Institut de Génétique et de Biologie Moléculaire et Cellulaire

CNRS-INSERM-Université De Strasbourg

Thèse

Présenté en vue de l'obtention du grade de

Docteur De l'Université de Strasbourg

Discipline : Science du vivant

Aspects moléculaires et cellulaires de la biologie

Par

Lama Al-Qusairi

Mécanismes physiopathologiques de la myopathie myotubulaire chez les souris déficientes en myotubularin

La commission d'examen

M. le Professeur *Jean-Louis Mandel*	Co-directeur de thèse
M. le Docteur *Jocelyn Laporte*	Co-directeur de thèse
M. le Professeur *Michel Koenig*	Rapporteur interne
Mme. Le Docteur *Pascale Guicheney*	Rapporteur externe
M. le Professeur *Urs Ruegg*	Rapporteur externe
Mme. Le Docteur *Buj-Bello Anna*	Examinateur

Contents

List of figures

List of Abbreviations

AD-CNM : Autosomal dominant centronuclear myopathy

AR-CNM : Autosomal recessive centronuclear myopathy

AChR: acetylcholin receptor

BAR: Bin/Amphiphysin/Rvs

CaM : calmodulin

CNM : centronuclear myopathies

CK : creatine kinase

CMT: Charcot-Marie-Tooth neuropathy

CME: clathrin-mediated endocytosis

CICR: calcium induced calcium release

CC: coiled-coil

CSQ or Casq: calsequestrin

CDD: central core disease

DHPR: Dihyhropyridine Receptor

DR or D: divergent regions

E-C: excitation–contraction

EM: Electron microscopy

ES: embryonic stem

GEFs: guanine nucleotide-exchange factors

GED: GTPase effector domain

GST: glutathione transferase GST

HE: hematoxylin-eosin staining

KDa: kilo dalton

FKBP: FK506-binding protein

3D: tri-dimensional

MH: Malignant hyperthermia

MD: myotonic dystrophy

MG: Myasthenia gravis

MDa: mega Dalton

MVB: multivesicular body

NADH-TR: NADH-tetrazolium reductase

NMJ: neuromuscular junction

PtdIns: phosphatidylinositol

PH/GRAM: Pleckstrin Homology/ Glucosyltransferase, Rab-like GTPase Activator and Myotubularins

PRD: Proline-Rich Domain

PDK1: phosphatidylinositol-dependent kinase 1

PTP: protein tyrosine phosphatase

PH: Pleckstrin-Homology

PDGF: platelet-derived growth factor

PDZ-BM: PDZ binding motif

PLN: phospholamban

RID: Rac-Induced recruitment Domain

RTKs: receptor tyrosine kinases

rAAV: recombinant adeno-associated vector

RyR: Ryanodine Receptor

SID: SET interacting domain

SET: Suvar3-9, Enhancer-of-zeste, Trithorax

SH3: SRC-Homology-3

SR: sarcoplasmic reticulum

SERCA: Sarco-Endoplasmic Reticulum Ca^{2+} pumps SERCA

SLN: sarcoplipin SLN

TrnR: Transferrin Receptor

TGN: trans-Golgi network

T-tubules: transversal tubules

VICR: voltage induced calcium release

VPS: vacuolar protein sorting

XLMTM: X-linked myotubular myopathy

YMR: yeast myotubularin related protein

Mécanismes physiopathologiques de la myopathie myotubulaire chez les souris déficientes en MTM1

La myopathie myotubulaire liée au chromosome X (XLMTM, OMIM 310400) appartient au groupe des myopathies centronucléaires et représente une maladie congénitale sévère de l'enfant. Le muscle squelettique est le tissu le plus touché par la maladie. Le phénotype est caractérisé par une hypotonie généralisée et une paralysie qui aboutissent à la mort de l'enfant pendant la première année de la vie par insuffisance respiratoire. L'examen histologique des muscles des patients révèle la présence de fibres de petite taille, arrondies, avec des noyaux en position centrale, ressemblant à des myotubes fœtaux, d'où le nom de la maladie (Wallgen-Pettersson et al., 1995).

Le gène *MTM1*, muté dans cette maladie, code pour la myotubularine, une lipide phosphatase exprimée de façon ubiquitaire qui régule la voie de l'endocytose (Laporte et al., 1996 ; Tronchère et al., 2004 ; Tsujita et al., 2004). Des souris mutantes (KO) pour le gène *Mtm1* ont été générées dans notre laboratoire et développent des signes cliniques et pathologiques similaires à l'homme, notamment une faiblesse et une hypotrophie musculaire (Buj-Bello et al., 2002), ce qui en fait un bon modèle pour analyser les mécanismes physiopathologiques de la maladie. Des études récentes montrent que la myotubularine est partiellement localisée au niveau des triades dans le muscle squelettique, une structure membranaire composée de tubule transverse (T-tubule) et de réticulum sarcoplasmique, où le couplage excitation-contraction (EC) se produit. Selon des travaux de surexpression dans le muscle, il a été suggéré que MTM1 pourrait réguler l'homéostasie des membranes et en particulier, des tubules transverses (Buj-Bello et al., 2008). Au cours de ma thèse, j'ai cherché principalement à comprendre les mécanismes moléculaires pouvant conduire à la faiblesse musculaire chez les patients en utilisant ce modèle murin.

J'ai tout d'abord caractérisé le phénotype clinique et histologique des souris déficientes en myotubularine sur le fond génétique 129PAS, différent de celui qui avait été précédemment étudié, C57/Bl6 (Buj-Bello et al., 2002). Dans ce fond, les souris KO manifestent une myopathie plus sévère et d'évolution plus homogène que dans l'autre fond

génétique, menant au décès en moyenne à 40 jours post-nataux. L'analyse histologique des muscles squelettiques révèle une atteinte des myofibres déjà à l'âge de 2 semaines. Ceci est caractérisé par une hypotrophie et par la présence d'anomalies structurales comme le changement de distribution des mitochondries et l'altération de la position des noyaux. Ces anomalies deviennent plus prononcées avec la progression de la maladie.

Je me suis intéressée plus particulièrement à l'étude de la morphologie et de la fonction de la triade, car nous avions 1) des données sur la localisation de la myotubularine dans la triade et 2) des résultats sur le transcriptome du muscle squelettique de souris *Mtm1* KO qui montraient une dérégulation potentielle de certains gènes impliqués dans l'homéostasie du calcium (ceci en collaboration avec le Dr. Beggs, Boston). En examinant par microscopie électronique des muscles normaux et mutants pour le gène *Mtm1* après marquage spécifique des T-tubules, j'ai mis en évidence la présence d'anomalies structurales des triades et des tubules transverses dans le muscle déficient en Mtm1. Deux types d'altérations sont décrites : la présence de tubules longitudinaux d'une part et la présence de triades qui sont dépourvues de T-tubules ou absence de triades de l'autre part. Ces altérations sont aussi présentes au stade précoce de la myopathie, ce qui suggère qu'elles contribuent à la pathogenèse de la maladie.

Deuxièmement, j'ai étudié les répercussions fonctionnelles de ces anomalies en étudiant le niveau d'expression de plusieurs gènes impliqués dans le cycle calcique par PCR quantitative et immunoblot. J'ai ainsi montré, notamment, qu'il existe une diminution du niveau protéique du récepteur à la ryanodine (RyR1) et du récepteur à la dihydropyridine (DHPRα), deux protéines clés dans le couplage excitation-contraction. L'analyse des muscles de souris mutantes à stade précoce de la maladie ne montre pas une altération de ces deux marqueurs. Cela suggère que ces altérations sont secondaires à la dérégulation des triades qui apparemment semblent arriver en premier dans la pathogenèse de XLMTM.

Finalement, afin de déterminer les conséquences fonctionnelles de ces altérations moléculaires dans le cycle calcique, j'ai collaboré avec l'équipe du Dr. Vincent Jacquemond (Lyon, France) pour la réalisation d'analyses électrophysiologiques sur des fibres isolées à partir du muscle FDB (*flexor digitorum brevis*) de souris sauvages et mutantes pour le gène *Mtm1*. Cette étude a montré qu'il existe un défaut de sortie du calcium du réticulum

sarcoplasmique suite à la dépolarisation de la membrane. Ces résultats montrent pour la première fois que des défauts dans le couplage excitation-contraction pourraient être à l'origine de la faiblesse musculaire liée à la myopathie myotubulaire.

Le deuxième projet sur lequel j'ai travaillé pendant ma thèse a été d'essayer de comprendre les mécanismes responsables de l'hypotrophie des fibres musculaires déficientes en *Mtm1*, un phénomène précoce dans l'évolution de la maladie chez la souris et qui représente un facteur de pronostic chez les patients (Pierson et al., 2008), et d'y remédier en apportant des facteurs trophiques. Dans ce but, j'ai croisé une lignée de souris transgéniques qui exprime l'IGF1 (insulin like growth factor 1) sous un promoteur muscle spécifique avec la lignée déficiente en myotubularine (Musaro et al., 2001). L'IGF1 joue un rôle important dans la prolifération et dans la différentiation des cellules satellites du muscle et sa surexpression dans le muscle squelettique induit une hypertrophie des fibres (Engert et al., 1996 ; Lawlor et al., 2000). Les résultats montrent que, contrairement à nos attentes, le muscle des souris KO *Mtm1* ne répond pas à l'effet anabolisant de l'IGF1. Afin d'élucider ce point, j'ai commencé à analyser la voie de signalisation de la PI3K/AKT, activée par ce facteur de croissance. En utilisant des analyses protéomiques, j'ai mis en évidence une accumulation du récepteur à l'IGF1 (IGF1R) dans le muscle mutant, en plus de l'augmentation du niveau protéique d'AKT, un acteur principal dans la voie de signalisation à l'IGF1. Ces analyses suggèrent que la voie de l'IGF1 pourrait être altérée dans les muscles des souris déficientes en *Mtm1*. J'ai étudié en suite certains gènes impliqués dans le système ubiquitine-protéasome (SUP), qui a été décrit comme étant impliqué dans l'atrophie musculaire induite dans plusieurs conditions comme la dénervation, le diabète ou le traitement aux glucocorticoïdes (Jagoe et al., 2001). J'ai évalué par PCR quantitative le niveau d'expression de plusieurs gènes impliqués dans l'atrophie musculaire observée chez les souris déficientes en *Mtm1*. Les résultats montrent une augmentation de l'expression de MURF1 et MAFbx à un stade tardif mais aussi à stade précoce, au début de l'apparition de signes histologiques. Cela suggère que l'activation de SUP contribue de manière significative à la pathogénèse de XLMTM.

Le travail effectué au cours de ma thèse suggère deux points :

i) L'absence de MTM1 dans le tissu musculaire conduit à une altération de la machinerie calcique qui pourrait provoquer une faiblesse musculaire.

ii) La présence d'une altération de la voie de signalisation à l'IGF1 et de l'activation de la SUP qui pourraient être impliqués dans l'atrophie musculaire.

Bibliographie

1- WALLGEN-PETTERSSON C. *et al*. The myotubularmyopathies: differential diagnosis of the X-linked recessive, autosomal dominant, and autosomal recessive forms and present state of DNA studies. J. Med. Genet. 32 (1995),

2- LAPORTE J. *et al.* gene mutated in X-linked myotubularmyopathy defines a new putative tyrosine phosphatase family conserved in yeast. Nat Genet. 1996 Jun;13(2):175-82.

3- TRONCHERE H. *et al*. Production of phosphatidylinositol 5-phosphate by the phosphoinositide 3-phosphatase myotubularin in mammalian cells. J. Biol. Chem., Vol. 279, Issue 8, 7304-7312, February 20, 2004

4- TSUJITA K. *et al.* Myotubularin regulates the function of the late endosome through the gram domain-phosphatidylinositol 3,5-bisphosphate interaction. J BiolChem.2004 Apr 2;279(14):13817-24. Epub 2004 Jan 12.

5- BUJ-BELLO A. *et al.* The lipid phosphatasemyotubularin is essential for skeletal muscle maintenance but not for myogenesis in mice. Proc NatlAcadSci U S A. 2002 Nov 12;99(23):15060-5. Epub 2002 Oct 21.

6- BUJ-BELLO A. *et al.* AAV-mediated intramuscular delivery of myotubularin corrects the myotubularmyopathy phenotype in targeted murine muscle and suggests a function in plasma membrane homeostasis. Hum Mol Genet. 2008 Jul 15;17(14):2132-43.

7- PIERSON CR. *et al.* Myofiber size correlates with MTM1 mutation type and outcome in X-linked myotubularmyopathy.NeuromusculDisord. 2007 Jul;17(7):562-8. Epub 2007 May 29. Erratum in: NeuromusculDisord. 2008 Jun;18(6):519.

8- MUSARO A. *et al*. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet. 2001 Feb;27(2):195-200.

9- ENGERT JC; *et al.* Proliferation precedes differentiation in IGF-I-stimulated myogenesis. J Cell Biol. (1996).

10- LAWLOR MA. *et al.* Insulin-like growth factor-mediated muscle cell survival: central roles for Akt and cyclin-dependent kinase inhibitor p21. Mol Cell Biol. 2000 Dec;20(23):8983-95.

11- JAGOE RT. *et al.* What do we really know about the ubiquitin-proteasome pathway in muscle atrophy?
Curr Opin Clin Nutr Metab Care. 2001 May;4(3):183-90.

I. Excitation contraction coupling in skeletal muscle

In order to achieve skeletal muscle contraction, the action potential, which is generated in the brain by motor neurons, is transmitted through motor nerves to all muscle cells of the motor unit (figure 1). The signal transmission from the motornerve into the muscle fiber occurs in a region called neuromuscular junction (NMJ). The arrival of an action potential at the axon terminal of the motornerve triggers a cascade of biochemical events resulting in the release of a neurotransmitter, the acetylcholine, into the synaptic cleft and the initiation of the action potential at the sarcolemma of muscle fiber (Fagerlund et al. 2009). The signal transmission from the sarcolemma to the actin/myosin apparatus is mediated in part by a second messenger, the calcium ions

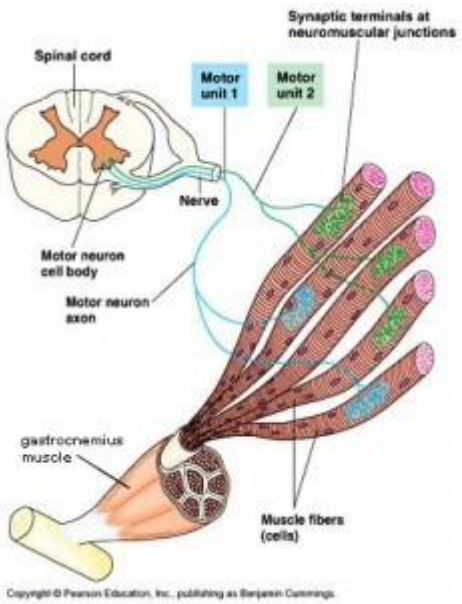


Figure (1): S*chematic representation of two motor units that innervate two different sets of myofibers within the same muscle* (from http://kamulagingapain.blogspot.com/2009/10/unit-motor.html).

Ca^{2+} entry at the plasma membrane provides a mechanism for signaling in a variety of cellular process as calcium concentration ($[Ca^{2+}]$) is in a milli-molar range in the extracellular space, which is 100 fold higher than the cytosolic calcium $[Ca^{2+}]$. Intracellular Ca^{2+} serves as a second messenger to regulate many physiological processes in several cell types. Processes involving Ca^{2+} signaling include secretion, synaptic transmission, fertilization, nuclear pore regulation, transcription and many others (Fill et al. 2002). An important role attributed to the calcium as second messenger is during muscle contraction, where the strong and fast

intracellular calcium variation plays a crucial role in signal transmission between the action potential and the muscle contraction.

Excitation-contraction (E-C) coupling refers to the process initiated with an action potential at the sarcolemma (excitation) and terminated by force generation and shortening within the striated muscle fiber (contraction) (Costantin 1975). This occurs through an intracellular calcium release (Costantin 1975).

It is known that a small increase in cytosolic calcium $[Ca^{2+}]c$ induces a massive intracellular sarcoplasmic reticulum (SR) Ca^{2+} release events in both skeletal and cardiac muscle (Endo 1977). This mechanism is identified as calcium induced calcium release (CICR). Findings in the past 30 years have identified CICR as the physiological cardiac signal leading to the Ca^{2+} release from the intracellular Ca^{2+} store (Fill et al. 2002). In skeletal muscle, the mechanism which is responsible for the EC coupling is the voltage gated calcium release (VGCR).

In this chapter, general findings about the structure related to VGCR and the cellular machinery which regulates this mechanism will be highlighted.

I-1. Triad description and biogenesis

Myofibers are 50-100 µm in diameter and several millimeters to centimeters long, they have devised ways to overcome spatial problems in using Ca^{2+} as secondary messenger:

1. Muscle fibers contain large internal Ca^{2+} stores with the ability to release Ca^{2+} within each micro-meter (µm) of the fiber (figure 2 right panel).
2. They have also the ability to maintain this store under the control of the action potential, which ensures Ca^{2+} release simultaneously within the whole interior of the muscle fiber.

To achieve this, the sarcolemma forms regular invaginations which travel between the myofibrils, termed transversal tubules (T-tubules). The T-tubules cross tightly close to the sarcoplasmic reticulum, in a region called terminal cisternae/junctional SR. The close association of one T-tubule with two terminal cisternae at each side forms the triad (figure 2).

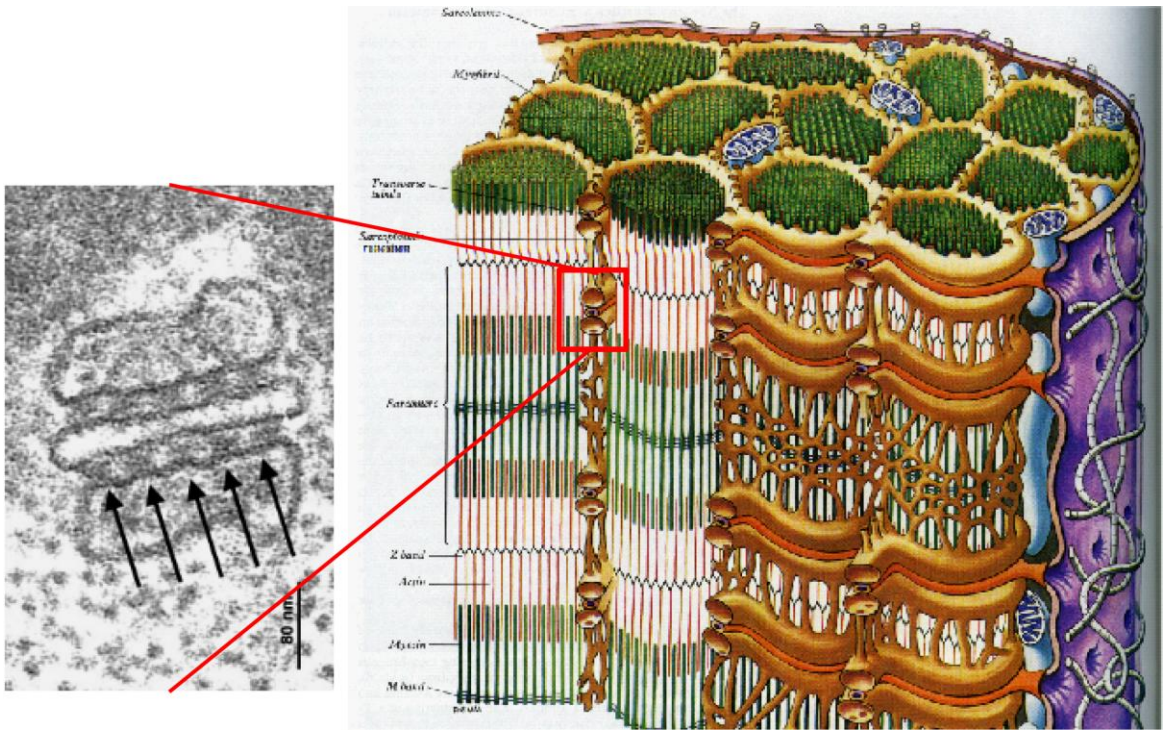


Figure (2) : *Triad organization in skeletal muscle.* Right panel, schematic representation of the muscle sarcomere and surrounding membranes. T-tubules shown in orange are specialized invaginations of the sarcolemma. The elaborated sarcoplasmic reticulum network is shown in yellow. Note the close proximity of T-tubules and terminal cisternae, (modified from D. Fawcett). Left panel, electron micrograph of a triad junction. A central T-tubular element is flanked on either side by a terminal

cisternae element of the sarcoplasmic reticulum. The arrows point to electron-dense junctional feet corresponding to RyR, (from C. Franzini-Armstrong).

Triads are highly specialized to allow for orchestration of EC coupling. A large set of specialized proteins take part in this process. These include:

1. Dihydhropyridine Receptor (DHPR), a voltage gated calcium channel that is present in T-tubule membranes (Fosset et al. 1983), (Curtis et al. 1984).
2. Ryanodine Receptor (RyR), a Ca^{2+} release channel that is localized on the junctional face of SR. RyRs appear as "feet" when observed by electron microscopy (figure 2 left panel) (Inui et al. 1987).
3. Calcium buffering proteins such as calsequestrin in the lumen of the SR (MacLennan et al. 1971).
4. Calcium channel regulators such as calmodulin, FKBP12 and many others (Tripathy *et al.* 1995), (Chen et al. 1994).
5. Sarco-Endoplasmic Reticulum Ca^{2+} ATPase pumps (SERCA), which perform rapid removal of the cytosolic calcium after fiber shortening (MacLennan et al. 1985).

The biogenesis of triad and the role of these proteins and their implication in EC coupling will be discussed.

I-1-A. Triad biogenesis

I-1-A-i. *Sarcoplasmic reticulum biogenesis*

The SR represents the main calcium store in striated muscle. It is highly specialized to ensure the simultaneous release of intracellular calcium in the entire cytosol of the muscle cell. SR biogenesis was observed using electron microscopy (EM) during muscle differentiation in mouse (Takekura et al. 2001). These observations were also supported by studies employing chicken embryo (Flucher et al. 1993). In mouse, the SR is detected from an early stage at embryonic day 14 (E14) with punctuate RyR clusters evident that are located in the periphery of the myofiber (Takekura et al. 2001). At this stage, the content of the feet (RyR) in the junctional SR is poor, however, some SR elements without any feet are observed. At day E16, RyR containing elements become abundant and start to be associated

with the edges of A bands (A-I junctions) of the newly formed sarcomeres. This specific association results in a distinct banding pattern of a discrete SR network at the I-band with thin longitudinal connecting SR elements (Takekura et al. 2001). During the next days (E17 and E18), junctional SR acquires a predominant transverse distribution taking their final position by forming one triad at each side of the Z-line (two SR sacs in each triad) (Takekura et al. 2001). During the maturation of SR membranes, the frequency of feet increases, in particular, between E16 and E18, when all junctions become completely filled by feet. The width of the junctional gap without feet is about "5,7 nm" and it becomes "9,6 nm" when it is occupied by feet. At birth, the RyR containing elements take the mature form (Takekura et al. 2001).

I-1-A-ii. *Transverse tubule biogenesis*

The T-tubules are invaginations of the plasma membrane, which are present exclusively in striated muscle. Their role is to maintain the SR calcium store under the tight control of membrane depolarization via the voltage sensor DHPR (Fosset et al. 1983). Morphological studies in chicken and mouse embryos have revealed that the T-tubules start their formation after the SR (Flucher et al. 1993), (Takekura et al. 2001). In mouse embryos, the first defined tubules can be observed at E15. At this stage they take the aspect of short cylinders invaginated by the plasma membrane that penetrate within the myotubes (Takekura et al. 2001). At E16, the newly formed T-tubules travel deeper within the myofiber, maintaining a connection with the surface by short transverse segments however they stay predominantly longitudinal (Takekura et al. 2001). During the last days of gestation (E17, 18, 19), T-tubules continue their development to invade the entire fiber; the majority of them are longitudinal with some transverse connecting elements (Takekura et al. 2001), (Flucher et al. 1993). In the postnatal period, the transverse connections take more regularity to achieve over time the normal transverse distribution (Takekura et al. 2001), (Flucher et al. 1993). Maturation of T-tubules is achieved in mouse 3 weeks after birth (Takekura et al. 2001), (Franzini-Armstrong et al. 1991).

I-2. Proteins implicated in VGCR in skeletal muscle

I-2-A. DHPR, the voltage sensor

I-2-A-i. *DHPR role in VGCR and its triadic organization*

The role of DHPR in E-C coupling is voltage sensing and signal transmission to RyR (Rios et al. 1987). It is arranged in tetrads on the junctional T-tubules (Block et al. 1988), (Franzini-Armstrong et al. 1991). The orientation and spacing of these tetrads match the disposition of junctional feet, which span the gap between SR and T-tubules at regular intervals (figure 3) (Block et al. 1988), (Wagenknecht et al. 1989). The tetrads form rows in which the position of DHPR alternates between adjacent rows, in a way in which DHPR in one row faces an empty space in the neibouring row (Block et al. 1988). Consequently, only half of the RyR molecules matches DHPR channels (Block et al. 1988). This model is consistent with the ratio of RyR to DHPR measured in skeletal muscle (Margreth et al. 1993), (Anderson et al. 1994).

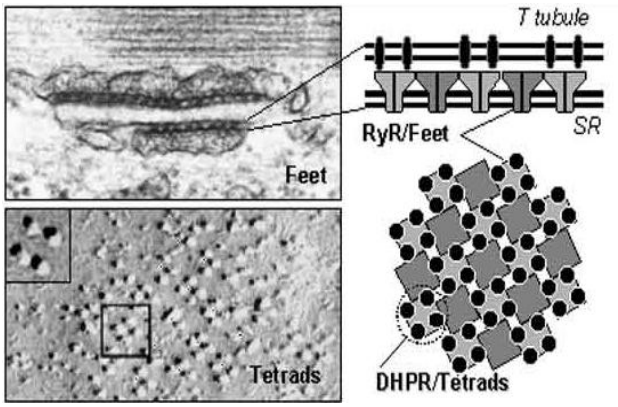


Figure (3): *Molecular organization of RyRs and DHPRs in skeletal muscle triads.* Left: Electronmicrographs of transverse section and freeze-fracture showing feet and tetrads. Note the alternate DHPR tetrads. Right: Corresponding models of RyR and DHPR assembly in the junction, (from http://physiologie.i-med.ac.at/flucher/ECC.htm).

Block *et al* have suggested a direct interaction between the voltage sensor DHPR and RyR. This interaction modulates the activation gate of calcium release channel to allow the massive release of calcium from the SR (Block et al. 1988). Thanks to the physical coupling between RyR and DHPR, the signal transmission in skeletal muscle fiber occurs within 2 ms instead to 100 ms in cardiac muscle (CICR mode) (Fill et al. 2002).

I-2-A-ii. *The DHPR channel model*

Although DHPR is considered as both a Ca^{2+} channel and a voltage sensor (Tanabe et al. 1987), and in contrast with heart muscle, only 5% of these receptors are functional Ca^{2+} channels in skeletal muscle. The prominent role of DHPR in this tissue is to interact physically with ryanodine receptor resulting is its opening (Rios et al. 1987).

Biochemical studies have demonstrated that the DHPR hetero-oligomer complex is composed of five subunits that are associated non-covalently. The DHPR subunits are α 1 (175 KD), β (53 KD), γ (33 KD), and the α2-δ dimer (220 KD) (Curtis et al. 1984), (Takahashi et al. 1987). The skeletal muscle isoforms are encoded by *Cacna1s, Cacnb1, Cacng1* and *Cacna2-d* genes respectively (Curtis et al. 1984), (Takahashi et al. 1987). Takahashi and co-authors suggested that the DHPR oligomer contains one subunit of each protein. This model is based on a central channel-forming element consisting of the alpha 1 subunit, which interacts with three other subunits (figure 4) (Takahashi et al. 1987). This structural model proposes that:

1. The α1 subunit, which is embedded in the membrane, contains four repeats of transmembrane domains. This subunit contains the calcium antagonist binding sites for the dihydropyridine.
2. The beta subunit is cytosolic and associates with the intracellular domain of α1.
3. The gamma subunit, a membranous peptide, interacts independently with α1.
4. The dimer alpha 2-delta, in which α2 is weakly associated to alpha 1, exposes the delta subunit on the outer face of the complex.

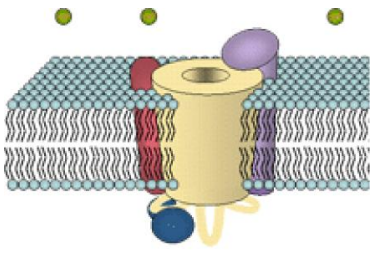


Figure (4): *DHPR model as proposed by Takahashi et al.* (Takahashi et al. 1987). The alpha subunit (in yellow) is the pore forming. The cytosolic domain of alpha subunit interacts with the beta subunit (in blue). The gamma (in red) and α2–δ dimer (in violet) are anchored in the T-tubule membrane and associate with the pore forming subunit, (from Grabner Laboratoty website).

I-2-A-iii. *Description of DHPR subunits*

I-2-A-iii-a. The alpha subunit

In addition to the role of DHPR α as the pore forming element, it also represents the voltage sensor component of the channel. This role is possible via the topological structure of this subunit. DHPR alpha subunit is organized in four repeated homology units, with each repeat containing six membrane-spanning segments predicted to form alpha helices (Noda et al. 1984) (figure 5). The fourth segment of each repeat (known as S4) contains a regularly spaced array of positively charged amino acids that constitute the voltage sensor (Noda et al. 1984). Upon depolarization, the positively charged amino acids are translocated, which induces a conformational change in the DHPR alpha subunit (Noda et al. 1984). This leads to its interaction with RyR1 in skeletal muscle or DHPR opening in cardiac muscle.

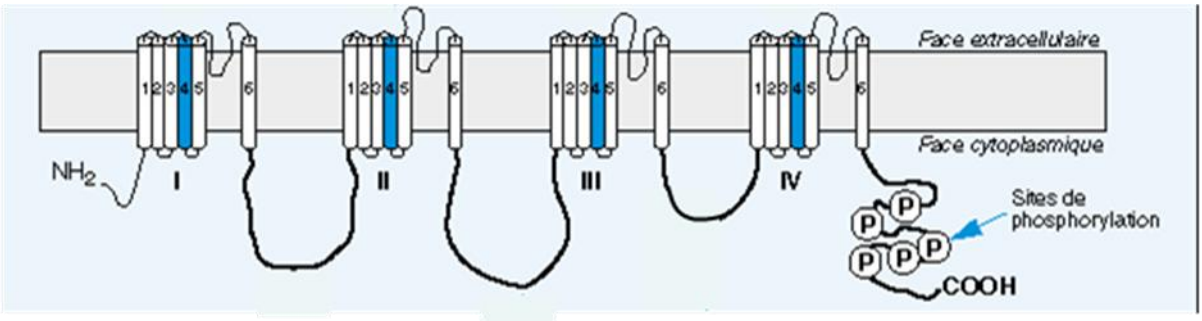


Figure (5): *Schematic representation of the skeletal muscle DHPR alpha subunit as presented by Tanabe (1995).* The four units of homology are displayed linearly; each unit contains six transmembrane segments (S1 to S6), represented here by cylinders. The S4 is the voltage sensor element (in bleu), (modified from N. Monnier and J. Lunardi, 2000, Annales de biologie Clinique).

The role of α1s in excitation-contraction was analyzed in dysgenic mice carrying an homozygous mutation that leads to absence of α1s expression (Beam et al. 1986). Myotubes derived from skeletal muscle of dysgenic embryos do not exhibit EC coupling (Adams et al. 1990), (Klaus et al. 1983). The role of DHPR α subunit in this process is to trigger RyR activation, which occurs by two different ways in the skeletal and cardiac muscles depending on the alpha subunit isoforms.

To date, two DHPR α1 isoforms have been identified, the skeletal α1S and the cardiac α1C isoforms (Curtis et al. 1984), (Chang et al. 1988). The α1S isoform is highly expressed in fast-twitch skeletal muscle compared to slow-twitch muscle, and it is absent from cardiac tissue (Froemming et al. 2000). The α1C isoform is specific to cardiac muscle but is also detected at low and intermediate levels in fast and slow twitch skeletal fibers respectively (Froemming et al. 2000). Interestingly, in the neonatal life, the predominant isoform in both types of skeletal muscle fibers is α1C. During muscle development, α1C is gradually replaced by the skeletal isoform (Froemming et al. 2000).

The molecular basis of the physiological difference between the two isoforms resides in the loop region between DHPR transmembrane segments II and III. This region of 126 aa is critical for RyR1 (RyR muscle skeletal isoform) activation (Tanabe et al. 1990).

I-2-A-iii-b. Role of DHPR β1a subunit

Four beta subunit genes have been identified in mammals, β1 to β4 (Powers et al. 1992), (Perez-Reyes et al. 1992), (Castellano et al. 1993)a, (Castellano et al. 1993)b. Alternative splicing of the β1 gene produces at least three isoforms (β1a, β1b, β1c), the β1a is specifically expressed in skeletal muscle while the β1b and β1c are expressed in brain, heart and spleen (Powers et al. 1992).

Zebrafish null mutants for β1a, obtained from a large-scale mutagenesis screen, were called "*relaxed*" (Haffter et al. 1996), (Schredelseker et al. 2005). Although *relaxed* zebrafish are viable, they display complete skeletal muscle paralysis (Granato et al. 1996). The absence of β1a in mice is much more severe, resulting in death due to asphyxia at birth (Gregg et al. 1996). This discrepancy can be explained by the difference in membrane expression of α1s in both mutants. The amount of α1s targeted to the T-tubule membrane is decreased by half in the *relaxed* zebrafish muscle (Schredelseker et al. 2005), however, α1s is undetectable in murine myotubes lacking β1a, as shown by immunofluorescence labeling (Gregg et al. 1996). In addition, freeze fracture electron microscopy has shown that the normal arrangement of DHPR in arrays of tetrads is completely lost in zebrafish β1a mutants (Schredelseker et al. 2005).

Thus, this indicates that beta subunit is essential for the correct transport, insertion and localization of α1s, what is termed the membrane expression of alpha1 subunit. However,

using overexpression approaches in rat myotubes, Flucher *et al.* (2000) have suggested that the targeting of α1s to the triad does not require the β1 subunit, but depends exclusively on the C-terminus of α1s protein which harbors a targeting signal (Flucher et al. 2000).

I-2-A-iii-c. Role of the DHPR γ1 subunit

The γ1 subunit is exclusively expressed in skeletal muscle (Wissenbach et al. 1998). Mice deficient for γ1 (*Cacng1*-KO) develop normally but several functional alterations have been observed (Freise et al. 2000). Although force response measurements of isolated muscles from γ1 deficient mice are normal (Ursu et al. 2001), the Ca^{2+} influx through DHPR was enhanced, leading to increase in calcium release from the SR (Freise et al. 2000). This indicates that γ1 plays an inhibitory role of DHPR function where is able to reduce Ca^{2+} influx through DHPR and consequently Ca^{2+} release (Freise et al. 2000), (Andronache et al. 2007). It may thus regulate the Ca^{2+} influx generated by the hyperdepolarization of the plasma membrane as seen in some stress conditions (Andronache et al. 2007).

I-2-A-iii-d. Role of the DHPR α2-δ subunits

The role of α2–δ in skeletal muscle has not been established however, it appears that the cardiac α2 subunit enhances the calcium channel activity of the DHPR (Mikami et al. 1989). Interestingly, mutations in α2 subunit lead to malignant hyperthermia (MH), a muscular disorder caused by the hyperactivity of the RyR1 channel (Iles et al. 1994), (Monnier et al. 2000). More details about MH will be discussed later.

I-2-B. RyR, the calcium release channel

I-2-B-i. *The RyR family*

Three ryanodine isoforms have been identified in mammals (RyR1, RyR2 and RyR3), which are encoded by three different genes (Marks et al. 1989), (Tunwell *et al.* 1996), (Hakamata et al. 1992). RyR1 represents the skeletal muscle isoform, however it is also detected in brain (Froemming et al. 2000), (Giannini et al. 1995). RyR2 is the cardiac specific isoform, but is also present at lower levels in brain (Froemming et al. 2000), (Giannini et al. 1995). Finally, RyR3 expression is specific to the nervous system, with low levels detected in

adult skeletal muscle (Giannini et al. 1995), (Flucher et al. 1999), (Froemming et al. 2000). Both RyR1 and RyR2 isoforms are expressed in skeletal muscle during neonatal life (Froemming et al. 2000).

RyR1 or RyR2 deficiency results in embryonic lethality in mice (Takeshima et al. 1995), (Takeshima et al. 1998). In contrast, RyR3 KO mice appear relatively healthy and have normal striated muscle (Clancy et al. 1999). Sequence divergence at the amino acid level may explain the functional specificity of the RyR subtypes. Amino acid sequence analysis has revealed the presence of several divergent regions. These regions are referred to D1, D2 and D3 or DR1, DR2 and DR3 (Sorrentino et al. 1993). Several roles in EC coupling are attributed to these regions including, the RyR channel regulation (the case of DR1), tetrad array and the interaction with DHPR (the case of DR2) (Radermacher et al. 1994), (Sheridan et al. 2006).

I-2-B-ii. *Structure of ryanodine receptor*

RyR is the largest ion release channel with a molecular mass of 2,3 MDa. Four identical subunits of 564 kDa assemble to form a tetrameric complex in the junctional SR membrane (Zorzato et al. 1990), (Block et al. 1988). Cryo-electron microscopy and 3D reconstructions (figure 6) demonstrated that RyR1 is composed of two main structural components: a membrane transversing segment, “the basal platform”, and a large cytoplasmic domain formed by 80% of the protein. The cytoplasmic domain, which has a square shape, represents the feet structures observed by electron microscopy (Block et al. 1988). (Wagenknecht et al. 1989).

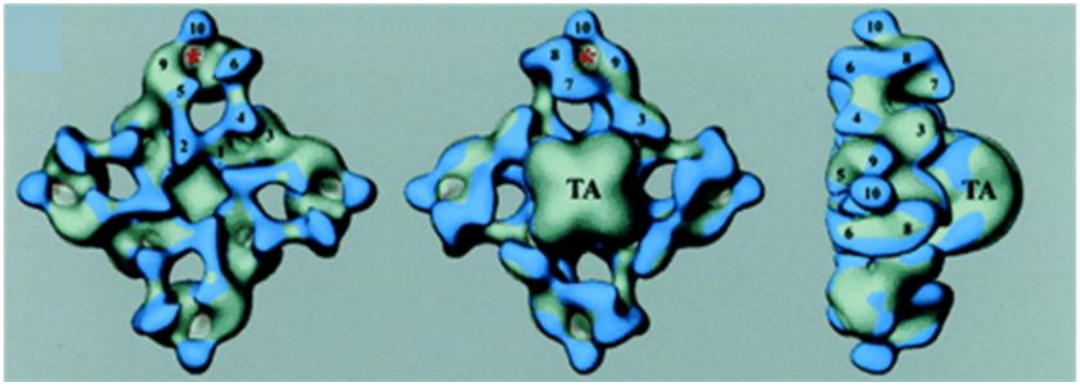


Figure (6): *Schematic representation of the 3D structure of RyR3 channel illustrating the two main domains of the channel.* The cytoplasmic face (left), the sarcoplasmic face (center) and a side view (right). TA: transmembrane assembly, (from Liu *et al.* 2000).

I-2-C. The Sarco-endo plasmic reticulum Ca^{2+} ATPase pump

The sarcoplasmic reticulum in skeletal muscle is a sophisticated organelle, specialized in calcium release to cytosol and subsequent rapid re-uptake. Ca^{2+} release is orchestrated by RyR1 and its associated proteins, and Ca^{2+} removal from the cytosol is fulfilled by the **s**arco-**e**ndoplasmic **r**eticulum **c**alcium **A**TPase (SERCA) pumps. To ensure a perfect coordination between both steps, RyR1 channels are localized at the junctional side of the SR and SERCA pumps are inserted at the longitudinal side of SR. In this section, SERCAs and their implication in Ca^{2+} re-uptake will be highlighted.

