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Regeneração muscular na miopatia centronuclear associada a mutações no gene *DNM2*

Skeletal muscle regeneration in *DNM2*-related centronuclear myopathy

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Resumo

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O músculo esquelético possui grande capacidade regenerativa após sofrer lesões, por causa da presença das chamadas células-satélite, que permanecem no tecido em estado quiescente, mas que, na presença de uma lesão, são capazes de proliferar e formar e/ou reparar miofibras. As células-satélite são importantes para o crescimento e manutenção do músculo adulto. Porém, em diversas doenças neuromusculares, a quantidade, a função e a capacidade proliferativa destas células podem estar comprometidas.

As miopatias centronucleares (CNM) são um grupo de doenças musculares caracterizadas por fraqueza muscular generalizada e o posicionamento dos núcleos na porção central da miofibra. A forma autossômica dominante (AD-CNM) é causada por mutações no gene *DNM2.* A proteína dinamina 2 é expressa ubiquamente e está envolvida no remodelamento de membranas, no tráfego intracelular e na dinâmica do citoesqueleto. Consequentemente, os mecanismos fisiopatológicos também são diversos e não completamente compreendidos, principalmente o fato de ser uma doença músculo-específica.

Nesta tese de doutorado, buscamos investigar as células-satélite no contexto da miopatia centronuclear. Para isto, utilizamos o camundongo modelo KI-*Dnm2*^{R465W}, portador da mutação mais frequente em pacientes humanos. Como na miopatia centronuclear não há um processo degenerativo em atividade, induzimos nos camundongos a lesão muscular por choque elétrico, em protocolo desenvolvido nesta tese, comparativamente a injeção de cardiotoxina. Verificamos que o número de células satélite no músculo gastrocnêmio do camundongo KI-*Dnm2*^{R465W} é reduzido em relação aos animais selvagens. Em consequência disto, o potencial regenerativo do animal mutante é reduzido e o músculo não se recupera completamente.

Investigamos também os efeitos funcionais de duas mutações, p.R465W e p.E650K, em mioblastos imortalizados. Examinamos o potencial miogênico *in vitro*, a propriedade migratória e a capacidade de endocitose. Verificamos que o potencial miogênico destas células é afetado pelas mutações, porém de maneiras distintas. Mostramos também que ambas as mutações impactam negativamente na capacidade migratória dos mioblastos, o que em parte justifica as alterações no potencial miogênico dos mesmos. Por fim, verificamos que a capacidade endocítica em mioblastos é alterada a depender da mutação, o que indiretamente também pode afetar a capacidade de diferenciação miogênica.

Palavras-chave: miopatia centronuclear; dinamina 2; regeneração muscular; células-satélite.

Abstract

ALMEIDA, Camila de Freitas. **Skeletal muscle regeneration in *DNM2*-related centronuclear myopathy**. 2019. 155p. Ph.D. thesis in Sciences (Biology - Genetics) – Biosciences Institute, University of São Paulo, São Paulo, 2019.

The skeletal muscle has a remarkable regenerative capacity upon injury, due to the presence of the satellite cells, which remain quiescent in the tissue, but, when required, they are able to proliferate and form and/or repair myofibers. Moreover, satellite cells are important to muscle growth and maintenance. However, in many neuromuscular disorders, the amount, function, and proliferative capacity of these cells are impaired.

Centronuclear myopathies (CNM) are a group of muscle diseases characterized by generalized muscle weakness and myofibers with central nuclei. The autosomal dominant form (AD-CNM) is caused by mutations in the *DNM2* gene. Dynamin 2 protein is ubiquitously expressed and is involved in membrane remodeling, intracellular trafficking, and cytoskeleton dynamics. Therefore, the pathophysiological mechanisms are equally diverse e not completely understood, mainly the fact to be a muscle-specific disease.

In the present Ph.D. thesis, we sought to investigate the satellite cells in the context of centronuclear myopathy. For this, we used the mouse model KI-*Dnm2*^{R465W}, bearing the most frequent mutation found in human patients. Since in centronuclear myopathy there is no evident degenerative process ongoing, we induced muscle lesion by electrical shock, a protocol developed for this thesis, comparatively to cardiotoxin injection. We verified that the number of satellite cells in gastrocnemius muscle is reduced in the KI-*Dnm2*^{R465W} mouse in relation to wild-type animals. As a result, the regenerative potential of the mutant mouse is decreased and the muscle is not able to fully recover.

In addition, we investigated the functional consequences of two mutations, p.R465W and p.E650K, in immortalized myoblasts. We examined the myogenic potential in vitro, the migratory property, and the endocytosis capacity. We found that both mutations impact on the myogenic potential, but in different ways. We also show that both mutations impair the migratory capacity of myoblasts that justify, in parts, the alterations in their myogenic potential. Finally, we verified that the endocytosis capacity is affected in a mutation-dependent manner, which may also indirectly disturb the myogenic differentiation efficiency.

Keywords: centronuclear myopathy; dynamin 2; muscle regeneration; satellite cells.

I. Chapter 1 - Introduction

I.1. The skeletal muscle tissue

I.1.1. Function and structure

Humans have approximately 600 muscles in their bodies, accounting for 40 % of total body mass in adult. There are three main muscle types: cardiac, smooth and skeletal muscle. Striated skeletal muscle is the most common and contributes for many and diverse mechanical and metabolic activities, providing body sustenance, force generation and movement (FRONTERA; OCHALA, 2015).

The skeletal muscle is organized in a very ordered and hierarchical architecture. It is composed by bundles of myofibers (or muscle fibers) associated with connective tissue. Muscle fibers are post-mitotic and multinucleated cells, delimited by the sarcolemma - the plasma membrane - surrounded by a layer of connective tissue named endomysium. A group of muscle fibers is aligned to each other and packed by a layer of connective tissue, the perimysium. Finally, these bundles of myofibers are wrapped by the epimysium, forming the muscle (Figure 1).

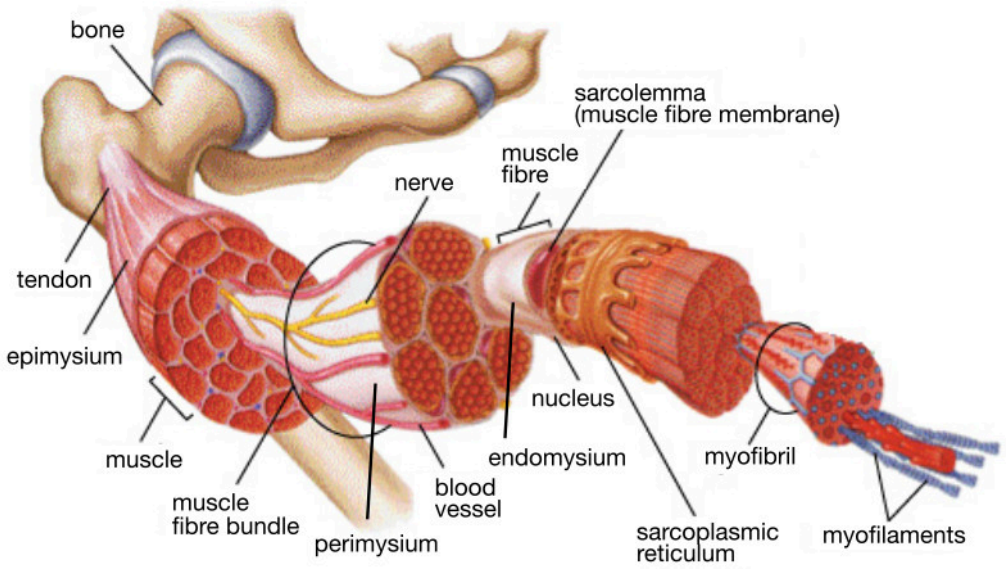

Figure 1 Skeletal muscle structure

The skeletal muscle is formed by bundles of myofibers, held together by layers of connective tissue and permeated with nerves and blood vessels. Nuclei are located at myofibers' periphery. The sarcoplasm is filled with myofilaments of contractile proteins. Adapted from *The Muscular System Manual: The Skeletal Muscles of the Human Body,* 3rd ed. Joseph E. Muscolino. Elsevier Health Sciences.

The intracellular organization of muscle cells is also unique. The sarcoplasm is filled with filaments of contractile proteins, the myofibrils, composed mainly by actin and myosin proteins. The myofilaments are formed by repeated segments of thick filaments of myosin and thin filaments of actin, named sarcomere, the muscle contractile unit. The sarcomere is the segment delimited by two Z-lines where actin thin filaments anchor. It contains an A-band (the superposition of actin and myosin) that separates two I-bands which are composed by actin. The H-band is formed only by myosin thick filaments (CLARK et al., 2002) (Figure 2).

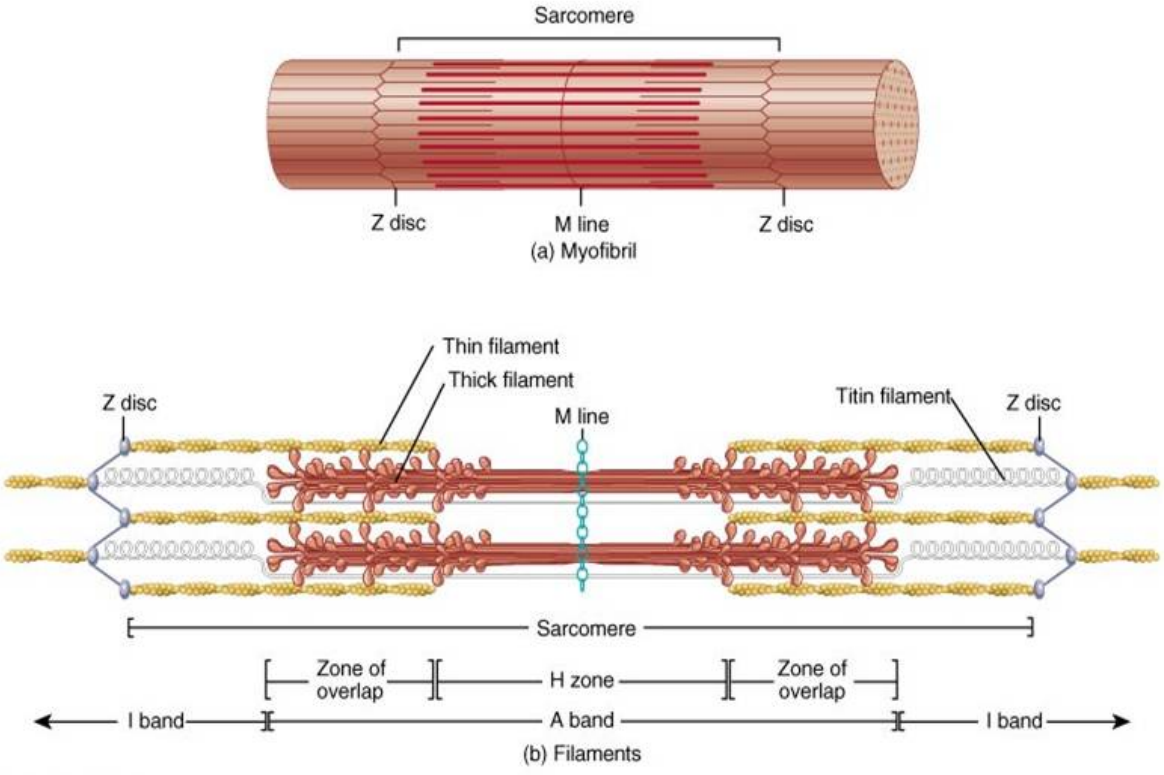

Figure 2 The sarcomere

Sarcomeres are formed of actin and myosin filaments that slide over each other during muscle contraction. Extracted from Tortora & Derrickson *Introduction to the human body 10th edition*

The sarcoplasmic reticulum (SR) surrounds the myofibrils and it exerts functions of transport and storage, regulating the flux of ions Ca^{2+} which are required for muscle contraction. Transversal invaginations of plasma membrane form a muscle-specific structure named T-tubule which together with the sarcoplasmic reticulum form the triad. This triad is essential for excitation-contraction coupling (ECC), the communication between the nervous system and calcium release. The action potential propagates through the T-tubule and reaches calcium channels on the SR promoting calcium release from SR and initiation of muscle

contraction (Figure 3). During contraction, the myosin filaments change conformationally through ATP hydrolysis, and slide over actin filaments, shortening the sarcomere and consequently the whole muscle fiber (FRONTERA; OCHALA, 2015).

