suv4-20h1) are required to promote the facultative heterochromatin which is under dynamic transitions between constitutive heterochromatin and euchromatin. The Suv4-20h1 is a paradigm of such enzyme which is required to restrict MyoD gene expression in QSCs (Boosanay et al., 2016).

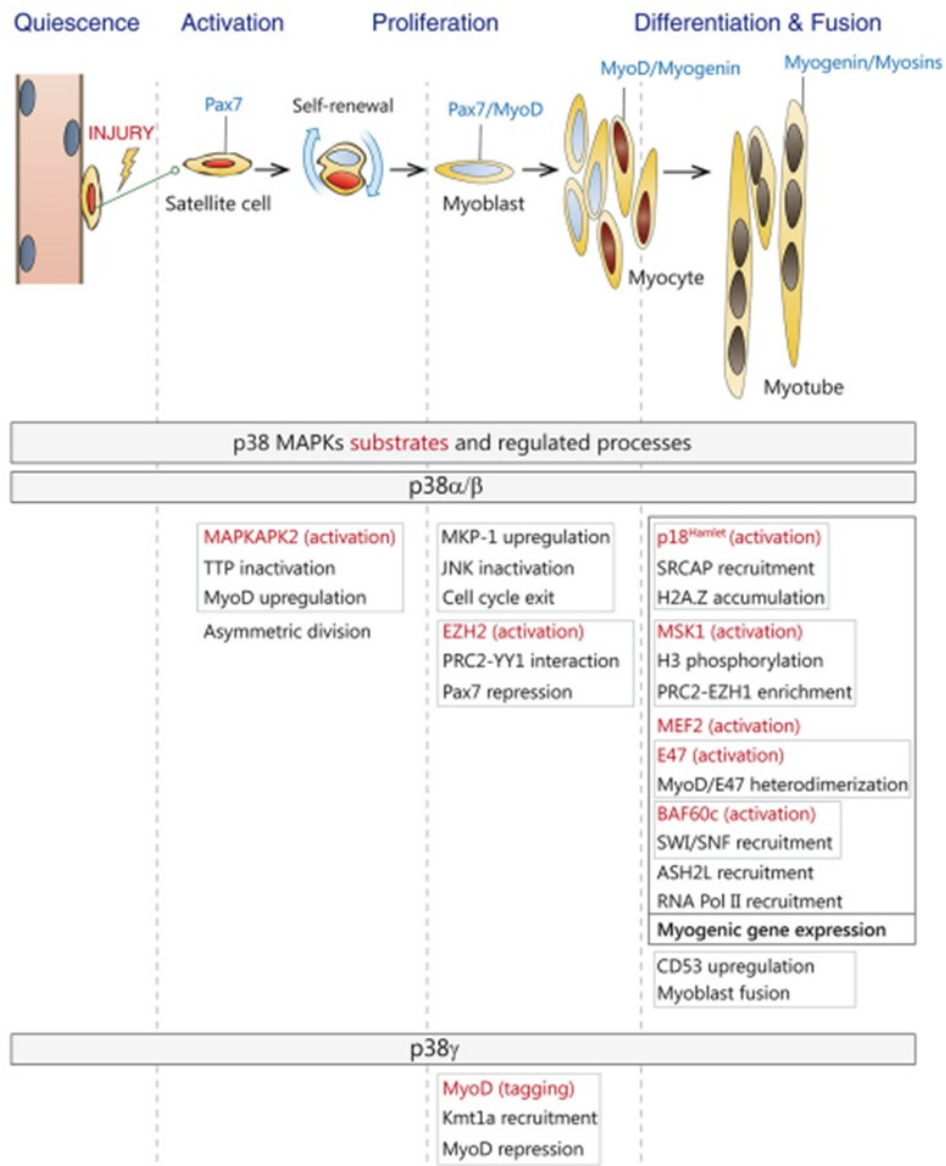
The ability of satellite cells to maintain quiescence in the resting state is essential for the long-term conservation of the satellite cell pool. The **Notch signaling** is very critical for the **maintenance of quiescence** of SCs (Bjornson et al., 2012; Mourikis et al., 2012). Notch signaling activity is higher in quiescent satellite cells than in activated myogenic cells (Mourikis et al., 2012). Sex hormones induce Mind bomb1 expression in myofibres at puberty, which activates Notch signalling in cycling juvenile SCs and causes them to be converted into adult quiescent SCs (Kim et al., 2016). Quiescent satellite cells have a **low metabolic rate**, but their activation and entry into the cell cycle are characterized by major metabolic changes. Isolated satellite cells cultured *in vitro* experience a switch from oxidative to glycolytic metabolism (Ryall et al., 2015). *In vitro* cultures could however dictate the metabolic status of the cells and thus further full *in vivo* studies need to address this issue (L'honoré et al., 2014).

### Chapter 3: Activation

Upon **exposure to signals** from a damaged environment, satellite cells exit their quiescent state and start to proliferate (satellite cell activation). Adult satellite cells are primed to respond rapidly to activation signals (Rodgers et al., 2014), but are held back by expression of inhibitors such as the insulin binding protein, Igfbp6, or by Dach1 that can act as a corepressors of Six transcription factors (Pallafacchina et al., 2010). The niche **insulates** the SC from a majority of extracellular stimuli, allowing them to become activated only in specific conditions associated with damage or disease (Bischoff, 1990). Upon muscle damage, quiescent SCs are exposed to extrinsic, **pro-myogenic stimuli**, including fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which activate intrinsic pathways that stimulate proliferation (Almada and Wagers, 2016; Yin et al., 2013a). Once SC activation, **down-regulation** of genes encoding **adhesion molecules** and changes in transcripts for extracellular matrix components will affect the response to growth factors (Montarras et al., 2013; Pallafacchina et al., 2010).

Proper satellite cell activation is crucial to ensuring an adequate regenerative response after muscle injury. It has been reported that the **first cell division** is the **rate-limiting step** in the conversion of satellite cells to a highly proliferative state (Rodgers et al., 2014), which probably explains why the peak of satellite cell expansion occurs approximately 3–4 days after muscle injury in vivo (Hardy et al., 2016). The long delay in initiation of satellite cell division may hint at the possibility that QSCs transit through one or more intermediate cell states on the path to full activation (Almada and Wagers, 2016).

Cell response to external stimuli requires signal transduction mechanisms and the mitogen-activated protein kinases (MAPKs) seem to participate in most signal transduction pathways. Different stimuli can activate p38 MAPK in SCs (Segalés et al., 2016). **p38 $\alpha$ / $\beta$  MAPK** is crucial for awakening QSCs into the proliferative state (Figure 7). Asymmetric localization of the Par complex activates p38 $\alpha$ / $\beta$  MAPK in only one daughter cell, in which MyoD is induced by inhibiting the RNA-destabilizing protein tristetraprolin, allowing cell cycle entry and generating a proliferating myoblast. In contrast, MyoD induction is prevented in the other daughter cell by the absence of p38 $\alpha$ / $\beta$  MAPK signaling, renewing the quiescent satellite cell pool (Troy et al., 2012).



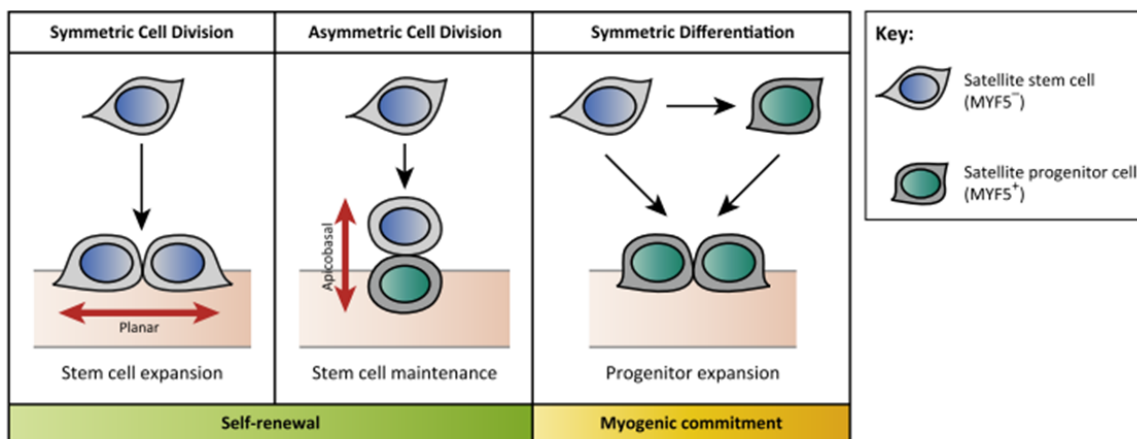
**Figure 7** : Roles of p38 MAPKs in satellite cell-driven myogenesis. (Top) Satellite cells in adult muscles are in a quiescent state. Upon injury, satellite cells are activated, undergo asymmetric division and generate a self-renewing daughter cell and a committed progenitor or myoblast which enters the cell cycle. Myoblasts proliferate, differentiate and fuse to form myotubes and new myofibers during adult muscle regeneration. Canonical markers for each stage are indicated-(Bottom) Known substrates and regulated processes by p38α/β and p38γ MAPKs during the different stages of myogenesis. Direct substrates are indicated in red. Linked processes are boxed in gray or black. From (Segalés et al., 2016)

Once MyoD is expressed, it has the potential to broadly alter the epigenome in myoblasts and myotubes, in addition to regulating the expression of skeletal muscle genes (Cao et al., 2010). MyoD promotes the myogenic satellite cells to enter the first cell cycle S phase after transitioning out of quiescence by regulating the gene *Cdc6* that is vital to endowing chromatin with the capability of replicating DNA (Zhang et al., 2010).

## Chapter 4: Self-Renewal

A hallmark of stem cells is the ability to **self-renew** (a process of duplication without losing developmental potential). For effective restoration of structure and function in the face of repeated injury, the pool of quiescent satellite cells must be replenished. Evidence for self-renewal has come from studies of myoblast and single fiber transplantation assays into adult muscle, where the satellite cells gave rise to both differentiated myonuclei and functional satellite cells (Collins et al., 2005; Montarras et al., 2005).

Stem cells can divide and self-renew in two fashions: asymmetric cell division and symmetric cell division (Figure 8). The asymmetric architecture of the stem cell niche dictates that stem cells normally divide asymmetrically (apico-basal division) into a new stem cell (self-renewal) and a committed progenitor (differentiation). In **asymmetric** cell division, one parental stem cell gives rise to two functionally different daughter cells: one daughter stem cell and another daughter cell destined for differentiation (Wang et al., 2013). In **symmetric** cell division (planar division), one parental stem cell divides into two daughter stem cells of equal stemness. In either fashion, the number of stem cells is maintained at a constant level.



**Figure 8** : Modes of Satellite Stem Cell Division. Satellite stem cells can self-renew via symmetric or asymmetric cell divisions. A symmetric cell division along the planar axis (with respect to the myofiber) generates two stem cell daughters. Asymmetric cell divisions along the apicobasal axis give rise to a stem cell and a committed myogenic progenitor cell. Alternatively, satellite stem cells can directly express myogenic commitment factors (such as MYF5) to commit to the myogenic lineage and expand the progenitor population that will participate in muscle repair. From (Chang et al., 2016).

The choice of asymmetric versus symmetric division is largely correlated to the **mitotic spindle orientation** relative to the longitude axis of the myofiber (Yin et al., 2013a). Polar segregation of cellular components (cell polarity) intrinsically determines asymmetric cell division and subsequent divergent cell fates. One well-characterized paradigm of

asymmetric spatial organization of cellular components also described in *Drosophila* neuroblast divisions (Chang et al., 2016), is the asymmetric segregation of the Par-complex and Notch-inhibitor Numb to opposite poles (Shinin et al., 2006). A recent study showed also that the **dystrophin** has an essential role in the regulation of satellite cell **polarity**. Consequently, dystrophin-deficient satellite cells have a reduced number of asymmetric divisions, while also abnormal division patterns, impaired mitotic spindle orientation and prolonged cell divisions (Dumont et al., 2015b), exacerbating thus the exhaustion of SCs observed in Duchenne muscular dystrophy. Intriguingly a recent study, using intravital imaging in live mice, described that muscle progenitors undergo predominantly planar divisions along the regenerating myofiber and in constant contact with the basal lamina (Webster et al., 2016).

The symmetric versus asymmetric outcome of satellite stem cell division is regulated by cumulative signals from a variety of cell types in the local milieu. For example, enhanced **Wnt7a signaling**, upregulated by new-formed myofibers, during regeneration promotes symmetric expansion of satellite stem cells by stimulating the Frizzled-7 planar-cell-polarity pathway (Le Grand et al., 2009).

The aforementioned heterogeneity in the stemness of SCs showed that only the Pax7+Myf5- population, which represents the 10% of sublamina Pax7+ cells, undergoes asymmetric division whereby the Pax+Myf5+ give rise to committed myogenic progenitors (Kuang et al., 2007). Other modes of asymmetric divisions distinct from asymmetric satellite stem cell divisions (Wang et al., 2013), have been observed in myoblasts, which give rise to either MyoD- reserve cells or Pax7-MyoG+ differentiating myocytes (Zammit et al., 2004).

Self-renewing cells are **slow-dividing** cells as it has been shown by pulse-chase experiments using thymidine analogs or histone fluorescent marks (H2B-GFP) (Chakkalakal et al., 2014; Fuchs, 2009; Rocheteau et al., 2012; Schultz, 1996). After a chase period, rapidly dividing cells dilute out the label and terminally differentiating cells are lost in the homeostatic flux of tissue turnover (Fuchs et al., 2004). Thus, label-retaining cells (LRCs) can be visualized in stem cell niches (Shinin et al., 2006).

Furthermore, **oxygen availability** affects the self-renewal of SCs so that it is enhanced in hypoxic conditions (Liu et al., 2012).

## Chapter 5: Fusion

Adult skeletal muscle fiber is “a symplast<sup>6</sup>, **multinuclear** structure” (Shenkman et al., 2010) developed in ontogenesis by the fusion of the myoblasts (muscle progenitor cells). The

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<sup>6</sup> from Anc Greek: συν (syn) = together + πλάσμα (plasma) = something formed or molded

nuclei of a muscle fiber (myonuclei) are those located at the periphery of fiber in the space between myofibrils and sarcolemma. A descriptive study in the myotome of *Xenopus laevis* based on electron microscope observations claimed that endoreplication and amitotic divisions form syncytium during early myotome development (stage 45) in *Xenopus laevis* (Boudjelida and Muntz, 1987). In *Drosophila* embryos a well-characterized system for myoblast fusion, mononuclear myocytes fuse with each other, giving rise to the syncytium. Although these embryonic mononucleated myoblasts are committed to a muscle-specific program of differentiation, often undergo only a single cell division subsequent to fusion (Abmayr and Pavlath, 2012). Studies in vertebrate models confirmed the classical view of inability of true muscle nuclei to divide while SCs nuclei are capable of undergoing mitosis in growing animals and such mitoses are followed by the fusion of one or both daughter SC nuclei into the associate fiber to become true muscle nuclei (Moss and Leblond, 1970, 1971).

The inability of muscle nuclei to reenter the cell cycle is associated with the existence of mechanisms of inhibition and suppression of cell cycle event. Such components of the mammalian cell cycle machinery are the tumor suppressors, Rb and Arf (Ink4a), as it was demonstrated that inactivation of both Rb and Arf in primary myotubes leads to cell cycle reentry accompanied with dedifferentiation of postmitotic myotubes (Pajcini et al., 2010).

Myoblast fusion is important not only for skeletal muscle formation during development, but also for postnatal muscle growth and regeneration of skeletal muscle. After muscle injury SCs first become activated, divide and differentiate, and only later fuse to the existing myofiber (Yin et al., 2013a). Other data show that satellite cells fuse with the myofiber concomitantly to cell division, and only when the nuclei of the daughter cells are inside the myofiber, do they complete the process of differentiation (Marti et al., 2013).

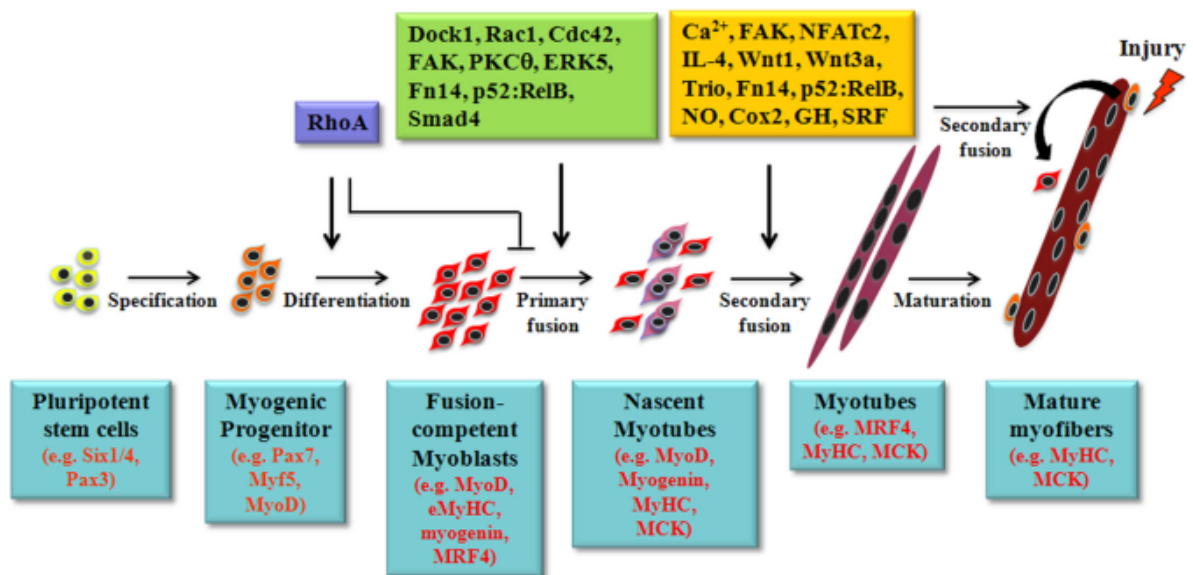
At the cellular level, the fusion process is characterized by the **alignment** of myoblast and myotube membranes and **rearrangements of actin cytoskeleton** at contact sites followed by membrane fusion, which occurs in two stages. Initially, myoblast-myoblast fusion (which is referred to as “**primary fusion**”) results in the formation of nascent myotubes. In the second phase, myoblasts fuse with nascent myotubes (“**secondary fusion**”), which results in nuclear accretion and growth of the myotubes (Abmayr and Pavlath, 2012; Rochlin et al., 2010).

Myoblast fusion follows an ordered set of cellular events, including elongation, cell migration, recognition/adhesion and membrane fusion (Pavlath, 2010a). Cell **migration** process is required for an attractive interaction between myoblasts. During secondary fusion attractive guidance cues emanating from the myotubes, orient myoblast migration towards to the myotubes (Schejter, 2016). Myogenic cells destined to fuse must first recognize and adhere to each other. **Cell adhesion proteins** are critical mediators of this process,



including cell surface proteins (integrins, M- and N-cadherin, ADAM12), transmembrane lipids (cholesterol and phosphatidylserine), intracellular domain-associated signaling or adaptor proteins (actin, beta-catenin, kindlin-2, myoferlin, creatine kinase B, diacylglycerol kinase, Rac1, focal adhesion kinase, syntrophin) that accumulate at **cell-cell contact sites** either in a symmetrical or an asymmetrical manner. Following adhesion, downstream signaling pathways are activated, leading to localized **actin changes** at cell-cell contact sites (Hindi et al., 2013).

An array of cell signaling pathways plays critical roles in myoblast fusion (Figure 9). Some of these pathways are activated as a result of the recruitment of specific cell-surface proteins between fusion partners, whereas others are activated as part of the myogenic differentiation program, but they contribute to the fusion process.



**Figure 9 :** The roles of different signaling molecules in primary and secondary myoblast fusion during myogenesis. Muscle progenitor cells first undergo myogenic commitment and differentiation to become fusion-competent myoblasts. The initial commitment to differentiation requires the activity of the RhoA GTPase. Active RhoA interferes with myoblast fusion, and so it is deactivated before fusion occurs. A number of signaling molecules and pathways are activated in fusion-competent myoblasts that regulate primary myoblast fusion, which results in nascent myotubes. Additional signaling molecules are then recruited, which lead to fusion of additional mononucleated myoblasts with nascent myotubes. Secondary fusion also plays a critical role in the regeneration of injured myofibers. Major signaling molecules involved in primary and secondary myoblast fusion based on experimental evidence are depicted along the top. Specific myogenic markers expressed at different stages in cells of myogenic lineage during myogenesis are noted along the bottom. From (Hindi et al., 2013)

In yeast and *Drosophila* systems, **actin fusion focus**, a specialized actin structure, is assembled to promote plasma membrane emerging. The primary proposed function of the

actin focus is to provide **physical force** to bring the partner plasma membranes in close juxtaposition and help overcome the energy barrier to membrane fusion (Martin, 2016). In *Drosophila* embryo, myotubes are generated through an **asymmetric fusion** process between two distinct cell types: a fusion-competent myoblast (FCM) and a founder cell (FC) (Chen et al., 2007). These two cell types express distinct surface adhesion molecules, the protein Dumb-founded (Duf) in the FCM while the protein Sticks and Stones (Sns) in the FC. The actin focus proper is only present in the FCM, at the center of an invasive membrane protrusion, a podosome-like structure that invades into the FC/myotubes (Kim et al., 2007). Recently, a study with live observations in *Drosophila* myoblast fusion identified a dense array of **actin-based filopodia** that emanate from the entire surface of myotubes and come in contact with the approaching myoblasts. They proposed that the filopodia are necessary to prime the heterotypic adhesion process between the two cell types, possibly by Sns-Duf adhesive interactions (Segal et al., 2016).

In mammalian myoblast, examination of the actin cytoskeleton in cell culture has shown F-actin (polymerized actin) structures decorating the interface of juxtaposed myoblasts sites of fusion. Myoblast fusion in mice similarly requires actin-binding proteins (ABPs)-dependent actin assembly suggesting a possible similar organization of the actin cytoskeleton. However, there is currently no evidence for actin foci during muscle biogenesis in vertebrates *in vivo* (Martin, 2016). Knockout mouse studies focusing on myoblast fusion include demonstrations of the essential involvement of **actin regulators** such as DOCK1 and N-WASp, homologs of which are well-established contributors to myoblast fusion in the *Drosophila* system (Schejter, 2016).

While actin effectors are not muscle-specific, a variety of **transmembrane** proteins, involved in the cell fusion process and machinery components important for resealing a disrupted sarcolemma, have also been described in mice (Demonbreun et al., 2015). Among them are the ferlin family of proteins (including dysferlin and myoferlin) (Posey et al., 2011), EHD-containing family of proteins (Doherty et al., 2008), the sarcolemmal membrane repair machinery component, the MG53 protein (Cai et al., 2009). The recently discovered protein which drew attention is the Transmembrane protein 8c (Tmem8c) otherwise named Myomaker (Millay et al., 2013).

Myomaker is a **muscle-specific membrane protein** which is expressed in myotome, in limb buds and axial skeletal muscles during mouse embryogenesis thereafter declined postnatally and re-expressed during muscle regeneration. Mouse myomaker knock-out die perinatally because of an absence of multinucleated myofibers (Millay et al., 2013). *Ex vivo* investigation of the myomaker functions in primary cell cultures revealed a block of myoblast fusion in its absence whereas its overexpression strikingly increases the fusion and

myomaker is ultimately associated with the actin cytoskeleton to exert its fusogenic function (Millay et al., 2013). In consequence with its fusogen role, loss of myomaker in adult SC resulted in a complete block of regeneration with the myoblasts unable to fuse. The presence of E-boxes in the promoter region of myomaker and functional analysis confirmed the binding of MyoD and myogenin and indicated myomaker as a novel important effector of mammalian myoblast fusion (Millay et al., 2014). Interestingly myomaker promotes the **heterologous fusion** between fibroblasts and myoblasts forming chimeric myotubes and this activity is conserved in vertebrate species whereas other related family members (TMEM8a and TMEM8b) lack fusogenic activity (Millay et al., 2016). Upstream regulators of myomaker, described in cardiac tissue, is the protein inhibitor of activated STAT-1 (Pias1) as it was found that Pias1 expression in embryonic, cardiac myoblasts enhances the induction of cardiac muscle genes MyoD, Myogenin and Myomaker (Constanzo et al., 2016).

## Chapter 6: Heterogeneity in Satellite Cell Population

Different muscles exhibit distinct characteristics, including anatomical structure, contractile and metabolic properties, fiber composition, blood supply, pattern of innervation and embryonic origin. Moreover, they have different regenerative capacities and are differentially affected in genetic disorders. Numerous studies have begun to clarify the developmental, cellular and molecular bases of this diversification (Biressi and Rando, 2010).

Satellite cells are **defined by their anatomical location** between the basal lamina and the fiber sarcolemma which justify their name. Increasing evidence suggests that satellite cells represent a heterogeneous population of cells which differ in their embryonic origins, in their gene expression signatures, myogenic differentiation tendency, stemness and lineage potential to assume non myogenic fates (Yin et al., 2013).

Adult SCs in resting conditions in contrast to perturbed ones, have a different **molecular signature** as defined by the expression of Myf5, CD34, Mcadh and criteria of **cell size** and **granulosity** (Beauchamp et al., 2000; Montarras et al., 2013). Moreover, adult SCs have a different molecular signature in different muscle groups as for example regarding the expression of either Pax3 or Pax7. Pax 3-expressing SCs are abundant in the diaphragm and in most trunk muscles and less frequent in hind limb muscles (Relaix et al., 2006).

Heterogeneity in myogenic destiny exists with some differentiating rapidly, whereas others are responsible for maintaining the pool (Rantanen et al., 1995). BrdU administration in growing rats confirmed the presence of two populations of satellite cells with differing **rates of division** and proliferative capacities (Schultz, 1996) The slow-cycling and fast-cycling cells described in Schultz's study may correspond to Myf5-not expressing and Myf5-expressing SCs which respectively contribute or not to the pool of quiescent SCs (Kuang et

al., 2007). The same study demonstrated that only Myf5-not expressing SCs could undergo asymmetric cell divisions, giving rise to a Myf5-not expressing satellite stem cell and a Myf5-expressing committed progenitor cell (Kuang et al., 2007) confirming further heterogeneity in the stemness of SCs. In 2014, Chakkalakal et al. using a mouse strain to report the proliferative history, showed that the fast dividing cells are committed to differentiation which is in agreement with the notion that cell cycle kinetics defines the **stemness** (Chakkalakal et al., 2014).

The predominant fate of satellite cells *in vivo* is the myogenesis in young, healthy muscle. Although SCs are unipotential, a fraction of SCs possess a transdifferentiation potential, thus linking intrinsic SC heterogeneity to **multipotentiality**. The capacity of SCs to differentiate into alternative lineages, such as fibrogenic, adipogenic and osteogenic lineages, has been suggested by *in vitro* analyses of cells associated with muscle fibers (Asakura et al., 2001). More recently it has been shown *in vivo* by lineage tracing the potential of SCs to give rise to brown adipocytes. The microRNA, the **miR-133**, is expressed in SCs and is upregulated through the myogenic progression to repress the Prdm16 gene expression restricting thus the adipogenic fate. The switch towards the adipogenic lineage is achieved by inhibiting the miR-133 during muscle regeneration or by **cold exposure** leading in the *de novo* generation of satellite cell-derived brown **adipocytes** (Yin et al., 2013b).

Further heterogeneity in the stem cell population is reflected in recent studies that show a **non-random DNA segregation** during asymmetric cell division. Non-random DNA segregation was shown to be a property of a subset of SCs and is associated with divergent fates of descendant SCs (Rocheteau et al., 2012). Rocheteau et al. showed that template DNA strand segregation is executed by satellite cells with high levels of Pax7, showing thereby that two subpopulations of SCs exist concerning the Pax7 level expression (High and Low). In the great majority of the cases, the daughter cell inheriting the older templates retained the more immature phenotype, whereas the daughter inheriting the newer templates exhibited signs of differentiation. This aligns with the **immortal DNA strand hypothesis**, a proposed mechanism by which adult stem cells minimize genomic mutation (Charville and Rando, 2013; Rando, 2007). The fundamental idea behind this hypothesis is that, by retaining the immortal strands within the stem cell progeny, errors in DNA duplication would be passed on to non-stem, more differentiated, and shorter-lived daughter cells. An alternative explanation is that asymmetric cell divisions and cell fate are codirected by **epigenetic differences** between sister chromatids (Lansdorp, 2007). A recent study using a novel methodology that utilizes cancer sequencing data to estimate the rate of accumulation of mutations in healthy stem cells of the colon, blood, head and neck tissues, showed that in some tissues, mutations in stem cells accumulate at rates strikingly similar to those expected

without the protection from the immortal strand mechanism (Tomasetti and Bozic, 2015). The non-random segregation of DNA during stem cell replication might be a mechanism operating for a small, rarely-dividing pool of stem cells at expense of the mutation-directed beneficial evolution.

## Chapter 6: Satellite Cell Fate in Ageing

Aging of the satellite cell is characterized by a **decline** in stem cell **numbers** and **functionality** and the main consequence of satellite cell aging is the progressive reduction of SC regenerative function (Sousa-Victor and Muñoz-Cánoves, 2016). The process of stem cell aging is a consequence of the combined effects of age-dependent alterations in the environment and age-associated intrinsic dysregulations of the stem cell itself (Dumont et al., 2015a).

Aged satellite cells, in comparison with adult satellite cells, spend **less time** in a quiescent state and they have increased cycling activity in resting conditions. The disruption of quiescence affects the self-renewal but does not impede the myogenic commitment (Chakkalakal et al., 2012). *In vitro* studies showed that myogenic cells from aged muscles have a **delayed response** to activation cues (Barani et al., 2003). Furthermore, **impaired differentiation** ability *in vitro* has been reported for aged SCs (Biressi and Rando, 2010). Aged SCs have an increased tendency to enter **alternative differentiation programs** by adopting an adipogenic and fibroblastic fate leading thus to the replacement of aged muscle by fibrotic connective and adipose tissue (Biressi and Rando, 2010). The aged systemic environment promotes a myogenic to fibrogenic conversion and this is associated with increased components of aged serum containing Frizzled receptors resulting in **enhanced Wnt signaling** in myogenic cells during aging (Brack et al., 2007).

When old murine muscle stem cells are exposed to a young environment or to growth factors, their capacities to proliferate and differentiate are partly restored (Conboy et al., 2005), suggesting that functional deregulations with age may be **reversible**. However, recent studies suggest that in geriatric ages, when the sarcopenic phenotype is exacerbated, there are intrinsic alterations in the satellite cell, which **irreversibly** compromise its function, and cannot be reversed by exposure to youthful cues (Sousa-Victor and Muñoz-Cánoves, 2016).

A proportion of satellite cells displays a loss of reversible quiescence by switching to an irreversible pre-senescence state, caused by derepression of p16(INK4a) (Sousa-Victor et al., 2014a). Upon regenerative or proliferative pressure, satellite cells from geriatric muscles thus fail to activate and proliferate and instead undergo full **senescence** and irreversibly withdraw from the cell cycle (geroconversion) and they do so even in a youthful

environment (Sousa-Victor et al., 2014b). However recent studies also showed that muscle precursor cells from adult and aging humans do not undergo senescence (Bigot et al., 2015).

The quiescent stem cells display continuous basal autophagy, but as this activity declines with age, results in toxic cellular waste accumulation and entry into senescence (García-Prat et al., 2016; Wen and Klionsky, 2016). **Autophagy** seems to be a decisive factor to prevent senescence, since overexpression of Atg7, a crucial autophagy-related protein needed for autophagosome formation and pharmacological treatment in old mice with rapamycin, a well-known autophagy-inducing regime, **prevented** senescence and restored regeneration in aged muscles (García-Prat et al., 2016).

The **epigenetic profile** of QSCs changes, as they age. Age-associated DNA methylation that it acts through suppression of genes controlling cell quiescence has been recently shown in humans for SPRY1 gene, which is an inhibitor of fibroblast growth factor (FGF) signalling (Bigot et al., 2015). Aged muscle secretes higher levels of Fgf2 into the satellite cell niche, leading to repression of the quiescence in elderly stem cells. Inhibition of FGF receptor-1 (FGFR1) or overexpression of (Sprouty1) Spry1 can rescue age-related defects (Chakkalakal et al., 2012). However aged SCs possess a cell-autonomous intrinsic defect in self-renewal arises from an impaired response to FGF ligands and elevated p38 $\alpha$  $\beta$  MAPK activity that cannot be rescued by exposure to a young environment (Bernet et al., 2014).

## Part 5: Skeletal Muscle Mass – Hypertrophy and Atrophy

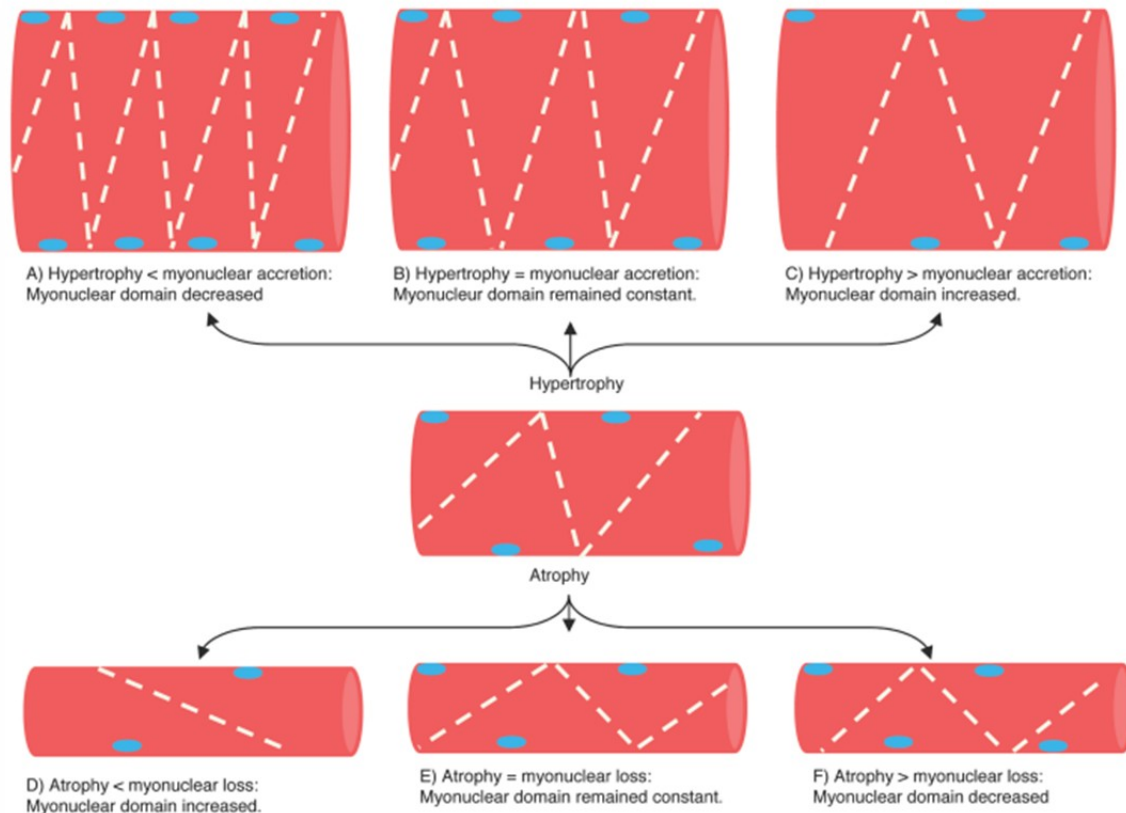
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Postnatal growth of skeletal muscle in mammals occurs rapidly and continues until adulthood. Postnatal muscle growth is achieved by an increase in number (hyperplasia) and size (hypertrophy) of myofibres in rat, but mainly by hypertrophy of myofibres in mouse (White et al., 2010). In developing muscle, satellite cells undergo extensive proliferation and most of them fuse with myofibers, thus contributing to the increase in myonuclei during early postnatal stages (Pallafacchina et al., 2013). In adult life, the regulation of muscle mass and fiber size essentially reflects protein turnover named as the balance between protein synthesis and degradation within the muscle fibers. However, skeletal muscle fibers are multinucleated structures, thus protein turnover may also be affected by cell or nuclear turnover, i.e. addition of new myonuclei, due to fusion of satellite cells, or loss of myonuclei, due to nuclear apoptosis (Schiaffino et al., 2013).

