Identification and characterisation of new genes associated to multiminicore disease

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)
Submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

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2015

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Date of defense: January 20th, 2015

Université Pierre et Marie Curie

Freie Universität

Ecole Doctorale Complexité du Vivant

Laboratory Pathophysiology of striated muscles

Identification and characterisation of new genes associated to multiminicore disease

By Laurianne Davignon

Doctorate of Molecular Biology

Co Directed by Dr Ana Ferreiro and Pr Michael Gotthardt

Thesis defense: January 20th, 2015

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ACKNOWLEDGMENTS

I would like to thank the members of my jury – Florence Ruggiero, Susana Quijano-Roy, Simone Spüler, Stefanie Grunwald and Onnik Agbulut – who accepted to read this manuscript and judge my work. I would also like to thank them for their availability, as fixing a date and a location has not been that simple! I would also like to express my gratitude to Florence Ruggiero and Susana Quijano-Roy who accepted to be my scientific experts and for the time they dedicated to the editing of my manuscript.

I am using the opportunity to express my gratitude to the MyoGrad program for providing me funding and the scientific network to conduct these investigations for the past three years. I would also like to thank David Sassoon and Jean Marie Dupret for welcoming me in their respective laboratories for my PhD. I would like to thank Patrick Vicart and more particularly Ana Ferreiro for welcoming me into their newly fused group. Ana thank you for initiating me to the genetics of congenital myopathies and sharing the realities of patients conditions. Getting « stuck » is also part of the learning process and I am grateful for your patience, which gave me the time and the independence to manage this project on my own. I am looking forward submitting our paper now! I would also thank Michael Gotthardt, who has always been available and actively involved in scientific discussion; while I was showing up in Berlin, especially on short notice. This manuscript is also a mix of several comments and suggestions. I am grateful to the people who contributed to this final version: Sonia, Fany, Nathalie and Corinne for their help in refining the introduction. Thanks to Isabelle who has been going through the English and inconsistencies.

I am grateful to the people of Ana's group, and especially Claire, without whom, this project would have never seen the day! Le 105, a huge THANK YOU! It has been an amazing two years with you guys. I would like more particularly to thank « les Relaixxx » and « les petits poulets » for their expertise, kindness and their time for brainstorming. Obviously, Daniel, Vincent, Bruno, Maria, Sonia, Shin, Sestina, Nathalie… I've to say that I will miss our scientific beer sessions, where we discussed the craziest ideas that were a goldmine for me! I would also thank Charline, Bernie and Julie for being always around while I started culturing cells. Frederic,

thank you for sharing a bit of the « magic » you do have with molecular biology. Vanessa, thank you for being amazingly supportive that is impossible to transpose on paper. John and Antoine, well … thank you for pushing me; I will always be an appreciative monkey!

To the BFA unit, thank you for demonstrating me that a 6-month-lengh period is sufficient to move an entire lab and restart efficient work! Thank you for easing our settling and making us feeling comfortable and part of the group; Alain and Eva, for providing me a spot in your office while we were in transition. Isabelle, wish you that the Mister T challenge would be up to your expectations! Brigitte, thank for your time, discussing protocols and professional prospects. I'm glad that we finally succeeded to set up the PLA experiment together. Corinne, Nathalie and Anne, « mes mamans poules » for your support !! Alice, Fany and Zabeth: le chocolat Lindt est mon ami !!

A PhD also requires the support of people from the « outside ». Thank you to Zunho, Yak, Steeve and Ben, for their incredible support while I was writing. To my buddies from Strasbourg: Lena, Claire, Serena, Hub, Olga and Karim. My friends in Paris: Anne-Lyse, Kirik, Lapin, Julien & Angelica, Le Gouas. My deepest THANK YOU to Sylvia who has been there, days and nights listening and giving advices... I wish for you to find out the postdoc position you deserve. Last but not least, my family. They can be sometimes overwhelmed with my crazy ideas, but would invariably being supportive.

Papa, Maman, Cyrielle … croyez le ou non, mais j'ai fini mes études !

SUMMARY

ABBREVIATIONS

ASC-1	Activating Signal Cointegrator 1
BSA	Bovin Serum Albumin
CM	Congenital myopathies
CMD	Congenital muscular dystrophies
DMEM	Dulbecco's Modified Eagle's medium
E-C coupling	Excitation-contraction coupling
ECM	Extra Cellular Matrix
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HS	Horse serum
MmD	Multiminicore disease
MRF	Muscle Regulatory Factors
NGS	Next Generation Sequencing or Masse Parallel Sequencing
NIMA	Never In Mitosis A
NMD	Nonsense Mediated Decay
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
SC	Satellite cell
SRF	Serum Response Factor
SNP	Single Nucleotide Polymorphism
TCA	Transcriptional CoActivator
TRIP4	Thyroid Receptor Interacting Protein 4
WB	Western Blot
WES	Whole Exome Sequencing

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PREFACE

The object of my PhD was the study of a heterogenous congenital myopathy termed multiminicore disease. This condition affects mostly skeletal muscles although its most severe form can also present with cardiac involvement. My work is based on the molecular and post-gene investigations of a previously unreported form of multiminicore disease without cardiac involvement. Thus, I will present in this manuscript the skeletal muscle tissue, its contractile function and the protein network participating to this mechanism (Section I and II). Since our findings underline the contribution of the muscle repair to the pathophysiology of congenital myopathies, the main factors regulating myogenesis and muscle regeneration are compiled in Section III.

The current knowledge regarding genes mutated in congenital muscular disorders and their classification is provided in Section IV. The strategies used for the identification of causative genes and mutations have drastically evolved within the last 10 years. I provide an overview of the different sequencing "generations" and discuss their limitations in Section V.

The second part of this manuscript reports the work I conducted during my PhD. It is organised with first a presentation of the material and methods I used and developed in the laboratory during my thesis. The investigations conducted and the results I obtained are mentioned prior to a discussion of my current interpretations and the perspectives of this project.

INTRODUCTION

I. SKELETAL MUSCLE TISSUE

This manuscript is treating about congenital disorders affecting specifically and exclusively the skeletal muscle tissue. Theferore, here is a short introduction regarding the muscle tissues classification. The contribution of skeletal muscle to human body movements and the healthy and resting histological presentation of this tissue is also hereafter provided.

I.1 Muscle tissue (s)

Muscle tissue is one of the four major soft tissues of the human body. The exact number of muscles is difficult to assess due to the different classifications used, but there are approximately 642 muscles mostly organised in pairs due to the bilateral organisation of the human body. According to their organisation, muscles can be subdivided into smooth and striated muscles (Figure 1). Differing by their cellular organisation but showing the same organised contractile filament architecture, cardiac and skeletal muscles are assembled together in the striated muscle subgroup. The skeletal muscles represents the most important fraction of muscle tissue and correspond to a third of our body mass.

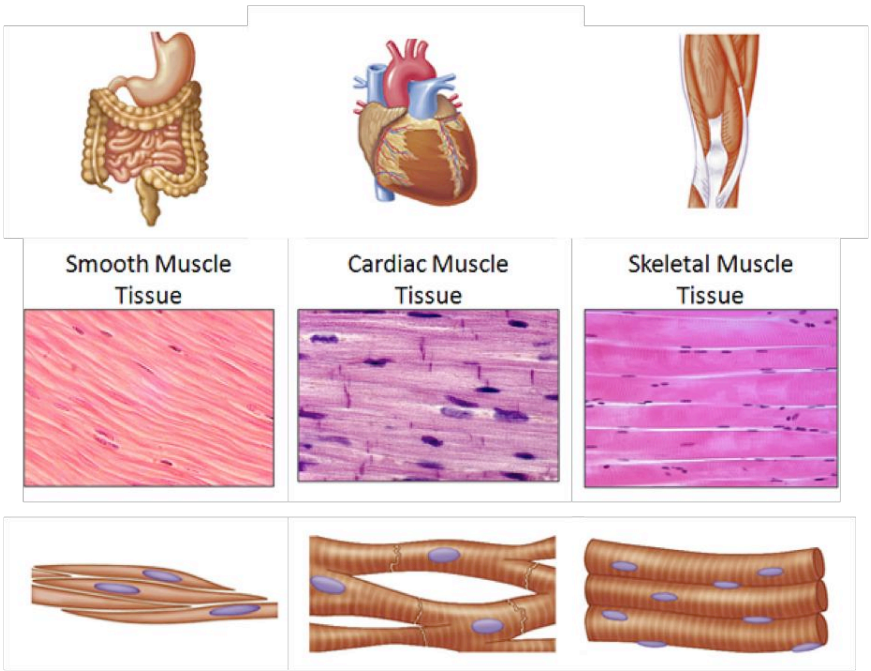


Figure 1: Smooth, cardiac and skeletal muscle tissues. Here are presented schematically the differences between the subgroups of muscles. Example of the location and organs constituted by the different types of muscles (top panel), longitudinal histological sections providing an overview of the tissue structure (middle panel) and scheme of the cellular organisation of each type muscle (bottom panel). Figure adapted from © The McGraw Hills companies, Inc.

I.2 Skeletal muscle

Skeletal muscles are voluntary muscles and therefore their contraction is controlled by the voluntary nervous system. Skeletal muscle contraction is responsible for skeleton or body motion that refers to voluntary movements. Skeletal muscles also mediate automatic or semi-automatic functions such as posture, head maintenance and most importantly breathing resulting in the strict requirement of this tissue for human survival. The neuromuscular junction (NMJ) makes the connection between a motor nerve and a muscle fibre and represents the first chemical synapsis described (Dale et al., 1936) and one of the best-studied (Sanes and Lichtman, 1999).

Skeletal muscle is a unique tissue composed of fused muscle cells which can be considered as forming individual syncytia termed muscle fibres or myofibres (Konigsberg *et al*., 1960). These cells share the same highly organised cytoplasm, whereas nuclei are located in a peripheral position under a basal membrane called sarcolemma. Rare quiescent cells located in a satellite position (between the sarcolemma and the basal lamina), termed thereafter satellite cells, can be also observed along the myofibres. Satellite cells represent the main pool of muscle progenitor cells, already committed into the muscular lineage and contributing to muscle regeneration (Chargé and Rudnicki, 2004; Collins *et al*., 2005).

Each myofibre is wrapped individually in a connective tissue envelope, termed the endomysium (Figure 2). The perimysium is the connective tissue surrounding a fascicle composed of 20 to 40 myofibres. Finally, a third connective tissue envelope termed epymisium protects each whole muscle. While blood vessels circulating between fascicles ensure the distribution of oxygen and nutrients, nerve fibres ensure the transduction of the motor signal from motor nerves to each muscle fibre. At both extremities, the myotendinous junction links muscle fibres to collagen filaments. Grouped, these filaments form the tendon, which is attached to bone's periosteum (Figure 2).

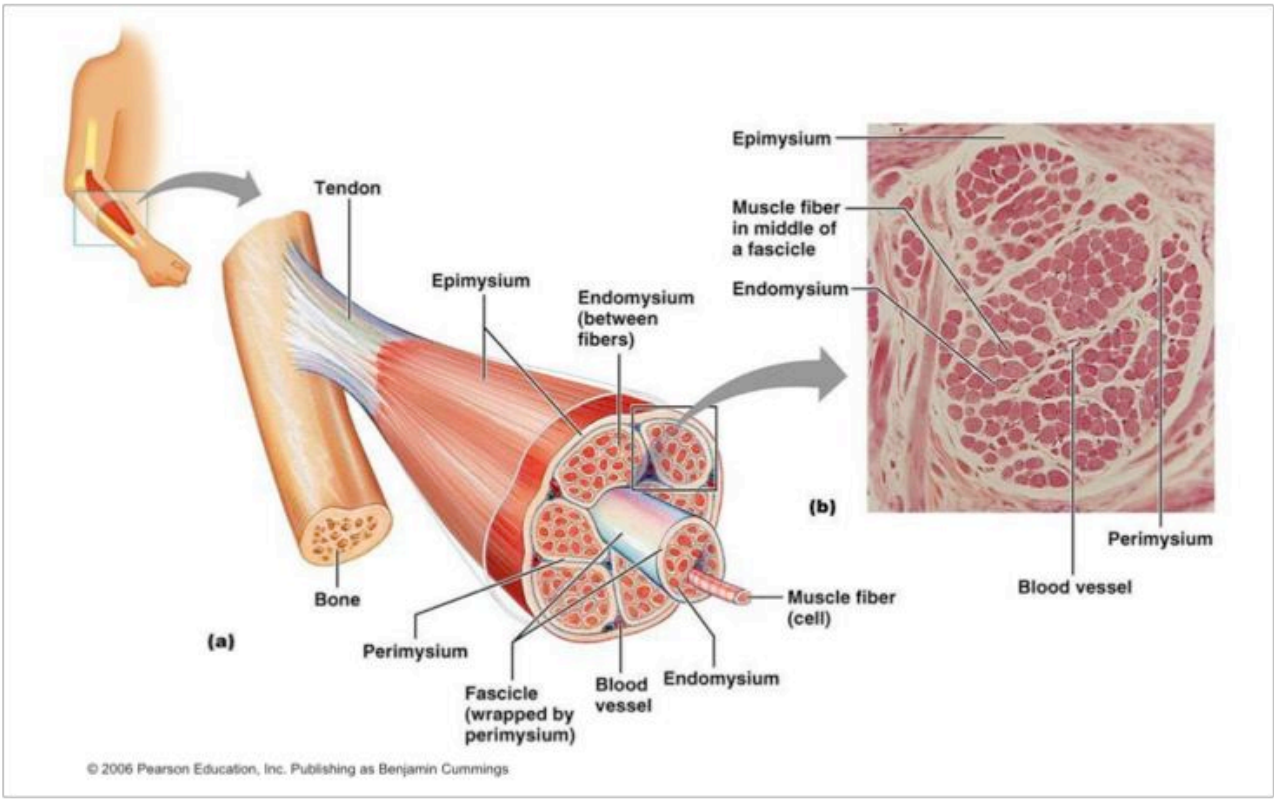


Figure 2: Skeletal muscle organisation. a) A scheme of the skeletal muscle and b) hematoxylin eosin staining of a transversal muscle section presenting the organisation of the different surrounding envelopes of connective tissues. Also, blood vessels localisation is mentioned and the tendinous structure linking muscle to bone is presented. Figure from © 2006 Pearson Education, Inc.

II. SKELETAL MUSCLE CONTRACTION

Skeletal muscle is composed of a unique highly structured and contractile network required for muscle contraction. Impairment of the proteins constituting these filaments is associated with muscular disorders. An overview of the three types of sarcomeric filaments and the excitation-contraction coupling mechanism mediating voluntary muscle contraction are presented in the object of this section.

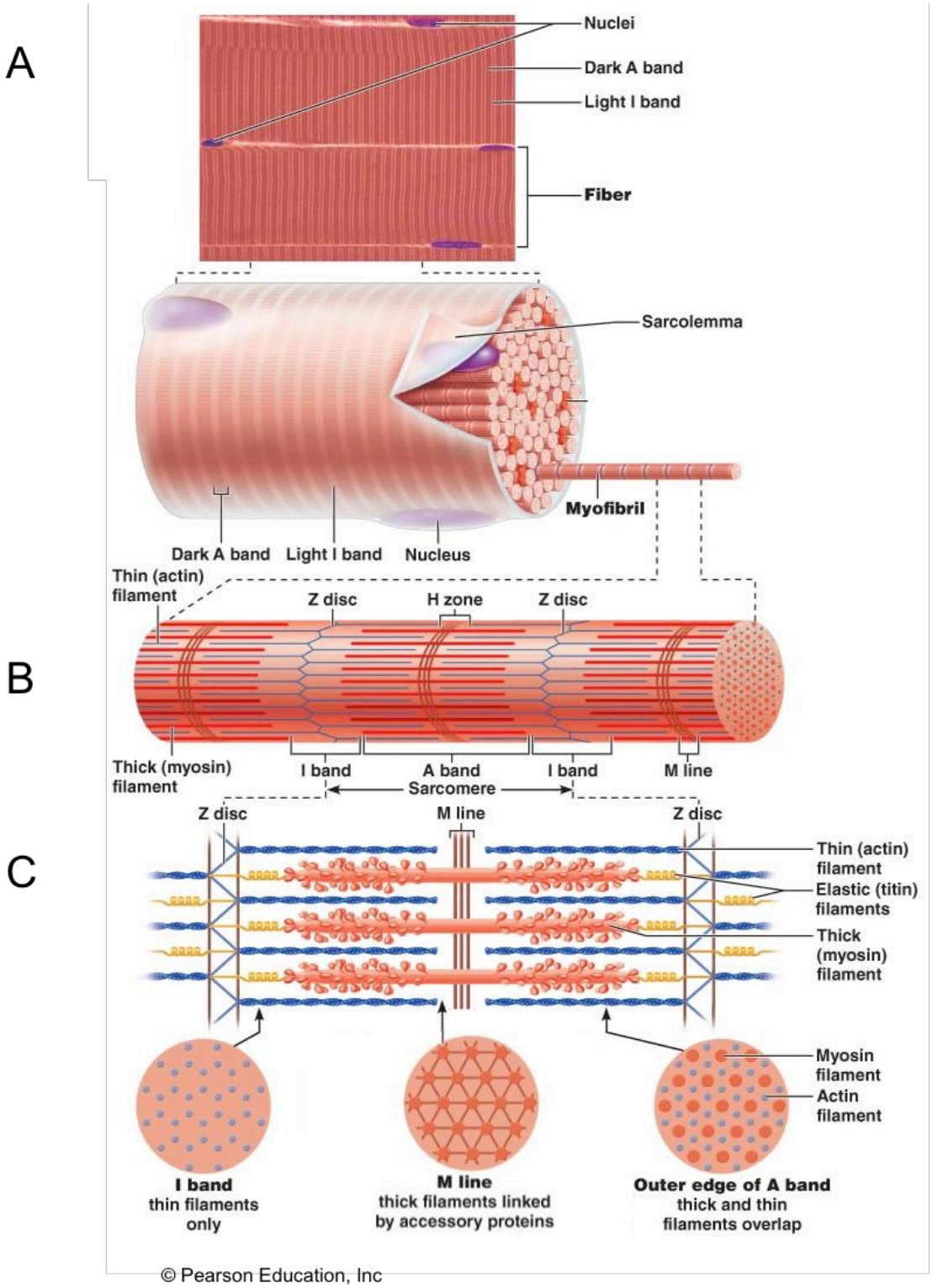


Figure 3: The sarcomere as the minimal contraction unit of striated muscles. From the top to the bottom panel, see the magnification of the muscle fibre to the sarcomere and its key components. Panel A represents a muscle fibre and the alternation of dark and light bands. Panel B presents the sarcomere structure and its different zones. Panel C presents the antiparallel arrangement of filaments of myosin (pink) and actin (blue). Note the presentation of titin protein as the third major component of the sarcomere (yellow). Figure adapted from ©Pearson Education, Inc.

As mentioned previously, the muscle fibre is a highly organised syncytium. The sarcoplasm (muscle cytoplasm) includes numerous parallel and aligned myofibrils. These myofibrils compose the contractile protein network providing the specific striated pattern in skeletal and cardiac muscles (Figure 3A). This pattern is due to the alternation of dark and light bands visible by electron microscopy: A-bands (Anisotropic) and I-bands (Isotropic) respectively. Anchoring platforms located in the middle of each band and termed M-line and Z-disk respectively, ensure the maintenance of the striated structure (Figure 3B). These anchoring platforms are essential for both contractile and non-contractile protein localisation, contributing to muscle contraction.

II.1 Muscle contraction unit: the sarcomere

The sarcomere is defined as the 2 µm-length minimal contraction unit delimited by two Z-disks. A sarcomere is thus composed of an A-band and two half I-bands. Thin filaments, mainly composed of actin, form the I-bands, while the main component of the A-bands are thick filaments of myosin (Hanson and Huxley, 1953). The giant protein titin is the third major component of the sarcomere and is responsible for the elasticity of the structure (Figure 3C).

II.1.a Thick filaments and associated proteins

Myosin II (also termed conventional myosin) is a hexamer composed of a dimer of heavy chains (MyHC, approximately 200 kDa) and two pairs of light chains (MyLC, approx. 20 kDa) termed RLC and ECL for Regulatory and Essential light chains (Figure 4). The myosin heavy chains are composed of 2 parts: the C-terminal α-helical coiled-coil tails, able to dimerize and assembly into thick filaments, and the mobile N-terminal heads (Rayment et al., 1993), which emerge at the surface of the structure to bind the light chains and contain the ATP- and actin-binding sites.

According to the development phase and muscle fibre specificity, different isoforms of "conventional" Myosin II can be expressed. Two developmental isoforms MYHC-embryonic (MYH3) and MYHC-perinatal (MYH8) can be considered, while there are 3 adult skeletal muscle isoforms: MYHC IIa (MYH2), MYHC IIb (MYH4) and MYHC IIx/d (MYH1). The MYHC-beta/slow (MYH7) is also expressed in cardiac muscle. Myosin isoforms differ by their ATPase activities and therefore defined muscle fibre functional properties. Slow myosin heavy chains are found in type I fibres rich in oxidative enzymes and higly resistant to fatigue. Myosins of type II characterised fast contracting muscle fibres, rich in glycolytic markers.

Also, the myosin binding protein C (MyBP-C, Starr and Offer, 1971) and the highly homologous MyBP-H proteins, known to localise at the A band. MyBP-C has been involved in thick filaments assembly and accessibility of myosin heads, thus participating in muscle contraction (Gilbert et al., 1999; Gruen et al., 1999). Recently, MyBP-C N-terminal domains have been described as decorating thin filaments (Luther et al., 2011; Mun et al., 2011) enhancing its critical requirement for muscle contraction (van Dijk et al., 2014).

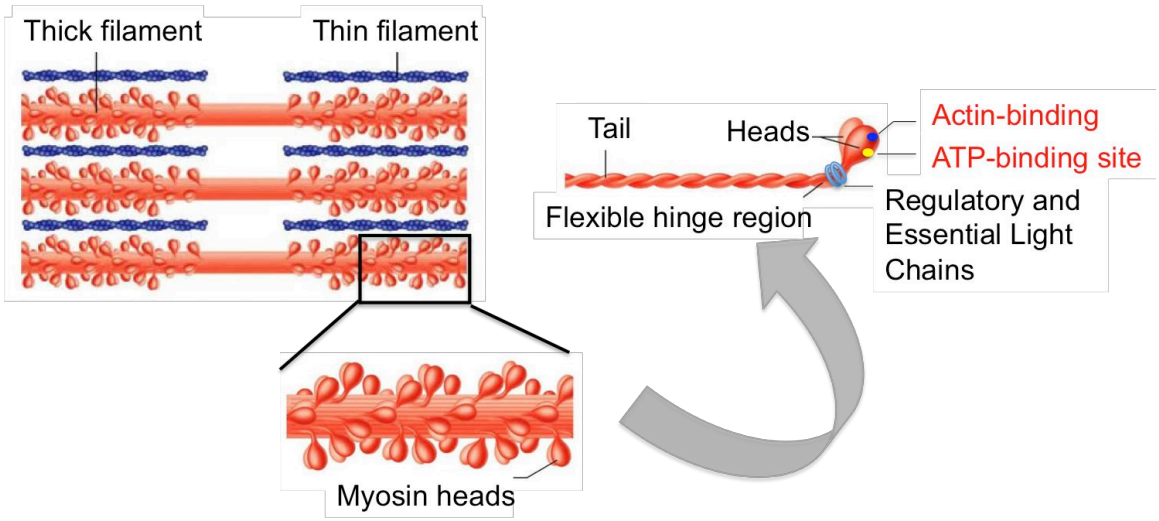


Figure 4: Assembly of myosin II-containing thick filaments. Thick filaments are the rigid skeletons localised in the middle of the sarcomere (top panel) and composed of a network of multiple myosin hexamers due to myosin heavy chain tail domains, from where emerge myosin heavy chain heads. Visualisation of the assembly of myosin II hexamer (right panel): tail domains ensure the rigidity of the complex, while the mobile domains (the heads) are responsible for the binding to the Light Chains subunits. Myosin heavy chains are also the sites for ATP- and actin-binding. Figure adapted from © Pearson Education.

II.1.b Thin filaments and associated proteins

The actin monomer is a 42 kDa globular protein (G-Actin) that can polymerise to form F-Actin, the actin filament. This microfilament consists of a two-stranded helical polymer (Hanson and Lowy, 1963) stabilised on both sides (helical grooves) by a "backbone" of tropomyosin (Corsi and Perry, 1958). Tropomyosin is a homodimer filament capable of movement along the actin filament, thus uncovering myosin-binding sites. Another component of the thin filament is troponin which consists of 3 subunits termed T (*tropomyosin binding)*, I (*inhibitory)* and C (*calcium binding)* and is essential for the complex function (Greaser and Gergely, 1971). When the complex conformation changes upon Ca^{2+} influx, tropomyosin slides and releases myosin-binding sites (Narita et al., 2001, Figure 5).

As actin polymerisation is a continual process, thin filaments are also a polarised structure. Thus, actin has a fast growing "barbed" end localised in the Z disk, and a slow growing "pointed" end (Carlier and Pantaloni, 1997). Tropomodulin is an actin-capping protein of the pointed end (Weber, 1994) and therefore contributes to maintain the final length of F-actin. Tropomodulin binds to tropomyosin and nebulin (Kostyukova et al., 2006; McElhinny et al., 2001). Nebulin is a giant protein, which spans the entire length of the thin filament, stabilising and scaffolding the entire structure (Pappas et al., 2010, 2011). Nebulin interacts also with the barbed-end actin-capping protein Cap Z (Pappas et al., 2008).

During Excitation-Contraction (E-C) coupling, the Ca^{2+} released triggers the accessibility of myosin-binding domains at the surface of actin filaments (mechanism more extensively described in II.2). In an ATP dependent manner, myosin heads are then able to generate new cross bridges on actin (Figure 5). This motion in an antiparallel manner of the filaments is responsible for the shortening of the sarcomere.

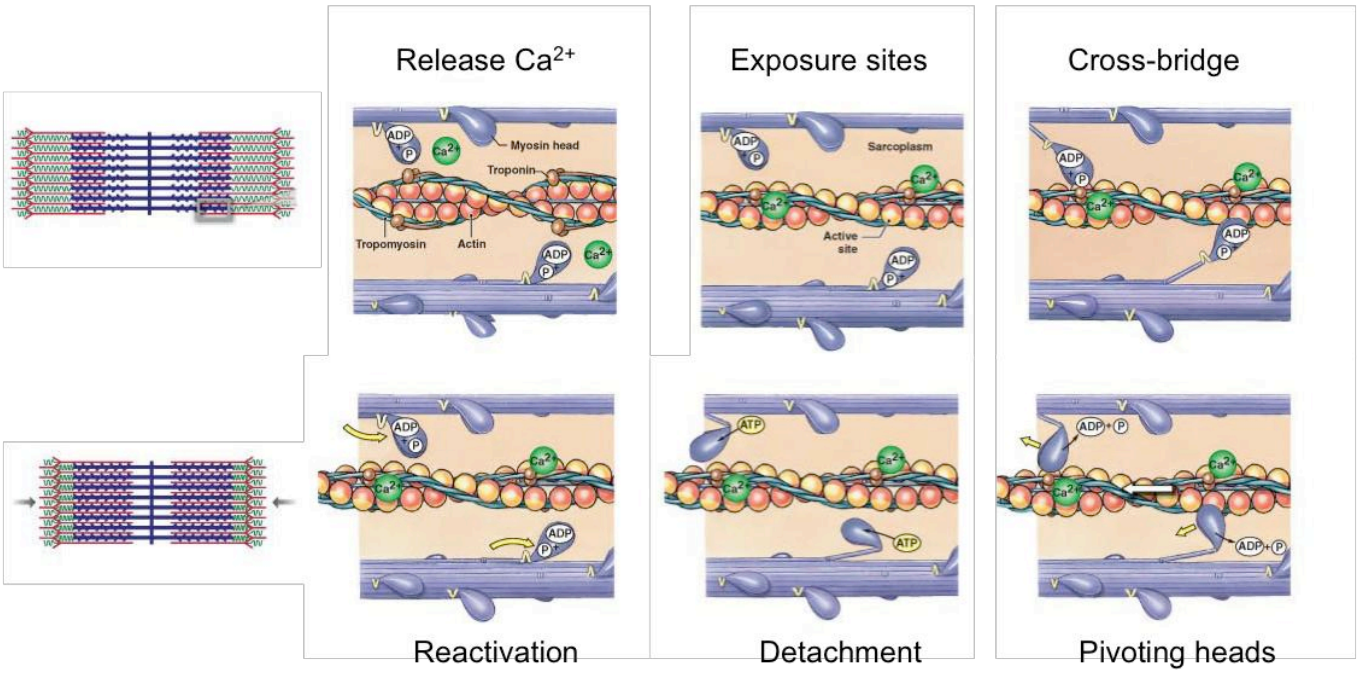


Figure 5: Muscle contraction and proteins participating to the motion of actin and myosin filaments. Upon Ca^{2+} realease and its binding to the trimeric troponin complex, tropomyosin filaments located in the groove of the actin filament slide and release myosin binding domains at the surface of actin proteins. In an ATP dependent manner, myosin heads are capable of anchoring to further actin molecules. Therefore, the actin and myosin filaments moving in an antiparallel manner shorten the sarcomere, which leads to muscle contraction. Figure adapted from ©2011 Pearson Education, Inc.

II.1.c Titin filament as a third component of the sarcomere

The giant titin protein is the largest protein known in the animal kingdom (its canonical form has a predicted size of 4200 kDa, Bang et al., 2001) and is considered as the third major component of the muscular myofibrillar system. Titin is a 1 µm elastic molecule that anchors to the Z-disk and the M-line and therefore covers half a sarcomere (Labeit et al., 1990). Due to its size, titin binds numerous structural and functional partners along the sarcomere (Figure 6, Chauveau et al., 2014). First, the N-terminal part anchors in the Z disk where it binds, among others, telethonin and actin (Gautel et al., 1996; Gregorio et al., 1998; Linke et al., 1997; Mues et al., 1998). The I-band includes the titin PEVK domain (termed after the amino acid presentation in the motif), critical for sarcomere stiffness (Freiburg et al., 2000; Linke et al., 1998) and responsible for titin-actin binding (Linke et al., 2002). The A-band domain of titin consists mainly of repeats of two sequence motifs named type I and type II homologous to fibronectin type III and immunoglobulin-C2 domains respectively (Benian et al., 1989). At the A-band, titin is able to bind myosin (Houmeida et al., 1995) and also its partner MyBP-C partner (Labeit et al., 1992). Finally, the M-line includes the titin C-terminal pseudo-kinase domain, whose activity is widely discussed (Bogomolovas et al., 2014; Gotthardt et al., 2003; Mayans et al., 1998). The M-line is also stabilised by the ternary complex formed by titin and its partners myomesin, obscurin and obscurin-like (Agarkova and Perriard, 2005; Fukuzawa et al., 2008; Pernigo et al., 2010).

Titin is known to play a major role as a blueprint for myofilament assembly and also contributes to sarcomere maintenance (Freiburg and Gautel, 1996; Gotthardt et al., 2003; Labeit et al., 1992; Miller et al., 2003). However, titin remains a complex and giant protein whose function is not fully understood.

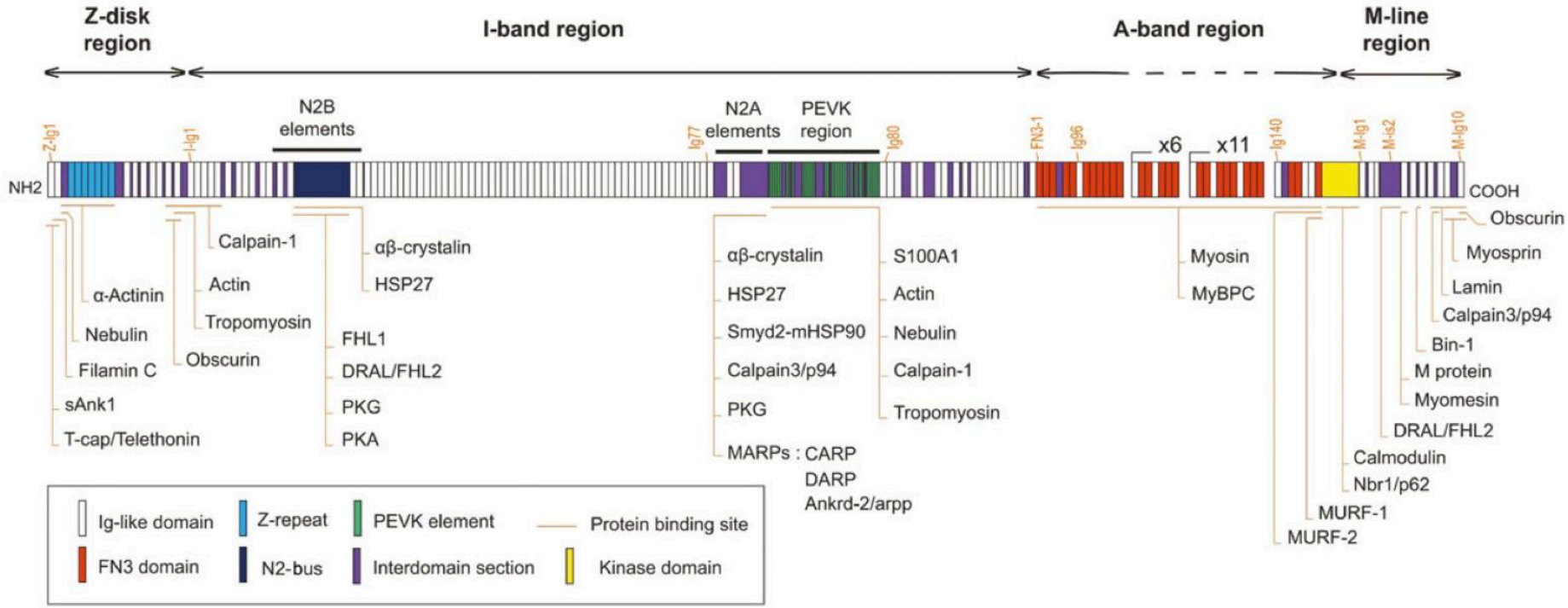


Figure 6: Titin binding sites and partners along the sarcomere. Titin is the largest mammalian protein and therefore interacts with numerous partners. The latest overview is provided in this scheme. Figure from (Chauveau et al., 2014).

II.2 The triadic junction

Muscle contraction is driven by the voluntary nervous system. Thus, muscle contraction is initiated by an action potential generated at the neuromuscular junction and propagated along the muscle fibre. This action potential requires an intermediate messenger to mediate muscle contraction; this role is played by calcium ions. Due to the length and the thickness of myofibres, the simultaneous release of calcium along the whole fibre needs a precise excitation – calcium release coupling. These spacial constraints are overtaken by a specific membrane transtalk structure: the triad. The triadic junction is a highly organised interface between the myoplasm (muscle cytoplasm) and the extracellular compartment. A triad is composed of the sarcolemmal membrane invagination termed T-tubule surrounded by two sarcoplasmic reticulum (SR; muscle endoplasmic reticulum) cisternae buttons. Two calcium channels mediate calcium release: the Dihydhropyridine Receptor (DHPR) a voltage-gated calcium channel and the Ryanodine Receptor (RyR) a calcium-gated release channel (Figure 7). DHPR and RyR are localised face to face in the T-tubule and the SR membranes respectively (Curtis and Catterall, 1984; Fleischer et al., 1985; Fosset et al., 1983; Inui et al., 1987). *RYR1*, *RYR2*, and *RYR3* encode for RyR channels. *RYR1* and *RYR2* are respectively expressed in skeletal muscle and myocardium (Fill and Copello, 2002), whose activation mechanisms are different. In skeletal myofibers, RyR1s and DHPRs are physically coupled (Figure 7), while in cardiac fibres, RyR2s are activated by the influx of Ca^{2+} through DHPRs after depolarisation; this mechanism is termed Calcium Induced Calcium Release (CIRC) (Protasi, 2002). In both cases, RyRs activation leads to the release of Ca^{2+} from the SR lumen into the cytosol. Released Ca^{2+} thereafter mediates the motion of contractile filaments, which in turn is responsible for muscle contraction; this process is therefore termed Excitation-Contraction Coupling (E-C Coupling). The calcium homeostasis of the system is mediated by calsequestrin that sequesters Ca^{2+} in the SR lumen (MacLennan and Wong, 1971), and the Sarco-Endoplasmic Reticulum Calcium ATPase pumps (SERCA), responsible for Ca^{2+} recapture (MacLennan et al., 1985).