I-2-C-i. *SERCA pump family*

The SERCA pump belongs to the family of P-type ATPases, that includes also the plasma membrane Ca^{2+} ATPase. Three SERCA genes have been identified in mammals: ATP2A1, ATP2A2 and ATP2A3, which encode SERCA1, 2 and 3, respectively. SERCA1 is the skeletal muscle Ca^{2+} pump and is alternatively spliced into SERCA1a and SERCA1b, the adult and fetal isoform respectively (Brandl et al. 1986), (Brandl et al. 1987). The SERCA2 encoding gene is induced during the terminal differentiation of myoblasts into myotubes (Zarain-Herzberg et al. 1990). This gene encodes for three isoforms: SERCA2a, which is predominantly expressed in cardiac and slow switch skeletal muscle (Zarain-Herzberg et al. 1990), SERCA2b that is ubiquitously expressed (Zarain-Herzberg et al. 1990), (Gunteski-Hamblin et al. 1988), and SERCA2c that is expressed in cardiac and also in non-muscle cells (Dally et al. 2006), (Gelebart et al. 2003). The SERCA3 gene encodes for six isoforms (3a-3f), which appear to be present in muscle at low levels, but they are also expressed in non-muscle cells (Anger et al. 1993), (Martin et al. 2002), (Bobe et al. 2004).

I-2-C-ii. *SERCA structure and mechanism of action*

SERCA pumps are composed of transmembrane and cytoplasmic domains. The primary description of SERCA structure was obtained by predicted domain structure and hydrophobicity blots (MacLennan et al. 1985). The cytoplasmic domain of SERCA is composed of three globular domains, A, P and N. Domain A represents the transduction domain, domain P is the phosphorylated domain, and the N domain represents the nucleotide (ATP) binding domain, (figure 7). The transmembrane region contains ten hydrophobic

helices (M1 to M10). Transmembrane and cytoplasmic domains are connected by intermediated region, which is called the stalk (MacLennan et al. 1985), (Brandl et al. 1986).

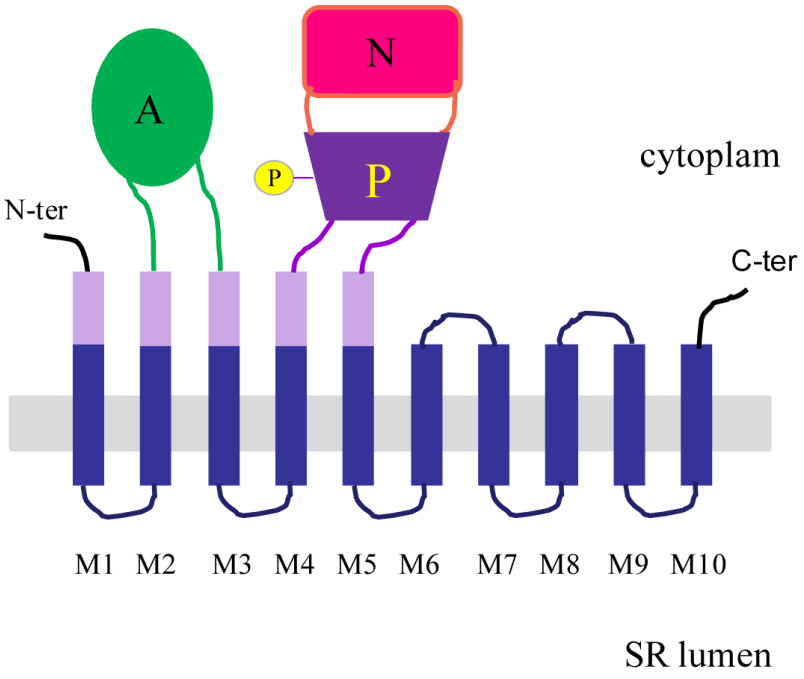


Figure (7): S*chematic representation of the model as suggested by Brandl, 1986.* SERCA pumps are composed of a transmembrane region and cytoplasmic region connected by a stalk (light violet). The transmembrane domain (bleu) is composed of 10 transmembrane helices (M1 to M10). The cytosolic section contains the A (green), P (dark violet) and N (pink) domains.

SECRA pumps use the energy derived from ATP hydrolysis to transport ions across a biological membrane. It pumps two Ca^{2+} ions from the cytoplasm into the SR/ER lumen for each molecule of ATP hydrolyzed (Inesi et al. 1980). SERCA pumps perform conformational change, triggered by an increase in the cytosolic Ca^{2+} concentration, which open the Ca^{2+} binding sites to the cytosol or the SR lumen (Inesi et al. 1980), (Inesi 1987) (figure8).

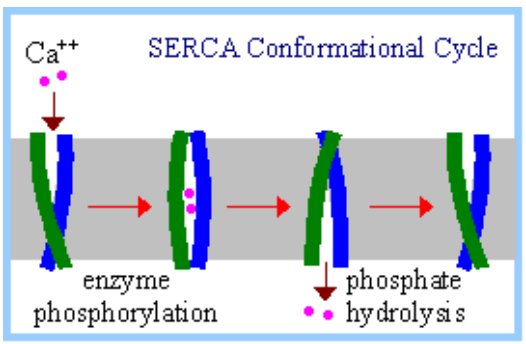


Figure (8): *Schematic representation of SERCA mechanism of action showing one conformational cycle starting from left,* (from Joyce J. Diwan. Website).

In one conformational cycle

- The binding of two calcium ions from the cytosolic face stabilizes the conformation that allows the enzyme phosphorylation using ATP (MacLennan et al. 1997), (Inesi et al. 1990).

- The dissociation of the two Ca^{2+} ions into the SR lumen promotes hydrolysis of Pi from the enzyme, which induce a conformational change (recovery) that renders the Ca^{2+} binding sites accessible again from the cytosol, (figure 8) (MacLennan et al. 1997), (Inesi et al. 1990).

I-2-D. Other proteins involved in the EC coupling mechanism

I-2-D-i. *Calsequestrin*

Calsequestrin (Casq) is the main Ca^{2+} binding protein present in the SR lumen. It associates with the junctional SR membranes via junctin (another SR protein) generating luminal densities/accumulations in close proximity to the junctional SR membrane, to maintain a high level Ca^{2+} content in junctional SR (figure 9) (Wei et al. 2009), (Franzini-Armstrong et al. 1987).

Two isoforms of mammalian Casq have been identified (Jorgensen et al. 1979), (Campbell et al. 1983). *Casq1* and *Casq2* genes encode for the skeletal muscle Casq1 and the cardiac muscle Casq2 isoforms respectively (Fliegel et al. 1987), (Scott et al. 1988). Both genes are expressed in slow twitch skeletal muscle, where Casq2 represents 25% of total Casq, and both proteins bind free Ca^{2+} with similar affinity (Damiani et al. 1990). In fast twitch fibers, Casq1 is the only isoform in adult fibers, although some Casq2 is present until shortly after birth (Sacchetto et al. 1993).

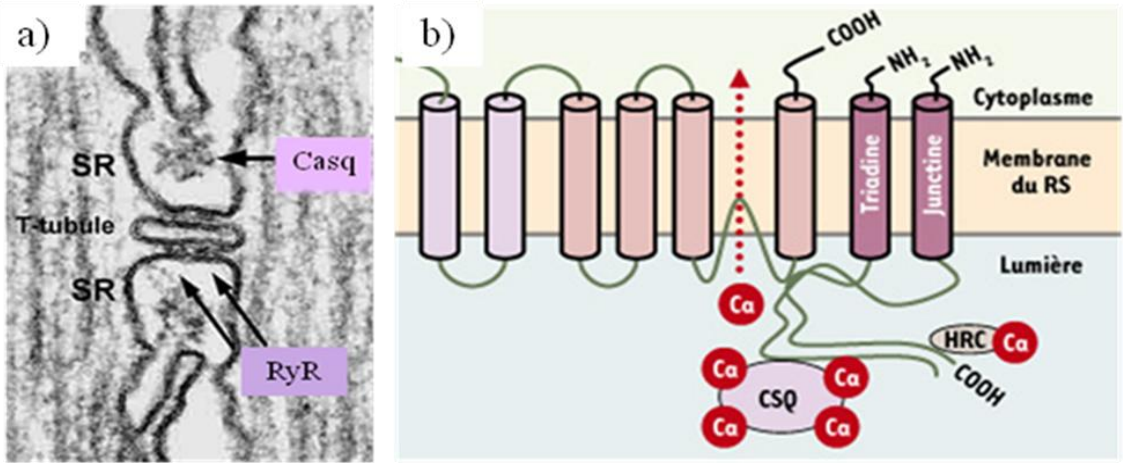


Figure (9): *Calsequestrin in the triad.* A) EM from toadfish swimbladder muscle showing Casq accumulation in the lumen of SR, note the cytoplasmic domains of RyRs (the feet), (from Franzini-Armstrong C.). B) Casq association to the SR membrane via junction, (from Lacompagne A.). Note the interaction of triadin and junctin with the C-terminal loop of RyR.

Casq contains a large number of acidic amino acids that permit it to coordinately bind 40-50 Ca^{2+} molecules (MacLennan et al. 1971). By binding to free calcium, Casq increases the capacity of SR. This Ca^{2+} binding is under the control of RyR, as the activation of RyR induces Ca^{2+} dissociation from Casq, which is then released by RyR channel (Ikemoto et al. 1991). On the other hand, Casq activates RyR channel by increasing the open probability (Kawasaki et al. 1994), (Ohkura et al. 1998). An interesting model has suggested that Casq is always associated to RyR and the phosphorylation state of Casq determines the amount of Ca^{2+} released (Herzog et al. 2000). The phosphorylation of Casq at low luminal [Ca^{2+}] enhances junctin binding and increases its Ca^{2+} binding capacity (Beard et al. 2008).

Additional information about the role of Casq1 has emerged by the study of the mutant mice. Although the *Casq1*-KO mice are viable and fertile, they exhibit several functional and structural changes (Paolini et al. 2007). As expected, deletion of Casq1 has a predominant effect on fast twitch muscles. Even though force development is not affected, the time required for maximal contraction or relaxation is prolonged (Paolini et al. 2007). Ca^{2+} imaging revealed a decrease in Ca^{2+} release, which consists with the role of Casq in RyR activation and in SR loading capacity (Paolini et al. 2007).

I-2-D-ii. *Triadin and junctin*

Triadin (95 kDa) and junctin (26 kDa) belong to the family of the single membrane-spanning endoplasmic reticulum proteins (Knudson et al. 1993), (Jones et al. 1995). The transmembrane domain separates the protein into a small N-terminal cytoplasmic domain and a long SR luminal domain (figure 9b) (Knudson et al. 1993), (Jones et al. 1995). They are inserted at the junctional face of terminal cisternae, where they interact with triadic components. Triadin binds to the RyR and DHPR (Caswell et al. 1991), while junctin binds to RyR and calsequestrin (Zhang et al. 1997), (Wei et al. 2009).

Studies addressing the role of triadin and junctin in Ca^{2+} release report conflicting results. On the one hand, Ohkura and co-workers have shown by the [H^3]-ryanodine binding assay, that triadin generates an inhibitory effect on RyR and even antagonize the stimulatory effect of calsequestrin through interfering its association to the junctional face of the membrane (Ohkura et al. 1998), (Guo et al. 1995). On the other hand, Wei *et al.* have demonstrated that triadin and junctin activate the RyR channel (Wei et al. 2009). In addition, it has been suggested that binding of triadin to RyR is important to rapid calcium release (Goonasekera et al. 2007).

I-2-D-iii. *Calmodulin*

Calmodulin (**cal**cium-dependent **modul**ator prote**in)** is ubiquitously expressed and highly conserved in vertebrates (Friedberg 1990). It is implicated in many cellular processes through its interaction with a wide spectrum of proteins (Crivici et al. 1995). Wagenknecht and co-workers have demonstrated by 3D-reconstitutions, that CaM binds to the cytoplasmic domain of the RyR channel (figure 10) (Wagenknecht et al. 1997).

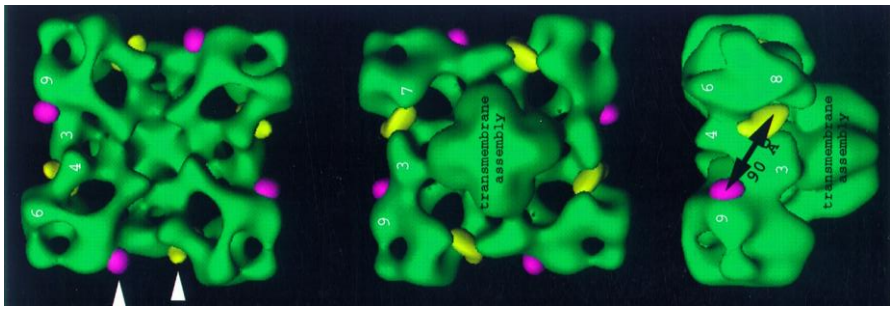


Figure (10): *Solid-body representations of 3D reconstruction of RyR with CaM (yellow) and FKBP (pink).* The cytoplasmic face (left), the sarcoplasmic face (center) and a side view (right), (from Wagenknecht T., JBC, 1997).

Tripathy and coworkers have shown using single-channel measurements that CaM regulates RyR activity depending on cytosolic Ca^{2+} concentrations, where it enhances RyR opening at low $[Ca^{2+}]c$ (< 0,2 µM) and inhibits RyR opening at high calcium $[Ca^{2+}]c$ (> 50 µM). CaM modulates RyR channel opening by reducing the mean duration of channel opening and/or the number of single open events (Tripathy et al. 1995).

CaM can also regulate DHPR activity in a similar manner to RyR1. Peterson and co-workers demonstrated that upon intracellular Ca^{2+} increase, Ca^{2+} binds to CaM and induces a conformational change, that leads to DHPR inhibition as CaM associated to DHPR (Peterson et al. 1999). This inhibition, which is mediated by CaM binding to the C-terminal tail of the DHPRα1 subunit, would suggest that CaM acts as a Ca^{2+} sensor for Ca^{2+}-dependent inactivation of DHPR (Peterson et al. 1999).

These findings indicate that CaM binds to both RyR and DHPR to induce their activation at low cytosolic Ca^{2+} levels and their inactivation at high cytosolic Ca^{2+} concentrations resulting in a feedback mechanism that controls SR Ca^{2+} release.

I-2-D-iv. *FKBP12*

FKBP12 was identified in 1992 by Jayaraman and co-workers as a protein of 12 kDa binds to the immunosuppressant FK506 (Jayaraman et al. 1992). Using electron cryo-microscopy followed by 3D reconstruction, it was shown that FKBP12 binds to the periphery of the cytoplasmic face of RyR complex and that each RyR subunit contains one binding site for FKBP12 (figure 10) (Wagenknecht et al. 1997). FKBP12-KO mice die during embryogenesis or neonatal life due to severe dilated cardiomyopathy with ventricular septal defects (Shou et al. 1998).

FKBP12 role on RyR regulation was elucidated by single channel measurement, which has showed that FKBP12 association has an inhibitory effect on channel function by reducing open probability and decreasing mean open time of RyR, which leads to RyR stabilization (Ahern et al. 1994), (Brillantes et al. 1994). Removal of FKBP12 gives rise to subconductance states, which are characterized by open events with abnormally reduced current amplitude (Ahern et al. 1994), (Brillantes et al. 1994). In addition, FKBP12 plays a unique role in the synchronization of the action of neighboring RyR channels which is termed "coupled gating" (Marx et al. 1998).

I-2-D-v. *Phospholamban and sarcolipin*

PLN (52 aa) and SLN (31aa) are functionally homologous to each other, and are encoded by two different genes, *Pln* and *Sln* genes (Fujii et al. 1987), (Odermatt et al. 1997). PLN and SLN are alpha-helical proteins inserted in the SR membrane (figure 11a) (Hellstern et al. 2001). The insertion of SLN in SR membrane depends mainly on its interaction with SERCA but, in the absence of SERCA, can also be inserted in SR via a targeting signal located in the highly conserved C-terminal region (Gramolini et al. 2006). Contrary to PLN, SLN expression varies among species. PLN protein is abundant in ventricles and atria of small and big mammals, whereas SLN protein is found in atria of small mammals but in skeletal muscle (in both fast and slow twitch muscles) of big mammals (Vangheluwe et al. 2005).

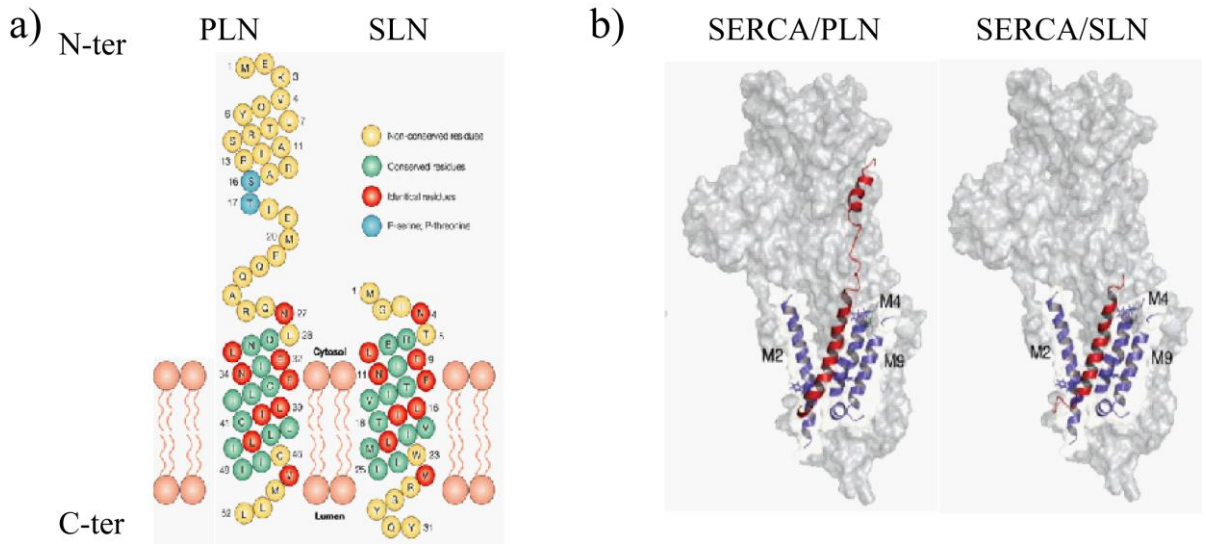


Figure (11): *Primary structure of PLN and SLN and their position in a complex with SERCA pumps.* a) primary structure of PLN and SLN shows the conserved residues between both proteins (modified from D. H. MacLennan & E. G. Kranias, Nat Rev, 2003). b) Ribbon representation of SLN and PLN (red) and their position in SERCA/SLN and SERCA/PLN complexes. This representation demonstrates that these two SERCA regulators occupy the same interaction site, (from D. H. Maclennan and C. Toyoshima).

The study of SERCA regulation by SLN or/and PLN has been addressed by overexpression experiments and transgenic models. SLN deficient mice appear normal and proteomics analysis of the mutant heart showed that the expression level of genes involved in the Ca^{2+} handling machinery was unaffected (Babu et al. 2007). However, functional analysis of atria revealed an enhanced Ca^{2+} uptake, which results in enhanced contractility, suggesting

that SLN inhibits SERCA by decreasing its affinity to Ca^{2+} (Babu et al. 2007). The overexpression of SLN in soleus muscle showed that it induces a reduction in Ca^{2+} uptake, and when SLN and PLN were co-expressed together with SERCA1a or SERCA2a, they induced a super-inhibition state (Tupling et al. 2002), (MacLennan et al. 2003).

SLN and PLN activity is regulated by phosphorylation. In fact, SLN is phosphorylated by serine/threonine kinase 16 (Gramolini et al. 2006). PLN, in turn, is phosphorylated by cAMP dependent kinase (PKA) and Calmodulin dependent kinase (CaMKII), (Kirchberber et al. 1975), (Kranias 1985). SLN and PLN phosphorylation removes the inhibitory effect on SERCA and increases calcium uptake (Kranias 1985), (Gramolini et al. 2006).

I-3. Human diseases related to mutations in RyR/DHPR/SERCA genes

Mutations within DHPR, RyR and SERCA are at the origin of several muscle disorders, which are characterized by structural and/or physiological defects. In this section, the most important physiological and genetic findings will be briefly presented.

I-3-A. Diseases related to RyR1

I-3-A-i. *Malignant hyperthermia (MH):* is an autosomal dominant genetic disease, which was first described in 1960 for a familial case presenting an hypermetabolic status and hyperthermia with sustained muscle contraction (Denborough 1998). This occurs upon anesthesia with suxamethonium and/or volatile halogenated anesthetic agents (Denborough 1998). The association between MH and RyR mutations is reported in pigs and humans (Fujii et al. 1991), (MacLennan et al. 1990). Two mutation hot spots are identified in the RyR1 gene, the central region corresponding to an interaction domain with FKBP12 (regulator of RyR function) and the N-terminal region, which is implicated in the regulation of channel function (Monnier et al. 2000) (figure 12).

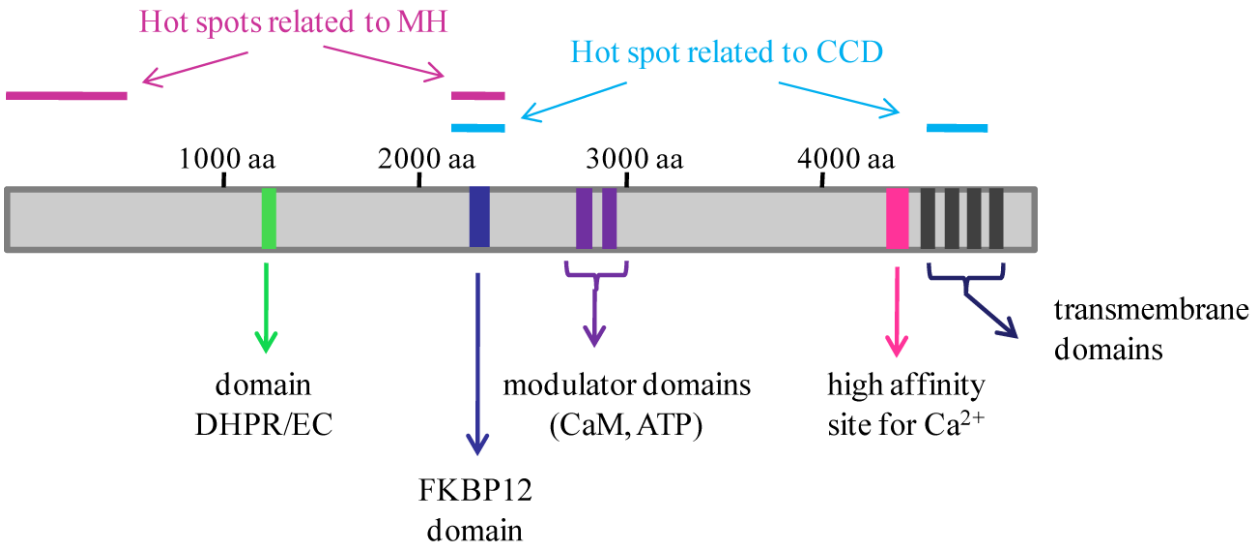


Figure (12): *Schematic representation of RyR1 protein with the most important functional domains and the hotspots*. MH: malignant hyperthermia, CCD: central core disease, (modified from N. Monnier, J. Lunardi, 2000, Annales de biologie Clinique).

In MH patients, the mutations within RyR1 generate a hyperexcitability state, i.e. an increased sensitivity to activator agents (cafein, halothane and activating concentration of

Ca^{2+}) and decreased sensitivity to inhibitor conditions (inhibitor concentrations of calmodulin and calcium) (Richter et al. 1997). As a consequence, these mutations lead to a rapid and sustained increase in myoplasmic Ca^{2+}**,** resulting in long contractures.

I-3-A-ii. *Central core disease (CCD):* is a rare inherited disease with dominant or recessive transmission. It is non-progressive myopathy presenting in infancy and characterized by hypotonia and proximal muscle weakness (Zhang et al. 1993), (McCarthy et al. 2000). Histological examination of muscle biopsies from CCD patients reveals the presence of areas with reduced oxidative activity in type 1 (slow) muscle fibers, the "central cores" , which extend through the entire fiber length (figure 13). Some CCD Patients are susceptible to MH episodes (Dirksen et al. 2002), (Denborough et al. 1973).

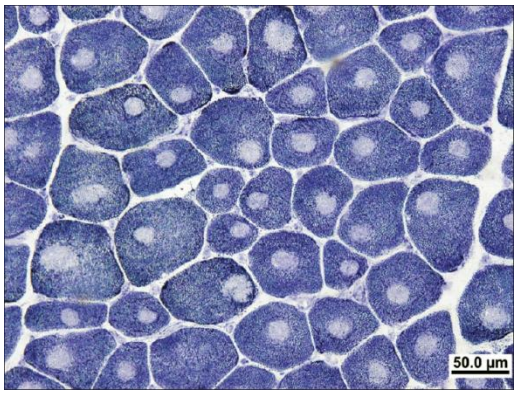


Figure (13): *Muscle biopsy from a patient with central core disease: NADH-TR staining, transverse muscle section.* Predominance of type1 fibers with cores in virtually all fibers, (from Chieko Fujimura-Kiyono, 2008, Neurology India).

Mutations in the C-terminal region of the *Ryr1* gene are related mainly to CCD (Monnier et al. 2000). It is likely that these mutations lead to muscle weakness and cores development by decreasing SR Ca^{2+} release. This may happen either by an increased activation of the RyR1 channel, which leads to excessive leakage of Ca^{2+} in the cytoplasm (leaky channel hypothesis), or by reducing the coupling between RyR1 and DHPR (EC coupling hypothesis). Both cases lead to decrease in voltage dependent calcium release. (Dirksen et al. 2004), (Lyfenko et al. 2007).

I-3-A-iii. *Multi-minicore disease (MmD):* is a congenital myopathy with an autosomal recessive transmission. Muscle biopsy revealed the presence of multiple core lesions, which extend through a few sarcomeres (Engel et al. 1971), (Ferreiro et al. 2002). It

has been reported that MmD is due to mutations in RyR1 (Ferreiro et al. 2002). Moreover, some of MmD cases can represent an early stage of the autosomal recessive CCD.

Moreover, mutation within RyR1 is related to **centronuclear myopathy** as documented in the next chapter (Jungbluth et al. 2007). In addition to mutations within the *Ryr1* gene, aberrant splicing of the RyR1 pre-mRNA has been reported in **myotonic dystrophy** (MD) (Kimura et al. 2005), a disease caused by a CTG repeat expansion in the MDPK (myotonic dystrophy protein kinase) gene (Brook et al. 1992).

I-3-B. Diseases related to DHPR

I-3-B-i. *Malignant hyperthermia (MH):* Mutations in the *Cacna1s* gene and the *Cacna2* gene (encoding for the alpha 1 and alpha 2 subunits of DHPR, respectively) are reported to be related to MH (Monnier et al. 1997), (Iles et al. 1994). Functional characterization of the R1086H mutation at the III-IV loop of DHPR alpha 1 subunit (figure 14) revealed that this mutation leads to an increase in RyR1 sensitivity by abolishing the inhibitory effect of DHPR III-IV loop on RyR1 (Weiss et al. 2004).

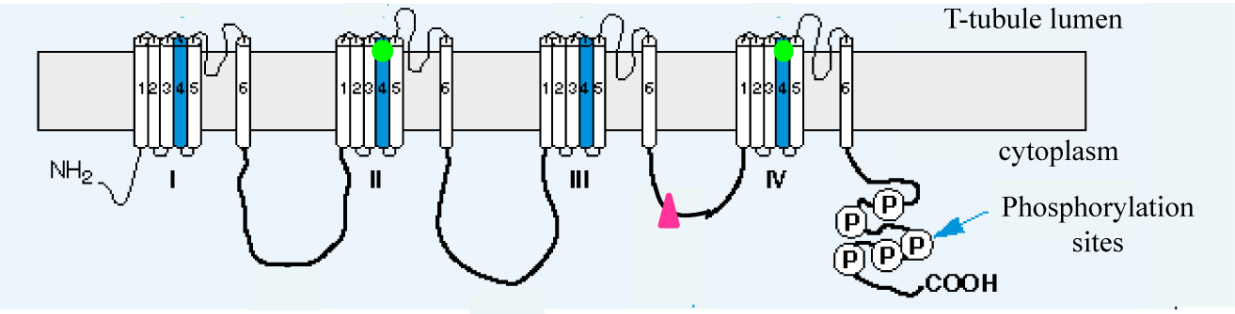


Figure (14): *Schematic representation of the structure of the skeletal muscle DHPR alpha1 subunit with disease causing mutations.* The four units of homology are displayed linearly; each unit contains six transmembrane segments, represented here by cylinders. The segment S4 of each unit (in blue) represents the voltage sensor. The purple mark indicates the R1086H mutation associated to MH and the green marks indicate mutations associated with HypoPP-1, (modified from N. Monnier, J. Lunardi, 2000, Annales de biologie Clinique).

I-3-B-ii. *Hypokalemic periodic paralysis (HOKPP):* is an autosomal dominant muscle disease characterized by acute and reversible attacks of muscle weakness. This may happen because of the ingestion of a meal rich in carbohydrates or the rest after exercise (Elbaz et al. 1995). HOKPP is due to mutations in the alpha subunit of DHPR, more

specifically, in the transmembrane segment S4 in domain II or IV of the alpha subunit of DHPR (figure 14) (Jurkat-Rott et al. 1994), (Elbaz et al. 1995). These mutations lead to a decrease in the DHPR capability to sense depolarization as the S4 segment of each domain is involved in the voltage sensing, which results in reduced calcium current (Sipos et al. 1995).

I-3-C. Diseases associated to SERCA gene family members

Brody myopathy: mutations within SERCA1a and 1b lead to the rare autosomal recessive Brody myopathy (Odermatt et al. 1996). Patients manifest exercise-induced impairment of skeletal muscle relaxation with stiffness and over-shortening of muscles (Brody 1969). Interestingly, SERCA $1^{-/-}$ mice exhibit slow limb movements and contractures similar to that observed in Brody patients (Pan et al. 2003). Electron microscopy examination of the mutant murine diaphragm revealed the presence of fibers with prominent hypercontraction regions of the myofibrils (Pan et al. 2003). Patients with Brody disease have a normal lifespan, as the function of calcium pumping is partially fulfilled by SERCA2a. Contrary to what is observed for human, mouse SERCA1 function is not compensated by another Ca^{2+} pumps. Thus its deletion results in a dramatic reduction in Ca^{2+} reuptake and consequent impaired skeletal muscle relaxation (Pan et al. 2003).

II. Centronuclear myopathies

Congenital myopathies are a group of muscular disorders characterized by early onset and a wide spectrum of clinico-pathological features. They are classified into several subgroups depending on clinical, pathological and molecular aspects; one of these subgroups includes centronuclear myopathies (CNM), rare genetic muscle diseases (Sewry et al. 2008). CNM myopathies will be described in this chapter, and the proteins implicated in these diseases will be discussed.

II-1. Clinical description and genetic of centronuclear myopathy

The clinical picture of CNM is variable depending on the implicated gene and the causative mutation. However, all forms share some common features. Skeletal muscle represents the primary affected tissue and muscle weakness can be either progressive or non-progressive, being generalized or restricted to specific muscles. In addition, serum creatine kinase (CK) level is normal or slightly increased. The pathological hallmark of CNM is the presence of a high proportion of hypotrophic myofibers with central nuclei and abnormal distribution of oxidative enzyme activity as shown by NADH-tetrazolium reductase (NADH-TR) staining, which is either central or radial instead to be homogenous (Wallgren-Pettersson et al. 1995), (Zanoteli et al. 2009). Type 1 oxidative fibers are predominant and signs of necrosis or excessive regeneration are usually absent in all forms of centronuclear myopathies. In contrast to X-linked CNM, the autosomal forms are rarely fatal in childhood (Jungbluth et al. 2008), (Pierson et al. 2005).

II-1-A. Autosomal dominant centronuclear myopathy (AD-CNM)

The autosomal dominant CNM form is due to mutations within the dynamin 2 gene (*DNM2*) (Bitoun et al. 2005), (Bitoun et al. 2007), a ubiquitously expressed large GTPase involved in endocytosis and membrane tubulation (Warnock et al. 1996), (see (Praefcke et al. 2004) for review). Muscle weakness in patients appears usually during childhood or adulthood but some neonatal cases have been reported (Bitoun et al. 2005), (Bitoun et al. 2007). Patients present relatively mild clinical symptoms summarized by slow progressive, distal or proximal, muscle weakness and wasting. Facial hypotonia with mild or severe ptosis is a frequent feature (figure 15a) (Schessl et al. 2007), (Zanoteli et al. 2000), (Fischer et al. 2006). Some clinical cases present cardiac or respiratory defects (Bataille et al. 1991),

(Zanoteli et al. 1998). Muscles contain hypotrophic type 1 fibers with the characteristic presence of radial sarcoplasmic strands surrounding the perinuclear halo, in particular, in adult cases (Jeannet et al. 2004) (figure 15c).

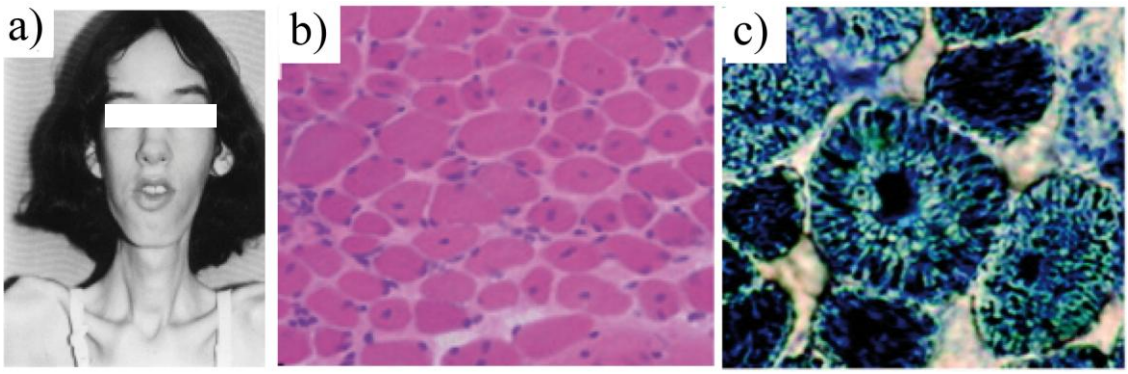


Figure (15): *The main characteristic of AD-CNM pathology:* a) A patient with DNM2 mutation with childhood onset, not the typical long, thin, and triangular face with mild ptosis. (from Zanoteli, 1998, J Neurol Sci). b,c) muscle biopsy from DNM patient with early onset show the centrally located nuclei by hematoxylin-eosin staining and the radial distribution of the sarcoplasmic reticulum, (from Bitoun, Ann Neurol, 2007).

II-1-B. Autosomal recessive centronuclear myopathy (AR-CNM)

The onset of the AR-CNM ranges between birth and childhood with patients exhibiting mild, proximal, slowly progressive muscle weakness. One implicated gene is *BIN1* which encodes amphiphysin 2 (Nicot et al. 2007), a protein with a role in endocytosis in neuronal tissues (David et al. 1996), and in the organization of transverse tubules in skeletal muscle (Lee et al. 2002). Interestingly, in *Bin1*-KO mice no defect in skeletal muscle was reported. However, a severe cardiomyopathy was responsible for the lethal phenotype of murine *BIN1* deficiency (Muller et al. 2003). Despite these observations, no cardiac defects are observed in patients (Nicot et al. 2007). Muscle biopsies from patients show type 1 predominance and accumulation of NADH-TR-positive-staining regions around the central nuclei (Nicot et al. 2007) (figure 16).

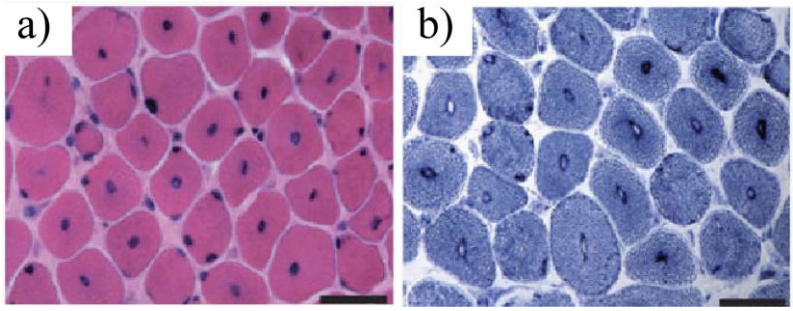


Figure (16): *Muscle biopsy from a patient shows the main characteristic of AR-CNM.* a) The central nuclei are present in virtually every fiber (seen by hematoxylin-eosin staining, HE). b) The NADH-TR staining of muscle shows that centrally located nuclei are surrounded by a rim of darkly stained material, (from Nicot AS. *et al*. Nature Genetics, 2007).

II-1-C. X-linked myotubular myopathy (XLMTM)

The X-linked form of centronuclear myopathy affects 1 of 50 000 newborn males (Jungbluth et al. 2008). Males suffering from XLMTM are born with generalized hypotonia and respiratory difficulties (figure17a), and the majority of them are completely or partially ventilator-dependent after birth and during early childhood. Polyhydramnios and reduced fetal movements are frequent during gestation. Muscle weakness is non-progressive and no defects in cognitive development have been observed (Wallgren-Pettersson et al. 1995). In addition to the muscle involvement, non-muscular complications have been reported in long-term XLMTM survivors (>1 year of age), including renal and hepatic dysfunction and coagulation defects (Herman et al. 1999). Most cases present neonatally, however muscle weakness appears in some cases late during the life (Hoffjan et al. 2006). The gene responsible for the disease is *MTM1*, which encodes for a protein known as myotubularin (Laporte et al. 1996). More than 200 *Mtm1* mutations have been associated to XLMTM, which are distributed along the coding sequence (Laporte et al. 2000), (Biancalana et al. 2003). MTM1 is the founder member of a large family of lipid phosphatases, the myotubularin family, and may be implicated in membrane-associated processes (Tsujita et al. 2004), (Buj-Bello et al. 2008). MTM1 can catalyze two substrates both *in vivo* and *in vitro*, PI3P and PI3,5P2 by removing the phosphate from the D3 position to produce PI and PI5P (Blondeau et al. 2000), (Schaletzky et al. 2003).

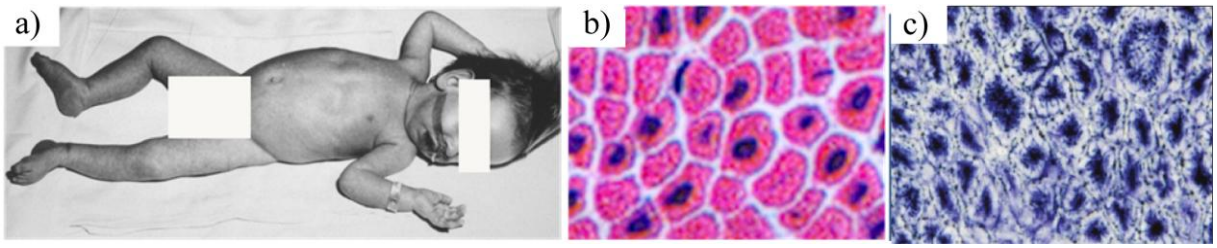


Figure (17): *The major pathological features of X-linked centronuclear ("myotubular") myopathy.* a) Newborn patient with mutation in the myotubularin *(MTM1)* gene. Note the generalized hypotonia and the inverted V-shaped mouth (from MedLink Neurology). b) HE staining showing rounded muscle fibers with central nuclei. c) NADH-TR staining showing the accumulation of the mitochondria forming a halo around the nucleus in the center of the fiber, (from Chieko Fujimura-Kiyono, Neurology India, 2008).

Finally, although carrier females are usually asymptomatic, some of them exhibit a mild muscle involvement due to additional genetic defects such as skewed X-inactivation (Wallgren-Pettersson 2000), (Kristiansen et al. 2003) or structural X-chromosomal abnormalities (Dahl et al. 1995).

In addition to these three genes mutated in centronuclear myopathies, some sporadic cases indicate the involvement of other genes. Jungbluth et al. has reported the case of a 16 year-old female with a *de novo* dominant mutation in the ryanodine receptor 1 gene (*RYR1*), a key player in the excitation–contraction coupling mechanism of skeletal muscle (Block et al. 1988), (Nakai et al. 1997). This patient exhibits the characteristics of AD-CNM, with the addition of complications during embryonic life such as polyhydramnios and reduced fetal movements. The muscle biopsy from this patient differs from those of typical AD-CNM, because of the absence of radial distribution of the sarcoplasmic reticulum and presence of both hypotrophic type II and hypertrophic type I fibers (Jungbluth et al. 2007). Another study has reported the implication of a novel member of the myotubularin family in CNM (hJUMPY/ MTMR14). MTMR14 acts as a PtdIns3P and PtdIns(3,5)P2 3-phosphatase *in vitro* and *ex vivo* (Tosch et al. 2006). The reported mutations affect the enzymatic function of the lipid phosphatase. The mode of disease inheritance and pathogenic involvement of this mutation remain unclear (Tosch et al. 2006).