The muscle cell is multinuclear and each nucleus controls transcriptional activity in the surrounding territory of cytoplasm called **myonuclear domain**<sup>7</sup> (MND) (Pavlath et al., 1989). MND size varies with the fiber type and is inversely proportional to the muscle fiber oxidative capacity. Accordingly to the constant myonuclear domain model (Van der Meer et al., 2011) is that the number of myonuclei should change in proportion to size during atrophy and hypertrophy (Figure 10). During muscle fiber hypertrophy, **change in MND size precedes** change in myonuclei count, suggesting that the myonuclei have the ability to enhance their synthetic capacity according to cell size, functional and metabolic needs. MND size has a **"ceiling" limit** during hypertrophic process beyond which extra myonuclei are donated by satellite cell to support further muscle growth (Qaisar and Larsson, 2014; Van der Meer et al., 2011). The higher number of nuclei should contribute to the increase in protein synthesis because total protein synthesis is the product of synthesis per nucleus and the number of nuclei (Gundersen, 2016). The incorporation of SCs into the growing myofiber was confirmed by nuclei labeling (3H-thymidine) study after compensatory hypertrophy induction (Schiaffino et al., 1972, 1976).

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<sup>7</sup> defined as the cytoplasmic volume per myonucleus



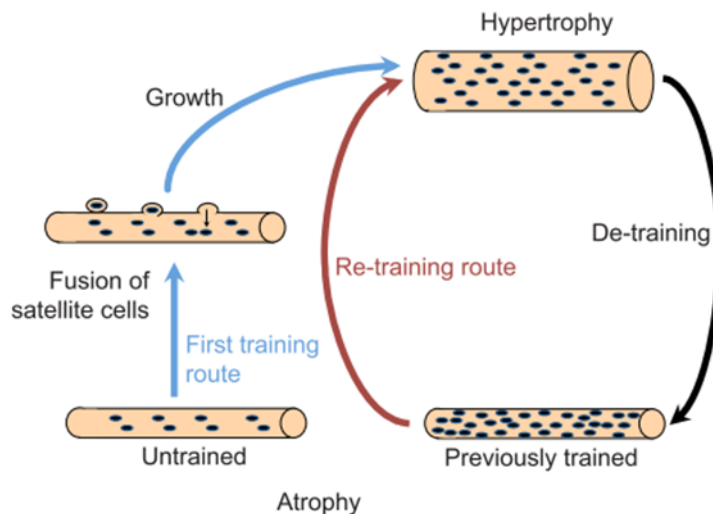
**Figure 10** : Possible scenarios of changes in myonuclear domain size with different types of muscular adaptation. From (Van der Meer et al., 2011).

However, recent studies showed that the **satellite cell fusion precedes** the growth phase. *In vivo* imaging to study live myonuclei belonging to distinct muscle fibers observed that new myonuclei are added before **any major increase in size** during overload exercise (Bruusgaard et al., 2010). Thus, the myonuclear domain is temporarily decreased during the growth phase. Respectively, in several different atrophy models, using *in vivo* time-lapse imaging, it has been observed that the number of myonuclei remained constant with no signs of apoptosis (Bruusgaard and Gundersen, 2008). Alike, the myonuclear domain is **temporarily decreased** during the atrophic phase. Such a study concludes that disuse atrophy is not a degenerative process, but is rather a change in the balance between protein synthesis and proteolysis in a permanent cell syncytium.

Recent studies showed that during muscle unloading and reloading, an increase in cross sectional area of 59% is not accompanied with an increase in the number of myonuclei (Bruusgaard et al., 2012; Jackson et al., 2012). The concept that under atrophic conditions, no apoptosis have been observed in the myonuclei, this allows the hypertrophic growth to happen without the recruitment of myonuclei during re-training, not because more nuclei are not needed in large fibers, but because the nuclei are already there (Gundersen, 2016).



A new cellular model for hypertrophy and atrophy is the **muscle memory** describing a cellular memory in skeletal muscle in which hypertrophy is ‘remembered’ and ‘stored’ in the myonuclei (Gundersen, 2016). According to this model, “previously untrained fibers recruit myonuclei from activated satellite cells before hypertrophic growth. Even if subsequently subjected to acute atrophy, the higher number of myonuclei is retained, and the myonuclei seem to be protected against the elevated apoptotic activity observed in atrophying muscle tissue. Fibers that have acquired higher number of myonuclei grow faster than naive fibers when subjected to overload exercise, thus the myonuclei represent a functionally important “memory” of previous strength” (Gundersen, 2016) (Figure 11).



**Figure 11** : A new model for the cell biology of hypertrophy and atrophy. For naive fibers and preceding hypertrophic growth, myonuclei are recruited from satellite cells, temporarily reducing the myonuclear domain volume, leading to a large fiber with many myonuclei. Upon subsequent atrophy the myonuclei are maintained, leading to a small fiber with a high myonuclear density and small myonuclear domains. Such fibers can hypertrophy without recruiting new nuclei, and this re-training route seems to be faster than the first training route. The permanently higher number of myonuclei represents the muscle memory. From (Gundersen, 2016).

## Chapter 1: Hypertrophy

The plasticity of skeletal muscle extends also in the means of adapting its structure and function following to mechanical environment. The nature of the stimulus ultimately dictates the adaptation in skeletal muscle in response to exercise. For instance, **resistance exercise** training is generally characterized by **increases in muscle mass** and muscle fiber cross sectional area planned to resist the stress of lifting heavy loads. In contrast, **endurance exercise** training is generally characterized by **metabolic adaptations** planned to enhance energy selection and resist fatigue (Snijders et al., 2015). Resistance training is

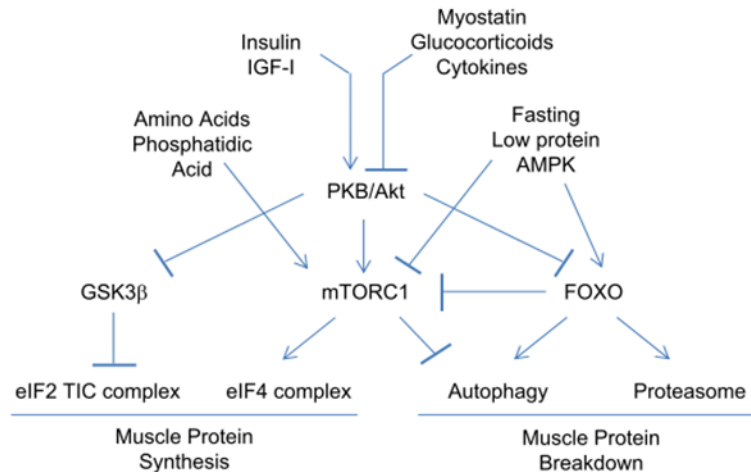
the only *bona fide* intervention to increase muscle mass that can be performed on a population-wide basis. Mechanical loading also results in strong adaptive responses in a host of other tissues including bone, tendon and the extracellular matrix in muscle, protecting these tissues against future injury (Marcotte et al., 2015). It has been reported that muscles can remain hypertrophic after several months of detraining (Harris et al., 2007)

Skeletal muscle mass is primarily dictated by the regulation of muscle protein synthesis. Increased structural protein synthesis serves to add **new contractile filaments** to preexisting sarcomere units. The increase in muscle proteins during muscle hypertrophy can be achieved either by **increasing** RNA and protein synthesis from the existing nuclei or maintaining the same level of RNA and protein synthesis from each nucleus and **adding** new nuclei to the fibers. Since the adult muscle fiber nuclei (myonuclei) are unable to divide, the new nuclei, which are incorporated by the fiber, originate from outside the fiber. Satellite cells are the major **donors** of new nuclei, being myogenic precursor cells, important for muscle development, for muscle regeneration and possibly also for muscle hypertrophy in response to exercise, training and hormonal stimulation (Blaauw and Reggiani, 2014).

The necessity of SCs for hypertrophic muscle growth and maintenance is not widely agreed as a study in satellite cell-depleted muscle did not show attenuated hypertrophy following overload (Fry et al., 2014; McCarthy et al., 2011). These studies indicate that satellite cells are not required to maintain a significantly increased muscle mass for an extended period of time. However the growth plateau, in the absence of satellite cells, is associated with excessive ECM accumulation suggesting that SCs are required for healthy remodeling of the ECM during muscle adaptation through regulation of fibroblast activity (Fry et al., 2014). A very recent study, where the aforementioned experiments were repeated, failed to support the previous conclusions and showed that the hypertrophy was prevented in plantaris muscle lacking SCs concluding that the hypertrophic response to mechanical overload is dependent on satellite cells (Egner et al., 2016). It is not demonstrated if other stem cells in skeletal muscle contribute to muscle hypertrophy (Blaauw and Reggiani, 2014).

## Chapter 2: Signalling

Multiple signal transduction networks guide and coordinate the muscle protein balance (Figure 12) to maintain protein homeostasis (proteostasis). Genetics, hormones, and environmental stimuli each influences proteostasis control, altering capacity and/or efficiency of muscle growth. Molecular mechanisms that regulate load-induced skeletal muscle hypertrophy and that might be exploited for developing new interventions designed to increase muscle mass.



**Figure 12** : Signaling pathways coordinate muscle protein balance. Anabolic and catabolic stimuli are integrated through the PKB/Akt-mTORC1 signaling to regulate mechanisms that control muscle protein synthesis and breakdown. FOXO, Forkhead box class O; mTORC1, mechanistic target of rapamycin complex 1; eIF4, eukaryotic initiation factor 4. From (Anthony, 2016)

## Hormones

The first molecular regulators of skeletal muscle mass and strength identified were hormones, such as testosterone, IGF-1 and growth hormone, and it is sex-associated (Smith and Mittendorfer, 2016). Even though hormones set our baseline muscle size and strength, they play **little role** in the adaptive response to resistance training (Marcotte et al., 2015). Hypertrophic growth induced by humoral/hormonal stimuli, such as IGF-1 and androgens, also induces satellite cell activation.

### Insulin-like growth factor-1

Insulin-like growth factor-1 (IGF-1), also known as **mechano-growth factor**, is a circulating growth factor that is produced and released systemically by the liver and locally by the muscle (Philippou and Barton, 2014). IGF-1 has long been recognized as one of the critical factors for coordinating muscle growth, enhancing muscle repair, and increasing muscle mass and strength. The major muscle phenotype induced by IGF-1 **overexpression** is hypertrophy characterized by an **increase** in the cross-sectional area of the myofibers and a shift toward a more oxidative fiber type (Musarò et al., 2001). This overexpression has been shown to modulate the proliferative capacity of the satellite cells (Chakravarthy et al., 2000) and to increase SC fusion (Jacquemin et al., 2004). Mice overexpressing IGF-1 exhibit a proportional **gain in muscle force and size** demonstrating thus that the addition of extra

myonuclei is a prerequisite for maintenance of specific force during muscle hypertrophy (Qaisar et al., 2012).

#### Interleukin 6

Traditionally, Interleukin (IL-6) was considered to be an inflammatory cytokine. IL-6 is also referred to as a “myokine” as it is also produced by skeletal muscle in response to exercise. An exercise induced increase in circulating levels of IL-6 has been observed to enhance fat-oxidation, improve insulin-stimulated glucose uptake (Snijders et al., 2015). In addition, muscle hypertrophy is blunted in mice **lacking** IL-6, and this **reduced** growth was linked to a compromised activation and fusion of satellite cells (Serrano et al., 2008). Chronic exposure to IL-6 in disease states or age-related increases in serum IL-6 appears to be detrimental, impairing muscle protein synthesis, and contributing to the loss of skeletal muscle mass (Snijders et al., 2015).

#### Interleukin 4

The cytokine IL-4 was identified as a molecular signal that controls myoblast fusion with myotubes and it is regulated by NFATc2 transcription factor which controls myoblast fusion at a specific stage of myogenesis after the initial formation of a myotube and is necessary for further cell growth. Myofibers **lacking** IL-4 or the IL-4 alpha receptor subunit form normally but are **reduced** in size and myonuclear number. IL-4 is expressed by a subset of muscle cells in fusing muscle cultures and requires the IL-4 alpha receptor subunit on myoblasts to **promote fusion** and **growth** (Horsley et al., 2003). In postnatal muscle, IL-4 is a downstream target of Srf transcription factor as it is downregulated upon Srf-loss (Charvet et al., 2006) and the IL-4 overexpression in Srf-deleted muscles restores SC recruitment and muscle growth (Guerci et al., 2012a).

#### mTOR Signaling

Mechanical stimuli can **regulate** the rate of protein synthesis through changes in **translational efficiency** and/or translational capacity. Translational efficiency has three potential stages for regulation (initiation, elongation and termination), and mechanical stimuli appear to primarily affect the stage of initiation (Hornberger, 2011). mTOR (mammalian target of rapamycin) is an evolutionarily conserved protein kinase that is important in relaying information from nutrients, growth factors and mechanical loading to drive protein synthesis and cell growth. mTOR exists in two distinct multi-protein complexes: the mTORC1 and the mTORC2 complexes. mTORC1 directly regulates protein synthesis and is sensitive to rapamycin. Inhibiting mTORC1 with rapamycin has been shown to block acute amino acid-

and resistance exercise-induced increases in muscle protein synthesis (Marcotte et al., 2015). Mechanical stimulation is sufficient for the activation of mTORC1 signaling and subsequently for the mechanically-induced changes in protein synthesis and compensatory growth.

The effect of mTORC1 on the translation machinery and protein synthesis is mediated through inhibition of 4E-binding protein 1 and activation of the ribosomal protein S6 kinase. Deletion of S6 kinase reduces myoblast size to the same extent as that observed with mTOR inhibition by rapamycin without affecting the myonuclei number (Ohanna et al., 2005). mTORC1 induces protein synthesis by phosphorylating and inhibiting 4E-BP1 and thereby relieving the repression of eukaryotic translation initiation factor 4E and cap-dependent translation. It also regulates the **ribosome biogenesis** by the phosphorylation of the ribosomal protein S6 that stimulates mRNA translation and by the modulation of ribosomal RNA synthesis (Ma and Blenis, 2009). Ribosome biogenesis is a central mechanism used by skeletal muscle to regulate protein synthesis and control skeletal muscle mass in response to anabolic and catabolic stimuli (Chaillou et al., 2014).

The IGF-1-Akt pathway increases muscle protein synthesis via inhibiting glycogen synthase kinase 3b (an inhibitor of eIF2 ternary complex formation) and activating mechanistic target of mTORC1 signalling (Schiaffino and Mammucari, 2011).

## Myostatin Signaling

Myostatin (Mstn), a member of the **transforming growth factor- $\beta$**  superfamily, is a highly conserved, muscle-specific, secreted protein that controls **organ size**. It is a **negative regulator** of skeletal muscle growth as inhibition of myostatin gene early in development results in both hyperplasia and hypertrophy (McPherron et al., 1997). Postnatal Mstn-inactivation can induce skeletal muscle hypertrophy (Whittemore et al., 2003), while its overexpression or systemic administration causes muscle atrophy (Rodriguez et al., 2014). Resistance exercise leads to myostatin downregulation potentially playing a role in the growth of muscle fibers after exercise (MacKenzie et al., 2013).

Myostatin functions at the crossroad between the protein synthesis and degradation signaling pathways in muscle. The cross-talk between myostatin and the intracellular AKT/mTOR signaling pathway controls protein synthesis and muscle hypertrophy in adults (Rodriguez et al., 2014). Expression of constitutively active AKT (also known as protein kinase B) in skeletal muscle rapidly leads to muscle hypertrophy (Blaauw et al., 2009), thus myostatin negatively regulates the activity of the **Akt pathway**.

Myostatin binds and activates a heterodimeric receptor complex comprising activin type II Ser/Thr kinase receptors (ActRIIB and ActRIIA) and type I receptor, activin receptor-

like kinase 4 and 5 (ALK4 and ALK5) (Rebbapragada et al., 2003) which in turn leads to the phosphorylation and activation of Smad2 or Smad3 and formation of heterodimers with Smad4. Smad TFs translocate into the nucleus and activate the transcription of the target genes involved in muscle growth (Marshall et al., 2008) and wasting. Inhibition of Smad2/3 or TGF- $\beta$  signalling promotes muscle hypertrophy independent from satellite cell activation but partially **dependent** of the mTOR signaling (Sartori et al., 2009).

The role of myostatin in the activation of SCs is conflicting since there are studies showing activation and others not. Post-developmental inactivation of myostatin leads to hypertrophy with little or no myonuclear accretion (Amthor et al., 2009). Some data have suggested that satellite cells might also contribute to muscle hypertrophy following myostatin loss. BrdU labeling *in vivo* showed activated and increased Mstn-null SCs than SCs from wild-type mice (McCroskery et al., 2003). Mstn-overexpression promotes the terminal differentiation program and causes a decrease in the number of embryonic muscle progenitors. In the long term, this leads to a decrease in the number of myofibers within muscle masses (muscle hypotrophy) (Manceau et al., 2008).

**Myostatin inhibition** may induce myofiber hypertrophy by increasing the cytoplasm/myonuclear ratio (MND domain) before activating satellite cells. The large MNDs found in fast muscles of Mstn-null mice were correlated with the decrement in specific force and myosin content in Mstn-null muscles. Thus, myostatin inhibition not being able to maintain the appropriate MND for optimal function leading to a **pathological hypertrophy** suggesting that there is a critical volume of individual myonuclei that could support efficiently the hypertrophic growth (Qaisar et al., 2012).

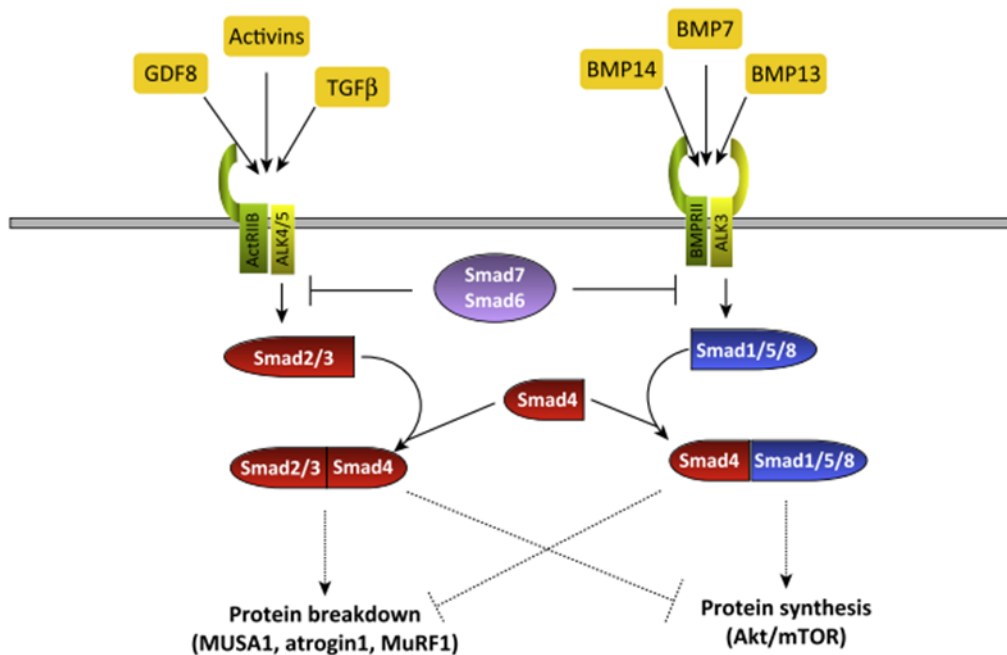
## BMP Signaling

The bone morphogenetic protein (BMP) subfamily, the parallel signaling axis of the TGF- $\beta$  superfamily is the **dominant pathway** controlling muscle mass, even more so than myostatin (Sartori and Sandri, 2015). BMP cytokines preferentially binds to a combination of type II receptors that includes BMP type II receptor (BMPRII), ActRIIA, and ActRIIB, before promoting recruitment of type I receptors (ALK3, ALK6 and ALK2). Similar to Smad2 and Smad3, BMP-dependent Smad proteins (Smad1/5/8) also form a transcriptional complex with Smad4 to effect transcriptional regulation of ID gene family that controls **cell growth** and **differentiation** (Sartori et al., 2014).

Enhanced BMP signalling (constitutively active ALK3 expression) trigger muscle hypertrophy and prevent denervation-induced muscle atrophy by inducing hypertrophy of denervated myofibers (Sartori et al., 2013; Winbanks et al., 2013). Inhibition of BMP signalling, by Noggin (BMP antagonist) overexpression or knockdown of Smad 1/5, induces muscle

atrophy via the HDAC4-myogenin-E3 ligases (Sartori et al., 2013; Winbanks et al., 2013). Moreover inhibition of BMP signaling reverted the hypertrophic phenotype of myostatin-knockout mice (Sartori et al., 2013). Enhancing BMP–Smad1/5 signaling is protective in models of neurogenic atrophy, by **suppressing** the activity of the **atrophy-inducing HDAC4–myogenin–E3 ubiquitin ligase axis**. The BMP signaling axis is a *bona fide* regulator of skeletal muscle growth by **stimulating** mTOR-dependent anabolic mechanisms (Winbanks et al., 2013).

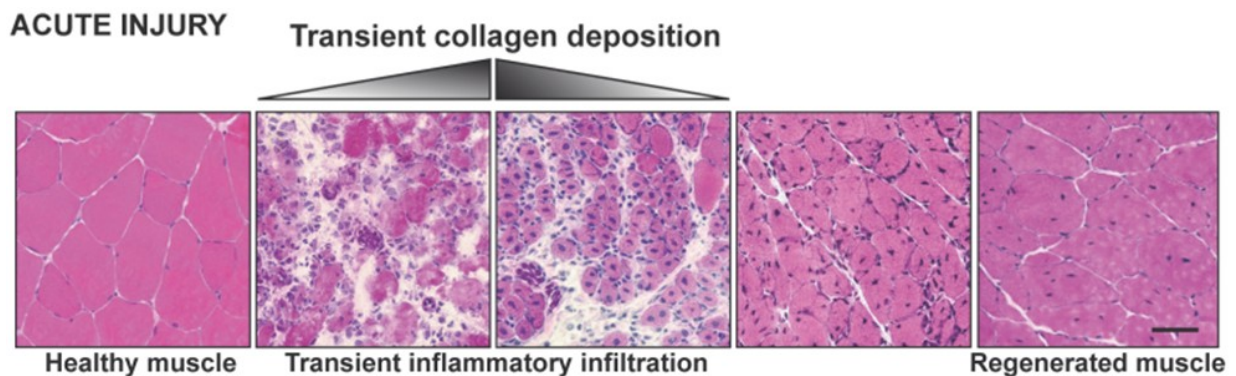
The ability of myostatin or BMP signalling to regulate muscle growth is dependent on **competition** for Smad4 binding (Figure 13). When BMP signaling is high, Smad4 shifts binding toward Smad1/5/8 and this either results in the targeting of a different set of genes or reverses the expression effects of myostatin (Marcotte et al., 2015). In addition to Smad4, ligands from both subgroups also compete for access to some type II receptors. Competition between ligands either in binding type II receptors or in the recruitment of type I receptors has been proposed as one of the mechanisms that regulate the signaling of the two branches of TGF $\beta$  superfamily (Sartori et al., 2014).



**Figure 13** : Interplay between myostatin and bone morphogenetic protein (BMP) signaling pathways. Smad4 is shared between the Smad2/3 and Smad1/5/8 transcription factors and is required for their actions. Therefore, it is feasible that a decrease of activated Smad2/3 would inevitably release Smad4, which would be recruited toward the BMP signaling pathway to promote hypertrophy and counteract atrophy. Vice versa, when the BMP pathway is blocked or myostatin expression is increased, more Smad4 would be available for Smad2/3, which would then transactivate an atrophy response. This model would also explain why these two branches share common targets (e.g. the Akt–mTOR axis) but with opposite regulation. Therefore, in normal circumstances, a balance between these competing pathways is required for maintenance of muscle mass. The broken lines indicate mechanisms that are as yet unknown. From (Sartori et al., 2014)

## Part 6: Skeletal Muscle Regeneration

Adult skeletal muscle is a high plastic tissue with a remarkable capacity to regenerate following a variety of injuries, ranging from acute traumatic injuries, such as the strain injuries common in sport medicine, to chronic degenerative diseases, such as the muscular dystrophies, characterized by repeated cycles of segmental necrosis and regeneration. Muscle regeneration and repair occur in four interrelated and time-dependent phases: degeneration, inflammation, regeneration and remodeling-repair (Figure 14). Injury of myofibers results in the rapid necrosis, due to an influx of extracellular calcium, which induces calcium-dependent proteolysis of the myofibers that drives the tissue degeneration. The presence of necrotic fibers activates a defined inflammatory response that is characterized by the sequential invasion of muscle by inflammatory cell populations. The inflammatory response is followed by regenerative phase, characterized by satellite cell activation and by presence of regenerating fibers. The final phase is a period during which the maturation of the regenerated myofibers, the contraction and reorganization of the scar tissue and the recovery of the functional performance of injured muscle occur (Yin et al., 2013a).



**Figure 14** : Muscle histology in acute muscle regeneration. Tibialis anterior muscles of mice were injected with cardiotoxin and samples were taken at different stages of the regeneration process. A representative sample showing the inflammatory phase, characterized by a transient increase in collagen deposition, and subsequently the resolving phase of healing, with progressive recovery of the normal tissue morphology (hematoxylin and eosin). Adapted from (Mann et al., 2011)

Satellite cells are the primary stem cells responsible for muscle regeneration however other stem cells able to regenerate muscle (e.g. bone marrow stem cells, muscle side



populations, PW1 + /Pax7 – interstitial cells, muscle-derived stem cells, mesoangioblasts and pericytes) have been explored (Yin et al., 2013a).

## Chapter 1: Inflammation Responses

Inflammatory and immune cells have crucial role in the regeneration process. Acute muscle injury causes an immediate transient wave of neutrophils followed by a more persistent infiltration of M1 (pro-inflammatory) and M2 (anti-inflammatory/pro-regenerative) macrophages. Macrophages infiltrating injured skeletal muscle are not only involved in the phagocytosis of necrotic fibers, but may also release factors that promote muscle regeneration. **M1 macrophages** appear soon after injury and rapidly decline, have a pro-inflammatory role and **stimulate satellite cell proliferation**, while **M2 macrophages** have an anti-inflammatory role and **promote myoblast fusion** and myotubes hypertrophy (Chazaud, 2014).

### Regulatory T cells

T cells were also identified in dystrophic and injured skeletal muscle, however recent studies revealed a **specific population** of regulatory T cells (Treg cells), the “muscle Tregs”. The crucial observation supporting the role of Treg cells in the context of muscle injury is that muscle regeneration is **compromised** by conditional depletion of Tregs in a mouse line expressing a human diphtheria toxin receptor under control of Foxp3 (a lineage-specific transcription factor). Tregs not only **promote the M1-to-M2 switch** in macrophages but also directly act on muscle satellite cells. Indeed, **SC proliferation** is stimulated *in vitro* by co-cultured activated Treg cell. The cytokine, IL-33, has been involved in the control of Treg cells in different tissues. IL-33 increases shortly after injury in skeletal muscle and acts on cells containing the ST2 (suppression of tumorigenicity 2) receptor. ST2 is one of the more strongly upregulated receptors in Tregs isolated from injured muscle compared to Tregs present in lymphoid tissue. Treg cells devoid of ST2 show impaired accumulation in injured muscle, less effective clearing of the muscle infiltrate and delayed muscle regeneration (Schiaffino et al., 2016).

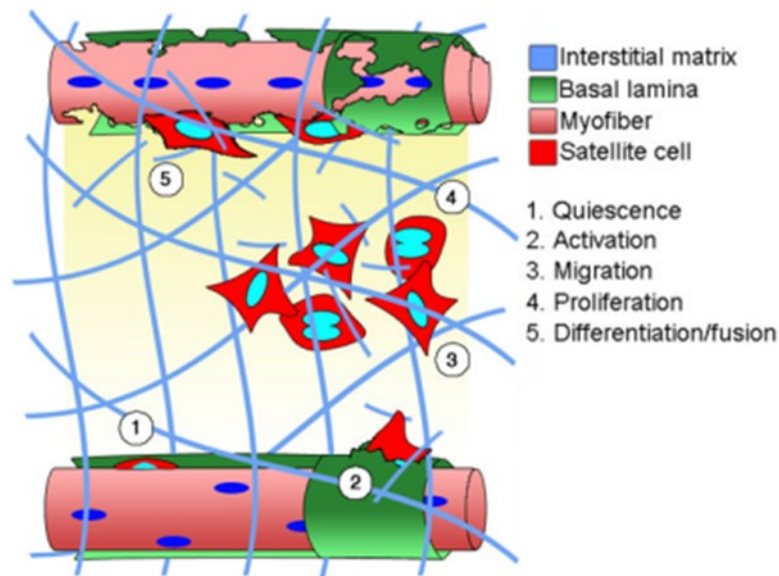
## Chapter 2: ECM, Fibroblast and SC Cross-talk

Muscle regeneration requires the coordinated interaction of multiple cell types. Satellite cells are regulated by their surrounding niche, which includes multiple cell types. In addition to inflammatory and satellite cells, efficient muscle repair also requires the migration and proliferation of fibroblasts, in order to **produce new temporary ECM components**

(Mann et al., 2011) to assure the normal tissue architecture. Skeletal muscle fibers make up about 95% of a muscle's cross-sectional area and the ECM, only about 1–9%. Fibroblasts, a cell population in abundance in regenerating muscle, produce a large array of ECM proteins, such as collagen, fibronectin, matrix metalloproteinases and proteoglycans (Chapman et al., 2016) in order to reconstitute the ECM. These elements serve to stabilize the tissue, and to ensure the myofiber maintains a similar position as they act as a **scaffold to guide** the regenerative new fibers (Vracko and Benditt, 1972). Moreover, the satellite cells also utilize the basement membranes of pre-existing necrotic fibers (**ghost fibers**) to orient their migration and division (Mourikis and Relaix, 2016; Webster et al., 2016). Basement membranes and temporary ECM components are also crucial for guiding the formation of neuromuscular junctions (Mann et al., 2011). Finally, in addition to ECM remodeling, **angiogenesis** facilitates the development of a new vascular network at the site of injury, while newly formed muscle fibers undergo growth and maturation (Mann et al., 2011).

In 2011, Murphy et al. identified for the first time that muscle connective tissue fibroblasts are a major cellular component of regenerating muscle. In this study, mouse lines were developed in which they were able to inducibly ablate satellite cells by targeting Pax7+ cells or all connective tissue fibroblasts by inducibly ablating Tcf4+ cells. When satellite cells were ablated, skeletal muscle regeneration was inhibited. However, when connective tissue fibroblasts were ablated, satellite cell dynamics were also altered, resulting in premature differentiation of satellite cells and poorly regenerated muscle fibers with decreased diameters. Therefore, **reciprocal interactions** between fibroblasts and satellite cells prevent premature differentiation of satellite cells allowing satellite cell expansion and thus proper muscle regeneration (Murphy et al., 2011).

ECM signaling is very critical for SC in regulating cellular determination, differentiation, proliferation, survival, polarity and migration (Figure 15). Numerous studies have identified roles for the ECM proteoglycans in modulating the **bioavailability** and signaling potential of key **growth factors** including FGF-2, HGF and TGF- $\beta$ . The activity of the matrix metalloproteases is crucial in **releasing** these growth factors from their associate proteoglycans allowing for their interaction in cell signaling and mechanotransduction (Lund and Cornelison, 2013). The SC-ECM interactions are reciprocal, as it has been shown in a recent study that SCs are able to **autoregulate their niche** by secreting collagen VI. Collagen VI, a muscle ECM component, contributes to establish a three-dimensional support with specific mechanical properties important for satellite cell **self-renewal** (Urciuolo et al., 2013). Also fibronectin (ECM glycoprotein) bound to Syndecan-4 and Frizzled-7, a co-receptor complex in satellite cells, stimulates the ability of Wnt7a to induce the symmetric expansion of SCs through the planar-cell-polarity pathway (Bentzinger et al., 2013).



**Figure 15** : Schematic representation of the local matrix interactions during multiple phases of satellite cell activity in vivo. Adapted from (Lund and Cornelison, 2013)

### Chapter 3: SC Migration

The migration of SCs from nearby viable muscle towards the injured site is an important aspect of the regeneration response as it provides an important means of augmenting the population of myogenic cells and permits the myogenic population to contribute en masse to the centripetal wave of regeneration from the time it is initiated at the muscle periphery. A variety of muscle trauma conditions stimulate migration including ischemia (Phillips et al., 1987; Schultz et al., 1988), thermal injury (Morgan et al., 1987; Phillips et al., 1990), crushing (Schultz et al., 1985; Watt et al., 1994) and snake venom toxin (Klein-Ogus and Harris, 1983; Maltin et al., 1983).

Focal crush injury at one end of a muscle results in the activation and movement of satellite cells **beneath the basal lamina** from distant uninjured tissue toward the crush site (Schultz et al., 1985). If the injury stimulus is perpendicular to the direction of the myofibers, some cells are **able to cross** the basal lamina of uninjured myofibers and migrate transversely through the muscle toward the injury (Klein-Ogus and Harris, 1983; Maltin et al., 1983; Phillips et al., 1990). However a study using intravital imaging showed that stem cells do not migrate from uninjured muscle fibers to regenerate injured tissue and the muscle progenitors migrate bi-directionally in ghost fibers **along** the long axis (Webster et al., 2016). Thus, the new fibers would be forced to form in the alignment with the axis of damaged fiber.

The **chemotaxis** of SCs is driven by the chemical signals from the damaged fiber, the ECM and the **inflammatory** cells recruited in the local area of injury. The extracellular matrix

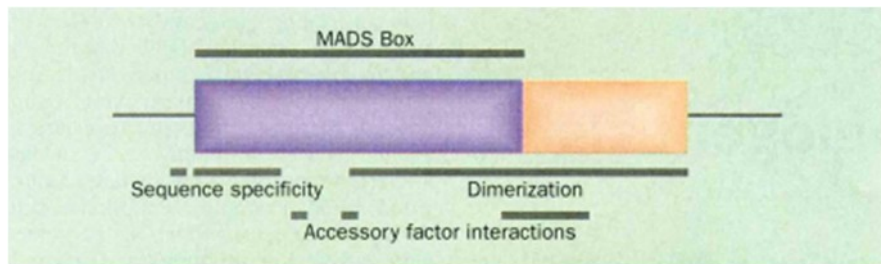
forms the substratum for migration. The primary *in vivo* substrate for SC migration is the exterior lamina of the host myofiber, engaged by the  $\alpha 7\beta 1$  laminin receptor on SCs. SCs express multiple **guidance ligand** and **receptors** to drive their migration. In activated SCs members of all four major classes of guidance receptors (including semaphorins and plexins, Ephs and ephrins, netrins and Dcc/Unc5 family members, Robos and Slits) were detected (Siegel et al., 2009). In this study with 3D timelapse analysis of SC motility, they proposed a model in which soluble **motogens** such as **HGF**, released by damaged areas of the myofiber, promote SC motility, while **repulsive interactions** with comparatively intact areas of the myofiber maintain cell motility in the absence of injury (Siegel et al., 2009). Activation of **Wnt7a/Fzd7 signaling** markedly stimulates the motility of SCs and myogenic progenitors by inducing polarization and enhancing **directionality** of migration (Bentzinger et al., 2014).

## 2. Serum Response Factor

The 67-kD serum response factor (Srf) is an **ubiquitous** transcription factor (TF), widely expressed from flies to humans (Posern and Treisman, 2006). The serum response factor was first identified as the nuclear protein binding to a short DNA sequence element, the **serum response element** (SRE), mediating the transient transcriptional activation of c-fos and cytoskeletal actin genes after serum induction (Treisman, 1987). Srf belongs to the MADS (MCM1-Agamous-Deficiens-SRF) box transcriptional factors family. The **MADS-box transcription factors** constitute a family of proteins that includes the metazoan transcription factors SRF and MEF2<sup>8</sup>, the yeast transcription factors MCM1 and ARG80, and a large number of plant homeotic gene products (Shore and Sharrocks, 1995). The MADS-box motif (Figure 16) has been highly conserved through evolution and comprises the DNA-binding domain and a part of the dimerization domain in Srf-like transcription factors. The N-terminal half of the MADS box determines DNA specificity among the different family members. The carboxy-terminal half of the MADS box forms part of the dimerization surface, however efficient dimerization requires an additional 30 residues C-terminal MADS box, which is also necessary for recruitment of accessory proteins (Treisman, 1995).

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<sup>8</sup> four members of the MEF2 family : MEF2A, MEFB, MEFC, and MEFD



**Figure 16** : Simplified schematic representation of SRF and MCM1 DNA-binding domains. The DNA-binding domain is shown as a box, with the MADS-box in purple. Regions of the domain shown biochemically to determine DNA-binding specificity, to mediate dimerization and accessory factor interactions are indicated. Adapted from (Treisman, 1995)

Human SRF is a 508 amino-acid (aa)-long protein (Norman et al., 1988) and murine Srf contains 504 aa (Belaguli et al., 1997). Srf consists of an N-terminal regulatory domain (aa 1-142), the MADS box (aa 142-171), and a carboxyl-terminal transactivation domain (aa 266-508). The **N-terminal regulatory domain** is **phosphorylated** by casein kinase II and ribosomal S6 kinase (Johansen and Prywes, 1995) and contains the nuclear localization signal (NLS) (Rech et al., 1994).

