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Study of the role of RhoA in skeletal muscle plasticity

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Science *is like* ***love*** *: take the risk to share your idea !*

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Abbreviation

BMP = Bone Morphogenetic Protein

Col = Collagen

CSA = Cross Section Area

ECM = Extra Cellular Matrix

EdU = 5-ethynyl-2′-deoxyuridine

eMHC = embryonic Myosin Heavy Chain

EPs = Embryonic Progenitors

ERK = Extracellular signal-Regulated Kinases

FAPs = Fibroblast Adipocites progenitros

FC = Founder cells

FCM = Fusion Competent myoblast

Fn = Fibronectin

GAPs = GTPase-accelerating proteins

GEFs = Guanine nucleotide exchange factors

GTP = Guanosine-5'-triphosphate

HSA = Human Skeletal Actin

IGF1 = Insulin Growth Factor 1

IPA = Ingenuity Pathway Analysis

MAPK = Mitogen-Activated Protein Kinase

MLC2 = Myosin Light Chain2

MMPs = Matrix Metallo Proteases

MuRF1 = muscle RING-finger protein-1

MyHC = Myosin Heavy Chain

NFAT = Nuclear factor of activated T-cells

OV = Overload

PKB (AKT) = Protein kinase B

PL = Plantaris

RhoA = Ras homolog family member A

SC = Satellite cell

SHG = Second Harmonic Generation

SO = Sham Operated

SUnSET = surface sensing of translation

TA = Tibialis Anterioris

TMX = Tamoxifen

TnC = TenascinC

Abstract (English)

RhoA is a small GTPase protein that regulates numerous processes such as, cell cycle, motility and adhesion and has been poorly studied in muscle tissue. RhoA activation responds to mechanical stimuli and mechanical force transmitted and mediated by cell-matrix and cell-interaction (Lessey et al. 2012)(Burridge et al. 2019) controlling mechanical stretch response, activating downstream mediators important to translate environmental cues in biochemical signals. Thanks to this work we deciphered the role of RhoA in skeletal muscle *in vivo* during embryonic development, postnatal growth and adult muscle maintenances and plasticity.

Deletion of *RhoA* in adult myofibers allowed us to characterize the role of RhoA in skeletal muscle tissue. We showed that the absence of RhoA prevented an optimal hypertrophic response to increased load. Although the synthesis of new proteins, occurring upon overload-induced hypertrophy, was not affected, satellite cells (SC) fusion to growing fibers was impaired. These data suggest that SC fusion is the main event that may contribute to the compromised growth of mutant muscles.

Microarray analysis, performed one week after hypertrophy surgery on control and mutant *Plantaris* muscles, evidenced that the genes involved in the extracellular matrix remodelling, such as *Mmp9/13,* are among the most downregulated ones in the mutant muscles upon overload. Moreover, concerning extra cellular matrix (ECM) muscle proteins, two important defects have been evidenced: the substrates of Mmps were accumulated in the mutant muscles in comparison to the control and the mutant fibrillar collagen, visualized by using the second harmonic generation (shg) microscopy, emerged differentially organized in comparison to the control. Furthermore the inhibition of Mmp activity altered hypertrophic growth suggesting that the decreased expression of Mmp could account for RhoA mutant altered overload-induced hypertrophy.

Transcriptomic analysis showed also a decrease in the expression of potent chemoattracting chemokines such as *Cx3Cl1* and *Ccl3*. We correlated lower chemokine expression with diminution of macrophage infiltration in *Plantaris* muscles upon overload-induced hypertrophy. Furthermore, we demonstrated the functional role of macrophages (by depletion experiments) to achieve a correct muscle hypertrophic growth.

Finally, we found a decline of Erk1/2 activity in RhoA mutant muscles compared to the control ones upon hypertrophy. Our data suggest that RhoA can directly stimulate Erk1/2 within the myofiber compartment because we found it activated in cultured myotubes treated with Rho Activator. Interestingly, Erk1/2 inhibition in cultured myotubes showed that the increase of Mmps and of Chemokines expressions triggered by RhoA activation were dependent on Erk1/2.

Thanks to *RhoA* deletion in embyonic progenitors (EPs), we showed that RhoA was not essential for the embryonic myogenesis and for the muscle development. Nevertheless, RhoA was required for efficient fusion in post-natal stages.

The deletion of *RhoA* in adult satellite cells allowed us to further characterize RhoA function during adult muscle maintenance: importantly we showed that the absence of RhoA impaired skeletal muscle regeneration. *RhoA*-lacking satellite cells did not display proliferation defects nor differentiation defects; however, their fusion capacities were strongly impaired. Surprisingly we evidenced that there were no main defects in actin cytoskeleton assembly nor in cells contractility, both important for SC fusion. Transcriptomic analysis performed on control and mutant primary satellite cells in proliferation and differentiation states will help us to decipher new important players of RhoA signalling involved in SC fusion.

Abstract (French)

Résumé en français

Le muscle squelettique adulte est un tissu plastique qui est capable de se régénérer suite à une lésion et dont la masse varie en fonction de la quantité de nutriments ou de l'activité. Le muscle est composé de myofibres plurinucléées post-mitotiques et de cellules souches musculaires (CS) localisées sous la lame basale. A l'état basal, les CSs sont quiescentes. En réponse à différents stimuli, elles s'activent, prolifèrent, se différencient et fusionnent entre elles pour former de nouvelles myofibres (régénération) ou avec des fibres en croissance (hypertrophie). Enfin les CSs s'auto-renouvellent afin de maintenir le pool de cellules souches. Le but de mon projet est d'identifier les voies de signalisation qui contrôlent la plasticité du muscle et en particulier l'étude de la GTPase RhoA au cours de ce mécanisme.

RhoA est une protéine qui régule de nombreux processus cellulaires comme le cycle cellulaire, la motilité et l'adhésion. RhoA est peu étudiée dans le muscle squelettique *in vivo*. Son rôle a été montré *in vitro* pour réguler la différenciation et la fusion de lignées de myoblastes. RhoA est importante aussi pour le réarrangement du cytosquelette d'actine et son activation répond aux stimulations mécaniques. Dans les myotubes, RhoA est activée en réponse à un étirement des cellules. Et *in vivo,* il a été montré que l'expression de RhoA diminue après l'atrophie induite par l'inutilisation du muscle. Toutes ces données suggèrent que l'expression et l'activité de RhoA sont fortement liées aux signaux mécaniques et que RhoA est capable de traduire des signaux environnementaux (mécaniques) en signaux biochimiques. Le but de ce travail est de caractériser le rôle de RhoA dans le muscle squelettique *in vivo* pendant le développement embryonnaire, la croissance postnatale, la préservation et les adaptations du muscle adulte.

Afin de étudier le rôle de RhoA dans le muscle adulte *in vivo*, nous avons développé deux modèles murins permettant la délétion conditionnelle et inductible de RhoA dans les myofibres (HSA-CREert2:RhoAfx/fx) ou dans les cellules satellites (Pax7- CREert2:RhoAfx/fx) adultes. Et un modèle murin permettant la délétion de RhoA dans les progéniteurs embryonnaires (EP) musculaires (Myf5-CRE:RhoAfx/fx). Grâce à la suppression de RhoA dans les EP (à partir du 9eme jour après la naissance), nous avons pu montré que RhoA n'est pas indispensable à la myogenèse embryonnaire et au développement musculaire. À E18.5 et et en post-natal, la naissance des souris mutantes ne respecte pas le ratio mendélien. La perte d' embryon mutant a dû se produire très tôt durant le développement, peut-être en raison d'une fuite d'expression de la Cre dans d'autres tissus. Les mutants survivants n'ont pas montré de phénotype musculaire evident. Après la naissance, les muscles mutants présentent une fusion réduite des cellules satellites, mettant en évidence un défaut de croissance post-natale. De plus après 14 jours de régénération musculaire suite à une lesion

causée par une injection de cardiotoxine chez des souris adultes, le processus de fusion est altéré (moins de myonoyaux / fibre), démontrant également un défaut de fusion au cours de la régénération suite à une lésion musculaire.

Ces résultats indiquent que RhoA n'est pas cruciale pour la myogenèse embryonnaire. Cependant le défaut de fusion des CSs observé après la naissance indique qu'elle est nécessaire pour une fusion efficace des cellules myogéniques seulement en post-natal. L'ensemble de ses résultats suggère que certaines des voies de signalisation nécessaires pour contrôler la fusion pendant le développement et la myogenèse adulte sont distinctes en fonction du stade développemental et que la signalisation RhoA n'est indispensable qu'après la naissance.

Pour caractériser davantage la fonction de RhoA pendant la préservation du muscle adulte, nous avons délété RhoA dans les cellules souches musculaires adultes (CS). En particulier nous avons utilisé la lignée de souris Pax7-Cre^{ert2}: RhoA$^{fx\ /\ fx}$ permettant une suppression spécifique et inductible de RhoA (après traitement au tamoxifène). La mobilisation des CSs est induite par une blessure à la cardiotoxine. Nous avons montré que l'absence de RhoA altère la régénération du muscle squelettique. À différents stades de la régénération, les myofibres régénérées mutantes étaient plus petites que les contrôles. Quatre jours après la blessure, les CSs sans RhoA étaient moins nombreux, revenant à un niveau contrôle dans les jours qui ont suivi. Cependant les cellules satellites dépourvues de RhoA ne présentaient pas de défauts de prolifération ou de différentiation. Par contre, les capacités de fusion des CSs mutants RhoA étaient fortement altérées, comme en témoigne la diminution du nombre de myonoyaux par myofibres à tous les différents moments analysés.Un défaut de fusion a également été trouvé au cours de l'hypertrophie induite par une surcharge mécanique, un modèle dans lequel la croissance des myofibres dépend en partie de la fusion des CSs. Dans cette situation, les myofibres expriment RhoA et seules les CSs sont mutantes. Cela suggère que RhoA est nécessaire dans les deux partenaires de fusion pour obtenir une fusion efficace. Tous les résultats ont été confirmés *ex vivo* sur des cellules satellites primaires, montrant que le phénotype observé est cellule musculaire dépendent. Les cultures primaires de CSs montrent aussi un défaut de fusion. Pour l'instant, nous n'avons pas mis en évidence de défaut dans l'assemblage du cytosquelette d'actine. En fait les CSs mutant et contrôles ne montrent pas de différence dans l'organisation des filaments d'actine lorqu'elle est observées en microscopie confocale. De même, la contractilité des cellules n'est pas affectée, car il n'y a pas de différence dans la phosphorylation de la chaine légère de myosine (MLC2), protéine important dans la régulation de la contractilité des cellules musculaires. La contractilité et l'assemblage du cytosquelette sont deux événements important pour la fusion, mais des analyses complémentaires sont nécessaires. Les culture primaires de CSs seront ultérieurement utilisées pour préciser les

mécanismes impliqués dans le défaut de fusion: une analyse transcriptomique sur des cellules mutantes et contrôles en prolifération et en milieu de différenciation a été effectué. L'analyse transcriptomique a identifié des potentielles cibles qui peuvent être impliquées dans le défaut de fusion comme par exemple la Chordin et l'adenilate cyclase. Le premier est un inhibiteur des BMPs, stimulant la proliferation des myoblastes et inhibant leur différentiation. Le deuxième (l'adenilate cyclase) a été montré très important pour la fusion des cellules du trophoblaste. Des analyses supplémentaires nous aideraient à identifier de nouveaux effecteurs de la signalisation RhoA impliqués dans la fusion des CSs, en identifiant les voies moléculaires par laquelle RhoA peut contrôler la fusion.

Enfin, nous nous sommes intéressés au rôle joué par RhoA dans les fibres musculaires adultes. Nous avons utilisé une lignée de souris HSA-Cre^{ert2}: RhoA$^{fx\ /\ fx}$, pour obtenir une délétion de RhoA spécifique et inductible dans les myofibres adultes. Afin de perturber l'homéostasie musculaire nous avons utilisé un modèle d'hypertrophie compensatoire. Nous avons montré que l'absence de RhoA dans les myofibres empêche une réponse optimale à la surcharge mécanique et que RhoA est requis pour l'hypertrophie correcte du muscle. À l'état basal, aucun phénotype évident n'a été observé. Trois semaines après l'hypertrophie compensatoire des muscles *Plantaris*, le poids du muscle mutant *Plantaris* hypertrophié et la taille des myofibres ont montré une diminution significative.

Nous avons vérifié que la synthèse globale des protéines n'est pas affectée, grâce à la technique SUnSET. En plus la signalisation Akt, verifiée par western blot, n'est pas modifiée par la délétion de RhoA. Une semaine et trois semaines après l'OV, nous avons montré que la suppression de RhoA dans les myofibres n'affectait ni le nombre de CSs (exprimant RhoA) ni leur proliferation. Surtout, l'absence de RhoA dans les myofibres altère la fusion des CSs aux myofibres en croissance, suggérant que ce défaut est l'événement principal pouvant contribuer à la croissance réduite observée chez les mutants.

Afin de découvrir les voies moléculaires altérées dans les muscles mutants lors de la croissance induite par une surcharge mécanique, nous avons effectué un analyse transcriptomique sur les muscles *Plantaris* contrôles et mutants une semaine après l'hypertrophie. L'analyse du transcriptome des muscles *Plantaris* contrôles et mutants, une semaine après l'hypertrophie, a mis en évidence que les gènes fortement up-régulés pendant la surcharge mécanique dans le contrôle sont significativement down-régulés chez le mutant. Parmi les gènes dont l'expression était fortement diminué dans le muscle mutant il y a des gènes impliqués dans le remodelage de la matrice extracellulaire (MEC), tels que Mmp9 / 13, Adam8, Adamtslike1, ainsi que de puissantes chémokines chimioattractantes de macrophages comme CX3CL1 et CCL3L3. En parallèle, nous

avons réalisé une étude transcriptomique sur des myotubes exprimant ou non RhoA afin d'identifier les effets cellulaires autonomes de la perte de RhoA. De manière inattendue, il y avait un très petit recoupement entre les données *in vitro* et *in vivo* suggérant que ces gènes dépendaient de la contraction in vivo et / ou pouvaient être exprimés par d'autres cellules que les myofibres. Fait intéressant, sur une culture de myoblastes *in vitro*, l'expression de MMP9 et MMP13 a été induite par étirement unidirectionnel. Après purification des myofibres isolées, avant et après la surcharge mécanique induisant l'hypertrophie, leur expression était clairement up-régulée par les forces mécaniques, mais était significativement diminuée dans les myofibres mutantes, ce qui suggère que leur expression dépend de RhoA et des signaux mécaniques dans les myofibres. Les MMPs ont deux rôles principaux: hydrolyser les composants de la MEC et libérer des cytokines bioactives et ainsi que des facteurs de croissance qui sont "piégés" dans la MEC. Nous avons mis en évidence par la technique de "second harmonic microscopy" (SHG) une mauvaise organisation du collagène fibrillaire, le composant le plus abondant de la MEC musculaire. De plus, par western blot nous avons mis en évidence une accumulation de protéines des composants de la matrice (comme la Ténascine C et la Fibronectine, deux substrats de MMP9 et MMP13) dans les *Plantaris* mutants après OV par rapport aux contrôles.

Etant donné que, dans les muscles mutants qui ont un défaut de croissance, les protéines musculaires de la matrice extra cellulaire (substrats MMPs) ont été retrouvées accumulées et qu'en imagerie SHG il a été mis en évidence une mauvaise organisation du collagène fibrillaire on peut supposer que les MMPs jouent probablement un rôle important dans l'absence de croissance observée chez les mutants RhoA. Il est intéressant de noter que l'inhibition des MMPs au cours du processus d'hypertrophie, obtenue par l'injection d'un puissant inhibiteur de MMPs à large spectre (GM6001), reproduit le phenotype des mutants RhoA, suggérant une implication de la diminution de l'expression des MMPs dans la croissance musculaire compromise des mutants RhoA.

L'analyse transcriptomique a également montré une expression réduite de chemokines chemioattractantes telles que Cx3Cl1 et Ccl3. Ces derniers sont de puissants chimioattractants des macrophages et sont induit par l'hypertrophie du muscle squelettique (comme nous le démontrons en mesurant son niveau d'ARNm sur des myofibres isolées sur OV.

Nous avons corrélé une expression de chemokines plus faible avec une diminution de l'infiltration des macrophages dans le muscle *Plantaris* au moment de la surcharge mécanique. Plus précisément, le nombre de macrophages une semaine après l'OV est réduit dans la section de muscle *Plantaris* mutant par rapport au contrôle. De plus, nous avons démontré la nécessité fonctionnelle des macrophages (par des expériences de déplétion) pour une hypertrophie correcte.

Enfin, nous avons trouvé la signalisation Erk1 / 2 réduite après hypertrophie dans les muscles mutants RhoA par rapport aux contrôles. Des expériences *in vitro* suggèrent que RhoA peut directement stimuler Erk1/2 dans les myotubes et que le contrôle de l'expression de Mmp13 et de Cx3Cl1 par RhoA dépend de l'activité de Erk1/2.

Ces résultats suggèrent que RhoA est important pour maintenir globalement un microenvironnement musculaire approprié et régule son influence dynamique sur les fonctions des populations cellulaires résidentant dans le muscle.

L'ensemble de mes résultats devrait permettre une meilleure compréhension du rôle de RhoA dans la plasticité du muscle.

Introduction

Part I: Skeletal muscle and muscle stem cells

1. Skeletal muscle system

1.1 General characteristics

Human body is composed of more then 600 muscles. The main functions of muscular system are:

- **Mechanical**: converting the chemical energy to mechanical energy, muscular system allows the body to maintain a posture and to produce movements;
- **Metabolic**: the muscle is the primary site for glucose storage and uptake, but it is also a reservoir of amino acids stored as protein (Argilés et al. 2016);
- **Vital** functions, as the respiration and the digestion, are accomplished by the muscles.

In the vertebrate there are three types of muscles: smooth muscle, cardiac muscle and skeletal muscle. Smooth muscles are composed of mononucleated cells that contract in an involuntary way. They coat the internal cavity of the organs and allow the movement of the blood in the vessels, of the food in the digestive system and of the air in the lungs.

Cardiac muscle is composed of specific cells called cardiomyocytes. They contract in an involuntary and rhythmic way, allowing blood circulation and consequently organ oxygenation and nutrition.

On the contrary, skeletal muscles are attached by tendons to the bones and they contract voluntarily, allowing all the movements that we daily do and the maintenance of an erect posture.

Skeletal muscle is one of the most abundant organ of human body, comprising approximately 40% of the total body weight. Skeletal muscle is mainly composed by contractile material. The functional cellular unit responsible for skeletal muscle contraction is the myofiber. Muscle tissue is also composed of blood vessels that bring oxygen and nutrients, nerves that support neuromuscular activity and connective tissue representing the extra cellular matrix (ECM).

1.2 Myofibers

The myofibers are multinucleated and post mitotic cells. Their dimensions in mouse are approximately of 10 μm in diameter and some cm in length. They are defined as specialized syncytium (from the ancient greek syn=with/together and kytos=cellula), which means that they come from the fusion of myoblasts during development. Indeed, they contain hundreds of nucleus, sharing a common undivided cytoplasm and disposed at the periphery in a healthy state. It is estimated that each muscle fiber is made of thousands of myofibrils and contains billions of

myofilaments. The myofilaments are assembled together in a very orderly and characteristic pattern forming sarcomeres that are the basic contractile units of the skeletal muscle.

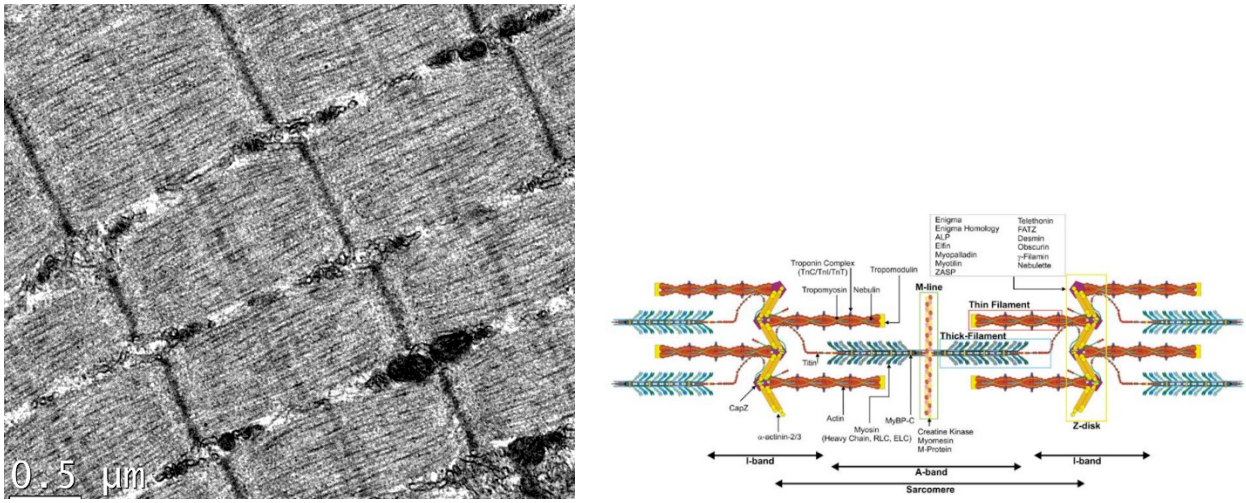

Figure1: Electronic microscopy image (Imag'ic Platform, Cochin Institute) and schematic organization of sarcomere (Au 2004)

Ultrastructural images of sarcomeres allow us to visualize their striped aspect. Structural features of the sarcomere include transversal thick and thin filaments, and longitudinal Z-disk and M-line. The region of the sarcomere containing the Z-disk and thin filaments is the I-band, while the remaining area is the A-band. The A-band comprises the area where the thick and thin filaments interdigitate, an area of thick filament alone and the M-line. The **thin filament** is composed primarily of **actin,** a protein with a double helical structure. The polymerization of actin occurs upon two molecules of the filamentous protein nebulin acting as a 'molecular ruler' for actin filament assembly and regulating the length of the thin filament. The other principal proteins associated to actin are: **tropomyosin** that blocks the interaction between actin and myosin during relaxed state; **tropomodulin** that acts as a flexible linker between tropomyosin, actin and nebulin; **troponin complex** (composed of TnC, TnI and TnT) that, in a calcium-dependent manner, controls the position of tropomyosin and hences the binding of myosin to actin for muscle contraction (Au 2004).

The main constituent of the **thick filament** is **myosin**. The complete myosin molecule is a complex of six polypeptide chains consisting of two 'heavy chains' and two pairs of different light chains. Myosin protein contains a tail composed of the two heavy chains rolled up and two heads composed of the association of heavy and light chains. The direction of the myosin heads changes polarity at the M-line, allowing interaction with the thin filaments anchored to the the adjacent Z-disk (Au 2004).

Actin and myosin are the most abundant proteins in the single fiber and they contribute to the muscle contraction sliding on top to each other.

The well-structured process leading to muscle contraction, called excitation-contraction coupling, begins when an action potential causes depolarization in the myofiber membrane. The depolarization is spread via invaginations of the muscle cell membrane called the transverse (T) tubules that help to expand depolarization signals to the entire muscle fiber. Depolarization of the T tubules elicits a conformational change in the dihydropyridine receptors, which causes the opening of nearby ryanodine receptors on the sarcoplasmic reticulum (SR), the storage site for calcium within muscle cells. When calcium is released from the SR, it binds to troponin C. This binding provokes a conformation change, which shifts tropomyosin, allowing the myosin heads to attach to the actin filaments creating what is known as a cross-bridge. Then the cross-bridge cycling begins. When ATP binds to an ATP binding domain on the myosin head, it leads the myosin to dissociate from the actin, breaking the cross-bridge. ATP is then hydrolyzed into ADP and P, which causes the myosin heads to change their conformation and to move toward the positive end of the actin, cocking the myosin head. The phosphate is released, and the ADP bound myosin binds to a new location on the actin filament. Once that ADP is released it causes the return of myosin to its original position, pulling on the actin filament. Therefore, the sarcomere and the muscle fiber contract. These cycles continue until calcium levels in the myofibers fall, causing tropomyosin to cover again the myosin binding sites of the actin filaments. The triggers that control the movement are transmitted from motor neurons to their target muscle cells at the neuromuscular junctions (NMJ). The NMJs contain many proteins distributed within the complex structure of the motor nerve terminal and the postsynaptic muscle membrane. The chemical transmitter, released from the nerve, acts on the muscle evoking the contraction (Slater 2017).

1.3 Fiber type

There are different genes coding for the myosin heavy chain that constitute the diverse types of fibers. The fiber identity is first established during embryonic development by intrinsic myogenic control mechanisms and it is later modulated by neural, hormonal factors and functional demands. The diversity of their physiological properties is very important because it allows the participation of a muscle in activities with various metabolic and mechanical demands. During the last few decades, muscle fibers have been classified using different criteria including: (1) color of muscle fibers (red *vs* white) that correlates with myoglobin content and their vascularization, (2) contractile properties of the motor units in response to electrical stimulation, (3) speed of shortening during a

single twitch (fast *vs* slow), (4) degree of fatigability during sustained activation (fatigable *vs* fatigue-resistant), (5) predominance of certain metabolic or enzymatic pathways (oxidative *vs* glycolytic), (6) enzyme-histochemical stain reaction (based mainly on ATPase staining techniques but also on oxidative enzymes such as succinate dehydrogenase or SDH), (7) calcium handling by the sarcoplasmic reticulum (slow *vs* fast), (8) protein isoform expression, (9) mitochondrial content and (10) myofibers size among others (Schiaffino et al. 2011a).

In mammalian, four types of myosin heavy chain isoforms expressed in four different fiber types have been identified in skeletal muscle: Myh7 isoform coding in fiber type I (oxidative metabolism and slow contracting fibers), Myh2 isoform coding in fiber type IIA and Myh4 in fibers IIB (glycolytic metabolism and fast contraction), Myh1 in fibers IIX (glycolitic metabolism and fast contraction intermediate between the type IIA and IIB).

Today is well known that the common view of diversity based on the old classification of fast-twitch muscles, characterized by glycolytic metabolism and specialized for phasic activity, and slow-twitch muscles, rich in myoglobin and oxidative enzymes and specialized for more continuous activity, is simplistic. Firstly, because we cannot assume a direct link between fast/slow properties and mitochondrial content because, as revealed by electron microscopy, there are mitochondria-rich fast twitch fibers. Secondly because there are hybrid fibers that can express different isoforms of myosin at the same time (Murgia et al. 2017). The discovery of specific antibodies that recognize the different types of myosin constitutes an essential tool to avoid or at least to reduce the mistakes coming from work procedures using the revelation of ATPase reaction staining (pH, reagent etc.) (Schiaffino 2018).

Since there is a correlation between biochemical and histological properties, it was shown already in 1960 that the fibers in a motor unit are homogeneous: a motorneuron (MN) innervates muscle fibers of a single type. One motor unit is composed by one MN and the fibers that it innervates (Edström et al. 1968). MNs are neuronal cells located in the central nervous system (CNS) controlling a variety of downstream targets. There are two main types of MNs, (i) upper MNs that originate from the cerebral cortex and (ii) lower MNs that are located in the brainstem and spinal cord. Among them there are the spinal MNs (SpMNs) that are located in the ventral horn of the spinal cord and control effector muscles in the periphery. MNs form coherent groups connecting to a unique muscle target defined as MN pools. Somatic MNs can be divided into 3 groups: (i) alpha, (ii) beta, and (iii) gamma according to the muscle fiber type they innervate within a specific muscle target. The diversity of MNs mirrors the multiplicity of targets they innervate. Indeed they present differences in term of size and excitability (Stifani 2014).

1.4 Extracellular matrix (ECM)

Skeletal muscle is composed of bundle of fibers. An individual muscle is surrounded by a connective tissue called epimysium. Group of fibers within that muscle are arranged in bundles and surrounded by another layer of connective tissue known as the perimysium. A single muscle fiber is surrounded by a cell membrane or basal lamina (KJAeR 2004). All these layers simply classify the ECM organization in the muscle that is more complex. Myofibers are attached to the bones by tendons and the specialized region including the muscle-tendon connection is called myotendinous junction (MTJ). The MTJ is thus a major site of force transmission from myofibrils to the extracellular matrix and adapts to mechanical stress by increasing the muscle-tendon contact area (Charvet et al. 2012). Structurally, the MTJ consists of subsarcolemmal, transmembrane and extracellular protein complexes.

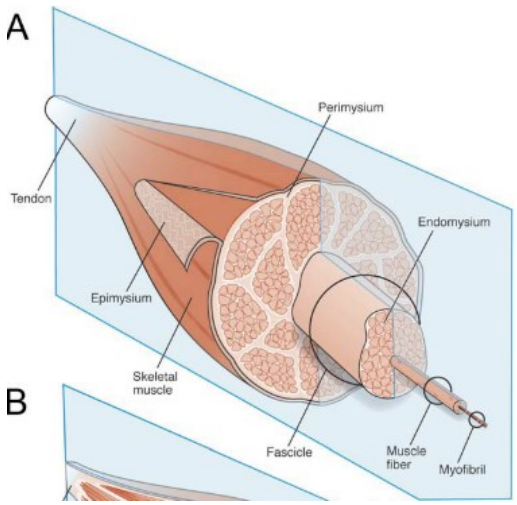

Figure 2: Anatomic organization of skeletal muscle (Gillies et al. 2011)

The ECM is a complex meshwork of proteins whose biochemical and biophysical properties allow to integrate the informations coming from workload stimuli and to convert them into mechanical capacities.

The ECM, that forms the intramuscular connective tissue, has multiple **functions**. Firstly, it provides a basic mechanical support for vessels and nerves, creating the scaffold tissue. Secondly, the connective tissue ensures the passive elastic response of muscle contributing to the force transmission. Thirdly, it serves as an adhesive substrate for cell migration and binding morphogens and growth factors (Naba et al. 2016). Up to now it is well known that ECM has mechanical properties, capable to modulate the stiffness of the tissue, playing an important role in the tissue structural features and force transmission. Moreover, ECM plays also a dynamic role: it directs

resident cell function upon a plethora of stimuli and it releases factors and molecules embedded inside the matrix itself.

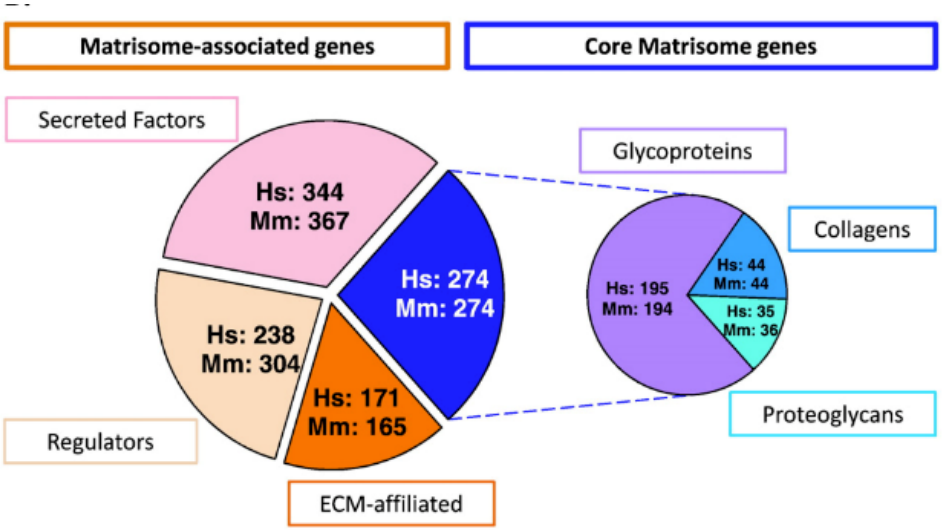

Figure 3: Extracellular matrix composition remodeling, where "Hs" indicates genes in the human genome and "Mm", genes in the murine genome (Naba et al. 2016)

Naba et al. have characterized "the matrisome" composing the extracellular matrix. They compared 14 different tissues and they found out that ECM was composed of 33% of secreted factor, 27% of regulators (ECM-crosslinking and ECM-modifyng enzymes), 14% of ECM-affiliated proteins and 24% of glycoproteins, collagens and proteoglycans representing the core matrisome.

ECM composition in the muscle

The basement membrane (BM), that coats the skeletal muscle myofibers, is composed of two layers: an internal felt-like basal lamina (BL) directly linked to the plasma membrane and an external fibrillar reticular lamina. The BL contains non-fibrillar collagen, non-collagenous glycoproteins and proteoglycans. The fibrils of the reticular lamina are collagenous and they are embedded in an amorphous proteoglycan-rich ground substance (Sanes 2003).

The BL consists primarily of type IV collagen and laminin. This later is the major non-collagenous protein in basal lamina. Laminins are bound by integrins and β-dystroglycan to the BM. Laminins can bind directly to Collagen type IV, but may also be indirectly linked by fibronectin. β-dystroglycans complex interacts with the cytoskeleton and it is important to provide a mechanical linkage between the contractile components of skeletal muscle (i.e. actin cytoskeleton) and the interconnected layers of the intramuscular connective tissue (Csapo et al. 2020). As well, integrins are thought to act in a bidirectional manner: allowing intracellular signalling molecules to regulate external adhesion and transferring external stimuli to affect cellular processes. In the BL, there are

several other key components like the **proteoglycans** (PG). A number of PGs are present in muscle ECM and many belong to the family of small leucine-rich proteoglycans (SLRPs). SLRPs consist of a core protein with attached glycosaminoglycans (GAGs) chains and they bind to growth factors. Growth factors in skeletal muscle can be stored and released by negatively charged GAGs. Importantly, enzymes in ECM can cleave GAG chains with their associated growth factors, allowing their interaction to cell surface receptors and their implication in signalling and in mechanotransduction (Gillies et al. 2011). Proteoglycans form a complex once they have bound Collagen. The location of collagen crosslinks determines the structural organization of skeletal or muscle ECM.

The fibrillar reticular lamina is composed principally of collagen and fibronectin. **Collagen** is the central component of ECM. The collagen superfamily contains a total of 28 different members, among which types I, III, IV, V, VI, XII, XIII, XIV, XV, XVIII, and XXII have been shown to be present in mature skeletal muscle. Fibrillary collagen type I and type III are the most abundant and they predominate in adult endo-, peri- and epi-mysium, confering tensile strength and rigidity to the muscle. In particular, collagen type IV is a helical molecule and produces a network structure that, as mentioned before, constitutes the basis of the basal lamina.

With its multidomain structure, fibronectin plays a role of “master organizer” in matrix assembly, forming a bridge between cell surface receptors i.e. integrins and compounds such as collagen, proteoglycans and other focal adhesion molecules. Fibronectin plays an essential role also in the assembly of fibrillin-1 into a structured network (Halper et al. 2013). Fibronectin can provide sufficient signals to control attachment, spreading and formation of focal adhesions and of extracellular matrix (Woods et al. 2000). For example fibronectin binds syndecan-4 forming a complex that is important to regulate satellite cells homeostasis in their niche (Bentzinger et al. 2013).

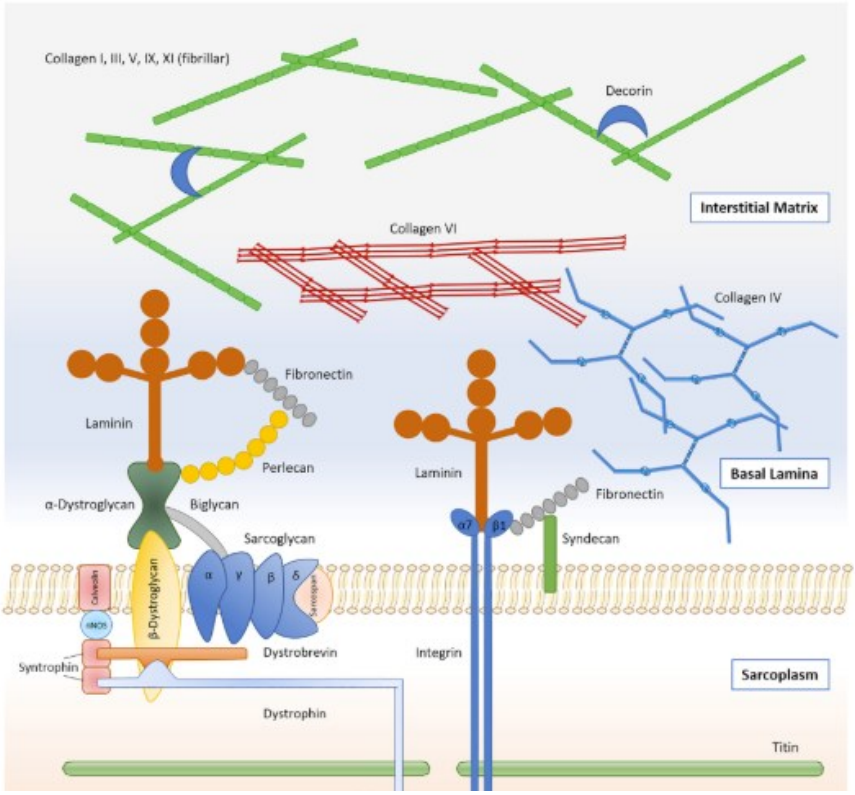

Figure 4: Schematic representation of principal component of muscle extracellular matrix (Csapo et al. 2020)

Moreover, in the ECM are secreted **matricellular proteins** that do not play a structural role. Although these proteins do not provide structural support, they are vital in ECM signalling and maintaining ECM organization. In skeletal muscle they include: osteopontin, secreted protein acidic and rich in cysteine (SPARC), thrombospondin and tenascin-C. Osteopontin has cytokine-like functions and it is usually not present in healthy skeletal muscle but it appears during muscle regeneration. Although the osteopontin is typically secreted by inflammatory cells, also myoblasts can secrete it (Uaesoontrachoon et al. 2008). SPARC has been shown to bind collagen and influences its assembly in the ECM (Bradshaw 2009). Thrombospondin is a large multifunctional homotrimeric matrix glycoprotein whose action primarily serves to inhibit angiogenesis: it is considered as an important endogenous negative regulator of angiogenesis preventing excessive capillarization in the muscles (Malek et al. 2009). Tenascin-C is localized at the neuromuscular junction (NMJ) and it is important for its formation, maturation and stabilization (Cifuentes-Diaz et al. 2002). It has been recently shown that Tenascin-C has a critical role in the correct maintenance of muscular stem cells niche microenvironment (Tierney et al. 2016).

ECM composition and organization change according to the health of the muscle, exercise and ageing. ECM homeostasis is maintained through finely tuned anabolic and catabolic processes that are governed by various growth factors, proteoglycans and enzymes responsible for collagen

degradation. The constant ECM homeostatis requires an accurate regulation of its synthesis and degradation. A wide variety of mononuclear cell types, resident into the muscle, are involved in the maintenance of ECM. The primary mononuclear cells in normal muscle responsible for producing the majority of ECM components are the **fibroblasts**. They are sensitive to mechanical loading and they link mechanical signals to gene expression alterations.

The two superfamilies of enzymes involved in the regulation of ECM homeostasis are the metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs).

At the beginning, MMPs family was identified as enzymes degrading fibrillar collagen (Brinckerhoff and Matrisian 2002). Most of them are secreted proteins; however, there are MMPs that have trans-membrane domains and short cytoplasmic tails. Proteolysis driven by MMPs can: create space for cells migration, produce specific substrate-cleavage fragments with independent biological activity, regulate tissue architecture through effects on the ECM and intercellular junctions, and regulate vascularization. MMPs can modify the activity of signalling molecules directly or indirectly by ECM protein cleavage revealing cryptic functions (Giannelli et al. 1997; Page-McCaw et al. 2007). MMP substrates include peptide, growth factors, tyrosine kinase receptors, cell-adhesion molecules, cytokines and chemokines as well as other MMPs and unrelated proteases. MMPs have many important functions during embryonic development, morphogenesis and remodelling of several tissues, as it has been shown in numerous experiments using MMPs null mice which displayed lethality and maturation defects (Page-McCaw et al. 2007). In skeletal muscle MMPs have regulatory roles in muscle growth and development and they are also important for muscle repair processes after traumatic injury or disuse myopathy. One of the most important and predominant MMPs associated with skeletal muscle is MMP2, type IV collagenase. Recent studies assert the primary role for MMP13 in myoblast migration (Lei et al. 2013), in supporting postnatal growth and in the resolution of acute damage (L. R. Smith et al. 2020). The involvement of MMP9 in skeletal muscle regeneration has been defined too (Li et al. 2009).

The activities of MMPs are also under the control of enzyme TIMPs. TIMP1 binds to active forms of MMPs forming non-covalent complexes, whereas TIMP2 stabilizes the inactive form of the enzyme and thus inhibits the formation of active proteolytic enzyme. Disturbances in the ratio of specific MMPs and their inhibitors may conduct to physiological dysfunctions that will result in clinical disorders (Carmeli et al. 2004).

Table 1

Mammalian MMPs and substrates.

MMP	Non-traditional substrate	Resultant effector	Matrix substrates
Minimal domain			
MMP-7/Matrilysin	Pro-a-defensin [41•]	α-Defensin	Proteoglycans, laminin, fibronectin,
	Decorin [13]	Bioavailable TGFβ	gelatin, collagen III/IV/V/IX/X/XI,
	Cell surface bound Fas-L [27•,28•]	Active soluble Fas-L	fibrin/fibrinogen, entactin, tenascin,
		Inactive soluble Fas-L	vitronectin
	B4 integrin [31]	Release of B4 integrin	
	E-cadherin [32,33•]	Bioactive E-cadherin ectodomain	
	Plasminogen [60,61•]	Angiostatin	
	Pro-TNFα [39•]	Bioavailable TNFα	
	Pro-MMP-2,7	MMP-2,7	
MMP-26/ Matrilysin-2/Endometase	α1-proteinase inhibitor [62]	Inactive serpin	Gelatin, collagen IV, fibronectin,
	MMP-9 [63]	Pro-MMP-9 [63]	fibrinogen [62–64]
Collagenases			
MMP-1/Collagenase-1	Perlecan [12]	Bioavailable FGF	Collagen I/II/III/VII/X, gelatin,
	IGFBP-2,3	Bioavailable IGF	entactin, aggrecan, tenascin
	α1-antichymotrypsin	Inactive serpin	
	α1-proteinase inhibitor	Inactive serpin	
	Pro-MMP-1,2	MMP-1,2	
	Pro-TNFα	Bioavailable TNFα	
MMP-8/Collagenase-2	α1-proteinase inhibitor	Inactive serpin	
	Pro-MMP-8	MMP-8	
MMP-13/Collagenase-3	α1-antichymotrypsin	Inactive serpin	
	Pro-MMP-9,13	MMP-9,13	
Stromelysins			
MMP-3/Stromelysin-1	Perlecan [12]	Bioavailable FGF	Proteoglycans, laminin, fibronectin,
	Decorin [13]	Bioavailable TGFβ	gelatin, collagen III/IV/V/IX/X/XI,
	Pro-HB-EGF [18]	HB-EGF	fibrin/fibrinogen, entactin, tenascin,
	Pro-IL1-B [21]	IL1-β	vitronectin
	Plasminogen [59]	Angiostatin	
	E-cadherin [33•,65]	Bioactive E-cadherin ectodomain	
	IGFBP-3	Bioavailable IGF	
	α1-antichymotrypsin	Inactive serpin	
	α1-proteinase inhibitor	Inactive serpin	
	Pro-MMP-1,3,7,8,9,13	MMP-1,3,7,8,9,13	
	Pro-TNFα	Bioavailable TNFα	
MMP-10/Stromelysin-2	Pro-MMP-1,8,10	MMP-1,8,10	
MMP-11/Stromelysin-3	α-1-proteinase inhibitor	Inactive serpin	Laminin, fibronectin, aggrecan
	IGFBP-1	Bioavailable IGF	
Gelatinases			
MMP-2/Gelatinase A	Decorin [13]	Bioavailable TGFβ	Gelatin, elastin, fibronectin, collagen
	Pro-TGF-B2 [20•]	TGF-β2	I/IV/V/VII/X/XI, laminin, aggrecan,
	Pro- IL1-B [21]	Active IL1-β	vitronectin
	MCP-3 [36••]	Inactive chemoattractant	
	IGFBP-3/5	Bioavailable IGF	
	ProTNFα	TNFα	
	FGF-R1 [23]	Bioactive FGF-R1 ectodomain	
	Pro-MMP-1,2,13	MMP-1,2,13	
MMP-9/Gelatinase B	Unknown [37••]	Bioavailable VEGF	
	Pro-TGF-B2 [20•]	TGF-β2	
	Pro-IL1-B [21]	IL1-β	
	Cell-surface bound IL-2Ra [22•]	Release of IL-2Rα	
	Plasminogen [60,61•]	Angiostatin	
	α1-proteinase inhibitor [66•]	Inactive serpin	
	Pro-TNFα	TNFα	
Membrane associated			
MMP-14/MT1-MMP	Pro-MMP-2,13	MMP-2,13	Gelatin, fibronectin, vitronectin,
	Cell-surface bound CD44 [67]	Release of CD44	collagen, aggregan
	Cell-surface bound tissue transglutaminase (tTG) [68]	Release of tTG	
MMP-15/MT2-MMP	Cell surface bound tTG [68]	Release of tTG	
MMP-16/MT3-MMP	Pro-MMP-2	MMP-2	
	Cell surface bound tTG [68]	Release of tTG	
MMP-24/MT5-MMP	Pro-MMP-2 [69,70]	MMP-2	
MMP-17/MT4-MMP	Pro-MMP-2 [71]	MMP-2	Gelatin [71]
MMP-25/MT6-MMP	Pro-MMP-2 [72]	MMP-2	Gelatin, collagen IV, fibrin, fibronectin,
			Laminin-1 [73]
MMP-23			Gelatin [74]
Other MMPs			
MMP-12/ Metalloelastase	Plasminogen [57,58]	Angiostatin	Elastin, fibronectin, fibrin/fibrinogen,
			laminin, proteoglycan,
MMP-19/RASI			Gelatin, tenascin, fibronectin,
			Collagen IV, laminin, entactin,
			Fibrin/fibrinogen, aggrecan,
			COMP [75,76]
MMP-20/Enamelysin			Amelogenin, Aggrecan, COMP [74]
MMP-28/Epilysin			

Figure 5: Principal MMPs substrates and relative outcomes (McCawley and Matrisian 2001)

2. Development of skeletal muscle

2.1 Myogenesis

During the process of gastrulation, 5-6 days after fertilization, three cell layers called ectoderm, mesoderm and endoderm, are formed in the mouse blastocyst. Then, each tissue comes from a distinct layer, in a spatio-temporal dependent mechanism (depending on the distance from the primitive node) that influences cell lineage choice.

The mesoderm is anatomically separated in paraxial (somitic), lateral and intermediate with respect to its position towards notochord. In the course of development, local oscillations in gene expression and morphogen gradients induce pairwise condensations of paraxial mesoderm into somites (a process called somitogenesis), which develop progressively from head to tail.
In the medial somite, the most ventral part forms the mesenchymal sclerotome, the most dorsal portion of the somite remains epithelial and becomes the dermomyotome.
The sclerotome contains precursors to cartilage and bone, contributing to the formation of axial skeleton. The dermomyotome gives rise to skeletal muscles of the body, with the exception of some head muscles. The lips of the dermomyotome mature into the myotome, located between the dermomyotome and the medioventrally located sclerotome; it is a primitive muscle structure containing mononucleated post-mitotic myocytes (Bentzinger et al. 2012; Biressi et al. 2007). Lateral dermomyotome muscle progenitors differentiate early and subsequently develop into intercostal muscles and muscles of the body wall.
Dermomyotome can be subdivided into an epaxial (dorso-ventral) and hypaxial (ventro-lateral) regions. Committed muscle cells from epaxial region give rise to the deep back muscles. At the limb level, myogenic progenitors delaminate from hypaxial region of dermomyotome and migrate distally to the limb buds where they start to express the myogenic determination genes. Hypaxial dermomyotome cells give rise to various muscles including tongue muscles, limb, associated shoulder muscles and the muscular diaphragm depending on their position along the rostro caudal axis.
The craniofacial muscles, unlike muscles of the trunk and limbs, which are all from somitic origin, arise from three distinct regions of the embryo, namely the occipital somites, precordal (cranial to the notochord) and paraxial (flanking the notochord) head mesoderm. Craniofacial skeletal muscles are associated with head (including extra-ocular muscles, jaw and facial muscles) and neck structures as well as oesophagus (Sambasivan et al. 2011a).

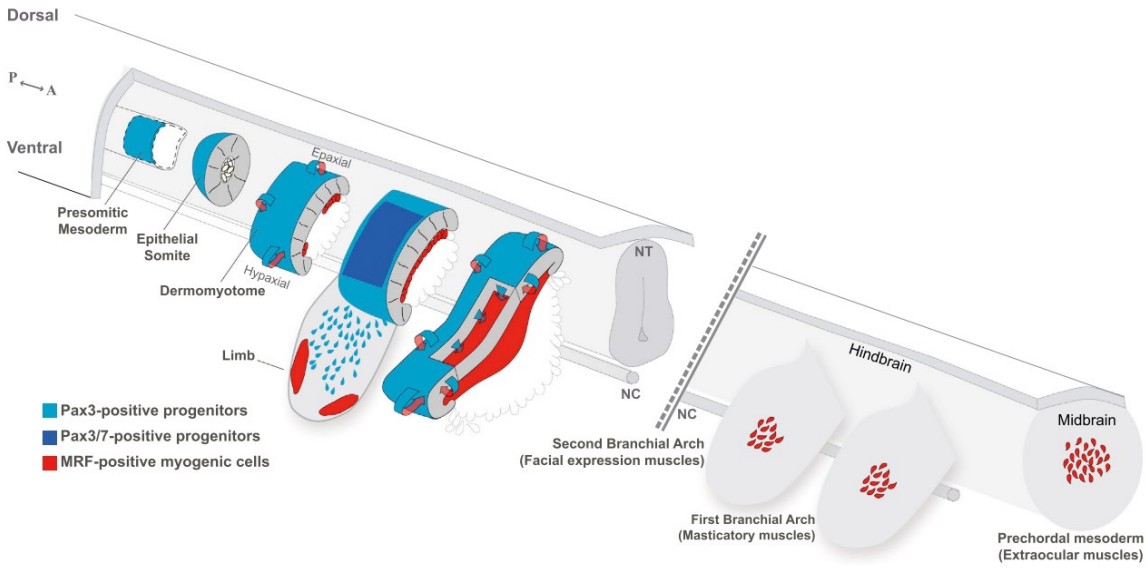

Figure 6: Representative schema of myogenesis (Buckingham and Rigby 2014)

The somitogenesis starts at embryonic day 8 (E8.0) in the mouse. At this time point, skeletal muscle development begins in the embryo. Once the myogenic progenitors have reached their final destination, they will give rise to terminally differentiated myocytes and form the primary myotome. During the primary myogenesis, the first multinucleated muscle fibers are formed from embryonic progenitors, which differentiate by fusing with each other. This will establish the scaffold of the different skeletal muscles. This phase is usually considered to take until Embryonic Day 14.5 (E14.5) in the mouse (Biressi et al. 2007). Secondary myogenesis depends on fusion of fetal progenitors. It results in secondary fibers, in a second wave of 'secondary' muscle fibre formation. This step is important for the growth and maturation (acquisition of mature fibre type identity) of muscles during embryonic development, concomitant with the innervation. Secondary (fetal) fibers are originally smaller and surround primary fibers with a donut like configuration.

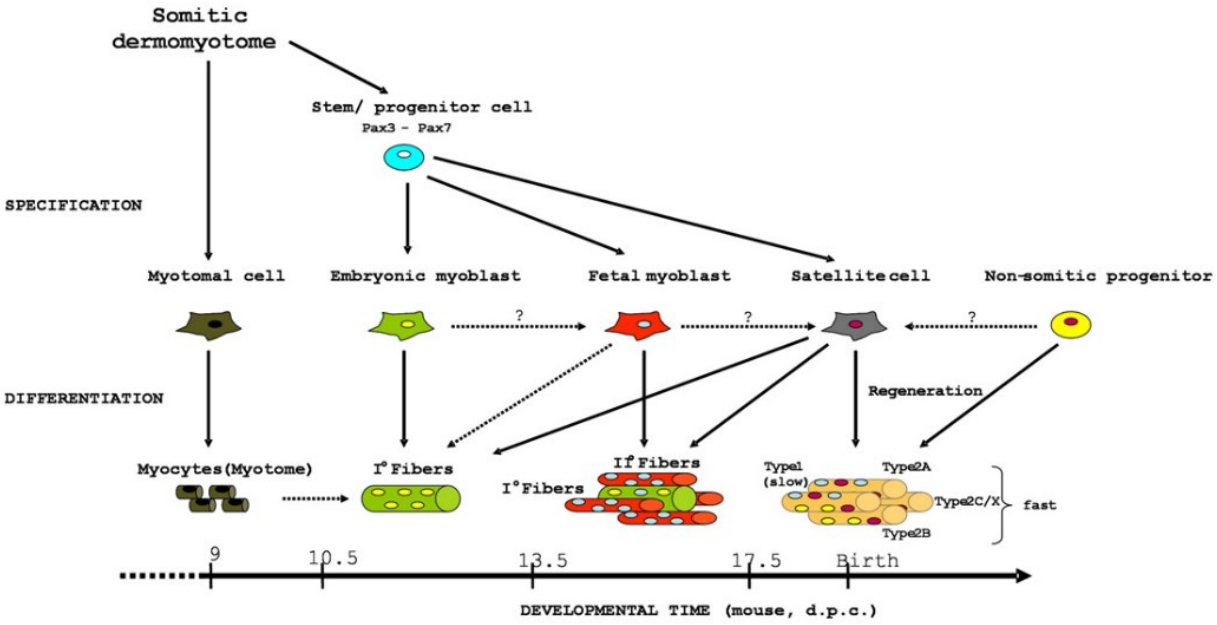

Figure 7: Schematic representation of skeletal muscle formation (Biressi et al. 2007)

At the same time, a basal lamina begins to form around each fiber and it is only after its formation, at the end of this phase, that adult muscle progenitors (also called satellite cells) can be morphologically identified as mononucleated cells laying between the basal lamina and the fiber plasma membrane (Cossu et al. 2005). Transcription factors like Six1 and Six4 are required for the correct satellite cells positioning during embryonic myogenesis, maybe affecting genes coding for extracellular matrix proteins (Wurmser et al. 2020), in synergy with Notch signalling shown to be required for efficient Pax7+ cells homing (Bröhl et al. 2012).

During the perinatal period, there is a considerable growth of muscle, mediated by the satellite cells. Around day 21 after birth, at the end of postnatal growth, satellite cells enter into quiescence, but they can be activated if the muscle tissue is damaged or in response to further growth demands. Many works from different laboratories identified specific features of embryonic, fetal myoblasts and satellite cells and characterized them as distinct classes of myogenic cells. Even if all canonical myogenic cells (embryonic and fetal myoblasts and satellite cells) may be committed to myogenesis in the newly formed somite, terminal differentiation occurs only for a fraction of somitic cells. The existence of successive phases of muscle growth involving distinct progenitors can be explained by the need of maintaining a precursor pool of dividing myogenic cells (to cope with the growing size of the embryo) and at the same time to generate contracting and post-mitotic skeletal muscle fibers (to allow early movements of the embryo) (Cossu et al. 2005). Different transcription factors are required to regulate the identity of myotomal versus later myogenic progenitors. Moreover, another

important secreted factors like TGFβ are required to regulate skeletal muscle histogenesis, due to its different sensitivity from embryonic or foetal myoblast (Cusella-De Angelis et al. 1994).

2.2 Gene regulation of myogenesis

MRFs

During embryonic development, specific signalling molecules are required to specify the myogenic cell fate and differentiation of pluripotent embryonic cells.

In 1987, Davis et al. discovered that transfection of fibroblasts with ***MyoD*** expression vector converts them to stable myoblasts (Davis et al 1987). In the same years three other basic helix–loop–helix factors - **Myf5**, **Myogenin**, and **MRF4** (also known as Myf6) were discovered and were shown to be able also to induce myoblast traits in non-muscle cell lines (T. Braun et al. 1989; Wright et al. 1989).

These Myogenic Regulatory Factors (MRF) are bHLH transcription factors that control myogenic fate acquisition in the vertebrate embryos. They contain a conserved basic DNA binding domain that binds the E box, a DNA motif, which contains the sequence CANNTG. This DNA motif is present in the promoters of many skeletal muscle specific genes, and is controlled by the binding of MRF.

The *MRF* genes are expressed exclusively in the skeletal muscle lineage and have been shown to play key regulatory roles in the development of the skeletal muscle lineage. In the mouse embryo, each factor has a distinct pattern of expression during the formation and maturation of skeletal muscle, yet Myf5, MyoD and MRF4 share the common property to commit pluripotent embryonic cell in the myogenic lineage, while Myogenin is involved in their differentiation.

Myf5

For the first time in 1992, Braun et al. described the phenotype of mouse embryo mutant for Myf5. Mice lacking Myf5 were unable to breathe and died immediately after birth, owing to the absence of the major distal part of the ribs and associated skeletal muscle. Skeletal muscle from newborn mice revealed no morphological abnormalities, but the appearance of myotomal cells in early somites was delayed by several days in mutant embryo (Braun et al. 1992). Skeletal abnormalities specific to the ribs in homozygous *Myf5* mutant mice came as a surprise. Tajbakhsh et al. have shown that the myogenic precursors migrating from the dermomyotome to the limb buds are $Myf5^{-}$, and start to express Myf5, the earliest MRF, after the migration in peripheral position. This observation means that the somite-derived myogenic precursor cells migrating into the limb are already a distinct determined cell population and that Myf5 is only turned on after the migration

step (Tajbakhsh et al. 1994). Moreover, the effect of neighboring genes, such as *MRF4*, for *Myf5* mutant phenotype have to be taken into account (Kaul et al. 2000). Analysis of other mutant alleles of *Myf5*, where MRF4 is still expressed, showed that *Myf5*$^{-/-}$ mice have no detectable defects, showing that the lethality of the former *Myf5* mutants is due to both MRF4 and Myf5 null expression.

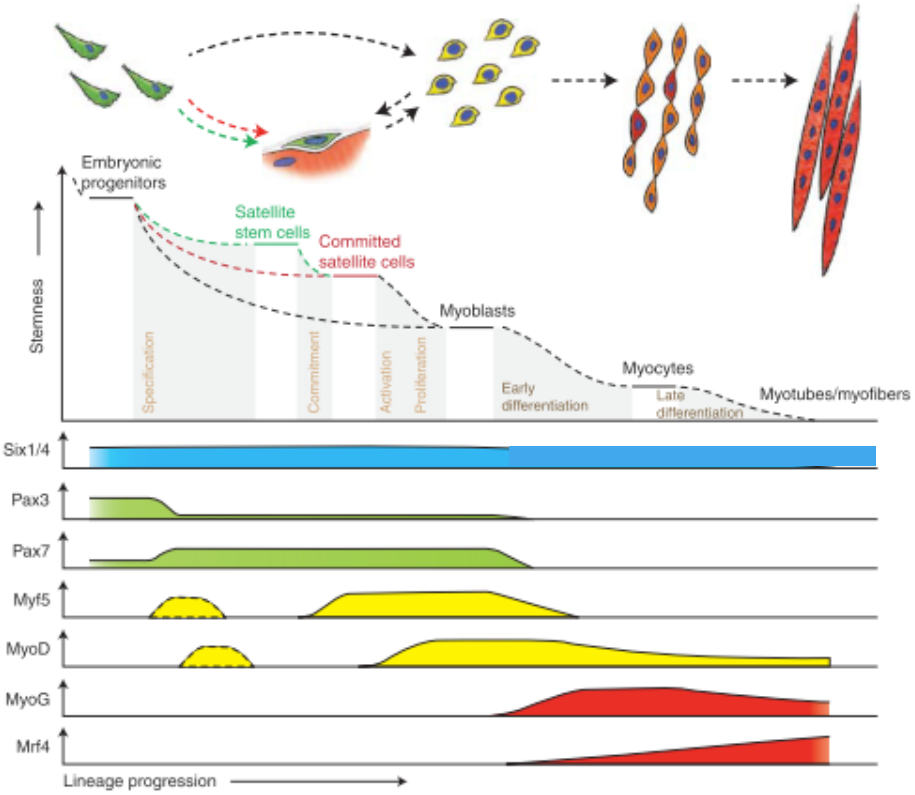

Figure 8: Hierarchy of transcription factors regulating progression through the myogenic lineage (adapted from (Bentzinger et al. 2012)

MyoD

In 1992, Rudnicki et al. studied mice lacking MyoD and surprisingly discovered that they were viable and fertile, with no muscle abnormalities. However *Myf5* expression was significantly increased in muscles of mutant animals, and since Myf5 is normally repressed at midgestation, they suggested that Myf5 may partially substitute for the function of MyoD in mutant animals (Rudnicki et al. 1992). Further analysis showed that *MyoD*$^{-/-}$ embryos displayed normal development of paraspinal and intercostal muscles, but muscle development in limb buds and brachial arches was delayed by about 2.5 days (Kablar et al. 1997). In absence of MyoD, the progression of adult satellite cells through the myogenic program was affected in a post natal muscle regeneration paradigm, while their self-renewal was not (Megeney et al. 1996). Thus satellite cells coming from

MyoD deficient mice proliferated more compared to wild type satellite cells but had differentiation defects (Cornelison et al. 2000). In parallel, another work evidenced the importance of MyoD for correct proliferation of cultured myoblasts in addition of the failure of MyoD mutant cells to efficiently differentiate (Montarras et al. 2000). In addition, ten years later, MyoD was described to interact with the Cdc6 promoter and to control its expression and the engagement of myoblasts or primary cells in the cell cycle (Zhang et al. 2010).

Myogenin

In 1993 Hasty et al. showed that mice lacking Myogenin survived during fetal development, but died immediately after birth. They showed a severe reduction of all skeletal muscles and accumulation of mononucleated cell populations, arrested in the differentiation pathway and unable to fuse. Myogenin has a crucial role for biochemical and morphological differentiation of skeletal muscle cells (Hasty et al. 1993). Another study investigated Myogenin absence on embryonic and fetal development and showed that Myogenin affected secondary myogenesis more severely than primary myogenesis and that it was not required for the initial aspects of myogenesis. Indeed, Myogenin is not essential for commitment of cells to the myogenic lineage but is important for their embryonic myogenesis terminal differentiation. Moreover it is not essential for *MyoD* expression (Venuti et al. 1995), but *Myogenin* expression is under the control of MyoD, Myf5, MRF4 genetic cascade.

MRF4

MRF4 is the only muscle regulator factor that is expressed at high levels both in adult muscle and in embryonic myotomes, suggesting that MRF4 regulates maturation events and maintainance of the adult muscle phenotype. *MRF4* mRNA was found in the myotomal compartment of the somites. During late fetal muscle development its expression increases and is maintained in the adult too (Hinterberger et al. 1991). On the basis of its expression pattern, it has been proposed that MRF4 may regulate skeletal muscle maturation and several aspects of adult myogenesis. Two mutant mice were generated to delete *MRF4* gene, showing variable phenotypes. In one study, viable mutant mice exhibited multiple rib anomalies, including extensive bifurcations, fusions, and supernumerary processes, and a dramatic increase of Myogenin expression, as a compensatory response (Zhang et al. 1995). In the other study, mutant mice died postnatally within a few minutes, caused by a lack of distal rib structures. These mice presented a pronounced down-regulation of *Myf5* transcription and a very similar phenotype to *Myf5* null mice (Braun et al. 1995). These different phenotypes were explained later by Olson et al. as depending on the sequences of the mutated genes and as an effect

in cis-regulatory interactions with the neighbour *Myf5* gene (Olson et al. 1996). More recently mutant mice for *MRF4* or mutant for *Myf5* and *MyoD* unrevealed the embryonic function of *MRF4* as determination gene, as already proposed for *Myf5* and *MyoD* (Kassar-Duchossoy et al. 2004).

PAX genes

Different teams investigated the factors controlling *MRF* expression that could regulate skeletal muscle origin.

The upstream level in the genetic hierarchy controlling myogenesis is dominated by the paired-homeobox transcription factors Pax3 and Pax7, that define progenitor cell population during all the stages of skeletal muscle formation (Kassar-duchossoy et al. 2005). The paired-domain is a large, highly conserved sequence of about 130 amino acids. All known paired-domains of *Drosophila* and vertebrates are located close to the amino terminus of their proteins.

Pax genes are of particular interest from a developmental as well as an evolutionary point of view because their presence is conserved in vertebrates. In humans, these genes are of a considerable importance because they are associated with severe developmental defects (Noll 1993).

In mice, spontaneous mutation for *Pax3* leads to exencephaly, spina bifida, and defects in neural crest cell derivatives (Epstein et al. 1991). Mouse embryos homozygous for the Splotch *Pax3* loss-of-function mutation do not develop the hypaxial domain of the somite and consequently do not form limb or diaphragm muscles, but epaxial-derived muscles are less affected (Bober et al. 1994). Pax3 is classified genetically as an upstream regulator of MRFs. Several teams showed that Pax3 was expressed in myogenic cells in somites and in migratory muscle precursor cells in the developing limbs. Splotch mutant mice missed myogenic transcription factors expression (MyoD, myf5 and Myogenin) in the limbs (Bober et al. 1994) concluding that it is a key regulator of somitic migrating myogenesis (Maroto 1997).

From embryonic day 9.75 (E9.75), almost all dermomyotomal cells express Pax3 and Pax7, although Pax3 is expressed at higher levels in the dorsal and ventral dermomyotome lips, whereas Pax7 is preferentially expressed in the central dermomyotome (Kassar-Duchossoy et al. 2005).

This work showed that Pax3/7 expressions do not disappear in *Myf5* null embryo, evidencing these two transcription factors as markers of MPCs. Moreover, Pax^{+}/MRF^{-} population remains also during later limb development, meaning that they represent a persistent stem cell pool. To investigate the precise role and the hierarchy of these factors in programming skeletal myogenesis, Tajbakhsh et al. have performed experiment on *Pax3/Myf5* double mutant mice. Mutant embryos are devoid of all body muscles and lack expression of MyoD and of all other downstream myogenic factors, while craniofacial myogenesis is preserved (Tajbakhsh et al. 1997).

In the Pax subfamily, Pax3 constitutes a unique subgroup with Pax7 (Mayran, Pelletier, and Drouin 2015). As well *Pax7* mutant mice were generated and showed defect in cephalic neural crest differentiation, dying shortly after weaning. Even though homozygous animals born died shortly after weaning, no obvious phenotype was detected in skeletal muscle on transversal whole embryo sections (Mansouri et al. 1996). Some years later, Rudnicki's group showed that Pax7 was expressed specifically in satellite cells residing in adult muscle. Also if overall organization of muscle fibers was not affected, muscles from *Pax7* null mice were smaller and progressively lost satellite cells (Seale et al. 2000). Furthermore expression of Pax7 prevented cells from apoptosis (Kassar-duchossoy et al. 2005) as revealed by the increase of apoptotic cells in *Pax7* mutant mice (Relaix et al. 2006). However, only Pax3, but not Pax7, is expressed in long-range migrating cells, which form the initial limb musculature. On the contrary Pax3 is expressed in quiescent adult satellite cells, with different frequency according to the different muscles (Relaix et al. 2006). So, it seems that if Pax3 is implicated in the determination of myogenic cell fate, Pax7 is more important for the myogenesis after birth. Pax3/Pax7 positive cells constitute the main proliferating population of the myotome, providing a major source of myogenic cells for the formation of skeletal muscle fiber. *Pax7/Pax3* double mutant starts to form the somite until E10.5 under the control of Myf5 and MRF4. But as the development proceeds skeletal muscle formation is severely compromised, with almost no muscle detected at E13.5. Moreover MyoD expression is strongly compromised and the mutant progenitors lose their myogenic fate (Relaix et al. 2005).

Pax3 and Pax7 functions partially overlap as shown by replacing *Pax3* alleles by *Pax7* ones. Indeed, Pax7 can substitute for Pax3 function in dorsal neural tube, neural crest cell, and somite development, but not in the formation of muscles involving long-range migration of muscle progenitor cells (Relaix et al. 2004). Importantly Pax7/Pax3 positive cells represent the pool of myogenic progenitors that allows muscle growth during development and that remains the committed myogenic population required for adult myogenesis.

Interplay between the different myogenic factor

Specific genetic mutations of the *MRF* family have revealed their necessity for skeletal muscle development and underlined the interplay between them.

Indeed, Rudnicki et al. have shown that mice lacking both *MyoD* and *Myf5* have no detectable skeletal muscle-specific mRNAs. Histological examination of these mice revealed a complete absence of skeletal muscle (Rudnicki et al. 1993). These data illustrate the functional redundancy of these two transcription factors. Rawls et al. analysed the phenotype of *MRF4/Myogenin* double mutant mice. Even if these genes are not absolutely essential for myoblast commitment, they are indispensable to support normal myogenesis *in vivo* (Rawls et al. 1998). At last, in triple-mutant

mice lacking *Myogenin*, *MyoD*, and *MRF4,* differentiated muscle fibers failed to form *in vivo* and myoblasts from neonates of this triple-mutant genotype were unable to differentiate *in vitro*, showing that *Myf5* alone is not sufficient to allow a correct myogenesis (Valdez et al. 2000).

Other transcription factors important for the regulation of embryonic myogenesis

Serum response factor (Srf)

Srf is a conserved transcription factor during evolution. It is encoded by a single gene and is at the intersection of multiple signalling pathways controlling the transcription of immediate early response genes and muscle-specific genes. *In vivo*, the importance of Srf as a crucial transcriptional regulator was evidenced by the generation of a total knockout Srf-/- mice reported by Nordheim's lab (Arsenian et al. 1998). They showed the requirement of *Srf* to have a normal gastrulation process, identifying Srf as an essential regulator of mammalian mesodermal formation during embryonic development. Srf is expressed at E6.5 in ectorderm and endoderm. At E7.5 Srf protein is detected in all three germ layers of wild type embryos. Interestingly, at E8.5, this ubiquitous distribution becomes regionalized and Srf protein expression is found to be high in the developing heart, but barely detectable in other tissues. At E10.5, Srf protein expression is detected in the developing myotome. All these studies underline the importance of Srf protein during mouse early embryogenesis. A potential role for the involvement of Srf in myogenesis was first inferred by *in vitro* experiments where the authors showed the requirement of this factor for MyoD expression and consequently myogenic differentiation and maintenance of muscle fibres (Gauthier-Rouviere et al. 1996). These results suggested a crucial function of Srf in the early events of myoblast formation or fusion.

Six

Six family genes have been first identified in *Drosophila* where they participate to eyes development. In vertebrate *Six* genes are grouped in three classes: *Six1* and *Six2*, *Six 3* and *Six6*, *Six4* and *Six5*. It has been shown that Six1 and Six4 bind MEF3 site and induce *Myogenin* expression in C2C12 and in mouse embryos during embryonic development (Spitz et al. 1998). Thanks to *Six1–/–* embryos, Six1 has been shown to play a specific role in hypaxial muscle differentiation since the mutant embryo have impaired primary myogenesis, characterized at E13.5 by a severe reduction and disorganisation of primary myofibers in most body muscles (Laclef et al. 2003). To disclose the role and the relationship between *Six* members family, *Six1Six4* double KO mice were generated. They were characterized by severe and general muscle hypoplasia, absence of hypaxial musculature. It was shown that Six1 and Six4 directly bind the regulatory elements of

Pax3, Myf5, MyoD and Myogenin, controlling several steps of hypaxial myogenesis (Grifone et al. 2005).

2.3 Pax and MRFs transcription factors in post-natal and adult muscle

All the transcription factors that we described before are necessary for a correct embryonic myogenesis and have also a crucial role at later stages, during post-natal growth or adult myogenesis.

Maybe the most important is Pax7 that is considered as a marker of quiescent adult satellite cells. The normal appearance of newborn *Pax7* null muscles suggests that embryonic and fetal myogenesis are unaffected in mutant animals, identifying a unique requirement for *Pax7* in the satellite cell lineage. *Pax7* null mice die within 2-3 weeks after birth, making impossible studying their phenotype in the adult (Seale et al. 2000). Nonetheless between 5 and 10% of mutant animals survive until adulthood. Thanks to these animals, Oustanina et al. have evidenced that Pax7 is crucial for the maintaining of satellite cells number and in turn for a correct post-natal growth and adult muscle regeneration (Oustanina et al. 2004). These data were confirmed by Kuang et al. who evidenced that satellite cells in mutant *Pax7* mice lost their regenerative potential (Kuang et al. 2006). In the last years, genetically engineered ablation of satellite cells, using an inducible $Pax7^{Cre-ERT2}$ allele to activate a *Diphteria* toxin sequence in a $Rosa26^{DTA/+}$ cross (Lepper et al. 2011) or administration of the toxin to $Pax7^{DTR/+}$ mice where an allele of *Pax7* encodes the *Diphtheria* toxin receptor (Sambasivan et al. 2011b), resulted in loss of muscle regeneration. Some years later, von Maltzahn et al. using a conditional mutation of the gene in $Pax7^{CreERT2/flox}$ mice after tamoxifen administration reported an impaired muscle regeneration after 21 days and obtained a much more severe regenerative phenotype when they increased exposure to tamoxifen (von Maltzahn et al. 2013). In addition, after a second injury, regeneration was very severely compromised, underling *Pax7* importance for expansion of satellite cells population.

Less is known about *Pax3* in the adult. It is expressed in quiescent satellite cells present in diaphragm and trunk muscles and its frequency varies between muscles and is not related to fiber type. Together with *Pax7*, *Pax3* is required to achieve a correct myogenic differentiation of adult satellite cells (Relaix et al. 2006).

MRF4 expression level is maintained in the adult, together with decreased expression of the other three MRFs. It is the predominant MRF expressed in adult muscle. However, the function of MRF4 in adult muscle is not fully known. A recent paper showed that the inhibition of MRF4 in adult muscle induced an increase in muscle fibers size. Indeed they demonstrated that MRF4 interacted

with MEF2, antagonizing its transcriptional activity and inhibiting myofibers hypertrophy (Moretti et al. 2016).