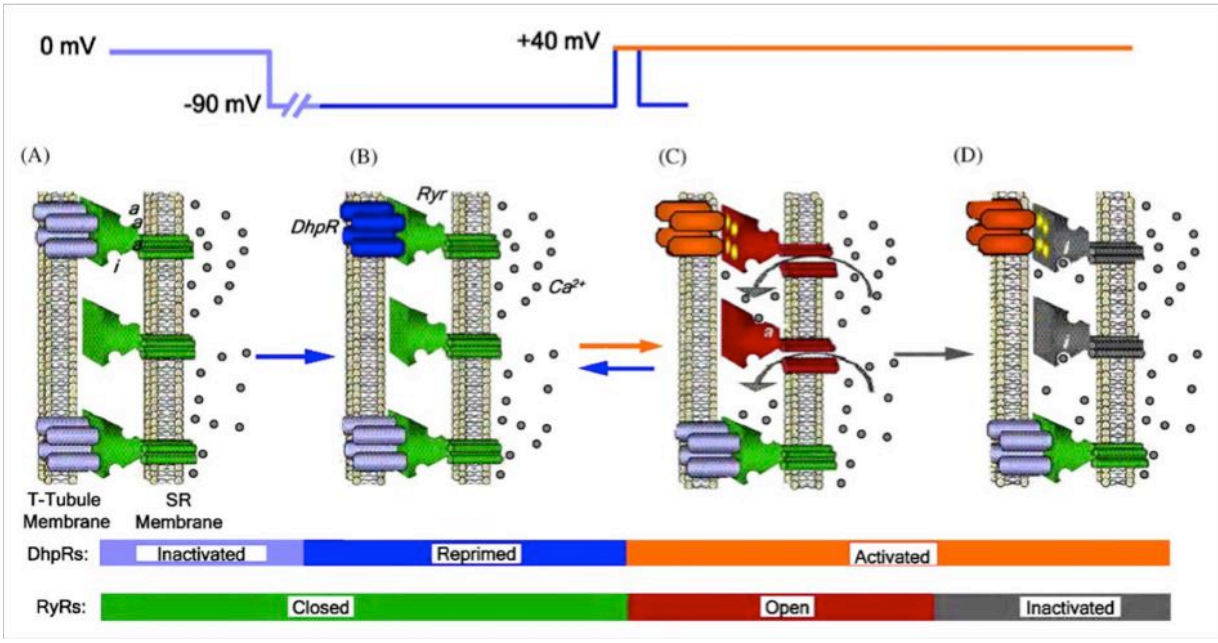


Figure 7: Modelisation of the Ca^{2+} release mechanism in skeletal muscle. Two main actors of this triad structure are the DHPRs and RYRs calcium channels located in the T-tubule and SR membranes respectively. A) At resting potential, DHPRs are inactivated and RYRs are closed. B) Upon membrane action potential, a part of DHPRs channels are reprimed. After this repriming event, C) activated DHPRs would in turn activate RYRs channels, responsible for Ca^{2+} release. RYRs channels can be activated upon DHPR activation (coupled channels) or by local elevation of Ca^{2+} (visualised by Ca^{2+} binding to "a"). According to depolarisation length, DHPRs can be desactivated and RYRs channels closed – visualised by the reverse transition C) to B). Over a longer depolarisation event, DHPRs remain activated, by a Ca^{2+} induced inactivation (visualised by Ca^{2+} binding to "i") RYRs close. Scheme from Klein and Schneider, 2006

III. MYOGENESIS

Muscle regeneration takes place after muscle injury and recapitulates partially the myogenic process that occurs in pre- and post-natal development to generate skeletal muscle structures. This process is structured in multiple ordered steps that require the sequential activation of cell autonomous muscle-specific transcription factors. The sequential activation of these factors and their requirement for myogenesis have mostly been investigated during muscle development. *In vitro* and *in vivo* these factors are crucial for differentiation and myogenesis respectively.

Knowledge about myogenesis has dramatically increased during the past decade. However, most of the precise mechanisms leading to this sequential process remain largely unknown. I will present in this section the current knowledge regarding muscle repair and myogenesis, a physiological mechanism potentially involved in the pathophysiology of multiminicore disease and investigated during my PhD.

III.1 Muscle specific transcriptional factors

III.1.a Paired box (Pax) transcription factors

Pax3 and Pax7 are structurally closely related transcription factors and constitute the subgroup III of the Paired-homeobox (Pax) family. Pax3/7 contribute to the expansion of muscle progenitors and are also required for differentiation during developmental and adult regeneration myogenesis (Collins *et al*., 2009). Pax3/7 may recruit a H3K4 methyltransferase complex, activating myogenic genes (Diao *et al*., 2012; McKinnell *et al*., 2008).

Although both proteins are expressed in muscle primitive progenitors, their contribution to muscle commitment is not redundant. Pax3 is required for early processes such as migration of myogenic precursors in limb buds and body wall. *Splotch* mutant mice lacking functional Pax3 present limb structures devoid of muscle due to impaired migration of precursors (Daston *et al*., 1996). Pax7 contribution is restricted to adult age. $Pax7^{-/-}$ animals develop normally but fail to grow and regenerate (Seale *et al*., 2000). Recent studies are in agreement with the requirement of Pax7 to muscle progenitor survival (Oustanina *et al*., 2004; Relaix *et al*., 2006). Indeed, Pax3 can be expressed in some satellite cells but cannot compensate the absence of Pax7 (Kuang *et al*., 2006; Relaix *et al*., 2005, 2006). An elegant study presents the spacio-temporal contribution of both proteins to the fates of myogenic progenitors during mouse development (Hutcheson *et al*., 2009).

III.1.b Muscle Regulatory Factors

A subgroup of basic Helix-loop-Helix (bHLH) transcription factors constitutes the Muscle Regulatory Factors (MRFs) family (Davis and Weintraub, 1992). Originating from a common ancestral gene, their spatial and temporal expression has been analysed to establish the hierarchy between them (Atchley *et al*., 1994). The specification and differentiation of myogenic progenitors also depend on the sequential expression of these factors (Figure 8). MRFs bind E-box motifs (CANNTG, where N denotes any base) within muscle-specific gene enhancers and interact with non-myogenic MEF2 factors (Molkentin *et al*., 1995) to induce transcription. Although the consensus E-box motif is highly represented into the genome (more than 10 million sites), only a small fraction is effectively bound by MRFs in both overlapping and distinctive manners (Blais *et al*., 2005; Cao *et al*., 2010). Most of the knockout experiments show the requirement of the MRFs for muscle differentiation, while knock-in experiments highlight their non-overlapping, although sometimes partially redundant, functions.

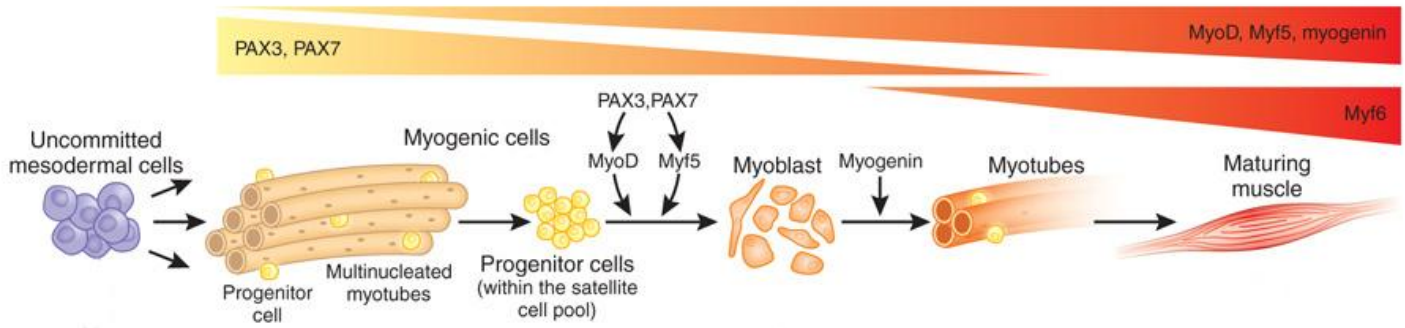


Figure 8: Key myogenic transcription factors during myogenesis and muscle regeneration. Scheme of expression pattern adopted of Pax3/7 and MRFs. Progenitor cells derivate from the mesodermal cell pool and therefore express Pax3/7. At the adult stage, these cells contribute to the satellite cell pool and maintain solely Pax7 expression. Upon Pax3/7 action, MyoD and Myf5 determining MRF are expressed. Cells proliferate and give rise to myoblasts that will exit cell cycle while expressing the differentiation MRF myogenin, undergo terminal differentiation and fuse in myotubes. During development, these newly formed myotubes generate a mature muscle. When muscle injury occurs, the myoblasts will either repair existing myofibres or generate new muscle fibres. Figure adapted from (Hettmer and Wagers, 2010)

MyoD and Myf5 are the earliest factors involved in the determination of myogenic cells and thus are considered as determinant MRFs. Myogenin (MyoG) appears later during myogenic differentiation and determines a "non-return" state closely associated with terminal differentiation, and therefore is termed differentiation factor. Myf6 (previously MRF4) function is not yet clearly elucidated and appears both as a determination and a differentiation factor (Kassar-Duchossoy *et al*., 2004).

MyoD was the first bHLH factor identified, able to ectopically induce the expression of skeletal muscle genes in non-muscle cells (Davis *et al*., 1987; Weintraub *et al*., 1989). In murine embryos, Myf5 (E8.0) is expressed prior to MyoD (E10.5) in somatic primitive progenitors (Cossu *et al*., 1996), while the opposite occurs in avian embryos. However, determinant Myf5 and MyoD transcriptional factors exhibit a redundant function: while independent inactivation of MyoD or Myf5 results in a relatively normal myogenesis process, combined inactivation leads to a complete absence of skeletal muscles (Braun *et al*., 1992; Rudnicki *et al*., 1992, 1993). Although MyoD and Myf5 present some overlapping function, both mark alternative lineages. The epaxial myotome, that leads to trunk musles, preferentially expresses Myf5 while the hypaxial myotome, at the origin of limb muscles, would express MyoD in larger amounts (Kablar et al., 1997). These alternative and independent lineages have been highlighted in recent $Myf5^{-/-}$ mice models (Gensch et al., 2008; Haldar et al., 2008)

Myogenin (MyoG) is a marker of myogenic commitment and terminal differentiation as evocated by the mutually exclusive expression of MyoG and Pax7 (Olguin and Olwin, 2004). Myogenin knock out mice present perinatal lethality due to a severe impairment of all muscle structures (Hasty et al., 1993; Nabeshima et al., 1993). Myogenin expression is initiated after the determinant MRFs as double knockout $Myf5^{-/-}$,$MyoG^{-/-}$ or $MyoD^{-/-}$,$MyoG^{-/-}$ animals recapitulate the drastic phenotype observed in the $MyoG^{-/-}$ model (Rawls et al., 1995). Thus, Myogenin acts a unique and downstream MRF, mediating terminal differentiation during foetal myogenesis.

Myf6 function has remained elusive, but seems to have a role in both determination and differentiation, consistently with its biphasic expression: first from E9.0 to E11.5 and then reappearing in differentiated muscle fibres at E16 (Bober et al., 1991). $Myf6^{-/-}$,$MyoD^{-/-}$

mutants show a severe muscle deficit similar to the one observed in myogenin mutants, anticipating a potential compensatory role for Myf6 with regard to MyoD (Rawls et al., 1998). However, due to the close vicinity of *Myf5* and *Myf6* genes, the transgenic mice generated often recapitulate a combined $Myf5^{-/-}$,$Myf6^{-/-}$ phenotype (Braun and Arnold, 1995). Several mutants show the requirement of Myf6 to generate skeletal muscle in $Myf5^{-/-}$,$MyoD^{-/-}$ model (Kassar-Duchossoy et al., 2004).

Also, Myf6 expression is maintained in mature myofibres highlighting its role in both differentiation and maintenance. Indeed, Myf6 possibly downregulates MyoG since Myf6 knock out mice present a strong upregulation of MyoG (Zhang et al., 1995).

III.2 Myogenic determination and differentiation

Most of the spatial information regarding MRFs expression has been provided by *in vivo* experiments. However, the hierarchical relationship and function of the different MRF members has been established in *ex vivo* studies using satellite cells and in *in vitro* C2C12 myoblastic cell line. Adult muscle regeneration is characterised by the activation of the satellite cells, which are the quiescent resident muscle stem cells. Once activated, these cells become myogenic precursors. Activation is followed by the expansion of the myogenic progenitor cell pool prior to cell cycle withdrawal, which is concomitant with differentiation. Terminal differentiation is characterised by the fusion of mononucleated cells and the expression of contractile proteins.

III.2.a Induction of MRF

Upon activation, myogenic precursors are rapidly characterised by the expression of both Myf5 and MyoD, by dissociation of Myf5 sequestered mRNP granules (Crist et al., 2012) and initiation of MyoD transcription (Smith et al., 1994), promoting expansion of the myogenic precursors. Knock out of either MyoD or Myf5 in *mdx* mice induces muscle regenerative defects (Megeney et al., 1996; Ustanina et al., 2007). In addition to the promotion of myogenic progenitor expansion, MyoD is responsible for the initiation of the muscle differention process, depending on the cellular environment (Blais et al., 2005; Cao et al., 2010). Indeed, *in vitro* experiments performed on MyoD$^{-/-}$ and Myf5$^{-/-}$ cells showed proliferative defects ultimately leading to differentiation impairments (Gayraud-Morel et al., 2007; Sabourin et al., 1999).

On the opposite, Myogenin induces cell cycle exit and promotes terminal differentiation (Liu et al., 2012). Indeed, knock-in expression of Myogenin driven under the control of Myf5 locus, in Myf5$^{-/-}$,MyoD$^{-/-}$ background, leads to the generation of skeletal muscles but mice die perinatally due to a reduced number of healthy muscle fibres (Wang and Jaenisch, 1997).

III.2.b Cell cycle and progenitors expansion

The cell cycle is divided in four periods (or phases), called G1, S, G2 and M (Figure 9) leading to the division of a cell into 2 daughter cells. The cell fate transition (G1) is followed by DNA replication (S), synthesis of mitotic proteins (G2) and cell division (M) providing each daughter cell with the same genetic inheritance (or material) prior to return into G1 for both generated cells. A specific G0 phase refers to non-cycling cells that can, upon activation, reenter into G1 and cycle. Cyclins and cyclin-dependent-kinases (CDKs), whose functions are modulated by CDK inhibitors (CKIs), play an important role in regulating these phase transitions. Two classes of inhibitors are involved: INK4 (inhibitor of CDK4), inhibiting the catalytic subunits of CDK4 and CDK6, and the Cip/Kip family, which acts more broadly on cyclin D-, E- and A-dependent kinases by binding to both cyclin and CDK subunits.

The early myogenic transcription factors MyoD and Myf5 present distinct and contrasting expression patterns during the cell cycle (Kitzmann et al., 1998) with non redundant functions as shown by the defects observed in MyoD$^{-/-}$ or Myf5$^{-/-}$ *mdx* animals (Megeney et al., 1996; Ustanina et al., 2007). Myf5 protein levels peak in G0, prior to an important drop in early G1. Its expression finally rises again at the end of G1 and remains stable through mitosis (Kitzmann et al., 1998). At the end of mitosis, Myf5 phosphorylation leads to its degradation. The modulation of this MRF is supposed to promote initiation of differentiation (Lindon et al., 1998).

MyoD is preferentially expressed during mid-G1 (Kitzmann et al., 1998) and degraded by the ubiquitin proteasome system in late G1 in a CyclinE/CDK2 dependent manner (Song et al., 1998; Tintignac et al., 2000). The maintenance of MyoD beyond the G1 phase can interfere with cell cycle progression and blocks the G1/S transition (Crescenzi et al., 1990; Sorrentino et al., 1990).

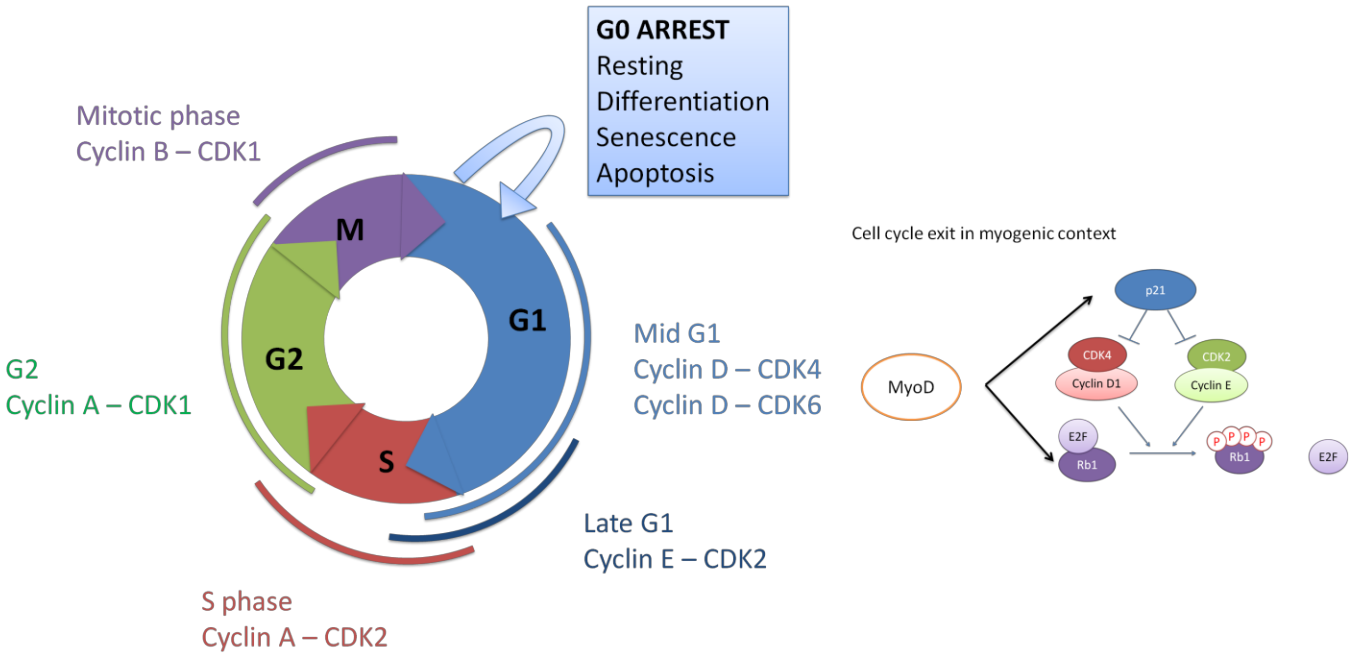


Figure 9: Cell cycle phases and associated proteins in a myogenic context. The four phases of the cell cycle are represented. G1 restricts cell to resting condition or entering the cell cycle. The S phase allows the replication of the genomic material and G2, the synthesis of mitotic proteins. During the M phase, the cell undergoes numerous changes ranging from chromatin condensation to cytokinesis. Cell cycle exit is mediated in a myogenic context by MyoD, which promotes p21 and Rb transcription (black arrows). Rb phosphorylation is mediated by p21 activity. P21 inhibits CDKs activity and prevens Rb phosphorylation required for E2F cell cycle progression transcription factor release.

III.2.c Cell cycle withdrawal

The cell cycle plays a crucial role in myogenesis. Cell cycle components mediating transitions through the different cycle checkpoints are now well known. However, signalling events associated to cell cycle exit are not yet clearly understood. They may drive the cell to quiescence, senescence or differentiation depending on so far poorly defined mechanisms. Upon terminal differentiation, the ultimate fusion of myoblasts and the expression of muscle-specific proteins such as contractile proteins lead to the formation of muscle fibres. It is commonly accepted that terminal differentiation requires cell cycle exit. This statement has been verified *in vitro*. Indeed, overexpression of cyclins D, E or A inhibits MRF transcriptional activity (Rao et al., 1994; Skapek et al., 1995, 1996) leading to inhibition of terminal differentiation. Conversely, MyoD is responsible for the transcriptional induction of Rb and also Cdkn1a (p21) (Halevy et al., 1995; Martelli et al., 1994), which favour the establishment of a postmitotic status within the cell (Figure 9, Walsh and Perlman, 1997).

III.2.d Induction of differentiation and fusion

Terminal differentiation is ultimately characterised by an irreversible loss of proliferative capacity coupled to the activation of skeletal muscle related genes. This activation is based on the combined regulation of MyoD and Myogenin (Blais et al., 2005). MyoD favours chromatin accessibility at muscle-specific loci by recruiting ATP-dependent chromatin-remodelling complexes such as SWI/SNF (de la Serna et al., 2005) and the p300 histone acetyltransferase (Dilworth et al., 2004; Puri et al., 1997). Importantly, while binding to a large number of genes along the genome (Cao et al., 2010), MyoD facilitates DNA accessibility for Myogenin (Blais et al., 2005; Cao et al., 2006). Thereby the remodelling function of MyoD promotes the transcriptional activation mediated by Myogenin (Bergstrom and Tapscott, 2001; Ohkawa et al., 2006). MyoD targets include late muscle differentiation genes as well as cell cycle withdrawal actors such as Cdkn1a (Cao et al., 2010; Puri et al., 1997).

III.3 Embryonic myogenesis

III.3.a Specification of domains

Vertebrate myogenesis has been extensively investigated in three main vertebrate models: birds (chick and quails), mammals (mouse) and fish (zebrafish). In vertebrates embryos, trunk and limbs skeletal muscles derive from somites, segments of the paraxial mesoderm formed following the anterior-posterior axis on either side of the neural tube and notochord (Bryson-Richardson and Currie, 2008). Adjacent tissues signalling are responsible for determination of somitic derivates. The paraxial mesoderm changes to transitory somatic compartments: dermomyotome, and sclerotome (Figure 10) leading to the specification of several deriving tissues such as skeletal muscle, cartilage, endothelia and connective tissue. Later the dermomyotome splits in dermatome and myotome to generate trunk, dermis and muscle. This last specification seems to depend upon cell division and spindle orientation (Ben-Yair and Kalcheim, 2005). Signalling pathways promoting these various tissues start to emerge. Notch signalling promotes smooth muscle differentiation at skeletal muscle expense (Ben-Yair and Kalcheim, 2008). High levels of Sonic Hedgehog (Shh), expressed by the neural tube and the notochord, favour Nkx3.2 expression in the sclerotome and cartilaginous differentiation (Cairns et al., 2008).

III.3.b Specification of muscle primitive progenitors

Vertebrate muscles structures are composed of the trunk, limbs and head muscles. Head muscles originate from the unique convergence of various cell populations and respond to different specification pathways. Limbs and trunk muscles originate from the same multipotent cells population. However, cells movement and the onset of lineage initiating markers differ and would be described thereafter.

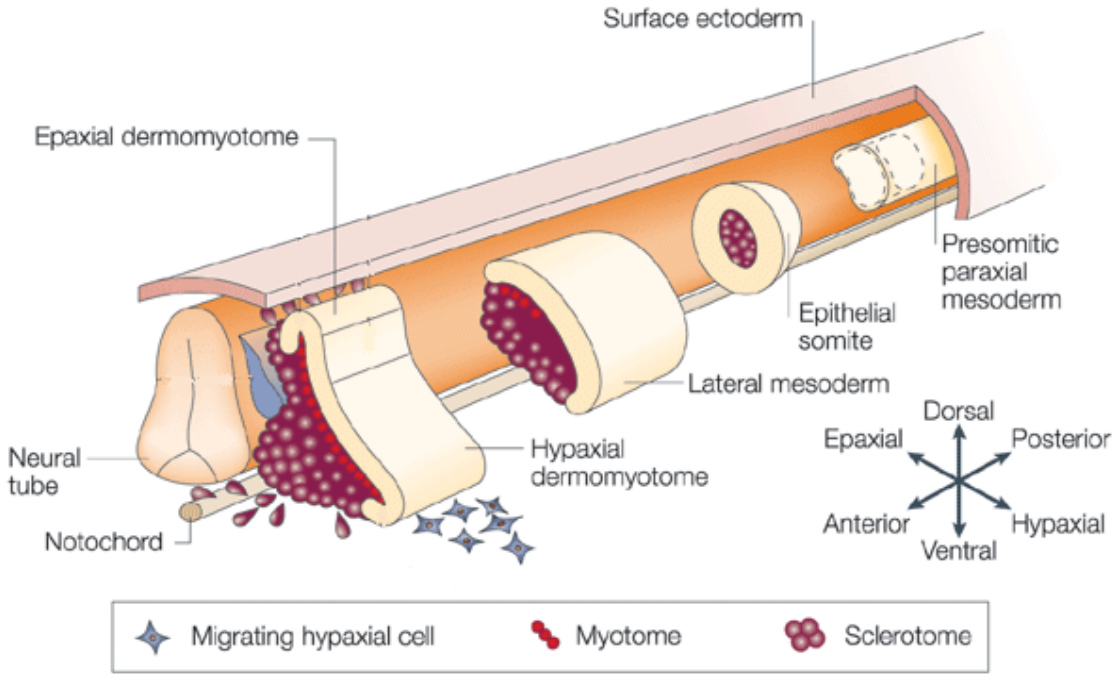


Figure 10: Somite segmentation. Following the anterior posterior axis, somites give rise to dermomyotome and sclerotome. The dermomyotome is at the origin of muscle cell progenitors. Figure adapted from (Parker et al., 2003).

Myogenesis is initiated by delamination of $Pax3^{+}$ myogenic progenitors cells from dermomyotome lips to the myotome, first skeletal muscle in amniotes (Figure 11a). At E10.5 in mice and 3.5 days in chicks, the central domain of the dermomyotome undergoes an epithelial-to-mesenchymal transition (EMT) following the anterio-posterior gradient (Gros et al., 2004; Hutcheson et al., 2009). Resident muscle progenitor cells $Pax3^{+}$ engulf the myotome (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005) that either proliferate to generate a maintained pool of progenitors or undergo myogenic differentiation to constitute the primitive trunk muscles (Figure 11c). While delamination occurs, $Pax3^{+}$ multipotent progenitors cells from the hypaxial dermomyotome migrate as single cells (Figure 11b) into limb buds mesenchyme (Birchmeier and Brohmann, 2000; Schienda et al., 2006). In chick embryos these cells also participate to vascular and lymphatic endothelia as well as muscle lineage (He et al., 2003). In mice, the expression of Pax7 restrict the $Pax3^{+}/Pax7^{+}$ to muscle lineage and thus, to limb muscles (Hutcheson et al., 2009).

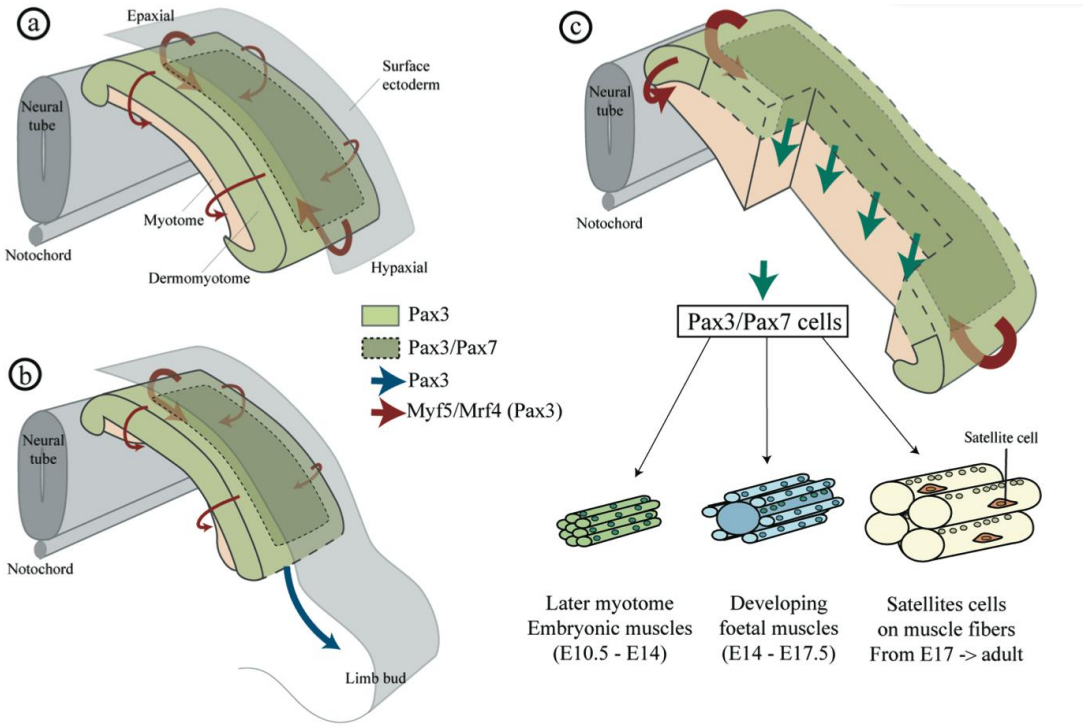


Figure 11: Amniotes delamination of trunk and limb muscle progenitors in a mouse embryo. (a) Dermomyotome is composed of Pax3^{+} multipotent cells that express Myf5 and Myf6 (Mrf4). The edge of this epithelial structure delaminates to form the myotome. (b) Also, cells from the hypaxial dermomyotome migrate and colonise the limb bud. (c) Cells from the central region of the dermomyotome express Pax3/7 engulf the myotome. The following phases of muscle growth and progenitors proliferation depend of this population. Figure from Lagha et al., 2008.

III.3.c Embryonic pathways regulating muscle progenitor cell fate

Embryonic myogenesis is dependent upon numerous pathways also termed positional signals arising from neighbouring tissues: neural tube, notochord and ectoderm (Figure 12). Activation of myogenesis depends of the activation of Muscle Regulatory Factors, such as Myf5 and MyoD. The neural tube preferentially express Wnt1 promoting activation of Myf5 in an axial manner, while Wnt7a is expressed in the dorsal ectoderm and enhances MyoD expression in the hypaxial region (Tajbakhsh et al., 1998). Also sonic hedgehog (Shh), expressed in the floor plate of the neural tube and the notochord, promotes Myf5 in the epaxial region (Borycki et al., 1999a; Gustafsson et al., 2002). Bone Morphogenetic Proteins (BMP) are expressed in the mesoderm and the limb bud ectoderm and mediates the

proliferation maintenance by Pax3 expression while inhibiting MyoD (Amthor et al., 1998). To note, Pax3 expression has also a complementary function in the regulation and activation of MyoD under axial (neural tube and notochord) and surface ectoderm signals (Borycki et al., 1999b).

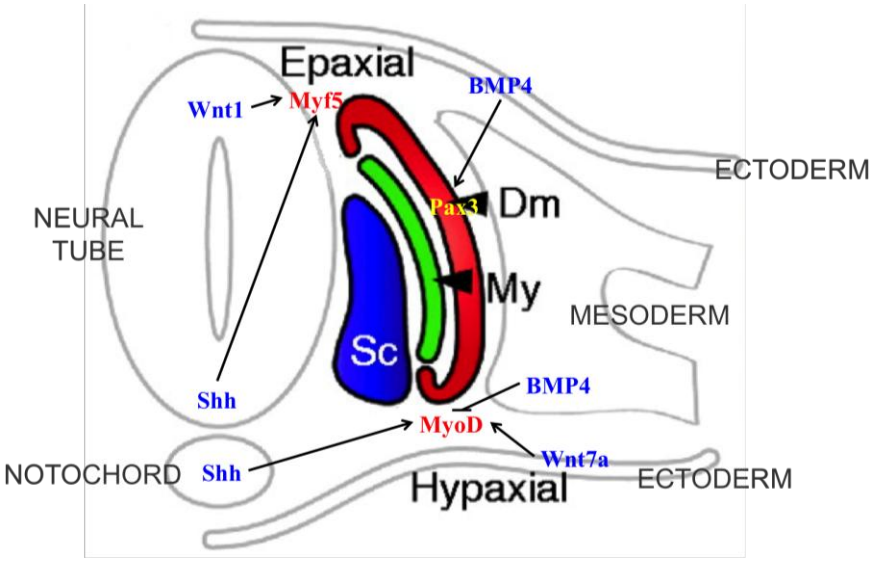


Figure 12: Embryonic pathways mediating positional location during embryonic myogenesis. Among them, Wnt, sonic hedgehog (Shh) and bone morphogenetic proteins (BMP) pathways have been widely investigated. Wnt promotes muscle lineage in the epaxial and hypaxial regions of the dermomyotome as well as Shh. BMP promote preferentially proliferation by activating Pax3 and repressing MyoD in the hypaxial region. Pax3 (Pax protein) is quoted in yellow, early MRF factors are labelled in red. Pathways are quoted in blue. Dm dermomyotome, My: myotome, Sc: sclerotome.

III.3.d Foetal myogenesis and muscle growth

Muscle progenitors cells derived from a progenitor pool of $Pax3^{+}/Pax7^{+}$ committed cells restricted to the central part of the dermomyotome and limb buds, that contribute to myogenesis (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Ben-Yair and Kalcheim, 2005). Double mutant *Pax3/Pax7* mice show a startling muscle deficit (Relaix et al., 2005). Cell lacking Pax3/7 expression do not initiate myogenic determination program and die or differentiate in different lineages. Downstream effectors of Pax3/7 genes drive developmental myogenesis. During foetal myogenesis, a fraction of the muscle progenitors would undergo cell cycle withdrawal and provide the first "wave" of fusion, leading to the development of embryonic myofibres at the origin of muscles. Then, during postnatal myogenesis, muscle

would growth due to the combination of hypertrophy and massive fusion of early satellite cells (approximately 30% of muscle cells) that represent the second "wave" of fusion. The remaining satellite cell pool (approximately 2-5% of muscle cells) would be maintained in a quiescent state through entire life and reactivate (proliferate and differentiate) during muscle injury to contribute to muscle regeneration (Figure 13).

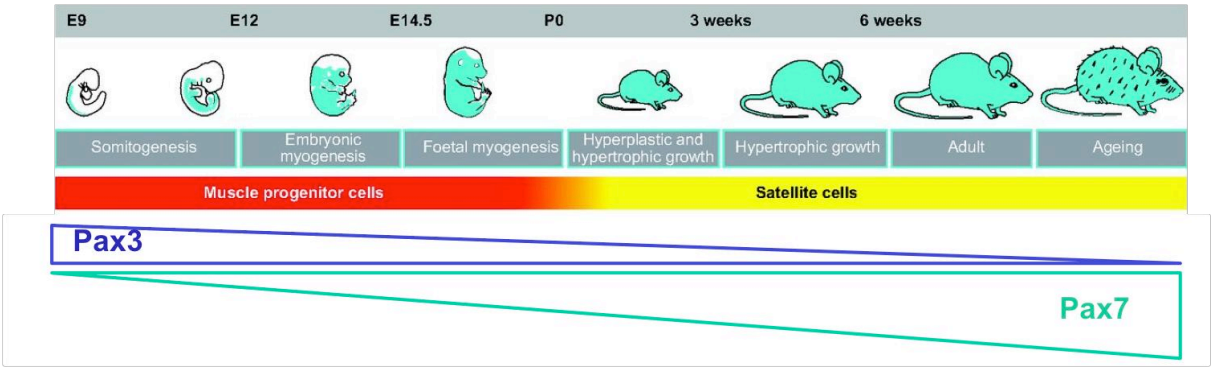


Figure 13: Developmental stages in mouse embryo and contribution of progenitor cells. The different stages of the murine development are quoted according to the time along development: (E) stands for embryonic and (P) postnatal days of life. Muscle progenitors Pax3/7 status is mentioned. Figure adapted from Relaix and Zammit, 2012.