The Srf gene is localized in the chromosome 17 in mouse and in 6 in human. Srf mRNA transcript has 7 exons. Murine Srf mRNA levels were the highest in adult skeletal and cardiac muscle, but barely detected in liver, lung, and spleen tissues (Belaguli et al., 1997).

#### SRF target genes

*In vivo* and *in silico* experiments suggest the presence of over 200 Srf-regulated target genes. Known Srf target genes are characterized by the presence of single or multiple copies of the Srf binding consensus element  $CC(A/T)_6CG$ , the **CArg box**, in their promoter elements (Sun et al., 2006).

Srf controls transcription of many cellular CArG-dependent “**immediate-early**” genes (IEGs) (Posern and Treisman, 2006), whose mRNA may appear in cells within minutes after stimulation, without the need for *de novo* protein synthesis (Bahrami and Drabløs, 2016). The CArG box has been identified in a number of post-replicative **muscle-specific genes** (Table 1), including cardiac and skeletal muscle actins (Minty and Kedes, 1986), dystrophin (Klamut et al., 1990), and myosin light chain 1/3 (Ernst et al., 1991). Srf activity can be differentially controlled by **signal-regulated** or **tissue-specific regulatory cofactors** (Posern and Treisman, 2006).

**Table 1** : Cytoskeleton-contractile SRF target genes. From (Miano et al., 2007)

Cytoskeleton					
Cytoplasmic	Membrane	Contractile	Physiological Association	Hypothetical	
ACTB <sup>a</sup>	AKAP12A	ACTA1 <sup>c</sup>	ARHE	ANKRD1	MYO1
ACTG1 <sup>a</sup>	AOC3	ACTA2 <sup>d</sup>	ATP2A2 <sup>#</sup>	ANLN	MSN
ACTR3 <sup>b</sup>	DMD	ACTC <sup>f</sup>	BARX2	ANXA2	NEBL
CAPZA3 <sup>b</sup>	DTNA	ACTG2 <sup>d</sup>	BIN1	ANXA3	NES
CFL1 <sup>a</sup>	ENAH <sup>c</sup>	ACTN1 <sup>d</sup>	CA3	ARPC4 <sup>b</sup>	PFN2 <sup>a</sup>
CFL2 <sup>a</sup>	ITGA1 <sup>a</sup>	CALD1 <sup>d</sup>	CALB1	BVES	RHOQ
CNN1 <sup>a</sup>	ITGA5 <sup>c</sup>	CRYAB	CASQ1 <sup>#</sup>	CDV1	RHOQ
CORO1A <sup>b</sup>	ITGA9 <sup>c</sup>	MYH11 <sup>d</sup>	CKM	CMYA1	TCAP
CSR1	ITGB1 <sup>c</sup>	MYH4 <sup>c</sup>	CPT1B	CNN2	TMOD3
CSR2	ITGB1BP2 <sup>c</sup>	MYH6 <sup>f</sup>	DMPK <sup>#</sup>	DSP	TNS1
DES <sup>a</sup>	SDC2 <sup>c</sup>	MYH7 <sup>f</sup>	FHL1	EPLIN	TPM4 <sup>a</sup>
DSTN <sup>a</sup>	SDC4 <sup>c</sup>	MYL3	FHL2	FLNB <sup>b</sup>	VASP <sup>c</sup>
FLNA <sup>b</sup>	SPTB <sup>c</sup>	MYL4	MYOD1 <sup>c</sup>	FNBP1 <sup>a</sup>	ZYX <sup>c</sup>
FLNC <sup>b</sup>	TGFB111	MYLKv6 <sup>d</sup>	MYOG <sup>c</sup>	FNBP3 <sup>a</sup>	
GSN <sup>a</sup>	TJP1	MYLKv7 <sup>d</sup>	NKX2-5 <sup>f</sup>	JPH2	
HSPB7	TLN <sup>c</sup>	SMTNA <sup>d</sup>	NPPA	KRT14	
KRT17	VCL <sup>c</sup>	TNNC1 <sup>e,f</sup>	OPHN1	KRT18	
PDLIM5	VIL1	TNNT2 <sup>f</sup>	PLN <sup>7</sup>	KRT19	
PFN1 <sup>a</sup>		TPM2 <sup>d</sup>	SLC2A1	KRT7	
SNX2			SLC8A1 <sup>#</sup>	KRT8	
TAGLN1 <sup>d</sup>			SRF	LDB3	
TPM1 <sup>a</sup>				LPP	
TRIP6				LRRC10	
TTN				MYL9	
TUFT1				MYO1B	

## Part 1: Srf Cofactors-Signalling

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Like many transcription factors, Srf exhibits combinatorial interactions with other TFs. There are at least two pathways (Figure 17), involving different cofactors, for serum induction that converge on the SRE. In the fibroblast model system, Srf target genes can be distinguished on the basis of their relative sensitivity to MEK-ERK and RhoA-actin signaling pathways (Gineitis, 2001).

### MAPK Signalling/Ternary Complex Factors

Srf forms a ternary complex at the c-fos SRE with members of a family of Ets domain accessory proteins, the ternary complex factors (**TCFs**), which bind to a conserved **Ets motif** adjoining the SRF-binding site (Treisman, 1994). These proteins, which include SAP-1, Elk-1 and ERP-1/NET/SAP-2., contain two conserved N-terminal regions required for DNA binding and ternary complex formation with Srf and a conserved C-terminal activation domain containing potential **MAP kinase consensus sites** (Price et al., 1995). For full SRE function, the ternary complex is essential (Shaw et al., 1989). Genomic footprinting studies show that the ternary complex is present at the SRE even in unstimulated cells, suggesting that SRE activation involves regulation of transcriptional activation rather than DNA binding (Herrera et al., 1989). Functional studies demonstrated that recruitment of Srf cofactors is gene-specific (Esnault et al., 2014).

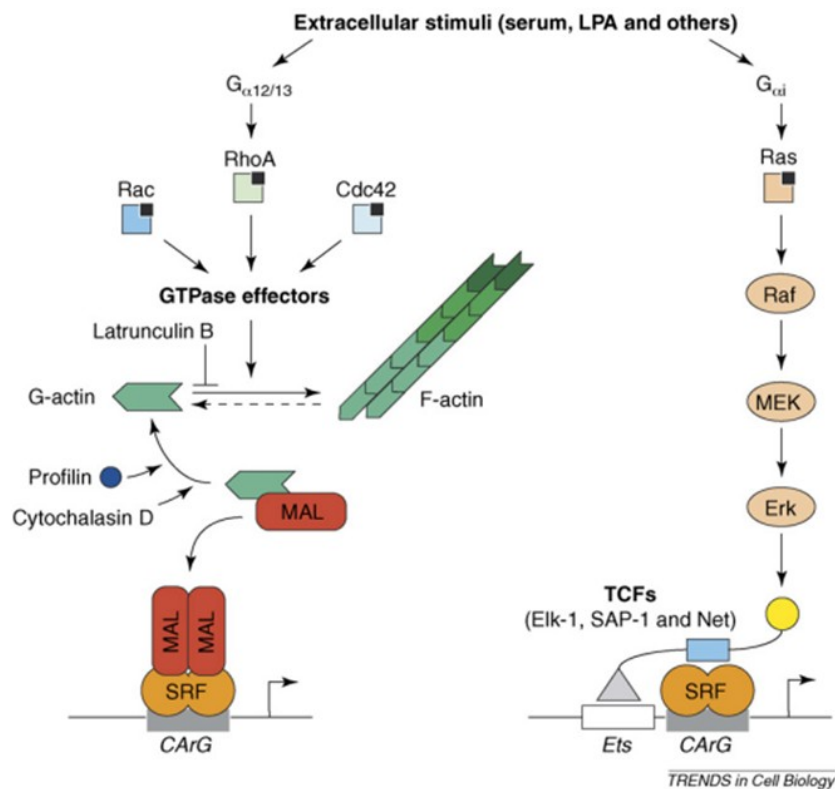
At least one route by which signals arrive at the SRE is via the MAP kinase pathway and is dependent on the Srf/TCF ternary complex (Figure 17). Activation of the MAP kinase pathway, through Ras, Raf, MEK and ERK, phosphorylates TCFs (Posern and Treisman, 2006), which bind to their own Ets motif site and Srf in a 'grappling hook' model (Treisman et al., 1992).

### RhoA-Actin Signalling

Srf activity is also regulated by the Rho family of small GTPases (Hill et al., 1995) through the recruitment of myocardin related transcription factors (**Mrtfs**) at Srf target genes (Olson and Nordheim, 2010). **Rho GTPases** regulate the Srf and its cofactors Mrtfs via their ability to **induce actin polymerization** (filamentous actin). Alterations in actin dynamics (G-actin/F-actin) are required for RhoA-mediated Srf activation, which is inhibited upon treatment of cells with the G-actin binding drugs demonstrating that G-actin level controls Srf activity (Sotiropoulos et al., 1999). The cytoplasmic concentration of G-actin is reflected by

the concentration of Mrtf retained in the cytoplasm. Thus, Mrtfs act as “**actin sensors**” in the cytoplasm, through their ability to bind actin via their **RPEL domains** (Guettler et al., 2008; Miralles et al., 2003; Vartiainen et al., 2007). Following serum stimulation, the release and nuclear translocation of cytoplasmic Mrtfs induce Srf-directed target gene activation (Figure 17).

The RhoA-actin pathway controls a subset of Srf target genes, including the immediate-early genes, such as  $\beta$ -actin, vinculin, and srf, and the cardiac and smooth muscle  $\alpha$ -actin genes (Posern and Treisman, 2006). Mrtf targets (>2600 MRTF-binding sites) encode regulators of the cytoskeleton, transcription, and cell growth (Esnault et al., 2014). Constitutively active forms of both RhoA and other Rho family GTPases such as Cdc42 and Rac1 can activate Srf in the absence of extracellular signals (Hill et al., 1995).



**Figure 17** : Model of two principal pathways regulating SRF activity in non-muscle cells. Stimulation activates both Rho-dependent (left) and Ras-dependent (right) signalling. Activation of the MAP kinase pathway through Ras, Raf, MEK and ERK phosphorylates TCFs, which bind to their own Ets DNA recognition site and SRF in the ‘grappling hook’ model. Signalling through Rho family GTPases (squares, with small black squares indicating GTP) and the actin treadmilling cycle (left) results in the dissociation of MAL from actin, which then binds and activates SRF. From (Posern and Treisman, 2006)



## micro RNA Regulation

Several studies show a major role of Srf in regulating and being regulated by various miRNAs. SRF regulates certain microRNAs that play a role in cardiac and skeletal muscle development. **Cardiac-specific overexpression** of Srf (Srf-Tg) led to altered expression of a number of microRNAs. Interestingly, downregulation of miR-1, miR-133a and upregulation of miR-21 occurred by 7 days of age in these mice, long before the onset of cardiac hypertrophy, suggesting that SRF overexpression impacted the expression of microRNAs which contribute to **cardiac hypertrophy** (Zhang et al., 2011). A high level overexpression of Srf leads to the drastic development of **fibrosis** associated with an increased expression especially of connective tissue growth factor (CTGF) and a strong downregulation of miR-133a, revealing a key role of the **SRF/CTGF/miR-133a axis** in the regulation of cardiac fibrosis (Angelini et al., 2015).

## Part 2: Actin: a Known Srf-Target Gene

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Actins form a major component of the cytoskeleton of eukaryotic cells. In vertebrates the two major cytoskeletal actins,  **$\beta$  and  $\gamma$** , are encoded by a pair of evolutionarily related genes which are expressed **in all cell types** (Rubenstein, 1990). Mammalian cytoskeletal actin genes are members of a set of genes whose transcription is **rapidly** and **transiently** activated following stimulation of susceptible cells with growth factors and mitogens (Elder et al., 1984). The promoters of these genes contain a region of striking **homology** to the human c-fos SRE (Mohun et al., 1987).  $\beta$  and  $\gamma$  cytoplasmic actin genes (ACTB and ACTG1) expression are Srf-dependent (Miano et al., 2007).

Actin is also a component of muscle **sarcomere**. A pair of actin genes is expressed in the striated muscles (skeletal muscle and heart) of vertebrates. One of these genes ( **$\alpha$ -skeletal**) encodes the major actin species in adult skeletal muscle actin and the other gene ( **$\alpha$ -cardiac**) encodes the major actin species in the adult heart (Gunning et al., 1983a). The cardiac actin gene has also been shown to be expressed at high levels in embryonic and fetal skeletal muscle. In adult skeletal muscle, however, there is only a low level of cardiac actin mRNA, showing that the **cardiac** actin gene is subject to **developmental** regulation (Paterson and Eldridge, 1984). The different actin genes are unlinked in mammalian genomes (Gunning et al., 1984) and are also unlinked to other contractile protein genes (Czosnek et al., 1982). The presence of the CARG-box in the upstream regions of  $\alpha$ -actins (Minty and Kedes, 1986) suggests a Srf-binding site and Srf-dependent regulation (Miano et al., 2007).

### G-actin/F-actin

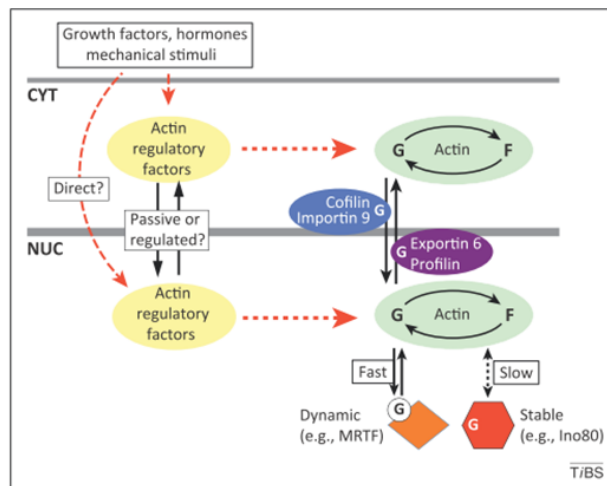
Actin is an **ATPase** that cycle between monomeric (G-actin) and polymerized (F-actin) states. The four subdomains of actin form two lobes, separated by a deep cleft that binds nucleotide and a bivalent cation, and the molecule adopts differing conformations according to whether ATP or ADP is bound. Nucleotide binding is not required for polymerization by itself but stabilizes the molecule (Kabsch et al., 1990). F-actin is a polar structure with pointed (-) and barbed (+) ends. In **actin treadmilling cycle**, ATP-loaded G-actin monomers are assembled at the barbed end of the F-actin microfilaments, disassembled as ADP-monomers at the pointed end, and subsequently recycled (Posern and Treisman, 2006). Polymerization of monomeric globular actin (G-actin) into a filamentous actin (F-actin) is influenced by local intracellular concentrations of ATP-bound G-actin and by

the activity of many **actin-binding proteins** (ABPs). G-actin forms complexes with different ABPs, including the nucleating factors profilin, formins and the actin-related protein 2/3 (ARP2/3) complex (Olson and Nordheim, 2010).

### Nuclear Actin

Actin has been known as a major component of the cytoskeleton and as a key player in many cellular processes including cell migration, division and shaping. Actin is also present in the cell nucleus (Figure 18), where it has been linked to many processes that **control gene expression**. Nuclear actin and numerous actin-binding proteins have been identified as an important component of **transcriptional machineries** and **chromatin remodeling complexes** (Treisman, 2013). The activity of the Mrtf transcriptional coactivators is controlled via their dynamic interaction with the cytoplasmic and nuclear monomeric G-actin pools. Actin is actively **shuttled** between cytoplasm and nucleus. Nuclear actin levels are maintained by active nuclear import and export of actin. Cytoplasmic actin is imported to nuclei by Importin 9, while nuclear actin is exported to the cytoplasm by Exportin 6 (Miyamoto and Gurdon, 2013). This controls the nuclear exit of other G-ABPs, such as MRTFs, which are exported bound to G-actin.

In addition, recent studies indicate that nuclear actin and actin-binding proteins play vital roles in transcriptional activation during cell differentiation and reprogramming (Miyamoto and Gurdon, 2013).



**Figure 18** : Cytoplasmic and nuclear actin dynamics. Potential relationships between the cytoplasmic and nuclear actin pools, actin regulatory factors, and the functions of nuclear actin are shown. The cytoplasmic and nuclear actin pools (indicated by green ovals) are in dynamic communication. Those functions of nuclear actin that rely on transient interactions with target proteins are expected to be influenced by short-term alterations in actin dynamics, whereas those functions in which actin functions as part of stable complexes with nuclear proteins are refractory to them. Whether signals can be transmitted directly to the nuclear actin regulatory machinery, or if they are transmitted to cytoplasmic machinery which then

translocates to the nucleus, remains to be determined. Abbreviations: CYT, cytoplasm; F, polymerized F-actin; G, monomeric G-actin; MRTF, myocardin-related transcription factor; NUC, nucleus. From (Treisman, 2013)

## Chapter 1: Linking Actin Dynamics and Srf Transcriptional Activity

Changes in actin dynamics mediate signal-induced Srf transcriptional activity. Stabilization of F-actin by the actin-binding drug jasplakinolide is sufficient to activate Srf in the absence of extracellular stimuli, whereas **overexpression** of actin **inhibits** Srf (Sotiropoulos et al., 1999). Actins that cannot polymerize with the use of actin-binding drugs that do not promote F-actin formation, are effective inhibitors of signaling to Srf (Olson and Nordheim, 2010; Posern et al., 2002; Sotiropoulos et al., 1999). Actin mutants that enhance F-actin formation can activate Srf-dependent transcription when overexpressed (Posern et al., 2002). These results present direct evidence for participation of monomeric actin in the signaling pathway to Srf. Moreover, Srf can be activated by overexpression of the actin-binding protein profilin (an actin-binding protein involved in the polymerization process), and this is blocked by profilin mutations that prevent actin binding (Geneste et al., 2002; Sotiropoulos et al., 1999). Constitutively active forms of regulators of actin polymerization, such as LIMK, mDia, the Wiskott Aldrich Syndrome protein (WASP) family, and vasodilator-stimulated phosphoprotein (VASP), can potentiate Srf activity in the absence of extracellular (Sotiropoulos et al., 1999; Tominaga et al., 2000). Depletion of G-actin is both necessary and sufficient for activation both of Srf reporters and a subset of Srf target genes. The link between the actin **cyto-architecture** and **gene activity** assures many dynamic cell functions such as cell migration, guided movement, engulfment, adhesion and contraction (Olson and Nordheim, 2010).

### Signal Regulation of Actin Dynamics

The Rho family of Ras-related GTPases, RhoA, controls diverse cellular processes concerned with the cytoskeleton, actin polymerization, myosin-based contractility, focal adhesion formation, transformation and cytokinesis (Olson and Nordheim, 2010). RhoA controls the formation of actin assembly by interacting with a set of **effector proteins** that includes the **Rho kinases** (Geneste et al., 2002). Activation of Rho GTPases promotes actin polymerization by two downstream signalling modules, one involving the Rho-associated kinase (ROCK)–LIM kinase–cofilin pathway (cofilin is an ABP that can stimulate actin depolymerization and thereby enhance actin polymerization elsewhere), and the other mediated by the Diaphanous group of formin proteins (mDia proteins). RhoA controls the

assembly of the actin cytoskeleton in response to **extracellular signals** (Figure 19). Activation of Rho leads to the accumulation of filamentous actin (F-actin) through both filament stabilization and *de novo* polymerization with concomitant depletion of cellular levels of monomeric actin (G-actin) (Olson and Nordheim, 2010).

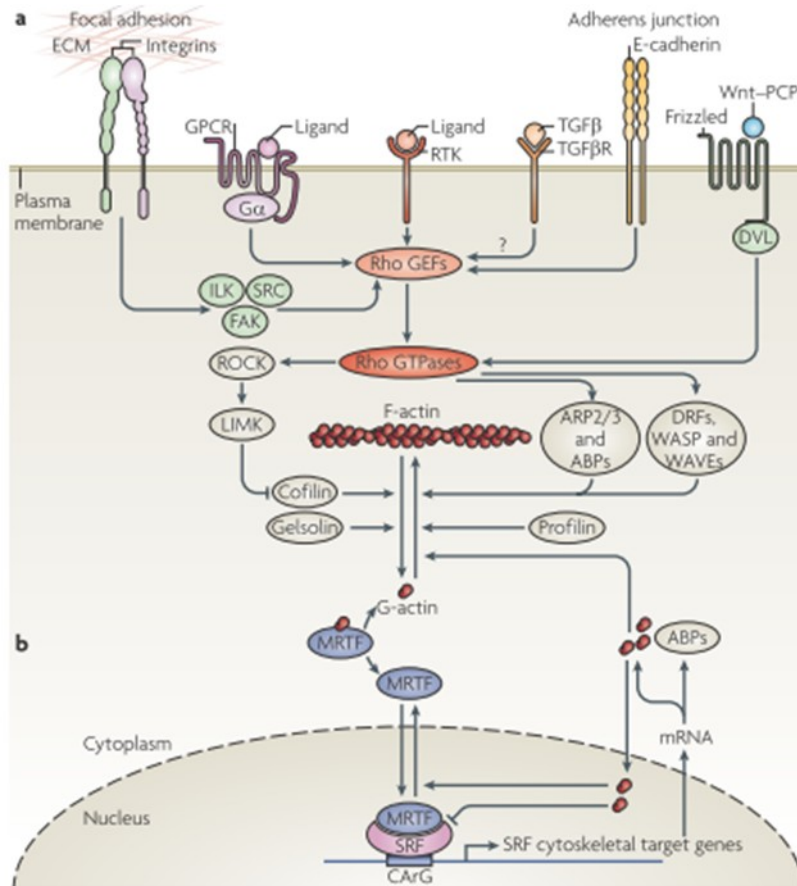


Figure 19 : Receptors affecting actin dynamics and MRTF-mediated regulation of SRF target genes. a | Cytoskeletal actin microfilament dynamics are affected by the activation of six classes of plasma membrane receptor. b | Activation of SRF class II target genes. Nuclear MRTF can be complexed by nuclear G-actin, which inhibits MRTF-mediated stimulation of SRF-dependent transcription and facilitates MRTF nuclear export. SRF class II target genes that are transcribed as a result of MRTF–SRF activation include actin itself and many genes that modulate actin dynamics. These newly made proteins, with increasing time and concentration, might stimulate cytoplasmic actin polymerization, complex cytoplasmic MRTF or elevate levels of nuclear G-actin to downregulate MRTF-mediated transcription and stimulate nuclear export of MRTF. From (Olson and Nordheim, 2010)

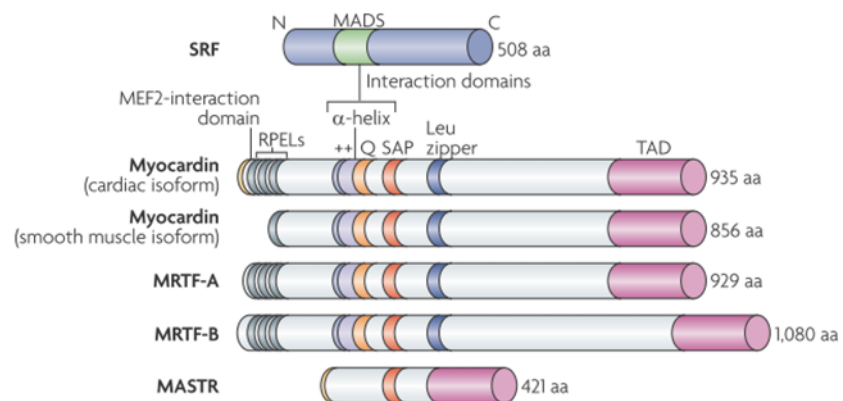
## Chapter 2: MRTFs

The link between Rho-actin signalling and Srf transcriptional activity is the MRTFs. The activity of the MRTF transcriptional coactivators is controlled via their dynamic **association** with the cytoplasmic and nuclear monomeric G-actin pools (Treisman, 2013),

which are **redistributed** from the cytoplasm to the nucleus in response to Rho-induced actin polymerization.

Members of the MRTF family (Myocardin, Mrtf-A, Mrtf-B, and MASTR) play important roles in differentiation and remodeling of cardiac, smooth, and skeletal muscle cells. **Myocardin** (a heart-specific Srf coactivator), the founding member of the Mrtf family (Wang et al., 2001, 2003), is expressed specifically in the **cardiovascular** system, whereas other Mrtf family members (Figure 20) display more widespread expression patterns (Olson and Nordheim, 2010). Myocardin shares homology with Mrtf-A (also known as MAL, MKL1 and BSAC) and Mrtf-B (also known as MKL2 and MAL16) in a series of conserved domains (Pipes et al., 2006). MASTR, a SAP domain TF is a muscle-enriched MEF2 coactivator (Creemers et al., 2006).

Myocardin-dependent cardiac and smooth gene expression is regulated by GATA transcription factors (Oh et al., 2004), BMP and TGF- $\beta$ /Smad signalling (Callis et al., 2005), the Rho/Mrtf/Srf axis, JAK-STAT3 signalling (Liao et al., 2015) and Calcineurin/transient receptor potential channel (TRP)/nuclear factor of activated T-Cell (NFAT) signaling (Li et al., 2016; Lighthouse and Small, 2016).



**Figure 20** : Structure of myocardin family members. Functional domains of homology among the myocardin family proteins are shown and the numbers of amino acids are indicated. Myocardin-related transcription factors (MRTFs) are potent transcriptional coactivators that associate with serum response factor (SRF) through a basic region (++) and an adjacent Glu-rich domain (Q). Between these domains is a short  $\alpha$ -helical region with similar secondary structure to a domain in the ternary complex factor protein ELK1, known as a B box, which mediates their interaction with SRF. Myocardin family proteins contain Arg-Pro-X-X-X-Glu-Leu (RPEL) motifs, which mediate their interaction with globular actin (G-actin). A dimerization motif resembling a Leu zipper mediates homo- and hetero- dimerization of myocardin and MRTFs. Alternative usage of 5' exons in the myocardin gene gives rise to proteins with different amino termini. A cardiac-specific splice variant of myocardin contains a unique amino-terminal sequence that confers the ability to interact with the myocyte-specific enhancer factor 2 (MEF2) transcription factor, a MADS-box transcription factor related to SRF. This MEF2-interaction domain is also contained in a divergent member of the

myocardin family called MEF2-activating SAP transcriptional regulator (MASTR). MASTR lacks the SRF-interaction domain. Adapted from (Olson and Nordheim, 2010)

### Actin-Mediated Regulation of MRTF Nuclear Shuttling

At low actin polymerization states, Mrtfs are held in an inactive state in the cytoplasm by reversible complex formation with G-actin (Posern et al., 2004). Thus, Mrtfs are *bona fide* G-ABPs. Stimulation of Rho GTPases feeds G-actin into the F-actin filament, thereby liberating Mrtfs from G-actin and allowing the nuclear import of Mrtf and subsequent activation of Srf-dependent transcription. Nuclear G-actin also modulates Mrtf functions in multiple ways. First, nuclear export of Mrtfs is facilitated by nuclear G-actin-binding is inhibited upon signal-induced depletion of the G-actin pool. Second, nuclear G-actin prevents nuclear Mrtf from activating Srf target genes, so that liberation of the nuclear actin–Mrtf complex is required to stimulate Srf. Thus, cellular G-actin regulates Mrtfs at three levels: **nuclear import**, **nuclear export** and **nuclear activation or inactivation** of Mrtf–Srf-dependent transcription (Vartiainen et al., 2007). Thereby, the actin–Mrtf–Srf circuit allows for the precise modulation of gene expression in concert with cytoskeletal assembly and disassembly (Olson and Nordheim, 2010).

A recently described pathway that regulate nuclear actin show that MICAL-2, an **atypical actin-regulatory nuclear protein** which promotes the **depolymerization** of nuclear actin upon **redox stimuli**, also facilitates the depletion of nuclear G-actin, which enables Mrtf to accumulate in the nucleus and to stimulate Mrtf–Srf-dependent gene expression (Lundquist et al., 2014).

## Part 3: Srf Activity in Muscle Tissue

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### The Muscle CArG-box

The SRE found in the promoter region of the human c-fos gene, shares sequence similarity with the CArG box, a ten nucleotide motif (CC[AT]<sub>6</sub>GG) which is **conserved** amongst vertebrate striated **muscle actin** genes (Minty and Kedes, 1986) and they are **functionally interchangeable** (Taylor et al., 1989) showing that a common mechanism is utilized in both muscle-specific gene expression and serum-responsive gene transcription probably by **binding the same TF**. The CArG box binding factor is the Srf that binds to the cardiac and skeletal  $\alpha$ -actin promoters (Boxer et al., 1989).

Several different promoter elements have been identified as important for muscle-specific gene transcription. Some bind complexes containing muscle-specific transcription factors: for example, the E-box (CANNTG) binds members of the myogenic family of bHLH proteins (Arnold and Braun, 1996). In contrast, other sequences in muscle-specific promoters bind ubiquitous factors whose activity can at least in some cases be modified according to the promoter context. An example is the CArG box, which binds the ubiquitous transcription factor Srf. In muscle-specific promoters, the CArG box acts as a **strong constitutive promoter element** (Sartorelli et al., 1990; Wei et al., 1998). In contrast, in immediate-early gene promoters, such as that of c-fos, the CArG box functions as a growth factor-inducible promoter element and has only low basal activity (Taylor et al., 1989; Treisman, 1990). Thus, the involvement of a transcription factor in muscle-specific transcription need not imply that the factor is expressed exclusively in muscle cells. It is however possible that the Srf interacts with the MRFs for muscle-specific gene expression (Sartorelli et al., 1990), as it was shown by **physical interaction** between Srf and the MRFs MyoD and MyoG-E12 heterodimers (Groisman et al., 1996).

### Chapter 1: Srf-Expression Pattern in Mouse Development

Staining of sectioned embryos with Srf-specific antiserum revealed expression at E6.5 in ectoderm as well as endoderm. At **E7.5**, Srf protein could be seen in **all three germ layers** of wild type embryos. Interestingly, at **E8.5**, this ubiquitous distribution became a regionally localized one and Srf protein expression was found to be high in the developing **heart** (especially in the myocardium), but barely detectable in other tissues. At **E10.5**, distinct Srf protein expression was detected in the developing **myotome** (Arsenian et al., 1998). *In*



*situ* hybridization analysis revealed enrichment of Srf transcripts in the **smooth muscle of vessels** in mouse embryos (Belaguli et al., 1997). These Srf expression patterns in mouse embryos are **congruent** with those found in chicken embryos (Croissant et al., 1996).

Srf germline-mutants mice have an **embryonic lethality at E12.5** because of severe gastrulation defect with scarcity of **mesoderm formation**. The availability of the Cre/loxP technology allowed the generation of “floxed Srf” mice to investigate the inactivation of the Srf gene in an organ and time specific manner (Wiebel et al., 2002).

## Chapter 2: Srf-loss in Cardiac Muscle

A conditional mutant of Srf by using a  $\beta$ -MHC-Cre transgenic mouse line was used to study the role of Srf in embryonic and postnatal cardiac functions. Heart-specific deletion of Srf in the embryo results in lethal cardiac defects between E10.5 and E13.5, as evidenced by abnormally thin myocardium, dilated cardiac chambers, poor trabeculation and a disorganized interventricular septum. At E9.5, a marked reduction in the expression of essential regulators of heart development, including Nkx2.5, GATA4, myocardin, and the Srf target gene c-fos was found prior to overt maldevelopment. Thus, Srf is crucial for **cardiac differentiation and maturation**, acting as a global regulator of multiple developmental genes (Parlakian et al., 2004).

In human heart samples, expression of **four different isoforms** of SRF was found, with 67-kDa full-length SRF being the predominant isoform. Interestingly, in failing hearts, it was found robust expression of a low-molecular-mass (~52 kDa) SRF isoform, which is encoded by an alternatively spliced form of SRF lacking exons 4 and 5. Overexpression of this isoform into cells inhibits SRF-dependent activation of cardiac muscle genes (Davis et al., 2002).

Mice model of tamoxifen inducible cardiac specific Srf knockout led to heart failure with **dilated cardiomyopathy**, characterized by a **reduced contractility**, a left ventricular dilation accompanied by a **downregulation of energetic proteins** and changes in cytoarchitecture (Parlakian et al., 2005). This work provided therefore a mouse model of morphological and clinical features of acquired dilated cardiomyopathy in humans.

## Chapter 3: Srf-loss in Smooth Muscle

Inactivation of Srf in smooth muscle cells (SMCs) through SM22  $\alpha$ -actin-Cre mediated excision during embryonic development, leads to a decrease in the number of peri-vascular progenitor cells as well as SMC lacking organizing actin/intermediate filament bundles at E10.5, suggesting a crucial role for Srf in **vascular SMC differentiation** (Miano et al., 2004).

In 2008, Franco et al. found that Srf expression is also restricted to **vascular endothelial cells** (ECs) of small vessels such as capillaries in the mouse embryo. EC-specific Srf deletion led to aneurysms, hemorrhages and reduced capillary density from E11.5 and lethality at E14.5 (Franco et al., 2008). This work revealed a crucial role of Srf downstream of vascular endothelial growth factor (VEGF) and FGF signalling during sprouting **angiogenesis**, regulating **EC migration**, actin polymerization, tip cell morphology, EC junction assembly and vascular integrity (Franco and Li, 2009).

### Gastrointestinal Tract

Tamoxifen inducible Sm22-Cre mice lead to down-regulation of many smooth muscle-specific genes in urinary bladder and gastrointestinal tract (GI) (Coletti et al., 2016). In the GI tract, Srf is the central regulator of genes involved in **apoptosis**, **dedifferentiation**, proliferation, and migration of cells. Abnormal expression of Srf is common in several GI diseases. Normal expression of the protein is essential for GI SMC differentiation. Loss or reduction of Srf may trigger **myopathy**, hypertrophy of SMCs or GI **cancers**, while overexpression of the protein may be linked to ulcers (Ro, 2016a).

## Chapter 3: Srf-loss in Skeletal Muscle

*In vitro* studies attempting to explore the impact of Srf-loss in myogenic cell lines showed that: 1) Srf is involved in the process of **skeletal muscle differentiation** as inhibition of Srf through microinjection of purified Srf antibodies, prevents the expression of two myogenic differentiation markers, myogenin and troponin T (Soulez et al., 1996a; Vandromme et al., 1992). 2) Srf activity is **required** for the expression of the muscle-determining factor **MyoD** in proliferating and cultured under differentiating conditions myoblast (Gauthier-Rouviere et al., 1996; Soulez et al., 1996b).

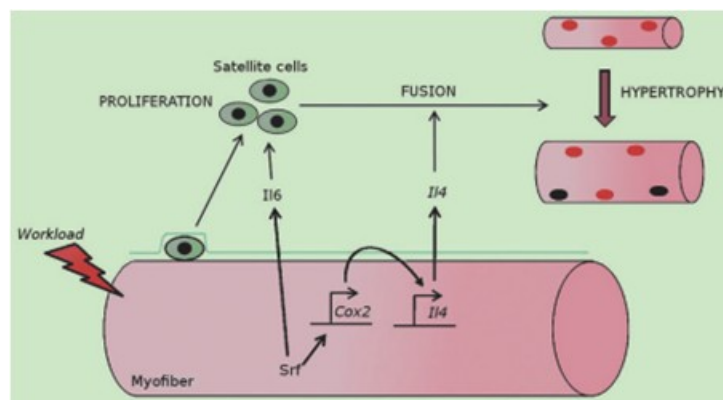
Subsequent *in vivo* studies using two Cre transgenes, with different temporal patterns of expression in the muscle developmental pathway, were conducted to delete Srf in myogenic cells. Early MyoG-Cre is activated by **E9.5** before the first round of myoblast fusion, whereas the late muscle creatine kinase-Cre is activated later during muscle fiber differentiation. These mice formed muscle fibers but they showed a **compromised hypertrophic growth** necessary for the formation of fully functional skeletal muscle and led to early postnatal lethality probably due to respiratory failure. The finding that two skeletal muscle Cre transgenes activated at different times in development lead to similar phenotypes, albeit with differing severity, suggests that the Srf mutant phenotype reflects a **late function** of Srf in hypertrophic growth, for example, in myoblast fusion, rather than an early developmental role (Li et al., 2005). Nevertheless to exclude a role of Srf in early

myogenesis, an approach of the Cre transgene under the Myf5 promoter needs to be conducted in order to delete Srf in early myogenic development. MyoD appears at **E10.5** (see in the previous description about MyoD) when the first myogenesis had occurred which means that myoblast formation and fusion was completed (Sassoon et al., 1989), thus it seems evident that in the Li's study from Olson's lab, there is no mention for MyoD expression. **However** the observed phenotype of **hypotrophy** in Srf-depleted skeletal muscle shows similarity in the MyoD-null phenotype which results in hypotrophy of skeletal muscle (postnatal growth is attenuated) (Megeney et al., 1996). But given that they did not report for MyoD expression we cannot have a response concerning the inhibition of MyoD by the Srf *in vivo* as reported in the *in vitro* studies.