Concerning Myf5, Tajbakhsh's lab has shown that the adult *Myf5* null mice exhibit a subtle progressive myopathy. They do not regenerate properly and have adipocytes and fibrosis accumulations. However the number of *Myf5* null satellite cells is not affected, but their proliferation is impaired (Gayraud-Morel et al. 2007).

As mentioned before *Myogenin* null mice die at birth, so Knapp et al. generated inducible mutant mice that lost *Myogenin* after embryonic muscle development. The mutant mice were 30% smaller than control mice but presented normal skeletal muscles, suggesting that the absence of Myogenin disrupts general body growth (Knapp et al. 2006). In addition, when *Myogenin* is lost upon denervation in adult mice, atrogenes expression is blocked and the mutant muscles are more resistant to atrophy (Moresi et al. 2010).

Finally MyoD is expressed transiently in adult satellite cells, only after their activation and it is undetectable in quiescent stem cells (Yablonka-Reuveni et al. 1994).

3. Satellite cells

3.1 General definition

Satellite cells (SCs) are the adult muscle stem cells. They have been described for the first time by A. Mauro in 1967, using electron microscopy. They were called *satellite* because of their peripheral localization, "wedged" between the plasma membrane of the muscle fiber and the basement membrane. They were identified in different animal species (frog, rat, mice) suggesting that skeletal muscle fibers of vertebrates, in general, contain satellite cells (Mauro 1961).

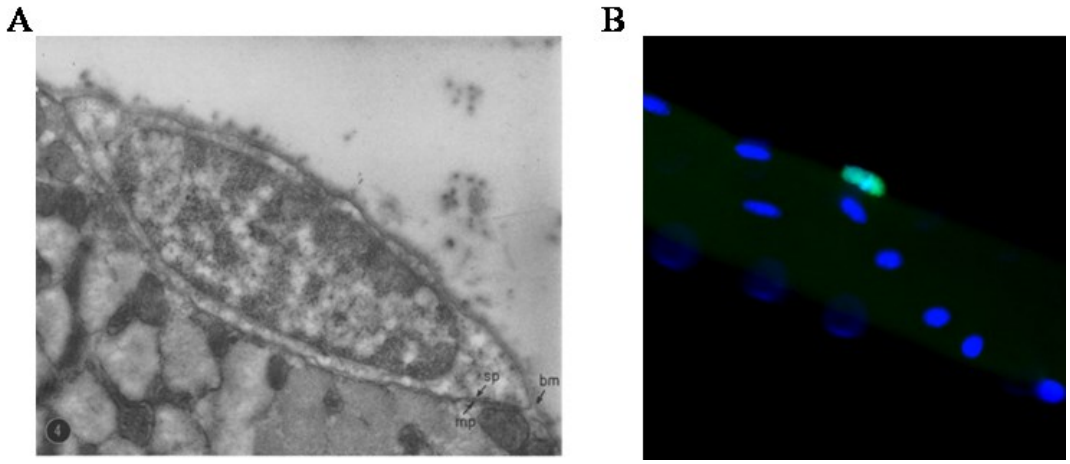

Figure 9: Satellite cell. A) first image of satellite cell by A.Mauro (1961). B) Picture of Pax7 positive cell (green) on single isolated myofiber

Some years later, Snowet al. demonstrated that SCs are in a mitotically quiescent state in young uninjured muscle, and are able to survive to muscle damage. Muscle damage induces SC proliferation and fusion in order to regenerate injured myofibers (Snow 1977).

At birth in mice, satellite cells represent approximatively 30% of sublaminar muscle nuclei followed by a decrease to approximatively 5% in a 2-month-old adult mice (White et al. 2010). This decline in SC number reflects the fusion of satellite cells with present myofibers during the postnatal growth of skeletal muscle. In young mice, mononuclear SCs are around 5% of the total nuclei present in each muscle and their proportion varies with age and muscle groups.

3.2 Origin of muscle progenitors

To determine the origin of satellite cells many studies were performed. Thanks to a *Pax3*-derived Cre driver line, it has been shown that the majority of, even if not all, limb muscle satellite cells arises from cells expressing Pax3 specifically in the hypaxial somite and their migratory derivatives

(Schienda et al. 2006). Lepper et al. in 2010 demonstrated, using the *Pax7*^{CreERT2} allele combined with R26R$^{LacZ\ Cre}$ reporter for inducible lineage tracing, that satellite cells present in adult *tibialis* muscles come from Pax7-expressing cells at E11.5. So, the cells expressing Pax7 at E11.5 are the same that contribute to the pool of adult stem cells in adult *tibialis* muscle and are the same that contribute to the adult muscle regeneration after injury (Lepper et al. 2010). So, one portion of the Pax3/Pax7 positive cells emerging during embryonic myogenesis do not differentiate at any time but will establish the pool of adult stem cells. Going further through a more deep knowledge about the origin and the adult fate of satellite cells, other teams generated lineage tracing models allowing to follow the fate of *Myf5* (Kuang et al. 2007), *MyoD* (Kanisicak et al. 2009) or *Mrf4* expressing cells (Sambasivan et al. 2013). In summary 10% of the Pax3/Pax7 positive population never expresses Myf5 during their ontogeny but all adult satellite cells originate from MyoD+ progenitors expressing MRF4 as well.

In addition some satellite cells may also be derived from cells associated with the vascular system including embryonic dorsal aorta (De Angelis et al. 1999).

3.3 Heterogeneity in satellite cells population

It is well known that the heterogeneity is a feature of stem cells in the tissue. Since body muscles are heterogeneous, also adult skeletal muscle stem cells population is heterogeneous. Indeed, differences in gene expression of SCs sorted from different muscles have been reported. For example, SCs from the *diaphragm* express higher levels of Pax3 compared to hindlimb muscles, with the exception of the *gracilis* muscle (Relaix et al. 2006).

Satellite cells are not identical since they exhibit different level of quiescence, they exist in different metabolic states and they express various grade of their hallmark genes (Yin et al. 2013). Some molecular markers are sufficiently specific to identify SC population, such as Pax7 for the adult stem cells in the muscle. However, many markers are expressed at low level distinguishing different SC subpopulations. Examination of the expression of satellite cell markers CD34, M-cadherin and Myf5 in freshly prepared single fibers suggests that a subpopulation of satellite cells exhibits heterogeneous expression of these markers and that the minority of CD34/Myf5 negative cells may be involved in maintaining the stem cells lineage (Beauchamp et al. 2000). Moreover the 10% of cells that do not express *Myf5* (and had never expressed *Myf5* during the development) are considered as a subpopulation with an higher stemness, more prone to self renew (Kuang et al. 2007). In 2014 Chakkalakal et al. have identified two subpopulations of satellite cells based on their proliferative history. Indeed, SCs that retained H2B-GFP label, are more able to proliferate and self

renew, expressing high level of p21 and p27 protein cell cycle inhibitors. On the contrary the unlabelled SCs are more prone to differentiate (Chakkalakal et al. 2014).

Different experiments suggest the presence of subsets of SCs with different self-renewal abilities. In skeletal muscle, Pax7 is a paired-box transcription marker considered specific of quiescent and activated satellite cells. Tajbakhsh's lab discovered two different subpopulations of satellite cells expressing high or low level of Pax7-nGFP (Rocheteau et al. 2012). The $Pax7^{high}$ population is in a more dormant state with a lower metabolism and are less primed for myogenic commitment as compared to the $Pax7^{low}$ population.

Since 1996, it has been evidenced that satellite cells may adopt an asymmetric way to divide during their proliferation, giving rise to a population that continues to cycle and to another population that remains quiescent to maintain a reserve stem cell population (Schultz 1996). Currently we know that during the satellite cell division the DNA segregate in a not equivalent way in the daughter cells, establishing which cells continue to proliferate and which ones replenish the stem cell pool, retaining the "immortal DNA strands" (Shinin et al. 2006).

The heterogeneity emerges over development as recently demonstrated by Pala et al. This team published for the first time the metabolic profile of satellite cells in different states and conditions such as young, old and post-mortem, evidencing significant alterations in metabolic signatures (Pala et al. 2018). Some of the differences observed in SCs gene expression reflect the different SC transient states. The transition in different SC states is influenced by local cues. That underlines the importance and the complexity of the SC niche.

The capacity of SCs to differentiate into alternative lineages, such as fibrogenic, adipogenic and osteogenic lineages, has been suggested by different studies. Asakura et al. have demonstrated the plasticity of satellite stem cells, because they are able to differentiate into osteocytes or adipocytes following treatment with bone morphogenetic proteins (BMPs) or adipogenic inducers (Asakura et al. 2001). Muscle stem cells in old mice tend to pass from a myogenic to a fibrogenic lineage following a hyper-activation of Wnt signalling (Brack et al. 2007). Despite the plasticity of satellite cells *in vitro*, it is important to note that the myogenic fate is the predominant satellite cells fate *in vivo* since fibrosis or adipose infiltration, is not normally observed in young healthy muscle. Finally a recent description of myogenic Pw1+/Pax7− interstitial cells ("PICs"), a new and anatomically identifiable population of muscle progenitors in postnatal growing muscle, further increases the level of complexity of SCs plasticity (Mitchell et al. 2010).

3.4 Niche (environment) and cross talk with other cells types

Satellite cells reside in a specific niche represented by a determined microenvironment. A large amount of evidences suggests that satellite cell fate is strongly influenced also by the interactions with the ECM niche in which they reside. Indeed, as a dynamic environment, the stem cell niche transmits mechanical and chemical signals that act to protect quiescent stem cells or that induce their activation, proliferation and differentiation (Yin et al. 2013). The niche contains a multitude of cellular and acellular elements that regulate satellite cell functions, influencing the balance between differentiation and self-renewal. In this manner, skeletal muscles regenerative capacity and, in general, tissue homeostasis are preserved. *In vitro*, satellite cell survival is greater when cultured on hydrogels that mimic the physiological stiffness of muscle strongly supporting the importance of biophysical and biochemical niche features (Gilbert et al. 2010). In an immediate level the satellite cells are in direct contact with the myofiber. Myofiber, in a paracrine manner, secrete chemical compounds that regulate satellite cell behaviour. For example, myofibers secrete SDF-1, which serves as ligand to the cell surface receptor CXCR4 on satellite cells and SDF-1/CXCR4 signalling stimulates satellite cell migration (Ratajczak et al. 2003). Moreover upon mechanical stimuli, myofiber secretes IL-4 and IL-6, respectively to regulate satellite cell fusion and proliferation during skeletal muscle hypertrophy (Guerci et al. 2012).

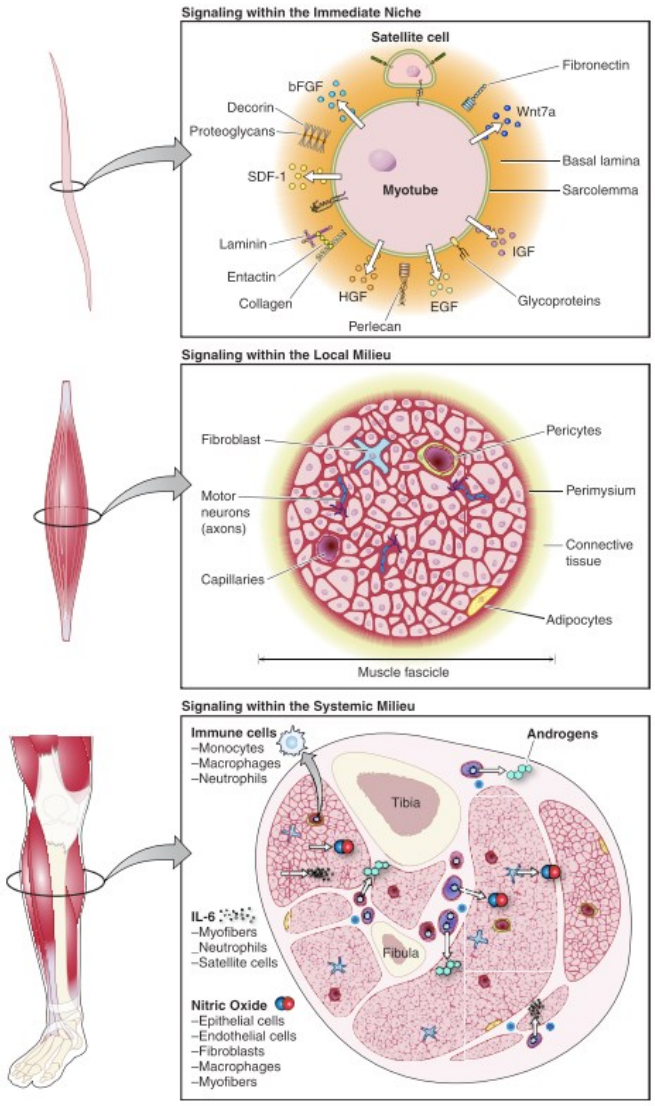

Figure 10: Complete overview of skeletal muscle environment (Yin et al. 2013)

Satellite cells basally express integrins (e.g. integrin α7β1) that bind laminins present in the basal lamina and apically express M-cadherin (Mcad) that contributes to adhesive interactions with the myofiber plasma membrane. M-cadherin is a classical calcium-dependent cell adhesion molecule. Even if its deletion had no apparent defect in muscle development or regeneration (Hollnagel et al. 2002), Krauss's team showed that the removal of both *N-cadherin* and *M-cadherin* in mice led to a break in SC quiescence with long-term expansion of a regeneration-proficient SC pool (Goel et al. 2017).

Satellite cells are also in contact with the ECM proteins. The binding between β-integrin on satellite cells and laminin present in ECM is fundamental in order to maintain quiescent the satellite cells in their niche (Rozo et al. 2016). Syndecan-4, Frizzled-7 co-receptor, expressed on satellite cells, binds fibronectin to activate planar-cell-polarity signalling and to stimulate satellite cells symmetric

division. The protein fibronectin is identified as an important regulator of satellite cell niche, essential to maintain their quiescence (Bentzinger et al. 2013). Interestingly a recent study evidenced that the loss of *Syndecan-3* in satellite cells prevents self-renewal and re-homing of satellite cells to their niche, maintaining a pool of activated/proliferating cells that largely ameliorates muscular dystrophy in *mdx* mice (Pisconti et al. 2016). A nice work revealed that the extracellular matrix protein collagen VI, expressed by satellite cells, is a key component of their niche influencing satellite cells self-renewal capacity (Urciuolo et al. 2013).

The receptors present on satellite cells are also activated by growth factors that are in an inactive state in the matrix, and can swiftly activated by proteolytic enzymes present in the ECM such as thrombin, serine proteases and matrix metalloproteinases. Beyond ECM and their componing proteins, there are also other cell types that reside in muscle tissue and that are indispensable for its functions. The major population found consist of the fibroblasts. They secrete growth factors, such as FGFs, and contribute also to the skeletal muscle ECM set by depositing collagen, laminin, fibronectin, heparan sulfate proteoglycans, tenascin and NCAM. In some pathological conditions, increased level of connective tissue is linked to an increase of fibroblast number. Several studies reveal that these intramuscular adipocytes and fibroblast can arise from muscle residing fibroblast/adipocyte progenitors (FAPs) (Uezumi et al. 2010). At steady state, they are preserved in an undifferentiated state, but upon injury they are important for a correct regeneration process (Joe et al. 2010). One year later, Murphy et al. confirmed the crucial role of fibroblasts by identifying them with the Tcf4 marker and describing them as an important component of the niche regulating satellite cell expansion during regeneration (Murphy et al. 2011). More recently, Wosczyna et al. underlined the importance of the FAPs in the muscle to maintain both skeletal muscle homeostasis and the muscular SC pool (Wosczyna et al. 2019). The regeneration potential of satellite cells *in vivo* might be largely determined by the niche and the microenvironment. Olwin's lab identified a side population (SP) characterized by ABCG2, Syndecan-4 and Pax7 expression that contributes to skeletal muscle regeneration, suggesting a control by a specialized niche (Tanaka et al. 2009).

Motoneuron, blood vessels and immune cells, all of them with their associated secreted factors, also reside within skeletal muscle. Recently, muscle satellite cells were shown to interact with endothelial cells making a direct contact with capillaries. This interaction regulates satellite cells quiescence. In particular, Notch ligand Dll4, expressed on endothelial cells, activate Notch signalling in satellite cells, enhancing the reserve satellite cells pool (Verma et al. 2018).

Concerning the immune cells, they are very few in a healthy muscle, but upon injury they are attracted to the damaged tissue and diffusible factors that they produce are essential for a correct muscle tissue response.

3.5 Behaviour of satellite cells

a. Quiescence

Satellite cells are quiescent in a resting muscle and they exist in a non-cyclic state.

In 2014, Rodgers et al. showed that stem cell quiescent state is composed of two distinct functional phases, G0 and an 'alert' phase that they termed G-Alert. Indeed, they demonstrated that the satellite cells of intact muscles, in a mouse that was subjected to an injury in another muscle, are in an intermediate state between the one of injured muscle and the one of a resting muscle in a non-injured mouse. The satellite cells referred as "alert" SCs are able to enter in cell cycle faster, eliciting enhanced muscle regeneration. Moreover, this SCs alert-response depends on mTORC1 signalling (Rodgers et al. 2014).

Several studies have been conducted to characterize quiescent SCs transcription profile, to unveil their quiescence signature, in order to explain the unique mechanism allowing SCs to stay in a non-cyclic state. Fukada et al. compared gene expression profiles of quiescent and of activated satellite cells, demonstrating that in quiescent state there was an enrichment of negative regulator of cell cycle like *p57* and *Sprouty*. The expression of Calcitonin receptor (*CalcR*) was found upregulated in quiescent satellite cells and its addition on cell culture delays SCs cell cycle entry and differentiation, keeping them in their niche (in the case of treatment of isolated myofibers) and preventing their activation (Fukada et al. 2007). Likewise the same team found upregulated genes that positively control myogenesis like *Gli2* or *Meox2*, that upregulates *Myf5* and *Pax7* respectively (Gustafsson et al. 2002; Mankoo et al. 2003).

The same lab some years later confirmed the crucial role of Calcitonin receptor in the maintaining of satellite quiescence. In the absence of Calcitonin receptor satellite cells escape dormancy, acting through cAMP-PKA pathway, and emerge from their niche via cAMP- PKA and cAMP-Epac pathway. Moreover, they do not proliferate correctly but their myogenic differentiation is not affected (Yamaguchi et al. 2015). More recently, Zhang et al. add another important element in the molecular mechanisms by which CalcR signalling regulates quiescence in muscle SCs, showing that CalcR-activated PKA phosphorylates Lats1/2, the main effector of Hippo signalling. In this way, nuclear accumulation of Yap1 is inhibited and thereby also the expression of Hippo-target genes, including cell-cycle-related molecules (Zhang et al. 2019).

Another lab evidenced the importance of Calcitonin receptor in maintaining satellite cells in a quiescent state, by the interaction with CollagenV, a critical component of the quiescent niche (Baghdadi et al. 2018). Notch signalling is critical for the maintenance of the quiescence state.–A constitutively active form of Notch1 expression in embryonic stage blocked the differentiation of muscle progenitors and maintained a self-renewing population of muscle stem/progenitors

(Mourikis et al. 2012). Moreover, experiments performed on *Rbpj* null mice (Notch nuclear receptor) showed that it is necessary to inhibit myogenic progenitors differentiation, demonstrating that Notch functions are accomplished via canonical signalling. Furthermore, cell adhesion and basal lamina surrounding satellite cells are disrupted in absence of Notch signalling, evidencing its crucial role for a correct satellite cells microenvironment (Bröhl et al. 2012).

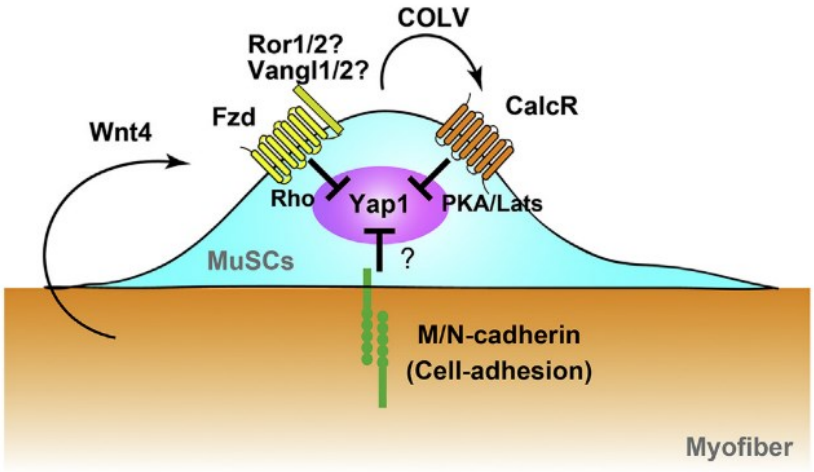

Figure 11: Molecules involved in conservation of satellite cells quiescence

There is also an epigenetic signature specific of quiescent stem cells. The chromatin of quiescent satellite cells is maintained in a permissive state with a large number of genes marked by H3K4me3 (expressed) and few marked by H3K27me3 (repressed). The level of H3K27me3 rapidly increases upon SC activation, converting the chromatin to a more repressed state and depicting this epigenetic modification as a chromatin feature of stem cell potential (Liu et al. 2013).

Satellite cell quiescence is also maintained by microRNAs (miRNAs). Cheung et al. showed that the ablation of Dicer, a key mediator of miRNA processing, in satellite cells caused the exit from their quiescence state which is essential in the survival of proliferating myogenic progenitors (Cheung et al. 2012). In particular they focused on miR-489 among the 22 quiescent specific miRNAs and they demonstrated that its overexpression was sufficient to extend SCs quiescent state delaying their activation. However other studies revealed that muscle- specific miRNAs, such as miR-1/206 and miR-133, were important for the maintenance of the myogenic program and to facilitate the transition into differentiation (Ma et al. 2015). In particular, miR-206 regulates skeletal muscle cells proliferation repressing Pax7 (Chen et al. 2010). Interestingly miR-31 is sequestered with *Myf5* mRNA in mRNP granules present in quiescent cells. Upon activation this granules are

dissociated: miRNA-31 levels are reduced and Myf5 proteins are promptly translated (Crist et al. 2012).

Other signalling pathways have been explored in the context of satellite cell quiescence. For example, Angiopoietin 1 (Ang1) and its receptor Tie-2 regulate the quiescent state where Tie-2 is expressed by quiescent satellite cells. Ang1/Tie- 2 signalling, through ERK1/2 pathway, decreases the myogenic precursor cells proliferation and differentiation, promoting their entry in G0 phase. The authors suggest that Ang1 (produced among others by neighboring interstitial cells) binding to Tie-2 is involved in the regulation of the return to a quiescent state of a subset of satellite cells in regenerating muscle (Abou-Khalil et al. 2009).

Satellite cell environment is crucial to maintain them in a quiescent state. Although niche molecules regulating quiescence are not fully elucidated, Brack's lab showed that when *Wnt4* is ablated in the myofibers and consequently missing in SC niche, the SC quiescence is lost. In addition they showed that Wnt4 signals via RhoA to control SC niche retention, regulating stiffness and cytoskeleton mechano-properties (Eliazer et al. 2019).

The importance of N-cadherin and M-cadherin to maintain satellite cells quiescence has been recently investigated. Indeed in *N-cadherin* and *M-cadherin* double mutant mice, satellite cells proliferate actively at basal state, breaking their quiescent state, but they are able to give a pseudo regeneration process (Goel et al. 2017). Moreover satellite cells express some cell-surface transmembrane proteins like Syndecan that function as co-receptor for different signals and regulate their activation (Cornelison et al. 2004).

In summary, cellular quiescence is defined as a transient and reversible cell cycle arrest. It is characterised by cell size decrease, nucleo-cytoplasmic ratio increase, 2N genome content, low RNA and protein synthesis, altered metabolism and gene expression profile. Quiescence has been considered for some time to be a default state corresponding to the absence of proliferation and differentiation. It is now clear that cellular quiescence is an actively maintained state with new functional markers continually being identified (Evano et al. 2018).

b. Motility

The activation of satellite cells is not restricted in the site of muscle damage. Already in 1985, it was shown that all the satellite cells of the myofibers respond to a focal injury (Schultz et al. 1985). Today, thanks to more innovative techniques, we know that the activated satellite cells are not immobile, but migrate following specific paths defined by myofibers scaffold (Webster et al. 2016). Moreover, activated satellite cells not only may move along the entire length of a myofiber, but

migrate also between fibers (Yin et al. 2013). These observations enable us to speculate that the whole myofiber could provide the right niche for SCs.

Myofibers harvest is enough to trigger satellite cells activation (Machado et al. 2017) and nice timelapse video of satellite cells on their host myofiber showed that they exit their sublaminal niche adhering to the external of the myofibers (Siegel et al. 2009). Several factors enhance satellite cell activation and migration. For instance, satellite cells express on their surface the functional receptor CXCR4. The specific ligand for this receptor is the α-chemokine stromal-derived factor 1 (SDF-1) secreted by fibroblasts. The CXCR4-SDF-1 axis is important for chemoattracting satellite cells to the site of injury (Ratajczak et al. 2003). Another paper showed that the cultured myoblasts express chemokine receptors CCR2 and CCR5 to regulate myoblast proliferation and migration upon injury (Yahiaoui et al. 2008).

c. Activation / Proliferation

When the muscle is exposed to signal such as a lesion/injury, the niche that insulates the satellite cells, protecting them from the extracellular stimuli, is broken. In this way the satellite cells exit from their quiescent state and get active. We can define the activation as the first cell division of the stem cells once they leave quiescent state. Moreover, even when the muscle is exposed to mechanical cues (and the niche is preserved) the muscle stem cells get active, suggesting that the activation is mediated by mechanical stimulus too. The differences between activation processes caused by the two different procedures, like regeneration or hypertrophy, are not fully elucidated. Generally, the activation process is studied upon muscle lesion where the myofibers are destroyed and an inflammatory response is activated. Cytokine, chemokine and factor released have an important impact on satellite cells activation. Growth factors, secreted by myofibers or released from the extracellular matrix injured, impact satellite cell activation. Several pathways are activated downstream of growth factors, building a complex intracellular crosstalk regulating SCs activation. In C2C12 cell line, the chemotactic activity of the factor hepatocyte growth factor (HGF) promotes myoblast migration and proliferation (Villena et al. 2004). HGF acts through the cell surface receptor c-Met found on both quiescent and activated satellite cells (Tatsumi et al. 1998). It was demonstrated that the activation of HGF/c-Met pathway stimulates quiescent satellite cells to enter the cell cycle, (thus myoblasts proliferation increases) and inhibits myogenic differentiation (Miller et al. 2000). HGF has dual functions depending on the protein recruited on the receptor. C-met, once phosphorylated, by binding of Grb2 activates MAPK/Erk signalling promoting proliferation and by binding to PI3K promotes differentiation (Leshem et al. 2002).

Several laboratories have reported that fibroblast growth factors (FGF) signalling promotes satellite cells activation and expansion as well as cell cycle exit to generate either a self-renewed quiescent stem cell or a terminally differentiated myonuclei (Pawlikowski et al. 2017). Dimerization of FGFs with FGF receptors leads to tyrosine phosphorylation of FGF receptor and activates the downstream Ras/MAPK pathway, inducing proliferation and blocking differentiation.

Insulin-like growth factor 1 (IGF-1) plays important role in the regulation of satellite cell activity (Philippou et al. 2007). The increase of IGF-1 expression following injury is associated to an increase of satellite cells expression markers (Hill et al. 2003). In addition it has been proposed that the isoform mIGF-1 enhanced the distal mobilization of stem cells of haematopoietic origin to promote stem cell- mediated muscle regeneration (Mourkioti et al. 2005).

Among the molecules implicated in regulating satellite cell behaviour, there are those that belong to p38α/β MAPK family. They function as molecular switchs to activate the quiescent satellite cell. Activation of p38α/β is required for satellite cell activation (MyoD induction and cell cycle entry) as demonstrated by inhibition of p38α/β. However, it is also required for muscle cell line differentiation, underling its fine regulating role depending on the different stimuli and substrates (Jones et al. 2005).

Notch and Wnt signallings stand among the best known signalling pathways involved in satellite cell activation and proliferation. A recent study showed that *in vivo* β-catenin over-activation, *via* adenomatous polyposis coli (APC) genetic deletion specifically in satellite cells, did not result in muscle satellite cell exit from quiescence at the basal state. But APC lost in satellite cells impaired regeneration process preventing satellite cells expansion and increasing their death. So, Wnt signalling should be dampened to allow correct cell cycle progression of myoblast (Parisi et al. 2015).

Therefore, a correct timing of activation together with a proper level of canonical Wnt pathway is crucial during muscle regeneration. Le Grand's laboratory recently demonstrated that an adequate level of Wnt/β-catenin signalling is fundamental to control muscle progenitor function during muscle regeneration. They showed that satellite cell-specific β-catenin loss-of-function and gain-of-function mutations resulted in the alteration of the regeneration process *in vivo*. Satellite cell descendants deprived of canonical Wnt signalling differentiate less efficiently (reduced generation of Myogenin+ cells), while constitutive activation of Wnt/β-catenin leads to precocious growth arrest and premature differentiation (Rudolf et al. 2016). Several studies suggested that a correct timing of canonical Wnt pathway activation is important for proper muscle regeneration and that the crosstalk between Wnt pathway and Notch signalling must be finely regulated. Otto et al. asserted that Wnt inhibition caused satellite cells proliferation decrease and that β-catenin was found in

activated MyoD+ myoblast and it was lost in differentiated Myogenin+ myocytes. Moreover, they evidenced distinct classes of Wnt proteins that had opposite effects on proliferation rate of satellite cells (Otto et al. 2008). The sophisticated temporal and spatial regulation of these proteins is illustrated by conflicting results obtained by Brack team. They suggested that Wnt signalling is important in the late stage of adult myogenesis, promoting myogenic commitment and terminal differentiation. Furthermore, they demonstrated that there was a temporal balance between Notch and Wnt signalling during the early phase of regeneration. Notch signalling seems to antagonize Wnt signalling effects in early phase of proliferation to achieve a correct myogenic lineage progression and a later differentiation (Brack et al. 2008). The most known primary targets of canonical Notch signalling are Hes and Hey families of myogenic transcriptional repressors. Mice *Hey1/HeyI* double KO displayed defects in the generation of quiescent satellite cells pool during regeneration (Fukada et al. 2011). Interestingly, recently, it has been shown that their expressions were critical for skeletal muscle stem cells in a hypertrophy model too (Noguchi et al. 2019). At the transcription level, SCs express MyoD, a hallmark of activated satellite cells. At the same time proteins involved in cell cycle are activated to allow satellite cells proliferation.

The factors that induce muscle SC activation in overloaded muscle are unknown, but some potential mechanisms have been identified. One possibility ~~is~~ that SCs perceive mechanical cues in a direct manner; another possibility is that non SCs, including myofibers, sense the mechanical stress and subsequently release factors which act on SCs, for example disrupting their quiescent state. Serrano et al. identified cytokine interleukin-6 (IL-6) as an essential regulator of muscle stem cell-mediated hypertrophic muscle growth (Serrano et al. 2008). IL-6 family-mediated Stat3 activation might be a common mechanism for SC proliferation in both regenerating and overloaded muscle.

However, the cellular source and cytokines expression regulation, controlling SC activation/proliferation, seem to be different in overloaded muscle and in regenerating muscle.

d. Differentiation and Self renewal

Satellite cells that underwent differentiation are called myocytes. The first step of differentiation process is the exit from cell cycle. This cell cycle exit requires the upregulation of specific cyclin-dependent kinase inhibitors. Whereas the return to quiescence requires p27Kip1, differentiation requires the concerted upregulation of p21Cip1 (Cdkn1a), p19Arf (Cdkn2a) and p57 (Dumont et al. 2015). In addition, myoblast differentiation is regulated by the expression of myogenic bHLH transcription factors. In 2010, Cao et al. conducted a wide genome analysis comparing myoblasts with myotubes gene expression, evidencing that the gene set enriched in myotubes is associated to muscle formation and function (cytoskeleton and sarcomere proteins), whereas genes associated

with myoblasts are related to cell proliferation and cell communication (cell surface proteins) (Cao et al. 2010).

Satellite cells, once activated, if do not differentiate, can choose to return to quiescence, thus they self-renew. Satellite cells after their activation perform symmetric division or asymmetric division. If the symmetric division is necessary to replenish the pool of satellite cells, asymmetric division is characterized by the segregation of different cell fate determinants into the daughter cells. The reasons that push satellite cells to choose one or other division remain still unclear. It is well known that stem cell subpopulations are able to undergo both symmetric and asymmetric modes of self-renewal to maintain satellite cell numbers through repetitive rounds of regeneration. Previously in the text, describing the heterogeneity of satellite cells, I evidenced the differences between subpopulation of cells, that are more prone to self renew, such as the satellite cells that never express Myf5 (Kuang et al. 2007) or the satellite cells that have different Pax7 expression level (Rocheteau et al. 2012). Several signalling pathways have been reported to influence the balance between symmetric and asymmetric divisions. Among them Wnt planar cell polarity (PCP) pathway is implicated in symmetric satellite stem cell expansion (Le Grand et al. 2009). It was shown that Wnt7a stimulates satellite cells symmetric planar division, driving the expansion of the satellite stem cell pool. Moreover, Rudnicki team's data support the importance of the protein Vangl2 as a polarity effector and as a regulator of myoblast pool amplification. Indeed cell polarization is typically accompanied by migration and then cytoskeletal reorganization. The same lab showed that the small GTPases Rac1, Cdc42 and RhoA are essential for the induction of cell motility by Wnt7a (Bentzinger et al 2014).

Numb protein, that is an inhibitor of Notch signalling, by its asymmetrical localization, regulates satellite cell fate. In particular, Numb causes SCs exit from cell cycle and enhances myogenic differentiation (Conboy et al. 2002). Asymmetric division is controlled by two major interacting events: (1) the unequal distribution of polarity proteins and cell fate determinants; and (2) mitotic spindle orientation. Importantly, Par complex is essential in cell polarity and asymmetric division. It colocalizes with phospho-p38 enhancing MyoD and Myogenin expressions (Troy et al. 2012).

In skeletal muscle, Stat3 regulates myogenic differentiation (Wang et al. 2008). Investigating the role of Stat3 in satellite cells by using shRNA approach, Sacco's laboratory has shown that Stat3 signalling promoted myogenic lineage progression, thereby inhibiting the expansion of the satellite cells during skeletal muscle repair (Tierney et al. 2014). The same year Rudnicki's laboratory has related that pharmacological inhibition of Stat3 stimulated symmetric expansion of satellite cells *in vitro* and enhances muscle regeneration *in vivo* (Price et al. 2014). However, one year later, another laboratory using a genetic mouse model to ablate specifically Stat3 in satellite cells, showed that

Stat3 deletion impaired SC self-renewal upon muscle injury, without affecting SC proliferation, assigning a previously unknown role to Stat3 (Zhu et al. 2016).

e. Fusion

Once myocytes are differentiated, they fuse one to each other resulting in the formation of nascent myotubes or they fuse to mature myotube to supply the accretion and the growth of the myotube itself.

Fusion between two cells is a key event for a lot of biological process, used by different species and in mammals in different tissues. Fusion is a crucial dynamic process in secretion, protein and lipid trafficking, enveloped virus infection. It is essential in fertilization, immune response, bone resorption, placenta formation, tissue and organ developments, including skeletal muscle. All these situations share general biophysical pathway for membrane rearrangements but they differ in specific fusion activating proteins.

However, even though a lot of cells gets in contact, not all of them fuse, remaining mononucleated. This observation suggests that cell fusion is a tightly regulated process that involves a complex regulation. Elaborated mechanisms allow the cells to go further the simply contact merging their own membranes to promote their fusion.

Many studies were conducted in *Drosophila* to clarify the mechanisms of fusion process (Duan et al. 2019). The musculature formation in *Drosophila* occurs twice during its life span: embryogenesis and metamorphosis (when is generated the adult musculature). Most of the studies concerning myoblast fusion are based on the study of larval body wall muscles formation, which are functionally equivalent to skeletal muscles in vertebrates (Schulman et al. 2015).

Fusion process is composed of different steps: at first the **recognition of two partners**. The two cells have to migrate toward each other and bring themselves close enough (within 10 nm of one another) to allow the start of cells adhesion machinery. At second the induction of close membrane apposition through **F-actin protrusions** has to occur. The third step is the rearrangement of membrane lipid bilayer (hemifusion) and finally pore formation and cell cytoplasm **fusion**.

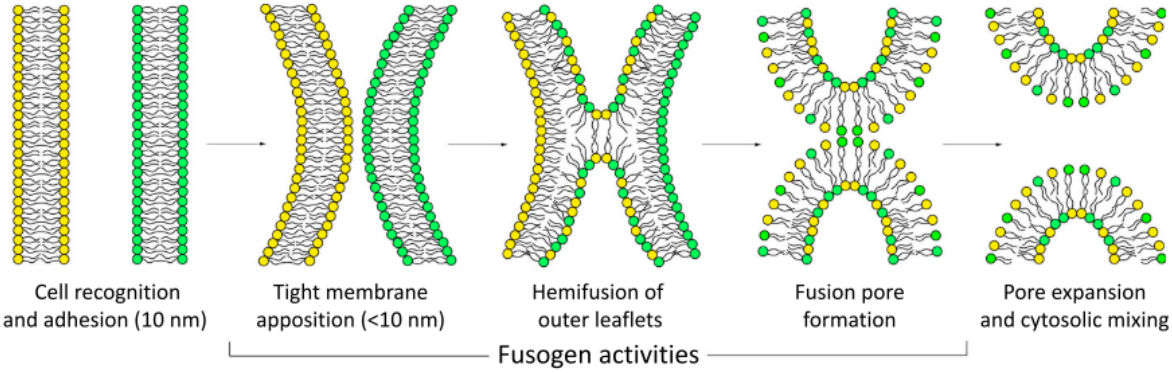

Figure 12 : Essential steps of the cell membrane fusion (Petrany and Millay 2019)

In *Drosophila,* fusion occurs between two types of muscle cells, muscle founder cells (FC) and fusion-competent myoblasts (FCMs). This two different type of cells are specified from their somatic mesodermal origin thanks to specific signalling pathway regulatory activities (Notch and Ras) (Carmena et al. 2002). Interactions between these two muscle cell types are mediated by heterotypic cell adhesion molecules (CAM). Each cell expresses specific CAM. Dumb- founded (Duf) (also known as Kirre) is identified on founder cells, Sticks and Stones (Sns) on FCMs. Duf interacts with Rols7 (rolling pebbles7 also known as antisocial) that allows its right localization on the membrane.

Beyond CAM protein, essential for myoblast recognition, a crucial role for myoblast fusion is played by actin cytoskeleton and actin polymerization regulators. Sens et al. showed an asymetric organization of actin foci, forming a thick actin wall on FCM cell and a thin F-actin shealt on FC (Sens et al. 2010).

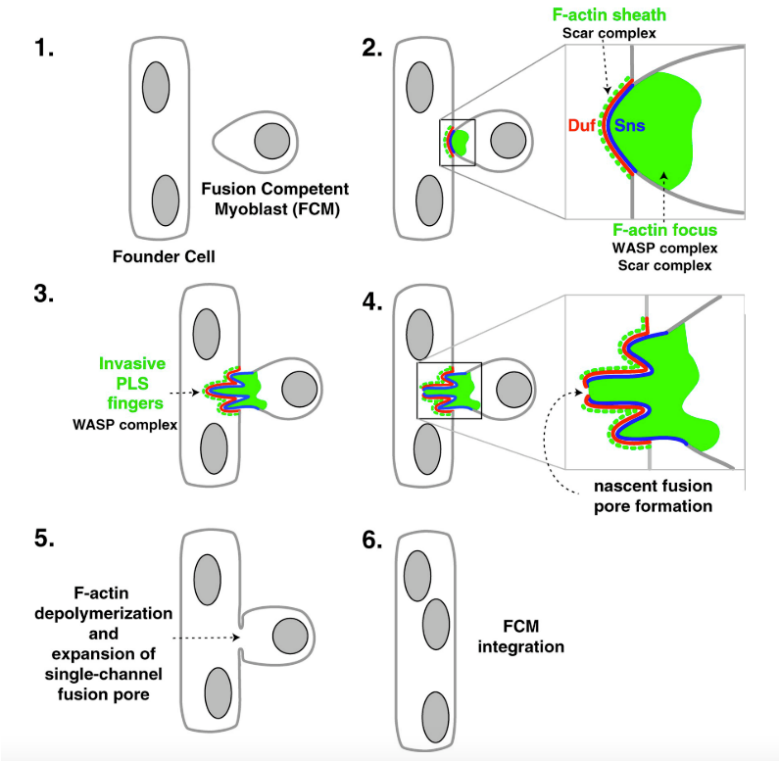

Figure 13 : Fusion events between founder cell and competent myoblast in *Drosophila*

There are many proteins involved in actin rearrangement machinery. The first gene discovered essential for myoblast fusion in embryonic *Drosophila* development is Myoblast city (Mbc) (Rushton et al. 1995). In 2002, Brugnera et al. proposed that DOCK180 (the homologus of Mbc) forms a complex with ELMO to act as GEF for Rac, a small GTPase protein important for myoblast fusion (Brugnera et al. 2002). Rac regulates actin cytoskeleton together with WASP (Wiskott-Aldrich syndrome) and WAVE proteins. WASP regulates Arp2/3-mediated actin polymerization promoting the nucleation of actin monomers to form branched actin filament (Pollitt et al. 2009).

In FCM cells, Blow is one of the cytoplasmic proteins necessary for myoblast fusion. It enhances filament actin branching and thereby the formation of densely packed, shorter and stiffer actin filaments. These actin filaments consequently generate sufficient mechanical force to promote cell membrane protrusion (Jin et al. 2011).

D. A. Kesper et al. described in 2007 the “fusion restricted myogenic adhesion” structures (FuRMas), formed during myogenesis in the *Drosophila* (Kesper et al. 2007). In fact, during recognition and adhesion steps many proteins are required at the cell-cell contacts to form a connective structure between FC and FCM. Actin cytoskeleton emerges important not only for cellular physiologic function but also for cell-cell fusion. FCM assembles a podosome-like structure (composed of an F-actin core and an adhesion molecules ring) that invades the FC and that evolves in a channel fusion pore formation and, at the end, in the fusion of the two cells (Sens et al. 2010).

Moreover, this group, thanks to the analysis of double mutant embryos of *Scar* (a *WASP* family member) and *Sltr* (FCM-specific protein Solitary (Sltr)/dWIP), has shown that both complexes were required for cytoskeleton rearrangement and F-actin foci formation at the site of fusion.

Parallel studies underlined the active role of FC in opposing an active resistance to FCM pushing forces. At the site of fusion, known as the **fusogenic synapse**, in response to applied mechanical forces, there is an accumulation of MyoII that is considered as a mechanical biosensor of myoblast fusion. This accumulation increases cortical stiffness and provides resistance to invasive protrusion (Kim et al. 2015). At the fusogenic synapse, an attacking fusion partner invades its receiving fusion partner with actin-propelled membrane protrusions, whereas the receiving fusion partner mounts a myosin II (MyoII)-mediated mechanosensory response. The pushing and resisting forces from the two fusion partners bring the two cell membranes into close proximity and put the fusogenic synapse under high mechanical tension in order to promote fusogene engagement and cell membrane merge. Although multiple long and narrow invasive protrusions coming from the attacking fusion partner are known to be required for cell-cell fusion, it is unclear how these protrusions are spatially constructed and shaped in order to generate high mechanical tension at the fusogenic synapse.

Another protein involved in mechanical FC response to FCM invasion is **spectrin** (founded in myoblast cell line, too). Spectrin is a membrane skeletal protein that accumulates specifically in FCs in a dynamic and transient manner interacting with Duf at the fusogenic synapse. In a MyoII dependent manner it enhances myoblast fusion working as cellular fence to restrict cell adhesion molecules and as a cellular sieve to construct the invasive protrusions coming from the attacking cell (Duan et al. 2019). More recently, it has been shown that Dynamin, a large GTPase protein, bundles actin filament increasing their thickness and their mechanic stiffness, and regulates actin cytoskeletal dynamics to promote invasive protrusions in cell fusion (Zhang et al. 2020).

Unfortunately, little is known about the membrane trafficking of CAM and lipids movement in fusing cells. Further studies are necessary to better understand the destabilization of the two lipid bilayers and the pore formation.

In mammals, in contrast to *Drosophila*, there is no evidence of founder cells and feeder subpopulation. Myoblast fusion is a critical event in different circumstances: during embryonic development in order to generate new myofibers or in adults in order to support myofibers maintenance and facilitate myofibers growth (upon hypertrophy or after repair after injury).

The differentiation state of the myoblasts reduces their migration capacity, increasing their possibility to get in contact and enhancing the fusion process. In fact, myogenic differentiation program contributes to the fusion process. Once the fusion begins, other cells migrate in pre-

determined direction, following factor gradient. This event suggests that migration is directed in response to chemotactic factors secreted by muscle cells (Jansen et al. 2006).

Mechanical forces play an essential role in cell-cell adhesion and cell-cell fusion, and are essential to overcome repulsive force. Due to hydration repulsion, the energy barrier is elevated and in this moment specialized proteins come in play; these proteins are called fusogenes and they are required to initiate the fusion events (Sampath et al. 2018).

How can we define the fusogenes? Fusogenes should have three important characteristics: they have to be necessary for fusion, they have to be present on the fusion membrane at right time and place and they have to be sufficient to fuse membranes that normally do not fuse.

Recently, the first muscle specific fusogene has been discovered: Myomaker. Millay's team described for the first time this seven-pass transmembrane protein. It is expressed during embryonic development and in adult only during muscle regeneration (Millay et al. 2013). When it is absent, fusion between myoblasts in culture or during mouse development does not occur. In overexpression experiments of Myomaker in fibroblasts, the last ones were induced to fuse with myoblasts (but not with another fibroblast) because Myomaker have to be expressed in both fusing cells (Quinn et al. 2017). During hypertrophy, Myomaker comes especially from the myoblast and not from myofibers, and when it is deleted in satellite cells, the fusion and the muscle growth are impaired (Goh et al. 2017). Meanwhile three independent groups discovered Myomerger, a second muscle specific fusion protein, involved in pore formation. When Myomaker and Myomerger are co-expressed in fibroblast, they are sufficient to induce fusion in non-fusogenic fibroblasts. Moreover, Myomaker and Myomerger mutants do not have differentiation defects.

A crucial step for cell fusion is the **cell-cell adhesion** occurring thanks to cell-cell adhesion molecules. Numerous molecules are localized at the contact site like M-cadherin, Integrin and Adam12. They can be also accumulated at the cell junction and then dissipate once that membrane fuse like E-cadherin complex (Lafuste et al. 2005). Additionally, cell-contact sites are characterized by changes in the cell membranes and phospholipids composition / assembling.

β1-integrin subunit in myogenic cells is an essential component for primary myoblast fusion (referred to myoblast-myoblast fusion) and sarcomere assembly in mice (Schwander et al. 2003). One of the potential mediator by which integrins regulate myoblast fusion is the non-receptor protein tyrosine kinase focal adhesion kinase (**Fak**). Rando's lab evidenced that satellite cells lacking Fak did not fuse *in vitro* and *in vivo* upon regeneration, especially acting by its downstream target Caveolin3 and β1D-integrin isoform (Quach et al. 2009). **M-cadherin** localization, but neither N-cadherin nor β1-integrin, is regulated by RhoA to achieve a correct myoblast fusion (Charrasse et al. 2006). Moreover, M-cadherin–dependent adhesion activates Rac1 and leads to the

formation of multiproteic complex containing M-cadherin, the Rho-GEF Trio and Rac1. This complex mediates cell adhesion and contact formation, two essential events for myoblast fusion (Charrasse et al. 2007).

GPCR BAI3 is also required for myoblast fusion and it functions by activating Rac1 pathway through ELMO–Dock (Hamoud et al. 2014). In addition the ferlin family member **Dysferlin**, a protein located at the cell membrane of muscle cells, is important for myoblast fusion as shown by the negative effect of its deficiency in human muscle cells (De Luna et al. 2006).

All these proteins are important not only to enhance the contact between two cells, but also to start signalling transduction pathway. Among the signalling pathway described for having a role in myoblast fusion, there is the MAPK **ERK5**. Indeed, inhibition of ERK5 blocks cells fusion, but it does not affect specific muscle gene expression such as MyoD or MyHC, activating instead the transcription factor SP1 and consequently Kfl2 and Kfl4 (Sunadome et al. 2011).

Many *in vitro* studies have demonstrated that fusion of myoblasts into multinucleated myotubes is regulated by calcium-dependent processes (Constantin et al. 1996). An increased amount of calcium activates **NFAT** (nuclear factor of activated T cells) family of transcription factors. Experiments on *NFATc2-/-* mice showed defects in the ability of muscle cells to fuse with the myofiber and to add nuclei to the myofiber after freeze damage (Horsley et al. 2001). NFATc2 controls IL-4 secretion by nascent myotubes. **IL-4** through IL-4R on myoblasts induces secondary fusion (fusion between myoblasts with nascent myotubes). This action allows the accretion of nuclei within nascent myotubes and, along with protein accumulation, the formation of a large, mature myotube (Horsley et al. 2003). Moreover, IL-4 seems to be regulated by Cox2 that is itself directly regulated by Serum Responsive Factor (**Srf**). An elegant experiment showed that the activation of Srf in myofibers in response to overload leads to the increased production of IL-6 and IL-4, which enhance respectively the proliferation of satellite cells and their fusion to existing myofibers (Guerci et al. 2012).

Moreover the **Nfkb** pathway is involved in myoblast fusion too. When it is activated by low concentration of TWEAK, it increases the fusion between cultured myoblasts (Enwere et al. 2012).

The fusion between myoblasts is strongly dependent on **remodeling of the actin cytoskeleton**. The formation of a subplasmalemmal actin wall, during myoblast alignment, is a critical event for myoblast fusion and it requires the function of **non-muscle myosin type IIA** (Duan et al. 2009). The two components of the small G-proteins family **Rac1** and **Ccd42** are essential for cytoskeleton rearrangement and actin fibers recruitment to the myoblast contacts fusion site (Vasyutina et al. 2009).

On primary murine myoblasts, has been discovered for the first time by Randrianarison-Huetz et al. finger-like actin protrusions at the site of fusion. Characterized as essential structures for the fusion

process, the molecular mechanism underlying their formation remains however unknown (Randrianarison-Huetz et al. 2018).

The last step of the fusion is the cell membrane mixing with a local disruption and rearrangement of lipid monolayer. Recently phospholipid membrane constituents such as phosphatidylserine (PS) have been shown to be implicated in mammalian myoblast fusion. Hochreiter-Hufford et al. demonstrated that the apoptotic cells induced signalling via the phosphatidylserine receptor BAI1. BAI1 signals through ELMO/Dock180/Rac1 proteins in order to promote fusion of healthy myoblasts (Hochreiter-Hufford et al. 2013). Another study underlined the importance of hemifusion step to finalize correctly fusion process. In particular the requirement of Annexins (Anx1 and Anx5) for an efficient lipid mixing and the requirement of Dynamin (DNM2) for pore formation on cultured cells have been reported (Leikina et al. 2013). Finally it has been asserted that GRAF1, actin and lipid dynamics co-regulator, previously described as a RhoA inhibitor (Doherty et al. 2011), regulates myoblast fusion by promoting endocytic recycling-dependent membrane recruitment of the fusogenic Ferlin proteins to the plasma membrane (Lenhart et al. 2014). What governs the transition between hemifusion and complete fusion remains not fully elucidated and seems to depend on several factors. However, it is clear that all these proteins play their role in cooperation to complete the intricate and largely uncharacterized myoblast fusion sequence.

Part II : Skeletal muscle regeneration

1. General features of skeletal muscle regeneration

Adult skeletal muscle has a remarkable ability to regenerate after injury. Injuries can be due to traumatic lesions such as extensive physical activity or to myotoxin exposure or to genetic defects such as muscular dystrophies. Muscle injury is followed by myofiber necrosis accompanied by increased calcium influx or calcium release from the sarcoplasmic reticulum, which in turn activates calcium-dependent proteolysis and drives tissue degeneration. Immediately after, it induces an inflammatory response and satellite cells activation. The phase of degeneration is followed by the phase of regeneration called adult myogenesis that recapitulates the embryonic myogenesis. It includes satellite cells proliferation, differentiation and fusion in order to allow new myofiber formation. The last phase is composed of the maturation and the remodeling of newly formed myofiber. Satellite cells have been implicated as the primary stem cells responsible for regenerating muscle. This concept was confirmed for the first time by Murphy et al. when they demonstrated SC requirement in muscle regeneration using *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (Murphy et al. 2011). In fact, when satellite cells are ablated, the regeneration of injured *tibialis* muscle is dramatically impaired accompanied with fibrotic tissue increase.

The regenerative process is coordinated by a network of interactions from a variety of cell types. De Micheli et al. generated a transcriptomic atlas of adult mouse hindlimb muscles upon regeneration and they broadly identified 12 different cell populations. Multiple non-myogenic cell populations have been defined by their expression patterns, including FAPs, tenocytes, smooth muscle cells (SMCs), mesenchymal progenitors, endothelial cells, neuro-muscular cells and at least five different immune cells that participate to muscle regeneration (De Micheli et al. 2020). Moreover, each population differs in their gene expression along all the regeneration process. We can find among the cell types of notably importance in regeneration process the **fibroblasts**. They have been described in muscle connective tissue and they express PDGFRα and TCF4. Indeed, their partially ablation in injured muscle (using *Tcf4*$^{CreERT2/+;}$*R26R*$^{DTA/+}$ mice) affects regeneration process principally leading to premature activation and differentiation of Pax7+ satellite cells, with a consequent depletion of satellite cells pool (Murphy et al. 2011). More recently, another group investigated the involvement of **FAPs** in skeletal muscle regeneration, determining their necessity in homeostatic maintenance and normal growth. FAPs are required in the early stages of skeletal muscle regeneration. They support satellite and hematopoietic cells expansion in the area of injury and they are required for long-term homeostatic maintenance of adult skeletal muscle as well as homeostatic maintenance of the muscular stem cells pool (Wosczyna et al. 2019).

2. Inflammation

Tissue damage immediately triggers the inflammatory response. A crucial function is carried out by chemokines that regulate the trafficking of leukocytes in response to injury as well as during normal physiological events. Immediately after the tissue lesion, the first cells recruited are the inflammatory ones. Concurrently with the flux of myeloid cells in muscle tissue after acute injury, myogenic cells undergo a stereotypical pattern of expression in order to regulate transcription factors and other genes involved in muscle repair and regeneration. In the absence of any immune challenge, immunity system functions are focused on recruitment of cells that clear tissue debris. The first cells to arrive on site are the neutrophils that start the pro-inflammatory response to attract monocytes, before dying within a couple of days. Neutrophils induce muscle membrane damage through the release of myeloperoxidase (MPO). Immediately after, the macrophages are recruited at site of lesion. Initially macrophages type 1 (M1) come: they are charged to clean the tissue phagocytising tissue debris.

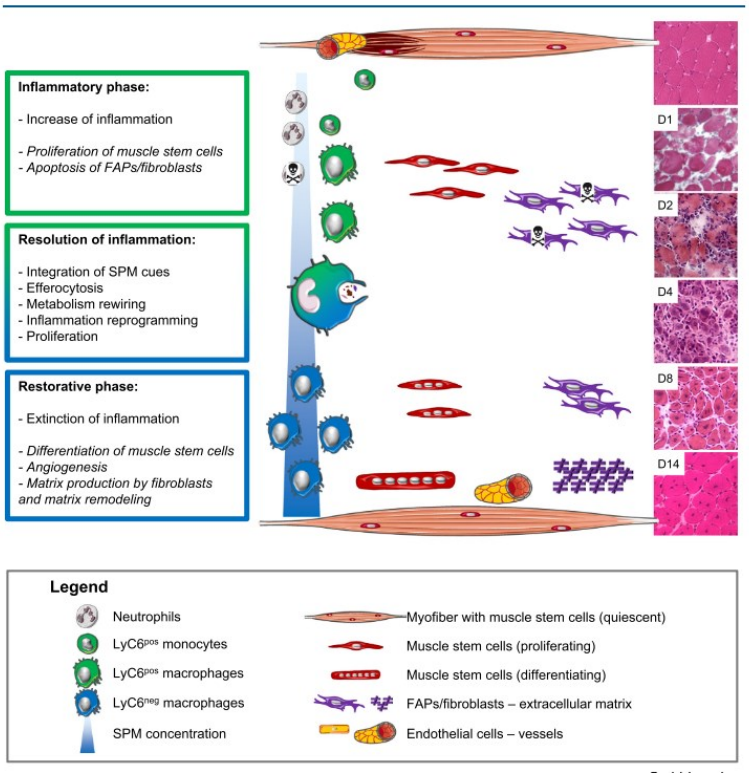

Figure 14 : Sequential roles of macrophages during inflammatory response in mouse skeletal muscle regeneration

M1 macrophages can also promote muscle damage *in vitro* and *in vivo* by the production of cytotoxic levels of nitric oxide (NO) generated by inducible nitric oxide synthase (iNOS).

Later, as a second wave, there are the macrophages type 2 (M2) that exhibit an anti-inflammatory/restorative profile (Chazaud 2020). Indeed, once M1 macrophages reach their peak concentration in injured muscle, they are replaced by a population of M2 macrophages that can attenuate the inflammatory response and promote tissue repair. Actually, the shift in macrophage phenotype (from M1 to M2 populations), that is a milestone event in muscle regeneration, occurs when there is the change from the proliferative phase to the early differentiation phase of adult myogenesis. Following muscle injury, the shift of the macrophages phenotype from M1 to M2, appears to be important in order to change the muscle tissue environment from a first one that promotes the removal of cellular debris and increases satellite cell activation and proliferation to a second one that promotes muscle differentiation and growth (Tidball et al. 2010). Several studies support the crucial role of macrophages for a correct muscle regeneration process. Indeed, using a loss of function approach, it has been shown that muscle regeneration is impaired in absence of the major chemokine receptor expressed on intramuscular macrophages: the CX3CR1 (Zhao et al. 2016). In *in vitro* cultured human muscle cells, it has been demonstrated that the pro-inflammatory macrophages inhibit myoblast fusion, while anti-inflammatory macrophages strongly promote myoblast differentiation by increasing their commitment into differentiated myocytes and enhancing the formation of mature myotubes (Saclier et al. 2013).

In vivo Arnold et al. have shown that monocyte circulating depletion impairs skeletal muscle regeneration (Arnold et al. 2007). Macrophages take part at the inflammation muscle environment as secreting cells that modulate SC behaviours. It has been shown that macrophages secrete the metalloproteinase ADAMTS1 that, by cleaving and so inhibiting Notch1, lead to SC activation (Du et al. 2017). In addition NO, released by several cells during the inflammation, can activate Mmps which in turn allow growth factor matrix release and the activation of satellite cells for tissue repair (Filippin et al. 2009). Finally, Varga et al. have evidenced that PPARγ regulates in repair macrophages the expression of secreted factor TGF-β family member, GDF3. In turn, it regulates the restoration of skeletal muscle integrity by promoting muscle progenitor cell fusion (Varga et al. 2017).

Part III: Adult skeletal muscle plasticity

1. Muscle fiber type plasticity

Skeletal muscle is a plastic tissue that can change its physiological properties depending on external stimuli. Pre-existing myofibers can change their contractile and metabolic characteristics upon different external factors. As previously described, skeletal muscle is composed of different fiber types that express different myosin isoforms. Myofibers are not fixed units but represent highly versatile entities: they are able to respond to different functional demands changing their phenotypic profiles. The fiber type transitions do not proceed in immediate jumps from one extreme to the other, but occur in a graded and orderly sequential manner. For the myosin heavy chain, this event entails a sequence going from the fastest (MHCIIb) to the slowest (MHCI) isoform, and vice-versa (Pette et al. 1997). One of the main modulators of muscle plasticity, that influences the fibers phenotype and triggers a series of intracellular signaling pathways, is the **physical exercise**. We can classify exercise in two categories: the endurance exercise (aerobic/oxidative) and resistance exercise (strength/glicolitic).

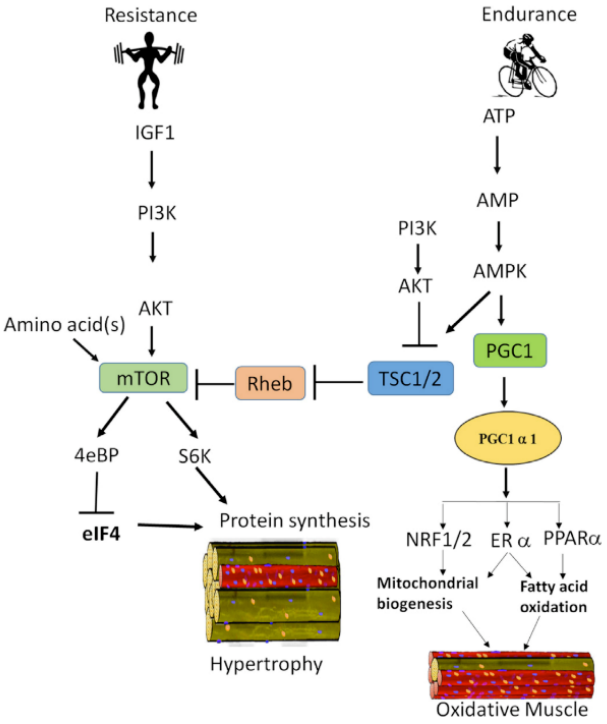

Figure 15: Signalling pathway controlling fiber types.

Endurance exercise (EE) is characterized by repeated, sustained, low intensity contractions for a prolonged period of time without getting fatigue. In this case the fiber type II changes result in more oxidative metabolism and slow twitch myosin expression (IIb->IIa). A hallmark response of endurance training is mitochondrial biogenesis, mainly promoted by the activation of PGC-1α and its downstream target. In summary the EE increases the muscle oxidative and regenerative potential by increasing the proportion of slow myosin, mitochondria, capillaries, nuclei and the satellite cells pool. These adaptations allow the athletes to exercise long for a given intensity or increase the intensity for a given exercise time in an energy efficient way.

Resistant exercise (RE) involves low-frequency, high intensity contractions against an external resistance with the aim of increasing the muscle bulk. It is generally believed that the hypertrophy of existing muscle fibers following RE is due predominantly, even if not entirely, to the addition of new sarcomeres and myofibrils in parallel alongside with the increase of contractile motor proteins amount (actin and myosin). The net protein accretion is accompanied by an increase in satellite cells number, even though the role of satellite cells in overload-induced muscle hypertrophy is still debated.

The diversity of muscle fibers allows them to perform specialized functional tasks. Thus the type I fibers with high oxidative capacity and capillary density are more suitable for endurance exercise, while type IIb fibers with low oxidative capacity and capillary density are suitable for short term resistance exercise. Type IIa are the intermediate fiber types that allow high power generation at a considerable velocity with good endurance (Qaisar et al. 2016).

In addition, the immobilization or the limb suspension can also influence the myofiber type (Sun et al. 2013).

Among the other extrinsic factors that control myofibers type, there are the thyroid hormones that could regulate MyH genes expression (Salvatore et al. 2014). Moreover, motoneurons stimulation changes correlate with changes in fiber type. The innervation changes according to specific muscle fiber type (Stifani 2014). Denervation could induce fibers atrophy and in addition a switch toward a slow phenotype.

Which are the signalling pathways and transcription factor regulating fibers twitch and myosin expression upon regeneration, upon denervation or following exercise? Among the key regulators of myofiber conversion there are Nuclear Factor of Activated T cells (NFAT) and MEF, that are regulated by Calcineurin and in turn promote slow-fiber-specific gene expression (Chin et al. 1998). Recently it has been shown that NFAT activity was suppressed by RhoA and that could affect myofiber reprogramming upon exercise and metabolic disease (Koo et al. 2017).

The Ca2+/calmodulin-dependent protein phosphatase, Calcineurin, is known as the master regulator in the change from fast to slow twitch fiber type. It has been shown that Pgc1α is a target of Calcineurin signaling and that in turn, in cooperation with Mef2, activates slow fiber gene promoters (Lin et al. 2002). Calcineurin is a downstream mediator of IGF1 thus it stimulates hypertrophy (Musarò et al. 1999). Beyond target genes downstream Calcineurin there is NFAT, resulting in a master regulator of myofiber conversion (Koo et al. 2017). As well, Ca2+/calmodulin-dependent protein (CAMK) is relevant for skeletal muscle fiber adaptation to exercise. It acts synergistically with Calcineurin, activating fiber specific genes by Pgc1α activation.
As well Hippo signaling, that modulates YAP, TAZ and TEAD, three important transcription factors for mechano-signal transduction, regulates slow muscle genes expression (Tsika et al. 2008). Moreover, concerning the effect of mechanical cues on myofibers twitch, Lee et al. showed that overload-induced hypertrophy of *Plantaris* muscles for two weeks, induces in an increase in type IIa and IIb fiber frequency with a corresponding decrease in relative abundance of IIx fibers. However they did not discern the pathway involved (Lee et al. 2016). Recently Peterson's lab confirmed a shift from a fast to a slower fiber type in *Plantaris* muscle using a progressive weighted- wheel-running (PoWeR) protocol that approximates exercise training in humans (Dungan et al. 2019).

2. Increase within skeletal muscle mass: hypertrophy

Skeletal muscle is a dynamic and post-mitotic tissue with unique plasticity since it is able to react to different stimuli adapting its size. Half of body weight increase, occurring during postnatal growth (7-8 fold), is caused by the increase in skeletal muscle size. Postnatal muscle growth is achieved by an increase in number (hyperplasia) and size (hypertrophy) of myofibers in rats, but mainly by hypertrophy of myofibers in mice (White et al. 2010). In adult in general, muscle mass is sensitive to factors such as nutritional status, hormonal balance, physical activity/exercise and injury or disease, among others (Frontera et al. 2015). Thereby multiple factors, like environmental signals, genetics, hormones and the nutrition, contribute to muscle mass control.

2.1 Mice model of muscle hypertrophy

In mouse, different procedures are used to reproduce muscle hypertrophy, which are able to mimic a chronic workload increase. One method is to prevent, by removing (**surgical ablation**) or not (**tenotomy**), the functionality of synergistic muscle (*gastrocnemius* and *soleus*), in order to stimulate the *Plantaris* muscle to compensate the workload. *Plantaris* muscle, remaining attached to the bone by tendons, is charged of all the tension, resulting in overload-induced hypertrophy.

Importantly, in this kind of surgery experiments there could be a possible inflammation and muscle swelling induced by the surgery, above all in the early phase of hypertrophy process. To avoid these secondary effects linked to the surgical procedures, Cui et al. developed a physiological animal model of resistance exercise. This model employs a cage top on a regular mouse cage where an individually housed mouse performs squat-like activity against controlled load during feeding, recapitulating many of the known responses to a single bout, short-term, and long-term resistance exercise training in humans (Cui et al. 2020). In addition to **voluntary weightlifting exercise** another accepted model leading to muscle hypertrophy is the wheel running. The **wheel running** is an endurance-training model in which the rodents have a strong intrinsic drive to run spontaneously large distances in their active phase. By applying resistance to the running wheel, muscle hypertrophy can be induced: both *Plantaris* and *Soleus* muscles are stimulated. **Treadmill running** is a forced exercise model for endurance training in rodents and it is widely used to generate an eccentric training and produce greater muscle hypertrophy. However, close attention must be paid to the negative effects of treadmill running because alterations in neuroendocrine tissues and immune responses, commonly associated with chronic stress, were observed in male Sprague-Dawley rats (Moraska et al. 2000).

It is well known that androgens have a positive effect on muscle hypertrophy. Already in 1938 Papanicolaou and Falk have described an hypertrophy of the temporal muscles in the guinea pig after testosterone administration (Papanicolaou et al. 1934). However, male sexes sensitivity is not equally expressed in all muscles. In order to characterize androgens signalling in skeletal muscle Chambon et al., using a genetic mouse model in which the androgen receptor (AR) is selectively ablated in myofibers, discovered that the post-natal hypertrophy of perineal muscles was mediated by AR and IGF-IEa downstream signalling. On the contrary in limb muscles, myocytic AR deficiency did not affect the post-natal hypertrophy of fibers, but myofibrillar organization of sarcomeres and muscle strength (Chambon et al. 2010). Anyway, the mechanisms of androgen action in skeletal muscle remain poorly understood.

2.2 SCs participation

In the adult, the two major mechanisms related to muscle growth and to an accretion in sarcoplasmic volume are: increased protein synthesis in order to add new contractile filaments to pre-existing sarcomere units and the fusion of new nuclei which come from activated satellite cells. Since 1989, the concept of **myonuclear domain**, that is a fixed cytoplasmic volume transcriptionally governed by one myonucleus, was postulated (Pavlath 1989). Today, myonuclear domain theory and the satellite cells-mediated myonuclear accretion (occurring during adaptation to

overload), are extensively discussed. Different scenarios that could happen upon hypertrophy, together with myofibers cross section area increase, are proposed:

A) finely regulating the myonuclear domain, the contribution of SCs is indispensable to increase the number of myonuclei and to support the muscle growth (Egner et al. 2016).

B) the number of myonuclei remains the same, there is no myonuclei accretion.

Supporting this last scenario, McCarthy et al. demonstrated that effective hypertrophy (increase in muscle weight and in cross section area) occured after the ablation of SCs in adult mice (Pax7-DTA mice). In this way myonuclear domain increases and myofiber hypertrophy is not accompanied by an accretion of myonuclei. They suggested that the principal role of SCs during hypertrophy process is to regenerate damaged fibers rather than to support myonuclear accretion (Mccarthy et al. 2011). Moreover Kirby et al. showed an high transcriptional level of resident myonuclei during hypertrophic mechanical load increase, above all in absence of SCs (Kirby et al. 2016). To clarify previous conflicting arguments, Murach et al. demonstrated that the age is a critical variable. They showed that only when SCs depletion occurred in 2 months aged mice (and not in > 4 months of age), overload-induced CSA growth is impaired (Murach et al. 2017). Only in aged mice, but not in young mice, hypertrophy can occur with the increase of CSA, but without the addition of new myonuclei coming from SCs.

Further evidences confirming this scenario came from other groups who succeed in inducing significant hypertrophy just by manipulating the important signalling pathway of muscle fiber size regulation. They obtained increased muscle mass in absence of muscle overload or damage and without myonuclear accretion (Amthor et al. 2009; Blaauw et al. 2009). Moreover, it is important to relate muscle size to muscle function (muscle specific force) in the different mouse model used to modulate muscle mass. For example both *Igf1* overexpression and *Myostatin* ablation lead to an increase of myofibers size and cross section area; furthermore in *Myostatin-/-* mice, the myonuclear domain (MND) is not conserved and the specific force decreases, contrary to $mIgf1^{+/+}$ mice where the MND is maintained and the specific force increases compared to wild type *Edl* muscle (Qaisar et al. 2012). In this way some authors suggested that the nuclear accretion, preceding the muscle growth, is indispensable in order to have a functional skeletal muscle, closer to physiological hypertrophy situation. Indeed recently, Masschelein et al. have show, that mechanical load (performed with running wheel with increased resistance) increases muscle grip force and intrinsic muscle endurance capacity and that it is accompanied by satellite cells recruitment to the growing myofibers (Masschelein et al. 2020). This study underlines that SC fusion and the concomitant myonuclear accretion is typical in a physiological setting of increased mechanical load.

During overload process, SC niche is almost completely retained and SCs start to proliferate 48 hours after tenotomy. The factors that induce SCs activation in over-loaded muscles are still unknown. One possibility is that SCs receive mechanical cues in a direct manner. Another possibility is that non-SCs, including myofibers, sense the mechanical stress and subsequently release the activation factor which acts on SCs or disrupts their quiescent state (Fukada et al. 2020). Overload disrupts the extracellular matrix (ECM) and might initiate SC activation by releasing cytokines that are retained in the ECM structure or by reducing the amount of ligands available for quiescence signaling.

Further studies have been accomplished to support the contribution of satellite cells during hypertrophy, corroborating the scenario A and underling the critic role of satellite cell fusion during hypertrophy. Different mice model with fusion incompetent satellite cells were used in order to investigate the role of satellite cell recruitment upon skeletal muscle overload. Notably, Goh et al. by using muscle stem cells lacking Myomaker (a muscle-specific membrane protein necessary for fusion of embryonic and adult myogenic precursors) demonstrated that the muscle response to hypertrophy was blunted. Therefore satellite cells capable to fuse are required for a correct hypertrophic growth (Goh et al. 2017). In addition, loss of Srf (a master regulator of SC fusion) in satellite cells impairs overload-induced muscle growth. These data provide once again the evidence that the hypertrophy is compromised when SCs are unable to fuse (Randrianarison-Huetz et al. 2018).

We can conclude that myonuclei are recruited before growth under many hypertrophic conditions and precede muscle growth.

From zebrafish to mouse, skeletal muscle tissue adapts its physiological environment by altering its excitability, its contractile and its metabolic phenotype, depending on external stimuli. In human both endurance and resistance exercises promote maintenance of muscle mass and function. High-resistance strength-training exercise increases muscle size and strength.

2.3 Secreted factor important for muscle hypertrophy regulation

Igf1

Insulin like growth factor (Igf1) is implicated in the enhancement of hypertrophy in adult animal. It is mainly produced by the liver. There are two isoforms of this important protein: particularly in rodents, they are Igf1-Ea and Igf1-Eb. The overexpression of Igf1 in mice, specifically in muscle cells using *MLC/Igf1* transgenic mice line, is able to induce muscle hypertrophy (Musarò et al. 2001). Some studies affirmed that the hypertrophic effect of sustained Igf1Ea overexpression is evident in growing muscle during post-natal development or regeneration, but is not in normal adult

muscle (Shavlakadze et al. 2013). Some others asserted that only the isoform Igf1Ea had an effect on hypertrophy in 6 months old mice (Barton 2006). Also in human skeletal muscle it has been shown that the isoforms Igf1-Ea and Igf1-Eb (this last renamed mechano growth factor (MGF) are induced by stretch, but respond in a different way to high resistance exercise depending on the age (Hameed et al. 2003). This concept underlines the age-related desensitization of the muscle to mechanical loading.