These proliferation and differentiation mechanisms are affected by several signalling pathways such as TGFβ signalling: Myostatin overexpression promoting the differentiation of muscle progenitor pool and can lead to hypotrophic muscles (Manceau et al., 2008). Notch signalling is required for maintaining Pax3 status, promoting self-renewal of myogenic progenitors as overexpression of Delta1 (Notch ligand) in chick muscle progenitors maintains their undifferentiated and proliferative status (Delfini et al., 2000). Inversely, inhibition of Rbpj (transcriptional mediator of Notch) or reduction of Delta1 leads to premature differentiation of muscle progenitors (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). In post migration phases, progenitors cells start to express Pax7, mediating their commitment to muscle lineage. Although these cells still transcribed Pax3, its expression is down regulated before birth (Horst et al., 2006; Relaix et al., 2006) enhancing the sole contribution of Pax3 to embryonic myogenesis.

III.4 Adult myogenesis

At the adult stage, skeletal muscle remains an extraordinary tissue capable of entirely regenerating upon muscle injury. Diverse mononucleated cell populations have been characterised in skeletal muscle tissue. The main resident quiescent stem cell population is the satellite cell (SCs) pool, committed into the muscle lineage. Additional non-conventional cell populations presenting myogenic properties are the extensive focus of investigations as potential therapeutic targets. However the contribution of the later to growth and muscle regeneration remains probably feeble in physiological conditions (Cossu and Biressi, 2005; Péault et al., 2007; Sambasivan et al., 2011). In comparison, SCs have shown an extraordinary capacity to proliferate in order to generate within a few weeks new myofibres and to ensure the satellite cell pool self-renewal (Chargé and Rudnicki, 2004; Collins et al., 2005). For the past two decades, intensive studies have contributed to understand the contribution of this small population – 2 to 5% of total muscle cells – to the total repair of muscle tissue (Zammit et al., 2002), with important therapeutic implications (Marg et al., 2014).

By definition, satellite cells constitute the pool of muscle stem cells (Figure 14). A few satellite cells engrafted in muscle are capable of successfully proliferating to generate new muscles fibres and reconstitute the satellite cell niche for subsequent regeneration rounds (Collins et al., 2005; Sacco et al., 2008).

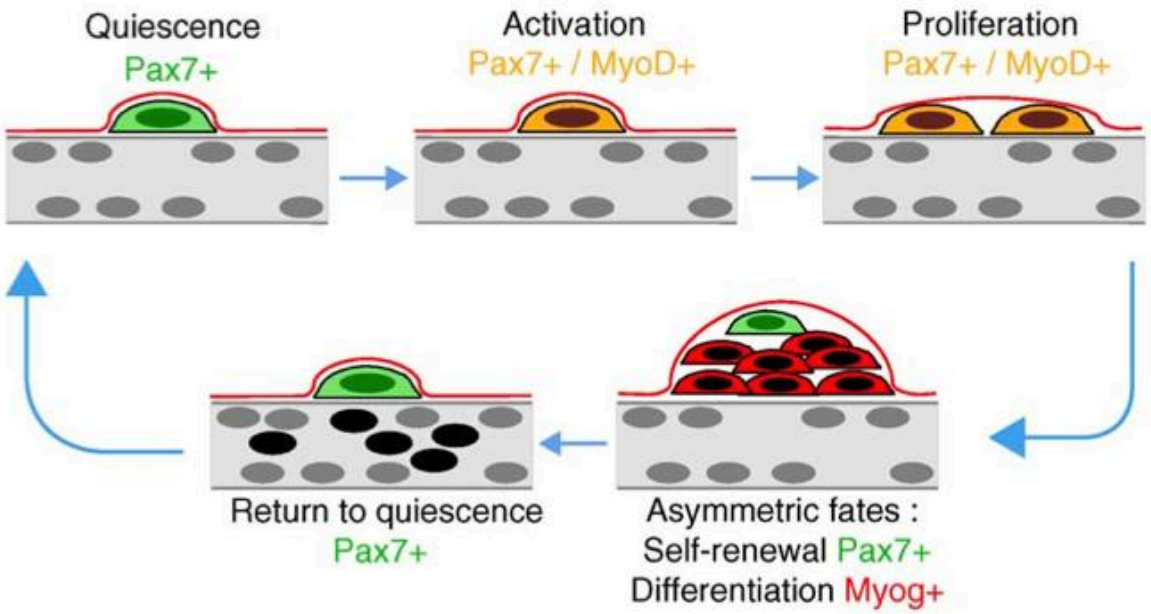


Figure 14: Satellite cells are the main muscle stem cells. A quiescent satellite cell (Pax7^{+}) will upon activation express MyoD prior to proliferation. Once two daughter cells are obtained, cells can follow different cell fates: the daughter cells maintaining Pax7 expression will downregulate MyoD expression and contribute to the replenishment of the satellite cell pool, while the second will recapitulate the expression of the different MRFs and participate to muscle regeneration. Scheme from (Relaix and Marcelle, 2009)

III.4.a Quiescent satellite cells

Satellite cells (SC) derive from Pax-positive myogenic progenitors from the dermomyotome region (Armand et al., 1983; Gros et al., 2005; Schienda et al., 2006). Localised between the myofibre basal lamina and the endomysium (Mauro, 1961), SCs are actively maintained in a dormant state (G0 phase) under resting conditions (Fukada et al., 2007). Quiescent SCs express characteristic markers such as Pax7 (Seale et al., 2000; Zammit et al., 2006) which is important for SC maintenance and proliferation (Kuang et al., 2006; Oustanina et al., 2004; Seale et al., 2000). Only a few SCs still express Pax3, which seems to play a key role in preventing myogenic differentiation and apoptosis (Hirai et al., 2010; Montarras et al., 2005; Relaix et al., 2006). Among the MRF factors, quiescent SCs express solely Myf5, while MyoD cannot be detected (Cornelison et al., 2000; Tajbakhsh et al., 1996).

III.4.b Activation

Satellite cells are able to sense their environment. In response to muscle injury, SCs rapidly initiate their reactivation (Cheung and Rando, 2013). This efficient reactivation is favoured by the unique permissive chromatin state of satellite cells (Liu et al., 2013) and also by the sequestration of Myf5 transcripts through miRNA-31 in mRNP granules – which are transcriptionally inactives cytoplasmic bodies composed of proteins and sequestered mRNA – (Crist et al., 2012). Satellite cells are activated by mitogens originated from damaged muscle fibres and infiltrated inflammatory cells. This activation is facilitated by the overexpression upon injury of several growth factors like HGF (Tatsumi et al., 1998), FGF (Floss et al., 1997), IGF (Musarò, 2005). Of note, the location of satellite cells in the vicinity of endothelial cells may favour their reactivation in a local but also systemic manner. A recent study shows that murine quiescent satellite cells are more prone to reactivate when muscle was injured in the contralateral leg (Rodgers et al., 2014).

III.4.c Satellite cell heterogeneity

When activated, satellite cells re-enter the cell cycle, and become myogenic precursor cells also termed myoblasts. Once proliferation is achieved, cells undergo differentiation and fuse to each other or to pre-existing myofibres to repair muscle damage or return to a quiescent state (Chargé and Rudnicki, 2004; Collins et al., 2005). The presence or absence of early myogenic markers allows tracking the fait of the different cell populations (Figure 14). $Pax7^{+}/MyoD^{-}$ is found in quiescent cells, which upon activation will acquire a $Pax7^{+}/MyoD^{+}$ status and proliferate. Once entering differentiation, cells lose Pax7 and become $Pax7^{-}/MyoD^{+}$ and subsequently $MyoD^{+}/MyoG^{+}$(Dhawan and Rando, 2005; Relaix et al., 2006). Cells recapitulating $Pax7^{+}/MyoD^{-}$ revert to a quiescent state (Zammit et al., 2004, 2006), able to reactivate after further injury demonstrating their stem cell function (Collins et al., 2005; Sacco et al., 2008). This regeneration strategy has been visualised in amphibians (Chen et al., 2006; Morrison et al., 2006) where the major mechanism was thought to be the reversion and undifferentiation of fragmented muscle fibre cells (Brockes and Kumar, 2002).

Satellite cells have been long considered as a unique population of committed muscular stem cells. However, it appears that these cells are heterogeneous: they possess different "stemness" properties, and respond differentially to muscle injury. The SC pool is composed of a majority of highly proliferative cells undergoing myogenic differentiation, while a certain subpopulation (approximately 20%) divides slowly and is more prone to replenish the SC pool (Schultz, 1996; Shinin et al., 2006). Their myogenic differentiation capacities seem to be linked to their myogenic gene expression signature. Using a transgenic *Tg:Pax7-nGFP* mice, Rocheteau et al., (2012) showed also the existence of two resident Pax7 populations. Pax7-$nGFP^{Low}$ cells undergo random DNA segregation while Pax7-$nGFP^{High}$ cells were less primed for reactivation and divide asymmetrically. Pax7-$nGFP^{High}$ cells were thus capable of generating daughter cells with distinct cell fates: the cells maintaining previous profile Pax7-$nGFP^{High}$ and contributing to replenish the pool, and Pax7-$nGFP^{Low}$ cells contributing to regeneration (Conboy et al., 2007; Shinin et al., 2006). This asymmetric division was consistent with the validation of the "immortal strand hypothesis" proposed decades ago by John Cairns, where asymmetric division is characterised by the strict retention of the template DNA into the daughter cell maintaining the self-renewing marks. This phenomenon seems to be promoted by Notch; for instance Numb (a Notch inhibitor) is located asymmetrically in dividing myoblasts, promoting differentiation (Conboy and Rando, 2002; Shinin et al., 2006).

The SC population is also reportedly composed of 10% of pure stem cells: $Pax7^{+}/Myf5^{-}$ capable of dividing asymmetrically in two subpopulations: $Pax7^{+}/Myf5^{-}$ and $Pax7^{+}/Myf5^{+}$ daughter cells (Figure 15), contributing respectively to stem cell self-renewal and to the generation of committed progenitor cells able to differentiate (Kuang et al., 2007). However, the existence of a potential stem cell pool that has never committed in the muscle lineage is contradicted by another group showing that 98% of the SCs are committed progenitors that had expressed MyoD (Kanisicak et al., 2009).

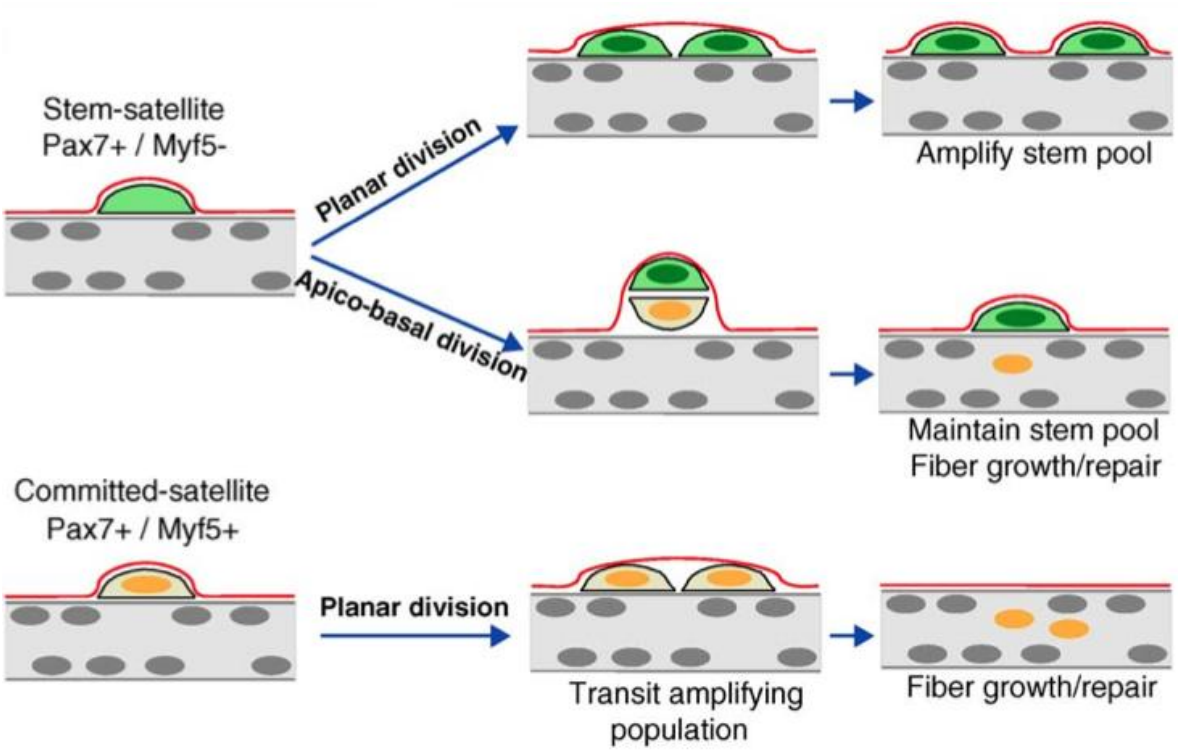


Figure 15: Committed satellite cells. Stem-satellite cells can either divide symmetrically to replenish the progenitor pool or divide asymmetrically and contribute to replenishment and regeneration. Committed satellite cells divide in a symmetrical manner to contribute exclusively to muscle regeneration. Scheme from (Relaix and Marcelle, 2009)

III.4.d Satellite cells and their niche

The stem cell microenvironment is important to define the activation state of satellite cells. Also, a stem cell niche is required to maintain their quiescence, proliferation, differentiation and self-renewal properties (Ohlstein et al., 2004; Scadden, 2006). Inside the niche, cell-to-cell and cell-to-matrix interactions are required to maintain equilibrium while the biochemical environment and autocrine/paracrine signalling via FGF, Wnt and Notch pathways modulate stem cell specification. This niche is impaired during myogenic injury as well as during ageing.

Changes in the stem cell niche are known to influence SCs quiescence and function. For example, FGF signalling activation results in loss of quiescence and decreased regenerative capacities (Chakkalakal et al., 2012). SC properties are strongly linked to the basal lamina of muscle fibres. The extra cellular matrix and in particular collagen VI are critical for SC pool maintenance and can modulate SC behaviour (Urciuolo et al., 2013). With ageing SCs regenerative potential declines due to changes in the stem cell niche (Brack et al., 2007; Carlson and Conboy, 2007) but also to intracellular impairment. Recent studies have shown that ageing SCs lose their reversible quiescence, switching to senescence. SCs from geriatric and sarcopenic human and murine muscles were upregulated for p16 (Cdkn2a), a senescence master gene which irreversibly affects regeneration and self-renewal properties of those cells (Sousa-Victor et al., 2014). Furthermore, epigenetics mark changes accumulate and may lead to the functional decline observed in ageing quiescent SCs (Liu et al., 2013).

Wnt signalling plays key roles in myogenic differentiation, from embryonic to postnatal myogenesis and in adult regeneration (Polesskaya et al., 2003). The canonical Wnt pathway promotes satellite cell proliferation (Otto et al., 2008) favouring symmetric division, surprisingly contributing to muscle regeneration and to the replenishment of satellite cell pool (Le Grand et al., 2009). During ageing, the canonical Wnt signalling by contrast promotes the conversion of satellite cells to the fibrogenic lineage (Brack et al., 2007).

Activation of Notch signalling prevents myoblast differentiation and promotes self-renewal by upregulating the HES1 protein, a MyoD inhibitor (Kuroda et al., 1999). Notch promotion of muscle regeneration has also been observed altered in aged muscle (Conboy et

al., 2003). Absence of Notch signalling in adult quiescent satellite cells activates spontaneously differentiation, depleting the SC pool and preventing further muscle regeneration (Bjornson et al., 2012; Mourikis et al., 2012). Notch signalling also promotes production of proteins such as MEGF10 (Holterman et al., 2007).

Alltogether, these informations raise the possibility that satellite cells and their microenvironment could emerge as a pathophysiological mechanism in congenital disorders. Indeed, the potential dysregulation of muscle regeneration as a pathophysiological mechanism in muscular dystrophies as been evocated (Gnocchi et al., 2008). Moreover, evidence brought by the functional relevance of *MEGF10* and *SEPN1* genes, mutated in multiminicore disease, for satellite cell regulation, tends to link muscle regeneration to congenital myopathies such as multiminicore disease.

IV. CONGENITAL MUSCULAR DISORDERS

Congenital muscular disorders affect predominantly skeletal muscle tissues and are commonly subdivided into two main entities: Congenital Muscular Dystrophies (CMDs) and Congenital Myopathies. Depending on the morphological and histological features, different pathophysiological mechanisms are involved. Despite impressive progress in the genetic characterisation of congenital muscle diseases in the last years, in a significant number of patients and families the known genes have been excluded and the genetic cause of disease remains unknown.

Congenital muscular dystrophies are most often associated with sarcolemma network impairment. In these conditions, the transmembrane protein complex mediating the link between the cytoskeleton and the extra cellular matrix is affected and there is a variable degree of muscle fibre degeneration. Generally speaking, muscular dystrophies are characterised by repetitive regeneration processes ultimately leading to the exhaustion of muscle regeneration capacities. Patient muscle biopsies present a loss of muscle fibres jointly replaced by fibro-adiposis (a combination known as “dystrophic pattern”) and inflammatory response markers.

Most congenital myopathies are associated with the mutations of the genes coding for the contractile or triadic junction proteins evocated in Section II (Skeletal muscle contraction) and that are essential for muscle function. Typically, the tissue integrity is preserved and there is no major muscle fibre loss; however, the muscle fibre architecture and the contractile network itself present abnormalities. My work has focused on a clinically heterogeneous type of congenital myopathy: the multiminicore disease (MmD), for which our laboratory has identified most of the mutant genes. I investigated more specifically an unreported form of multiminicore disease associated with a mixed phenotype, including histological or clinical findings commonly observed in other congenital conditions, namely the congenital myopathy cap disease and Collagene VI-related CMDs. Therefore, all these conditions will be more extensively described in this section.

IV.1 General presentation

Congenital muscular disorders are rare inheritied heterogenous disorders, typically apparent from birth or early infancy (before the age of two). Genetic investigations and histologic diagnosis are required to define the condition subtype. Hypotonia and muscle weakness are commonly present at birth and during infancy. Muscle weakness and delayed motor milestones are the common presenting symptoms observed leading to consultation. A significant number of patients are never able to walk. Accordingly to the condition, progressive and restrictive respiratory insufficiency, orthopaedic complications (such as joint contractures and spinal rigidity) or heart disease can be observed.

Recent improvement in our knowledge of the pathophysiological mechanisms involved has open the way to the investigations of potential therapeutic strategies in congenital muscular disorders, which are extensively described by Collins and Bönnemann, 2010. However, no specific treatments are available so far for any of these conditions. Therapeutic approaches are essentialy based on supportive care to preserve functional ability, to provide respiratory support and to prolong patient life expectancy (Wang et al., 2010). This includes correction of skeletal abnormalities such as scoliosis, foot deformities and contractures in order to maintain ambulation. These proceedings are coupled to stretching, bracing and orthopaedic procedures. Pulmonary complications are also a major concern, often requiring physiotherapy and non invasive ventilation from an early age (Wallgren-Pettersson et al., 2004). Permanent ventilation via a tracheostomy can be required in the most severe cases.

IV.2 Congenital muscular dystrophies

IV.2.a General presentation

Congenital muscular dystrophies are clinically and genetically heterogeneous disorders. The exact prevalence of CMD is difficult to estimate due to the frequency of some mutations in certain populations presenting a founding effect. The point prevalence ranges from 0.68 to 2.5 per 100,000 (Mercuri et al., 2009; Norwood et al., 2009; Peat et al., 2008). Also, this number might be underestimated regarding the absence of diagnosis for the mildest forms of muscle disease.

CMD are known to be caused by defects of four major groups of proteins: i) the proteins of the extracellular matrix (ECM), ii) the proteins involved in the post-translational modification of the alpha-dystroglycan external receptor; and proteins with elusive function localised in the iii) the inner nuclear envelope and the iv) the endoplasmic reticulum (Figure 16). A recapitulative table (Annexe 1) presents the genes associated to date with CMD.

The genes encoding structural proteins from the ECM mutated in CMD are: *LAMA2*, *COL6A1*, *COL6A2*, *COL6A3*, *ITGA7*. Regarding the clinical presentation observed in patients, a specific attention will be provided to Collagenopathies (see bellow). The main mutant enzymes associated with alpha-dystroglycan glycosylation are: *POMT1*, *POMPT2*, *FKTN*, *FKRP*, *LARGE*, *POMGNT1*, *ISPD*. In both groups, the interaction between the ECM and the cytoskeleton is impaired and destabilises the sarcoplasmic membrane. Also, dystroglycans play a key role in the formation of the neuromuscular junction and contribute to the physiological location of the acetylcholine receptor (AChR) at the synaptic button. Additionally, alpha dystroglycan glycosylation impairment is frequently associated with mental retardation symptoms associated with structural brain lesions such as lissencephaly and cerebellar cysts among others.

SEPN1 encodes Selenoprotein N. This particular protein contains a specific Sec (selenocysteine) residue in its catalytic domain that confers it highly reactive redox properties

to the protein. Selenoprotein N is located in the endoplasmic reticulum where it is thought to mediate redox homeostasis (its function will be detailed further in the next section). *LMNA* encodes Lamin A/C, a B-type lamin which forms the intermediate filament network of the nuclear lamina apposed to the nucleoplasmic face of the inner nuclear membrane.

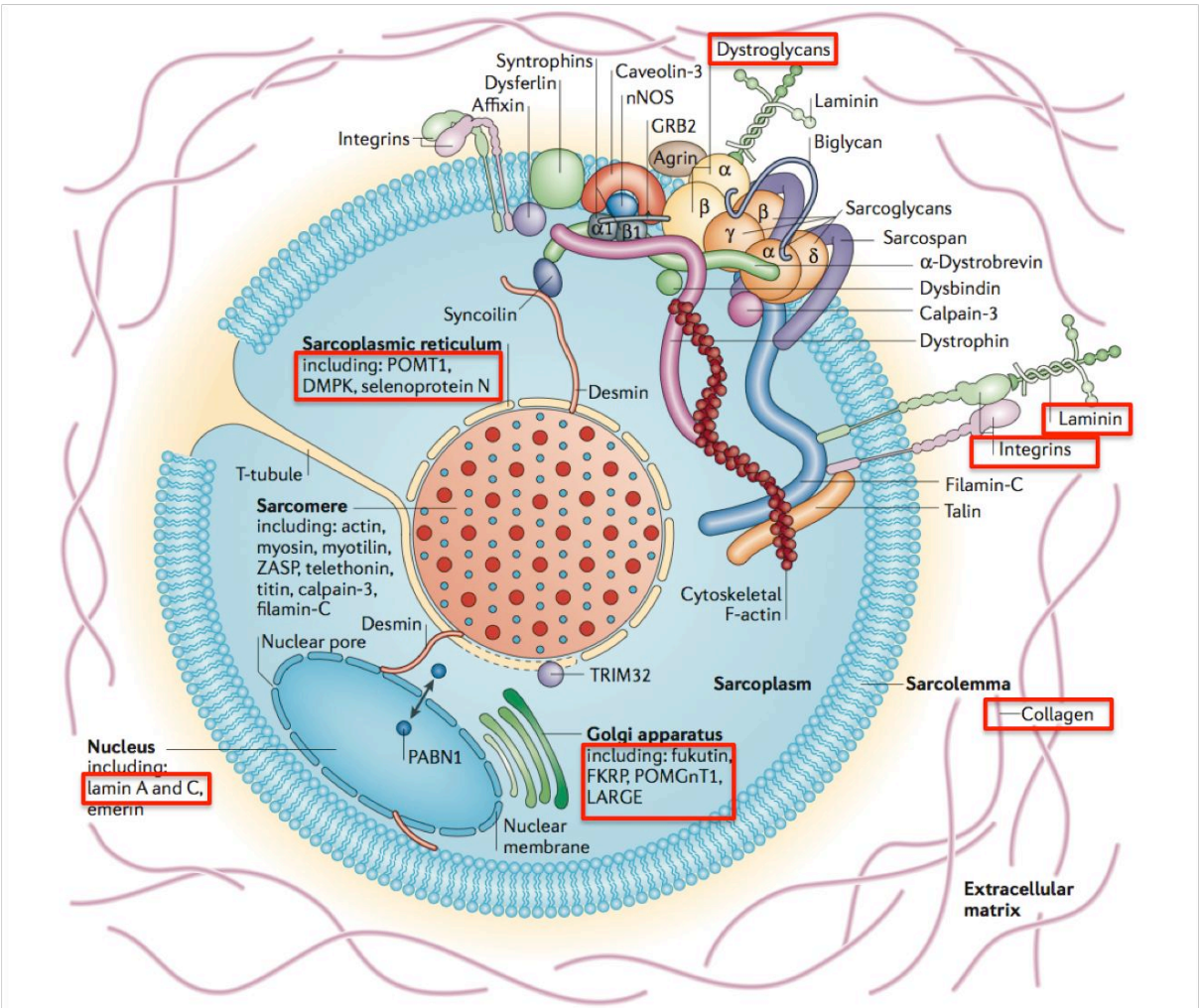


Figure 16: Main proteins involved in congenital muscular dystrophies and their location within muscle fibres. Proteins mutated in CMD (red frame) and their location in the different cell compartments: nucleus, Golgi apparatus, sarcoplasmic reticulum, sarcolemma or the extracellular matrix. Figure from Davies and Nowak, 2006.

IV.2.b Muscle collagenopathies

The collagen VI-related muscular conditions Bethlem myopathy and Ulrich congenital muscular dystrophy (UCMD) are due to mutations in Type VI Collagen. This protein is encoded by 3 genes: *COL6A1*, *COL6A2* (separated by 150kb on chromosome 21q22.3) and *COL6A3* located on chromosome 2q37 (Heiskanen et al., 1995; Weil et al., 1988). Most often, Bethlem myopathy and UCMD are inherited in an autosomal dominant and autosomal recessive manner, respectively. However, in studies that have illustrated original cases (Baker et al., 2005; Pace et al., 2008) some heterozygous mutations impact on the assembly and excretion of the collagen VI (Figure 17) which tends to correlate with the severity of the disease, and cause dominant UCM (Briñas et al., 2010; Camacho Vanegas et al., 2001; Demir et al., 2002, 2004). Intermediate phenotypes have led to consider a continuous spectrum of collagen VI-related muscle disorders. Also, Collagen VI mutations have been described in autosomal dominant limb-girdle muscular dystrophy and autosomal recessive myosclerosis (Merlini et al., 2008a; Scacheri et al., 2002).

Bethlem myopathy is characterised by a combination of progressive proximal muscle weakness and distal joint contractures (finger, elbow, knee and ankles). The first signs typically appear at the end of the first decade of life, and evolution of the disease is slow, although eventually most adult patients will require assistance with ambulation (Jöbsis et al., 1999). Patients with UCMD typically present from birth with neonatal hypotonia, multiple proximal joint contractures and distal joint hyperextensibility. Patients present also a skin phenotype including skin hyperextensibility, hyperkeratosis pilaris and/or keloid scars. Motor milestones are delayed and ambulation is frequently not achieved. A severe respiratory involvement often develops during childhood. Muscle histology reveals type 1 fibre-atrophy and progressive dystrophic features (Schessl et al., 2008).

A potential dysregulation of mitochondrial permeability transition pore (PTP) opening has led to preliminary Cyclosporine A trials in $Col6^{-/-}$ mice (Bernardi and Bonaldo, 2008; Maraldi et al., 2009) and patients (Merlini et al., 2008b). However, additional *in vitro* investigations on patients cells highlight the need for a better comprehension of this defect (Hicks et al., 2009). As Collagen VI is secreted by fibroblasts in muscle (Zou et al., 2008), a recently released paper reports the efficient engraftment of adipose-derived stem cells and their effective secretion of collagen VI in $Col6a1^{-/-}$ animal models (Alexeev et al., 2014).

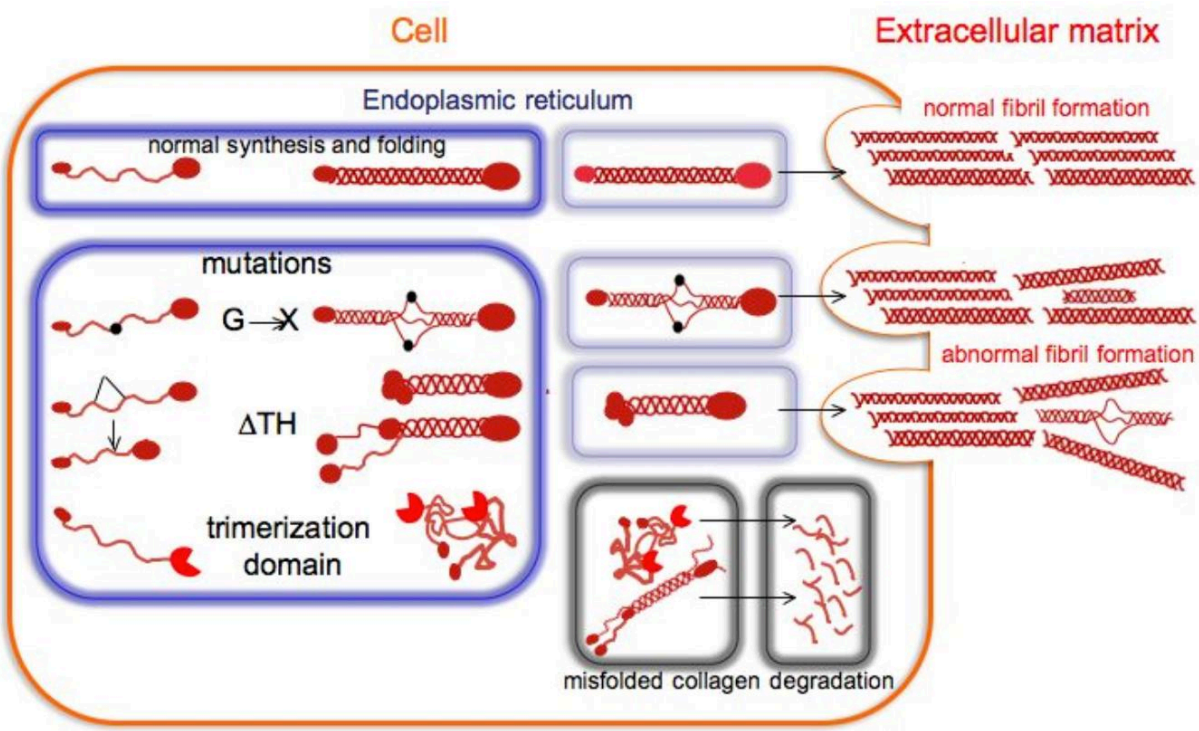


Figure 17: Collagen 6 assembly. This complex process starts with the trimerisation of α-chains encoded by COL6A1, COL6A2, and COL6A3. Intracellularly two monomers align in an antiparallel manner prior to tetramerisation; the structure is maintained by disulphide brigdes of cysteine residues. After secretion, tetramers associate end-to-end forming a microfillamentous network characteristic of the extracellular matrix (Colombatti et al., 1995; Furthmayr et al., 1983). Collagen 6 mutations can be null-mutations; however mutations such as insertions/deletions (indels) or amino acid substitutions can lead to the synthesis of abnormal α-chain unable to assemble and to be secreted. Figure from Bonod-Bidaud and Ruggiero, 2013.

IV.3 Congenital myopathies

IV.3.a General presentation

Classically, these conditions differ from muscular dytrophies by the type of histological lesions observed. In congenital myopathies the crosstalk with the extracellular matrix and the sarcolemma integrity is maintained and the tissues exhibit little or no necrotic or regenerative fibres. However, the muscle fibre internal architecture itself is altered and presents some characteristic lesions, each of which defines an individual form of congenital myopathy. The most frequent lesions are i) intracytoplasmic inclusions, ii) cores, iii) central nuclei, or iv) fibre type disproportion (Bönnemann and Laing, 2004; North, 2008; North et al., 2014). A recapitulative table (Annexe 2) provides the list of the known genes responsible for these congenital myopathies.

These conditions can be subdivided in 4 main groups according to the histological abnormalities described above. Mutated genes identified in congenital myopathies are then clustered regarding the histological defects observed: i) protein accumulation (*ACTA1*, *MYH2*, *MYH7*, *TPM2*, *TPM3*, *TNNT1*, *NEB*, *CFL2*), ii) the presence of cores (*RYR1*, *SEPN1*, *TTN*, *MEGF10* and hardly ever *ACTA1*, *MYH7*, *CFL2*), iii) centralised nuclei (*MTM1*, *BIN1*, *DNM2*, *RYR1*) or iv) fibre size disproportion (*ACTA1*, *SEPN1*, *TPM3*, *RYR1*, *MYH7*). Most of these genes encode contractile proteins associated to one of the three major components of the sarcomere: the thin, the thick and the titin filaments. Additionally, some of these proteins and particularly RyR1 are (or are thought to be) associated to the triadic junction, which is the membrane crosstalk platform responsible for Excitation-Contraction coupling. Other pathophysiological mechanisms are however involved in other forms of congenital myopathies, such as part of the phenotypical forms of multiminicore disease, which will be the object of the next section.

IV.3.b Cap disease

Cap disease is a rare condition originally identified in 1981 (Fidzianska et al., 1981) whose prevalence remains unknown. Patients present characteristic histological abnormalities, namely the accumulation of unorganised contractile proteins under the sarcolemma, localised in the peripheral part of the myofibre and taking the shape of a cap. These structures are assumed to originate from abnormal fusion and/or muscle protein synthesis (Fidziańska, 2002). The proportion of myofibres with caps seems to correlate with the severity of the condition. Patients exhibit muscle hypotonia, weakness, skeletal dysmorphism and respiratory insufficiency since early childhood, which can be fatal in the most severe forms. Patients may also present with a high-arched palate, ptosis and long faces; they can also developed spinal lordosis or scoliosis.

So far, known mutated cap disease genes encode the thin (actin) filaments. Heterogygous mutations in *TPM2* and *TPM3*, encoding tropomyosins, were recently identified (Clarke et al., 2009; Lehtokari et al., 2007; Malfatti et al., 2013; Ohlsson et al., 2008, 2009; De Paula et al., 2009; Waddell et al., 2010). Cap disease has also been associated with mutations in *ACTA1*, coding for actin (Hung et al., 2010) thus enlarging the spectrum of mutated genes. The potential pathophysiological mechanism relies on contractility defects due to impairments in actin-myosin affinity and Ca^{2+} sensitivity (hyper- and hypo-sensitivity). Also, predictions associate mutations to impaired assembly of thin filaments (Marttila et al., 2012, 2014; Tajsharghi et al., 2012).

IV.4 Specific focus: Multiminicore disease

Multiminicore disease (MmD) is an early onset, autosomal recessive congenital myopathy that was named after the observation of histological abnormalities called "minicores" in muscle fibres. Minicores are multiple localised foci of sarcomere disorganisation (visible by electron microscopy) and mitochondria depletion, with expand only along a few sarcomeres along the myofibre longitudinal axis (Engel et al., 1971). Typically, no dystrophic signs (i.e. fiber necrosis or regeneration, significant endomysial fibrosis) are observed in MmD patients. One particularity of MmD is its clinical heterogeneity, with at least 5 different phenotypical forms (Ferreiro et al., 2000). Their main common features are neonatal hypotonia, delayed motor development and generalized muscle weakness predominantly axial. Interestingly, our laboratory has identified several genes associated with this condition, some of which are also associated with other congenital myopathies and muscular dystrophies. Of note, the correlation between phenotype and genotype is not established. The proteins encoded by the genes responsible for this condition are involved in different pathways.

