Novel interventions to recover the regenerative capacity of aged skeletal muscle by targeting the interactions in the stem cell niche

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“ Never underestimate the power of dreams and the influence of the human spirit. We are all the same in this notion. The potential for greatness lives within each of us “

Wilma Rudolph.

To my family and loved ones.

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Résumé

Le muscle squelettique possède une capacité de régénération remarquable qui lui est conférée par ses cellules souches appelées cellules satellites. Or, cette capacité régénérative des cellules souches du muscle diminue nettement avec l'âge. Par conséquent, il est nécessaire de développer des stratégies visant à améliorer la réparation du muscle chez les personnes âgées ; en particulier pour accélérer leur rétablissement après une lésion musculaire, liée à une chute ou une intervention chirurgicale ayant affecté le muscle par exemple. Les causes de la perte de fonctionnalité des cellules satellites avec l'âge sont multi-systémiques. Nous avons donc décidé d'étudier les changements liés à l'âge à plusieurs niveaux de l'environnement des cellules souches, afin de trouver des manières de restaurer leur capacité de régénération de façon synergique.

Grâce à un criblage protéomique, nous avons identifié la fibronectine comme étant une molécule de structure et de signalisation de la matrice extracellulaire musculaire qui est perdue avec l'âge. Les cellules hématopoïétiques et endothéliales se sont révélées être les principales sources de fibronectine ; et la perte de fibronectine dans les muscles âgés en régénération est essentiellement liée à l'incapacité du muscle âgé à recruter ces cellules lors d'une lésion musculaire. Nos travaux ont révélé que la fonctionnalité des cellules souches âgées est altérée à cause de leur perte d'adhésion et de leur mort cellulaire au cours d'un processus appelé anoïkis, mais que ces altérations pouvaient être inversées grâce à un traitement par la fibronectine *ex vivo* et *in vivo*. Au niveau moléculaire, la perte de fibronectine est un événement déclencheur qui perturbe les voies de signalisation intracellulaires dans les cellules souches. Le traitement à la fibronectine permet de rétablir des voies de signalisation connues pour être perturbées avec l'âge, comme p38 et ERK ; ainsi que la signalisation par FAK dont nous avons nouvellement révélé des altérations liées à l'âge. Nous avons ainsi démontré une perturbation de la régulation paracrine des cellules souches au cours du vieillissement liée à l'incapacité à remodeler la matrice extracellulaire efficacement lors d'une lésion du muscle âgé ; ainsi que la possibilité d'intervenir sur ce mécanisme de manière thérapeutique par l'injection de fibronectine afin d'améliorer la régénération du muscle âgé.

Dans un deuxième projet, nous avons étudié les communications cellulaires entre les cellules satellites et des cellules de soutien non-myogéniques appelées progéniteurs fibro/adipogéniques (FAPs). Le devenir adipogénique des FAPs est très étroitement régulé par le microenvironnement du muscle et par la capacité myogénique de régénération. Nous avons mis en évidence que la fonctionnalité des FAPs et leur communication avec les cellules satellites sont altérées avec l'âge. Afin de mieux comprendre cette communication, nous avons identifié WISP1 comme protéine secrétée par les FAPs et qui promeut la prolifération et la myogenèse des cellules satellites. La production de WISP1 est altérée durant le remodelage du muscle âgé faisant de WISP1 un facteur de communication paracrine entre les FAPs et les cellules satellites, et responsable de leur perte de communication avec l'âge. Le traitement de souris âgées par WISP1 a permis de restaurer la fonctionnalité des cellules satellites ainsi que la régénération du muscle. Ces résultats révèlent ainsi la possibilité de cibler la communication des cellules satellites avec leur environnement afin d'améliorer la perte de régénération musculaire liée à l'âge.

Dans un dernier projet, nous avons révélé chez les cellules satellites âgées des altérations de signalisation par un peptide appelé apeline, dues à une réduction de son niveau circulant ainsi qu'à une diminution de l'expression de son récepteur APJ chez les cellules satellites âgées. Nous avons

démontré que le traitement à l'apeline permet de restaurer la fonctionnalité des cellules satellite ainsi que la régénération du muscle âgé.

Ainsi, ces approches ont ainsi permis d'identifier de nombreuses possibilités permettant d'améliorer la régénération du muscle en ciblant les interactions des cellules satellites avec leur niche. Nos résultats ouvrent la voie au développement des stratégies thérapeutiques intégrant différents niveaux de communication et de signalisation dans la niche pour stimuler la fonctionnalité des cellules satellites âgées.

Mots clés : muscle strié squelettique, niche de cellules souches, vieillissement, régénération, adipogenèse ectopique, cellules satellites, cellules souches musculaires, FAPs, matrice extracellulaire, apeline.

Abstract

The remarkable ability of skeletal muscle to regenerate upon injury is conferred by tissue-resident stem cells called satellite cells. With age, the regenerative capacity of muscle stem cells (MuSCs) dramatically declines. Developing strategies to enhance muscle repair in elderly people is therefore required; in particular to accelerate their recovery from injuries following falls or from surgical interventions affecting muscle tissues. As the causes of MuSC dysfunction with age are multi-systemic, we decided to dissect age-related changes at different levels of the MuSC environment, in order to uncover synergistic ways to restore their regenerative capacities. This thesis describes three major interventions at the extracellular matrix, cell-cell interaction and tissue/systemic level that successfully restored skeletal muscle regeneration in aged mice.

Using a proteomic screen, we identified fibronectin as a structural and signaling molecule of the extracellular matrix that is lost in the aged muscle niche. Loss of fibronectin in aged regenerating muscle primarily arises from perturbed cellular turnover of hematopoietic and endothelial cells which were shown to be the major fibronectin producers in muscle upon injury. Our work also uncovered that the function of old MuSCs is impaired by loss of adhesion and cell death by anoikis, and can be rescued by fibronectin treatment both *ex vivo* and *in vivo*. At the molecular level, loss of fibronectin is an upstream trigger leading to perturbed MuSC signaling. Fibronectin treatment rescues perturbations of pathways, such as p38 and ERK, that were previously known to be altered in aged MuSCs, as well as the newly discovered age-related perturbations of FAK signaling. Altogether we demonstrated that aging impairs the remodeling of the extracellular matrix of the MuSC niche, and thereby triggers multiple dysfunction of old MuSCs that can be rescued therapeutically.

In a second project, we dissected the cellular cross-talk between MuSCs and non-myogenic support cells called Fibro-Adipogenic Progenitors (FAPs). We uncovered that the adipogenic fate of FAPs is tightly correlated to the muscle micro-environment and the myogenic regenerative capacity in different models of regeneration and aging. The function of FAPs and their cross-talk with MuSCs are impaired with age. We identified the secreted protein WISP1 as a paracrine communication factor between FAPs and MuSCs which is perturbed with age. Treating aged mice with WISP1 restored stem cell function and muscle regeneration, highlighting the possibility to target the cross-talk of MuSC with other cells of the niche to ameliorate their function.

Our last project identified altered signaling of the small circulating peptide apelin in aged MuSCs through lowered circulating levels of apelin and down-regulation of its receptor APJ in aged MuSCs. We demonstrated that apelin treatment rescued muscle stem cell function and regeneration in aged mice, highlighting that MuSC dysfunction can also be targeted systemically.

Taken together, these approaches reveal the multiple possibilities to ameliorate muscle repair by targeting MuSC interactions with their niche. Our results also pave the way to develop integrated therapeutic strategies to boost old MuSC function.

Key words: skeletal muscle; stem cell niche; aging; repair; regeneration; ectopic adipogenesis; satellite cells; MuSCs; FAPs; extracellular matrix; apelin.

Table of content

List of figures

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

Chapter VII

List of tables

Chapter III

Chapter IV

Chapter V

List of abbreviations

-/-	Null
+/-	Heterozygous
AAV	Adeno-associated virus
Acadm	Acyl-CoA dehydrogenase medium
Ach	Acetylcholine
Acox	Acyl-coenzyme A oxidase
Acs/l	Acyl-CoA synthesase short-/long-chain
Acss	Acetyl-coenzyme A synthetase
ActR	Activin receptor
Adam12	A disintegrin and metalloprotease 12
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AIDS	Acquired immune deficiency syndrome
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Ang	Angiopoietin-1
AP	Alkaline phosphatase
aP2	Adipocyte protein 2
APJ	Apelin receptor
Apl / Apln	Apelin
atf	Activating Transcription Factor
ATP	Adenosine triphosphate
BaCl2	Barium dichloride
BAT	Brown adipose tissue
BDNF	Brain-derived neurotrophic factor
bFGF	Beta-fibroblast growth factor
BMP	Bone-morphogenic proteins
Bmpr	BMP receptor
BSA	Bovine serum albumine
C/EPB	CAAT/Enhancer-Binding-Proteins
Ca	Calcium
CacR	Calcium sensing receptor
CCN	Connective tissue growth factor (CTGF), Cystein rich protein (Cyr61), and Nephroblastoma overexpressed gene (nov)
Cdkn	Cyclin-dependent kinase inhibitor
cDNA	Complementary Deoxyribonucleic acid
chrebp	Carbohydrate-responsive element-binding protein
Col	Collagen
cox	Cytochrome c oxidase
Cpt	Carnitine palmitoyltransferase
CR domain	Cysteine-rich domain
cRNA	Complementary ribonucleic acid
CT	Scanner tomography
CTGF	Connective tissue growth factor
Ctrl	Control
CTX	Cardiotoxin
DHEA	Didehydroepiandrosterone

DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DN	Dominant negative
DNA	Deoxyribonucleic acid
DOMS	Delayed onset muscle soreness
dpi	Days post injury/injection
DTA	Diphtheria toxin A
DTR	Diphtheria toxin receptor
DXA	Dual-energy absorptiometry
ECM	Extracellular matrix
EDL	Extensor digitorum longus
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EMA	European medicines agency
EMCL	Extra-myocellular lipids
eMHC	Embryonic myosin heavy chain
Emr1	EGF-like module containing mucin-like hormone receptor 1
ESCEO	European Society for Clinical and Economical Aspects of Osteoporosis and Osteoarthritis
EWGSOP	European Working Group on Sarcopenia in Older People
FAK	Focal adhesion kinase
FAP	Fibro/Adipogenic Progenitor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor receptor
FN	Fibronectin
FNIH-SP	Foundation for the National Institutes of Health Sarcopenia Project
Foxo	Forkhead box O
Frzd7	Frizzled-7
Gastroc.	Gastrocnemius
GDF	Growth differentiation factor
GFP	Green fluorescent protein
GH	Growth hormon
gln synth	Glutamine synthetase
glut4	Glucose transporter type 4
GPCR	G protein coupled-receptor
GO	Gene Ontology
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
h	Hours
Hadh	Hydroxyacyl-CoA dehydrogenase
HDACi	Histone deacetylase inhibitor
HIF	Hypoxia inducible factor
HS	Horse serum
HSMM	Human Skeletal Muscle Cells and Myoblasts
HxKx	Histone-x-Lysine-x
ICD code	International Classification of Diseases code
IGF	Insulin-like growth factor
IL	Interleukin
IMAT	Intermuscular adipose tissue
IMCL	Intra-myocellular lipids

iPSC	Induced pluripotent stem cells
Itga	Integrin-α
Itgb	Integrin-β
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JNK	C-Jun N-terminal kinase
KO	Knock-out
LAM	Laminin
LCFA	Long chain fatty acid
LIFE study	Lifestyle Interventions and Independence for Elders study
Lin^+	Lineage positive cells (endothelial and hematopoietic)
LRC	Label retaining cells
Luc	Luciferase
M1	Pro-inflammatory macrophages M1
M2	Anti-inflammatory macrophages M2
MAPK	Mitogen-activated protein kinase
Mcad	M-cadherin
MCP-1	Monocyte chimoattractant protein 1
mdx	*Mdx* mutation of *Dmd* gene
MgCl2	Magnesium chloride
MHC	Myosing Heavy chain
min	Minutes
miR	Micro-RNA
MMP	Matrix metalloproteinases
MRF	Myogenic regulatory factor
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mRNP	Messenger ribonucleoprotein
mTOR	Mammalian target of rapamycin
MuSC	Muscle stem cell
Myh	Myosing Heavy chain
MyoD	Myogenic differentiation factor
NAD	Nicotinamide adenine dinucleotide
NG2	Neuroglial 2
Ngf	Nerve growth factor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nicotinamide-riboside
OCT	Optimum cutting temperature coumpound
p70S6K	ribosomal protein S6 kinase
Pax7	Paired boy protein
PBS	Phosphate-buffered saline
PBTX	Triton X-100 in Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFRα	Platelet-derived growth factor receptor alpha
PDGFRβ	Platelet-derived growth factor receptor beta
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PICS	PW1+/Pax7- interstitial cells
PPAR	Peroxisome-proliferator activated receptor

PRDM16	PR Domain-Containing Protein 16
PW1	PW1 gene/paternally expressed gene 3
qPCR	Quantitative polymerase chain reaction
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAT	Satellite cells
Sca1	Stem cell antigen 1
Sham	Sham / control
siRNA	Small interfering ribonucleic acid
Sirt	Sirtuin
Sma	Smooth muscle actin
sod	Superoxide dismutase
SPPB	Short Physical Performance Battery
srepb-1c	Sterol regulatory element-binding protein 1c
SRF	Serum response factor
Srpy	Sprouty
Synd	Syndecan
TA	Tibialis anterior
T-cells	Lymphocytes T
Tcf4	Transcription Factor 4
Td	Td-Tomato
tfam	Mitochondrial transcription factor A
TGF	Transforming growth factor
TIMP	Tissue inhibitors of matrix metalloproteinases
TNF	Tumor necrosis factor
Treg	Lymphocytes T, regulatory
ulk2	Unc-51 Like Autophagy Activating Kinase 2
UPR	Unfolded protein response
UPS	Ubiquitin-Proteasome system
VEGF	Vascular endothelial growth factor
VO2	Oxygen volume
vol	Volume
WAT	White adipose tissue
WISP1	Wnt1 inducible signaling pathway protein 1
WT	Wild type
YFP	Yellow fluorescent protein
β-gal	β-galactosidase

CHAPTER I. Introduction

I. Generalities about skeletal muscle

I.1. Function and structure of skeletal muscle

Three kinds of muscle tissues reside in our organism: cardiac, smooth and skeletal muscles. Cardiac and smooth muscles are known as “involuntary” muscles because of their inability to control their movement. Unlike skeletal and cardiac muscles, smooth muscles are not striated. They are located in the walls of various organs and their involuntary and rhythmical contractions allow movements to control organ functions, such as digestion, and blood circulation. Cardiac muscle is striated like skeletal muscles; and like smooth muscles, its contractions are not under conscious control. However, pacemaker cells allow heart contraction even in the absence of nervous input.

Skeletal muscles are striated and controlled by the somatic (voluntary) nervous systems. They constitute a large proportion of the body, since they represent 35 to 40% of body weight while they contain 50 to 75% of all body proteins. Their integrated contractile activity enables locomotor activity but also postural behavior and breathing via the diaphragm muscle. In addition, skeletal muscles play important physiological functions since their strong metabolic demands represent an important determinant of whole body energy expenditure, metabolism of amino acids and insulin sensitivity (Samuel and Shulman, 2012, Zurlo et al., 1990). Since the early 2000’s, skeletal muscle endocrine role was revealed, as different myokines such as IL-6, IL-15, BDNF, FGF-2, irisin and kynurenine were identified as secreted by muscle and acting on various organs (Pedersen, 2011, Pratesi et al., 2013, Bostrom et al., 2012, Schlittler et al., 2016). And these myokines often regulate the beneficial effects of exercise on other tissue (Schnyder and Handschin, 2015).

Skeletal muscles are attached to bones by tendons. Epimysium is the external muscle connective tissue sheath that fuses with the tendon and that encompasses the fascicles composing the muscle (**Fig.1**). Muscle fascicles are made of several groups of myofibers and are wrapped by the perimysium sheath. At the cellular level, myofibers are the primary component of skeletal muscle and they are surrounding by the endomysium. These cells are multinucleated syncytia whose post-mitotic nuclei are located at the myofiber periphery.

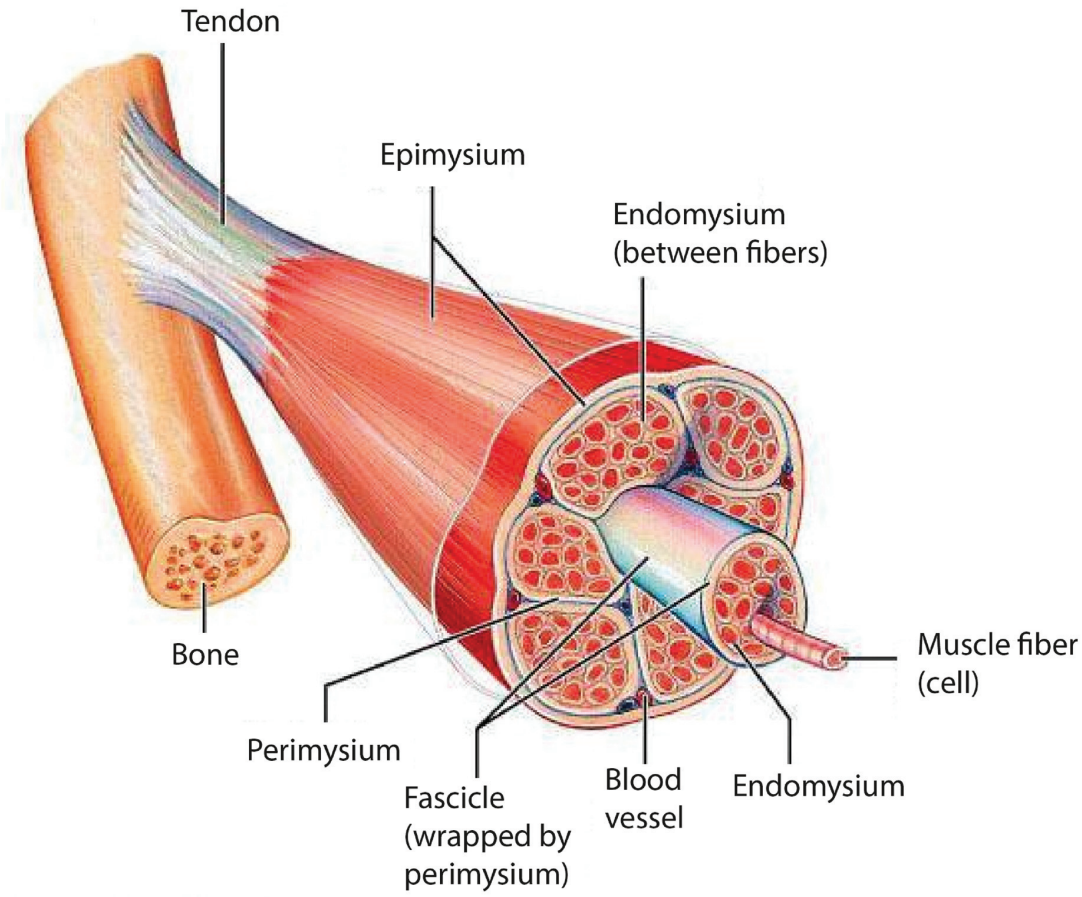

Figure 1. The skeletal muscle structure. Skeletal muscles are attached to at least one bone by the tendon and are irrigated by blood vessels. They are made of groups of muscle fibers surrounded by the perimysium, also called fascicles. Myofibers are themselves surrounded by the endomysium. *Source: Faith's anatomy and physiology blog*

(https://anatomyphysiologyfaithc.files.wordpress.com/2015/01/skeletal-muscle.jpg).

At the molecular level, the contractile units of muscle are the sarcomeres, a complex multiproteic network of anchoring and contractile proteins, out of which actin and myosin constitute the key components (Hwang and Sykes, 2015). Actin and myosin form the thin and thick filaments, respectively. Actin filaments are capped at the Z-line, interleaved between myosin filaments located between two Z-bands; thus forming the sarcomere unit. The head domain of Myosin Heavy Chain proteins (MYH) directly interacts with actin filaments and ATP hydrolysis generates a conformational change of MYH heads allowing myosin filaments to slide over actin filaments, thus generating muscle contraction characterized by a shortening of the Z-band. The interaction between MYH heads and actin filaments is made possible by Ca^{2+} influx which binds to troponin and thus, unmasks actin filament binding sites by releasing tropomyosin. Ca^{2+} influx in myofibers is subsequent to acetylcholine (Ach) release by the motor neuron at the neuromuscular junction. Ach binding to myofiber membrane receptors triggers an action potential which travels along the membrane down to the T tubule, an invagination conformation of the sarcolemma in close proximity to the sarcoplasmic reticulum (Frontera and Ochala, 2015, Gehlert et al., 2015). This membrane depolarization is sensed by the dihydropyridine receptor (DHPR) and leads to Ca^{2+} release from the sarcoplasmic reticulum via the ryanodine receptor, allowing MYH to actin filaments binding, and thereby muscle contraction.

In each adult mammalian myofiber, one of the four different isoforms of MYH is expressed, categorizing muscle fibers according to their contraction speed, and indirectly according to their metabolic source of ATP. The slow-twitch type I fibers are characterized by the lowest contraction speed and rely on oxidative mitochondrial metabolism for efficient resistance to fatigue, whereas the fast-twitch type IIb fibers are characterized by high intensity short-duration contractions and rely on glycolytic metabolism (Schiaffino and Reggiani, 1994, Spangenburg and Booth, 2003, Gundersen, 2011). Type IIa and IIx have intermediate contractile and metabolic properties, with IIa fibers having a fatigue resistant oxidative phenotype and IIx fibers having faster contraction speed and mixed metabolism. The relative proportion of the various fiber types in a given muscle is determined by the contractile characteristics which are directly controlled by the firing frequency of afferent motor neurons. Postural muscles tend to be slow-twitch fatigue resistant while locomotor muscle have faster high-intensity characteristics depending on their exact function. However, fiber type determination relies on a combination of factors: genetics, exercise training, innervation, mechanical conditions, and paracrine-autocrine signaling as well as circulating hormones (Pette and Staron, 2001, Gundersen, 2011, Schiaffino, 2002). Training and various diseases contribute to fiber type composition, as well. Fiber type determination also has species-specific characteristics as rodent muscles are generally rich in fast IIb fibers while this fiber type is only very rarely expressed in humans (Scott et al., 2001, Smerdu et al., 1994).

I.2. The myogenic program

Satellite cells, the quintessential muscle stem cells, were discovered in 1961 (Mauro, 1961). During the mouse embryonic development, satellite cells arise around E17.5 from a subpopulation of myogenic precursors of the somites (somatic dermomyotome) expressing Pax7 and/or Pax3 (Bryson-Richardson and Currie, 2008, Messina and Cossu, 2009). The cells do not express myogenic regulatory factors (MRFs) and migrate toward their niche, the sub-laminal position (on the surface of the myofiber beneath the basal lamina). After birth, satellite cells rapidly proliferate to accommodate muscle growth and provide additional nuclei to fibers, while acquiring molecular features of adult satellite cells, such as the cell surface markers Integrin α7, CD34, syndecan 3 and syndecan 4, M-Cadherin, CXCR4 (Yin et al., 2013b) (**Fig. 3**).

As later described in section **II.2.**, adult quiescent satellite cells express Pax7, alone or together with Myf5, according to their commitment state (Kuang et al., 2007). Myf5 was also suggested to regulate satellite cell proliferation rate as well as homeostasis (Gayraud-Morel et al., 2007, Ustanina et al., 2007). Adult myogenesis mostly relies on the sequential expression MRFs, coordinated through progressive activation and repression of these transcription factors (**Fig. 6**). Once activated, satellite cells proliferate and commit toward the myogenic lineage through the upregulation of the myogenic differentiation factor 1 (MyoD), generating what we call the myoblasts. MyoD is indeed required for the differentiation potential of myoblasts (Cornelison et al., 2000, Sabourin et al., 1999). During later commitment, myoblasts down-regulate Pax7 and while starting to express myogenin (Olguin and Olwin, 2004, Zammit et al., 2002). This early differentiated state is called myocytes. Mononucleated myocytes then start expressing myosin heavy chain and fuse together to form *de novo* myofibers, or fuse to existing fibers (Kuang and Rudnicki, 2008). Multinucleated fibers then express high levels of desmin and MRF4 (Le Grand and Rudnicki, 2007). Interestingly, reciprocal inhibitions between Pax7 and MRF control the myogenic program. Indeed, sustained expression of Pax7 inhibits progression toward differentiation by regulating MyoD activity (Olguin and Olwin, 2004, Olguin et al., 2007). Conversely, myogenin is thought to be involved in Pax7 down-regulation, thus ensuring fine-tuned progression of myoblasts into the myogenic program (Olguin et al., 2007).

I.3. Skeletal muscle plasticity & molecular regulatory networks

Skeletal muscle is a very plastic tissue and both its structural and functional malleability relies on internal and external stimuli, as well as on its use. Muscle plasticity notion is defined at three levels by the tight regulation of muscle mass and muscle metabolism, together with the capacity of skeletal muscle to regenerate. In this part, we will present the regulation of muscle mass metabolism in high levels; as muscle regeneration will be further detailed in part **III**.

Unlike post-natal and young muscles which rely on cellular turnover for growth, adult muscle growth is primarily regulated by a balance of anabolic and catabolic signaling that, in turn, modulates protein turnover. However, muscle hypertrophy is not spontaneous at adult age but can still can occur as a consequence of exercise. While high-load exercise, with strength training can induce muscle hypertrophy, endurance exercise is more prompt to lead to changes of cellular content leading to metabolic adaptation (Hoppeler, 2016). Indeed, three months of heavy-resistance training is enough to significantly increase muscle fibers size in young adult men, in the absence of myonuclei number increase; but this increased size cannot be maintained after the end of the training program (Kadi et al., 2004b). Similarly, myofiber cross-sectional area and myofibrils volume were increased of 8-10% after 6 weeks of heavy-resistance training (Luthi et al., 1986); while endurance training over the same time period could increase the mitochondrial content of about 30% (Hoppeler et al., 1985). Physiological stressors appearing during exercise can be classified as follows: mechanical load, neuronal activation, hormonal adjustments and metabolic disturbances (Fluck and Hoppeler, 2003).

Maintenance of muscle homeostasis at the protein level involves a fine-tuned balance between protein degradation and protein synthesis. Understanding molecular networks regulating muscle plasticity, both at the muscle mass and metabolic levels is key to further develop strategies for the prevention or treatment of muscle wasting.

Skeletal muscle adaptation to endurance exercise

What we call the endurance phenotype is obtained as a result of an accumulation of many bouts of endurance exercise, each of which lead to gene expression changes in the trained muscle as well as increased mitochondrial function and content, eventually (Hoppeler, 2016, Joseph et al., 2006). The transcription coactivator PGC1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) appears as a central regulator of transcriptional changes resulting from endurance training. PGC1α regulates mitochondrial biogenesis by inducing of the expression of mitochondrial genes from both nuclear and mitochondrial DNA (Wu et al., 1999). The major roles of PGC1α in glycogen storage,

lipid catabolism and lipid anabolism are also established, placing PGC1α as a major regulator of exercise capacity and post-exercise refueling (Summermatter et al., 2011, Wende et al., 2007, Chan and Arany, 2014). PGC1α is rapidly upregulated after exercise and quickly comes back to basal level when the activity stops; but high PGC1α levels can be maintained in chronically trained skeletal muscles (Pilegaard et al., 2003, Russell et al., 2003). AMP-activated protein kinase (AMPK) also plays a key role in long-term adaptation to exercise (Mounier et al., 2015). AMPK balances energy demands and nutrient supply via the control of anabolic versus catabolic processes in cells. Contrary to short term actions of AMPK on acute phosphorylation of various regulatory proteins, its long-term regulatory actions are achieved by direct phosphorylation of PGC1α, thus mediating the transcription of the glucose transporter GLUT4 (to enhance fuel intake and storage) and genes involved in mitochondrial biogenesis (Jager et al., 2007, McGee and Hargreaves, 2010, Mounier et al., 2015). Muscle adaptation to exercise is also influenced by Ca^{2+}-dependent signaling. Ca^{2+} binds to calmodulin which in turns, interacts with calcium decoders (such as calcineurin and calmodulin-dependent protein kinase) subsequently modifying PGC1α expression through the activation of diverse transcription factors (Norrbom et al., 2004). Although Ca^{2+} signaling is extremely fast, it is thought that calcium decoders mediate skeletal muscle adaption by participating to the memory of previous activation events (Hoppeler, 2016). Reactive oxygen species are also induced upon exercise and are thought to regulate their auto-protection through NF-κB-mediated activation of anti-oxidant (such as manganese superoxide dismutase), as well as to promote AMPK-induced PGC1α (Gomez-Cabrera et al., 2005, Hoppeler, 2016).

Muscle cells also respond to beta-adrenergic signaling to promote PCG1α-dependent VEGF expression, and thus induce angiogenesis in response to the increased demand of oxygen and blood flow to sustain the metabolic adaptation to exercise (Chan and Arany, 2014). In addition, myofibers are known to store VEGF in vesicles, and can release the angiogenic factor into the extracellular space upon contraction events to further enhance muscle capillary supply (Hoier et al., 2013). VEGF is also a transcriptional target of the hypoxia inducible factor (HIF-1), a major cellular stressor and regulator of oxygen homeostasis that is stabilized in hypoxic conditions. As HIF-1 also regulates the expression of genes involved in glycolysis, glucose uptake and angiogenesis, many endurance training protocols have been established to induce hypoxia and maximize HIF-1 downstream effects (Millet et al., 2010). Nevertheless, HIF-1 induction upon exercise as well as its beneficial downstream roles for adaptation are questioned (Lindholm et al., 2014, Mason and Johnson, 2007). Skeletal muscle endurance exercise also promotes energy metabolism regulation. In particular, endurance training leads to increase in intracellular free long-chain fatty acids (LCFAs), as well as to an upregulation of peroxisome-

proliferator activated receptor delta (PPARδ) which induce lipid metabolism and adapt skeletal muscle to the metabolic use of fatty acids (Kannisto et al., 2006).

Positive regulators of skeletal muscle mass

As already mentioned, skeletal muscle tissue is the main reservoir of proteins; and muscle mass is tightly controlled by a fine-tuned balance between protein catabolism and anabolism. Many pathways involved in muscle mass control converge towards mTOR complex 1 (Mammalian target of rapamycin complex 1, mTORC1), a key regulator of translation initiation and elongation (Sandri, 2008). IGF-1 (Insulin-like Growth Factor 1)-Akt signaling is one of the best characterized pathway regulating muscle growth. IGF-1 is secreted by the liver under the control of Growth-Hormone (GH), but different IGF-1 splicing products are also produced directly by skeletal muscle. Overload has been shown to induce IGF-1 overexpression (Goldspink, 1999, McCall et al., 2003), leading to muscle adaptation through the induction of hypertrophy and increase of muscle strength (Alzghoul et al., 2004, Musaro et al., 2001, Schiaffino and Mammucari, 2011). IGF-1 is known to activate PI3K-Akt pathway, which in turns stimulates mTORC1 and protein synthesis (Sandri, 2008, Schiaffino et al., 2013). In mammals, there are three *Akt* genes, *Akt1* and *Akt2* being strongly expressed in skeletal muscle (Yang et al., 2004). Several studies have reported Akt activation upon muscle contractile activity, and in particular stretch and functional overload both in rodents and humans (Turinsky and Damrau-Abney, 1999, Bodine et al., 2001, Sakamoto et al., 2004, Sakamoto et al., 2003, Sakamoto et al., 2002). A downstream effector of mTORC1 is the ribosomal protein S6 kinases (p70S6K) which activates protein synthesis. mTORC1 also acts through the inactivation of 4E-BP1, a repressor of protein synthesis (Sandri, 2008). Akt-mTOR pathway is tightly regulated through positive and negative feedbacks. In particular, mTORC1 (also called raptor, and rapamycin-sensitive mTOR complex) negatively regulates IGF-1 signaling, and mTORC2 (also called rictor, and rapamycin-non sensitive mTOR complex) enhances it. Last, it is important to note that the anabolic effects in skeletal muscle depend on the availability of essential amino acids, and leucine particularly (Hoppeler, 2016). The intracellular sensor of amino acid signaling to mTORC1 are recently coming to light and in particular the Ras-related GTPases (guanosine triphosphatases), or Rag, which induce mTORC1 activation (Sancak et al., 2008, Bar-Peled and Sabatini, 2014). Rag proteins are themselves regulated by various sensors such as folliculin or Ragulator/v-ATPase that sense lysosomal amino-acids like arginine (Zoncu et al., 2011, Duran and Hall, 2012). Leucine differently signals on GATOR1 and GATOR2 that activate Rag proteins (Bar-Peled et al., 2013), and Sestrin 2 was recently uncovered to be the leucine sensor regulating GATOR2 (Saxton et al., 2016, Wolfson et al., 2016).

While it is clear that Akt-mTORC1 axis is key for skeletal muscle growth, it remains to be defined how mechanical stress is transduced into Akt activation, although many studies report evidences of mechanical sensors and additional signaling pathways. Mechanical stress induced by functional overload is sensed by mechano-sensors, out of which focal-adhesion kinase (FAK) was shown to play a critical role (Hoppeler, 2016). In particular, it was demonstrated that FAK is required for IGF-1-induced muscle hypertrophy and that it can activate p70S6K in an Akt-independent way (Crossland et al., 2013, Klossner et al., 2009). The transcription factor serum response factor (SRF) was reported to be required for overload-induced muscle hypertrophy, although it is still debated whether its role is Akt-dependent or not (Schiaffino et al., 2013). β-agonists (such as clenbuterol and formoterol), androgens (such as testosterone) were also showed to lead to Akt phosphorylation and to have anabolic effects (White et al., 2013, Koopman et al., 2010, Kline et al., 2007). Bone-morphogenic proteins (BMPs) were also identified to cross-talk with the Akt/mTOR pathway through Smad1/5/8 leading to positive regulation of muscle mass (Sartori et al., 2014, Sartori et al., 2013).

Negative regulators of skeletal muscle mass

Muscle wasting occurs as a consequence of inactivity, denervation or secondary to severe diseases such as AIDS, cardiac failure, diabetes, cancer or renal disease. Aging can also spontaneously lead to loss of skeletal muscle mass and function, a recently medically-recognized disease called sarcopenia (Lloyd, 2016). Although muscle atrophy is often multisystemic, suppression of anabolic pathways and induction of protein degradation pathways are common to these conditions.

Myostatin is one of the best known negative regulator of muscle growth. Myostatin is a member of the transforming growth factors β (TGF-β) family of proteins, and its roles are mostly studied in the context of myostatin inhibition-induced muscle hypertrophy (myostatin mutations, use of a myostatin inhibitor that is follistatin…), both through hyperplasia (increase in the number of cell number) and hypertrophy (increase in cell size) (Lee, 2004, Sandri, 2008). Indeed, it was demonstrated that myostatin plays a dominant role in regulating cellular turnover, as it inhibits satellite cell activation and differentiation (Iezzi et al., 2004, McFarlane et al., 2008, McFarlane et al., 2006). Of note, it was reported that gain of muscle mass in myostatin-null mice does not correlate with gain of muscle strength, contrasting with IGF-1 overexpression models were both hypertrophy and force gain are observed (Alzghoul et al., 2004, Musaro et al., 2001, Schiaffino and Mammucari, 2011, Amthor et al., 2007). Like activin A and growth differentiation factor 11 (GDF-11), myostatin binds to activin type II receptors ActRIIA/B. The Smad2/3 complex is phosphorylated and activated subsequent to ligand binding to ActRIIA/B. Smad2/Smad4 and Smad3/Smad4 then inhibit muscle growth, but their

transcriptional targets mediating this effect are not known. However, it was suggested that myostatin could interfere with the Akt/mTOR pathway either directly (Sartori et al., 2009, Trendelenburg et al., 2009) or through Smad complexes (Remy et al., 2004, Conery et al., 2004); thus blocking protein anabolism. Although it has been shown that myostatin administration inhibits protein synthesis and leads to muscle loss, the role of myostatin in directing atrophy is not well understood (Lee, 2004, Taylor et al., 2001, Schiaffino et al., 2013). A model was further built in which myostatin/activin/TGF-β cross-talk with the positive regulators of muscle mass BMPs, which signal through Smad1/5/8, interfere with the Akt/mTOR and negatively regulate the muscle ubiquitin ligase MUSA-1 required for muscle atrophy (Sartori et al., 2014, Sartori et al., 2013). It was proposed that Smad4 is released and recruited to Smad2/3 or Smad1/5/8 when BMP pathway is blocked or levels of phosphorylated Smad2/3 are weak, respectively (Sartori et al., 2013)

Forkhead box O (FoxO) family of transcription factors are central players of muscle atrophy. When active and located in the nucleus, FoxO promotes the expression of atrophy-related genes called atrogenes; out of which the muscle-specific ubiquitin ligases atrogin-1/MAFbx and MuRF1 are the most induced, leading to protein degradation through the ubiquitin-proteasome system (Yauk et al.) (Sacheck et al., 2007). FoxO also regulates autophagy, another process involved in protein degradation (Bonaldo and Sandri, 2013). In particular, FoxO3 upregulates glutamine synthetase inducing glutamine production, which inhibits mTOR signaling and thus, promotes autophagy (Sandri, 2012).

Cross-talks between protein synthesis and breakdown are largely reported, referring to the IGF-1 / Akt / FoxO signaling. Indeed, atrogenes upregulation is normally blocked by Akt through negative regulation of FoxO (Sandri et al., 2004, Stitt et al., 2004, Lee et al., 2004). Conversely, there are evidences that FoxO suppresses protein synthesis (Demontis and Perrimon, 2010, Lee et al., 2010, Reed et al., 2012).

Other pathways are involved in negative regulation of muscle mass; such as inflammation, and in particular inflammatory cytokines like IL-6 and TNFα which are important triggers of muscle wasting and can induce protein degradation and blockade of the IGF-1/Akt pathway (Peterson et al., 2011, Dogra et al., 2007). Glucocorticoids that are used to treat inflammatory diseases or are elevated in several conditions can also trigger muscle wasting through mTORC1 pathway suppression, stimulation of *FoxO* transcription or increase *myostatin* transcription (Schakman et al., 2013).

Therefore, maintenance of skeletal muscle mass and function tightly depends on the fine-tuned balance of the process described above. And processes presented as negative regulator of muscle mass remain key for muscle homeostasis. For instance, as we will see later, age-related alterations of muscle autophagy and mitophagy is deleterious for muscle function in sarcopenia.

II. Satellite cells

Another feature of muscle plasticity is marked by its ability to self-repair, which relies on the regenerative capacity of the quintessential adult stem cells, the satellite cells.

II.1. Satellite cells and their specific marker Pax7

Pax7 is the canonical marker of all quiescent and activated satellite cells (Seale et al., 2000), and the contribution of satellite cells to muscle development and muscle regeneration has been extensively studied.

Mouse models of conditional depletion of satellite cells by diphtheria toxin *in vivo* demonstrated the absolute requirement for satellite cells (targeted by their Pax7 expression) in skeletal muscle regeneration (Lepper et al., 2011, Murphy et al., 2011, Sambasivan et al., 2011). Although it is not excluded that other muscle cell types can participate to muscle regeneration (Joe et al., 2010, Murphy et al., 2011), these cells are not myogenic in an autonomous way in the presence of satellite cells.

Pax7 expression requirement for satellite cell function has long been debated in different contexts. Indeed, contrary to the $Pax7^{-/-}$ mice in C57/B6 background that die at 2-3 weeks of age (Seale et al., 2000), $Pax7^{lacZ/lacZ}$ mice in 129Sv/J background (Kuang et al., 2006) can live up to 6 weeks and allow to study muscle growth and regeneration in the absence of Pax7. Expression of Pax7 in satellite cells is essential for muscle growth and regeneration, and required in the satellite cell progenitors to express markers of functional satellite cells, such as Syndecan4, CD34 (Kuang et al., 2006). Absence of muscle regeneration in $Pax7^{lacZ/lacZ}$ mice reveals that presence of other resident or circulating stem cells in muscle is not sufficient to compensate the loss of satellite cell function. Of note, this study also revealed the presence of a few myogenic interstitial $Pax3^{+}$ cells as a new myogenic population that is distinct of the satellite cell lineage (Kuang et al., 2006). Later, it was reported that while Pax3 and Pax7 are determinant for muscle embryonic development (Relaix et al., 2005), dual tamoxifen-induced conditional inactivation of Pax3 and Pax7 expression in satellite cells does not impair adult muscle regeneration (Lepper et al., 2009). Consequently, expression of Pax7 in satellite cells has been suggested to only be required up to the juvenile period when progenitor cells transition into quiescence, suggesting different Pax7-dependency of neonatal progenitors and adult satellite cells. However, this observation was more recently challenged by another study which revealed that on the contrary, Pax7 is absolutely essential for the normal function of satellite cells during muscle regeneration in both neonatal and adult skeletal muscle (von Maltzahn et al., 2013). Here, Pax7

deletion using the same alleles generated by Lepper *et al.* (Lepper et al., 2009) leads to cell cycle arrest and down-regulation of the myogenic regulatory factors (MRFs). Consequently, the total number of satellite cells is dramatically decreased, and muscle regeneration markedly impaired. The difference between both studies results mostly relies on the paradigm of tamofixen treatment. It seems that an IP injection of tamofixen (followed by CTX-induced muscle injury) (Lepper et al., 2009) only leads to a partial deletion, where some satellite cells escape deletion and are able to repopulate the regenerating muscle with Pax7 expressing satellite cells for partial regeneration, as it was shown that as few as one transplanted satellite cell confers regenerative potential (Cosgrove et al., 2014, Sacco et al., 2008). Nevertheless, the idea that a continuous tamoxifen exposure through tamoxifen diet after CTX injury (von Maltzahn et al., 2013) could prevent the regrowth of residual Pax-7 expressing cells does not correlate with the absence of Pax7 detection in the first study, both at the mRNA and protein level in muscles 10 days after a first CTX injury or even 6 days after a second one (Lepper et al., 2009), although it is difficult to exclude the possibility that a few $Pax7^{+}$ escaper cells may have been missed from the analysis. The contradictory observations could originate from the different time points used for measurement. Nevertheless, as in healthy muscle Pax7 deletion did not decrease the size of the satellite cell pool, it was suggested that Pax7 deletion prevents the self-renewal of satellite cells and induces satellite cell loss through terminal differentiation, which dramatically impairs muscle regeneration after injury (von Maltzahn et al., 2013). Although the first study reported that in adult mice, Pax7 depleted satellite cells seem to be functional and able to proliferate; self-renewal capacity of these cells was not tested (Lepper et al., 2009). A third study confirmed Pax7-dependency of muscle regeneration, using a prolonged tamoxifen treatment starting before injury and extended administration during the regeneration process (Gunther et al., 2013). Today, Pax7 requirements for satellite cell self-renewal and muscle repair is well acknowledged, and those three studies have enlightened the potential limitations of the Cre-lox technology (Brack, 2014). Its efficiency indeed depends on Cre-activation, gene recombination efficiency and loss of the proteins in the targeted cell population, and should be analyzed on a high number of targeted cells in each mouse. One should be cautious about how to interpret non-recombined cells, even when considered rare, as “escaper” cells can have the capacity to repopulate and out-compete the recombined cells, especially if targeted cells show high proliferative and regenerative capacities such as satellite cells.

II.2. Stemness of satellite cells and heterogeneity

Satellite cells belong to the adult stem cell type, also called tissue stem cells. Like any other stem cell type (embryonic stem cells, induced pluripotent stem cells (iPSCs), germinal stem cells, fetal stem cells, cord blood stem cells), adult stem cells have the ability to self-renew to maintain the stem cell pool and to differentiate into a specialized cell (Wagers and Weissman, 2004).

The stemness of satellite cells first originated from evidence showing that satellite cells were able to form multinucleated myotubes *in vitro* and to participate to muscle regeneration *in vivo* (Snow, 1978, Schultz et al., 1985, Zammit et al., 2002). Self-renewal capacity of satellite cells was then demonstrated *in vivo* by showing enhanced muscle regeneration after single fiber (containing 7-20 satellite cells) (Collins et al., 2005) or single cell transplantation (Sacco et al., 2008). Recently, the long-term self-renewal of satellite cells has been shown after 7 rounds of serial transplantations *in vivo* (Rocheteau et al., 2012). In contrast to this ability to regenerate muscle from a single stem cell, satellite cell depletion models using $Pax7^{CreER}$-DTA do not lead to 100% depletion of $Pax7^{+}$ satellite cells but alter regeneration of muscle (Sambasivan et al., 2011, Fry et al., 2014), suggesting an heterogeneity in the regenerative potential of satellite cells.

In spite of their common Pax7 expression, satellite cells are a heterogeneous population, both in their function and gene expression profiles. Origin of satellite cells heterogeneity have been discussed extensively (Kuang et al., 2007, Rocheteau et al., 2012, Chakkalakal et al., 2014); and it appears that heterogeneity could arise from satellite cell development where the satellite cell preferential fate is already established at the quiescent state or could rather arise after the first cell division following an injury (Troy et al., 2012). Nevertheless, as satellite cells self-renew and return back to quiescence after activation, events occurring during muscle development or adulthood result in global heterogeneity of the satellite cell pool at the quiescent state. The analysis of the satellite cell pool from Myf5-Cre/ROSA-YFP mice showed that satellite cells are composed of stem cells that have never expressed Myf5 ($Pax7^{+}$/$Myf5^{-}$, 10%) and of committed progenitors that have already initiated an activation of myogenic differentiation in the history ($Pax7^{+}$/$Myf5^{+}$) (Kuang et al., 2007) (**Fig. 2a**). Prospective isolations have shown that whereas $Pax7^{+}$/$Myf5^{+}$ cells preferentially differentiate, $Pax7^{+}$/$Myf5^{-}$ cells contribute to the satellite cell reservoir by asymmetric division, mostly in an apical-basal orientation (Kuang et al., 2007). Another level of satellite cell heterogeneity and stemness properties has been described based on Pax7 expression level using Tg:Pax7-nGFP mice (Rocheteau et al., 2012). In this study, Pax7-$nGFP^{Hi}$ cells are less primed for differentiation than Pax7-$nGFP^{Low}$ cells and their first mitosis is delayed. Pax7-$nGFP^{Hi}$ cells display more stem-like markers, perform template DNA strand segregation and can give rise to Pax7-$nGFP^{Low}$ cells after several passages *in vitro* and after

serial transplantations *in vivo*. Others have used a TetO-H2B-GFP reporter to quantify the proliferative history of satellite cells and identified two subpopulation based on label retention (Chakkalakal et al., 2014). Adult satellite cells retaining the H2B-label (called labelled retaining cells, or LRC) express high levels of $p21^{cip1}$ (*Cdkn1a*) and $p27^{kip1}$ (*Cdkn1b*) and are able to self-renew; while non-retaining H2B-label cells (nonLRC) are restricted to differentiation. It was shown that both subpopulations with distinct potential are established at birth and maintained throughout life, as LRC cells were shown to give rise to either LRC or nonLRC cells.

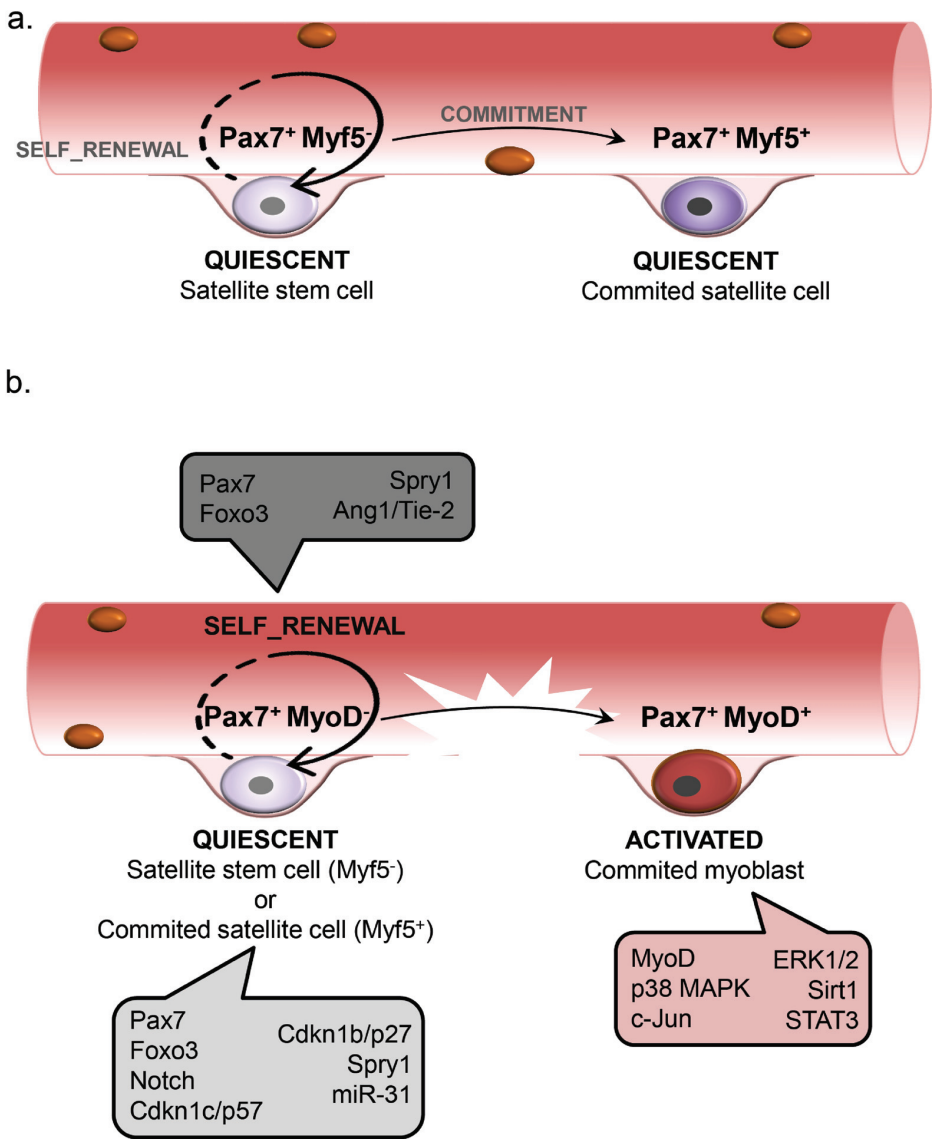

Figure 2. The myogenic differentiation program. (a) Subpopulations of quiescent satellite cells according to their Myf5 expression. In adult muscle, most satellite cells (90%) have already expressed Myf5 and have committed to the myogenic lineage but a subpopulation of quiescent satellite cells have never expressed Myf5 (10%) and are thus considered as the "satellite stem cells". **(b)** Regulators of satellite cell quiescence, activation and self-renewal during the activation process.

II.3. Maintenance of satellite cell quiescence in the adult muscle

Satellite cells quiescence is maintained by the complex cellular and paracrine environment of the muscle stem cell niche. An adult stem cell niche is a close micro-environment made of blood vessel, neurons, other tissue resident cells (some of them being called support cells) and extracellular matrix providing them with structural, physical, metabolic and soluble cues (Yin et al., 2013b, Lutolf and Blau, 2009). Disrupting the niche (upon injury or for isolation of satellite cells by flow-cytometry for instance) rapidly initiate changes leading to activation and *in vitro* culturing of muscle stem cells amplify the activation and differentiation process (Montarras et al., 2005, Gilbert et al., 2010). In alignment with most studies in the field, we will hereafter consider freshly isolated satellite cells as quiescent (Liu et al., 2013, Fukada et al., 2007, Pallafacchina et al., 2010).

In adult and healthy muscle, satellite cells are found below the basal lamina in the G_0 phase (mitotically quiescent). *Pax7* expression maintenance is essential for blocking the myogenic program, and control of its transcriptional activation plays an important role in the regulation of quiescence (Olguin and Olwin, 2004, Olguin et al., 2007). Quiescent satellite cells are distinguished from activated satellite cells and committed transient amplifying progenitors by a distinct transcriptomic profile, and studies have provided an estimation of more than 500 differentially regulated genes (Fukada et al., 2007, Pallafacchina et al., 2010, Liu et al., 2013). Absence of *MyoD* expression is part of the quiescence phenotype (Cooper et al., 1999) (**Fig. 2b**). At the single gene levels, quiescent satellite cell were reported to upregulate cell cycle regulators such as cyclin-dependent kinase inhibitor 1C (*Cdkn1c/p57*), regulator of G-protein signaling 2 and 5 (*Rgs2-5*), *Cdkn1b*/*p27*, the retinoblastoma tumor suppressor protein (*Rb*), as well as sprouty 1 (*Spry1*), the negative regulator of FGF (Fukada et al., 2007, Liu et al., 2013). Several myogenic inhibitors were also reported to be upregulated in quiescent satellite cells, such as bone morphogenic proteins (*Bmp2*, *Bmp4*, *Bmp6*). At the gene set level, it was shown that quiescent satellite cells are enriched in signatures for cell-cell adhesion, extracellular matrix, copper ion binding, lipid transporter activity (Fukada et al., 2007). The potential of satellite cells to control their niche through cell adhesion and matrix remodeling was further supported by Pallafacchina *et al.*, who uncovered a specific signature of quiescent satellite cells for oxidative stress protection (Pallafacchina et al., 2010). Tie-2 receptor is also particularly enriched in quiescent and reserve satellite cells, and its downstream signaling favors return to G_0 as well as an increased *Pax7* expression (Abou-Khalil et al., 2009).

Notch signaling is also well known to be involved in the maintenance of satellite cell quiescence and the regulation of satellite cell activation and proliferation (Bjornson et al., 2012). While inhibition of Notch signaling leads to myogenic differentiation, constitutive activation of Notch1 maintains Pax7 expression and down-regulates Myf5 and MyoD (Conboy and Rando, 2002, Wen et al., 2012).

Supporting the role for Notch signaling in quiescence, Notch3 is upregulated in freshly isolated satellite cells compared to activated satellite cells and protein levels decline rapidly after activation (Fukada et al., 2007, Mourikis et al., 2012). Furthermore, satellite cell specific deletion of recombining binding protein-Jκ (RBP-Jκ), a nuclear factor downstream of Notch regulating the transcription of target genes (such as Pax7, Hes and Hey (Wen et al., 2012)), leads to satellite cell depletion and impaired regeneration (Bjornson et al., 2012). The inability to regenerate properly was attributed to the rapid activation and direct differentiation of satellite cell, and lack of cell division; suggesting a role for Notch in self-renewal as well. In addition, *Notch 1* and *Notch 3* transcription was showed to be activated by Foxo3; which is enriched in quiescent satellite cells (Gopinath et al., 2014).

Quiescence is also controlled by microRNAs (miRNA)-induced inhibition of myogenic regulatory factor transcription, such as *Myf5*. Although miRNA control of satellite cell function is extremely poorly understood, a few miRNAs such as miR-1, miR-206 and miR-133 were found to regulate proliferation and differentiation (Townley-Tilson et al., 2010). MiR-31 was, however, identified to regulate quiescence through its sequestration into messenger ribonucleoprotein (mRNP) granules together with *Myf5* mRNA (Crist et al., 2012).

The epigenetic landscape of quiescent satellite cells also differs from that of activated cells. Quiescent satellite cells possess about 50% of their genes marked by H3K4me3, specific of an active transcription, thus suggestive of a permissive and active chromatin (Liu et al., 2013). Activation process leads to H3K27me3 gain, associated with transcriptional repression; and this epigenetic control was identified in particular for genes highly expressed in quiescent cells and further down-regulated upon activation. This study revealed that epigenetic control of satellite cell quiescence may facilitate the activation process. On the opposite, it was recently revealed that maintenance of heterochromatin state in satellite cells nuclei regulates quiescence (Boonsanay et al., 2016). In particular, Suv4-20h1, a H4K20 dimethyltransferase involved in heterochromatin formation, was shown to transcriptionally repress MyoD. Loss of Wuv4-20h1 resulted in MyoD relocalization at the centre of nuclei, where transcription takes place, while allowing a generalized loss of heterochromatin state of satellite cells nuclei and activation; thus leading to stem cell depletion and loss of long-term regenerative capacity, eventually (Boonsanay et al., 2016, Li and Dilworth, 2016). Altogether, both study suggest a specific spatio-temporal requirement of chromatin conformation control for satellite cells homeostasis.

II.4. Satellite cells and fiber plasticity.

Satellite cells during exercise

Extensive studies in mice and humans have reported acute increase in satellite cell numbers after a single bout of exercise (reviewed in (Martin, 2012, Snijders et al., 2015)). Interestingly, this increase was observed in various types of training, whether they induce muscle damage (eccentric exercise), or not (single session of resistance, endurance) (Snijders et al., 2015). Increased satellite cell number is preceded by satellite cell activation occurring less than 10h after a single bout of exercise (Snijders et al., 2012), is observed as soon as 24h after exercise, reaches a maximum after 72h and satellite cell number then comes back to basal level (Snijders et al., 2015).

Chronic resistance and endurance exercise both lead to an increase of satellite number of the same extent (Martin, 2012, Smith and Merry, 2012, Verney et al., 2008). Muscle mass, however, is only increased through hyperplasia as a result of long-term resistance training but not after endurance training, therefore suggesting that regulation of satellite cell activation is not sufficient to promote hyperplasia and myonuclear accretion (Hoppeler, 2016, Martin, 2012). As endurance training does not induce myofiber micro-injuries like resistance training (Snijders et al., 2015), it remains to be elucidated if activated satellite cells enter the regenerative program or rather return to quiescence after endurance exercise. In the latter case, satellite cell activation could potentially serve as an alert, in case of a future injury. Another option is that they could help remodeling the muscle in a non-hypertrophic way, in the absence of the high intensity contraction induced by resistance training (Joanisse et al., 2013, Joanisse et al., 2015). Furthermore, it would be interesting to evaluate if the molecular triggers of satellite cell activation are initially similar or whether they differ and prime satellite cells for nuclear addition or any different fate.

Satellite cells and hypertrophy

It is of common knowledge that myonuclei can be added under various conditions of hypertrophy. However, it is still debated whether satellite cell addition is required for hypertrophic growth. First reports indicated that nuclei addition was necessary for overload-induced hypertrophy, and suggested that nuclei addition precedes muscle growth (Schiaffino et al., 1976, Rosenblatt and Parry, 1992, Rosenblatt et al., 1994). However, these experiments used satellite cell ablation through X-ray irradiation, which likely affect other critical cell types than satellite cells, and intense debates resulted in the conclusion that previous studies could unfortunately not allow to confirm whether or not satellite cells are necessary for muscle hypertrophy (Bodine, 2007, Hikida, 2007, O'Connor and

Pavlath, 2007, O'Connor et al., 2007). Later on, mouse models with hyper-muscularity (Akt or Ski transgenic or myostatin KO) demonstrated that muscle hypertrophy can arise without nuclei addition, although maintenance of long-term hypertrophy as well as correlations with gain of muscle force was not confirmed (Amthor et al., 2007, Blaauw et al., 2009, Bruusgaard et al., 2005, Amthor et al., 2009).

It is suggested that myonuclei addition is required only in case of extreme hypertrophy or that muscle growth can work without hyperplasia when the total number of myonuclei is already elevated. The "ceiling" theory thus emerged according to which a hypertrophy over about 30% (or over a certain cytoplasmic volume per myonucleus) requires hyperplasia (Kadi et al., 2005, Petrella et al., 2008, Gundersen, 2011, Gundersen, 2016).

More recent and accurate model of satellite cell ablation, using Pax7^{-DTA} mice which ablate more than 90% of satellite cells, demonstrated that hyperplasia was not necessary for mechanical overload (ablation of synergistic muscles)-induced short term hypertrophy (McCarthy et al., 2011) However, muscle regeneration induced by this model was blunted. The hypertrophy observed was less than 15%, fitting the "ceiling" hypothesis. This hypertrophy was further shown to be maintained for an extended period of time (Fry et al., 2014). Overloading was characterized by an activation of the remaining (10%) satellite cells that was not sufficient to replenish the muscle niche, and accompanied by an excessive ECM deposition. *In vitro*, muscle progenitor conditioned medium was able to significantly reduce the ECM gene expression by muscle fibroblasts, therefore suggesting that satellite cells act as a modulator of ECM remodeling during hypertrophy, supporting other reported evidences (Joanisse et al., 2013, Joanisse et al., 2015). Similarly, satellite cell ablation did not prevent re-growth after muscle atrophy (Jackson et al., 2012).

Satellite cells in maintenance of skeletal muscle homeostasis

Skeletal muscle "memory" is a terminology used to describe a phenomenon reported for various conditions where muscle tasks would be executed faster if they have already been performed in the past. In humans, muscle memory is estimated to last as long as the myonuclei turnover (Gundersen, 2016), which is thought to be at least 15 years (Spalding et al., 2005). In mice, this effect supposedly lasts for about 10% of the lifespan (Egner et al., 2013). Concerning exercise, resistance-trained athletes can undergo long period of inactivity and rapidly regain muscle mass and strength (Staron et al., 1991). In fact, myonuclei addition induced by overload exercise leads to a persistent elevated myonuclei number which subsequently protects muscles from disuse atrophy in mice (Bruusgaard et al., 2010). Similarly, early anabolic exposure leading to fiber hypertrophy and hyperplasia primes skeletal muscle for rapid overload-induced hypertrophy (Egner et al., 2013). On the

opposite, deleterious effects of TNF-α for muscle mass maintenance also marks skeletal muscle for a long time (Sharples et al., 2015). In particular, C2C12 treated with TNF-α at early proliferative lifespan showed reduced differentiation capacities and maturation 30 population doublings later. Furthermore, muscle progenitor cell memory was associated there with epigenetic changes (methylation) at the MyoD locus.

Pax7 lineage tracing studies have confirmed that satellite cells contribute to myofibers at adulthood in the uninjured skeletal muscle, thus re-enforcing the role of satellite cells in maintenance of muscle physiology (Keefe et al., 2015). The specific role of satellite cells in adult muscle homeostasis remains, however, largely unclear and further investigations will be required in the future. As a matter of fact, satellite cell ablation barely impaired overall myofiber size (EDL and extraocular muscles were, however, a bit affected) (Keefe et al., 2015), which meets another study's conclusion demonstrating that satellite cell ablation does not affect sarcopenia outcome at older ages (Fry et al., 2015). Muscle adaption to aeroby (increase of mitochondrial content, lipid droplet accumulation and fiber vascularization) neither seems affected by satellite cell depletion (Jackson et al., 2015). Yet, in this study, mice whose muscles were depleted of satellite cells still showed decreased voluntary wheel running performances as well as reduced coordination, although the wheel running did not affect the total number of satellite cells nor myonuclei. Intrafusal plantaris fibers, serving as proprioceptors, showed, however, increased extracellular matrix micro-environment and decreased cross-sectional area, potentially revealing a new role of satellite cells for motor coordination.

Altogether, those studies open up the road for further research development aiming at understanding the role of satellite cell for homeostasic regulation through life and their implication in the maintenance of muscle plasticity.

Satellite cells and nutritional status

Satellite cells are particularly sensitive to nutritional availability and many efforts are being done to understand how muscle stem cells respond to nutritional compounds and are affected by the body metabolic status, in the context of physical performance, metabolic disease or malnutrition research. It has been described very early on that malnourished children displayed a reduced number of satellite cells, and that the phenotype could be ameliorated by nutritional rehabilitation (Hansen-Smith et al., 1979). In birds, it was further shown that postnatal starvation impaired satellite cell function, in particular in a context of postnatal growth (Halevy et al., 2000, Halevy et al., 2003, Mozdziak et al., 2002). Unfortunately, it seems that early malnutrition also affects future function of satellite cells. In mice, it was demonstrated that prenatal under-nutrition leads to reduced number of satellite cells at adolescent age and reduced regenerative capacities (Woo et al., 2011). Conversely,

postnatal mice fed with high fat diet also led to reduced muscle stem cell frequency and regenerative function; suggesting that both prenatal under-nutrition and post-natal over-nutrition affect the development and numerical balance of muscle-resident progenitor cells. In chicken, restricting protein synthesis in satellite cells *ex vivo* by modulating the concentration of methionine and cysteine affected their proliferation and differentiation (Powell et al., 2013, Harthan et al., 2014). Besides, although the underlying mechanisms are not understood today, many studies have reported reduction of satellite cell function in rodents models of type I or II diabetes, or fed with high fat diet (reviewed in (Fujimaki et al., 2015)). The causes leading to alterations of satellite cell function with diabetes are indeed poorly understood; but epigenetic changes, increased oxidative stress, chronic low-grade inflammatory profile and impaired extracellular matrix remodeling have been proposed to participate to loss of satellite cell regenerative capacities (D'Souza et al., 2013). Recently, evidence revealed that Notch signaling was increased in satellite cells from human patients suffering from type I diabetes, as well as in rodent models, along with elevated levels of the Notch ligand DLL1 in muscles (D'Souza et al., 2016).

Nutritional ergogenic aids have long been used in sport to enhance physical performances. How nutritional supplements modulate muscle stem cell function is, however, only partly elucidated. Studies have demonstrated the absence of effect of herbal compounds extracts often used as dietary supplements on satellite cell function *in vitro* (Fernyhough, 2004). Additional studies have demonstrated that compounds such as disodium 5'-inosinate, disodium 5'-guanylate, disodium inosinate, L-citrulline, caffeine, creatine, whey proteins and taurine showed pro-proliferative and/or differentiation effects of satellite cells in vitro (Guo et al., 2014, Oishi et al., 2015, Fernyhough et al., 2010, Farup et al., 2014, Kim et al., 2015).

Collectively, these studies reveal the need of additional investigations to better understand the fine-tuned control of satellite cell function by nutritional status; and thus elaborate strategies to sustain muscle health when nutrition is unbalanced.

III. Skeletal muscle regeneration

III.1. Clinical implications of muscle regeneration

Muscle injuries represent up to 55% of sport injuries (Garrett, 1990, Canale et al., 1981). While exercise induces satellite cell activation as previously described, muscle injuries cause different extent of traumatic damage to myofibers that require muscle regeneration. Muscle injuries can touch myofibers only (*in situ* necrosis), but are generally broader and affect both muscle fibers and blood vessels that rupture (shearing type muscle damage) (Jarvinen et al., 2013). Amongst shearing damages, contusions and strain represent over 90% of all muscle injuries, while lacerations are rarer (Jarvinen et al., 2005). Contusions, caused by heavy extrinsic compressive force, are often non-reported because quite non-specific; and their symptoms include soreness, pain and limited movements (Beiner and Jokl, 2001). Strain injuries are a consequence of the intrinsic force of the exercise itself and vary in their severity which is classified in three grades. The mildest injury is a consequence of overtraining relative to the muscle fitness level called “delayed onset muscle soreness” (DOMS), but it is still debated whether it can be considered as a strain injury as muscle fibers are nor torn (Jarvinen et al., 2013). Lacerations originate from a sport trauma or mostly from surgery and represent a common clinical condition requiring muscle regeneration as part of the patient recovery. Yet, it is acknowledged that all shearing damages (strain, contusion or laceration injury) will have similar regenerative response (Beiner and Jokl, 2001, Jarvinen et al., 2013, Jarvinen et al., 2005, Garg et al., 2015).

After muscle injury, immobilization can be recommended but should not last more than 4-6 days to prevent excessive scar formation and thus fibrosis, and re-rupture at the injury site. Then early mobilization should occur gradually and as soon as possible to boost regeneration and angiogenesis. Not only does mobilization allow correct orientation of the regenerating myofibers, but loading also helps counteracting the catabolic signaling triggered by inflammation (Jarvinen et al., 2013, Jarvinen et al., 2005, Kannus et al., 2003).

Muscle regeneration is also induced in genetic myopathies where structural proteins of skeletal muscle are mutated. For example, Duchenne Muscular dystrophy (DMD) is an X-linked disease affecting 1 boy out 4000 new borns. The disease is caused by a mutation in the *DMD* human gene encoding the 3600 amino-acids long dystrophin protein. Dystrophin is expressed in myofibers and builds the connection between the cell cytoskeleton and the extracellular matrix through the dystrophin-associated glycoprotein complex. Lack of dystrophin protein in dystrophic patients leads to multiple cycles of degeneration and regeneration, following the classical phases hereafter described (Section **III.2.**). However, contrary to healthy muscles, chronic regeneration in dystrophic muscles is

accompanied by elevated and imbalanced inflammation, fibrosis and fat infiltration, as well as satellite cell dysfunction (Mann et al., 2011, Pessina et al., 2015, Dumont et al., 2015a, Chang et al., 2016, Klingler et al., 2012, Zhou and Lu, 2010).

III.2. Phases of muscle regeneration

Remodeling of muscle stem cell niche after muscle injury

In homeostasis, satellite cells reside in their stem cell niche where they are attached to myofibers and the extracellular matrix (ECM) through their receptors M-Cadherin and CD34 (Beauchamp et al., 2000, Irintchev et al., 1994), and integrin α7, integrin β1 and dystroglycan (Blanco-Bose et al., 2001, Cohn et al., 2002), respectively (**Fig. 3**). Satellite cells thus receive structural, physical and signaling cues from the ECM, as their receptors (such as syndecan receptors) can also serve as soluble factor sequesters to regulate quiescence or activation (Cornelison et al., 2001, Pisconti et al., 2010, Xian et al., 2010).

At a higher level, satellite cells also receive endocrine and paracrine cues from other muscle resident cell types present in the niche (**Fig. 4a**). Numerous endothelial cells, fibroblasts, immune cells, pericytes and other mesenchymal cells such as the fibro/adipocytes progenitors (FAPs) surround the myofibers, and allow signal exchange with the satellite cells. Close vascularization also brings systemic cues to the local satellite cell environment (Bentzinger et al., 2013a, Pannerec et al., 2012). Upon muscle injury, the muscle stem cell niche is however disrupted, together with sequential activation of the different muscle resident cells; and new paracrine and systemic cues as well as cell-cell interactions are then involved through the fine-tuned and balanced presence of those cells in parallel to myogenic commitment of satellite cells (**Fig 4b**).

Time-dependent phases of muscle regeneration.

Muscle regeneration can be easily studied and monitored in mice using established models; such as induction of muscle damage and repair following barium chloride (BaCl2), nonetoxin-, cardiotoxin (CTX)- or glycerol-intramuscular injections, or freeze injury (Lukjanenko et al., 2013, Ownby et al., 1993, Pisani et al., 2010a, Hardy et al., 2016, Bernet et al., 2014, Cosgrove et al., 2014). Following an injury, muscle repair is sustained by fiber regeneration *per se* together with the participation of all stem cell niche components. We can then describe the repair process in five interrelated phases; which

will be further detailed in the following paragraphs (Bentzinger et al., 2013a, Charge and Rudnicki, 2004, Musarò, 2014).

1) Muscle degeneration & Necrosis

Muscle degeneration and necrosis is the first event following muscle injury, and it has been mostly described in CTX models (Maltin et al., 1983). Necrosis involves the influx of calcium ions together with the loss of myonuclear and contractile material, leading to fiber destruction and debris production. This phase stimulates inflammatory response and repair.

2) Inflammation

Right after damage, granulocytes and monocytes invade the injured muscle. A fined-tuned temporal involvement of different immune cells then follow to get rid of cellular debris and promote the myogenic program.

A detailed description of this process will be provided in parts: **III.3.**

3) Satellite cell activation and myofiber regeneration

Satellite cell activation is the process of breaking quiescence and promoting cell-cycle entry. After activation, satellite cells will strongly proliferate, commit to the myogenic lineage and fuse into damaged fibers or fuse together in order to form *de novo* fibers. A subpopulation of activated satellite cells will escape the myogenic program and return back to quiescence in order to replenish the satellite cell pool.

A detailed description of satellite cell activation and self-renewal will be provided in part: **III.4.**

Satellite cell differentiation during regeneration follows the classical myogenic program and MRF expression, as previously described in part: **I.2.**

4) Niche remodeling

Satellite cell activation is accompanied by activation of other muscle resident progenitors (endothelial, pericytes, fibroblasts, mesenchymal cells) whose role is to support satellite cell function, while remodeling the extracellular matrix and vascular networks.

A detailed description of this process will be provided in parts: **III.5.** & **IV.3.**

5) Maturation and functional repair.

Although immune response is resolved in a few days after muscle injury, and regenerating myofibers fill out the entire muscle space by less than two-weeks (**Fig. 4b**), muscle regeneration is only complete

when the innervation network and functional performances are recovered which can take another 10 days in mice (CTX-models) (Mitchell and Pavlath, 2004, Mozdziak et al., 2001). Indeed, while the first phases of muscle regeneration are independent of neuronal influence, fiber growth and maturation requires nerve activity. Protein turnover as well as gene expression controlling isoforms of myosin heavy chains depends on the formation of new neuromuscular junctions (NMJs) between surviving axons and newly formed fibers. In addition, neuronal influx indirectly influences satellite cell function.

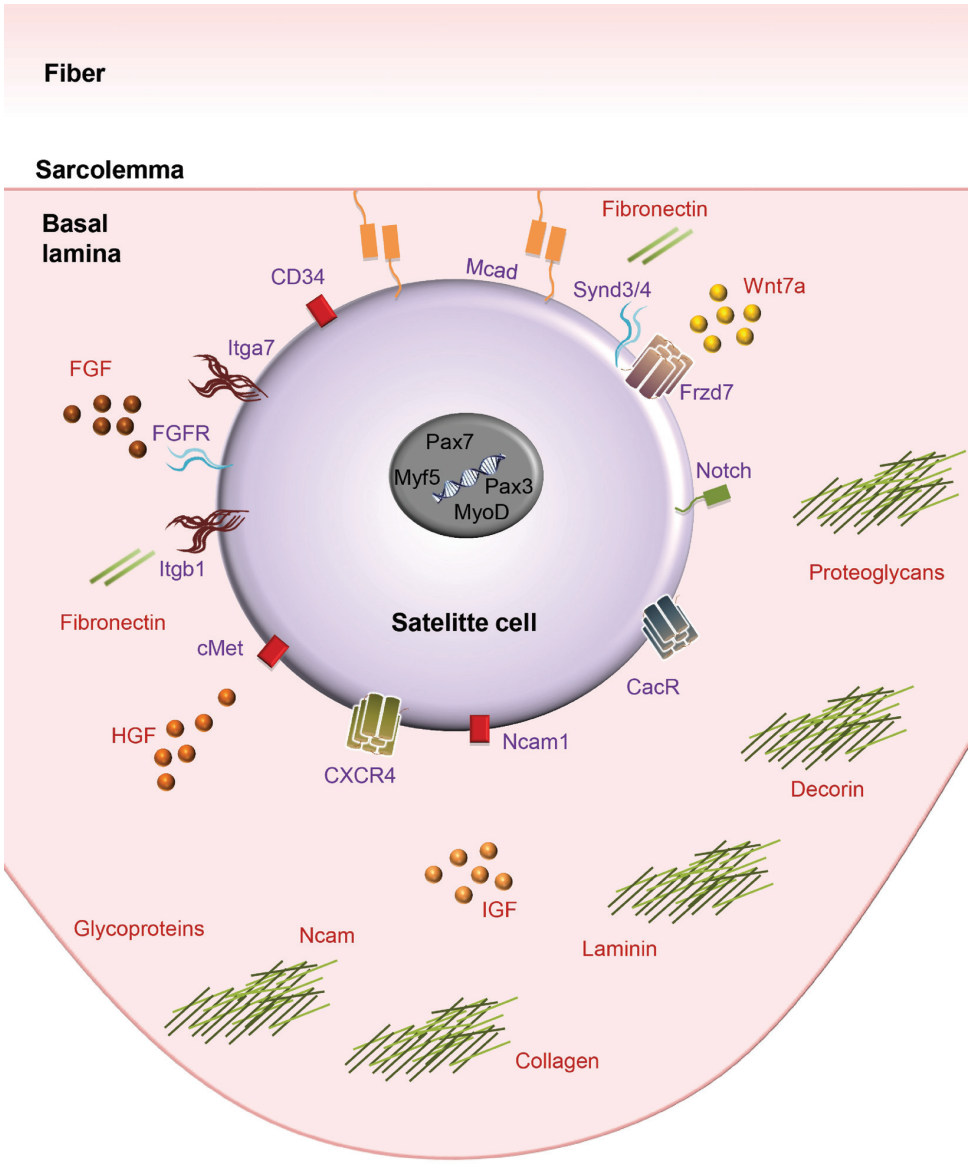

Figure 3. Satellite cell immediate micro-environment. (a) Satellite cells express numerous cell surface receptor allowing anchoring to the extracellular matrix and myofibers. **(b)** The extracellular matrix and basal lamina provide immobilzation of soluble cues to maintain quiescence or promote activation.