Igf1 treatment in culture leads to an increase of myotube diameter, promotes proliferation and differentiation of myoblast (Cheng 1995) and enhances myoblast fusion with existing myotubes. *In vivo* on aged detrained rats muscles, the infusion of Igf1 restores the loss of muscle mass and increases the proliferation of the satellite cells (Chakravarthy et al. 2000). In the same way on human primary culture of myoblast, Igf1 promotes fusion with an increased number of myonuclei per myotubes (Jacquemin et al. 2004). Moreover Igf1 has an effect on protein synthesis (Philippou et al. 2007). In mice the hypertrophy generated upon Igf1 overexpression is mediated by the over activation of Akt signalling (Schiaffino et al. 2011), one of the major pathway involved in muscle hypertrophy that will be discussed later.

Myostatin

Myostatin (Msn) is a member of TFG-β superfamily and it acts as inhibitor of muscle growth. Disruption of *Myostatin* gene leads to development of enlarged muscles in mice (McPherron et al. 1997). The enlargement is due to hyperplasia and hypertrophy, but is not accompanied by an increase in fibers strength. On the contrary the overexpression of *Myostatin* gene results in muscle atrophy (Durieux et al. 2007).

Myostatin signals via the Activin type II receptors (ActRIIA and ActRIIB) and Activin type I receptors (ALK4 and ALK5) to phosphorylate responsive Smad proteins (Smad2 and Smad3, Smad2/3). This phosphorylation enables Smad proteins to form a transcriptional complex with Smad4 to regulate target genes transcription. Moreover, it has been shown that Myostatin inhibits activation of the Akt/mammalian target of rapamycin (mTOR)/p70S6 protein synthesis pathway, inhibiting both myoblasts differentiation and myotubes hypertrophy (Trendelenburg et al. 2009).

BMPs

Bone morphogenetic proteins (BMPs) belong to TGF-β superfamily. Initially, they were detected by their ability to direct ectopic bone formation, but they are also involved in many developmental processes. Recently it has been shown that they have a crucial role in skeletal muscle growth and

maintenance. BMPs interact with type I and type II cell surface receptors. There are three different BMP type II receptors (BMPR-II, ActR-IIA et ActR-IIB) and four BMP type I receptors (BMPRI-A, BMPRI-B, ActR-I and ActR-II). BMP receptor activation starts an intracellular cascade signalling by triggering SMAD1/5/8 phosphorylation. Phosphorylated SMAD1/5/8 forms a heteromeric complex with SMAD4 to regulate specific genes transcription. This complex recognises specific BMP responsive elements (BRE) localized at the promoter level of target genes. An established target gene for BMPs is ID1 (inhibitor of differentiation) (Korchynskyi and Ten Dijke 2002).

Moreover, BMP signalling pathway could be negatively regulated by SMAD6 that inhibits SMAD1/5/8 phosphorylation. Other proteins can, as well, act as antagonist of BMP signalling, recognizing and interacting with BMP receptor in order to block the downstream pathway. Among these proteins, there are Noggin, Chordin, Dan and Gremlin. Certainly numerous pharmacological agents, like blocking antibodies or kinase inhibitor, have been developed to modulate BMP signalling at different levels (Gomez-Puerto et al. 2019).

Besides the canonical SMAD pathway, BMPR activation can also induce non-SMAD signalling. Non-SMAD signalling is characterized by intracellular activation of p38 and JNK MAP kinases and small GTPases such as Rho and Rac (Zhang 2017).

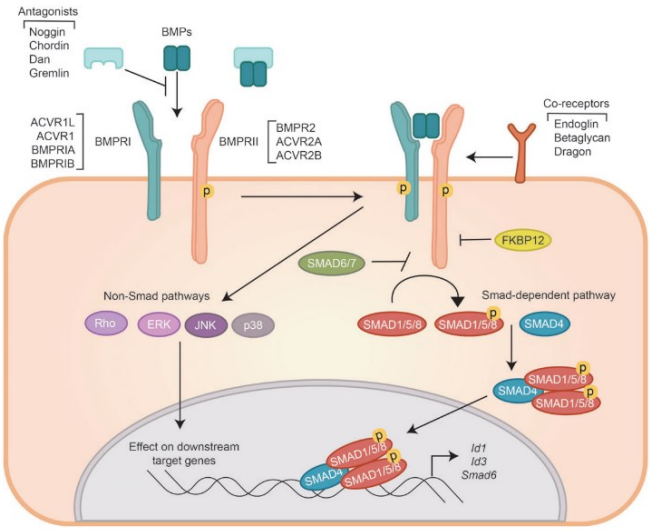

Figure 16: BMPs signalling pathways

Wang et al. have shown that the inhibition of BMP signalling by Noggin treatment impairs fetal chick muscle development, decreasing the number of Pax7+ cells and the myofibers size (Wang et al. 2010). On primary satellite cells, BMP inhibits myogenic differentiation and Noggin treatment enhances myoblast differentiation and myotube formation (Friedrichs et al. 2011). These data

suggest that BMP is crucial during muscle development in order to coordinate the equilibrium between cells proliferation and differentiation.

Likewise, BMPs are important during post-natal muscle growth. Indeed, Stantzou et al. have shown that the inhibition of BMP signaling in mice three days after birth (via the overexpression of both Noggin and SMAD6) induces a delayed muscle growth correlated with a decrease in satellite cells number and proliferation (Stantzou et al. 2017). In 2013, for the first time the role of BMPs was discovered in homeostasis of adult muscle and its relationship with muscle mass regulation was described (Winbanks et al. 2013). Sartori et al. have shown that the inhibition of BMP pathway, (through or loss of SMAD4 in the muscle or the overexpression of Noggin or pharmaceutical treatment), leads to muscle atrophy evindenced by smaller fibers and decreased muscle strength. Moreover, they revealed an increase of MUSA1 activity. However, the overexpression of BMP receptor is sufficient to induce muscle hypertrophy. Moreover, they observed that in *Mstn*$^{-/-}$ mice, characterized by hypertrophy and hyperplasia, SMAd signalling is increased and that the BMP signalling inhibition completely reverts hypertrophic phenotype, showing that it is predominant on Myostatin signalling. In the same article they demonstrated that SMAD4, a protein shared between the two pathway, is the limiting factor to activate one or the other pathway and that Myostatin and BMP are in competition to activate SMAD 4 and the downstream genes (Sartori et al. 2013).

Androgens

Among the hormone regulator of skeletal muscle mass there is the testosterone. Testosterone is a well-known anabolic steroid: it increases muscle protein synthesis and muscle mass; after the onset of puberty it is most likely responsible for the greater increase in boys' lean mass in comparison to the girls one (Smith et al. 2014). The anabolic effects of testosterone on muscle mass are well known. In human, has been shown that testosterone administration induces an increase in muscle volume related to an hypertrophy of myofibers (Sinha-Hikim et al. 2002). Moreover the same team evidenced that testosterone-induced muscle fibers hypertrophy is associated with an increase in satellite cell number, a proportionate increase in myonuclear number and some changes in satellite cell ultrastructure (Sinha-Hikim et al. 2003). However the biological mechanisms of androgen action in skeletal muscle are poorly understood (Chen et al. 2005).

Interleukins

Traditionally, Interleukin 6 (IL-6) was considered to be an inflammatory cytokine. However, it is well established that IL-6 is also produced by skeletal muscle in response to exercise. For this reason, it is also known as "myokine". IL-6 following physical exercise is systemically released in a

temporal manner. Its concentration rapidly increases and remains elevated from few hours to a few days. This temporal increase in IL-6 appears to have a positive impact on muscle health, augmenting glucose uptake and improving muscle stem cell function (Snijders et al. 2015). Indeed, satellite cells from *IL-6* knock-out mice have a reduced proliferative capacity, both *in vitro* and *in vivo*. *IL-6* knock-out mice shows a blunted muscle fiber hypertrophic response and a less SC-mediated myonuclear accretion compared to wild-type mice, following overload induced muscle hypertrophy (Serrano et al. 2008).

IL-4 has been identified as one of the myoblasts recruiting factor that leads to further nuclear addition and to myotube size increase. Il-4 expression is regulated by the transcription factor NFAT and its receptor is expressed in a wide range of tissues. Muscles lacking Il-4 or its receptor are smaller and the reduced cross-section area of myofibers results from defects in the ability of muscle cells to fuse with the myofiber *in vivo*. Il-4 promotes fusion and muscle growth (Horsley et al. 2003). Moreover, Guerci et al. have shown that IL-4 overexpression in Srf-deleted muscles, which display defective overload-induced hypertrophy and satellite cells mobilization to pre-existing fiber, restores SC recruitment and muscle growth (Guerci et al. 2012).

2.4 Pathways involved in regulation of skeletal muscle hypertrophy

A highly conserved signalling pathway that plays a major role in the regulation of skeletal muscle growth, involves insulin-like growth factor 1 (**IGF1**) and a cascade of intracellular components that mediate its effects. A central component in this cascade is the kinase **Akt**, also called protein kinase B (PKB). Akt in turn controls both protein synthesis, via the kinases mammalian target of Rapamycin (mTOR) and Glycogen Synthase Kinase 3b (GSK3b), and protein degradation, via the transcription factors of the FoxO family (Schiaffino et al. 2011b). Bodine et al. have found that hypertrophy of myotubes induced *in vitro* by Igf-1 depends on a pathway initiated by PtdIns-3-OH kinase (PI3K) and the PtdIns-regulated kinase Akt (Bodine et al. 2001). In summary, following to exercise, Igf1 together with Insulin, binds their respective receptors and activates PI3K. The kinase phosphoryls the lipidic chain releasing PIP3 that recruits Akt on the membrane. In this way Akt is activated. In the model of compensatory hypertrophy of the *Plantaris* muscle *in vivo,* Akt activity and its downstream targets increase underling its crucial role. As mentioned before, Akt phosphoryation leads to activation of Glycogen Synthase Kinase 3β (GSK-3β), leading to its inhibition and the upregulation of protein synthesis (Jefferson et al. 1999).

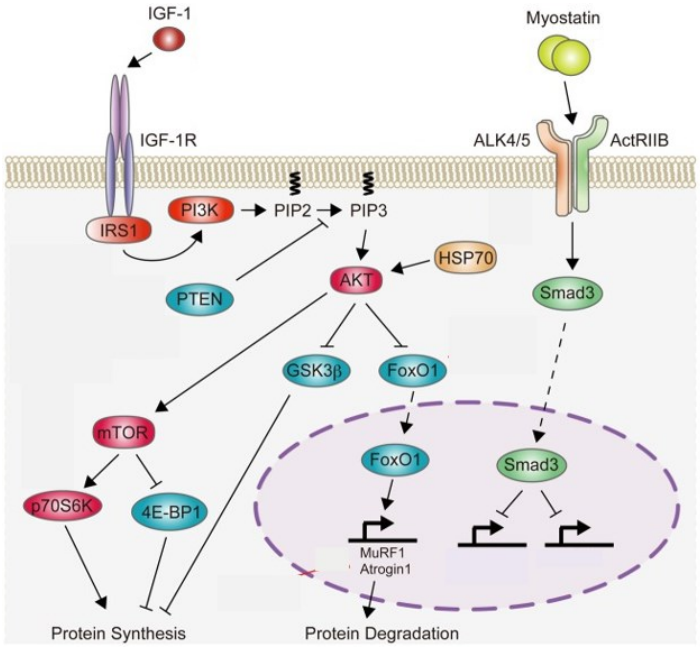

Figure 17: Principal signalling pathway controlling skeletal muscle hypertrophy

Since GSK3 normally has acted to inhibit the translation initiation factor eIF2B, the inhibition of GSK3 by Akt may promote translation initiation and protein synthesis, and thus may contribute to hypertrophy (Rommel et al. 2001). Interestingly a novel mechanism mediating the effect of GSK3b on muscle growth has been recently described. GSK3b is able to phosphorylate Nebulin at two Ser sites in the C-terminal region of Nebulin localized to the Z-disk, thus preventing the interaction of Nebulin with neuronal Wiscott-Aldrich Syndrome Protein (N-WASP), a ubiquitously expressed member of the WASP family which is involved in actin assembly. IGF1-Akt signalling, by inhibiting GSK3b, allows the interaction of N-WASP with the unphosphorylated Nebulin; the consequent recruitment of N-WASP to the Z-disk promotes actin nucleation and elongation of actin filaments. This process appears to be relevant to muscle growth *in vivo* because long-term knockdown of *N-WASP* in adult mouse muscles causes atrophy of the transfected muscle fibers (Takano et al. 2010). At the same time Akt phosphorylates mTOR. The Akt/mTOR pathway is activated in, and it is required for, muscle hypertrophy *in vivo*. The mammalian target of Rapamicin (**mTOR**) is a Ser/Thr kinase that forms two structurally and functionally complexes: mTORC1 and mTORC2. mTORC1 contain Raptor and is Rapamycin sensitive, mTORC2 contain the protein Rictor and is required for Akt signalling. The two complexes have different roles in embryonic myogenesis, since different phenotypes have been evidenced by the deficiency of one or the other complex in mice skeletal muscle progenitors (at E8). Raptor protein (component of mTORC1) gets activated at E11.5 and its absence impairs a correct development of skeletal muscle fibers. The

principal downstream targets of mTORC1 are the ribosomal protein S6 kinases (p70S6K) and a repressor of the cap-binding protein eIF4E (4E-BP1). Both of them have been shown to promote protein synthesis through increases in translation initiation and elongation. Indeed phosphorylation of p70S6K leads to its activation and to the promotion of protein synthetis pathways. Consequently, the phosphorylation of PHAS-1/4E-BP1 releases it from an inhibitory complex with the translation initiation factor eIF4E, thereby permitting the binding of eIF4E to eIF4G and promoting translation initiation (Lin et al. 1994). In an independent manner, Akt activates mTOR that as well plays a central role in hypertrophic response induced by mechanical load.

In cultured myoblast *in vitro,* it has been shown that Akt is necessary for a correct protein synthesis, for a correct myoblast proliferation, differentiation and fusion even if its absence does not abrogate completely all these processes. *In vivo*, when Raptor protein is absent in satellite cells, skeletal muscle presents serious problem of regeneration after Ctx injection. Mainly, there is a delay in Pax7+ cells activation, principally affecting protein synthesis (Rion et al. 2019).

A recent article shows that the defining component of complex mTORC1 Raptor is necessary to mediate mechanical induced hypertrophy, not influencing the rate of protein synthesis, but maybe controlling the quality of newly synthesized proteins or maybe increasing autophagy of proteins that should normally drive a hypertrophic response (You et al. 2019). Moreover already in 2001 Bodine et al. have asserted that Rapamycin treatment *in vivo* almost completely prevents the hypertrophic increase in *Plantaris* muscle weight and fiber size (Bodine et al. 2001). On the contrary mTORC2 is not Rapamicyn sensitive and regulates Akt posphorylation necessary for its complete activation (Sarbassov et al. 2006).

The activity of Igf1-Akt pathway is controlled by several feedback loops and can be modulated by a variety of factors. Negative feedback involves S6K, which inhibits IRS by phosphorylation at multiple sites, thus inducing its degradation and its altered cell localization. Positive feedback involves mTORC2, which phosphorylates Akt at serine 473, a phosphorylation required for maximum activation of Akt in addition to phosphorylation at threonine 308 by PDK1.

Moreover, mechanical signals via Integrin β1 and Integrin-linked kinase (ILK), lead to phosphorylation of the Igf1 receptor modulating PI3K-Akt pathway in muscle cells (Wang et al. 2008).

In addition, although it is well established that the release of growth factors, acting locally, induces mTOR activation, it has been shown that mechanical stimuli can activate mTOR pathway independently of PI3K/Akt and growth factors. Hornberger et al. showed that mechanically stimulation (passive stretch) of skeletal muscle *ex vivo,* results in the activation of mTOR-dependent signalling events (Hornberger et al. 2004). The intermittent passive stretch, considered as the source

of mechanical stimulation, involves Phospholipase D (PLD) and the lipid second messenger Phosphatidic Acid (PA) for mTOR signalling activation. In particular Phosphatidic Acid, generated by Phospholipase D (PLD) directly binds and activates mTOR (Hornberger et al. 2006). Furthermore, inhibition of PI3K–Akt activity do not prevent the activation of mTOR signalling by eccentric muscle contractions, indicating that PI3K–Akt is not the only one upstream regulatory pathway and that mTOR following eccentric contraction can also be directly activated independently of PI3K/Akt signalling by PA synthesized by PLD (O'neil et al. 2009). In addition, it has been recently demonstrated on C2C12 that PA and mTOR co-localize in macropinosome upon mechanical stretch or osmotic shock, suggesting that tension fluctuation-induced macropinosome functions as a platform for mTOR recruitment and its activation via PLD activity (Lin et al. 2019).

The molecular **mechanosensor**, that triggers growth processes in response to mechanical loading, are nowadays elusive. An attractive emerging concept in muscle biology is that signals dependent on muscle activity, and specifically on mechanical load, may arise in the sarcomere, the basic unit of the contractile machinery of striated muscles, and from there they are transmitted to the nucleus to affect gene expression (Sandri 2008).

In 2012, it was proposed that a sensor for mechanical cues was the transcription Serum Response Factor (Srf). Indeed, it was shown that Srf activity in the myofibers is required for muscle mass growth in response to increased loading (Guerci et al. 2012). The authors proposed that Srf may be a central transcription factor required for the translation of mechanical signals into a myofiber transcriptional program. These events are governed by local milieu modifications that consequently are interpreted by satellite cells in order to support muscle growth. Moreover, disuse muscle atrophy leads to a sustained decrease in the global activity of Srf. Moreover, Srf overexpression induces a significant resistance to atrophy, highlighting its key role as a mediator of mechanotransduction in muscle (Collard et al. 2014).

Among signallings that regulate gene expression in response to the mechanical cues, Hippo pathway has been identified. The core effector of Hippo cascade signalling are the transcriptional co-activators Yap/Taz, found initially in fruit fly as regulator of organ size (Gateff 1978). Many studies have been reported on the crosstalk between Yap/Taz with Akt-Tsc-mTOR signalling and Myostatin-Smad signalling (Gabriel et al. 2016). Moreover, other studies found that energy stress and the key exercise kinase AMPK regulate Yap, suggesting that Yap should be affected by exercise and diet (DeRan et al. 2014). Recent studies have related Yap activity directly to muscle fiber hypertrophy. Goodman et al. showed that the overexpression of Yap induces muscle hypertrophy. Moreover they evidenced that Yap acts via mTORC1-independent mechanism (Goodman et al. 2015). Independently by Goodman's study, another team established that Yap

silencing leads to a reduction in muscle mass affecting protein synthesis and increasing atrogenes expression. Surprisingly, when Yap is overexpressed *in vivo*, muscle mass and related myofibers size increase. This YAP positively regulation of muscle mass is associated to TEAD transcription factor interaction (Watt et al. 2015). In addition, more recently it has been shown that in healthy skeletal muscle, nuclear Yap increases significantly in response to *in vivo* muscle physiological loading. In mature isolated muscle fibers cultured on stiff substrate nuclear Yap increases. As well, in a context of potential preactivated state of mechano-signalling and increased stiffness of pathological muscle, Yap gene expression increases in dystrophic muscle (Iyer et al. 2019). Interestingly Elosegui-Artola et al. proposed that stiff environment to a mechanical connection between the nucleus and the cytoskeleton are linked. Forces transmission allows Yap to reach the nucleus: the induced nuclear flattening stretches nuclear pores, reduces their mechanical resistance to molecular transport and increases YAP nuclear import (Elosegui-Artola et al. 2017). In conclusion, Yap and TAZ may connect mechanical signals to other transcription factors or metabolic pathways in order to harmonize mechanical state of the cell and its behaviours (Panciera et al. 2017).

During the transition from rest to exercise, ATP turnover can rise potentially more than 200-fold. To maintain homeostasis during such a large step change in ATP hydrolysis, systems have evolved to sense energy levels and to initiate the signalling processes that regulate both short- and long-term adaptations of energy metabolism. Exercise increases the concentrations of AMP and ADP in contracting skeletal and cardiac muscle, and exercise depletes glycogen, especially in muscle. In this system, glucose, glycogen, AMP and ADP are sensed principally by the heterotrimeric AMPK complex. Therefore, it comes as no surprise that **AMPK** is a key mediator of the adaptation to endurance exercise, particularly in skeletal muscle (Kjøbsted et al. 2018). AMPK is an heterotrimeric protein that is activated by a complex mechanism involving an increase in the AMP/ATP ratio and allosteric modification and phosphorylation by one or more upstream kinases, including LKB1. AMPK is one of the most widely studied proteins implicated in muscle glucose transport in response to cellular energy status changes. The exercise training induces the activation of AMPK. It has been shown that AMPK phosphorylates Pgc1α directly and these direct phosphorylations of the Pgc1α protein at Threonine-177 and Serine-538 are required for the Pgc1α - dependent induction of the Pgc1α promoter (Jäer et al. 2007). The transcriptional coactivator Pgc1α controls the mitochondrial biogenesis and metabolic homeostasis. The overexpression of *Pgc1α* in skeletal muscle leads to a slow-twich fiber type conversion (Lin et al. 2002) and in *Pgc1α* muscle-specific knockout mice mRNA and/or protein content of mitochondrial respiratory chain proteins and ATP synthase are reduced. Upon endurance exercise, Pgc1α controls skeletal muscle adaptation

by the regulation of VEGF expression and of mitochondrial metabolic and anti-oxidant proteins. In this way it influences the substrate utilization, exerting an anti-inflammatory effect (Handschine et al. 2008). Multiple upstream signalling molecules could activate Pgc1α and in turn elicit, in skeletal muscle, exercise-induced gene responses.

Moreover AMPK responds to energy stress by suppressing cell growth and biosynthetic processes, in part through inhibition of the rapamycin-sensitive mTOR (mTORC1) pathway (Gwinn et al. 2008).

Similarly, p38 mitogen activated protein kinase (**p38 MAPK**) is activated by various stimuli including contraction, insulin, environmental stress and pro-inflammatory cytokines. Studies on C2C12 myocytes show that p38 MAPK, in response to increased muscle activity, stimulates Pgc-1 α gene transcription as part of the mechanisms for skeletal muscle adaptation (Akimoto et al. 2005).

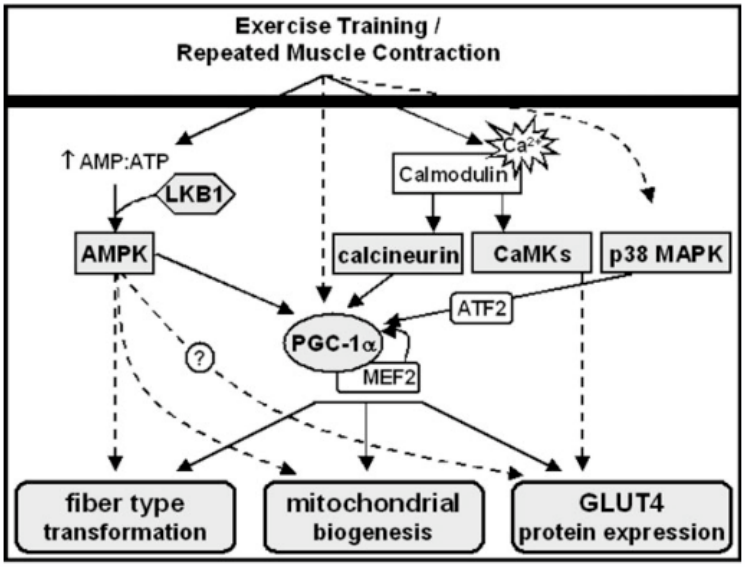

Figure 18: Important signalling pathway triggered by exercise (Röckl, Witczak, and Goodyear 2008)

2.5 Hypertrophy and other cell types

Since in absence of satellite cells the hypertrophy ~~is~~ has not been completely blunted, a potential contribution of other circulating cells to adult muscle hypertrophic growth is suggested, even if clear demonstrations are still missing. Different interstitial stem cells, like PW1-positive interstitial cells, mesangioblasts or pericytes, show myogenic potential and possibly therefore contribute to muscle growth (Blaauw et al 2014). I already described the importance of interleukins during

hypertrophy process. It is known that IL4 and IL-6 are secreted by satellite cells and **macrophages** and also that the macrophages number increases upon overload (OV) hypertrophy. DiPasquale et al. have shown that ablation of macrophages leads to a reduction of muscle mass 14 days after *Plantaris* muscle surgical ablation, not affecting mTOR pathway. However the authors did not investigate whether macrophages control SCs behaviour during OV (DiPasquale et al. 2007). In addition, another group evidenced the importance of macrophages during muscle reload. In fact the depletion of macrophages by antibody treatment prevents muscle fibre growth upon hindlimb reload (Tidball et al. 2007). Peterson lab showed an increase of macrophages following training in humans (Walton et al. 2019).

Vessel-associated stem cells, **mesoangioblasts**, are known to have a strong myogenic (Minasi et al. 2002) and angiogenic potential (Sachdev et al. 2012). They respond to the release of cytokines from inflammatory cells, a typical event during the early phases of hypertrophy. In addition considering that angiogenesis is stimulated during hypertrophy induced by overexpression of Akt (Blaauw et al. 2009), it could be hypothesized that other resident interstitial cells could mediate and could have un important role in the adaptations of muscle during muscle hypertrophy.

Moreover, hypertrophy requires an ECM remodelling. Indeed the **fibroblasts** present in the muscle communicate with myogenic precursor cells and finely regulate the collagen deposition during hypertrophy muscle remodelling (Fry et al. 2017).

In addition, the content of **FAPs**, cells that have an adipogenic and fibrogenic potential in culture, increases in exercise trained mice. However further investigations are needed to elucidate FAPs function following exercise (Collao et al. 2020). In a recent study was suggested that exercise alters FAPs population in a pathology-dependent manner when control mice are compared to high fat diet fed mice (D'Souza et al. 2019).

3. Decrease in muscle mass: atrophy

Atrophy occurs in specific muscles with inactivity (for example, during plaster cast immobilization) or denervation (for example, in patients with spinal cord injuries). Muscle wasting is a physiological response to fasting or malnutrition. It occurs systemically in older people (a condition known as sarcopenia) and in many diseases including chronic obstructive pulmonary disorder, cancer-associated cachexia, diabetes, renal failure, cardiac failure, Cushing syndrome, sepsis, burns and trauma (Cohen et al. 2014). Muscle atrophy is characterized by a decrease of myofibers size and consequently a reduction of muscle mass and muscle functions. Muscle homeostasis depends on a fine equilibrium between protein synthesis and protein degradation: in atrophy condition this

equilibrium is disrupted. Indeed, in atrophied muscle the protein degradation is prevalent on protein synthesis. The contractile proteins, that are approximately the 80% of total myofibers volume, are the principal proteins to be degraded.

Muscle atrophy is an active process controlled by specific signalling pathways and transcriptional programs. The comparison of different muscle atrophy models leads to the identification of a subset of genes that are commonly up- or downregulated in atrophied muscle. These common genes were called atrophy-related genes or **atrogenes** (Sacheck et al. 2007).

Upon atrophic conditions or during detraining, the nuclei in the myofiber are not lost: the fibers lose proteins but maintain their number of nuclei. Upon re-training these atrophied fibers grow faster, suggesting that they skip the step of myonuclei recruitment. These observations introduce an important concept in skeletal muscle plasticity that is the muscle memory. Many experiments suggest that there is a form of cellular memory in the muscle cells: the muscle mass previously obtained is easily regained and the cellular mechanism for this 'memory' is related to the number of myonuclei (Gundersen 2016). Gundersen suggested that the hypertrophic growth could occur without recruitment of myonuclei during re-training. This is not due to the fact that more nuclei are not necessary in larger fibers, but rather to the fact that the nuclei are already there, considering the concept of muscle memory.

Igf1/Akt signalling pathway is deregulated during muscle atrophy and the cross talk between protein breakdown and protein synthesis is unbalanced. Normally Akt phosphorylates Fork-head Box (Fox) O transcription factors (**FoxOs)**, promoting the export of FoxOs from the nucleus to the cytoplasm. The reduced activity of Akt pathway observed in different models of muscle atrophy results in decreased levels of phosphorylated FoxO in the cytoplasm and in a marked increase of nuclear FoxO protein (Stitt et al. 2004). Among the most induced genes target of FoxO there are several atrogenes encoding for muscle-specific ubiquitin ligases, like **Atrogin-1/MAFbx and MuRF**. These promoting genes are responsible for the increased protein degradation through the ubiquitin-proteasome system. Recently, were discovered a new ubiquitin ligase named MUSA1 (muscle ubiquitin ligase of the SCF complex in atrophy-1) involved in Smad/ BMP signalling (Sartori et al. 2013) and another FoxO-dependent ubiquitin ligase termed SMART (Milan et al. 2015). Actually, Atrogin and Murf are considered the best markers of muscle atrophy and they can be considered as master genes of muscle wasting.

Atrogenes are also regulated by **NFκB-dependent signalling** that is activated by inflammatory cytokines, such as TNFα. A study showed that muscle-specific overexpression of IKKβ in transgenic mice led to severe muscle wasting, mediated at least in part by the ubiquitin-ligase MuRF1 (Cai et al. 2004). The critical role of NFKB and its crosstalk with other pathways, involved

in muscle wasting, are still investigated. In parallel cells turnover, myonuclear accretion and myonuclear loss are fundamental for the size of muscle fibers.

Another principal mechanism controlling protein degradation is the **autophagy** and recent evidences suggest that the autophagy-lysosome system is activated during atrophy. Autophagy, or cellular self-digestion, is a cellular pathway involved in protein and organelle degradation. There are three major types of autophagy in eukaryotic cells: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Microautophagy involves the direct engulfment of cytoplasm at the lysosome surface by invagination, protrusion and septation of the lysosome membrane. In contrast, during macroautophagy, portions of cytoplasm are sequestered into a de novo-formed double-membrane vesicle termed an autophagosome. Subsequently, the completed autophagosome fuses with the lysosome/vacuole and the inner single-membrane vesicle is released into the lumen. In either case, the membrane of the resulting autophagic body is lysed to allow the contents to be broken down, and the resulting macromolecules are transported back into the cytosol through membrane permeases for reuse. In contrast, CMA does not involve a similar type of membrane rearrangement; instead, it translocates unfolded, soluble proteins directly across the limiting membrane of the lysosome (Yang et al. 2009).

In skeletal muscle it was showed that FoxO3 is necessary and sufficient for the induction of autophagy, controlling the transcription of autophagy-related genes, including LC3 and Bnip3 (Mammucari et al. 2007). In addition, Runx1, a DNA binding protein, was identified as a protein required to avoid muscle wasting and muscle atrophy that are induced by myofibrillar disorganization and autophagy (Wang et al. 2005).

In cancer-induced muscle, wasting autophagic markers are increased suggesting an induction of autophagy (Aversa et al. 2016).

Moreover, autophagy is a critical biological process during physiologic muscle adaptation to standard exercise. In rodents skeletal muscle, acute endurance exercise activates autophagy at the gene and protein level (He et al. 2012). In contrast in human muscle upon acute exercise, the content of autophagosomes decreases, suggesting that other degradative pathways are involved for the maintenance of human muscle functions in response to acute resistance exercise or that autophagosome degradation process is faster than its formation process (Fritzen et al. 2016).

4. Aging

Reduction in lean body mass and concomitant increase in fat mass are among the most striking and consistent changes associated with aging. Skeletal muscle and bone mass are the principal components of lean body mass that declines with age. Loss of skeletal muscle mass occuring during aging is called sarcopenia (Evans 2010). The etiology of sarcopenia is multifactorial and complex. Factors that are implicated for sarcopenia include: decreased physical activity level, decreased androgen concentrations, specific nutritional deficiencies (dietary protein and vitamin D), chronic inflammation, insulin resistance, and a number of other factors. Loss of muscle results in a decrease in strength, metabolic rate, and aerobic capacity and thus in its global functional capacity. Loss of skeletal muscle associated with aging begins relatively early and continues until the end of life. Both muscle hypotrophy (decrease in muscle fibers size) and hypoplasia (decrease in fibers number) occur during aging (Lexell et al. 1983).

Anyway, the biology of sarcopenia remains elusive. Various mechanisms are proposed to explain the change in total muscle mass observed, including:

(a) lack of regular physical activity (‘‘use it or lose it’’);

(b) change in protein metabolism (a deficit between protein synthesis versus protein degradation). Inadequate nutrient intake and low protein synthesis are common in older adults and they have been proposed as factors responsible for lower muscle strength in people with sarcopenia. Beyond the reduced protein synthesis during aging there is an age-related accumulation of non-functioning proteins, not efficiently removed from the muscle, that could increase the amount of non-contractile material in muscle, which might explain why muscle strength declines to a greater degree than total muscle mass in sarcopenia (Marcell 2003).

(c) Alterations in the endocrine milieu (decrease in growth hormone (GH) and testosterone and an increase in cortisol and cytokines), since aging is characterized by an increase in inflammatory markers and its related factors. Aging-related inflammation in the absence of infection is low-grade, chronic and systemic, resulting in inflammatory responses that contribute to degeneration of tissue. Some cytokines contribute to a predisposition to sarcopenia by triggering the ubiquitin–protease system (Mitch et al 1996).

(d) Loss of neuromuscular function (denervation versus reinnervation). Muscle loss age-related is linked to degenerative changes, like changes in fibers innervation. The gradual loss of motoneurones, taking place during aging, affects fiber type. Larsson et al. suggested a preferential loss of type II fibers with aging, starting potentially in early adulthood. Type II fibers show selective atrophy (with a preservation of type I fiber area) with aging (Larsson 1983).

(e) Altered gene expression. Evidences showed that sarcopenia might be affected by a genetic predisposition (Willems et al. 2017).

(f) Apoptosis and other factors may also contribute in part to sarcopenia.

Moreover, another cause of sarcopenia that has been proposed is the failure of satellite cell activation in the muscle (Ryall et al. 2008).

Part IV: Small GTPase protein RhoA

1. General characteristics

Ras homolog family member A (RhoA) belongs to the mammalian Rho-like GTPases proteins. Rho family proteins constitute one of the five major branches of rat sarcoma (Ras) superfamily that has been shown to regulate a wide spectrum of cellular functions. The Rho family of small GTPases currently consists of over 20 GTP-binding protein members divided into 6 subfamilies based on their primary aminoacid sequence, structural motifs and biological function. These include the RhoA-related subfamily, the Rac1-related subfamily, the Cdc42-related subfamily, the Rnd subfamily, the RhoBTB subfamily and the Miro subfamily. The RhoA related subfamily is composed of RhoA, RhoB and RhoC members that exhibit significant aminoacid sequence identity (~85%) (Wennerberg et al. 2004).

RhoA gene was discovered for the first time in 1985 by Madaule et al. (Madaule et al. 1985). The striking evolutionary conservation among distant eukaryotes from the mollusc to human suggested that it has important biological function.



Figure 19: Phylogenetic tree of the Rho- family GTPases and representatives of other Ras-superfamily GTPases (Wennerberg et al. 2004)

In human and in mouse RhoA small GTPase protein is encoded by the *RhoA* gene. The gene is located on chromosome 9 in mouse and consists of four exons. These small monomeric GTP-binding proteins usually have between 190 and 250 residues and measure 20–30 kDa in size.

A B

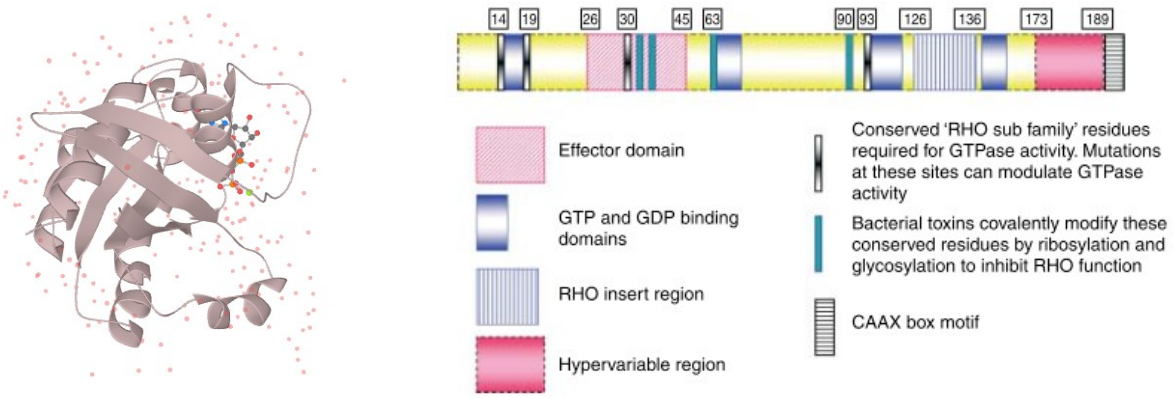

Figure 20: A) RhoA protein cristallography B) Core structure of RHO GTP binding proteins domain

The available functional and structural data show that Rho–GTP-binding proteins are made-up of an effector domain, four separate guanosine phosphate binding regions that span the length of the core structure, a hypervariable region and a CAAX box motif (C, Cys; A, aliphatic residue; X, any residue). The effector domain (residues 26–45) changes conformation between the GTP bound and GDP bound states. All Rho proteins have conserved residues at Gly14, Thr19, Phe30 and Gln93 which are required for GTP binding and hydrolysis. Modifications of Gly14 to Val and Gln63 to Leu result in inactivation of GTPase activity. The N-terminus region also contains switch 1 (residues 27–40) and switch 2 (residues 59–78) regions which change their conformation between GTP- and GDP-bound states and may facilitate changes in effector region required for binding to downstream targets. The hypervariable region, made-up of residues 173–189, is the region of most diversity between distinct Rho family members. It may contain sites for palmitoylation and one polybasic region which can determine cell membrane association. The C-terminal CAAX box motif dictates post-translational prenylation, proteolysis and carboxymethylation of the prenylated Cys residue. The conserved residue Asn41 within the Rho effector region undergoes inhibitory ADP ribosylation by C3 transferases (Lartey et al. 2009).

RhoA is ubiquitously expressed, in almost all the tissues. At cellular level its localization is cytoplasmic, but in its active state translocates to the membrane (Bros et al. 2008).

2. Regulation

Like all members of the Ras superfamily, the Rho GTPases function as **molecular switches**, cycling between an inactive GDP-bound state and an active GTP-bound state. Like all members of the Ras superfamily, the activity of the Rho GTPases is determined by the ratio of their GTP/GDP-bound forms in the cell. Although the Rho switch itself is straightforward, it is very carefully regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). They regulate the ratio of the two forms. In fact, the GEFs enhance the exchange of bound GDP for GTP and the GAPs increase the intrinsic rate of hydrolysis of bound GTP. The conformation of the GTP-bound protein results in increased binding affinity for downstream effector proteins. In addition, the Rho-like GTPases are regulated further by guanine nucleotide dissociation inhibitors (GDIs), which can inhibit both the exchange of GTP and the hydrolysis of bound GTP. GDIs confer dissociation of RhoA from the membrane and maintain it in the cytosol. GDIs also inhibit nucleotide exchange and GTP hydrolysis of RhoA (Bros et al. 2008). Rho GTPases subcellular location is critical for their functions and depends on C-terminal lipid modifications and sequences, and as well as on RhoGDIs interaction (Wennerberg et al. 2004). The affinity of RhoA for GDI is also regulated by phosphorylation of a C-terminal Serine in RhoA. Indeed phosphorylation of RhoA by cAMP-dependent protein kinase A (PKA) increases the affinity of RhoA for GDI, enhancing its translocation to the cytoplasm (Lang et al. 1996). Moreover AMP kinase α1 (AMPKα1) phosphorylates RhoA in vascular smooth muscle cells in response to estradiol, leading to RhoA inhibition (Gayard et al. 2011).

Recently has been reported that, in response to EGF, RhoA is directly phosphorylated by ERK and its activity is enhanced (Tong et al. 2016). Moreover, **post-translational modifications** of RhoA residues, that are respectively localized in its switch I and switch II regions, may give rise to RhoA activity changes. The ribosylation of Rho proteins in the effector-binding domain by C3 transferase (a bacterian ADP-ribosyl transferase), inactivates them and it is prevalently used as an inhibitory approach to study Rho proteins functions. In addition, mutations in RDB binding domain are experimental approaches used to study Rho operational fields. Among the mutations used there are RhoAV14 and RhoAN19, showing respectively stronger or lower affinity of RhoA for target protein.

RhoA activity is also controlled by **protein ubiquitination**: the ubiquin ligase Smurf1 directly catalyzes RhoA ubiquitination, leading to RhoA degradation and consequently to a deregulation of lamellipodia formation (Wang et al. 2003).

The regulation of RhoA occurs also at the **transcriptional level**. A recent paper discovered Myc–Skp2–Miz1 transcriptional complex critical for RhoA gene expression and for the regulation of cell

migration (Chan et al. 2010). Another group discovered, for the first time, binding site for NFkB and STAT6 on RhoA human promoter. In response to cytokine, their binding regulates RhoA expression (Goto et al. 2010). Finally Gilkes et al. identified on *RhoA* gene hypoxia-inducible factors 1 (HIF) binding site which interaction induces RhoA expression and enhances focal adhesions formation in human breast cancer cells (Gilkes et al. 2014).
Moreover there are several **microRNAs** shown to regulate RhoA expression in response to various stimuli in given cells (Kim et al. 2018).

3. RhoA signalling pathway

The RhoA signalling pathway is central to the mechanotransduction because it plays a key role in regulating the actin cytoskeleton and its response to mechanical force.
The cytoskeleton consists of three types of filaments: actin microfilaments, microtubules and various types of intermediate filaments. Cytoskeleton is not a static scaffold supporting cell shape: indeed, it is a very plastic structure. Actin filaments are polymers of repeated units of the actin protein. Based on the cells demand, they can exist in a monomer state (globular actin) or polymeral state (filamentous actin). The polymerization of actin filaments drives many types of cell extension. When actin filaments are highly cross-linked, they can give rise to a relatively rigid cell cortex. However, cytoskeleton can be rapidly remodeled to allow cell protrusion and changes in cell shape (Lessey et al. 2012). Cytoskeleton rearrangement is rapidly influenced by cell-cell crosstalk and soluble external factors. Upstream of the Rho family, only a few elements have been defined. Pathways leading to the cytoskeletal changes are initiated by agonists that bind to a variety of receptors. Each of the three following molecules, Bradykinin, Bombesin and LPA bind to a serpentine-type receptor that is linked to a heterotrimeric G protein in order to activate respectively the formation of filopodia, ruffles and stress fibers (Zigmond 1996). G-proteins are associated with G-protein-coupled receptors (GPCR). The multiple functions of GPCR receptor are mediated by heterotrimetric G-protein (composed by three subunit: alpha, beta and gamma) and by the small GTPases proteins that are monomeric. It has been shown that GPCR physically interacts with small GTPase proteins to mediate downstream signal (Dong et al. 2013). The α subunits of heterotrimeric G proteins are classified into four subfamilies based on the homology of their amino acid sequences: Gs, Gi, Gq, and G12. The G12 subfamily is composed of Gα12 and Gα13. GPCR-dependent activation of RhoA is primarily mediated through the Gα12/13 heterotrimeric proteins (Suzuki et al. 2009). These cell surface receptors act like an inbox for messages in the form of light energy, peptides, lipids, sugars and proteins. For example, **LPA** (lipid lysophosphatidic acid) is an

important serum component involved in maintaining microtubules stability. Among the intracellular signalling pathways, LPA involves the small GTP-binding Rho protein (Cook et al. 1998).

In fibroblasts, extracellular stimuli have been shown to activate the Rho GTPase cascade at different points. Progresses have been made in establishing the connection between growth factor receptors and Rho-like GTPases. Under serum-free condition, Rho activity is decreased and Integrins are not significantly clustered, therefore focal adhesions are not readily detectable. The addition of LPA or serum activates Rho within minutes to generate contractile force, leading to clustering of Integrins and formation of focal adhesions and stress fibers (Ren et al. 1999).

In contrast, there is an adhesion-dependent downregulation of Rho following activation by LPA or serum (Ren et al. 1999). Cell adhesion to ECM seems being able to activate Rho. This point is controversial because it is difficult to decouple Rho activity changes from the structural contributions of ECM, both important for the formation of focal adhesions.

Several lines of evidence suggest that Rho is also regulated by **Integrin-mediated cell adhesion** to ECM (Schwartz et al. 1996). Integrin stimulation enhances stress fibers and focal adhesions formation in serum-starved Swiss 3T3 cells, indicating that Rho is activated by Integrins (Barry et al. 1997). Moreover, Rho protein inhibition or activation, inhibits or activate respectively the Integrin-mediated MAP kinase (Renshaw et al. 1996). Therefore, Integrins are mechanosensitive receptors that trigger rapid cytoskeletal remodelling and they are able to adapt cellular stiffness upon applied tensional force. Recently, it has been shown that Rho is required for Syndecan-4-mediated adaptive cell stiffening (Chronopoulos et al. 2020).

Increase in RhoA activity could be due to GAP inhibition or GEF activation. In particular it has been shown that mechanical forces through Tensin on Integrin promote FAK activation and MEK/ERK pathway downstream in order to activate GEF and RhoA activity. On the contrary, it has been shown that in cells exposed to rigid environments GAP proteins activity is decreased, contributing to the elevated activity of RhoA. However, an important role is played by the nucleus, that it is considered as a physical structure connected to the cytoskeleton and that contributes to mechanotransduction (Lessey et al. 2012).

Newly formed focal adhesions generate signals to downregulate Rho in order to prevent excessive formation of focal adhesions. Downregulation of Rho may also play a role in the disassembly of focal adhesions required for cell migration. The actin cytoskeleton and microtubules suppress Rho activity possibly by controlling the localization of GAPs.

Recentely, it has been described evolutionarily conserved actin-binding protein, called STARS (STriated muscle Activator of Rho Signalling), that is expressed specifically in cardiac and skeletal muscle cells and that activates Rho-signalling events (Arai et al. 2002).

Principal direct downstream effectors

Upon GTP binding, RhoA exerts its function through downstream effectors. The principal Rho effector molecules are ROCK, mDia, Rhotekin, Rhophilin, PKN and Citron.

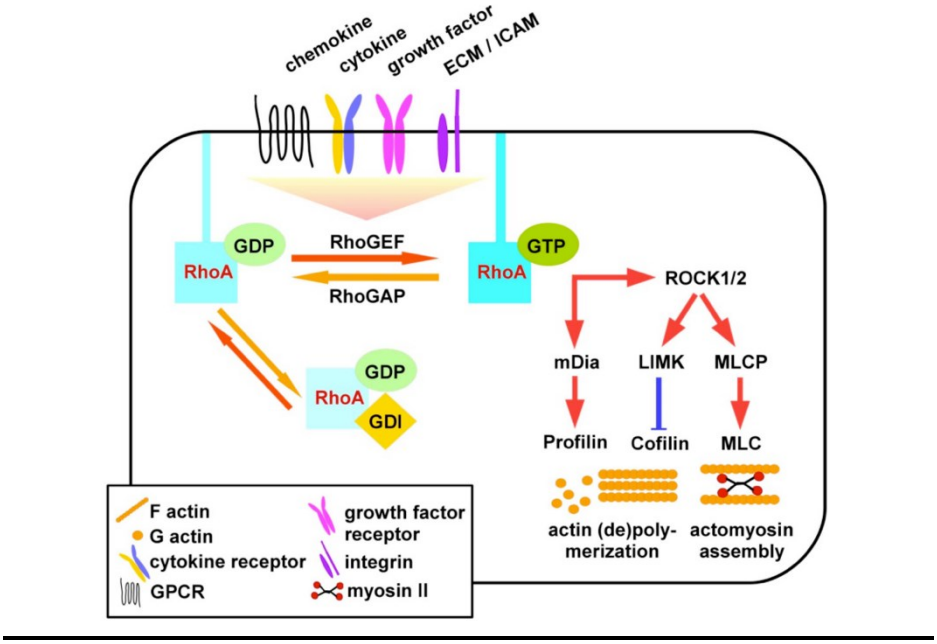

Figure 21: Principal RhoA signaling pathway

ROCK. RHO-associated coiled-coil containing protein Kinase is one of the most important RhoA effector. Rho-kinase is a serine/threonine kinase. It is composed of an N-terminal catalytic domain, a central coiled-coil domain and a C-terminal PH domain interrupted by a Cys-rich region. Rho-kinase requires both N- and C-terminal extension segments in addition to the core catalytic domain for its activity. Rho activates Rho-kinase by binding to the C-terminal portion of the coiled-coil domain (Amano et al. 2010). Other small GTPases besides Rho - Rnd3/RhoE, Gem and Rad - can bind Rho-kinase outside of the Rho-binding region and can inhibit its function (Riento et al. 2003a). There are two isoforms of ROCK in mammals named ROCK1 and ROCK2. So far, several functional differences between ROCK1 and ROCK2 have been reported. ROCK1 is specifically cleaved by caspase-3, whereas ROCK2 is cleaved by Granzyme B (Coleman et al. 2001; Sebbagh et al. 2005). RhoE preferentially binds ROCK1, but not ROCK2, whereas the myosin phosphatase target subunit (MYPT) binds only ROCK2 (Komander et al. 2008; Wang et al. 2009) . Gene silencing experiments also suggest different cellular functions for these two proteins: ROCK1 appears to be essential for the formation of stress fibers, whereas ROCK2 appears to be necessary for phagocytosis and cell contraction, depending on MLC phosphorylation (Yoneda et al. 2005).

Two principal compound inhibitors are used to investigate ROCK functions: Fasudil and Y-27632 both act in an ATP-competitive manner.

Principally ROCK activated by RhoA-GTP, phosphorylates myosin bind subunit of myosin phosphatase (MYPT1) enhancing the phosphorylation of Myosin light chain (MLC) (Kimura et al. 1996). Therefore MYPT1 phosphorylation at two inhibitory sites, resulting in a decrease in MYPT1 phosphatase activity and an increase in phosphorylated MLC. It has been shown that phosphorylation of MLC induces a conformational change in myosin, thereby increasing its binding to actin filaments and subsequently the formation of stress fibers (Riento et al. 2003).

Moreover, *in vitro* ROCK is able to phosphorylate directly MLC (Amano et al. 1996). The amount of myosin phosphorylation depends mainly on the balance of two enzymes: the Ca^{2+} -dependent MLC kinase (MLCK) and MLC phosphatase (MLCP). In smooth muscle cells, ROCK phosphorylates MLC in Ca^{2+}-free condition, and is essential for the cell contractility (Swärd et al. 2000).

Thus, the main function of ROCK signalling to form and to maintain stress fibers and focal adhesions is mediated by MLC phosphorylation, but Rock phosphorylates also another target: LIMK. *In vitro* study evidenced the LIMK site, phosphorylated by ROCK (Ohashi et al. 2000). Rock-mediated LIMK phosphorylation results in increased Cofilin phosphorylation and in turn its inactivation. Cofilin is an actin-depolymerizing factor and regulates actin dynamics. Its inactivation brings to the stabilization of actin filament. In conclusion, Rho-kinase seems to induce and to maintain stress fibers by increasing contractility via MLC phosphorylation and by stabilizing actin filaments through LIMK activation that results in Cofilin phosphorylation. LIMK2 (but not LIMK1) signalling, downstream of RhoA and ROCK, regulates astral microtubule organization. Both LIMK1 and LIMK2 are key regulators of the centrosomes and they provide functional links between the actin cytoskeleton and spindle dynamics (Heng et al. 2012).

In addition, RhoA/ROCK signalling promotes migration by limiting inappropriate protrusions at the rear of migrating cells and perhaps by reducing the formation of new Integrin adhesion complexes (Worthylake et al. 2003). Recently, it has been shown that Rock phosphorylates Par-3 blocking Par complex formation and in turn modulating cell polarity (Nakayama et al. 2008).

Rho-kinase is highly expressed in the nervous system, implying an important role in neuron and/or glial cells architecture. In addition, Rock has an important role also during both the progression of the cleavage furrow formation and the disassembly of intermediate filaments, during cytokinesis (Amano et al. 2010).

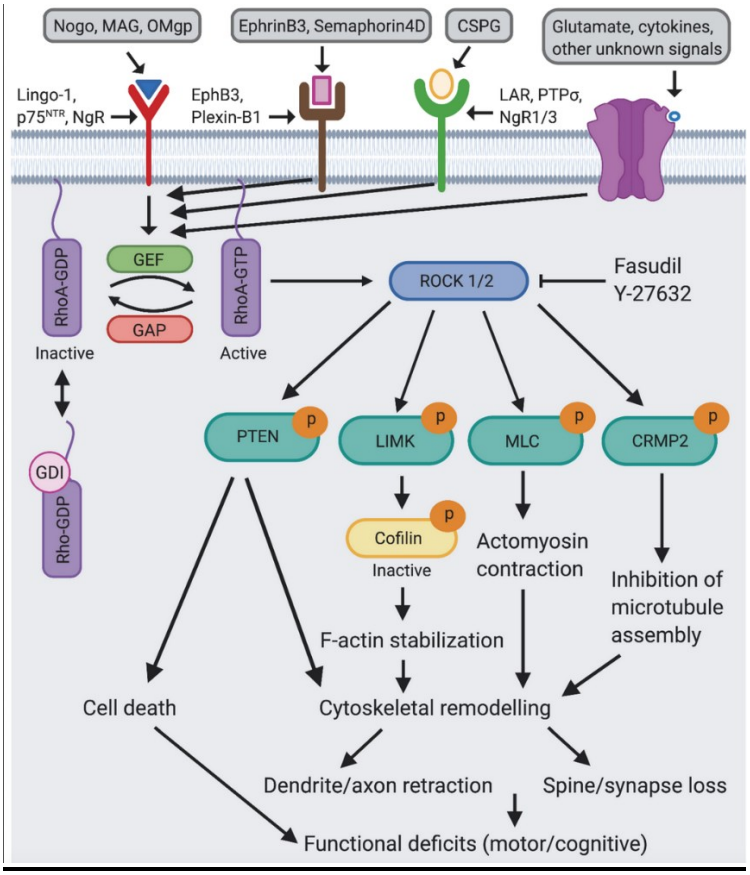

Figure 22: RhoA-ROCK axis downstream target

mDIA: mammalian Diaphanous downstream effector of Rho was discovered in 1997 as homolog of Drosophila. It binds selectively RhoGTP-bound form in order to regulate actin filament formation (Watanabe et al. 1997). mDia belongs to the family of Formin related protein: the C-terminal part is the actin assembly apparatus and contains three structural and functional elements: (a) the profilin-binding FH1, (b) the actin-binding FH2, and (c) the diaphanous autoregulatory domain (DAD). The N-terminal part is the regulatory region that mediates intramolecular interactions with the C-terminus to maintain Formins in an autoinhibited state. In the N-terminus part there is the GDB domain, which directly binds Rho- family GTPases in the GTP-bound state. Moreover, it conteins also other three distinct structural domains, a) the diaphanous inhibitory domain (DID), which binds the C-terminal autoinhibitory DAD segment and also interacts with Rho GTPases; b) the dimerization domain (DD); and c) a coiled-coil (CC) region (Goode et al. 2007). mDia is prevently involved in actin filament polymerization. It binds directly to barbed ends of actin filaments and promotes burst polymerization (Higashida et al. 2007). Recently, mDia is involved in the regulation of microtubules, as evidenced by its direct interaction with them causing their stabilization (Bartolini et al. 2008). mDia in cooperation with LIMK is required to induce Srf activity in neuronal cell lines, underling the importance of RhoA/mDia pathway to regulate the transcription factor

activation (Geneste et al. 2002). mDia is also involved in filopodia formation when it is a target of other Rho proteins (besides RhoA) (Goh et al. 2012). In a Rho-independent way, mDia exhibits a mechanosensitive actin nucleation (Higashida et al. 2013).

Between the biological functions involving mDia, there are also endocytosis and phagocytosis. Brandt et al. showed the importance of mDia to bind cytoskeleton protein and to regulate the actin filament assemblying at the phagocytic cup formation (Brandt et al. 2007). mDia, in colocalization with RhoA, is essential in mammalian cell cytokinesis (Watanabe et al. 2008). Although the majority of actin filaments at the cleavage furrow originates from pre-existing filaments, it is likely that some *de novo* actin polymerization is required. Maybe this is the role of mDia (Kato et al. 2001).

Study on p140mDia1 null mice evidenced a defect T-cell development and function, suggesting a crucial role for mDia1 in the dynamic regulation of the actin cytoskeleton in activated T-cells (Eisenmann et al. 2007).

PKN: is a serine/threonine protein kinase that has a catalytic domain homologous to protein kinase C (PKC) family members and an unique regulatory region containing antiparallel coiled-coil (ACC) domains (Mukai 2003). The PKN enzyme family includes three isoforms enzymes isolated as PKNα, PKNβ and PRK2/PKNγ. PKN is the first identified serine/threonine protein kinase that can bind to small GTPase Rho and that can be activated by themselves (Shibata et al. 1996). It can also be activated by unsaturated fatty acids such as arachidonic acid, linoleic acid and oleic acid *in vitro* (Mukai 1994). PKN is involved in several cellular functions including cell-cell adhesion of keratinocytes (Calautti et al. 2002) and tumoral cell line cell migration (Lachmann et al. 2011). Moreover, PKN induces smooth muscular cells migration and proliferation by regulating vascular remodeling (Singh et al. 2012).

PKN increases glucose transport (Standaert et al. 1998) and plays a crucial role in controlling the mitotic timing of *Xenopus* egg cycling (Misaki 2001). However, other functions *in vivo* remain to be elucidated.

Rhophilin: is a RhoA target that specifically binds GTP state of Rho protein. It was first cloned in 1996 (Watanabe et al. 1996). To date, two Rhophilin isoforms, Rhophilin-1 and Rhophilin-2 are known in mammals (Thumkeo et al. 2013). Rhophilin1 is highly expressed in germ cells of the mouse testis and localizes in the principal piece of the sperm tail (Nakamura et al. 1999). Given its tissue specific expression, the function of Rhophilin-1 is difficult to dissect and remains relatively unknown. In human, Rhophilin-2 may function normally in a Rho pathway to limit stress fiber formation and/or to increase the turnover of F-actin structures in the absence of high levels of RhoA activity (Peck et al. 2002). Neither Rhophilin-1 nor Rhophilin-2 alter RhoA-induced SRE activity.

Rhothekin: it was identified for the first time in 1996 as a potent RhoA effector sharing a significant homology to the Rho-binding domains with PKN and Rhophilin (Reid et al. 1996). Considering its high interaction with Rho, Rhotekin Rho Binding Domain was used to affinity-precipitate cellular GTP-Rho (Ren et al. 1999). Currently, there are two known isoforms of Rhotekin, i.e. Rhotekin1 and Rhotekin2. In contrast to the ubiquitous expression of Rhotekin1, Rhotekin2 expression is apparently limited to a particular lineage of lymphocytes (Collier et al. 2004). To date, there is no report about the role of Rhotekin *in vivo*. Experiments *in vitro* evidenced a crucial role of Rhotekin in the central nervous system. It has been shown that Rhotekin is required for maintenance and for survival of neurons. Moreover, it positively regulates differentiation and neurite outgrowth (Iwai et al. 2012) underling its importance for the neurite outgrowth regulation (Yuasa et al. 2012). In rat embryonic fibroblast, Rhotekin was identified as a candidate regulator organizing Septin structures (Ito et al. 2005). Finally in cancer cell line, Rhotekin seems to have an antiapoptotic effect mediated by the activation of NfKb target genes (Liu et al. 2004).

Citron: identified as a novel partner for GTP-bound form of Rho protein in 1995, Citron kinase has extensive homologies with Rho proteins effectors already known (Madaule et al. 1995). *Citron* deficient mice display defect in neurogenesis: a massive apoptosis and an abnormal cytokinesis impair the development of central neural system. The phenotype of the *Citron* kinase knockout mice, although severe, is not lethal (Di Cunto et al. 2000). There are 2 isoforms of Citron: the longer form, Citron-K with kinase domain and the shorter form, Citron-N without kinase domain (Madaule et al. 1998). During cytokinesis, Citron kinase colocalizes with phosphorylated MLC at the cleavage furrows. Citron phosphorylates MLC directly at both sites (whereas ROCK affects these sites indirectly through phosphorylation and inhibition of MLC phosphatase). This data suggest that Citron kinase is involved in regulating bi-phosphorylation of MLC during cytokinesis (Yamashiro et al. 2003). Eda et al. too, evidenced the localization of Citron kinase at the cleavage furrow and its interaction with RhoA in order to manage the actin cytoskeleton rearrangement during HeLa cell division (Eda et al. 2001). The inhibition of Citron kinase, but not ROCK, blocks cytokinesis in HeLa cells. On the contrary, recent work performed on Drosophila showed that Citron ortholog does not need RhoA interaction to localize to the cleavage site during cytokinesis (Bassi et al. 2011). Moreover this function is principally mediated by coiled coil domain that is critical for its localization and its function during cytokinesis (Watanabe et al. 2013). On the other hand, Citron-N localize at the Golgi organelle, being essential for its maintenance and acting in a ROCK dependent manner (Camera et al. 2003).

4. RhoA-related function

Rho GTPase plays crucial roles in various cellular processes. Members of the Rho subfamily were involved primarily in the regulation of cytoskeletal organization in response to extracellular growth factors. Currently, they are known to be involved in diverse cellular events such as membrane trafficking, transcriptional regulation, cell transformation, cell cycle progression, chemotaxis, cell polarization, cell migration, cell-cell adhesion and development. Their function is mainly determined by their level of expression, their state of phosphorylation, their subcellular localization and their binding to GTP.

a) Cytoskeleton organization

The actin cytoskeleton of animal cells is composed of actin filaments and many specialized actin-binding proteins (Zigmond 1996). Actin cytoskeleton can be assembled in different patterns: a) if it is assembled in long bundles, F-actin supports finger-like protrusions of the plasma membrane known as filopodia. Filopodia are finger-like protrusions that contain a tight bundle of long actin filaments in the direction of the protrusion. They are found primarily in motile cells and neuronal growth cones. b) If it is assembled as a meshwork, it supports sheet-like protrusions known as ruffles or lamellipodia. Lamellipodia are thin protrusive actin sheets that dominates the edges of cultured fibroblasts and many motile cells. Membrane ruffles observed at the leading edge of the cell result from lamellipodia that lift up off the substrate and fold backward. c) If it is assembled in bundles coupled to attachment plaques, actin stress fibers exert force against the substratum. Actin stress fibers are bundles of actin filaments that traverse the cell and are linked to the ECM through focal adhesions. Focal adhesions are the regions where stress fibers are anchored to the plasma membrane and where the cell adheres most tightly to the substratum. The cytoplasmic components of focal adhesions include cytoskeletal proteins such as Actinin, Vinculin, and Talin in addition to signalling molecules such as the Focal Adhesion Kinase (FAK).

Therefore, it is important that the polymerization and the rearrangement of cortical actin are tightly regulated. GTPase proteins control both the formation (actin polymerization) and the spatial organization (filament bundling) of actin filament.

In particular RhoA promotes actin-myosin contractility and thereby the formation of stress fibers and focal adhesions, regulating cell shape, cell attachment and cell motility. Rac1 promotes lamellipodia formation, which are curtain-like extensions composed of thin protrusive actin sheets at the leading edge of migrating cells. Cdc42 causes formation of filopodia which are thin, finger-like cytoplasmic extensions that contain tight actin bundles and might be involved in the recognition of the extracellular environment (Wennerberg et al. 2004).

Actin polymerization in eukaryotic cells occurs through the coordinated activities of proteins that regulate filament severing and filament capping. The two major actin polymerization factors are Arp2/3 and Formin. Arp2/3, activated by Rac and Cdc42, acts via WAVE (a WASP family protein) and WASP, and initiates a branched filament network. Whereas Formins are activated by Rho and promote linear elongation of actin filaments thanks to profilin/actin complex that acts at barbed ends (Jaffe et al. 2005).

LPA-induced stress fiber formation is mediated by Rho (Ridley et al. 1992). Ridley and Hall have shown that RhoA is essential to coordinate the assembly of focal adhesions and stress fibers. (Wennerberg et al. 2004). This role was evidenced for the first time in 1990 on fibroblast since the treatment with recombinant RhoA or a constitutively activated mutant, has induced the formation of stress fibers (Paterson et al. 1990). Moreover in 1992, Ridley and Hall discovered that the ends of the newly formed actin stress fibers induced by Rho were associated with the focal adhesions (Ridley et al. 1992). However, a recent paper shows that RhoA function is redundant with related RhoB and RhoC GTPases for the actomyosin regulation in mouse primary fibroblast (Melendez et al. 2011).

Cytoskeletal rearrangements play a crucial role in numerous processes such as cell motility, cytokinesis and phagocytosis, for which the assembly/disassembly of peripheral actin filaments is used to promote localized changes in the structure of the plasma membrane. Indeed, the mechanisms of phagocytosis used by the complement receptor is mediated by Rho (Caron et al. 1998).

Actin cytoskeleton participates in the regulation of cell morphology (in terms of shape and polarization), in the regulation of cell behavior (such as proliferation) and in the regulation of cell movement. Concerning this last point, RhoA was described to control monocyte transmigration regulating cytoskeletal dynamics (Worthylake et al. 2001).

Similar to actin filaments also the microtubules with their intracellular organization contribute to cell polarity, morphology and division. So, RhoA through mDia stabilizes microtubules in the cell (Palazzo et al. 2001). Therefore, in response to external cues, Rho GTPases contributes to morphogenesis by regulating both the actin and microtubule cytoskeletons and in turn cell-cell adhesion complex assembly.

Several lines of evidence indicate that the Rho-family GTPases are implicated in adherens junction assembly. Fuchs's lab has demonstrated that the intercellular junction formation is preceded by filopodia formation. And the adhesion junction clusterization is catalyzed by active and dynamic process driven by actin filament polymerization (Vasioukhin et al. 2000).

Rho proteins are also involved in regulating secretion, pinocytosis and clathrin coat-mediated endocytosis. It is possible that the effects of Rho GTPases on phospholipid metabolism may provide a point of intersection between the coordinated control of membrane flow and cytoskeletal organization.

b) Nuclear transcription activation

The serum response element (SRE) is found in many promoters including those of genes encoding cytoskeleton components, particularly actin. The transcription factor Srf acts at the SRE in complex with two eventual co-factors: (a) the ternary complex factor (TCF) regulated by the Ras/MAP kinase pathway and (b) the myocardin-related transcription factors (MTRFs) regulated by Rho. This last assertion was demonstrated in 1995 by Hill et al. that described Srf as a target of Rho signalling (Hill et al. 1995). Specifically, RhoA mediates SRE activity in response of serum stimuli. Moreover ROCK, the direct target of RhoA, stimulate the transcriptional activity of c-fos serum response element in order to regulate cytoskeleton organization (Chihara et al. 1997). Recently, it has been shown that Rho-actin signalling regulates the subcellular localization of the Srf coactivator MAL. MAL/MRTF (myocardin-related transcription factors) is expressed in a wide range of embryonic and adult tissues (Wang et al. 2002). MAL binds to monomeric G-actin via RPEL domains. The actin polymerization results in MAL nuclear translocation, suggesting that MAL is sensitive to decreased cellular G-actin levels (Guettler et al. 2008). Rho-mediated changes in the actin cytoskeleton promote this translocation, allowing MAL-Srf interaction and promoting target genes activation (Miralles et al. 2003; Posern et al. 2006).

Actin polymerization occurs not only in the cytoplasm but also in the nucleus. Staus et al. have demonstrated that RhoA cellular localization is related to MRTF activity and that mDia, a direct target of RhoA, regulates nuclear actin dynamics (Staus et al. 2014).

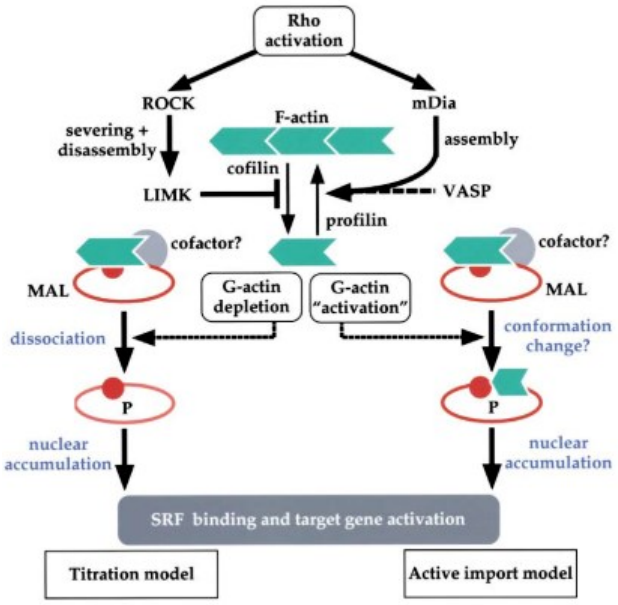

Figure 23: The Rho-actin signal pathway and models for MAL nuclear accumulation

Dependently to F-actin, RhoA is able to regulate Sox9 activity. Kumar et al. have demonstrated that RhoA-mediated modulation of actin polymerization regulates the ability of the transcription factor Sox9 both to activate chondrocyte-specific markers and to maintain its own expression in chondrocytes via a positive feedback loop (Kumar et al. 2009).

It is well known that RhoA activation is required for **YAP/TAZ** activation in order to regulate the maintenance of stress fibers (Dupont et al. 2011). A nice work showed that the exposure to a stiff environment leads cells to establish a mechanical connection between the nucleus and the cytoskeleton. Exerted through focal adhesions, the force reaches the nucleus and extends nuclear pores in order to facilitate molecular transport and directly drives YAP nuclear translocation (Elosegui-Artola et al. 2017). Recently, it has been shown in human gastric cancer cell line that YAP, as a transcriptional co-activator, upregulates the expression of ARHGAP29, a Rho GTPase activating protein. ARHGAP29 is a known regulator of the RhoA-LIMK-cofilin pathway and its overexpression causes actin filament depolymerization (Qiao et al. 2017).

Rho GTPases also affect gene transcription through signal transduction pathways not involving the actin cytoskeleton. RhoA binds MEKK1, a mitogen-activated protein kinase kinase kinase (MAP3K) and their association regulates cellular function (Gallagher et al. 2004). The Rho-like GTPases are implicated in the activation of the nuclear transcription factor NF-κB. In addition, activation of NF-κB by Rho proteins is induced by its translocation to the nucleus and in particular Rho proteins target IκBα phosphorylation (Perona et al. 1997).

Recently, Messina's laboratory evidenced the increase of RhoA activity during embryonic myogenesis and its decrease in foetal stage. Moreover, RhoA overexpression in fetal myoblast

blocks Nfix expression, an important transcription factor essential for skeletal muscle development, defining RhoA/ROCK-ERK signalling as one of the major signalling pathways that regulates the temporal progression of prenatal myogenesis (Taglietti et al. 2018).

c) Other functions

Rho proteins stimulate fibroblast **proliferation** and cell cycle progression (Olson et al. 1995), principally suppressing cyclin-dependent-kinase inhibitor p21Waf1/Cip1 and enhancing the entry in S phase (Hecht et al. 1975). More recently on fibroblast cell line it has been shown that inhibition of Rho blocks ERK activity and cyclin D1 production (Welsh et al. 2009).

5. RhoA and myogenesis *(in vitro)*

The first study to investigate the role of RhoA during skeletal myogenesis, was conducted in the 1990s by Carnac et al. on C2.7 myoblasts cell line. For the first time they discovered a link between RhoA protein activity and the expression of the key muscle regulator MyoD. They found that RhoA is required for the expression of MyoD. When they blocked RhoA activity, using the general inhibitor lovastatin or more specific antagonists of RhoA proteins such as C3-transferase or dominant negative RhoA constructs, they showed a dramatic decrease in MyoD protein levels and promoter activity. Moreover RhoA-dependent transcriptional activation of MyoD requires functional Srf, suggesting that RhoA promotes expression of myogenic transcription factors via activation of Srf (Carnac et al. 1998). The same year another laboratory supported the concept that RhoA signalling, via Srf, serves as an obligatory muscle differentiation regulatory pathway (Wei et al. 1998). In addition to these studies, several reports have confirmed the requirement of RhoA in the promotion of skeletal myogenesis, as evidenced by the fact that it regulates myotube formation, expression of myogenic markers and accumulation of plurinucleated myotubes (Nishiyama et al. 2004; Takano et al. 1998).

Moreover, RhoGTPases are also involved in regulation of critical switch to decide the adipogenesis-myogenesis fate. Truly, in absence of p-190-B RhoGAP (Sordella et al. 2003) the adipogenic differentiation of cultured mouse embryo-derived fibroblasts (MEFs) is blocked, being more prone to myogenic differentiation. These data provide the evidence that p190-B RhoGAP is a regulator of levels of Rho activation that, in response to insulin/IGF signaling, is necessary for adipogenesis-myogenesis fate determination.

Another team two years later showed that the overexpression of GEF protein in differentiated C2C12 cells promoted skeletal myogenesis and increased myotube formation. Moreover it was able to inhibit the adipogenic program of adipocyte cell line (Bryan et al. 2005).

However, other studies in cultured cells suggest that RhoA activity must be perfectly regulated in a finely coordinated time-dependent manner to ensure appropriate skeletal muscle formation. Additionally, conflicting and controversial roles of RhoA signalling, in myogenic differentiation, have underlined the complex regulation of Rho in skeletal muscle differentiation. In order to elucidate the role of RhoA in myoblast fusion, rat myoblast expressing constitutively activated RhoA, were induced to differentiate. Even if the expression of specific muscle genes was not affected and the differentiation process was enhanced, myotube formation was inhibited (Meriane et al. 2000).

