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Defining the role of APC and canonical WNT signaling in embryonic and adult myogenesis

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“Je suis de ceux qui pensent que la science est d’une grande beauté. Un scientifique dans son laboratoire est non seulement un technicien : il est aussi un enfant placé devant des phénomènes naturels qui l’impressionnent comme des contes de fées. Nous ne devrions pas laisser croire que tout progrès scientifique peut être réduit à des mécanismes, des machines, des rouages, quand bien même de tels mécanismes ont eux aussi leur beauté. Je ne crois pas non plus que l’esprit d’aventure risque de disparaître dans notre monde. Si je vois quelque chose de vital autour de moi, c’est précisément cet esprit d’aventure, qui semble qui me paraît indéracinable et s’apparente à la curiosité. Sans la curiosité de l’esprit, que serions-nous ? Telle est bien la beauté et la noblesse de la science : désir sans fin de repousser les frontières du savoir, de traquer les secrets de la matière et de la vie sans idée préconçue des conséquences éventuelles.”

Marie Curie

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TABLE OF CONTENTS

ABSTRACT ..... **I**

LIST of ABBREVIATIONS ..... **III**

INTRODUCTION **1**

I. Skeletal muscle **1**

1. The formation of skeletal muscle 1

The origin of trunk and limb muscles: somitogenesis 2

From the somite to the muscle: the dermomyotome 4

Extrinsic determination of the dermomyotome 6

Primary and secondary myogenesis 7

Upstream regulators that govern myogenesis 8

The induction of myogenesis: the MRFs 11

Transcriptional mechanisms of MRFs 13

Head muscle formation 16

2. Adult skeletal muscle: an overview 19

Anatomy of adult skeletal muscle 19

Skeletal muscle contraction 20

Metabolism 21

3. Skeletal muscle regeneration 22

Satellite cells: definition and identification 23

Embryonic origin of satellite cells 25

Transcriptional control of adult myogenesis 26

Beyond transcription: miRNA-mediated control of myogenesis 28

Satellite cells heterogeneity 28

The immediate satellite cells niche 30

Interaction between satellite cell and its microenvironment 32

Extrinsic control of adult myogenesis 33

4. Muscle regeneration in pathological context: muscular dystrophies 38

II. WNT signaling **41**

1. Extracellular regulation of Wnt signaling 42

Wnt ligands 42
WNT secretion 42
Extracellular regulators of Wnt signaling 44
Alternative Wnt pathways 47
2. Canonical Wnt signaling 50
Canonical Wnt receptors: Frizzled and LRP6 50
Intracellular signal transduction 51
β-catenin destruction complex 52
Wnt-dependent regulation of transcription: the TCF/LEF family 53
β-catenin and chromatin regulation 55
Canonical Wnt signaling in organogenesis 57
Canonical Wnt signaling in adult stem cells homeostasis 59
III. Adenomatous Polyposis Coli 63
1. APC: general features 63
Structure 63
Genetic tools to study APC 64
2. APC and canonical Wnt signaling 65
APC and β-catenin destruction complex 65
Regulation of β-catenin subcellular localization 66
3. APC functions not related to canonical Wnt signaling 67
APC and cell migration 67
APC and cell division 68
Nuclear functions of APC 68
4. Contribution of APC to cancer 69
Aberrant activation of Wnt target genes 69
Chromosomal instability 70
IV. OBJECTIVES OF THE PROJECT 73
RESULTS 75
I. APC is required for skeletal muscle formation and regeneration 75
II. Canonical Wnt signaling is required for myogenic differentiation during embryonic and adult myogenesis 101

DISCUSSION 105

APC, canonical Wnt signaling and tumorigenesis 106

APC, canonical Wnt signaling and cell death 107

Dose-dependent effects of canonical Wnt signaling activation 108

Regulation of canonical Wnt signaling levels 108

Canonical Wnt signaling and muscle aging 110

Canonical Wnt signaling and implication in muscular dystrophies therapies 110

APC and asymmetric cell division 111

APC and chromatin dynamics 112

ANNEX 115

BIBLIOGRAPHY 133

ABSTRACT

The Wnt/β-catenin signaling pathway, also called canonical Wnt signaling, is implicated in a large variety of biological processes, including embryonic axis determination, organogenesis and adult stem cells homeostasis. Canonical Wnt signaling regulates the stability of β-catenin, a transcriptional co-activator that, in absence of Wnt ligands, is targeted to proteasomal degradation by a multiproteic complex comprising the Adenomatous Polyposis Coli (APC) tumor suppressor. Activation of canonical Wnt signaling blocks β-catenin degradation and results in its accumulation into the nucleus, where it induces the expression of Wnt target genes.

During embryonic myogenesis, the process of skeletal muscle formation, a proportion of pluripotent dermomyotomal cells restrict their fate to acquire a myogenic identity and differentiate into contractile myofibers, the functional units of skeletal muscle.

Myogenesis can take place also in adult skeletal muscle. Indeed, upon acute injury or in pathological conditions, quiescent muscle-specific stem cells, called satellite cells, become activated and give rise to myogenic progenitors that massively proliferate, differentiate and fuse to form new myofibers and restore tissue functionality. In addition, a proportion of proliferating progenitors returns back to quiescence and replenish the pool of satellite cells in order to maintain the regenerative potential of skeletal muscle.

The role of canonical Wnt signaling in the cell fate choice that drives multipotent dermomyotomal cells toward the myogenic lineage remains elusive. Similarly, a possible involvement of the Wnt/β-catenin cascade has been hypothesized in satellite cells during adult skeletal muscle regeneration, but different approaches came to contradictory results. In this study, we use genetic mouse models to investigate the precise role of canonical Wnt signaling in embryonic and adult myogenesis. *In vivo* constitutive overactivation of Wnt/β-catenin signaling following conditional deletion of APC, the major intracellular negative regulator of the pathway, results in complete abrogation of skeletal muscle formation and regeneration. By combining *ex vivo* and *in vitro* approaches, we show that canonical Wnt signaling hyperactivation alters cell cycle progression and results in programmed cell death. Conversely, conditional inactivation of β-catenin does not perturb the proliferative ability of myogenic progenitors but rather affects their differentiation.

Collectively, our results demonstrate at least two distinct roles of the Wnt/β-catenin cascade in skeletal muscle. First, during myogenic initiation, canonical Wnt signaling must be inhibited to allow proper activation of myogenesis, in particular to elicit myogenic commitment of dermomyotomal cells and activation of adult satellite cells. Second, in myogenic progression, the Wnt/β-catenin pathway is required in both embryonic and adult muscle progenitors for proper differentiation and myofibers formation.

RESUME

La voie de signalisation Wnt/β-caténine (Wnt canonique) est impliquée dans une grande variété de fonctions biologiques, entre autres dans l'établissement des axes embryonnaires, l'organogenèse et l'homéostasie de cellules souches adultes. En absence de signaux Wnt, un complexe multiprotéique comprenant le suppresseur de tumeur Adenomatous Polyposis Coli (APC) marque la β-caténine pour la dégradation protéasomique. L'activation de la voie Wnt canonique induit un arrêt de la dégradation de la β-caténine, qui s'accumule dans le noyau, où elle active l'expression de gènes cibles de Wnt.

Au cours de la myogenèse embryonnaire, processus pendant lequel le muscle squelettique est formé, une partie des cellules pluripotentes du dermomyotome acquièrt l'identité musculaire, se différencie et forme les myofibres, les unités fonctionnelles du muscle squelettique.

La myogenèse n'est pas confinée qu'à la période embryonnaire. En effet, elle peut être réactivée dans le muscle adulte suite à une lésion ou dans certaines conditions pathologiques. Dans ce contexte, les cellules souches du muscle squelettique, appelées cellules satellites, sortent de leur quiescence et génèrent des progéniteurs myogéniques qui prolifèrent et se différencient en formant de nouvelles myofibres pour réparer le tissu. De plus, une partie des progéniteurs myogéniques retournent à l'état quiescent, renouvelant ainsi la population de cellules souches résidentes.

Le rôle de la voie de signalisation Wnt/β-caténine dans l'engagement des cellules pluripotentes du dermomyotome vers le destin myogénique demeure méconnu. De même, la fonction de la voie Wnt canonique dans les cellules satellites au cours de la régénération du muscle squelettique adulte reste à l'heure actuelle controversée, car différentes approches sont parvenues à des conclusions contradictoires.

Grâce à des modèles génétiques murins, nous avons caractérisé le rôle précis de la voie Wnt canonique au cours de la myogenèse embryonnaire et adulte. Nous montrons in vivo que l'hyperactivation constitutive de la voie de signalisation Wnt/β-caténine induite par l'inactivation conditionnelle d'APC, le principal régulateur négatif de la cascade, se traduit par un défaut majeur de formation et de régénération du muscle squelettique. Nos résultats ex vivo et in vitro démontrent que l'hyperactivation de la voie Wnt canonique altère la progression du cycle cellulaire et entraîne la mort par apoptose. De plus, l'inactivation conditionnelle de la β-caténine n'affecte pas la prolifération des progéniteurs myogéniques mais perturbe leur différenciation.

Globalement, nos résultats suggèrent deux rôles différents de la voie de signalisation Wnt/β-caténine dans le muscle squelettique. D'une part, l'inhibition de la voie Wnt canonique est nécessaire au cours de l'initiation de la myogenèse pour permettre l'engagement myogénique des cellules pluripotentes du dermomyotome et l'activation des cellules satellites. D'autre part, la voie de signalisation Wnt/β-caténine est requise à la fois dans les progéniteurs musculaires embryonnaires et adultes pour leur différenciation et la formation des myofibres.

LIST of ABBREVIATIONS

ADP	Adenosine diphosphate
APC	Adenomatous Polyposis Coli
ASEF	APC-stimulated guanine nucleotide exchange factor
ASH2	Absent, small, or homeotic discs 2
ATP	Adenosine triphosphate
BCL-9	B-cell CLL/lymphoma 9
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BrdU	5-bromo-2'-deoxyuridine
c-Myc	Myelocytomatosis oncogene
CAMKII	Calcium/calmodulin-dependent protein kinase II
CDK	Cyclin-dependent kinase
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
Cdkn1b	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
Cdkn1c	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
Cdkn2a	Cyclin-dependent kinase inhibitor 2C ($p16^{INK4a}$)
ChIP	Chromatin immunoprecipitation
CIN	Chromosomal instability
CK1	Casein kinase 1
CRD	Cys-rich domain
CreERT2	Causes recombination recombinase – Estrogen receptor 2
CREB	cAMP response element-binding protein
CTR	Calcitonin receptor
CTX	Cardiotoxin
DKK	Dickkopf WNT signaling pathway inhibitor 1
DM	Dermomyotome
DMD	Duchenne Muscular Dystrophy
DVL	Dishevelled
ECM	Extracellular matrix
EDL	Extensor Digitorum Longus
EMT	Epithelial-to-mesenchymal transition
ERK	Elk-related tyrosine kinase
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FACS	Fluorescence-activated cell sorting
FAP	Familial adenomatous polyposis
FAPs	Fibro/adipogenic progenitors
FGF	Fibroblast growth factor
FZD	Frizzled
GSK3	Glycogen synthase kinase 3
GTP	Guanosine-5'-triphosphate
HAT	Histone acetyltransferase
HGF	hepatocyte growth factor
iPS	Induced pluripotent stem cells
IWP	Inhibitor of Wnt production
LEF1	Lymphoid enhancer binding factor 1
LGR	Leucine-rich repeat G protein-coupled receptor
LoxP	Locus of Crossover in P1

LRP	Low density lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MLL2	Myeloid/lymphoid or mixed-lineage leukemia 2
MMP	Metalloproteinase
MRF	Myogenic Regulatory Factor
mTORC	Mechanistic target of rapamycin (mTOR) complex 1
MYF5	Myogenic factor 5
MYOD	Myogenic differentiation (Myod1)
PAX	Paired box
PCNA	Proliferating cell nuclear antigen
PCP	Planar cell polarity
PKC	Protein kinase C
PLC	Phospholipase C
PSM	Presomitic mesoderm
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein)
RbBP5	retinoblastoma binding protein 5
RHOA	Ras homolog family member A
ROCK	Rho kinase
RSPO	R-spondin
SC	Satellite cell
sFRP	Secreted frizzled-related protein
SHH	Sonic hedgehog
siRNA	Small interfering RNA
SIX	Sine oculis-related homeobox
SMAD	Small mother against decapentaplegic
SWI/SNF	SWItch/Sucrose NonFermentable nucleosome remodeling complex
TA	Tibialis anterior
TCF	T cell factor
TNF	Tumor necrosis factor
TOPGAL	Tcf optimal-promoter beta-galactosidase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VANGL2	Van gogh like 2
WIF	Wnt inhibitory factor
Wls	Wntless homolog
WNT	Wingless-type MMTV integration site family, member 1
WRE	Wnt responsive element

INTRODUCTION

I. Skeletal muscle

A characteristic feature of all animals is their ability to move. All Metazoa, with the exception of some invertebrates such as basal Porifera and Placoza, possess muscle cells. The musculature accounts not only for locomotion, but also for vital functions such as feeding, breathing, vision, reproduction, blood circulation and many others.

Vertebrates have two types of muscles: smooth and striated. Smooth muscle is responsible for the contractility of organs such as blood vessels, gastrointestinal tract, lungs, uterus and bladder. Striated muscle comprises cardiac and skeletal muscles. It owes its name to its highly organized internal structure that produces regular striations visible by light microscopy. Cardiac muscle is responsible for heart contraction and therefore it controls blood flow throughout the body, whereas skeletal muscle is anchored to bone by tendons and it controls skeleton movement and posture.

Skeletal muscle is the most abundant tissue of vertebrate body, accounting for approximately 40% of body weight in humans. Skeletal muscles can have distinct metabolic and contractile properties and are tailored for specific functions.

Adult muscle is a highly plastic tissue that can adapt upon extrinsic stimuli. Changes in mechanic signals or nutrient availability result in modifications of muscle fiber size and metabolic properties. Furthermore, the presence of muscle stem cells called satellite cells allows skeletal muscle to regenerate after injury. These cells lie in close contact with muscle fibers and upon injury leave their quiescent state, proliferate and undergo myogenic differentiation recapitulating many aspects of muscle formation during embryogenesis.

1. The formation of skeletal muscle

Myogenesis, the process of muscle formation, is the result of a very well orchestrated coordination of transcriptional cascades and signaling pathways that spatio-temporally direct cell proliferation, differentiation, migration and morphological changes (see Bryson-Richardson and Currie, 2008). The integration of extrinsic cues and cell autonomous events determines the commitment of pluripotent mesodermal cells into **myoblasts**, i.e. myogenic proliferating cells. This transition is driven by the induction of

muscle-specific genes that restrict cell fate to the myogenic lineage and activate a transcription cascade that induces cell cycle exit and conversion of myoblasts into postmitotic differentiated cells called **myocytes**. Fusion between myocytes and activation of the terminal differentiation genetic program under the control of muscle-specific genes result in the generation of multinucleated syncytial cells, **myotubes**. Myotubes grow and form mature myofibers and concomitant development of connective tissue, blood vessels and innervation leads to the formation of functional muscles.

The induction of myogenic identity is under the control of upstream regulators, namely transcription factors belonging to the **Paired-box** and the **Sine Oculis-Related Homeobox** families (except in head myogenesis, see the paragraph *Head muscle formation*), whereas the muscle-specific genetic program is controlled by transcription factors called **Myogenic Regulatory Factors** (**MRFs**), comprising Myf5, MyoD, Myogenin and MRF4.

Trunk and limb myogenesis starts at midgestation (embryonic day 9 in mice) in the **dermomyotome**, a transient embryonic structure derived from the paraxial mesoderm, and comprises two phases. In the first phase, called primary myogenesis, post-mitotic cells delaminate from the dermomyotome and differentiate into the first muscle cells of the embryo. In the second phase, called secondary or fetal myogenesis, proliferating muscle progenitors migrate from the central region of the dermomyotome and differentiate and fuse with the primary myofibers thereby giving rise to trunk muscles.

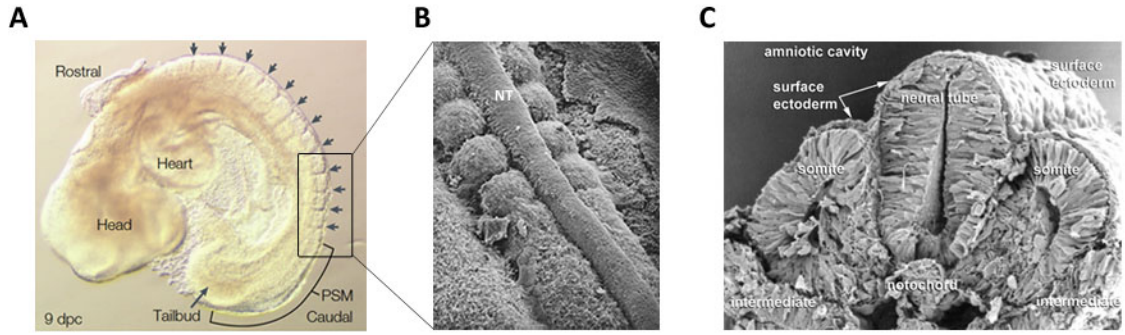
A proportion of these myogenic cells migrate to colonize the limb buds and form limb muscle. Through a tight balance between self-renewal and differentiation, proliferating myogenic progenitors persist in fetal and postnatal life and are responsible for muscle growth.

Head muscle, namely craniofacial muscle, originates from the cranial mesoderm. Due to its distinct embryonic origin, head myogenesis slightly differs from trunk and limb myogenesis in terms of signaling pathways and genetic cascades. Nonetheless they partially share common aspects (see section *Head muscle formation*).

The origin of trunk and limb muscles: somitogenesis

Skeletal muscle of body trunk and limbs derives from somites, as demonstrated for the first time by chick-quail grafts experiments (Chevallier, 1979). Somites are segments of

paraxial mesoderm that form sequentially at regular intervals (90 minutes in chicken, 120 minutes in mouse and 4-5 hours in human) from head to tail at either side of the neural tube (see Dequéant and Pourquié, 2008). Somite formation starts early in embryogenesis, when the neural folds reach the center of embryo axis and form the neural tube. The first somites appear at the rostral side of the embryo, where future somitic cells condensate and ultimately split apart from the paraxial mesoderm to become discrete spheres with an outer epithelial layer and an inner mesenchyme



(Figure 1).

Figure 1. Anatomical features of somitogenesis. A, Phase contrast photo of E9.0 mouse embryo showing the epithelial somites the and caudal presomitic mesoderm (PSM). Arrows indicate somites boundaries (from Saga and Takeda, 2001). **B,** Scanning electron microscopy image of neural tube (NT) somites at each side of NT and the PSM (bottom right) (from Gilbert 2006). **C,** Scanning electron microscopy image of a transverse section of a day 25 human embryo, showing the epithelial organization of somites (source: http://embryology.med.unsw.edu.au/).

The first model for somitogenesis, the so-called “Clock and Wavefront” mechanism, was proposed by Cooke and Zeeman in 1976 and did not include any precise cellular or molecular components. According to this general scheme, the Clock consists of cell-autonomous oscillators and the Wavefront corresponds to a signal that moves across the presomitic mesoderm (PSM) in an anterior-to-posterior direction and arrests the oscillations of the clock, thereby progressively defining the borders of future somites. Further investigation in chick, zebrafish and mouse confirmed this model by identifying a group of genes with oscillating levels in the PSM resulting from cyclic mRNA expression and degradation (Palmeirim et al., 1997; see Oates et al., 2012). These transcriptional oscillations occur with the same periodicity as somite emergence from the PSM, leading to the hypothesis that cyclic genes cause somite segmentation, thus they were defined as the “segmentation clock”. The majority of genes participating to the segmentation clock are targets of the Wnt, Notch and FGF signaling pathways and their periodicity seems to be controlled by negative feedback loops (Dale et al., 2003; Morimoto et al., 2005). Regarding the wavefront, a posterior-to-anterior gradient of

FGF signaling is present in PSM cells and FGF8-coated beads grafts in the chick embryo demonstrated that high levels of FGF pathway activation define the identity of posterior PSM cells and that progressively lower levels of FGF signaling activation through the embryo axis allow PSM cells to respond to the segmentation clock and become a somite. Further experiments showed that a Wnt3a tail-to-head gradient is present in the PSM and that Wnt signaling is required to establish the FGF gradient (see Dequéant and Pourquié, 2008). This evidence led to the formulation of a model in which specific thresholds of Wnt and FGF signaling pathways define a determination front that moves posteriorly along the embryo during somitogenesis. Retinoic acid was also shown to be involved in the positioning of the front determination since it is present as an anterior-to-posterior gradient opposing the Wnt/FGF gradient. These findings led to a revision of the "Clock and Wavefront" model that is depicted in Figure 2. A very recent work reported that inhibition of BMP in PSM without cyclic expression of clock genes is sufficient for somitogenesis (Dias et al., 2014). However, in this setup the rostro-caudal subdivision is lacking. Therefore the authors proposed that the clock genes are responsible for somite patterning rather then formation.

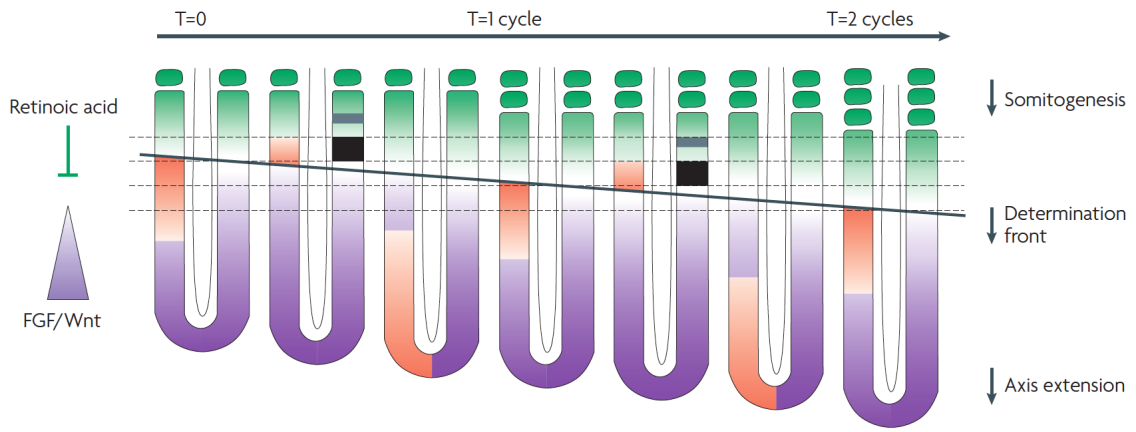


Figure 2. Schematic representation of the "Clock and Wavefront" somitogenesis model. The opposing FGF/Wnt (purple) and retinoic acid (green) gradients determine the position of the determination front (black line), a particular combination of signaling levels that allow cells residing in this region to become competent to respond to the segmentation clock (depicted in orange). The size of the future somites (dotted lines) is defined by the distance travelled by the wavefront during one oscillation of the segmentation clock. From Dequéant and Pourquié, 2008.

From the somite to the muscle: the dermomyotome

Epithelial somites are marked by the expression of the Paired-box transcription factor Pax3. During somite maturation, the most ventral part down-regulate Pax3 and starts to express Pax1, which is induced by Shh signaling from the notochord (Fan and Tessier-Lavigne, 1994). This domain of the somite undergoes a epithelial-to-

mesenchymal transition (EMT) and gives rise to the **sclerotome**, which ultimately contributes to vertebrae and rib cartilage, and to the **syndetome**, a group of cells adjacent to the sclerotome that originate the tendons of trunk muscle (Gilbert, 2006) (Figure 3).

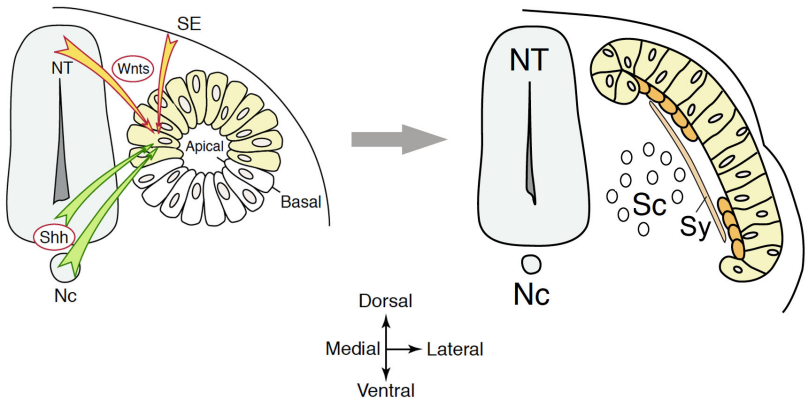


Figure 3. From the somite to the dermomyotome. During somite maturation, the most ventral portion of the somite (white) receives Shh from the nothocord and undergos EMT, giving rise to the sclerotome (Sc), and the Syndetome (Sy). Adapted from Tajbakhsh, 2003

The most dorsal region of the somite retains its epithelial organization longer than the central part and is referred to as the **dermomyotome**, a multipotent structure that contributes to the formation of back dermis, skeletal muscle, endothelial cells, a part of smooth muscle and brown adipose tissue (Ben-Yair and Kalcheim, 2005; Pouget et al., 2006; Esner et al., 2006; Enerbäck, 2009). The dermomyotome is a transient epithelium that can be subdivided into a central sheet and four lips (dorsomedial, ventrolateral, rostral, caudal). During development, cells from the four lips undergo EMT, acquire a myogenic fate and generate the **myotome**, the first differentiated muscle cells (Figure 4). The central sheet generates proliferating multipotent progenitors that express Pax3 and Pax7 and either delaminate dorsally, downregulate Pax3 and Pax7 and commit to the dermic lineage, or transiently maintain Pax3 and Pax7 and migrate toward the underlying myotome, where they differentiate into myogenic cells (Ben-Yair and Kalcheim, 2005). The fate choice between these two lineages is influenced by the orientation of cell division: in early dermomyotome, symmetric planar divisions mediated by Leu-Gly-Asn repeat-enriched protein (LGN) ensure the expansion of multipotent progenitors. During dermomyotome maturation there is a shift in cell division orientation, and in late dermomyotome cell division perpendicular to the epithelium provides a correct proportion of dermic and myogenic cells (Ben-Yair et al., 2011).

In the dermomyotome, two regions can be distinguished: the **epaxial** domain, which gives rise to the back muscle, and the **hypaxial** domain, from which originate the body wall muscle, the limb muscle and some migratory muscles such as the tongue and the diaphragm (Figure 4, see Bryson-Richardson and Currie, 2008).

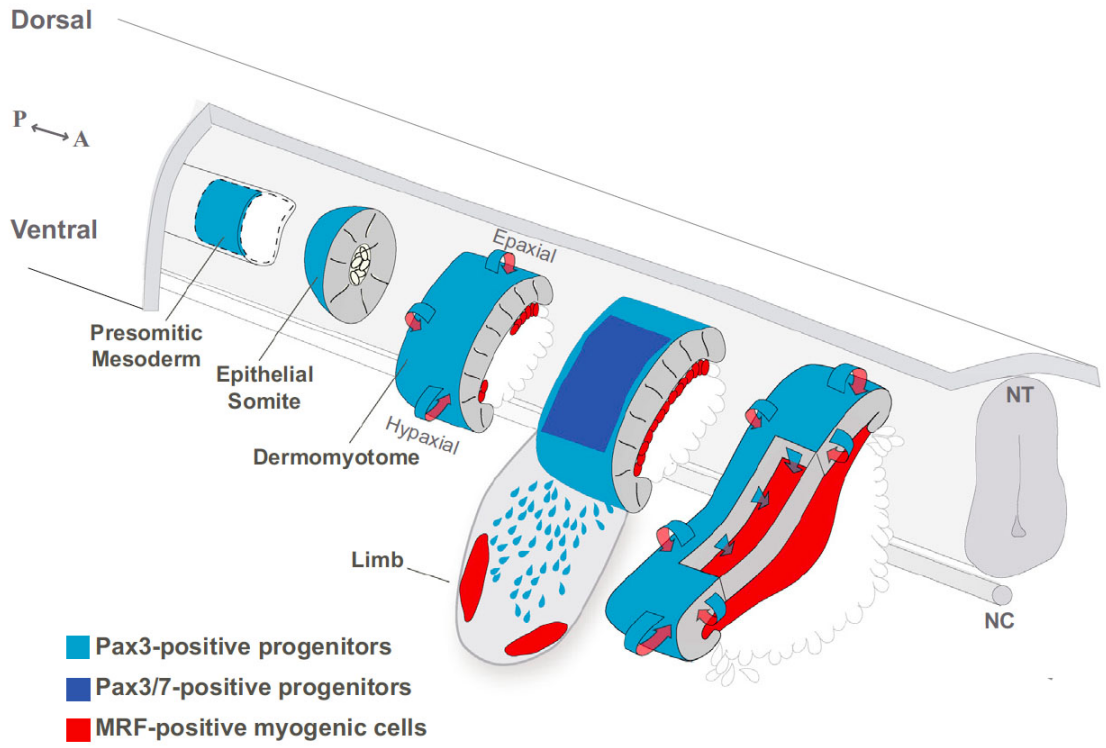


Figure 4. Schematic representation of the morphogenetic movements during primary and secondary myogenesis. Image from Buckingham and Rigby, 2014.

Extrinsic determination of the dermomyotome

The major role in determining dermomyotome identity is played by extrinsic signals secreted by the surrounding structures. During dermomyotome specification, signaling gradients from the neural tube and notochord interact and convert the dorsal portion of the somite into a particular pattern of gene expression that defines the dermomyotome.

A great contribution in understanding the mechanisms underlying dermomyotome specification came from experiments based on inactivation of genes belonging to signaling pathways in mouse embryos and on chimeric grafts in avian embryos. By surgical ablation or by injection of protein-secreting cells in chick embryos, it was shown that BMP4 induces *Wnt1* and *Wnt3a* expression in the neural tube (Marcelle et

al., 1997). In turn, these two secreted factors are necessary for the induction of the dermomyotome. Indeed, in *Wnt1*/*Wnt3* compound mutant mouse embryos, the dermomyotome is reduced in size and lacks the medial compartment, as shown by the reduction of Pax3 and Myf5 expression domains (Ikeya and Takada, 1998). Chick-quail grafts demonstrated that Wnt1 is released by the dorsal neural tube and is necessary and sufficient to induce the dermic lineage, whereas Shh from the notochord inhibits dermis formation and promotes cartilage and muscle development (Olivera-Martinez et al., 2001). Analysis of the expression pattern of Wnt receptors belonging to the Frizzled family confirmed this evidence: *Fzd1* and *Fzd6* are expressed by the medial (epaxial) domain of the dermomyotome and mediate the induction of *Myf5*. The identification of functional Wnt and Shh responsive elements in *Myf5* promoter revealed that its expression depends on a synergy between the Wnt/β-catenin pathway and Shh signaling (Borello et al., 2006). Furthermore, it has been reported that Wnt1 and Wnt7a proteins can stimulate Protein kinase A (PKA) signaling and CREB-mediated transcription of *Pax3*, *Myf5* and *MyoD* in the somite, providing evidence for a non-canonical Wnt signaling pathway in embryonic myogenesis (Chen et al., 2005).

Another signaling pathway involved in dermomyotome determination is the BMP cascade. BMP4 secreted from the lateral plate mesoderm (i.e. the mesoderm lying the farthest away from the neural tube) and it inhibits myogenic differentiation of muscle progenitors migrating ventrally into the myotome so that they retain their progenitor features until they are in the correct position to differentiate (Pourquié et al., 1996; Reshef et al., 1998).

In addition to positive signals that induce the dermomyotome in the dorsal somites, inhibitory regulators prevent the dermomyotomal fate in inappropriate locations. For instance, in the chick embryo Shh released by the notochord avoids mislocalized expression of *Wnt11*, a marker of medial dermomyotome (Marcelle et al., 1997).

Primary and secondary myogenesis

During muscle formation, two subsequent phases of myogenesis can be distinguished.

In a first phase, called **primary myogenesis**, postmitotic cells from the four lips of the dermomyotome activate the expression of the myogenic determination factors *Myf5* and *Myf6* (MRF4), loose their epithelial organization and delaminate underneath the remaining dorsal epithelial dermomyotome. Here they differentiate and give rise to the primary myotome, a structure composed of aligned myocytes and myotubes that

serve as scaffold for following waves of myogenesis (Kahane et al., 1998; Gros et al., 2004). The first activation of *Myf5* seems not to be under the control of Pax3, but rather to be induced by Wnt and Shh signaling secreted from the neural plate (Buckingham and Relaix, 2007). Myf5 in turn promotes the expression of MyoD and together they activate a genetic program that drives the myogenic differentiation.

In a second phase, called **secondary myogenesis**, new myogenic progenitors arise from the dermomyotome and sustain fetal and postnatal myogenesis. Lineage tracing studies in chick embryos based on dividing cells labeling revealed that myogenic precursors expressing Pax3 and Pax7 continuously delaminate from the dermomyotome epithelium and persist as a proliferating population. These cells continuously proliferate within the primary myofibers and eventually differentiate, thereby contributing to trunk and limb muscles growth during fetal and postnatal life (Ben-Yair and Kalcheim, 2005; Gros et al., 2005). This evidence has been confirmed also in mouse models (Kassar-Duchossoy et al., 2005; Relaix et al., 2005) and recently it was shown that the balance between proliferation and differentiation of this Pax3/Pax7-positive cell population is temporally and spatially controlled by the Notch, BMP and Shh signaling (Vasyutina et al., 2007; Wang et al., 2010; Kahane et al., 2013). At the end of fetal development, a proportion of Pax3/Pax7-expressing myogenic progenitors locate underneath the basal lamina of myofibers, thereby giving rise to adult satellite cells (Gros et al., 2005; Relaix et al., 2005). The homing of fetal muscle progenitors to the satellite cell niche is controlled by the Notch pathway. Notch signals stimulate the expression of basal lamina components and adhesion molecules by Pax3/7-positive cells, inducing them to contribute to their own microenvironment and to adhere to myofibers (Bröhl et al., 2012).

Upstream regulators that govern myogenesis

As mentioned above, commitment of somitic pluripotent cells to the myogenic lineage is induced by the activation of muscle-specific genes. Combination of mouse gene knockout and lineage tracing approaches defined the genetic hierarchies that govern myogenesis. A panel of transcription factors have been shown to act upstream of muscle-specific genes and spatio-temporally determine myogenic commitment and progression. These upstream regulators mainly belong to the **Paired-Homeobox Family** and to the **Sine Oculis-Related Homeobox Family** and are described hereafter.

The Paired-box (PAX) Family

Among the nine members of the PAX family, only Pax3 and Pax7 play a major role in muscle formation. Both transcription factors are not muscle specific, but this section focuses only on their roles in myogenesis (For a review of Pax3 and Pax7 functions in other tissues see Buckingham and Relaix, 2007).

Pax3 is initially expressed in epithelial somites and later during embryogenesis it becomes restricted to the epithelial dermomyotome (see Blake and Ziman, 2014). Pax3 is maintained in migrating limb muscle progenitors during fetal and postnatal myogenesis and it is downregulated at the onset of myogenic differentiation. Loss-of-function mutation of *Pax3* in mouse (called Splotch) is lethal at embryonic day 15 and it abrogates hypaxial muscle formation, but epaxial muscle is less affected (Bober et al., 1994; Tremblay et al., 1998). Comparison of *Pax3* mutant embryos and compound *Pax3;Myf5;MRF4* null embryos revealed that both Myf5 and Pax3 act as upstream regulators in the myogenic program (Tajbakhsh et al., 1997). However, Pax3 is required for *Myf5* expression in hypaxial dermomyotome whereas it is dispensable in epaxial dermomyotome, where Myf5 can drive MyoD transcription independently of Pax3, as summarized in Figure 5 (Bajard et al., 2006).

Compared to Pax3, **Pax7** function is limited to later development, Pax7 being expressed only in epithelial dermomyotome just prior to its epithelial-to-mesenchymal transition. The phenotype of *Pax7* null mice is milder than the Splotch mutants: they die only two weeks after birth and present dramatic defects in muscle size and function. Indeed, whereas primary myogenesis is not altered, fetal and postnatal muscle growth is completely abolished (Seale et al., 2000).

Genetic ablation of the Pax3 and Pax7 cell populations demonstrated that the Pax7 lineage arises from Pax3 expressing cells and that in absence of Pax7 expressing cells only late myogenesis is perturbed (Hutcheson et al., 2009). This led to the conclusion that both Pax3 and Pax7 act in fetal myogenesis, whereas only Pax3 is also required in early myogenic induction. Double *Pax3;Pax7* null embryos display a more severe phenotype than Pax3 or Pax7 single mutants, since the primary myotome is formed but muscle formation is completely abrogated. This demonstrates that Pax3 and Pax7 can compensate for each other, and genetic replacement experiments showed that Pax7 can fully restore Pax3 function in trunk muscle. Nonetheless, in limb muscle the compensation is only partial, indicating that these two factors present both shared and distinct molecular functions (Relaix et al., 2004). Similar results have been obtained in adult muscle stem cell (Relaix et al., 2006), and a recent large-scale study showed that, at least in adult muscle stem cells-derived myoblasts, Pax3 and Pax7 share only a small

proportion of genomic binding sites and they diverge in their affinity for different binding motifs (Soleimani et al., 2012a).

Despite their crucial requirement for muscle formation, little is known about the molecular mechanism by which Pax3 and Pax7 drive embryonic and fetal myogenesis. For instance, they have been shown to directly regulate a relatively small number of muscle specific genes. It is important to consider that Pax3 and Pax7 are not exclusively expressed in the muscle lineage, but they are also found in the neural tube and in the neural crests. Furthermore, like other members of the Pax family, Pax3 and Pax7 have been described to be poor transcriptional activators that need cofactors to efficiently induce transcription of target genes. Therefore, their key role in myogenic determination might rely on a particular combination of coactivators, which to date still remain to be identified.

Concerning the transcriptional activity of Pax3, the first Pax3 target gene to be identified was *c-Met*, a tyrosine kinase receptor required for the delamination and migration of myogenic precursors (Epstein et al., 1996). Pax3 has been shown to activate two regulatory elements responsible for the hypaxial and limb expression of *Myf5* (Bajard et al., 2006), although *Myf5* transcription is regulated by a large number of enhancers, which are activated in a spatio-temporal manner presumably by several other factors (see Buckingham and Rigby, 2014). More recently, Pax3 binding sites in the *Fgfr4* promoter have been identified by *in vivo* ChIP-on-chip experiments. Based on this evidence and on the analysis of the knockout of *Sprouty2*, a modulator of the FGF pathway, it has been proposed that Pax3 controls the balance between self-renewal and differentiation of myogenic progenitors by regulating FGF signaling (Lagha et al., 2008). Although *in vivo* transcriptomic data provided a list of putative target genes, our knowledge on Pax3 transcriptional activity remains incomplete (Lagha et al., 2010).

Similarly to Pax3, Pax7 has been shown to directly activate the transcription of few genes. Among them, *Nfix*, a transcription factor required for the expression of several muscle-specific genes during fetal myogenesis, has been proposed as a potential target of Pax7 (Messina et al 2010). Based on *in vitro* experiments, other genes suggested to be regulated by Pax7 are MyoD and Myf5 (Hu et al., 2008; Soleimani et al., 2012a). A possible mechanism by which Pax7 regulates gene expression is the recruitment of a histone modifier, the Wdr5-Ash2L-MLL2 methyl transferase complex, that drives the tri-methylation of lysine 4 of histone H3 (H3K4) at least in the promoter region of the *Myf5* gene (McKinnell et al., 2008).

The Sine Oculis-Related Homeobox (SIX) Family

The SIX transcription factors have been first identified in Drosophila, where together with their cofactors of the Eyes Absent (Eya) and Dachshund (Dach) families, they play a crucial role in eye development (Kawakami et al., 2000). In mouse, out of the 6 *Six* genes, *Six1*, *Six4* and *Six5* are expressed in overlapping domains in somites, limb buds, dorsal root ganglia and branchial arches. Six1 and Six4 control different stages of myogenesis by binding through their homeodomain to consensus DNA sequences called MEF3, present in the promoter of several muscle-specific genes. At the onset of myogenesis in the hypaxial dermomyotome, they directly regulate *Pax3* expression, as it has been demonstrated by the analysis of *Six1;Six4* and *Eya1;Eya2* double mutants, in which hypaxial muscle is completely absent (Grifone et al., 2005, 2007). During limb myogenesis, Six proteins participate with Pax3 to the activation of *Myf5* (Giordani et al., 2007). Later in myogenic differentiation, Six1 and Six4 control *MyoD* induction by binding to regulatory elements of its promoter (Relaix et al., 2013) as well as *Myog* and *MRF4* (Spitz et al., 1998; Grifone et al., 2005).

Unlike Pax factors, Six proteins are expressed also in differentiated skeletal muscle, where they have been proposed to determine the metabolic specialization of fast-type muscles (Niro et al., 2010; Sakakibara et al., 2014).

The induction of myogenesis: the MRFs

The acquisition of the myogenic fate is achieved by the activation of a genetic program governed by a group of transcription factors referred to as **Myogenic Regulatory Factors** (**MRFs**) that include Myf5, MyoD, MRF4 and Myogenin. MRFs are basic helic-loop-helic (bHLH) transcription factors that, together with a large variety of cofactors, activate the transcription of muscle-specific genes that allow myogenic progenitors to differentiate into functional muscles.

Myogenic differentiation 1 (MyoD)

The first MRF to be discovered is MyoD, which is referred to as the master regulator of the skeletal muscle gene expression program. Forced expression of *MyoD* (by transfection of a plasmid carrying *Myod* cDNA or infection with a viral vector coding for *Myod*) into mouse, monkey, chicken, human and rat fibroblasts, as well as in melanoma, neuroblastoma, liver and adipocyte cell lines is sufficient to transform cells

into myoblasts (Davis et al., 1987; Weintraub et al., 1989). The three other MRFs were identified due to their sequence similarity with the *MyoD* gene.

Myogenic factor 5 (Myf5)

As MyoD, Myf5 was reported to be sufficient to induce the myogenic program into mouse fibroblasts (Braun et al., 1989). Later studies showed that Myf5 is the first MRF to be expressed during embryonic myogenesis, being transiently transcribed throughout the presomitic mesoderm and in the (Ott et al., 1991). However this low expression does not result in the commitment of the presomitic cells to the myogenic program, thus its precocious role remains to be elucidated. Although Myf5 is a myogenic determination factor, it has been reported to be transiently expressed by a subpopulation of cells in the developing nervous system (Tajbakhsh et al., 1994). This complex expression pattern might be due to the complexity of its regulatory region, which comprises a large number of enhancers distributed on approximately 100kb upstream the gene.

Myogenin (Myog)

Another MRF is Myogenin. It was first detected in differentiating myogenic cell lines and in skeletal muscles extracts and, similarly to MyoD and Myf5, its exogenous expression was capable of converting fibroblasts into myoblasts (Edmondson and Olson, 1989). Genetic invalidation of the *Myog* gene in mouse embryos did not perturb somitogenesis or the expression of MyoD, but impaired muscle differentiation: myogenic cells persist as mononucleated myocytes and did not fuse to form myofibers (Hasty et al., 1993). This evidence, together with further analysis of *Myog* promoter revealed that Myogenin is downstream of MyoD and Myf5 in the genetic cascade that regulates myogenesis, being crucial for terminal differentiation (Cheng et al., 1993; Gerber et al., 1997).

Myogenin regulatory factor 4 (MRF4)

The last MRF to be identified is MRF4 (also known as Myf6), which has a role in terminal differentiation, where its expression is controlled by Myogenin (Rhodes and Konieczny, 1989; Miner and Wold, 1990; Hasty et al., 1993). However, a function also in myogenic specification cannot be excluded, since it has been reported to be

expressed quite early during myotome formation and it can compensate for MyoD and Myf5 absence (Kassar-Duchossoy et al., 2004).

Genetic hierarchy within the MRFs

Analysis of *MyoD*-null and *Myf5*-null embryos demonstrated that the absence of either *MyoD* or *Myf5* resulted in apparently normal muscle development (Braun et al., 1992; Rudnicki et al., 1992). However, combined *Myf5* and *MyoD* mutation led to total lack of skeletal muscle fibres and myoblasts, suggesting that either Myf-5 or MyoD is required for the determination of skeletal myoblasts *in vivo* (Rudnicki et al., 1993). Importantly, the first *Myf5*-null mice were also deficient for *MRF4* expression, due to a cis-repression by the nLacZ knock-in cassette in the *Myf5* gene. The generation and analysis of new *Myf5:MyoD* double-null embryos with functional *MRF4* revealed the presence of differentiated skeletal muscle at the embryonic stages of development and identified *MRF4* as a determination gene (Kassar-Duchossoy et al., 2004). As currently understood, at least for trunk myogenesis, Myf5 and MRF4 act upstream of MyoD and Myf5, MRF4 and MyoD act upstream of Myogenin (Figure 5, for review, see Buckingham and Rigby, 2014).