Titin (encoded by *TTN*) is the largest mammal protein identified so far. Considered as the third major component of the sarcomere, its various spliced isoforms are essential for sarcomeric stiffness and elasticity and therefore rule the muscle fibre contraction capacities. Titin mutations are associated with different skeletal and cardiac muscle conditions, most of them autosomal dominant and starting in adulthood (Chauveau et al., 2014). In the laboratory, we have indentified forms of multiminicore myopathy associated to a large range of childhood-onset cardiac diseases which are due to autosomal recessive *TTN* mutations (Carmignac et al., 2007; Chauveau et al., 2013). Today, the frequency of *TTN* mutations in human conditions is increasingly recognised (Chauveau et al., 2013; Herman et al., 2012; Lopes et al., 2013). Recent investigations reinforce the complexity of the spectrum of *TTN* mutations and their heterogeneous associated clinical phenotypes (Evilä et al., 2014).

RYR1 encodes the skeletal muscle ryanodine receptor, a calcium channel of the sarcoplasmic reticulum that is essential for Ca^{2+} release during excitation-contraction coupling at the triadic junction. The RyR1 calcium flux is redox sensitive, which links this protein with the Selenoprotein N (Jurynec et al., 2008). Originally, *RYR1* mutations were

associated with the pharmacogenetic condition malignant hyperthermia (Brandt et al., 1999; Manning et al., 1998; Sambuughin et al., 2001) and also to Central Core Disease (Monnier et al., 2001; Quane et al., 1993; Tilgen et al., 2001; Zhang et al., 1993; Zorzato et al., 2003). Other forms of congenital myopathy, including MmD (Ferreiro et al., 2002a; Monnier et al., 2003), core-rod disease (Monnier et al., 2000; Scacheri et al., 2000), centronuclear myopathy (Wilmshurst et al., 2010), or fibre type uniformity (Sato et al., 2008) have also been described.

SEPN1 encodes the glycoprotein Selenoprotein N (SelN) (Lescure et al., 1999), the first selenoprotein associated to a human genetic disease. After primarily being related to rigid spine muscular dystrophy (Moghadaszadeh et al., 2001), *SEPN1* mutations have also been identified as a cause of multiminicore disease (MmD), desmin-related myopathy with Mallory body-like inclusions and Congenital Fiber Type Disproportion (CFTD) (Clarke et al., 2006; Ferreiro et al., 2002a, 2004). This large spectrum of histological presentations associated with *SEPN1* mutations contrasts with the highly homogeneous clinical presentation in all patients; thus they are thought to represent one unique condition known as "SEPN related myopathy" (Arbogast and Ferreiro, 2010). The protein is localised in the endoplasmic reticulum membrane (Okamoto et al., 2006), however its function remains incompletely understood. Expressed at low levels in adult tissues, the protein is present at higher concentrations during development. It has been shown in a zebrafish model that Selenoprotein N contributes to slow muscle lineage (Jurynec et al., 2008). Moreover its depletion has a dramatic impact on satellite cell dynamics under regeneration conditions which correlates with an exhaustion of this pool of cells in patients (Castets et al., 2011). Experiments conducted by our group have determined the key role of this protein in redox homeostasis and in cell survival upon oxidative stress conditions (Arbogast et al., 2009). Additionally, patient cells show calcium homeostasis abnormalities consistent with an oxidative stress-related RyR dysfunction. Pharmacological therapeutic approaches using an antioxidant such as N-acetylcysteine represent a promising treatment in SEPN1- and RYR1-related myopathies (Arbogast et al., 2009; Dowling et al., 2012).

The transmembrane protein encoded by *MEGF10* is mutated in autosomal recessive skeletal muscle disorders including discrete rare cases with multiminicores (Boyden et al., 2012; Hartley et al., 2007; Logan et al., 2011; Pierson et al., 2013). MEGF10 is expressed in the brain and spinal cord, but also in muscle tissues including skeletal, cardiac and smooth

muscles (Nagase et al., 2001). In zebrafish, mutations lead to severe motor impairment due to widespread myofibrillar disorganisation. *MEGF10* regulates satellite cells myogenesis and therefore MEGF10 conditions are the first "satellite cell conditions" described. Overexpression of MEGF10 leads to increased proliferation in a C2C12 cell line, consistent with the profound satellite cell pool exhaustion associated with premature differentiation observed in patient cells (Holterman et al., 2007; Mitsuhashi et al., 2013; Nagase et al., 2001).

Thus, mutations associated to *SEPN1* and *MEGF10* are consistent with a relevant pathophysiological mechanism related to satellite cell pool exhaustion due to premature differentiation that enhances the contribution of myogenesis and muscle regeneration to congenital myopathies.

Multiminicore has also been observed in rare cases with *ACTA1* and *DNM2* and *NEB* mutations associated to histological features typical of other congenital myopathies, such as centralised nuclei and nemaline rods (Jungbluth et al., 2001; Kaindl et al., 2004; Romero et al., 2009; Schessl et al., 2008). Additional mutations in *MYH7*, *DOK7*, and *CCDC78* genes in cases with multiminicore lesions have also been lately described (Clarke et al., 2013; Lorenzoni et al., 2013; Majczenko et al., 2012; Muelas et al., 2010). The proteins encoded by *MYH7* and *DOK7* genes participate to muscle contraction and postsynaptic organisation respectively. *CCDC78* gene function remains elusive, although the corresponding protein seems to localise at the triadic junction. Nonetheless, no clear pathophysiological pattern has emerged yet.

V. METHODS OF INVESTIGATIONS IN MONOGENIC DISORDERS

During my PhD, I focused my interest on multiminicore disease and the identification of new mutated genes in this monogenic disorder. I will provide an overview of the strategies developed over the last thirty years, which are at the origin of modern molecular biology. Although these techniques are still largely used for medical purposes, the emerging of massive parallel sequencing techniques is considerably changing this field. I will mention the current available strategies and their limitations.

V.1 Human genome and monogenic disorders

In the past 50 years, significant progresses in molecular biology have allowed major technological breakthroughs that end up in the generation of scoped databases carrying whole genome sequences from various organisms: from bacteria to human beings. Those powerful technologies were also developed to be tools at the service of medicine.

The human genome is composed of 23 pairs of chromosomes: 22 autosomal and two sex determining pairs (male: XY, female: XX) This set is localised in the cell nucleus, therefore termed nuclear genome and composed of 3 $x10^9$ base pairs. The approximated 23,000 human genes are encoded by less than 3% of the genome; which do the 37-mitochondrial coding genes complete. Current identification of human genetic disorders requires the screening – also termed genotyping – of these coding sequences based on the reading of DNA sequence according to Dr F. Sanger method (1977).

Monogenic disorders are caused by a single variation (mutation) carried by an individual. This mutation can be *de novo* or inherited according to Mendel's laws: transmission from parents to offspring. Consequently, this mutation can be pursued in the family by different techniques that would be described hereafter.

V.2 Classical strategies used in human mutation screening

In the past 30 years, extensive work has been conducted to identify human mutations at the origin of monogenic disorders. This work is based on the emergence of a robust technique allowing the amplification of DNA fragments: the Polymerase Chain Reaction (PCR, Mullis et al., 1986; Nelson, 1991). Empirical techniques based on PCR amplification (first and second generation) remain reliable and have therefore been the technical bases of my PhD investigations. Hereafter are presented the techniques commonly used and associated to the sequencing of "first generation".

V.2.a Linkage analysis

Linkage analysis consists on the approximative localisation of a gene relative to another genomic DNA sequence termed "genetic marker", which position is already determined. Genetic markers are polymorphic DNA sequences (size or sequence) carried by each individual and transmitted through generations according to Mendel's laws. Comparison of these highly variable markers between individuals is at the origin of phylogeny. Therefore, causative genes are mapped by measuring recombinaison against a panel of these genetic markers spread over the genome. In absence of any recombination event between the causative gene and the marker, the two are "linked" that refines the quantitative trait locus (QTL).

Prior to the identification of the mutated gene a linkage analysis is performed on genomic DNA from the different relatives of an affected family. This strategy allows to retricted investigations to genomic regions, which segregate into the family with the pathology. This long-standing concept (Sturtevant AH, 1913) used today two types of marker: Single Nucleotide Polymorphisms (SNPs) and microsatellites.

Microsatellites, also termed simple sequence repeats (SSR), are composed of 2 to 10 nucleotides repeat motifs – most commonly AC tandem repeats – and represent 3% of the genome and distributed among exonic and intronic regions (Dib et al., 1996; Lander et al.,

2001; Subramanian et al., 2003; Tóth et al., 2000). Recently, the use of SNPs, more profitable in terms of cost efficiency, has been favoured (Shen et al., 2005; Syvänen, 2005) as they are more frequent across the genome (Last NCBI, US National Library Medicine update from July 23, 2013 listed 62,676,337 SNPs). SNPs by definition correspond to single nucleotide variations in the population and are commonly termed alleles. Most of these SNPs exist as unique set of two alleles. However, SNPs are not necessarily distributed uniformaly along the genome, making these techniques not equivalent but complementary (Ball et al., 2010; Varela and Amos, 2010).

V.2.b Positional cloning

Positional cloning is an empirical strategy to assess a genetic diagnosis. It allows narrowing a candidate region, like those obtained by linkage analysis, until the gene and its mutation are found. This strategy relies on two-steps identification. The first step requires the mapping of the individual's genome based on the isolation of partially overlapping DNA segments from genomic libraries cloned in bacterial systems. This makes possible the progression toward a contiguous locus of interest. In a second step, the sequential screening of additional markers in the locus narrows the window of investigations to a unique candidate gene. Further sequencing and comparison to databases would confirm the molecular defect. Alternatively a "candidate gene" approach may shorten the process of investigation. Locus associated genes with expression patterns consistent with the disease phenotype or showing a function related to the phenotype could be considered as candidates.

V.2.c Concrete case: Investigation in consanguineous families

Maintenance of biodiversity during the reproduction process of all organisms is achieved through genetic mixing. Each parent provides half of its genetic material to generate a new organism carrying a unique genetic combination. During meiosis (process contributing to gametes generation by reducing the number of chromosomes to the half), several chromosomal recombinations occur leading to the transmission of a unique subset of characters.

From a genetic point of view, a consanguineous family is an interesting subpopulation to study. Strikingly, an individual receiving from his two parents a deleterious gene variant would necessarily received the associated genetic markers surrounding the mutation that would be informative for its localisation. In consanguineous families, autosomal recessive conditions are investigated as a priority. In this situation, both parents received from a common ancestor the deleterious gene variant. Their affected child - carrying the two parental deleterious gene variants – is homozygous for the mutation and the set of surrounding genetic markers (haplotype). Thus, homozygocity mapping would permit the identification of homozygous loci. Loci shared between patients and healthy siblings would be discarded. On the contrary, homozygous loci that would be identified in patients and not healthy siblings (non carriers or heterozygous) would be retained for further investigations. The more generations between the common ancestor and the parents, the few loci should be shared and the smaller they would be. Afterwards, a candidate gene research is commonly performed to identify the causative gene.

V.3 Second generation sequencing

In the last decade, the combination of major technical improvements and the development of high-performance computing (driven by Moore's Law 1965), leads to the emergence of the Next Generation Sequencing (NGS) also termed second-generation sequencing which allows the sequencing of the entire genome of an individual. This approach is widely used in numerous biological fields and currently dominates clinical genetics investigations (Mardis, 2008; Morozova and Marra, 2008).

The NGS technology is based on the previous strategy: amplification by PCR and sequencing. The main breakthrough comes from the possibility to attach small DNA molecules to solid surfaces or beads, thus allowing millions of sequencing reactions to happen in a parallel fashion. Moreover, only a small amount of DNA is now required to generate fragments "libraries". First, DNA is fragmented into small sequences spared from 40 to 250 bp long. Then, DNA fragments undergo poly(A) end repair that provide anchorage for ligation of adapters sequences. These adapter sequences enable the clonal amplification of these fragments, generating the library. Adapters also carry the docking site for support-specific primers and a unique signature that enable the sequencing of several sets of signatures (different individuals) in the same process. In the specific case of our investigations, a whole exome sequencing (WES) has been performed. The enrichment in exons fragments termed "exon capture" is performed prior to amplification and sequencing steps; its description is provided Figure 18.

The sequencing process relies on the sequentially identification of signals emitted from the template strand fragment re-synthetised, termed "read". Millions of reads generated are computationally reassembled using a reference genome as a scaffold. While processing hundreds of amplicons only required few hours, analysis requires considerable amount of time. Three main suppliers offer equipment for this technique: Roche 454 GS FLX Genome Analyser commercialised by Roche, Solexa 1G sequencer from Illumina and AB SOLiD by Applied Biosystems. Our Canadian collaborators are using the last equipment that provided the data I analysed; therefore only a scheme of the AB SOLiD system is provided (Figure 19).

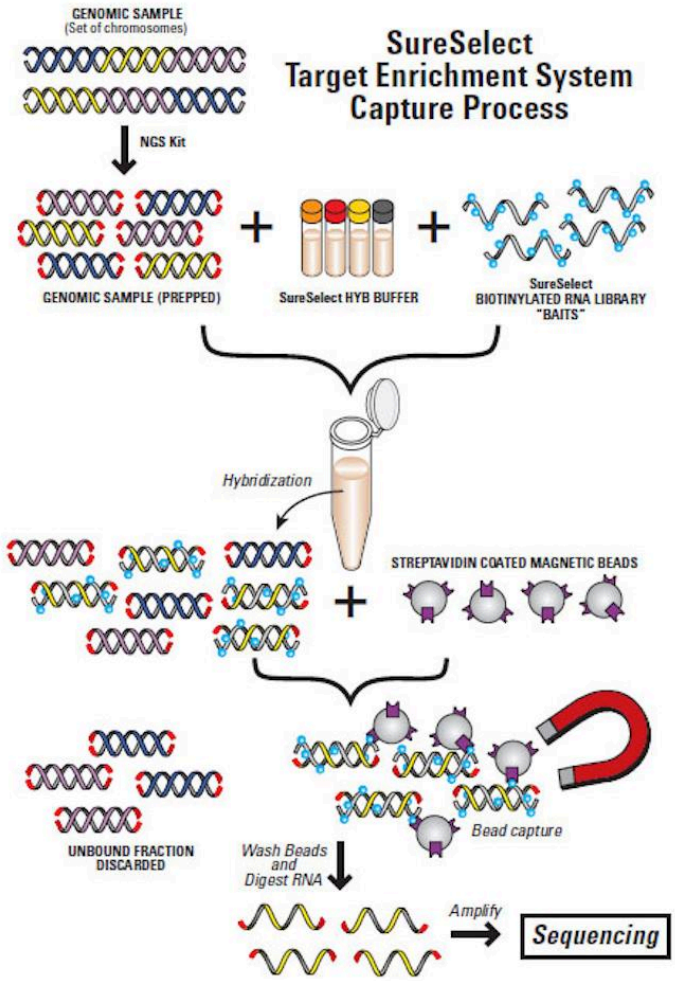


Figure 18: Preparation of fragment libraries and exonic enrichment. Genomic DNA is fragmented and fragments undergo an end repair step, facilitating the ligation of adapters. An optional step is described here: the exonic enrichment step. A library of biotinylated RNA fragments is allowed to bind complementary fragments of the DNA sample of interest. These biotinylated RNA-DNA complexes are recognised by streptavidin coated magnetic beads. Fragments that are not bind to streptavidin beads are assumed to be intergenic fragments (non transcribed elements) and discarded by washing. The enriched fragments are then detached from the magnetic beads by degradation of the RNA baits. Released fragments undergo amplification prior to sequencing. Figure from Sure Select, Agilent Technologies.

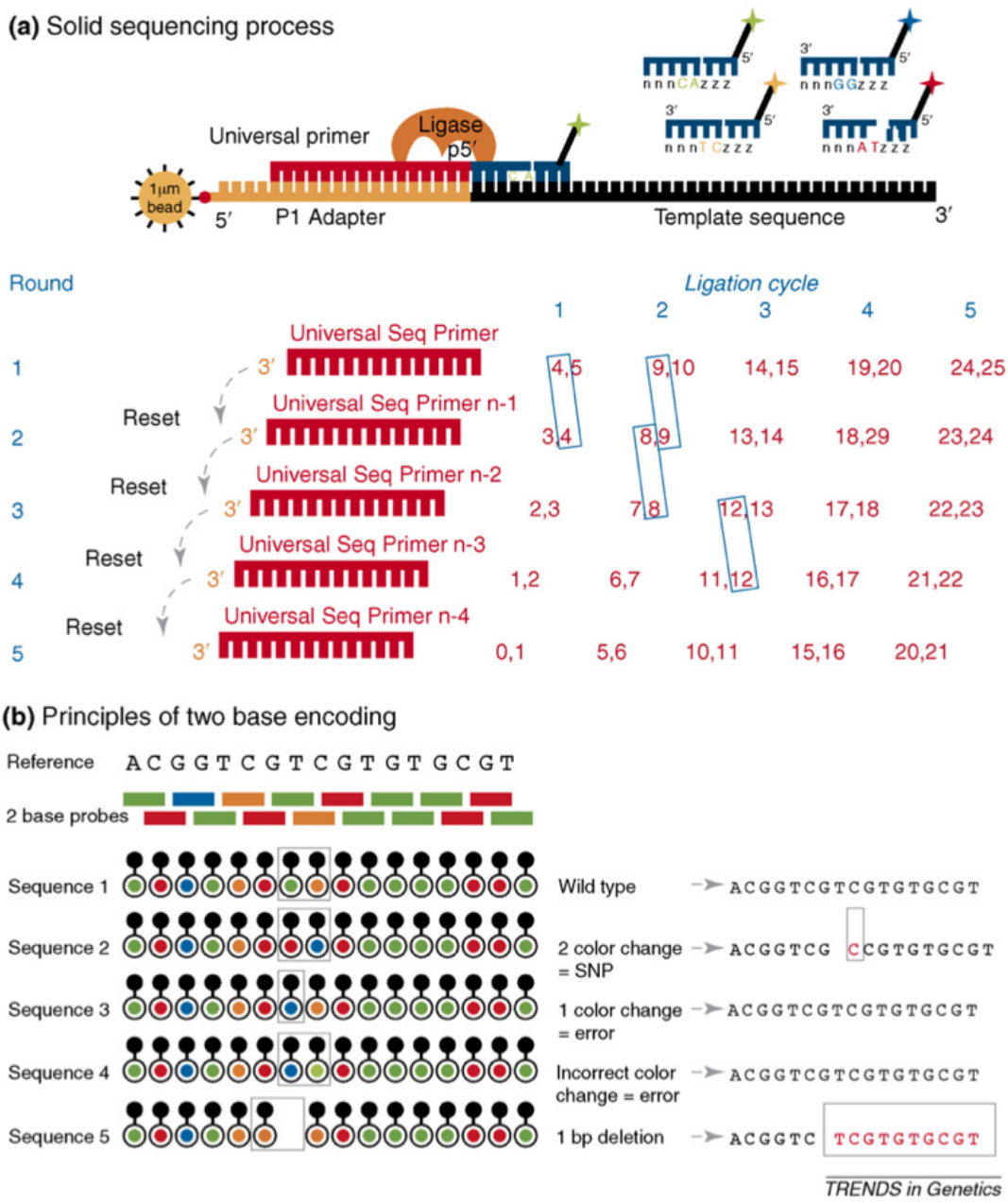


Figure 19: AB SOLiD sequencing strategy and 2 base encoding processing. AB SOLiD is termed after Sequencing by Oligo Ligation and Detection. A run is processed in 5 days and produced 3 to 4 Gb of data with average read length of 30-50 bp. This technique requires 5 rounds of sequencing to recapitulate a continuous sequence. (a) Sequencing process starts with the annealing of a universal primer to the adapter sequence. A complementary labelled 8mer oligonucleotide is added, ligated, prior to imaging. The fluorochrome code is based on the 2 bases (position 4 and 5) of these degenerated oligomers. By chemical cleavage (between position 5 and 6), the fluorochrome is released, facilitating the following 8mer binding. The following fluorescent signal would provide information for fragments bases position 9 and 10. (b) The technique relies on the nucleotide identities to discriminate base calling errors from true polymorphisms and indels events with more than 95% of confidence. Figure from Mardis, 2008.

V.4 Third generation sequencing

The next generation sequencing, coming from the second generation, is already termed "current generation" and would soon become "last generation sequencing". Numerous improvements of such a high throughput sequencing strategy are constantly achieved. However, this emerging technology is still under optimisation. These techniques are described by two reviews (Pareek et al., 2011; Schadt et al., 2010).

This new generation of sequencing aims to reduce costs, time, DNA amounts, errors rates etc. The 3rd generation sequencing is based on the direct detection of a signal while a nucleotide is incorporated (or cleaved). Most of these techniques do not require anymore the generation of adapter – DNA fragments libraries, getting read of the PCR amplification steps (cost and reagents) and of the associated biases. Two techniques are based on nucleotide incorporation and can detect, during the time course of the polymerase activity 1) the phospholinked nucleotides incorporation: SMRT (Single Molecule Real Time) or 2) the hydrogen ions (H_30^+) released by DNA synthesis: Ion Torrent. One of the major benefits of this technique is the abolition of time consuming "washing steps". In the future, several concepts aim to go without any synthesis and to detect bases electronically using a Scanning Tunnelling Microscope (SMT) where the ssDNA is passing through a pore. The ssDNA sequence can be detected while it is degraded by an exonuclease: Nanopore Sequencing or simply by passing the DNA molecule through a "transistor" system (alternation of metal and dielectric material). The proof of concept of this last technique developed by IBM has not yet been published.

VI. AIMS OF MY PROJECT

Congenital myopathies have been in the last decades the object of massive genetic investigations. While for other muscle conditions such as Duchenne muscular dystrophy, clinical trials start to emerge, these early onset conditions remain without cure, and their potential complications have a strong impact on the quality of life and the life expectancy of these patients.

My work focused on a specific form of congenital myopathy, termed multiminicore disease (MmD). Our laboratory is at the origin of the identification of most of the multiminicore – related genes and has collected DNA samples from over 200 patients and their relatives over the world. We estimate that at least 30% of MmD cases remain without molecular diagnosis. At the age of massive parallel sequencing, these uncharacterised MmD forms deserve intensive investigations.

During my PhD, my objective was to identify new mutated genes in MmD and to characterise the role of the resultant protein in muscle physiology and pathophysiology. These approaches have significant clinical impact, opening the possibility of molecular diagnosis and prenatal screening to affected families. Furthermore, they improve our understanding of muscle function. On these purpose I conducted investigations based on linkage analyses and massive parallel sequencing data previously generated in the lab.

As a main project, I conducted investigations to characterise the role of a novel transcriptional coactivator. Therefore I developed an *in vitro* model that was a mandatory basis for the identification of novel pathways potentially useful as therapeutic targets. Although transcriptomic analyses, did not allow me to identify affected pathways, I have been able to show the requirement of this protein to muscle differentiation.

MATERIAL & METHODS

I. GENETIC INVESTIGATIONS

I.1. Patients DNA samples and consent

Investigations were performed in 5 genetically informative consanguineous families presenting with one or more affected children compatible with an autosomal recessive transmission and non-linked to known MmD genes. These families were addressed to our laboratory for identification of new causative gene associated to MmD. Patients presented with the classical form of MmD. Minicores had been identifiy by the referring histopathology laboratories by standard histological investigations (oxidative histochemistry and electron microscopy) on diagnostic muscle biopsies. DNA from all the family members was obtained from peripheral blood samples following standad methods, after informed consent.

I.2. Linkage analyses

Linkage analyses were performed using the Affymetrix Human Mapping 250K Nsp Array (approximately 262 000 SNPs) and the Illumina Human panel V, 6056SNPs. Patients, their parents and healthy siblings were investigated. Linkage analyses were performed under the assumptions of autosomal recessive inheritance, an equal recombination frequency for male and female subjects, a disease-gene frequency of 0.0001, one liability class and penetrance of 1. Logarithm of odds (LOD) scores were calculated for equal allele frequencies using MERLIN program (Abecasis et al., 2002). Haplotypes were constructed under the assumption of the minimum number of recombinations.

I.3. Next Generation Sequencing

The Whole Exome Sequencing (WES) was performed for selected patients using genomic DNA in collaboration with M. Samuels' lab, CHU Sainte-Justine, Montreal, Canada. The library was established with the Sure Select Human All Exons 50Mb Kit (Life

Technologies) according to the manufacturer's protocol, including approximately 180,000 annotated protein-coding exons plus selected non-coding RNA. Captured fragments were sequenced in single-end 50-base mode using AB SOLiD4 (Life Technologies) chemistry. Reads were aligned to the reference genome GRCh37 and analysed with NextGene v.2.16 software (Soft Genetics, Inc). The analysis was carried on a minimal 6 reads coverage and variation present in 80% of reads, with a mutation score of 10. Unknown variations were also compared to databases such as UCSC (Kent et al., 2002) and Exome Variant Server (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA). Collectively, these complementary approaches favour the investigation of candidate genes carrying variants unknown, unreported and localised in loci segregating with the condition in families.

I.4. Positional cloning and Sanger sequencing

Variants of interest were re-sequenced by Sanger sequencing in patients tested. After verification of the segregation in the family, the presence of the variant was investigated in 96 DNA samples from a cohort with non-neuromuscular disorders DNA samples with similar ethnical and regional origins.

Sanger sequencing was used to investigate *TRIP4* gene coding sequence (exons and surrounding 100 bp-length intronic sequences) as a positional candidate gene. Sequencing was performed in the orignical family and then in additional series of patients with MmD (32 patients), Collagene VI-like (19 patients) or Cap disease (7 patients). Fragments were amplified with the GC Rich PCR System dNTPack (Roche). The primers used are listed in Annexe 3.

II. CELL CULTURE

II.1. Human material

Primary skin and muscle cell cultures were obtained after informed constent from patients III.1 (myoblasts from a diagnostic muscle biopsy) and II.1 and II.2 (fibroblasts) and

for the healthy mother I.1. Myoblasts were maintained in proliferation in F-10/DMEM GlutaMAX (4,5g/L glucose, Pyruvate) 20% FBS 2% Penicillin-Streptomycin and changed at confluence for the differentiation medium in DMEM GlutaMAX (4,5g/L glucose, Pyruvate) 2% HS 2% Penicillin-Streptomycin. Fibroblasts were maintained in proliferation in DMEM (1g/L glucose, L-glutamine, Pyruvate) 10% FBS 2% Penicillin-Streptomycin. Cells were cultured and sampled for mRNA and proteic purpose.

II.2. Murine myoblastic cell line

C2C12 cells were cultured in DMEM GlutaMAX (4,5g/L glucose, Pyruvate) 10% FBS 1% Penicillin-Streptomycin (Sigma). At confluence (85-90%), the medium was changed for the differentiation medium DMEM GlutaMAX (4,5g/L glucose, Pyruvate) 2% HS 1% Penicillin-Streptomycin.

II.3. RNA silencing

For the *Trip4* knockdown experiments C2C12 cells were plated 24 hours prior to transfection. Cells were then transfected in OptiMEM (GIBCO) with Lipofectamine RNAiMax (Invitrogen) and siRNAs or scramble sequences (OriGene). To obtain an efficient silencing, cells are transfected with a mix of two siRNA (A) 5' AGCACAAACUCAUCAAUAACUGUCT 3' and (B) 5' GCAGACUAGAUGAGACAAUACAAGC 3' targeting murine Exon 7 and 11 respectively. Maximun downregulation was achieved 48 hours post transfection. Except specific mention, all the experiments carried out started 48h post transfection.

III. TRANSCRIPTOMIC ANALYSES

III.1. RT PCR & q RT PCR

RNA was extracted from cultured cells using the RNEasy Mini Kit (Qiagen) according to the manufacturers' insctructions. Genomic DNA was eliminated by deoxyribonuclease

treatment (RNase-Free DNase set, Quiagen) and samples were immediately stored at -80°C. cDNA was obtained with the SuperScript III Kit (Invitrogen) according to the manufacturers' protocol. Semi-quantitative PCR was performed on human cells cDNA with SuperMix Platinium (Invitrogen). Quantitative PCR was performed on a Light Cycler 480 II (Roche) using a compatible SYBR Green I Master 2x (Roche) in 20 µL reaction mix. The primers used are reported in Annexe 3.

III.2. Microarray

Total RNA samples were collected from C2C12 knocked down for *Trip4* at 48h (proliferation condition) or 72h (early differentiation) post transfection; under the same conditions as previously described (III.1). Purification and quality were tested on an Agilent RNA6000 Nano LabChip II in a Bioanalyzer 2100 (Agilent). The RNA integrity number was ranged between 9.5 and 10.

Microarray experiments were performed using MouseWG-6 v2.0 whole-genome Expression BeadChips (Illumina) cointaning approximatively 45,000 probes (over 26,000 established and annotated coding transcripts). cRNA strands were synthetised using Ambion kit and purified on Qiagen columns. Briefly, the resulting double-stranded complementary DNA was used to synthethise biotin-labelled complementary RNA. After purification, complementary RNA was fragmented and hybridized to chips for 20 hours. Chips were washed and scanned. The image was then analysed with BeadScan software to obtain raw data and metrics for quality control.

Data were quantile normalised using BeadStudio software. The working lists were created by filtering probes with detection P values < 0.05 for all the chips and discarding overlapping probes. Each dataset was derived from three biologically independent replicate samples. Independent samples were compared by computing fold ratios and were filtered at a 0.8 fold ratio for Venn Diagrams and pathways analysis. For pathway analysis, GenBank accession numbers were mapped to the Ingenuity database (IPA, http://www.ingenuity.com) to retrieve relevant biological processes.

III.3. Luciferase assays

C2C12 cells were co transfected with a total equal amount of plasmids material containing 1/ either a plasmid carrying the *Trip4* transcript (pCITO-Trip4) or an empty control vector pCITO. Cells were also transfected with reporter constructs as 2/ a Renilla internal reporter and 3/ pGCL2 Luciferase reporter constructs under the control of a minimal promoteur (pGCL2-LUC), the desmin promoter (DES-LUC) or the p21 promoter (p21-LUC). Cells were transfected and incubated for 48h. Cells were treated with 100 mM thyroid hormone 6h, harvested and assayed for luciferase. Enzyme activity was measured in a Centro XS LB 960 Microplate Luminometer (Berthold Technologies) and normalised to the TK Renilla expression accordingly to manufacturers conditions (Dual-Luciferase reporter Assay System, Promega).

IV. PROTEIN ANALYSES

IV.1. Western Blotting

Cellular samples were collected in Cell Lysis Buffer (Sigma-Aldrich) and completed with 1% Protease inhibitor Coktail (Sigma-Aldrich). C2C12 cell lines extracts (15-20 µg) were separated on precast PAGE Criterion TGX 4-15% gels (BioRad) and transferred on PVDF Trans-Blot Turbo membranes (BioRad). Membranes were blocked in PBS 1x, 0.2% Tween-20, 0.25% SDS-20 and 2% skimmed milk. Membranes were probed with primary antibodies 4 hours at room temperature, washed twice and then probed with a fluorescent-coupled secondary antibody for an hour. Primary cultured cells extracts (30 µg) were separated and transfered under the same conditions. Membranes were blocked in PBS 1x, 0.01% Tween-20 and 3% skimmed milk prior to similar antibodies incubation conditions. Immunolabelled proteins were detected using the Odyssey CLx system. The antibodies used are listed in Annexe 4. Quantification of western blots was carried out using ImageJ and normalised to Tubulin or Histone H3 levels.

IV.2. Animals tissues sampling

Six weels old adult mice were sacrificed according to ethical proceeding by cervical dislocation. Tissues were collected and immediately frozen in cooled isopentane. Tissues were then grinded prior to resuspension. Tissue extracts (30 µg) were then separated by western blotting accordingly to the protocol mentioned herebefore. Quantification of western blots was carried out using ImageJ and normalised to GAPDH.

IV.2. Immunofluorescence

Cells were washed in PBS 1x prior to fixation – permeabilisation in methanol for 5 minutes at -20°C. Cells were then blocked in PBS 1x, 2% BSA, 0,1% Triton X100 for one hour at room temperature followed by the incubation with primary antibodies for 4 hours, washing and incubation with secondary antibodies for one hour at room temperature. Finally, samples were mounted in Vectashield and imaged on a Zeiss LSM 700 confocal microscope using the ZEN 2009 software. Antibodies references and dilutions are provided in Annexe 4.

VI. STATISTICAL ANALYSES

Values are presented as +/- SEM. Statistics were calculated using the PRISM software. P-values <0.05 were considered statistically significant (* $p< 0.05$, ** $p< 0.01$).

RESULTS

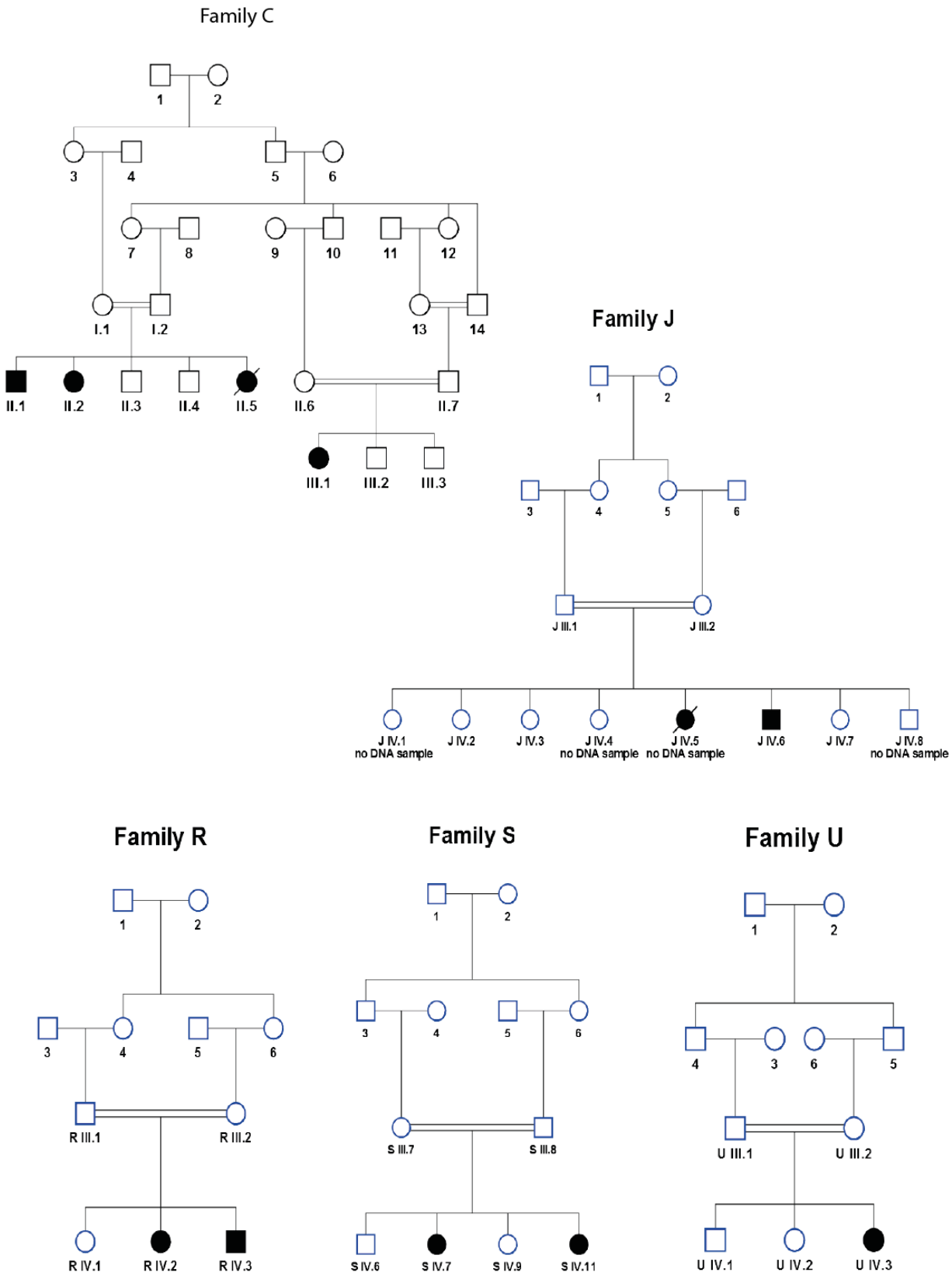


Figure 20: Pedigree of the 5 families investigated. DNA samples were collected from parents and children on the last two generations. Mention for non-available DNA sample is provided underneath concerned individuals. Pedigrees generated on Haplo Painter software.