In mouse C2C12 myoblast, RhoA activity decreases in a biphaisc manner during myogenic differentiation (Nishiyama et al. 2004). In the same work, RhoAV14 stable transfected C2C12 are not able to form myotubes, although differentiation marker genes expression is not affected. Moreover, RhoA negatively regulates myoblast fusion via ROCK that in turn regulates FOXO1a localization. Several studies have demonstrated that when Foxo1 is phophorylated by Rock, it can not interact with MRTF-A/Smad1/4. In this way this complex remains associated to the Id3 promoter, enhancing its gene expression (Iwasaki et al. 2008). Furthermore, during C2C12 differentiation, RhoA mRNA level, protein level and activity decrease. However C3 transferase treatment (RhoA activity inhibition) on myocytes, affects myotube morphology (maybe in a Rock independent manner) but it does not influence cells differentiation (Castellani et al. 2006). If the C3 transferase treatment takes place in proliferating myoblast MyoD expression is blunted, suggesting a role of RhoA in maintaining myoblast identity.

The role of RhoA as an important regulator of myoblast fusion is confirmed in several experiments. Indeed, the expression of a constitutive active RhoA cause a decrease and a delocalization of M-cadherin protein, required for the fusion process, enhancing its degradation (Charrasse et al. 2006). Moreover the transiently decrease of RhoA expression at the onset of fusion process is mediated by RhoE, an essential regulator of myoblast fusion (Fortier et al. 2008). Cadherin–mediated cell-cell adhesion plays an important role in skeletal myogenesis and Cadherin-dependent adhesion is required for a variety of cellular events during skeletal myogenesis. Using C2C12 myoblast cells, Charrasse et al. have demonstrated that N-cadherin-dependent cell-cell adhesion increases RhoA activity and induces the activity of a- skeletal actin, MyoD and myosin light chain 1A promoters. These data potentially link cell-cell adhesion with downstream signalling of RhoA, namely Srf, to induce muscle-specific gene expression of MyoD and other proteins required for skeletal myogenesis (Charrasse et al. 2002).

Recently RhoA-ROCK mediated MLC phosphorylation is described as a downstream event of the Ca^{2+} channel Piezo1 activation. The Piezo1-mediated Ca^{2+} influx promotes RhoA/ROCK-

dependent phosphorylation of MLC2 and subsequent actomyosin assemblies, thereby controlling cell fusion and the polarized elongation of multinucleated myotubes (Tsuchiya et al. 2018).
In C2C12, RhoA was described as a central mediator of the mechanotrasduction during skeletal muscle differentiation. Indeed upon cyclic mechanical stretch, RhoA activity increases and it is regulated by β1D-integrin (Zhang et al. 2007).
Recently it has been described a novel mechanism of regulating RhoA activity by ROS (Aghajanian et al. 2009). In myoblast, RhoA was shown as a mediator of mitochondrial ROS–triggered F-actin accumulation during plasma membrane repair, a crucial cellular pathway for myofibers homeoastasis after local injury. Indeed mitochondria are able to repair damaged cell membrane without producing new ATP, but through calcium removal and ROS production, activating RhoA and initiating F-actin remodeling at the site of plasma membrane injury (Horn et al. 2017).

6. RhoA *in vivo*: development

Skeletal myogenesis is a multistep process temporally and spatially regulated in the embryo. *Trio* knockout mice provided evidences that Rho GTPases are involved in the process of myogenesis during murine embryogenesis. In these animals, Trio-mediated activation of Rho family GTPases is blocked. Indeed, in absence of Trio (a GEF protein), late embryonic development is impaired, the secondary myogenesis is absent and the embryo die at birth (O'Brien et al. 2000).
The ability of the Rho family of GTPases to orchestrate actin-filament rearrangements lets us think that it has a potential role in morphogenesis. Brakebusch observed that RhoA-null mice die during embryogenesis (Pedersen et al. 2012). Already in 1993, was shown that the sperm-induced cytoplasmic division of Xenopus embryos was inhibited by microinjection into the embryos of a RhoA-GDI, affecting the cytoplasmic division through the actomyosin system (Kishi et al. 1993).
RhoA is important for the mouse blastocist formation. Kono et al have shown that embryo treated with ROCK inhibitor fail in inner cell mass (ICM) formation, evidenced in particular by the involvement of Hippo signalling (Kono et al. 2014). Supporter confirmation of the importance of RhoA during development came from experiment of *ROCK* deficient mice that died *in utero* before 9.5 days post-coitum, mostly as a result of vascular defects (Kamijo et al. 2011).
In vivo mouse model is useful to understand better RhoA involvement in development. Mice with a tissue-restricted deletion of RhoA have revealed interesting cell type specific differences in RhoA function. Herzog et al. published that embryo missing RhoA in neuroepithelium, so in the spinal cord from E10.5, died at late embryonic stages, never observing live-born mutants. In particular in their experiment, RhoA is important for a correct cell proliferation and adhesion (Herzog et al. 2011). RhoA is also important for the apical contriction occurred during lens pit invagination. Its

ablation in lens epithelium results in a more open shape of the lens pit due to reduced contraction at the apical side. Moreover, RhoA Trio-mediated activation plays a key role in the apical localization of Shroom3, an actin-binding cytoskeletal protein (Plageman et al. 2011). In 1997 an interesting experiment conducted in Drosophila has associated RhoA mutation with the loss of cell polarity, proposing that RhoA, like Dishevelled (Dsh), acts downstream of Frizzled receptor (Strutt et al. 1997). More recently mice strains with specific deletions of *RhoA* in the central nervous system showed RhoA importance for apical-basal polarity (Katayama et al. 2011). Surprisingly cardiomyocyte-restricted deletion of the *RhoA* had no effect on heart development and consequently it does not affect mouse viability. However it causes increased infarct size and myocardial damage in an ischemia/reperfusion (I/R) model (Xiang et al. 2011).

The different phenotype observed in different tissues could be related to the concomitant expression of other Rho members that have RhoA redundant functions.

7. RhoA *in vivo:* skeletal muscle tissue

Very few data are published about the role of RhoA in skeletal muscle *in vivo*, principally because muscle specific *RhoA* knock-out mouse model are not available.

Given the RhoA implication in mechanotrasduction signalling, it has been shown in *Plantaris* rat that RhoA mRNA and protein expression increase upon chronic functional overload (McClung et al. 2003). In addition, it has been demonstrated that also during disuse and recovery of rat *Plantaris* muscle, RhoA mRNA and protein decrease upon suspension and increase upon reload. In parallel, the RhoA localization changes too, being cytosolic during disuse and becoming membranous during the reload (McClung et al. 2004). Moreover, for the first time Chockalingam et al. showed that, during muscle atrophy, RhoA activity, and its protein are not at all detectable. Its strong decrease occurs in parallel with dystrophin-glycoprotein complex (DGC) decrease, suggesting the importance of RhoA to mediate its signalling (Chockalingam et al. 2002). In double knockout *dystrophin/utrophin* mice, RhoA expression increases in skeletal muscle stimulating osteogenic potential rather than adipogenic potential of the muscle derived stem cells. By treating mice with ROCK inhibitor, muscle regeneration is ameliorated (Mu et al 2013). More recent data have linked the increase of ROCK activity to the increase of insulin sensitivity, suggesting that the short-term exercise increases both insulin sensitivity and glucose tolerance, through the increased Rock mediated IRS1 phosphorylation (Ser 632/635), in rats (Muñoz et al. 2018). Recently the same group asserted that the *soleus* muscle rat showed the greatest ROCK activity (Muñoz et al. 2020). ROCK activity could be related to fiber type as suggested by Koo et al. that founded an increase of oxidative fibers following the deletion *in vivo* of upstream activator of ROCK. They showed that

NFAT activity was suppressed by ROCK phosphorylation and that this event could affect myofiber reprogramming upon exercise and metabolic disease (Koo et al. 2017).

Additionally, in human *quadriceps* muscle biopsies, eight weeks after hypertrophy-stimulating resistance training, RhoA mRNA and protein level were found significantly increased, underling the involvement of RhoA signalling in response to changes in muscle loading. Moreover, data on human muscles upon bed rest confirming inactivation of RhoA signalling pathway in atrophy condition (Mahmassani et al. 2019).

A recent paper has shown the involvement of Wnt4-RhoA signalling axis for the maintenance of SCs quiescence, showing that the heterozygote disruption of RhoA in SCs leads to an increase of SCs proliferation and early activation.(Eliazer et al. 2019). However further investigation are required to fully elucidate the signalling pathway involved.

8. RhoA *in vivo*: cardiac muscle

The role of RhoA in heart has been investigated by several groups. Conflicting results have been obtained. On one hand in rat cardiomyocites has been shown that RhoA inhibition critically influence mechanical stress–induced hypertrophic responses (Aikawa et al. 1999). Moreover transfection of neonatal rat ventricular myocytes with RhoA antisense oligonucleotide attenuated stretch-induced FAK and ERK1/2 phosphorylation, demonstrating that RhoA/ROCK signalling plays a crucial role in coordination of cytoskeleton reassembling upon stretch (Torsoni et al. 2005). *In vivo,* ROCK inhibition, achieved by Fasudil drug administration, blunts the hypertrophic response to angiotensin II of coronary artery supporting the involvement of RhoA/ROCK signalling in development of hypertrophy *in vivo* (Higashi et al. 2003). On the other hand, recent studies have shown no inhibition of pressure overload-induced hypertrophy in mice in which ROCK1, the RhoA target suggested to mediate hypertrophy in neonatal rat ventricular myocytes, is genetically deleted (Shi et al. 2008). However in *ROCK1–/–* mice the fibrosis in the myocardium is reduced in parallel with a reduced expression of a variety of ECM proteins (Zhang et al. 2006).

Moreover, the importance of RhoA is associated to vascular system in old animals. RhoA expression increases in arteries rat 19 months old and RhoA localizes in the membrane, suggesting an increase of its activity and an involvement of RhoA in age-related responses to vascular change (Miao et al. 2001).

Objectives

The aim of my project is to understand the role of RhoA in skeletal muscle plasticity. RhoA expression has been shown decreased by hindlimb suspension and increased by reloading in rat skeletal muscle (McClung et al. 2004) or upon surgical ablation induced overload (McClung et al. 2003). Data on human muscles upon bed rest confirm inactivation of RhoA signalling pathway in atrophy condition (Mahmassani et al. 2019). This data suggest that *in vivo* RhoA activation respond to mechanical stimuli and mechanical force transmitted and mediated by cell-matrix and cell-interaction (Lessey et al. 2012). However, nobody has investigated in details RhoA pathway and its downstream effectors involved in mouse hypertrophy model. In order to carry out the project objectives, we generate a genetic mouse model in which RhoA is deleted in a specific and inducible manner in the adult myofibers. We are going to describe the phenotype of mutant muscle in hypertrophic condition and to propose the possible mechanism underlying the mutant defective growth.

Additionally during embryonic myogenesis, it has been shown that RhoA is important to regulate the switch between embryonic and foetal myoblast, although its role is not fully elucidate (Taglietti et al. 2018). In order to clarify RhoA function, we delete RhoA in embryonic progenitor and we describe before and after birth the mutant phenotype obtained. Preliminary data that we attain will be useful to report the role played by RhoA during embryonic development and in later stages of muscle growing.

Finally *in vitro,* it has been shown that RhoA activity increases upon mechanical stretch on C2C12 myotubes (Zhang et al. 2007). These data suggest that RhoA controls mechanical stretch response in myotubes. Moreover, RhoA has to be finely regulated to have a correct differentiation and fusion process highlighting its crucial role in myogenesis (Castellani et al. 2006; Charrasse et al. 2006). The mentioned *in vitro* study was performed on myoblast cell line, but no work was conducted on primary cells. So, we generated a mouse model in which RhoA is deleted in specific and inducible manner in adult satellite cells. We are going to study mutant satellite cells behaviour in *ex vivo* culture condition and following acute injury *in vivo*. The results carried on will be essential to elucidate the not yet investigated role of RhoA in adult muscle stem cells and to contribute to address the question of RhoA place in skeletal muscle plasticity.

Results

Part I: RhoA in myofibers controls skeletal muscle microenvironment to allow hypertrophic growth.

RhoA within myofibers controls satellite cell microenvironment to allow hypertrophic growth

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Abstract

Adult skeletal muscles adapt their fiber size to workload. Here we showed that RhoA within myofibers is needed for skeletal muscle hypertrophy. The impaired growth was not associated with a protein synthesis defect but rather with altered SCs fusion to the growing myofibers. We evidenced that the most downregulated genes in overloaded mutant muscles as compared to controls are genes involved in extracellular matrix remodelling, like *Mmp9/13*. We showed that their decreased expression in mutants is associated with un-degraded ECM matrix and fibrillar collagen disorganization. Moreover Mmps are functionally required for muscle hypertrophic growth as the inhibition of their activity affected muscle growth by inhibiting SC recruitment and thus phenocopying RhoA mutants. In addition, we showed that the expression of potent chemo-attracting chemokines (*Cx3Cl1* and *Ccl3*) is reduced in mutants upon hypertrophy and is associated with diminution of macrophage infiltration which functional necessity was demonstrated by depletion experiments. Finally, altered Erk1/2 signalling in mutant muscles may participate to the compromised hypertrophic growth. This findings unravel the putative implication of RhoA within myofibers in the building of a permissive micro-environment for muscle growth and for SC accretion through ECM remodelling and macrophage recruitment.

Introduction

Skeletal muscle is one of the most abundant tissue and the most plastic structure in the vertebrate body. The cellular muscle unit is the myofiber which is mainly composed by contractile material. Physiological demands such as exercise or working overload lead to an increase of muscle mass due to a hypertrophic growth of myofibers. In the adult the two major mechanisms, related to muscle growth and to increase of the sarcoplasmic volume are: i) an increase of protein synthesis to add new contractile filaments to pre-existing sarcomere units and ii) the fusion of new nuclei provided from resident muscle stem cells, termed satellite cells (SCs). SCs in response to increased workload, exit the quiescent state. Afterwards they proliferate and subsequently they differentiate and fuse to growing myofibers.

Furthermore, at the cellular level, skeletal muscle tissue homeostasis requires the coordinated function of diverse cell types. Among them there are blood vessels that bring oxygen and nutrients, nerves that support neuro-muscular activity, and connective tissue involved in the extra cellular matrix (ECM) building. During the regenerative process, numerous studies have shown that a network of interactions of SCs with a variety of cell types including immune cells (macrophages for instance), endothelial cells and fibro/adipogenic progenitors (FAPs) orchestrates muscle regeneration. For example, macrophages play various roles during skeletal muscle regeneration. At the early stages of injury, pro-inflammatory macrophages stimulate SCs proliferation and later on, after their conversion into anti-inflammatory macrophages, they support their differentiation and their fusion into myofibers (Juban et al., 2017). However, the contribution of the muscle resident cells other than SC and myofibers to muscle hypertrophy and the role of muscle microenvironment have been poorly studied so far.

In addition, modifications of muscle tissue microenvironment, such as ECM remodelling, occurring upon increase workload have not been fully explored. Three major roles of ECM are: i) basic mechanical support for vessels and nerves and to create scaffold tissue ii) implication in the passive elastic response of muscle contributing to the force transmission iii) localization of embedded molecules such as growth factors and glycoproteins that regulate dynamically cell functions. In addition ECM provides a structural support to the integrity to the SCs niche, physically separating the stem cell pool from other tissue resident cells, and could thus play an important role in SC accretion during hypertrophy. The proteins mostly present in ECM are Collagens, Fibronectin and Tenascin-C among others. Interestingly it has been shown that these proteins are crucial components of satellite cells niche regulating their self renewal (Bentzinger et al., 2013; Urciuolo et al., 2013) and their regenerative potential (Tierney et al., 2016).

The major enzymes responsible for the physiological breakdown of ECM are matrix metalloproteinases (Mmps). One of the most characterized Mmp in SCs and muscle tissue is Mmp9 (Chenette et al., 2016; Rayagiri et al., 2018). Mmp9 depletion is deleterious to muscle mass (Mehan et al., 2011) while constitutive muscle-specific over-expression promotes growth (Dahiya

et al., 2011) supporting the notion that a precise regulation of muscle ECM is necessary during periods of adaptation to ensure optimal muscle growth. Although how ECM is remodelled upon increased workload and how it is regulated remains unclear.

Ras homolog family member A (RhoA) is a small GTPase protein that oscillate between the GTP bound and GDP bound states, regulating a wide spectrum of cellular functions. RhoA controls contractility, via ROCK, actin polymerization and actin cytoskeleton reorganization. RhoA is a mechanosensor present in the cytosol that translate physical forces into biochemical signalling pathways and transmits the signal in the nucleus, activating specific transcription factor (Lessey et al., 2012)(Burridge et al., 2019). Among them RhoA modulate Srf activity by controlling the nuclear accumulation of its coactivator, myocardin-related transcription factor A (Mrtf-A) by actin dynamics (Miralles et al., 2003). In muscle tissue, mRNA and protein expressions of RhoA have been shown to increase upon chronic functional overload and hypertrophy-stimulating resistance training in rat and human, respectively (McClung et al., 2003; Carson et al., 2002). *In vitro*, cyclic stretch activated RhoA in cultures myotubes (Zhang et al., 2007). However, the role of RhoA in skeletal muscle physiology and in the control of muscle mass has not been investigated.

In this study, we assessed the role played by RhoA within myofibers during skeletal muscle hypertrophy by inducing compensatory hypertrophy of *plantaris* muscles harbouring a conditional and inducible deletion of RhoA in myofibers and showed compromised hypertrophic growth in absence of RhoA. The impaired hypertrophic growth was not associated with a protein synthesis defect but rather with altered SC fusion to the growing myofibers. We evidenced that the most downregulated genes in overloaded mutant muscles as compared to controls are genes involved in extracellular matrix remodelling, like *Mmp9/13*. We showed that a decreased expression of these genes in mutants is associated with un-degraded ECM matrix and fibrillar collagen disorganization and that the inhibition of their activity affected muscle growth by inhibiting SC recruitment and thus phenocopying RhoA mutants. In addition we showed that the expression of potent chemoattracting chemokines (*Cx3Cl1* and *Ccl3*) is reduced in myofibers lacking RhoA upon hypertrophy and is associated with diminution of macrophage infiltration which functional necessity was demonstrated by depletion experiments.

All together these data evidence a new role of RhoA within myofibers in the regulation of muscle microenvironment upon overload in order to achieve a correct SC niche and SC behaviour via ECM remodelling and regulation of inflammation and thus permit a correct hypertrophic growth. Understanding the pathways involved in the control of muscle mass, in particular in response to increased mechanical load, may be essential to identify and design treatments able to alleviate loss of muscle mass during disuse or aging.

Results

RhoA is required in myofibers for overload-induced hypertrophy

In order to investigate the signalling pathways that may play a functional role in the control of adult skeletal muscle growth in response to increased load, we performed a transcriptomic study of genes expressed in *plantaris* muscles at the basal state (Sham Operated, SO) and 1 week after overload-induced hypertrophy (OV 1w). We identified genes differentially expressed before and after hypertrophy (fold change 1.2 and p-value < 0.05). Analysis of the Canonical Pathways using a Gene Onthology software (Ingenuity Pathway Analysis) pointed out RhoA signalling (Z-score = 3.71 and -log(p value) = 6,61) among the first 10 pathways ranked by their significance with predicted activation (Z-score ≥ 2) during overload-induced hypertrophy (Table 1). We thus hypothesized that RhoA signalling could have a crucial role during skeletal muscle hypertrophy.

To examine the contribution of RhoA during overload-induced muscle hypertrophy, we generated a mouse model in which *RhoA* is deleted in a specific and inducible manner only in the adult myofiber compartment by injecting tamoxifen to *HSA-Cre^{ERT2}:RhoA$^{lx/lx}$* mice (refered as mutant) and performed overload induced hypertrophy (OV) of the *plantaris* muscle. During the OV procedure control (Ctl) and mutant (Mut) mice were injected with tamoxifen at day 3 and day 6 (Figure 1A). At the steady state (Sham Operated, SO), a significant (60%) RhoA loss was achieved at the protein and transcript levels in different muscles including *gastronemius* and *plantaris* (Figure 1B, 1C). Because different cell types are present in the muscles and myofiber myonuclei represent roughly 50% of the total nuclei (Dos Santos et al., 2020), we checked the expression of *RhoA* transcripts in isolated control and mutant *plantaris* single myofibers. We showed an efficient 85% decrease upon tamoxifen treatment, even more compared to RhoA transcript level in bulk muscle (Figure 1D, SO condition). No differences of muscle weight and myofiber Cross Section Area (CSA) were observed before hypertrophy between control and mutant *plantaris* (Figure 1E, 1F, 1G). Following compensatory hypertrophy procedure, *RhoA* expression increased at the onset of the hypertrophy process (one week after surgery) in muscle tissue and isolated myofibers (Figure 1D). This is in agreement with the increased *RhoA* expression observed in rat muscles overloaded (McClung et al., 2003) and its decreased expression in human muscles upon disuse (Mahmassani et al., 2019), further suggesting an involvement of RhoA in muscle overload-induced hypertrophy. Three weeks following overload-induced hypertrophy, *plantaris* muscle mass and CSA were increased in controls as compared to unloaded muscles (SO Ctl). However, the extent of hypertrophic growth was decreased in mutant *plantaris* muscles. They displayed a 15% increase in muscle weight (after 3w of OV) instead of 30% in controls and no significant increase of their CSA (Figure 1E, 1F, 1G). In addition, the increase in muscle mass was mainly caused by myofiber hypertrophy as the total number of myofibers did not vary after OV (Figure 1H). These data show that RhoA in the myofibers is necessary for optimal overload-induced myofiber hypertrophy and that its absence causes a muscle growth defect.

RhoA is dispensable for protein synthesis control upon hypertrophy

Hypertrophic growth is associated with an increase of protein synthesis and a decrease of protein degradation at the onset of compensatory hypertrophy procedure, and Akt signalling plays a central role in this control by activating mTOR and reducing FoxO-mediated atrogenes transcription (Sandri et al., 2004). We first tested whether the impaired overload-induced hypertrophy of mutant *plantaris* muscles could be attributed to impaired global protein synthesis. The rate of total protein synthesis was measured *in vivo* using the SUnSET technique that relies on the incorporation of Puromycin in newly translated proteins 30 min prior the end of the experiment. We showed a similar increase in both control and mutant muscles one week following overload with no significant difference between the two groups (Figure 2A). We then analysed Akt signalling, and observed no difference in Akt phosphorylation between controls and mutants, with a six fold increase upon hypertrophy (Figure 2B). By phosphorylating FoxO transcription factors, Akt activation prevents their nuclear localisation and the transcription of their target genes, among which the atrogenes encoding *MuRF1* and *Atrogin-1* (*MAFbx)* ubiquitin ligases. In line with the activation of Akt activity, *MuRF1* and *MAFbx* expressions decreased in a similar manner in control and mutant *plantaris* 1 week following compensatory hypertrophy and came back to basal levels (SO) after 3w (Figure 2C). Altogether these data suggest that the absence of RhoA in the myofibers does not affect protein synthesis and degradation pathways upon overload-induced hypertrophy.

Loss of RhoA in myofibers impairs SC behaviours and in particular their fusion

In addition to the increase of the cytoplasmic volume depending on protein content, compensatory hypertrophy of the muscle relies on the accretion of new nuclei through the mobilization of satellite cells. We hypothesized that RhoA in the myofibers may control the behaviour of satellite cells under overload-induced hypertrophic conditions. We investigated whether RhoA loss altered the number of SCs expressing Pax7 and not displaying a deletion of *RhoA*. Before overload, the number of Pax7+ SCs was identical between controls and mutants (Figure 3A, SO). One week after OV, there was a significant increase in Pax7+ SCs in both genotypes, but the number of SCs was slightly lower in mutant *plantaris* muscles. We further assessed the proliferative potential of SCs by determining the number of SCs that entered S-Phase (*in vivo* EdU injection 24h and 4h prior the end of the experiment – Pax7+EdU+ cells). Consistent with total SC quantification, the number of Pax7+EdU+ proliferating SCs was lower in mutant muscles one week after OV (Figure 3B). However the proportion of proliferating SCs (Pax7+EdU+/Pax7+ cells) was not significantly affected (Figure 3C). Overall, following an increased load, RhoA in myofibers may slightly affect the proliferative and/or the activation potential of SCs.

We next investigated whether *RhoA* deletion in the myofiber could affect the myogenic differentiation potential of SCs and their fusion to the mutant myofibers. Upon OV, *Myogenin* transcript levels did not differ between control and mutant *plantaris* muscles, with a sharp increase one week post OV that came back to SO levels after 3 weeks of OV (Figure S1). Thus the loss of RhoA within myofibers did not affect the engagement of SCs in myogenic differentiation. Then, the fusion potential of SCs to the growing myofibers was assessed by counting the number of myonuclei within the dystrophin-labelled myofiber sarcolemma (Figure 3D). The number of myonuclei per myofiber was similar before overload between control and mutant and significantly increased 1 week and 3 weeks after compensatory hypertrophy in controls and to a lesser extend in mutant muscles 3 weeks after OV (Figure 3E). Indeed, in mutant *plantaris*, the number of myonuclei were significantly decreased as compared to controls 1 and 3 weeks after overload (Figure 3E).

To further assert the impaired fusion capacities of satellite cells to the mutant myofibers *in vivo*, we chronically injected mice subjected to compensatory hypertrophy with EdU and we tracked EdU+ nuclei incorporated into growing myofibers (Figure 3F, 3G). Three weeks after overload, the percentage of EdU+ myofibers was significantly reduced in mutants compared to controls (Figure 3G). These data suggest that within myofibers RhoA is needed for an efficient mobilisation of SCs during hypertrophy by controlling their number and their recruitement to the growing myofibers. The impaired behaviour of SCs in absence of RhoA could explain, at least in part, for the impaired growth observed in mutant.

RhoA control of SC behaviours is environment dependent

To investigate whether the altered behaviours of SCs, especially cell fusion, in RhoA-deleted muscles subjected to compensatory hypertrophy are cell autonomous and unrelated to the local environment and/or mechanical stimuli, we set up an *in vitro* assay. This assay was designed to assess whether the absence of RhoA within myotubes (Adeno-Cre-mCherry transduced $RhoA^{lx/lx}$ myotubes) affects fusion with RhoA-expressing mononucleated myocytes (expressing GFP) (Figure S2A, S2C). Surprisingly the lack of *RhoA* expression in myotubes (Figure S2B) did not affect the ability (dual-labelling) of myocytes to fuse to myotubes *in vitro* (Figure S2D). These data suggest that the defect on SC recruitment to *RhoA* mutant myofibers observed *in vivo* relies on muscle environment linked to the *in vivo* overload context, conditions that cannot be reproduced *in vitro*. This highlights the importance of mechanical cues and *in vivo* microenvironment in the control of muscle compensatory growth. To further analyse how RhoA in the myofibers may control the hypertrophic growth, we focused on *in vivo* studies.

Mmps and chemokines expression is affected in absence of RhoA within myofibers

In order to decipher the molecular mechanisms underlying the growth defect of RhoA-deleted muscles, we performed a genome wide microarray analysis of gene expression from control and mutant *plantaris* muscles, before and one week after overload-induced hypertrophy procedure. Gene expression profiling evidenced a massive change in their expressions (SO vs OV 1w), suggesting that an important change in gene expression occurs in muscles upon increased load. Different expression groups were revealed. There are more than 6000 genes up- and down-regulated in control and in RhoA mutant muscles upon overload compared to SO (fold change > 1.2; p-value < 0.05) (Figure 4A). Upon OV condition, 569 genes were differentially expressed between control and mutant *plantaris* (233 genes down regulated and 336 genes up regulated) (Figure 4A). Among the 233 down-regulated genes in overloaded mutant muscles as compared to overloaded controls, we focused our attention on up-regulated genes in controls after hypertrophy (and to a lesser extend in mutants) (Figure 4B, highlighted by a red box). The thirty most differentially expressed genes of this category (in overloaded conditions, Mut OV vs Ctl OV) are shown in Figure 4C. Among those genes, there are genes encoding proteins involved in extracellular matrix remodelling, like *Mmp9* gelatinase, *Mmp13* collagenase and *Adam8* metalloproteinase, and important chemokines like *Cx3cl1* and *Ccl3* (Figure 4C). Their respective mRNA expressions were evaluated by RT-qPCR and we confirmed that the expression of those genes was strongly induced upon overload in controls and that it was significantly reduced in the mutant (Figure 4D).

To investigate whether these gene expression alterations could be linked to a lack of RhoA in myofibers or from other cell types expressing RhoA present in the muscle, we isolated single myofibers and we checked the expression of these genes in control and mutant myofibers before and following compensatory hypertrophy. In line with the data on bulk RNA, RT-qPCR on isolated single myofibers from *plantaris* muscles one week after hypertrophy showed that *Mmp9, Mmp13, Adam8, Cx3Cl1* and *Ccl3* gene expression levels were strongly increased in controls following hypertrophy and were significantly diminished in mutant overloaded myofibers (Figure 4E).

In addition, we performed a transcriptomic analysis of gene differentially expressed between *RhoA*$^{lx/lx}$ myotubes expressing (Adeno mCherry transduced) or not RhoA (Adeno Cre-mCherry transduced). Strikingly, there was a very little overlap (41 genes) between the genes differentially expressed in myotubes expressing or not RhoA and the genes differentially expressed in control and RhoA mutant overloaded *plantaris* muscles (Mut OV 1w vs Clt OV 1w). Only 25 genes had their expression levels changed in the same direction (up or down) (Figure S3A, Table S1). *Mmp9, Mmp13, Adam8, Cx3Cl1* and *Ccl3* genes were not among them and thus their expression was not affected by *RhoA* deletion in basal culture conditions (Figure S3B, Table S1). Together, these findings further support the notion that the absence of RhoA in the myotubes cannot recapitulate the *in vivo* hypertrophic setting. One parameter that is lacking *in vitro,* as compared to

compensatory hypertrophy *in vivo*, is the absence of mechanical load applied to the myotubes that could be associated with low RhoA activity. Indeed it has been shown that stretch can activate RhoA in myotubes (Zhang et al., 2007). In order to activate RhoA in standard static culture conditions, we treated myotubes with a RhoA activator (Rho Activator II, CN03), and we monitored gene expression levels after 8h. Importantly the expressions levels of *Mmp13, Adam8,* and *Cx3Cl1* were significantly increased following RhoA activation (Figure S3C). These observations are in line with our data obtained in isolated myofibers (Figure 4). They suggested that at least a part of the impaired expression of these genes *in vivo* can be attributed to the specific absence of RhoA in myofibers and is thus cell autonomous. We hypothesized that the decreased expression of those genes in myofibers upon overload might participate to the incorrect hypertrophic response of mutant muscles.

Pathways impaired by RhoA depletion in overloaded myofibers

To obtain further insights on the pathways that might be impaired by the deletion of RhoA, we focused our attention on the Upstream Regulators predicted activated or inhibited following hypertrophy in control but not in mutant muscles using IPA analysis (Z-score ≥ 2 or ≤ 2, and p-value < 0.05). Several Upstream Regulators were involved in the remodelling of ECM such as Mmp9, Periostin (Postn), Tenascin-C (TnC), Cysteine-rich angiogenic inducer 61 (Cyr61), Lysyl oxidase homolog 2 (LoxL2), Urokinase-type plasminogen activator (Plau) (Table 2). Mmp9 stood as an interesting molecule because: i) it is among the genes whose expression levels were the most decreased in mutants OV as compared to controls OV (Figure 4C) ii) it is among the upstream regulator predicted activated only in the controls and iii) it is connected to the predicted activation/inhibition of other several Upstream Regulators (Table 2). Matrix Metalloproteinases (Mmp) are proteolytic enzymes that hydrolyse components of the extracellular matrix (ECM) participating to ECM remodelling and release many bioactive cytokines and growth factors being entrapped in the extracellular matrix (Page-McCaw et al., 2007). Among the Upstream Regulators listed in Table 2, there are several Mmp9 substrates such as TnC, Fgf, Tnf, Cxcl1 and SerpinA4, and molecules linked to the activity of Mmp substrates such as ItgA9, ItgaV and TgfβR1. For instance the release and activation of the non-matrix cytokines Tnfα and the matrix-associated growth factor Fgf by Mmp9 have been reported in the litterature and consistently their activities were predicted to be activated in our IPA analysis (Z-scores ≥ 2) (Table 2) (Allen, Teitelbaum, & Kurachi, 2003). Moreover the cleavage of TnC by Mmp9 generates integrin-activating peptides that is consistent with a Z-score = 2.42 for TnC, a Z-score = 3.56 for ITGA9 and a Z-score = 2.18 for ITGAV (Fujita et al., 2020). In the case of SerpinA4, its cleavage by Mmp9 inactivates serpin and accordingly its activity was predicted to be decreased (Z-score = -2.17) (Table 2) (Liu et al., 2000).

In addition, several Upstream Regulators, only predicted activated in controls after hypertrophy, were associated with inflammation such as IL3, IL17RA and Gm-csf (implicated in the

functional activation and survival of macrophages) and chemokines Ccl2, Cxcl1, chemokine receptors CCR1 (Ccl3 receptor) and CCR2 (Ccl2 receptor) participating to macrophage recruitment (Table 2).

Finally, several signalling pathways including Erk1 (MAPK3), Jnk (MAPK8), Stat3, Nfkb (RelA and Sn50 peptide) were predicted to be activated in control overloaded *plantaris* and not in mutant muscles (Table 2). We thus checked by Western blot their activity (Erk1/2, Stat3, JNK, RelA) upon hypertrophy in control and mutant muscles. A difference between control and mutant was only observed for Erk1/2 activity, evidenced by its phosphorylation status. Indeed, the increase of P-Erk1/2 following OV is significantly less in mutant muscles as compared to controls (Figure 5A). In order to investigate whether RhoA can activate directly Erk1/2 within myofibers, we treated control myotubes *in vitro* with a Rho activator. Interestingly, a short activation of Rho GTPases (3h) was sufficient to induce an increase of P-Erk1/2 in a dose dependent manner (Figure 5B) suggesting that the decrease of P-Erk1/2 observed in the overloaded mutant muscles could be partially attributed to the lack of RhoA in myofibers.

Altogether, these data suggest that, upon hypertrophy, RhoA in the myofibers might affect pathways involving extracellular matrix remodelling, Mmps, Erk1/2 and macrophage recruitment/activity.

RhoA in myofiber impairs ECM organization and Mmps activity

Based on the transcriptomic and IPA analyses, we hypothesized that extracellular matrix remodelling and Mmp activity might be affected by RhoA loss in the growing myofibers. First, the global amount of collagen present in *plantaris* muscle upon overload was quantified by PicroSirius Red staining (Figure 6A). We demonstrated that collagen fiber content increased upon overload in control and mutant muscles, however no difference in the global collagen amount was observed between controls and mutants (Figure 6A). To further analyse ECM organization, we investigated the biophysical aspect of ECM performing second harmonic generation (SHG) imaging, allowing the visualization of fibrillar collagen, but not its quantitative assessment, of thick (300 μm) sections of *plantaris* muscles from controls and mutants before and three weeks after overload (Figure 6B). By image analysis, we observed that the fibrillar collagen is less linear and appears more tortuous in mutants after OV compared to controls, as quantified by calculating the ratio of fibrillar collagen length to the Feret's diameter (Figure 6C). Moreover it accumulated between the myofibers rather than surrounding them. These data suggest that in hypertrophic conditions RhoA impacts ECM organization, in terms of tortuosity and assembly.

As Mmps are key proteolytic enzymes that hydrolyze components of the extracellular matrix, we next quantified the protein amount of ECM substrates of Mmp9 and Mmp13 such as Collagen I α 2, Tenascin-C and Fibronectin by Western blot (Figure 6D). We observed that Collagen I α 2, Tenascin-C and Fibronectin protein amounts augmented with overload in both

control and mutant muscles and that there was a further significant accumulation of these proteins in mutant overloaded muscles. Interestingly the sharp increase upon overload in both genotypes was also seen at transcript level indicating that this may be mainly due to increased transcription of *Collagen I α 2, Tenascin-C* and *Fibronectin* genes (Figure 6E). Importantly, there was no difference in transcript level between overloaded control and mutant *plantaris* suggesting that the increase in Collagen I α 2, Tenascin-C and Fibronectin proteins amounts observed in the mutants might be due to their decreased cleavage by Mmp9 and Mmp13 (Figure 6E).

Thus, we could thus speculate that in absence of RhoA, Mmp expression decrease might affect the correct ECM protein hydrolysis and that a wrong ECM rearrangement could impair muscle growth.

Mmps are functionally involved in compensatory hypertrophy

In order to get insights on the functional implication of Mmps in the hypertrophic process, we treated mice using a broad spectrum Mmp inhibitor GM6001 (targeting Mmp 1, 2, 9, 8, 3) and we assessed its effect on compensatory hypertrophy (Figure 7A). One week post-overload, Fibronectin protein amount, a substrate of several Mmps, increased in GM6001-treated muscles, showing the *in vivo* efficacy of the drug (Figure 7B). *Plantaris* muscles of GM6001-treated mice were less hypertrophied three weeks after overload than muscles of vehicule-injected mice as assessed by their decreased weight and CSA (Figure 7C and 7D). Importantly, this altered growth was accompanied by a reduction of the number of myonuclei per myofiber (Figure 7E). Altogether these results show the important role of Mmps to achieve optimal hypertrophy in response to increased load. Mmp inhibition phenocopied the altered growth and SC fusion observed in muscle lacking RhoA and Mmp9/13 expressions were decreased in RhoA mutants upon overload. Our findings suggest that the blunted hypertrophy of *RhoA* deleted muscles may be partially due a decreased Mmp activity.

Mmp processing can release ECM-tethered growth factors and bioactive cytokines. They can also indirectly activate different signalling pathways. Therefore we wondered whether the decreased activation of Erk1/2 observed in RhoA mutant overloaded muscles (Figure 5A) could be partially attributed to the decreased Mmp expression/activity. Importantly, GM6001- and vehicule-treated muscles displayed similar levels of P-Erk1/2 upon overload suggesting that the alteration of Erk signalling is not downstream of the Mmps (Figure 7F).

RhoA in overloaded myofibers impacts macrophage recruitment implicated in muscle growth

In absence of RhoA, we showed a decreased expression of *Ccl3* and *Cx3cl1* by the growing myofibers (Figure 4D and 4E). These two chemokines/chemoattractants are implicated in

inflammation by recruiting and activating macrophages. In addition, IPA analysis highlighted several Upstream Regulators activated in overloaded controls linked to the inflammatory response (Table 2). Therefore, we investigated the number of macrophages (F4/80+) present in the muscle following compensatory hypertrophy. One week after overload, there was a 10-fold increase in F4/80+ macrophage number in control muscles that came back almost to SO levels three weeks after OV, showing a transient and strong inflammatory response in the muscle following mechanical load increase (Figure 8A). Strikingly, the number of macrophages was significantly reduced in mutants compared to controls 1 week post OV (Figure 8A). This prompted us to hypothesize that RhoA in the myofibers may influence macrophages recruitment (by controlling indirectly *Ccl3* and *Cx3cl1* expression levels) in the muscle tissue following mechanical load increase.

It has been shown that macrophages are very important to ensure the proper regeneration of muscle by controlling SC behaviours such as proliferation and fusion (Arnold et al., 2007). To investigate whether macrophages may play a functional role during hypertrophy, we depleted the macrophages by injecting intraperitoneally Clodronate liposomes that induce macrophage death (Figure 8B). At first, we checked macrophage depletion by counting the number the macrophages on *plantaris* section one week post overload. Clodronate injection reduced the number of F4/80+ macrophages by 30% at 1 week and by 50% at 3 weeks post OV (Figure 8C). This partial ablation was sufficient to blunt hypertrophic growth of *plantaris* muscles three weeks after overload as evidenced by the reduced *plantaris* weight in macrophage-depleted mice (Figure 8D). Moreover macrophage depletion led to decreased number and fusion of SCs (Figure 8E, 8F), strongly suggesting that, in hypertrophic conditions, macrophages modulate SC behaviour. These results are reminiscent of those observed in RhoA mutant overloaded muscles, in which SC behaviours are altered (Figure 3), and suggest that RhoA within the myofiber may partially control muscle growth by favouring the recruitment of macrophages in overloaded muscles.

Discussion

Taking advantage of a genetic model that allows the invalidation of *RhoA* in myofibers and not SCs, we demonstrated that RhoA is needed for skeletal muscle hypertrophy and for the recruitment of SCs to the growing fibers. We provide evidence for the implication of RhoA within myofibers in the building of a permissive microenvironment for muscle growth and for SC accretion through ECM remodelling and macrophage recruitment. At the molecular level, we propose that, in response to mechanical load, RhoA controls in a cell autonomous manner Erk1/2 activation, the expression of regulators of ECM such as *Mmp9/13* and of macrophage chemo-attractants such as *Ccl3* and *CX3cl1* (Figure 9).

Hypertrophic growth of skeletal muscle relies on both the increase of net protein content and the addition of new nuclei in the growing myofibers provided by SCs. We showed that, in absence of RhoA, the hypertrophy defect is not accompanied by a deregulation of protein synthesis/protein degradation balance and by a compromised Akt signalling, but mostly by a deficiency of SC fusion. We propose that, upon overload-induced hypertrophy, the altered recruitment of SC to *RhoA* deleted myofibers may be responsible for their impaired growth. Indeed, even if the role of SCs in skeletal muscle hypertrophic growth has been debated, the necessity of competent fusing SCs to have a correct muscle hypertrophy has been clearly demonstrated using genetic models in which SCs are incompetent for fusion. For instance, SC deletion of *Myomaker*, a muscle-specific membrane protein necessary for fusion of embryonic and adult muscle progenitors, compromised muscle hypertrophic response (Goh et al., 2017). In addition, loss of Serum response factor (Srf) in SCs, a master regulator of F-actin scaffold and SC fusion, impaired overload-induced muscle growth and the restoration of the fusion potential of Srf mutant SCs (through maintenance of F-actin architecture) was sufficient to rescue growth (Randrianarison-Huetz et al., 2018).

RhoA is an important small GTPase protein regulating contractility, actin cytoskeleton rearrangement, actin polymerization through its downstream effectors ROCKinases (Rho-associated coiled-coil protein kinases) and mammalian diaphanous protein (mDIA) amongst many others. Moreover RhoA activation responds to mechanical stimuli and mechanical force transmitted and mediated by cell-matrix and cell-interaction (Lessey et al., 2012). In cultured C2C12 myotubes, it has been shown that cyclic mechanical stretch increased RhoA activity (Zhang et al., 2007) suggesting that RhoA participates to the mechanical stretch response in myotubes, activating downstream mediators important to translate environmental cues in biochemical signals. Trying to disclose the molecular mechanism underlying the lack of hypertrophic growth of *RhoA* deleted myofibers, we overlapped the data of microarray experiments performed *in vivo* on overloaded

plantaris control and mutant muscles and the data of microarrays performed on myotubes expressing or not RhoA (respectively Adeno m-cherry transduction and Adeno-CRE transduction of differentiated myotubes). Surprisingly, the intersection set was very little, suggesting that the pathways regulated by RhoA during overload procedure *in vivo* are very different from those operating in myotubes cultured in standard static conditions. None of the following genes *Mmp9, Mmp13, Adam8, Ccl3, Cx3Cl1,* which were differentially expressed between control and mutant overloaded whole *plantaris* muscles and isolated myofibers, had their expression affected by *RhoA* deletion in myotubes. Additionally, we were not able to reproduce the fusion defect of control myocytes with RhoA mutant myotubes *in vitro*. This discrepancy between *in vivo* and *in vitro* settings highlights the need of mechanical inputs to trigger RhoA-dependent muscle hypertrophy. In addition, in response to increased load, RhoA in the myofibers may affect other cell types present in the muscle (FAPs, macrophages), but absent *in vitro*, that may participate to muscle growth. Altogether, this underlines the importance of *in vivo* model to analyse RhoA contribution to muscle hypertrophy.

It has been shown that Rho GTPases, and in particular RhoA, are potent activators of Srf transcriptional activity (Hill et al., 1995; Sotiropoulos et al., 1999). In addition, we have previously shown that Srf within the myofibers is required for muscle hypertrophy in response to workload by exerting a paracrine control on SC recruitment to the growing fibers through Ptgs2/Il4 (Guerci et al., 2012). We wondered whether a decrease of Srf activity could be responsible for the impaired hypertrophic growth of *RhoA* deleted muscles. Several data argue against this possibility. First, our *in vivo* and *in vitro* transcriptomic data on muscles upon overload and on myotubes suggested that RhoA governs downstream signalling pathways independently from Srf, as almost none of the *bona fide* Srf target genes are down regulated when RhoA is absent. For instance, *Ptgs2* (a Srf direct target gene functionally implicated in muscle hypertrophic growth), which expression increases during OV in control muscles and is blunted in Srf mutants, displays a similar expression in control and RhoA mutant overloaded *plantaris*. In cultured myotubes, RhoA deletion did not affect the expression of Srf target genes such as *Egr1*, *Myl9, Cnn2, Ptgs2* and *Acta1* (Guerci et al., 2012; Randrianarison-Huetz et al., 2018). Second, several genes such as *Mmp9, Adam8* and *Cx3Cl1* had their expression augmented following chemical activation of RhoA *in vitro* on cultured myotubes and their expression decreased in overloaded RhoA mutant muscles. Importantly, the expression of those “RhoA responsive” genes remained unaffected by constitutive Srf activation in myotubes (induced by Ad-SrfVP16 transduction) (Guerci et al., 2012). Thus, we propose that the absence of muscle growth of RhoA mutant is likely not due to a decrease of Srf activity and that Srf-independent signalling pathways are operating downstream RhoA upon hypertrophy.

In the present study we showed that among the genes mostly down-regulated in overloaded muscles in absence of RhoA, there are genes involved in the extracellular matrix remodelling, like *Mmp9* and *Mmp13*. In addition, *in vivo* Mmps inhibition by a broad-spectrum Mmp inhibitor GM6001 impaired overload-induced hypertrophy and SC accretion, and phenocopied RhoA mutants. Interestingly, Mmp inhibitor GM6001 was shown to alter SC number following muscle laceration-induced injury (Bellayr et al., 2013). Thus, we propose that *Mmp9* and *Mmp13* decreased expressions may contribute to the altered muscle growth of RhoA mutants by in part modulating SC fusion. Many studies were conducted to evidence the importance of Mmps on muscle growth. Fiber size is decreased in adult hindlimb muscles from *Mmp9* null mice (Mehan et al., 2011) and muscle specific overexpression of *Mmp9* significantly increased skeletal muscle fiber cross-section area, levels of contractile proteins and force production in isometric contractions (Dahiya et al., 2011). Paradoxically, inhibition of Mmp9 was shown to improve Soleus muscle regeneration and to ameliorate development of excessive fibrosis (Zimowska et al., 2012) and continuous unregulated high Mmp9 activity disrupted SC niche through ECM damages (Chenette et al., 2016). In the context of hypertrophic growth, Peterson laboratory recently proposed that Mmp9 levels in muscle fibers should be finely regulated (through miRNA secreted by SCs among others) to have a correct ECM integrity and to facilitate the long-term hypertrophic response, too much or not enough Mmp9 being deleterious (Fry et al., 2020). *Mmp13* null mice did not exhibit any histological or functional muscle deficits, however muscle regeneration and muscle hypertrophy caused by increased IGF-1 were impaired (Smith et al., 2020). Mmp9 and 13 have also been shown to modulate myoblast migration *in vitro* (Lei et al., 2013; Lewis et al., 2000). It is reasonable to assume that the remodelling of ECM matrix by Mmps will enable more efficient movement of myoblasts toward myofibers and could thus contribute to SC recruitment upon growth.

While searching for addition players responsible for the impaired growth of RhoA mutant muscles, we focus our interest on two potent macrophage attractants: *Cx3cl1* and *Ccl3* whose expressions are increased upon overload in controls and not in mutants. Data obtained in control and mutant isolated myofibers and in myotubes treated with a Rho activator further demonstrated that RhoA stimulated the expression of these chemokines in a myofiber cell-autonomous manner. We do not know whether Cx3cl1 and Ccl3 influence SCs directly, but we can strongly relate their decreased expressions to a significant impairment of macrophages recruitment in RhoA mutants. Indeed, compensatory hypertrophy procedure induced a transient accumulation of macrophages in *plantaris* muscle that was blunted in mutants. In human, it has been shown that resistance exercise increases the expression of chemotactic factors including *Cx3cl1* and mobilizes monocytes (Della Gatta et al., 2014; Jajtner et al., 2018; Strömberg et al., 2016) and that macrophages positively correlate with myofibers hypertrophy (Walton et al., 2019). Knowing the multifaceted roles of macrophages during muscle regeneration to support tissue recovery, in particular by promoting SC

differentiation (Chazaud, 2020), we proposed that macrophages may as well participate to muscle hypertrophy by facilitating SC recruitment to myofibers. By depleting macrophages in control mice using clodronate liposomes, we showed that macrophages were required for muscle hypertrophy. Our results are in agreement with those of DiPasquale et collegues in which macrophages depletion also negatively affected muscle hypertrophic growth (DiPasquale et al., 2007). However we provide additional data concerning the positive action of macrophages on SC number and fusion.

It is important to note that the absence of RhoA in the myofibers is sufficient to affect the behaviour of SCs expressing RhoA, highlighting the importance of RhoA signalling in myofibers in the remodelling of SC microenvironment upon hypertrophy. How the satellite cells niche is remodelled during hypertrophy process remain poorly examined. It was already proved that, after increased load, myofibers secrete myokines like Il-4 and Il-6 that are able to influence satellite cells proliferation and fusion (Guerci et al., 2012, Serrano et al, 2008, Horsley et al, 2003). In addition, important transcriptional modifications have been reported shortly after compensatory hypertrophy, this study (1wk) and Carson ‘s study (3 days post OV in rat) (Carson et al., 2002) which concern several genes encoding components of extracellular matrix (such as *Collagens*, *Fibronectin* and *TenascinC*). By affecting the expression of *Mmp9* gelatinase and *Mmp13* collagenase, RhoA might promotes an important global ECM rearrangement and modification of SC niche upon overload that could influence SC behaviour such as fusion. While the global collagen deposition (quantified by the Sirius red staining) was not modified in overloaded mutant muscles compared to controls, we show the protein accumulation of several Mmp9/13 ECM protein substrates such as Collagen1α2, TenascinC and Fibronectin. Moreover, analysis of collagen fibrils structure in overloaded mutant muscles, by second harmonic microscopy, displayed a significant decrease in fibrillar collagen tortuosity as compared to the control. These data suggest that the correct rearrangement of ECM/collagen, rather than its quantity accumulation, is crucial to build a SC microenvironment appropriated for muscle growth and SC fusion. Interestingly, both Tenascin-C and Fibronectin have been shown to remodel and adapt SC niche (Tierney et al., 2016) and to regulate a feedback signal to promote symmetric division and replenish SCs pool (Bentzinger et al., 2013), respectively. In both cases, the proteins are produced by SCs that autoregulate their own niche. Because in our instance SCs are “wild type”, we could speculate that this auto-regulation is not sufficient alone but should be associated with enzyme activity for ECM remodelling meaning from the myofiber. Of note, as macrophages are decreased in RhoA mutant muscles upon hypertrophy compared to controls, on top of a myofibers intrinsic deficit, one part of the defective ECM remodelling occurring in mutants could be attributed to macrophages which also secrete Mmp9 in a context of muscle regeneration (Rahman et al., 2020).

In addition to matrix proteins, Mmps process growth factor and bioactive cytokines entrapped in the matrix releasing them and activating downstream signalling pathway. Among the signalling pathways predicted reduced in RhoA mutant muscles compared to the controls, we show a decrease of Erk1/2 signalling in overloaded mutant muscles. Importantly, this pathway was activated in cultured myotubes treated with a Rho Activator suggesting that RhoA can stimulate Erk1/2 within the myofiber compartment. In line with our data, MAPK/Erk1/2 activation downstream of the RhoA–ROCK signaling pathway was described during osteogenic differentiation in response to rigidity (Khatiwala et al., 2009). We can speculate that the transmission of mechanical signal in myofibers occurs via Rho and Erk1/2 signaling. It was showed in many cells types that Mmp9 was upregulated in response to an activation of MAPK/Erk signaling (Wang et al., 2017; Genersch et al., 2000; Lakka et al., 2002). In keratinocytes it was showed that MEK/ERK signaling was necessary for baseline Mmp9 promoter function (Iyer et al., 2005). Moreover Erk1/2 pathway together with Jnk 1/2 elicit AP-1 activity that is a crucial transcriptional factor for Mmp9 expression (Xia et al., 2015). We could adapt and propose this scenario also for muscle cells. However it remains undisclosed whether in RhoA mutant muscles, Mmp9 regulation could also occur independently to Erk signalling.

In conclusion we have described an important role for RhoA within myofibers in regulating muscle hypertrophic growth and SC accretion. We propose that it is achieved through a modification of SC microenvironment by a fine ECM remodelling and by inflammatory cells recruitment. Our work suggests that RhoA signalling is important to regulate adult muscle stem cells fusion and MMPs function. It will be important in future work to define more comprehensively the molecular pathway involved and the specific cellular contributions.

Materials and methods

Mouse protocols

RhoA$^{flox/flox}$ mice are homozygous for RhoA floxed alleles harboring LoxP sites flanking exon 3 of endogenous *RhoA* gene (Lauriol et al., 2014). HSA-*Cre-ER*^{T2} transgenic mice express Cre-ERT2 recombinase under the control of human skeletal actin promoter (Schuler, Ali, Metzger, Chambon, & Metzger, 2005). Tg :Pax7-nGFP transgenic mice express nuclear localized EGFP under the Pax7 promoter (Sambasivan et al., 2009).

To investigate the effect of myofiber-specific RhoA-deletion in adult muscle, the mouse strain following mice were generated: HSA-*Cre-ER*^{T2}:*RhoA*$^{flox/flox}$. In all experiments, 3-month-old HSA-*Cre-ER*^{T2}:*RhoA*$^{flox/flox}$ mice were given five intraperitoneal (IP) tamoxifen (TMX, 1 mg/day; MP Biomedicals) injections to induce *RhoA* deletion and were referred as mutant mice (Mut). *RhoA*$^{flox/flox}$ mice injected with TMX were used as control mice (Ctl).

No statistical differences in body weights were observed after tamoxifen in HSA-*Cre-ER*^{T2}:*RhoA*$^{flox/flox}$ and *RhoA*$^{flox/flox}$ mice up to 2 months post TMX injection.

Mice were genotyped by PCR using the following primers: Cre-F: 5'-CCTGGAAAATGCTTCTGTCCG-3'; Cre-R: 5'-CAGGGTGTTATAAGCAATCCC-3'; RhoAlox-F: 5'-AGG GTT TCT CTG TAC GGT AGTC-3'; RhoAlox-R: 5'-GCA GCT AGT CTA ACC CAC TACA-3'.

Compensatory hypertrophy (OV) of *plantaris* muscles of control (Ctl) and mutant (Mut) mice was induced through the incapacitation of *soleus* and *gastrocnemius* muscles by sectioning their tendon. This procedure was achieved in both legs. During the process of CH, Mut mice were injected with TMX at day 3, 5 and 7 post OV. At the indicated time (1 and 3 weeks post OV), *plantaris* muscles were dissected and subsequently processed for histological analyses. When indicated mice were administered intraperitoneally with 50 or 20 μg/g EdU (Life Technologies) or with 20 ug/g GM6001 (Merck Millipore).

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 and French Ministry of Research (number A751402).

Macrophage depletion,

Clodronate liposome and control Liposome (Clophosome-A and control liposome anionic) were purchased from ChemQuest. Mice were injected with Clodronate- or control liposomes (0,15 ml administrated intraperitoneally i.p.) 1 days prior compensatory hypertrophy. Additionally, liposomes (Clodronate- or control) were continued to be administrated day 1, 4, 6, 9, 12, 15 and 18 post surgery (0,1 ml, i.p.). The muscle were harvested 7 days or 21 days post compensatory hypertrophy.

SunSET

The SUnSET, or SUrface SEnsing of Translation, technique allows estimation of global protein synthesis in tissue of living animals, specifically involves the use of an anti-puromycin antibody for the immunological detection of puromycin-labelled peptides. Briefly we injected mice with puromycin (Sigma) at 0.04 µ.04/g body mass via an intraperitoneal (IP) 30 minutes before samples harvesting. Western blot was then performed with a mouse anti-puromycin antibody (1/1000 Merck Millipore) and HRP-conjugated anti-mouse secondary antibody. Puromycin incorporation was measured by Fusion FX system (Vilber company) with comassie blue staining as loading control.

Multiphoton imaging and characterization of ECM topography and architecture

Plantaris muscle was excised and placed in 2% paraformaldehyde for 1h at 4°C and then included in agarose low gelling 6%. Muscles was then sliced in thick sections (300 µm) using a vibratome. An microscope,multiphoton Leica SP5 was used to observe collagen fibers by second harmonic generation microscopy, as described (Stearns-Reider et al., 2017). From images, the tortuosity was calculated using Feret's diameter divided by collagen fibrils length (ImageJ software) of single collagen fibrils.

Primary muscle cell culture and adenoviral transduction

Primary cultures were derived from hindlimb muscles of *RhoA*$^{flox/flox}$ *mice* harboring Pax7-*nGFP* transgene that allowed the prospective selection by flow cytometry (Fluorescence Activated Cell Sorting or FACS) of satellite cells as described in (Randrianarison-Huetz et al., 2018). In standard conditions, myoblasts were grown in growth medium (DMEM/F12, 2% Ultroser 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 *RhoA* allele, *RhoA*$^{flox/flox}$ myoblasts were differenciated for 3 days and then transduced with Ad-mCherry or Ad-CREmCherry adenoviruses (100 MOI, Vector Biolabs).

To generate a stable *RhoA*$^{flox/flox}$ cell line expressing GFP, RhoA$^{flox/flox}$ myoblasts were infected twice with the GFP lentiviruses (100 MOI, Vector Biolabs) and 8 mg /ml of polybrene then selected by FACS sorting.

Cell mixing fusion assays

To analyze fusion, myotubes (at day 2 of differentiation) were transduced with Ad-CREmCherryGFP or Ad-mCherry. Two days post transduction, they were co-cultured with GFP expressing myocytes (myoblasts incubated overnight in DM). Two days later, cells cultured were fixed for 8 min in 4% PFA and nuclei were stained using DAPI and then mounted in Dako Fluorescence Mounting Medium and kept at 4°C until image acquisition. Fusion events were

scored by counting the dual-labeled cells (green cells GFP+/ mCherry+). The number of fusion events was normalized by the total number of nuclei.

Muscle section immunostaining

Plantaris muscles were collected and snap-frozen in liquid nitrogen-cooled isopentane. Eight µm-thick muscle sections were fixed in 4% PFA for 8 min at room temperature and blocked in over night at 4°C in PBS 1x, 10% Horse serum, 0.5% Triton X-100. Then, they were incubated with primary antibodies over night at 4°C in PBS 1x, 10% Horse serum, 0.5% Triton X-100. The following primary antibodies were used: mouse anti-dystrophin (Novocastra, NCL-Dys2, 1/50), anti-f4/80 (Invitrogen, MA1-91124, 1/400) or rabbit anti-laminin (Sigma, L9393, 1/200). After washes in PBS 1x, sections were incubated with secondary antibodies for 1 h at room temperature. The following secondary antibodies were used goat anti-mouse IgG1 Alexa 488 (ThermoFisher, A21121, 1/1000) and donkey anti-rabbit Alexa 546 (LifeTechnologies, A10040, 1/1000). Nuclei staining were performed using DAPI. Muscles sections were then mounted in Dako Fluorescence Mounting Medium and kept at 4°C until image acquisition.

For Pax7 staining, muscle sections were fixed in 4% PFA for 8 min at RT and permeabilized in ice-cold methanol for 6 min. Muscle sections were treated with Antigen Unmaking Solution pH 6 (Vector, H-3300) 15 min at 95°C and cooled on ice for 30 min. Blocking and incubation with primary and secondary antibodies were conducted as described in the previous paragraph. Primary mouse anti-Pax7 antibody (Santa Cruz, sc-81648) was used at dilution 1/50.

EdU detection was performed using Click-iT® EdU Alexa Fluor® 647 kit, according to the manufacture's instructions (Life Technologies).

Western blot analysis

Plantaris muscles were lysed in RIPA buffer (Sigma) and proteins were separated through denaturating SDS-PAGE electrophoresis using Mini-Protean TGX precast gels 4-15% (Biorad) and transferred on PVDF or Nitrocellulose (0,2 µm, Biorad) membrane using the Trans-Blot turbo transfer system (Biorad). Membranes were blocked with 5% skinned milk in TBS-1% Tween (TBST) 1h at room temperature and probed overnight at 4°C with primary antibodies in TBST 5% BSA. The following antibodies were used: mouse anti-RhoA (Santa Cruz, sc-418, 1/150), rabbit anti-P-Akt (Cell Signaling, #9271S, 1/1000), anti-CollagenIa2 (Abcam, ab34710, 1/1000) Fibronectin (Sigma F3648, 1/1000), erk (Cell Signaling, #9102, 1/1000), P-erk (Cell Signaling, #9101, 1/1000), rat anti-TenascinC (Invitrogen, MA1-26778, 1/200), and mouse anti-GAPDH (Cell Signaling, D16H11, 1/1000). Following washing in TBST, membranes were hybridized with goat anti-mouse and goat anti-rabbit secondary antibodies coupled to HRP (ThermoFisher, 62-6520 and A27036, 1/10 000). Proteins were revealed using SuperSignal West Femto substrate (ThermoFisher).

Image acquisition

Digital images were acquired using a Olympus BX63F microscope with 10x objective (UplanFL, numerical aperture 0.3) and 20x objective (UPLSAPO, 0.75), ORCA-Flash4.0 LT C11440-42U camera (Hamamatsu), or using a Zeiss Axiovert 200M microscope with 5x objective (PLANFLUAR, 0.25) and 20x objective (LD PLANNEOFLUAR, 0.4), CoolSnap-HQ camera (Photometrics), or using a Spinning Disk Leica DMI6000 confocal microscope with a 100x oil-immersion objective (HCX PL APO, 1.47), CoolSnap-HQ^2 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

Myofiber cross section area (CSA) was analyzed by using immunostaining of dystrophin, marking myofiber sarcolemma, and then using MuscleJ tool (Mayeuf-Louchart et al., 2018). Between 600 and 800 myofibers were analyzed. For the quantification of the number of nuclei per myofibers Image J was used and at least 500 myofibers were counted per muscle.

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent and reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen). cDNA was synthesized from 1 ug of RNA. Quantitative PCR analysis was performed using a Light Cycler (Roche) according to the manufacturer's instructions using a SYBR Green I kit (Roche). Values were normalized using *Hydroxymethylbilane synthetase* (*Hmbs*). The following primers were used: RhoA-F 5'- AACCTGTGTGTTTTCAGCACC-3'; RhoA-R 5'-ACCTCTGGGAACTGGTCCTT-3'; MuRF-1-F 5′-GAGAACCTGGAGAAGCAGCT-3'; MuRF-1-R 5′-CCGCGGTTGGTCCAGTAG-3'; MAFbx-F 5′-GTCCAGAGAGTCGGCAAGTC-3'; MAFbx-R 5′-GTAGCCGGTCTTCACTGAGC-3', Mmp9-F 5'- TCCTACTCTGCCTGCACCACTAAAG-3' ; Mmp9-R 5'- CTGTACCCTTGGTCTGGACAGAAAC-3', Mmp13-F 5'-AGTTGACAGGCTCCGAGAAA-3' ; Mmp13-R 5'-CACATCAGGCACTCCACATC-3' ; Adam8-F 5'- GCAGGACCATTGCCTCTACC-3' ; Adam8-R 5'- TGGACCCAACTCGGAAAAAGC-3' ; Ccl3-F 5'-TTCTCTGTACCATGACACTCTGC-3'. Ccl3-R 5'-CGTGGAATCTTCCGGCTGTAG-3' ; Cx3Cl1-F 5'-ACGAAATGCGAAATCATGTGC-3' ; Cx3Cl1-R 5'-CTGTGTCGTCTCCAGGACAA-3', col1a2F 5'- GTAACTTCGTGCCTAGCAACA-3' ; col1a2R 5'- CCTTTGTCAGAATACTGAGCAGC3' Tnc-F 5'-CACACACCGCATCAACATCC-3' ; Tnc-R 5'-GACGACTTCTGCAGCTTGGA-3', Fibronectin-F: 5'-GGCCACACCTACAACCAGTA-3' ; Fibronectin-R: 5'- TCGTCTCTGTCAGCTTGCAC-3' ; Hmbs-F: 5'-TGCACGATCCTGAAACTCTG-3'; Hmbs-R 5'-TGCATGCTATCTGAGCCATC-3'.

Affymetrix microarrays

Microarray analysis was performed from four *plantaris* muscles coming from four different mice per group. Four conditions (groups) were analyzed : SO Ctl, 1wOV Ctl, SO Mut, 1wOV Mut. Total RNAs were obtained using TRIzol reageant and DNAse treatment (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 plateform (Institut Cochin, Paris). Probe data normalization and gene expression levels were processed using the Robust Multi-array Average (RMA) algorithm in expression Console software (Affymetrix). Gene ontology analysis was performed using Ingenuity (IPA) software. Full data are available on GEO : GSE105125 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE105125.

Statistical analysis

Quantitative data sets were analyzed using an unpaired non-parametric Mann Whitney test , Student t-Test with Welsh correction or a two-way ANOVA with Bonferroni's multiple comparisons test using GraphPad Prism 6 software. Statistical significance was set at a p value < 0.05.

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Figure 1

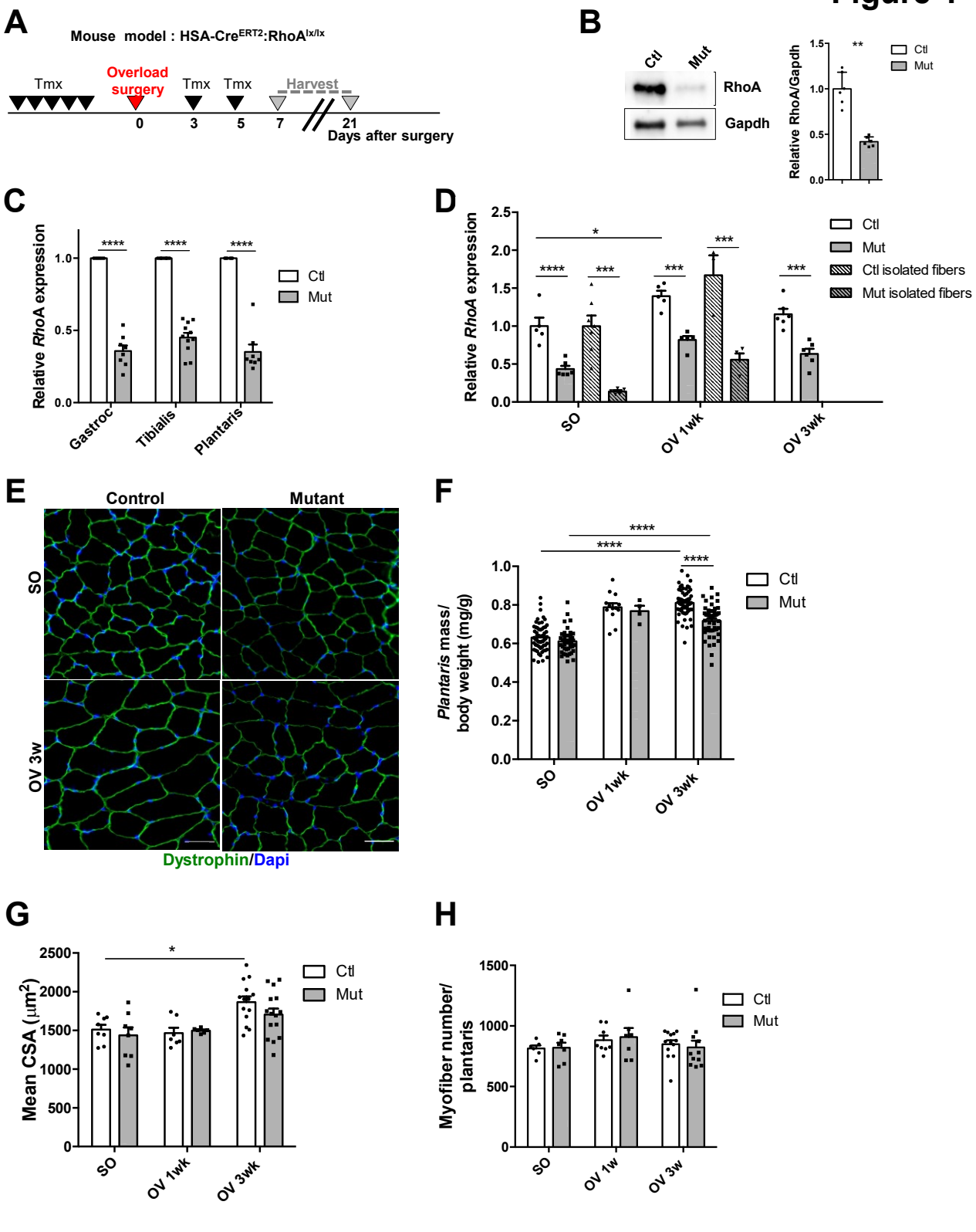

Figure 1. RhoA loss within myofibers impairs overload-induced hypertrophy. **(A)** RhoA mutant mice were injected with Tmx 1 week (wk) before OV procedure. *Plantaris* muscles were isolated 1 and 3 wk after surgery. **(B)** RhoA protein was analysed by Western blot in control and mutant *gastrocnemius* muscles. Gapdh was used as a loading control. **(C)** Analysis of *RhoA* mRNA expression by qRT-PCR in control and mutant *gastrocnemius, tibialis anterior* and *plantaris* muscles. Data were normalized by *Hmbs* expression and relative to control (n=6-11). **(D)** Analysis of *RhoA* mRNA expression by qRT-PCR in control and mutant *plantaris* muscles or isolated myofibers before (SO) and after 1 and 3 wk OV. Data were normalized by *Hmbs* expression and relative to control SO (n=3-7). **(E)** *Plantaris* muscle sections immunostained for Dystrophin (green) and nuclear staining with DAPI for control and mutant mice before (SO) and after 3 wk of OV. **(F)** Ratio of *plantaris* mass (mg) to body weight (g) before (SO) and after 1 and 3 wk OV in control and mutant mice n=5-20 **(G)** Mean CSA before (SO) and after 1 and 3 wk OV in control and mutant mice (n=6-14). **(H)** Mean myofiber number before (SO) and after 1 and 3 wk OV in control and mutant mice. Data and mean ± SEM. *pvalue <0.05, *** pvalue<0.001, **** pvalue<0.0001.

Figure 2

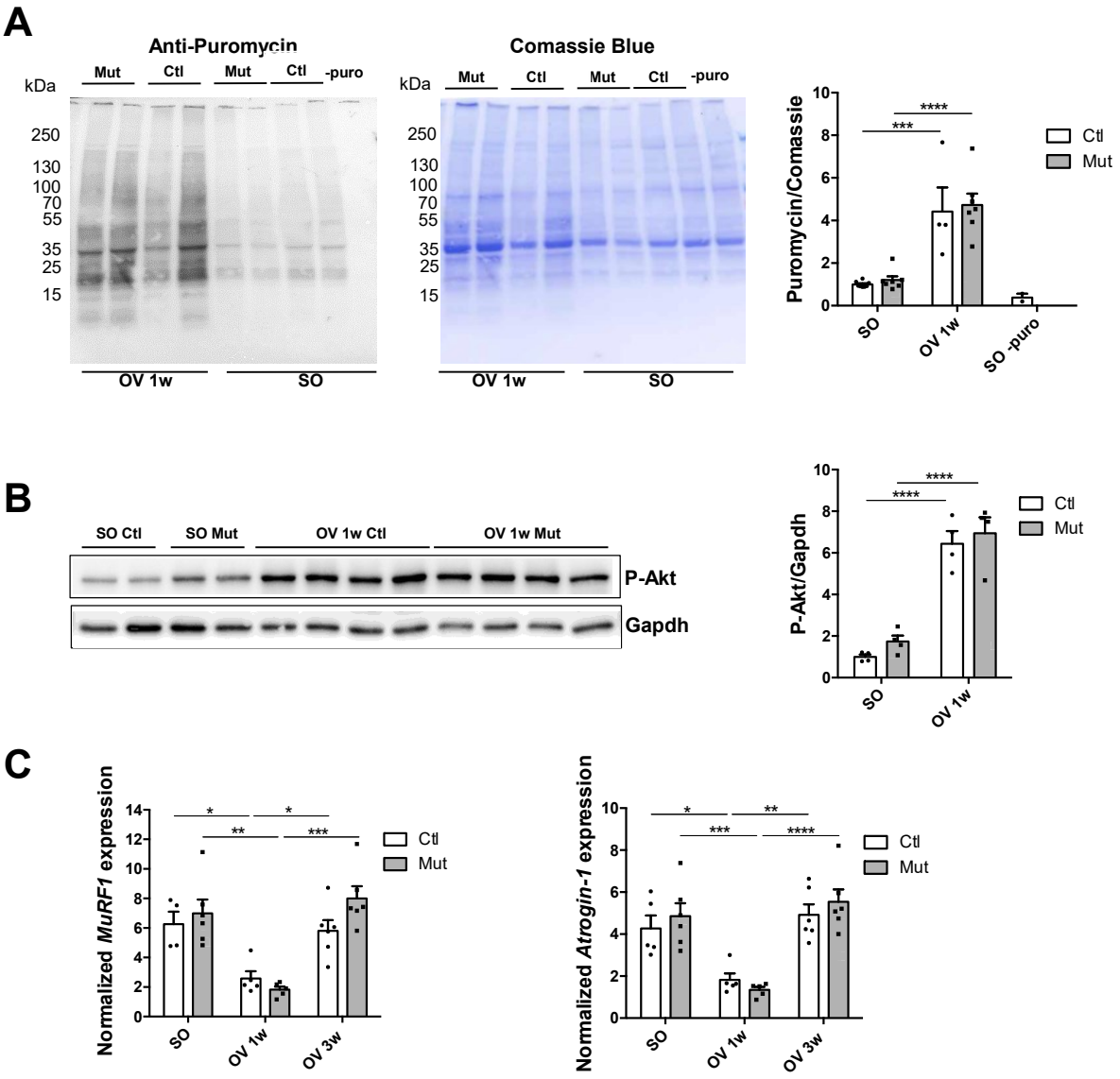

Figure 2. RhoA is not required for Akt dependent signalling and protein synthesis. **(A)** Western Blot SUnSET measurements were performed on control and mutant plantaris muscles before (SO) and after 1wk OV injected with Puromycin 30 min before harvesting. Puromycin-labelled peptides were quantify by Western Blot using anti-Puromycin antibody. Coomassie was used as a loading control (n=4-8). Ratio of the quantification of Puromycin to Coomassie is shown in the right panel. **(B)** Phosphorylated Akt was analysed by Western blot in control and mutant plantaris muscles before (SO) and after 1wk OV. Gapdh was used as a loading control (n=4). Ratio of the quantification of P-Akt to Gapdh is shown in the right panel. **(C)** *MuRF1* and *Atrogin-1* mRNA expressions were analysed by qRT-PCR in control and mutant *plantaris* muscles before (SO) and after 1 and 3 wk OV (n=4-6). Data were normalized by *Hmbs* expression. Data and mean ± SEM. *pvalue <0.05, ** pvalue<0.01, *** pvalue<0.001, **** pvalue<0.0001.