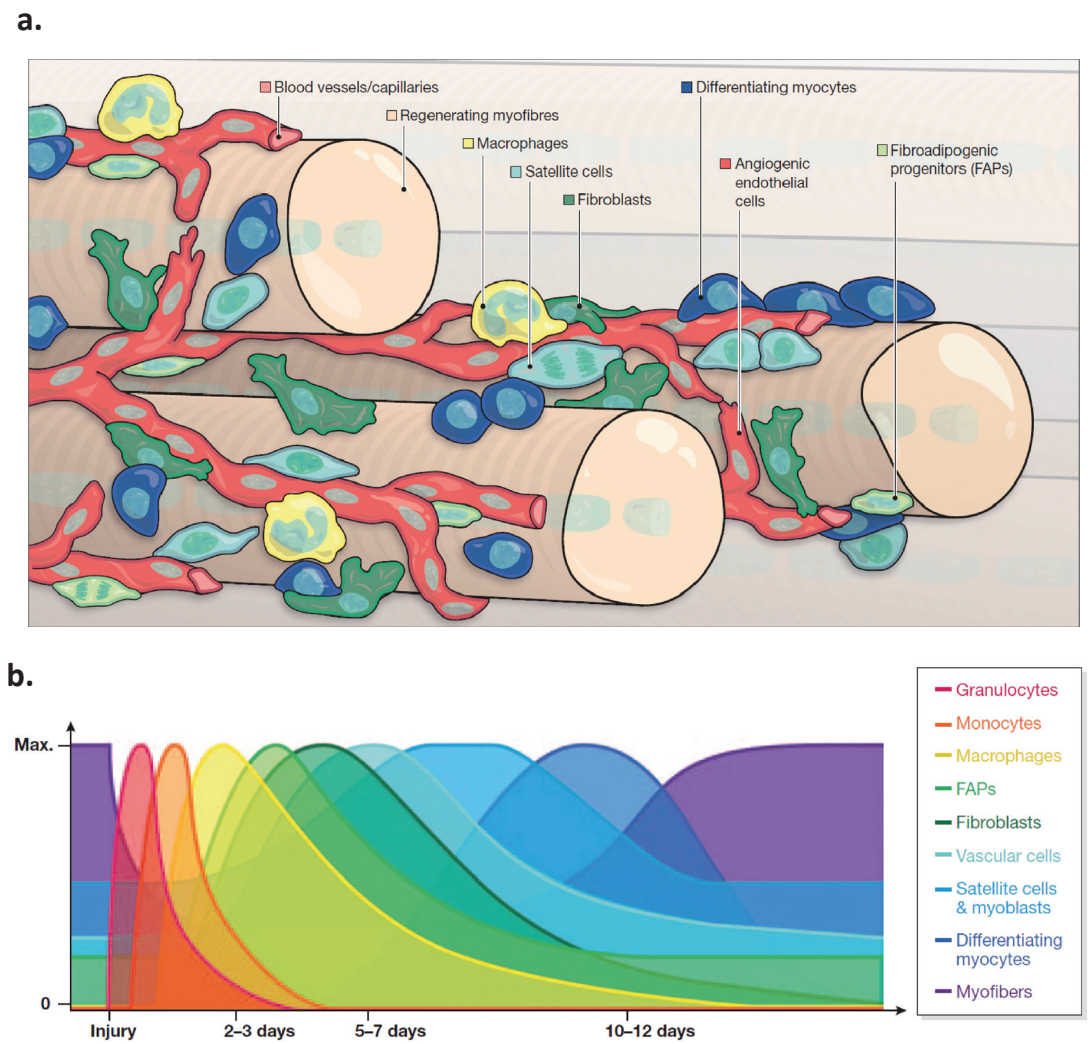

Figure 4. Stem cell niche cell type and their temporal participation to regeneration. (a) Satellite cells reside in their stem cell niche and receive structural, mechanical, paracrine and endocrine cues from the extracellular matrix and other uscle resident cells. **(b)** Fine-tuned temporal presence of immune cells, fibroblast-like and endothelial cells, and myogenic cells during the repair process. Extracted from (Bentzinger et al., 2013a).

III.3. Immune response to muscle injury

Inflammation is a major event following muscle injury and required for efficient muscle repair, mostly acting through the secretion of cytokines and growth factors (Charge and Rudnicki, 2004, Huard et al., 2002, Saini et al., 2016). In response to injury, about 100 000 immune cells can be found per mm^3 of injured muscle (Saini et al., 2016, Wehling et al., 2001). No later than 2h after muscle injury, neutrophils invade the site of injury but their function remains largely unclear (**Fig. 5**) (Dumont et al., 2008). Neutrophils can exert phagocytosis of necrotic debris, and are known to secrete IL-1 and IL-8 which potentially attracts macrophages.

Less than 24h after injury, $Ly6C^+$ monocytes are recruited from the blood and differentiate into pro-inflammatory macrophages (or M1), which reach peak activation in the first 2 to 3 days and proceed to the elimination of muscle cell debris arising from necrosis (Villalta et al., 2009). M1 are known to sustain satellite cell proliferation while inhibiting their fusion (Saclier et al., 2013). When the initial phagocytic phase has cleared the injured muscle from dead cells, M1 macrophages are replaced by $Ly6C^-$ M2 anti-inflammatory macrophages to sustain myogenesis and turn down inflammation. These M2 macrophages peak 4-5 days after muscle damage. The importance of the sequential involvement of pro-inflammatory macrophages and anti-inflammatory macrophages for muscle repair after an injury is well established, and occurs when myoblasts switch from proliferation to differentiation (Arnold et al., 2007, Perdiguero et al., 2012, Perdiguero et al., 2011, Segawa et al., 2008). Early monocytes and macrophages secrete IL-6, a pro-myogenic factor (Zhang et al., 2013). The pro-inflammatory factor TNF-α is also released by neutrophils, along with IL-1β and IFN-γ, and sustains inflammation by promoting M1 differentiation. TNF-α shows further actions on myoblasts, as it promotes satellite cell activation, proliferation and *MyoD* repression through NF-κB signaling (Langen et al., 2004, Chen et al., 2007, Li, 2002). Nevertheless, TNF-α also promotes p38 activation, potentially regulating satellite cell activation and / or myoblast differentiation (Jones et al., 2005, Perdiguero et al., 2007). p38α/β MAPK was also shown to coordinate M1 to M2 transition (Perdiguero et al., 2012, Perdiguero et al., 2011), and perturbation of this timing impairs muscle regeneration (Arnold et al., 2007, Perdiguero et al., 2011, Vidal et al., 2008). One mechanism of anti-inflammatory macrophages for sustaining muscle repair is the secretion of IGF-1 (Tidball and Welc, 2015), known to stimulate both satellite cell activation (Chakravarthy et al., 2000, Machida et al., 2003) and myofiber anabolic affects through upregulation of MRFs (Schiaffino and Mammucari, 2011). The innate immune system and more particularly eosinophils were also reported to be involved in muscle regeneration, through IL-4 and IL-13 secretion (Heredia et al., 2013).

The classical model of M1/M2 macrophages classification is now being challenged. A recent study established transcriptional profile of macrophages during muscle repair and uncovered four

main phases of macrophage polarization during regeneration (Varga et al., 2016). Ly6C$^+$ macrophages that first invade the site of injury are enriched in acute-phase proteins and inflammatory genes. Resolution of inflammation is then preceded and sustained by metabolic changes of those macrophages which shift from glycolytic to oxidative metabolism, during which AMPKα1 plays a key role (Mounier et al., 2013). This is followed by proliferation of Ly6C$^-$ macrophages. The latter actively participate to healing through the secretion of factors involved in intercellular communications, and in particular, ECM-regulating molecules.

The adaptive immune system is also involved in muscle repair, mostly by T-cells; although their clear role still remains to be determined (Saini et al., 2016). It is possible that T-cell modulate satellite cell function through the secretion of factors like FGF-2, TNF-α and TGF-β. However, regulatory T cells (Treg) were proposed to regulate the accumulation of myeloid cells in the injured muscles and promote the pro-inflammatory to anti-inflammatory switch (Burzyn et al., 2013). Indeed, Treg cell infiltration into damaged skeletal muscles coincides with the timing of M1 to M2 transition. This Treg population was described as unique since the TCR (T cell receptor)-repertories of injured-muscle and lymphoid organ Treg cells were distinct (Burzyn et al., 2013). Treg cell ablation strongly impaired muscle regeneration, and this influence on muscle repair was suggested to be both indirect, through the modulation of other myeloid cell action, and direct. Muscle Treg cells were found next to the regenerating fibers and expressed Amphiregulin, (Areg), an EGF family member which improves satellite cell differentiation *in vitro* and *in vivo*. IL-33, a member of the IL-1 family of cytokines, was demonstrated to specifically regulate Treg homeostasis in muscle, as no other immune cell population invading the site of injury expressed Il1r1, the IL-33 receptor (Kuswanto et al., 2016). IL-33 was shown to spike in muscle a few hours (6-12hours) after injury, thereby leading to high accumulation of Tregs 2 to 4 days after injury. This timing is actually consistent with another proposed role of Tregs to sustain satellite cell proliferation, while delaying their differentiation (Castiglioni et al., 2015). Contradictory suggestions regarding the influence of Treg cells on satellite cell differentiation (Burzyn et al., 2013, Castiglioni et al., 2015) might suggest distinct subpopulation or timed-expression of various cytokines during the repair process.

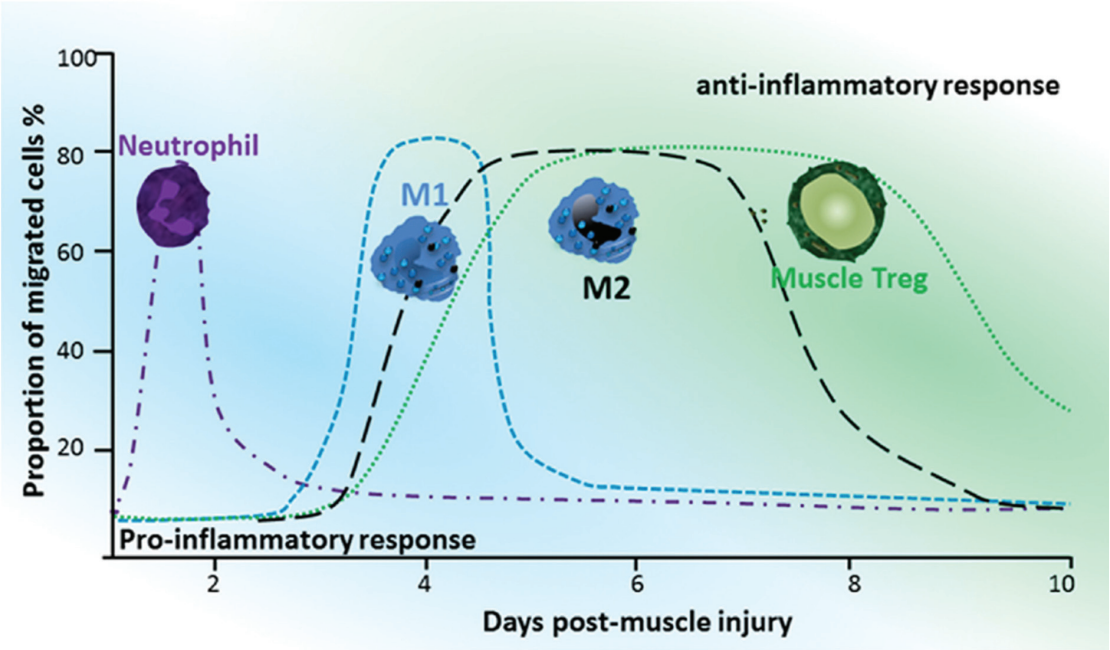

Figure 5. Inflammatory response during muscle regeneration. Fine-tuned sequential events of major immune cell type involved in inflammation and resolution of inflammation after muscle injury. Extracted from (Saini et al., 2016)

III.4. Satellite cell activation and self-renewal

Activation

In parallel to immune response, satellite cells quickly break quiescence and start to expand. The molecular signals which trigger satellite cell activation upon injury have only been partially elucidated. Multiple factors have been demonstrated to regulate satellite cell proliferation and entry into myogenic differentiation, many of which are released by the damaged muscle itself (**Fig. 3**). Extensive reports have suggested the role of extrinsic signals and micro-environment-secreted factors. TNFα, a factor released upon injury by macrophages, monocytes and injured myofibers promotes entry to the cell cycle and G1-to-S phase transition (Chen et al., 2007, Li, 2002). Hepatocyte Growth Factor (HGF), a molecule that is present in muscles and released after injury, has been shown to activate quiescent satellite cells *in vivo* through its binding to c-met receptor on satellite cells (Tatsumi et al., 1998). Nitric oxide (NO) synthesis by sarcolemma-bound nitric oxide synthase-I (NOS-I) in response to stretching leads to the release of hepatocyte growth factors and thus, satellite cell activation (Wozniak and Anderson, 2007). Interestingly, the mechanisms of satellite cell activation by NO and HGF is modified by stretching: in the presence of stretching, satellite cell activation is attenuated by NOS inhibitor and by HGF; while in the absence of stretching activation is stimulated by NOS inhibition and HGF. In addition, FGF (fibroblast growth factor), which can bind to syndecan-3 and syndecan-4 at the satellite cell surface, has been demonstrated to have similar actions (Floss et al., 1997). FGF-2 is thus well known to be induced in injured muscles and promote satellite cell activation (Chakkalakal et al., 2012, Yablonka-Reuveni et al., 1999, Olwin et al., 1994). IGF-1, which can act as a hormone or a paracrine growth factor, was also suggested to play an active role in muscle regeneration (Musaro, 2005).

Sphingosine-1-phosphate and activation of the Wnt signaling are required for satellite cell entry into cell cycle, demonstrating the importance of intrinsic signals (Nagata et al., 2006, Otto et al., 2008). PI3K-Akt signaling appears central for satellite cells activation. While IGF-1-stimulated proliferation of primary rat satellite cells is accompanied by PI3K-Akt stimulation (Chakravarthy et al., 2000), PI3K-Akt signaling is also downstream of c-met upon HGF binding, leading to mTORC1 activation and subsequent satellite cell activation (Rodgers et al., 2014). Foxo1 was also showed to transduce IGF-1 signaling to down-regulation of $p27^{Kip1}$, resulting in cell cycle entry (Machida et al., 2003). MAPK signaling is another hub of activation. TNFα, HGF and FGF also activate p38α/β MAPK (Mapk14/11), a signaling necessary to satellite cell activation and the first marker of activation (Jones et al., 2005, Chen et al., 2007). It was also shown that p38α/β MAPK signaling is activated by PAR, whereby PAR-3 and PKCλ form a complex with p38α/β MAPK (Troy et al., 2012). Of note, p38α MAPK signaling is also

necessary for satellite cell differentiation but through different signaling pathway than for proliferation (Perdiguero et al., 2007). In addition, c-Jun N-terminal kinase (JNK), another MAPK signaling pathway, was shown to promote satellite cells proliferation (Perdiguero et al., 2007), and Erk1/2 (Mapk3/1) that is activated upon FGF-2 signaling, is required for G1-to-S phase transition (Jones et al., 2001, Yablonka-Reuveni et al., 1999). Recently, it was demonstrated that APC (Adenomatous polyposis coli) is essential in satellite cells to lower Wnt signaling and allow their cell cycle progression (Parisi et al., 2015). Deletion of APC signaling induced over-activation of β-catenin, thus leading to satellite cell death upon activation. These results were consistent with the need for balanced β-catenin levels in satellite cells, and with the induction of differentiation by canonical Wnt signaling (Rudolf et al., 2016, Jones et al., 2015).

Satellite cell activation requirements go beyond proper extrinsic signals and intracellular signaling cascades. As this process is strongly energy-demanding, satellite cells must sustain the bioenergetics demands to increase proliferation. To do so, macro-autophagy is induced in quiescent stem cells under Sirt1 control, to enter the cell cycle and soon show higher metabolic activities (Tang and Rando, 2014). Using inducible satellite cell specific Sirt1-KO mice, it was shown that satellite cell activation was delayed in the absence of Sirt1. The role of Sirt1 in satellite cell metabolism reprograming further influencing the gene expression program of activating satellite cells was later emphasized (Diaz-Ruiz et al., 2015, Ryall et al., 2015). While stem cells are thought to rely on glycolysis as a primary source of ATP to limit oxidative stress (Folmes et al., 2012), the metabolic flexibility of satellite cells required for their specific fates (quiescence, activation, proliferation or return to quiescent) is only starting to be understood. A recent study showed that metabolic changes accompany satellite cells throughout their myogenic program, and further mechanistically translate into epigenetic changes supporting the regenerative function (Ryall et al., 2015). When activated, satellite cells undergo a metabolic shift from oxidative to glycolytic metabolism, which reduces NAD^+ levels and thus Sirt1 activity. The reduction of Sirt1 activity promotes H4K16 acetylation and subsequently, cascade of myogenic genes transcription, but constitutive ablation of SIRT1 in satellite cells leads to premature differentiation (Ryall et al., 2015). Consistently, the role of Sirt1 and mitochondrial oxidative respiration in the maintenance of satellite cell function was further demonstrated, as loss of NAD^+ content impacts mitochondrial activity and leads to senescence (Zhang et al., 2016), which is considered as a quiescence break (Sousa-Victor et al., 2014). Altogether, these study suggest that Sirt1, together with NAD^+ level, is a central regulator of satellite cell function for both the maintenance of stem cell quiescence and acute induction of metabolic reprogram during activation, and that Sirt1 activity is reduced during high expansion and differentiation of satellite cells.

A pre-activation state was recently uncovered in satellite cells from non-injured muscles as a consequence of systemic HGF signaling. Downstream activation of mTORC1 via PI3K-Akt has been

shown to be sufficient to activate a new satellite cell phase, between quiescence and activation, described as the “alert” phase (Rodgers et al., 2014). It has indeed been shown, that satellite cells that are distant from an injury enter this transition phase. Alert satellite cells are not cycling, but still are larger and have higher mitochondrial activity than quiescent satellite cells and share intermediary transcriptomic profile between activation and quiescence. This enables to promote faster activation and functional myogenic engagement than quiescent satellite cells upon injury.

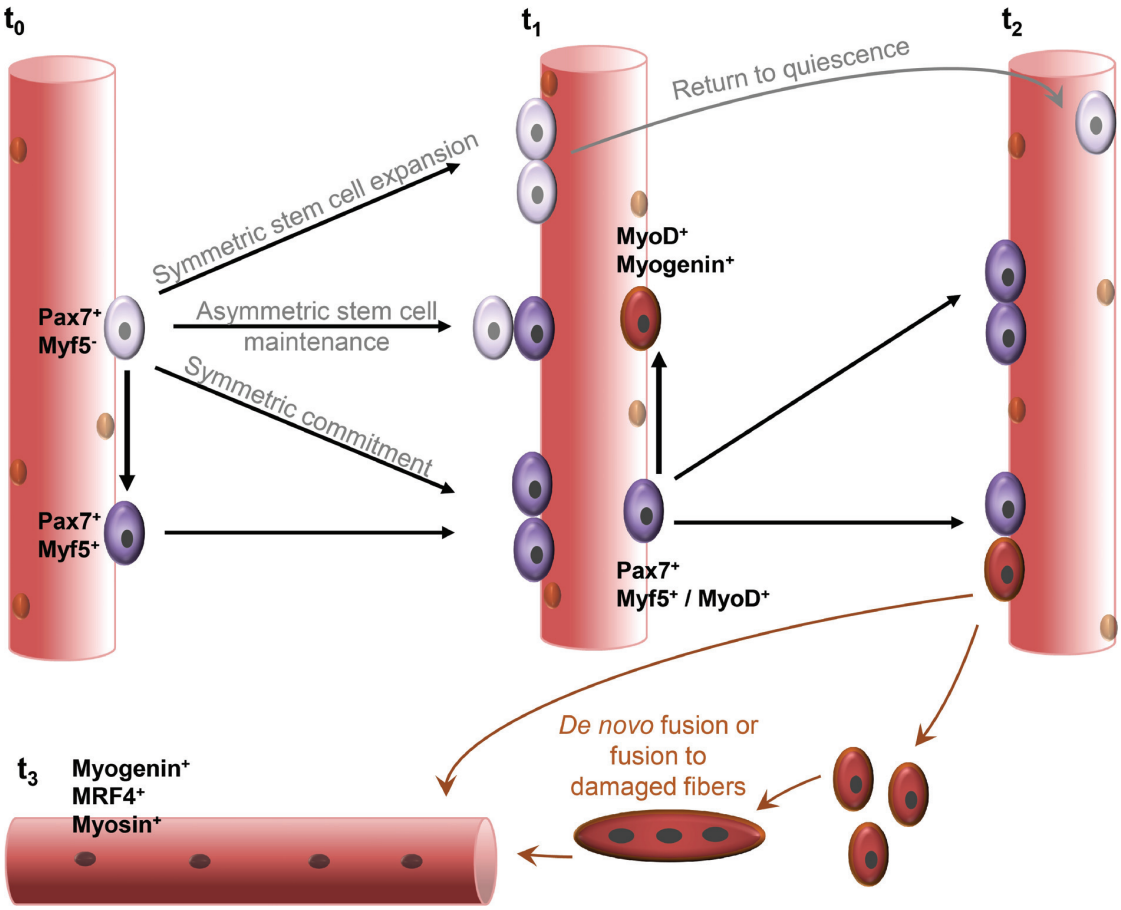

Figure 6. Myogenic fate of satellite cells. T_0, quiescent state on non injured fibers; t_1, activated satellite cells after an injury events, and various types of division leading to a balanced satellite cell expansion and commitment; t_2, committed satellite cells entering the differentiation program sequentially expressing MyoD, myogenin and MRF4 and Myosin while they fuse to form fibers.

Satellite cell fate decision: satellite stem cells vs. committed progenitors

Once activated and in proliferation, satellite cells give rise to two-daughter cells after each round of cellular division, and a first fate determination occurs where progeny participate to either self-renewal or myogenic commitment. While, as previously described in section **II.2.**, $Pax7^{+}/Myf5^{+}$ more likely give rise to two committed progenitors through symmetrical division, $Pax7^{+}/Myf5^{-}$ satellite stem cells are more prone to participate to the stem cell pool replenishment by either giving two daughter stem cells through planar symmetrical cell division or one stem and one progenitor cell through apico-basal asymmetrical division (**Fig. 6**) (Dumont et al., 2015a, Kuang et al., 2007, Le Grand et al., 2009). Balance between myogenic commitment, stem cell maintenance and stem cell expansion is thus critical to maintain regenerative capacities of muscle through life.

Symmetrical proliferation of satellite cells is controlled through the planar cell polarity pathway under the control of Wnt7a, a ligand that is upregulated during muscle regeneration and bind to Wnt-receptor Frizzled-7 on satellite cells (Le Grand et al., 2009). It was further demonstrated that after activation, satellite cells can remodel their niche through the transient autologous expression of fibronectin, a glycoprotein found in the extracellular matrix, which binds to the satellite cell receptor Syndecan4 (Bentzinger et al., 2013b). Syndecan4 forms a complex with Frizzled-7, thus allowing fibronectin to stimulate satellite cell expansion through the activation of Wnt7a signaling. Although most evidence suggests that canonical Wnt pathway is involved in promotion of satellite cell differentiation (von Maltzahn et al., 2012), a study indicated a role for Wnt proteins (in particular Wnt1, Wnt3a and Wnt5a) in supporting satellite cell proliferation (Otto et al., 2008).

More studies report various regulations of asymmetrical division, mostly based on either unequal distribution of cell fate determinant in the two daughter cells or specific mitotic spindle orientation. $Pax7^{Hi}$ satellite cells, expressing more stem cell markers, perform non-random asymmetrical-template DNA strand segregation, preferentially distributing Pax7 transcription factor the daughter cells inheriting from the old DNA template and myogenin to the one inheriting from the new DNA strand (Rocheteau et al., 2012). Non-random DNA segregation was further shown to be regulated by cell adhesion cues, DNA-stand retaining cells more likely associated with low adhesive surface (Yennek et al., 2014). Similar to this myogenic asymmetric distribution, Notch3 receptor is enriched in $Myf5^{-}$ daughter cell (supporting the role of Notch in regulating quiescence) while $Myf5^{+}$ daughter cell more likely inherits the Notch ligand Delta1 (Kuang et al., 2007). Asymmetrical commitment was also attributed to enrichment of p38α/β MAPK signaling (phospho-p38) in the committed daughter cell, as a consequence of an asymmetrical presence of the partitioning-defective protein 3 PAR-3/, a key regulator of cell polarity establishment (Troy et al., 2012). Par-3 polarization leading to asymmetrical division is also regulated by Mark2, upon its interaction with dystrophin

(Dumont et al., 2015b, Keefe and Kardon, 2015). Dystrophin is a structural protein linking the cytoskeleton to the extracellular matrix through the dystrophin-associated glycoprotein complex. This work actually revealed new intrinsic satellite cell dysfunction of dystrophin-deficient muscles; which display impaired mitotic spindle orientation (as observed from Aurora kinase localization during cell division), present impaired polarity (loss of apicobasal division) and thus a lack of asymmetric stem cell division (lack of Myf5+ committed daughter cells), causing reduced regeneration capacities. Recently, a role for primary cilia, an organelle anchored in the cytoplasm and emanating from the cell surface, in allocating self-renewal fate to satellite cells was uncovered (Jaafar Marican et al., 2016). When entering the cell cycle, it is suggested that satellite cells lose their primary cilia, which later reappeared in $Pax7^{+}$/$Myogenin^{-}$ satellite cells suggested to be primed for self-renewal. Although the use of myogenin as a marker of self-renewal together with the use drugs targeting primary cilia reassembly that potentially target larger cytoskeleton can be discussed, primary cilia disappearance upon activation and asymmetric re-appearance during cell division certainly reveals a new role in regulation of satellite cell fate.

Physical cues are also critical to sustain self-renewal, thus re-enforcing the importance of the muscle stem cell niche. *Ex vivo* studies have indeed showed the beneficial effects of using soft hydrogel substrates that mimic the elasticity of muscle (12 kilo Pascal) on the satellite cells self-renewal and function upon transplantation (Gilbert et al., 2010, Cosgrove et al., 2014). Oxygen level was also found to promote asymmetric self-renewal division, upregulation of *Pax7* and subsequent down-regulation of myogenic transcription factors (Liu et al., 2012b). As a consequence, *ex vivo* culture of primary myoblasts under hypoxic conditions activated Notch signaling pathway, thus leading to increased engraftment upon transplantation *in vivo.*

Satellite cell fate decision: cell cycle exit

After activation and possibly cell division, satellite cells either exit the cell cycle to return back to quiescence, or to switch to terminal myogenesis (**Fig. 6**) (Zammit et al., 2004).

The subpopulation of cells that stop dividing without entering myogenic differentiation, but yet retain the ability to proliferate and differentiate in a future round of activation was observed very early on *in vitro* and called “reserve cells” (Kitzmann et al., 1998, Yoshida et al., 1998, Abou-Khalil et al., 2013). Numerous lineage tracing experiments using mouse models to permanently or temporally control genetic recombination (“MRF”-Cre^{ERT2} mice or MRF-Cre-reporter mice) have allowed uncovering the capacity of satellite cells engaged in the myogenesis program to re-enter quiescence. As a matter of fact, 90% of quiescent cells have expressed *Myf5* in their history (Kuang et al., 2007),

30% of MyoD$^+$ cells can revert and down-regulate MyoD, 4% of Myog$^+$ and none of MRF4$^+$ cells can do so (Zammit et al., 2004, Lepper, 2015, Southard et al., 2014).

Transcriptional regulation seems to be key for myogenesis inhibition and quiescence. Indeed, it was shown that *Pax7* and *myogenin* expression are exclusive during the myogenic commitment, and *Pax7* overexpression promotes cell cycle exit toward quiescence by blocking *MyoD* expression (Olguin and Olwin, 2004). mRNA translation was also shown to be controlled by the translation initiation factor eIF2α phosphorylation that allows translation of selected mRNA only, thereby preventing activation of quiescent satellite cells or allowing return to quiescence of activated cells (Zismanov et al., 2016). Consistent with a role of ERK pathway for activation, Spry1 which is an inhibitor of ERK signaling and downstream targets and negative feedback modulator of the FGF signaling cascade (Kim and Bar-Sagi, 2004), is enriched in quiescent satellite cells (Fukada et al., 2007), down-regulated with activation and induced again in cells exiting the cell cycle to return to quiescence (Shea et al., 2010). Spry1 requirement for reversible quiescence is explained by its inhibitory effect on ERK signaling. On the contrary, other studies have demonstrated that muscle stem cell quiescence is regulated by the ERK1/2 pathway, and in particular downstream of Ang1/Tie-2 signaling (Abou-Khalil et al., 2009, Reed et al., 2007). Satellite cells returning to quiescence express the Tie-2 receptor at their surface, and bind the Angiopoietin-1 ligand expressed by vessel cells; thus further promoting return to G0 (Abou-Khalil et al., 2009, Abou-Khalil et al., 2010, Mounier et al., 2011).

Different mechanisms have been elucidated to regulate myogenesis. It has been proposed that Notch1 and Numb are cell fate determinants in a model where Numb inhibits Notch signaling in one daughter cell after asymmetric division allowing it to undergo differentiation; whereas the other daughter cell keeps active Notch and continue proliferating after activation (Conboy and Rando, 2002). Numb is an antagonist of Notch1, leading to direct ubiquitination and degradation of Notch1 (Beres et al., 2011, Conboy and Rando, 2002). Recently it was shown that Numb-deficient muscles show impaired muscle regeneration and reduced satellite cell proliferation (George et al., 2013). Numb ablation also leads to an up-regulation of p21 and Myostatin, two inhibitors of myoblast differentiation (Langley et al., 2002, Zhang et al., 1999). Also, p38α specifically controls fusion and differentiation by promoting cell cycling arrest through JNK down-regulation, together with MRF transcriptional cascade (Perdiguero et al., 2007).

In contrast to PCP-non canonical pathway, several studies have shown that canonical Wnt signaling, in contrast, promotes satellite cell differentiation (reviewed in (von Maltzahn et al., 2012)). Exogenous addition of Wnt3a could promote pre-mature differentiation, and inhibition of GSK3-β also enhanced differentiation (Brack et al., 2008, van der Velden et al., 2006). In fact, it was suggested that induction of differentiation is induced by a switch from Notch to Wnt canonical pathway, as GSK3-β is

maintained in an active form by Notch thus blocking Wnt canonical pathway (Brack et al., 2008). Supporting this evidence, Wnt3a was further shown to promote cell cycle arrest and myogenin activation, together with follistatin expression (Jones et al., 2015). The critical role of β-catenin in the regulation of muscle regeneration was recently supported by the demonstration that both the absence and activation perturbed muscle regeneration leading to attenuated or precocious satellite cell differentiation, respectively (Rudolf et al., 2016). It was therefore suggested that a controlled level of β-catenin is required to regulate the fine-tuned satellite cell expansion and differentiation. These results were contrasting with a previous study stating that Wnt/β-catenin signaling was not required for satellite cell function (Murphy et al., 2014). In the latter study, Wnt/β-catenin was deleted ahead of muscle injury with a recombination of 94%, and potentially allowing low Wnt/β-catenin activity at in the first stages of regeneration together with escaper cells rescuing sufficient Wnt signaling to induce differentiation at later stage.

III.5. ECM remodeling and muscle niche cells contributions

ECM composition

Collagen proteins are the major components of the skeletal muscle extracellular matrix, in particular collagen IV and collagen VI (Gillies and Lieber, 2011). Fibronectin, laminin, proteoglycans such as decorin and biglycan, and glycoproteins such as α-dystroglycan are also abundant in the extracellular space and can interact with collagen proteins (Gattazzo et al., 2014, Gillies and Lieber, 2011). Altogether, this network of proteins form the basis for muscle architecture and elasticity, and facilitate satellite cell adhesion via binding to their receptors such as integrin α7β1 and syndecan-4 (Gattazzo et al., 2014). ECM also contains non-structural secreted proteins called matricellular proteins, including osteopontin (also called secreted-protein acidic and rich in cysteine (SPARC)), thrombospondin, tenascin-C, connective tissue growth factors (CTGF), which participate to ECM signaling and help maintain its organization (Vial et al., 2008, Morales et al., 2011, Gillies and Lieber, 2011). To allow muscle adaptation (growth, regeneration…), the extracellular matrix must be plastic; with matrix remodeling enzymes regulating extracellular molecule turnover. These enzymes, also called matrix metalloproteinases (MMP) are themselves tightly controlled by tissue inhibitors of matrix metalloproteinases (TIMPs).

ECM remodeling

Extracellular matrix remodeling participates to repair by ensuring the complete restoration of muscle architecture. ECM genes, such as pro-collagen, matrix-metalloproteinases, biglycan and periostin are expressed in a coordinated way during muscle regeneration, together with inflammatory factors, growth factors and myogenic genes (Goetsch et al., 2003). In addition to structural scaffold formation to allow fiber re-organization, production of extracellular matrix components such as fibronectin (FN) and collagen-VI (Col-VI) also serve to boost satellite cell function and fate, and thus muscle regeneration (Bentzinger et al., 2013b, Urciuolo et al., 2013, Tierney et al., 2016). While extracellular matrix is degraded in the first phases of regeneration, macrophages (in particular M2 macrophages) are known to further secrete ECM molecules such as FN and Col-VI (Schnoor et al., 2008, Gratchev et al., 2001, Chang et al., 2012). Furthermore, cytokines, and in particular TGF-β1, also regulate the transient tissue scar formation, and participate to extracellular matrix remodeling (Charge and Rudnicki, 2004, Vidal et al., 2008).

Muscle resident fibroblasts and mesenchymal cells are the other main sources of extracellular matrix (Yang et al., 2011), and their contribution during muscle regeneration has been revealed critical.

However, evidences suggest that their role during muscle regeneration is not restricted to ECM production but also involves in regulation of myogenesis (Fiore et al., 2016, Mozzetta et al., 2013, Murphy et al., 2011).

Muscle resident fibroblasts and mesenchymal progenitors during regeneration.

The use of Tcf4 as a novel marker of fibroblasts recently allowed dissecting interactions between satellite cells and fibroblasts during muscle regeneration, demonstrating that fibroblasts are important components of muscle stem cell niche (Murphy et al., 2011). Using mice expressing $Tcf4^{CreERT2}$ and $R26^{DTA}$ alleles, ablation experiments of $Tcf4^{+}$ fibroblasts during muscle regeneration resulted in impaired muscle regeneration, partly explained by a premature satellite cell differentiation; which suggested that $Tcf4^{+}$ fibroblasts may support myoblast proliferation. Notably, the phenotype was observed in spite of the partial deletion of muscle fibroblasts (as *Tcf4* is expressed in only 40% of muscle fibroblasts), which stresses the importance of ECM-producing cells to sustain efficient regeneration. In the context of development, the role of $Tcf4^{+}$ fibroblasts in supporting myogenesis was proposed via a mechanism promoting slow-MHC expression and fiber maturation (formation of multinucleated syncytia) (Mathew et al., 2011). It is possible that $TCF4^{+}$ fibroblasts exert distinct functions according to the stage of satellite cell myogenic progression, through various mechanisms.

PDGFRα+ mesenchymal progenitors called Fibro/adipogenic progenitors (FAPs) because of their adipogenic potential and their ability to differentiate into fibroblast-like cells, are also highly activated and strongly proliferate after injury (see section **IV.4.** for further details on FAPs). (Joe et al., 2010, Liu et al., 2012a, Rodeheffer, 2010, Uezumi et al., 2010, Uezumi et al., 2011). Of note, FAPs can also enter an alert phase, as satellite cells do, remotely from the site of injury (Rodgers et al., 2014). Amongst other muscle resident cell types, FAPs are enriched in fibrotic and ECM-related gene expression such as *α-Smooth muscle actin* (*α-SMA*), *CTGF*, *Col1a* and *Col3a1* (Uezumi et al., 2011). FAPs also secrete IL-6, a pro-myogenic factor; and *in vitro* evidence has shown that they support satellite cell differentiation (Joe et al., 2010, Mozzetta et al., 2013). Ablation of the adipogenic lineage during muscle regeneration, using aP2-Cre mice crossed with ROSA26-iDTR mice, also resulted in impaired muscle regeneration, re-enforcing the central role played by adipogenic/fibrogenic progenitors and lineage cells (Liu et al., 2012a).

It is however, important to note that while fibroblasts and mesenchymal cells are required for efficient muscle regeneration, fibrogenic triggers must be restricted to these cells. In *mdx* mice and DMD patients, physio-pathological increase of TGF-β signaling was shown to induce a mesenchymal-

fibrogenic transition of muscle specialized cells (such as satellite cell, endothelial or hematopoietic cells), thus hampering muscle regeneration (Biressi et al., 2014, Pessina et al., 2015).

Endothelial cells, pericytes & PICS

Angiogenesis accompanies muscle regeneration in order to recover the vascular network and blood supply to the muscle (Luque et al., 1995). Not only is angiogenesis crucial for satellite cell survival but cross-communications also occur between satellite cells and vessel cells (Abou-Khalil et al., 2010). In skeletal muscle, satellite cells and in particular proliferating satellite cells, are predominantly found in close proximity of capillaries. Consistent with this observation, endothelial cells show pro-proliferative effect at least *in vitro*, via the secretion of IGF-1, HGF, bFGF, PDGF-BB and VEGF. Reciprocally, satellite cells were shown to be pro-angiogenic (Christov et al., 2007, Abou-Khalil et al., 2010). A role of VEGF in this reciprocal cross-talk was further sustained, as both satellite cells and endothelial cells secrete and support each other in a VEGF-dependent way (Abou-Khalil et al., 2010, Rhoads et al., 2009).

Peri-endothelial cells, also called pericytes, are cells embedded within the vascular basement membrane. However, their peri-endothelial location leads to confusion and most of the time, vascular smooth muscle cells, mesenchymal progenitors, fibroblasts, macrophages and epithelial cells are considered as pericytes when found in such position (Armulik et al., 2011). Skeletal muscle also possesses a large variety of pericytes (or perivascular cells) in the niche, whose fate and roles are diverse. ADAM12 (a disintegrin and metalloprotease 12) marker was found to identify a distinct subpopulation of $Sca1^+/PDGFR\alpha^+$ perivascular cells that are can be activated during muscle injury to serve a source of fibrotic scar tissue (Dulauroy et al., 2012). Depletion of $ADAM12^+$ cells using inducible expression of diphtheria toxin under the control of the Adam12 promoter reduced interstitial collagen accumulation three weeks after muscle injury. Unfortunately, muscle regeneration efficiency was not assessed in the study to uncover an eventual pro-myogenic role of those pericytes.

Subpopulations of pericytes are, however, myogenic and defined as mesoangioblasts. Mesoangioblasts are bone-marrow derived circulating myogenic progenitor cells identified in adult mice and derived from embryonic precursors in the dorsal aorta, which possess the ability to proliferate *in vitro* and contribute to mesoderm tissues *in vivo* upon transplantation (De Angelis et al., 1999, Ferrari et al., 1998, Minasi et al., 2002). Prospective isolations have identified human mesoangioblasts as a subset of muscle pericytes marked by common pericyte markers such as neuro-glial 2 proteoglycan$^+$ ($NG2^+$), $PDGFR\beta^+$, αSMA^+ and alkaline phosphatase$^+$ (AP^+) (Dellavalle et al., 2007). These cells do not express any myogenic markers but can efficiently and spontaneously form myofibers both *in vitro* and *ex vivo* upon transplantation in *mdx* mice, a model for human Duchenne Muscular

Dystrophy, caused by a mutation in the *dystrophin* gene. The myogenic natural contribution of AP^+ pericytes to myofiber formation was further demonstrated using mice transgenically labelled with an inducible Alkaline Phosphatase Cre^{ERT2} (Dellavalle et al., 2011). AP^+ cells showed the ability to fuse into myofibers during development and to join the satellite cell compartment present at adult age. In adult mice, AP^+ pericytes could participate to muscle regeneration, but at a very low extent. We could hypothesize that pericytes might represent in adult muscle a latent myogenic population that is only recruited once satellite cell function is altered. Cell therapy against muscular dystrophy using intra-arterial delivery of mesoangioblasts have shown successful results in mice and dogs (Sampaolesi et al., 2006, Sampaolesi et al., 2003), and are now in Phase I/II clinical trials in humans using HLA-identical donors. Using a Nestin-GFP/NG2-DsRed transgenic mouse, the pericyte compartment was further subdivided into two populations called type-1 (Nestin-GFP^-/NG2-$DsRed^+$) and type-2 (Nestin-GFP^+/NG2-$DsRed^+$) pericytes (Birbrair et al., 2013a, Birbrair et al., 2013c). While both cell types co-localize with other pericyte markers (CD146, PDGFRβ), and are also found in close proximity to blood vessel endothelial cells, only $Nestin^+$ type-2 pericytes were found myogenic *in vitro* and *ex vivo* upon transplantation, thus participating to muscle regeneration.

Similarly, a novel muscle resident population was identified using PW1 as a marker and defined as $PW1^+$/$Pax7^-$ interstitial cells (PICs) (Mitchell et al., 2010). PICs are highly myogenic *in vitro*, as well as *ex vivo* upon transplantation. PICs share transcriptional similitudes with mesenchymal progenitors, and were consistently shown to possess 2 distinct fates : $PDGFR\alpha^+$ PICs are adipogenic, while its myogenic subpopulation is defined by PDGFRα negativity (Pannerec et al., 2013). However, PIC contribution during adult regenerative myogenesis remains to be determined.

IV. Fibrosis and adipogenesis

IV.1. Muscle fibrosis and intramuscular fatty infiltration, clinical facts

Intramuscular fat infiltration

In humans, intramuscular fat accumulation is associated with age-related sarcopenia (Song et al., 2004, Goodpaster et al., 2001a) and is believed to be causal in the pathophysiology of muscle decline and metabolic impairment in elderly people (Marcus et al., 2012, Tuttle et al., 2012, Vettor et al., 2009). Intramuscular fat content is even considered as a strong predictor of poor mobility in elderly people (Marcus et al., 2012). In particular, intramuscular fat accumulation is often directly linked to an elevation of plasma fatty acid levels or dietary fat content, and is believed to be one of the causes leading to insulin resistance and type II diabetes (Hulver and Dohm, 2004, Krssak et al., 1999, Morino et al., 2006, Shulman, 2000).

Unfortunately, the literature contains misleading conclusions on intramuscular fat infiltration and its causes and consequences on human health. Indeed, there are four types of intramuscular fat, each requiring distinct quantification methods and each correlating with various health outcomes (metabolic *vs.* muscle function), and each located in different anatomical locations (between muscles, between the fascicles of a single muscle, or within muscle fibers) (**Fig. 7**).

Fat quantification methods can be invasive (muscle biopsies followed by histology analysis or lipid extraction) or non-invasive. Four major non-invasive methods exist:

- Dual-energy absorptiometry (DXA). This method is the most commonly used because the most widely available. It can differentiate tissues (fat, bone, non-fat) based on differences in the attenuation of X-rays emitted at two different energy levels. However, DXA provides low-quality images, underestimates the age-related loss of thigh muscle mass and does not distinguish single muscles.
- Imaging by computed tomography scanner (CT). This more sophisticated and powerful X-ray method provides high quality images and quantifications based either on images themselves or retrieved from X-ray attenuation values.
- Imaging by magnetic resonance imaging (MRI). Without any radiation, MRI differentiates tissues based on magnetic relaxation. It also provides high quality images allowing quantifications, but fat content and muscle mass can also be retrieved from magnetic resonance attenuation values.

- Spectroscopy. Based on magnetic resonance, spectroscopy can assess intra- and extra-cellular lipids (hydrogen resonance).

Subcutaneous adipose tissue can be easily distinguished and quantified by imaging methods. This fat depot is usually removed from images from any muscle fat quantification. In humans, subcutaneous fat deposition is not linked with insulin sensitivity (Goodpaster et al., 2003), but it responds to high fat diet in mice by growing through hyperplasia (contrary to visceral adipose tissue which does not respond to high fat diet but uses hypertrophy to grow) (Joe et al., 2009).

The fat depot located between different muscle groups and made of adipocytes is called intermuscular adipose tissue (IMAT) (Gallagher et al., 2005, Marcus et al., 2012). However, many studies also use "IMAT" determination for intramuscular adipose tissue, which creates a first point of confusion. IMAT can unfortunately not be sampled by biopsies and is only quantified using imaging methods. While IMAT is thought to be linked with diabetes and insulin resistance (Goodpaster et al., 2003, Miljkovic-Gacic et al., 2008), most evidence in the literature positively correlate IMAT content with age and negatively correlate it with physical activity, muscle strength and physical performances (Marcus et al., 2012, Song et al., 2004, Miljkovic-Gacic et al., 2008, Manini et al., 2007). IMAT is also increased in skeletal muscle atrophy conditions, such as after spinal cord injury for instance (Gorgey and Dudley, 2007). Unfortunately, studies on IMAT are limited as no rodent model has ever reported its presence nor performed characterizations.

Rare are the data reporting the presence of ectopic adipocytes within a single muscle, in the interstitial space, in human muscles. Those adipocytes are mostly observed in rotator cuff muscles after tendon rupture (Samagh et al., 2013, Osti et al., 2013, Itoigawa et al., 2011) or in patients suffering from inclusion body myositis or myopathies (Uppin et al., 2013). This can only be assessed by histology analysis. On the contrary, mice data have provided rich literature on intramuscular ectopic adipocytes which are linked with muscle regenerative capacities. Indeed, intramuscular adipocytes arise during muscle regeneration in a transient way or rather invade the muscle when muscle regeneration is impaired, upon satellite cell ablation for instance (Yamanouchi et al., 2006, Pagano et al., 2015, Liu et al., 2012a, Uezumi et al., 2010, Sambasivan et al., 2011). Nevertheless, no histological human data have related the presence of those ectopic adipocytes with age to our knowledge. In mice, it was reported that ectopic adipocytes arising during muscle regeneration were more numerous with age and that this correlated with a poor regenerative capacity (Liu et al., 2012a).

Skeletal muscle can also contain fat in the form of lipids droplets, located either within fibers or between fibers and called intra- (IMCL) or extra-myocellular lipids (EMCL), respectively. This fatty infiltration is formed by an accumulation of neutral lipids such as triglycerides and ceramides within myofibers (Ingram et al., 2012, Krssak et al., 1999, Schrauwen-Hinderling et al., 2006). These lipids can

be quantified by magnetic resonance spectroscopy or by histology if muscle tissue is available. IMCL have long been strongly associated with metabolic disorders such as insulin resistance, obesity and type-2 diabetes (Goodpaster et al., 2000, Kelley et al., 2002, Pan et al., 1997, van Loon et al., 2004). Ironically, endurance-trained muscles of athletes also show high IMCL content while presenting enhanced insulin sensitivity, a phenomenon called the "athlete's paradox" (Goodpaster et al., 2001b, van Loon et al., 2004). It was further demonstrated that insulin sensitivity could be rescued by moderate chronic exercise (Dube et al., 2008). Age is also acknowledged to be a key parameter inducing elevation of IMCL content and perturbation of lipid droplet localization in fibers (Crane et al., 2010, Cree et al., 2004, Nakagawa et al., 2007, St-Onge, 2005).

While imaging methods to quantify IMAT can be considered as accurate, reports have drawn conclusion from intramuscular fat quantification by using regression models to retrieve muscle attenuation values and correlate it to fat content. Unfortunately, there is no confirmation about the link between muscle attenuation values and fat content, as it is very likely that those values are also perturbed by water content and fibrosis in the muscle (Goodpaster et al., 2001a). Yet, muscle attenuation is associated with decline in muscle performance, but no type of fat is properly related to such decline (Goodpaster et al., 2001a, Therkelsen et al., 2016, Cawthon et al., 2009, Cesari et al., 2009).

Muscle fibrosis

Fibrosis is defined as the excessive and unregulated deposition of extracellular matrix components, and often (but not always) accompanies fat deposition in diseases. Transient matrix deposition normally occurs in muscle after injury to serve satellite cell function, but chronic injuries accompanied by chronic inflammation also often result in fibrosis (Mann et al., 2011). Contrary to fat infiltration, fibrosis is very well modeled in mice. In fact, fibrosis is a hallmark of myopathies, and in particular Duchenne Muscular Dystrophies, and fibrous tissue participates to progressive muscle dysfunction both in humans (Klingler et al., 2012, Zhou and Lu, 2010) and mice (Mann et al., 2011, Pessina et al., 2015). Fibrotic deposition also hinders repair after acute muscle injuries such as contusion, laceration and strain injuries, and in fact, many anti-fibrotic drugs approved by FDA to blunt fibrosis in other clinical settings (many of them targeting the TGF-β/Smad pathway) are being tested in rodent muscle (Garg et al., 2015). However, reduced fibrosis alone after muscle injury does not seem sufficient to improve muscle regeneration and enhance function. This stresses out that finding the right balance between preservation of beneficial effects of scar formation and limitation of the deleterious effects of fibrosis is key for allowing positive clinical outcomes. In addition, fibrosis is also a hallmark of muscle aging and results in increased stiffness and muscle weakness, and therefore

remains a cellular mechanism to target in the context of multi-modal intervention targeting the different perturbations of muscle aging (see section **V.1.** for further details).

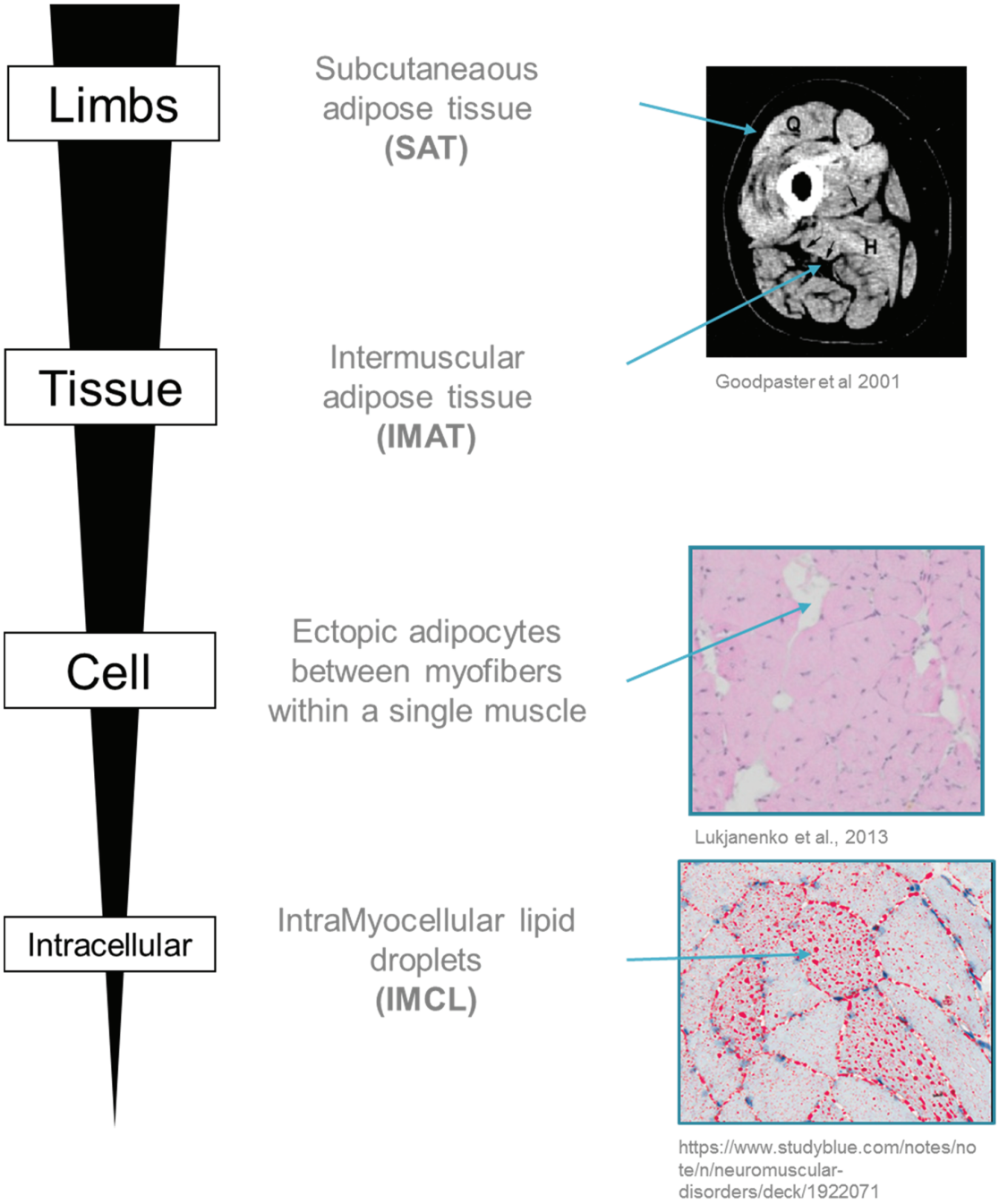

Figure 7. Types of intramuscular fat infiltration. Classification of intramuscular fat according to the localization and nature.

IV.2. Cellular players of intramuscular fat infiltration

The sources of fat infiltrating skeletal muscle are largely unknown. Nevertheless, extensive studies have tried to uncover the origin of lipid accumulation in rodent muscles, as well as of ectopic adipocytes found in the interstitial space of a single muscle.

Non-myogenic capacities of satellite cells

A number of studies have demonstrated non-myogenic differentiation capacities of satellite cells. When grown in adipogenic medium for 2 weeks, human satellite cells initially isolated from single fibers or primary myoblasts derived from mouse satellite cells differentiated into adipocytes (De Coppi et al., 2006, Asakura et al., 2001), and such adipogenic potential increases with age (Taylor-Jones et al., 2002). Other studies demonstrated the spontaneous adipogenic differentiation of mouse satellite cells on isolated single fiber cultured on matrigel without the use of adipogenic factors (Asakura et al., 2001, Shefer et al., 2004). Clonal analysis revealed that individual satellite cells from freshly isolated single myofibers produced mutually exclusive myogenic and non-myogenic but adipogenic clones (Shefer et al., 2004). As Pax7, PPARγ and C/EBPα are initially expressed in all clones and then undergo extinction depending on clonal commitment, it was suggested that satellite cells possess mesenchymal plasticity, committing either to myogenesis or to a mesenchymal alternative differentiation program. As for studies on their adipogenic potential, fibroblast conversion of muscle myogenic cells in various physio-pathological conditions has also been extensively studied using C2C12 cell lines, *mdx* primary cells or isolated fibers upon fibrotic triggers or not (Li and Huard, 2002, Alexakis et al., 2007, Li et al., 2004). Unfortunately, many technical limitations challenge the conclusions drawn by all those studies concerning non-myogenic capacities of myogenic progenitors. Indeed, method employed such as progenitor isolation from isolated fibers, enzymatic digestion and pre-plating or even non-stringent fluorescent-activated flow cytometry, are at high risk of carrying over non satellite cell contaminants or inducing non physiological transformation through cell passages. In addition, and in particular when assessing fibrotic capacities, discrepancies can easily arise from the interpretation of physiological extracellular matrix remodeling capacities of satellite cells versus excessive production, thus called fibrosis. Conclusive studies using fate tracing of satellite cells or pure isolation permitted by flow-cytometry are unfortunately rare.

On the opposite, using lineage tracing, a study showed in the late 2000's that satellite cells do not spontaneously adopt non-myogenic fates (Starkey et al., 2011). Using MyoD lineage tracing, it was shown that satellite cells expressing MyoD do not undergo adipogenic differentiation. It was suggested that the non-myogenic clones observed in single muscle fiber cultures could result from unintentional

contamination with non-myogenic progenitors Pax7$^-$/MyoD$^-$ coming from muscle interstitium. The possibility that Pax7$^+$/MyoD$^-$ cells, which were not traced in that study, could exhibit adipogenic potential was not evaluated. Nevertheless, satellite cells freshly isolated by flow-cytometry from muscles were shown not to undergo adipogenic differentiation *ex vivo* (Joe et al., 2010). When cultured in adipogenic medium and embedded within their native niche (on isolated fibers), 0,1% of cells deriving from Pax7-expressing cells showed a brown adipogenic potential, giving rise to PRDM16$^+$ brown adipocytes (Yin et al., 2013a). In fact, it was demonstrated that satellite cells are multipotent and express microRNA miR-133 that directly represses the expression of *Prdm16* to sustain their myogenic fate. The role of PRDM16 in determining brown fat *vs.* skeletal muscle fate was already known, as it had been demonstrated that brown adipocyte and myogenic progenitors arise from a common Myf5$^+$ precursor whose bidirectional fate is controlled by PRDM16 (Seale et al., 2008). Notch1 was also recently revealed as a molecular switch promoting brown adipogenesis in satellite cells (Pasut et al., 2016). Deletion of Pax7 in satellite cells results in loss of proliferation and premature differentiation (von Maltzahn et al., 2013), but ectopic activation of Notch1 in Pax7-deficient satellite cells successfully restores their proliferative potential and induced PRDM16 expression (Pasut et al., 2016).

The use of Pax7$^{Cre\text{-}ER}$;ROSA26 strain to trace the lineage of Pax7, the marker of the "stem satellite cells", revealed, that old Pax7$^+$ cells tend to deviate from their myogenic lineage to a fibrotic fate in response to an elevated Wnt signaling (Brack et al., 2007), emphasizing that the restricted myogenic fate of satellite cells might depend on their stemness level when traced in the different studies, as well as on their environment. It was further demonstrated in Pax7^{CreER};R26R^{YFP};mdx^{5Cv} mdx, that *mdx* YFP$^+$ satellite cells or satellite cell progeny loose the expression of myogenic marker; while enhancing the expression of fibrogenic genes (Col1a1, Fibronectin) (Biressi et al., 2014). This phenotype is induced by the enhanced Wnt signaling in *mdx* muscles, which results in an increased TGF-β2 signaling in satellite cells responsible for their fibrogenic conversion. This result was further confirmed by a recent study showing the fibro-mesenchymal transition of *mdx* satellite cells, as well as endothelial and hematopoietic cells, in response to increased TGF-β signaling (Pessina et al., 2015).

Identification and characterization of FAPs.

Although it is still equivocal whether satellite cells possess non-myogenic differentiation properties, prospective analyses in mice have characterized muscle resident mesenchymal progenitors devoid of myogenic activity and which express PDGFRα in mice (Uezumi et al., 2010). These progenitor cells show adipogenic potential *in vitro* and *in vivo* after transplantation forming adipocytes (Joe et al.,

2010, Liu et al., 2012a, Rodeheffer, 2010, Uezumi et al., 2010), but can also differentiate into collagen-I expressing cells *in vitro* (especially upon TGF-β induction) (Joe et al., 2010) and *in vivo* (Uezumi et al., 2011). They have consequently been called Fibro/Adipogenic Progenitors (FAPs). FAP origin is not clear yet, but it was demonstrated that FAPs do not arise from Myf-5 expressing cells (Joe et al., 2010) nor Pax-3 lineage (Liu et al., 2012a). Fat cells which invade skeletal muscle do not express UCP1, suggesting that FAPs differentiate into white adipocytes (Liu et al., 2012, Pisani et al., 2010a). The osteogenic and chondrogenic capacity of FAPs is, however, debated. While the mesenchymal origin of FAPs was supported by their ability to differentiate into osteoblasts and smooth muscle cells under specific culture conditions (Uezumi et al., 2010), another study reported that FAPs could not form osteogenic nodules nor chondrocytes when cultured in osteogenic or chondrogenic conditions (Joe et al., 2010). The discrepancy could originate from the different osteogenic media used for differentiation. Indeed, the positive osteogenic differentiation was only reported in response of BMP7 (Uezumi et al., 2010).

The identity of FAPs, as PDGFRα+ mesenchymal progenitors, remains, however, to be deeper characterized. Indeed, FAPs are today placed as central key players in the muscle niche and considerable efforts have been done in the past few years to better determine their function. However, their main and recognized markers ($CD34^+/Sca1^+/PDGFR\alpha^+$) are unfortunately only partially specific and many groups have used different markers to describe various muscle cell populations resembling FAPs. $Tie2^+/PDGFR\alpha^+/Sca1^+$ mesenchymal cells were, for instance, described as the progenitors of ectopic osteogenesis in a mouse model of BMP2-induced heterotopic ossification (Wosczyna et al., 2012). Also, the adipogenic potential of $GFP^+/CD31^-/CD45^-/PDGFR\alpha^+/Sca\text{-}1^+$ isolated from $Tie2^{Cre};R26^{NG/+}$ mice was demonstrated *in vivo* after transplantation into wild-type mice with or without BMP2-induced injury. This population is very similar to FAPs by their interstitial localization and marker expression, and both population very likely overlap. Nevertheless, an intramuscular injection of glycerol into tibialis anterior muscles of the $Tie2^{Cre};R26^{NG/+}$ mice induced the accumulation of GFP^- adipocytes, suggesting that progenitors of ectopic adipocytes do not belong to the Tie2 lineage (Wosczyna et al., 2012), contrary to what was first suggested (Uezumi et al., 2010, Wosczyna et al., 2012). In humans, muscle resident mesenchymal $PDGFR\alpha^+$ cells also revealed an osteogenic potential *in vitro* and *in vivo*, as well as an important role of miRNAs during osteogenic differentiation (Oishi et al., 2013). Mechanistically, the activation of PDGFRα through PI3K-Akt and MEK2-MAPK signaling was further shown to induce the proliferation of $PDGFR\alpha^+$ cells, which was blocked upon treatment with the tyrosine kinase inhibitor imatinib; and a pathological accumulation of these cells in muscle was also described in human diseased muscles (Uezumi et al., 2014). FAPs were also characterized in human skeletal muscles as $CD15^+/PDGFR\alpha^+/CD56^-$ and *de bona fide* committed to white adipocytes *in vitro* (Arrighi et al., 2015). Interestingly, the study also revealed for the first time a metabolic difference between adipocytes derived from FAPs and adipocytes derived from subcutaneous adipose tissue.

While adipogenic potentials were comparable, and resulted in similar triglyceride synthesis and lipolysis function in both types of adipocytes, FAP-derived adipocytes were, however, not responsive to insulin (Arrighi et al., 2015). Based on nestin marker expression, type-1 Nestin$^-$ pericytes present adipogenic and fibrogenic potentials both *in vitro* and *ex vivo* after transplantation (Birbrair et al., 2013a, Birbrair et al., 2013c). Those type-1 pericytes isolated from Nestin-GFP/NG2-DsRed transgenic mice are also positive for FAP markers as cells participating to fat accumulation are characterized as Nestin$^-$/Ng2$^+$/CD34$^+$/Sca1$^+$/PDGFRα$^+$ (Birbrair et al., 2013a). Similarly, fibrotic ADAM12$^+$ perivascular cells induced upon injury express both Sca1 and PDGFRα (Dulauroy et al., 2012). However, ADAM12$^+$ cells are mostly a source of pro-fibrotic cells but show poor adipogenic capacities in contrast to ADAM12$^-$ perivascular cells. Altogether, this re-enforces the hypothesis that FAPs likely represent a heterogeneous population of muscle resident mesenchymal cells and supports the need to find defined markers identifying adipogenic progenitors, fibrogenic progenitors and progenitors possessing dual fates, eventually. Indeed, PDGFRα$^+$/CD34$^+$/Sca1$^+$ characterization may only emphasize FAPs belonging to adipogenic progenitor classification, as PDGFRα was traced in other tissues and marking adipogenic progenitors. PDGFRα$^+$/CD34$^+$/Sca1$^+$ cells (but negative for pericyte markers) cells have been discovered in mice white adipose tissue (WAT) as bipotential adipocyte progenitors capable of brown adipocyte differentiation upon β3-adrenoceptor activation (Lee et al., 2012). Using Pdgfrα-Cre reporter mice, the adipocyte cellular lineage was characterized *in vivo* in white adipose tissue as Lin$^-$/Sca1$^+$/CD34$^+$/PDGFRα$^+$ adipocyte progenitors expressing CD24 and losing CD24 expression while committing to the adipocyte lineage (Berry and Rodeheffer, 2013).

Other potential sources of intramuscular adipocytes.

Different muscle resident cell types, not defined as FAP-like cell populations, have also been identified with adipogenic capacities, thus as potential source of ectopic adipogenesis in muscle. For instance, inducible brown adipocytes progenitors were identified in skeletal muscle and white fat as Sca1$^+$/CD45$^-$/Mac1(CD11b)$^-$ (Schulz et al., 2011). In addition, muscle side population cells (defined by their ability to exclude Hoeschst 33342 dye) and myo-endothelial cells (defined as CD45$^-$/CD34$^+$/Sca1$^+$ CD45$^-$/CD31$^+$/Sca1$^+$) are highly enriched for PDGFRβ and negative for PDGFRα and show adipogenic capacities *in vitro* (Huang et al., 2014, Tamaki et al., 2002, Uezumi et al., 2006).

FAPs are now acknowledged to be the main source of muscle fibrosis and ectopic adipogenesis. However, the interplay between both fates together with the overlap between all adipogenic muscle populations thus remains to be clarified. Yet, only a lineage tracing using PDGFRα-Cre reporter mice will allow to confirm such assumptions.

IV.3. FAPs during muscle regeneration

FAPs cross-talk with satellite cells

FAPs are strongly activated upon muscle injury, and are capable of entering both the "alert state" and proliferation (Joe et al., 2010, Rodgers et al., 2014). The fate of FAPs is then thought to be, however, tightly regulated by the muscle micro-environment and likely depends on multiple interactions with other component of the muscle stem cell niche.

Intramuscular cardiotoxin (CTX) injection is an established model of muscle regeneration, as CTX is a snake venom toxin which destroys myofibers and activates repair and regeneration (Ownby et al., 1993). It has been shown that intramuscular injections of glycerol could efficiently induce ectopic adipogenesis in muscle (Joe et al., 2010, Pisani et al., 2010a, Pisani et al., 2010b, Uezumi et al., 2010). Glycerol injection was also reported to induce muscle damage and regeneration, and mimic myopathies in rabbit (Kawai et al., 1990) and in mice (Abraham and Shaw, 2006, Arsic et al., 2004). In spite of the great proliferation of satellite cells and FAPs in response to muscle damage, the fate of FAPs has been shown to be regulated by the different environments triggered by specific models of muscle injury. Indeed, FAPs isolated from glycerol-injured muscles and transplanted in CTX-injured muscles do not mature to adipocytes (Uezumi et al., 2010). On the opposite, glycerol-injured muscles offer an environment prone to commitment to adipogenesis of FAPs isolated from CTX-injured muscles (Joe et al., 2010).

It seems that regenerating myofibers have a strong inhibitory effect on adipogenesis through direct interaction with FAPs (Uezumi et al., 2010), and ectopic adipogenesis occurs in injury models (exercise and CTX) after satellite cell ablation (Sambasivan et al., 2011). Nevertheless, ectopic adipogenesis was also reported in efficient regenerating models (Yamanouchi et al., 2006, Pagano et al., 2015)). Similarly, many studies have also reported an increased fibrosis in models where satellite cell function is impaired (Murphy et al., 2011, Fry et al., 2014), consistent with a model supporting that FAP differentiation is blunted by an efficient muscle regeneration (Rodeheffer, 2010). In overload-induced muscle hypertrophy, ablation of satellite cells also increases ECM deposition and number of $Tcf4^+$ fibroblasts (Fry et al., 2014). Increased fibrosis was also observed in a homeostatic context after satellite cell ablation (Fry et al., 2015). *In vitro*, muscle myogenic progenitors negatively regulated the ECM gene expression level by muscle fibroblast after 24h of conditioned-medium or transwell co-cultures suggesting a paracrine signaling by the muscle progenitors to modulate fibrosis (Fry et al., 2014). It is therefore possible that satellite cell modulation of adipogenesis and fibrotic deposition by FAPs/fibroblasts occurs through distinct mechanisms, contact inhibition or paracrine signaling. Myo-endothelial cells present in the muscle also regulate the fate of muscle FAP-like adipocyte progenitors

(Huang et al., 2014). While myo-endothelial cells can efficiently engraft and fuse into myofibers *in vivo* after transplantation into injured muscles, their normal contribution in developing or regenerating muscles is minor. Rather, it was shown that myo-endothelial cells act as repressors of adipogenesis within muscles through Bmpr1a signaling, in cell-autonomous and cell-cell communication ways. Indeed, ablation of Bmpr1a reduced myo-endothelial proliferation capacities, converts their fate from myogenic to adipogenic and also reduced adipogenic inhibition of adipogenic progenitors by myo-endothelial cells in direct co-cultures (Huang et al., 2014). In agreement with the idea that excessive ectopic adipogenesis is detrimental for muscle regeneration (Cordani et al., 2013, Liu et al., 2012a, Mozzetta et al., 2013, Uezumi et al., 2010), it was shown that mature adipocytes inhibited myogenic differentiation through paracrine signaling whereas pre-adipocytes promoted it (Takegahara et al., 2014).

Conversely, co-culture experiments between FAPs and SATs revealed that FAPs support myogenesis by enhancing myoblast terminal differentiation (Joe et al., 2010). This triggered a model in which FAPs participate to muscle regeneration by sustaining myogenic capacities of satellite cells (Rodeheffer, 2010). The critical role of the adipogenic and fibrogenic lineages during muscle repair was emphasized by dramatic regeneration impairment upon ablation of either the AP2 adipogenic lineage or the $Tcf4^{+}$/$PDGFR\alpha^{+}$ fibroblasts (Liu et al., 2012a, Murphy et al., 2011). Fibro/adipogenic progenitors from neural crest origin were also described to sustain the masseter regenerative response in mice, as mesoderm FAPs do for the tibialis-anterior muscle (Lemos et al., 2012), although masseter muscles also contain a fraction of mesoderm-derived FAPs (Paylor et al., 2014). Neural-crest FAPs were able to self-renew conserving their adipogenic potential after serial transplantation, potentially suggesting that FAPs present stemness (Paylor et al., 2014). While no FAP ablation was ever done to confirm FAP requirements during muscle regeneration, a study established a parallel between absence of ectopic adipogenesis, decreased PDGFRα expression and impaired muscle repair (Pagano et al., 2015). More precisely, a new study revealed that mice pharmacological treatment with nilotinib (a tyrosine kinase inhibitor, acting downstream of TGF-β receptor and promoting apoptosis of *mdx* FAPs (Lemos et al., 2015)) after acute injury lead to reduced expansion of FAPs, together with a decline of their fibrogenic profile and full muscle transient collagen-made scar formation (Fiore et al., 2016). Nilotinib treatment also resulted in a reduced myoblast expansion *in vivo*, and impaired regeneration. This decline in satellite cell proliferation was further suggested to be non-cell autonomous, but rather a consequence of reduced FAPs activity; as FAPs were demonstrated to promote activation and proliferation of satellite cells on isolated fibers, in a paracrine and FAPs quantity-dependent manner. Recent evidences arising from studies in various physiological conditions and models, tend to suggest a general ability of FAPs or adipocyte progenitors to communicate with satellite cells through paracrine signaling (Fiore et al., 2016, Im et al., 2014, Mozzetta et al., 2013). Nevertheless, while IL-6 and IL-10 are suggested

candidates (Joe et al., 2010, Lemos et al., 2012), no FAPs-secreted factors have been identify to signal on satellite cells in healthy muscles. And yet, whether FAPs support satellite cells proliferation or differentiation (amongst many other possible actions such as supporting satellite cells survival, activation, self-renewal…), remains today debated (Fiore et al., 2016, Joe et al., 2010, Mozzetta et al., 2013). In rabbits, adipose stem cell extracts were shown to induce rabbit muscle progenitor proliferation in transwell co-culture assay (Im et al., 2014). However, FAPs did not induce SAT proliferation, but were rather thought to act on satellite cells differentiation (Joe et al., 2010). Studies on *mdx* mice have shown that the action of *mdx* FAPs on muscle regeneration by mediating the effect of histone deacetylase inhibitor (HDACi), is age- and disease stage-dependent, while the similar intrinsic myogenic potential of satellite cells is similar in young and old *mdx* mice (Mozzetta et al., 2013). *Mdx* FAPs translated the effect of HDACi through the secretion of follistatin which enhanced satellite cell myogenic capacities. The beneficial effect of HDACi on muscle regenerative potential in young *mdx* mice was further explained by the capacity of HDACi to derepress a latent myogenic program in FAPs (Saccone et al., 2014). This newly uncovered bipotency of FAPs is regulated by HDAC-myomi-BAF60 networks. In fact, FAPs were able to respond to HDACi by downregulating the SWI/SNF chromatin remodeling complex subunits BAF60A and B, thus inhibiting their fibro/adipogenic fate, and upregulating BAF60C and MyoD leading to the activation of the myogenic program (Faralli and Dilworth, 2014, Saccone et al., 2014). On top of activating a myogenic reprogramming, HDACi were able to enhance the ability of FAPs to improve SATs differentiation in co-culture settings. The bipotency was observed only in FAPs from young *mdx* mice or in FAPs isolated from CTX-injured wild-type muscles, but neither in FAPs from uninjured wild-type skeletal muscle or old *mdx* mice. Therefore, it was suggested that the latent myogenic fate of FAPs could only be activated under HDACi treatment in a muscle regenerative environment that allows chromatin remodeling and thus, sensitivity to HDACi. In the study, FAPs were isolated from muscles as Sca1+/Integrin α7- cells (Saccone et al., 2014), a population that also comprises the PDGFRα- PICs that were shown to be myogenic, although rare (Pannerec et al., 2013). It would be then interesting to deeper understand whether HDACi-induced pro-regenerative effects of FAPs is restricted to the myogenic PDGFRα$^-$ PICs or truly relies on bipotency of the PDGFRα$^+$ FAPs that are devoid of myogenic differentiation in absence of regenerative cues. That finding is nevertheless supported by another study where FAPs, isolated as Sca1$^+$/CD34$^+$/ PDGFRα$^+$ cells, where demonstrated to adopt the myogenic lineage (Pax7 expression) in cancer cachectic muscles (He et al., 2013). Cachectic muscles are characterized by myofiber damages in mice and humans. Muscle wasting in cachectic mice was partly explained by an NF-κB dependent up-regulation of Pax7 in satellite cells and in non-myogenic cells that were isolated upon markers that define the FAP population, thus blocking myogenic differentiation and impairing muscle regenerative capacity (He et al., 2013).