I. COHORT PRESENTATION

A unique cohort of 5 consanguineous and highly informative families (Figure 20) was investigated in order to identify new genes associated with multiminicore disease. All patients presented with histological abnormalities consistent with multiminicore disease. They also presented with congenital or infantile-onset muscle dysfunction of variable severity, associated in all cases with scoliosis and/or respiratory insufficiency, compatible with the classical form of MmD. None of the 5 families had cardiac disease. The pedigrees and parental consanguinity strongly suggested an autosomal recessive inheritance. Implication of the known MmD genes had been excluded through linkage and/or sequencing studies.

II. IDENTIFICATION AND CHARACTERISATION OF A TRANSCRIPTIONAL COACTIVATOR MUTATED IN AN UNREPORTED FORM OF CONGENITAL MYOPATHY

I focused the main part of my PhD on the characterisation of the physiological and pathophysiological relevance of a novel gene, mutated in one large family (Family C) and associated with an unreported form of congenital myopathy. This part of my project will be the object of a publication as first author. Family C is a consanguineous family of French origin presenting several consanguinity loops (Figure 20). The three affected children are distributed in two different branches, which were collected separately.

Patients have an unreported clinical and histological presentation with features of multiminicore disease associated with clinical findings commonly associated to collagenopathies and with histological lesions typical of other congenital myopathies (centronuclear myopathy, cap disease) as well as some dystrophic findings. A recognisable phenotype with variable degrees of severity was observed in all the affected patients. Implication of the known MmD genes, and particularly *RYR1*, *TTN* and *SEPN1* had been excluded.

II.1 Original phenotypical presentation

The clinical phenotype in all the patients was consistent with a particularly severe form of congenital myopathy (Figure 21, Table 1). They presented from birth with neonatal hypotonia particularly severe in axial (neck and trunk) muscles, severe head lag, poor antigravity movements in limbs and, in 3 out of the 4 patients, feeding and respiratory

difficulties which needed intermittent nasogastric feeding and respiratory assistance from the first months of life. Motor development was severely delayed, but some degree of motor maturation was observed during the first decade. Only the mildest case (Patient II.2) achieved independent ambulation at age 4 years and was able to walk short distances with waddling gait until the age of 11 years. There were no congenital contractures but a generalised joint hyperlaxity. This was associated with a skin phenotype (mild hyperelasticity, follicular hyperkeratosis, dry skin with scratch lesions) of variable severity, which cosegregated, strictly with the muscle phenotype in the family. The female patients showed also a particular aspect of lower limbs, with abundant adipous tissue having a pseudo-infiltrative aspect ('big legs'). Joint hyperlaxity, spinal rigidity, keratosis pilaris and increased adiposity in lower limbs were reminiscent of collagen 6-related myopathies, but the absence of keloid scars and of marked contractures (including finger flexors) even at the beginning of the third decade were important differential diagnosis findings.

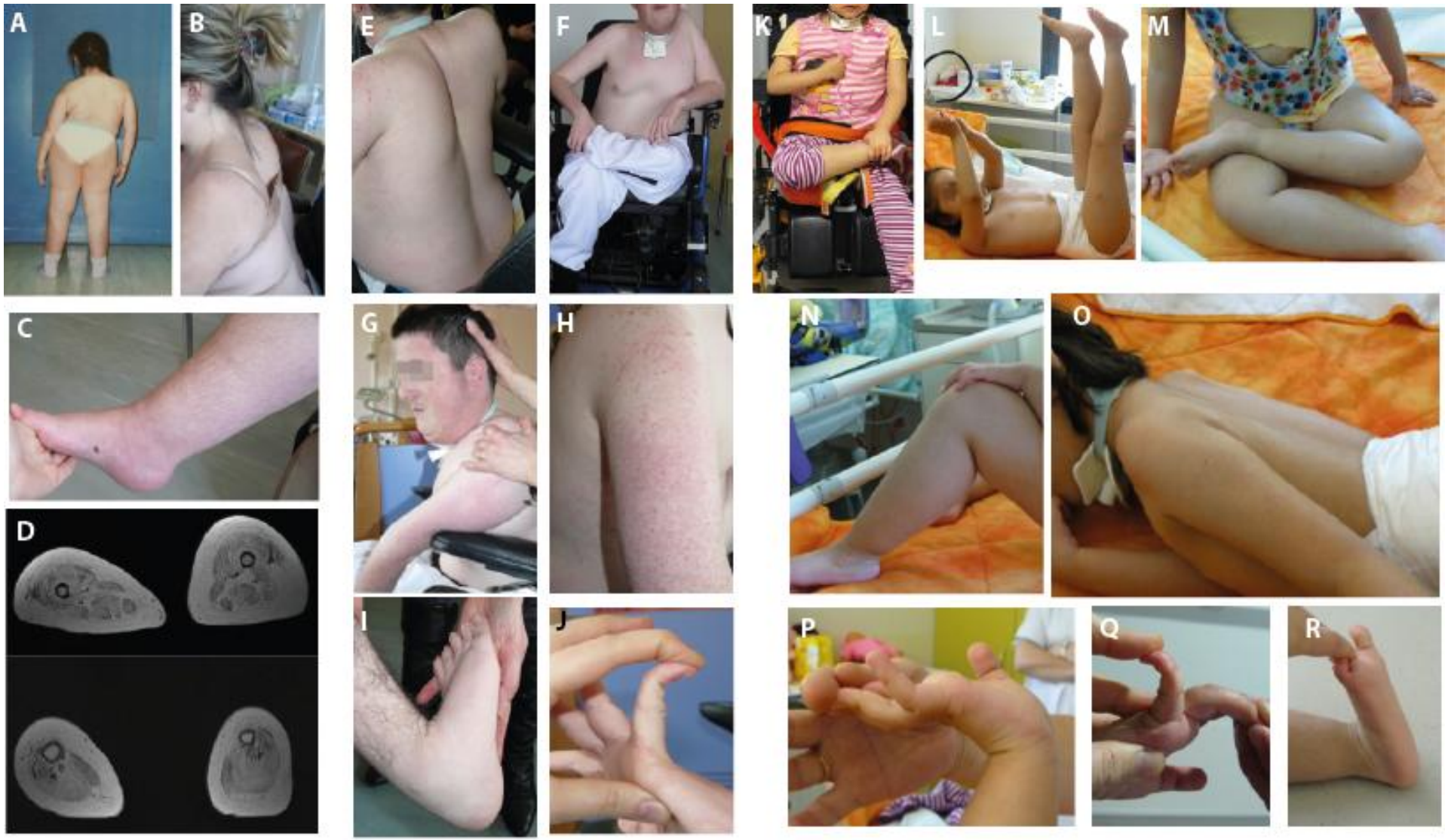
Importantly, all the patients presented with a particularly severe respiratory failure, which required assisted ventilation from the first year of life in 3 patients (two of them tracheotomised) and from the age of 11 years in the mildest case. The youngest sibling in the first branch of the family (II.3), who was severely affected, required intubation during a respiratory infection at age 15 months, could not be extubated and died at 16 months.

The surviving patients are aged at present 24, 23 and 9 years. They are wheelchair-bound and require full assistance for all daily life activities. In the two siblings having reached the second decade of life, a slow muscular and respiratory deterioration has been observed. All cases show severe muscle weakness predominantly involving axial and proximal muscles. Neck flexors are particularly weak (0 to 1 on the MRC scale), leading to head lag, poor head control and cervical rigid spine in patients II.1 and II.2. Weakness of trunk muscles requires compensation by a peculiar posture in order to maintain the sitting position. The oldest patients developed severe scoliosis, which required spinal fusion in their early teens. Limb muscle weakness is more marked in proximal muscles (quoted globally around 2). Joint hyperlaxity persists in all cases, associated in the older patients to mild lower limb contractures that appeared towards the middle of the second decade and remain remarkably mild for non-ambulant patients.

CPK level was normal or nearly normal (<2xN). EMG performed in patient II.1 showed a myopathic pattern with normal conduction velocities. Electroencephalography (II.1 and II.3) was normal, as well as a neonatal brain CT scan performed in patient II.1. A muscle CT scan performed at age 19 years in patient II.2 showed major diffuse fatty degeneration of most muscle groups, with relative preservation of abductors only. There was no heart disease, and cardiological examinations (echocardiography, electrocardiography) were normal except for an incomplete right branch block in patient II.1 at 20 years of age. Thyroid hormone levels were normal. Patient II.1 showed normal testosterone and dihydrotestosterone but moderately elevated delta-4 androstenedione levels (2469 pg/ml, N<2000). Immunocytochemical analysis of collagen 6 secretion by primary skin fibroblast cultures was considered normal in patient II.2 and in a first sample from patient II.1. A second culture sample from this same patient showed mild abnormalities whose pathological significance was unclear. Muscle collagen 6 immunostaining was normal.

Muscle biopsies performed at ages 5 months (II.1), 6 years (II.2) and 4 years (III.1) showed variable degrees of combination of lesions characteristic of congenital muscular dystrophies and of various forms of congenital myopathy (Figure 22). Samples showed fiber size variability, type 1 fiber predominance, rounded fibers with mild increase of endomysial connective tissue and more marked adipous replacement. Abundant minicore lesions were associated with a variable but significant number of centrally located nuclei. In patient II.1, light and electron microscopy studies disclosed the presence of typical cap lesions in a significant proportion of fibers. Immunostaining for dystrophin and merosin was normal. Linkage studies excluded linkage to the SEPN1, RYR1, COL6A1/A2 and COL6A3 loci.

The parents and the two healthy siblings of patients II.1, 2 and 3 were examined and showed no muscle, respiratory or skin phenotype.



Patient II.2 : 9 and 19 years

Patient II.1 : 20 years

Patient III.1 : 5 years

Figure 21: Clinical presentation in Family C. Patients II.2 (A-D), II.1 (E-J) and III.3 (K-R). Major axial muscle weakness led to severe scoliosis that required arthrodesis in patients beyond their first decade of life (A, B, E) and was associated with cervical spine rigidity (G: maximum neck flexion) and dorsal hyperlordosis (B, E, O). Note the identical spontaneous position of II.1 and III.3 (unknown to each other): they form a triangle with their lower body to support their highly hypotonic trunk. Limb muscles retained partial antigravity strength (L). Only patient II.2 was able to walk independently short distances between the ages of 4 and 11 years (A). Joint hyperlaxity was present in all patients (F, G, I, J, K, M, P, Q, R). Strikingly, limb joint contractures were absent in non-ambulant Patient III.3 (P, R) and mild and localised in II.1 and II.2 (C, I). The severity of the skin phenotype was variable but all patients showed some degree of follicular hyperkeratosis, associated in II.1 and II.2 with major skin dryness and scratch lesions (H), which cosegregated with the muscle phenotype in the family. The female patients showed an increase in subcutaneous adipous tissue particularly in legs (C, M, N). Lower limb MRI in II.2 (at 20 y) showed major and diffuse adipous replacement of muscles, involving predominantly the quadriceps, the posterior compartment of the thigh and the soleus (D).

Patient	II.1	II.2	II.3	III.1
Gender	M	F	F	F
Perinatal signs	Acute foetal hypoxia (DIP2), APGAR 6/8, neonatal hypotonia (>axial), poor antigravity movements, feeding difficulties (intermitent nasogastric feeding in the first 2 years), neonatal ventilatory assistance	Neonatal hypotonia (>axial), poor antigravity movements	Hydramnios, shoulder dystocia with left brachial plexus lesion, APGAR 3/5/7, neonatal hypotonia (>axial), poor antigravity movements, weak cry,poor deglutition, transient nasogastric feeding at age 7m	Neonatal hypotonia, feeding difficulties, respiratory difficulties, gastro-oesophageal reflux, gastrostomy
Early motor development	Delayed motor milestones	Delayed motor milestones (walked unsupported at 4 y.	Delayed motor milestones	Delayed motor milestones
Evolution of motor performance	Improving until age 14y, slowly progressive afterwards	Improving until age 7y, then stable or slowly progressive	Stable	Stable
Maximum motor ability	At 14y: Standing unsupported, walking a few steps indoors with support, eating alone	At 7y: walking unsupported >50m (waddling gait), could raise from a chair with difficulties. Gait loss at 11y	Poor head control at 8m, partial limb antigravity movements, no sedestation at 15m	Partially rolls over, sits without support, crawls on her buttocks at 5 y
Scoliosis	+++, from age 8y. Arthrodesis at 15y	++, from age 9y. Arthrodesis at 12y	-	- at 5 y
Rigid spine	++	++	-	-
Respiratory involvement	From birth Frequent respiratory infections from age 2 months, profilactic antiobiotic treatment Night ventilation from 11m Tracheotomy at 17m. Ventilated 23/24h at age 20y	Short episodes of obstructive apnea at age 2y Frequent respiratory infections from age 2 months, profilactic antiobiotic treatment Night ventilation from age 11y Thorax CT: basal right atelectasia	From birth. Weak cough, frequent respiratory infections from age 2 m, Hypoventilation Intubation at 15 m (severe respiratory decompensation) Deceased at 16 m	Night ventilation, tracheotomy at 10 m
Latest FVC	22% at 15y (sitting)	27% at 19y (sitting)	-	- (tracheo)
Joint contractures	Mild After age 14y Hips, ankles	Mild to moderate From age 15y Hips, knees, ankles	No	None at 5y
Joint hyperlaxity	++	++	++	+++
Skin phenotype	+++	++	+++	+
Dysmorphic features	Funnel thorax Pectus excavatus Valgus feet	Flat thorax Pectus excavatus Valgus feet	Unknown	High arched palate Flat thorax
Other	Obesity Learning difficulties Delayed puberty Testicular ectopia	Obesity 'Big legs'	Unknown	'Big legs'

Table 1: Clinical features. Intrafamilial variations in the disease observed

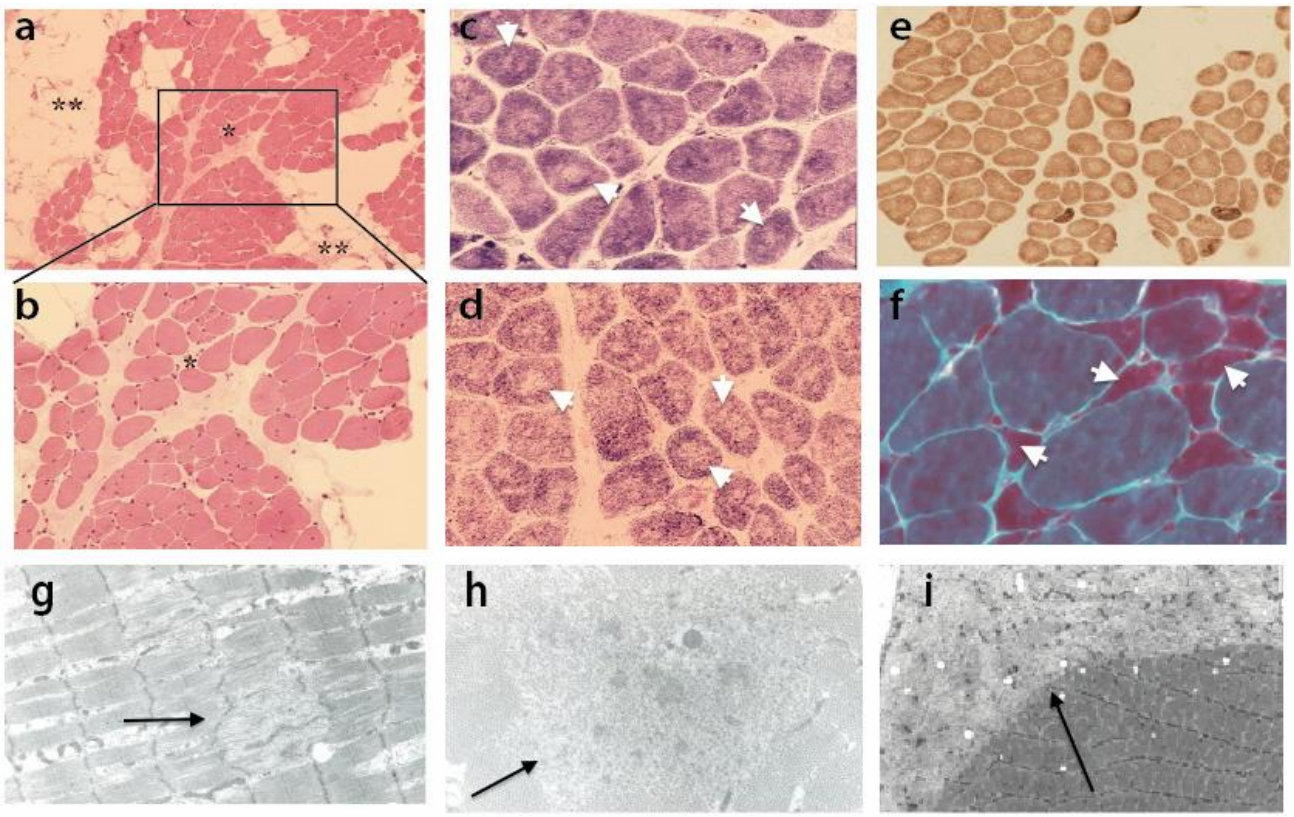


Figure 22: Histological presentation. Hematoxylin eosin stainings (a 10x, b 20x) show fibro-adipous (: fibrotic, ** adipous) replacement and fibres with nuclear centralisations (arrowheads). DPNH (c, 40x) and SDH (d, 40x) on transversal frozen sections disclosed multiple light areas lacking oxidative activity (arrowheads) that corresponded on EM to short zones of mitochondrial depletion and sarcomere disorganisation (arrows) termed minicores (g, h). ATPase pH 9,4 staining (e, 20x) showing major predominance of type I fibres (light coloration). Gomori trichrome (f, 63x) and EM studies (i) revealed subsarcolemmal accumulation of disorganised proteins forming cap lesions (i, arrow) in a variable proportion of small, relatively angular muscle fibers (f, arrowheads)*

II.2 Novel locus associated to a human condition

The branch A was primarily addressed to the laboratory with the diagnosis of MmD for genetic investigations. After exclusion of the known MmD and cap disease genes, this branch was investigated by linkage analysis (homozygosity mapping) using the Illumina 6056SNPs, which leds to the identification of two loci of interest: 15q22 and 22q11 (3.2 and 2.36 Mb respectively). SNPs in these two loci were identified in a homozygous state in patients, inherited from a common ancestor, and heterozygous in the unaffected family members (homozygosity by descent). After subsequent identification of the second branch of the family, a screening of selected SNPs within the two loci was undertaken to discriminate between both chromosomal regions. The youngest patient III.1 being heterozygous for the locus Chr22 (3 SNPs screened: rs742163, rs738401 and rs9613266), linkage of the disease to this locus was excluded. Also, this second analysis restricted the locus on Chr 15 to 2.6 Mb, and increased its LOD Score (> 3.8, Figure 23), thus **confirming the identification of a new locus for human congenital myopathies disorders.**

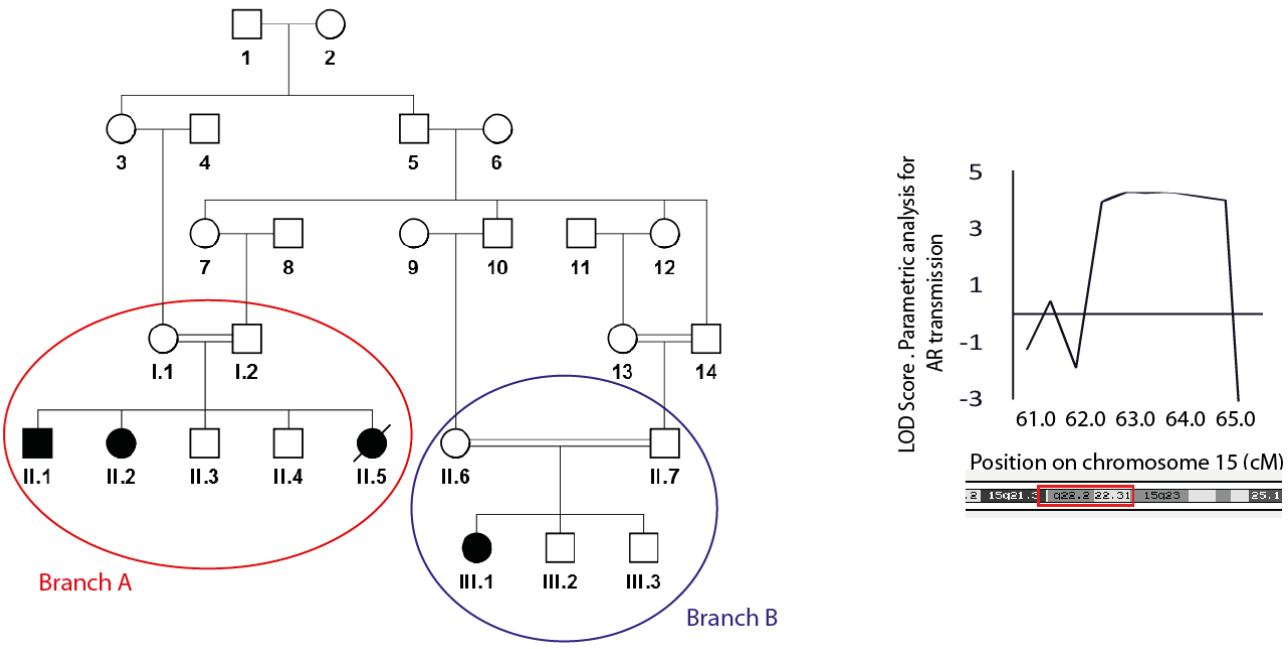


Figure 23: Family C pedigree and identification of a novel locus associated with a human condition. On the left is represented the pedigree of Family C, with the two branches of the family identified sequentially. On the right, the graph presents the LOD score obtained for Chr15q22.

II.3 Positional candidate genes

The new locus located on Chr15 had never been associated to human conditions. Among of the 32 positional candidate genes located in this region, a prioritisation was necessary. None of them present a muscle specific expression or were previously associated with muscle function. Thus, prioritisation was established accordingly to functional relevance of the proteins encoded. Ten genes were sequenced (Table 2) and two variants were identified.

Gene	Position	Unknown variants
TRIP4	chr15: 64,680,003-64,747,502	Chr15 : 64,701,875 G>A
ZNF609	chr15: 64,752,941-64,795,259	None
PLEKHO2	chr15: 65,134,082-65,160,201	Chr15 : 65,152,202 G>A
ANKDD1A	chr15: 65,214,120-65,251,041	None
RASL12	chr15: 65,345,675-65,369,028	None
CLPX	chr15: 65,442,784-65,477,563	None
PTPLAD1	chr15: 65,822,827-65,870,693	None
RAB11A	chr15: 66,161,797-66,184,329	None
MEGF11	chr15: 66,191,066-66,546,075	None
MAP2K1	chr15: 66,694,220-66,783,882	None

Table 2: List of candidate genes investigated in the 3Mb locus at Chr15q22. The genomic position of the 10 genes sequenced and the variations identified and segregating with the condition in the family are reported in this table.

The first variant 65,152,202 G>A is splice region variant (rs369451494) located in the donor site of *PLEKHO2* and is predicted to alter exon 4 splicing. In patient cells, this variation leads to the expression of alternatively spliced messengers missing exon 4 which represent 30% of total mRNA while 70% of the messengers maintained their original coding sequence (Annexe 5). This low expression of the variant transcripts is probably not compatible with haploinsufficiency, while the absence of phenotype in the heterozygous carriers is not suggestive of a dominant negative effect. This, coupled with the proposed implication of the protein in pathways *a priori* not relevant for muscle disease (cytokine/cytokine receptor interaction), suggest that the pathophysiological relevance of this

variant in the current condition is weak or null and prompted us to analyse other positional candidate genes.

Subsequently, we identified a homozygous nonsense variant at Chr15: 64,701,875 G>A, located in the *TRIP4* coding sequence (exon 7 out of 13). A *TRIP4* unique transcript (NM_016213.4) codes for a transcriptional coactivator (NP_057297.2). The nonsense variation observed (c.G950A) generates a premature stop codon (PTC) which predicts truncation of the associated protein (p.W297X). The variant segregates in the family with the phenotype and was not found in 86 healthy DNA controls. The protein is ubiquitously expressed and poorly described in the literature, and has never been implicated in muscle physiology so far. The predicted impact of this nonsense change was compatible with severity of the condition and its autosomal recessive transmission, so we focused our further investigations on this gene.

II.4 Relevance of the TRIP4 mutation

II.4.a A nonsense mutation leading to messenger RNA degradation

The nonsense mutation identified in the *TRIP4* gene is located in the Exon 7 (Figure 24A). As visualised in Figure 24B, all the patients in the family carried the c.G950A variation at the homozygous state, inherited from their parents who carried the variant at the heterozygous state. Further investigations are in agreement with a potential Nonsense Mediated Decay (NMD) of this mutated messenger RNA. Indeed, the location and the type of mutation fulfil the NMD criteria (Brogna and Wen, 2009). Furthermore, quantitative PCR confirmed a massive loss of the mutant mRNA, leading to a residual expression of 10% in homozygous patient fibroblasts cDNA (II.2: 8.8%; II.1: 12% and III.1: 9%) compared to healthy controls. Consistently, 53% cDNA was detected in a healthy heterozygous mother (individual I.2, Figure 24C). Also, we excluded by RT-PCR with primer set encompassing the entire *TRIP4* coding sequence a potential excision of the mutated exon (Figure 24D). No alternatively spliced isoform was detected suggesting the absence of compensatory splicing mechanisms such as exon skipping (Figure 24E). Similar results were observed in cDNA

extracted from the youngest female patient myoblasts (in proliferating or differentiating conditions) compared to controls. These results suggest a haploinsufficiency mechanism by which the homozygous patients would be in a situation comparable to a human KO for TRIP4.

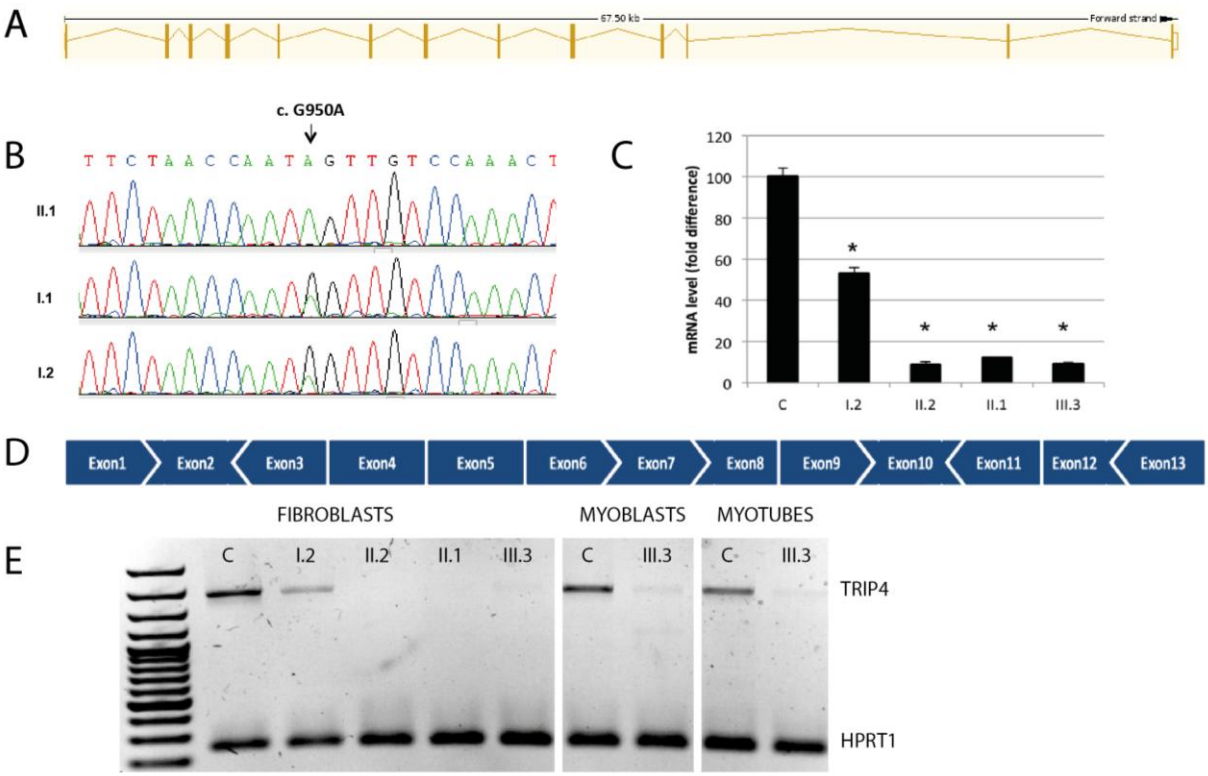


Figure 24 The homozygous TRIP4 nonsense mutation is associated to a major reduction mRNA expression. (A) Human TRIP4 gene leads to the expression of a unique coding messenger (NM_016213), modelisation from Ensembl (Flicek et al., 2014). (B) The variation c.G950A is shown at the homozygous state in genomic DNA from patient II.1 and at the heterozygous state in his parents II.1 and II.2. (C) qPCR quantification on primary fibroblast cultures showing a decreased expression of TRIP4 messenger in affected patients (II.2: 8.8% residual expression, II.1: 12% and III.3: 9%) and a 53% residual amount in the heterozygous mother (I.2). (D) TRIP4 reading frame. (E) Gel electrophoresis from RT-PCR TRIP4 templates demonstrated the absence of alternative spliced isoforms (expected canonical transcript from 1.7 kilo base pairs) in patient fibroblasts and in profilerating or differentiating primary muscle cell cultures. Famliy C pedigree is provided Figure 20.

II.4.b The nonsense mutation leads to the complete absence of protein

TRIP4 codes for a unique protein: Activating Signal Cointegrator 1 (ASC-1), which is known as a steroid hormone nuclear receptor coactivator (Lee et al., 1995). The protein sequence is relatively conserved through evolution (Figure 25A) and the nonsense variant identified in *TRIP4* was predicted to code for a prematurely truncated ASC-1 protein (p.W297X, length reduced to 49%, Figure 25B). However, in patients the minimal residual expression of *TRIP4* mutant mRNA leads to absence of the truncated protein and to total loss of protein expression, as observed by western blot in primary patient cells. Consistently, fibroblasts from the healthy heterozygous mother showed a protein level reduced by 50% (Figure 25C).

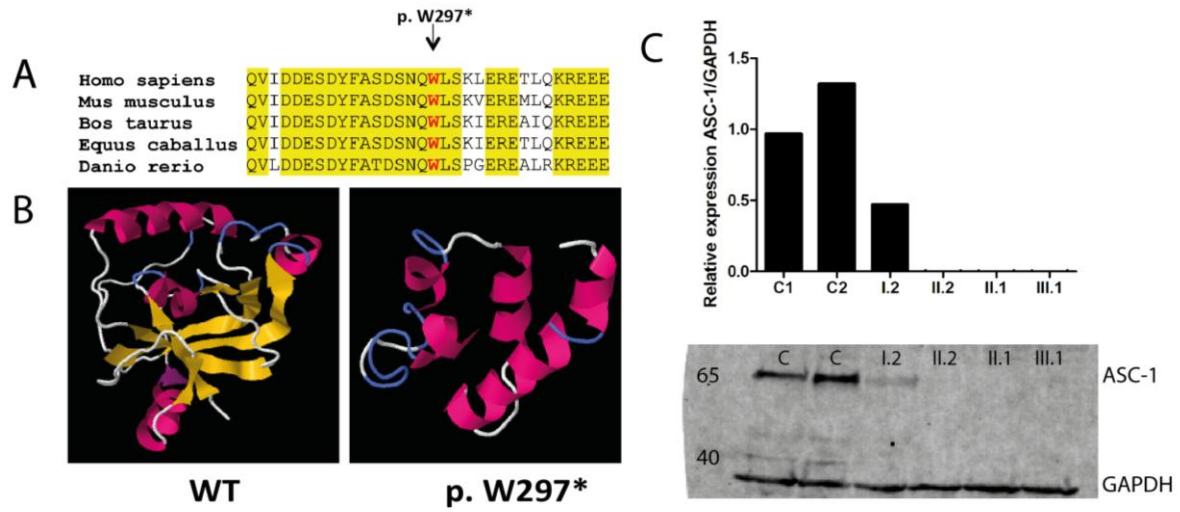


Figure 25: Nonsense mutation associated to an absence of protein. (A) Alignment of the ASC-1 protein sequence (NP_057297.2) and conservation of the amino acid. (B) The predicted structure of the WT and the truncated protein. (C) Western Blot analysis of protein samples reveals the absence of protein in patient fibroblasts (II.2, II.1 and III.1) and a 50% decrease in heterozygous mother fibroblasts (I.2) compared to two different of control samples (C).

II.4.c Absence of ASC-1 impairs the intracellular localisation of known protein partners

ASC-1 is known to be part of a complex named thereafter ASC-1 complex (Jung et al., 2002). The complex is composed of four subunits where ASC-1 plays a key role for the association with its partners ASCC2 and ASCC3, while ASCC1 does not interact directly with ASC-1 but associates to ASCC3 (Figure 26C). I investigated the impact of ASC-1 absence on the assembly of the complex using patient primary fibroblast cultures. The punctuated profile associated to normal ASCC3 labelling appears altered in the absence of ASC-1. Compared to control fibroblasts (Figure 26A), the amount of ASCC3 spots is decreased in patient fibroblasts (Figure 26B). Also ASCC2 and ASCC3 appear to be in the vicinity of each other, however the colocalisation observed in control fibroblasts (arrow heads) has not been observed in patients cells suggesting a possible destabilization of the protein complex. This remains to be confirmed by a more accurate technique such as Proximity Ligation Assay (PLA, currently under optimization). Moreover ASCC2 localises differently in patient fibroblasts (Figure 26E). Quantification of the observed localisation patterns is provided in the graph (Figure 26F). While in 91% of the control fibroblasts, nuclear ASCC2 presents as a difuse punctuate profile (Figure 26D, arrow) this pattern was observed in only 47% of the patient cells. 27% of the patient cells exhibit a “ring-like” peripheral enhancement (Figure 26E, white arrowhead) that is only observed in 9% of control fibroblasts. Additionnaly, 26% of the patient fibroblasts nuclei show also a difuse nuclear ASCC2 labelling, which seems to be associated to an increase of the protein expression in the nucleoplasm (Figure 26E, yellow arrowhead). These alterations confirm the existence of structural and functional consequences of ASC-1 absence in patient cells and thus support the pathogenicity of the *TRIP4* mutation identified.