Figure 3

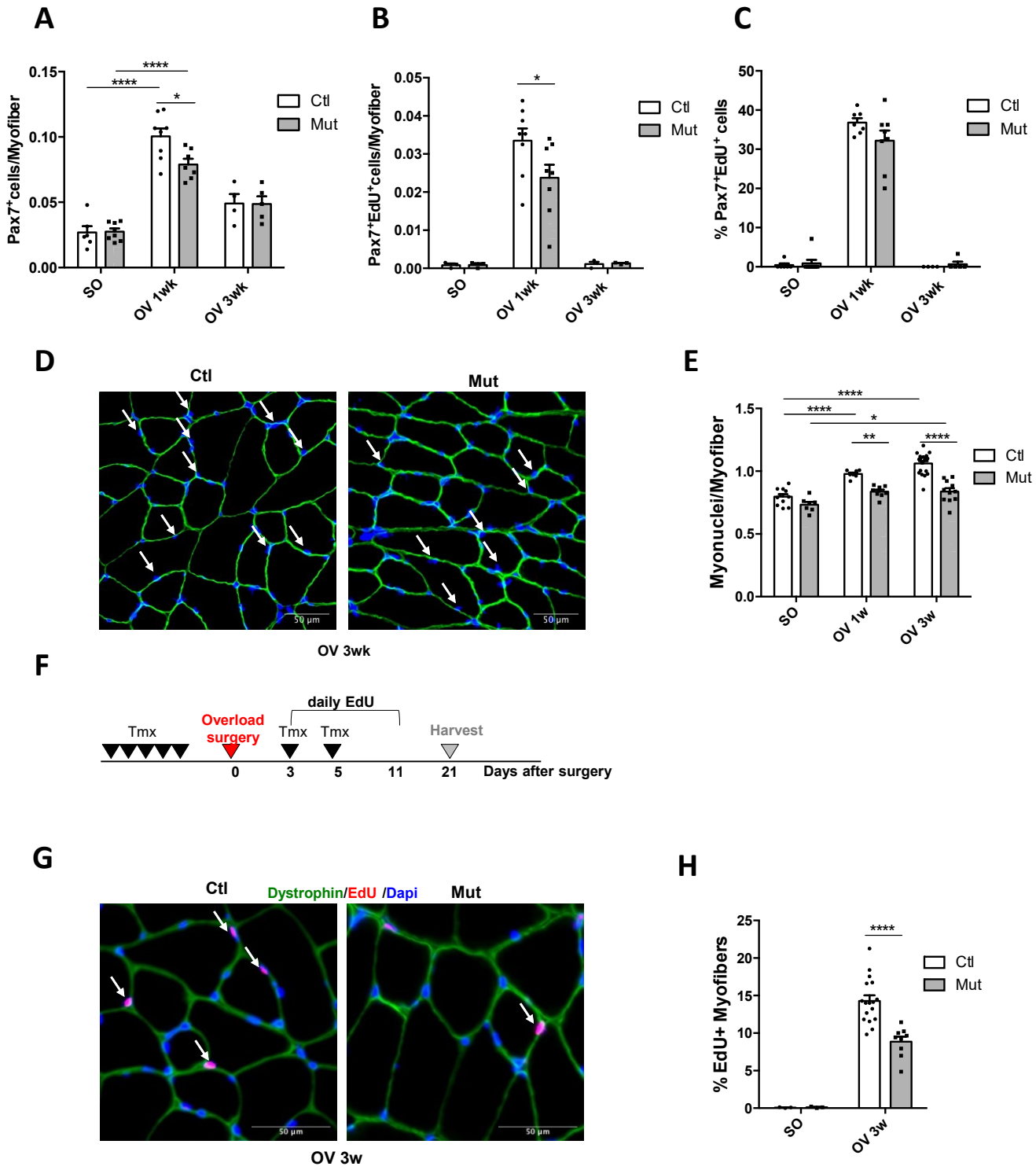

Figure 3. RhoA loss within myofibers impairs SC fusion. **(A)** Number of Pax7+ cells per myofiber in control and mutant *plantaris* muscle sections before (SO) and after 1 and 3 wk OV (n=4-8). **(B)** Number of Pax7+EdU+ cells per myofiber in control and mutant *plantaris* muscle sections before (SO) and after 1 and 3 wk OV (n=3-8). **(C)** Percentage of Pax7+EdU+ among Pax7+ cells in control and mutant *plantaris* muscle sections before (SO) and after 1 and 3 wk OV (n=4-8). **(D)** *Plantaris* muscle sections immunostained for Dystrophin (green) and nuclear staining with DAPI for control and mutant mice after 3 wk of OV. White arrows indicated the myonuclei within dystrophin+ sarcolemma. **(E)** Number of myonuclei (DAPI) within sarcolemma per myofiber before (SO) and after 1 and 3 wk OV (n=7-19). **(F)** Ctl and Mut were injected daily with EdU from day 3 to 11 post OV. Plantaris were isolated after 3 wk OV **(G)** *Plantaris* muscle sections immunostained for Dystrophin (green), EdU (red) and nuclear staining with DAPI for control and mutant mice before (SO) and after 3 wk of OV. **(H)** Percentage of EdU+ myofibers in control and mutant plantaris sections (SO) and after 3 wk OV (n=9-14). Data and mean ± SEM. *pvalue <0.05, ** pvalue<0.01, **** pvalue<0.0001.

Figure 4

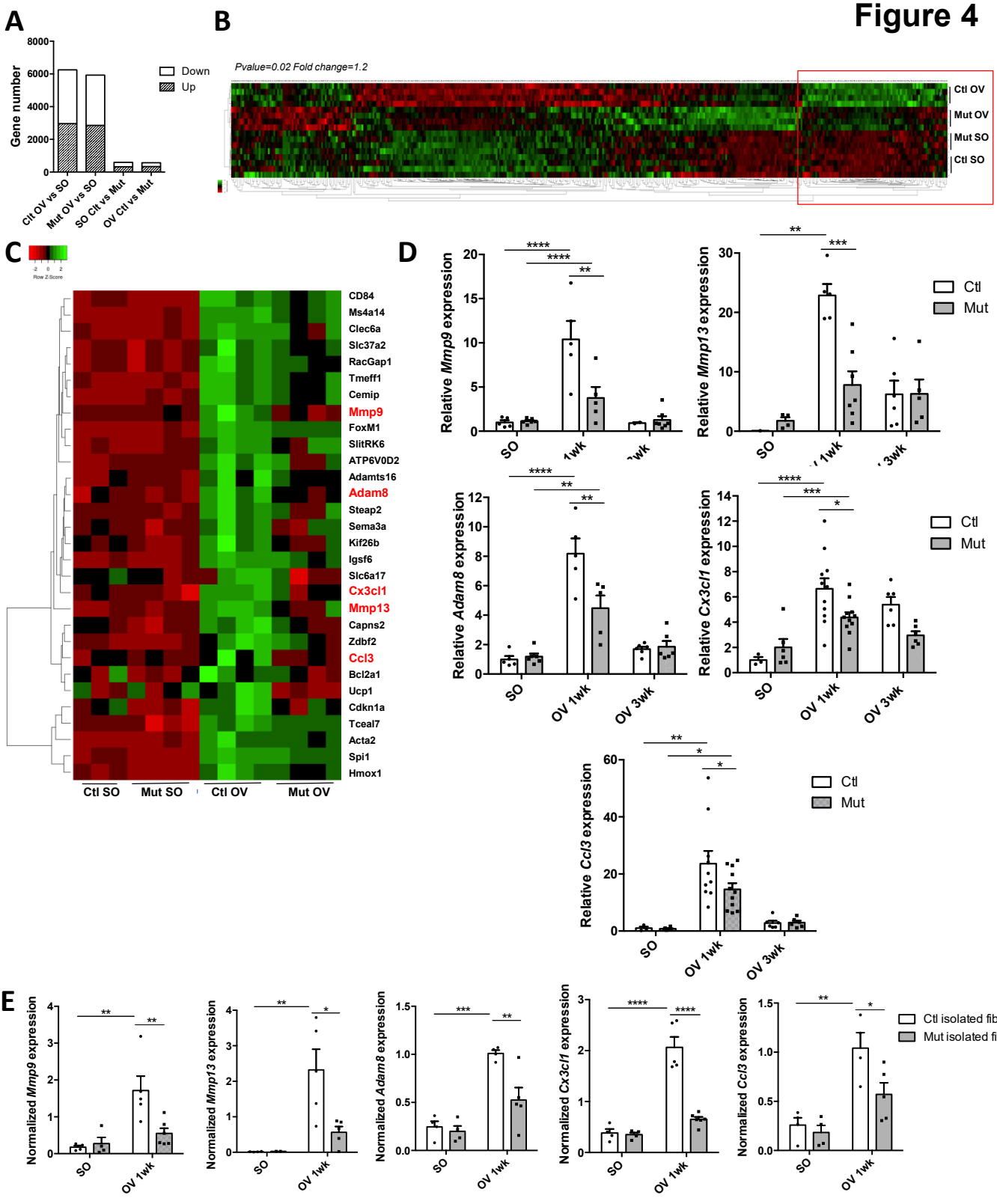

Figure 4. Transcriptomic analysis of control and RhoA mutant overloaded *plantaris* muscles. (A) Affimetrix analysis has been performed from RNA extracted from Clt and Mutant *plantaris* muscles submitted (OV) or not (SO) to overload-induced muscle hypertrophy. Number of genes differentially expressed (fold change > 1,2; p-value inf 0,05) depending of the condition SO/OV and of the RhoA expression Ctl/Mut. **(B)** Heat map representing up and down regulated genes between Ctl and Mut plantaris muscle before and after 1 wk OV. Genes whose expressions increase upon OV in controls are highlighted with a red box. **(C)** Close-up on the top 30 differentially expressed genes (fold change) between Ctl OV and Mut OV. **(D)** Analysis of *Matrix metallopeptidase 9* (*Mmp9*), *Matrix metallopeptidase 13* (*Mmp13*), *Disintegrin and metalloproteinase domain-containing protein* 8 (*Adam8*), *C-C motif chemokine Ligand 3* (*Ccl3*) and *C-X3-C Motif Chemokine Ligand 1* (*Cx3Cl1*) mRNA expression by qRT-PCR in control and mutant *plantaris* muscles before (SO) and after 1 and 3 wk OV (n=3-10). Data were normalized by *Hmbs* expression. **(E)** Analysis of *Matrix metallopeptidase 9* (*Mmp9*), *Matrix metallopeptidase 13* (*Mmp13*), *Disintegrin and metalloproteinase domain-containing protein* 8 (*Adam8*), *C-C motif chemokine Ligand 3* (*Ccl3*) and *C-X3-C Motif Chemokine Ligand 1* (*Cx3Cl1*) mRNA expression by qRT-PCR in control and mutant isolated myofibers from *plantaris* muscles before (SO) and after 1 and 3 wk OV (n=4-6). Data were normalized by *Hmbs* expression. Data and mean ± SEM. *pvalue <0.05, ** pvalue<0.01, *** pvalue<0.001, **** pvalue<0.0001.

Figure 5

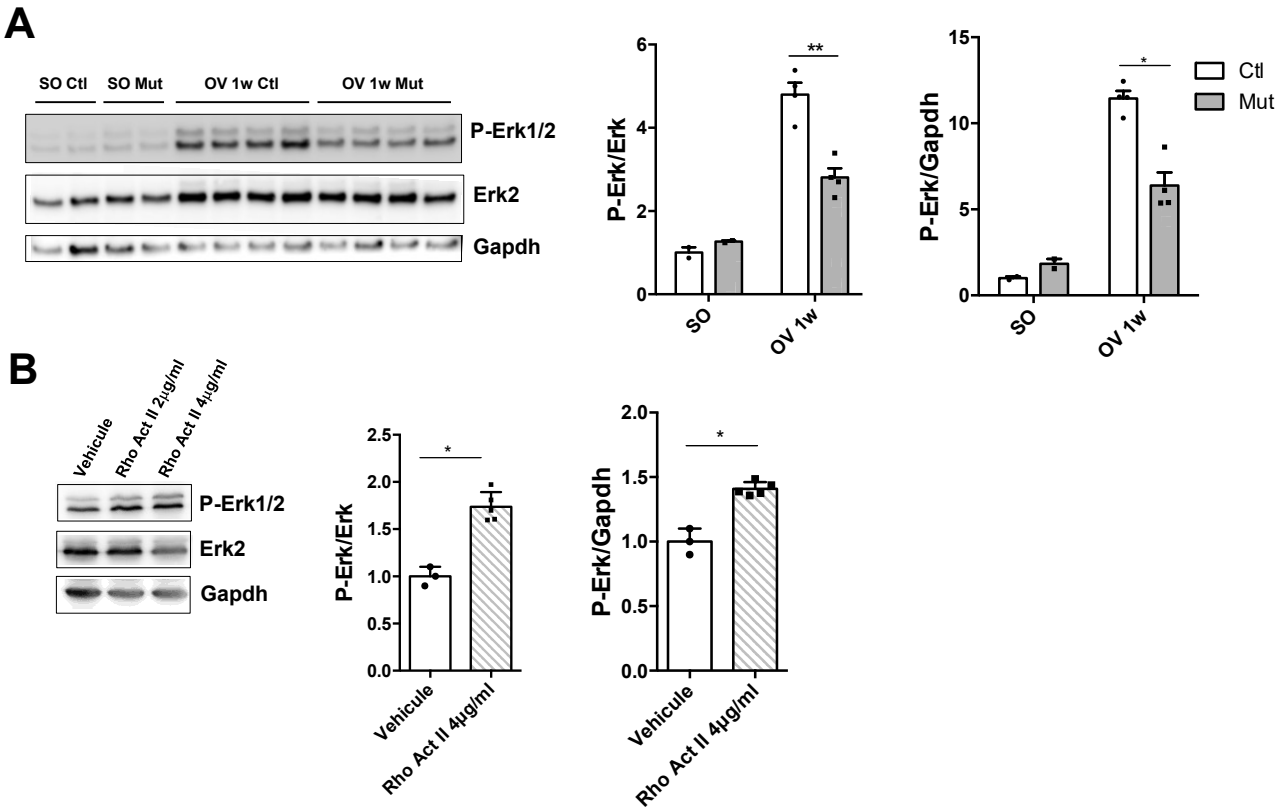

Figure 5. RhoA loss within myofibers impairs Erk1/2 activation upon overload-induced hypertrophy. **(A)** Phosphorylated Erk1/2 and total Erk1/2 were analysed by Western blot in control and mutant *plantaris* muscles before (SO) and after 1wk OV (n=4). Gapdh was used as a loading control. Ratio of the quantification of P-Erk1/2 to Erk1/2 relative to Ctl SO and ratio of the quantification of P-Erk1/2 to Gapdh are shown in the right panels. **(B)** Phosphorylated Erk1/2 and total Erk1/2 were analysed by Western blot in control myotubes treated with Rho Activator II (CN03) for 3h (n=3-5). Gapdh was used as a loading control. Ratio of the quantification of P-Erk1/2 to Erk1/2 and ratio of the quantification of P-Erk1/2 to Gapdh are shown in the right panels. Data and mean ± SEM. *pvalue <0.05, **pvalue <0.01.

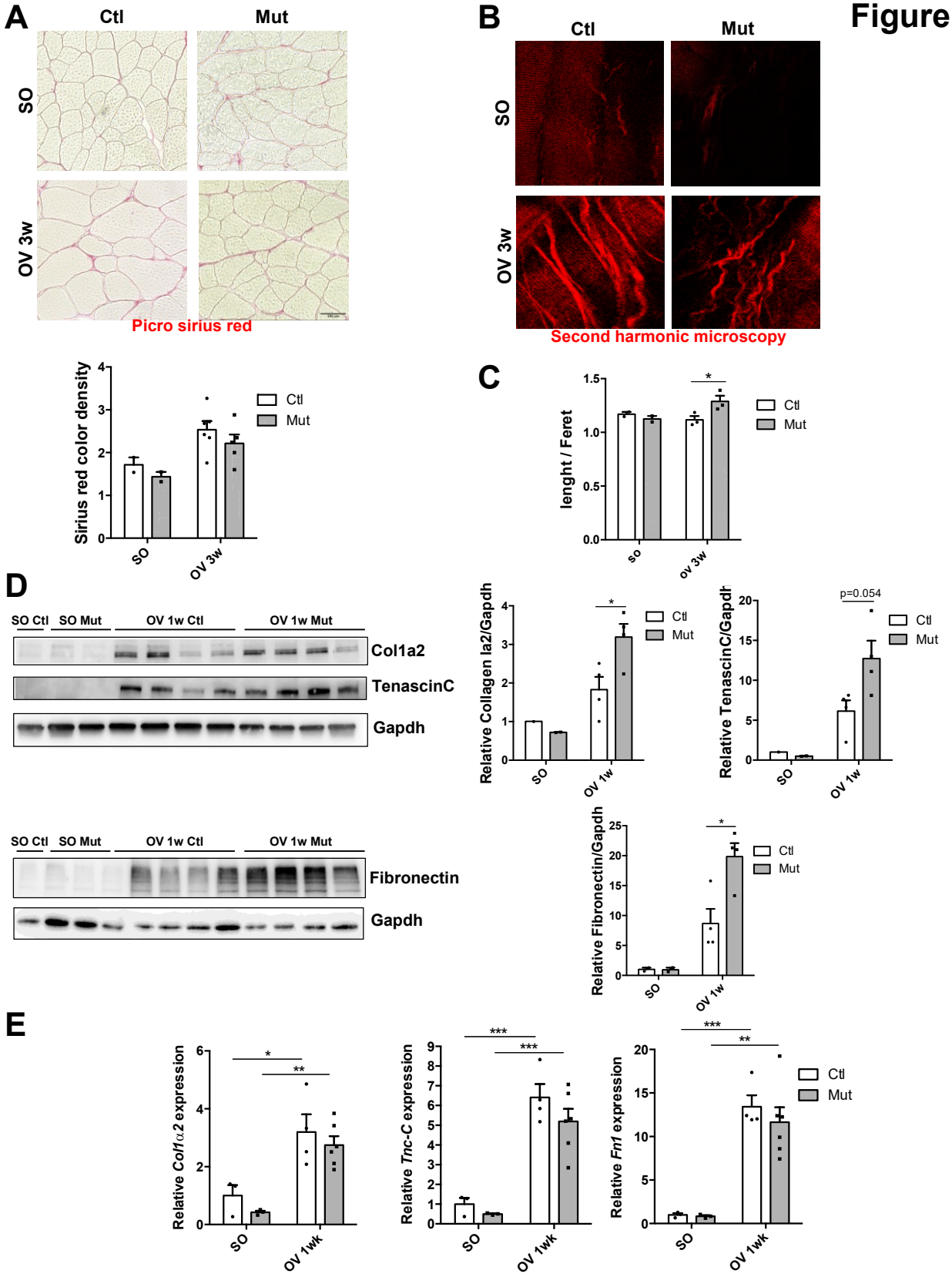

Figure 6. RhoA loss within myofibers impairs ECM organization upon overload-induced hypertrophy. **(A)** Picro sirius red staining of *plantaris* muscle sections from control and mutant mice before (SO) and after 3 wk of OV. Quantification of color density was performed using CaseViewer (n=2-5). **(B)** Visualization of fibrillar collagen using second harmonic generation (SHG) imaging on thick *plantaris* muscle sections from control and mutant mice before (SO) and after 3 wk of OV. **(C)** Tortuosity quantification (ratio length to Ferret of one collagen fiber) from control and mutant mice before (SO) and after 3 wk of OV (n=3). **(D)** Collagen 1a1, Tenascin-C and Fibronectin were analysed by Western blot in control and mutant plantaris muscles before (SO) and after 1wk OV. Gapdh was used as a loading control (n=3-4). Ratio of the quantification of Col1a1, TenascinC and Fibronectin to Gapdh and relative to control SO is shown in the right panel. **(E)** Analysis of *Collagen 1a1* (*Col1a1*), *TenascinC* (*Tnc*), and *Fibronectin* (*Fbn*) mRNA expression by qRT-PCR in control and mutant *plantaris* muscles before (SO) and after 1 and 3 wk OV (n=3-4). Data were normalized by *Hmbs* expression. Data and mean ± SEM. *pvalue <0.05, *** pvalue<0.001, **** pvalue<0.0001.

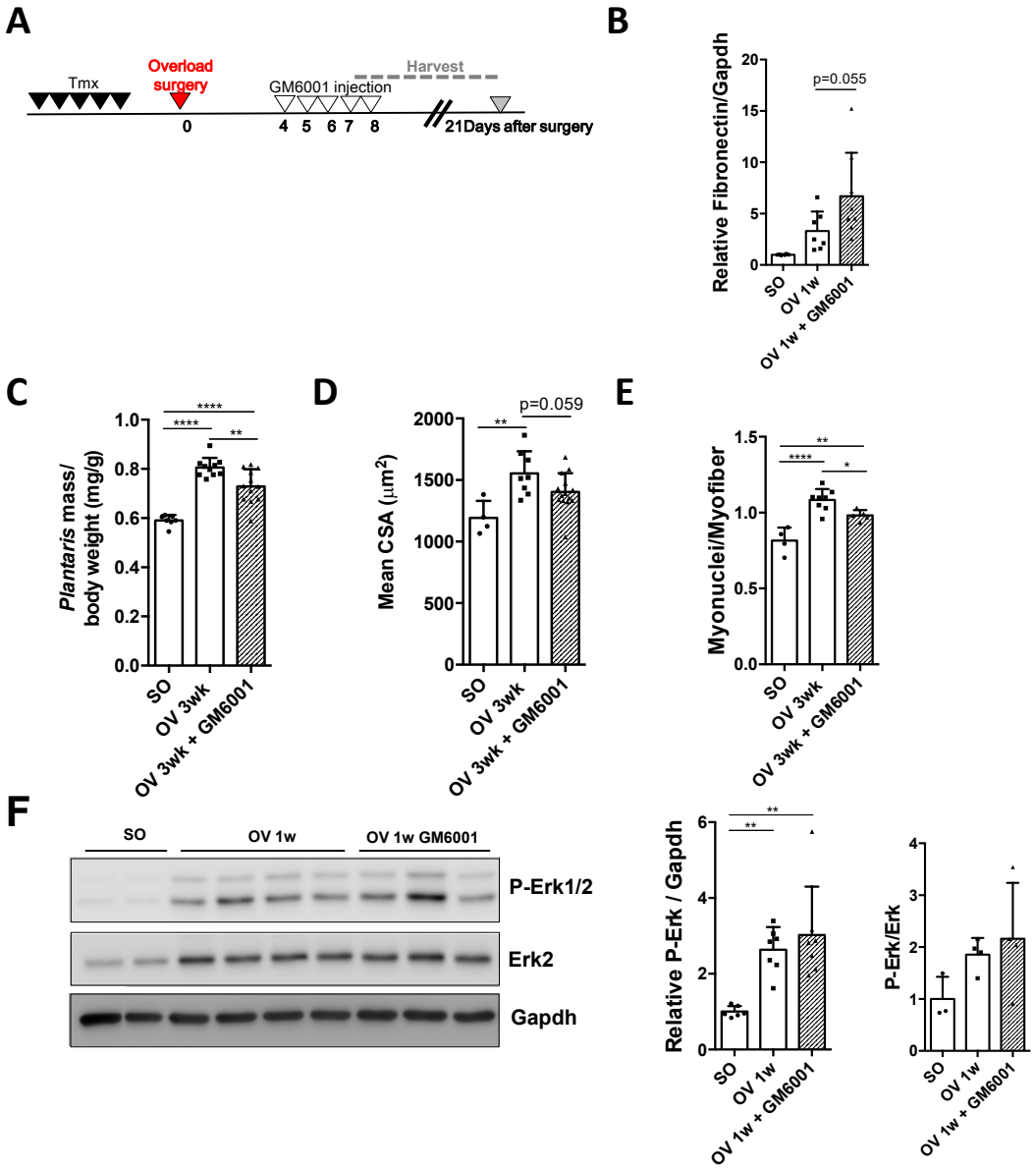

Figure 7. Mmps activity are important for overload-induced hypertrophy. **(A)** Control mice were submitted to OV procedure and were injected with Mmp inhibitor (GM6001) or vehicule from day 4 to 8 post surgery. *Plantaris* muscles were isolated 1 and 3 weeks after surgery. **(B)** Fibronectin was analysed by Western blot in control and mutant plantaris muscles before (SO) and after 1wk OV. Gapdh was used as a loading control (n=6-8). Ratio of the quantification of Fibronectin to Gapdh relative to Ctl SO is shown. **(C)** Ratio of *plantaris* mass (mg) to body weight (g) before (SO) and after 3 wk OV in control treated or not with GM6001 (n=7-14). **(D)** Mean CSA (scare micrometers) before (SO) and after 3 wk OV in control mice treated or not with GM6001 (n=4-14). **(E)** Number of myonuclei within sarcolemma per myofiber in *plantaris* muscle sections before (SO) and after 3 wk OV from control mice treated or not with GM6001 (n=4-8). **(F)** Phosphorylated Erk1/2 and total Erk1/2 were analysed by Western blot in control mice treated or not with GM6001 before and 1 wk post OV. Gapdh was used as a loading control (n=3-8). Ratio of the quantification of P-Erk1/2 to Erk1/2 and ratio of the quantification of P-Erk1/2 to Gapdh are shown in the right panels. Data and mean ± SEM. *pvalue <0.05, ** pvalue<0.01, **** pvalue<0.0001.

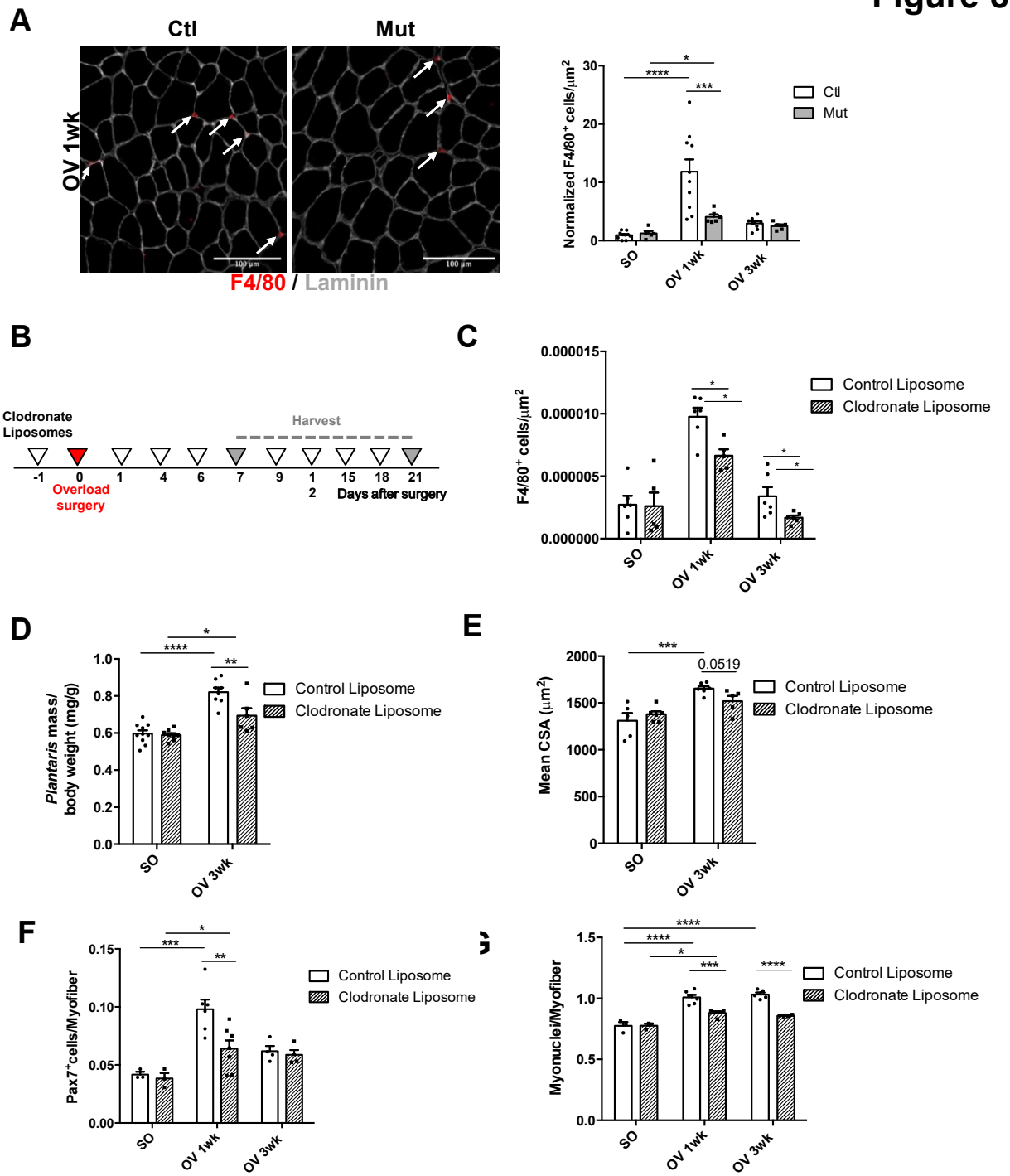

Figure 8. RhoA loss whithin myofiber impairs macrophage recruitment. **(A)** *Plantaris* muscle sections immunostained for F4/80 (red), laminin (grey) and nuclear staining with DAPI for control and mutant mice after 1 wk of OV. White arrows indicated f4/80+/DAPI macrophages. Number of macrophages (F4/80+) per µm^2 before (SO) and after 1 and 3 wk OV relative to Ctl SO are quantified in the right panel (n=5-6). **(B)** Control mice were injected with Clodronate-loaded liposomes or control liposomes one day before surgery. After they were submitted to OV procedure and were injected with Clodronate loaded liposomes or control liposomes day 1, 4, 6, 9,12,15,18 post surgery. *Plantaris* muscles were isolated 1 and 3 wk after surgery. **(C)** Number of macrophages (F4/80+) per µm^2 before (SO) and after 1 and 3 wk OV in plantaris sections from Ctl mice treated with Clodronate loaded liposomes or control liposomes (n=5-6). **(D)** Ratio of *plantaris* mass (mg) to body weight (g) before (SO) and after 3 wk OV in control mice treated with Clodronate loaded liposomes or control liposomes (n=6-10). **(E)** Mean CSA (mm2) before (SO) and after 3 wk OV in Ctl mice treated Clodronate loaded liposomes or control liposomes (n=5-6). **(F)** Number of Pax7+ cells per myofiber in *plantaris* muscle sections before (SO) and after 1 and 3 wk OV from control mice treated with Clodronate loaded liposomes or control liposomes (n=3-7). **(G)** Number of myonuclei within sarcolemma per myofiber in *plantaris* muscle sections before (SO) and after 1 and 3 wk OV from control mice treated treated with Clodronate loaded liposomes or control liposomes (n=4-8). Data and mean ± SEM. *pvalue <0.05, ** pvalue<0.01, ** pvalue<0.01, *** pvalue<0.001, **** pvalue<0.0001.

Figure S1

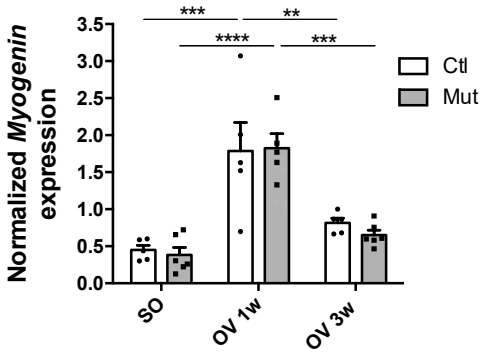

Figure S1. RhoA loss within myofibers does not affect *Myogenin* expression. *Myogenin* mRNA expression was analysed by qRT-PCR in control and mutant *plantaris* muscles before (SO) and after 1 and 3 wk OV (n=5-6). Data were normalized by *Hmbs* expression. Data and mean ± SEM. <0.05, ** pvalue<0.01, *** pvalue<0.001, **** pvalue<0.0001.

Figure S2

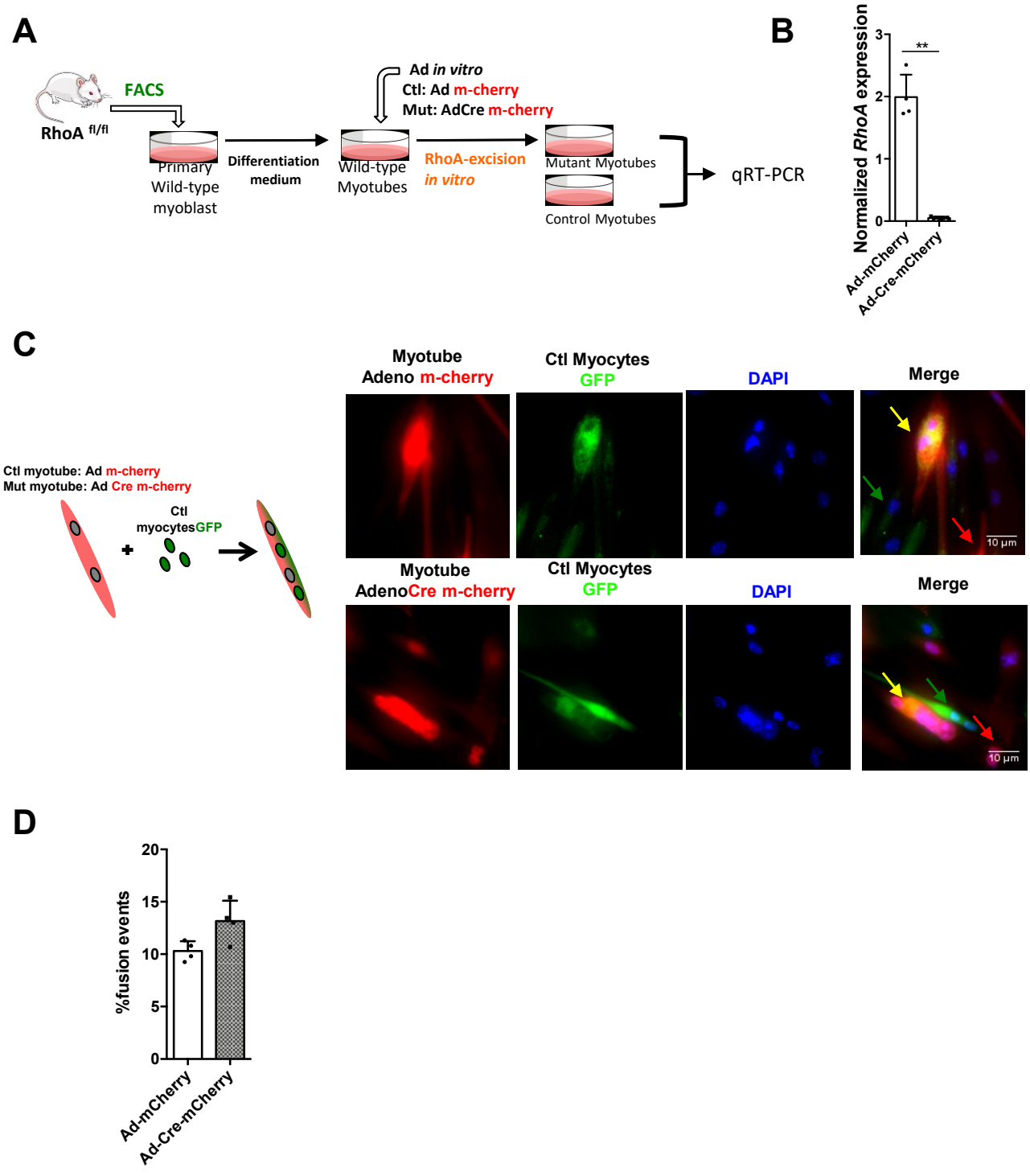

Figure S2. RhoA loss within myotubes *in vitro* does not affect myoblast fusion. **(A)** FACS-sorted control RhoA$^{flox/flox}$ cells were cultured in rich medium and then induced to differentiate. Differentiated myotubes were transduced with Ad-mCherry or Ad-Cre-mCherry to induce RhoA deletion.Transduced myotubes were used to extract Rna and perform qRT-PCR **(B)** *RhoA* expression was analysed by qRT-PCR *in RhoA*$^{lx/lx}$ myoblasts transduced with Ad-mCherry or Ad-Cre mCherry to induce RhoA loss. Data were normalized by *Hmbs* expression (n=4). **(C)** Myotubes transduced with Ad-mCherry (Ctl, red) or Ad-Cre mCherry (Mut, red) were mixed with Ctl myocytes labeled in green (expressing GFP). After 48h of co-culture, myotubes were analysed for dual labeling. **(D)** Myotubes transduced with Ad-mCherry (Ctl, red) or Ad-Cre mCherry (Mut, red) were mixed with Ctl myocytes labeled in green (expressing GFP). The percentage of dual-labeled cells per total number of nuclei (fusion events) was scored (n=3). Data and mean ± SEM. ** pvalue<0.01.

Figure S3

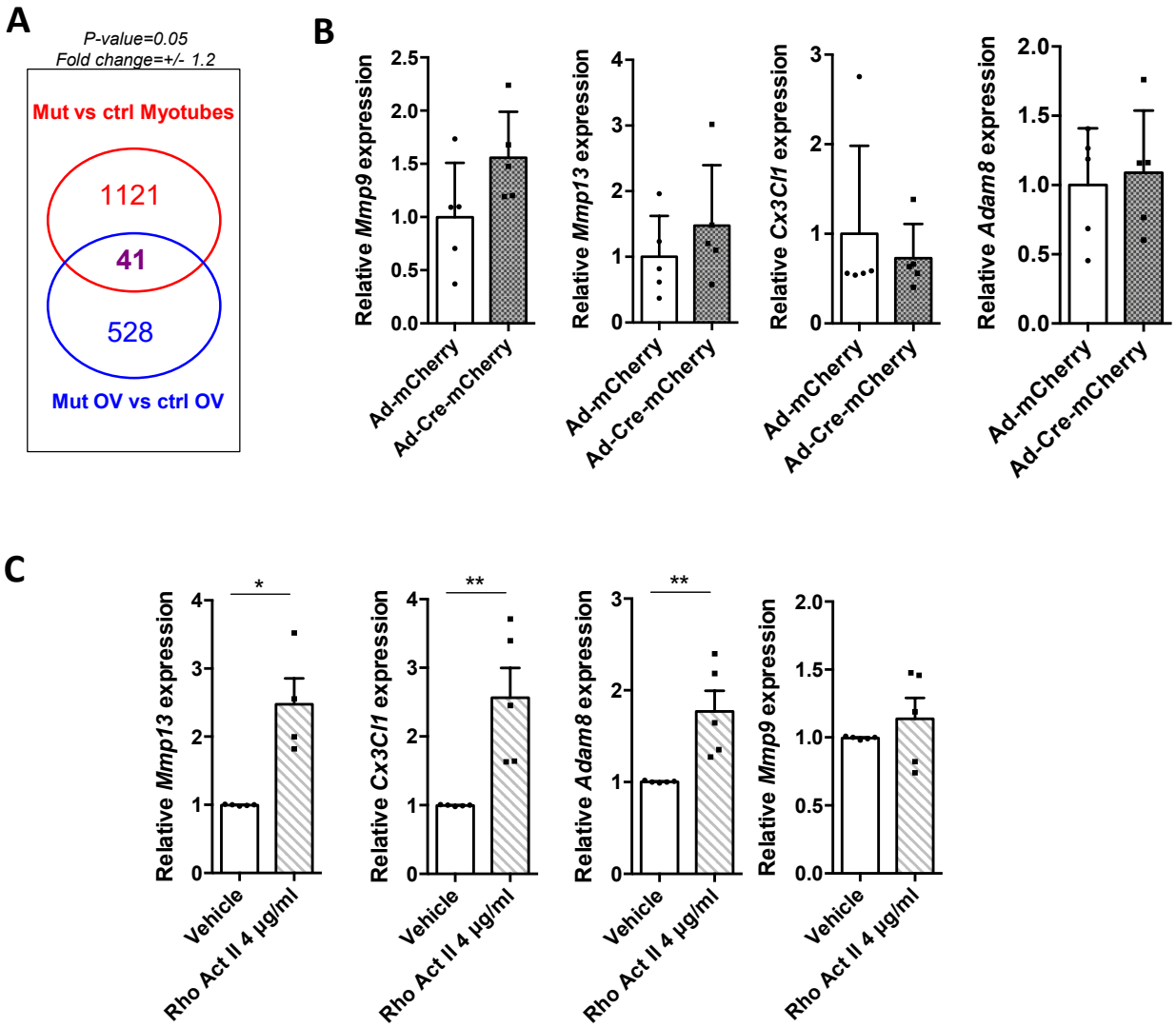

Figure S3. **(A)** Affymetrix analysis has been performed from mRNA from Myotubes transduced with Ad-mCherry (Ctl) or Ad-Cre-mCherry (Mut). Venn diagram showing the intersection between genes differentially regulated by RhoA (fold change > 1,2; p-value inf 0,05) in Myotubes, and plantaris overload muscles (Mut OV vs Ctl OV). **(B)** Mmp9, Mmp13, Adam8 and Cx3Cl1 mRNA expression was analysed by qRT-PCR in RhoAflox/flox myotubes transduced with Ad-mCherry or Ad-Cre mCherry to induce RhoA loss. Data were normalized by Hmbs expression (n=4). **(C)** Analysis of *Mmp13*, *Cx3Cl1*, *Adam8* and *Mmp9* mRNA expression by qRT-PCR in control myotubes treated with Rho Activator II (CN03) and/or Erk inhibitor (Binimetenib) for 8h (n=3-4). Data were normalized by Hmbs expression. Data and mean ± SEM.

Figure 9

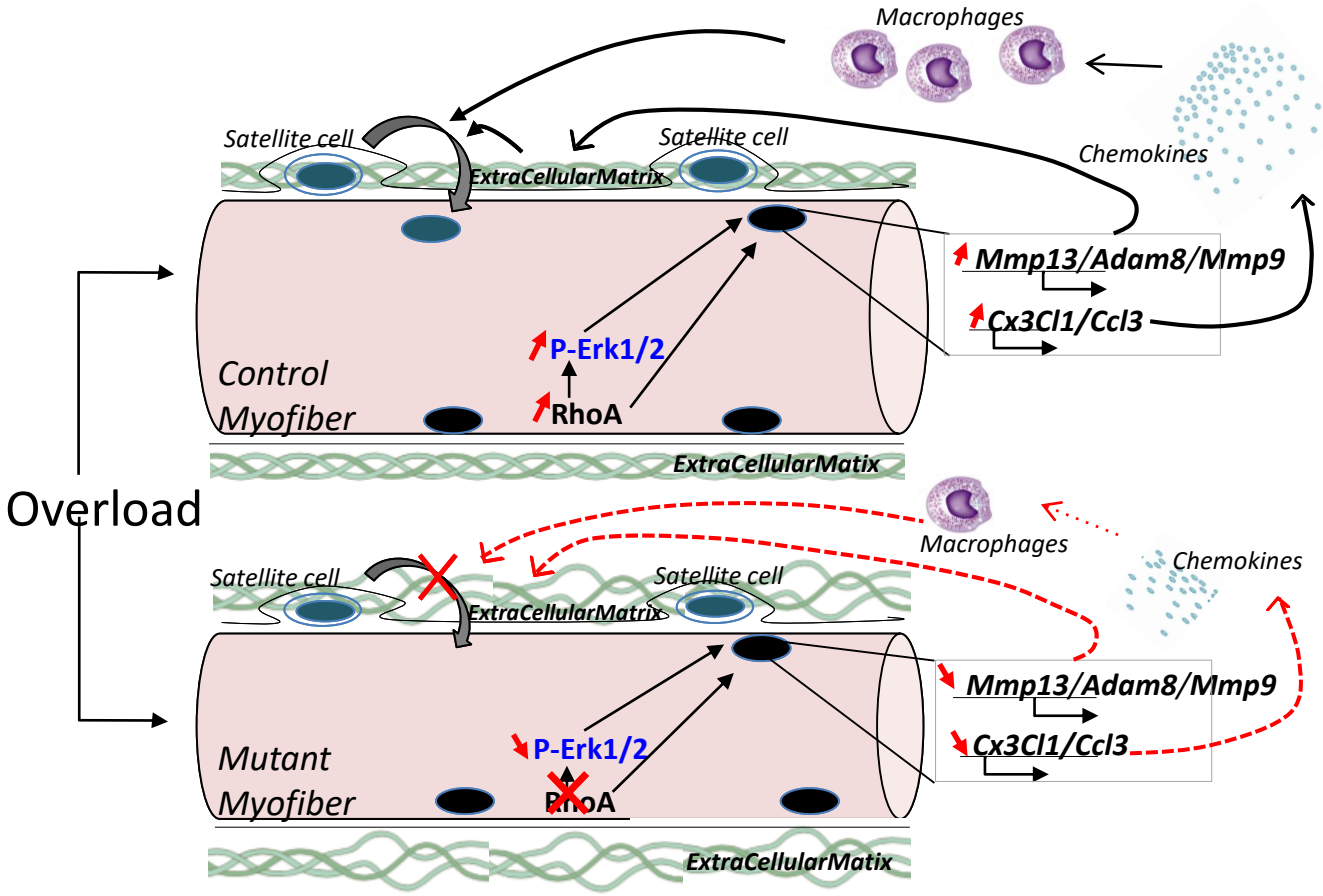
Macrophages
Chemokines
Satellite cell
Satellite cell
ExtraCellularMatrix
Mmp13/Adam8/Mmp9
Cx3Cl1/Ccl3
P-Erk1/2
RhoA
Control
Myofiber
ExtraCellularMatix
Overload
Macrophages
Chemokines
Satellite cell
Satellite cell
ExtraCellularMatrix
Mmp13/Adam8/Mmp9
Cx3Cl1/Ccl3
P-Erk1/2
RhoA
Mutant
Myofiber
ExtraCellularMatix

Table 1 - Microarray GO analysis 1 week after overload control SO vs OV

Canonical pathways	-log(p-value)	zScore
Integrin Signaling	1.07E01	4.07
IL-8 Signaling	8.25E00	4.12
FcÎ³ Receptor-mediated Phagocytosis in Macrophages and Monocytes	8.18E00	5.25
Remodeling of Epithelial Adherens Junctions	7.69E00	2.23
Leukocyte Extravasation Signaling	7.25E00	3.75
Regulation of Actin-based Motility by Rho	6.92E00	4.23
RhoA Signaling	***6.61E00***	***3.71***
Rac Signaling	6.33E00	4.15
Glioma Signaling	6.09E00	2.14
Phospholipase C Signaling	5.82E00	3.16
Actin Nucleation by ARP-WASP Complex	5.81E00	3.88
Cdc42 Signaling	5.7E00	2.54
Dendritic Cell Maturation	5.37E00	3.44
Colorectal Cancer Metastasis Signaling	5.2E00	3.44
Signaling by Rho Family GTPases	5.17E00	3.47
p70S6K Signaling	5.15E00	2.56

Table 2 – Predicted activated/inhibited Upstream Regulators in Control only 1 week after overload SO vs OV

Function	Upstream Regulator		Activation Z-score	p-value of overlap	Link with Mmps	Link with inflammation
Modulation of ECM	**Mmp9**	Peptidase	2.167	1.24E-04		
	Postn	Secreted extracellular matrix protein	2.199	4.17E-02		
	TnC	matricellular protein	2.427	1.73E-04	Mmp9 substrat Generation of integrin-activating peptides	
	Cyr61	matricellular protein	2.457	1.27E-05		
	Loxl2	enzyme	2.392	7.54E-03		
	Plau	peptidase/in ECM	2.825	1.02E-03		
Integrin Receptor	**ITGA9**	transmembrane receptor	3.569	5.45E-03	Generation of integrin-activating peptides from TnC	
	ITGAV	transmembrane receptor	2.218	9.51E-04		
Growth Factor	**Pgf**	growth factor VEGF sub-family	2.929	1.45E-03		
	Fgf	group/growth factor	2.128	9.91E-03	Mmp9 substrat/Active	
	Fgf7	growth factor	2.643	6.68E-04	Mmp9 substrat/Active	
	Gdf2	growth factor	2.923	2.71E-02		
	EgfR	kinase/ growth factor receptor	2.721	2.24E-03		
Cytokines	**Faslg**	cytokine	2.583	3.56E-02	Cleaved by Mmm7	
	Tnf (family)	group/cytokine	3.455	9.01E-04	Mmp9 substrat	
	TgfbR1	cytokine receptor	2.308	3.35E-03	Generation of active Tgfb by Mmp9	
	Il3	cytokine	2.389	1.76E-03		Activation of macrophages
	IL17Ra	cytokine receptor	2.686	3.56E-02		
	Gm-csfR	group/cytokine receptor	3.229	8.76E-03		Macrophage survival
Chemokines	**Ccl2**	chemokine chemoattractant	3.630	4.59E-03		Recrutes monocytes
	CcR1	Ccl3 chemokine receptor	2.387	1.49E-06		Macrophage chemoattractant
	CcR2	CCL2 chemokine receptor	2.452	3.26E-03		Recrutes monocytes
	CxCR4	CXCL12/Sdf1 chemokine receptor	2.798	7.82E-03		
Signaling	**Mapk3/Erk1**	Kinase	2.790	1.19E-02		
	Mapk8	Kinase	2.344	6.34E-04		
	Stat3	transcription regulator	3.036	3.26E-07		
	Rock2	kinase	2.393	3.44E-03		
	RelA	transcription regulator Nfkb	2.408	2.18E-02		
	Sn50 peptide	Nfkb inhibitor	-2.608	2.11E-02		
Other	**SerpinA4**	protease inhibitor	-2.171	3.67E-03	Mmp9 substrat/Inactive	

Table S1 –Common genes differentially expressed in Myotubes expressing or not RhoA and in Control vs Mutant overloaded-*plantaris* that were similarly regulated (up or down)

	Myotubes lacking RhoA vs Clt		**Overloaded-*plantaris* Mut vs Clt**	
Gene Symbol	***Fold Change***	***p-value***	***Fold Change***	***p-value***
AMMECR1	-1,92	0,009	-1,233	0,00182
BCL2A1	-1,63	0,0051	-1,623	0,0148
CSNK1G1	-1,76	0,004	-1,235	0,00366
Cst12	-1,33	0,0027	-1,221	0,00989
LRP11	-1,91	0,0082	-1,25	0,022
RHOA	-1,68	0,0181	-1,204	0,00418
SLX4	-1,43	0,0054	-1,283	0,0117
ZNF8	-1,44	0,0144	-1,216	0,00272
1700001F09Rik (includes others)	1,28	0,0481	1,306	0,0171
AKAP1	2,78	0,0262	1,25	0,031
ARPC5L	1,46	0,0229	1,358	0,0146
CHCHD1	1,35	0,0414	1,353	0,0218
COG4	2,17	0,0284	1,267	0,0121
COX19	1,69	0,0458	1,263	0,00886
CRYGD	1,48	0,0129	1,369	0,021
LONP1	1,5	0,044	1,246	0,0104
MRPL12	1,58	0,0065	1,219	0,000396
MRPL53	1,72	0,0404	1,248	0,0032
NDUFB5	1,23	0,0274	1,21	0,00386
NEURL2	1,81	0,0152	1,271	0,00937
PIPOX	1,24	0,0384	1,267	0,0319
POLR2F	2,32	0,0366	1,302	0,00096
PPA1	2,56	0,0391	1,226	0,0141
TIMM10	1,86	0,0108	1,217	0,0458
TWF2	2,38	0,014	1,26	0,00321

Part II: The role of RhoA in satellite cells during skeletal muscle plasticity

Mouse skeletal muscle development begins at embryonic day 8 (E8.0) when the somitogenesis starts. The embryonic development of skeletal muscle is governed by specific signalling molecules. The main genes regulating myogenesis in vertebrate are the MRF proteins: Myf5, MyoD, Myogenin and MRF4. At E10, from the mesoderm cell layer give rise the dermomyotome. The lips of the dermomyotome mature into the myotome, located between the dermomyotome and the medio-ventrally located sclerotome; it is a primitive muscle structure containing committed muscle cells. (Bentzinger et al. 2012; Biressi et al. 2007). Once that the myogenic progenitors reach their final destination, they give rise to terminally differentiated myocytes and form primary myotome. Primary myogenesis establishes the scaffold of muscles and it is followed by secondary myogenesis. From lateral myotome muscle progenitors differentiate early and subsequently develop into intercostal muscles and muscles of the body wall. The fine regulated expression of MRF is crucial for a correct myogenesis. Among them Myf5 is the earliest MRF expressed in myogenic progenitors once migrating in their peripheral position. Using a lineage tracer under the control of Myf5 promoter it was shown that in adult 90% of Pax7+ cells express once Myf5. 10% of cells that do not express Myf5 (and never expressed during the development) are considered a subpopulation with an higher stemness, more prone to self renew (Kuang et al. 2007). This means that the cells that express Myf5 give rise to committed myogenic progenitors, a subpopulation of future adult stem cells.

The adult skeletal muscle is a high plastic tissue able to adapt to external stimuli. The adult Pax7+ stem cells called Satellite Cells (SCs) are located between the basal lamina and sarcolemma. SCs are maintained in a quiescent state and in response to muscle damage or upon increased workload, get activate, start to proliferate, differentiate and fuse to form new myofibers (during regeneration) or to supplement existing growing muscle fibers (during hypertrophy). This process is called adult myogenesis and it mostly recapitulates embryonic myogenesis. Typically, adult muscle SCs self-renew to replenish and maintain the stem cell pool.

Following engagement into differentiation (could be of the embryonic muscle precursor cells (EP) or of SCs), a critical event of myogenesis is the fusion of myoblasts to generate new multinucleated myofibers. The fusion process follows an ordered set of cellular events that includes cell migration, alignment, adhesion and membrane fusion. Actin cytoskeleton plays an essential role in the fusion conserved from *Drosophila* to mammals. Conditional mutation of key controller of cytoskeletal and particularly actin rearrangements such as Rac and Cdc42 showed that they are essential to allow the myoblast fusion process to occur (Vasyutina et al. 2009). As well myoblast deletion of Srf, a

transcription factor controlling the expression of target genes involved in cytoskeletal organization (among others) blocked their fusion. RhoA small GTPase protein is responsible for actin cytoskeleton assembly and involved in regulation of cell proliferation, cell contact, cell migration and eventually in cell fusion. This last hypothesis is not yet demonstrated and it is based on previous mentioned report on the other member of Rac family. Thus, it is interesting to investigate the role of RhoA in myoblast fusion. Published data on cultured muscle cell line C2C12 showed that RhoA expression and activity should be finely regulated to have a correct fusion process and expression of key genes involved in skeletal muscle differentiation. However very little is known on the role played by RhoA in primary myoblast and *in vivo*. In mouse embryo it was showed that RhoA/ROCK axis defines the identity of embryonic myoblasts and that RhoA activity is selectively activated during embryonic myogenesis and shuts down at the foetal stage, suggesting its finely regulation during muscle development (Taglietti et al. 2018). Recently it was showed on primary satellite cells that RhoA pathway is important to maintain SCs quiescence and to have a normal regeneration (Eliazer et al. 2019). In muscle tissue it is described that RhoA activation is triggered in response to mechanical force. However, considering all the muscle tissue, there is no discrimination among the satellite cells and the other cell types present in the muscle

With this rational we want to investigate the role of RhoA in skeletal muscle development and in adult myogenesis. In particular we used two different Cre lineage mouse model i) *Myf5*Cre:RhoA$^{lx/lx}$, where the deletion occur in a constitutive way in embryonic progenitors (EPs); ii) Pax7^{CreErt2}:RhoA$^{lx/lx}$ which permit specific and inducible (upon tamoxifen treatment) deletion of *RhoA*, in the adult muscle stem cells.

1. Deletion in EPs

1.1 Validation of the mouse model

In order to investigate the role played by RhoA during embryonic development, we deleted RhoA gene in muscle progenitors using the genetic mouse model *Myf5*Cre:RhoA$^{lx/lx}$. In our model RhoA was deleted in embryonic progenitors (EPs) at E8 in a constitutive manner.

In order to validate the mouse model, we verified *RhoA* deletion in *Plantaris* muscle of nine weeks old animals. Because the deletion occurred in embryonic progenitors, all the muscle cells arised from (i.e. the future satellite cells and myofibers), were mutant as well. As showed in the Figure 24 the gene expression of *RhoA* was significantly reduced in mutant muscle compared to the control. Specifically there is a 53% less of *RhoA* mRNA transcript in mutant *Plantaris* muscle compared to the control (Figure 24A). The decrease of *RhoA* expression obtained in this model was comparable

to that obtained when *RhoA* was deleted in myofibers (results part I). However, lack of *RhoA* was not total (100%). This result could be explained by i) the fact that we are not sure that the *RhoA* deletion occurred in all the myogenic cells, ii) the fact that in the muscle there are other cells that express RhoA, that could blend the real result. To validate the mouse model in a tidier manner, we should verify *RhoA* expression on embryonic progenitors that can be isolated by surface antigen reactivity by FACS from embryo EPs or adult SCs.

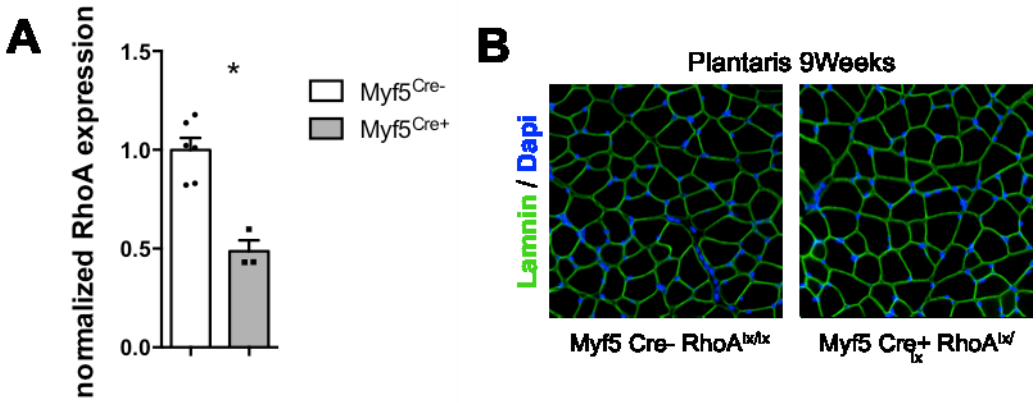

Figure 24: A. Analysis of RhoA mRNA expression by qRT-PCR in control and mutant *Plantaris* muscles. Data were normalized by Hmbs expression and relative to control (n=3-6 muscles). B. *Plantaris* muscle sections immunostained for Laminin (green) and nuclear staining with DAPI for control and mutant mice 9 wk old

1.2 RhoA Mutant mice before and after birth do not respect Mendelian ratio

When *RhoA* was deleted in EPs, the Mendelian ratio was not respected. Crossing males carring Cre gene and $RhoA^{lx/wt}$ alleles with female with no Cre and $RhoA^{lx/lx}$, the expected Mendelian ratio for each genotype should be 25% and it was not the case. Indeed, we obtained 13.91% of mice Cre+ $RhoA^{lx/lx}$, 26.08% of mice Cre+ $RhoA^{lx/wt}$, 29.56% of mice Cre- $RhoA^{lx/lx}$ and 30.43% of mice Cre- $RhoA^{lx/wt}$ instead of 25% expected (Figure 25). The real number of mutant Cre+ $RhoA^{lx/lx}$ is less compared to the predicted. In order to verify if the death rate was already altered before birth, we looked at ratio of the different genotypes at 18.5 days after fertilization, just before birth and they were not mendelian.

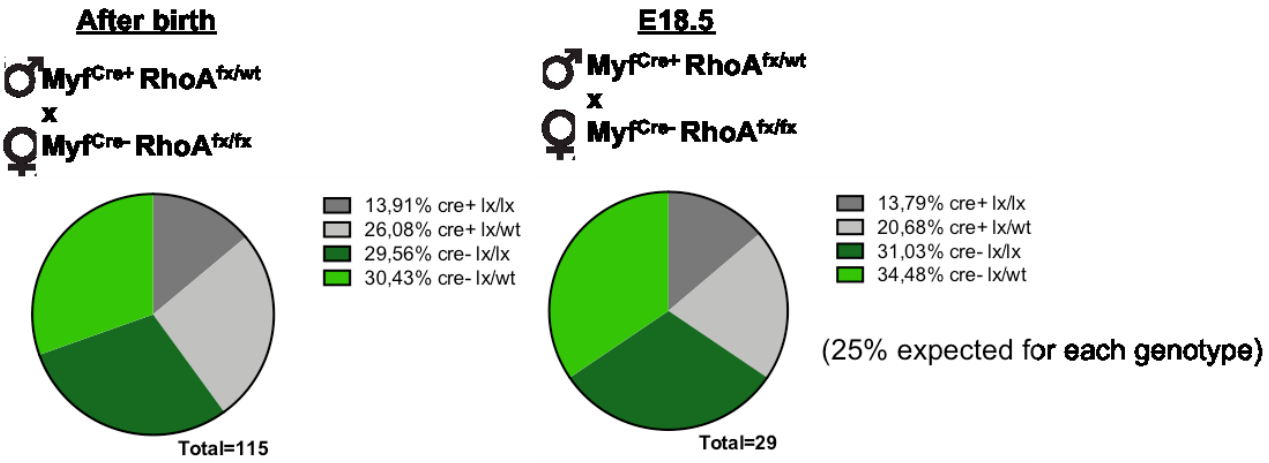

Figure 25: Representation on Mendelian ratio of different genotype crossing resulted from Cre+ RhoA$^{lx/wt}$, and Cre- RhoA$^{lx/lx}$ after birth and at E18.5

The results showed that already before birth the Mendelian ratio was not respected, because we obtained only 13.79% of mutant embryo rather then 25%. Even if the total number of embryo collected is rather low (n=29), we can conclude that there is a higher mortality of mutant mice already before birth at a premature stage of development. Because we did not find any residual embryo in pregnant female mice (E18.5), we suppose that the loss of some mutant embryo should have occurred very early in development, maybe due to a leakage of Cre expression in other tissues. We can assert that it is not due to muscle defects because quadruple knock out mouse model of *Six1*,*Six2*, *Six4* and *Six5* generated in our laboratory show severe muscle defects with an absence of muscle masses (myofibers disorganization and only reduced dorsal muscle mass), and have a correct mendelian ratio at E18.5, dying just after birth. These data suggest that the absence of muscle tissue cannot be responsible for the total loss of embryo at E18.5 (P. Maire personal communication). Myf-5 expression begins to be detected at embryonic day 8 (E8) in the mesencephalon and coincides with the appearance of the first differentiated neurons. This suggest a crucial role of Myf-5 in neurogenesis as well as myogenesis, and introduces a new lineage marker for the developing brain (Tajbakhsh et al. 1995). It is well described that RhoA is indispensable for the developing mouse nervous system (Herzog et al. 2011). Thus, we can speculate that the expression of Cre under Myf5 promoter could lead to *RhoA* deletion in another tissue, like brain, causing the early death of mutant embryo.

However, it could be interesting to investigate the embryo phenotype at earlier time point to evaluate possible precocious death.

1.3 Myf5^{Cre};RhoA$^{lx/lx}$ embryo and adult mice present different phenotype: fusion defect is evidenced only at post natal stage

The Figure 26, showing a control and mutant limb cross section at E18.5 stained for Laminin, proved that muscles correctly form before birth.

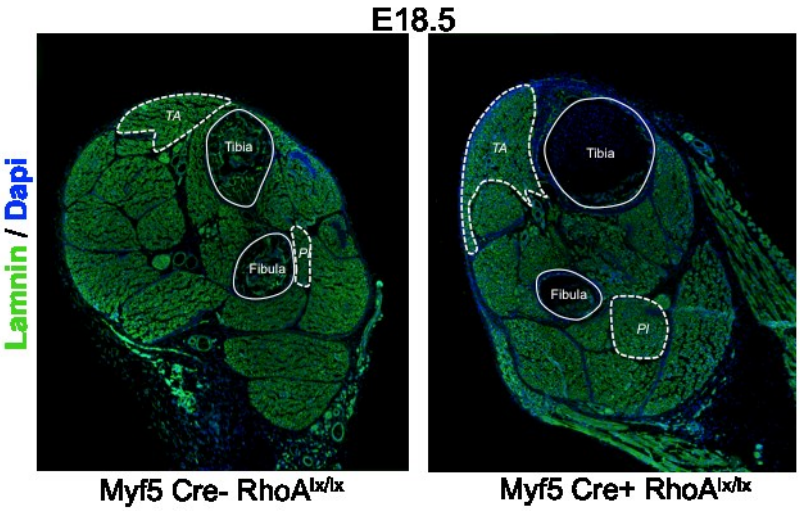

Figure 26: Posterior limb transversal section sections immunostained for Laminin (green) and nuclear staining with DAPI for control and mutant embrio at E18.5.

We can clearly identify *Plantaris (Pl)* and *Tibialis Anterioris (TA)* muscles on the section even if the cryoconserved limbs are cutted at different level of depth. Anyway, we are able to distinguish in both control and mutant the different muscle masses.

Surviving young adult mutants do not exhibit phenotypic defects in global muscle structure (Figure 24B), suggesting that RhoA during embryonic development is not required to control muscle formation or maybe that its absence is compensated by other member of Rho family to supply its function.

Next we analysed the *Plantaris* muscle weight and myofibers number evidencing no difference between control and mutant (Figure 27A-B). However, there is a tendency to smaller CSA in the mutant compared to the control, even if it did not reach significance (Figure 27C).

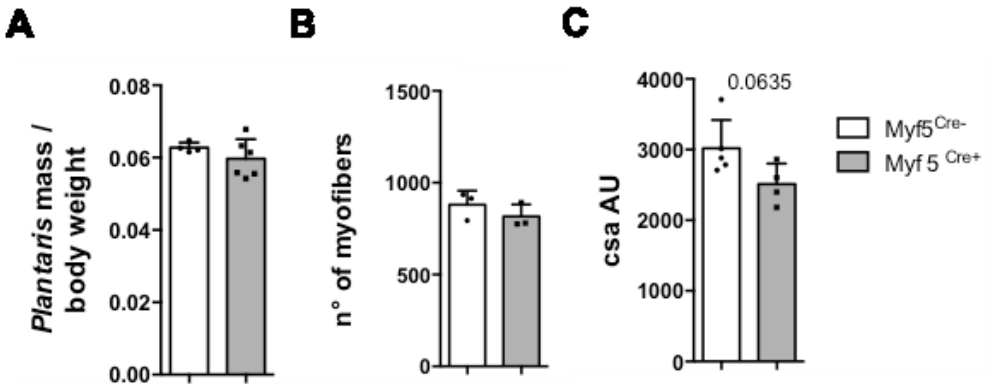

Figure 27: A. Ratio of *Plantaris* mass (milligrams) to body weight (grams) of control and mutant mice (n=4-6 muscles) B. Mean myofiber number in control and mutant mice C. Mean CSA (scare micrometers) in control and mutant mice. Mice used are 9 weeks old.

It will be important to add other mice samples for CSA data, to confirm or not any difference between control and mutant mice. If a difference is evidenced it is possible that this difference comes from: i) an abnormal fiber type distribution that takes place in the mutant (oxydative fibers are smaller, I and IIa, while glycolytic fibers IIb are bigger) and/or ii) decreased SC recruitment that implies a fusion defect. We showed a more oxydative phenotype of mutant muscles, with a tendency increase in fiber TypeI (Figure 28). However, the percentage of type I in *Plantaris* is negligible so we investigated the type IIb that is the most representative fiber type in *Plantaris* muscle. Importantly, the percentage of type IIb fibers significantly decrease in mutant. Since the fiber type IIB are the biggest one, this could contribute to the reduction of the average CSA.

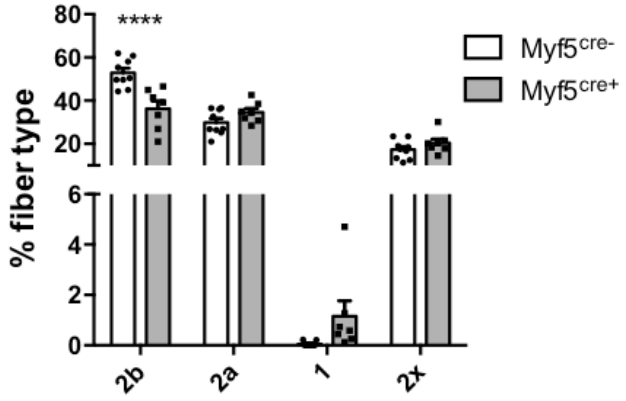

Figure 28: Percentage of fiber types in *Plantaris* muscle on male control and mutant 9 weeks old mice

As previously hypothesized the second possibility is that the smaller CSA could be due to a defect SCs accretion during post-natal muscle growth of the mutant. Once we ascertain that the number of Pax7 positive cells at E18.5 and at day4 post birth are similar between control and mutant (Figure 29A), we investigated the number of myonuclei per myofibers to study the fusion events. Counting the Dapi+ nuclei stained inside the laminin ring at E18.5 and D4 (subtracting Pax7+ nuclei to the total number of nuclei) we observed that the nuclei per myofibers were similar between control and mutant *Plantaris* muscles. However, later on, during post-natal growth at D35 and D60 using Dystrophin/Dapi staining, the number of myonuclei per myofibers was significantly decreased in mutant muscle (Figure 29B). These data underline that the fusion process is impaired only during the post-natal growth and not at embryonic stage and that impaired fusion could account for the smaller CSA of mutant muscles (9 wk).

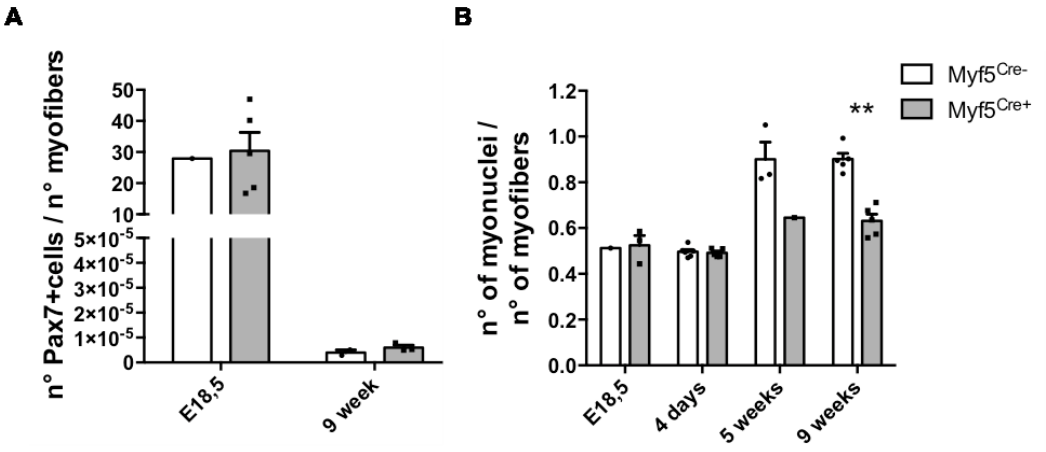

Figure 29: A. Number of Pax7+ cells per myofiber in control and mutant *Plantaris* muscle sections at E18.5 and 9 weeks after birth (n=1-6 muscles) B. Number of myonuclei (DAPI) within dystrophin ring per myofiber at E18.5 and 4 days, 5 and 9 weeks after birth (n=1-6 muscles).

Surely, the addition of more samples is indispensable to confirm the data at E18.5 and 35D.

In order to investigate SCs fusion in adult muscle, we perturbed homeostatic condition in adult mice by performing Cardiotoxin (CTX) injury on *TA* muscle. CTX is the most common used myotoxin extracted from snake venom. Intramuscular injection of CTX provoked a monophasic necrosis of the mouse tissue followed by sequential and synchronous regeneration. We analysed the phenotype at 14D after injection and we investigated the fusion process at this time point of regeneration process. We observed that the fusion was impaired: 35% decrease of the number of myonuclei per myofibers in mutants compared to the controls (Figure 30).