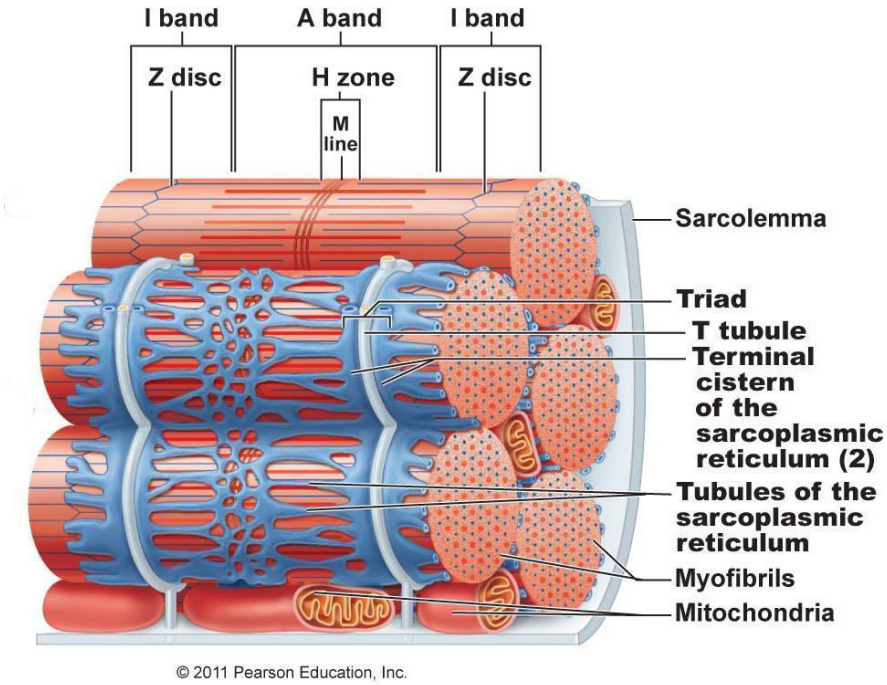

Figure 3 Transversal (T)-tubule and triad
Sarcolemma invaginations form the T-tubules which together with the sarcoplasmic reticulum form the triad. Extracted from Human anatomy and physiology 9e Elaine Marieb Katja Hoenh.

The muscle plasma membrane is also very specialized and it is the location of many muscle-specific protein complexes responsible for the connection between the intracellular cytoskeleton and the extracellular matrix (ERVASTI, 1993) required to stabilize the fiber and force transmission during contraction. Among these protein complexes, the dystrophin-glycoprotein complex (DGC) is composed of several proteins, including β-dystroglycan that is anchored to the plasma membrane and interacts with dystrophin in the cytoplasm and with the extracellular α-dystroglycan. Dystrophin interacts with the cytoskeletal actin and α-dystroglycan, and, finally attaches the whole complex to the extracellular laminin (IBRAGHIMOV-BESKROVNAYA et al., 1992; YOSHIDA; OZAWA, 1990).

I.1.2. Myogenesis and regulatory factors

Skeletal muscle development can be divided as follows: cell fate determination (myoblasts formation); myoblasts proliferation; alignment and fusion of myoblasts; myotube formation; myotube maturation and fiber formation (Figure 4).

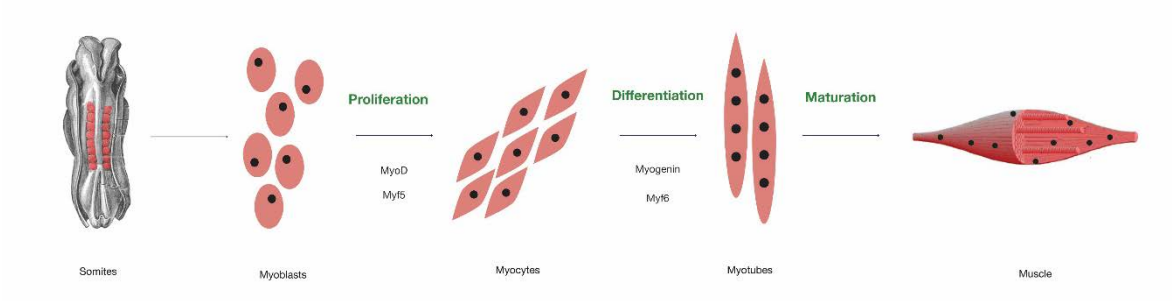

Figure 4 Skeletal muscle development

Skeletal muscle originates from somites. Myoblasts proliferate and express MYF5 and MYOD, then they start to differentiate and fuse; myogenin and MYF6 control the terminal differentiation.

Each step of muscle development is highly controlled by regulatory factors that act in a precise spatiotemporal order (PARKER; SEALE; RUDNICKI, 2003) (Figure 5). The myogenic precursors that give rise to muscles from trunk and limbs derive from somites, transient spheres of paraxial mesoderm flanking the neural tube and notochord in the embryo. Head and neck muscles have their origin in the anterior paraxial mesoderm, which does not form somites (BUCKINGHAM, 2017; CHAL; POURQUIÉ, 2017). During early steps of embryogenesis, the transcription factor PAX3 is responsible for the determination of cell fate, survival, self-renew and migration of muscle progenitor cells (BUCKINGHAM, 2017; HUTCHESON et al., 2009; MAGLI et al., 2013). Under the influence of the signaling factors SHH, WNT and BMP secreted by the notochord and neural tube, the somites transit into sclerotome (precursor of tendons, ribs and vertebral column) and dermomyotome from which cells delaminate and give rise to the dermis and myotome. It is at this point that the expression of myogenic regulatory factors (MRFs) is first detected (OTT et al., 1991). PAX3 is also directly or indirectly involved in the activation of the MRFs MYF5, MYOD, MRF4 (or MYF6) and MYOG (myogenin), which are the coordinators of the myogenic program, determining cell fate and regulating the terminal differentiation of committed cells (ASFOUR; ALLOUH; SAID, 2018; ZAMMIT, 2017). Then, myogenesis progresses through different sets of proliferation and differentiation, known as primary and secondary myogenesis.

In the primary myogenesis, the myofibers arise from PAX3+ progenitors in the dermomyotome (HORST et al., 2006; HUTCHESON et al., 2009), forming the templates for future myofibers. In secondary myogenesis, there is a downregulation of PAX3 and myogenic progenitors start to upregulate MRFs and express PAX7, which is a PAX3 orthologue, more active during post-natal growth and regeneration. The PAX7+ cells either fuse with each other or fuse to the preexisting myofibers, sustaining muscle growth in the fetal period (CHAL; POURQUIÉ, 2017; ONTELL; KOZEKA, 1984).

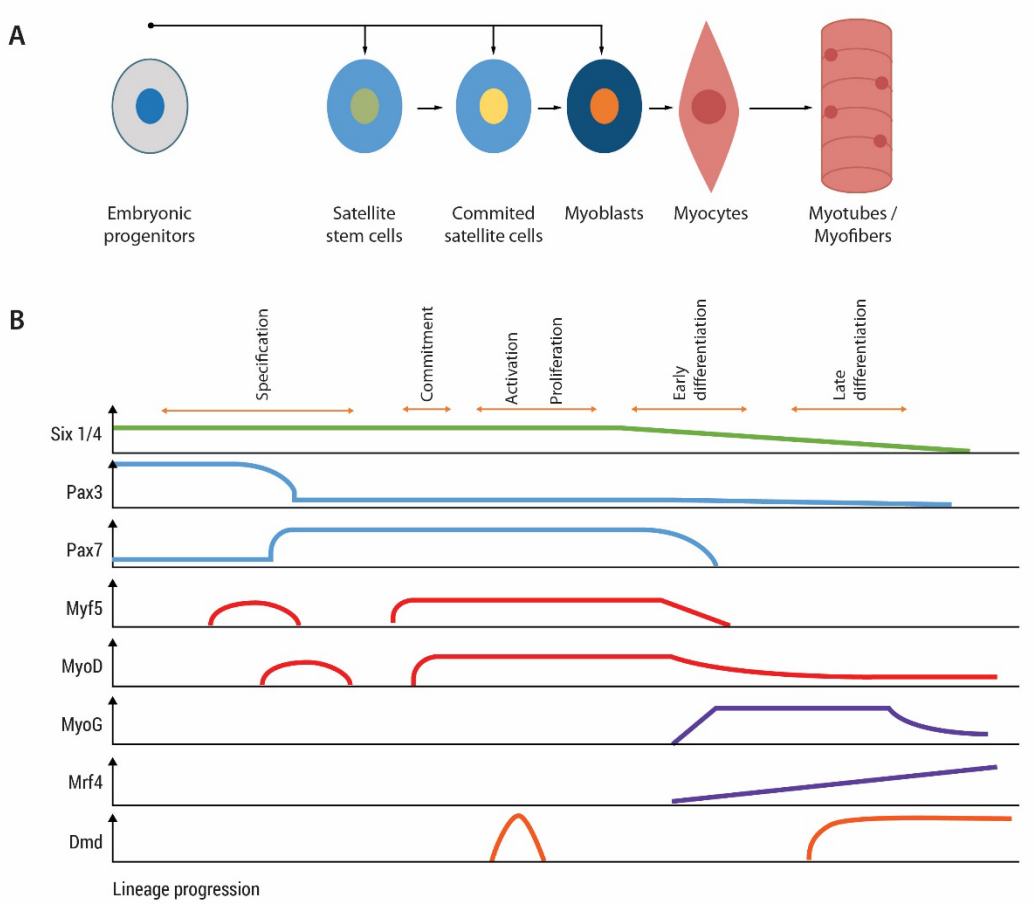

Figure 5 Cellular and molecular hierarchy of muscle development

(A) During development, an embryonic progenitor directly originates satellite stem cells, committed satellite cells, and myoblasts, which afterwards form a mature fiber. Some of them remain as satellite cells. In adult myogenesis, satellite cells can form myoblasts that will go through a similar process observed in development. (B) Expression of transcription factors involved in myogenesis. Extracted from Almeida et al, 2016.