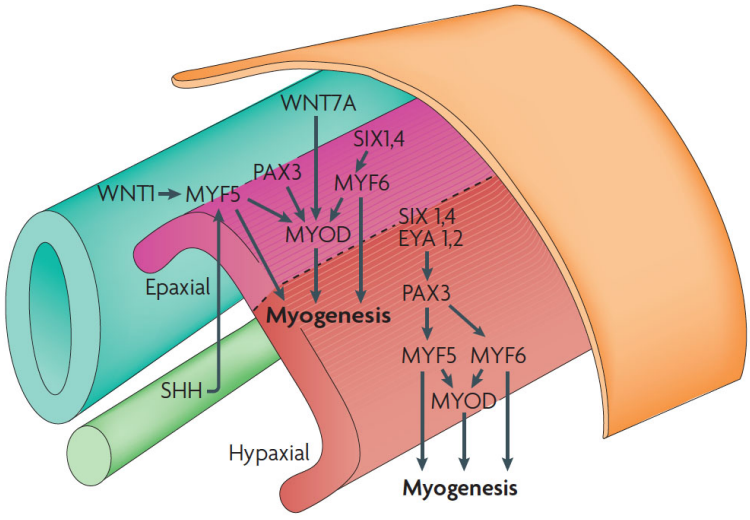


Figure 5. Schematic representation of the genetic hierarchy of upstream regulators and MRFs. Whereas in the epaxial dermomyotome (pink) Myf5 is not under the control of Pax3, in the hypaxial dermomyotome (red), Myf5 lies downstream of Pax3. Image from Bryson-Richardson and Currie, 2008.

Transcriptional mechanisms of MRFs

Regulatory regions of muscle specific genes, such as contractile proteins or muscle specific enzymes responsible for muscle functions, harbor specific consensus DNA

sequences, called **E-boxes**, located close to the transcription start site. MRFs recognize and bind E-boxes and recruit cofactors, chromatin modifiers and the RNA transcription machinery to activate transcription of these loci.

Transcriptional induction of muscle differentiation genes

The transcriptional mechanism by which MRFs induce myogenesis has been largely characterized for MyoD, which cooperates with cofactors and chromatin modifiers to spatio-temporally regulate the activation of muscle specific genes.

To activate transcription of its target, MyoD binds to 2 E-boxes and therefore it forms heterodimers with other MRFs or with **E-proteins**, bHLH factors that are ubiquitously expressed (see Tapscott, 2005). E-proteins can also be sequestered by other bHLH proteins that act as inhibitors of myogenesis by limiting their availability for the interaction with MRFs. This is believed to prevent inappropriate expression of myogenic genes (Berkes and Tapscott, 2005). Morevover, based on ChIP-seq analysis, it has been proposed that Snail1 and Snail2 occupy MyoD binding sites in proliferating myoblasts, therefore imparing MyoD transcriptional activity (Soleimani et al., 2012b). However, at the onset of differentiation, downregulation of Snail allows MyoD to bind these loci and induce their transcription.

An important observation is that although MyoD binds to a huge number of sites throughout the genome (ChIP-seq analysis identified about 25 000 binding sites), only a small subset of genes (1956) is regulated during differentiation (Cao et al., 2010). A recent large-scale study revealed that E-boxes binding is determined by their accessibility, which depends on lineage specific chromatin compaction (Fong et al., 2012). However, how the epigenetic specificity of myogenic lineage is established remains poorly understood. A combination of *in vivo* and *in vitro* observations suggested that the Polycomb Ezh2 methyltransferase complex is associated to and represses myogenic genes, at least *Myh4* (also known as MHCIIb) and *MCK*, in early myotome by mediating lysine 27 of histone 3 (H3K27) methylation, a repressive chromatin mark. As development proceeds, Ezh2 is downregulated and therefore released form chromatin, H3K27 becomes hypomethylated, and this enhances the accessibility of E-boxes for MyoD, which binds to these regions and activate transcription (Caretti et al., 2004).

MyoD actively participates to chromatin remodeling by recruiting p300, an acetyltransferase complex that mediates histone acetylation, which together with histone hypomethylation creates a permissive chromatin context for transcription activation (Puri et al., 1997; Sartorelli et al., 1997). MyoD has been proposed to have also a repressive function, by interacting with histone deacetylases to prevent

inappropriate activation of the myogenic program to early during myogenesis (Mal et al., 2001).

Control of cell cycle

During myogenesis, terminal differentiation is intimately linked to cell cycle exit. This evidence is supported by the early observation that MyoD-dependent conversion of fibroblasts into myogenic cells is inhibited when cyclins are ectopically expressed by a mechanism that partially implies pRb, a key regulator of cell cycle progression (Rao et al., 1994; Skapek et al., 1996). Inactivation of p21 (Cdkn1a) and p57 (Cdkn1c), two factors involved in the control of cell cycle arrest, leads to severe defects in skeletal muscle formation due to increased apoptosis and lack of expression of markers of terminal differentiation (Zhang et al., 1999). Further *in vitro* transcriptomic studies confirmed that the onset differentiation correlates with upregulation of genes that promote cell cycle exit and downregulation of cyclins and cyclin-dependent kinases (CDKs), as depicted in Figure 6 (Shen et al., 2003).

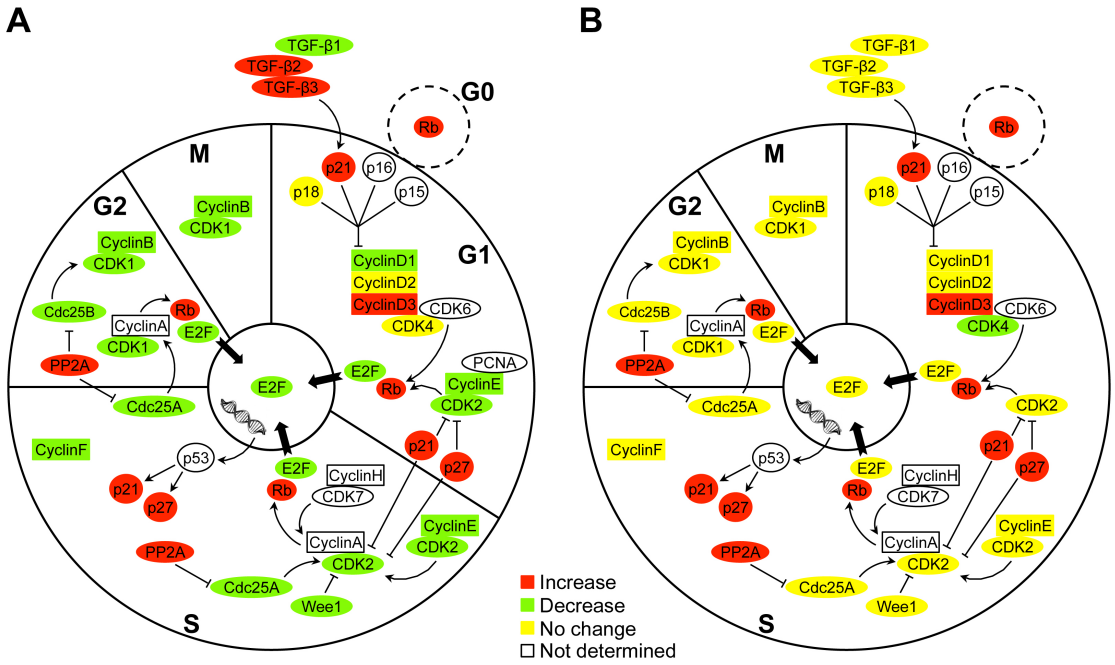


Figure 6. Evolution of cell cycle regulation during myogenic differentiation. Representation of changes in the expression level of cell cycle regulator after **1 day** of differentiation versus proliferation (**A**), and after **6 days** versus 1 day of differentiation (**B**). From Shen et al., 2003

Protein stability and transcriptional activity of MyoD and Myf5 vary throughout cell cycle and this underlies the fact that their function is coupled to cell cycle dynamics, although no precise mechanisms involved in this regulation have been described (for a review see Singh and Dilworth, 2013). Similarly, the exact role of MyoD, and to a lesser

extent Myf5, in controlling cell cycle progression and exit has widely been investigated but remains controversial. It has been proposed that MyoD promotes cell cycle exit by inducing p21 expression (Halevy et al., 1995). However, a more recent study suggested that in adult myogenesis MyoD positively regulates *Cdc6*, which is required for DNA replication and S phase progression (Zhang et al., 2010).

A possible explanation for this dual role of MyoD in cell cycle regulation comes from the evidence that during muscle differentiation MyoD induces the expression of *Myog*, which promotes terminal arrest of proliferation by repressing genes that positively regulate cell cycle progression and by inducing p21 (Liu et al., 2012b). Genome-wide analysis of promoter occupancy of MyoD and Myog highlighted that these two factors share a large number of target genes (Cao et al., 2006). This led to draw a two-step model in which MyoD participates to chromatin opening and transcription initiation and Myog further potentiates the activation of target genes. In this perspective, MyoD is responsible for the expansion of proliferating muscle progenitors in order to generate a correct amount of cells, whereas Myog accounts for their terminal differentiation.

Head muscle formation

Head muscles comprise the axial, laryngoglossal, branchial and extraocular subgroups. With the exception of the tongue and some neck muscles, which derive by migration of myogenic precursors from the rostral-most somites, all other head muscles derive from the cranial paraxial mesoderm (Figure 7). Table 1 summarizes their location, origin and function.

Unlike trunk paraxial mesoderm, cranial mesoderm is not segmented, therefore molecular markers rather than anatomical features are used to distinguish different regions. Indeed, cranial mesoderm is a multipotent structure composed of several cell populations that give rise not only to head skeletal muscle, but also to cardiomyocytes of the anterior heart field, angiogenic cells and bone cells forming the posterior part of the skull (see Noden and Francis-West, 2006).

Another important element that differs from somitic myogenesis is the genetic regulation of myogenic program in craniofacial myogenesis. Although myogenic differentiation is achieved through the action of MRFs as in trunk and limb muscles with only minor differences (for details see Sambasivan et al., 2011), the induction of myogenesis in the head depends on distinct signaling pathways and upstream regulators.

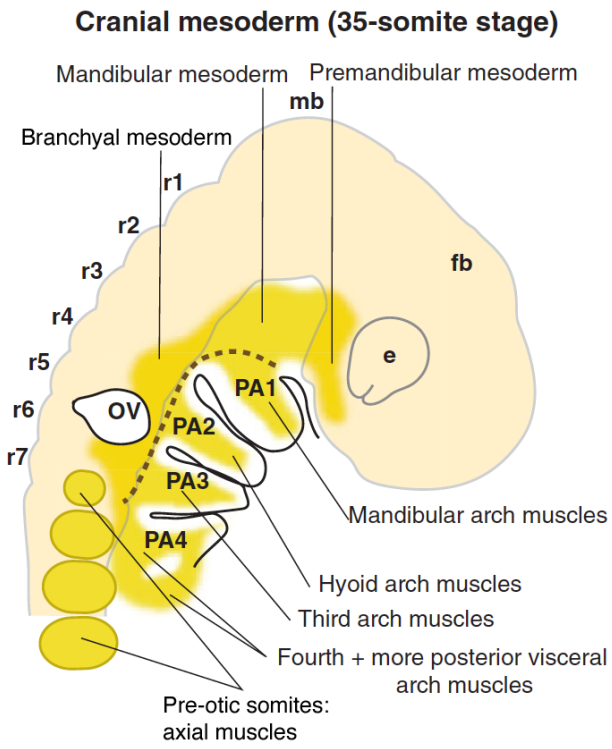


Figure 7. Schematic representation of the cranial mesoderm in the mouse embryo. The cranial mesoderm can be subdivided into premandibular, mandibular and branchyal mesoderm. e: eye; fb: forebrain; mb: midbrain; PA1-4: pharyngeal arch; r1-7: rhombomeres (neural tube segments). Adapted from Sambasivan et al., 2011

Whereas somitic myogenesis is induced by Wnt signals secreted from the neural tube, cranial myogenic induction requires the Wnt inhibitor Frzb and the BMP antagonists Noggin and Gremlin released by the neural crests and the surrounding tissues (Tzahor et al., 2003).

Regarding the upstream regulators, Pax3, which is absolutely required for the activation of MRFs in trunk and limb myogenesis, is not expressed in head paraxial mesoderm (Hacker and Guthrie, 1998) and its function is carried out by two transcription factors, Pituitary homeobox 2 (Pitx2) and T-box 1 (Tbx1). Pitx2 directly regulates *Myf5* and *MyoD* expression in extraocular muscles (Zacharias et al., 2011) and it partially participates to the formation of branchial muscles (Dong et al., 2006), which depends also on Tbx1 (Kelly et al., 2004). Similarly to somitic myogenesis, Pax7 is expressed by myogenic progenitors that account for fetal and postnatal muscle growth.

Cranial myogenesis is intimately linked to cardiogenesis, at least for branchial muscles. Interestingly, second heart field cardiomyocytes and branchial arch-derived muscles share Pitx2 and Tbx1 as critical regulator for their developmental programs

(Nowotschin et al., 2006) and they arise from a common progenitor (Lescroart et al., 2010). Furthermore, the proteins Musculin (Msc, also known as MyoR) and Transcription factor 21 (Tcf21, also known as capsulin) are also shared by cardiac and branchial myogenic progenitors. Double *Msc;Tcf21* knockout mouse embryos demonstrated that these two bHLH factors are required for *Myf5* and *MyoD* expression (Lu et al., 2002). However, their mechanism of action and their relationship with MRFs still remain unclear.

Muscle	Location	Origin	Function
Axial	Neck	Pre-otic somites	Head movement
Laryngoglossal	Neck and mouth	Pre-otic somites, branchial mesoderm	Larynx, mouth and tongue movement
Branchial	Mandible, maxilla and pharynx	Mandibular mesoderm	Jaws and pharynx movement
Extraocular	Eye	Premandibular + branchial mesoderm	Eye movement

Table 1. Location, origin and function of head muscles. From Noden and Francis-West, 2006.

2. Adult skeletal muscle: an overview

The outcome of embryonic and fetal myogenesis is the formation of more than 600 muscles located throughout the human body and responsible movement of head, trunk and body, as well as several other physiological functions.

Anatomy of adult skeletal muscle

Each muscle is composed of thousands of long multinucleated cells, called **myofibers**, generated by fusion between myogenic progenitors. Each myofiber has a specialized cell membrane, called **sarcolemma**, and is surrounded by a layer of extracellular matrix called **basement membrane**. Groups of ten to hundreds of myofibers are organized in bundles enveloped by a thin layer of connective tissue named **perimysium**. Several bundles form a muscle and are enclosed in an external layer of connective tissue called **epimysium**. Each end of muscles is attached to the bones by the tendons and contraction of muscle is responsible for the movement of the skeletal structure to which they are associated (Figure 8).

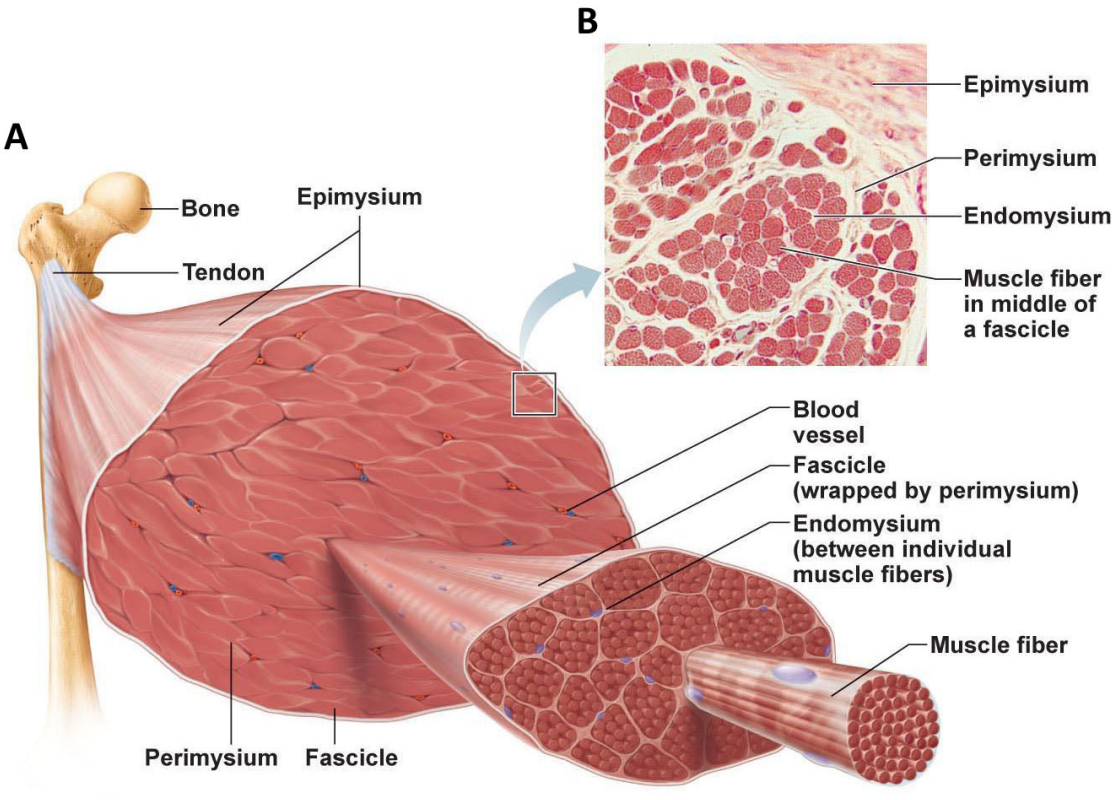


Figure 8. Skeletal muscle anatomy. A, Illustration of skeletal muscle components. **B,** Photomicrograph of a cross-section of skeletal muscle. From Marieb and Hoehn, 2011

In addition to myofibers, several other components are present within the muscle and participate to its functionality.

The **vascular system**, organized in a broad network of blood capillaries and associated pericytes, provides the correct supply of nutrients and oxygen as well as the clearance of metabolic waste.

Motoneurons are connected to the sarcolemma through a particular synapse called neuromuscular junction and are responsible for the transmission of signals required for muscle contraction. Sensory receptors are also present within the muscle to detect muscle lengthening and to convey information about spatial body position to the brain. They are called **muscle spindles** and are composed of about ten specialized muscle fibers innervated both by sensory neurons and motoneurons.

Immune cells reside within the muscle to eliminate pathogens and dying cells, and additional circulating immune cells infiltrate during muscle injury or in pathological conditions, where they stimulate tissue repair.

Satellite cells are mononucleated muscle-specific stem cells located in close contact with the myofibers, between the sarcolemma and the **basal lamina** (i.e. the internal layer of the basement membrane), and confer to skeletal muscle the ability to regenerate upon injury.

Finally, **interstitial cells** are a heterogeneous population of cells intercalated between myofibers. They secrete extracellular matrix proteins and growth factors and during regeneration they can interact with satellite cells.

Skeletal muscle contraction

During embryonic terminal muscle differentiation, a highly ordered sequence of transcriptional, translational and post-translational events directs the assembly of the myofibrils, the contractile structures of myofibers composed of repetitive units called **sarcomeres** (see Braun and Gautel, 2011). Each side of the sarcomere is delimited by a structure called Z disc, in which crosslinker proteins such as α-actinin and titin anchor interdigitated and antiparallel filaments of actin and myosin (Figure 9).

Muscle contraction is governed by the level of calcium, which is stored in the myofiber endoplasmic reticulum and released in the myofiber cytoplasm after transmission of a membrane depolarization signal from the motoneuron to the myofiber. Together with ATP, calcium ions regulate the binding between myosin and actin filaments. Indeed, in

presence of calcium, actin binding sites become accessible on the myosin heads, which are associated with ADP. The binding of myosin heads to actin filaments releases ADP and produces a conformational change that brings the myosin heads toward the center of the sarcomere and lets the actin and myosin filaments slide along each other. At this step myosin heads bind ATP, release the actin filaments and ATP hydrolysis into ADP brings the myosin heads to the starting conformation. Cycles of ATP hydrolysis and exchange allow further sliding of the actin and myosin filaments and shorten the distance between the two Z-discs, thus resulting in muscle contraction (see Lodish et al 2004).

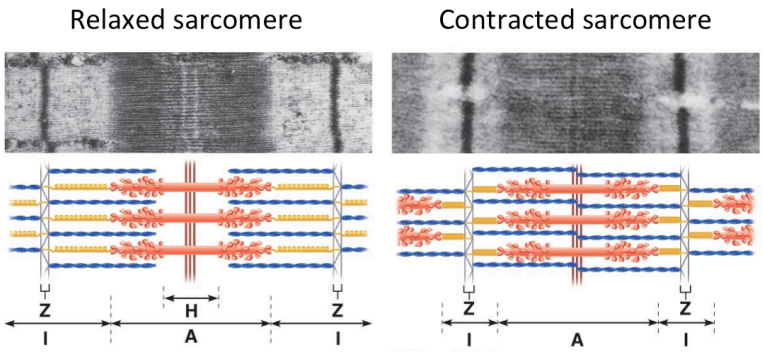


Figure 9. Skeletal muscle contraction. Electron microscopy photo and schematic representation of relaxed and contracted sarcomeres. Actin filaments are depicted in blue, myosin in red and crosslinker proteins in yellow. From Marieb and Hoehn, 2011

Metabolism

Based on metabolic and contractile properties, two types of myofibers can be distinguished: slow/oxidative and fast/glycolytic. Distinct myosin heavy chain isoforms are associated to these physiological features (see Schiaffino and Reggiani, 2011).

Slow muscle fibers are more resistant to fatigue and produce the ATP necessary for contraction through the respiratory chain of mitochondria. Slow muscles express myosin heavy chain type 1 (official symbol MYH7, also known as MYHCI or slow).

Fast myofibers have a stronger but short-lasting contraction force and produce energy through glycolytic enzymes present in their sarcoplasm. Fast muscles express myosin heavy chain type 2, of which 3 isoforms exist: MYH2 (MYHCIIA), MYH1 (MYHCIIX) or MYH4 (MYHCIIB). MYH2-expressing myofibers are considered as “mixed” fibers, since they produce a quite rapid contraction but they have an oxidative metabolism,

whereas MYH1 and MYH4 myofibers are glycolytic and exert stronger and faster contractions.

Due to its plasticity, skeletal muscle can undergo changes in metabolism and contraction properties through the action of several factors, among them physical exercise, muscle disuse and aging.

3. Skeletal muscle regeneration

Skeletal muscle is a stable tissue with infrequent myonuclei turnover, due to repair of physiological minor lesions caused by everyday muscle use (Schmalbruch and Lewis, 2000). Myofibers are irreversibly post-mitotic, but the presence of muscle stem cells associated to myofibers ensures their regeneration. Indeed, upon severe muscle trauma inflicted by intense exercise, muscle injury or chronic muscle degenerative pathologies (see section 4. Skeletal muscle in pathological context), a multi-step regeneration process is activated to repair the tissue and restore its functionality. After a first phase of myofibers necrosis and inflammatory response, satellite cells exit their quiescent state to proliferate and give rise to a population of transient amplifying myogenic progenitors (also called adult myoblasts) that differentiate and fuse to form new myofibers (Figure 10). In parallel, revascularization, reinnervation and deposition of new extracellular matrix ensure the perfect functionality of the regenerated muscle. The self-renewal ability of satellite cells replenishes the stem cell pool of the tissue, thereby allowing subsequent rounds of muscle regeneration.

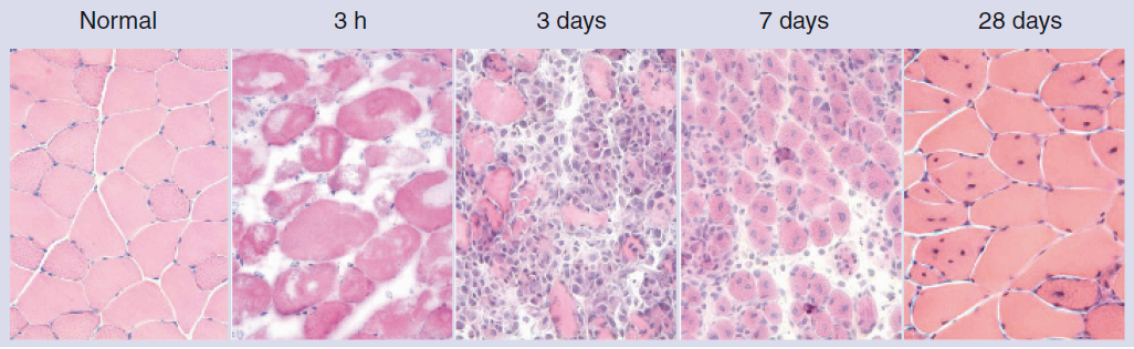


Figure 10. Regeneration of Tibialis Anterior (TA) muscle after acute injury. Hematoxylin and eosin staining on sections of uninjured (normal) muscle and at different time-points after notexin-induced injury. Note that after a necrosis (3h) and invasion of inflammatory cells and proliferation of myogenic cells (3 days), new myofibers are generated (7 days). 28 days post injury the tissue integrity is restored and newly formed myofibers can be distinguished by the presence of centrally located nuclei. From Gayraud-Morel et al., 2011.

Satellite cells: definition and identification

Anatomical features

Satellite cells are the stem cells of skeletal muscle. They have been first identified in 1961 during the characterization of frog myofibers by electron microscopy (Mauro, 1961). In his brief communication, Mauro reports "[…] the presence of certain cells, intimately associated with the muscle fiber, […] which we have chosen to call *satellite cells.* […]" (Figure 11). Due to their close association with to the myofibers, satellite

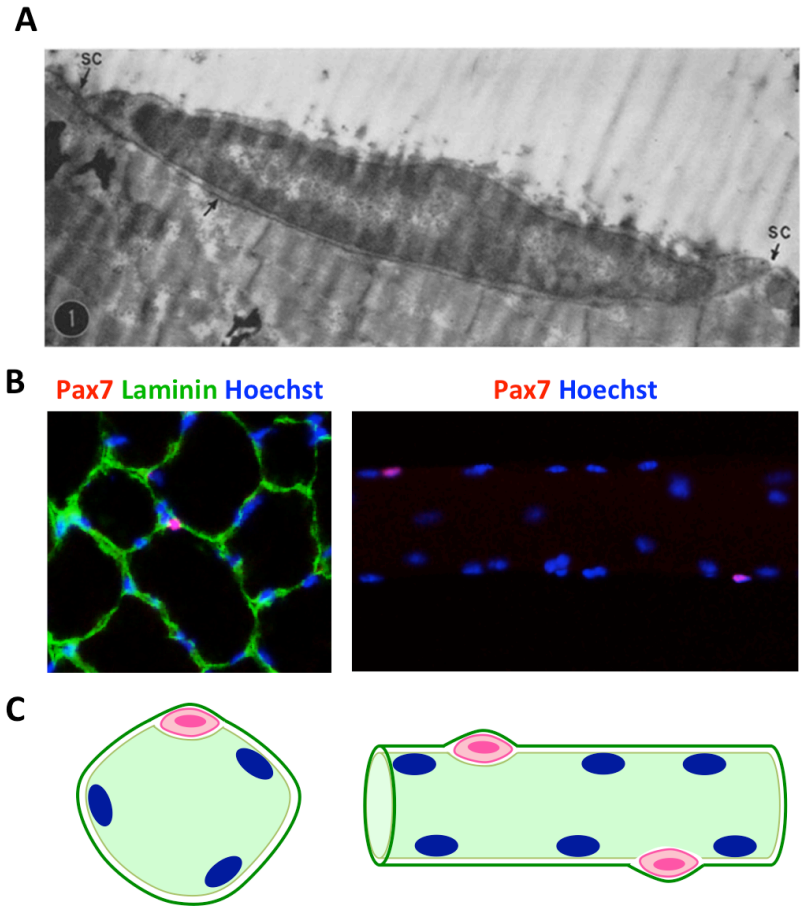


Figure 11. The satellite cell. A, Electron microscopy photo showing a longitudinal view of a satellite cell in close contact with the host myofiber (Mauro, 1961). **B,** *Left panel:* cross-section of a portion of Tibialis Anterior muscle immuno-stained with antibodies against Pax7 (red), one of the most widely used marker for adult satellite cells, and Laminin (green), a component of the myofibers basal lamina. *Right panel*: Longitudinal view of a portion of a single myofibers isolated from Extensor Digitorum Longus (EDL) muscle and immuno-stained for Pax7 (red). Nuclei are stained with Hoechst (blue). **C,** Schematic representation of a cross section (*left panel*) and a longitudinal view (*right panel*) of a myofibers with satellite cells in their niche (pink) and myonuclei (blue). The basal lamina is represented as an external green thick layer, and the sarcoplasm in light green, delimited by the sarcolemma, visible as a thin light green line.

cells soon became good candidates for the solution of what Mauro defined “the vexing problem of skeletal muscle regeneration”. Indeed, it was already well known that skeletal muscle regenerates after injury, but the cellular origin of new adult myofibers was still obscure.

The first evidence that satellite cells, and not myonuclei, participate to myofiber regeneration came from *ex vivo* experiments in which single myofibers were isolated from adult rats and cultured for several days. In this setup, continuous observation of satellite cells associated to the isolated fibers revealed that 22 hours after isolation, they start to divide synchronously and give rise to a progeny that after 5 days in culture fuse and form multinucleated myotubes within the basement membrane of the isolated fiber (Bischoff, 1975). Further *in vivo* experiments based on radioactive labeling and cell tracing during skeletal muscle regeneration confirmed that satellite cells are activated upon injury and generate muscle progenitors that build new myofibers (Snow, 1977).

Molecular markers

After their anatomical identification, satellite cells have been widely studied in order to unravel their molecular features. During the last decades, a number of surface molecules and transcription factors have been found to be specifically expressed by satellite cells and are represented in Figure 12.

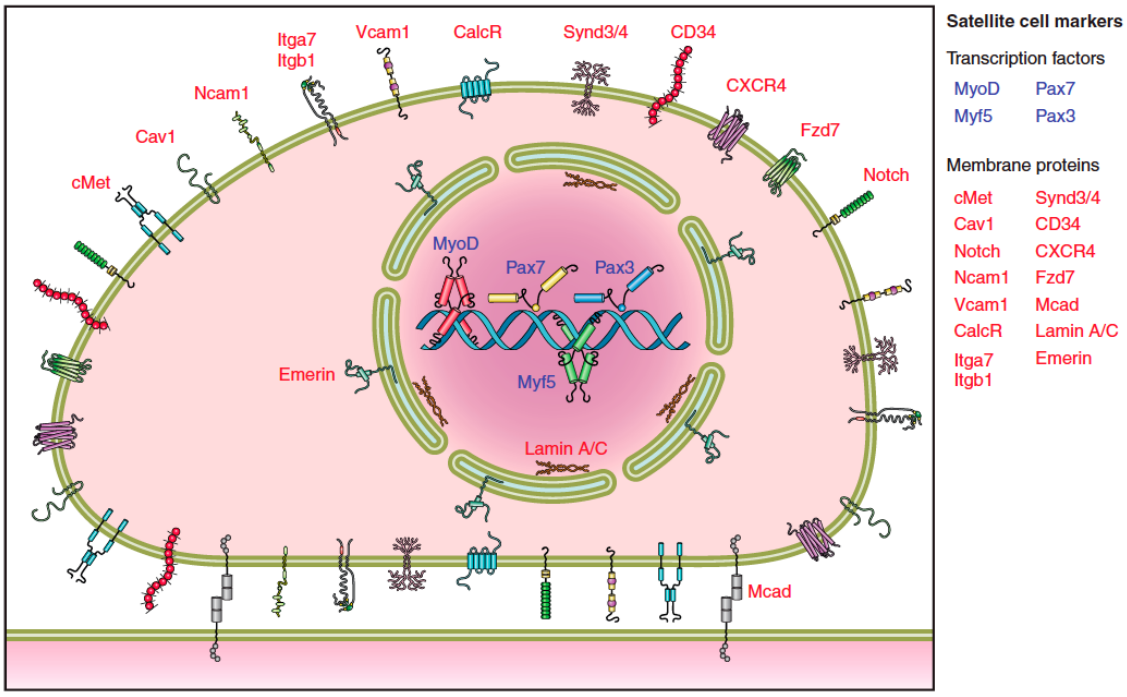


Figure 12. Satellite cells markers. Whereas some of these markers are expressed both by quiescent and activated adult satellite cells (such as Pax7 or Syndecan3/4), others are specific of a particular status. From Yin et al., 2013.

Pax7 is considered as the best satellite cell marker because in the adult it is specifically expressed by both quiescent and activated satellite cells in many species.

Satellite cells specifically express also two transmembrane heparan sulfate proteoglycans, Syndecan-3 and Syndecan-4, which act as coreceptors facilitating the transduction of several signaling pathways (Cornelison et al., 2001).

Other frequently used markers are the membrane proteins α7-integrin, M-cadherin, CD34, although they are expressed also by other cell types, thus they are used in combination with anatomical or other molecular information (reviewed in Yin et al., 2013).

Recent transcriptomic comparison of quiescent and activated satellite cells identified the calcitonin receptor (CTR) as a marker for quiescent satellite cells and suggested that calcitonin signaling (thus indirectly calcium levels) maintains satellite cell quiescence by negatively regulating cell cycle entry (Fukada et al., 2007).

The identification of molecular markers allowed to further characterize satellite cells and to answer, at least partially, to several questions that arose after their discovery: what is the origin of satellite cells? What are the molecular mechanisms and the signals underlying satellite cells quiescence, activation, differentiation and self-renewal? What is the relationship between the satellite cell and its surrounding environment?

Embryonic origin of satellite cells

The idea that satellite cells are myoblasts that have not fused during embryonic myogenesis and remained as mononucleated “dormant” cells was one of the first hypotheses proposed after their anatomical identification to explain their origin. Chick-quail chimeric somite transplantations provided the first experimental evidence that indeed satellite cells derive from the somite (Armand et al., 1983). Analysis of the molecular features of adult satellite cells led to determine that they are characterized by the expression of Pax7, and that *Pax7* null mice display a complete absence of satellite cells (Seale et al., 2000). The identification of a Pax3/Pax7 expressing population of proliferating myogenic progenitors that continuously arise from the dermomyotome during embryonic myogenesis further strengthened the hypothesis of the somitic origin of satellite cells (see the paragraph *Primary and secondary myogenesis*). The final demonstration came form lineage tracing experiments, in which no satellite cells were generated when the embryonic *Pax7* lineage was ablated by

using the conditional expression of the diphtheria toxin (Hutcheson et al., 2009). However, what instructs a proportion of the myogenic progenitors to become satellite cells instead of differentiating and become myofibers still remains mysterious.

Transcriptional control of adult myogenesis

Upon myofiber damage, satellite cells leave their quiescent state and start to proliferate to give rise to a progeny of transient amplifying myoblasts. These cells are myogenic progenitors that undergo a transcriptional cascade that results in their differentiation and fusion between them to form new myofibers or with the remaining damaged myofibers to repair them (Figure 13). The transcriptional events largely recapitulate embryonic myogenesis, with only minor differences.

Quiescent satellite cells express Pax7 (Figure 13). Although for a first study concluded that Pax7 is dispensable in adult satellite cells (Lepper et al., 2009), 2 more recent reports unequivocally demonstrated that conditional *Pax7* gene inactivation altered

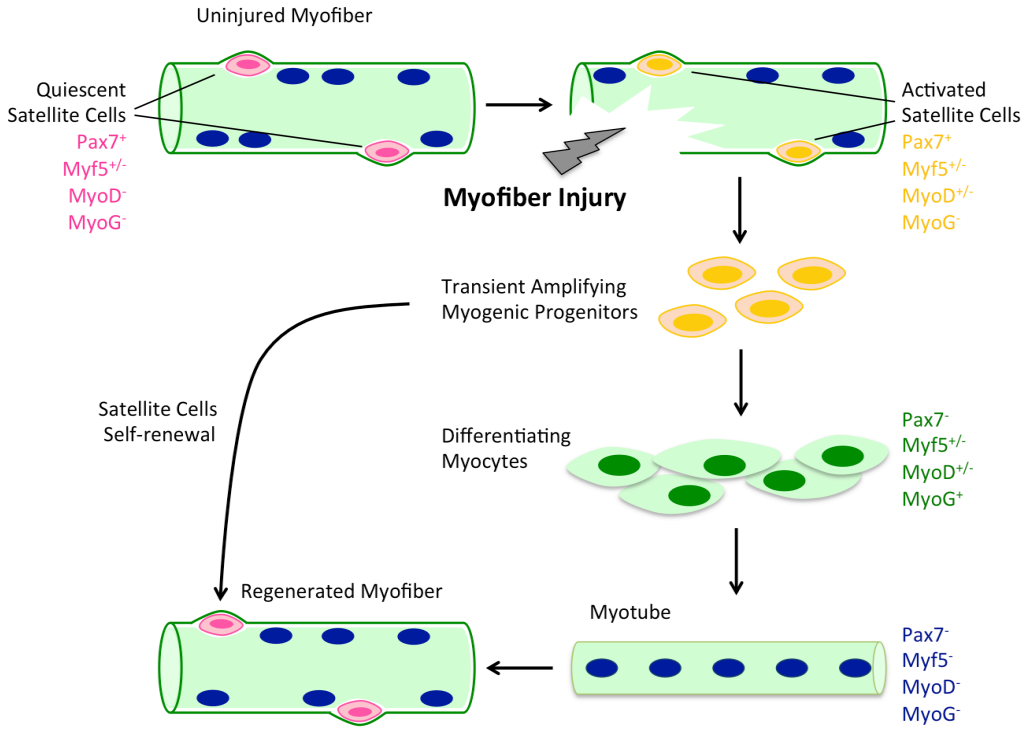


Figure 13. Schematic representation of skeletal muscle regeneration. Skeletal muscle regeneration is orchestrated by a genetic cascade that involves Pax7 and the MRFs and drives each step of satellite cell activation, generation of transient amplifying progenitors, differentiation and formation of new myofibers. Satellite cell self-renewal replenishes the stem cell pool of the tissue after regeneration

the molecular signature of satellite cells in intact muscle and dramatically perturbed injury-induced muscle regeneration (Günther et al., 2013; von Maltzahn et al., 2013). Combined analysis of gene expression and of genome-wide of Pax7 binding-sites further supports the role of Pax7 in adult myogenesis, where it regulates a panel of genes responsible for proliferation and inhibition of differentiation (Soleimani et al., 2012a).

Among Pax7 target genes in adult satellite cells, *Myf5* is one of the most important. Indeed, Pax7 binds the *Myf5* promoter and analysis of the *Myf5-LacZ* reporter mouse line showed the *Myf5* locus is transcriptionally active in adult quiescent satellite cells (Beauchamp et al., 2000). However, whether Myf5 protein is present in quiescent satellite cells is still under debate. Satellite cell activation correlates with *Myf5* and *MyoD* upregulation (Cooper et al., 1999). Myf5 is expressed before MyoD and is necessary for myoblasts proliferation, whereas MyoD seems to drive cells toward differentiation. Therefore, it has been proposed that the balance between Myf5 and MyoD determines myoblasts behavior (see Yin et al., 2013).

Immediately after injury, transient amplifying progenitors expand in number (Figure 13). During this phase, precocious differentiation is prevented by a repression of *Myog* transcription. Indeed, MyoD recruits a histone lysine methyltransferase to the *Myog* promoter, which establishes repressive epigenetic marks thereby inhibiting transcription (Gillespie et al., 2009). Formation of this repressive MyoD complex is induced by p38-γ/MAPK signaling, which mediates MyoD phosphorylation, thereby enhancing its affinity for the methyltransferase KMT1A. Interestingly, it has been shown that p38-γ pathway can be activated by TNF-α, which is known to be secreted during skeletal muscle injury (Palacios et al., 2010). p38-mediated phosphorylation also regulates the activity of Mef2, a key MyoD cofactor that potentiate MyoD transcriptional activity and participates to a feed-forward circuit that governs the transcriptional cascade responsible for myogenic differentiation (see Tapscott, 2005). Similarly to myogenic progression during muscle development, MyoD induces *Myog* expression and this event correlates with irreversible cell cycle exit and the activation of terminal differentiation (Grounds et al., 1992).

Six1, a key regulator of embryonic myogenesis (see page 11), plays an important role in skeletal muscle regeneration since it regulates several aspects of adult myogenesis. During satellite cells activation and myoblasts differentiation Six1 directly activates expression of *MyoD* and *Myog*, respectively (Le Grand et al., 2012). Moreover, Six1 regulates satellite cell self-renewal by tuning ERK signaling (see below, “Satellite cell self-renewal).

Beyond transcription: miRNA-mediated control of myogenesis

The genetic cascades described above cannot fully explain how few transcription factors can direct the complex myogenic differentiation in a precise and fine spatio-temporal manner. Among the other levels of regulation of myogenesis, microRNAs play a crucial role. Conditional knockout of *Dicer* (the enzyme responsible for microRNA generation) in the myogenic lineage results in severe and diffuse muscle hypoplasia and perinatal lethality (O'Rourke et al., 2007), and in the last decade a large number of microRNAs have been implicated in several aspects of myogenesis.

The microRNA families miR-1/206 and miR133a/133b are exclusively present in skeletal muscle and therefore they are referred to as **myomiR**. In skeletal muscle, microRNAs are regulated by MRFs and in turn modulate the expression of MRFs, thereby building complex regulatory networks. An example is miR-206, which is directly regulated by MyoD and targets *Pax7* in activated satellite cells to allow their myogenic progression (Liu et al., 2012a). Moreover, miR-206 and miR-30a are induced by MyoD and Myogenin and inhibit *Snail* expression, promoting the activation of MyoD target genes involved in myogenic differentiation (Soleimani et al., 2012b). For a more detailed description of microRNAs in myogenesis see Williams et al., 2009.

Satellite cells heterogeneity

For a long time, satellite cells have been considered a homogeneous population of muscle stem cells. Careful molecular characterization revealed a certain level of heterogeneity in terms of expression of satellite cells specific markers and in the degree of commitment.

Regarding their molecular features, one of the most important differentially expressed genes is *Myf5*. Lineage tracing experiments used *Myf5-Cre;Rosa-YFP* mice, in which whenever *Myf5* locus is transcribed, the Cre recombinase irreversibly activates the expression of YFP, thereby labeling cells that experienced *Myf5* expression at some moment of their developmental history. By this approach two subpopulations of satellite cells have been identified in intact adult muscle: a more committed subgroup that express both Pax7 and YFP, and a more stem subgroup that represents only 10% of total satellite cells and expresses only Pax7 but not YFP, therefore it has never expressed Myf5 (Kuang et al., 2007). Interestingly, transplantation of FACS-isolated $Pax7^{+}/YFP^{-}$ cells into satellite cells-devoid muscles efficiently supported their regeneration, as demonstrated by the presence of YFP positive fibers and of Pax7

expressing cells located in the typical satellite cell sublaminar position. Conversely, $Pax7^{+}/YFP^{+}$ were only able to differentiate and form new myofibers, but were inefficient in generating satellite cells. Furthermore, upon division $Pax7^{+}/YFP^{-}$ satellite cells can give rise to both $Pax7^{+}/YFP^{-}$ and $Pax7^{+}/YFP^{+}$, whereas $Pax7^{+}/YFP^{+}$ only generate $Pax7^{+}/YFP^{+}$, suggesting that $Pax7^{+}/YFP^{-}$ have a broader lineage potential. These data demonstrate that uniquely $Pax7^{+}/YFP^{-}$ satellite cells can truly be defined stem cells.

In several tissues it has been shown that during asymmetric stem cell division, the daughter cell that retains stem properties inherits older template DNA strands, whereas the daughter cell that differentiates receives newly synthetized DNA strands (see Yennek and Tajbakhsh, 2013). This mechanism is referred to as **non-random template DNA segregation** and it is thought to prevent accumulation of genetic mutations in stem cells and preserve their genome integrity. Interestingly, two studies reported that only a subpopulation of satellite cells performs non-random template DNA segregation during mitosis (Shinin et al., 2006; Conboy et al., 2007). Recently, this feature has been correlated to Pax7 expression level. Indeed, within quiescent satellite cells two subpopulations have been identified by cytometric analysis, and it has been proposed that high levels of Pax7 ($Pax7^{Hi}$) correspond to a less committed state (Rocheteau et al., 2012). When $Pax7^{Hi}$ cells divide, they display a higher frequency of asymmetric template DNA segregation compared to cells expressing lower levels of Pax7 ($Pax7^{Lo}$). However, the relevance of these observations is questionable, since the two subpopulations seem to have comparable efficiency in participating to regeneration and satellite cell repopulation when transplanted into injured muscles. Moreover, it is not clear how $Pax7^{Hi}$ satellite cells could represent a less committed state since the authors report that they express higher levels of *Myf5*, the key commitment gene.

Satellite cells are heterogeneous also regarding their cell cycle progression. An early examination of satellite cell-mediated postnatal muscle growth showed that 80% of satellite cells have a fast cell cycle progression, whereas the remaining 20% divide more slowly and are referred to as “reserve cells” (Schultz, 1996). More recently, FACS isolation of fast- and slow-cycling satellite cells from adult muscle followed by analysis of their myogenic and self-renewal potential revealed that only the slow-dividing subpopulation can give rise to a progeny with long-term self-renewal ability *in vitro* (Ono et al., 2012). Furthermore, slow-dividing but not fast-dividing satellite cells efficiently contributed to regeneration when transplanted into injured muscles. Transcriptomic analysis revealed that slow-cycling satellite cells are marked by the specific expression of Id1, a Notch target gene that negatively regulates bHLH factors

activity, and other genes (Ono et al., 2012). A very recent study showed that slow-dividing satellite cells express higher levels of p27 (Cdkn1b), which is required for their long-term maintenance *in vivo* (Chakkalakal et al., 2014).