II-2. Proteins implicated in centronuclear myopathies

II-2.-A. Dynamin

II-2-A-i. *The dynamin family*

Dynamin 2 (DNM2) is a member of the dynamin superfamily of GTPases that play a role in membrane tubulation and fission. This returns to three virtues, first, their GTPase–dependent oligomerization, second, their low GTP and GDP binding affinity, and finally the lipid membrane association of some members (Praefcke et al. 2004). DNM2 belongs to the classical family of dynamins. In mammals, this family contains three genes; *DNM1*, which is expressed exclusively in neurons, where it is mainly associated with membranous organelles (Nakata et al. 1991), (Noda et al. 1993), *DNM2,* which is ubiquitously expressed (Cook et al. 1994), and *DNM3* that is expressed in testis, brain, lung (Nakata et al. 1993), (Cook et al. 1996).

Classical DNMs display three essential structural domains (figure 18), a large GTPase domain (about 300 amino acids) located at the N-terminal region, a central MID domain and the C-terminal GTPase effector domain (GED). This minimal structure is supported by additional auxiliary domains involved in the targeting of DNMs to special membrane compartments. Such domains include the lipid binding Pleckstirn-Homology (PH) domain, which binds to PI(4,5)P2 and the Proline-Rich Domain (PRD), which binds to SRC-Homology-3 (SH3) containing proteins including amphiphysin (David et al. 1996), profillin (Witke et al. 1998) and syndapin 1 (Qualmann et al. 1999), members of the membrane remodeling system (Praefcke et al. 2004). Most of the mutations described in *Dnm2* leading to CNM are located within the PH and the MID domains (Bitoun et al. 2005), (Bitoun et al. 2007).

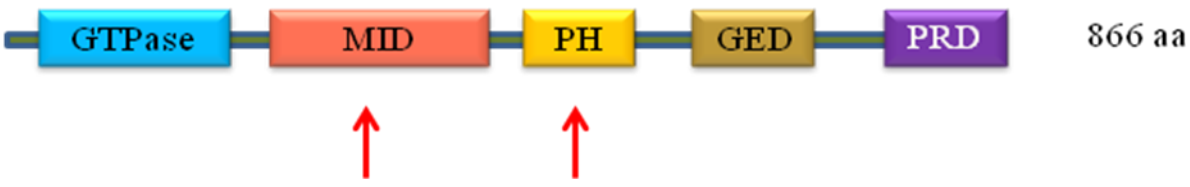


Figure (18): *Schematic representation of Dynamin 2 protein domains.* Arrows indicate the domains with the mutations implicated in human disease (AD-CNM and dominant intermediate Charcot-Marie-Tooth neuropathy (DI-CMT).

In addition to its implication in CNM, *Dnm2* mutations are implicated in CMT. Mutations within the PH domain of DNM2 cause the dominant intermediate CMT form (DI-CMTB) (Zuchner et al. 2005), and the dominant axonal CMT form (CMT type 2) (Fabrizi et al. 2007), (Bitoun et al. 2008). Generally, in all forms of CMT, patients present distal muscle weakness with sensory dysfunction.

II-2-A-ii. *Regulation of dynamin's GTPase activity*

Dynamin GTPase activity is enhanced by self-oligomerization, which is in contrast to small GTPases that require guanine nucleotide-exchange factors (GEFs) to stimulate the GTPase function. Self-oligomerization of DNM is mediated by the interaction of the GED domain with the GTPase and MID domains in intra-chain and in inter-chain reactions (Muhlberg et al. 1997), (Zhang et al. 2001). Interestingly, oligomerization in turn is regulated by membrane recruitment of DNM to its site of action (David et al. 1996), and this step is fulfilled by the DNM PRD domain binding to the SH3 domain of amphiphysin (David et al. 1996). In addition, the PRD domain acts as a negative regulator on GTP hydrolysis, and binding to amphiphysin may overcome the inhibitory effect of the PRD (Zhang et al. 2001). It has been suggested that the GTPase activity of DNM can determine the ability of the enzyme to tubulate liposomes and/or lead to membrane fission. This would suggest that at slow hydrolysis activity, the enzyme can tubulate membranes, whereas at high GTPase activity it can pinch them (Praefcke et al. 2004) (figure 19).

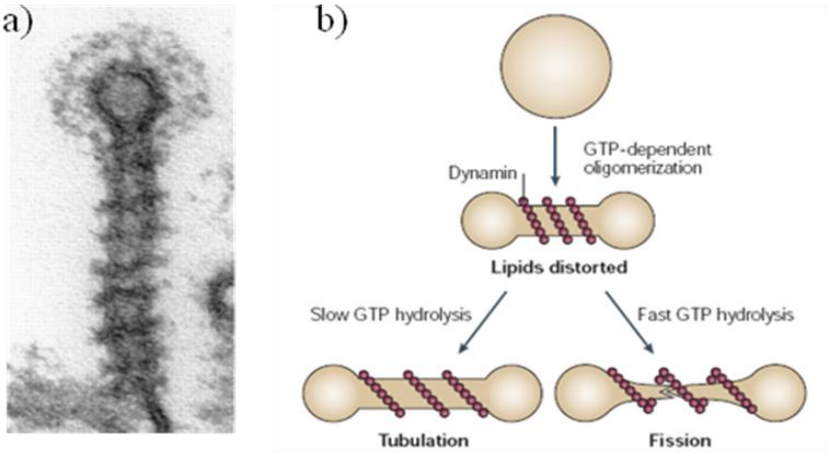


Figure (19): *Dynamin tubulation of cellular membrane*. a) Micrograph of long invaginating tubules decorated with dynamin collars and connected to a terminal clathrin-coated pit. This structure is observed in synaptosomes treated with GTPγS, this non hydrolysable GTP locks dynamin in the GTP-bound state and prevents him to perform membrane fission (from Takei et al., Nature, 1995). B) A

schematic view of dynamin binding to a lipid vesicle, which leads to the formation of a helix on a lipid tube. Slow or uncooperative GTPase activity leads to an extension of the helix, which results in stretching and subsequent tubulation. Fast and cooperative dynamin GTPase activity leads to a rapid extension of the helix and fission of the tube, (from Praefcke GJK. And McMahon HT. 2004).

II-2-A-iii. *Cellular roles of Dynamin 2*

II-2-A-iii-a. Dynamin and its role in membrane fission

Dynamin has an established role in clathrin-mediated endocytosis (CME). The first report implicating DNM in membrane trafficking involved temperature-sensitive mutants of the dynamin 1 homologue in drosophila (*Shibire* flies). Analysis of the temperature-induced paralysis demonstrated a reversible accumulation of endocytic vesicles (Poodry et al. 1979), (Koenig et al. 1989). Deletion of *Dnm2* in mouse embryonic stem (ES) cells by homologous recombination has suggested that DNM2 is required for CME, transport of proteins from the trans-Golgi-network (TGN) to the plasma membrane and platelet-derived growth factor (PDGF)-stimulated macropinocytosis, but is not required for other endocytic pathways (Liu et al. 2008).

The process of clathrin-mediated endocytosis can be dissected into: 1) recruitment of the clathrin coat, 2) invagination of the coated membrane patch and 3) fission of the coated bud, a process that requires dynamin (David et al. 1996), (Shupliakov et al. 1997). The action of DNM begins with its recruitment to the endocytic patch via amphiphysin (David et al. 1996), (Shupliakov et al. 1997). Recruitment of DNM to membrane stimulates its oligomerization (Hinshaw et al. 1995), which in turn enhances the membrane binding capacity of DNM (Klein et al. 1998), (Lemmon et al. 2000). During this step, DNM polymers form collars around the clathrin-coated pits (figure 19) (David et al. 1996), (Sweitzer et al. 1998). Stimulation of the DNM GTPase activity via amphiphysin interaction, membrane binding and by self-assembly around the membrane invagination necks allow DNM to pinch off vesicles from the plasma membrane (Hinshaw et al. 1995), (Sweitzer et al. 1998), (Takei et al. 1999).

II-2-A-iii-b. Role for dynamins in actin nucleation

Although DNMs have an established role in endocytosis, they also have a role in actin dynamics. DNM1 has been reported to participate with profilin proteins in the regulation of actin assembly (Witke et al. 1998). In addition, DNM2 binds cortactin, an essential component of the actin assembly apparatus, where it promotes the formation and the stabilization of the actin network that drives protrusion at the leading edge of migrating cells (Weaver et al. 2001). Cortactin binds to filamentous actin (F-actin) and the ARP2/3 complex, which represents the essential molecular machine for nucleating actin filament assembly (Weed et al. 2000).

In addition, Dynamin plays a coordinator role at the interface between endocytosis and actin assembly by its interaction with the F-actin binding protein (Abp1) (Kessels et al. 2001). It has been reported that growth factor activations can result in recruitment of Abp1 to the endocytic machinery residing in dynamin-containing pits (Kessels et al. 2001).

II-2-A-iii-b. Dynamin implication in microtubules dynamics

This implication is manifested in two roles, its role in cytokinesis and in centrosome adhesion. In eukaryote cells, cytokinesis requires the concerted efforts of microtubules and the actin cytoskeleton as well as membrane remodeling to establish the cleavage furrow and complete daughter cell separation (Konopka et al. 2006). *Drosophila* and *C. elegans* express only one dynamin homologue, the *Shibire* gene and *dyn-1* respectively. *Shibire* protein localizes at the invaginated membranes within the *drosophila* embryo and mutations within its GTPase domain disrupt cellularization (Pelissier et al. 2003). In addition, DNM2 is a bona fide component of the centrosome. At the centrosome, DNM2 interacts directly with gamma-tubulin via its middle domain, where this interaction mediates centrosome cohesion (Thompson et al. 2004). The knocking down of DNM2 in rat fibroblast results in splitted centrosome (Thompson et al. 2004).

II-2-B. Amphiphysin

II-2-B-i. *The amphiphysin family*

Two amphiphysin genes (*Amph1* and *Amph2*) have been identified in mammals. *Amph1*, discovered by Lichte and coworkers in 1992, is highly expressed in nerve terminal, where it localizes to the cortical cytoplasm and participates in synaptic vesicle endocytosis (Lichte et al. 1992), (David et al. 1996). *Amph2* (also called *BIN1*) characterized by Butler *et al*. contains several alternative splicing variants. Larger variants (*N-Amph2,* N for neuronal) are similar to *Amph1*, and are predominantly detected in axon initial segments and nodes of Ranvier (Butler et al. 1997). Smaller variants (*M-Amph2*, M for muscular) lacking the clathrin binding domain, are highly expressed in skeletal muscle where they localize to the T-tubule stucture (Butler et al. 1997). In contrast to the other isoforms, the BAR domain of M-Amph2 contains stretch of basic residues which ensures its targeting to PI4,5P2, a phosphoinositide enriched in plasma membrane compartments (Lee et al. 2002).

II-2-B-ii. *Amphiphysin 2 protein domains*

Amphiphysin proteins are able to tubulate lipid membranes via their BAR (Bin/Amphiphysin/Rvs) domain. This domain is located at the N-terminus of the molecule and forms a banana shaped structure, through homodimerization, that induces membrane curvature (figure 20) (Takei et al. 1999), (Peter et al. 2004). This faculty of the BAR domain is conferred by the positively charged residues located at the concave surface of the molecule, which bind to negatively charged membranes (Peter et al. 2004). In *Bin1* related myopathy, two missense mutations have been identified in the BAR domain that affect its membrane tubulation capacity (Nicot et al. 2007).

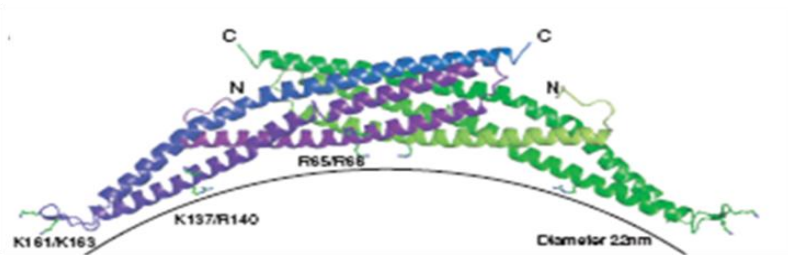


Figure (20): *Ribbon model of the BAR domain of drosophila amphiphysin.* The BAR domain is a homodimer of α-helical bundles. The two subunits are colored green and purple. The ends and oncave surface have several basic residues, some of which are shown here, (from Zimmerberg J. *et al.,* Curr Biol, 2004).

The central region of N-AMPH2 consists of a proline-rich domain (PRD), which is involved in the association with clathrin and the clathrin-adapted protein, AP-2 (McMahon et al. 1997), (Slepnev *et al.* 1998). The C-terminus region contains a SH3 domain which interacts with the PRD motif present in DNM proteins, resulting in recruitment of DNM to specific membrane compartments (McMahon et al. 1997), (David et al. 1996). Amphiphysin can tubulate membranes independently or in cooperation with DNMs (Takei et al. 1999), however, a partial truncation of the C-terminal SH3 domain leads to centronuclear myopathy. This reveals the importance of DNM2 as a scaffold protein in the stabilization of AMPH2 induced tubules, as the SH3 domain is involved in DNM2 interaction (Nicot et al. 2007), (Lee et al. 2002).

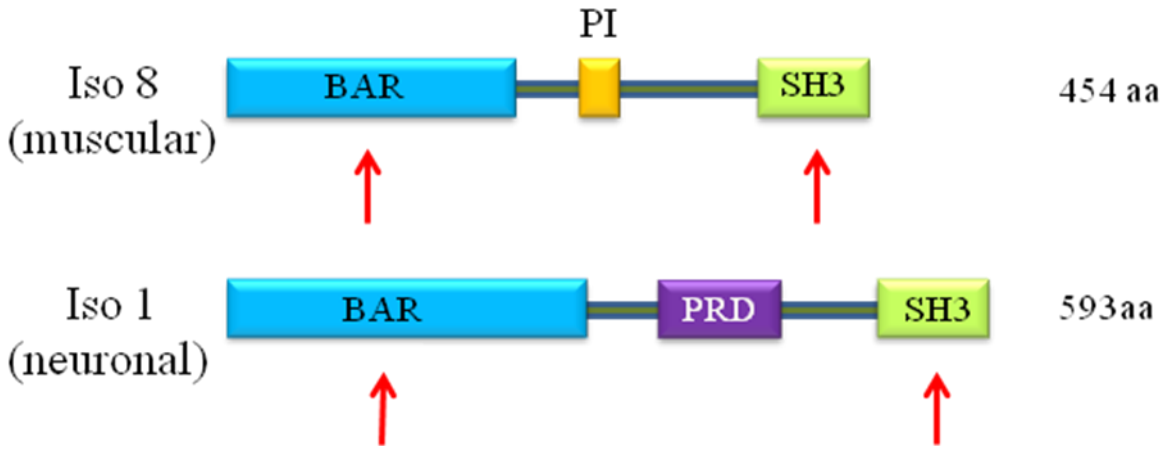


Figure (21): *Schematic representation of the muscular (Iso 8) and the neuronal (Iso 1) isoforms of amphiphysin 2 protein.* Arrows show the mutation hot spots related to the CNM.

II-2-B-iii. *Cellular roles of amphiphysin proteins*

II-2-B-iii-a. Common role of non-muscle specific and muscle specific amphiphysins

The two isoforms of amphiphysin cooperate with dynamin 2 in the endocytosis pathway. AMPH roles include:

- DNM recruitment to membrane remodeling patches (Shupliakov et al. 1997).
- Interaction with the dynamin PRD domain via the SH3 domain to unblock its inhibitory effect on DNM GTPase activity (Zhang et al. 2001).
- Membrane tubulation via the BAR domain (Takei et al. 1999).

In addition, several studies have reported a link between amphiphysin1 and a neuronal disease of autoimmune origin (Stiff-man Syndrome) that is associated with breast cancer (De Camilli et al. 1993).

II-2-B-iii-b. The role of muscle-specific amphiphysin 2

Mutations within the *Amph* gene in *Drosophila melanogaster* render the flies flightless (Razzaq et al. 2001). This is not due to synaptic transmission defects as was expected, but to a severe structural disorganization of the excitation-contraction coupling machinery in skeletal muscle and in particular, of transverse-tubules (T-tubules) (Razzaq et al. 2001).

The importance of AMPH2 in T-tubule biogenesis was elucidated by Lee *et al* in studies employing C2C12 cells. In this cell line, upon differentiation into myotubes, plasma membrane levels of PI4,5P2 are increased by 10 fold. This results in enrichment of M-Amph2 at the plasma membrane where it can bend membrane in cooperation with partner proteins (Lee et al. 2002). In the *Bin1* mutant mice, newborn homozygous KO pups die due to a cardiac hypertrophy, and do not exhibit a defect in synaptic junction (Muller et al. 2003). Electron microscopy (EM) analysis of KO cardiac muscle revealed that the sarcomere structure was severely affected, with diffuse Z-line and lack of the A-band (Muller et al. 2003). In contrast to *Drosophila* mutants, mice deficient for *Amph2* show no alteration in the ultrastructure of skeletal muscle fibers (Muller et al. 2003). Interestingly, amphiphysin 2 is located in the cytosol of skeletal muscle but it is predominantly nuclear in cardiac muscle (Muller et al. 2003). AMPH2 was shown to interact with c-Myc (Elliott et al. 1999), and as c-Myc overexpression leads to cardiac hypertrophy (Jackson et al. 1990), AMPH2 is proposed to have a regulatory role in heart development (Muller et al. 2003).

II-2-C. Myotubularin

II-2-C-i. *Myotubularin (MTM) family*

MTM1 is the founder member of a large family of phosphoinositide phosphatases (figure 22), the MTM family that belongs to the protein tyrosine phosphatase (PTP) superfamily (Laporte et al. 1996). Myotubularin family has one member present in yeast (YMR1 or spMTM) (Laporte et al. 1996). In human, there are 15 members named MTM1 and MTM-related 1 to 14 (MTMR1-14) (Laporte et al. 2003), (Tosch et al. 2006). These members are divided into six phylogenetic subgroups, which share common functional and/or structural features (figure 22). Each subgroup is represented in *Caenorhabditis elegans* and *D. melanogaster* by one member (Laporte et al. 2003).

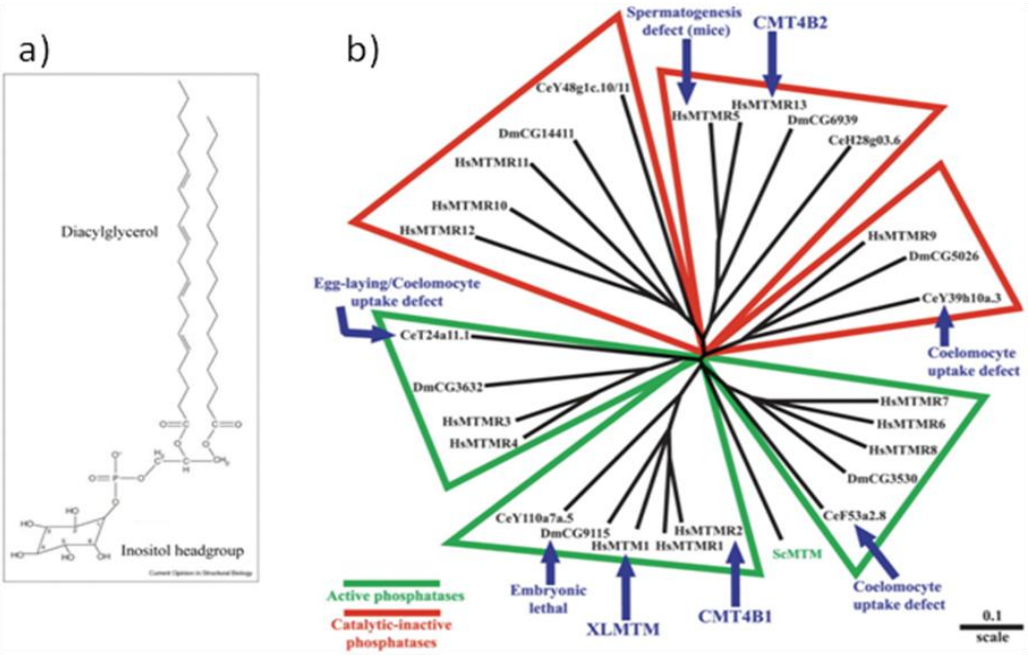


Figure (22): *Phosphoinositide structure and phylogenetic relationship of myotubularins.* a) Chemical structure of phosphatidylinositol (PtdIns) which is composed of fatty acid tail and an inositol head group, both are connected by a diacylglycerol link. The hydroxyls at positions D-3, D-4 and D-5 of the inositol headgroup can be phosphorylated (from Begley MJ. Curr Opin Struct Biol. 2005). b) Myotubularins in higher eukaryotes are classified into six subgroups with one protein for each subgroup in *Drosophila melanogaster* and *Caenorhabditis elegans*. Phenotypes related to myotubularins loss-of-function in human genetic disorders and animal models are indicated: Charcot–Marie–Tooth type 4B1 and 4B2 (CMT4B1 and CMT4B2 neuropathies), X-linked myotubular myopathy (XLMTM). (Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Sc, *Saccharomyces cerevisiae*), (from Laporte, J. *et al*. Hum. Mol. Genet. 2003).

In mammals, the MTM family members appear to be ubiquitously expressed with the exception of MTMR7, which is restricted to brain (Laporte et al. 1998), and MTMR14, which is expressed in skeletal and cardiac muscle (Shen et al. 2009). Several MTM members are implicated in human disease, *MTM1* is mutated in XLMTM (Laporte et al. 1996), *MTMR2* and *MTMR13* mutations lead to CMT4B1 and CMT4B2 neuropathies, respectively (Bolino et al. 2000), (Senderek et al. 2003), and *MTMR14* mutations which may lead to CNM (Tosch et al. 2006), (Shen et al. 2009).

II-2-C-ii. *Myotubularin protein domains*

The characteristic feature of the MTM family is the PTP active site. The PTP contains three catalytically essential residues, the **cystein** and the **arginin** in the CX5R motif, which forms a loop at the base of the substrate binding pocket, where they dephosphorylate the substrate, and the **aspartate** residue, usually located near the top of the substrate pocket and is involved in the release of the dephosphorylated product (Begley et al. 2005). Interestingly, some myotubularins contain substitutions of one or more of these amino acids, which render them dead (inactive) phosphatases (Laporte et al. 2003). The presence of dead phosphatases early in the evolution suggests that they have an important role independent of the phosphatase function (Begley et al. 2005), (Laporte et al. 2003). Cooperation between catalytically active (9 members) and dead (6 members) myotubularins has been proposed. To date, four active-inactive pairs have been identified, MTM1 and MTMR12 (Nandurkar et al. 2003), MTMR2 and MTMR5 (Kim et al. 2003), MTMR2 and MTMR13 (also known as SBF2) (Robinson et al. 2005), and MTMR7 and MTMR9 (Mochizuki et al. 2003). The inactive myotubularins may play the role of adaptors, regulating the intracellular localization of the active MTM, as it is the case for MTMR5 and MTMR12 inactive phosphatases, or may enhance the catalytic activity of active partners, as demonstrated for MTMR13 and MTMR9 inactive phosphatases (Begley et al. 2005).

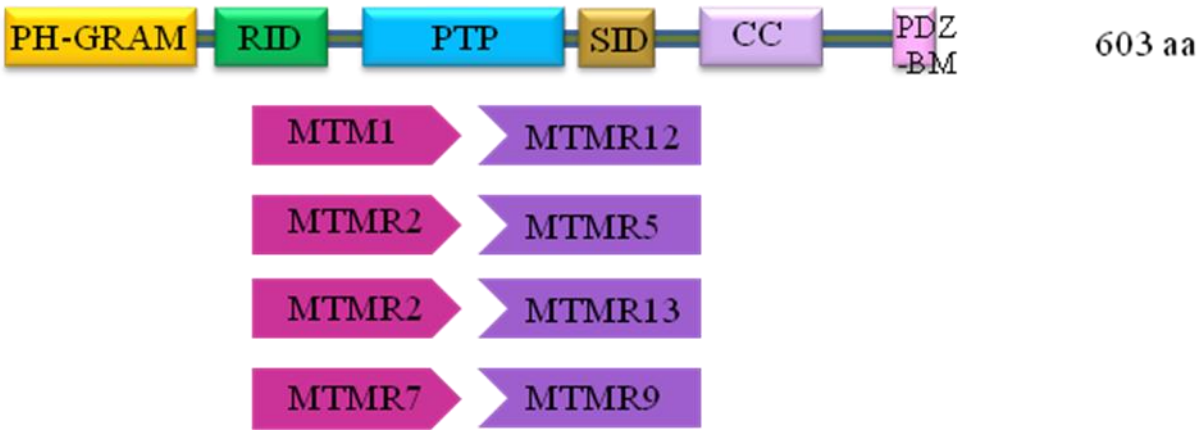


Figure (23): *Schematic representation of MTM1 protein domains and the four active (purple)-inactive (violet) identified pairs of myotubularin.*

In addition to the PTP domains, five other domains have been identified in MTM1, the PH/GRAM, RID, SID, the coiled-coil (CC) and PDZ-BM. The **PH/GRAM** domain, (Pleckstrin Homology/ Glucosyltransferase, Rab-like GTPase Activator and Myotubularins) is a phosphoinositide-protein interaction domain, identified in several proteins involved in membrane-associated processes (Doerks et al. 2000). It has been demonstrated that the PH/GRAM domain of MTM1 binds to PI3,5P2 and PI5P, and this interaction is important for its phosphatase activity (Tsujita et al. 2004), (Schaletzky et al. 2003). Moreover, mutations within the GRAM domain are associated with XLMTM (Herman et al. 2002). and mutation within the PH domain, which reduces the ability of MTM1 to bind PI5P, gives rise to XLMTM (Laporte et al. 2000). The lipid binding capacity of these phosphatases is involved in:

1-**Regulation of the enzymatic activity.** It has been reported that PI5P binding to MTM1, via the PH domain, has an allosteric effect on its catalytic activity, which is mediated by the oligomerization of MTM1 into a heptameric ring structure (Schaletzky et al. 2003).

2-**Specific membrane targeting.** The PI binding could serve as a strategy to compartmentalize the enzymatic activity to membrane compartments enriched in either PI3,5P2 or PI5P (Schaletzky et al. 2003).

The **RID** (Rac-Induced recruitment Domain) plays an essential role in the recruitment of MTM1 to specific plasma membrane domains upon Rac1 activation (Laporte et al. 2002). The **SID** (SET interacting domain), is a protein-protein interaction domain formed by paired-helical motif. It was originally identified in the inactive phosphatase MTMR5 and then in all

MTM family. SET (Suvar3-9, Enhancer-of-zeste, Trithorax) containing-proteins are implicated in gene silencing due to histone lysine methyltransferase activity (Yeates 2002). This proposed for the first time a link between epigenetics and myotubularins (Cui et al. 1998). Via the SID domain, MTMR5 interacts with the Hrx SET domain (the mammalian homologue of *Drosophila* thrithorax) and MTM1 may interact with the inactive MTMR12 (Nandurkar et al. 2003). The **PDZ-BM** (PDZ binding motif) presents at the C-terminal end of some MTM family members, potentially is necessary for the interaction with PDZ domain containing proteins which act as scaffolds for the formation of signaling complex (Zimmermann 2006). The **CC** domain is implicated in the interactions between active and dead myotubularins, (Kim et al. 2003), (Mochizuki et al. 2003). Moreover, the myotubularin CC domain plays a role in the target specificity of the phosphatase (Srivastava et al. 2005). This has been shown for the calcium activated potassium channel (K_{Ca} 3.1), which interacts with MTMR6 CC domain but not with MTM1 CC domain (Srivastava et al. 2005).

The myotubularin having the phosphoinositide phosphatase activity plays an essential role (with other enzymes) in keeping a spatial and temporal equilibrium of phosphoinositides (PtdIns) within the cell. The next chapter will summarize the major functions of PtdIns in the animal cells.

II-2-C-iii. *The biological roles of phosphatidylinositol phosphates*

Phospholipids are important building blocks in eukaryotic cells, as they constitute cellular membranes. Phosphatidylinositol phosphates (PtdIns) represent a small fraction of cellular phospholipids. PtdIns molecules are composed of three chemical elements, a fatty acid tail which can be anchored to a membrane and an inositol head group, these two elements are connected by a diacylglycerol link. The inositol sugar ring can be phosphorylated at three positions thus, 7 different PtdIns can be formed. The interconversion between the PtdIns and the corresponding PtdIns is performed by a large repertoire of phosphoinositide kinases and phosphatases, providing a dynamic equilibrium between the seven PtdIns. These distinct configurations confer to PtdIns the ability to define membrane identities, to bind a wide spectrum of proteins and to partake in several pathways, (Nicot et al. 2008), (Balla et al. 2009) (figure 24). In this chapter, the biological roles of phosphoinositides will be briefly discussed.

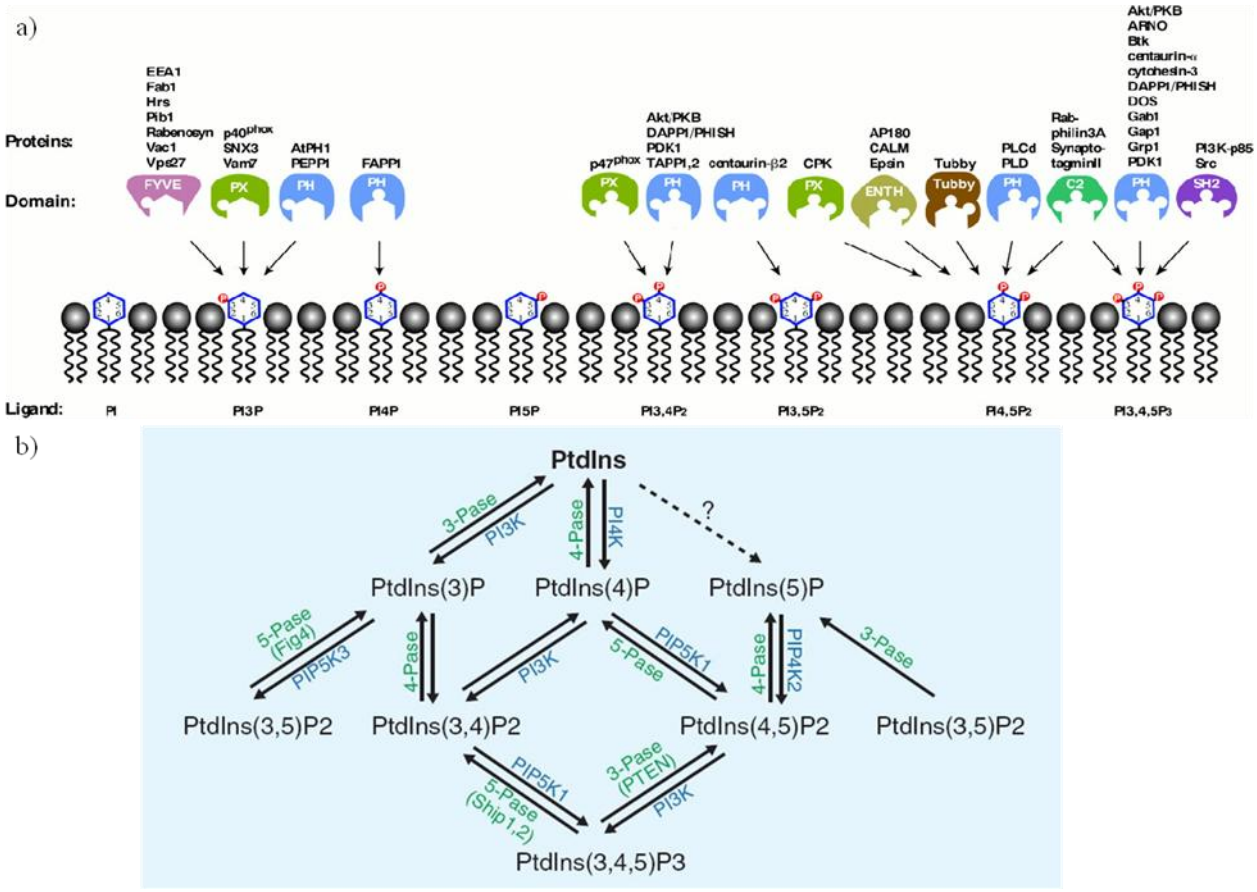


Figure (24): *phosphoinositides species and their interconversion enzymes.* a) The seven forms of PtdIns showing the variety of PtdIns binding domains and the wide spectrum of associated proteins. (from Overduin et al.,Mol Interv, 2001). b) Schematic representation of the PtdIns metabolic cycle with the PI kinases indicated in blue, and the PI phosphatases in green (from Vicinanza M. *et al.*, EMBO, 2008)

II-2-C-iii-a. PtdIns in the control of vesicular trafficking

The main role of PtdIns in this process is the spatially and temporally activation, recruitment and/or assembly of the molecular machineries performing the biogenesis and the transport of the different compartments of this pathway (Vicinanza et al. 2008). PtdIns(4,5)P2 pools are detected at the plasma membrane and at Golgi complex (figure 25) where they involved in several membrane trafficking events. This includes i) the biogenesis of exocytic vesicles (Eberhard et al. 1990), (Hay et al. 1993). ii) the clathrin mediated endocytosis where PtdIns(4,5)P2 may serve as a recruitment signal for coat components (Gaidarov et al. 1996), (Gaidarov et al. 1999). iii) the clathrin independent endocytosis which is regulated by Arf6 (Aikawa et al. 2005). Moreover, PtdIns(4,5)P2 seems implicated in the cytoskeleton remodeling events necessary to vesicle traffiking (Yin et al. 2003). Specially, as new PtdIns(4,5)P2 effectors has been identified by genome wide lethality screen to be

cytoskeleton modulators as they act in the recruitment of proteins implicated in actin polymerization in yeast (Audhya et al. 2004).

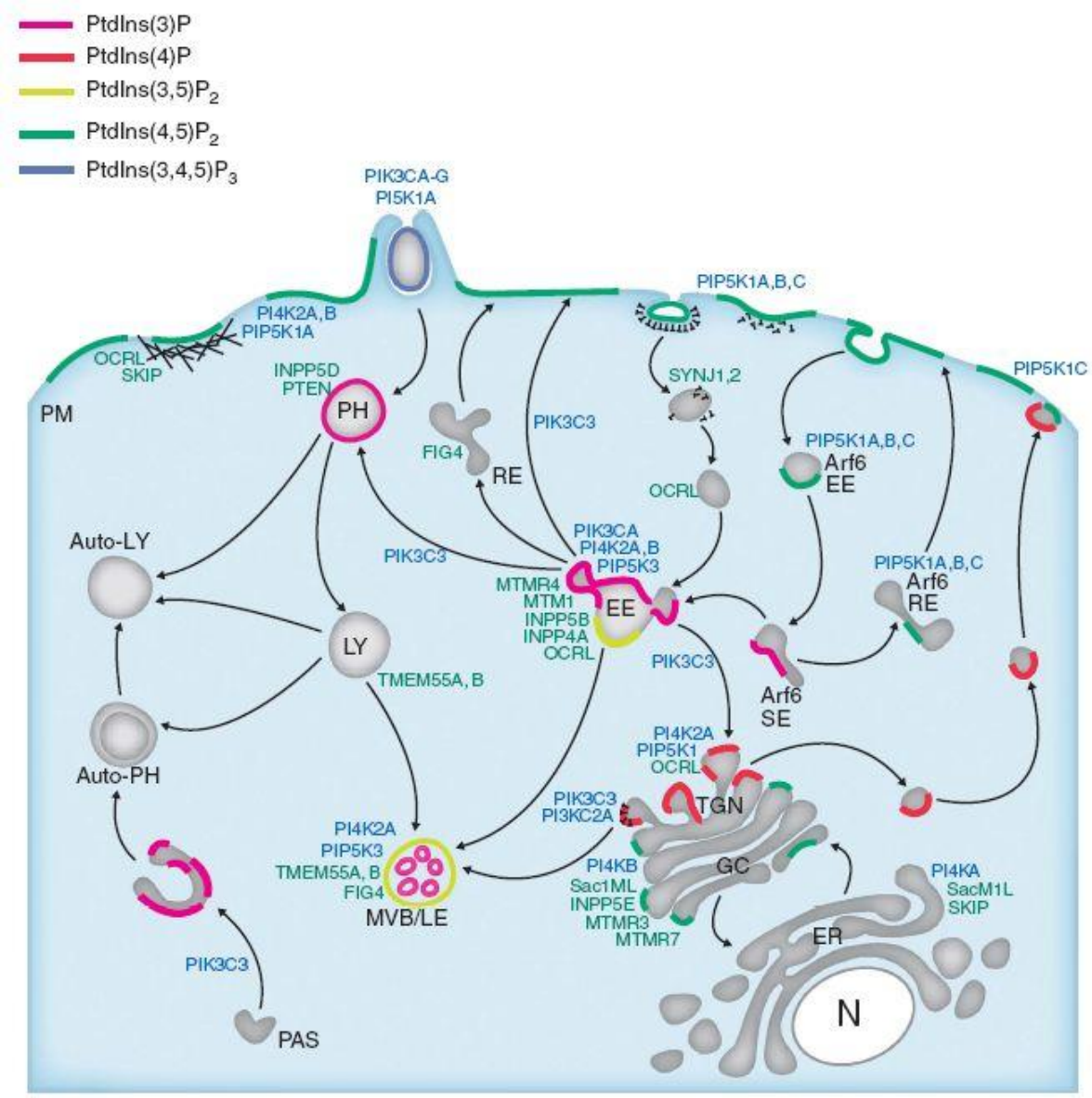


Figure (25): *Subcellular distribution of the PtdIns and PtdIns-metabolizing enzymes.* The localization of the different PIKs (blue) and PtdIns phosphatases (green), as well as the predominant PtdIns species (as visualized by PtdIns-binding protein domains) in the different cell compartments. It is noted that many of the PtdIns-metabolizing enzymes are present in more than one cellular compartment, and their overall distributions do not completely fit with the PtdIns map, as indicated using the PI-binding protein probes. PM, plasma membrane; EE, early endosome; SE: sorting endosomes; RE, recycling endosome; LY, lysosome; MVB/LE, multivesicular body/late endosome; PAS, pre-autophagosomal structure; PH, phagosome; TGN, trans-Golgi network; GC, Golgi complex; ER: endoplasmic reticulum; N, nucleus, (from Vicinanza M. *et al.*, EMBO, 2008).

PtdIns3P localizes at the early endosome and the internal vesicles of the multivesicular body (MVB) and plays a role in the recruitment of the endocytic machinery (Gillooly et al. 2000) (figure 25). PtdIns(3,5)P2 is enriched at the early endosome and in the outer membrane of MVB, and proposed to be involved in the regulation of vacuolar volume as its levels are elevated in yeast in response to osmotic stress (Dove et al. 1997). Where PtdIns(3,5)P2 plays a major role in controlling the size and shape of the vacuole and in mediating its fragmentation (Dove et al. 2007), (Bonangelino et al. 2002). The regulation of the PtdIns3P and PtdIns(3,5)P2 equilibrium is maintained by several kinases and phosphatases in human cells. The significance of a deregulation in this balance is highlighted by the number of diseases associated with such an event (figure 26) (Nicot et al. 2008). It has been shown that defect in PtdIns(3,5)P2 production which is fulfilled by PIP5K3/PIKfyve is related to Corneal Fleck Dystrophy, characterized by enlarged vesicles of unknown origin in the cornea (Li et al. 2005). However, mutations related to phosphatases which catalyze PtdIns3P and PtdIns(3,5)P2 such as MTMR2 and SAC3/FIG4 cause several forms of Charcot-Marie-Tooth (CMT) neuropathy (Bolino et al. 2000), (Chow et al. 2007). This in addition to MTM1 which gives rise to XLMTM when mutated (Laporte et al. 1996).