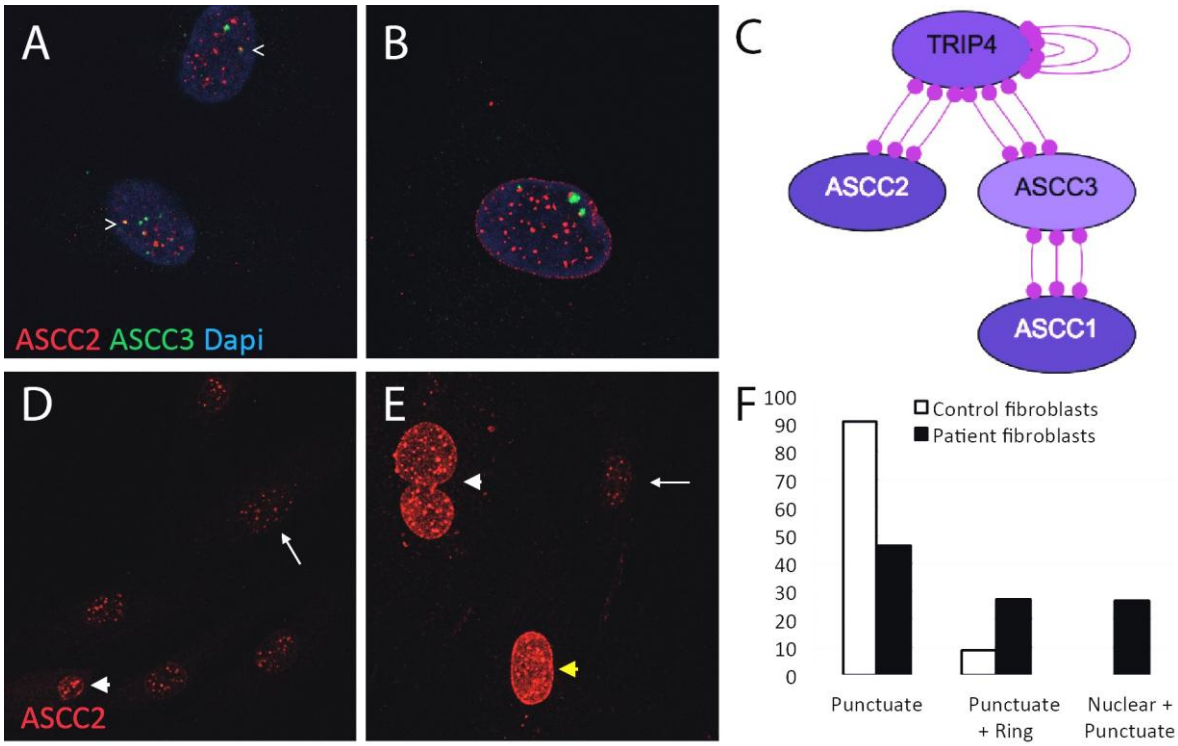


Figure 26: Mislocalisation of ASC-1 protein partners. ASC-1 complex is composed of four proteins (C), modelisation from FANTOM4 (Kawaji et al., 2011). Control fibroblasts (A,D) stained with antibodies targeting ASC-1 direct partners: ASCC2 and ASCC3 show a nuclear punctuate profile. Patient fibroblasts (B,E) present a particular ASCC2 labelling which can be punctuated (arrow), associated to a peripheral "ring-like" phenotype (white arrow head) or to a diffuse nucleoplasmic labelling of the protein (yellow arrow head). Quantification of these phenotypes is recapitulated in graph F. Labelling performed on P14 and P9 fibroblasts from control and patient respectively. Over 200 nuclei have been analysed per condition.

II.5 ASC-1 as a novel actor in muscle physiology

Accordingly to the literature, ASC-1 is ubiquitously expressed (Jung et al., 2002; Lee et al., 2002), however the precise expression profile of the protein was not known. Therefore I conducted western blot analysis to determine the expression profile of ASC-1 in adult murine tissues compared to GAPDH expression (similar results obtained with ASC-1 relative expression compared to alpha tubulin, data not shown). In basal adult skeletal muscle tissues ASC-1 protein is clearly expressed although its level is low compared to other tissues such as lung or liver (from 15 to 35 fold higer expression, Figure 27A). ASC-1 protein is preferentially expressed in axial muscles (diagraphm and paravertebral muscles) with levels, which are from two to four fold compared to limb muscles. This pattern is consistent with the axial involvement observed in patients. Interestingly, ASC-1 is also expressed in skin that could explain the skin phenotype observed in patients. Regarding the strong expression profile of ASC-1 protein in lung, the characterization of patient respiratory involvement and particularly the existence of primary lung disease should be considered. ASC-1 expression profile in the myocardium is similar to diaphragm (Figure 27B). Being closely monitored, patients do not exhibit any cardiac involvement suggesting a restricted involvement of ASC-1 in skeletal muscle function. However, all patients are under 25 years old and thus, the development of a cardiac disease later in life cannot be excluded.

The absence of specific antibodies prevents the determination of ASC-1 localization in skeletal muscle tissue. The transcriptional expression of *Trip4* remains unchanged during the differentiation of the C2C12 murine myoblastic cell line (collaboration with Jyostna Dhawan's laboratory). However, the protein expression tends to increase during this process, suggesting an enhanced translation of the transcripts or a stabilization of the protein during serum withdrawal (Figure 27C). These results would be consistent with an ASC-1 role in myogenic differentiation.

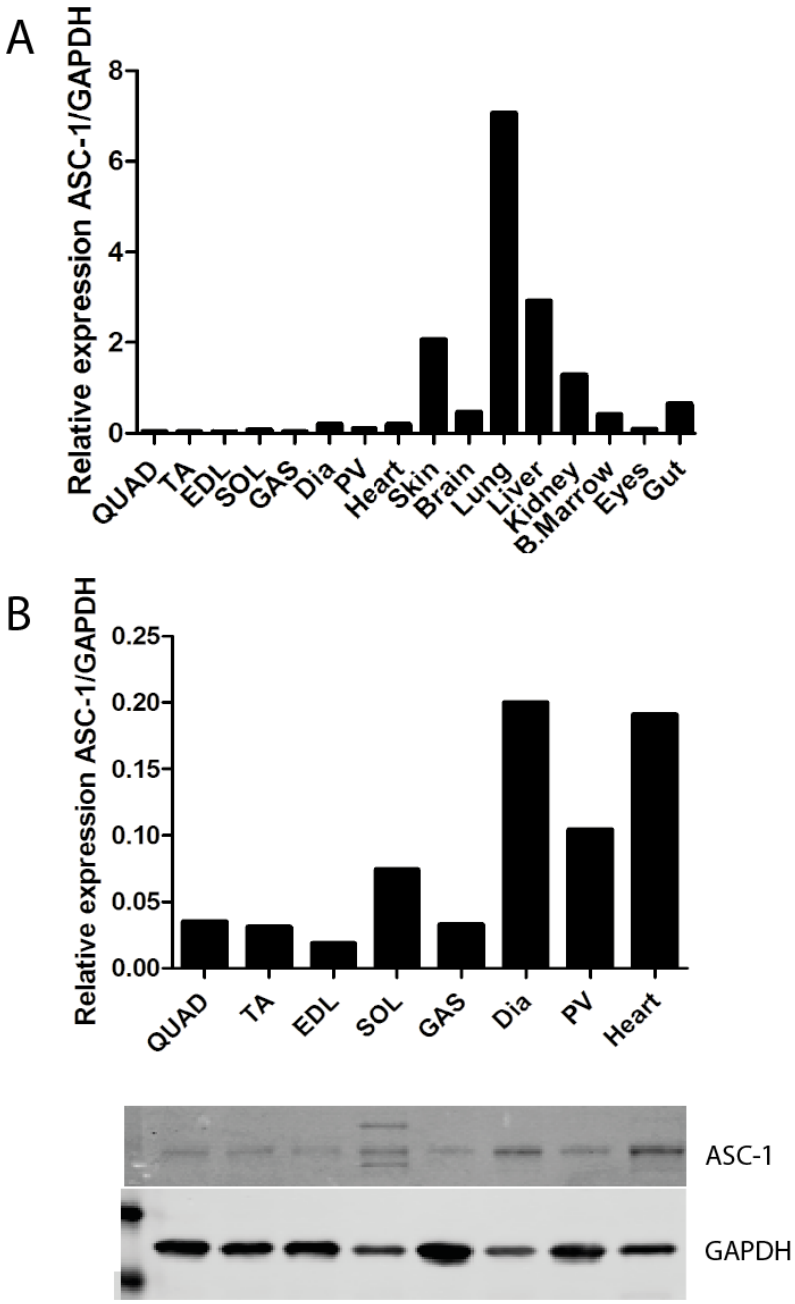


Figure 27: Relative expression of ASC-1 in adult tissues and protein expression during in vitro *C2C12 differentiation. (A) Western blot quantification performed on adult mice presenting the relative amount of ASC-1 in differentiated tissues. (B) Focus on skeletal muscle tissues and myocardium presenting the relatively higher expression of ASC-1 in axial muscles (10x compared to QUAD). (C) Western Blot quantification shows the relative expression of ASC-1 in differentiating C2C12 (N=3). D0 stands for confluent cells in proliferation media, prior to serum withdrawal; D1 to D4 stand for days post low serum differentiation induction. QUAD: quadriceps, TA: Tibialis anterior, EDL: Extensor digitorum longus, SOL: soleus, GAS: gastrocnemius; Dia: diaphragm, PV: paravertebral.*

The current knowledge regarding the impaired mechanisms associated to congenital muscular disorders involves 1/ the expression and integrity of contractile proteins and 2/ the defective proliferative and differentiative potential of progenitor cells. ASC-1 being a transcriptional coactivator, it could contribute to both mechanisms. To explore these questions I developed an *in vitro* model of ASC-1 deficiency.

II.6 Function of ASC-1 in an *in vitro* model

Little is known about ASC-1 protein and its related complex whose function remains elusive. In order to understand the contribution of ASC-1 to myogenesis and to identify a cell phenotype, I generated a transient knock down system. RNA silencing or interference is based in the introduction of small interfering RNA (siRNA) which binds to a complementary messenger RNA and targets it to degradation prior to translation. Therefore, this strategy has been used to knock down *Trip4* in a C2C12 cell line. This system allows investigations of proliferative and differentiative capabilitites *in vitro* as a basis to understand the human condition.

II.6.a Transcriptomic analysis of a transient knock down model

Regarding the transcriptional activity of ASC-1 (Jung et al., 2002), I conducted a whole-genome transcriptomic analysis to identifiy target genes or pathways affected by the loss of ASC-1 expression and to decipher the role of the protein during differentiation. I chose two time points: late proliferation and early differentiation (Figure 28A). After verification of silencing efficiency (-80% messenger and -80 to -90% of protein expression), samples were compared to scrambled controls (Figure 28B). Results show the absence of strong modulation (fold change > 1.5) of any transcripts carried on this MouseWG-6 v2.0 whole-genome Expression BeadChips (Illumina) chip. These results maybe partly explained by the limits of our cell model which might not allow enough time for larger quantitative amplification of the effects of ASC-1 absence. But they are also in agreement with the

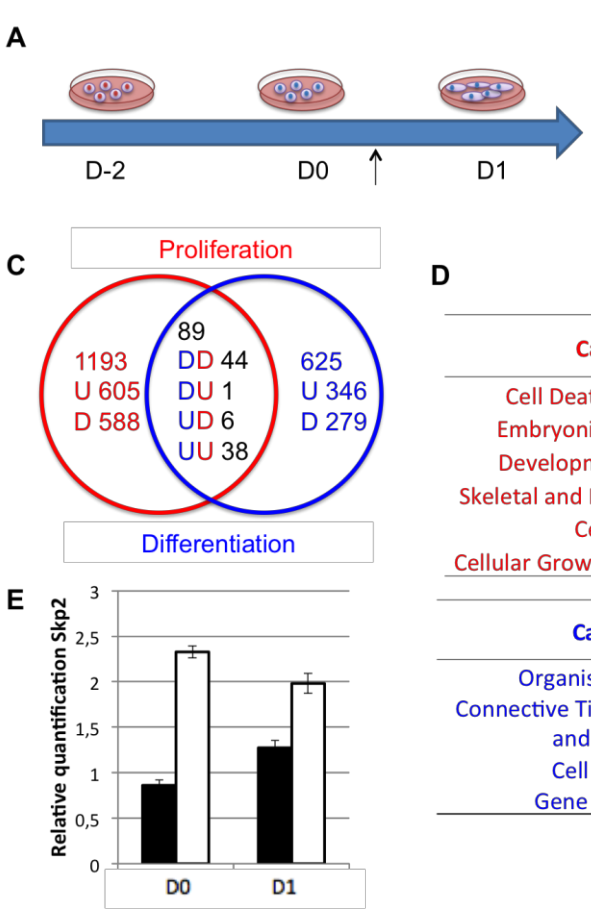
proposed function of ASC-1 as a coactivator that would not necessarily have an on-off effect but rather finely modulate transcriptional activities of possibly numerous transcriptional complexes.

The transcripts significantly modulated (fold change >0.8 and <1.2, p-value < 0.05, Figure 28C) were processed through Ingenuity software in order to identify pathways or functions affected in this model (Figure 28D). In proliferative conditions, function associated to cell death and survival was predicted downregulated, while functions associated with developmental and skeletal muscle disorders were upregulated. Additionally, the cell cycle and proliferative functions were upregulated. These modulations are in favour of an increased propensity to proliferate for cells transiently knocked down for *Trip4*, under proliferative conditions (Figure 28D).

In early differentiative conditions, fewer transcripts appear to be significantly modulated. The functions predicted as upregulated were associated to the connective tissue, cell signaling and transcription. At this early stage, skeletal muscle development pathways were not predicted significantly modulated, although the machinery associated to gene expression was positively regulated (Figure 28D). Nonetheless, results suggested a downregulation of major sarcomeric proteins including titin (*Ttn,* -55%), myosin (*Myh3,* -70%), troponins (*Tnnt1,* -50%, *Tnnt3* -49%), tropomyosin (*Tpm2,* -49%), Myomesin (*Myom1* -20%)) and also *Ryr1* (-39%) in the absence of ASC-1. These evidences are in agreement with the non-strict requirement of ASC-1 for muscle gene expression but suggest a quantitative impact on expression of muscle specific proteins.

Interestingly, 44 and 38 transcripts were significantly down- and upregulated respectively in both conditions. These transcripts were not predicted by either IPA (Ingenuity) nor DAVID Bioinformatic Database (Huang et al., 2009) as belonging to any common pathways. Also, I considered commonly modulated transcripts as targets from a similar transcriptional factor. My preliminary investigations (ENCODE ChIP-Seq Significance Tool) did not led to the identification of a common motif within the regulatory regions of these genes. One of the most significantly modulated targets identified was the *Skp2* transcript, whose upregulation has been validated by qPCR (Figure 28E). *Skp2* encodes an F-box protein associated to the ubiquitin ligase complex SCF responsible for p21 degradation (Bornstein et al., 2003). *Skp2* transcript was upregulated in both proliferation

(2.32 ± 0.12 vs 0.86 ± 0.11) and early differentiation (1.98 ± 0.21 vs 1.27 ± 0.16) in the *Trip4* knocked down cells. This is in agreement with a contribution of ASC-1 to cell cycle exit.



D

Category	p-Value	Prediction	Activation z-score
Cell Death and Survival	8,26E-04	Decreased	-2,647
Embryonic Development	1,39E-02	Decreased	-2
Developmental Disorder	2,14E-03	Increased	2,191
Skeletal and Muscular Disorders	2,14E-03	Increased	2,191
Cell Cycle	2,73E-03	Increased	2,193
Cellular Growth and Proliferation	4,72E-06	Increased	2,199

Category	p-Value	Prediction	Activation z-score
Organismal Survival	1,33E-02	Decreased	-2,218
Connective Tissue Development and Function	7,07E-04	Increased	2,05
Cell Signaling	9,08E-03	Increased	2,188
Gene Expression	1,00E-03	Increased	2,374

Figure 28: Microarray analysis performed in a C2C12 transient knock down model. (A) Cells were plated 24h prior to transfection. D0 corresponds to proliferation condition; D1 corresponds to 24hrs after induction of differentiation by medium change (arrow). (B) qPCR and Western blot analyses: efficient silencing of Trip4 mRNA and ASC-1 protein in the samples used for microarray. (C) Venn diagrams of transcripts modulated (p value < 0.05) where U and D stand for upregulated and downregulated respectively. (D) Ingenuity predictions for pathways modulated; full lists are provided in Annexe 6. (E) qPCR validation of Skp2 transcript in both conditions (black= scramble, white= knock down).

II.6.b ASC-1 has no major impact on myoblast proliferation in vitro

Since transcriptomic analysis results suggested a modulation of cell cycle exit by ASC-1, I investigated the proliferative potential of our *in vitro* ASC-1 knock down model from 6 to 54 hours post siRNA transfection (Figure 29A, B and C). TRIP4 knock-down does not alter the proliferative potential of C2C12 myoblasts as visualized by bright field observation or by counting the number of cells per optical field (Figure 29A and 25B). Although the number of cells remains unchanged, the proliferative marks carried by cells seem to be different (Figure 29C), although so far we only have preliminary results from this experiment, which will need to be repeated in order to, provide a reliable interpretation of this alteration. Six hours post transfection, knocked-down cells proliferative marks are strongly decreased compare to controls. The proportion of cells expressing Ki67, a cellular marker for proliferation which is present during all active phases of the cell cycle (G1, S, G2, and mitosis) but absent from resting cells (G0), is divided by two (siRNA: 19%, scramble: 48% and control: 47%). The proportion of cells expressing the phosphorylated form of the histone H3 ($pH3^{+}$), a marker for mitosis, is reduced to 6% (while in controls scramble: 20% and control: 28%). This trend is inverted at 30 hours post-transfection. Knocked-down cells express in a higher proportion the Ki67 marker at 30 and 54 hours (68% and 47%) compared to controls (scramble: 55% and 22% and control: 48% and 21%), while the effective rate of dividing cells remains unchanged, consistently with the similar proliferative profile illustrated in Figure 29A and 29B.

These preliminary results would be compatible with a potential role of ASC-1 in modulating cell cycle withdrawal upon confluence (achieved at 54 hours). Therefore, we analysed a potential role of ASC-1 on the expression of the key growth arrest and differentiation inductor protein p21 using luciferase assays in C2C12 cells overexpressing ASC-1 which were incubated for 6 hours in T3-devoid or T3-supplemented medium (Figure 29D and 29E). The overexpression of ASC-1 leads to the specific induction of luciferase in a p21 reporter construct (median: 2.18) compared to a desmin reporter (median 0.36) or a minimal reporter (median: 0.06). In this *in vitro* assay, the specific induction of p21 reporter construct was favoured by the addition of thyroid hormone (median: 3.02), consistently with the role of ASC-1 as a steroid hormone receptor coactivator potentially mediating the contribution of thyroid hormone to muscle differentiation.

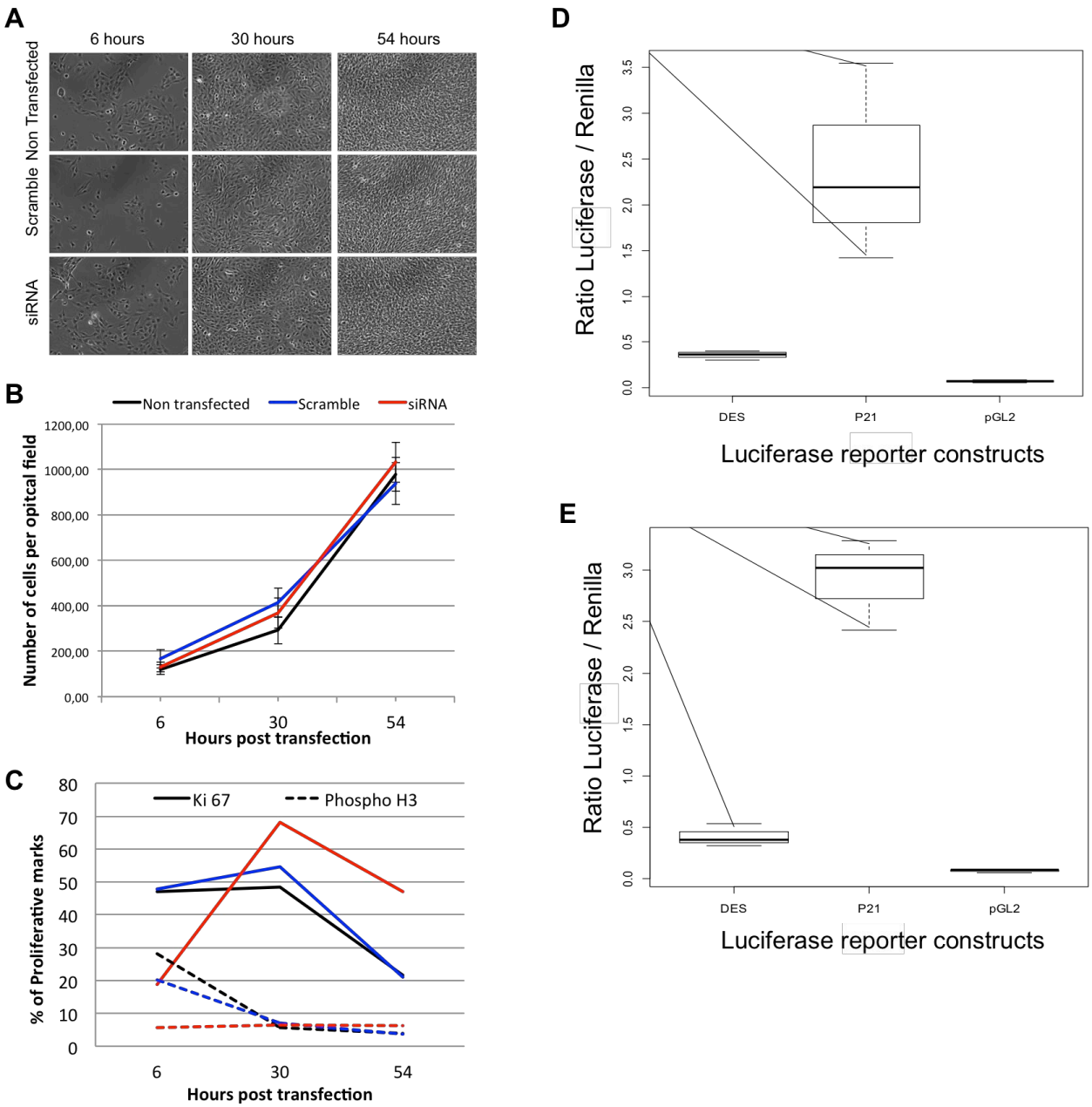


Figure 29: TRIP4 knockdown impact on C2C12 proliferation and cell cycle exit. (A) C2C12 cells were transfected with siRNA or scramble RNA, non-transfected cells serving as controls, and were imaged 6, 30 and 54 hours post transfection. (B) The number of cells counted shows that transfection protocols and TRIP4 knockdown has no impact on proliferation (N=3). (C) Percentage of cells labelled cells with proliferative markers Ki67 and Phospho H3 reveals a potential alteration of the cell cycle regulation of knocked down cells (N=1). Luciferase assays (D, E) performed in C2C12 overexpressing ASC-1 show the fold of activation of the Desmin (DES), p21 and empty (pGL2) Luciferase reporter constructs in basal conditions (D) and after 6 hours of thyroid hormone induction (E, N=3).

II.6.c ASC-1 transient knock down induces a delay in late myogenic differentiation

Consistently with the results above, and with the fact that ASC-1 expression profile tends to increase during C2C12 differentiation, I investigated the role of ASC-1 in late myogenic differentiation. As the absence of the protein does not alter C2C12 proliferation, cells were confluent enough to go through efficient differentiation upon serum deprivation. C2C12 cells transiently knocked for *Trip4* or transfected with scramble sequences were pushed toward differentiation until D4 (4 days after serum deprivation that corresponds to 7 days post transfection) (Figure 30A). Although ASC-1 knocked-down cells are able to fuse, terminal differentiation is markedly impaired (Figure 30B).

The reduction by 70% of the protein expression in differentiative conditions (Figure 30C) is sufficient to drive an altered delayed differentiation. Labelling of Pax7 positive nuclei (Figure 30D) is comparable in knocked-down and in scramble cells, suggesting that there is no impact in the commitment of the cells. In addition, quantification of the hypophosphorylated (activated) form of MyoD (Figure 30E) was unchanged, revealing a correct initiation of differentiation. However, the number of Myogenin-positive nuclei was mildly reduced at D3 (Figure 30F, statistical analysis in progress). Expression of MRFs such as MyoD and Myogenin is usually associated with cell cycle exit and terminal differentiation, which is marked by mononucleated cells fusion and the expression of contractile proteins such as the Myosin Heavy Chain. In Trip4 knocked-down cells, MHC expression appeared to be reduced by 50% at D4 (statistical analysis in progress, Figure 30G) and the formation and growth of myotubes impaired, suggesting that ASC-1 has a relevant role in terminal muscle differentiation and/or myotube growth (Figure 30B). Additional experiments to quantify and characterize this defect are in progress.

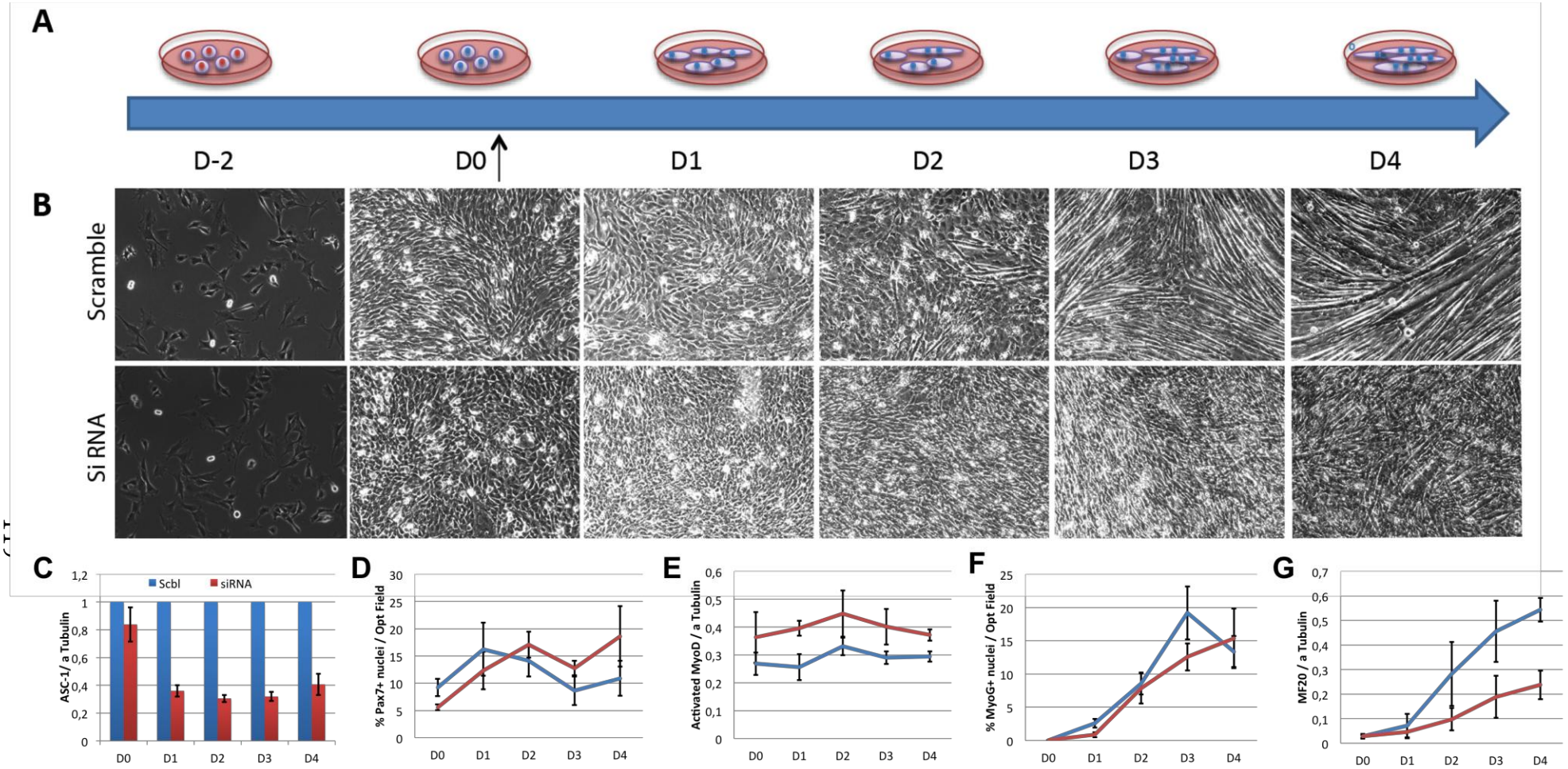


Figure 30: ASC-1 transient silencing altered C2C12 late differentiation. (A) C2C12 cells were transfected 24 hours after plating (D-2) and proliferated for 48 hours to reach confluence (D0) prior to differentiation medium change (arrow). Cells were harvested daily until D4, period of optimum silencing efficiency. (B) Brightfield pictures showing defective myotube formation and growth in knocked down C2C12 compared to control cells transfected with a scramble construct. Key proteins for muscle differentiation were also followed in this assay. Western blot (C, E, G) and IHC (expand) (D, F) analyses showed the effective ASC-1 silencing (C), the maintenance of the myogenic potential of cells (D, E), a mild decrease in MyoG at D3 (F) and a delayed expression of MF20 (G) suggesting a delayed differentiation in knocked down condition. Western blot samples are the pool of triplicates, N=3, while more than 1000 nuclei were counted in immunolabelling experiments, N=4. Errors bars are presented as SEM.

II.7 TRIP4 mutation as a privative condition

This work is the first description of ASC-1 contribution to muscle physiology and to human pathology. In order to better understand its contribution to congenital muscle conditions, we looked for additional *TRIP4* mutations in undiagnosed forms of congenital myopathies or early-onset conditions in which patients present with similar clinical features. We collected three cohorts of patients presenting with similar clinical and histological features, which are recapitulated in Table 3. Two series of patients with MmD or with cap lesions similar to those observed in Family C patients were analysed. Additionally, regarding the peculiar dystrophic phenotype also observed in patients, a unique cohort of patients presenting with a "Collagene 6-like" phenotype, including skin abnormalities and joint hyperlaxity, were investigated. Patients genomic DNAs were tested for exonic *TRIP4* mutations using genomic DNA. So far, no additional mutations were identified, suggesting that mutations in TRIP4 are not a prevalent cause of congenital muscle disease.

Condition	Number of patients investigated
Multiminicore disease	32 - No mutation identified
Collagene 6-like	19 – Under investigation
Cap disease	7 - Under investigation

Table 3: List of the three different cohorts investigated regarding the phenotypical presentation of patients and the number of genomic DNA samples tested for TRIP4 mutations.

III. INVESTIGATIONS IN A SERIE OF PATIENTS WITH MmD AND SCOLIOSIS: IDENTIFICATION OF NEW CAUSATIVE GENES

III.1 Strategies used

During my PhD I also contribute to the identification of new causative genes in the highly informative families presented in Figure 20. For this purpose we combined the data generated by two powerful techniques commonly used in the diagnosis of these unlabelled conditions (Rabbani et al., 2012) .In order to ease the reading of this chapter I summarise below (Figure 31) a diagram of the flow of investigations used for each family.

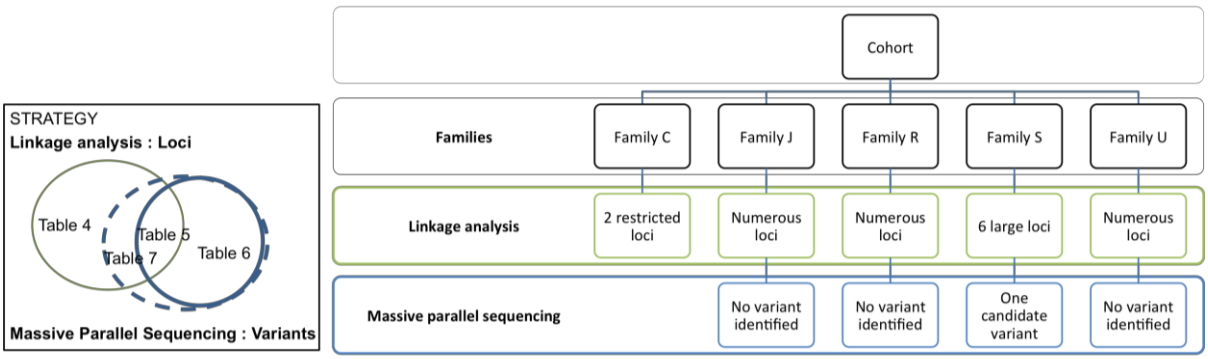


Figure 31: Summary of the genetic investigations conducted and the current diagnosis status of each family. The analyses performed prior to my arrival in the laboratory and which represent the basis of my work are presented: linkage analysis (green) and massive parallel sequencing (blue). The scheme on the left recapitulates the analyses conducted and the associated referenced table. The chart flow on the right presents the analyses performed per family and the resulting interpretations.

Prior to my arrival in the laboratory, linkage analyses using the homozygosity mapping approach had been performed (Table 4). Families C, J, R and U were investigated by 250K Nsp chips (Affymetrix) in collaboration with the Centre National de Génotypage, Evry, France. In Family S, DNA samples were collected later on and analysed using a 6056 SNPs chip (Illumina).

Family	Chip used	Loci	Size
Family J	Human Mapping 250K Nsp array, Affymetrix	14	96.18 Mb from 0.28 to 59.5 Mb
Family R	Human Mapping 250K Nsp array, Affymetrix	25	128.81Mb from 0.19 to 28.47 Mb
Family S	6056 SPNs Human Panel V, Illumina	6	69.6 Mb from 1.8 to 29.1 Mb
Family U	Human Mapping 250K Nsp array, Affymetrix	27	180.43 Mb from 0.38 to 39.97 Mb

Table 4: Homozigosity mapping and loci identified. The table presents per family, the strategy used (Affymetrix or Illumina) and the number of loci segregating with the condition in each family. A mention of the global size encompassing these loci and a range scale is provided in the last column.

The 250K Nsp chip (Affymetrix) sensitivity did not allow the identification of clearcut loci. In the three families tested, Family J, R and U, multiple loci (more than 10 per family) of various sizes (from 0,19 to approximately 60 Mb) were identifed. Despite the informativity of the families, the homozygosity mapping performed in these families did not allow identification of positional candidate genes for further investigations. A massive parallel sequencing strategy was therefore favoured.

The 6056 SNPs chip (Illumina) carrying fewer markers has proven to be more productive on Family S, allowing the identification of 6 loci of various sizes (ranging from 1.8 to 29.1 Mb) in the family. However, the size of the loci identified in Family S was not compatible with a subsequent positional candidate gene study. A massive parallel sequencing has therefore also been undertaken for identification of a new causative gene.

Whole Exome Sequencing analyses conducted on NextGene software allowed the identification of numerous missense variations and a few in frame indels (Table 5). I took

over and contributed to the analysis of these WES results, defining priorities among the variants based on expression and functional data as well as on their position in loci previously identified by linkage analyses. This approach allowed restricting further investigations to 1 or 2 candidate genes per family and the identification of a candidate gene in Family S (Table 5).

Family	Total	Missense	Indels	Nonsense	In Loci	Conclusion
Family J	23	16	7	0	1	SNP
Family R	29	24	5	0	1	Variant
Family S	17	13	4	0	2	Candidate gene
Family U	16	12	4	0	2	Familial polymorphisms

Table 5: Results of WES investigations for homozygous variants. The table summarises per family the total number of variants identified by WES and unknown in databases. A short description of variant type is also provided (missense, indels and nonsense). According to the linkage analyses previously performed, few candidate variations were retained: all these variants are missense. Each variant was re-sequenced for patients and siblings.

From this prioritised variant list, I have not been able to identifiy relevant variations in Families J, R or U. Therefore I enlarged my investigations by considering each of the analysis separately: either the massive sequencing, or the homozygosity mapping. First, I analysed the WES results in a blind manner (Table 6). Therefore I established a second list of prioritised variants regarding 1/ the conservation of the amino acid, 2/ the conservation of the reading frame (for indels), 3/ the consistency of the transmission mode (autosomal chromosomes) and 4/ the function attributed to the encoded protein. Variants, which were not recapitulating these items, were not investigated. From this second analysis recapitulated in Table 6, no additional relevant variant has been identified.