It has been shown that the distal regulatory region (DRR) of the mouse and human MyoD gene contains a conserved Srf binding CArG-like element and Srf binding to the CArG element is involved in the transcriptional activation of MyoD in skeletal myoblasts and during muscle regeneration (L'honore et al., 2003). MyoD DRR represents a new hybrid element composed of binding sites for both Srf and MEF2 factors enables a molecular relay **from Srf-driven to MEF2-driven activation** of MyoD transcription when progressing from myoblast proliferation to differentiation into myotubes (L'honore et al., 2007).

### In Postnatal Muscle

A murine model with a postmitotic myofiber-targeted Srf disruption (Human Skeletal Actin-Cre) display severe skeletal muscle mass reductions due to a postnatal muscle growth defect resulting in highly **hypotrophic** adult myofibers. Srf-depleted myofibers also **failed** to regenerate following injury (Charvet et al., 2006). Tamoxifen-inducible deletion of Srf from myofibers **blunts overload-induced hypertrophy**, and impairs satellite cell proliferation and recruitment to pre-existing fibers (Guerci et al., 2012a). They showed that Srf-depleted myofibers impaired production of secreted factors Il-6, Il-4, and Cox2 which drive overload-induced muscle growth through a paracrine control (Figure 21) of satellite cell functions (affecting both satellite cell proliferation and fusion) (Guerci et al., 2012a).



**Figure 21** : Schematic model, in response to increased workload, Srf within myofibers modulates IL6 and Cox2/IL4 expression and, therefore, exerts a paracrine control of satellite cell proliferation and fusion, respectively, which in turn support skeletal muscle hypertrophy. From (Guerci et al., 2012b)

A recent study from our lab showed that Srf is a key **mediator** of **mechanotransduction** in skeletal muscle atrophy through the actin-Mrtf-Srf axis. Inhibition of the actin-Mrtf-Srf axis exacerbates the decreased Mrtf-Srf-dependent transcription activity observed in disuse atrophy (Collard et al., 2014).

### Srf in Ageing Skeletal Muscle

Contrary to the cardiac muscle, there is an **age associated decrease** in Srf expression in mice and human muscles. Srf is downregulated in skeletal muscle suggesting that this naturally occurring decrease could contribute to the muscle phenotype observed during the ageing process and in **sarcopenia** (Coletti et al., 2016). Srf **loss** in adult myofibers display **premature ageing** accompanied with a wide spectrum of alterations including atrophy, **fibrosis**, lipid accumulation and a perturbed regeneration (Lahoute et al., 2008). All these features are characteristic of aged skeletal muscle, suggesting that Srf is required for the **maintenance of an adequate “niche”** for the efficient recruitment of muscle satellite cells during overload-induced hypertrophy (Guerci et al., 2012a) and injury-induced regeneration (Lahoute et al., 2008).

## Objectif of the Study

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The adult skeletal muscle is highly plastic tissue which means that it can adapt its metabolism and size/muscle mass in response to physiological demands such as resistance or endurance exercise, mechanical overload and caloric restriction or disuse. It has also a remarkable capacity to regenerate upon muscle damage leading to an *ad integrum* reconstitution of muscle structure and function. The functional unit of muscle activity is the muscle fiber (myofiber) a multinucleated, postmitotic, high-performance structure. The adult skeletal tissue also contains muscle-specific stem cells, named as satellite cells (SCs), with an important and indispensable role in the muscle homeostasis and adaptation to internal and external stimuli in adult life.

**The aim of this study is to better understand the plasticity of muscle tissue based on SCs and how the SC fate is controlled by the serum response factor.**

The serum response factor (Srf) is a widely expressed transcription factor, member of the MADS box transcription factor family, which binds the core sequence of SRE/CArG boxes [CC(A/T)<sub>6</sub>GG]. Results obtained with specific Srf knock-out models, using the Cre-LoxP system, emphasize a crucial role for Srf in postnatal cardiac, skeletal and smooth muscle homeostasis (Coletti et al., 2016). Specifically in the adult skeletal muscle, our lab showed premature aging in skeletal muscles lacking Srf in the myofibers (Lahoute et al., 2008), Srf-dependent control of muscle mass upon disuse atrophy via the actin–Mrtf–Srf pathway (Collard et al., 2014), and Srf-requirement within myofibers for satellite cell-mediated hypertrophic muscle growth via a gene network in which Srf modulates interleukin-6 and cyclooxygenase-2/interleukin-4 signalling (Guerci et al., 2012a). However an *in vivo* study of the Srf role in adult muscle-specific stem cells has not been conducted up to now.

Why does Srf need to be studied in the satellite cells? Because Srf was initially identified to mediate the signal-induced transcriptional activation of immediate-early genes and Srf-binding sites, the CArG box, was found in muscle-specific promoters and in genes implicated in cell adhesion and migration.

Previous *in vitro* studies in C2C12 and C2 myogenic cell lines pointed out a Srf-function in the proliferation and differentiation of myoblasts (Gauthier-Rouviere et al., 1996; Soulez et al., 1996a; Vandromme et al., 1992). Moreover a CArG-like element is contained in the distal regulatory region of the MyoD gene, a master regulator of myogenic determination (L'honore et al., 2003).

In the present study the SCs were challenged by two means: 1) an injury-induced TA muscle regeneration and 2) overload-induced compensatory hypertrophy in plantaris muscle. The two models differ in the destruction or not of the myofiber niche.

With the present study, we hope to further address the question of the Srf-role in the SC functions contributing to adult skeletal muscle plasticity.

## Results

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### Part 1: Srf controls satellite cell fusion during skeletal muscle hypertrophy through the maintenance of actin architecture

A part of this study, on which the present PhD work was conducted, is presented in the following article currently prepared for soon submission.

**Srf controls satellite cell fusion during skeletal muscle hypertrophy through the maintenance of actin architecture**

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**Abstract**

Satellite cells (SCs) are muscle adult stem cells which are mobilized when muscle homeostasis is perturbed such as hypertrophy and muscle regeneration. We investigated the role played by the transcription factor Srf in the control of SCs behaviours *in vivo* using a mouse model of conditional deletion of Srf in SCs. Deletion of Srf in SCs compromised hypertrophy in *plantaris* myofibers. Unexpectedly the loss of Srf in SCs is neither accompanied with altered MyoD expression, nor altered proliferation or differentiation but with a fusion defect. We show that SC-specific Srf deletion leads to downregulation of alpha-skeletal actin target gene and an impairment of actin cytoskeleton. By restoring the network of polymerized actin with the overexpression of an alpha-actin isoform in the SCs, fusion event is rescued showing thus the importance of the actin cytoskeleton maintenance in heterotypic myogenic fusion *in vivo* between a control myotube and a mutant myoblast. However the homotypic fusion between mutant myoblasts was not affected by actin architecture restoration suggesting the implication of additional Srf targets. We provide new genetic evidence that SC fusion to growing myofiber is required for an efficient hypertrophic myofiber growth and that myoblast fusion is the limiting step for the hypertrophic growth.

## Introduction

Skeletal muscle is the most abundant tissue and the most flexible structure in the vertebrate body. Mature adult myofibers can adapt their size/muscle mass to physiological demands such as exercise or working overload (Blaauw and Reggiani, 2014). Skeletal muscle is composed of the postmitotic myofibers and the resident muscle stem cells termed as satellite cells (SCs) situated adjacent to the myofiber and they are responsible for postnatal myofiber growth and repair following injury. During neonatal and juvenile development, satellite cell fusion to growing myofiber results in the increase of myofiber size as their number remains constant. In adult skeletal muscle, under normal conditions, satellite cells are quiescent and satellite cells fusion occurs sporadically to compensate for the muscle turnover caused by daily wear and tear. However, upon injury or mechanical load stimuli satellite cells undergo adult myogenesis (Yin et al., 2013a).

In response to activation cues, SCs exit the quiescent state. Afterwards they proliferate and subsequently they differentiate and fuse either between them to form multinucleated myofibers or to preexisting myofibers. The myogenic potential of satellite cells is tightly regulated by specific Paired-box and bHLH transcription factors (Dumont et al., 2015a). Quiescent satellite cells are identified by the expression of Pax7 (Seale et al., 2000), a paired-homeobox transcription factor (TF). Once activated, they upregulate MyoD and Myf5 determination bHLH TFs and their engagement into the myogenic differentiation is marked by the expression of myogenin (MyoG) differentiation factor while their maturation and growth is regulated by the MRF4 TF expression (Moretti et al., 2016). In parallel with the myogenic progression a reserve population of Pax7-expressing satellite cells down-regulates MyoD and undergoes self-renewal division in order to replenish the satellite cell pool (Almada and Wagers, 2016). Although it was clearly demonstrated the requirement of satellite cells in muscle regeneration (Lepper et al., 2011; Sambasivan et al., 2011b), the role of SCs in the hypertrophic growth is still a matter of debate (Egner et al., 2016; McCarthy et al., 2011). Even if many intrinsic and extrinsic factors governing satellite cell functions have been discovered, SC adaptations during regeneration and overload-induced hypertrophy are also coordinated by additional players and pathways not yet well explored.

The serum response factor (Srf) is a member of the MADS box family of transcription factors, binds the core sequence of CArG boxes in the promoter regions of its target genes (Posern and Treisman, 2006) and it is a crucial factor for muscle-specific gene expression such as  $\alpha$ -actin and myosin sarcomeric proteins (Esnault et al., 2014; Miano et al., 2007). The Srf binding sites, the CArG box, were found in many muscle specific target genes such as MyoD (L'honore et al., 2007) and sarcomeric proteins and in genes implicated in cell migration and adhesion (Esnault et al., 2014). Srf is expressed in adult skeletal myofibers

and regulates the muscle mass adaptations to atrophic and hypertrophic conditions (Collard et al., 2014; Guerzi et al., 2012a). However, Srf was identified as the TF which mediates the transcriptional activity in response to serum and mitogenic factors (Treisman, 1987). Previous in vitro studies in C2C12 muscle cell line have shown that Srf activity is required for myoblast proliferation and differentiation as in the absence of Srf, the fusion is blocked and subsequently the transition of myoblasts to myotubes is prevented (Soulez et al., 1996b; Vandromme et al., 1992). Moreover Srf knockdown and dominant negative constructs prevent the expression of the muscle-determining factor MyoD (Gauthier-Rouviere et al., 1996), however in vivo data are lacking in the description of Srf role in muscle stem cells.

The Srf activity is regulated by the ternary complex factor (TCF) family of Ets domain proteins activated by mitogen activated protein (MAP) kinase phosphorylation and a family of signal-regulated SRF cofactors, the myocardin-related transcription factors (MRTFs) controlled by Rho-family GTPases and monomeric actin signalling pathway (Olson and Nordheim, 2010). Actin is also a component of muscle sarcomere and a known Srf target gene (Charvet et al., 2006; Parlakian et al., 2005). A pair of actin genes is expressed in the striated muscles (skeletal muscle and heart) of vertebrates and shares high homology in the amino-acid sequence. One of these genes ( $\alpha$ -skeletal) encodes the major actin species in adult skeletal muscle actin, and the other gene ( $\alpha$ -cardiac) encodes the major actin species in the adult heart (Gunning et al., 1983a). The cardiac actin gene has also been shown to be expressed at high levels in embryonic and fetal skeletal muscle. In adult skeletal muscle, however, there is only a low level of cardiac actin mRNA, showing that the cardiac actin gene is subject to developmental regulation (Paterson and Eldridge, 1984). Actin is implicated in many cell functions such as migration and fusion.

The fusion process is characterized by the alignment of myoblast and myotube membranes and rearrangements of actin cytoskeleton at contact sites followed by membrane fusion, which occurs in two stages. Initially, primary fusion between myoblast-myoblast results in the formation of nascent myotubes. In the secondary fusion, myoblasts fuse with nascent myotubes, which results in nuclear accretion and myotubes growth. In drosophila organisms, actin fusion focus (Martin, 2016) and actin-based filopodia (Segal et al., 2016) are the molecular structures mediating the myoblast fusion. In mammalian myoblasts Cdc42 and Rac1 are major activators of vinculin, F-actin, Vasp, and the Arp2/3 complex for the cytoskeletal remodeling that occurs before myoblast fusion (Vasyutina et al., 2009).

In the present study we were interested in addressing the role of Srf in adult muscle stem cells (satellite cells) in the course of compensatory hypertrophy (CH) in plantaris muscle. We used a mouse model of conditional and inducible deletion of Srf in the satellite cells leaving the myofiber intact. We show here that plantaris myofibers harboring Srf-

depleted SCs do not display an optimal hypertrophic response when subjected to an experimental overload. SCs lacking Srf are able to proliferate and differentiate however they are not able to fuse with the preexisting myofiber indicating the requirement of satellite cell-mediated fusion to an efficient muscle hypertrophic growth. Transcriptomic analysis revealed the actin cytoskeleton and signalling implication in the Srf-control in myoblast fusion. Overexpression of an isoform of alpha-skeletal actin in Srf-mutant SCs rescue the actin pool of polymerized F-actin and partially restores the fusion event with the nascent myofibers showing thus the importance of the actin cytoskeleton maintenance in myogenic cell functions as myoblast fusion *in vivo*.

## Materials and Methods

### Mouse protocols

Mice homozygous for *Srf* floxed alleles (Parlakian et al., 2004) (abbreviated to *Srf<sup>flox/flox</sup>*), *Pax7<sup>CreERT2/+</sup>* mice (Lepper et al., 2009) and *Pax7-nGFP* transgenic mice (Sambasivan et al., 2009) have been described elsewhere.

To investigate the effect of satellite cell-specific *Srf*-deletion in adult muscle, the mouse strain following mice were generated: *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP*. In all experiments, 2-month-old *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* mice were given five intraperitoneal (IP) tamoxifen (TMX, 1 mg/day; MP Biomedicals) injections to induce *Srf* deletion and were referred as mutant mice (Mut). Both *Pax7<sup>+/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* mice injected with tamoxifen and non-injected *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* mice were initially used as control mice. However as all muscle phenotypes investigated were identical between these two controls, un-injected *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* mice were used as control mice (Ctl) in the presented experiments

To assay if the overexpression of alpha-actin could rescue some of the outcomes of the *Srf*-loss, we used a transgenic mouse model (*CMV-flx-CAT-flx-Actc1*) allowing the inducible and conditional overexpression of exogenous rat *alpha-cardiac actin* (*Actc1*) when bred with a Cre driver mouse line. The transgenic construction is composed of  $\beta$ -actin promoter and cytomegalovirus (CMV) enhancer ensuring the transgene expression. The cDNA of the rat alpha-cardiac actin (*Acta1*) is downstream the chloramphenicol acetyl transferase (CAT) gene flanked by loxP sites. Poly-adenylation sites present downstream of the CAT gene preclude the expression of alpha-cardiac actin. *CMV-flx-CAT-flx-Actc1* mice were bred with the *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* mice in order to obtain *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP:CMV-flx-CAT-flx-Actc1* mice in which TMX injections permits both *Srf*-loss, the deletion of the CAT cassette and the concomitant over-expression of *Actc1* in satellite cells. TMX-injected *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP:CMV-flx-CAT-flx-Actc1* were referred as double mutant mice (Mut/Act+).

Mice were genotyped by PCR using the following primers: GFP-F: 5'-CGACGTAAACGGCCACAAGTTC-3'; GFP-R: 5'-GACGTTGTGGCTGTTGTAGTTG-3'; CRE-F: 5'-CCTGGAAAATGCTTCTGTCCG-3'; CRE-R: 5'-CAGGGTGTTATAAGCAATCCC-3'; ACTtg-F: 5'-CGAGGGACCTAATAACTTCG-3'; ACTtg-R: 5'-GCCGATAAACTTGTGCTT-3'; SRFlox-F: 5'-TTCGGAAGTCCCGGGCACTAAA-3'; SRFlox-R: 5'-CTGTAAGGGATGGAAGCAGA-3'.

Compensatory hypertrophy (CH) of *plantaris* muscles of control, mutant and double mutant mice was induced in control, *Srf* mutant through the incapacitation of *soleus* and

*gastrocnemius* muscles by sectioning their tendon. This procedure was achieved in both legs. During the process of CH, mice were injected with TMX at day 2 and 4 post CH. At the indicated time (1, 3 and 5 weeks post CH), *plantaris* muscles were dissected and subsequently processed for histological analyses.

Muscle tissue injury in control, mutant and double mutant mice was produced by a single intramuscular injection of 30  $\mu$ l of Cardiotoxin (CTX) solution (6  $\mu$ l; Latoxan) into *tibialis anterior* muscle. During the process of regeneration, mice were injected with TMX at day 2 and 4 post CTX. Mice were allowed to recover for 30 days and *tibialis anterior* muscles were harvested.

All animal experiments were conducted in accordance with the European guidelines for the care and use of laboratory animals and were approved by the institutional ethic committee (number 00315.1).

### **Single-fiber culture**

Individual fibers were isolated from *EDL* muscles of 2-month old control mice as described (Le Grand et al., 2012). Myofibers and associated satellite cells were either fixed in 4% paraformaldehyde immediately after their isolation (quiescent satellite cells) or kept in culture for 24h in DMEM, 20% Fetal Calf Serum, 10% Horse Serum, 1% chicken embryo extract (activated satellite cells) before fixation in 4% PFA.

### **Primary muscle cell culture and infections**

Primary cultures were derived from hindlimb muscles of control (Ctl), mutant (Mut) and double mutant (Mut/Act+) mice all harboring Pax7-nGFP transgene that allowed the prospective selection by flow cytometry (Fluorescence Activated Cell Sorting or FACS) of satellite cells.

Methods for muscle dissection, digestion and SC extraction were previously described (Montarras et al., 2005). Mononucleated muscle-derived cells were isolated from hind limb muscle of 6–8-wk-old mice. Muscles were mulched into a smooth pulp and digested in DMEM containing Collagenase D and Trypsine. Pax7/GFP positive satellite cells were isolated with BD FACSAria III cell sorter.

In standard conditions, myoblasts were grown in growth medium (DMEM/F12, 2% Ultrosor G (PALL Life Sciences), 20% Fetal Calf Serum) on plastic dishes coated with 0,02% Gelatin. For differentiation, myoblasts were seeded in Matrigel-coated dishes and cultured in differentiation medium (DMEM/F12, 2% Horse Serum).

To induce in culture the excision of the floxed *Srf* allele, *Srf*<sup>flx/flx</sup> myoblasts were transduced twice with Ad-GFP or Ad-CreGFP (100 MOI). Two days after the first transduction, GFP-positive myoblasts were selected and collected by cell sorting with BD FACSAria III.

### **Proliferation assays**

To detect S-phase entry, control and mutant satellite cells were plated immediately after sorting, cultured for 5 days in growth medium and pulsed with EdU (10  $\mu$ M, Life Technology) for 2 h prior to fixation by 4% PFA and the subsequent EdU detection was performed using Click-iT® EdU Alexa Fluor® 647 kit, according to the manufacture's instructions (Life Technologies).

For cell cycle analysis, control and mutant myoblasts were collected, fixed in 70% cold ethanol, washed by PBS, and then resuspended in the staining buffer containing 50  $\mu$ g/ml propidium iodide (PI) and 100  $\mu$ g/ml RNase (Invitrogen). The cell cycle profiles were acquired using BD Accuri™ C6 cytometer (BD Biosciences) and processed with NovoExpress software (ACEA).

### **Cell migration assay**

Migration of primary mouse muscle cells was quantified using time-lapse microscopy. Myoblasts were seeded in gelatin-coated in 8-wells Ibidi plates and maintained in rich medium. The next day, cells were filmed using a Zeiss inverted Axio Observer Z1 microscope with a LCI PlnN 10 $\times$ /0.8 W DICII objective and an incubation chamber at 37 °C, 5% CO<sub>2</sub>, 3% O<sub>2</sub>. Live cells were monitored every 6 min for 6h with Bright field and Metamorph 7.7.5 software. Cell velocities were calculated in micrometers per minute using ImageJ (NIH) software by tracking the paths of cells. At least 100 cells were tracked for each sample.

### **Cell mixing fusion assays**

To analyze heterotypic fusion between myoblasts (MB), control (Ctl) or mutant (Mut) MB were loaded with 6  $\mu$ M Green Cell Tracker (Molecular probes) for 30 min and were co-cultured with control (Ctl), mutant (Mut) or double mutant (Mut/Act+) MB loaded with 6  $\mu$ M Orange Cell Tracker (Molecular probes) for 30 min in differentiation medium. For heterotypic fusion between control myotubes (MT Ctl) and myoblasts, control myotubes at day 2 of differentiation were loaded with Green Cell Tracker and then were co-cultured with control (Ctl), mutant (Mut) or double mutant (Mut/Act+) MB loaded with Orange Cell Tracker. Two days after cell mixing, fusion events were scored by counting the dual-labeled cells. The number of fusion events was normalized by the total number of nuclei for MB-MB fusion and the total number of cells for MT-MB fusion.

### **Microarray**

Microarray analysis was performed on three independent Ad-GFP and Ad-CreGFP transductions. Total RNAs were obtained at the myoblast stage (D0), at the onset of

differentiation (D1) and three days after the switching to differentiation medium (D3) using RNeasy Mini Kit (Qiagen) and treated by DNase (Qiagen). RNA integrities were certified on bioanalyzer (Agilent). Hybridation to Mouse Gene 2\_0-ST arrays (Affimetrix) and scans (GCS3000 7G expression Console software) were performed on the Genomi'c platform (Institut Cochin, Paris). Probe data normalization and gene expression levels were processed using the Robust Multi-array Average (RMA) algorithm in expression Console software (Affimetrix). Gene ontology analysis was performed using Ingenuity (IPA) software.

### **RNA extraction and qRT-PCR**

RNA extraction and quantitative real-time (qRT)-PCR analysis were performed as described previously (Guerci et al., 2012). Values were normalized using *Hydroxymethylbilane synthetase (Hbms)*. The following primers were used: Acta1-F: 5'-CTGAGCGCAAGTACTCAGTGTGGA-3'; Acta1-R: 5'-TTCCAAAAACAGGCGCCGGCTGCA-3'; Srf-F: 5'-CACCTACCAGGTGTCGGAAT-3'; Srf-R: 5'-GCTGTGTGGATTGTGGAGGT-3'; MyoD-F: 5'-GCAGATGCACCACCAGAGTC-3'; MyoD-R: 5'- TTCCTGGGTCCAGCCTCAAC-3'; Myogenin-F: 5'-GCAATGCACTGGAGTTTCG-3'; Myogenin-R: 5'-ACGATGGACGTAAGGGAGTG-3'; Hmbs-F: 5'-TGCACGATCCTGAAACTCTG-3'; Hmbs-R: 5'-TGCATGCTATCTGAGCCATC-3'.

### **Western blotting analysis**

Western blotting was performed as described previously (Lahoute et al., 2008). Immunoblots were hybridized with antibodies against Srf (Santa Cruz) and Tubulin (Millipore).

### **Quantification of F/G-actin ratio**

The ratio of filamentous (F-) to globular (G-) actin was determined using the G-actin/F-actin In Vivo Assay Kit (Cytoskeleton). Briefly, myoblasts were harvested and lysates were cleared by centrifugation at 500g for 5 min. Subsequently, supernatants were centrifugated at 100,000 g for 1h at 37°C, which resulted in F-actin in the pellet and G-actin in the supernatant. The F-actin containing pellet was resuspended and solubilized in F-actin depolymerization buffer at a volume equal to the G-actin-containing supernatant volume. Equivalent volumes of supernatant and pellet were resolved by SDS-PAGE and subjected to immunoblot analysis using an anti-pan-actin antibody (Cytoskeleton). The F/G-actin ratio was quantified by using FusionCapt Advance software (Vilber Lourmat).

### **Immunostaining**

*Plantaris* and *tibialis anterior* muscles were collected and snap-frozen in liquid nitrogen-cooled isopentane. Eight µm-thick Muscle sections of were fixed in 4% paraformaldehyde for 8 min and immunostainings were performed as described (Collard et al., 2014). The primary antibodies used were against mouse monoclonal dystrophin (Novocastra/clone NCL/Dys)



dilution 1/50, mouse monoclonal Pax7 (Santa Cruz/sc-81648) dilution 1/50, rabbit polyclonal Myogenin (Santa Cruz/clone F5D/sc-576) dilution 1/100. Nuclei staining were performed using DAPI.

Muscle cells cultured in dishes were fixed for 8 min in 4% paraformaldehyde and then permeabilized and blocked in PBS with 0,1% Triton X-100, 5% Horse Serum for 1h at TA. Cells were the incubated overnight at 4°C with the following primary antibodies against MyoD (Santa Cruz) dilution 1/100, Myogenin (Santa Cruz) dilution 1/100, MHC embryonic (MF20, Alexis biochemicals) 1/50 diluted in the same buffer. After incubation 1h at RT with fluorescent secondary antibody (Invitrogen), with DAPI to stain nuclei and/or with Phalloïdin Alexa 488 (Thermofisher) to stain F-actin, cells were mounted in Fluorescent Mounting Medium (Dako).

### **Image acquisition**

Digital images were acquired using a Zeiss Axiovert 200M microscope with 10x, 20x or 40x magnification, cooled CCD CoolSNAP-HQ2 camera (Photometrics) and Metamorph 7.7.5 (Molecular Devices). Images were composed and edited in ImageJ. Background was reduced using brightness and contrast adjustments applied to the whole image.

### **Morphometric analysis and Phalloidin quantification**

Myofiber CSA was analyzed by using immunostaining of dystrophin, marking myofiber sarcolemma, and then using Metamorph 7.7.5. Between 600 and 800 myofibers were analyzed.

Phalloidin signal intensity per cell was quantified using ImageJ. At least 150 cells were analyzed.

### **Statistical analysis**

The significance of differences between means was assessed with a Student's t-test. P values of < 0.05 were considered statistically significant.

## Results

### **Srf in SCs is required for overload-induced hypertrophy**

We first examined Srf expression in quiescent and activated adult muscle stem cells, the satellite cells (SCs). Single-fibers and their associated SCs were isolated from EDL muscles at the steady state and were fixed immediately to evaluate the expression of Srf in quiescent SCs expressing Pax7, a reliable biomarker for SCs. Srf was barely expressed in quiescent SCs (Figure 1A and 1B). In contrast, when single-fibers were maintained 24 hours in culture to allow the activation of the associated SCs, Srf expression was detected in all activated SCs (Figure 1B).

To investigate the role played by Srf in satellite cells, compensatory hypertrophy (CH) of the plantaris muscle was performed in *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* mutant mice previously injected with tamoxifen to induce SC-specific Srf loss (Figure 1C). Non injected *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* mice were used as controls. Compensatory hypertrophy presents the advantage to mobilize SCs without any destruction of the myofibers, allowing the study of mutant SC behaviors in a wild-type environment. Following tamoxifen treatment, efficient loss of Srf was achieved at the transcript and at protein levels in FACS-sorted SCs from control and mutant muscles (Figure S1). At the steady state, no differences in muscle weight and in myofiber cross-section area (CSA) were observed between control and mutant plantaris muscles (Figure 1D, 1E, F). Following hypertrophy, a significant increase in plantaris muscle mass and in CSA 3 and 5 weeks post overload were scored in control overloaded muscles as compared to unloaded muscles (C) (Figure 1D, 1E, 1F). However in muscles lacking Srf in their SCs, the extend of this hypertrophic growth was strongly reduced, displaying only a 12% increase in mass/CSA versus 30% in control 3 weeks post CH (Figure 1E, 1F). These changes were not accompanied with modifications of the myofiber number (Figure 1G), indicating that the increase in muscle mass in this muscle growth model was mainly due to myofiber hypertrophy or due to increased myonuclei.

These data show that even though some limited growth can occur in mutant muscles, Srf in SCs is necessary for optimal overload-induced myofiber hypertrophy to take place.

### **Srf inactivation in SCs does not affect their expansion**

Srf has been shown to control the proliferation of several cell types including C2C12 muscle cell line (Soulez et al., 1996b). To investigate whether SCs functions were altered by Srf loss upon CH, the number of SCs expressing Pax7 was first quantified. Before overload, no difference in SC number was observed between control and mutant plantaris muscles

(Figure 2B) precluding a role for Srf in the maintenance of the quiescent SC pool, which is consistent with the poor expression of Srf in quiescent SCs (Figure 1B). A significant 2.5 fold increase in the number of Pax7+ cells was observed 1 week post CH in both control and mutant plantaris muscles that returned to its original level 5 weeks post CH (Figure 2A, AB). Importantly, following overload induced hypertrophy, there was no difference in SC number between control and mutant muscles suggesting that Srf loss did not hamper the growth response of SCs to mechanical cues.

The proliferation potential of Srf-deleted SCs was further assessed in vitro by determining the EdU incorporation rate (which specifically marks the S-phase of cycling cells) of control and mutant FACS-sorted primary myoblasts. In agreement with our observations in vivo, the proliferation rates of control and of mutant myoblasts were similar (Figure 2C). We further showed that the distributions of control and Srf-deleted myoblasts in the different cell cycle phases analyzed by FACS-quantification of DNA content did not differ (Figure 2D). Additional experiments were conducted in which Srf loss was achieved in vitro by transducing primary *Srf<sup>flox/flox</sup>* myoblasts with adenovirus expressing Cre recombinase (Ad-Cre) or GFP (Ad-GFP) (Figure S2A). Again no difference in the proliferation capacities of Ad-GFP and Ad-Cre transduced myoblasts was observed in BrdU incorporation experiments (Figure S2B). Altogether these data show that Srf in myogenic precursor cells is dispensable for their proliferation in a cell autonomous manner.

### **Srf controls SC motility but it does not influence their myogenic differentiation potential**

We then hypothesized that other SC functions (such as motility, differentiation, fusion) could be modified by Srf loss and could account for the impaired hypertrophic growth of mutant muscle. After activation signals, SCs become extremely motile and move from beneath the lamina to the surface of the myofiber as it was monitored by intravital imaging in the muscle regeneration model (Webster et al., 2016). Nevertheless such a study has not been performed in the hypertrophy model. Since Srf is a central regulator of genes involved in cell migration (Pipes et al., 2006), the motile functions of Srf-deleted SCs were monitored in vitro using time-lapse video-microscopy. The motility of mutant myoblasts was strongly decreased as compared to control (Figure 2E). Accordingly Ad-Cre transduced *Srf<sup>flx/flx</sup>* myoblasts displayed also a diminished motility by more than 2 fold (Figure S2C). These results demonstrate that Srf is needed for satellite cell motility.

We next investigated whether Srf deletion in SCs could affect their myogenic differentiation potential. Indeed, previous in vitro studies in C2C12 muscle cell line indicated that Srf activity was required for the expression of MyoD, a MRF required for myogenic cell

determination, and of Myogenin (Gauthier-Rouviere et al., 1996; Vandromme et al., 1992). We first assessed the number of Myogenin-expressing cells *in vivo* during overload-induced hypertrophy procedure. After one week of CH, the number of Myogenin-positive cells was increased in a similar way in both control and mutant plantaris muscles (Figure 3A). These data do not support a role for Srf in SCs differentiation engagement. We then thought to determine in primary cultured muscle cells (cell sorted from control or mutant muscles) whether Srf deletion could affect MyoD and Myogenin expressions. In contrast to what was shown in C2C12, the expression of MyoD and of Myogenin at the protein and transcript levels did not differ between control and mutant primary myoblasts under proliferation conditions or after induction of differentiation (Figure 3B, 3C, S3A, S3B). We even observed an increased proportion of nuclei expressing Myogenin in mutant cells 3 days after the induction of differentiation (Figure 3C). Similar MyoD and Myogenin expressions were also observed when Srf loss was achieved *in vitro* by Ad-Cre transduction of *Srf<sup>fllox/fllox</sup>* myoblasts (Figure S3C, S3D). The expression of a later differentiation marker (the sarcomeric myosin heavy chain, MyHC) was monitored in control and mutant cells upon differentiation using MF20 antibody (Figure 3D). Three days post differentiation the proportion of nuclei in MyHC+ cells was identical between control and mutant cells (Figure 3E). Taken together, these data show that the loss of Srf in the SCs does not impair their engagement in the myogenic differentiation.

### **Srf is needed for SC fusion capacities**

To determine whether the lack of Srf in SCs might impact cell fusion, myonuclei numbers inside the sarcolemma were counted in control and mutant plantaris muscles at various time post CH by performing Dystrophin/Dapi staining (Figure 1D). Significant increases in myonuclei numbers were observed in control muscles at 1, 3 and 5 weeks post-CH. Whereas the myonuclei numbers was similar before overload between control and mutant, following overload, the myonuclei number did not vary in mutant muscles and was significantly decreased as compared to control (Figure 4A). The blunted fusion capacity of SCs lacking Srf show that Srf within SCs is needed for their efficient recruitment to the growing myofiber.

Fusion is unrolled in two and overlapped phases: the primary fusion that occurs between myoblasts resulting in the formation of nascent myotubes and the secondary fusion between a myoblast and a nascent myotube. To discern whether these fusions are defected by Srf loss, we set up various *in vitro* assays to specifically assess fusion between myoblasts and fusion between myoblasts and myotubes. This later setting models what occurs *in vivo* during overload-induced hypertrophy. Fusion was first monitored during the course of

differentiation. Mutant cells presented an altered capacity to form multinucleated myotubes 3 days after differentiation induction (Figure 4B). Their fusion index, which represents the proportion of the total cell population that has fused, was strongly decreased as compared to control cells, showing a reduced efficiency of the first fusion phase (Figure 4C). Accordingly, the mean number of nuclei in differentiated MyHC<sup>+</sup> cells was strongly reduced in mutant cells (Figure 4D). We then evaluated whether heterotypic myoblast fusion, between control and mutant myoblasts, was also affected by performing mixing experiments of cells labeled with different dyes. We could confirm that mutant myoblasts were unable to fuse in a homotypic manner as we observed a 60% decrease in fusion events (dual labeling) between mutant myoblasts as compared to control myoblasts (Figure 4E). Furthermore mutant myoblasts exhibited an altered heterotypic fusion with control cells (Figure 4E) indicating that efficient fusion occurs only when Srf is expressed in both fusing myoblasts.

The secondary and asymmetric fusion was specifically studied by mixing differently labeled control or mutant myoblasts with control myotubes. The lack of Srf in myoblasts was accompanied with a sharp decrease in the proportion of dual labeled cells (Figure 4F) showing that Srf within myoblasts is required for their fusion to control myotubes, a process that leads to nuclei accretion of new nuclei into myofibers upon overload-induced hypertrophy.

Altogether these results show that Srf within myoblasts is needed for both primary and secondary fusion phases. In addition our results suggest that the decreased ability of SCs lacking Srf to fuse with the growing fibers could account for the defective hypertrophic growth of mutant muscles.

### **The impaired actin cytoskeleton organization in Srf-deleted myoblasts is partially rescued by the compensatory expression of an alpha actin isoform**

To identify Srf target genes and Srf-dependent pathways/biological functions that could control SC motility and fusion, we performed a microarray analysis of gene expression using proliferating myoblasts (D0) and differentiating cells (early phase D1 and later phase D3) expressing (Ad-GFP) or not Srf (Ad-Cre). We identified the set of 145 genes whose expression was altered by Srf loss in both myoblasts and in differentiating cells (Figure 5A, Table S1). Analysis of the potential biological roles of such genes by an ontology program (Ingenuity) pointed out an over-representation of genes involved in the regulation of actin cytoskeleton rearrangements (Figure 5B) pointing out a putative implication of actin cytoskeleton integrity in the perturbed functions of SCs lacking Srf. In agreement with our *in silico* analysis, several genes encoding sarcomeric proteins ( $\alpha$ -actins, myosin light chain) and genes involved in actin cytoskeleton treadmilling have been identified among Srf targets

genes (Esnault et al., 2014). Moreover, analysis of Srf knock-out models in skeletal and cardiac muscles have highlighted actin genes as major hits of Srf (Charvet et al., 2006; Parlakian et al., 2005). In the present transcriptomic study, we observed that the expression of several actin genes in proliferating and differentiating cells was altered by Srf deletion. In particular alpha skeletal-actin (Acta1) expression was strongly diminished in Srf-deleted muscle cells (Figure 5C). Cytoskeletal scaffold structures of actin microfilaments undergo dynamic changes in polymerization of monomeric globular actin (G-actin) into a filamentous actin (F-actin). Hence, we quantified the total amount of F-actin in control and in mutant myoblasts by a phalloidin staining (Figure 5D) and showed a significant decrease of total F-actin in mutant cells (Figure 5E). Furthermore, we quantified soluble G-actin and insoluble F-actin fractions in muscle cells. In agreement with phalloidin staining data, the F/G-actin ratio was decreased 2.5 fold in myoblasts lacking Srf (Figure 5F, 5G). Together these results show that Srf controls the maintenance of actin cytoskeleton in cultured muscle cells.

