**Doctoral Thesis in agreement with the cotutelle contract between:**

# Université Pierre et Marie Curie

**and**

# Freie Universität Berlin

# Link between signalling pathways, cell cycle and mechanical forces during foetal myogenesis

**Joana Helena Esteves de Lima**

**Supervised by Dr Delphine Duprez and Prof Dr Carmen Birchmeier**

Doctoral Thesis in agreement with the cotutelle contract between:

# Université Pierre et Marie Curie

Doctoral School: Ecole Doctorale Complexité du Vivant
Laboratoire de Biologie du Développement - Institut de Biologie Paris Seine

and

# Freie Universität Berlin

Department of Biology, Chemistry and Pharmacy of the Freie Universität
Laboratory: Max Delbrück Center for Molecular Medicine

## Link between signalling pathways, cell cycle and mechanical forces during foetal myogenesis

Joana Helena Esteves de Lima

Supervised by Dr Delphine Duprez and Prof Dr Carmen Birchmeier

Publically presented and defended on the 28th September 2015 in Paris and on the 16th October 2015 in Berlin

PhD Jury composed of:

Dr Delphine Duprez (Director of research, thesis co-supervisor)

Prof Dr Carmen Birchmeier (Professor, thesis co-supervisor)

Dr Pascal Maire (Director of research, reviewer)

Dr Pascal de Santa Barbara (Director of research, reviewer)

Dr Gisèle Bonne (Director of research, representative of the UPMC)

Prof Dr Simone Spuler (Professor, representative of the FU)

Prof Dr Sigmar Stricker (Professor, FU guest)

Dr Stefanie Grunwald (FU Post-doc)

*To my parents and sister*

# Declaration

I hereby declare that I have done my doctoral thesis entitled "Link between signalling pathways, cell cycle and mechanical forces during foetal myogenesis" independently and with no other sources or aids than cited.

Berlin, 15th July 2015

_____

(signature)

# Acknowledgements

I thank Dr Delphine Duprez for accepting me in her laboratory and for entrusting me with this project, for being always present for scientific discussion and for supervising and correcting all the writing of my PhD thesis and manuscripts.

I thank Prof Dr Carmen Birchmeier for supervising and correcting my PhD thesis, for reading and commenting on the manuscripts and for being always available for discussing the project.

I thank Dr Pascal Maire and Dr Pascal de Santa Barbara for accepting to be the reviewers of my thesis. I thank Dr Gisèle Bonne, Prof Dr Simone Spuler, Prof Dr Sigmar Stricker and Dr Stefanie Grunwald for accepting to be part of the members of my PhD jury.

I thank the founders of the MyoGrad Programme that made possible this enriching PhD in cotutelle: Prof Dr Simone Spuler, Prof Dr Thomas Voit and Dr Helge Amthor. I thank Susanne Wissler for essential support at the administrative level during all my PhD.

I thank Dr Emmanuelle Havis for being always available for scientific discussion, technical support and for reading and commenting on the manuscripts.

I thank Dr Ines Lahmann for scientific and technical supervision at the MDC laboratory in Berlin.

I thank Marie-Ange Bonnin for being always available to help me and for being of major technical assistance during my entire thesis.

I thank Adeline Bourgeois for teaching me many of the techniques used in our laboratory.

I thank Dr Claire Fournier-Thibault for scientific discussion and tutorship during my PhD.

I thank all the other members of the Duprez's laboratory that contributed to scientific discussion and technical help, namely Dr Benjamin Charvet, Sonya Nassari, Stephanos Papaefstathiou, Ludovic Gaut, Cédrine Blavet, Dr Estelle Hirsinger, Dr Cécicle Milet and Mickael Orgeur. I thank all the members of the Birchmeier's laboratory, in particular to Dr

Michael Strehle, Dr Thomas Muller, Carola Griffel, Mandy Terne, Séverine Kunz and Jie-Shin Chen.

I thank Jürgen Stumm for kindly translating the summary of my thesis into German.

I thank Dr Thierry Jaffredo and Dr Olivier Haccard for allowing me to use material belonging to their laboratories essential for some of my experiments.

I thank Dr Pascal de Santa Barbara for molecular tools required to answer some of the questions addressed in the part 2 of the results.

I thank Sophie Gournet for helping with illustrations of my thesis and manuscripts.

# Table of Contents

# Summary

Foetal myogenesis relies on PAX7+ muscle progenitors that provide the source of cells for muscle growth during development and for the generation of the satellite cell pool. We aimed to decipher the signals that regulate the balance between myogenic differentiation and proliferation. We performed an exhaustive analysis of the cell cycle phases of myogenic cells during foetal myogenesis. I defined that PAX7+ cells in the S/G2/M phases were enriched at the contact points to the tendons. BMP and NOTCH signals increase the number of PAX7+ cells during foetal development, but affect differentiation in a positive and negative manner, respectively. I revealed that BMP and NOTCH increase the number of PAX7+ cells independently of each other. However, they act antagonistically during differentiation. Thus, the interplay between NOTCH and BMP signalling differs in proliferation and differentiation. Because muscle is a mechanical tissue, we tested the importance of muscle contraction for foetal myogenesis in chick embryos. I found that the block of muscle contraction during foetal myogenesis mimicked a NOTCH loss-of-function, *i.e.* decreased the number of foetal muscle progenitors and shifted the balance between proliferation and differentiation towards a differentiation fate. Mechanical forces provided by muscle contractions are sensed in myonuclei by the transcriptional co-activator YAP1 that regulates expression of the NOTCH ligand JAGGED2 in muscle fibres. This JAGGED2 signal keeps the muscle progenitors in an undifferentiated state and suppresses differentiation.

# Resumé

La myogenèse fœtale repose sur les cellules progénitrices musculaires PAX7+ qui assurent la croissance musculaire au cours du développement et qui sont à l'origine des cellules satellites. Nous avons cherché à interpréter les signaux régulant la myogenèse fœtale et leur lien avec le cycle cellulaire. Nous avons effectué une analyse exhaustive du cycle cellulaire des cellules myogéniques au cours de la myogenèse fœtale. Nous avons aussi identifié que les cellules PAX7+ progressant dans le cycle cellulaire (phases S, G2, et M) sont régionalisées aux extrémités des muscles. Les voies de signalisation BMP et NOTCH régulent positivement le nombre de cellules PAX7+ pendant le développement fœtal mais ont un effet différent sur la différenciation musculaire. Nous avons montré que les voies de signalisation BMP ou NOTCH augmentent le nombre de cellules PAX7+ de manière indépendante. Nous avons aussi identifié des interactions antagonistes entre ces deux voies lors de la différenciation musculaire. Nous avons testé l'importance de la contraction musculaire pendant la myogenèse fœtale chez l'embryon de poulet. Le blocage des contractions musculaires mime un phénotype de perte de fonction NOTCH, à savoir une diminution du nombre de cellules progénitrices musculaires avec une tendance à la différenciation musculaire. Nous avons aussi montré que les forces mécaniques produites par les contractions musculaires sont détectées par le co-activateur transcriptionnel YAP1 qui régule l'expression d'un ligand de NOTCH au sein des fibres musculaires, qui à son tour va maintenir le pool de cellules progénitrices musculaires fœtaux.

# Zusammenfassung

PAX7+ Muskelvorläuferzellen spielen in der fötalen Myogenese eine entscheidende Rolle, indem sie das Muskelwachstum während der Entwicklung sowie die Bildung des Satellitenzellpools tragen. In der vorliegenden Arbeit war es unser Ziel, die Signalmechanismen zu entschlüsseln, welche die fötale Myogenese im Zusammenhang zum Zellzyklus regulieren. Dazu analysierten wir Zellzyklusstadien myogener Zellen während der fötalen Myogenese. Wir konnten eine Anhäufung PAX7+ Zellen in späten Zellzyklusstadien an den Enden fötaler Muskeln zeigen. BMP und NOTCH Signalwege üben eine positive Regulation auf die Anzahl PAX7+ Zellen während der fötalen Entwicklung aus, mit unterschiedlichen Konsequenzen für die fötale Muskeldifferenzierung. Wir konnten zeigen, dass während der Proliferation von Pax7+ Zellen BMP- und NOTCH unabhängig voneinander agieren, während der Differenzierung jedoch beide Signalwege eine antagonistische Wirkung aufeinander ausüben. Da die Muskulatur zu den mechanischen Geweben gehört haben wir die Bedeutung von Muskelkontraktion auf die fötale Myogenese im Huhnembryo untersucht. Wir haben beobachtet, dass ein Verhindern von Muskelkontraktion während der fötalen Myogenese einen NOTCH Funktionsverlust imitiert, d.h. in einer Abnahme fötaler Muskelvorläufer mit Verschiebung hin zur Muskeldifferenzierung resultiert. Wir konnten weiter zeigen, dass mechanische Kräfte durch Muskelkontraktion in den Myonuclei durch den Transkriptionscoaktivator YAP1 erkannt werden. Dieser reguliert die Expression von NOTCH Liganden in Muskelfasern, welche den Pool an fötalen Muskelvorläufern aufrechterhalten.

**Gene Nomenclature**

The current established gene nomenclature is as follows: for mouse (lower case), for chick and human (upper case). Because we mainly worked with the chick model the "results" and "discussion and perspectives" were written according to the established nomenclature for ulterior publication of the manuscripts.

Gene nomenclature is standardised to lower case in the "state of the art" to facilitate reading, due to a major prominence of work performed in mouse. Although, findings obtained using different model organisms are cited.

Gene expression is systematically displayed in *"italic"*.

**Abbreviations**

BMP - Bone Morphogenetic Protein

EMT - Epithelial to Mesenchyme Transition

MRFs - Muscle Regulatory Factor

bHLH - basic Helix Loop Helix

EEE - Early Epaxial Enhancer

Pax - Paired homeobox transcription factors

Six - Sine oculis homeobox

Eya - Eyes absent homologue

HGF - Hepatocyte Growth Factor

SF - Scatter Factor

Lbx1 - Ladybird homeobox 1

Msx1 - Msh homeobox one

Sim - Single-minded homolog

TGFβ - Transforming Growth Factor β

MCK - Muscle Creatine Kinase

PkCθ – Protein kinase C θ

PDGFR Platelet-Derived Growth Factor Receptor

PDGF - Platelet-Derived Growth Factor

MyHC - Myosin Heavy Chain

Nfix - Nuclear factor I X

CDK - Cyclin-Dependent Kinase

pRb - Retinoblastoma protein

Id - Inhibitor of differentiation proteins

Nog - Noggin

CDKI - Cyclin-Dependent Kinase Inhibitors

FGF - Fibroblast Growth Factor

Vgll - Vestigial-like transcriptional co-activators

Jag - Jagged

Dll - Delta-like

DSL - Delta-Serrate-Lag domain

NICD - Notch Intracellular Domain

EGF - Epidermal Growth Factor

RBP-J - Recombination signal Binding Protein for immunoglobulin kappa J region

HESR - hairy and enhancer of split-related

BrdU - BromodeoxyUridine

EdU - EthynyldeoxyUridine

Cdc6 - Cell division cycle 6

GDFs - Growth/Differentiation factors

BMPR - BMP Receptor

ActR - Activin Receptor

Alk - Activin-like receptor

R-Smads - Regulatody Smads

I-Smads - Inhibitory Smads

PH3 - Phosphorylated Histone H3

pSmad - Phosphorylated Smad

DMB - Decamethonium Bromide

PB - Pancuronium Bromide

mdg - muscular dysgenesis

Yap - Yes-associated protein

RASSF - Ras Association Domain Family Proteins

Amot - Angiomotin

MCAT - Muscle CAT elements

Ctgf - Connective-tissue growth factor

Cyr61 - Cysteine-rich protein 61

Tead - TEA domain transcription factor

NMJ - Neuro-Muscular Junctions

shRNA - short-hairpin RNA

siRNA - small interfering RNA

Fucci - Fluorescent ubiquitination-based cell cycle indicator

MLC - Myosin Light Chain

ESCs - Embryonic Stem Cells

NSCs - Neural Stem Cells

Ach - Acetylcholine

AchR - Acetylcholine Receptor

Delta1DN - Delta1 dominant-negative

# State of the art

# State of the art

## Synopsis

The skeletal muscle is the most abundant type of tissue in the vertebrate's body. Muscle activity is controlled by motoneurons, which provide the electrical signals to allow muscle contraction. The mechanical forces produced by the skeletal muscle are transmitted to the bone by the tendon, which connects to muscle through the myotendinous junction and to bone via the enthesis. Skeletal muscles are organised in myofibres that are multinucleated cells formed by the fusion of post-mitotic mononucleated myoblasts. The contractile proteins in the fibres are organised in smaller units, the sarcomeres, which are mainly composed of actin and myosin. Muscle fibres are heterogeneous; they can be classified as fast or slow fibres according to their physiological and metabolic characteristics. Slow muscle fibres present high mitochondria content and therefore high level of oxidative enzymes, conferring resistance to fatigue. In contrast, fast muscle fibres have a high glycolytic enzymatic content and are capable of higher force production but are less resistant to fatigue.

A particular characteristic of the skeletal muscle is the presence of residual stem cells (satellite cells) that will contribute to the homeostasis and regeneration of the tissue during adult life. These cells are located at the periphery of the myofibres, between the basal lamina and the membrane of the fibre. Satellite cells adopt this position at the end of foetal myogenesis. Muscle formation occurs in two phases, embryonic and foetal myogenesis. Correct development, homeostasis and regeneration of the skeletal muscle rely on muscle progenitors. Several studies suggest that similar mechanisms regulate the balance between muscle progenitor proliferation and differentiation during muscle development and repair. The study of signals that regulate the behaviour of the muscle progenitor pool is thus of great importance.

In vertebrates, skeletal muscles of the trunk and limbs derive from the somites. The limbs of chick embryos can be easily manipulated without disturbing the entire embryo, and constitute a good model to study muscle development. During this project, I addressed how signalling pathways and mechanical forces regulate the muscle progenitor pool and skeletal muscle growth during foetal myogenesis.

# State of the art

## 1 – Skeletal muscle development

### 1.1 – Skeletal muscle specification in the head, trunk and limbs

Skeletal muscles of the trunk and limbs derive from somites (Chevallier et al., 1977; Christ et al., 1974). Somites are transient mesodermal structures that align symmetrically on the sides of the neural tube and develop in an anterior to posterior manner. These structures, that are initially presented as round epithelial structures are exposed to signals from surrounding tissues that will instruct their further fate (Fig. 1A). Dorsally, the dermomyotome is produced and will generate muscles of the trunk and limb, the dermis of the back and endothelial cells. The ventral somite will form sclerotome and syndetome that will give rise to cartilage/bones and tendons, respectively (Fig. 1A) (reviewed by Buckingham and Rigby, 2014).

Wnt signals provided by the ectoderm and dorsal neural tube induce the formation and maintenance of the dermomyotome (Fig. 1) (reviewed by Christ and Brand-Saberi, 2002). Also in cultured somites, Wnt proteins maintain the expression of dermomyotomal markers (Capdevila et al., 1998; Wagner et al., 2000). The dermomyotome is further regionalized in a medio-lateral manner which is also regulated by external signals. The epaxial lip of the dermomyotome (medial part) requires signals from the axial structures, namely the neural tube and the notochord, to form the epaxial muscles. In the absence of both neural tube and notochord, epaxial muscles do not develop (Christ, 1970; Rong et al., 1992). The signals required for the epaxial myogenesis are sonic hedgehog (Shh), secreted from the notochord, and Wnt proteins from the dorsal neural tube (Fig. 1B) (reviewed by Christ and Brand-Saberi, 2002). The muscles that originate from the epaxial lip of the dermomyotome are the muscles of the ribs, the base of the skull and some dorsal vertebral muscles. Hypaxial muscles are generated from the lateral dermomyotome and correspond to muscles of the abdomen, diaphragm, tongue, limbs and a subset of vertebral muscles. The specification and maintenance of the hypaxial lips of dermomyotomes depend on Wnt and Bone Morphogenetic Protein 4 (BMP4) that are secreted from the ectoderm (reviewed by Christ and Brand-Saberi, 2002).

The primary myotome contains the first differentiated skeletal muscle cells. Initially, cells at the borders of the dermomyotome give rise to the primary myotome that is organised

in a thin layer under the dermomyotome (Denetclaw and Ordahl, 2000; Gros et al., 2004; Kahane et al., 2002). In a second phase, cells that originate from the central part of the dermomyotome undergo epithelial-to-mesenchyme transition (EMT) and invade the primary myotome (Gros et al., 2005; Kassar-Duchossoy, 2005; Relaix et al., 2005).

Myogenesis relies on the presence of muscle progenitor cells that express the paired homeobox transcription factors Pax3 and Pax7. Pax3/7 proteins play a role in tissue specification in several contexts and are not expressed in a muscle-specific manner. Studies performed in mouse and chick showed that a resident muscle progenitor pool is present in the central dermomyotome and co-expresses *Pax3* and *Pax7*. These progenitor cells are maintained during further development and are a source to generate all trunk and limbs skeletal muscles and associated satellite cells (Fig. 1A) (Relaix et al., 2005, Kassar-Duchossoy., 2005; Gros et al., 2005). While *Pax7* expression is restricted to the central area of the dermomyotome, *Pax3* is expressed in both epaxial and hypaxial lips (Fig. 1A), but is expressed stronger in the latter (Kassar-Duchossoy, 2005; Relaix et al., 2004). This is in accordance with the fact that in *Pax3*-mutant mice all the muscles of the limbs are absent whereas some trunk muscles are still formed (Relaix et al., 2004). The *Pax3/Pax7*-double mutant mice lose all trunk and limb skeletal muscles and only form the primary myotome (Relaix et al., 2005). *Pax7* is not essential during development, but becomes important in the postnatal muscle where it is expressed in quiescent satellite cells. *Pax7*-mutant mice lack satellite cells that progressively die after birth which compromises muscle homeostasis and regeneration (Oustanina et al., 2004; Seale et al., 2000).

Skeletal muscle differentiation relies on the myogenic regulatory factors (MRFs). All secreted factors required to specify and maintain the dermomyotome activate the myogenic program by triggering the expression of MRFs. The family of MRFs is composed of the determination factors Myf5, MyoD and Mrf4, and the differentiation factor Myogenin (Ott et al., 1991; Pownall and Emerson, 1992; Sassoon, 1993). This family of basic-helix-loop-helix (bHLH) transcription factors bind to ubiquitous bHLH E-proteins and form heterodimers that recognize the E-box consensus sequence (CANNTG) on promoters and enhancers of muscle-related genes promoting their expression. MRFs that are determining factors act redundantly, for instance, in the absence of *MyoD* muscle is formed and *Myf5* expression is upregulated (Rudnicki et al., 1992). However, in the absence of all the three determination factors no skeletal muscle forms (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1992). *Myogenin* has no major effect in the specification of the early myogenic lineage, but in its absence

myoblast differentiation and myofibre formation are impaired (Hasty et al., 1993; Nabeshima et al., 1993).

The first MRF expressed in somites is *Myf5* that initially appears in the epaxial dermomyotome. *Myf5* expression is modulated by Wnt1 and Shh signals produced by the neural tube and the notochord, respectively (Fig. 1B). These signals activate the early epaxial enhancer (EEE) in the Myf5 promoter that contains Tcf and Gli binding sites (Fig. 1B) (reviewed by Buckingham and Rigby, 2014). Cells in the epaxial lip of the dermomyotome that start to express *Myf5*, and sequentially *Mrf4* and *MyoD*, will be the first to contribute to

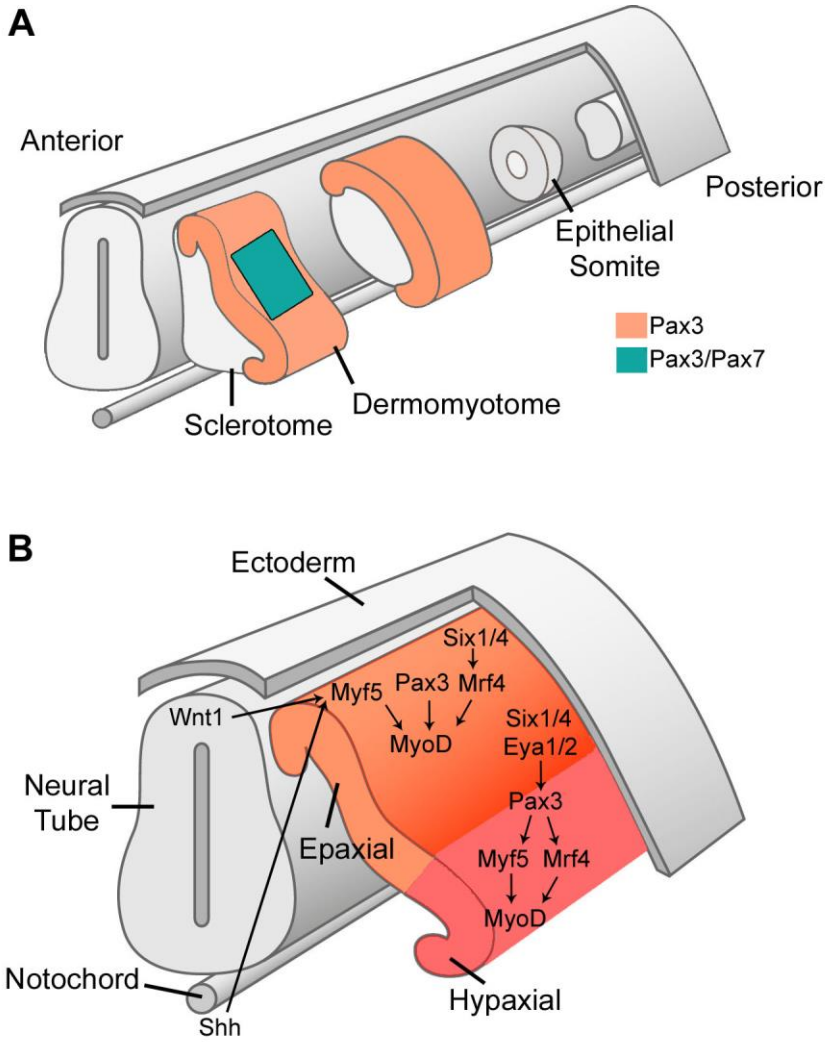



**Figure 1** – **Myogenic lineage specification.** (A) Sequential steps of the dermomyotome formation. Expression pattern of the Pax genes in the different regions of the dermomyotome. (B) Schematic regulatory cascade of the myogenic lineage in the hypaxial and epaxial lips of the dermomyotome (adapted from Bryson-Richardson and Currie, 2008).

the primary myotome (reviewed by Buckingham and Rigby, 2014). Activation of *Myf5* via the EEE is independent of Pax3, which explains the formation of the primary myotome in *Pax3/Pax7*-double mutant mice (Relaix et al., 2005). In the hypaxial dermomyotome, Pax3 directly regulates *Myf5* that in turn activates *MyoD* (Fig. 1B) (Bajard et al., 2006). *Myf5*-mutant mice do not present morphological skeletal muscle anomalies despite a delayed myotome formation, indicating that its activity is compensated by other MRFs (Braun et al., 1992).

Expression of *Pax3* in the dermomyotome is regulated by members of the sine oculis homeobox (Six) and eyes absent homologue (Eya) protein families (Fig. 1B). Ectopic expression of *Six1* and *Eya* activates *Pax3* expression in chick embryos (Heanue et al., 1999). In addition, Six1 and Six4 as well as Eya1 and Eya2 induce *Pax3* expression in the hypaxial lip of the murine dermomyotome (Fig. 1B) (Grifone et al., 2005, 2007) which initiates the myogenic program (Bajard et al., 2006). Six1 and Six4 directly bind to regulatory sequences of *Mrf4* and activate its expression in epaxial muscle (Grifone et al., 2005). Together with Myf5, Mrf4 activates *MyoD* in epaxial muscle (Fig. 1B).

Head skeletal muscles originate from distinct mesodermal structures, the cranial paraxial mesoderm and the prechordal mesoderm. Cranial paraxial mesoderm gives rise to the myoblasts that will colonize the first and second branchial arches that form most head muscles. These muscles regulate jaw movement, facial expression as well as pharyngeal and laryngeal contraction. The extraocular muscles are responsible for eyes movement and derive from the most anterior cranial mesoderm and from cells of the prechordal mesoderm. The muscles responsible for the movement of the head and postural stabilization originate from the cervical and occipital somites and follow a developmental program that is similar to that of the trunk muscles. Occipital somites contribute to tongue muscle and also form intrinsic pharyngeal muscles. Tongue muscle precursors derive from the caudal part of occipital somites while intrinsic pharyngeal muscles derive from their rostral part (reviewed by Noden and Francis-West, 2006).

The MRFs are essential for formation of all skeletal muscle. However, upstream cascades required to initiate their expression differ in the trunk and head. Mice mutant for the transcription factors *Tbx1* or *Pitx2* display several abnormal head muscles (Dong et al., 2006; Lu et al., 1999). Tbx1 or Pitx2 promote activation of *Myf5*, *Mrf4* and *MyoD* which also initiate the myogenic program in the head (reviewed by Bryson-Richardson and Currie,

2008). In contrast to trunk, Pax3 does not play a role in head muscle development. Thus, in *Pax3/Myf5*-double mutant mice no skeletal muscle in the trunk and limbs form but head muscles are spared (Tajbakhsh et al., 1997).

# State of the art

## 1 – Skeletal muscle development

### 1.2 – Limb muscle formation

Limb muscles derive from waves of migrating progenitor cells that reside in the hypaxial lips of the dermomyotomes (Chevallier et al., 1977; Christ et al., 1974). In mouse embryos, the migrating muscle progenitors express only *Pax3* and activation of *Pax7* occurs once the cells reach the limbs. In chick embryos, migrating muscle progenitors co-express *Pax3* and *Pax7* (reviewed by Duprez, 2002; reviewed by Biressi et al., 2007a). The first muscle progenitors start to be observed in forelimb buds at embryonic day 2.5 (E2.5) in the chick (Fig. 2) and at E10.5 in the mouse (reviewed by Duprez 2002; reviewed by Biressi et al., 2007a; Tozer et al., 2007).

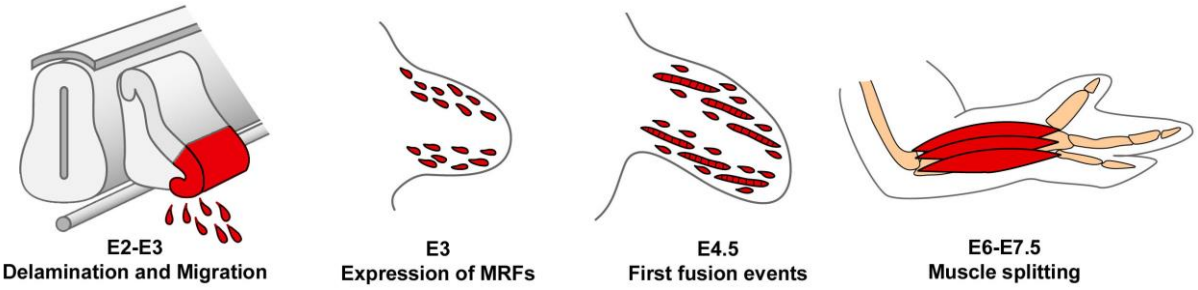



**Figure 2 – Stages of development of chick forelimb muscles.** Chick limb skeletal muscle progenitors delaminate from the hypaxial lip of the dermomyotome and migrate to the limb bud between E2 and E3. Dorsal and ventral muscle masses are established at E3 and the muscle progenitors start to express the muscle regulatory factors (MRFs). The first fusion events occur at E4.5 and muscles split between E6 and E7.5.

Cells in the hypaxial dermomyotomes at limb levels undergo EMT and migrate towards the limb bud (Fig. 2). These cells, strongly express *Pax3* (Fig. 3) (Relaix et al., 2004; Kassar-Duchossoy et al., 2005). In the hypaxial lip of the dermomyotome, *Pax3* expression is maintained by the Six and Eya family of transcription factors (Fig. 1B). In both, *Six1/Six4* or *Eya1/Eya2* double mutant mice, *Pax3* expression is lost which results in an impaired colonisation of the limb by the muscle progenitors (Grifone et al., 2005; 2007). Pax3

regulates the expression of the tyrosine kinase Met, and activation of Met regulates delamination of Pax3+ cells from the hypaxial dermomyotome (Fig. 3) (Bladt et al., 1995; Daston et al., 1996; Dietrich et al., 1999). In addition, β-catenin, an effector of the Wnt signalling pathway, is required to maintain *Pax3* and *Met* expression levels in the dermomyotome (Daston et al., 1996). Delamination and oriented migration of muscle progenitors requires the interaction of Met with its ligand HGF (hepatocyte growth factor), also named SF (scatter factor), that is expressed in the lateral plate-derived limb mesenchyme (Fig. 3) (Bladt et al., 1995). Limbs and diaphragm muscles are absent in *Met* and *HGF* mutant mice, and the tongue muscle is very small (Bladt et al., 1995; Dietrich et al., 1999).

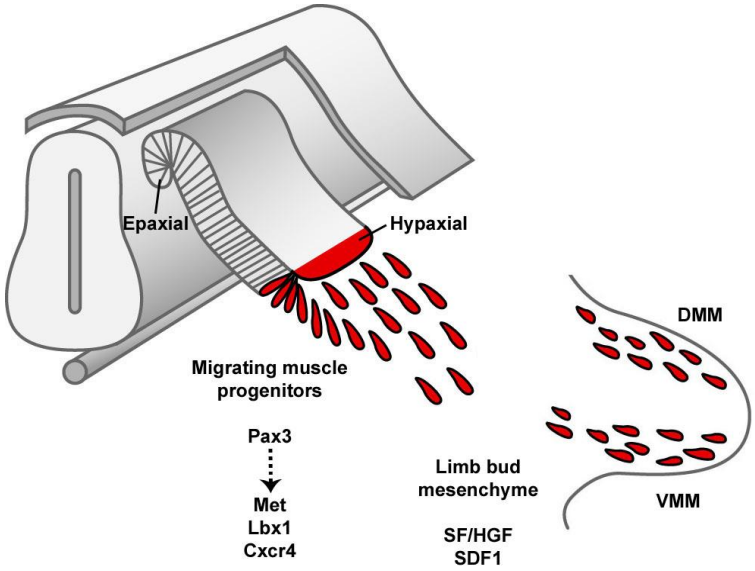



**Figure 3 – Regulation of the migrating muscle progenitor cells.** Limb muscle progenitors delaminate from the hypaxial lip of the dermomyotome via activation of Met. *Met* expression depends on Pax3. The Met ligand *SF/HGF* is expressed in the limb bud. *Lbx1* expression in the migrating progenitors is regulated by Pax3 and mediates the oriented migration towards the limb bud by promoting *Cxcr4* expression. Limb mesenchyme expresses the Cxcr4 ligand *Sdf1*. DMM, dorsal muscle masses; VMM, ventral muscle masses.

Long-range migratory muscle progenitors of the hypaxial lips of limb dermomyotomes and of the occipital and cervical somites express the *ladybird homeobox 1* (*Lbx1*) transcription factor (Jagla et al., 1995). *Lbx1* expression depends on Pax3 and is required for the migration of the myogenic cells into the limb (Fig. 3). In *Lbx1*-mutant mice the muscle progenitors are able to delaminate from dermomyotomes but cannot invade correctly the limb bud and show a complete loss of the dorsal muscles in forelimbs

(Brohmann et al., 2000; Gross et al., 2000; Schäfer and Braun, 1999). The control of migratory muscle progenitors by Lbx1 occurs in part through activation of the chemokine receptor *Cxcr4* in these cells (Fig. 3) (Vasyutina et al., 2005). Conversely, the ligand of Cxcr4, the *stromal derived factor1* (*Sdf1*), is expressed in the limb mesenchyme (Fig. 3). In *Cxcr4*-mutant mice, reduced number of muscle progenitors reach the limb and apoptosis is increased, showing that Cxcr4 controls migration and survival of these muscle progenitors (Vasyutina et al., 2005).

Once the migratory cells reach the limb, they downregulate *Pax3* expression and start to express *Myf5* (Mankoo et al., 1999). In particular, in the chick, activation of the myogenic program in the limbs occurs at E3 (Delfini et al., 2000). This implies that the undifferentiated state of the Pax3+ cells is maintained during migration. Transcriptional repressors like the msh homeobox one (Msx1) and single-minded homolog (Sim) prevent the premature differentiation. *Msx1* is expressed in the hypaxial lip of the dermomyotome and in migrating Pax3+ cells (Houzelstein et al., 1999). In addition, *Myf5* expression in the limb is activated after *Msx1* downregulation. Overexpression of *Msx1* in myoblast cell cultures inhibits myotube differentiation (Song et al., 1992) and in *Msx1*-mutant mice, *Myf5* expression is increased in the developing limbs (Wang et al., 2011). These studies show that Msx1 prevents activation of the myogenic program. *Sim1* has been shown to be expressed in the hypaxial lip of the dermomyotome (Coumailleau and Duprez, 2009; Pourquié et al., 1996). The expression of *Sim2* is more restricted and is observed in progenitors of the ventral limb muscle masses (Coumailleau and Duprez, 2009; Havis et al., 2012). Sim2 binds to the *MyoD* promoter and represses *MyoD* expression in mouse limbs which results in the repression of myogenic differentiation. Conversely, *MyoD* expression is upregulated in *Sim2*-mutant mice (Havis et al., 2012).

## 2 – Embryonic, foetal and adult myogenesis

Skeletal muscle formation occurs in successive and overlapping phases. During the embryonic phase, a first wave of myogenesis that relies on Pax3+ cells takes place and Pax3+ cells give rise to embryonic myoblasts. The first fusion events occur between embryonic myoblasts and give rise to embryonic fibres that function as scaffold for subsequent muscle growth. Embryonic myogenesis takes place at E2.5 in the chick and at E11.5 in the mouse (Fig. 4) (reviewed by Duprez, 2002; reviewed by Biressi et al., 2007a). During foetal myogenesis, a second wave of progenitors differentiates. Foetal progenitors express *Pax7* and give rise to foetal myoblasts. Foetal myoblasts fuse with themselves and with pre-existing embryonic fibres allowing the muscle growth. Foetal myogenesis starts at E6 in the chick and at E14.5 in mice (Fig. 4) (reviewed by Duprez 2002; reviewed by Biressi et al., 2007a). By the end of foetal myogenesis, the pool of satellite cells (Pax7+ cells) that is required for the peri-natal muscle growth and adult homeostasis is established. Foetal muscle progenitors adopt a satellite cell-like position under the basal lamina at E17 and E16 in the chick and mouse, respectively (Fig. 4) (reviewed by Biressi et al., 2007a).

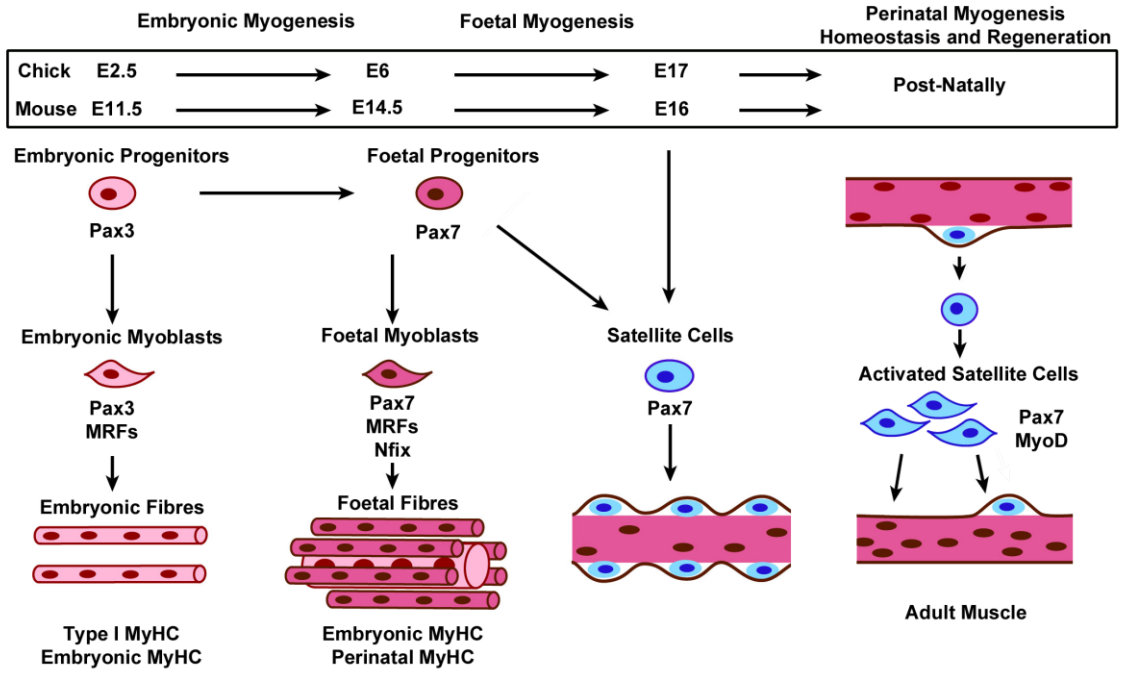



**Figure 4** – **Distinct phases of muscle development.** The illustration shows equivalent time-points of chick and mouse myogenesis. The scheme also indicates molecular markers for the different myogenesis phases.

During limb muscle formation, the co-existence of embryonic and foetal muscle progenitors that undergo differentiation at different developmental stages suggests that they might respond differently to external cues. *In vitro* studies demonstrate that embryonic and foetal muscle progenitors display different requirements for the cytokine transforming growth factor β (TGFβ) 1 and 2. TGFβ inhibits myotube formation in cultured foetal but not embryonic myoblasts (Cusella-De Angelis et al., 1994). Similarly, BMP4 or the phorbol ester TPA inhibit foetal but not embryonic differentiation (Biressi et al., 2007b; Cossu et al., 1988; Cusella-De Angelis et al., 1994). BMP4, TPA or TGFβ also inhibit differentiation of cultured satellite cells or C2C12 cells (C2C12 is a myogenic cell line that derives from adult murine satellite cells) (Biressi et al., 2007b; Dahlqvist, 2003). Thus, foetal and adult myoblasts respond in a similar manner to these signals. Few proliferating cells are present in the limb when embryonic fibres are first formed. In the vicinity of the first fibres, the number of proliferating cells increases (Cusella-De Angelis et al., 1994). Ectodermal TGFβ is abundant in the limb at this stage. This suggests that due to TGFβ, an environment with little mitogenic activity exists in the limb which leads to the first wave of myogenesis and formation of the embryonic fibres. According to this model, the first fibres potentiate the proliferation of the surrounding cells, the foetal progenitors that previously did not differentiate because they were exposed to TGFβ. The levels of TGFβ subsequently decrease, which allows the fusion of foetal myoblasts (Cusella-De Angelis et al., 1994).

Embryonic and foetal myoblasts also differ in their dependence of β-catenin for proliferation and muscle fibre formation (Hutcheson et al., 2009). In mouse limbs, ablation of *β-catenin* in embryonic progenitors (Pax3+ cells) does not impair embryonic myogenesis. However, when *β-catenin* is depleted in foetal progenitors (Pax7+ cells) less muscle fibres form. Precocious differentiation of foetal progenitors and consequent loss of the foetal progenitor pool might account for this result (Hutcheson et al., 2009). A constitutively active form of β-catenin increases the number of Pax7+ cells and leads to a reduction in muscle fibre numbers, possibly by blocking terminal differentiation (Hutcheson et al., 2009). This is consistent with *in vitro* studies that showed that β-catenin inhibits myogenic differentiation and fusion (Gavard et al., 2004; Perez-Ruiz et al., 2008).

A genome-wide gene expression analysis of purified embryonic and foetal myoblasts from mouse embryos showed that these two populations express different genes, which can contribute to their different sensitivities to TGFβ, BMP4 and TPA (Biressi et al., 2007b). This study revealed that a subset of genes is differentially expressed in embryonic or foetal myoblasts. In particular, foetal myoblasts display high levels of *Pax7*, *PkCθ* (protein kinase C θ), *β-enolase*, *MCK* (muscle creatine kinase), several laminins and the components of Notch signalling pathway *Notch1* and *Jagged1*. The presence of high levels of *PkCθ* in foetal myoblasts might be related to their increased sensitivity to TGFβ. The high expression levels of *Smad6* and *Smad7* in embryonic compared to foetal myoblasts could explain their insensitivity to of BMP4 and TGFβ. In addition, high levels of *platelet-derived growth factor receptor α* and *β* (*PDGFRα/β*) are present in embryonic but not foetal myoblasts (Biressi et al., 2007b). Structural muscle genes are also differently expressed in embryonic and foetal muscle fibres. In the mouse, embryonic fibres present a general slow phenotype, expressing mainly the slow type I MyHC (myosin heavy chain) but also an embryonic MyHC (fast) isoform (Fig. 4). Foetal fibres display a fast phenotype and express mainly the embryonic and

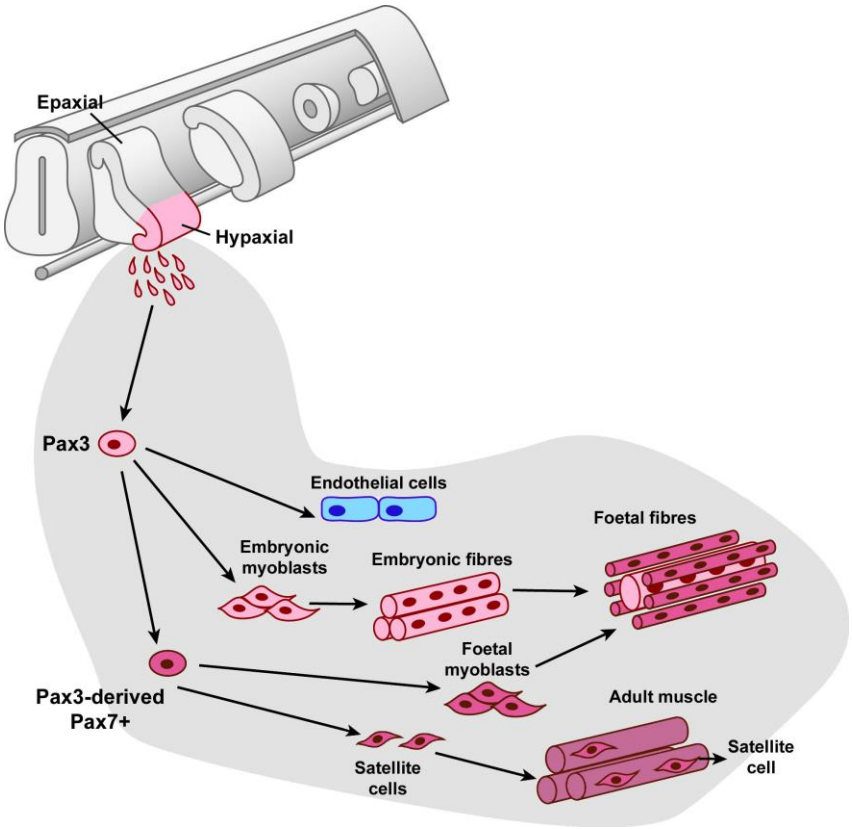



**Figure 5 – Origin of embryonic and foetal muscle progenitors.** Scheme illustrating the origin of limb embryonic and foetal myoblasts from the same original population (scheme adapted from Hutcheson et al., 2009).

perinatal fast MyHC isoforms (Fig. 4) (reviewed by Biressi et al., 2007a). The transcription factor *nuclear factor I X* (*Nfix*) is highly expressed in foetal but not embryonic myoblasts (Fig. 4) (Biressi et al., 2007b). *Nfix* expression is activated by Pax7 in foetal muscle, activates foetal-specific genes and represses embryonic-related genes, regulating thus the switch between embryonic and foetal myogenesis (Messina et al., 2010). Pax7 is sufficient to activate *Nfix* expression and binds directly to the *Nfix* promoter. However, Pax7 is not required for induction of *Nfix*, since *Nfix* expression levels are only modestly reduced in *Pax7*-mutant mice (Messina et al., 2010). Conditional mutation of *Nfix* mice in skeletal muscle results in dramatic disorganisation of the muscle fibres and poorly assembled sarcomeres, demonstrating a role of Nfix in sarcomere organisation (Messina et al., 2010).

Whether embryonic and foetal myoblasts derive from equivalent progenitors is an open question that was only recently started to be addressed. Lineage ablation studies in the mouse showed that embryonic and foetal progenitors derive from the same Pax3+ cell population that migrates from the dermomyotome to the limb. Once in the limb, Pax3+/Pax7- cells form the pool of embryonic progenitors that contributes to primary myogenesis. Pax7+ cells are derived from the population of Pax3+ cells that downregulate *Pax3* and form the pool of foetal progenitors (Fig. 5) (Hutcheson et al., 2009).

The satellite cells adopt a specific position between the basal lamina of the fibres and the cell membrane (Mauro, 1961). In both chick and mouse, satellite cells adopt this position during late stages of foetal myogenesis (Fig. 4). The correct positioning of the satellite cells depends on their ability to assemble a basal lamina, which requires Notch signalling (Bröhl et al., 2012). Postnatally, satellite cells are highly mitotic to allow peri-natal muscle growth but in the adult, satellite cells are mostly in a quiescent state (reviewed by Parker et al., 2003). Recent studies proposed that the acquisition of the quiescent state of muscle progenitors occurs early during development and possibly even before they adopt a satellite cell-like position (Picard and Marcelle, 2013).

Activated and quiescent satellite cells express *Pax7*, and in *Pax7*-mutant mice the satellite cells are lost, which severely impairs the regenerative capacity of the muscle (Seale et al., 2000). Therefore, the maintenance of the satellite cell pool has to be tightly regulated to ensure muscle homeostasis. Satellite cells were reported to be heterogeneous. Two sub-populations of satellite cells, fast and slow–cycling, have been identified (Schultz, 1996). Quiescent satellite cells do not express MRFs, however, *Myf5* transcripts are present in the

majority of quiescent satellite cells (Fig. 6) (Beauchamp et al., 2000). These transcripts are sequestered in cytoplasmic granules, and appear not to be translated (Crist et al., 2012). Lineage tracing studies identified a sub-population of satellite cells that have never expressed *Myf5*. These cells have a lower propensity to differentiate than the general pool of satellite cells, and a higher propensity to self-renew. They were therefore proposed to function as a stem cell reservoir that sustains the pool of non-commited satellite cells (Kuang et al., 2007).

# State of the art

## 3 – Regulation of the muscle progenitor pool

### 3.1 – The balance between proliferation and differentiation

The mechanisms involved in muscle formation and growth rely on a tight control of the cell cycle. I define here exit or withdrawal from the cell cycle as an irreversible process, that is accompanied by terminal differentiation and distinguish it from a reversible stop in proliferation that is observed in quiescence. Proteins that play a central role in cell cycle regulation are required to switch between myoblast proliferation and differentiation. In addition, MRFs and cell cycle-associated factors control each other to ensure appropriate muscle development. Most studies on cell cycle regulation during myogenesis were performed *in vitro* and rely frequently on the use of the C2C12 cell line. Very little is known about cell cycle regulation during myogenesis *in vivo*.

During development, proliferating myoblasts are characterized by the presence of *Pax3* and/or *Pax7* and the early MRF *Myf5* (Fig. 6). *MyoD* starts to be expressed in proliferating myoblasts, and strong expression of MyoD forces cells into cell cycle exit and myogenic differentiation (Fig. 6, 7). The activity of MyoD in proliferating myoblasts was proposed to be inhibited to allow the progression through the cell cycle (Fig. 7) (reviewed by Kitzmann and Fernandez, 2001). In proliferating myoblasts, MyoD is associated with histone deacetylases (HDCA), which inhibit trans-activation of muscle-related genes (reviewed by Ciemerych et al., 2011). In addition, the inhibitor of differentiation proteins (Id) are

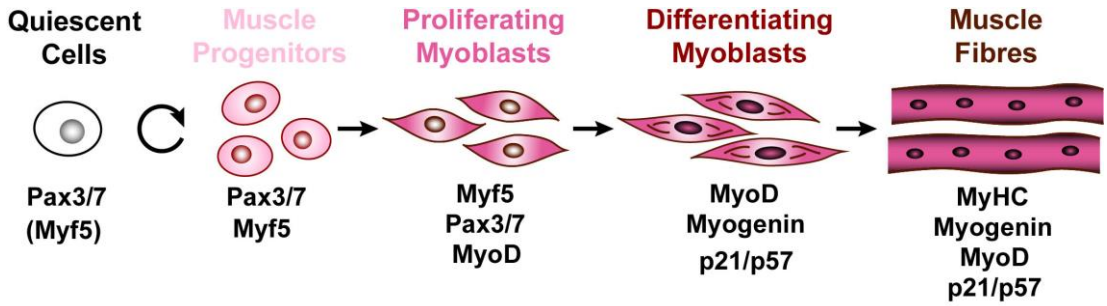



**Figure 6 – Progression of myogenic cells towards differentiation.** Different molecular markers characterising the distinct stages of myogenic differentiation are illustrated. Muscle progenitors and satellite cells can be considered "quiescent cells". Brackets around Myf5 indicate that a subset of satellite cells have expressed *Myf5* in their developmental history.

expressed in proliferating myoblasts. Id proteins are helix-loop-helix proteins that lack DNA binding capacity. They bind to E-proteins, in particular to the E2A family members, inhibit heterodimerization between E-proteins and MyoD and therefore block the activation of myogenic genes (Fig. 7) (reviewed by Kitzmann and Fernandez 2001). In proliferating myoblasts, MyoD protein levels are higher during G1 phase and are degraded in the late G1 (Kitzmann et al., 1998). Cyclin E-CDK2 (cyclin-dependent kinase 2) complexes phosphorylate MyoD at Ser200, which marks MyoD for degradation and inhibits its activity (Fig. 7) (reviewed by Kitzmann and Fernandez 2001).