The MRFs consist of the four muscle-specific transcription factors MYF5, MYOD, MYOG and Myogenic Regulatory Factor 4 (MRF4 or MYF6). They act in distinct phases of muscle development, regulating proliferation, cell cycle arrest and activation of muscle-specific genes for sarcomere assembly and terminal differentiation (ASFOUR; ALLOUH; SAID, 2018; HERNÁNDEZ-HERNÁNDEZ et al., 2017; ZAMMIT, 2017). The MRFs share a common protein

structure composed of three domains: an N-terminal cysteine/histidine-rich domain, a central region with a basic helix-loop-helix (bHLH) motif and a C-terminal serine/threonine-rich domain (OLSON, 1990). The bHLH domain leads DNA binding to the E-box consensus sequence CANNTG, found in promoter and enhancer sequences of many muscle-specific genes (BERKES; TAPSCOTT, 2005).

MYF5, MYOD, and MYF6 have overlapping functions as shown by distinct gene inactivation studies, that demonstrated that muscle formation is possible even in the absence of one of them. In *Myod*$^{-/-}$ mice, an elevation of *Myf5* expression compensates the role of *Myod*, forming apparently normal musculature (RUDNICKI et al., 1992). A similar situation is observed in *Myf5*-null mice (BRAUN et al., 1992). Mice mutant for *Myf6* develops a normal musculature with upregulation of myogenin expression, demonstrating that MYF6 acts as a negative regulator of myogenin (PATAPOUTIAN et al., 1995; ZHANG; BEHRINGER; OLSON, 1995).

In opposition, in myogenin-null mice, myogenesis fails, with the presence of only a few myofibers and predominance of undifferentiated myoblasts (HASTY et al., 1993; NABESHIMA et al., 1993; RAWLS et al., 1995), showing that myogenin is indispensable for late steps in embryogenesis, especially for the expression of myosin heavy chain and actin. Mice lacking both *Myf5* and *MyoD* have a complete absence of muscle formation, although *Myf6* being able to specify the myotome in early development, indicating that *Myf6* is also capable to initiate myogenesis (KASSAR-DUCHOSSOY et al., 2004; RUDNICKI et al., 1993). Double-mutant mice for *MyoD* and *Myf6* have a muscle phenotype similar to myogenin-null ones, despite normal expression of myogenin, suggesting that *MyoD* and *Myf6* have a redundant function and myogenin alone is insufficient for normal myogenesis (RAWLS et al., 1995).

Taken together, all these studies establish *MyoD* and *Myf5* important for myogenic determination, while myogenin and *Myf6* are more engaged with terminal muscle differentiation. The correct balance in the expression of all these four genes is required for complete and normal muscle formation.

I.1.3. Muscle regeneration and Satellite cells

Although being a post-mitotic tissue, skeletal muscle has a remarkable capacity to regenerate upon injury. This regenerative potential is mainly due to the presence of muscle-resident stem cells, named satellite cells. Satellite cells were described for the first time more than 50 years ago, in a seminal work by Mauro, residing between the plasma membrane and the basal lamina (MAURO, 1961).

Skeletal muscle repair can be divided into three phases: 1) degeneration/inflammation, defined by rupture and necrosis of myofibers and invasion of inflammatory cells; 2) repair phase, in which dead cells are phagocyted and new fibers and scar tissue start to be produced; 3) remodeling phase, when muscles fibers are reorganized, scar tissue is remodeled and muscle function is restored (TURNER; BADYLAK, 2012). The whole process is completed within weeks (Figure 6).

Muscle regeneration is triggered by the presence of myonecrosis that is characterized by segmented sarcoplasm about 24 hours post-injury. The lesion destroys the plasma membrane integrity, increasing ions calcium influx, activating autodigestion mechanisms (HUARD; LI; FU, 2002). Concomitantly, there is the infiltration of inflammatory cells, which exert many functions in all phases of regeneration (Figure 6) (GROUNDS, 2014).

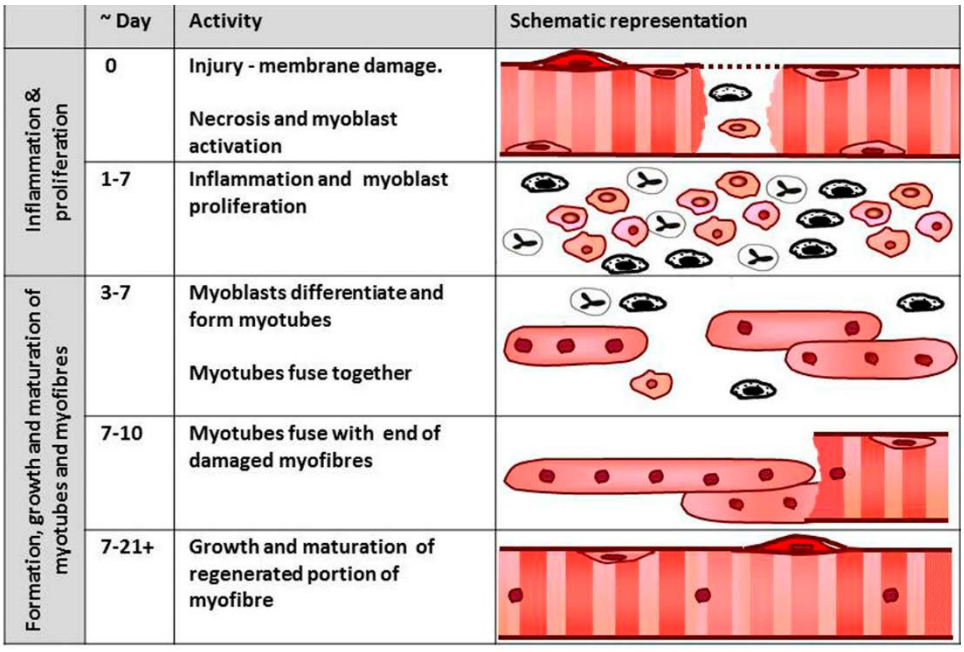

Figure 6 Cellular events in response to muscle injury
The injury is featured by membrane fragmentation. Within one day of damage, the first inflammatory cells appear. Myoblast proliferation starts at day one and continues up to day seven. Differentiation and fusion occur from days 3 to 10. Afterwards, inflammation decreases and the new myofibers maturate. Regenerated myofibers have their nuclei positioned at the central portion. Modified from Grounds, 2014.

Degeneration and inflammation develop within the first days after injury. The front of inflammatory cells includes macrophages, neutrophils, mast cells, and lymphocytes. They secrete enzymes and cytokines promoters of inflammation, but also other molecules that will work both on tissue cleaning from cellular debris and activation of muscle precursor cells and hence myogenesis (BRUNELLI; ROVERE-QUERINI, 2008; HUARD; LI; FU, 2002; KHARRAZ et al., 2013).

The muscular regeneration itself starts generally after the third day and requires the action of the satellite cells (SCs), the muscle stem cells, although other cell types can also contribute (PARKER; SEALE; RUDNICKI, 2003). Satellite cells originate during development as reservoir cells and are responsible for post-natal growth, maintenance, and repair of injuries caused both by exercise and disease (ALMEIDA et al., 2016). These cells represent from 3% to 11% of the total number of myonuclei in adult muscle, varying from species to species. Neonate mice have about 32% of nuclei of SCs, but this number drops to 5% in adult mice (ALLBROOK; HAN; HELLMUTH, 2007; BISCHOFF; HEINTZ, 1994).

SCs are recognized by their location and, more specifically, by the expression of many molecular markers, especially the transcription factor PAX7, the canonical satellite cell regulator (SEALE et al., 2000). Experiments in which Pax7-expressing cells were conditionally ablated in adult tissue showed impaired muscle regeneration after acute injury, highlighting the absolute necessity of these cells for muscle repair (LEPPER; PARTRIDGE; FAN, 2011; SAMBASIVAN et al., 2011).

In uninjured muscle, satellite cells stay in a quiescent state, characterized by inhibited cell division and undifferentiated morphology, controlled by several transcriptional, signaling and epigenetic pathways. Quiescent stem cells are marked by the presence of PAX7 and absence of MYOD and other markers related to cell proliferation (YOSHIDA et al., 1998; ZAMMIT et al., 2004). Generally, all the mechanisms are devoted to sustain PAX7 expression and repress MRFs expression.

NOTCH signaling pathway plays a major role in promoting SC quiescence, demonstrated by different works (BJORNSON et al., 2012; BUAS; KADESCH, 2010; CONBOY; RANDO, 2002; JIANG et al., 2014; MOURIKIS et al., 2012). Upon satellite cell activation, NOTCH rapidly declines. Experimental abrogation of NOTCH signaling leads SCs to differentiate,

without passing through proliferation and thereby leading to stem cell pool depletion. This result also demonstrates the role of NOTCH for self-renew (MOURIKIS et al., 2012). Ectopic expression of members of the NOTCH pathway showed an upregulation of PAX7, which in turn suppresses MYOD (WEN et al., 2012), thus reinforcing the role of NOTCH for the stability of quiescence. In its turn, NOTCH is regulated by the FOXO3 transcription factor, which stimulates NOTCH receptors expression, helping the maintenance of quiescence (GOPINATH et al., 2014).

In spite of all these mechanisms to keep satellite cells in a quiescent state in adult muscle, when required, they are promptly activated and start to proliferate in order to repair injured fibers and/or form new myofibers. The subsequent cellular and molecular steps are coordinated by transcription factors analogously to embryonic development, only with some specific differences (Figure 5) (GROUNDS, 2014; RUDNICKI et al., 2008).

The activation of SCs is a transient process: the damaged fibers as well as the inflammatory cells release growth factors, like fibroblast growth factor (FGF), tumor necrosis factor-α (TNF-α) and hepatocyte growth factor (HGF), known to activate signaling pathways related to cell cycle (LI, 2003; TATSUMI et al., 1998; YABLONKA-REUVENI; SEGER; RIVERA, 1999). The entry on S phase is stimulated by FGF2, through the stimulation of ERK1/2 pathway (JONES et al., 2001).