Alpha cardiac (Actc1) and alpha skeletal (Acta1) actins have redundant roles in cardiac and skeletal muscles. For instance Actc1 overexpression can functionally replace Acta1 in Acta1 knock out mice (Nowak et al, 2009) highlighting their similar role in maintenance of adequate muscle functions. Thus, we hypothesized that the perturbed organization of actin cytoskeleton in Srf-deleted muscle cells could be counteracted by alpha cardiac actin (Actc1) overexpression using sorted SCs from tamoxifen-treated *Pax7<sup>CreERT2/+</sup>:Srf<sup>flox/flox</sup>:Pax7-nGFP:CMV-flx-CAT-flx-Actc1* mice (MutAct) (Figure S4A). We first validated, in FACS-sorted SCs isolated from double mutant mice injected with Tamoxifen, the efficient loss of endogenous Srf expression and the concomitant overexpression of exogenous Actc1 transcripts (Figure S4B). Strikingly, the overexpression of Actc1 in Srf-deleted myoblasts was sufficient to restore to control levels both the total amount of F-actin (Figure 5C, 5D) and F/G-actin ratio (Figure 5E, 5F) and therefore to preserve actin cytoskeleton organization in muscle cells lacking Srf.

### **Alpha actin overexpression rescues the heterotypic fusion defect caused by the Srf-loss in myoblasts**

We next asked whether the reestablishment of actin cytoskeleton in Srf mutant SCs by alpha actin overexpression could rescue impaired SC's functions such as motility and fusion. Cell tracking experiments showed a similar decrease of motility in double mutant and mutant myoblasts (Figure S4C) precluding the implication of alpha actin-mediated F-actin stabilization in the impaired motile functions of SCs lacking Srf.

We then assessed the fusion capacity of double mutant cells during the course of differentiation. As observed for Srf mutant cells, double mutant cells displayed an unaltered

engagement in differentiation evidenced by their MyoD, Myogenin and Myosin Heavy Chain expressions that were similar or even higher than control cells (Figure S4D, S4E, S4F, S4G). In addition, upon differentiation, the fusion index and the mean number of nuclei per myosin expressing cell were reduced to a similar extent in double mutant and in Srf mutant cells suggesting that actin overexpression did not alleviate the homotypic fusion defect of cells lacking Srf (Figure 6A, 6B, 6C). Most interestingly, the impaired heterotypic fusion between control and Srf mutant myoblasts was fully rescued by alpha-actin overexpression, as assessed by counting the fusion event in cell mixing experiments 3 days post differentiation (Figure 6D). Finally, actin overexpression was sufficient to restore the fusion defect of Srf mutant myoblasts with control myotubes (Figure 6E). Overall, these data suggest that the maintenance of F-actin network in Srf mutant myoblasts by alpha actin over-expression is sufficient to rescue their heterotypic fusion with control myoblasts/myotube while being insufficient to permit homotypic fusion.

### ***Alpha actin overexpression restores overload-induced muscle hypertrophic growth of Srf mutant muscles***

To gain in vivo insights into the possible rescue of Srf-deleted myoblast fusion by alpha actin overexpression, overload-induced hypertrophy and muscle regeneration procedures, recapitulating heterotypic and homotypic fusion events respectively, were conducted on control, mutant and double mutant mice. In our genetic models, Srf deletion, and/or concomitant actin overexpression, occurs only in Pax7-expressing SCs of mutant/double mutant muscles but never in their myofibers. Hence, the nuclear accretion that occurs in vivo during overload-induced hypertrophy, a situation in which myofibers remain intact, corresponds to the heterotypic fusion between mutant/double or mutant myoblasts and control myotubes. On another hand the in vivo procedure that models homotypic fusion is the regeneration. Upon Cardiotoxin (CTX)-induced injury, SCs are activated, proliferate, differentiate and fuse between them to form new myofibers, though fusion occurs exclusively among cells harboring the same genotype (control, mutant or double mutant).

Control, mutant and double mutant tibialis anterior (TA) muscles were subjected to CTX-induced regeneration. Thirty days post-regeneration, Dystrophin/Dapi staining of regenerated muscle sections revealed that the overall structure of Srf mutant muscles was severely affected as compared to control muscles (Figure 7A). Indeed mutant muscles presented newly formed fibers that are 10 times smaller (Figure 7B) and 3 times more numerous than control muscles (Figure S5A). Moreover mutant regenerated muscles displayed a strong decrease of their myonuclei number (Figure 7C). Of note, the number of SCs was not affected by Srf loss 30 days post injury (Figure S5B) implying that Srf

expression does not have an impact on the number of SCs. Together these data suggest a strong deficiency of Srf-deleted SCs in their ability to fuse that could account for the small sized and the increased number of newly formed regenerated fibers present in mutant muscles.

We next assessed whether alpha actin overexpression could counteract the altered regeneration and defective homotypic fusion of regenerated Srf mutant muscles. Thirty days post injury, when double mutant, mutant and control muscles were compared, only a very modest improvement of fusion was scored in double mutant as compared to Srf mutant muscles that was not accompanied with a significant change in the mean CSA (Figure 7B, 7C). These data are in agreement with the absence of amelioration of the homotypic fusion defect that was observed *in vitro* in double mutant myoblasts when compared to Srf mutant cells.

As heterotypic fusion between mutant/double mutant myoblasts and control myotubes models the nuclear accretion that occurs *in vivo* during overload-induced hypertrophy, we determined the impact of alpha actin overexpression on the defective hypertrophy of Srf mutant muscles. Three and 5 weeks post-CH, actin overexpression rescued to control levels the overall growth of Srf mutant muscles. Indeed, double mutant plantaris muscle mass and mean CSA were significantly higher than those of Srf mutant muscles and were comparable to those of control muscles (Figure 7D, 7E). Furthermore myonuclei number was significantly increased in double mutant as compared to Srf mutant and reached 60% of the control level 5 weeks post CH (Figure 7F).

Taken together these data show that while maintenance of F-actin scaffold within SCs lacking Srf did not ameliorate homotypic fusion, it was sufficient to efficiently drive heterotypic fusion *in vitro* and *in vivo* and to restore the impaired hypertrophic growth of Srf mutant muscles.



## Discussion

Our study aims to understand the plasticity of skeletal muscle tissue in the hypertrophic context based on SCs and we focus on Srf transcription factor in the control of SC functions. We used the genetic mouse model *Pax7<sup>CreERT2/+</sup>;Srf<sup>flox/flox</sup>* permitting the analysis of the Srf role in SCs. We show that Srf is required for SC fusion to the growing myofibers under CH to achieve an efficient myofiber hypertrophic growth. Srf deletion in SCs abrogates the fusion capacity of mouse myoblasts. Moreover Srf-loss in SCs leads to downregulation of a-skeletal actin target gene and an impairment of actin cytoskeleton. Overexpression of an alpha-actin isoform in the SCs restores the network of polymerized actin and rescues the SC-mediated hypertrophic myofiber growth while it fail to do so under muscle regeneration showing that Srf regulates unknown target genes effectors of myoblast fusion that act in coordination with the actin cytoarchitecture maintenance.

Srf is expressed in adult skeletal myofibers and its expression varies accordingly to mechanical stimuli, such as it is increased in the CH and decreases in the atrophy, rendering thus Srf a mechanical sensor (Collard et al., 2014; Gordon et al., 2001). Moreover Srf mediates the transcriptional activation of immediate early genes (IEGs) upon serum stimulation and muscle-specific genes during muscle differentiation (Posern and Treisman, 2006) highlighting Srf as a good candidate for the transcriptional control of signal-induced SC recruitment.

In several contexts, Srf affects cell proliferation either by enhancing it (Koegel et al., 2009) or compromising it (Sun et al., 2009; Werth et al., 2010). The myocardin related transcription factors (Mrtfs) are Srf coactivators involved in cell proliferation and myogenesis of cardiac, smooth and skeletal muscle (Olson and Nordheim, 2010). Skeletal muscle-specific deletion of Mrtf-A and Mrtf-B (dKO) during skeletal muscle development demonstrate a decrease in the proliferation of dKO embryonic myoblasts and an increase in apoptosis (Cenik et al., 2016). In contrast to these previous data, our results show that SCs lacking Srf respond to mechanical cues by expanding their number similarly to control SCs. Moreover Srf does prevent the myoblast proliferation contrary to previous observations in myogenic cell lines (Soulez et al., 1996a) where Srf is indispensable for myoblast proliferation.

In contrast with the previous observations in C2C12 cell line where Srf knockdown and dominant negative lead to a shutdown of MyoD expression (Gauthier-Rouviere et al., 1996), in our *in vivo* SC-specific genetic deletion of Srf (Srf KO) and in primary cell culture models, Srf is not indispensable for the MyoD expression. SC-specific deletion of both MASTR (a member of Mrtf family) and Mrtf-A lead to excessive proliferation of SCs due to down-regulation of MyoD expression (Mokalled et al., 2012). It has been proposed that a member of the MADS box family, the MEF2 muscle-enriched transcription factor and its

associated MASTR cofactor cooperate with Mrtf-A (Srf cofactor) to control the transcriptional regulation of MyoD expression (Creemers et al., 2006; Meadows et al., 2008; Mokalled et al., 2012). Thus it is possible that in the absence of Srf in SCs, MyoD is controlled by the MASTR/MEF2 pathway.

Since Srf-binding sites have identified in muscle-specific promoters and Srf is required for differentiation and growth of skeletal, cardiac and smooth muscles (Li et al., 2005; Miano et al., 2004; Parlakian et al., 2004), therefore it is expecting a Srf-directed regulation in the myoblasts differentiation. Previous *in vitro* studies showed that Srf is required for the myoblast-myotube transition and that Srf loss prevented the expression of both myogenin and MyoD as also the expression of muscle-specific marker troponin T (Gauthier-Rouviere et al., 1996; Vandromme et al., 1992). In our system, cultured primary myoblasts under differentiation conditions do not demonstrate a downregulation neither in MyoD nor MyoG expression indicating that the engagement of myoblasts into the differentiation program is not affected by the Srf loss. In addition, MyHC is expressed in the absence of Srf likewise to controls. These results reveal that Srf does not abolish the differentiation myogenic program. However the fusion which is the end up step of terminal differentiation is highly compromised with only small myotubes containing limited nuclei are formed. Our results are in accordance with the Mrtf dKO in skeletal muscles, where fusion of myoblasts into myotubes was not completely prevented by the loss of Srf cofactors Mrtf-A and Mrtf-B (Cenik et al., 2016). In addition we did not observe any change in myomaker (TMEM8C) expression a known mammalian myoblast fusogen (Millay et al., 2013) as it was also the case of Mrtf-A and Mrtf-B double knockout (Cenik et al., 2016).

Srf-mutant myoblasts present a major fusion defect. Primary homotypic fusion (between myoblasts) as well as secondary (between myoblasts and nascent myotubes) homotypic fusion is both affected in Srf-mutant myoblasts. In addition, heterotypic fusion is not improved as shown in heterotypic mixture of mutant and control myoblasts/myotubes demonstrating therefore that the symmetric expression of Srf is required in a pair of fusing cells.

Actin is a structural sarcomeric protein and part of RhoA-actin-Mrtf-Srf signalling cascade (Nordheim, 2014). Actin exchanges between monomeric G-actin and polymerized F-actin in order to maintain the functional actin network, mediating thus cellular processes such as migration, adhesion and fusion (Martin, 2016; Segal et al., 2016). Srf-mutant myoblasts present an altered actin network with paucity of F-actin which contribute to decreased fusion (Nowak et al., 2009).

Transcriptomic analyses revealed an over-representation of genes implicated in actin cytoskeleton rearrangement and signalling. Acta1 gene expression is highly decreased,

comparing to controls. From many aspects adult myogenesis recapitulates molecular pathways activated in embryonic myogenesis (Yin et al., 2013a). In vertebrate embryos, the alpha-cardiac actin gene encodes a major sarcomeric component in both skeletal and cardiac muscle (Latinkić et al., 2002; Paterson and Eldridge, 1984). Indeed the *Actc* gene is the predominant sarcomeric isoform in early fetal skeletal muscle development (Ilkovski et al., 2005) showing that the cardiac actin gene is subject to developmental regulation (Gunning et al., 1983b). Based on these assumptions, we used an available transgenic mouse model of *Actc* overexpression. Overexpression of alpha-cardiac (*Actc*) isoform in *Srf*-depleted SCs, restores the F-actin pool and rescues the heterotypic fusion. The underlying possible mechanism for the rescue of heterotypic/asymmetric fusion could be the restoration of the mechanical invading force which helps to overcome energy barriers for membrane apposition and drives cell membrane fusion (Kim et al., 2015) or the appropriate cellular distribution of signalling molecules and/or contractile proteins molecules to mediate the fusion (Tran et al., 2012). Compensatory alpha-actin expression in *Srf*-depleted SCs is not however sufficient for the homotypic/symmetric fusion suggesting other alpha-actin-independent mechanisms downstream *Srf*-targets.

Nevertheless overexpression of the alpha-actin isoform does not rescue the motile functions of SCs suggesting thus a non alpha-actin dependent control of cell motility. The non-muscle isoforms of beta- and gamma-actin or myosin, all being *Srf*-target genes (Esnault et al., 2014; Miano et al., 2007) could be involved in the process of cell movement (Callan-Jones and Voituriez, 2016). Pharmacological induction (jasplakinolide-treatment) of F-actin polymerization in an attempt to maintain the F-actin network in wild-type and *Srf*-mutant myoblasts could confirm/phenocopy the observed phenotype from the genetic model of *Actc* overexpression.

The *in vivo* model of homotypic or symmetric fusion study is the muscle regeneration where activated SCs and their descendant myoblasts either fuse between them or to nascent myotubes/pre-existing myofibers to reconstitute the under lesion muscle. Clearly the regeneration process is impaired in TA muscles harboring *Srf*-mutant SCs as the fusion process did not take place. The double mutant SCs seem to slightly ameliorate the fusion capacity with no impact in the size of new-formed myofibers. However in the *in vivo* model of asymmetric fusion, CH, double mutant SCs being able to fuse to the growing myofiber results in the increase in the myofiber size as in controls. Our study shows that the actin network maintenance is important but not sufficient for the fusion process in mammalian myoblasts, and other *Srf*-target genes are implicated and needed for SC functions. Such targets could be involved in a number of cellular processes, including lipid metabolism such as caveolins and cavinins, the structural proteins of caveolae, cause alterations in cell membrane

composition (lipodystrophy) and they are characterized by the presence of CArG boxes in their promoter (Swärd et al., 2016). Lipid modifications correlate with a reduction in plasma membrane rigidity resulting in fusion impairment (Blondelle et al., 2015).

Satellite cells are the major donors of new nuclei, being myogenic precursor cells, important for muscle development, for muscle regeneration and possibly also for muscle hypertrophy in response to exercise, training and hormonal stimulation (Blaauw and Reggiani, 2014; Montarras et al., 2013). The role of satellite cells in muscle hypertrophy has long been a debated issue (Blaauw and Reggiani, 2014; Gundersen, 2016; Pallafacchina et al., 2013; Snijders et al., 2015). Studies with a transgenic Pax7-DTA mouse model, where the Pax7+ SC depletion is achieved with TMX treatment, showed that satellite cells are not required for muscle growth over a short period (McCarthy et al., 2011) but they are required to overcome/negatively regulate the excessive ECM accumulation over a prolonged period resulting in attenuated hypertrophy (Fry et al., 2014). *Egner et al.* repeated these experiments analyzing the muscles by immunohistochemistry and *in vivo* and *ex vivo* imaging and they found that overload hypertrophy was prevented in the satellite cell-deficient mice, in both the plantaris and the extensor digitorum longus muscles. In consequence they claim that there is currently no model in which functional, sustainable hypertrophy has been unequivocally demonstrated in the absence of satellite cells.

We provide here new genetic evidence showing that when SCs are not able to fuse, hypertrophy is impaired. *Srf*-mutant mice show no signs of fusion and the moderated increased CSA could be attributed to an increase in the protein synthesis in *Srf*-expressing myofibers. *In vivo* overexpression of alpha-actin rescues the fusion in the CH model. We conclude that SC myonuclear accretion is required however for an optimal hypertrophic growth and it indicates the necessity of SC fusion to the growing myofiber.

Taken together these data show that while maintenance of F-actin scaffold within SCs lacking *Srf* did not ameliorate the homotypic fusion, it was sufficient to efficiently drive heterotypic fusion *in vitro* and *in vivo* and to restore the impaired hypertrophic growth of *Srf* mutant muscles. More generally these data strongly support the need of SC-mediated fusion in the hypertrophy of myofiber.

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Figures

Figure 1

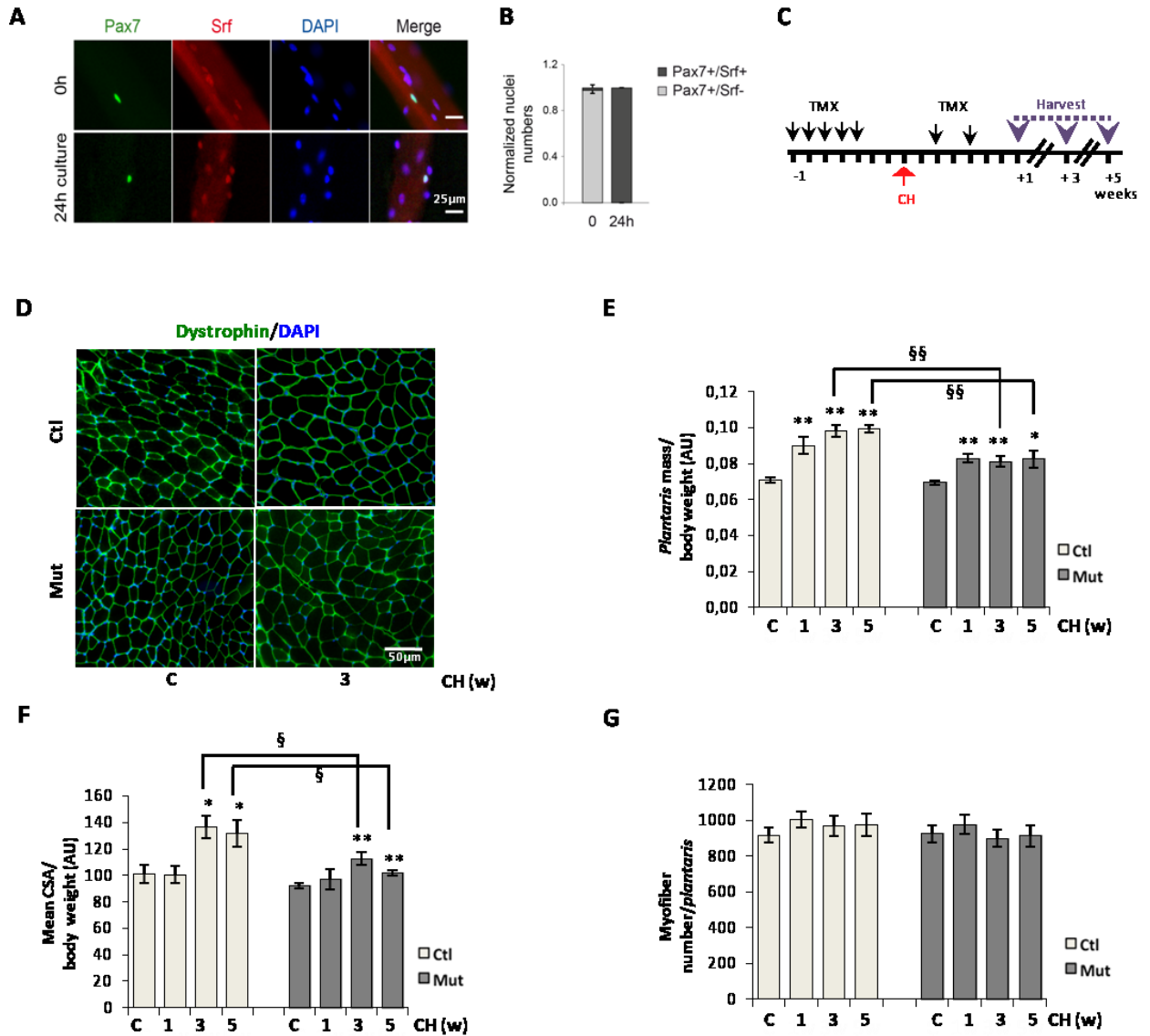


Figure 1: *Srf* loss in SCs results in compensatory hypertrophy deficiency in *plantaris* muscle

(A) Immunostaining for Pax7 (green) and Srf (red) to illustrate Srf expressing satellite cells (Pax7+Srf+) on single fibers fixed immediately after isolation (0h – quiescent SCs) or maintained in culture for 24h (activated SCs).

(B) Proportion of satellite cell displaying or not Srf expression (Pax7+/Srf+ and Pax7+/Srf- respectively).

**(C)** Srf mutant mice were injected with tamoxifen (TMX) one week before compensatory hypertrophy (CH) procedure and after CH. Plantaris muscles were isolated 1, 3 and 5 weeks post surgery.

**(D)** Plantaris muscle sections immunostained for Dystrophin (green) and nuclear staining with DAPI for control (Ctl) and Srf mutant (Mut) mice before (c) and after 3 weeks of CH.

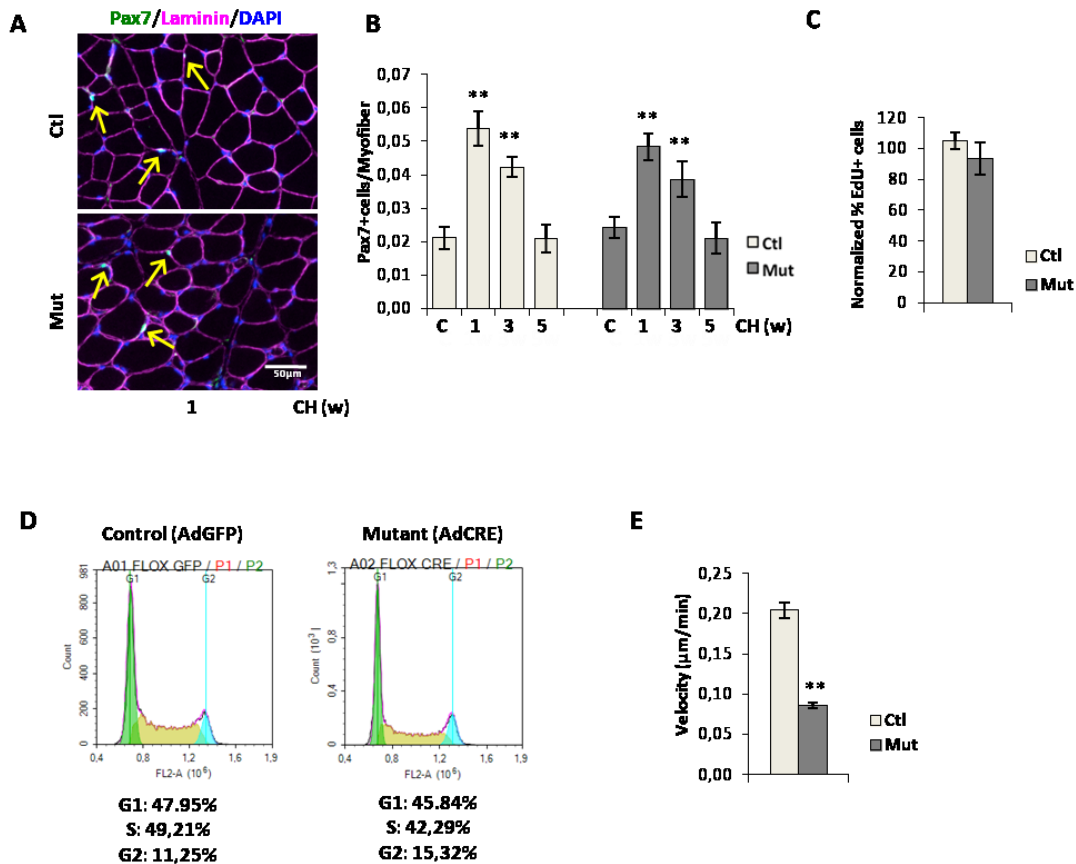
**(E)** Ratio of plantaris mass to body weight before (c) and after 1, 3 and 5 weeks of CH in control and mutant mice (n=5 to 14).

**(F)** Ratio of CSA to body weight before (c) and after 1, 3 and 5 weeks of CH in control and mutant mice (n=5 to 14).

**(G)** Mean myofiber number before (c) and after 1, 3 and 5 weeks of CH in control and mutant mice (n=5 to 14).

Data are mean  $\pm$  s.d. \*p< 0.05 versus c, \*\*p< 0.01 versus c, §p< 0.05, §§p< 0.01. AU, arbitrary units.

**Figure 2**



**Figure 2: *Srf* loss within satellite cells does not impact their proliferation but impairs their motility**

**(A)** Plantaris muscle section immunostained for Pax7 (green) and laminin (magenta) and nuclear staining with DAPI (blue) from control and mutant mice one week post CH.

**(B)** Number of Pax7+ cells per myofiber were quantified in control and mutant plantaris muscle sections before (c) and after 1, 3 and 5 weeks of CH (n=5 to 13).

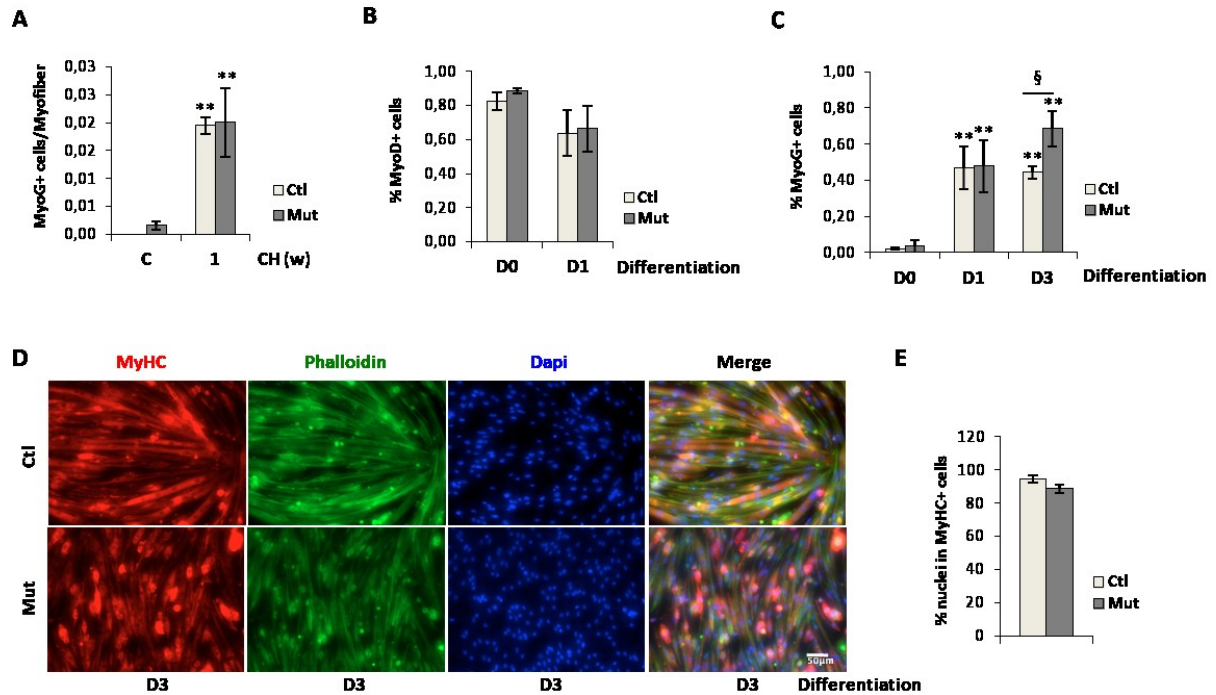
**(C)** Normalized percentage of EdU+ cells in control and mutant FACS cell-sorted satellite cells cultured in rich medium for 5 days (n=6).

**(D)** Cell-cycle distribution of control (AdGFP) and mutant (AdCRE) myoblasts grown in rich medium, were analyzed by flow cytometry after propidium iodide staining. Percentages of cells in G1-phase, in S-phase, and in G2M-phase from one representative experiment are presented and have been processed with NovoExpress software (ACEA).

**(E)** Mean velocity (µm/min) of control and mutant myoblasts determined by time-laps video-microscopy (at least 100 cells analyzed per genotype).

Data are means ± s.d. \*\*p< 0.01 versus c or versus Ctl.

**Figure 3**



**Figure 3: *Srf* loss does not affect myogenic differentiation of satellite cells**

**(A)** Number of Myogenin+ (MyoG+) cells per myofiber were quantified in control and mutant plantaris muscle sections before (c) and after 1 week of CH (n=4 to 5).

**(B)** Percentage of MyoD+ cells in control and mutant FACS cell-sorted satellite cells cultured in rich medium (D0, myoblasts) or after one day (D1) following differentiation induction (n=3).

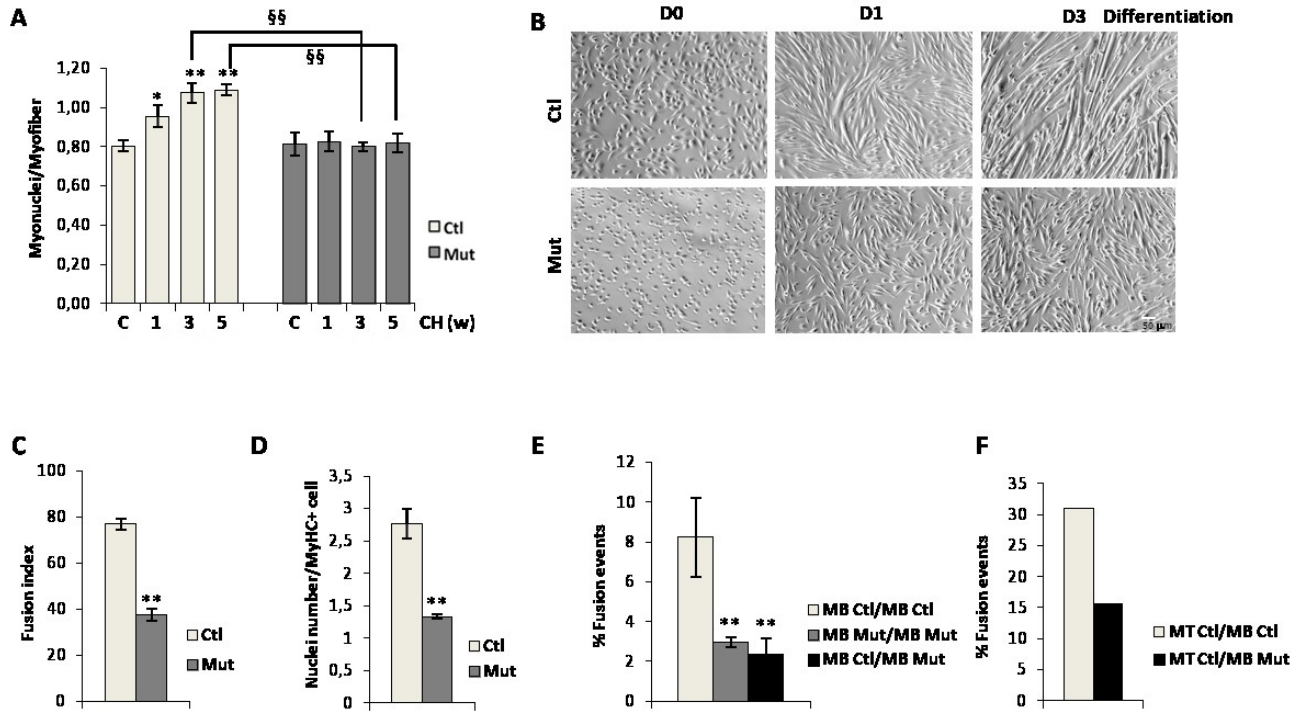
**(C)** Percentage of Myogenin+ cells (MyoG+) in control and mutant FACS cell-sorted satellite cells cultured in rich medium (D0, myoblasts) or after one (D1) and three (D3) days following differentiation induction (n=3).

**(D)** Immunostaining for Myosin Heavy Chain (MyHC) (red), nuclear staining with DAPI (blue) and F-actin staining with Phalloidin (green) on control and mutant cells at day 3 post-differentiation induction.

**(E)** Percentage of nuclei in MyHC+ cells in control and mutant cells three days following differentiation induction (n=3).

Data are mean  $\pm$  s.d. \*\*p< 0.01 versus c or D0, §p< 0.05.

**Figure 4**



**Figure 4: Srf controls satellite cell fusion**

**(A)** Number of DAPI-stained nuclei within the dystrophin-positive sarcolemma was counted before (c) and after 1, 3 and 5 weeks of CH in control and mutant plantaris muscles and are expressed per myofiber (n=5 to 9). Data are mean  $\pm$  s.d. \*p< 0.05 versus c, \*\*p< 0.01 versus c §§p< 0.01.

**(B)** Phase contrast representative pictures of FACS cell-sorted control and mutant satellite cells cultured in rich medium (D0, myoblasts) or after one (D1) and three (D3) days following differentiation induction.

**(C)** Control and Srf mutant myoblasts were induced to differentiate for 3 days. After staining of nuclei with DAPI, the proportion of nuclei within multinucleated cells (fusion index) was calculated (n=3).

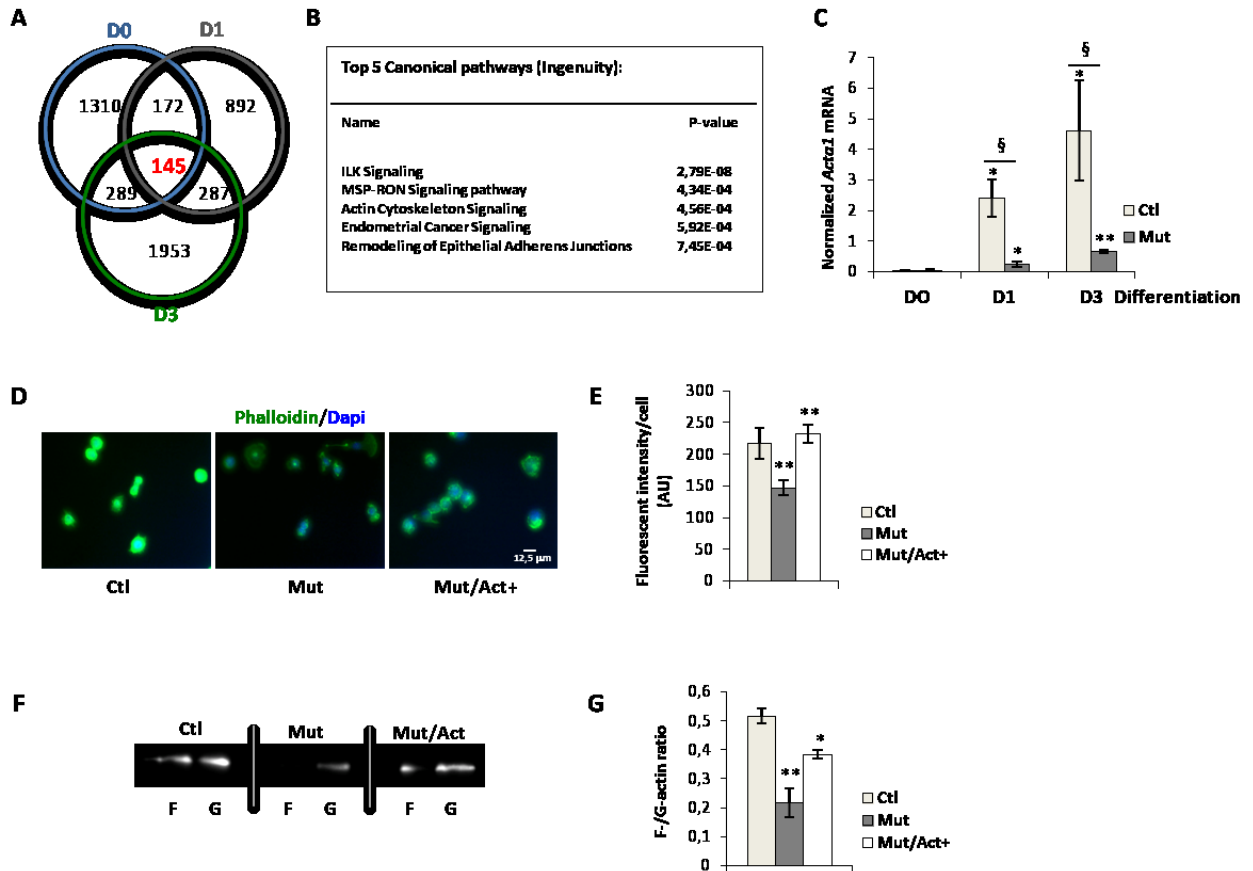
**(D)** The mean number of nuclei per MyHC+ cells was counted in control and mutant cells that were induced to differentiate for 3 days (n=3).