One of the major cell cycle regulators involved in myoblast differentiation is the retinoblastoma protein (pRb). pRb belongs to the pocket protein family of transcriptional regulators (pRb, p130, p107), inhibits progression of the cell cycle from G1 into S phase and contributes thus to cell cycle exit (reviewed by De Falco et al., 2006). In its active/hypophosphorylated form, pRb physically interacts and inhibits the activity of E2F transcription factors, which activate the expression of genes associated with cell cycle progression (Fig. 7) (Nevins, 1992). This contributes to the entry into a terminally differentiated state. In addition to its role in balancing proliferation and differentiation, *in vitro* studies show that pRb also contributes to cell fate determination (reviewed by Ciemerych et al., 2011). In skeletal muscle, pRb inhibits cell cycle progression and contributes thus to cell cycle withdrawal. pRb also binds to MyoD and Myogenin promoters thereby activating their expression (reviewed by Ciemerych et al., 2011).

*pRb* transcripts are stable during the cell cycle (Classon and Dyson, 2001), but activity of the pRb protein is regulated post-transcriptionally by cyclin-CDK complexes (Fig.7) (reviewed by De Falco et al., 2006). In proliferating myoblasts, mitogenic cues contribute to activation of cyclin-CDK complexes that positively regulate cell cycle progression. Particularly, the activated G1 phase-associated cyclin-CDK complexes cyclin D-CDK4/6 and cyclin E-CDK2 phosphorylate pRb inactivating it, which releases its binding to the E2F. The liberated E2F factors will then promote transcription of cell cycle progression genes (Fig.7) (reviewed by Ciemerych et al., 2011). In addition, Id2 binds to the active form of pRb and inhibits its activity, which contributes to the maintenance of the proliferative state of myoblasts (Fig. 7) (Iavarone et al., 1994).

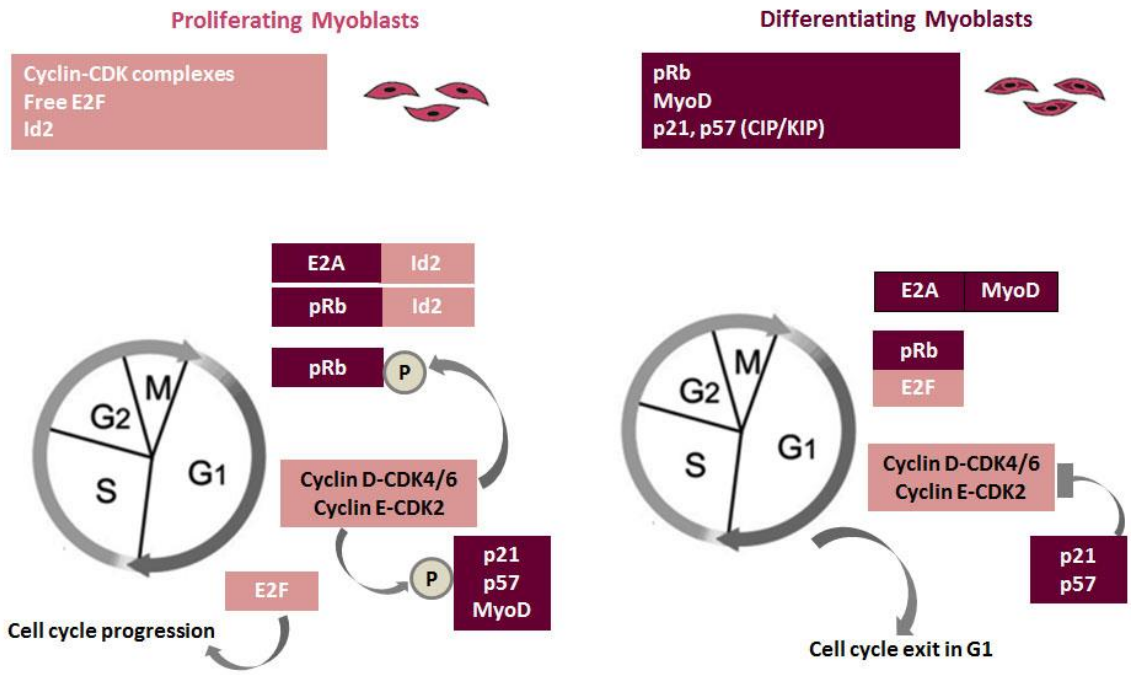



**Figure 7 – Schematic representation of the main cell cycle regulators in proliferating and differentiating myoblasts.** In proliferating myoblasts in the G1 phase of the cell cycle, active cyclin-CDK complexes together with high levels of Id2 and free E2F promote cell cycle progression. In differentiating myoblasts, high levels of MyoD, pRb and of the CDKI p21/p57 promote a terminally differentiated state.

The decision between cell cycle progression and differentiation is made during the G1 phase (Fig. 7). In differentiating myoblasts, the activity of the main positive regulators of the cell cycle, cyclin-CDK complexes, have to be inhibited to achieve the cell cycle exit and differentiation. The p21, p27 and p57 proteins, members of the CIP/KIP family of CdK inhibitors (CDKI), inhibit the activity of cyclin-CDK complexes and play a critical role in cell cycle exit (Fig. 7). Inhibition of cyclin-CDK complexes stabilizes pRb in its active hypo-phosphorylated form (Fig. 7) (reviewed by De Falco et al., 2006). MyoD activates *p21* and *p57* expression by directly binding to their promoters and enhancers (Figliola et al., 2008; Vaccarello et al., 2006; Zalc et al., 2014). Conversely, by inhibiting MyoD phosphorylation, high levels of p57 stabilize MyoD protein (Fig. 7) (Reynaud et al., 1999). MyoD also activates the expression of *pRb* and contributes thus to the cell cycle arrest (reviewed by Ciemerych et al., 2011).

The late MRF *Myogenin* starts to be expressed in myoblasts shortly before they assume a post-mitotic state (Fig. 6) (Andrés and Walsh, 1996). p21 can be detected in myoblasts when they reach a post-mitotic state (Andres and Walsh, 1996), and p21 and p57 positive myoblasts do not co-express Ki67, a cell cycle progression marker (Zalc et al., 2014). In *p21/p57*-double mutants, myoblasts fail to fuse and instead, continue to proliferate and undergo apoptosis (Zhang et al., 1999). In these mutants the muscle differentiation marker *Myogenin* is expressed, and the number of Myogenin-positive cells that display mitotic activity is increased, demonstrating that the myogenic differentiation program can be initiated even when cell cycle exit is impaired (Zalc et al., 2014). In addition, in *MyoD*-mutant mice, myogenic differentiation is delayed and *Myogenin* expression is impaired. This is accompanied by increased *p57* expression in the forming muscle masses indicating that cell cycle exit and muscle differentiation can be uncoupled (Zalc et al., 2014). Once the myoblasts reach a post-mitotic state, genes that encode proteins of the contractile apparatus like MyHC are expressed, and then the post-mitotic myoblasts fuse (Andres and Wash, 1996).

The INK family of CDKI (p15, p16, p18 and p19) participate in the maintenance of the quiescent state of myoblasts. In contrary to the CIP/KIP proteins, expression of INK genes is not influenced by MyoD. INKs block the activity of CDK4 and CDK6 by inhibiting their binding to cyclin D, which blocks cell cycle re-entry and contributes to the maintenance of quiescence (reviewed by Kitzmann and Fernandez 2001).

In summary, the maintenance of the muscle progenitor pool relies on the accurate balance between cell cycle progression and terminal differentiation and depends on the interplay between cell cycle-associated proteins and MRFs.

# State of the art

## 3 – Regulation of the muscle progenitor pool

### 3.2 – Signalling pathways

The balance between proliferation and differentiation of the muscle progenitors during the different myogenesis steps is critical to achieve muscle growth and maintain the undifferentiated pool of muscle progenitors. Several signalling pathways have been identified to play a role in the maintenance of the muscle progenitor pool *in vivo*. Notch signalling is associated with the maintenance of muscle progenitors, inhibition of terminal differentiation and formation and positioning of satellite cells (Bröhl et al., 2012; Delfini et al., 2000; Hirsinger et al., 2001; Mourikis et al., 2012a; Schuster-Gossler et al., 2007; Vasyutina et al., 2007a). Studies on the role of BMP signalling pathway in myogenesis have revealed two distinct functions in embryonic and foetal myogenesis. In embryonic myogenesis, activation of BMP pathway increases the muscle progenitor pool and blocks differentiation whereas in foetal development, activation of BMP leads to muscle growth with increased numbers of muscle progenitors and fibres (Amthor et al., 1998, 1999; Wang et al., 2010). Fibroblast growth factor (FGF) signalling activates proliferation and blocks myogenic differentiation *in vitro* (reviewed by Olson, 1992). In addition, *in vivo* activation of FGF signalling by FGF4 in chick limbs decreases *Pax3* and *MyoD* expression levels and results in less muscle fibres (Edom-Vovard et al., 2001). The role of Wnt signalling pathway in myogenesis is quite controversial, and *in vitro* studies demonstrate either a role in promoting or blocking myogenesis (Gavard et al., 2004; Goichberg et al., 2001; Perez-Ruiz et al., 2008). However, *in vivo* analyses suggest a role of Wnt in the maintenance of foetal myoblasts (Hutcheson et al., 2009) and satellite cell expansion (Le Grand et al., 2009).

# State of the art

## 3 – Regulation of the muscle progenitor pool

### 3.2 – Signalling pathways

#### 3.2.1 – Notch signalling pathway

Notch signalling controls decisions on fate and lineage progression in many cell types. The signalling is achieved when the cell that displays the transmembrane Notch receptor contacts with the signal-sending cell that expresses the Notch ligand (Fig. 8A). As a result, the Notch receptor is cleaved and the notch intracellular domain (NICD) is released and transported to the nucleus where it interacts with the recombination signal binding protein for immunoglobulin kappa J region (RBP-J), the major transcriptional mediator of the Notch signal. In mammals, four Notch receptors (Notch1/2/3/4) and five canonical ligands (Jagged1/2 (Jag) and Delta-like1 (Dll1)/3/4) exist. The different receptor/ligand combinations mainly differ in quantitative but not qualitative output changes. Although, a few reports indicate that signalling output might vary depending on the receptor or the receptor/ligand combination (reviewed by Andersson et al., 2011). Commonly, ligands in the signal sending-cell can activate Notch in trans but also inhibit Notch in cis. This cis-inhibition occurs via a decrease of Notch receptors presented at the cytoplasmic membrane of the signal sending-cell (reviewed by Andersson et al., 2011; Matsuda and Chitnis, 2009; Pérez et al., 2005). The Dll3 ligand has been rarely observed on the cell surface, it is unable to activate Notch receptors, and might function as cis-inhibitor rather than trans-activator (Ladi et al., 2005). The Notch receptors can be modified post-translationally mainly in the epidermal growth factor-like (EGF) sequences present in the extracellular domain. These modifications are not required for the signal transduction but modify the interaction of Notch receptors with their ligands. In particular, the EGF repeats can be modified by the addition of *O*-fucose by the *O*-fucosyltransferase 1 (Pofut1) which allows the subsequent glycosylation by Fringe proteins. Fringe-modified variants of Notch have increased Dll1 affinity and decreased affinity to Jag1 (reviewed by Andersson et al., 2011).

Activation of Notch target genes depends on the translocation of NICD to the nucleus and its interaction with RBP-J, which belongs to the CBF1/Supressor of Hairless/LAG-1 (CSL) family of DNA binding proteins (Fig. 8A). The bHLH family of transcriptional repressors hairy and enhancer of split-related (HESR) are important targets of Notch

signalling pathway (Fig.8 A). Different target genes of the HESR family can be activated in different contexts and thus expression of none of the HESR is a mandatory readout of Notch activation. A comparison of the changes in gene expression after NICD overexpression in different cell types (reviewed by Andersson et al., 2011) showed that the most commonly upregulated gene is *Hey1*. In stem cells *Hes5* is upregulated frequently in response to Notch signals. The response of other HESR genes is mainly context dependent (reviewed by Andersson et al., 2011).

Several studies demonstrated that Notch signalling is required for the maintenance of the muscle progenitor pool in chick and mouse embryos (Delfini et al.,2000; Hirsinger et al., 2001; Schuster-Gossler et al., 2007; Vasyutina et al., 2007a; Mourikis et al., 2012a). *In vitro*, Notch signalling inhibits myogenic differentiation in cultured primary myoblasts and in myogenic cell lines (reviewed by Vasyutina et al., 2007b). In particular, overexpression of *Delta1* in C2C12 cells leads to decreased *MyoD* expression levels which might depend on a RBP-J-mediated upregulation of *Hes1* (Kuroda et al., 1999). The first *in vivo* evidence for a role of Notch signalling in muscle development of vertebrates comes from studies in the chick. Activation of Notch by *Delta1* overexpression using the RCAS virus system leads to a decrease of *MyoD* expression and a decrease in muscle fibres number (Delfini et al., 2000). The inhibition of myogenic differentiation upon Notch activation was also observed in chick somites (Hirsinger et al., 2001). In the mouse, conditional ablation of Notch signalling by mutation of *RBP-J* in muscle progenitors leads to an uncontrolled myogenic differentiation and depletion of the muscle progenitor pool (Vasyutina et al., 2007a). In the limbs of the *RBP-J* conditional mutant mice at E11.5, the ratio of Pax3+/MyoD+ cells was increased but no significant changes in the proportion of proliferative or apoptotic Pax3+, Myf5+ or MyoD+ cells were observed. However, a 24h pulse-chase of bromodeoxyuridine (BrdU) incorporation assay showed that BrdU+ cells co-expressed more frequently MyoD and less frequently Pax3 than cells in control mice, showing that proliferating muscle progenitors will more likely give rise to MyoD+ than to Pax3+ cells (Vasyutina et al., 2007a). Therefore, loss of the muscle progenitor pool in *RBP-J* conditional mutant embryos is mostly due to a precocious shift towards differentiation. In accordance, the phenotype of the *RBP-J* conditional mutant embryos is rescued by the additional mutation of *MyoD*, which rescues the progenitor pool (Bröhl et al., 2012).

*Dll1*-mutant mice die during embryogenesis, whereas a hypomorphic *Dll1*-mutant mouse develops until birth but presents severe developmental muscle deficits that resemble the ones observed in *RBP-J* mutants (Schuster-Gossler et al., 2007). The embryos display a severe reduction in the skeletal muscle masses, a decline in the number of muscle progenitors during development but no evidence of increased cell death. This suggests that the loss of the skeletal muscle growth is again associated with the uncontrolled premature differentiation of the muscle progenitors (Schuster-Gossler et al., 2007).

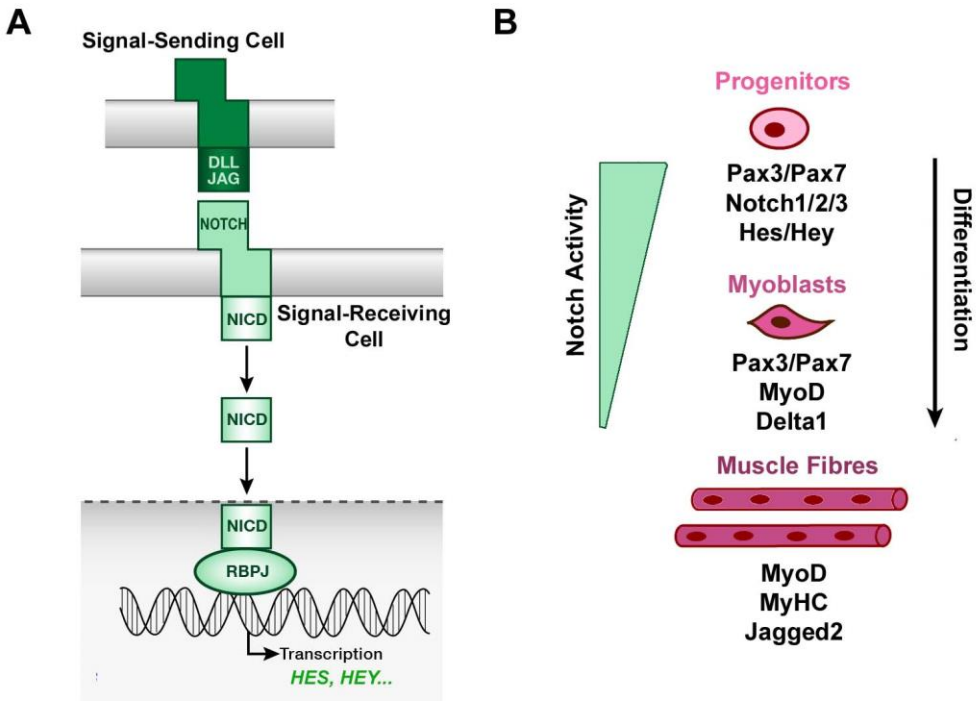



**Figure 8 – Role of Notch signalling pathway in myogenesis.** (A) Simplified scheme of Notch signalling pathway, requiring cell-cell interaction. (B) Illustration of the Notch activity in myogenic cells in different stages of differentiation, where muscle progenitors display highest Notch activity. The scheme (B) was elaborated by bringing together results obtained from studies performed in mouse and chick embryos.

The constitutive activation of Notch signalling (NICD) in Myf5+ muscle progenitors generates mouse embryos that lack skeletal muscle but where the myogenic progenitor pool is maintained. Despite the absence of committed muscle cells, the NICD-overexpressing muscle progenitors progressively assume the embryonic, foetal and satellite cell fate (Mourikis et al., 2012a). The survival of muscle progenitors depends on the presence of differentiating myoblasts (Kassar-Duchossoy et al., 2005). Forced activation of Notch seems

to be sufficient to maintain the pool of muscle progenitors even when committed myoblasts are absent, suggesting that Notch provides an important signal during the crosstalk between myoblasts and muscle progenitors.

Expression of various components of the Notch pathway was defined in the muscle of the developing forelimbs in E5 chick embryos. *Notch1* was expressed outside of muscle fibres in cells that are actively proliferating, while the ligands *Delta1* and *Jagged2* were mainly present in non-proliferating muscle cells (Fig. 8B) (Delfini et al., 2000). Later in development, at E7, *Jagged2* is only detected in muscle fibres (MF20+ cells, MF20 recognises sarcomeric myosin heavy chains) (Fig. 8B) (Delfini et al., 2000). In addition, the Notch target gene Hes1 is expressed in muscle progenitors of the limb (Fig. 8B) (Vasyutina et al., 2007a). Recent studies demonstrated that Notch signalling declines during the progression of muscle differentiation (Mourikis et al., 2012b). These analyses were performed using progenitor and myoblasts from embryonic (E12.5), foetal (E17.5), postnatal (P8) and adult mice. Notch signalling is active in proliferating myoblasts and very high in activated satellite cells. Within the pool of satellite cells, different sub-populations that express various amounts of GFP driven by the Pax7 promoter (low, medium, high) could be distinguished. These cells also vary in the potential to self-renew, *i.e.* Pax7-GFP$^{high}$ cells have a higher potential to self-renewal than Pax7-GFP$^{low}$ cells (Mourikis et al., 2012b). The Pax7-GFP$^{low}$ cells express higher levels of *MyoD* and *Myogenin* and are thus committed to differentiation (Fig. 8B) (Mourikis et al., 2012b). As the cells go from a stem to a committed state (from Pax7-GFP$^{high}$ to Pax7-GFP$^{low}$) they shift the expression levels of Notch signalling components, *i.e.* increase of *Dll1* and decrease of Notch receptors (*Notch1/2/3*) expression (Fig. 8B) (Mourikis et al., 2012b). These studies show that Notch activity is associated with proliferating myoblasts and satellite cells with self-renewal potential, while Notch activity is low in post-mitotic myoblasts and differentiating satellite cells, which is accompanied by an increase in the expression levels of Notch ligands (Fig. 8B).

*RBP-J*-deficient satellite cells lose their quiescence and undergo differentiation, thus Notch signalling is required to maintain satellite cells in a quiescent state (Mourikis et al., 2012a). Notch receptors *Notch1/2/3* as well as Notch/RBP-J target genes *Hes1*, *Hey1* and *HeyL* are highly expressed in quiescent satellite cells and drastically drop after injury in activated satellite-cells (Mourikis et al., 2012a). This shows a dual role for Notch signalling; Notch maintains the self-renewal capacity of satellite cells after injury, but also preserves the quiescent state of satellite cells and inhibits uncontrolled differentiation and exhaustion of the

stem cell pool. Since *MyoD* expression is important to exit quiescence by activating *cell division cycle 6* (*Cdc6*), it was proposed that the shut-down of Notch signalling after muscle injury releases the *MyoD* suppression. Four to five days after injury, expression of Notch target genes increases again, possibly due to the presence of committed Dll1+/Myogenin+ satellite cells that signal to the Pax7+ self-renewing satellite cells (reviewed by Mourikis and Tajbakhsh, 2014).

In summary, Notch signalling positively regulates the number of muscle progenitors that express Notch receptors and target genes. For differentiation, Notch needs to be inhibited and determined myoblasts start to express Notch-ligands. Thus, how muscle progenitors that receive Notch signals crosstalk with myoblasts that express Notch ligands has started to be understood.

# State of the art

## 3 – Regulation of the muscle progenitor pool

### 3.2 – Signalling pathways

#### 3.2.2 – BMP signalling pathway

BMPs are growth factors of the TGFβ superfamily, which also includes Activins, Nodals and Growth/Differentiation factors (GDFs). TGFβ family members play a role in cell growth, differentiation and morphogenesis. BMPs act as morphogens, *i.e.* they are secreted and can induce distinct responses depending on their concentration. Dimerization of BMPs is required for activation of the BMP receptors that possess intracellular serine/threonine kinase domains. Two type II and two type I receptors are assembled in the receptor complex (reviewed by Massagué, 2012). In the presence of the ligand, the type II receptor phosphorylates the type I receptor (Fig. 9A). The kinase activity of type I receptors depends on this phosphorylation in a domain rich in glycine and serine (GS domain). The type II receptors that mediate BMP signals are mainly the BMP receptor II (BMPRII) but also the Activin receptor type IIa and IIb (ActRIIa/IIb). The type I subunits of the BMP receptor complexes are mainly the BMP receptors Ia and Ib (BMPRIa/Ib) (also named Activin-like receptor 3 (Alk3) and Alk6, respectively) but also in certain contexts the receptors Alk1 and Alk2 (reviewed by Moustakas and Heldin, 2009). The activated type I receptors phosphorylate and activate the regulatory Smad proteins (R-Smads), the effectors of the pathway, that translocate to the nucleus and function as transcription factors (Fig. 9A). The BMP type I receptors (Alk3/6/1/2) signal via Smad1/5/8 (Fig.9 A), while the type I receptors that bind TGFβ signal via Smad2/3 (reviewed by Moustakas and Heldin, 2009). The Smad proteins function in trimers, where two phosphorylated R-Smads bind to the common mediator (co-Smad) Smad4. Smad4 is required for trimerization of Smad1/5/8 and Smad2/3 (reviewed by Massagué et al., 2012). The third class of Smads, the Inhibitory-Smads (I-Smads) Smad6 and Smad7, bind to the type I receptor and interfere with phosphorylation and activation of R-Smads. Smad7 blocks the activity of the different TGFβ type I receptors whereas Smad6 has higher affinity for BMPRIa/Ib (Fig. 9A) (reviewed by Moustakas and Heldin, 2009).

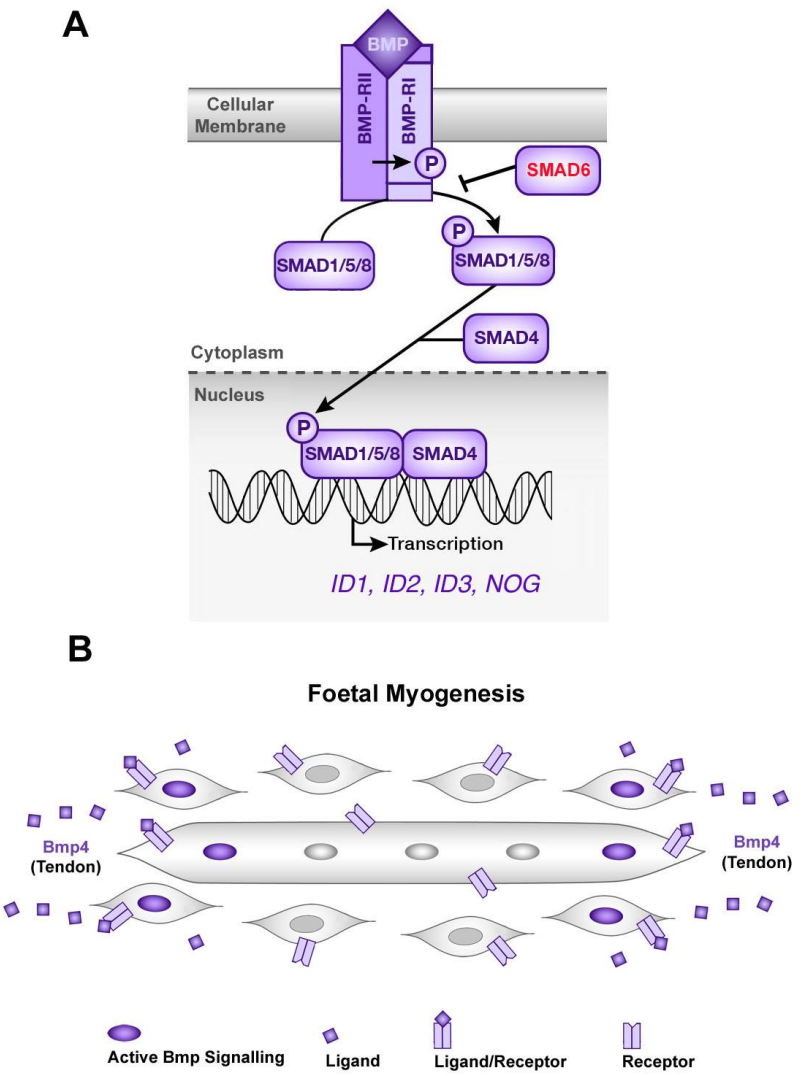



**Figure 9 – BMP signalling during foetal myogenesis.** (A) Simplified scheme of the BMP signal transduction cascade. (B) Illustration of myogenic cells that receive BMP signals mainly located to the tips of the muscle fibres during foetal myogenesis.

*In vivo* studies on the role of BMP signalling in muscle development were mainly performed in the chick. Expression of *BMP4* in the lateral plate of the developing chick embryo preserves muscle progenitors in the hypaxial lip of the dermomyotome in an undifferentiated state. Upon BMP exposure, these cells increase *Pax3* and reduced *MyoD* expression (Pourquié et al., 1996). BMPs from the surface ectoderm of the limb maintain muscle progenitors (Pax3+ cells) in a proliferative state (Amthor et al., 1998). When the ectoderm is removed, *Pax3* expression is decreased and *MyoD* is upregulated, which results in premature differentiation of muscle progenitors and subsequent muscle loss. The same is observed in trunk somites when the ectoderm is removed (Amthor et al., 1999). Low

concentration of exogenous BMPs can mimic the effect of the ectoderm and maintain Pax3+ cells in somites and limbs (Amthor et al., 1998; 1999).

BMP signalling is positively regulating the number of muscle progenitors and fibres during foetal myogenesis in the chick limb (Wang et al., 2010). Gain and loss-of-function experiments using RCAS showed that ectopic expression of *BMP4* triggers muscle growth (more Pax7+ cells and more muscle fibres) while blockade of the pathway by overexpression of *Noggin* leads to a decreased muscle size (less Pax7+ cells and less muscle fibres) (Wang et al., 2010). Subsets of muscle progenitors (Pax7+) and terminally differentiated cells (MF20+) observed with pSmad1/5/8 locate at the tips of muscles (Fig. 9B). This is in accordance with the restricted expression of *BMP4* in tendons close to the muscle tips (Fig.9 B) (Wang et al., 2010). Muscle progenitors positive for pSmad1/5/8 also co-expressed a proliferation marker, phospho-histone H3 (PH3), suggesting that the BMP signals promote cell proliferation (Wang et al., 2010).

Recent studies have demonstrated the role of BMP signalling in the balance between proliferation and differentiation of activated satellite cells that express high levels of *BMPRIa* and are positive for pSmad1/5/8 (Friedrichs et al., 2011; Ono et al., 2010). BMP4 promotes an osteogenic fate in cultured C2C12 cells but sustains proliferation and prevents commitment and differentiation in primary satellite cells by up-regulating *Id1* (Friedrichs et al., 2011; Ono et al., 2010). Consistently, inhibition of BMP signalling by dorsomorphin treatment (that blocks phosphorylation of Smad1/5/8), noggin (Nog) or small interfering RNA (siRNA)-mediated downregulation of BMPRIa leads to precocious differentiation. Once satellite cells commit for differentiation, the expression of *Chordin* and *Noggin*, two antagonists of the pathway, is activated (Friedrichs et al., 2011; Ono et al., 2010).

In adult mice, BMP promotes muscle growth by inducing muscle hypertrophy (increase in the cell size) and inhibition of BMP signalling causes muscle atrophy (decreased muscle size) (reviewed by Sartori et al., 2014). BMP induces muscle hypertrophy by stimulating protein synthesis through activation of the Akt/mTOR pathway, and blockade of mTOR prevents the BMP-induced muscle hypertrophy (reviewed by Sartori et al., 2014). In addition, BMP signalling negatively regulates the expression of *MUSA1*, a ubiquitin-ligase that leads to degradation of muscle proteins (Sartori et al., 2013), which also contributes to the hypertrophy.

# State of the art

## 3 – Regulation of the muscle progenitor pool

### 3.2 – Signalling pathways

#### 3.2.3 – Crosstalk between Notch and TGFβ/BMP signalling pathways

The Notch and BMP signalling pathways play similar roles in the maintenance of the muscle progenitor pool during foetal myogenesis. Mechanisms of crosstalk between these two pathways started to be explored in different developmental processes, including myogenesis.

Notch and TGBβ/BMP inhibit myogenic differentiation of C2C12 cells and the two factors can cooperate (Fig.10) (Blokzijl, 2003; Dahlqvist, 2003). While exogenous BMP4 inhibited myogenic differentiation of C2C12 cells, blocking Notch signalling by a pharmacological agent (L-685,458) or a dominant-negative RBP-J interfered with this result (Dahlqvist et al., 2003). This indicates that BMP4-mediated inhibition of myogenic differentiation *in vitro* requires active Notch signalling. In addition, exogenous BMP4 increases the expression of Notch target genes *Hes1* and *Hey1*, which suggests a synergistic effect of the two pathways in the inhibition of differentiation (Fig. 10) (Dahlqvist et al., 2003). Exogenous TGFβ in C2C12 cells also increases the expression of *Hes1*, effect that is no longer observed in the presence of a dominant-negative RBP-J (Fig. 10) (Blokzijl et al., 2003). In addition, *in vivo* experiments in the chick embryo showed that activation of TGFβ signalling by overexpression of the constitutively active Alk5 also up-regulates *Hes1* (Fig. 10) (Blokzijl et al., 2003).

In these studies, direct interactions between NICD and Smad3 (the effector of the TGFβ signalling pathway) or Smad1 (the effector of the BMP signalling pathway) were observed (Fig. 10) (Blokzijl et al., 2003; Dahlqvist et al., 2003). In addition, NICD, Smad1 and Smad3 can be recruited to Notch/RBP-J binding sites on DNA, which potentiates the activation of Notch target genes. In particular, Smad1/NICD complexes bind to the *Hey1* promoter (Fig. 10) (Dahlqvist et al., 2003). In accordance, the activation of Notch reporter constructs, which respond to transfection with NICD, is potentiated by BMP4 (Dahlqvist et al., 2003). Furthermore, Smad3/NICD but not Smad3 activated a reporter construct carrying RBP-J binding sites (Blokzijl et al., 2003). These results indicate that BMP/TGFβ and Notch

signalling pathways synergistically interact to potentiate the inhibition of myogenic differentiation (Fig. 10).

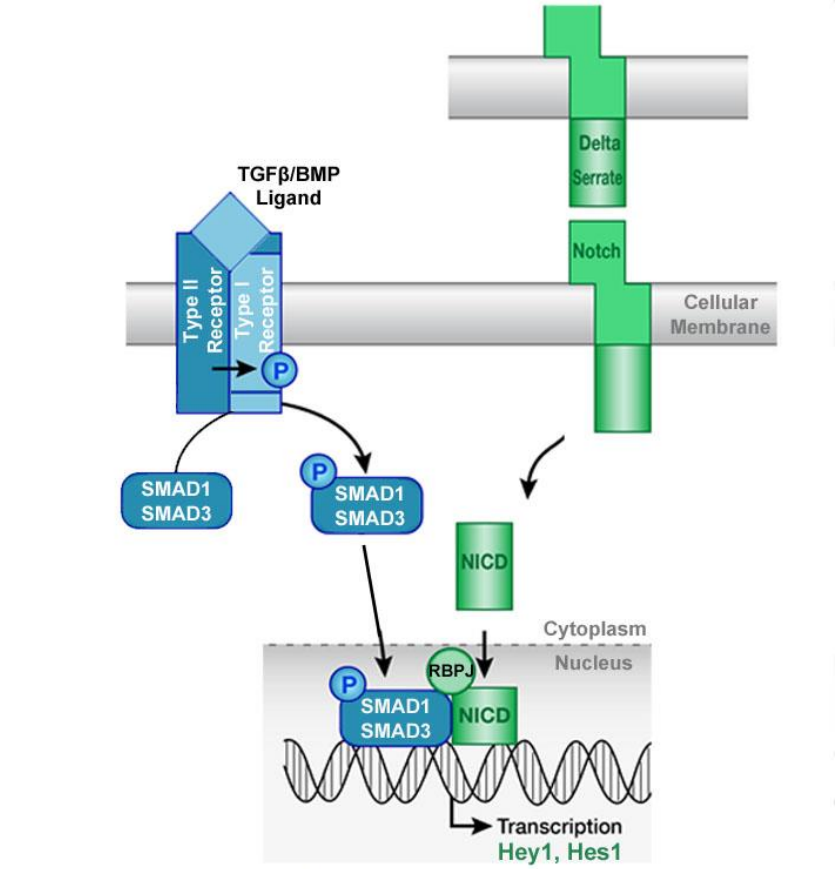



**Figure 10 – Synergism between Notch and TGFβ/BMP signalling pathways.** Scheme illustrating the intracellular crosstalk observed between Notch and TGFβ/BMP signalling pathways which results in a functional synergism. Results described in the literature are summarized.

A similar crosstalk between Notch and BMP pathways also exists in other cell types like mouse regulatory T cells, mouse neuroepithelial cells, endothelial cells and neural stem cells (reviewed by Guo and Wang, 2009). Antagonistic effects between these two pathways have also been described. For instance, branching morphogenesis of the prostate gland is repressed by BMP7 that also reduces *Notch1* expression (Grishina et al., 2005). Overexpression of TGFβ1 in 10T1/2 fibroblasts leads to the activation of smooth muscle-related genes and decreased expression of *Notch3*, whereas overexpression of Notch3 interfered with TGFβ1-mediated expression of smooth muscle markers (Kennard et al., 2008). In aged muscles, TGFβ signalling (via Smad3) is active while Notch signalling

activity is decreased compared to younger muscles (Carlson et al., 2008). The forced activation of Notch in aged muscles prevents the binding of Smad3 to promoters of the cell cycle and senescence regulators *p21*, *p27*, *p16* and *p15*, which interferes with the expression of these genes in aged muscles (Carlson et al., 2008).

In conclusion, the majority of published studies on the crosstalk between Notch and TGFβ/BMP signalling demonstrate that these two pathways synergise to inhibit differentiation during myogenesis.

# State of the art

## 4 – Muscle as mechanical tissue

### 4.1 – Importance of muscle contraction during development

During development, the mechanical environment plays an important role in the formation of different organs like heart, lung, kidney, bone and tendon. Particularly in the musculoskeletal system, the mechanical forces produced by muscle contraction are crucial for the correct development of the skeleton and tendons (reviewed by Shwartz et al., 2013). How the mechanical signals are sensed by cells and regulate processes like cell growth, cell shape rearrangement, differentiation and apoptosis are important question in developmental biology.

The role of muscle contraction in skeleton and tendon development has been investigated in mice and chick. Mouse models that lack limb muscles, *i.e. Pax3*-mutant or *Myf5/MyoD*-double mutant mice, display a generalised decrease in bone formation and altered deposition of ossified structures which results in an abnormal bone morphology (reviewed by Nowlan et al., 2010). Chick embryos can be exposed to paralysing agents like decamethonium bromide (DMB) that induces a rigid muscle paralysis or pancuronium bromide (PB) that exerts flaccid paralysis, which results in increased or decreased muscle tension, respectively. DMB-treated embryos display thinner and aberrantly curved bones in the trunk and shorter bones in the limbs (reviewed by Nowlan et al., 2010). In addition to the effects of muscle contraction on skeletogenesis, tendon development is also influenced by it. Initiation of tendon development in the limb is muscle-independent, but the following morphogenesis requires muscle interaction (Kardon, 1998). This muscle-dependent phase of tendon development seems to rely on muscle contraction, and chick embryos exposed to DMB decrease the expression of tendon markers (Havis et al., unpublished data).

Whereas the role of muscle contraction in the formation of bone and tendon is well studied, little is known about the relevance of mechanical forces for the integrity of developing skeletal muscle. In chick embryos, limb myogenesis is initiated in the absence of muscle contraction (achieved by ablation of the neural tube, including motoneurons). However, the integrity of muscles is not maintained and muscle begins to degenerate by apoptosis at E10 (Rong et al., 1992). Muscular dysgenesis (mdg) in mice interferes with excitation/contraction coupling and precludes contraction. Muscle formation is initiated in

these animals but the muscles start to degenerate around E14 (Pai 1965a; 1965b). Exerting muscle paralysis in chick embryos with DMB during foetal developmental stages, *i.e.* after limb muscles are assembled, decreases the muscle cross-sectional area and lowers muscle force (Hall and Herring, 1990). Thus mechanical forces play a role in the maintenance of the skeletal muscle during development.

## 4 – Muscle as mechanical tissue

### 4.2 – The mechano-sensitive gene Yap and its role in myogenesis

The Yes-associated protein (Yap) is an effector of the Hippo pathway. The Hippo pathway was first described in *Drosophila melanogaster* where it suppresses cell proliferation and tumour growth and regulates organ size (reviewed by Zhao et al., 2011). Hippo is one of the Serine/Threonine kinases in the signalling cascade, and its homologues in mammals are Mst1/2 (Fig. 11). When Mst proteins are phosphorylated they become activated and phosphorylate their direct substrates Lats1/2 (Fig. 11) (Wts in *Drosophila*). When activated, Lats1/2 proteins phosphorylate Yap and its paralogue Taz (homologues of Yorkie (Yki) in *Drosophila*) that are then sequestrated in the cytoplasm. Consequently Yap and Taz are unable to shuttle into the nucleus and their transcriptional activity is blocked (Fig. 11) (reviewed by Zhao et al, 2011). Upstream mechanisms regulating Mst activity are poorly understood. Mst1/2 can be activated by binding to Ras association domain family proteins (RASSF) that form complexes with Ras proteins and inhibit cell growth (reviewed by Zhao et al, 2011). Increased Yap activity is observed in many types of cancers. *Yap* overexpression in the liver increases its size and sustained Yap activity leads to tumour formation. The same is observed in mouse deficient for *Mst1/2*, which present increased Yap activity (reviewed by Zhao et al, 2011). In summary, the Hippo pathway determines organ size and inhibits tumorigenesis by controlling Yap.

Protein interactions in the Hippo pathway are mainly mediated by binding between proteins containing WW domains and proline-rich motifs (PPxY/F). Yap transcripts can be alternatively spliced, and originate variants that contain one or two WW domains, both variants interact with the PPxY/F motifs present in Lats (reviewed by Wackerhage et al., 2014). Cytoplasmic-nuclear shuttling of Yap is regulated by several mechanisms. Phosphorylation of Yap by Lats1/2 leads to its cytoplasmic retention and allows binding to the 14-3-3 protein, which interacts with α-catenin in adherens junctions (Fig. 11). Once Yap is phosphorylated, it can also be ubiquitylated and degraded (reviewed by Wackerhage et al., 2014). Yap activity and location in the cytoplasm is also achieved by phosphorylation-independent mechanisms like the binding to angiomotin proteins (Amot) (Fig. 11) (reviewed by Zhao et al, 2011). Amot proteins are expressed in mechanical tissues like skeletal and

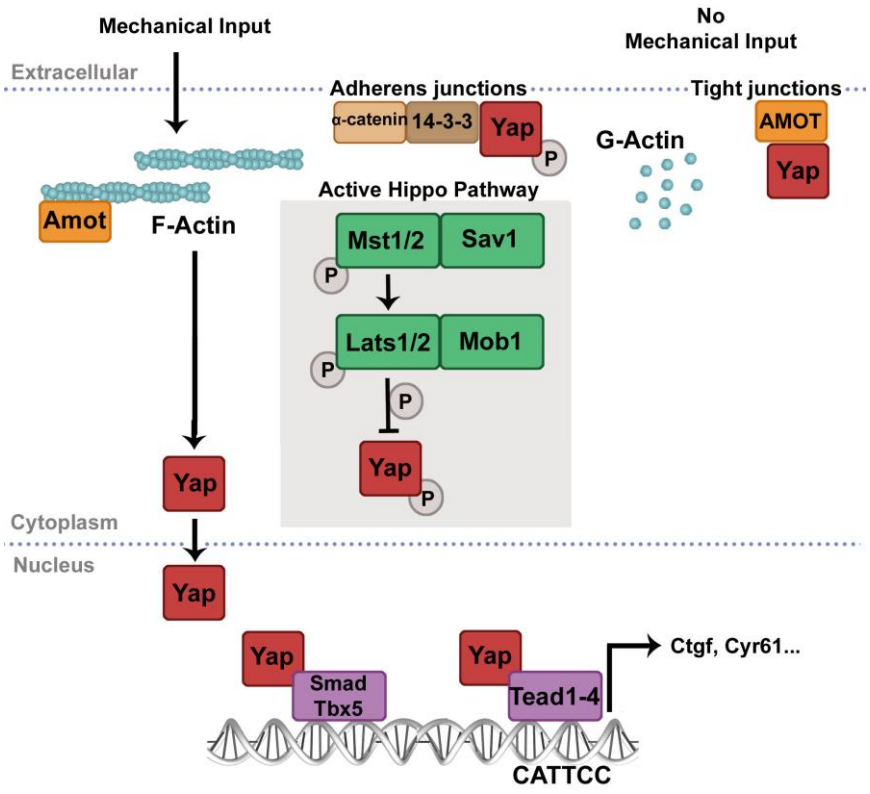



**Figure 11 –Signals regulating Yap activity.** Scheme illustrating the regulation of Yap by the Hippo pathway and by mechanical input.

cardiac muscles (reviewed by Wackerhage et al., 2014). The sequestration of Yap in the cytoplasm by Amot proteins depends on mechanical stimuli (Fig. 11). Mechanical signals and cultures on stiff substrates or low densities increase F-actin polymerisation (Fig. 11). Under these conditions, Amot proteins bind to F-actin and release Yap which is then free to shuttle to the nucleus (Fig. 11) (reviewed by Wackerhage et al., 2014).

Yap is a transcriptional co-activator that is unable to directly bind DNA. Therefore, it needs to interact with transcription factors to exert its function as trans-activator (Fig. 11). Through its Tead-binding domain, Yap interacts with the transcription factors Tead1/2/3/4 (TEA domain transcription factors). Tead recognizes the MCAT elements (CATTCC) on gene promoters or enhancers, and modulates their expression by binding to trans-activators like Yap or Vgll1/2/3 (vestigial-like), or inhibitors like Vgll4 (Fig. 11) (reviewed by Zhao et al, 2011; reviewed by Wackerhage et al., 2014). Yap can also bind through its WW domain to other transcription factors like Smad1 or Tbx5 (Fig. 11) (reviewed by Zhao et al, 2011). However, in mammary epithelial cells Yap/Tead1 co-occupy the same binding sites in 80% of the cases, indicating that this interaction is of major functional importance (Zhao et al.,

2008). Direct target genes of Yap/Tead complexes have been identified, like connective-tissue growth factor (Ctgf) and cysteine-rich protein 61 (Cyr61), both involved in promoting cell proliferation (Fig. 11) (reviewed by Zhao et al, 2011).

Components of the Hippo pathway are ubiquitously expressed in adult mice (Watt et al., 2010). In particular, Yap mRNA and protein are present in skeletal muscles of adult mice and in C2C12 myotubes (Watt et al., 2010). During mouse foetal development (E17), the levels of Yap mRNA and protein in the muscles are abundant but decline during post-natal maturation (Watt et al., 2015). These findings raised the question whether Yap might function in skeletal muscle.

In C2C12 cells, plated satellite cells or satellite cells in floating myofibre cultures, Yap promotes proliferation and inhibits differentiation (Judson et al., 2012; Watt et al., 2010). These experiments relied on overexpression of a constitutively-active Yap variant (hYap1 S127A) that cannot be phosphorylated on Ser127 and is constitutively nuclear. This resulted in increased expression levels of *Pax7* and *Myf5* and decreased expression of *Myogenin*, which suggests that active Yap plays a role in the expansion of stem and progenitor cells (Judson et al., 2012; Watt et al., 2010). In addition, in satellite cells in floating myofibre cultures Yap activity (nuclear Yap protein and mRNA levels) is low when the cells are quiescent (freshly-isolated), and increases when the cells are activated (48h in culture) and proliferate (72h in culture) (Pax7+/MyoD+) (Judson et al., 2012). Once the satellite cells are activated, Yap expression levels remain high until cells re-enter quiescence or terminally differentiate.

Yap localisation has been also studied in the adult muscle. Yap is present in some but not all myofibre nuclei and in the fibre membrane, particularly close to the neuro-muscular junctions (NMJ) (Watt et al., 2015). Loss-of-function experiments using an adeno-viral vector carrying a short-hairpin RNA (shRNA) targeting Yap administrated in limb muscles leads to muscle atrophy, *i.e.* a decrease in muscle mass and a reduced myofibre diameter (Watt et al., 2015). The muscle atrophy is associated with a reduced protein synthesis but not increased protein degradation (Watt et al., 2015). Conversely, increased Yap activity in muscles promotes hypertrophy. These experiments showed for the first time that Yap positively regulates the adult skeletal muscle mass. Because Yap is accumulated at the membrane close to the NMJ, Yap activity was also evaluated after denervation. Denervated muscles show an increase in phosphorylated and non-phosphorylated Yap protein and nuclear

accumulation. This might provide a compensatory mechanism that prevents the excessive muscle loss after denervation.

Functions of Yap in adult skeletal muscle started to be understood, but a role of Yap in muscle development has not been addressed and in general, developmental functions of Yap have been little studied. *Yap*-deficient mice die at E8.5 due to defects in yolk sac development (reviewed by Barry and Camargo, 2013). Yap localises to nuclei of cells of the trophectodermal lineage, which forms the extra-embryonic tissues, but is present in the cytoplasm of blastocyst cells, indicating that the Hippo pathway might participate in cell fate decisions at early developmental stages (reviewed by Barry and Camargo, 2013). In chick embryos, Yap/Tead downstream of the classical Hippo pathway regulate the neural progenitor pool, increase cell proliferation by inducing *cyclin D1* expression, and inhibit differentiation by suppressing *NeuroM* (Cao et al., 2008). In contrast, downregulation of Yap by shRNA resulted in cell death of the neural progenitors (Cao et al., 2008).

# Results

**Part of the results I obtained during my PhD were:**

**Published in Developmental Biology journal:**

**Joana Esteves de Lima**, Marie-Ange Bonnin, Adeline Bourgeois, Alice Parisi, Fabien Le Grand and Delphine Duprez. (2014). Specific pattern of cell cycle during limb foetal myogenesis Dev. Biol. 392, 308–323. (doi:10.1016/j.ydbio.2014.05.015)

**Submitted to BMC Developmental Biology journal:**

Adeline Bourgeois, **Joana Esteves de Lima**, Benjamin Charvet, Koichi Kawakami, Sigmar Stricker and Delphine Duprez. (2015). Stable and bicistronic expression of two genes in somite- and lateral plate-derived tissues to study chick limb development.