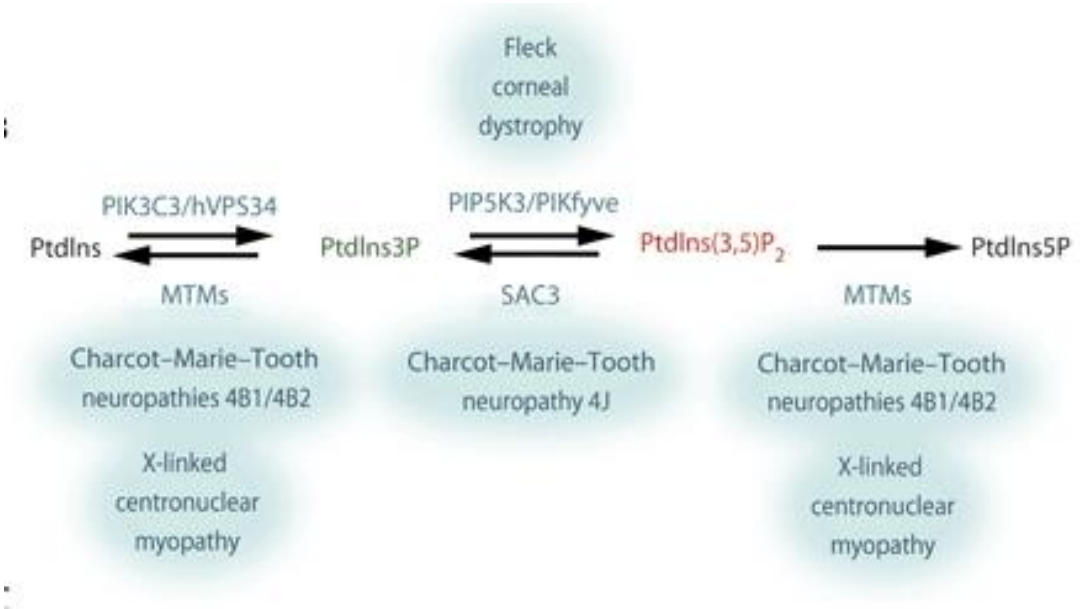


Figure (26): *The implication of PtdIns interconverting proteins in several human diseases.* The interconversion of PtdIns3P and PtdIns (3,5)P2 is catalyzed by kinases such as PIK3C3 and PIP5K3 and phosphatases including SAC3 and myotubularins (MTM1 and MTMR2). Associated human diseases are shown in blue, (from Nicot AS.and Laporte J., traffic, 2008).

II-2-C-iii-b. PtdIns in the control of ion transport proteins

Ion transport proteins serve to transfer ions between the extra and intra cellular mediums or between different organelles within the cell. They are usually under tight control by associated proteins and/or small molecules such as ions or PtdIns. It was shown in the early 80's that plasma membrane Ca^{2+} pumps are activated by the phosphorylation of associated PtdIns (Varsanyi et al. 1983). The phosphoinositide which found to be the most implicated in ion channels, pumps, and exchangers regulation is PtdIns4,5P2. This PtdIns has been found to regulate both cardiac Na^{+} Ca^{2+} exchanger (NCX1) and cardiac K_{ATP} potassium channels (Hilgemann et al. 1996). PtdIns4,5P2 has also been reported to be associated with members of the transient receptor potential ion channels family (Rohacs et al. 2005). This is believed to occur through an interaction of PtdIns4,5P2 with a charged domain on the cytoplasmic face of the channel (Hilgemann et al. 2001). More recently, PtdIns3P was found to be implicated in the regulation of the Ca^{2+} activated K channel (KCa 3.1) in T cells (Srivastava et al. 2005). In addition, PtdIns3,4P2 and PtdIns3,5P2 are shown to regulate RyR1 calcium release channel in skeletal muscle (Shen et al. 2009).

The way by which PtdIns regulate ion transporters remains elusive. However, several mechanisms have been suggested, they include: i) A direct interaction of PtdIns with a basic sequence in the cytoplasmic tail of the channel might lead to a change in its conformation that can alter the channel activity or affect the association of the channel to another regulator protein. ii) An interaction of PtdIns with a protein, which in turn regulates the channel activity. (see (Balla et al. 2009) for review)

II-2-C-iii-c. The PI3K/AKT signaling pathway

PtdIns3P is an essential component of several signaling pathways as it forms the bridge between the cell membrane and the intracellular signaling components. The role of PI3K in this pathway begins when PI3K is activated by receptor tyrosine kinases (RTKs) such as (Insulin and Insulin-like growth factor receptors). Consequently, PI3K phosphorylates and converts the lipid second messenger PtdIns(4,5)P2 to PtdIns(3,4,5)P3, which recruits and activates phosphatidylinositol-dependent kinase 1 (PDK1). Active PDK1 triggers a long cascade of kinases which regulating several processes including cell metabolism, protein synthesis or degradation (Clemmons 2009).

Finally, phosphoinositides have also described roles in the nuclear process such as the modulation of chromatin structure (Jones et al. 2004).

II-2-C-iv. *Myotubularin cellular function*

Since the identification of MTM1 1996 (Laporte J., 1996), several studies have emerged exploring its cellular function. These studies have attributed to myotubularin a role in membrane remodeling events such as the endocytosis and membrane homeostasis in skeletal muscle. This section contains an outline of observed findings.

II-2-C-iv-a. In cellular models

Although deletion of spMTM (the yeast orthologue of MTM1) does not have an obvious phenotype, overexpression of human MTM1 in yeast leads to a cell growth defect, enlarged vacuoles and decreased PtdIns3P level (Blondeau et al. 2000). This vacuolar phenotype is similar to that reported in strains carrying mutations within members of the VPS (vesicle protein sorting) class of protein kinase such as VPS34p (Takegawa et al. 1995). Tsujita *et al.* have reported that overexpression of MTM1 in COS-7 cells induces enlarged endosomal structures and impaired growth factor trafficking from the late endosome to the lysosome, supporting a role of MTM1 in vesicular transport pathways (Tsujita et al. 2004).

Moreover local overexpression of MTM1 in HeLa cell endosome compartments, leads to localized loss of PtdIns3P pool and to microtubule-dependent tubularization of the Rab5a endosome compartment (the early endosome) (Fili et al. 2006). This maturation defect of endosomes prevents Transferrin Receptor (TrnR) recycling and slows epidermal growth factor receptor (EGFR) trafficking (Fili et al. 2006). The association of MTM1 with the early endosome was confirmed by Cao *et al*. who demonstrated that myotubularin shares with RabGTPases and hVPS PI3 kinases the control of endosomal function and morphology (Cao et al. 2007). *In vivo* and *in vitro* studies have shown that MTM1 and Rab GTPases (Rab5 and Rab7) bind to the same domain of hVPS15 (PI3 kinase) via a competitive binding mechanism (Cao et al. 2007). In the model proposed by Cao, the active Rab GTPases bind to and activate the hVPS kinase complex leading to the PtdIns3P synthesis. The accumulation of PtdIns3P and the presence of active hVPS complex serve to recruit MTM1 which dephosphorylates PtdIns3P and turn off its signal. This leads to the release of downstream effectors of PtdIns3P signal and breaks the membrane binding of MTM1. These events create a new environment for a next step of endosome maturation (Cao et al. 2007).

II-2-C-iv-b. In Caenorhabditis elegans

In C. elegans, the mtm1 orthologue seems to be expressed in several engulfing cell types where it localizes at the plasma membrane (Zou *et al.* 2009). It has been suggested that a physiological function of mtm1 in C.elegans is to negatively regulate the engulfment of cell bodies by modulating PtdIns3P level at the plasma membrane of the engulfing cell (Zou *et al.* 2009). In addition, mtm1, with other myotubularins, plays an essential role in the endocytic process in coelomocytes (scavenger cells located in the pseudocoelomic cavity that continuously and nonspecifically endocytose fluid from the body cavity (Fares et al. 2001)). Moreover, mtm1 inactivation can recover the endocytic defect induced by Let-512 deletion (Xue et al. 2003), (Let-512 is the only VPS34 PI kinase homologue, it gives rise to endocytosis defect and larval lethality when mutated (Roggo et al. 2002). Interestingly, the larval lethality induced by Let-512 deletion can be rescued by mtmr6 inactivation (Xue et al. 2003). However, the overexpressed mtm1 localizes to the cytoplasm and is not associated to membranes as observed for mtmr6 (Xue et al. 2003).

II-2-C-iv-c. In zebrafish

Dowling *et al* (2009) have recently generated a zebrafish model for XLMTM by knocking down *mtm1* with antisense morpholinos. Zebrafish expresses *mtm1* and 12 out of the 14 myotubularin-related genes identified in human (Dowling et al. 2009).

mtm1 downregulation leads to a dorsal curvature in embryos (described in zebrafish as a myopathic phenotype) (Dowling et al. 2009). In addition, motor function (as characterized by spontaneous embryo coiling frequency, chorion hatching and touch-evoked escape) was severely affected in these mutants (Dowling et al. 2009). Muscle fibers are clearly hypotrophic and contain abnormal (large and rounded) nuclei. Electron microscopy (EM) analysis of the perinuclear zone has shown structural abnormalities, including aberrant mitochondria, areas devoid of organelles, and the presence of unusual membranous structures (Dowling et al. 2009). Interestingly, functional analysis by electrical stimulation showed defective excitation–contraction (E-C) coupling, possibly due to abnormalities in the sarco-tubular network, as shown by EM imaging (Dowling et al. 2009).

Finally, using this model, it has been shown for the first time in skeletal muscle that the level of PtdIns3P is increased in the absence of *mtm1* (Dowling et al. 2009).

II-2-C-iv-c. In mice

Myotubularin is ubiquitously expressed in mouse tissues, however its level is higher in striated muscles than in the nervous system. In addition, the *Mtm1* gene is regulated during skeletal muscle development, as its expression in skeletal muscle is upregulated during the first week of the postnatal life (Buj-Bello et al. 2008). Myotubularin is localized in muscle fibers at the sarcolemma and the I-band including the triad (Buj-Bello et al. 2008). Further investigation by EM immunogold staining has confirmed the localization of overexpressed MTM1 at the triads. By subcellular fractionation of myofibers, it has been shown that MTM1 is located in both the cytosolic and membrane fractions (Buj-Bello et al. 2008).

The mouse model of XLMTM was generated by homologous recombination and described by Buj-Bello *et al*. (2002) The mutant allele of *Mtm1* gene contains an exon 4 deletion, which creates a frameshift and a premature stop codon. This results in absence of MTM1 protein in all mouse tissues. Mice lacking MTM1 are viable, and they develop in the postnatal life, a rapidly progressive and generalized centronuclear myopathy. Muscle mass and strength decline rapidly, leading to death at 6-12 weeks of age probably due to respiratory insufficiency and cachexia. In addition, skeletal muscle is the primary tissue affected by the absence of MTM1, as shown by the use of a conditional mouse line with muscle-specific deletion of *Mtm1* (Buj-Bello et al. 2002) (figure 27).

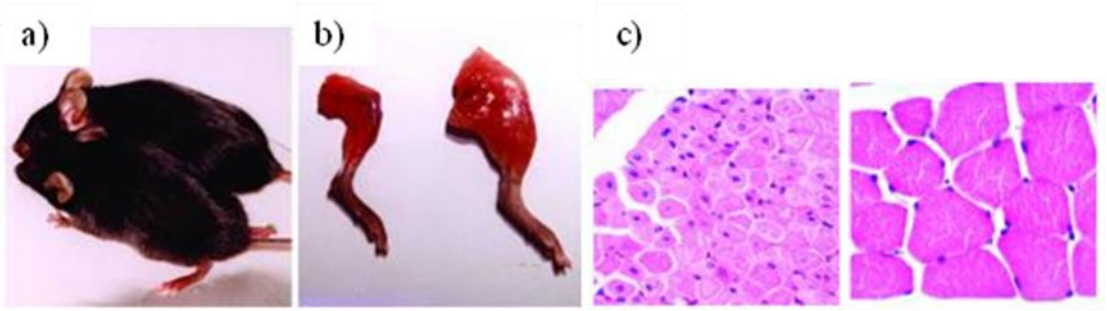


Figure (27) : *XLMTM pathology in Mtm1$^{-/-}$ mouse.* a) *Mtm1*-KO mouse (left) shows signs of kyphosis, because of a weakness in paravertebral muscles, and hindlimb paralysis. b) Illustration of the important muscle mass reduction in the hind limb of a KO mouse (*left*) compared with a control (right). c) HE staining of quadriceps cross sections from KO (*left*) and wild-type (right) mice at 6 weeks of age, (from Buj-Bello A., PNAS, 2002).

At birth, *Mtm1* KO mice appear normal (although they are lighter than wild-type mice by 10% at postnatal day 8) and signs of muscle weakness begin at about 4 weeks of age (Buj-Bello et al. 2002). Contrary to previous suggestions that the XLMTM phenotype represents an arrest in the myogenesis at the myotubular stage (van Wijngaarden et al. 1969), (Spiro et al. 1966)), myogenesis occurs normally in KO mice giving rise to mature myofibers with typical peripheral nuclei. During disease progression, hypotrophy and ultrastructural alterations appear in myofibers. This includes myofibril disorganization, Z-line streaming and abnormal pattern of oxidative enzyme activity. During this disorganization process the position of nuclei within myofibers is altered, which become internal and/or central, the hallmark of CNM. There are no major signs of necrosis, apoptosis, fibrosis or inflammation, and regenerating myotubes are uncommon. In addition, large vacuoles are occasionally observed in murine XLMTM muscle fibers as the disease progresses (Buj-Bello et al. 2008).

The overexpression of *Mtm1* in wild-type skeletal muscle by using a rAAV induces the accumulation of membrane structures, including vacuoles and needle-like structures within myofibers (figure 28a) (Buj-Bello et al. 2008). Interestingly, these vacuoles are derived from sarcolemma and/or T-tubules, as they are positive for caveolin-3, dystrophin and dihydropyridine receptor (DHPR), and negative for laminin 2 staining. Needle-like structures were occasionally associated with honeycomb figures (figure 28b), which are usually due to the abnormal proliferation of the T-tubule system (Angel A., Myology, 2004). EM analysis of the needle-like structures has shown that they are composed of highly organized parallel membrane arrangements taking the shape of saccules and not of tubules (figure 28d). More interestingly, they harbor accumulations of the overexpressed MTM1 protein (figure 28c) (Buj-Bello et al. 2008).

These elaborated analyses of MTM1 containing structures provide an evidence for myotubularin implication in the biogenesis and/or the remodeling of membranous structures in skeletal muscle.

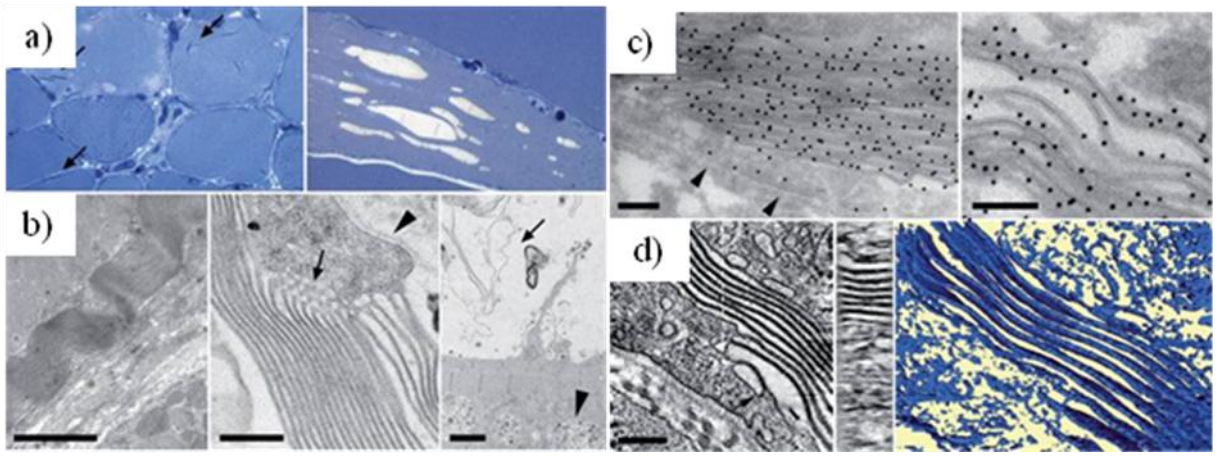


Figure (28) : *Effect of overexpression of myotubularin in skeletal muscle.* a) Presence of needle-like structures (left panel, arrows) and vacuoles (right panel) in wild-type muscle overexpressing myotubularin. Semithin cross-sections of tibialis anterior were stained with toluidine blue (×630, left and ×1000, right). b) Electron micrographs illustrate the nature of myotubularin-induced needle-like structures (left and middle panels). They resemble myelin-like structures and are occasionally associated with honeycomb-like structures (arrow, middle panel). Note that the basal lamina (arrowhead) is not included. Vacuoles often contain degenerative membrane aggregates (arrow, right panel). Occasional autophagic vacuoles are also present (arrowhead). Bars represent 2 µm in the left and right panels and 400 nm in the middle panel. c) Immunogold detection with an anti-myotubularin antibody shows labeling of the sarcolemma (arrowheads) and an intense signal on the membrane aggregates. Bars indicate 200 nm. d) Left panel: central section of an electron tomogram from a cellular region containing such a membrane assembly. Membrane sheets clearly appear as being composed of two bilayers. Central panel: section through the membrane assembly normal to the section plane as indicated by the dotted line in the left panel. This cross-section demonstrates that the lipid assemblies are flat layers and not tubular structures. Right panel: surface representation of the lipid layers which evidences the twisted nature of the close to parallel lipid assemblies. The bar represents 200 nm in the left and central panels and 125 nm in the right panel, (from Buj-Bello A., HMG, 2008).

Materials and Methods

In this part the material and techniques employed to perform non-published experiments are presented.

I. Primers for Q-RT-PCR (from 5' to 3')

Arpc2:	F- CGACTTCCTTAAGGTGCTCA	and	R- CTCCTCAGTTCCCAAGAGTT
Rab5a:	F- CTGCTGTTGGCAAATCAAGC	and	R- CGTTCTTGACCAGCTGTATC
Anx2 :	F- GTCCCTGTACTACTACATCC	and	R- AGTCTCTAGAACGCCAGGTT
Sln:	F- AGAGACTGAGGTCCTTGGTA	and	R- CTGGATGAGAACTCCAGAAG
Pln:	F- CAGCTAAGCTCCCATAAGAC	and	R- TAGTGGAGGCTCTCCTGATA
Atrogin 1:	F- CAACAGACTGGACTTCTCGA	and	R- GTGTAGAGAGTCTGGAGAAG
Murf1:	F- ATGAAGTGATCATGGACCGG	and	R- CTTCTGTCCTTGGAAGATGC
Nedd4:	F- GAGTGACATTGTATGACCCG	and	R- GACATCCACTTGACCTAGGA
Znf216:	F- GGGAATCCTAGGACAAATGG	and	R- TGGGCTGAGAAACTGATGGA
Runx1:	F- CTCGGCAGAACTGAGAAATG	and	R- CTCTATGGTAGGTGGCAACT
Rrad:	F- GAGGGCGTTTACAAGGTACT	and	R- GCTTCTTCTCCATCCACAGT

II. Protein extraction

To extract whole muscle proteins, muscle was homogenized using a Ultra-Turrax (IKA-WERKE) homogenizer in 50 mM Tris, 10% glycerol, 50 mM KCl, 0.1% SDS, 2% Triton X 100 and a set of protease inhibitors: 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF (phenylmethylsulphonyl fluoride), 1 µM pepstatin, 10 µM leupeptin. The homogenate was kept at 4°C for 2 hours, and clarified by centrifugation at 13000 rpm for 10 min. Protein concentration of the supernatant fraction was quantified with the Biorad Protein Asssay (Biorad laboratorie GmbH).

III. SLN polyclonal antibody generation

The anti-SLN antibody recognizes a 12 residue peptide (MERSTQELFINFC, the last cystein was adding for conjugation) corresponding to the N-terminal region of the mouse and rat SLN sequence. The peptide was synthesized by the IGBMC platform for peptides and was conjugated by the C-terminal cystein to ovalbumin, which acts as a carrier protein.

III-1. *Coupling the Sln peptide to ovalbumin:* 300 µl of the Sln peptide (10 mg/ml) was combined to 200 µl of ovalbumin (Imject maleimide activated ovalbumin; Thermo Scientific) and allowed to mix gently for 2h at room temperature (RT). The coupled proteins were dialyzed overnight in phosphate buffer saline (PBS) at 4°C and then dialyzed in fresh PBS for 2h at 4°C. The conjugated peptide was then injected into two rabbits by the IGBMC antibody platform. Serums from the immunized rabbits were collected and purified by affinity chromatography.

III-2. *Purification of Sln antibody:* To purify Sln antibody from rabbit sera, 100 µl of Sln peptide (20 mg/l) was bound to a Sulfolink Coupling gel (Pierce) by incubating 30 min at RT. Following binding, 1 ml of cysteine (50 mM) was added and was allowed to mix gently for 15 min and then incubated for 30 min at RT. The precleared serum (centrifuged for 10 min at 2000 rpm) was added to the column and allowed to bind. The column was washed with 20 ml PBS and the antibodies were eluted with 5 ml of 0,1 M glycine pH2.8. Fractions of 0,5 ml were collected, neutralized immediately with 25 µl of Tris 1M pH9.5, dialyzed overnight in PBS and stored at -20°C.

IV. Immunoblot analysis

Protein homogenates (50 µg) were analyzed by SDS-PAGE followed by protein transfer on a nitrocellulose membrane employing standard techniques. The membranes were saturated with tris buffer saline (TBS), 3% Tween20, 7% dry milk for 1 hour (h) at RT, incubated with the primary antibody diluted in TBS 3%, Tween20, overnight at 4°C, and with the secondary antibody diluted also in TBS 3%, tween20, for 2 h at RT.

Antibodies employed for immunoblot analysis:

Rabbit anti AKT antibody (Cell Signaling Technology) used at 1/1500,

Rabbit anti mTOR (7C10) antibody (Cell Signaling Technology) used at 1/1500,

Rabbit anti FoxO3α antibody (Cell Signaling Technology) used at 1/1000,

Rabbit anti Phospho-FoxO3α (ser 318/321) (Cell Signaling Technology) used at 1/1000,

Rabbit anti IGF1Rα (N-20) antibody (Santa Cruz Biotechnology) was used at 1/300,

Mouse anti Cav3 (clone26) (BD Biosciences) was used at 1/2500,

Rrad antibody was kindly provided by Dr., Khan CR. (University of Michigan, USA) and used at 1/200.

V. Bioinformatic analysis of Rrad genomic sequence to identify potential transcription factor binding sites (TFBSs)

We followed a four step approach:

Step 1: Extraction of orthologous sequences

Step 2: Conservation

Step 3: Identification of potential TFBSs in a chosen reference sequence

Step 4: If conserved, TFBS validation

V-1. *Extraction of orthologous sequences:*

We searched for the genomic sequence of the Rrad gene in UCSC Genome Browser in the following organisms: human, rat, mouse, cow, dog, opossum, chicken, xenopus, zebra fish, tetraodon. Only three orthologue sequences were found, the human *(Homo sapiens)*, mouse *(Mus musculus)* and bull (*Bos taurus*) *Rrad* sequences.

V-2. *Conservation*

We performed a multi-alignment analysis for the three sequences using ClustalX software and PromAn software designed by the bioinformatics platform at the IGBMC. The multi-alignment results were visualized by the GenDoc program. All software employed were provided by the IGBMC bioinformatics platform.

V-3. *Identification of potential TFBS in a chosen reference sequence*

The M. musculus *Rrad* sequence served as the reference sequence for the identification of potential TFBSs, as the DNA microarray analysis performed in the XLMTM mouse model showed an important upregulation of *Rrad* mRNA. The Genomatix MatInspector tool was employed to screen the Rrad sequence for TFBSs available in the Genomatix database. Parameters were set in such a way that TFBS were selected by the MatInspector tool if they exhibited matrix similarity >70% and core similarity >70%.

The table below illustrates what is assigned the core and matrix for a TFBS. The nucleotide distribution of the RUNX matrix which is built from published data is depicted. Capital letters and red marked nucleotides indicate the high conservation positions which represent the core of the matrix, which is formed in turn by less conserved nucleotides (shown in blue).

	1	2	3	4	5	6	7	8	9
A	2	0	0	0	0	0	0	1	7
C	9	0	0	4	0	0	1	4	0
G	0	1	12	0	12	12	0	1	1
T	1	11	0	8	0	0	11	6	4
	c	t	G	Y	G	G	t	y	w

Core

matrix

This table shows the 12 binding sites of RUNX which are described in the literature are used to build RUNX matrix. The matrix reveals that the GYGG in the 3, 4, 5 and 6 positions are crucial to have a RUNX binding site, while 1, 2, 7, 8 and 9 positions can be occupied by several possible nucleotides.

V-4. *Bioinformatic validation of TFBSs*

Potential TFBSs for RUNX were validated in the case that these TFBSs selected by MatInspecter were conserved between the three orthologou genomic sequences. This step was done manually.

Objectives

The MTM1 gene was identified in 1996 using a positional cloning strategy, as the causative gene for the X-linked myotubular myopathy (Laporte 1996). MTM1 is a phosphoinositide phosphatase involved in membrane trafficking events. In order to analyze the proper function of MTM1 in skeletal muscle and its physiological implications, we generated a mouse model. Fortunately, *Mtm1*-KO mouse manifest the main pathological features of XLMTM, in particular, muscle atrophy, decrease of muscle strength and reduced life expectancy (Buj-Bello 2002). This animal tool has driven many fundamental projects aiming to understand MTM1 biological roles in skeletal muscle, but has allowed also the development of therapeutic assays including gene therapy using viral delivery systems.

When I joined the MTM team, DNA microarray data had just been obtained in collaboration with Dr. AH Beggs (Boston, USA). This analysis revealed the deregulation of several pathways including, Ca^{2+} homeostasis and endocytosis. Additionally, a MTM1 antibody had been developed in our lab, which allowed the identification of the subcellular localization of MTM1. Myotubularin was found at the sarcolemma and triads, where excitation-contraction coupling occurs. However, nothing was known about the molecular mechanism of XLMTM pathogenesis.

The objectives which I aimed to perform during my PhD were:

1) *Characterization of Mtm1-KO mice from a 129-PAS background.*

Two mouse strains were used as Mtm1-KO models, the previously described BlackC57/6 strain and the one on a 129-PAS background, which exhibits more homogenous features related to the onset and the course of the disease. My first objective was to characterize the Mtm1-KO mice of this background.

2) *Analysis of the molecular mechanism involved in XLMTM pathogenesis*

Depending on the data available when I joined the MTM team; I focused my analysis on the endocytosis and the Ca^{2+} homeostasis pathways as they are potentially implicated in the XLMTM pathogenesis.

3) Analysis of the overexpression of IGF1 in Mtm1-KO mice

The overexpression of insulin like growth factor 1 (IGF1) by crosslinking was a therapeutical approach to correct the defect induced by MTM1 absence. In this project my intention was to analyse a potential rescue induced by IGF1 overexpression in *Mtm1*-KO mice.

I. Molecular mechanisms implicated in XLMTM pathogenesis

I-1. Selection of the pathway involved in XLMTM pathogenesis from DNA microarray data

I-1-A. Introduction

Studies performed using a mouse model of XLMTM have shown that MTM1 absence primarily affects the skeletal muscle. However, the pathological mechanism underlying this disease remained unknown. Two points have made it difficult to assign a function to MTM1 and in turn to decipher the mechanism implicated in XLMTM:

i) Although *MTM1* is ubiquitously expressed, mutations within the *MTM1* gene give rise to a skeletal muscle-specific defect.

ii) The phosphoinositides (including myotubularin substrates) serve as secondary messengers in several processes such as membrane trafficking, signaling pathways and the regulation of ion channels.

In order to identify potential pathways involving MTM1, transcriptome analysis of *Mtm1* deficient skeletal muscle using DNA microarrays was performed (study by Anna Buj-Bello in collaboration with Alan Beggs group, Boston). This study identified 611 deregulated genes that were then segregated into different pathways. The work presented herein involves Q-RT-PCR analysis for two of these implicated pathways; the endocytosis and the calcium homeostasis pathways. The findings observed warranted further investigation into the Ca^{2+} homeostasis mechanism.

I-1-B. Results

I-1-B-i. *Transcriptome profile of Mtm1-KO mice*

As myotubular myopathy is a progressive disease in mice, we collected DNA microarray data from skeletal muscle of mice at 2 and 6 weeks of age. We selected specifically these ages as *Mtm1*-KO mice do not show any clinical sign of myopathy at 2 weeks, whereas, at 6 weeks, they exhibit the main signs of XLMTM. Affymetrix mouse 430A (containing 22,690 genes and ESTs) GeneChips were used and included quadriceps muscles from 5-6 different *Mtm1*$^{-/-}$and WT mice, at 2 and 6 weeks of age. Gene expression was

considered as deregulated for a 2 or higher fold change and a P value < 0.05. Although, no deregulated genes were apparent in skeletal muscle of 2 week-old mice, 611 deregulated genes were identified in *Mtm1*-KO skeletal muscle at 6 weeks of age. There were 591 upregulated genes and 20 downregulated genes (analysis performed by Sanoudou D., Beggs group, Boston). These deregulated genes are implicated in several cellular pathways (figure 29).

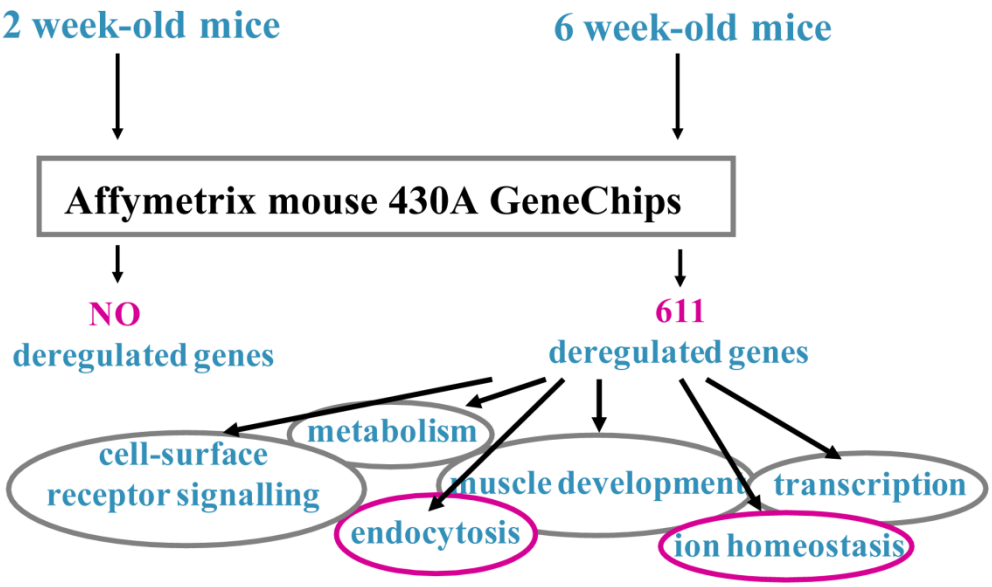


Figure (29): *The deregulated pathways in skeletal muscle of Mtm1-KO mice, as identified by transcriptome analysis: transcription, cell surface receptor signaling, metabolism, ion transport and muscle development.*

Two of these pathways were selected for further investigation:

i) endocytosis; a pathway with an established role for MTM1 (see Table 1 for deregulated genes of this pathway)

ii) Ca^{2+} homeostasis; MTM1 localizes at triads, structures with a direct role in Ca^{2+} signaling and muscle contraction (see Table 1 for deregulated genes of this pathway).

a)

genes implicated in endocytosis	Fold change
Rin 1	4.31
Annexin 2	3.81
Arf 6	3.02
Rab 33	3.00
Annexin 1	2.85
Arpc1	2.57
Rab 13	2.37
Dynein	2.30
Clathrin	2.11
Arpc3	2.06
Kinesin Kif22	2.06
VPS 29	2.05
Arf gap3	2.018
Arf 4	2.00
Rab 5a	2.00
Arpc2	2.00

b)

genes implicated Ca^{2+} homeostasis	Fold change
Sarcolipin	72.42
Rrad	61.65
S100a4	9.99
S100a13	3.77
CamK2D	3.27
Nucleobindin 2	2.98
S100a10	2.97
Casq2	2.86
Annexin 7	2.58
Reticulocalbin	2.08

Table (1): *Upregulation of genes implicated in endocytosis (a) and Ca^{2+} homeostasis (b) at 6 weeks of age.*

We followed up several deregulated genes from these two pathways by analyzing three groups (at 2, 4 and 6 weeks of age) of WT and *Mtm1*-KO mice.

I-1-B-ii. *Deregulation of genes involved in endocytosis*

Endocytosis involves several subcellular compartments including the early, late and recycling endosomes as well as lysosomes. MTM1 has been described to have a role in the endocytic pathway (see paragraph II-2-C-iv. in the introduction). To further confirm the deregulation of this pathway, the expression levels of Rab5a, Anxa2 and Actin-related protein 2 (Arp2) were determined by Q-RT-PCR. RAB5a, is implicated in several endocytic processes with a predominant role in the formation of clathrin coated pits and their subsequent fusion with the early endosome (Somsel Rodman et al. 2000). Anxa2, is involved in the biogenesis of multivesicular transport intermediates destined for late endosomes (Mayran et

al. 2003). Arpc 2, a subunit of the Arp 2/3 complex that is required for nucleating new actin filaments and creating a branched actin network (Insall et al. 2009). Q-RT-PCR was performed from quadriceps muscle of WT and MTM1 KO mice at 2, 4 and 6 weeks.

The analysis performed with 6 extracts from week-old mice showed a 3.16 (+/- 0.39) fold increase in Rab5a expression. Moreover, the expression levels of Anxa2 and Arpc2 were increased by 2.86 (+/- 0.39) and 1.73 (+/- 0.16) fold respectively (Figure 30a). These findings suggest a deregulation of endocytosis at the final phase of the disease as analysis of the expression of these genes at earlier phases, in 2 and 4 week-old mice, showed no significant differences in gene expression between KO and WT muscle (figure 30b and 30c).

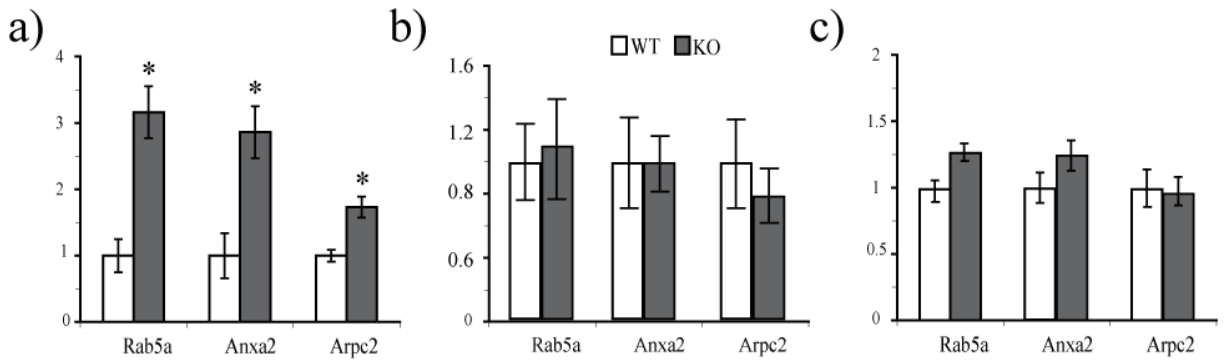


Figure (30): *Expression levels of genes involved in endocytosis in WT and Mtm1-KO mice as determined by Q-RT-PCR.* a) In 6 week-old mice, P=0.01 for Rab5a, P=0.02 for Anxa2 and Arpc2, (n= 3WT and 3 KO). b) In 4 week-old mice, no significant differences were detected, (n=5 WT and 5 KO). c) In 2 week-old mice, we also found no significant differences between genotypes (n= 2WT and 3KO). Expression values were normalized against GAPDH expression level. * = (P<0.05)

I-1-C. Discussion

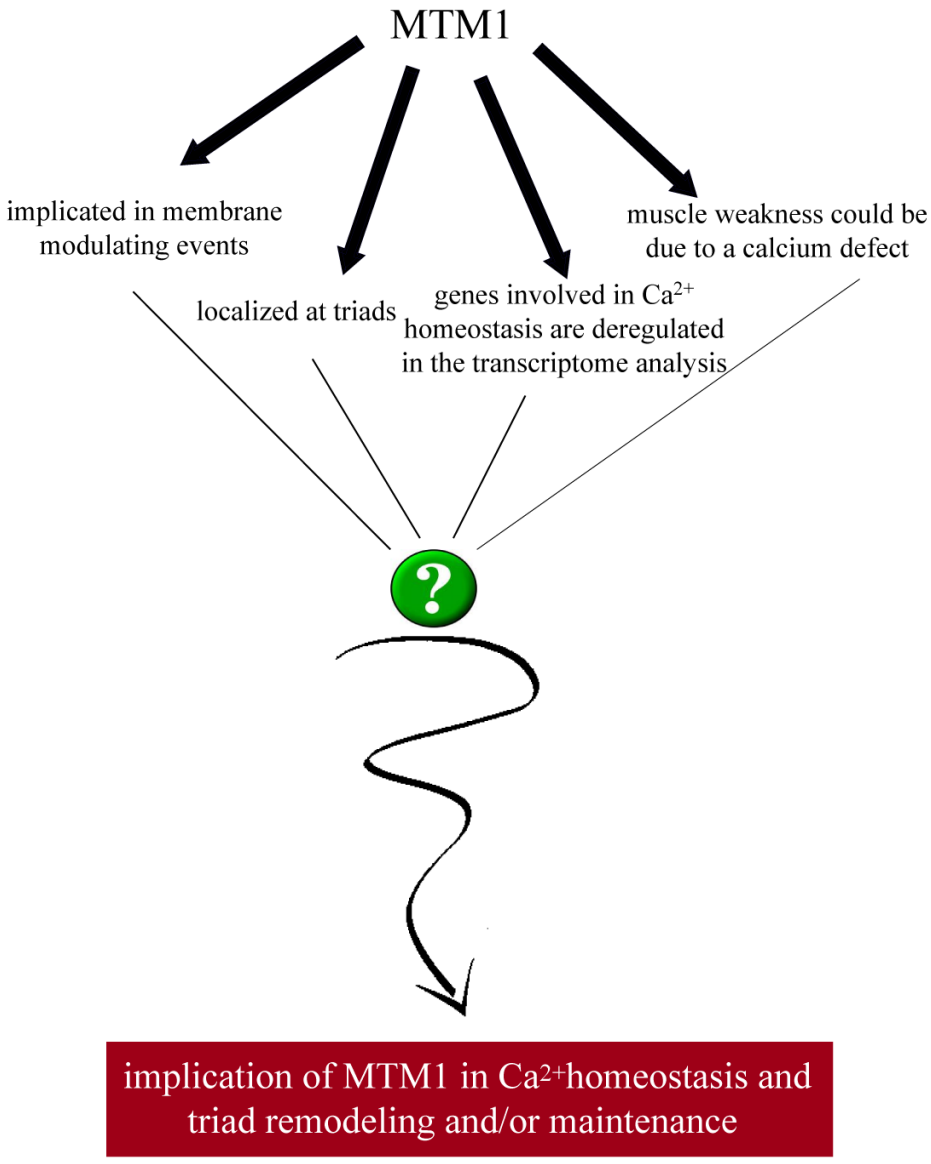
DNA microarray analysis on *Mtm1*-KO mice at two ages revealed that gene expression remains unchanged during the asymptomatic phase of the disease. However, at later phase, there is an active transcriptional response to the absence of MTM1. These molecular findings seem in agreement with the progressive nature of XLMTM in mice. Six pathways with a potential role in XLMTM were identified. We decided to look into two of them: Ca^{2+} homeostasis and endocytosis. We analyzed deregulated genes implicated in endocytosis and found that Rab5a, Anxa2 and Arpc2 expression is upregulated at a late stage, but remains unchanged at earlier phases. This indicates that although there may be a defect in endocytosis, it is unlikely to be involved in the pathogenesis of the disease. Thus we

abandoned further investigations into this pathway and focused on Ca^{2+} homeostasis and its potential role in XLMTM.

I-2. Analysis of the calcium regulating machinery in *Mtm1*-KO mice

I-2-A. Introduction

The findings presented so far show that even though endocytosis is deregulated at a late phase of the disease it is unlikely to be involved in the pathogenesis of XLMTM. This observation, in addition to others has led us to propose a hypothesis based on the rational outlined below.



I-2-B. Results

I-2-B-i. *T-tubule disorganization and defective Ca^{2+} release in Mtm1-KO mice*

The publication entitled "T-tubule disorganization and defective excitation-contraction coupling in muscle fibers lacking myotubularin lipid phosphatase" presents lines of evidence that support this hypothesis.

My contribution in this project involved:

1- The characterization of the phenotype of *Mtm1*-KO 129PAS mice during disease progression by analyzing: mouse body weight, muscle strength (not shown), and pathology using classical histological approaches. I have demonstrated that during the late phase of the disease, skeletal muscle mass and myofiber area are severely decreased, and the ultrastructure of muscle fibers is altered, as shown by the presence of internal nuclei and mitochondrial disorganization. However, during the asymptomatic phase of the disease (2 weeks of age), and contrary to what was shown in the previously described C57Bl6 strain, I have shown that muscle fibers are already altered, with a decrease in the area cross section, a small but significant increase in the percentage of internal nuclei and alterations in mitochondrial positioning.