The immediate satellite cells niche

Somatic stem cells of adult tissues reside in a particular microenvironment called niche. The niche plays a pivotal role in the preservation of stem cell features, namely their self-renewal ability, tissue specificity and pluripotency. To this end, the niche provides signaling molecules and physical cues that, in the case of satellite cells, prevent improper quiescence withdrawal or differentiation in homeostatic conditions.

The observation that satellite cells are localized between the basal lamina and the sarcolemma led to hypothesize that these two elements are the major components of their niche.

The basal lamina

The basal lamina is a layer of extracellular matrix that envelops the myofibers and is connected to the sarcolemma and the myofiber cytoskeleton through the dystrophin-glycoprotein complex (see Michele and Campbell, 2003). The basal lamina is composed by a sheet of laminin (namely the α2β1γ1 isoform), secreted by the myofibers and connected to a collagen IV network, secreted by muscle fibroblasts (see Gillies and Lieber, 2011). A further layer of extracellular matrix, called interstitial matrix, fills the space between myofibers and is composed of collagens (mainly collagen VI secreted by muscle fibroblasts) and proteoglycans (Figure 14). In addition to physical support, elasticity and mechanical force transduction during muscle contraction, the basal lamina and interstitial matrix provide a reservoir of signaling molecules ready to be released upon muscle injury and promote satellite cell activation (see below, *Extrinsic control of adult myogenesis*).

Satellite cells adhere to Laminin through α7/β1 integrin, which is expressed by quiescent and activated satellite cells. Integrins transduce extracellular mechanical signals into intracellular transcriptional and post-transcriptional cascades. The role of α7/β1 integrin in quiescence remains elusive, but it has been shown to be important in activate satellite cell migration (Siegel et al., 2009). However, this evidence is based on *in vitro* analyses and their relevance to the *in vivo* situation has not been reported yet.

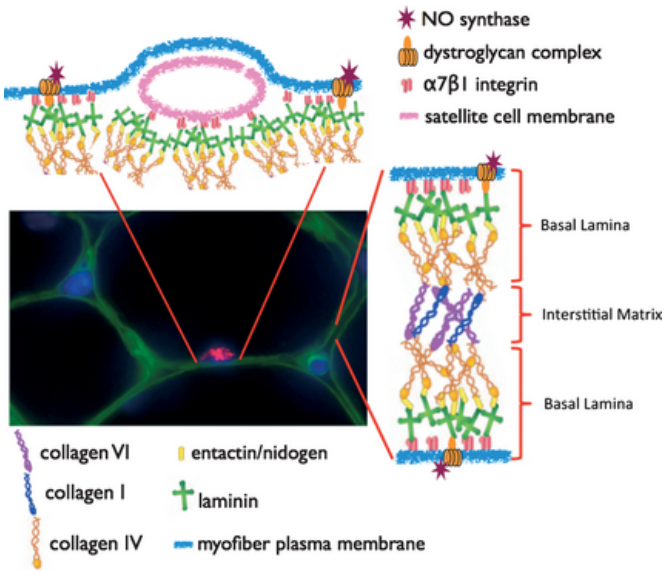


Figure 14. Satellite cell interaction with the myofiber. A satellite cell (identified by immunofluorescent staining for Pax7) under the basal lamina (marked by Laminin immunofluorescent staining, in green) on a muscle cryosection. The schematic drawing illustrates the components of the extracellular matrix within the satellite cell niche. From Lund and Cornelison, 2013.

Although the main sources of ECM components are interstitial cells and myofibers, recently it has been shown that upon activation, satellite cells participate to the remodeling of their niche by secreting the glycoprotein Fibronectin, which facilitates Wnt7 binding to its receptor Frizzled7 and therefore symmetric satellite stem cells expansion (Bentzinger et al., 2013, see below, "Satellite cell self-renewal").

The sarcolemma

The sarcolemma, the fiber plasma membrane, chemically and electrically isolates satellite cells from the myofibers cytoplasm (the sarcoplasm). An early study demonstrated that the contact of satellite cells with the sarcolemma of intact myofibers reduces the mitogenic response of satellite cells, pointing out a role for sarcolemma integrity in maintenance of satellite cells quiescence (Bischoff, 1990). Further analysis of the sarcolemma revealed that it hosts the nitric oxide synthase (NOS), which is anchored to the dystrophin-glycoprotein complex and that this enzyme acts as a mechanosensor: rapidly after myofiber stretching, it produces and releases nitric oxide (NO), which is in turn contributes to satellite cell activation. Indeed NO activates matrix metalloproteinases (MMPs) that mediate the release of HGF, a key event for satellite cell activation (Wozniak and Anderson, 2007. See below, “Satellite cells activation and myogenic progression”).

Interaction between satellite cell and its microenvironment

Interstitial cells

The majority of interstitial cells in skeletal muscle are Tcf4-expressing (Tcf4+) **connective tissue fibroblasts**. Their best-characterized function is to secrete extracellular matrix components that constitute the basal lamina and interstitial matrix. Adult myogenesis is accompanied by a transient increase in Tcf4+ fibroblasts, and conditional cell ablation experiments showed that they are required for proper muscle regeneration (Murphy et al., 2011). Indeed, they support myoblasts proliferation and inhibit their precocious differentiation. Reciprocally, proliferating myoblasts support initial expansion of Tcf4+ fibroblasts, whereas in later phases of regeneration the number of fibroblasts appears to be negatively regulated by differentiating myocytes. Therefore, the dynamic interplay between satellite cells and connective tissue fibroblast is crucial for proper injury-induced adult myogenesis.

Another abundant interstitial population is represented by the so-called **fibro/adipogenic progenitors** (**FAPs**). They have been identified as Sca-1 expressing progenitors and they demonstrated a dual fibrogenic and adipogenic potential *in vitro* (Joe et al., 2010). FAPs number increased after muscle injury and *in vitro* co-culture of FACS-sorted satellite cells and FAPSs enhanced myogenic differentiation. This led to the conclusion that during muscle regeneration the expansion of FAPs supports adult myogenesis. FAPs and Tcf4-expressing fibroblasts are very similar in terms of anatomical position, gene expression and functions during muscle regeneration. Whether these are overlapping populations remains yet to be elucidated. Ongoing lineage tracing experiments and molecular transcriptomic studies should soon formally answer this question.

Blood vessels

In intact muscle, satellite cells are non-randomly distributed throughout the tissue. Indeed, they display preferential localization in proximity to capillaries (Christov et al., 2007). This distribution has functional purposes: blood vessels are the source of oxygen and nutrients, and endothelial cells have been shown to secrete growth factors (IGF-1, HGF, bFGF, PDGF-BB, and VEGF) that support satellite cell proliferation and survival. Moreover, the interaction of satellite cells with endothelial cells is thought to be important for their self-renewal (see below, “Satellite cells self-renewal”).

Immune cells

Although in healthy muscle only a small number of immune cells are present, few hours after acute skeletal muscle injury, circulating immune cells are recruited to the tissue from still unknown signals (McLennan, 1996). Experimental reduction of the number of immune cells during skeletal muscle injury impairs proper tissue repair, demonstrating their requirement for the process (see Saclier et al., 2013).

Early studies using co-cultures of macrophages and myoblasts as well as macrophage-conditioned medium on myoblasts increased both muscle cell proliferation and differentiation (Cantini et al., 1994). Further molecular *in vivo* characterization revealed that **macrophages** play several distinct roles during muscle regeneration. In the first phase, they remove by phagocytosis the necrotic myofibers and in parallel they stimulate satellite cells activation and proliferation. As regeneration proceeds, macrophages undergo a pro- to anti-inflammatory switch that changes their behavior and the set of cytokines that they secrete and favor differentiation and fusion of myogenic progenitors (Deng et al., 2012; Saclier et al., 2013b). In parallel, activated satellite cells have been reported to secrete interleukins and TNF-α, which attract macrophages thereby promoting their infiltration in the first hours after injury (Chazaud et al., 2003). Besides secreted factors, another important element in macrophage-satellite cell interaction is cell-cell contact, which protects satellite cells from apoptosis after muscle injury (Sonnet et al., 2006).

Extrinsic control of adult myogenesis

Signaling molecules secreted by the immediate niche and by the satellite cell microenvironment regulate each phase of adult myogenesis. The interaction between secreted molecules and their receptors at the surface of satellite cells ultimately results in the regulation of the transcription of genes involved in proliferation, migration and differentiation. This orchestrates the first transient expansion of myogenic progenitors, the following myogenic differentiation and concomitant self-renewal of the stem cell pool. To be successful, skeletal muscle regeneration requires the coordination between these phases. Therefore, extrinsic signals cooperate and cross-regulate each other to ensure the correct spatio-temporal balance between the satellite cell proliferation, differentiation and self-renewal.

Satellite cells quiescence

In uninjured muscle, satellite cells are quiescent (G0 phase) and transcriptionally inactive, as demonstrated by their highly condensed chromatin (Mauro, 1961; Günther et al., 2013). A considerable limitation to the molecular characterization of this quiescent state resides in the fact that removal of satellite cells from their niche activates them.

Two studies demonstrated that **Notch** signaling is responsible for the maintenance of satellite cells quiescence (Bjornson et al., 2012; Mourikis et al., 2012). However, it remains unclear whether Notch signaling exerts its function by directly promoting cell quiescence or rather indirectly by inhibiting differentiation.

More recently, **microRNA-489** has been proposed to control of satellite cell quiescence. mir-489 is transcribed from the CTR locus, a marker of quiescent satellite cells, and it has been postulated to target the Dek proto-oncogene in satellite cells, to prevent entry into the cell cycle. However, the precise molecular mechanisms underlying mir-489 function remain elusive (Cheung et al., 2012).

For a long time, the nature of satellite cell quiescence has been elusive. A very recent study begun to shed light on this aspect demonstrating that two distinct phases within quiescence exist, G_0 and G_{Alert} (Rodgers et al., 2014). Indeed, in a muscle contralateral to an injured muscle satellite cells are in a particular status that slightly differs from the normal widely described G_0 quiescence. In this status, named G_{Alert}, cells display what the authors called an alert phenotype, which is characterized by a small but significant increase in metabolic and transcriptional activity, a more pronounced propensity to enter cell cycle and higher level of mTORC signaling activation. Therefore, G_{Alert} corresponds to a more responsive state in which cells can rapidly react to muscle injury. G_0 and G_{Alert} reversible phases form a quiescence cycle and the transition between the two phases is regulated by HGF/cMet-mediated mTORC pathway activation.

Satellite cells activation and myogenic progression

Satellite cells express a large variety of membrane receptors and it has been shown that several ligands are secreted upon myofibers injury. For instance, **hepatocyte growth factor** (**HGF**) is sequestered within the surrounding extracellular matrix by proteoglycans (Tatsumi and Allen, 2004). Muscle injury triggers the activation of MMPs and other proteases that mediate the diffusion of these factors. HGF binding to its receptor c-Met at satellite cell surface directly promotes satellite cell activation and

proliferation and inhibit myogenic differentiation (Gal-Levi et al., 1998). In addition, it has been reported that *Hgf* transcription is induced in satellite cells after injury, suggesting that HGF can act in both an autocrine and a paracrine manner (Sheehan et al., 2000). Similarly, inactive precursors of **fibroblast growth factors** (**FGFs**) have been found in the extracellular matrix and some FGFs have been implicated in stimulation of myogenic precursors proliferation and inhibition of differentiation (see Yin et al., 2013).

Besides its role in maintenance of satellite cells quiescence, **Notch** signaling is also involved in their activation and in myoblast proliferation. Indeed, the activated form of Notch1 is detected *in vivo*, both on myofibers and satellite cells rapidly after injury, and *in vitro* activation of satellite cells is accompanied by upregulation of *Delta*, the Notch ligand (Conboy and Rando, 2002). Moreover, Notch stimulation increases myoblast proliferation and inhibits differentiation whereas siRNA-mediated downregulation of *Notch* decreases proliferation and overexpression of the Notch inhibitor Numb enhances differentiation. Consistently with this notion, myogenic differentiation requires Notch inhibition, and during satellite cells division, asymmetric distribution of Numb determines which daughter cell upregulates differentiation markers such *Desmin* (Conboy and Rando, 2002).

In vitro studies proposed an interplay between the Notch and the **BMP** pathways (Dahlqvist et al., 2003). In particular, BMP4 and Notch synergize to activate two Notch targets genes, *Hes1* and *Hey1*, and BMP4 stimulation blocks myogenic differentiation in a mechanism that requires functional Notch signaling. Further analysis of BMP signaling revealed that activated satellite cells are responsive to BMP since upon activation they upregulate the BMP receptor BMPR-1A (also known as Alk-3), and this activate the pathway, since concomitant phosphorylation of Smad1/5/8 was reported (Ono et al., 2011). *In vivo* blockade of BMP signaling immediately after skeletal muscle injury results in defective regeneration, however deeper investigation is needed to elucidate the exact role of BMP signaling in adult myogenesis.

Satellite cells self-renewal

The ability of adult stem cells to give rise to daughter cells with identical stem properties is called self-renewal. Transplantation of a single intact myofiber (directly after isolation) into a radiation-ablated muscle endogenously defective for myogenesis led to generation of hundreds of myofibers bearing thousands of myonuclei and over two hundreds associated satellite cells, demonstrating the self-renewal ability of satellite cells (Collins et al., 2005).

An important step in understanding the mechanisms by which satellite cells self-renew was the observation that the stem subpopulation of satellite cells ($Pax7^+/YFP^-$, see paragraph *Satellite cells heterogeneity*) can divide symmetrically, giving rise to two identical daughter stem cells, or asymmetrically, giving rise to one stem cell and one committed ($Pax7^+/YFP^+$) cell (Kuang et al., 2007). Interestingly, the cell division plane correlates with cell fate: planar divisions (where the division plane is parallel to myofibers axis) produce mostly two identical satellite stem cells, whereas apical-basal divisions (with division plane perpendicular to myofibers axis) are asymmetric. Further investigation on asymmetric satellite stem cell division revealed that Wnt7a is secreted during muscle regeneration and it promotes symmetric expansion of the stem subpopulation of satellite cells. Indeed, it regulates the subcellular distribution of the planar cell polarity pathway effector Vangl2, which in turn favors satellite stem cells planar division (Le Grand et al., 2009).

Satellite cells can self-renew also by reverting their myogenic commitment. Indeed, on single myofibers isolated and cultured for several days (a widely used paradigm to study satellite cells activation and myogenic progression), all satellite cells upregulate MyoD after 24hours and start dividing. However, after 72 hours the proportion of $Pax7^+/MyoD^+$ cells decreases and about 23% of satellite cells downregulate MyoD, express only Pax7 and exit cell cycle (Zammit et al., 2004). A recent work proposed that oxygen levels regulate the balance between commitment and self-renewal (Liu et al., 2012c). *In vitro*, hypoxic conditions increase the number of $Pax7^+/MyoD^-$ at the expense of the $Pax7^+/MyoD^+$ fraction, suggesting that low levels of oxygen favor satellite cell self-renewal.

The notion that committed satellite cells can return to a more stem state has been confirmed also *in vivo* during injury-induced adult myogenesis, where it has been reported that ERK signaling is active in proliferating myoblasts, and Sprouty 1, an ERK inhibitor, is expressed by Pax7 quiescent satellite cells, downregulated upon activation and re-expressed by Pax7 positive cells that withdraw cell cycle to re-enter quiescence. Therefore it has been concluded that Sprouty 1-mediated inhibition of ERK signaling is responsible for satellite cell return to quiescence (Shea et al., 2010). ERK signaling has been implicated in satellite cell self-renewal also in a study that investigated the cross-talk between myoblasts and their microenvironment during adult myogenesis. The authors reported that ERK signaling is activated by the interaction of Ang1, secreted by smooth muscle cells and by satellite cells themselves, with its receptor Tie2 on quiescent satellite cells and it decreases proliferation and differentiation, thereby promoting return to quiescence of a subset of transient amplifying myoblasts (Abou-Khalil et al., 2009). Interestingly, ERK activation in satellite cells has been reported to

be controlled by Six1, which directly regulates the expression of a phosphatase that dampens ERK activation, thus controlling return satellite cell self-renewal by quiescence re-entry (Le Grand et al., 2012).

These studies considered the self-renewal of the entire satellite cell population. Therefore, it will be interesting to investigate how ERK signaling influences only a subpopulation of satellite cells and what distinguishes these cells from the other proliferating myoblasts.

4. Muscle regeneration in pathological context: muscular dystrophies

Muscular dystrophies are inherited degenerative diseases characterized by progressive muscle wasting and weakness, resulting in gradual loss of locomotion ability and respiratory difficulty. In the most severe forms, heart or respiratory failure leads to premature death (Emery, 2002). In many dystrophies, satellite cells cannot compensate for the loss of muscle tissue. The constant myofiber degeneration process ultimately exhausts the stem cell pool leading to replacement of myofibers by fibrotic tissue and loss of contractile properties of muscle. Moreover, disease-related systemic factors have been proposed to diminish the myogenic potential of satellite cells (Trensz et al., 2010; Mann et al., 2011).

The most frequent muscle dystrophy is the Duchenne Muscular Dystrophy (DMD), which affects around 1 in 3,600 boys and is caused by recessive mutations that affect the gene located on the X chromosome that codes for dystrophin. Dystrophin is a large (427 kDa) protein that contributes to the formation of a multiproteic complex, the dystrophin-glycoprotein complex (DGC), that links the myofiber cytoskeleton to the extracellular matrix. This connection is required for force transmission between neighboring myofibers during muscle contraction. In the absence of dystrophin, the DGC is highly unstable, thus contraction force is not properly transmitted throughout the muscle and this increases susceptibility of myofibers to contraction-induced injury. As a result, dystrophic muscle experience continuous cycles of myofibers damage and regeneration, until the satellite cell pool is completely exhausted and the damaged tissue can no longer be repaired. Regeneration failure is accompanied by increasing deposition of fibrotic tissue and progressive loss of contractile ability (see Kharraz et al., 2014).

Although genetic alterations responsible for most of muscular dystrophies have been identified, to date, no therapy successfully blocks dystrophic muscle degeneration. Corticosteroids administration is the only treatment available, but it only delays the pathological progression. Two main strategies have been developed to find a possible therapy for dystrophies: to repair the mutated gene and/or restore its expression (gene therapy), and to replace the mutated gene with an exogenous wild-type version (cell therapy).

Gene therapy

Antisense oligonucleotide-mediated exon-skipping approaches have been developed in order to cure dystrophies in which mutations disrupt the reading frame and produce truncated non-functional proteins. These strategies have been widely investigated for the dystrophin gene (*Dmd*), which is the largest gene in human genome (2.4Mb). In this case, targeting the exon carrying the mutation with antisense oligonucleotides that hide it from the splicing machinery results in an mRNA coding for smaller but still functional protein. Clinical trials have been completed and despite some aspects remain to be optimized, results showed efficient increase in dystrophin expression and improvement of patients conditions (see Benedetti et al., 2013).

Other strategies to correct premature stop codon mutations seek to identify molecules, called read-through compounds, that interfere with ribosome proofreading activity and allow the insertion of an amino acid instead of a premature stop codon (see Berardi et al., 2014). Furthermore, recent advances in endogenous gene repair have been achieved by using endonucleases that can be engineered to target a custom DNA sequence and thus specifically remove the mutated regions producing a double-strand break (DSB) that is repaired by the DNA repair pathways (Rousseau et al., 2011; Ousterout et al., 2013). The major advance in adopting this approach is that it can lead to permanent gene correction, whereas other strategies like exon-skipping act only transiently and therefore imply continuous lifelong treatments.

Cell therapies

Strategies to replace the mutated genes with exogenous versions imply cellular or viral vectors. Due to their remarkable ability to regenerate skeletal muscle, satellite cells have been long considered obvious candidates for cell-based therapies. Pioneering experiments in a mouse model lacking dystrophin, the *mdx* mouse line, were very encouraging since they showed that wild-type myoblasts could be injected into dystrophic muscles and could give rise to dystrophin positive myofibers (Partridge et al., 1989). This strategy was further improved by using satellite cells isolated by FACS and directly injected in dystrophic muscles (Montarras et al., 2005). However, clinical trials were unsuccessful for several reasons (Berardi et al., 2014). First, transplanted cells have been shown to experience high mortality and limited migratory capacity. Systemic delivery of donor cells is not possible because myoblasts cannot cross the vessel wall, whereas intramuscular injections are more efficient but the poor migratory ability of injected myoblasts imposes a huge number of localized injections, which cannot be performed for muscle such diaphragm or heart. Moreover, immune

rejection is another major issue, and autologous strategies using satellite cells isolated from the patient, genetically corrected *in vitro* and re-transplanted are inefficient since *in vitro* culture of satellite cells leads to loss of their myogenic potential. A recent finding, however, could partially solve this problem. Indeed, it has been reported that satellite cell-derived myoblasts cultured on a substrate that mimics muscle elasticity retain their myogenic potential and efficiently contribute to muscle regeneration when transplanted into satellite cell-depleted muscles (Gilbert et al., 2010). In addition, sustained activation of Notch signaling in canine satellite cells *ex vivo* before transplantation into mice was shown to enhance donor muscle cell engraftment (Parker et al., 2012).

Yet, autologous myoblast transplantation may be a safe route to treat muscular dystrophies that impact specific muscle groups. For instance, it has been recently shown that local injection of autologous myoblasts in pharyngeal muscles is a safe and efficient strategy for oculopharyngeal muscular dystrophy (OPMD) patients, in which pharyngeal muscles are selectively involved (Périé et al., 2014).

The identification of other cells with myogenic potential distinct from satellite cells widens therapeutic possibilities. For instance, interstitial cells associated with blood vessels have been identified in mice and dogs and have been reported to have a myogenic potential both *in vitro* and *in vivo* (Sampaolesi et al., 2003, 2006). These cells, called mesoangioblasts, can cross the endothelium, enabling them to be delivered systemically by intra-arterial transplantation. Recently, mesangioblasts were isolated from dystrophic (mdx) mice and genetically corrected by introduction of a human artificial chromosome harboring the entire human dystrophin genetic locus (Tedesco et al., 2011). Autologous systemic delivery resulted in successful engraftment and generation of dystrophin expressing myofibers. In humans, vessel-associated cells called pericytes display share many features with mesoangioblasts and have been shown to generate myofibers when transplanted into dystrophic mice (Dellavalle et al., 2007). Based on all these evidence, mesoangioblasts are currently being used in a phase I/II clinical trial.

Recently, strategies based on induced pluripotent stem cells start to be explored and turned out to be successful (Tedesco et al., 2012). Therefore it is crucial to understand what controls muscle stem cells myogenic potential and long-term self-renewal in order to enhance efficiency of reprogramming and of the maintenance of myogenic stemness.

II. WNT signaling

Wnt proteins are secreted growth factors that bind to specific transmembrane receptors belonging to the **Frizzled** family, and to several co-receptors, including the low-density lipoprotein receptor-related protein (**LRP**), receptor Tyr kinase-like orphan receptor (**ROR**), protein Tyr kinase 7 (**PTK7**), receptor Tyr kinase (**RYK**), muscle skeletal receptor Tyr kinase (**MUSK**) and the **Proteoglycan** families.

Wnt binding to their receptors/co-receptors can induce distinct intracellular molecular cascades that result in modification of gene expression and/or cytoskeletal changes. Depending on the involvement of β-catenin, WNT signaling pathways have been classified as **canonical** (β-catenin-dependent, also called **Wnt/β-catenin**) and **non-canonical** (β-catenin-independent). In turn, non-canonical Wnt signaling comprises the Planar Cell Polarity (**Wnt/PCP**) Pathway and the Calcium-dependent (**Wnt/Ca^{2+}**). Frizzled (Fzd) receptors can transduce both canonical and non-canonical Wnt signaling, but their association with distinct co-receptors dictates which pathway is activated (see van Amerongen et al., 2008).

To date, 19 Wnt ligands and more than 15 receptors and co-receptors have been identified in vertebrates. Moreover, secretory components, extracellular antagonists and post-translational modifications together with the large number of ligands and receptors confer to Wnt signaling tremendous complexity (for details visit the Wnt Homepage: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/).

Wnt proteins are conserved throughout the animal kingdom and are involved in a large variety of biological processes, ranging from body axes specification and organ formation during embryogenesis to adult stem cells homeostasis (Croce and McClay, 2008; Holland et al., 2013). Furthermore, alteration of Wnt signaling can have dramatic pathological consequences: hyper-activation of canonical Wnt signaling is detected in 90% of colorectal cancers and has been demonstrated to be a causal event, and a number of mutations in Wnt pathways components have been associated to several pathologies (Clevers and Nusse, 2012; Chien et al., 2009).

1. Extracellular regulation of Wnt signaling

Wnt ligands

The first component of Wnt signaling to be identified was *Wnt1*, which was originally named Integration-1 (*Int-1*) since it was discovered as a preferential integration site for the Mouse Mammary Tumor Virus in virally induced breast tumors (Nusse and Varmus, 1982). Few years after the discovery of *Int-1*, the homology with the *Wingless* (wg) *Drosophila* gene was demonstrated, thus the *Int-1* gene was renamed *Wnt1* as a fusion of wg and *Int-1* (Rijsewijk et al., 1987). Since then, 19 different Wnt ligands subdivided in 12 subfamilies have been identified and are well conserved among Metazoa.

One of the most complex features of Wnt signaling is the fact that the same Wnt ligand can bind to different receptors and activate distinct downstream pathways. As a rough approximation, Wnt1, Wnt3a and Wnt8 have been implicated mainly in activation of canonical Wnt signaling, whereas Wnt5a and Wnt11 most frequently activate Wnt-independent pathways (Kikuchi et al., 2011). However, context-specificity has to be considered and ongoing research attempts to shed light on this aspect.

WNT secretion

Wnt proteins are cysteine-rich glycoproteins that are processed and modified by addition of carbohydrate and lipid chains in the endoplasmic reticulum, and further secreted through vesicles emanating from the Golgi network. Although several aspects of Wnt secretion have been clarified, some details still remain to be elucidated.

Intracellular processing

Lipid modifications of Wnt proteins are necessary for proper secretion and efficient binding to their receptors. Purification of Wnt3a and Wnt8 from mouse and *Drosophila* cell lines revealed that a palmitoyl group is covalently bound to a conserved cysteine of both proteins and removal of this post-translational modification leads to inefficient Wnt signaling downstream transduction (Willert et al., 2003). Further determination of the structure of Wnt8/Frizzled8 complex demonstrated that the palmitoleic lipid participates to the binding of the ligand to its receptor (Janda et al., 2012).

The enzyme responsible for Wnt palmitoylation is Porcupin, a transmembrane O-acyltransferase present within the endoplasmic reticulum membrane that was identified in *Drosophila* and is conserved in vertebrates (Kadowaki et al., 1996; Hofmann, 2000). Depending on the Wnt ligands and the species considered, different cysteine and serine residues have been reported to undergo palmitoylation and some aspects still remain controversial (Takada et al., 2006; Galli et al., 2007; Franch-Marro et al., 2008; Gao and Hannoush, 2014). However, it is well established that Porcupin-mediated Wnt palmitoylation is absolutely required for Wnt secretion, since inhibition of this post-translational modification results in Wnt retention in the endoplasmic reticulum (Takada et al., 2006; Franch-Marro et al., 2008; Gao and Hannoush, 2014).

Palmitoylated Wnt is then transferred to the Golgi network through the action of proteins belonging to the p24 family (Buechling et al., 2011). Within the Golgi network, Wnt interacts through the palmitoyl group with Wntless (WIs), a transmembrane protein that seems to mediate the sorting of Wnt-containing vesicles to the plasma membrane, although the exact mechanism by which WIs acts remains unclear (see Clevers and Nusse, 2012).

Wnt secretion and gradient formation

Due to their lipid modifications, Wnt proteins are highly hydrophobic and have high affinity for the phospholipid bilayers of cell membranes and tend to attach to the cell surface. Therefore, Wnt ligands display low mobility within the extracellular space and their secretion implies mechanisms that limit the interaction between Wnt and the cell membrane to enhance their diffusion. A mechanism responsible for Wnt extracellular diffusion is the transport within lipoprotein particles or other membranous vesicles such exosomes, reported in *Drosophila* as well as in human cells (Panáková et al., 2005; Neumann et al., 2009; Gross et al., 2012).

Although several molecular mechanisms have been proposed to allow the generation of Wnt long-range gradients, this phenomenon has been demonstrated to occur only within the wing imaginal disc of *Drosophila* embryos, where the carrier protein Secreted Wingless-interacting molecule (Swim), a member of the Lipocalin family, interacts with Wnt and masks the palmitate group, thereby increasing Wnt solubility and mobility (Mulligan et al., 2012). Similarly, components of the extracellular matrix have also been implicated in Wnt gradient formation within the wing imaginal disc (Glise et al., 2005; Gorfinkiel et al., 2005). However, in the large majority of tissues Wnt signaling is thought to act in an autocrine or paracrine manner due to its low mobility and high hydrophobicity.

Extracellular regulators of Wnt signaling

Fine-tuning of Wnt signaling is achieved by a combination of multiple levels of regulation, including extracellular regulation of Wnt receptors by secreted and membrane agonists and antagonists, transcriptional regulation through feedback loops, intracellular control of signal transduction. Redundancy and complexity of the mechanisms controlling Wnt signaling allow an extremely precise modulation of the distinct Wnt pathways, a crucial aspect if we consider the number of cellular processes regulated by Wnt signals or the dramatic consequences of Wnt signaling alterations.

A great contribution to this fine and complex regulation comes from secreted factors. One of the best-characterized families of Wnt secreted inhibitors is the **Dickkopf** (**DKK**) family. Out of the four vertebrate DKK proteins (DKK1-4), DKK3 diverges in terms of protein similarity and is the only member that does not affect Wnt signaling (Krupnik et al., 1999; Mao and Niehrs, 2003).

Another family of Wnt inhibitor is the **secreted frizzled-related proteins** (**sFRPs**) family, which in mammals comprises 5 members (see Bovolenta et al., 2008). Unlike DKKs, which inhibit uniquely β-catenin-dependent Wnt signaling, sFRPs can regulate both Wnt/β-cat and Wnt/PCP pathway, as demonstrated by gene inactivation strategies in *Xenopus* and mouse (Li et al., 2008; Satoh et al., 2008; Matsuyama et al., 2009; Gibb et al., 2013).

Finally, a third secreted Wnt inhibitor is **Wnt-inhibitory factor 1** (**WIF1**). Although its mechanism of action is still poorly understood, some evidence suggests that it can influence both canonical and non-canonical Wnt signaling.

Regarding secreted Wnt agonists, the best-characterized factors belong to the **R-spondin** family. In mammals, the 4 R-spondin proteins have been first implicated in amplification of canonical Wnt signaling (Kazanskaya et al., 2004; Kim et al., 2008) and more recently also of Wnt/PCP pathway (Jin and Yoon, 2012; see also Ohkawara et al., 2011).

An increasing number of studies report alterations in the expression of these factors in several diseases. For instance, DKKs and sFRPs have been proposed to act as tumor suppressors, and R-spondins control many developmental and physiological processes, highlighting the importance of this level of Wnt signaling regulation (Cruciat and Niehrs, 2013).

All these secreted factors act at the extracellular level by controlling the interaction between Wnt ligands and their receptors in several manners, which are described hereafter.

Inhibition by competition for receptor binding

In this mode of action, which has been reported at least for DKKs and sFRPs, the interaction between the inhibitor and Wnt receptors or co-receptors prevents Wnt binding and therefore signal transduction is blocked (Figure 15). For instance, it has been reported both *in vitro* and *in vivo* (in *Xenopus* embryos) that DKK1 and DKK2 bind to LRP5 and LRP6 with high affinity, thereby impairing LRPs interaction with Frizzled (Semënov et al., 2001; Bafico et al., 2001; Li et al., 2002). Receptor heterodimers are therefore unavailable for Wnt ligands and signal transduction is inhibited.

In addition to Wnt sequestration (see next paragraph), sFRPs have been proposed to abolish Frizzled/LRP interaction by binding to Frizzled through a cysteine-rich domain that displays high sequence similarity with Frizzled CRD (Rattner et al., 1997; Bafico et al., 1999). However, sFRPs/Frizzled interaction has been reported *in vitro* by overexpression experiments, therefore its physiological role remains controversial, since it has also been proposed that sFRP1/Frizzled2 interaction could activate non-canonical Wnt signaling (Rodriguez et al., 2005; Bovolenta et al., 2008).

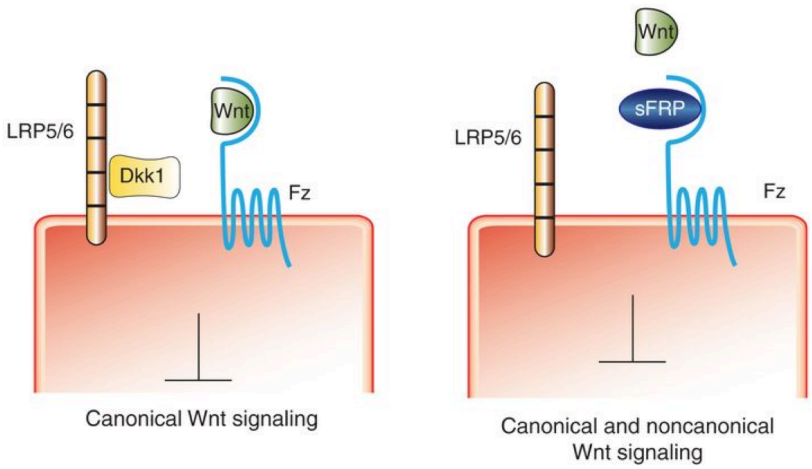


Figure 15. Wnt signaling inhibition by competition for receptor binding. Schematic representation of the mode of action of DKK and sFRP, which bind to Frizzled or LRP thereby inhibiting Wnt binding. Adapted from Cruciat and Niehrs, 2013.

Inhibition by ligand sequestration

As shown in Figure 16, sFRPs negatively regulate Wnt signaling mainly by binding to Wnt ligands and prevent their interaction with receptors and co-receptors. sFRPs/Wnt interaction involves the CRD domain of sRFPs (Rattner et al., 1997; Bafico et al., 1999). Early analysis of sFRPs in *Xenopus* embryos suggested that the *Xenopus* sFRP ortholog Frzb binds to Wnt8 and inhibits canonical Wnt signaling (Leyns et al., 1997; Wang et al., 1997). However, more recent studies revealed that sFRP/Wnt interaction is tissue-

specific and can vary among species and developmental stages (see Cruciat and Niehrs, 2013).

Similarly, WIF1 was reported to bind and inhibit *Drosophila* Wingless and *Xenopus* Wnt8 (Hsieh et al., 1999). Further experiments in chick embryos revealed that WIF1 can interact with a number of Wnt ligands, including Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt9a and Wnt11, and its interaction with Wnt3a during inhibits canonical Wnt signaling during cartilage development (Surmann-Schmitt et al., 2009).

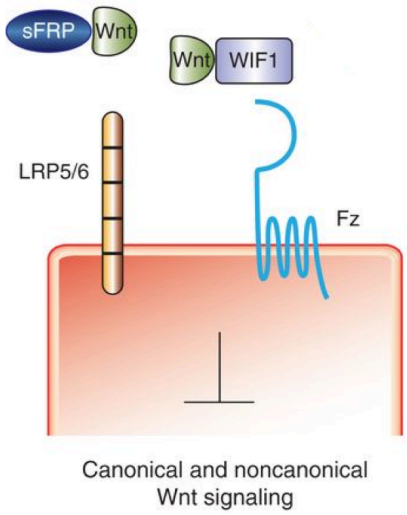


Figure 16. Wnt signaling inhibition by ligand sequestration. WIF and sFRP can interact with Wnt, thereby preventing its binding to Frizzled and LRP. From Cruciat and Niehrs, 2013

Amplification by inhibition of receptor degradation

Recently, two works identified a novel negative feedback loop of Wnt signaling regulation acting at the receptor level in adult mouse intestine and in mouse and *Zebrafish* embryos. Indeed, upon Wnt signaling activation, ZNFR3 and RNF43, two transmembrane ubiquitin ligases, are upregulated and mediate Frizzled targeting for degradation, thereby inhibiting Wnt signal transduction by decreasing Frizzled availability at the cell membrane (Hao et al., 2012; Koo et al., 2012). Interestingly, these two works showed also that R-spondins induce recruitment of ZNFR3 and RNF43 on LGRs (R-spondins receptors) and subsequent internalization of the entire RSPO/LGR/ZNFR3 (or RNF43) complex (Figure 17). Therefore, R-spondins enhance both Wnt/β-cat and Wnt/PCP signaling pathways by inducing clearance of ZNFR3 and RNF43 from the membrane and subsequent stabilization of Frizzled.

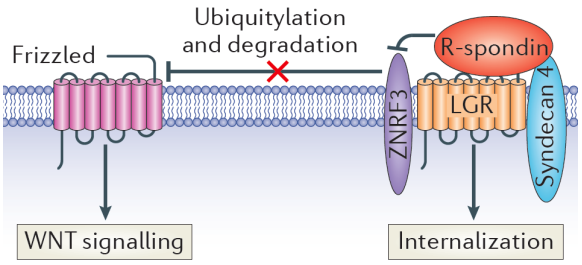


Figure 17. Proposed model for R-spondin/Wnt synergy. R-spondin binding to LGR recruits the ubiquitin ligase ZNRF3, thereby inhibiting Frizzled targeting to proteasomal degradation and increasing its presence at the plasma membrane. In this way Frizzled availability for Wnt binding is increased. From Niehrs, 2012. A more detailed description of the mode of action of the R-spondin/Lgr5/Rnf43 module can be found in de Lau et al., 2014, where an animation of the process is available.

Amplification by induction of endocytosis

Both Wnt/β-cat and Wnt/PCP signaling pathways require endocytosis. Indeed, in β-catenin-dependent signaling transduction, endocytosis of the Wnt/Frizzled complex participates to β-catenin stabilization (see the paragraph *β-catenin destruction complex*), and inhibition of clathrin-mediated endocytosis compromises Wnt/PCP activation although the precise function of this process still remains obscure (see Niehrs, 2012). Recently, it has been reported that *in vitro* stimulation with recombinant Rspo3 results in its interaction with Syndecan 4 and internalization of Rspo3 and Frizzled in clathrin-coated vesicles in *Xenopus* cells (Ohkawara et al., 2011). This led to draw a model in which R-spondins potentiate Wnt/PCP signaling pathway by binding to Syndecans, which in turn mediate clustering with Wnt receptors and further endocytosis. However, this has model needs to be confirmed in other organisms.

Alternative Wnt pathways

As mentioned above, the involvement of β-catenin in the intracellular transduction is used to distinguish canonical and non-canonical Wnt signaling. Based on evidence gathered in different cell types and animal models, three main pathways, Wnt/β-catenin have been defined. However, Wnt signals can induce context-specific intracellular events and most likely we are far from a comprehensive description of all the actors involved in Wnt signal transduction. Whereas a full section of this chapter is dedicated to Wnt/β-catenin pathway (page 50), β-catenin-independent pathways are briefly described hereafter.

The planar cell polarity pathway (Wnt/PCP)

The Wnt/PCP pathway is involved in the establishment of apical-basal cell polarity and it contributes to supply positional information during cell migration and

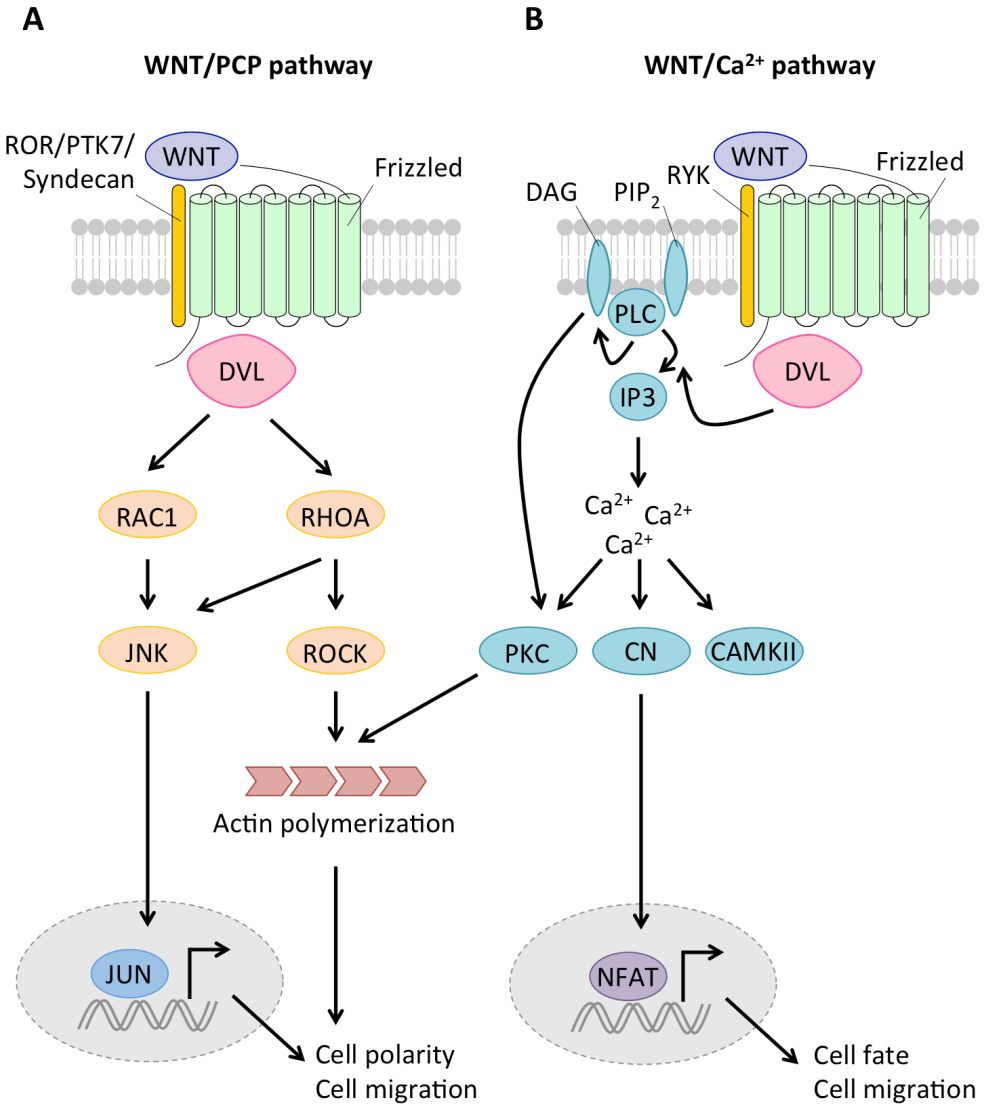


Figure 18. Schematic representation of alternative Wnt signaling pathways. A, The planar cell polarity (Wnt/PCP) pathway. **B,** The Calcium-dependent (Wnt/Ca^{2+}) pathway. Refer to the text for a detailed description of the two pahways.

morphogenetic movement. The WNT/PCP pathway polarizes the cell by inducing differential distribution of proteins and effectors along the apical-basal axis of the cell.

In this signaling pathway, Wnt ligands binding to Frizzled receptors and ROR or PTK7 or Syndecan co-receptors activates small GTPases RAC1 and RHOA, which in turn activate ROCK and JNK, two kinases that trigger a phosphorylation cascade (Figure 18). This ultimately results in actin polymerization and microtubule stabilization driving cytoskeletal changes and/or in activation of gene transcription mediated by transcription factors activated by JNK (Kikuchi et al., 2011).

Wnt/PCP pathway was first identified in *Drosophila*, where a series of mutants displaying disorganization of cuticular structures and cellular hair on wings blade, or misorientation of sensory cells in the compound eye were characterized (Simons and Mlodzik, 2008). Genetic analysis led to identify the genes responsible for these phenotypes and functional characterization defined the mode of transduction of what is today referred to as the Wnt/PCP pathway. Further investigation in vertebrates unraveled the presence of orthologs and of other proteins participating to this type of Wnt-dependent signaling. In vertebrates the Wnt/PCP pathway is involved in a variety of processes, including gastrulation, inner ear formation, cilia development, epithelial organs development (Simons and Mlodzik, 2008). More recently, aberrant Wnt/PCP activation has been correlated to malignant features of tumors such as invasion and metastasis (see Anastas and Moon, 2013).

The calcium-dependent pathway (Wnt/Ca^{2+})

As depicted in Figure 18, in the Wnt/Ca^{2+} pathway, Wnt binding to Frizzled and RYK induces the production of inositol triphosphate (IP3) and diacylglycerol (DAG) by the membrane-associated enzyme Phospholipase C (PLC). Accumulation of IP3 triggers the release of intracellular calcium ($Ca2^{+}$), which in turn activates calcitoneurin (CN), $Ca2^{+}$- and calmodulin-dependent kinase (CAMKII) and protein kinase C (PKC). As a result, the NFAT, CREB and NFkB transcriptional regulators are activated and induce target genes expression (De, 2011).

Although many aspects of this pathway still remain to be elucidated, it has been shown to be involved in early embryogenesis and organogenesis, as well as in some pathological conditions such as neurodegeneration and cancer (seeDe, 2011).

2. Canonical Wnt signaling

β-catenin-dependent Wnt signaling is also called canonical Wnt signaling since it was the first Wnt pathway to be described. After the discovery of Wnt1, epistasis experiments performed mainly in *Drosophila* embryos led to identify progressively all the other components of canonical Wnt signaling pathway and to determine their hierarchy, which was further confirmed for mouse and human homologs (see Nusse and Varmus, 2012).