# Results

## Specific pattern of cell cycle during limb foetal myogenesis

This is a summary from part of the results I obtained during my PhD thesis and that are published in Developmental Biology journal.

**Joana Esteves de Lima**, Marie-Ange Bonnin, Adeline Bourgeois, Alice Parisi, Fabien Le Grand and Delphine Duprez. (2014). Specific pattern of cell cycle during limb foetal myogenesis. Dev. Biol. 392, 308–323.

I contributed to this work by performing all the mouse *in vivo* analysis, the Fucci/RCAS vector construction, the chick *in vivo* and *in vitro* analysis, cell quantification, analysis and interpretation of the data and writing of the manuscript.

### Synopsis

Objectives: Myogenesis relies on an undifferentiated pool of muscle progenitors that is required to sustain muscle growth during development and that gives rise to the satellite cells. Therefore, the balance between proliferation and differentiation has to be tightly regulated during development. In this study we aimed to characterise the cell cycle state of the myogenic cell lineage during foetal myogenesis.

Methods: We used the previously established Fucci system (fluorescent ubiquitination-based cell cycle indicator) (Sakaue-Sawano et al., 2008) in which cells in the G1 phase of the cell cycle express a red fluorescent reporter (mKO2-hCdt1; Fucci red) and cells in the S, G2 and M phases express a green fluorescent reporter (mAG-hGem; Fucci green). We used a Fucci red/green double-transgenic mouse line to detect cells in different phases of the cell cycle during foetal myogenesis.

Results: We observed that cell proliferation is not randomised in chick limbs during foetal myogenesis. Fucci green reporter expression indicates that cells in the S/G2/M phases of the cell cycle accumulated around the muscles and at muscle tips. We analysed if this was also true for muscle progenitors. In muscles, we identified an increased number of Pax7+ cells in S/G2/M phases at muscle tips compared to the centre of the muscles, which might

suggest that the environment at the muscle tips promotes expansion of muscle progenitors and non-myogenic cells.

Pax7+ cells expressed mainly the Fucci green reporter while MyoD+ cells expressed mostly Fucci red. Pax7+ cells rarely expressed the G1 phase marker Fucci red. This might reflect the fact that Fucci system is not expressed or expressed at very low levels in the early G1 cell cycle phase, and that visible expression of Fucci red is associated with a lengthening of the G1 cell cycle phase (Roccio et al., 2013).(Sakaue-Sawano et al., 2008). Therefore, this indicates that Pax7+ cells display a short G1 phase and are thus similar to neural stem cells (Roccio et al., 2013). Since Pax7+ cells were rarely labeled by Fucci red, we used Fucci green for further characterisation of the cell cycle state of Pax7+ cells. By combining Fucci green and EdU (ethynyldeoxyuridine) labelling, we conclude that around 30% of the Pax7+ cells could be detected by Fucci green expression and should thus correspond to the Pax7+ cells in early G1 phase of the cell cycle.

Conclusion: The Fucci system allowed us to show that actively cycling cells are distributed in a non-random manner in the limbs and inside the muscles. The Fucci system does not completely cover all the phases of the cell cycle and in particular does not allow the visualisation of cells that go very rapidly through G1. However, the Fucci green reporter can be used to detect proliferating cells that are in the S, G2 and M phases.

# Results

## Stable and bicistronic expression of two genes in somite- and lateral plate-derived tissues to study chick limb development

This is a summary from part of the results I obtained during my PhD thesis and that were submitted to BMC Developmental Biology journal.

Adeline Bourgeois, **Joana Esteves de Lima**, Benjamin Charvet, Koichi Kawakami, Sigmar Stricker and Delphine Duprez. (2015). Stable and bicistronic expression of two genes in somite- and lateral plate-derived tissues to study chick limb development.

I contributed to this work by constructing the pT2AL-Tol2-p57MRE/βactin-Tomato-2A-GFP-Tol2 vector, performing chick limb somite electroporation, histological analysis and imaging of the electroporated limbs.

### Synopsis

Objectives: Electroporation of somites at the axial level of the limbs allows the misexpression of genes in limb somite derivatives (Bonnet et al., 2010; Wang et al., 2011). This targets the cells that will give rise to myogenic and endothelial cells of the limbs. In contrast, electroporation of the lateral plate targets other limb cells like cartilage, bone, ligaments and tendons (Gros et al., 2005). The objective of this study was to establish a technique to misexpress genes in muscle cells at different stages of differentiation and also to misexpress genes in lateral plate-derived tissues.

Methods: We used vectors that take advantage of the Tol2-transposase system-mediated integration into the genome for stable integration. These allow a continuous misexpression of the gene-of-interest even at later developmental stages and differ thus from transient misexpression. Therefore, we intended to design vectors containing two cDNAs of interest between two Tol2 transposons. To target muscle cells at different stages of differentiation, we used the CMV/βactin promoter for a general misexpression in the myogenic lineage, the p57MRE/βactin promoter for expression in myoblasts and the MLC promoter for expression in differentiated muscle cells. To perform analysis on a cellular

level, we aimed to misexpress in the same cell the gene-of-interest together with a reporter gene. For this, we took advantage of the 2A peptide that allows expression of a bicistronic mRNA (Szymczak et al., 2004). A single peptide is produced by the bicistronic mRNA, and auto-cleavage of the 2A peptide subsequently produces equal amounts of the two proteins. The final plasmids obtained were: pT2AL-Tol2-CMV/βactin-Tomato-2A-GFP-Tol2, pT2AL-Tol2-p57MRE/βactin-Tomato-2A-GFP-Tol2 and pT2AL-Tol2-MLC-Tomato-2A-GFP-Tol2. Each one of these plasmids was electroporated in association with a transient vector containing the transposase (pCMV/βactin-T2TP) to allow genomic integration.

Results: After electroporation of somites at the limb level, the ubiquitously expressed CMV/βactin promoter targeted both muscle progenitors (PAX7+ cells) and differentiated cells (MF20+ cells), the p57MRE/βactin promoter targeted mononucleated (MF20-) cells and muscle fibres (MF20+), and the MLC promoter targeted differentiated cells (MF20+). With these vectors we observed a simultaneous expression of Tomato and GFP at the different stages of the muscle differentiation. Lateral plate electroporation with the vector containing the CMV/βactin promoter allowed Tomato and GFP expression in cartilage, tendons and connective tissues, but never in myogenic PAX7+ or MF20+ cells.

Conclusion: In conclusion, we achieved to generate bicistronic vectors that are able to drive stable gene expression in the myogenic lineage at different steps of differentiation. I used this system to study the interaction between differentiated muscle cells and muscle progenitors (see Results 2). In addition, these bicistronic vectors can be electroporated in somitic and lateral plate mesoderm and provide a tool to study the interaction between lateral plate-derived tissues (connective tissue, tendon, cartilage) and myogenic cells.

# 1 - The interplay between NOTCH and BMP signalling pathways is different during proliferation and differentiation during foetal myogenesis

# Results

## 1 – The interplay between NOTCH and BMP signalling pathways is different during proliferation and differentiation during foetal myogenesis

### Synopsis

Objectives: BMP and NOTCH signalling positively regulate the number of muscle progenitors but have different outcomes on muscle differentiation (Amthor et al., 1998; 1999; Delfini et al., 2000; Hirsinger et al., 2001; Vasyutina et al. 2007a; Schuster-Gossler et al., 2007; Wang et al., 2010; Mourikis et al., 2012a). Downregulation of Notch signalling is required for myogenic differentiation, but BMP potentiates muscle differentiation in foetal myogenesis and is required for myotube formation *in vitro* (Delfini et al., 2000; Bröhl et al., 2012; Vasyutina et al., 2007a; Mourikis et al., 2012a; Wang et al., 2010; Umemoto et al., 2011). We aimed to define the interplay between NOTCH and BMP signalling in proliferation and in differentiation during foetal myogenesis *in vivo* and *in vitro*.

Methods: The RCAS virus broadly infects chick dividing cells and can be used to misexpress genes in chick limbs *in vivo* and in primary cultures of chick foetal myoblasts *in vitro*. Myoblast culture allowed us to separately investigate proliferation and differentiation conditions.

Results: BMP and NOTCH positively regulate the number of foetal muscle progenitors *in vivo*. *In vitro*, BMP and NOTCH stimulate *PAX7* expression and act independently of each other. Forced NOTCH activation leads to an increased number of muscle progenitors without activating BMP signalling in chick limbs. In addition, in proliferating cultures of chick foetal myoblasts NOTCH activation increases *PAX7* and *MYF5* but does not alter the expression of BMP target genes. This effect is not modified by the presence of a BMP inhibitor. Activation of BMP in chick limbs increases *NOTCH1* expression and the number of PAX7+ cells. In myoblast cultures, BMP activation also increases expression of *PAX7*, *MYF5* and of the NOTCH target gene *HEYL*. Thus, *PAX7* expression could be regulated indirectly by the up-regulation of *HEYL* expression. However, blocking NOTCH activity with DAPT in myoblast cultures in the presence of BMP did not change expression of *PAX7*. These results show that NOTCH and BMP have a convergent effect on *PAX7* expression, but they act independently of each other.

During differentiation, BMP and NOTCH have antagonist effects and activation of one pathway decreased the activity of the other. Activation of BMP in differentiation cultures of myoblasts decreases expression of the NOTCH target genes *HES1* and *HEYL* and conversely, blocking BMP increases *HES1* and *HEYL*. This suggests a role of BMP in inhibiting NOTCH in differentiating myoblasts. In addition, activation of NOTCH decreases expression of BMP target genes *ID1*, *ID2* and *NOG*. Thus, NOTCH and BMP interactions differ during proliferation and differentiation. These results are consistent with the spatial regulation of NOTCH and BMP activity *in vivo*. *NOTCH* is widely expressed in muscle progenitors while BMP is active only in muscle progenitors at the muscle tips and in differentiated fibres (Wang et al., 2010; Mourikis et al., 2012a). Our results suggest that BMP inhibits NOTCH signalling during differentiation.

Conclusion and Perspectives: NOTCH and BMP signalling have similar effects on PAX7+ muscle progenitors and differently regulate muscle differentiation. The interplay between the two signalling pathways is different in proliferating and differentiating cells.

BMP and NOTCH regulate *PAX7* expression positively and independently of each other. At the muscle tips, where BMP and NOTCH are active, the number of PAX7+ cells present in the S/G2/M phases of the cell cycle increase (Esteves de Lima et al., 2014). To test if the forced activation of both pathways could potentiate an increase in *PAX7* expression compared to activating NOTCH or BMP alone, we will simultaneously activate NOTCH (DLL1/RCAS) and BMP (BMP4/RCAS).

In addition we would like to determine whether the changes of PAX7 mRNA levels in myoblast culture are correlated with differences in the number of PAX7+ cells. For this, we will perform PAX7 immunohistochemistry in myoblast cultures.

# Results

## 1 - The interplay between NOTCH and BMP signalling pathways is different during proliferation and differentiation during foetal myogenesis


### Abstract

NOTCH and BMP are two signalling pathways known to positively regulate the pool of muscle progenitors during developmental, postnatal and regenerative myogenesis. During foetal myogenesis, NOTCH and BMP positively regulate the number of muscle progenitors, have opposite effects on muscle differentiation and display distinct pattern of activity in muscles. These two signalling pathways are often studied independently in the context of myogenesis. However, synergistic or antagonist interactions have been described between NOTCH and BMP in various developmental processes. We analysed the interactions between NOTCH and BMP signalling pathways during foetal myogenesis using chick *in vivo* and *in vitro* systems. During proliferation of foetal muscle cells, NOTCH and BMP displayed a convergent effect to promote/maintain muscle proliferation but acted independently of each other. NOTCH signalling pathway did not induce BMP activity and NOTCH acted independently of BMP to maintain the pool of muscle progenitors. BMP signalling activated primary NOTCH target genes, but NOTCH was not required for BMP function on proliferating foetal myoblasts. During foetal muscle differentiation, NOTCH and BMP displayed antagonist interactions, consistent with their opposite effects on muscle differentiation. These antagonist interactions in differentiating foetal myoblasts suggested that BMP in differentiated muscle cells blocked NOTCH signalling to allow muscle differentiation. We conclude that the interplay between NOTCH and BMP intracellular signalling pathways is different in proliferating and differentiating foetal muscle cells. These interactions are fully consistent with the endogenous pattern of NOTCH and BMP activities in limb foetal muscles and with their respective functions in foetal muscle proliferation and differentiation.

# Introduction

## 1 - The interplay between NOTCH and BMP signalling pathways is different during proliferation and differentiation during foetal myogenesis

Skeletal muscle formation occurs in successive and overlapping waves of myogenesis. During development, embryonic myogenesis will set the muscle architecture, while foetal myogenesis will assure muscle growth (Biressi et al., 2007; Stockdale, 1992). Adult muscle progenitors other named satellite cells are responsible for perinatal muscle growth, homeostasis and muscle regeneration. The pool of satellite cells is established during foetal myogenesis. Consequently, the understanding of the source and the nature of molecular pathways regulating foetal myogenesis is important. The NOTCH and BMP signalling pathways have been described as being involved in foetal myogenesis in mouse and chick models. NOTCH and BMP have a convergent and positive effect on muscle progenitors, but have opposite effects on muscle differentiation (Mourikis et al., 2012a; Schuster-Gossler et al., 2007; Vasyutina et al., 2007a; Wang et al., 2010). Components of the BMP and NOTCH signalling pathways display a distinct expression pattern in foetal muscles. NOTCH signalling appears to be active homogenously in all foetal progenitors (Delfini et al., 2000; Mourikis et al., 2012a; Vasyutina et al., 2007a), with *NOTCH1* expression in all foetal muscle progenitors and *JAG2* expression in myosin positive cells with no spatial restriction in chick limbs (Delfini et al., 2000; Mourikis et al., 2012a). In contrast, BMP signalling is preferentially active in a sub-population of foetal muscle progenitors close to muscle tips (Wang et al., 2010). Moreover, in contrast to NOTCH, which is never active in differentiated muscle cells (Delfini et al., 2000, Vasyutina et al., 2007a; Mourikis et al., 2012a), BMP is active in a subpopulation of myonuclei in differentiated foetal muscle cells (Wang et al., 2010).

During development, NOTCH signalling is known to promote muscle progenitor proliferation, while inhibiting muscle differentiation (Delfini et al., 2000; Schuster-Gossler et al., 2007; Vasyutina et al., 2007a, Mourikis et al., 2012a). NOTCH gain- and loss of-function experiments have been performed in head, somite and limb muscles during development in chick and mouse models (Czajkowski et al., 2014; Delfini et al., 2000; Hirsinger et al., 2001; Mourikis et al., 2012a; Vasyutina et al., 2007a; Schuster-Gossler et al., 2007). All these *in*

*vivo* developmental studies converged to the same results, *i.e.* NOTCH signalling was active in muscle progenitors to maintain the pool of progenitors and its downregulation was required for muscle differentiation. Modification of NOTCH activity in muscle cell cultures also led to similar conclusions (Kopan et al., 1994; Kuroda et al., 1999). The transmembranar ligands, DELTA-like or JAGGED ligands activate canonical NOTCH signalling in adjacent cells. Upon ligand activation, NOTCH receptor undergoes a proteolytic cleavage to release the NOTCH intracellular domain (NICD). This NICD fragment translocates to the nucleus where it binds to the RBPJ transcription factor, which is the primary transcriptional mediator of NOTCH signalling. The binding of NICD to the RBPJ releases the repressor transcriptional activity of RBPJ. The NICD-RBPJ complex in association with others proteins binds to defined DNA binding regions (CGTGGGAA motif) and activates NOTCH target genes (Andersson et al., 2011). The bHLH transcription factors of the HES (Hairy and enhancer of split) and HEY (Hairy/enhancer-of-split related with YRPW motif) families are known to be primary and direct target genes of NOTCH. HES and HEY proteins are transcriptional repressors and have been shown to repress cell differentiation and determine cell fate (Andersson et al., 2011). However, transcriptomes performed in different cell types upon forced NOTCH activation, indicated a diversity in the immediate downstream NOTCH response (Andersson et al., 2011). Consequently, there is no universal NOTCH target gene and they vary in different cell contexts (Andersson et al., 2011). In mouse *Hes1*, *Hey1* and *HeyL* are relatively highly expressed in Pax7+ foetal muscle progenitors versus differentiated muscle cells (Mourikis et al., 2012a). However, *HeyL* is one of the major responding genes to NICD in mouse foetal muscle cells (Mourikis et al., 2012a). In mouse, Hes and Hey proteins repressed the transcription of differentiation muscle genes (Bröhl et al., 2012; Buas and Kadesch, 2010), to maintain muscle cells in a progenitor state. In mouse limbs, Hes1 has been shown to directly repress the transcription of the cell cycle exit gene $p57^{kip2}$ (Zalc et al., 2014). In double $Hey1^{-/-}/HeyL^{-/-}$mutant mice it was shown that Hey1 and HeyL are essential for generation and maintenance of the satellite cell number (Fukada et al., 2011). *HES1* and *HEYL* are thus considered as primary target genes of NOTCH activity.

BMPs (Bone Morphogenetic Proteins) were first identified as potent bone inducer. Since then, BMPs have been shown to be involved in numerous cell processes during development (Massagué, 1998). Similarly to NOTCH signalling pathway, BMP ligands have been shown to positively regulate the number of foetal and adult muscle progenitors in chick, mouse and zebrafish models (Friedrichs et al., 2011; Ono et al., 2010; Patterson et al., 2010; Wang et al., 2010). However, the BMP effect on foetal muscle differentiation is different

from that of the NOTCH signalling. NOTCH inhibited muscle differentiation, while BMP did not inhibit muscle differentiation in embryos (Delfini et al., 2000; Mourikis et al., 2012a; Wang et al., 2010). BMP gain-of-function experiments increased the number of foetal muscle fibres, while inhibition of BMP signalling led to small muscles during foetal myogenesis in chick limbs (Wang et al., 2010). BMPs belong to the TGF-β (Transforming growth factor-beta) family of cytokines. The canonical BMP signalling involves BMP ligands, two types of receptors (Type I and Type II) and the signal transducers SMAD proteins. Upon ligand activation, the BMP receptor phosphorylates the receptor-regulated SMADs (R-SMADs), including SMAD1, SMAD5 and SMAD8. Activated R-SMADs interact with the common mediator (co-SMAD) SMAD4 and accumulate into the nucleus where the SMAD complex binds to defined DNA sequences (GGAGCC motif) to regulate target gene transcription. SMAD complex associates with other factors to activate gene transcription (Massagué et al., 1998). The ID (inhibitor of differentiation) proteins are the best recognized BMP/SMAD1/5/8 primary response (Miyazawa et al., 2002). *ID1*, *ID2*, *ID3* and *NOG* have been shown to be direct target genes of SMAD1/5 in human endothelial cells (Morikawa et al., 2011). These helix-loop-helix (HLH) ID proteins that lack DNA-binding domains are known to negatively regulate cell differentiation and stimulate cell cycle progression during neurogenesis, osteogenesis or stem cell maintenance (Lasorella et al., 2014). In muscle, ID proteins have been shown to negatively regulate muscle differentiation in cultured muscle cells (Melnikova et al., 1999). ID proteins bind directly to MYOD and thus repress MYOD activity (Benezra et al., 1990).

NOTCH and BMP signalling pathways convert extracellular information into specific transcriptional response in the nucleus to modify gene transcription in muscle cells. It is now recognized that BMP and NOTCH signalling pathways exhibit crosstalk or share points of intersection. Synergistic or antagonist interactions have been highlighted in numerous developmental, differentiation or pathological processes (Blokzijl, 2003; Carlson et al., 2008; Dahlqvist, 2003; Grishina et al., 2005; Kennard et al., 2008). However, in myogenesis these two pathways have been mostly studied independently. In the present manuscript, we analysed the interplay between NOTCH and BMP signalling pathways in foetal myogenesis using chick *in vivo* and *in vitro* systems. We found that the interplay between NOTCH and BMP signalling pathways is different in proliferating and differentiating foetal muscle cells. These interactions are consistent with the endogenous patterns of activity of both pathways and with their respective functions in foetal muscle proliferation and differentiation.

# Results

## 1 - The interplay between NOTCH and BMP signalling pathways is different during proliferation and differentiation during foetal myogenesis

### Forced-NOTCH activity increased the number of PAX7+ foetal progenitors in chick limbs without activating BMP signalling pathway

DELTA1/RCAS overexpression in chick limbs has been shown to activate NOTCH signalling and block muscle differentiation, while increasing the number of PAX7+ progenitors in chick limbs (Bonnet et al., 2010; Delfini et al., 2000; Havis et al., 2012; Esteves de Lima et al., in preparation). BMP signalling also increased the number of PAX7+ cells during foetal myogenesis (Wang et al., 2010). In order to define whether forced-activation of NOTCH activity induced BMP signalling, we examined pSMAD1/5/8 expression in PAX7+ cells after DELTA1/RCAS overexpression. We observed that pSMAD1/5/8 was not increased in PAX7+ cells upon NOTCH activation (Figure 1A-J). *ID2* has also been reported as a primary BMP/SMAD1/5 target gene (Morikawa et al., 2011). During chick foetal myogenesis, *ID2* was expressed in close association with BMP4 expression sites (Figure S1A-D). *ID2* expression was also upregulated and downregulated in BMP gain- and loss- of function experiments, respectively, in chick embryos (Figure S1E-K), indicating that *ID2* expression is a readout of BMP activity. Consistent with the absence of increase of pSMAD1/5/8, DELTA1-activated NOTCH did not modify *ID2* expression in chick limbs (Figure 1K-M). We conclude that forced-NOTCH activity increased the number of PAX7+ cells without activating BMP signalling pathway, in chick limbs.

### NOTCH gain- and loss-of-function experiments modified muscle gene expression in proliferating foetal myoblasts, without modifying the activity of BMP signalling pathway

In order to dissociate proliferation to differentiation processes, we turned to an *in vitro* system of chick foetal myoblasts. Chick foetal myoblasts cultured in proliferation conditions displayed *PAX7, MYF5, MYOD* and *MYOG* transcript expression (Figure S2). At the

transcript levels, *PAX7* and *MYF5* expression levels were downregulated upon differentiation (Figure S2). DELTA1-activated NOTCH in chick foetal myoblasts did not modify the proportion of the different phases of cell cycle (Fig 2A,B, Fig S3). DELTA1-activated NOTCH in chick foetal myoblasts led to an increase of *PAX7* and *MYF5* expression levels, while inhibiting *MYOD* and *MYOG* expression levels compared to control cultures (Figure 2D). These changes of mRNA levels in proliferating myoblast cultures are consistent with the maintenance of *PAX7* and *MYF5* expression and the inhibition of *MYOD* and *MYOG* observed in chick limbs after DELTA1/RCAS overexpression (Delfini et al. 2000; Bonnet et al., 2010; Havis et al., 2012). NOTCH activity was assessed with the expression of the primary NOTCH target genes, *HES4* (equivalent of *Hes1* in mouse) and *HEYL*, which both have been shown to be upregulated in muscle cells upon activation of NOTCH (NICD) during mouse foetal myogenesis (Mourikis et al., 2012a). DELTA1/RCAS induced a massive increase of *HEYL* expression and modest increase of *HES4* expression in chick proliferating foetal myoblasts compared to control myoblasts (Figure 2D). This is consistent with the mouse limb situation where *HeyL* and not *Hes1* was the major responding gene to NICD in foetal muscle cells (Mourikis et al., 2012a). NOTCH activation did not induce any increase in the expression levels of the primary BMP target genes, *ID1*, *ID2* and *NOG* in proliferating foetal myoblast cultures (Figure 2D), consistent with the *in vivo* situation (Figure 1). We conclude that DELTA1-activated NOTCH induced the expected changes in muscle gene expression, while not affecting the BMP signalling, in proliferating myoblast cultures.

Blocking NOTCH signalling using the DAPT chemical inhibitor induced a dramatic loss of NOTCH activity assessed by the decrease of *HEYL* expression level compared to control cultures (Figure 2E). NOTCH inhibition led to the expected changes for muscle gene expression, *i.e.* a concomitant decrease of the *PAX7* and *MYF5* and increase of *MYOD* and *MYOG* mRNA levels in proliferating foetal myoblasts (Figure 2E). Inhibition of NOTCH activity did not modify the expression levels of BMP readout genes, *ID1*, *ID2* and *NOG* (Figure 2E), indicating that BMP signalling was not modified in the absence of NOTCH function in proliferating foetal myoblasts.

Although BMP signalling did not appear to be modified upon NOTCH gain- and loss-of-function experiments, we blocked BMP activity, using the BMP inhibitor LDN, in DELTA1/RCAS-treated myoblasts. We observed that the block of BMP activity did not significantly modify the expression levels of *PAX7*, *MYF5* and *MYOD* genes compared to

DELTA1/RCAS infected myoblasts (Figure S4). This showed that the loss of BMP activity had no effect on the ability of NOTCH signalling to increase *PAX7* and *MYF5* expression.

We conclude that NOTCH gain- and loss-of-function experiments in proliferating myoblasts affect muscle gene expression, independently of BMP activity.

**Forced-BMP activity increased the expression of NOTCH target genes in foetal limbs and in proliferating foetal myoblasts, while block of BMP had no effect on NOTCH activity**

We showed that NOTCH modified the number of foetal muscle progenitors in limbs and *PAX7* and *MYF5* expression levels in foetal myoblasts, independently of BMP activity (Figures 1, 2). In order to analyse the participation of NOTCH signalling in the BMP effect on foetal myogenesis, we analysed the expression of NOTCH readout genes after BMP4/RCAS expression in chick limbs. BMP4/RCAS-infected chick limbs induced ectopic BMP activity assessed with the increase of pSMAD1/5/8 in foetal myoblasts (Wang et al., 2010) and the ectopic expression of the primary BMP target gene, *ID2*, compared to control limbs (Fig. S1G-J). We observed that ectopic BMP4 increased the expression of the *NOTCH1* receptor in foetal muscles in chick limbs, compared to control limbs (Figure 3A-C). *JAG2* was not affected in BMP4/RCAS-infected foetal muscles, (Figure 3D, E). We concluded that forced-BMP activity increased *NOTCH1* expression in foetal limbs.

In chick foetal myoblasts, retroviral BMP4/RCAS expression increased the transcript levels of the primary BMP target genes, *ID1*, *ID2* and *NOG* compared to control cultures (Figure 4). Upon BMP-forced expression, *PAX7* and *MYF5* expression was upregulated (Figure 4D). This is consistent with the increase in the number of PAX7+ progenitors observed in BMP4/RCAS limbs (Wang et al., 2010). We observed that BMP4/RCAS activated the relative mRNA levels of the transcriptional readout of NOTCH activity, *HEYL* compared to control cultures (Figure 4D). We conclude that BMP forced-activity increased NOTCH activity in proliferating myoblasts. Conversely, blocking BMP activity following SMAD6/RCAS overexpression decreased the relative expression levels of *PAX7* and *MYF5* genes in foetal myoblasts compared to control cultures (Figure 4E). The decrease of *PAX7* and *MYF5* expression upon BMP inhibition in foetal myoblast cultures is consistent with the decrease in the number of PAX7+ foetal muscle progenitors in BMP loss-of-function

experiments in chick limbs (Wang et al., 2010). Consistent with the blockade of BMP activity, SMAD6/RCAS-infected myoblasts displayed a decrease of the relative mRNA levels of BMP target genes, *ID1*, *ID2* and *NOG* versus control cultures (Figure 4E). However, inhibition of BMP activity did not modify the expression of the transcriptional readout of NOTCH activity, *HEYL* (Figure 4E). Using the Fucci system, we observed that BMP gain-of-function experiments did not modify the proportion of the phases of the cell cycle, while BMP loss-of-function experiments increased the number of cells in G1 phase compared to control cultures (Figure 4, Figure S3).

We conclude that BMP gain-of-function experiments increased NOTCH activity in limb foetal muscles and in foetal myoblasts cultured in proliferation conditions.

**Forced-BMP activity increased the relative expression levels of *PAX7* and *MYF5* genes independently of NOTCH activity in proliferating foetal myoblasts**

The upregulation of NOTCH target genes following BMP gain-of-function experiments (Figure 3, 4) suggested that NOTCH could mediate the positive effect of BMP on foetal muscle progenitors. In order to determine whether the increase of NOTCH activity was required for the BMP effect on *PAX7* and *MYF5* expression, we blocked NOTCH activity in BMP gain-of-function experiments in foetal myoblast cultures. Inhibition of NOTCH activity (using the DAPT inhibitor) did not modify the BMP effect on *PAX7* and *MYF5* expression nor the BMP effect on *ID1*, *ID2* and *ID3* (Figure 5). The drastic diminution of *HEYL* expression assessed the blockade of NOTCH activity with the DAPT inhibitor. This result indicated that active NOTCH was not required for the positive BMP effect on *PAX7* and *MYF5* expression in proliferative foetal myoblasts. However, we observed that the inhibition of NOTCH activity increased *MYOD* and *MYOG* expression in BMP4/RCAS-infected cultures when compared to BMP4/RCAS-infected myoblasts (Figure 5). This effect is consistent with the increase of *MYOD* and *MYOG* expression observed when NOTCH activity is blocked (Figure 2). We conclude that BMP-forced activity increased *PAX7* and *MYF5* gene expression independently of NOTCH activity in proliferating foetal myoblasts.

**NOTCH gain-of-function experiments inhibit BMP target genes in foetal myoblasts cultured in differentiation conditions**

NOTCH and BMP signalling pathways positively regulate the foetal progenitor pool, independently of each other. We next investigated putative crosstalk between these two signalling pathways in foetal myoblasts cultured in differentiated conditions. NOTCH-forced activation blocked muscle differentiation assessed by the inhibition of myotube formation (Figure 6A, B) and the dramatic loss of *MYOD* and *MYOG* expression (Figure 6C) in foetal myoblasts cultured in differentiation conditions. This result observed in foetal myoblasts is fully consistent with the inhibition of *MYOD, MYOG* and myosin expression in DELTA1/RCAS infected chick limbs (Delfini et al., 2000; Bonnet et al., 2010). Upon DELTA1/RCAS overexpression, the relative expression of the primary NOTCH target genes, *HES4* and *HEYL* was upregulated in differentiated myoblasts compared to control cultures (Figure 6C). In this experimental design, we observed that the transcriptional readout of BMP activity, *ID1*, *ID2* and *NOG* expression, was downregulated compared to control cultures (Figure 6C). We conclude that the inhibition of myogenic differentiation upon NOTCH activation is concomitant with a downregulation of BMP activity. However, the blockade of NOTCH activity with the DAPT inhibitor did not modify BMP activity or muscle gene expression (Figure 6D).

**BMP gain- and loss-of-function experiments modified NOTCH activity in foetal myoblasts cultured in differentiation conditions**

NOTCH-forced activity inhibited BMP signalling (Figure 6). We next determined whether BMP gain- and loss-of-function experiments would affect NOTCH activity in foetal myoblasts cultured in differentiation conditions. BMP4/RCAS-infected myoblasts cultured in differentiation conditions led to a decrease in *MYF5, MYOD* and *MYOG* expression and to an inhibition of myotube formation compared to control cultures (Figure 7). This result in myoblast cultures is consistent with previous *in vitro* studies (Friedrichs et al., 2011; Katagiri et al., 1994; Ono et al., 2010) but is somehow different to the foetal muscle phenotype observed in BMP4/RCAS-infected limbs that display increased muscle fibre number (Wang et al., 2010). One likely explanation for the difference of the BMP4 effect on muscle differentiation between *in vitro* and *in vivo* situations could be the upregulation of *NOG* expression, a potent inhibitor of muscle differentiation (Wang et al., 2010), observed in

BMP4/RCAS-infected myoblasts (Figure 7D) and not in BMP4/RCAS-infected limbs. (Figure S5). Nevertheless, NOTCH activity assessed by *HES4* and *HEYL* expression was downregulated in BMP4/RCAS-infected myoblasts cultured in differentiation conditions (Figure 7D). Conversely, in BMP loss-of-function experiments (following SMAD6 overexpression) we observed an upregulation of NOTCH activity assessed by *HES4* and *HEYL* expression. BMP gain- and loss-of-function experiments led to *JAG2* downregulation and upregulation, respectively, consistent with the expression of *HES4* and *HEYL* (Figure 7E). SMAD6/RCAS-infected myoblasts cultured in differentiation conditions led to an inhibition of muscle markers expression (Figure 7E) and myotube formation (Figure 7C), which is consistent with the inhibition of foetal myogenesis observed in NOGGIN/RCAS-infected limbs (Wang et al., 2010).

We conclude that NOTCH signalling is inhibited upon BMP-forced activation and increased upon BMP blockade in foetal myoblasts cultured in differentiation conditions.

# Figures

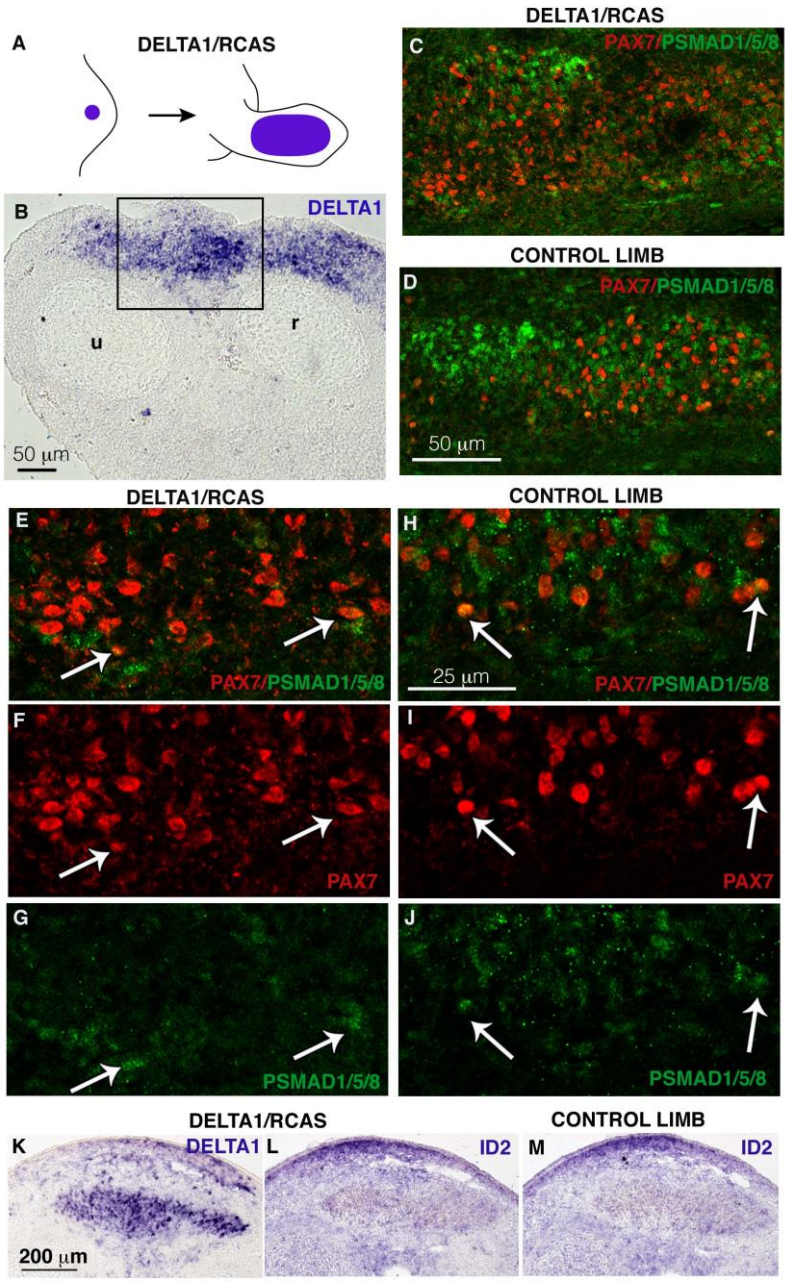



Figure 1

**Figure 1 - DELTA1-activated NOTCH did not activate BMP signalling in PAX7+ fœtal progenitors in chick limbs.** (A) DELTA1/RCAS expressing cells were grafted into the presumptive right forelimbs of E3.5 chick embryos. The grafted embryos were fixed three days later at E6.5. Transverse sections from grafted (B,C,E,F,G) and control (D,H,I,J) limbs were analysed at the same proximo-distal levels in order to allow comparison. Transverse

sections of grafted limbs were hybridized with *DELTA1* probe to visualise the virus spread (B). Immunohistochemistry was performed with the PAX7 and PSMAD1/5/8 antibodies on DELTA1/RCAS infected limbs (C,E,F,G) and compared with control limbs of the same embryos (D,H,I,J). PSMAD1/5/8 expression was not activated in PAX7+ cells in DELTA1-activated NOTCH limbs. *In situ* hybridization with *ID2* probe was performed on DELTA1/RCAS infected limbs (K,L) and compared to control limbs (M). *ID2* expression was not activated in DELTA1-activated NOTCH limbs.

**Figure 2 - NOTCH gain- and loss-of-function experiments in proliferating foetal myoblasts did not modify BMP signalling.** (A) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7, MYF5, MYOD* and *MYOG*), components of the NOTCH pathway (*HES4, HEYL, NOTCH1, JAG2*) and components of the BMP pathway (*ID1, ID2, NOG*) in DELTA1/RCAS-infected myoblasts cultured in proliferation condition. (B) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7, MYF5, MYOD* and *MYOG*), components of the NOTCH pathway (*HES4, HEYL, NOTCH1, JAG2, DLL1*) and components of the BMP pathway (*ID1, ID2, NOG*) in DAPT-treated myoblasts cultured in proliferation condition. For each gene, the mRNA levels of control culture were normalised to 1. *$p<0.05$; ** $p<0.01$; *** $p<0.001$; Error bars indicate s.e.m. (C-E) Representative fields of chick foetal myoblasts infected with Fucci/RCAS as control (C), with Fucci/RCAS and DELTA1/RCAS (D) or with Fucci/RCAS and DAPT (E), showing merged pictures of phase contrast, Fucci red (G1 phase) and Fucci green (S/G2/M phases) cells.

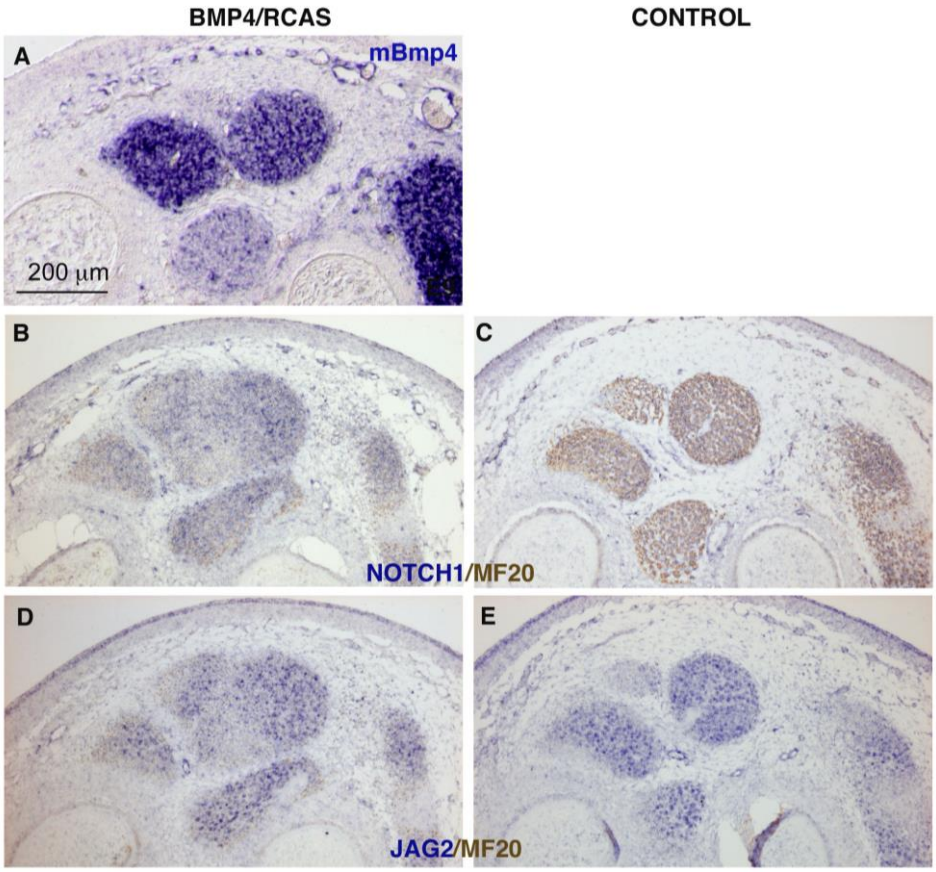



Figure 3

**Figure 3 - Ectopic BMP activated the expression of components of the NOTCH pathway in foetal muscles in chick limbs.** (A-E) BMP4/RCAS expressing cells were grafted into the presumptive right forelimbs of E5.5 chick embryos. The grafted embryos were fixed four days later at E9.5. Sections from grafted (A,B,D) and control (C,E) limbs were analysed at the same proximo-distal level in order to allow comparison. Transverse sections of grafted limbs were hybridized with mouse BMP4 probe to visualise the virus spread (A), with chick NOTCH1 (B) and JAG2 (D) probes. Transverse sections of control limbs hybridized with chick NOTCH1 (C) and JAG2 (E) probes. *NOTCH1* was activated in BMP4/RCAS-infected limbs. Scale bar, 200 µm.

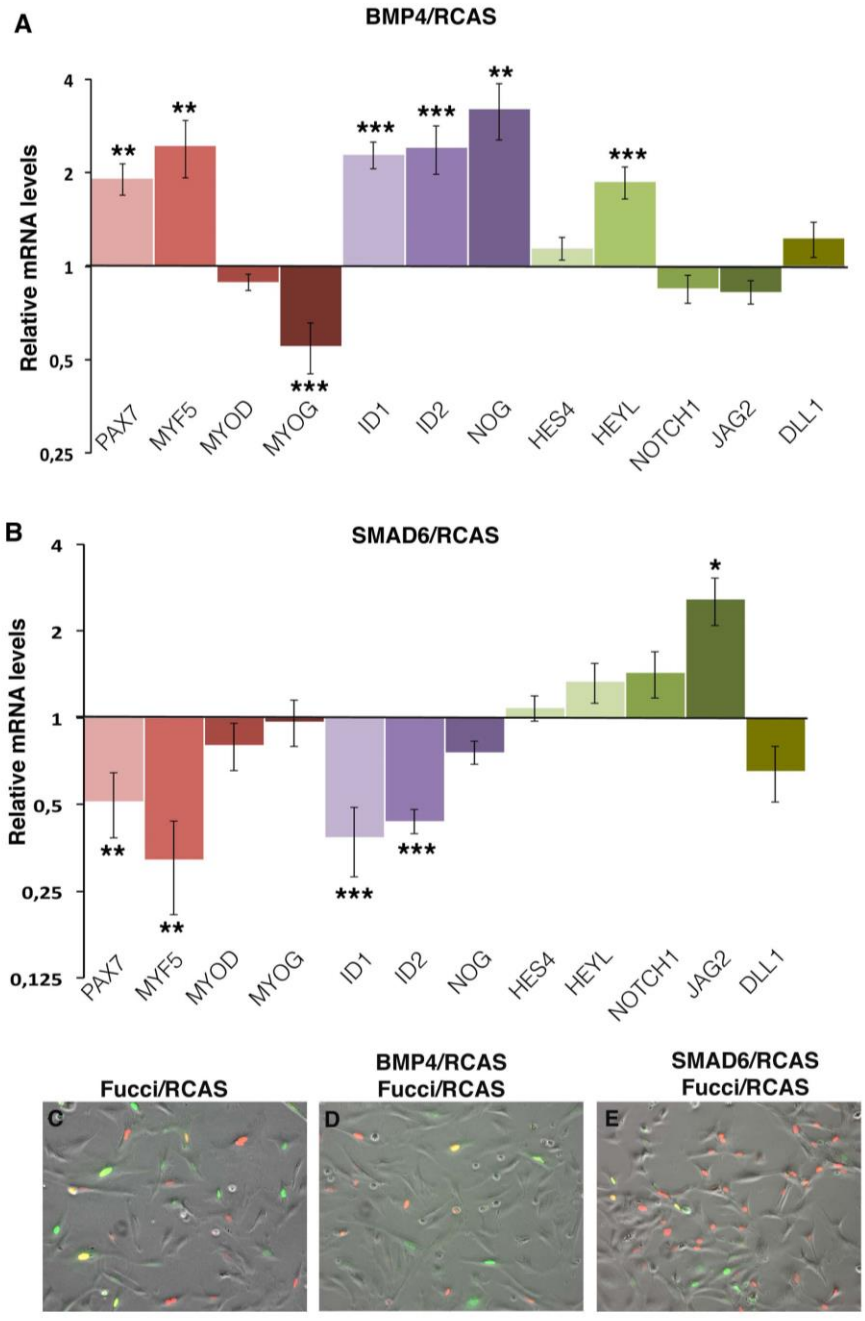



Figure 4

**Figure 4 - NOTCH activity following BMP gain- and loss-of-function experiments in chick foetal myoblasts cultured in proliferation conditions.** (A) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7*, *MYF5*, *MYOD* and *MYOG*), components of the BMP pathway (*ID1*, *ID2*, *NOG*) and components of the NOTCH pathway (*HES4*, *HEYL*, *NOTCH1*, *JAG2*, *DLL1*) in mBMP4/RCAS infected myoblasts cultured in proliferation condition. (B) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7*, *MYF5*, *MYOD* and *MYOG*), components of the BMP pathway (*ID1*, *ID2*, *NOG*) and components of

the NOTCH pathway (*HES4, HEYL, NOTCH1, JAG2, DLL1*) in SMAD6/RCAS-infected myoblasts cultured in proliferation condition. For each gene, the mRNA levels of control culture were normalised to 1. *p<0.05; ** p<0.01; *** p<0.001; Error bars indicate s.e.m. Representative fields of chick foetal myoblasts infected with Fucci/RCAS as control (C), with mBMP4/RCAS and Fucci/RCAS (D) or with Smad6/RCAS and Fucci/RCAS (E), showing merged pictures of phase contrast, Fucci red (G1 phase) and Fucci green (S/G2/M phases) cells.

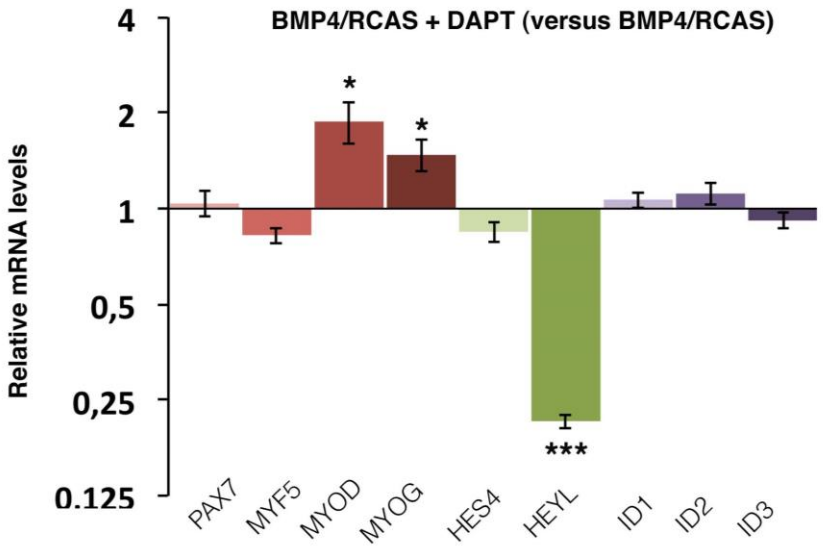



Figure 5

**Figure 5 - Inhibition of NOTCH signalling pathway did not affect the BMP positive effect on *PAX7* and *MYF5* expression in proliferating foetal myoblasts.** (A) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7*, *MYF5*, *MYOD* and *MYOG*), primary targets of the BMP pathway (*ID1*, *ID2*, *ID3*) and primary targets of NOTCH pathway (*HES4*, *HEYL*) in mBMP4/RCAS-infected myoblasts treated or not treated with the DAPT inhibitor and cultured in proliferation conditions. For each gene, the mRNA levels of mBMP4/RCAS-infected myoblast culture were normalised to 1. *p<0.05; ** p<0.01; *** p<0.001; Error bars indicate s.e.m.