2- The morphological study of T-tubules by electron microscopy and immunofluorescence assays. I have demonstrated that myotubularin deficient myofibers have a disorganized sarcotubular system, in particular, there are alterations in the morphology of triads, with presence of longitudinally orientated T-tubules and triads devoid of T-tubules. Moreover, I have shown that these alterations become more pronounced with disease progression.

3- The expression analysis of genes involved in the excitation-contraction (EC) machinery by Q-RT-PCR and western blot. I have shown that the expression of several of these genes is deregulated, in particular, RyR1 level is severely reduced. Interestingly, I have demonstrated that the expression of these genes is not significantly altered at early stages of the disease (2 weeks of age). This suggests that the molecular deregulation of the EC machinery may appear as a consequence of triad and T-tubule alterations.

4- The coordination of the collaboration with the group of Vincent Jaquemond (Lyon, France) for electrophysiological studies.

I-2-B-ii. *Expression analysis of genes involved in calcium uptake*

This part will be published with the data concerning the transcriptome of skeletal muscle from XLMTM patients and mice (manuscript in preparation).

I-2-B-ii-a.Introduction

DNA microarray data obtained from WT and *Mtm1*-KO mice has demonstrated that sarcolipin (*Sln)* expression is dramatically upregulated (70-fold up) in KO mice. Sln is known to negatively regulate the activity of SERCA pumps alone or in cooperation with its functional homolog, phospholamban (*Pln*) (Babu et al. 2006), (Asahi et al. 2003). The SERCA pump, in association with Sln and Pln, constitutes the Ca^{2+} uptake system in striated muscle (for more details see I-2-C-ii and I-2-C-v. in the introduction). Our aim was to analyze the RNA and protein levels of Sln and Pln and to address a potential functional effect on Ca^{2+} uptake in *Mtm1*-KO muscle fibers.

I-2-B-ii-b. Results

We analyzed mRNA levels of *Sln* in tibialis anterior (TA) muscle by Q-RT-PCR at a late phase (in 5 week-old mice), and we found that Sln expression is upregulated by 192 +/- 46 fold (figure 31a). Interestingly, *Sln* mRNA was also increased by 6.6 (+/- 1.3) fold in 2 week-old mice (figure 31b). Similar results were obtained from quadriceps muscle (not shown). We also evaluated the mRNA levels of *Pln*, however, we detected no deregulation neither at a late stage nor at an early stage (data not shown).

To quantify the protein level of Sln, we generated rabbit polyclonal antibodies against a SLN peptide, as published by Vangheluwe P. *et al*. (2005). SLN is a 31 residue protein (4 kDa) located in the sarcoplasmic reticulum. It contains a transmembrane domain, the N-terminal end is exposed in the cytosol and the C-terminal tail resides in the SR lumen. In order to quantify SLN levels in myotubularin deficient muscle, microsomal preparations of quadriceps from 5 week-old WT and *Mtm1* KO mice were analyzed by Western blotting with the anti-SLN antibody in collaboration with the group of Dr. Wuytack (Leuven University, Belgium). As expected, SLN level is dramatically increased in *Mtm1*-KO muscle whereas it is undetectable in WT muscle (figure 31c). PLN levels remained unchanged in mutant muscle (figure 31c). We tried to look at the subcellular localization of SLN in the *Mtm1*-KO muscle

by immunofluorescence staining, but unfortunately, the antibody was not suitable to this application.

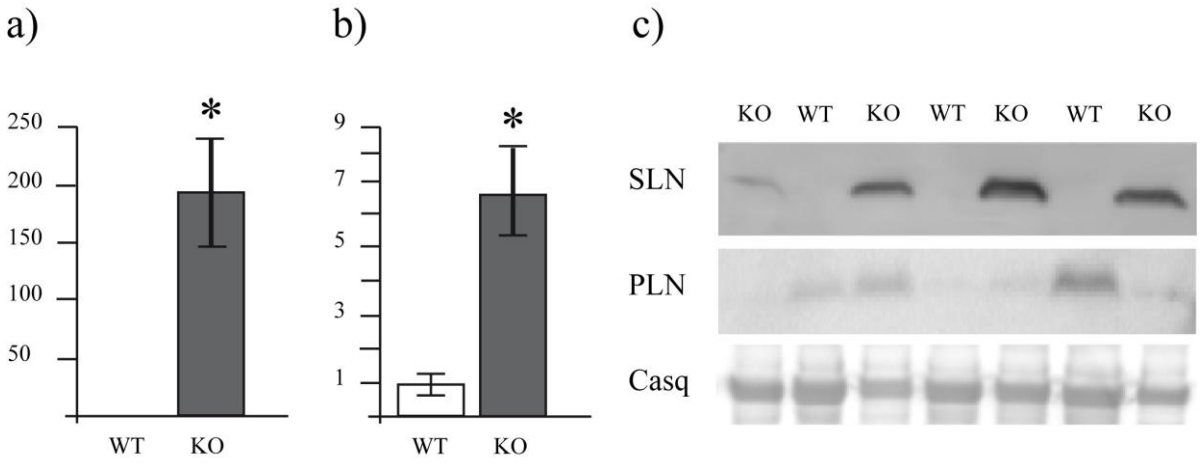


Figure (31): *Sln upregulation in Mtm1-KO muscle.* a) Q-RT-PCR analysis of *Sln* expression in TA muscle from 5 week-old animals (n=3 WT and 3 KO), P=0.01. b) Q-RT-PCR analysis of *Sln* expression in TA muscle from 2 week-old animals (n=5 WT and 3 KO), P=0.006. c) Western blot analysis of quadriceps muscle from 5 week-old mice, showing high levels of SLN in *Mtm1* null mice, with undetectable levels in WT mice (each lane represents a microsomal fraction from different WT or KO mice). PLN level seems unchanged in mutant muscle. An anti-calsequestrin (Casq) antibody was used as internal control for the microsomal preparation (this Western blotting is a courtesy of Dr. Wuytack laboratory). * = ($p<0.05$)

I-2-B-ii-c. Discussion

In this study, we demonstrate that the expression level of *Sln* is dramatically increased at both an early and a late stages of the disease. This indicates that SLN may play a role in the pathogenesis of XLMTM, at least in the murine model. In contrast to the mouse model, transcriptome analysis performed on muscle biopsies from XLMTM patients did not shown any *Sln* deregulation (Noguchi et al. 2005). This may be due to differences in the normal levels of sarcolipin between small and big mammals (Vangheluwe et al. 2005). This argument is supported by the presence of the same discrepancy in nemaline myopathy (Sanoudou et al. 2003), (Witt et al. 2006).

As SLN is an inhibitor of SERCA pumps, its overexpression induces a decrease in calcium uptake and in turn affects contractility (Babu et al. 2006), (Ottenheijm et al. 2008). Our electrophysiological analysis of isolated muscle fibers from WT and *Mtm1*-KO mice, has demonstrated that the calcium uptake capacity was significantly depressed (by 30%)

following 5 ms-long pulses, but it was unchanged after longer pulses (10 and 20 ms-long). It is unlikely that this decrease of calcium removal is due to SLN-inhibition of SERCA, as indicated by the normal values of Ca^{2+} uptake after longer pluses. However, this decrease of calcium uptake observed at 5 ms-long pulses may be due to the decreased amount of Ca^{2+} released in *Mtm1*-KO fibers, regarding the Ca^{2+} dependence property of Ca^{2+} uptake. This discrepancy between the known effect of *Sln* overexpression and the results of our functional measurements may be explained by the regulation of the activity of SLN by phosphorylation, as phosphorylation of SLN removes its inhibitory effect (Gramolini et al. 2006). Thus, more analyses are required to assess the phosphorylation state of SLN in our model.

In addition, we cannot rule out a direct or indirect role of myotubularin or even of its substrates/products in the control of SERCA pumps. It was previously reported that the plasma membrane Ca^{2+} ATPase (PMCA) in human platelets, which belongs to the same family of SERCA Ca^{2+} ATPase, is regulated by PI3K or PI4K (Rosado et al. 2000). Moreover, SERCA pump was found to be associated with PI which enhances its activity when phosphorylated (Varsanyi et al. 1983). This indicates a potential role for phosphoinositides in the regulation of Ca^{2+} pumps. In this context, it will be of interest to analyze the activity of SERCA pumps in the presence of phosphoinositides or myotubularins.

II-IGF1 signaling in Mtm1 deficient mice and characterization of atrophic factors involved in myotubular myopathy pathogenesis

II-1. Introduction

X-linked myotubular myopathy (XLMTM) is a rare congenital disease characterized by generalized muscle weakness. Patients muscle biopsies exhibit small and rounded myofibers with centrally located nuclei (Wallgren-Pettersson et al. 1995). This disease is due to mutations within the *MTM1* gene, that encodes a ubiquitous phosphoinositide phosphatase, named myotubularin (MTM1) (Laporte et al. 1996). MTM1 is involved in the maintenance of muscle structure and membrane homeostasis, in particular, T-tubule organization in skeletal muscle (Buj-Bello et al. 2008), (Al-Qusairi et al. 2009). Mice deficient in MTM1 exhibit the main pathological features of the disease, including muscle hypotrophy and decrease in muscle strength. *Mtm1* null mice look normal at birth and start to develop a CNM phenotype by the age of 3-4 weeks (Buj-Bello et al. 2002), (Al-Qusairi et al. 2009). The observed atrophy is due to a decrease in myofibers diameter not due to a decrease in fiber number or to necrosis (Buj-Bello et al. 2002). In addition, this hypotrophy affects both fiber types but is more obvious in slow twitch (type I) fibers (Buj-Bello et al. 2002). However, the cellular pathway(s) involved in the XLMTM related-atrophy remains elusive.

Insulin like growth factor 1 (IGF1) is both a mitogen and a differentiation factor. Low concentrations of IGF1 enhance satellite cell proliferation and high concentrations stimulate the differentiation of myoblast to form postmitotic myotubes (Clemmons 2009). Overexpression of IGF1 in C2C12 cells enhances the level of transcription factors implicated in muscle differentiation, like MyoD and myogenin, and induces the formation of myotubes (Coolican et al. 1997). Its overexpression in transgenic mice results in myofiber hypertrophy and increased muscle mass (Coolican et al. 1997), (Musaro et al. 2001). Direct administration of IGF1 into atrophic muscle results in increased muscle mass and enhanced satellite cell proliferation (Chakravarthy et al. 2000). It has been shown that IGF1 treatment can counter muscle atrophy induced by several pathologies. For example, AAV-mediated co-delivery of IGF1 and micro-dystrophin increases muscle mass and strength, and rescues the dystrophic phenotype of mdx mice (Abmayr et al. 2005).

IGF1 is known for its capacity to induce protein synthesis and prevent protein breakdown via two main pathways, the PI3K/AKT (Bodine et al. 2001) and the Calcineurin /GATA pathways (Musaro et al. 1999). IGF1R is composed of two extracellular alpha chains and two signal transducing beta chains cross-linked by disulfide bridges. IGF1 binding to the receptor induces a conformational change leading to activation of its intrinsic tyrosine kinase which resides in the beta subunit. This allows the autophosphorylation of additional tyrosine kinases that act as docking sites for downstream signaling proteins such as the Insulin Receptor Substrate-1 (IRS-1). IRS-1 binds to the receptor and is subsequently phosphorylated (Clemmons 2009). IRS-1 is a docking protein for several downstream effectors including p85, the regulatory subunit of PI-3 kinase (PI-3K). PI-3K activation leads to phosphoinositide generation in the plasma membrane, which recruits and activates AKT kinase (PKB). This is considered as the predominant pathway of IGF-I-mediated hypertrophy, (see figure 32). When activated, AKT leads to the activation of mammalian target of rapamycin (mTOR) resulting in the formation of a signaling component termed TORC1, which in turn activates protein synthesis (Wang et al. 2006).

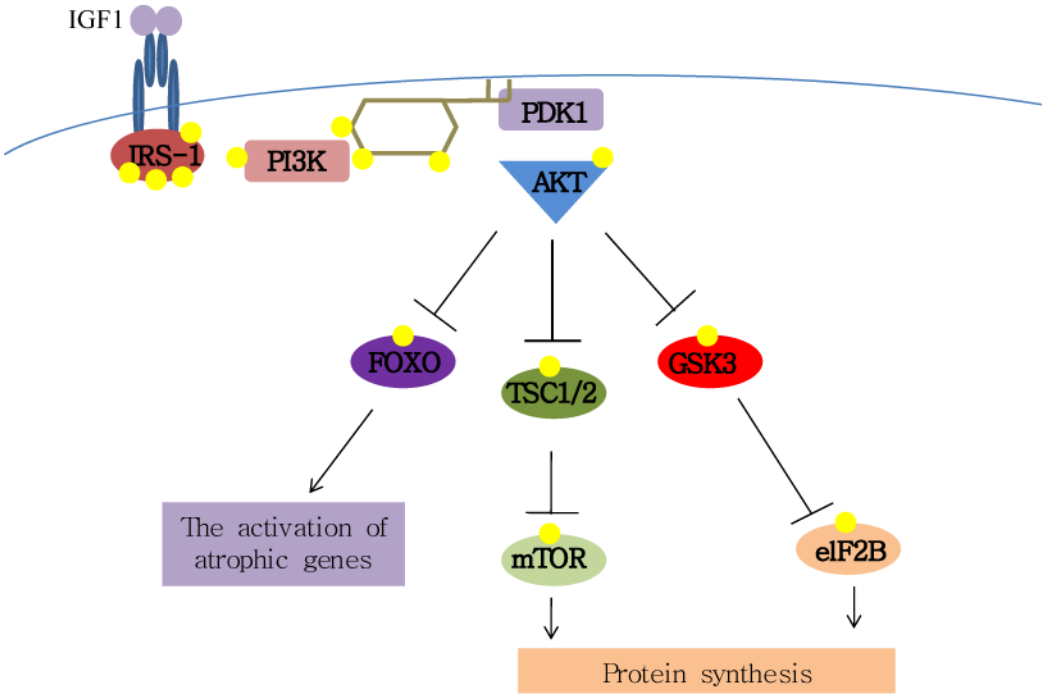


Figure (32): *IGF1/AKT canonical pathway*

In addition, the hypertrophic effect of IGF1 can be mediated by the Calcineurin /GATA pathway (Musaro et al. 1999). Calcineurin is a ubiquitous calcium-activated serine phosphatase composed of a hetero-trimeric complex including a catalytic subunit (CnA), a

regulatory subunit (CnB) and the calcium sensor calmodulin (Crabtree 1999). IGF1 induces the expression and nuclear translocation of calcineurin (Musaro et al. 1999). In the nucleus, calcineurin is associated with GATA2 and NF-AT to activate the transcription program that results in fiber hypertrophy (Musaro et al. 1999).

We overexpressed IGF1 in the skeletal muscle of two murine strains lacking *Mtm1*, in an attempt to reverse the muscle hypotrophy induced by the absence of *Mtm1*. Although *Mtm1* mutant mice from both backgrounds develop generalized and progressive CNM, signs of disease appear earlier in the 129-PAS strain compared to C57Bl6 (B6). Surprisingly, we found that the *Mtm1*-KO mice do not respond to IGF1 activation at least in the 129-PAS background. This led us to investigate the IGF1 signaling pathway in *Mtm1*-KO skeletal muscle, and to explore the atrophic mechanism/s responsible for the decrease in muscular mass observed in XLMTM.

II-2. Results

II-2-A. *Effect of IGF1 overexpression in Mtm1-KO mice*

In order to overexpress IGF1 in skeletal muscle of *Mtm1* deficient mice, heterozygous females for the *Mtm1* mutation were crossed with transgenic male from FVB/N strain overexpressing IGF1 under the myosin light chain (MLC) promoter, a skeletal muscle-specific promoter (Buj-Bello et al. 2002), (Musaro et al. 2001). In this study, we used two *Mtm1*-KO strains, the 129-PAS strain and the C57Bl6 strain in which the phenotype is less severe (Buj-Bello et al. 2002), (Al-Qusairi et al. 2009). We first evaluated the effect of IGF1 overexpression by measuring the whole body weight of WT, WT overexpressing IGF1 (WT^{IGF1+}), KO and KO overexpressing IGF1 (KO^{IGF1+}) mice. The analysis showed in the 129-PAS strain, an increase in the whole mass of PAS-WT^{IGF1+} mice by about 14% however, the body weight of PAS-KO^{IGF1+} mice was comparable to that of PAS-KO animals (figure 33a). Then, we searched for subtle increases in muscle mass by measuring the weight of isolated skeletal muscles from mice of this background. We found an increase in the weight of WT quadriceps by 47,6 % (58 +/- 2 in WT^{IGF1+} compared to 39 +/- 9 in WT quadriceps) (figure 33c). As MLC promoter is activated exclusively in fast twitch fibers, no increase was observed in the weight of the slow twitch (soleus) muscle (2 +/- 0,1 in WT^{IGF1+} and 2 +/- 0,2 in WT soleus) (figure 33d). Thus, the quadriceps and the soleus muscles can serve as positive and negative controls respectively for IGF1 action. As for the analysis of whole body weight,

the weight of isolated muscles from KO^{IGF1+} indicated no effect of IGF1 overexpression (the weight of the quadriceps from KO mice was 30 +/- 1 and from KO^{IGF1+} mice was 30 +/- 2) (figure 33d). Together, this shows that IGF1 overexpression has no effect on the muscle mass of 129-PAS Mtm1-KO mice. In addition, IGF1 overexpression did not alter the lifespan of KO^{IGF1+} mice (figure 33b).

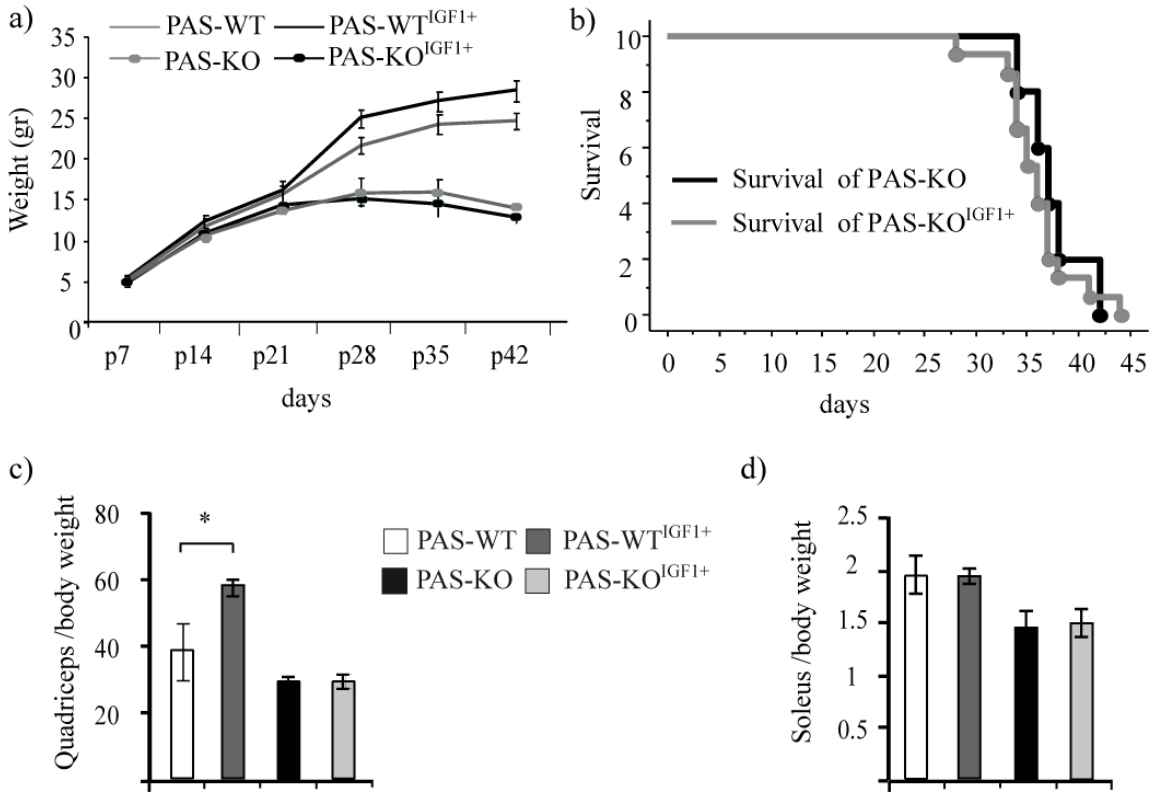


Figure (33): *The effect of IGF1 overexpression on WT and Mtm1-KO mice (129-PAS strain).* a) Growth curve of the four groups of mice, n=7 and n=10 for WT and WT^{IGF1+} respectively, n=6 and n=16 for KO and KO^{IGF1+} respectively, ANOVA test for WT and WT^{IGF1+} gives p< 0,001. b) Survival of PAS-KO and PAS-KO^{IGF1+} mice, n=5 and n=9 respectively. c,d) Weight of isolated quadriceps and soleus muscles from the four groups of 4 week-old mice, normalized by the whole body weight to eliminate the variability due to differences between littermates, n=7 and n=8 for WT and WT^{IGF1+} respectively, n=5 for each KO and KO^{IGF1+} , *=(p = 0.04).

We also initiated the analysis of the effect of IGF1 overexpression in C57Bl6 mice lacking *Mtm1*. Due to the low reproduction rate of B6 mice, we have not yet been able to analyze the phenotype of *Mtm1*-KO mice overexpressing IGF1 but, very preliminary results may suggest that the genetics background affect the response to IGF1 overexpression. Again,

we analyzed the whole body weight of B6-WT, B6-KO, B6-WT overexpressing IGF1 (B6-WT^{IGF1+}) and B6-KO overexpressing IGF1 (B6-KO^{IGF1+}). The analysis showed a significant increase in whole body weight of B6-WT^{IGF1+} mice by 12,5% (figure 34a). However, B6-KO^{IGF1+} mice showed an increase in whole body weight by 8,8% (figure 34a) but ANOVA analysis showed that it did not reach significance. Isolated muscle weight measurements revealed an increase in the weight of quadriceps from B6-WT^{IGF1+} by 9% (63 +/- 0,2 in WT^{IGF1+} compared to 58 +/- 1 in WT mice) (figure 34c). While there was no increase in the weight of soleus muscle as expected (2,6 +/- 0,3 in WT^{IGF1+} compared to 2,6 +/- 0,2 in WT mice) (figure 34d). The weight of quadriceps muscle from B6-KO^{IGF1+} was increased by 17%, whoever, a significant test could not be applied due to a small number of mice (n=2 for B6-KO^{IGF1+}). Thus, we cannot yet conclude about the effect of IGF1 overexpression in the B6 strain (more mice have to be included in this study). As observed in the 129-PAS study, IGF1-overexpression did not impact on the lifespan of B6-KO^{IGF+} (figure 34b)

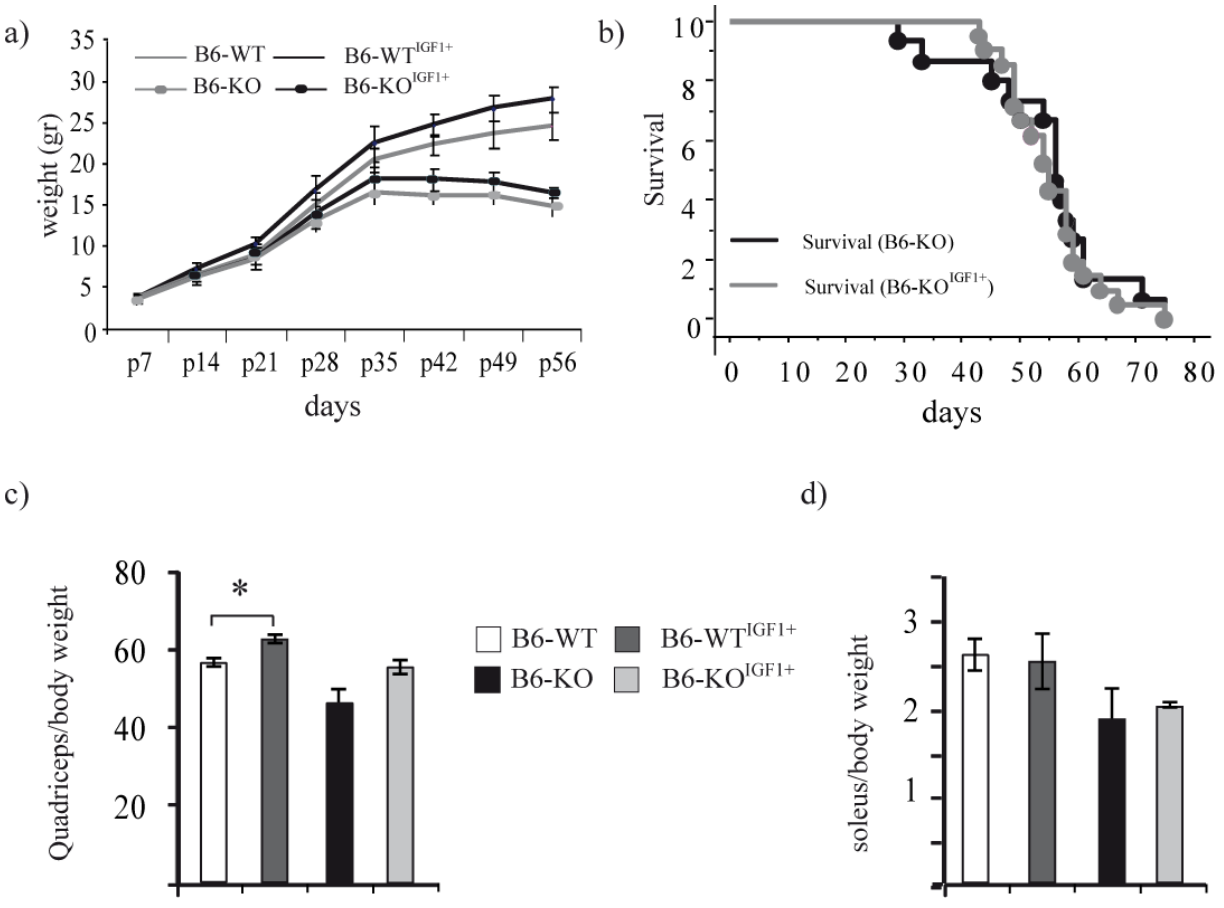


Figure (34): *The effect of IGF1 overexpression on WT and Mtm1-KO mice from the C57Bl6 strain.* a) Growth curve of the four groups of mice (for B6-WT and B6-WT^{IGF1+}, n=14 and n=12 respectively, p < 0,0003), (for B6-KO and B6-KO^{IGF1+}, n=8 each, p=0,17). b) Survival of B6-KO and B6-KO^{IGF1+},

n=11 and n=14 for each group, respectively. c,d) Weight of isolated quadriceps and soleus muscles from the four groups of 4 week-old mice, normalized against the whole body weight, n=4 and n=3 for B6-WT and B6-WT^{IGF1+} respectively, n=3 and n=2 for B6-KO and B6-KO^{IGF1+} respectively , * = (p = 0.02).

II-2-B. *Analysis of IGF1 signaling pathway in Mtm1-KO mice*

The data presented above suggest that mice deficient in *Mtm1* do not respond properly to IGF1 overexpression. To elucidate the cause(s) of this defect, we analyzed the expression level of several components of the IGF1 pathway in tibialis anterior (TA) muscle from WT and KO mice by western blotting (WB) and immunofluorescence analysis (IF). In this part of our study, we chose to analyze the 129-PAS strain. We observed a 5 fold increase in the level of IGF1R and almost a 2 fold increase in AKT levels in *Mtm1*-KO versus WT (figure 35a). IF analysis showed that the overexpressed IGF1R is localized at the plasma membrane and also in the cytoplasm, as in the WT muscle (figure 35b). However, the protein level of mTOR and Foxo3a remained unchanged (figure 35a). In addition to IGF1 canonical pathway, studies using *Cav3*-KO mice have shown that caveolin3 (Cav3) is an enhancer of insulin signaling in skeletal muscle (Oshikawa et al. 2004). This may occur by the direct stimulation of the insulin receptor (IR) kinase activity (Yamamoto et al. 1998). We analysed the protein levels of Cav3 by WB and we found an increase by about 2 fold in KO muscle, which mainly localises, as expected ,at the sarcolemma (figure 35b).

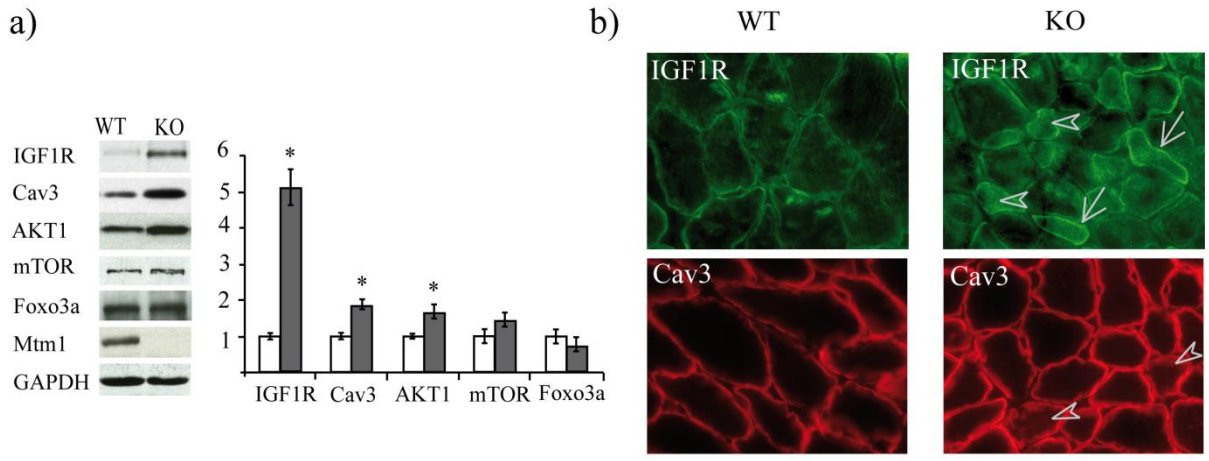


Figure (35): *Levels of several IGF-I/AKT pathway components is altered in Mtm1-KO mice*. a) WB analysis for proteins implicated in the IGF1 pathway in 5 weeks old mice (left panel). Quantification of protein levels (right panel) which reflects a significant increase in the IGF1R (5,1 +/- 0,5), Cav3 (1.9 +/- 0.13) and AKT1 (1,7 +/- 0,19) proteins. (n= 3 WT and n=3 KO for Cav3 and n= 4 WT and n=

4 KO for the other proteins, p = 0.0002, 0.004 and 0.008 for IGF1R, Cav3 and AKT1 respectively, the level of proteins was normalized by the level of GAPDH). b) Immunofluorescence analysis of transversal sections of TA muscle with anti-Cav3 and anti-IGF1R specific antibodies from 5 week-old WT and KO mice. Arrows indicate sarcolemmal localization and arrow heads indicate cytoplasmic distribution. * = ($p<0.05$)

II-2-C. *Role of the proteasome pathway in XLMTM-related atrophy*

Muscle atrophy induced by several conditions such as fasting, denervation, diabetes, disuse, glucocorticoids treatment, involves protein degradation via several pathways, including the proteasome pathway (Jagoe et al. 2001). This process requires a series of transcriptional adaptations such as an increase in the gene expression of atrogenes, genes involved in protein-degradation in skeletal muscle (Jagoe et al. 2002). Several atrogenes have been identified as hallmarks of muscle atrophy, including Atrogin1, Murf1 and Nedd4, which all have a E3 ubiquitin ligase activity (Gomes et al. 2001), (Bodine et al. 2001), (Harvey et al. 1999). Recently, ZNF216 was identified as a ubiquitin recognition factor implicated in muscle atrophy (Hishiya et al. 2006). The expression of these atrogenes (not in the case of Nedd4) is activated by FOXO transcription factors (Sandri et al. 2004), (Hishiya et al. 2006). In fact, the activation of the IGF1 pathway induces the phosphorylation of Foxo transcription factors. This phosphorylation keeps them in an inactive state as they cannot cross the nucleus pores. The absence of IGF1 signaling results in nuclear import of Foxo proteins, which can in turn activate the expression of atrogenes (Sandri et al. 2004).

In this study, we have evaluated the phosphorylation levels of Foxo3a by a specific antibody (figure 36a). We observed a decrease in the levels of phosphorylated Foxo3a. Consequently, we analyzed the expression of Foxo3a target genes and Nedd4 using quantitative RT-PCR. The data obtained from WT and KO mice at late stage of the disease, suggest that both Atrogin1 and MurF1 were increased by about 3 fold and 3,6 fold, respectively. Similarly, the expression levels of Nedd4 and Znf216 both were increased by 1,5 fold (figure 36b). Interestingly, the expression profile of these genes was altered even at an early stage of the disease, at the beginning of the hypotrophy (figure 36c).

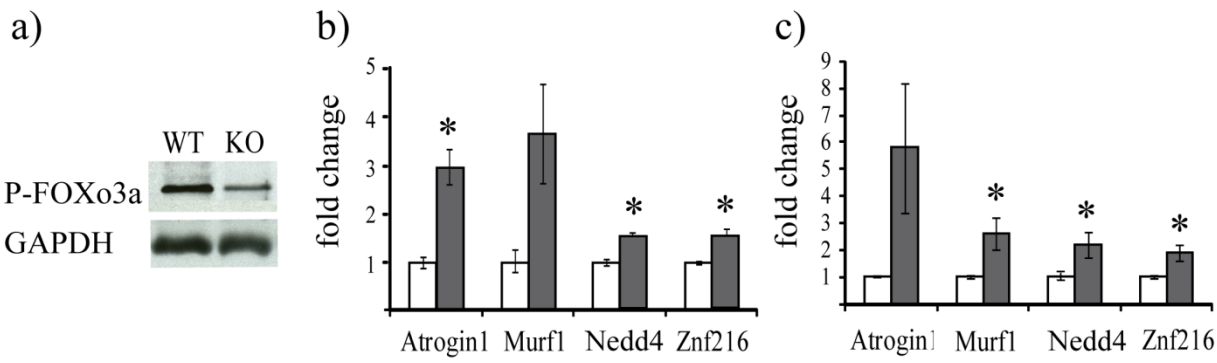


Figure (36): *Expression level of atrogenes in Mtm1-KO muscle*. a) Western blotting analysis showing a decrease in the phosphorylated form of Foxo3a. b) Q-RT-PCR shows the increase in expression of atrogenes: *Atrogin1* (2,9 +/- 0.35), *Murf1* (3,6 +/-1,03), *Nedd4* (1,5 +/- 0,04) and *Znf216* (1,5 +/- 0,13) at a late stage of the disease in TA muscle (p= 0.02 and 0.07 for atrogin1 and Murf1 respectively, p=0.001 and 0.04 for Nedd4 and Znf216 respectively). c) Q-RT-PCR shows the expression level of atrogenes in TA muscle of KO mice in the asymptomatic phase (2 weeks of age), atrogin1 (5,8 +/-2), Murf1 (2,6 +/-0,6), Nedd4 (2,2 +/- 0,5) and Znf216 (1,9 +/- 0,3), (p=0.09 and 0.04 for atrogin1 and Murf1 respectively, p= 0.004 and p=0.02 for Nedd4 and Znf216 respectively). (n=4 wt and n=4 KO) for each panel. * = ($p<0.05$)

II-3. Discussion

II-3-A. *Effect of IGF1 overexpression in Mtm1-KO mice*

We overexpressed IGF1 in an attempt to rescue the hypotrophic phenotype observed in *Mtm1*-KO mice. To address the effect of IGF1 overexpression, we used two genetic backgrounds as they display some differences in the response to myotubularin deficiency. Interestingly, we found that the strain which exhibits the more severe phenotype (129-PAS) does not respond to IGF1 overexpression. Whoever, due to the small number of C57Bl6 mice used in the study, we cannot yet conclude about the effect of IGF1 overexpression on *Mtm1*-KO mice from this strain. These observations suggest that the IGF1 signaling is not properly activated in *Mtm1*-KO muscle at least in 129-PAS strain. However, we cannot rule out that a high level of IGF1 overexpression can have a hypertrophic effect in these mutant mice. In addition, the effect of IGF1 on muscle strength remains to be quantified.

II-3-B. *Mtm1 related hypotrophy and IGF1 pathway*

A decrease in IGF1/AKT signaling can lead to muscle atrophy (Bodine et al. 2001), (Sandri et al. 2004). Moreover, IGF1 treatment of atrophied myofibers blocks the atrophy program and stops the degradation of muscle fiber proteins (Sacheck et al. 2004). In our study, we show that muscles deficient in MTM1 do not increase in mass in response to IGF1 overexpression. This observation led us to investigate IGF1 signaling and we observed alterations in the protein level of several components of this pathway, and in particular, a major increase in IGF1R level. In fact, an accumulation of epidermal growth factor receptor (EGFR) in *Mtm1* deficiency conditions was previously described in human cells expressing siRNA against MTM1, where EGFR protein was increased and accumulated in the early endosome (Cao et al. 2008). In this study, we show an accumulation of IGF1R in the cytoplasm and also at the sarcolemma. There are several possible explanations for this, i) a defect in the endocytosis of cell surface receptors, ii) a compensatory mechanism aiming to amplify the IGF1 effect, iii) a defect in the phosphorylation of the receptor as it is endocytosed upon phosphorylation.

To elucidate the reason for this increase in IGF1R, we plan to analyse IGF1R levels at earlier stages of the disease (ie, one week) and also to analyse its phosphorylation status. In addition to an increase in IGF1R level in *Mtm1* mutant muscle, we observed an increase in AKT and Cav3. Similarly, analysis at earlier stages will help us to understand if these alterations are due to a compensatory mechanism or primary defect. Moreover, we plan to analyse the phosphorylation status of AKT to determine the level of IGF1/AKT activation in this model.

II-3-C. *The implication of the ubiquitin-proteasome system (UPS) in MTM1-related hypotrophy*

In this study, our results suggest that the hypotrophy observed in the *Mtm1*-KO mouse is may be due to the activation of the UPS pathway. Our analysis indicated that the expression of several atrogenes is upregulated, in particular, E3 components of the UPS pathway, including atrogin1, Murf1 and Nedd4 levels were increased. The upregulation of Murf1 can account for the sarcomere disorganization, which is observed in *Mtm1*-KO mice, as it has been shown that Murf1 targets include proteins implicated in sarcomere structure, such as titin (Centner et al. 2001). We have previously shown that the ryanodine receptor (RyR1) and the

subunit alpha of DHPR are present at low levels in KO muscle, whereas their mRNA levels remain unchanged (Al-Qusairi et al. 2009). This may result from Nedd4 overexpression, as it has been shown that, it is involved in the degradation of several transmembrane and cell surface proteins (Koncarevic et al. 2007).

Moreover, our analysis has demonstrated that at the age of 2 weeks, prior to the appearance of muscle weakness, the muscular mass of *Mtm1*-KO mice is already significantly decreased (Al-Qusairi et al. 2009). At the same age, the expression levels of analyzed atrogenes was shown to be elevated. This suggests that the observed hypotrophy is not a secondary mechanism but may be involved in the early pathogenesis of murine XLMTM. In this context, it will be interesting to analyze the factor/s which trigger the atrophic pathway.