The rapid activation of SCs suggests that these cells are somewhat primed to respond as quickly as possible. MYOD and MYF5 are the first and most important regulators of SCs proliferation and differentiation once they are activated. Therefore, their expression must be actively repressed during quiescence, whereas quickly upregulated following activation. In fact, many mechanisms have been uncovered in this sense.

Although MYOD protein is undetectable in quiescent satellite cells, yet PAX7 and FOXO3 induce its mRNA expression. Then, its translation is somehow repressed. In practice, TTP (tristetraprolin), an mRNA destabilizing protein, drives *Myod* transcripts to mRNA decay (HAUSBURG et al., 2015). In addition, p38α and p38β MAPK, which are essential for SCs awakening, induce MYOD protein translation by inhibiting TTP and allowing cell cycle entry (JONES et al., 2005). By this, TTP ensures that MYOD is stable only when the satellite cell is activated.

Similarly, *Myf5* is also subjected to transcriptional and post-transcriptional control. The microRNA miRNA-31 was shown to sequestrate *Myf5* mRNA into cytoplasmic granules in quiescent SCs. Upon activation, these granules disassemble and *Myf5* mRNA is immediately available for strong protein expression (CRIST; MONTARRAS; BUCKINGHAM, 2012). *Myf5* gene transcription is regulated by CARM1 (a methyltransferase), which methylates PAX7 and leads it to associate with HMT (histone methyltransferase) complex and then induce *Myf5* transcription in activated SCs (KAWABE et al., 2012; MCKINNELL et al., 2008).

Following activation, SCs proliferates intensely to give rise to the new cells that will repair the lesion. Most of the satellite cells are a short-term repopulating cell (KUANG et al., 2007), while a subset is capable of long-term self-renew (GUREVICH et al., 2016; KUANG et al., 2007; ROCHETEAU et al., 2012). So, in order to maintain the stem cell pool, it is necessary that a fraction of cells return to the quiescent state and ensure a sufficient number of cells for future lesions. This is accomplished by some mechanisms discussed below.

Satellite cells can undergo either symmetric or asymmetric division. In asymmetrical divisions that occur in an apical-basal orientation, one cell daughter will commit to the muscle program (the one closer to the myofiber) and the other will remain quiescent (the one closer to the basal lamina). This asymmetry is achieved by the distribution of different factors between daughter cells. PAX7+ cells that never expressed MYF5 contributes to the stem cell reservoir, while PAX7+/MYF5+ cells preferentially differentiate (KUANG et al., 2007). PAX7+/MYOD- cells are self-renewing, PAX7+/MYOD+ cells are proliferative, while PAX7-/MYOD+ ones are committed to myogenic differentiation (HALEVY et al., 2004; OLGUIN; OLWIN, 2004; ZAMMIT et al., 2004). The same is valid for MYOG: PAX7-/MYOG+ cells progress through myogenic differentiation and PAX7+/MYOG- cells are destined to self-renew (YENNEK et al., 2014).

Still, self-renew can also occur by symmetrical divisions: *Wnt7a* drives symmetrical division of satellite cells in regenerating muscle, stimulating the expansion of both cells contributing to muscle repair and self-renew (LE GRAND et al., 2009).

Besides MRFs, other proteins are involved in satellite cell fate determination. Sprouty1 (SPRY1) is a marker of quiescent satellite cells (FUKADA et al., 2007) and its disruption in PAX7+ satellite cells impairs their return to quiescence and consequently, hampers the

reestablishment of the SC pool after injury by 50%. During the proliferation phase, *Spry1* expression is reduced, but those cells meant to go back to quiescence, reacquire *Spry1* expression and inhibit cell cycle (SHEA et al., 2010). With aging, DNA methylation suppresses *Spry1*, thereby reducing the ability to return to quiescence and to the stem cell pool in aged muscles (BIGOT et al., 2015).

It was found that dystrophin, a protein generally expressed in mature myofibers, participates in cell polarity definition and that dystrophin deficiency leads to a loss in the number of asymmetrical divisions and ultimately, to a reduced stem cell pool and poor regeneration (DUMONT et al., 2015). More recently, epidermal growth factor receptor (EGFR) and aurora kinase A (Aurka) were identified as regulators of asymmetrical divisions. EGF treatment of dystrophin-deficient mouse rescued the SC polarity and enhanced regeneration, helping to restore muscle strength (WANG et al., 2019).

I.1.3.1. Induced lesion approaches

Generally, the degenerative and regenerative phases following an injury are very similar among the different types of muscle and various causes of the lesion, but the extension and dynamic of each phase can vary according to the type of muscle, the source of lesion or the animal model (IRINTCHEV; WERNIG, 1987; LEFAUCHEUR; SÉBILLE, 1995; MITCHELL; MCGEACHIE; GROUNDS, 1992; PAVLATH et al., 1998).

Currently, there are many available techniques to induce muscle degeneration in animal models to the study of muscle regeneration in healthy and diseased conditions. They are divided into three classes of agents: myotoxins, such as cardiotoxin (CTX), notexin (NTX); chemicals, like barium chloride ($BaCl_2$); and physical, like denervation, transplantation, irradiation, crush and freeze injury. Generally, all these injury models induce a phase of intense necrosis followed by muscle regeneration and tissue remodeling. Many studies take advantage of these models to drive general conclusions about muscle regeneration.

However, each model has its particularities and may impact the muscle differently, inducing a different regeneration process too. In *Myf5*-null mice injured by freeze injury and cardiotoxin injection, both methods helped to identify a perturbed regeneration in this mouse

model. Nonetheless, one specific role of *Myf5* was identified only in the freeze-injury model. Although the loss of satellite cells at the damaged area by freeze-injury was comparable to CTX injection, in the latter the satellite cells were more evenly distributed. So, in the freeze injury model, the cells contributing to regeneration must come from long distances, which showed to be more difficult in the absence of *Myf5* (GAYRAUD-MOREL et al., 2007), underscoring the need to carefully take in account the method of lesion employed.

Cardiotoxin injection is the most popular method, probably because of the easiness to employ this method, reproducibility, consistency and perhaps tradition in literature, although all its biological properties are unknown. CTX is a protein kinase C-specific inhibitor isolated from snake venom; it appears to provoke the depolarization and contraction of muscle fibers, disrupting the membrane and to lyse different types of cell. But there are probably potential side effects of this toxin still to be determined (CHARGÉ; RUDNICKI, 2004).

By comparing four different lesion methodologies (freeze, NTX, CTX, and $BaCl_2$), a study found that despite causing similar necrosis and complete recovery one month after, significant differences were detected between them. For example, the injection of CTX, NTX, and $BaCl_2$ stimulated monophasic necrosis, followed by regeneration, while the freeze-injury model presented an asynchronous regeneration, with all regeneration phases present at the same time (HARDY et al., 2016).

Satellite cells population was also differently affected. In freeze injury, they observed a loss of 95% of satellite cells, while in the other methods, about 60% of the cells survived 18 hours after injection. Three months post-injury, in the freeze and $BaCl_2$ models the SCs numbers returned to normal counts, in contrast to myotoxins models, in which the number of SCs increased substantially (HARDY et al., 2016). Therefore, despite in all models, the muscle regeneration is successful, the time course can vary significantly and this must be considered in the experimental design.

I.2. Neuromuscular disorders

The term *neuromuscular disorders* encompasses a vast group of disorders affecting primarily the peripheral nerves and/or the muscle fibers. They are divided into groups and subgroups: spinal amyotrophies, neuropathies, congenital muscular dystrophies (several subtypes), progressive muscular dystrophies (many forms), myotonic dystrophy, metabolic myopathies, congenital myopathies and others (LAING, 2012; REED, 2002). A complete list of all the genes identified in neuromuscular disorders can be found at http://www.musclegenetable.fr/.

This present work was done on a congenital myopathy, so the next sessions are dedicated to this topic.

I.2.1. Congenital myopathies

The congenital myopathies comprise a group of genetic skeletal muscle disorders, typically present at birth or in early childhood and characterized by hypotonia and skeletal muscle weakness with stable or slowly progressive clinical course. Late onset or adult forms were also identified. The clinical presentation is heterogeneous and not certainly benign, including severe forms and even fatal ones. Five major categories are recognized: nemaline myopathy, core myopathies, myosin storage myopathy, congenital fiber disproportion and centronuclear myopathies (NORTH et al., 2014).

Traditionally, the congenital myopathies have been identified by specific histological findings on muscle biopsy (NORTH et al., 2014; REED, 2002). The genetics includes X-linked and autosomal inheritance, both dominant and recessive, involving many different genes. The same gene can be related to different forms, as well as one form can be caused by multiple genes.

In nemaline myopathy, the most common histological finding is the presence of rods, structures formed mainly by alpha-actinin and desmin. Seven genes were associated with this form and they code for components of thin filaments of the sarcomere. Thus, the mutations lead to a loss in force generation during contraction (LAING, 2012; NANCE et al., 2012).

Central core myopathies are characterized by the presence of areas without oxidative and glycolytic activity, reflecting the absence of mitochondria, and central foci of abnormal

myofibrils. Several genes were associated with these diseases, but the most frequent ones are *RYR1* and *SEPN1* (NANCE et al., 2012; NORTH et al., 2014; REED, 2002).

Myosin storage myopathy is characterized by the accumulation of ATPase and hyaline bodies in type I muscle fibers (DYE et al., 2006). In congenital fiber-type disproportion myopathy, there is a relative type 1 fiber hypotrophy compared to type 2 fibers (KISSIEDU; PRAYSON, 2016).

The centronuclear myopathies will be detailed in the next topic.

I.2.1.1. Centronuclear myopathies

Congenital myopathies with central nuclei or centronuclear myopathies (CNM) are classically identified by histological findings on muscle biopsies, mainly by the presence of many muscle fibers with nuclei arranged in rows in the central portion of the fiber without evident signs of degeneration or regeneration and in absence of other histopathological features of congenital myopathies (ROMERO, 2010).

The clinical presentation is very heterogeneous: patients present from severe hypotonia at birth to mild late-onset muscle weakness. The first group of patients with moderate and mild forms were identified in the 60s, and after, the severe cases of male newborns, with X-linked inheritance (ROMERO; BITOUN, 2011).

Three main forms are identified according to the inheritance pattern: recessive X-linked caused by mutations in *MTM1* (myotubularin) (XL-MTM) (LAPORTE et al., 1996; THOMAS; WALLGREN-PETTERSSON, 1996); autosomal recessive caused by *BIN1* (amphiphysin) mutations (AR-CNM) (NICOT et al., 2007); and autosomal dominant form due to *DNM2* (dynamin 2) mutations (AD-CNM) (BITOUN et al., 2005) and *BIN1* (BÖHM et al., 2014). *RYR1* mutations (mainly autosomal recessive) are also found (BEVILACQUA et al., 2011; WILMSHURST et al., 2010). Very rare cases were linked to *CCDC78* (MAJCZENKO et al., 2012) and *MTMR14* (TOSCH et al., 2006) are associated with late-onset autosomal dominant mutations. More recently, the genes *TTN* (CARMIGNAC et al., 2007; CEYHAN-BIRSOY et al., 2013) and *SPEG* (AGRAWAL et al., 2014) were found mutated in recessive cases.