Canonical Wnt signaling relies on the regulation of cytoplasmic β-catenin protein stability. In unstimulated conditions, β-catenin is retained in a multiprotein complex, named β-catenin destruction complex**,** in which two kinases phosphorylate β-catenin. Phosphorylated β-catenin is further ubiquitinated and subsequently degraded by the proteasome. Upon Wnt binding to their receptors Frizzled and co-receptors LRP6, β-catenin is no longer ubiquitinated and degraded, thus it accumulates within the cytoplasm and translocates into the nucleus, where it associates to TCF and LEF transcription factors to activate transcription of target genes.

Canonical Wnt receptors: Frizzled and LRP6

Canonical Wnt signal transduction is mediated by the formation of a ternary complex comprising a Wnt ligand, a Frizzled receptor and a LRP6 co-receptor.

In human and mouse, the **Frizzled** family comprises 10 members (Frizzled1 to 10) subdivided in 5 subgroups. Frizzled are seven-pass transmembrane receptors with a large extracellular cysteine-rich domain (CRD) that mediates the interaction with Wnt. The first Frizzled was identified in *Drosophila*, where a recessive mutation of the frizzled (fz) gene resulted in misorientation of hair and ommatidia (units of fly compound eye). Early biochemical analyses suggested that frizzled codes for a protein with seven transmembrane domains (Vinson et al., 1989). In search for genes similar to frizzled, another seven-pass transmembrane protein was identified and named Dfz2 (Bhanot et al., 1996). Increased cytoplasmic β-catenin levels were reported upon overexpression of Dfz2, which was also shown to directly interact with Wnt Drosophila homolog Wingless. All these evidence determined that Frizzled proteins are Wnt receptors, and further experiments in mouse and other species led to the identification of all ten 10 mammalian members of the Frizzled family (see MacDonald and He, 2012).

Frizzled receptors can transduce both canonical and non-canonical Wnt signaling, but their association with distinct co-receptors dictates which pathway is activated (see van Amerongen et al., 2008). In the case of β-catenin-dependent Wnt signaling, the specificity is determined by LRP5 and LRP6, two members that constitute a unique subgroup of the Low Density Lipoprotein Receptor (LDLR) family. These two single-pass membrane receptors have an extracellular domain that binds Wnt, and recently multiple Wnt-binding sites have been identified, suggesting that a single molecule of LRP6 could bind simultaneously several Wnts. Indeed, the stoichiometry of the Wnt/Frizzled/LRP complex is to date under investigation, although current models suggest a 1:1:1 ratio (see MacDonald and He, 2012).

Intracellular signal transduction

The conformational changes induced in Frizzled by Wnt binding as well as the molecular details of the first events triggered by Wnt/Frizzled/LRP interaction remain to date poorly understood. However, it seems that the recruitment of the scaffold protein Dishevelled (DVL) by the intracellular domain of LRP and Frizzled is a crucial step in intracellular signal transduction (see Gao and Chen, 2010). One of the roles of Dvl is to stabilize the Frizzled/LRP dimerization (Bilić et al., 2007). Moreover, Dvl recruits GSK3α and CK1γ, two kinases anchored to the plasma membrane, which phosphorylate the cytoplasmic tail of LRP (Zeng et al., 2005, 2008). Dvl recruits also Axin (Cliffe et al., 2003), to the plasma membrane, where it binds to phosphorylated LRP. This event induces the localization of the β-catenin destruction complex to the plasma membrane and promotes further changes within the complex that ultimately result in β-catenin stabilization (see next section).

It is important to mention that Dvl has the ability to interact with a large variety of partners that regulate its activity and that Dvl participate to the transduction of both canonical and non-canonical Wnt signals. Therefore it represents a point of convergence and regulation of several pathways (see Gao and Chen, 2010).

β-catenin destruction complex

As mentioned above, in absence of Wnt ligands, cytoplasmic β-catenin is captured by a destruction complex in which it is continuously phosphorylated and targeted to proteasomal degradation by ubiquitination.

The first component of the β-catenin destruction complex to be identified was **Adenomatous polyposis coli** (APC), a multi-functional protein that is believed to act as scaffold, although its precise molecular role in Wnt signaling remains controversial (see section III. Adenomatous polyposis coli).

The assembly of β-catenin destruction complex is coordinated by **Axin**, a scaffold protein that interacts with all other components of the complex (Behrens et al., 1998; Kishida et al., 1998; Liu et al., 2002). Vertebrates have two Axin genes, Axin1 and Axin2 (also known as Conductin), which are functionally indistinguishable and diverge only for their expression pattern (see Stamos and Weis, 2013). Mutation in Axin1 gene has been detected in hepatocellular carcinoma, where it causes aberrant nuclear accumulation of β-catenin and constitutive transcriptional activation of canonical Wnt target genes (Satoh et al., 2000). Axin acts as a negative regulator of canonical Wnt signaling since it promotes β-catenin degradation. Intriguingly, Axin2 expression is directly induced by Wnt/β-catenin stimulation and this might constitute a negative feedback loop (Lustig et al., 2002).

Two kinases are present within the β-catenin destruction complex: **Casein kinase Iα** (**CK1α**) and **Glycogen synthase kinase 3β** (**GSK3β**). CK1α mediates the first β-catenin phosphorylation on Ser45. This event primes β-catenin for further phosphorylation by GSK3β on Thr41, Ser37, and Ser33 (Liu et al., 2002). Importantly, besides β-catenin phosphorylation, GSK3β-mediated phosphorylation promotes the ubiquitination and subsequent proteasomal degradation of a large variety of proteins (Kim et al., 2009).

Several models have been proposed to explain how β-catenin is stabilized after Wnt binding to Frizzled. First, it has been suggested that β-catenin destruction complex is disassembled rapidly after Wnt stimulation, either by Axin recruitment to the membrane by Dvl (Cliffe et al., 2003), or by disruption of interaction between Axin and GSK3β (Liu et al., 2005b). A large number of studies supported the idea that β-catenin stabilization depends on GSK3 inhibition (see Metcalfe and Bienz, 2011). For instance, *in vitro* evidence favored the hypothesis that GSK3β-mediated β-catenin phosphorylation is impaired directly by phosphorylated LRP6, although this study did not elucidated how this inhibition occurred (Cselenyi et al., 2008). A more recent work suggested that the β-catenin destruction complex is recruited to the cell membrane by association with the LRP and the whole signalosome (Wnt/Frizzled/LRP/β-catenin

destruction complex) is internalized by endocytosis. GSK3 is thereby confined to endosomal vesicles and can no longer phosphorylate β-catenin (Taelman et al., 2010).

Notably, all these models were based on artificial conditions such as overexpression of truncated forms of Wnt components or cell-free in vitro assays. Conversely, a recent detailed biochemical characterization of endogenous dynamics of Wnt signal transduction revealed that the intact β-catenin destruction complex is recruited to the membrane and the first key event for β-catenin stabilization is the displacement of β-TrCP from the complex (Li et al., 2012). This study proposed the model for Wnt signal transduction depicted in Figure 19.

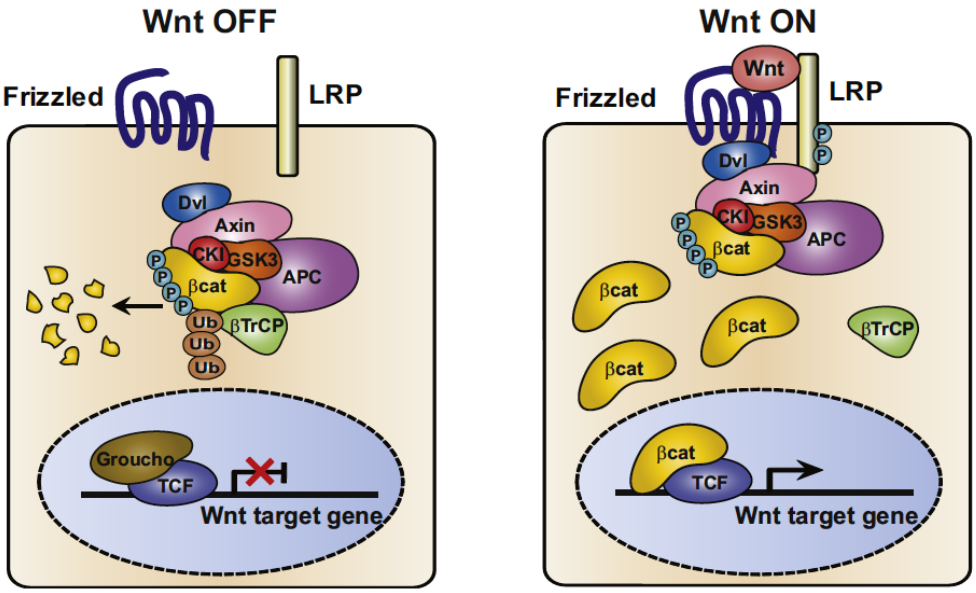


Figure 19. Model for canonical Wnt signaling transduction. In absence of Wnt/Frizzled/LRP interaction (*left panel*), the β-catenin destruction complex targets β-catenin to proteasomal degradation. Upon Wnt stimulation, β-TrCP is displaced from the β-catenin destruction complex, therefore newly synthetized β-catenin is no longer degraded, but it accumulates and enters the nucleus, where it activates TCF/LEF-mediated transcription of target genes.

Wnt-dependent regulation of transcription: the TCF/LEF family

The ultimate output of canonical Wnt signaling activation is the transcriptional regulation of target genes. Although the best known Wnt target genes are tissue specific, Axin2 and Lef1 are considered as general readouts of canonical Wnt signaling activation.

Wnt-dependent regulation of transcription occurs through the TCF/LEF family of transcription factors (TCFs). In vertebrates, this family comprises 4 high-mobility group (HMG) DNA binding proteins: TCF1, LEF1, TCF3 and TCF4. Although they have been discovered in the immune system (TCF stands for T Cell Factor and LEF for Lymphoid Enhancer binding Factor 1), a large number of studies reported their implication in virtually all body tissues (see Archbold et al., 2012).

TCFs bind to specific DNA sequences referred to as Wnt response elements (WREs), present within the regulatory regions of Wnt target genes. The HMG box of TCFs contacts the minor groove of the DNA and causes a strong bending in the helix (approximately 130°). This event has been postulated to bring into proximity chromatin domains otherwise lying at far distance such as transcriptional enhancers (see Mosimann et al., 2009).

TCF/LEF transcriptional switch

When Wnt signaling is inactive, β-catenin is not present in the nucleus and TCFs are bound to the DNA, where they repress transcription through the binding with co-repressors belonging to the Groucho/transducin-like enhancer of split (Gro/TLE) repressor family (see Cadigan and Waterman, 2012). A first model based on evidence gained in *Drosophila* and *C. elegans* suggested that when β-catenin enters into the nucleus, it associates to TCFs and this event displaces their co-repressors, thereby converting TCFs into transcriptional activators (Cavallo et al., 1998; Shetty et al., 2005). However, due to the presence of four distinct TCFs in vertebrates the mechanism seems to be more complicated than in invertebrates, in which only one TCFs ortholog exists (and is called Pangolin in *Drosophila* and Pop1 in *C. elegans*). Indeed, each vertebrate TCF has its own specificity in terms of regulation of target genes transcription. For instance, studies in *Xenopus* embryos suggested that LEF1 and TCF1 are essentially activators, whereas TCF3 and TCF4 displayed repressor functions (Houston et al., 2002; Liu et al., 2005a). Experiments in zebrafish and mouse embryos confirmed the repressor role of TCF3 (Kim et al., 2000; Merrill et al., 2004) and evidence from different tissues and cell types supported the activation role of LEF1 (Kratochwil et al., 2002; Warsito et al., 2012; Schuijers et al., 2014). TCF1 and TCF4 have been associated to both transcriptional activation and repression and this dual function might rely on the fact that tissue-specific variants are produced by alternative splicing and by transcription from alternative promoters (Mao and Byers, 2011).

In general terms, the emerging idea is that transcription activation occurs through a switch from repressor to activator TCFs and this switch is induced by β-catenin.

Although the molecular mechanisms involved in this switch have been only partially elucidated, it has been shown in Xenopus embryos and mammalian cells that Wnt-dependent phosphorylation of TCF3 induces its dissociation from DNA and concomitant replacement by TCF1 (Hikasa et al., 2010).

β-catenin and chromatin regulation

Besides promoting the exchange of TCF factors, β-catenin recruits chromatin modifiers to the regulatory region of Wnt target genes thereby providing a further level of complexity in the control of target genes transcription. It has been demonstrated in several cellular contexts that β-catenin interacts with chromatin remodelers such histone acetyltransferases, histone methyltransferases and SWI/SNF nucleosome remodeling complexes (Figure 20). Considering the elevated number of nuclear β-catenin interacting partners, a sequential recruitment is more likely to occur in order to mediate a progressive transition from a repressive to an activating chromatin conformation.

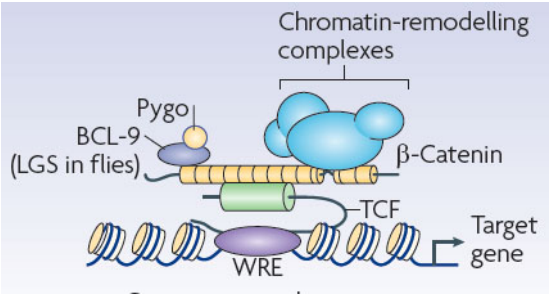


Figure 20. β-catenin interaction with chromatin modifiers. β-catenin regulates the transcription of Wnt target genes by interacting with TCF transcription factors and by recruiting chromatin remodelers to the regulatory region of Wnt target genes. Adapted from Mosimann et al., 2009.

<u>β-catenin and histone acetylation</u>

Histone acetylation is commonly associated to transcription activation (see Tollervey and Lunyak, 2012). *In vitro* experiments showed that two histone acetyltransferases (HAT), p300 and CBP, interact with β-catenin and are recruited to specific target genes, where they enhance β-catenin-mediated transcription (Miyagishi et al., 2000; Hecht et al., 2000; Ma et al., 2005). Further analysis demonstrated that p300 and CBP can bind TCF and their silencing results in a reduction of Wnt targets transcription (Li et al., 2007). The recruitment of CBP correlates with widespread histone acetylation at Wnt target loci and this event is upstream to transcription activation, suggesting a mechanism by which β-catenin binding to TCFs recruits histone acetyltransferases to promote a chromatin landscape which is permissive for transcription (Parker et al., 2008). This notion is confirmed by the fact that TLEs (TCFs co-repressors) associate to

histone deacetylases that repress transcription (Winkler et al., 2010; Courey and Jia, 2001).

β-catenin and histone methylation

In addition to acetylation, β-catenin also favors histone methylation to activate transcription. In particular, it was shown that trimethylation of lysine 4 of histone 3 (H3K4me3) increases following induction of Wnt targets transcription, whereas it declines when the Wnt/β-catenin pathway is inhibited (Sierra et al., 2006). Pull-down of β-catenin followed by mass spectrometry analysis demonstrated that in the nucleus β-catenin participates to a multiproteic complex that comprises SET1-related histone methyltransferases (precisely the MLL and MLL2, as well as 3 subunits of the SET1 complex: MEN-1, RbBP5 and Ash2) and HATs (Sierra et al., 2006). ChIP experiments following Wnt signaling stimulation further demonstrated that recruitment of these factors to Wnt target genes depend on the activation status of the pathway. In turn, H3K4me3 is required to recruit Pygopus and BCL-9, two co-activators of the β-catenin/TCF transcription complex that are necessary for efficient induction of target genes expression (Fiedler et al., 2008).

Methylation of other lysines of histone 3 and 4 was implicated in canonical Wnt-mediated transcriptional activation. For instance, H3K79me3 and H4K20me1 were identified in association with induction of Wnt target genes transcription and enzymatic complexes responsible for these modifications were shown to interact with β-catenin (Li et al., 2011; Mohan et al., 2010).

β-catenin and chromatin remodeling

Besides post-transcriptional modifications of histone tails, another important event required for transcription activation is chromatin opening. During this process, nucleosomes position and structure are modified by multiproteic complexes that use ATP hydrolysis to make gene regulatory regions accessible to transcription factors and co-activators. β-catenin contributes to this type of remodeling by recruiting chromatin modifiers such as Brg-1 (also known as SMARCA4), a member of the SWI/SNF complex (Barker et al., 2001). This event has been shown to be crucial for expression of Wnt target genes in several models, including yeast (Barker et al., 2001), zebrafish (Eroglu et al., 2006), and mammals (Park et al., 2009). More recently, Brg-1 has been proposed as a therapeutic target in intestinal cancer (Holik et al., 2014). Another component of

the SWI/SNF complex that showed interaction with β-catenin is the ISW1, although its role is still controversial (Eckey et al., 2012; Sierra et al., 2006).

As a concluding remark, it should be stressed that the majority of these mechanisms have been described by using *in vitro* systems and immortalized cell lines, mainly due to technical issues. Nonetheless, a deep analysis of context- and tissue-specificity of β-catenin interacting partners and more generally of its functions in target gene transcription is lacking. Yet, technical improvement of last years will probably allow exploring this interesting aspect.

Canonical Wnt signaling in organogenesis

As mentioned above, canonical Wnt signaling regulates many biological aspects. β-catenin null embryos present dramatic gastrulation defects and die early during embryogenesis (Haegel et al., 1995). Conditional genetic manipulation of canonical Wnt signaling during development demonstrated that the pathway is required for the formation of tissues deriving from all the three primary embryonic germ layers. In many cases, canonical Wnt signaling drives cell fate choices by inducing genes required for the differentiation of progenitors into functionally specialized cell types. Another function of canonical Wnt signaling during organogenesis is the control cell proliferation. Importantly, canonical Wnt signaling can play multiple roles within the same tissue in distinct phases of development, thus highlighting the great complexity of this signaling pathway. Hereafter I will focus on most prominent examples. Yet, for a comprehensive review of canonical Wnt signaling involvement in organogenesis please see Grigoryan et al., 2008.

Ectoderm-derived tissues

Components of the Wnt/β-catenin pathway are expressed during development of **hair follicle**, and inhibition of canonical Wnt signaling perturbs its formation (Millar et al., 1999). This evidence is based on observations of several mouse models, in which ectopic expression of Dkk1 or inactivation of β-catenin in the epidermis (under the control of Keratine 14 promoter) impairs hair follicle formation (Huelsken et al., 2001; Andl et al., 2002). This phenotype is due to the fact that inactivation of canonical Wnt signaling converts hair follicles into epidermal cells by perturbing the expression of hair follicle-specific genes. Conversely, expression of a constitutively active form of β-

catenin results in excessive hair follicles formation, and this phenotype depends on the level of stabilized β-catenin (Närhi et al., 2008). Therefore, canonical Wnt signaling controls the cellular identity of hair follicle.

During spinal cord development, canonical Wnt signals promote proliferation of neuroepithelial cells both in mouse and in zebrafish embryos (Bonner et al., 2008; Ille et al., 2007). More specifically, patterning of the central nervous system is dependent on Wnt1 activity (Thomas and Capecchi, 1990), since inactivation of Wnt1 results in failure of midbrain and rostral hindbrain development (McMahon et al., 1992). β-catenin gene deletion in the Wnt1-Cre lineage results in dramatic brain malformation and failure of craniofacial development (Brault et al., 2001).

Mesoderm-derived tissues

During early **skeletogenesis**, mesoderm-derived osteochondrogenic progenitors give rise to two distinct lineages: the osteogenic lineage that contributes to bone formation, and the chondrogenic lineage, that ultimately forms cartilage. Canonical Wnt signaling is responsible for the fate choice that drives the osteochondrogenic progenitors toward the osteogenic lineage, as shown by β-catenin loss-of-function and gain-of-function conditional mutations and ectopic ligand overexpression (Day et al., 2005; Hill et al., 2005). Besides early commitment to the osteogenic lineage, canonical Wnt signaling directly regulates Osteoprotegerin, a major effector of bone cells terminal differentiation, thus indicating that the Wnt/β-catenin pathway has multiple roles during skeleton differentiation (Glass II et al., 2005).

Similarly to osteogenesis, the Wnt/β-catenin pathway is active also during **dermis** development, another mesodermal derivative, where it controls the specification and differentiation of the dermal lineage (Atit et al., 2006; Ohtola et al., 2008).

During embryogenesis, a bipotent gonad arises from the coelomic epithelium and is driven toward either female or male differentiation. Wnt4 null female embryos display defective ovary development and partial masculinization (Vainio et al., 1999). Further analysis revealed that Wnt4 activates the β-catenin pathway to regulate gonad development in two ways. In early coelomic epithelium, Wnt/β-catenin pathway promotes cell proliferation (Chassot et al., 2012), whereas later during gonad fate determination, it induces ovarian differentiation (Chassot et al., 2008; Maatouk et al., 2008). Interestingly the Wnt agonist Rspondin plays a major role in this activation, as shown by sex reversal of $Rspo1^{-/-}$ XX mice (Chassot et al., 2008, 2012).

Endoderm-derived tissues

Canonical Wnt signaling plays multiple roles during endoderm anterior-posterior patterning. Evidence in *Xenopus* embryos indicated that the Wnt/β-catenin pathway is active in the posterior endoderm, where it positively regulates the posterior identity by inducing the hindgut fate. Conversely, in the anterior endoderm the pathway is inhibited by the Wnt antagonist sFRP5 and this inhibition determines the foregut fate (Li et al., 2008; McLin et al., 2007). Accordingly, data from mouse embryos confirm a crucial role of Wnt/β-catenin inhibition in the induction of foregut identity. sFRP1 and sFRP2 are expressed in the gastric progenitors and allow their epithelial differentiation by blocking canonical Wn signaling (Kim et al., 2005).

Besides fate specification, canonical Wnt signaling also regulates endoderm proliferation. For instance, during pancreas development, in early phases forced β-catenin stabilization blocks pancreatic progenitors proliferation, whereas later it enhances the expansion of pancreas exocrine compartment (Heiser et al., 2006).

Canonical Wnt signaling in adult stem cells homeostasis

Canonical Wnt signaling has been widely implicated in homeostasis of adult stem cells, where alterations of the activation level of the pathway is often associated to an imbalance between cell proliferation and differentiation.

Intestinal stem cells

One of the best-studied aspects of the Wnt/β-catenin pathway is its involvement in **intestine** homeostasis. Small intestine epithelium is organized in numerous protrusions into the intestinal lumen, called villi and composed of differentiated cells, and invaginations, called crypts and representing the stem cell reservoir. Each crypt extends from the base of the villus to the underlying connective tissue and that host quiescent and transient amplifying stem cells (Figure 21). The intestinal epithelium undergoes continuous renewal through a process in which transient amplifying progenitors rapidly divide, progressively migrate from the crypt toward the tip of the villus and differentiate to replace all villi cells every 4-5 days in mice. Canonical Wnt signaling plays multiple roles during intestine renewal. First, Wnt/β-catenin pathway is required for transient amplifying cells generation and proliferation. Indeed, Tcf4 knockout mice display total absence of transient amplifying cells, whereas the differentiated villi cells are not altered, and conditional inactivation of Tcf4 or β-

catenin in adult mice blocks transient amplifying cells proliferation (Korinek et al., 1998). Further transcriptomic analysis identified a set of Wnt/Tcf4 target genes responsible for the determination of transient amplifying cell identity (Van der Flier et al., 2007; van de Wetering et al., 2002). Among the intestinal Wnt targets, Lgr5 draws much attention since it was identified as a specific marker of cycling stem cells present at the base of the crypt (Barker et al., 2007). Lgr5 is one of the receptors for R-spondin and it is required for R-spondin-dependent amplification of canonical Wnt signaling (de Lau et al., 2011). Intestinal stem cells, as well as the proliferating progenitors, are rapidly lost following conditional intestinal deletion of Lgr4 and Lgr5, highlighting the crucial role of Lgr/Rspo and canonical Wnt signaling in maintenance of these cell populations (de Lau et al., 2011). Several recent studies focused on the identification of genes specifically expressed by Lgr5+ intestinal stem cells. However, it remains still to be determined whether among them there are direct Wnt targets (Koo and Clevers, 2014). Interstingly, Lgr5 also marks stem cells of other compartments, such as stomach (Barker et al., 2010), hair follicle (Jaks et al., 2008) and in ovarian and tubal epithelia (Ng et al., 2014).

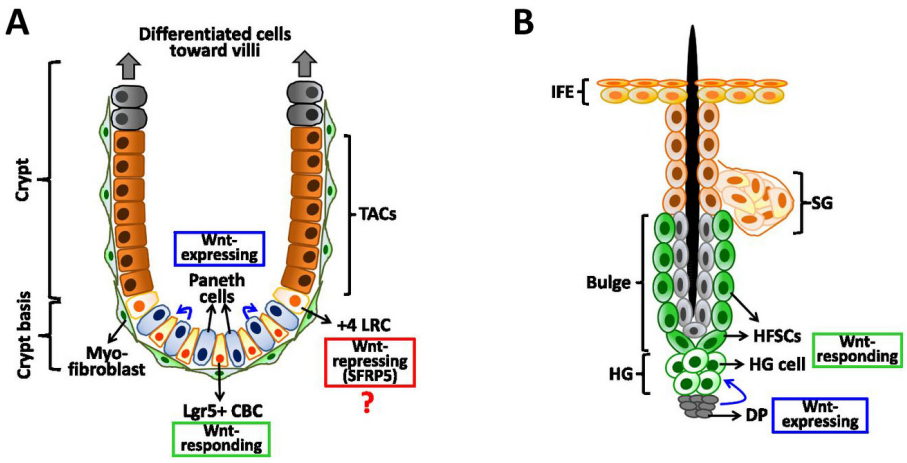


Figure 21. Intestine and skin stem cell niches. A, Schematic representation of the intestinal crypt and the different cell populations. TAC: transient amplifying cells; LRC: label retaining cells (slow-dividing cells); CBC: crypt base columnar cells (cycling cells). **B,** Schematic representation of the hair follicle and epidermis. IFE: interfollicular epidermis; SG: sebaceous gland; HFSCs: hair follicle stem cells; HG: hair germ; DP: dermal papilla. Adapted from Lien and Fuchs, 2014.

Skin stem cells

Another compartment in which canonical Wnt signaling controls stem cell dynamics is the skin. Adult skin is composed of the epidermis, a stratified epithelium in which

sebaceous glands and hair follicles are interspersed and are responsible for sebum secretion and skin hair production respectively (Figure 21). Unlike interfollicular epidermis and sebaceous glands that undergo constant turnover, hair follicles repeatedly oscillate between a resting state (telogen), active growth (anagen) and regression (catagen). Interestingly, transiently forced β-catenin activation in adult epidermis induces resting hair follicles to enter active growth (Celso et al., 2004), suggesting that inhibition of the Wnt/β-catenin pathway might be required for hair follicle maintenance during the inactive phases of hair cycle. This is confirmed by the fact that Tcf3 is expressed by slow-cycling stem cells present within the hair follicle, where it represses Wnt target genes transcription (Nguyen et al., 2009; Lien et al., 2014). However, hair follicle stem cells are responsive to canonical Wnt signals and during hair regeneration, the Wnt/β-catenin pathway is activated and activate genes that drive the hair fate (Lien et al., 2014). A recent study further demonstrated that β-catenin activation within the hair follicle during hair regeneration not only controls orientation of cell division, but it also induces canonical Wnt signaling activation in neighboring cells by secreting Wnt ligands (Deschene et al., 2014).

Neural stem cells

Recent focus on adult neural stem cells revealed that canonical Wnt signaling is active in adult hippocampus. *In vitro* overexpression of *Wnt3a* enhances proliferation of adult hippocampal progenitors and *in vivo* inhibition of canonical Wnt signaling blocks adult hippocampal neurogenesis (Lie et al., 2005). Further analysis confirmed these roles of canonical Wnt signaling on both proliferation and differentiation of adult neural stem cells. First, in a mouse model for schizophrenia, expression of a constitutive active form of β-catenin restores normal proliferation level in cortical progenitors (Mao et al., 2009). Second, *in vivo* results in adult hippocampal progenitors revealed that the Wnt/β-catenin pathway directly activates the expression of NeuroD1, a master gene in neural differentiation (Kuwabara et al., 2009). More recently, the presence of Wnt/β-catenin-responsive neural stem cells in the adult hyppocampus has been demonstrated by lineage-tracing experiments (Bowman et al., 2013).

Mammary gland stem cells

Interestingly, in some cases canonical Wnt signaling can sustain stem cell self-renewal without altering proliferation. It is the case in the mammary gland, where stem cells ensure massive proliferation and tissue remodeling during pregnancy and lactation.

Indeed, genetic overactivation of the Wnt/β-catenin pathway does not affect the proliferation rate of mammary gland stem cells, but it rather favors their self-renewal at the expense of differentiation (Zeng and Nusse, 2010). Furthermore, lineage-tracing analysis revealed that Wnt/β-catenin-responsive cells constitute a stem cell population that persists over multiple pregnancies and retains a long-term proliferative potential (van Amerongen et al., 2012).

These few examples highlight the importance of canonical Wnt signaling functions in tissue formation and homeostasis. Based on these and other evidence, it is not surprising that deregulation of this pathway can have dramatic consequences such as malignant transformation. Indeed, mutations of members of the Wnt/β-catenin have been involved in tumorigenesis of several tissues and in some cases they can be the direct cause of cancer initation (see Clevers and Nusse, 2012). Much effort has been put into to development of therapies to override cancerous hyperactivation of canonical Wnt signaling. A comprehensive description of these approaches has been reported elsewhere (see Anastas and Moon, 2013).

III. Adenomatous Polyposis Coli

The Adenomatous polyposis coli (*Apc*) gene was first identified as one of the most frequent loci mutated in familial adenomatous polyposis (FAP) and in both inherited and sporadic colorectal cancers (Nishisho et al., 1991; for a review see Zeineldin and Neufeld, 2013). The high incidence of *Apc* mutations in colon tumors (the third cause of cancer mortality worldwide, globocan.iarc.fr/Pages/fact_sheets_cancer.aspx) prompted researchers to deeply investigate the functions of *Apc* gene product. After about thirty years of intense studies, it is now well established that *Apc* gene codes for a large multifunctional and ubiquitously expressed tumor suppressor able to interact with several partners in multiple subcellular localizations and involved in a large variety of cellular processes.

1. APC: general features

Structure

APC is a 310kDa protein composed of approximately 2800 amino acids. The most N-terminal part of APC contains a homodimerization domain of about 55 amino acids and an armadillo domain that participates to interaction with a large variety of proteins involved in cytoskeleton dynamics (see section 3. APC functions not related to canonical Wnt signaling).

The central portion of APC comprises several domains of interaction with β-catenin and Axin. In particular, APC contacts β-catenin through two regions, containing a series of 15-amino acids and 20-amino acids repeats, respectively. Other 16-amino acids repeats called SAMP motifs lie in between the 20 amino acids repeats and are required for Axin binding (Figure 22). Lastly, the C-terminal extremity of APC interacts with microtubules and other proteins that regulate actin dynamics.

Several nuclear export signals (NES) have been identified within the C-terminal and central domains, whereas two nuclear localization signals (NLS) have been found to be interspersed within the Axin-binding region (Figure 22). This evidence supports a role of APC in the nucleus (see paragraph *Nuclear functions of APC*).

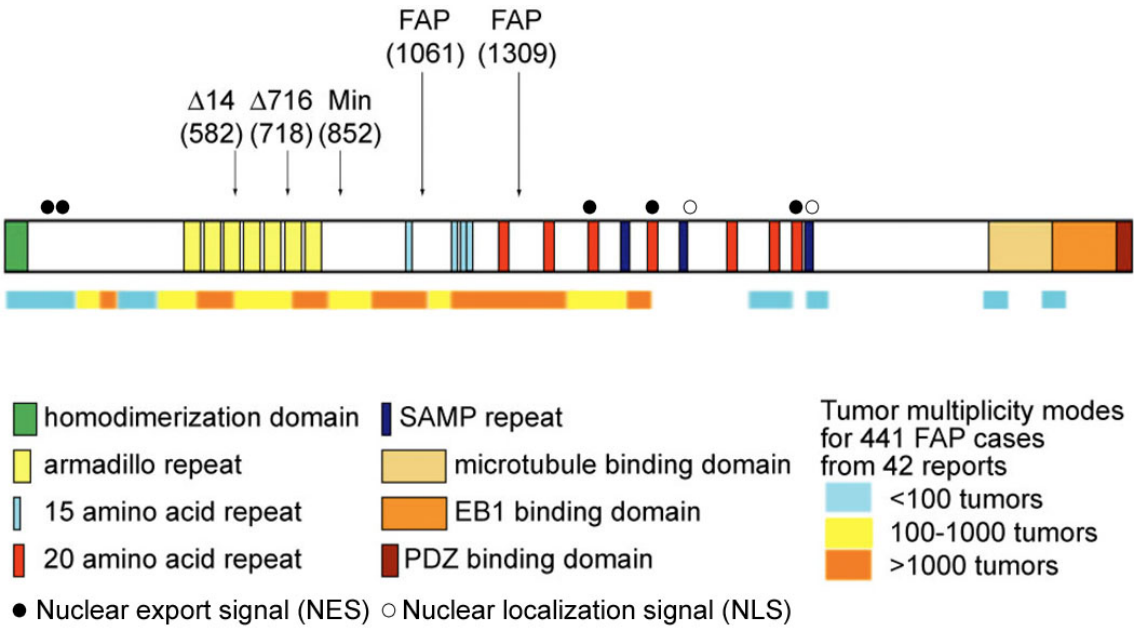


Figure 22. Schematic representation of human APC domains. Short arrows indicate the orthologs of the three most common APC mutated alleles of mouse models: Min, Δ14 and Δ716. Min: first model to be generated, it harbors a nonsense mutation at codon 850 in mouse (corresponding to codon 852 in human). Δ14: deletion of the exon 14 produces a truncation mutation at the codon 580 (corresponding to human codon 582). Δ716: deletion of exon 15. Long arrows indicate the most frequent mutations found in Familial adenomatous polyposis (FAP) patients. The color bar below indicates the genotype-phenotype correlation of sites of protein truncation to disease severity. Adapted from Kwong and Dove 2009.

Genetic tools to study APC

The large size of APC (~12 Mb for the transcript, ~310kDa for the protein) entails technical limitations. Expression of tagged full-length APC has been challenging, such as recognition of endogenous APC with different antibodies, which gave contrasting results in terms of subcellular localization of APC. Furthermore, lethality of homozygous germline *Apc* loss of function dictated the need for conditional alleles. Based on the extremely high similarity (92% at the amino acid level) between human and rodent *Apc* gene, a number of genetic mouse models with APC loss-of-functions mutations have been developed in order to generate in vivo systems that recapitulate human pathogenesis.

The first *Apc* mutant allele able to reproduce the human phenotype of FAP and colorectal cancer was identified in a screen of ethylnitrosurea-mediated (ENU) mutagenesis (Moser et al., 1990; Su et al., 1992). This allele was called Apc^{Min} (where Min stands for multiple intestinal neoplasia) and harbors a premature stop codon after

codon 850, which results in a truncated APC protein only retaining the armadillo domain.

In order to generate conditional mutation of *Apc* in genetically targeted tissues, floxed *Apc* alleles have been generated. In particular, LoxP sites have been introduced in introns flanking exon 14, so that Cre recombinase-mediated excision produces a stop codon after codon 580 (Shibata et al., 1997; Colnot et al., 2004). This $Apc^{\Delta 14}$ largely recapitulates the Apc^{Min} allele when recombination is induced in the intestine and has been widely used to disrupt APC function in several tissues.

Other *Apc* mutations have been generated to study particular domains of the protein or to reproduce alterations found in human cancers. A full updated list of these alleles can be found in Zeineldin and Neufeld, 2013.

2. APC and canonical Wnt signaling

APC was first identified as a negative regulator of canonical Wnt signaling. In this context, APC is part of the β-catenin destruction complex, in which it has been postulated to act as a scaffold, possibly by facilitating the interaction between β-catenin and the kinases responsible for its phosphorylation and subsequent targeting for proteasomal degradation. Moreover, APC can influence canonical Wnt signaling by regulating the subcellular localization of between β-catenin as well as its transcriptional activity.

APC and β-catenin destruction complex

Early studies demonstrated that APC is necessary and sufficient to decrease β-catenin levels (Munemitsu et al., 1995). However, the precise role of APC within the β-catenin destruction complex remains elusive. Despite direct APC interaction with β-catenin and Axin has been shown to be required for phosphorylated β-catenin targeting to degradation, a direct involvement of APC in β-catenin phosphorylation has not been reported yet (Su et al., 2008). Indeed, addition of the APC portion containing the β-catenin- and Axin-binding sites to an *in vitro* reconstituted destruction complex did not increase β-catenin phosphorylation. Accordingly, colon cancer cell lines expressing a

truncated APC that lacks the Axin-binding domains display high levels of phosphorylated β-catenin (Sadot et al., 2002).

It has been proposed that APC could also be crucial for the recruitment of the β-catenin destruction complex to the plasma membrane through its interaction with Amer1 (also known as WTX), a plasma membrane-bound protein identified by tandem-affinity purification of the β-catenin interacting partners (Major et al., 2007; Grohmann et al., 2007). More recently, further analysis of Amer1 implication in canonical Wnt signaling showed that Amer1 also induces LRP6 phosphorylation, a key event in Wnt signal transduction (Tanneberger et al., 2011).

Regulation of β-catenin subcellular localization

A possible mechanism by which APC participates to the regulation of canonical Wnt signaling is the regulation of β-catenin subcellular localization. Early studies identified conserved nuclear export signals within the APC N-terminal and central portions, and postulated a possible role of APC as a chaperone for β-catenin nuclear export (Henderson, 2000; Rosin-Arbesfeld et al., 2000). It has been hypothesized that APC participates to β-catenin destabilization by mediating its export to the cytoplasm, thereby promoting β-catenin binding to the destruction complex. However, more recent studies reported evidence in favor of a role for APC in β-catenin retention in the cytoplasm. This hypothesis is supported by live-cell microscopy and fluorescence recovery after photobleaching (FRAP) experiments that showed that APC and Axin increase cytoplasmic β-catenin without affecting its nuclear import and export (Krieghoff et al., 2006).

Another possible function of APC in regulating canonical Wnt signaling might occur at the chromatin level. Chromatin immunoprecipitation (ChIP) assays revealed that APC binds to regulatory regions of Wnt target genes and perturbs β-catenin interaction with Lef1, thereby impairing target gene transcription (Sierra et al., 2006). Furthermore, mutation of the three APC nuclear localization signals resulted in increased Wnt/β-catenin induction *in vivo* (Zeineldin et al., 2012).

3. APC functions not related to canonical Wnt signaling

As mentioned above, APC has several distinct subcellular localizations and a variety of interacting partners. In addition to its involvement in the regulation of canonical Wnt signaling, APC has been implicated in cell migration, mitotic spindle formation chromosome segregation during cell division, DNA repair. It is noteworthy to highlight that in some context an interplay between Wnt signaling and other APC functions has been postulated or directly demonstrated, thus proposing APC as an important node of integration of distinct cellular processes.

APC and cell migration

Cell migration requires a dynamic and directional regulation of actin and microtubules networks. In this process, cells use energy (GTP) to modify their cytoskeleton and orient their movement.

The C-terminal portion of APC directly interacts with microtubules. This interaction was first suggested by the evidence that APC colocalizes with microtubules plus ends at the plasma membrane, where it organizes in granules, and it dynamically moves along the microtubules (Näthke et al., 1996; Mimori-Kiyosue et al., 2000). Further analysis revealed that this interaction has a functional role: APC forms a complex with EB1, a microtubule-associated protein (MAP) that binds a subset of microtubules in interphase and mitotic cells, and mDia, a Rho GTPase effector, to actively stabilize microtubules plus ends, thereby promoting cell migration (Wen et al., 2004). *In vivo* analysis of intestinal crypts showed that loss of APC function correlates with impaired migration of intestinal progenitors along the crypt axis (Sansom et al., 2004). However, this study did not determine whether this phenotype was driven by the overactivation of canonical Wnt signaling resulting from APC removal or directly by the absence of APC. Indeed, a recent report demonstrated that during axon elongation canonical Wnt signaling regulates microtubule directionality by modulating APC localization at plus ends (Purro et al., 2008). This evidence supports a mechanism of regulation of cell migration that involves both APC and canonical Wnt signaling, suggesting that these two aspects are intimately linked.

In addition to microtubule dynamics, APC also controls actin cytoskeleton. Indeed, the armadillo domain present within the N-terminal portion of APC interacts with regulators of actin polymerization, such as Asef, a guanidine exchange factor, and IQGAP1, a Rho GTPase effector (Kawasaki et al., 2000; Watanabe et al., 2004). Furthermore, the C-terminal region of APC directly interacts with actin filaments

(Moseley et al., 2007) and actin assembly/disassembly assays demonstrated that APC actively participates to actin polymerization by synergizing with mDia and recruiting actin monomers to growing filaments (Okada et al., 2010). Thus APC has been postulated as a key regulator of cell migration that coordinates tubulin and actin cytoskeleton dynamics.

APC and cell division

Microtubules are responsible for mitotic spindle formation and chromosome segregation during cell division. APC associates to microtubules during mitosis and plays a crucial role in chromosome segregation. One of the first evidence supporting this notion came from the observation that wild-type APC localizes at the kinetochore, the structure by which chromatids are attached to the mitotic spindle, and that C-terminus truncation mutations of APC correlate with defective chromosome segregation and aneuploidy *in vitro* (Fodde et al., 2001; Kaplan et al., 2001). This was corroborated by further evidence that in addition to perturbed kinetochore function, APC loss leads to deregulation of mitotic spindle checkpoint, a process that prevents chromosome missegregation by enabling cell division only if chromosomes are correctly attached to the mitotic spindle (Dikovskaya et al., 2012). Furthermore, although it was reported that APC localizes at the centrosomes during mitosis, its precise function in this context still remains elusive (see Lui et al., 2012).

Recently, a novel role for APC in mitosis has been proposed. Based on proteomic analysis of chromatin composition following APC depletion, it has been postulated that APC is required to recruit chromatin modifiers that regulate mitotic chromatin compaction (Dikovskaya et al., 2012).

Nuclear functions of APC

Accumulating evidence demonstrates that APC shuttles between the nucleus and the cytoplasm through nuclear import and export signals present within N-terminal and central part of the protein (see Lui et al., 2012). In addition to the regulation of the β-catenin transcriptional complex mentioned above, other nuclear functions of APC have been reported. For instance, ChIP assays revealed that APC is enriched in transcriptionally active regions and participates to double strand breaks repair (Kouzmenko et al., 2008). Further *in vitro* experiments suggested that in response to

DNA replication chemical stress, APC acts as a scaffold to recruit several factors involved in DNA repair at stalled replication forks (Brocardo et al., 2011).

4. Contribution of APC to cancer

Apc is considered as a tumor suppressor since loss-of-function mutations correlate with cancer onset or progression in several tissues. For instance, hereditary germline mutations of one *Apc* allele cause familial adenomatous polyposis (FAP), a precancerous condition characterized by the presence of a high number of colonic polyps. In most cases, loss of heterozygosity (LOH) by somatic mutation of the second APC allele is believed to rapidly induce colorectal tumor initiation. *Apc* loss of function is also an early event in sporadic colorectal cancer onset (see Half et al., 2009).

In addition to its involvement in intestinal cancer, *Apc* loss of function was also detected in up to 50% of kidney carcinomas and *Apc* expression was found to be repressed through promoter hypermethylation in 30-40% of breast cancers. However, it is still unclear whether *Apc* mutations are the primary cause of cancer development (Sansom, 2009). Besides epidemiological observation of *Apc* mutation incidence in human cancers, genetic studies in mouse models confirmed the tumor suppressor function of *Apc*. Indeed, tissue-specific inactivation of *Apc* promotes tumor formation in intestine (Sansom et al., 2004a), liver (Colnot et al., 2004), kidney (Qian et al., 2005) and mammary gland (Méniel et al., 2005).

The most common mutations of *Apc* gene in human colorectal cancers occur in a region called mutation cluster region (MCR), which spans the codons 1286 to 1513 (Miyoshi et al., 1992; see also Zeineldin and Neufeld, 2013). These mutations generate truncated proteins that lack the domains of interaction with cytoskeleton as well as the β-catenin- and Axin-binding regions (Figure 22). Therefore, both the Wnt-antagonist function and the cytoskeleton-related roles of APC are affected and are thought to be implicated in tumorigenesis.

Aberrant activation of Wnt target genes

Mutations in several components of canonical Wnt signaling that induce aberrant β-catenin stabilization and overactivation of the pathway have been linked to tumor

onset and progression (see Anastas and Moon, 2013). Accordingly, *Apc* mutations have been implicated in tumorigenesis due to their ability to trigger a rapid increase in canonical Wnt signaling (Morin et al., 1997).

Large-scale analysis allowed identifying a set of Wnt target genes with aberrant expression in cancer cell lines and in intestinal tumors. Among them, *Myc* has been shown to play a pivotal role in small intestine tumorigenesis, where it acts as a transcriptional regulator. In this context, it has been shown to be responsible for upregulation of Cyclin D1 and repression of p21 (Cdkn1a) and p27 (Cdkn1b) (Daksis et al., 1994; Gartel et al., 2001; Yang et al., 2001). This enhances cell cycle progression and is thought to favor mitotic errors and chromatin instability, thus leading to malignant transformation. The complete rescue of the malignant phenotype of *Apc* loss by simultaneous deletion of *Apc* and *Myc* genetically demonstrated that Myc is the major effector downstream β-catenin (Sansom et al., 2007).