FAPs cross-talk with immune cells

Cross-talks of FAPs within the muscle stem cell niche are not limited to satellite cells. In fact, numerous evidences have highlighted close relationships between FAPs and immune cells. New insights on the role of FAPs in supporting muscle regeneration were first uncovered in relation to eosinophils (Heredia et al., 2013). IL-4/13 signaling was demonstrated to be activated in FAPs in response to cytokine secretion by eosinophils that invade the muscle upon injury. This IL-4/IL-13 activation promotes FAP proliferation, which then sustain myogenesis by participating to muscle debri clearance through phagocytosis, while inhibiting FAP differentiation (Heredia et al., 2013). These findings were later confirmed by another study in which the use of glucocorticoids (dexamethasone) stimulates FAPs adipogenic differentiation *in vitro* and *in vivo*, acting through the suppression of IL-4 expression in muscles (Dong et al., 2014). This brought a new dimension in the understanding of dialogues and inter-relationships between components of the stem cell niche and systemic factors. The cross-talk between immune cells invading the muscle after injury and FAPs, was recently reinforced by a new study revealing the apoptotic effect of TNFα, produced by pro-inflammatory macrophages, on FAPs which limits regeneration-induced fibrosis, eventually (Lemos et al., 2015). Indeed, pharmacological TNFα blockade leads to survival of FAPs *in vitro* and *in vivo*, as well as collagen deposition during regeneration. A few days after injury, macrophages then switch from pro-inflammatory to anti-inflammatory phenotypes, which produce TGF-β and show pro-regenerative activities (Arnold et al., 2007, Perdiguero et al., 2012, Perdiguero et al., 2011, Segawa et al., 2008). Yet, the study also revealed anti-apoptotic and pro-fibrotic effects of TGF-β, sustaining the importance of a well-organized temporal succession of events to allow effective regeneration. Indeed, *mdx* mice present chronic muscle damage and it was reported that both pro- and anti-inflammatory macrophages are present in *mdx* muscles (Lemos et al., 2015, Villalta et al., 2009). It was shown in vitro that the combination of TNFα and TGF-β treatments blunts TNFα pro-apoptotic effect, which could explain the excessive collagen deposition observed in muscle of *mdx* mice (Lemos et al., 2015, Munoz-Canoves and Serrano, 2015, Tidball and Wehling-Henricks, 2015). Pharmacological treatment with nilotinib, a tyrosine kinase inhibitor blocking the action of p38 downstream of TGF-β, was able to counteract TGF-β survival effect *in vitro*, increase FAP apoptosis *in vitro* and *in vivo*, as well as to decrease collagen deposition in an *mdx* model of muscle microdamage (Lemos et al., 2015).

Reciprocally, not only do immune cells regulate FAPs function and survival, but FAPs were also shown to trigger inflammatory response upon injury. Indeed, FAPs were recently identified as the major source of IL-33 upon muscle injury, a cytokine that is required for Treg function in sustaining regenerative myogenesis (Kuswanto et al., 2016).

Altogether, foreseeable heterogeneity in the FAPs compartment as well as their various roles and new uncovered interactions with the muscle stem cell niche really place FAPs as central players during muscle regeneration and open the need to better characterize their mechanism of action.

IV.4. Regulators of muscle adipogenesis and fibrosis

Muscle ectopic adipogenesis is regulated by various molecular signaling pathways, as well as physiological triggers. However, the confusing literature on intramuscular types of fat and sources makes it extremely difficult to decipher what specifically modulate adipocyte generation within a single muscle. In particular, regulation of FAP adipogenic commitment is even more poorly described. A recent study revealed that the endothelial protein C and activated protein C (APC)-receptor CD201 identifies human muscle mesenchymal PDGFRα^{+} cells with high adipogenic potential, and is not express in human CD56^{+} myogenic progenitors (Uezumi et al., 2016). Human mesenchymal progenitors also expressed the protease activated receptor-1 (Par1), a CD201 co-receptor known to transduce intracellular signaling upon APC binding (Mohan Rao et al., 2014). Together with adipogenic cues, activated protein C treatment enhanced adipogenic capacities of the progenitors and concomitantly activated Akt and Erk pathways.

Considering the important role of skeletal muscle in maintenance of metabolic homeostasis, together with the energy storage function of adipocytes, metabolic cues and nutrient availability are central triggers of muscle adipogenesis. Indeed, high glucose conditions increase adipogenic differentiation of primary rat muscle primary cells *in vitro* (isolated by preplating) (Aguiari et al., 2008) and lead to elevated intracellular lipid accumulation in porcine satellite cells (Yue et al., 2010). In human, while intramuscular fat infiltration is associated with insulin resistance, dietary creatine intervention decreased fat mass in dystrophic patients (Tarnopolsky et al., 2004, Prior et al., 2007). Similarly, neural crest-derived FAPs showed higher adipogenic response to muscle damage upon high fat diet (Paylor et al., 2014).

Terminal adipogenic differentiation of both WAT and BAT is controlled by a transcriptional cascade, mainly involving the early expression of CAAT/Enhancer-Binding-Proteins (C/EBPβ and C/EBPδ), which induce the expression of the master adipogenic regulators C/EBPα and Peroxisome Proliferator-Activated Receptor gamma (PPARγ), and consequently their action on the transcription of genes involved in terminal adipocyte maturation (Huang et al., 2009, Lowe et al., 2011, Rosen et al., 2000, Kajimura et al., 2010). PPARγ knock-down of rat muscle crude of myogenic and non-myogenic progenitors reduced adipogenesis after bupivacaine hydrochloride treatment *ex vivo* (a treatment used to induce muscle injury), and promoted myogenic differentiation (Takegahara et al., 2014).

Rodent models of rotator cuff tear, whose regeneration is often affected by massive intramuscular invasion of adipocytes, characterized a role for mTOR in regulating ectopic adipogenesis via SREBP-1 and PPARγ, as rapamycin-treated animals showed significantly reduced fat infiltration (Joshi et al., 2013).

Signaling pathways controlling adipose tissue development are well characterized, including the Wnt and BMP (Bone morphologic proteins) pathways (Huang et al., 2009, Prestwich and Macdougald, 2007). However, the signaling pathways regulating the development of early mesenchymal precursors into ectopic adipocytes in other organs than adipose tissue remain only partially understood (Lowe et al., 2011, Rosen et al., 2000).

Wnt proteins are thought to regulate the adipogenic *vs.* osteogenic fate of mesenchymal stem cells (Pretheeban et al., 2012). In particular, Wnt10b, through the canonical β-catenin-dependent Wnt pathway, maintains pre-adipocytes in their progenitor state by blocking C/EBPα and PPARγ activity (Ross et al., 2000, Kawai et al., 2007). Interestingly, rotator cuff muscle fatty degeneration associated with tendon rupture in humans was associated with decreased *Wnt10b* expression (Itoigawa et al., 2011). In mouse muscles, Wnt signaling is increased with age triggering both fibrogenic conversion of satellite cells through upregulation of TGF-β2 (Brack et al., 2007) and increase adiposity in primary muscle progenitors (Vertino et al., 2005). On the contrary, Wnt5a and Wnt5b favor adipogenic differentiation by inhibiting the canonical β-catenin-dependent Wnt pathway and activating PPARγ (van Tienen et al., 2009).

BMPs are central players of adipogenic and osteogenic differentiation of progenitors. In particular, BMP-2/4 can induce commitment of C3H10T1/2 pluripotent stem cells into adipocytes (Huang et al., 2009). BMP7 triggers brown adipocyte differentiation of $Sca1^+$ muscle interstitial progenitors (Tseng et al., 2008, Schulz et al., 2011). On the opposite, BMP signaling can also inhibit adipogenesis. In myo-endothelial cells, Bmpr1 ablation induced adipogenic conversion (Huang et al., 2014). Supporting the mesenchymal origin of FAPs, presence of BMP7 in osteogenic medium lead to (and is required for) osteogenic differentiation of FAPs (Uezumi et al., 2010). As for as $Tie2^+$/$PDGFR\alpha^+$/$Sca1^+$ mesenchymal cells, they are able to commit both into the osteogenic and adipogenic lineage upon BMP2 induction (Wosczyna et al., 2012).

Similar to BMPs, TGF-β signals via Smad specific proteins and often both pathways can cross-talk. TGF-β is a key inducer of fibrogenic differentiation and thus appears as an inhibitor of adipogenic commitment (Uezumi et al., 2011, Petruschke et al., 1994). Conversely, TGF-β promotes adipogenic progenitors proliferation (Zamani and Brown, 2011). In mice, the treatment with SB431542, a small molecule inhibitor of TGF-β, resulted in significant reduction of fibrosis and ectopic fat infiltration in a model of rotator cuff tear (Davies et al., 2016). Interestingly, TGF-β inhibition led to a reduction of FAPs number through apoptosis. The role of myostatin, a member of the TGF-β superfamily of proteins, in

adipogenesis is, however, discussed. Myostatin has indeed alternatively shown pro- (Artaza et al., 2005, McPherron and Lee, 2002) and anti-adipogenic properties in mesenchymal cells and pre-adipocytes (Li et al., 2011, Rebbapragada et al., 2003). Interestingly, *mdx* FAPs secrete follistatin upon HDACi treatment (a myostatin inhibitor) to signal on satellite cells in a paracrine way (Saccone et al., 2014). Yet, and because HDACi also reduces FAP adipogenic differentiation, it is possible that follistatin acts on FAPs in an endocrine way to blunt their adipogenic fate.

It was also demonstrated that Nitric Oxide, which is known to improve muscle regeneration in *mdx* mice (Wehling et al., 2001), negatively regulates FAP differentiation into adipocytes *in vitro* and NO treatment of *mdx* mice leads to a reduction of FAPs, collagen expression and fat deposition in dystrophic muscles (Cordani et al., 2013). *In vitro*, NO treatment down-regulates *PPARγ* expression through an increased expression of miR-27b, a key inhibitor of adipocyte differentiation (Lin et al., 2009). *In vivo*, fibrosis is indirectly affected by NO treatment consistently with a reduction of miR-133a levels, a negative regulator of Col1A1 (Castoldi et al., 2012).

Multiple other signals, interleukin, myokines, growth factors such as IGF-1, IL-17, FGF-1, FGF-2, activin can influence adipogenic fate of stem cells (Lowe et al., 2011). FAPs, in particular, see their adipogenic fate blocked by IL-4, an interleukin present in the muscle upon injury, and which sustains, however, their proliferation (Heredia et al., 2013, Dong et al., 2014).

It also seems that physical cues during muscle regeneration interact with the propensity of FAPs to differentiate into adipocytes. Indeed, inducing hindlimb-unloading after muscle injury in mice decreased PDGFRα expression, while impairing both muscle regeneration and ectopic adipogenesis (Pagano et al., 2015).

Similarly, cues other than TGF-β are well known to regulate fibrosis. In skeletal muscle, connective tissue growth factors are elevated in animal models and human patients suffering from muscular dystrophies and can trigger pro-fibrotic effects (Sun et al., 2008, Vial et al., 2008). PDGFs also appear as good candidates for fibrosis regulation (Andrae et al., 2008), and in fact, PDGF-AA induces FAP proliferation in vitro, similar to TGF-β, while upregulating *Col3a1* and *α-SMA* expression (Uezumi et al., 2011). Other evidence is based on human dystrophic muscles which present high levels of PDGF ligands and receptors proteins (Zhao et al., 2003), and the fact that PDGFRα knock-in mice present systemic muscle fibrosis (Olson and Soriano, 2009).

The molecular triggers and interplay governing fibrosis, adipogenesis and muscle regeneration remain to be understood. Also, whether fibrosis and adipogenesis play a key role or are rather an independent program taking place during muscle healing will also have to be clarified to develop therapeutic strategies and rescue muscle regenerative capacities in various diseases.

V. Skeletal muscle aging

Aging leads to major changes in body composition that can dramatically affect the quality of life of elderly people. With age, there is a progressive loss of lean mass and a progressive gain in fat mass, which starts around the age of 25 (Janssen et al., 2000). While lean muscle mass represents around 35-40% of our body mass at young ages, this percentage decreases down to about 25% by the age of 75-80 years (Short et al., 2004). It is reported that muscle mass decline is of 0.5-1% per year from the age of 30 years, 1-2% from the age of 50 years, and that strength also declines at 1.5% per year and at 3% from the age of 60 years. Therefore, loss of muscle mass is not sufficient to fully explain the age-related change in muscle force (Zembron-Lacny et al., 2014, Hughes et al., 2001).

Aging of skeletal muscle is a systemic process and is accompanied by numerous intrinsic and environmental changes (**Fig. 8**). We will hereafter cite the major contributors to skeletal muscle aging, and specifically provide additional details concerning age-related changes affecting the cellular compartment of the muscle stem cell niche in part **V.1.** The overview of the current knowledge about sarcopenia will be provided in section **V.2.** To better introduce the subject of this thesis, the two last sections (**V.3.** and **V.4.**) will be specifically dedicated to provide a literature review about age-related muscle stem cell dysfunction and aging of the muscle stem cell niche.

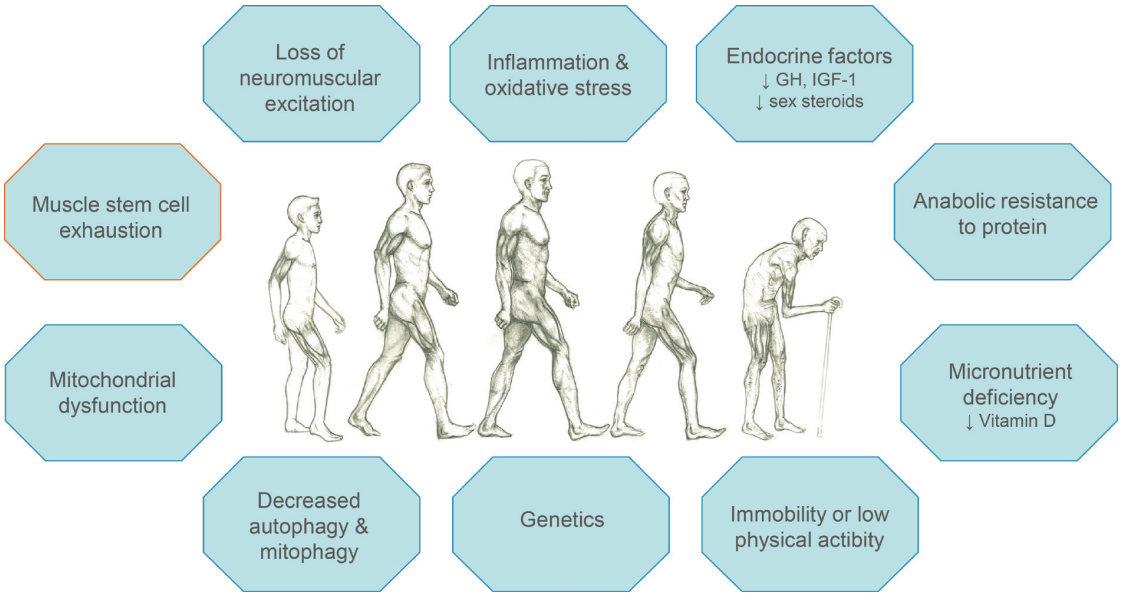

Figure 8. The causes of sarcopenia are multi-factorial. Adapted from (Ali and Garcia, 2014).

V.1. Pathophysiological causes of muscle decline during aging

Muscle characteristics are modified with age. Indeed, a shift in fiber composition towards slow fibers with loss with fast IIb fibers is observed with age, accompanied with a decreased muscle and fiber cross-sectional area that correlates with muscle mass (Verdijk et al., 2010, McGregor et al., 2014). Fiber arrangement has also been reported to contain alterations with age, such as decreased pennation angle and muscle fascia length (Stenroth et al., 2012). In addition, muscle force generation is dramatically impaired with age (Lowe et al., 2001, Thompson and Brown, 1999, Zhong et al., 2006) and this effect was partly attributed to ATPase activity uncoupling to force generation (Lowe et al., 2004, Lowe et al., 2002). Many phenotypic changes have also been reported in aged skeletal muscles, such as alterations in contractile protein turnover, oxidative modifications, glycation and carbonylation, as well as nitration (Thompson, 2009). Nevertheless, their implication in skeletal muscle dysfunction is not well established. More and more evidences link altered autophagy and mitophagy, either at the full muscle level or at the satellite cell level, with functional defects with age (Garcia-Prat et al., 2016, Ko et al., 2016, Wohlgemuth et al., 2010). Recently, stimulation of mitophagy in full muscle of rodents through nutritional intervention with urolithin A, resulted in enhanced exercise capacities of old animals (Ryu et al., 2016).

Skeletal muscle mitochondrial capacity and energy is associated with gait speed and declines with age, as it has been reported that mitochondrial DNA, mRNA and ATP production all decrease with age (Coen et al., 2013, Short et al., 2005). Together with cardiovascular impairment, the capacity of skeletal muscle to use oxygen and drive the overall body aerobic capacity rapidly declines with age and is a strong predictor of mobility in elderly (Fiser et al., 2010, Fleg et al., 2005).

Defects in motor unit and neuromuscular activation also accompany muscle aging, and the number of motor units itself is reduced in elderly people (Hakkinen et al., 1996, McNeil et al., 2005). It was recently showed neuromuscular decline both in rats and humans likely leads to higher susceptibility of muscles to sarcopenia (Pannerec et al., 2016). Age-related declines in cholesterol synthesis were suggested to be triggers of neuromuscular dysfunction.

Age-related hormonal changes are thought to act as key determinants of skeletal muscle decline during aging, and in particular testosterone, estrogens, growth hormone and IGF-1 decline (Baumgartner et al., 1999, Gooren, 1998, Morley, 2003), whose positive effect on muscle mass was previously provided in section **I.3** . Low-grade chronic inflammation defined by an increase of cytokines (such as IL-6 and TNF-α) circulating levels is considered as an underlying mechanism of sarcopenia, potentially acting on protein degradation, cell apoptosis and muscle strength in a more global way (Ferrucci et al., 2002, Toth et al., 2006, Visser et al., 2002).

Life style factors remain predominant predictors of loss of skeletal muscle mass and function. It is well documented that sedentary elderly are more at risk of suffering from loss of muscle mass and strength, and that inactivity worsens muscle decline (Burton and Sumukadas, 2010, Denison et al., 2015, Roubenoff, 2000). Together with deregulated protein turnover (Balagopal et al., 2001, Thompson, 2009), malnutrition of elderly is also a considerable contributor to muscle decline as it is defined by a decline of food intake and so energy intake, and protein consumption in particular (Chapman et al., 2002, Campbell et al., 2001, Calvani et al., 2013, Morley et al., 2010). Low vitamin D status is another recognized important contributor (Visser et al., 2003). Obesity, type-II diabetes and insulin resistance are also important triggers of skeletal muscle loss and likely interplay with age-related loss of muscle function (Kalyani et al., 2014, Guillet and Boirie, 2005).

As previously stated in section **IV.1.**, fat infiltration is a recognized acknowledged hallmark of skeletal muscle aging (Marcus et al., 2012, Song et al., 2004, Miljkovic-Gacic et al., 2008, Manini et al., 2007, Crane et al., 2010, Cree et al., 2004, Nakagawa et al., 2007, St-Onge, 2005). Excessive extracellular matrix deposition is also considered as a marker of aged muscles and is thought to induce elevated stiffness of skeletal muscle, which potentially leads to muscle satellite cells dysfunction in case of an injury, muscle weakness and atrophy (Kragstrup et al., 2011, Ryall et al., 2008, Thompson, 2009). Fibrosis is very well modeled in mice (Mann et al., 2011), and numerous studies have reported changes in collagen subtypes, alterations in collagen synthesis and degradation, and formation of collagen cross-links all of which affect the mechanical properties of skeletal muscle (Kragstrup et al., 2011). In muscles of elderly individuals, increase of fibrosis remains, however, quite speculative as no direct evidence exist (McGregor et al., 2014). Yet, the increased fibrosis observed in old rodent muscles has been associated with the aged-related loss of nNOS expression (Richmonds et al., 1999, Samengo et al., 2012), together with elevation of muscle resident CD163+ M2a macrophages with age (Wang et al., 2015). Indeed, it was shown that old muscles present increased number of CD68+ M1 macrophages and CD163+ M2a macrophages; but the expression of an nNOS transgene in skeletal muscle was able to abrogate this macrophage invasion with age, suggesting that muscle derived-nitric oxide negatively regulated intramuscular macrophage content (Wang et al., 2015). It is known that M2a macrophages can hydrolyze arginine and produce ornithine, which can then be metabolized into proline (necessary for collagen production), thus leading to fibrosis (Wehling-Henricks et al., 2010). In agreement, rescue of nNOS expression in old mice could blunt the aged-related collagen deposition in muscle. Besides, M2a macrophage intramuscular elevation with aging, as well as increased fibrosis, could also be blocked by young bone-marrow transplantation at the adult age (Wang et al., 2015). Altogether, the

study demonstrated that aging of bone-marrow results in muscle invasion by M2a macrophages, which is exacerbated by the loss of nNOS expression in old muscles, leading to fibrosis, eventually.

V.2. Sarcopenia

Sarcopenia occurs when loss of muscle mass and strength becomes debilitating. Sarcopenia is rooted in the Greek words *sarx* and *penia* and means loss of flesh. Sarcopenia prevalence is poorly reported but estimates range between 8% and 40% of elderly people aged over 60 years (Abellan van Kan, 2009, Kim KM, 2015, Fielding et al., 2011). Even more, it is thought that 50% of elderly over 80 years are affected by sarcopenia (Zembron-Lacny et al., 2014). Quality of life is strongly affected in elderly suffering from sarcopenia, ranging from difficulties in getting out of bed or walking up stairs to loss if independence and mobility, with high risks of falls. Consequently, sarcopenia is a strong predictor if hospitalization, disability and death (Newman et al., 2006, Fielding et al., 2011). In addition, healthcare costs associated with sarcopenia are extremely high (estimated at $18.5 billion per year in the U.S.A. in 2004) (Janssen et al., 2004), although more precise and updated health economic analyses are required.

Sarcopenia is thus defined by the progressive loss of skeletal mass and strength, independently of any other underlying condition. Loss of skeletal muscle strength is not necessarily caused by a loss of skeletal muscle mass only, so sarcopenia definition therefore includes loss of muscle mass outcomes, together with loss of skeletal muscle strength and physical performance (Zembron-Lacny et al., 2014). So far, neither consensus clinical definition of sarcopenia exist nor standardized measurement to define the outcomes. Huge efforts are thus being made to propose clear operational definitions, define target population, suggest standardized clinical methods and potential endpoints in order to accelerate consideration of sarcopenia by regulatory and health authorities. Expert groups like the European Working Group on Sarcopenia in Older People (EWGSOP) and the Foundation for the National Institutes of Health Sarcopenia Project (FNIH-SP) try to develop consensual definitions and diagnostics in this sense. Various measurement methods to quantify muscle mass (CT, MRI, DXA, Bioimpedance analysis…), muscle strength (handgrip strength, knee flexion/extension, peak expiratory flow…) and physical performance (Short physical performance battery, usual gait speed, timed get-up-and-go test, stair climb power test…) are also being proposed and in their way for standardization (Cruz-Jentoft et al., 2010). A decision tree was therefore proposed by the EWGSOP for sarcopenia screening and assessment (Zembron-Lacny et al., 2014).

2016 has been marked by a turning point in research on sarcopenia. On the 28th of 2016, Aging in Motion (a coalition initiated by Alliance for Aging Research and actively supporting and pushing for greater levels or research in the sarcopenia area), announced that sarcopenia was granted an ICD-10-CM code (#M62.84) by the Centers for Disease Control and Prevention; thus providing recognition as an official disease (Lloyd, 2016). This will give a new acceleration in the research against sarcopenia, allow establish clear clinical guidelines for both diagnosis and treatment and finally, help the development and approval of new treatments by regulatory agencies such as FDA or EMA.

When considering the broader geriatric syndrome, sarcopenia is often studied in parallel to frailty (Cesari et al., 2014). Frailty is a term used to define a combination of geriatric-linked symptoms contributing to increase the risks of negative health events such as falls, hospitalizations, disability, and death. While it is clear that sarcopenia might act as a strong inducer of frailty and that both are interconnected, some groups are in favor of distinguishing the physical frailty (sarcopenia) and frailty as a global geriatric syndrome (Cesari et al., 2014, Cruz-Jentoft et al., 2010). Indeed, frailty is also defined by non-physical impairments that are fatigue, sedentary behavior, weight loss, and although debated, cognitive impairment and social isolation.

While it is expected that granting an ICD-10-CM code for sarcopenia will bring new guidelines, so far no standardization exists to assess the efficacy of new therapeutic treatments for sarcopenia. Nevertheless, a meeting of the European Society for Clinical and Economical Aspects of Osteoporosis and Osteoarthritis (ESCEO) recently brought new conclusions and suggestions about a potential outcome tree that could be used (Reginster et al., 2016). It would not only take into account ameliorations of pathophysiological manifestations of sarcopenia, but also outcomes considered as impacts of the new disease such as socio-economic costs and quality of life and independence.

Many efforts have been performed by multiple groups to develop strategies aiming at preventing sarcopenia or limiting its symptoms, such as anti-inflammatory drugs or endocrine therapies, hormone replacement and anti-oxidant or nutritional strategies, and in particular vitamin D supplementation (Brioche et al., 2016, Sakuma and Yamaguchi, 2012). To date, exercise is seen as the most effective strategy to prevent sarcopenia, and endurance, resistance, stretching together with proprioception trainings are all recommended (Brioche et al., 2016, Phu et al., 2015). As the **Chapter VI** of this thesis will focus on a molecule produced in response to exercise, we will hereafter provide additional details on the beneficial effect of physical training on muscle physiology and function.

It is indeed well acknowledge that resistance training leads to a gain of muscle mass and strength (Hoppeler, 2016, Luthi et al., 1986). Nevertheless, elderly men and women subjected to a 3-

4 months endurance training consisting of sessions of 20-45 minutes at 60 to 80% of the peak heart rate showed increased muscle size and strength, as well as increased protein synthesis (Konopka et al., 2011, Short et al., 2004). Effect of exercise in elderly on proteolysis is, however, less known. While it is estimated that exercise had no effect on proteolysis, a few studies suggest a possible activation of the ubiquitin/proteasome system (UPS) upon exercise (Brioche et al., 2016). Yet, it is commonly accepted that autophagy is decreased with age in humans and rodents, but can be rescued with exercise (Carnio et al., 2014, Kim et al., 2013, Zampieri et al., 2015). Thus, in addition to mitochondrial, metabolic and cardiovascular improvements (Brioche et al., 2016), exercise helps maintain muscle protein balance in elderly people. Although no data exist in humans, exercise has been showed to reduce myonuclear apoptosis in old animals (Luo et al., 2013, Song et al., 2006, Wohlgemuth et al., 2010). Reduction in apoptotic fiber nuclei might be attributed to the higher activation of satellite cells in response to exercise, together with an increase of the satellite cell pool upon training. Indeed, 3 to 6 months of resistance training (3 days a week, at 80% of the peak heart rate) lead to satellite cells activation together with gain of muscle function in elderly (Leenders et al., 2013, Verdijk et al., 2009). Satellite cells increase was also demonstrated to occur in elderly after endurance exercise (Verney et al., 2008). A multicenter, single blinded randomized trial called Lifestyle Interventions and Independence for Elders study or LIFE study, was launched in 2010 in order to evaluate whether physical activity can prevent mobility disability. 1635 sedentary adults aged 70-89 years with functional limitations were subjected to either a moderate-intensity physical activity program or to a health education program and followed for about 2.7 years. This study showed that the moderate-intensity physical program reduced mobility disability, when assessed by the loss of ability to walk 400m, compared to the health education program (Pahor et al., 2014). However, no reduction in serious fall injury was observed although results suggested evidence that the physical activity program could decrease the prevalence of fall-related fractures in men (Gill et al., 2016). The study will also assess the effects of physical intervention of mild cognitive impairment or dementia.

V.3. Satellite cells in the aged skeletal muscle

Decrease of the number of satellite cells with age

It is today commonly accepted that the satellite cell pool size diminishes with age. In mice, satellite cells represent 30% of myonuclei in the neonate, and this number drops to 4% in the adult and 2% in the old mouse (Shefer et al., 2006). The lower number of satellite cells with aging translates well in human skeletal muscles (Renault et al., 2002). Satellite cell proportion in human was estimated by electron microscopy at 15% of myonuclei at birth, dropping to 4% in the adult (Schmalbruch and Hellhammer, 1976). When compared to adult age (20-32 year-old), the number of satellite cells per muscle fiber in elderly (70-83 year-old) has been shown to decrease by 24% in women and 37% in men (Kadi et al., 2004a). In humans, loss of satellite cells predominantly affects type II muscle fibers, coinciding with the preferential development of age-related atrophy in fast-twitch fibers (Verdijk et al., 2007, Verdijk et al., 2014). The onset of satellite cell loss appears, however, staggered compared to muscle dysfunction as the satellite cell pool seems maintained until around 70 years of age (Dreyer et al., 2006, Roth et al., 2000) before declining at later ages (Renault et al., 2002, Kadi et al., 2004a, Verdijk et al., 2014). In mice, the numeric and function decline of satellite cells do not appear at the same age. For instance, it was demonstrated that while old and geriatric mice possess the same satellite pool size, geriatric satellite cells only presented dramatic functional impairments (Cosgrove et al., 2014). This potentially explains the diversity and debates about intrinsic functional defects of old satellite cells described in the last 15 years.

Satellite cell regenerative dysfunction with age

It is widely accepted that muscle regeneration capacity decreases with age. Nevertheless, the causes leading to impairments in the old satellite cells are still not fully elucidated. It was first shown that satellite cell abundance but not *in vitro* myogenic potential decreases with age, and aged muscle niche can still support efficient myogenesis upon enrichment with FGF (Shefer et al., 2006). The similar differentiation capacities of old satellite cells contrasted, however, with the reported delay in differentiation that was attributed to an impaired proliferation of old satellite cells. Delayed proliferation had indeed been previously described by others (Conboy et al., 2005).

Old myofibers show a decline in the number of their associated $Pax7^+$ cells, but present other cells under the basal lamina which do not express Pax7 nor myogenic markers (Collins et al., 2007). This decrease of myogenicity during aging is accompanied by apoptosis of non-myogenic cells. However, transplantation of single old myofibers resulted in efficient satellite cell renewal and muscle

regeneration, suggesting that only a minority of muscle satellite cells are responsible for muscle regeneration, and that these stem cells retain their intrinsic potential throughout life (Collins et al., 2007). Not only activation and emergence of satellite cells from suprabasal lamina are delayed in old muscles, but the migration speed of old satellite cells is less than half the speed of young satellite cells (Collins-Hooper et al., 2012). It was suggested that this slow migration is likely due to an impaired dynamism of the blebbing process. Delay in old satellite cells activation was further confirmed in geriatric cells (Sousa-Victor et al., 2014). In humans as well, it was shown that satellite cell activation significantly declines with age (Carlson et al., 2009). Altogether, these study have sequentially highlighted that multiple features of satellite cells engagement into the myogenic program were altered with age. This demonstrates the need of developing therapeutic strategies targeting various features of satellite cell dysfunction, and/or uncovering common upstream triggers that could be targeted to restore old satellite function throughout all steps of the myogenic program.

Many evidences suggest a link between the functional decline of old satellite cells (in particular imbalance between asymmetric and symmetric divisions of muscle stem cells and senescence switch) and exhaustion of the pool (reviewed in (Almada and Wagers, 2016, Brack and Munoz-Canoves, 2015, Dumont et al., 2015a) and further detailed in section **V.4.**). Nevertheless, proofs of concept have demonstrated the possibility of overcoming age-related muscle stem cells dysfunction.

Satellite cells and sarcopenia

The impact of satellite cell number and function decline on sarcopenia and frailty was recently challenged. Indeed, while there is a body of evidence showing that satellite cells fusion is not an absolute requirement for muscle hypertrophy (at least for induction or early stages of muscle growth), less is known about the role of satellite cells during muscle atrophy or sarcopenia (as previously discussed in **II.4**) (Biressi and Gopinath, 2015).

It was recently shown that satellite cell depletion at adult age followed by natural aging of the mice did not exacerbate the aging phenotype, as assessed by single muscle mass, fiber-typing and fiber cross-sectional area, and grip strength (Fry et al., 2015). This idea was also confirmed by another study demonstrating that although satellite cells participate to myonuclei turnover, their genetic ablation does not affect fiber size nor muscle strength (Keefe et al., 2015). As expected, satellite cell depletion strongly impaired muscle regeneration. In addition, ECM deposition was increased in satellite cell-depleted muscles, and this observation has already been correlated with decreased whole muscle force by the same authors, in conditions where single myofiber force was unchanged (Fry et al., 2014). It is therefore possible that, preserving the satellite cell pool is critical to limit ECM deposition and in turn, allow the beneficial effects of exercise on muscle function. Like in humans, endurance exercise

showed beneficial effect in aged mouse muscles by limiting the reduction of satellite cell number and myogenic capacity (Shefer et al., 2010). It is very likely that satellite cells are required for anabolic effects in response to treatment or exercise, since exercise, like any muscle injury, has detrimental effect on muscle in absence of satellite cells (Sambasivan et al., 2011). Indeed, regeneration is quite important in elderly humans that can have micro or macro-trauma in muscle, more than mice do. It would have thus been interesting to study the effect of satellite cell depletion in old mice subjective to physical challenges.

V.4. Causes of age-related regenerative dysfunction of satellite cells.

Age-related changes in niche factors

In vitro studies and *in vivo* mesenchymal stem cells engraftment experiments after muscle injury demonstrated that the old systemic milieu and the local niche within muscles show similarity in their inhibitory effect on regeneration (Carlson and Conboy, 2007). On the contrary, whole EDL muscle graft transplantations in mice showed similar myotube formation in response to engraftment in young EDL grafted in young mice and in old mice, suggesting that an old host systemic environment is not inhibitory to myogenesis (Shavlakadze et al., 2010). While the diverse use of assays described in the past decade makes it difficult to define the contribution of the local and systemic control of satellite cell function, it is clear, however, that aging leads to multiple changes in the muscle stem cell niche and systemic environment which accompany the regenerative decline of satellite cells.

The exposure to the circulation of young mice in parabiosis experiments between young and old mice restored proliferation and regenerative capacity of aged satellite cells after muscle injury, notably by restoring the upregulation of the Notch ligand Delta after injury (Conboy et al., 2005). The decline of Notch activation in old satellite cells is accompanied by an increased TGF-β production leading to high levels of pSmad3 and upregulation of cyclin-dependent kinase inhibitors (Carlson et al., 2008). It was shown that Notch and TGF-β pathways antagonize each other, suggesting that the deregulated balance between these signaling pathways could impair muscle regeneration potential in the aged (Carlson et al., 2008). Old satellite cells also present an activated Wnt signaling, leading to an increased muscle fibrosis and impaired muscle regeneration (Brack et al., 2007). Myogenic lineage tracing using permanent β-galactosidase (β-gal) expression only in $Pax7^{+}$ myogenic cells in the adult showed that myogenic progenitors tend to deviate from their myogenic lineage in the aged

environment, and convert to a fibrotic lineage as about 17% of old progenitors are non-myogenic in vitro. This is associated with an enhanced Wnt signaling in aged progenitors which may be conveyed by the systemic environment, as serum of old mice activated Wnt signaling in satellite cells. It was later demonstrated that C1q complement, whose concentration increased in serum of aged mice, was responsible for Wnt activation associated with age (Naito et al., 2012). While C1q-dependent activation of Wnt was showed to be mediated by cleavage of Lrp5/6, upregulation of the Wnt receptor Frizzled 1 was also reported in aged satellite cells (Doi et al., 2014). In addition, serum content in oxytocin, an hormone involved in lactation, was also demonstrated to be reduced in aged mice, together with its receptor level at the surface of old satellite cells (Elabd et al., 2014). Oxytocin activates satellite cells proliferation through the MAPK/ERK pathway, and systemic administration of oxytocin to old mice successfully improved muscle regeneration. Altogether these examples illustrate that both the local environment and the systemic milieu contributes to regeneration efficiency.

Conversely, the aged systemic and local niche may also upregulated factors blunting regeneration. The increased expression of FGF-2 by old myofibers exemplifies how an age-dependent change in the muscle niche influences satellite cell function (Chakkalakal et al., 2012). Indeed, FGF-2 induces old satellite cells to enter cell cycle, thus disrupting quiescence and leading to the satellite cell pool depletion, eventually. GDF-11 (also known as BMP-11), a protein homologous to myostatin at 89% in its amino-acids sequence and thought to share the same signaling pathways, has been shown to increase in serum of aged mice (Egerman et al., 2015). And like myostatin, GFD-11 impairs satellite cell function and regeneration efficiency (Egerman et al., 2015, Lee and Lee, 2013, Souza et al., 2008). Those findings conflicted with another study revealing a loss of GDF-11 circulating in the serum and showing that GDF-11 treatment of old mice rescued muscle regeneration (Sinha et al., 2014). This discrepancy was further discussed and likely arises from distinct GDF-11 measurement methods and protocols to treat old mice (Brun and Rudnicki, 2015).

Aging also affects the fine-tuned timing of inflammatory response that is critical for muscle regeneration. It was indeed shown that regeneration is delayed in old mice as a consequence of reduced inflammatory response leading to a delayed inflammation, and weaker chemotactic stimuli produced by old damaged myofibers themselves (Shavlakadze et al., 2010). It was further shown that old muscles present reduced accumulation of Treg cells after injury, caused by impaired recruitment of Treg to muscles, impaired proliferation and retention of those cells (Kuswanto et al., 2016). As previously described (section **III.4.**), Treg play a critical role during muscle regeneration, mostly through their regulation of the pro- to anti-inflammatory switch and controlling satellite cell function (Burzyn et al., 2013, Castiglioni et al., 2015). FAPs are the major producers of IL-33 (although production by satellite cells was not assessed), the cytokine involved in Treg recruitment, and it was

shown that IL-33 expression was significantly reduced in old injured muscles. This reduction was attributed to a decreased number of IL-33-expressing FAPs in muscle upon injury, but IL-33 administration to old mice could restore Treg accumulation into muscles and ameliorate regeneration (Kuswanto et al., 2016). In humans, media conditioned with whole blood lymphocytes isolated from young and old individuals and further activated *ex vivo,* demonstrated opposite effects on C2C12 (Al-Dabbagh et al., 2015). Young lymphocyte secretome promoted proliferation and migration of the myogenic cells, but old lymphocyte secretome rather promoted fast differentiation. Interestingly, satellite cell sensitivity to splenic T cells is also affected by aging. Indeed, while T cell conditioned media promoted young satellite cell proliferation and migration without affecting their myogenic commitment, old satellite cell proliferation and migration remained, in contrast, unchanged and even showed a decreased myogenesis (Dumke and Lees, 2011).

Molecular triggers affecting aged satellite cells

Most studies described above have suggested that age-related satellite cells dysfunction is reversible. However, transplantation assays, parabiosis or rescue of local micro-environment mostly demonstrated a partial improvement of muscle regeneration in old mice. This suggests the presence of cell-autonomous alterations and the idea that ageing is a multisystemic process.

Recent studies revealed intrinsic alterations in old and geriatric satellite cells that are causative of the muscle regeneration decline with ageing and that cannot be rejuvenated by a young host environment (Cosgrove et al., 2014, Sousa-Victor et al., 2014, Bernet et al., 2014). The differences with studies where a young systemic milieu is sufficient to rejuvenate old muscle stem cells (Conboy et al., 2005), first rely on the age of satellite cells (old vs. geriatric) (Sousa-Victor et al., 2014). In addition, limiting-dilution assays of old satellite cells transplanted in young recipients showed a decreased regenerative capacity (by two-third) of old transplanted cells compared to young transplanted cells only when very few cells (as few as 10) were transplanted (Cosgrove et al., 2014). Therefore, this suggests that the intrinsic defect of old satellite cells is masked when high number of cells is transplanted into young mice, supporting the idea that only a subset of satellite cells retains the long-term functional stemness through life (Collins et al., 2007, Cosgrove et al., 2014). Nevertheless, whereas it was first demonstrated that despite the reduced number of $Pax7^{+}$ cells in old muscles, engrafting a single old myofiber leads to self-renewal and regeneration as efficiently as engrafting a young myofiber containing more satellite cells (Collins et al., 2007); a more recent study presented opposite results (Bernet et al., 2014). There, heterochronic myofiber transplantations showed that the number old myofiber-associated GFP-marked satellite cells that fuse to host young myofibers and reach the satellite cells position is 2-fold reduced as compared to the engraftment of young donor-

derived satellite cells (Bernet et al., 2014). Such diverging results might be explained by the different timings used to transplant the old myofibers and injure the young host muscle. In the first setting, the host muscle was injured 4 weeks after old satellite cells engraftment (Collins et al., 2007); whereas in the last study, old satellite cells associated with myofibers where transplanted concomitant to BaCl2 injury (Bernet et al., 2014), possibly revealing differences in the capacity of old satellite cells to engraft and self-renew when transplanted in the quiescent state and in the activated state. Another difference likely accounting for the differences observed between those studies is the young recipient donor (mouse background, healthy muscle phenotype *vs. mdx* phenotype…). When, however, a very high number of adolescent, young adult and old mouse satellite cells where transplanted in young *mdx* regenerating muscles (transplantation was done 2 days after muscle injury), engraftment capacity of the satellite cells was clearly reduced with age (Price et al., 2014).

The comparison of muscle properties of young, adult, old and geriatric wild-type mice revealed that sarcopenia, described by the sharp regenerative decline, occurs at geriatric age (Sousa-Victor et al., 2014). Such declining functions could not be explained by a reduction of the satellite cell pool, as the number of satellite cells was similar in old and geriatric muscles. It was demonstrated that geriatric satellite cells lose reversible quiescent, and thus their ability to activate, proliferate and self-renew; leading to the depletion of satellite cell pool over time when exposed to proliferative pressure (Li and Belmonte, 2014, Sousa-Victor et al., 2014, Cosgrove et al., 2014). The quiescence-to-senescence switch was explained by the loss of H2A monoubiquitination by PRC1 in the INK4a locus, causing the derepression of $p16^{INK4a}$, a master regulator of cellular senescence. Induction of $p16^{INK4a}$ expression in geriatric satellite cells was also observed in humans, supporting this first reported evidence of muscle stem cell senescence. In addition, the senescent phenotype and the cell-autonomous self-renewal defect of old satellite cells were also accompanied by an elevated p38α/β MAPK signaling and increased cell cycle inhibitors (Cosgrove et al., 2014, Bernet et al., 2014, Bentzinger and Rudnicki, 2014). Despite the increased p38α/β MAPK signaling in old satellite cells, the percentage of cells with asymmetric phospho-p38 was reduced as compared to the percentage in young satellite cells, consistant with the role of asymmetric activation of p38α/β MAPK in driving satellite cell activation and self-renewal (Troy et al., 2012). The elevated p38α/β MAPK signaling pathway in old satellite cells was assessed at very early time points, 1h after fiber isolation (Bernet et al., 2014) or analyzed by flow cytometry on freshly isolated satellite cells (Cosgrove et al., 2014), when young satellite cells do not present any p38α/β MAPK activation yet. Considering the reported delay in activation of old satellite cells (Collins-Hooper et al., 2012, Sousa-Victor et al., 2014), this suggests that their increase in p38α/β MAPK occurs at the quiescent state, which is supported by the same observation in uninjured muscle section (Bernet et al., 2014).

A synergistic action of a biochemical cue through the inhibition of p38α/β signaling, and a biophysical cue using cultures on soft hydrogel substrates allowed to maintain Pax7 expression in old satellite cells and to increase *ex vivo* expansion of old satellite cells, thus improving engraftment, reconstitution of the satellite cell reservoir as well as muscle strength after transplantation (Cosgrove et al., 2014). Indeed, the beneficial effects of using soft hydrogel substrates that mimic the elasticity of muscle on the satellite cell self-renewal *ex vivo* had already been demonstrated and allow to study stem cell function *in vitro* (Gilbert et al., 2010). It has furthermore been demonstrated that muscle fibers freshly isolated from old muscle showed increased stiffness, that was associated with higher muscle fibrosis and which was responsible for decreased old satellite cell proliferative capacities (Lacraz et al., 2015). In addition, it was also shown that the FGF-receptor 1 was desensitized in old muscles, as FGF-2, a signal present in the muscle niche known to promote satellite cell expansion (Jones et al., 2005), failed to activate FGFR1-dependent p38α/β MAPK signaling (Bernet et al., 2014). This result was consistent with the previously reported increased amount of FGF-2 in old skeletal muscles (Chakkalakal et al., 2012). Constitutive FGFR1 signaling allowed to partially rescue the self-renewal of old satellite cells. A model was proposed where the desensitized FGFR1, along with the elevated p38α/β MAPK signaling, fails to restrict p38 signaling to one cell and establish a polarity, thus leading to symmetrical division where both daughter cells are committed to myogenic differentiation (Bernet et al., 2014, Bentzinger and Rudnicki, 2014). Altogether, these 3 recent studies provide strong evidence of intrinsic changes in satellite cells associated with age. Although, it is also possible that the altered aged environment is responsible for the increased p38α/β MAPK signaling, such as cellular stress or inflammatory responses, thus leading to permanent changes that are considered as intrinsic, as previously suggested (Chakkalakal et al., 2012, Brack et al., 2007). Another age-related change observed at the satellite cell level, the activation of JAK/STAT signaling, was also reported in adult and old satellite cells compared to adolescent (Price et al., 2014). Inhibition of JAK/STAT signaling (Jak2 or Stat3) in adult satellite cells by siRNA or inhibitors increased their symmetric divisions, and thus the number of satellite cells; as well as enhancing their engraftment into injured muscles. Similarly, direct intramuscular injections of JAK/STAT inhibitors ameliorated muscle regeneration and muscle force after injury (Price et al., 2014). The findings were concomitantly supported by another study showing that Stat3, promotes myogenic lineage progression of satellite cells (Tierney et al., 2014). Interestingly, IL-6 that is elevated with age, is an activator of Stat3. Genetic or pharmacological inhibition of Stat3 resulted in MyoD repression and satellite cells expansion, and transient Stat3 inhibition could therefore be a therapeutic strategy to enhance muscle repair in aged mice.

Altogether, those studies demonstrate the possibility to pharmacologically intervene on the intrinsic aged-related signaling of stem cells. Further, recent studies remarkably enlightened metabolic dysfunction in satellite cells, paving up the way for new therapeutic approaches such as nutritional

intervention. Indeed, failure of basal autophagy was identified in old human and mouse satellite cells, accounting for their senescent switch (Garcia-Prat et al., 2016). In particular, old quiescent satellite cells present impaired basal mitophagy accompanied by elevated reactive oxygen species (ROS) content; which were shown to be responsible for the loss of H2A monoubiquitilation at the INK4a locus leading to p16INK4a induction and senescence, eventually. Indeed, while blocking autophagy in young quiescent satellite cells using Atg7ΔPax7 mice caused premature senescence, loss of satellite cells *in vivo* and impairments of regenerative functions; geriatric satellite cells quiescence and subsequent regenerative functions could be rescued both by inhibition of autophagy (by genetic overexpression of Atg7 or pharmacological treatment with rapamycin) or downstream ROS inhibition (using a vitamin E analogue) (Garcia-Prat et al., 2016). Similarly, another link between senescence and mitochondrial dysfunction was also uncovered, together with the possibility to prevent senescence by treating old mice with nicotinamide-riboside (NR), a precursor of NAD^+, in a SIRT1-dependent manner (Zhang et al., 2016). Mechanistically, it was shown that NR promotes the mitochondrial unfolded protein response (UPR) and prohibitin signaling pathways to block senescence. At the full muscle level, long-term nutritional supplementation of mid-age rodents with urolithin A, a metabolite derived from ellagitannins present in pomegranate, nuts and berries, induced mitophagy in old animals and ameliorated their exercise capacity (Ryu et al., 2016).

Altogether, these studies imply that age-related satellite cell dysfunction can be reversed by correcting metabolic defects, intervening with pharmaceutical inhibitors, or restoring the local or systemic micro-environment. The possibility to indirectly intervene on old satellite cells by targeting their structural and cellular micro-environment has however, poorly been investigated.

CHAPTER II. Rationale & experimental strategy of the thesis

I Rationale and motivation

Since the discovery of the first mammalian satellite cell in 1961 (Mauro, 1961), many efforts have been done to study myogenesis and satellite cell biology (Scharner and Zammit, 2011). However, the investigation of skeletal muscle physio-pathology in various conditions (such as age, myopathies, and metabolic diseases) has demonstrated the presence of non-muscle tissue such as fibrotic depots and fat, raising the question of their cellular origin. Extensive research has been pursued to assess whether satellite cells could enter a non-myogenic lineage under myogenic cues or spontaneously (Alexakis et al., 2007, Asakura et al., 2001, Li et al., 2004, Li and Huard, 2002, Shefer et al., 2004, Starkey et al., 2011). In parallel, many efforts identified and defined numerous muscle resident mononucleated cells distinct from satellite cells (as reviewed in (Judson et al., 2013, Pannerec et al., 2012)). Side population cells, PICs, pericytes, mesenchymal stem cells and fibroblasts were identified surrounding muscle fibers and altogether, present a vast range of lineage fates. In particular, fibro/adipogenic progenitors (FAPs) were identified by two groups in 2010 (Joe et al., 2010, Uezumi et al., 2010). While the first group used double positivity for Sca1 and CD34 to mark the non-satellite cells muscle resident progenitors and identify their dual fate, the second group used PDGFRα to prospectively identify those mesenchymal progenitors and assess their adipogenic capacities. In addition, Joe et al. noted that FAPs expressed PDGFRα (Joe et al., 2010). The following year, the second group published another study confirming by clonal study that FAPs possess a dual fate (Uezumi et al., 2011), and the overlap of populations described by both studies.

Ectopic fat infiltration has always been considered detrimental to muscle function, and observed in particular in degenerative contexts (Samagh et al., 2013, Osti et al., 2013, Itoigawa et al., 2011, Sambasivan et al., 2011, Uppin et al., 2013). In particular, it is well acknowledged that the regenerative capacity of skeletal muscle is reduced with age. As such, increased adiposity is observed in aged muscles (Marcus et al., 2012, Song et al., 2004, Miljkovic-Gacic et al., 2008, Manini et al., 2007, Crane et al., 2010, Cree et al., 2004, Nakagawa et al., 2007, St-Onge, 2005). Impaired muscle regeneration with age is partly due to the decline of satellite cell regenerative capacities themselves,

together with a depletion of their pool (Almada and Wagers, 2016, Brack and Munoz-Canoves, 2015). In humans, the slow muscle healing together with increased inflammation translate into low muscle performance and excessive muscle fatigue in elderly following trauma or chirurgical laceration of muscles (in the context of abdominal surgery, arthroplasty, or hip/knee replacement for instance) (Bautmans et al., 2014). Conversely, low recovery of elderly people after muscle trauma exacerbates the age-related muscle weakness. Therefore, it is necessary to develop strategies to boost muscle repair and maintain efficient regenerative capacities throughout life.

Significant amount of studies have enlightened multiple changes in the old muscle environment leading to loss of positive triggers to satellite cells (Conboy et al., 2005, Elabd et al., 2014), or conversely negatively influencing old satellite cell function (Brack et al., 2007, Naito et al., 2012, Chakkalakal et al., 2012, Carlson et al., 2008). Efforts have also resulted in better understanding the molecular and metabolic satellite cell autonomous alterations of muscle stem cells with age functions (Bernet et al., 2014, Cosgrove et al., 2014, Garcia-Prat et al., 2016, Price et al., 2014, Sousa-Victor et al., 2014, Zhang et al., 2016). The demonstration that age-related cell autonomous changes are reversible in these studies was the proof of the possibility to further develop therapeutic strategies to restore the function of aged satellite cells.

As micro-environmental changes can lead to satellite cell autonomous permanent dysfunction, we interrogated how the old satellite cell niche is impaired at different levels. A large part of this thesis therefore consisted in examining satellite cell aging in the context of their local and systemic environment and evaluate innovative potential strategies to restore the regenerative capacity of skeletal muscle in the context of aging. This thesis aimed at answering the following major questions: What are the molecular, cellular or metabolic mechanisms of ectopic adipogenesis during muscle regeneration? Are FAP fate, function and cross-talk with satellite cells affected by age, and can we target the satellite cell cross-talk with FAPs to ameliorate muscle regeneration? Is the extracellular matrix remodeling during muscle repair changed with age, and can we target satellite cell interaction with their close micro-environment to enhance their regenerative capacities? Is sensitivity of satellite cells to external stimuli perturbed with age, and can we improve satellite cell responsiveness to their local or systemic micro-environment?

II. Objectives

1st axis of the thesis: Understanding the molecular mechanisms of ectopic adipogenesis during muscle regeneration by comparing two mouse models of muscle regeneration.

When we started this project, the recent discovery of intramuscular adipocytes progenitors had provided strong bases to develop research projects aiming at limiting muscle marbling by fat and restoring muscle repair. We therefore started this work by interrogating the etiology of ectopic adipogenesis during regeneration (and this work was pursued during the beginning of my PhD studies). In particular, we wanted to understand if the transcriptional mechanisms that drive ectopic adipogenesis can be uncoupled from the myogenic response. How does inflammation and cellular dynamics during remodeling of the niche balance myogenesis and adipogenesis? Are there specific metabolic pathways favoring adipogenesis or myogenesis? This work was the basis to understand how FAPs could cross-talk with their environment during muscle regeneration and how their differentiation is regulated during the time course of repair.

The manuscript will be provided in **Chapter III**.

2nd axis of the thesis: Evaluating how aging impairs the remodeling of the extracellular matrix and examining the downstream consequences on functional alterations at the satellite cell level.

We were interested in the very close satellite cell environment and asked how aging affects the environment that provides structural and signaling cues to the muscle stem cells. In particular, how is the extracellular matrix of the satellite cell niche changed with age and injury? Do satellite cells display cell autonomous dysfunctions that make them less responsive to environmental cues? Can defects of the extracellular matrix be targeted therapeutically to restore regenerative potential?

The manuscript will be provided in **Chapter IV**.

3rd axis of the thesis: Examining FAP function with age and interrogating how the cross-talk between satellite cells and FAPs is modified in the aged niche.

Very little is known about how the cross-talk between satellite cells and other niche cells, in particular FAPs, evolves with age; and ageing of FAPs itself had never been studied either. Is the satellite cell cross-talk with FAPs changed with age? Is FAP function perturbed with age? What are the signals between FAPs and satellite cells and are they lost with age? Can we intervene on this cross-talk

to ameliorate muscle regenerative function? Does altered FAP function influence ectopic adipogenesis in old regenerating muscles?

The manuscript will be provided in **Chapter V**.

4th axis of the thesis: Understand how the circulating and paracrine peptide apelin regulates satellite cell function and muscle regeneration in the context of aging.

When evaluating age-related changes at the satellite cell level in order to examine potential defects of the response to their micro-environment, we uncovered a strong downregulation of APJ, the receptor to the circulating peptide apelin, in aged satellite cells. This finding opened-up many questions that we decided to tackle through a collaboration with Philippe Valet and Cédric Dray's team (Toulouse, France). Does apelin play a role during muscle regeneration? Can we use apelin therapeutically to ameliorate regeneration in old muscles? Are satellite cells responsive to apelin? How are the levels of apelin regulated with age in the muscle and systemically? What are the sources of apelin?

The manuscript will be provided in **Chapter VI**.

III. Gobal experimental strategy

In order to answer the questions described above, we established a comprehensive and integrative experimental strategy allowing us to study many complementary aspects of the regulation of muscle stem cells.

We used young adult age or old but non-geriatric wild-type male mice as a model of natural ageing, which were challenged with glycerol-induced muscle injury to model muscle regeneration. Muscle regeneration was studied at different time points (3, 7 or 14 days after injury (dpi)), as a model to study both regeneration efficiency and ectopic adipogenesis with age (**Fig. 9**). We validated that muscle regeneration was impaired in aged mice in our model by showing a significantly reduced upregulation of several myogenic markers at the mRNA level (Pax7, MyoD, Myogenin and the embryonic myosing heavy chain Myh3) (**Fig. 10a**). This result was confirmed at the histological level, since old regenerative fibers were smaller at 14dpi (**Fig. 10b,c**). We then undertook a combination of mRNA and proteomic screens in order to profile changes in full muscle, both in uninjured or

regenerating conditions (Fig. 9). These results, which are described in the different manuscripts of this thesis, were complemented with transcriptomic analyses on progenitors sorted by flow cytometry from young and aged mice, in order to decipher age-related changes directly in satellite cells and FAPs, in the quiescent and in the activated/proliferative state (Fig. 11).

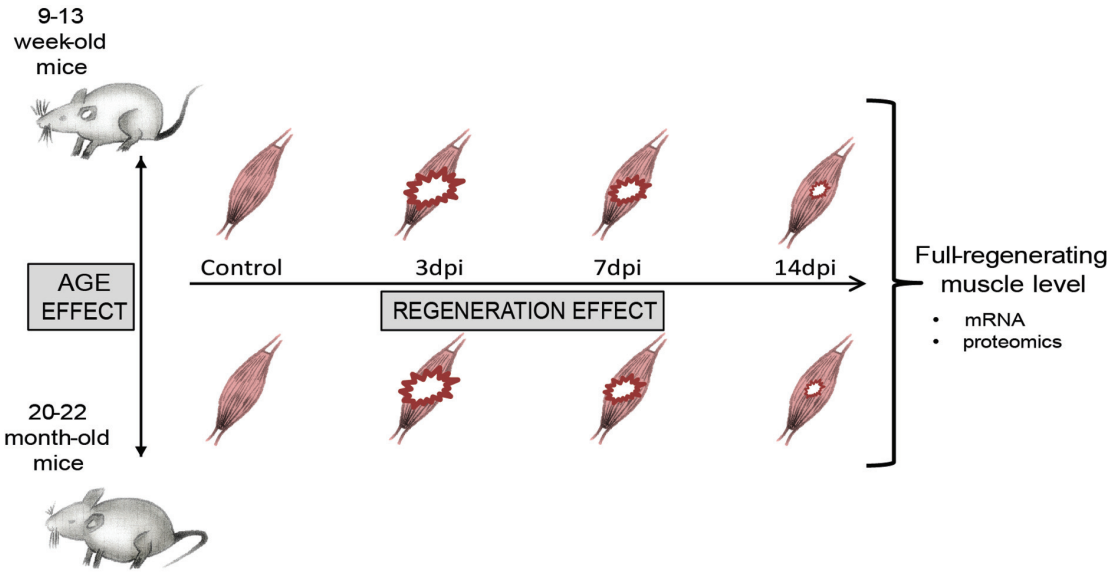

Figure 9. Global experimental strategy to study regenerating muscles with during aging. Young and old muscles were injured and muscles were collected at 3, 7 or 14 days post injury (dpi), or non-injured as controls. Muscles were them subjected to transcriptomic and proteomic anaylses.

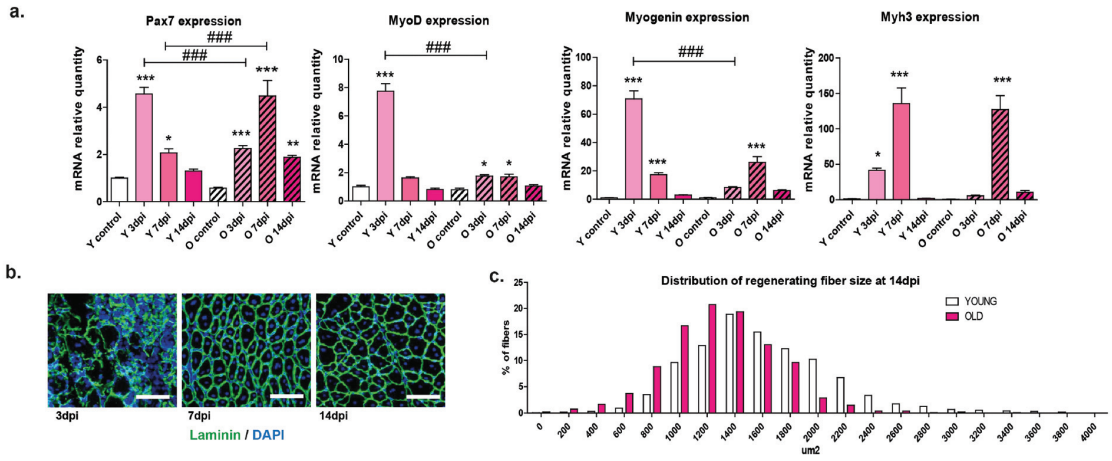

Figure 10. Muscle regeneration is impaired in old mice following glycerol-induced injury. (a) Young (9-13 week-old) and old (20-22 month-old) mice were injured using an intramuscular injection of 50% (v/v) glycerol into *tibialis anterior* muscles, and muscles were collected at 3, 7 or 14 days post injury (dpi), or non-injured as controls. mRNA levels of myogenic markers (Pax7, MyoD, Myogenin and Myh3) were quantified by qPCR. **(b)** Regenerating muscle sections were stained for laminin (green) and DAPI (blue) to visualize regenerating fibers with central nuclei. **(c)** Distribution of cross-sectional area of fibers with central nuclei at 14 dpi in young and old mice. Y=young; O=old; n=8; *: p-value *vs*. control of respective age < 0.05; **: p-value *vs*. control of respective age < 0.01; ***: p-value *vs*. control of respective age < 0.001; ###: p-value *vs*. young at respective time points < 0.001.

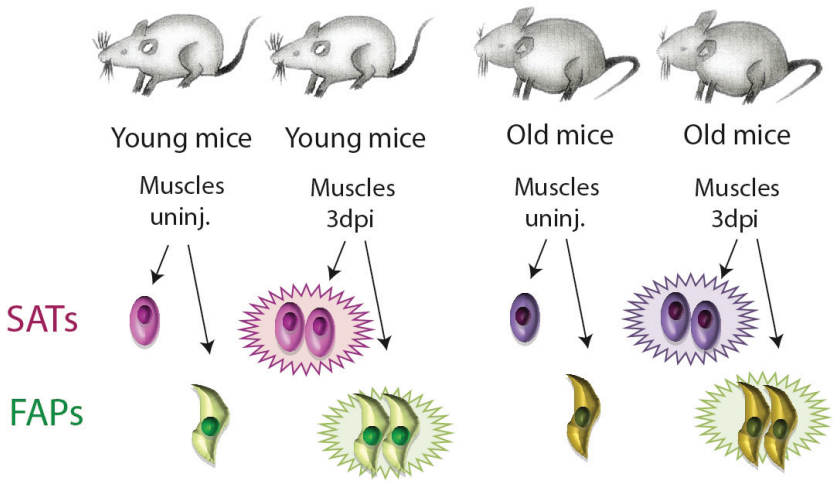

Figure 11. Global experimental strategy to profile satellite cell and FAP activation and aging. Satellite cells and FAPs were freshly isolated from resting or 3dpi injured young (9-13 week-old) and old (20-22 month-old) muscles, and subjected to micro-array anaylsis.

CHAPTER III. Genomic profiling reveals that transient adipogenic activation is a hallmark of mouse models of skeletal muscle regeneration.

Rationale

Intramuscular (CTX) injection is an established model of muscle regeneration, as CTX is a snake venom toxin which destroys myofibers and activates repair and regeneration (Ownby et al., 1993). Glycerol injection was also reported to induce damage and regeneration and mimic myopathies in rabbit (Kawai et al., 1990) and in mice (Arsic et al., 2004). It was also shown that intramuscular injections of glycerol could efficiently induce ectopic adipogenesis in muscle (Pisani et al., 2010b, Joe et al., 2010, Pisani et al., 2010a, Uezumi et al., 2010). The muscle resident fibro/adipogenic progenitors (FAPs) are considered as the source of ectopic adipocytes and fibrotic tissue, and similar to satellite cells, they are activated and strongly proliferate after muscle injury (Joe et al., 2010, Uezumi et al., 2010, Uezumi et al., 2011). FAPs also promote myogenic differentiation of satellite cells, suggesting that they play an active role in muscle regeneration (Joe et al., 2010). Importantly, FAP fate was suggested to be regulated by the muscle micro-environment, as using reciprocal transplantation of FAPs isolated after glycerol or CTX injury into either CTX- or glycerol-injured muscles, respectively; it was found that only glycerol-injured muscles were permissive to ectopic adipocyte formation (Uezumi et al., 2010). Consequently, when we started this work in 2012, it was widely believed that ectopic adipocyte formation in muscle was the consequence of the specific micro-environment triggered by glycerol-induced injury, which could not be recapitulated in other experimental models of muscle injury.

An understanding of the etiology of skeletal muscle ectopic adipogenesis is not only key for the future development of therapeutic strategies aiming at limiting fat infiltration, but is also very interesting for the fundamental knowledge of FAP biology and the global muscle stem cell niche. In this chapter, we therefore aimed at understanding the molecular mechanisms of ectopic adipogenesis during muscle regeneration, using young mouse models of Glycerol- vs. CTX-induced muscle injury. To this end, we performed a comprehensive molecular and histological profiling of four different timepoints of muscle regeneration.

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My contribution

I designed, conducted and interpreted all the in vivo, molecular and histological analyses of this work with the technical support of Eliane Pierrel. I also interpreted the bioinformatic analyses of the genomics data performed by Sophie Brachat, and wrote the paper under supervision.

Publication

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Genomic Profiling Reveals That Transient Adipogenic Activation Is a Hallmark of Mouse Models of Skeletal Muscle Regeneration

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Abstract

The marbling of skeletal muscle by ectopic adipose tissue is a hallmark of many muscle diseases, including sarcopenia and muscular dystrophies, and generally associates with impaired muscle regeneration. Although the etiology and the molecular mechanisms of ectopic adipogenesis are poorly understood, fatty regeneration can be modeled in mice using glycerol-induced muscle damage. Using comprehensive molecular and histological profiling, we compared glycerol-induced fatty regeneration to the classical cardiotoxin (CTX)-induced regeneration model previously believed to lack an adipogenic response in muscle. Surprisingly, ectopic adipogenesis was detected in both models, but was stronger and more persistent in response to glycerol. Importantly, extensive differential transcriptomic profiling demonstrated that glycerol induces a stronger inflammatory response and promotes adipogenic regulatory networks while reducing fatty acid β-oxidation. Altogether, these results provide a comprehensive mapping of gene expression changes during the time course of two muscle regeneration models, and strongly suggest that adipogenic commitment is a hallmark of muscle regeneration, which can lead to ectopic adipocyte accumulation in response to specific physio-pathological challenges.

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Introduction

Skeletal muscle is a highly plastic tissue, which responds to exercise or disuse by modulating the mass and composition of contractile proteins [1,2]. In addition, muscle fibers have a strong regenerative capacity as muscle injuries trigger the proliferation and activation of satellite cells, a specific type of stem cells expressing the marker Paired box protein 7 (Pax7) and committed to the myogenic lineage [3–5], which subsequently fuse to injured fibers to promote their efficient repair. Other cell types are also involved in muscle repair during the different phases of the healing process [4,6]. In particular, immune cells are recruited to degenerating muscle to allow the removal of cellular debris and support myogenesis [7,8]. In addition, a novel type of Pax7-negative myogenic progenitors expressing the marker PW1 also participate to muscle regeneration [9]. Despite the ability of healthy skeletal muscle to regenerate, several pathological conditions such as muscular dystrophies or aging impair satellite cell homeostasis and myofiber regeneration [10,11], thereby weakening muscle plasticity and integrity. In such diseases, excessive cycles of degeneration/regeneration prime the muscle for fibrosis and ectopic adipocyte accumulation, leading to an exhaustion of the regenerative capacity and ultimately to impaired muscle contraction.

Muscle ectopic adipogenesis is particularly prominent in myopathies such as Duchenne muscular dystrophy, where young boys with dystrophin mutations have important fat infiltration that can reach up to 50% of muscle content in the gluteus muscle [12]. Intra-muscular fat accumulation also occurs in sarcopenia where marbling of skeletal muscle by adipose tissue plays an important role in contractile and metabolic dysfunction [13,14]. It has been recently demonstrated that fat cells which invade skeletal muscle originate from mesenchymal progenitors distinct from satellite cells and expressing the platelet-derived growth factor receptor alpha (PDGFRα) [15–17]. Using lineage tracing, PDGFRα has also recently been recognized as a general marker for adipogenic progenitors giving rise to mature fat cells in white and brown adipose tissues [18,19]. Interestingly, muscle-resident PDGFRα-positive progenitors can also give rise to collagen-type I expressing cells, indicating that ectopic adipogenesis and fibrosis are regulated in parallel from common fibro/adipogenic progenitors (FAPs) [20,21]. In order to differentiate into pathological fat or fibrotic depots, FAPs require external triggers, that remain to be characterized, but rely on the muscle environment rather than the progenitors themselves [15,17]. Human PDGFRα-positive FAPs have also recently been demonstrated to have osteogenic potential, and could contribute to pathological calcification of skeletal muscle occurring during Myositis Ossificans [22]. How-

ever, FAPs also seem to positively influence myogenesis and muscle regeneration as they are activated upon muscle damage and show increased expression of IL-6 [20], a factor that promotes myogenesis [4,23,24]. In addition, when co-cultured with myogenic progenitors *in vitro*, FAPs induce myogenic differentiation in a dose-dependent manner, suggesting that they might play an active role in muscle regeneration [20]. Furthermore, it has recently been demonstrated that IL-4/IL-13 signaling is activated in FAPs in response to IL-4 secretion by eosinophils upon injury, thus promoting FAP proliferation to support myogenesis and inhibiting their commitment toward adipogenesis [25].

Many mouse models have been developed to study skeletal muscle regeneration, out of which the intramuscular injection of toxins such as cardiotoxin (CTX) has been one of the most extensively studied [26]. In contrast, the intramuscular injection of glycerol has recently been recognized as a new model of regeneration which promotes ectopic adipogenesis in muscle [16,17,20,27]. Although the model of intramuscular glycerol injection has been technically refined recently [16], understanding the causes and mechanisms of ectopic fat cell deposition remains an open question. Towards that goal, we conducted a comprehensive profiling of the molecular and histological responses occurring after muscle damage induced by CTX and glycerol at 4 different time points. Our results demonstrate that the myogenic response overlaps to a large extent in both injury models. Surprisingly, an adipogenic response was also detected in both models, although glycerol induced stronger and more prolonged adipocyte formation. Altogether, our data provide a comprehensive correlation between the molecular and histological changes differentially occurring during glycerol and CTX regeneration, and the transcriptional signatures of these two injury models constitute a key resource to further understand muscle regeneration and ectopic adipogenesis.

Materials and Methods

Animals

All animal experiments were approved by the Kantonales Veterinäramt Basel Stadt, Switzerland. Adult C57BL/6J male mice were purchased from The Jackson Laboratory, maintained at 22°C in a 12-h light–12-h dark cycle with unrestricted access to regular diet and water and injured at 12 to 14 weeks old.

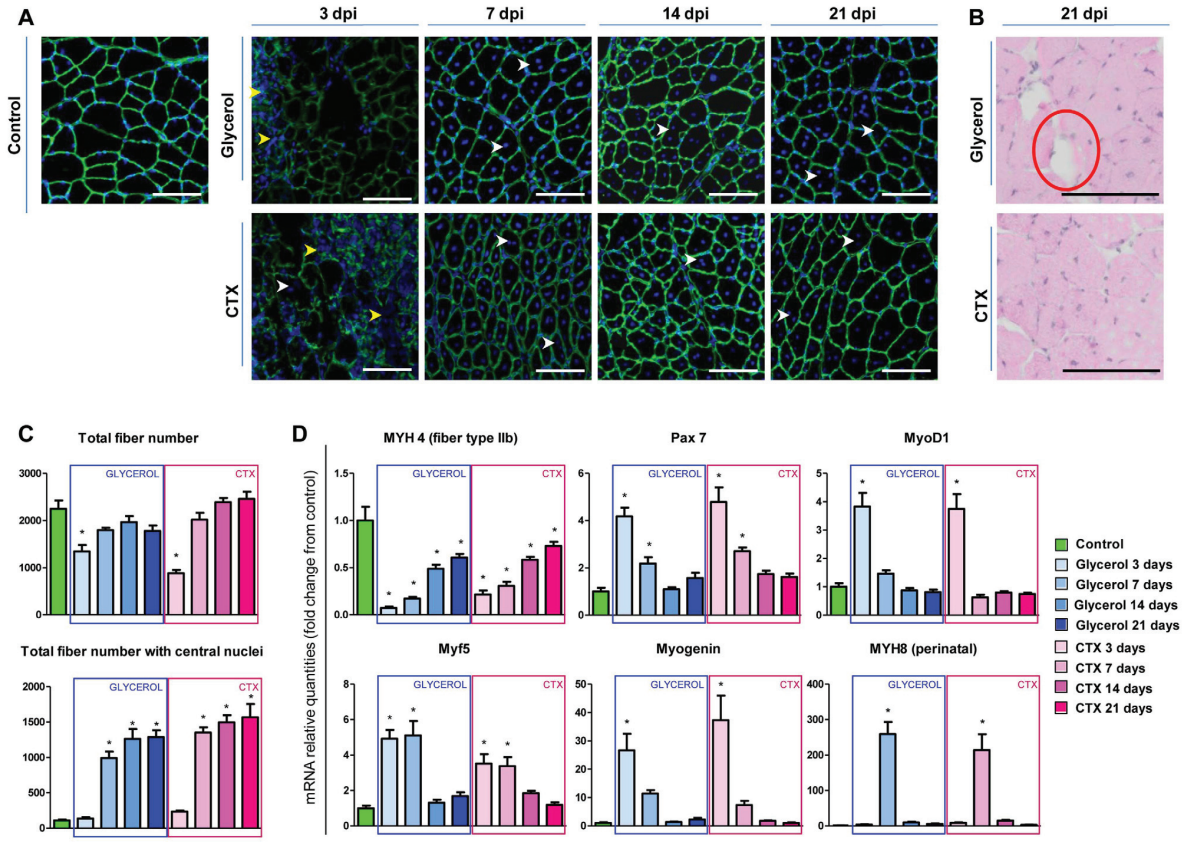

Figure 1. Glycerol and CTX induce similar kinetics of degeneration and regeneration. Control uninjured tibialis anterior muscle, and tibialis anterior muscles injected with either 25 µl of 50% (v/v) glycerol or 10 µM CTX were sectioned and stained with laminin and DAPI 3, 7, 14 or 21 days after injection (dpi) (A), or with hematoxylin-eosin at 21 dpi (B). Cryosections were performed at the mid-belly part of tibialis anterior. Scale bars, 100 µm. Yellow arrow: immune cell nuclei, white arrow: central nuclei, red circle: fat cell-like structure. (C) Quantitative analysis of total myofibers and of myofibers with at least one central nuclei from laminin/DAPI stained sections. (D) qPCR analysis of the mRNA levels of different markers of muscle regeneration. Data are expressed as mean ± s.e.m., n = 5–6/group. * p-value <0.05 *vs.* control. MYH, Myosin Heavy Chain.
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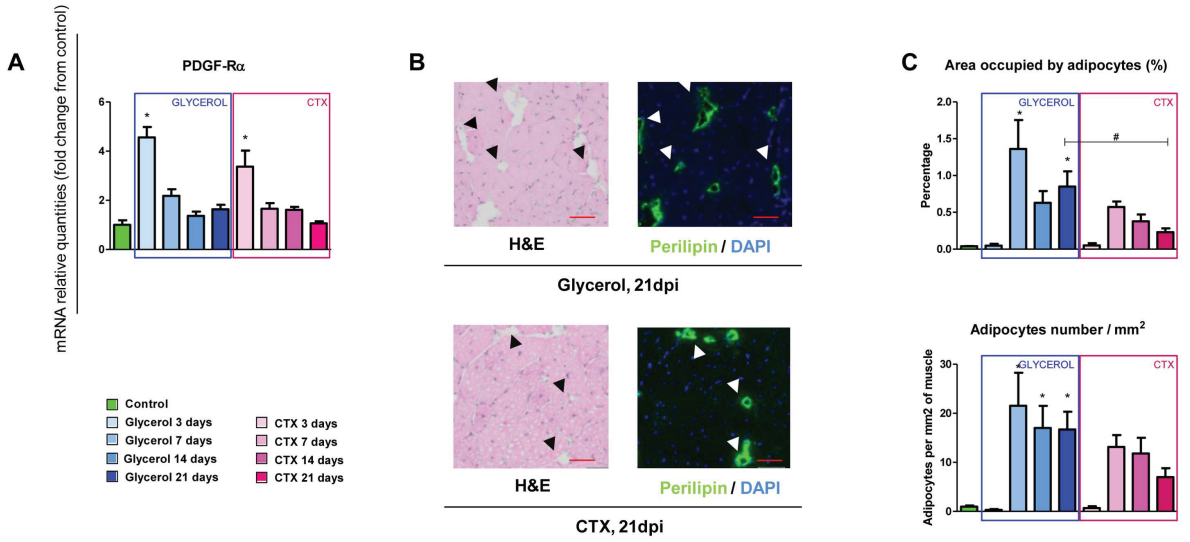


Figure 2. Ectopic adipogenesis occurs in both glycerol- and CTX-induced muscle regeneration. (A) qPCR analysis of the mRNA level of the platelet-derived growth factor receptor alpha (PDGFRα). (B) Cryosections were performed at the mid-belly part of TA and subjected to H&E and perilipin staining at each time points after injection. Representative perilipin (green) /DAPI (blue) fluorescent stainings at 21 dpi are shown next to an H&E staining of the same region. Scale bars, 50 μm. (C), Quantitative analysis of perilipin expression assessed by counting and measuring the area of all perilipin expressing cells per section. Data are expressed as mean ± s.e.m., n = 5–6/group. * p-value <0.05 *vs.* control, # p-value <0.05 in Glycerol *vs.* CTX at same time points.
doi:10.1371/journal.pone.0071084.g002

Muscle injury and muscle preparation

25 μl of 50% v/v glycerol or 25 μl of 10 μM cardiotoxin (CTX) was injected through two injections of 12.5 μl into tibialis anterior (TA) muscle, using a 22 gauge needle (Hamilton). The intramuscular injections were performed under anesthesia using isoflurane inhalation and mice were injected intra-peritoneally with 0.1 mg/kg of Buprenorphine (Temgesic), one hour before injury and the day after, as analgesic. Mice were sacrificed at the indicated time points with CO_2 (air mixture 85:15) in an inhalation chamber. TA muscles were cut into two parts, one part being frozen into liquid nitrogen for total RNA extraction and the other part being embedded into OCT, frozen for 2 seconds in liquid nitrogen and then frozen in isopentane cooled with liquid nitrogen for histological analysis.