Epidemiological data about CNM is limited, but the estimated incidence of all CNM forms is of 24 per one million births. Most of the cases are of XL-MTM (69.7%), and the remaining 30.3% is distributed among the other genes: 8.3% DNM2, 8.3% RYR1, 2.8% BIN1 and 11% of unknown genetic origin. Based on survival curves, the estimated prevalence is of 45% for XL-MTM and 55% for all no-XL-MTM forms (data calculated for the United States, Europe, Japan, and Australia). Translating into numbers, it means that currently exist approximately 4600 CNM patients in these regions (VANDERSMISSEN et al., 2018).

i. X-linked myotubular myopathy (XL-MTM)

X-linked myotubular myopathy is caused by *MTM1* mutations and affects mainly male newborns (LAPORTE et al., 1996), but there is an increasing number of female carriers manifesting symptoms reported on literature (SAVARESE et al., 2016). The term myotubular was coined because of the resemblance of myofibers with immature myotubes present during development (SHER et al., 1967; SPIRO, 1966). This also raised the hypothesis that these fibers never completed the maturation process, but this was never proved (ROMERO, 2010) since *Mtm1* knock-out mice have a normal muscle development and maturation (BUJ-BELLO et al., 2002).

Affected males usually present severe symptoms: hypotonia at birth, external ophthalmoplegia, generalized muscle weakness, and respiratory failure, leading to death in the first year of life. They require ventilatory support and a feeding tube. Individuals presenting milder symptoms and receiving intense medical care can survive until adolescence (JUNGBLUTH; WALLGREN-PETTERSSON; LAPORTE, 2008; ROMERO; BITOUN, 2011).

In the muscle biopsy, it is observed a high number of small fibers, large central nuclei, and predominance of type I fibers (Figure 7A and B). Central areas stain darker for periodic acid Schiff (PAS) and reduced staining for myofibrillar ATPase reaction. The reaction for oxidative enzymes activity shows increased activity in the central region of fibers, surrounded by a pale halo at the periphery (ROMERO, 2010; ROMERO; BITOUN, 2011; SEWRY; WALLGREN-PETTERSSON, 2017) (Figure 7C).

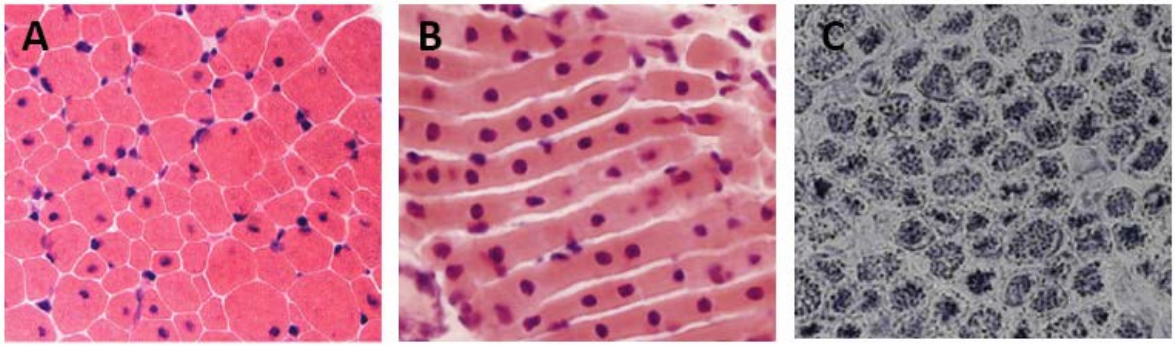

Figure 7 XL-MTM histopathology

Hematoxylin and eosin staining in (A) transversal section and (B) longitudinal section, showing central nuclei and small size fibers. (C) Oxidative enzymes staining highlights fibers with a dark central region, frequently surrounded by a pale peripheric halo. Modified from Romero, 2010 and Sewry and Wallgren-Petterson, 2017.

MTM1 gene has 15 exons and more than 300 mutations were already described, leading to absence or reduced activity of the protein (BIANCALANA et al., 2003; HERMAN et al., 2002; LAPORTE et al., 2000; TSAI et al., 2005). *MTM1* gene codes for myotubularin, a phosphoinositide phosphatase involved in phosphoinositides metabolism and trafficking (BLONDEAU et al., 2000).

Myotubularin acts in a broad range of cellular processes, particularly membrane trafficking. MTM1 dephosphorylates PI3P and PI3,5P2 and produces PI and PI5P, important second messengers in membrane trafficking and endocytosis. It was suggested that PI3P may play a role during myogenesis and cell differentiation (TAYLOR; MAEHAMA; DIXON, 2002).

Myotubularin has also been involved in endosomal trafficking (TSUJITA et al., 2004), excitation-contraction coupling (AL-QUSAIRI et al., 2009), in functions at the neuromuscular junction (DOWLING et al., 2012), autophagy (FETALVERO et al., 2013), cytoskeletal organization (HNIA et al., 2011) and satellite cell proliferation and survival (LAWLOR et al., 2012). The depletion of *Mtm1* expression in the muscles of adult mice causes a phenotype of myotubular myopathy, showing that myotubularin is also necessary for proper muscle function in adult muscle and not only for development (JOUBERT et al., 2013). This diversity of functions of myotubularin could be an explanation for the gravity of the disease.

ii. Autosomal recessive centronuclear myopathy (AR-CNM)

The classical autosomal recessive form of CNM is due to *BIN1* mutations. *RYR1, TTN, CCDC78, MTMR14* and *SPEG* mutations cause a CNM-like phenotype. Cases related to

mutations in these genes are sparse, except for *RYR1* mutations which are more frequently identified in CNM-like patients.

BIN1 codes for amphiphysin 2, a ubiquitous protein, mostly expressed in brain and muscles. Only a few cases have been described with recessive *BIN1* mutations (CLAEYS et al., 2010; NICOT et al., 2007). The clinical features can be positioned as intermediaries between XL-MTM and AD-CNM forms (ROMERO, 2010). Patients present delayed motor milestones, difficulties to run, walk and climb stairs. Muscular atrophy, diffuse weakness, ptosis, facial diplegia, and ophthalmoplegia are common features (ROMERO; BITOUN, 2011). Muscle biopsies show a predominance of centronuclear small type I fibers. By oxidative histochemical staining, its observable fibers with a central clear zone with a dark surround (ROMERO, 2010; ROMERO; BITOUN, 2011) (Figure 8). Amphiphysin 2 plays a role in T-tubule organization and in muscle maturation and mutations disrupt these properties (COWLING et al., 2017; NICOT et al., 2007).

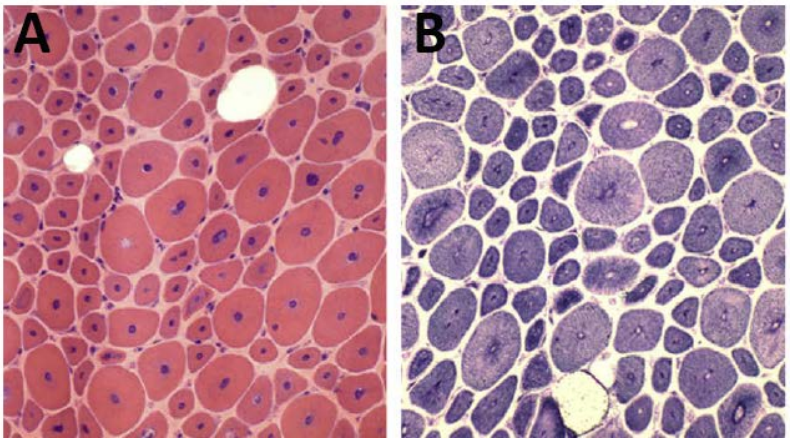

Figure 8 AR-CNM histopathology
(A) Hematoxylin and eosin staining in transversal section showing central nuclei, small rounded fibers and accumulation of endomysial tissue. (B) Oxidative enzymes staining reveals a central zone with dark border corresponding to the nuclear area. Modified from Romero, 2010.

iii. Autosomal dominant centronuclear myopathy (AD-CNM)

The clinical spectrum in the autosomal dominant form of centronuclear myopathy is wide. It varies from severe sporadic forms manifesting from birth (BITOUN et al., 2007), to mild forms with childhood and adolescence-onset (BITOUN et al., 2005), but the most frequent are the late-onset forms. AD-CNM phenotype can include delayed motor milestones,

facial and muscular weakness, ptosis and ophthalmoplegia (FISCHER et al., 2006; HANISCH et al., 2011). Muscle weakness progresses slowly and for most patients; cardiac and respiratory functions are normal. Loss of ambulation may occur only after the fifth decade of life (FISCHER et al., 2006).

In severe cases of neonatal onset CNM, the patients present generalized weakness, hypotonia, facial weakness with open mouth, ptosis and ophthalmoplegia (BITOUN et al., 2007), the progression is also slow (MELBERG et al., 2010; SUSMAN et al., 2010), but it can be fatal (JUNGBLUTH et al., 2010). A strength improvement can occur during childhood (SUSMAN et al., 2010); however, some children develop respiratory problems later (BITOUN et al., 2007; MELBERG et al., 2010).

The most prominent histological features are the central location of many nuclei, no signs of degeneration/regeneration, predominance of type I small fibers, hypotrophy of slow muscle fibers, slight deposition of endomysial connective tissue, and the radial distribution of sarcoplasmic strains from the central nuclei, identified by nicotinamide adenosine dinucleotide-tetrazolium reductase staining (NADH-TR) (JEANNET et al., 2004; SEWRY; WALLGREN-PETTERSSON, 2017) (Figure 9).

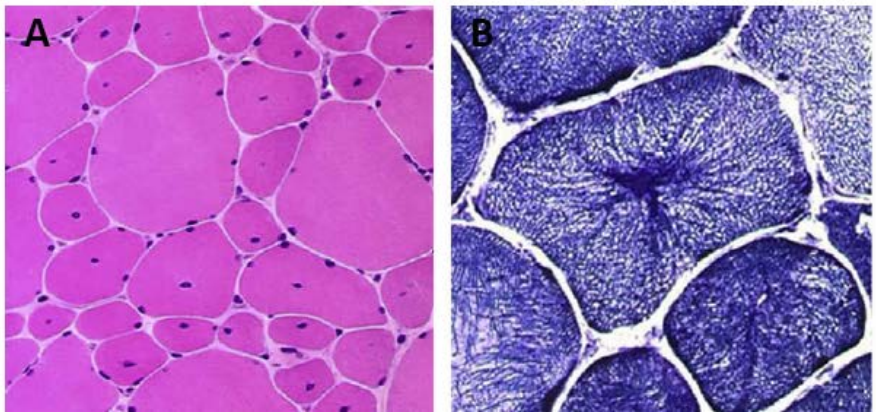

Figure 9 AD-CNM histopathology

(A) Hematoxylin and eosin staining in transversal section showing central nuclei in small fibers (B) Oxidative enzymes staining shows radial sarcoplasmic strands. Modified from Romero, 2010.