**(E)** Control or mutant myoblasts (MB Ctl or MB Mut) were labeled in green (green cell tracker) and mixed to control or mutant myoblasts labeled in red (orange cell tracker). After 48h of co-culture in differentiation media, myotubes were analyzed for dual labeling. The percentage of dual labeled cells per total number of nuclei (DAPI+) was scored (n=3).

**(F)** Control myotubes (MT Ctl) were labeled in green (green cell tracker) and mixed with control or mutant myoblasts (MB Ctl or MB Mut) labeled in red (orange cell traker). After 48h of co-culture, myotubes were analyzed for dual labeling. The percentage of dual labeled cells per total number of cells was scored.

For C, D, E, F data are mean  $\pm$  s.d. \*\*p< 0.01 versus Ctl.

**Figure 5**



**Figure 5: Alpha actin overexpression restores the impaired actin cytoskeleton organization of *Srf*-deleted myoblasts**

**(A)** Venn diagram showing the intersection between of genes differentially regulated by *Srf* ( $p$  value < 0,05) in myoblasts (D0), in myocytes at the onset of differentiation (D1) and in differentiated cells (D3) expressing or not *Srf* (*Srf*<sup>Δ/Δ</sup> myoblasts transduced with AdGFP or AdCre). In red is indicated the number of genes (145) than are modulated by *Srf* independently of the differentiation state of muscle cells.

**(B)** Top five canonical pathways identified by gene ontology analysis using Ingenuity of the 145 common genes whose expressions are *Srf*-dependent.

**(C)** *Alpha skeletal actin* mRNA (*Acta1*) expression was analysed by qRT-PCR in FACS cell-sorted control and mutant satellite cells cultured in rich medium (D0, myoblasts) or after one (D1) and three (D3) days following differentiation induction. Data (mean ± s.d.) were normalized by *Hmbs* expression (n=3).

**(D)** Staining for F-actin (Phalloidin, green) and for nuclei (DAPI, blue) on control (Ctl), *Srf*-deleted (Mut) myoblasts and *Srf*-deleted myoblasts overexpressing alpha actin (Mut/Act+).



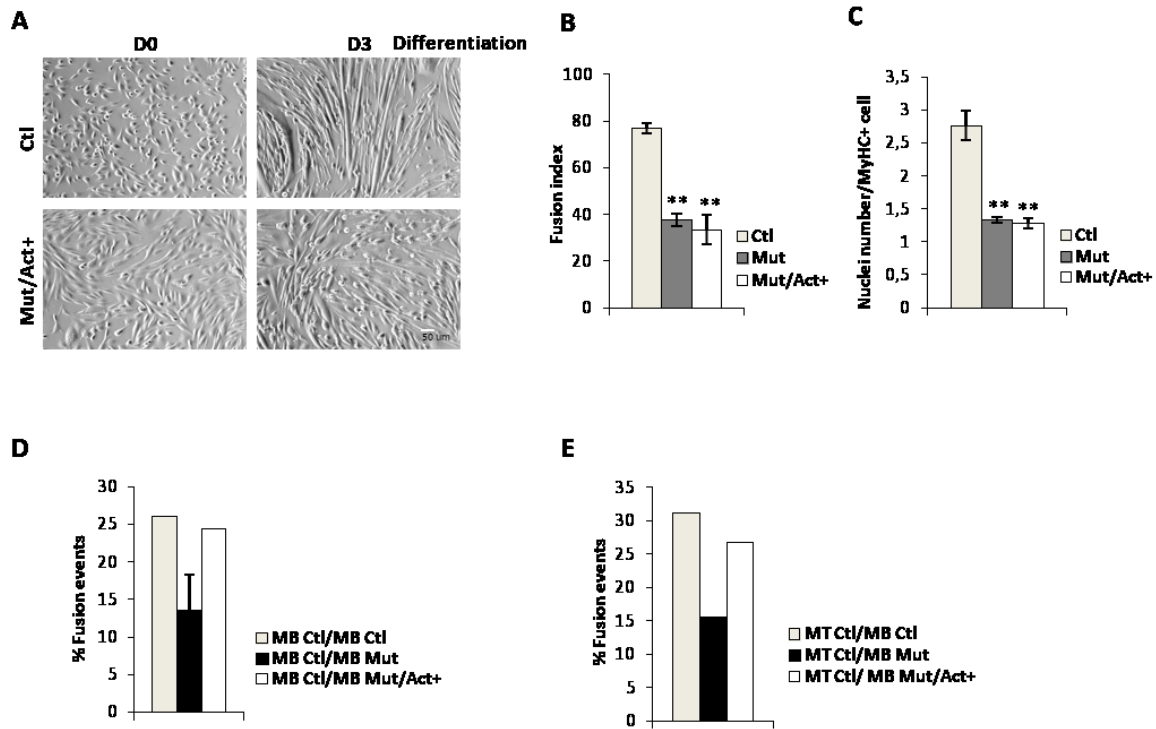
**(E)** Quantification of F-actin by measuring the total phalloidin fluorescence intensity per cell (Image J) in control (Ctl), *Srf*-deleted (Mut) myoblasts and *Srf*-deleted myoblasts overexpressing alpha actin (Mut/Act+) (at least 150 cells analyzed per genotype).

**(F)** Representative immunoblot showing actin in the insoluble (F) versus soluble (G) fractions in control (Ctl), *Srf*-deleted (Mut) myoblasts and *Srf*-deleted myoblasts overexpressing alpha actin (Mut/Act+).

**(G)** Quantification of the F-/G-actin ratio from immunoblots (n=4).

Data are mean  $\pm$  s.d. \*p< 0.05 versus Ctl or D0, \*\*p< 0.01 versus Ctl or D0, §p< 0.05.

**Figure 6**



**Figure 6: Alpha actin overexpression in myoblasts lacking Srf restores asymmetric fusion**

**(A)** Phase contrast representative pictures of FACS cell-sorted control (Ctl) and double mutant (Mut/Act+) satellite cells cultured in rich medium (D0, myoblasts) or after three (D3) days following differentiation induction.

**(B)** Control, Srf mutant (Mut) and double mutant (Mut/Act+) myoblasts were induced to differentiate for 3 days. After staining of nuclei with DAPI, the proportion of nuclei within multinucleated cells (fusion index) was calculated (n=3).

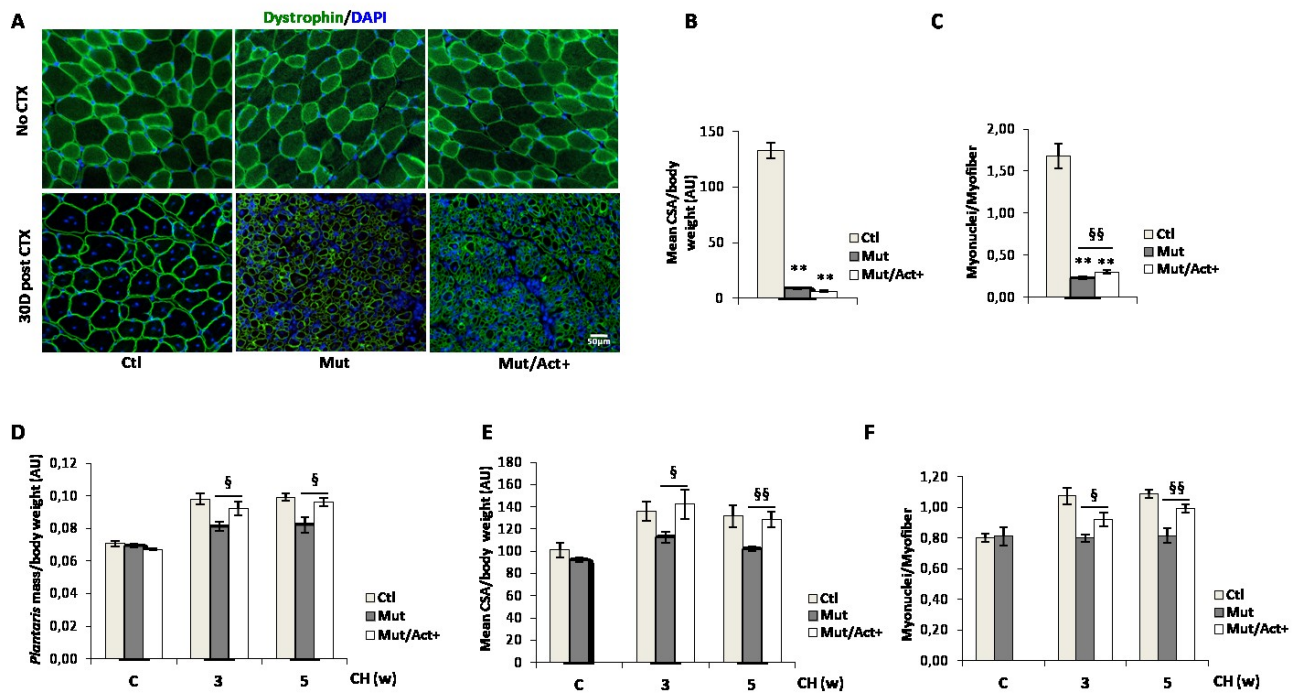
**(C)** The mean number of nuclei per MyHC+ cells was counted in control, Srf mutant (Mut) and double mutant (Mut/Act+) cells that were induced to differentiate for 3 days (n=3).

**(D)** Control, mutant or double mutant myoblasts (MB Ctl, MB Mut or MB Mut/Act+) were labeled in green (green cell tracker) and mixed to control myoblasts labeled in red (orange cell tracker). After 48h of co-culture in differentiation media, myotubes were analyzed for dual labeling. The percentage of dual labeled cells per total number of nuclei was scored.

**(E)** Control myotubes (MT Ctl) were labeled in green (green cell tracker) and mixed with control, mutant or double mutant myoblasts (MB Ctl, MB Mut or MB Mut/Act+) labeled in red (orange cell tracker). After 48h of co-culture, myotubes were analyzed for dual labeling. The percentage of dual labeled cells per total number of cells was scored.

Data are mean  $\pm$  s.d. \*\*p< 0.01 versus Ctl.

**Figure 7**



**Figure 7: Alpha actin overexpression in Srf-deleted satellite cells rescues fusion and hypertrophic growth upon overload**

**(A)** Tibialis anterior muscle sections immunostained for Dystrophin (green) and nuclear staining with DAPI for control (Ctl), Srf mutant (Mut) and double mutant (Mut/Act+) mice untreated (No CTX) and 30 days following cardiotoxin-induced muscle injury (30D post-CTX).

**(B)** Ratio of CSA to body weight of regenerated tibialis anterior muscles 30 days post CTX-induced injury in control (Ctl), Srf mutant (Mut) and double mutant (Mut/Act+) mice (n=4 to 7).

**(C)** Number of DAPI-stained nuclei within the dystrophin-positive sarcolemma was counted in regenerated tibialis anterior muscles 30 days post CTX-induced injury of control (Ctl), Srf mutant (Mut) and double mutant (Mut/Act+) mice are expressed per myofiber (n=5 to 11).

**(D)** Ratio of plantaris mass to body weight before (c) and after 3 and 5 weeks of CH in control (Ctl), Srf mutant (Mut) and double mutant (Mut/Act+) mice (n=5 to 12).

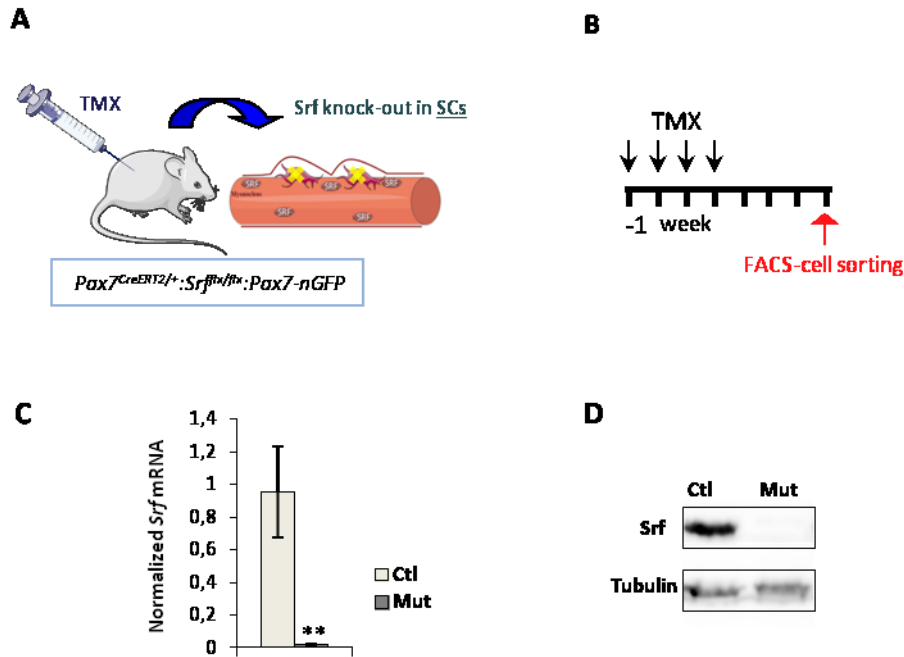
**(E)** Ratio of CSA to body weight before (c) and after 3 and 5 weeks of CH in control (Ctl), Srf mutant (Mut) and double mutant (Mut/Act+) mice (n=5 to 12).

**(F)** Number of DAPI-stained nuclei within the dystrophin-positive sarcolemma was counted before (c) and after 3 and 5 weeks of CH in control (Ctl), Srf mutant

(Mut) and double mutant (Mut/Act+) mice plantaris muscles and are expressed per myofiber (n=5 to 9).

Data are mean  $\pm$  s.d., \*\*p < 0.01 versus Ctl, §p < 0.05, §§p < 0.01. AU, arbitrary units.

**Figure S1**



**Figure S1: Validation of Srf deletion in satellite cells**

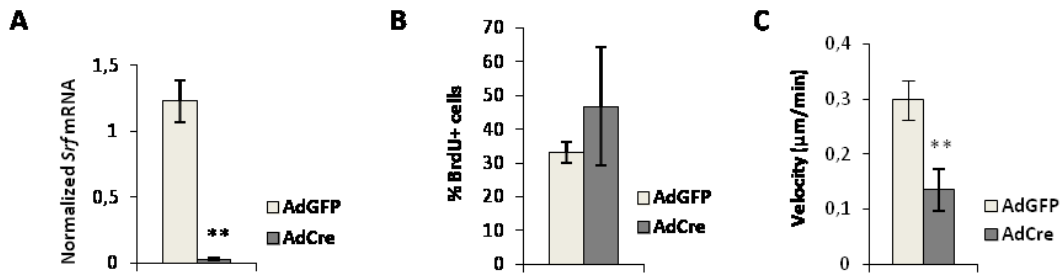
**(A)** Schematic representation of the mouse model used. *Pax7<sup>CreERT2/+</sup>; Srf<sup>flx/flx</sup>; Pax7-nGFP* injected with tamoxifen (TMX) were referred as Mutants and mice untreated with TMX were used as Controls.

**(B)** Srf mutant mice were injected with tamoxifen (TMX) one week before FACS cell sorting of nGFP expressing satellite cells. Sorted SCs when the maintained in culture in complete medium.

**(C)** *Srf* mRNA expression was analysed by qRT-PCR in control and mutant cell-sorted satellite cells maintained in culture. Data (mean ± s.d.) were normalized by *Hmbs* expression. \*\*p< 0.01 versus Ctl.

**(D)** Srf protein was analyzed by western blotting in control and mutant cell sorted satellites cells maintained in culture. Tubulin was used as a loading control.

**Figure S2**



**Figure S2: Ex vivo *Srf* loss does not impact myoblasts proliferation but impairs their motility**

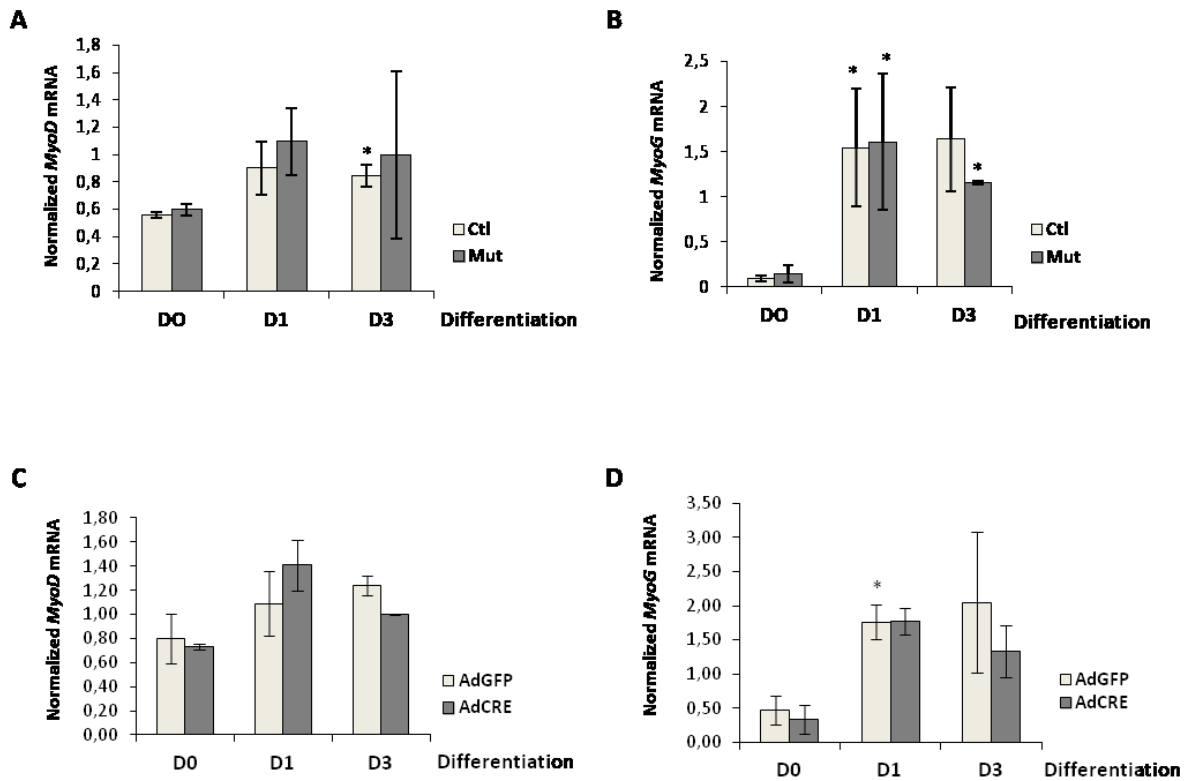
**(A)** *Srf* mRNA expression was analysed by qRT-PCR in *Srf*<sup>flx/flx</sup> myoblasts transduced with Ad-GFP or Ad-Cre to induce *Srf* loss. Data (mean ± s.d.) were normalized by *Hmbs* expression.

**(B)** Normalized percentage of BrdU+ cells in *Srf*<sup>flx/flx</sup> myoblasts transduced with Ad-GFP or Ad-Cre (n=3).

**(C)** Mean velocity (μm/min) of *Srf*<sup>flx/flx</sup> myoblasts transduced with Ad-GFP or Ad-Cre determined by time-laps video-microscopy (at least 100 cells analyzed per genotype).

Data are mean ± s.d. \*\*p < 0.01 versus AdGFP.

**Figure S3**



**Figure S3: *Srf* loss does not impact *MyoD* and *Myogenin* expressions**

**(A)** *MyoD* mRNA expression was analysed by qRT-PCR in FACS cell-sorted control and mutant satellite cells cultured in rich medium (D0, myoblasts) or after one (D1) and three (D3) days following differentiation induction. Data (mean  $\pm$  s.d.) were normalized by *Hmbs* expression (n=3).

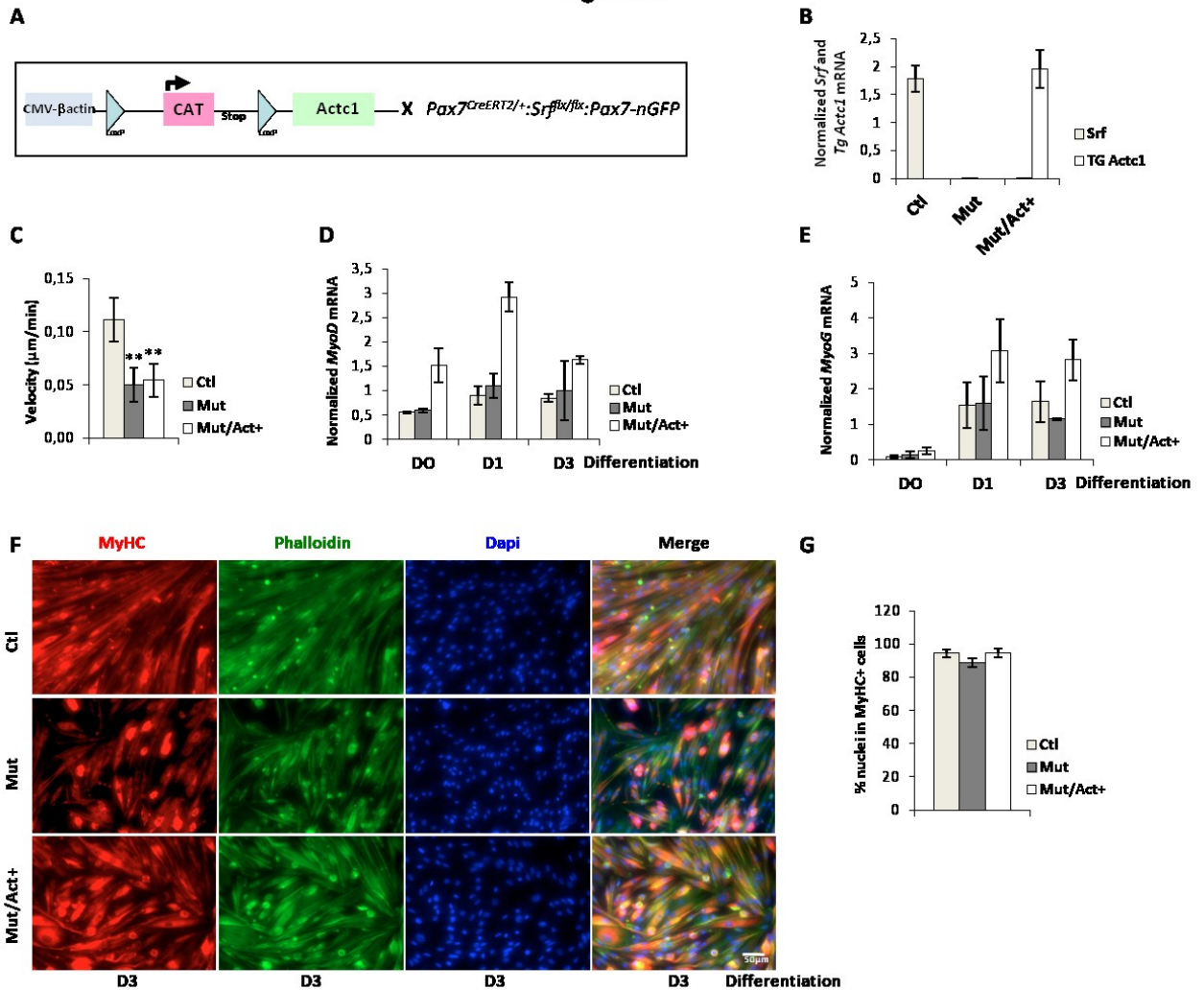
**(B)** *Myogenin* mRNA (*MyoG*) expression was analysed by qRT-PCR in FACS cell-sorted control and mutant satellite cells cultured in rich medium (D0, myoblasts) or after one (D1) and three (D3) days following differentiation induction. Data (mean  $\pm$  s.d.) were normalized by *Hmbs* expression (n=3).

**(C)** *MyoD* mRNA expression was analysed by qRT-PCR in *Srf*<sup>flx/flx</sup> myoblasts transduced with Ad-GFP or Ad-Cre to induce *Srf* loss and cultured in rich medium (D0, myoblasts) or after one (D1) and three (D3) days following differentiation induction. Data (mean  $\pm$  s.d.) were normalized by *Hmbs* expression (n=3).

**(D)** *Myogenin* mRNA (*MyoG*) expression was analysed by qRT-PCR in *Srf*<sup>flx/flx</sup> myoblasts transduced with Ad-GFP or Ad-Cre to induce *Srf* loss and cultured in rich medium (D0, myoblasts) or after one (D1) and three (D3) days following differentiation induction. Data (mean  $\pm$  s.d.) were normalized by *Hmbs* expression (n=3).

Data are mean  $\pm$  s.d. \*p < 0.05 versus D0.

Figure S4



**Figure S4: Alpha actin over-expression does not restore the reduced motility of Srf mutant cell and does not affect myogenic differentiation**

**(A)** Schematic description of the genetic model allowing the concomitant invalidation of Srf and Acta1 overexpression in satellite cells upon tamoxifen injection (*Pax7<sup>CreERT2/+</sup>:Srf<sup>flx/flx</sup>:Pax7-nGFP:CMV-flx-CAT-flx-Actc1* mice).

**(B)** *Srf* mRNA and exogenous rat *Actc1* expressions were analysed by qRT-PCR in control (Ctl), Srf mutant (Mut) and double mutant (Mut/Actc1) cell-sorted satellite cells maintained in culture. Data (mean ± s.d.) were normalized by *Hmbs* expression.

**(C)** Mean velocity (µm/min) of control, Srf mutant and double mutant myoblasts determined by time-laps video-microscopy (more than 100 cells analyzed per genotype). Data are means ± s.d. \*\*p < 0.01 versus c or versus Ctl.

**(D)** *MyoD* mRNA expression was analysed by qRT-PCR in FACS cell-sorted control, mutant and double mutant satellite cells cultured in rich medium (D0, myoblasts) or after one (D1)

and three (D3) days following differentiation induction. Data (mean  $\pm$  s.d.) were normalized by *Hmbs* expression (n=3).

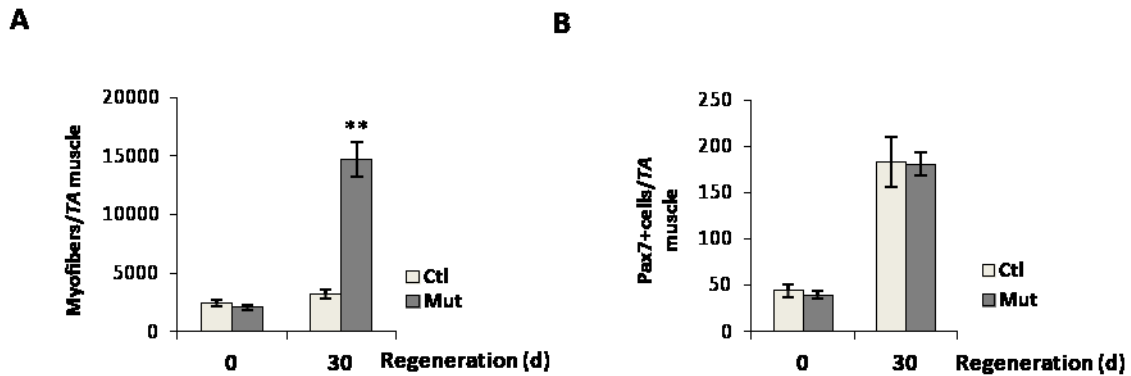
**(E)** *Myogenin* mRNA (*MyoG*) expression was analysed by qRT-PCR in FACS cell-sorted control, mutant and double mutant satellite cells cultured in rich medium (D0, myoblasts) or after one (D1) and three (D3) days following differentiation induction. Data (mean  $\pm$  s.d.) were normalized by *Hmbs* expression (n=3).

**(F)** Immunostaining for Myosin Heavy Chain (MyHC) (red), nuclear staining with DAPI (blue) and F-actin staining with Phalloïdin (green) on control, mutant and double mutant cells at day 3 post-differentiation induction.

**(G)** Percentage of nuclei in MyHC+ cells in control, mutant and double mutant cells three days following differentiation induction (n=2).



**Figure S5**



**Figure S5: Regeneration of control and Srf mutant muscle**

**(A)** Mean myofiber number of control (Ctl) and Srf mutant (Mut) *tibialis anterior* muscles before and 30 days post CTX-induced injury (n=5 to 6).

**(B)** Number of Pax7+ cells per myofiber were quantified in control (Ctl) and Srf mutant (Mut) *tibialis anterior* muscles before and 30 days post CTX-induced injury (n=5 to 6).

**Table S1**

Molecule Name	Full Name	Entrez Gene ID for Mouse	
061009L1BRik	RIKEN cDNA 061009L1B gene		66838
1700086L19Rik	RIKEN cDNA 1700086L19 gene		74284
1700095B10Rik	RIKEN cDNA 1700095B10 gene		67360
2810410L24Rik	RIKEN cDNA 2810410L24 gene		100042332
4833423E24Rik	RIKEN cDNA 4833423E24 gene		228151
4933403O08Rik	RIKEN cDNA 4933403O08 gene		71030
ABHD17C	abhydrolase domain containing 17C		70178
ABRA	actin-binding Rho activating protein		223513
ACTA2	actin, alpha 2, smooth muscle, aorta		11475
ACTB	actin, beta		11461
ACTR3	ARP3 actin-related protein 3		74117
Adam4/Gm4787	a disintegrin and metallopeptidase domain 4	214321 11498	
APOE	apolipoprotein E		11816
ARHGAP29	Rho GTPase activating protein 29		214137
ARL4A	ADP-ribosylation factor-like 4A		11861
ASGR1	asialoglycoprotein receptor 1		11889
ASPA	aspartoacylase		11484
AZIN2	antizym inhibitor 2		242669
BLVRB	biliverdin reductase B (flavin reductase (NADPH))		233016
C20orf24	chromosome 20 open reading frame 24		67388
C82005J03Rik	RIKEN cDNA C82005J03 gene		319979
C8orf4	chromosome 8 open reading frame 4		69068
C9orf16	chromosome 9 open reading frame 16		73737
CA3	carbonic anhydrase 3		12350
CAMK2N1	calcium/calmodulin-dependent protein kinase II inhibitor 1		66259
CD68	CD68 antigen		12514
CNN2	calponin 2		12798
COL4A1	collagen, type IV, alpha 1		12826
COPRS	coordinator of PRMT5, differentiation stimulator		66423
CSRP1	cysteine and glycine-rich protein 1		13007
CSTF2T	cleavage stimulation factor, 3' pre-RNA subunit 2, tau		83410
CTNNA1	catenin (cadherin associated protein), alpha 1		12385
DCCR	dicarbonyl-L-xylulose reductase		67880
DIO2	deiodinase, iodothyronine, type II		13371
DIRAS2	DIRAS family, GTP-binding RAS-like 2		68203
DUSP2	dual specificity phosphatase 2		13537
ECM1	extracellular matrix protein 1		13601
EEF1A2	eukaryotic translation elongation factor 1 alpha 2		13628
EGR1	early growth response 1		13653
EMCN	endomucin		59308
EME1	essential meiotic endonuclease 1 homolog 1 (S. pombe)		268465
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)		13866
F630040K05Rik	RIKEN cDNA F630040K05 gene		433111
FAF1	Fas-associated factor 1		14084
FAM167A	family with sequence similarity 167, member A		219148
FAM171A2	family with sequence similarity 171, member A2		217219
FAM53B	family with sequence similarity 53, member B		77938
FBXO4	F-box protein 4		106052
FERMT2	fermitin family homolog 2 (Drosophila)		218952
FHL1	four and a half LIM domains 1		14199
FHL2	four and a half LIM domains 2		14200
FLNA	filamin, alpha		192176
FRAS1	Fraser syndrome 1 homolog (human)		231470
GADD45G	growth arrest and DNA-damage-inducible 45 gamma		23882
GCHFR	GTP cyclohydrolase I feedback regulator		320415
Gm6978	predicted gene 6978		629480
Gm7056	predicted gene 7056		631105
Gm20603	predicted gene 20603		100503553
GML	GPI anchored molecule like protein	15202 625599	
GNA13	guanine nucleotide binding protein, alpha 13		14674
GNPMB	glycoprotein (transmembrane) nmb		93695
GSTM1	glutathione S-transferase, mu 1		14863
Gucy2g	guanylate cyclase 2g		73707
HPSE	heparanase		15442
IFITM3	interferon induced transmembrane protein 3		66141
IGFBP7	insulin-like growth factor binding protein 7		29817
IL2RG	interleukin 2 receptor, gamma chain		16186
ILK	integrin linked kinase		16202
ITGB1BP2	integrin beta 1 binding protein 2		26549
KCNAB2	potassium voltage-gated channel, shaker-related subfamily, beta member 2		16498
KLF5	Kruppel-like factor 5		12224
		18048 16622 16612 16616 16619 13646	
		18050 16624 16623 16613 16615 16617	
		16618 13648	
KLK3	Kallikrein related peptidase 3		

Molecule Name	Full Name	Entrez Gene ID for Mouse	
KRT18	keratin 18		16668
LARGE	like-glycosyltransferase		16795
LAYN	layilin		244864
LBH	limb-bud and heart		77889
LDLR	low density lipoprotein receptor		16835
LILRB3	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	18733 18726 18729 18725 18722 100038909 100041146	
LIMA1	LIM domain and actin binding 1		65970
LSP1	lymphocyte specific 1		16985
MAPKAPK2	MAP kinase-activated protein kinase 2		17164
MARVELD1	MARVEL (membrane-associating) domain containing 1		277010
MARVELD2	MARVEL (membrane-associating) domain containing 2		218518
ME3	malic enzyme 3, NADP(+)-dependent, mitochondrial		109264
MED14	mediator complex subunit 14		26896
MED12L	mediator of RNA polymerase II transcription, subunit 12 homolog (yeast)-like		329650
MGST2	microsomal glutathione S-transferase 2		211666
MPDU1	mannose-6-phosphate utilization defect 1		24070
MRPS6	mitochondrial ribosomal protein S6		121022
MS4A10	membrane-spanning 4-domain, subfamily A, member 10		69826
MSRB3	methionine sulfoxide reductase B3		320183
MYADM	myeloid-associated differentiation marker		50918
MYL9	myosin, light polypeptide 9, regulatory		98932
MYO18B	myosin XVIIIb		74376
NAB2	Ngf-A binding protein 2		17937
Ngp	neutrophilic granule protein		18054
NMB	neurokinin B		68039
NUDT14	nudix (nucleoside diphosphate linked moiety X)-type motif 14		66174
NXT2	nuclear transport factor 2-like export factor 2		237882
OXC11	3-oxoacid CoA transferase 1		67041
PARP8	poly(ADP-ribose) polymerase family, member 8		52552
PCDH19	protocadherin 19		279653
PDLIM7	PDZ and LIM domain 7		67399
PFAS	phosphoribosylformylglycinamide synthase (FGAR amidotransferase)		237823
PIK3CB	phosphatidylinositol 3-kinase, catalytic, beta polypeptide		74769
PLA2G10	phospholipase A2, group X		26565
Plp1	phospholipid phosphatase 1		19012
PLTP	phospholipid transfer protein		18830
PPP2R3A	protein phosphatase 2, regulatory subunit B', alpha		235542
PRSS23	protease, serine, 23		76453
PTGES3L	prostaglandin E synthase 3 (cytosolic)-like		73635
REEP6	receptor accessory protein 6		70335
RFTN2	rafflin family member 2		74013
RSU1	Ras suppressor protein 1		20163
SASH1	SAM and SH3 domain containing 1		70097
SBK3	SH3 domain binding kinase family member 3		381835
SDHD	succinate dehydrogenase complex, subunit D, integral membrane protein sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C		66925
SEMA3C			20348
Serpina1f	Serpina1f		68348
SH3BP2	SH3-domain binding protein 2		24055
SLC9A3R2	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2		65962
Snora41	small nucleolar RNA, H/ACA box 41		100217464
Sorbs2os	sorbin and SH3 domain containing 2, opposite strand		319940
SRF	serum response factor		20807
SVIL	supervillin		225115
SYT13	synaptotagmin XIII		80976
Tac4	tachykinin 4		93670
TAGLN	transgelin		21345
TBC1D1	TBC1 domain family, member 1		57915
TGFBI1	transforming growth factor beta 1 induced transcript 1		21804
TMEM109	transmembrane protein 109		68539
TNC	tenascin C		21923
Tpm1	tropomyosin 1		22003
Tpm4	tropomyosin 4		326618
TRAF3IP3	TRAF3 interacting protein 3		215243
TSC22D1	TSC22 domain family, member 1		21807
TSPO2	translocator protein 2		70026
UBALD1	UBA-like domain containing 1		207740
UGDH	UDP-glucose dehydrogenase		22235
Vmn1r214 (includes others)	vomeranasal 1 receptor 214	171273 100312485	
WDR1	WD Repeat Domain 1		22388
ZBBX	Zinc Finger Protein B-Box Domain Containing		213234
ZBTB41	Zinc Finger and BTB Domain Containing 41		226470
ZNF365	Zinc Finger Protein		216049
ZNF878	Zinc Finger Protein		170938

**Table S1: List of the genes whose expressions in muscle cells depend on Srf**

Transcriptomic analysis of genes regulated by Srf in myoblasts (*Srf<sup>Δx/lx</sup>*) transduced with AdGFP or AdCre adenoviruses (D0) and during the course of differentiation (D1 and D3).