It is noteworthy to highlight that despite Cyclin D1 has been long considered a direct target of canonical Wnt signaling in colorectal cancer, careful *in vivo* analysis revealed that Cyclin D1 expression was not affected by β-catenin nuclear accumulation and conditional Cyclin D1 depletion in the intestine did not rescue the phenotype caused by *Apc* mutation (Sansom et al., 2005).

An important consideration is that different mutations in the *Apc* gene do not trigger the same level of canonical Wnt signaling activation. For instance, whole-gene deletion of Apc leads to lower Wnt target genes induction compared to Apc^{Min} truncation (Cheung et al., 2010). To some extent a correlation between the type of *Apc* germline mutation in FAP mouse models and the number of polyps formed has been demonstrated (Figure 22, see also Zeineldin and Neufeld, 2013). However, contradictory lines of evidence have been reported in distinct mouse models and the exact contribution of APC and canonical Wnt signaling to tumor initiation is still under debate.

Chromosomal instability

As described above, APC interacts with cytoskeletal proteins during mitotic spindle formation and chromosome segregation. Therefore, it is not surprising that APC loss affects these processes, thereby leading to chromosome instability (CIN), which includes aneuploidy and chromosomal aberrations and is a key hallmark of many cancers (Fodde et al., 2001; Kaplan et al., 2001). Whether this feature is a cause or rather a consequence of malignant transformation still remains to be determined. For

instance, about 50% of APC-mutated colon adenomas are not aneuploid but progress nevertheless to carcinoma (Rusan and Peifer, 2008).

Interestingly, a recent study analyzed *in vivo* the orientation of mitotic spindle in intestinal stem cells and found that mutation of one *Apc* allele is sufficient to perturb cell division alignment in precancerous tissues (Quyn et al., 2010). This would produce a permissive context for further aberrant cell division and the accumulation of mitotic errors. Moreover, some truncated forms of APC have been reported to act in a dominant negative fashion by tethering the wild-type APC and thereby causing chromosome instability and mitotic aberrations (Kwong and Dove, 2009).

In conclusion, to date *Apc* mutation is thought to promote tumor initiation and progression at least in two ways (Figure 23). To one hand it induces exacerbated expression of Wnt target genes that lead to an increase in the proliferation rate. To the other hand it perturbs cytoskeletal dynamics, thereby causing mitotic errors and chromosome alterations. However other cellular processes are likely to be altered in absence of APC and intense research is ongoing to clarify the involvement of this important tumor suppressor in cancer.

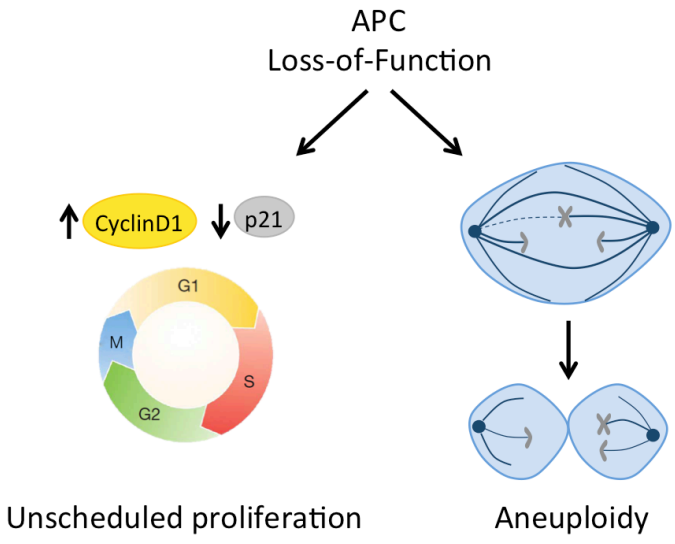


Figure 23. Mechanisms by which APC participates to tumorigenesis. Canonical Wnt signaling overactivation mediated by APC loss results in Myc-dependent upregulation of *CyclinD1* and repression of p21. This events generates a faster G1/S progression and favors a cell division more prone to mitotic errors. In parallel, APC loss at the kinetochore and at the mitotic spindle alters chromosome segregation, inducing aneuploidy.

IV. OBJECTIVES OF THE PROJECT

Background

Due to the major involvement of APC in tumorigenesis, great effort has been put in understanding how its mutation leads to malignant transformation. A very well accepted paradigm based on cancer models indicates that APC loss-of-function inevitably results in enhanced proliferation, thus increasing the incidence of mitotic errors underlying tumor initiation. According to this model, canonical Wnt signaling hyper-activation following APC loss induces the expression of genes that promote cell cycle progression. This suggests that APC ensures correct cell proliferation by negatively regulating the Wnt/β-catenin pathway. However, whether this model can be extended to normal tissue development and homeostasis has poorly been determined. This question becomes even more relevant if we consider that the paradigm of APC and canonical Wnt signaling as gatekeepers of cell proliferation has been developed in constantly and fast renewing tissues such as the murine intestine, in which the epithelium undergoes complete self-renewal every 5 days, or the skin, which has a turnover of approximately 10 days in mice.

Canonical Wnt signaling has been implicated in embryonic and adult myogenesis, although many aspects and molecular details remain elusive or controversial. However, the role of APC in this context has never been explored.

Conditional knockout of β-catenin in the early mouse somitic cells perturbs epithelial organization of somite and abrogates dermomyotome and myotome formation (Hutcheson et al., 2009). Overexpression of canonical Wnt ligands in chick embryos resulted in increased cell proliferation within the dermomyotome and the myotome and enhanced expression of Pax3 and Pax7 (Galli et al., 2004; Otto et al., 2006). By contrast, when the β-catenin gene is invalidated in progenitors that already committed to the myogenic lineage, muscle formation is not perturbed, suggesting that canonical Wnt signaling is required for somitogenesis and dispensable for myogenic differentiation (Hutcheson et al., 2009). Finally, the Wnt/β-catenin pathway has been shown to drive terminal myogenic differentiation by determining the fast and slow patterning of avian wing muscles (Anakwe et al., 2003). However, **its involvement in the transition of dermomyotome cells from multipotency to muscle-specific commitment has not been addressed yet.**

Regarding adult myogenesis, the picture is even more controversial. *In vivo* observations proposed that the Wnt/β-catenin pathway is active in differentiating

progenitors, where it has been postulated to promote myogenic differentiation (Brack et al., 2008). However, myofibers isolated and cultured in vitro in presence of canonical Wnt ligands displayed enhanced satellite cell proliferation (Otto et al., 2008). **These contrasting results highlight the need for an appropriate model to carefully study the precise function of canonical Wnt signaling during adult muscle regeneration**. Moreover, adult skeletal muscle provides an interesting paradigm of slow-renewing tissue. Indeed, in intact adult muscle, only ~2% of myonuclei are replaced every week and satellite cells are maintained in a quiescent state. Therefore, skeletal muscle can be used to shed light on the role of APC and canonical Wnt signaling in a tissue with slow turnover.

The main objective of my PhD project was to investigate the role of APC during embryonic and adult myogenesis and to determine whether its regulation of canonical Wnt signaling is required for proper skeletal muscle formation and regeneration. In other words, is APC-mediated modulation of canonical Wnt signaling required for fate choice of dermomyotome pluripotent cells? How do APC and canonical Wnt signaling control this process? Are they required for adult muscle stem cells activation and myogenic progression? Do they affect the proliferation or differentiation of embryonic and adult muscle stem cells?

Experimental approach

To answer these questions, we developed *in vivo* and *in vitro* models to genetically address the role of APC and canonical Wnt signaling during skeletal muscle formation and regeneration.

We conditionally inactivated the APC and β-catenin genes in pluripotent dermomyotome progenitors *in vivo* and we tested their ability to progress along the myogenic lineage by analyzing skeletal muscle development at multiple embryonic stages.

Similarly, we used mouse models of conditional gene inactivation specifically in adult satellite cells and we performed *in vivo* adult muscle regeneration assays to determine the regenerative ability of satellite cells devoid of APC and/or β-catenin. Through *ex vivo* and *in vitro* models we characterized the cellular and molecular consequences of APC and β-catenin loss and we gained insights into the molecular mechanism underlying APC function by combining large-scale and candidate-gene approaches.

RESULTS

I. APC is required for skeletal muscle formation and regeneration

The majority of the results obtained during my PhD are summarized in the article reported hereafter, which has been submitted to the *Science* journal.

Science AAAS

Submitted manuscript: Confidential

Title: APC is required for muscle stem cell proliferation and skeletal muscle formation and regeneration

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Abstract: The tumor suppressor Adenomatous Polyposis Coli (APC) is a crucial regulator of many stem cell types. In constantly cycling stem cells of fast turnover tissues APC loss results in the constitutive activation of a Wnt target gene program that massively increases proliferation and leads to malignant transformation. However, APC function in skeletal muscle, a tissue with a low turnover rate, has never been investigated. Here we show that conditional genetic disruption of APC in embryonic and adult muscle progenitors respectively results in a near complete absence of skeletal muscle formation and in the abrogation of adult muscle regenerative potential. We demonstrate that APC removal in adult muscle stem cells abolishes cell cycle entry and leads to cell death. By using double knockout strategies, we further prove that this phenotype is attributable to over-activation of β-catenin signaling. Our results demonstrate that APC dampens canonical Wnt signaling to allow cell cycle progression in muscle stem cells and radically diverge from previous observations concerning stem cells in actively self-renewing tissues.

Main Text: The APC gene codes for a large protein with multiple cellular functions and interactions (*1-3*). APC is an essential component of the canonical Wnt signaling pathway and is required for the formation of a cytoplasmic complex that targets β-catenin for proteasomal degradation when Wnt signals are absent (*4*). APC also participates in several cellular processes cell adhesion and migration (*5*), actin dynamics (*6*) and chromosome segregation (*7*). In humans, APC mutations lead to the

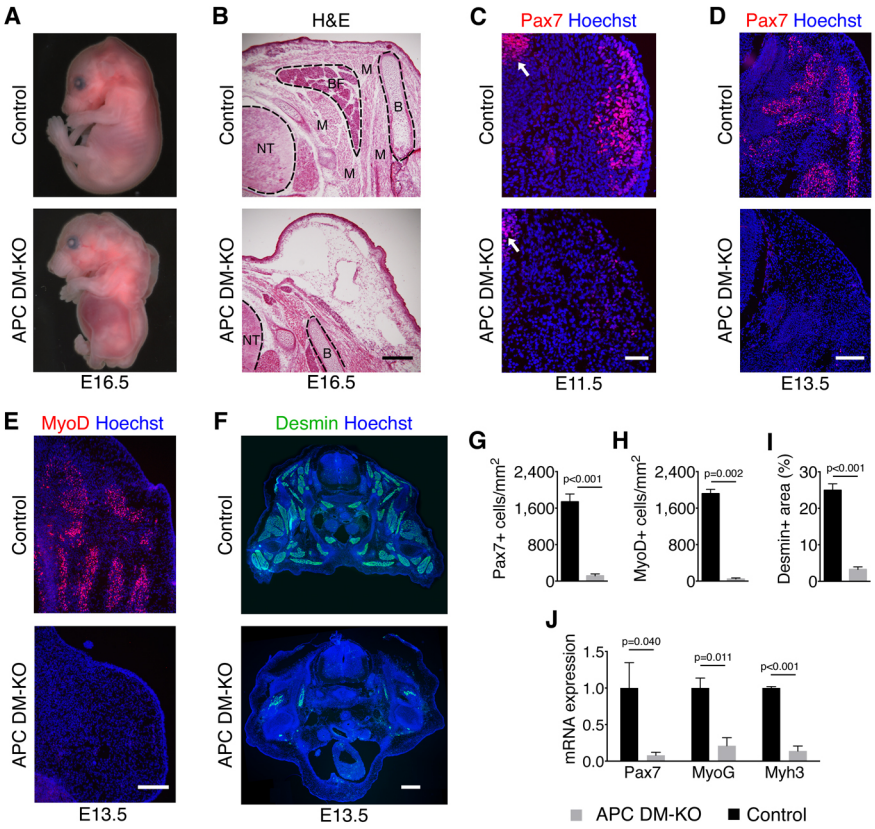

Fig. 1. **APC is required for the formation of skeletal muscle. A**, E16.5 control ($Myf5^{Cre}$) and APC DM-KO ($Myf5^{Cre}$;$APC^{lox/lox}$) whole embryos. **B,** Transverse sections of E16.5 embryos at the thoracic level stained with hematoxylin and eosin (H&E). BF: brown fat, B: bone, M: muscle, NT: neural tube. **C,** Transverse sections of E11.5 dorsal regions at the forelimb level immunostained for Pax7. Arrows indicate Pax7 expressing cells from the neural tube. **D, E,** Transverse sections of E13.5 dorsal thoracic regions immunostained for Pax7 (**D**) and MyoD (**E**). **F,** Whole transverse sections of E13.5 embryos stained for Desmin. **G-H**, Quantification of Pax7+ (**G**) and MyoD+ (**H**) cells per mm^2 in E13.5 embryos. **I**, Quantification of relative Desmin+ area in E13.5 embryos. **J,** RT-qPCR analysis of mRNA expression of Pax7, Myogenin, and Embryonic Myosin Heavy Chain (Myh3). Nuclei stained with Hoechst. Scale bars: 100µm in **B**, 50µm in **C**, **D**, **E**, 200 µm in **F**. Error bars: SEM.

second most common cause of cancer death (*11*). More specifically, in high turnover tissues, such as in the intestine, loss or mutation of APC leads to uncontrolled proliferation and accumulation of aberrant cells and leading to carcinogenesis (*9*,*10*). Due to its role in controlling cell cycle progression of several stem cell compartments, APC was a good candidate to regulate muscle stem cells proliferation and quiescence, which to date are poorly characterized. In skeletal muscle, a tissue with slow turnover, the formation and repair of differentiated skeletal muscle fibres depend on Pax7 expressing (Pax7+) myogenic stem/progenitor cells. During skeletal muscle development, Pax7+ myogenic progenitors arise from the mesoderm and are maintained as a proliferating population that sustains successive phases of myogenesis (*12*,*13*). In adulthood, a pool of quiescent Pax7+ muscle stem cells called satellite cells ensures myofibres regeneration following injury (*14*,*15*). Although a diversity of signals have been shown to regulate skeletal muscle formation and regeneration, the molecular mechanism underlying cell cycle progression of muscle stem cells remains to be elucidated.

We first investigated the genetic requirement for APC in muscle development *in vivo*. Since APC homozygous null embryos die before gastrulation(*16*), the APC gene was deleted in myogenic precursors by crossing the $APC^{flox/flox}$ mouse line(*8*) with the $Myf5^{Cre}$ driver (*17*). The $Myf5^{Cre}$ lineage comprises embryonic myogenic progenitors (*18*,*19*) and analysis of transverse sections at the rostral level of E10.5 embryos revealed a strong accumulation of β-catenin protein within the dermomyotome (DM) of conditional APC mutants (APC DM-KO), demonstrating efficient APC gene disruption *in vivo* (Fig. S1, A and B). APC DM-KO embryos were observed at normal Mendelian ratios at E13.5 and E16.5; however, we failed to obtain any live APC DM-KO mice at postnatal day 7, suggesting earlier lethality due to muscle dysfunction (Fig. S1C). E16.5 APC DM-KO embryos were alive, as their hearts were beating, but presented major morphogenetic defects, with flaccid dorsal skin, shortened limbs and dorsal tissues hypoplasia (Fig. 1A). Histological analysis of E16.5 APC DM-KO embryos showed a lack of differentiated tissue originating from the Myf5 lineage: skeletal muscle and interscapular brown adipose tissue (*20*), whereas other tissues including bone and epidermis had a normal morphology (Fig.1B). Muscle formation requires the generation of Pax7+ progenitor cells that give rise to MyoD+ myoblasts (*21*). Myoblasts terminally differentiate and fuse to form multinucleated myofibres. Dysfunction of one or more of these processes could contribute to lack of muscle formation and to APC DM-KO lethality. Strikingly, no Pax7+ cells were detected in E11.5 APC DM-KO embryos in the myotomal regions, showing that the generation of the embryonic muscle

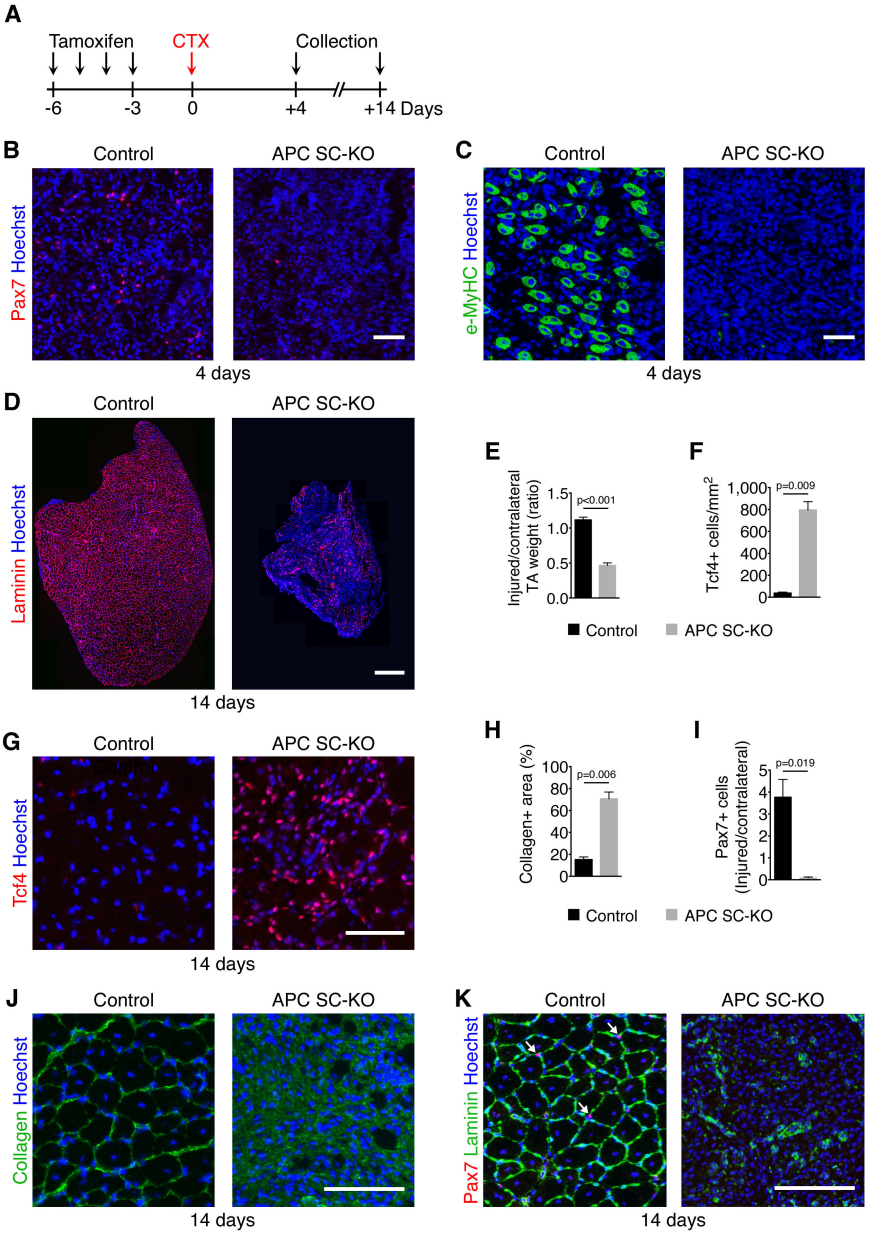

Fig. 2. APC is required for adult skeletal muscle regeneration. A, Tamoxifen and cardiotoxin (CTX) regimen and regeneration assay scheme for control ($Pax7^{CreER}$) and APC SC-KO ($Pax7^{CreER}$;$APC^{lox/lox}$) mice. **B**-**C**, Immunostaining for Pax7 (**B**) and e-MyHC (**C**) on TA cryosections 4 days after injury. **D-K,** Analysis of TA muscles 14 days post unjury. **D**, Whole sections of TA muscles immunostained for Laminin. **E**, TA weight 14 days after injury normalized by non injured TA weight. **F,** Quantification of Tcf4+ cells per mm^2. **G,** Tcf4 immunostaining on TA cryosections. **H-I,** Quantification of relative Collagen+ area (**H**) and of sub-laminar Pax7+ cells (**I**, normalized by Pax7+ cells of non-injured TA). **J-K**, Type 1 Collagen (**J**) and Pax7 and Laminin (**K**) immunostaining on TA cryosections. Arrows in **K** indicate satellite cells. Scale bars: 50µm in **B**, **C**, **G**, **J**, **K,** 200µm in **D**. Nuclei are stained with Hoechst. Error bars: SEM.

progenitors pool is completely abrogated in absence of APC (Fig. 1C). To determine whether the observed phenotype reflected a transient delay in myogenesis or rather a permanent defect, we analyzed myogenic progression at a later developmental stage. At E13.5, the number of Pax7+ myogenic progenitors was drastically reduced in the trunk of APC DM-KO fetuses compared to control littermates (Fig. 1, D and G). Similarly, MyoD+ myoblasts were rarely detected in APC DM-KO embryos (Fig. 1, E and H). This was accompanied by a near total absence of Desmin+ myofibres at the thoracic (Fig. 1, F and I), limb and head levels (Fig. S2) in APC DM-KO embryos. RT–qPCR on E13.5 dorsal tissues confirmed a change in gene expression consistent with the absence of differentiated myofibres in APC DM-KO embryos compared to controls (Fig. 1J). We conclude that APC is required for embryonic myogenesis.

To understand APC function in adult regenerative myogenesis, we used inducible gene inactivation in the adult. APC gene was conditionally deleted in satellite cells by crossing $APC^{flox/flox}$ mice with tamoxifen-inducible $Pax7^{CreERT2}$ mice (*22*) (APC SC-KO). After four daily tamoxifen injections to 2-months old animals (see Methods), we observed an absence of APC protein and the accumulation of β-catenin protein in satellite cells of APC SC-KO Extensor Digitorum Longus (EDL) myofibres (Fig. S3, A and B). Following APC loss, satellite cells did not leave their quiescent state, since less than 1% were Ki67+ in both APC SC-KO and control mice one month following induction of Cre activity (not shown). Consistent with the low turnover rate of adult satellite cells, uninjured APC SC-KO Tibialis Anterior (TA) muscles appeared undistinguishable from controls 1 month after APC gene disruption (Fig. S3, C and D). To determine if APC mutant satellite cells can support injury-induced myogenesis, we injured TA muscles by cardiotoxin injection (*23*) (Fig. 2A). The early satellite cell-dependent events of regenerative myogenesis were abrogated following APC loss, as indicated by the total absence of accumulating Pax7+ progenitors (Fig. 2B) and of newly formed myofibres expressing embryonic myosin heavy chains (e-MyHC) (Fig. 2C) four days post injury. Two weeks after injury, control muscles were fully regenerated and composed of functional myofibres (1,629 ± 278 regenerated myofibres per mm^2), whereas APC SC-KO muscles dramatically failed to regenerate (9 ± 7 regenerated myofibres per mm^2) (Fig. 2D) with the remaining tissue accounting for less than half the contralateral uninjured muscle weight (Fig. 2E). In APC SC-KO mice, muscle tissue was replaced by a scar tissue composed of Tcf4+ fibroblasts (Fig. 2, F and G) and a Collagen Type I matrix (Fig. 2, H and J). To rule out the possibility that the excessive Tcf4+ cells derived from APC mutant satellite cells that lost their identity, we performed regeneration assays in Control and APC SC-KO adults bearing the double-reporter Z/EG allele (*24*). In control

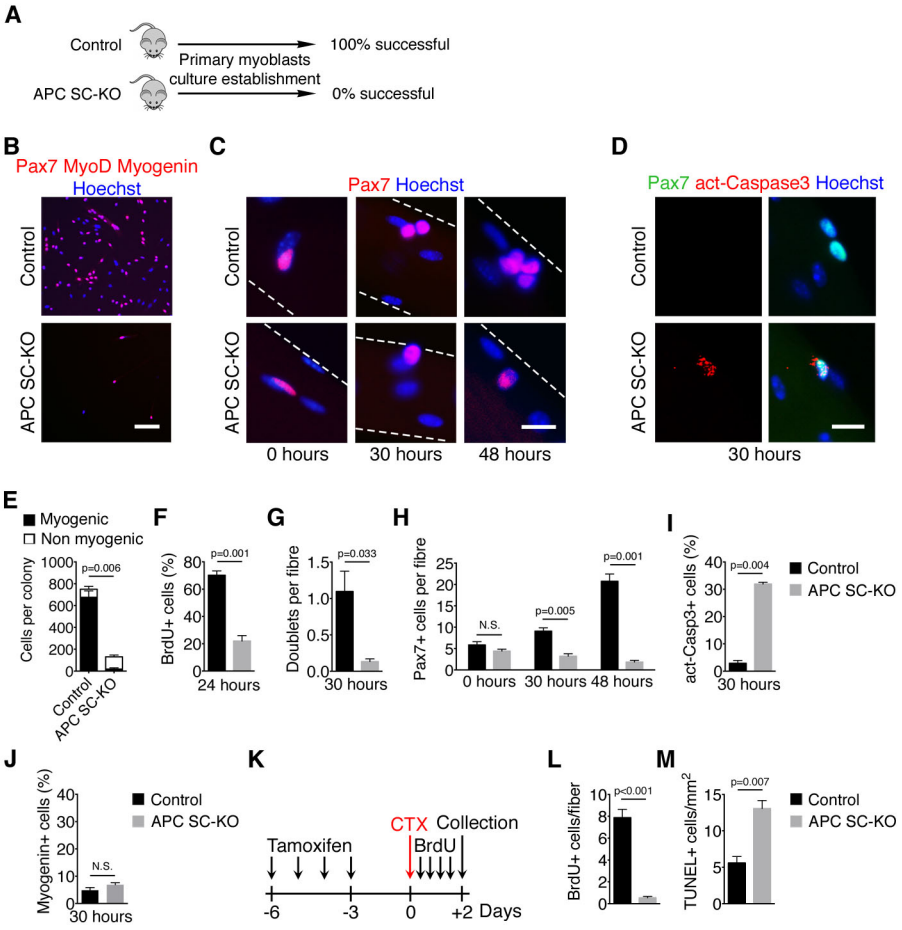

Fig. 3. APC-null satellite cells loose their myogenic potential and undergo apoptosis. A, Scheme of success ratio in generation of primary myoblasts culture. **B,** Pax7, MyoD and Myogenin immunostaining on myogenic colonies obtained from single EDL fibres cultured on matrigel for 7 days. **C,** Pax7 immunostaining on single EDL fibres isolated and fixed (0 hours) or cultured in floating conditions for 30 or 48 hours. **D,** Pax7 and activated Caspase3 (act-Casp3) immunostaining on EDL single fibres after 30 hours culture. **E,** Quantification of myogenic and non-myogenic cells per colony obtained in **B.** P-value refers to the myogenic proportion. **F,** Quantification of BrdU-incorporating satellite cells on EDL fibres after 24 hours culture. **G,** Quantification of satellite cells doublets per fibre after 30 hours culture. **H,** Quantification of **C**. **I,** Quantification of act-Casp3+ satellite cells after 30 hours culture. **J,** Quantification of the percentage of satellite cells expressing Myogenin after 30 hours culture. **K,** Tamoxifen, cardiotoxin (CTX) and BrdU regimen and regeneration assay scheme for control and APC SC-KO mice. **L,** Quantification of BrdU-incorporating satellite cells on regenerating fibres 2 days after injury. **M,** Quantification of apoptotic cells by TUNEL staining on TA sections 2 days after injury. Scale bars: 100µm in **B**, 10µm in **C** and **D**. Error bars: SEM.

conditions, all regenerated myofibres derived from the Pax7 lineage, while APC SC-KO muscles did not contain any eGFP+ cells (Fig. S4). Furthermore, we did not detect any Pax7+ cells in APC SC-KO TAs 14 days post injury, indicating that adult-specific APC mutant satellite cells cannot self-renew and repopulate the niche (Fig. 2, I and K). Thus, APC expression by satellite cells is required for injury-induced myogenesis in adulthood.

To determine whether the observed phenotype was cell-intrinsic or depended on the muscle microenvironment, we tried unsuccessfully to grow *in vitro* satellite cells-derived primary myoblasts isolated from APC SC-KO muscles using a widely used protocol that routinely works in our hands (*25*) (Fig. 3A). We then isolated single myofibres with their associated satellite cells from EDL muscle and plated them on matrigel. In these conditions, satellite cells leave their niche and migrate away from the fibres to generate myogenic colonies. Compared to controls, conditional APC-inactivated satellite cells were defective in expansion and failed to establish myogenic colonies (Fig. 3, B and E). To identify the cellular events involved in this process, we studied satellite cells behavior in their niche at the surface of isolated single myofibres in “floating” culture conditions (Fig. 3C). We observed that satellite cells with targeted APC gene disruption did not incorporate BrdU and did not divide after respectively 24hrs and 30hrs of culture (Fig. 3, F and G). As a consequence, while the number of myofibre-associated satellite cells was not different between control and APC SC-KO myofibres directly after isolation, APC-null satellite cells were unable to amplify and give rise to Pax7+ cells clusters (Fig. 3H). More specifically, we observed that satellite cells of APC SC-KO myofibres expressed the active form of Caspase-3 (Fig. 3, D and I), indicating that they were undergoing programmed cell death. Importantly, we did not observe accelerated differentiation (Myogenin+ cells) in APC-null satellite cells (Fig 3J). To test whether cell cycle arrest and programmed cell death are responsible for the regeneration failure of APC SC-KO mice, we performed BrdU incorporation assays following CTX injury and analyzed satellite cell at the earliest time point (48 hours post injury) (Fig. 3K). We observed that, in contrast to control satellite cells, APC-null satellite cells did not incorporate BrdU, demonstrating that they do not enter into S phase *in vivo* (Fig. 3L). TUNEL staining further revealed an increase in the number of apoptotic cells in APC SC-KO muscles compared to controls (Fig. 3M). Taken together our results suggest that APC is necessary for satellite cells survival at the time of exit from quiescence.

To understand the molecular mechanisms underlying the requirement of APC by satellite cells, and since APC SC-KO satellite cells did not grow *in vitro*, we silenced APC in proliferating wild-type satellite cells-derived primary myoblasts (Fig. S5A) and analyzed global gene expression 48h after transfection using Affymetrix mRNA microarrays. APC knockdown led to downregulation of 337 and upregulation of 291 transcripts relative to mock treated cells, with a false discovery rate (FDR) of less than 5% (Data available on the GEO database, accession number GSE57898). Differentially expressed genes had enriched gene ontology terms belonging to regulatory pathways associated with nucleotide binding, chromosomes and cell cycle (Table S1). Ingenuity Pathway Analysis demonstrated enrichment of programmed cell death pathway along with reduction of cell cycle, cellular assembly and DNA replication pathways (Fig. S5B). Overall, changes in gene expression were consistent with apoptosis of APC-deficient primary myoblasts and indicated a link with impaired cell cycle progression. More specifically, we observed that transcription of Proliferating Cell Nuclear Antigen (PCNA) and several factors that associate with PCNA during DNA replication were downregulated following APC silencing (Fig. S5C). On single myofibres, quiescent satellite cells do not express PCNA while almost all activated satellite cells express high levels of PCNA proteins (Fig. S5D). APC-null satellite cells were unable to express PCNA proteins (Fig. S5E), and in these conditions PCNA silencing on single myofibres impaired satellite cell amplification, similarly to APC silencing (Fig. S5F).

Since APC is a multi-functional protein, we aimed to understand which signaling cascade could explain the observed phenotype. By analyzing our microarray data with the Upstream Regulators tool of the Ingenuity software, we identified WNT3A as one of the pathways activated following APC silencing (Fig. S6A). Indeed, 23 known canonical Wnt target genes were upregulated in APC-depleted primary myoblasts (Fig. S6B). We then determined how canonical Wnt signaling is regulated satellite cells *ex vivo* and *in vivo*. We found that β-catenin was uniquely cytoplasmic (inactive signaling) in quiescent satellite cells and in satellite cells undergoing their first division. However, strong β-catenin nuclear accumulation (active signaling) was detected in the progeny of satellite cells at the time of fate choice decision and cell cycle exit (Fig. S7A). Accordingly, expression of the Wnt reporter allele $Axin2^{LacZ}$ was not detected in uninjured muscles but strongly marked myogenin-expressing differentiating satellite cells during muscle regeneration (Fig. S7B). These observations suggest that Wnt/β-catenin signaling is inactive during satellite cells quiescence and activation, and becomes activated upon myogenic commitment. Thus, APC-null satellite cells present unscheduled aberrant activation of canonical Wnt signaling.

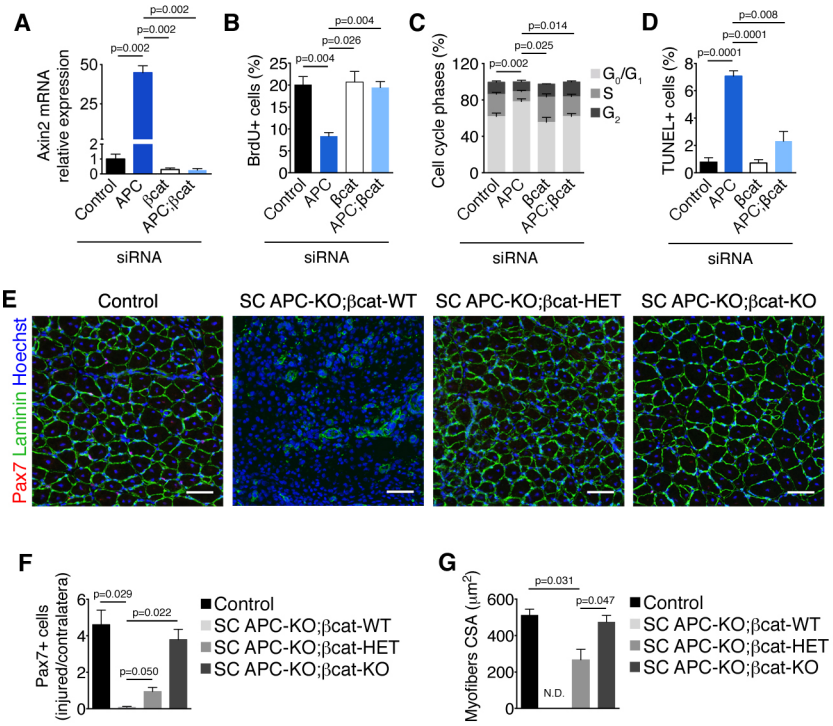

Figure 4. APC controls satellite cell proliferation and survival by dampening Wnt/β-catenin signaling. **A,** RT-qPCR analysis of the canonical Wnt target Axin2 expression shows that Wnt/β-catenin over-activation is blunted in APC;β-catenin double silenced cells. **B,** Quantification of BrdU+ primary myoblasts 48 hours after siRNA transfection. **C,** Distribution of cells in each cell cycle phase assessed by quantification of Propidium Iodide (PI) incorporation on primary myoblasts 48 hours after siRNA transfection. P-value refers to S phase. **D,** Quantification of apoptotic index by TUNEL staining on primary myoblasts 48 hours after siRNA transfection. **E,** TA cryosections 14d after injury immunostained for Pax7 and Laminin. Nuclei stained with Hoechst. Scale bar, 50µm. **F,** Quantification of sub-laminar Pax7+ cells 14 days after injury (normalized by Pax7+ cells of non-injured TA). **G,** Quantification of TA muscle fibres cross sectional area (CSA) 14 days after injury. Error bars: SEM.

To test whether the phenotype of APC-depleted cells was a direct consequence of canonical Wnt signaling over-activation, we simultaneously silenced APC and inhibited the Wnt/β-catenin pathway by combining APC and β-catenin siRNAs *in vitro* (Fig. S8A and Fig. 4A). Strikingly, while simple APC silencing blocked BrdU incorporation and decreased the percentage of S-phase cells in proliferating primary myoblasts, silencing of both APC and β-catenin restored normal cell cycle progression (Fig. 4, B and C). This was associated with the rescue of adequate G1-S checkpoint genes expression in APC;β-catenin double-silenced cells (Fig. S8B). In this experimental setup, we observed that the induction of programmed cell death in primary myoblasts following APC silencing requires β-catenin, as quantified by TdT-mediated dUTP Nick End Labeling (TUNEL) analysis (Fig. 4D). To determine whether β-catenin inactivation could compensate for APC loss *in vivo*, we conditionally deleted both genes in adult satellite cells. We used the $Pax7^{CreERT2}$ allele to recombine both APC floxed alleles along with one (SC APC-KO; βcat-HET) or two (SC APC-KO; βcat-KO) β-catenin floxed alleles (*26*) in adult mice. Strikingly, CTX-induced muscle regeneration was completely restored in TA muscles with APC;β-catenin double inactivated satellite cells (Fig. 4E), as assessed by quantification of satellite cell numbers (Fig. 4F) and regenerated fibre size (Fig. 4G) two weeks after injury. Importantly, recombination of only one β-catenin allele resulted in a partial rescue of APC genetic disruption. In this context muscle regeneration occurred but satellite cells could not repopulate their niche and the regenerated myofibres were smaller as compared to single or double inactivated TAs. These results genetically demonstrate that cell cycle arrest and apoptosis following APC inactivation are triggered by β-catenin over-activation. Therefore, APC is required for satellite cell functions during muscle repair by dampening β-catenin signaling.

Here we report for the first time that APC is absolutely necessary for both embryonic and adult myogenesis. We describe that APC inactivation in stem cells of a low turnover tissue results in inhibition of proliferation and programmed cell death. This is in contrast with previous observations of several tissues with high turnover rate, in which APC acts as a tumor suppressor. Interestingly, these constantly renewing tissues have high tumor incidence and it has been demonstrated, at least in some cases, that tumors derive from residing stem cells (*27*). In these cells, β-catenin hyperactivation following APC loss promotes unscheduled proliferation leading to neoplastic transformation (*28*,*29*). Unexpectedly, this paradigm cannot be extended to skeletal muscle. This not only supports the evidence that tissue-specific stem cells have different susceptibility to cancer initiation, but also suggests that these differences rely on tissue-specific functions of APC and Wnt/β-catenin pathway. Clinically, APC or other

canonical Wnt signaling regulators can be targeted (*30*) to drive the expansion of human satellite cells or induced pluripotent stem (iPS)-derived myogenic cells for skeletal muscle tissue repair with far-reaching medical impact.

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Supplementary Materials:

-Materials and Methods

-Supplementary Figure S1-S7

-Supplementary Tables 1-3 (Tables available only in the electronic version of the manuscript)

Materials and Methods

Mice. Experimental animal protocols were performed in accordance with the guidelines of the French Veterinary Department and approved by the University Paris-Descartes Ethical Committee for Animal Experimentation. All experiments were performed on age and sex matched mice, with an equal ratio of male to female mice. Generation and genotyping of the $Myf5^{Cre}$, $Pax7^{CreERT2}$, $APC^{flox/flox}$, b-$catenin^{flox/flox}$ and Z/EG lines have been described previously.

Induction of Cre activity and Cardiotoxin injury. Intraperitoneal (i.p.) injections of tamoxifen from MP Biomedicals at 5 µL per gram body weight of 20 mg mL^{-1} diluted in corn oil were administrated to 2-months old mice daily for 4 days prior to injury. Formuscle injury experiments, mice were anaesthetized by i.p. injection of Ketamin-Xylazin (Centravet) at 10 µL per gram body weight of 80-10 mg mL^{-1} diluted in saline. Mouse legs were cleaned with alcohol and Tibialis Anterior muscles were injected with 35µL of cardiotoxin solution (Latoxan) (12µM, diluted in saline) using an insulin needle (3/10cc Insulin Syringe from Becton Dickinson). For *in vivo* proliferation assays, 4 i.p. injections of BrdU at 5µl per gram of body weight of 10 mg mL^{-1} diluted in saline.

Histology and immunohistochemistry. For cryosections, embryos and skeletal muscles were embedded in Tissue-Tek O.C.T. Compound (Gentaur), frozen on 2-methylbutane-cooled liquid nitrogen, and processed for cryostat sectioning. 10-µm sections were collected from the thoracic, limb and head regions of embryos or the midbelly of muscles. Immunohistochemistry was performed by fixation with 4% PFA/PBS, processing for antigen retrieval with the Antigen Unmasking Solution (Vector) at 95°C for 15 minutes in a thermostated microwave, permeabilization with 0.2% Triton X-100 in PBS, blocking with 5% Heat inactivated serum/0.2% Triton X-100/1% BSA/PBS, incubation with primary antibody overnight at 4°C, then incubation with Alexa-Fluor secondary antibodies for 1 h at room temperature. Nuclei were counterstained with Hoechst and slides mounted in fluorescent mounting medium (DAKO). Primary antibodies are as follows: mouse b-catenin (BD Bioscience) goat Collagen Type I (Southern Biotech.), rabbit Desmin (Abcam), rabbit GFP (Life Tech.), rabbit Ki67 (Abcam), rabbit Laminin (Sigma), mouse MyoD (Dako), mouse embryonic-MyHC (DSHB), mouse Pax3 (DSHB), mouse Pax7 (DSHB), rabbit Tcf4 (Cell Signaling Tech.). The TdT-mediated dUTP nick end labelling (TUNEL) reaction was performed by using the In situ cell death detection kit (Roche) following manufacturer's instructions for tissue cryosections.

Single myofibres isolation and primary myoblasts preparation. Single myofibres were isolated from the EDL muscle by Collagenase type I digestion and gentle triturating, as

previously described[25]. Isolated myofibres were cultured in suspension for up to 2 days in 6-well plates coated with horse serum to prevent fibre attachment. Alternatively, myofibres were let to adhere to Petri dishes coated with 20% Matrigel (BD Biosciences) in DMEM (Life Technologies) and cultured for 7 days. Fibres were incubated in plating medium consisting of 15% Fetal Bovine Serum (Hyclone) and 1% Chick Embryo Extract (Accurate Chemicals) in DMEM. For isolation of primary myoblasts, skeletal muscles of the hindlimbs of 6- to 8-week-old mice were dissected, with care to take off as much fat and connective tissue as possible. Muscles were transferred to a sterile 6cm Petri dish on ice, mulched into a smooth pulp and incubated in CollagenaseB/DispaseII/CaCl2 solution (Roche, respectively 1.5 U/mL; 2.4 U/mL and 2M in DMEM). Following a 15 minutes incubation at 37°C, the muscle pulp was triturated with a heat polished glass Pasteur pipettes, and this incubation/trituration step was repeated once. Tissue digestion was stopped with addition of FBS, cells were filtered, washed twice with PBS and resuspended in growth medium consisting of Ham's F10 (Life Technologies) supplemented with 20% FBS and 2.5 ng/µL of basic FGF (RnD systems). After 2 hours of pre-plating in a non-coated 10cm plate, the medium was transferred onto collagen-coated Petri dishes. Cultures were maintained in growth medium until cells reach 80% confluence. To enrich cell cultures in myoblasts and eliminate contaminating fibroblasts, cell dishes were tapped. Thanks to differential adhesion, this led only myoblasts to be detached and replated onto new plates

RNA interference. Wild-type primary myoblasts were transfected with the following Silencer Select Pre-designed small interfering RNA (siRNA) sequences (Life Technologies): APC: 5'-CAGCUGACCUAGCCCAUAAtt, b-catenin: 5'-CACUUGCAAUAAUUACAAAtt-3'. siRNA transfection was performed using Lipofectamine 2000 and Optimem (Life Technologies) following manufacturer's protocol.

Immunocytochemistry. Cells were fixed with 4% PFA/PBS, permeabilized with 0.2% Triton X-100/PBS, blocked with 5% Heat inactivated serum/PBS, incubated with primary antibody for at least 1 hour, then incubated with Alexa-Fluor secondary antibodies for 1 hour. Nuclei were counterstained with Hoechst and slides mounted in Fluorescent Mounting Medium (Dako). Primary antibodies are as follows: rabbit APC (Abcam), mouse BrdU (Dako), rabbit activated-Caspase3 (Cell Signaling Tech.), rabbit activated- b-catenin (Cell Signaling Tech.), mouse MyoD (Dako), rabbit Myogenin (SCBT Inc.), mouse Pax7 (DSHB), rabbit PCNA (Bethyl Lab.). Secondary antibodies were conjugated with Alexa-488 or Alexa-550 fluorochromes. The TdT-mediated dUTP nick end labelling (TUNEL) reaction was performed by using the In situ cell death detection

kit (Roche) following manufacturer's instructions for adherent cell layers. For cell proliferation studies, cells were incubated for 40 minutes with culture medium containing 30µM BrdU and processed for immunostaining with Pax7 and BrdU antibodies after fixation and treatment with 2N HCl for 20 minutes.

Image acquisition and quantitative analysis. Immunofluorescent stainings were analyzed with a Zeiss Axiovert 200M microscope with x20 magnification and with a Nikon AZ100 macroscope with x5 magnification. NIS-Element, Metamorph and Photoshop softwares were used for image acquisition and processing in compliance with Nature's guide for digital images. All quantifications were done with ImageJ software.