To date, 28 mutations were found in the *DNM2* gene as causative of AD-CNM (Figure 10). In addition, one mutation (p.Phe379Val) was found in homozygous state, causing a lethal congenital syndrome (KOUTSOPOULOS et al., 2013). A set of ten mutations located in the

middle domain cause Charcot-Marie Tooth (CMT) neuropathy that affects motor and sensory nerves, which the main feature is impaired myelination of neurons (SIDIROPOULOS et al., 2012). Hereditary spastic paraplegia is a third phenotype related to *DNM2* mutation (SAMBUUGHIN et al., 2015). There is no overlap between AD-CNM and CMT mutations.

Genotype-phenotype correlations are not clear, except for de novo mutations on the C-terminal portion of the PH domain: all are associated with severe neonatal phenotypes (BITOUN et al., 2007). For some mutations, a classification into three distinct classes was proposed: p.Ala618Thr and p.Ser619Trp/Leu are linked to the severe and neonatal onset phenotype; p.Glu368Lys is associated with intermediate clinical manifestation; and p.Arg369Trp, p.Arg465Trp, p.Arg522His/Cys and p.Arg523Gly mutations are present in patients with milder clinical manifestations (BÖHM et al., 2012).

For a long time, mutations only in *DNM2* were found in AD-CNM. However, surprisingly in 2014, Böhm et al. identified five new heterozygous mutations in *BIN1* in five families with AD-CNM, in which no *DNM2* mutation had been found (BÖHM et al., 2014).

The subjacent pathological mechanisms of AD-CNM and CNMs, in general, are not completely understood, but some studies have been contributing to relevant conclusions about the molecular basis of this disease. In the next session, dynamin 2 features and the pathophysiological mechanisms proposed for the CNMs will be detailed.

I.2.2. Dynamin 2 and Pathophysiological mechanisms in CNM

i. The protein dynamin 2 and its functions

Human *DNM2* gene is located on chromosome 19, has 114 kb, divided into 22 exons and produces, by alternative splicing, four main isoforms. The *DNM2* transcript is ubiquitously expressed, but it is more abundant in the brain and skeletal muscle (DIATLOFF-ZITO et al., 1995; DURIEUX et al., 2010a).

The dynamin superfamily embodies an assortment of multi-domain large GTPases, which exert a broad range of cellular functions, mainly vesicle formation and trafficking. Dynamin 2 is one of the three classical dynamins and is formed by five distinct domains. The

N-terminal GTPase domain makes the hydrolysis of GTP (CHEN et al., 2004), while the middle domain (MD) participates in DNM2 self-assembly and in conformational change induced by GTP hydrolysis (SMIRNOVA et al., 1999). The pleckstrin homology (PH) domain binds to phosphoinositides at the plasma membrane, directing dynamin to the membrane (KLEIN et al., 1998). The GTPase Enhancing Domain (GED) is proposed to participate in DNM2 self-oligomerization (SEVER; MUHLBERG; SCHMID, 1999). Finally, the proline-rich domain (PRD) interacts with the SH3 domains of the many proteins with which DNM2 interplays (HEYMANN; HINSHAW, 2009) (Figure 10).

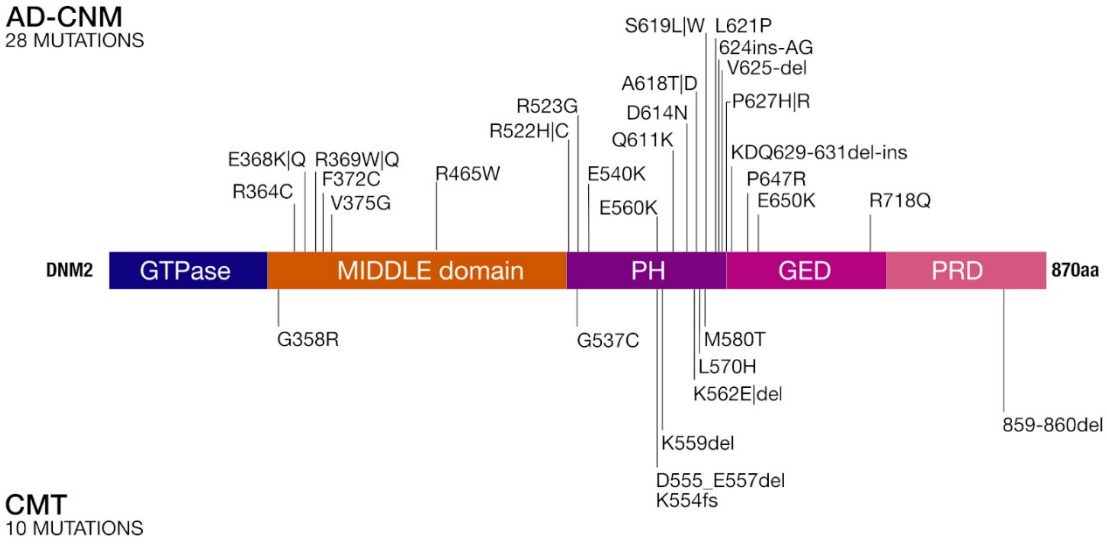

Figure 10 Dynamin 2 structure and mutations.

Dynamin 2 is formed by five conserved structural domains: an N-terminal GTPase, a middle domain, a pleckstrin homology domain that binds phosphoinisitides at the membrane, a GTPase effector (GED) domain that promotes the self-assembly and GTP hydrolysis, and a proline-rich domain (PRD) that binds to several SH3-domain containing proteins. To date, 28 mutations were reported as causative of CNM and 10 mutations are the cause of CMT.

The first property of dynamins to be discovered was its ability to promote membrane fission (ANTONNY et al., 2016). This was first observed in *Drosophila shibire* mutants in which dynamin mutation led to the accumulation of vesicles bound to the plasma membrane (VAN DER BLIEK; MEYEROWRTZ, 1991). Subsequently, dynamin 2 was proved to be a facilitator of clathrin-coated vesicles budding and thus in clathrin-mediated endocytosis and vesicle trafficking in general (METTLEN et al., 2009). DNM2 oligomerizes into helical polymers around

the neck of the nascent vesicle, and by GTP hydrolysis changes its conformation, constricting the plasma membrane and scissoring it (Figure 11).

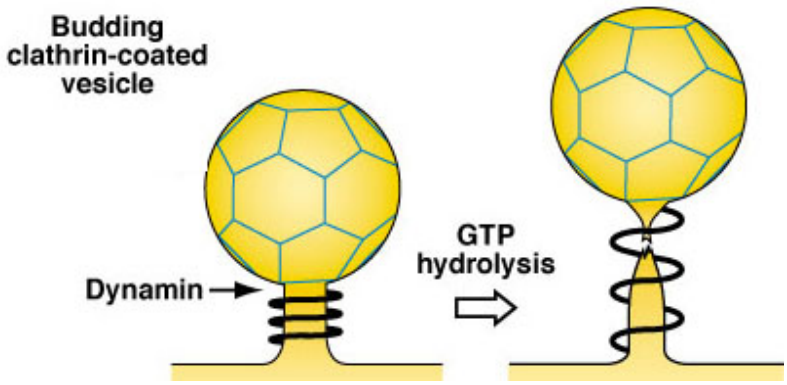

Figure 11 Dynamin 2 in endocytosis
Dynamin 2 is a facilitator of clathrin-coated vesicles budding. It oligomerizes into helical polymers around the neck of the nascent vesicle, and by GTP hydrolysis changes it conformation, constricting the plasma membrane and scissoring it.

DNM2 also plays a role in clathrin-independent endocytosis, intracellular trafficking of membranes, exocytosis, actin and microtubule dynamics, the cohesion of the MTOC (MicroTubule Organizing Centre), and apoptosis (DURIEUX et al., 2010a).

In figure 12 are represented the diverse cellular compartments in which DNM2 had been found playing a role (Figure 12). This gives an idea about the complexity that might be involved in the pathophysiological mechanisms underlying the dominant centronuclear myopathy and why there is still a lot to unravel about the functions of dynamin 2 for all tissues, especially for the skeletal muscle.

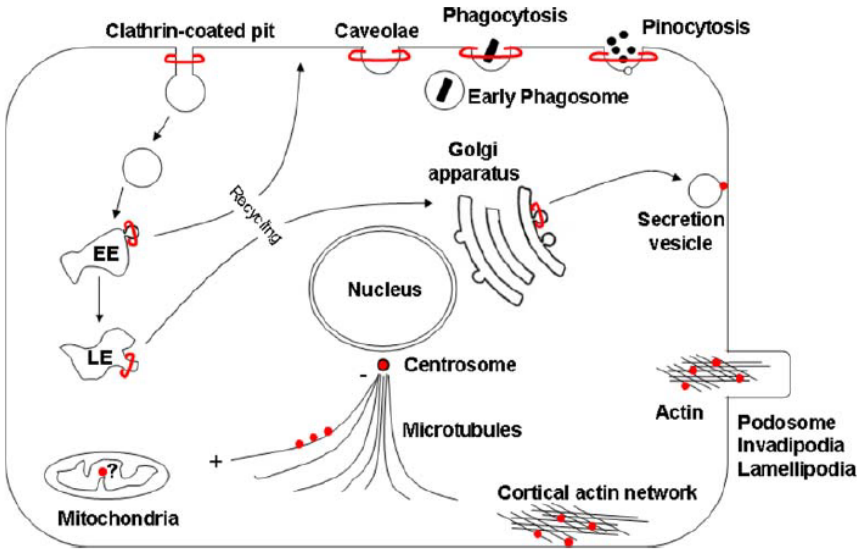

Figure 12 DNM2 functions
The multiple cellular localizations reported for DNM2 are represented in red.EE: early endosome; LE: late endosome. Extracted from Durieux et al, 2010.

The specific roles of dynamin in skeletal muscle are not yet determined, but the localization of DNM2 in muscle cells can give some clues. DNM2 localizes at the plasma membrane, the I-band, and the perinuclear regions. It also partially co-localizes with the microtubule network, at the neuromuscular junction and nearby sarcomeric Z-disks close to triads (COWLING et al., 2011; DURIEUX et al., 2010b) where it participates in T-tubule organization (TINELLI; PEREIRA; SUTER, 2013).