III-Overexpression of RUNX1 and its target Rrad in skeletal muscle lacking MTM1

III-1. Introduction

Protein degradation is accomplished through three mechanisms during muscle atrophy: 1) the ubiquitin/proteasome mechanism, 2) autophagy, 3) and the action of caspase proteases. However, under disease conditions, muscular tissues develop mechanisms to retard disease progression. Such mechanisms include the activation of RUNX1 gene expression which limits muscle atrophy upon denervation by preventing autophagy (Wang et al. 2005). Runx proteins are a family of transcription factors that share a Runt homology domain (RHD) and play important roles in embryonic development and cancerogenesis (Bae et al. 2006). Mutations within RUNX1 are associated with acute myeloid leukemia (AML) (Look 1997). In normal skeletal muscle, RUNX1 is expressed at low level however, muscle denervation induces its upregulation (Zhu et al. 1994). Interestingly, innervated WT and *Runx1*-KO muscles appear similar but, denervated *Runx1*-KO muscle is more atrophied than denervated WT muscle and contains a high number of autophagosomes (Wang et al. 2005). Analysis of the transcriptional profile of denervated WT and denervated *Runx1*-KO muscle using DNA microarray, identified 29 genes as potential RUNX1 targets, including genes implicated in muscle structure and electrical activity (Wang et al. 2005). (see figure 37)

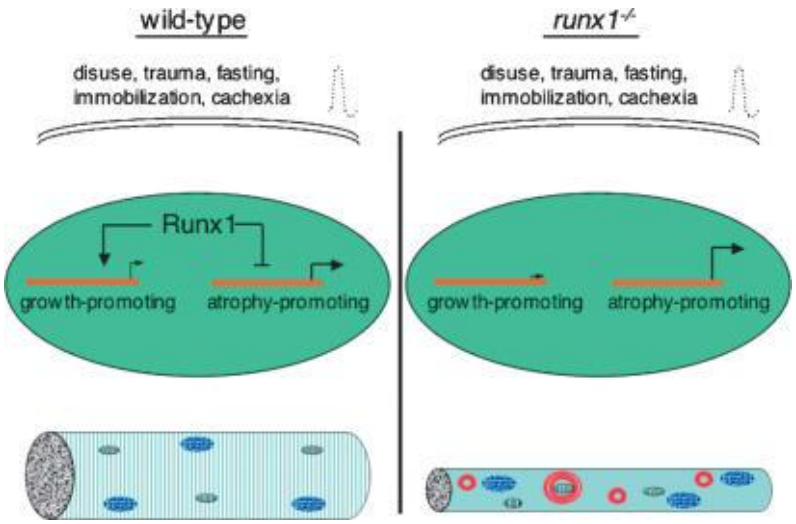


Figure (37): *Proposed model for the role of RUNX1 in muscle injury*. In wild-type mice, induction of RUNX1 limits the extent of muscle wasting and preserves the structural integrity of myofibrils. In the absence of Runx1, muscle disuse leads to the disorganization of myofibrils, excessive autophagy, and

severely atrophied myofibers. RUNX1 activates and represses genes in denervated myofibers, and misregulation of these genes in *Runx1* mutant muscles is responsible for the severe muscle wasting observed, (from Wang, 2005, Mol Cell Biol).

One of the potential targets of RUNX1 is *Rrad*, a small GTPase that is overexpressed in the skeletal muscle of patients with type II diabetes (Ras associated with diabetes) (Reynet et al. 1993). *Rrad* represents the prototypic member of a novel *Ras*-related GTPases family, the *Rrad*, *Gem*, and *Kir* (RGK) family which displays a *Ras*-related core domain (see figure 38) (Reynet et al. 1993), (Maguire et al. 1994), (Chen et al. 2005). In skeletal muscle, Rrad interacts with β-tropomyosin and the cytoskeleton in a GTP-dependent manner. These observations suggests that it may be involved in skeletal muscle motor function and cytoskeletal organization (Zhu et al. 1996). In addition, it is considered a modulator of L-type calcium channels dihydropyridine receptor (DHPR) (Beguin et al. 2001), (Finlin et al. 2003). DHPR is the voltage sensor calcium channel that mediates signal transduction between depolarization and calcium release in cardiac and skeletal muscles. More specifically, Rrad has an inhibitory effect on DHPR function, but the mechanism is not clear. This seems to happen either by a decrease in the membrane expression of the DHPR alpha subunit (Beguin et al. 2005), or by a direct association to the beta subunit of DHPR without perturbing the localization of the DHPR alpha subunit (Finlin et al. 2003), (Finlin et al. 2006).

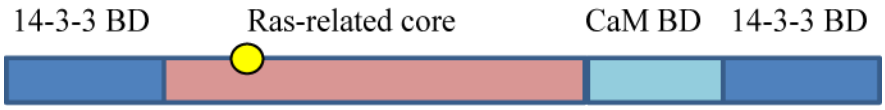


Figure (38): Schematic representation of the RGK protein family structure showing the Ras-like core domain (purple), N and C-terminal regions (dark blue), which have a 14-3-3 binding site, and the calmodulin binding domain (light blue). The GTP binding site is shown by yellow circle. (modified from Béguin P., 2005, J Mol Biol)

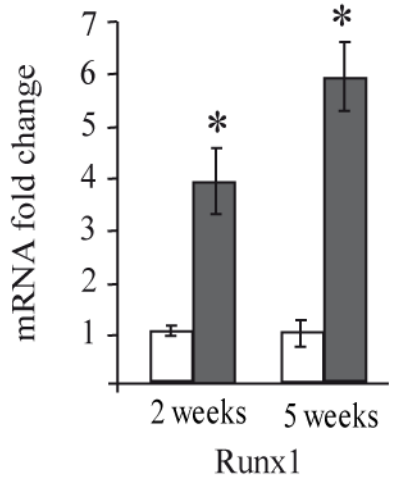
We have studied *Runx1* expression and its potential target *Rrad* in *Mtm1*-KO muscle during disease progression. We also demonstrate using bioinformatics tools that the *Rrad* genomic sequence contains two potential binding sites for the RUNX protein.

III-2. Results

III-2-A. *Analysis of Runx1 expression level in Mtm1-KO mice during disease progression*

DNA microarray analysis of *Mtm1*-KO mice has demonstrated the deregulation of 3 transcription factors, including activating transcription factor 3 (*Atf3*), Paired-like homeodomain transcription factor 2 (*Pitx2*) and *Runx1*, which were upregulated by 4, 2 and 5 fold respectively. We focused our analysis on Runx1 due to its implication in the inhibition of muscle autophagy (Wang et al. 2005). We analyzed the expression levels of Runx1 by Q-RT-PCR and found an increase in the mRNA level by about 6 fold at a late phase of the disease (as showed DNA microarray data) and about 4 fold in 2 week-old mice lacking MTM1 (Figure 4a). In addition, the expression of several RUNX1 targets was increased as shown by the DNA microarray data obtained from WT and *Mmt*1-KO muscles (n=3 WT and n=3 KO, D., Sanoudou and AH. Beggs) (Figure 39b).

a)



b)

genes deregulated in myotubular myopathy and potentially regulated by RUNX1	fold change
Ras-related associated with diabetes (Rrad	61,65
myosin, heavy polypeptide 3, skeletal muscle, embryonic (Myh3)	23,72
keratin complex 1, acidic, gene 18 (Krt1-18))	27,28
RIKEN cDNA 1110025L05 gene (1110025L05Rik)	5,17
RIKEN cDNA 5830411E10 gene (5830411E10Rik)	3,01
double cortin and calcium/calmodulin-dependent protein kinase-like 1 (Dcamkl1)	2,56
four and a half LIM domains 1 (Fhl1)	2,25
calcium channel, voltage-dependent, gamma subunit 1 (Cacng1)	2,25
thrombospondin 1 (Thbs1)	2,24

Figure (39): *Upregulation of Runx1 mRNA level and potential RUNX1-targets.* a) Q-RT-PCR analysis of TA muscle shows RUNX1 overexpression at early (3.9 +/- 0.36) and at late (5.8 +/- 0.66) stages of the disease (p= 0.0006 and 0.0003 for muscle from 2 and 5 week-old mice respectively, n=5 WT and

n= 3 KO at 2 weeks of age, n=3 WT and n=3 KO at 5 weeks). b) Table showing several genes proposed to be regulated by RUNX1 (Wang et al. 2005), which expression is increased in *Mtm1*-KO mice at a late stage of the disease as shown by DNA microarray data of WT and *Mtm1*-KO mice. * = ($p<0.05$)

III-2-B. *Identification of putative RUNX responsive elements in the Rrad promoter*

Previous studies have shown that RUNX transcription factors are either gene activators or suppressors depending on the associated co-factors. It has been suggested that *Rrad* is a potential target of RUNX1 (Wang et al. 2005). The DNA microarray data performed on *Mtm1*-KO muscle at a late stage of the disease, has shown the upregulation of Rrad expression by about 62 fold. These data are further confirmed by Q-RT-PCR analysis, were we found that Rrad expression is dramatically increased by about 32 fold, in *Mtm1*-KO muscle at a late phase of the disease (in 5 week-old mice) (figure 40a). Moreover, WB analysis confirmed this upregulation (figure 40b). More interestingly, even in the clinically asymptomatic stage, *Rrad* expression was increased by 9 fold (figure 40a).

It is likely that the increase of *Rrad* expression is due to an increase of RUNX1 expression, we then aimed to determine whether there is a transcription factor binding site (TFBS) for RUNX1 in the *Rrad* regulatory sequence (analysis performed in collaboration with Laurent Bianchetti from IGBMC bioinformatics platform). For this, we extracted the sequences of the *Rrad* gene from *Homo sapiens*, *Bos taurus* and *Mus musculus* genomes, analyzed the conservation of *Rrad* sequences between the three orthologues using multi-alignment PromAn software and searched for the potential TFBS in the mouse genomic sequence via the Genomatix MatInspector tool and Genomatix database. As parameters, we chose Matrix similarity >70% and Core similarity >70% (see figure 40d and c). Finally, we selected the TFBS predictions conserved between orthologous sequences. We found a 1.1 kb sequence upstream and a 1.2 kb sequence downstream of the *Rrad* mouse gene that are highly conserved between the three orthologues. These regions, in addition to the coding sequence, were screened for potentials TFBS.

Using this approach, we identified 52 matches, 11 of which were conserved in all orthologues. This study allowed the identification of two potential RUNX binding sites, one situated in the second intron starting at +1023 base, and another situated at +514 base after the

last exon. Figure 40c shows a schematic representation of TFBS for RUNX1 and other transcription factors on Rrad genomic sequence.

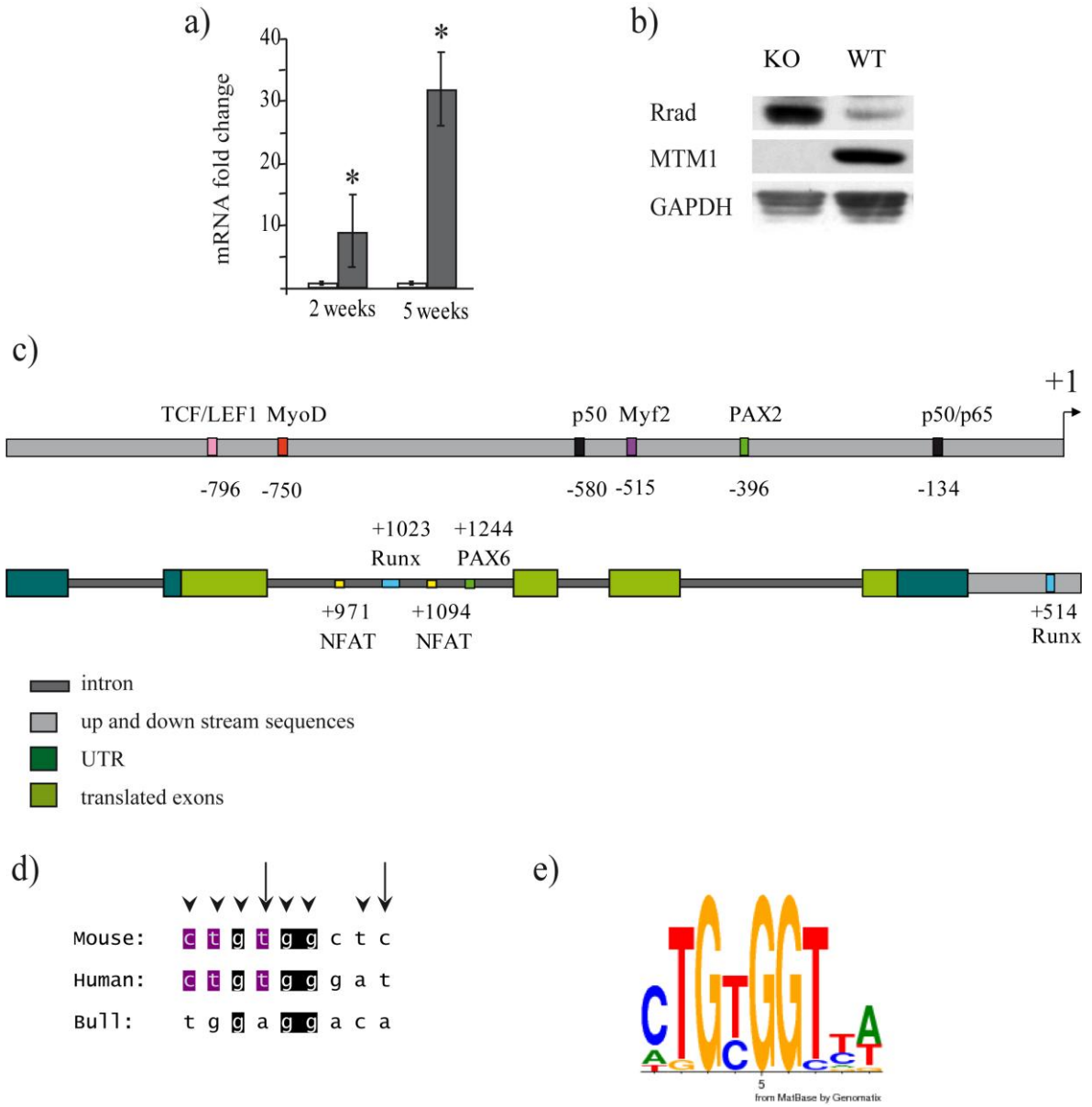


Figure (40): *Rrad upregulation in Mtm1 KO muscle and the prediction of RUNX binding sites in the Rrad genomic sequence.* a) Q-RT-PCR analysis in TA muscle showed an increase of *Rrad* mRNA level at early (9.1 +/- 2.7) and also late (32 +/- 5.8) stages of the disease, p = 0.006 for both ages (n=5 WT and n=3 KO at 2 weeks of age, n=3 WT and n=3 KO at 5 weeks), * = ($p<0.05$). b) Western blotting shows an increase of RRAD protein level at late stages of the disease, (n = 7 KO and n= 8 WT). c) Schematic representation of *Rrad* genomic sequence in mouse shows its five exons with the translated (light green) and untranslated (dark green) regions. Potential transcription factors including RUNX1, which regulate *Rrad* expression and their predicted sites are indicated. d) The multiple alignment of mouse, human and bull sequence showing the potential TFBS of RUNX, arrowhead

means a complete match between the orthologues sequences and the matrix and arrow indicates that 2 out of 3 positions in the orthologues sequences are reported as present in RUNX matrix. e) RUNX matrix obtained from Genomatix site.

III-C. Discussion

In this study, we show that the expression of *Runx1* is upregulated at a late and at an early stages of the disease in Mtm1$^{-/-}$ mice. Previous study analyzing *Mtm1* null mice has suggested that autophagic vacuoles appear absent from the mutant muscle as revealed by hematoxylin-eosin (HE) staining (Buj-Bello et al. 2002). Skeletal muscle examination of *Mtm1*-KO mice by electron microscopy has confirmed this observation. One possible mechanism employed by injured muscle to delay disease progression is potentially mediated by RUNX1 (Wang et al. 2005). Our analysis revealed that *Runx1* expression is enhanced from the early phase of the disease and is further increased at the later phase. Interestingly, many genes potentially regulated by RUNX1 were found to be activated in Mtm1$^{-/-}$ KO skeletal muscle as showed the DNA microarray analysis. This fits with the reported anti-autophagic effect of RUNX1. Molecular analysis of autophagy in our mouse model would be important to exclude the implication of this process in XLMTM pathogenesis. In addition, RUNX1 seems to be implicated in apoptosis, which may occur through different pathways in a cell type-specific manner. More specifically, RUNX1 has been shown to have anti-apoptotic effect in MEG-01 cells (megakaryoblast), by the regulation of *PIK3CD* expression, which encodes for the catalytic subunit of PI3K (Edwards et al. 2009). However, RUNX1 seems to induce apoptosis in hepatic cells by cooperation with Foxo3 (Wildey et al. 2009). On the same line, no apoptotic nuclei were detected in Mtm$^{-/-}$ mice using TUNNEL assay (Buj-Bello et al. 2002).

A putative target gene of RUNX1 is *Rrad*, which is overexpressed in many pathologies. In skeletal muscle Rrad expression is regulated by several factors such as insulin (Wu et al. 2007). Developmental studies performed in Dr. Garry laboratory have shown that the expression of Rrad is restricted to muscle lineages and it is enhanced in the last weeks of mouse embryonic development (Hawke et al. 2006). This study has also demonstrated that the *Rrad* promoter contains four E-boxes which are regulated by myogenic factors like MyoD, Myf-5 and MEF2. Interestingly, the expression of these factors in C2C12 cells but not in COS-7 cells activates the expression of a reporter driven by the *Rrad* promoter. This suggests that factors specific to muscle cells are crucial for the activation of *Rrad* expression (Hawke et

al. 2006). Moreover, *Rrad* was identified as a potential target gene for RUNX1 by DNA microarray analysis (Wang et al. 2005). Our bioinformatics analysis has revealed several TFBS within conserved regions in the *Rrad* promoter, the coding sequence and the downstream 3' UTR region. We identified two potential RUNX1 binding sites, one in the second intron and another in the downstream sequence. Functional studies employing a reporter gene driven by the Rrad sequence and/or chromatin immunoprecipitation experiments are required to confirm this prediction. In addition, we have observed synchronization in the overexpression of RUNX1 and its target Rrad. Indeed, both show a progressive upregulation during XLMTM pathogenesis. Together, these findings reinforce the hypothesis that RUNX1 is crucial for *Rrad* expression during muscle injury. However, the role of Rrad in the XLMTM pathological process remains to be elucidated.

In an attempt to understand the physiopathology of XLMTM, several hypotheses have been put forward. It was proposed that the myotubular myopathy is due to an arrest in muscle maturation, since apparently undifferentiated myofibers were observed in patient muscle biopsies (Spiro et al. 1966), (van Wijngaarden et al. 1969). Alternatively, it was suggested that this muscle disorder is due to a defect in motor neuron maturation (Coers et al. 1976), (Fidzianska et al. 1994). This was supported by observations of nerve muscle co-culture that were derived from patients. Indeed, patient and control myotubes behave similarly in co-culture; they display similar features in relation to their morphology, differentiation and contractile activity (Dorchies et al. 2001). The generation of *Mtm1*-null mice and *Mtm1*-conditional mice, that do not express MTM1 in skeletal muscle specifically ruled out the implication of MTM1 in the early myogenesis or in the maturation of motor neuron at least in mice (Buj-Bello et al. 2002). It is now clear that the primary tissue implicated in XLMTM pathogenesis is the skeletal muscle and that MTM1 deficiency leads to defects in the maintenance of muscle structure, however, it has no effect on myotube differentiation (Buj-Bello et al. 2002).

In this chapter, aspects of the XLMTM pathology, the molecular mechanisms of MTM1 pathogenesis, and how our novel findings relate to what is known about MTM1 function will be discussed.

I. Analysis of MTM1 deficiency at the whole organism level

I-1. Interaction between the genetic background and XLMTM phenotype in the mouse model

This study involved the characterization of the XLMTM pathology in the 129-PAS *Mtm1* deficient mice. Analysis revealed that this mouse strain is more sensitive to MTM1 absence compared to the previously described C57Bl6 strain (B6) (Buj-Bello et al. 2002). Although both backgrounds develop generalized and progressive centronuclear myopathy, signs of disease appear earlier in 129-PAS mutant mice. Indeed, at the age of 2 weeks, B6 mice show no signs of myopathy (Buj-Bello et al. 2002), whereas, examination of 129-PAS muscle, at the same age, reveals a significant difference between WT and KO animals. The 129-PAS *Mtm1*-KO mice have smaller muscles with a higher percentage of centralized nuclei in comparison to WT at this age. This might be explained by the fact that 129-PAS mice grow faster than B6 as shown in figure (41). The 129-PAS mice exhibit a rapid gain in whole body weight during the first three weeks after birth, reaching adult body weight at the age of 5

weeks, whereas B6 mice grow more slowly and reach a stable weight at around 7 weeks of age. This may correlate with the fact that, B6 *Mtm1*-KO mice live longer than 129-PAS-KO mice.

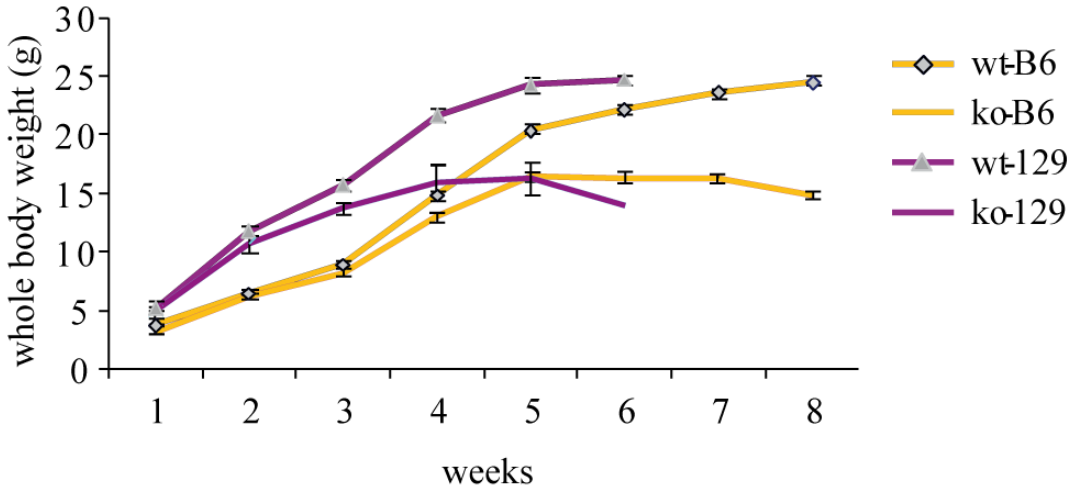


Figure (41) : *Histogram comparing growth curves of WT and Mtm1-KO animals in B6 and 129-PAS genetic backgrounds.*

I-2. Myotubular myopathy, when time makes the rules

It was believed that XLMTM may be due to a defect in differentiation/ maturation of skeletal muscle. This hypothesis was excluded after the analysis of the *Mtm1*-KO mouse, in which myogenesis was observed to occur normally with CNM symptoms appearing 3-4 weeks after birth (Buj-Bello et al. 2002). This may be explained by the difference in the timing of myogenesis between big and small mammals. More specifically, muscle maturation (defined by the peripheral position of the nuclei and the organized distribution of sarcomeres and triads) is achieved in humans at about 16 weeks of gestation however, in mice, this happens 2-3 weeks after birth (Novilla MN., 1996), (Franzini-Armstrong et al. 1991). *Mtm1*-KO mice of both genetic backgrounds develop clinical features similar to that described in patient at a later phase of the disease.

Another discrepancy in XLMTM pathogenesis between human and mouse is the progression of the disease. Newborn patients with MTM1 deficiency have a severe, generalized and non-progressive muscle dysfunction, which leads to death in the absence of respiratory assistance (Herman et al. 1999). In contrast, *Mtm1*-KO mice are healthy at birth and they progressively develop a generalized muscle dysfunction, and muscle pathology

appears to coordinate progressively beyond what is observed in patients, in particular, the development of vacuoles and the widespread defects of contractile apparatus organization.

In murine XLMTM, we have shown that muscle weakness is probably a consequence of a calcium release defect that may be directly caused by triad morphological anomalies. Interestingly, the maintenance/integrity of triad structures can be compromised by defective Ca^{2+} release/uptake cycles, as it have been shown in mice lacking DHPR, RyR, or both, that the number of triad are considerably decreased (Felder et al. 2002). In other words, the interaction between Ca^{2+} cycle and the triads seems to play a major role in the progression of XLMTM in mice (figure 42). Moreover, a defect in the structure of T-tubules has been also reported in patients, however, it still not known whether this defect leads to depressed calcium release (Dowling et al. 2009). It is reasonable to think that the decreased muscle force observed in patients is due to a decrease in calcium release, as appears to be the case in *Mtm1*-KO mice. Myotubular myopathy is non-progressive in humans, which indicates the presence of compensatory mechanism(s) which are able to prevent further muscle deterioration. This might be achieved by overexpression of related myotubularins or other proteins implicated in T-tubule organization and/or Ca^{2+} homeostasis. Although MTM1 and MTMR1 share an important similarity (~ 90%) (Laporte et al. 1998), it is unlikely that MTMR1 compensate for the absence of MTM1 as a deletion of both genes is associated with a classic severe phenotype of XLMTM (Zanoteli et al. 2005).

In this context, it would be interesting to screen the expression level of candidate genes in muscle biopsies from XLMTM patients such as related myotubularin or protein implicated in triad structure or function. In contrast to what has been shown in mice, DNA microarray data collected from patients at different ages show that the transcriptional alterations were more present in the younger patients (0-12 months) than in older patients (4-6 years) (unpublished findings, AH.Beggs). Only ten deregulated genes were detected in the group of older patients versus 85 deregulated genes in the group of younger patients. This might either be due to the fact that patients who survive longer have less severe phenotype, or indicate the stabilization of the disorder by compensatory mechanisms. In addition, another gene expression profiling of young XLMTM patients (< 9 months) performed in Nishino group, has revealed several sets of deregulated genes (Noguchi et al. 2005). The genes encoding for proteins implicated in cytoskeleton remodeling and extracellular matrix were

found to be upregulated, in addition to genes specific to type 1 fiber (Noguchi et al. 2005), whereas, genes encoding for energy metabolism, muscle contraction and type 2 fiber proteins were downregulated (Noguchi et al. 2005).

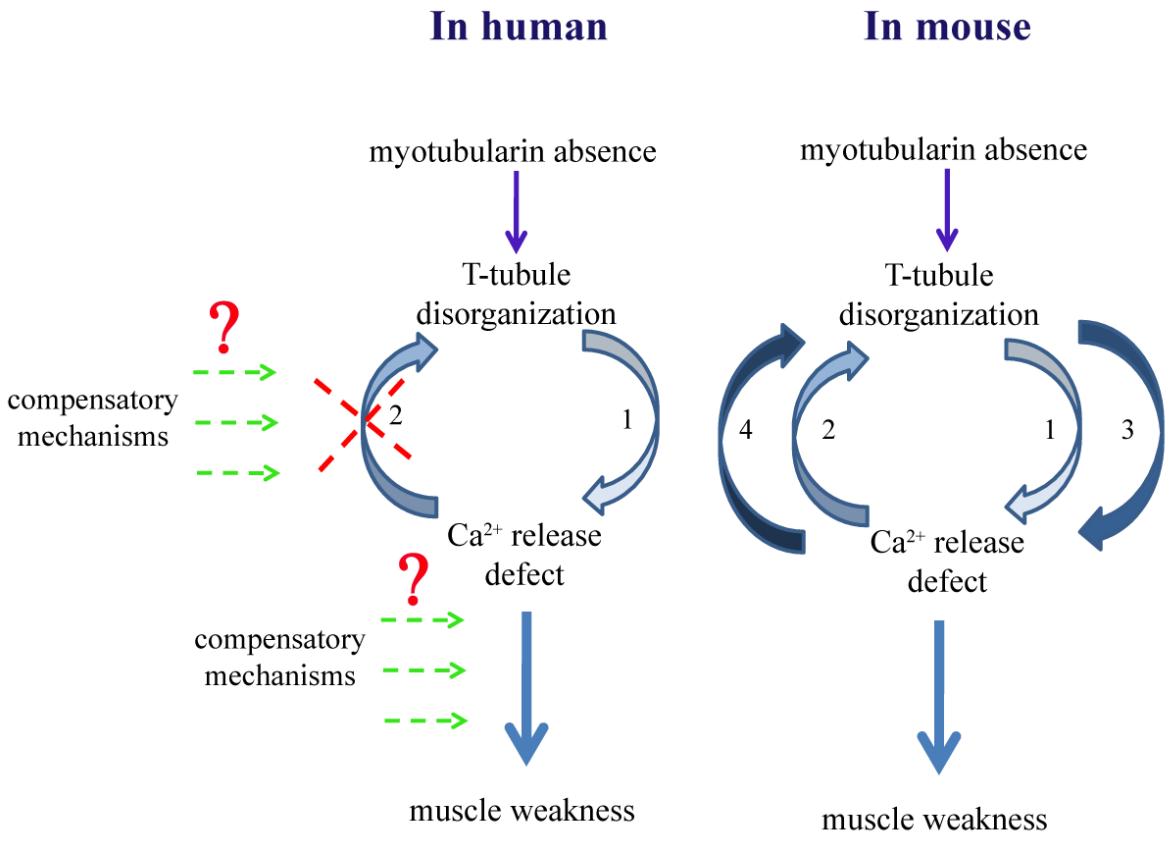


Figure (42): *Proposed model of disease progression in human and mouse through the relationship between T-tubules and calcium release/uptake.* In murine XLMTM (right panel), myotubularin absence results in disorganization of T-tubules which in turn gives rise to altered calcium homeostasis. Then, calcium homeostasis defect intensifies the disorganization of T-tubule system resulting in a repeated loop of cause-effect. In human (left panel), potential compensatory mechanisms prevent further deterioration of the phenotype.

II-What does the mouse model tell us ?

II-1. Alteration of triad structure and its functional impact

Skeletal muscle contraction/ relaxation occurs through cycles of Ca^{2+} release and re-uptake by the sarcoplasmic reticulum (SR). This mechanism is accomplished in part by a specialized membranous structure, the triads. The triad morphology ensures the perfect positioning of the voltage sensor (DHPR) and the Ca^{2+} release channel (RyR1) in a face-to-face configuration.

Although MTM1 through its action on phosphoinositide is a component of the machinery implicated in membrane trafficking, it is unlikely that the canonical endocytosis pathway is involved in the pathogenesis of XLMTM as endocytotic markers are not deregulated until the last phase of disease progression. As MTM1 localizes to triads in skeletal muscle, it is possible that its absence may affect T-tubule morphology. Indeed, observation of muscle sections from *Mtm1*-KO mice revealed dramatic T-tubules changes. Similar alterations in T-tubule morphology were reported in XLMTM patients and in zebra fish lacking MTM1 (Dowling *et al.* 2009). We found that, these invaginations were observed to have lost their orientation taking false routes which may not meet the SR terminal cisternae (see figure 43). Further analysis is needed to confirm this suggestion by co-labeling T-tubules with potassium ferrocyanide and the junctional SR by electron-immunogold using antibodies against RYR1. In addition to the mislocalized T-tubule, triads that were deprived of T-tubule elements were also observed. Interestingly, these anomalies precede the alterations of EC coupling (as shown by analysis of EC markers, RyR, DHPR α and β subunits). We suggest that T-tubule disorganization is a direct consequence of MTM1 loss. T-tubule anomalies in turn may result in a defect in RyR1-mediated Ca^{2+} release, as some RyR1 channels would no longer be activated as they have lost their interaction with DHPR. This Ca^{2+} release reduction is also likely due to a reduced number of RyR1 channels (see figure 43).

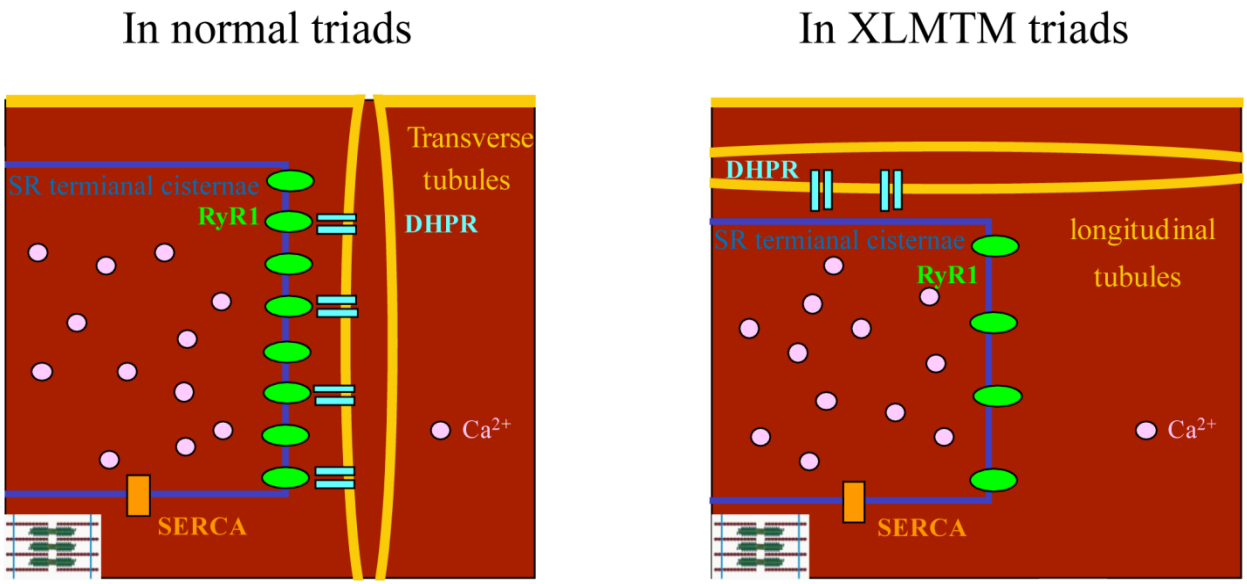


Figure (43): S*chematic representation of triads in normal and XLMTM muscle.* In normal triads, the interaction of the RyR1 channel (in green) with DHPR (in light blue) leads to RyR1 opening and release of Ca^{2+} that is sequestered in the SR. In *Mtm1*-KO mice triads, T-tubules are often absent or longitudinally oriented, thus RyR1 loses its activator (DHPR), which results in a defect in calcium release. This defect is likely to be enhanced by a decrease in the protein level of RyR1 and DHPRα1.

II-2. Insights into the molecular mechanisms underlying triad biogenesis

Although the events characterizing triads biogenesis are morphologically defined as previously seen in the introduction (section I-1), the molecular mechanism (s) involved in its biogenesis and maintenance remain elusive. In this section a physiological role of MTM1 will be proposed and discussed with others proteins which are implicated in triad anomalies when mutated either in genetic diseases or in animal models.

II-2-A. *Role for MTM1*

Although MTM1 is ubiquitously expressed, its absence leads to a skeletal muscle disorder. This indicates that MTM1 has a muscle-specific role, which cannot be compensated by another protein. Recently, immunological and EM analysis revealed that MTM1 overexpression in mouse muscle leads to the formation of exogenous membrane structures (Buj-Bello *et al.* 2008). This includes vacuoles that are derived from sarcolemma and/or T-tubules; as they have positive staining for caveolin-3, dystrophin and dihydropyridine receptor

(DHPR), and negative staining for laminin 2 (Buj-Bello *et al.* 2008). Moreover, these exogenous membrane structures contain MTM1 protein (Buj-Bello *et al.* 2008). This indicates a direct or indirect role for MTM1 in the generation of new membrane structures in skeletal muscle (Buj-Bello *et al.* 2008).

As discussed above, MTM1 seems to be involved in T-tubule biogenesis or maintenance. Since MTM1 expression is induced in the postnatal life in mouse and *Mtm1*-KO muscles display less altered T-tubules at an early stage than at a late stage, it is likely that MTM1 plays a role in the maturation and/or the maintenance of T-tubules rather than in the early biogenesis.

During myofiber growth, the T-tubule network grows up to occupy the entire muscle fiber. It is likely that T-tubule growth/extension happens by adding new membrane structures coming from internal regions of the muscle fiber. An old theory about T-tubule biogenesis proposed by Yuan *et al* suggested that new membrane materials derived from the sarcoplasmic reticulum are added to T-tubules (figure 44) (Yuan *et al.* 1991). It is also possible that the growth of T-tubules is ensured by new membranes coming from endosomes. MTM1 may play a role in the formation and/or remodeling of growing T-tubules by regulating the incorporation of these membranes and/or turnover of existing tubular membranes. To confirm this hypothesis, it will be important to follow up the biogenesis and remodeling of T-tubules in muscle fibers by using markers such as MTM1, CAV3 and BIN1.

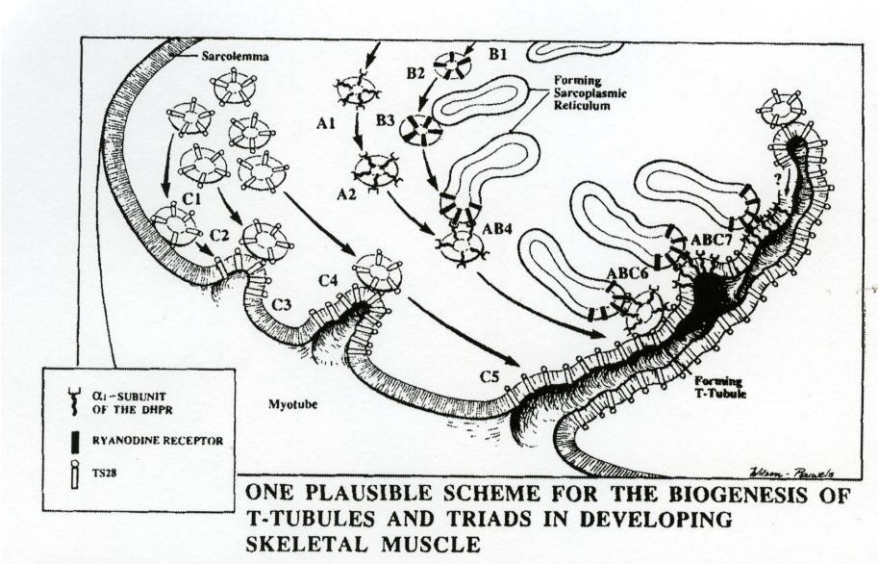


Figure (44): The model of Yuan et al. shows that vesicles coming from internal regions (SR) and plasma membrane of a fiber contribute to T-tubule growth/extension. (Yuan S., J Cell Biol, 1991).

Alternatively, it has been proposed that the product of MTM1, PI5P, is a specific allosteric activator of the phosphatase and it induces the oligomerization of MTM1 to form a heptameric ring with 12.5 nm of diameter (Schaletzky *et al.* 2003). This seems interesting as the diameter of T-tubules measured in murine skeletal muscle is 10-12 nm (Felder *et al.* 2002). Could MTM1 form such a ring to maintain the newly formed T-tubule in skeletal muscle? To answer this question it would be important to define the ultrastructural localization of MTM1 on the triad (T-tubule and/or SR), and to address also the formation of MTM1 heptameric rings in muscle fibers.

In this context, other points that remain to be resolved are: **1)** to know whether MTM1 could be recruited to the T-tubule membrane by binding to PI3,5P2 and PI5P via its PH/GRAM domain (Tsujita *et al.* 2004), (Schaletzky *et al.* 2003); **2)** to know if MTM1 role in T-tubule maturation/maintenance is mediated by its phosphatase activity, as it is the case for its function in endocytosis.

II-2-B. Other proteins involved in triad biogenesis

Our suggestion that MTM1 is involved in triad maturation/maintenance adds a new component to a previously described list of proteins involved in triad biogenesis, maturation or maintenance. These proteins include, caveolin 3 (CAV3), the skeletal muscle specific isoform of amphiphysin 2 (AMPH2), mitsugumin 29 (MG29) and mitsugumin 72 (MG72).

Mutations within CAV3 and AMPH2 are associated with skeletal muscle disease such as limb-girdle muscular dystrophy type 1C and the autosomal recessive form of centronuclear myopathy (Minetti *et al.* 1998), (Nicot *et al.* 2007). Moreover, animal models lacking CAV3 or AMPH2 exhibit a skeletal muscle defect associated to alterations in T-tubule morphology and EC coupling (figure a-f) (Galbiati *et al.* 2001), (Razzaq *et al.* 2001). In addition to the skeletal muscle defect, mutations within *Cav3* are also associated with cardiomyopathy and *Bin1* deletion is lethal in mouse due to cardiac hypertrophy (Gazzerro *et al.* 2009), (Catteruccia *et al.* 2009), (Muller *et al.* 2003). Since CAV3 and AMPH2 have the capacity to induce the formation of caveolae and tubules, respectively (Smart *et al.* 1999), (Takei *et al.* 1999), they are good candidates of T-tubule network formation in skeletal muscle.