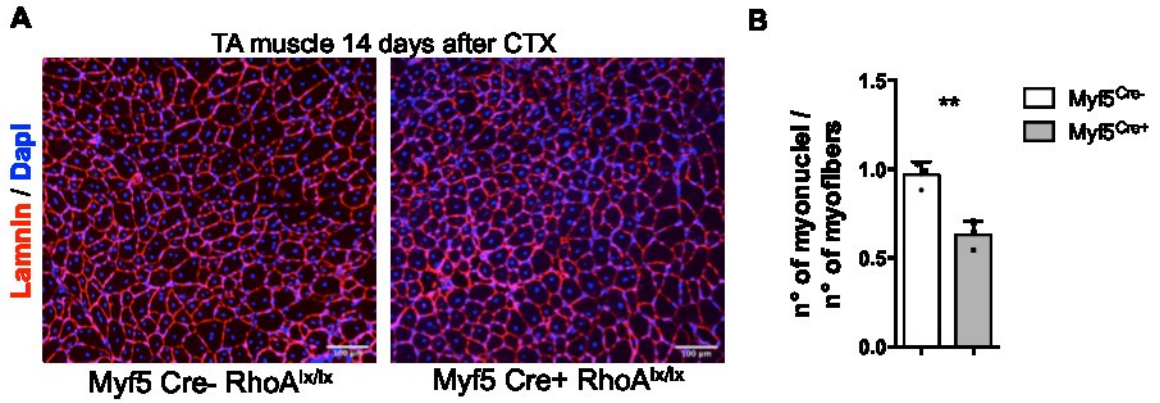

Figure 30: A. *Tibialis* muscle sections immunostained for Laminin (red) and nuclear staining with DAPI for control and mutant mice 14 days after CTX injection. B. Number of myonuclei (DAPI) within dystrophin ring per myofiber at 14 days after CTX injury (n=3 muscles)

Mutant *TA* muscles appear smaller compared to the control (Figure 31A). Even if it will be needed to check the average CSA, we observe by eyes that the regenerated myofibers are tinier (Figure 30A).

Next in order to exclude other possible defects we investigated whether the lack of RhoA in embryonic progenitors could affect SCs niching in adult muscle. Pax7 homing occurs during fetal myogenesis and a right position of SC is crucial to have a correct regeneration (Relaix et al. 2005). However, when we quantified the percentage of SCs outside the basal lamina in adult *TA* muscles it did not change between control and mutant (Figure 31B). Next we verified the number and the proliferation of SCs 14 days after CTX injection and there is no difference between control and mutant (Figure 31). These data suggest that the absence of *RhoA* in embryonic progenitors does not affect the SCs niching in the adult stage. Moreover, the arised adult SCs do not display problems in later stage (14 days) of regeneration (as their number and their proliferation is not affected) that could have justify smaller regenerated mutant muscle.

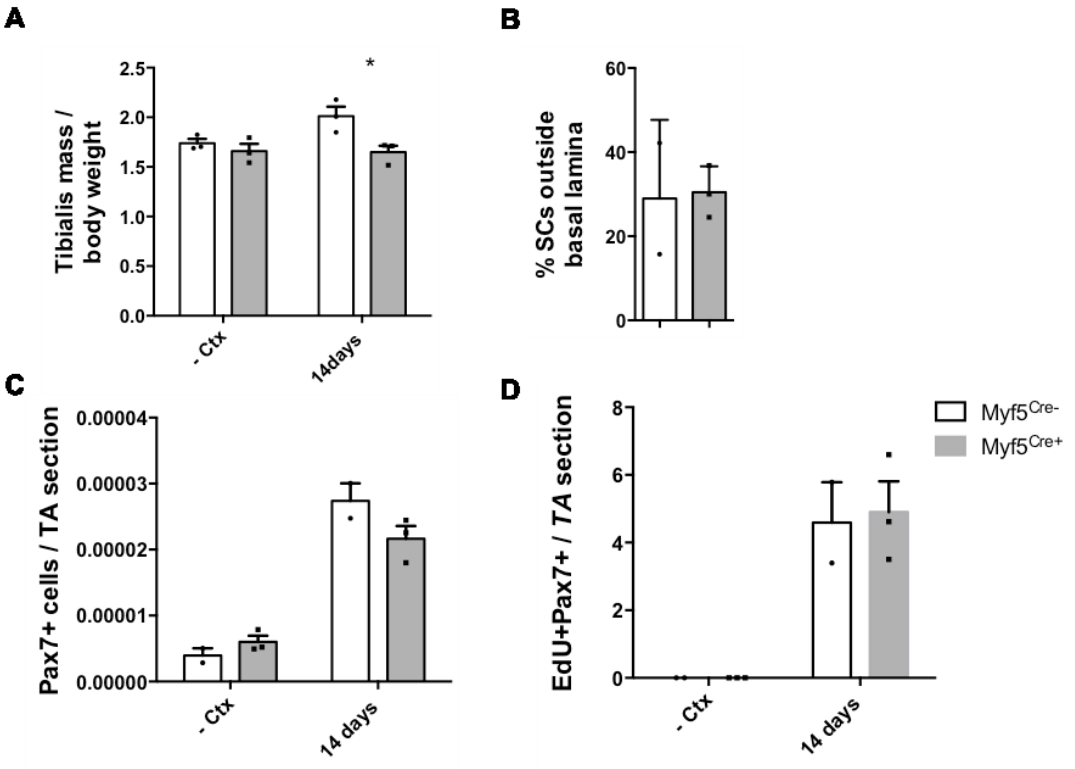

Figure 31: A. Ratio of Tibialis mass (milligrams) to body weight (grams) before CTX injury and 14 days after CTX injury in control and mutant mice (n=3-4 muscles). B. Quantification of SCs outside the basal lamin on control and mutant Tibalis muscle section at basal state. C. Number of Pax7+ cells and D. Pax7+Edu+cells per Tibialis muscle section (with more than 70% of regeneration in injured case) in control and mutant before and 14 days after Ctx lesion (n=2-3 muscle)

Altogether, these results evidence that RhoA is not essential for the embryonic myogenesis and for muscle development but that it is required for efficient fusion in post-natal stages. Indeed, we showed that RhoA controlled adult stem cells fusion, but not EPs fusion. Although GTP-bound activated Rho was increased at E12.5 and E14.5, at E16.5, it decreased, suggesting that Rho GTPases were selectively activated during embryonic myogenesis and shut down at the foetal stage (Taglietti et al. 2018). However, the constitutive RhoA deletion could incite its compensation by other members of the same family. Moreover, previous report demonstrated that embryonic, fetal and postnatal myogenic cells differed in their cellular properties, gene expression and response to signalling molecules (Biressi et al. 2007). This supports our hypothesis that suggests a major discrepancy in the signalling pathways that control fusion during embryonic and post-natal myogenesis. We can conclude that the signalling pathways required to control fusion during development and adult myogenesis are distinct and that RhoA signalling is only indispensable after birth. We thus wondered what could be the function of RhoA in adult satellite cells *in vivo*. Because *Myf5* mouse model allow deletion in progenitors and so in both derived satellites cells and myofibers in adult, we used a more accurate mouse model.

2. Deletion in adult Satellite cells

2.1 Validation of the mouse model

In order to decipher the role of RhoA in adult SCs we used a second mouse strain in which the deletion of *RhoA* was induced only in the adult satellite cells, in two months old mice. Precisely the mouse model used was Pax7$^{CreErt2/+}$:RhoA$^{lx/lx}$:Pax7-nGFP. After tamoxifen (TMX) administration, the Cre mediated excision of *RhoA* exon3 was achieved. As controls we used the same mouse strain not injected with tamoxifen. Even if Pax7$^{+/+}$:RhoA$^{lx/lx}$:Pax7-nGFP gave rise to the same results, control mice Cre- (Pax7$^{+/+}$) and Pax7$^{CreErt2/+}$ were not pooled together and for clarity reasons only the results concerning the mice line Pax7 $^{CreErt2/+}$:RhoA$^{lx/lx}$ more or less tamoxifen injection were shown.

Following TMX injection, efficient loss of RhoA was achieved at the transcript level and at the protein level in FACS sorted SCs (Pax7:nGFP) from control and mutant muscles (Figure 32).

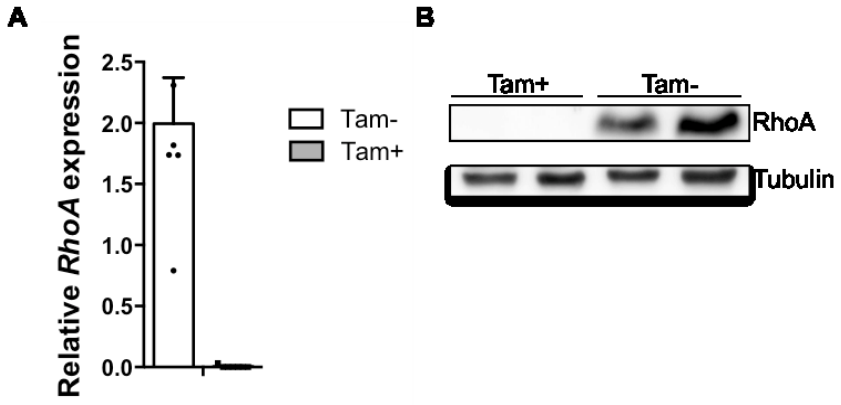

Figure 32: A. RhoA expression was analysed by qRT-PCR in RhoA$^{lx/lx}$ FACS sorted SCs from mice injected (Tam+) or not (Tam-) with Tamoxifen to induce RhoA deletion. Data were normalized by Hmbs expression (n=5 mice). B. RhoA was analysed by Western blot in control and mutant sorted satellite cells maintained in culture. Tubulin was used as a loading control (n=2 mice)

2.2 Similar number of SCs at basal state in control and mutant muscles

After validating the mouse model, we showed that at steady state there are no differences between control and mutant TA muscles. Indeed, we counted the number of SCs and their proliferation, assessed by EdU intraperitoneal injection 24hr and 5hr before muscle harvest.

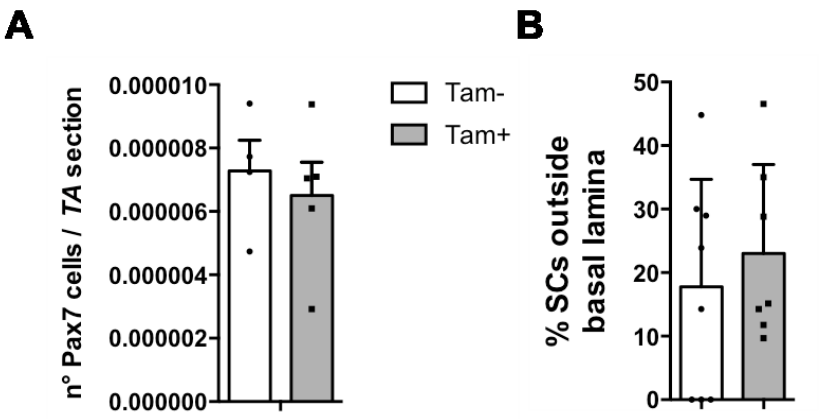

Figure 33: A. Number of Pax7+ cells per *Tibialis* muscle section in control and mutant at basal state (n=4-5 muscles). B. Quantification of SCs outside the basal lamina in control and mutant *Tibialis* muscle sections at basal condition (n = 7-8 muscle).

The results showed that the absence of RhoA in adult SCs did not affect their number (Figure 33A). The rate of their proliferation was zero (Figure 42B) meaning that there is not an abnormal SCs activation at basal state. As well, we assessed their niching counting the satellite cells located outside the basal lamina. Elsewhere was described that RhoA regulates niche-retention of quiescent SCs and that when RhoA is disrupted in adult SCs, 35% of SCs were located outside the niche (Eliazer et al. 2019). On the contrary, our data showed that SCs lacking RhoA were well located, the majority of them being inside the basal lamina (Figure 13B). The divergence between these studies could be explained by the differences between the mouse model used ($Pax7^{CreErt2}$:$RhoA^{lx/lx}$:Pax7-nGFP vs Tg: $Pax7^{CreER/+}$; $RhoA^{lx/+}$) and between the experimental context. Indeed, we verified the number of Pax7 cells and their location at 21 and 37 days after tamoxifen injection and we injected the EdU at given time point while it was chronically injected in study of Brack's laboratory.

2.3 Cadiotoxin approach to investigate adult myogenesis

2.3.1 Loss of RhoA affects a correct muscle regeneration

In order to mobilize the SCs, we injected Cardiotoxin (CTX) into *TA* muscle to chemically induce injury. We studied the regeneration process at different time point as showed by the illustrated timeline (Figure 34). *TA* muscles were harvested at 3, 4, 8, 14 and 30 days after CTX-injury for further analyses.

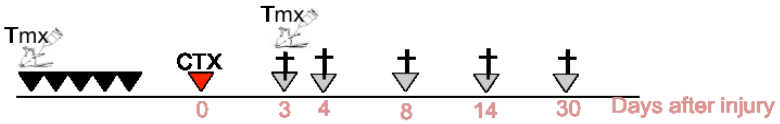

Figure 34: RhoA mutant mice were injected with TMX 1 week before and the third day after CTX injury. *TA* muscles were harvested 3, 4, 8, 14 and 30 days after muscle damage.

Although at basal state there were no evident differences between control and mutant SCs, the regeneration process of mutant muscle was impaired (Figure 35).

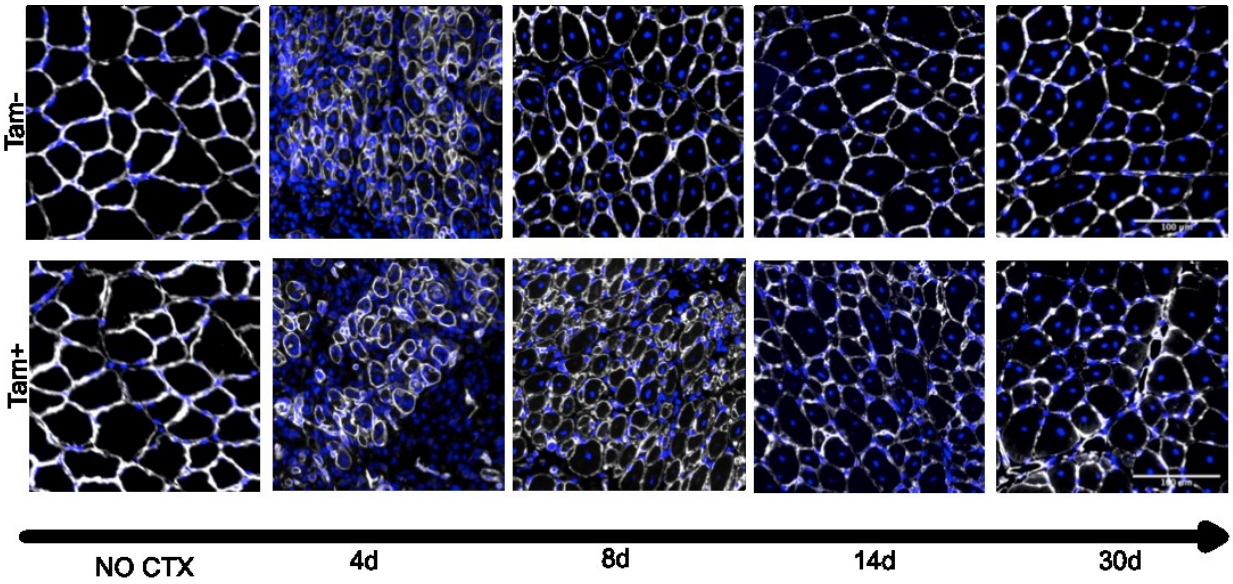

Figure 35: Representative images of *TA* muscle sections immunostained for Laminin (gray) and nuclear staining with DAPI for control and mutant mice at steady state, 4, 8, 14 and 30 days after CTX injury.

At 8, 14 and 30 days after CTX injury, mutant *TA* muscle masses normalized per mouse body weight were smaller (Figure 36). Of note, the phenotype 14 days after CTX injury reminded the one observed in *Myf5*-Cre model, confirming that the defect emerged in adult stage.

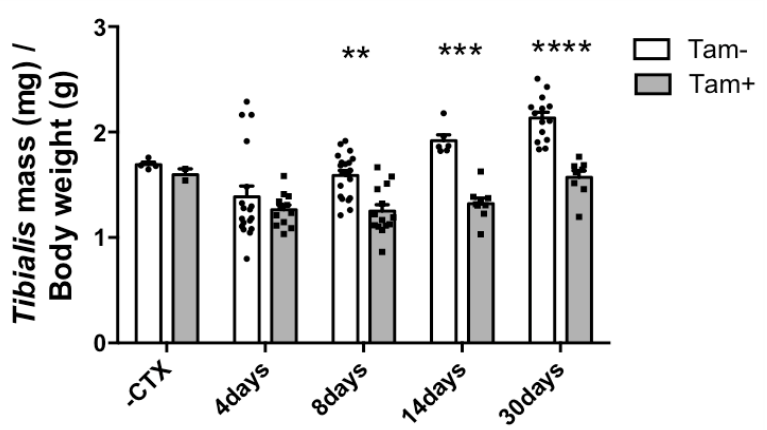

Figure 36: Ratio of *Tibialis* mass (milligrams) to body weight (grams) before CTX injury and 4, 8, 14 and 30 days after CTX injury in control and mutant mice (n=2-20 muscles).

To further determine whether the decreased muscle mass in mutant mice resulted from the reduced myofibers size or from reduced myofiber number, we quantify both parameters (Figure 37). The absolute number of myofibers per muscle counted at different time points during the regeneration process did not change between control and mutant (Figure 37A). On the contrary the CSA of regenerated myofibers was strongly diminished in muscle lacking RhoA in satellite cells (Figure 37B).

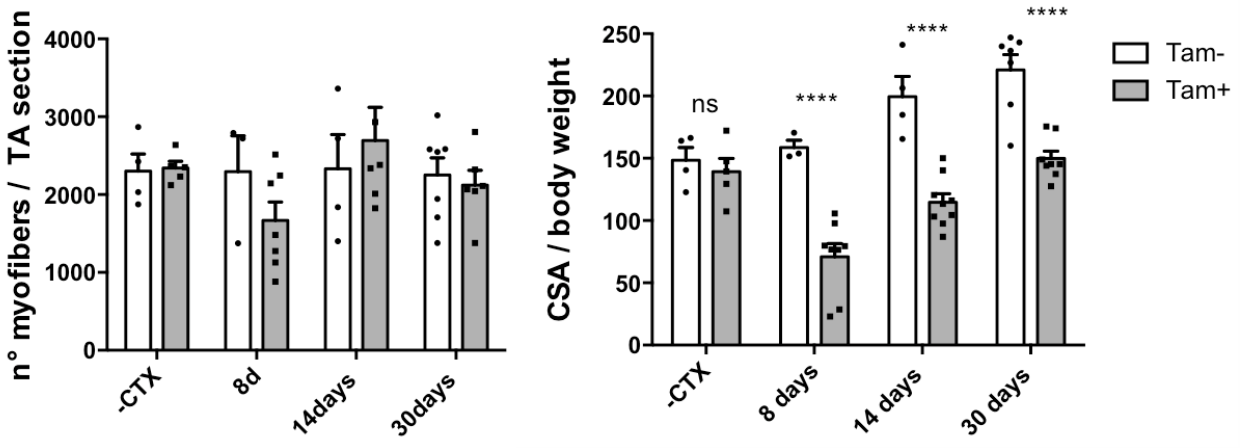

Figure 37*:* A. Myofibers number counted in the regenerated area (>70% of total area) on *TA* section B. Mean CSA (scare micrometers) of regenerated fibers in control and mutant mice normalized per body weight (n=2-20 muscles)

The time point at 4 days is not shown because we are not sure that laminin+ myofibers are indeed myofibers, looking at hematoxylin/eosin staining (Figure 38A). Indeed empty fibers, renamed ghost fibers, surrounded by basal lamina were described at 1 day post injury (Webster et al. 2016). At

basal state and before 3 days post injury, embryonic myosin heavy chain (eMHC) was not expressed in control nor in mutant *TA*. eMHC staining of mutant and control *TA* muscles three days after CTX did not achieve to clearly disclose its expression because of the high background signal and the presence of diffuse necrosis of the tissue and inflammatory infiltration. For this reason, to identify the newly formed myofibers we immunostained the regenerative muscles with eMHC 4 days after CTX injury. eMHC is expressed during embryonic development but is also transiently expressed in adult muscle during regeneration, being a robust marker of muscle regeneration (Forcina et al. 2020). As revelead by immunofluorescence staining eMHC had a different expression pattern in the mutant compared to the control at 4 days post injury (Figure 38A).

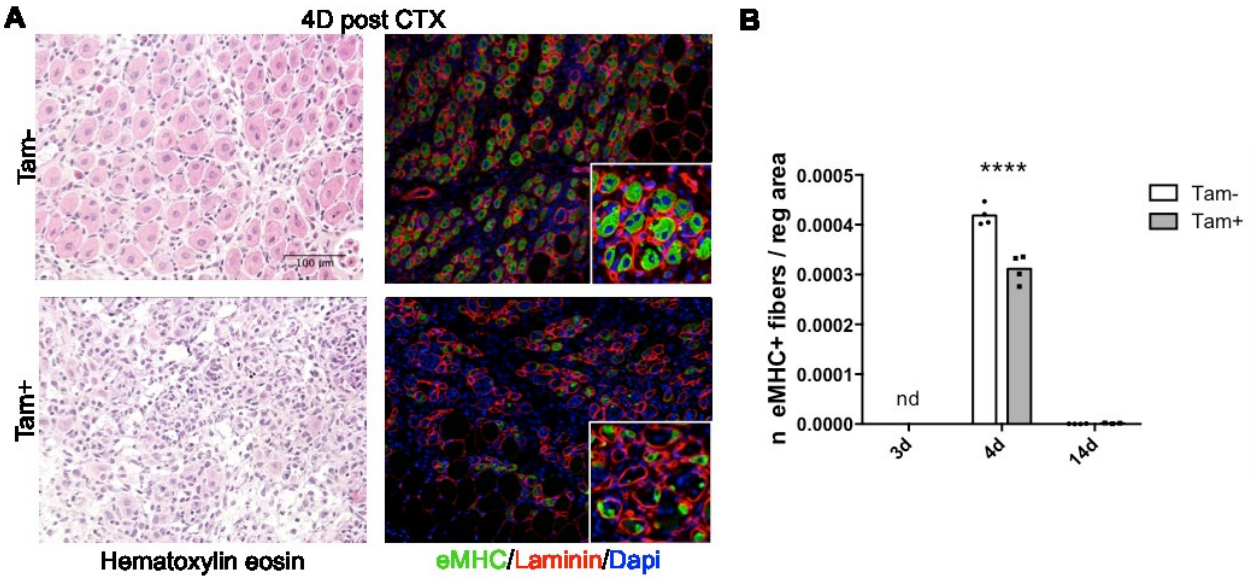

Figure 38. A Representative image of Hematoxylin /Eosin and eMHC staining of *TA* muscle sections at 4 days after CTX injury. B. Quantification of eMHC+ fibers normalized per regenerated area (n=4 muscles).

Moreover, we quantified the number of myofibers eMHC+ not considering laminin positive demarcation. Unfortunately, as previously justified we were not able to quantify the staining at 3 days. However, there is a significative difference between control and mutant at 4 days after CTX injury. Fourteen days after injury the difference was equalized and decreased in both genotypes. The quantification of CSA of eMHC+ myofibers will help to precisely characterize CSA at early time point of regeneration even if the difference appears visible by eye.

In agreement with eMHC staining at 4 days post CTX, *eMHC* mRNA expression was significantly reduced in the mutant 3 days after CTX injury (Figure 39).

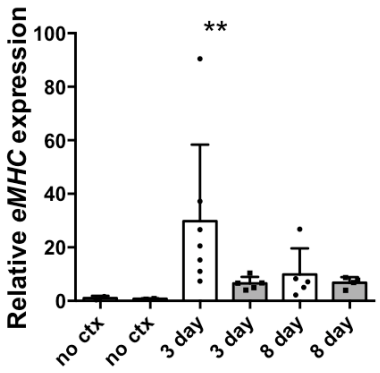

Figure 39*: eMHC* mRNA expression by qRT-PCR in control and mutant muscles from *TA* muscles before, 3 and 8 days after CTX injection (n=2-7 muscles)

All these data suggest a default in mutant muscle repair. The transient decrease of eMHC transcript and protein levels during early phase of regeneration process could be due to a defect in myofibers maturation. It will be interesting to check the expression of adult myosin, when complete regeneration is occurred, to verify if this decrease affects the functionality of mutant regenerated muscle.

2.3.2 Loss of RhoA in adult SCs affects transiently their number at the beginning of regeneration process

Because the regeneration of mature myofibers is dependent of SCs, we investigated the behaviour of SCs lacking RhoA as compared to controls. At first, we counted, based on Pax7 expression, the absolute number of satellite cells on *TA* muscle sections (only when there are more that 70% regeneration) or the number of satellite cells normalized per regeneration area (Figure 40A-B).

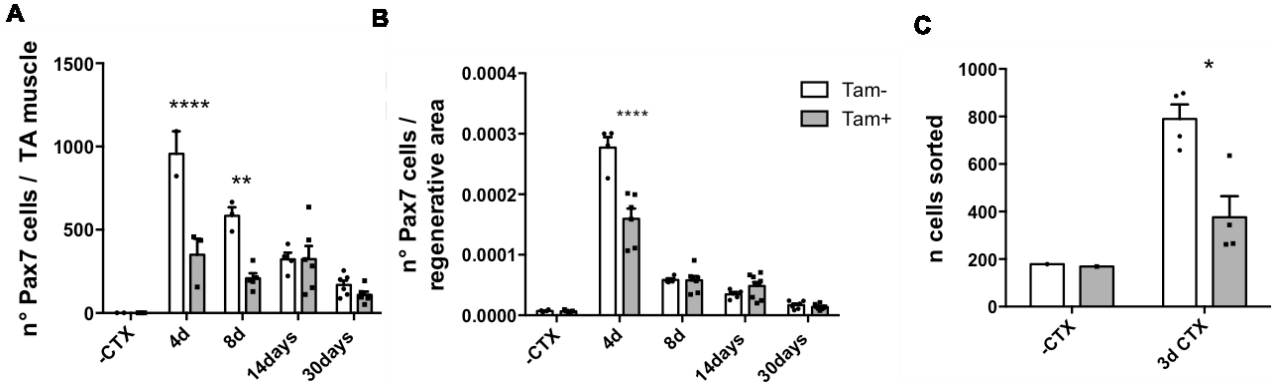

Figure 40: A. Absolute number of Pax7+ cells per TA muscle section entity. B. Number of Pax7+ cells normalized per regenerative area on TA transversal section at 4, 8, 14 and 30 days post CTX injury (n=2-20 muscles). C. Number of GFP FACS sorted control and mutant SCs before and after 3 days of CXT injury

At steady state, as previously verified, both control and mutant displayed the same number of Pax7+ cells. Four days after injury, SCs lacking RhoA were less in the number, coming back in a normal range in the next days. If we consider the absolute number of cells, it remained less also at 8 days after CTX injury (Figure 40A-B).

The immunofluorescence staining was representative of an arbitrary fixed area in the muscle and moreover at early time point cells counting could give rise to some errors due to un-specific signal appeared in injured tissue. So, to verify the number of SCs at an early regeneration time point, 3 days after CTX injury, *TA* derived SCs were purified (based on Pax7-nGFP transgene expression) and counted by FACS. Similarly to IF staining, the number of Pax7+ cells at steady state did not change between control and mutant. After CTX, satellite cells number increased 4 fold in the control, but was significantly less in the mutant (Figure 40C). Compared to previous data from our laboratory, the number of collected satellite cells by FACS was rather low and could probably be due to inefficient tissue digestion or to the loss of cells during the washing during experimental process. Anyway, the difference evidenced between control and mutant could be taken in account considering that both samples were treated in the same way.

a) Is the decreased number of RhoA mutant SCs is caused by apoptosis ?

This transient decrease of SCs number could be correlated to a default in a correct balance between apoptosis and proliferation. In order to understand if the reduced number of Pax7+ cells observed in the mutant at early stages of regeneration could be attributed to an altered cell survival, we performed TUNEL assay. The Apoptotag kit labels apoptotic cells, detecting single and double stranded breaks associated with apoptosis. On muscle sections 4 days after CTX injury, no satellite cells were apoptotic both in the control and in the mutant, as there are no cells double stained for Pax7 and TUNEL (Figure 41). Following tissue injury, it is normal to find nuclei TUNEL+ because of extensive tissue damage, however they were not SCs. It was shown that 18 hours after CTX injection 40% of SCs were lost, but that in a wild type mouse, four days after injury there was no apoptosis observed (Hardy et al. 2016). These data support the fact that there are no SCs Pax7+ TUNEL+ in our experiments. However, we did not quantify the myonuclear apoptosis (Tunel+ myonuclei). Indeed, myonuclear loss implicated disadvantages to achieve a correct muscle recovery during regeneration process (Fry et al. 2016). The absence of TUNEL+ SCs suggests that the apoptosis does not contribute to the decline of SCs number observed at four days post injury.

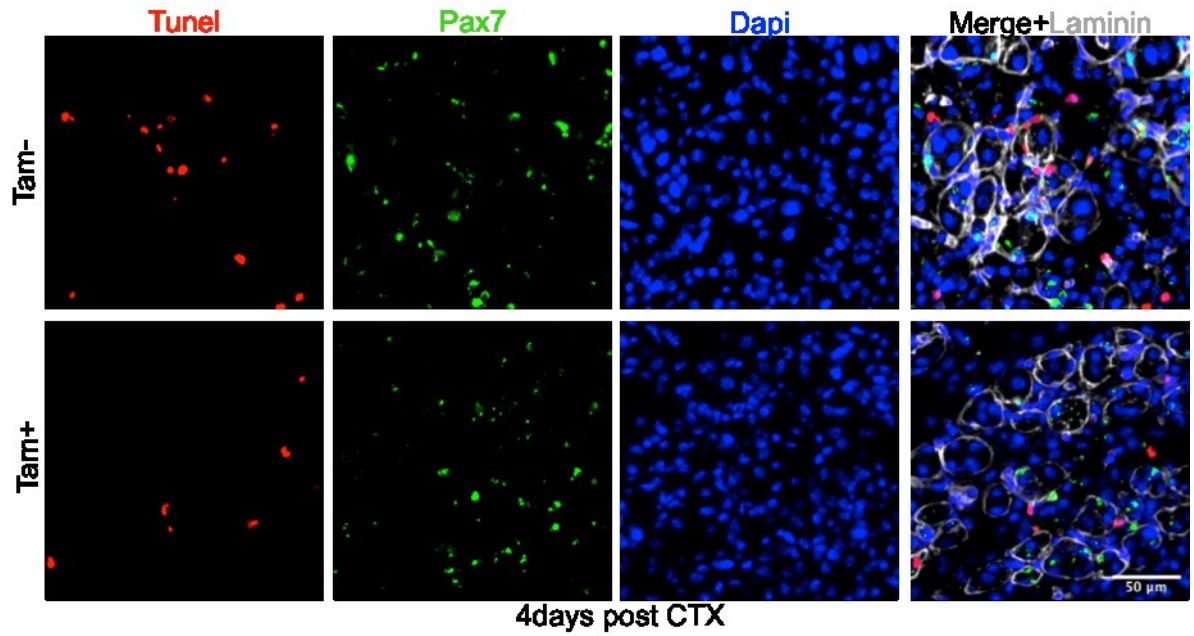

Figure 41: Immunostaining for Pax7 (green) and TUNEL (red) to illustrate apoptotic cells 4 days after CTX in control and mutant *TA* muscles.

b) Is the decreased number of RhoA mutant SCs caused by an activation delay ?

The reduced number of SCs could suggest a delay in the activation of SCs lacking RhoA.

In order to inestigate SCs activation, we examined SCs on freshly isolated single myofibers. We cultured control and mutant single myofibers to follow SCs activation and progression toward differentiation. Indeed, single fibers isolation is considered as a useful tool (*in vitro* approach) to investigate behaviour of *in situ* SCs. However, different parameters should be taken in account such as the isolation protocol used (mechanical / enzymatic) and the culture condition (plate coating / medium composition) (Renzini et al. 2018). In our instance, fresh single myofibers were isolated by enzymatic digestion (collagenase Type A), they were kept floating in DMEM/calf serum and they were fixed after twenty hours. The percentage of Pax7+/MyoD-, Pax7+/MyoD+ and Pax7-/MyoD+ were quantified to indicate activated and proliferating satellite cells (Pax7+/MyoD+) and satellite cells engaged in differentiation process (Pax7-/MyoD+). Preliminary results suggested that there was a shift in mutant satellite cells activation as pointed out by the more evident difference between the percentage of Pax7-/MyoD+ SCs, that is higher in control compared to the mutant (Figure 42).

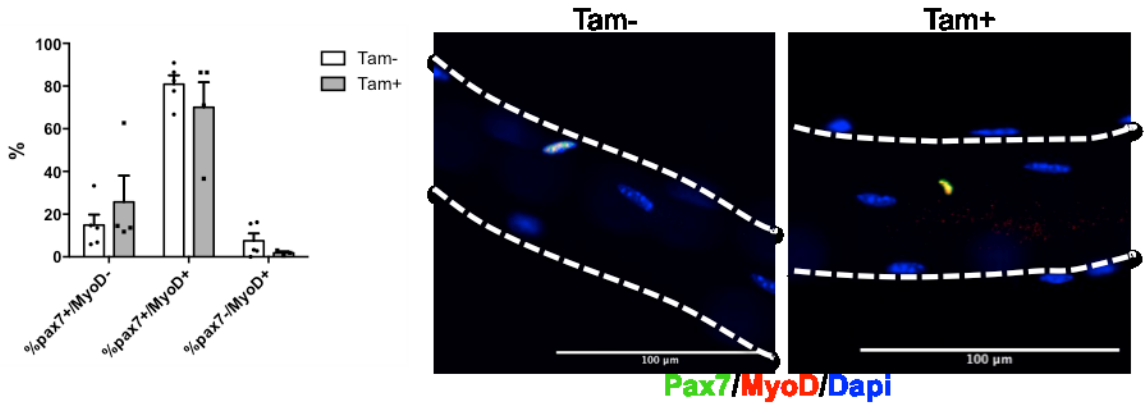

Figure 42: Quantification of Pax7+/MyoD-, Pax7+/MyoD+ and Pax7-/MyoD+ cells on single isolated myofibers after 20hr in culture medium (n=3-5 mice)

However, because these are preliminary data, it is too soon to definitively conclude. Moreover, it would be interesting to check SCs proliferation rate doing an EdU pulse on isolated myofibers in order to "snap" the first cycle entry of SCs virtually retained in their own niche. This experiment approach will give us an idea on mutant RhoA SCs activation.

c) Do RhoA lacking SCs proliferate less?

Considering the activation step as the first entry in the cell cycle, we wondered if the reduced number of satellite cells RhoA-depleted came from an alteration in their proliferation, i.e. if they proliferated less. In order to investigate the proliferation potential of satellite cells *in vivo* upon CTX-injury, we injected intraperitoneally EdU, 24hr and 5hr before mouse sacrifice. EdU is a nucleoside analog of thymidine that incorporate into DNA during active DNA synthesis, staining the S-phase entry of the cells. By a double staining EdU and Pax7 on *TA* muscle cryosections, we asserted the number of proliferating satellite cells. The percentage of Pax7+/EdU+ cells revealed no difference between control and mutant satellite cells entering in S phase. Moreover, we observed an extensive satellite cells proliferation at day 4 after CTX that progressively came back to quiescence state (day 30) (Figure 43).

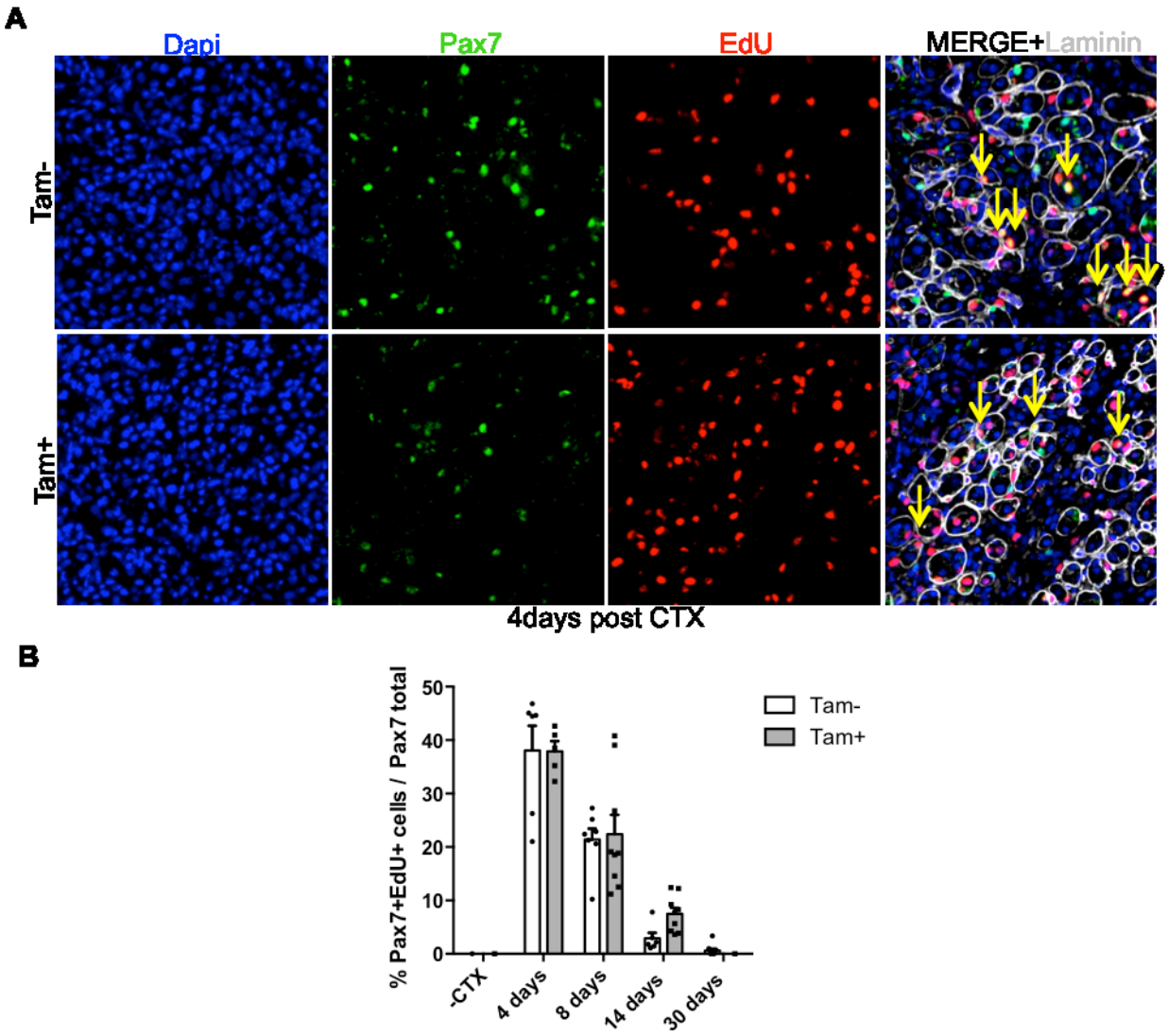

Figure 43: A. Immunostaining for Pax7 (green) and EdU (red) to illustrate proliferating satellite cells (yellow narrow) 4 days after CTX in control and mutant *TA* muscles. *B.* Quantification (n=2-20 muscles)

Although these results were convincing and robust, Ki67 staining was required to characterize all the proliferating SCs and not only the entered in S phase of cell cycle 24hr before muscle harvesting.

To confirm the *in vivo* results, EdU incorporation was assessed in proliferating myoblast *ex vivo*. Thus, we verified the proliferation potential of *Rho*-deleted SCs *ex vivo* on FACS sorted control and mutant primary myoblasts in which *RhoA* deletion occured *in vivo* after TMX injection. After 5 days in culture, the primary sorted cells received a pulse of EdU for two hours. We assessed that the proliferation rate of control and mutant myoblast was similar (Figure 44).

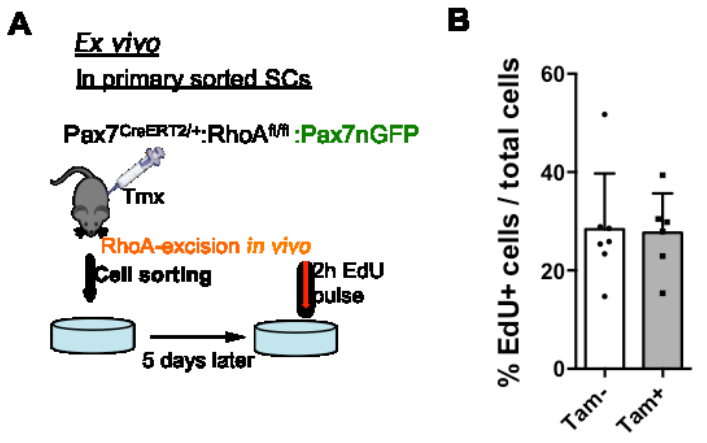

Figure 44: A. Experimental model for primary cells isolation and culture protocol B. Quantification of EdU+ myoblast after 5 days in culture and a pulse of EdU of 2hr.

In addition upon isolation and culturing *ex vivo*, mutant and control myoblast cycle entry was measured by EdU incorporation (constant exposure of cells to EdU) from 43 hours until 96 hours after plating. Unfortunately, four days after plating we had only one experiment and we observed a reduction of number of cells, maybe suggesting a toxicity of EdU constantly in the medium. But at least at 43 and 48 hours after plating there was no difference between control and mutant. However the percentage obtained for the cycling cells was lower to what was obtained in other similar experiments already published, where the proliferation rate of quiescent in culture was around 20% twenty hours after plating (Rodgers et al. 2014). Indeed, depending on mouse strain and EdU concentration used, results could be different. Anyway, these are preliminary results that should be confirmed adding new samples and adjusting experimental condition.

2.3.3 Does RhoA loss in SCs affect their differentiation potential ?

In vivo

We verified *Myogenin* expression three days and eight days after CTX injection. Even if *Myogenin* expression level increased significantly at 3 days in both genotypes, it had a tendency to be lesser in mutant compared to the control, suggesting a default in differentiation process. In later stages *Myogenin* transcript decreased synergistically with the progress of regeneration course (Figure 45A). Nevertheless, there was not anymore difference between control and mutant mice. These last results were confirmed at 14 days after CTX injury, when Myogenin protein level was investigated by immunofluorescence on cryosections. No difference in MyoG+ cells was evidenced between control and mutant (Figure 45B).

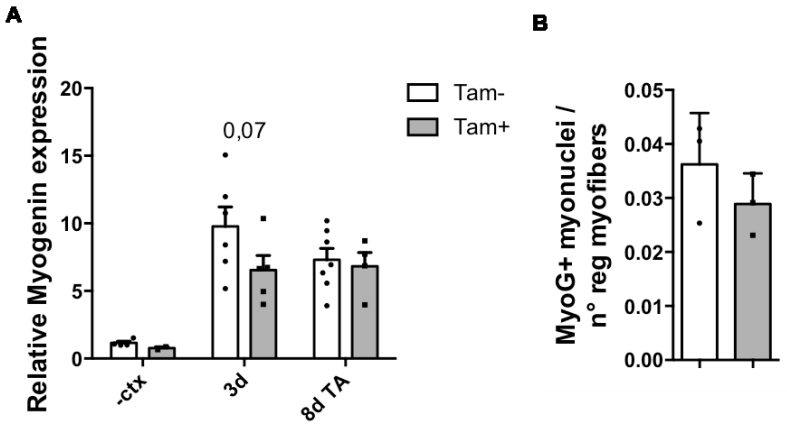

Figure 45: A. *Myogenin* mRNA expression by qRT-PCR in control and mutant *Tibialis* muscles before, 3 and 8 days after CTX injection (n=2-9 muscles) B. Number of Myogenin+ myonuclei normalized per number of fibers in regenerative area on *TA* transversal section at 14 days post CTX injury (n=3 muscles)

These preliminary results suggest that there could be a mild deficiency in differentiation process in the early phases. Therefore, *Myogenin* expression and Myogenin protein level should be explored between 3 and 8 days after injury to better disclose if there is a shift of the differentiation events.

In vitro

To gain further insights, the differentiation was investigated *in vitro* on cultured primary myoblasts. The myogenic potential of primary sorted RhoA mutant satellite cells was investigated. *MyoD* and *Myogenin* transcript levels did not differ between control and mutant under proliferation (day 0) and differentiation conditions (day 1 and day 3) (Figure 46A-B). In addition, we monitored the expression of late differentiation marker (Myosin Heavy Chain, MyHC) in control and mutant cells at day 3 post differentiation using MF20 antibody. As shown by the differentiation index (i.e. the number of cells MyHC+ normalized on the number of total nuclei), the absence of RhoA did not impair the engagement on myogenic differentiation as all the mutant cells were MyHC+ (Figure 46 C).

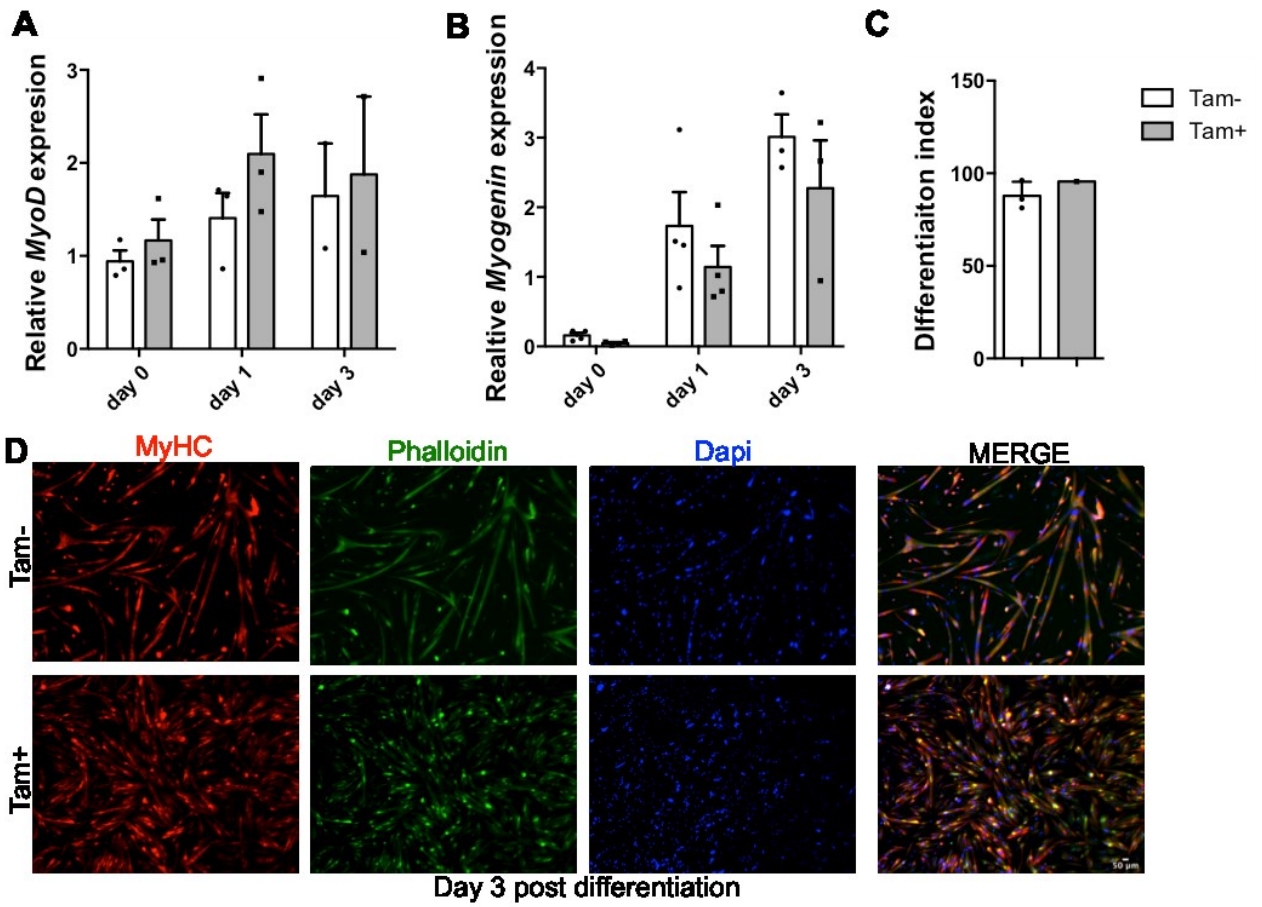

Figure 46: *MyoD* (A) and *Myogenin* (B) mRNA expression by qRT-PCR in control and mutant primary proliferating myoblast (Day0) and differentiating myocytes (Day1-Day3) (n=2 cultures). C. Percentage of MyHC+ cells on total number of nuclei in control and mutant cells 3 days after differentiation induction (n=2 cultures). D. Immunostaining for MyHC, F-actin staining with Phalloidin and nuclear staining with DAPI on control and mutant cells 3 days after differentiation induction.

2.3.4 In vitro investigation of myoblast motility

Coordination of migration and fusion of SCs is essential to have a correct regeneration process (Webster et al. 2013). We wondered if RhoA plays a role in myoblast migration that could explain the poor regeneration phenotype of mutants. Cell migration was monitored by live cell imaging (time-lapse microscopy) and velocity was measured. Mean velocity (µm/s) was calculated for individual myoblast (control and mutant) cultured for 4 hours and 30 minutes (Figure 47A). There was not a significant difference among the control and the mutant cell migration. Interestingly RhoA, together with other members of Rac family (Cdc42 and Rac) is involved in lamellipodia and filopodia formation that determine the direction of the migration at the cell's edge. We observed that the traces of migration path of mutant and control myoblast recorded for 4 hours and 30 minutes were different. Control migratory path were more linear with an elevated distance between the point of origin and the point of end of record, contrary to the mutant (Figure 47B). All these

data indicate that RhoA is important for a correct and efficient migration even though velocity is not affected.

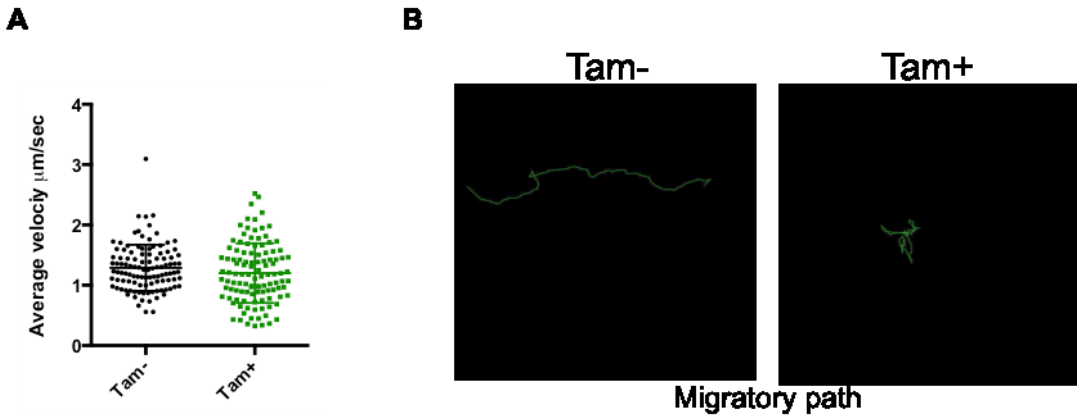

Figure 47: A. Mean velocity (micrometers per seconds) of control and mutant myoblasts determined by time-lapse videomicroscopy (n=200 cells) B**.** Representative migratory path of control and mutant myoblast.

2.3.5 Could the impaired regeneration of mutant muscles be caused by impaired fusion capacity of SCs?

In vivo (Cardiotoxin approach)

Next, we investigated the fusion of satellite cells, a crucial step after differentiation to allow the formation of newly regenerated myofibers.

To determine whether the fusion capacity of RhoA depleted satellite cells is affected, myonuclei number inside the sarcolemma are counted in *TA* muscle section at different time points after CTX injury, by performing laminin/dapi staining. In control the number of myonuclei per myofibers comes back to the steady state already eight days after CTX injection. In contrast in mutant muscles this number is significantly decreased as compared to control. These data suggest that the lack of RhoA in SCs elicits an important fusion defect and in turn an impaired muscle regeneration (Figure 47). Interestingly this phenotype reminds the *Myf5*^{Cre} adult phenotype, where the fusion was analysed 14 day after CTX injection.

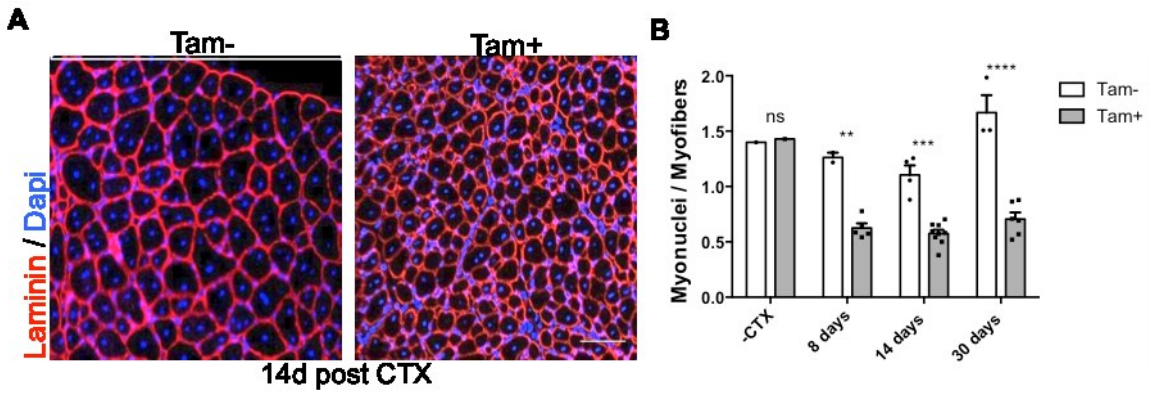

Figure 47: A. Representative images of *TA* muscle sections immunostained for Laminin (red) and nuclear staining with DAPI for control and mutant mice at 14 days after CTX injury. B. Number of myonuclei (DAPI) within laminin ring per myofiber at 8, 14 and 30 days after CTX injury (n=1-7 muscles).

In vivo (Overload approach)

We conducted *Plantaris* compensatory hypertrophy approach (overload) to investigate the fusion event in another context. Whether regeneration experiment recapitulate homotypic fusion event between mutant myoblasts, the overload induced hypertrophy recapitulate heterotypic fusion between wild type myofibers and mutant satellite cells (in our model Pax7^{CreERT2}). We found that the absence of RhoA in SCs affectes the growth of *Plantaris* muscle after hypertrophy (Figure 48A). This defect is correlated with an impaired fusion of mutant satellite cells to wild type growing myofibers (assessed by EdU staining within myofibers) (Figure 48B-C). We did not investigate SCs activation in this experimental approach, even if the signals triggering SC activation during regeneration process and during hypertrophy process are different and maybe governed by different pathway. Indeed, upon CTX injury muscle tissue is completely destroyed, while during overload approach SC niche is conserved. Despite these differences RhoA seems to be required for both heterotypic and homotypic fusions. The fact that the fusion events are strongly affected suggests that RhoA is necessary in both skeletal muscle compartments to achieve a correct fusion. However, *in vitro* experiment reproducing heterotypic fusion between control myotubes and mutant myoblast are needed to imply the necessity of RhoA in both myogenic partner in order to fuse.

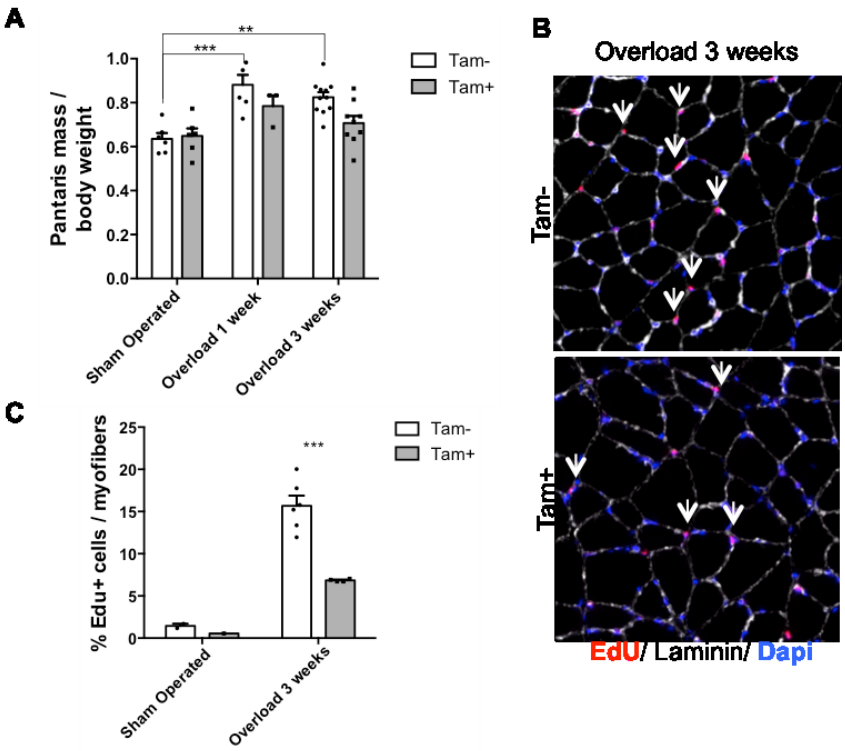

Figure 48: A. Ratio of *Plantaris* mass (milligrams) to body weight (grams) in sham operated mice, & week and 3 weeks after overload in control and mutant mice (n=5-11 muscles). B. Rappresentative images of immunostaining for EdU (red), Laminin (gray) and Dapi to illustrate proliferating myofibers (white narrow) 3 weeks after overload in control and mutant *Plantaris* muscles C. Quantification of precedent illustrated staining

In vitro

In order to further investigate mutant SC fusion potential, we performed *in vitro* experiments on primary myoblasts.

Control and mutant SCs were isolated by FACS, placed in culture and let them differentiate for three days. RhoA mutant cells are unable to form "classical" myotubes (Figure 48A). The number of myonuclei per myotubes, interpreted as the number of nuclei in differentiated cells with more than two myonuclei, was much lower in the mutant compared to the control (Figure 48B). Accordingly, the fusion index, that represents the proportion of mononucleated cells that fused, was lower compared to the control (Figure 48C). Collectively this data shown that RhoA loss in SCs strongly affects fusion events.

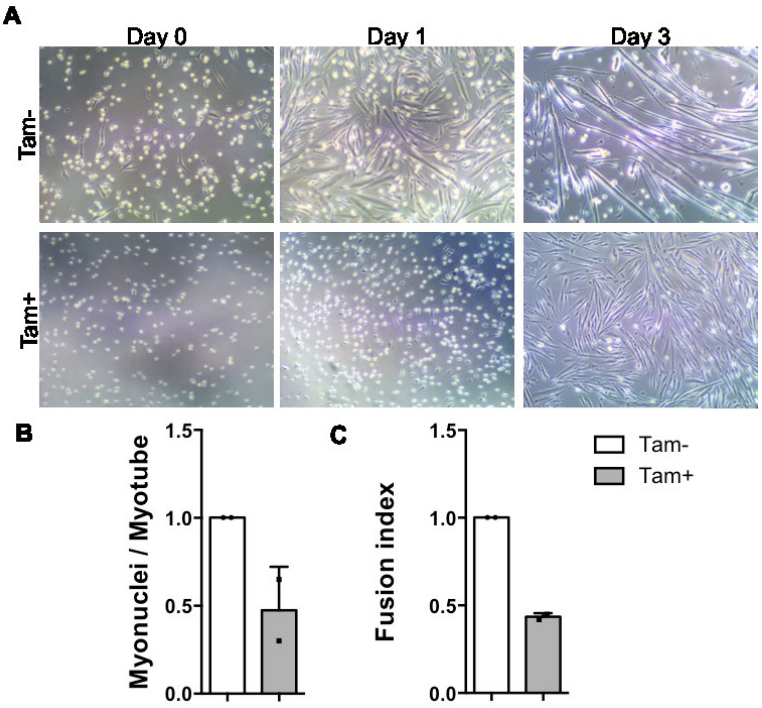

***Figure 49**:* A. Phase-contrast representative pictures of FACS sorted control and mutant SCs cultured in proliferation medium (Day0) or in differentiation medium (Day1 and day3) B. Normalized number of nuclei MyHC+ in control and mutant cells at day 3 of differentiation C. Proportion of nuclei within multinucleated cells in control and mutant cells 3 days after differentiation induced.

2.3.6 Which could be the molecular mechanisms underlying mutant SCs fusion defect?

Actin cytoskeleton organization.

A recent publication from our laboratory showed the role of actin cytoskeleton in myoblast fusion, describing finger-like actin-based protrusions formed at the site of fusion, using metal-replica EM on unroofed myoblasts. The absence of these structures in Srf mutant myoblasts is correlated with their impaired fusion capacity (Randrianarison-Huetz et al. 2018). So, the formation of the actin-based protrusion at the cell-cell contact between fusing cells is regulated by Srf, which is a downstream transcriptional effector of RhoA. In addition, RhoA is a major regulator of cell contractility and actin cytoskeletal reorganization, inducing the formation of stress fibers and focal adhesions. Thus, we wonder whether RhoA mutant myoblasts evidence obvious cytoskeleton defects that could implicate the fusion deficiency. Using confocal microscopy on control and mutant proliferating myoblasts stained for F-actin we did not observe recognizable differences. There is a lot of variability among cells in term of shapes and there is not a primary and reproducible defect in mutant cells. By confocal images, actin cytoskeleton (stained by Phalloidin reaction) did not show aberrations in mutant cells. Actin cables and cortical actin are clearly distinguishable in both control and mutant (Figure 50A). Phalloidin intensity signal measured by

ImageJ software normalized by the surface area of the cells was not reproducibly different between control and mutant cells. Indeed, depending on the cell culture, F-actin amount was increased or decreased in *RhoA* deleted myoblasts compared to controls (37-115 cells counted) (Figure 50B).

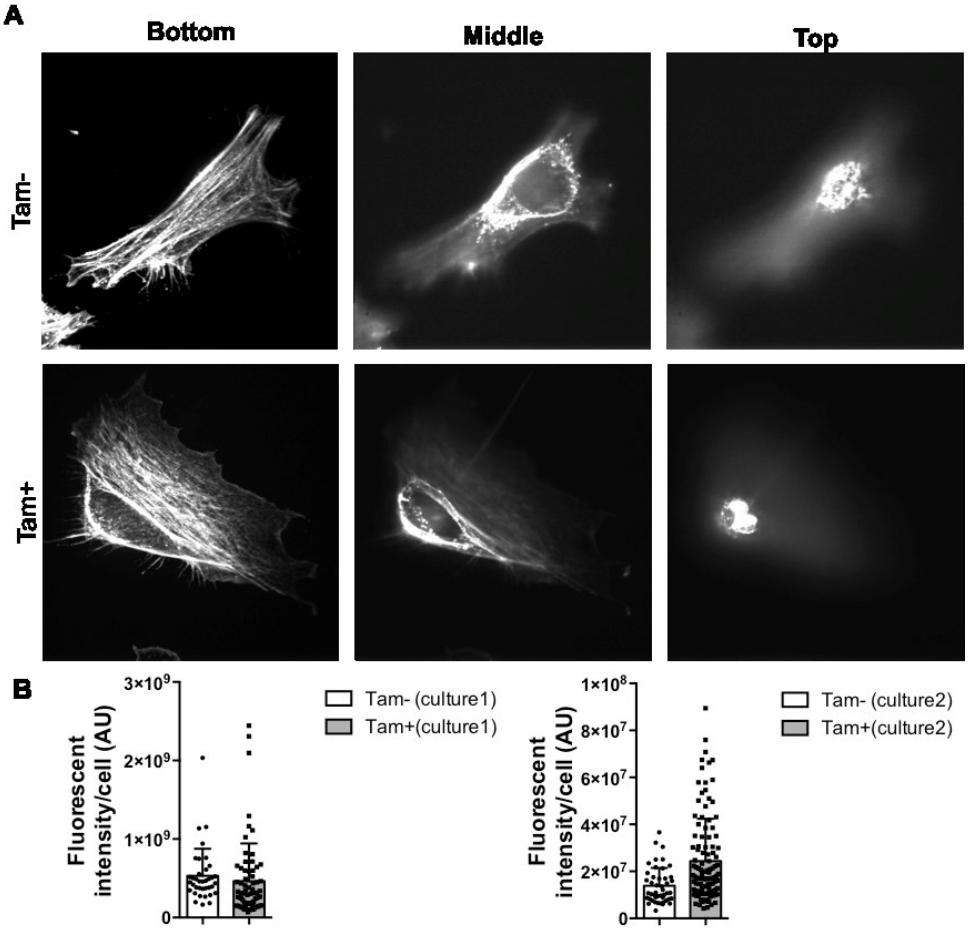

Figure 50: A. Representative confocal projection of z-sections of F-Actin staining (Phalloidin) taken from the adherent (ventral), cell bottom and middle and proceeding to the media-facing top of the control and mutant myoblasts B. Quantification of F-actin by measuring the total Phalloidin fluorescence intensity per cells (ImageJ)in control and mutant myoblasts.

In order to quantify the proportion of monomeric globular actin (G-actin) and filamentous actin (F-actin) we should quantify soluble G-actin and insoluble F-actin fractions in muscle cells, as a way of verify the actin polymerization, more than the total quantity. Perspective studies include:

- the investigation of actin cytoskeleton by metal-replica EM on unroofed myoblasts, in order to visualize the ultrastructure of actin cytoskeleton and so obtain additional insight into the precise organization of the actin-based protrusions in mutant myoblast.
- the analysis of RhoA mutant actin cytoskeleton on micro-pattern that confine the cells in standard shape, in order to compare different cultures among them.

Contractility

It is well known that one of the major downstream target of RhoA is RhoKinase (ROCK). The major substrate of ROCK is MLC2 and MYPT, leading to an increase in MLC2 phosphorylation and consequently to an increase of actomyosin contraction. In *Drosophila* it has been shown that MLC and cortical rigidity mediate the invasion of fusion partners and that its mutation leads to myoblast fusion impairment (Kim et al. 2015).

Unpublished data from our laboratory reported that treating control myoblasts with Blebbistatin (a potent inhibitor of myosin II ATPase activity) strongly impaired their fusion capacities (K.Kobon and V.Randrianarison-Huetz personal communication). So, we hypothesized that the decreased fusion capacity of mutant myoblasts could be due to a defect in cell contractility originated from impairment of MLC2 phosphorylation expected after perturbation of RhoA-ROCK axis.

Surprisingly the total intensity (mean intensity x surface, to delete eventual bias due to different cell shape) of pMLC2 signal, quantified by immunofluorescence, did not change between control and mutant (Figure 51A). As well pMLC2 protein quantity assessed by Western blot was not affected in the mutant. In both genotypes pMLC2 protein level decreased after ROCK inhibitor treatment, underling the specificity of antibody used, but not difference was evidenced between control and mutant (Figure 51B). These data suggest that there are other compensatory molecules, beyond RhoA signalling, that trigger MLC2 phosphorylation and that the fusion defect is maybe independent to Myosin2 phosphorylation. However, these are preliminary results that should be confirmed in additional myoblast cells cultures. Perspective studies will involve biophysicist collaborators in order to measure myoblast cortical tension using single cell rheometer. This will help us to discern whether impaired mechanical tension could partially account for the defective fusion of RhoA mutant myoblasts.

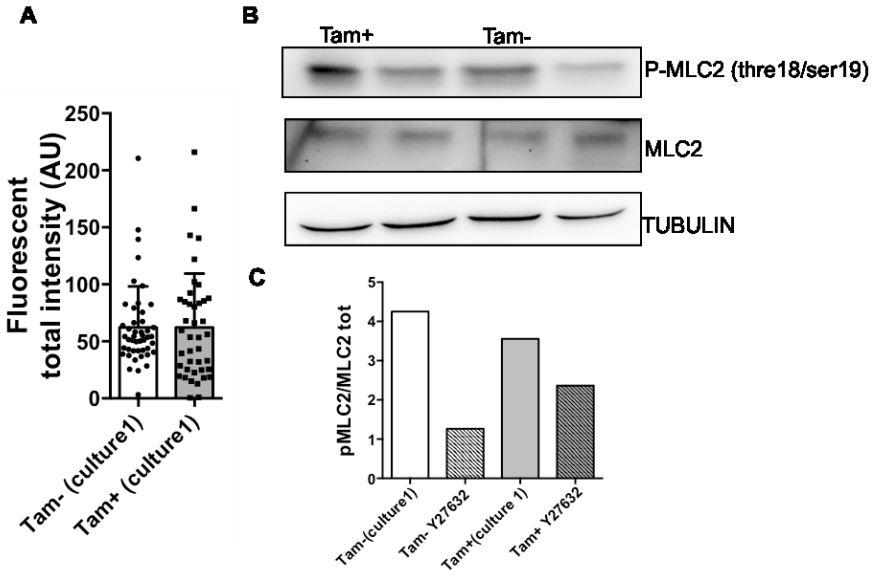

Figure 51: *A.* Quantification of pMLC2 signal by measuring the total fluorescence intensity per cells (ImageJ) in control and mutant myoblasts B. Immunoblot showing total MLC2 and pMLC2 in control and mutant with or without ROCK inhibitor treatment. Tubulin was used as a loading control C. Quantification of pMLC2/MLC ratio from immunoblot already normalized by tubulin

Transcriptomic study to identify new partners involved in myoblast fusion

In order to point out genes and signalling pathways involved in the impaired fusion of *RhoA* deleted myoblasts, we performed a microarray analysis on myoblast expressing or not RhoA at three stages: proliferating myoblasts (D0), myocytes engaged in differentiation (D1) and differentiated myotybes (D3) (Figure 52A). We identified only 17 genes whose expression was altered by the loss of *RhoA* in all conditions - proliferating myoblast (Day0), differentiating myocytes (Day1) and in myotubes and (Day3) (Figure 52B).

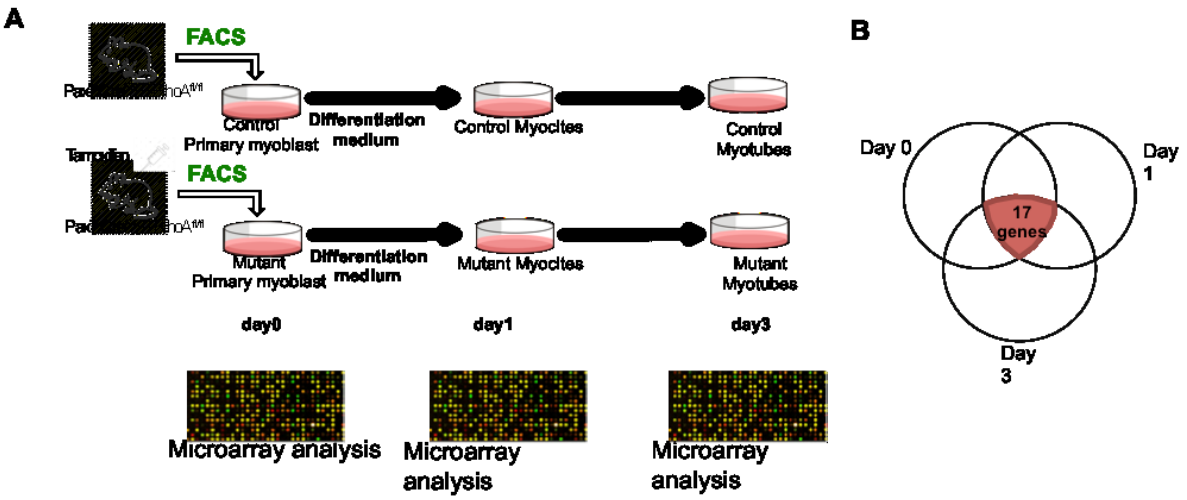

Figure 52: A. Experimental plan B. Venn diagram showing the intersection between genes differentially regulated by RhoA in myoblast (Day0), myoblast at the onset of differentiation (Day 1) and differentiated cells (Day 3)

In Table 1 are summerized only the nine genes that are downregulated or upregulated in the mutant during all the time point.

		day0		day1		day3	
		Expr p-value(A1)	Expr Fold Change(A1)	Expr p-value(A2)	Expr Fold Change(A2)	Expr p-value(A3)	Expr Fold Change(A3)
C920021L13Rik	RIKEN cDNA C920021L13 gene	1,55E-02	1,256	4,23E-02	1,202	2,53E-02	1,23
CCDC180	coiled-coil domain containing 1	1,08E-02	1,298	3,55E-02	1,227	4,49E-02	1,214
CHRD	chordin	3,22E-02	-1,38	4,04E-02	-1,357	4,08E-02	-1,356
Dhrs2	dehydrogenase/reductase mer	1,88E-02	1,193	2,54E-02	1,18	2,48E-02	1,181
ESCO1	establishment of sister chroma	3,73E-02	2,015	2,61E-02	2,135	2,21E-02	2,194
Fate1	fetal and adult testis expressed	1,03E-03	1,26	5,98E-03	1,196	4,92E-03	1,203
Olfr881	olfactory receptor 881	2,59E-02	-1,102	3,30E-03	-1,15	4,81E-02	-1,088
PEAR1	platelet endothelial aggregatio	1,87E-02	1,363	8,17E-03	1,434	1,49E-02	1,382
Rsl1 (includes others	regulator of sex limited protein	1,76E-02	1,371	2,81E-02	1,332	9,15E-03	1,428

Table 1: Top genes that are downregulated or upregulated in the mutant cells during all the differentiation.

We can focus on Chordin gene (*Chrd*) that is downregulated in mutant in the course of all the time points. Elsewhere it was described that Chordin acts as a BMP inhibitor and so is necessary to promote satellite cell cycle exit, commitment towards the differentiated myoblast, subsequently accelerating fusion (Friedrichs et al. 2011). We could speculate that in absence of RhoA the fine-tuning of BMP signalling is perturbed. Moreover, in Gene Ontology analysis (IPA analysis, Ingenuity) at Day1, BMP2/4 was identified as an upstream regulator significantly deregulated in the mutant (but with not predicted Z-score), supporting our hypothesis. The genes that are differently expressed between control and mutant are respectively 980 at Day0, 423 at Day1 and 614 at Day3, evidencing the divergence with the intersection set. These data suggest that the function

orchestrated by RhoA are different depending on muscle cell stage. Depending on the specific cell function and cellular context, RhoA acts on different signalling pathways. This hypothesis is corroborated by the fact that among the Canonical Pathway predicted by gene ontology program as differently regulated between control and mutant, there is a very little overlap between Day0, Day1 and Day3 (Table 2).