Immunohistochemistry and microscopy

Frozen muscle tissues were sectioned (10 μm thickness) using a cryostat HM 560 (Microm-Thermo Fisher Scientific Inc). Muscle sections were subjected to hematoxylin-eosin (H&E) staining and the slides were mounted in Pertex (Histolab). Immunostainings were performed using polyclonal antibodies against perilipin or laminin detected using goat anti-rabbit IgG antibodies conjugated to Alexa 488 or 555 (Molecular Probes), respectively. For perilipin immunostaining, cryosections were allowed to dry during 10 minutes, and fixed in PFA 4% during 10 minutes. After 2 quick washes in PBS, samples were permeabilized during 10 minutes in 0.5% Triton X-100 (Sigma-Aldrich) in PBS (0.5% PBTX), and blocked for 1 hour at room temperature in blocking solution (2% Goat Serum (Sigma-Aldrich) in 0.5% PBTX). Cryosections were stained during 3 hours at room temperature using monoclonal anti-perilipin A/B antibody produced in rabbit (Sigma-Aldrich) diluted at 1/250 in the block solution. After 3 washes of 5 minutes in PBS, slides were incubated during 1 hour at room temperature with the secondary antibody diluted at 1/200 in the blocking solutions. Slides were washed again, and mounted in Prolong Gold Medium with DAPI (Invitrogen). For laminin immunostaining, cryosections were allowed to dry during 10 minutes and were permeabilized during 10 minutes in PBS and blocked for 45 minutes at room temperature in the blocking solution (10% Goat Serum (Sigma-Aldrich), 1% bovine serum albumin (BSA) (Sigma) in 0,5% PBTX). Cryosections were stained during 3 hours at room temperature using monoclonal anti-Laminin antibody produced in rabbit (Sigma-Aldrich) diluted at 1/200 in the blocking solution. After 3 washes of 10 minutes in PBS, slides were incubated during 1 hour at room temperature with secondary antibody diluted at 1/200 in the blocking solutions. Slides were washed again, and mounted in Prolong Gold Medium with DAPI (Invitrogen). Stained tissues were photographed using Olympus VS120 Virtual Microscopy Slide Scanning System and analyzed using the VS-ASW FL software measurement tools.

Image analysis

The number of myofibers with central nuclei was calculated from laminin/DAPI stainings on all fibers of the section using an automated image processing software developed internally (Astoria). Cells expressing perilipin and their surface were counted manually on the whole cryosection.

RNA extraction & preparation for profiling

Total RNA was extracted from the frozen tibialis part, using an RNeasy Fibrous Tissue Mini Kit (Qiagen), and eluted in 40 μl RNase free water. RNA concentration and quality was measured by a ND-1000 spectrophotometer (NanoDrop, Thermo Scientific). In case RNA quality was not acceptable (ratio 260/230<1.5), samples were cleaned using a RNeasy Plus Micro Kit (Qiagen), eluted in 15 μl and the new concentration and ratios measured again. RNA final quality was assessed on an Agilent 2100

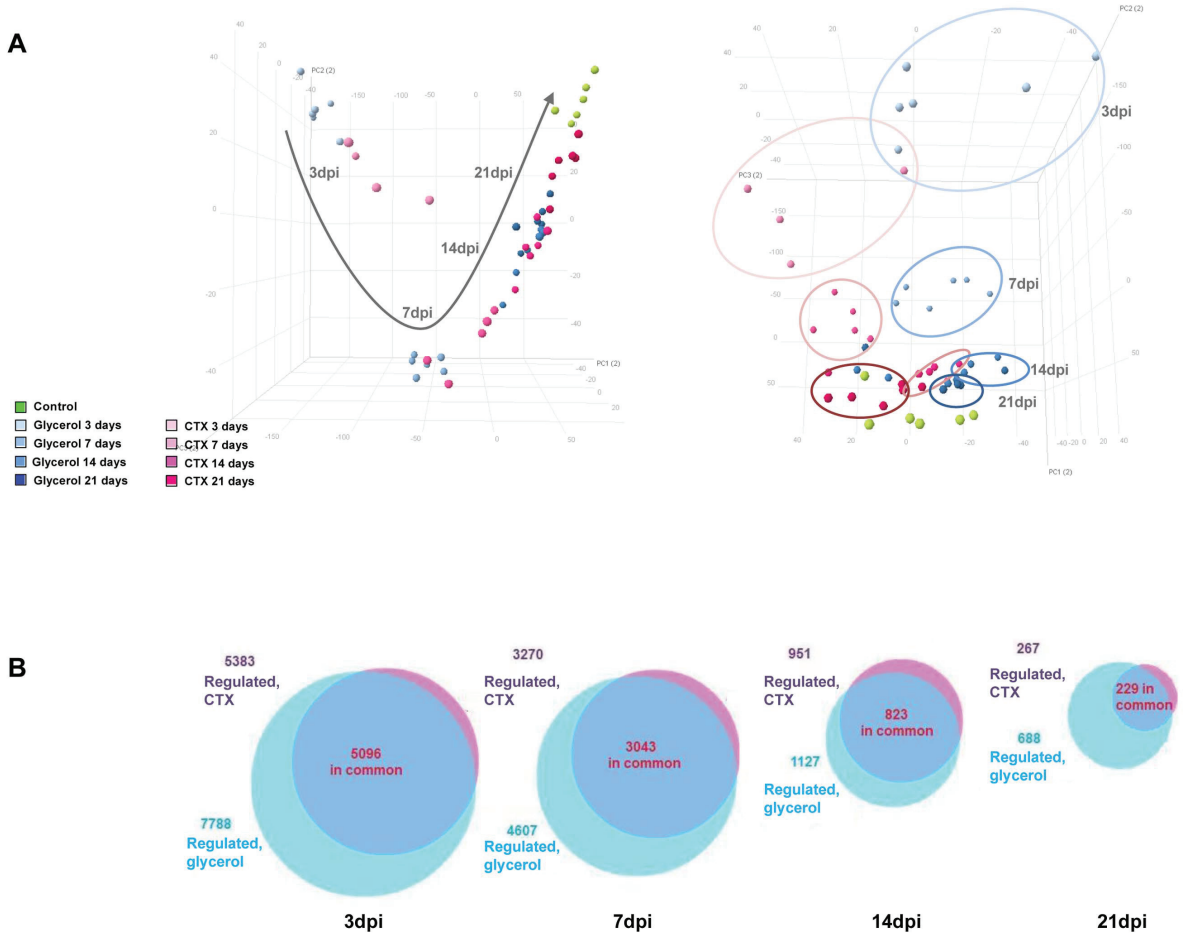


Figure 3. Overview of transcriptomic analysis showing time-dependent genome wide regulation. (A) Principal component analysis diagram showing most variation between time points along the first principal component axis (left graph), and differentiating between models along the third principal component axis (right graph). (B) Venn diagrams comparing the number of regulated genes in the glycerol and CTX models *vs.* control at 3 dpi, 7 dpi, 14 dpi and 21 dpi time points. Gene expression changes were considered significant when the absolute Fold Change >2 and adjusted p-value <0.01.
doi:10.1371/journal.pone.0071084.g003

Bioanalyzer using the Agilent RNA 6000 Nano kit, and processed for microarray when RIN>7.

Microarray processing and data analysis

RNA samples were subjected to microarray analysis on Affymetrix GeneChip Mouse Genome 430 2.0 chips (Affymetrix, Santa Clara, CA) according to the manufacturer's recommendations. All statistical analyses were performed using R/Bioconductor (www.bioconductor.org). Quality control was performed using both AffyQCreport and arrayQCmetrics packages. Data was RMA normalized using RMA and scaled to a 2% trimmed mean of 150. Probes with normalized expression values below 50 in all groups were filtered out. Differential gene expression was performed using a linear model approach (Limma). Genes with a fold change higher than 2 and an adjusted P-value below 0.01 (Benjamini and Hochberg multiple testing correction) were considered regulated. Venn diagrams were drawn using the BioVenn online tool (http://www.cmbi.ru.nl/cdd/biovenn/) [28]. Gene set enrichment analysis (GSEA) [29], was performed on fold change ranked list of all non filtered probesets collapsed to gene symbols [30] using the Broad (www.broadinstitute.org/gsea) and the Molecular Signature v3.0 gene sets databases. GSEA results were further analyzed using the enrichment map tool [31] and visualized in Cytoscape [32]. Other visualizations were performed using Tibco Spotfire. Pathway maps were generated using Pathvisio [33]. Data was submitted to the Gene Expression Omnibus repository and is available under the accession number GSE45577.

Reverse Transcription and qPCR

Total RNA extracts were diluted at 100 ng/µl and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and then diluted 1/20. Quantitative PCR reactions were performed on a BioRad thermocycler (association of CFX384^{TM} Real-Time System and C1000^{TM} Thermal Cycler) with HotGoldStar DNA polymerase (qPCR Master Mix Plus, Eurogentec). The amplification curves were analyzed by the BioRad CFX Manager software. Specific

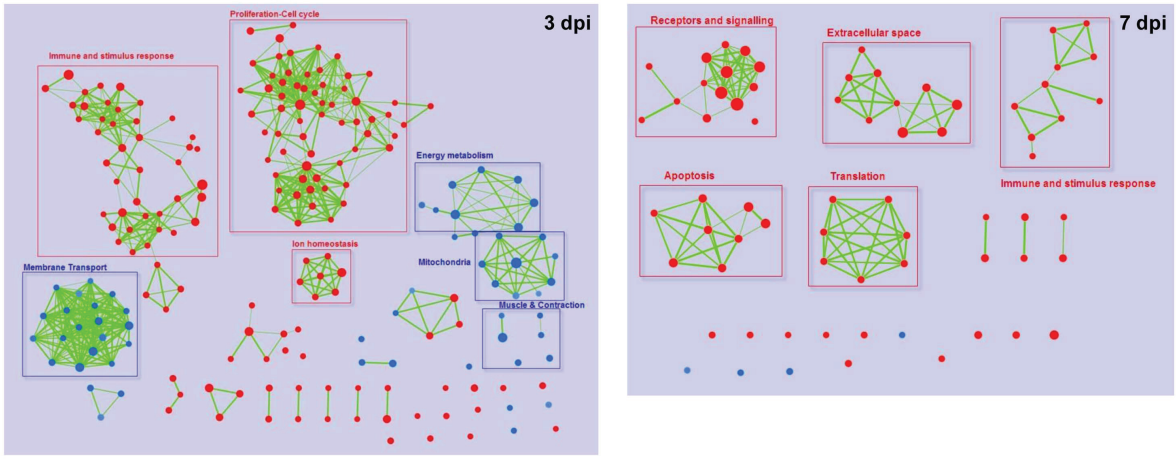


Figure 4. Gene set enrichment mapping of glycerol- *vs.* CTX-injected muscle. Gene set enrichment analysis was performed on glycerol-injected compared to CTX-injected muscles 3 and 7 days after injection, and clustered according to gene set ontology. The size of nodes is proportional to the number of genes contained in the gene set. Red nodes: gene sets upregulated in glycerol *vs.* CTX model, blue nodes: gene sest downregulated in glycerol *vs.* CTX model, green bar: link between two gene sets sharing regulated genes.
doi:10.1371/journal.pone.0071084.g004

Taqman probes (Applied Biosystems by Life Technologies) are listed in Table S1. 18 S was amplified as qPCR normalization control.

Statistical Analysis of qPCR and histology data

Statistical significance was assessed by the Student's t-test for binary comparisons. For comparison of more than 2 groups, one-way ANOVA followed by Bartlett's test was used. All data are expressed as mean value +/− s.e.m.; and unless otherwise indicated, $n=6$ in each CTX and glycerol group; and $n=5$ in the control group were analyzed. A p-value smaller than 5% was considered statistically significant.

Results

Glycerol and CTX induce similar myofiber damage and degeneration, followed by rapid muscle regeneration

In order to compare the molecular profiles of muscle regeneration after glycerol and CTX injection, the Tibialis Anterior muscle of adult wild-type mice was injected with 25 µl of 50% glycerol or 10 µM CTX and compared to a control muscle 3, 7, 14 or 21 days after injection. The dose of glycerol was selected from a pilot study showing that 25 µl of 50% glycerol was able to induce levels of myofiber damage in a slightly lower, yet comparable range than our established model of CTX-induced degeneration (figure S1) [34].

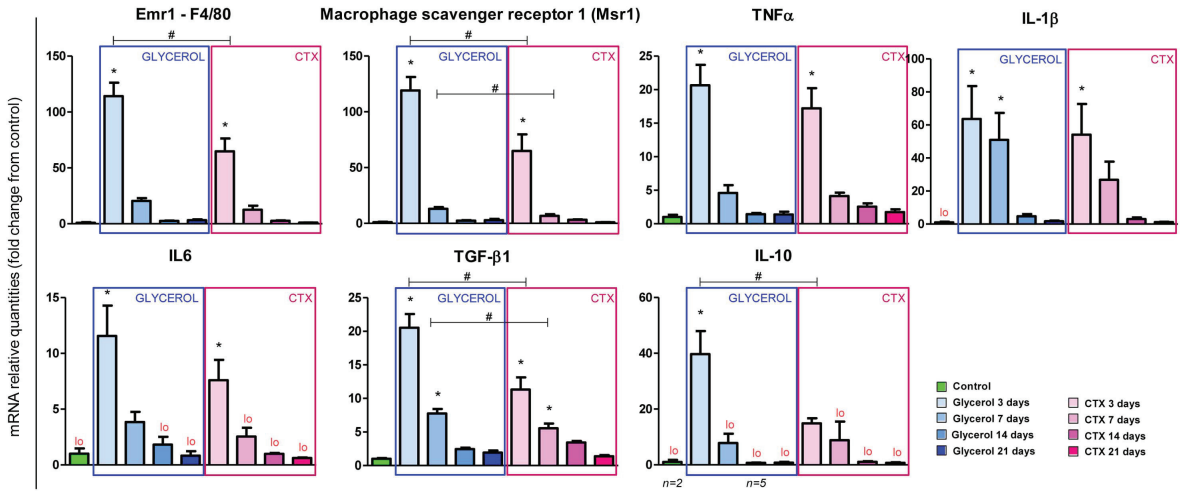
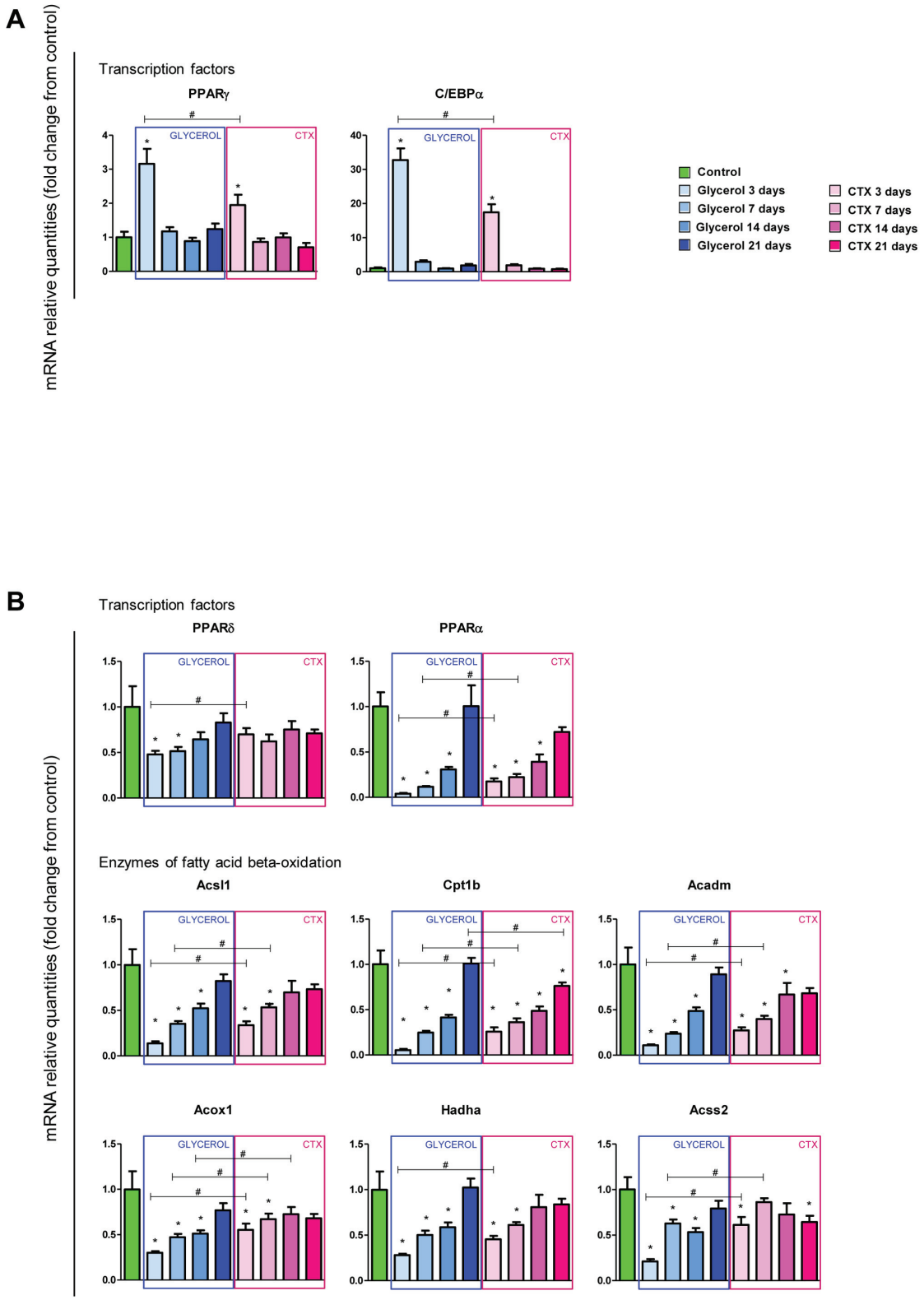


Figure 5. The inflammatory signature is stronger in response to glycerol than to CTX. qPCR analysis of the mRNA levels of various macrophage markers and cytokines. Data are expressed as mean ± s.e.m., n = 5–6/group. * p-value <0.05 *vs.* control, # p-value <0.05 in Glycerol *vs.* CTX at same time points. Emr1; EGF-like module containing mucin-like hormone receptor 1; TNFα, tumor necrosis factor alpha, IL, interleukin; TGF-β1, transforming growth factor beta 1.
doi:10.1371/journal.pone.0071084.g005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mRNA relative quantities (fold change from control)
Transcription factors
PPARγ
C/EBPα
GLYCEROL
CTX
Control
Glycerol 3 days
Glycerol 7 days
Glycerol 14 days
Glycerol 21 days
CTX 3 days
CTX 7 days
CTX 14 days
CTX 21 days
B
mRNA relative quantities (fold change from control)
Transcription factors
PPARδ
PPARα
Enzymes of fatty acid beta-oxidation
Acsl1
Cpt1b
Acadm
Acox1
Hadha
Acss2

Figure 6. Adipogenesis and β-oxidation are differentially regulated in muscle after glycerol or CTX injection. qPCR analysis of the mRNA levels of different adipogenic (A), or in fatty-acid oxidation (B) regulators. Data are expressed as mean ± s.e.m., n = 5–6/group. * p-value <0.05 *vs*. control, # p-value <0.05 in Glycerol *vs*. CTX at same time points. Acadm, acyl-CoA dehydrogenase medium; Acs/l, acyl-CoA synthesase short-/long-chain; Acss, Acetyl-coenzyme A synthetase; Acox, Acyl-coenzyme A oxidase, Palmitoyl; C/EBP: CCAAT/ Enhancer binding protein; Cpt, carnitine palmitoyltransferase; Hadh, hydroxyacyl-CoA dehydrogenase; PPAR, peroxisome proliferator activated receptor.
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As expected, the control muscle was composed of mature differentiated myofibers with multiple nuclei lying exclusively at the periphery of the cell (figure 1A). Both glycerol and CTX injection lead to a complete destruction of myofibers in the degenerating zones at 3 dpi, characterized by a dramatic reduction of the number of myofibers concomitant with an important infiltration of immune cells. Consequently, myofibers started regenerating 7 days post injection in both models, as many newly formed fibers with centrally located nuclei were observed, and the gradual regeneration of myofibers was almost complete after 3 weeks in both models. At 21 dpi, a differential process could be observed between the two models as glycerol-treated mice had much more cellular structures devoid of eosin-positive cytoplasm, which are reminiscent of mature white adipocytes containing triglycerides in a large lipid droplet (figure 1B). In addition, the regeneration capacity seemed slightly better with CTX than with glycerol as the number of regenerating fibers with centralized nuclei increased more rapidly and the total number of fibers recovered more rapidly (figure 1C).

When the molecular profiles of key markers of muscle function and regeneration were measured at the mRNA level, both models initially induced a profound loss of myosin heavy chain IIB (MYH4), which gradually recovered during the regenerative process over 14 to 21 days (figure 1D). As expected, both CTX and glycerol induced Pax7 expression at 3 dpi when satellite cells proliferate in response to muscle injury, and Pax7 levels gradually decreased during regeneration as the new pool of satellite cells engaged in myogenic differentiation and lost the stem cell marker Pax7. Consequently, the downstream myogenic transcription factors MyoD, Myf5 and Myogenin were also strongly induced at 3–7 dpi, but neither Pax7, nor MyoD/Myf5/Myogenin showed differential regulation in CTX vs. glycerol-injected muscles. The embryonic myosin heavy chain MYH8 was also strongly activated at 7 dpi in both models as muscle regeneration transiently re-activates embryonic myogenic programs occurring during development [4,35]. Altogether, these molecular profiles demonstrate that CTX and glycerol induce similar satellite cell activation and myogenic differentiation.

In vivo ectopic adipogenesis is detected in both models of regeneration but with a stronger amplitude and persistence after glycerol injury

In order to study ectopic adipogenesis in the two models of muscle regeneration, we first evaluated the expression of the PDGFRα, a marker which is specifically expressed by fibro/ adipogenic progenitors (FAPs) [17]. As previously reported [15,17,20], PDGFRα mRNA levels increased 3 days after injection of both glycerol and CTX, and returned to the basal expression level at 7 dpi (figure 2A), confirming that FAP activation and proliferation occurs at similar levels after glycerol and CTX injection. Adipogenic differentiation of FAPs was then evaluated through a perilipin staining which labels lipid droplets and allows outlining ectopic adipocytes (figure 2B). As previously reported [16,17,36], perilipin expression was detected in the interstitial spaces of muscles treated with glycerol and corresponded to cellular structures devoid of eosin-positive cytoplasm in H&E sections. Surprisingly, ectopic adipocytes also formed during regeneration after CTX injury, suggesting that a transient adipogenic response during muscle regeneration might be more frequent than previously appreciated [17]. Most adipocytes appeared 7 days after glycerol and CTX injections at a similar level, and then slowly decreased in number during muscle regeneration (figure 2C). However, the adipocytes that differentiated in muscles injected with glycerol were generally bigger and persisted for a longer time than in muscles injected with CTX in which the number of fat cells decreased after 7 dpi.

Glycerol injection regulates more genes than CTX at the genome wide level

In order to understand the molecular mechanisms of regeneration and ectopic adipogenesis, we performed a differential profiling of the glycerol and CTX models at the 3, 7, 14 and 21 dpi time points using Affymetrix expression arrays. The numerous variables contributing to inter-sample variations, averaged over all the genes analyzed were reduced through a principal component analysis (PCA) to three variables that account for the major sources of variation in the dataset. Time after injury was the predominant variable, as samples were grouped by time point in the first dimension of the PCA diagram without any distinction between the glycerol and CTX model (figure 3A). The most distant samples in the PCA diagram were the 3 dpi samples, indicating that both glycerol and CTX injections induce a major gene expression change at 3 dpi which likely reflects the massive physiological events and differences in cell-type composition occurring during the first days after injury. Consistently, the number of regulated genes was maximal at 3 dpi (5000–8000 genes, figure 3B). During the regeneration process, samples moved in the PCA space towards the control group while the number of regulated genes gradually decreased down to 250–700 regulated genes at 21 dpi, demonstrating that damaged muscles gradually return to normal physiological conditions within the 21 days of repair. In addition to the prominent effect of time on gene expression patterns, samples were separated according to model (*i.e.* Glycerol *vs*. CTX) along the 2nd and 3rd component of the PCA (figure 3A). The vast majority of genes were commonly regulated in both models, but glycerol also induced a specific set of genes while very little genes were specifically regulated by CTX (figure 3B, Table S2). Altogether, these data demonstrate that glycerol and CTX regulate similar pathways during regeneration for which time after injury is the key factor. However, glycerol also triggers a specific set of genes which likely accounts for additional biological processes.

Glycerol specifically triggers a stronger expression of anti-inflammatory cytokines and activates adipogenic networks while repressing fatty acid oxidation

In order to assess the main biological functions of the numerous genes regulated in muscle after glycerol and CTX-injections, we performed a Gene Set Enrichment Analysis (GSEA) for the 3 dpi and 7 dpi time points, and analyzed the different sets of genes that were specifically enriched in glycerol- *vs*. CTX-treated muscles. The significantly enriched genesets were analyzed using the enrichment map tool and plugged into Cytoscape, a platform used to visualize complex networks with integration of p-value data.

Using this approach, genesets sharing a significant number of genes are connected, generating a summarized network view of the specific biological processes that are differentially regulated between the 2 conditions (figure 4). Consistent with observations at the individual gene level, more gene sets were differentially regulated between glycerol- and CTX-injected muscles at 3 dpi than at 7 dpi, demonstrating that the differential responses of muscles upon glycerol- and CTX-induced injuries initiate early on during the regenerative process.

The strong regulation of sets of genes involved in immune and stimulus response (figure 4) was consistent with the crucial role of inflammation in the muscle regeneration process [4,6]. Indeed, 3 days after glycerol injection, muscles were characterized by a stronger signature for immune cells and proliferation/cell cycle than after CTX injection, and the differences between both models were partially (immune cell gene sets) or totally (proliferation gene sets) attenuated at 7 dpi (figure 4).

The importance of the sequential involvement of pro-inflammatory macrophages (or M1) and anti-inflammatory macrophages (or M2) for muscle repair after an injury is well established [7,8]. In order to investigate if the inflammatory response is differentially involved in both models after injury, we further analyzed inflammatory molecular signatures in muscle using qPCR. As expected, the mRNA levels of macrophage markers, such as Emr1/F4/80, which is also expressed in eosinophils [37], and the macrophage scavenger receptor MSR-1 massively increased at 3 dpi, and then rapidly decreased at 7 dpi (figure 5A). Interestingly, Emr-1 and MSR-1 were more strongly induced by glycerol than CTX with a 115–120-fold increase in the glycerol model and only 65-fold increase in the CTX model, suggesting that macrophages could trigger a stronger inflammatory response to glycerol injury and thereby potentially exacerbate glycerol-induced adipogenesis. We also analyzed different cytokine expression profiles (figure 5A). As expected, both pro-inflammatory cytokines such as the tumor necrosis factor alpha (TNFα), the interleukins 1 beta (IL-1β) and 6 (IL6) and anti-inflammatory cytokines such as the transforming growth factor beta 1 (TGF-β1) and the interleukin 10 (IL10) were detected in muscle 3 days after injection of glycerol and CTX, potentially resulting from an active presence of M1 and M2 macrophages in injected muscles. However, no IL-4/IL-13 signal was detected in muscles at the mRNA levels (results not shown). The similar expression of pro-inflammatory cytokines in muscles at 3 and 7 dpi in both models demonstrates that both models trigger a similar early inflammatory response. In contrast, glycerol-injected muscles had a stronger anti-inflammatory cytokine mRNA expression as TGF- β1 and IL-10 inductions were approximately 2 fold higher in response to glycerol *vs.* CTX, suggesting that glycerol may elicit stronger M2 macrophage activation. These cytokine mRNA profiles data illustrate that glycerol induces a higher expression of anti-inflammatory cytokines a few days after injury. These results likely illustrate a different extent of inflammatory response between both models, which could influence the cytokine balance and confound signals sent to satellite cells, myoblasts and adipocyte progenitors, and possibly influence adipogenesis.

Many gene sets involved in muscle contractile function, ion and metabolite transport (membrane transport gene sets), and muscle metabolism were down regulated in glycerol- *vs.* CTX-injected muscles 3 days after injection (figure 4), indicating a stronger molecular signature for muscle and metabolic impairment in the glycerol model. To further characterize the difference between both muscle regeneration models at the molecular level, we performed a pathway analysis of the genes specifically or differentially regulated by glycerol, which highlighted that adipogenesis and fatty acid oxidation are differentially regulated by glycerol and CTX at 3 dpi (figures S2, S3 and S4). The most prominent findings were confirmed by qPCR at the single gene level. Among these, the Peroxisome Proliferator Activated Receptors (PPARs) were preferentially regulated in the glycerol model. PPARγ, the master adipogenic regulator [38,39], was strongly over-expressed at 3 dpi in glycerol- *vs.* CTX-injected muscles with a 3.2-fold increase in the glycerol model vs 1.6-fold increase only in response to CTX, suggesting a prominent engagement of glycerol-activated adipocyte progenitors in the adipogenic program (figure 6A). Concomitantly, the transcription factor CCAAT/enhancer binding protein α (C/EBPα) was also more strongly activated by glycerol than CTX at 3 dpi (32- *vs.* 17-fold activation, respectively). As adipogenic differentiation is controlled by a transcriptional cascade which relies extensively on PPARγ and C/EBPα [38–40], we analyzed downstream functional adipogenic markers.

In contrast, both PPARα and PPARβ/δ, which are prominently expressed in skeletal muscle and control the expression of genes involved in fatty acid catabolism [41,42], were down-regulated in glycerol- *vs.* CTX-injected muscles (figure 6B, figures S2, S3, S4). In particular, the acyl-CoA synthesase long-chain 1 and short-chain 2, carnitine palmitoyltransferase 1, acyl-CoA, dehydrogenase medium, acyl-coA oxidase 1 and hydroxyacyl-CoA dehydrogenase were dramatically down-regulated 3 days after injection. Their expression gradually increased during muscle regeneration, and muscles almost recovered the basal mRNA level 21 days after injury. Interestingly, glycerol induced a stronger down-regulation of all 6 genes than CTX with a decrease of 70-95%, whereas CTX induced a decrease of only 40–75%, illustrating a differential metabolic shift occurring in response to glycerol or CTX injection.

Altogether, the microarray-based expression data and subsequent qPCR validation confirm that glycerol induces stronger adipogenic differentiation which may relate to the stronger up-regulation of some cytokines and immune cell markers in glycerol injected muscles, and to the increased expression of adipogenic regulators concomitant with a down-regulation of genes controlling fatty acid oxidation. The data also highlight that other biological processes which are differentially regulated in glycerol vs. CTX model at the mRNA level, such as immune response, cell proliferation and muscle metabolism, may also influence ectopic adipogenesis during muscle regeneration.

Discussion

Intramuscular adipogenesis has emerged as a physiopathological condition of growing interest, due to the correlation between ectopic fat in skeletal muscle and the reduction of muscle function and metabolic homeostasis [43–45]. In the present study, we have compared the molecular and cellular processes occuring during glycerol- and CTX-induced regeneration. Both glycerol and CTX induce acute muscle degeneration followed by efficient muscle repair with similar time courses, when assessed at the histological level or by the Principal Component Analysis (PCA) of the global gene expression changes measured in both models. Consistent with the well described mechanisms of muscle regeneration [4,6,46], both models induced the transient loss of adult myosin heavy chains (MYH), which recovered after temporary compensation by embryonic MYH. Satellite cell activation also lead to similar activation of the myogenic trasncription factors MyoD1, Myf5, Myogenin. Despite similar or slightly lower levels of degeneration at the histological level in glycerol than CTX-injected muscle, gene set enrichment analysis suggested that the molecular and cellular remodeling during degeneration could be stronger upon glycerol

injection. Although the basis to this observation remains unclear, one possibility is that glycerol and CTX may differentially damage the cell membrane and the intracellular components.

Several studies have shown that PDGFRα-positive fibro adipogenic progenitors (FAPs) proliferate upon muscle injury and differentiate into ectopic adipocytes in muscle under certain physio-pathological conditions [20,21,47]. As previously reported [16,17], glycerol-induced regeneration promoted FAP proliferation and ectopic adipocyte formation in muscle in our experiments. Previous studies have shown that adipogenic potential, and more recently FAP proliferation, are induced in various models of muscle injury [17,48]. It was, however, suggested that the muscle environment is permissive to terminal adipogenic differentiation in muscles injected with glycerol but not with CTX [17]. Consistently, we also observed that PDGFRα expression was transiently elevated 3 days after glycerol and CTX injection when the PDGFRα-positive fibro-adipogenic progenitors (FAPs) proliferate. However, our results demonstrate that CTX injection can still transiently prime muscle towards adipogenic commitment, albeit at a lower extent than glycerol. The transient adipogenic activation observed in both models is supported by an activation of the adipogenic transcription factors PPARγ and C/EBPα, limited to the first few days post injury, and the appearance of perilipin-positive cells in muscle. These adipocytes are larger and more persistent in the glycerol than in the CTX model, as the number of perilipin-expressing cells and the fat area gradually decrease in the CTX model during muscle regeneration. Upon muscle injury, activated FAPs express high levels of IL-6 and co-culture experiments have demonstrated that myogenic differentiation was more efficient in the presence of FAPs [20]. Since we observed an adipogenic response in the two regeneration models tested, it is possible that ectopic adipogenesis may be a hallmark of muscle regeneration that could be required for efficient muscle repair.

At the molecular level, glycerol induced a wider genomic signature than CTX, which most likely accounts for the induction of a stronger adipogenic commitment. Gene set enrichment analysis also revealed the exacerbated regulation of various biological processes by glycerol, which was particularly prominent at early time points. Gene sets involved in proliferation/cell cycle and immune response were strongly enriched 3 days after glycerol injection, illustrating that the cell proliferation required to induce the early steps of tissue repair is stronger after glycerol injection and may participate to stronger ectopic adipogenesis. In particular, glycerol-injected muscles were characterized by a stronger anti-inflammatory signature defined by increased TFG-β1 and IL-10 levels, suggesting that the M1 to M2 macrophage transition occurring during regeneration may differ in the two models and thereby differentially affect adipogenesis. Finally, a differential regulation of metabolic pathways also very likely contributes to the stronger adipogenic response induced by glycerol. In particular, gene sets involved in energy metabolism and mitochondrial function, and many enzymes of fatty acid β-oxidation pathways were down-regulated in glycerol-injected muscles. In contrast, a stronger adipogenic signature was prominent in glycerol over CTX muscle. These results therefore strongly suggest that a shift in the balance between fatty acid storage and utilization contributes to stronger ectopic adipogenesis in glycerol-injected muscle. Interestingly, a molecular pathway analysis demonstrated that PPARs could account for the transcriptional integration of this orchestrated metabolic response. PPARγ, the master regulator of adipogenic differentiation [39], was more strongly regulated by glycerol than CTX. In contrast, PPARα and PPARβ/δ, the PPARs primarily involved fatty acid catabolism [41,42], were down regulated in glycerol- *vs.* CTX-injured muscles.

Altogether, our data demonstrate that transient adipogenic activation is an integral response of skeletal muscle regeneration which is differentially modulated according to physio-pathological conditions through inflammatory and metabolic cues. In addition, these results also provide a comprehensive transcriptomic resource of the genes commonly or differentially regulated during muscle regeneration and ectopic muscle adipogenesis which will likely turn useful for further characterization of these processes.

Supporting Information

Figure S1 Effect of glycerol dosage on muscle degeneration. Control uninjured tibialis anterior muscle, and tibialis anterior muscles injected with 25 µl 25% (v/v), 25 µl 50% (v/v) or 50 µl 50% (v/v) glycerol, or 25 µl 10 µM CTX were sectioned and stained with laminin and DAPI, 3 days after injection (dpi). The total area occupied by laminin-surrounded fibers was measured by histomorphometry.
(PDF)

Figure S2 Differential regulation of fatty acid beta oxidation pathway in glycerol *vs.* control models. Relative gene expression in glycerol-injected muscles vs. control muscles was mapped on Wikipathways. Colors represent $\log_2$ of Fold Change (logFC); blue, $-2<\text{logFC}<0$; red, $0<\text{logFC}<2$, blue and red intensity increases with the amplitude of regulation. Each rectangle represents a probe and is separated into 4 sections, describing the fold change values at 3, 7, 14 and 21 dpi as indicated in the legend. Lpl, lipo-protein lipase; Acs/l, acyl-CoA synthesase short-/long-chain; Acad, acyl-CoA dehydrogenase; Hadh, hydroxyacyl-CoA dehydrogenase; Gyk&Gk2, glycerol kinases; Gpd2, mitochondrial gylcerol 3-phosphate dehydrogenase 2; Tpi1, triosephosphate isomerase 1; Crat, carnitine O-acyltransferase; Cpt, carnitine palmitoyltransferase; Chkb, choline kinase β; Slc25a20, solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase); member 20.
(PDF)

Figure S3 Differential regulation of fatty acid beta oxidation pathway in CTX *vs.* control models. Relative gene expression in CTX-injected muscles vs. control muscles was mapped on Wikipathways. Colors represent $\log_2$ of Fold Change (logFC); blue, $-2<\text{logFC}<0$; red, $0<\text{logFC}<2$, blue and red intensity increases with the amplitude of regulation. Each rectangle represents a probe and is separated into 4 sections, describing the fold change values at 3, 7, 14 and 21 dpi as indicated in the legend. Lpl, lipo-protein lipase; Acs/l, acyl-CoA synthesase short-/long-chain; Acad, acyl-CoA dehydrogenase; Hadh, hydroxyacyl-CoA dehydrogenase; Gyk&Gk2, glycerol kinases; Gpd2, mitochondrial gylcerol 3-phosphate dehydrogenase 2; Tpi1, triosephosphate isomerase 1; Crat, carnitine O-acyltransferase; Cpt, carnitine palmitoyltransferase; Chkb, choline kinase β; Slc25a20, solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase); member 20.
(PDF)

Figure S4 Differential regulation of fatty acid beta oxidation pathway in glycerol *vs.* CTX models. Relative gene expression in glycerol-injected muscles vs. CTX-injected muscles was mapped on Wikipathways. Colors represent $\log_2$ of Fold Change (logFC); blue, $-2<\text{logFC}<0$; red, $0<\text{logFC}<2$, blue and red intensity increases with the amplitude of regulation. Each rectangle represents a probe and is separated into 4 sections, describing the fold change values at 3, 7, 14 and 21 dpi as indicated in the legend. Lpl, lipo-protein lipase; Acs/l, acyl-CoA synthesase short-/long-chain; Acad, acyl-CoA dehydrogenase; Hadh, hydroxyacyl-CoA dehydrogenase; Gyk&Gk2, glycerol

kinases; Gpd2, mitochondrial gylcerol 3-phosphate dehydrogenase 2; Tpi1, triosephosphate isomerase 1; Crat, carnitine O-acyltransferase; Cpt, carnitine palmitoyltransferase; Chkb, choline kinase β; Slc25a20, solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase); member 20.
(PDF)

Table S1 Reference of Taqman probes used for qPCR.
(DOC)

Table S2 List of regulated genes in the different models. Lists of genes are separated in the different tabs by positive or negative regulation and by time points in the different models (Glycerol *vs.* sham, CTX *vs.* Sham or Glycerol *vs.* CTX). Genes with a fold change higher than 2 and an adjusted P-value below 0.01 (Benjamini and Hochberg multiple testing correction) were considered regulated.
(XLS)

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Author Contributions

Conceived and designed the experiments: LL JNF ELT. Performed the experiments: LL EP. Analyzed the data: LL SB JNF. Contributed reagents/materials/analysis tools: EP SB. Wrote the paper: LL JNF.

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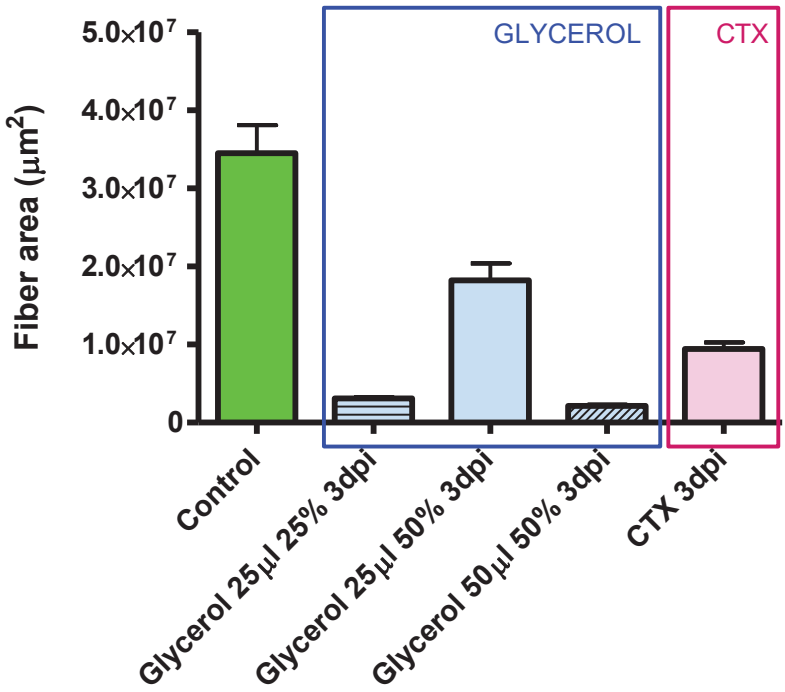
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CTX
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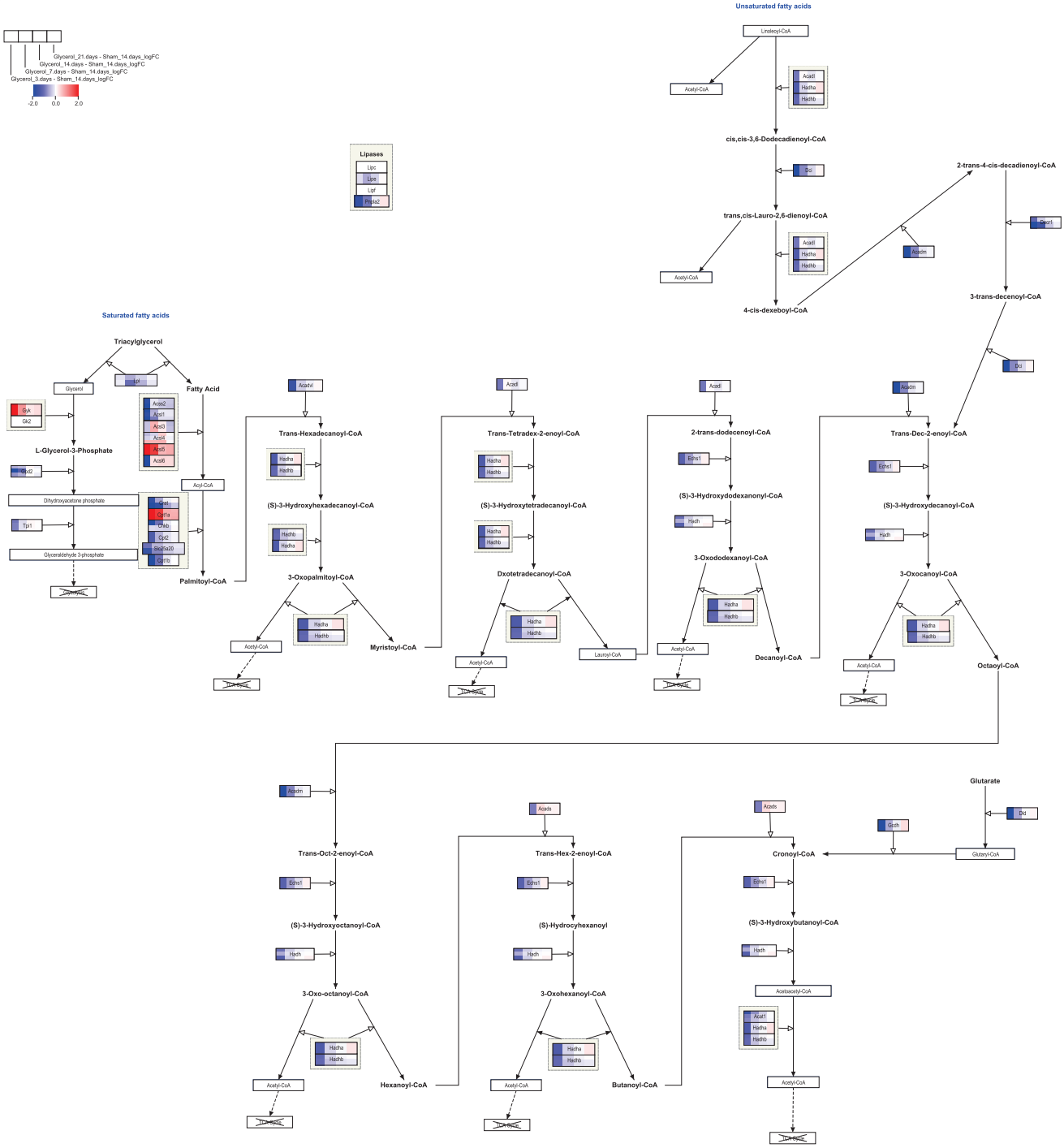
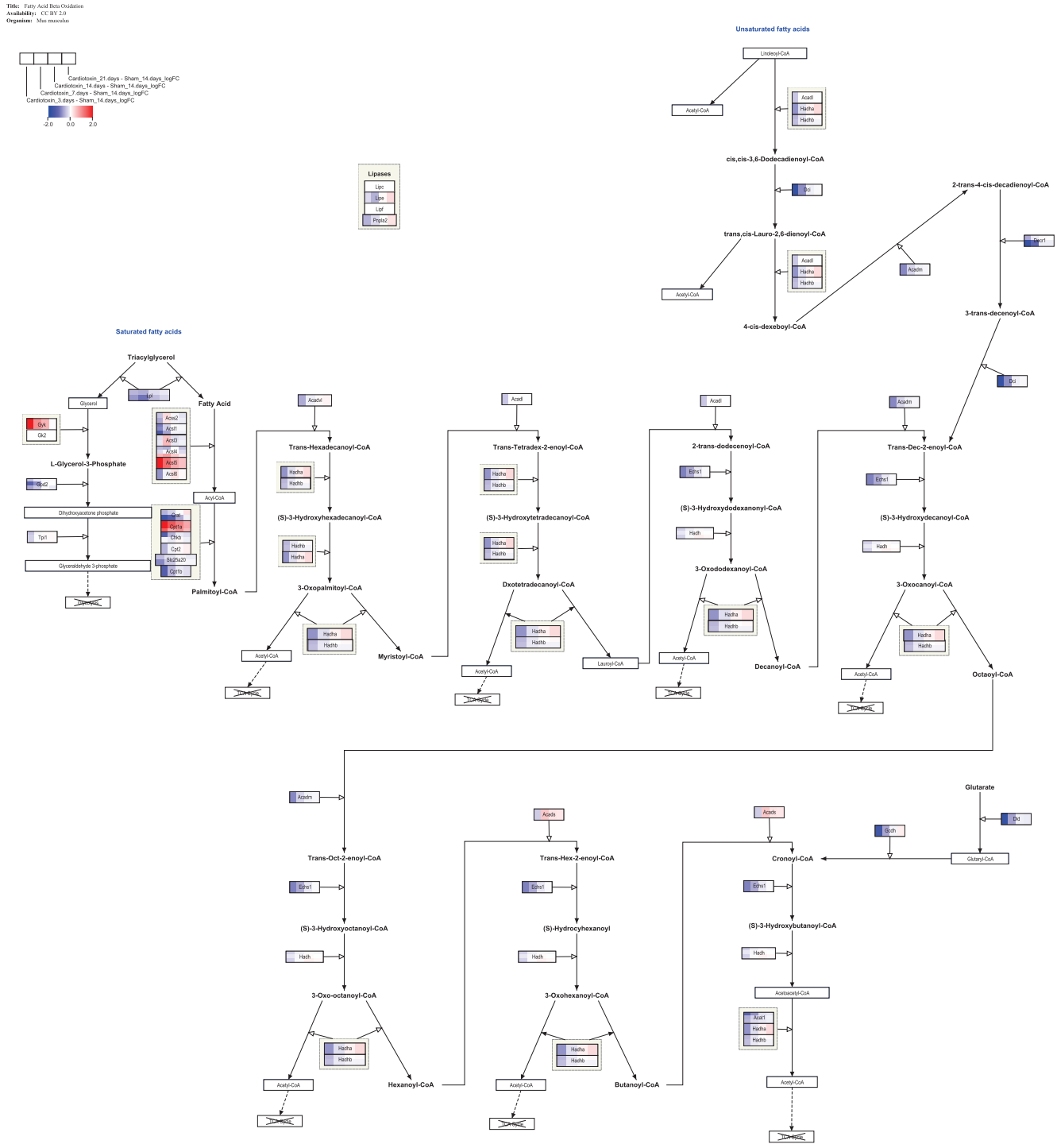
4.0×10⁷
3.0×10⁷
2.0×10⁷
1.0×10⁷
0
Control
Glycerol 25μl 25% 3dpi
Glycerol 25μl 50% 3dpi
Glycerol 50μl 50% 3dpi
CTX 3dpi

Title: Fatty Acid Beta Oxidation
Availability: CC BY 2.0
Organism: Mus musculus

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Availability: CC BY 2.0
Organism: Mus musculus
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Cardiotoxin_7.days - Sham_14.days_logFC
Cardiotoxin_3.days - Sham_14.days_logFC
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0.0
2.0
Lipases
Lpc
Lipe
Lipf
Pnpla2
Unsaturated fatty acids
Linoleoyl-CoA
Acetyl-CoA
Acadl
Hadha
Hadhb
cis,cis-3,6-Dodecadienoyl-CoA
Eci2
trans,cis-Lauro-2,6-dienoyl-CoA
4-cis-dexeboyl-CoA
2-trans-4-cis-decadienoyl-CoA
Decr1
Acadm
3-trans-decenoyl-CoA
Eci1
Saturated fatty acids
Triacylglycerol
Pnpla2
Glycerol
Fatty Acid
Gyk
Gk2
L-Glycerol-3-Phosphate
Gpd2
Dihydroxyacetone phosphate
Tpi1
Glyceraldehyde 3-phosphate
Glycolysis
Acsbg1
Acsl1
Acsl3
Acsl4
Acsl5
Acsl6
Acyl-CoA
Palmitoyl-CoA
Acadvl
Trans-Hexadecanoyl-CoA
(S)-3-Hydroxyhexadecanoyl-CoA
3-Oxopalmitoyl-CoA
Myristoyl-CoA
Trans-Tetradex-2-enoyl-CoA
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Dxotetradecanoyl-CoA
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Hexanoyl-CoA
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3-Oxohexanoyl-CoA
Butanoyl-CoA
Cronoyl-CoA
(S)-3-Hydroxybutanoyl-CoA
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Acat1
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Dld
Glcdh
Glutaryl-CoA

Figure S3. Fatty acid oxidation pathway in glycerol *vs.* CTX.

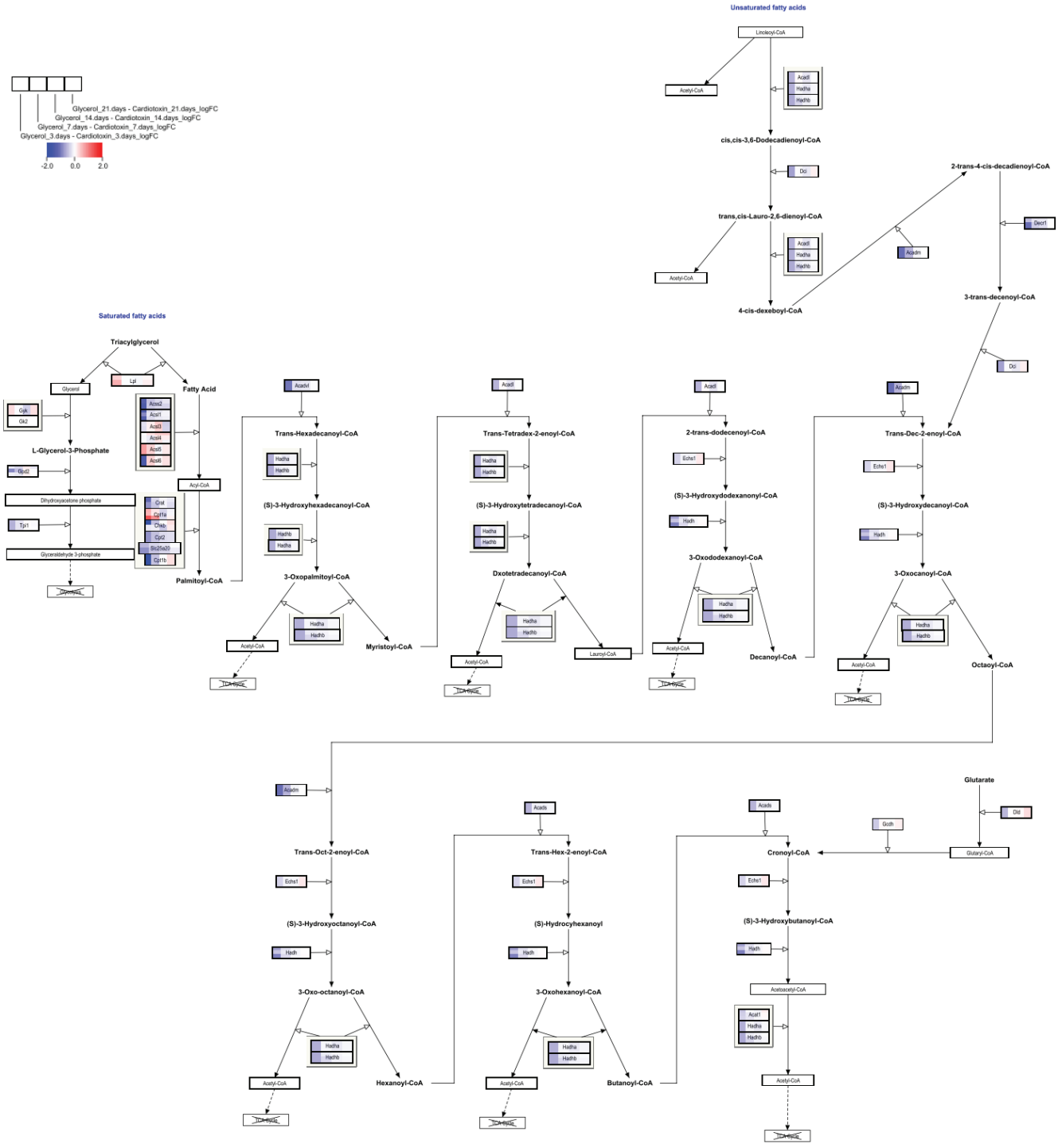

Table S1. Taqman® gene expression assays used (Applied Biosystems by Life Technologies).

Gene	Reference
18S rRNA	4319413E
Acadm	Mm01323360_g1
Adipoq	Mm00456425_m1
Acox1	Mm01246831_m1
Acsl1	Mm00484217_m1
Acss2	Mm00480101_m1
Cpt1b	Mm00487200_m1
C/EBPα	Mm01265914_s1
Ear5	Mm00658916_s1
Ednra	Mm01243722_m1
Emr1-F4/80	Mm00802529_m1
Exp	Mm00514768_m1
Fasn	Mm00662319_m1
Hadha	Mm00805228_m1
Hadhb	Mm01210656_m1
IL-1β	Mm01336189_m1
IL-4	Mm00445259_m1
IL-6	Mm04446190_m1
IL-10	Mm00439614_m1
IL-13	Mm00434204_m1
Msr1	Mm00446214_m1
Myf5	Mm00435125_m1
Myh4	Mm01332541_m1
Myh8	Mm01329494_m1
MyoD1	Mm00440387_m1
Myogenin	Mm00446194_m1
Pax7	Mm00834082_m1
PDGF-Rα	Mm00440701_m1
PPARα	Mm00440939_m1
PPARδ	Mm00803184_m1
PPARγ	Mm00440945_m1
Retn	Mm00445641_m1
TGF-β1	Mm01178820_m1
TNFα	Mm00443258_m1

CHAPTER IV. Loss of fibronectin from the aged stem cell niche affects the regenerative capacity of skeletal muscle in mice

Rationale

Numerous studies have explored intrinsic and extrinsic changes of satellite cells leading to their loss and functional decline with age (see **Chapter I**, section **V.4.**). Changes in the satellite cell environment during aging inhibits the production of appropriate signals required to enter the myogenic program in response to injury, or conversely enrich the niche in inhibitory signals (Chakkalakal et al., 2012, Conboy et al., 2005, Elabd et al., 2014, Carlson et al., 2008). Recent studies have also enlightened cell-autonomous intrinsic dysfunction of very old satellite accompanied by abnormally elevated pathways (such as p38α/β MAPK, JAK/STAT) (Bernet et al., 2014, Cosgrove et al., 2014, Price et al., 2014, Sousa-Victor et al., 2014, Tierney et al., 2014). Following the hypothesis according to which changes in stem cell niche could drive cell-autonomous satellite cell dysfunction with age, we aimed at understanding the local upstream triggers leading to loss of function in aged satellite cells. To this end, we interrogated in this chapter how aging affectsthe satellite cell structural and signaling micro-environment through the extracellular matrix.

Authors

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My contribution

I designed, performed and interpreted all the experiments on aged mice that identified the perturbed regulation of fibronectin and ECM with age (Fig. 1), and all the ex-vivo analyses of aging, FN treatment and molecular signaling in MuSC (Fig. 4/5) I also had a direct contribution in the design and interpretation of all other experiments reported and in writing the publication.

Publication

For Supplementary Tables 1, 2 and 3: See online (http://www.nature.com/nm/journal/v22/n8/full/nm.4126.html#supplementary-information)

Note

News and Views (Tierney and Sacco, 2016) and Previews (Rodgers, 2016) published about our work are provided in the **Appendices**.

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Loss of fibronectin from the aged stem cell niche affects the regenerative capacity of skeletal muscle in mice

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Age-related changes in the niche have long been postulated to impair the function of somatic stem cells. Here we demonstrate that the aged stem cell niche in skeletal muscle contains substantially reduced levels of fibronectin (FN), leading to detrimental consequences for the function and maintenance of muscle stem cells (MuSCs). Deletion of the gene encoding FN from young regenerating muscles replicates the aging phenotype and leads to a loss of MuSC numbers. By using an extracellular matrix (ECM) library screen and pathway profiling, we characterize FN as a preferred adhesion substrate for MuSCs and demonstrate that integrin-mediated signaling through focal adhesion kinase and the p38 mitogen-activated protein kinase pathway is strongly de-regulated in MuSCs from aged mice because of insufficient attachment to the niche. Reconstitution of FN levels in the aged niche remobilizes stem cells and restores youth-like muscle regeneration. Taken together, we identify the loss of stem cell adhesion to FN in the niche ECM as a previously unknown aging mechanism.

Extrinsic signals that originate in the immediate cellular environment, commonly known as the stem cell niche, are critical for the regulation of MuSCs[1]. Following injury, the stem cell niche in muscle is subject to a coordinated flux of various cell types that interact directly with MuSCs or that release regulatory growth factors and ECM. These niche interactions regulate the activation, self-renewal, differentiation and return to quiescence of MuSCs. Recent work has revealed a fundamental role of structural elements in the niche. Tissue stiffness, which is largely dependent on the composition of the ECM, is a critical fate determinant for MuSCs[2–4]. Moreover, the ECM molecules collagen VI and FN have been shown to provide signals that are essential for MuSC self-renewal during the regeneration of adult muscle[5–7].

The MuSC niche can be severely perturbed by chronic degenerative diseases of skeletal muscle that are accompanied by aberrant deposition of ECM and altered support cell dynamics[8]. De-regulated niche signals eventually lead to stem cell dysfunction and inefficient tissue repair. Of note, a number of multisystemic conditions—such as aging, diabetes, obesity and cancer cachexia—are also accompanied by a loss of MuSC function and consequently by a decline of the regenerative capacity of skeletal muscle tissue[9–12]. In the elderly, this problem is also paralleled by a loss of MuSC numbers, resulting in dramatically delayed or incomplete healing of muscle following injury or surgery[13–15]. Impaired musculoskeletal recovery leads to prolonged immobility that in turn exacerbates the loss of muscle mass that often accompanies aging. Thus, inefficient muscle healing in the elderly is a major clinical problem, and therapeutic approaches for restoring MuSC function are needed.

It remains controversial whether intrinsic or extrinsic signals are the causative mediators of MuSC aging[16]. Changes in the niche may lead to long-lasting or irreversible cellular effects that could ultimately be interpreted as intrinsic MuSC aging. Notably, several studies have shown that a number of pathways are constitutively activated in aged MuSCs. This includes the p38 mitogen-activated protein (MAP) kinase and fibroblast growth factor (FGF)–ERK MAP kinase cascades, as well as signaling through the Janus kinase (JAK)–STAT transcription factor pathway[17–21]. Reduction of signaling through these pathways by using pharmaceutical inhibitors can restore MuSC self-renewal and promote muscle healing in aged mice. These observations raise the question of whether changes in the stem cell niche lead to an upstream induction of these signaling cascades.

Here we describe that loss of FN from the aged-niche ECM in regenerating muscles impairs MuSC function by affecting integrin signaling through PTK2 protein tyrosine kinase 2 (PTK2; also known as FAK) and MAP kinase pathways. Restoration of FN levels in muscle from old mice (aged muscle) rescues MuSC function and improves muscle healing. Thus, loss of stem cell adhesion to niche-derived FN

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is a root cause for MuSC aging that can be targeted to restore the regenerative capacity of muscle tissue in the elderly.

RESULTS

Loss of fibronectin from the aged niche

To interrogate the effect of age-induced changes on MuSCs in the stem cell niche, we performed microarray profiling on freshly isolated cells from 9- to 10-week-old young animals and 20-month-old aged animals 3 d following muscle injury. The viability between young and aged cell populations using our flow cytometry isolation protocol was comparable (**Supplementary Fig. 1a**,**b**). As previously reported, we observed significant enrichment of components of the JAK–STAT and MAP kinase pathways in aged MuSCs, as compared to those in young cells, whereas expression of genes involved in cell cycle regulation was lower in aged cells[17–22] (**Fig. 1a–c** and **Supplementary Table 1**). Notably, we also found that expression of components of the ECM–receptor pathway, including integrins and syndecans, was de-regulated in aged MuSCs, as compared to that in the young MuSCs (**Fig. 1d** and **Supplementary Table 1**). This observation suggested that the ECM composition of the niche is affected by aging. To test this hypothesis we used a spectrum of ECM-protein-specific slow-off-rate-modified aptamers[23] on homogenates from uninjured muscles (uninj.) and from muscles that were collected 3, 7 and 14 d post injury (d.p.i.) (**Fig. 1e**). Consistent with increased baseline fibrosis in aged muscles[8], the majority of ECM molecules were present at higher levels in the uninjured aged mice than in the young control mice. Notably, as compared to muscles from young mice (young muscle), aged muscle showed an impaired ability to globally upregulate ECM molecules over a 14-d regeneration time course following injury (**Fig. 1e**,**f**). As previously reported[5], FN levels were strongly increased following injury in young muscles and, in contrast to the other ECM components, declined sharply at 14 d. Moreover, we observed that old regenerating muscles contained only 48% of FN protein, as compared to that in young muscles, at 3 d.p.i. and 43% of FN at 7 d.p.i. (**Fig. 1g**). Quantitative PCR (qPCR) analysis revealed lower levels of *Fn1* mRNA at 3 d.p.i. but not at 7 d.p.i. in aged muscles, as compared to that in young muscles (**Supplementary Fig. 1c**), suggesting a substantial delay between *Fn1* gene expression and the accumulation of protein. In summary, young regenerating muscle shows a sharp peak in FN levels following injury, whereas this response is blunted in aged muscle.

To determine in which muscle-resident cell populations the expression of FN is affected by aging, we isolated MuSCs, fibro-adipogenic progenitors (FAPs) and lineage-positive (Lin^+) cells (including immune, hematopoietic and endothelial cells) from muscles at 3 d.p.i. using flow cytometry. Of note, we observed 45% lower numbers of Lin^+ cells in aged muscles than in young muscle controls (**Supplementary Fig. 1d**). qPCR analysis revealed a 77% reduction of *Fn1* mRNA expression in aged MuSCs and a trend toward lower levels in aged Lin^+ cells, as compared to those in young cells (**Supplementary Fig. 1e**). To determine the overall contribution of the different cells populations to FN expression in young and aged muscles, we adjusted *Fn1* mRNA expression by cell abundance (**Fig. 1h**). This revealed that the contribution of Lin^+ cells to FN expression was orders of magnitude higher than that for all of the other cell types. Notably, aging lowered these levels by 55%. These observations demonstrate that Lin^+ cells are major contributors to FN expression and that they are lost from aged regenerating muscles.

To directly address the effect of loss of FN from the niche, we generated FN-knockout (KO) mice by crossing animals carrying a tamoxifen-inducible *Cre* allele under the *ROSA26* promoter to animals with a *loxP*-flanked ('floxed') *Fn1* allele[24,25] (which we hereafter refer to as iFN-KO mice). 4 weeks after the tamoxifen injection, the iFN-KO and control (Ctrl) mice were injured. 5 d later the muscles were collected, and *Fn1* mRNA levels were assessed by qPCR (**Fig. 1i**). *Fn1* transcripts were found to be 89.9% lower in iFN-KO mice than in control mice. Immunostaining tissue sections revealed a strong reduction in FN protein in iFN-KO mice as compared to that in control mice at 5 d.p.i. (**Supplementary Fig. 2**). Notably, the lower FN concentration in iFN-KO mice was sufficient to phenocopy the age-related loss of MuSCs and was associated with a significantly lower number of paired box 7 (Pax7)-positive cells (to 77% of that measured in muscles from control mice) (**Fig. 1j**). Thus, FN is essential for the maintenance of MuSCs during muscle healing.

Fibronectin is a preferred adhesion substrate for MuSCs

MuSCs can adhere to FN through integrins and the syndecan-4–frizzled-7 (Sdc4–Fzd7) co-receptor complex[5,26]. This led us to investigate how FN compares to other ECM molecules with respect to cell adhesion. For this purpose we used an array containing 36 different ECM conditions spotted onto a layer hydrogel (**Fig. 2a**)[27,28]. Primary mouse-MuSC-derived myoblasts were seeded on these arrays, and the percentage of cells adhering to the different ECMs was determined after 3 h, 6 h and 24 h. Myoblasts could adhere most efficiently on spots that contained either FN or FN in combination with other ECMs at all of the time points tested (**Fig. 2b**). The best conditions at 3 h and 6 h were spots with FN and laminin or with FN and collagen I at a 1:1 ratio. At the 24 h mark, myoblasts were able to adhere more broadly over the array, but FN-containing spots were still preferred.

With the best ECM-protein-binding condition being FN alone, human myoblasts also showed a pronounced preference for adhesion to ECM components that were mixed with FN at all of the time points tested (**Fig. 2c**). Taken together, these results demonstrate that FN is a preferred binding substrate for mouse and human muscle progenitors and that its loss from the aged stem cell niche could result in a de-regulation of signaling pathways that are modulated by cell adhesion.

Adhesion to fibronectin modulates MuSC aging pathways

To get a comprehensive understanding of the signaling pathways that are affected by adhesion of MuSCs to FN, we undertook a phosphorylation-profiling approach. To this end, we used an antibody array consisting of 1,318 site-specific antibodies covering a wide spectrum of signaling molecules. Mouse MuSC-derived myoblasts were grown on FN for 72 h and compared to cells grown on collagen I (Col), an excellent adhesion substrate that is commonly used in primary myoblast culture. Following background subtraction, phospho-specific signals were normalized to the signals of the antibodies that were raised against the unphosphorylated antigen. A total of 64 proteins were found to be more than 10% changed in their phosphorylation levels after comparing myoblasts that were grown on FN to those grown on Col (**Supplementary Table 2**). Notably, among the factors affected by growth on FN were the cell matrix receptor β1-integrin and several components involved in the ERK and p38 mitogen-activated protein kinase (MAPK) and cell cycle regulatory pathways, which are also altered in MuSCs as a consequence of aging[17,20,21] (**Fig. 1b**,**c**). Other factors whose phosphorylation levels was altered in response to growth on FN mapped to classes such as cytokine–cytokine receptor, phosphatidylinositol, Wnt, regulation of actin cytoskeleton, phosphatidylinositol 3-kinase (PI3K)–protein kinase B (Akt) pathway and cell cycle pathway.

To further verify that FN modulates the p38 and ERK MAPK aging pathways, we performed western blot analysis using extracts from myoblasts that were grown on Col, FN or laminin (LAM) for 72 h (**Fig. 3a** and **Supplementary Fig. 3**). Phosphorylation and total levels of p38 were lower in cells that were grown on FN than on Col or LAM. Growth on LAM led to increased ERK phosphorylation and total ERK levels. In contrast, total levels of ERK were reduced by growth on FN. These experiments demonstrate that sustained exposure to FN is able to curb signaling through two of the major aging-related pathways in MuSCs.

Adhesion signaling is known to coordinate the activity of a number of transcription factors[29]. Because we observed strong changes in

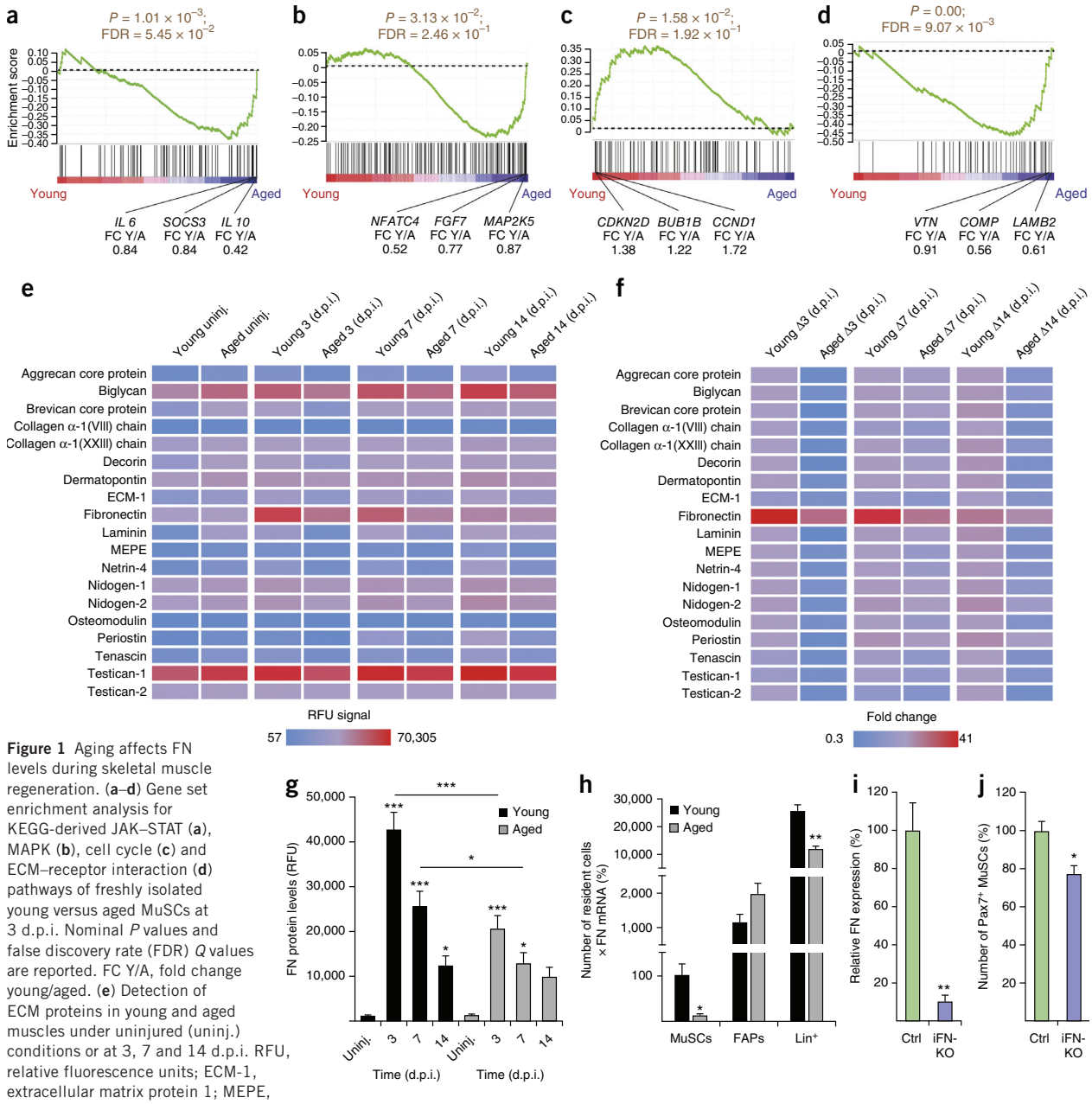

Figure 1 Aging affects FN levels during skeletal muscle regeneration. (**a**–**d**) Gene set enrichment analysis for KEGG-derived JAK–STAT (**a**), MAPK (**b**), cell cycle (**c**) and ECM–receptor interaction (**d**) pathways of freshly isolated young versus aged MuSCs at 3 d.p.i. Nominal *P* values and false discovery rate (FDR) *Q* values are reported. FC Y/A, fold change young/aged. (**e**) Detection of ECM proteins in young and aged muscles under uninjured (uninj.) conditions or at 3, 7 and 14 d.p.i. RFU, relative fluorescence units; ECM-1, extracellular matrix protein 1; MEPE, matrix extracellular phosphoglycoprotein. Data represent means. (**f**) Changes (Δ) of the RFU signal shown in **e** relative to the uninjured condition in young and aged muscles. Data represent means. (**g**) RFU signal for FN in young and aged muscles. (**h**) FN expression by different cell populations in young and aged muscles at 3 d.p.i. (**i**) qPCR for FN expression in muscles of control (Ctrl) or iFN-KO mice at 5 d.p.i. (**j**) Number of Pax7+ MuSCs per unit area in control and iFN–KO mice at 5 d.p.i. Unless otherwise specified, data are means + s.e.m. In **a**–**d**, $n = 6$ mice for young and $n = 5$ for aged. In **e**–**g**, $n = 8$ mice for young, aged uninj. and aged at 3 d.p.i.; $n = 5$ for aged at 7 d.p.i.; and $n = 7$ for aged at 14 d.p.i. In **h**, $n = 5$ mice for young and $n = 6$ for aged. In **i**,**j**, $n = 3$ mice per group for Ctrl and iFN–KO. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test (**g**) or by Student's *t*-test (**h**–**j**).