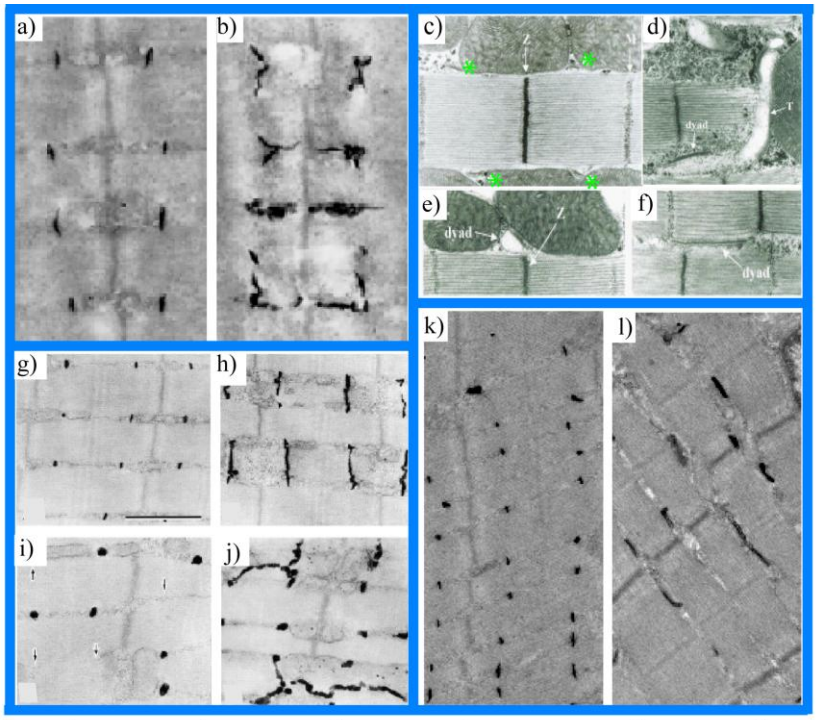
Caveolin proteins are known to be associated to caveolae, which are subcompartments of the plasma membrane that have the aspect of 50-100 nm vesicular invaginations (Lisanti *et al.* 1994). In contrast to other plasma membrane regions that are composed mainly of

phospholipids, caveolae are composed of cholesterol-sphingolipid rich raft domains (they contain approximately four times more cholesterol than the plasma membrane) (Smart *et al.* 1999). In skeletal muscle, Cav3 is located at the sarcolemma and the T-tubule system (Parton *et al.* 1997). In particular, caveolin 3 is found in both developing and mature T-tubules and plays a role in the early and late biogenesis of this structure (Rosemblatt *et al.* 1981), (Parton *et al.* 1997; Carozzi *et al.* 2000).

Amphiphysin proteins are able to tubulate lipid membranes via their BAR (Bin/Amphiphysin/Rvs) domain, which makes them effective to form T-tubules in skeletal muscle. In addition, AMPH2 can tubulate membranes separately or in cooperation with DNM2, another protein involved in the autosomal dominant form of CNM (Takei *et al.* 1999), (Bitoun *et al.* 2005). The muscle specific function of AMPH2, that is, its role in T-tubule biogenesis is provided by exon 11 (Ramjaun *et al.* 1998). Exon 11 is important for Bin1 recruitment to T-tubule membranes as it encodes for a basic sequence which can bind specifically PI4,5P2 and PI4P (Lee *et al.* 2002). Interestingly, the levels of PI4,5P2 and the protein levels of AMPH2 increase simultaneously during myogenesis in C2C12 myotubes (Lee *et al.* 2002), (Wechsler-Reya *et al.* 1998). The cooperation between AMPH2 and DNM2 (see introduction II-2-B-ii) occurs via an interaction of the AMPH2 SH3 domain with the proline rich domain of DNM2. However, this interaction cannot occur prior to AMPH2's association to the T-tubule membrane, as the region encoded by exon 11 binds to the SH3 domain when it is not membrane bound (Kojima *et al.* 2004). Indeed, Exon 11 binding to PI4,5P2 is necessary to release the SH3 domain enabling it to bind DNM2, which restricts the action of AMPH2 on developing T-tubules (Kojima *et al.* 2004). Myotubes expressing a mutant AMPH2 construct lacking exon 11 failed to form a normal T-tubule network (Kojima *et al.* 2004).

In addition, COS-1 overexpressing an AMPH2 having a mutation in the BAR domain failed to form tubules in culture, however, a mutation in the SH3 domain leading to premature codon stop result in BIN1 which is able to tubulate membrane but unable to recruit DNM2 to the newly formed tubules (Nicot *et al.* 2007). Interestingly, the mutation leading to loss of the interaction between BIN1 and DNM2 results in the most severe phenotype in patients (Nicot *et al.* 2007). Surprisingly, no skeletal muscle defect was reported in *Bin1* $^{-/-}$ mice, this may due to the embryonic lethality and to the fact that *Bin1*-KO skeletal muscle from embryo (at 18.5 E) were analyzed at low magnification (x200) which does not allow the detection of tiny

structure such as T-tubules. In this context, a muscle-specific *Bin1* KO mouse (in progress in our laboratory) would be a good tool to analyze the role of AMPH2 in T-tubule biogenesis, In addition, it will be interesting to study whether MTM1 interacts with AMPH2 and/or DNM2, which could give clues on a possible common pathway for the 3 proteins implicated in centronuclear myopathy.



	sk. muscle myopathy in human	cardiac implication in human	sk. muscle myopathy in rodent	cardiac implication in rodent	membrane binding	membrane curvature	T-tubules organization	alteration EC
CAV3	+	+	+	+	cholesterol	caveolae	✓	mild (mouse)
AMPH2	+	+	not reported	+	PI	tubules	✓	severe (*Droso*)
MG 29	not reported	-	+	-	transmembrane	-	✓	mild (mouse)
MTM1	+ +	-	+ +	-	PI	-	✓	severe (mouse)

Figure (45): Implication of CAV3, AMPH2, MG29 and MTM1 in the organization of the T-tubule system in skeletal muscle. The table describes some properties of the four proteins and their implication in genetic diseases.

a,b) EM images from control and *Cav3* mutated mice respectively, note the altered appearance of T-tubules, (from Galbiati F., 2001).

c-f) EM images from *Drosophila* show normal muscle (c) note the dyads (green asterix) localized in proximity to the Z-line (similar to vertebrate). d-f) from *Bin1* null *Drosophila* show the alteration of T-tubules, resulting in dilated (d), mislocalized (e) and longitudinal (f) tubules, (from Razzaq A., 2001).

g-h) EM images from control (g,h) and *MG29*-KO mice (i,j) demonstrate the alterations in T-tubules organization, not the absence of T-tubule (i) and the longitudinal orientation (j), (from Nishi M., 1999).

k,l) EM images from control (k) and *Mtm1*-KO mice (l) show the severe alteration in T-tubule organization (from Al-Qusairi L., 2009).

In addition to these proteins, which are involved in the biogenesis of T-tubules, other proteins are involved in the biogenesis of SR and terminal cisternae and the junction between T-tubules and SR. This is the case of mitsugumin 29 (MG29) and mitsugumin 72 (MG72), which is also known as junctophilin 1. Both MG are transmembrane proteins that are associated with triads in skeletal muscle (Komazaki *et al.* 1999), (Takeshima *et al.* 2000). MG29 is expressed early during myogenesis even before the apparition of triads, while MG72 expression is enhanced after birth (Komazaki *et al.* 1999), (Ito *et al.* 2001).

MG29 first associates to newly formed SR vesicles and then to T-tubules, which appear later (Komazaki *et al.* 1999). This observation implicates MG29 in the formation of junctional SR and T-tubules (Komazaki *et al.* 1999). Although *MG29*-KO mice look comparable to WT, decreased muscle mass and a slight decrease in the force generation capacity was observed (Nishi *et al.* 1999). EM analysis of mutant muscle has revealed morphological alterations in triad structures, including a swollen SR, longitudinal T-tubules and triads deprived of T-tubule components (figure 3g-j) (Nishi *et al.* 1999).

MG72 mice die shortly after birth due to defects in jaw muscles resulting in no milk suckling (Ito *et al.* 2001). Electron microscopy analysis of skeletal muscle from newborn mice has revealed several anomalies in triad morphology which lead to defect in EC coupling (Ito *et al.* 2001). These anomalies include, reduced number of triads, swollen junctional SR, partially vacuolated longitudinal SR and irregular orientation of SR network (Ito *et al.* 2001). This specific role of MG72 is also supported by its ability to induce junctions between the endoplasmic reticulum and the plasma membrane when overexpressed in nonmuscle cells (Takeshima *et al.* 2000). This in addition to the fact that the expression of MG72 is enhanced

after birth indicates a role of MG72 in the late biogenesis/maturation of triads (Ito *et al.* 2001).

Mitsugumin proteins may spine the SR via their C-terminal transmembrane domain and associate with the T-tubule/plasma membrane via their N-terminal domain, which binds to membrane phospholipids including, sphingomyelin and phosphatidylcholine, as suggested for MG72 (Takeshima *et al.* 2000).

By analyzing these findings, one can imagine a process in which BIN1 tubulates membranes (in cooperation with DNM2) and CAV3 contributes to the formation of small caveoles that will be incorporated into the basal part of T-tubules (as proposed by Carozzi 2000). During late myogenesis, muscle fibers grow rapidely and there is the need of adding new membrane structures, which might be derived from endosomes, to T-tubules for elongation; MTM1 may play a role in regulating this latter process. Myotubularin could also be implicated in the remodeling of longitudinal tubules to become transverse. Alternatively, myotubularin may play a role in the maintenance of the T-tubule structure by forming heptameric rings around the tubular membrane that interact with cytoskeletal proteins. On the other hand, mitsugumin proteins would be important for the positioning of junctional SR to the proximity of transversal tubules. In this context, it will be interesting to analyse the localization and level of mitsugumin proteins in *Mtm1* KO muscle. Figure 46 illustrate the proposed roles of MTM1 with the other proteins involved in T-tubule biogenesis.

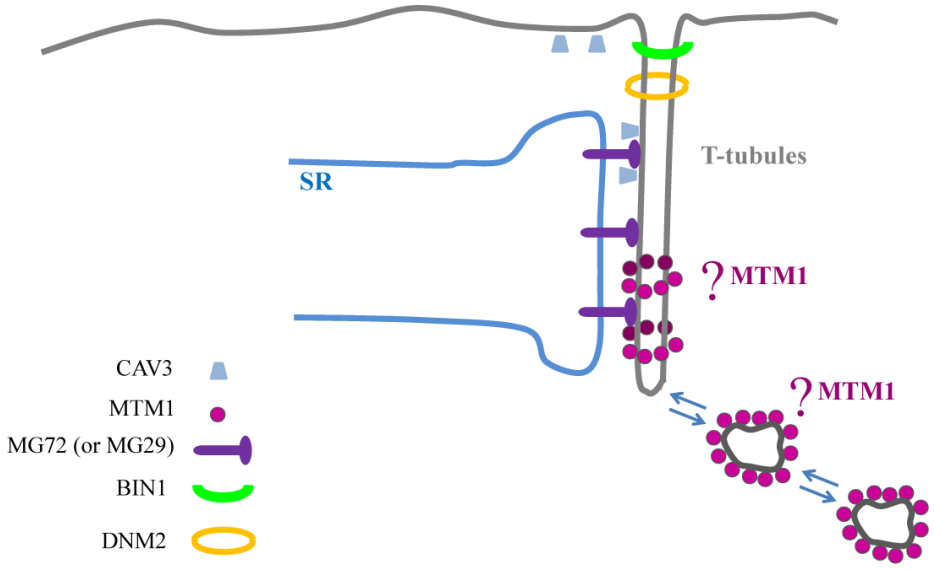


Figure (46): Schematic representation of the proteins thought to be involved in the biogenesis of T-tubules with a proposed role for MTM1.

II-3. Deregulation of proteins involved in EC coupling

II-3-A. RyR1 and DHPRα1s protein level

Mutations leading to a dramatic decrease of RyR1 protein level are usually associated with a recessive form of congenital myopathies with cores (Monnier *et al.* 2008). The expression of one normal *Ryr1* allele supplies muscle with enough amounts of RyR1 channel (Monnier *et al.* 2008). However, in skeletal muscle of XLMTM mice, we found a 70% decrease in RyR1 protein, which is thus likely to be responsible, at least in part, for the reduction in calcium release. Moreover, we also observed a 30% decrease in DHPR alpha subunit level, which, together with alterations of the triad structure, may contribute significantly to the decreased SR calcium release (figure 3).

Muscle weakness in RyR1-related diseases is usually proposed to be caused by a decrease in SR Ca^{2+} release, according to two alternative models: the uncoupled or the "leaky" Ca^{2+} channel model (Dirksen *et al.* 2002). Contrary to the uncoupled model, muscle fibers with "leaky" RYR1 channels are characterized by the presence of increased cytosolic [Ca^{2+}]. No changes in neither sarcoendoplasmic [Ca^{2+}] nor cytosolic [Ca^{2+}] were detected in myotubularin deficient fibers. Thus, we propose that RyR1 channels in *Mtm1*-KO muscle are partly uncoupled to DHPR as a consequence of triad morphology alterations.

Although the mRNA level of *RyR1* and *Cacna1s* were normal in mutant muscle, decreased protein levels may result from altered protein stability, possibly due to defects in RYR1-DHPR interaction. In addition, the increased expression of atrogenes (Atrogin 1, Murf1, Nedd4 and ZNF216) in *Mtm1* deficient muscle could also contribute to RyR1 and DHPRα1s degradation. Accordingly, Nedd4 has been implicated in the turnover of transmembrane and cell surface proteins (Koncarevic *et al.* 2007).

II-2-B. DHPRβ protein level

We have observed an increase in DHPRβ1 protein level (6-fold up) at a late but not an early stage of the disease in XLMTM mice. These data suggest that DHPRβ1 upregulation arises from a compensatory mechanism and plays no primary role in XLMTM pathogenesis. As described in the introduction, β1 plays three essential roles in the EC coupling mechanism:

- It increases the membrane expression of α1s.
- It participates in the arrangement of DHPR in tetrads.

- It enhances EC coupling by enhancing the charge movement and by stabilizing the interaction with RyR1.

Two models have been proposed for the action of β1a in excitation-contraction coupling. In the first model, β1a would act as an additional RyR1 binding entity: although several α1s regions are involved in RyR1 interaction, they do not provide sufficient stability to the RyR1-α1s complex. Thus, β1a would add additional binding site(s) for efficient DHPR-RyR1 association (Tanabe *et al.* 1990), (Cheng *et al.* 2005). In the second model, β1a would play a role as an allosteric modifier: in this scenario, α1s does not acquire its functional conformation unless associated with the β1a subunit, and consequently, it would not be able to produce charge movements and bind to RyR1 (Schredelseker *et al.* 2009).

Our electrophysiological data has revealed a 37% decrease in DHPR calcium current of flexor digitorum brevis (FDB) muscle fibers from *Mtm1* KO mice at 5 weeks of age (which correlates with the 30% reduction of DHPR alpha level in these muscles, as shown by western blot analysis). Surprisingly, electrophysiological measurements in FDB myofibers from mice at 4 weeks of age, when skeletal muscles exhibit a significant decrease in muscle strength, revealed that the function of DHPR is not altered (data not shown). We suggest that the overexpression of the beta subunit might increase the membrane expression of the alpha subunit and stabilize the function of DHPR until a late phase of the disease, when the overexpressed beta subunit can no longer compensate the defect.

II-4. Primary defects in mitochondrial organization in XLMTM

Defects in mitochondrial position were previously reported in XLMTM patients (Wallgren-Pettersson et al. 1995) and in mice lacking myotubularin (Buj-Bello et at, 2002; 2008). In this study, we show that the position of mitochondria is already altered in myofibers from 2 week-old mice. It has been shown that deregulations in cytosolic calcium level may result in mitochondria abnormalities and is particularly evident in central core myopathy (Dirksen *et al.* 2002). Since the level of proteins involved in calcium homeostasis, such as RyR and DHPR alpha and beta subunits, is unchanged at this early stage of the disease, it is likely that these defects results from other mechanisms.

Recent studies have shown that Charcot-Marie-Tooth (CMT) mutations of MTMR2 and XLMTM mutations of MTM1 induce neurofilament light chain (NLF) aggregation in cell lines (Goryunov *et al.* 2008). NFL, also responsible for a CMT form (CMT2E), is an

intermediate filament that interacts with MTMR2 (Previtali *et al.* 2003). It might be possible that myotubularin interacts with members of the intermediate filament family in muscle, which are known to link the contractile apparatus to the sarcolemma and membranous organelles, such as mitochondria (Capetanaki *et al.* 2007).

II-5. Relation between hypotrophy/atrophy and XLMTM: role of growth factors and proteasome pathway

Myofiber hypotrophy is a common feature of CNM. Histological analysis of muscle from patients and mice deficient in MTM1 revealed a consistent decrease in myofiber diameter. Interestingly, we have shown that there is a decrease in the weight of isolated muscles at the asymptomatic phase of the disease when muscle strength appears normal. Similarly, T-tubule defects were already observed at this stage of the disease. We suggest that the hypotrophy and the T-tubule defect may act in parallel or one before the other in order to induce muscle weakness. It is known that one week-old mutant mice are already smaller by about 10% (Buj-Bello et al. 2002). In this context, it will be interesting to analyze the structure of triads in these animals to see if hypotrophy precedes or not the ultrastructural defects.

Muscle atrophy can be induced by several conditions including fasting, denervation, diabetes, disuse, glucocorticoids treatment, involves protein degradation via several pathways, including the proteasome pathway which can be activated by a decrease in PI3K/AKT signaling (Jagoe et al. 2001), (Bodine *et al.* 2001). Initial DNA microarray data collected from XLMTM patients and *Mtm1*-KO mice did not suggest an implication of the AKT/Foxo pathway in the muscle fiber atrophy observed (Noguchi et al. 2005) and (unpublished data, AH.Beggs). We decided however, to investigate a potential implication of this pathway in the pathology, as the *Mtm1*-KO mice do not exhibit a significant increase in muscular mass in response to IGF1 overexpression at least in 129-PAS background. Our molecular analysis in mice has revealed defects in the IGF1/AKT/Foxo pathway with the upregulation of atrogene expression, including atrogin1, Murf1, Nedd4 and ZNF216, which represent components o the ubiquitin proteasome system (UPS). This upregulation was detected even during the asymptomatic phase (at 2 weeks of age), which indicates that the activation of the UPS machinery may play a role in XLMTM pathogenesis. The upregulation of atrogenes may be due to a decrease in Foxo3 phosphorylation as shown by western blot analysis. The

implication of AKT/Foxo signaling in muscle atrophy was previously established (Sandri et al. 2004), (Crossland et al. 2008).

In addition to the implication of Foxo3 in the UPS, Foxo3 is also a critical player in autophagy (Sandri et al. 2004), (Mammucari et al. 2007). Moreover, a functional link has been established between MTM1 and autophagy due to the implication of PI3P in this process (Lindmo et al. 2006). However, it has been recently reported that among all the enzymaticaly active myotubularins, only MTMR14/Jumpy and MTMR6 play a role in autophagy by regulation of PI3P levels at the autophagosome membrane (Vergne et al. 2009). Hematoxylin-Eosin (HE) staining of *Mtm1* null muscle did not detect autophagic vacuoles in the *Mtm1*-KO muscle. Instead, vacuoles observed in this case are probably originated from SR dilatation as they do not contain cellular debris (Buj-Bello et al. 2002). Our observations of muscle preparations from WT and *Mtm1*-KO mice by electron microscopy confirm this suggestion. Nevertheless, we cannot exclude a direct or indirect role of MTM1 in autophagosome biogenesis. It would be interesting to analyze autophagic markers, including Beclin-1, autophagy-related genes 5, 8 and 12 (Atg5, Atg8 and Atg12) in muscle from *Mtm1*-KO mice compared to WT animals.

In addition to the control of autophagy by the AKT/Foxo pathway and phosphoinositides, the autophagic program can be inhibited by the activation of the RUNX1 transcription factor (Wang et al. 2005). RUNX1 induces the expression of genes implicated in muscle structure and electrical activity, and its overexpression preserves myofiber integrity in the case of muscle damage (Wang et al. 2005). In this study, we have shown the upregulation of RUNX1 and its targets genes. In line with these observation, one can put forward the hypothesis that, if *Mtm1*-KO mice do not exhibit autophagy this may be due to a RUNX1-mediated process.

III. Myotubularins and ion channels

Although the regulatory role of PIPs for ion transport proteins was initially reported in 1983, the link between myotubularins and ion channels is new. The first myotubularin implicated was the *C.elegans* MTMR6 ($_{Ce}$MTMR6) which was found to interact with the Ca^{2+} activated potassium channel (K_{Ca} 3.1) in a yeast two hybrid screen performed by the Skolnik group (Srivastava et al. 2005). They showed also that the mammalian MTMR6 interacts with

K_{Ca} 3.1 and regulates its activity (Srivastava et al. 2005). This interaction which occurs via the CC domain of MTMR6, enables the phosphatase to dephosphorylate a PI3P pool of the microdomain found adjacent to the channel (Srivastava et al. 2005). The decrease in PI3P levels results in K_{Ca} 3.1 inhibition, which implies a direct role for PI3P in the regulation of this potassium channel (Srivastava et al. 2005).

In addition, recent studies performed in *Mtmr14*-KO mice have shown that PI3,5P, the main substrate of MTMR14, activates RyR1 in skeletal muscle (Shen et al. 2009). Absence of MTMR14 in mouse, which results in the accumulation of PI3,5P2, leads to a hyperactivation of RyR1 and a depletion of the SR Ca^{2+} content (Shen et al. 2009). It has been reported that mutations within the *MTMR14* gene may cause centronuclear myopathy in humans, and mild muscle weakness in mice (Tosch et al. 2006), (Shen et al. 2009). Histological analysis of the *Mtmr14*-KO muscle revealed a slight alteration in triad structure including swollen SR (Shen et al. 2009).

As MTM1 also dephosphorylates PI3,5P2, it is not unreasonable to speculate that MTM1 deletion may lead to a similar effect that is, accumulation of PI3,5P2 accumulation and hyperactivation of RyR1. However, in *Mtm1*-KO mice we observed a decrease in Ca^{2+} release which is mainly due to a dramatic decrease (3 fold) of RyR1 protein levels. This indicates that MTM1 and MTMR14 trigger muscle weakness by distinct mechanisms. It is likely that MTM1 is implicated in T-tubules maturation and/or maintenance whereas, MTMR14 plays a role in the regulation of PI3,5P2 levels at the SR. However, we cannot exclude a role for MTM1 in the regulation of calcium channels at the triads.

Moreover, we demonstrated here a dramatic upregulation of the sarcoplipin (SLN) mRNA and protein levels in the XLMTM murine model. mRNA levels of Sln were also elevated in the asymptomatic phase. Surprisingly, measurement of Ca^{2+} uptake did not reveal inhibition of SERCA activity as anticipated. This can be potentially explained by: i) A shift in the phosphorylation state of SLN could abolish the effect of Sln on SERCA. ii) As SERCA associates with a phosphoinositide that resides in a hydrophobic pocket within the pump it is possible that phosphorylation of this PI enhances Ca^{2+} uptake by increasing the affinity of SERCA to bind calcium (Varsanyi et al. 1983). It will be interesting to monitor the impact of MTM1 and its substrates on SERCA activity. Moreover, it will be important to identify the molecular pathway leading to such a dramatic upregulation.

IV. Towards XLMTM therapy

The main characteristics of XLMTM are severe muscle weakness and hypotrophy. To date, no specific treatment is available that can improve the course of the XLMTM disease. Several potential therapeutic approaches will be discussed, some of them being under investigation in preclinical models.

IV-1. Gene therapy

IV-1-A. Overexpression of myotubularins

It has been demonstrated in our laboratory that adeno-associated virus (AAV) mediated intramuscular injection of the *Mtm1* gene in the muscle-specific *Mtm1*-KO mice rescues the XLMTM phenotype at the age of 4 weeks, when KO muscle exhibits the main pathological features (Buj-Bello et al. 2008). Recovery traits included increase in muscle mass and also the correction of the histological defect characterized by the central position of the nuclei within the myofiber (Buj-Bello et al. 2008). Interestingly, 4 weeks following injection, KO muscle has the capacity to generate isometric force comparable to WT muscle (Buj-Bello et al. 2008).

In addition to gene transfer experiments performed in the mouse model, similar assays have been performed in the zebra fish model for the disease (Dowling et al. 2009). Zebra fish embryos were injected with myotubularin morpholino to reduce MTM1 protein levels in addition to mRNA of myotubularin, MTMR1, or MTMR2 (MTMR1 and MTMR2 share high similarity with MTM1) (Laporte et al. 1998) to rescue the phenotype (Dowling et al. 2009). All three tested myotubularins were able to restore muscle strength which is evaluated in this case by the ability of embryos to hatch from their chorions by 60 hours post fertilization (hpf) (Dowling et al. 2009). The effect of MTMR1 and MTMR2 overexpression in *Mtm1*-KO mice is currently being evaluated in our lab however, preliminary results suggest that the murine MTMR1 and 2 are both able to rescue the phenotype induced by *Mtm1* deficiency.

Moreover, AAV-mediated gene therapy has given promising results in clinical trials for Leber's congenital amaurosis eye disease, where a subretinal injection resulted in persistent improvement in vision of all treated patients (Maguire et al. 2009), (Cideciyan et al. 2009). In addition to the local application of AAV-mediated treatment, other strategies are

under investigation. In particular, the AAV-mediated ApoA-1 gene delivery through intravenous injection in mouse model of apolipoprotein (Apo) resulted in full recovery of the phenotype (Vaessen et al. 2009). Together, these observations point to gene therapy employing MTM1 and/ or the related myotubularins as promising targets for XLMTM treatment.

IV-1-B. Manipulation of growth factor pathways

Skeletal muscle mass following differentiation is regulated by a balance between protein synthesis and protein degradation. This balance can be shifted towards protein synthesis (hypertrophy) or degradation (atrophy) through endogenous and exogenous signaling factors. As this balance is shifted towards protein degradation in XLMTM, where muscle atrophy predominates, manipulation of growth factor pathways could potentially reverse this inbalance. Several pathways are implicated in the control of skeletal muscle mass such as the IGF1/PI3K/AKT, the β-adrenergic and the Myostatin (Mstn) pathway (Bodine et al. 2001), (Kim et al. 1992), (Lee et al. 1999). In this study, we overexpressed IGF1 specifically in skeletal muscle of *Mtm1*-KO mice. Our results suggest that *Mtm1*-KO mice, at least of the 129-PAS strain, do not respond to IGF1 overexpression. We cannot rule out that higher levels of overexpressed IGF1 could potentially induce a hypertrophic effect. Actually, IGF1 gene transfer by an AAV delivery system has been initiated by our team (by Buj-Bello A.) to evaluate a possible effect of IGF1 overexpression in *Mtm1*-KO mice.

The Myostatin pathway also represents a good candidate for manipulation as Mstn is a negative regulator of muscle mass. A project aiming to evaluate the effect of a dominant negative form of Mstn in MTM1 KO muscle by AAV delivery is underway (by Buj-Bello A. and Relizani K.). Preliminary observations of muscle mass and myofiber diameter appear promising however; further analysis is required to pinpoint the effect of Mstn inhibition and in turn, of the Mstn pathway in the XLMTM mouse model. Alternatively to the AAV-mediated Mstn inhibitor delivery, an intraperitoneal injection of a soluble activin receptor (ActRIIB) may be employed for recovery, as ActRIIB is the receptor for Mstn (Morrison et al. 2009). Such an approach seems promising because injection of soluble ActRIIB in SOD1 ^{G93A} transgenic mice, a mouse model for amyotrophic lateral sclerosis (ALS), prior to the symptomatic phase, induces a delay in disease onset with an increase in muscle weight and function (Morrison et al. 2009). Soluble AcrtRIIB represents a putative target for therapy of

muscle disorders characterized by the presence of atrophy. Finally, a strategy aiming for complete rescue of the myopathic phenotype via a co-injection of MTM1 and the Mstn dominant negative mutant is being set-up (by Buj-Bello A.).

IV-1-C. The manipulation of genes involved in the T-tubules biogenesis

Our analysis suggests that alterations in T-tubules are a primary defect in XLMTM pathogenesis. This observation prompts us to speculate that rescue of T-tubules morphology might result in a recovery of Ca^{2+} release and increased muscle strength. T-tubules dynamics is established and controlled by sophisticated mechanisms which implicate several protein complexes depending on the developmental phase of the muscle. These processes are involved in T-tubules biogenesis, maturation and/or maintenance.

Given the role of MTM1 and AMPH2 in T-tubules biogenesis, and the potential implication of DNM2 in this process, a rescue of XLMTM using AAV delivery of these genes and also using chimeric myotubularins is under investigation in the team (by Cowling B. and Amoasii L.).

IV-1-D. Meganuclease based genome surgery

Meganucleases, like restriction enzymes, cleave double stranded DNA with high specificity. They differ from restriction enzymes however, as their recognition sequence is longer (3-8 bp for the restriction enzyme, 12-40 for the meganuclease) (Belfort et al. 1997). Meganucleases can be engineered to cleave a chosen sequence, thus, they are turning out to be a potentially powerful tool for genome surgery (Arnould et al. 2007). A novel strategy is based on highly specific (22bp recognition site) engineered meganuclease that are designed to cleave very close to mutation sites in a given disease, and thus stimulate homologous recombination ex vivo (or even perhaps in vivo) to repair the mutated gene by recombination between the chromosomal locus and an extra-chromosomal repair matrix (Arnould et al. 2007). However, several challenges remain to be resolved as in patient's cells this mechanism can be affected by several factors including vectorization, cleavage activity, homologous recombination efficiency, and the chromatin status of the targeted locus, and this relies also on efficiency of stem cell therapy for each disease. One may propose that as the muscle mass

to be treated in a neonatal patient with XLMTM is much smaller than in a ten year old child with DMD, XLMTM could provide a test case for applying such approach to a muscle disease.

IV-2. Chemical therapy

IV-2-A. To increase muscle mass

As previously mentioned, the PI3K/AKT and the β-adrenergic pathway represent pathways involved in the control of skeletal muscle mass. It is possible to manipulate the PI3K/AKT pathway in an IGF1-independent manner via the β-adrenoreceptor pathway (figure 47) (Lynch et al. 2008). Therapeutic assays in the mouse model of Duchenne muscle dystrophy DMD, mdx mice, have demonstrated that administration of the β-adrenoreceptor agonist to mdx mice increases muscle mass (Choo et al. 1989), (Agbenyega et al. 1995), (Fowler et al. 2004). However, the effect on muscle force was less consistent, this was countered by the newer generation of β-agonist drugs (formoterol) (Lynch et al. 2008). Formoterol induced an increase in the muscular mass and strength of mdx mice (Harcourt et al. 2007).

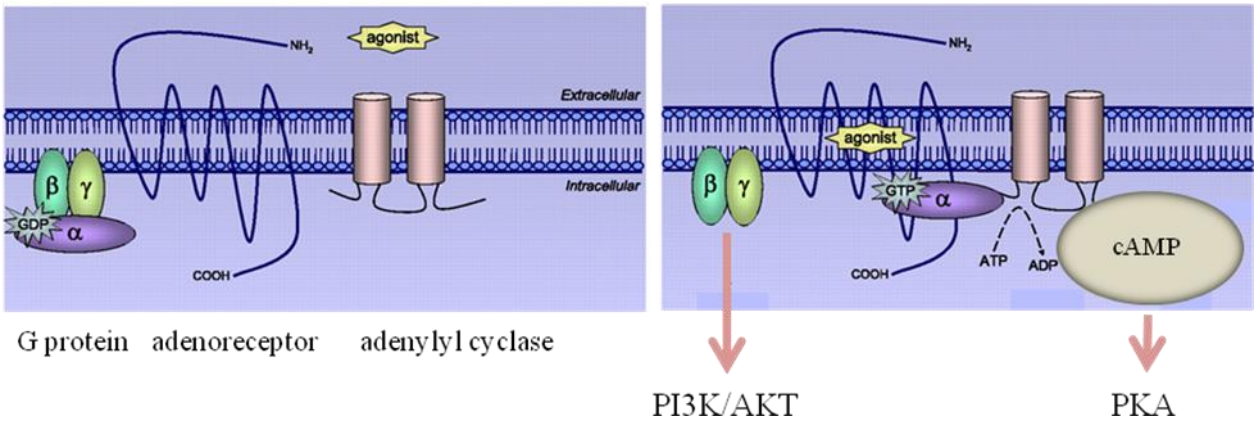


Figure (47): *The β2-adrenoreceptor signaling and its anabolic effect through the PI3K/AKT and PKA pathways.* a) The general β2-adreneceptor signaling pathway involves the receptor, a heterotrimeric G protein, and the membrane-bound adenylyl cyclase which catalyse the conversion of ATP to cAMP. b) The activation of the β2-adrenoceptor occurs through the binding of an appropriate agonist, which in turn results in the association of a heterotrimeric G protein with the third intracellular loop of the β2-adrenoceptor. This association results in GDP dissociation from and GTP binding to the α-subunit of the G protein, causing a conformational change in the heterotrimeric protein allowing both the α- and

βγ-subunits to activate downstream signaling targets including protein kinase A (PKA) and the PI3K/AKT, respectively (modified from Lynch, G. S. *et al*. Physiol. Rev., 2008)

Recent studies using DNA microarray analysis of skeletal muscle following β-agonist (formoterol) treatment have shown an effect on the expression of a wide spectrum of genes (Pearen et al. 2009). These genes are mainly implicated in skeletal muscle hypertrophy and in particular in the myostatin pathway and also in transcription, metabolism and mitochondrial activity (Pearen et al. 2009). In line with these findings, it will be of interest to assess the effect of formoterol treatment on $Mtm1^{-/-}$ mice.

IV-2-B. To improve Ca^{2+} homeostasis

Ca^{2+} release is activated in skeletal muscle by direct interaction between RyR and DHPR following depolarization however, in cardiac muscle SR calcium release is triggered via the activation of RyR by the entry of extracellular calcium through DHPR (Fill et al. 2002). Our results suggest that the muscle weakness observed in XLMTM is due to a decrease in Ca^{2+} release from SR with no alteration of the SR Ca^{2+} content. This renders possible the increase of Ca^{2+} release from the SR or alternatively the increase of Ca^{2+} entry from the extracellular space. Thus, it will be interesting to investigate the effect of drugs which induce RyR and/or DHPR activation. In this context, β-agonist can also have an effect, which is mediated here by cAMP/PKA. It has been shown that PKA phosphorylates RyR1 resulting in an increase in the activation of the channel (Reiken et al. 2003). Moreover, PKA can activate SERCA pumps and enhance Ca^{2+} removal (Dutta et al. 2002).

It seems that until we find an optimal way to express myotubularin in skeletal muscle of XLMTM patients, exploring the effect of β-agonist drugs on myotubular myopathy seems a priority. This work will also involve optimization of drug dosages to avoid muscle tremor and cramps, headaches, peripheral vasodilatation linked to β-agonist administration (Prather et al. 1995).

IV-2-C. To reexpress a functional protein using Readthrough strategy

A potential strategy to correct the deleterious effect of a nonsense mutation in monogenic diseases is expression of functional full–length protein using the mRNA carrying the causative mutation. This approach is referred to as the "readthrough" strategy (Linde et al. 2008). Drugs affecting ribosome decoding site, such as aminoglycoside (Kulyte et al. 2005), (Diop et al. 2007) can be used to achieve this as binding of aminoglycosides to ribosomes induces a subtle difference in the conformation of the rRNA decoding site, which results in translational readthrough by the insertion of an amino acid at the stop codon in eukaryotes (figure 48) (Fan-Minogue et al. 2008).

It has been shown that the treatment of mdx mice with gentamycin (an aminoglycoside) results in a partial re-expression of full-length dystrophin protein and a reduction of the dystrophic symptoms (Barton-Davis et al. 1999). Moreover, this treatment was successful in three out of four DMD patients carrying PTCs mutation in the dystrophin gene (Politano et al. 2003). Several factors were shown to affect the response to readthrough treatment such as the identity of the stop codon (UGA more efficient than UAG; UAG more than UAA), and also its neighboring nucleotides (Manuvakhova et al. 2000), (Howard et al. 2000), this, in addition to the chemical composition of the aminoglycoside itself (Diop et al. 2007). However, the potential renal and otic toxicity have limited its clinical applications.

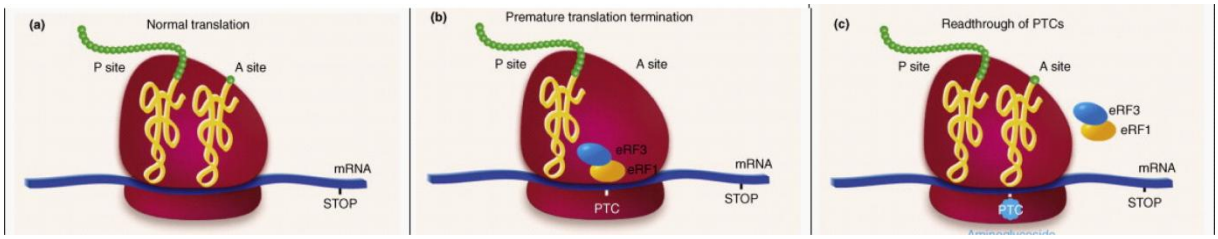


Figure (48): *The effect of readthrough drugs on protein translation.* **(a)** Normal protein synthesis. Recognition occurs between the codon of the mRNA and the anticodon of the amino-acyl-tRNA at the A site of the rRNA . The peptidyl tRNA binds to the P site. During translation, the nascent polypeptide binds to the amino acid in the A site and the ribosome moves along the mRNA three nucleotides at a time, transferring the tRNA polypeptide from the A to the P site. **(b)** Termination of protein synthesis due to a premature translation termination (PTC). When the ribosome encounters a PTC, there is no

corresponding tRNA and the translation is stalled. This leads to the binding of the release factors (eRF1 and eRF3), resulting in translation termination and release of the polypeptide. At this stage, non-sense mediated RNA decay (NMD) might be elicited. **(c)** Readthrough of PTC by aminoglycosides or PTC124. The aminoglycosides can bind the A site of the rRNA. This binding alters the RNA conformation and the accuracy between codon–anticodon pairing is reduced. The reduced accuracy occasionally enables readthrough of the PTC by incorporation of an amino acid, generating full-length proteins, (Liat Linde, Trends in genetics, 2008).

PTC124 was identified in a high-troughput screen. It is a small organic molecule with no antibiotic properties. PTC124 can selectively promote readthrough of disease-causing PTCs but not normal termination codons (Welch et al. 2007). This drug may serve a new strategy to recover a wide variety of monogenetic disease with PTCs. A recent Phase IIa clinical trial in cystic fibrosis patients who have a nonsense mutations within the cystic fibrosis transmembrane conductance regulator (CFTR) gene, has shown that oral administration of PTC124 can improve a major biological parameter of the disease such as chloride transport as measured by nasal epithelium electrophysiology and also some clinical parameters (Kerem et al. 2008).

This opens a new perspective for XLMTM subjects as 25% of mutations identified within the Mtm1 gene give rise to mRNA with premature termination codons (PTCs), which result in truncated non-functional protein (Laporte et al. 2000). PTC124 administration may thus potentially be a promising treatment for 25% of XLMTM patients. However, as there are also a large proportion of missense disease causing mutations in XLMTM, one will have to test first whether read through over certain stop codons may lead to full length but non functional protein, using appropriate cell models, and perhaps avoid stop codons that are located in functionally crucial parts of the protein. As XLMTM is a very severe neonatal disease, one might expect that clinical improvement might be easier to measure than in a slowly progressing disease.

IV-3. Stem cell therapy

Different stem cell populations, from both embryonic and adult origins have the potential to generate skeletal muscle cells. These include stem cells which are derived from muscle, bone marrow or circulating blood (Mouly et al. 2005). Even though this strategy seems promising in mouse models, its efficiency remains to be proven as there is massive cell death of myoblast observed following injection (Mouly et al. 2005). However, more recent clinical assays using muscle-derived CD133+ cells or umbilical cord blood stem cells report promising findings (Torrente et al. 2007), (Zhang et al. 2005). Patients stem cells might first be corrected using viral vectors (as for gene therapy of severe immunodeficiencies) or by meganuclease based homologous recombination (see above), for autologous transplantation. Although this strategy gives some hope, further studies are required to optimize their application in muscle disease.

Conclusion

Myotubular myopathy (XLMTM) is a severe congenital disorder that affects skeletal muscle and is due to mutations in the *MTM1* gene, which encodes myotubularin, a lipid phosphatase implicated in endocytosis. Mice deficient for *Mtm1* have been generated and reproduce clinical and pathological aspects of the disease. The aim of thesis was to provide clues about the pathophysiological mechanisms underlying this disease.

For that, I have characterized the *Mtm1* KO model in a novel genetic background, 129PAS, and shown that these mice develop a more severe phenotype than previously characterized C57Bl6 mutant mice, with earlier appearance of pathological signs and lethality.

I was particularly interested in analyzing the excitation-contraction coupling machinery at the ultrastructural, molecular and functional level. I have shown that the organization of triads, and in particular T-tubules, is altered in myotubularin deficient muscle fibers, with either presence of longitudinal tubules or absence of T-tubules, at an early stage of the disease. I also found that the expression of some genes implicated in calcium homeostasis is deregulated, with a striking reduction in RyR1 level. At the electrophysiological level, we provide for the first time evidences that depolarization-induced sarcoplasmic reticulum calcium release is strongly diminished in mutant myofibers. These results suggest a muscle-specific role for myotubularin in the maturation/maintenance of the T-tubule system, and that defects in excitation-contraction coupling might be responsible for the muscle weakness observed in this disease.