Ingenuity Canonical Pathways					
mutant vs control Day0	**p-value**	**mutant vs control Day1**	**p-value**	**mutant vs control Day3**	**p-value**
Cleavage and Polyadenylation of Pre-mRNA	1,8E-02	Valine Degradation I	5,9E-03	Hypoxia Signaling in the Cardiovascular System	3,7E-03
Selenocysteine Biosynthesis II (Archaea and Eukaryotes)	3,1E-02	Leucine Degradation I	1,4E-02	Unfolded protein response	5,5E-03
Purine Nucleotides Degradation II (Aerobic)	4,0E-02	Prostanoid Biosynthesis	1,8E-02	Serotonin Receptor Signaling	7,6E-03
Thio-molybdenum Cofactor Biosynthesis	4,9E-02	TNFR2 Signaling	2,5E-02	Endoplasmic Reticulum Stress Pathway	7,6E-03
Threonine Degradation II	4,9E-02	Retinoate Biosynthesis I	2,9E-02	FXR/RXR Activation	8,5E-03
Methylglyoxal Degradation VI	4,9E-02	**Relaxin Signaling**	**3,5E-02**	**Relaxin Signaling**	**3,2E-02**
		Isoleucine Degradation I	3,9E-02	LXR/RXR Activation	2,2E-02
		GDP-L-fucose Biosynthesis I (from GDP-D-mannose)	4,2E-02	Acute Phase Response Signaling	2,2E-02
				CD27 Signaling in Lymphocytes	2,2E-02
				4-1BB Signaling in T Lymphocytes	2,6E-02
				Complement System	3,0E-02
				Induction of Apoptosis by HIV1	3,5E-02
				IL-1 Signaling	3,7E-02
				GDP-L-fucose Biosynthesis I (from GDP-D-mannose)	4,1E-02

Table 2: Top canonical pathway identified by gene ontology analysis using Ingenuity of the genes differentially expressed between control and mutant cells at Day0, Day1 and Day3

For instance, we found “Relaxin signaling” as significantly deregulated in mutant at Day3 and at Day1 of differentiation progress. The peptide hormone Relaxin mediates many biological actions in a wide range of tissus. Relaxin binds to the G protein-coupled receptor relaxin family peptide receptor 1 (RXFP1) to activate a variety of downstream signal transduction pathways. This signalling cascade is complex and also varies in diverse cellular backgrounds. Among the intracellular signalling pathway activated by relaxin is noteworthy cyclic adenosine monophosphate (cAMP). Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger that is synthesised from adenosine triphosphate by adenylate cyclases (ACs), and is degraded by phosphodiesterases (PDEs) (Valkovic et al. 2019). IPA analysis found Adenylaye Cyclase 8 (*ADCY8)* gene as component of the deregulated Relaxin pathway. In addition, Adenylaye cyclase 8 (*ADCY8)* gene is significantly reduced in mutant compared to the control at Day3. Interestingly phosphodiesterase6d and 8b (respectively coded by *PDE6D* and *PDE8B* genes and also highlighted in relaxin pathway by IPA) are significantly upregulated in the mutant myotubes (Day3) compared to the control. The cAMP signaling pathway is the major route to trigger trophoblast fusion and its activation results in phosphorylation of specific intracellular target proteins, in transcription of fusogenic genes and assembly of macromolecular protein complexes constituting the fusogenic machinery at the plasma membrane (Gerbaud et al. 2015). Even if its role in myoblast fusion is not

characterized, it could be a potential candidate to investigate in RhoA mutant fusion deficient myoblast.

Next, we focused our attention on the Upstream Regulators predicted activated (Z-score>2) or inhibited (Z-score<2) in mutant compared to the control using IPA analysis (p-value < 0.05). During all the time points, we noticed that interstingly at three days of differentiation, among the upstream regulator significantly predicted inhibited, there was Adam12. Adam12 is a disintegrin and metalloprotease identified at first time in 1995 as a potent stimulator of myoblast fusion (Yagami-Hiroshimasa et al. 1995). More recently it was shown that Adam12 and α_9 Integrin mediate a cell– cell interaction selectively involved in fusion of mononucleated myoblasts to preformed myotubes (Lafuste et al. 2005). Some extracellular matrix protein genes like the proteoglycan (collagen associated) Decorin (*DNC*) and the tyrosine-rich acidic matrix protein Dermapontin (*DPT*) were found increased in the mutant at Day3. Even if their link with Adam12 is not yet characterized, we can speculate that Adam12, as upstream regulator, controls matrix protein and Integrin expression (*Integrin subunit β like 1* gene is up regulated in mutant at Day1), regulating adhesion and fusion of primary myoblast.

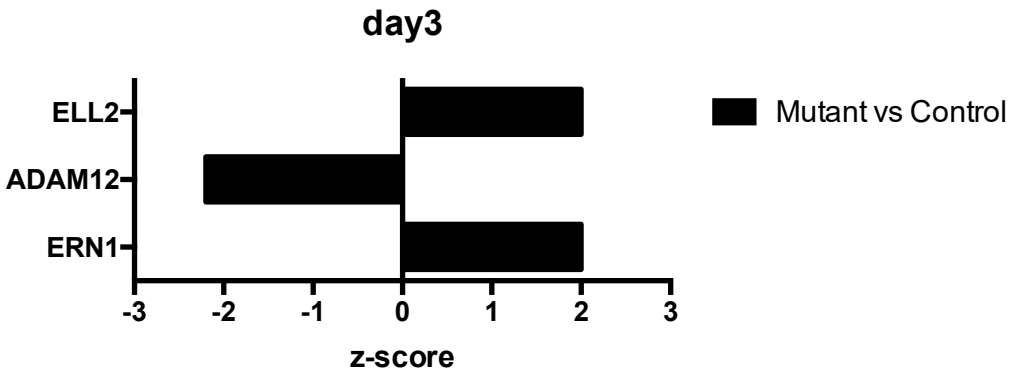

Figure 53: Upstream regulator identified by gene ontology analysis of the genes differentially expressed between control and mutant cells at Day3, using Ingenuity.

Finally, in order to identify the genes potentially involved in mutant myoblast defective fusion we focused on the genes differentially expressed between control and mutant at Day0, at Day1 and at Day3. Among the genes most down regulated in mutant, we did not find genes already described as important for myoblast fusion, like *Myomaker*, *Myomixer*, *Srf*, *Dynamin* or *Piezo*. Surprisingly, no genes involved in the actin cytoskeleton signalling came out, evidencing little overlap with Srf mutant cells already described in the lab, where the components and the regulators of actin network were strongly perturbed. Array data corroborate the previous experimental data showing no apparent defect in cytoskeleton of RhoA mutant cells. In this way, RhoA seems regulate myoblast

fusion via other pathways, also independently from Srf. Indeed, there is a very little overlap between the genes differentially expressed in Srf mutant cells and those in RhoA mutant cells. Even more, we are pushed to explore the genes up and downregulated in the mutant to discover novel crucial participants of myoblast fusion.

Discussion and perspectives

Skeletal muscle is the most abundant tissue and the most plastic structure in the vertebrate body. Skeletal muscle is composed mainly by contractile material. The functional unit responsible for skeletal muscle contraction is the sarcomere. Ordered patterns of sarcomeres compose the myofiber, the cellular muscle unit. In addition to myofibers, skeletal muscle contains a specific small population of mononucleated cells that are the resident muscle stem cells, termed satellite cells. Other tissue components, that strongly influence muscle function, are blood vessels that bring oxygen and nutrients, nerves that support neuro-muscular activity and connective tissue participating to the extra cellular matrix (ECM) building.

Skeletal muscle is a dynamic tissue with unique plasticity since it is able to react to different stimuli by adapting its size and to regenerate following a lesion. Physiological demands, such as exercise or working overload, lead to an increase of muscle mass due to an hypertrophic growth of myofibers. In response to activation cues, that could be as well local injury, SCs exit the quiescent state. Afterwards they proliferate and subsequently they differentiate and fuse to growing myofibers or in case of lesion they are able to restore destroyed myofibers, regenerating the muscle. In both cases satellite cells play a crucial role.

No data have been published on the role of RhoA in skeletal muscle regeneration and in hypertrophic growth *in vivo*. In cultured differentiating muscle cell line it has been shown that RhoA activity must be tightly regulated in a finely coordinated time-dependent manner to ensure appropriate skeletal muscle differentiation and fusion (Charrasse et al. 2006).

Several papers describe and underline the importance of activation of RhoA in response to mechanical force *in vitro* and *in vivo* (Zhang et al. 2007; Muñoz et al. 2018). In muscle tissue, RhoA protein and transcript expressions have been reported to be increased and decreased upon enhanced workload and disuse, respectively (McClung et al. 2004). Even in our mouse model we observe an activation of RhoA signaling pathway in hypertrophy-induced overloaded muscle.

This work aims to understand the role of RhoA in adult skeletal muscle plasticity. We took advantage of specific genetic models that allow us to delete RhoA in two distinct muscle compartments: myofibers ($HSA^{CreErt2}$) and satellite cells ($Pax7^{CreErt2/+}$). Muscle homeostasis was perturbed by two means overload-induced hypertrophy or lesion-induced regeneration. I will try to discuss the different results obtained, to attempt to give a global and total elucidation of RhoA function in skeletal muscle biology.

1. Impaired hypertrophic growth in muscle lacking RhoA within myofibers: SCs fusion is the limiting cellular event

When *RhoA* is deleted in the myofibers and *Plantaris* compensatory hypertrophy is induced, mutant muscles diplay a blunted growth. Thus RhoA is necessary to achieve a correct muscle hypertrophy.
There are two different mechanisms that could be affected in an impaired muscle growth: the synthesis of new protein, to support the expansion of the contractile units and the recruitment of satellite cells, to supply new nuclei further supporting the increase of the myofiber cytoplasmic volume. Once asserted that the global newly protein synthesis was not affected, we focused on satellite cells.
Satellite cells in our mouse model normally express RhoA because the deletion occurs specifically in the myofibers. However their number is slightly reduced in the mutant shortly after the onset of hypertrophy. Even if they do not present defect in proliferation rate, their apoptosis remain to be investigated. Next we investigated the fusion of satellite cells on growing mutant myofibers, that is strongly affected in mutant muscles.
Both these altered satellite cells functions could be responsible to sharp growth defect of mutant muscle.

Activation / proliferation of SCs in muscle hypertrophy

A decreased number of Pax7+ cells (at 1 week post OV) could suggest a defect in SCs activation. Freshly isolated single myofibers after hypertrophy surgery could help to decipher eventually early defect in the satellite cells activation process. Of note, satellite cells activation upon hypertrophy differs from its activation upon local injury, principally because MuSC niche is relatively or completely retained in overloaded muscle, whereas is completely lost and destroyed upon cardiotoxin injury. Recent work highlighted the difference between satellite cells activation during hypertrophy or during regeneration, asserting upon hypertrophy, the importance of Heyl expression, a Notch effector gene, to suppress MyoD expression (contrary to adult myogenesis upon regeneration) and to allow effective MuSC proliferation on myofibers and beneath the basal lamina. If impaired SCs activation could account for a defective regeneration process, it could be not the case during hypertrophy (Fukuda et al. 2019). It is not likely that the slight decreased number of SCs observed in RhoA mutants account for their reduced muscle growth. It has been shown that when Srf is deleted in the myofibers, satellite cell proliferation is reduced. Moreover, when satellite cell proliferation in normalized by Il6 overexpression this not improve muscle hypertrophy, meaning that the reduced proliferation of satellite cells is not the limiting step responsible for the lack of muscles hypertrophy. Importantly, the rescue of fusion alone was sufficient to restore

muscle growth (Guerci et al. 2012). So we can deduce that the mild reduction in satellite cells number in RhoA mutant muscles, that is smaller than the one observed in Srf mutants, is probably not responsible for their altered growth response.

Fusion

Recent study demonstrated the necessity of competent fusing satellite cells to have a correct muscle hypertrophy. In fact, Qingnian Goh et al. using mice model with muscle stem cells lacking Myomaker, a muscle-specific membrane protein necessary for fusion of embryonic and adult MPs, demonstrated that the muscle hypertrophic response was blunted. Thus satellite cells competent for fusion are required for a correct hypertrophic growth (Goh et al. 2017). In addition loss of Srf, a master regulator of SC fusion, in SCs impaires overload-induced muscle growth, providing further evidence that the hypertrophy is compromised when SCs are unable to fuse (Randrianarison-Huetz et al. 2018). In our RhoA mutant mouse model, the blunted muscle growth is as well associated with less myonuclei per myofibers and with no affection of protein synthesis or degradation. So, the impaired hypertrophic growth in absence of RhoA in myofibers may mainly result from blunted satellite cells fusion.

A still open debate concerns the importance of the correlation between the muscle size and muscle function (muscle specific force). Recently Masschelein et al. showed that mechanical load (performed with running wheel with increased resistance) increases muscle grip force and intrinsic muscle endurance capacity and it is accompanied by satellite cells recruitment to the growing myofibers (Masschelein et al. 2020). This study underlines that SC fusion and concomitant myonuclear accretion is typical in a physiological setting of increased mechanical load. Is RhoA loss in myofibers, that is responsible to less hypertrophy and decreased number of myonuclei per myofibers, accompanied by a decrease in muscle force? It will be interesting to measure the force of hypertrophied *Plantaris* mutant and control muscles to check whether less hypertrophy in the mutant is correlated to a lesser strength of the muscle, correlating the satellite cells recruitment to a functional muscle overload.

2. Which are the upstream and downstream effectors of RhoA participating to impaired mutant muscle hypertrophy?

2.1 Mechanical signals

It is described that RhoA is a mechanosensor present in the cytosol that translates physical forces into biochemical signalling pathways and transmits the signal in the nucleus, activating specific transcription factor. RhoA activation responds to mechanical stimuli and mechanical force

transmitted and mediated by cell-matrix and cell-interaction (Lessey et al. 2012; Burridge et al. 2019). *In vitro* it has been shown that upon mechanical stretch, on C2C12 myotubes, RhoA activity increases (Zhang et al. 2007) suggesting that RhoA controls mechanical stretch response in myotubes, activating downstream mediators important to translate environmental cues in biochemical signals.

Trying to disclose the molecular mechanism we generated an *in vitro* model where RhoA is deleted in the primary myotubes already differentiated (that should mimick the myofiber). Moreover, in order to investigate the heterotypic fusion among control satellite cells and mutant myotubes (reproducing what happened during hypertrophy model *in vivo*), we set up an *in vitro* assay to assess whether the absence of RhoA within myotubes (AdenoCre m-cherry transduced) affects fusion with RhoA expressing mononucleated myocytes (expressing GFP). Surprisingly we are not able to reproduce the fusion defect *in vitro* since the fusion between control myocites and mutant myotubes is not affected. This data suggest that the pathways regulated by RhoA depend on mechanical stretch and on *in vivo* mechanical cues and microenvironment. We thought that *in vitro* there is a complete decoupling between the mechanical cues and the environment response and that the events happening during overload condition cannot be reproduced on standard petri cultured myotubes where external mechanical stimuli are absent.

Usually mechanic stretch of myotubes *in vitro* is used to model muscle hypertrophy (Vandenburgh et al. 1979) even if the *in vitro* model do not take in account the contribution of other cells present in skeletal muscle and cannot be compared to mouse model. This underline the importance of *in vivo* model to analyse the effect of mechanotransduction in muscle cells mediated by RhoA. However, in order to carry out rescue experiments with eventual target molecules, it will be interesting to model *in vitro* the fusion defect of mutant myoblast and wild type myoblast. A partial solution is to perform the same experiment of mixed culture on stretched condition (where myotubes are cultivated on flexcell and are exposed to unidirectional stretch) in order to undergo the myotubes to a mechanical force and in turn activate RhoA. This could be able to, at least in part, reproduce *in vivo* condition and the RhoA activation.

2.2 Chemokines and macrophages influence SCs behaviours

A microarray analysis performed on mutant and control *Plantaris* muscle evidenced the genes mainly affected by *RhoA* loss. We found i) genes involved in the extracellular matrix remodelling, like *Mmp9* and *Mmp13* and ii) genes coding for two potent macrophage attractants such as *Cx3Cl1* and *Ccl3l3*.

The myofibers are sensitive to mechanical stimuli and translate them in paracrine signal that regulate satellite cells function. It has been described that myofibers secrete myokines that influence proliferation and fusion of satellite cells (Guerci et al. 2012). It could be envisaged that the absence of RhoA in the myofibers negatively affects myokine or chemokine secretion, impairing a correct satellite cells behaviour. This hypothesis is supported by our data that show decrease of chemokine expression *Cx3Cl1* and *Ccl3* in mutant myofibers in a cell-autonomous manner.

In a myoblast cell line, it has been shown that chemoattractant protein-1 (MCP-1; CCL2), macrophage inflammatory protein-1α (MIP-1α; CCL3) and MIP-1β (CCL4) induce phosphorylation of extracellular signal-regulated kinase ERK 1/2 (mitogen-activated protein kinase: MAPK) and increase myoblast proliferative responses. In particular, chemokine treatment enhances myoblast proliferation and migration *in vitro* and slows down the myogenic differentiation *in vivo* upon injury (Yahiaoui et al. 2008). In our case we do not know whether Cx3Cl1 and CCl3l3 influence SCs directly, but we can strongly relate their decreased expression to a significant impairment of macrophages recruitment, as displayed by a significantly decrease of macrophages number in mutant muscle sections upon overload compared to the controls. Indeed Cx3Cl1 and Ccl3l3 are two potent macrophage chemo-attractants and it has been shown on human myotubes that mechanical loading induces neutrophils chemotaxis (Tsivitse et al. 2005). Although the multifaceted roles of macrophages are well described during muscle regeneration (where they solve the tissue recovery) (Chazaud 2020), their role during hypertrophy model is not elucidated. We clearly showed that adaptation to increased workload alters muscle macrophages content. Using clodronate liposome in order to specifically deplete macrophages in control mice upon muscle overload, we showed that the transient reduction of macrophages affects number of proliferating and fusing SCs, blunting the hypertrophic response. Relying on published work (DiPasquale et al. 2007), macrophages depletion should not affect protein synthesis during hypertrophy and we showed that it correlates with decreased number of fusing SCs, phenocopying RhoA mutants.

In order to define the absolute requirement of monocyte-derived macrophages in skeletal muscle hypertrophy we could take advantage of chemokine/chemokine receptor deficient mice, namely *Cx3Cl1* and *Cx3Cr1* knockout (KO) animals. These mice lacking respectively the chemokine (CX3C motif) ligand 1 (CX3CL1) (also known as fractalkine) or its receptor (Cx3Cr1) should be submitted to hypertrophy model to study SCs behaviour in overload condition, in order to test the potential direct effect of chemokines on SCs.

Moreover, because the chemokines are secreted molecules it will be interesting to test their role in culture via conditioned medium. Medium of control myotubes containing secreted molecules could be used for mutant myotubes and control myocites co-culture, cultured in stretch condition in order

to mimic mechanical tension. In this condition it will be interesting to observe if the fusion between myotubes and myocyte is affected. Moreover, if we consider that the main function of chemokines is to recruit the macrophages we could propose to set a co-culture of macrophages and myotubes (or use conditioned medium from activated macrophages) in which will be added myocytes. Next we want to score whether the fusion among myocytes and myotubes is enhanced in standard or in stretched condition. Similar experiments on cultured myoblasts were performed by B. Chazaud.

Moreover, the implication of decreased chemokine expression in the lack of growth in RhoA-deleted muscles could be addressed *in vivo* by injecting control and mutant *Plantaris* muscles with an AAV driving the target chemokine expression prior to hypertrophy surgery. In the condition of robust target chemokine overexpression, the muscle mass and the satellite cells behaviour analysis will help us to understand if the defect in the mutant could account to deficient chemokine expression and macrophage recruitment and if its overexpression is sufficient to rescue de mutant phenotype.

We can propose that the mechanical load elicits the myofibers to produce chemokine chemoattractants that are indispensable to macrophages recruitment in the muscle. In turn macrophages are necessary to achieve a correct muscle growth mainly affecting satellite cells behaviour.

2.3 Mmps and their substrates

Mmps have been described as important modulators of extracellular matrix (degrading matrix proteins) and important signalling regulators (cleaving and activating growth factor). Skeletal muscle hypertrophy is associated with extensive transcriptional regulation of genes encoding for ECM remodelling proteins including Mmps (Zhang et al. 2014 and our study). We were able to evaluate the global activity of Mmps upon hypertrophy using Mmp sense probe *in vivo* and we asserted that it is significantly increased one week after overload in *Plantaris* muscles, suggesting that they are directly involved in ECM remodelling during hypertrophy.

At the same time, we demonstrated that the chemical inhibition of Mmps blunts the hypertrophic growth.

Concerning Mmp9, contrasting reports have described its favourable or deleterious role depending on the physiological and pathological situation (Mehan et al. 2011; Dahiya et al. 2011). Interestingly *Mmp9* null mice showed decrease in fiber size and change in fiber type in adult mouse hindlimb muscles (Mehan et al. 2011). Moreover *Mmp9* specific overexpression in the muscle increases muscle mass (Dahiya et al. 2011). However, how Mmp9 is regulated in muscle fibers is not clear. Recently it has been proposed that, in the muscle, *Mmp9* expression is finely regulated

(through miRNA secreted by SCs among others) to have a correct ECM integrity and to "facilitate the long-term hypertrophic response" (Murach et al. 2020). Too much or not enough Mmp9 is deleterious for muscle tissue. This concept could be applied also to other Mmps, like mt-Mmp1 or Mmp2, strongly expressed in the muscle, which activity is regulated in multi-phases manner during skeletal muscle tissue development (Ohtake et al. 2006).

In our mouse model the absence of RhoA is associated to a decrease of *Mmp9* (among others metalloproteinases) expression. Less Mmps as previously mentioned could be associated to smaller myofibers. Moreover, in our instance their decrease is also related to a wrong fibrillar collagen organization and to an accumulation of matrix protein that seem remain un-degraded.

Mutant collagen fibrils structure displays a significant increase in collagen fibers tortuosity when it is compared to the control after overload. K.M.Stearns-Reider et al. described collagen fiber orientation and their perpendicular alignment to the direction of the myofibers. In addition they evidenced that collagen fibril structure and architecture in the skeletal muscle affected ECM stiffness and in turn SCs myogenic potential (Stearns-Reider et al. 2017; Gilbert et al. 2010). Wrong ECM protein rearrangement may produce an environment not suitable to support a correct myofibers growth. We could speculate that the reduction of the metalloproteinases in mutant muscles influences satellite cells niche, entrapping satellite cells in an "uncomfortable" environment. We can speculate as well that the collagen biophysical properties, together with its quantity accumulation, are crucial for SC niche, affecting their behaviour, like motility and fusion to the growing myofibers. It has been described that Mmps mediate remodelling events of SC niche, modulating lamininα1 deposition and in turn regulating SCs propagation, differentiation and self renewal, principally acting via Integrinα6 (Rayagiri et al. 2018). In line with our observations, this result underlines the important role of Mmps in stem cells niche rearrangement.

Among the genes mostly downregulated in the mutant we found *Mmp13*. It has been shown that Mmp13 affects primary satellite cells migratory capacity in 2D and in 3D (Smith et al. 2020). Video microscopy assessed the high satellite cell motility on single living myofibers (Siegel et al. 2009). Satellite cells migrate along all myofiber and during hypertrophic condition they choose at some point where fuse. It is reasonable to assume that many different Mmps are required to degrade the variety of protein substrates surrounding matrix to make the movement of myoblasts toward the fibers more efficient. We suspect that less Mmp13 affects satellite cells migration on myofiber. We can speculate that impaired migration could impede the satellite cell to reach the right position where it should fuse. However, this data is missing a cause of the impossibility to visualize *in loco* the fusion of satellite cell on hypertrophied myofiber.

Metalloproteinases substrates, like TenascinC and Fibronectin, need to be cleaved and correctly rearranged to accomplish their functions and to create a functional niche. Less *Mmp9* expression in the mutant is correlated with less Tenascin-C and Fibronectin degradation and their consecutive accumulation, that may change ECM properties. Tenascin-C has been shown to remodel and to adapt stem cells niche (Tierney et al. 2016) and Fibronectin has been shown to regulate a feedback signal to promote symmetric division and replenish SCs pool (Bentzinger et al. 2013). In both case the proteins are produced by SCs that autoregulate their niche. Because in our instance SCs are "wild type", we could speculate that this autoregulation is not sufficient alone to form a functional niche, but should be associated with enzyme activity (meaning from the myofiber) for a correct ECM remodelling.

It would be interesting to investigate by Shg technique the rearrangement of proteins, like Tenascin-C, Fibronectin or CollagenIα2 in the SCs niche in control and mutant muscle and in control muscles treated with wide range Mmps inhibitor. This will allow a better characterization of the effect of Mmps absence/inhibition on their substrates assembly in a 3D visualization.

Moreover, it has been shown that macrophages secrete Adamts1 (A Disintegrin-Like And Metalloproteinase With Thrombospondin Type 1 Motif) and by targeting Notch1, regulate satellite cells activation in regenerating muscle (Du et al. 2017). The connection between macrophages and Mmps is further supported by recently report showing that Mmp-9 is secreted by macrophages in young but not in aged muscle in order to enhance the regeneration process (Rahman et al. 2020). Based on these data we cannot exclude that part of decreased Mmp expression found *in vivo* in mutant muscles is due to less macrophages, in addition to a myofibers intrinsic deficit. Therefore, verification of Mmps expression in overloaded muscles depleted for macrophages would be informative to test that hypothesis.

2.4 Erk 1/2 signaling

Among the signalling pathways significantly reduced in mutant RhoA compared to the control, we found Erk1/2. Erk is a potent Map kinase involved in numerous cellular functions. In other cell types it has been shown a crosstalk among Mmps and RhoA in order to mediate Erk activation (Meriane et al. 2006). In osteogenic differentiation ECM rigidity activates MAPK/Erk1/2, downstream of RhoA–ROCK signaling pathway (Khatiwala et al. 2009). The transmission of mechanical signal trough ECM occurs via mechanical sensitivity of Rho signalling. We can speculate that one of the principal mediator of RhoA signalling in response to mechanical cues is Erk, as its phosphorylation is decreased in mutant RhoA muscles. This hypothesis is confirmed by *in vitro* approach in which myotubes treated with RhoA activator drug present an increase in Erk1/2

phosphorylation. *In vitro* results showed that the increase of Erk 1/2 activity occurs in the myotubes, suggesting that the increase that we see *in vivo* upon hypertrophy came from the myofibers. Importantly, phosphorylation increases in conditions of boosted RhoA activity *in vitro* meaning that it is due to RhoA activation.

Interestingly in many cell lines *Mmp9* expression was increased in response to an upregulation of MAPK/Erk signaling (Wang et al. 2017; Genersch et al. 2000; Lakka et al. 2002). In keratinocytes it was shown that MEK/ERK signaling is necessary for baseline *Mmp-9* promoter function (Al. 2005). Moreover Erk1/2 pathway together with Jnk 1/2 elicit AP-1 activity that is a crucial transcriptional factor for Mmp-9 expression (Xia et al. 2015). We could adapt and speculate this scenario for muscle cells too and we propose that RhoA activation mediates activation of Erk signalling and based on the literature that Erk could elicit *Mmp9* transcription. However, these hypothesises remain to be confirmed. *In vitro* essay should be performed inhibiting Erk signalling upon RhoA activation to verify if *Mmp9* expression is negatively affected. However, remain undisclosed if in RhoA mutant muscle Mmp regulation could also occur independently to Erk signaling.

2.5 New overload-related genes by RhoA activity

Recently in the laboratory it was performed a single nucleus RNA-seq (snRNA-seq) experiment, using a droplet-based platform on purified nuclei from control and mutant *Plantaris* muscles with and without overload. Using Seurat software and Uniform Manifold Approximation and Projection (UMAP) clustering, we were able to detect different clusters of myonuclei present only in the ShamOperated (SO) condition or only in the overload (OV) condition. Moreover, in mutant mouse upon OV this cluster of myonuclei disappears (Figure 54).

Perspectives studies will aim to characterize the expression profile of these nuclei to identify specific markers of nuclei that respond to overload. We could hypothesize that these myonuclei:

i) are myonuclei that react to mechanical stimuli upon overload. The absence of this cluster in the mutant OV could be explained by the fact that the myonuclei present in mutant myofibers do not respond to mechanical stimuli because they lack the mecanoresponsive RhoA signalling.

ii) that come from newly fused satellite cells. The cluster that we found in OV condition in control but not in mutant could correspond to the nuclei that are fused and that are absent in mutant OV in which we have shown that SC fusion to growing myofibers was impaired.

It will be interesting develop a myogenic trajectory between the satellite cells and this new identified cluster of myonuclei in order to provide new insights into the continuum of myogenic cell-states during overload.

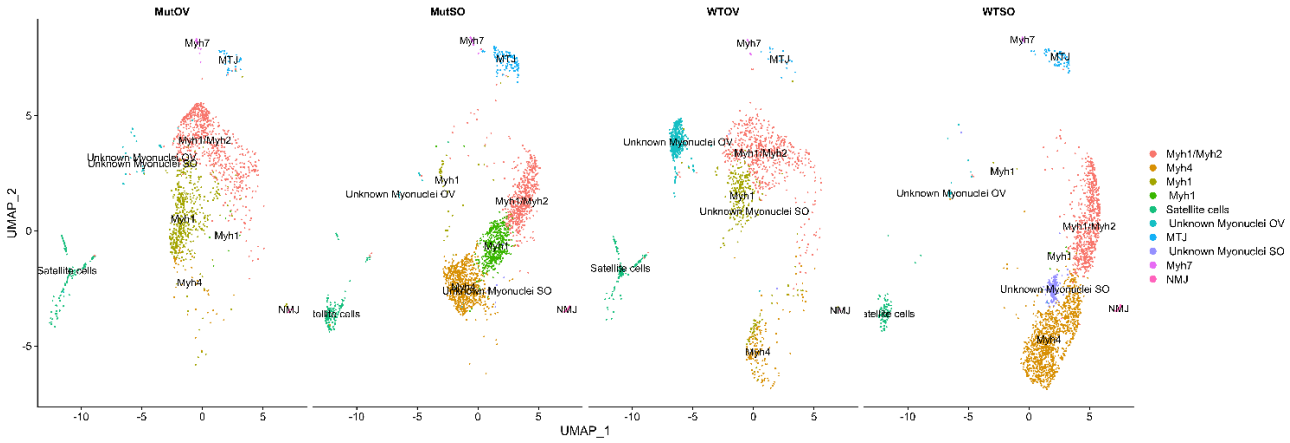

Figure 54: UMAP of myonuclei and satellite cells in SO and OV condition in mutant and control muscles.

In conclusion our proposed model is the following (Figure 55):

during adult muscle hypertrophy, the absence of RhoA in the myofibers affects *Mmps* expression. Less Mmps causes a fibrillar collagen disorganization and ECM proteins accumulation. This wrong rearrangement of SC niche, in turn influences SC behaviour and in particular negatively impacts their fusion to the growing myofibers. Myofibers lacking RhoA express less chemokines that attract less macrophages in the growing muscles. During the hypertrophic process, RhoA within the myofibers activates Erk1/2 pathway. However further experiments are necessary to define whether Ek1/2 signalling controls *Mmps* and *chemokine* expressions.

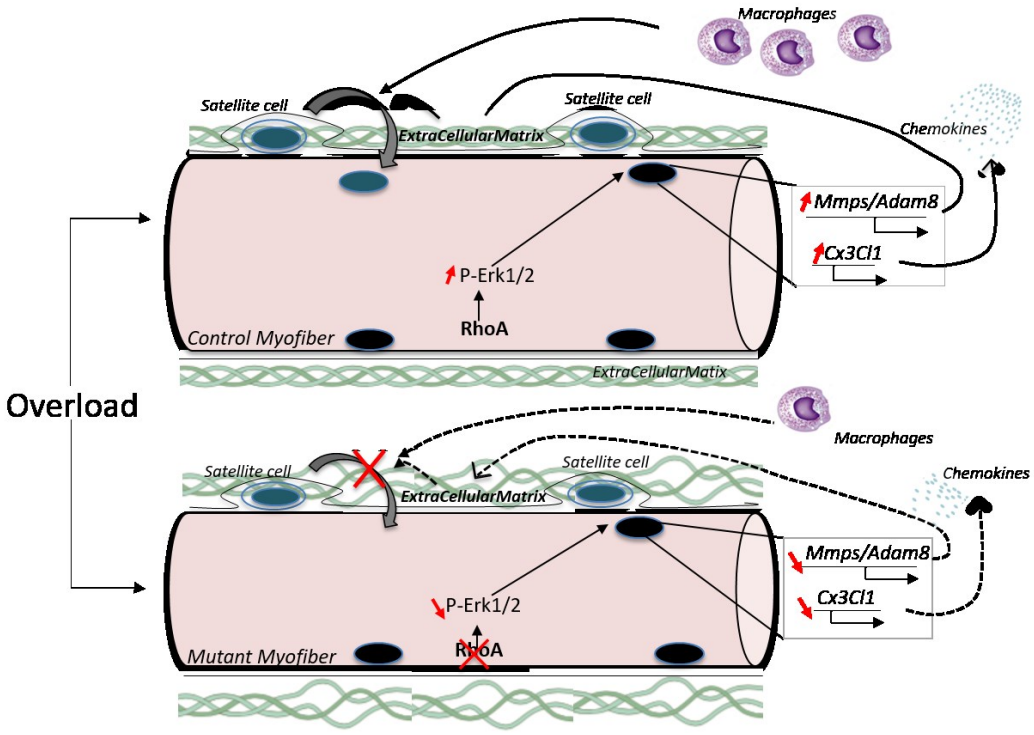

Figure 55: Proposed model

3. RhoA deletion in embryonic progenitors affects fusion only after birth

The loss of RhoA in embryonic progenitors (E9 of development) leads to higher mortality as indicated by the fact that the mendelian ratio is not respected neither before birth (E18.5) nor after birth. We thought that this letality was not due to a problem in muscle development, as at birth the muscles were completely formed and at E18.5 all muscle masses were present in limb sections. At E18.5 we did not find dead embryo in pregnant female mouse, so the death should occur in an earlier developmental stage. To confirm this hypothesis, we should analyse embryo at earlier stages such as E11 or E14. One possibility is that the precocious death is due to a defect in vital organ, like heart or brain. A publication from M. Buckingham laboratory described Myf-5 expression as a lineage marker for the developing brain (Tajbakhsh et al. 1995). It is well described that RhoA is indispensable for the development of mouse nervous system and the absence of RhoA under a neuronal promoter led to a mortality at E14 with no live born mice (Herzog et al. 2011). Thus, we can speculate that the expression of Cre under Myf5 promoter could lead to *RhoA* deletion in another organs, like brain, causing the early death of mutant embryo. Anyway the efficacy of the Cre used remains to be investigated (Haldar et al. 2007) and in addition the reason why there is so much variability in its expression.

Three possibilities are envisaged for the surviving mice:

1) The constitutive deletion of RhoA elicits the overexpression of other member of Rac family in order to compensate its absence. However recently paper showed that RhoA function is redundant with related RhoB and RhoC GTPases for the actomyosin regulation in mouse primary fibroblast (Melendez et al. 2011).

It will be interesting to verify the expression and activity of RhoB or RhoC in the muscle of surviving young mice.

2) The deletion is not 100% efficient and some wild type EPs remain. In these muscle-cells the residual RhoAGTPase will be enough to no elicit a strong phenotype.

3) RhoA is not required for muscle embryonic development. On the contrast RhoA is important for the post-natal growth. Indeed, the nuclei per myofibers observed at E18.5 and D4 were similar between control and mutant *Plantaris* muscles, whereas later on, during post-natal growth at D35 and D60 the number of myonuclei per myofibers was significantly decreased in mutant muscle. These data underline the fact that the fusion process is impaired only during the post-natal growth and not at embryonic stage. Moreover, we confirmed this hypothesis thanks to the cardiotoxin-induced regeneration experiments that allowed us to evaluate the fusion during the adult myogenesis. We evidenced that the regeneration process was impaired and the number of

myonuclei per myofibers in the mutant was decreased, underlining the major defect in myoblast fusion.

Previous report showed the importance of RhoA/ROCK axis to maintain the repression of Nfix transcription factor through the inhibition of Erk Kinase. Mainly this axis is important to regulate the transition between embryonic and foetal myogenesis (Taglietti et al. 2018). We can speculate that the deletion of RhoA should induce an activation of Nfix from E9 and so a premature foetal switch. However, Taglietti et al blocked ROCK (using Y27632) or activated RhoA *ex vivo*, on embryonic progenitors (E12.5 or E16.5): a completely different approach compared to our genetic mouse model. Moreover, it should be considered that RhoA activation is finely tuned (GTP-bound activated Rho was increased at E12.5 and E14.5, at E16.5 it decreases to allow its selective activation during embryonic myogenesis and it shuts down at the foetal stage). So its spatial/temporal activation is very important, suggesting that its deletion at fixed time point and/or during all the time could elicit unexpected and complex mechanism deregulation. In the same report, it was showed that the role of RhoA/ROCK axis role was not conserved in adult satellite cells. This is not surprising if we consider that embryonic, fetal and postnatal myogenic cells differ in cellular properties, gene expression and response to signaling molecules (Biressi et al. 2007). This is in accordance with our hypothesis stating that the signalling pathways required to control fusion during embryonic and post-natal myogenesis are distinct. Taken together we suggest that the inhibition of RhoA at early stage of myogenesis is not deleterious for a correct muscle development. Detailed analysis of muscle at embryonic stage and at juvenile stage (foetal vs embryonic vs adult Myosin staining i.e.) should be performed to confirm that the development occurred correctly. However, RhoA is indispensable after birth to govern fusion.

4. RhoA deletion in adult satellite cells affect muscle regeneration and hypertrophy

SCs were mobilized by two different means *in vivo*: i) through the lesion of muscle tissue induced by CTX injection, that leads to the complete muscle tissue destruction, and ii) through the increased workload applied to myofibers, keeping myofibers intact.

We showed that RhoA in SCs is needed to achieve correct muscle regeneration and hypertrophy. We then focused on the specific SC behaviors affected by *RhoA* deletion.

4.1 RhoA deletion in adult satellite cells transiently affects their number

The loss of RhoA in adult skeletal muscle stem cells did not affect their number at steady state neither their homing. This explains the absence of phenotype at basal state. However, when the muscle is exposed to acute injury the loss of RhoA transiently affectes SCs number. We tried to explain the decrease of satellite cells 4 days after cardiotoxin injury, evaluating different hypothesis:

- Activation

In order to decipher if the decreased number of Pax7-expressing SCs at the early stages of regeneration is due to delayed SC activation, single fiber cultures were performed. Preliminary results suggested that there was a shift in mutant satellite cells activation as pointed out by the percentage of Pax7-/MyoD+ SCs, that is higher in control compared to the mutant. It could seem, that in absence of RhoA satellite cells do not respond quickly to the activation after injury. However, the activation stimuli that they receive in culture is not the same received *in vivo*: Facs purified SCs isolated after CTX injection and kept in culture will help to decipher early MyoD and Pax7 expression in the following hours after injury.

Differently from our preliminary results, it was recently shown that RhoA activation reinforces SC quiescence and when it was disrupted in satellite cells induces an abnormal activation of satellite cells in uninjured muscle (Eliazer et al. 2019). The difference between these experiments can be due to two main reasons:

i) the mouse models used are different: in both cases the deletion occurs specifically in the satellite cells but our mice are *RhoA*$^{lx/lx}$ whether the mice reported by Brack et al. are *Rho*A$^{lx/+}$. They reported a mild reduction of RhoA activity in SCs, but there is no report on residual RhoA expression. On the contrary in our case we have a complete (almost 100%) reduction of RhoA expression. Even if we did not verify RhoA activity,

ii) the way in which SC activation is assessed by counting SC proliferation at basal state is different. They have injected BrdU for 14 days before mice sacrifice and they have observed BrdU incorporation in satellite cells *in vivo* once muscles were harvested. On the contrary we have measured satellite cells activation based on Pax7 and MyoD expression on freshly isolated fibers. Because the experimental conditions are so different, I do not think that the results could be comparable, leaving unsolved the role of RhoA in SCs activation.

- Proliferation

SCs upon injury undergo an extensive proliferation: almost the 50% of satellite cells are cycling. In absence of RhoA the total proliferation rate of satellite cells is not affected. Indeed, there are similar percentages of cycling satellite cells in mutant and in control. RhoA is described as being involved in the proliferation of cancer cells (X. Liu, Chen, and Liu 2014), but our evidences do not confirm

this role in muscle stem cells. Moreover, we investigated by *in vivo* EdU injection the satellite cells entry in S-phase. Even if we did not investigate the kinetic of cell cycle progression.
In addition, mutant satellite cells kept five days in culture after FACS sorting did not evidence any difference in cycling (2hr EdU pulse). Maybe by isolating SCs after injury and by monitoring EdU incorporation for a longer time period, we will be able to follow the cell cycle enter and evidence more subtle differences among control and mutant. Another envisaged possibility is to perform EdU pulse on isolated fibers in order to evaluate *in situ* satellite cells proliferation. Until now, accordingly with data obtained in C2C12 (Castellani et al. 2006) and with the present data, we can assert that RhoA does not affect primary cells proliferation.

4.2 SCs differentiation is not affected in absence of RhoA

In vivo evidences seem to indicate a delay in differentiation progress. This is mainly suggested by eMHC expression data. eMHC staining reveals a strong difference in expression pattern between control and mutant. eMHC is expressed during embryonic development but it is also transiently exceptionally expressed in adult muscle during regeneration, being a robust marker of muscle regeneration (Forcina et al. 2020). Less eMHC transcript and protein level evidenced a delay in myogenic progression of SCs lacking RhoA. However, to exclude that was not due to a decreased number of satellite cells at the beginning of the regeneration process, we performed *in vitro* experiments. Primary satellite cells differentiation showed that two principal markers of differentiation, *Myogenin* and MyHC, are not affected in mutants. This is in line with published data on C2C12 cell line where both constitutive RhoA activation (RhoAV14) and RhoA activity inhibition (C3 transferase treatment) did not affect differentiation (Castellani et al. 2006).
RhoA activity must be tightly regulated in a finely coordinated time-dependent manner to ensure appropriate skeletal muscle formation. Indeed it has been shown in mouse C2C12 myoblast that RhoA activity decreases in a biphasic manner during myogenic differentiation (Nishiyama et al. 2004). However, we showed for the first time that the absence of RhoA in primary murine myoblasts do not affect their myogenic differentiation.

4.3 The principal defect in SCs lacking RhoA is fusion

Both CTX injury and hypertrophy *in vivo* reveal that satellite cells lacking RhoA are unable to fuse among them or to growing myofiber. 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. In absence of RhoA the number of myonuclei per myofiber, at 8, 14 and 30 days post CTX-injury was highly diminished compared to controls. *In vitro* differentiation of mutant primary myoblasts are able to recapitulate the *in vivo* phenotype showing a decreased fusion index and number of myonuclei per myotubes. Reports already published on C2C12 myoblast cell line have asserted that RhoA must be deactivated before myoblast fusion (Charrasse et al. 2006). Somehow our results confirm that RhoA should be finely regulated during myogenesis process because when it is constantly deleted it is as well deleterious for the fusion.
In the case of hypertrophy protocol *in vivo,* the fusion of mutant satellite cells to the growing myofibers is as well impaired, suggesting that even if the myofiber is wild type, mutant satellite cell is not able to fuse. We can suggest that the absence of RhoA in satellite cells renders them uncompetent to fuse neither to myoblasts (regeneration situation) nor to myofibers, mutant (during muscle regeneration) or control (during muscle hypertrophy).

In vitro culture mixing should be performed to reproduce heterotypic fusion between control myotube and mutant myoblasts. Comparison with control culture will help to decipher the principal difference at cytoskeleton level (investigating F-actin protrusions on myoblasts) and cell-contact level (investigating Cadherin and Integrin protein, referred as essential component of fusion). Moreover, it will be interesting to perform mixed culture of control and mutant myoblasts to verify if the expression of RhoA in one of the two fusing partners is sufficient to allow primary fusion.

In conclusion, *in vivo* data suggest that when RhoA is absent in SCs they are not able to fuse anymore (or at least their capacity to fuse is reduced) both with another mutant myoblast and with a control or mutant myofibers. This suggest that RhoA expression, in *in vivo* conditions, is necessary in satellite cells in order to allow their primary and secondary fusion.

4.4 The fusion defect of RhoA deleted myoblasts is not correlated with actin desorganization and altered cellular contractility

One of the principal function of RhoA is the actin cytoskeleton remodelling. In particular RhoA promotes actin-myosin contractility and thereby the formation of stress fibers and focal adhesions, in order to regulate cell shape, cell attachment and cell motility. One of the principal effector of RhoA is ROCK. ROCK is a kinase that phosphorylates myosin bind subunit of myosin phosphatase (MYPT1) enhancing the phosphorylation of Myosin light chain (MLC) (Kimura et al. 1996). So MYPT1 phosphorylation, at two inhibitory sites, results in a decrease of MYPT1 phosphatase

activity and an increase in phosphorylated MLC. Moreover, it has been shown *in vitro* that ROCK is able to phosphorylate directly MLC. Phosphorylation of MLC has been shown to induce a conformational change in myosin, thereby increasing its binding to actin filaments and subsequently the formation of stress fibers (Riento et al. 2003). In addition, ROCK activates the LIM domain-containing kinase (LIMK), resulting in actin reorganization through inhibition of the globular G-actin state and depolymerization of filamentous F-actin.

In *Drosophila,* it has been evidenced the active role of founder cell, opposing an active resistance to pushing forces of fusion competent myoblast. At the fusogenic synapse, mechanical forces are applied and they increase cortical stiffness and provide resistance to invasive protusion (Kim et al. 2015). The pushing and resisting forces from the two fusion partners bring the two cell membranes into close proximity and put the fusogenic synapse under high mechanical tension to promote fusogen engagement and cell membrane merge. The fusion event is also strongly dependent on remodeling of the actin cytoskeleton which is regulated by RhoA among others. It was shown that MyoII is important to enhance myoblast fusion working as cellular fence to restrict cell adhesion molecules and a cellular sieve to constrict the invasive protrusions from the attacking cell (Kim et al. 2016).

In murine myoblast cell line it has been showed that MyosinII is indispensable to forme cortical actin wall during myoblast alignment that supports bipolar membrane alignment and temporally regulates trafficking of vesicles to the nascent fusion sites, to critically regulate myoblast fusion (Duan et al. 2009). Recently it has been shown that RhoA-ROCK mediated MLC phosphorylation is a downstream event of the Ca^{2+} channel Piezo1 activation. So the PIEZO1-mediated Ca2+ influx promotes RhoA/ROCK-dependent phosphorylation of MLC2 and subsequent actomyosin assemblies, thereby controlling cell fusion and the polarized elongation of multinucleated myotubes (Tsuchiya et al. 2018). Data in our lab showed that the inhibition of cell contractility by treating primary myoblasts with Blebbistatin impairs their fusion. All these data suggest the importance of MLC2 and cellular contractility for a correct fusion process.

Surprisingly in mutant RhoA myoblast, MLC2 phosphorylation is not affected. pMLC2 total fluorescent intensity and pMLC2 protein level in proliferating primary myoblast do not change between controls and mutants. However, we wonder whether the MyosinII dependent cortical tension in the cell lacking RhoA is affected, so we would like to measure rigidity/ elasto-viscosity of RhoA mutant and control myoblasts in collaboration with physicists (Atef Asnacios, Université de Paris).

In addition, RhoA controls both the formation (actin polymerization) and the spatial organization (filament bundling) of actin filament, regulating the actin assembling in long bundles and

supporting finger-like protrusions of the plasma membrane known as filopodia. In particular RhoA promotes the formation of stress fibers and focal adhesions, regulating cell shape, attachment and motility. In primary murine myoblasts it was discovered for the first time by our lab that finger-like actin protrusions are formed at the site of fusion. They have been described as essential structures for the fusion process (Randrianarison-Huetz et al. 2018). Surprisingly RhoA mutant myoblasts do not present evident defects in F-actin cytoskeleton organization and F-actin amount, as it is showed by Phalloidin total fluorescence intensity quantification and by confocal images of cells stained for Phalloidin. The formation of F-actin bundles do not appear strongly affected by the absence of RhoA.

Although actin cytoskeleton should be further investigated and MLC phosphorylation should be checked in other cultures, these two elements do not seem to be the major defect responsable for the impaired fusion of myoblast lacking RhoA.

As perspective studies, we would like to investigate by metal-replica electron microscopy (EM) on unroofed muscle cells, the structures forming filopodia-like actin based protrusions in RhoA mutant cells in order to investigate if they are absent or not well assembled in fusion incompetent RhoA mutant myoblasts.

The first important step of the cells fusion is cell migration. The cells have to migrate one to each other and bring themselves close enough (within 10 nm of one another) to allow the cells adhesion machinery to start. We show in preliminary data that cell migration, but not motility, was mildly affected in RhoA mutants. Even if further analyses are needed, we can speculate that this indispensable step can participate to the impaired mutant myoblast fusion. In order to disclose if this is a limiting step we envisage to plate mutant myoblasts in confluent high concentrations. This experiment will allow us to decipher whether the fusion defect could be attributed or not to cell “meeting” incapacity.

4.5 In search for signalling pathways responsible for the fusion defect of myoblasts lacking RhoA

Regeneration and hypertrophy processes are governed by a countless of signalling pathways that involve numerous transcription factors and signalling molecules. Previous work in the lab explored Serum Response transcription Factor (Srf) functions during adult muscle hypertrophy and regeneration, identifying it as a master regulator of satellite cells fusion, principally controlling actin cytoskeleton architecture (Randrianarison-Huetz et al. 2018).

Srf activity is regulated by myocardin-related transcription factor A (MRTF-A) cofactors. MRTF-A binds to monomeric G-actin and actin polymerization results in MRTF-A nuclear translocation (Guettler et al. 2008). RhoA-mediated changes to the actin cytoskeleton (actin treadmilling cycle), promote nuclear MRTF-A translocation, allowing its interaction with Srf and promoting target genes activation (Miralles et al. 2003; Posern et al. 2006).

In order to understand the pathways regulating fusion that involve RhoA/Srf signalling we compared the phenotype obtained deleting Srf or RhoA in satellite cells.

Both RhoA and Srf are not essential for SC proliferation nor for their differentiation. Moreover, fusion propensity of SCs lacking Srf and SCs lacking RhoA was blunted *in vivo* and *in vitro*. However, if myoblast lacking Srf displayed reduced elastoviscosity, less pMLC2 (personal communication from K. Kobon) and have an impaired actin cytoskeleton organization (with the absence of formation of actin-based finger-like protrusions at the site of cell-cell fusion), RhoA mutant myoblasts did not reproduce all these defects, suggesting that the fusion defect in RhoA mutant is Srf independent.

We performed a microarray analysis on primary control and mutant Srf or mutant RhoA muscle cells, on differentiation condition. The overlap of the data set is very little: among the genes differently expressed in mutant RhoA compared to the control we do not evidence important Srf target genes, meaning that its "signature" is not affected. The canonical pathway pointed out as among the major affected in Srf mutant is actin cytoskeleton signaling. On the contrary, gene ontology analysis using Ingenuity do not identify, among the canonical pathways affected by the lacking of RhoA, signaling correlated with actin cytoskeleton.

Interestingly a pathway found significantly deregulated in RhoA mutant at Day3 and at Day1 of differentiation progress is the Relaxin signaling. In this pathway is included adenylate cyclase which gene expression is significantly reduced in mutant compared to the control at Day3.

The cAMP signaling pathway is the major route to trigger trophoblast fusion and its activation results in phosphorylation of specific intracellular target proteins, in transcription of fusogenic genes and assembly of macromolecular protein complexes constituting the fusogenic machinery at the plasma membrane (Gerbaud et al. 2015). Even if its role in myoblast fusion is not characterized, it could be a potential candidate to be investigated in RhoA mutant myoblast fusion deficient.

In order to identify target molecules involved in defective fusion capacities, based on encouraging array data, we will quantify the total amount of this important intracellular second messanger in myotubes. If as expected the total amount of the protein is reduced in mutant, we could treat the mutant cells with a potent activator of adenylate cyclase and of biosynthesis of Amp cyclic (such as

the Forskolin) to see if it is able to rescue the fusion defect. Our work will help to decipher new important molecules under RhoA control that regulate myoblast fusion.

Annexes

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Article

Srf controls satellite cell fusion through the maintenance of actin architecture

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Satellite cells (SCs) are adult muscle stem cells that are mobilized when muscle homeostasis is perturbed. Here, we show that serum response factor (Srf) is needed for optimal SC-mediated hypertrophic growth. We identified Srf as a master regulator of SC fusion required in both fusion partners, whereas it was dispensable for SC proliferation and differentiation. We show that SC-specific Srf deletion leads to impaired actin cytoskeleton and report the existence of finger-like actin–based protrusions at fusion sites in vertebrates that were notoriously absent in fusion-defective myoblasts lacking Srf. Restoration of a polymerized actin network by overexpression of an α-actin isoform in Srf mutant SCs rescued their fusion with a control cell in vitro and in vivo and reestablished overload-induced muscle growth. These findings demonstrate the importance of Srf in controlling the organization of actin cytoskeleton and actin-based protrusions for myoblast fusion in mammals and its requirement to achieve efficient hypertrophic myofiber growth.

Introduction

Adult skeletal muscle possesses a tremendous degree of plasticity, as it can adapt its size to various stresses such as functional overload, and can efficiently regenerate after injury. Skeletal muscle tissue contains multinucleated postmitotic myofibers and a small resident muscle stem cell population, known as satellite cells (SCs). In undamaged muscle, SCs are maintained in a quiescent state and express the transcription factor Pax7. In response to muscle lesion or increased load, activated cells divide to form a pool of proliferating myoblasts (MBs) that coexpress Pax7, Myf5, and MyoD. Cells committed to myogenic lineage progression and differentiation exit the cell cycle, decrease expression of Pax7, and express MyoD and Myogenin. Most SCs progress along the myogenic lineage and fuse to form new myofibers (during regeneration) or supplement existing growing muscle fibers (during hypertrophy), and a subset of SCs maintain Pax7 expression and revert back to quiescence to replenish the SC pool (Dumont et al., 2015; Almada and Wagers, 2016). Thus, both repair and growth of multinucleated skeletal muscle cells are dependent on the fusion of muscle progenitor cells.

The fusion process follows an ordered set of cellular events that includes cell migration, alignment, adhesion, and membrane fusion. Many molecules, including secreted factors, membrane receptors, and intracellular molecules, participate in MB fusion (Hindi et al., 2013). In *Drosophila melanogaster*, fusion is accompanied by extensive rearrangement of the actin cytoskeleton, at the site of MB contact, highlighted by the formation of an actin-based invasive podosome-like protrusion structure (Sens et al., 2010; Kim et al., 2015a; Martin, 2016; Schejter, 2016), which in turn may provide the necessary pushing force to bring plasma membranes in close apposition for fusion to occur (Kim et al., 2015b). In addition, actin-based filopodia were shown to facilitate MB and myotube (MT) recognition/adhesion before MB fusion in adult *Drosophila* myogenesis (Segal et al., 2016). The essential role of the actin cytoskeleton in fusion is conserved in mammals, in which the actin regulators Rac1, Cdc42, and N-Wasp are required for the fusion process during muscle development (Vasyutina et al., 2009; Gruenbaum-Cohen et al., 2012). However, there is no evidence for discrete actin-based structures associated with the fusion process in vertebrates during muscle development, adult muscle regeneration, or hypertrophy or in primary muscle cell cultures.

Serum response factor (Srf) transcription factor controls the expression of target genes involved in cell growth, migration, and cytoskeletal organization (Esnault et al., 2014). Among Srf

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targets, some are specifically expressed in skeletal muscle, including *MyoD* and several genes encoding sarcomeric proteins (*α-actins* and *myosin light chain*; Pipes et al., 2006). The ability of Srf to regulate transcription depends on its association with cofactors (Posern and Treisman, 2006; Gualdrini et al., 2016), such as the Myocardin-related transcription factors (MrtfA and MrtfB). The Rho family of small GTPases and actin dynamics have been shown to control the nuclear accumulation of Mrtf-A/B and therefore modulate Srf activity (Posern and Treisman, 2006; Nordheim, 2014). Thus, the actin/Mrtf/Srf pathway forms a conserved homeostatic feedback system to ensure that actin levels are appropriate to support the actin dynamics required for complex cell behavior by controlling the expression of numerous genes involved in actin cytoskeletal structures and treadmilling, while being regulated itself by actin dynamics (Esnault et al., 2014; Kalo et al., 2015).

In mammals, the deletion of Srf in muscle progenitors during embryonic development impairs muscle formation (Li et al., 2005). Experiments performed using the C2C12 muscle cell line have shown that functional Srf is required for MyoD expression, MB proliferation, and differentiation (Gauthier-Rouviere et al., 1996; Soulez et al., 1996; Carnac et al., 1998). The use of mouse genetic models with the specific loss of *Srf* in myofibers showed that Srf is required for postnatal and adult muscle growth in vivo (Li et al., 2005; Charvet et al., 2006; Guerci et al., 2012) and that the decrease of Srf activity plays a functional role in disuse muscle atrophy (Collard et al., 2014). However, there are no data on the role played by Srf in SC behavior in vivo during adult muscle remodeling. Srf activity may be required to control SC cell fate in vivo in various situations of stress by controlling genes involved in cell proliferation (immediate early genes), myogenic differentiation (*MyoD*, *α actins*, and *myosins*), and actin architecture.

Here, we assessed the role played by Srf in SCs under stress conditions by inducing compensatory hypertrophy (CH) of plantaris muscles harboring a conditional and inducible deletion of Srf in the SCs and showed compromised hypertrophic muscle growth in the absence of Srf. We showed that Srf in SCs is not essential for *MyoD* expression, SC proliferation, or differentiation, in contrast to what was reported in the C2C12 cell line. However, the motility and fusion capacities of SCs lacking *Srf* were blunted and were accompanied by impaired actin cytoskeleton. Both homotypic (between two cells harboring the same genotype) and heterotypic (between a control and mutant cell) fusion events were defective, demonstrating the requirement for Srf in both fusion partners. We showed that the lack of Srf perturbed actin cytoskeleton organization in primary cells. We used metal-replica EM on unroofed muscle cells and demonstrated the existence of actin-based finger-like protrusions at the site of fusion, which were absent in fusion-deficient MBs lacking Srf. Strikingly, reestablishment of the actin scaffold in Srf mutant SCs by the overexpression of α-cardiac actin (*Actc1*) restored the heterotypic fusion capacity between control MBs/MTs and mutant MBs, both in vitro and in vivo (upon overload), and overload-induced muscle growth. However, homotypic fusion was not rescued by the maintenance of the actin architecture in cultured mutant cells and in vivo (upon regeneration), suggesting that additional Srf target genes are involved. Altogether, our data provide evidence for the importance of the actin cytoskeleton and actin-based protrusions in MB fusion in vertebrates and for the requirement of SC fusion in growing myofibers to obtain efficient hypertrophic myofiber growth.

Results

Srf in SCs is required for overload-induced hypertrophy

We first examined Srf expression in quiescent and activated adult muscle stem cells. Single fibers and their associated SCs were isolated from extensor digitorum longus (EDL) muscles at steady state and were immediately fixed to evaluate Srf expression in quiescent SCs expressing Pax7 by immunostaining (Fig. 1 A). Srf was poorly expressed in quiescent SCs, as <20% of $Pax7^+$ cells were also Srf^+. In contrast, single fibers maintained for 24 h in culture, to allow the activation of the associated SCs, showed Srf expression in all activated SCs (Fig. 1, A and B).

We investigated the role played by Srf in SCs by inducing CH of the plantaris muscle in control and mutant mice (Fig. 1 C). In our CH procedure, SCs were mobilized without destruction of the myofibers, allowing the study of mutant SC behavior in a wild-type environment. Tamoxifen (TMX) treatment induced efficient loss of Srf transcripts and protein (Fig. S1, C and D) in FACS-sorted SCs from control and mutant muscles (Fig. S1, A and B). At steady state (sham operated [SO]), there was no difference in muscle weight and myofiber cross section area (CSA) between control and mutant muscles (Fig. 1, D–F). After hypertrophy, the plantaris muscle mass and CSA were greater for control overloaded muscles 3 and 5 wk after CH than for unloaded muscles (SO; Fig. 1, D–F). However, the extent of hypertrophic growth was strongly reduced in mutant muscles, displaying only a 10% increase in CSA and 20% in mass versus 50% in controls 3 wk after CH. These changes were not accompanied by modifications of myofiber number (Fig. 1 G), indicating that the increase in muscle mass was mainly caused by myofiber hypertrophy. These data show that Srf expression by SCs is necessary for optimal overload-induced myofiber hypertrophy.

Srf is dispensable for SC proliferation

Srf has been shown to control the proliferation of several cell types including the C2C12 MB cell line (Soulez et al., 1996). We next investigated whether Srf loss altered SC function upon CH by quantifying the number of SCs that express Pax7. Before overload, SC numbers were identical between control and mutant plantaris muscles (Fig. 2 B). 1 wk after CH, there was a significant increase in the number of $Pax7^+$ cells in both control and mutant plantaris muscles, which returned to their original level 5 wk after CH (Fig. 2, A and B). Importantly, no difference in SC number was observed between control and mutant muscles after CH, suggesting that Srf loss did not hamper SC growth response to mechanical cues.

We further assessed the proliferative potential of *Srf*-deleted SCs in vitro by determining S (EdU incorporation), G2, and M phases (phospho-histone-$H3^+$ [$PH3^+$] nuclei) of control and mutant FACS-sorted MBs. In agreement with in vivo observations, the percentages of EdU^+ and $PH3^+$ cells of control and mutant MBs were equivalent, indicating similar proliferation rates (Fig. 2, C and D). We conducted additional experiments in which *Srf* loss was achieved in vitro by transducing primary $Srf^{flox/flox}$ MBs with an adenovirus expressing either the Cre recombinase (Ad-Cre) or GFP (Ad-GFP; Fig. S1 E). Again, there was no difference in the proliferative capacities of Ad-GFP– and Ad-Cre–transduced MBs in BrdU incorporation experiments (Fig. S1 F) and in their distribution in the different cell cycle phases analyzed by FACS quantification of DNA content (Fig.

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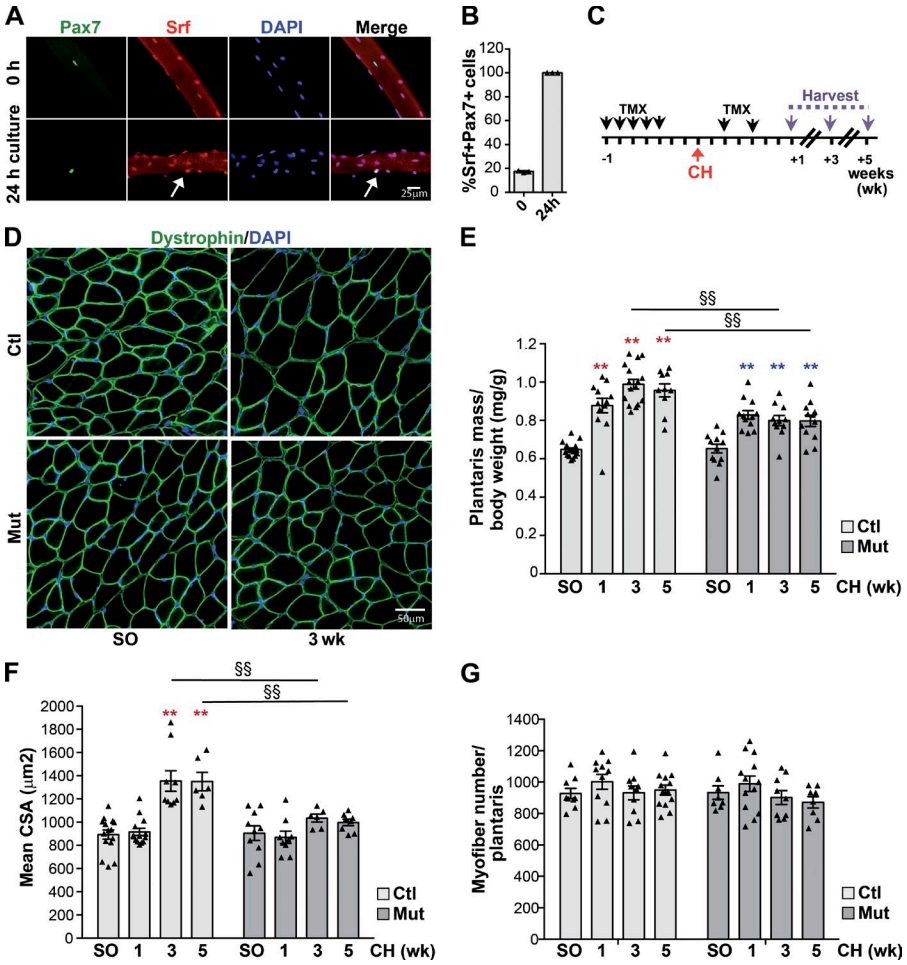

Figure 1. ***Srf* loss in SCs results in CH deficiency in plantaris muscle. (A)** Immunostaining for Pax7 (green) and Srf (red) on single fibers fixed immediately after isolation (0 h) or maintained in culture for 24 h. White arrows indicate SC expressing both Srf and Pax7. **(B)** Proportion of SCs displaying Srf expression ($Pax7^+Srf^+$; $n = 3$). **(C)** Srf mutant mice were injected with TMX 1 wk before CH procedure and after CH. Plantaris muscles were isolated 1, 3, and 5 wk after surgery. **(D)** Plantaris muscle sections immunostained for dystrophin (green) and nuclear staining with DAPI for control and Srf Mutant mice before (SO) and after 3 wk of CH. **(E)** Ratio of plantaris mass (milligrams) to body weight (grams) before (SO) and after 1, 3, and 5 wk of CH in control and Mutant mice (n = 10–16 muscles from n = 6–9 mice). **(F)** Mean CSA (square micrometers) before (SO) and after 1, 3, and 5 wk of CH in control and mutant mice (n = 6–15 muscles from n = 5–9 mice). **(G)** Mean myofiber number before (SO) and after 1, 3, and 5 wk of CH in control and mutant mice (n = 8–14 muscles from n = 6–9 mice). Data are mean ± SEM. **, $P < 0.01$ versus SO; §§, $P < 0.01$.

S1 G). Altogether, these data show that Srf in SCs is dispensable for their proliferation in a cell autonomous manner.

Srf controls SC motility but is not essential for the myogenic differentiation potential of SCs

We reasoned that other SC functions such as motility, differentiation, and fusion could be modified by *Srf* loss and account for the impaired hypertrophic growth of mutant muscle. As Srf is a central regulator of genes involved in cell migration (Esnault et al., 2014), we monitored the motile functions of *Srf*-deleted SCs in vitro using time-lapse videomicroscopy. The motility of mutant and Ad-Cre transduced $Srf^{flox/flox}$ MBs was decreased in comparison to control cells (Figs. 2 E and S1 H), demonstrating that Srf is needed for SC movement.

We next investigated whether *Srf* deletion could affect the myogenic differentiation potential of SCs. Indeed, previous in vitro studies in the C2C12 muscle cell line indicated that Srf activity was required for *MyoD* and *Myogenin* expression and myogenic cell determination and differentiation (Vandromme et al., 1992; Gauthier-Rouviere et al., 1996). We first assessed the number of Myogenin-expressing cells in vivo during overload-induced hypertrophy. After 1 wk of CH, the number of $Myogenin^+$ cells increased similarly in both control and mutant plantaris muscles (Fig. 3 A). In primary cultured muscle cells, MyoD and Myogenin protein and transcript levels did not differ between control and mutant MBs under proliferation conditions or after induction of differentiation (Fig. 3, B and C; and Fig. S1, I and J), in contrast to previous observations in C2C12 cells. We monitored the expression of a late differentiation marker (the sarcomeric myosin heavy chain [MyHC]) in control and mutant cells upon differentiation using the MF20 antibody (Fig. 3 D). The proportion of nuclei in $MyHC^+$ cells was identical between control and mutant cells 3 d postdifferentiation (Fig. 3 E). Collectively, these data show that Srf inactivation in SCs does not impair their engagement in myogenic differentiation.

Srf is crucial for SC fusion capacities

We examined whether the lack of Srf in SCs affects cell fusion by counting the number of myonuclei (DAPI staining) inside the sarcolemma (dystrophin immunostaining) in control and mutant plantaris muscle sections at various times after CH (Fig. 1 D). The number of myonuclei per myofiber was similar before overload between control and mutant muscles and increased significantly in control muscles at 1, 3, and 5 wk after CH. In contrast, myonuclei number did not change in mutant muscles after overload and was significantly reduced compared with controls (Fig. 4 A). To further determine the fusion capacities of SCs in vivo, we assessed fusion through EdU labeling of proliferating cells and by tracking the EdU^+ nuclei incorporated into dystrophin myofibers (Fig. S2, A and B). 3 wk after CH, the percentage of EdU^+ myofibers was blunted in mutant compared with control muscles (Fig. S2 C). Such decreased SC recruitment to the growing myofibers may be attributable to the

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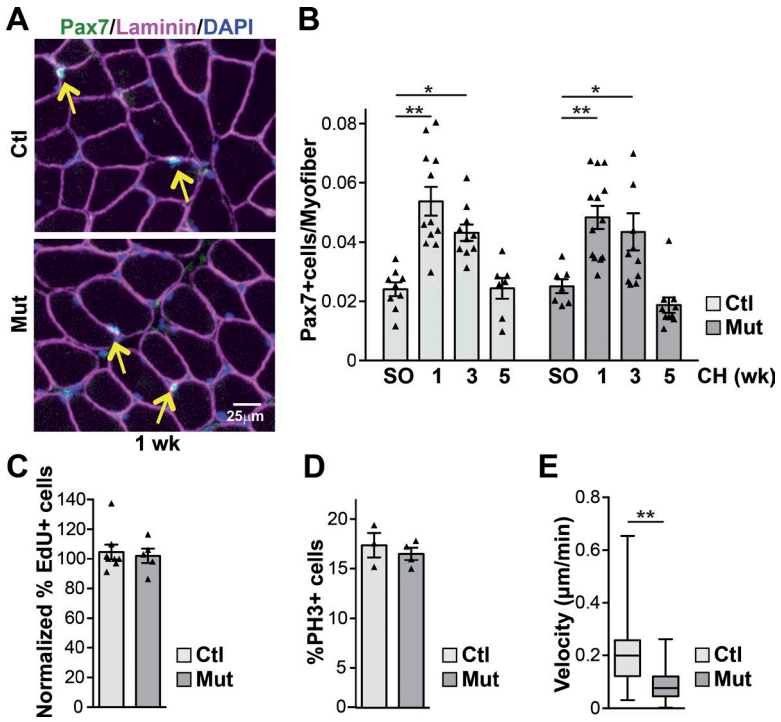

Figure 2. ***Srf* loss within SCs does not affect their proliferation but impairs their motility. (A)** control and Srf mutant plantaris muscle section immunostained for Pax7 (green), laminin (magenta), and nuclear staining with DAPI 1 wk after CH. Yellow arrows indicate Pax7-expressing SCs. **(B)** Number of Pax7$^+$ cells per myofiber in control and mutant plantaris muscle sections before (SO) and after 1, 3, and 5 wk of CH (n = 7–12 muscles from n = 4–7 mice). **(C)** Normalized percentage of EdU$^+$ cells in control and mutant FACS-sorted SCs cultured in rich medium for 5 d (n = 8–5 mice). **(D)** Percentage of PH3$^+$ cells in control and mutant FACS-sorted SCs cultured in rich medium for 5 d (n = 3–4 mice). **(E)** Mean velocity (micrometers per minute) of control and mutant MBs determined by time-lapse videomicroscopy (one representative experiment). Data are means ± SEM. *, P < 0.05; **, P < 0.01.

dampened fusion capacity of SCs lacking Srf, as both number and proliferative capacity of SCs were unaffected by *Srf* loss.