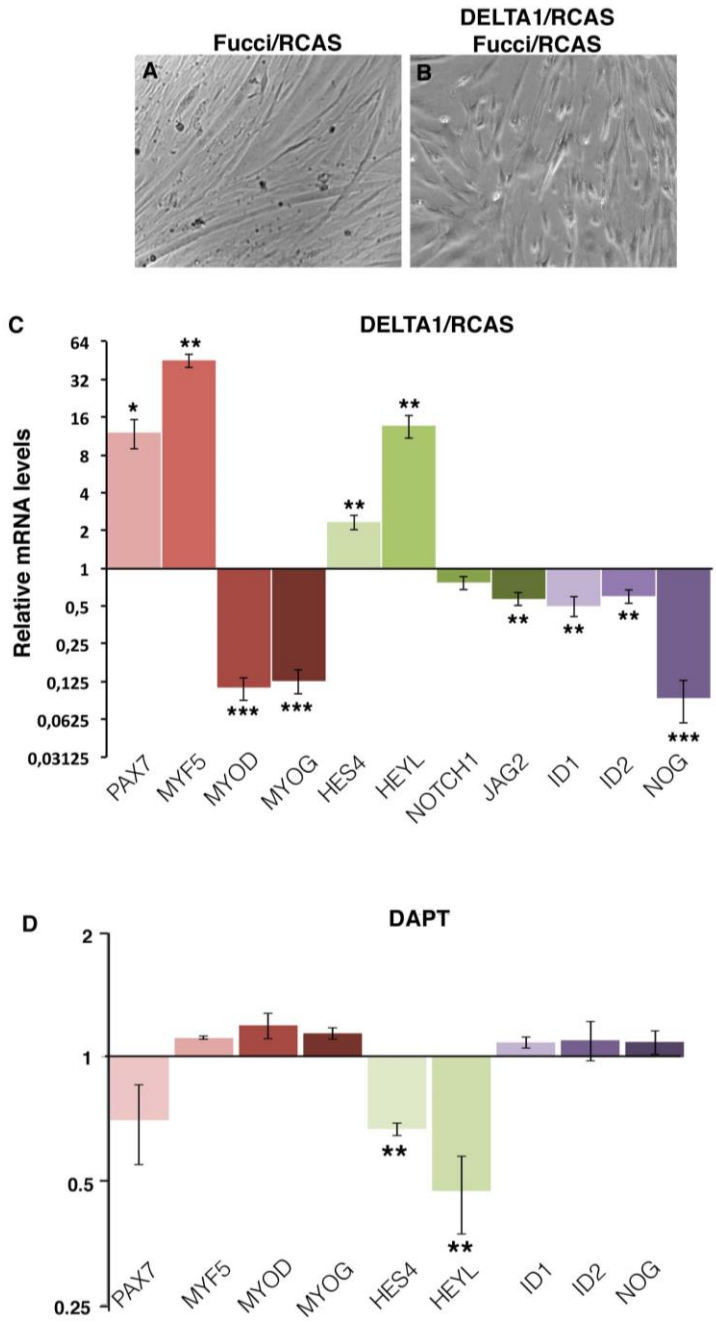



Figure 6

**Figure 6 - DELTA1-induced NOTCH decreases BMP activity in foetal myoblasts cultured in differentiation conditions.** (A,C) Representative fields showing phase contrast of chick foetal myoblasts infected with Fucci/RCAS as control (B) or with DELTA1/RCAS and Fucci/RCAS cultured in differentiation conditions. (C) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7*, *MYF5*, *MYOD* and *MYOG*), components of the NOTCH pathway (*HES4*, *HEYL*, *NOTCH1*, *JAG2*) and primary genes of the BMP pathway

(*ID1, ID2, NOG*) in DELTA1/RCAS-infected myoblasts cultured in differentiation conditions. For each gene, the mRNA levels of Fucci/RCAS-infected myoblasts (control) were normalised to 1. (D) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7*, *MYF5*, *MYOD* and *MYOG*), primary targets of the NOTCH pathway (*HES4*, *HEYL*) and primary genes of the BMP pathway (*ID1, ID2, NOG*) in DAPT-treated myoblasts cultured in differentiation conditions. For each gene, the mRNA levels of control myoblasts were normalised to 1. *p<0.05; ** p<0.01; *** p<0.001; Error bars indicate s.e.m.

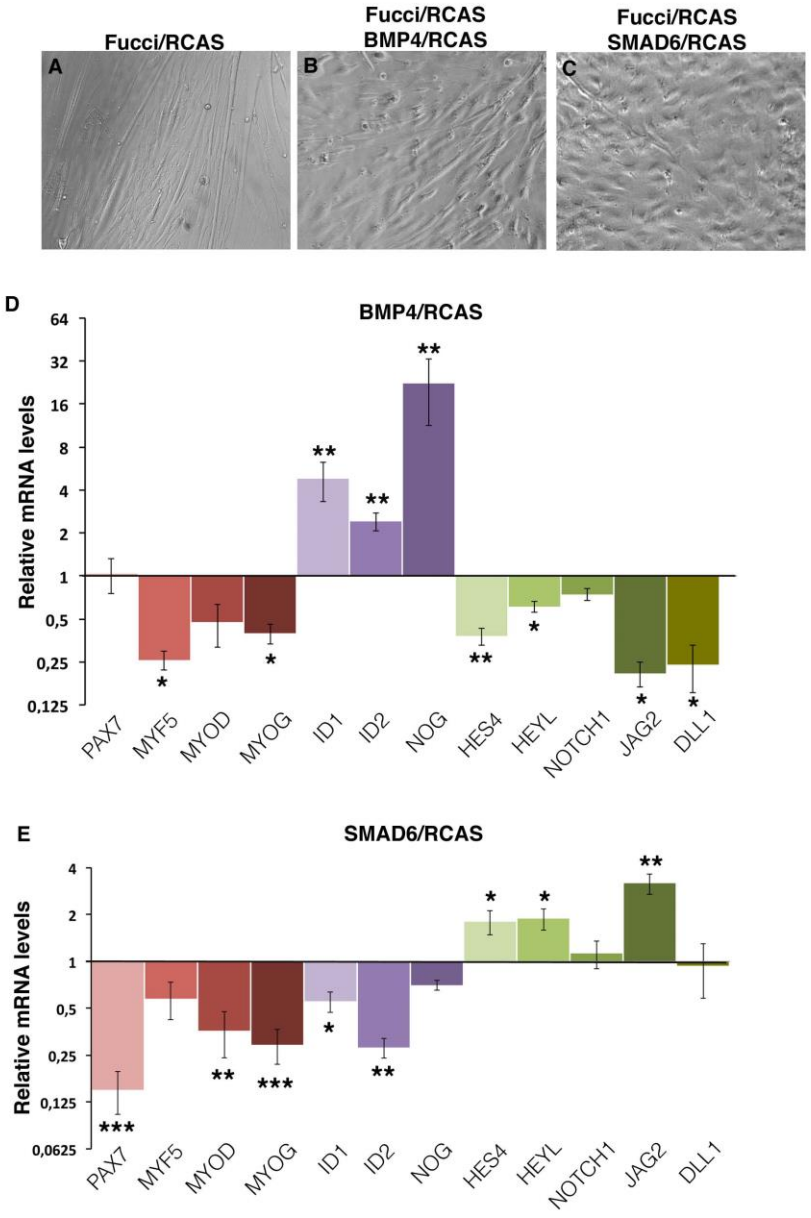



Figure 7

**Figure 7- NOTCH activity in BMP gain- and loss-of-function experiments in foetal myoblasts cultured in differentiation conditions.** (A-C) Representative fields in phase contrast of chick foetal myoblasts infected with Fucci/RCAS as control (A), with BMP4/RCAS and with Fucci/RCAS (B) or with Smad6/RCAS and Fucci/RCAS (C), and cultured in differentiation conditions. (D) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7*, *MYF5*, *MYOD* and *MYOG*), primary BMP target genes (*ID1*, *ID2*, *NOG*) and components of the NOTCH pathway (*HES4*, *HEYL*, *NOTCH1*, *JAG2*, *DLL1*) in BMP4/RCAS-infected myoblasts cultured in differentiation conditions. For each gene, the

mRNA levels of Fucci/RCAS infected myoblasts were normalised to 1. (B) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7, MYF5, MYOD* and *MYOG*), components of primary BMP target genes (*ID1, ID2, NOG*) and components of the NOTCH pathway (*HES4, HEYL, NOTCH1, JAG2, DLL1*) in SMAD6/RCAS-infected myoblasts cultured in differentiation conditions. For each gene, the mRNA levels of Fucci/RCAS infected myoblasts were normalised to 1. *$p<0.05$; ** $p<0.01$; *** $p<0.001$; Error bars indicate s.e.m.

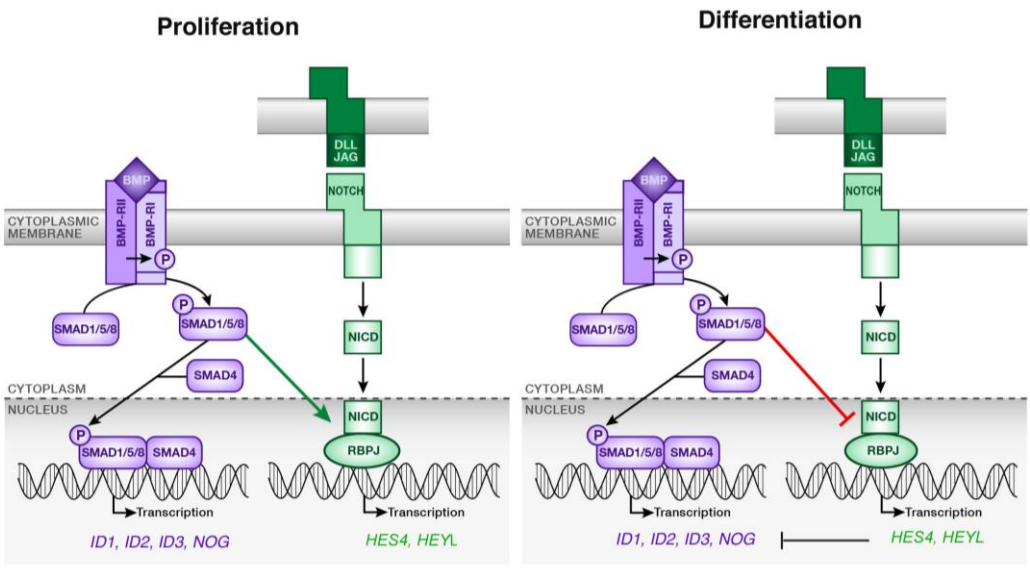



Figure 8

**Figure 8 - The interactions between BMP and NOTCH signalling pathways are different during proliferation and differentiation in foetal myoblasts.** Schematic representation of the BMP and NOTCH intracellular signalling pathways in proliferation and differentiation. In proliferation conditions, BMP activates the expression of NOTCH target genes, *HES4* and *HEYL* in chick myoblasts. In differentiation, BMP inhibits the expression of NOTCH target genes, *HES4* and *HEYL*, while inhibition of BMP activity increases the expression of NOTCH target genes, *HES4* and *HEYL*, in chick myoblasts.

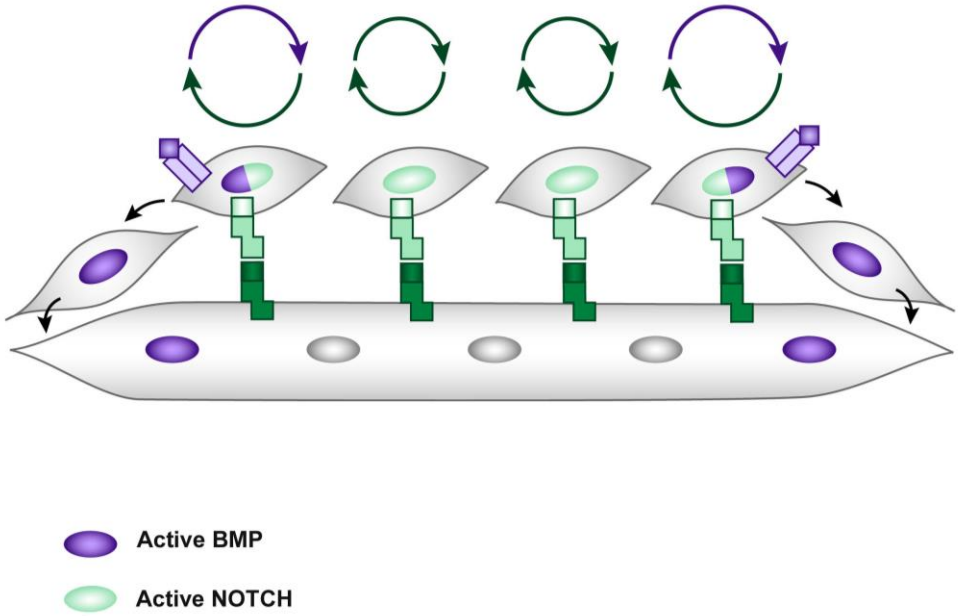



Figure 9

**Figure 9 – Interplay between BMP and NOTCH signalling pathways during foetal myogenesis**. Components of the NOTCH pathway (DELTA1, JAG2 and NOTCH1) are homogeneously distributed in chick foetal muscles. The *DELTA1* and *JAG2* ligands are expressed in differentiated muscle cells (myoblast and muscle fibres), while the *NOTCH1* receptor is excluded from differentiated muscle cells. The homogeneous production of NOTCH ligands (JAG2) from muscle fibres allows a general promotion of adjacent muscle progenitors positive for NOTCH activity. BMP signalling is restricted in chick foetal muscles, in PAX7+ muscle progenitors and in myonuclei, at muscle tips. At muscle tips, PAX7+ cells displayed active BMP and NOCTH signalling, which can possibly account to increase the proliferation of PAX7+ cells in this region.

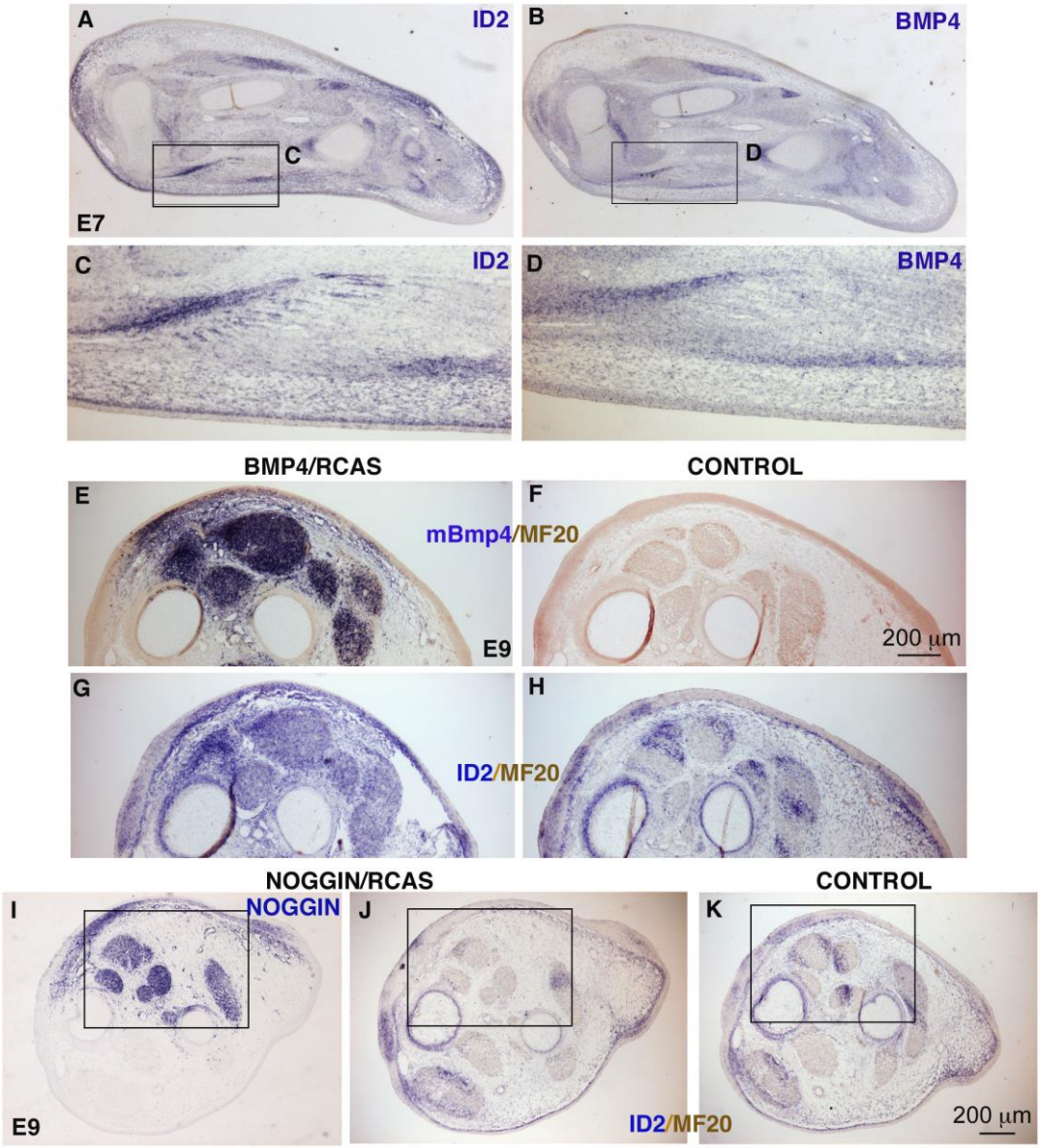



Figure S1

**Figure S1 - *ID2* is a transcriptional readout of BMP activity in chick limbs.** (A,C) *In situ* hybridization experiments showing *ID2* endogenous expression in chick foetal muscles. Longitudinal sections of limbs of E7 (A-D) chick embryos were hybridized with ID2 probe (A,C) or with the BMP4 probe (B,D). (A) is adjacent section of (B). (C,D) are high magnifications of a ventral limb muscle of (A,B). (E-H) BMP4/RCAS-infected (E,G) and contralateral (F,H) forelimbs from E9 chick embryos were cut transversely and hybridized with the mouse BMP4 probe (E,G) and with the chick ID2 probe (F,H) and then immunostained with the MF20 antibody to visualise muscles. (E,F) and (G,H) are adjacent

sections. *ID2* expression is upregulated in BMP4/RCAS limbs (G) compared to contralateral limbs (H). (I-K) NOGGIN/RCAS-infected (I,J) and contralateral (K) forelimbs from E9 chick embryos were cut transversely and hybridized with the chick NOGGIN (I) and ID2 (J,K) probes and then immunostained with the MF20 antibody to visualise muscles. (I,J) are adjacent sections. *ID2* expression is downregulated in NOGGIN/RCAS limbs (J) compared to contralateral limbs (K). The downregulation of *ID2* in NOGGIN expressing regions is squared and compared with similar regions in contralateral limbs.

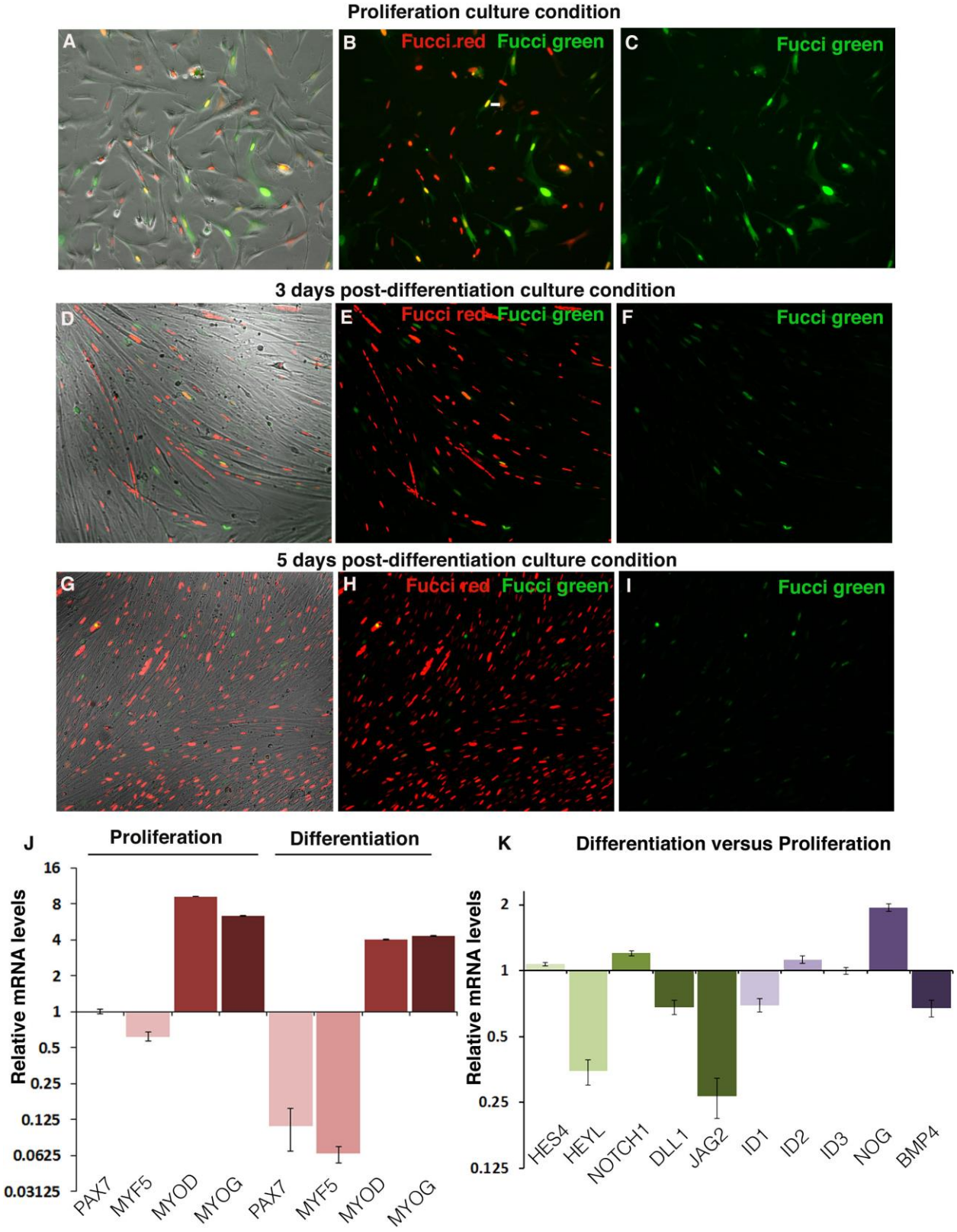

Proliferation culture condition
A
B
Fucci red
Fucci green
C
Fucci green
3 days post-differentiation culture condition
D
E
Fucci red
Fucci green
F
Fucci green
5 days post-differentiation culture condition
G
H
Fucci red
Fucci green
I
Fucci green
J
Proliferation
Differentiation
Relative mRNA levels
16
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
PAX7
MYF5
MYOD
MYOG
PAX7
MYF5
MYOD
MYOG
K
Differentiation versus Proliferation
Relative mRNA levels
2
1
0.5
0.25
0.125
HES4
HEYL
NOTCH1
DLL1
JAG2
ID1
ID2
ID3
NOG
BMP4

**Figure S2**

**Figure S2 - Foetal myoblast culture mimics foetal myogenesis *in vivo***

(A-C) Foetal myoblasts cultured in proliferation conditions. Myoblasts were transfected with Fucci/RCAS virus and left for 5 days in cultures. Representative field of proliferating myoblasts showing merged picture (A), Fucci red and Fucci green (B) and Fucci green (C).

(D-I) Foetal myoblasts cultured in differentiation conditions. Myoblasts were transfected with Fucci/RCAS virus were left in culture until cells reached confluence and then placed in differentiation medium and fixed 3 days (D-F) or 5 days (G-I) after. Representative fields of differentiated muscle cells showing merged picture (D,G), Fucci red and Fucci green (E,H) and Fucci green (F,I), 3 days (D-F) and 5 days (G-I) post differentiation.

(J) RT-q-PCR analyses of the mRNA expression levels of muscle markers (*PAX7, MYF5, MYOD* and *MYOG*) in proliferation and differentiation conditions. The mRNA levels of *PAX7* in proliferation conditions was normalised to 1, and the relative mRNA levels of the other muscle genes was calculated versus PAX7 expression in proliferating conditions. Error bars indicate s.e.m. There was a clear decrease of *PAX7* and *MYF5* expression in differentiation versus proliferation conditions. However, the relative mRNA levels of *MYOD* and *MYOG* did not change between proliferation and differentiation conditions.

(K) RT-q-PCR analyses of the relative mRNA expression levels of components of the NOTCH pathway (*HES4, HEYL, NOTCH1, DLL1, JAG2*,) and components of the BMP pathway (*ID1, ID2, NOG, BMP4*) in differentiating myoblasts versus proliferating myoblasts. The mRNA levels of each gene in proliferating myoblasts were normalised to 1. *$p<0.05$; ** $p<0.01$; *** $p<0.001$; Error bars indicate s.e.m. During the muscle differentiation process, there was a significant downregulation of the primary NOTCH target gene, *HEYL* expression levels.

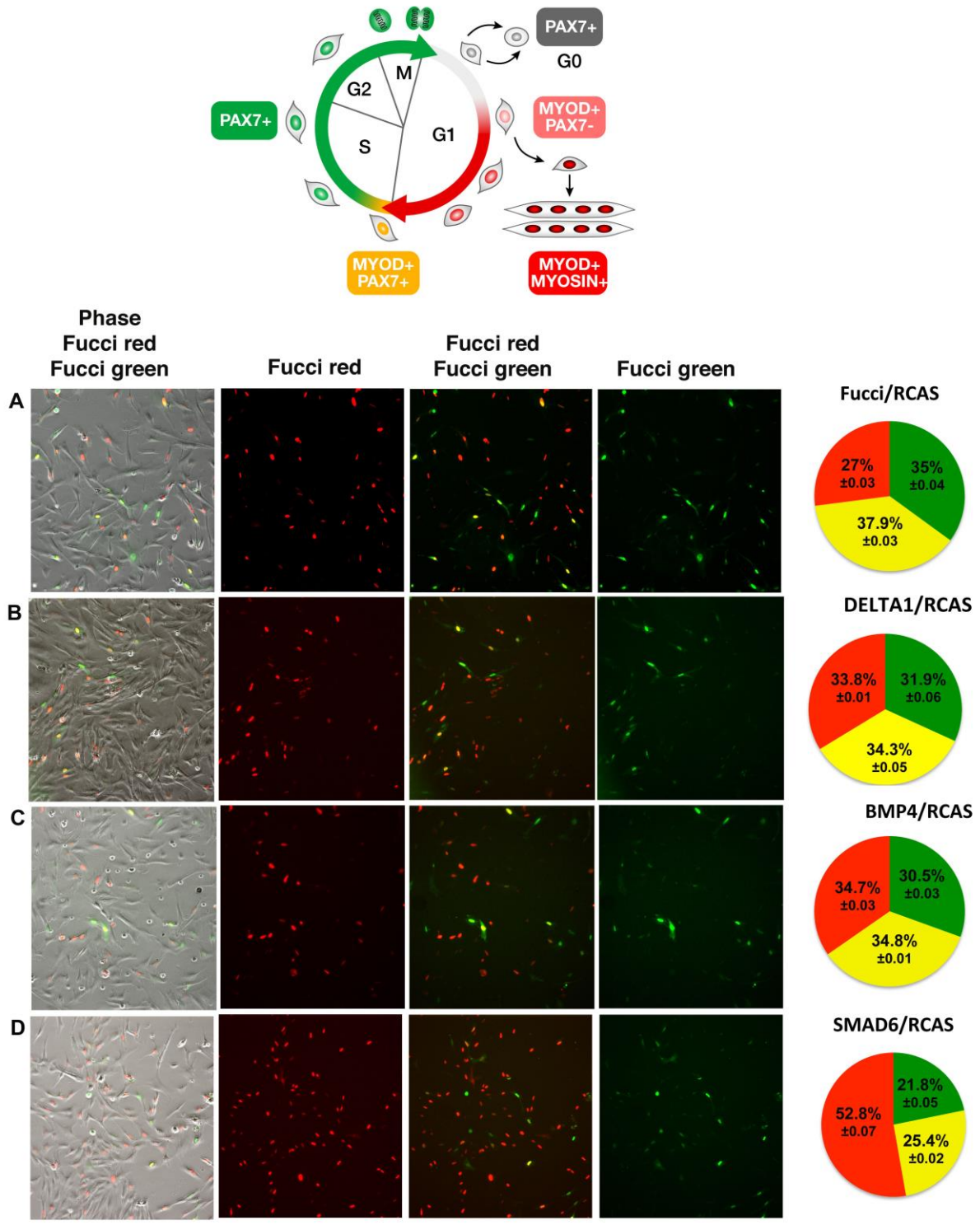



**Figure S3**

**Figure S3 - Analysis of the cell cycle phases in proliferating myoblasts using the Fucci system in NOTCH and BMP misexpression experiments.** Myoblasts were transfected with (A) Fucci/RCAS, (B) DELTA1/RCAS + Fucci/RCAS, (C) BMP4/RCAS + Fucci/RCAS or (D) SMAD6/RCAS + Fucci/RCAS, cultured in proliferation conditions and fixed before

confluence. Representative fields of proliferating myoblasts showing merged pictures, Fucci red (G1 phase), Fucci red and Fucci green (G1/S transition phases) or Fucci green (S/G2/M phases) for each condition. DELTA1-Forced NOTCH activation (B) and BMP-forced activation (C) did not change the number of cells in each of the phase of cell cycle compared to control myoblasts (A). (D) SMAD6/RCAS-infected myoblasts showed a significant increase in the number of Fucci red cells compared to control myoblasts (A). This showed that block of BMP signalling increased the proportion of muscle cells in G1 phase in chick foetal myoblast culture.

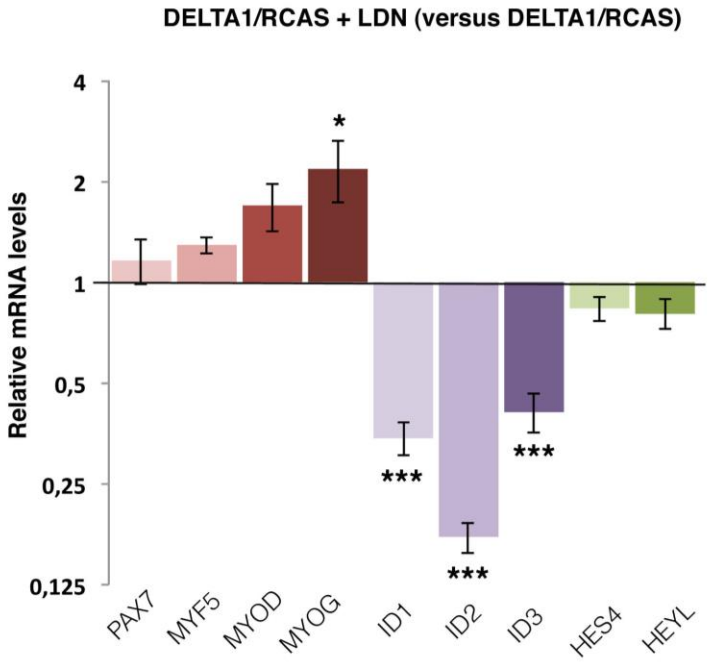



Figure S4

**Figure S4 - Inhibition of BMP signalling pathway did not affect the NOTCH effect on *PAX7* and *MYF5* expression in proliferating foetal myoblasts.** (A) RT-q-PCR analyses of the expression levels of muscle markers (*PAX7*, *MYF5*, *MYOD* and *MYOG*), primary targets of the BMP pathway (*ID1*, *ID2*, *ID3*) and primary targets of NOTCH pathway (*HES4*, *HEYL*) in DELTA1/RCAS-infected myoblasts treated or not treated with the LDN inhibitor and cultured in proliferation conditions. For each gene, the mRNA levels of DELTA1/RCAS-infected myoblasts were normalised to 1. *p<0.05; ** p<0.01; *** p<0.001; Error bars indicate s.e.m.

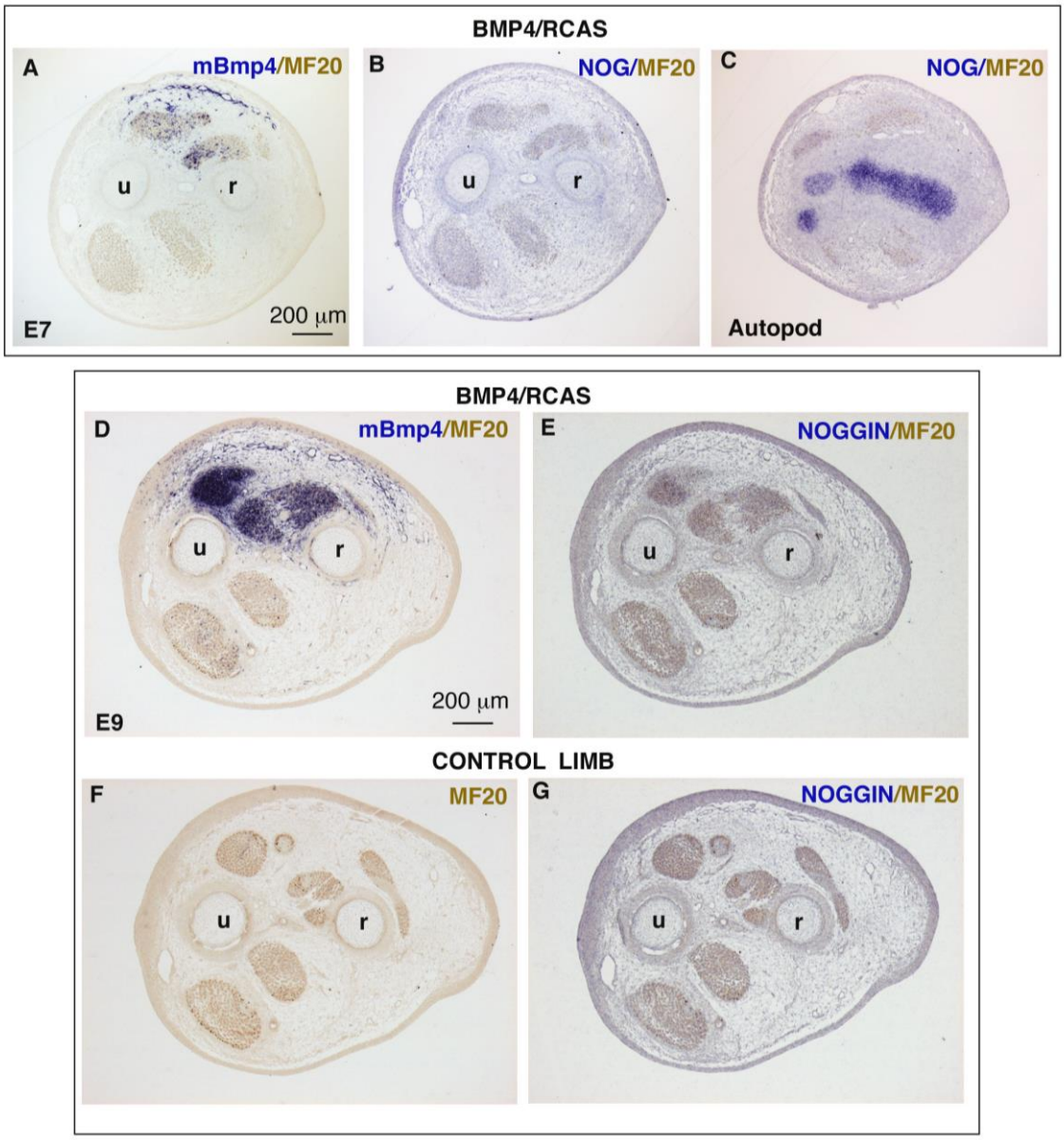



Figure S5

**Figure S5 - Ectopic BMP signalling did not induce ectopic expression of *NOG* in chick limbs**. (A-F) BMP4/RCAS-infected (A-F) and contralateral (G,H) forelimbs from E7 (A-C) and E9 (E-H) chick embryos were cut transversely and hybridized with the mouse BMP4 probe (A,E,G) and with the chick NOG probe (B,C,F,H) and then immunostained with the MF20 antibody to visualise muscles. (A,B), (E,F) and (G,H) are adjacent sections. (C) is a distal section of the BMP4/RCAS infected limbs, to show that NOG was expressed in digits. *NOG* expression was not detected in zeugopod regions (H) and not activated by ectopic BMP4 (B,F).

| | | |
|---|---|---|
| PAX7 | Fw | AGAAGAAGGCCAAGCACAGCATAG |
| | Rv | ATTCGACATCGGAGCCTTCATCCA |
| MYF5 | Fw | ACCAGAGACTCCCCAAAGTG |
| | Rv | TCGATGTACCTGATGGCGTT |
| MYOD | Fw | CGACAGCAGCTACTACACGGAAT |
| | Rv | CTCTTCCCATGCTTTGGGTC |
| MYOG | Fw | AGGCTGAAGAAGGTGAACGAAG |
| | Rv | CAGAGTGCTGCGTTTCAGAGC |
| HES4 | Fw | ATGGAAAAACCCACCGCTTC |
| | Rv | CATTTACCTTCCGGTGCTCG |
| HEYL | Fw | CCAAGCTGGAGAAGGCAGA |
| | Rv | CCAGAGCACGAGCATCCA |
| NOTCH1 | Fw | TACGACTGCACCTGTCTGC |
| | Rv | TTGTTCCCTGGACAGTCATCG |
| JAGGED2 | Fw | CAAGTGGCTGGGAAGGAGAA |
| | Rv | TGCATCGGCCACCATTATGA |
| DELTA1 | Fw | CTTCACCTGTGGAGAGCGT |
| | Rv | ATTCCCCAGGTTTGTCGCA |
| ID1 | Fw | CCGGAGGGTCTCTAAAGTGG |
| | Rv | GCAGGTCCCAGATGTAGTCG |
| Id2 | Fw | GAAGAACGGCCTTTCGGAG |
| | Rv | TCATGTTGTACAGCAGGCTCA |
| ID3 | Fw | GCTGGAGGAACCCATGAATCT |
| | Rv | TCCCGCAATTTGGAGTAGCA |
| NOGGIN | Fw | TGGATCTAATCGAGCACCCG |
| | Rv | TGAGGCTCCTTAGCAAGGTC |
| BMP4 | Fw | CTTCGTCTTCAACCTCAGCA |
| | Rv | GACAGCGGCTTCATCACT |

**Table S1**

**Table S1** - List of RT-q-PCR used in this study.

# Discussion

## 1 - The interplay between NOTCH and BMP signalling pathways is different during proliferation and differentiation during foetal myogenesis

### NOTCH and BMP are two signalling pathways that have convergent effects during muscle proliferation and divergent outcomes for muscle differentiation *in vivo* and *in vitro*

Myogenesis can be studied *in vivo* and *in vitro*. *In vitro* culture systems are very powerful to dissociate proliferation from differentiation processes. However, muscle cell cultures do not always mimic *in vivo* myogenesis. Muscle cell cultures select muscle cells that simultaneously express PAX7 and MYOD (Esteves de Lima et al., 2014). In addition there is an increase in the proportion of muscle cells that are in G1/S transition (Fucci yellow in Figure S2; Esteves de Lima et al., 2014). Foetal muscle cells expressing both PAX7 and MYOD are very rare in vivo (Relaix et al., 2005) as there is very few muscle cells in G1/S transition in foetal limbs (Esteves de Lima et al., 2014). We believe that the muscle cell population in a transient state between muscle progenitor (PAX7+) and myoblast (MYOD+) is amplified *in vitro*. These myogenic cells that are at the same time progenitors and myoblasts can, nevertheless, be used to study muscle cell proliferation.

NOTCH and BMP have a similar effect on PAX7+ foetal muscle cells *in vivo* and *in vitro*. NOTCH and BMP gain-of-function experiments in chick limbs lead to an increase in the number of PAX7+ cells compared to control limbs (Figure 1; Wang et al., 2010, Esteves de Lima et al., in preparation). This effect can also be observed *in vitro* with the increase of the expression levels of *PAX7* and *MYF5* genes upon NOTCH or BMP gain-of-function experiments in chick proliferating foetal myoblasts (Figures 2, 4). Conversely, NOTCH or BMP loss-of-function experiments lead to the opposite effect, *i.e.* a decrease in the number of PAX7+ cells in limb foetal myogenesis (Wang et al., 2010; Vasyutina et al., 2007a). Consistently with the *in vivo* experiments, the expression levels of *PAX7* and *MYF5* genes were decreased when inhibiting NOTCH or BMP activity in chick proliferating foetal myoblasts (Figures 2, 4). Despite the amplification of the PAX7+/MYOD+ cell population *in vitro*, NOTCH and BMP appeared to have consistent effects *in vitro* and *in vivo*.

Proliferating myoblasts undergo differentiation in a low serum-containing medium (Figure S2). *In vitro* foetal myotubes do not reach the *in vivo* maturation but can display spontaneous contractile activity (Spector and Prives, 1977). During the differentiation process of foetal myoblasts, the activity of the NOTCH pathway is downregulated assessed by the downregulation of *HES4* and *HEYL* transcript levels compared to proliferating myoblasts (Figure S2). This is consistent with the requirement of NOTCH inhibition to allow foetal muscle differentiation *in vivo* (Vasyutina et al. 2007a; Mourikis et al., 2012a). NOTCH gain-of-function experiments in foetal myoblasts inhibited myotube formation consistent with *in vivo* experiments. We believe that the NOTCH function on foetal muscle cells is very robust and reflects the *in vivo* situation during proliferation and differentiation. BMP gain- and loss-of-function experiments in proliferating foetal myoblasts also mimicked the *in vivo* situation. However, in differentiating myoblasts, BMP has a different effect *in vitro* and *in vivo*. This could be explained by the fact that *in vitro*, but not *in vivo*, activation of BMP increases the expression of *NOG*, an antagonist of the pathway, known to inhibit muscle differentiation.

**BMP and NOTCH interplay during foetal muscle cell proliferation**

BMP and NOTCH signalling pathways have a similar positive effect on foetal muscle progenitors. We have shown that NOTCH-induced activity does not activate BMP signalling in PAX7+ foetal muscle progenitors *in vivo* nor in proliferating foetal myoblasts. pSMAD1/5/8 and *ID2* gene expression was not activated in PAX7+ cells upon DELTA1-induced NOTCH activation. The expression of primary BMP target genes was also not modified upon NOTCH gain- and loss-of-function experiments in foetal myoblasts. Moreover, blocking BMP did not modify the NOTCH effect on *PAX7* expression. We believe that NOTCH signalling pathway promotes foetal muscle cell proliferation independently of BMP signalling pathway. BMP-forced activity induced an increase in the expression of NOTCH target genes in chick limbs and foetal myoblasts. The ability of BMP to activate transcription of primary NOTCH target genes has been observed in numerous systems (Bai et al., 2007; Itoh et al., 2004; Moya et al., 2012; Quillien et al., 2011; Takizawa, 2003). *HEY1* and *JAG1* have been identified as direct transcriptional target genes of BMP-SMAD1/5 in human endothelial cells (Morikawa et al., 2011). Several molecular mechanisms have been described to explain NOTCH target gene activation by BMP. One mechanism described in endothelial cells is based on direct interaction of activated SMADs (R-SMAD1/5 and

SMAD4) with NICD to induce transcription of primary NOTCH target genes such as *Hes1* and *Hey1* (Larrivée et al., 2012; Li et al., 2011; Moya et al., 2012). Another mechanism has been described in neurogenesis, where the direct binding of ID to HES1 released the negative auto-regulation of HES1 on its own promoter, which is accompanied by an increase in *Hes1* transcript levels and activity (Bai et al., 2007). However, in proliferating chick foetal myoblasts, the inhibition of NOTCH activity did not modify the BMP effect on *PAX7* and *MYF5* genes. This suggests that BMP does not require NOTCH activity to exert its effect on *PAX7* expression.

The fact that both NOTCH and BMP have a positive effect on PAX7+ muscle cells independently of each other does not exclude a potentialisation effect of both pathways. This would be consistent with the endogenous activity of BMP and NOTCH in foetal muscles. Active BMP signalling is restricted in a subpopulation of foetal muscle progenitors at muscle tips close to tendons (Wang et al., 2010), while NOTCH activity is present in all foetal progenitors (Mourikis et al., 2012a). Consequently, at muscle tips, PAX7+ progenitors display both BMP and NOTCH activities (Figure 9). PAX7+ progenitors have been shown to be preferentially in S/G2/M phases compared to those in the middle of muscles (Esteves de Lima et al., 2014). A cooperative effect between NOTCH and BMP signalling pathways could explain the increase of muscle progenitor proliferation at muscle extremities.

**BMP and NOTCH interaction during foetal muscle differentiation**

Although NOTCH and BMP have a convergent effect to increase muscle cell proliferation, both signalling pathways have different outcomes for foetal muscle differentiation. NOTCH inhibited muscle differentiation *in vivo* (Bonnet et al., 2010; Delfini et al., 2000; Hirsinger et al., 2001; Mourikis et al., 2012a; Schuster-Gossler et al., 2007; Vasyutina et al., 2007a) and *in vitro* (Kopan et al., 1994; Kuroda et al., 1999), while BMP promoted muscle differentiation in chick foetal limbs (Wang et al., 2010). NOTCH activity is high in muscle progenitors and low in differentiated muscle cells (Vasyutina et al., 2007a; Mourikis et al., 2012a). In contrast to NOTCH, and in addition to being active in a subpopulation of muscle progenitors, BMP activity is also observed in differentiated foetal muscle cells in chick limbs (Wang et al., 2010). Consistent with the presence of BMP and absence of NOTCH activity in differentiated muscle cells *in vivo*, we observed antagonistic interactions between the two signalling pathways in myoblasts cultured in differentiation

conditions (Figures 6-8). NOTCH or BMP forced-activity inhibited primary BMP or NOTCH target genes, respectively in differentiating myoblasts.

BMP inhibited NOTCH activity in chick foetal myoblasts in differentiation conditions. Moreover, blockade of BMP activity increased the expression of NOTCH target genes. The NOTCH inhibition by BMP has been described during branching morphogenesis in the prostate gland, where BMP negatively regulated NOTCH activity (Grishina et al., 2005) and during zebrafish retinogenesis, where ID2 limited NOTCH activity (Uribe et al., 2012). The antagonism was also observed in olfactory nerve formation where BMP activity was required for neuron differentiation by inhibiting NOTCH activity (Maier et al., 2011). The function of active BMP in a subset of myonuclei at muscle tips is not known. Based on our *in vitro* data, we believe that active BMP signalling at the tips of differentiated muscle cells inhibits NOTCH signalling to lock the muscle differentiation process (Figure 9). Consistent with this idea, inhibition of BMP signalling in differentiating myoblasts released this inhibition and increased NOTCH activity.

NOTCH also inhibited the BMP activity in foetal myoblasts in differentiation conditions, suggesting that NOTCH inhibits the muscle differentiation promoting effect of BMP. The underlying molecular mechanism of BMP inhibition by NOTCH is less described; but Hey1 has been shown to promote Id protein degradation in mouse endothelial cells and antagonise BMP function (Itoh et al., 2004).

In summary, we showed that NOTCH and BMP interactions are different in proliferating and differentiating foetal muscle cells. During proliferation NOTCH and BMP have a convergent effect on muscle progenitors, but act independently of each other. During differentiation, NOTCH and BMP have an opposite effect on muscle differentiation and display antagonist interactions.

# Material and Methods

## 1 - The interplay between NOTCH and BMP signalling pathways is different during proliferation and differentiation during foetal myogenesis

### Chick embryos

Fertilized chick eggs from commercial sources (JA 57 strain, Institut de Sélection Animale, Lyon; White Leghorn from HAAS, Strasbourg) were incubated at 38.5°C. Embryos were staged according to days *in ovo*.

### Production and grafting recombinant/RCAS expressing cells

The DELTA1/RCAS, BMP4/RCAS and NOGGIN/RCAS-expressing cells were prepared for grafting as previously described by (Delfini et al., 2000; Wang et al., 2010; Esteves de Lima et al., 2014). Cell pellets of approximately 50-100µm in diameter producing DELTA1/RCAS were grafted into limb buds of E3.5 chick embryos and BMP4/RCAS or NOGGIN/RCAS-producing cells, were grafted into E5.5 chick embryos. At several times after grafting, embryos were harvested and processed for immunohistochemistry or *in situ* hybridization to tissue sections.

### Cell Culture

Chick primary myoblasts were obtained from hind limbs of E10 chick embryos, mechanically dissected into small pieces and cultured in a minimal essential medium complemented with 10% of foetal calf serum as previously described (Spitz et al., 1997; Havis et al., 2012). Chick primary myoblasts were transfected with RCAS-Delta1, RCAS-Bmp4 or RCAS-Smad6 at a confluence of 30% using the Calcium Phosphate Transfection Kit (Invitrogen), overnight at 37°C and 5% $CO_2$. Transfection with RCAS-Fucci (Esteves de Lima et al., 2014) was used as control. Cells were let in culture in proliferation conditions for at least 5 days in order to allow a virus infection of virtually 100%, and collected for RT-q-PCR analysis at a

maximum confluence of 80%. For differentiation conditions a minimal essential medium complemented with 2% of foetal calf serum (Spitz et al., 1997; Havis et al., 2012) was added to the cells when they reached confluence. Cells were kept in culture with differentiation medium for 5 days and collected for RT-q-PCR analysis. LDN-193189 (Sigma) stock solution was prepared at 10mM and was added to the cells at a final concentration of 2uM for 24h. DAPT (Sigma) stock solution was prepared at 10mM and was added to the cells at a final concentration of 5uM for 24h.