Family	Total	Prioritised variants				Non investigated
		HTZ	Artefacts	Segregation	SNPs	
Family J	23	2	2	4	2	12
Family R	29	2	0	1	12	13

Family U	16	0	1	0	4	9

Table 6: Blind analysis of the WES investigations for homozygous variants. The table associates per family the total number of variants identified by WES, the newly priorised and the non-investigated variants. Relevance of autosomic variants was established regarding the amino acid and reading frame conservation. Prioritised variants were further investigated by Sanger sequencing and are presented here with their final status: heterozygous variant, artefact, or non-segregation with the phenotype in the family. The identification of a variant as a familial polymorphism or as present in our control cohort (over 160 control chromosomes) screening led to consider it as a SNPs.

In 2011, when these studies were performed, the WES strategy appeared as a powerful technique; however, numerous technical improvements had been provided since then. Although the investigations conducted were highly enriched in coding sequences, the low coverage of certain variants (fewer than 6 reads) prevented their analyses. Thus, I considered the linkage analyses performed as a more reliable method and I investigated the presence of any WES variants with low coverage in the loci of interest, which had not been revealed by the initial analysis due to the threshold strength applied to the NextGENE software (Table 7).

Family	Total	HTZ	Artefacts	Segregation	SNPs
Family J	2	0	2	0	0
Family R	6	0	4	1	1
Family U	4	0	1	1	2

Table 7: Additional low coverage variants not retained for the NextGENE analysis and located in loci of interest. The total number of variants tested is listed as well as the status of each variant post Sanger sequencing analysis: most of the variants were artefacts; none of them had been identified at the heterozygous state in patients. In total five vaiants were missed by the first stringent analysis. Two variants do not segregate with the condition in the family and three were identified in the control cohort.

These two additional analyses contribute to the analyses of additional variants, however no causative variants had been identified in Families J, R or U and their molecular diagnosis remains elusive.

III.2 Families status and candidate genes

By crossreferencing the results of the linkage analysis and the massive parallel sequencing, I have been able to restrict in Families J, R, S and U the investigations of numerous (from 16 to 29) variants to one or two candidate missense per family. This strategy led to the identification of a candidate gene in Family S. The variant segregates in the family and was not found in 160 control chromosomes. The variant localised in Exon3, codes for p.A59V in a N-GTP binding protein widely studied in cancer for its role in stemness maintenance and its functional relevance for p53 expression (PolyPhen 2 Possibly Damaging 0.589; PROVEAN -1.851 Neutral). The recent identification of an additional branch of the family can potentially improve our understanding of the segregation of this variant (Figure 32). Further analyses are in progress in the laboratory to assess the pathogenicity of this variant.

In Families J, R and U the diagnosis remains elusive. The two variants prioritised in Family U appear to be familial polymorphisms while the candidate variant investigated in Family J was identified at the heterozygous state in 10% of the controls samples that I tested. The pathogenicity of these variants was thus considered unlikely.

The candidate variant prioritised in Family R codes for a p.Q920H (PolyPhen 2 Benign; PROVEAN -0.271 Neutral) change in a tubulin glutamylase. The variant segregates with the condition and I did not identify this variant in any of the 160 chromosomes tested by Sanger sequencing nor in databases. However, the protein truncation is associated to a congenital "cone first" retinal disease without any description of muscle weakness. The relevance of this protein for the cilia function and the restricted ocular phenotype associated to its defects are not in favour of its involvement in a human skeletal muscle condition.

In conclusion, the results of these complex analyses suggest that the origin of congenital myopathies is probably not restricted to exonic mutations, and that other DNA regions not covered by WES have a so far underestimated role.

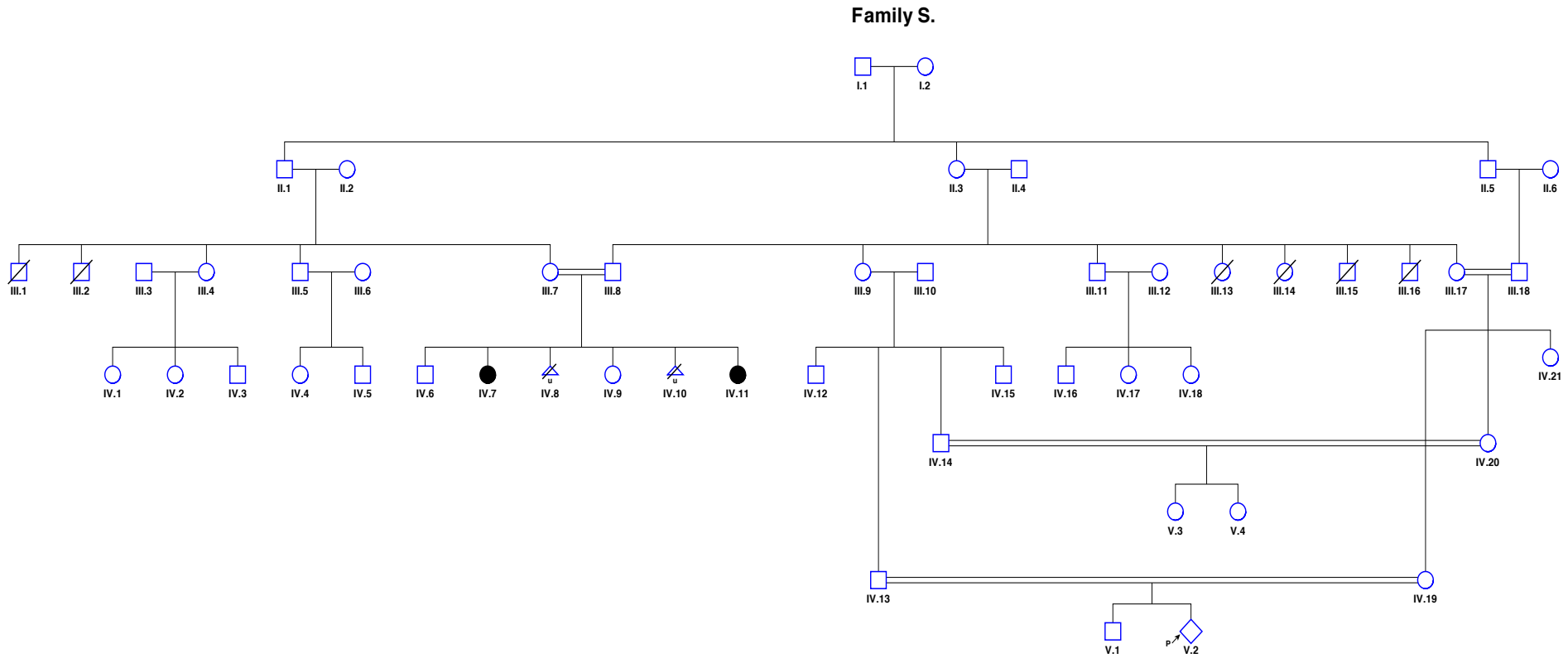


Figure 32: Enlarged pedigree for the family S. The newly identified branch of the family provided us with the historical and nearby reported familial history. A postnatal diagnosis for V.2 would be started collectively, with DNA sampling and consent of the parents (IV.13 and IV.19) and the unaffected brother (V.1).

DISCUSSION

Multiminicore disease is a phenotypically and genetically heterogeneous condition. The emerging characterisation of mixed phenotypes challenges myology. A more detailed correlation genotype – phenotype is required to better understand muscle physiology. With numerous strategies emerging, the possibility to provide genetic diagnosis to families with unlabelled congenital myopathies and to develop therapeutic approached becomes possible. Importantly, identification of new actors in muscle physiology and pathophysiology improves our understanding of these complex mechanisms.

Identification of *TRIP4* as a novel MmD gene: towards a reassessment of the classification of congenital muscle disorders

This work contributed to the identification of a new candidate gene and to the characterisation of a novel gene responsible for multiminicore disease. Regarding the latter, we report i) a novel condition with a unique clinical presentation ii) a new culprit gene and iii) a novel pathophysiological mechanism. The extensive analyses conducted on three out of the five families investigated excluded the known genes, confirming their genetic originality and predicting the existence of additional genetic defects in this condition.

Multiminicore disease is a form of congenital myopathy, which is most commonly associated to contractile protein mutations. This is associated to specific histological abnormalities that distinguish these conditions from congenital muscular dystrophies. However, multiminicore disease is emerging as a condition, which blurs the known concepts and boundaries in the field. First, most of the genetically diagnosed cases of MmD are associated to mutations in *SEPN1* and *RYR1* genes, encoding non-contractile proteins (Ferreiro et al., 2002a, 2002b). The recent identification of *MEGF10* as a mutated gene in MmD expanded the spectrum of underlying pathophysiological mechanisms (Boyden et al., 2012; Hartley et al., 2007; Logan et al., 2011; Pierson et al., 2013). *MEGF10* and *SEPN1* mutations are known to promote differentiation coupled to premature exhaustion of the progenitor pool (Castets et al., 2011; Holterman et al., 2007; Mitsuhashi et al., 2013; Nagase et al., 2001). Increasing cellular evidences highlight the relevance of muscle repair and

muscular progenitor differentiation balance as mechanisms also implicated into myopathy-related pathways. According to our *in vitro* experiments in C2C12, the absence of ASC-1 protein is associated to an altered myotube terminal differentiation, which represents the first description of delayed differentiation in a congenital myopathy. Therefore, *TRIP4* mutations represent a novel pathophysiological mechanism enlarging the spectrum of multiminicore disease-related pathways.

The classification of congenital myopathies based on clinical presentation and histological abnormalities remains a powerful technique to prioritise diagnosis strategies. Nonetheless, numerous mutated genes can be responsible for one condition, whereas one mutated gene can be responsible for different conditions (Kaplan and Hamroun, 2013). *SEPN1*-related conditions are a perfect illustration of this, since they can present histologically with patterns typical of four different diseases, including 3 congenital myopathies and a congenital muscular dystrophy (Arbogast and Ferreiro, 2010). The novel phenotype reported here in the *TRIP4* mutated family includes histological lesions normally linked to three phenotypically and genotypically different congenital myopathies and overlapping with congenital muscular dystrophy features. These recently identified culprit genes, aside from enlarging the pathophysiological mechanisms associated to congenital muscular conditions; stress the need to re-evaluate their classification. While the more "classical" forms of congenital muscular disorders are now investigated in diagnosis laboratories (Bönnemann et al., 2014; North et al., 2014), the most conflicting cases presenting with mixed phenotypes or peculiar conditions are currently the object of intensive investigations. If the use of NGS strategies keeps its promise and provides a complete catalogue of the exonic mutations by 2020, a comprehensive overview of the entire human gene coding conditions would be conceivable. A comprehensive classification will therefore be required. Some suggestions regarding gene clustering and networks, also termed hubs, are emerging (Barabási et al., 2011). This approach recommends considering subcellular connectivity and the intracellular network affected rather than clustering diseases according to the function of the mutated protein. This classification based on the molecular relationship between different actors affected in different phenotypic presentations rejoins the concept of disease module and diseasome (Goh et al., 2007). This stresses the need for systematic mapping of links between conditions. A better comprehension of molecular links could help to identify therapeutic approaches. A relevant example of this is the recent therapeutic use of N-acetylcysteine (NAC). This antioxidant has been successfully administrated in a *Ryr1*

zebrafish model (Dowling et al., 2012) and *in vitro* on *SEPN1*-mutated patient cells (Arbogast et al., 2009). Recently, this molecule has also been used in an in vitro model of intermediate filament myopathy and shown to decrease mutated desmin aggregates (Segard et al., 2013). Therefore, widening current knowledge regarding molecular networks could provide significant advances in the development of therapeutic approaches in even non-overlapping phenotypes.

The analysis of DNA samples from 58 patients presenting with compatible phenotypes did not allow the identification of additional *TRIP4* mutations, suggesting that defects of this gene are not a highly frequent cause of congenital muscle conditions. We propose the systematic investigations of *TRIP4* coding sequence, which is technically feasible since the gene is encoded by 13 small exons, in all unlabelled congenital muscular disorders. Identification of additional mutations in *TRIP4* gene will contribute to a better understanding of the correlations genotype – phenotype.

TRIP4 deficiency and muscle disease: potential genotype-phenotype correlations

The mixed phenotypical presentation in the *TRIP4* mutant family challenges myology and our comprehension of the underlying mechanisms. Regarding its function as a transcriptional coactivator (Jung et al., 2002; Kim et al., 1999; Lee et al., 1995, 2002), ASC-1 protein may act as an upstream element of the commonly established pathophysiological cascades. I suggest below a few physiopathological hypotheses based on the current knowledge of the defective pathways involved in multiminicore and cap diseases or in Collagene VI muscular dystrophy.

Common dysfunctions are associated with cap disease (previously associated with mutations of the tropomyosin or actin-coding genes TPM2, TPM3 or ACTA1) and MmD. The suspected pathophysiological mechanism in cap disease considers the impaired assembly and Ca^{2+} sensitivity of the actin filaments upon contraction (Marttila et al., 2012, 2014; Tajsharghi et al., 2012). Patients presenting with multiminicore lesions associated to *SEPN1*

or *RYR1* mutations also have an impaired calcium homeostasis most probably impacting on the regulation of the excitation-contraction coupling (Arbogast et al., 2009). This suggests that calcium mechanism could be a common pathophysiological mechanism between cap and multiminicore disease. Further investigations are required to assess the potential calcium homeostasis impairment in *TRIP4*-associated conditions. This would guide further pathophysiological and therapeutic investigations.

ACTA1 mutations were reported in cap disease (Hung et al., 2010) and also multiminicore disease (Jungbluth et al., 2001; Kaindl et al., 2004). Additionally, multiminicore disease is also associated to titin mutations, the third major component of the sarcomere contractile network, contributing to contraction and binding to actin filaments. Further research will clarify a potential role of ASC-1 as a regulator of muscle-specific gene expression, and the impact of its absence on the quantitative expression of contractile proteins, namely tropomyosin, actin or titin among others.

The link between Collagene VI conditions and *TRIP4* mutation remains elusive. Investigations conducted on patient fibroblasts did not highlight any impairment of the Collagene VI secretion, excluding a strict requirement of ASC-1 for the transcription of this ECM component. However, the dystrophic-like aspect of patient biopsies would be compatible with an impairment of muscle progenitor regulation also suspected in the *MEGF10*- and *SEPN1*-related forms of multiminicore disease (Castets et al., 2011; Holterman et al., 2007; Mitsuhashi et al., 2013; Nagase et al., 2001).

Finally, impairment of splicing processes are known to be involved in human conditions (Faustino and Cooper, 2003). ASC-1 and its associated protein complex could be involved in mediating exon retention (Auboeuf et al., 2004), this mechanism being already associated to a large range of muscular and cardiac conditions such as Myotonic Dystrophy (Sobczak et al., 2013) characterized by the sequestration of splicing effector MBNL1 in CUG repeats RNAs and more recently the identification of *Rbm20* a possible *Ttn* splicing effector mutated in a cardiomyopathy (Guo et al., 2012; Li et al., 2013). Understanding the contribution of ASC-1 to muscle physiology and pathophysiology remains a mandatory objective in order to develop therapeutic approaches.

ASC-1 as a novel key player in muscle physiology and pathophysiology

TRIP4 gene exists as a single copy located on Chr 15 in humans and Chr 9 in mice. Several transcripts are ubiquitously detectable by Northern Blot. However, a unique transcript leads to the expression of ASC-1 protein in human. In mice, an ubiquitous and a testis-specific transcripts share the same coding sequence, the latter being expressed at puberty in mice (Lee et al., 2002). ASC-1 was originally investigated as thyroid receptor β transcriptional interactor in a yeast model (Lee et al., 1995). In humans, a *TRIP4* SNP has been recently identified as a novel susceptibility locus in Alzheimer disease in the Spanish population (Ruiz et al., 2014). However, *TRIP4* remains a poorly characterised gene and the protein function is far from fully understood.

My work provides the first evidence of the implication of ASC-1 in a pathophysiological pathway associated to a severe form of congenital myopathy. While ASC-1 protein and its partners are ubiquitously expressed, their relevance for muscle physiology is not understood. *Trip4* transcription is initiated during development at E11.5 in mice embryos according to the EMBRYS database (Yokoyama et al., 2009). By in situ hybridisation, *Trip4* mRNA is not detectable earlier (at E9.5 or E10.5). The transcript appears in muscle structure at E11.5, suggesting a role of ASC-1 in early myogenesis. No data are available regarding the initiation of transcription of the ASC-1 partners.

Western blots performed on murine adult tissues revealed the low expression of ASC-1 protein in basal skeletal muscles, suggesting (but not proving) that the main role of ASC-1 is possibly not adult muscle maintenance (Figure 27A). The correlation between the ASC-1 expression profile and the restricted muscle phenotype remains difficult to clarify. However the relative higher expression observed in axial tissues (diaphragm and paravertebral muscles) correlates well with the predominantly axial muscle weakness in patients (Figure 27B). Compared to the other striated muscles, murine myocardium ASC-1 expression is as high as in diaphragm. Although the *TRIP4*-mutant patients are young and thus the development of cardiac disease in future years cannot be excluded, their consistent absence

of myocardial abnormalities so far suggests that the underlying pathophysiological mechanism is restricted to skeletal muscles.

Interestingly, the high ASC-1 expression in murine lung brings into consideration the patients' respiratory involvement. Respiratory insufficiency in myopathies is commonly considered as a consequence of respiratory muscle weakness. However, this high protein expression raises the question of a potential primary involvement of the lung tissue in this condition. Recently, a possible primary pulmonary involvement has been considered in *SEPN1* patients, after the abnormal lung development observed in the *Sepn1*$^{-/-}$ mouse model (Moghadaszadeh et al., 2013). The severity of the respiratory involvement, which represents the main cause of death in both conditions, supports the hypothesis of a common pathophysiological mechanism that could involve both skeletal muscles and lung as primary targets of *SEPN1* and *TRIP4* insufficiency.

Additionally, the significant expression of the ASC-1 protein in murine skin is consistent with the skin phenotype in humans. Regarding the continuous renewal of this tissue, a common underlying mechanism between skin and muscle can be hypothesised. Two distinct cell populations participate to epidermal renewal and repair after wounding (Ito et al., 2005). Characterising the ASC-1 expression in these cell populations and its expression profile should contribute to the understanding of the pathway affected.

The only *in vivo* investigation carried in *C. elegans* associated this gene to starvation stress response in dauer larvae (or "enduring" larvae, which is a stress resistance stage of *C. elegans* development) and proposed a contribution to cell survival in the aging process (Cherkasova et al., 2000). The authors report that extended-life nematodes surexpress significantly the ASC-1 homolog *dur123* (Cherkasova et al., 2000). The comprehension of the function of this protein remains to be clarified in mammals. Of note, while *Trip4* transcription is not significantly activated during satellite cell activation (Fukada et al., 2007; Li et al., 2012), *Trip4* is repressed in ageing quiescent satellite cells (Liu et al., 2013). These evidences suggest a potential contribution of ASC-1 to maintenance of muscle progenitors, cell survival and responsiveness to muscle damage. Investigating the regenerative potential of patients' muscles by labelling and quantifying progenitor muscle cells (Pax7+) will contribute to a better characterisation of the possible underlying pathophysiological mechanism.

ASC-1 (p65) is known to be the central component of a 650-kDa tetrameric complex (Figure 27C), termed ASC-1 complex (Jung et al., 2002), which corresponds to a nuclear receptor coregulator complex (Millard et al., 2013). ASCC2 (p100), which binds directly to ASC-1, remains the most uncharacterised protein. Predictions associate ASCC2 amino acid sequence to a CUE domain supposed to be involved in the ubiquitin/proteasome degradation pathway. But this protein is the only member of the complex whose localisation is restricted to the nucleus. The complex is also composed of the additional partners ASCC1 (p50) and ASCC3 (p200), each presenting characteristic RNA-interaction domains (Jung et al., 2002). ASCC3/HELIC1 is an 3'-5' DNA helicase that also interacts with the ALKBH3 complex to promote DNA alkylation repair and prevent cancer cell proliferation (Dango et al., 2011). ASCC3 is the only known partner for ASCC1. ASCC1 carries a KH domain responsible for nucleic acid-protein binding (Jung et al., 2002). *ASCC1* mutations are associated to Barrett Esophagus/Esophegeal Adenocarcinoma susceptibility (Orloff et al., 2011). In a human stomach cancerous cell line, silencing of either ASC-1 or ASCC1 represses the transcription of a gastrin paracrine pathway (Almeida-Vega et al., 2009), suggesting the requirement of these two subunits for the complex function. ASC-1 is thought to be the docking protein of the ASC-1 complex. This hypothesis is in agreement with our preliminary stainings performed in patient fibroblasts, which suggests a destabilisation of the complex in the absence of ASC-1. Further investigations using Proximity Ligation Assay (PLA) will clarify the relevance of the ASC-1 protein for the effective interactions between the different protein partners (Söderberg et al., 2008).

The ASC-1 protein comprises two identified functional domains (Figure 33). A unique zinc finger, including a cysteine arrangement which shows a strong similarity to the viral transactivator E1A protein domain ($CX_2CX_{12\text{-}13}CX_2CX_4C$) (Lee et al., 1995) and is sufficient to mediate the interaction with numerous partners. The domain has been characterised after its homology to the viral E1A transactivator (Webster and Ricciardi, 1991; Webster et al., 1991) that binds TATA box-binding proteins (Geisberg et al., 1994, 1995). As a transcriptional coactivator, ASC-1 zinc finger mediates numerous interactions with 1) nuclear receptors, 2) transcriptional integrators and also 3) elements of the basal transcriptional machinery. ASC-1 binds steroid hormone nuclear receptors (TR, RXR, RAR, ERα) in a ligand-independent manner *in vitro* (Kim et al., 1999; Lee et al., 2002). However, ASC-1 requires functional nuclear receptors AF-2 domain to transactivate these proteins and

promote transcription (Kim et al., 1999). ASC-1 complex also appears to bind key transcription factors involved in numerous pathways such as serum response factor (SRF), activating protein 1 (AP-1) and nuclear factor κB (NFκB) (Jung et al., 2002). Moreover, ASC-1 zinc finger binds to the basal transcription machinery of RNA Polymerase II: TBP, TFIIA, transcriptional integrators SRC-1 and the histone acetyltransferase CBP/p300 (Kim et al., 1999). Recent evidences support that post-translational modifications of ASC-1 enhance its binding to these integrators (Yoo et al., 2014).

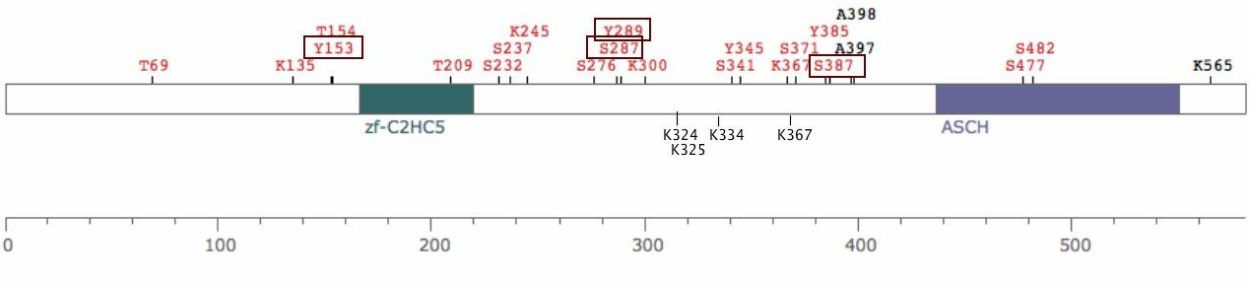


Figure 33: Schematic representation of ASC-1 domains and predicted posttranslational modifications. The human protein includes 581 amino acids. The zinc finger domain (PFMA predictions: 168-218) mediates the interactions with the nuclear receptors, transcriptional cointegrators and the basal transcriptional machinery. The function of the ASCH domain (PFAM predictions: 437-531) remains elusive. Figure from the Phosphosite freeware (Hornbeck et al., 2012) where human (red) and murine (blue) modifications predictions are reported (top panel). The modifications framed were quantified by Mass Spectroscopy. The modifications quoted are phosphorylation (residues Y, T, S), ubiquitination (K) and acetylation (K656). The ufmylation marks recently reported by (Yoo et al., 2014) are also presented (bottom).

ASC-1 also contains a unique C-terminal domain, whose β barrel conformation presents strong similarities with a PUA RNA binding domain. The amino acid GxKxxxxR motif (where x is any amino acid) defines an entirely new domain termed ASCH domain (Iyer et al., 2006). Our current knowledge regarding transcription regulation associates dual functions to numerous factors (Allemand et al., 2008; Han et al., 2006). The ASC-1 complex is also suspected to be a bi-functional complex. The unique structure of its ASCH domain is predicted to mediate RNA binding (Iyer et al., 2006; Park et al., 2011). Functioning as a transcriptional coactivator complex, initiating the transcription of class II genes, ASC-1

might also coordinate pre-mRNA processing. More importantly, steroid hormones are known to affect splicing decisions in a promoter dependent manner (Auboeuf et al., 2002). p50 and p200 are structurally assimilated to splicing factors and investigations performed in HeLa cells provide evidence that the ASC-1 complex promotes transcription and represses splicing in a steroid hormone- and promoter- dependent manner (Auboeuf et al., 2004). To my knowledge, little is known regarding the regulation of this mechanism. Numerous motifs identified as splicing regulatory elements (SRE) are responsible for these splicing activites in a factor- and location-dependent manner. The exonic SREs are now largely identified, however intronic SREs are still currently under investigations (Wang et al., 2012). A complete picture of these regulatory motifs might be required to investigate further their regulatory mechanisms.

Although the functional domains of ASC-1 subunits are described, the understanding of their complex function is not fully clarified so far. In our experiments, ASC-1 protein expression tended to increase during C2C12 differentiation while transcription is not modulated, suggesting a posttranscriptional regulation. ASC-1 is interacts with Nek6, a NIMA-related serine/threonine kinase, in human cells and is strongly phosphorylated *in vitro* (Vaz Meirelles et al., 2010). ASC-1 posttranslational modifications (Figure 33) such as phosphorylation and ufmylation may affect its stability and modulate its cellular localisation and its affinity for transcriptional factors and protein partners. Proteins of the ASC-1 complex (except p100, ASCC2) are able to shuttle into the cytoplasm (Jung et al., 2002). In HeLa cells, ASC-1 was observed shuttling to the cytoplasm when media was deprived of serum (Kim et al., 1999) suggesting a potential function in cellular growth arrest. However, this shuttling has not been observed in MCF7 breast cancer derived cells, suggesting a possible cell line specificity (Yoo et al., 2014).

ASC-1 function and relevance in muscle physiology is unknown. Based on the data available in the literature and in phenotypical similarities with SEPN1-related myopathy, we considered two hypotheses: a) ASC-1 absence could lead to the impairment of proliferation or b) to a defective differentiation process. The transcriptomic analysis performed aimed to identify pathways or targets strongly modulated in the absence of ASC-1. Because patient primary cells typically show high variability and a combination of primary and compensatory mechanistic changes, we used for this experiment a murine C2C12 cell line, for its unlimited accessibility and its reproducibility. The transient silencing protocol leads to the effective and

efficient downregulation of the *Trip4* transcripts and a decrease of about 80 to 90% of the ASC-1 protein expression, sufficient to observe a cellular phenotype.

Accordingly to the literature, we established our preliminary hypotheses on a link between ASC-1 and early differentiation. Therefore, we conducted transcriptional analyses in late proliferation and early differentiation conditions, to better understand the contribution of ASC-1 to this process. The transcriptomic analysis allowed the identification of numerous transcripts whose relatively mild modulation (Fold change between 0.8 and 1.2) is consistent with an ASC-1 role as a general transcriptional coactivator not indispensable for general transcription. The limited time-frame imposed by our transient knock-down may also prevent further quantitative amplification of the fold changes. Of note, while the relevance of miRNA for muscle differentiation is currently emerging (Dmitriev et al., 2013), only 56 miRNA probes were included in this transcriptomic analysis. Thus, other ASC-1 targets possibly remain to be characterised.

While the transcriptional investigations results suggest a contribution of ASC-1 to cell proliferation (IPA Predictions, Figure 28D), silencing of ASC-1 does not impair the C2C12 proliferation rate (Figure 29A and 29B, Phospho H3 labelling 29C). However, preliminary immunostainings suggest that the loss of ASC-1 is associated with a maintenance of proliferative marks (Ki67+, Figure 29C) and a modulation of *Skp2* transcripts by qPCR (Figure 28E). Consistently, overexpression of ASC-1 induces the expression of a p21 luciferase reporter. Validation of Skp2 and p21 protein expression profile will be essential to confirm a role of ASC-1 in cell cycle exit. Additionally, Skp2 activity is also linked to the degradation of p27, whose mobilisation is required for satellite cell quiescence and maintenance (Chakkalakal et al., 2014). Investigating the p27 status of satellite cells depleted of ASC-1 will allow deciphering the contribution of ASC-1 to maintain the dormance of mononucleated stem cells.

While the Ingenuity predictions did not point out any modulation of muscle-specific pathways, even at the early differentiation time point chosen for the transcriptomic analyses we observed a downregulation of expression of major sarcomeric proteins (including titin myosin heavy chain, troponins, tropomyosin, Myomesin and RyR1) in the absence of ASC-1. These results, which are being confirmed by qRT-PCR and protein studies, are consistent with the results of our in vitro differentiation experiment, which revealed the requirement of ASC-1 for the late differentiation and myotube growth in C2C12. In agreement with this, the

expression of MyoD or Myogenin was not impacted by the absence of ASC-1 (Figure 30E and 30F), but MHC protein levels were severely reduced in ACS-1 knocked-down myotubes (Figure 30G). Taken together, these results reveal ASC-1 as a novel key regulator of late myogenic differentiation, muscle growth and cell cycle exit. Further experiments regarding the mechanisms underlying this role, and particularly a potential implication of SRF (cf. Perspectives) are in progress.

Search for new genes in MmD: efficiency and limitations of the current genetic methods of investigation

My work was based on the genetic investigations in 5 highly informative and consanguineous families. During my PhD, I analysed and crossed the data from linkage and whole exome sequencing (WES) analyses. This work led to the identification of a variant in a novel positional candidate gene which is currently the object of further investigations in our lab and will eventually generate an original article of which I will be a co-author. More importantly, this has been an enriching training that gave me the opportunity of learning these novel techniques of genetic analysis and a good appreciation of their limitations.

The initial linkage analyses were performed prior to the generalisation of next generation sequencing and brought about important information. Linkage studies by homozygosity mapping remain a powerful tool for the whole-genome screening of consanguineous families suspected of an autosomal recessive condition. The identification of two loci of 5,56Mb in Family C illustrates the strength of the strategy. However, in some cases, this approach is not powerful enough. Due to the proximity of the consanguinity loops, the ancestral haplotypes segregating within the families can be large and numerous, as observed in Families J, R, S and U, preventing classical positional candidate gene studies. Nevertheless, linkage studies in these families confirmed the exclusion of known MmD genes, underlining the originality of the genetic conditions in this set of patients.

Additionally, the use of two different chips provides an opportunity for comparing their respective performances. While the high SNPs density carried by the 250K Nsp chip could have been expected to be highly sensitive, it appeared that its resolution was not as good as that of the 6056 SNPS chip. This difference could arise from the rate of genotyping errors. As visualised in the low coverage variants analysis (Table 6), 2 variants out of the 12 (16%) identified by WES and supposedly included within the loci defined by the 250K Nsp chip turned out not to co-segregate with the phenotype in the families after verification by Sanger sequencing. Thus, it appears, in the specific cases investigated here, that the high density of markers does not necessarily improve the balance between the amount of information collected and the efficiency/strength of the analysis (Evans and Cardon, 2004).

In the past five years, Next Generation Sequencing has emerged as a powerful technique for molecular investigations. This new tool has significantly impacted on the recent advances in medical genetics (Rabbani et al., 2014). Whole-exome sequencing (WES) covers almost the entire exome and has been at the origin, in the last years, of the identification of more than 180 new mutated genes in rare genetic disorders (Boycott et al., 2013). However, it is now known that WES can be ineffective in some cases. Indeed, published data report about 60% efficiency for the identification of disease mutations (Gilissen et al., 2012), which might even be overestimated. For example in a recent study of 140 families with a severe autosomal recessive nemaline myopathy non-associated with known genes, only 28% cases were mutated for a founder effect-related gene (*KLHL40)* (Ravenscroft et al., 2013). This is why we chose to combine WES with homozygosity mapping, a powerful approach to achieve, in an efficient and cost-effective manner, the identification of candidate genes in unlabelled autosomal recessive conditions (Rabbani et al., 2012). This strategy allowed the identification of a potential deleterious variant in Family S, whose pathogenicity is currently being investigated. In the other three highly informative families (Families J, R, S and U) analysed by WES in collaboration with the Université de Montreal, numerous reads once aligned covered non-coding regions, while other coding regions were missing as evocated in Table 7. In 2011, NGS was still at its early stages and has been since then the object of technical improvements, especially in the exon capture strategy, leading to 95% coverage of the coding sequences. These improvements are actually a strong argument in favour of a second WES analysis for Families J, R and U, currently remaining without diagnosis. More importantly, none of the variants identified by WES was in known congenital muscle disorders genes,

stressing once more the originality of the cases investigated and their potential for future identification of novel genes.

Interestingly, the variants identified were mostly missense and in frame insertions-deletions. Surprisingly, I identified neither nonsense nor frameshift mutations in those families, although recent data suggest that each individual carries privative nonsense variations. Also, the important number of missense variants identified analysed, associated with the billions of SNPs already indexed, and stresses the need to assess the pathogenicity of each variant. According to the literature and recent massive sequencing of the general population (1000 Genomes project), it has been estimated that up to 27 % of SNPs reported are missannotated or falsely considered as deleterious variants associated to human conditions (Bell et al., 2011). Therefore, caution and additional perspective is still required with this technique, and databases need to be readjusted.

The absence of gene identification in 75% of the families analysed by WES is consistent with lately published articles (Ravenscroft et al., 2013) but can also be partially explained by our current restricted knowledge regarding cellular processes. The most commonly used Next Generation Sequencing investigation is whole-exome sequencing (WES). Due to technical and knowledge limitations, whole-genome sequencing (WGS) remains a "future" technique. It is commonly accepted or predicted that by 2020, the complete spectrum of human mutations will be identified. However, efforts have been so far concentrated on coding sequences. Recent statistics suggest that 86% of mutations are localised in coding regions while solely 14% are intronic (11%) or in regulatory regions (3%; Cooper et al., 2010). In a yeast model the rate of mutagenesis has been associated with the accessibility of DNA and its transcription (Park et al., 2012). Regarding the dynamics of the human genome and the current breakthroughs in the gene regulation field, numerous possibilities remain to be explored. The effective transcription of non-coding genes remains unexplored by current WES strategies. Additionally, gene regulation itself appears as a potentially important physiopathological mechanism. miRNA, circRNA, lncRNA, eRNA (Lam et al., 2014) or pseudogenes are important elements of the transcriptional regulation. Other non-coding elements, such as transposable or regulatory sequences, are also potentially important. A review of these elements is provided by Cooper et al., 2010. Our comprehension

of the transcription regulation at the scale of the nucleus is also dramatically improving. The fascinating concept of the 3D transcriptional regulation (also termed ‘gene looping’) and transcriptional factories are emerging. These dynamic mechanisms remain to be investigated to understand their relevance for transcription initiation. In complex diseases, gene expression profiling (RNA-Seq) appears as a promising technique to uncover new RNA editing disequilibrium (Costa et al., 2013).