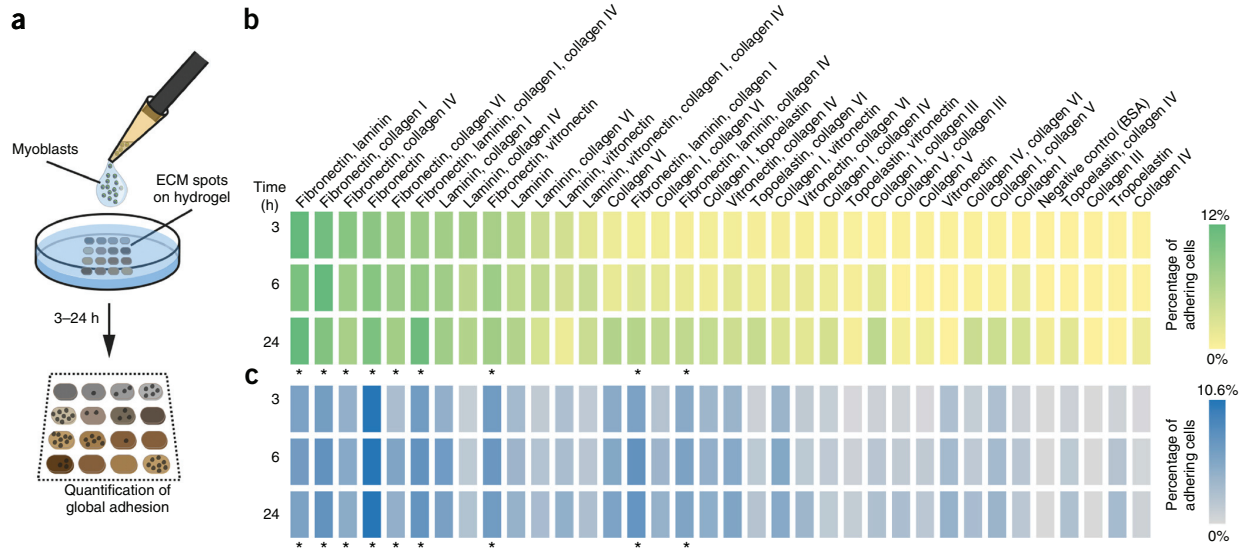

Figure 2 Fibronectin is a preferred adhesion substrate for mouse and human muscle progenitors. (**a**) Graphical overview of the experiment. (**b**,**c**) Quantification of the adhesion of mouse MuSC-derived myoblasts (**b**) and human myoblasts (**c**) on ECM hydrogel arrays 3 h, 6 h and 24 h after seeding. The total number of cells adhering to the array was set to 100%. Shades of green or blue color indicate changes in adherence. Asterisks indicate conditions containing FN. Values are means from $n = 3$ ECM hydrogel arrays per time point from independent experiments.

several signaling pathways after MuSCs were grown on FN for sustained periods of time, we decided to interrogate their transcriptional signature in comparison to that of cells grown on Col, using microarray analysis. Genes with a $\log_2$(fold change) ≥0.5 and an adjusted *P* value of 0.05 (**Fig. 3b**,**c** and **Supplementary Table 3**) were tested for enrichment by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The highest numbers of genes that were upregulated by FN were found in pathways related to focal adhesion, cell cycle, p53 signaling, ECM–receptor interaction and small-cell lung cancer, whereas the highest numbers of genes downregulated by FN were associated with the lysosome pathway. Taken together, our data suggests that growth of cells on FN alters the activity of a number of signaling systems involved in cell adhesion and cell–matrix interaction on the proteomic and transcriptomic levels (**Fig. 3d**). These pathways involve integrin-mediated adhesion signaling, the p38 and ERK MAPKs and cell cycle regulatory pathways, which have all previously been implicated in MuSC aging[17–22].

Lower adhesive capacity of aged MuSCs

Given the effects of FN on the adhesion signaling and aging pathways, we speculated that its prolonged loss from the aged regenerative niche might alter the adherence and survival capacity of MuSCs. To address this hypothesis we used flow cytometry to sort young and aged MuSCs, which we then plated on Col for 3 h, 6 h and 36 h directly after isolation. At the 3 h and 6 h time points, young and aged MuSCs were able to adhere equally well to the plates (**Fig. 4a**). After 36 h, however, the number of attached cells was reduced by 56% for the aged cells as compared to that for the young cells. To assess whether the mechanism leading to this difference involves anchorage-dependent programmed cell death (anoikis), we analyzed the cells by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) (**Fig. 4b**). This experiment revealed 40% higher numbers of $TUNEL^+$ aged cells at 36 h than young cells. Therefore, owing to their reduced adherence capacity, aged cells are more prone to anoikis.

The cytoplasmic nonreceptor tyrosine kinase FAK is a central hub in the adhesion signaling pathway that is activated by FN (**Fig. 3d**) and is a suppressor of anoikis[30]. To investigate whether FAK levels are affected by the aging process, we compared freshly isolated young and aged MuSCs by immunostaining. This revealed that aging leads to a downregulation of FAK expression (**Fig. 4c**,**d**). Thus, loss of FAK could be the reason for the reduced adhesive capacity and the increased amount of anoikis in aged MuSCs.

FN has been shown to activate FAK through integrins[29]. This indicates that a loss of integrin signaling would recapitulate aspects of the MuSC aging phenotype, including impaired adhesion and FAK signaling. Indeed, comparable to aged cells, myoblasts isolated from β1-integrin-knockout ($Itgb1^{-/-}$) mice[31] were severely defective in their adherence capacity as compared to those isolated from control mice (**Fig. 4e**,**f**). Of note, $Itgb1^{-/-}$ myoblasts were able to adhere better to Col than to FN or LAM. This indicates that ECM receptors other than β1-chain-containing integrin dimers are involved in the adhesion to Col. To determine whether β1-integrin (ITGB1) is required for FN-mediated FAK activation, we performed western blots from cells that were grown for 72 h on Col, FN or LAM. As expected, as compared to control cells, activating FAK phosphorylation by growth on FN was strongly perturbed in the $Itgb1^{-/-}$ cells (**Fig. 4g** and **Supplementary Fig. 4**). No differential FAK phosphorylation could be observed in cells that were grown on Col or Lam. These observations show that, similarly to aged cells, $Itgb1^{-/-}$ cells show impaired adhesive capacity and perturbed FAK signaling after exposure to FN.

Fibronectin rescues the adhesion capacity of aged MuSCs

Our data indicate that FN is a preferred adhesion substrate for MuSCs and that the concentration of this ECM component is decreased in the aged regenerative niche, leading to impaired FAK signaling and increased anoikis. Therefore, we decided to test whether aged MuSCs would show better attachment to FN, as compared to attachment in the presence of abundant adhesion substrates such as Col and LAM.

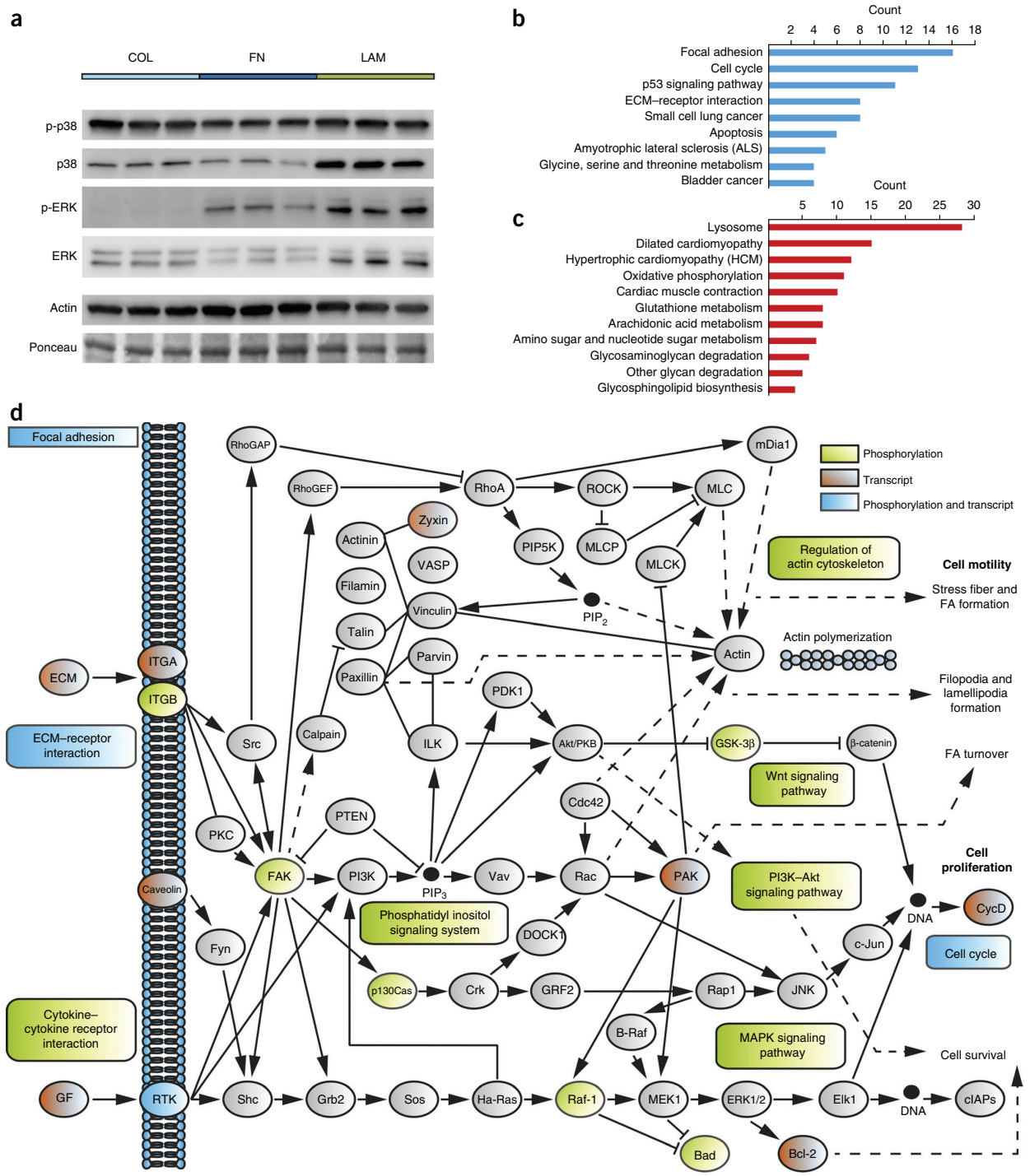

Figure 3 MuSC aging pathways are modulated by fibronectin. (**a**) Representative western blot analysis for the ERK and p38 MAP kinases from myoblasts grown on collagen I (COL), FN or laminin (LAM) for 72 h. Actin and a Ponceau-stained dominant band at the same molecular weight are shown as loading controls. (**b**,**c**) Gene Ontology analysis of statistically differentially regulated genes in microarray data from cells grown on FN versus those grown on Col for 72 h ($n = 3$ microarrays per condition). Graphs represent counts of up- (**b**) or downregulated (**c**) genes that are annotated to the indicated terms. (**d**) Mapping of proteins and genes that are differentially affected by FN to the KEGG adhesion–signaling system and its associated pathways. Light green indicates factors that are affected by FN at the proteomic level (phosphorylation), red denotes factors whose expression is changed at the transcript level, and blue indicates changes to factors at both levels.

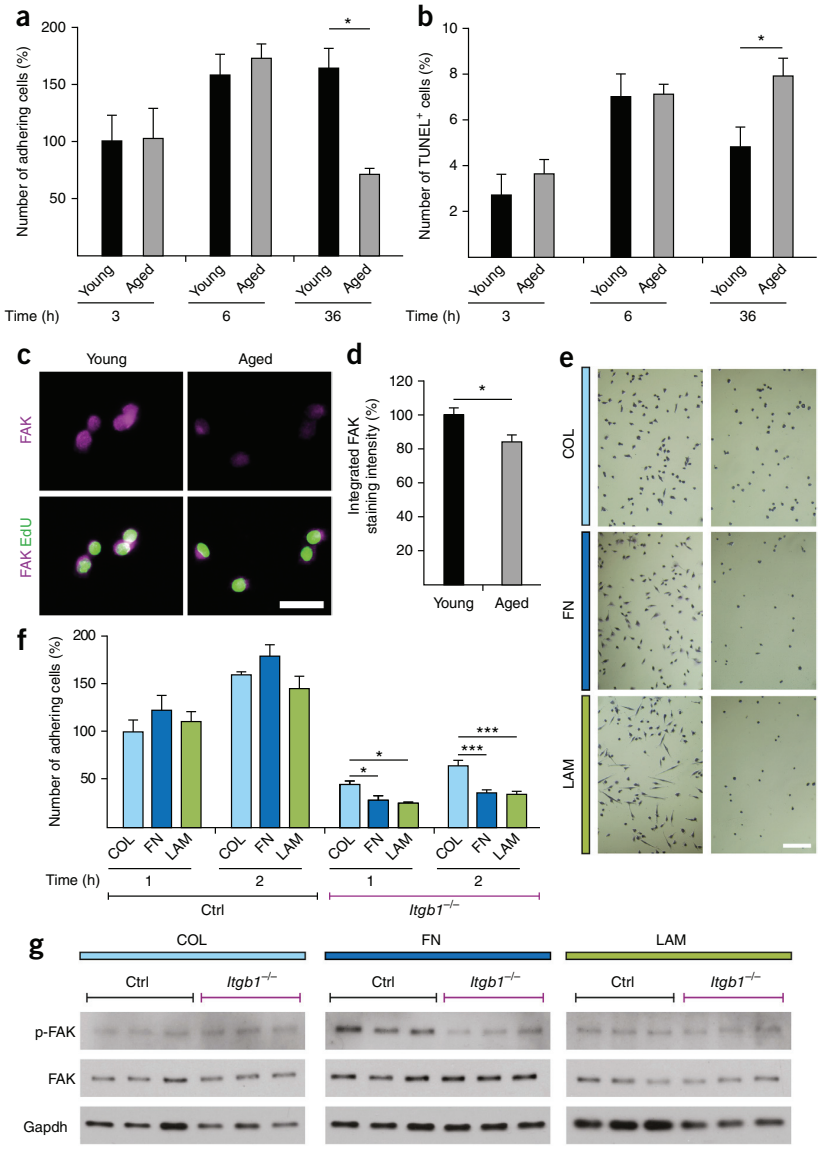

Figure 4 Impaired FN-mediated adhesion signaling in aged MuSCs. (**a**) Adhesion of freshly isolated young and aged MuSCs 3 h, 6 h and 36 h after seeding on Col. (**b**) Quantification of freshly isolated TUNEL$^+$ apoptotic young and aged MuSCs at 3 h, 6 h and 36 h following adherence. (**c**,**d**) Representative images (**c**) and quantification (**d**) of freshly isolated MuSCs from young and aged mice that were seeded on Col and were subsequently stained for FAK. Scale bar, 25 µm. (**e**) Representative images of crystal violet–stained β1-integrin-knockout (*Itgb1*$^{-/-}$) (right) and wild-type (Ctrl) (left) cells that were grown on Col (top), FN (middle) and LAM (bottom) 2 h after plating. Scale bar, 20 µm. (**f**) Adhesion capacity of *Itgb1*$^{-/-}$ and Ctrl cells on Col, FN and LAM at 1 h and 2 h following plating. (**g**) Representative western blots for FAK and phospho-FAK (pFAK) from *Itgb1*$^{-/-}$ and Ctrl cells that were grown for 72 h on Col, FN or LAM. Gapdh is shown as a loading control. Throughout, data are means + s.e.m. In **a**,**b**,**d**, $n = 3$ mice per group. In **e**,**f**, $n = 4$ independent cell culture replicates with $n = 1$ low-magnification image recorded per condition. In **g**, $n = 3$ myoblast lysates per condition, each from a different mouse. $^{***}P < 0.001$, $^{*}P < 0.05$; by two-way ANOVA followed by Bonferroni *post hoc* test (**a**,**b**,**f**) or by Student's *t*-test (**d**).

Indeed, FN significantly improved the attachment of freshly isolated aged MuSCs at all of the time points following plating (**Fig. 5a**). Young MuSCs showed a similar preference for FN over Col or LAM (**Supplementary Fig. 5**). Growth on FN also reduced the number of TUNEL$^+$ aged cells (**Fig. 5b**) and led to a minor but significantly higher number of proliferating cells that incorporated EdU (**Fig. 5c**). These data indicate that FN can overcome several age-related defects of MuSCs, including their impaired adhesion capacity, propensity for anoikis and reduced proliferative capacity.

To get further insights into the downstream mechanisms involved in the FN-mediated restoration of adhesive capacity of aged MuSCs, we plated cells on either Col or FN in the presence of a FAK inhibitor (**Fig. 5d**). Treatment with the FAK inhibitor, as compared to that with a vehicle (Veh) control, led to lower numbers of adherent young cells on both Col and FN. However, aged cells that were plated on Col lost their responsiveness to FAK inhibition. In contrast, growth on FN restored the sensitivity of aged cells to the FAK inhibitor to levels observed in the young cells. These data demonstrate that FAK activity is specifically lost in aged cells and restored by FN. Therefore, FAK signaling is required for the improved adherence of aged MuSCs to FN.

Under conditions of enhanced stress and cell detachment, FAK can translocate into the nucleus[32]. Given the critical role for FAK in MuSCs that we identified, we set out to determine whether there were any changes in the subcellular localization of FAK between the young and aged cells. When comparing the growth of freshly isolated young and aged MuSCs on Col, we observed a higher proportion of cells that had a high ratio of nuclear FAK to total cellular FAK (**Fig. 5e**,**f**). Notably, growth on FN was able to restore the subcellular localization of FAK to that found in young cells. Thus, aged MuSCs that are exposed to FN are under less stress, and their FAK signaling activity is restored to that observed in young cells.

Because our pathway-profiling assays (**Fig. 3**, **Supplementary Fig. 3** and **Supplementary Table 2**) showed that the ERK and p38 MAPKs are affected by FN, we decided to also interrogate these pathways for a possible role in the age-induced anchorage deficit of MuSCs. Pharmacological inhibition of the ERK MAPK pathway did not alter the number of young and aged cells that adhered to Col or FN (data not shown). In contrast, treatment with a p38 MAPK inhibitor, as compared to treatment with vehicle, led to a significantly higher number of adherent aged cells after growth on Col (**Fig. 5g**). In line with the suppression of the p38 pathway after prolonged exposure to FN (**Fig. 3b**), we observed that, as compared to vehicle-treated cells, p38 inhibitor had no further beneficial effects on the adhesion of aged cells on this substrate. These results demonstrate that ERK

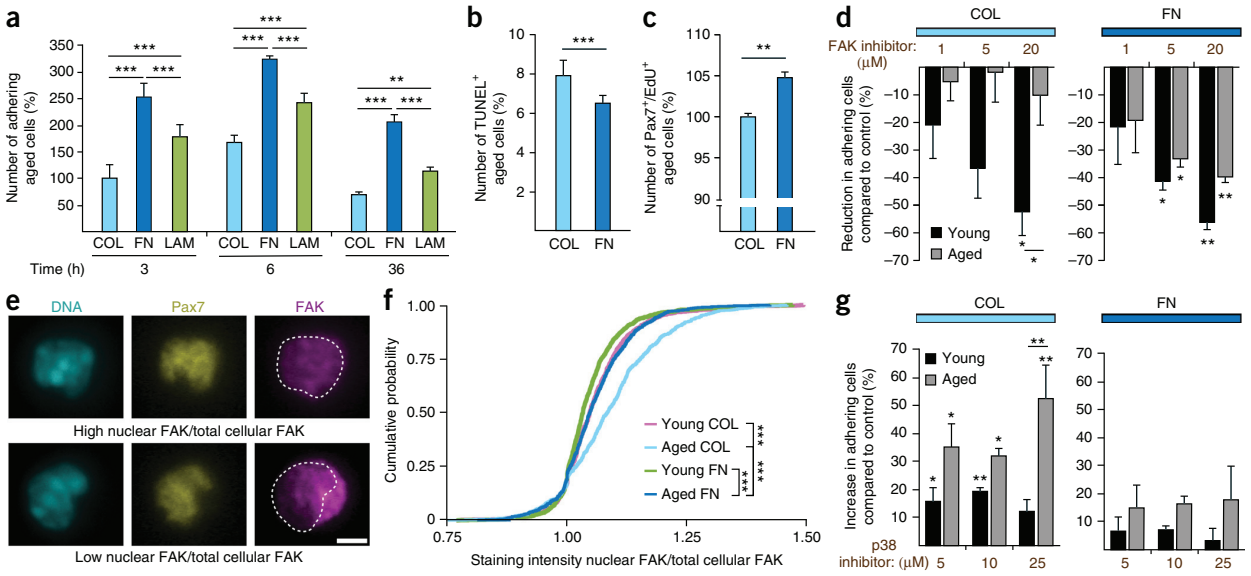

Figure 5 Exposure to FN rescues adhesion signaling in aged MSCs. (**a**) Adhesion of freshly isolated aged MuSCs on Col, FN and LAM at 3 h, 6 h and 36 h after seeding. (**b**) Quantification of freshly isolated TUNEL$^+$ aged MuSCs 36 h after isolation and plating on Col or FN. (**c**) Proliferation of freshly isolated aged MuSCs grown on Col or FN for 96 h. (**d**) Percentage reduction in numbers of MuSCs that were seeded on Col (left) or FN (right) for 36 h and then exposed to a FAK inhibitor or to vehicle (control). (**e**) Representative images of MuSCs showing differential FAK subcellular localization after growth on Col (top) or FN (bottom). Scale bar, 2.5 μm. (**f**) Cumulative probability of the ratio of nuclear FAK over total FAK in young and aged MuSCs that were plated on Col or FN for 6 h. Empirical cumulative distribution functions were built on the basis of $n = 3$ mice per condition. Kolmogorov–Smirnov distance (D) in young MuSCs on FN (young FN) versus aged MuSCs on FN (aged FN), aged FN versus aged MuSCS on Col (aged COL), and young MuSCs on Col (young COL) versus aged COL are 0.13, 0.12 and 0.21, respectively, and $^{***}P$ values are 1.05×10^{-6}, 5.17×10^{-6} and 1.55×10^{-14}, respectively. (**g**) Percentage increase in numbers of adhering MuSCs seeded on either Col or FN for 36 h and exposed to either the p38 inhibitor or vehicle. Throughout, bars represent means + s.e.m. For all experiments, unless otherwise noted, $n = 3$ mice. In **e**,**f**, $n \geq 60$ cells were analyzed per mouse, $n = 3$ mice per condition. Unless otherwise noted, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus vehicle-treated cells or as indicated; by two-way ANOVA followed by Bonferroni *post hoc* test (**a**,**d**,**g**) or by Student's *t*-test (**b**,**c**).

signaling does not affect the adhesive capacity of MuSCs, whereas the age-related induction of the p38 pathway diminishes adhesion and cell survival.

FN treatment rejuvenates MuSCs and improves regeneration

To demonstrate that FN can also improve adhesion signaling and MuSC function in aged skeletal muscle, we injected mice with purified mouse FN at 2 d.p.i. and analyzed the regenerating tissue 3 d later (**Fig. 6a**). Injection of FN, as compared to injection with vehicle, led to a significantly higher abundance of FAK puncta in aged Pax7$^+$ MuSCs (**Fig. 6b**). No significant effect of FN on total FAK levels was observed in muscles of young mice (**Supplementary Fig. 6a**). Similar to its effect on freshly isolated MuSCs *in vitro* (**Fig. 5e**,**f**), FN treatment also restored FAK subcellular localization *in vivo* in Pax7$^+$ cells to that seen in young cells (**Fig. 6c**). Moreover, by using immunostaining to detect the cell proliferation marker Ki67, we observed that MuSCs from muscles of FN-treated aged mice are more proliferative, as compared to MuSCs from muscles of vehicle-treated mice (**Fig. 6d**). No effect of FN treatment on MuSC proliferation was observed in muscles from young mice (**Supplementary Fig. 6b**). To investigate whether the FN-mediated restoration of adhesion signaling and proliferation of aged MuSCs also results in a higher number of cells available for differentiation and muscle repair, we quantified the abundance of cells that were positive for the myogenic commitment marker MyoD1 (also known as MyoD). This revealed higher numbers of Pax7$^+$MyoD$^+$ and Pax7$^-$MyoD$^+$ cells in muscles from aged FN-treated mice, as compared to those from aged vehicle-treated mice (**Fig. 6e**,**f**). No positive effects on the abundance of Pax7$^+$MyoD$^+$ and Pax7$^-$MyoD$^+$ cells were observed as a consequence of FN injections, as compared to vehicle injections, in young muscles (**Supplementary Fig. 6c**,**d**). Taken together, these observations demonstrate that FN treatment of aged muscle is able to rescue FAK signaling in MuSCs and thereby restores their proliferative and myogenic potential.

To examine whether FN treatment can improve the function of MuSCs sufficiently to restore tissue healing in the aged, we injected muscles twice over a longer period of regeneration (**Fig. 6g**). Staining for developmental myosin heavy chain (devMHC), a marker of the earliest stage of muscle fiber formation, revealed that aged muscles injected with FN contained less number of immature fibers at 7 d.p.i. than those injected with vehicle (**Fig. 6h**). No changes in the abundance of devMHC$^+$ fibers were observed between young FN- or vehicle-treated muscles (**Supplementary Fig. 6e**). Finally, in contrast to injection with vehicle, injection of aged mice with FN accelerated muscle regeneration and led to significantly larger muscle fibers at 7 d.p.i. (**Fig. 6i**). No effect on muscle fiber size was observed between young FN-injected or vehicle-treated mice (**Supplementary Fig. 6f**). Taken together, these results demonstrate that restoration of FN levels during muscle regeneration to those seen in young animals is able to rescue the niche-dependent loss of MuSC function that is associated with aging.

DISCUSSION

Old age in mammals is accompanied by a loss of MuSC function and number, leading to impaired healing of skeletal muscle following

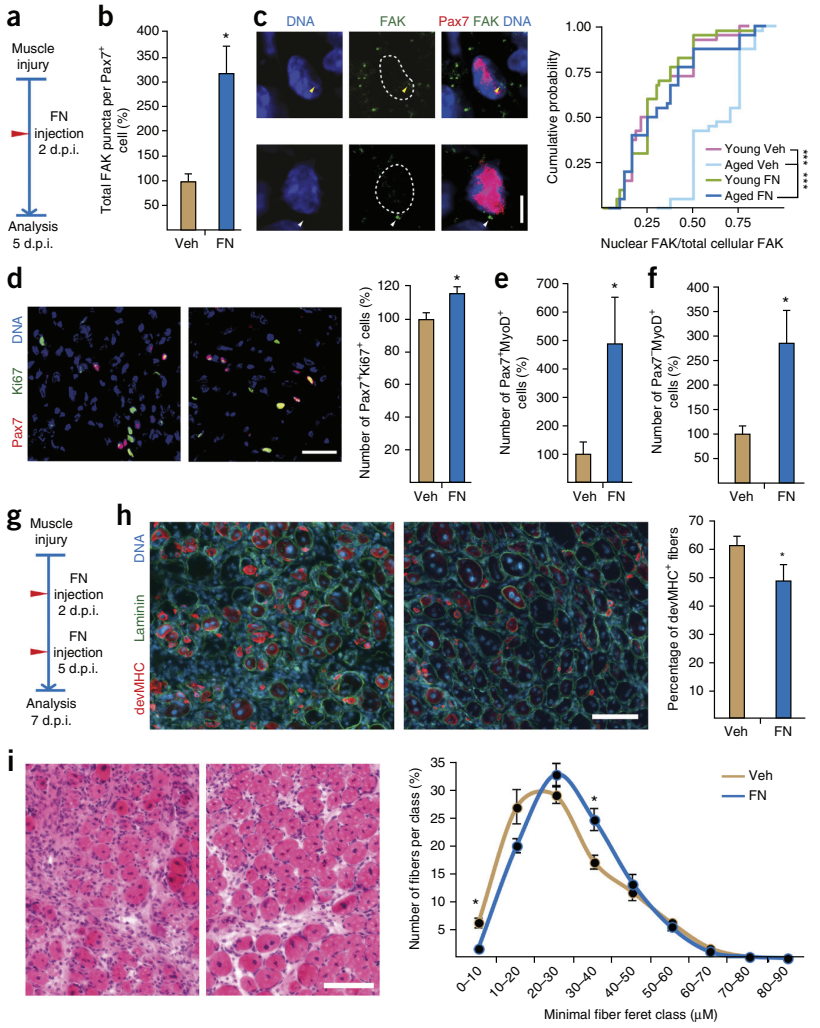

Figure 6 Fibronectin treatment restores the regenerative capacity of aged muscles. (**a**) Experimental protocol used for **b**–**f**. (**b**) Quantification of FAK levels in Pax7$^+$ MuSCs in tissue sections of vehicle (Veh)- or FN-treated muscles. (**c**) Representative images of nuclear (yellow arrowheads) (top) or cytosolic (white arrowheads) (bottom) FAK puncta in Pax7$^+$ cells (left) and quantification of the cumulative probability of the ratio (per cell) of nuclear FAK puncta/total FAK puncta. Kolmogorov–Smirnov distance (*D*) in aged FN versus aged COL, and in young COL versus aged COL, are 0.725 and 0.7, respectively. ***P values are 1.48×10^{-9} and 6.15×10^{-9}, respectively. (**d**) Representative images of Pax7 and Ki67 staining in tissue sections from vehicle- (left) or FN-treated (middle) mice, and quantification of Ki67$^+$ cells within the Pax7$^+$ cell population (Pax7$^+$Ki67$^+$) (right). (**e**,**f**) Quantification of Pax7$^+$MyoD$^+$ (**e**) and Pax7$^-$MyoD$^+$ (**f**) cells per unit area in muscles of vehicle- or FN-treated muscles. (**g**) Experimental protocol used for **h**,**i**. (**h**) Representative images of muscle sections stained for developmental myosin heavy chain (devMHC) and laminin in vehicle- (left) or FN-treated (middle) aged mice, and quantification of the percentage of devMHC$^+$ fibers in muscles after the indicated treatments (right). (**i**) Representative H&E-stained images of muscle cross-sections from vehicle- (left) or FN-treated (middle) mice and quantification of fiber size on the basis of laminin staining (right). Throughout, bars and data points represent means + s.e.m. and ± s.e.m., respectively. Mice used were $n = 3$ (**e**, FN; **f**, FN; **i**) or $n = 4$ (**b**–**e**, Veh; **f**, Veh; **h**) per condition. In **c**, $n = 10$ cells were analyzed per mouse. In **d**,**h**,**i**, quantification was performed on stitched images covering the entire cross section of the tibialis anterior muscle of each mouse. **$P < 0.01$, *$P < 0.05$; by Student's *t*-test (**b**,**d**–**f**,**h**,**i**). Scale bars, 5 μm (**c**), 25 μm (**d**) and 100 μm (**h**,**i**).

injury[13–16]. Aging is a multisystemic process, and it has been suggested that changes in circulating factors could be the principal mediators of MuSC dysfunction[33]. However, it has remained unclear whether such alterations in the systemic environment act directly on the stem cells or whether they lead to local changes in the niche that indirectly affect MuSC function. In support of the latter hypothesis, our study revealed that structural regulatory elements in the stem cell niche in skeletal muscle are profoundly changed as a consequence of the aging process and that these alterations de-regulate the majority of pathways that have previously been associated with MuSC aging[17–22].

In contrast to the higher baseline fibrosis seen in uninjured aged muscles, we observed that following injury, old muscles failed to upregulate a transitional regenerative FN-rich extracellular matrix. Concomitantly, ubiquitous deletion of FN in iFN-KO mice leads to a reduction of MuSC numbers that reiterates the aging phenotype. We found that FN is a preferred adhesion substrate for MuSCs that regulates the p38 and ERK MAPK aging pathways through ITGB1 and FAK. Restoration of attachment to FN in the aged niche reactivates FAK signaling in MuSCs and thereby restores the regenerative capacity of old skeletal muscle.

Our results revealed that Lin$^+$ cells—which include immune, hematopoietic and endothelial cells—express high levels of FN and are extremely abundant in young regenerating muscles early after injury. Coinciding with the notably lower overall FN content in aged muscles, the contribution of Lin$^+$ cells to *Fn1* mRNA expression is much lower as compared to that in young tissue. In addition, our results show lower levels of *Fn1* mRNA expression in aged MuSCs than in young MuSCs. It has been shown that self-renewal in the muscle lineage is dependent on autologous regulation by FN that is derived from MuSCs themselves[5,7]. Therefore, decreased local FN production by MuSCs further exacerbates the age-associated decline in niche FN and is likely to affect the maintenance of MuSCs in the tissue during regeneration. It is also possible that aging affects FN expression in muscle fibroblasts. However, there are no suitable surface markers available for the flow cytometric isolation of muscle fibroblasts[34]. Thus future studies will have to address the spatiotemporal contribution of individual FN-secreting cell populations to the MuSC niche using specific *Cre* drivers and reporter alleles.

Aged muscles have been demonstrated to contain higher levels of FGF2, which induces a break in MuSC quiescence, leading to regenerative failure[17]. FGF signaling is known to activate the JAK–STAT, phosphatidylinositol, PI3K–Akt and MAPK pathways[35]. Notably, we found that most of these pathways are also affected by FN. FGF signaling is regulated by integrins that can serve as FN receptors[36]. Our

results demonstrate that signaling pathways downstream of integrins are de-regulated as a consequence of loss of environmental FN in aged cells. Thus, these observations indicate that changes in the content of FN in the MuSC niche are closely connected to age-related alterations in FGF signaling[37].

At a very advanced age in mice, MuSCs enter a senescent state that is marked by high expression of the cell cycle inhibitor $p16^{INK4A}$ and failure of autophagy[22,38]. Of note, $p16^{INK4A}$ has also been shown to induce anoikis in several different cell types[39]. In line with our results, this study demonstrated that $p16^{INK4A}$-mediated anoikis is strongly inhibited by FN but not by LAM. On the basis of these observations we speculate that restoration of the FN content of the niche could also be a strategy to overcome MuSC senescence in geriatric individuals.

Taken together, we discovered FN as a structural element in the stem cell niche that is critical for the maintenance and function of MuSCs during muscle regeneration. Loss of FN from the niche affects a substantial number of pathways and cellular mechanisms that have been implicated in MuSC aging[17–22]. Our work reveals that alterations in the ability of stem cells to adhere to the niche are a root cause of MuSC aging and a promising target for the rejuvenation of skeletal muscle tissue.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: microarray data have been deposited under accession number GSE81096 and GSE81225.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.J.J., N.H., C.P.-C., M.R., S.K., M.S., L. Li, S.M., U.L., F.S., N.A.D., A.P., C.-M.F. and J.v.M., designed and conducted experiments, and analyzed results; E.M. and F.R. performed data analysis; G.J. provided support with high-content image acquisition and analysis; M.A.R. provided critical reagents, edited the manuscript and helped with data interpretation; R.F. provided critical reagents; D.H.W. helped with data acquisition and analysis; P.S. and P.D. helped with experimental design, data interpretation and editing of the manuscript; and L. Lukjanenko, J.N.F. and C.F.B. designed and conducted experiments, analyzed results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mice. All mice were housed under standard conditions and allowed access to food and water *ad libitum*. *Itgb1*$^{-/-}$ myoblasts and controls were isolated from both genders of mice, otherwise only male mice were used. Unless otherwise indicated, the strain C57BL/6JRj (Janvier) was used. Young mice were between 9 and15 weeks of age, and aged mice were 20–24 months old. For isolation of cells from regenerating muscle, tibialis anterior, gastrocnemius and quadriceps muscles were injected with 50 µl, 100 µl or 50 µl of 50% vol/vol glycerol in phosphate-buffered saline (PBS), respectively[40]. Animal experiments were approved by the Vaud Cantonal Commission (Switzerland) for animal experimentation under licenses VD2620 and VD2947, the Institutional Animal Care and Use Committee (IACUC) of the Carnegie Institution for Science (USA) under the permit number A3861-01 and the Landesamt für Verbraucherschutz Abteilung Gesundheitlicher und technischer Verbraucherschutz (Germany) under Reg.-Nr 03-010/15. Fibronectin-floxed mice were provided by R.F., and the *ROSA26–CreER*^{T2} mice were provided by F. Stewart (Technische Universität Dresden, Germany)[24,25]. Experiments with iFN-KO and the respective control animals were performed in accordance with University of Ottawa guidelines for animal handling and animal care, as determined by the University of Ottawa Animal Care Committee. iFN-KO and Ctrl mice were treated with four daily intraperitoneal injections of 100 mg per kg body weight (mg/kg) tamoxifen (Sigma) in corn oil at 2–3 weeks of age. Tibialis anterior muscle injury in iFN-KO mice was induced at 6–7 weeks of age by a single injection of 50 µl of 10 µM cardiotoxin (Sigma) solution in 0.9% saline. For *in vivo* FN treatment, tibialis anterior muscles of young and aged mice were injured with a single injection of 50 µl of 20 µM cardiotoxin solution in 0.9% saline. Mice were then injected with 0.5 mg/ml mouse FN solution (Biopur) or vehicle (Veh) at 2 and 5 d.p.i. Veh was 50 mM Tris and 0.5 M NaCl at pH 7.5 in water. Muscles were isolated and analyzed at 5 or 7 d.p.i.

Slow-off-rate-modified aptamer assay. Muscles samples were pulverized using the cryoPREP impactor system (Covaris). The muscle powder was then subjected to mechanical lysis using a Polytron homogenizer, and the proteins were extracted in 50 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.5% Triton X-100. Protein concentration was determined by a bicinchoninic acid (BCA) assay assay (Pierce), and samples were diluted at 250 ug/ml. Protein extracts were analyzed by using DNA-aptamer-based recognition on the SOMAscan platform (Somalogic, Boulder, CO, USA), as described[23]. Median normalized relative fluorescence units (RFUs) were $\log_2$-transformed before applying principal component analysis and linear models. Statistical analyses were performed in R 3.1.3 (R Foundation for Statistical Computing).

ECM arrays and coating. Mouse primary myoblasts and human primary myoblasts (HSMM, Lonza) between passage 4 and 10 were seeded on MicroMatrix 36 arrays (Microstem) containing ECMs spotted onto a 10% polyacrylamide hydrogel of 10 kPa in stiffness. After 3 h, 6 h and 24 h, the number of adhering cells was determined by visual counting. For ECM coating, dishes were covered with collagen (C7774, Sigma), fibronectin (F2006, Sigma) or laminin (L6274, Sigma).

Antibody arrays and western blot. Mouse primary myoblasts were grown on FN- or Col-coated dishes for 72 h, trypsinized, collected by centrifugation and frozen in dry ice. Phospho Explorer Antibody Arrays were analyzed by Full Moon BioSystems. Briefly, as described in the manufacturer's instructions, proteins were extracted by using nondenaturing lysis buffer, concentration was adjusted between samples, and protein extracts were biotinylated, coupled to the antibodies on the array and detected by dye-conjugated streptavidin using a microarray scanner. Cells for western blot analysis were grown for 3 h or 72 h on Col-, FN- or LAM-coated dishes and lysed in RIPA buffer (Sigma) following collection. After adjustment of protein concentrations (as determined by BCA assays) samples were boiled in Laemmli buffer and interrogated by standard western blot procedures. Antibodies used were: rabbit p44/42 MAPK (Cell Signaling #9102), rabbit phospho-p44/42 MAPK (Cell Signaling #4370), rabbit p38 MAPK (Cell Signaling #9212), rabbit phospho-p38 MAPK (Cell Signaling #9211), rabbit FAK (Cell Signaling #13009), rabbit pFAK (Cell Signaling #3284), mouse β-actin (Sigma A5441) and mouse Gapdh (RDI/Fitzgerald, RDI–TRK5G4–6C5). Antibodies for western blots were diluted 1/1,000, except for β-actin, which was used at 1/5,000. Antibody validation is provided on the manufacturers' websites.

Quantitative PCR. RNA was extracted from frozen muscles or freshly sorted cells by using miRNeasy Mini Kit or RNeasy Micro Kit (Qiagen), respectively. RNA samples were subjected to reverse transcription using random primers (High Capacity cDNA Reverse Transcription Kit, ABI). SYBR and Taqman quantitative PCR was performed on a LightCycler 480. The following primers were used: *Fn1* sense: GGCCACACCTACAACCAGTA, *Fn1* antisense: TCGTCTCTGTCAGCTTGCAC. Primers for housekeeping genes were: *Actb* sense: CAGCTTCTTTGCAGCTCCTT, *Actb* antisense: GCAGCGATATCGTCATCCA. Taqman probe for *Fn1* was Mm01256744_m1 (Applied Biosystems). For qPCR using whole regenerating muscles, reference genes were selected based on their stability across time points of regeneration from microarray data: *Atp5b* sense: ACCTCGGTGCAGGCTATCTA, *Atp5b* antisense: AATAGCCCGGGACAACACAG, *Eif2a* sense: CACGGTGCTTCCCAGAGAAT, *Eif2a* antisense: TGCAGTAGTCCCTTGTTAGCG, *Psmb4* sense: GCGAGT CAACGACAGCACTA, *Psmb4* antisense: TCATCAATCACCATCTGGCCG.

Flow cytometry, MuSC *in vitro* assays and myoblast culture. For isolation of cell populations, muscles were collected and digested with Dispase II (2.5 U/ml; Roche), Collagenase B (0.2%; Roche) and $MgCl_2$ (5 mM) at 37 °C. Cells were then incubated at 4 °C for 30 min with antibodies against CD45 (Invitrogen, MCD4501 or MCD4528; dilution for both 1/25), CD31 (Invitrogen, RM5201 or RM5228; dilution for both 1/25), CD11b (Invitrogen, RM2801 or RM2828; dilution for both 1/25), CD34 (BD Biosciences, 560230 or 560238; dilution for both 1/60), Ly-6A–Ly-6E (Sca1) (BD Biosciences, 561021; dilution 1/150), α7-integrin (R&D, FAB3518N; dilution 1/30) and CD140a (eBioscience, 12–1401–81 or 17–1401–81; dilution for both 1/30). Antibody validation is provided on the manufacturer's website. FACS isolation was performed on a Beckman–Coulter Astrios Cell sorter. MuSCs were $CD45^-CD31^-CD11b^-Sca1^-CD34^+Itga7^+$; FAPs (fibro-adipogenic progenitors) were $CD45^-CD31^-CD11b^-Sca1^+CD34^+PDGFRa^+$; and Lin^+ cells were $CD45^+CD31^+CD11b^+$. For high-throughput imaging, MuSCs were distributed into 96-well plates at a density of 1,765 cells/cm^2. Freshly sorted MuSCs were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM), 20% heat-inactivated FBS, 10% inactivated horse serum, 2.5 ng/ml basic FGF (bFGF; Invitrogen), 1% penicillin–streptomycin (P–S), 1% L-glutamine, 1% Na pyruvate (Invitrogen). EdU was added to the medium at 10 µM for 6 h before fixation. For long-term culture, myoblasts were maintained in Ham's F-10 (Gibco), 20% heat-inactivated FBS, 2.5 ng/ml bFGF (Invitrogen) and 1% P–S. Small molecular inhibitors were: FAK inhibitor (F14, CAS 4506–66–5, #sc–203950) and p38 inhibitor (SB 203580 hydrochloride, R&D systems TOCRIS #1402). *Itgb1*-KO myoblasts and controls were isolated from *Pax7*$^{CE/+}$;*Itgb1*$^{f/f}$;*R26R*$^{YFP/YFP}$ mice[31,41,42] and *Pax7*$^{CE/+}$;*R26R*$^{YFP/YFP}$, respectively[37]. Primary human myoblasts from adult donors were obtained from Lonza (HSMM) after the supplier received informed consent from the donors and after consent was obtained from the Vaud ethics commission for human research (CER–VD) under protocol 281/14.

Immunostaining and image analysis. Adhesion of freshly isolated MuSCs was assessed at different time points by fixation in 4% paraformaldehyde (PFA) followed and counterstaining with the nuclear dye DAPI. $TUNEL^+$ cells were quantified using the *In situ* Cell Death Detection Kit, TMR red (Roche #12 156 792 910). EdU incorporation was revealed by using the Click-0iT assay (Molecular Probes) according to manufacturer's instructions. Briefly, cells were fixed for 15 min in 4% PFA, permeabilized for 20 min in PBS containing 0.5% (vol/vol) Triton X-100 (PBTX), stained with the Click-iT reaction mix and counterstained with DAPI. For immunostaining, cells were blocked for 1–2 h in 5% goat serum, 1% BSA and 0.2% PBTX, before incubation with primary and secondary antibodies. Image acquisition was performed using the ImageXpress (Molecular Devices) platform. Quantifications were done using the MetaXpress software. The number of Pax7-, MyoD- and Ki67-positive cells was determined by counting of immunostainings in muscle sections, as previously described[5]. *In-situ* quantifications were done using the ImageJ software[43] (U. S.

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National Institutes of Health) over the entire cross-sectional area of the muscle. For minimal fiber feret measurements, only centralized fibers on LAM-stained sections were considered. Antibodies were: mouse Pax7 (DHSB; for tissue sections, undiluted hybridoma culture supernatant and purified at 2.5 µg/ml)[5], mouse devMHC (DSHB, F1.652; for tissue sections, dilution 1/500), rabbit FAK antibody (Abcam, ab40794; for tissue sections, dilution 1/200; for isolated cells, dilution 1/40), rabbit Ki67 (Abcam, ab833; for tissue sections, dilution 1/200), rabbit laminin (L9393 Sigma; for tissue sections, dilution 1/1,000), rabbit fibronectin (Abcam, ab23750; for tissue sections, dilution 1/1,000)[5]. Where no citation is provided, antibody validation and references can be found on the manufacturer's website.

Microarrays. For isolation of RNA from freshly sorted activated MuSCs, the RNeasy Micro Kit (Qiagen) was used. RNA samples were then subjected to 3′ microarray analysis on Illumina MouseRef–8_V2 chips. 3 ng of total RNA were used to produce cRNA in a two-round amplification protocol, using first Messageamp II aRNA amplification kit (AM1751, Life Technologies, Inc.) followed by Messageamp II–biotin enhanced aRNA amplification kit (AM1791, Life Technologies, Inc.). 750 ng of cRNA were hybridized for 16 h at 55 °C on Illumina MouseRef–8 v2 microarrays. Quality of total RNA was checked by using the Bioanalyzer 2100 with Total RNA Pico kit, and quality of cRNA was checked by using the Bioanalyzer 2100 with the Total RNA Nano kit (Agilent Technologies). Quantifications were done using the Quant-iT RiboGreen RNA Assay Kit (Life Technologies, Inc.). For microarrays comparing FN and Col, cells were grown for 72 h on FN- or Col-coated dishes. RNA was extracted with the Agencourt RNAdvance Tissue Kit (Beckman Coulter, Inc). cRNA was produced with the Illumina TotalPrep-96 Kit (Life Technologies Inc). 15 µg of cRNA were fragmented prior to a 16-h hybridization at 45 °C on an Affymetrix Mouse Genome 430 2.0 Array. Quality of total RNA and cRNA was checked by using the Bioanalyzer 2100 with the Total RNA Nano kit (Agilent Technologies). Quantification was done by using the Quant-iT RiboGreen RNA Assay Kit assay (Life Technologies Inc). Microarray data have been deposited to Gene Expression Omnibus under accession number GSE81096 and GSE81225.

Transcriptomic analysis. The robust-multiarray-average (RMA) approach was used for the creation and normalization of the summarized Affymetrix probe set signals. We applied a nonspecific filter to discard probe sets with low variability; we retained 19,050 Affymetrix probe sets whose s.d. was greater than the median of the s.d. of all of the probe sets. Illumina expression signals were quantile-normalized. We applied a nonspecific filter to discard probe sets with low variability and retained 12,848 Illumina probe sets whose s.d. was greater than the median of the s.d. of all of the probe sets. For differential expression analysis and pathway analyses, genes (represented by probe sets) were tested for differential expression using the moderated *t*-statistic as implemented in LIMMA[44] for both data sets. We exploited DAVID Bioinformatics Resources 6.7 (ref. 45) to assess whether the differentially expressed genes were related to specific KEGG pathways. Gene Set Enrichment Analysis (GSEA), using the Broad Institute algorithm v2.2.0 (ref. 46), was conducted on the preranked gene lists defined according to the LIMMA results and the gene sets derived from the KEGG pathway database (c2.cp.kegg.v5.0.symbols.gmt) were tested.

Statistical analysis. All wild-type mice were randomized according to body weight before interventions, and no mouse was excluded from the study, except for those that died a natural death during the course of the experiment. Sample size determination was based on the expected effect size and variability that was previously observed for similar readouts in the investigators' labs. *In vivo* treatments were not blinded, but imaging readouts were analyzed in a blinded manner. Genome-wide statistical analyses and Kolmogorov–Smirnov tests were performed using R version 3.1.3 and relevant Bioconductor packages as described in the sections above. Gene Set Enrichment Analyses were performed using the Broad Institute algorithm v2.2.0. All other statistical analyses were performed using GraphPad Prism (GraphPad Software) assuming normal distribution of the variables measured. Statistical significance for binary comparisons was assessed by a Student's *t*-test after checking that variances do not differ between groups or by a Welch correction when variances differed between groups. All exploratory and signaling experiments were analyzed by using two-tailed tests, and *in vivo* phenotypic rescue experiments were tested using a one-tailed test. For comparison of more than two groups, one-way or two-way ANOVAs were used, according to the experimental design, and followed by Bonferroni multiple-comparison testing. All measurements that were fully independent were analyzed using unpaired statistics, whereas experiments in which treatments were performed with primary cells isolated from the same animal(s) were analyzed by using paired statistics. All data are expressed as mean + s.e.m. or mean ± s.e.m. For plotting the cumulative probability of the ratio of nuclear FAK puncta over total FAK puncta per cell in tissue resident MuSCs, 0.5 pseudo puncta were added to the entire data set before performing statistics.

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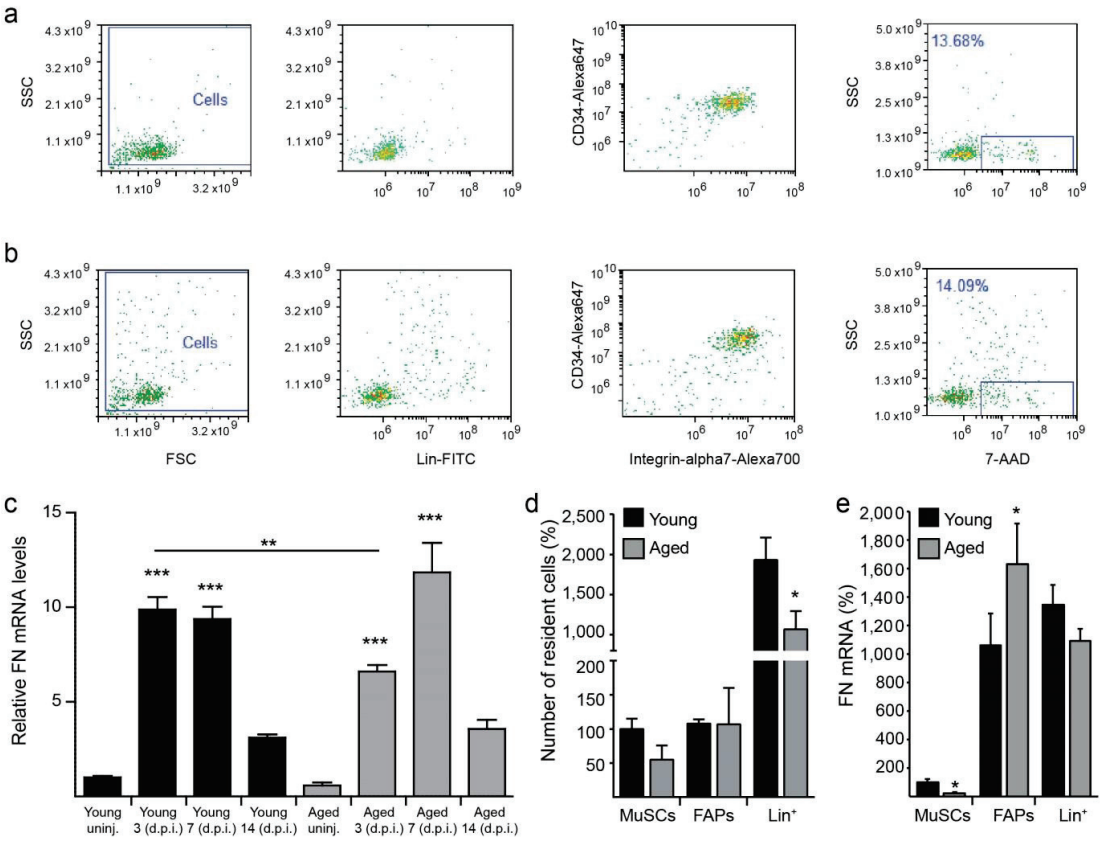
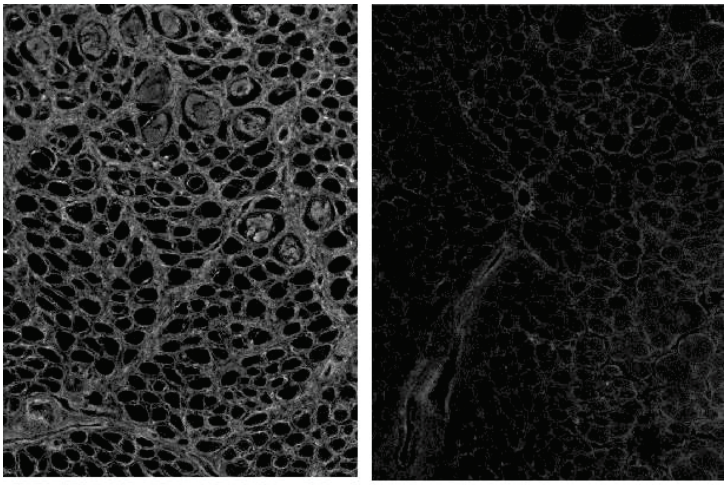
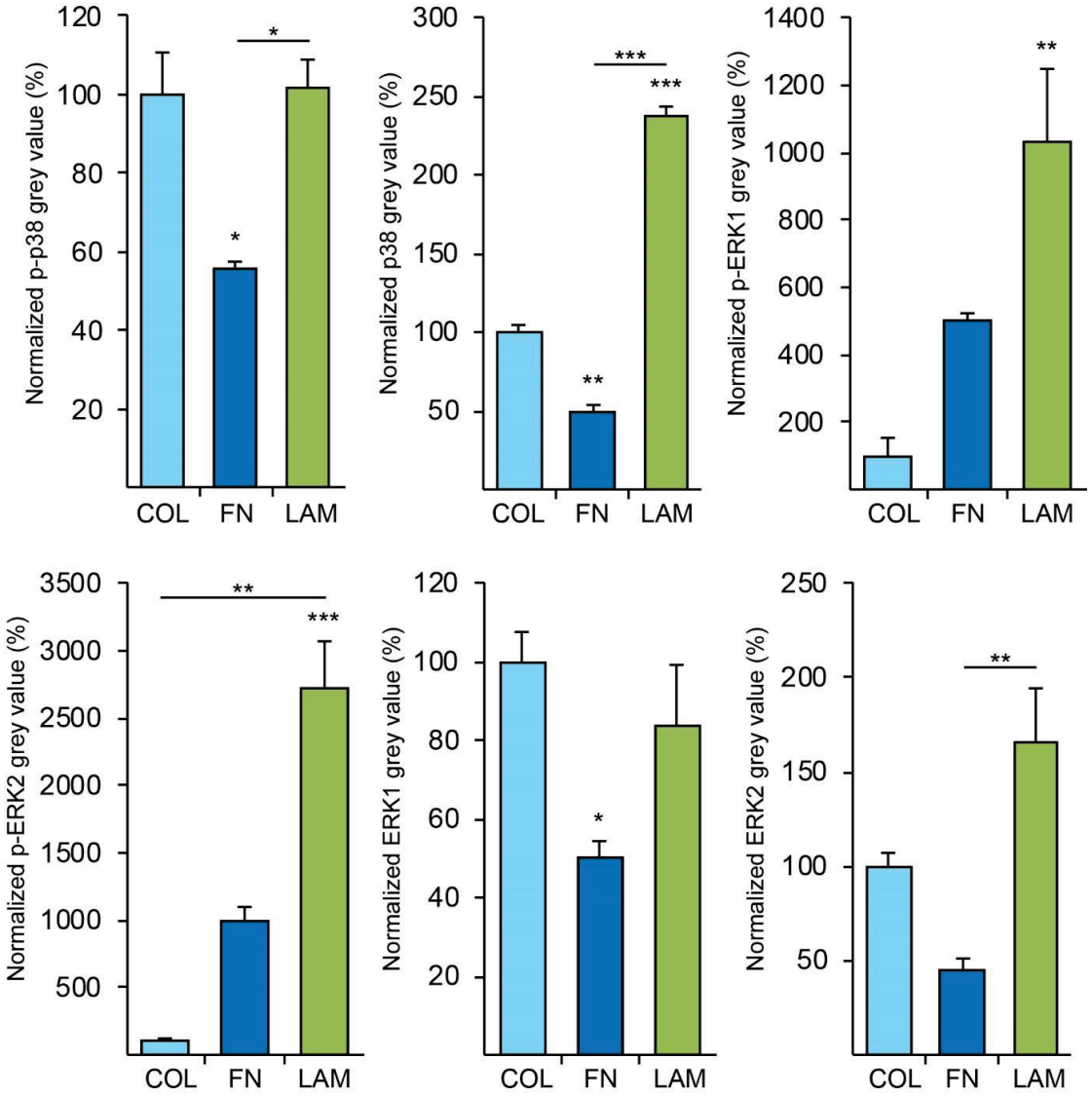


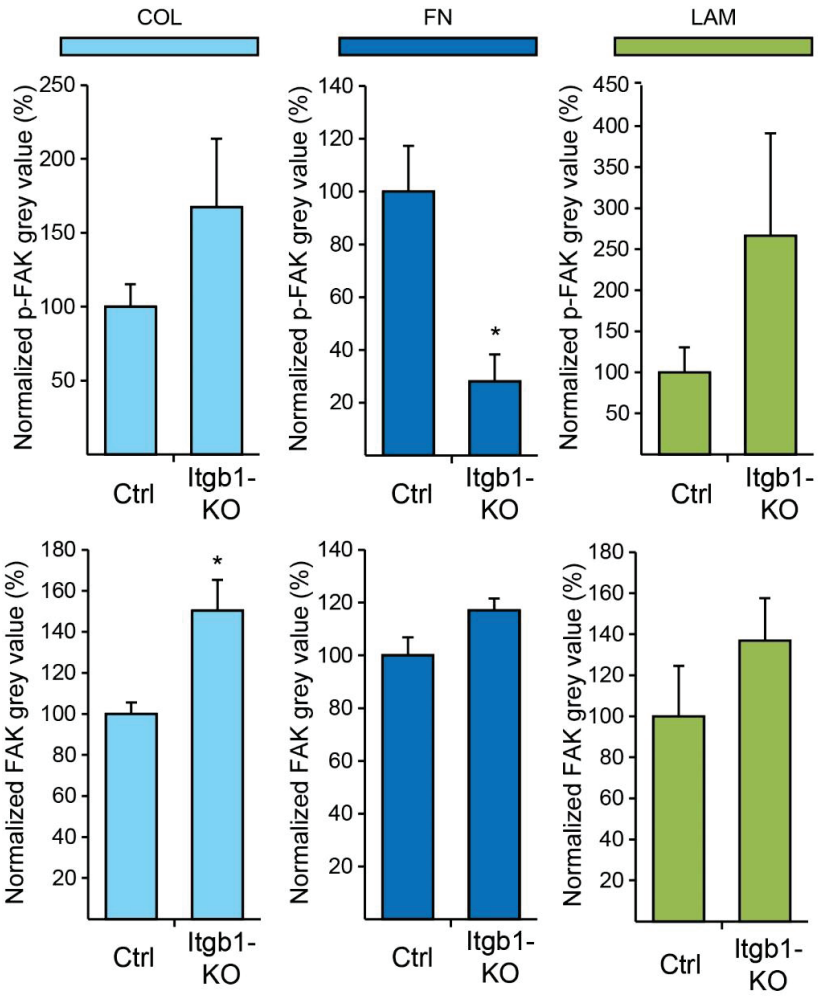
Supplementary figure 1 MuSC viability following FACS and FN levels in young and aged tissue. (**a, b**) MuSCs were gated for lineage negativity (Lin–FITC), and positively for CD34 (CD34–Alexa647), Integrin–alpha7 (Integrin–alpha7–Alexa700) and for the viability dye 7–AAD (7–aminoactinomycin D). Based on 7–AAD staining, 13,68 % of aged (**a**) and 14.09 % of young (**b**) cells that were identified as not viable. No major differences in viability were observed between the conditions. (**c**) qPCR for FN expression from RNA isolated from uninjured muscles or 3, 7 and 14 days post injury. Bars are normalized to Young uninj. (**d**) Number of isolated cells from young and aged muscles at 3 d.p.i. quantified by flow cytometry. (**e**) qPCR for FN expression from RNA isolated from different cell types isolated from muscles 3 d.p.i. Expression values were quantified on equal amounts of total RNA. Throughout, bars represent means + s.e.m.. All replicates are biological. n = 8 (**c**), n = 4 (**d**); n = 5 for young and n = 6 for aged (**e**). *** $P < 0.001$, ** $P < 0.01$,* $P \leq 0.05$ versus uninj. or comparison indicated; one–way ANOVA followed by Bonferroni post–test (**c**) or Student's t–test (**d, e**).



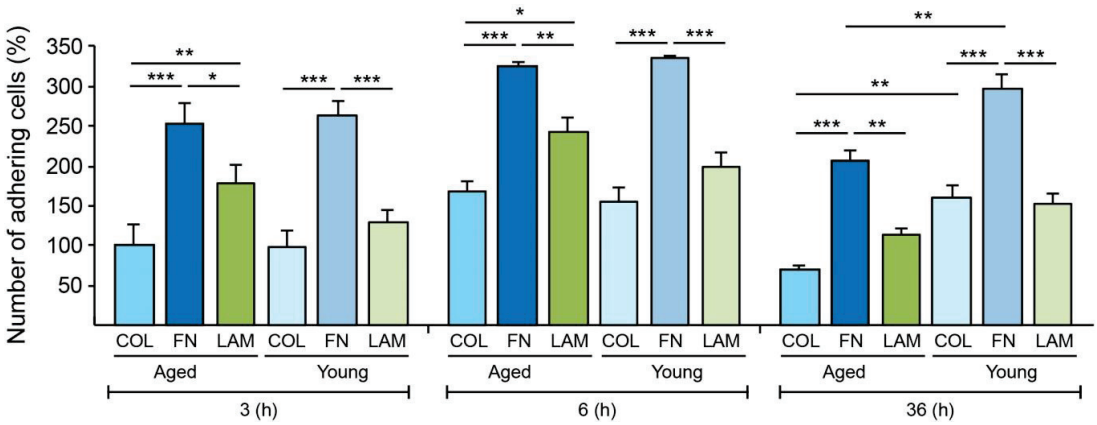
Supplementary figure 2 FN tissue levels in iFN–KO mice. Immunostaining for Fibronectin (FN) from muscles of control (Ctrl) or FN knockout mice (iFN–KO) five days following injury. Images were acquired with the same exposure time.



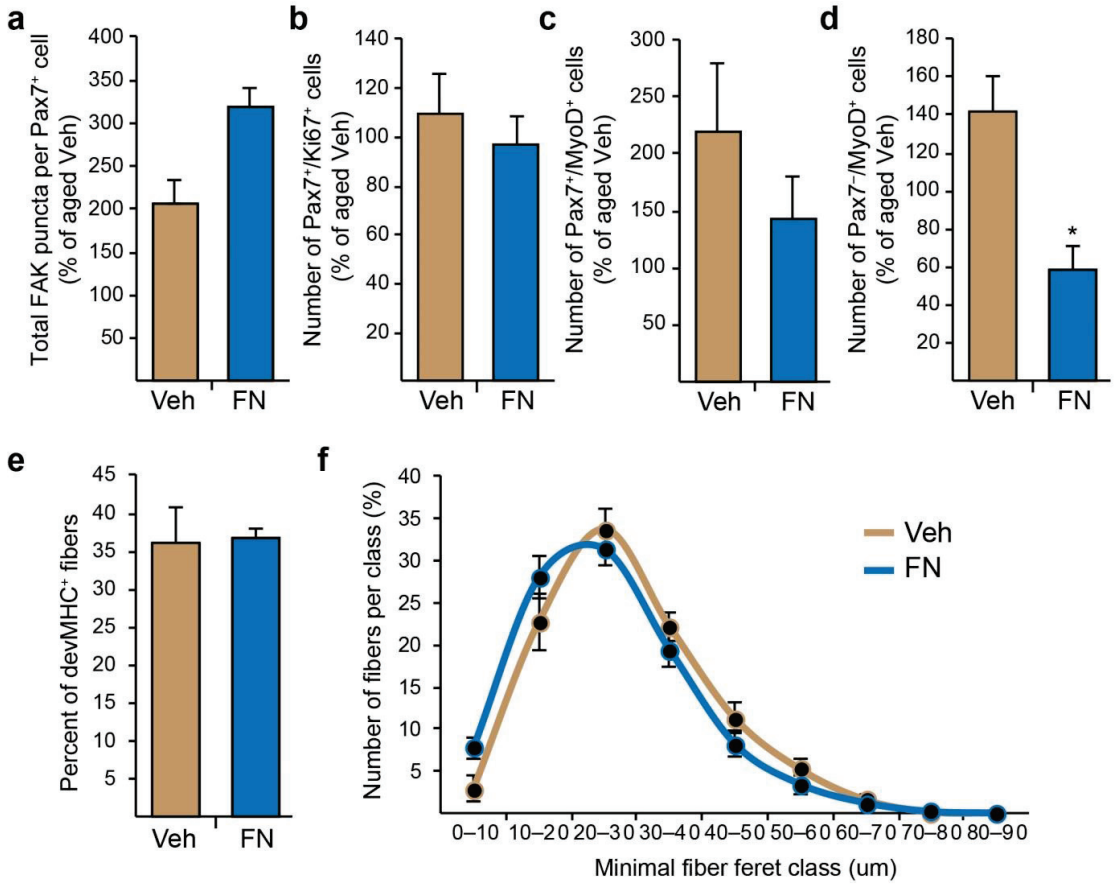
Supplementary figure 3 Quantification of western blot grey values for figure 3a. Grey values were background corrected and normalized to actin. Bars represent means + s.e.m.. *n* = 3 cell lysates from independent experiments per condition. One–way ANOVA followed by Bonferroni post–test. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.



Supplementary figure 4 Quantification of western blot grey values for figure 4g. Grey values were background corrected and normalized to GAPDH. Bars represent means + s.e.m.. *n* = 3 cell lysates from independent experiments per condition. Student's *t*–test *P* value is * $P < 0.05$.



Supplementary figure 5 Adhesion capacity of freshly isolated aged and young MuSCs. Cells were analyzed 3 h, 6 h and 36 h after seeding on COL, FN or LAM. Bars represent means + s.e.m.. *n* = 3 mice. two–way ANOVA followed by Bonferroni post–test. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.



Supplementary figure 6 FN treatment has limited effects on muscle regeneration in young mice. (**a**) Quantification of FAK levels in Pax7^{+} MuSCs in tissue sections of vehicle (Veh) or FN treated young mice at 5 d.p.i. (**b–d**) Numbers of Pax7 and Ki67 positive (Pax7^{+}Ki67^{+}) cells, Pax7 and MyoD (Pax7^{+}MyoD^{+}) positive cells, and Pax7 negative MyoD positive (Pax7^{-}MyoD^{+}) cells per area in muscle cross sections of Veh or FN treated young mice at 5 d.p.i. (**e**) Quantification of the percentage of devMHC^{+} fibers in muscles of young Veh or Col treated mice at 7 d.p.i. (**f**) Fiber size quantification from young Veh or FN treated mice at 7 d.p.i. Throughout, bars and data points represent means + s.e.m. and ± s.e.m., respectively. n = 3 (**c** FN), n = 4 (**a**, **b**, **c** Veh, **d**) or n = 5 (**e, f**) mice per condition. * $P < 0.05$. Student's t–test.

CHAPTER V. Aging disrupts the communication between fibro-adipogenic progenitors and muscle stem cells by inhibiting the production of WISP-1

Rationale

Very little is known about the mechanisms of ectopic adipogenesis associated with aging. Since we hypothesized in **Chapter III** that transient ectopic adipogenesis is a hallmark of regeneration (Lukjanenko et al., 2013) and it is recognized that regenerative capacities are reduced with age (Brack and Munoz-Canoves, 2015), we asked how aging modulates the response to ectopic adipogenesis in the context of regeneration. Those questions are not only important for the general understanding of muscle regenerative biology, but exploring the mechanisms of muscle regeneration and ectopic adipogenesis during aging also appears as a significant approach to evaluate the cross-communications taking place between niche cells with age.

FAPs are at the origin of ectopic adipocytes arising during muscle regeneration, and they cross-talk with satellite cells in the first days following muscle injury. It is thought that FAP differentiation in adipocytes is inhibited by regenerating fibers through cell-cell contacts (Uezumi et al., 2010), potentially explaining why ectopic adipogenesis or fibrosis is important in models where regeneration is inefficient (Murphy et al., 2011, Sambasivan et al., 2011, Uezumi et al., 2010). Conversely, FAPs very likely support myogenesis throughparacrine signaling (Fiore et al., 2016, Im et al., 2014, Mozzetta et al., 2013, Joe et al., 2010). Nevertheless, the secreted signaling molecules participating in the cross-talk between satellite cells and FAPs remain to be identified. In addition, it is debated whether FAPs support satellite cell proliferation or differentiation (Fiore et al., 2016, Joe et al., 2010, Mozzetta et al., 2013), and whether FAPs can modulate other features of satellite cell function (activation, self-renewal, migration…) has never been studied. In addition, no study has explored the possibility to intervene on niche-resident cells to ameliorate satellite cell function in the context aging. In order to answer these open questions, we tested the detailed role of FAPs in supporting myogenesis and how aging influences the function of FAPs and their cross-talk with satellite cells. In a second step, we searched for novel therapeutic strategies to restore the cross-talk between aged FAPs and satellite cells to ameliorate muscle regeneration. WISP1 was identified as a matricellular protein secreted by FAPs upon injury and lost during aging. WISP1 was previously known to promote progenitor cell

proliferation and suggested to play a role in tissue repair in other tissues (Maiese, 2014), but its role in skeletal muscle was, however, unknown. We therefore interrogated whether WISP1 could mediate the communication between satellite cells and FAPs, and thereby promote muscle regeneration.

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My contribution

I designed, conducted and interpreted all the in vivo, molecular, cellular and histological analyses of this work with the technical support of Sonia Karaz. I also interpreted the bioinformatic analyses of the genomics data performed by Eugenia Migliavacca, and wrote manuscript.

Publication

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Aging Disrupts the Communication between Fibro-Adipogenic Progenitors and Muscle Stem Cells by Inhibiting the Production of WISP-1

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Environmental and molecular triggers leading to loss of regenerative function in aged skeletal muscle is largely studied in muscle stem cells. In contrast, the impact of aging on the cross-communication between muscle stem cells and other niche-resident cell types, in particular the fibro/adipogenic progenitors (FAPs), remains largely unknown. In this study, we demonstrate that aging severely affects intrinsic FAP function, and impairs their ability to support the myogenic function of satellite cells. Through transcriptomic profiling of aged FAPs, we identify WISP1 as a novel matricellular protein secreted by FAPs upon muscle injury and lost during aging. WISP1 directly regulates satellite cell function by promoting their early adhesion and proliferation. Treatment of aged mice with WISP1 reconstitutes the muscle stem cell niche and rescues muscle regeneration. Altogether, our results demonstrate that altered cellular communication from FAPs to satellite cells plays an important role in aging of the stem cell niche and can be targeted via WISP-1 to prevent age-related regenerative failure.