This list represents the 145 genes that are regulated by Srf in myoblasts (D0) and in differentiating cells (D1 and D3) with a p value < 0.05 versus AdGFP.

## Part 2: Srf role in Satellite Cells during Skeletal Muscle Regeneration

Skeletal muscle has a remarkable ability to regenerate after injury. Responding to injury, skeletal muscle undergoes a highly orchestrated degeneration and regenerative process that takes place at the tissue, cellular and molecular levels (Yin et al., 2013a). Muscle regeneration depends on satellite cells (SCs), the resident muscle stem cells. Upon muscle trauma, SCs become activated, which promotes their subsequent proliferation, differentiation into fusion-competent myoblasts while a subset of activated SCs escape the myogenic destiny and self-renew to replenish the pool of muscle satellite cells thereby ensuring that the capacity to respond to future injuries is maintained in the muscle. Myogenic cells differentiate and fuse to pre-existing damaged fibers or fuse between them to form *de novo* myofibers. This process recapitulates many phases of embryonic myogenesis while the SC-mediated fusion during functional overload mimics the postnatal growth in muscles without (or limited) *de novo* formation of myofibers.

As in the case of CH model we used the mouse strain of  $Pax7^{CreERT2/+};Srf^{flox/flox};Pax7-nGFP$  conditional genotype to study the regenerative capacity of skeletal muscles harboring Srf-depleted SCs. After tamoxifen (TMX) administration the Cre-mediated excision of the Srf exon 2 is achieved and these are referred as the mutant (Mut) mice. We used as control either mice ( $Pax7^{CreERT2/+};Srf^{flox/flox};Pax7-nGFP$ ) not injected with tamoxifen (Ctl) or  $Pax7^{+/+};Srf^{flox/flox}$  mice TMX-injected. Both control mice give the same results (muscle mass, CSA, myofibers, Pax7+cells, MyoG+cells). The results of control mice were not pooled together and heterozygous controls' results will be shown for clarity reasons. In the study of the regenerative capacity of mice' skeletal muscles, myotoxins isolated from snake venoms (here cardiotoxin is used) are widely used. Cardiotoxin (CTX) is a small basic membrane-active polypeptide with endogenous phospholipase A2 activity and their myotoxic activity results from their ability to disrupt the integrity of skeletal muscle sarcolemma, thereby inducing a calcium influx that culminates in cell death. The basal lamina of the muscle fiber is not damaged, and the regeneration of the damaged muscle fiber takes place within the "old" basal lamina tube (Harris 2003).

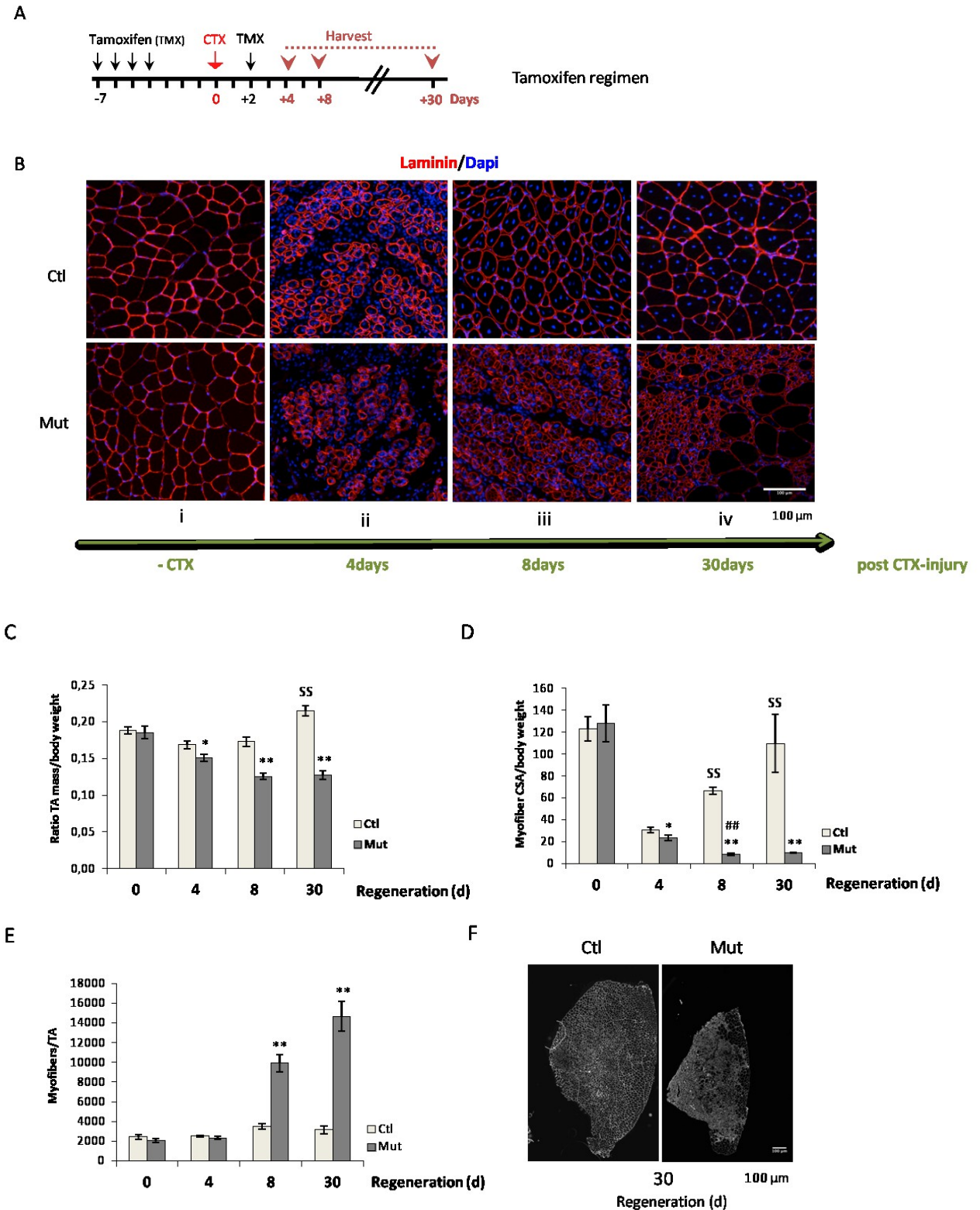
### 1. Conditional Inactivation of Srf in Satellite Cells Leads to Impaired Muscle Regeneration

To assess the regenerative capacity of skeletal muscles harboring Srf-depleted SCs, we injected CTX into *tibialis anterior* (TA) muscles to chemically induce injury. Mice were sacrificed and TA muscles were harvested at 4, 8 and 30 days after CTX-injury (Figure 22A) for further histological analyses. Laminin (an ECM glycoprotein) staining contours the

unaffected myofibers and the regenerated centrally nucleated myofibers. At the steady uninjured state (-CTX) control and mutant TA muscles (transversal sections) look similar without any signs of wasting (Figure 22B i). One month after CTX-injury control muscle (Figure 22B Ctl-iv) show a complete recovery with the normal myofiber caliber. The presence of centrally-nucleated myofibers indicates the recent regenerative process that it took place. The deficiency in the regeneration response in the mutant mice is evident (Figure 22B Mut-iv). Srf-mutant mice have an impaired regeneration, as we can observe it from the 8th day where the new formed myofibers have a diminished size (Figure 22B Mut-iii), a phenotype which is more obvious 30 days after the CTX-injury (Figure 22B Mut-iv). Interestingly, as depicted by the laminin staining mutant new-formed fibers present a completely different phenotype in their shape with “marguerite”-like appearance mainly at 8 days post CTX-injury. At the end of regeneration mutant mice seem to have numerous, atrophic new formed myofibers as concluded by the histological analyses.

Inactivation of Srf in satellite cells did not affect the weight of uninjured TA muscle (Figure 22C right). However the TA muscle mass (counted as TA muscle mass per mouse body weight) growth phase normally occurred during the regeneration process, between 8 and 30 days in our control, is affected in the mutant mice (Figure 22C). To further determine whether the decreased muscle mass in mutant mice resulted from the reduced myofiber size or number or both and quantify the observations of the histological readout, we enumerated the total number of regenerating myofibers and calculated the myofiber cross-sectional area (CSA). The number and the size of myofibers were counted during the different time points of phenotypic examination of regeneration in TA muscle. Myofiber CSA increases from day 4 to day 8, until the day 30 after CTX-injury in control mice. Mutant mice show ~8-fold smaller size of new-formed myofibers (CSA) compared to control at day 8 and this difference is more marked at day 30 (Figure 22D). Also mutant mice exhibit ~5-fold more myofiber number at day 30 (Figure 22E) while the number of myofibers in the control mice remains constant during the regeneration process, thereby implying a genetic control in the regulation of myofiber number. Decreased myofiber size and abnormal numerous myofiber numbers characterize the diminished growth phase through the regeneration in TA muscles-harboring Srf-depleted SCs. Therefore mutant muscles remain smaller compared to control and to their pre-injury state (Figure 22F).

These results show a defective regeneration after CTX-injury on TA muscle-harboring Srf-depleted SCs.



**Figure 22** : Impaired muscle regeneration. A) Tamoxifen (TMX)-regimen and experimental procedure illustrated in a timeline. B) Kinetics of cardiotoxin (CTX)-induced regeneration in *tibialis anterior* (TA) muscles of control and mutant mice, at steady state (-CTX), 4, 8 and 30 days post CTX-injury. Laminin staining (depicted in red) on TA cryosections permits the visualization of the myofibers and the progress in the regeneration process. Hoechst staining (nuclei dying in bleu) permits the distinction of the regenerating myofibers as they are centrally-nucleated compared to undamaged myofibers with peripherally-located nuclei. C)

Ratio of TA muscle mass to mouse body weight during the regeneration process. D) Myofiber caliber as counted as cross-sectioned area (CSA) on TA cryosections by a laminin immunofluorescent staining and normalized to body weight. E) Myofiber number counted in the regenerating area ( $\geq 50\%$  of total area) on TA sections. F) TA muscle size 30 days post CTX-injury. Numerical data are presented as mean  $\pm$  s.d. (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$  indicated in the mutant series versus control at each time point. (S)  $p < 0.05$ , (SS)  $p < 0.01$  indicated in the control series compared to control from chronically previous time point. (#)  $p < 0.05$ , (##)  $p < 0.01$  indicated in the mutant series compared to mutant from chronically previous time point. Student's t-test was used for statistical analyses. At least  $n = 3$  mice per group.

## 2. Srf-loss has an Impact in Satellite Cell Pool at Early Stages of Regeneration

Trying to understand which function of SCs is affected upon Srf-loss, we enumerated the number of SCs, based on the Pax7 expression, on TA muscle sections. At the steady state (-CTX) both control and mutant TA muscles have the same number of Pax7+ cells, showing that Srf inactivation in SCs does not affect their number at the basal state. At all time points of regeneration we observe a severe diminution of Pax7+ cells in the mutants compared to control mice (Figure 2A), when they were normalized to regenerating myofiber numbers. M-cadherin was used as another SC marker for counting the SCs (Yin et al., 2013a) and we confirmed the same diminution of M-cadherin+ cells at 8 days post CTX-injury (Figure 23B).

In our case the normalizing question arises because the mutant TA muscles remain smaller but also they possess much more myofibers compared to control. When we normalize per muscle entity we do not observe anymore the diminution of Pax7+ cells absolute numbers at 8 and 30 days post CTX-injury (Figure 23C left). M-cadherin+ cells absolute number, at 8 and 30 days post CTX-injury, confirms the same result (Figure 23D). Nevertheless at 4 days post CTX-injury, the number of Pax7+ cells, as they were counted in whole, under regeneration area of TA muscle section, is much lower compared to control. The percentage of lesion at least of 60% and the counted area, in the TA transversal section where the SCs were enumerated, were taken in considerations for counting and comparing the absolute number of Pax7+ cell population.

Whatever the way of normalization, the reduced number of Pax7+ cells in the mutant mice at the early stage of 4 days of regeneration indicates a defect in their maintenance which is associated with early events in the SC functions. At this stage the effect of the Srf-mutant new formed myofibers in the SCs could be neglected due to a non *ad integrum* reconstitution of the damaged muscle.

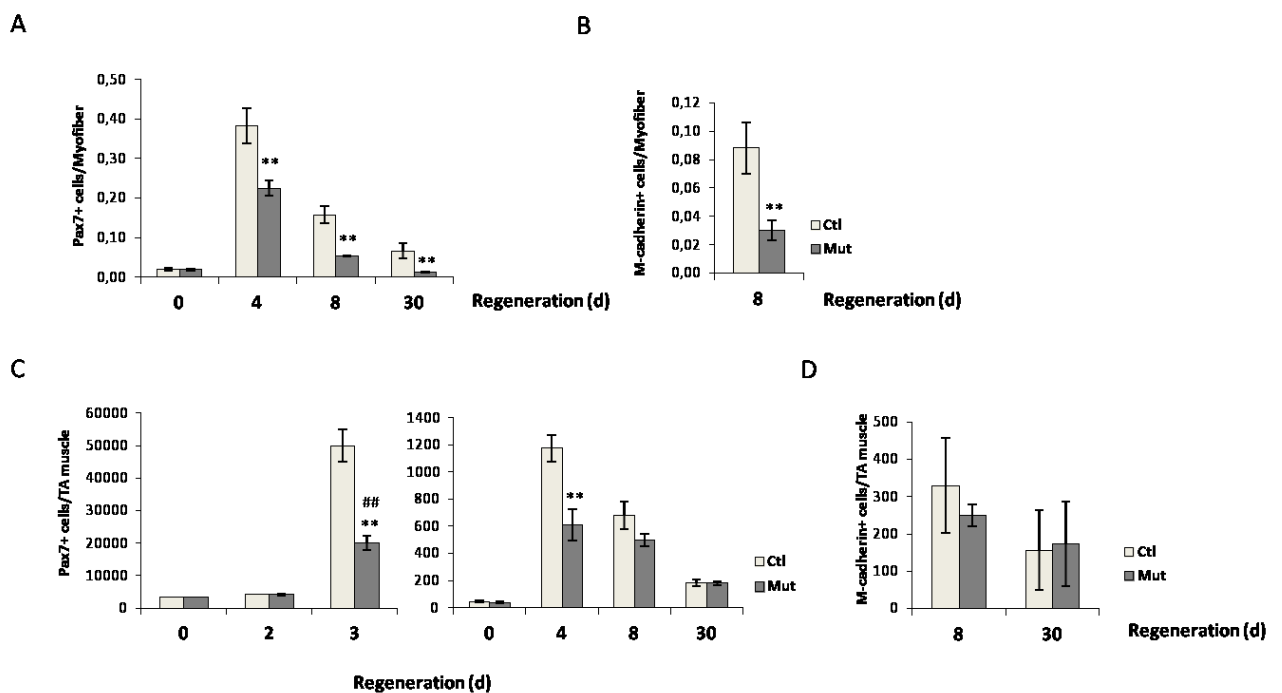
FACS-purified SCs quantification analysis will shed light on the number of Pax7+ cells at earlier stages after CTX-injury in TA muscle, as the IF staining approach is not optimal due to increased auto-fluorescence/not specific signal observed in tissue lesions. TA-derived SCs



are purified by FACS based on the Pax7-GFP transgene expression and the obtained values could be compared to the IF counts (Pax7+ cells/muscle) as both techniques are based on the fluorescent signal (direct or indirect).

Similarly to IF staining, FACS-purified SCs quantification analysis shows the same number of SCs between control and mutant mice at the steady state. Control SC numbers at day 2 after CTX-injury are almost as many as at the pre-injured state (-CTX) and they are highly increased at day 3 post CTX-injury, a time point where the mutant SCs hardly increase their number (Figure 23C right). Therefore, FACS analysis confirmed the observed phenotype in the mutant mice, at day 4 post CTX-injury from IF staining.

Taken together, all these results show that the initial phases of SC recruitment during injury-induced regeneration are impaired by the Srf-loss as it was shown by the reduced numbers of mutant SCs despite the fact that progressively this phenotype is not further seen. Such initial events could be the activation and/or proliferation of SCs.

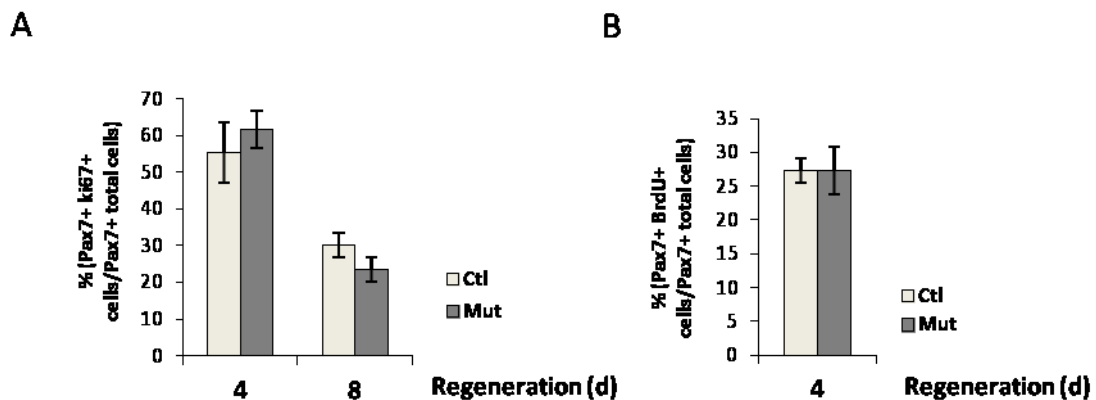


**Figure 23** : Satellite cells enumeration. A) Pax7+ cells normalized per regenerating myofiber on TA transversal sections at 4, 8 and 30 days post CTX-injury. B) M-cadherin+ cells normalized per regenerating myofiber at day 8 post CTX-injury. C) (right) FACS-purified SCs counts from uninjured TA muscles and at 2 and 3 days post CTX-injury. (left) Absolute number of Pax7+ cells per TA muscle entity. D) Absolute number of M-cadherin+ cells per TA muscle at day 8 post CTX-injury. Numerical data are presented as mean  $\pm$  s.d. \*P < 0.05, \*\*P < 0.01 indicated in the mutant series versus control at each time point. (#) p < 0.05, (##) p < 0.01 indicated in the mutant series compared to mutant from chronically previous time point. Student's t-test was used for statistical analyses. At least n = 3 mice per group.

### 3. Normal Proliferation of Srf-depleted Satellite Cells

The reduced number of Srf-depleted SCs could be a consequence of altered proliferation or cell cycle progression. Ki67 nuclear antigen marks proliferating cells in all phases of the cell cycle, except in the G0 phase (Scholzen and Gerdes, 2000). By a double IF staining of Pax7 and ki67 on TA muscle sections, we checked for the proliferation potential of SCs *in vivo* under injury signal. Percentage (%) of Pax7+ ki67+ cells reveals no difference in the cycling SCs in both control and mutant mice at day 4 and 8 post CTX-injury (Figure 24 A). Furthermore the S-phase entry was assessed by a double Pax7 and BrdU staining 4 days post CTX-injury, a time point where extensive proliferation takes place. BrdU was administered IP twice, 24h and 6h before sacrifice at day 4 post CTX-injury. Likewise to ki67 proliferation marker, mutants SCs incorporate BrdU (a thymidine analogue) at the same manner with the controls, meaning that they do not present any defect in the S-phase entry upon Srf-loss (Figure 24 B). Consistent to the *in vivo* results are the EdU-incorporation by proliferating myoblasts *in vitro* (5 day-culture post FACS-isolation) presented previously in CH model (see in the article Figure 2C).

Therefore, these results confirm our results from CH model that Srf does not seem to affect the proliferation potential of SCs, with emphasis in the S-phase entry.

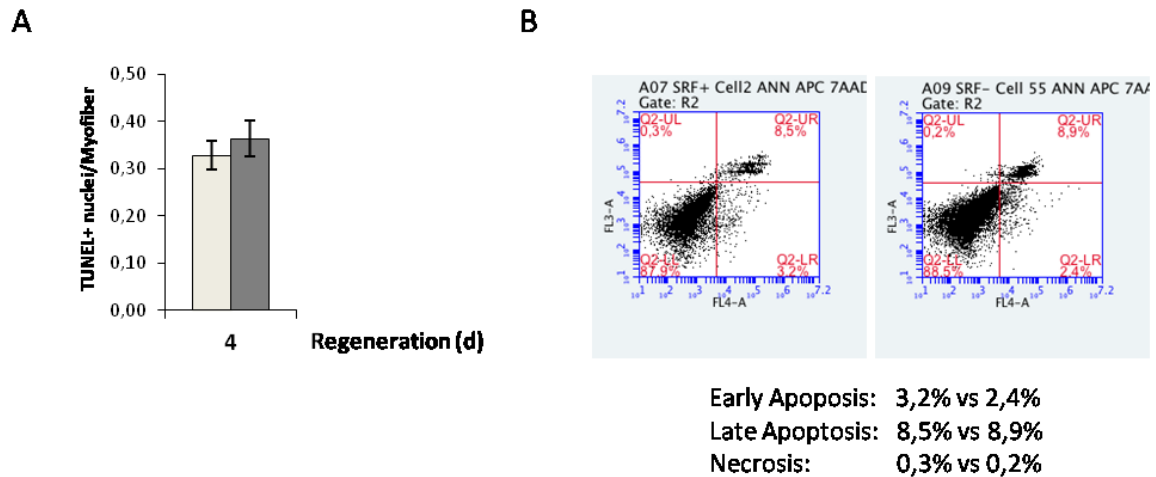


**Figure 24** : Normal proliferation of Srf-depleted SCs. A) Percentage (%) of Pax7+ki67+ cycling cells of the total Pax7+ population at 4 and 8 days post CTX-injury. B) BrdU pulse 24h and 6h before sacrifice. Percentage (%) of Pax7+BrdU+ cells of the total Pax7+ population at 4 days post CTX-injury.

### 4. Do Srf-depleted Satellite Cells Undergo Apoptosis?

The reduced number of Pax7+ cells observed in the mutant mice at the early stages of regeneration could be attributed from an altered cell survival. To test this hypothesis we performed a TUNEL assay to detect the apoptosis based on labeling of DNA strand breaks,

in the regenerating muscle at day 4 post CTX-injury. Although we did not achieve a double staining with the Pax7 marker to estimate the apoptotic fraction in the Pax7+ population, the overall TUNEL staining in the regenerating area in TA muscles was not more positive in the mutant muscles (Figure 25A). Further apoptosis detection with Annexin V labeling on cultured SCs and flow cytometry did not showed more apoptotic cells in the mutant SCs compared to control (Figure 25B).



**Figure 25** : Apoptosis assessment. A) TUNEL+ nuclei per myofiber in the regenerated area on TA muscles at day 4 post CTX-injury. B) Annexin staining in control and mutant cultured myoblasts under proliferation conditions. Numerical data are presented as mean  $\pm$  s.d. Student's t-test was used for statistical analyses. At least n = 3 mice per group.

Transcriptomic analysis was conducted in control versus mutant SCs isolated from the TA muscle which was CTX-injured 3 days before FACS-sorting. This transcriptomic analysis revealed many genes and pathways involved in cell death and apoptosis (data not shown). Further analysis needs to be done to clarify whether SCs lacking Srf undergo apoptosis resulting in the decreased number of Pax7-expressing SCs at day 3 and 4 post CTX-injury.

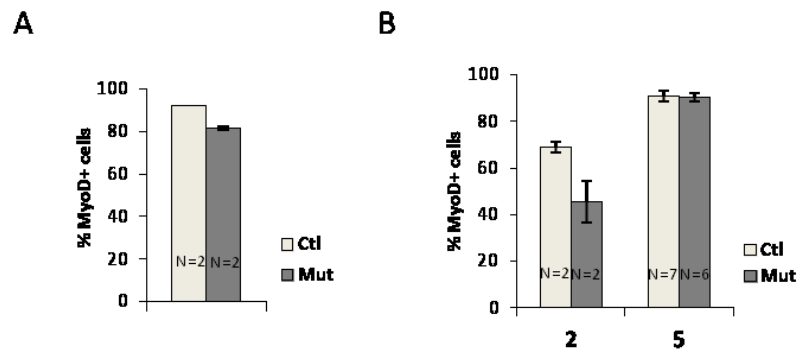
## 5. An Activation Delay in SCs lacking Srf?

We next hypothesized that SCs lacking Srf are not able to efficiently respond to activation cues, as control SCs do upon injury for driving their expansion, leading thus to the reduced descendants cells. The activation of satellite cells from a state of quiescence and their subsequent progression along the myogenic lineage are controlled by various transcription factors MRFs, and the MyoD is one of them, which is highly upregulated in activated SCs. In the CH model, it has been shown that MyoD is not affected upon Srf inactivation. FACS-purified SCs from TA muscle, 2 days-damaged after CTX-injury were kept

in culture for 2 days before MyoD staining. We observed the same percentage of MyoD+ cells in both control and mutant mice (Figure 26A). These are preliminary data (N=2).

Moreover, FACS-purified SCs from uninjured TA muscle were cultured for 2 and 5 days. The percentage of satellite cell-derived MyoD+ myoblasts is lower in mutants than controls after 2 days in culture post FACS-isolation whereas the percentage of MyoD+ myoblasts is the same in both controls and mutants after 5 days in culture (Figure 26B). These data are preliminary and they needed to be confirmed in the appropriate *ex vivo* model of isolated myofibers for such kind of experiments.

For the present time we can only suppose that there is not a major defect in the activation of Srf-depleted SCs but a delay which could eventually result in the diminished number of Pax7+ cells at early stages of their expansion.



**Figure 26** : Activation assessment. A) Activated SCs were isolated from injured TA muscles at 2 days post CTX-injury and cultured for subsequent 2 days before MyoD immunostaining. % MyoD+ cells normalized to total nuclei. Preliminary data with N=2 per group. B) Quiescent SCs were isolated from uninjured TA muscles and cultured for subsequent 2 and 5 days before MyoD immunostaining. % MyoD+ cells normalized to total nuclei. Preliminary data with N=2 per group at day 2. Numerical data are presented as mean  $\pm$  s.d.

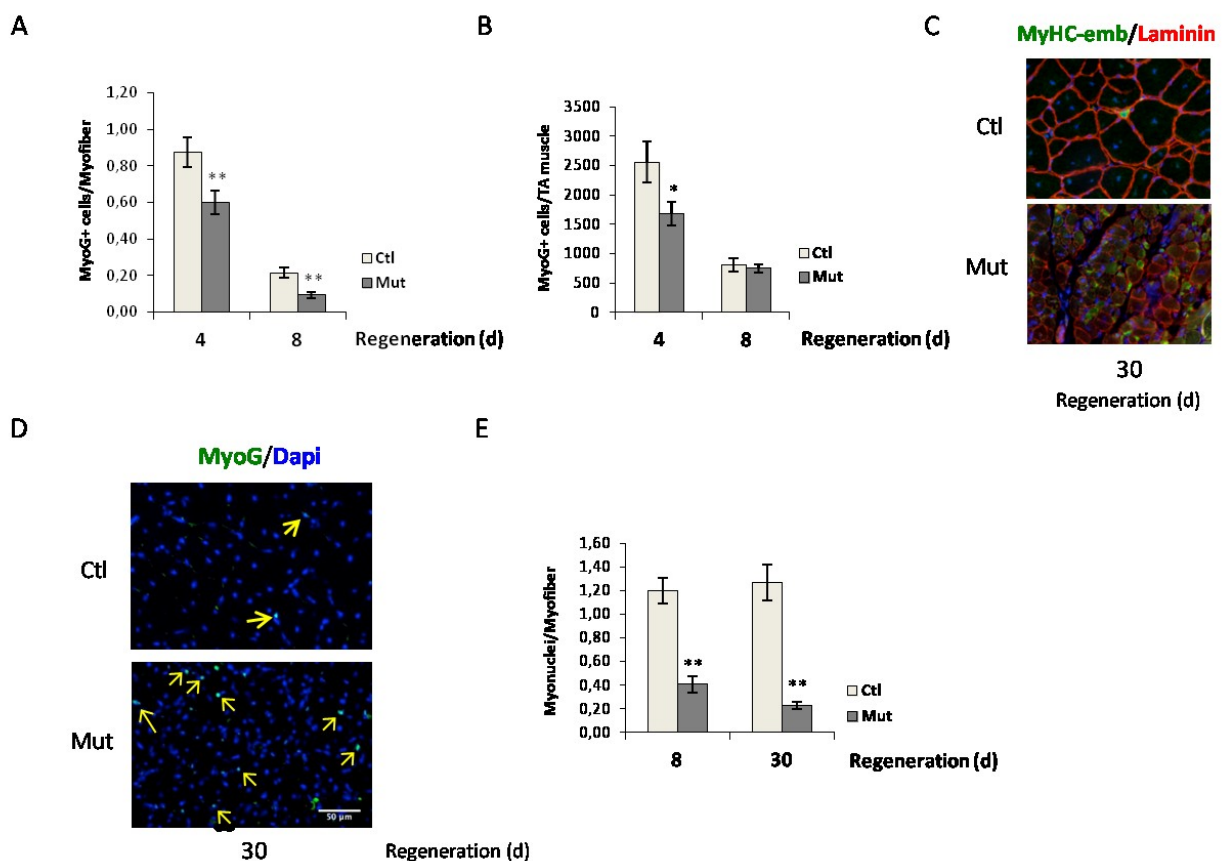
## 6. Normal Early Differentiation of Srf-depleted Myoblasts but Immature and Fusion-Deficient Srf-depleted Myoblasts

We showed with the CH model that the Srf-loss in the SCs does not affect their engagement in the myogenic lineage regarding the expression of MyoG *in vivo* (1 week post CH) and *in vitro*. MyoG+ cells per myofiber are less in mutant at 4 and 8 days post CTX-injury (Figure 5A) as they are the Pax+ cells. Similarly the absolute number of MyoG+ cells does not differ in control and mutant at day 8 of regeneration but it is reduced at day 4 of regeneration (Figure 5B). MyoG+ cells are the descendants of SC-derived Pax7+ cells and the reduced number of MyoG+ cells at day 4 is a consequence of the reduced Pax7+ cells at the same time point.

Checking for the expression of embryonic myosin heavy chain (MyHC) which is a marker of late differentiation, we observe that at 8 days we have more MyHC-emb+ myofibers in the mutant muscles in comparison to control (data not shown). Also MyHC-emb expression persists in the mutants at 30 days post CTX-injury, showing an immaturity of the new-formed myofibers. Moreover at day 30 of regeneration, sparse MyoG+ cells is found in control muscles whereas MyoG+ cells are still present (Figure 5D), indicating thus a retard in terminal differentiation.

As previously described in the CH model and with the *in vitro* studies, the fusion capacity of Srf-depleted myoblasts is highly decreased in the mutant muscles at 4 and 8 days post CTX-injury. It is noteworthy that many “empty” myofibers, with no nuclei inclusion, are observed on TA transversal sections.

All these results show a defect in the terminal differentiation with the accumulation of immature myocytes not capable to fuse and to reconstitute the damaged muscle.



**Figure 27** : Aberrant terminal differentiation of Srf-depleted SCs. A) MyoG+ cells normalized per regenerating myofiber on TA transversal sections at 4 and 8 days post CTX-injury. B) Absolute number of MyoG+ cells per TA muscle entity at 4 and 8 days post CTX-injury. C) IF detection of embryonic myosin heavy chain (MyHC-emb) on TA muscle sections at day 30 post CTX-injury. D) IF of myogenin expression on TA muscle sections at day 30 post CTX-injury. E) Fusion index as counted by the ratio of number of myonuclei to number of regenerating myofiber at 8 and 30 days post CTX-injury. Numerical data are presented as

mean  $\pm$  s.d. (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$  indicated in the mutant series versus control at each time point. Student's t-test was used for statistical analyses. At least  $n = 3$  mice per group.

## 7. The Srf-loss in SCs Does Not Impair their Self-Renewal. Srf is Needed in SCs to Sustain the Muscle Homeostasis upon Consecutive Regeneration Demands

Another aspect of SC pool maintenance is the self-renewal required in muscle homeostasis for ensuring future lesions. 30 days post CTX-injury control and mutant mice seem to have the same number of Pax7+ cells per muscle (see Figure 23C). However the Srf-depleted SCs display an increased cycling shown by the percentage of Pax7+ ki67+ cells at day 30 after CTX-injury (Figure 28A). At this time point we cannot neglect the niche-effect and as the myofiber-niche is also Srf mutant, this could have an impact in the self-renewal capacity of the Srf-depleted SCs further affecting their return to quiescence.

The use of CH model helped us to distinguish between SC- or myofiber-dependent effect, in the elevated number of cycling Pax7+ cells at 30 days after CTX-injury, as the myofiber is preserved wild type. 5 weeks post CH-induction the percentage of EdU+ cells<sup>9</sup> seems to be similar in control and mutant Pax7+ cells (Figure 28B) implying thus a myofiber-dependent effect in the proliferation of Srf-depleted myoblasts at late stages of regeneration.

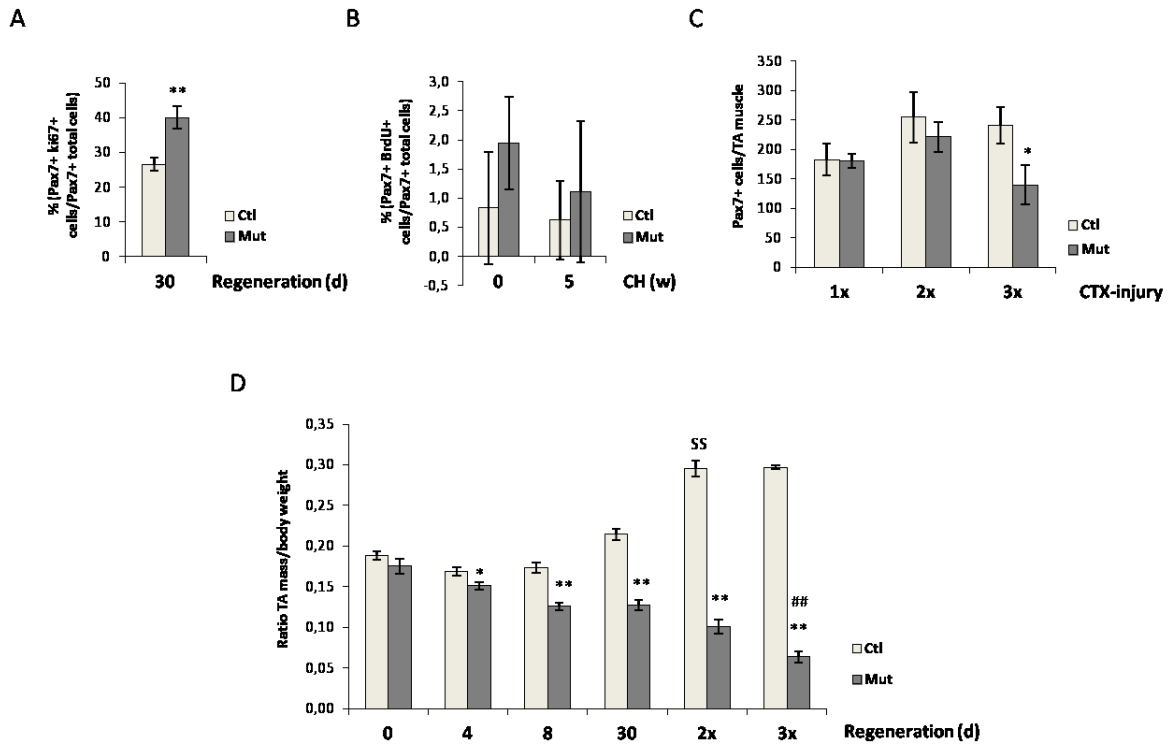
Further we wanted to test if the reduced number of Pax7+ SCs at early stages and the increased ratio of cycling cells at day 30 could lead to an exhaustion of SCs if they are subjected to intense proliferation under stress conditions like repeated cycles of regeneration. 2 times of repeated CTX-injury with one month interval leads to normal absolute number of Pax7+ cells in both control and mutant muscles 30 days after the last CTX-injury, therefore confirming that the self renewal took place from the first round of regeneration. At 3 times of repeated CTX-injury with one month interval between each CTX-injury we see a significant decrease at absolute number of Pax7+ cells (Figure 28C). We need to repeat these experiments in order to assess the ki67 cycling although preliminary data with small number of animals show that the ki67 cycling does not seem to be altered (data not shown).