Quantitative real-time PCR (qPCR). Total RNA was extracted from either mouse tissue or cultured cells with TRIzol Reagent (Life Technologies) and treated with DNA-free kit (Life Technologies) to remove any possible DNA contamination. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) with 500ng of RNA as input. Gene expression was assessed on LightCycler 480 Real-Time PCR Systems (Roche) using LightCycler 480 SYBR green I Master (Roche) with specific primers. Transcripts levels were determined by absolute quantification using a 4-point standard curve and relative gene expression was calculated by normalization against 18S and cyclophylin reference genes. Sequences of the primers used for real-time PCR were as follows: 18S-Fwd: GTAACCCGTTGACCCCATT, 18S-Rev: CCATCCAATCGCTAGTAGCG, APC-Fwd: GCAGCTACAGGGAAGTATTGAA, APC-Rev: TCTCCTGAACGGCTGGATAC, Axin2-Fwd: AAGAGAAGCGACCCAGTCAA, Axin2-Rev: CTGCGATGCATCTCTCTCTG; b-catenin-Fwd: ATGGAGCCGGACAGAAAAGC, b-catenin-Rev: CTTGCCACTCAGGGAAGGA; Cdc6-Fwd: CCCGGGATTGTGAAGTAAAA, Cdc6-Rev: GGAGATAACCGGGGAGTGTT, Cdkn1a-Fwd: TTGTCGCTGTCTTGCACTCT, Cdkn1a-Rev: AATCTGTCAGGCTGGTCTGC, Chek1-Fwd: TCGTGAACGCTTACTGAACAA, Chek1-Rev: ACAGCTATCACTGGGCTGGT, Cyclophilin-Fwd: AAGAAGATCACCATTTCCGACT, Cyclophilin-Rev: TTA CAG GAC ATT GCG AGC, MyH3-Fwd: AGAGGAGAAGGCCAAAAGG, MyH3-Rev CCTTCCAGCTCAAACTCCAG, Myogenin-Fwd: GAAAGTGAATGAGGCCTTCG, Myogenin-Rev: ACGATGGACGTAAGGGAGTG, Pax7-Fwd: CTGGATGAGGGCTCAGATGT, Pax7-Rev: GGTTAGCTCCTGCCTGCTTA.

Microarray and bioinformatics. Total RNA was prepared from primary myoblasts using TRIzol Reagent (life Technologies). After validation of the RNA quality with Bioanalyzer 2100 (using Agilent RNA6000 nano chip kit), 50 ng of total RNA is reverse transcribed following the Ovation PicoSL WTA System (Nugen). Briefly, the resulting double strand cDNA is used for amplification based on SPIA technology. After purification according to Nugen protocol, 5ug of single strand DNA is used for generation of Sens Target DNA

using Ovation Exon Module kit (Nugen). 2.5µg of Sens Target DNA are fragmented and biotin labelled using Encore Biotin Module kit (Nugen). After control of fragmentation using Bioanalyzer 2100, cDNA is then hybridized to GeneChip® Mouse Gene 1.0 ST (Affymetrix) at 45°C for 17 hours. After overnight hybridization, chips are washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned using the GCS3000 7G. The scanned images are then analyzed with Expression Console software (Affymetrix) to obtain raw data and metrics for Quality Controls. The observations of some of these metrics and the study of the distribution of raw data show no outlier experiment. Affymetrix probe-set data were normalized using the robust multi-array average (RMA) method using R. Gene expression levels were compared using one-way ANOVA. Lists of differentially expressed genes are presented in Supplementary Table 1. Differentially expressed genes ($P < 0.01$, fold change >1.5) were functionally annotated according to gene ontology terms and enriched terms were calculated using DAVID[33] (Supplementary Table 2). Networks were generated with Ingenuity pathways analysis (Ingenuity Systems, http://www.ingenuity.com) using the Upstream Regulators and the Diseases&Functions tools that predict the activation status of signaling cascades and biological processes. The heat map with microarray data was created by using the matrix2png web interface (http://www.chibi.ubc.ca/matrix2png/bin/matrix2png.cgi).

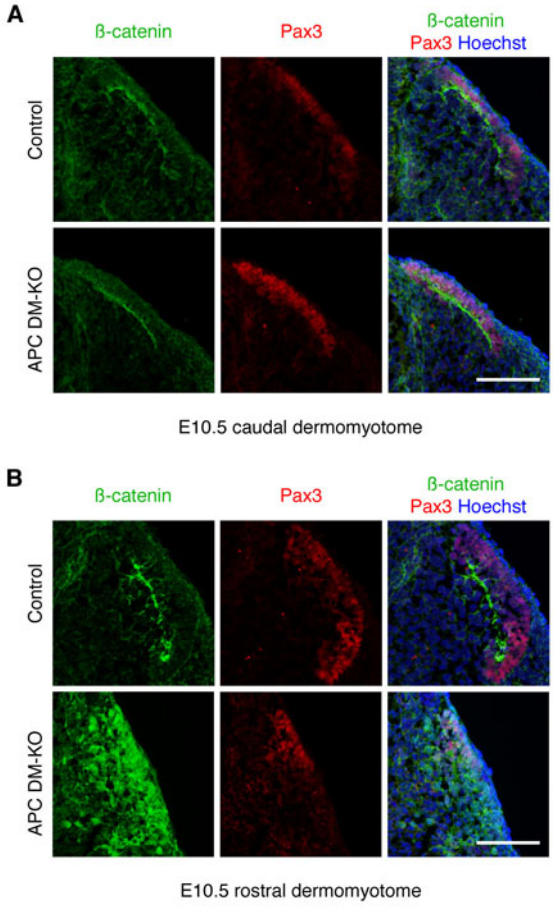
Western blot analysis. Cells were lysed in RIPA Buffer (SIGMA) supplemented with Complete Protease Inhibitor Cocktail (Roche). Equal amounts of proteins were prepared with NuPAGE LDS Sample Buffer and Reducing Agent (Life Technologies), separated on NuPAGE® Novex® 3–8% Tris-Acetate Gels (Life Technologies) and transferred on nitrocellulose membranes (Biorad). After blocking in 5% milk, 0.1% Tween-20/TBS, membranes were incubated with primary antibodies overnight and then with HRP-conjugated secondary antibodies for 1 hour. Specific signals were detected by chemiluminescence system (GE Healthcare). The following primary antibodies were used: rabbit APC (provided by K. Neufeld, University of Kansas, USA), mouse HSC70 (SCBT).

Flow cytometric analysis for cell cycle. For cell cycle analysis cells were trypsinized, collected in cold PBS and fixed in 70% ethanol overnight at 4°C. After PBS wash, cells were incubated with 50ug/ml Propidium Iodide (Sigma-Aldrich), 50ug/mL RNase A (Roche) and 0.1% Tween-20 in PBS for 15 minutes and then analyzed using an Accuri C6 flow cytometer (BD Biosciences). Cell cycle phases were determined by using the cell cycle function of FlowJo software Tristar).

Statistical analysis. Each histological analysis of embryo or adult skeletal muscle was performed on at least four samples per genotype. No randomization or blinding was

used and no animals were excluded from analysis. For cell culture studies, at least three independent experiments were performed in duplicate and at least six random fields were imaged per sample. Data are presented as mean ± s.e.m. Differences between groups were tested for statistical significance using an unpaired two-tailed Student's t-test. $P<0.05$ was considered statistically significant. All statistical analyzes were performed using GraphPad Prism software.

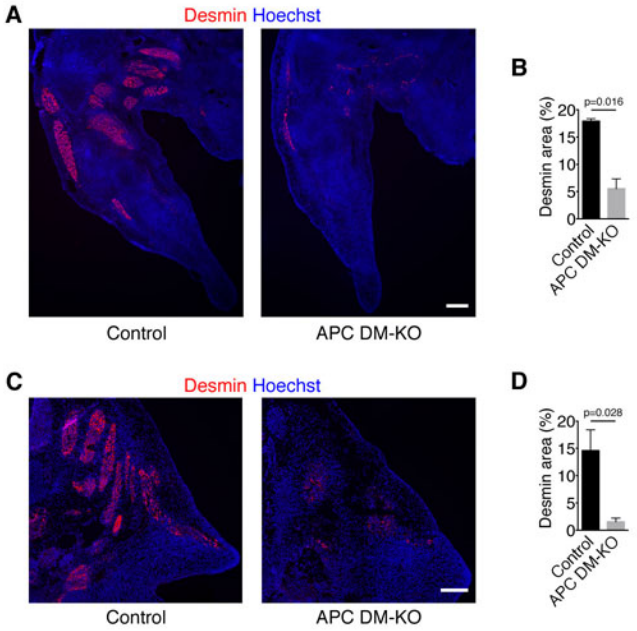
Supplementary Figures S1-S8



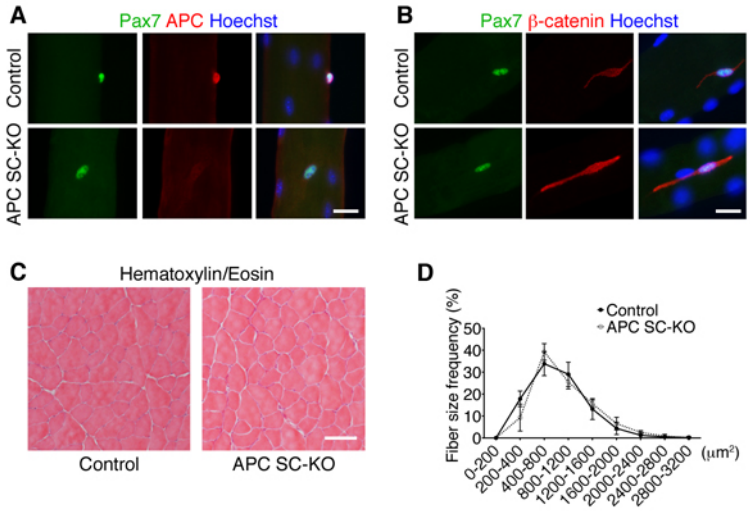
C

Age	Total pups	Frequency	Cre⁻ APC$^{+/LoxP}$	Cre⁺ APC$^{+/LoxP}$	Cre⁻ APC$^{LoxP/LoxP}$	Cre⁺ APC$^{LoxP/LoxP}$
<E16.5	31	Observed	8 (25%)	7 (22%)	8 (25%)	9 (28%)
		Expected	8 (25%)	8 (25%)	8 (25%)	8 (25%)
P7	48	Observed	21 (44%)	19 (40%)	8 (17%)	0 (0%)
		Expected	12 (25%)	12 (25%)	12 (25%)	12 (25%)

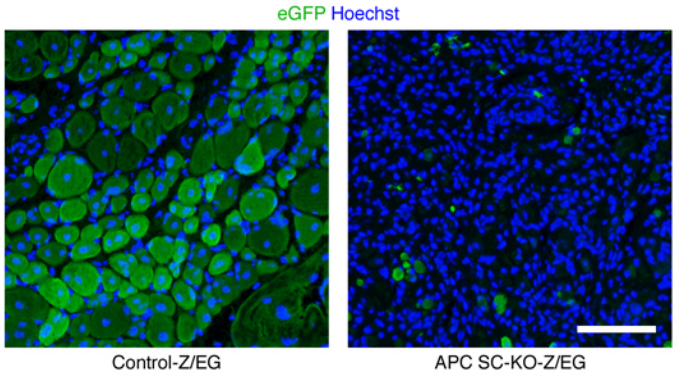
Supplementary Fig. 1. Embryonic APC gene disruption in the Myf5 lineage results in β-catenin stabilization and is lethal perinatally. A-B, Transverse sections of E10.5 caudal (**A**) and rostral (**B**) somites immunostained for β-catenin and Pax3. In the Myf5^{Cre} driver line, the Cre recombinase is expressed when dermomyotome cells are specified into the myogenic lineage. In earlier caudal dermomyotome, APC recombination has not taken place yet, as demonstrated by the evidence that β-catenin expression pattern of APC DM-KO embryos is comparable to controls. By contrast, in later rostral tissues of APC DM-KO embryos, β-catenin accumulated in the nucleus of DM cells and this is accompanied by alteration of dermomyotome architecture. This shows that APC recombination occurs during dermomyotome maturation, when myogenic progenitors acquire their myogenic identity. **C,** Expected and observed frequencies of litters genotypes. Whereas APC DM-KO embryos were observed at expected Mendelian ratios, no live APC DM-KO pups were obtained due to perinatal lethality. Scale bars: 50µm. Nuclei stained with Hoechst.



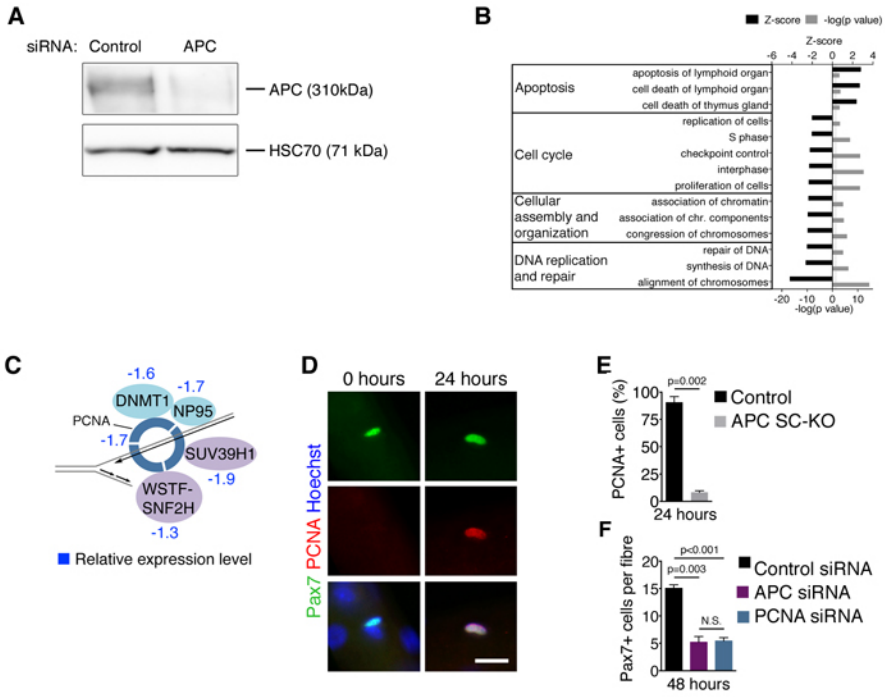
Supplementary Fig. 2. APC gene disruption in the Myf5 lineage abrogates head and limb muscle formation. A, Desmin immunostaining on longitudinal sections of E13.5 limbs. **B,** Quantification of relative Desmin area on E13.5 limbs. **C,** Desmin immunostaining on transverse sections of E13.5 head. **D,** Quantification of relative Desmin area on E13.5 head. Both limb and head muscle formation is defective following APC gene disruption. Scale bars: 100µm. Nuclei stained with Hoechst.



Supplementary Fig. 3. Conditional APC gene disruption in the Pax7 lineage results in β-catenin stabilization in adult satellite cells and does not affect skeletal muscle tissue integrity. Both control and APC SC-KO mice were subjected to tamoxifen (TM) administration for four consecutive days. **A,** Pax7 and APC immunostaining on single EDL myofibres seven days after TM treatment. Control satellite cells express APC, whereas satellite cells associated with myofibres isolated from APC SC-KO animals are negative for APC immunostaining, demonstrating efficient APC gene disruption. **B,** Pax7 and β-catenin immunostainings on single EDL myofibres shows that β-catenin is stabilized following APC knockout. **C,** Hematoxylin and Eosin staining on uninjured TA cryosections TM treatment. **D,** Distribution of myofibres cross sectional area (CSA) shows no significant difference between non injured TAs of control and APC SC-KO TAs 30 days after TM treatment.



Supplementary Fig. 4. APC-null satellite cells do not participate to adult muscle regeneration. GFP immunostaining on transverse sections of TAs isolated from Pax7^{CreER};Z/EG (Control-Z/EG) and Pax7^{CreER}APC$^{lox/lox}$;Z/EG (APC SC-KO-Z/EG) 14 days after injury. After TM-induced recombination of the Z/EG reporter allele, satellite cells constitutively express eGFP and their progeny can be traced during regeneration. 14 days after injury, newly formed fibres are positive for eGFP in control TAs, demonstrating that they arise from satellite cells. In APC SC-KO-Z/EG TAs eGFP is barely expressed, demonstrating that APC-null satellite cells do not give rise to new myofibres, nor trans-differentiate in other cell types. Nuclei stained with Hoechst. Scale bar: 50µm.



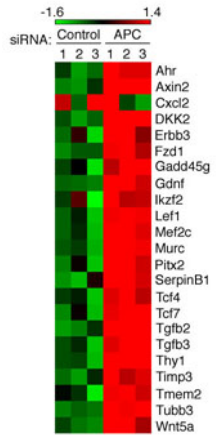
Supplementary Fig. 5. APC loss affects DNA replication and cell cycle progression. A, APC western blotting shows a decrease in APC protein level on proliferating primary myoblasts transfected with a siRNA targeting APC. **B,** Representation of 14 most relevant biological functions and their categories predicted by microarray analysis to be activated (z-score > 2) or inhibited (z-score < -2) following APC silencing as compared with control proliferating myoblasts. A full list of all biological functions and associated z-scores is reported in Supplementary Table 3. **C,** Schematic representation of DNA replication fork and the associated factors. Numbers indicate the fold changes of the expression levels obtained by microarray between control and APC-silenced primary myoblasts. **D,** Pax7 and PCNA immunostaining on EDL single myofibres isolated from wild type animals and directly fixed (0 hours) or cultured for 24 hours. Nuclei stained with Hoechst. Scale bar, 10µm. **E,** Quantification of the percentage

of satellite cells expressing PCNA on single fibres isolated from control and APC SC-KO EDL and cultured for 24 hours. **F**, Quantification of the number of satellite cells per fibre on EDL single fibres transfected with Control, APC or PCNA siRNA and cultured for 48 hours.

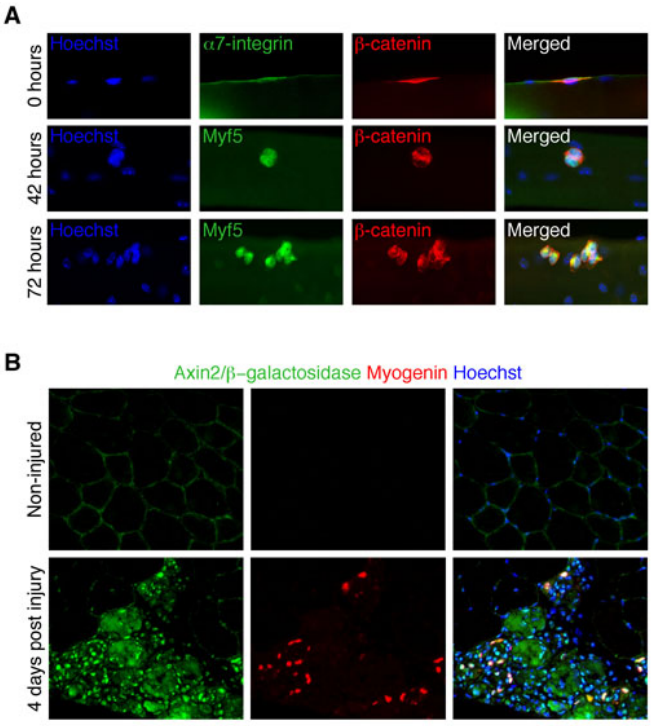
A

Upstream Regulator	Activation Z-score	p-value of overlap
NUPR1	6.595	3.39E-24
TP53	5.902	5.27E-45
CDKN2A	5.056	7.41E-22
TGFB1	4.383	1.62E-21
let-7	4.353	7.07E-24
WNT3A	4.180	1.05E-10
SMARCB1	3.990	7.07E-11
CDKN1A	3.935	8.69E-44
tretinoin	3.856	8.23E-05
TCF3	3.785	8.58E-14

B

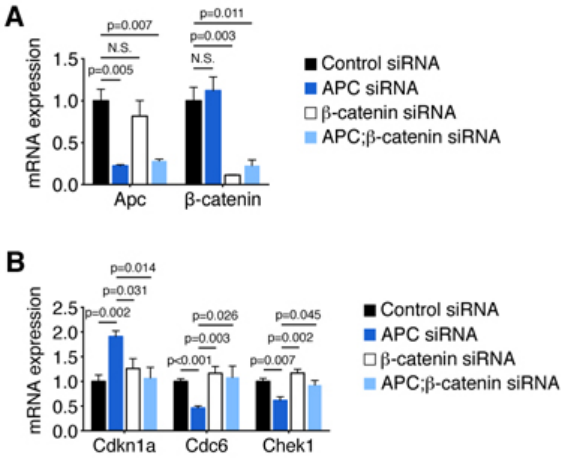


Supplementary Fig. 6. APC loss activates canonical Wnt signalling. A, Table depicting the 10 upstream regulators with highest activation z-score identified by pathway analysis of microarray comparing Control and APC-silenced primary myoblasts. **B.** Heat map representing the expression level of genes belonging to canonical Wnt pathway (Wnt3a) identified by pathway analysis of microarrays comparing control and APC-silenced primary myoblasts. 1, 2, 3 indicate three independent experiments. Each row is normalized to have mean zero, variance one.



Supplementary Fig. 7. Canonical Wnt signaling is activated during satellite cell myogenic progression. A, Immunofluorescent staining for a7-integrin, Myf5 and b-catenin on single EDL fibres isolated and fixed (0 hours) or cultured for 42 and 72 hours. b-catenin is detected only in cytoplasm of quiescent satellite cells and accumulates in the nucleus only after 72 hours of culture. This indicates that b-catenin subcellular localization is modulated during myogenic lineage progression. **B,** b-galactosidase and Myogenin immunostaining on sections of non-injured TA muscles and injured TA muscles sampled 4 days post CTX injection from Axin2$^{LacZ/+}$ mice, in which the lacZ gene coding for the b-galactosidase

protein has been inserted within the Axin2 locus. This enables to monitor canonical Wnt signaling activity. In non-injured muscles b-galactosidase is not expressed. Conversely, 4 days after injury, differentiating myocytes that express Myogenin are also positive for b-galactosidase, suggesting that canonical Wnt signaling is only active in differentiating cells and not in quiescent satellite cells.



Supplementary Fig. 8. Simultaneous knockdown of APC and β-catenin restores G1/S progression. A, RT-qPCR analysis of APC and β-catenin mRNA level confirms efficient reduction of expression levels of genes targeted by siRNA transfection in primary myoblasts. **B,** RT-qPCR analysis of three markers of G1/S transition. Cdkn1a induction and concomitant decrease of cdc6 and chek1 expression following APC knockdown reflect a G1/S arrest.

II. Canonical Wnt signaling is required for myogenic differentiation during embryonic and adult myogenesis

Conditional knockout of APC in early dermomyotome progenitors revealed that canonical Wnt signaling is not active during cell fate choice to allow proper myogenic commitment. However, the severity of the phenotype resulting from APC loss prevented further investigation of the role of Wnt/β-catenin pathway during myogenic differentiation. Similarly, our results obtained in adult muscle harboring conditional APC deletion in satellite cells demonstrated that APC-mediated inhibition of canonical Wnt signaling is required for proper satellite cell activation and cell cycle progression. Interestingly, we found that the Wnt/β-catenin pathway is activated in committed and differentiating myogenic cells during adult myogenesis, suggesting a functional role in this context. Therefore we undertook a canonical Wnt signaling loss-of-function in both embryonic and myogenic progenitors and we analyzed their differentiation ability in absence of β-catenin. During my PhD I obtained some preliminary results, presented hereafter, which served as the starting point for a postdoctoral project carried out by Dr. Anja Rudolf in our team.

Conditional knockout of β-catenin perturbs myogenic differentiation during embryonic myogenesis

To genetically address the role of canonical Wnt signaling during embryonic myogenesis, we crossed β-catenin$^{flox/flox}$ mouse line with the Myf5^{Cre} driver line. Immunofluorescent staining revealed a total absence of β-catenin protein within dermomyotomal myogenic progenitors of conditional β-catenin mutants (β-cat DM-KO) at embryonic day (E) 10.5 (Fig. 1A). This demonstrates that in this model Cre-mediated recombination efficiently abrogates β-catenin expression specifically in dermomyotomal myogenic without altering other lineages.

We analyzed myogenic lineage progression and differentiation at E13.5, when the wave of primary myogenesis is accomplished. At this stage, β-cat DM-KO embryos displayed reduced body size as compared to control littermates and showed strong defects at the dorsal level, with evident sparse hemorrhages (Fig. 1B). This is consistent with previous observations that β-catenin is required for development of dorsal dermis deriving from the dermomyotome (Atit et al., 2006). At the muscular level, the morphology of developing muscles was comparable in control and β-cat DM-KO embryos (Fig. 1C). Similarly, the number of Pax7-expressing myogenic progenitors

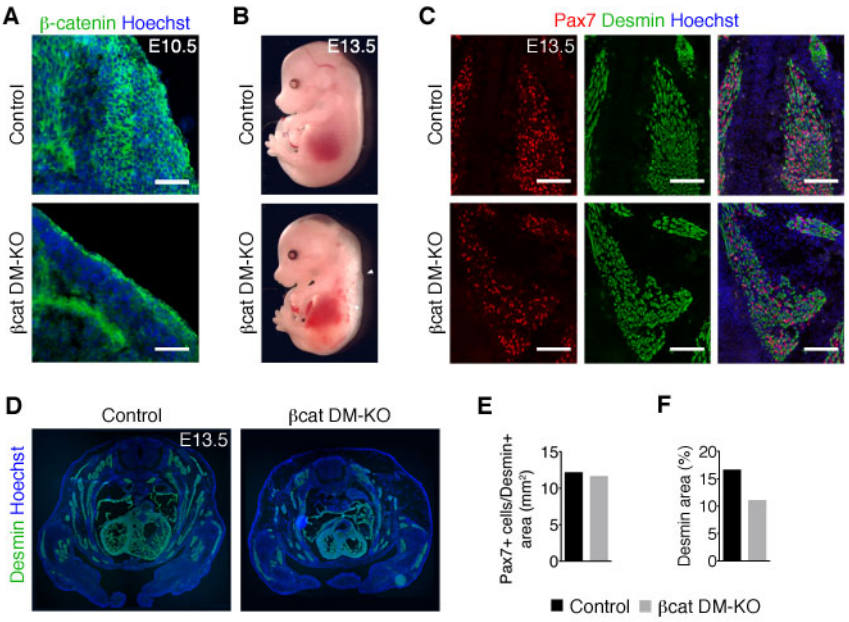


Fig. 1. β-catenin is required for embryonic myogenic differentiation. **A,** Transverse sections of E10.5 control ($Myf5^{Cre}$) and β-cat DM-KO ($Myf5^{Cre}$; β-$catenin^{lox/lox}$) embryos immunostained for β-catenin showing specific loss of β-catenin expression in the dermomyotome. **B,** E13.5 control and β-cat DM-KO whole embryos. **C,** Transverse sections of E13.5 dorsal regions at the forelimb level immunostained for Pax7 and Desmin. **D,** Whole transverse sections of E13.5 embryos stained for Desmin. **E,** Quantification of the number of Pax7+ cells within each developing muscle at E13.5, normalized by the muscle (Desmin+) area. **F,** Quantification of relative Desmin+ area in E13.5 embryos. Nuclei stained with Hoechst. Scale bars: 100μm in **A**, 50μm in C. Quantification performed only on 1 control and 1 β-cat DM-KO embryo.

within each muscular mass was not affected following β-catenin loss (Fig. 1, C and E). However, when we measured the total area occupied by desmin-expressing differentiated myofibers we detected a slight decrease in β-cat DM-KO embryo compared to control littermates (Fig. 1D, F).

These preliminary results are in line with what observed following dermomyotome-targeted APC knockout, since they show that β-catenin is dispensable for the generation of Pax7-expressing myogenic progenitors. Furthermore, they suggest that a wave of Wnt signaling activation is required in committed progenitors to allow proper myogenic differentiation.

Conditional knockout of β-catenin in satellite cells affects their myogenic differentiation

In order to determine whether canonical Wnt signaling is also required for adult myogenic differentiation, we inactivated the β-catenin gene specifically in adult satellite cells (β-cat SC-KO) by crossing β-$catenin^{flox/flox}$ mice with $Pax7^{CreERT2}$ mice, in

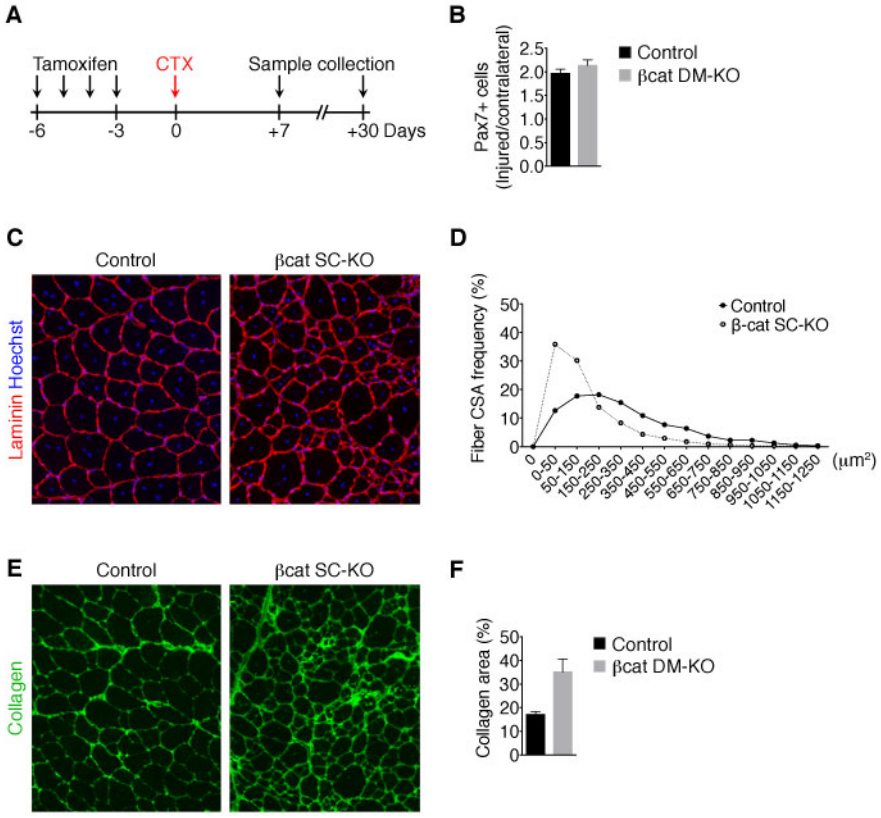


Fig. 2. β-catenin is required for myogenic differentiation during adult muscle regeneration. **A,** Tamoxifen and cardiotoxin (CTX) regimen and regeneration assay scheme for control ($Pax7^{CreER}$) and β-cat SC-KO ($Pax7^{CreER}$;$β\text{-catenin}^{lox/lox}$) mice. **B,** Quantification of sub-laminar Pax7+ cells (normalized by Pax7+ cells of contralateral non-injured TA) 30 days after injury. **C,** cryosections of TA muscles immunostained for Laminin 30 days after injury. **C,** Transverse sections of E13.5 dorsal regions at the forelimb level immunostained for Pax7 and Desmin. **D,** Distribution of cross sectional area (CSA) of TA myofibers 30 days post injury. **E,** Type I collagen immunofluorescent staining on TA cryosections 30 days post injury. **F,** Quantification of relative Collagen+ area in E. These results were obtained in collaboration with Dr. Anja Rudolf.

which the Cre recombinase is activated by tamoxifen. As depicted in Fig. 2A, after 4 daily tamoxifen injections to 8-week old mice, we injured Tibialis Anterior (TA) muscles by cardiotoxin (CTX) injection and analyzed muscle regeneration 1 month post injury. We did not detected any change in the number of Pax7-expressing satellite cells following β-catenin knockout, suggesting that muscle stem cell self-renewal does not require canonical Wnt signaling (Fig. 2B). However, β-cat SC-KO muscles displayed reduced fiber size, as demonstrated by the shift in myofibers cross-sectional area (CSA) distribution compared to controls (Fig. 2, C and D). This was accompanied by an increase in collagen deposition, a clear sign of defective muscle regeneration (Fig. 2, E and F).

From these results we conclude that canonical Wnt signaling sustains myogenic differentiation during adult muscle regeneration.

Conclusion and perspectives

Altogether, these preliminary lines of evidence suggest that canonical Wnt signaling plays similar roles in embryonic and adult myogenesis by promoting myogenic cell differentiation. It will be interesting to characterize the molecular mechanisms underlying this function. One possibility would be that β-catenin/TCFs transcriptional complex directly activates the expression of genes responsible for myogenic differentiation. To test this hypothesis it will be interesting to determine the genomic occupancy of β-catenin as well as its interacting partners. Another possible mechanism by which the Wnt/β-catenin pathway could drive myogenic differentiation might be the regulation of other cellular processes such as cell cycle exit. Indeed, during myogenic lineage progression, the engagement to myogenic differentiation and the irreversible proliferation arrest are intimately linked (Shen et al., 2003; Singh and Dilworth, 2013; Esteves de Lima et al., 2014). Hence, we could speculate that cell cycle entry during satellite cell activation and exit during myogenic differentiation could be regulated by fluctuations of canonical Wnt signaling that would act as a cell cycle switch.

DISCUSSION

The results obtained during my PhD enabled to determine for the first time the role of APC in embryonic and adult myogenesis. Through a combination of *in vivo*, *ex vivo* and *in vitro* approaches, we demonstrated that APC is required in muscle progenitors to enter cell cycle and undergo myogenic commitment. We further proved that APC exerts this function mainly by negatively regulating canonical Wnt signaling. This previously unappreciated role of APC highlights its tissue-specificity and suggests that the paradigm of APC as a tumor suppressor defined in tissues with fast turnover cannot be extended to the slow self-renewing adult skeletal muscle.

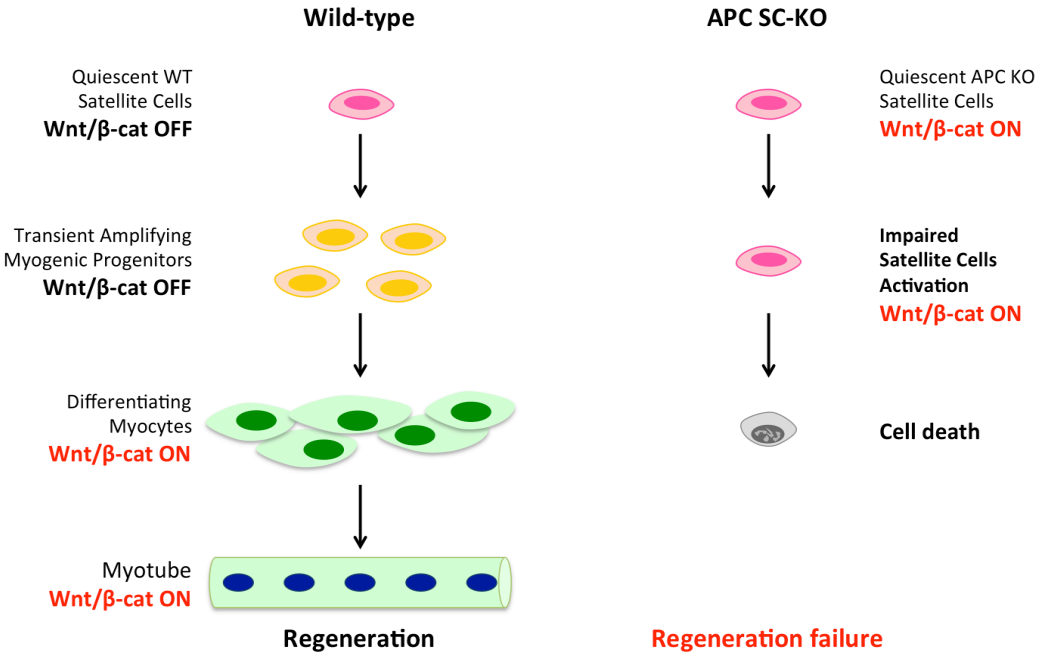


Figure 1. APC is required for satellite cell activation and for myogenic progenitors proliferation. In wild-type animals, canonical Wnt signaling is not active in quiescent satellite cells and in transient amplifying myogenic progenitors. By contrast, differentiating myocytes display active canonical Wnt signaling, and our preliminary results suggest that this activation is required for myogenic differentiation. In APC SC-KO animals, constitutive overactivation of canonical Wnt signaling impairs their activation and induces cell death. Our results suggest that in myogenic cells the Wnt/βcatenin pathway induces cell cycle arrest, therefore we hypothesize that the role of canonical Wnt signaling is to sustain myogenic differentiation by promoting cell cycle exit.

APC, canonical Wnt signaling and tumorigenesis

Irrefutable evidence from cancer models demonstrated that canonical Wnt signaling overactivation following APC loss induces unscheduled proliferation by increasing the expression level of cyclins and other genes related to cell cycle progression, thereby favoring malignant transformation (see Niehrs and Acebron, 2012). Conversely, as a mirror image, forced inactivation of β-catenin-dependent transcription as well as APC overexpression in colorectal cancer cells result in G1 phase cell cycle arrest (Baeg et al., 1995; van de Wetering et al., 2002). Lineage tracing and reporter experiments in skin and intestine compartments revealed that canonical Wnt signaling sustains stem and progenitors cell proliferation during normal tissue homeostasis (see Grigoryan et al., 2008). Collectively, these observations led to develop the notion of mitogenic Wnt/β-catenin pathway, which proposes canonical Wnt signaling as a key positive regulator of cell cycle progression in both normal and cancerous cell contexts.

In our experimental setup, as expected, APC gene inactivation massively activated canonical Wnt signaling in myogenic progenitors, but contrary to what previously reported in other systems, this resulted in defective G1/S phase transition and subsequent cell death. One of the mechanisms by which canonical Wnt signaling has been proposed to regulate cell cycle progression is the upregulation of c-Myc, a transcription factor that acts both as an activator and a repressor of several components of the cell cycle machinery. In particular, during small intestine tumorigenesis, Wnt-induced c-Myc induce Cyclin D1 and inhibits p21 (Cdkn1a), the major negative regulator of G1/S checkpoint, thereby disrupting the ability of cells to arrest proliferation even when mitotic errors are accumulating. This unscheduled cell cycle progression is thought to favor malignant transformation. Interestingly, in our transcriptomic analysis we did not detect any upregulation of c-Myc transcript levels following APC loss in myogenic cells. Conversely, we found augmented Cdkn1a transcription, which was consistent with arrested G1/S transition. Moreover, this was accompanied by a dramatic decrease of virtually all cyclins and cell cycle regulators. This evidence suggests that the transcriptional program activated by canonical Wnt signaling in myogenic progenitors differs from other systems, pointing out the poorly explored tissue-specificity of the Wnt/β-catenin pathway.

This divergence between skeletal muscle and other tissues in terms of canonical Wnt signaling requirement for cell cycle progression could reside in the particularly prolonged quiescence of adult satellite cells and in the low turnover rate of the skeletal muscle tissue. Indeed, although compartments such as intestine (Buczacki et al., 2013) and skin (Tumbar et al., 2004) contain quiescent or infrequently dividing stem cells, their fast spontaneous turnover imposes continuous engagement of stem

cells to feed tissue self-renewal. By contrast, skeletal muscle is a highly stable tissue with very low turnover, and satellite cells exit their quiescent state only upon particular stimuli such as intense exercise or injury. Therefore, we could hypothesize that the molecular control of cell cycle diverges in stem cells with such different cell cycle dynamics. In line with this, canonical Wnt signaling could have been co-opted by the myogenic lineage to regulate cell cycle exit of differentiating cells rather than the proliferation of myogenic progenitors. This could explain why in the myogenic lineage, canonical Wnt signaling is inactive in quiescent and proliferating progenitors, whereas its activation coincides with cell cycle exit and concomitant myogenic differentiation.

Intriguingly, besides its slow self-renewal rate, skeletal muscle also displays extremely infrequent tumorigenesis. Rhabdomyosarcomas, i.e. tumors arising from skeletal muscle, with their incidence of approximately 3% of childhood cancers, are considered relatively rare tumors (Punyko et al., 2005). This low propensity to tumor initiation of satellite cells could be intimately linked to their particular regulation of cell cycle dynamics, which diverges from stem cells of fast renewing tissues and is able to lock muscle stem cells in a prolonged, yet reversible, quiescence.

APC, canonical Wnt signaling and cell death

A striking aspect of our study is the evidence that APC loss imperatively triggers satellite cell death, presumably as a consequence of cell cycle arrest at the G1/S transition. We clearly demonstrated that this cell demise requires active canonical Wnt signaling, since simultaneous invalidation of APC and β-catenin restores satellite cell viability. Nonetheless, it still remains unclear how canonical Wnt signaling hyper-activation drives satellite cell death. The most likely explanation might be that the Wnt/β-catenin pathway would directly control the expression of p21 and that p21 accumulation would trigger cell cycle arrest in the G1 phase, as it has already be demonstrated in other cell contexts (Robson et al., 1999). G1-arrested cells would be then driven toward cell death by a mechanism possibly implying the G1/S checkpoint. However, in other cell types, when this checkpoint is activated, it induces cell death only when DNA damage is not resolved (Sancar et al., 2004). Therefore, other mechanisms overriding this aspect must be involved in APC-depleted satellite cells. Interestingly, increased apoptosis following APC loss or canonical Wnt signaling overactivation has already been reported in the intestinal compartment (Sansom et al., 2004; Wong et al., 1998). Yet, it was associated to enhanced proliferation and the underlying molecular mechanism has not been clarified.

Dose-dependent effects of canonical Wnt signaling activation

Our *in vivo* results obtained combining APC and β-catenin gene inactivation in adult satellite cells revealed that removal of a single allele of β-catenin partially rescued the phenotype caused by APC loss, whereas regeneration was completely restored by loss of both β-catenin copies. This suggests that canonical Wnt signaling has a dose-dependent effect on satellite cells activation and cell cycle progression. This feature has already been described in other systems and might be a general property of canonical Wnt signaling. In adult epidermis, different levels of β-catenin can induce aberrant *de novo* follicles formation from distinct cell populations of the skin, suggesting that cell types within the same tissue have different sensitivity to canonical Wnt stimulation (Silva-Vargas et al., 2005). Similarly, during hematopoiesis canonical Wnt signaling is activated at different levels in distinct hematopoietic precursors and this has a functional relevance. Indeed, by using a combination of APC alleles that produce different levels of canonical Wnt signaling activation, it has been shown that each hematopoietic lineage requires a specific optimal Wnt dosage for proper expansion and differentiation (Luis et al., 2011). A Wnt/β-catenin activation gradient is present along the axis of intestinal crypts, with highest levels at the crypt bottom, and this gradient has been proposed to define the borders of the proliferative region (van de Wetering et al., 2002; Gregorieff et al., 2005; Hirata et al., 2013).

The level of canonical Wnt activation seems also to play a role during tumorigenesis. Observation of frequency and type of germline and somatic APC mutations in colorectal cancers from Familial Adenomatous Polyposis (FAP) patients revealed non-random selection of the second mutation during tumor initiation (Albuquerque et al., 2002). In particular, mutations that completely abolish the ability to regulate β-catenin stability (thereby leading to maximal canonical Wnt hyperactivation) are extremely infrequent. Therefore, it seems that selection favors mutations that promote a level of Wnt/β-catenin that is optimal for tumor formation.

It would be interesting to investigate more deeply whether different levels of canonical Wnt signaling activation have different outputs during myogenic lineage progression, by using appropriate APC truncated alleles or β-catenin gain-of-function mutations.

Regulation of canonical Wnt signaling levels

Canonical Wnt signaling participates to multiple steps of embryonic and adult myogenesis, such as somitogenesis, myotome induction and fiber type patterning

(Hutcheson et al., 2009; Borello et al., 2006; Anakwe et al., 2003). Our analysis of β-catenin conditional knockout contributed to show that it is required for myogenic differentiation during both muscle development and regeneration. However, the Wnt/β-catenin pathway can also have deleterious consequences when improperly activated, as shown by our results obtained by APC gene inactivation in embryonic and adult myogenesis. Therefore, its activation must be tightly regulated, not only in in terms of dosage, but also a spatio-temporal manner.

The great number of Wnt ligands and of possible receptors/co-receptors combinations, as well as the variety of agonists, antagonists and regulators, highlights the complexity of the Wnt/β-catenin pathway. Therefore, a comprehensive analysis of Wnt regulation *in vivo* is challenging. Nonetheless, our analysis of Wnt-reporter activity during adult muscle regeneration revealed that canonical Wnt signaling is active only in committed myogenic myocytes. Several mechanisms could ensure this precise timing. One possibility would be to modulate Wnt ligand availability. This option is quite intriguing, if we consider that the extracellular matrix (ECM) surrounding the myofibers has been largely described as a "reservoir" of secreted factors, which can be rapidly released upon muscle injury by metalloproteases and other ECM components (see Lund and Cornelison, 2013). Moreover, components of the extracellular matrix of the Drosophila wing imaginal disc have been implicated in Wnt gradient formation (Glise et al., 2005; Gorfinkiel et al., 2005).

Another strategy could be to regulate Wnt ligands transcription and/or secretion. This leads us to a still unsolved question: what is the source of canonical Wnt signaling responsible for the regulation of myogenic differentiation during embryonic and adult myogenesis? Both resting and regenerating muscles have been reported to express Wnt ligands (Polesskaya et al., 2003). However, the precise source of Wnt ligands and which downstream pathway each ligand activates still remains unknown. Increasing biochemical evidence suggests that Wnt ligands rarely diffuse at long ranges due to their high hydrophobicity. However, wide-range modes of delivery able to circumvent this issue have been recently reported. For instance, Wnt can be loaded into exosomes (Panáková et al., 2005; Neumann et al., 2009; Gross et al., 2012) or, as shown during chick somitogenesis, into migrating cells (Serralbo and Marcelle, 2014). Therefore, canonical Wnt signaling could act in an autocrine or paracrine manner, the two modes of signaling being not mutually exclusive.