Skeletal muscle regenerative capacity relies on the activity of tissue-resident stem cells called satellite cells. As a consequence of aging, the regenerative function of satellite cells is dramatically reduced, leading to impaired muscle repair upon injury [1-3]. The functional decline of satellite cells with age most often leads to a decline in the number of satellite cells. Aged satellite cells have decreased activation, adhesion, migration, proliferation and self-renewal, and gradually switch to a senescent phenotype, that further exacerbates the exhaustion of the satellite cell pool [4-9]. Many molecular and metabolic perturbations have been described in satellite cells as determinants of their functional impairments. These include decreased autophagy and mitochondrial functions [10, 11], and constitutive activation of the p38-mitogen-activated protein kinase (MAPK), ERK/MAPK and JAK/STAT signaling pathways [9, 12-14]. However, satellite cells are also responsive to triggers coming from the systemic or paracrine environment. For example, several positive extra-cellular regulators of satellite cell function such as Notch ligands or oxytocin are lost with age [6, 15, 16]. In contrast, aging also induces the production of several secreted proteins, such as pro-inflammatory cytokines, fibroblast growth factor (FGF)-2, Wnt ligand C1q, or transforming growth factor (TGF-) β [9, 13, 16-19], which alter the activation of satellite cells from quiescence to proliferation and self-renewal and thereby adversely affect muscle regenerative capacity. The inability to transiently remodel the extra-cellular matrix of the satellite cell niche during regeneration also impairs satellite cell function and regeneration during aging [7, 20], highlighting that other cell types also contribute to the functional decline of satellite cells with age.

In the muscle stem cell niche, satellite cell are surrounded by muscle resident cells such as endothelial cells, pericytes, fibroblasts, immune and mesenchymal cells [21, 22]. However, the cellular communication between satellite cells and the different cell types is only partly understood. It is known that satellite cells communicate with peri-endothelial and endothelial cells in the niche to regulate both satellite cell proliferation and angiogenesis [23]. The cross-talk between satellite cells and the fibrogenic lineage has been largely reported and is critical for proper regeneration [24]. In particular, the fibro/adipocyte progenitors (FAPs) are $CD34^+/Sca1^+/PDGFR\alpha^+/Integrin\ \alpha7^-$ mesenchymal progenitor cells residing in the skeletal muscle stem cell niche between myofibers [25, 26]. FAPs are adipogenic *in vitro* and *in vivo* but can also differentiate into collagen-

l producing cells [25-29]. While their pathogenic differentiation to fat or fibrosis is believed to be regulated by a specific permissive micro-environment of diseased muscle [26], FAPs also positively participate to muscle repair by supporting myogenesis [25, 30, 31]. Although no PDGFRα-lineage ablation has been reported so far to characterize the role of FAPs in muscle homeostasis and repair *in vivo*, the supportive role of FAPs on myogenesis and regeneration has been inferred from ex vivo co-cultures wih satellite cells. FAPs activate upon muscle injury in the same time frame as satellite cells, and support their myogenic function [21, 25]. In addition, other adipogenic or fibrogenic lineages also participate to muscle regeneration as ablation of the AP2 adipogenic lineage or the Tcf4^{+} fibrogenic lineage have both lead to impaired muscle regeneration [27, 32]. We have previously shown that transient ectopic adipogenesis is a hallmark of several models of efficient muscle regeneration [33], re-enforcing the fact that the activity of FAPs is important for regeneration. A recent study has also demonstrated that limiting FAP expansion through nilotinib treatment (a tyrosine-kinase inhibitor acting downstream of TGF-β receptor) resulted in reduction of myoblast expansion in a non-cell autonomous way. This was attributed to defects in cross-talk of FAPs with satellite cells, leading to impaired proliferation. While increasing evidence from recent reports suggests that FAPs exert their support to satellite cells in a paracrine manner, the signaling molecules mediating this beneficial cross-talk remain unknown [30, 31, 34].It is also debated whether FAPs support satellite cell proliferation or differentiation. This highlights that the cross-talk between satellite cells and FAPs is emerging but poorly understood, and that the impact of aging on this cross-talk remains largely unknown.

In this study, we demonstrate that FAP activity is impaired during aging and that aged FAPs fail to efficiently support satellite cell function and myogenesis. The matricellular secreted protein WISP1 was identified as a secreted mediator between FAPs and satellite cells during regeneration, which is lost in aged FAPs. WISP1 treatment rescued satellite cell function and regenerative capacity in old mice, demonstrating that cellular cross-talks in the muscle stem cell niche can be targeted therapeutically to recover the regenerative failure associated with aging.

RESULTS

Aging causes an intrinsic dysfunction of FAPs.

Aging profoundly delays muscle regeneration, and adversely affects satellite cell function [2, 3, 7]. As previously reported, we observed both a decline in the number of satellite cells with age (**Fig. 1a**) as well as intrinsic defects of freshly-sorted old satellite cells (**Fig. 1b-e**). Activation of aged satellite cells is altered through slower entry into the first cell cycle (**Fig. 1b**) and through delayed transition to myogenic commitment (**Fig. 1c**) 36 hours after isolation from non-injured muscles. As a consequence, the proliferation of old satellite cells is also decreased after their activation (**Fig. 1d**), and their differentiation potential declines (**Fig. 1e**).

Fibro/Adipogenic Progenitors (FAPs) play an important role in the muscle stem cell niche and the timed regulation of their proliferation and apoptosis is key to muscle regeneration following an injury [25, 26, 37]. In order to better understand how aging influences the cooperation of muscle stem cells with their environment, we asked whether altered FAP function could participate to the loss of regenerative capacity in the aged muscle stem cell niche. The number of FAPs quantified by flow cytometry increased in the tibialis anterior muscle of aged animals (**Fig. 1f**). When isolated from non-injured muscles and analyzed *ex vivo*, aged FAPs showed a dramatic reduction in their proliferative capacity (**Fig. 1g**). Under specific physiological cues, FAPs can differentiate to the adipogenic or fibrogenic lineages [25, 26, 29]. Differentiation of old FAPs to mature lipid-droplet expressing adipocytes was impaired compared to young FAPs, both in adipogenic medium (**Fig. 1h**) and spontaneously (**Supp. 1**). In contrast, old FAPs had a fibrogenic differentiation in α-smooth muscle actin positive cells similar to young FAPs (**Fig. 1i**), suggesting that aging differentially affects certain cellular fate of FAPs or different subpopulations of FAPs.

FAPs can give rise to ectopic adipocytes between muscle fibers during regeneration [25, 26], and transient ectopic adipogenesis is a hallmark of muscle regeneration in various models of muscle injury [33]. In order to assess how FAP function correlates with ectopic adipogenesis during aging, we performed a time-course of glycerol-induced muscle regeneration in young and old mice, and analyzed adipocyte formation by Oil-red-O and perilipin stainings (**Fig. 2a-c**). As expected, we observed a progressive formation of adipocytes during muscle regeneration, reaching its maximum at 14 days post injury (dpi) in young mice (**Fig. 1b**). Consistent with the altered function of aged FAPs ex vivo, ectopic Oil Red O-positive adipocyte formation in regenerating muscle was strongly blunted in old mice at all time points analyzed. This result was further confirmed by the quantification of perilipin-positive adipocytes, which demonstrated lower adipocyte infiltration in aged muscle at 14dpi (**Fig. 1c-d**), and were replicated in an additional independent cohort of young and old regenerating muscles (results not

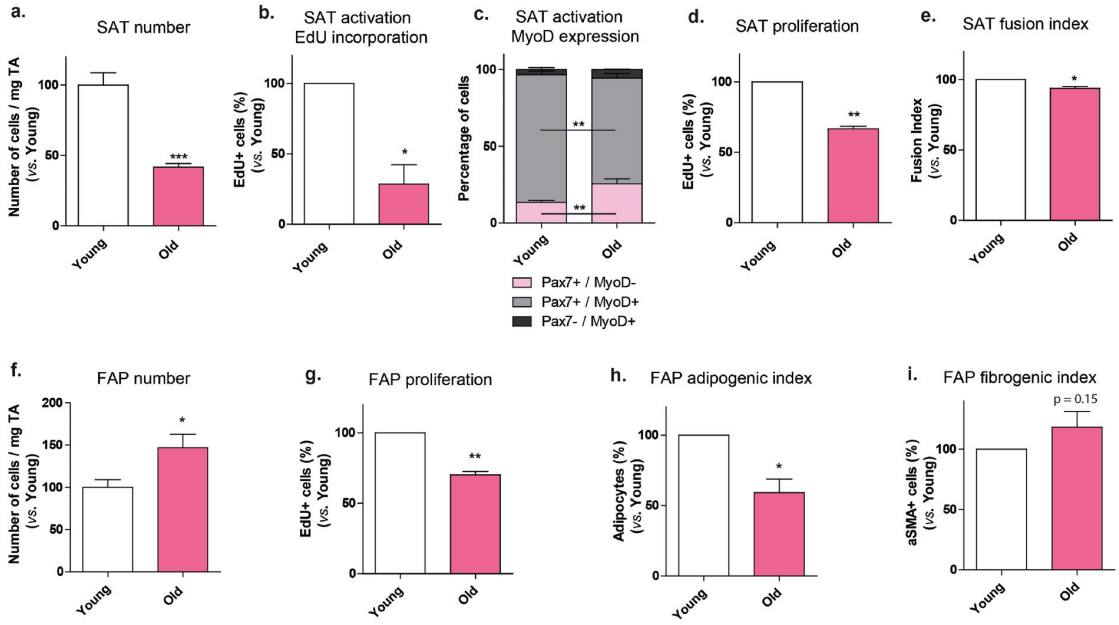

Figure 1. Intrinsic alterations of aged FAPs and satellite cells ex vivo. (a) Quantification of the number of satellite cells (SATs) sorted from the tibialis anterior (TA) muscle of young (9-13 week-old) and old (20-22 month-old) mice. **(b-c)** Young *vs*. aged satellite cell *ex vivo* activation capacity assessed by EdU incorporation (b) or MyoD expression (c) 36h after isolation. **(d)** Proliferation capacity and **(e)** fusion index of young *vs*. aged satellite cells, 3 and 6 days after isolation, respectively. **(f)** Quantification of the number of FAPs sorted from TA muscle of young (9-12 week-old) and old (20-22 month-old) mice. **(g)** Young *vs*. aged FAP *ex vivo* proliferation capacity assessed by EdU incorporation 6 days after isolation. **(h)** Young *vs*. aged FAP adipogenic differentiation capacity after 7 days in adipogenic differentiation medium. **(i)** Young *vs*. aged FAP fibrogenic differentiation capacity after 6 days in growth medium. p: p-value, *: p-value *vs*. age/genotype respective control < 0.05, **: p-value *vs*. age/genotype respective control < 0.01, ***: p-value old *vs*. young < 0.001. n=3 (b, c, d, e, g, h, i), n>5 (a-f) independent biological replicates.

shown). Altogether, these results reveal that intrinsic FAP function declines with age both *in vivo* and *ex vivo*.

Old FAPs are less efficient to support satellite cell myogenic function.

FAPs are activated upon injury, and can support satellite cell function [25, 30, 31], although their exact function on the different phases of satellite cell activation, proliferation and commitment remains unclear. To investigate the support function of FAPs at each stage of satellite cell commitment, we isolated satellite cells from tdTomato (Td) mice, which constitutively express a nuclear TdTomato fluorescent protein under a CAG promoter [35], and tracked their fate with the fluorescent reporter when co-cultured with wild-type (WT) FAPs, or satellite cells as a control (**Fig. 3a**). As we recently showed that satellite cell adhesion to the extra-cellular matrix prevents cell death by anoikis in the early steps of activation [7], we investigated the potential role of FAPs on satellite cell function at early time points after isolation. Strikingly, we observed a significant increase (35%) of adhering Td^+ satellite cells after 12h of co-culture with FAPs compared to Td^+ satellite cells cultured alone or with WT satellite cells, demonstrating that FAPs support satellite cell adhesion during early activation (**Fig. 3b**). FAPs did not increase the entry of Td+ satellite cells into cell cycle at 36h (**Supp. 2a**), and FAPs did not affect the proportion of $Pax7^+$ satellite cells activating MyoD expression (results not shown). When the effect of FAPs on Td+ satellite cell function was analyzed after 3 days of co-culture, we observed that FAPs did not influence the rate of satellite cell proliferation (**Supp. 2b**), but maintained a positive effect on the total satellite cell pool as a consequence of early adhesion and survival (**Fig. 3c**). The co-culture with FAPs did not affect the rate of Td+ cell fusion into myotubes (**Supp. 2c**), but increased the total number of Td+ nuclei in differentiated cells expressing myosin heavy chain (**Fig. 3d**). Altogether, these results indicate that FAPs support satellite cells by increasing their adhesion and survival at the early phases of activation, and thereby enhance myogenic differentiation.

To understand how aging of FAPs influences their myogenic support to satellite cells, we co-cultured freshly isolated Td^+ satellite cells in the presence of young and old FAPs during 12

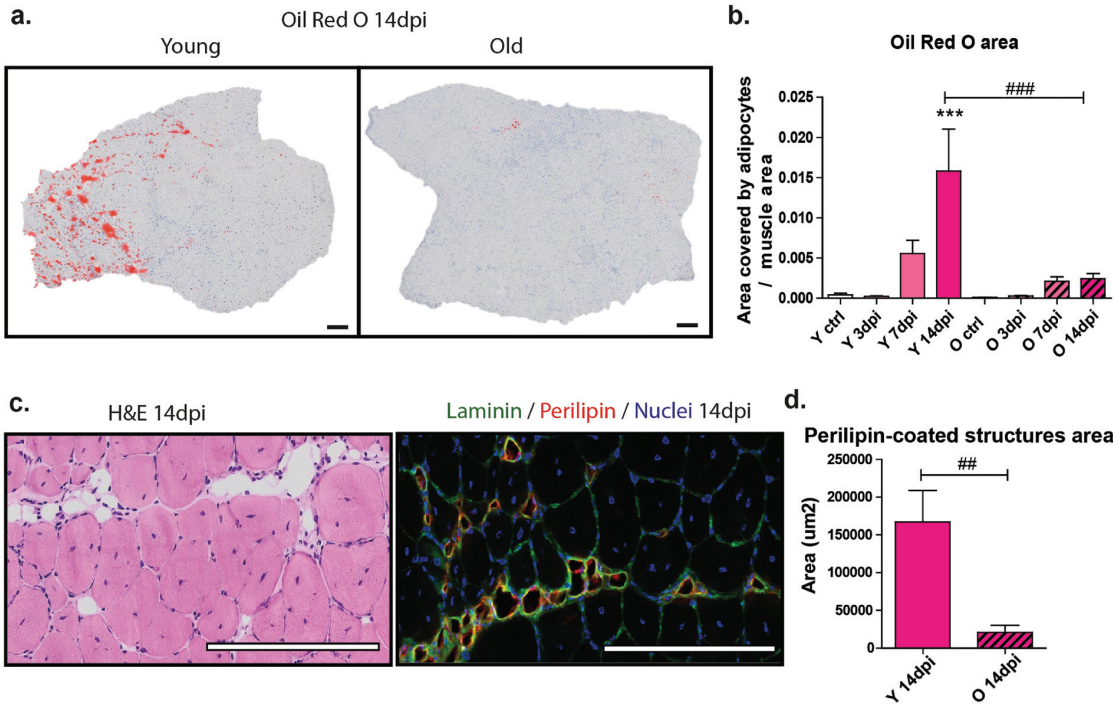

Figure 2. Transient ectopic adipogenesis during muscle regeneration is reduced in old mice. Tibialis anterior muscles from young (Y) and old (O) wild-type mice **(a-d)** were injured using an intramuscular injection of glycerol and collected at 3, 7 or 14 days post injury (dpi). **(a)** Representative oil-red O staining of muscle sections at 14 days post-injury, young (left) and old (right) **(b)** Quantification of area covered by oil-red O structures at 14 dpi in young and old muscles. **(c)** Hematoxylin and eosin (H&E) (left) and perilipin (right) stainings of young muscle sections at 14 dpi (Red = perilipin, Green = laminin, Blue = hoechst). **(d)** Quantification of area covered by perilipin-delimited adipocytes at 14dpi in young and old muscles. In (b,d), ***: p-value *vs*. age respective control < 0.001; ##: p-value old *vs*. young at respective time points < 0.01; ###: p-value old *vs*. young at respective time points < 0.001; n>7 mice per group. Scale bar = 200µM.

hours, and quantifying the number of Td^+ satellite cells (**Fig. 3e**). The ability of old FAPs to support satellite cell expansion was decreased by 39% compared to young FAPs. In order to further model the cross-talk of age-matched satellite cells and FAPs, we co-cultured young and old WT satellite cells either alone, or in the presence of young and old FAPs throughout the whole myogenic differentiation program *ex vivo*. Similar to what happens during the phase of satellite cell adhesion and activation (**Fig. 3e**), old FAPs partially lost their support on myogenesis compared to young FAPs (**Fig. 3f**). Conversely, young FAPs were able to partially rescue the myogenic defects of old satellite cells, demonstrating that satellite cell dysfunction with age can be reversed through cellular interactions with a youthful stem cell niche. Interestingly, old FAPs maintained some capacity to support the function of aged satellite cells, but only to a partial extent compared to young FAPs. (**Fig. 3f**). Thus, these results demonstrate that FAPs present intrinsic impairments in their ability to support myogenesis during aging, but still participate to a beneficial cellular communication with satellite cells in the aged stem cell niche.

WISP1 is a matricellular molecule secreted by activated FAPs which is lost during aging.

In order to understand how FAPs and satellite cell cross-talk in the aged stem cell niche, we first asked whether FAPs could support satellite cell function through soluble factors by comparing direct co-cultures of FAPs and satellite cells, and satellite cells grown alone in medium conditioned by freshly isolated young FAPs or satellite cells (**Fig. 4a**). FAP conditioned medium was sufficient to mimic the direct co-culture with FAPs and increased the number of differentiated satellite cells, whereas conditioned medium from satellite cells had no autocrine effect (**Fig. 4a**).Thus, the beneficial effects of FAPs on myogenesis are mediated, at least in part, by secreted factors produced by FAPs and sensed by satellite cells. To uncover molecular candidates mediating the beneficial effects of FAPs in the context of aging,young and old FAPs and satellite cells were isolated from either non-injured

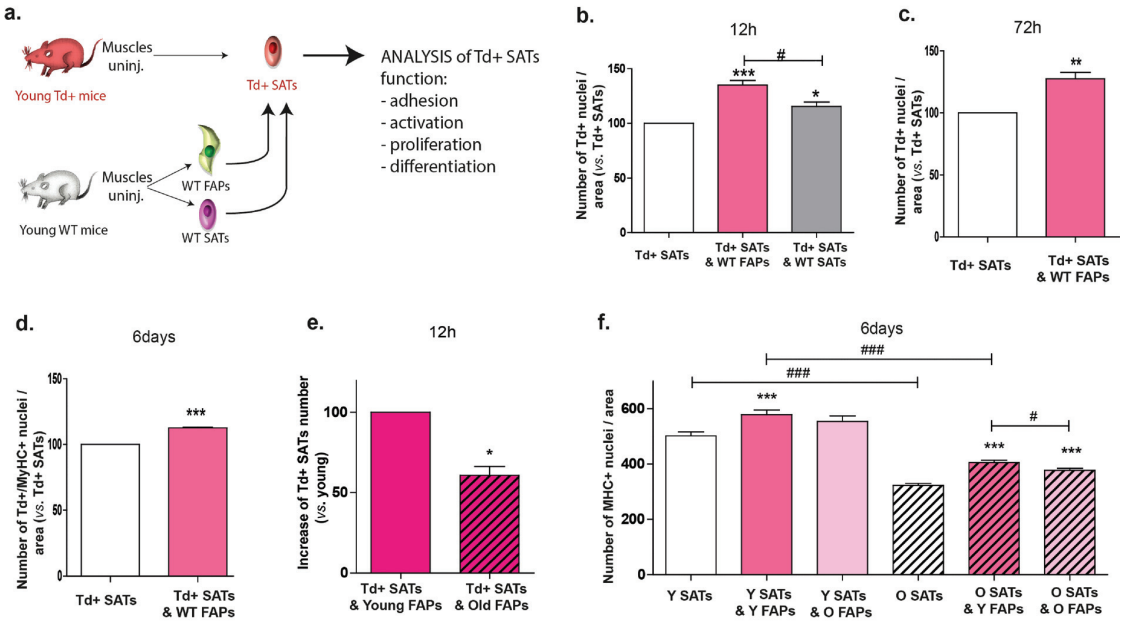

Figure 3. The myogenic support of FAPs to satellite cells is impaired with age. (a) Study design for (b-d). Satellite cells were freshly isolated from young Td-Tomato+ (Td+) non-injured muscles and seeded alone or together with freshly isolated wild-type (WT) FAPs or satellite cells. **(b)** Number of adhering Td+ satellite cells 12h after seeding. **(c)** Proliferation capacity of Td+ satellite cells 3 days after seeding assessed by EdU incorporation over the last 3h. **(d)** Differentiation capacity of Td+ satellite cells assessed by the total number of Td+ nuclei in MHC+ cells. **(e)** Number of young Td-Tomato+ (Td+) satellite cells co-cultured with freshly isolated young or old WT FAPs 12h after sorting. **(f)** Number of differentiated young and old WT satellite cells alone or in co-culture with young or old FAPs. (b-e): n=3, *: p-value *vs*. Td+ SATs < 0.05; **: p-value *vs*. Td+ SATs < 0.01; ***: p-value *vs*. Td+ SATs < 0.001; #: p-value Td+ SATs & WT FAPs *vs*. Td+ SATs & WT SATs < 0.05. (f): ***: p-value *vs*. SATs alone of the respective age < 0.001; #: p-value SATs & O FAPs *vs*. SATs & Y SATs < 0.05; ###: p-value O SATs (or O SATs & O FAPs) *vs*. Y SATs (or Y SATs & Y FAPs) < 0.001. Representative graph of two independent experiments performed with cells pooled from several mice.

muscles (where they are in a non-proliferative quiescent state), or muscles that were injured 3 days before (where they are highly activated), and subjected to transcriptomic analysis (**Fig. 4b**). We first analyzed the gene ontology (GO) terms enriched in FAPs *vs*. satellite cells, to assess biological function specific to each cell type. Transcriptomic signatures differentiating satellite cells and FAPs isolated from non-injured muscles were strongly enriched in "organ development", "cell adhesion", and "extracellular matrix organization" development terms (**Supp. Table 1**). Interestingly, it appeared that most of the differentially regulated genes from the "extracellular matrix" GO term 0031012 were upregulated in FAPs (**Supp. 3**). In particular, FAPs were strongly enriched in numerous collagen and laminin genes (Col16a1, Col15a1, Col6a1, Col5a1, Col4a2, Col4a1, Col14a1, Col3a1, Col1a2, Col6a2, Lama2, Lamb1-1, Lamc1), potentially participating to satellite cell adhesion in direct co-cultures. We also noticed that FAPs were enriched in signaling molecules such as Fgf10, Wnt5a, Igf1, and Tgf-beta-like genes. On the opposite, satellite cells were significantly enriched in Wnt4, Wnt6 and Integrin beta 4 receptor, amongst others. The transcriptomic signature of aging FAPs strongly affected many biological functions, such as "epithelial cell proliferation", "angiogenesis", and "TGF-beta receptor signaling" (**Supp. Table 2**), confirming that aging alters the physiology and cellular functions of FAPs.

Given that FAPs communicate with satellite cells through secreted factors, we next examined which signaling proteins were secreted by activated FAPs. Out of the 321 genes significantly upregulated with a fold-change > 2 during activation of young FAPs, we identified 20 signaling proteins (**Fig. 4c-d**). In order to understand why aged FAPs fail to efficiently support satellite cell function and myogenesis in the aged niche, the genes upregulated in activated FAPs were filtered for differential regulation with age and activation. 10 genes were induced by at higher levels in young than aged activated FAPs, out of which one encodes a secreted protein: Wnt1 inducible signaling pathway protein 1 (WISP1). WISP1 was also the most down-regulated gene when old activated FAPs were directly compared to young activated FAPs

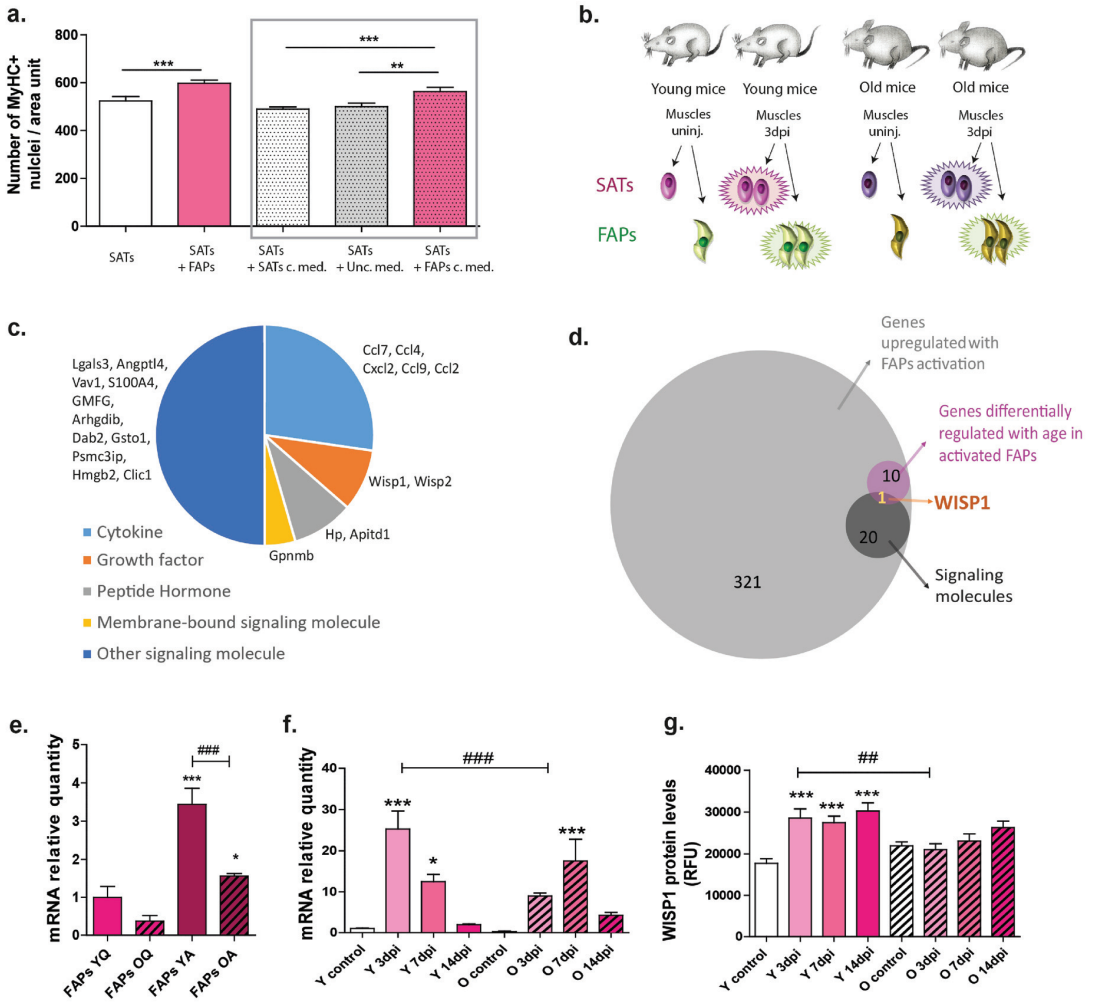

Figure 4. Transcriptomic profiling identifies WISP1 as a novel protein secreted by activated FAPs and down-regulated with age. (a) Young WT satellite cells differentiation alone (white), in direct co-culture with FAPs (pink) or when cultured in medium conditioned by satellite cells (gridded white), FAPs (gridded pink) or no-cells (gridded gray). Representative graph of three independent experiments performed with cells pooled from several mice. **(b)** Study design for (c-d): satellite cells and FAPs were freshly isolated from non-injured muscles (non-proliferating / quiescent-like cells) or 3 days after muscle injury (activated cells), of young (9-13 week-old) and old (20-22 month-old) mice, and subjected to genome-wide transcriptomic profiling. n>5. **(c)** Pie chart of sub-classification of the secreted proteins upregulated during FAP activation. **(d)** Venn diagram of all genes upregulated during FAP activation, and of genes differentially upregulated during activation with age. These gene lists were then filtered for genes encoding proteins annotated as "signaling molecules" in PantherDB. **(e)** WISP1 mRNA expression by qPCR in freshly isolated FAPs from young (Y) and old (O) mice, either from uninjured muscles (quiescent cells, or Q) or 3 days after muscle injury (activated cells, or A). Data are normalized to YQ FAPs; n>4. **(f)** WISP1 mRNA expression by qPCR in TA muscle of young and old mice without injury or 3, 7 and 14 days post injury (dpi). Data are normalized to young non-injured muscles (control), n=8. **(g)** WISP1 protein level in regenerating muscles. n=8 mice per group. For (a,e,f,g), *: p-value *vs.* quiescent/control of respective age/culture condition < 0.05, **: p-value *vs.* quiescent/control of respective age/culture condition < 0.01, ***: p-value *vs.* quiescent/control of respective age/culture condition < 0.001, ##: p-value old *vs.* young at respective time points < 0.01, ###: p-value old *vs.* young at respective time points < 0.001.

independently of the levels during quiescence (**Supp. Table 3**). WISP1 is a secreted extra-cellular matrix associated protein, also known as a connective tissue growth factor (CTGF), and is the 4th member out of 6 of the CCN family of matricellular proteins ("Connective tissue growth factor, Cystein rich protein, and Nephroblastoma overexpressed gene" family). WISP1 has been shown to regulate various biological processes such as neuronal development and neurogenesis, adipose-, mesenchymal- or cancer-stem cell proliferation, cell survival and angiogenesis

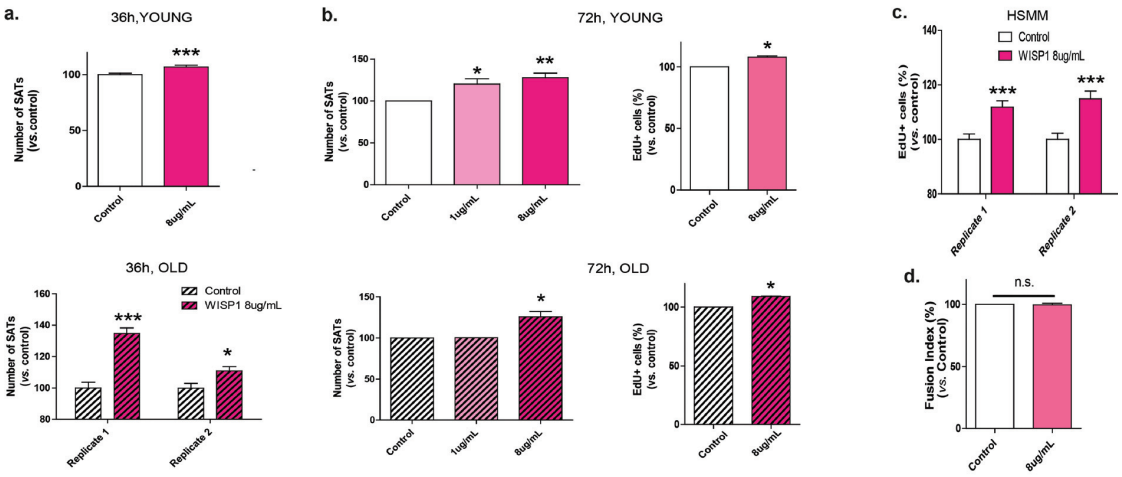

Figure 5. WISP1 improves satellite cell function ex vivo. Adhesion **(a)** and proliferation **(b)** of satellite cells freshly sorted from young and old mice and treated with WISP1 *ex vivo*. n=1 (a, young; b, old, number of SATs, 1µg/mL), n=2 (a, old; b, young, number of SATs, 1µg/mL; b, old, EdU+ cells), n=3 (b, old, number of SATs, 8µg/mL; b, young, number of SATs), n=4 (b, young, number of SATs, 8µg/mL) biological replicates. **(c)** Proliferation of human skeletal muscle myoblasts (HSMM) assessed by EdU incorporation over the last 5h after a 3 day treatment with WISP1. n=2 independent experiments. **(d)** Young mouse satellite cells were differentiated and treated with WISP1 during 48h and the fusion index was defined as the percentage of nuclei in MHC positive cells. n=3 independent experiments with independent biological replicates. Data are normalized to non-treated cells, n.s: p-value *vs*. PBS > 0.05, *: p-value *vs*. PBS < 0.05, **: p-value *vs*. PBS < 0.01, ***: p-value *vs*. PBS < 0.001.

(Reviewed in [38]). A role of WISP1 has been described in the musculoskeletal system for bone development and fracture repair [39, 40], and in regulation of cartilage homeostasis [41]. However, the role of WISP1 in skeletal muscle remains unknown.

To validate the differential expression of WISP1 in FAPs and interrogate whether it is also expressed by other cell types of the muscle stem cell niche upon injury, we repeated the isolation of satellite cells and FAPs from young and old non-injured muscles or muscles at 3dpi. We also isolated the lineage positive cells (Lin^+), comprising immune and hematopoietic cells, together with endothelial cells; which represent an active and very abundant cell population in the muscle stem cell niche after injury [7]. We also included $Sca1^+/CD34^+/PDGFR\alpha^-$ cells (hereafter called $PDGFR\alpha^-$), potentially comprising the myogenic PICS, and various pericyte populations [42-44]. As expected, we validated by qPCR that WISP1 was strongly upregulated during activation in young FAPs, but that this induction was blunted in aged FAPs (**Fig. 4e**). $PDGFR\alpha^-$ cells and satellite cells also expressed WISP1 in the non-proliferative state with a smaller upregulation upon injury (**Supp. 4a**). Lin^+ cells, however, expressed only very low levels. Altogether, these results showed that activated FAPs are the major producers of WISP1 in muscle, and this expression is strongly affected with age. Notably, we observed a strong decrease of WISP1 mRNA levels in old uninjured muscle compared to young (**Supp. 4b**). In addition, as WISP1 has previously been identified in the systemic circulation, we evaluated WISP1 content in the serum of aged sarcopenic rats [45], and observed a strong reduction of circulating WISP1 protein levels during aging (**Supp. 4c**). Thus, WISP1 concentration is strongly decreased with age at the cell, tissue, and systemic level. mRNA expression of WISP1 in the entire muscle was also strongly upregulated after injury, and gradually recovered during the time course of muscle regeneration (**Fig. 4f**). Similar to what was observed in FAPs, the induction of WISP1 mRNA was blunted and delayed in aged muscle. At the protein level, WISP1 was also upregulated in young muscles after injury, with high levels persisting until 14dpi, and this upregulation was strongly delayed and significantly reduced in old regenerating muscles (**Fig. 4g**). Altogether, these results demonstrate that WISP1 is a novel secreted protein produced by FAPs, whose expression is induced during muscle regeneration but blunted during aging.

WISP1 enhances satellite cell function and ameliorates muscle regeneration in old mice.

In order to test if the production of WISP1 by FAPs could be sensed by satellite cells to promote their activity during regeneration, we tested whether WISP1 could modulate satellite cell adhesion and proliferation as CCN proteins

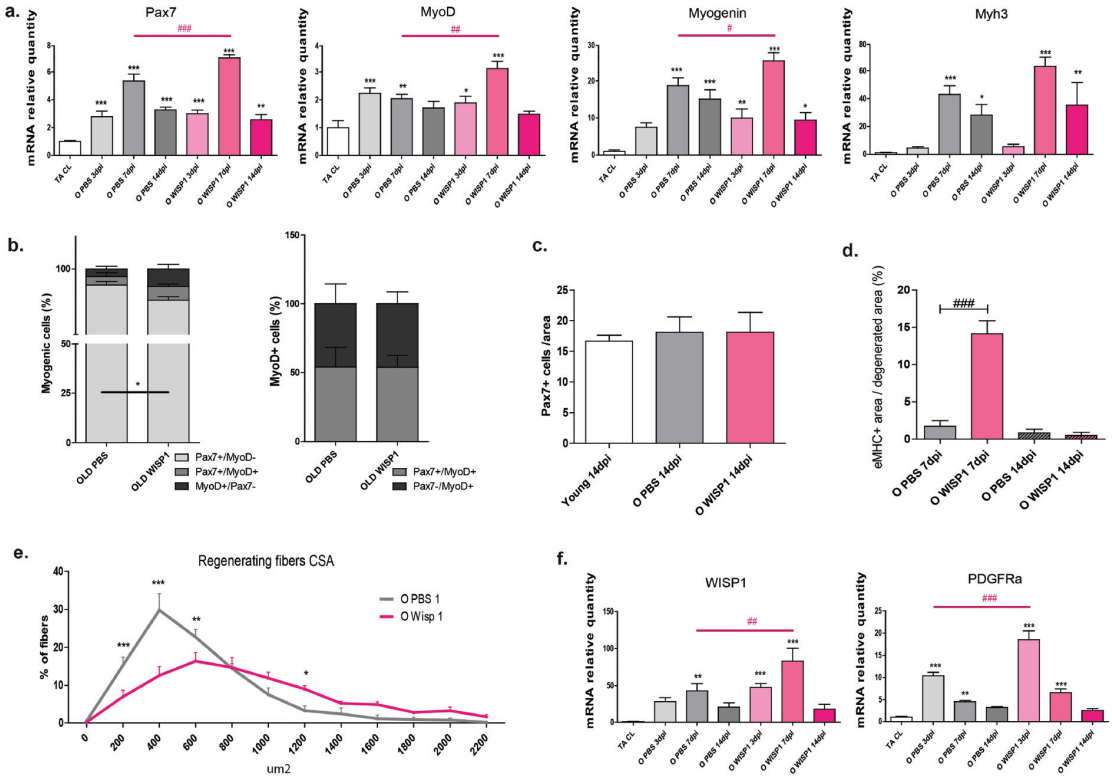

Figure 6. WISP1 treatment in vivo rejuvenates muscle regeneration in old mice. (a) qPCR of different myogenic markers (Pax7, MyoD, myogenin, Myh3) in regenerating muscles of old mice treated daily with PBS or 1mg/kg/day of WISP1 during 3, 7 and 14 days post injury (dpi). **(b)** Classification of satellite cells in the injured area based on their Pax7 and MyoD expression by immunohistochemistry at 7dpi. **(c)** Quantification of Pax7 positive cells in muscle sections at 14dpi. **(d)** Quantification of the area covered by regenerating embryonic myosin heavy chain (eMHC) positive fibers in muscle sections at 7 and 14dpi. **(e)** Distribution of the cross-sectional area of regenerating myofibers at 7dpi quantified from a histological staining for laminin. **(f)** PDGFRα and WISP1 expression assessed by qPCR in regenerating TA muscles. (a, e) Data were normalized to gene expression in contralateral non-injured tibialis anterior muscles (TA CL), *: p-value *vs*. TA cl < 0.05, **: p-value *vs*. TA cl < 0.01, ***: p-value *vs*. TA cl < 0.001; #: p-value WISP1 *vs*. PBS treated mice < 0.05, ##: p-value WISP1 *vs*. PBS treated mice < 0.01, ###: p-value WISP1 *vs*. PBS treated mice < 0.001. (b) *: p-value O WISP1 *vs*. O PBS for the indicated fiber size class < 0.05, **: p-value O WISP1 *vs*. O PBS for the indicated fiber size class < 0.01, ***: p-value O WISP1 *vs*. O PBS for the indicated fiber size class < 0.001. (c) ###: p-value WISP1 *vs*. PBS treated mice < 0.001. (d) *: p-value WISP1 *vs*. PBS treated mice < 0.05. n>4.

have already been reported to regulate adhesion, proliferation and cell-survival in other contexts [38]. Young and old satellite cells were freshly isolated and treated with WISP1 *ex vivo* during adhesion, activation or proliferation. 36h hours after isolation, WISP1 increased satellite cell adhesion in both young and aged satellite cells (**Fig. 5a**), and this effect was more prominent in old satellite cells that have a high propensity for cell death by anoikis during this time frame [7]. WISP1 did not accelerate the activation of young and old satellite cells into the cell cycle (results not shown), but the effects of WISP1 on satellite cell expansion were maintained until the proliferation phase, 3 days after isolation, both in young and old satellite cells (**Fig. 5b left**). WISP1 treatment also enhanced satellite cell proliferation after activation as demonstrated by the higher incorporation of EdU in young and aged satellite cells treated with WISP1 (**Fig. 5b right**). The proliferation of human primary myoblasts (HSMM) was also enhanced by WISP1 treatment (**Fig. 5c**), demonstrating that WISP1 has the potential to also enhance muscle stem cell function in humans. To sustain an efficient myogenic function, satellite cells must have the possibility to exit the cell cycle and commit to differentiation. Importantly, WISP1 did not impair differentiation capacity as the fusion index of satellite cells treated with WISP1 was not altered (**Fig. 5d**). Altogether, these results demonstrate that WISP1 enhances young and old satellite cell function *ex vivo*.

To investigate if WISP1 could also ameliorate satellite cell function *in vivo*, we injured tibialis anterior muscle of old mice, treated the

mice by intraperitoneal injections of either WISP1 or vehicle (PBS) and analyzed the regenerating muscles at 3, 7 and 14 days after injury. Satellite cell expansion during regeneration is delayed in aged mice and peaks 7 days after injury. The mRNA levels of Pax7, MyoD and Myogenin were significantly higher in WISP1 treated muscles than in PBS treated muscles at 7dpi, demonstrating that satellite cell function is enhanced upon WISP1 treatment (**Fig. 6a**). Myh3 expression level also tended to be higher at 7dpi in WISP1 treated muscles, suggesting that the effects of WISP1 on stem cell function promote a faster repair process in aged mice. At the histological level, WISP1 enhanced the transition of satellite cells to the myogenic lineage as the proportion of $MyoD^-$ satellite cells was significantly reduced while the MyoD+ lineage increased in old WISP1 treated muscles at 7dpi (**Fig. 6b left**).The proportion of committed $MyoD^+$ cells also expressing Pax7, thus retaining a capacity to self-renew, was unchanged (**Fig. 6b right**); suggesting that WISP1 improves old satellite cell commitment, without losing $Pax7^+$ satellite cells. The number of $Pax7^+$ satellite cells was unchanged at 14dpi in the muscle of WISP1 treated mice, confirming that WISP1 does not deplete the satellite cell pool when regeneration has progressed (**Fig. 6c**). In order to evaluate if the increased satellite cell function upon WISP1 treatment *in vivo* translates into better regeneration in old mice, we next quantified the surface covered by regenerating fibers expressing embryonic myosin heavy chain (eMHC). This area was significantly larger in WISP1 treated muscles at 7dpi (**Fig. 6d**), thereby demonstrating that WISP-1 promotes faster expression of the transient MHC allowing myofiber growth during regeneration. To further assess muscle regeneration efficiency, we also quantified the cross-sectional area of regenerating muscle fibers (fibers with centralized nuclei). At 7dpi, WISP1 treated muscles presented significantly fewer small regenerating fibers ($<600\mu m^2$) and more fibers above $1000\mu m^2$, demonstrating that WISP1 enhanced regeneration by accelerating the functional recovery of myofiber size (**Fig. 6e**).

Interestingly, we observed that WISP1 expression was further enhanced in old regenerating muscles treated with WISP1, potentially revealing a positive-feedback regulation loop in muscle (**Fig. 6f**). In addition, because WISP1 is predominantly expressed by activated FAPs, we evaluated PDGFRα expression by qPCR, and observed a significantly stronger upregulation of this FAP-specific marker at 3dpi, suggesting that WISP1 treatment might also regulate FAP activity upon injury in an autocrine manner. Altogether, we demonstrated that WISP1 production by aged FAPs is impaired during muscle regeneration and that restoring WISP1 therapeutically enhances old satellite cell function and ameliorates muscle regeneration in old mice.

DISCUSSION

Aging leads to depletion of the satellite cell pool and loss of muscle regenerative function, both in mice and humans [1, 3, 4]. Extensive efforts have been done to understand the causative factors, either cell-autonomous or originating from the satellite cell environment, driving satellite cell dysfunction with age [8-14, 46]. How the cross-talk between satellite cells and other niche-resident cells changes with age is, however, more elusive.

Muscle regeneration relies on a succession of fine-tuned biological interventions and events, each of which pave the way to satellite cell myogenic commitment in a regulated kinetic manner [21]. In particular, timing of immune cells infiltration to the site of injury, such as neutrophils, pro-inflammatory and anti-inflammatory macrophages, followed by regulatory lymphocytes T (T_{reg}) is critical for cleaning cellular debris and regulating satellite cell commitment [47]. Extracellular matrix remodeling is also critical to ensure the repair of muscle architecture, provide to satellite cells structural support and serve as signaling cues [48-50]. FAP cross-talk between satellite cells is today, unequivocal, but the mechanisms of their myogenic support function (in particular their effect on satellite cell proliferation or differentiation) is still debated [25, 30, 31]. In this study, our results suggested that FAPs action was performed in a time-dependent manner. We found that quiescent-like FAPs are enriched in extracellular matrix molecules and support satellite cells attachment at early time points. Later, FAPs cross-talked with satellite cells in a paracrine way to enhance the number of differentiated cells. While old muscles present increased infiltration of macrophages in non-injured states [19], damage-induced recruitment of inflammatory cells is delayed [51, 52]. Similarly, fibrosis is increased in old mice leading to alteration of muscle mechanical properties [53, 54]; but, little is known about age-related changes of the fate of fibroblasts or mesenchymal cells, including FAPs, during muscle regeneration. We had previously demonstrated that muscle regeneration is accompanied by a transient ectopic adipogenesis suggesting that adipocytes formation is a hallmark of muscle repair [33]. It has been reported that ectopic adipogenesis could be stronger in old animals during muscle regeneration [27, 55]. Using two independent mouse cohorts and two quantification methods, we actually demonstrated that young mice with more efficient muscle repair, activate stronger

ectopic adipogenesis than old mice during muscle regeneration. While this apparent controversy could arise from the different models of injury (CTX *vs*. glycerol) as well as the age of old mice analyzed, it is actually likely that altered ectopic adipogenesis at late time points could be a remnant mark of perturbed FAP function in the first days following muscle injury. Supporting this point, hindlimb-unloading during muscle regeneration has recently been reported to alter muscle regeneration and abolish ectopic adipogenesis [56]. Interestingly, decreased adipogenesis was accompanied by reduced expression of PDGFRα, also suggesting decreased FAP activity in this model of regenerative failure associated to unloading. Similarly, preventing FAP expansion upon damage using nilotinib treatment lead to impaired muscle regeneration [30]. Coinciding with the reduced ectopic adipogenesis and regeneration we observed in old mice, our results revealed for the first time that aged FAPs present cell-autonomous proliferation and adipogenic impairments, together with decreased capacities to support satellite cell function *ex vivo*. FAPs fail to produce IL-33 during aging, and thus recruit T_{reg} at the site of injury, leading to reduced muscle repair efficiency [51].

We discovered that FAPs secrete the Wnt1 signaling pathway protein 1 (WISP1) upon activation and that the induction of WISP-1 is blunted with age. Similarly, old muscles failed to upregulate WISP1 at early time points upon muscle damage. WISP1 is part of the CCN family that have been involved in tissue repair [57]. In particular, WISP1 expression has been shown to be elevated after cardiac ischemia [58], damage of lung epithelium [59], bone fracture [39, 60] and upon oxidative stress of neuronal tissue [61, 62]. WISP1 can signal directly on satellite cells to improve both their adhesion/survival and proliferation, the latter being dramatically reduced with age [6, 7, 63]. The role of WISP1 on cell adhesion, survival and proliferation is also important in other biological settings such as cancer, bone, neuronal and cardio-vascular systems [38, 64, 65]. Similar to our findings, WISP1 has also been reported as a signaling factor allowing paracrine cross-communication [66, 67]. Although the mechanisms underlying WISP-1 rescue of old satellite cell function remain to be uncovered, it is possible that WISP1 acts both in a satellite cell autonomous way by potentiating other local signals from the niche. CCN proteins possess four conserved functional domains through which they act as co-factors for the extracellular matrix, growth factors and cytokines [38]. In addition to possessing proliferative effects on their own, CCN proteins bind to and enhance the signaling of growth factors such as VEGF, FGF and PDGF [68, 69], which are known to promote satellite cell function and to influence muscle regeneration [13, 23, 70, 71]. Interestingly, some CCN proteins have been shown to interact with integrins αvβ3, α6β1 via their cysteine-rich domains [72-75], and integrin β1 activity is critical for satellite cell function, and is deregulated during aging [20]. The capacity of some CCN proteins to bind fibronectin [76] and the importance of fibronectin for satellite cell adhesion, survival and signaling [7, 24] suggests that WISP1 could integrate interactions between satellite cells and the extra-cellular matrix in the muscle stem cell niche.

Taken together, we revealed important age-related dysfunction of FAPs leading to decreased support to satellite cells. We discovered WISP1 as a FAP-secreted molecule involved in the cross-talk with satellite cells that is lost in the muscle niche after injury. WISP1 has a strong therapeutic potential as it rescues defective adhesion and proliferation properties of old satellite cells *ex vivo*, and ameliorates muscle regeneration *in vivo*. Our work establishes that targeting the cellular cross-talk between FAPs and satellite cells is a promising approach to ameliorate muscle repair in elderly.

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Author contributions

L.L., S.K., F.S., A.P., S.M designed and conducted experiments, and analyzed the data. E.M. and F.R. performed genomic data analysis. G.J. provided support with high-content image acquisition and analysis. P.D. supervised genomic experiments. L.L., C.F.B. and J.N.F. designed and interpreted experiments, supervised the project and wrote the manuscript

Competing financial interests

The authors declare competing financial interests: all authors are employees of the Nestlé Institute of Health Sciences S.A., Switzerland

MATERIEL & METHODS

Animals. All animals were housed under standard conditions and allowed access to food and water ad libitum. All mice were of C57BL/6JRj (Janvier) was used. Young male mice were between 9-13 weeks old and aged male mice were ≥20 month. Heterozygote $ROSA^{nT\text{-}nG}$ (B6;129S6-Gt(ROSA)$^{26Sortm4(CAG\text{-}tdTomato*,\text{-}EGFP*)Ees}$/J), hereafter called Td-Tomato mice were purchased from Jackson Labs and used for isolation of

satellite cells bearing a nuclear TdTomato fluorescent marker [35]. Muscle regeneration was induced by intramuscular injections of 50µl of 50% v/v glycerol into tibialis anterior (TA) muscles. Mice were sacrificed 3, 7 or 14 days post-injury (dpi) and regenerating TA muscles were analyzed at the molecular and histological levels. For young and old WT animals, control groups were non-injured animals. For isolation of muscle progenitors, tibialis anterior, gastrocnemius and quadriceps muscles were intramuscularly injected with 50µl, 50µl and 100µl of 50% v/v glycerol, respectively. Mice were sacrificed at 3 dpi, and muscles were processed for cell isolation by flow-cytometry. For *in vivo* WISP1 treatment, 1mg/kg/day of mouse recombinant WISP1 protein (R&D # 1680-WS) was injected intra-peritoneal daily after muscle injury. Control mice were injected with the corresponding volume of PBS. WISTAR rats were purchased from Janvier, sacrificed at 8, 18 or 24 month-old and serum was collected.

Mouse progenitor isolation. For isolation of cell populations, muscles were collected uninjured or 3 days after glycerol injection and digested with Dispase II (2.5 U/ml) (Roche), Collagenase B (0.2%) (Roche) and MgCl2 (5 mM) at 37 °C. Cells were then incubated at 4 °C for 30 min with antibodies against CD45 (Invitrogen, MCD4501 or MCD4528; dilution for both 1/25), CD31 (Invitrogen, RM5201 or RM5228; dilution for both 1/25), CD11b (Invitrogen, RM2801 or RM2828; dilution for both 1/25), CD34 (BD Biosciences, 560230 or 560238; dilution for both 1/60), Ly-6A–Ly-6E (Sca1) (BD Biosciences, 561021; dilution 1/150), α7-integrin (R&D, FAB3518N; dilution 1/30) and CD140a (eBioscience, 12–1401–81 or 17–1401–81; dilution for both 1/30). Antibody validation is provided on the manufacturer's website. FACS isolation was performed on a Beckman Coulter Astrios Cell sorter. Satellite cells (SATs) were isolated by flow-cytometry as: $CD31^-/CD11b^-/CD45^-/Sca1^-/CD34^+/Integrin\ \alpha7^+$. Fibro/Adipogenic progenitors (FAPs) were isolated as: $CD31^-/CD11b^-/CD45^-/Sca1^+/CD34^+/PDGFR\alpha^+$. Lineage positive cells (Lin+) were isolated as: $CD31^+/CD11b^+/CD45^+$. $CD31^-/CD11b^-/CD45^-/Sca1^+/CD34^+/PDGFR\alpha^-$ cells were also collected and named $PDGFR\alpha^-$ cells.

Cell quantification in muscle tissue. Tibialis anterior muscles from young and old mice were collected uninjured, and processed for flow-cytometry as described above. Cell suspension was analyzed by flow-cytometry together with an equal volume of Beckman Coulter FlowCount fluorosphere beads containing precisely 978 beads/ µl, and satellite cells and FAPs were counted by analyzer and normalized to cell suspension volume.

Slow off-rate modified aptamer assay. Muscle samples were pulverized using the cryoPREP impactor system (Covaris). The muscle powder was then subjected to mechanical lysis using a Polytron homogenizer and proteins were extracted in 50 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.5% TritonX. Protein concentration was determined by a BCA assay and samples were diluted at 250ug/mL. Rat serum and mouse protein extracts were analyzed using DNA aptamer+based recognition on the SOMAscan platform (Somalogic, Boulder, CO, USA), as described previously [36]. Median normalized relative fluorescence units (RFU) were log2 transformed before applying principal component analysis and linear models. Statistical analyses were performed in R 3.1.3.

Quantitative PCR. RNA was extracted from frozen muscles or freshly sorted cells using miRNeasy Mini Kit or RNeasy Micro Kit (Qiagen), respectively. RNA samples were subjected to reverse transcription using random primers (High Capacity cDNA Reverse Transcription Kit, ABI). Quantitative PCR on full muscle was performed using the SybR Green I master kit (Roche) on a LightCycler 480. Reference genes (ATP5b, EIF2a and PSMB4) were selected based on their stability across timepoints of regeneration from micro-array data. qPCR probes were designed as follows : ATP5b forward: ACCTCGGTGCAGGCTATCTA, ATP5b reverse: AATAGCCCGGGACAACACAG; EIF2a forward: CACGGTGCTTCCCAGAGAAT, EIF2a reverse: TGCAGTAGTCCCTTGTTAGCG; PSMB4 forward: GCGAGTCAACGACAGCACTA, PSMB4 reverse: TCATCAATCACCATCTGGCCG; Pax7 forward: AAGTTCGGGAAGAAAGAGGACGAC, Pax7 reverse: GAGGTCGGGTTCTGATTCCACATC; MyoD forward: GCAGATGCACCACCAGAGTC, MyoD reverse: GCACCTGATAAATCGCATTGG; Myog forward: xxxx GTGCCCAGTGAATGCAACTC, Myog reverse: CGCGAGCAAATGATCTCCTG; Myh3 forward: ACAGTCAGAGGTGTGACTCAGC, Myh3 reverse: TCCGACTTGCGGAGGAAAG; WISP1 forward: CAGTGAGCCCAAGAGTCAGG, WISP1 reverse: TCGTCTCTGTCAGCTTGCAC; PDGFRα forward: AGTGGCTACATCATCCCCCT, PDGFRα reverse: CCGAAGTCTGTGAGCTGTGT. Quantitative PCR on isolated progenitors was performed using Taqman probes (ABI) in SybR Green I master mix (Roche) on a LightCycler 480. Reference genes (Ap1m1 and Ywhaq) were selected based on their stability across cell types and states from micro-array data. The Taqman probes used were: mWISP1 (ThermoFisher Scientific, Mm01200484_m1, #4331182), mAp1m1 (ThermoFisher Scientific, Mm00475912_m1, #4448489) and mYwhaq (ThermoFisher Scientific, Mm01231061_g1, #4448489).

Cell culture. Satellite cells and FAPs were isolated using a Beckman/Coulter Astrios Cell sorter, and were distributed into 96 well plates. Freshly sorted cells were grown in 20mM glucose DMEM, 20% heat-inactivated FBS, 10% inactivated horse serum, 2.5ng/ml bFGF (Invitrogen), 1% P/S + 1% L+-Glutamine, 1% Na-pyruvate (Invitrogen). For satellite cell activation experiments, 1µM EdU was added to the medium directly after cell sorting, and cells were let to activate for 36h. For satellite cell proliferation, 1µM EdU was added in the medium the third day after sorting for 2-5h. Satellite cell differentiation was induced after four days of growth, by switching to differentiation medium (20mM glucose DMEM, 5% inactivated horse serum, 1% P/S) for 2 days. FAPs were either let to spontaneously differentiate for 13 days in growth medium, or switched to adipogenic differentiation medium on the sixth day for another seven days (20mM glucose DMEM, 20% heat-inactivated FBS, 1% P/S, 0,25µM dexamethasone, 1µg/ml insulin, 5µM troglitazone, 0.5mM isobutylmethylxanthine). To assess fibrogenic capacity,

FAPs were grown for 6 days in growth medium. For co-cultures, the same number of satellite cells and FAPs/satellite cells were seeded in wells. When conditioned medium was used, all cells were freshly isolated the same day, and transfer of conditioned medium to satellite cells was performed after 1 day, then daily during the entire protocol. Human Skeletal Muscle Myoblasts were purchased from Lonza. HSMM cells were seeded at 2000 cells / well in 96 well-plates, and grown in HSMM Amsbio growing medium during 3 days for proliferation assays. For differentiation assays, HSMM cells were seeded at 30000 cells / well in 96 well-plates, and switched to differentiation medium the following day (DMEM-F12, 2% inactivated FBS, 1% P/S) during 2 days. Cells were treated with WISP1 by adding of mouse (R&D # 1680-WS) or human (Peprotech #120-18) recombinant WISP1 protein in the medium, and medium was changed daily.

Immunohistochemistry and image analysis. TA muscles were frozen in isopentane cooled with liquid nitrogen, and further sectioned at 10µm on a cryostat Hematoxylin and Eosine (H&E) staining was performed by placing the dried slides in Harris-hematoxylin during 1 min, followed by differentiation in 1% acid-alcohol and washing, and 1min bath in eosine-phloxine (10g/L). Oil-Red-O staining was performed on air-dried slides by incubating them in 50% ethanol during 30min, followed by 15min incubation in 2.5g/L oil-red-O solution in 70% ethanol, 1 minute washing in 50% ethanol then water; and slides were counterstained with Mayer's hematoxylin. For laminin-eMHC immunostaining, cryosections were allowed to dry during 10 minutes and blocked for 45 minutes at room temperature in the blocking solution (PBS, 4% BSA, 1% FBS). Cryosections were stained during 3 hours at room temperature using monoclonal anti-laminin antibody produced in rabbit (Sigma-Aldrich #L9393) and anti-eMHC produced in mouse (DSHB #F1.652) diluted at 1/100 and 1/500 in the blocking solution, respectively. For perilipin staining, sections were fixed during 10 minutes with PFA 4%, permeabilized during 10 minutes in PBTX 0,5%, blocked in PBS, 4% Goat-serum. A rabbit polyclonal anti-mouse perilipin antibody (Sigma #P1873) and a chicken polyclonal anti-human laminin antibody (Life Span Bioscience #LC-C96142-100) were then incubated on the sections during 3h at room temperature, diluted 1/300 and 1/200 in the blocking solution, respectively. Slides were then incubated during 1 hour at room temperature with secondary antibodies and counterstained with Hoechst 33342. For Pax7 and MyoD stainings, sections stained using an in-house optimized protocol on a Ventana slide stainer (Roche). Briefly, slides were fixed with PFA 4% during 8 minutes at 35°C, and permeabilized in PBTX 0,5% at room temperature for 10 minutes. Antigen retrieval was performed with two successive incubations of 4 minutes at 95°C in citric acid 0.01M, and sections were further blocked in PBS, 4% BSA, followed by 30 minutes blocking with a goat-anti-mouse FAB diluted 1/100 (Jackson #115-007-003). Mouse anti-mouse Pax7 (DHSB, purified), rabbit-anti mouse MyoD (Santa-Cruz #sc-304) and chicken anti-human laminin (Life Span Bioscience #LC-C96142-100) antibodies were then incubated at 2.5 µg/ml, 1/100 and 1/200 in blocking solution, respectively. Pax7 signal was further amplified using a goat-anti mouse IgM1-biotin (Jackson) followed by a streptavidin treatment, together with other secondary antibodies and Hoechst counterstain. Stained tissues were photographed using Olympus VS120 Virtual Microscopy Slide Scanning System and analyzed using the VS-ASW FL software measurement tools. The number of Pax7- and MyoD-positive cells was determined manually by counting of immunostainings in muscle sections in five random areas of the injured region, and the area covered by eMHC-positive fibers and degenerated area was determined manually across the entire sections. The size of myofibers with central nuclei was calculated from laminin/DAPI stainings on all fibers of the section, and Oil-Red-O positive structures segmentation and area determination were performed across the entire sections, using an automated image processing algorithm developed internally using the MetaXpress software (Molecular Devices).

Immunocytochemistry and image analysis. EdU incorporation was revealed using the Click-iT assay (Molecular Probes) according to manufacturer's instruction. Briefly, cells were fixed during 15 minutes in 4% PFA, permeabilized during 20 minutes in PBTX 0.5%, stained with the Click+-iT reaction mix and counterstained with DAPI. For MHC staining, cells were fixed during 10 minutes in 4% PFA, permeabilized using cold EtOH/MetOH (v/v) during 5 min, incubated during 1h with the primary antibody anti-MHC 1/200 (Millipore clone A4.1025) in PBS, 1% Horse Serum at room temperature, and incubated during 30 min with the secondary antibody Alexa488 anti-mouse IgG diluted at 1/1000 (Life Tech. A-10680) and Hoeschst 33342 in PBS, 1% Horse Serum at room temperature. Fusion index was determined as the percentage of nuclei located within MHC positive fibers; and when indicated, the total number of nuclei located within MHC positive fibers is also reported. For Pax7 and MyoD immunostaining, cells were blocked for 1–2 h in 5% goat serum, 1% BSA and 0.2% PBTX, before incubation with primary mouse anti-mouse Pax7 (DHSB, purified, 2.5 µg/ml), rabbit-anti mouse MyoD antibody (Santa-Cruz #sc-304, 1/100)) and secondary antibodies. For α-SMA staining, FAPs were fixed and permeabilized, blocked in PBS, 5% GS, 1% BSA, and incubated with a mouse IgG2a anti-mouse α-smooth muscle actin antibody (#Sigma A5228, 1/150). For adipogenic differentiation, cells were fixed for 10 minutes in PFA 4%, then incubated with Bodipy 493/503 (LifeTechnologies) (1/1000 of the 1mg/ml stock solution of bodipy in ethanol), and counterstained with Hoechst 33342. Image acquisition was performed using the ImageXpress (Molecular Devices) platform. Quantifications were done using the MetaXpress software.

Microarrays. For isolation of RNA from freshly sorted activated MuSCs and FAPs, the RNeasy Micro Kit (Qiagen) was used. RNA samples were then subjected to 3' microarray analysis on Illumina MouseRef–8_V2 chips. 3 ng of total RNA were used to produce cRNA in a two-round amplification protocol, using first Messageamp II aRNA amplification kit (AM1751, Life Technologies, Inc.) followed by Messageamp II–biotin enhanced aRNA amplification kit (AM1791, Life Technologies, Inc.). 750 ng of cRNA were hybridized for 16 h at 55 °C on Illumina MouseRef–8 v2 microarrays. Quality of total RNA was checked by using the

Bioanalyzer 2100 with Total RNA Pico kit, and quality of cRNA was checked by using the Bioanalyzer 2100 with the Total RNA Nano kit (Agilent Technologies). Quantifications were done using the Quant-iT RiboGreen RNA Assay Kit (Life Technologies, Inc.).

Transcriptomic analysis. Illumina expression signals were quantile-normalized. We applied a nonspecific filter to discard probe sets with low variability and retained 12,848 Illumina probe sets whose s.d. was greater than the median of the s.d. of all of the probe sets. For differential expression analysis and pathway analyses, genes (represented by probe sets) were tested for differential expression using the moderated t-statistic as implemented in LIMMA44 for both data sets. Venn diagrams where built from DE genes (http://www.cmbi.ru.nl/cdd/biovenn/index.php) (Hulsen et al., 2008). We used the Pantherdb platform to identify protein classes (signaling molecules) within lists of DE genes (http://pantherdb.org/).

Statistical analysis. All wild-type mice were randomized according to body weight before interventions. Sample size determination was based on the expected effect size and variability that was previously observed for similar readouts in the investigators' labs. *In vivo* treatments were not blinded, but imaging readouts were analyzed in a blinded manner. Genome-wide statistical analyses and Kolmogorov–Smirnov tests were performed using R version 3.1.3 and relevant Bioconductor packages as described in the sections above. GO terms were tested on genes differentially expressed in the indicated conditions as: adj.p < 0.05. Genes upregulated during FAP activation were filtered as: adj. p-value<0.001, Fold-change>2. Genes differentially upregulated in FAPs during activation with age were filtered as: adj. p-value [Activation young] < 0.001, Fold-change [Activation young] > 2; adj. p-value [interaction: Activation*Age] < 0.25; adj. p-value [old activated FAPs *vs*. young activated FAPs] < 0.1. All other statistical analyses were performed using GraphPad Prism (GraphPad Software) assuming normal distribution of the variables measured. Statistical significance for binary comparisons was assessed by a Student's t-test after checking that variances do not differ between groups or by a Welch correction when variances differed between groups. All exploratory and signaling experiments were analyzed by using two-tailed tests, and *in vitro* phenotypic analysis experiments of young and old wild-type SATs and FAPs were tested using a one-tailed test. For comparison of more than two groups, one-way or two-way ANOVAs were used, according to the experimental design, and followed by Bonferroni multiple-comparison testing. All data are expressed as mean + s.e.m.

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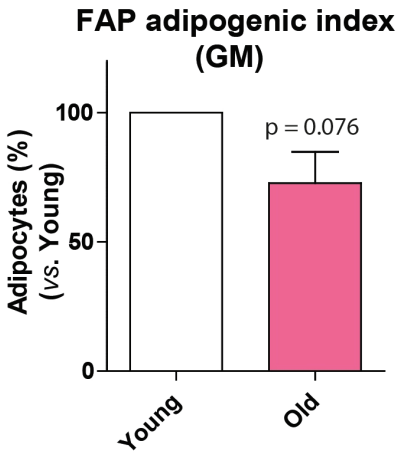
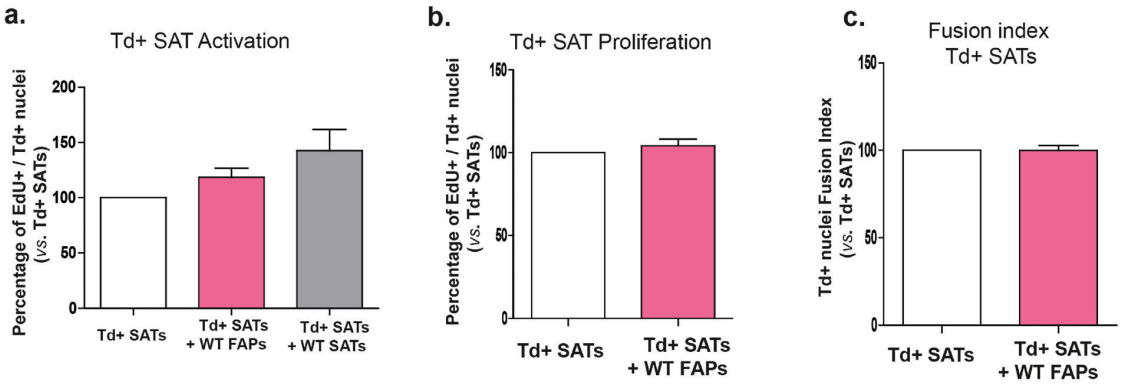
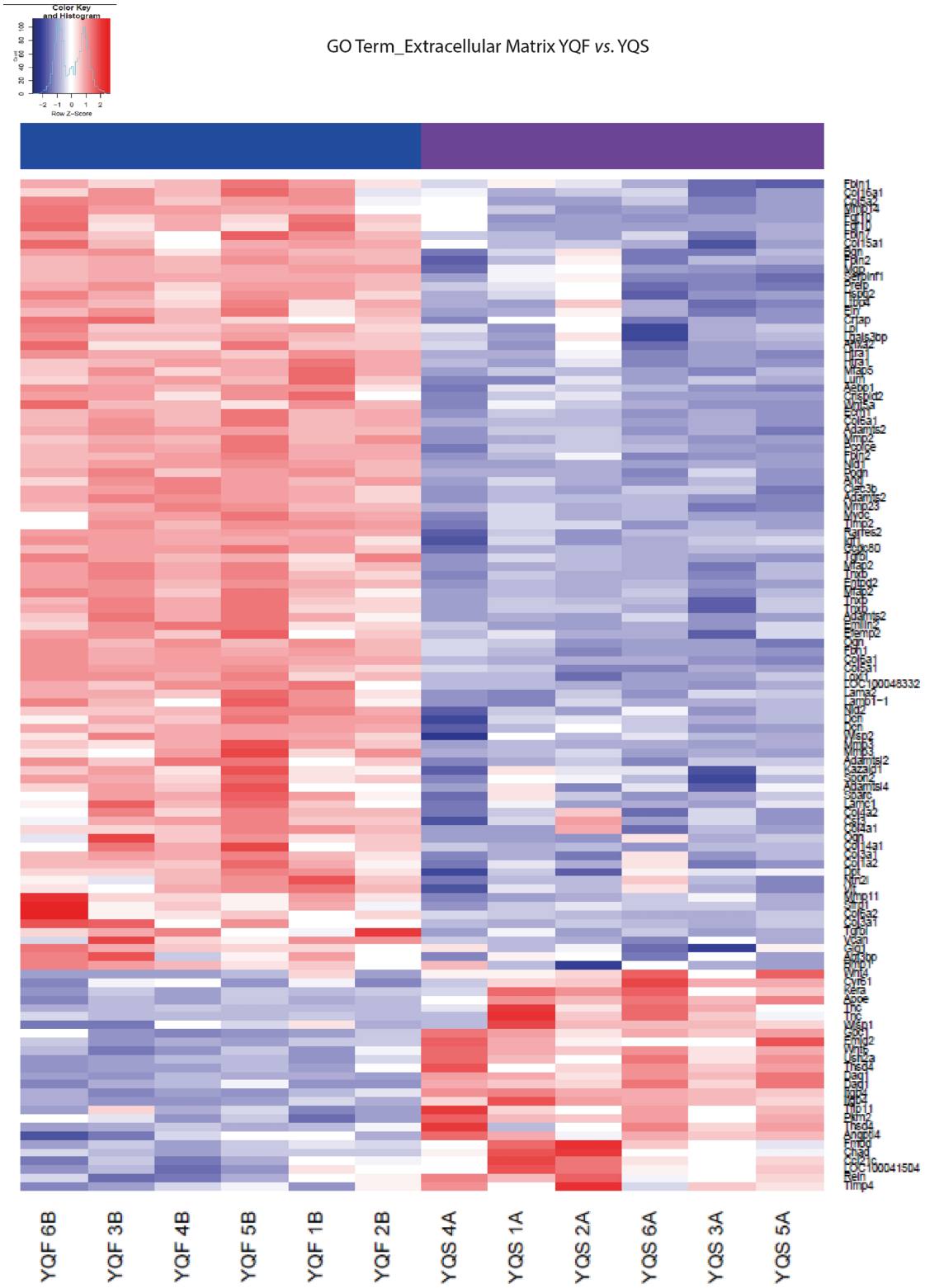


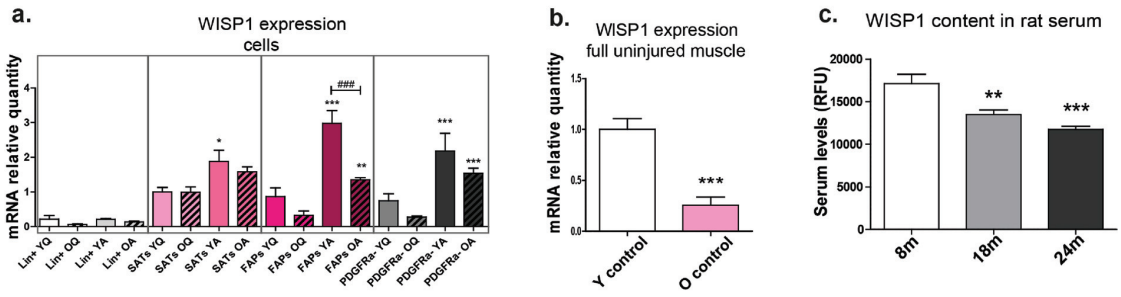
Supplementary figure 1. Spontaneous adipogenic differentiation capacity of young and old FAPs in growth medium. n = 3, p: p-value.



Supplementary figure 2. Satellite cells were freshly isolated from young Td-Tomato$^+$ (Td$^+$) non-injured muscles and seeded alone or together with freshly isolated wild-type (WT) FAPs or satellite cells. **(a)** Quantification of activated Td$^+$ satellite cells assessed by the incorporation of EdU 36h after seeding. **(b)** Proliferation capacities of Td$^+$ satellite cells 3 days after seeding assessed by the total number of cells. **(c)** Differentiation capacities of Td$^+$ satellite cells assessed by fusion index. n=3.



Supplementary figure 3. Heatmap of genes of the gene ontology (GO) term "Extracellular matrix" significantly regulated in young quiescent FAPs *vs.* SATs. Y = young, Q = quiescent, F = FAPs, S = Satellite cells.



Supplementary figure 4. (a) WISP1 expression in freshly isolated cells, assessed by qPCR. Satellite cells, FAPs, Lineage cells (Lin+) ($CD31^+/CD11b^+/CD45^+$); and $PDGFR\alpha^-$ cells ($CD31^-/CD11b^-/CD45^-/Sca1^+/CD34^+/PDGFR\alpha^-$) were isolated from young (Y) and old (O) mice, either from uninjured muscles (quiescent cells, or Q) or 3 days after muscle injury (activated cells, or A). Data are normalized to YQ SATs; *: p-value *vs*. young quiescent respective cell type < 0.05, **: p-value *vs*. young quiescent respective cell type < 0.01, ***: p-value *vs*. young quiescent respective cell type < 0.001; ###: p-value old *vs*. young activated respective cell type < 0.001, n>4. **(b)** WISP1 expression in uninjured TA muscles of young and old mice. Data are normalized to young non-injured muscles (control), ***: p-value *vs*. young < 0.001, n=8. **(c)** WISP1 protein level in rat serums measured using aptamer-based detection. **: p-value *vs*. 8 month-old rats < 0.01, ***: p-value *vs*. 8 month-old rats < 0.001, n>9 rats per group.