**RNA isolation, reverse transcription and quantitative real-time PCR**

Total RNAs were extracted from chick myoblast cultures. 500ng to 1µg RNAs were reverse-transcribed using the High Capacity Retrotranscription kit (Applied Biosystems). RT-q-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences used for RT-q-PCR are listed in Table S1. The relative mRNA levels were calculated using the 2^-ΔΔCt method (Livak and Schmittgen, 2001). The ΔCts were obtained from Ct normalized with GAPDH levels in each sample. For mRNA level analyses of myoblasts in each condition, 7 to 11 independent RNA samples originating from independent experiments were analysd in duplicate. Results were expressed as Standard Error of the Mean (SEM). Data were analysed by paired student *t*-test. Asterisks in figures indicate the different *p* values (* <0.05; ** <0.01 and *** <0.001).

***In situ* hybridisation to tissue sections**

Normal and grafted forelimbs were fixed in Farnoy (60% ethanol, 30% formaldehyde 40% and 10% acetic acid) and processed for *in situ* hybridization to wax tissue sections, as previously described (Wang et al., 2010). The grafted right and control left forelimbs from the same manipulated embryos were positioned in the same orientation for transverse sectioning to allow comparison. The digoxigenin-labelled mRNA probes were used as previously described: mouse BMP4, BMP4, NOG (Wang et al., 2010), DLL1 (Delfini et al., 2000), ID2 (EST clones).

**Immunohistochemistry**

Chick forelimbs were fixed with paraformaldehyde 4% overnight at 4°C and processed for cryostat sections (12 µm). Immunohistochemisry of forelimbs sections was performed as previously described (Esteves de Lima et al., 2014). Active BMP signalling was detected using the polyclonal PSMAD antibody recognizing the complex BMP-activated receptor-phosphorylated SMAD 1/5/8 (Cell Signaling). 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.

# 2 – Muscle contraction activates YAP and NOTCH signalling and thus regulates the pool of muscle progenitor cells during foetal myogenesis

# Results

## 2 – Muscle contraction activates YAP and NOTCH signalling and thus regulates the pool of muscle progenitor cells during foetal myogenesis

### Synopsis

Objectives: NOTCH signalling is a major regulator of muscle stem cells in development and in the adult. NOTCH loss-of-function in muscle progenitors leads to a precocious differentiation and depletion of the progenitor pool during development (Vasyutina et al., 2007a). The most important function of NOTCH is to supress MyoD expression and activity, which maintains the progenitor pool in an undifferentiated state (Bröhl et al., 2012). In this study, we aimed to identify upstream regulators of NOTCH during myogenesis. Mechanical forces from muscle contractions are known to regulate important aspects of the morphogenesis of the musculoskeletal system during development. Even though the influence of mechanical forces on development of bone, cartilage and joints has been intensely studied, little is known whether these forces affect muscle development and in particular the maintenance of the muscle progenitor.

Methods: We examined foetal muscle development in the absence of muscle contraction by using the neuromuscular blocking agent DMB. DMB is an agonist of acetylcholine (Ach) and acts as a depolarising agent of the muscle fibre membrane. DMB binds to nicotinic acetylcholine receptors in the neuromuscular junction which results in a rigid paralysis of skeletal muscle (Lee and Jones, 2002; Osborne et al., 2002). We used the electroporation technique (Bourgeois et al., submitted) to misexpress a dominant-negative form of DLL1 specifically in differentiated cells (MLC promoter) to block NOTCH ligands. In addition, the RCAS virus system was used to misexpress components of the NOTCH pathway in chick limbs.

Results: We demonstrated that activating NOTCH by overexpressing DLL1 increases the number of muscle progenitors and, like previously observed, decreases muscle differentiation (Delfini et al., 2000), reinforcing the idea of NOTCH positively regulates the size of the muscle progenitor pool by inhibiting differentiation.

Embryos treated with DMB displayed a decreased number of muscle progenitors (diminished *PAX7* and *MYF5*, and decreased PAX7+ cell number) and an increase in the

committed myoblast population (increased *MYOD* and *MYOG*). This mimics the effects of the NOTCH loss-of-function, and consistently, we observed a reduction of NOTCH activity in DMB-treated embryos (decreased *HEYL*, *HES5*, *NOTCH2* and *JAG2*). Moreover, forced activation of NOTCH attenuated the reduction in the number of muscle progenitors after DMB treatment. This shows that Notch signalling responds to mechanical forces and rescues the loss of muscle progenitors in immobilised embryos.

We showed that muscle fibres and not muscle progenitors are primary responding to mechanical forces. Upon immobilization of the muscle, expression of the NOTCH ligand *JAG2* is lost in foetal muscle fibres. We hypothesised that the reduction of *JAG2* in fibres would lead to a decreased NOTCH activity in associated muscle progenitors. To block NOTCH ligands expressed in the fibres, we overexpressed dominant-negative DLL1 specifically in differentiated muscle cells. This demonstrated that a decreased expression of NOTCH ligands in differentiated cells results in a decrease in the number of PAX7+ cells.

In the absence of muscle contraction, the activity of the mechanosensitive gene YAP was decreased in muscle fibres (decreased expression of the YAP target *CTGF* and loss of nuclear YAP). Since YAP/TEAD complexes bind to regulatory sequences of the *JAG1* and *JAG2* genes in other systems (Zhao et al., 2008), we hypothesised that YAP might regulate expression of *JAG2* in muscle fibres. We found that YAP binds to sequences, upstream of the *JAG2* coding sequence, which contain TEAD binding sites (CATTCC). In addition, forced YAP activity in cultures of chick foetal myoblasts increased *JAG2* expression and activated NOTCH signalling (increased expression of *HEYL*).

Conclusion and Perspectives: In this project, we showed that muscle contraction regulates the size of the muscle progenitor pool by activating NOTCH signalling. We found that the muscle fibres are primary affected by immobilisation. The fibres display decreased YAP activity, decrease in *JAG2* expression and an increased cell death. We also provide evidence that YAP directly binds regulatory sequences and regulates the transcription of *JAG2* gene.

We would like to determine whether forced YAP activity in muscle fibres suffices to rescue *JAG2* expression in fibres in the absence of mechanical forces. For this, we will electroporate a constitutively-active form of Yap (mYapS112A) in differentiated muscle cells. The mYapS112A cannot be phosphorylated at serine S112 and is therefore always located in the nucleus and continuously active. We expect that forced activation of YAP in

muscle fibres of immobilised embryos will maintain *JAG2* expression and rescue the myogenic progenitor pool.

# Results

## 2 – Muscle contraction activates YAP and NOTCH signalling and thus regulates the pool of muscle progenitor cells during foetal myogenesis


### Abstract

Muscle development, postnatal growth, homeostasis and regeneration rely on muscle stem cells. The NOTCH signalling pathway is a central molecular input controlling the maintenance of the muscle stem cells. However, the upstream mechanisms regulating NOTCH signalling are not known in the context of foetal myogenesis. We show in this study that forced NOTCH activation increases the number of foetal muscle progenitors. Inhibition of muscle contraction mimics a NOTCH loss-of-function phenotype, *i.e.* diminishes NOTCH activity and dramatically decreases the number of foetal muscle progenitors. Forced NOTCH activation prevents the diminution in the number of foetal muscle progenitors in immobilized embryos. Muscle fibres rather than muscle progenitors first respond to the loss of mechanical forces. Muscle fibres display loss of the expression of the NOTCH ligand *JAG2*, loss of YAP activity, and apoptosis in immobilized embryos. Moreover, NOTCH ligand activity in muscle fibres was required to maintain the pool of foetal muscle progenitors in chick embryos. The recruitment of YAP to regulatory regions of the *JAG2* gene in foetal limbs combined with *JAG2* activation by constitutive active YAP in foetal myoblast cultures indicate that YAP positively regulates *JAG2* expression in muscle fibres. Our results show that mechanical forces provided by muscle contractions are sensed in myonuclei by the transcriptional co-activator YAP1 that regulates the expression of the NOTCH ligand *JAG2* in muscle fibres, which in turn maintains the pool of foetal muscle progenitors.

# Introduction

## 2 – Muscle contraction activates YAP and NOTCH signalling and thus regulates the pool of muscle progenitor cells during foetal myogenesis

Skeletal muscle formation, growth and regeneration rely on muscle stem cells. A major goal of muscle research is to understand the mechanisms underlying the regulation of skeletal muscle stem cells. NOTCH is a major signalling pathway involved in the maintenance of the muscle stem cells. However, upstream regulators of NOTCH signalling have not been identified in the context of developmental myogenesis.

The paired homeobox transcription factors, PAX3 and PAX7, define the pool of muscle stem cells during developmental, postnatal and regenerative myogenesis (Gros et al., 2005; Kassar-Duchossoy, 2005; Relaix et al., 2005). In the absence of both PAX3 and PAX7, muscle development is arrested (Relaix et al., 2005). The entry into the muscle differentiation program is controlled by the muscle regulatory factors (MRFs) that are a family of bHLH (basic Helix-Loop-Helix) transcription factors composed of the specification factors MYF5, MYOD and MRF4 and the differentiation factor MYOG (Ott et al., 1991; Pownall and Emerson, 1992; Sassoon, 1993).

Skeletal muscle formation occurs in distinct but overlapping phases that include embryonic, foetal and perinatal myogenesis. Embryonic myogenesis relies on muscle progenitors identified by the expression of *PAX3* and gives rise to the first muscle fibres, while foetal myogenesis depends on *PAX7*-expressing muscle progenitors and is associated with muscle growth (Hutcheson et al., 2009; Kassar-Duchossoy, 2005; Relaix et al., 2005). By the end of foetal myogenesis, PAX7+ cells adopt a satellite cell position under the basal lamina of the muscle fibres and will contribute to perinatal muscle growth, adult muscle homeostasis and muscle repair (reviewed by Biressi et al., 2007).

The NOTCH signalling pathway is a major regulator of all skeletal muscle stem cells. NOTCH signalling has been shown to be involved in the regulation of embryonic, foetal and adult muscle stem cells (Mourikis and Tajbakhsh, 2014). Activation of the NOTCH signalling pathway requires cell-cell communication between a signal-emitting cell that expresses the NOTCH ligand and a signal-receiving cell that expresses the NOTCH receptor.

When activated by the ligand, the intracellular domain of the NOTCH receptor is cleaved and translocates into the nucleus, where together with the transcription factor RBP-J, activates the transcription of the bHLH transcriptional repressor genes, *HEY* and *HES* (reviewed by Andersson et al., 2011). Ectopic activation of NOTCH supresses myogenic differentiation in muscle cell cultures and in chick and mouse embryos (Conboy and Rando, 2002; Delfini et al., 2000; Hirsinger et al., 2001; Kopan et al., 1994; Mourikis et al., 2012a). NOTCH loss-of-function experiments in mice induced a loss of muscle progenitor pool due to premature muscle differentiation (Bröhl et al., 2012; Czajkowski et al., 2014; Schuster-Gossler et al., 2007; Vasyutina et al., 2007a). During developmental myogenesis, active NOTCH signalling is associated with proliferating muscle progenitors, while NOTCH ligands are expressed in differentiated muscle cells (Bröhl et al., 2012; Czajkowski et al., 2014; Delfini et al., 2000; Hirsinger et al., 2001; Mourikis et al., 2012a; Schuster-Gossler et al., 2007; Vasyutina et al., 2007a). There have been numerous studies to identify NOTCH target genes in muscle progenitors (Bröhl et al., 2012; Mourikis et al., 2012a), but upstream regulators of the NOTCH signalling pathway have not been identified during foetal myogenesis.

Similar to NOTCH, the YAP (yes-associated protein) has been shown to promote satellite cell proliferation and inhibit muscle differentiation in cell cultures (Judson et al., 2012; Watt et al., 2010). *In vitro* studies using C2C12 cells demonstrated that YAP activity was high in proliferating myoblasts and decreased in myotubes (Watt et al., 2010). YAP and its paralogue TAZ (transcriptional co-activator with PDZ-binding motif; WWTR1) are effectors of the Hippo pathway that controls organ size, cell growth, apoptosis and tumour suppression (reviewed by Zhao et al., 2011). The Hippo kinases MST1 and MST2, when activated, phosphorylate their direct substrates LATS1/2. Once activated, LATS1 and LATS2 phosphorylate YAP/TAZ to mediate their cellular localisation and subsequently determine their activity. When the Hippo pathway is inactive, YAP/TAZ are dephosphorylated and are able to translocate into the nucleus to exert their function as transcriptional co-activators (reviewed by Zhao et al., 2011). Several transcription factors such as TEAD, TBX5, SMAD1, β-catenin and p73 have been identified to interact with YAP/TAZ and control gene expression (Alarcón et al., 2009; Cao et al., 2008; Mahoney et al., 2005; Murakami et al., 2005; Strano et al., 2001; Wackerhage et al., 2014; Zhao et al., 2008). However, YAP and TEAD1 showed a co-occupancy in 80% of promoters in mammary epithelial cells (Zhao et al., 2008), suggesting that YAP/TEAD interaction could constitute the major molecular mechanism of YAP-mediated regulation of gene transcription. The TEAD transcription factors recognise and bind MCAT elements (CATTCC), which are enriched in regulatory

regions of muscle-related genes (reviewed by Wackerhage et al., 2014). In addition to being involved in muscle stem cell proliferation (Judson et al., 2012; Watt et al., 2010), YAP has been recently shown to be a critical regulator of skeletal muscle fibre size in adult mice (Goodman et al., 2015; Watt et al., 2015).

In addition to being the nuclear effector of Hippo signalling, YAP has been identified as a sensor of external mechanical signals mediating cellular responses (Aragona et al., 2013; Dupont et al., 2011; Mendez and Janmey, 2012; Porazinski et al., 2015). YAP acts downstream of mechanical overload to induce muscle hypertrophy (Goodman et al., 2015). During development, mechanical forces produced by the muscles are essential for the correct establishment of the musculoskeletal system. Although the influence of the mechanical forces for cartilage, joint, bone and tendon development has been previously addressed (Nowlan et al., 2010; Shwartz et al., 2013), the molecular consequences of muscle contractions on muscle development itself are largely unknown. Paralyzed muscles in muscular dysgenesis (*mdg/mdg)* mice degenerate from foetal stages due to the absence of excitation-contraction coupling (Pai, 1965a, 1965b; Powell, 1990). In chick embryos lacking innervation (achieved by neural tube ablation), limb myogenesis initiated but the muscles then degenerate and undergo apoptosis at E10 (Edom-Vovard et al., 2002; Rong et al., 1992). This suggests that muscle contraction might be required for proper muscle development, however it is unclear how the mechanical input would be sensed by muscle cells and transduced into molecular signals that regulate proliferation, differentiation and apoptosis.

In this study, we explore the interplay between mechanical signals and molecular forces in the regulation of foetal muscle progenitors. We show that muscle contractions are required for the maintenance of the muscle progenitor pool during foetal myogenesis via NOTCH signalling. Muscle fibres rather than muscle progenitors respond first to mechanical forces. We further provide evidence that, downstream of mechanical forces, YAP positively regulates the expression of the NOTCH ligand *JAG2* in muscle fibres, which maintains the pool of muscle progenitors.

# Results

## 2 – Muscle contraction activates YAP and NOTCH signalling and thus regulates the pool of muscle progenitor cells during foetal myogenesis

### DELTA1-induced activation of the NOTCH signalling pathway increases the number of muscle progenitors.

The role of the NOTCH signalling pathway in developmental myogenesis has mostly been addressed in the context of NOTCH loss-of-function. We aimed to examine if NOTCH activation would modulate the number of muscle progenitors. Overexpression of DELTA1 using the RCAS virus system in chick limbs is known to activate the NOTCH pathway and inhibit the expression of muscle differentiation genes including *MYOD, MYOG* expression (Bonnet et al., 2010; Delfini et al., 2000). The effect on PAX7+ muscle progenitors has not been addressed in these conditions. We therefore grafted DELTA1/RCAS-producing cells in forelimbs of E3.5 chick embryos (Figure 1A). The ectopic *DLL1* expression showed the extent of the virus spread (Figure 1B). In *DLL1* overexpressing regions, we observed an increase in the number of the PAX7+ cells (Figure 1C-F). In DELTA1/RCAS infected muscles, there was a significant increase of 36%, in the number of PAX7+ cells compared to contralateral muscles (Figure 1I). Terminal muscle differentiation was inhibited in DELTA1/RCAS-infected limb muscles (Figure 1G, H), as previously shown (Delfini et al., 2000). However, muscle area (including PAX7+ and MF20+ cells) was not significantly altered (Figure 1I). The pool of PAX7+ cells was maintained at E9.5, six days after grafting DELTA1/RCAS producing cells, despite inhibition of muscle differentiation (Supplementary Figure 1). These results showed that DELTA1-activated NOTCH increased the number of muscle progenitors and maintained the pool of foetal progenitors over time, in the absence of muscle differentiation.

### Inhibition of muscle contraction reduces the number of foetal muscle progenitors

Mechanical forces produced by skeletal muscle contraction are known to be required for the establishment of the musculoskeletal system during development (Shwartz et al.,

2013). The consequences of immobilization for muscle formation have not been studied. We investigated the effect of inhibition of muscle contraction during foetal myogenesis. We set up an experimental design to block muscle contraction in chick embryos (Figure 2A). DMB (decamethonium bromide) was administrated *in ovo* at E7.5 and at E8.5. At E7.5, the final muscle pattern was established and limb foetal muscles were individualized (Tozer et al., 2007). Limbs from embryos exposed to DMB or to a buffer control solution (see materials and methods) were harvested 12h (at E8) or 48h (at E9.5) after DMB application at E7.5 (Figure 2A). RT-q-PCR analyses for muscle markers showed a significant decrease in the relative expression levels of *PAX7* and *MYF5* mRNAs in DMB-treated limbs, as early as 12h after application and with a more prominent effect at 48h (Figure 2B). In contrast, the expression levels of *MYOD* and *MYOG* were increased in limbs upon DMB exposure (Figure 2B). Consistent with the decrease of *PAX7* expression in DMB-treated limbs, the number of PAX7+ cells was decreased in limb muscles of immobilized embryos compared to those of control embryos (Figure 2C-H). In the absence of muscle contraction, muscle fibre assembly was disorganised (Figure 2C-F). In addition to the relative increase of *MYOD* mRNA levels (Figure 2B), increase in *MYOD* expression was also observed by *in situ* hybridization in DMB-treated limbs (Figure 2I, J). In order to confirm the muscle phenotype obtained with the rigid paralysing agent DMB, we repeated the experiments using the pancuronium bromide (PB) drug that exerts a flaccid skeletal muscle paralysis (Nowlan et al., 2010). PB-treated limbs presented a similar muscle phenotype as DMB-treated limbs, *i.e.* a decrease in the number of PAX7+ cells, disorganised muscle fibres and increased *MYOG* expression (Figure S2). To test if the DMB treatment had a non-specific effect on muscle development, independently of its paralysis effect, we treated primary cultures of chick foetal myoblasts with DMB. There was no change in the relative mRNA levels of the muscle genes *PAX7*, *MYOD* and *MYOG* in DMB-treated foetal myoblasts compared to control myoblast cultures (Supplementary Figure 3). This showed that DMB did not have any off target effect on muscle differentiation. We concluded that inhibition of muscle contraction following either rigid or flaccid paralysis with DMB or PB drugs, respectively, reduces the pool of the foetal muscle progenitors and altered muscle differentiation.

**NOTCH activity is decreased in limbs in the absence of muscle contraction**

The concomitant diminution of the muscle progenitor pool and increase of *MYOD* and *MYOG* expression in immobilized chick embryos was reminiscent of a NOTCH loss-of-function phenotype previously described in mice (Bröhl et al., 2012; Vasyutina et al., 2007a). In order to determine if NOTCH activity was modified in muscles that lack contraction, we examined the mRNA levels of components of the NOTCH signalling pathway in DMB-treated limbs. The primary NOTCH target genes, *HES5* and *HEYL*, are considered to be the transcriptional readout of NOTCH activity (Andersson et al., 2011). Moreover, *HeyL* is the major responding gene following Notch activation in limb foetal muscle cells in mice (Mourikis et al., 2012a). RT-q-PCR analyses showed that the relative mRNA levels of *HES5* and *HEYL* genes were significantly downregulated in limbs of DMB-treated embryos (Figure 3A), indicating that NOTCH signalling was decreased. In chick limbs, *HES5* is expressed in foetal muscles in a salt and pepper fashion (arrows) and in endothelial cells (arrowheads) (Figure 3B). In immobilized embryos, *HES5* expression was decreased in limb muscles, while *HES5* expression was maintained in endothelial cells (arrowheads) (Figure 3C). The expression levels of the *NOTCH2* receptor and *JAG2* ligand were also decreased in immobilized embryos (Figure 3A). Despite a first decrease in the *DLL1* mRNA levels 12h after DMB exposure, *DLL1* was significantly increased 48h after DMB exposure (Figure 3A). This *DLL1* increase observed in chick DMB-treated limbs was consistent with the increase in the expression of *MYOD* and *MYOG* (Figure 2) and was reminiscent of the concomitant increase of *MyoD* and *Dll1* in mouse foetal myoblasts (Mourikis et al., 2012a). This is also consistent with the major function of Notch during mouse foetal myogenesis, which is to repress *MyoD* expression (Bröhl et al., 2012). These results showed that NOTCH activity assessed by *HES5* and *HEYL* expression was decreased in limb muscles in immobilized embryos.

**DLL1-mediated activation of NOTCH prevented the diminution of the muscle progenitor pool in the absence of muscle contraction**

The inhibition of muscle contraction led to a diminution in the number of muscle progenitors and to a decrease of NOTCH activity in foetal limbs. In order to determine whether NOTCH signalling was involved in the reduction of the muscle progenitor pool in immobilized embryos, we performed NOTCH rescue experiments. We forced NOTCH

activity by performing grafts of DELTA1/RCAS-producing cells in chick limbs and treated the embryos with DMB as described previously (Figure 2A). In DELTA1/RCAS-grafted limbs of immobilized embryos, we observed an amplification of the pool of muscle progenitors compared to that of DMB-treated embryos (Figure 4). We detected a significant increase of 77% in the number of PAX7+ cells in the NOTCH-activated muscles of immobilized embryos compared to muscles of immobilized embryos (Figure 4G). This showed that forced NOTCH activity was able to prevent the diminution in the number of PAX7+ cells in limb muscles of immobilized chick embryos. We conclude that the NOTCH signalling pathway is involved in the diminution of the foetal muscle progenitor pool during immobilization.

**NOTCH ligand activity in differentiated myogenic cells is required to maintain the pool of muscle progenitors**

In immobilized embryos, NOTCH activity was lost in limb muscles and forced NOTCH activity was able to prevent the diminution in the number of foetal muscle progenitors. However, because NOTCH signalling in muscle progenitors is activated by a NOTCH ligand produced by muscle fibres (Delfini et al., 2000; Hirsinger et al., 2001), we wanted to determine whether the loss of muscle contraction would affect primary muscle progenitors or muscle fibres. The NOTCH ligand *JAG2* was strongly expressed in foetal muscle fibres in chick limbs (Figure 3D), as previously shown (Delfini et al., 2000). In immobilized embryos, *JAG2* expression was drastically decreased in limb muscle fibres (Figure 3E). This suggested that the loss of NOTCH activity in limb muscles of immobilized embryos could be a consequence of the loss of NOTCH ligands in muscle fibres. To test this, we first determined whether the decreased number in muscle progenitors in DMB-treated embryos was due to increased cell death. While apoptotic figures were very rare in control muscles (Figure 5A-C, arrowhead), we observed an increased number of apoptotic cells in foetal muscles of immobilized embryos (Figure 5D-F). However, these apoptotic figures were observed in MF20+ cells and not in PAX7+ progenitors (Figure 5D-F, arrows). This showed that some foetal muscle fibres, but not muscle progenitors were undergoing cell death in the absence of muscle contraction. In addition, we defined the proliferative state of PAX7+ cells in DMB-treated embryos. PAX7+ cells were still co-localising with EdU in immobilized

embryos (Figure 5G-L). This showed that immobilization induced apoptosis in foetal muscle fibres and did not affect the proliferative capacity of PAX7+ cells.

The dramatic loss of *JAG2* expression in muscle fibres (Figure 3) and the absence of cell death in PAX7+ cells (Figure 5) suggested that the diminution of muscle progenitors in immobilized embryos could be due to the loss of the NOTCH ligand in muscle fibres. In order to test this hypothesis, we blocked NOTCH ligand activity specifically in muscle fibres (Figure 6). We performed chick somite-electroporation using a vector that can be integrated into the genome in the presence of the transposase (Figure 6A, B) (Bourgeois et al., submitted). This vector co-expresses the Tomato reporter gene and a dominant-negative DELTA1 under the control of the mouse Myosin Light Chain (MLC) promoter that drives expression in differentiated muscle cells (Wang et al., 2011). This dominant-negative DELTA1 has been shown to prevent the signal from the ligand-expressing cells and consequently blocked NOTCH signalling in signal-receiving cells (Henrique et al., 1997). The embryos were electroporated at E2.5 and processed at E8.5 (Figure 6B). The non-electroporated limbs of the same embryos were used as controls (Figure 6B). We observed that blocking the signal of the NOTCH ligands in differentiated muscle cells decreased the number of PAX7+ cells by 35%, while the muscle area and shape were not affected in electroporated limbs compared to contralateral limbs (Figure 6). This showed that the reduction of NOTCH-ligand activity in the differentiated muscle cells led to a decrease in the number of adjacent muscle progenitors. We conclude that the NOTCH ligand in muscle fibres is required to maintain the pool of muscle progenitors and that muscle fibres primary respond to mechanical forces.

**YAP is active in muscle fibres and in a subpopulation of muscle progenitors in chick limb foetal muscles**

Immobilization of embryos induced a downregulation of *JAG2* expression in muscle fibres that decreases the muscle progenitor pool (Figures 2, 3, 6). We then asked which signal could sense mechanical forces and regulate *JAG2* expression in muscle fibres. The mechanosensitive YAP protein was a good candidate to fulfil this role. *YAP1* transcripts were expressed ubiquitously in chick limbs, but with stronger intensity in different regions, including tendons (Figure 7A, arrows) and skin (Figure 7A). Consistent with the pattern of *YAP1* mRNA expression, YAP protein was also observed ubiquitously in chick limbs with

higher intensity in tendons (Figure 7B, C, arrows) and skin (Figure 7B, C). In muscles, *YAP1* expression was detected in MF20+ muscle fibres (Figure 7D, E, arrowheads) and outside of MF20+ muscle fibres (Figure 7D, E, arrows). Since YAP protein subcellular localisation reflects its activity, we further analysed whether YAP protein was localised in the nucleus of muscle cells. We observed that all myonuclei of MF20+ cells displayed nuclear YAP (Figure 7G-L arrowheads), while only a subset of PAX7+ cells showed nuclear YAP (Figure 7M-O, arrowheads). Proliferating myoblasts have been shown to be associated with increased nuclear YAP in cell culture (Judson et al., 2012; Watt et al., 2010). In order to examine if nuclear YAP visualised in a subset of muscle progenitors was associated with the cell cycle state of PAX7+ cells, we performed EdU labelling in E9.5 chick embryos. Both YAP+ and YAP- cells could be detected in the PAX7+/EdU+ cell population, suggesting that active (nuclear) YAP was not directly related to the cell cycle state of PAX7+ cells (Supplementary Figure 4). This showed that YAP was active in all myonuclei of muscle fibres and in a subset of muscle progenitors unrelated to cell cycle state.

**Embryo immobilisation decreased YAP activity in muscle fibres**

We next analysed YAP activity in muscles of immobilized embryos. We first analysed the expression of two recognized YAP target genes *CTGF* and *CYR61* (Lai et al., 2011; Zhao et al., 2008) in limbs of immobilized embryos (Figure 8A). We observed that the *CTGF* and *CYR61* expression levels were significantly decreased in DMB-treated limbs, 12 and 48 hours after DMB exposure (Figure 8A). We next examined the expression pattern of the YAP target gene *CTGF* by *in situ* hybridization in contraction-deprived muscles. In control limbs, *CTGF* was observed in muscle fibres, regionalised at the muscle tips (Figure 8B, D, arrows) and in cartilage elements (Figure 8B, arrowheads). In immobilized embryos, *CTGF* expression was lost in muscle fibres (Figure 8C, E), while *CTGF* expression was maintained in cartilage (Figure 8C, E, arrowheads), indicating a specific loss of YAP activity in muscle fibres in the absence of mechanical forces. Consistent with the loss of *CTGF* expression in muscle fibres, nuclear YAP, observed in myonuclei of control limb muscles (Figure 8F-H, arrows), was lost in myonuclei of muscle fibres of immobilized embryos (Figure 8I-K, arrowheads). We did not observe any obvious loss of nuclear YAP in PAX7+ cells (Figure 8L-Q). This showed that there was a specific loss of YAP activity in foetal muscle fibres of immobilized embryos.

**YAP is recruited to *JAG2* regulatory sequences in chick limb muscles**

In immobilized embryos, muscle fibres displayed a loss in YAP activity, a decrease of *JAG2* expression and increased apoptosis. In order to define whether YAP directly regulated *JAG2* transcription, we performed a ChIP assay in limbs of E9.5 chick embryos. YAP/TEAD complexes occupy 80% of the promoters in human mammary epithelial cells (Zhao et al., 2008). We identified regulatory sequences upstream of the *JAG2* coding sequence that contained MCAT elements (CATTCC), which are binding sites for TEAD complexes (Davidson et al., 1988). We observed that YAP was recruited to Region 2 and Region 3, which both contained a MCAT element and which are located around 4 kb and 1 kb upstream of the *JAG2* coding sequence, respectively (Figure 9). Region 4, located 9 kb upstream of the *JAG2* coding sequence containing 3 MCAT elements, did not show any YAP occupancy. This showed that there was an *in vivo* YAP recruitment to the *JAG2* promoter regions in foetal skeletal muscles.

We next examined if YAP increased *JAG2* expression. We first tested this in cultured chick foetal myoblasts. We transfected the myoblasts with a constitutively active form of the murine Yap (mYapS112A). The mYapS112A cannot be phosphorylated at the level of Ser112 and is systematically translocated into the nucleus (Xin et al., 2013). Transfection of mYapS112A/RCAS in chick foetal myoblasts inhibited myotube formation, as previously described in mouse myogenic cells (Judson et al., 2012). Active YAP induced a decrease of *MYOD* and *MYOG* and an increase of *PAX7* expression levels in cultured myoblasts. In these conditions, *JAG2* and *HEYL* expression was also activated by mYapS112A (Supplementary Figure 5). This showed that forced activation of YAP increased *JAG2* expression and activated the NOTCH pathway. The *JAG2* positive regulation by YAP combined with the *in vivo* YAP recruitment to the *JAG2* promoter indicated that YAP was a good candidate for activating *JAG2* transcription in foetal muscle fibres in chick limbs.

# Figures

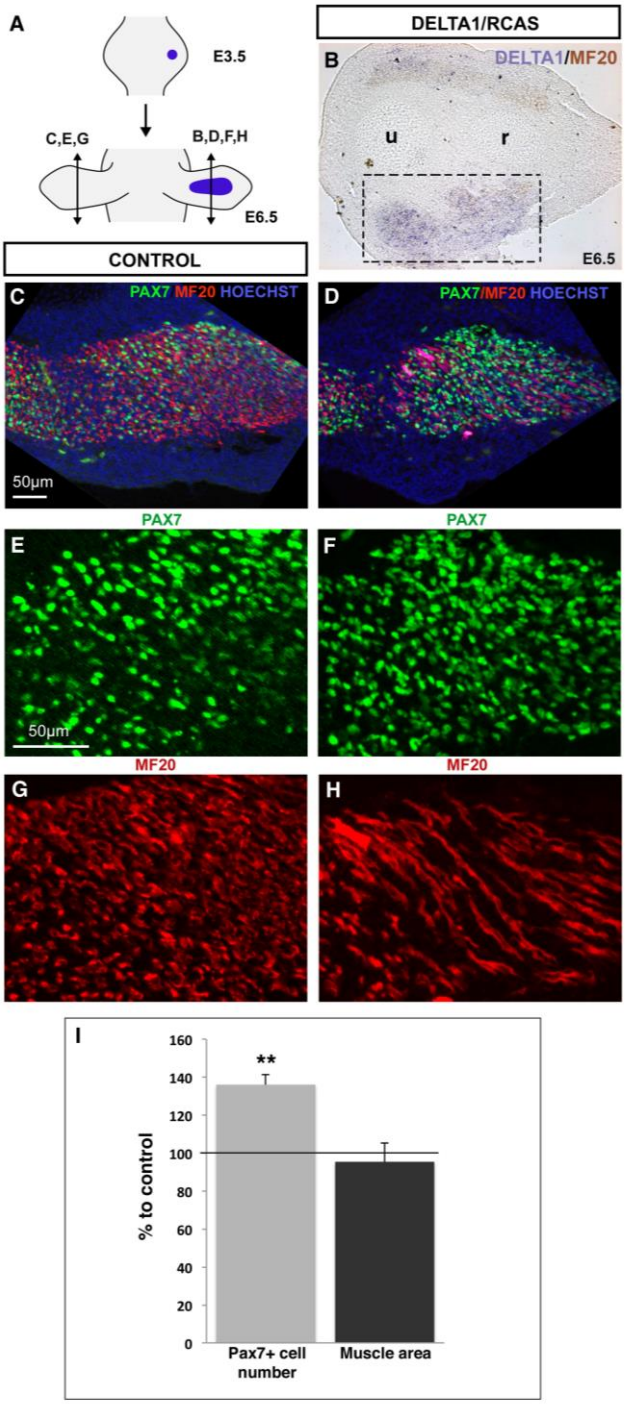



Figure 1

**Figure 1 - DELTA1-activated NOTCH increased the number of PAX7+ foetal muscle progenitors in chick limbs.** (A) DELTA1/RCAS-producing cells were grafted into limb buds of E3.5 chick embryos. Grafted-embryos were fixed three days later at E6.5. (B-H) DELTA1-grafted (B,D,F,H) and contralateral (C,E,G) limbs were cut transversely and

analysed for *DELTA1* expression by *in situ* hybridization (B) and for muscle markers by immunohistochemistry (C-H). Ectopic *DELTA1* expression visualized in ventral muscle masses (B) increased the number of PAX7+ cells (D,F) compared to ventral muscle masses in contralateral limbs (C,E) and affected muscle differentiation (H versus G). (I) The quantification of the number of PAX7+ cells and of muscle area were performed in DELTA1-grafted and contralateral limbs and presented as percentage of the contralateral limbs (control). Error bars indicate SD. Asterisks indicate the p-value, ** $p < 0.01$.

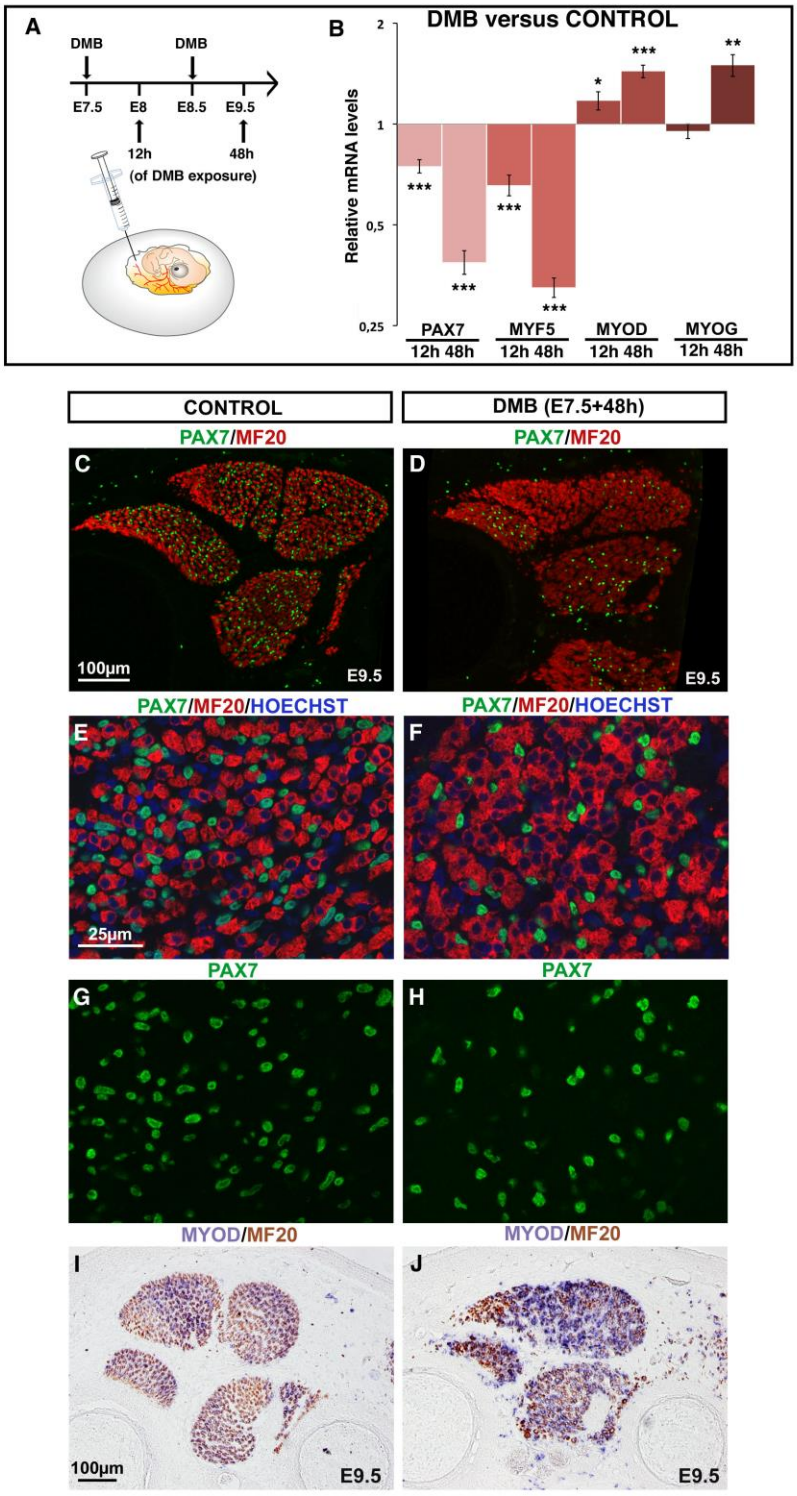



**Figure 2**

**Figure 2 - Inhibition of muscle contraction led to a decrease in the number of foetal muscle progenitors.** (A) Chick embryos were treated with DMB at E7.5 and E8.5, in order to block muscle contraction. Embryos were processed 12h (at E8) or 48h after DMB exposure (at E9.5). (B) RT-q-PCR analyses of muscle gene expression levels of 12h and 48h

DMB-treated limbs compared to control limbs. For each gene, the mRNA levels of control limbs were normalized to 1. The *PAX7* and *MYF5* expression levels decreased in DMB-treated limbs, while those of *MYOD* and *MYOG* were upregulated in DMB-treated limbs compared to the control. Asterisks indicate the p-value, *$p<0.05$; ** $p<0.01$, ***$p<0.001$; Error bars indicate SEM. (C-J) Control (C,E,G,I) and DMB-treated (D,F,H,J) limbs were transversely sectioned and analysed for muscle markers by immunohistochemistry (C-H) or *in situ* hybridization (I,J). DMB-treated embryos displayed a diminution in the number of PAX7+ cells in limb muscles (D,F,H) compared to control embryos (C,E,G). DMB-treated muscles visualised with MF20 labelling were affected compared to control muscles (D,F versus C,E). (I,J) *MYOD* expression was upregulated in limb muscles of DMB-treated embryos (J) compared to control muscles (I).

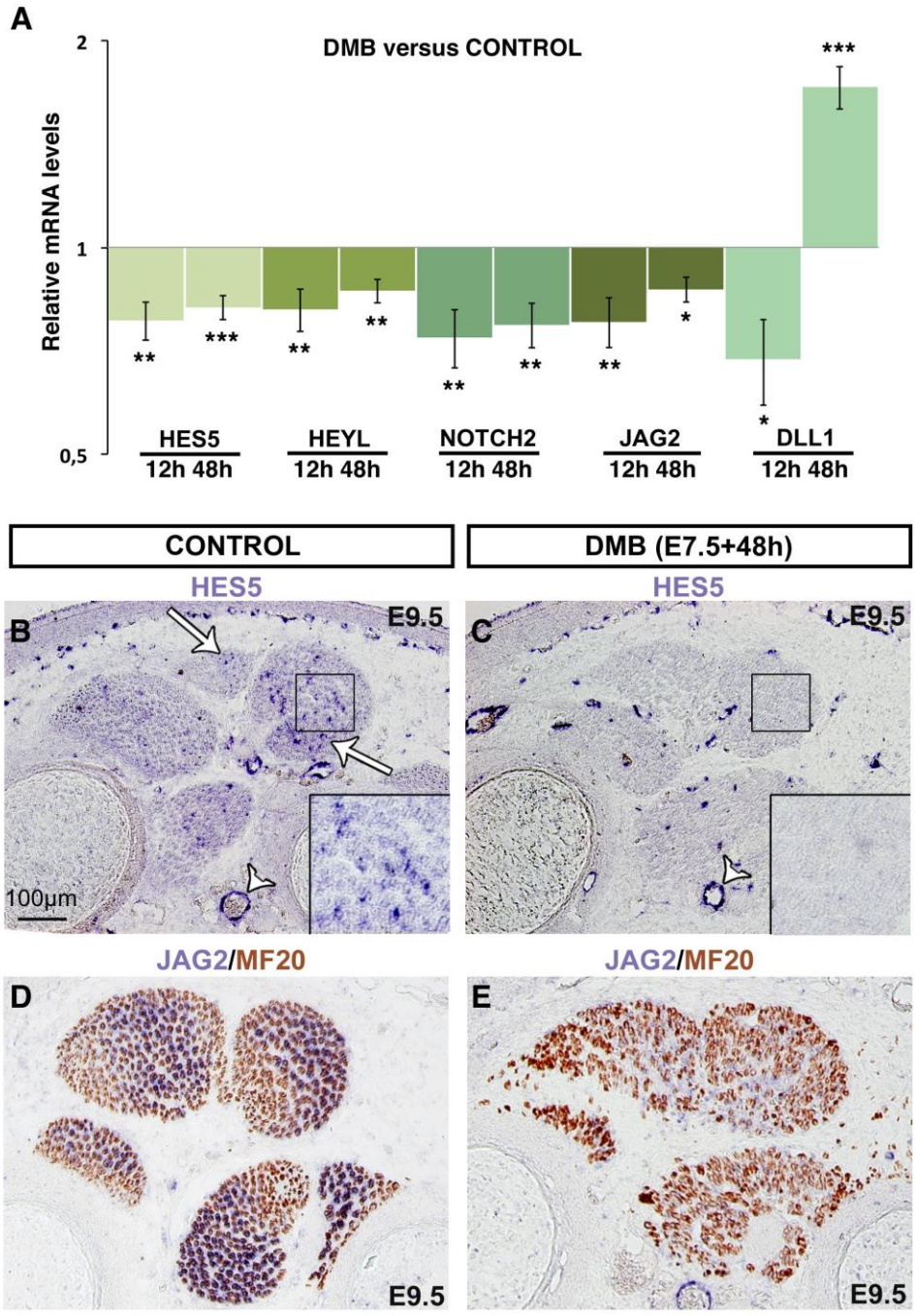



Figure 3

**Figure 3 - The inhibition of muscle contraction decreased NOTCH activity.** (A) RT-q-PCR analyses of the expression levels of NOTCH signalling components in limbs of 12h and 48h DMB-treated embryos. For each gene, the mRNA levels of control limbs were normalized to 1. The relative expression levels of *HES5*, *HEYL*, *NOTCH2* and *JAG2* were downregulated in limbs when muscle contraction was blocked, 12h and 48h after DMB application. The relative expression of *DELTA1* was first downregulated (12h) but then

increased after 48h of DMB exposure compared to control. Asterisks indicate the p-value, *$p$<0.05; *$p$<0.01; ***$p$<0.001; error bars indicate SEM. (B-E) Transverse limb sections of E9.5 DMB-treated (C,E) or control (B,D) embryos were hybridized with JAGGED2 (B,C) or HES5 (D,E) probes. *JAGGED2* and *HES5* expression was downregulated in limb muscles of DMB-treated embryos (C,E) compared to control embryos (B,D).

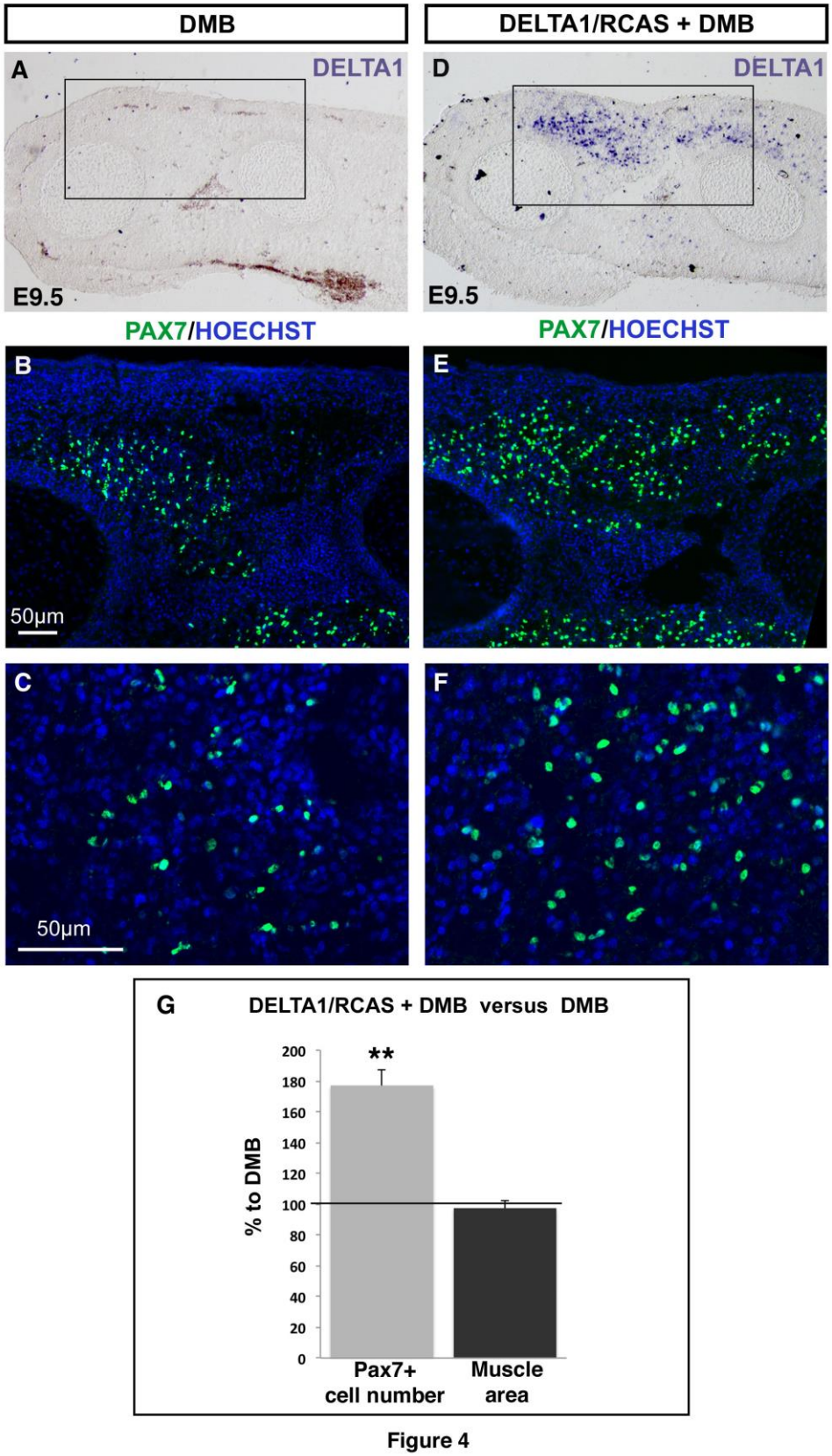



Figure 4

**Figure 4 - Forced-NOTCH activity prevented the diminution of PAX7+ foetal muscle progenitors in the absence of muscle contraction.** Transverse sections of contralateral (A-C) and DELTA1/RCAS grafted (D,F) limbs of DMB-treated embryos were hybridized with

DELTA1 probe to visualised ectopic *DELTA1* expression (A,D) and analysed for foetal muscle progenitors (B,C,E,F). In limb regions showing ectopic DELTA1 expression, visualised here in dorsal limb regions (D), we observed an increase in the number of PAX7+ cells (E,F) compared to contralateral limbs (B,C), in the absence of muscle contraction. (G) The quantification of PAX7+ cell number and muscle area was performed in DELTA1-grafted and contralateral limbs (in the absence of muscle contraction) and presented as percentage of contralateral. Asterisks indicate the p-value, $**p<0.01$; error bars = SD.