CONCLUSION & PERSPECTIVES

During my PhD, I aim to identify and characterise new genes responsible for the genetically undiagnosed cases of multiminicore disease. This project is based on the genetic investigations of five highly informative consanguineous families with clinical presentation consistent with this condition. The work I conducted contributes to enlarge the spectrum of genes mutated in multiminicore disease and also to widen the clinical presentations associated to this condition. The combination of known and newly identified genes functions highlights the relevant contribution of differentiation and regeneration mechanisms to congenital myopathies. Previously restricted to alterations of contractile proteins, multiminicore disease narrows the gap between congenital muscular dystrophies and myopathies pathophysiological mechanisms.

The cross referencing of linkage analysis and whole exome sequencing leads to the identification of a missense variation in a novel candidate gene, known in the literature as an actor of the progenitors stemness maintenance. The validation of the missense pathogenicity would be the object of further investigations resume in the laboratory. This candidate gene identification would facilitate postnatal diagnosis in a newly identified branch of the family. Also, further experiments regarding the role of this protein in muscle physiology would enlarge our understanding of muscle physiology and congenital myopathies pathophysiology.

Also, this work brings the first relevant contribution of an unaware transcriptional coactivator to muscle physiology and pathophysiology. My project leads to the characterisation of an unreported form of congenital myopathy and the preliminary *in vitro* evidences assess the role of ASC-1 in muscle physiology. To my knowledge, the work I conducted, that would be the object of a first authorship publication, is the first description of transcriptional impairment in congenital myopathies. The current technical limitations prevent the determination of the protein localisation. Therefore I recently generated a tagged construct that would be a useful tool to follow the protein delocalisation upon various condition variations in a muscular context. This tagged construct would also provide a useful support to immunoprecipitate ASC-1 uncharacterised partners. Also, this construct could contribute to the characterisation of the nucleic acids sequences targeted by the protein, as the functional domains seem to bind both RNA and DNA fragments.

In the C2C12 model I developed, I have been able to visualise the role of this protein in the promotion of muscular differentiation. This work is the first study regarding the

physiological relevance of this transcriptional coactivator. First, these investigations are in favour of a proliferation – differentiation balance impairment upon depletion of ASC-1. Additional evidences appear to link ASC-1 to the promotion of late differentiation. However, the transient silencing model I developed appears being limited for further investigations. The development of a stable knocked down model (shRNA) would recapitulate a more physiological absence of ASC-1 and contribute to a better characterisation of the protein role in differentiating conditions.

The transcriptomic analysis conducted reveals the catalysing activity of ASC-1 regarding the low modulation of significantly regulated transcripts. These analyses question the potential pathways modulated by ASC-1 in a muscular context. The Ingenuity predictions associated the depletion of ASC-1 to the downregulation of the SRF pathway. This gene contributes to muscle physiology (Sakuma and Yamaguchi, 2013). Interestingly, a transactivation of SRF by ASC-1 has been reported *in vitro* in Hela cells (Jung et al., 2002). The SRF knockout being lethal, a post mitotic model recapitulates under regenerative conditions, a premature aging phenotype associating adiposis and fibrosis accumulation (Lahoute et al., 2008) associated to growth immaturity and sarcomere disorganisation (Charvet et al., 2006). Additionally, a recent study highlight the requirement of *Srf* for the hypertrophic growth of skeletal muscle (Guerci et al., 2012). In the muscular context, the contribution of ASC-1 to SRF transactivation should thus be investigated. The use of an SRE (SRF element) reporter construct in the overexpressing system I developed will therefore answer this question.

According to the literature, ASC-1 binds several nuclear receptors, adding a potential hormonal component to the skeletal muscle pathophysiology of congenital disorders. Steroid hormones play a critical role in the establishment of muscle mass and the regulation of myogenesis versus adipogenesis. Regarding the historical background of ASC-1 interactions with steroid hormones nuclear receptors, THRβ is the most well described (Lee et al., 1995). The preliminary cell cycle exit investigations conducted in the overexpressing system are in favour of the ASC-1 contribution to T3 signalling in the muscular context. A review treating the current comprehension of thyroid hormones in skeletal muscle has been recently released (Salvatore et al., 2013). Thyroid hormone (TH) is a crucial determinant of striated muscle differentiation, regeneration (Leal et al., 2014) specification and adaptation (Little and

Seebacher, 2014; Yu et al., 2000). Also, the role of TH in skin that was controversial has been recently associated, while *Thra* and *Thrb* are deleted, to hair growth and wound healing defects (Contreras-Jurado et al., 2014). This contribution of TH to skin repair could explain the skin phenotype observed in patients. In a recent paper the authors report, despite THR expression in their C2C12 model, the difficulties to obtain a resolutive image of MRFs or MHC changes upon T3 induction (Ohn et al., 2013). This work emphases the requirement of an *in vivo* system to follow the T3 paracrine modulation. These observations also reinforce the potential contribution of ASC-1 to numerous pathways and the resulting differentiation delay caused by another partner pathway modulation.

These preliminary results do not exclude the requirement of the protein for embryonic and foetal development. Therefore the development of an *in vivo* model would be useful to go further in the investigations of the protein function during muscular development. The zebrafish model appears to be a highly recommended model to investigate human conditions at a larger scope (Ablain and Zon, 2013). Also a system widely used to address various regenerative questions in different tissues (Gemberling et al., 2013) and the latest investigations about salamanders/zebrafish regeneration are in favour of myogenic investigations in this system (Rodrigues et al., 2012).

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ANNEXES

ANNEXE 1: Congenital muscular dystrophies

Genes mutated in congenital muscular dystrophies. In this table are presented the list of the current conditions known as congenital muscular dystrophies and their mode of inheritance (AD: autosomal dominant, AR: autosomal recessive). The gene and its location are provided, except in the few conditions in which inly a locus has been associated to the condition so far. The last column recapitulates the proteins encoded by these mutated genes. Adapted from (Kaplan and Hamroun, 2013)

CONGENITAL MUSCULAR DYTROPHIES				
Disease name (OMIM number)	**Inheritance**	**Chromosome**	**Gene symbol (OMIM number)**	**Protein**
Congenital muscle dystrophies due to structural defects				
Congenital muscular dystrophy with merosin deficiency (607855)	AR	6q2	LAM2A (156225)	Laminin alpha 2 chain of merosin (laminin 2)
Bethlem myopathy (158810)	AD	21q22.3	COL6A1 (120220)	Collagen type VI subunit alpha 1
Bethlem myopathy (158810)	AD	21q22.3	COL6A2 (120240)	Collagen type VI subunit alpha 2
Bethlem myopathy (158810)	AD	2q37	COL6A3 (120250)	Collagen type VI subunit alpha 3
Bethlem myopathy (158810)	AR	21q22.3	COL6A2 (120240)	Collagen type VI subunit alpha 2
Ulrich Syndrome (254090)	AR	21q22.3	COL6A1 (120220)	Collagen type VI subunit alpha 1
Ulrich Syndrome (254090)	AR	21q22.3	COL6A2 (120240)	Collagen type VI subunit alpha 2
Ulrich Syndrome (254090)	AR	2q37	COL6A3 (120250)	Collagen type VI subunit alpha 3
Myosclerosis (255600)	AR	21q22.3	COL6A2 (120240)	Collagen type VI subunit alpha 2
Congenital muscular dystrophy with integrin defect (613204)	AR	12q13	ITGA7 (600536)	Integrin alpha7
Congenital muscle dystrophies due to sarcomeric defects				
Rigid spine syndrome (602771)	AR	Xq26.3	FHL1 (300163)	Four and half LIM domain 1
Congenital muscular dystrophy with telethonin defect	AR	17q12	TCAP (604488)	Titin cap (telethonin)
Congenital muscle dystrophies due to compartments specific proteins defects				
Rigid spine syndrome (602771)	AR	1p36	SEPN1 (606210)	Selenoprotein N1
Congenital muscular dystrophy due to LMNA defect (L-CMD)	AD	1q21.2	LMNA (150330)	Lamin A/C
Congenital muscle dystrophies due to defective glycosylation				
Fukuyama congenital muscle dystrophy (253800)	AR	9q31-q33	FKTN (607440)	Fukutin
Walker-Warburg syndrome (253801)	AR	9q31-q34	FKTN (607441)	Fukutin
Walker-Warburg syndrome (236670)	AR	9q34	POMT1 (607423)	Protein O mannosyltransferase 1
Walker-Warburg syndrome (613150)	AR	14q24.3	POMT2 (607439)	Protein O mannosyltransferase 2
Walker-Warburg syndrome (613153)	AR	19q13.32	FKRP (606596)	Fukutin related protein
Walker-Warburg syndrome (253280)	AR	1p34.1	POMGNT1 (606822)	O mannose beta 1,2 N acetylglucosaminyl transferase
Walker-Warburg syndrome (614643)	AR	7p21.2	ISPD (614631)	Isoprenoid synthase domain containing
Walker-Warburg syndrome (614830)	AR	3p22.1	GTDC2 (614828)	Glycosyltransferase like domain containg 2
Walker-Warburg syndrome	AR	11q13.2	B3GNT1 (605517)	UDP GlcNAc betaGal beta 1,3 N

(615287)				acetylglucosaminyltransferase 1
Muscle eye brain disease (253280)	AR	1p34.1	POMGNT1 (606822)	O mannose beta 1,2 N acetylglucosaminyl transferase
Muscle eye brain disease (613153)	AR	19q13.32	FKRP (606596)	Fukutin related protein
Muscle eye brain disease (613150)	AR	14q24.3	POMT2 (607439)	Protein O mannosyltransferase 2
Muscle eye brain disease (615350)	AR	3p21.31	GMPPB (615320)	GDP mannose pyrophosphorylase B
Congenital muscular dystrophy with hypoglycosylation of dystroglycan (613153)	AR	19q13.32	FKRP (606596)	Fukutin related protein
Congenital muscular dystrophy with hypoglycosylation of dystroglycan (613154)	AR	22q12	LARGE (603590)	Like glycosyl transferase
Congenital muscular dystrophy with hypoglycosylation of dystroglycan (608799)	AR	20q13.13	DPM1 (603503)	Dolichyl phosphate mannosyltransferase 1, catalytic subunit
Congenital muscular dystrophy with hypoglycosylation of dystroglycan and severe epilepsy (615042)	AR	9q34.13	DPM2 (603564)	Dolichyl phosphate mannosyltransferase 2, regulatory subunit
Congenital muscular dystrophy with hypoglycosylation of dystroglycan (300884)	XR	Xq23	ALG13(300776)	UDP N acetylglucosaminyltransferase subunit
Congenital muscular dystrophy with hypoglycosylation of dystroglycan WWWS/MEB like (15181)	AR	1q42.3	B3GALNT2 (610194)	Beta 1,3 N acetylgalactosaminyltransferase 2
Congenital muscular dystrophy with hypoglycosylation of dystroglycan and mental retardation (615351)	AR	3p21.31	GMPPB (615320)	GDP mannose pyrophosphorylase B
Congenital muscular dystrophy with hypoglycosylation of dystroglycan type A10 (615041)	AR	12q14.2	TMEM5 (605862)	Transmembrane protein 5
Congenital muscular dystrophy with hypoglycosylation of dystroglycan type A12 (615249)	AR	8p11.21	POMK-*SGK196* (615247)	Protein O mannose kinase
Other Congenital muscle dystrophies				
Congenital muscle dystrophies with joint hyperlaxity	AR	3p23-21	?	
Congenital muscle dystrophies with mitochondrial structural abnormalities (magaconial type) (602541)	AR	22q13	CHKB (612395)	Choline kinase beta
Congenital muscle dystrophies (604801)	AR	1q42	?	

ANNEXE 2: Congenital myopathies

Congenital myopathies and their main mutated genes. The mode of inheritance (AD: autosomal dominant, AR: autosomal recessive) of the known conditions is listed hereafter. The transcriptomic analysis conducted reveals the catalysing activity of ASC-1 The transcriptomic analysis conducted reveals the catalysing activity of ASC-1 The transcriptomic analysis conducted reveals the catalysing activity of ASC-1 Adapted from Kaplan and Hamroun, 2013

CONGENITAL MYOPATHIES				
Disease name (OMIM number)	**Inheritance**	**Chromosome**	**Gene symbol (OMIM number)**	**Protein**
Congenital myopathies with protein accumulation				
Nemaline Myopathy (609284)	AD	1q21.2	TPM3 (191030)	tropomyosin 3
Nemaline Myopathy (256030)	AR	2q22	NEB (161650)	Nebulin
Nemaline Myopathy (161800)	AD	1q42.1	ACTA1 (102610)	actin, alpha1 skeletal muscle
Nemaline Myopathy (609285)	AD	9p13	TPM2 (190990)	tropomyosin 2 beta
Nemaline Myopathy with Escobar syndrome (26500)	AR	9p13	TPM2 (190990)	tropomyosin 2 beta
Nemaline Myopathy (605355)	AR	19q13	TNNT1(191041)	Troponin T type 1 slow skeletal
Nemaline Myopathy (609273)	AD	15q22.31	KBTBD13(613727)	Kelch repeat and BTB (POZ) domain containing 13
Nemaline Myopathy (610687)	AR	14q12	CFL2 (601443)	Cofilin 2 (muscle)
Severe autosomale recessive Nemaline Myopathy (615348)	AR	2p22.1	KLHL40 (615340)	Kelch like family member 40
Hyaline body myopathy, recessive (255160)	AR	3p22.2-p21.32	?	?
Hyaline body myopathy dominant (myosin storage myopathy) (608358)	AD	14q12	MYH7(160760)	Myosin heavy chain 7 cardiac muscle b
myosin storage myopathy and cardiomyopathy recessive	AR	14q12	MYH7(160760)	Myosin heavy chain 7 cardiac muscle b
myosin lia myopathy dominant (inclusion body myopathy) (605637)	AD	17p13.1	MYH2 (160740)	Myosin heavy chain 2 cskeletal muscle adult
myosin lia myopathy, recessive	AR	17p13.1	MYH2 (160740)	Myosin heavy chain 2 cskeletal muscle adult
Cap myopathy (190900)	AD	9p13	TPM2 (190990)	tropomyosin 2 beta
Cap myopathy (609284)	AD	1q21.2	TPM3 (191030)	tropomyosin 3
Cap myopathy	AD	1q42.1	ACTA1 (102610)	actin, alpha1 skeletal muscle
Congenital myopathies with fiber size variation				
Myopathy congenital with fiber-type disproportion (255310)	AD	1q42.1	ACTA1 (102610)	actin, alpha1 skeletal muscle
Myopathy congenital with fiber-type disproportion (255310)	AR	1p36	SEPN1 (606210)	Selenoprotein N1
Myopathy congenital with fiber-type disproportion (255310)	AD	1q21.2	TPM3 (191030)	tropomyosin 3
Myopathy congenital with fiber-type disproportion (255310)	AR	19q13.1	RYR1 (180901)	Ryanodine receptor
Myopathy congenital with fiber-type disproportion (255310)	AD	14q12	MYH7(160760)	Myosin heavy chain 7 cardiac muscle b
Congenital myopathies with central nuclei				

Myotubular myopathy (310400)	XR	Xq28	MTMT1 (300415)	Myotubularin 1
Centronuclear myopathy, dominant (160150)	AD	19p13.2	DNM2 (602378)	Dynamin 2
Centronuclear myopathy, recessive (255200)	AR	2q14	BIN1 (601248)	Amphiphysin 2
Centronuclear myopathy, recessive (255200)	AR	19q13.1	RYR1 (180901)	Ryanodine receptor
Congenital myopathies with cores				
central core disease, dominant (117000)	AD	19q13.1	RYR1 (180901)	Ryanodine receptor
central core disease, recessive (transient multiminicore myopathy) (117000)	AR	19q13.1	RYR1 (180901)	Ryanodine receptor
Multiminicore disease with external ophtalmoplegia (255320)	AR	19q13.1	RYR1 (180901)	Ryanodine receptor
Multiminicore disease , classical form (255320)	AR	1p36	SEPN1 (606210)	Selenoprotein N1
Early onset myopathy, areflexia, respiratory distress and dysphagia (614399)	AR	5q23.2	MEGF10 (612453)	multiple EGF like domains 10
Recessive congenital myopathy with minicores (614399)	AR	5q23.2	MEGF10 (612453)	multiple EGF like domains 10
Congenital myopathy with fatal cardiomyopathy (611705)	AR	2q31	TTN (188840)	Titin
Other Congenital myopathies				
Congenital neuromuscular disease with uniform type 1 fiber (117000)	AD	19q13.1	RYR1 (180901)	Ryanodine receptor
Congenital skeletal myopathy and fatal cardiomyopathy	AR	11p11.2	MYBPC3 (600958)	Cardiac myosin binding protein C
Compton North congenital myopathy	AR	12q11-q12	CNTN1 (600016)	Contactin1
Sarcotubular myopathy	AR	9q31	TRIM32 (602290)	Tripartite motif containing 32 (ubiquitin ligase)
Congenital myopathy related to PTPLA	AR	10p12.33	PTPLA *HACD1* (610467)	protein tyrosine phosphatase like (3 hydroxyacyl CaoA dehydratase)

ANNEXE 3: Primers for *TRIP4* sequencing and quantification

Primers for *TRIP4* exon amplification and Sanger sequencing (Human)		
Exon 1	GGAGGGCACAAGGAAGTAGA	GAGTAAGGGGTTCAAAGTTAGG
Exon 2	CTAGCACCTAGCATAGTACTTG	TCTCGATCTCCTGACCACG
Exon 3 Part1	CTTAGTCCCAGCCACTTGTT	AGCTTCAATCTATAAGCACTCAC
Exon 3 Part2	TAGATGGGCAGAAATCAGGC	ATCGCACCACTGCACTCC
Exon 4	CTACATTTACCCTCCACTAGC	CCTGTAGTTCCAGCTGCTC
Exon 5	CCATTAAGTCATTCAGGGACATG	CTGAGAATGAGTGAATAAACTGC
Exon 6	ACCTGTCATGTGTCAAGCACT	GCCTCCTATGTAGAGCATCT
Exon 7	CAGAAGGTAGAGGCTGGAC	AGCCTGGGCAACAAAGCGA
Exon 8	GGAGTGGGGAGTAGGGAA	GAATAAGGTTGGCAGGTGTG
Exon 9	CCACCACGCCCAGCTAAT	CTCTAAGATCTTCCACTGCC
Exon 10	GTTGTAGGGATCAGGTGAAAAG	GGATCTCAGAGTTTACACTAGTC
Exon 11	TTGTGGGGTCCTTGAACTTTGATG	TTTCAAGACCAGCCTGGCCAACA
Exon 12	GGATTGTAGAAAGGTGAAGG	CTGATCCAAAGGTAGTGGG
Exon 13	GTTCCCATTGCTTCCTTATTC	ACAGGATTCCTCAAACATCC

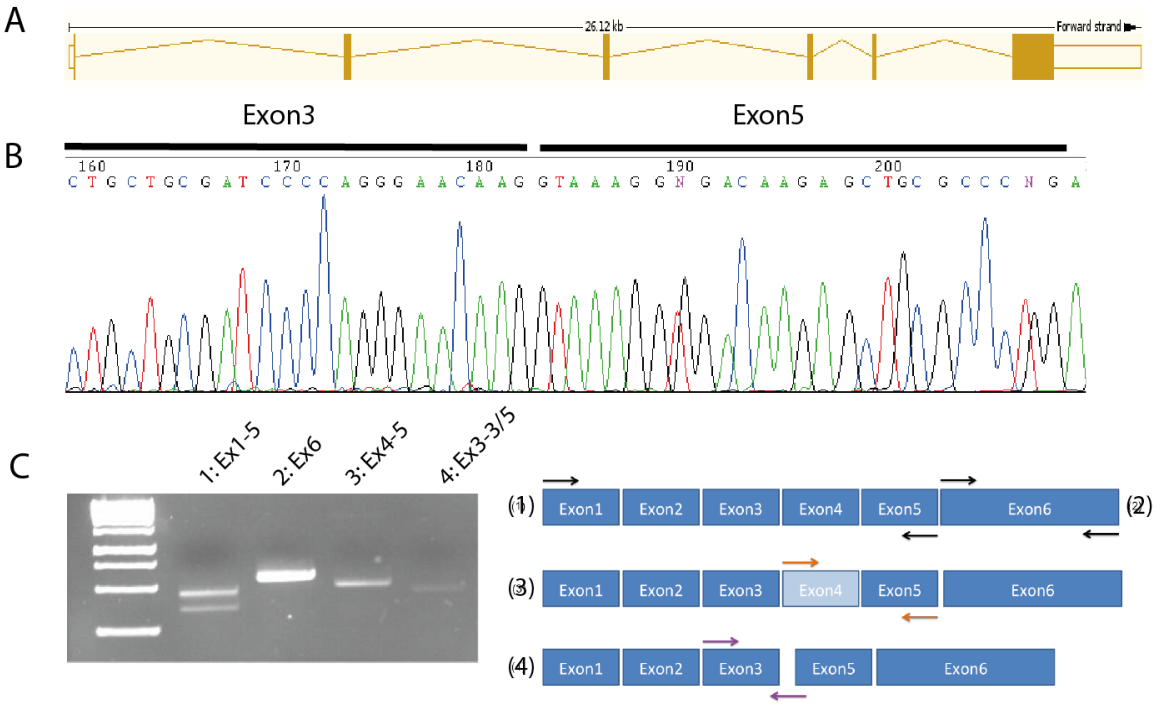
Primers for TRIP4 and Trip4 quantification			
Human	TTGCAGCTCAGCTGGTT	CTCCACTATGCTGTATAGTTCC	RT PCR
Mouse	TAAAGCGGAGTAGGAGGAAAGG	GGAAGCAGGACTGCAAGTTTGT	qPCR

ANNEXE 4: Antibodies

Antigen	Species and supplier	Western Blot	Immunofluoresence
Anti-ASC-1	Rabbit, ab 70627 Abcam	1:1,000 (h) to 5,000 (m)	/
Anti ASCC2	Rabbit D16 Santa Cruz		1: 50
Anti ASCC3	Goat S20 Santa Cruz		1: 50
Anti Ki67	Mouse IgG1 556003 BD Pharmingen	/	1: 100
Anti MyoD	Rabbit C20 Santa Cruz	1 :5,000	1:50
Anti Myogenin	Mouse IgG1 F5D DSHB	1 :1,000	1:50
Anti Myosin Heavy Chain	Mouse IgG2b MF20 DSHB	1 : 5,000	1: 50
Anti Phospho Histone H3	Rabbit 06-570 Millipore	/	1:500
Anti p21	Rabbit C19 Santa Cruz	1 :500	1: 100
Anti Pax7	Mouse IgG1 sc-81648 Santa Cruz		1: 50
Anti tubulin	Mouse IgG1, Sigma	1 :10,000	/
Anti GAPDH	Mouse	1 :5,000	/
Anti Histone H3	Rabbit, H0164 Sigma	1 : 5,000	/
Anti Goat IgG Alexa Fluor 488	Donkey, A11055 Life Technologies	/	1:1,000
Anti Mouse IgG Alexa Fluor 488	Donkey, A21202 Life Technologies	/	1:1,000
Anti Mouse IgG2b Alexa Fluor 633	Goat, A21146 Life Technologies	/	1:1,000
Anti Rabbit IgG Alexa Fluor 555	Donkey, A31572 Life Technologies	/	1:1,000
Anti Mouse IgG Alexa Fluor 680	Donkey, A10038 Life Technologies	1 : 7,500	/
Anti Rabbit IgG Alexa Fluor 790	Goat, A11369 Life Technologies	1 : 7,500	/

ANNEXE 5: PLEKHO2 variant predictions

Branching site mutation is associated to the expression of an alternatively spliced variant in patient fibroblasts. (A) Human PLEKHO2 gene codes for a canonical transcript NM_025201.4 (modelisation from Ensembl Flicek et al., 2014) and shorter isoform missing the Exon 2 NM_001195059.1. (B) The alternatively spliced variant resulting from the donor branching site variation that does not carry Exon 4. (C) RT-PCR performed with different sets of primers show the relative expression of the different isoforms (primer set Ex1-Ex5, line 1), the amplification of both variants (primer set Ex6, line 2). The alternative splicing resulting from the branching site mutation is visualised by the amplification of the wild type isoform (primer set Ex4-Ex5, line 3) and its alternative isoform (primer set Ex3-Ex3/5, line 4).



ANNEXE 6: Predicted altered function by IPA

PROLIFERATION - FUNCTIONS

Category	Diseases or Functions Annotation	p-Value	Predicted Activation State	Activation z-score	# Molecules
Cellular Growth and Proliferation	proliferation of cells	4,72E-06	Increased	2,199	267
Developmental Disorder	dystrophy of muscle	2,14E-03	Increased	2,191	5
Skeletal and Muscular Disorders	dystrophy of muscle	2,14E-03	Increased	2,191	5
DNA Replication, Recombination, and Repair	homologous recombination of DNA	2,73E-03	Increased	2,193	5
Cell Cycle	homologous recombination of DNA	2,73E-03	Increased	2,193	5
Cell Death and Survival	cell death of melanoma cell lines	8,26E-04	Decreased	-2,647	26
Cell Death and Survival	apoptosis of melanoma cell lines	4,12E-03	Decreased	-2,371	13
Embryonic Development	degeneration of embryoblast	1,39E-02	Decreased	-2	4
Tissue Morphology	degeneration of embryoblast	1,39E-02	Decreased	-2	4

DIFFERENTIATION - FUNCTIONS

Category	Diseases or Functions Annotation	p-Value	Predicted Activation State	Activation z-score	# Molecules
Tissue Morphology	quantity of connective tissue cells	7,07E-04	Increased	2,05	17
Connective Tissue Development and Function	quantity of connective tissue cells	7,07E-04	Increased	2,05	17
Gene Expression	transactivation of RNA	1,00E-03	Increased	2,374	31
Cell Signaling	protein kinase cascade	9,08E-03	Increased	2,188	25
Protein Synthesis	quantity of hdl cholesterol in blood	9,10E-03	Increased	2,425	6
Digestive System Development and Function	morphology of digestive system	1,70E-03	Decreased	-2,19	35
Organismal Survival	neonatal death	1,33E-02	Decreased	-2,218	26

ABSTRACT

Identification and characterization of a new gene associated with an unreported congenital myopathy phenotype

Congenital myopathies are rare genetic disorders characterized by neonatal hypotonia, delayed motor development and muscle weakness, associated with characteristic histological changes in the structure of muscle fibres visible on the patients' muscle biopsies. Our laboratory is particularly interested in the study of core myopathies, which are emerging as the most prevalent form of congenital myopathy, and especially of multi-minicore disease (MmD), which is characterised by multiple focal short areas of mitochondria depletion and sarcomere disorganisation (cores) within muscle fibers. Our group identified most of the genes associated to this genetically and phenotypically heterogeneous condition. However, at least 30% of multiminicore disease cases are not associated with the known genes and remain genetically uncharacterized.

During my PhD, my objective was to identify and characterize new genes responsible for this condition. The study of a large consanguineous family by homozygosity mapping allowed the identification of a homozygous nonsense mutation in the coding sequence of a transcriptional coactivator (named thereafter TCA), which had never been associated with a muscle condition. The 3 affected patients presented with a novel, very severe form of congenital myopathy with an unreported histological pattern associating minicores, nuclear internalization and cap lesions. qPCR and western blotting showed absence of messenger and protein on patient samples, suggesting NMD (nonsense mediated decay). The increased TCA expression profile in murine axial skeletal muscles is consistent with the clinical presentation. Also, I found increased protein expression during *in vitro* C2C12 myoblastic cell line differentiation, which is compatible with a contribution to myogenesis. Subsequently, I performed microarray analysis on a transient TCA silencing model, which disclosed a tendency to downregulation of muscle and contractile proteins (in differentiation conditions), and an upregulation of cell cycle proteins (in proliferative conditions), suggesting a role of TCA in regulating the proliferation/differentiation balance in muscle. Consistently, Dual Reporter Luciferase assays performed on proliferative C2C12 identified p21 as an activated target of TCA suggesting a role of the protein in the cell cycle exit regulation more specifically.

Thus, we report a novel congenital muscle condition with a unique histological pattern, stressing the histological overlap of different forms of congenital myopathies and muscular dystrophies. We characterize a new gene in human genetic conditions and a novel regulator of the proliferation/differentiation balance in muscle.

In parallel, to identify other genes associated with MmD, I investigated four highly informative and consanguineous families with MmD non-associated with the known genes. By crossing homozygosity mapping data and massive parallel sequencing, I identified a candidate gene, which encodes a protein potentially implicated in cell stemness and linked to p53 activity. Confirmation of the pathogeneicity of this change and gene are in progress in our laboratory.

A novel transcriptional coactivator is pivotal in regulating the balance between proliferation and differentiation of myogenic progenitors and is mutated in a novel form of congenital myopathy. Davignon *et al*. – *in preparation*

RESUME

Identification et caractérisation d'un nouveau gène impliqué dans une nouvelle forme de myopathie congénitale

Les myopathies congénitales sont des pathologies génétiques rares qui se caractérisent par une hypotonie néonatale, un retard moteur et une faiblesse musculaire associés à des défauts de structure des fibres musculaires visibles sur les biopsies des patients. Notre laboratoire s'intéresse à l'étude des myopathies à cores et plus spécifiquement à la myopathie à multiminicore (MmD) qui se caractérise par une réduction de l'activité mitochondriale en de multiple points focaux et une désorganisation des sarcomères (cores) au sein de la fibre musculaire. Notre groupe est à l'origine de l'identification de la plupart des gènes mutés dans cette pathologie. Néanmoins, 30% des cas restent à ce jour sans diagnostique moléculaire.

L'objectif de ma thèse a été d'identifier et de caractériser de nouveaux gènes responsables de cette pathologie. L'étude de liaison réalisée dans une famille consanguine a permis d'identifier une mutation homozygote tronquante dans la région codante d'un coactivateur transcriptionnel (appelé TCA) dont la fonction n'a jamais été associée au muscle. Les 3 patients affectés sont atteints d'une nouvelle forme de myopathie congénitale sévère. Le profil histologique des fibres musculaires présente à la fois des minicores, une centralisation nucléaire et des lesions de type « cap ». L'analyse du contenu ARN (qPCR) et protéique (WB) des cellules de patients a révélé l'absence de l'ARN messager de TCA (suggérant un mécanisme d'éliminiation de type NMD – nonssense mediated decay) ainsi que l'absence de la protéine. Le profil d'expression de TCA dans les muscles axiaux de souris adultes est en accord avec la présentation clinique des patients. De plus, l'augmentation de l'expression de la protéine au cours de la differentiation dans une lignée murine myoblastique (C2C12) est en faveur de son implication dans la myogenèse. Par des analyses transcriptomiques sur un modèle d'extinction transitoire de TCA, j'ai pu mettre en évidence une diminution de l'expression des protéines musculaires contractiles (en différenciation) et une augmentation des protéines du cycle cellulaire (en prolifération) suggérant que TCA joue un rôle dans la balance proliferation/differenciation au sein du tissu musculaire. Finalement, des expériences d'expression d'un gène rapporteur Luciférase dans les cellules C2C12 en proliferation ont permis d'identifier p21 comme cible de TCA ce qui oriente son rôle plus spécificiquement vers une régulation de la sortie de cycle cellulaire.

Nous présentons ici une nouvelle forme de myopathie congénitale avec un profil histologique original qui met en évidence l'existence de nombreux points communs entre les différentes formes de myopathies mais également avec les dystrophies musculaires. Nous avons identifié un nouveau gène impliqué dans les maladies génétiques humaines qui se trouve être un acteur de la balance prolifération/différenciation au sein du muscle.

En parallèle, j'ai étudié 4 familles consanguines informatives présentant une forme non étiquetée de myopathie à multiminicores. En croisant les données issues des analyses de liaison et de séquençage à haut débit, j'ai pu identifier un gène candidat. La protéine codée par ce gène est probablement impliquée dans la maintenance des cellules progénitrices et liée à l'activité de p53. La pathogénicité de cette mutation due à changement d'acide aminé est actuellement à l'étude dans le laboratoire.

Article en préparation : A novel transcriptional coactivator is pivotal in regulating the balance between proliferation and differentiation of myogenic progenitors and is mutated in a novel form of congenital myopathy. Davignon *et al*.

ABSTRAKTE

Identifikation und Charakterisierung eines neuen Genes das mit einem unbeschriebenen Phänotyp der Kongenitalen Myopathie korreliert.

Kongenitale Myopathien sind seltene genetische Erkrankungen zu deren Symptomen neonatale Hypotonie, eine verspätete motorische Entwicklung und Muskelschwäche gehören. Sie sind mit charakteristischen histologischen Veränderungen in der Muskelfaserstruktur assoziiert. Unser Labor beschäftigt sich mit der Erforschung der Kongenitalen Myopathien – insbesondere der Multiminicore Krankheit (MmD), die durch multiple fokale kurze Areale mit mitrochondrialem Abbau die zur Auflösung des Sarkomers führen charakterisiert ist. Unsere Arbeitsgruppe identifizierte die meisten der mit dieser Krankheit assoziierte Gene. Dennoch sind 30% der Multiminicore Erkrankungen sind nicht mit diesen Genen assoziiert und noch nicht genetisch charakterisiert.

Das Ziel meiner Promotionsarbeit war die Identifikation und Charakterisierung neuer MmD Gene. Als Vorarbeiten hatte unser Labor eine Großfamilie mit drei Patienten untersucht, die ungewöhnliche Minicores mit Kerninternalisierungen und Cap-Läsionen aufwiesen, als Zeichen einer bisher unbekannten, schweren Form der Kongenitalen Myopathie. Die zugrundeliegende homozygote Nonsens-Mutation des Transkriptionscoaktivators (TCA) wurde zuvor nicht mit der Kongenitalen Myopathie in Verbindung gebracht. Der Verlust von TCA mRNA und Protein wurde in Realtime PCR und Western Blot dokumentiert und impliziert nonsense mediated decay (NMD) als moleklulare Grundlage der Erkrankung. Das Expressionsprofil von TCA im Skeletmuskel in vivo als auch in vitro (C2C12). Letztere wiesen eine erhöhte Expression von TCA während der Differzierung auf. Dies unterstützt die Hypothese der Beteiligung von TCA an der Myogenese. Nachfolgend untersuchte ich TCA defiziente C2C12 Zelllinien mit Hilfe einer Microarray Analyse. Die Ergebnisse zeigten eine Runterregulierung von kontraktilen Muskelproteinen und gleichzeitig die Hochregulierung von Zell-Zyklus-Proteinen. Dies suggeriert die Beteiligung von TCA an der Regulierung der Proliferation und der Differenzierung im Skelettmuskel. Zudem zeigten Reporter-Luciferase-Assays in proliferierenden, TCA überexpremierenden C2C12 Zellen eine direkte und aktivierende Interaktion zu p21, was die Rolle von TCA auf das Ende des Zell-Zyklus spezifiziert.
Wir beschreiben somit eine neue kongenitale Muskelkondition mit einem einzigartigen histologischen Muster, die eine histologische Überlappung zwischen verschiedenen Kongenitalen Myopathien und Muskeldystrophien aufzeigt. Zudem charakterisierten wir unter humanen genetischen Bedingungen ein neues Gen, welches die Proliferation und Differenzierung des Muskels reguliert. Weitergehende Untersuchungen des Gens werden in unserem Labor bereits durchgeführt.
Parallel untersuchte ich fünf weitere Familien mit MmD. Dabei identifizierte ich ein weiteres Gen dessen Protein potentiellen Einfluss auf den Differenzierungsmechanismus hat und mit der Aktivität von p53 verknüpft zu sein scheint. Die Pathogenität der Veränderung und das Gen werden in unserem Labor weiter untersucht.

Eine Veröffentlichung mit dem Titel „A novel transcriptional coactivator is pivotal in regulating the balance between proliferation and differentiation of myogenic progenitors and is mutated in a novel form of congenital myopathy." ist in Bearbeitung. Davignon *et al*.