Supplementary table 1_Top GO Term_FAPs vs. SATs, cell type effect

GO.ID	Term	Annotated	Significant	Expected	classicFisher	elimFisher	wei01Fisher	classicKS	elimKS	wei01KS
GO:0044707	single-multicellular organism process	3763	580	407.01	1.80E-26	3.10E-02	1.00E+00	2.80E-15	8.78E-01	1.00E+00
GO:0032501	multicellular organismal process	3835	583	414.8	5.60E-25	6.57E-02	1.00E+00	5.30E-14	9.66E-01	1.00E+00
GO:0048731	system development	2580	428	279.06	2.40E-24	5.18E-02	1.00E+00	1.10E-12	6.89E-03	1.00E+00
GO:0048513	organ development	1894	335	204.86	4.60E-23	1.04E-02	1.00E+00	2.20E-12	4.04E-02	1.14E-01
GO:0044767	single-organism developmental process	3432	523	371.21	9.20E-22	3.74E-01	1.00E+00	6.20E-14	2.35E-01	1.00E+00
GO:0032502	developmental process	3452	525	373.37	1.20E-21	3.62E-01	1.74E-01	7.20E-14	4.56E-01	4.10E-01
GO:0007275	multicellular organismal development	2949	462	318.97	4.40E-21	8.75E-02	2.76E-02	1.10E-11	5.50E-01	3.66E-02
GO:0048856	anatomical structure development	3088	478	334	7.70E-21	5.48E-01	1.00E+00	1.00E-12	7.90E-01	1.00E+00
GO:0007155	cell adhesion	679	148	73.44	9.50E-18	9.10E-06	1.20E-05	1.60E-11	3.74E-03	3.21E-03
GO:0030198	extracellular matrix organization	157	57	16.98	1.90E-17	8.40E-06	7.20E-06	1.60E-10	1.90E-04	1.90E-04
GO:0009888	tissue development	1137	214	122.98	2.00E-17	1.77E-01	2.12E-01	2.20E-11	1.21E-01	8.16E-02
GO:0043062	extracellular structure organization	158	57	17.09	2.60E-17	1.00E+00	1.00E+00	1.80E-10	4.83E-01	4.83E-01
GO:0022610	biological adhesion	687	148	74.31	2.90E-17	1.00E+00	1.00E+00	5.00E-11	8.72E-01	1.00E+00
GO:0016477	cell migration	745	154	80.58	3.10E-16	6.00E-04	1.36E-03	1.60E-09	2.00E-03	6.64E-03
GO:0072358	cardiovascular system development	699	147	75.6	3.40E-16	1.55E-01	1.55E-01	2.00E-11	1.32E-01	5.87E-01
GO:0072359	circulatory system development	699	147	75.6	3.40E-16	1.55E-01	1.00E+00	2.00E-11	1.32E-01	1.00E+00
GO:0044699	single-organism process	8387	1031	907.15	1.20E-15	8.58E-01	1.00E+00	2.20E-06	6.61E-01	1.00E+00
GO:0048870	cell motility	790	159	85.45	1.20E-15	4.89E-01	2.71E-01	1.20E-09	4.48E-01	6.01E-01
GO:0051674	localization of cell	790	159	85.45	1.20E-15	4.89E-01	1.00E+00	1.20E-09	4.48E-01	1.00E+00
GO:0040011	locomotion	918	177	99.29	1.80E-15	7.26E-01	4.14E-01	3.70E-10	5.97E-02	6.99E-01
GO:0009653	anatomical structure morphogenesis	1597	267	172.73	8.90E-15	2.42E-01	2.70E-01	5.60E-11	6.34E-02	1.42E-02

Supplementary table 1. Top Gene Ontology (GO) terms of genes regulated in FAPs vs. SATs.

Supplementary table 2_Top GO Term_FAPs, age effect

GO.ID	Term	Annotated	Significant	Expected	classicFisher	elimFisher	wei01Fisher	classicKS	elimKS	wei01KS
GO:0065007	biological regulation	6584	48	32.7	4.20E-05	5.81E-02	1.00E+00	7.37E-03	3.95E-01	1.00E+00
GO:0050794	regulation of cellular process	5944	45	29.52	4.70E-05	4.14E-02	1.00E+00	7.24E-02	6.94E-01	3.38E-01
GO:0050678	regulation of epithelial cell proliferation	208	7	1.03	7.30E-05	2.99E-01	1.00E+00	9.70E-04	3.08E-02	4.47E-01
GO:0050789	regulation of biological process	6311	46	31.35	1.00E-04	4.21E-02	1.00E+00	8.61E-03	3.19E-01	1.00E+00
GO:0070228	regulation of lymphocyte apoptotic proces	51	4	0.25	1.20E-04	1.36E-02	9.46E-03	7.29E-02	7.29E-02	1.36E-01
GO:0044092	negative regulation of molecular function	604	11	3	1.60E-04	2.53E-02	1.00E+00	2.43E-01	3.08E-01	2.61E-01
GO:0001525	angiogenesis	320	8	1.59	1.70E-04	9.11E-03	9.11E-03	5.23E-02	1.31E-01	3.51E-01
GO:0050673	epithelial cell proliferation	240	7	1.19	1.80E-04	3.94E-01	1.00E+00	5.07E-03	8.40E-02	9.42E-01
GO:0043433	negative regulation of sequence-specific DNA binding transcription factor activity	110	5	0.55	2.10E-04	2.10E-04	1.23E-03	4.17E-01	4.17E-01	2.78E-01
GO:0007179	transforming growth factor beta receptor signaling	114	5	0.57	2.50E-04	2.71E-02	2.71E-02	2.01E-02	2.01E-02	3.44E-02
GO:0070887	cellular response to chemical stimulus	1239	16	6.15	2.60E-04	2.83E-02	1.00E+00	1.23E-02	2.31E-01	2.89E-01
GO:0070227	lymphocyte apoptotic process	63	4	0.31	2.60E-04	2.31E-02	1.00E+00	2.78E-01	2.78E-01	1.00E+00
GO:0042221	response to chemical	1820	20	9.04	3.30E-04	4.78E-02	1.00E+00	1.87E-03	4.32E-01	2.27E-01
GO:0001568	blood vessel development	456	9	2.26	3.90E-04	4.55E-01	2.39E-01	4.99E-02	1.03E-01	4.96E-01
GO:0071559	response to transforming growth factor beta	130	5	0.65	4.60E-04	4.35E-02	1.00E+00	1.72E-02	1.72E-02	1.00E+00
GO:0071560	cellular response to transforming growth factor	130	5	0.65	4.60E-04	4.35E-02	1.00E+00	1.72E-02	1.72E-02	2.47E-01

Supplementary table 2. Top Gene Ontology (GO) terms of genes regulated in quiescent FAPs with age.

				YAF vs. YQF Activation effect			Interaction Age * Treatment			OAF vs. YAF Effect of age in activated FAPs		
Probe Id	Accession	Symbol	Amean	log_2(FC)	p-value	BH adj. p-value	log_2(FC)	p-value	BH adj. p-value	log_2(FC)	p-value	BH adj. p-value
ILMN_2952275	NM_007482.2	**Arg1**	7.774136	1.57418	1.61E-07	5.37E-06	1.110655	0.001121808	0.179146479	1.130296667	2.04E-05	0.020123961
ILMN_2632712	NM_009689.2	**Birc5**	7.391934	1.546418333	8.11E-14	1.30E-10	-0.50471167	0.001089471	0.178674974	-0.44682	0.000107574	0.051584051
ILMN_2742912	NM_201367.2	**Gpr176**	7.259477	1.439868333	3.49E-09	2.67E-07	-0.77940833	0.001608279	0.199178049	-0.622685	0.000516491	0.080925328
ILMN_2612206	NM_023223.1	**Cdc20**	7.414382	1.724106667	1.83E-12	9.41E-10	-0.80067	0.000143918	0.092453149	-0.62642833	4.38E-05	0.031814026
ILMN_1235697	NM_026526.2	**N6amt2**	9.317762	1.575351667	4.93E-09	3.58E-07	-0.89838	0.001204227	0.179609402	-0.706145	0.000438063	0.080118423
ILMN_2970623	NM_025495.1	**Cenpp**	7.221673	1.366705	2.53E-08	1.25E-06	-0.94690167	0.000460104	0.147785252	-0.76514333	0.000109332	0.051584051
ILMN_2844996	NM_134156.1	**Actn1**	11.03137	1.121933333	2.16E-06	4.48E-05	-1.06105	0.000334277	0.134212027	-0.83001667	0.000110836	0.051584051
ILMN_1224635	NM_024169.3	**Fkbp11**	10.729	2.33442	7.83E-12	2.45E-09	-0.918625	0.001551561	0.199178049	-0.89775	5.34E-05	0.036133989
ILMN_2898319	NM_153543.1	**Aldh1l2**	7.424884	1.291073333	3.45E-08	1.62E-06	-1.12440667	4.37E-05	0.043221189	-1.14651167	2.44E-07	0.001042847
ILMN_2492264	NM_018865.2	**Wisp1**	7.972638	2.15291	1.92E-08	1.01E-06	-1.20416167	0.002757801	0.243394442	-1.52888167	4.22E-06	0.009030832

Supplementary table 3. Identification of genes both significantly regulated in Old activated FAPs *vs*. Young activated FAPs and significantly upregulated with activation in young FAPs: adj. p-value [Activation young] < 0.001, Fold-change [Activation young] > 2; adj. p-value [interaction: Activation*Age] < 0.25; adj. p-value [old activated FAPs *vs*. young activated FAPs] < 0.1. Y = young, O = Old, Q = quiescent, A =activated, F = FAPs, FC = fold-change.

CHAPTER VI. Apelin is a novel exerkine which reverses sarcopenia

Rationale

When looking for specific satellite cell changes with age that could potentially unravel an age-related deregulated sensitivity to their local or systemic environment, we identified APJ, the receptor for the small circulating peptide apelin, as the gene the most strongly down-regulated with age in satellite cells in our transcriptomic data. APJ is a G protein coupled receptor (GPCR) which couples to the protein $G_{i/0}$ (Chaves-Almagro et al., 2015), a G-protein previously shown to promote muscle regeneration and stem cell function (Minetti et al., 2014, Minetti et al., 2011). The family of Apj ligands is composed of Apelin-36, 17 or 13 (three isoforms of 36, 17 or 13 amino acid residues, respectively, which are cleaved from the same precursor protein produced by a single apelin gene, pyroglutaminated-Apelin 13, a post-translationally modified Apelin-13 (Lee et al., 2000, Tatemoto et al., 1998), and the recently identified Elabela peptide (also called Apela, or Toddler) which is homolog to Apelin but encoded by a separate gene (Deng et al., 2015, Pauli et al., 2014). The apelin isoforms are differentially expressed across tissues, but Apelin-17 and (pyr)-13 are the most abundant forms found in the circulation (Azizi et al., 2008). Apelin has been involved in cardiomyocyte contraction (Kuba et al., 2007, Szokodi et al., 2002), vasodilatation (Tatemoto et al., 2001), angiogenesis (Masri et al., 2004, Saint-Geniez et al., 2003) and in various functions of the central nervous system such as neuroprotection or analgesia (Xu et al., 2009, Yang et al., 2014).

Apelin can be produced in adipocytes and myofibers (Bertrand et al., 2013, Boucher et al., 2005), and is therefore considered to be an adipokine and a myokine. The metabolic effects of Apelin were demonstrated through its role on glucose transport through the intestinal epithelium, in response to glucose itself (Dray et al., 2013). Apelin was also shown to enhance glucose utilization by skeletal muscle (Dray et al., 2008), and to promote fatty acid oxidation and insulin sensitivity in muscle (Attane et al., 2012, Yamamoto et al., 2011), via a mechanism requiring AMPK (Castan-Laurell et al., 2012).

Following our observation of loss of APJ expression in aged satellite cells, we collaborated with the team of Philippe Valet and Cédric Dray (INSERM, Toulouse, France), which focuses on apelin biology in metabolic tissues, and to study the role of apelin the context of muscle aging and sarcopenia. The specific aim of the collaboration was to assess whether apelin was able to ameliorate

muscle repair in aged mice, and to understand the molecular and cellular mechanisms through which this peptide regulates satellite cell function. We found that both apelin and APJ are strongly upregulated during muscle regeneration, but that this upregulation is blunted with age. Apelin supplementation of 3, 12 and 24 month-old mice during muscle regeneration successfully enhanced muscle repair.

Authors

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My contribution

I discovered the regulation of APJ in aged satellite cells and designed, performed and analyzed the *in vivo* muscle regeneration experiments (**Fig. 5 and S4**). I also wrote the corresponding sections of the manuscript and brought the overall expertise on muscle regeneration and stem cell biology for data interpretation.

Publication

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Apelin is a Novel Exerkine which Reverses Age-Associated Sarcopenia.

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Sarcopenia is the progressive loss of skeletal muscle mass and function with age, and is considered as the main driver of loss of autonomy in the elderly. It has become essential to identify the molecular, cellular and endocrine perturbations leading to sarcopenia and potentially serving as targets to develop therapeutic interventions. In this study, we identified that the circulating peptide apelin is secreted by skeletal muscle in response to exercise and lost during aging. Apelin levels and upregulation upon muscle contraction were reduced in aged mice and sarcopenic patients. Apelin knock-out mice early lost muscle mass and developed sarcopenia prematurely. Apelin supplementation successfully rescued loss of muscle mass and function of old mice in an AMPK-dependent manner. In addition, apelin treatment also boosted muscle stem cell function and resulted in enhanced regenerative capacities of old mice. Altogether, our work establishes that apelin is as a novel biomarker of muscle function and exercise success in elderly people, and a promising preventive and therapeutic strategy against sarcopenia.

Preserving independence and physical function of the aged population not only preserves the lifestyle of elderly people but also considerably reduces the direct and indirect healthcare costs linked to dependence [1]. Indeed, low muscle mass and strength in the elderly, termed sarcopenia, contributes to the progressive loss of autonomy and is tightly correlated with the development of other age-associated pathologies such as osteoporosis, heart failure or cognitive diseases [2-4]. Consequently, age-associated loss of mobility and locomotion can be considered as one of the most powerful predictive factors for the frailty-dependence transition and mortality [5, 6]. Pharmacological strategies to treat muscle wasting have been proposed, essentially by hormonal therapy (such as testosterone, growth hormone and DHEA) but resulted in disappointing effects since most of the treatments exhibited important side effects without gain of strength [7]. Conversely, by alternatively activating both myofiber metabolism and satellite cell activation, physical exercise emerges as the most interesting strategy to counteract age-associated muscle atrophy. However, implementation as a standard of care remains challenging in the elderly population because of poor compliance [8]. Among the different mechanisms leading to sarcopenia, loss of the metabolic capacities of muscle fibers, and particularly mitochondrial alterations, promotes muscle wasting by different molecular pathways such as lack of energy supply, decreased proteostasis or radical oxygen species production [9]. Weakened muscle function in elderly people is exacerbated by the inefficient repair of damaged fibers upon muscle injuries induced by trauma or falls. This decreased regenerative capacity of aged muscle results from loss of the number and function of satellite cells, the muscle stem cells [10-13]. Although age-related muscle loss is inevitable, it is critical to develop therapies and/or interventions that could prevent or reverse loss of skeletal muscle and satellite cell function.

In this context, we identified apelin, a 13 to 36 amino acid peptide, as an exercise-induced myokine that triggers both muscle fiber metabolism and satellite cell activation during aging. The apelin receptor APJ is a G-protein coupled receptor that couples to $G_{\alpha i}$ [14], and apelin promotes beneficial physiological functions on vasodilatation, cardiomyocyte contraction, angiogenesis and neuropreotection [15-20]. Metabolic properties of apelin have also been described in the context of insulin resistance. By stimulation of AMPK and Akt downstream its receptor APJ in muscle cell, apelin increases mitochondrial function and biogenesis and thereby prevents obesity and diabetes [21-23]. We report that apelin is produced by skeletal muscle in the context of exercise-associated contraction both in pre-clinical models and humans. Chronic treatment of aged mice with apelin or viral overexpression of apelin in muscle induces myofiber hypertrophy and improves muscle strength and function by enhancing mitochondrial metabolism and restoring satellite cell function.

RESULTS

The production of apelin by skeletal muscle in response to physical activity is blunted during aging.

In order to evaluate whether apelin could be linked to physical decline in the elderly, we first measured circulating levels of the apelin peptide in a human cohort of elderly sarcopenic women [24]. Age-associated loss of skeletal muscle evaluated by appendicular lean mass measurement by DXA was associated to a robust decrease in plasma apelin levels (**Fig. 1a**). Compared to other cytokines or hormones (leptin, insulin, IL6, IL8, Ngf, MCP-1), the decrease of plasma apelin was specifically associated to sarcopenia independently of weight and fat mass. Similarly, in a mouse model of aging, plasma apelin levels were progressively reduced with age and associated with a loss of apelin mRNA expression specifically in skeletal muscle (**Fig. 1b** and **1c**). Age-associated loss of apelin was confirmed and specifically demonstrated in isolated mouse muscle fibers by mRNA expression and anti-apelin immunofluorescence labeling (**Fig. 1d** and **1e,** respectively). Apelin induces an auto-regulatory positive feedback loop on the expression of its receptor APJ. Consequently, we also observed a down-regulation of APJ expression in muscle fibers from aged mice (**supplemental S1a**). The loss of apelin production fromaged muscle was also confirmed in the context of human biology by demonstrating that myotubes differentiated from an aged donor showed both lower apelin mRNA expression and lower apelin release in the medium (**Fig. 1f** and **supplemental S1b**). Altogether, these data highlight the relationship between aging and skeletal muscle apelin production and led us to investigate the regulatory processes controlling muscle apelin production.

As we previously identified physical exercise as a potential enhancer of muscle apelin production in obese people [22], we first measured apelin production in human muscle cells stimulated by forskolin (**Fig. 1g**) or by electric stimulation (**Fig. 1h**) in order to mimic the effects of muscle contraction *in vitro*. Apelin secretion in culture media was significantly increased after short-term forskolin treatment (20 minutes) or electric contraction in human cells from young donors (**Fig. 1g-h** black bars), whereas this regulation was dramatically altered with age (**Fig. 1g-h**, white bars). We confirmed a dramatic age-dependent decrease of muscle apelin release in bloodstream after contraction in vivo by evaluating apelin arteriovenous difference following sciatic nerve stimulation-induced muscle contraction in aged mice (**Fig.1i**, hatched bars). Along the same line, middle aged and aged mice failed to increase plasma apelin levels during acute physical exercise (**Fig. 1j**). However, a four-weeks training (30 min of treadmill running/day) enhanced muscle apelin expression (**Fig. 1l**) and consequently increased plasma apelin (**Fig. 1k**) in an age-dependent manner, suggesting that loss of exercise-induced muscle apelin production is reversible at middle age but lost in the elderly. Similarly, 5 day-chronic forskolin treatment induced an age-dependent rescue of apelin expression in young and aged-donor myotubes (**Fig. 1m**). Finally, the link between apelin and muscle utilization/contraction has been definitively demonstrated by the inhibition of apelin expression in skeletal muscle of young mice immobilized during 14 days (**Fig. 1n**). Altogether, these results demonstrate that apelin is produced locally in skeletal muscle in response to exercise and lost during aging.

Apelin/APJ deficiency dramatically accelerates muscle ageing

The age-related loss of apelin production in muscle led us to investigate whether apelin is necessary and sufficient to maintain muscle mass and function during aging. Apelin deficient mice ($apln^{-/-}$) died prematurely (**supplemental S2a**, red curve) compared to wild type mice, which drove us to perform experiments with 12-month-old mice. Lack of apelin induced a decrease of lean tissue mass specifically associated with a reduction of skeletal muscle mass and muscle fiber diameter (**Fig. 2a-c**, red bars), without affecting other tissues such as the heart. Moreover, apelin deficiency affects muscle fiber size without

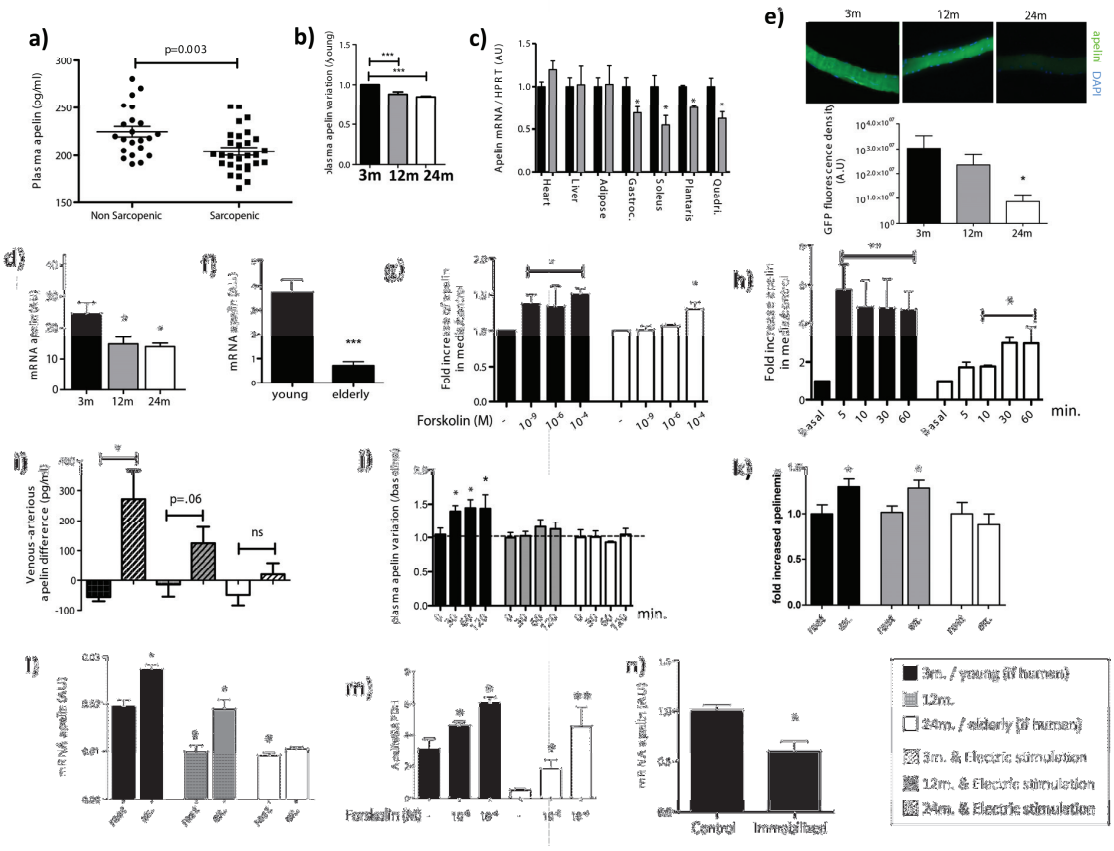

Figure 1: Apelin is an exercise-induced myokines that is lost with age. (a) Apelinemia measured in plasma of elderly people affected (n=28) or not (n=22) by sarcopenia. **(b)** Apelinemia measured in plasma of 3, 12 and 24 month-old mice. n=10. **(c)** Apelin expression in different tissues of 3 month-old (black bars, n=5) and 12 month-old (grey bars, n=6) mice. Gastroc. = gastrocnemius, quadri = quadriceps, adipose = adipose tissue. **(d)** Apelin expression in fibers isolated from EDL of 3, 12, and 24-month-old mice. **(e)** Apelin (green) / Nuclei (blue) staining of EDL-isolated fibers (top), and quantification of fluorescence intensity (bottom). n=4 in average. **(f)** mRNA Apelin expression in fibers 14 days after induction of differentiation human myoblasts from young (19 year-old) and old (79 year-old) donors. **(g)** Apelin concentration in medium of fibers differentiated from young and old human myoblasts after 30 minutes acute stimulation by forskolin**. (h)** Apelin concentration in medium of fibers differentiated from young and old human myoblasts after 5, 10, 30 or 60 minutes (min.) of electrical stimulation (30V, 0,1mA)**. (i)** Differential measurement of apelin concentration in plasma collected from carotid artery and femoral vein of 3, 12 or 24 month-old mice in resting condition (plain bars) or after sciatic nerve electrical stimulation (hatched bars). **(j)** Relative plasma apelin concentration in 3, 12 or 24 month-old mice subjected to acute exercise at 70% of the VO2 max., at baseline, or after 30, 60 or 120 min. of exercise. n=4. **(k-l)** Relative plasma apelin concentration (k) or full muscle apelin mRNA levels(l) in 3, 12 or 24 month-old mice subjected subjected (ex.) or not (rest) to 28-days exercise program (1 hour running on treadmill with 10% positive inclination, 6 days a week). **(m)** Apelin concentration in medium of fibers differentiated from young and old human myoblasts after 24 hours long term stimulation by forskolin**.** n=4. **(n)** mRNA expression in soleus muscles of young mice immobilized during 15 days. n=4.

distinction of fiber type (**Fig. 2c**). To appreciate the consequence of such an alteration on muscle function, we measured the strength developed by *plantaris* or *tibialis anterior* muscles after tetanic sciatic nerve electrical-stimulation in anesthetized mice. Both muscles displayed reduced contractile capacity (7 to 12 %) in apelin deficient mice (**Fig. 2d-e**). Similarly, *in vivo* experiments demonstrated that apln$^{-/-}$ mice have lower grip strength than WT controls, as well as declined resistance and endurance running capacities (**Fig. 2f-h**). Furthermore, we also confirmed the crucial role of apelin in muscle physiology during aging by targeting APJ, the apelin receptor (**Fig. 2b, f, g** and **h**, orange bars). APJ knock-out mice are embryonic lethal [25], but APJ heterozygous knock-outs (APJ$^{+/-}$) had decreased muscle mass with normal heart mass (**Fig. 2b**, orange). Contrary to apelin deficient mice, resistance exercise capacities were not altered by deletion of

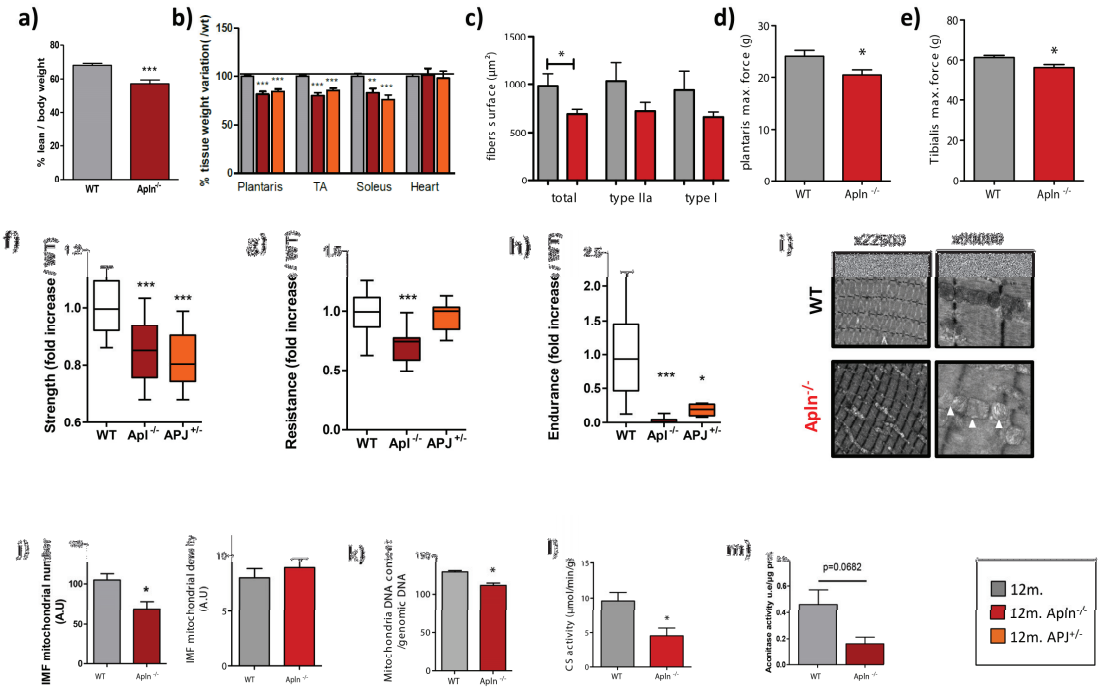

Figure 2: Apelin/APJ signaling deficiency leads to premature muscle alterations in mice. (a) Lean mass of 12 month-old wild-type (WT) and apelin knock-out ($Apln^{-/-}$) mice measured by EchoMRI. **(b)** Mass of different muscles expressed relatively to body weight of 12 month-old WT, $Apln^{-/-}$ and mice heterozygous for APJ ($APJ^{+/-}$). **(c)** Average muscle fiber cross-sectional area per fiber type in 12 month-old WT and $Apln^{-/-}$ mice. **(d,e)** Maximal *plantaris* (d) or *tibialis anterior* (e) force following sciatic nerve electric stimulation. **(f-h)** Physical performances of 12 month-old WT, $Apln^{-/-}$ and $APJ^{+/-}$ mice assessed by grip strength (force, (f)), resistance (latency to falls in inverted grid tests, (g)), and endurance (maximal speed on treadmill, (h)). **(i)** Intermyofibrillar mitochondria in soleus muscles imaged by electronic microscopy. **(j)** Quantification of intermyofibrillar mitochondria number (left) and density (right). **(k)** Quantification of mitochondrial DNA in soleus muscles relative to genomic DNA. **(l-m)** citrate synthase (l) and aconitase (m) enzymatic activity measured in isolated mitochondria.

one APJ allele, whereas strength and endurance were both significantly decreased in $APJ^{+/-}$ mice (**Fig. 2f-h**). Taken together, these results demonstrate that apelin and its receptor are necessary for maintaining muscle mass and function during aging.

Previous mechanistic studies from our group and others have demonstrated that apelin enhances muscle mitochondrial function in the context of obesity [21]. To test whether loss of apelin could induce sarcopenia by altering mitochondria, we analyzed mitochondrial shape, quantity and function in apelin deficient mice (**Fig. 2i- m**). Intramyofibrillar mitochondria number was reduced in $apelin^{-/-}$ mice (**Fig. 2i-k**) and was accompanied with an accumulation of mitchondrial inclusions (**Fig. 2i**, white arrows). Interestingly, mitochondrial activities such as citrate synthase or aconitase were also dramatically reduced (**Fig. 2m, l**). Together with reduced PGC1α expression in the muscle of apelin KO mice (**supplemental S2b**), these results strongly suggest that apelin participates to mitochondriogenesis. Altogether, these results demonstrate that loss of apelin is sufficient to induce sarcopenia and that altered muscle apelin production during aging could be a direct cause of age-associated muscle weakness.

Apelin supplementation reverses age-associated sarcopenia

As plasma and muscle apelin levels progressively decline with age, we tested whether restoring apelin levels in aged mice through systemic pharmacological supplementation or genetic AAV-mediated muscle over-expression is sufficient to prevent or rescue sarcopenia. Middle-aged and aged mice were treated by daily intraperitonally of the apelin 13 peptide at 0.5 umol/kg/d during 28 days. Apelin-treatment significantly enhanced *plantaris* and *tibialis* anterior muscle mass (**Fig. 3a-b**; hatched bars), and the cross sectional areas of *plantaris* muscle fibers (**Fig. 3c**; hatched bars) both in middle-aged and aged mice. Additionally, pharmacological apelin treatment induced *plantaris* fiber hypertrophy as demonstrated by the shift of fiber diameter distribution obtained in aged mice (**Fig.**

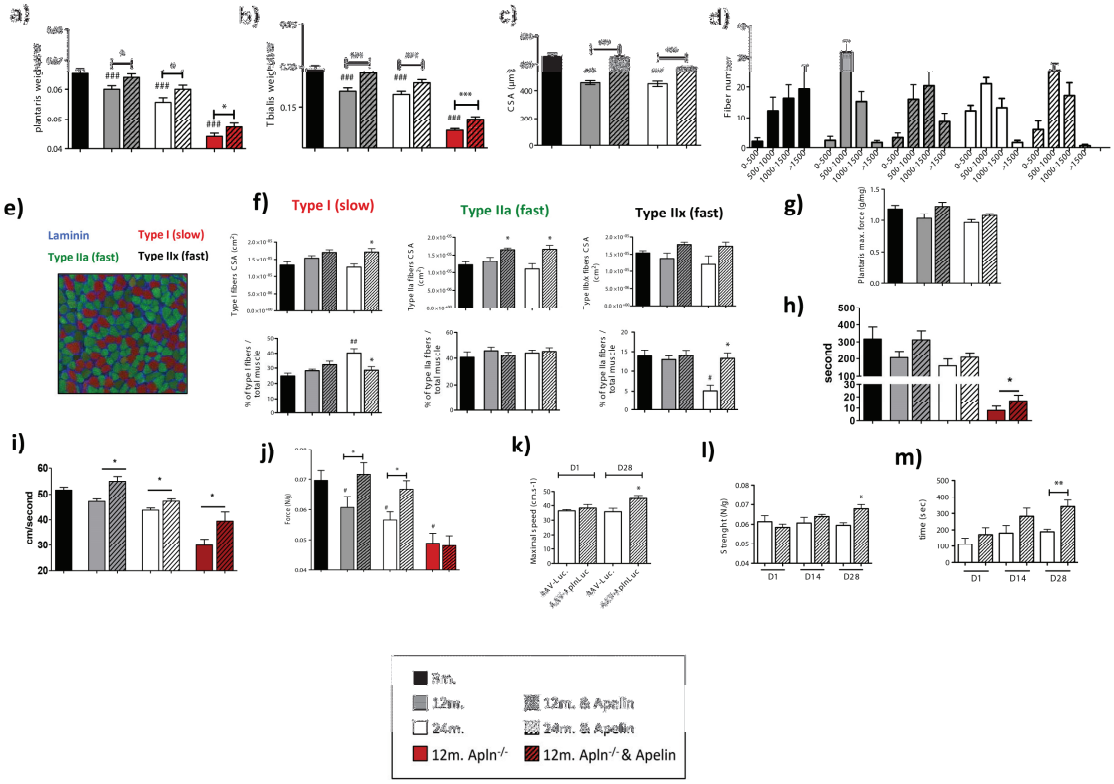

Figure 3: Apelin supplementation restores age-related muscle alterations. (a,b) Plantaris (a) and tibialis anterior (b) muscle weight relatively to body weigth in 3, 12, 24 month-old WT or Apln$^{-/-}$ mice supplemented (hatched bars) or not (plain bars) with apelin. **(c,d)** average (c) or size distribution (d) of muscle fibers cross-sectional area. **(e)** Immunohistochemistry fiber-typing; type I (red), type IIa (green), type IIx (black), laminin (blue). **(f)** Average fiber cross-sectional area (top graphs) and repartition (bottom graphs) per fiber type. **(g)** Maximal *plantaris* force following sciatic nerve electric stimulation in 3, 12, 24 month-old WT mice supplemented (hatched bars) or not (plain bars) with apelin. **(h-j)** Physical performances of 3, 12, 24 month-old WT mice supplemented (hatched bars) or not (plain bars) with apelin assessed by resistance (latency to falls in inverted grid tests, (h)), endurance (maximal speed on treadmill, (i)), and grip strength (force, (j)). **(k-m)** Physical performances of 24 month-old WT mice intramuscularly injected with AAV-Apelin-Luc vectors (hatched bars) or AAV-Luc vectors (plain bars) assessed by endurance (k), grip strength (l) and resistance (m).

3d, hatched bars). Apelin treatment also normalized the shift of fiber type happening during aging by simultaneously increasing the percentage of type IIx fibers and decreasing the percentage of slow fibers (type I) (**Fig. 3e** and **f**). Of note, apelin treatment also rescued APJ expression at the full muscle level (**supplemental S3a**).

Ex vivo, the *plantaris* muscle of aged apelin-treated mice displayed enhanced contractile capacities compared to controls (**Fig. 3g**). In addition, apelin administration in aged mice led to a significant increase of muscle function evaluated by resistance and endurance exercise capacities as well as strength (**Fig. 3h-j** respectively). Apelin treatment also rescued endurance and resistance in Apln$^{-/-}$ mice, demonstrating that the phenotype of apelin KO mice is reversible and does not result from long-term adaptations that cannot be reversed. The protective effect of apelin on aged muscle was confirmed by specific overexpression of apelin in 24 month-old mice hindlimb muscles through intramuscular injection of an adeno-associated-virus coding for apelin, and for which we validated apelin overexpression in muscle (**supplemental S3b-c**). Intramuscular injection of AAV-apelin in the hind leg resulted in increased *tibialis anterior* and *plantaris* muscle mass (**supplemental S3d-e**). No increase of soleus mass was observed (**supplemental S3f**), possibly because this muscle was not targeted by the intramuscular injection. As previously observed with the pharmacological approach, apelin overexpression in muscle induced a significant increase in endurance (**Fig. 3k**), strength (**Fig. 3l**) and resistance (**Fig. 3m**); demonstrating that local apelin production is determinant for muscle physiology during aging. Together with the regulation of muscle apelin production by

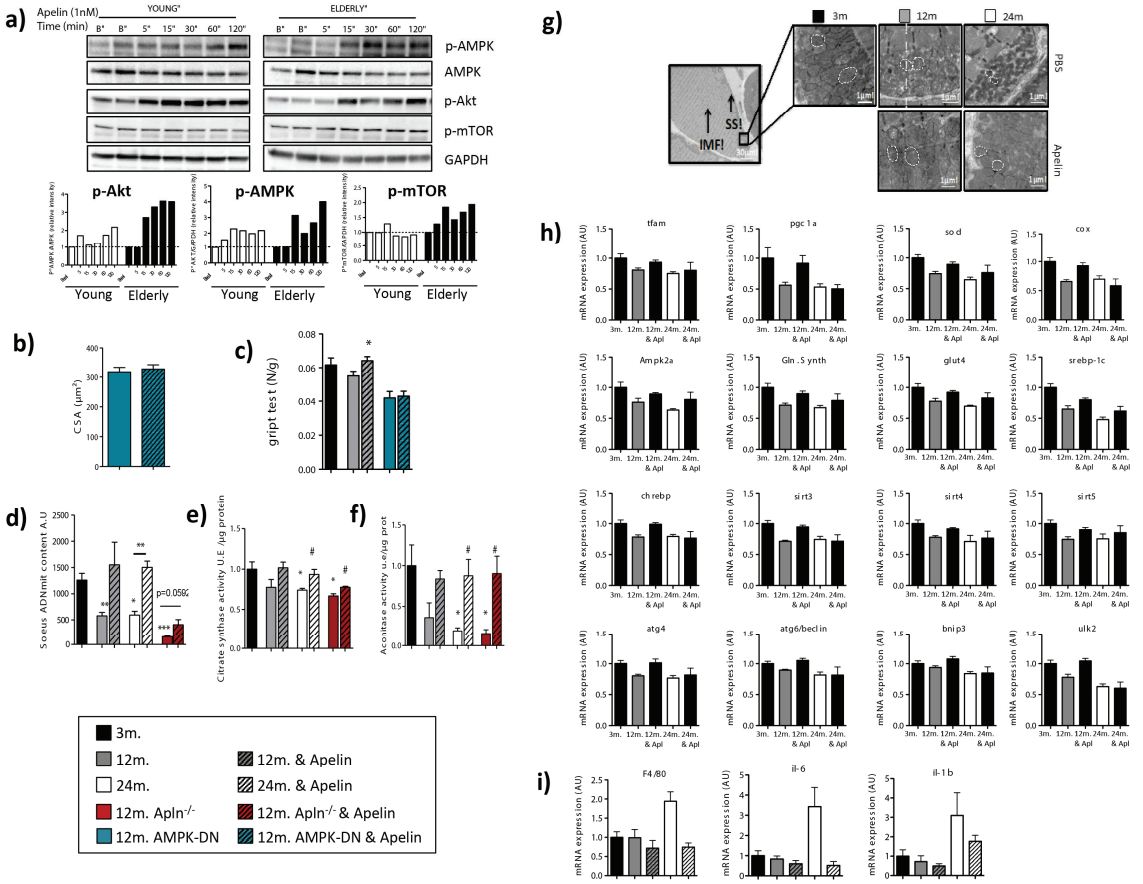

Figure 4. Apelin signaling in skeletal muscle requires AMPK. (a) Western blot for phosphorylated-AMPK Thr172 (p-AMPK), total AMPK, phosphorylated-Akt Ser 308 (p-Akt), phosphorylated-mTOR (p-mTOR) relatively to GAPDH of human myotubes differentiated from young and old donor myoblasts and treated with Apelin for different durations (Top). Western Blot quantification graphs for p-Akt, p-AMPK, p-mTOR (Bottom). **(b,c)** Average muscle fiber cross-sectional area (b) and grip strength (c) of 12 month-old APMK-DN mice treated (hatched bars) or not (plain bars) with apelin. **(d-f)** *Soleus* mitochondrial DNA content (d), and citrate synthase (e) and aconitase (f) enzymatic activity of 3, 12, 24 month-old WT and 12 month-old Apln$^{-/-}$ mice supplemented (hatched bars) or not (plain bars) with apelin. **(g)** Subsarcolemal mitochondria in soleus muscles of 3, 12, 24 month-old WT treated with PBS or apelin, imaged by electronic microscopy. **(h,i)** mRNA levels of different genes involved in metabolism quantified by Fluidigm Biomark.

exercise, this result raises the question of a potential cumulative effect of apelin and physical exercise during aging on muscle physiology. We thus performed apelin treatments combined with exercise during 28 days in aged mice. No additive effect of apelin with exercise was observed on muscle function since apelin-treated mice did not display a gain in muscle mass or force compared to trained mice only (**supplemental S3g-j**). Age-related muscle fatigue is molecularly characterized by accumulation of reactive oxygen species and alterations of metabolic function in fibers as well as poor cell renewal. Consequently, this result suggests that apelin could target these pathways and led us to investigate the consequences of APJ activation in fiber metabolism and satellite cell activation.

Apelin/APJ promote AMPK-induced mitochondriogenesis in sarcopenic skeletal muscle fibers

We previously showed the pivotal role of muscle AMPK in apelin signaling in the context of obesity [21, 23]. To determine the role of AMPK in apelin signaling during ageing, we first performed *in vitro* short-term apelin treatment in human muscle cells from young and aged donors (**Fig. 4a**). AMPK and Akt phosphorylation increased after 5 minutes of apelin (1nM) treatment in young cells. Surprisingly, this effect was maintained in aged cells suggesting that, like other AMPK or Akt

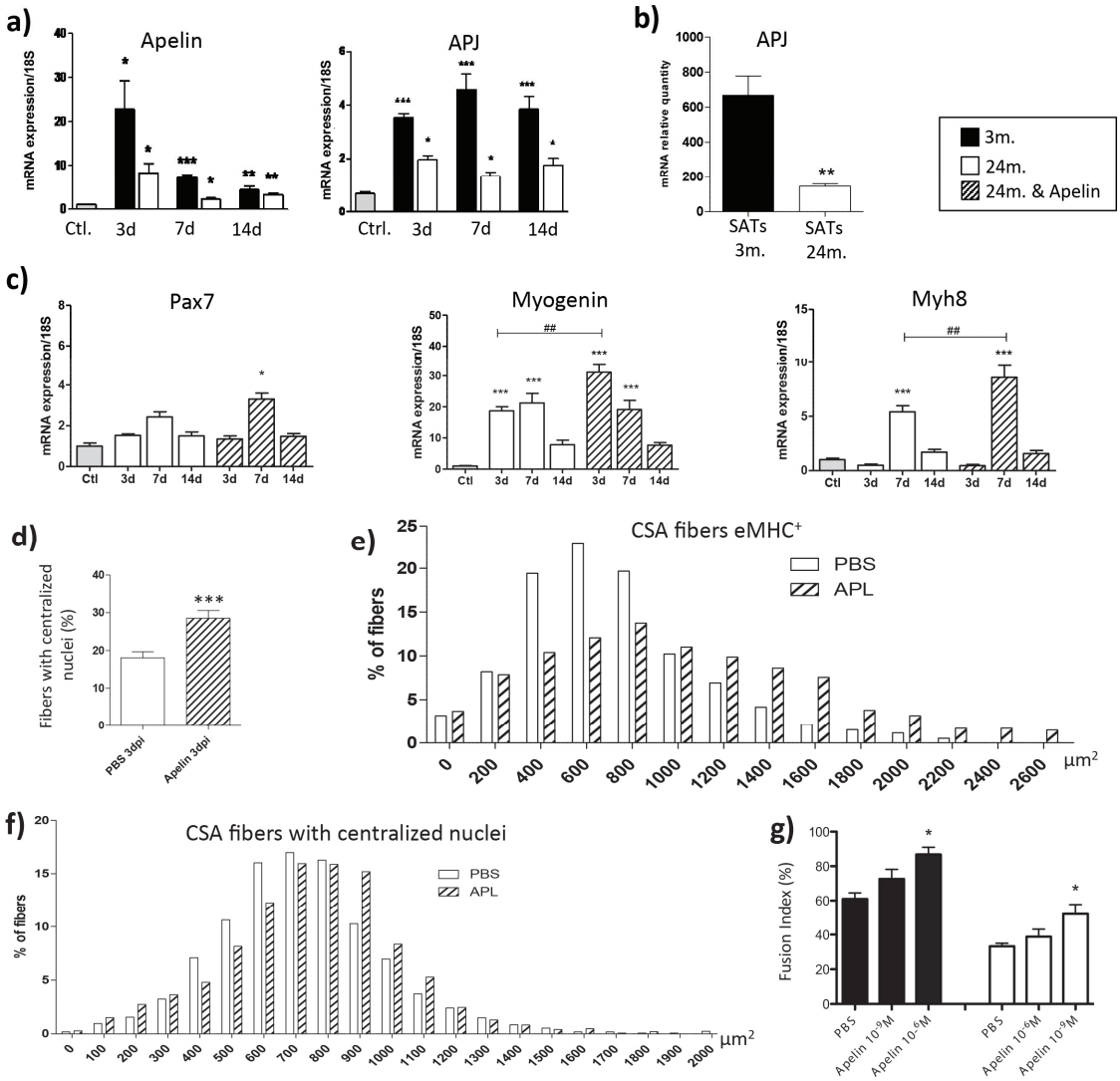

Figure 5. Apelin treatmemt enhances muscle regeneration. (a) Tibialis anterior muscles of 3 and 24 month-old mice were injured with CTX and collected at 3, 7 and 14 days (d) after injury. Apelin and APJ expression in regenerating muscles. **(b)** APJ expression level in young and old freshly isolated satellite cells. **(c-f)** 24-month old mice were treated with PBS (plain bars) or apelin (hatched bars) during a time course of muscle regeneration following CTX-induced injury. (c) qPCR expression of *Pax7*, *myogenin* and *Myh8* myogenic markers in regenerating muscles. (d) Quantification of regenerating fibers with centralized nuclei 3 days after muscle injury. (e) Cross-sectional area distribution of regenerating fibers expressing the embryonic myosin heavy chain (eMHC$^+$) 3 days after muscle injury. (f) Cross-sectional area distribution of regenerating fibers with centralized nuclei 14 days after muscle injury. **(g)** Fusion index of muscle fibers differentiated from human young (black bars) and old (white bars) myoblasts upon apelin treated *ex vivo*.

activators such as exercise or AICAR, apelin still stimulates these pathways. Since myoblast-derived cells in vitro may not fully recapitulate the perturbations of aging at the molecular pathway level, the crucial role of AMPK for apelin signaling was further tested by chronic apelin treatment of 12 month-aged mice lacking AMPK activity specifically in skeletal muscle (AMPK-DN) (**Fig. 4 b and c**, blue hatched bars). Apelin failed to enhance myofiber cross-sectional area and grip strength in AMPK-DN, demonstrating that AMPK is required to mediate the beneficial effects of apelin on muscle strength and hypertrophy during aging. Chronic disuse of muscle, sedentary behavior and aging each independently result in a decline in mitochondrial content and function, leading to the production of free radicals and cell death. Downstream of AMPK, chronic apelin treatment enhanced mitochondrial biogenesis, as suggested by increased mitochondrial DNA content in muscles of aged and apln$^{-/-}$ mice treated with apelin (**figure 4d**); as well as mitochondrial

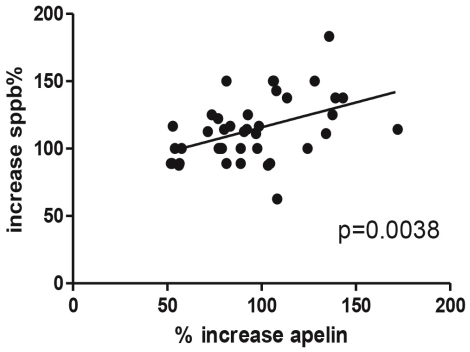

Figure 6. Apelin could serve as a biomarker to score success of exercise-based programs. Correlation between SPPB score increase of elderly following 6 months of exercise program, and increase of apelin plasma concentration.

activity as shown by improved citrate synthase and aconitase activity (**Fig 4e and f**). Finally, all these improvements led to better mitochondrial morphology by enlarging the size of the organite in aged mice, definitively arguing for a beneficial role of apelin in muscle mitochondrial physiology (**Fig 4g**).

In order to uncover the molecular mechanism of improved mitochondrial physiology in response to apelin, we performed a transcriptomic approach using a microfluidic array to analyze gene expression in isolated *plantaris* muscle of mice treated chronically with apelin (**Fig 4h)**. In the middle-aged group, apelin increased the expression of genes related to mitochondrial biogenesis (*tfam* and *pgc-1α*), oxidative damage response (*sod* and *cox*), metabolism (*ampk, akt, gln synth., pfk, glut4, srepb-1c and chrebp*), sirtuins (*sirt3, sirt4* and *sirt5*) and autophagy (*atf4, atf6/beclin, bnip-3, ulk2*). Surprisingly, apelin did not stimulate the transcription of these genes in 24-month-old mice but prevented the activation of inflammatory genes (*IL-1ß, F4/80* and *il-6*) characteristic of chronic low grade inflammation (**Fig 4i**). This result indicates that apelin could trigger different molecular mechanisms accordingly to the age and physiological state of the muscle, and suggests that normalizing chronic low grade inflammation could be an upstream mechanism through which apelin rescues the decline of aged muscle.

Apelin targets satellite cells to enhance muscle regeneration

It has been widely reported that exercise leads to muscle stem cell activation [26-29]. As apelin was upregulated by muscle contraction and exercise, we questioned a potential role of apelin on muscle stem cell function. Using an intra-muscular injection of cardiotoxin (CTX), we injured *tibialis anterior* muscles in young and aged mice, and observed a strong upregulation of apelin and APJ at the mRNA level 3 days after muscle injury in young mice (**Fig 5a**), suggesting a potential role for the apelin/APJ signaling axis during muscle regeneration. While apelin mRNA levels returned to basal levels throughout muscle regeneration, APJ mRNA levels remained elevated until at least 14 days after injury. Decline of muscle regenerative capacities with age is well acknowledged [11, 12], and poor recovery of elderly people after muscle trauma can aggravate their already weakened muscle function. Interestingly, we observed that both apelin and APJ upregulation following muscle injury were reduced in aged mice (**Fig 5a**), suggesting that altered apelin signaling could also be linked to regenerative failure in the elderly. We next isolated satellite cells from uninjured muscles of young and old mice by flow-cytometry, and demonstrated that the apelin receptor was expressed on satellite cells and dramatically decreased with age (**Fig 5b**).

Consequently, we treated cardiotoxin-injured aged mice with apelin (0.5 µmol/kg, ip) and analyzed the effect of this supplementation on muscle regeneration. Apelin supplementation increased the expression of myogenic markers such as *Pax7*, *myogenin* and the embryonic myosin heavy chain *Myh8* following muscle injury, demonstrating a better regenerative capacity in apelin-treated mice (**Fig 5c**). At the histological level, regenerating fibers with centralized nuclei appeared more rapidly in apelin-supplemented mice (**Fig 5d**), and the cross-sectional area of regenerating fibers (either expressing the embryonic myosin heavy chain eMHC or with centralized nuclei) was increased (**Fig 5e,f**, respectively). Altogether, these results showed that muscle regeneration was significantly ameliorated in old mice upon apelin supplementation. Apelin supplementation also induced beneficial effects on muscle regeneration in young (**supplemental S4a,c**) and 12 month-old (**supplemental S4b,d**) mice, both at the molecular and histological levels. Finally, apelin enhanced myogenic differentiation of human myoblasts from an aged donor in vitro (**Fig 5g**), suggesting that the beneficial effects of apelin on regeneration and myogenesis also extend to the human setting.

Apelin is a new biomarker of successful exercise in the elderly

By acting simultaneously on muscle metabolic and functional plasticity, contraction-induced apelin production in muscle could constitute a biomarker of successful aging regarding physical exercise and myogenesis. To

support this hypothesis, we measured plasma apelin in a group of elderly patients who underwent a regular physical exercise during one year (LIFE-Study) [8]. The functional response of physical activity quantified by the increase in the Short Physical Performance Battery (SPPB) from baseline to the end of the exercise program positively correlated with the change in apelin levels over the first 6 months of the study (**Fig. 6**). Compared to their baseline level of plasma apelin before the exercise program, individuals increasing their blood levels of apelin 6 months after the beginning of the trial therefore had the most optimized SPPB score after 1 year of physical activity. This result indicates that plasma apelin levels measured in the early phases of an exercise intervention in elderly could represent a new biomarker to predict physical exercise success or the need for pharmacological intervention.

DISCUSSION

Age-associated muscle weakness is described as one of the most powerful predictive factors for disability, age-related pathologies, and ultimately death [5, 6]. Consequently, it appears fundamental to identify and characterize individuals at risk of developing sarcopenia early and propose strategies in order to prevent frailty and disability, and thereby delay institutionalization and death. In this context, our study demonstrates a new regulatory loop between muscle contraction, apelin production and prevention of sarcopenia. Interestingly, our results reveal that the inability of skeletal muscle to efficiently produce apelin during aging plays a causal role in loss of muscle mass and strength. Finally, we also provide evidence that apelin is a promising therapeutic target to treat physical decline, as well as a new tool for diagnosis of sarcopenia and prognosis of exercise success in elderly populations.

The first evidence showing the crucial role of the apelin peptide in muscle physiology during aging came from our analysis of apelin and APJ deficiency in mice. Lack of apelin or its receptor in middle-aged mice induced a dramatic loss of muscle mass and revealed alterations in muscle functions leading to a strong acceleration of muscle weakness. Interestingly, muscle atrophy was not observed in young apelin KO mice emphasizing a specific role of apelin at accelerating physical decline during aging and thereby causing sarcopenia independently of muscle growth. Consequently, we hypothesized that apelin did not act on muscle physiology as an anabolic factor such as testosterone or insulin growth factor-1 [30, 31], but rather preserved processes de-regulated during aging such as metabolism, oxidative stress, innervation or inflammation. In a context of metabolic diseases, we and others have previously demonstrated that apelin signals in skeletal muscle and improves metabolic function through an AMPK-mitochondriogenesis dependent axis [21, 23]. Furthermore, APJ drives the activation of intracellular pathways such as AMPK, mTOR, P70S6 kinase in various cellular contexts other than skeletal muscle [16, 32-34]. During aging, alterations of mitochondrial metabolism in muscle is widely established and AMPK, P70S6 kinase are major actors of this deficiency [35-37]. Our results show that apelin supplementation in old mice is associated with an AMPK-dependent improvement of different parameters related to muscle rejuvenation such as mitochondrial function and biogenesis, antioxidant enzymes, autophagy and inflammation without displaying an overactivation of all these physiological processes. Even if other muscle atrophy-associated processes such as innervation or neoangiogenesis need to be investigated and cannot be ruled out to explain the effects of apelin; these results confirmed the pivotal aspect of AMPK-dependent mitophormesis in skeletal muscle during aging [37, 38], and most importantly identified a new paracrine mechanism that remains sensitive to exercise and therapeutic intervention during aging. Complementary investigations will be required to distinguish the direct effects of apelin from the consequences of metabolic improvements triggered by apelin.

Another aspect of our work also described a beneficial effect of apelin during muscle regeneration in aged mice and human cell lines. Apelin triggers mechanisms allowing the expansion and differentiation of satellite cells and leads to an improvement of regenerative processes. However, by using a mouse model controlling the inducible depletion of satellite cells, Fry et al. demonstrated that regenerative processes do not affect sarcopenia in sedentary mice [39]. Nevertheless, apelin could be a promising therapeutic intervention to enhance muscle repair after trauma or hip/knee replacement surgery where muscle stem cells are directly mobilized, in particular in aged populations with poor regenerative capacity. In addition, it is interesting that apelin treatment and exercise can both promote beneficial effects on myofiber metabolism and muscle stem cell function. Apelin, which is produced by myofibers in response to contraction during exercise, is therefore both an autocrine myokine enhancing myofiber mitochondrial function and a paracrine myokine mediating the communication between myofibers and satellite cells in the muscle stem cell niche. This dual action is particularly beneficial in the context of exercise as resistance training induces

low-grade acute muscle damage by disrupting the architecture of myofibers, and thereby triggers a mild regenerative response through muscle stem cell activation. Apelin production upon muscle contraction can therefore both enhance the beneficial metabolic adaptations of exercise in myofibers and accelerate the recovery from the mechanical stress of contraction by enhancing stem cell function. .

Using both human and rodent models, our results confirmed previous data reporting apelin expression in skeletal muscle [21, 23, 40] and further establish apelin as a bona fide myokine secreted systemically in response to exercise, as previously suggested at the transcriptional level [22, 41]. We demonstrate for the first time that muscle contraction induces apelin expression and secretion into bloodstream both in rodents and in humans. Additionally, our results demonstrate that basal and exercise-induced production of apelin by muscle is blunted during aging and can be used as a molecular biomarker of muscle function in aging. The regulatory loop between apelin, exercise and myogenesis defines apelin as a newly identified "exerkine" that can be used as a potential biomarker of exercise success, especially during aging. Compared to other exercise-induced secreted factors such as interleukin-6 [42], apelin has the potential to be more specific as it is not confounded by immune cell production. However, different apelin isoforms (apelin-13, 17 or 36 amino acids) are present in plasma or biological fluids arguing for the need to better characterize the forms produced by contractile muscle by more accurate techniques to further gain specificity in the use of apelin as a biomarker of age-related exercise success.

Altogether, we uncovered a new and central role of apelin in the maintenance of skeletal muscle function and the response to exercise during aging. Our results pave the road for new approaches in the medical management of physical decline as apelin can be used both as a biomarker of sarcopenia and a therapeutic strategy to treat age-associated muscle weakness. Treatments with recombinant apelin or APJ agonists are currently being tested in clinical trials in healthy volunteers and patients suffering from idiopathic pulmonary hypertension, COPD or obesity (such as NCT01590108, NCT02150694 and NCT02259686, [43]). While these studies test administration routes and assess safety primarily, they also pave the road for future trials in the context of muscle function and amelioration of disability. The opportunity to combine the systemic detection of low apelin production with the corresponding therapeutic intervention targeting only the subset of responder patients within the larger elderly population at risk will be of invaluable benefit in the era of precision medicine.

METHODS

Human subjects. 70+ year-old patients from the Multidomain Alzheimer Preventive Trial (MAPT) were given a sarcopenic index based on their appendicular lean mass and fat mass measured by DEXA [24]. Patients were stratified in four subgroups based on their sarcopenic index and plasma apelin concentration was measured in the two extreme groups. Plasma apelin concentration before and after an exercise program was performed in subjects from the Lifestyle Intervention and Independence for Elders (LIFE) studies [8].

Apelin assay plasma sample. Arterial or Venous total blood were sampled in fasted mice and human, centrifuged, then serum was collected and frozen at -80°C. Serum apelin, insulin and leptin concentrations were measured using a nonselective apelin-12 EIA kit (Phoenix Pharmaceuticals; Belmont, CA), an ultrasensitive mouse/human insulin ELISA (Mercodia, Uppsala, Sweden) and mouse/human leptin ELISA (R&D system) respectively.

Animals. Animals were handled in accordance with principles and guidelines established by the Institute of medical Research. C57Bl6/J Wild-Type (WT) from 3 to 24 months-old mice were obtained from Janvier Loboratory (St-Berthevin France). Mice deficient in AMPK activity (DN AMPK) were kindly provided by the laboratory of Pr. Birnbaum (University of Pennsylvania Medical School; Philadelphia, USA). Apelin-deficient ($apelin^{-/-}$) mice were generated as described previously [15]and backcrossed to C57Bl6/J WT mice 10 times. APJ-deficient ($APL^{+/-}$) mice were generated from cross between C57Bl6/J littermates WT and mice heterozygous for the APJ mutation (Deltagen, San Carlos, CA, USA). Mice were housed conventionally in a constant temperature (20–22°C) and humidity (50%–60%) animal room, with a 12/12 h light/dark cycle and free access to food and water. All mice were fed with normal diet. All mice were euthanized in a fed state by cervical vertebra dislocation.

Apelin supplementation by chronic intra-peritoneal injection. 3 to 24 months-old WT mice, 12 months-old $apelin^{-/-}$ and DN AMPK mice were injected daily with apelin-13 (Phoenix Biotech) at 0.5 µmol/kg/day intra-peritoneal as previously described for 28 days (ref). Age and genotype-matched control mice were PBS injected during the same period. All mice were euthanatized 24 h after the last apelin injection.

Apelin over-expression. Adeno-associated virus vector was constructed as follow: *apelin* gene was integrated to AAV genome under luciferase promoter dependency with secretion signal. 24 months-old mice were both leg shaved and anesthetized with ketamin-xylamin and injected in gastrocnemius and tibialis anterior muscles of each leg (10^{11}vg/muscle). AAV production was followed every 7 days through luciferase activity-induced bioluminescence 15 minutes after i.p injection of luciferin (promega, Ref, Concentration), the luciferase substrate.
One week after injection was needed to AAV skeletal muscle cell integration and reaching maximal protein production (estimated through luciferase activity). The four following weeks were considerate to apelin

impregnation. Thus, mice were euthanized 35 days post-injection.

Acute and chronic physical training. Before entrainment began, mice were submitted to acclimation phase, they were placed on treadmill for 5 minutes (0cm/sec speed without inclination), then for 5 minutes at slow speed (10cm/sec). The next day, maximal speed capacity is measured by rising speed to 5 cm/sec every 2 min until exhaustion. 24h after maximal speed capacity test, entrainments begin. 3, 12 and 24 months-old WT and 12 months apelin$^{-/-}$ mice run for 30 minutes, 65% maximal speed, 10% slope, 6 days a week for 1 or 28 days (respectively for acute and chronic training).

Skeletal muscle function exploration. *In vivo* muscle strength was measured by grip test: mice were allowed to grasp onto the horizontal grid connected to a dynamometer by using 4 legs and pulled backwards 5 times. The force applied to the grid each time before the animal lost its grips was recorded in Newton. Average of the 5 measures is normalized to the whole body weight of each mouse as previously described**.** *Inverted grid test or four limb hang test* uses a wire grid system to non-invasively measure of mice's ability to exhibit sustained limb tension to oppose their gravitational force, in other words, measure endurance of whole body skeletal muscle. Mice were placed on a wire grid which is then inverted. The latency to fall is recorded as previously described. We tested locomotion and *running performance* by testing maximal speed capacity of mice on treadmill with the test described above.

Sciatic nerve electrical stimulation. Mice were anesthetized with pentobarbital i.p injection warmed by heat lamp, knee and foot were fixed. Distal tendon of TA and plantaris were isolated from other surrounded muscles, cute and binded with silk attached to a force transducer (AD Instruments, 50g and 500g respectively for plantaris and TA) using PowerLab system (26T, ADInstruments) and softward (LabChart 4 ADIstruments). Bipolar electrical probes were placed on isolated sciatic nerve and stimulated using supramaximal square wave pulse. Force generation capacity was evaluated by measuring the maximal force generated in response to isometric contraction under electrical stimulation (frequency of 75-150 Hz, train of stimulation of 200 msec). Maximal isometric force was determined at L0 (length at which maximal tension was obtained during the tetanus). Force was normalized to the muscle mass as an estimate of specific force. Fatigue resistance was determined after 2 minutes of resting period. Sciatic nerves were stimulated at 10 Hz during 500 msec, every 2 sec, for 1 minute. All contractions were made at an initial length L0. Contractions were expressed as a percentage of the initial maximal (isometric) force. (Duration corresponding to a force decreased by 50% was noted as fatigue.

Venous-arterial difference measurements. Mice were anesthetized with pentobarbital and warmed on the back on a 37°C isothermal pad. We shaved the neck and groin and we made skin incision in order to visualize left carotid artery and left femoral vein. Catheters were placed in both vessels then arterial and venous blood was collected through catheters to 0.5 ml tube maintained at 4°C on ice. System were stabilized for 10 minutes before installation of contractility device with isolation of left TA tendon attached to force transducer as described above and left sciatic nerve isolation. System with catheter, probes and TA tendon attached with silk was stabilized for 10 minutes, then new tubes were placed to catheters extremities and blood was collected for 10 minutes and considerate as "baseline condition". Then, sciatic nerve was stimulated at XXXX Hz during, every XXX for 10 minutes leading to skeletal muscle contraction, while new tubes were placed to catheters extremities and arterial and venous blood were collected and considerate as "stimulation condition".

Cardio toxin injury-induced skeletal muscle regeneration. Injuries were performed as previously described. Mice were intra-peritoneal injected with, buprenorphin (Centravet, 0.1 mg/kg) 30 minutes before injury and the day after. Mice were anesthetized with isofluran inhalation and shaved legs then 10 µM cardiotoxin (CTX, losartan, #L8102) were injected through two injections of 25 µl into left tibialis anterior (TA) muscle and two injections of 50 µl into left gastrocnemius muscle, using a 22 gauge needle (Hamilton). Mice were euthanized 3, 7 and 14 days after injury by cervical dislocation, muscles were cut in two parts, one being frozen into liquid nitrogen for total RNA extraction and the other part being embedded into OCT, frozen for 2 seconds in liquid nitrogen and then frozen in isopentan cooled with liquid nitrogen for histological analysis.

Human cell culture. Two muscle cell lines derived from young and aged human donors (19 and 79 year-old; cell lines #155 and #379, respectively) were given to us by Dr. Vincent Mouly (Genethon, Paris).

Mouse muscle stem cell isolation and micro-array. For isolation of satellite cells, muscles were collected uninjured and digested with Dispase II (2.5 U/ml), Collagenase B (0.2%) and MgCl2 (5 mM) at 37 °C as previously desbribed [44]. Cells were then incubated at 4 °C for 30 min with antibodies against CD45, CD31, CD11b, CD34, Ly-6A–Ly-6E (Sca1) and α7-integrin. FACS isolation was performed on a Beckman Coulter Astrios Cell sorter. Satellite cells were isolated by flow-cytometry as: CD31-/CD11b-/CD45-/Sca1-/CD34+/Integrin α7+. RNA was extracted from freshly sorted satellite cells using RNeasy Micro Kit (Qiagen). RNA samples were then subjected to 3' microarray analysis on Illumina MouseRef–8_V2 chips. 3 ng of total RNA were used to produce cRNA in a two-round amplification protocol, using first Messageamp II aRNA amplification kit (AM1751, Life Technologies, Inc.) followed by Messageamp II–biotin enhanced aRNA amplification kit (AM1791, Life Technologies, Inc.). 750 ng of cRNA were hybridized for 16 h at 55 °C on Illumina MouseRef–8 v2 microarrays. Quality of total RNA was checked by using the Bioanalyzer 2100 with Total RNA Pico kit, and quality of cRNA was checked by using the Bioanalyzer 2100 with the Total RNA Nano kit (Agilent Technologies). Quantifications were done using the Quant-iT RiboGreen RNA Assay Kit (Life Technologies, Inc.). Illumina expression signals were quantile-normalized. We applied a nonspecific filter to discard probe sets with low variability and retained 12,848

Illumina probe sets whose s.d. was greater than the median of the s.d. of all of the probe sets. For differential expression analysis and pathway analyses, genes (represented by probe sets) were tested for differential expression using the moderated t-statistic as implemented in LIMMA44 for both data sets.

Mitochondrial DNA content. Total DNA was extracted from soleus and plantaris muscles using a commercial kit (DNeasy; QIAGEN). The content of mitochondrial (mt) DNA was calculated using real-time quantitative PCR by measuring the threshold cycle ratio of a mitochondrial encoded gene (COX1) and a nuclear-encoded gene (cyclophilin A) as previously described.

Citrate synthase and aconitase activity. Frozen cryostat sections were dropped into 100 µl of ice-cold extraction buffer to 5 mM sodium phosphate buffer, pH 7.2, containing 4 mM magnesium acetate and a proteinase inhibitor, aprotinin, as indicated by the manufacturer (Boehringer Mannheim, Meylan, France). Following 1h of centrifugation in the cold at 1,500 *g*, pellets were recovered for citrate synthase (CS) enzymatic activity measurements with 100 ml CS buffer [5 mM HEPES, pH 8.7, 1 mM EGTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, Triton X-100 (0.1%)], followed by incubation for 60 min at 4 °C to ensure complete enzyme extraction from mitochondria. All assays were performed in 96-well plates, with a final volume of 200 µl. Protein concentrations were determined using a commercial kit (Bradford Bio-Rad Protein Assay Kit). Determination of CS activity was assayed, according to previously described methods. Aconitase activity was performed as previously described. The photochrome was measured at 525 nm using spectrophotometer.

Microscopic image analysis and quantification. For electron microscope analysis, soleus muscle was cut into small pieces and fixed as previously reported. The tissue was then cut and mounted on copper grids and observed with a Hitachi HU 12A transmission electron microscope equipped with a high-resolution camera.

qPCR. Total RNAs (1 µg) were isolated from muscle using GeneJet RNA Purification kit (K0732, Fermentas, Thermo Scientific) and were reverse transcribed using random hexamers and Superscript II reverse transcriptase (Multiscribe, Applied Biosystem). Real time PCR was performed as previously described starting with 6.25 ng cDNA and both sense and antisense oligonucleotides in a final volume of 10 µl in a 384 well plate using the SYBR green universal PCR master mix (Eurogentec). Analysis of HPRT RNA was performed to normalize gene expression.
Fluidigm by Biomark qPCR was performed with 6.5 ng of 96 cDNA and 5µl (100µM) both 96 sense and antisense oligonucleotides.

Western blotting. Muscle and cells samples were lysed (precellys 24, Ozyme France) and loaded (30mg protein per lane) on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane (GE Healthcare, life Sciences, Whatman). Membranes were blotted with anti-phospho-AMPKα, phospho-Akt, phospho-mTOR, phospho-P70S6K, phospho-FOXO, and respective anti-total protein antibody (Cell Signaling Technology). Immunoreactive proteins were detected using the Clarity Western ECL Substrate (BioRad) and quantified by Image Lab software (BioRad).

Immunohistology. *In situ* determination of fiber type was performed as previously described. Briefly, 10µm muscles frozen sections were immune labeled for the different myosin heavy chains (MHC): primary antibodies were MHC-1 (BA-D5), MHC-2a (SC-71), eMHC (F1.652) from Developmental Studies Hybridoma Bank (DSHB, University of Iowa, IA) and laminin (abcam). Sections were blocked 1 h in PBS plus 4% BSA, 2% goat serum, 0.01% Triton X-100. Sections were then incubated overnight with primary antibodies against. After washes in PBS, sections were incubated 1 h with secondary antibodies anti-Ig2b AF 488, anti-IgG1 AF 546, anti-rabbit AF 350 (Life Technology). Slides were finally mounted in ProLong Gold antifade Reagent (Molecular probes by Life Technology). Images were captured using a digital camera (Nanozoomer, Hamamatsu) attached to a motorized fluorescence microscope and morphometric analyses were made using the VS-ASW FL software measurement tools. For laminin-eMHC immunostaining, cryosections were allowed to dry during 10 minutes and blocked for 45 minutes at room temperature in the blocking solution (PBS, 4% BSA, 1% FBS). Cryosections were stained during 3 hours at room temperature using monoclonal anti-laminin antibody produced in rabbit (Sigma-Aldrich #L9393) and anti-eMHC produced in mouse (DSHB #F1.652) diluted at 1/100 and 1/500 in the blocking solution, respectively. Stained tissues were photographed using Olympus VS120 Virtual Microscopy Slide Scanning System and analyzed using the VS-ASW FL software measurement tools. The area covered by eMHC-positive fibers and degenerated area was determined manually across the entire sections. The size of myofibers with central nuclei was calculated from laminin/DAPI stainings on all fibers of the section, using an automated image processing developed internally using the MetaXpress software (Molecular Devices).