In the next session, the muscle-specific roles of dynamin 2 that have been revealed from studies of DNM2 mutants will be exposed.

ii. Pathophysiological hypotheses

The heterogeneity seen in the genetics, histopathology and clinical features in CNMs is also reflected in the variety of hypotheses raised to explain the development of these disorders. The three main proteins implicated in the CNMs – myotubularin, dynamin 2 and amphiphysin 2 – are involved in membrane remodeling and trafficking issues, which are relevant to cellular processes like endocytosis, intracellular vesicle trafficking, and autophagy. The impairment of these functions would consist of a major mechanism common to all the CNMs (COWLING et al., 2012), besides more specific mechanisms restrained to each form.

Studies performed in different animal models for centronuclear myopathies have suggested several pathogenic mechanisms, including triad abnormalities (AL-QUSAIRI et al., 2009; DOWLING et al., 2009; TOUSSAINT et al., 2011), calcium homeostasis deregulation (FRAYSSE; GUICHENEY; BITOUN, 2016; KUTCHUKIAN et al., 2017), defects in neuromuscular junction (DOWLING et al., 2012; GIBBS et al., 2013; ROBB et al., 2011), clathrin-mediated endocytosis impairment (BITOUN et al., 2009), actin kinetics impairment (GONZÁLEZ-JAMETT et al., 2017) mitochondria and desmin cytoskeleton alterations (FRANCK et al., 2019; HNIA et al., 2011), and satellite cells defects (LAWLOR et al., 2012).

The localization of DNM2 is altered in mutant DNM2 models, with cytosolic accumulation in vitro (JAMES et al., 2014; KENNISTON; LEMMON, 2010; KIERDASZUK et al., 2013; KOUTSOPOULOS et al., 2011; LIU; LUKIYANCHUK; SCHMID, 2011) and in vivo (CHIN et al., 2015; DURIEUX et al., 2010b; GONZÁLEZ-JAMETT et al., 2017). In fibroblasts and muscle from CNM-DNM2 patients, there is a normal expression of both mRNA and protein of the mutated and wild-type dynamin 2 (BITOUN et al., 2005, 2009; ECHANIZ-LAGUNA et al., 2007; KIERDASZUK et al., 2013; KOUTSOPOULOS et al., 2013) that favors for a dominant-negative effect of the heterozygous mutations, resulting in loss of function. Nonetheless, some studies point out that several DNM2 mutations increase oligomer stability and GTPase activity (KENNISTON; LEMMON, 2010; WANG et al., 2010). Furthermore, overexpression of wild-type or mutant-DNM2 leads to a CNM-like phenotype in mice and alterations in T-tubules in Drosophila (CHIN et al., 2015; COWLING et al., 2011; LIU et al., 2011). Therefore, some authors hypothesize that DNM2 mutations imply a gain of function effect. Nevertheless, the debate is ongoing and it is still not possible to draw unambiguous conclusions.

Both BIN1 and DNM2 participate together in the endocytic process, suggesting endocytosis impairment as a pathogenic consequence in autosomal CNM. Indeed, the impairment in clathrin-mediated endocytosis was reported in patient-derived fibroblasts with DNM2 mutations (BITOUN et al., 2009; KOUTSOPOULOS et al., 2011), however, this was not yet demonstrated in human myoblasts. In opposition, other studies did not confirm these observations (LIU; LUKIYANCHUK; SCHMID, 2011; SIDIROPOULOS et al., 2012). And it still obscures how the impairment of endocytosis has a muscle-specific impact since it is a ubiquitous cellular process. The impact of DNM2 mutations on other membrane trafficking processes, like in the endosomal pathway and exocytosis have not been explored either.

On fibroblasts transfected with DNM2 mutants, it was observed a decrease on activation of MAPK ERK1/2 pathway by EGF stimulation (BITOUN et al., 2009), a process known to be dependent on endocytosis and DNM2 (ANDRESEN et al., 2002; KRANENBURG; VERLAAN; MOOLENAAR, 1999). The same was not true for CNM patient-derived fibroblasts, but this needs to be clarified on muscle cells (BITOUN et al., 2009).

Alterations in the autophagy pathway have also been associated as a mechanism shared by CNMs (AL-QUSAIRI et al., 2013; FETALVERO et al., 2013). Autophagy is important to the degradation of defective proteins and organelles, infection defense and adaptation to changes in metabolism (MIZUSHIMA, 2007; SANDRI et al., 2013). Autophagy is particularly important for neurons and muscles, since their high energy demand, and, together with the ubiquitin-proteasome system, autophagy acts in the pathogenesis of muscle atrophy (SANDRI et al., 2013). Autophagy pathway involves several steps, carefully regulated, starting with autophagosome assembly, which fuses with lysosomes, resulting in the final degradative structures, the autolysosomes (MIZUSHIMA, 2007). Autophagy has emerged as an important primary and secondary mechanism for many neuromuscular diseases, including the CNMs (MERLINI; NISHINO, 2014).

Considering the links between endocytic and autophagic pathways, it was also expected to find alterations of autophagy in CNM-DNM. In fact, in the heterozygous mouse model for CNM-DNM2, it was demonstrated an upregulation of genes related to ubiquitin-proteasome and autophagy pathways (DURIEUX et al., 2010b). The homozygous animals showed an even pronounced phenotype regarding the autophagy, manifesting increased glycogen storage, hepatomegaly, hypoglycemia and early death (DURIEUX et al., 2012).

Dynamins have a critical role in actin network by elongating (GU et al., 2010), remodeling (MOOREN et al., 2009) and stabilizing (YAMADA et al., 2013) actin filaments. Dynamin 2 has emerged as a player in actin network remodeling because of its oligomerization and GTPase properties and its interactions with actin-related proteins. There are three actin isoforms in skeletal muscle: alpha-actin in the composition of the sarcomere, and beta- and gamma-actins present in other cytoskeletal elements that regulate trafficking and stabilization of plasma membrane proteins (KEE; GUNNING; HARDEMAN, 2009). Both dynamin 2 and gamma-actin localize in areas of intense membrane remodeling and trafficking (COWLING et

al., 2011; DURIEUX et al., 2010b; PAPPONEN et al., 2009). Additionally, mice lacking gamma-actin in muscles develop a CNM-like phenotype, suggesting a link between actin and centronuclear myopathy pathogenesis (SONNEMANN et al., 2006).

Recently, it was shown that DNM2 mutations expressed in human-derived myoblasts suppress *de novo* actin polymerization and insulin-induced insertion of GLUT4 – a glucose receptor – into the plasma membrane and its endocytosis. Moreover, myofibers isolated from the knock-in mouse model of CNM-DNM2 exhibit the same effects seen on human transfected myoblasts. Taken together, these findings confirm a prominent role of DNM2 in actin cytoskeleton dynamics and consolidate one more layer for the understanding of CNM pathophysiology (GONZÁLEZ-JAMETT et al., 2017).

The middle domain of DNM2 is essential for its centrosomal localization and binding to gamma-tubulin (THOMPSON et al., 2004) and it is where most of the mutations concentrate in. DNM2 mutants have abnormal centrosomal labeling in fibroblasts, so the mutations could be hindering its proper localization to the main MTOC or its interaction with gamma-tubulin (BITOUN et al., 2005).

In addition, microtubule and actin networks play a central role in nuclear positioning in muscle cells (MORRIS, 2003). Nuclear positioning in skeletal muscle is a sophisticated process; during fiber maturation, nuclei must be precisely moved, distributed and organized at the fiber's periphery (BRUUSGAARD et al., 2003; CADOT; GACHE; GOMES, 2015). In skeletal muscle, the number of nuclei determines the final fiber's size and each nucleus controls the transcription of a determined volume of cytoplasm, named as the myonuclear domain (LANDING; DIXON; WELLS, 1974; WHITE et al., 2010). Although the central position of nuclei is a hallmark of the CNM, this has not been much explored in the literature. In recent work, the morphometry, number, and positioning of the nuclei were investigated in the tibialis anterior muscle of KI-*Dnm2* mice. They found a lower number of nuclei per unit of fiber length that can explain the hypotrophy seen in the heterozygous mice, but at a specific age (3 weeks). The lack of nuclei accretion with growing was attributed to a reduced number of satellite cells on TA muscle (FONGY et al., 2019). Furthermore, the spatial organization of myonuclei is affected, like the nuclear orientations and distance between neighbor nuclei, and the authors raised the possibility of an altered anchoring at the periphery, but this needs to be further

addressed, taking into account the effects of DNM2 mutations on microtubule and actin networks. Finally, it is underscored the need to further explore the consequences of satellite cell's deficiency in this context (FONGY et al., 2019).

The role of satellite cells during development and skeletal muscle regeneration is established and consolidated as a consensus, in opposition to their participation in adult muscle maintenance and hypertrophy that is a target of controversy. For muscular dystrophies, muscle disorders in which there is chronic degeneration, the role of satellite cells both for disease progression and the potential therapeutic target is outstanding. Considering the centronuclear myopathies, the number of investigations aiming at the study of these cells is limited.

The mouse model for XL-MTM showed a reduced satellite cell number as evidenced by reduced PAX7 expression and number of myogenic cells isolated from muscle and quantified by flow cytometry. Furthermore, with aging, the number of SCs declined. In vitro and in vivo studies demonstrated a reduced proliferative capacity and increased susceptibility to apoptosis, probably triggered by altered Ca^{2+} homeostasis due to MTM1 deficiency (LAWLOR et al., 2012). In addition, depletion of MTM1 from mouse muscle postnatally led to a 90% reduction in the number of SCs (JOUBERT et al., 2013). These studies argue for the exhaustion of the SC population contributing to disease progression in XL-MTM.

As the muscle fiber is a syncytium, cell fusion is an essential step for muscle formation, in which actin cytoskeleton passes through an intense remodeling. By targeting *Dnm2* with siRNA, Leikina et al. showed inhibited syncytium formation by C2C12 mouse myoblasts (LEIKINA et al., 2013). Using a knock-out conditional mouse model, Shin et al. ablated the expression of DNM2 in myoblasts and observed impaired myotube formation. They also targeted clathrin-mediated endocytosis by using a siRNA against clathrin and the number of myotubes formed was reduced (SHIN et al., 2014). Thus, dynamin and CME showed to be important for myoblast fusion.

Excitation-contraction coupling is central for muscle functioning through the regulation of Ca^{2+} flux in the sarcoplasmic reticulum. For this, an adequate T-tubule structure is crucial. It is already documented the participation of MTM1 (AL-QUSAIRI et al., 2009; DOWLING et al., 2009), BIN1 (LEE, 2002; RAZZAQ, 2001; TOUSSAINT et al., 2011) and DNM2

(CHIN et al., 2015; GIBBS et al., 2014) proteins in T-tubule biogenesis and maintenance; thus the ECC impairment could be a pathological mechanism shared by all the CNMs and with a clear and direct explanation for the muscle weakness suffered by the patients.

The over-expression of mutant R465W-DNM2 in the muscles of adult mice induced a CNM-like phenotype, accompanied by T-tubule disorganization, suggesting a role for DNM2 in adult muscle maintenance (COWLING et al., 2011). Afterward, another group reported hyperactivity of other DNM2 mutants resulting in T-tubule fragmentation in mice and fly muscles and consequently disturbing ECC (CHIN et al., 2015).