Canonical Wnt signaling and muscle aging

Muscle strength and mass decline during aging. This decline is accompanied by a progressive reduction of the number of satellite cells as well as in the gradual loss of their stem properties (García-Prat et al., 2013; see Renault et al., 2002). As a consequence, the regenerative potential of skeletal muscle decreases with age and several pathways have been implicated in this progressive loss (Carlson et al., 2008; Chakkalakal et al., 2012). Among them, canonical Wnt signaling has been proposed to play a pivotal role. Aged (~24-month-old) satellite cells have been reported to display increased canonical Wnt signaling compared to young (~6-month-old) satellite cells, as shown by higher levels of Axin2 transcription and of Wnt-reporter (TOPGAL) activity (Brack et al., 2007). In the same study, aged satellite cells-derived cultures or young satellite cells-derived cultures treated with canonical Wnt3a similarly contained high percentages of non-myogenic fibroblastic cells. Therefore, the authors proposed that enhanced canonical Wnt signaling is responsible for the progressive increase in fibrous connective tissue observed during muscle ageing by converting myogenic progenitors into fibrotic cells. However, a more recent study clearly demonstrated that the decline in regenerative ability of geriatric (~28- to 32-month-old) muscle is due to a conversion of the quiescent state of satellite cells into irreversible senescence (Sousa-Victor et al., 2014). This switch is mediated by an increase in the $p16^{INK4a}$-Rb axis, which induces G0 irreversible cell-cycle arrest. Interestingly, in our transcriptomic analysis the $p16^{INK4a}$ (Cdkn2a) pathway was predicted to be upregulated following APC knockdown (Fig. S6). This lets us speculate that the increase in canonical Wnt signaling during aging could be responsible for $p16^{INK4a}$-dependent satellite cell senescence, although a direct regulation of Cdkn2a expression by the Wnt/β-catenin pathway has not been demonstrated yet.

Canonical Wnt signaling and implication in muscular dystrophies therapies

One of the most promising tools for future personalized cell-therapy is the use of patient-derived induced pluripotent stem (iPS) cells. This approach has also been recently undertaken for muscle dystrophies and encouraging results in mouse models already laid the foundations for further clinical studies. For instance, human iPS generated from Limb-girdle muscular dystrophy (LGMD2D) patients have been used to obtain myogenic progenitors that have been expanded and corrected in vitro and subsequently transplanted in a mouse model for LGMD2D. This efficiently restored α-sarcoglycan expression, the gene mutated in this pathology, as well as muscle functionality: transplanted cells generated new myofibers and replenished the pool of

residing progenitors that was depleted by the pathological condition (Tedesco et al., 2012). Similar results have been obtained for Duchenne muscular dystrophy (DMD) (Darabi et al., 2012; Tedesco et al., 2012). Nonetheless, many aspects of this strategy still need be improved. Therefore our results concerning canonical Wnt signaling as a major regulator of cell cycle progression and differentiation of myogenic progenitors might contribute to the improvement of in vitro expansion of iPS-derived myogenic precursors or its efficiency of differentiation after *in vivo* transplantation.

Due to its prominent involvement in tumor initation and progression, canonical Wnt signaling is therapeutic target for cancer. A number of small molecules have been developed to modulate the Wnt/β-catenin pathway in preclinical models (see Anastas and Moon, 2013). For instance, high-throughput screens based on the effect on Wnt-reporter activation identified novel inhibitors of canonical Wnt signaling, such as IWP, which interferes with Wnt secretion (Chen et al., 2009), or XAV939, pyrvinium, which enhances β-catenin phosphorylation (Thorne et al., 2010). In addition to small molecules, blocking antibodies and peptides have been shown to specifically inhibit canonical Wnt signaling and delay cancer progression (see Anastas and Moon, 2013). By contrast, Wnt activating compounds are far less numerous, most probably due to the fact that the main goal of cancer therapies is to dampen the Wnt/β-catenin pathway. Among them, lithium chloride (LiCl) is one of the first GSK3β inhibitor to be identified as Wnt activators.

Modulation of canonical Wnt signaling could be of great interest not only to optimize cell therapies for muscular dystrophies, but also to limit or delay fibrous degeneration typical of these conditions. Indeed, it has been proposed that the serum of dystrophic mice has a higher canonical Wnt activity compared to control mice, and this has been postulated to be directly involved in aberrant fibrosis, since canonical Wnt ligands stimulate proliferation of interstitial cells as well as their collagen production (Trensz et al., 2010).

APC and asymmetric cell division

Our work demonstrates that the APC-dependent downregulation of canonical Wnt signaling plays a major role in embryonic and adult myogenic commitment. However, we cannot exclude that APC could affect other molecular aspects involved in this process. The pivotal role of APC in the control of division orientation and (a)symmetry has been well established in several systems. In particular, in Drosophila male germline stem cells, APC contributes to mitosis orientation thereby ensuring the correct

positioning of daughter cells, one remaining in the stem niche and one moving apart from the niche and committing to differentiation (Yamashita et al., 2003). Similarly, mitotic spindle orientation within the intestinal crypt requires APC and it determines the asymmetric distribution of template DNA strand segregation (Bellis et al., 2012; Quyn et al., 2010). Both these features have been shown to be important for the balance between muscle stem cells self-renewal and myogenic commitment. Indeed, the orientation of mitotic spindle determines whether satellite cell division will be symmetric, thereby amplifying the stem cell pool, or asymmetric, generating one stem cell and one committed daughter (Kuang et al., 2007). Furthermore, the stem subpopulation of satellite cells performs non-random template DNA segregation during mitosis (Conboy et al., 2007; Shinin et al., 2006). Interestingly, loss of only one APC allele perturbs mitotic spindle orientation and template DNA strand segregation in the intestine (Quyn et al., 2010). It will be interesting to carefully analyze APC subcellular localization during satellite cells division, and to determine whether APC is involved in these aspects of satellite cells. Satellite cell death following APC loss prevented this kind of analysis. However, satellite cells inactivated for both APC and β-catenin are viable, therefore our double knockout model provides a great tool to further characterize APC involvement in mitotic spindle orientation and asymmetric satellite cell division.

APC and chromatin dynamics

Several studies proposed multiple roles for APC at the chromatin level, ranging from regulation of β-catenin transcriptional complex to DNA repair upon replication stress (see Lui et al., 2012). In particular, APC has been postulated to drive mitotic chromatin compaction by recruiting histone modifiers and chromatin remodelers (Dikovskaya et al., 2012). Quiescent satellite cells are characterized by abundant heterochromatin (Mauro, 1961; Günther et al., 2013). However, transition from quiescence to active proliferation and further differentiation is accompanied by remarkable changes in chromatin condensation and composition, consistent with the increase in gene transcription and metabolic activity required for these steps (Simone et al., 2004; Fukada et al., 2007; Saccone and Puri, 2010). Although the phenotype caused by APC loss is rescued by concomitant β-catenin depletion, we cannot exclude the possibility that APC plays a role in chromatin dynamics during myogenic lineage commitment and differentiation. Preliminary evidence obtained by mass-spectrometry analysis of the nuclear proteome of APC-silenced primary myoblasts revealed a decrease in the abundance of proteins related to chromatin condensation belonging to the Structural

Maintenance of Chromosome (SMC) family compared to control cells (data not shown). However, further investigation is needed to confirm these preliminary observations and to assess whether this phenomenon is directly caused by the absence of APC, or if it is rather an indirect consequence of impaired cell cycle progression of APC-depleted cells.

ANNEX

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Specific pattern of cell cycle during limb fetal myogenesis

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ABSTRACT

Tight regulation of cell proliferation and differentiation is required to ensure proper growth during development and post-natal life. The source and nature of signals regulating cell proliferation are not well identified in vivo. We investigated the specific pattern of proliferating cells in mouse limbs, using the Fluorescent ubiquitynation-based cell-cycle indicator (Fucci) system, which allowed the visualization of the G1, G1/S transition and S/G2/M phases of the cell cycle in red, yellow or green fluorescent colors, respectively. We also used the retroviral RCAS system to express a Fucci cassette in chick embryos. We performed a comprehensive analysis of the cell cycle state of myogenic cells in fetal limb muscles, adult myoblast primary cultures and isolated muscle fiber cultures using the Fucci transgenic mice. We found that myonuclei of terminally differentiated muscle fibers displayed Fucci red fluorescence during mouse and chick fetal development, in adult isolated muscle fiber (ex vivo) and adult myoblast (in vitro) mouse cultures. This indicated that myonuclei exited from the cell cycle in the G1 phase and are maintained in a blocked G1-like state. We also found that cycling muscle progenitors and myoblasts in G1 phase were not completely covered by the Fucci system. During mouse fetal myogenesis, Pax7+ cells labeled with the Fucci system were observed mostly in S/G2/M phases. Proliferating cells in S/G2/M phases displayed a specific pattern in mouse fetal limbs, delineating individualized muscles. In addition, we observed more Pax7+ cells in S/G2/M phases at muscle tips, compared to the middle of muscles. These results highlight a specific spatial regionalization of cycling cells at the muscle borders and muscle–tendon interface during fetal development.

Introduction

An open question is to understand how the balance between proliferation and differentiation is maintained over time in order to ensure progressive tissue growth during development. The spatial and temporal organization of cell cycle is not characterized in vivo.

Embryonic, fetal and peri-natal myogenesis occur in successive and overlapping phases. The myogenesis processes involve different muscle progenitor populations, which are defined throughout development by the expression of the *paired-box* transcription factors, *Pax3* and *Pax7* (Hutcheson et al., 2009; Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Schienda et al., 2006). Pax3+ cells define the embryonic muscle progenitors, while Pax7+ cells identify fetal muscle progenitors and satellite cells (Hutcheson et al., 2009; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). Once specified to a muscle fate, muscle progenitors use a common muscle program, which involves the myogenic regulatory factors (MRFs). The four members of this family of DNA binding proteins (Myf5, MyoD, Mrf4 and Myogenin) induce the expression of a variety of genes involved in the contractile properties of mature skeletal muscle cells. While embryonic myogenesis involves specification and determination processes, fetal myogenesis is based on muscle growth (Messina et al., 2010). Moreover, it is by the end of fetal myogenesis that the satellite cells are generated and acquire their specific location underneath the basal lamina (Brohl et al., 2012). Perinatal muscle growth is due to an increase in the number of myonuclei and to muscle hypertrophy (White et al., 2010). In the adult, satellite cells have a stem cell potential (Seale et al., 2000). Under resting conditions satellite cells are quiescent, while after damage they become activated, proliferate

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and reactivate the myogenic program via the MRFs (reviewed in Buckingham and Relaix, 2007).

A tight control of cell proliferation is crucial for proper skeletal muscle formation, particularly during fetal myogenesis. Different signaling pathways regulate fetal muscle progenitors, including Notch, Wnt and Bmp pathways (Biressi et al., 2007; Hutcheson et al., 2009; Vasyutina et al., 2007; Wang et al., 2010). Recently, a transcription factor, Nfix, has been identified as a major regulator of the switch between embryonic and fetal myogenesis, by regulating directly the transcription of several fetal specific genes and concomitantly inhibiting embryonic specific genes (Messina et al., 2010). Several arguments exist to say that fetal muscle progenitors are not all equivalent (Hauschka, 1974; Schafer et al., 1987; Biressi et al., 2007). It has been observed that a subpopulation of fetal muscle progenitors responding to Bmp signaling are preferentially located at the tips of muscles, close to the tendons in chick embryos (Wang et al., 2010). Lastly, two distinct muscle progenitor populations have been described during fetal development, which display different rates of cycling in chick and mouse embryos (Picard and Marcelle, 2013), although there is no indication of spatial regionalization for these two cell populations. All together these data suggest a heterogeneity of proliferating fetal muscle progenitors.

Adult satellite cells are a heterogeneous population as observed through multiple parameters. Cre/LoxP lineage tracing studies identified a sub-population of satellite cells that had never expressed *Myf5* and functioned as a stem cell reservoir (Kuang et al., 2007). These Pax7+/Myf5-satellite cells give rise to Pax7+/Myf5+ committed cells through apical–basal oriented divisions, which asymmetrically generate a basal Pax7+/Myf5-cell and an apical Pax7+/Myf5+ cell (Le Grand et al., 2009; Kuang et al., 2007). ex vivo work on FACS-sorted satellite cells demonstrated that while the vast majority of activated satellite cells were fast-dividing cells, slow-dividing cells were observed as a minority population (Ono et al., 2012). In addition, clusters of activated satellite cells are more concentrated at the extremities of isolated adult muscle fibers (Wang et al., 2010).

in vitro studies have highlighted the role of cell cycle components during the muscle differentiation process (reviewed in Ciemerych et al., 2011). In particular, pRb, (retinoblastoma protein), which is associated with cell cycle exit and terminal differentiation, has been shown to up-regulate *MyoD* transcriptional activity and to induce the expression of late muscle differentiation markers (Gu et al., 1993). Moreover, MyoD promotes the transcriptional activation of the Cdk inhibitors (CKI) from the CIP/KIP family, p21, p27 and p57, in differentiating myoblasts, contributing to cell cycle arrest (Cenciarelli et al., 1999; Figliola and Maione, 2004; Otten et al., 1997). MyoD activity is higher during G1, while it starts to be degraded as the cells enter S phase and proceed with the cell cycle (Kitzmann et al., 1998). While MyoD is associated with G1 phase and cell cycle withdrawal, Myf5 expression starts in late G1 phase and is higher throughout the S/G2/M phases and in the quiescent G0 state (Kitzmann et al., 1998; Lindon et al., 1998). However, the organization of cell cycle is poorly characterized in vivo. It has been shown that the number of proliferating Pax7+ cells decreases over development in both mouse and chick embryos (He et al., 2005; Picard and Marcelle, 2013).

The Fucci (Fluorescent ubiquitination-based cell cycle indicator) system has been developed based on the fact that several proteins oscillate through the different phases of the cell cycle, due to the activity of ubiquitin ligase complexes (Sakaue-Sawano et al., 2008). Both Cdt1 and Geminin proteins are involved in the licensing of the DNA replication and therefore their activities are tightly regulated (McGarry and Kirschner, 1998; Nishitani et al., 2000; Wohlschlegel et al., 2000). Geminin is ubiquitinated during G1 phase by the APC^{Cdh1} complex, while Cdt1 is tagged to degradation by the SCF^{Skp2} ubiquitin ligase complex at the S phase (Li et al., 2003; McGarry and Kirschner, 1998), allowing a non-co-localization of these proteins except at the G1/S transition phase. By fusing human Geminin to the red fluorescent protein monomeric Kosabira Orange 2 (mKO2) and Cdt1 to the green fluorescent protein monomeric Azami-Green (mAG), it has been established a color system that covers the different cell cycle phases (Sakaue-Sawano et al., 2008). Two transgenic mouse lines expressing ubiquitously mKO2-hCdt1 (Fucci red) or mAG-hGem (Fucci green) have been established (Sakaue-Sawano et al., 2008).

In this paper, we analyzed the pattern of cell cycle in limbs during mouse and chick fetal myogenesis using the Fucci system. We also analyzed the Fucci state of adult myoblasts and isolated fibers.

Materials and methods

Mouse lines and chick embryos

The Fucci mice expressing either Cdt1-KO2 (FucciTG mouse #596, Fucci red) or Gem-AG (FucciTG mouse #504, Fucci green) were obtained from the RIKEN Brain Science Institute, (Japan). Mouse embryos were collected after natural overnight matings. For staging, fertilization was considered to take place at midnight. Fertilized White Leghorn chick eggs (HAAS, Kaltenhouse) were incubated at 38.5 °C. Chick embryos were staged according to days in ovo.

RCAS-Fucci-2A plasmid construction and grafting RCAS-Fucci-2A-expressing cells

The Fucci-2A cassette containing mKO_2-hCdt1 and eGFP-hGem sequences was established using the 2A peptide, which allows the production of two proteins under the same promoter (Szymczak et al., 2004; Feillet et al., 2014). The Fucci-2A cassette was excised from the pPRIHy Fucci-2A vector by performing a double digestion with ClaI and AccI restriction enzymes, and inserted into the RCAS-BP(A) vector, digested with ClaI and further dephosphorilated with the Antartic Phosphatase enzyme, using the T4 DNA Ligase.

Chicken embryonic fibroblasts (CEFs) obtained from E10 chick embryos were transfected with RCAS-Fucci-2A at a confluence of 50% using the Calcium Phosphate Transfection Kit (Invitrogen), overnight at 37 °C and 5% CO_2. Pellets of approximately 50–100 μm in diameter were grafted into limb buds at E3.5. The embryos were harvested 5 days after grafting at E8.5, and processed for immunohistochemistry to tissue sections.

Adult primary myoblast culture

Skeletal muscles of hindlimbs of 2 month-old Fucci green and red mice were dissected, transferred to a sterile 6 cm Petri dish on ice, mulched into a smooth pulp and incubated in CollagenaseB/DispaseII/$CaCl_2$ solution (1.5 U/ml, 2.4 U/ml, and 2 M, respectively, in DMEM; Roche). After a 15 min incubation at 37 °C in the culture incubator, the muscle pulp was triturated with heat-polished glass Pasteur pipettes, and this incubation/trituration step was repeated. The tissue digestion was stopped with the addition of FBS, cells were filtered and washed twice with PBS, re-suspended in growth medium consisting of Ham's F10 supplemented with 20% FBS and 2.5 ng/μl of bFGF, and let to adhere onto a non-coated 10 cm plate for 2 h. At the end of the preplating procedure, the media was transferred onto collagen-coated Petri dishes. Cultures were maintained in growth medium until cells reached 80% confluence. The myoblast population was enriched by differential adhesion

compared with fibroblasts during the first week of culture. Primary myoblasts used in this study were 95% pure (as assessed by Pax7/MyoD double staining) at passage four.

Isolated muscle fibers

Single myofibers were isolated from the EDL muscles of 2-month-old Fucci green and red mice as previously described (Le Grand et al., 2012). Myofibers were cultured in suspension in 6-well plates coated with horse serum to prevent fiber attachment. Fibers were incubated in plating medium consisting of 15% FBS and 1% chick embryo extract (CEE; Accurate Chemicals) in DMEM. At 0 and 72 h of culture, individual fibers were picked and fixed in 1% PFA, permeabilized with 0.2% Triton X-100 in PBS, and then processed for immunostaining.

Immunohistochemistry

Forelimbs from RCAS-Fucci-2A-manipulated E8.5 chick embryos and E15.5 or E18.5 Fucci mouse embryos were fixed in 4% paraformaldehyde, overnight at 4 °C and then processed in gelatin/sucrose for 12 μm cryostat sections. Immunohistochemistry of forelimbs sections was performed as previously described (Wang et al., 2010). The Fucci green reporter (mAG fluorescence) was detected using the Anti-monomeric Azami-Green 1 (MBL, PMO52). The Fucci red reporter (mKO2 fluorescence) was observed without any additional labeling. The monoclonal antibodies, MF20 that recognizes sarcomeric myosin heavy chains and Pax7 that recognizes muscle progenitors, developed by D.A. Fischman and A. Kawakami, respectively, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology Iowa City, IA 52242. The anti-MyoD monoclonal antibody was obtained from Dako (M3512) and the anti-Ki67 antibody (mouse IgG1) was obtained from BD Pharmingen (556003). The type I collagen polyclonal rabbit antibody was obtained from (Calbiochem, 234167). After overnight incubation with the primary antibody at 4 °C, secondary antibodies conjugated with Alexa-488 (Invitrogen, A1 1008 α-rabbit, A2 1121 α-mouse IgG1, A2 1141 α-mouse IgG2b) and Alexa-555 (Invitrogen, A2 1127 α-mouse IgG1, A21147 α-mouse IgG2b) were applied for 1 h at room temperature. Amplification with biotinilated secondary antibodies was carried out when needed by performing incubation with anti-mouse IgG2b biotinilated antibody (Southern Biotech, 109008) for 1 h and Cy5-Streptavidin (far-red) (Invitrogen, 434316) for 45 min, both at room temperature. Hoechst (Molecular Probes) staining was performed with a dilution of 1/20,000 in PBS 1 × for 10 min at room temperature.

EdU staining

Intra-peritoneal injection of 300 μl of 5 mg/ml EdU solution (Invitrogen) was performed on pregnant Fucci green mice. Embryos were dissected 1.5 or 5 h after EdU injection.

EdU (final concentration 10 μM) was added in culture medium of adult myoblast cultures in proliferation conditions for 24 h before fixation.

Image capturing

After immunohistochemistry, images of the sectioned samples were obtained using a Leica DMI6000 B microscope or a Leica SP5 confocal system.

Quantification and statistical analyses

Quantification of the number of myogenic cells in vivo (tissue sections) and in vitro (cell culture) in each phase of the cell cycle was carried out in double mKO2-hCdt1/mAG-hGem transgenic mouse embryos. Forelimbs of E15.5 Fucci red/green mouse embryos were processed for cryostat transverse and longitudinal sections and immunohistochemistry. To quantify the number of Pax7+ or MyoD+ cells, several sections of three different muscles (Flexor Digitorium Profundus, Extensor Carpi Radialis Longus, Extensor Carpi Radialis Brevis) were analyzed in 3 different embryos. To determine the number of proliferating muscle progenitors at the tips and middle of muscles, images were captured from muscles of forelimbs of 3 different mAG-hGem transgenic mouse embryos labeled with Pax7 (E15.5). For enumeration of primary myoblasts and myotubes isolated from two Fucci red/green mice, pictures of cultures stained with Pax7 and MyHC antibodies were assembled and counted. Results shown are the mean of biological triplicates from three independent cultures. Statistical analysis was performed with Excel software using the two-tailed unpaired Student's *t* test to determine *p*-values. Statistical significance was set at $^{***}p < 0.001$. Cell quantification (in vivo and in vitro) was done using the Cell Counter plug-in of the free software ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012).

Results

Myonuclei of post-mitotic differentiated cells displayed Fucci red fluorescence during mouse fetal myogenesis

In order to characterize the cell cycle state of limb cells during fetal development, we used the E15.5 Fucci mouse embryos, at a stage when the musculo-skeletal pattern is established. We first analyzed the regionalization of cells in G1 phase on transverse forelimb sections of mKO2-hCdt1 transgenic mice (Fucci red mice). One striking observation was that red fluorescence appeared to be expressed strongly in muscles, which were labeled with the MF20 antibody recognizing myosins (Fig. 1A–C). Higher magnifications of transverse and longitudinal sections of muscles showed that myonuclei in terminally differentiated muscle fibers were systematically Fucci red (Fig. 1D–I, arrowheads). Because myonuclei were not cycling (Moss and Leblond, 1970), the persistence of red fluorescence suggested that myonuclei were in a blocked G1-like phase in muscle fibers. We also observed Fucci red+ nuclei located outside muscle fibers, which displayed a lower red fluorescence compared to that of myonuclei in muscle fibers (Fig. 1G–I, arrows). Next, we analyzed forelimb muscles of mAG-hGem transgenic mice (Fucci green mice), to visualize cells in S/G2/M phases (Fig. 1J–L). We observed that cells displaying green fluorescence were always excluded from muscle fibers (Fig. 1M–O, arrows). These cells could be muscle progenitors, myoblasts or muscle connective tissue cells. We conclude that the myonuclei of Myosin+ cells were in a G1-like state in mouse limb fetal muscles. The Myosin- cells could be observed in all phases of cell cycle.

Fucci state of MyoD+ cells during mouse fetal myogenesis

We next analyzed the cell cycle state of MyoD+ cells in mouse fetal limbs using the Fucci system (Fig. 2). We observed that 64.6% of the MyoD+ nuclei were positive for Fucci red, while only 7% of MyoD+ cells displayed Fucci green fluorescence (Fig. 2A–H). We also observed a relative high proportion of MyoD+ cells (28,1%) that were negative for the Fucci system (Fig. 2G and H). Because myonuclei in

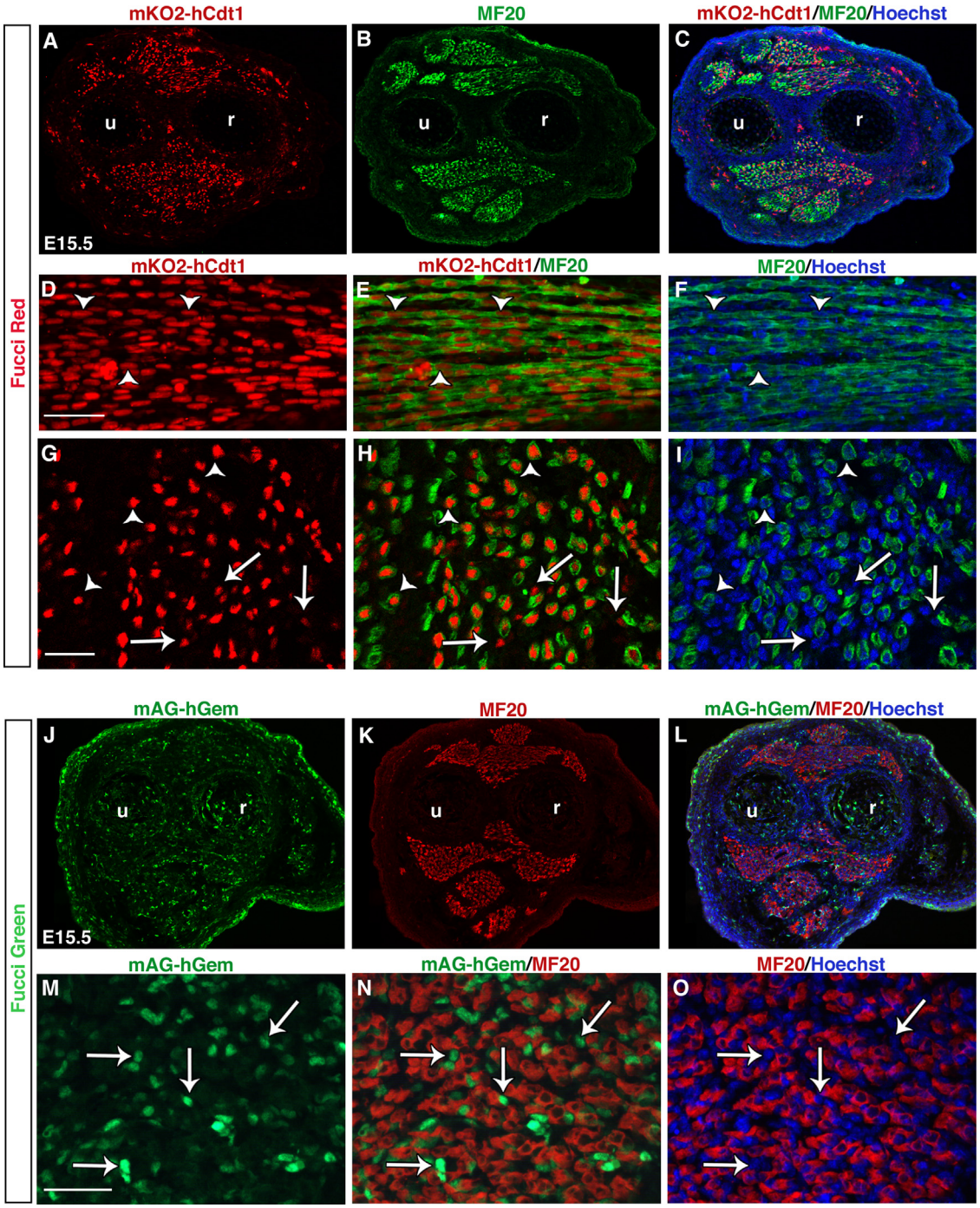

Fig. 1. Fucci transgene expression in fetal muscles of mouse forelimbs. Limbs from mKO2-hCdt1 (Fucci red, A–I) or mAG-hGem (Fucci green, J–O) E15.5 mouse embryos were sectioned and immunostained with the with the MF20 antibody that recognizes myosins. (A–C) Transverse limb sections from Fucci red embryos were immunostained with the MF20 antibody (green). Higher magnification of longitudinal (D–F) and transverse (G–I) muscle sections showed that myonuclei in MF20-positive fibers systematically displayed Fucci red fluorescence (D–I, arrowheads). (G–I) Fucci red+ nuclei were also observed in mononucleated cells outside MF20+ fibers (G–I, arrows). (G, H) Fucci red nuclei in mononucleated cells displayed a weaker signal (arrows) compared to that of myonuclei in multinucleated cells (arrowheads). (J–O) Transverse limb sections from Fucci green embryos were immunostained with the MF20 antibody (red). Fucci green+ cells in S/G2/M phases were systematically located outside of the muscle fibers (M–O, arrows). Scale bars=50 μm.

muscle fibers were all Fucci red+ (Fig. 1), we analyzed the Fucci state of MyoD+/Myosin- cells, by performing double staining with MyoD and MF20 antibodies in Fucci red or green embryos (Fig. 2I–N). MyoD+ nuclei displaying Fucci red fluorescence were observed in differentiated muscle fibers (Fig. 2I–K, arrowheads) or outside Myosin+ cells (Fig. 2I–K, arrow). These Fucci red+/MyoD+/Myosin- cells displayed lower red fluorescence (Fig. 2J, arrow) compared to that of Fucci red+/MyoD+/Myosin+ cells (Fig. 2J, arrowheads). 15.1% of MyoD+/Myosin- cells were Fucci red. MyoD+ Fucci green+ cells were consistently located outside of the muscle fibers (Fig. 2L and M, arrows). The size of the MyoD+/Myosin- pool in S/G2/M phases (Fucci green) was 65.6%. We conclude that MyoD+/Myosin+ cells were systematically Fucci red+, while the MyoD+ cells outside muscle fibers were mostly in S/G2/M phases.

Pax7+ cells labeled with the Fucci system were mainly in S/G2/M phases during mouse fetal myogenesis

In order to define the Fucci state of fetal muscle progenitors, we analyzed Pax7 expression in mKO2-hCdt1, mAG-hGem and mKO2-hCdt1/mAG-hGem mice (Fig. 3). A first clear observation was that the Pax7+ cells were barely displaying Fucci red fluorescence (0.6%), while 40.5% of Pax7+ cells were Fucci green+ (Fig. 3A–I). Another striking observation was that 57.6% of Pax7+ cells were negative for the Fucci system (Fig. 3G–I). Consistently,

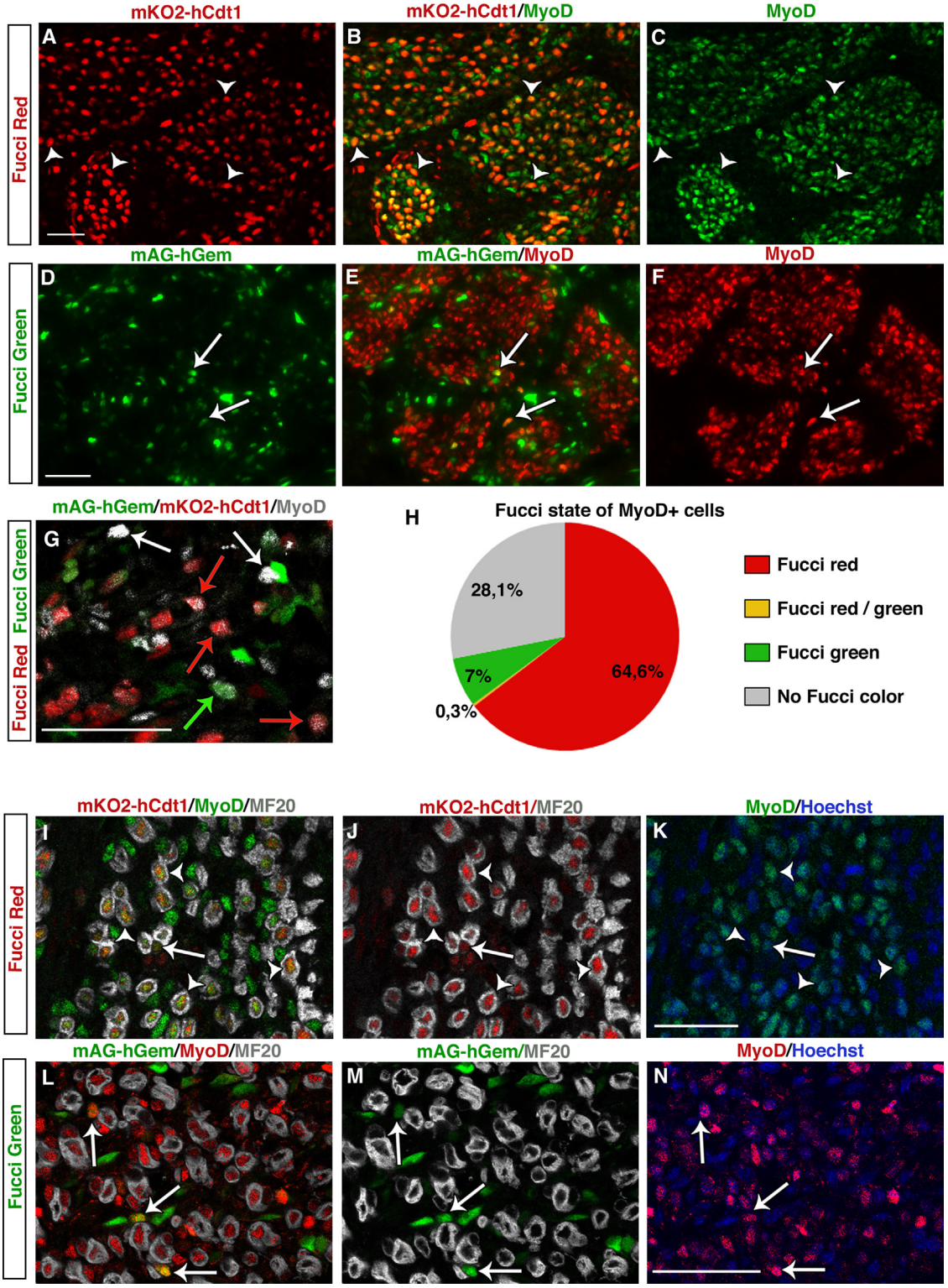

Fig. 2. Cell cycle state of MyoD+ cells during mouse fetal myogenesis using the Fucci system. Limbs from mKO2-hCdt1 (Fucci red, A–C, I–K), mAG-hGem (Fucci green, D–F, L–M) or double mKO2-hCdt1/mAG-hGem (Fucci red and green, G) E15.5 mouse embryos were sectioned and immunostained with the MyoD antibody only (A–G) or with the MyoD and MF20 antibodies (I–N). MyoD+ cells were mainly Fucci red (A–C, arrowheads), and also Fucci green (D–F, arrows). (G) MyoD+ cells in double mKO2-hCdt1/mAG-hGem muscles. Red arrows point to Fucci red+/MyoD+ cells, green arrow points to Fucci green+/MyoD+ cells and white arrow points to MyoD+ cells negative for the Fucci system. (H) Quantification of the proportion of MyoD+ cells observed in the different colors of the Fucci system. (I–K) MyoD+ nuclei displaying red fluorescence were either inside of MF20+ fibers (arrowheads) or outside MF20+ fibers (arrows). (L–N) The MyoD+ cells in S/G2/M phases (Fucci green) were always MF20-negative (arrows). (O) Quantification of the proportion of Fucci red+ or Fucci green+ cells among the MyoD+ cells. Scale bars=50 μm.

Pax7+ cells were never Myosin+ (Fig. 3J–O, arrows) as previously established (Relaix et al., 2005). Pax7+ cells in S/G2/M phases (Fucci green+) were also always excluded from MF20+ cells (Fig. 3M–O, arrows). We conclude that Pax7+ cells labeled with the Fucci system were found mostly in S/G2/M phases.

Link between the Fucci system and the proliferation markers, Ki67 and EdU

A relatively high percentage of MyoD+ and Pax7+ cells did not express any Fucci reporter (Figs. 2H, 3I). It has been previously shown that there is gap of Fucci fluorescence between the M and G1 phases in human and zebrafish cell cultures, indicating that the early G1 phase of cell cycle was not covered by the Fucci system (Sakaue-Sawano et al., 2008; Sugiyama et al., 2009). In order to estimate the percentage of cycling cells not labeled by the Fucci system in fetal muscles, we first used the Ki67 proliferation marker that labels all cell cycle phases except the G0 phase (Scholzen and Gerdes, 2000) and quantified the percentage of cells that did not display any Fucci reporter among the Ki67+ cell population. We estimated that 43.9% of Ki67+ cells were not covered by the Fucci system, in muscles (Supplementary Fig. S1A–I). We believe that this proportion of Ki67+/Fucci- cells include cycling cells that are in early G1 phase. In order to define cycling muscle progenitors not labeled with the Fucci system, we turned to EdU incorporation experiments in Fucci green mouse embryos (Fig. 4). We used the Fucci green-only mice, because there were very little Fucci red+ cells among the total Pax7+ cell population (0.6%, Fig. 3I). We performed two different pulses of EdU incorporation, 1.5 and 5 h. We first observed that 30.4% and 55.2% of the Pax7+ cell population was EdU+, after 1.5 h and 5 h of EdU pulses, respectively (Fig. 4A–G). Among the cycling EdU+/Pax7+ cells, we observed that around 30% of the cycling Pax7+ cells were not Fucci green, after 1.5 and 5 h of EdU exposure (Fig. 4A–F, H). We conclude that around 30% of the cycling Pax7+ cells were in early G1 and consequently not covered by the Fucci system.

Specific pattern of proliferating cells in mouse fetal limbs

In order to determine whether there was a specific spatial regionalization of dividing cells in fetal limbs, we analyzed limbs from double mKO2-hCdt1/mAG-hGem transgenic E15.5 mouse embryos (Fucci red/green mice). As mentioned previously (Figs. 1 and 2), in transverse limb sections, Fucci red+ cells were mainly visualized in individualized muscles (Fig. 5A–C). Because post-mitotic muscle fibers displayed myonuclei in a G1-like state (Figs. 1, 5A–C) and early G1 phase is not covered by the Fucci system (Fig. 4), we analyze the pattern of proliferating cells that were in S/G2/M phases. Cells in S/G2/M phases were non-homogeneously distributed in mouse limbs (Fig. 5D). Proliferating cells in S/G2/M appeared to delineate individualized muscles and cartilage elements (Fig. 5D–F, Supplementary Fig. S2). Cells in S/G2/M phases also displayed a specific hierarchized pattern in skin, being localized in the inner layer (basal layer) (Supplementary Fig. S2). High magnification of a group of ventral muscles showed an accumulation of dividing cells surrounding muscles (Fig. 5G–I, arrows). We also observed a high density of Fucci green cells (S/G2/M phases) in tendons, which were visualized by type I Collagen expression (Fig. 5J–O, arrows). In order to define whether this increase in the number of dividing cells in tendons was maintained during development, we analyzed limbs at late fetal stages, from E18.5 Fucci mouse embryos. We clearly observed an increased number of dividing cells in tendons compared to muscles (Supplementary Fig. S3A–C). We conclude that proliferating cells in S/G2/M phases display a specific spatial organization in mouse limbs, with a high number of proliferating cells at the muscle–tendon interface during fetal development.

Proliferating fetal muscle progenitors are regionalized in muscles

We previously observed that muscle progenitors displaying active Bmp signaling at muscle tips proliferate (Wang et al., 2010). However, we could not conclude whether the Pax7+ cells were proliferating more at the tips of muscle than in other muscle regions. Since the percentage of fetal muscle progenitors visualized in G1 phase was very low (0.6%, Fig. 3I), we only considered muscle progenitors in S/G2/M phases to quantify the number of cycling Pax7+ cells. Using the Fucci system, we analyzed the specific location of Pax7+ cells in S/G2/M phases inside individualized muscles. We observed the presence of a higher number of Fucci green+/Pax7+ cells at muscle tips compared to middle muscle regions (Fig. 6A–C). We confirmed this observation by quantifying the number of Fucci green+/Pax7+ cells at the tips versus middle of muscles (Fig. 6D–L). The proportion of muscle progenitors that were going through the S/G2/M phases was significantly higher at muscle tips compared to the middle of muscle (Fig. 6D–L). In order to define whether this specific regionalization of dividing muscle progenitors at muscle tips was maintained during development, we analyzed limb muscles at late fetal stages, in E18.5 mAG-hGem mouse embryos. At the end of fetal development, the number of proliferating cells had drastically decreased in muscles (Supplementary Fig. S3A–C). However, Pax7+ cells in the S/G2/M phases could still be observed at muscle tips (Supplementary Fig. S3D–F). We conclude that fetal muscle progenitors in S/G2/M phases were localized more at muscle tips compared to the middle of muscles.

Use of the Fucci system in the chick model

In order to introduce the Fucci system as a tool for the chick model, we used the RCAS retrovirus system, which has been widely used to misexpress genes in chick embryos. The RCAS replication-competent retrovirus infects dividing cells and spreads efficiently in chick limb tissues, such as cartilage, tendon and muscles (Duprez et al., 1998, 1996; Edom-Vovard et al., 2002; Wang et al., 2010). RCAS-Fucci-2A was transfected into chick embryonic fibroblasts (CEFs). Analysis at the cellular level revealed the presence of cells that displayed only red (G1 phase), yellow (G1–S transition) or green (S/G2/M phases) fluorescence in fibroblast cell culture (Supplementary Fig. S4). In addition time-lapse imaging of CEFs showed cell cycle-dependent changes in fluorescence in cells, exampled with a red to green conversion with an intermediary overlapping phase (yellow). This red to green conversion reflected the transition between G1 and S phase (Supplementary Fig. S5). This result demonstrated that the expression of human Cdt1 and Geminin proteins was oscillating in chick fibroblasts, in vitro. In order to visualize the cell cycle state of chick limb cells, RCAS-Fucci-2A-expressing CEFs were grafted into forelimb buds of E3.5 chick embryos. Grafted-forelimbs were analyzed 5 days later in order to allow virus spread. E8.5 RCAS-Fucci-2A infected limbs displayed red and green fluorescence (Fig. 7A–C). In chick limb sections, we observed cells in G1 phase (red cells), in G1/S transition phase (yellow cells) and in S/G2/M phases (green cells) (Fig. 7D–F). This showed that the Fucci cassette was also functional in chick embryos, in vivo, in addition to being functional in chick cell culture (Supplementary Figs. S4 and S5). In order to analyze the cell cycle state of chick myogenic cells, we used the MF20 and Pax7 antibodies to follow muscle fibers and muscle progenitors, respectively. It has to be noted that since we are overexpressing the Fucci cassette using the RCAS system, the Fucci system will be active only in virus-infected cells and not in all

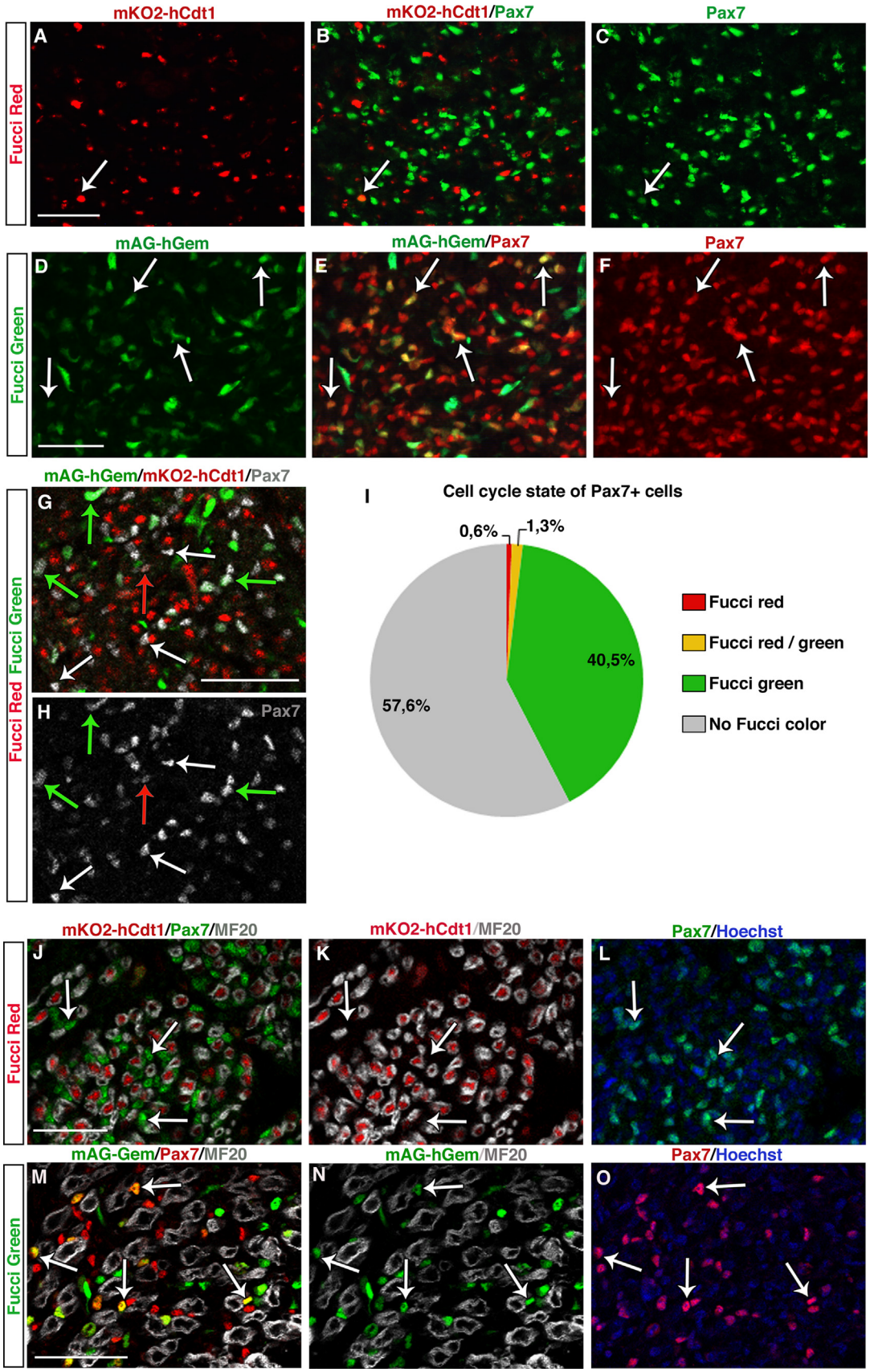

Fig. 3. Cell cycle state of Pax7+ cells during mouse fetal myogenesis using the Fucci system. Limbs from mKO2-hCdt1 (Fucci red, A–C, J–L), mAG-hGem (Fucci green, D–F, M–O) or double mKO2-hCdt1/mAG-hGem (Fucci red and green, G, H) E15.5 mouse embryos were sectioned and immunostained with the Pax7 antibody only (A–H) or with both Pax7 and MF20 antibodies (J–O). (A–C) Most Pax7+ cells were negative for Fucci red. Only occasional Pax7+ cells were found to be Fucci red positive (arrows). (D–F) Pax7+ cells were observed in S/G2/M phases of cell cycle (arrows). (G–H) Pax7+ cells in double mKO2-hCdt1/mAG-hGem muscles. Green arrows point to Fucci green+/Pax7+ cells, red arrow points to a rare Fucci red+/Pax7+ cell, and white arrows point to Pax7+ cells negative for the Fucci system. (I) Quantification of the proportion of Pax7+ cells observed in the different phases of cell cycle using the Fucci system. (J–L) Pax7+/Fucci red- cells were consistently observed outside MF20+ cells (J–L, arrows), while myonuclei were in a G1-like state (K). (M–O) Pax7+ cells were mainly in S/G2/M phases and were always MF20- cells (M,N, arrows). Scale bars=50 μm.