Statistical analysis. Experiments were performed at least three times and each time in duplicate, at minimum. Data are expressed as the mean ± the standard error of the mean (s.e.m.). All statistical analyses were performed using GraphPad Prism (GraphPad Software) assuming normal distribution of the variables measured. Statistical significance for binary comparisons was assessed by a Student's t-test after checking that variances do not differ between groups or by a Welch correction when variances differed between groups. For comparison of more than two groups, one-way or two-way ANOVAs were used, according to the experimental design, and followed by Bonferroni multiple-comparison testing. p-values less than 0.05 (*), 0.01 (**) or 0.001 (***) were taken as statistically significant

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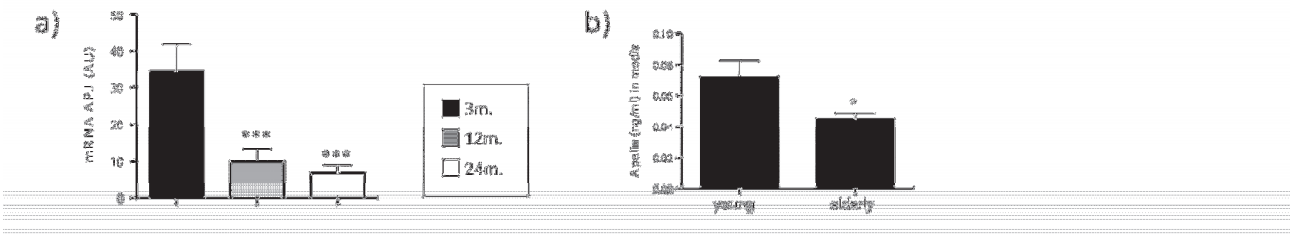
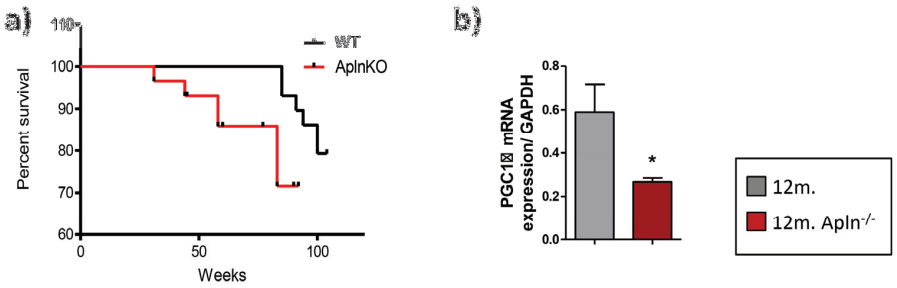
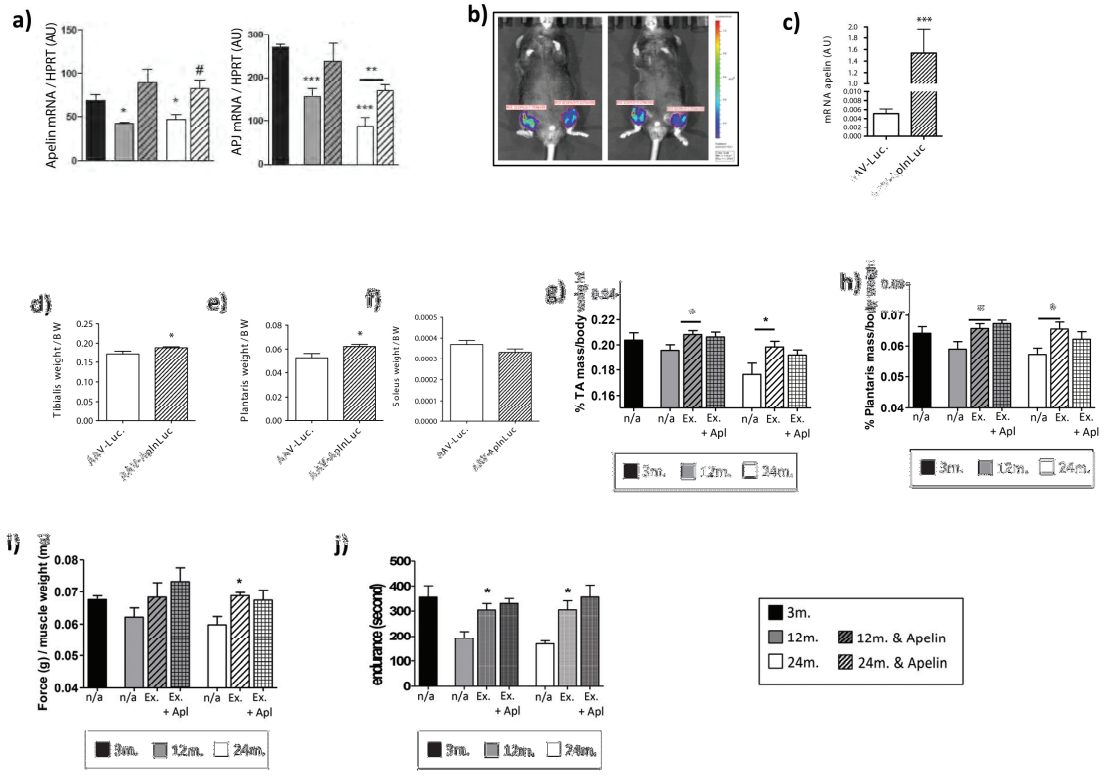


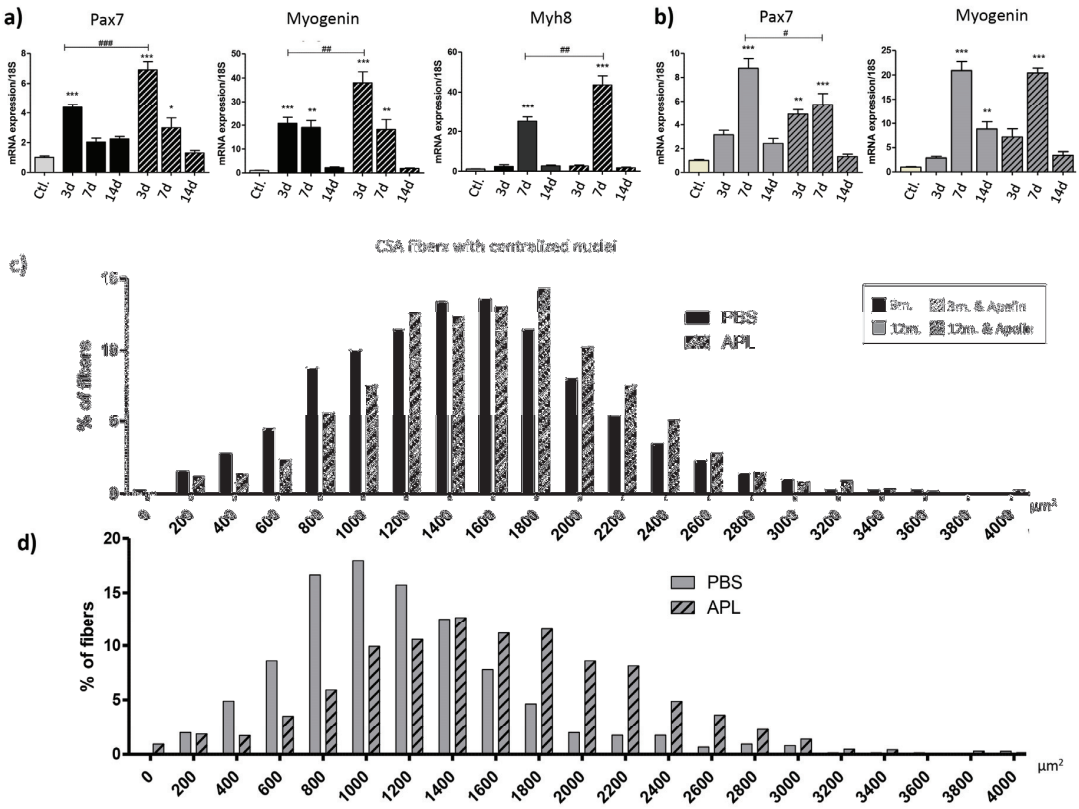
Supplemental S1. **(a)** APJ expression in fibers isolated from EDL of 3, 12, and 24-month-old mice. **(b)** Apelin concentration in medium of fibers 14 days after induction of differentiation human myoblasts from young (19 year-old) and old (79 year-old) donors.



Supplemental S2. **(a)** Lifespan curves of WT and Apln$^{-/-}$ mice. **(b)** PGC1α expression in full muscle of 12 month-old WT and Apln$^{-/-}$ mice.



Supplemental S3. (a) Apelin and APJ expression in muscles of 3, 12, 24 month-old WT mice supplemented (hatched bars) or not (plain bars) with apelin. **(b)** Maximal *tibialis anterior* force following sciatic nerve electric stimulation in 3, 12, 24 month-old WT mice supplemented (hatched bars) or not (plain bars) with apelin. **(c)** Luciferase activity of AAV vectors one week after intramuscular injection in *tibialis anterior* and *gastrocnemius*. **(d)** Apelin expression in gastrocnemius five weeks after AVV-vector injection. **(e-g)** *Tibialis anterior* (e), *plantaris* (f) and *soleus* (g) weight relatively to body weight of 24 month-old mice injected or not with AAV-Apelin. **(h,i)** *Tibialis anterior* (h) and *plantaris* (i) weight relatively to body weight of 3, 12 or 24 month-old mice with no challenged (plain bars), exercised (hatched bars), exercised and supplemented with apelin (squared bars) during 28 days. **(j,k)** Force (j) and endurance (k) of 3, 12 or 24 month-old mice with no challenged (plain bars), exercised (hatched bars), exercised and supplemented with apelin (squared bars) during 28 days.



Supplemental S4. 3 (black bars) and 12-month old mice (grey bars) were treated with PBS (plain bars) or apelin (hatched bars) during a time course of muscle regeneration following CTX-induced injury. **(a,b)** qPCR of myogenic markers (pax7, myogenin and myh8) in 3 (a) and 12 (b) month-old regenerating muscles. **(c,d)** Cross-sectional area distribution of 3 (c) and 12 (d) month-old regenerating fibers possessing centralized nuclei 14 days after injury.

CHAPTER VII. Discussion & Conclusion

I. Why targeting the muscle stem cell niche?

Impaired healing capacity of the aged skeletal muscle has long been acknowledged to rely on both exhaustion of the satellite cell pool and loss of regenerative function of the old muscle stem cells. The interdependence between numerical and functional decline of satellite cells with age is not fully understood. In mice, the number of satellite cells dramatically reduces from the neonate to the adult, and old mice possess 50% less satellite cells than adult mice (Shefer et al., 2006, Brack et al., 2005, Chakkalakal et al., 2012). Many studies have highlighted perturbations in the balance between satellite cell commitment and self-renewal after an injury in old mice, likely accounting for the progressive depletion of the muscle stem cell pool (reviewed in (Blau et al., 2015)). Conversely, loss of satellite cell number from the niche is progressive through life and stabilizes at geriatric ages, while cell autonomous dysfunction becomes even more dramatic (Bernet et al., 2014, Sousa-Victor et al., 2014). Regenerative functional decline of old satellite cells has been defined by impaired satellite cell activation, migration, proliferation, self-renewal and switch to senescence (Collins-Hooper et al., 2012, Conboy et al., 2005, Cosgrove et al., 2014, Price et al., 2014, Sousa-Victor et al., 2014).

In our results, we could reiterate the loss of satellite cell number, activation (both at the cell cycle entry and MyoD expression levels) and proliferation with age. Interestingly, at the activation onset (36h hours after satellite cell isolation), we observed a dramatic decrease of the number of old satellite cells *ex vivo*, suggesting a poor adhesion capacity. Our results actually revealed that, when let to adhere *ex vivo*, freshly isolated cells from resting muscles initially adhere efficiently but rapidly undergo cell detachment from their substrate. We observed that de-adhesion was considerably more important in old satellite cells and tightly correlated with a higher cell death; demonstrating that old satellite cells undergo anchorage-dependent programmed cell death called anoikis. Age-related susceptibility to apoptosis has been reported in human muscle stem cells (Fulle et al., 2013), but we further uncovered here for the first time that aging affects cell death by inhibiting the cross-talk between adhesion and survival.

Our exploration of intrinsic signaling defects of aged satellite cells during aging focused on the cytoplasmic focal adhesion kinase (FAK) as it plays an important role in cell adhesion and suppression of anoikis (Paoli et al., 2013, Lim, 2013). We demonstrated that FAK signaling is a regulator of satellite

cell adhesion and is dramatically decreased with age. Like us, many groups had previously reported changes in stress pathways in old satellite cells. In particular, constitutively activated p38/MAPK, FGF-ERK/MAPK, JAK-STAT and $p16^{INK4a}$ (Bernet et al., 2014, Chakkalakal et al., 2012, Cosgrove et al., 2014, Price et al., 2014, Sousa-Victor et al., 2014). Demonstrations have shown that blunting these increased stress pathways could rescue the reversible quiescence and the balance between satellite cell commitment and self-renewal, thus partly restoring old satellite cell regenerative capacities. Failure of basal autophagy in old satellite cells was demonstrated to induce $p16^{INK4a}$ activation and senescence (Garcia-Prat et al., 2016). Interestingly, a link between increased $p16^{INK4a}$ and induction of anoikis was established in human cancer cells, and addition of fibronectin could protect the cells from anoikis (Plath et al., 2000). In addition, induction of autophagy has been reported in response to epithelial cell de-adhesion, promoting survival and enhancing the chance of cells to reattach (Fung et al., 2008, Horbinski et al., 2010). Notably, we found that decreased FAK and elevated p38/MAPK and FGF-ERK/MAPK pathways are both concomitantly affected by age and rescued by fibronectin, suggesting that FAK may be a novel regulator of common aging pathways.

Altogether, these studies suggest the possible existence of common upstream extracellular triggers leading to deregulation of intra-cellular signaling pathways and satellite cell autonomous dysfunction with age. Nevertheless, these upstream triggers have only been partly elucidated. In particular, increased FGF-2 and IL-6 in the aged muscles have been proposed to account for the elevated ERK/MAPK and JAK-STAT pathways, respectively (Chakkalakal et al., 2012, Tierney et al., 2014, Toth et al., 2006).

These evidence point to a possible inter-correlation between the different alterations described in old satellite cells, sustaining our interest in searching for common triggers of satellite cell dysfunction. In this thesis, we dissected changes in the larger stem cell niche (**Fig. 12**), in order to open-up the way for novel intervention or prevention strategies to ameliorate healing properties of old muscles. In this view, we used old but non-geriatric mice as models, in order to establish proofs of concept for aged patients in the early phases of physical decline which could benefit from interventions preventing further decline. We expect two major advantages in targeting the satellite cell niche to restore the regenerative function. First, we hypothesized that changes in the muscle stem cell niche might appear as a common upstream cause leading to multiple cell-autonomous defects in the aged satellite cells. Therefore, targeting the niche could be a global strategy to simultaneously restore different dysfunctions and potentially achieve higher efficiency. Second, understanding the cross-talk between satellite cells and their niche, and in particular with other cellular component of the niche, might provide knowledge translatable to other tissue-specific adult stem cell niches.

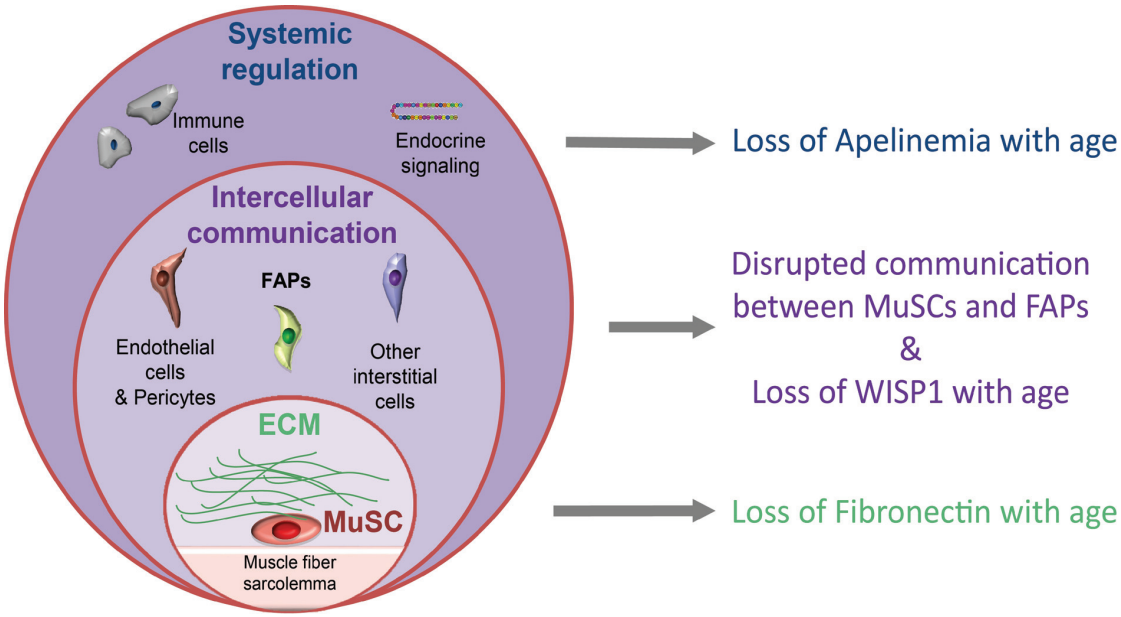


Figure 12. Newly identified multi-level alterations of the satellite cell niche with age that we targeted to restore regenerative capacities of old MuSCs.

II. Targeting the extracellular matrix to restore multiple cell autonomous defects of old satellite cells

The extracellular matrix is the micro-environment in closest proximity to satellite cells, to which it provides structural support as well as signaling cues (Fig. 3). Extracellular matrix remodeling is critical during muscle regeneration to provide a structural scaffold to regenerating fibers (Goetsch et al., 2003), but also to promote satellite cell function (Bentzinger et al., 2013b, Urciuolo et al., 2013, Tierney et al., 2016). The regulation of satellite cell intracellular signaling, and thus satellite cell function, by binding of ECM molecules to membrane receptors has been characterized in certain contexts, although we still don't understand the complexity of these interactions. For example, activation of PI3K and Rac1-MAPK downstream of integrin receptor activation was shown to induce satellite cell activation and differentiation (Liu et al., 2011, Wang et al., 2008). The transmembrane heparin sulfate proteoglycan Syndecan3 was reported to cross-talk with Notch signaling, and thus regulate satellite cell self-renewal (Pisconti et al., 2010). Fibronectin and Collagen-VI also boost satellite cell function and muscle regeneration (Bentzinger et al., 2013b, Urciuolo et al., 2013, Tierney et al., 2016). In particular, fibronectin binds to Syndecan4 that forms a complex with Frizzled-7, and thus potentiates Wnt7a-induced satellite cell expansion (Bentzinger et al., 2013b). Satellite cells are also very sensitive to the matrix rigidity. Bio-mimicking skeletal muscle elastic stiffness (12 kPa) enhances satellite cell self-renewal and maintens stemness (Cosgrove et al., 2014, Gilbert et al., 2010). Aging increases stiffness of isolated myofibers (Lacraz et al., 2015), and the excessive extracellular matrix deposition in skeletal muscle with age leads to increased muscle rigidity (Gao et al., 2008, Rosant et al., 2007). Altogether, these evidence place the extracellular matrix as a central regulator of satellite cell function. The dynamics of its remodeling during muscle repair with age had, however, never been investigated.

Using a proteomic screen performed on young and old regenerating muscles, we found that fibronectin, a protein of the extracellular matrix, was strongly upregulated at 3 and 7 days after injury, and that this upregulation was blunted in the old regenerating niche. In line with the reported fibrosis in old muscles (Kragstrup et al., 2011), we observed increased levels of numerous extracellular matrix proteins at baseline; but fibronectin levels were unchanged suggesting a particular role of this ECM protein during muscle repair. We demonstrated that fibronectin is the preferred adhesion molecule for mouse and human myoblasts, and rescues the anoikis phenotype of old satellite cells, as well as

their proliferation defects *ex vivo* and *in vivo*. Physiologically, distinct cellular processes have been identified in various cell types to protect from anoikis, and adhesion to the extracellular matrix is the most efficient trigger to prevent anoikis (Paoli et al., 2013, Frisch and Ruoslahti, 1997, Reddig and Juliano, 2005). Intercellular adhesion is also known to promote cell survival. Interestingly, we have also demonstrated in this thesis that FAPs promote satellite cell adhesion, therefore possibly enhancing their survival.

Mechanistically, fibronectin rescued old satellite cell adhesion in an integrin-β1 dependent manner, by restoring FAK signaling as well as blunting the elevated MAPK/p38 signaling, a major stress pathway constitutively active in old satellite cells (Bernet et al., 2014, Cosgrove et al., 2014) (**Fig. 13**). Our results therefore suggest that loss of fibronectin from the aged niche causes loss of adhesion and apoptosis of old satellite cells upon injury through the reduction of FAK signaling and the induction of MAPK/p38. Of note, constitutive activation of MAPK/p38 had been identified in old freshly isolated satellite cells or in satellite cells embedded in their niche on freshly isolated myofibers, in a state close to quiescence (Bernet et al., 2014, Cosgrove et al., 2014). This finding indicates the possibility that different upstream regulators may modulate p38 pathway in old resting muscles. Yet, the authors rescued old satellite cell self-renewal using p38 inhibitors at the onset of satellite cell activation and proliferation, and we also observed that aging induces a transcriptional MAPK signature in activated satellite cells. These observations are therefore in line with a role of fibronectin in modulating adhesion and proliferation through the maintenance of low p38 levels after injury. We could also hypothesize that fibronectin regulates satellite cell migration, as in pigs, FAK has been recently shown to regulate satellite cell migration and adhesion to fibronectin, thus further supporting our findings (Wang et al., 2016). Interestingly, another study published simultaneously to ours, highlighted that fibronectin potentiates young satellite cell response to FGF-2, a signal required for their activation, in a integrin-β1 dependent manner (Rozo et al., 2016) (**Fig. 13**). Notably, the authors showed that fibronectin also allowed integrin-β1 deficient satellite cells to respond to higher levels of FGF-2; suggesting that fibronectin can bind to other integrins to cooperate with FGF signaling, at least in integrin-β1 KO cells. Their results also demonstrated that the fibronectin receptor integrin-β1 sensitivity is altered with age, and that restoring its activity with an activating antibody also restores old satellite cell defective sensitivity to FGF-2 and regenerative potential (Bernet et al., 2014, Rozo et al., 2016). With age, integrin-β1 and its downstream effectors are abnormally localized in satellite cells (Rozo et al., 2016), and likely leads to some of the cell-autonomous dysfunction we could observed in old stem cells *ex vivo*. At the phospho-proteomic level, we showed that exposure of satellite cells to fibronectin activates integrin-β1; it would therefore be interesting to assess whether it can also restore its conformation and sensitivity. Collectively, our two studies indicate that changes in fibronectin content

in the old niche may also participate in the aged-related alterations in FGF signaling. In addition, considering the reported links between p16INK4a induction, anoikis and induction of autophagy, additional studies would be required to evaluate a possible cross-talk between p16INKa and integrin-signaling pathways (Fung et al., 2008, Horbinski et al., 2010, Plath et al., 2000, Garcia-Prat et al., 2016), together with a role in age-related satellite cell senescence. Therefore we believe that fibronectin is a promising candidate for developing global therapeutic strategies simultaneously targeting multiple aging pathways, with the potential of achieving high levels of functional restoration.

One direct application of our discovery could be the local intramuscular injection of fibronectin to facilitate functional recovery in localized trauma or surgeries involving muscle laceration such as knee or hip replacement. However, fibronectin is a large protein that cannot be delivered to the niche via systemic treatment, therefore making this application limited to localized muscle injuries amenable to intramuscular treatment. Conversely, the use of activating antibody targeting integrin-β1 could be used in chronic interventions, targeting similar mechanisms in a systemic manner. Our results have also provided a larger integrated view of the satellite cell niche which also opens strategies to directly stimulate local fibronectin production by targeting muscle resident cells through systemic interventions. We uncovered that the lineage cells (Lin^+), encompassing endothelial and hematopoietic cells, were the major source of fibronectin in damaged muscles, and that the loss of fibronectin in the aged niche results from a lower expansion of Lin^+ cells in the aged regenerating muscle. Of note, we also showed that old satellite cells dramatically decrease their expression of fibronectin 3 days after muscle injury. This local autocrine impairment likely exacerbates the local depletion of fibronectin in the stem cell niche compared to the rest of the muscle. It therefore remains unclear which cell type should be primarily targeted to rescue fibronectin levels by stimulating endogenous production. Anti-inflammatory macrophages are good candidates as they are known to secrete extracellular matrix molecules, including fibronectin (Schnoor et al., 2008, Gratchev et al., 2001, Chang et al., 2012). However, further investigation is required to identify the specific Lin^+ cell type accounting for altered fibronectin production in aging. Altogether, our work has emphasized the critical role of the extracellular matrix remodeling as a signaling trigger for satellite cell function during muscle regeneration. Moreover, it reinforced the comprehensive view of satellite cell aging where cellular, protein and molecular changes in satellite cells arise both from alterations of intra-cellular metabolism and extra-cellular signaling from the niche and the systemic environment which are sensed through membrane receptors and then cascaded inside the cell.

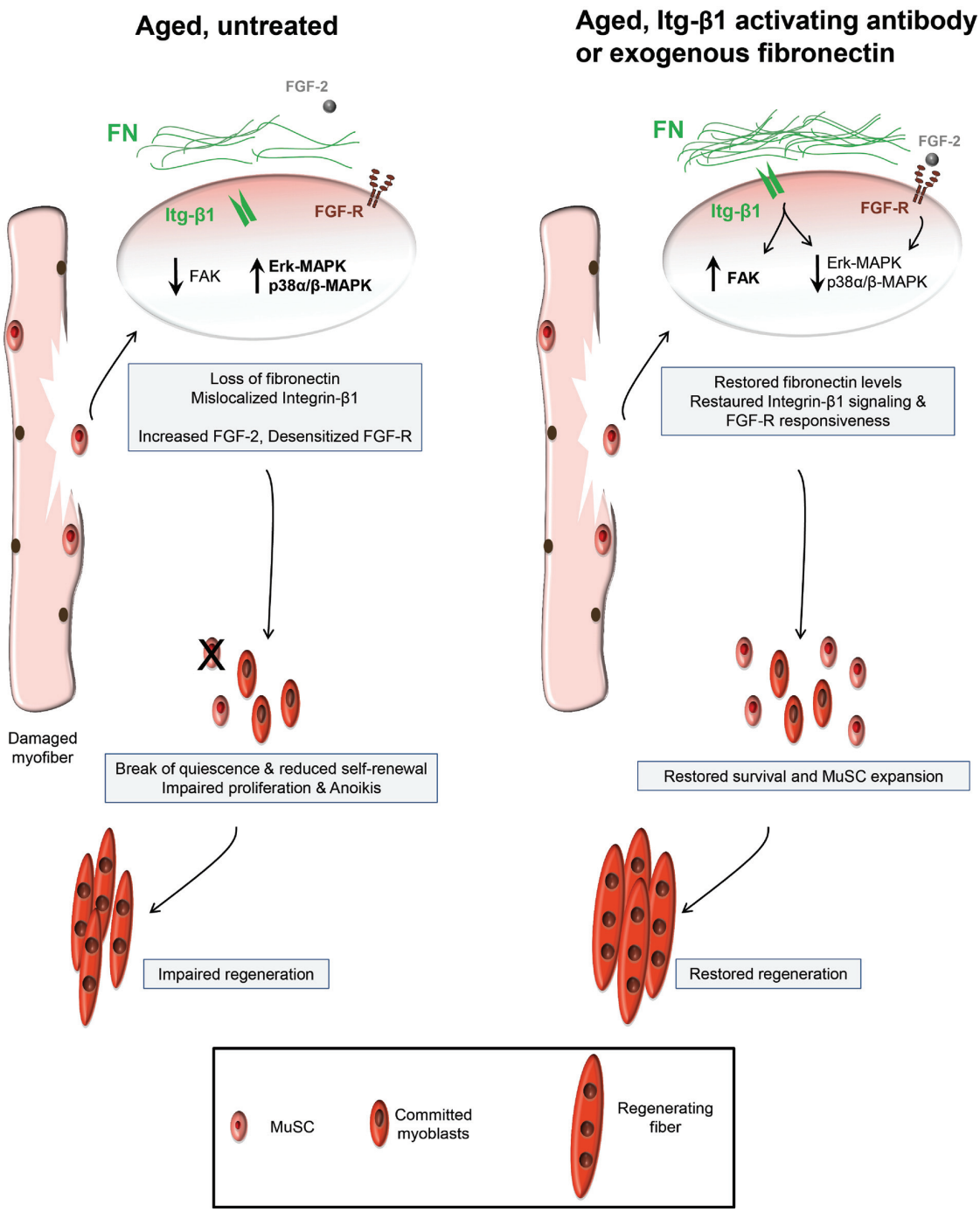


Figure 13. Targeting the interaction between the extracellular matrix and satellite cells ameliorated muscle regeneration. Aged muscles are affected by increased levels of FGF-2 and old satellite cells present a mislocalization of their integrin-β1 receptor and desensitized FGF receptor, both leading to quiescence break. Upon injury, fibronectin is also lost from the extracelular matrix, leading to anoikis. Therapeutic intervention using exogenous addition of fibronectin or activating antibodies against integrin-β1 successfully restore MuSC sensing of their niche, survival and self-renewal, resulting in enhanced regeneration.

III. FAPs as therapeutic targets

III.1. Ectopic adipogenesis, a residue of FAP activity upon injury?

Various cell types reside in the muscle stem cell niche, close to satellite cells (**Fig. 4a**). Their cross-talk with muscle stem cells during homeostasis or after injury is, however, only partly characterized. Amongst them, FAPs are mesenchymal progenitors located between fibers, and that get activated after injury concomitantly to satellite cells (Joe et al., 2010, Uezumi et al., 2010).

Although no PDGFRα-lineage tracing has been done to determine their ability to give rise to adipocytes *in vivo*, the characterization of FAP adipogenic potential *in vitro* and *ex vivo* upon transplantation was a breakthrough. Huge efforts had indeed been previously been done to uncover the source of intramuscular fat (Asakura et al., 2001, De Coppi et al., 2006, Shefer et al., 2004, Taylor-Jones et al., 2002). Together with a study demonstrating that MyoD+ cells do not spontaneously commit into adipocytes (Starkey et al., 2011), the discovery of FAPs provided invaluable insights to future projects aiming at understanding intramuscular fat infiltration. When I started this work, ectopic fat infiltration was considered (and still is today) negatively correlated with muscle function, mainly because adipose infiltration is observed in many muscle degenerative conditions such as dystrophies or rotator cuff tears (Samagh et al., 2013, Osti et al., 2013, Itoigawa et al., 2011, Sambasivan et al., 2011, Uppin et al., 2013). In addition, aging was reported to increase muscle adiposity in direct correlation with decreased muscle function (Crane et al., 2010, Cree et al., 2004, Nakagawa et al., 2007, St-Onge, 2005, Liu et al., 2012a, Song et al., 2004, Vettor et al., 2009). Although these observations relate to types of fat distinct from ectopic adipocytes (mainly IMCL and intermuscular fat), the discovery of FAPs opened-up new study perspectives to search for ways to limit intramuscular and ameliorate muscle function.

We hypothesized that FAPs represent a critical population in the muscle niche during aging to regulate the myogenic support of satellite cells and intra-muscular adipogenesis. We evaluated the etiology of FAP adipogenic commitment by profiling two mouse models of muscle regeneration. In order to make sure the regeneration efficiency would not be confounding, we validated that both models induced similar extent of muscle damage and that kinetics of regeneration were comparable in both models. Surprisingly, our results demonstrated that both models of regeneration triggered a transient ectopic adipogenesis, although at a higher extent in the glycerol model. Contrary to the absence of ectopic adipogenesis reported in the CTX model after FAP transplantation (Uezumi et al.,

2010), we observed ectopic adipogenesis in our CTX-injured muscles. It is possible that *in vivo* differentiation of endogenous FAPs is more permissive than *in vivo* differentiation after transplantation, thus potentially explaining the difference observed between our study and Uezumi et al. Since then, other studies have also reported the presence of ectopic adipogenesis in models of efficient muscle regeneration (Yamanouchi et al., 2006, Pagano et al., 2015), therefore confirming that ectopic adipogenesis is a hallmark of muscle regeneration.

At the genome wide transcriptional level, our results demonstrated that adipogenesis and fatty-acid β-oxidation were differentially regulated between models, a few days after injury. While myofiber disruption together with the proliferation and invasion of the numerous cell types involved in muscle regeneration makes it difficult to interpret full muscle metabolism at the transcriptomic level, our results suggested that the balance between fatty acid storage and utilization may be a trigger of FAP adipogenesis. It is likely that this change in full muscle metabolism reflects different relative abundance between cell types in the muscle, especially at early time points. Our genome analysis indeed revealed a significantly stronger inflammatory signature in response to glycerol-induced injury. In particular, the levels of the anti-inflammatory cytokines IL-10 and TGF-β1 were approximatively 2-fold higher in the glycerol injured muscles than in the CTX-injured muscles. Additional studies performed by flow-cytometry or protein quantification would be required to assess the balance between pro-inflammatory and anti-inflammatory macrophages or cytokines in the injured muscles, respectively. Nevertheless our observations raised the possibility that adipogenesis may be regulated by inflammatory triggers, and more particularly, anti-inflammatory signaling. In fact, cross-talks between FAPs and immune cells were later described and emphasized the central role of FAPs in the niche (Heredia et al., 2013, Kuswanto et al., 2016, Lemos et al., 2015). In particular, it was shown that FAP apoptosis is induced by TNF-α, secreted by pro-inflammatory macrophages, and this effect is blunted by the anti-inflammatory cytokine TGF-β; thus leading to FAP survival (Lemos et al., 2015). Our results could therefore indicate that the higher TGF-β levels induced by glycerol, participate to FAP survival, and therefore to stronger ectopic adipogenesis.

Altogether, our study sustains the hypothesis that ectopic adipogenesis is a hallmark of muscle regeneration and has provided hints on molecular signals potentially regulating ectopic adipogenesis. The transient adipocyte invasion results, however, from the regulation of FAP proliferation, survival and adipogenic commitment, and further studies will be required to dissect how each step of their differentiation program is modulated by the muscle environment. This study opens up a major question: Is ectopic adipogenesis required for muscle regeneration, or is it only the trace of FAP early activity during muscle regeneration? There is indeed growing evidence that FAPs and/or cells from

similar lineages are required for muscle regeneration as ablation of the adipogenic AP2 or fibrogenic Tcf4 lineages, both resulted in reduced regenerative capacities (Liu et al., 2012a, Murphy et al., 2011).

Interestingly, we have also demonstrated in this thesis that poor regenerative capacities of old muscles is accompanied by reduced ectopic adipogenesis (**Chapter V**, **Fig. 2**). This result is, however, in contrast to other groups having reported that ectopic adipogenesis was stronger in old animals during muscle regeneration (Ikemoto-Uezumi et al., 2015, Liu et al., 2012a). We believe the differences may arise from the different models of injury, as Ikemoto-Uezumi et al. and Liu et al. used a cardiotoxin (CTX)-induced model of muscle damage, while we used the glycerol model as we had previously demonstrated that it leads to muscle regeneration as efficiently as CTX and is accompanied by a stronger ectopic adipogenesis (Lukjanenko et al., 2013). In addition, each group has studied fat formation at various time points of muscle regeneration, 5 days post-CTX injury (Liu et al., 2012a) or 11 days post-CTX injury (Ikemoto-Uezumi et al., 2015), while we performed our quantification 14 days after glycerol injury. Quantification of ectopic adipocytes in our aged regeneration study was quantified using 2 independent staining procedures (Oil-red-O and perilipin), in 2 independent experiments and always lead to the conclusion that aging blunts ectopic adipocyte expansion after glycerol injury. In order to further confirm that we were detecting true adipocytes in muscle, we performed a time course of muscle regeneration in PPARγ KO mice (Nadra et al., 2010), since adipocyte differentiation is fully dependent on PPARγ (Rosen et al., 2000). Adipocyte formation during muscle regeneration measured by Oil-Red-O was fully inhibited in PPARγ KO mice (**Fig. 14a,b**), with PPARγ KO mice having a 99% reduction of Oil-Red O positive adipocytes compared to WT. Ectopic fat infiltration during muscle regeneration therefore fully requires PPARγ, thus ruling out the possibility that fat accumulates in the extra-cellular environment or in non-adipogenic cells. Altogether, our results demonstrate that transient ectopic adipocyte infiltration in response to muscle injury decreases with age along with regenerative capacity. This result suggests that ectopic adipogenesis might play a causal in sustaining muscle regeneration, possibly through pre-adipocyte or mature adipocyte cross-talk with the regenerating muscle. To further address this hypothesis, we quantified mRNA levels of Pax7 and MyoD in PPARγ KO regenerating muscles (**Fig. 14c**). The upregulation of these myogenic markers was significantly lower in PPARγ KO muscles 3 days after injury, suggesting a perturbation of early stages of myogenic commitment. Further investigations are currently being done in order to dissect the role of PPARγ in the cross-talk between satellite cells and FAPs or differentiated-FAPs, as well as in other cell types involved during muscle regeneration. In this context, macrophages would be interesting candidates to study as their polarization has been shown to be modulated by PPARγ (Wang et al., 2014). Similar to our observations, other groups have revealed correlations between perturbed ectopic adipogenesis and impaired muscle regeneration, either using hindlimb-unloading during muscle repair

(Pagano et al., 2015) or by blocking TGF-β signaling with nilotinib treatment (Fiore et al., 2016). Altogether, these studies suggest that the accepted idea that ectopic adipogenesis is detrimental for muscle regeneration should be revisited and that it is of major importance to discriminate the type of fat infiltration in muscle (IMCL, adipocytes, IMAT) when making functional correlations.

The cross-talk between other tissues and fat cells (either adipocyte progenitors or differentiated adipocytes) have been widely described; but fat cells have been reported both in positive and negative regulation of tissue function (Rosen and Spiegelman, 2014). In regenerative/tissue-renewal contexts, adipocyte-precursors have been involved in skin epithelial stem cell activation in the hair follicle (Festa et al., 2011, Donati et al., 2014) and the adipogenic lineage was shown to recruit fibroblasts during skin wound healing (Schmidt and Horsley, 2013). Cross-talk between adipose-derived stem cells and chondrocytes have also been recently found *in vitro*, potentially revealing similar cross-communications during cartilage repair (Zhong et al., 2016). Interestingly, PDGF signaling was often identified as a central regulator of the cross-talk between adipocytes and tissue-progenitor cells (Festa et al., 2011, Schmidt and Horsley, 2013, Zhong et al., 2016). Therefore, while we strongly believe that FAPs are key positive determinant for the efficiency of muscle repair, understanding if their functional role is limited to their pre-adipocyte state or extends after differentiation will be key in the future to develop specific and targeted strategies aiming at ameliorating muscle regeneration and function through FAPs. As fat depots in the mammalian body are metabolically different, it would be interesting to characterize FAP-derived adipocytes and assess, for instance their response to insulin or glucose, and profile their secretome. In fact, it was recently shown that human adipocytes derived from FAPs do not respond to insulin *ex vivo* (Arrighi et al., 2015).

III.2. FAPs as central players of the muscle stem cell niche

The evidence described above convinced us to focus on FAPs in the niche and evaluate if and how they could be targeted to improve muscle regeneration, in particular during aging. Different studies have attempted to derive non-myogenic cells, such as mesoangioblasts, pericytes or PICs, towards the myogenic lineage to enhance repair efficiency (Birbrair et al., 2013a, Dellavalle et al., 2011, Mitchell et al., 2010, Sampaolesi et al., 2006). FAPs were also targeted in strategies aiming at limiting their expansion and fibrosis (Lemos et al., 2015, Uezumi et al., 2014). In this thesis, and as a second axis to modulate the niche, we chose a different approach and focused on boosting the support function of FAPs to satellite cells. This approach had already been undertaken in *mdx* mice (Mozzetta et al., 2013), but was never explored in the aging context of aging.

As a first step, we determined the specific role of FAPs on satellite cells *ex vivo*. Their ability to support either satellite cell proliferation or differentiation was debated (Fiore et al., 2016, Joe et al., 2010, Mozzetta et al., 2013). Here we showed that FAPs participate to satellite cell myogenic support function by increasing their adhesion capacities at very early time points, maintaining a continuous effect allowing a persistent elevated number of myogenic cells throughout the differentiation process *ex vivo*. This role is all the more critical as we had also revealed in the first part of this thesis the importance of biological adhesion for satellite cell survival. Compared to satellite cells, we showed that the gene signature of FAPs was significantly enriched in extracellular matrix annotations. We therefore speculate that one role of FAPs could be to produce structural proteins of the extracellular matrix to serve as adhesion substrates for satellite cells.

We also revealed for the first time that FAP proliferation, differentiation and support function capacities were reduced as a consequence of age. This was likely accounting for the reduced ectopic adipogenesis we observed in aged regenerating muscles, as previously discussed. We believe that FAP activity is required for efficient muscle repair and likely leads to non-detrimental limited ectopic adipogenesis. Strategies ambitioning to limit FAP differentiation should only target pathologies where muscle homeostasis is highly disrupted (such as dystrophies or animal models of satellite cell ablation), allowing FAP activity to take over satellite cell activity. We propose that aged skeletal muscles maintain a balanced but reduced FAP-satellite cell activity, and that strategies should aim at boosting FAPs to restore the overall myogenic capacity of the aged niche.

In addition to their central role in the muscle stem cell niche, and their deregulated function with age, the mesenchymal-like nature of FAPs make them an interesting cell population to target. Although osteogenic and chondrogenic differentiation capacities of FAPs have been debated, most recent evidence confirmed mesenchymal stem cell identity of FAPs (Oishi et al., 2013, Uezumi et al., 2014, Uezumi et al., 2010). One therapeutic perspective would be to translate FAP boosting strategies to mesenchymal stem cells of other tissues (such as bone and skin) and thus enhance global recovery of elderly people after surgery for instance.

Future prospective analyses will also be necessary to better dissect the muscle stem cell niche and assess to what extent FAPs overlap and/or can be distinguished from other muscle resident cell types expressing PDGFRα and /or reported to show adipogenic and fibrogenic potentials (Birbrair et al., 2013b, Birbrair et al., 2013c, Dulauroy et al., 2012). It is likely that uncovering new markers could specify FAP subpopulations with distinct propensities to adopt the different lineages. Understanding how these subpopulations evolve with age would also bring rich information on the muscle stem cell niche. In white adipose tissue, the adipogenic lineage was characterized *in vivo* as Lin^- /$Sca1^+$/$CD34^+$/$PDGFR\alpha^+$ (Berry and Rodeheffer, 2013). $Sca1^+$/$CD34^+$/$PDGFR\alpha^+$ progenitors from white

adipose tissue were also found to be bipotent with the ability to differentiate in brown adipocytes *in vivo* and *in vitro* upon β3-adrenergic stimulation, but interestingly not *in vivo* after transplantation (Lee et al., 2012). As it has never been assessed, we could expect a capacity of FAPs to differentiate into brown adipocytes upon stimulation. Yet, the nature of ectopic adipocytes accompanying muscle regeneration *in vivo* should be prospected, as the description of FAP-derived adipocytes as adipocytes of the white adipose lineage has only been assessed *in vitro* in the absence of stimulation mimicking the physiological differentiation in brown adipocytes upon cold exposure (Arrighi et al., 2015, Joe et al., 2010).

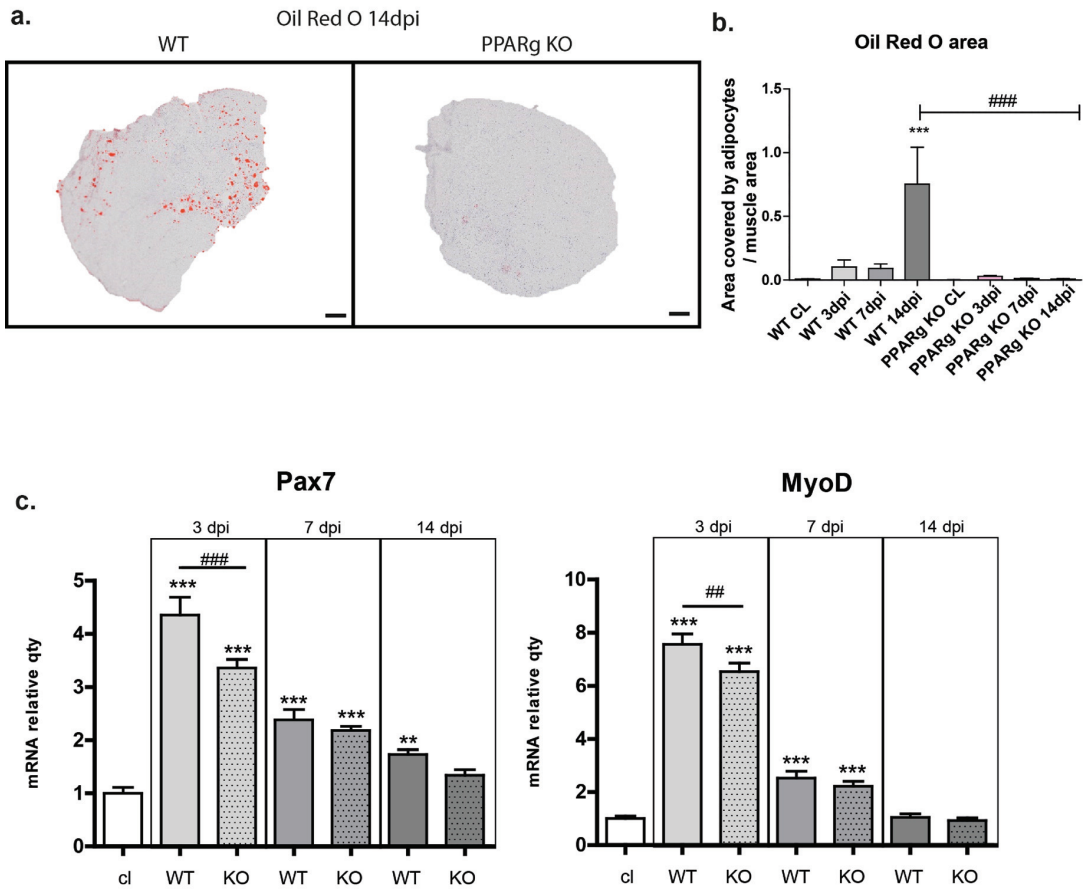


Figure 14. Ectopic adipogenesis is blunted in mice PPARγ deficient mice. Tibialis anterior muscles of young females (11-15 weeks old) PPARγ knock-out (KO) (C57Bl/6 – PPARγ$^{L2/L-}$;Sox2^{Cre}) and wild type (WT) counterparts (C57Bl/6 – PPARγ$^{L2/+}$) (Nadra et al., 2010), were injured using an intramuscular injection of glycerol and collected at 3, 7 or 14 days post injury (dpi). **(a)** Oil-red O staining of PPARγ KO (right) and WT counterparts (left) regenerating muscles at 14dpi. **(b)** Quantification of area covered by oil-red O structures in PPARγ KO and WT counterparts muscles. **(c)** qPCR quantification of Pax7 and MyoD expression in regenerating muscles. Data were normalized to contralateral TA of the 3dpi WT mice group (cl). In (b,c), **: p-value vs. genotype respective control < 0.01; ***: p-value vs. genotype respective control < 0.001; ##: p-value PPARγ KO vs. WT at respective time points < 0.01; ###: p-value PPARγ KO vs. WT at respective time points < 0.001; n>7. Scale bar = 200µM.

III.3. WISP1, a communication factor between satellite cells and FAPs.

Using FAP-conditioned medium, we demonstrated that FAPs are able to communicate with satellite cells through paracrine signaling, as it had been suggested but never demonstrated previously (Fiore et al., 2016, Im et al., 2014, Mozzetta et al., 2013, Joe et al., 2010). In order to understand the origin of old FAP dysfunction in their myogenic support function, we undertook a transcriptomic strategy and specifically interrogated the genes upregulated during activation of FAPs and whose upregulation was lost with age. Amongst those, the Wnt1 inducible signaling pathway protein 1 (WISP1) appeared as the unique secreted factor. WISP1 was predominantly expressed in activated FAPs, although young activated satellite cells also appeared to upregulate it at a lower extent. At the full muscle level, this translates into a dramatic reduction of this protein from the aged niche. We demonstrated that WISP1 regulates young and old satellite cells by promoting both their adhesion and proliferation, suggesting that WISP1 is a central signaling molecule involved in the cross-talk between satellite cells and FAPs. Our results also showed that WISP1 systemic treatment can restore muscle regeneration in aged mice, highlighting the possibility to target the cross-talk between satellite cells and FAPs to ameliorate regenerative capacities of old muscles. Importantly, WISP1 treatment did not result into satellite cell pool exhaustion as we showed that $Pax7^+$ cells have similarly replenished the pool at 14dpi. Investigations are therefore ongoing to evaluate satellite cell proliferation and commitment at different time points of muscle regeneration to better understand how WISP1 modulates satellite cell function *in vivo*.

Interestingly, the cross-talk between pre-adipocytes and adult stem cells has already been shown to be regulated by Wnt signaling in the hair follicle (Donati et al., 2014). In other contexts, WISP1 was also shown to be a paracrine factor triggering cross-communication between cell-types and promoting cell proliferation (Tanaka et al., 2001, Xu et al., 2000). It is still possible that WISP1 is produced by or can target other cell types within the old muscle stem cell niche. Specific inhibition of WISP1 secretion by FAPs (using blocking antibodies, siRNA or WISP1 knock-out cells) followed by FAP-conditioned medium cultures or FAP transplantation experiments in old injured muscles will be required to fully demonstrate that WISP1 is required for the FAP to satellite cell crosstalk.

WISP1 is a member of the CCN family of proteins that are known to be involved in tissue repair (Leask and Abraham, 2006). Similar to our observations in muscles, elevated levels of WISP1 have been reported after cardiac ischemia (Colston et al., 2007), damage of lung epithelium (Heise et al., 2011), bone fracture (French et al., 2004, Macsai et al., 2012) and upon oxidative stress of neuronal tissue (Wang et al., 2012b, Wang et al., 2012a). The reported roles and downstream signaling pathways

induced by WISP1 in various cellular systems are extremely broad (Maiese, 2014), and further investigations will be required to dissect its role in the muscle stem cell niche. In particular, as WISP1 is a pro-survival factors and as we have demonstrated stronger cell death in old satellite cells, effect of WISP1 on satellite cell anoikis should be investigated. Molecular signaling triggering WISP1-rescued satellite cell adhesion and proliferation will also need to be interrogated.

We can expect that WISP1 acts both through direct signaling to satellite cells and through niche effects. As a matricellular protein, WISP1 can indeed act as co-factor for the extracellular matrix, growth factors and cytokines. It would therefore be worth assessing if like other members of the CCN proteins (Chen et al., 2004, Gao and Brigstock, 2004, Kawaki et al., 2008, Leu et al., 2002), WISP1 has the ability to interact with integrin and evaluate if the ameliorated satellite cell function we observed could result from the rescue of integrin β1 signaling that is deregulated in old satellite cells (Rozo et al., 2016). Furthermore, converging toward our previous results and since some CCN proteins bind to fibronectin (Sipes et al., 1993), it would be interesting to interrogate if WISP1 and fibronectin can synerigistically promote satellite cell adhesion. FAP responsiveness to WISP1 will also have to be evaluated. Indeed, WISP1 is known to induce extracellular matrix proteins production by fibroblasts (Konigshoff et al., 2009, Venkatachalam et al., 2009), and we could expect that WISP1 enhances FAP-support function to satellite cells also by increasing their secretion of adhesion molecules. It is also likely that WISP1 promotes FAP expansion, or further induce their production of pro-myogenic factors such as IL-6, as it has been reported in fibroblasts (Klee et al., 2016). All those questions are currently being tackled through ongoing experiments that hopefully will allow us to refine the understanding of how WISP1 regulates FAP-satellite cell communication.

Altogether, our work has established that FAPs are a central and key population of the muscle stem cell niche, whose function is decreased in aged muscles. We believe our results pave the way for the development of innovative strategies aiming at targeting FAPs or other support niche cells to ameliorate muscle repair.

IV. Targeting satellite cell function through their systemic environment

When we interrogated the intrinsic changes induced by age in satellite cells, APJ, the apelin receptor, was identified as the most down-regulated gene. We found that both apelin and APJ were strongly upregulated during muscle regeneration, and that this response was blunted in aged mice, which suggested a potential role of the Apelin/APJ pair in muscle repair. Our results also demonstrated that apelin supplementation *in vivo* considerably ameliorated muscle regeneration in old mice. This indicates that although old mice displayed a strong down-regulation of APJ expression in muscles and satellite cells, these low levels of apelin receptor are sufficient to allow responsiveness to apelin treatment. Interestingly, apelin treatment restored APJ expression levels in aged muscles, demonstrating that apelin induces a positive feedback loop in the expression of its receptor. In addition, apelin treatment of younger mice (3 and 12 month-old) similarly ameliorated muscle regeneration, suggesting that apelin is also beneficial to enhance the repair of healthy muscles. Our work could therefore be applied for therapeutic interventions aiming at improving muscle healing and recovery of both young and elderly patients, after sport trauma or surgery.

At the progenitor cell level, apelin increases human myoblast fusion, and ongoing investigations have revealed that apelin also enhances freshly isolated satellite cell proliferation (data not shown). The mechanisms underlying apelin signaling in satellite cells still remain to be uncovered. Of note, our collaborators reported no muscle atrophy in young apelin knock-out mice, suggesting that apelin has no major role or can be substituted by other ligands during muscle development. APJ is a GPCR that couples to the protein $G_{i/0}$ but the signaling pathways activated downstream of APJ are broad (Chaves-Almagro et al., 2015). Interestingly, the role of protein $G_{i/0}$ in the regulation of satellite cell function is already known, as $G_{\alpha i2}$ has been shown to promote satellite cell proliferation and differentiation in a PKC/GSK3β dependent manner (Minetti et al., 2014). It was also demonstrated that $G_{\alpha i2}$ negatively regulates histone deacetylase (HDAC) activity and promotes the expression of myo-miRNAs miR-1, miR-27b and miR-206 involved in myogenesis. It would therefore be extremely interesting to evaluate whether apelin triggers the same signaling pathways in satellite cells. Apelin treatment lowered muscle expression of inflammatory markers, and in particular of IL-6, which is elevated with age and triggers elevated JAK/STAT signaling in satellite cells (Price et al., 2014, Tierney et al., 2014). Although the effect of apelin on decreasing inflammation markers was specific to old

mice, it is possible that apelin also ameliorates old satellite cell function through the reduction of local inflammation.

In this work, our collaborators also showed that the Apelin/APJ system preserved muscle mass and function through life. Loss of APJ in particular, accelerated muscle aging phenotype, and apelin supplementation of old mice reversed it, in an AMPK-dependent fashion. Interestingly, AMPK is a known regulator of inflammation in skeletal muscles (Liong and Lappas, 2015, Mounier et al., 2013) and in other tissues (O'Neill and Hardie, 2013) . While it is not to exclude that apelin signals through distinct pathways according to age, decreased inflammation markers observed in old mice treated with apelin could result from apelin-induced AMPK activation. Contrary to other proteins measured in plasma, decreased apelinemia in a human cohort of women was associated with loss of muscle (assessed by appendicular lean mass) independently of fat mass. It was also demonstrated that apelin is secreted by skeletal muscle into the blood stream upon exercise. Importantly, as part of the LIFE study (Pahor et al., 2014), an increase of plasma apelin concentration was associated with elderly people response to physical training. One application emanating from these results is the potential to use apelin as biomarker of muscle weakness in the management of sarcopenia. Apelin secretion in the bloodstream after exercise could further serve as an indication of training success, in preventive programs against sarcopenia.

Altogether, our results demonstrate that apelin influences muscle physiology and function during aging, as well as its regenerative capacities. Although additional studies will be required to distinguish direct effects of apelin on muscle fibers and satellite cells *vs.* the indirect effects of metabolic improvements resulting from apelin supplementation, the existence of ongoing clinical trials with apelin or synthetic APJ agonists for other indications (such as NCT01590108, NCT02150694 and NCT02259686, (Brame et al., 2015)) highlights that therapeutic strategies targeting apelin are safe and could be extended to the treatment or prevention of age-realted muscle dysfunction. In fact, two major strategies targeting muscle function could emerge from our work; one being apelin supplementation of elderly people to prevent or ameliorate symptoms of sarcopenia, and a second being acute apelin supplementation at the time of a surgery affecting muscle tissue. Long term apelin rescue could also be achieved through physical training programs, as already discussed, or through nutritional intervention. Indeed, the eicosapentaenoic omega-3 fatty acid, has been shown to induce apelin secretion by skeletal muscle cells (Bertrand et al., 2013).

It will also be interesting to know what are the upstream signaling pathways leading to apelin production by skeletal muscle, upon exercise or injury. For example, does apelinemia increase upon muscle injury and after exercise through similar mechanims? Understanding what the sources of apelin are in injured muscle, and the different contributions of apelin-secreting cells at the site of injury and

in the circulation during regeneration and aging, will also be key in the future to better understand the role of this peptide in the muscle stem cell niche. Dissecting the effect of apelin on satellite cell function and understanding the downstream signaling pathways will also be very important for our knowledge of the muscle stem cell niche. In addition, and in view of the potential elaboration of global preventive strategies against sarcopenia, future studies will have to assess how muscle stem cells in resting muscles respond to long term treatment. In particular, one should make sure apelin treatment does not induce a break of quiescence of old satellite cells, which could be detrimental for the maintenance of muscle regenerative function chronically.

Our work also opened-up many interesting questions that will have to be investigated in future studies. It has been proposed that satellite cell fusion is not required for early stages of hypertrophy (Biressi and Gopinath, 2015), although it does contribute in certain contexts. Considering the effect of apelin on myoblast differentiation, it would be interesting to question the involvement of satellite cell fusion for the apelin-induced rescue of sarcopenia. Conversely, we can wonder whether preventing muscle aging symptoms *per se* can prevent loss of satellite cell function. Lastly, as they express APJ, satellite cells in resting muscles are also potentially responsive to circulating apelin levels or apelin secretion by myofibers. Considering that changes in the micro-environment with age might lead to satellite-cell autonomous dysfunction (Chakkalakal et al., 2012, Tierney et al., 2014, Toth et al., 2006), we could also question if the loss of apelin observed with age can affect old quiescent satellite cells.

The interplay between sarcopenia, exercise and satellite cell function remains to be better defined. The role of the satellite cell pool maintenance in sarcopenia has been recently challenged, when experiments depleting satellite cells in sedentary mice did not exacerbate the loss of muscle mass during aging, while severely affecting the regenerative response after injury (Fry et al., 2015, Keefe et al., 2015). Nevertheless, it is acknowledged that exercise leads to satellite cell activation and that resistance exercise that is associated with gain of muscle strength can also induce micro-damages requiring regeneration (Martin, 2012, Smith and Merry, 2012, Snijders et al., 2015, Snijders et al., 2012, Schiaffino and Mammucari, 2011). The beneficial role of apelin on muscle regeneration is therefore interesting as satellite cells are still likely solicited in aging muscles after damaging events, and in particular in context of training programs developed to prevent sarcopenia. Apelin therapeutics would therefore present the major advantage to target both loss of muscle function and loss of satellite cell function, thus limiting exacerbation of muscle weakness. Conversely, exercise itself is beneficial to satellite cell function and the maintenance of their pool (Shefer et al., 2010). Altogher, these evidence highlight the promising benefits of the positive regulatory loop involving apelin, exercise and enhanced satellite cell function to prevent sarcopenia.

V. Conclusion

The results presented in this thesis provide a better understanding of the larger muscle stem cell environment and demonstrate how all levels of the niche are interrelated. Aging is a multisystemic process that requires a global therapeutic approach. Likewise, targeting satellite cell regenerative capacities likely requires several points of action in order to rescue multiple dysfunctions. Our work opens new approaches for interventions and provides evidence that targeting satellite cell interactions with their niche is a promising strategy to restore regenerative function. We anticipate that future studies on skeletal muscle aging will focus on the characterization of niche cell aging as well as on the interrelation between cell types as a global network. Our findings and the underlying concepts also bear potential for extension to other adult stem cell niches in tissues with high regenerative potential (for instance bone, liver and skin (Chan et al., 2015, Hsu et al., 2014, Miyajima et al., 2014, Worthley et al., 2015)). Further attention will be required to fully understand the various mechanisms of actions on which our proofs of concept rely, and to translate them into preventive and interventional therapies to ameliorate muscle repair. Such applications will only become a reality with further integrated efforts to develop bioengineering, pharmaceutical or nutritional combinations allowing the local or systemic targeting of the muscle stem cell niche.

Appendices

studies to investigate whether there is cross-talk between the vagus nerve and the VTA-induced reward signaling described by Ben-Shaanan *et al.*[7], as well as how this influences immune responses and disease.

The findings by Ben-Shaanan *et al.*[7] establish a causal link between the activation of the reward system in the VTA and the generation of beneficial immune responses, and they help to explain how placebo treatment—and perhaps other positive experiences—can potentially lead to improved clinical outcomes.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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The role of muscle stem cell–niche interactions during aging

Matthew Timothy Tierney & Alessandra Sacco

Two recent studies have shown that alterations in muscle stem cell–niche interactions during aging underlie the functional decline in regenerative potential. The reconstitution of this communication restores stem cell function and enhances skeletal muscle repair.

Aging is associated with a progressive decline in skeletal muscle mass and regenerative capacity. Weakened muscles increase the likelihood of injury, and ineffective repair processes propagate a vicious cycle that negatively affects quality of life. Consequently, interventions that are able to improve aged muscle regeneration are needed greatly. Skeletal muscle is maintained by resident stem cells, termed satellite cells or muscle stem cells (MuSCs), which reside in a quiescent state in healthy tissues. Here stem cell function is preserved while these cells are poised to sense stress or damage and can readily activate to repair an injury. The stem cell niche, composed of support-cell types, local growth factors and extracellular-matrix (ECM) molecules, is crucial to direct stem cell self-renewal and differentiation during muscle regeneration.

Recently, several groups have identified a progressive impairment in MuSC self-renewal that accompanies aging[1–7]. Several factors have been identified, including alterations in niche composition, impaired interactions with niche elements and distorted transduction through key signaling cascades. Collectively, this diminishes the size and function of the MuSC pool through lost quiescence, premature differentiation or senescence. Still, a comprehensive understanding of the deleterious changes to MuSCs and their niche with aging is lacking.

In this issue of *Nature Medicine*, Rozo *et al.*[8] and Lukjanenko *et al.*[9] provide evidence, primarily in mouse models, that MuSC-niche interactions are required for the maintenance of MuSC function and tissue repair. The authors show that the cell surface receptor β1-integrin and the ECM protein fibronectin are dysregulated in aged MuSCs, and that reconstituting their function restores muscle regenerative capacity (**Fig. 1**). Thus, the two studies identify two novel therapeutic targets for the treatment of muscle-wasting diseases.

Rozo *et al.*[8] report that the activity of β1-integrin, a crucial sensor of the MuSC niche, is localized aberrantly in aged MuSCs. They find that conditional genetic deletion of β1-integrin in young mouse MuSCs recapitulates multiple aspects of the aged phenotype, including disrupted quiescence, reduced proliferation and a bias toward differentiation that results in MuSC loss and impaired regeneration. The authors also found that β1-integrin deletion impaired the ability of MuSCs to respond to microenvironmental cues, namely, the potent mitogen FGF2. Treatment of aged MuSCs with β1-integrin-activating antibodies restored FGF2 sensitivity by increasing the association of FGF2 with its receptor, which promoted MuSC proliferation via the activity and polarization of several mitogen-activated protein (MAP) kinases. Fibronectin presentation improved MuSC responsiveness to FGF2 in culture in a β1-integrin-dependent manner, which emphasizes the cooperative nature of the MuSC-niche exchange. Additionally, the administration of β1-integrin-activating antibodies to mouse models rescued the regenerative potential of aged and dystrophic muscle via restored MuSC expansion, increased myofiber size and improved muscle performance.

In the companion article, Lukjanenko *et al.*[9] investigate the role of the ECM components in aged MuSCs. Through carrying out mouse young and aged MuSC gene-expression analysis and apatamer-based ECM binding assays in regenerating muscles, the authors show that after injury, fibronectin is not upregulated sufficiently in aged muscles, as compared to young muscles. Fibronectin produced by MuSCs themselves has been shown to form a complex with Wnt7a, a secreted signaling molecule, and its receptor to promote their symmetric expansion during muscle repair[10]. Here the authors show that the major producers of fibronectin—hematopoietic and endothelial cells—are less abundant in aged regenerating muscles. Conditional deletion of fibronectin in young mice recapitulated the aging phenotype, which reduced MuSC number. Assays done *in vitro* further showed that fibronectin is the preferred adhesion substrate for mouse and human myoblasts and is able to modulate several signaling pathways that are altered in aged MuSCs, including the MAP kinases p38 and ERK. Aged MuSCs had reduced adhesion to fibronectin, lower focal adhesion kinase

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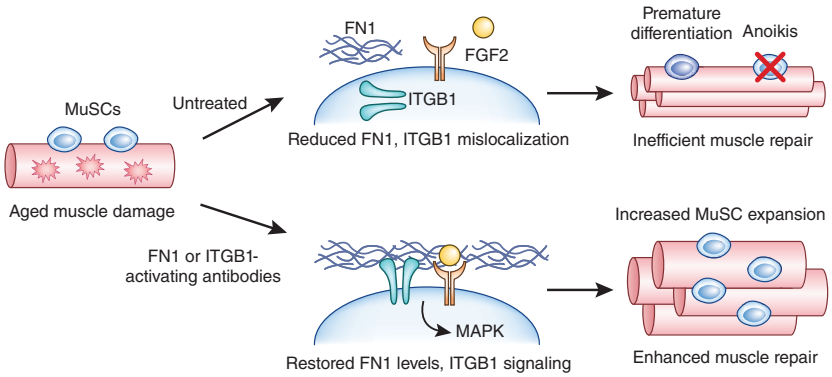

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Figure 1 Targeting MuSC-niche interactions improves aged MuSC self-renewal and skeletal muscle repair. In aged mice, Rozo *et al.*[8] and Lukjanenko *et al.*[9] demonstrate that the MuSC-niche components fibronectin (FN1) and β1-integrin are lost or dysregulated in aged mice. Restoration of fibronectin levels or β1-integrin activity can promote MuSC proliferation and self-renewal while inhibiting premature differentiation and anoikis, which improves muscle regeneration and performance.

(FAK) expression and increased anoikis, or adhesion-dependent programmed cell death. Consistently, β1-integrin was required for proper mouse and human myoblast adhesion to fibronectin and for FAK upregulation. Furthermore, the exposure of aged MuSCs to fibronectin in culture and the injection of fibronectin into aged muscles rescued these defects, increased the total number of MuSC progeny and accelerated muscle repair, as compared to vehicle-treated controls.

Both studies identify β1-integrin and fibronectin as key molecules that become lost or dysregulated with age and that are required for proper MuSC function. The deletion of these two genes in young mice impaired niche interactions and MuSC self-renewal, imitating aspects of premature aging in regenerating muscles. The nature of both molecules—fibronectin as being primarily deposited by non-muscle cells and β1-integrin as a receptor on the MuSC surface—suggests that both intrinsic and environmental factors are partially responsible for age-related defects in muscle repair. It will be important to explore how these two proteins are regulated in aged tissues, which could potentially enable the identification of pathways or epigenetic processes upstream that drive the functional decline in MuSCs with age.

Additionally, understanding how β1-integrin filters various external stimuli to initiate the appropriate downstream signaling cascades might lead to the identification or enable improvement of the specificity of clinically viable interventions that are designed to modify MuSC-niche interactions. Notably, the MAP-kinase pathways were implicated in both studies and are known to be major determinants of aged-MuSC behavioral defects[4,5]. Furthermore, it will be important to define potentially context-dependent, post-translational modifications of β1-integrin or fibronectin and the composition of β1-integrin-containing protein complexes that aid its proper localization.

A previous study has demonstrated that the aged-MuSC niche contains increased levels of FGF in comparison to young muscles[1], yet Rozo *et al.*[8] and others[4] have shown that aged MuSCs are insensitive to FGF signaling. It is possible that chronic exposure to FGF negatively affects the ability of aged MuSCs to respond appropriately to FGF during the repair process. Whether this is the result of a negative-feedback loop or stage-specific effects of FGF signaling during muscle homeostasis and repair will be a focus for future studies. Moreover, these aged-related changes to MuSCs may be substantially heterogeneous. Single-cell analyses and lineage-tracing experiments will provide insight into potential adaptations in MuSC-pool complexity with age.

Taken together, the findings reported in these two studies provide evidence that the development of therapeutic approaches aimed at targeting MuSC-niche interactions could counteract age-related regenerative defects. Future efforts aimed at developing pharmaceutical or bioengineering approaches to properly deliver molecules that are able to restore MuSC communication with their local microenvironment will accelerate the development of strategies to ameliorate muscle-wasting conditions.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Deteriorating Infrastructure in the Aged Muscle Stem Cell Niche

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Following an injury, the extracellular matrix (ECM) undergoes dramatic remodeling to facilitate tissue repair. In a new study, Lukjanenko and colleagues show how an age-associated change in this process affects the regenerative ability of muscle stem cells (MuSCs).

Introduction

One of the hallmarks of aging is a decline in the ability to heal from injury. Impaired healing represents an enormous medical problem and quality of life issue for the elderly (Gosain and DiPietro, 2004). Consequently, understanding how tissue repair changes with age is a topic with important implications. Much work has focused on direct comparisons between "young" and "aged" somatic stem cells, which play crucial roles in tissue repair, without elucidating the origin of the age-associated differences between them (Liu and Rando, 2011). In a recent report, Lukjanenko and colleagues (Lukjanenko et al., 2016) provide an explanation of why aged muscle stem cells (MuSCs) become defective at repairing muscle: the response of MuSCs to an age-associated change in the extracellular matrix (ECM) of the stem cell niche.

The importance of the ECM as it relates to stem cell function in normal tissue biology and repair cannot be overstated. The ECM is the infrastructure and structure of a tissue, the substrate upon which stem cells reside, and a conveyer and scaffold of signaling molecules (Frantz et al., 2010). Following injury, the ECM is dynamically remodeled to support the process of tissue repair. Fibronectin (FN), one of the core components of the ECM, is deposited in large quantities in damaged tissues following injury. Consistent with previous findings (Ashcroft et al., 1997; Bentzinger et al., 2013), Lukjanenko and colleagues report that aged mice had reduced FN deposition in injured muscle tissue. Among its many functions, FN serves as an adhesion substrate for stem and progenitor cells. Interestingly, in a competitive screen of ECM components, myoblasts bound more avidly to FN then to any other component of the ECM. Combined, these findings suggested that the reduction of FN, the preferred adhesion substrate of myoblasts, in injured aged muscle might affect the ability of MuSCs to repair muscle damage. In testing this idea, Lukjanenko and colleagues used a combination of model systems, myoblast cell lines, primary MuSCs, and in vivo muscle repair and found that many defective aspects of MuSC function and muscle repair in aged mice could be restored by increasing FN levels.

Lukjanenko and colleagues go on to show that the mechanism by which FN improves aged MuSC function is through FN's role as a biochemical signaling molecule. Interestingly, myoblasts cultured on FN displayed a reduction in signaling through the p38 and ERK pathways when compared to myoblasts cultured on laminin (a component of the ECM and a commonly used cell culture substrate). The p38 and ERK signaling pathways are known to be elevated in aged MuSCs with detrimental effects (Bernet et al., 2014; Chakkalakal et al., 2012). Culturing aged MuSCs on FN was sufficient to restore their cell adhesion properties and to reduce apoptosis—two aspects of aged MuSC function that were defective when they were cultured on collagen (another component of the ECM and a commonly used cell culture substrate). Together, these experiments suggest that FN provides signals that instruct the appropriate functional behaviors of MuSCs. Furthermore, the experiments suggest that dysfunction of aged MuSCs is the consequence of lower levels of FN in injured aged mice. Indeed, in testing this hypothesis, it was found that injection of purified exogenous FN into previously injured muscles restored several aspects of

MuSC function and muscle regeneration in aged mice.

This interesting set of findings supports a model in which changes in the aged stem cell niche impart a dysfunctional state in the aged stem cells that reduces their regenerative abilities. Importantly, normal or "young" niche signals can restore at least some aspects of stem cell function and tissue repair in aged mice.

It is well known that the structure, composition, crosslinking, and stiffness of the ECM changes in many tissues of the body through the course of aging (Labat-Robert and Robert, 1988). Using a collection of aptamers to measure the abundance of various ECM proteins, the authors detected several age-associated changes in uninjured muscle tissue. However, these modest changes in uninjured tissue were dwarfed by the reorganization of the ECM, and particularly of FN, that occurred following injury. Importantly, aged animals did not appear to have the same injury-induced reorganization of the ECM that young animals did. Because of the many important roles of the ECM, it is easy to envision how these differences could affect the function of stem cells and dramatically impact the ability to heal injuries.

So, what is responsible for the age-associated changes to the ECM? At least in terms of injury-induced FN deposition, the authors provide some critical clues. They made a very interesting observation that Lin^+ cells ($CD31^+$, $CD44^+$, $CD11b^+$), a mixture of circulating hematopoietic and immune cells and endothelial cells, were the largest contributors of FN in injured muscle. In aged mice, there was a dramatic decline in the number of these cells found in injured muscle. These findings are intriguing because FN deposition has important roles in the healing of many different types of tissues. Though it is difficult to assign FN deposition to a particular cell type, many are known to express FN, and soluble FN is an extremely abundant component of blood. If a population of circulating cells were responsible for the decline in FN deposition at sites of injury, this may be a mechanism that underlies the decline in healing that occurs throughout the body with age.

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Curriculum Vitae

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Professional Experience

PhD candidate in stem cell biology

Nestlé Institute of Health Sciences
& Ecole Polytechnique Fédérale de Lausanne
Oct 2012 – Dec 2016 (Expected)
Lausanne, Switzerland

- Thesis directors: Dr. Jerôme Feige & Prof. Johan Auwerx
- "Novel interventions to recover the regenerative capacity of aged skeletal muscle by targeting the interactions in the stem cell niche"

Research assistant

Novartis Institutes for BioMedical Research
Feb – July 2012
Basel, Switzerland
Master thesis

- Supervisor: Dr. Jerôme Feige
- "Profiling of regeneration and ectopic adipogenesis in mouse skeletal muscle"

Merck Serono SA
Aug 2011 – Jan 2012
Fenil-Sur-Corsier, Switzerland
Internship

- Supervisors: Dr. Matthieu Stettler
- Successfully established a scale-down model of shear-stress mimicking industrial-scale mammalian cell culture harvest

Macquarie University
Feb – June 2011
Sydney, Australia
Internship

- Supervisor: Prof Mark S. Baker
- Set-up an in-house purification kit of anti-human plasma protein IgY from chicken egg yolk

Savia Biotech SA
July 2010
Almeria, Spain
Internship

CNRS
June - July 2009
Bordeaux, France
Internship

Teaching

2007-2010 **Freelance**, Strasbourg, France
2012-2014 **EPFL,** Lausanne, Switzerland

Education and Training courses

Management of Biotech, Medtech and Pharma Ventures 2015 – 2017 (expected)	EPFL CAS program, modules attended so far: - Project Management - Marketing in Life Sciences
PhD candidate in stem cell biology Oct 2012 – Dec 2016 (expected)	Nestle Institute of Health Sciences & Ecole Polytechnique Fédérale de Lausanne (Lausanne, Switzerland)
Engineer in Biotechnology & **Master in Sciences of Medicine: Biotechnology And Therapeutic Innovations** Sept 2008 - Sept 2012	Ecole Supérieure de Biotechnologie de Strasbourg (ESBS) & University of Strasbourg (Strasbourg, France)

Laboratory techniques

- **Preclinical studies**: Mouse intraperitoneal injections, muscle injury by intramuscular injection, sciatic denervation.
- **Cell culture and flow cytometry**: Primary muscle progenitor isolation by FACS and culture, cell line culture, transfections **Microscopy & Immuno-histo/cyto-chemistry**: Bright/fluorescent field, image analysis, high content screening assays
- **Molecular biology & biochemistry**: qPCR, SDS-PAGE, Western blotting, Protein purification, site-directed mutagenesis
- **Bio-informatics & Computer skills**: Basic micro-array analysis, Microsoft Office, GraphPad prism, Adobe Photoshop, Adobe Illustrator, PaintShop Pro, ImageJ, MetaXpress, EndNote.

Publications and presentations in conferences

Publications

Lukjanenko, L., Karaz, S., Migliavacca, E., Sizzano, F., Jacot, G., Metairon, S., Raymond, F., Palini, A., Descombes, P., Feige, J. N., "Aging disrupts the communication between fibro-adipogenic progenitors and muscle stem cells by inhibiting the production of WISP-1" *(In preparation).*

Vinel, C., **Lukjanenko, L.,** Batut, A., Deleyruelle, S., Pradère, J.-P., Le Gonidec, S., Geoffre, N., Pereira, O., Mouisel, E., Mouly, V., Vigneau, M., Chopard, A., Pillard, F., Cesari, M., Pahor, M., Feige, J. N., Vellas, B., Valet, P. & Dray, C., "The new identified exerkine apelin reverses age-associated sarcopenia" *(In preparation).*

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Lukjanenko, L., Brachat, S., Pierrel, E., Lach-Trifilieff, E. & Feige, J. N., 2013, "Genomic profiling reveals that transient adipogenic activation is a hallmark of mouse models of skeletal muscle regeneration", *PLoS One*.

Tan, S. H., Mohamedali, A., Kapur, A., **Lukjanenko, L**. & Baker, M. S., 2012, "A novel, cost-effective and efficient chicken egg IgY purification procedure", *J Immunol Methods*.

Oral presentation

- Frontiers in Myogenesis, Pacific Grove, CA, USA, June 2016. "Loss of Fibronectin from the Aged Stem Cell Niche Affects the Regenerative Capacity of Skeletal Muscle"
- **Awarded Best Presentation third LIMNA Symposium**, Lausanne (Switzerland), March 2015. "Cross-talk between regeneration and ectopic adipogenesis during aging of skeletal muscle"

Posters

"Mechanisms of regeneration and ectopic adipogenesis during aging of skeletal muscle".

- **Gordon Conference Myogenesis,** Lucca (Italy), June 2015
- **EMBO Workshop_Molecular Biology of Muscle Development and Regeneration**, Lecce (Italy), May 2014
- **International Society for Stem Cell Research (ISSCR) Congress**, Florence (Italy), September 2013

Volunteering experience

The Consulting Society at EPFL, 2015-2016	Development of business relationship with consulting firms (Life Sc.)
Alumni Association ESBS, 2013-2014	Events organization

Languages

French	Mother tongue.
English	Excellent knowledge (writing/speaking)

Referees

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Hobbies and interests

Artistic Gymnastics (15 years practice, and judge in competitions), running and races, pastry, wine tasting