The knock-in mouse model for CNM-DNM2 showed an elevated cytosolic Ca^{2+} concentration, signaling for perturbed calcium homeostasis (DURIEUX et al., 2010b). Later, Ca^{2+} signaling and ECC dysfunctions were described in this same mouse model and correlated with muscle weakness (FRAYSSE; GUICHENEY; BITOUN, 2016; KUTCHUKIAN et al., 2017).

Papers published in the last years are delineating a scenario in which a close interaction and interdependence between MTM1, BIN and DNM2 are increasingly central for a range of cellular process for proper muscle function. And in this scenario, DNM2 is emerging as a key player, giving insights for a comprehensive understanding of CNM pathogenesis and providing venues for the development of therapeutic strategies.

DNM2 was identified as a modifier of XL-MTM. It was demonstrated that DNM2 expression is elevated in the *Mtm1* knock-out (KO) mouse model and in the fibroblasts of XL-MTM patients, raising the participation of DNM2 as a pathogenic element in XL-MTM. Afterward, it was generated an *Mtm1* KO mouse only expressing 50% of dynamin 2 who showed an improvement in survival and restoration of muscle functions due to reduced *Dnm2* levels (COWLING et al., 2014). This study provided a proof of concept of the therapeutic potential of the modulation of DNM2 levels for CNMs and strategies to clinically modulate DNM2 in vivo are currently in development (TASFAOUT et al., 2017, 2018).

Similarly, the therapeutic benefit of DNM2 downregulation proved to be beneficial for AR-CNM related to BIN1. $BIN1^{-/-}$ mice are unviable, but after genetic reduction of *Dnm2,* they became viable and survived for up to 18 months, showing normal muscle histology and ultrastructure. In this same study, the authors discovered BIN1 as a negative regulator of

DNM2 during muscle development and maturation, revealing a common pathway for these two proteins (COWLING et al., 2017).

DNM2 modulation is also beneficial for AD-CNM. Using allele-specific silencing RNA against the p.R465W mutation, Trochet et al. successfully reduced the levels of both mRNA and protein mutant in the HTZ mouse. This reduction was enough to restore the myopathic features in the animal (TROCHET et al., 2018).

Together, these studies suggest a shared disease mechanism by the CNMs that may be a therapeutic target. Moreover, they provide arguments in favor of the hypothesis that enhanced DNM2 activity is behind the disease development in autosomal-CNMs and XL-MTM.

I.2.3. Animal model

Almost one decade ago, a valuable tool for the study of AD-CNM was developed: a knock-in mouse model harboring the most frequent mutation found in human patients. This mouse bears the p.R465W mutation, carried by about 30% of all AD-CNM patients, and is named *KI-Dnm2*$^{R465W/+}$ or simply HTZ. The mutation is kept in the heterozygous state; homozygous animals die perinatally (DURIEUX et al., 2010b).

HTZ mice progressively develop a phenotype similar to human CNM, as evidenced by impairment of force generation, muscle atrophy and morphological abnormalities visible with oxidative histochemical staining. By three weeks of age, the muscles start to show an impairment of contractile properties. Progressive muscle atrophy is evident after two months of age: the tibialis anterior has a 20% reduction in muscle mass and fiber size is decreased by 14% (DURIEUX et al., 2010b). Muscle atrophy was shown to be triggered by the activation of autophagic genes, decreased autophagosome maturation and activation of proteasome pathways (DURIEUX et al., 2012). Hypotrophy is also correlated to a reduced number of nuclei in myofibers (FONGY et al., 2019).

Surprisingly, HTZ mice do not have an increased number of centralized nuclei, suggesting that nuclear centralization is not a major component of the pathophysiology in CNM. Even the homozygous mice, which have a severe phenotype, show no more than 10% of central nuclei, whereas human patients can present up to 90% of nuclei internalization,

suggesting that nuclear centralization is not a critical feature for disease physiopathology (DURIEUX et al., 2010b).

While mice knock-out for *Dnm2* is embryonically lethal, heterozygous null mice expressing 50% of wild-type *Dnm2* are viable with normal muscle phenotype (FERGUSON et al., 2009). However total ablation of *Dnm2* specifically on muscles caused a series of muscle abnormalities (TINELLI; PEREIRA; SUTER, 2013). Thus, these animal models demonstrate that dynamin 2 is fundamental for normal development and muscle function.

Dynamin models were also developed in other species: *Drosophila* in which there is an accumulation of pre-synaptic vesicles bind to the plasma membrane (VAN DER BLIEK; MEYEROWRTZ, 1991); *C. elegans* that shows abnormal development and locomotion (CLARK et al., 1997) and *Danio rerio,* which presents muscle weakness and abnormal neuromuscular junction (GIBBS et al., 2014).

VII. Chapter 7 - Final discussion and conclusions

In the present work, we investigated the regenerative potential and myogenic properties, as well as the functional aspects in the context of DNM2-related centronuclear myopathy.

The muscle regenerative potential is one of the most outstanding characteristics of this tissue. This process has been the subject of many works for many decades and there is still a lot to be unveiled in healthy and disease conditions. One important used approach to study muscle regeneration is by causing acute injury and flowing the subsequent cellular and molecular events. Acute lesions can be provoked either by chemicals, toxins or physical methods. Important differences have been revealed between them, highlighting the importance to carefully choose one method over the other, although the outcome can seem similar (HARDY et al., 2016).

Studies on gene therapy showed that the electroporation employed to DNA delivery on muscles have as a side effect an important muscle lesion (BALIGAND et al., 2012). Based on this, in Chapter 4, we presented the protocol we standardized to take benefit of this to induce muscle injury to the study of the regeneration events in muscle systems in which this mechanism is not activated. Our protocol showed to be simple to execute and efficient to provoke muscle degeneration and regeneration. With this system, we offer another alternative into the hall of injury methodologies that exclude the use of chemical compounds or toxins that may have off-target effects.

Dynamin 2 protein is expressed in all tissues, exerting diverse functions primarily related to membrane remodeling, especially on endocytosis, intracellular trafficking of membranes, and cytoskeleton dynamics. Mutations in the *DNM2* gene are linked to autosomal dominant centronuclear myopathy (AD-CNM), a disease that causes muscle weakness and mispositioning of nuclei in muscle fibers. Although many studies have already been published on the pathophysiological mechanisms of this disease, there is still lacking a comprehensive explanation for the muscle-specific impact of the mutations.

Defects in satellite cells population are an important component of several neuromuscular disorders. In the group of centronuclear myopathies, alterations in the biology

of satellite cells were found in X-linked myotubular myopathy, both in the animal model and muscle from human patients (LAWLOR et al., 2012). In a recent publication, it was found that the number of satellite cells is reduced in the tibialis anterior muscle of the KI-*Dnm2*^{R465W} mouse, the animal model for AD-CNM. But the functional consequences were not further explored.

In chapter 5, based on these observations, we sought the explore whether the regenerative capacity of KI-*Dnm2*^{R465W} mouse is compromised. For this, we used our protocol of muscle injury by electroporation and cardiotoxin injection to provoke muscle degeneration in the gastrocnemius muscle of KI-*Dnm2*^{R465W} (HTZ). We assessed histological parameters and the expression of genes important for muscle development and satellite cells function.

First, we saw that muscle mass after the lesion was reduced in HTZ and did not recover to normal values like in WT. In addition, the size and density of muscle fibers are decreased and not equivalent to healthy muscles. As a consequence, part of the muscle was replaced by fibrotic tissue, as evidenced by collagen deposition quantification. The number of new regenerating myofibers was also decreased in HTZ muscle, verified by the quantification of fibers expressing developmental myosin heavy chain.

We also quantified *Pax7* mRNA in the gastrocnemius muscles of KI-*Dnm2*^{R465W} mice and we found a reduction of 70% in its relative expression, as a result of the decreased number of satellite cells that we also quantified in muscle sections. Next, we measured the expression of the myogenic regulatory factors in injured muscles and we found that in HTZ animals they are all downregulated, suggesting that the regeneration in these mice is compromised. Moreover, the expression of *Spry1*, a regulator of quiescence, is upregulated in HTZ mice, suggesting that besides the reduced number of satellite cells, the muscle regeneration may also be impaired by a mechanism to maintain quiescence of its muscle stem cells. Taken together, our data provide evidence that dynamin 2 mutation also impacts on satellite cells and this impairs muscle regeneration, thus adding satellite cells defects as a pathophysiological component of AD-CNM.

In Chapter 6, we presented the functional consequences of two *DNM2* mutations on the myogenic potential of myoblasts *in vitro* system. For this, we used immortalized myoblasts obtained from the HTZ mouse, bearing the p.R465W mutation, and from a human patient

carrying the mutation p.E650K. First, the expression analysis of genes related to the myogenic program showed that, despite an apparently normal fusion, p.R465W myoblasts have an altered expression of *Pax7, Myf5, Myod,* and *Myog* genes as compared to WT cells, suggesting that the differentiation is somehow disturbed. Differently, p.E650K myoblasts showed, in general, a normal expression of myogenesis markers. However, we verified that they fused less, forming smaller myotubes.

Cell migration is an important step for myoblast fusion. So, we hypothesized that this property could be impaired in the mutated myoblasts. Indeed, we found that these cells developed a reduced speed and consequent coursed shorter distances in our migration assay. This was an additional phenotype that may be contributing to the reduced myogenic potential, given the role of dynamin 2 on actin dynamics and focal adhesion disassembly through endocytosis.

Finally, we tested the endocytosis capability on the mutant myoblasts, since this analysis has been made mostly on fibroblasts or other cell types and data from myoblasts was lacking. In the p.R465W myoblasts, we found a reduced endocytosis rate as compared to normal cells, in opposition to a recent publication in which it was described increased endocytosis (RABAI et al., 2019). Here, we used a different assay to measure the internalization of membrane receptors, but we also considered the size of the cells, which can account to the discrepancies between both studies. In p.E650K myoblasts, we verified a great variability among control cells, leading to the interpretation that significant alteration in the endocytosis process under the effect of this mutation could not be considered. Thus, the effect on endocytosis could be mutation-dependent, probably due to the different functions of the domains on which they localize. In the literature, there is a lot of controversy about the effects of DNM2 mutations in endocytosis, with some studies pointing to increased, normal or reduced endocytosis. The debate may persist for a while, but regarding the myogenic potential of the cells, our results can also be indirect evidence that an alteration in endocytosis is affecting cell migration, through an altered focal adhesion disassembly.

Collectively, our data support our hypothesis that dynamin 2 has a muscle-specific role, with important participation on satellite cells and myoblast differentiation, and these functions are impaired by mutations in centronuclear myopathies, contributing to the

development of the disease. Further studies are necessary to better explore the cellular and molecular mechanisms by which dynamin 2 participates on satellite cells biology.

VIII. References[2]

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