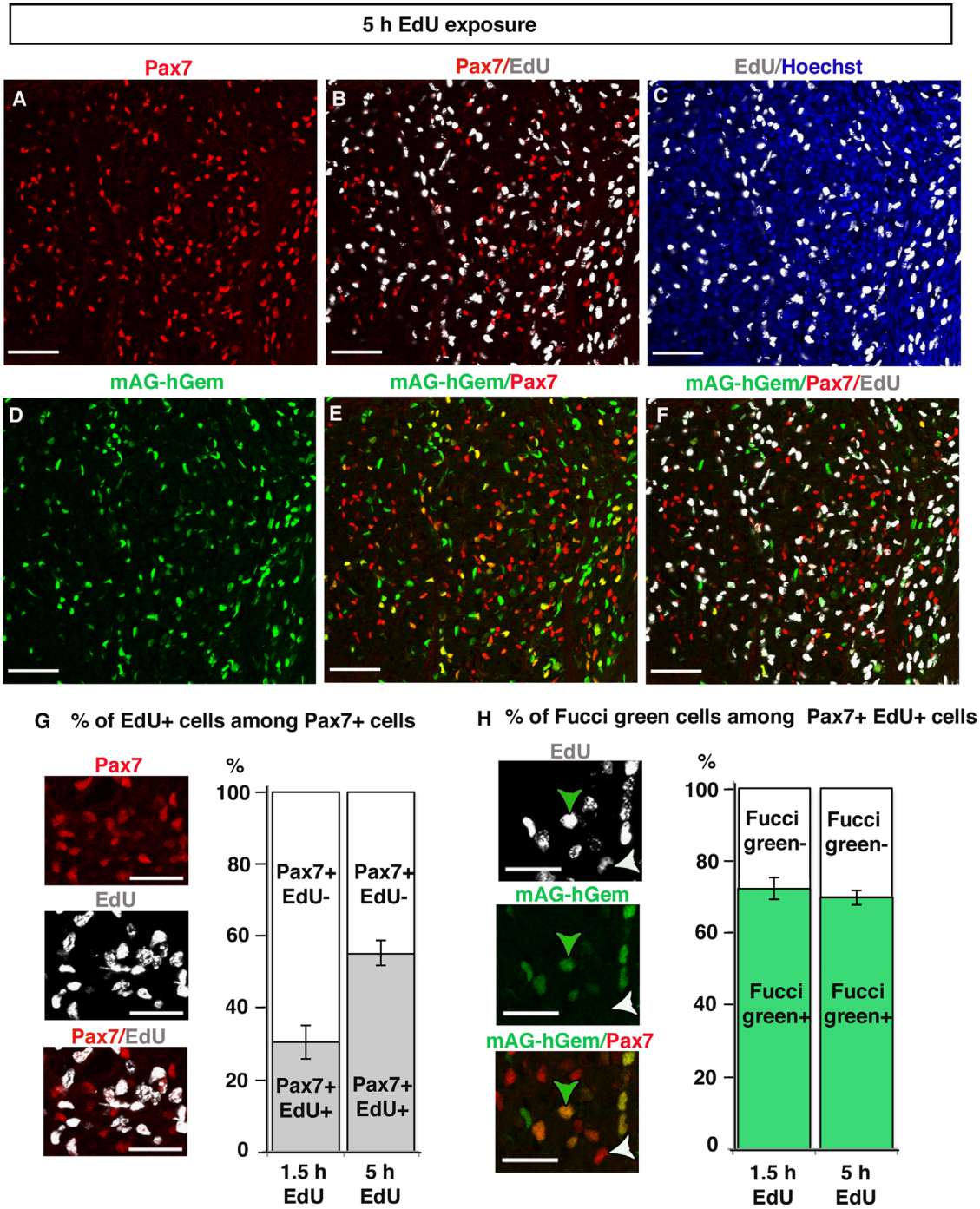

Fig. 4. The Fucci system did not cover all cycling cells labeled with EdU. mAG-hGem (Fucci green) adult pregnant mice were injected with EdU for 1.5 or 5 h before embryo fixation. (A–F) Representative transverse sections of forelimb muscles with 5 h EdU pulse were immunostained for Pax7 (red), EdU (gray) and mAG (green). (A–F) showed the same area of muscle with the different labeling. (G) Quantification of the proportion of the EdU+ and EdU- cells among the Pax7+ population after 1.5 and 5 h of EdU injection. Representative sections of muscle regions with 5 h EdU pulse showing Pax7/EdU+ cells and Pax7/EdU- cells. (H) Quantification of the proportion of the Fucci green+ and Fucci green– cells among the cycling Pax7+/EdU+ cells after 1.5 and 5 h of EdU exposure. Representative sections of muscles with 5 h EdU pulse showing Fucci green+/EdU+/Pax7+ cells (green arrowheads) and Fucci green- EdU+/Pax7+ cells (white arrowheads). 30% of cycling (EdU+) Pax7+ cells were not covered by the Fucci system, after 1.5 and 5 h EdU pulses. (A–F) Scale bars=50 μm; (G, H) scale bars=25 μm.

chick cells. This prevented us from any global quantitative analysis. However, we could clearly observe that muscle fibers displayed red myonuclei (Fig. 7G–I, red arrowheads). Fucci red+ cells could also be observed outside muscle fibers (Fig. 7G–I, red arrows). Cells in S/G2/M phases (green cells) were exclusively observed outside of muscle fibers (Fig. 7G–I, green arrows). Pax7+ cells labeled with the Fucci system were mainly observed in S/G2/M phases (Fig. 7J–L, green arrows). We conclude that the Fucci cassette (containing human proteins) works in chick cells in vitro and in vivo and that myonuclei of differentiated muscle fibers are in a G1-like state during chick fetal muscle development. These results are consistent with that observed in Fucci transgenic mouse embryos.

Fucci state of adult mouse myoblasts in vitro and satellite cells ex vivo

Adult skeletal muscles possess a remarkable regenerative capacity. This ability relies on the presence of adult muscle stem cells, named satellite cells that are required and sufficient for

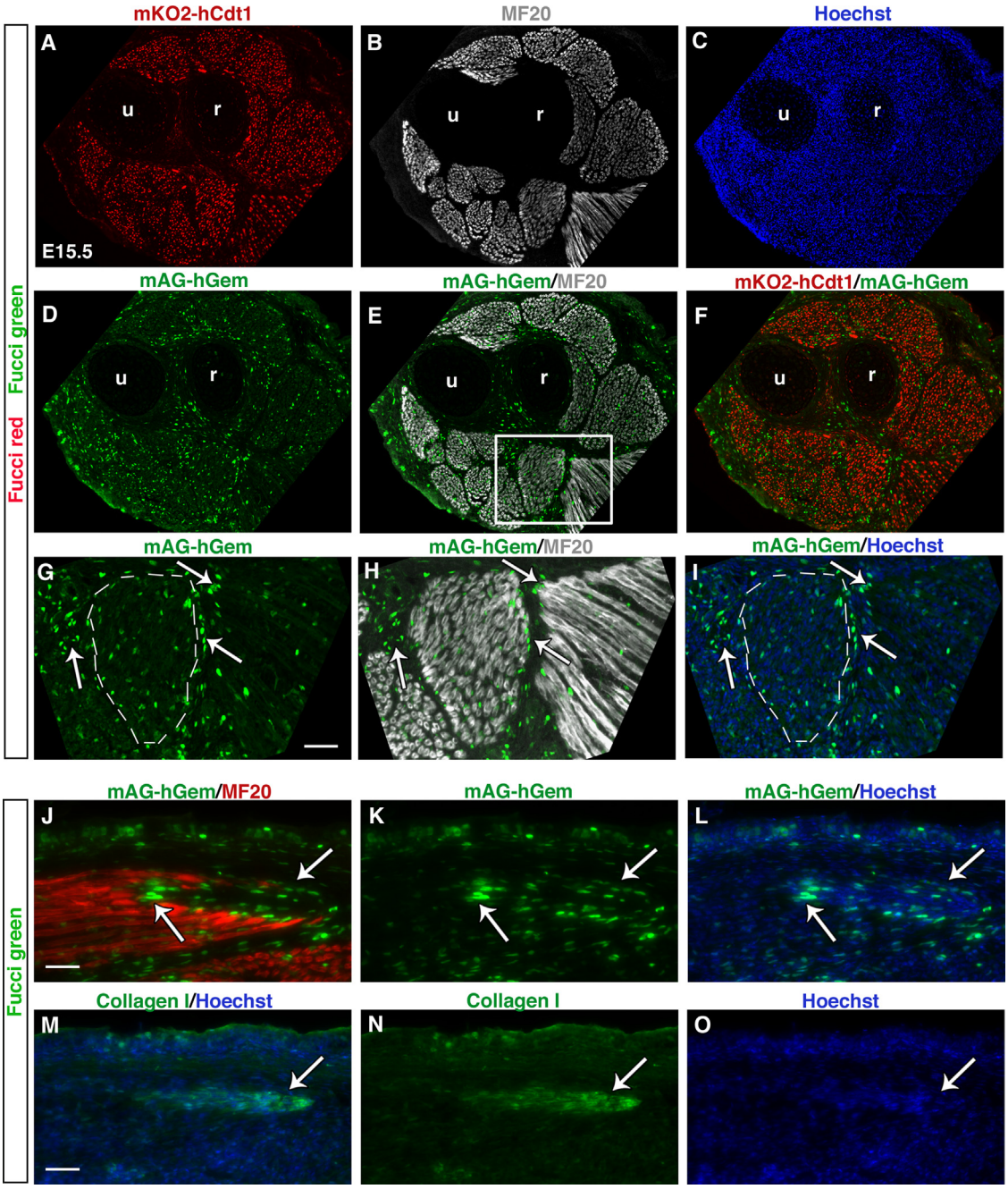

Fig. 5. Pattern of cell proliferation in E15.5 mouse limbs using the Fucci system. (A–I) Limb sections of mKO2-hCdt1/mAG-hGem (double Fucci red and green) E15.5 mouse embryos were immunostained with MF20 antibody. (A–F) Cells displaying Fucci red fluorescence were mainly located in muscles (A–C), while cells in S/G2/M phases were non-homogenously located in limbs (D–F). There was an increase of proliferating cells in S/G2/M phases delineating individualized muscles (low magnification D–F; high magnification G–I, arrows). (J–O) Longitudinal and adjacent limb sections were immunostained with the MF20 (J–L) and type I collagen (M–O) antibodies. The presence of proliferating cells in S/G2/M phases (J–L, arrows) was observed in tendons (M–O, arrows) close to muscle tips. Scale bars=50 μm.

muscle regeneration (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). In order to determine whether adult muscle progenitors displayed a Fucci distribution similar to fetal muscle progenitors, we performed primary cultures of adult myoblasts and cultures of isolated muscle fibers (Le Grand et al., 2012) from Fucci red/green mice. We first analyzed cell cycle in adult myoblasts in both proliferation and differentiation conditions (Fig. 8). In proliferation conditions, all adult myoblasts were Pax7+ and MyoD+ (Fig. 8A–F) (Cooper et al., 1999). In addition, adult myoblasts were dividing in proliferating conditions (Fig. 8G–I). Proliferating adult myoblasts could be observed in all cell cycle phases: G1 phase (red nuclei and arrows), G1/S transition (yellow nuclei and arrows) or in S/G2/M phases (green nuclei and arrows) or with no Fucci color (Fig. 8A–F, white arrows). Analysis of the proportion of cells in each cell cycle phase showed that adult myoblasts were mostly in G1 phase (55.3%) in proliferating culture condition (Fig. 8M). The 17.3% of cycling adult myoblasts displaying no Fucci color (Fig. 8) were likely in early G1 phase, since early G1 phase was not covered by the Fucci system (Sakaue-Sawano et al., 2008). The predominance of G1 phase (Fucci red) in dividing adult myoblasts was in contrast to the in vivo situation, where Pax7+ fetal progenitors labeled by the Fucci system were mostly in S/G2/M phases (Fig. 3) and where MyoD+/Myosin- myoblasts were also mostly in S/G2/M phases (Fig. 2). This highlights a clear

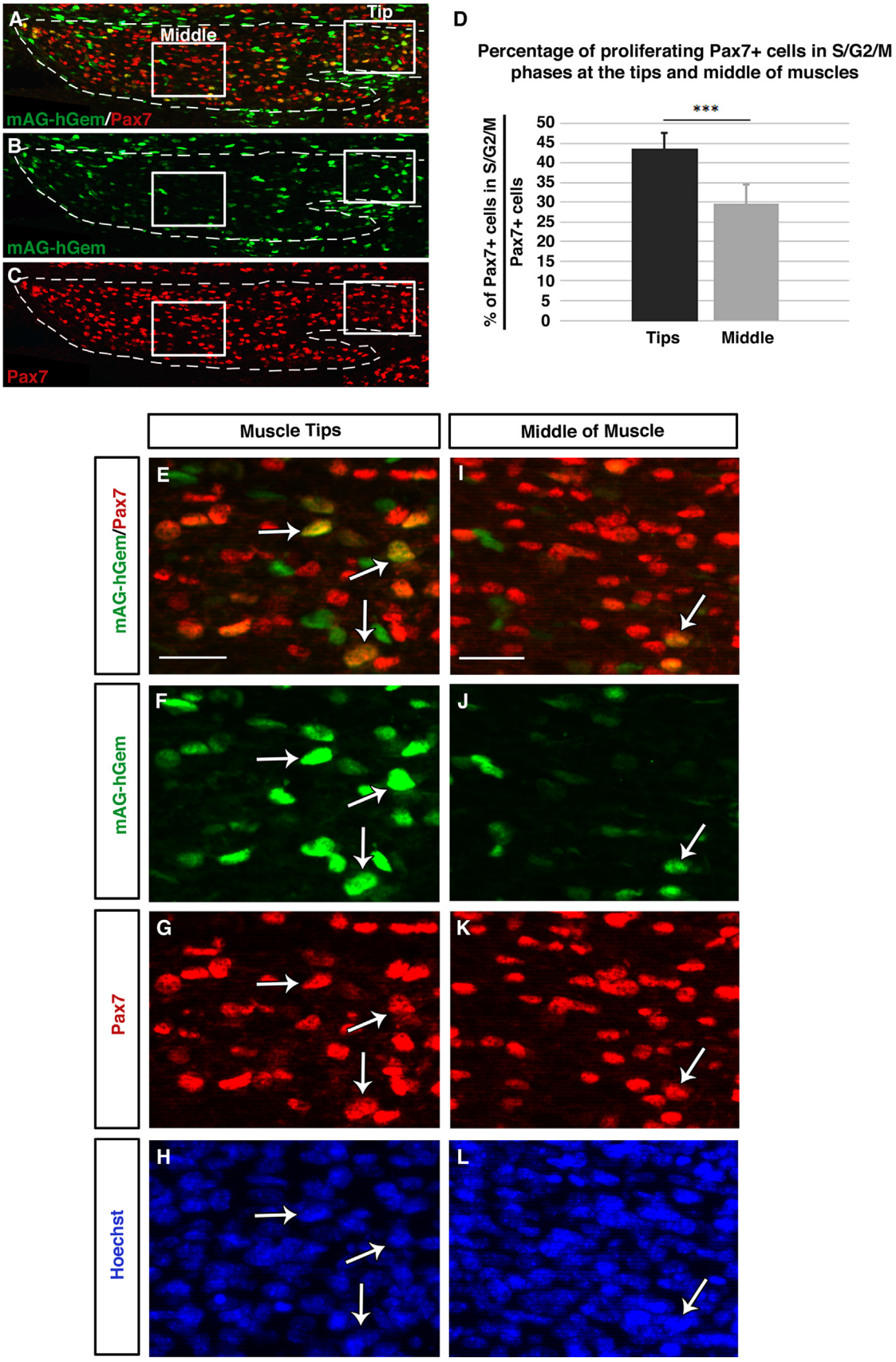

Fig. 6. Spatial regionalization of proliferative muscle progenitors in individualized muscles. (A–C) Limb muscle longitudinal sections from mAG-hGem E15.5 mice were immunostained with mAG (green) and Pax7 antibodies (red). (D) The percentage of proliferating Pax7+ cells in S/G2/M phases among Pax7+ cells was significantly higher at muscle tips compared to the middle of muscle, ***p-value < 0.001. (E–L) High magnified pictures of muscle tips (E–H) and middle of muscle (I–L), representative of the higher number of proliferating Pax7+ cells in S/G2/M phases (arrows) at muscle tips compared to that in the middle of muscles. Scale bars=25 μm.

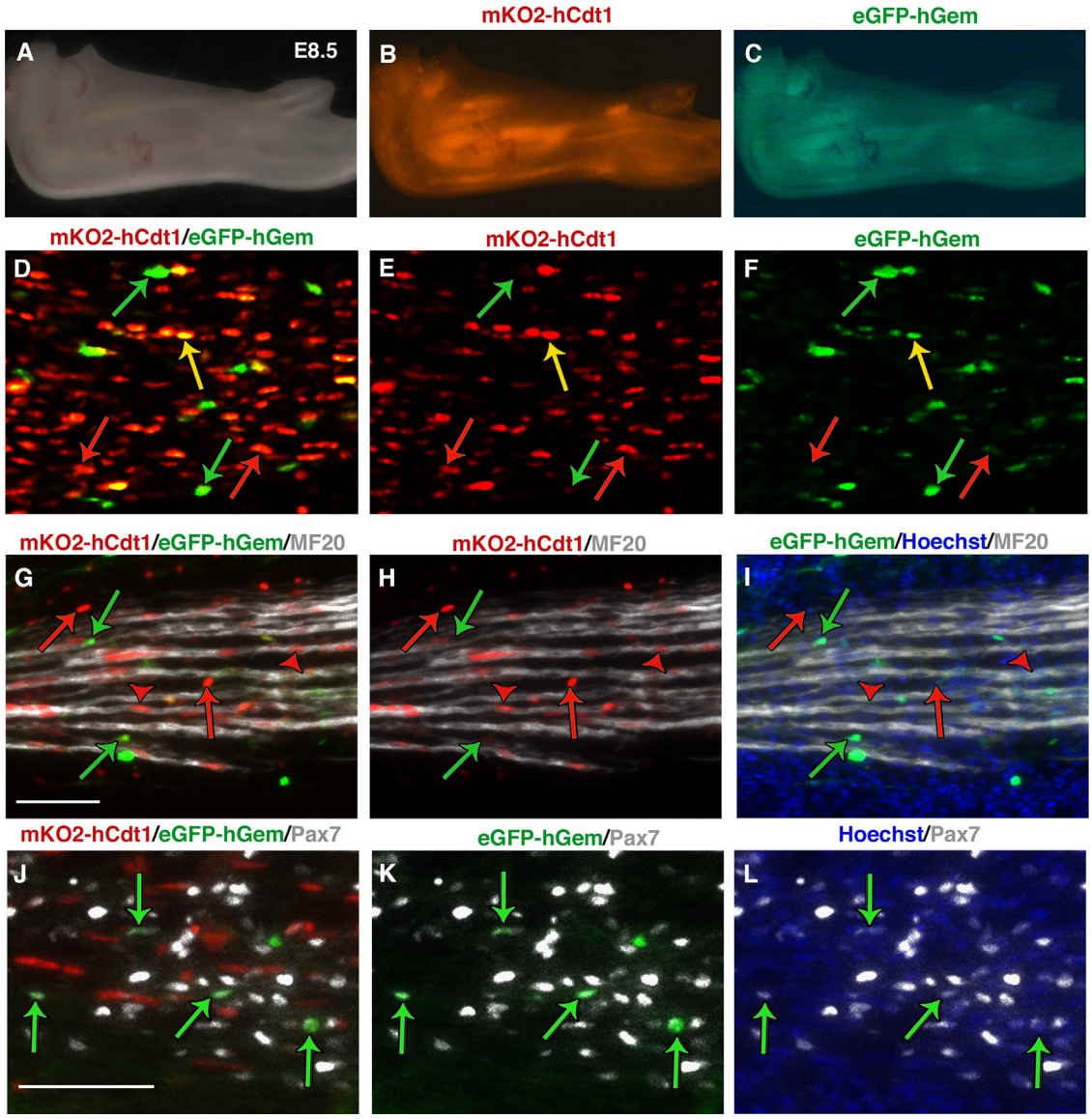

Fig. 7. RCAS-Fucci-2A in chick embryos. (A–C) Limbs from E3.5 chick embryos were grafted with fibroblasts expressing the RCAS-Fucci-2A retrovirus. Wholemount views of the same E8.5 limb expressing RCAS-Fucci-2A, 5 days after grafting: bright field (A), Fucci red (B) and Fucci green (C). (D–F) Sections of RCAS-Fucci-2A-infected limbs were performed. Limb cells could be observed in G1 (red arrows), in G1/S transition (yellow arrows) or in S/G2/M phases (green arrows). (G–L) Sections of RCAS-Fucci-2A-infected limbs were immunostained with MF20 (white) (G–I) or Pax7 (white) (J–L) antibodies. (G–I) Fucci red+ nuclei were observed in muscle fibers (G–I, red arrowheads) or outside muscle fibers (G–I, red arrows). Proliferating cells in S/G2/M phases (green) were systematically observed outside muscle fibers (G–I, green arrows). Pax7+ cells could be observed in S/G2/M phases (J–L, green arrows) but barely in G1 phase. Scale bars=50 μm.

difference between in vitro and in vivo situations for proliferating muscle progenitors and myoblasts. The culture medium contains a high level of growth factors including FGF, which is able to prevent myogenic differentiation and to allow expansion of muscle progenitor progeny. Thus, proliferating primary myoblasts expressing both Pax7 and MyoD are likely to be in an in vitro "blocked" state between stem cells and committed progeny that rarely exists in vivo. Upon serum removal, adult myoblasts differentiate and fuse into multinucleated myotubes. Under these conditions (4 days in differentiation medium), the majority of the myonuclei within myotubes were displaying Fucci red fluorescence (94.5%) (Fig. 8J–L and N). This was consistent with the in vivo situation during fetal myogenesis (Fig. 1).

In order to visualize satellite cells in their niche, adult muscle fibers can be isolated from an intact skeletal muscle and maintained in culture in "floating conditions". We performed single myofiber culture from EDL muscles of Fucci red or Fucci green adult mice. At T0 h, just after the fiber isolation procedure, all myonuclei displayed a red fluorescence (Fig. 9A–C). The G1-like state of myonuclei in isolated muscle fibers was consistent with the in vivo situation during fetal development (Figs. 1 and 2) and with adult myoblast cultures in differentiation conditions (Fig. 8). At T0 h Pax7+ cells were systematically negative for the Fucci red and green reporters (Fig. 9A–F, arrows), consistent with the G0 quiescent state of satellite cells in this culture system (Fukada et al., 2007). When cultured on single myofibers, satellite cells remain in their niche, and generate a heterogeneous progeny of proliferating and differentiating satellite cells after 72 h ex vivo (around four divisions) (Zammit et al., 2004). At this time point, Pax7+ cells were observed in S/G2/M phases but rarely in G1 phase (Fig. 9G–L, arrows). In clusters, MyoD+ cells could be observed either in G1 phase (Fig. 9M–O, arrows) or in S/G2/M phases (Fig. 9P–R, arrows). Myonuclei continued to display strong red fluorescence after 72 h of culture (Fig. 9G–I and M–O).

In summary, myonuclei were in a G1-like state both in cultured myotubes and in single myofibers similar to fetal myonuclei.

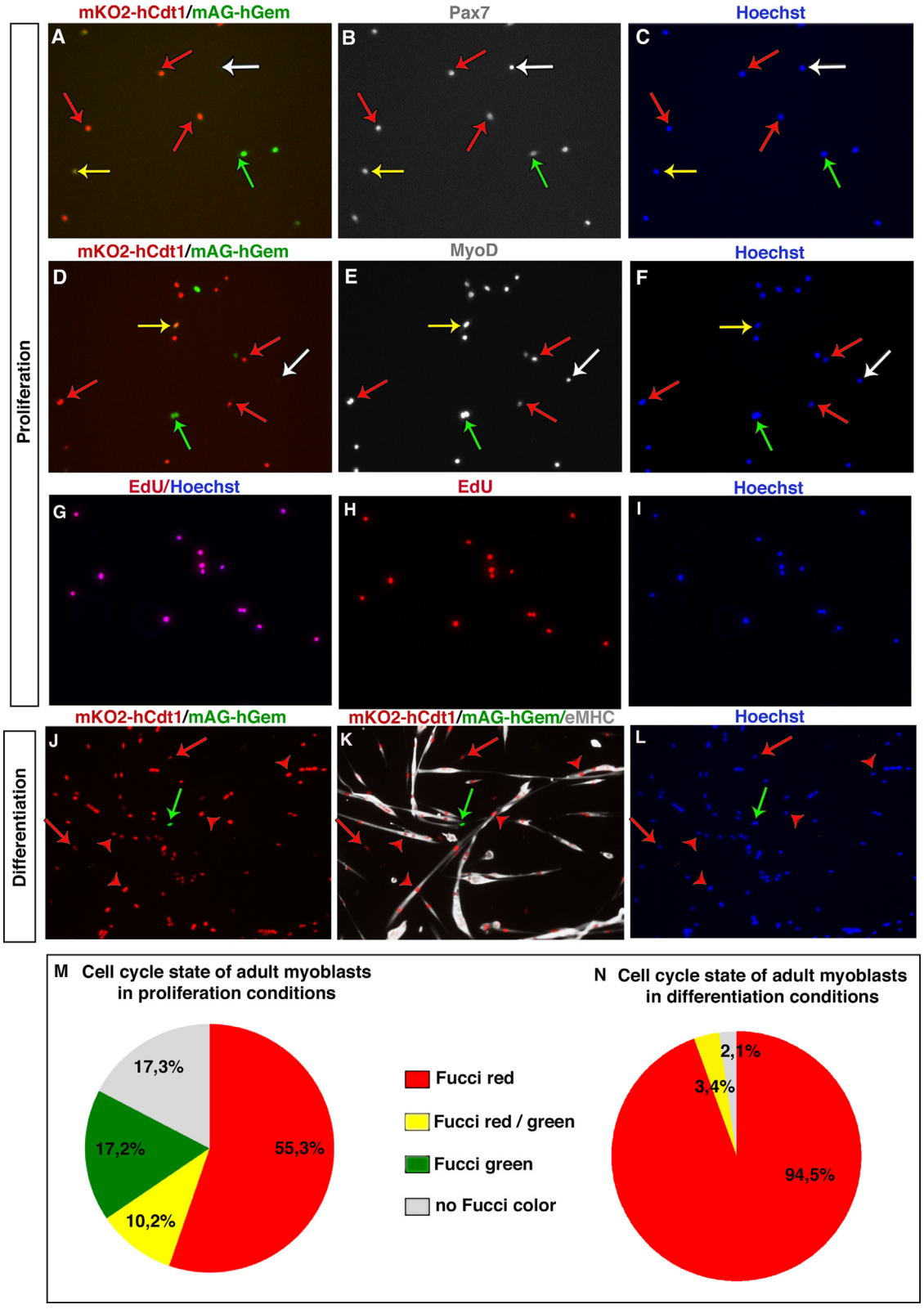

Fig. 8. Cell cycle state of adult myoblasts and myotubes in culture. Primary myoblasts from limb muscles of 2-month post-natal double Fucci red/green mice were cultured in proliferation conditions (A–I) or differentiation conditions (J–L). Myoblasts were immunostained with the Pax7 antibody (A–C), MyoD antibody (D–F) or EdU (G–I) in proliferation conditions, or with the MF20 antibody in differentiation conditions (J–L). (A–C) is the same field showing Fucci red and green cells (A), Pax7+ cells (B), and nuclei (C). All myoblasts were Pax7+ (B, C). (D–F) is the same field showing Fucci red and green cells (D), MyoD+ cells (E), and nuclei (E). All myoblasts were MyoD+ (E, F). (G–I) is the same field showing EdU+/Hoechst+ cells (G), EdU staining (H) and nuclei (I), after 24 h of EdU treatment. (J–L) is the same field showing Fucci red and green cells (J), Fucci red and green and MF20+ cells (K) and nuclei (L). All myonuclei were in G1 phase. (M, N) Quantification of the proportion of adult myoblasts in different phases of cell cycle using the Fucci system in proliferation conditions (M) and in differentiation conditions (N).

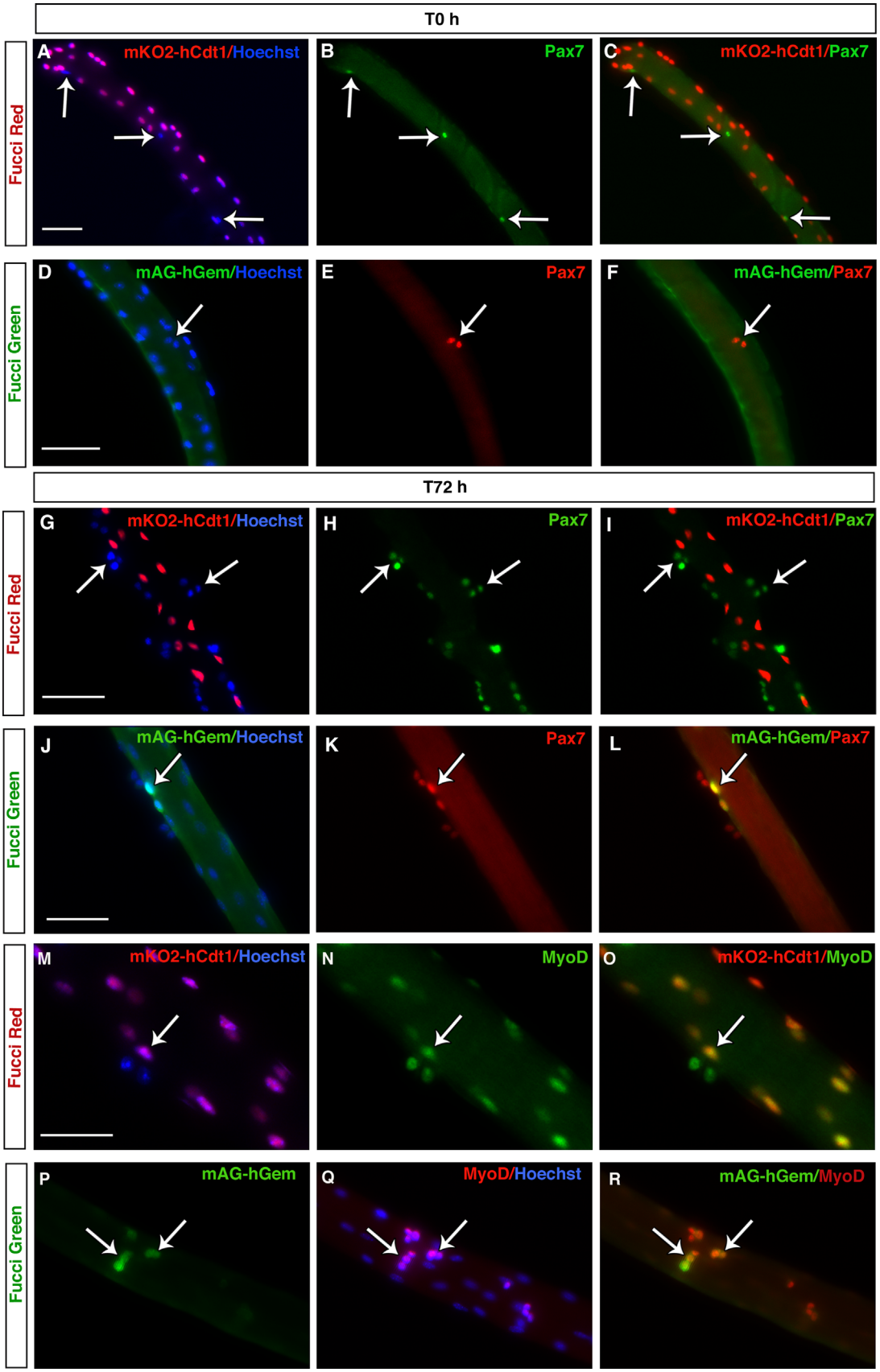

Fig. 9. Cell cycle state of myogenic cells in single myofiber cultures from Fucci transgenic mice. Isolation of single myofibers from EDL muscles of mKO2-hCdt1 (A–C, G–I, M–O) or mAG-hGem (D–F, J–L, P–R) 2-month-old mice. (A–F) Single fibers from Fucci red (A–C) or from Fucci green (D–F) mice were immunostained with Pax7 just after isolation (T0 h). At 0 h of culture, satellite cells labeled with Pax7 were negative for Fucci red (A–C, arrows) and for Fucci green (D–F, arrows). (G–R) Single fibers from Fucci red (G–I, M–O) or from Fucci green (J–L, P–R) mice were cultured for 72 h and then immunostained with Pax7 (G–L) or MyoD (M–R) antibodies. (A–C, G–I, M–O) mKO2 fluorescence (red) was observed without any additional labeling. (D–F, J–L, P–R) Single fibers from Fucci green mice were double-immunostained with mAG (green) and Pax7 (red, D–F) or MyoD (red, J–L) antibodies. Myonuclei were positive for Fucci red reporter (A, C). In 72 h clusters, Pax7+ cells were not Fucci red (G–I, arrows) but could be visualized with the Fucci green reporter (J–L, arrows). In clusters, MyoD+ cells could be visualized either with the Fucci red reporter (M–O, arrows) or with the Fucci green reporter (P–R, arrows).

Quiescent satellite cells on freshly prepared myofibers were negative for the Fucci system, while activated satellite cells in their niche exhibit a Fucci distribution similar to fetal muscle progenitors. In contrast to in vivo and ex vivo situations, in vitro cultured primary adult myoblasts showed a Fucci pattern independent of the presence of Pax7 or MyoD.

Discussion

In this paper we used the Fucci system to analyze the cell cycle state of limb cells during fetal development, with a particular focus on muscle. We found that the Fucci red system did not follow actively cycling cells in G1 phase since myonuclei were all Fucci red and the early G1 phase was not fully covered by the Fucci system. However, the Fucci green system allowed us to highlight a specific pattern of cell proliferation in mouse limbs, with an increase of cycling cells in S/G2/M phases in muscle-surrounding tissues and at muscle tips. We also analyzed the Fucci state of adult muscle cells using in vitro and ex vivo systems.

Myonuclei are in a G1-like state

A striking result of this study was the observation that the myonuclei of terminally differentiated muscle fibers displayed Fucci red fluorescence, suggesting that myonuclei were in a G1-like phase (Fig. 10). This particular state of cell cycle of post-mitotic muscle cells was observed in all the situations we analyzed. Myonuclei were Fucci red+ during mouse and chick fetal myogenesis, in cultured adult myotubes and isolated adult muscle fibers. This specific G1-like state of post-mitotic muscle cells has also been observed in Fucci transgenic zebrafish lines (Sugiyama et al., 2009). Because it is well established that terminally differentiated muscle fibers do not divide, this implies that myoblasts exit from the cell cycle in G1 during differentiation and then remain in G1-like phase during their lifetime. The cell cycle exit in G1 phase of myoblasts is consistent with old studies in cell cultures, based on radioactive-labeled-thymidine incorporation (Bischoff and Holtzer, 1968, 1969). The maintenance of Fucci red fluorescence in differentiated cells had already been observed in post-mitotic neurons in mouse Fucci embryos (Sakaue-Sawano et al., 2008) and in neural stem cells in differentiation conditions (Roccio et al., 2013). We also observed that the post-mitotic cells of the external layer (spinous layer) of the skin (reviewed in Fuchs, 2008) were also Fucci red (Supplementary Fig. S2). However, in cartilage elements, we did not observe a strict correlation between differentiated cells in diaphyses and Fucci red fluorescence (Supplementary Fig. S2D–F). We believe that muscle, neuronal and skin cells exit from cell cycle in G1 phase to differentiate and remain in a blocked G1-like phase. The maintenance of Fucci red fluorescence in post-mitotic cells could reflect the stability of the mKO2-hCdt1 transgene in post-mitotic cells or reflect a blocked G1-like state. The G1-like phase of myonuclei known to express MyoD and p57 is consistent with the association of MyoD activity and G1 phase and the role of p57 in timing cell cycle exit at G1/S transition (Kitzmann et al., 1998; Lindon et al., 1998; Gui et al., 2007). It is not clear what would be the physiological consequences/advantages of the maintenance of myonuclei in a G1-like state compared to a G0 state. In mammalian cells, the process of terminal differentiation is normally considered to be irreversible in physiological conditions. However, under experimental conditions, terminally differentiated mammalian myotubes have been shown to dedifferentiate and proliferate when stimulated with the appropriate signals (Blais et al., 2007; Mastroyiannopoulos et al., 2012; Odelberg et al., 2000; Pajcini et al., 2010). One conclusion from these studies is that the diminution of pRb is of critical importance in the induction of the myonuclear S-phase reentry (Blais et al., 2007; Pajcini et al., 2010). The experimental and rapid S-phase reentry of myonuclei is consistent with the physiological state of myonuclei in G1-like phase. In addition, the reversal of cellular differentiation to form proliferating progenitor cells is a common feature in the urodele amphibians during the appendage regeneration process (Brockes and Kumar, 2002).

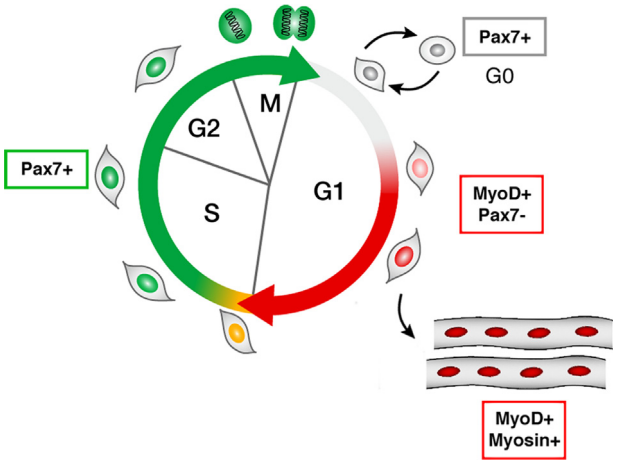

Fig. 10. Schematic representation of the cell cycle state of myogenic cells. Quiescent Pax7+ cells are in G0 phase and are not labeled by the Fucci system. The early G1 phase is not covered by the Fucci system. The Fucci system labels Pax7+ cells in S/G2/M phases and MyoD+ cells in G1 phase. MyoD+ cells exit cell cycle in G1 phase and retained a G1-like state leading to Fucci red myonuclei.

Muscle progenitor cells, cell cycle and quiescence in vivo

Another striking result of this study is the low percentage (0.6%) of Pax7+ cells in the G1 phase and the high proportion (57.6%) of Pax7+ cells not labeled by the Fucci system during mouse fetal myogenesis (Fig. 3I). We showed that a pool of cycling cells labeled with EdU or Ki67 did not express any Fucci reporter (Fig. 4, Supplementary Fig. S1). We believe that these cycling Fucci- cells are in early G1 phase of the cell cycle (Fig. 10), since it has been previously shown that this phase is not covered by the Fucci system (Sakaue-Sawano et al., 2008; Sugiyama et al., 2009). This shows that the Fucci system does not cover the complete cell cycle, specially the G1 phase, in muscle progenitors. In addition to the cycling Pax7+ cells not covered by the Fucci system, we also observed high proportions of Pax7+ cells that did not incorporate EdU after the different EdU pulses (Fig. 4). These non-dividing Pax7+/EdU- cells include to the quiescent fetal muscle progenitor population, already described by Picard and Marcelle (2013). The addition of cycling Pax7+ cells not covered by the Fucci system and quiescent Pax7+ cells explains the high proportion of (cycling and non cycling) Pax7+ cells not labeled by the Fucci system.

Although the early G1 phase is not covered by the Fucci system (Sakaue-Sawano et al., 2008), another non-exclusive explanation for the low percentage of Pax7+ cells in G1 is that fetal muscle progenitors display a short G1 phase during cell cycle. Since these cells are the exclusive source of myonuclei for fetal and peri-natal myofibers, it is likely that the Pax7+ population divides rapidly to allow muscle tissue growth during fetal and peri-natal myonuclear accretion phases. This feature (short G1 phase) is shared with embryonic stem cells (Fluckiger et al., 2006; Savatier et al., 1994; Stead et al., 2002) and stem cells in the crypts of the small intestine, which are rapidly dividing to fuel the active self-renewal of the epithelium (Clevers, 2013). A further hypothesis for the low percentage of Pax7+ cells in G1 during fetal myogenesis is that the increase of the Pax7+ cell pool occurs via

asymmetric division. One daughter cell will differentiate in G1 phase (express MyoD and no longer express Pax7) and the other will rapidly re-enter into the cell cycle (and maintain Pax7 expression). The fact that the proportion of (MyoD+ Myosin-) myoblasts displaying Fucci red (15.1%) is higher than that of (Pax7+) muscle progenitors (0.6%) favors the idea that asymmetric division could occur during fetal muscle growth. This mechanism has already been described to explain the self-renewal of the satellite cell population during adult muscle homeostasis (Kuang et al., 2007) and following muscle injury (Troy et al., 2012).

Regionalization of cell proliferation in limbs during fetal development

An important observation from this study is the specific pattern of cell proliferation in fetal mouse limbs. Limb cell proliferation does not appear to be a random and homogeneous process. We observed an increased density of cells in S/G2/M phases in muscle-surrounding tissues, corresponding to muscle borders and tendons. In addition to dividing non-muscle cells, there were also large numbers of muscle progenitors in S/G2/M phases at muscle tips. This increase of Pax7+ cells in S/G2/M phases at muscle tips could reflect an increase of cell proliferation or an augmentation of the length of the S, G2 or M phases in these muscle regions. The selective cell proliferation (myogenic and non myogenic cells) at the muscle–tendon interface suggests that signals in this region maintain cells in a proliferating state. This is consistent with the presence of enhanced active signaling pathways at the muscle–tendon interface such those of Bmp and Fgf (Edom-Vovard et al., 2001, 2002; Eloy-Trinquet et al., 2009; Wang et al., 2010). Our results suggest that during fetal myogenesis, localized signals regulate the developmental changes and regionalization of proliferating fetal muscle progenitors, supporting the idea that fetal muscle growth can be preferentially achieved at muscle tips. We believe that the muscle/tendon interface region behaves as a signaling center during fetal development. There are arguments to suggest that selective and regionalized cell proliferation also occurs during adult life. A greater concentration of satellite cells at the ends of growing fibers has been reported in muscles of post-hatching chicken (Allouh et al., 2008). An increase of satellite cell proliferation has also been observed close to the tendons in conditions of injury (Tsujimura et al., 2006). In addition, clusters of activated satellite cells are more concentrated at the extremities of isolated adult muscle fibers (Wang et al., 2010).

In summary, using the Fucci system we have shown that Pax7+ fetal (in vivo) and adult (ex vivo) muscle progenitors are mainly visualized in S/G2/M phases. Moreover, we showed that myonuclei of fetal and adult muscle fibers were maintained in a G1-like phase, preserving a strong Fucci red reporter expression, which can be of a wide use for imaging and analysis of myonuclei. Although, the Fucci system lacks the coverage of the cell cycle during early G1, our results reveal that Fucci can be used to study proliferating muscle cells in S/G2/M phases. Finally, our analysis of the spatial localization of proliferating cells in S/G2/M phases revealed a regionalization of the cell cycle during mouse limb fetal myogenesis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.05.015.

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