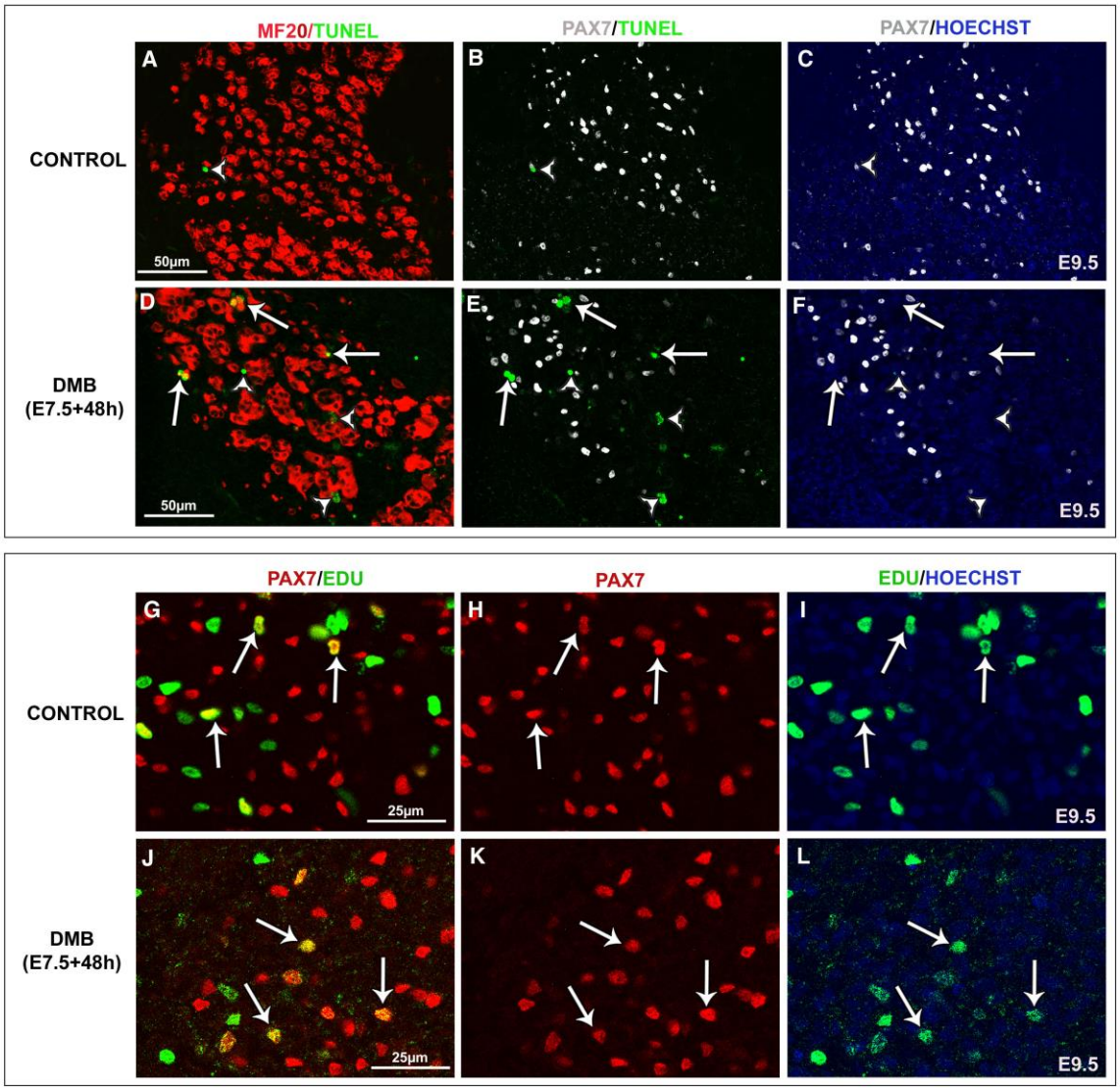



Figure 5

**Figure 5 - Muscle fibres but not muscle progenitors were undergoing apoptosis in immobilised embryos**. (A-F) Muscles of control and DMB-treated limbs were analysed for apoptosis by Tunel staining. (A-C, arrowheads) Control muscles were rarely visualised with Tunel staining. (D-F) Muscles of DMB-treated limbs displayed increased apoptosis (arrows and arrowheads), with muscle fibres displaying Tunel staining (D-E, arrows) but not muscle progenitors (E-F), compared to the control (A-C). (G-L) Muscles of control and DMB-treated limbs were analysed for cell proliferation by EdU incorporation. (G-I) Control limbs and (J-L) DMB-treated limbs displayed Pax7+ cells co-labelled with EdU showing active muscle progenitor proliferation.

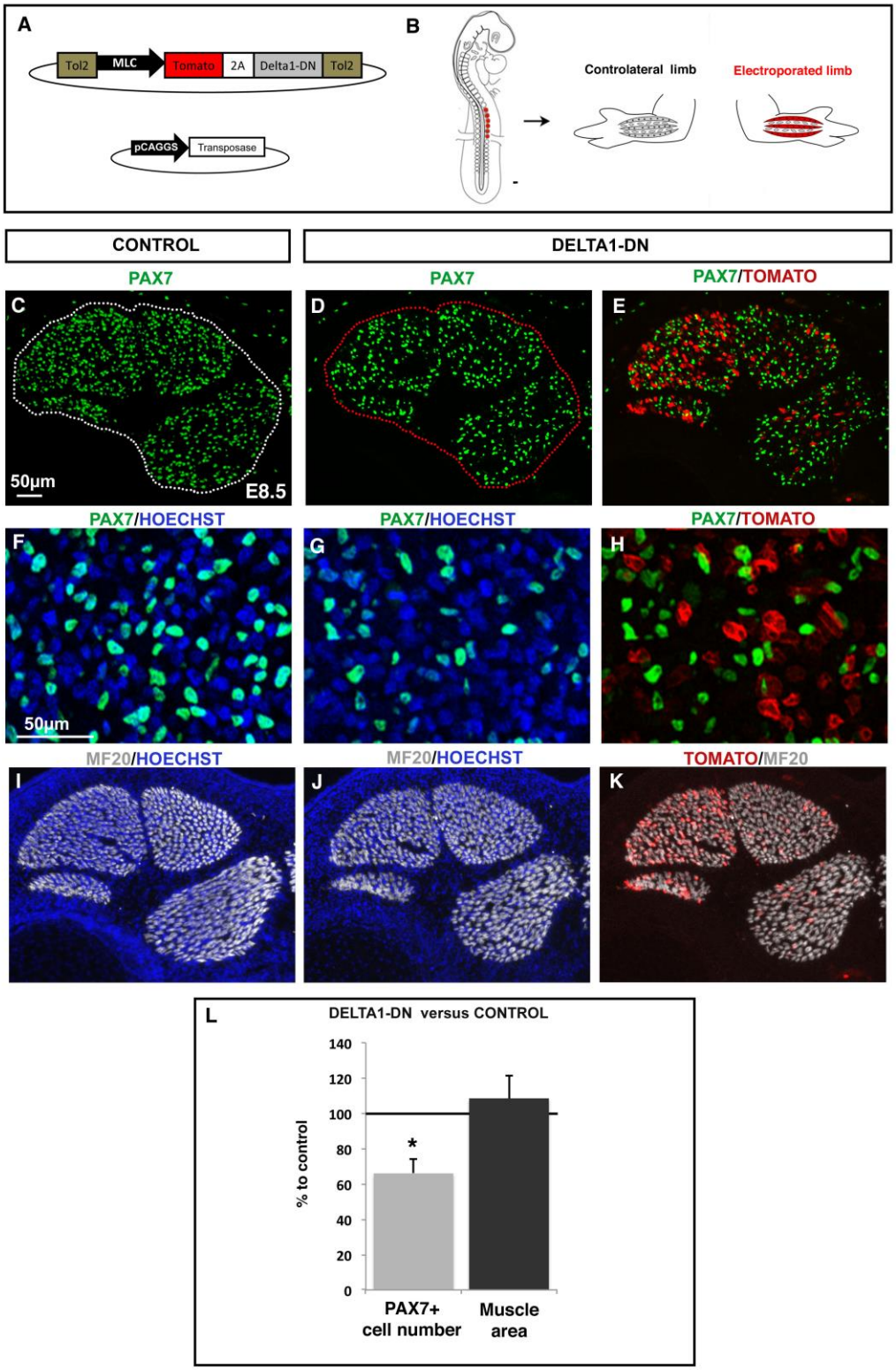



Figure 6

**Figure 6 - NOTCH-ligand activity in differentiated muscle cells was required to maintain the pool of PAX7+ foetal muscle progenitors**. (A) Schematic representation of the recombinant vector co-expressing the TOMATO reporter gene and a dominant form of

DELTA1 (DELTA1-DN) under the control of a muscle-specific promoter (MLC promoter) between two Tol2 transposons, and of the transient vector containing the transposase, allowing stable expression of the transgene. The use of the MLC promoter targeted transgene expression exclusively in differentiated muscle cells. (B) E2.5 chick embryos were electroporated at the level of limb somites in order to target limb muscle cells. Electroporated and contralateral limbs were analysed six days after electroporation, at E8.5. (C-K) Transverse limb sections of contralateral muscles (C,F,I) and electroporated muscles (D,E,G,H,J,K) were analysed for muscle markers. (D,E,G,H) Electroporated muscles visualised by the presence of Tomato expression (E,H) displayed a decrease in the number of PAX7+ cells (D,E,G,H) compared to contralateral limbs (C,F). The size and shape of the electroporated muscles was not affected (J,K versus I). (L) Quantification of the number of PAX7+ cells and of muscle area was performed in contralateral and electroporated limbs and presented as percentage of the contralateral limbs (control). Asterisk indicates the p-value, * $p < 0.05$; error bars = SD.

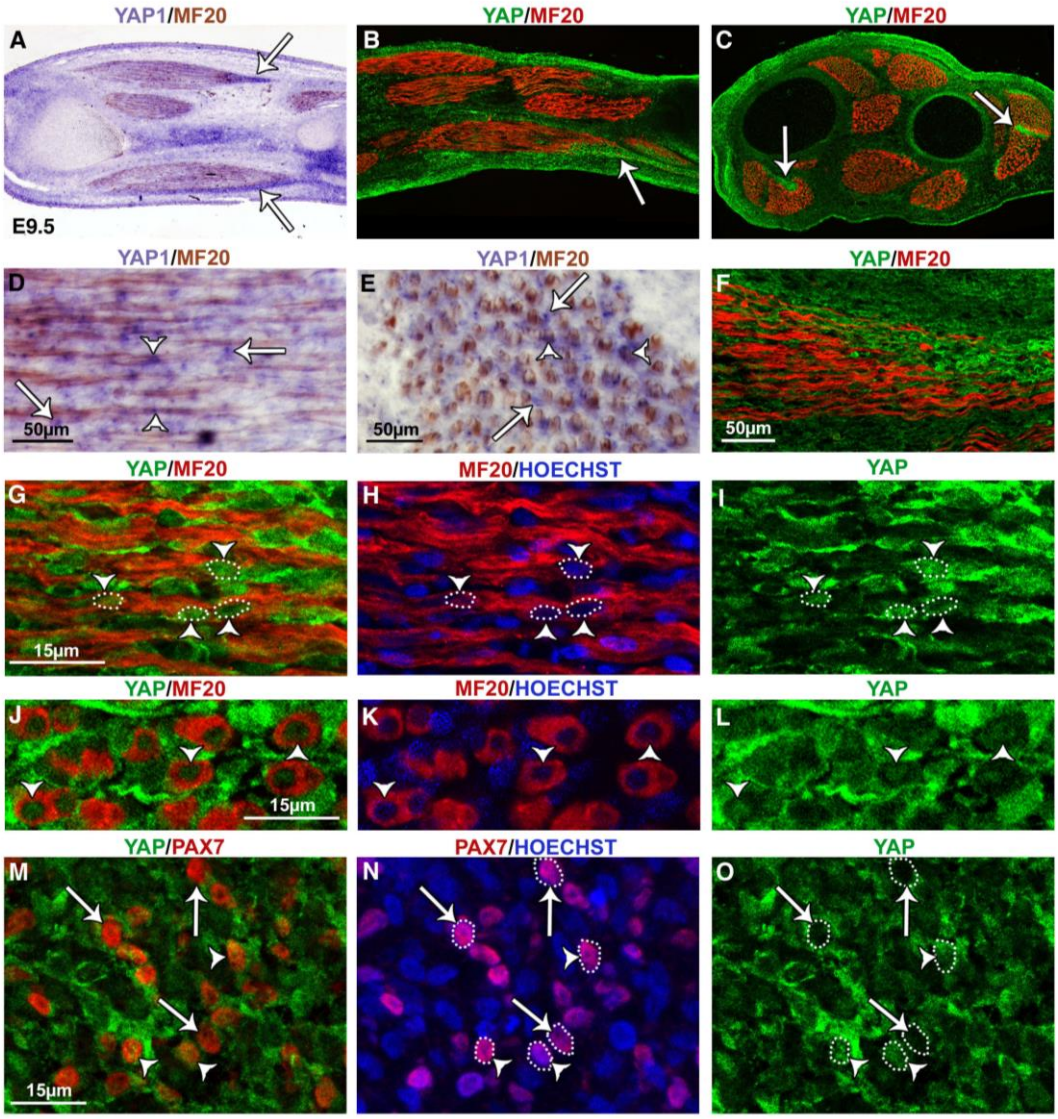



Figure 7

**Figure 7 - Endogenous expression and activity of the transcriptional co-activator YAP1 during limb foetal myogenesis.** Chick limbs from E9.5 embryos were analysed by *in situ* hybridization or immunohistochemistry for endogenous activity of YAP. (A) Longitudinal limb sections showed that *YAP1* expression was higher in the muscle tips at the level of the tendons (arrows) and in cartilage. (D,E) Longitudinal (D) and transverse sections (E) of limbs showed that in muscles *YAP1* was expressed in fibres (arrowheads) and outside of fibres (arrows). (B,C) Longitudinal (B) and transverse limb sections (C) immunostained with YAP showed a stronger YAP protein localisation labelling in the muscle tips at the level of the tendons. (F) Higher magnification of YAP localised in muscle tips at the level of the tendons.

(G-L) Longitudinal (G-I) and transverse limb sections (J-L) showed that in muscles YAP protein was systematically visualised in myonuclei (MF20+, arrowheads). (M-O) Transverse limb sections immunostained with PAX7 and YAP revealed a subpopulation of PAX7+ cells positive for YAP (arrowheads) and PAX7+ cells negative for YAP (arrows).

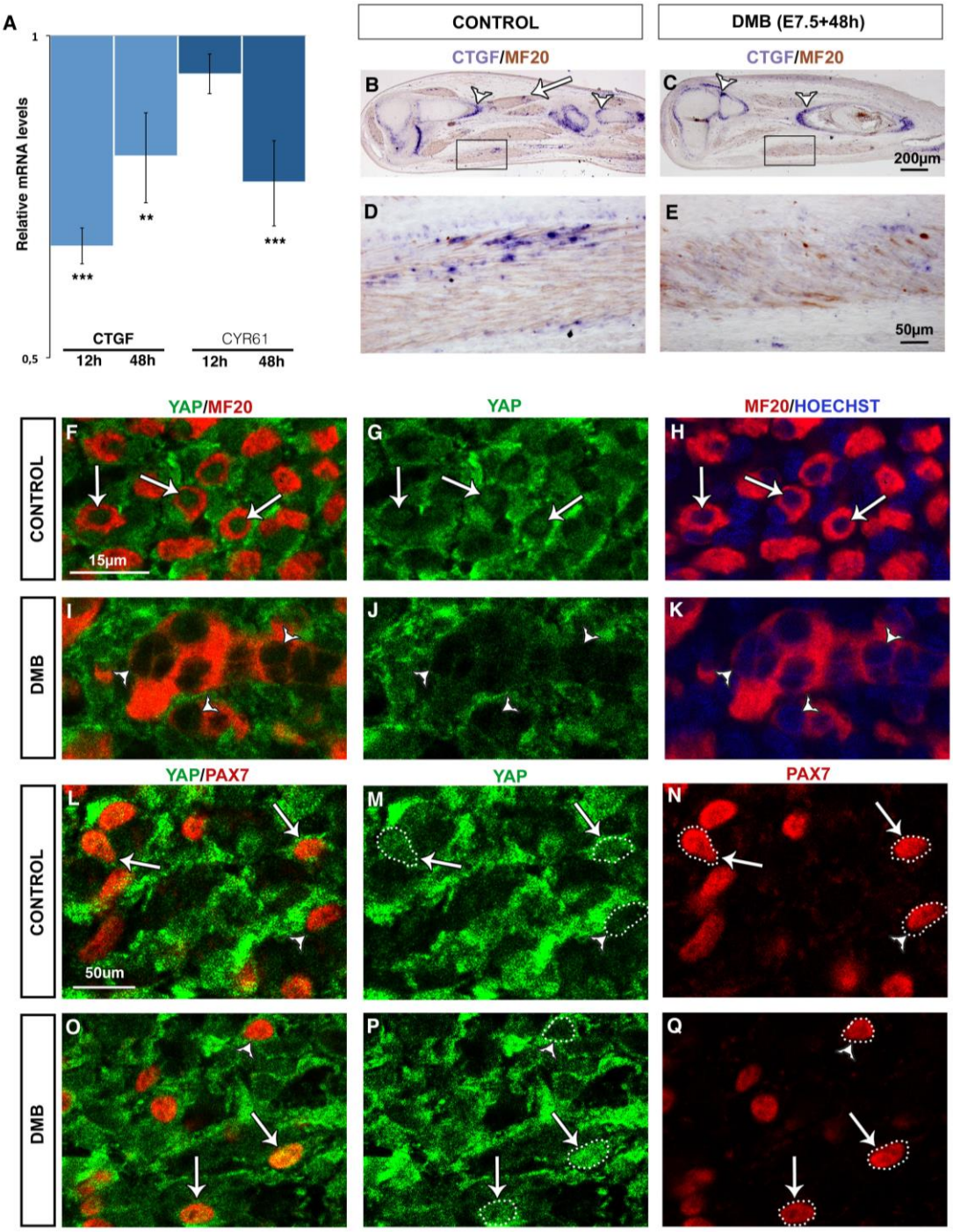



Figure 8

**Figure 8 - Block of the mechanical input led to a loss of YAP activity in muscle fibres.** (A) RT-q-PCR analyses of the expression levels of YAP target genes in limbs of 12h and 48h DMB-treated embryos. For each gene, the mRNA levels of control limbs were normalized to 1. The relative expression levels of *CTGF* and *CYR61* were downregulated in limbs when muscle contraction was blocked, after DMB application. Asterisks indicate the p-value,

*$p$<0.05; *$p$<0.01; ***$p$<0.001; error bars indicate SEM. Transverse limb sections of E9.5 DMB-treated (C,E) or control (B,D) embryos were hybridized with CTGF probe. *CTGF* expression was visualised in control limbs in muscle fibres close to the tips (B, arrows and D) in cartilage (B, arrowheads). *CTGF* expression was downregulated in limb muscles of DMB-treated embryos (C,E) but it was still visualised in cartilage (C, arrowheads). Transverse limb sections of E9.5 DMB-treated (I-K,O-Q) or control (F-H,L-N) embryos were immunostained with YAP and MF20 (F-K) or with YAP and PAX7 (L-Q). In limb muscles of DMB-treated embryos (I-K), the nuclear localisation of YAP protein in fibres (MF20+) is lost, compared to the control (F-H). In limb muscles of DMB-treated embryos (O-Q), the subpopulation of PAX7+ cells positive for nuclear YAP was still visualised, compared to the control (L-N).

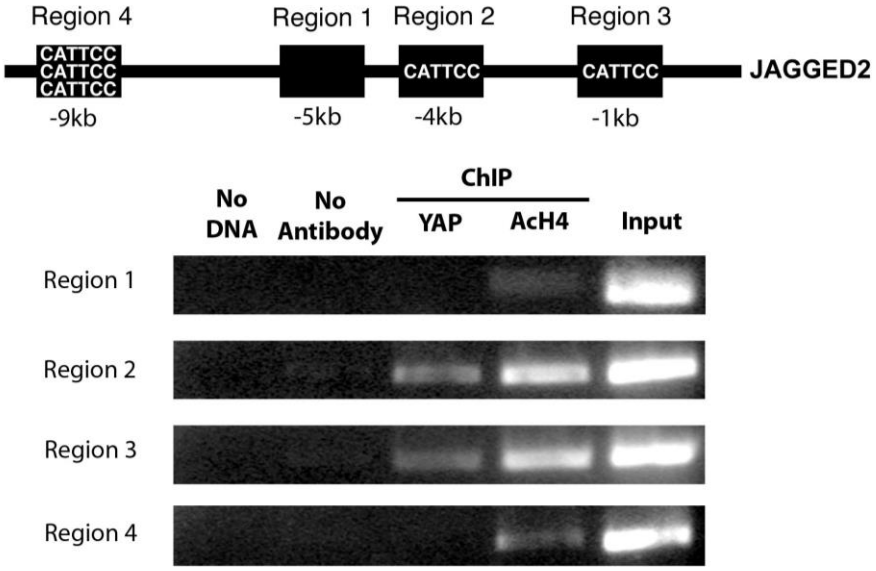



**Figure 9**

**Figure 9 - YAP was recruited to MCAT elements- containing regions upstream of *JAG2* coding sequence in foetal muscles.** ChIP assays were performed from limbs of E9.5 chick embryos with antibodies against YAP, AcH4 for positive control, or without any antibody as a negative control. ChIP products were analysed by PCR to study the presence of YAP on MCAT-containing regions upstream of *JAG2* coding sequence. We could detect the binding of YAP to the Regions 2 and 3 that contained a MCAT element. Region 1 did not contain a MCAT element and it was designed as a negative control. YAP was not detected binding to Region 1. Region 4 contains 3 MCAT elements but YAP binding was not detected. PCR amplifications were performed on chromatin isolated before immunoprecipitation (Input) as positive control or without DNA for negative control.

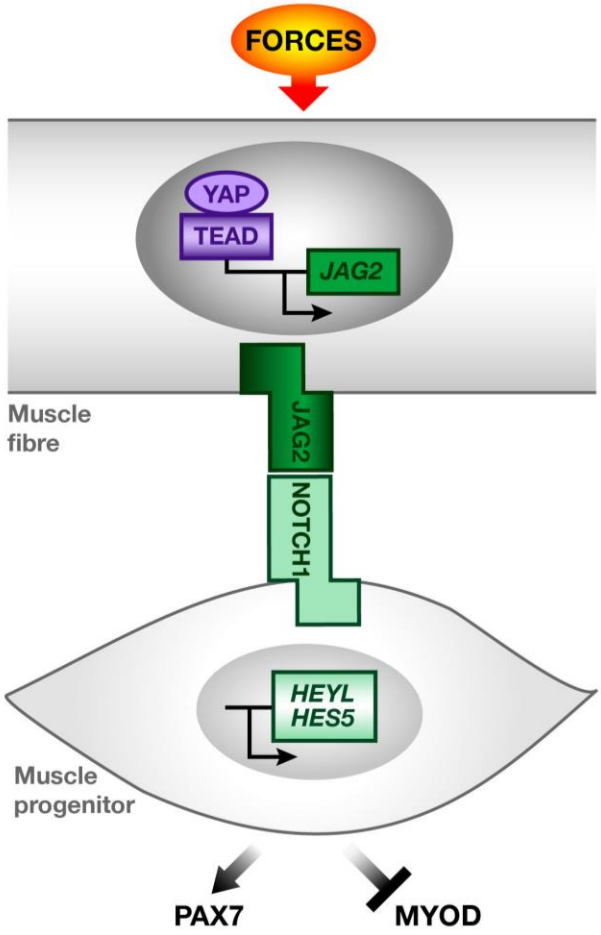
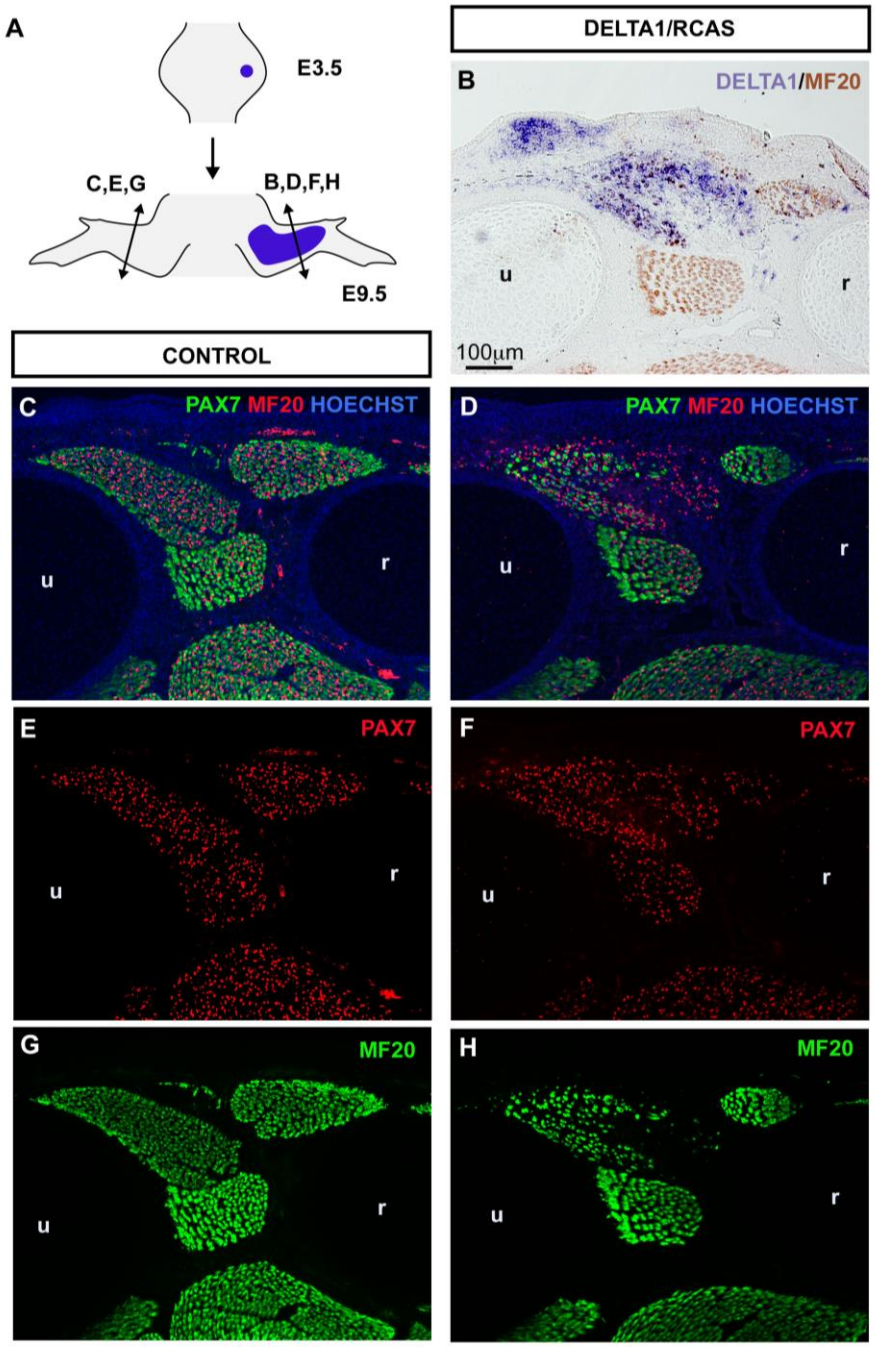



Figure 10

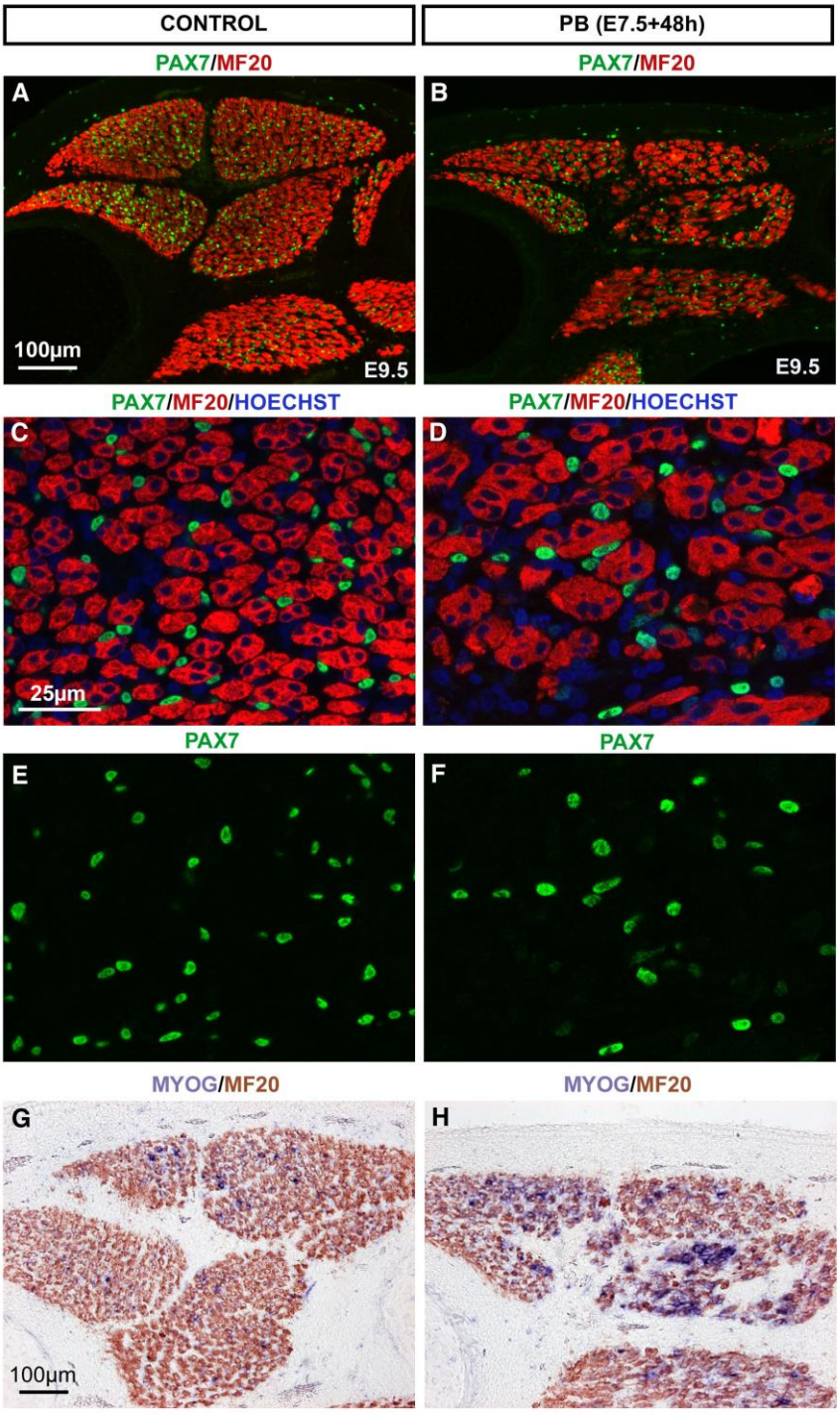
**Figure 10 – YAP activity is regulated by mechanical forces and influences the muscle progenitor pool via the NOTCH signalling pathway.** Mechanical forces are sensed in skeletal muscle fibres by the mechanosensitive gene YAP. YAP is able to bind regulatory regions of *JAG2* gene and influence its expression. The expression of *JAG2* in muscle fibres is required to signal to adjacent muscle progenitors and maintain high NOTCH activity (HES5, HEYL). NOTCH activity maintains the pool of PAX7+ muscle progenitors.




Supplementary Figure 1

**Supplementary Figure 1 - Continuous source of NOTCH ligand maintained the number of PAX7+ muscle progenitors despite the inhibition of muscle differentiation.** (A) DELTA1/RCAS-producing cells were grafted into limb buds of E3.5 chick embryos. Grafted-embryos were fixed six days later at E9.5. (B-H) DELTA1-grafted (B,D,F,H) and contralateral (C,E,G) limbs were cut transversely and analysed for *DELTA1* expression by *in situ* hybridization (B) and for muscle markers by immunohistochemistry (C-H). Ectopic

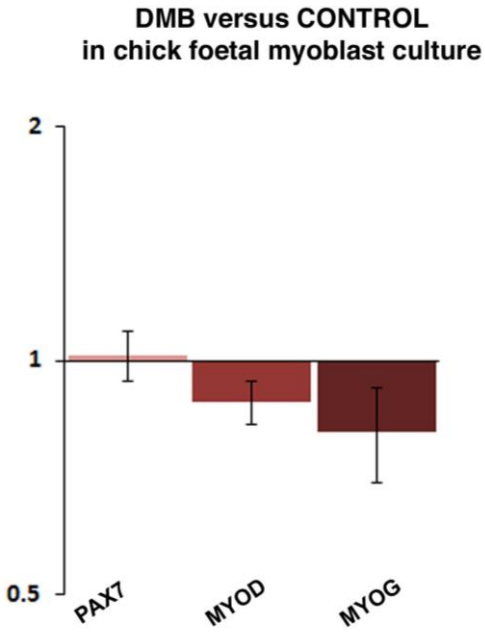
*DELTA1* expression visualized in dorsal muscle masses (B) maintained the pool of PAX7+ cells (D,F) compared to contralateral limbs (C,E) despite the decreased number of muscle fibres (H versus G).




**Supplementary Figure 2**

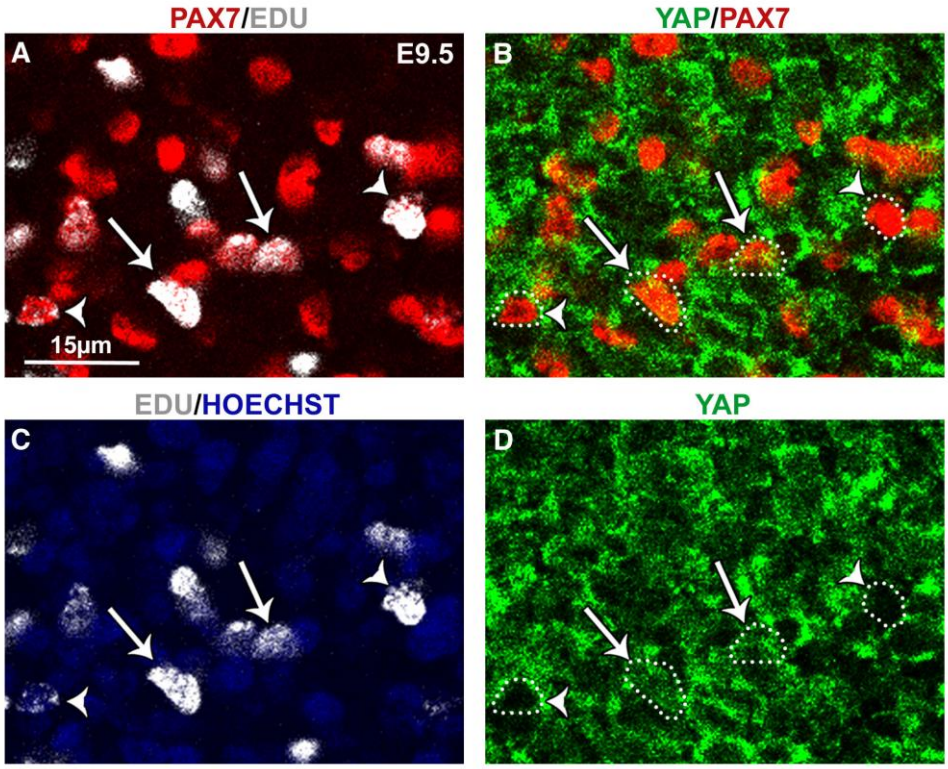
**Supplementary Figure 2 - Flaccid muscle paralysis decreased the number of PAX7+ muscle progenitors.** Chick embryos were treated with PB at E7.5 and E8.5, in order to block muscle contraction. Embryos were processed 48h after PB exposure (at E9.5). (C-J) Control (A,C,E,G) and PB-treated (B,D,F,H) limbs were transversely sectioned and analysed for muscle markers by immunohistochemistry (A-F) or *in situ* hybridization (G,H). PB-treated embryos displayed a diminution in the number of PAX7+ cells in limb muscles (B,D,F)

compared to control embryos (A,C,E). PB-treated muscles visualised with MF20 labelling were affected compared to control muscles (B,D versus A,C). (G,H) *MYOG* expression was upregulated in limb muscles of PB-treated embryos (H) compared to control muscles (G).



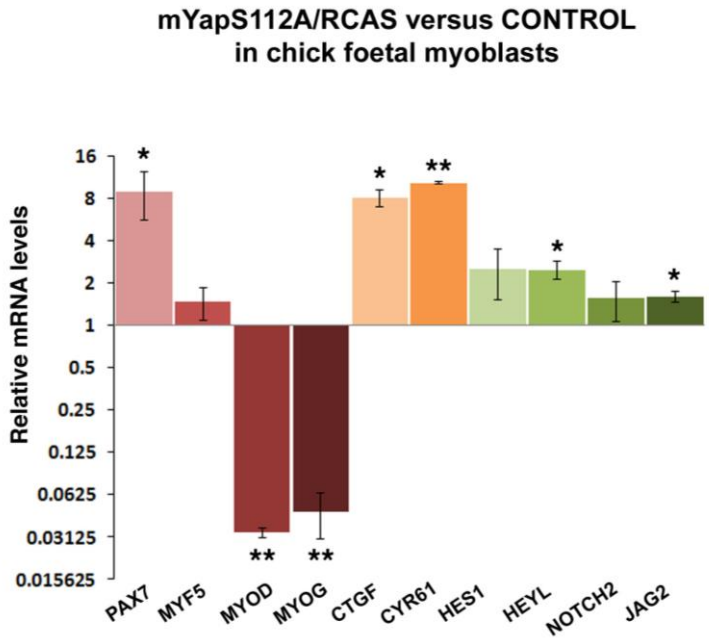

**Supplementary Figure 3**

**Supplementary Figure 3 – DMB treatment in primary cultures of chick foetal myoblasts did not change the expression of muscle genes.** RT-q-PCR analyses of the expression levels of muscle genes in primary cultures of foetal myoblasts treated with DMB. For each gene, the mRNA levels of cultures of foetal myoblasts treated with a control solution were normalized to 1. The relative expression levels of *PAX7*, *MYOD* and *MYOG* were not changed in cultures of foetal myoblasts treated with DMB compared to control. Error bars indicate SEM.




**Supplementary Figure 4**

**Supplementary Figure 4 - Endogenous YAP activity in PAX7+/EdU+ cell population.** Chick limbs from E9.5 embryos treated with EdU were analysed for immunohistochemistry with PAX7 and YAP antibodies. (A-D) Transverse sections of limbs immunostained with PAX7 and YAP showed that the PAX7+/EdU+ population were positive (arrows) or negative (arrowheads) for nuclear YAP.




**Supplementary Figure 5**

**Supplementary Figure 5 - Forced YAP activity increased *JAG2* expression and NOTCH activity in primary cultures of chick foetal myoblasts.** RT-q-PCR analyses of the expression levels of muscle genes and components of the NOTCH pathway in primary cultures of foetal myoblasts transfected with mYAPS112A/RCAS. For each gene, the mRNA levels of cultures of foetal myoblasts transfected with a control vector (Fucci/RCAS) was normalised to 1. The relative expression levels of YAP target genes *CTGF* and *CYR61* were significantly increased. The relative expression levels of *PAX7* were increased, while those of *MYOD* and *MYOG* were downregulated. *JAG2* and *HEYL* expression levels were increased upon forced YAP activation, compared to the control. Asterisks indicate the p-value, *$p$<0.05; *$p$<0.01; ***$p$<0.001; error bars indicate SEM.

| | | |
|---|---|---|
| PAX7 | Fw | AGAAGAAGGCCAAGCACAGCATAG |
| | Rv | ATTCGACATCGGAGCCTTCATCCA |
| MYF5 | Fw | ACCAGAGACTCCCCAAAGTG |
| | Rv | TCGATGTACCTGATGGCGTT |
| MYOD | Fw | CGACAGCAGCTACTACACGGAAT |
| | Rv | CTCTTCCCATGCTTTGGGTC |
| MYOG | Fw | AGGCTGAAGAAGGTGAACGAAG |
| | Rv | CAGAGTGCTGCGTTTCAGAGC |
| HES5 | Fw | ATACAGCCGAGCTTTTGCAG |
| | Rv | TGCAGAGCTTCTTTGAGGCA |
| HEYL | Fw | CCAAGCTGGAGAAGGCAGA |
| | Rv | CCAGAGCACGAGCATCCA |
| NOTCH2 | Fw | AATGGCTATGAGTGCCAGTGT |
| | Rv | ATTCACCGTGATGGCAAGGA |
| JAGGED2 | Fw | CAAGTGGCTGGGAAGGAGAA |
| | Rv | TGCATCGGCCACCATTATGA |
| DELTA1 | Fw | CTTCACCTGTGGAGAGCGT |
| | Rv | ATTCCCCAGGTTTGTCGCA |
| CTGF | Fw | AAGACACTTACGGCCCAGAC |
| | Rv | AGTAGTCTGCACCAGGCAAT |
| CYR61 | Fw | GGAACAACGAGCTGATTGCC |
| | Rv | CGGCTCGGATCCAAAAACAG |

**Supplementary Table 1**

**Supplementary Table 1 -** List of the primer pairs used for RT-q-PCR analysis in this study.

| | | |
|---|---|---|
| Region 1 | Fw | TGCATCCATTGCAAATAGGTCAAG |
| | Rv | TTCTCTGATGGACTGAACTGTGC |
| Region 2 | Fw | GCCCTAGCCCTTCAGACAAAG |
| | Rv | AAATCTCCCCGGAGGATCATAGG |
| Region 3 | Fw | GTGTTGCAATGCTCCCAGTG |
| | Rv | TTTGGCCGTAGGAGAACGTG |
| Region 4 | Fw | GTTTGAGGGGAGGGACTTGG |
| | Rv | AGGATCCCCAGATTAGTGCT |

**Supplementary Table 2**

**Supplementary Table S2** – List of PCR primers used in the ChIP experiment**.**

# Discussion

## 2 – Muscle contraction activates YAP and NOTCH signalling and thus regulates the pool of muscle progenitor cells during foetal myogenesis

### The foetal muscle progenitor pool is sensitive to mechanical forces

We have shown that the foetal muscle progenitor pool decreased in the absence of muscle contraction in chick embryos. Surprisingly, skeletal muscle is the component of the musculoskeletal system the less studied in unload conditions during development. Skeletal abnormalities, including delayed ossification, changes in the size and shape of cartilage elements and joint fusion have been described in immobilized chick embryos or in a mouse model with altered mechanical stimulation in the limbs due to the absence of limb skeletal muscles (Roddy et al., 2011; Rolfe et al., 2014). We show for the first time an effect of mechanical forces on PAX7 mRNA levels and on PAX7+ muscle progenitors during foetal myogenesis. Adult muscle stem cells are also sensitive to mechanical forces. 2-weeks hindlimb suspension induces a loss of muscle stem cells in mice (Mitchell and Pavlath, 2004). In young adult human, 2-weeks immobilisation induced a drop of *PAX7* mRNA expression, although with no obvious changes in the number of PAX7+ satellite cells (Carlson et al., 2009; Suetta et al., 2013). Conversely, a single bout of high intensity exercise (with no myofibre damage) increased the number of satellite cells, two days after exercise in human (Crameri et al., 2004, 2007). Moreover, muscle stimulation and exercises attenuate the age-related decline in the number of satellite cells in mouse models and in human (reviewed in Snijders et al., 2009). Muscle stem cells are thus sensitive to mechanical forces during development, homeostasis and ageing. This is consistent with the recognized importance of forces in the regulation of stem cell maintenance, proliferation and differentiation in fat, bone and hematopoietic system (Ivanovska et al., 2015). Taken together, mechanical forces could be a generic mechanism for stem cell maintenance.

**NOTCH is a signalling pathway responding to mechanical forces during foetal myogenesis**

We have shown that the loss of muscle contraction mimicked a NOTCH loss-of-function phenotype in foetal muscles. In immobilised chick embryos, there is a drop of NOTCH activity and the pool of foetal muscle progenitors is decreased with a shift towards differentiation, assessed by *MYOD* and *MYOG* upregulation. This shows that NOTCH is a signalling pathway responding to mechanical forces during foetal myogenesis. NOTCH pathway has been shown to be downregulated in skeletal muscles in unload conditions in old rats (Domingues-Faria et al., 2014). In adult muscle, NOTCH activity increases following physiological exercises (with no fibres damage) in young and old human subjects (Carlson et al., 2009). In zebrafish, swimming-induced exercises promote extensive transcriptional changes in fast muscles, including modification of NOTCH components (*Dll1, Jag1, Jag2, Notch 1* and *Noch2*) (Palstra et al., 2014). Mechanical stimuli following eccentric exercises or in hypertrophy models have been shown to increase NOTCH activity in muscles in animal models (Akiho et al., 2010; Arthur and Cooley, 2012; Tsivitse et al., 2009). However, it has to be mentioned that eccentric muscle contractions following downhill running results in extensive muscle injury. Overload hypertrophy can also lead to muscle regeneration (Blaauw and Reggiani, 2014). It is well established that NOTCH activity is upregulated during the process of muscle regeneration following injury and is required for proper muscle regeneration (reviewed in Tsivitse, 2010). Consequently, downhill running in adult mice induced NOTCH signalling in regenerating skeletal muscles (Tsivitse et al., 2009) and *Notch1* expression is upregulated in mechanically overload plantaris muscles in mice (Akiho et al., 2010).

NOTCH activity is also regulated by mechanical forces in other tissues during development. *Delta1* expression is decreased in bones of immobilised mouse embryos (Rolfe et al., 2014). In the cardiovascular system, Notch activity has been shown to be under the control of mechanical forces generated by the blood flow in heart endothelial cells (reviewed in Granados-Riveron and Brook, 2012) and in arteries (Jahnsen et al., 2015).

In summary, NOTCH activity is altered upon changes of mechanical input in different tissues during development and adult life. During foetal myogenesis, in addition to being downregulated after immobilisation, NOTCH activity is able to prevent the reduction of the foetal muscle progenitor pool in the absence of muscle contraction in chick embryos. Forced

NOTCH activity also restores the diminished regenerative potential in aged muscles in mice (Conboy et al., 2003). This data indicate that NOTCH is a central molecular pathway acting downstream of mechanical forces to regulate muscle progenitors.

**Muscle fibres and not muscle progenitors are primary responding to the loss of mechanical forces**

We observed a concomitant decrease in the number of muscle progenitors and of NOTCH activity in the absence of mechanical forces during foetal myogenesis. Although we observed a shift toward muscle differentiation, there is a clear diminution of global muscle masses, in immobilised chick embryos. This is consistent with muscle atrophy described in the contexts of muscle denervation or immobility during development (Edom-Vovard et al., 2002; Hall and Herring, 1990; Pai, 1965a, 1965b; Powell, 1990; Rong et al., 1992). A striking result from our study is that muscle fibres but not muscle progenitors appear to be primary affected by immobilisation conditions during foetal myogenesis. Some apoptotic figures were observed in muscle fibres but not in muscle progenitors. Moreover, proliferation could still be observed in PAX7+ muscle progenitors in immobilized embryos. In adult, muscle atrophy or wasting has been described in unload conditions, sarcopenia, neuromuscular diseases and cachexia (Arthur and Cooley, 2012; Brooks and Myburgh, 2014; Crameri et al., 2007). Conversely, muscle hypertrophy is observed following exercises and in overload conditions. The role of satellite cells in muscle atrophy and hypertrophy has long been a debated issue (Blaauw and Reggiani, 2014; Brooks and Myburgh, 2014). Our study provides evidences that foetal muscle fibres respond directly to the loss of mechanical forces. Moreover, we show that the loss of expression of the NOTCH ligand *JAG2* in muscle fibres induces a decrease in the number of adjacent muscle progenitors. We believe that immobilisation during myogenesis affects first muscle fibres and that NOTCH signalling pathway provides a molecular mechanism for cell-to-cell communication to explain the decrease of the muscle progenitor pool (Figure 10).

**YAP is molecular sensor of mechanical forces, which regulates the expression of *JAG2* in muscle fibres**

YAP has been identified as sensor and mediator of mechanical cues in different cell systems and during tissue morphogenesis (Aragona et al., 2013; Dupont et al., 2011; Porazinski et al., 2015). Thus, YAP was an obvious candidate to function as molecular sensor of the loss of mechanical forces during foetal myogenesis. YAP activity was decreased in foetal muscles in immobilized embryos. This shows for the first time that YAP is sensitive to mechanical stimuli in developing foetal muscles. Consistent with the loss of *JAG2* expression in foetal muscle fibres, YAP activity was lost in muscle fibres and not in muscle progenitors. This reinforces the idea that foetal muscle fibres and not muscle progenitors are first responding to mechanical forces during foetal myogenesis.