Srf mutant cells showed an altered capacity to form multinucleated MTs 3 d after induction of differentiation (Figs. 3 D and 4 B). Their fusion index, which represents the proportion of the total cell population that fused, was lower than that of control cells (Fig. 4 C). Accordingly, the mean number of nuclei in differentiated MyHC$^+$ cells was much lower in mutant than

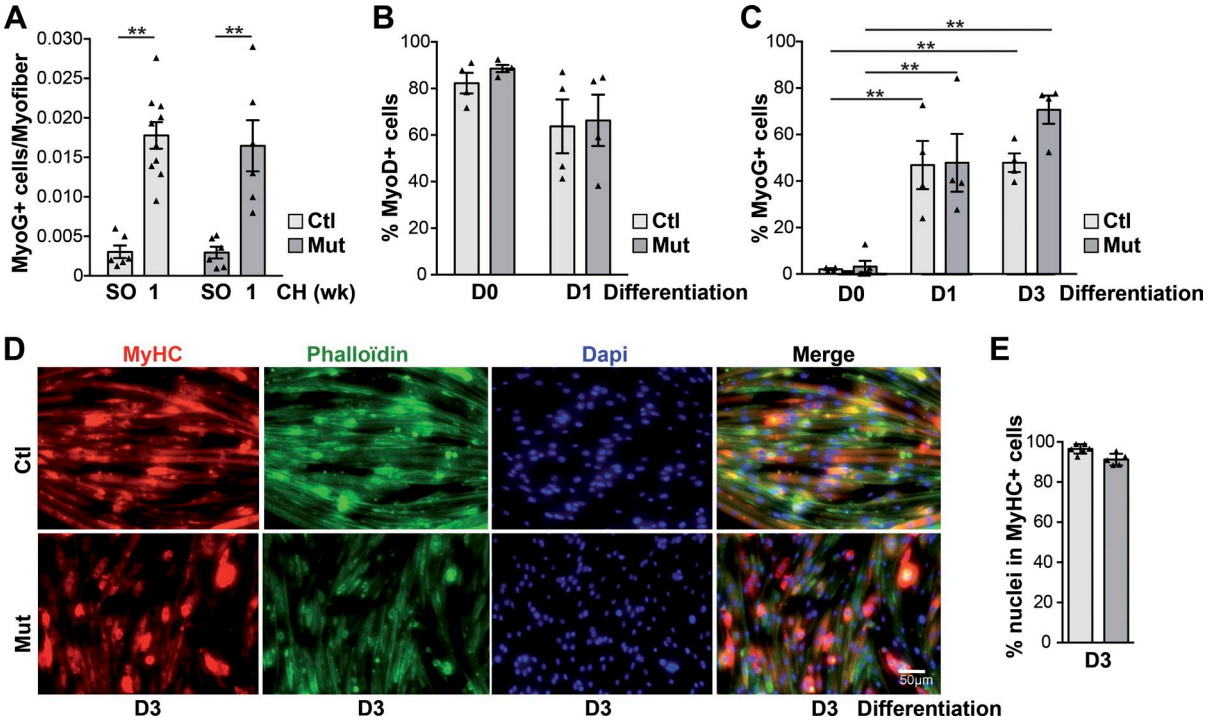

Figure 3. ***Srf* loss does not affect myogenic differentiation of SCs. (A)** Number of MyoG$^+$ cells per myofiber in control and Srf mutant plantaris muscle sections before (SO) and after 1 wk of CH (n = 6–10 muscles from n = 4–6 mice). **(B)** Percentage of MyoD$^+$ cells in control and mutant FACS-sorted SCs cultured in rich medium (D0, MBs) or 1 d after differentiation induction (D1; n = 4). **(C)** Percentage of MyoG$^+$ in control and mutant FACS-sorted SCs cultured in rich medium (D0) or 1 (D1) and 3 (D3) days after differentiation induction (n = 4–5). **(D)** Immunostaining for MyHC, nuclear staining with DAPI, and F-actin staining with phalloidin on control and mutant cells 3 d after differentiation induction. **(E)** Percentage of nuclei in MyHC$^+$ cells in control and mutant cells 3 d after differentiation induction (n = 5–6). Data are mean ± SEM. **, P < 0.01.

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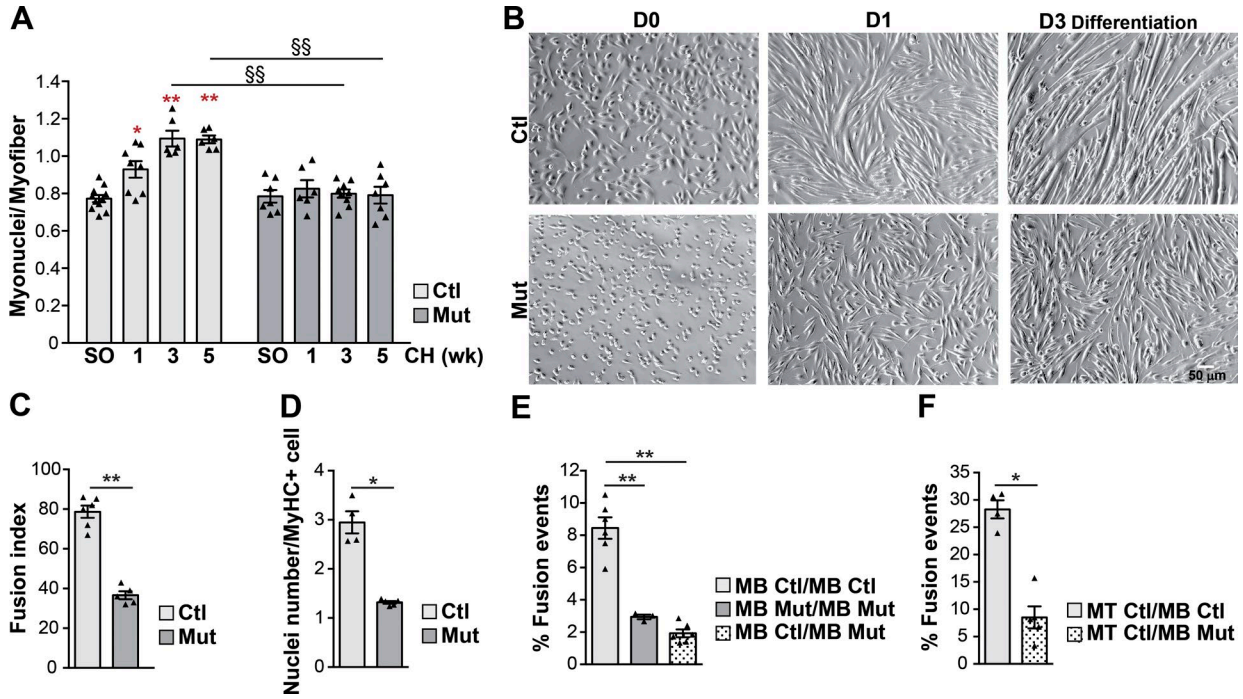

Figure 4. **Srf controls SC fusion. (A)** Number of nuclei (DAPI) within the dystrophin+ sarcolemma per myofiber before (SO) and after 1, 3, and 5 wk of CH in control and Srf mutant plantaris muscles (n = 6–11 muscles from n = 4–6 mice). Data are mean ± SEM. *, P < 0.05 versus SO; **, P < 0.01 versus SO; §§, P < 0.01. **(B)** Phase-contrast representative pictures of FACS-sorted control and mutant SCs cultured in rich medium (D0) or 1 (D1) and 3 (D3) days after differentiation induction. **(C)** Proportion of nuclei within multinucleated cells (fusion index) in control and mutant cells 3 d after differentiation (n = 5–6). **(D)** Mean number of nuclei per MyHC+ cell in control and mutant cells induced to differentiate for 3 d (n = 4–5). **(E)** Control and mutant MB were labeled with Orange Cell Tracker and mixed with control or mutant MBs labeled with Deep Red Cell Tracker. After 48 h of co-culture in differentiation medium, the percentage of dual-labeled MTs per total number of nuclei was scored (n = 3–6). **(F)** MT control were labeled with Orange Cell Tracker and mixed with MB control or MB mutant labeled with Deep Red Cell Tracker. After 48 h of co-culture, the percentage of dual-labeled MTs per total number of cells was scored (n = 4–5). For C–F, data are mean ± SEM. *, P < 0.05; **, P < 0.01.

control cells (Fig. 4 D). We then evaluated whether heterotypic MB fusion, between a control and a mutant MB, was also affected by performing cell mixing experiments of cells labeled with two different fluorescent dyes. We confirmed that mutant MBs were unable to fuse in a homotypic manner (between two mutant cells), as there were fewer fusion events (dual labeling) between mutant MBs than between control MBs (Fig. 4 E, lane MB Mut/MB Mut). Mutant MBs also exhibited highly diminished heterotypic fusion with control cells (Fig. 4 E, lane MB control/MB Mut). We then studied the fusion between MBs and MTs (MT/MB) by cell mixing of differently labeled cells. The lack of Srf in MBs was accompanied by a large decrease in fusion events (Fig. 4 F, lane MT control/MB mutant), showing that MBs require Srf for fusion to control MTs, a process that may lead to the accretion of new nuclei into myofibers upon overload-induced hypertrophy in vivo.

Altogether, these results show that Srf within MBs is indispensable for homotypic and heterotypic fusion events, indicating that Srf expression is required in both fusing cells. 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.

Srf-deleted MBs display impaired actin cytoskeleton organization and an absence of actin based finger-like protrusions at the site of fusion

We next sought to identify Srf target genes that could participate in the control of SC motility and fusion by performing a microarray analysis of gene expression in muscle cells expressing Srf (Ad-GFP) or not (Ad-Cre). We identified a set of 144 genes whose expression was altered by Srf loss in both proliferating MBs (D0) and differentiating cells (D1 and D3; Fig. 5 A and Table S1). Analysis of the potential biological functions of these genes by a gene ontology program (Ingenuity) pointed out an overrepresentation of genes involved in the regulation of actin cytoskeleton signaling (Fig. 5 B). We further focused our attention on several validated Srf direct genes including actin genes and genes implicated directly or indirectly in actin cytoskeleton regulation such as *Abra*, *Cnn2*, *Fermt2*, *FlnA*, *Tgfb1i1*, and *Wdr1* (Wang et al., 2011; Chong et al., 2012; Esnault et al., 2014). In line with the transcriptomic data, we confirmed by qRT-PCR that the expression of actin isoforms (including α-skeletal actin [*Acta1*], α-cardiac actin [*Actc1*], β-actin [*Actb*], γ-actin [*Actg*], and smooth muscle actin [*Acta2*]) and of *Abra*, *Cnn2*, *Fermt2*, *FlnA*, *Tgfb1i1*, and *Wdr1* genes was strongly diminished in cells lacking Srf (Fig. 5, C and D; and Fig. S2, D and E). Altogether, these expression studies show that, in muscle cells, expression of both structural components and regulators of actin network are affected by Srf deletion, suggesting that actin scaffold structures may be altered in Srf mutant cells, which may account for their perturbed functions.

We then quantified the total amount of F-actin per cell in control and mutant MBs by phalloidin staining (Fig. 6 A) and showed a significant reduction of total F-actin in mutant cells (Fig. 6 B). As the decrease of total F-actin per cell could be caused by the decrease of the total amount of actin in the cell or the

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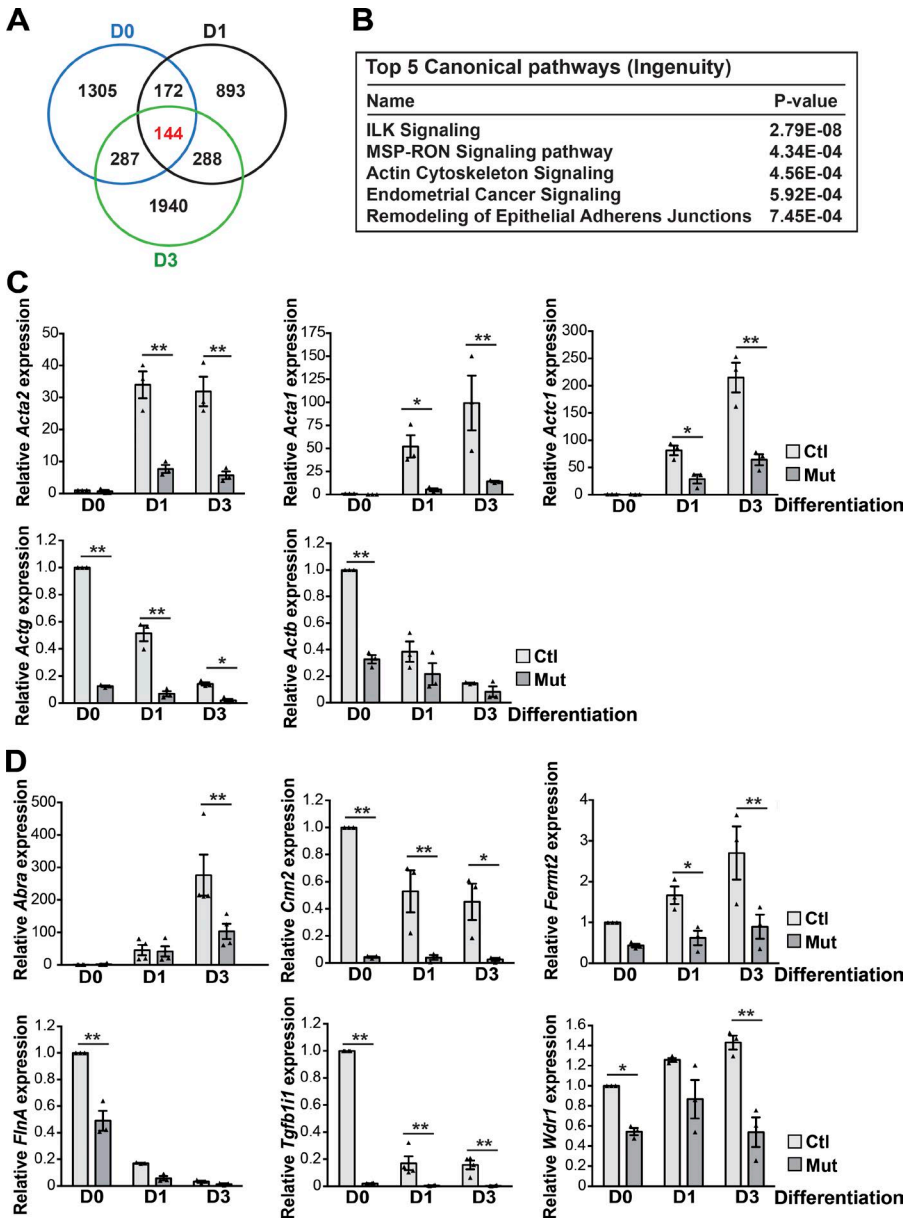

Figure 5. **Srf controls the expression of actin genes and genes implicated in actin cytoskeleton regulation. (A)** Venn diagram showing the intersections between genes differentially regulated by Srf ($P < 0.05$) in MBs (D0), myocytes at onset of differentiation (D1), and differentiated cells (D3). In red is indicated the number of genes (144) that are modulated by Srf independently of the differentiation state. **(B)** Top five canonical pathways identified by gene ontology analysis using Ingenuity of the 144 common genes whose expression is Srf dependent. **(C)** Analysis of *α-skeletal actin* (*Acta1*), *α-cardiac actin* (*Actc1*), *smooth muscle actin* (*Acta2*), *γ-actin* (*Actg*), and *β-actin* (*Actb*) mRNA expression by qRT-PCR in FACS-sorted control and Srf mutant SCs cultured in rich medium (D0) or 1 (D1) and 3 (D3) days after differentiation. Data were normalized by *Hmbs* expression and relative to D0 ($n = 3$). **(D)** Analysis of *Abra*, *Cnn2*, *Fermt2*, *FlnA*, *Tgfb1i1*, and *Wdr1* mRNA expression by qRT-PCR in FACS-sorted control and mutant SCs cultured in rich medium (D0) or 1 (D1) and 3 (D3) days after differentiation. Data were normalized by *Hmbs* expression and relative to D0 ($n = 3$). Data are mean ± SEM. *, $P < 0.05$; **, $P < 0.01$.

diminished proportion of the total pool of actin that is polymerized (F-actin), we first quantified total actin by Western blot using an antibody recognizing all actin isoforms and showed a sharp reduction of total actin quantity in mutant primary MBs compared with controls (Fig. 6, C and D). Furthermore, we quantified soluble G-actin and insoluble F-actin fractions in muscle cells; the F/G-actin ratio in MBs lacking Srf was reduced to 40% of control cell levels (Fig. 6, E and F). Thus, the decreased F-actin amount in mutant primary MBs could be attributed to both diminished total actin and altered regulation of actin polymerization.

We sought to obtain additional insights into the precise organization of the actin cytoskeleton in control and mutant primary MBs by confocal microscopy (Fig. 6 G) and metal-replica EM on unroofed muscle MBs (Fig. 6, H and I). The latter technique enabled us to visualize the ultrastructure of the actin cytoskeleton at the ventral membrane of the cell at high resolution. The actin cytoskeleton associated with the ventral membrane in control cells presented an organization that included both stress fibers formed from actin cables/bundles and branched actin (Fig. 6, G [bottom], H, H′, and H″; and Fig. S3, B, C, and C′). Srf mutant cells contained fewer and abnormally oriented actin cables (Fig. 6, G [bottom], I, I′, and I″; and Fig. S3, D, E, and E′). Accordingly, there was less intense cortical F-actin staining in mutant MBs than controls progressing toward the top of the cell on confocal *z*-sections (Fig. 6 G, middle and top). These results collectively show that Srf controls actin cytoskeleton organization (in actin cables) in cultured muscle cells.

It is possible that the altered actin organization of mutant MBs is responsible for their compromised fusion capacity. In *Drosophila*, podosome-like structures were shown to form at the "fusing" synapse (Martin, 2016). To visualize putative F-actin remodeling at the site of cell–cell fusion, we labeled primary myocytes with SiR-actin, a live fluorogenic cell F-actin probe, and performed time-lapse videomicroscopy upon differentiation. After tracking the fusion events using bright field (Fig. S3 A′ and Video 1), F-actin rearrangements were observed at the cell–cell contact at the fusion site (Fig. S3 A and Video 2). To visualize cellular and actin-based structures at the

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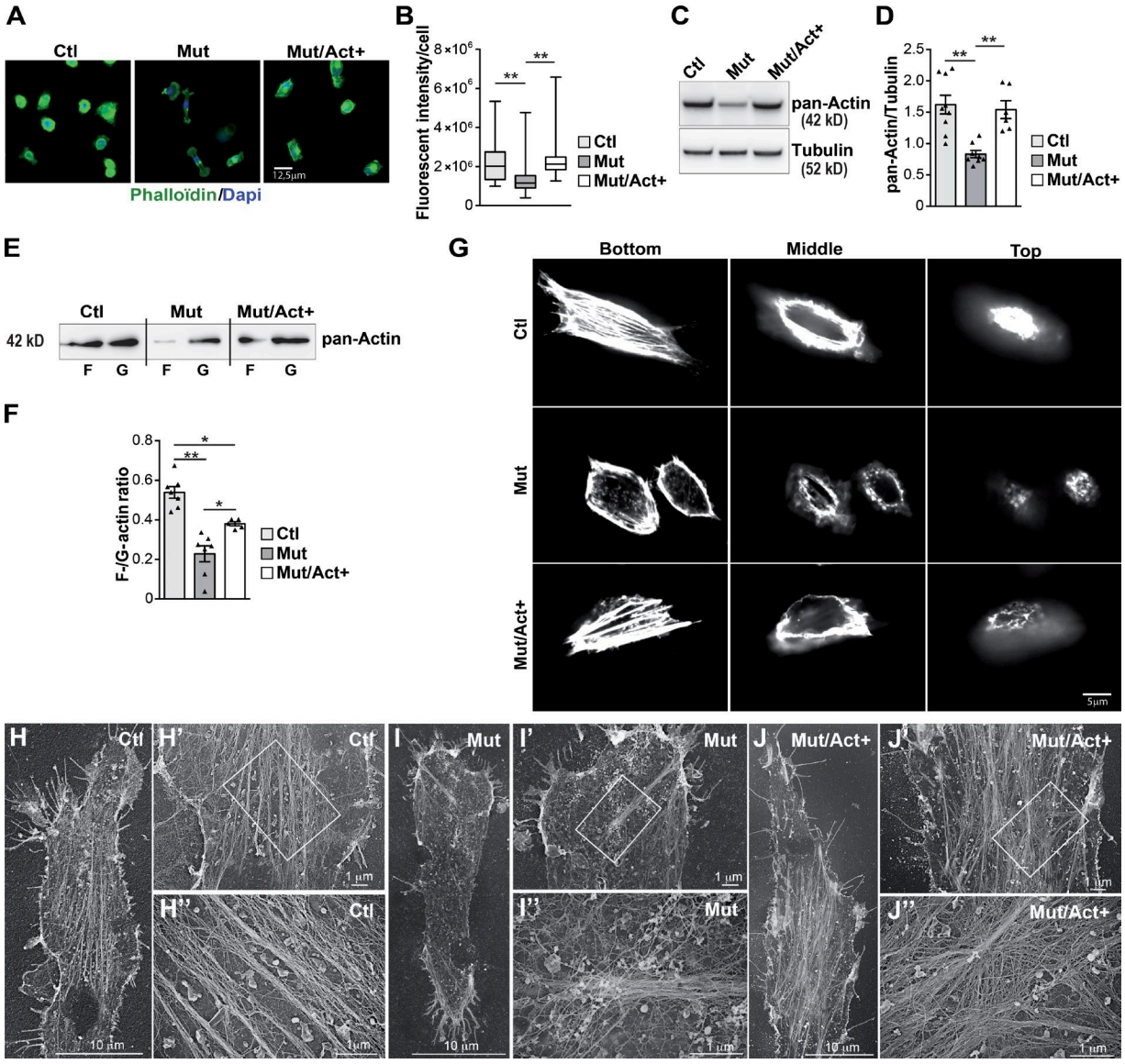

Figure 6. **α-Actin overexpression restores the impaired F-actin content of *Srf*-deleted MBs. (A)** Staining for F-actin (phalloidin) and nuclei (DAPI) on control, *Srf*-deleted (Mut), and *Srf*-deleted MBs overexpressing α-actin (Mut/Act+). **(B)** Quantification of F-actin by measuring the total phalloidin fluorescence intensity per cell (ImageJ) in control, mutant, and Mut/Act+ MBs (one representative experiment). **(C)** Representative immunoblot showing total actin (pan-Actin) in control, mutant, and Mut/Act+ MBs. Tubulin was used as a loading control. **(D)** Quantification of the pan-actin/tubulin ratio from immunoblots (n = 5–9). **(E)** Representative immunoblot showing actin in the insoluble (F) versus soluble (G) fractions in control, mutant, and Mut/Act+ MBs. **(F)** Quantification of the F-/G-actin ratio from immunoblots (n = 6–8). **(G)** Representative confocal projections of z-sections of F-actin staining (phalloidin) taken from the adherent (ventral) cell bottom and middle and proceeding up to the media-facing top of control, mutant, and Mut/Act+ MBs. **(H)** Survey view of the cytoplasmic surface of the plasma membrane from unroofed control MBs. **(H′)** Higher-magnification view from H. **(H″)** Higher-magnification view corresponding to the boxed regions in H′. **(I)** Survey view of the cytoplasmic surface of the plasma membrane from unroofed mutant MBs. **(I′)** Higher-magnification view from I. **(I″)** Higher-magnification view corresponding to the boxed regions in I′. **(J)** Survey view of the cytoplasmic surface of the plasma membrane from unroofed Mut/Act+ MBs. **(J′)** Higher-magnification view from J. **(J″)** Higher-magnification view corresponding to the boxed region in J′. Data are mean ± SEM. *, P < 0.05; **, P < 0.01.

basal membrane of fusing SCs at higher resolution, we used metal-replica EM in primary myocytes cultured for 24 h in differentiation medium. In control cells, we consistently observed the formation of finger-like actin-based protrusions at the site of prefusion. The electron density of these structures made them easily traceable even when located below the plasma membrane of the acceptor cell (Fig. 7, A, A′, and B). This feature allowed us to analyze "en face" the fusion process, which appears to be directional, as these protrusions occurred only in one cell (cell 1 in Fig. 7 A). The protrusions emanating from a first cell and which were composed of actin cables (white arrow) extended underneath the plasma membrane of the second cell (Fig. 7 B). Of note, we often observed branched actin filaments (indicated by an asterisk in Fig. 7 B) at the base of the actin cable forming the protrusion. In some instances, we observed complete fusion of the plasma membranes (Fig. 7, C and C′) and could visualize the protrusion below the fused membrane (Fig. 7 C′, yellow arrows). Strikingly, these finger-like structures were absent in fusion-deficient MBs lacking Srf and no fusion event was observed out of hundreds of contacting MBs

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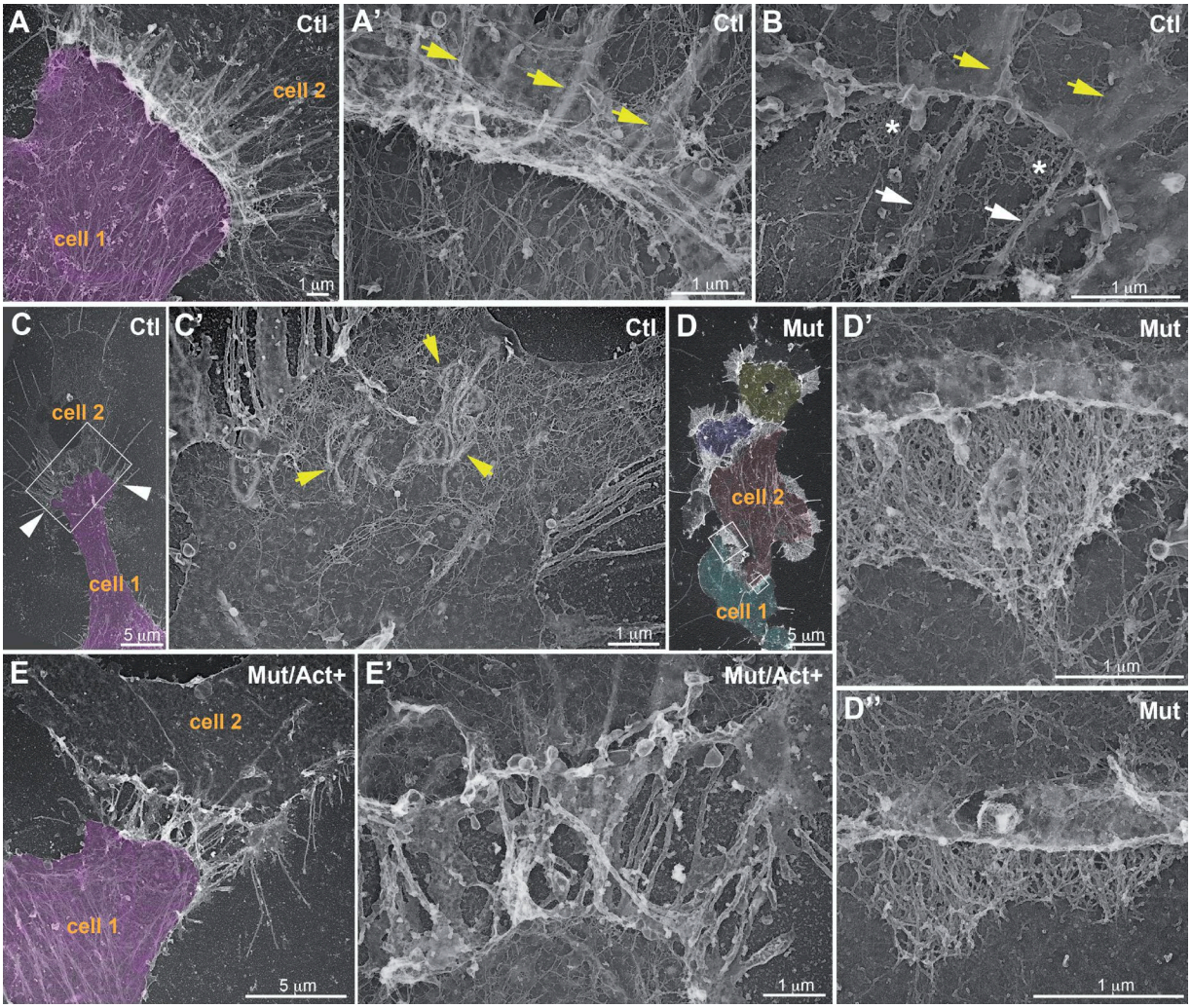

Figure 7. **Formation of finger-like actin-based protrusions at the site of fusion. (A)** Survey view of the cytoplasmic surface of the plasma membrane from control muscle cells differentiated for 24 h. Cell 1: Pseudocolored in purple forms finger-like protrusions that are traceable below the acceptor cell 2. **(A′)** Higher-magnification view from A of the finger-like protrusions denoted with yellow arrows. **(B)** Higher-magnification view of the finger-like protrusions from a double unroofed cell–cell contact. White arrows, actin cables from attacking cell 1; yellow arrows, finger-like protrusions traceable below cell 2; *, branched actin filaments. **(C)** Survey view of the cytoplasmic surface of the plasma membrane from two unroofed control muscle cells differentiated for 24 h that have successfully fused. Cell 1 presenting the protrusions is pseudocolored in purple. White arrowheads denote the fusion site. **(C′)** Higher-magnification view of the fusion site from boxed region in C. Yellow arrows denote finger-like protrusions below the fusion site. **(D)** Survey view of the cytoplasmic surface from mutant muscle cells differentiated for 24 h. Note clusters of MBs (each MB is a different pseudocolor) unable to fuse. **(D′ and D″)** Higher-magnification views of cell contact sites from the boxed regions in C. **(E)** Survey view of the cytoplasmic surface from Mut/Act$^+$ cells differentiated for 24 h. **(E′)** Higher-magnification view from E.

(Fig. 7, D, D′, and D″). Instead of actin cables, there was an accumulation of branched actin at the site of contact between mutant cells suggesting that the inability to form protrusions may be inefficiently compensated by branched actin accumulation (Fig. 7, D′ and D″).

Altogether, these observations show the presence of actin-based protrusions at the cell–cell contact between fusing cells that correlates with the fusogenic capacity of differentiating MBs and suggest that such structures may be required to drive fusion.

Impaired actin cytoskeleton organization in *Srf*-deleted MBs is rescued by the compensatory expression of an α-actin isoform

α-Cardiac (Actc1) and α-skeletal (Acta1) actins have redundant roles in skeletal muscle, as Actc1 overexpression can functionally replace Acta1 in *Acta1* knockout mice (Nowak et al., 2009). Thus, we hypothesized that the perturbed organization of the actin cytoskeleton in Srf-deleted muscle cells could be counteracted by Actc1 overexpression. We generated a mouse model in which *Srf* deletion and *Actc1* expression can be achieved specifically in SCs upon TMX treatment (Mut/Act$^+$ mice; Fig. S4, A and B). The overexpression of *Actc1* in *Srf*-deleted MBs was sufficient to restore the total amount of F-actin per cell (Fig. 6, A and B), the total amount of actin (Fig. 6, C and D), and the F/G-actin ratio (Fig. 6, E and F) to control levels. Confocal microscopy (Fig. 6 G, bottom) and metal-replica EM (Fig. 6, J, J′, and J″) revealed that Mut/Act$^+$ MBs contained more abundant F-actin structures at the ventral membrane, mainly organized in actin cables, than mutant cells, comparable to those in control MBs. Nevertheless, the amount of cortical actin, progressing toward the top of the cell, was similar in Mut/

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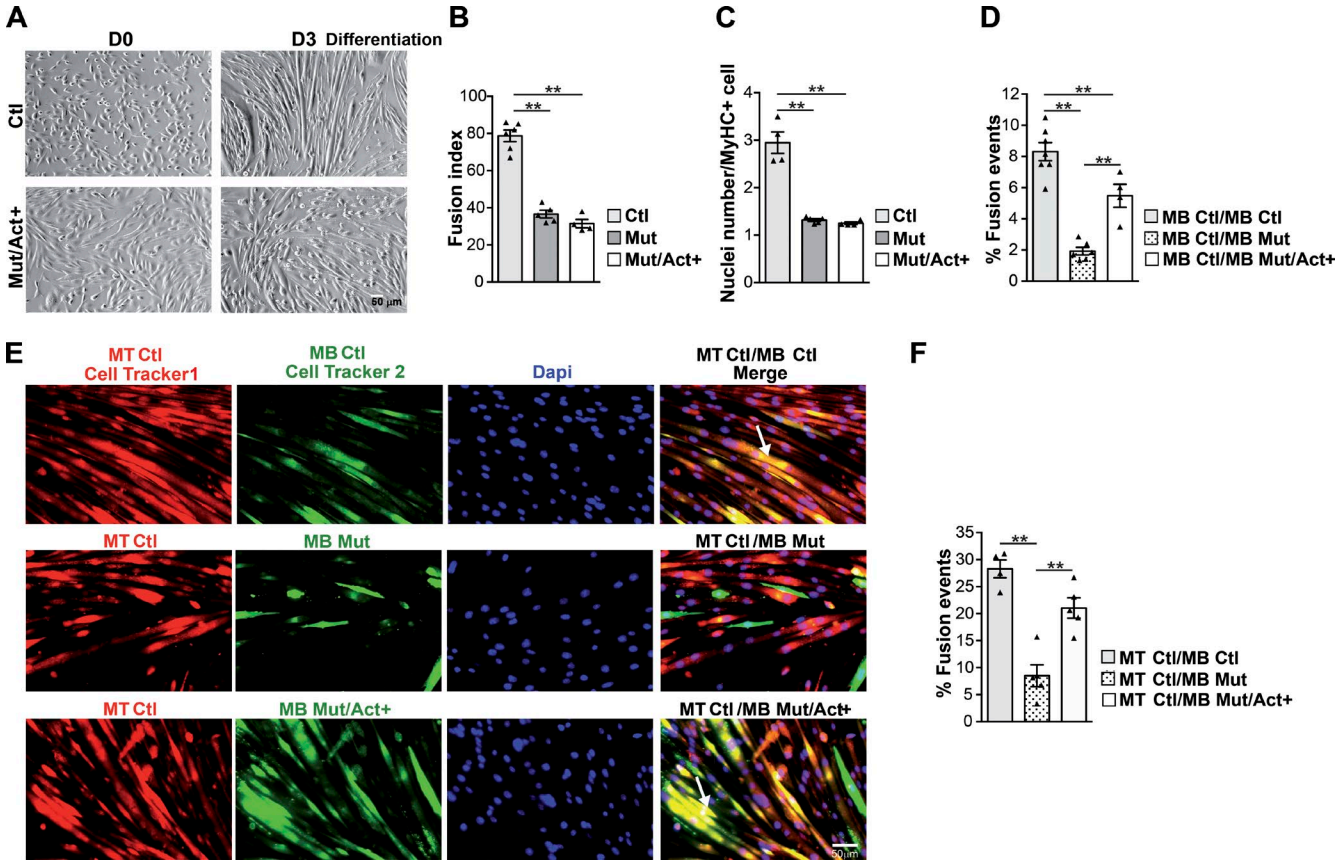

Figure 8. **α-Actin overexpression in MBs lacking Srf restores heterotypic fusion. (A)** Phase-contrast representative pictures of FACS-sorted control and Mut/Act+ SCs cultured in rich medium (D0) or 3 d after differentiation induction (D3). **(B)** Proportion of nuclei within multinucleated cells (fusion index) in control, mutant, and Mut/Act+ cells 3 d after differentiation (n = 4–7). **(C)** Mean number of nuclei per MyHC+ cell in control, mutant, and Mut/Act+ cells induced to differentiate for 3 d (n = 4–5). **(D)** MB control, MB mutant, or MB Mut/Act+ were labeled with Green Cell Tracker and mixed with control MBs labeled with Orange Cell Tracker. After 48 h of co-culture in differentiation medium, the percentage of dual-labeled MTs per total number of nuclei was scored (n = 4–7). **(E)** MT control were labeled with Cell Tracker 1 (Orange; visualized in red) and mixed with MB control, MB mutant, or MB Mut/Act+ labeled with CellTracker 2 (Deep Red; visualized in green). After 48 h of co-culture, MTs were analyzed for dual labeling (indicated by arrow). **(F)** MT control were labeled with Orange Cell Tracker and mixed with MB control, MB mutant, or MB Mut/Act+ labeled with Deep Red Cell Tracker. The percentage of dual-labeled cells per total number of cells was scored (n = 4–5). Data are mean ± SEM. **, P < 0.01.

Act+ and mutant MBs and less abundant than in control cells, suggesting that *Actc1* overexpression did not reestablish all actin structures (Fig. 6 G, middle and top). Collectively, these data demonstrate that α-actin isoform overexpression in SCs lacking Srf is sufficient to partially rescue the altered actin cytoskeleton organization observed in the mutant cells.

α-Cardiac actin overexpression rescues the heterotypic fusion defect caused by Srf loss in MBs

We next investigated whether the reestablishment of actin cytoskeleton in Srf mutant SCs by Actc1 overexpression could rescue defective SC functions, such as motility and fusion. Cell tracking experiments showed a similar decrease of motility in Mut/Act+ and mutant MBs relative to control cells (Fig. S4 C), precluding the involvement of α-actin–mediated F-actin stabilization in the impaired motile functions of SCs lacking Srf.

We then assessed the fusion capacity of Mut/Act+ cells during differentiation. As observed for Srf mutant cells, Mut/Act+ cells displayed an unaltered engagement in differentiation compared with control cells (Fig. S4, D, E, and F). However, upon differentiation, the fusion index and the mean number of nuclei per MyHC-expressing cell were reduced to a similar extent in Mut/Act+ and in Srf mutant cells, suggesting that actin overexpression did not alleviate the homotypic fusion defect of cells lacking Srf (Fig. 8, A–C). Although Mut/Act+ MBs could form numerous filopodia-like structures at the site of cell contact, these protrusions did not successfully extend below the ventral membrane of the contacting cell (Fig. 7, E and E′; and Fig. S3, F, G, and G′).

In contrast, impaired heterotypic fusion between control and Srf mutant MBs and between control MTs and mutant MBs was partially rescued in vitro by *Actc1* overexpression, as assessed by counting the fusion event in cell mixing experiments (Fig. 8, D [lane MB control/MB Mut/Act+], E, and F [lane MT control/MB Mut/Act+]). Importantly, the rescue of fusion in these cells is not accompanied by the restoration of their migratory capacities, highlighting the potential separation of the motility and fusion mechanisms. Overall, these data suggest that the maintenance of the F-actin network in Srf mutant MBs by α-actin overexpression is sufficient to rescue their heterotypic fusion with control MBs/MTs, providing genetic evidence for the requirement of actin-based protrusion in the control of SC fusion. However, actin overexpression was insufficient for homotypic fusion between Srf mutant SCs, indicating that additional Srf target genes must be expressed in at least one of the fusing cells to allow fusion to occur.

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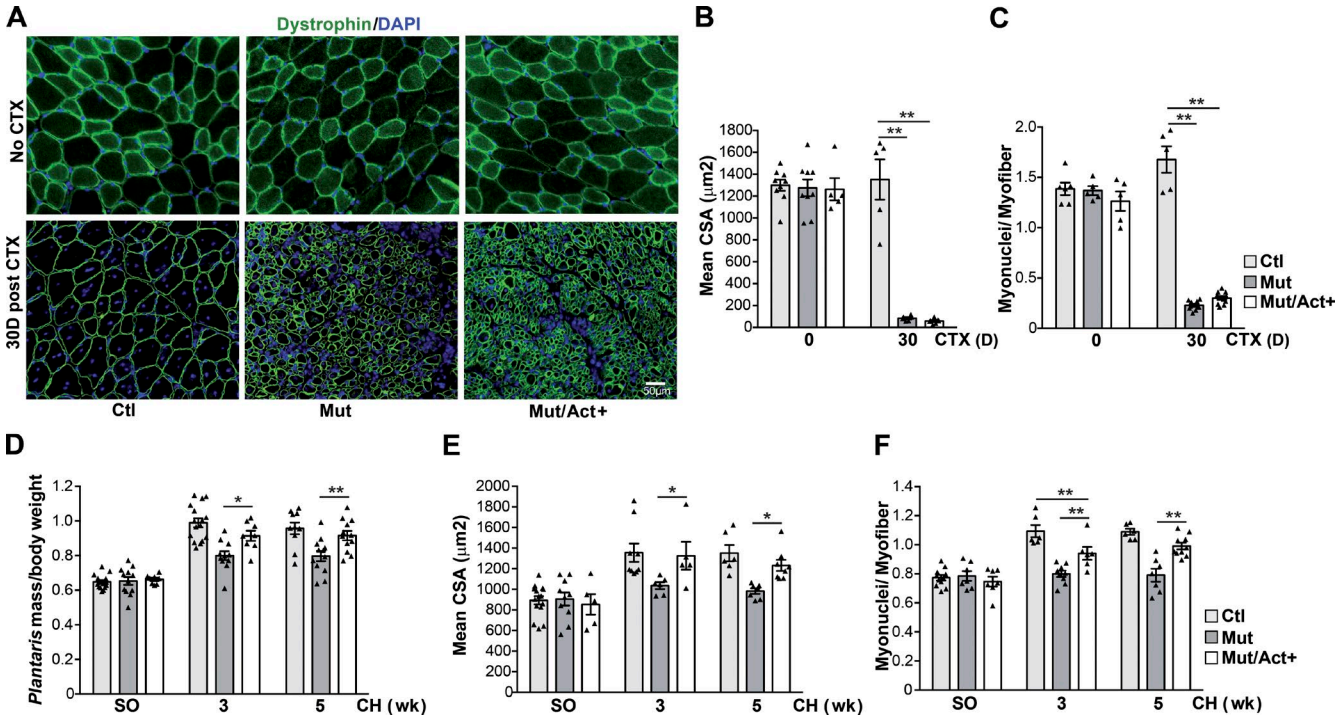

Figure 9. **α-Actin overexpression in Srf-deleted SCs rescues fusion and hypertrophic growth upon overload. (A)** TA muscle sections immunostained for dystrophin (green) and nuclear staining with DAPI for control, mutant, and Mut/Act+ mice untreated (No CTX) and 30 d after CTX-induced muscle injury (30D after CTX). **(B)** Mean CSA (square micrometers) of TA muscles untreated (0) and 30 d after CTX-induced injury in control, mutant, and Mut/Act+ mice (n = 5–9 muscles from n = 4–7 mice). **(C)** Number of nuclei (DAPI) within the dystrophin+ sarcolemma per myofiber in untreated (0) and 30 d after CTX-induced injury of control, mutant, and Mut/Act+ TA muscles (n = 5–11 muscles from n = 4–7 mice). **(D)** Ratio of plantaris mass (milligrams) to body weight (grams) before (SO) and after 3 and 5 wk of CH in control, mutant, and Mut/Act+ mice (n = 5–16 muscles from n = 4–9 mice). **(E)** Mean CSA (square micrometers) of plantaris before (SO) and after 3 and 5 wk of CH in control, mutant, and Mut/Act+ mice (n = 5–15 muscles from n = 4–9 mice). **(F)** Number of nuclei (DAPI) within the dystrophin+ sarcolemma per myofiber before (SO) and after 3 and 5 wk of CH in control, mutant, and Mut/Act+ plantaris muscles (n = 6–11 muscles from n = 4–6 mice). Data are mean ± SEM. *, P < 0.05; **, P < 0.01.

α-Cardiac actin overexpression in Srf mutant muscles does not rescue homotypic fusion in vivo upon regeneration

We conducted muscle regeneration experiments, recapitulating homotypic fusion events on control, mutant, and Mut/Act+ mice. Upon cardiotoxin (CTX)-induced injury, SC fusion occurs exclusively between cells of the same genotype. Dystrophin/DAPI staining of regenerated tibialis anterior (TA) muscle sections showed the overall structure of Srf mutant muscles to be severely affected relative to control muscles 30 d after CTX injection (Fig. 9 A). Indeed, the newly formed fibers in mutant muscles were 10 times smaller (Fig. 9 B) and three times more numerous than those in control muscles (Fig. S5 A). Moreover, there were far fewer myonuclei in mutant regenerated muscles than in control muscles (Fig. 9 C). Of note, the number of SCs was not affected by *Srf* loss 30 d postinjury (Fig. S5 B). Overall, these data suggest a strong deficiency of *Srf*-deleted SCs fusion that could account for the small size and the increased number of regenerated fibers present in mutant muscles.

We next assessed whether α-actin overexpression could counteract the altered regeneration and defective homotypic fusion of regenerated Srf mutant muscles. There was no improvement of fusion and mean CSA in Mut/Act+ relative to Srf mutant muscles 30 d postinjury (Fig. 9, B and C). These data are in accordance with the absence of an improvement of the homotypic fusion defect observed in vitro in Mut/Act+ MBs relative to Srf mutant cells (Fig. 8, B and C).

α-Cardiac actin overexpression restores overload-induced hypertrophic muscle growth of Srf mutant muscles

Heterotypic fusion between mutant or Mut/Act+ MBs and control MTs models the nuclear accretion that occurs in vivo during overload-induced hypertrophy. We thus determined the impact of α-actin overexpression on the defective hypertrophy of Srf mutant muscles. Actin overexpression rescued the overall growth of Srf mutant muscles to control levels 3 and 5 wk after CH. Indeed, Mut/Act+ plantaris muscle mass and mean CSA were significantly higher than those of Srf mutant muscles and were comparable to those of control muscles (Fig. 9, D and E). Furthermore, there were significantly more myonuclei in Mut/Act+ than in Srf mutant muscles, reaching 60% of control levels 5 wk after CH (Fig. 9 F). Accordingly, 3 wk after CH and after in vivo EdU labeling, the percentage of myofibers that incorporated EdU+ nuclei was significantly increased in Mut/Act+ muscles compared with Srf mutant muscles (Fig. S2 C). These data demonstrate that the maintenance of a filamentous actin scaffold within SCs lacking Srf was sufficient to efficiently drive heterotypic fusion in vitro and restore the impaired hypertrophic growth of Srf mutant muscles in vivo.

Discussion

Taking advantage of a genetic model that allows the specific deletion of Srf in adult muscle stem cells, we show in vivo and

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in vitro that Srf is a master regulator of SC fusion, whereas it is dispensable for their proliferation and differentiation. Furthermore, we used both metal-replica EM on unroofed muscle cells and a genetic model of α-actin overexpression to provide evidence on how actin cytoskeleton architecture affects SC fusion in mammals.

Srf activates *MyoD* expression, MB proliferation, and differentiation in the C2C12 muscle cell line (Gauthier-Rouviere et al., 1996; Soulez et al., 1996; Carnac et al., 1998), and the distal regulatory region of the *MyoD* gene contains a shared MEF2/Srf binding element (L'honore et al., 2003). In addition, skeletal muscle–specific deletion of Srf coactivators Mrtf-A and Mrtf-B during embryonic development impaired MB proliferation and increased apoptosis (Cenik et al., 2016). In contrast to these previous studies, our results show that SCs lacking Srf respond to mechanical cues by expanding their number similarly to control SCs and that their cell cycle progression is unaffected ex vivo. Moreover, *Srf* loss in primary cell culture did not perturb MyoD expression. The discrepancy between our results and those obtained with the C2C12 cell line and embryonic progenitors may be attributable to differences between cell lines and primary cells and muscle progenitors isolated from either adult muscles (our study) or embryonic muscles (study on Mrtfs deletion; Cenik et al., 2016). SC-specific deletion of both *MASTR* and *Mrtf-A* leads to excessive proliferation of SCs because of the down-regulation of *MyoD* expression (Mokalled et al., 2012). The authors of the study proposed that MEF2 transcription factor, and its associated MASTR cofactor, cooperate with Mrtf-A to regulate the transcriptional regulation of *MyoD* expression (Mokalled et al., 2012). Thus, it is possible that *MyoD* expression is mainly controlled by the MASTR/MEF2 pathway in *Srf*-deleted SCs.

Previous in vitro studies using the C2C12 cell line showed that the myogenic differentiation was impaired by inhibition of Srf (Gauthier-Rouviere et al., 1996; Soulez et al., 1996). However, in the present study, the number of Myogenin-/MyHC-expressing cells was not affected by *Srf* loss in cultured primary MBs under differentiation conditions or during overload-induced hypertrophy in vivo, indicating that Srf activity is not required for MB engagement into the differentiation program. In contrast, we identified Srf to be a major regulator of the fusion process. Indeed, the absence of Srf in SCs led to the formation of small MTs containing very few nuclei during differentiation in culture, small and numerous myofibers during in vivo regeneration, and the absence of nucleus accretion during myofiber hypertrophic growth. Our results are in accordance with the altered fusion observed in muscles lacking Srf cofactors Mrtf-A and Mrtf-B (Cenik et al., 2016).

Studies conducted in *Drosophila* and vertebrate systems led to a model for MB fusion. Three major phases accompany the MB fusion process: (a) the initial event of adhesive interactions between cells, (b) the recruitment of the actin-polymerization machinery to the membrane and the formation of transient actin-based structures, and (c) the formation of discontinuities and the merging of the apposed membranes (Kim et al., 2015a; Schejter, 2016). The contribution of the actin-polymerization machinery in mammalian MB fusion has been described using knockout models (Cdc42, Rac1, and N-WASp; Vasyutina et al., 2009; Gruenbaum-Cohen et al., 2012), but specific actin structures at the site of fusion, including cortical branched-actin and invasive extensions, have been described mainly in *Drosophila* (Sens et al., 2010; Dhanyasi et al., 2015; Segal et al., 2016). Our data provide the first description of discrete actin-based structures whose presence correlates with the fusion capacities of primary vertebrate MBs. Actin-rich protrusions have been previously described in differentiating C2C12 MBs, but there was no evidence of the presence or absence of such structures in fusion-incompetent cells (Shin et al., 2014). Using live imaging, we show the F-actin remodeling at the cell–cell contact of primary myocytes before fusion. EM analysis allowed us to directly visualize these structures and show that they form finger-like actin-based protrusions that are asymmetrically distributed and are composed of actin filament cables with branched actin networks at their base. These protrusions have the unique feature of crawling underneath the "receiving" cell, making them difficult to visualize by EM techniques that involve sectioning. We propose that these actin-based protrusions are functionally required for efficient fusion, as such structures are disassembled in cells that just fused and are virtually absent in fusion-incompetent Srf mutant cells.

Our results show that the absence of Srf expression leads to the blockade of fusion and the disruption of actin cytoskeleton organization. The major changes in the actin scaffold observed in Srf mutant cell—including less total actin and F-actin, fewer actin cables in the ventral part of the cell, and the absence of protrusion—can be attributed to Mrtf/Srf signaling being a key mediator of the cytoskeletal response to Rho family GTPase activation and actin cycling in wild-type cells (Esnault et al., 2014). Indeed, we showed that in addition to genes encoding actins, several regulators of actin cytoskeleton organization are controlled by Srf (including *Abra*, *Cnn2*, *Fermt2*, *FlnA*, *Tgfb1i1*, and *Wdr1*), and they all have been identified as direct Srf targets (Wang et al., 2011; Chong et al., 2012; Esnault et al., 2014). Several of these targets could be implicated in the formation of the actin-based protrusions. For instance, Fermt2 adaptor protein has recently been shown to associate with the Arp2/3 complex and induce Rac1-mediated membrane protrusions in fibroblasts (Böttcher et al., 2017). In addition, the actin regulator filamin-A has been shown to modulate filopodia formation through Cdc42 GTPase activation in lung carcinoma and melanoma cell lines (Chiang et al., 2017). Of note, both Rac1 and Cdc42 have been genetically linked to fusion defects in mammals (Vasyutina et al., 2009).

Remarkably, the overexpression of α-cardiac actin alone in Srf mutant cells restored actin architecture and rescued heterotypic fusion between a control MB/MT and a mutant MB and overcame the need for Srf expression in both fusion partners for efficient fusion, both in vitro and in vivo. In other words, fusion can take place between a control cell and a Srf mutant cell if its F-actin content and the organization of actin into cables are recovered. The possible underlying mechanism for this rescue could be restoration of the mechanical invading force that helps to overcome energy barriers for membrane apposition and drives cell membrane fusion (Kim et al., 2015b) or the appropriate cellular distribution of signaling molecules or contractile protein molecules required for fusion (Tran et al., 2012; Simionescu-Bankston et al., 2015).

Our study also shows that maintenance of the actin network in Srf-depleted SCs, achieved by the sole overexpression of α-cardiac actin, was not sufficient for the in vitro and in vivo (during muscle regeneration) reestablishment of homotypic fusion between two Srf mutant cells, suggesting that additional Srf targets involved in other cellular processes are required. In *Drosophila*, the initial invasive activity provided by actin foci triggers a mechanosensitive

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accumulation of myosin motors in the receiving cell to the site of invasion, which in turn generates cortical stiffness to facilitate fusion (Kim et al., 2015b). By controlling the expression of *Myl9* and *Myh9* (Medjkane et al., 2009), two components of the actomyosin network, Srf could contribute to the maintenance of mechanical tension/rigidity allowing, in concert with actin-based protrusions, productive invasion, and fusion.

The role of SCs in muscle hypertrophy as donors of new nuclei and contributors to muscle growth has been a highly debated issue (Pallafacchina et al., 2013; Blaauw and Reggiani, 2014; Snijders et al., 2015; Gundersen, 2016; Egner et al., 2017; McCarthy et al., 2017). Using a mouse model of SC ablation, McCarthy et al. (2011) showed that SCs were not required for plantaris muscle growth after overload. Egner et al. (2016) found that overload-induced hypertrophy was prevented in both SC-ablated plantaris and EDL muscles. A recent study suggested that this discrepancy may be attributed to the age of the mice, as young mice (<4 mo old) require SCs to undergo overload-induced myofiber hypertrophy, whereas mature mice (>4 mo old) do not require SCs but display an increased fiber number and size after CH (Murach et al., 2017). Our study (performed on 3-mo-old mice) provides new genetic evidence showing that hypertrophy is compromised when SCs are unable to fuse and is in agreement with the requirement of Myomaker, a known mammalian MB fusogen, in muscle hypertrophy (Goh and Millay, 2017). In vivo overexpression of α-actin in Srf mutant SCs rescued both fusion and overall growth in the overload-induced hypertrophy model, indicating the necessity of SC myonuclear accretion for optimal hypertrophic physiological muscle growth.

Collectively, our study reveals Srf as a key regulator of SC fusion and highlights the crucial role played by actin filaments to drive fusion in vitro and in vivo. Indeed, we report the existence of finger-like actin-based structures at the site of fusion only in fusion-competent mammalian cells. More generally, our data strongly support the requirement of SC-mediated fusion in myofiber hypertrophy.

Materials and methods

Mouse protocols

Srf$^{flox/flox}$ mice are homozygous for Srf floxed alleles harboring LoxP sites flanking exon 2 of endogenous *Srf* gene (Parlakian et al., 2004). *Pax7*$^{CreERT2/+}$ knock-in mice express Cre-ERT2 recombinase from the endogenous *Pax7* locus (provided by C.M. Fan, Carnegie Institution for Science, Washington, DC; Lepper et al., 2009), and Tg:*Pax7-nGFP* transgenic mice express nuclear localized EGFP under the Pax7 promoter (provided by S. Tajbakhsh, Pasteur Institute, Paris, France; BAC containing 55-kbp upstream Pax7 initiator ATG; Sambasivan et al., 2009).

To investigate the effect of SC-specific *Srf* deletion in adult muscle, the mouse strain *Pax7*$^{CreERT2/+}$*:Srf*$^{flox/flox}$*:Pax7-nGFP* was generated. In all experiments, 3-mo-old *Pax7*$^{CreERT2/+}$*:Srf*$^{flox/flox}$*:Pax7-nGFP* mice were given five i.p. injections of TMX (1 mg/d; MP Biomedicals) to induce *Srf* deletion and were referred to as mutant mice. Both *Pax7*$^{+/+}$*:Srf*$^{flox/flox}$*:Pax7-nGFP* mice injected with TMX and noninjected *Pax7*$^{CreERT2/+}$*:Srf*$^{flox/flox}$*:Pax7-nGFP* mice were initially used as control mice. However, because all muscle phenotypes investigated were identical between these two controls, uninjected *Pax7*$^{CreERT2/+}$*:Srf*$^{flox/flox}$*:Pax7-nGFP* mice were used as control mice (control) in the experiments here. No statistical differences in body weights were observed after TMX in *Pax7*$^{CreERT2/+}$*:Srf*$^{flox/flox}$*:Pax7-nGFP* mice.

To assay whether the overexpression of α-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 *α-cardiac actin* (*Actc1*) when bred with a Cre driver mouse line. The transgenic construction is composed of β-actin promoter and cytomegalovirus enhancer ensuring the transgene expression. The cDNA of the rat α-cardiac actin (Actc1) is downstream of the chloramphenicol acetyl transferase (CAT) gene flanked by loxP sites. Polyadenylation sites downstream of the CAT gene preclude the expression of α-cardiac actin. *CMV-flx-CAT-flx-Actc1* mice were bred with *Pax7*$^{CreERT2/+}$*:Srf*$^{flox/flox}$*:Pax7-nGFP* mice to obtain *Pax7*$^{CreERT2/+}$*:Srf*$^{flox/flox}$*:Pax7-nGFP:CMV-flx-CAT-flx-Actc1* mice, in which TMX injections permit *Srf* loss, the deletion of the CAT cassette, and the concomitant overexpression of *Actc1* in SCs. TMX-injected *Pax7*$^{CreERT2/+}$*:Srf*$^{flox/flox}$*:Pax7-nGFP:CMV-flx-CAT-flx-Actc1* were referred to as Mut/Act^{+} mice.

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′-GCCGGATAAAACTTGTGCTT-3′; Srflox-F, 5′-TTCGGAACTGCCGGGCACTAAA-3′; and Srflox-R, 5′-CTGTAAGGGATGGAAGCAGA-3′.

CH of plantaris muscles of control, mutant, and Mut/Act^{+} mice was induced through the incapacitation of soleus and gastrocnemius muscles by sectioning their tendon, in both legs. During the process of CH, mutant and Mut/Act^{+} mice were injected with TMX on days 2 and 4 after CH. At the indicated time (1, 3, and 5 wk after CH), plantaris muscles were dissected and processed for histological analyses. When indicated, mice were administered 25 µg/g EdU (Life Technologies).

Muscle tissue injury in control, mutant, and Mut/Act^{+} mice was achieved by a single intramuscular injection of 30 µl of 6 µM CTX (Latoxan) into TA muscle. During the process of regeneration, mutant and Mut/Act^{+} mice were injected with TMX on days 2 and 4 after CTX. Mice were allowed to recover for 30 d, and TA 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 ethics committee (00315.1).

Single-fiber culture

Individual fibers were isolated from EDL muscles of 2-mo-old control mice. EDL muscles were dissected by handling tendons only. Muscles were digested for 50 min at 35°C in 2 mg/ml collagenase I (Life Technologies) in DMEM/F12. When fibers were loosened, they were liberated using heat-polished glass Pasteur pipettes and selected by viewing under the microscope, and digestion was stopped with 20% FCS. Myofibers and associated SCs were either fixed in 4% PFA immediately after their isolation (quiescent SCs) or kept as nonadherent cultures for 24 h in DMEM, 20% FCS, 10% horse serum, and 1% chicken embryo extract (activated SCs) before fixation in 4% PFA. Myofibers were the stained against Pax7 (1/50; sc-81648; Santa Cruz Biotechnology) and Srf (1/100; sc 13029; Santa Cruz Biotechnology) and with DAPI. At least 30 Pax7^{+} cells were counted per experiment and in each condition.

Primary muscle cell culture and adenoviral transduction

Primary cultures were derived from hindlimb muscles of control, mutant, and Mut/Act^{+} of 6- to 8-wk-old mice all harboring the Pax7-nGFP transgene that allowed prospective selection of SCs by FACS. The dissection of the muscles was performed with care to take off as much fat and connective tissue as possible. The muscles were minced in DMEM/F12 supplemented with 2% antibiotic/antimycotic (15240-062; Gibco) in a sterile Petri dish on ice. The minced muscles were digested three

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times for 25 min at 37°C with 1 mg/ml collagenase D (Roche) and 0.1% Trypsin (15090-046; Gibco), and digestion was stopped by adding FCS (25% final). Cells were filtered through a 70-µm cell strainer and pelleted. Cells were then washed three times in DMEM/F12 and 2% antibiotic/antimycotic, resuspended in 1× PBS without Ca^{2+} and Mg^{2+}, 2% FCS, and 2% antibiotic/antimycotic, and finally filtered with a 40-µm cell strainer. Pax7/GFP-positive SCs were sorted on FACSAria III (BD) previously calibrated (fluorescence minus one and use of compensation beads) using the CYBIO Cochin Institute platform. Cells were collected in a FACS tube containing FCS and 2% antibiotic/antimycotic.

In standard conditions, MBs were grown in growth medium (DMEM/F12, 2% Ultroser G [PALL Life Sciences], and 20% FCS) on plastic dishes coated with 0.02% Gelatin. For differentiation, MBs were seeded in Matrigel-coated dishes and cultured in differentiation medium (DMEM/F12 and 2% horse serum).

To induce in culture the excision of the floxed *Srf* allele, $Srf^{flox/flox}$ MBs were transduced twice with adenoviruses Ad-GFP or Ad-CreGFP (100 MOI). 2 d after the first transduction, GFP^+ MBs were purified by cell sorting with BD FACSAria III.

Proliferation assays

To detect S-phase entry, control and mutant SCs were plated immediately after sorting, cultured for 5 d in growth medium, and pulsed with EdU (10 µM; Life Technologies) for 2 h before fixation with 4% PFA. EdU detection was performed using a Click-iT EdU Alexa Fluor 647 kit, according to the manufacturer's instructions (Life Technologies).

For cell cycle analysis, control and mutant MBs were collected, fixed in 70% cold ethanol, washed with PBS, and resuspended in the staining buffer containing 50 µg/ml propidium iodide and 100 µg/ml RNase (Invitrogen). Cell cycle profiles were acquired using a BD Accuri C6 cytometer (BD Biosciences) and processed with NovoExpress software (ACEA).

Western blot analysis

Cells were lysed directly in 1× Laemmli buffer, and proteins were separated through denaturing SDS-PAGE electrophoresis using Mini-Protean TGX precast gels (Bio-Rad) and transferred to PVDF membrane using the wet method (Bio-Rad). Membranes were blocked with 5% skim milk in TBS-1% Tween (TBST) for 1 h at RT and probed overnight at 4°C with primary antibodies in TBST and 5% BSA. The following antibodies were used: mouse anti-Srf (1/1,000; sc 13029; Santa Cruz Biotechnology), rabbit anti–pan actin (1/750; AAN01-A; Cytoskeleton), and mouse anti–α tubulin (1/4,000; T 6074; Sigma-Aldrich). After washing in TBST, membranes were hybridized with goat anti-mouse and goat anti-rabbit secondary antibodies coupled to HRP (1/10,000; 62-6520 and A27036; Thermo Fisher Scientific). Proteins were revealed using SuperSignal West Femto substrate (Thermo Fisher Scientific).

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). In brief, MBs were harvested, and lysates were cleared by centrifugation at 500 *g* for 5 min. Supernatants were centrifuged at 100,000 *g* for 1 h 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 that of the G-actin–containing supernatant. 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 using FusionCapt Advance software (Vilber Lourmat).

Cell migration assay

Migration of primary mouse muscle cells was quantified using time-lapse microscopy. MBs were seeded in gelatin-coated eight-well Ibidi plates and maintained in rich medium. The next day, cells were filmed using an inverted Axio Observer Z1 microscope (Zeiss) with a LCI PlN 10×/0.8 W DICII objective and an incubation chamber at 37°C and 5% CO_2. Live cells were monitored every 6 min for 6 h with bright-field and Metamorph 7.7.5 software. Cell velocities were calculated in micrometers per minute using ImageJ (National Institutes of Health) by tracking the paths of cells. At least 150 cells were tracked for each sample.

Live F-actin imaging

Control MBs were cultured in Matrigel-coated eight-well Ibidi plates. After 1 d of differentiation, they were stained with 75 nM SiR-Actin (Tebu-Bio) for 5 h before filming with an inverted Axio Observer Z1 microscope with a EC-PLAN NEOFLUAR 40×/0.75 objective and an incubation chamber at 37°C and 5% CO_2. Live cells were monitored every 10 min for 16 h with bright-field and Cy5 filter using Metamorph v.7.7.5.

Cell mixing fusion assays

To analyze heterotypic fusion between MBs, control or mutant MBs were loaded with 6 µM Orange Cell Tracker (Molecular Probes) for 30 min and co-cultured with control, mutant, or Mut/Act^+ MBs loaded with 6 µM Deep Red Cell Tracker (Molecular Probes) for 30 min in differentiation medium. For heterotypic fusion between MT control and MB, control MTs at day 2 of differentiation were loaded with Orange Cell Tracker and co-cultured with control, mutant, or Mut/Act^+ MBs loaded with Deep Red Cell Tracker. 2 d 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.

Muscle section and cell immunostaining

Plantaris and TA muscles were collected and snap-frozen in liquid nitrogen–cooled isopentane. 8-µm-thick muscle sections were fixed in 4% PFA for 8 min at RT and blocked overnight at 4°C in PBS 1×, 10% horse serum, and 0.5% Triton X-100, then incubated with primary antibodies overnight at 4°C in PBS 1×, 10% horse serum, and 0.5% Triton X-100. The following primary antibodies were used: mouse anti-dystrophin (1/50; NCL-Dys2; Novocastra), rabbit anti-laminin (L9393, 1/200; Sigma-Aldrich), and rabbit anti-myogenin (1/100; sc-576; Santa Cruz Biotechnology). After washes in PBS 1×, sections were incubated with secondary antibodies for 1 h at RT. The following secondary antibodies were used: goat anti–mouse IgG1 Alexa Fluor 488 (1/1,000; A21121; Thermo Fisher Scientific) and donkey anti-rabbit Alexa Fluor 546 (1/1,000; A10040; Life Technologies). Nucleus staining was performed using DAPI. Muscle sections were then mounted in Dako Fluorescence Mounting Medium and kept at 4°C until image acquisition.

For Pax7 staining, muscle sections were fixed in 4% PFA for 8 min at RT and permeabilized in ice-cold methanol for 6 min. Muscle sections were treated with Antigen Unmaking Solution, pH 6.0 (H-3300; Vector Laboratories) for 15 min at 95°C and cooled on ice for 30 min. Blocking and incubation with primary and secondary antibodies were conducted as described in the preceding paragraph. Primary mouse anti-Pax7 antibody (sc-81648; Santa Cruz Biotechnology) was used at dilution 1/50. EdU detection was performed using Click-iT EdU Alexa Fluor 647 kit, according to the manufacturer's instructions (Life Technologies).

Muscle cells cultured in dishes were fixed for 8 min in 4% PFA and then permeabilized and blocked in PBS with 0.1% Triton X-100 and 5% horse serum for 1 h at RT. Cells were incubated overnight at 4°C

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with the following primary antibodies: mouse anti-MyoD (1/100; SC-760; Santa Cruz Biotechnology), rabbit anti-myogenin (1/100; sc-576; Santa Cruz Biotechnology), mouse anti-MHC embryonic (1/50; MF20; Alexis Biochemical), and rabbit anti–phospho histone H3 (1/500; 3377; Cell Signaling Technology) diluted in the same buffer. After incubation for 1 h at RT with fluorescent secondary antibodies anti–mouse IgG1 Alexa 488 (1/1,000; A21121) and donkey anti-rabbit Alexa Fluor 546 (1/1,000; A10040; Life Technologies), cells were stained with DAPI (for nuclei) and phalloidin Alexa Fluor 488 (1/500; Thermo Fisher Scientific; for F-actin) and mounted in Fluorescent Mounting Medium (Dako).

Morphometric analysis and phalloidin quantification

Myofiber CSA was analyzed by using immunostaining of dystrophin, marking myofiber sarcolemma, and then using ImageJ. 600–800 myofibers were analyzed. For the quantification of the number of nuclei per myofiber, at least 500 myofibers were counted. Phalloidin signal intensity per cell was quantified using ImageJ. At least 150 cells were analyzed.

Image acquisition

Digital images were acquired using an Olympus BX63F microscope with 10× objective (UplanFL, numerical aperture 0.3) and 20× objective (UPLSAPO, 0.75), ORCA-Flash4.0 LT C11440-42U camera (Hamamatsu); an Axiovert 200M microscope (Zeiss) with 5× objective (PLANFLUAR, 0.25) and 20× objective (LD PLANNEOFL UAR, 0.4), cooled CCD CoolSNAP-HQ^2 camera (Photometrics); or a Spinning Disk Leica confocal microscope with a 100× oil-immersion objective (HCX PL APO, 1.47), cooled CCD CoolSNAP-HQ^2 camera (Photometrics) and Metamorph v.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.

Electron microscopy of unroofed cells

Adherent adult SCs were either cultured exclusively in proliferation medium or switched to fusion medium for 24 h before they were disrupted by sonication as described previously (Heuser, 2000). Glutaraldehyde/PFA-fixed cell cortices were further sequentially treated with OsO_4, tannic acid, and uranyl acetate before dehydration and hexamethyldisilazane drying (Sigma-Aldrich). Dried samples were then rotary-shadowed with platinum and carbon with a high vacuum metal coater (Leica). Platinum replicas were floated off the glass by flotation on hydrofluoric acid, washed several times in distilled water, and picked up on formvar/carbon-coated electromagnetic grids. The grids were mounted in a eucentric side-entry goniometer stage of a transmission electron microscope operated at 80 kV (model CM120; Philips), and images were recorded with a Morada digital camera (Olympus). Images were processed in Adobe Photoshop to adjust brightness and contrast and presented in inverted contrast. Anaglyphs were made by converting the −10° tilt image to red and the 10° tilt image to cyan (blue/green), layering them on top of each other using the screen blending mode in Adobe Photoshop, and aligning them to each other.

RNA extraction and quantitative real-time (qRT)–PCR

RNA extraction and qRT-PCR analysis were performed as described previously (Guerci et al., 2012). Values were normalized using *Hydroxymethylbilane synthetase* (*Hmbs*). The following primers were used: Srf-F, 5′-CACCTACCAGGTGTCGGAAT-3′; Srf-R, 5′-GCTGTGTGGATTGTGGAGGT-3′; MyoD-F, 5′-GCAGATGCACCACCAGAGTC-3′; MyoD-R, 5′-TTCCTGGG-TCCAGCCTCAAC-3′; Myogenin-F, 5′-GCAATGCACTGGAGTTCG-3′; Myogenin-R, 5′-ACGATGGACGTAAGG-GAGTG-3′; Acta1-F, 5′-CTGAGCGCAAGTACTCAGTGT-GGA-3′; Acta1-R, 5′-TTCCAA-AAACAGGCGCCGGCTGCA-3′; Acta2-F, 5′-GTCC-CAGACATCAGGGAGTAA-3′; Acta2-R, 5′-TCGGATACTTCAGCGTCAGGA-3′; Actc1-F, 5′-ACTCTCTTCCAGCCCTCTTTCATT-3′; Actc1-R 5′-GAGCCAGTGCAGTG-ATTTCCTT-3′; Actb-F, 5′-GTGGCATCCATGAA-ACTACAT-3′; Actb-R, 5′-GGCATAGAGGTCTTTACGG-3′; Actg-F, 5′-GGCTTACACTGCGCTTCTTG-3′; Actg-R, 5′-GAGTGCGGCGATTTCTTCTT-3′; ActTg-F, 5′-TGCTGGTTATTGTGCTGTCT-3′; ActTg-R, 5′-CTGTGGTCTCCTCGTCGT-3′; Abra-F, 5′-ATCGAGACGGAGAGGGACAA-3′; Abra-R, 5′-TTGCTGACAACCGTTCTGGT-3′; Cnn2-F, 5′-AATGGGCTTCCTG-TTTCTTCATCT-3′; Cnn2-R, 5′-TCGTGGGAAAGCAAA-CTTAGTCC-3′; Fermt2-F, 5′-AGTGGAATGTCAACTGGGAGATC-3′; Fermt2-R, 5′-GGACAACCGGACCT-CATCTG-3′; Flna-F, 5′-GATTGGGGAGGAGACGGTGAT-3′; Flna-R, 5′-TTTGCT-GGCTACCCTGAGGATAG-3′; Tgfb1i1-F, 5′-GCCTCTGTGGCTCCTG-CAATAAAC-3′; Tgfb1i1-R, 5′-CTTCTCGAAGAAGCTGCTGCCTC-3′; Wdr1-F, 5′-TGGAGCGG-GGCGTCTCTA-3′; Wdr1-R, 5′-AATCCGCTGGGTGCATACTTG-3′; Hmbs-F, 5′-TGCACGATCCTGAAACTCTG-3′; and Hmbs-R, 5′-TGCATGCTATCTGAGCCATC-3′.

Affymetrix microarrays

Microarray analysis was performed from three independent Ad-GFP– and Ad-CreGFP–transduced cell cultures. Total RNAs were obtained from cells at day 0 (corresponding to MBs), day 1 (corresponding to myocytes), and day 3 (corresponding to MTs) of differentiation, using RNeasy Mini kit (Qiagen) and DNase treatment (Qiagen). RNA integrity was certified on a bioanalyzer (Agilent). Hybridization to Mouse Gene 2.0-ST arrays (Affymetrix) and scans (GCS3000 7G Expression Console software) were performed on the Genom'ic platform (Institut Cochin, Paris, France). Probe data normalization and gene expression levels were processed using the robust multiarray average (RMA) algorithm in expression Console (Affymetrix). Gene ontology analysis was performed using Ingenuity (IPA) software. Full data are available on Gene Expression Omnibus: GSE105125.

Statistical analysis

Quantitative datasets were analyzed using unpaired nonparametric Mann–Whitney test (Fig. 2, C and D; Fig. 3 E; Fig. 4, C, D, and F; and Fig. S1, C–E), unpaired *t* test (Figs. 2 E and S1 H), one-way ANOVA with Bonferroni's multiple comparisons test (Fig. 4 E; Fig. 6, B, D, and F; Fig. 8, B–D and F; and Fig. S4, C and F), or two-way ANOVA with Bonferroni's multiple comparisons test (all other datasets) using GraphPad Prism 6 software. Statistical significance was set at $P < 0.05$.

Online supplemental material

Fig. S1 describes the validation of the genetic model used and the efficient loss of Srf in SCs. In addition, the phenotype of Srf-deleted SC (unaffected proliferation and differentiation and impaired motility) was confirmed by deleting Srf ex vivo using Ad-Cre transduction of Srfflox/flox MBs. Fig. S2 illustrates an alternative method to monitor the fusion defect of Srf mutant SCs in vivo. In search for the genes whose altered expression could participate in Srf mutant SC fusion defect, we quantified the expression of several genes encoding actin isoforms and regulators of actin cytoskeleton in a model of ex vivo Srf deletion (Ad-Cre transduction of Srfflox/flox MBs). Fig. S3, Video 1 (bright field), and Video 2 (Sir-Actin) show F-actin reorganization upon fusion by F-actin live imaging. Additional EM images of unroofed cells are represented. Fig. S4 describes the genetic model allowing the concomitant loss of Srf and Actc1 overexpression in SCs and the impact of Actc1 overexpression on Srf mutant MB motility and differentiation. Fig. S5 depicts the regeneration process (myofiber number and SC number) in Srf mutant muscles. Table S1 is a list of the genes whose expressions in muscle cells depend on Srf.

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Author contributions: V. Randrianarison-Huetz, A. Papaefthymiou, and S. Vassilopoulos designed and carried out experiments, analyzed results, and wrote the manuscript. G. Herledan, C. Noviello, U. Faradova, L. Collard, A. Pincini, and E. Schol conducted experiments and analyzed results. J.F. Decaux and P. Maire provided reagents and expertise. A. Sotiropoulos designed and carried out experiments, analyzed results, wrote the manuscript, and provided financial support.

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