Regarding the muscle growth in the terms of TA muscle mass is getting worse while the control mice show a distinct increase in TA muscle mass during the repeated cycles of regeneration (Figure 28D).

To end up, our results show that SCs lacking Srf are able to expand their number and self-renew (maintain the SC pool) under chemically stress signals but not for a long time. Srf

<sup>9</sup> The elevated standard deviation is not due to small number of animals as at least  $N=4$  per muscle group were analyzed at unloaded state and at least  $N=6$  at 5 weeks post CH-induction.

is required to a completed terminal differentiation and maturation with the fusion defect to be the limiting stage.



**Figure 28:** Srf is needed for SC maintenance. A) Percentage (%) of Pax7+ki67+ cycling cells at 30 days post CTX-injury. B) Percentage (%) of Pax7+EdU+ cells of the total Pax7+ population at 5 weeks post CH. C) Absolute number of Pax7+ cells per TA muscle entity after 1x, 2x and 3x of repeated cycles of CTX-injury with one month interval between each CTX-lesion. D) Ratio of TA muscle mass to mouse body weight during the repeated cycles of regeneration. Numerical data are presented as mean  $\pm$  s.d. (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$  indicated in the mutant series versus control at each time point. (S)  $p < 0.05$ , (SS)  $p < 0.01$  indicated in the control series compared to control from chronically previous time point. (#)  $p < 0.05$ , (##)  $p < 0.01$  indicated in the mutant series compared to mutant from chronically previous time point. Student's t-test was used for statistical analyses. At least  $n = 3$  mice per group.

### General Conclusion

SCs lacking Srf are able to undergo adult myogenesis and drive the regeneration in TA muscle as it was shown by the presence of new-formed, centrally-nucleated myofibers. However the regeneration mediated by Srf-depleted SCs is highly compromised. One month post CTX-injury mutant muscles remain smaller in size compared to control and to their pre-injury state. Decreased myofiber size and abnormal numerous myofiber numbers characterize the regeneration in TA muscles-harboring Srf-depleted SCs. 30 days post CTX-injury, an advanced stage of regeneration, the mutant muscles are composed of Srf-mutant

new-formed myofibers deriving from Srf-mutant SCs. Previous studies from our lab showed that myofiber lacking Srf (*HSA-Cre:Srf<sup>flox/flox</sup>*) failed to undergo normal regeneration and the poor regeneration in these mutant muscles was not linked to alterations in the satellite cell pool (Charvet et al., 2006).



## Discussion and Perspectives

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The adult skeletal muscle is composed of postmitotic, multinucleated muscle fibers and a reserve of undifferentiated progenitor cells, the satellite cells. The adult skeletal muscle is a highly plastic tissue as it adapts in response to changes in physiological demands, by actively regulating its muscle mass and metabolism within the myofiber. The muscle plasticity relies also on their tissue-specific stem cells, termed as satellite cells (SCs).

Although many transcription factors and signalling pathways implicated in SC function have been identified and characterized, there are many other molecular mediators involved in SC function and they are needed to be explored in an attempt to understand the adult skeletal muscle biology.

Serum Response Factor (Srf) is a widely expressed MADS box transcription factor (TF) that it has been characterized during myogenesis, postnatal growth, hypertrophy, regeneration and muscle wasting (Sakuma and Yamaguchi, 2013). Our study provides an *in vivo* and *in vitro* genetical model ( $Pax7^{CreERT2/+}; Srf^{flox/flox}; Pax7-nGFP$ ) for the study of Srf role in adult skeletal muscle satellite cells. In this study the SCs were mobilized *in vivo* by two means that differ in the nature of the signal inducing SC mobilization and in SC niche: 1) overload-induced hypertrophy and 2) regeneration.

The elucidation of the functions of SC lacking Srf, in the compensatory hypertrophy (CH) model, revealed mainly defects in motile functions and fusion. The fusion could be partially rescued by the compensatory expression of alpha cardiac actin, a known Srf-target gene which is downregulated upon Srf-loss implying the importance of the maintenance of actin cytoskeleton in cellular processes like the fusion.

The injury-induced regeneration is the widely accepted model and mostly used to challenge the satellite cells (SCs). SCs are the primary means by which the muscle of adult muscle is formed during regeneration (Bischoff, 1975). This process recapitulates many phases of embryonic myogenesis while the SC-mediated fusion during functional overload mimics the postnatal growth in muscles without (or limited) *de novo* formation of myofibers. Myotoxin (cardiotoxin)-induced injury leads to 5-fold overall increase in SC number at day 4 post-injury compared to steady state (Hardy et al., 2016). Therefore with the regeneration model we could check for other aspects in the function of SC upon Srf-loss, such extensive proliferation under chemical (myotoxic)-signal, inflammation-associated injury, fusion and self-renewal in a Srf-mutant niche (myofibers) and to what extent the SC behavior *in vivo* is SC-autonomous or niche-dependent. Thereafter comparing the regeneration readouts with

the CH model and *in vitro* studies could help us to obtain a spherical interpretation of the role of Srf in the SC functions.

### The Srf role in SC functions

#### 1. Does Srf Play a Role in Satellite Cell Proliferation?

The loss of Srf in SCs does not affect their number at the steady state (unloaded muscles and uninjured muscles) in both models which is consistent with the faint expression in quiescent SCs. Consequently PI and TA mutant muscles have no signs of wasting at the steady state. Upon exposure to activation signals SCs expand their number through cell proliferation. In the CH model, Srf is dispensable for the proliferation of Pax7-expressing SCs as assessed *in vivo* and *in vitro* while in the regeneration context the Srf loss leads to decreased number of Pax7-expressing SCs at early stages (3 and 4 day post CTX-injury).

There are the following major differences between the two models: 1. signal quality (mechanical or chemical) 2. degeneration/inflammation phase of regeneration 3. proliferation efficiency. Cardiotoxin-induced injury leads to 5-fold overall increase in SC number compared to steady state, at day 4 post-injury (Hardy et al., 2016) while in overload-induced hypertrophy leads to a 2.5 fold increase 1 week post CH-induction. Thus the SCs undergo extensive proliferation under the regeneration-stress comparing to mild cell proliferation under the mechanical-stress. Although under regeneration control and mutant SCs demonstrate the same percentage of ki67 cycling cells, Srf-depleted SCs are much lower at day 3 and 4.

It is possible that the SCs lacking Srf are slow-dividing cells which could result in the diminished number of Pax7-expressing SCs. In order to test this hypothesis, pulse-chase experiments *in vitro* using thymidine analogs or histone fluorescent marks would allow detecting of slowly dividing cells and following the kinetics of cell cycle progression over the chase period. SCs which are thought to divide slowly, would retain the label longer (Rocheteau et al., 2012; Shinin et al., 2006).

Srf coactivators, myocardin and Mrtfs have been implicated in anti-proliferative effects in a range of cell types. Double knockdown of Mrtf-A and Mrtf-B in NIH 3T3 fibroblasts under serum-starved conditions, an aberrant entry into the S and G2 phases was observed without subsequent cell division. Extended knockdown of Mrtfs leads to increased formation of micronuclei and enhanced aneuploidization (Shaposhnikov et al., 2013). Mrtf-A and -B depletion was also shown to induce oncogene-induced senescence with regression of hepatocellular carcinoma xenograft (Hampl et al., 2013). Mrtf-A and Mrtf-B dKO in developing (E17.5) skeletal muscle demonstrate a decrease in the proliferation in dKO

embryonic myoblasts as assessed by phospho-histone H3 (pH3) analysis which is a marker of mitosis (Cenik et al., 2016).

However in other cell types (keratinocytes) Srf seems to lead to an increase of cell proliferation (Koegel et al., 2009). Previous *in vitro* studies in C2C12 cell lines showed an impact of Srf-loss in myoblast proliferation (Soulez et al., 1996a). We cannot exclude that it could be due to a technical artifact derived from the microinjection of either the SRE oligonucleotide or Srf antibodies (Gauthier-Rouviere et al., 1996; Vandromme et al., 1992) or from transfection with Srf antisense RNA expression vector (Soulez et al., 1996a). A thorough detailed study needed to be done further testing the cell cycle progression by the cyclin A (marks S and G2 phases) and phospho-histone H3 (marks G2 and M phases) antibodies (Ponnusamy et al., 2016) to mark the transition between cell cycle phases.

Taken together all these previous bibliographic data show that Srf has a role in the regulation of cell cycle either by enhance/weaken it, or by inducing an eventual senescence. In the adult skeletal myogenic context Srf does not seem to have an impact on cell cycle (ki67, EdU, BrdU markers) as it is provided by our *in vivo* and *in vitro* data which is **in contrast to** the previous *in vitro* studies in C2C12 cell line (Gauthier-Rouviere et al., 1996; Soulez et al., 1996a).

## 2. Does Srf Affect the MyoD expression?

Previous *in vitro* studies in C2C12 cell line showed that Srf knock-down prevents the expression of MyoD in proliferating myoblasts but also in myoblasts cultured under differentiating conditions (Gauthier-Rouviere et al., 1996). Moreover, SC-specific deletion of MASTR and both MASTR and Mrtf-A (members of the MRTF family) showed to down-regulate the MyoD expression leading to increased proliferation of SCs (Mokalled et al., 2012).

Despite to these previous results, with our study SC-specific deletion of Srf and the derived primary cell culture (*ex vivo*) we show that the Srf is not indispensable for the MyoD muscle-specific TF.

## 3. Self-Renewal /Return to Quiescence

30 days post CTX-injury, the myofiber-niche is also Srf mutant with a completely different composition of numerous, small-sized myofibers. Therefore, it could not be surprising to have an impact in the self-renewal cell cycle of the Srf-depleted SCs. The growth fraction (Pax7+ ki67+) of Pax7+ population is elevated in Srf-mutant myoblasts. Do the Srf-depleted SCs fail to return to quiescence? Does is it a SC-dependent or myofiber/niche-dependent defect?

Indeed the stem cell niche is essential for quiescence and maintenance of the stem cell pool. It has been shown that induction of aged niche-derived Fgf2 disrupts stem cell quiescence during homeostasis leading to increased ki67+ SCS (Chakkalakal et al., 2012). A previous study from our lab showed that adult myofibers lacking Srf develop a broad spectrum of alterations similar to those which have been described in age-advanced skeletal muscles (Lahoute et al., 2008). It is noteworthy that the regenerated myofibers undergo a stage of growth phase which is also an important phase for the myofiber functional recovery. Another study from our lab showed that the Srf loss in the myofiber, subjected to CH, leads to decreased rate of proliferation (% Pax7+ ki67+ cells) and that secretion of signals by the myofibers during CH, functions in order to stimulate the proliferation of SCs. In the absence of such signals the SC proliferation ceases (Guerci et al. 2012). In the present model, 30 days post CTX-injury mutually SCs and myofibers are Srf-mutant and the rate of ki67 proliferation is increased showing thus that the SCs lacking Srf may not return to quiescence. Altered signal emission from the Srf-mutant myofibers act via Srf-lack in SCs to increase the cycling score in mutant myoblasts. Such a signal could be the angiopoietin I which is downregulated upon Srf loss within the myofiber (unpublished data). Ang1/Tie-2 signaling regulates SC self-renewal by controlling the return to quiescence (Abou-Khalil et al., 2009).

An adequate niche is critical for SC maintenance as disruption of quiescence leads to an exhaustion of SCs. By the repeated lesions we checked for this hypothesis and we found that after 3 times of CTX-injury (with one month interval between them) leads to decreased number of Pax7-expressing SCs. The CH model helped us to distinguish between SC- or myofiber-dependent effect, in the elevated number of cycling Pax7+ cells, as the myofiber is preserved wild type. 5 weeks post CH control and mutant SCs present the same EdU percentage.

Taken together, these results show even if there is not a cell-autonomous defect in the cycling capacities of SCs lacking Srf, Srf is an important player in the cross-talk between the SCs and the myofiber-niche.

#### 4. Apoptosis or Activation Delay?

##### Cell Viability

Although Srf does not seem to affect the proliferation capacity of myoblasts, the number of SCs is highly reduced at the early stages of SC recruitment. The reduction in the SC number could be a consequence of increased apoptotic activity due to lack of Srf as it is the case in many other muscle tissues (Park et al., 2015; Parlakian et al., 2004; Ro, 2016b). The transcriptomic analysis on sorted cells from damaged muscles (CTX+3days) pointed out an apoptotic signature. TUNEL staining on muscle sections 4 days post CTX-injury did not

display a difference in the overall apoptotic cells between control and mutant muscles likewise with the Annexin V labeling *in vitro*. We did not achieve to elucidate whether Srf-mutant SCs are more prone to undergo apoptosis. It is needed to test the apoptotic fraction in the Pax7+ population by a double staining of Pax7 and TUNEL or activated caspase-3 apoptotic markers on muscle sections at day 4 post CTX-injury.

*In vitro* cell growth curve analysis display also a diminution in the number of growing cells (data not shown) with any defect in the proliferation capacity (BrdU labeling). We did not detect detached, floating cells indicative of dying cells. Alternatively, there may be a cell cycle defect independent of apoptosis, such as a cell cycle arrest in G2 phase, resulting in a slower progression through the cell cycle. Or Srf-mutant SCs may need more time to attach to the culture dish and thus start to proliferate later.

In order to further assess whether Srf-mutant SCs are more prone to undergo cell death we should treat myoblasts in culture with cytotoxic compounds (cell death-inducing drugs) and to evaluate the eventual “fragility” of SCs lacking Srf in their maintenance. Further apoptosis assessment could be conducted on freshly-sorted cells from muscles under regeneration at day 3 or 4 post injury by Annexin V labeling.

#### Signal-induced Activation

Srf is among the specific transcription factors which have binding sites within regulatory regions of immediate-early genes (IEGs) involved in immediate-early response processes (immune responses or cellular stress). Expression of IEGs is quick and mainly transient, and their expression in interphasic cells is initiated by an extracellular signal, such as growth factors, mitogens, immunological and neurological signals, developmental, and stress (e.g. UV, toxins). Many IEGs are proto-oncogenes and their sustained expression can have profound effects on the regulation of the cell cycle and on cellular growth (Bahrami and Drabløs, 2016).

Upon exposure to signals from a damaged environment, satellite cells exit their quiescent state and start to proliferate (satellite cell activation). Adult satellite cells are primed to respond rapidly to activation signals (Rodgers et al., 2014), with the first cell division to be the time-limiting step in the conversion of satellite cells to a highly proliferative state (Rodgers et al., 2014).

It is plausible to hypothesize that SCs lacking Srf do not rapidly respond to activation and growth signals secreted within the first hours of muscle lesion leading to the decreased number of Pax7-expressing SCs at the early stages of regeneration. FACS-purified SCs from uninjured TA muscle were kept in culture for 2 and 5 days trying to imitate the initial phases of injury-induced *in vivo* activation by cell-sorting *ex vivo* activation. The activation state of

SCs is characterized by MyoD expression (Zammit et al., 2004). Preliminary data showed that the percentage of satellite cell-derived MyoD<sup>+</sup> myoblasts is lower in mutants than controls after 2 days in culture post FACS-isolation whereas the percentage of MyoD<sup>+</sup> myoblasts is the same in both controls and mutants after 5 days in culture. These experiments need to be repeated and test for MyoD activation even at earlier time points (e.g. 1 day in culture).

Single fiber isolation and culture will clarify whether the endogenous myofiber-associated SCs present the same activation and cell cycle kinetics. However in the CH model, at all time points examined, the number of Pax7-expressing cells are not different between control and mutant PI muscles. The signals triggering the SC activation upon injury or overload induction may be not mediated by the same stimulant factors (Mendez and Janmey, 2012; Tatsumi, 2010). Muscle degeneration begins with necrosis of damaged muscle fibers and precedes muscle regeneration. Myofiber necrosis is accompanied by increased calcium influx which in turn activates calcium-dependent proteolysis and drives tissue degeneration (Yin et al., 2013a). Myofiber necrosis also activates the complement cascade and induces inflammatory responses. Subsequent to inflammatory responses, chemotactic recruitment of circulating leukocytes occurs at local sites of damage (Tidball and Villalta, 2010). Thus the initial degeneration and inflammation phases of regeneration may result at differential impacts on SC-activation as render the local milieu more toxic/harmful for SCs lacking Srf. Indeed in different injury models lead to differential and considerable SC-loss few hours after injury-induction and myotoxins provoke an overall destruction of the tissue (Hardy et al., 2016).

In order to assimilate the *in vivo* conditions of SC-activation the model of co-culture of freshly-isolated SCs with M1 inflammatory macrophages could shed light in the cross talk between the two cell types and the possible activation defect of SCs lacking Srf. Pro-inflammatory M1 macrophages stimulate myogenic cell proliferation through mechanisms mediated by TNF-alpha and IL-6 (Arnold et al., 2007). Alternatively we could attempt culture of freshly-isolated SCs with crushed muscle saline extracts present in the cell culture medium (conditional medium) a widely-used method (Bischoff, 1997; Chakkalakal et al., 2012; Conboy et al., 2005; Guerci et al., 2012a). Crushing mimics muscle injury processes, and factors present in crushed muscle extracts are likely to be the factors released during normal tissue injury (Bischoff, 1986).

For the present time we can only suppose that there is not a major defect in the activation of Srf-depleted SCs but a delay which could eventually result in the diminished number of Pax7<sup>+</sup> cells at early stages of their expansion. This phenotype of Srf-depleted SCs is not “revealed” in the CH model where mild/gentle occurring conditions do not affect

the SC recruitment. Thus the SC mobilization depends on the kind of the signal (signal “quality”).

## 5. Terminal Differentiation and Maturation

In both *in vivo* models as also *in vitro*, the onset of myogenesis is not affected by the Srf-loss. In spite of previous *in vitro* studies in mouse C2 and rat C6 cell lines where Srf-inhibition prevented the MyoG expression (Vandromme et al., 1992), Srf-specific loss has no impact on MyoG expression.

Developing skeletal muscles express unique myosin isoforms, including embryonic and neonatal myosin heavy chains. These myosin isoforms are transiently expressed during embryonic and fetal development and disappear shortly after birth when adult fast and slow myosins become prevalent. However, these myosins are re-expressed during muscle regeneration and provide a specific marker of regenerating fibers in the pathologic skeletal muscle. Regenerating muscle fibers re-express developmental isoforms of myosin, troponin, and other muscle proteins confirming thus that the adult myogenesis recapitulates many aspects of embryonic myogenesis. Embryonic and neonatal MyHCs are detected in newly formed regenerating myofibers at 2–3 days after injury and persist for 2–3 weeks (Schiaffino et al., 2015).

30 days post CTX-injury, MyHC-emb+ newly formed myofibers abound in the mutant muscles as there are only rare in the controls. Also MyoG+ cells are found in the mutant muscles suggesting therefore that either there is a retard in the myogenic progression or Srf-depleted SCs do not switch to adult MyHC which is an index of terminally well-advanced differentiated myocytes. It is worthwhile to check for an eventual retard in the myogenesis and maturation of myoblasts lacking Srf by checking the myosins isoforms beyond the day 30 of regeneration.

## 6. Fusion Defect or Myofiber Branching?

The CH model revealed fusion-deficient SCs lacking Srf unable to fuse to growing myofiber. The fusion index, as measured as the ratio of myonuclei per myofiber, at 8 and 30 days post CTX-injury was highly diminished in comparison to control. At cellular level, the fusion proceeds in two stages. Initially, myoblast-myoblast fusion (primary fusion) results in the formation of nascent myotubes. In the second phase, myoblasts fuse with nascent myotubes (secondary fusion), which results in nuclear accretion and growth of the myotube (Hindi et al., 2013). During regeneration primary and secondary fusion take place. Extrapolating the *in vitro* conducted fusion mixture experiments helps us to describe the regeneration phenotype.

Primary homotypic or symmetric fusion between mutant myoblasts, and heterotypic or asymmetric fusion between control and mutant myoblasts mixture were affected. In the same manner secondary asymmetric fusion between control myotubes and mutant myoblasts mixture was also decreased. These experiments imply that the Srf is needed to be mutually expressed in both myogenic “partners” subjected to fuse. The fusion deficiency of Srf-mutant SCs leads to highly increased number of new-formed “myofibers” at day 30 post CTX-injury. If they are really myofibers (containing more than 2 myonuclei within the dystrophin contour) or mononucleated myocytes, longitudinal TA sections at 8 and 30 days (data not shown) showed the presence of multinucleated myofibers as also as mononucleated myocytes. However multinucleated myofibers are not as much elongated as in the control.

Interestingly, as depicted by the laminin and dystrophin staining, mutant new-formed fibers present a completely different phenotype in their shape with “marguerite”-like appearance mainly at 8 days post CTX-injury. Careful examination of transversal sections reveals at some foci, newly-formed myofibers appeared as “eccentric cycles” which means a forming myofiber within another myofiber with individual basal lamina (laminin staining). It is noteworthy that the CSA in mutant muscle not only does not change between 4 days and 8 days post CTX-injury but declines significantly. Also mutant mice exhibit ~5-fold more myofiber number at day 30. In many neuromuscular diseases muscle regeneration is aberrant and various abnormalities, such as variation in myofiber size, decreased myofiber number, fibrosis and branched myofibers, are observed (Pichavant and Pavlath, 2014). Branched myofibers are malformed cells which, instead of having a normal cylindrical shape, contain one or more offshoots of small daughter myotubes contiguous with the parent myofiber. I cannot exclude that Srf-deficient myoblasts that are not able to fuse properly lead to sprouted myofibers or the new-formed mutant myofibers are more prone to splitting. It could be an explanation for the decrease in the myofiber CSA from the day 4 to 8. Moreover such a phenotype was described *ex vivo* in some MyoD-null fibers with forked or small “sprouts” (Cornelison et al., 2000). It was described that the aberrant cytoarchitecture of branched myofibers likely arises from incomplete or aberrant fusion of small myotubes during muscle regeneration (Pavlath, 2010b; Schmalbruch, 1976). It is plausible to hypothesize that the fusion defect and the altered actin cytoskeleton of F-actin could result in susceptibility of mutant myofibers to splitting. To test this hypothesis it is needed to observe the longitudinal sections at higher resolution and magnification and further isolate single TA regenerating myofibers for an *ex vivo* observation (Pichavant et al., 2016).



## Underlying Mechanisms – Actin Player

### 1. Do the SCs participate in the Hypertrophic Growth of Myofiber?

Adult muscle fiber is a symplast, a multinuclear structure, developed in ontogenesis by the fusion of myoblasts (muscle progenitor cells). Myoblast fusion is important not only for skeletal muscle formation during development, but also for post-natal muscle growth and regeneration of skeletal muscle. Satellite cell-mediated hypertrophic growth in the myofiber under overload conditions mimics the post-natal growth with the SC nuclear accretion to lead to multinucleated myofibers and growth.

The role of satellite cells in muscle hypertrophy has long been a debated issue and the subject of many reviews (Blaauw and Reggiani, 2014; Gundersen, 2016; Pallafacchina et al., 2013; Snijders et al., 2015). Studies with a transgenic Pax7-DTA mouse model, where the Pax7+ SC depletion is achieved with TMX treatment, showed that satellite cells are not required for muscle growth over a short period (McCarthy et al., 2011) but they are required to overcome/negatively regulate the excessive ECM accumulation over a prolonged period resulting in attenuated hypertrophy (Fry et al., 2014). Egner et al. repeated these experiments and they found that overload hypertrophy was prevented in the satellite cell-deficient mice, in both the plantaris and the extensor digitorum longus muscles. In consequence they claim that there is currently no model in which functional, sustainable hypertrophy has been unequivocally demonstrated in the absence of satellite cells.

We provide new genetic data showing that when SCs are not able to fuse, hypertrophy is impaired. Srf-mutant mice show no signs of fusion and the moderated increased CSA could be attributed to an increase in the protein synthesis in Srf-expressing myofibers. *In vivo* overexpression of alpha-actin rescues the fusion in the CH model. We conclude that SC myonuclear accretion is required however for an optimal hypertrophic growth comparing to controls, and it indicates the necessity of SC fusion to the growing myofiber.

### 2. Actin-dependent rescue

The actins are a highly conserved protein family that plays crucial roles in cell biology, in division, motility, the cytoskeleton, and contraction. Higher eukaryotes have six different actins, each expressed from separate genes (Vandekerckhove and Weber, 1979).

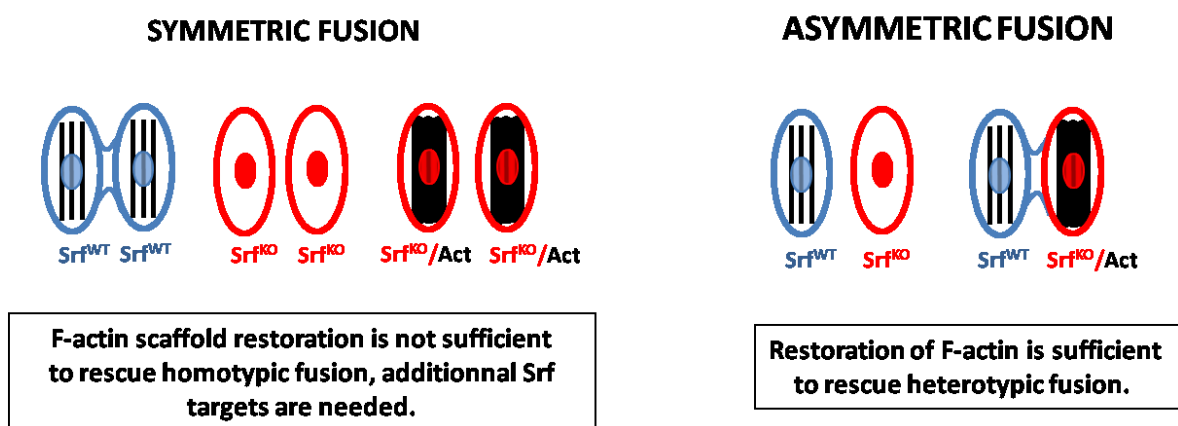
The activity of Srf is regulated by changes in actin dynamics. Analysis of Srf target gene set revealed hundreds of genes involved in actin filament dynamics, cell adhesion, extracellular matrix (ECM) synthesis and processing, cell motility, and other actin-linked

processes as well as a significant number of genes involved in microtubule-based cytoskeletal dynamics (Esnault et al., 2014). The alpha-skeletal actin (Acta1) is the prevalent isoactin in adult skeletal muscle. Alpha-cardiac actin (Actc1) is the major isoactin in adult heart and fetal skeletal muscle (Gunning et al., 1983a).

In Srf-depleted myoblasts the Acta1 is highly decreased, in comparison to controls, leading to aberrant actin network with paucity of F-actin. Overexpression of alpha-cardiac (Actc1) isoform in Srf-depleted SCs, restores the F-actin pool and rescues the heterotypic/asymmetric fusion. Therefore proper actin cytoskeleton maintenance is of critical importance to mediate the heterotypic cell fusion. In fact there are many reports of actin-mediated processes in myoblast fusion (Martin, 2016). In Drosophila model, cells utilize actin-propelled membrane protrusions to promote fusogenic protein engagement and fusion pore formation (Shilagardi et al., 2013) and actin-based filopodia emanating from the myotubes surface to prime the heterotypic adhesion process between the two cell types (Segal et al., 2016).

The underlying possible mechanism for the rescue of heterotypic/asymmetric fusion could be the restoration of the mechanical invading force which helps to overcome energy barriers for membrane apposition and drives cell membrane fusion (Kim et al., 2015) or the appropriate cellular distribution of signalling molecules and/or contractile proteins molecules to mediate the fusion (Tran et al., 2012).

The fact that the homotypic/symmetric fusion is not rescued indicates that other Srf-target genes (alpha-actin-independent) are required for a complete restoration of biological and mechanical properties in a pair of fusing cells (Figure 29).



**Figure 29** : Schematic model for the Srf and Actin-action in myoblast fusion.

Cell-ECM Adherence properties

Serum response factor controls multiple genes governing adhesion. Srf-null embryonic stem cells (ES), demonstrate that Srf deficiency causes impairments in ES cell adhesion and correlates with defective formation of cytoskeletal structures, namely actin stress fibers and focal adhesion (FA) plaques (Schratt et al., 2002). Focal adhesions (FAs) are dynamic adhesion structures that anchor the cell to the extracellular matrix. Mrf1s, co-regulators of the Srf, regulate expression of a set of genes encoding actin cytoskeletal/FA-related proteins and integrin-mediated regulation of FA components (Kishi et al., 2016). Srf-null stem cells displayed impaired expression of the integrin network and decreased adherence *in vitro* suggesting that Srf acts mainly through cell-matrix interactions and integrin signaling (Ragu et al., 2010). Srf knockout neutrophils fail to polymerize globular actin to filamentous actin, fail to migrate to sites of inflammation, show markedly reduced adhesion and although integrin expression levels are maintained with loss of Srf, integrin activation and trafficking are disrupted (Taylor et al., 2014).

Preliminary experiments with the (Ethylene-Diamine-Tetraacetic Acid) EDTA compound showed that mutant myoblasts need more time to detach from the cell dish substratum and also to adhere. Therefore we can hypothesize that myoblasts lacking Srf change the adherence properties profile which could encounter for the reduced motile functions. In the future studies it is need to explore the integrin expression pattern and further testing their role played by overexpression and knockdown constructs in control and mutant myoblasts.

### 3. Actin-independent

#### Cell Migration

Fusion generally occurs between muscle cells that were initially positioned at a distance from each other, and therefore requires mediation by a cell migration process, most commonly of myoblasts towards a myotube target (Schejter, 2016). The reduced motile capacity does not facilitate the “meeting” between the Srf-mutant myoblasts. In order to overcome this obstacle we plated mutant myoblasts and mutant myoblasts with the actin overexpression in confluent high concentrations. The fusion defect was not ameliorated in both cases meaning thus that cell migration defect might not account for the fusion incapacity.

Cell motility is highly perturbed in myoblast lacking Srf. Overexpression of the alpha-actin isoform does not rescue the motile functions of Srf-mutant myoblasts suggesting thus a non alpha-actin dependent control of cell motility. The non-muscle isoforms of beta- and gamma-actin or myosin, all being Srf-target genes (Esnault et al., 2014; Miano et al., 2007) could be involved in the process of cell movement (Callan-Jones and Voituriez, 2016). A

future perspective could be an attempt to overexpress the beta and/or gamma-actin or myosin aiming to elucidate the role of actin/myosin flows in both migration and polarization mechanisms (Callan-Jones and Voituriez, 2016; Kim et al., 2015).

#### Lipid Composition of Cell Membrane/Genes involved in Cell Metabolism

Cell-contact sites are further characterized by changes in the cell membranes and composition. For example, phosphatidylserine is transiently exposed at these sites (van den Eijnde et al., 2001) and required for myotubes formation. Additionally, lipid rafts containing cholesterol transiently accumulate at cell contact sites and are required for the accumulation of adhesion molecules and to maintain the proper rigidity of the lipid bilayers necessary for adhesion between the two myogenic cells. These intracellular molecules probably activate signal transduction pathways that ultimately lead to membrane fusion (Abmayr and Pavlath, 2012). Destabilization of the lipid bilayers, which makes them prone to fusion. Lipid modifications correlate with a reduction in plasma membrane rigidity resulting in fusion impairment (Blondelle et al., 2015).

The Mrtf-Srf target genes includes numerous transcriptional regulatory factors and a significant number of genes involved in metabolism (Esnault et al., 2014).

#### Perspectives

We have already identified the set of genes (transcriptomic studies) whose expression is altered by Srf loss (cells transduced with Adeno-Cre vs Adeno-GFP) in proliferating and differentiating myoblasts (day 1 and day 3 post differentiation). Gene annotation using Ingenuity software pointed out an over-representation of genes involved in cytoskeletal rearrangements, cellular movement, adhesion and cell death and survival.

We will focus our studies on the functional relevance of genes that can be related to the phenotypes of cells lacking Srf *in vitro* and *in vivo* and that are *bona fide* Srf targets (or have at least one Srf binding site in their promoter according to Genomatics software).

We have already obtained preliminary data concerning the Hic-5 gene. Hic-5 is a member of the LIM-protein family with homology to paxillin. It has been shown to regulate a range of biological processes including: senescence, integrin signaling, differentiation, and apoptosis. Interference experiments with Hic-5 expression in C2C12 cells showed its potential role in cell death and myotubes formation (Gao et al., 2007). In primary cultures of myoblasts, we efficiently knocked-down Hic-5 expression by siRNA transfection and showed that the cell fusion was blunted. These results identify Hic-5 as an interesting candidate whose decreased expression could account for the impaired fusion of muscle cells lacking Srf.

A very interesting perspective for future studies is the investigation of “rheological behavior” of Srf-mutant myoblasts and Srf-mutant myoblast overexpressing the alpha actin (Mut/Act+) collaborating with the lab of Dr. Sylvie Henon. The rheology science is a branch of physics that studies the stress/strain relationship of materials. It describes properties such as elasticity, plasticity or brittleness. Rheological study is the study of the flow of matter, primarily in a liquid state, but also as 'soft solids' or solids under conditions in which they respond with plastic flow rather than deforming elastically in response to an applied force. We expect that this study will shed light in the description of physical properties (elasticity, plasticity, viscosity) of a myogenic cell containing altered actin network when a pair of fusing cells are under the tension to overcome the energy barrier and the driving mechanical force to proceed to membrane merging and cell fusion. Actin flows have emerged as a highly conserved propulsion force in migrating cells, and have also been identified as key physical regulators of cell polarity (Callan-Jones and Voituriez, 2016).

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**Title:** Role of the Serum Response Factor (SRF) transcription factor in adult muscle stem cells

**Abstract:** The adult skeletal muscle is a high plastic tissue as it adapts its size upon overload and it is capable of regeneration upon muscle lesion. The skeletal muscle is composed of a specialized syncytium, the myofiber, which is the functional unit of the muscle and a small population of myogenic progenitors, residing adjacent to the myofibers, termed as satellite cells (SCs). SCs are the muscle-specific stem cells which endow the skeletal muscle with its remarkable capacity to repair and to maintain homeostasis during muscle turnover. In resting adult muscles, SCs are quiescent but they activate upon exposure to stimuli. The activated SCs (myoblasts) proliferate extensively and subsequently differentiate and fuse between them or pre-existing myofibers, a series of cellular events called myogenesis. In parallel to the myogenesis, a reserve population of SCs escapes the myogenic program and self-renews to replenish the SC pool. The current project aims to further characterize the signalling pathways involved in SC functions during muscle regeneration and compensatory hypertrophy (CH).

Srf is a muscle-enriched transcription factor with Srf-target genes implicated in cell proliferation, differentiation (sarcomeric proteins), adhesion, migration and cellular cytoskeleton. Studies in C2C12 mouse myogenic cell line showed that Srf loss prevent the myoblast proliferation and differentiation by down-regulating the expression of the myogenic determinant MyoD gene.

We used a genetic murine model for adult SC-specific Srf-loss in order to conduct *in vivo* and *ex vivo* studies for the Srf role in SCs. Compensatory hypertrophy and regeneration are the two means by which SCs were recruited. We show that loss of Srf in SCs affects the regeneration process and the CH suggesting the Srf role in the SC fate. Srf-depleted SCs display probably no defect in their proliferation and differentiation but reduced capacity in motility and fusion. Transcriptomic analysis revealed altered actin cytoskeleton and signalling. Srf-depleted SCs show reduced actin expression and altered actin cytoskeleton. Rescue of actin expression in Srf-depleted SCs partially restored the cytoskeleton organization and the fusion process. Interestingly by actin overexpression only the heterotypic/asymmetric fusion was established but not the homotypic/symmetric fusion. Therefore actin overexpression restored the hypertrophic growth in the CH (*in vivo* model of heterotypic fusion) but failed to do so in the regeneration (*in vivo* model of homotypic fusion).

This study contributed to the *in vivo* investigation of the Srf mechanistic role in adult SCs and underlined the importance of actin cytoskeleton maintenance in the fusion of myogenic cells.

**Keywords:** muscle satellite cells, serum response factor, skeletal muscle hypertrophy, skeletal muscle regeneration, actin cytoskeleton