Consistent with the YAP function in the regulation of cellular growth, YAP is elevated in activated satellite cells and has been shown to be involved in satellite cell proliferation, whilst inhibiting their differentiation in cell culture systems (Judson et al., 2012; Watt et al., 2010). Moreover, YAP is upregulated in alveolar rhabdomyosarcoma, an aggressive sarcoma of skeletal muscle (Crose et al., 2014). In addition to YAP function in cell proliferation, YAP has been recently shown to be upregulated by mechanical overload and to be critical for skeletal muscle hypertrophy in adult mice (Goodman et al., 2015; Watt et al., 2015). However, the contribution of muscle progenitors in these hypertrophy models is not clear. Moreover, the link between YAP and NOTCH signalling in muscle hypertrophy is not yet established. During foetal myogenesis, we showed that YAP is recruited to *JAG2* regulatory sequences in limb muscles. The YAP occupancy to JAG2 regulatory regions combined with (1) the concomitant downregulation of *JAG2* expression and YAP activity following immobilisation and (2) the *JAG2* activation upon constitutive active YAP in foetal myoblast cultures could indicate that YAP directly activates *JAG2* transcription in muscle fibres (Figure 10). Consistently, the YAP/TEAD complex has been shown to positively regulate *JAG1* expression and NOTCH activity in human hepatocellular carcinoma (Tschaharganeh et al., 2013). We provide a molecular mechanism downstream of mechanical forces that regulates the number of foetal muscle progenitors.

In conclusion, we have identified mechanical forces and the transcriptional co-factor YAP acting upstream of NOTCH signalling in the regulation of foetal muscle progenitors. Our results indicate that YAP senses the mechanical forces and regulates *JAG2* in muscle

fibres, which then activates NOTCH signalling in muscle progenitors to maintain the pool during foetal myogenesis.

# Materials and Methods

## 2 – Muscle contraction activates YAP and NOTCH signalling and thus regulates the pool of muscle progenitor cells during foetal myogenesis

### Chick embryos

Fertilized chick eggs were obtained from commercial sources: White Leghorn chick eggs (HAAS, Kaltenhouse) and chick eggs strain JA57 (Morizeau, Dangers), and incubated at 38.5°C. Chick embryos were staged according to days *in ovo.*

### Grafting of RCAS-DELTA1-expressing cells

Chicken embryonic fibroblasts (CEFs) obtained from E10 chick embryos were transfected with DELTA1/RCAS at a confluence of 50% using the Calcium Phosphate Transfection Kit (Invitrogen), overnight at 37°C and 5% $CO_2$. Pellets of cells of approximately 50–100 µm in diameter were grafted into limb buds of E3.5 embryos like previously described (Delfini et al., 2000). The embryos were harvested 3 or 6 days after grafting at E6.5 or E9.5, respectively.

### Construction of electroporation vector

The pT2AL-MLC-Tomato-2A-DELTA1DN was designed as follows: the dominant negative form of DELTA1 was amplified by PCR from the DELTA1/RCAS (Delfini et al., 2000) as a truncated form of DELTA1 that lacks all but 13 amino acids of the intracellular domain, in accordance to Henrique et al., 1997, with added stop codons. A restriction site for BstBI was added to the forward primer and a PmlI restriction site was added to the reverse primer. GFP was excised from the previously established stable vector pT2AL-MLC-Tomato-2A-GFP (Bourgeois et al., submitted) with the restriction enzymes BstBI and PmlI. A ligation using the purified Delta1-DN insert and the linearized vector was performed using the Rapid DNA

Ligation Kit (Roche). Primer sequences: Fw GACTTCGAAATGGGAGGCCGCT and Rv CACGTGTTACTATCACCTGCAGGCCTCG.

**Electroporation *in ovo***

Limb somite electroporation was performed as previously described (Bonnet et al., 2010; Bourgeois et al., submitted). The DNA solution was composed of the pT2AL-MLC-Tomato-2A-DELTA1DN vector and the transient transposase-containing vector pCAGGS-T2TP, which allows the stable integration of the MLC-Tomato-2A-DELTA1DN cassette into the genome, at a molar ratio of 3:1.

**Drugs administration *in ovo***

The stock solution for decamethonium bromide (DMB) (Sigma, D1260) was prepared at 10% in Hank solution (Sigma H9269). A DMB working solution was freshly prepared before each experiment at 0.5% in Hank solution with Penicillin-Streptomycin at 1% (Gibco, 15140). The stock solution for PB (Sigma, P1918) was prepared at 10mg/mL in Hank solution. A pancuronium bromide (PB) working solution was freshly prepared before each experiment at 8mg/mL in Hank solution with Penicillin-Streptomycin at 1%. The control solution was prepared using Hank solution with 1% of Penicillin-Streptomycin. 100µL of the working solution for DMB, PB or control solution was administrated in ovo at each of the time-points required.

**Cell culture**

Chick primary myoblasts were obtained from hind limbs of E10 chick embryos, mechanically dissected into small pieces and cultured in a minimal essential medium complemented with 10% of fetal calf serum as previously described (Havis et al., 2012; Spitz et al., 1997). Chick primary myoblasts were transfected with RCAS-mYapS112A, provided by Pascal de Santa Barbara (Université de Montpellier, France), at a confluence of 50% using the Calcium Phosphate Transfection Kit (Invitrogen), overnight at 37°C and 5% $CO_2$. Cultured cells were

let to confluence and prepared for RNA extraction. DMB treatment in cell culture was performed at a final concentration of 50µM for 48h.

**RNA extraction and quantitative real-time PCR**

Total RNAs were extracted from chick limbs at different time-points described for each experiment and from primary cultures of foetal myoblasts. 500ng to 1µg of RNA was reverse-transcribed using the High Capacity Retrotranscription kit (Applied Biosystems). RT-q-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences used for RT-q-PCR are listed in Supplementary Table 1. The relative mRNA levels were calculated using the 2^-ΔΔCt method (Livak and Schmittgen, 2001). The ΔCts were obtained from Ct normalized with GAPDH levels in each sample. 10 to 12 limbs for each condition were used as independent RNA samples originating from 3 to 4 independent experiments. 5 samples of cultured myoblasts were used as independent RNA samples originating from independent experiments. Each sample was analysed in duplicate. Results were expressed as Standard Error of the Mean (SEM). Data were analysed using the paired student *t*-test. Asterisks in figures indicate the different *p*-values (* <0.05; ** <0.01 and *** <0.001).

**EdU**

EdU (Invitrogen) was prepared at a stock solution of 5mg/mL. Chick embryos were administrated with EdU in ovo (100µL) at E9.5 for 1.5h.

**Immunohistochemistry**

Forelimbs of chicken embryos were fixed in 4% paraformaldehyde, overnight at 4°C and then processed in gelatin/sucrose for 12 µm cryostat sections. The monoclonal antibodies, MF20 that recognises 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 YAP mouse monoclonal antibody was obtained from Santa Cruz Biotechnology. Apoptosis was detected using the ApoTag kit (Millipore). After overnight incubation with the primary antibodies at 4°C, secondary antibodies conjugated with Alexa-488 and Alexa-555 (Invitrogen) were applied for 1 h at room temperature. Amplification with biotinylated secondary antibodies was carried out when needed by performing incubation with anti-mouse IgG1 or anti-mouse IgG2b (Southern Biotech) for 1 h and Cy5-Streptavidin (Invitrogen) for 45 min, both at room temperature. Hoechst (Molecular Probes) staining was performed with a dilution of 1/20000 in PBS 1X for 10 min at room temperature.

### *In situ* hybridization

Chick forelimbs were fixed in Farnoy (60% ethanol, 30% formaldehyde (stock at 37%) and 10% acetic acid) overnight at 4°C, and processed for *in situ* hybridization on wax tissue sections, as previously described (Wang et al., 2010). The digoxigenin-labelled mRNA probes were obtained as follows: DELTA1 and JAGGED2 (Delfini et al., 2000); HES5 was provided by Xavier Morin (Institut de Biologie de l'Ecole Normale Superieure, France), YAP1 was provided by Pascal de Santa Barbara (Université de Montpellier, France); CTGF (EST clones).

### Chromatin immunoprecipitation

ChIP assays were performed as previously described (Havis et al., 2006). 12 limbs from E9.5 chick embryos were homogenised using a mechanical disruption device (Lysing Matrix A, Fast Prep MP1, 40 sec at 6 m/s). 10 µg of the rabbit polyclonal YAP antibody (Santa Cruz Biotechnology) or 10 µg of the acetylated histone H4 (ACH4) antibody (Upstate Biotechnology) as a positive control were used to immunoprecipitate 20 µg of sonicated chromatin. ChIP products were analysed by PCR. Three pairs of primers were designed to amplify fragments upstream of the coding sequence of the *JAG2* gene that presented putative binding sites for YAP/TEAD (MCAT elements; CATTCC). One pair of primers was designed to amplify a fragment upstream of *JAG2* that did not present a MCAT element, as negative control. Primer pairs are summarized in the Supplementary Table 2.

## Image Capturing

After immunohistochemistry or *in situ* hybridisation, images of the sectioned samples were obtained using a Nikon epifluorescence microscope, a Leica DMI600B fluorescence microscope or a Leica SP5 confocal system.

## Quantification and statistical analyses

Forelimbs of chick embryos were processed for cryostat transverse and longitudinal sections and immunohistochemistry. To quantify the number of PAX7+ cells, several sections of the manipulated muscles were analyzed in 3 different embryos and respective controls. The area of the manipulated and control muscles was analised in the same sections where the counting of PAX7+ cells was performed. Data were analysed using the paired student *t*-test. Asterisks in figures indicate the different *p*-values (* <0.05 and ** <0.01). Cell quantification and muscle area measurements were done using 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

## 2 – Muscle contraction activates YAP and NOTCH signalling and thus regulates the pool of muscle progenitor cells during foetal myogenesis

### Additional information

**1 – BMP activity is not affected in muscle progenitors in the absence of muscle contraction**

Immobilised chick embryos displayed a diminution in the number of muscle progenitors. We showed that in the absence of muscle contraction NOTCH activity is decreased and that forced NOTCH activation can rescue the muscle progenitor pool. This suggests that NOTCH is responding to mechanical forces. BMP is another signalling pathway that positively regulates the number of muscle progenitors. We analysed if BMP signalling pathway was affected in immobilised embryos.

During foetal myogenesis, BMP is active in a subset of muscle progenitors and myonuclei at the muscle tips (Wang et al., 2010). We observed that in DMB-treated embryos, despite the decrease in the number of muscle progenitors, PSMAD1/5/8 was still observed in PAX7+ cells (Additional Figure 1C-H, arrows). Moreover, MF20+ cells also co-localised with PSMAD1/5/8 at the muscle tips in immobilized embryos (Additional Figure 1G-J, arrowheads). The relative expression of the BMP target genes *ID2* and *ID3* was not changed in immobilised embryos, while *ID1* expression was significantly downregulated in limbs of DMB-treated embryos compared to controls (Additional Figure 1K). These results indicate that BMP activity does not appear to be affected in muscle progenitors and fibres in the absence of mechanical forces.

We believe that the activity of NOTCH but not that of BMP is affected in the absence of mechanical forces and that this results in a decrease in the number of muscle progenitors.

**2 – Mechanical forces are required to maintain the F-actin striation of muscle fibres**

Mechanical forces derived from muscle contraction are required for correct foetal muscle development. In the absence of muscle contraction, YAP activity is decreased,

assessed with the loss of *CTGF* expression in muscle fibres and the loss of nuclear YAP in myonuclei. YAP activity can be modulated at the phosphorylation level by the Hippo pathway or by direct protein binding, both resulting in the retention of YAP in the cytoplasm. YAP has been shown to respond to mechanical stimuli and to be activated upon actin polymerization (reviewed by Zhao et al., 2011). During development muscle fibres start to display a striation derived from the organization of F-actin cables (Additional Figure 2A). We assessed the F-actin organisation in immobilised embryos. We observed that in DMB-treated embryos the muscle fibre striation was lost and actin bundles appeared to be thinner compared to the control (Additional Figure 2).

This suggests that in the absence of muscle contraction, the organisation of F-actin cables is impaired in muscle fibres and this could contribute to a decreased YAP activity.

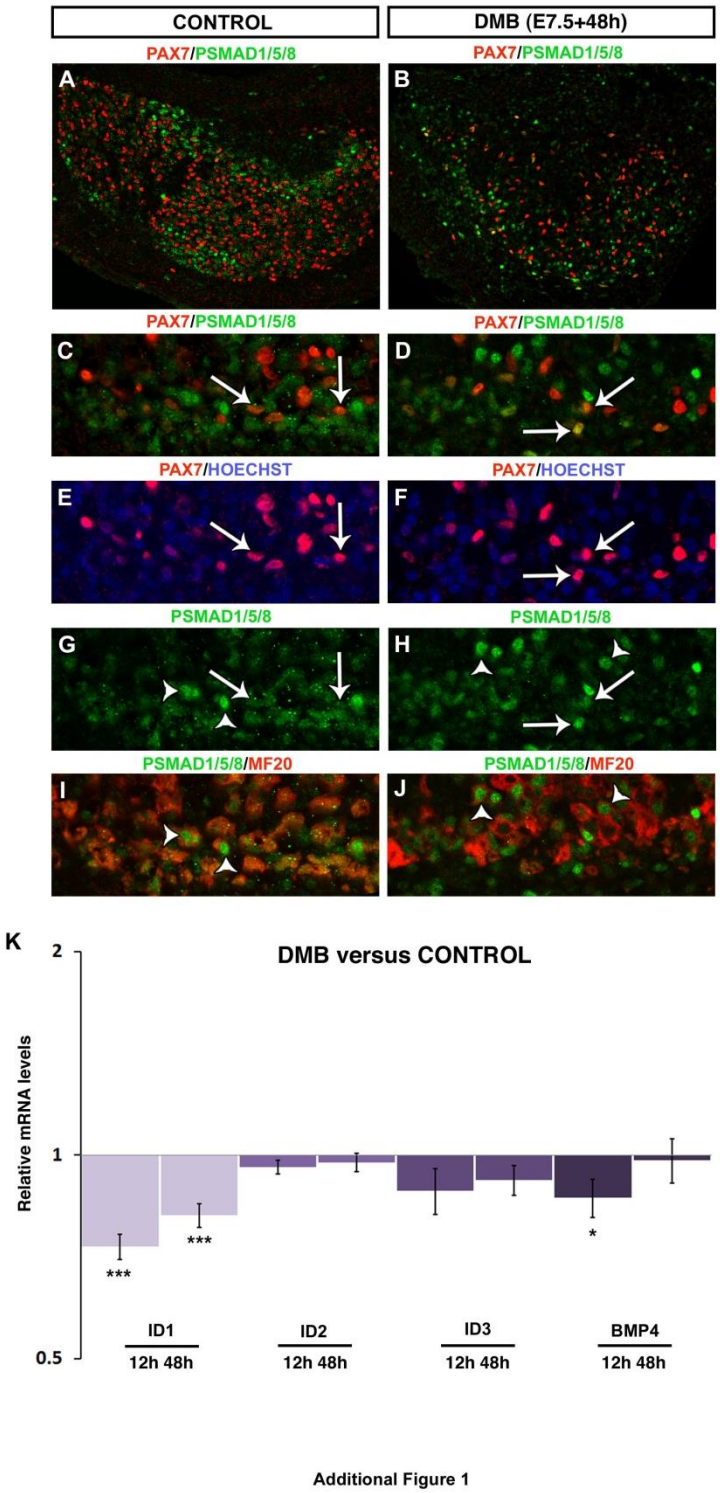
**3 – Mechanical forces are required for limb muscle differentiation**

Blocking mechanical forces during chick foetal stages lead to a decrease in the number of muscle progenitors due to a shift towards differentiation. Several studies performed in mouse and chick models lacking skeletal muscle contraction demonstrated that in the absence of muscle contraction limb muscle formation starts to occur but muscle undergoes degeneration during foetal stages (Edom-Vovard et al., 2002; Pai, 1965a, 1965b; Rong et al., 1992). We tested if mechanical forces are required for limb muscle differentiation at embryonic stages where first fusion events start to take place in the limbs.

Limbs of chick embryos immobilised by DMB treatment at E4.5 displayed a significant decrease in the expression levels of muscle genes *PAX7*, *MYF5*, *MYOD* and *MYOG* at 48h and 72h after DMB exposure, compared to the control (Additional Figure 3B). Consistently, we observed a drastic decrease in muscle mass (decreased MF20+ cells and *MYOD* expression) and in the number of muscle progenitors (PAX7+ cells) in DMB treated embryos compared to controls (Additional Figure 3C-H).

We conclude that mechanical forces influence muscle differentiation and that limb muscle masses are considerably smaller in immobilised embryos.

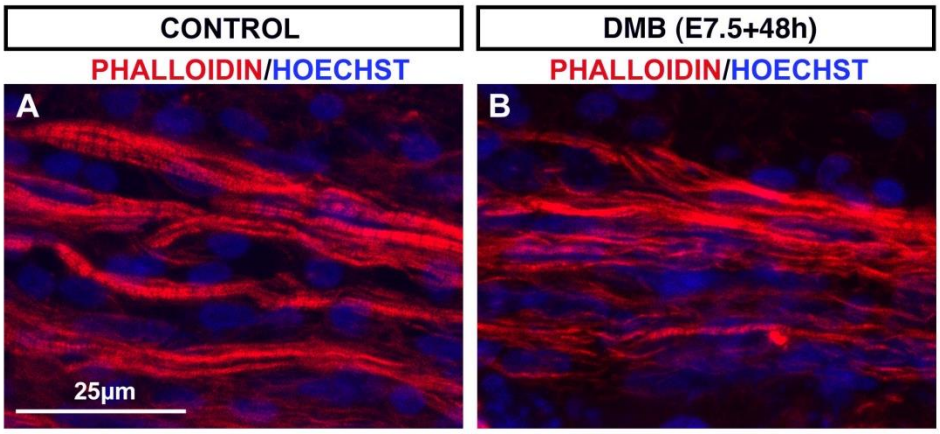
## Additional Figures




Additional Figure 1

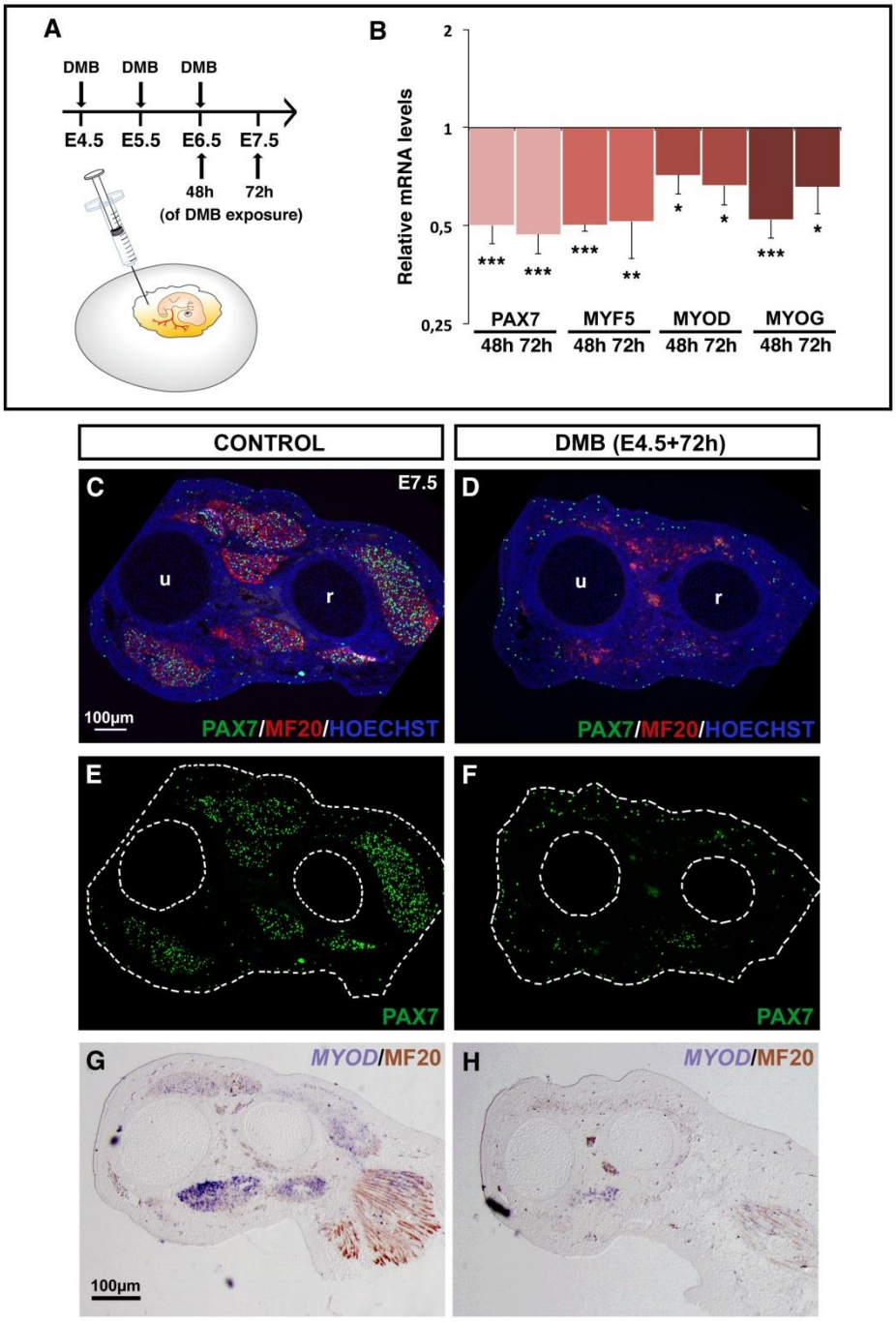
**Additional Figure 1 – BMP signalling is not affected in muscle progenitors in the absence of muscle contraction.** (A-J) Transverse limb sections of E7.5 DMB-treated (B, D, F, H, J) or control (A, C, E, G, H) embryos were immunostained with PAX7 or MF20 and

PSMAD 1/5/8 antibodies. (A-B) Low magnification showing PAX7+ cells co-localising with PSMAD1/5/8 at muscle tips in both DMB-treated (A) embryos and control (B). (C, E, G, I) and (D, F, H, J) are high magnifications of (A) and (B), respectively, showing PAX7+/PSMAD1/5/8+ (C-H) and MF20+/PSMAD1/5/8+ (G-J) cells in DMB-treated (D, F, H, J) and control embryos (C, E, G, I). (K) RT-q-PCR analyses of the expression levels of BMP signalling components in limbs treated with DMB at E7.5 for 12h (E8) or 48h (E9.5). For each gene, the mRNA levels of control limbs were normalized to 1. The relative expression levels of *ID2* and *ID3* were not changed while those of *ID1* were downregulated in limbs when muscle contraction was blocked, 12h and 48h after DMB application. The relative expression of *BMP4* was first downregulated (12h) but then increased after 48h of DMB exposure compared to control. Asterisks indicate the p-value, *$p$<0.05; *$p$<0.01; ***$p$<0.001; error bars indicate SEM.




**Additional Figure 2**

**Additional Figure 2 – Muscle fibres striation is misshaped in DMB-treated embryos.** (A-B) Longitudinal limb sections of E9.5 DMB-treated (B) or control (A) embryos were stained with PHALLOIDIN to visualise F-actin filaments. The F-actin striation is misshaped in DMB-treated embryos (B) compared to the control (A).




Additional Figure 3

**Additional Figure 3 – Mechanical forces are required for the limb muscle differentiation.** (A) Chick embryos were treated with DMB at E4.5, E5.5 and E6.5 in order to block muscle contraction. Embryos were processed 48h (at E6.5) or 72h (at E7.5) after DMB exposure. (B) RT-q-PCR analyses of muscle gene expression levels of 48h and 72h DMB-treated limbs compared to control limbs. For each gene, the mRNA levels of control limbs were normalized to 1. The expression levels of the myogenic genes *PAX7*, *MYF5*, *MYOD* and *MYOG* decreased in DMB-treated limbs, compared to the control. Asterisks

indicate the p-value, *p<0.05; ** p<0.01, ***p<0.001; Error bars indicate SEM. (C-F) Control (C, E, G) and DMB-treated (D, F, H) limbs were transversely sectioned and analysed for muscle markers by immunohistochemistry (C-F) or *in situ* hybridization (G-H). DMB-treated embryos displayed a drastic muscle loss and decreased number of PAX7+ cells in limb muscles (D, F) compared to control embryos (C, E). (G-H) *MYOD* expression was downregulated in limb muscles of DMB-treated embryos (H) compared to control muscles (G), consistent with the loss of muscle mass.

# Discussion and Perspectives

# Discussion and Perspectives

Muscle development, perinatal growth, homeostasis and repair rely on muscle progenitors. During development, in order to maintain the pool of muscle progenitors in an undifferentiated state and avoid premature muscle differentiation, the balance between proliferation and differentiation has to be precisely regulated. The main objective of this project was to decipher the molecular mechanisms regulating the number of foetal muscle progenitors *in vivo*:

For this I characterised the pattern of cell cycle of myogenic cells during foetal myogenesis, investigated the crosstalk between NOTCH and BMP signalling pathways in myogenic differentiation and identified mechanical forces as potential upstream regulator of signalling pathways required for the maintenance of the muscle progenitor pool.

# Discussion and Perspectives

## 1 – *In vivo* characterisation of the cell cycle state during foetal myogenesis

We observed that muscle progenitors and non-myogenic cells in the S/G2/M phases of the cell cycle are regionalised at the muscle tips during foetal myogenesis (Esteves de Lima et al., 2014). For these analyses, we used the Fucci system that has been developed as a tool to visualise the different phases of the cell cycle (Sakaue-Sawano et al., 2008). The developers of this system took advantage of the fact that different proteins oscillate during the cell cycle, and designed fusion proteins to visualise the G1 phase in red (mKO2-hCtd1) and the S/G2/M phases in green (mAG-hGem) (Sakaue-Sawano et al., 2008). To confirm that the increased number of Pax7+ cells in S/G2/M phases at the muscle tips is due to an increase in proliferation and not to an increase in the length of S/G2/M phases, we performed EdU labelling. Compared to the centre of muscles, in the muscle tips the number of Pax7+/EdU+ cells are increased (data not shown). Thus, the proliferative activity of muscle progenitors at the tips is increased. Several studies have demonstrated a regionalisation of proliferative signals in the muscle (Edom-Vovard et al., 2001; Wang et al., 2010; Esteves de Lima et al, unpublished data). The specific expression of *BMP4* in tendons restricts the location of progenitor cells which activate BMP signalling to the muscle tips (Wang et al., 2010). In addition, secreted signals that drive cell cycle progression like *FGF4* and *CTGF* are expressed in fibres at the muscle tips in chick (Edom-Vovard et al., 2001; Esteves de Lima et al., unpublished data). Indeed, increased number of satellite cells can be observed at the ends of growing fibres in the post-hatched chicken (Allouh et al., 2008). In addition, activated satellite cells are clustered at the end of isolated adult muscle fibres (Wang et al., 2010). Taken together, these results suggest that the muscle tips constitute a specialized environment that acts as a signalling centre in foetal muscle growth. In particular, the presence of such regionalised signals can explain the increased proliferation of muscle progenitors at the muscle tips compared to the centre of muscle.

The double transgenic mouse line for the Fucci system allowed us to evaluated cell cycle progression in developing mice. During foetal myogenesis, Pax7+ cells expressed mainly Fucci green, showing that a great part of the Pax7+ cells were progressing through the cell cycle. It was unexpected that so few Pax7+ cells expressed the Fucci red reporter (G1) (Esteves de Lima et al., 2014). Stem cells are characterised by a short G1 phase that increases

in length during the differentiation process (Roccio et al., 2013). Due to their short G1 phase, cultured embryonic stem cells (ESCs) almost exclusively express Fucci green (Roccio et al., 2013). In addition, neuronal stem/progenitor cells (NSCs) triggered to differentiate display an increased intensity of the Fucci red reporter, which correlates with the increase in the length of the G1 phase (Roccio et al., 2013). Thus, the Fucci red reporter protein needs considerable time to accumulate to a level needed to be visible, and it accumulates to such a level only when the G1 phase is long. Pax7+ cells represent the foetal pool of muscle progenitors, and we believe that they could therefore display similar characteristics as other stem cells and also possess a very short G1 phase.

We observed that the majority of the Pax7+ cells do not display any Fucci reporter (57.6%) (Esteves de Lima et al., 2014). To further define the pool of Pax7+/Fucci- cells, the percentage of cycling muscle progenitors in an early G1 phase (not labelled by Fucci) were determined by EdU labelling. This allowed us to define if any of these cells were quiescent. We could observe that 30% of the Pax7+/EdU+ population did not display the Fucci labelling. We assume that these correspond to the pool of cycling Pax7+ cells in an early G1 phase. This might suggest that the remainder, around 30% of the Pax7+ cells, are quiescent (Esteves de Lima et al., 2014). This is in agreement with previous studies showing that muscle progenitors attain quiescence before they adopt a satellite cell position (Picard and Marcelle, 2013).

Muscle fibre nuclei displayed a strong Fucci red staining intensity *in vivo*, in cultured myoblasts and in isolated muscle fibres (Esteves de Lima et al., 2014). A high intensity of the Fucci red reporter was previously observed in mouse post-mitotic neurons (Sakaue-Sawano et al., 2008) and in post-mitotic muscle cells of transgenic zebrafish (Sugiyama et al., 2009). Myoblasts exit the cell cycle from G1, after a long G1 phase (Bischoff and Holtzer, 1969). The length of the G1 phase before the exit of the cell cycle and differentiation could explain the high intensity of the Fucci red reporter in terminally differentiated muscle cells. The presence of Fucci red in these cells suggests that these terminally differentiated cells display a G1-like state. However, an alternative explanation might be that the fusion protein encoded by mKO2-hCdt1 (Fucci red) is more stable than endogenous hCdt1 and persists in terminally differentiated cells.

Satellite cells of freshly isolated muscle fibres from EDL (extensor digitorum longus) muscles of double transgenic Fucci mice are systematically negative for the Fucci system

(Esteves de Lima et al., 2014). This is in accordance with the quiescent state of satellite cells (Fukada et al., 2007). When these fibres are cultured, satellite cells become activated, proliferate and give rise to heterogeneous progeny that expresses MyoD, Pax7 or a combination of the two (Zammit et al., 2004). After 72h in culture, clusters of cells had formed and the Pax7+ cells in the cluster virtually never expressed the Fucci red reporter (G1 phase) but could be visualised with Fucci green (S/G2/M phases). This is similar to the Fucci expression in the developing muscle *in vivo*, and we assign the absence of Pax7+/Fucci red+ cells to the fact that these cells cycle too fast to accumulate Fucci red in G1. In addition, MyoD+ cells in satellite cell clusters expressed either Fucci red or Fucci green reporters. These results demonstrate that cultured satellite cells on fibres behave similar to muscle progenitors in regards to their expression of Fucci red/green.

We observed that the Fucci system does not completely cover the different cell cycle phases and might not faithfully allow to distinguish cycling cells in the G1 phase or terminally differentiated cells. However, the Fucci green reporter expressed during S/G2/M phases can be used to detect cells that are progressing through the cell cycle.

# Discussion and Perspectives

## 2 – Interaction between BMP and NOTCH pathways during foetal myogenesis

The BMP and NOTCH pathways positively regulate the number of muscle progenitors (Delfini et al., 2000; Vasyutina et al., 2007a; Amthor et al., 1998; 1999; Wang et al., 2010; Mourikis et al., 2012a). We showed that DLL1-mediated activation of NOTCH in myogenic cells of the chick limb significantly increased the number of PAX7+ cells and inhibited differentiation. DLL1-mediated activation of NOTCH occurs via activation of the canonical NICD/RBP-J pathway in the signal receiving-cell (Jarriault et al., 1998). While activation of BMP and NOTCH leads to an increased pool of muscle progenitors, the effect of the two pathways during differentiation diverges. During foetal myogenesis, BMP increases not only the number of progenitors but also the number of fibres and muscle size, whereas NOTCH increases the progenitor pool at the expense of muscle differentiation (Delfini et al., 2000; Vasyutina et al., 2007a; Wang et al ., 2010; Mourikis et al., 2012a).

Since both BMP and NOTCH play a similar role in the maintenance of the muscle progenitor pool, we examined whether crosstalk between the two signalling pathways exists. We showed that activation of NOTCH in myogenic progenitors does not activate BMP signalling (*i.e.* Smad1/5/8 phosphorylation) in PAX7+ cells. However, activation of BMP increases the expression of *NOTCH1* in muscle masses. Upon NOTCH activation, NICD is released, and antibodies specific for NICD exist and can be used to directly assess the activity of the signalling pathway. We were unable to directly define if *NOTCH1* upregulation was accompanied by increased NOTCH activity in PAX7+ cells, since the NICD antibody did not work in our hands. Nevertheless, these results suggest that in chick foetal muscles, BMP activates NOTCH by increasing *NOTCH1* expression. In order to further investigate the crosstalk between BMP and NOTCH signalling, we analysed primary cultures of chick foetal myoblasts under proliferation (high serum) and differentiation (low serum) conditions. Consistently with the *in vivo* situation, activation of BMP in cultured foetal myoblasts that are proliferating increased the expression of *HEYL*, a NOTCH target gene. BMP or TGFβ-mediated increase in the expression of NOTCH target genes has been observed in other cell types (Blokzijl, 2003; Dahlqvist, 2003). Activation of NOTCH signalling does not change the expression of BMP target genes in foetal myoblasts. Since activation of BMP increased the

number of PAX7+ cells *in vivo* and increased *PAX7* mRNA levels *in vitro*, we asked whether this effect could depend on the presence of active NOTCH. In cultured foetal myoblasts, forced BMP activity or forced BMP activity and simultaneous blockage of NOTCH have similar effects on *PAX7* expression. This indicates that BMP-mediated activation of *PAX7* is independent of NOTCH activity. *In vivo*, NOTCH is active in all muscle progenitors but BMP is active only in progenitors at the muscle tips (Delfini et al., 2000; Vasyutina et al., 2007a; Wang et al., 2010; Mourikis et al., 2012a). In this area, we observe an increase in the number of Pax7+ cells in S/G2/M phases compared to the centre of the muscle, in mouse embryos. This might be due to a synergistic effect of NOTCH and BMP in these regions.

Under differentiation conditions, forced BMP activity has a distinct effect on the expression levels of NOTCH target genes. In particular, myoblasts that differentiate respond to BMP by downregulating *HES1* and *HEYL*. Conversely, inhibition of BMP signalling by overexpressing *SMAD6* increases expression of *HES1* and *HEYL*. Thus, BMP inhibits NOTCH signalling in differentiating myoblasts. Furthermore, activation of NOTCH in differentiating cultures decreases expression of the BMP targets *ID1*, *ID2* and *NOG*. Thus, the BMP and NOTCH signalling pathways antagonize each other in differentiating myoblasts. *In vivo*, both pathways, NOTCH and BMP, are active in muscle progenitors at the muscle tips, whereas BMP but not NOTCH is also active in differentiated muscle cells in this area (Delfini et al., 2000; Vasyutina et al., 2007a; Schuster-Gossler et al., 2007; Mourikis et al., 2012a). Ectopic BMP in C2C12 cultures inhibits myotube differentiation but downregulation of endogenous BMP interferes with myoblast fusion, showing a role for BMP in differentiation (Furutani et al., 2011; Umemoto et al., 2011). At the muscle tips, BMP and NOTCH might interact in muscle progenitors to promote the expansion of the pool. However, in this area, BMP might contribute to the inhibition of NOTCH, required to achieve differentiation of myoblasts that still present active BMP but not NOTCH. Therefore, the muscle tips might be a preferred location for the expansion of the progenitor pool but also for myoblast differentiation and fibre growth.

My study reveals a distinct interaction between BMP and NOTCH signalling in proliferating and differentiating myogenic progenitors that allows insight into the distinct roles of these signalling pathways during myogenesis. The intracellular mechanisms underlying the crosstalk between these pathways in foetal myogenesis are not completely understood. In the literature, several studies highlighted an antagonism between NOTCH and BMP during cellular differentiation (Grishina et al., 2005; Maier et al., 2011; Uribe et al.,

2012). In particular, Hey1 has been shown to promote degradation of Id protein and inhibit BMP activity in endothelial cells (Itoh et al., 2004).

# Discussion and Perspectives

## 3 – Muscle progenitors and fibres communicate by NOTCH signals

Several studies suggested that the presence of differentiating myoblasts and muscle fibres are required to maintain the pool of muscle progenitors (Cusella-De Angelis et al., 1994; Kassar-Duchossoy, 2005; Zalc et al., 2014). In particular, in *Myf5/MyoD*-double mutant mouse embryos which lack differentiated myogenic cells, the number of Pax3+ progenitor cells co-expressing the cell cycle exit marker p57 increases, indicating that growth arrest is precocious when differentiated myoblasts are lacking (Zalc et al., 2014). Furthermore, NOTCH activity is decreased in the *Myf5/MyoD*-double mutant embryos, suggesting that NOTCH ligands expressed in differentiated myoblasts and/or fibres signal to muscle progenitors and maintain their undifferentiated and proliferative state (Zalc et al., 2014).

We aimed to investigate if NOTCH ligands expressed by differentiated myogenic cells can sustain the progenitor pool. In the chick at E5, *DLL1* and *JAG2* are expressed in differentiated myoblasts and at E7 *JAG2* is restricted to muscle fibres (Delfini et al., 2000). Variants of DLL and JAG with a truncation of the intracellular domain act as dominant-negative forms, compete with endogenous ligands for receptors and bind but do not activate NOTCH receptors (Chitnis et al., 1995; Henrique et al., 1997; Sun and Artavanis-Tsakonas, 1996). During NOTCH receptors maturation and activation, several proteolytic cleavage events take place. The first occurs during receptor maturation in the Golgi vesicles (S1 cleavage by Furin) and forms a bipartite heterodimeric NOTCH receptor. The second (S2) relies on the binding to the ligand, is carried out by ADAM metalloprotease and cleaves in the membrane-proximal extracellular domain (reviewed by Chitnis, 2006). Once the binding between the receptor and the ligand takes place, the internalisation of the ligand in the signal-sending cell is required to allow conformational changes in the NOTCH extracellular domain (pulling of the receptor), and this exposes the ADAM cleavage site (reviewed by Chitnis, 2006). In the absence of an intracellular domain, DLL1 fails to be internalised and therefore does not activate NOTCH (reviewed by Chitnis, 2006). The final cleavage of the NOTCH receptor occurs intracellularly by the γ-secretase complex and liberates NICD that is then free to translocate into the nucleus. DLL1/NOTCH is the best understood ligand/receptor pair, but

a similar activation mechanism, which depends on ligand internalisation, was also demonstrated for JAG (reviewed by Chitnis, 2006). Several researchers have used ligands that lack the intracellular domain for analysis of NOTCH functions. In the chick, forced expression of DELTA1-DN (DLL1 that lacks the intracellular domain) blocks NOTCH signalling during neurogenesis (Henrique et al., 1997). I expressed DELTA1-DN specifically in differentiated cells (MLC promoter) to block the function of NOTCH ligands present in differentiated muscle cells (myoblasts and fibres).

The overexpression of DELTA1-DN in differentiated cells and fibres does not affect muscle organisation. However, the number of PAX7+ progenitor cells is significantly decreased. This demonstrates that interfering with NOTCH ligands reduces the pool of muscle progenitors even when differentiated myoblasts and fibres are left intact. This is in agreement with previous results that demonstrated that forced NICD expression in Myf5+ myoblasts suffices to impair muscle differentiation in mouse. Despite of complete absence of differentiation, the pool of muscle progenitors in maintained throughout all myogenic stages in such animals (Mourikis et al., 2012a).

# Discussion and Perspectives

## 4 – Role of mechanical forces in muscle development

Mechanical forces produced by muscle contraction are involved in the normal development and establishment of the musculoskeletal system. The role of mechanical forces in myogenesis is poorly understood. We investigated the role of mechanical forces in the maintenance of muscle integrity during development.

Embryos exposed to drugs that immobilize the muscle for 48h (from E7.5 to E9.5) present disorganised muscle fibres with apoptotic figures, indicating that the muscle degenerates in the absence of mechanical forces. These observations are in agreement with previous observations that show that in the absence of innervation muscle degenerates in the chick limb (Edom-Vovard et al., 2002; Rong et al., 1992). In addition, skeletal muscles unable to contract also degenerate in mouse embryos (Pai, 1965a; 1965b). In the absence of muscle contraction, we observed a drastic decrease in the number of PAX7+ cells. This is associated with an increased expression of *MYOD* and *MYOG*. However, PAX7+ cells do not undergo apoptosis and are progressing through the cell cycle (EdU labelling) in the absence of mechanical forces. These results suggest that the loss of PAX7+ cells in the absence of muscle contraction is due to a change in the balance between proliferation/differentiation towards differentiation, rather than a loss of progenitor cells by apoptosis. The role of mechanical forces on the maintenance of muscle progenitors had not been addressed before. Mouse and human studies indicate that muscle stimulation and exercise attenuates the age-related decline of satellite cells and skeletal muscle mass (reviewed by Snijders et al., 2009). Thus, muscular activity stimulates satellite cells during ageing. In addition, high intensity exercise increases the number of satellite cells in young adults (Crameri et al., 2004). I showed for the first time in this study that mechanical forces are required to maintain the pool of foetal muscle progenitors.

# Discussion and Perspectives

## 5 – Link between mechanical forces and the NOTCH signalling

Loss of muscle contraction leads to a depletion of the muscle progenitor pool and resembles the NOTCH loss-of-function phenotype (Vasyutina et al., 2007a). In both cases, the muscle progenitor pool is lost because of precocious differentiation, which is accompanied by increased *MYOD* expression. During muscle development, muscle progenitors receive the NOTCH signals, while the differentiated myoblasts and myotubes present the NOTCH ligands (Delfini et al., 2000; Hirsinger et al., 2001; Mourikis et al., 2012a). We analysed whether NOTCH signalling was reduced when muscle contraction is blocked. Expression of several components of the NOTCH signalling pathway was significantly downregulated when the muscles could not contract. In particular, the NOTCH targets *HES5* and *HEYL*, the NOTCH ligand *JAG2* and the NOTCH receptor *NOTCH2* were expressed at reduced levels in these conditions. Further analysis using *in situ* hybridization showed that *JAG2* was downregulated in muscle fibres in the absence of muscle contraction and that *HES5* was downregulated in muscle masses but not in endothelial cells. DLL1 was regulated in a distinct manner, *i.e.* it was first downregulated 12h after the loss of contraction, but was upregulated subsequently. The increase in the expression of *MYOD* and *MYOG* in the absence of muscle contraction suggests the depletion of the muscle progenitor pool due to an increase in myogenic differentiation accompanied by increased *DLL1*.

The changes in muscle development in the absence of muscle contraction suggests that mechanical forces are required to maintain the expression of the NOTCH ligand *JAG2* and thus control the pool of muscle progenitors by regulating NOTCH signalling. To define whether NOTCH activity prevents the loss of the progenitor pool in immobilized embryos, we forced activation of NOTCH signalling by overexpressing *DLL1*. Ectopic *DLL1* attenuated the loss of the progenitor pool in the absence of mechanical forces. The link between NOTCH and mechanical forces has been little investigated. Muscle denervation, unloading or immobility result in muscle atrophy, and contradictory findings on the consequences of these conditions on NOTCH signalling were observed. Upon muscle denervation in the rat, the E3 ubiquitin ligase Nedd4 is upregulated which also targeted Notch1 for degradation and lead to a decreased NOTCH activity (Koncarevic et al., 2007).

Other studies performed in rat and mouse, revealed that the levels of NICD are increased upon muscle denervation, which is accompanied by an increase in *Hey1* expression (Liu et al., 2011; Nagpal et al., 2012). In addition, Notch1 protein levels were found to be unchanged after denervation of muscle in mice (Nagpal et al., 2012). Denervation which might stimulate repair mechanisms and increase the activity of satellite cells, could explain the increase in the NOTCH activity observed in the latter studies. During cardiac development, NOTCH was proposed to mediate signals that link hemodynamics (heart blood flow) and morphogenesis. Mechanical forces exerted by cardiac contractions were proposed to activate NOTCH in endocardial cells, a process required for correct ventricular trabeculation and assembly (reviewed in Granados-Riveron and Brook, 2012). In this study, I demonstrated that mechanical forces enhance NOTCH signalling by upregulating expression of *JAG2* in the muscle fibres. Thereby, mechanical forces maintain the pool of muscle progenitors during foetal muscle development.

# Discussion and Perspectives

## 6 – Mechanical forces are sensed in muscle fibres by YAP

When muscle contraction is abolished during foetal muscle development, NOTCH activity is reduced. YAP is a transcriptional regulator whose activity is known to be controlled by mechanical input. YAP is highly expressed in mouse foetal skeletal muscles, is present in adult myofibres and positively controls muscle mass (Watt et al., 2015). Subcellular location determines YAP activity, which needs to be present in the nucleus to exert its transcriptional function. We observed that nuclear YAP is present in muscle fibres and in a subset of PAX7+ cells. In the absence of muscle contraction, nuclear YAP is lost in fibres but not in muscle progenitors. In addition, expression of the YAP target gene *CTGF* is decreased in muscle fibres. These results suggest that the absence of mechanical forces decreases YAP activity in the fibres. As early as 12h after immobilization, expression of *JAG2* is downregulated in muscle fibres. Thus, the absence of mechanical forces is sensed by the fibres and results in gene expression changes that impair also the fibre-associated progenitor cells.

YAP binds to the DNA indirectly through its interaction with transcription factors like TEAD, and YAP/TEAD is known to directly bind to regulatory sequences in the *JAG1/2* gene in human mammary epithelial cells (Zhao et al., 2008). Downregulation of YAP in the hepato-carcinoma cell line HuH-7 leads to a decrease in JAG1 and NICD protein levels, and decreased *HES1* expression (Tschaharganeh et al., 2013). Thus, YAP controls NOTCH activity in the foetal muscle and in several other cell types. We think that the muscle fibres primarily sense muscle contraction, and that contraction-dependent effects on muscle progenitors are caused indirectly by changes in *JAG2* expression that signals to the progenitors. We searched for putative regulatory sequences upstream of chick *JAG2* gene which contain the sequence motif recognized by YAP/TEAD. We identified two sequences upstream of *JAG2* to where YAP is recruited in chick limbs. This suggests that YAP directly regulates *JAG2* gene. To confirm this, we plan to electroporate a constitutively active form of the mouse Yap (mYapS112A) (Xin et al., 2013) in differentiated muscle cells using a Tol2 vector system that allows stable integration into the genome (using the MLC promoter). Our preliminary results on cultured foetal myoblasts show that the forced activation of YAP

increases expression of its target genes *CTGF* and *CYR61*, in addition to components of the NOTCH signalling pathway like *JAG2*, *NOTCH2* and *HEYL*. Thus, YAP is able to induce *JAG2* expression in myoblasts. Further experiments will demonstrate whether activation of YAP upregulates the expression of NOTCH ligands, in particular *JAG2*, in muscle fibres *in vivo*. Finally, I will perform rescue experiments and determine whether forced YAP activation in immobilized muscles positively affects *JAG2* expression and the progenitor number *in vivo*.

Another open question is how YAP senses muscle contractions in the foetal muscle. Active Hippo signalling phosphorylates MST1/2, which phosphorylate LATS1/2 and in turn phosphorylate YAP; phosphorylated YAP is retained in the cytoplasm and thus inactive (reviewed by Zhao et al., 2011). MST1/2 have pro-apoptotic activity, in accordance with the role of the Hippo pathway as a regulator of organ size. In accordance, downregulation of *YAP* in chick neural progenitors *in vivo* leads to increased apoptosis (Cao et al., 2008). We tried to compare phosphorylation of MST1 protein in immobilized and mobile muscles using a commercial antibody, but this was unsuccessful. Activation of the MST1/2 proteins could explain the increase in apoptosis in the immobilized muscle fibres. Alternatively, muscle degeneration might be due to activation of muscle-specific E3 ubiquitin ligases. Such ligases contribute to muscle atrophy in the adult muscle (reviewed by Sartori et al., 2014). YAP is regulated independently of the Hippo pathway in mechanotransduction (Aragona et al., 2013; Dupont et al., 2011; Mana-Capelli et al., 2014). In particular, actin polymerisation in response to mechanical forces increases YAP activity; this is mediated by binding of Amot to F-actin which releases YAP that is then free to move to the nucleus (Mana-Capelli et al., 2014). I also observed that the striation of the immobilized muscles was abnormal. This suggests that F-actin is disorganised in the absence of mechanical forces, which might contribute to the changes in YAP activity.

# Conclusion

# Conclusion

Different signals operate in specific regions of the developing foetal muscle. In particular, the muscle tips are close with tendons and therefore exposed to a restricted source of signals. Muscle is known to influence tendon development, but the signals provided by tendons that influence myogenesis are not fully understood. In my thesis, I further highlighted the fact that muscle progenitors present at the tips are exposed to different signals than those in the centre of the muscle. We showed that muscle progenitors at the tips proliferate more than those in the centre. This might be due to the combined activity of BMP and NOTCH signalling at the tips, which can potentiate progenitor cells proliferation. With the experiments performed in cultured primary myoblasts, we defined a synergistic interaction between the BMP and NOTCH pathways in proliferation, and an antagonistic effect during differentiation. We showed that in differentiating myoblasts, activating either BMP or NOTCH inhibits the output of the other pathway. This is in agreement with a requirement of active BMP and inactive NOTCH during differentiation. The endogenous sites of BMP and NOTCH activity *in vivo* also are in accordance with this. My work demonstrates for the first time that the mechanisms by which BMP and NOTCH pathways crosstalk differ in proliferating and differentiating myoblasts.

How mechanical forces affect developmental processes, and how cells respond to different mechanical inputs is an important topic in biology. In particular, how mechanical signals from the external environment are sensed by the cells and transduced into a chemical or signalling output is an area of active research. I showed for the first time during my thesis that mechanical forces are required to maintain muscle progenitors in an undifferentiated state during development. Loss of mechanical forces leads to a precocious differentiation of the progenitors and thus to a depletion of the progenitor pool. This mimics a NOTCH loss-of-function phenotype. Consistently, NOTCH activity is lost in muscles in the absence of mechanical forces. The activity of other signalling pathways, like BMP, is not changed by immobilisation. Forced NOTCH activity rescues the pool of muscle progenitors in the absence of mechanical forces. This suggests that NOTCH signalling is regulated by mechanical forces.

YAP is a mechanosensitive gene that responds to changes in external mechanical input. We showed that YAP activity is lost in muscle fibres in the absence of mechanical

forces, demonstrating that fibres act as primary "sensors". We showed that YAP binds to upstream regulatory sequences of the NOTCH ligand *JAG2* in chick limbs. Since *JAG2* expression is drastically decreased in muscle upon immobilisation, we hypothesise that YAP regulates *JAG2* expression in fibres. Forced activation of YAP in myoblasts increases the expression of *JAG2* and activates NOTCH signalling. We still have to prove that this regulatory mechanism mediated by YAP is active also *in vivo*.

Thus, a crosstalk exists between differentiated muscle fibres that signal to the associated progenitors via NOTCH ligands. Our hypothesis is that *JAG2* expression is lost in fibres that are not exposed to mechanical forces. As a consequence, NOTCH activity in muscle progenitors is reduced which results in a depletion of the progenitor pool.

# References

# References


Akiho, M., Nakashima, H., Sakata, M., Yamasa, Y., Yamaguchi, A., and Sakuma, K. (2010). Expression profile of Notch-1 in mechanically overloaded plantaris muscle of mice. Life Sci. *86*, 59–65.

Alarcón, C., Zaromytidou, A.-I., Xi, Q., Gao, S., Yu, J., Fujisawa, S., Barlas, A., Miller, A.N., Manova-Todorova, K., Macias, M.J., et al. (2009). Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. Cell *139*, 757–769.

Allouh, M.Z., Yablonka-Reuveni, Z., and Rosser, B.W. (2008). Pax7 reveals a greater frequency and concentration of satellite cells at the ends of growing skeletal muscle fibers. J. Histochem. Cytochem. *56*, 77–87.

Amthor, H., Christ, B., Weil, M., and Patel, K. (1998). The importance of timing differentiation during limb muscle development. Curr. Biol. *8*, 642–652.

Amthor, H., Christ, B., and Patel, K. (1999). A molecular mechanism enabling continuous embryonic muscle growth-a balance between proliferation and differentiation. Development *126*, 1041–1053.

Andersson, E.R., Sandberg, R., and Lendahl, U. (2011). Notch signaling: simplicity in design, versatility in function. Development *138*, 3593–3612.

Andrés, V., and Walsh, K. (1996). Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J. Cell Biol. *132*, 657–666.

Aragona, M., Panciera, T., Manfrin, A., Giulitti, S., Michielin, F., Elvassore, N., Dupont, S., and Piccolo, S. (2013). A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. Cell *154*, 1047–1059.

Arthur, S.T., and Cooley, I.D. (2012). The effect of physiological stimuli on sarcopenia; impact of Notch and Wnt signaling on impaired aged skeletal muscle repair. Int. J. Biol. Sci. *8*, 731–760.

Bai, G., Sheng, N., Xie, Z., Bian, W., Yokota, Y., Benezra, R., Kageyama, R., Guillemot, F., and Jing, N. (2007). Id sustains Hes1 expression to inhibit precocious neurogenesis by releasing negative autoregulation of Hes1. Dev. Cell *13*, 283–297.

Bajard, L., Relaix, F., Lagha, M., Rocancourt, D., Daubas, P., and Buckingham, M.E. (2006). A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. Genes Dev. *20*, 2450–2464.

Barry, E.R., and Camargo, F.D. (2013). The Hippo superhighway: signaling crossroads converging on the Hippo/Yap pathway in stem cells and development. Curr. Opin. Cell Biol. *25*, 247–253.

Beauchamp, J.R., Heslop, L., David, S.W., Tajbakhsh, S., Kelly, R.G., Wernig, A., Buckingham, M.E., Partridge, T.A., and Zammit, P.S. (2000). Expression of CD34 and Myf5

defines the majority of quiescent adult skeletal muscle satellite cells. J. Cell Biol. *151*, 1221–1234.

Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L., and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell *61*, 49–59.

Biressi, S., Molinaro, M., and Cossu, G. (2007a). Cellular heterogeneity during vertebrate skeletal muscle development. Dev. Biol. *308*, 281–293.

Biressi, S., Tagliafico, E., Lamorte, G., Monteverde, S., Tenedini, E., Roncaglia, E., Ferrari, S., Ferrari, S., Cusella-De Angelis, M.G., Tajbakhsh, S., et al. (2007b). Intrinsic phenotypic diversity of embryonic and fetal myoblasts is revealed by genome-wide gene expression analysis on purified cells. Dev. Biol. *304*, 633–651.

Bischoff, R., and Holtzer, H. (1969). Mitosis and the processes of differentiation of myogenic cells in vitro. J. Cell Biol. *41*, 188–200.

Blaauw, B., and Reggiani, C. (2014). The role of satellite cells in muscle hypertrophy. J. Muscle Res. Cell Motil. *35*, 3–10.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature *376*, 768–771.

Blokzijl, A. (2003). Cross-talk between the Notch and TGF- signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. J. Cell Biol. *163*, 723–728.

Bonnet, A., Dai, F., Brand-Saberi, B., and Duprez, D. (2010). Vestigial-like 2 acts downstream of MyoD activation and is associated with skeletal muscle differentiation in chick myogenesis. Mech. Dev. *127*, 120–136.

Braun, T., Rudnicki, M.A., Arnold, H.H., and Jaenisch, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell *71*, 369–382.

Bröhl, D., Vasyutina, E., Czajkowski, M.T., Griger, J., Rassek, C., Rahn, H.-P., Purfürst, B., Wende, H., and Birchmeier, C. (2012). Colonization of the Satellite Cell Niche by Skeletal Muscle Progenitor Cells Depends on Notch Signals. Dev. Cell *23*, 469–481.

Brohmann, H., Jagla, K., and Birchmeier, C. (2000). The role of Lbx1 in migration of muscle precursor cells. Dev. Camb. Engl. *127*, 437–445.

Brooks, N.E., and Myburgh, K.H. (2014). Skeletal muscle wasting with disuse atrophy is multi-dimensional: the response and interaction of myonuclei, satellite cells and signaling pathways. Front. Physiol. *5*, 99.

Bryson-Richardson, R.J., and Currie, P.D. (2008). The genetics of vertebrate myogenesis. Nat. Rev. Genet. *9*, 632–646.

Buas, M.F., and Kadesch, T. (2010). Regulation of skeletal myogenesis by Notch. Exp. Cell Res. *316*, 3028–3033.

Buckingham, M., and Rigby, P.W.J. (2014). Gene Regulatory Networks and Transcriptional Mechanisms that Control Myogenesis. Dev. Cell *28*, 225–238.

Cao, X., Pfaff, S.L., and Gage, F.H. (2008). YAP regulates neural progenitor cell number via the TEA domain transcription factor. Genes Dev. *22*, 3320–3334.

Capdevila, J., Tabin, C., and Johnson, R.L. (1998). Control of dorsoventral somite patterning by Wnt-1 and beta-catenin. Dev. Biol. *193*, 182–194.

Carlson, M.E., Hsu, M., and Conboy, I.M. (2008). Imbalance between pSmad3 and Notch induces CDK inhibitors in old muscle stem cells. Nature *454*, 528–532.

Carlson, M.E., Suetta, C., Conboy, M.J., Aagaard, P., Mackey, A., Kjaer, M., and Conboy, I. (2009). Molecular aging and rejuvenation of human muscle stem cells. EMBO Mol. Med. *1*, 381–391.

Chevallier, A., Kieny, M., and Mauger, A. (1977). Limb-somite relationship: origin of the limb musculature. J. Embryol. Exp. Morphol. *41*, 245–258.

Chitnis, A. (2006). Why is delta endocytosis required for effective activation of notch? Dev. Dyn. *235*, 886–894.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature *375*, 761–766.

Christ, B. (1970). [Experiments on the locational development of the somites]. Verh. Anat. Ges. *64*, 555–564.

Christ, B., and Brand-Saberi, B. (2002). Limb muscle development. Int. J. Dev. Biol. *46*, 905–914.

Christ, B., Jacob, H.J., and Jacob, M. (1974). [Origin of wing musculature. Experimental studies on quail and chick embryos]. Experientia *30*, 1446–1449.

Ciemerych, M.A., Archacka, K., Grabowska, I., and Przewoźniak, M. (2011). Cell cycle regulation during proliferation and differentiation of mammalian muscle precursor cells. Results Probl. Cell Differ. *53*, 473–527.

Classon, M., and Dyson, N. (2001). p107 and p130: versatile proteins with interesting pockets. Exp. Cell Res. *264*, 135–147.

Conboy, I.M., and Rando, T.A. (2002). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev. Cell *3*, 397–409.

Conboy, I.M., Conboy, M.J., Smythe, G.M., and Rando, T.A. (2003). Notch-mediated restoration of regenerative potential to aged muscle. Science *302*, 1575–1577.

Cossu, G., Ranaldi, G., Senni, M.I., Molinaro, M., and Vivarelli, E. (1988). “Early” mammalian myoblasts are resistant to phorbol ester-induced block of differentiation. Dev. Camb. Engl. *102*, 65–69.

Coumailleau, P., and Duprez, D. (2009). Sim1 and Sim2 expression during chick and mouse limb development. Int. J. Dev. Biol. *53*, 149–157.

Crameri, R.M., Langberg, H., Magnusson, P., Jensen, C.H., Schrøder, H.D., Olesen, J.L., Suetta, C., Teisner, B., and Kjaer, M. (2004). Changes in satellite cells in human skeletal muscle after a single bout of high intensity exercise. J. Physiol. *558*, 333–340.

Crameri, R.M., Aagaard, P., Qvortrup, K., Langberg, H., Olesen, J., and Kjaer, M. (2007). Myofibre damage in human skeletal muscle: effects of electrical stimulation versus voluntary contraction. J. Physiol. *583*, 365–380.

Crist, C.G., Montarras, D., and Buckingham, M. (2012). Muscle satellite cells are primed for myogenesis but maintain quiescence with sequestration of Myf5 mRNA targeted by microRNA-31 in mRNP granules. Cell Stem Cell *11*, 118–126.

Crose, L.E.S., Galindo, K.A., Kephart, J.G., Chen, C., Fitamant, J., Bardeesy, N., Bentley, R.C., Galindo, R.L., Chi, J.-T.A., and Linardic, C.M. (2014). Alveolar rhabdomyosarcoma-associated PAX3-FOXO1 promotes tumorigenesis via Hippo pathway suppression. J. Clin. Invest. *124*, 285–296.

Cusella-De Angelis, M.G., Molinari, S., Le Donne, A., Coletta, M., Vivarelli, E., Bouche, M., Molinaro, M., Ferrari, S., and Cossu, G. (1994). Differential response of embryonic and fetal myoblasts to TGF beta: a possible regulatory mechanism of skeletal muscle histogenesis. Development *120*, 925–933.

Czajkowski, M.T., Rassek, C., Lenhard, D.C., Bröhl, D., and Birchmeier, C. (2014). Divergent and conserved roles of Dll1 signaling in development of craniofacial and trunk muscle. Dev. Biol. *395*, 307–316.

Dahlqvist, C. (2003). Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development *130*, 6089–6099.

Daston, G., Lamar, E., Olivier, M., and Goulding, M. (1996). Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. Dev. Camb. Engl. *122*, 1017–1027.

Davidson, I., Xiao, J.H., Rosales, R., Staub, A., and Chambon, P. (1988). The HeLa cell protein TEF-1 binds specifically and cooperatively to two SV40 enhancer motifs of unrelated sequence. Cell *54*, 931–942.

De Falco, G., Comes, F., and Simone, C. (2006). pRb: master of differentiation. Coupling irreversible cell cycle withdrawal with induction of muscle-specific transcription. Oncogene *25*, 5244–5249.

Delfini, M., Hirsinger, E., Pourquié, O., and Duprez, D. (2000). Delta 1-activated notch inhibits muscle differentiation without affecting Myf5 and Pax3 expression in chick limb myogenesis. Development *127*, 5213–5224.

Denetclaw, W.F., and Ordahl, C.P. (2000). The growth of the dermomyotome and formation of early myotome lineages in thoracolumbar somites of chicken embryos. Dev. Camb. Engl. *127*, 893–905.

Dietrich, S., Abou-Rebyeh, F., Brohmann, H., Bladt, F., Sonnenberg-Riethmacher, E., Yamaai, T., Lumsden, A., Brand-Saberi, B., and Birchmeier, C. (1999). The role of SF/HGF and c-Met in the development of skeletal muscle. Dev. Camb. Engl. *126*, 1621–1629.

Domingues-Faria, C., Chanet, A., Salles, J., Berry, A., Giraudet, C., Patrac, V., Denis, P., Bouton, K., Goncalves-Mendes, N., Vasson, M.-P., et al. (2014). Vitamin D deficiency downregulates Notch pathway contributing to skeletal muscle atrophy in old wistar rats. Nutr. Metab. *11*, 47.

Dong, F., Sun, X., Liu, W., Ai, D., Klysik, E., Lu, M.-F., Hadley, J., Antoni, L., Chen, L., Baldini, A., et al. (2006). Pitx2 promotes development of splanchnic mesoderm-derived branchiomeric muscle. Dev. Camb. Engl. *133*, 4891–4899.

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S., et al. (2011). Role of YAP/TAZ in mechanotransduction. Nature *474*, 179–183.

Duprez, D. (2002). Signals regulating muscle formation in the limb during embryonic development. Int. J. Dev. Biol. *46*, 915–926.

Edom-Vovard, F., Bonnin, M.-A., and Duprez, D. (2001). Misexpression of Fgf-4 in the Chick Limb Inhibits Myogenesis by Down-Regulating Frek Expression. Dev. Biol. *233*, 56–71.

Edom-Vovard, F., Schuler, B., Bonnin, M.-A., Teillet, M.-A., and Duprez, D. (2002). Fgf4 positively regulates scleraxis and tenascin expression in chick limb tendons. Dev. Biol. *247*, 351–366.

Esteves de Lima, J., Bonnin, M.-A., Bourgeois, A., Parisi, A., Le Grand, F., and Duprez, D. (2014). Specific pattern of cell cycle during limb fetal myogenesis. Dev. Biol. *392*, 308–323.

Figliola, R., Busanello, A., Vaccarello, G., and Maione, R. (2008). Regulation of p57KIP2 during Muscle Differentiation: Role of Egr1, Sp1 and DNA Hypomethylation. J. Mol. Biol. *380*, 265–277.

Friedrichs, M., Wirsdöerfer, F., Flohé, S.B., Schneider, S., Wuelling, M., and Vortkamp, A. (2011). BMP signaling balances proliferation and differentiation of muscle satellite cell descendants. BMC Cell Biol. *12*, 26.

Fukada, S., Uezumi, A., Ikemoto, M., Masuda, S., Segawa, M., Tanimura, N., Yamamoto, H., Miyagoe-Suzuki, Y., and Takeda, S. (2007). Molecular signature of quiescent satellite cells in adult skeletal muscle. Stem Cells Dayt. Ohio *25*, 2448–2459.

Fukada, S. -i., Yamaguchi, M., Kokubo, H., Ogawa, R., Uezumi, A., Yoneda, T., Matev, M.M., Motohashi, N., Ito, T., Zolkiewska, A., et al. (2011). Hesr1 and Hesr3 are

essential to generate undifferentiated quiescent satellite cells and to maintain satellite cell numbers. Development *138*, 4609–4619.

Furutani, Y., Umemoto, T., Murakami, M., Matsui, T., and Funaba, M. (2011). Role of endogenous TGF-β family in myogenic differentiation of C2C12 cells. J. Cell. Biochem. *112*, 614–624.

Gavard, J., Marthiens, V., Monnet, C., Lambert, M., and Mège, R.M. (2004). N-cadherin activation substitutes for the cell contact control in cell cycle arrest and myogenic differentiation: involvement of p120 and beta-catenin. J. Biol. Chem. *279*, 36795–36802.

Goichberg, P., Shtutman, M., Ben-Ze'ev, A., and Geiger, B. (2001). Recruitment of beta-catenin to cadherin-mediated intercellular adhesions is involved in myogenic induction. J. Cell Sci. *114*, 1309–1319.

Goodman, C.A., Dietz, J.M., Jacobs, B.L., McNally, R.M., You, J.-S., and Hornberger, T.A. (2015). Yes-Associated Protein is upregulated by mechanical overload and is sufficient to induce skeletal muscle hypertrophy. FEBS Lett. *589*, 1491–1497.

Granados-Riveron, J.T., and Brook, J.D. (2012). The Impact of Mechanical Forces in Heart Morphogenesis. Circ. Cardiovasc. Genet. *5*, 132–142.

Grifone, R., Demignon, J., Houbron, C., Souil, E., Niro, C., Seller, M.J., Hamard, G., and Maire, P. (2005). Six1 and Six4 homeoproteins are required for Pax3 and Mrf expression during myogenesis in the mouse embryo. Dev. Camb. Engl. *132*, 2235–2249.

Grifone, R., Demignon, J., Giordani, J., Niro, C., Souil, E., Bertin, F., Laclef, C., Xu, P.-X., and Maire, P. (2007). Eya1 and Eya2 proteins are required for hypaxial somitic myogenesis in the mouse embryo. Dev. Biol. *302*, 602–616.

Grishina, I.B., Kim, S.Y., Ferrara, C., Makarenkova, H.P., and Walden, P.D. (2005). BMP7 inhibits branching morphogenesis in the prostate gland and interferes with Notch signaling. Dev. Biol. *288*, 334–347.

Gros, J., Scaal, M., and Marcelle, C. (2004). A two-step mechanism for myotome formation in chick. Dev. Cell *6*, 875–882.

Gros, J., Manceau, M., Thomé, V., and Marcelle, C. (2005). A common somitic origin for embryonic muscle progenitors and satellite cells. Nature *435*, 954–958.

Gross, M.K., Moran-Rivard, L., Velasquez, T., Nakatsu, M.N., Jagla, K., and Goulding, M. (2000). Lbx1 is required for muscle precursor migration along a lateral pathway into the limb. Dev. Camb. Engl. *127*, 413–424.

Guo, X., and Wang, X.-F. (2009). Signaling cross-talk between TGF-β/BMP and other pathways. Cell Res. *19*, 71–88.

Hall, B.K., and Herring, S.W. (1990). Paralysis and growth of the musculoskeletal system in the embryonic chick. J. Morphol. *206*, 45–56.

Hasty, P., Bradley, A., Morris, J.H., Edmondson, D.G., Venuti, J.M., Olson, E.N., and Klein, W.H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature *364*, 501–506.

Havis, E., Anselme, I., and Schneider-Maunoury, S. (2006). Whole embryo chromatin immunoprecipitation protocol for the in vivo study of zebrafish development. BioTechniques *40*, 34–40.

Havis, E., Coumailleau, P., Bonnet, A., Bismuth, K., Bonnin, M.-A., Johnson, R., Fan, C.-M., Relaix, F., Shi, D.-L., and Duprez, D. (2012). Sim2 prevents entry into the myogenic program by repressing MyoD transcription during limb embryonic myogenesis. Development *139*, 1910–1920.

Heanue, T.A., Reshef, R., Davis, R.J., Mardon, G., Oliver, G., Tomarev, S., Lassar, A.B., and Tabin, C.J. (1999). Synergistic regulation of vertebrate muscle development by Dach2, Eya2, and Six1, homologs of genes required for Drosophila eye formation. Genes Dev. *13*, 3231–3243.

Henrique, D., Hirsinger, E., Adam, J., Roux, I.L., Pourquié, O., Ish-Horowicz, D., and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta–Notch signalling in the embryonic chick retina. Curr. Biol. *7*, 661–670.

Hirsinger, E., Malapert, P., Dubrulle, J., Delfini, M.-C., Duprez, D., Henrique, D., Ish-Horowicz, D., and Pourquié, O. (2001). Notch signalling acts in postmitotic avian myogenic cells to control MyoD activation. Development *128*, 107–116.

Houzelstein, D., Auda-Boucher, G., Chéraud, Y., Rouaud, T., Blanc, I., Tajbakhsh, S., Buckingham, M.E., Fontaine-Pérus, J., and Robert, B. (1999). The homeobox gene Msx1 is expressed in a subset of somites, and in muscle progenitor cells migrating into the forelimb. Dev. Camb. Engl. *126*, 2689–2701.

Hutcheson, D.A., Zhao, J., Merrell, A., Haldar, M., and Kardon, G. (2009). Embryonic and fetal limb myogenic cells are derived from developmentally distinct progenitors and have different requirements for -catenin. Genes Dev. *23*, 997–1013.

Iavarone, A., Garg, P., Lasorella, A., Hsu, J., and Israel, M.A. (1994). The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. Genes Dev. *8*, 1270–1284.

Itoh, F., Itoh, S., Goumans, M.-J., Valdimarsdottir, G., Iso, T., Dotto, G.P., Hamamori, Y., Kedes, L., Kato, M., and Dijke, P. ten (2004). Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. EMBO J. *23*, 541–551.

Ivanovska, I.L., Shin, J.-W., Swift, J., and Discher, D.E. (2015). Stem cell mechanobiology: diverse lessons from bone marrow. Trends Cell Biol.

Jagla, K., Dollé, P., Mattei, M.G., Jagla, T., Schuhbaur, B., Dretzen, G., Bellard, F., and Bellard, M. (1995). Mouse Lbx1 and human LBX1 define a novel mammalian homeobox gene family related to the Drosophila lady bird genes. Mech. Dev. *53*, 345–356.

Jahnsen, E.D., Trindade, A., Zaun, H.C., Lehoux, S., Duarte, A., and Jones, E.A.V. (2015). Notch1 Is Pan-Endothelial at the Onset of Flow and Regulated by Flow. PLoS ONE *10*, e0122622.

Jarriault, S., Le Bail, O., Hirsinger, E., Pourquié, O., Logeat, F., Strong, C.F., Brou, C., Seidah, N.G., and Isra l, A. (1998). Delta-1 activation of notch-1 signaling results in HES-1 transactivation. Mol. Cell. Biol. *18*, 7423–7431.

Judson, R.N., Tremblay, A.M., Knopp, P., White, R.B., Urcia, R., De Bari, C., Zammit, P.S., Camargo, F.D., and Wackerhage, H. (2012). The Hippo pathway member Yap plays a key role in influencing fate decisions in muscle satellite cells. J. Cell Sci. *125*, 6009–6019.

Kahane, N., Cinnamon, Y., and Kalcheim, C. (2002). The roles of cell migration and myofiber intercalation in patterning formation of the postmitotic myotome. Dev. Camb. Engl. *129*, 2675–2687.

Kardon, G. (1998). Muscle and tendon morphogenesis in the avian hind limb. Development *125*, 4019–4032.

Kassar-Duchossoy, L. (2005). Pax3/Pax7 mark a novel population of primitive myogenic cells during development. Genes Dev. *19*, 1426–1431.

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomès, D., Rocancourt, D., Buckingham, M., Shinin, V., and Tajbakhsh, S. (2004). Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature *431*, 466–471.

Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J.M., Fujisawa-Sehara, A., and Suda, T. (1994). Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J. Cell Biol. *127*, 1755–1766.

Kennard, S., Liu, H., and Lilly, B. (2008). Transforming Growth Factor-β (TGF-β1) Down-regulates Notch3 in Fibroblasts to Promote Smooth Muscle Gene Expression. J. Biol. Chem. *283*, 1324–1333.

Kitzmann, M., and Fernandez, A. (2001). Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts. Cell. Mol. Life Sci. CMLS *58*, 571–579.

Kitzmann, M., Carnac, G., Vandromme, M., Primig, M., Lamb, N.J., and Fernandez, A. (1998). The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. J. Cell Biol. *142*, 1447–1459.

Koncarevic, A., Jackman, R.W., and Kandarian, S.C. (2007). The ubiquitin-protein ligase Nedd4 targets Notch1 in skeletal muscle and distinguishes the subset of atrophies caused by reduced muscle tension. FASEB J. *21*, 427–437.

Kopan, R., Nye, J.S., and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development *120*, 2385–2396.

Kuang, S., Kuroda, K., Le Grand, F., and Rudnicki, M.A. (2007). Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle. Cell *129*, 999–1010.

Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H., and Honjo, T. (1999). Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. J. Biol. Chem. *274*, 7238–7244.

Ladi, E., Nichols, J.T., Ge, W., Miyamoto, A., Yao, C., Yang, L.-T., Boulter, J., Sun, Y.E., Kintner, C., and Weinmaster, G. (2005). The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. J. Cell Biol. *170*, 983–992.

Lai, D., Ho, K.C., Hao, Y., and Yang, X. (2011). Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF. Cancer Res. *71*, 2728–2738.

Larrivée, B., Prahst, C., Gordon, E., del Toro, R., Mathivet, T., Duarte, A., Simons, M., and Eichmann, A. (2012). ALK1 Signaling Inhibits Angiogenesis by Cooperating with the Notch Pathway. Dev. Cell *22*, 489–500.

Lasorella, A., Benezra, R., and Iavarone, A. (2014). The ID proteins: master regulators of cancer stem cells and tumour aggressiveness. Nat. Rev. Cancer *14*, 77–91.

Lee, C., and Jones, T. (2002). Molecular conformation–activity relationship of decamethonium congeners. Br. J. Anaesth. *88*, 692–699.

Li, F., Lan, Y., Wang, Y., Wang, J., Yang, G., Meng, F., Han, H., Meng, A., Wang, Y., and Yang, X. (2011). Endothelial Smad4 maintains cerebrovascular integrity by activating N-cadherin through cooperation with Notch. Dev. Cell *20*, 291–302.

Liu, X.-H., Yao, S., Qiao, R.-F., Levine, A.C., Kirschenbaum, A., Pan, J., Wu, Y., Qin, W., Bauman, W.A., and Cardozo, C.P. (2011). Nandrolone reduces activation of Notch signaling in denervated muscle associated with increased Numb expression. Biochem. Biophys. Res. Commun. *414*, 165–169.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif *25*, 402–408.

Lu, M.F., Pressman, C., Dyer, R., Johnson, R.L., and Martin, J.F. (1999). Function of Rieger syndrome gene in left-right asymmetry and craniofacial development. Nature *401*, 276–278.

Mahoney, W.M., Hong, J.-H., Yaffe, M.B., and Farrance, I.K.G. (2005). The transcriptional co-activator TAZ interacts differentially with transcriptional enhancer factor-1 (TEF-1) family members. Biochem. J. *388*, 217–225.

Maier, E., Nord, H., von Hofsten, J., and Gunhaga, L. (2011). A Balance of BMP and Notch Activity Regulates Neurogenesis and Olfactory Nerve Formation. PLoS ONE *6*, e17379.

Mana-Capelli, S., Paramasivam, M., Dutta, S., and McCollum, D. (2014). Angiomotins link F-actin architecture to Hippo pathway signaling. Mol. Biol. Cell *25*, 1676–1685.

Mankoo, B.S., Collins, N.S., Ashby, P., Grigorieva, E., Pevny, L.H., Candia, A., Wright, C.V., Rigby, P.W., and Pachnis, V. (1999). Mox2 is a component of the genetic hierarchy controlling limb muscle development. Nature *400*, 69–73.

Massagué, J. (1998). TGF-beta signal transduction. Annu. Rev. Biochem. *67*, 753–791.

Massagué, J. (2012). TGFβ signalling in context. Nat. Rev. Mol. Cell Biol. *13*, 616–630.

Matsuda, M., and Chitnis, A.B. (2009). Interaction with Notch determines endocytosis of specific Delta ligands in zebrafish neural tissue. Development *136*, 197–206.

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. J. Biophys. Biochem. Cytol. *9*, 493–495.

Melnikova, I.N., Bounpheng, M., Schatteman, G.C., Gilliam, D., and Christy, B.A. (1999). Differential Biological Activities of Mammalian Id Proteins in Muscle Cells. Exp. Cell. Res. *247*, 94 –104.

Mendez, M.G., and Janmey, P.A. (2012). Transcription factor regulation by mechanical stress. Int. J. Biochem. Cell Biol. *44*, 728–732.

Messina, G., Biressi, S., Monteverde, S., Magli, A., Cassano, M., Perani, L., Roncaglia, E., Tagliafico, E., Starnes, L., and Campbell, C.E. (2010). Nfix Regulates Fetal-Specific Transcription in Developing Skeletal Muscle. Cell *140*, 554–566.

Mitchell, P.O., and Pavlath, G.K. (2004). Skeletal muscle atrophy leads to loss and dysfunction of muscle precursor cells. Am. J. Physiol. Cell Physiol. *287*, C1753–C1762.

Miyazawa, K., Shinozaki, M., Hara, T., Furuya, T., and Miyazono, K. (2002). Two major Smad pathways in TGF-β superfamily signalling. Genes Cells *7*, 1191–1204.

Morikawa, M., Koinuma, D., Tsutsumi, S., Vasilaki, E., Kanki, Y., Heldin, C.-H., Aburatani, H., and Miyazono, K. (2011). ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif. Nucleic Acids Res. *39*, 8712–8727.

Mourikis, P., and Tajbakhsh, S. (2014). Distinct contextual roles for Notch signalling in skeletal muscle stem cells. BMC Dev. Biol. *14*, 2.

Mourikis, P., Gopalakrishnan, S., Sambasivan, R., and Tajbakhsh, S. (2012a). Cell-autonomous Notch activity maintains the temporal specification potential of skeletal muscle stem cells. Development *139*, 4536–4548.

Mourikis, P., Sambasivan, R., Castel, D., Rocheteau, P., Bizzarro, V., and Tajbakhsh, S. (2012b). A Critical Requirement for Notch Signaling in Maintenance of the Quiescent Skeletal Muscle Stem Cell State. STEM CELLS *30*, 243–252.

Moustakas, A., and Heldin, C.-H. (2009). The regulation of TGF signal transduction. Development *136*, 3699–3714.

Moya, I.M., Umans, L., Maas, E., Pereira, P.N.G., Beets, K., Francis, A., Sents, W., Robertson, E.J., Mummery, C.L., Huylebroeck, D., et al. (2012). Stalk Cell Phenotype Depends on Integration of Notch and Smad1/5 Signaling Cascades. Dev. Cell *22*, 501–514.

Murakami, M., Nakagawa, M., Olson, E.N., and Nakagawa, O. (2005). A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome. Proc. Natl. Acad. Sci. U. S. A. *102*, 18034–18039.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I., and Nabeshima, Y. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature *364*, 532–535.

Nagpal, P., Plant, P.J., Correa, J., Bain, A., Takeda, M., Kawabe, H., Rotin, D., Bain, J.R., and Batt, J.A.E. (2012). The Ubiquitin Ligase Nedd4-1 Participates in Denervation-Induced Skeletal Muscle Atrophy in Mice. PLoS ONE *7*, e46427.

Nevins, J.R. (1992). E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science *258*, 424–429.

Noden, D.M., and Francis-West, P. (2006). The differentiation and morphogenesis of craniofacial muscles. Dev. Dyn. *235*, 1194–1218.

Nowlan, N.C., Sharpe, J., Roddy, K.A., Prendergast, P.J., and Murphy, P. (2010). Mechanobiology of embryonic skeletal development: Insights from animal models. Birth Defects Res. Part C Embryo Today Rev. *90*, 203–213.

Olson, E.N. (1992). Interplay between proliferation and differentiation within the myogenic lineage. Dev. Biol. *154*, 261–272.

Ono, Y., Calhabeu, F., Morgan, J.E., Katagiri, T., Amthor, H., and Zammit, P.S. (2010). BMP signalling permits population expansion by preventing premature myogenic differentiation in muscle satellite cells. Cell Death Differ. *18*, 222–234.

Osborne, A.C., Lamb, K.J., Lewthwaite, J.C., Dowthwaite, G.P., and Pitsillides, A.A. (2002). Short-term rigid and flaccid paralyses diminish growth of embryonic chick limbs and abrogate joint cavity formation but differentially preserve pre-cavitated joints. J. Musculoskelet. Neuronal Interact. *2*, 448–456.

Ott, M.O., Bober, E., Lyons, G., Arnold, H., and Buckingham, M. (1991). Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. Dev. Camb. Engl. *111*, 1097–1107.

Oustanina, S., Hause, G., and Braun, T. (2004). Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. EMBO J. *23*, 3430–3439.

Pai, A.C. (1965a). Developmental genetics of a lethal mutation, muscular dysgenesis (mdg), in the mouse. I. Genetic analysis and gross morphology. Dev. Biol. *11*, 82–92.

Pai, A.C. (1965b). Developmental genetics of a lethal mutation, muscular dysgenesis (mdg), in the mouse. II. Developmental analysis. Dev. Biol. *11*, 93–109.

Palstra, A.P., Rovira, M., Rizo-Roca, D., Torrella, J.R., Spaink, H.P., and Planas, J.V. (2014). Swimming-induced exercise promotes hypertrophy and vascularization of fast skeletal muscle fibres and activation of myogenic and angiogenic transcriptional programs in adult zebrafish. BMC Genomics *15*, 1136.

Parker, M.H., Seale, P., and Rudnicki, M.A. (2003). Looking back to the embryo: defining transcriptional networks in adult myogenesis. Nat. Rev. Genet. *4*, 497–507.

Patterson, S.E., Bird, N.C., and Devoto, S.H. (2010). BMP regulation of myogenesis in zebrafish. Dev. Dyn. *239*, 806–817.

Pérez, L., Milán, M., Bray, S., and Cohen, S.M. (2005). Ligand-binding and signaling properties of the Ax[M1] form of Notch. Mech. Dev. *122*, 479–486.

Perez-Ruiz, A., Ono, Y., Gnocchi, V.F., and Zammit, P.S. (2008). beta-Catenin promotes self-renewal of skeletal-muscle satellite cells. J. Cell Sci. *121*, 1373–1382.

Picard, C.A., and Marcelle, C. (2013). Two distinct muscle progenitor populations coexist throughout amniote development. Dev. Biol. *373*, 141–148.

Porazinski, S., Wang, H., Asaoka, Y., Behrndt, M., Miyamoto, T., Morita, H., Hata, S., Sasaki, T., Krens, S.F.G., Osada, Y., et al. (2015). YAP is essential for tissue tension to ensure vertebrate 3D body shape. Nature *521*, 217–221.

Pourquié, O., Fan, C.-M., Coltey, M., Hirsinger, E., Watanabe, Y., Bréant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M., and Le Douarin, N.M. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. Cell *84*, 461–471.

Powell, J.A. (1990). Muscular dysgenesis: a model system for studying skeletal muscle development. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *4*, 2798–2808.

Pownall, M.E., and Emerson, C.P. (1992). Sequential activation of three myogenic regulatory genes during somite morphogenesis in quail embryos. Dev. Biol. *151*, 67–79.

Quillien, A., Blanco-Sanchez, B., Halluin, C., Moore, J.C., Lawson, N.D., Blader, P., and Cau, E. (2011). BMP signaling orchestrates photoreceptor specification in the zebrafish pineal gland in collaboration with Notch. Dev. Camb. Engl. *138*, 2293–2302.

Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2004). Divergent functions of murine Pax3 and Pax7 in limb muscle development. Genes Dev. *18*, 1088–1105.

Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature *435*, 948–953.

Reynaud, E.G., Pelpel, K., Guillier, M., Leibovitch, M.P., and Leibovitch, S.A. (1999). p57Kip2 stabilizes the MyoD protein by inhibiting cyclin E-Cdk2 kinase activity in growing myoblasts. Mol. Cell. Biol. *19*, 7621–7629.

Roccio, M., Schmitter, D., Knobloch, M., Okawa, Y., Sage, D., and Lutolf, M.P. (2013). Predicting stem cell fate changes by differential cell cycle progression patterns. Development *140*, 459–470.

Roddy, K.A., Prendergast, P.J., and Murphy, P. (2011). Mechanical Influences on Morphogenesis of the Knee Joint Revealed through Morphological, Molecular and Computational Analysis of Immobilised Embryos. PLoS ONE *6*, e17526.

Rolfe, R.A., Nowlan, N.C., Kenny, E.M., Cormican, P., Morris, D.W., Prendergast, P.J., Kelly, D., and Murphy, P. (2014). Identification of mechanosensitive genes during skeletal development: alteration of genes associated with cytoskeletal rearrangement and cell signalling pathways. BMC Genomics *15*, 48.

Rong, P.M., Teillet, M.A., Ziller, C., and Le Douarin, N.M. (1992). The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation. Development *115*, 657–672.

Rudnicki, M.A., Braun, T., Hinuma, S., and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell *71*, 383–390.

Sakaue-Sawano, A., Kurokawa, H., Morimura, T., Hanyu, A., Hama, H., Osawa, H., Kashiwagi, S., Fukami, K., Miyata, T., Miyoshi, H., et al. (2008). Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression. Cell *132*, 487–498.

Sartori, R., Schirwis, E., Blaauw, B., Bortolanza, S., Zhao, J., Enzo, E., Stantzou, A., Mouisel, E., Toniolo, L., Ferry, A., et al. (2013). BMP signaling controls muscle mass. Nat. Genet. *45*, 1309–1318.

Sartori, R., Gregorevic, P., and Sandri, M. (2014). TGFβ and BMP signaling in skeletal muscle: potential significance for muscle-related disease. Trends Endocrinol. Metab. *25*, 464–471.

Sassoon, D.A. (1993). Myogenic regulatory factors: dissecting their role and regulation during vertebrate embryogenesis. Dev. Biol. *156*, 11–23.

Schäfer, K., and Braun, T. (1999). Early specification of limb muscle precursor cells by the homeobox gene Lbx1h. Nat. Genet. *23*, 213–216.

Schienda, J., Engleka, K.A., Jun, S., Hansen, M.S., Epstein, J.A., Tabin, C.J., Kunkel, L.M., and Kardon, G. (2006). Somitic origin of limb muscle satellite and side population cells. Proc. Natl. Acad. Sci. U. S. A. *103*, 945–950.

Schultz, E. (1996). Satellite cell proliferative compartments in growing skeletal muscles. Dev. Biol. *175*, 84–94.

Schuster-Gossler, K., Cordes, R., and Gossler, A. (2007). Premature myogenic differentiation and depletion of progenitor cells cause severe muscle hypotrophy in Delta1 mutants. Proc. Natl. Acad. Sci. U. S. A. *104*, 537–542.

Seale, P., Sabourin, L.A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., and Rudnicki, M.A. (2000). Pax7 Is Required for the Specification of Myogenic Satellite Cells. Cell *102*, 777–786.

Shwartz, Y., Blitz, E., and Zelzer, E. (2013). One load to rule them all: Mechanical control of the musculoskeletal system in development and aging. Differentiation *86*, 104–111.

Snijders, T., Verdijk, L.B., and van Loon, L.J.C. (2009). The impact of sarcopenia and exercise training on skeletal muscle satellite cells. Ageing Res. Rev. *8*, 328–338.

Song, K., Wang, Y., and Sassoon, D. (1992). Expression of Hox-7.1 in myoblasts inhibits terminal differentiation and induces cell transformation. Nature *360*, 477–481.

Spector, I., and Prives, J.M. (1977). Development of electrophysiological and biochemical membrane properties during differentiation of embryonic skeletal muscle in culture. Proc. Natl. Acad. Sci. *74*, 5166–5170.

Spitz, F., Salminen, M., Demignon, J., Kahn, A., Daegelen, D., and Maire, P. (1997). A combination of MEF3 and NFI proteins activates transcription in a subset of fast-twitch muscles. Mol. Cell. Biol. *17*, 656–666.

Stockdale, F.E. (1992). Myogenic cell lineages. Dev. Biol. *154*, 284–298.

Strano, S., Munarriz, E., Rossi, M., Castagnoli, L., Shaul, Y., Sacchi, A., Oren, M., Sudol, M., Cesareni, G., and Blandino, G. (2001). Physical interaction with Yes-associated protein enhances p73 transcriptional activity. J. Biol. Chem. *276*, 15164–15173.

Suetta, C., Frandsen, U., Mackey, A.L., Jensen, L., Hvid, L.G., Bayer, M.L., Petersson, S.J., Schrøder, H.D., Andersen, J.L., Aagaard, P., et al. (2013). Ageing is associated with diminished muscle re-growth and myogenic precursor cell expansion early after immobility-induced atrophy in human skeletal muscle. J. Physiol. *591*, 3789–3804.

Sugiyama, M., Sakaue-Sawano, A., Iimura, T., Fukami, K., Kitaguchi, T., Kawakami, K., Okamoto, H., Higashijima, S., and Miyawaki, A. (2009). Illuminating cell-cycle progression in the developing zebrafish embryo. Proc. Natl. Acad. Sci. U. S. A. *106*, 20812–20817.

Sun, X., and Artavanis-Tsakonas, S. (1996). The intracellular deletions of Delta and Serrate define dominant negative forms of the Drosophila Notch ligands. Development *122*, 2465–2474.

Szymczak, A.L., Workman, C.J., Wang, Y., Vignali, K.M., Dilioglou, S., Vanin, E.F., and Vignali, D.A.A. (2004). Correction of multi-gene deficiency in vivo using a single "self-cleaving" 2A peptide–based retroviral vector. Nat. Biotechnol. *22*, 589–594.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell *89*, 127–138.

Takizawa, T. (2003). Enhanced gene activation by Notch and BMP signaling cross-talk. Nucleic Acids Res. *31*, 5723–5731.

Tozer, S., Bonnin, M.-A., Relaix, F., Di Savino, S., García-Villalba, P., Coumailleau, P., and Duprez, D. (2007). Involvement of vessels and PDGFB in muscle splitting during chick limb development. Dev. Camb. Engl. *134*, 2579–2591.

Tschaharganeh, D.F., Chen, X., Latzko, P., Malz, M., Gaida, M.M., Felix, K., Ladu, S., Singer, S., Pinna, F., Gretz, N., et al. (2013). Yes-associated protein up-regulates Jagged-1 and activates the Notch pathway in human hepatocellular carcinoma. Gastroenterology *144*, 1530–1542.e12.

Tsivitse, S. (2010). Notch and Wnt Signaling, Physiological Stimuli and Postnatal Myogenesis. Int. J. Biol. Sci. *6*, 268–281.

Tsivitse, S.K., Peters, M.G., Stoy, A.L., Mundy, J.A., and Bowen, R.S. (2009). The effect of downhill running on Notch signaling in regenerating skeletal muscle. Eur. J. Appl. Physiol. *106*, 759–767.

Umemoto, T., Furutani, Y., Murakami, M., Matsui, T., and Funaba, M. (2011). Endogenous Bmp4 in myoblasts is required for myotube formation in C2C12 cells. Biochim. Biophys. Acta BBA - Gen. Subj. *1810*, 1127–1135.

Uribe, R.A., Kwon, T., Marcotte, E.M., and Gross, J.M. (2012). Id2a functions to limit Notch pathway activity and thereby influence the transition from proliferation to differentiation of retinoblasts during zebrafish retinogenesis. Dev. Biol. *371*, 280–292.

Vaccarello, G., Figliola, R., Cramerotti, S., Novelli, F., and Maione, R. (2006). p57Kip2 is Induced by MyoD Through a p73-dependent Pathway. J. Mol. Biol. *356*, 578–588.

Vasyutina, E., Stebler, J., Brand-Saberi, B., Schulz, S., Raz, E., and Birchmeier, C. (2005). CXCR4 and Gab1 cooperate to control the development of migrating muscle progenitor cells. Genes Dev. *19*, 2187–2198.

Vasyutina, E., Lenhard, D.C., Wende, H., Erdmann, B., Epstein, J.A., and Birchmeier, C. (2007a). RBP-J (Rbpsuh) is essential to maintain muscle progenitor cells and to generate satellite cells. Proc. Natl. Acad. Sci. *104*, 4443–4448.

Vasyutina, E., Lenhard, D.C., and Birchmeier, C. (2007b). Notch Function in Myogenesis. Cell Cycle *6*, 1450–1453.

Wackerhage, H., Del Re, D.P., Judson, R.N., Sudol, M., and Sadoshima, J. (2014). The Hippo signal transduction network in skeletal and cardiac muscle. Sci. Signal. *7*, re4.

Wagner, J., Schmidt, C., Nikowits, W., and Christ, B. (2000). Compartmentalization of the somite and myogenesis in chick embryos are influenced by wnt expression. Dev. Biol. *228*, 86–94.

Wang, H., Noulet, F., Edom-Vovard, F., Le Grand, F., and Duprez, D. (2010). Bmp Signaling at the Tips of Skeletal Muscles Regulates the Number of Fetal Muscle Progenitors and Satellite Cells during Development. Dev. Cell *18*, 643–654.

Wang, H., Bonnet, A., Delfini, M.C., Kawakami, K., Takahashi, Y., and Duprez, D. (2011). Stable, conditional, and muscle-fiber-specific expression of electroporated transgenes in chick limb muscle cells. Dev. Dyn. *240*, 1223–1232.

Wang, J., Kumar, R.M., Biggs, V.J., Lee, H., Chen, Y., Kagey, M.H., Young, R.A., and Abate-Shen, C. (2011). The Msx1 Homeoprotein Recruits Polycomb to the Nuclear Periphery during Development. Dev. Cell *21*, 575–588.

Watt, K.I., Judson, R., Medlow, P., Reid, K., Kurth, T.B., Burniston, J.G., Ratkevicius, A., Bari, C.D., and Wackerhage, H. (2010). Yap is a novel regulator of C2C12 myogenesis. Biochem. Biophys. Res. Commun. *393*, 619–624.

Watt, K.I., Turner, B.J., Hagg, A., Zhang, X., Davey, J.R., Qian, H., Beyer, C., Winbanks, C.E., Harvey, K.F., and Gregorevic, P. (2015). The Hippo pathway effector YAP is a critical regulator of skeletal muscle fibre size. Nat. Commun. *6*, 6048.

Xin, M., Kim, Y., Sutherland, L.B., Murakami, M., Qi, X., McAnally, J., Porrello, E.R., Mahmoud, A.I., Tan, W., Shelton, J.M., et al. (2013). Hippo pathway effector Yap promotes cardiac regeneration. Proc. Natl. Acad. Sci. *110*, 13839–13844.

Yeo, S.-Y., and Chitnis, A.B. (2007). Jagged-mediated Notch signaling maintains proliferating neural progenitors and regulates cell diversity in the ventral spinal cord. Proc. Natl. Acad. Sci. *104*, 5913–5918.

Zalc, A., Hayashi, S., Aurade, F., Brohl, D., Chang, T., Mademtzoglou, D., Mourikis, P., Yao, Z., Cao, Y., Birchmeier, C., et al. (2014). Antagonistic regulation of p57kip2 by Hes/Hey downstream of Notch signaling and muscle regulatory factors regulates skeletal muscle growth arrest. Development *141*, 2780–2790.

Zammit, P.S., Golding, J.P., Nagata, Y., Hudon, V., Partridge, T.A., and Beauchamp, J.R. (2004). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J. Cell Biol. *166*, 347–357.

Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J.W., and Elledge, S.J. (1999). p21CIP1 and p57KIP2 control muscle differentiation at the myogenin step. Genes Dev. *13*, 213–224.

Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Yu, J., Lin, J.D., Wang, C.-Y., Chinnaiyan, A.M., et al. (2008). TEAD mediates YAP-dependent gene induction and growth control. Genes Dev. *22*, 1962–1971.

Zhao, B., Tumaneng, K., and Guan, K.-L. (2011). The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. Nat. Cell Biol. *13*, 877–883.