In addition, I have initiated a study in order to understand the mechanisms underlying the muscle hypotrophy in myotubular myopathy, and shown that there are alterations in the IGF1R/AKT/Foxo pathway, with increased expression of atrogenes. These results suggest that an unbalance between trophic and atrophic signals may lead to the decreased muscle mass observed in these mice and open a novel route to be explored in the pathogenesis of XLMTM.

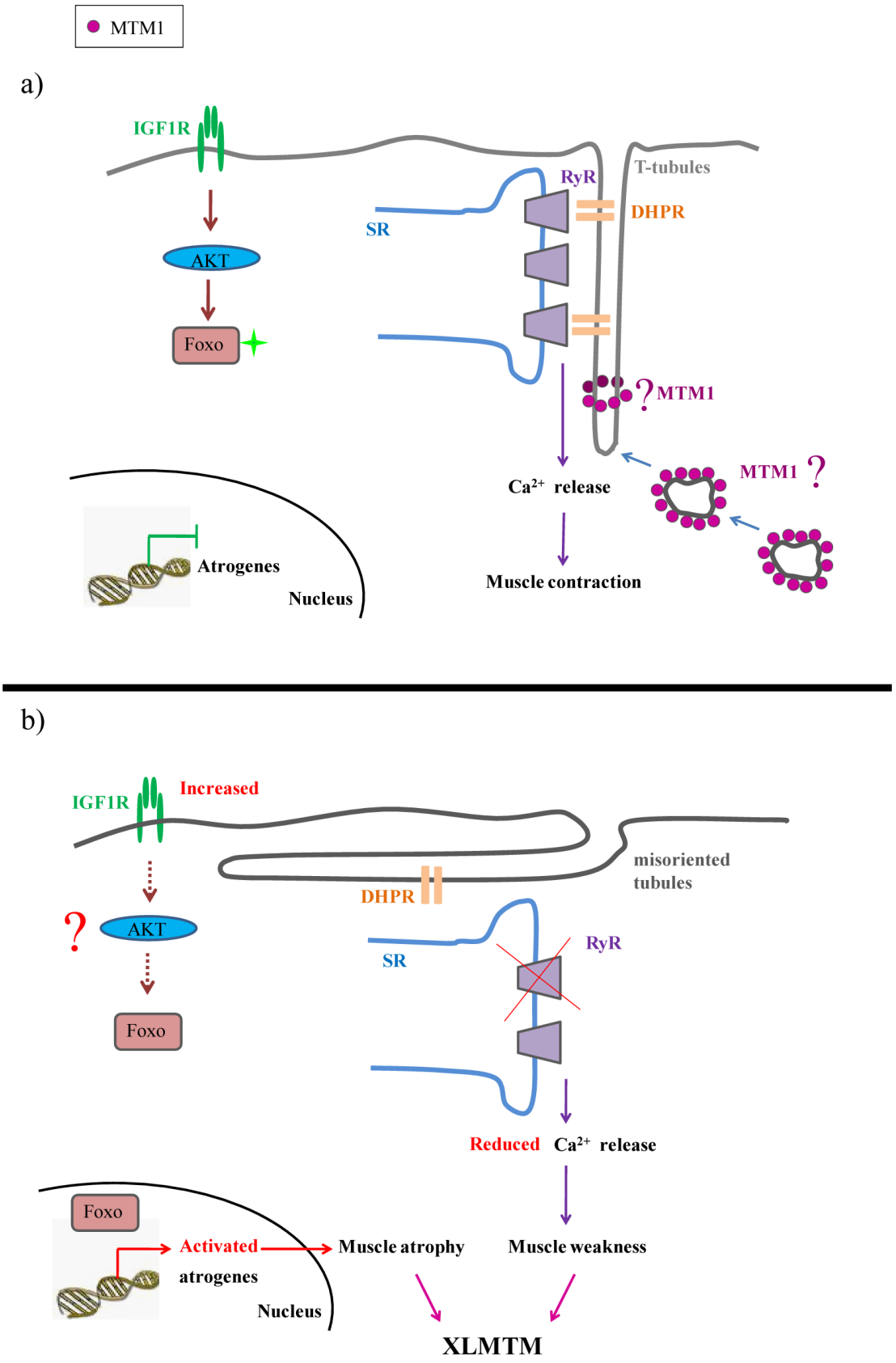


Figure (49): *A schematic representation of a normal muscle fiber (a) and a MTM1 lacking muscle fiber (b)*. This model suggests that the MTM1 is involved in the maturation/maintenance of T-tubule

system in skeletal muscle and its absence leads to decrease in Ca^{2+} release, which may result in muscle weakness. In addition, we observed alteration in IGF1/AKT/Foxo pathway characterized by the increase of IGF1R protein and also the activation of atrogenes expression which may result in the degradation of muscle proteins and the observed hypotrophy. However, the link between MTM1 and IGF1/AKT/Foxo pathway remains to be resolved.

Abmayr, S., P. Gregorevic, et al. (2005). "Phenotypic improvement of dystrophic muscles by rAAV/microdystrophin vectors is augmented by Igf1 codelivery." Mol Ther **12**(3): 441-50.

Adams, B. A., T. Tanabe, et al. (1990). "Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs." Nature **346**(6284): 569-72.

Agbenyega, E. T., R. H. Morton, et al. (1995). "Effect of the beta 2-adrenergic agonist clenbuterol on the growth of fast- and slow-twitch skeletal muscle of the dystrophic (C57BL6J dy2J/dy2J) mouse." Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **111**(3): 397-403.

Ahern, G. P., P. R. Junankar, et al. (1994). "Single channel activity of the ryanodine receptor calcium release channel is modulated by FK-506." FEBS Lett **352**(3): 369-74.

Aikawa, Y. and T. F. Martin (2005). "ADP-ribosylation factor 6 regulation of phosphatidylinositol-4,5-bisphosphate synthesis, endocytosis, and exocytosis." Methods Enzymol **404**: 422-31.

Al-Qusairi, L., N. Weiss, et al. (2009). "T-tubule disorganization and defective excitation-contraction coupling in muscle fibers lacking myotubularin lipid phosphatase." Proc Natl Acad Sci U S A.

Anderson, K., A. H. Cohn, et al. (1994). "High-affinity [3H]PN200-110 and [3H]ryanodine binding to rabbit and frog skeletal muscle." Am J Physiol **266**(2 Pt 1): C462-6.

Andronache, Z., D. Ursu, et al. (2007). "The auxiliary subunit gamma 1 of the skeletal muscle L-type Ca2+ channel is an endogenous Ca2+ antagonist." Proc Natl Acad Sci U S A **104**(45): 17885-90.

Anger, M., J. L. Samuel, et al. (1993). "The sarco(endo)plasmic reticulum Ca(2+)-ATPase mRNA isoform, SERCA 3, is expressed in endothelial and epithelial cells in various organs." FEBS Lett **334**(1): 45-8.

Arnould, S., C. Perez, et al. (2007). "Engineered I-CreI derivatives cleaving sequences from the human XPC gene can induce highly efficient gene correction in mammalian cells." J Mol Biol **371**(1): 49-65.

Asahi, M., Y. Sugita, et al. (2003). "Sarcolipin regulates sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA) by binding to transmembrane helices alone or in association with phospholamban." Proc Natl Acad Sci U S A **100**(9): 5040-5.

Audhya, A., R. Loewith, et al. (2004). "Genome-wide lethality screen identifies new PI4,5P2 effectors that regulate the actin cytoskeleton." Embo J **23**(19): 3747-57.

Babu, G. J., P. Bhupathy, et al. (2006). "Targeted overexpression of sarcolipin in the mouse heart decreases sarcoplasmic reticulum calcium transport and cardiac contractility." J Biol Chem **281**(7): 3972-9.

Babu, G. J., P. Bhupathy, et al. (2007). "Ablation of sarcolipin enhances sarcoplasmic reticulum calcium transport and atrial contractility." Proc Natl Acad Sci U S A **104**(45): 17867-72.

Bae, S. C. and Y. H. Lee (2006). "Phosphorylation, acetylation and ubiquitination: the molecular basis of RUNX regulation." Gene **366**(1): 58-66.

Balla, T., Z. Szentpetery, et al. (2009). "Phosphoinositide signaling: new tools and insights." Physiology (Bethesda) **24**: 231-44.

Barton-Davis, E. R., L. Cordier, et al. (1999). "Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice." J Clin Invest **104**(4): 375-81.

Bataille, J., F. Guillon, et al. (1991). "[Pathological anatomy of the heart in myopathies and infantile muscular atrophies]." Ann Med Interne (Paris) **142**(1): 5-8.

Beam, K. G., C. M. Knudson, et al. (1986). "A lethal mutation in mice eliminates the slow calcium current in skeletal muscle cells." Nature **320**(6058): 168-70.

Beard, N. A., L. Wei, et al. (2008). "Phosphorylation of skeletal muscle calsequestrin enhances its Ca2+ binding capacity and promotes its association with junctin." Cell Calcium **44**(4): 363-73.

Begley, M. J. and J. E. Dixon (2005). "The structure and regulation of myotubularin phosphatases." Curr Opin Struct Biol **15**(6): 614-20.

Beguin, P., R. N. Mahalakshmi, et al. (2005). "Roles of 14-3-3 and calmodulin binding in subcellular localization and function of the small G-protein Rem2." Biochem J **390**(Pt 1): 67-75.

Beguin, P., K. Nagashima, et al. (2001). "Regulation of Ca2+ channel expression at the cell surface by the small G-protein kir/Gem." Nature **411**(6838): 701-6.

Belfort, M. and R. J. Roberts (1997). "Homing endonucleases: keeping the house in order." Nucleic Acids Res **25**(17): 3379-88.

Biancalana, V., O. Caron, et al. (2003). "Characterisation of mutations in 77 patients with X-linked myotubular myopathy, including a family with a very mild phenotype." Hum Genet **112**(2): 135-42.

Bitoun, M., J. A. Bevilacqua, et al. (2007). "Dynamin 2 mutations cause sporadic centronuclear myopathy with neonatal onset." Ann Neurol **62**(6): 666-70.

Bitoun, M., S. Maugenre, et al. (2005). "Mutations in dynamin 2 cause dominant centronuclear myopathy." Nat Genet **37**(11): 1207-9.

Bitoun, M., T. Stojkovic, et al. (2008). "A novel mutation in the dynamin 2 gene in a Charcot-Marie-Tooth type 2 patient: clinical and pathological findings." Neuromuscul Disord **18**(4): 334-8.

Block, B. A., T. Imagawa, et al. (1988). "Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle." J Cell Biol **107**(6 Pt 2): 2587-600.

Blondeau, F., J. Laporte, et al. (2000). "Myotubularin, a phosphatase deficient in myotubular myopathy, acts on phosphatidylinositol 3-kinase and phosphatidylinositol 3-phosphate pathway." Hum Mol Genet **9**(15): 2223-9.

Bobe, R., R. Bredoux, et al. (2004). "Identification, expression, function, and localization of a novel (sixth) isoform of the human sarco/endoplasmic reticulum Ca2+ATPase 3 gene." J Biol Chem **279**(23): 24297-306.

Bodine, S. C., E. Latres, et al. (2001). "Identification of ubiquitin ligases required for skeletal muscle atrophy." Science **294**(5547): 1704-8.

Bodine, S. C., T. N. Stitt, et al. (2001). "Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo." Nat Cell Biol **3**(11): 1014-9.

Bolino, A., M. Muglia, et al. (2000). "Charcot-Marie-Tooth type 4B is caused by mutations in the gene encoding myotubularin-related protein-2." Nat Genet **25**(1): 17-9.

Bonangelino, C. J., J. J. Nau, et al. (2002). "Osmotic stress-induced increase of phosphatidylinositol 3,5-bisphosphate requires Vac14p, an activator of the lipid kinase Fab1p." J Cell Biol **156**(6): 1015-28.

Brandl, C. J., S. deLeon, et al. (1987). "Adult forms of the Ca2+ATPase of sarcoplasmic reticulum. Expression in developing skeletal muscle." J Biol Chem **262**(8): 3768-74.

Brandl, C. J., N. M. Green, et al. (1986). "Two Ca2+ ATPase genes: homologies and mechanistic implications of deduced amino acid sequences." Cell **44**(4): 597-607.

Brillantes, A. B., K. Ondrias, et al. (1994). "Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein." Cell **77**(4): 513-23.

Brody, I. A. (1969). "Muscle contracture induced by exercise. A syndrome attributable to decreased relaxing factor." N Engl J Med **281**(4): 187-92.

Brook, J. D., M. E. McCurrach, et al. (1992). "Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member." Cell **69**(2): 385.

Buj-Bello, A., F. Fougerousse, et al. (2008). "AAV-mediated intramuscular delivery of myotubularin corrects the myotubular myopathy phenotype in targeted murine muscle and suggests a function in plasma membrane homeostasis." Hum Mol Genet **17**(14): 2132-43.

Buj-Bello, A., V. Laugel, et al. (2002). "The lipid phosphatase myotubularin is essential for skeletal muscle maintenance but not for myogenesis in mice." Proc Natl Acad Sci U S A **99**(23): 15060-5.

Butler, M. H., C. David, et al. (1997). "Amphiphysin II (SH3P9; BIN1), a member of the amphiphysin/Rvs family, is concentrated in the cortical cytomatrix of axon initial segments and nodes of ranvier in brain and around T tubules in skeletal muscle." J Cell Biol **137**(6): 1355-67.

Campbell, K. P., D. H. MacLennan, et al. (1983). "Purification and characterization of calsequestrin from canine cardiac sarcoplasmic reticulum and identification of the 53,000 dalton glycoprotein." J Biol Chem **258**(2): 1197-204.

Cao, C., J. M. Backer, et al. (2008). "Sequential actions of myotubularin lipid phosphatases regulate endosomal PI(3)P and growth factor receptor trafficking." Mol Biol Cell **19**(8): 3334-46.

Cao, C., J. Laporte, et al. (2007). "Myotubularin lipid phosphatase binds the hVPS15/hVPS34 lipid kinase complex on endosomes." Traffic **8**(8): 1052-67.

Capetanaki, Y., R. J. Bloch, et al. (2007). "Muscle intermediate filaments and their links to membranes and membranous organelles." Exp Cell Res **313**(10): 2063-76.

Carozzi, A. J., E. Ikonen, et al. (2000). "Role of cholesterol in developing T-tubules: analogous mechanisms for T-tubule and caveolae biogenesis." Traffic **1**(4): 326-41.

Castellano, A., X. Wei, et al. (1993). "Cloning and expression of a neuronal calcium channel beta subunit." J Biol Chem **268**(17): 12359-66.

Castellano, A., X. Wei, et al. (1993). "Cloning and expression of a third calcium channel beta subunit." J Biol Chem **268**(5): 3450-5.

Caswell, A. H., N. R. Brandt, et al. (1991). "Localization and partial characterization of the oligomeric disulfide-linked molecular weight 95,000 protein (triadin) which binds the ryanodine and dihydropyridine receptors in skeletal muscle triadic vesicles." Biochemistry **30**(30): 7507-13.

Catteruccia, M., T. Sanna, et al. (2009). "Rippling muscle disease and cardiomyopathy associated with a mutation in the CAV3 gene." Neuromuscul Disord **19**(11): 779-83.

Centner, T., J. Yano, et al. (2001). "Identification of muscle specific ring finger proteins as potential regulators of the titin kinase domain." J Mol Biol **306**(4): 717-26.

Chakravarthy, M. V., B. S. Davis, et al. (2000). "IGF-I restores satellite cell proliferative potential in immobilized old skeletal muscle." J Appl Physiol **89**(4): 1365-79.

Chang, R. J. and H. Smilowitz (1988). "Identification of a approximately 170K subunit of the cardiac calcium channel using a monoclonal antibody to the skeletal muscle dihydropyridine receptor." Life Sci **43**(13): 1055-61.

Chen, H., H. L. Puhl, 3rd, et al. (2005). "Expression of Rem2, an RGK family small GTPase, reduces N-type calcium current without affecting channel surface density." J Neurosci **25**(42): 9762-72.

Chen, S. R., L. Zhang, et al. (1994). "Asymmetrical blockade of the Ca2+ release channel (ryanodine receptor) by 12-kDa FK506 binding protein." Proc Natl Acad Sci U S A **91**(25): 11953-7.

Cheng, W., X. Altafaj, et al. (2005). "Interaction between the dihydropyridine receptor Ca2+ channel beta-subunit and ryanodine receptor type 1 strengthens excitation-contraction coupling." Proc Natl Acad Sci U S A **102**(52): 19225-30.

Choo, J. J., M. A. Horan, et al. (1989). "Muscle wasting associated with endotoxemia in the rat: modification by the beta 2-adrenoceptor agonist clenbuterol." Biosci Rep **9**(5): 615-21.

Chow, C. Y., Y. Zhang, et al. (2007). "Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J." Nature **448**(7149): 68-72.

Cideciyan, A. V., W. W. Hauswirth, et al. (2009). "Human RPE65 gene therapy for Leber congenital amaurosis: persistence of early visual improvements and safety at 1 year." Hum Gene Ther **20**(9): 999-1004.

Clancy, J. S., H. Takeshima, et al. (1999). "Contractile function is unaltered in diaphragm from mice lacking calcium release channel isoform 3." Am J Physiol **277**(4 Pt 2): R1205-9.

Clemmons, D. R. (2009). "Role of IGF-I in skeletal muscle mass maintenance." Trends Endocrinol Metab **20**(7): 349-56.

Coers, C., N. Telerman-Toppet, et al. (1976). "Changes in motor innervation and histochemical pattern of muscle fibers in some congenital myopathies." Neurology **26**(11): 1046-53.

Cook, T., K. Mesa, et al. (1996). "Three dynamin-encoding genes are differentially expressed in developing rat brain." J Neurochem **67**(3): 927-31.

Cook, T. A., R. Urrutia, et al. (1994). "Identification of dynamin 2, an isoform ubiquitously expressed in rat tissues." Proc Natl Acad Sci U S A **91**(2): 644-8.

Coolican, S. A., D. S. Samuel, et al. (1997). "The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways." J Biol Chem **272**(10): 6653-62.

Costantin, L. L. (1975). "Contractile activation in skeletal muscle." Prog Biophys Mol Biol **29**(2): 197-224.

Crabtree, G. R. (1999). "Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT." Cell **96**(5): 611-4.

Crivici, A. and M. Ikura (1995). "Molecular and structural basis of target recognition by calmodulin." Annu Rev Biophys Biomol Struct **24**: 85-116.

Crossland, H., D. Constantin-Teodosiu, et al. (2008). "A potential role for Akt/FOXO signalling in both protein loss and the impairment of muscle carbohydrate oxidation during sepsis in rodent skeletal muscle." J Physiol **586**(Pt 22): 5589-600.

Cui, X., I. De Vivo, et al. (1998). "Association of SET domain and myotubularin-related proteins modulates growth control." Nat Genet **18**(4): 331-7.

Curtis, B. M. and W. A. Catterall (1984). "Purification of the calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubules." Biochemistry **23**(10): 2113-8.

Dahl, N., L. J. Hu, et al. (1995). "Myotubular myopathy in a girl with a deletion at Xq27-q28 and unbalanced X inactivation assigns the MTM1 gene to a 600-kb region." Am J Hum Genet **56**(5): 1108-15.

Dally, S., R. Bredoux, et al. (2006). "Ca2+-ATPases in non-failing and failing heart: evidence for a novel cardiac sarco/endoplasmic reticulum Ca2+-ATPase 2 isoform (SERCA2c)." Biochem J **395**(2): 249-58.

Damiani, E., P. Volpe, et al. (1990). "Coexpression of two isoforms of calsequestrin in rabbit slow-twitch muscle." J Muscle Res Cell Motil **11**(6): 522-30.

David, C., P. S. McPherson, et al. (1996). "A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals." Proc Natl Acad Sci U S A **93**(1): 331-5.

De Camilli, P., A. Thomas, et al. (1993). "The synaptic vesicle-associated protein amphiphysin is the 128-kD autoantigen of Stiff-Man syndrome with breast cancer." J Exp Med **178**(6): 2219-23.

Denborough, M. (1998). "Malignant hyperthermia." Lancet **352**(9134): 1131-6.

Denborough, M. A., X. Dennett, et al. (1973). "Central-core disease and malignant hyperpyrexia." Br Med J **1**(5848): 272-3.

Diop, D., C. Chauvin, et al. (2007). "Aminoglycosides and other factors promoting stop codon readthrough in human cells." C R Biol **330**(1): 71-9.

Dirksen, R. T. and G. Avila (2002). "Altered ryanodine receptor function in central core disease: leaky or uncoupled Ca(2+) release channels?" Trends Cardiovasc Med **12**(5): 189-97.

Dirksen, R. T. and G. Avila (2004). "Distinct effects on Ca2+ handling caused by malignant hyperthermia and central core disease mutations in RyR1." Biophys J **87**(5): 3193-204.

Doerks, T., M. Strauss, et al. (2000). "GRAM, a novel domain in glucosyltransferases, myotubularins and other putative membrane-associated proteins." Trends Biochem Sci **25**(10): 483-5.

Dorchies, O. M., J. Laporte, et al. (2001). "Normal innervation and differentiation of X-linked myotubular myopathy muscle cells in a nerve-muscle coculture system." Neuromuscul Disord **11**(8): 736-46.

Dove, S. K., F. T. Cooke, et al. (1997). "Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis." Nature **390**(6656): 187-92.

Dove, S. K. and Z. E. Johnson (2007). "Our FABulous VACation: a decade of phosphatidylinositol 3,5-bisphosphate." Biochem Soc Symp(74): 129-39.

Dowling, J. J., A. P. Vreede, et al. (2009). "Loss of myotubularin function results in T-tubule disorganization in zebrafish and human myotubular myopathy." PLoS Genet **5**(2): e1000372.

Dutta, K., M. W. Carmody, et al. (2002). "Depressed PKA activity contributes to impaired SERCA function and is linked to the pathogenesis of glucose-induced cardiomyopathy." J Mol Cell Cardiol **34**(8): 985-96.

Eberhard, D. A., C. L. Cooper, et al. (1990). "Evidence that the inositol phospholipids are necessary for exocytosis. Loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP." Biochem J **268**(1): 15-25.

Edwards, H., C. Xie, et al. (2009). "RUNX1 regulates phosphoinositide 3-kinase/AKT pathway: role in chemotherapy sensitivity in acute megakaryocytic leukemia." Blood **114**(13): 2744-52.

Elbaz, A., J. Vale-Santos, et al. (1995). "Hypokalemic periodic paralysis and the dihydropyridine receptor (CACNL1A3): genotype/phenotype correlations for two predominant mutations and evidence for the absence of a founder effect in 16 caucasian families." Am J Hum Genet **56**(2): 374-80.

Elliott, K., D. Sakamuro, et al. (1999). "Bin1 functionally interacts with Myc and inhibits cell proliferation via multiple mechanisms." Oncogene **18**(24): 3564-73.

Endo, M. (1977). "Calcium release from the sarcoplasmic reticulum." Physiol Rev **57**(1): 71-108.

Engel, A. G., M. R. Gomez, et al. (1971). "Multicore disease. A recently recognized congenital myopathy associated with multifocal degeneration of muscle fibers." Mayo Clin Proc **46**(10): 666-81.

Fabrizi, G. M., M. Ferrarini, et al. (2007). "Two novel mutations in dynamin-2 cause axonal Charcot-Marie-Tooth disease." Neurology **69**(3): 291-5.

Fagerlund, M. J. and L. I. Eriksson (2009). "Current concepts in neuromuscular transmission." Br J Anaesth **103**(1): 108-14.

Fan-Minogue, H. and D. M. Bedwell (2008). "Eukaryotic ribosomal RNA determinants of aminoglycoside resistance and their role in translational fidelity." Rna **14**(1): 148-57.

Fares, H. and I. Greenwald (2001). "Genetic analysis of endocytosis in Caenorhabditis elegans: coelomocyte uptake defective mutants." Genetics **159**(1): 133-45.

Felder, E., F. Protasi, et al. (2002). "Morphology and molecular composition of sarcoplasmic reticulum surface junctions in the absence of DHPR and RyR in mouse skeletal muscle." Biophys J **82**(6): 3144-9.

Ferreiro, A., N. Monnier, et al. (2002). "A recessive form of central core disease, transiently presenting as multi-minicore disease, is associated with a homozygous mutation in the ryanodine receptor type 1 gene." Ann Neurol **51**(6): 750-9.

Fidzianska, A. and H. H. Goebel (1994). "Aberrant arrested in maturation neuromuscular junctions in centronuclear myopathy." J Neurol Sci **124**(1): 83-8.

Fili, N., V. Calleja, et al. (2006). "Compartmental signal modulation: Endosomal phosphatidylinositol 3-phosphate controls endosome morphology and selective cargo sorting." Proc Natl Acad Sci U S A **103**(42): 15473-8.

Fill, M. and J. A. Copello (2002). "Ryanodine receptor calcium release channels." Physiol Rev **82**(4): 893-922.

Finlin, B. S., R. N. Correll, et al. (2006). "Analysis of the complex between Ca2+ channel beta-subunit and the Rem GTPase." J Biol Chem **281**(33): 23557-66.

Finlin, B. S., S. M. Crump, et al. (2003). "Regulation of voltage-gated calcium channel activity by the Rem and Rad GTPases." Proc Natl Acad Sci U S A **100**(24): 14469-74.

Fischer, D., M. Herasse, et al. (2006). "Characterization of the muscle involvement in dynamin 2-related centronuclear myopathy." Brain **129**(Pt 6): 1463-9.

Fliegel, L., M. Ohnishi, et al. (1987). "Amino acid sequence of rabbit fast-twitch skeletal muscle calsequestrin deduced from cDNA and peptide sequencing." Proc Natl Acad Sci U S A **84**(5): 1167-71.

Flucher, B. E., A. Conti, et al. (1999). "Type 3 and type 1 ryanodine receptors are localized in triads of the same mammalian skeletal muscle fibers." J Cell Biol **146**(3): 621-30.

Flucher, B. E., N. Kasielke, et al. (2000). "The triad targeting signal of the skeletal muscle calcium channel is localized in the COOH terminus of the alpha(1S) subunit." J Cell Biol **151**(2): 467-78.

Flucher, B. E., H. Takekura, et al. (1993). "Development of the excitation-contraction coupling apparatus in skeletal muscle: association of sarcoplasmic reticulum and transverse tubules with myofibrils." Dev Biol **160**(1): 135-47.

Fosset, M., E. Jaimovich, et al. (1983). "[3H]nitrendipine receptors in skeletal muscle." J Biol Chem **258**(10): 6086-92.

Fowler, E. G., M. C. Graves, et al. (2004). "Pilot trial of albuterol in Duchenne and Becker muscular dystrophy." Neurology **62**(6): 1006-8.

Franzini-Armstrong, C., L. J. Kenney, et al. (1987). "The structure of calsequestrin in triads of vertebrate skeletal muscle: a deep-etch study." J Cell Biol **105**(1): 49-56.

Franzini-Armstrong, C., M. Pincon-Raymond, et al. (1991). "Muscle fibers from dysgenic mouse in vivo lack a surface component of peripheral couplings." Dev Biol **146**(2): 364-76.

Freise, D., B. Held, et al. (2000). "Absence of the gamma subunit of the skeletal muscle dihydropyridine receptor increases L-type Ca2+ currents and alters channel inactivation properties." J Biol Chem **275**(19): 14476-81.

Friedberg, F. (1990). "Species comparison of calmodulin sequences." Protein Seq Data Anal **3**(4): 335-7.

Froemming, G. R., B. E. Murray, et al. (2000). "Comparative analysis of the isoform expression pattern of Ca(2+)-regulatory membrane proteins in fast-twitch, slow-twitch, cardiac, neonatal and chronic low-frequency stimulated muscle fibers." Biochim Biophys Acta **1466**(1-2): 151-68.

Fujii, J., K. Otsu, et al. (1991). "Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia." Science **253**(5018): 448-51.

Fujii, J., A. Ueno, et al. (1987). "Complete complementary DNA-derived amino acid sequence of canine cardiac phospholamban." J Clin Invest **79**(1): 301-4.

Gaidarov, I., Q. Chen, et al. (1996). "A functional phosphatidylinositol 3,4,5-trisphosphate/phosphoinositide binding domain in the clathrin adaptor AP-2 alpha subunit. Implications for the endocytic pathway." J Biol Chem **271**(34): 20922-9.

Gaidarov, I. and J. H. Keen (1999). "Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits." J Cell Biol **146**(4): 755-64.

Galbiati, F., J. A. Engelman, et al. (2001). "Caveolin-3 null mice show a loss of caveolae, changes in the microdomain distribution of the dystrophin-glycoprotein complex, and t-tubule abnormalities." J Biol Chem **276**(24): 21425-33.

Gazzerro, E., F. Sotgia, et al. (2009). "Caveolinopathies: from the biology of caveolin-3 to human diseases." Eur J Hum Genet **17**(12): 1692.

Gelebart, P., V. Martin, et al. (2003). "Identification of a new SERCA2 splice variant regulated during monocytic differentiation." Biochem Biophys Res Commun **303**(2): 676-84.

Giannini, G. and V. Sorrentino (1995). "Molecular structure and tissue distribution of ryanodine receptors calcium channels." Med Res Rev **15**(4): 313-23.

Gillooly, D. J., I. C. Morrow, et al. (2000). "Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells." Embo J **19**(17): 4577-88.

Gomes, M. D., S. H. Lecker, et al. (2001). "Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy." Proc Natl Acad Sci U S A **98**(25): 14440-5.

Goonasekera, S. A., N. A. Beard, et al. (2007). "Triadin binding to the C-terminal luminal loop of the ryanodine receptor is important for skeletal muscle excitation contraction coupling." J Gen Physiol **130**(4): 365-78.

Goryunov, D., A. Nightingale, et al. (2008). "Multiple disease-linked myotubularin mutations cause NFL assembly defects in cultured cells and disrupt myotubularin dimerization." J Neurochem **104**(6): 1536-52.

Gramolini, A. O., M. G. Trivieri, et al. (2006). "Cardiac-specific overexpression of sarcolipin in phospholamban null mice impairs myocyte function that is restored by phosphorylation." Proc Natl Acad Sci U S A **103**(7): 2446-51.

Granato, M., F. J. van Eeden, et al. (1996). "Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva." Development **123**: 399-413.

Gregg, R. G., A. Messing, et al. (1996). "Absence of the beta subunit (cchb1) of the skeletal muscle dihydropyridine receptor alters expression of the alpha 1 subunit and eliminates excitation-contraction coupling." Proc Natl Acad Sci U S A **93**(24): 13961-6.

Gunteski-Hamblin, A. M., J. Greeb, et al. (1988). "A novel Ca2+ pump expressed in brain, kidney, and stomach is encoded by an alternative transcript of the slow-twitch muscle sarcoplasmic reticulum Ca-ATPase gene. Identification of cDNAs encoding Ca2+ and

other cation-transporting ATPases using an oligonucleotide probe derived from the ATP-binding site." J Biol Chem **263**(29): 15032-40.

Guo, W. and K. P. Campbell (1995). "Association of triadin with the ryanodine receptor and calsequestrin in the lumen of the sarcoplasmic reticulum." J Biol Chem **270**(16): 9027-30.

Haffter, P., M. Granato, et al. (1996). "The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio." Development **123**: 1-36.

Hakamata, Y., J. Nakai, et al. (1992). "Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain." FEBS Lett **312**(2-3): 229-35.

Harcourt, L. J., J. D. Schertzer, et al. (2007). "Low dose formoterol administration improves muscle function in dystrophic mdx mice without increasing fatigue." Neuromuscul Disord **17**(1): 47-55.

Harvey, K. F. and S. Kumar (1999). "Nedd4-like proteins: an emerging family of ubiquitin-protein ligases implicated in diverse cellular functions." Trends Cell Biol **9**(5): 166-9.

Hawke, T. J., S. B. Kanatous, et al. (2006). "Rad is temporally regulated within myogenic progenitor cells during skeletal muscle regeneration." Am J Physiol Cell Physiol **290**(2): C379-87.

Hay, J. C. and T. F. Martin (1993). "Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca(2+)-activated secretion." Nature **366**(6455): 572-5.

Hellstern, S., S. Pegoraro, et al. (2001). "Sarcolipin, the shorter homologue of phospholamban, forms oligomeric structures in detergent micelles and in liposomes." J Biol Chem **276**(33): 30845-52.

Herman, G. E., M. Finegold, et al. (1999). "Medical complications in long-term survivors with X-linked myotubular myopathy." J Pediatr **134**(2): 206-14.

Herman, G. E., K. Kopacz, et al. (2002). "Characterization of mutations in fifty North American patients with X-linked myotubular myopathy." Hum Mutat **19**(2): 114-21.

Herzog, A., C. Szegedi, et al. (2000). "Surface plasmon resonance studies prove the interaction of skeletal muscle sarcoplasmic reticular Ca(2+) release channel/ryanodine receptor with calsequestrin." FEBS Lett **472**(1): 73-7.

Hilgemann, D. W. and R. Ball (1996). "Regulation of cardiac Na+,Ca2+ exchange and KATP potassium channels by PIP2." Science **273**(5277): 956-9.

Hilgemann, D. W., S. Feng, et al. (2001). "The complex and intriguing lives of PIP2 with ion channels and transporters." Sci STKE **2001**(111): re19.

Hinshaw, J. E. and S. L. Schmid (1995). "Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding." Nature **374**(6518): 190-2.

Hishiya, A., S. Iemura, et al. (2006). "A novel ubiquitin-binding protein ZNF216 functioning in muscle atrophy." Embo J **25**(3): 554-64.

Hoffjan, S., C. Thiels, et al. (2006). "Extreme phenotypic variability in a German family with X-linked myotubular myopathy associated with E404K mutation in MTM1." Neuromuscul Disord **16**(11): 749-53.

Howard, M. T., B. H. Shirts, et al. (2000). "Sequence specificity of aminoglycoside-induced stop condon readthrough: potential implications for treatment of Duchenne muscular dystrophy." Ann Neurol **48**(2): 164-9.

Ikemoto, N., B. Antoniu, et al. (1991). "Intravesicular calcium transient during calcium release from sarcoplasmic reticulum." Biochemistry **30**(21): 5230-7.

Iles, D. E., F. Lehmann-Horn, et al. (1994). "Localization of the gene encoding the alpha 2/delta-subunits of the L-type voltage-dependent calcium channel to chromosome 7q and analysis of the segregation of flanking markers in malignant hyperthermia susceptible families." Hum Mol Genet **3**(6): 969-75.

Inesi, G. (1987). "Sequential mechanism of calcium binding and translocation in sarcoplasmic reticulum adenosine triphosphatase." J Biol Chem **262**(34): 16338-42.
Inesi, G., M. Kurzmack, et al. (1980). "Cooperative calcium binding and ATPase activation in sarcoplasmic reticulum vesicles." J Biol Chem **255**(7): 3025-31.
Inesi, G., C. Sumbilla, et al. (1990). "Relationships of molecular structure and function in Ca2(+)-transport ATPase." Physiol Rev **70**(3): 749-60.
Insall, R. H. and L. M. Machesky (2009). "Actin dynamics at the leading edge: from simple machinery to complex networks." Dev Cell **17**(3): 310-22.
Inui, M., A. Saito, et al. (1987). "Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle." J Biol Chem **262**(4): 1740-7.
Ito, K., S. Komazaki, et al. (2001). "Deficiency of triad junction and contraction in mutant skeletal muscle lacking junctophilin type 1." J Cell Biol **154**(5): 1059-67.
Jackson, T., M. F. Allard, et al. (1990). "The c-myc proto-oncogene regulates cardiac development in transgenic mice." Mol Cell Biol **10**(7): 3709-16.
Jagoe, R. T. and A. L. Goldberg (2001). "What do we really know about the ubiquitin-proteasome pathway in muscle atrophy?" Curr Opin Clin Nutr Metab Care **4**(3): 183-90.
Jagoe, R. T., S. H. Lecker, et al. (2002). "Patterns of gene expression in atrophying skeletal muscles: response to food deprivation." Faseb J **16**(13): 1697-712.
Jayaraman, T., A. M. Brillantes, et al. (1992). "FK506 binding protein associated with the calcium release channel (ryanodine receptor)." J Biol Chem **267**(14): 9474-7.
Jeannet, P. Y., G. Bassez, et al. (2004). "Clinical and histologic findings in autosomal centronuclear myopathy." Neurology **62**(9): 1484-90.
Jones, D. R. and N. Divecha (2004). "Linking lipids to chromatin." Curr Opin Genet Dev **14**(2): 196-202.
Jones, L. R., L. Zhang, et al. (1995). "Purification, primary structure, and immunological characterization of the 26-kDa calsequestrin binding protein (junctin) from cardiac junctional sarcoplasmic reticulum." J Biol Chem **270**(51): 30787-96.
Jorgensen, A. O., V. Kalnins, et al. (1979). "Localization of sarcoplasmic reticulum proteins in rat skeletal muscle by immunofluorescence." J Cell Biol **80**(2): 372-84.
Jungbluth, H., C. Wallgren-Pettersson, et al. (2008). "Centronuclear (myotubular) myopathy." Orphanet J Rare Dis **3**: 26.
Jungbluth, H., H. Zhou, et al. (2007). "Centronuclear myopathy due to a de novo dominant mutation in the skeletal muscle ryanodine receptor (RYR1) gene." Neuromuscul Disord **17**(4): 338-45.
Jurkat-Rott, K., F. Lehmann-Horn, et al. (1994). "A calcium channel mutation causing hypokalemic periodic paralysis." Hum Mol Genet **3**(8): 1415-9.
Kawasaki, T. and M. Kasai (1994). "Regulation of calcium channel in sarcoplasmic reticulum by calsequestrin." Biochem Biophys Res Commun **199**(3): 1120-7.
Kerem, E., S. Hirawat, et al. (2008). "Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial." Lancet **372**(9640): 719-27.
Kessels, M. M., A. E. Engqvist-Goldstein, et al. (2001). "Mammalian Abp1, a signal-responsive F-actin-binding protein, links the actin cytoskeleton to endocytosis via the GTPase dynamin." J Cell Biol **153**(2): 351-66.
Kim, S. A., P. O. Vacratsis, et al. (2003). "Regulation of myotubularin-related (MTMR)2 phosphatidylinositol phosphatase by MTMR5, a catalytically inactive phosphatase." Proc Natl Acad Sci U S A **100**(8): 4492-7.

Kim, Y. S. and R. D. Sainz (1992). "Beta-adrenergic agonists and hypertrophy of skeletal muscles." Life Sci **50**(6): 397-407.

Kimura, T., M. Nakamori, et al. (2005). "Altered mRNA splicing of the skeletal muscle ryanodine receptor and sarcoplasmic/endoplasmic reticulum Ca2+-ATPase in myotonic dystrophy type 1." Hum Mol Genet **14**(15): 2189-200.

Kirchberber, M. A., M. Tada, et al. (1975). "Phospholamban: a regulatory protein of the cardiac sarcoplasmic reticulum." Recent Adv Stud Cardiac Struct Metab **5**: 103-15.

Klaus, M. M., S. P. Scordilis, et al. (1983). "Evidence for dysfunction in the regulation of cytosolic Ca2+ in excitation-contraction uncoupled dysgenic muscle." Dev Biol **99**(1): 152-65.

Klein, D. E., A. Lee, et al. (1998). "The pleckstrin homology domains of dynamin isoforms require oligomerization for high affinity phosphoinositide binding." J Biol Chem **273**(42): 27725-33.

Knudson, C. M., K. K. Stang, et al. (1993). "Biochemical characterization of ultrastructural localization of a major junctional sarcoplasmic reticulum glycoprotein (triadin)." J Biol Chem **268**(17): 12637-45.

Koenig, J. H. and K. Ikeda (1989). "Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval." J Neurosci **9**(11): 3844-60.

Kojima, C., A. Hashimoto, et al. (2004). "Regulation of Bin1 SH3 domain binding by phosphoinositides." Embo J **23**(22): 4413-22.

Komazaki, S., M. Nishi, et al. (1999). "Immunolocalization of mitsugumin29 in developing skeletal muscle and effects of the protein expressed in amphibian embryonic cells." Dev Dyn **215**(2): 87-95.

Koncarevic, A., R. W. Jackman, et al. (2007). "The ubiquitin-protein ligase Nedd4 targets Notch1 in skeletal muscle and distinguishes the subset of atrophies caused by reduced muscle tension." Faseb J **21**(2): 427-37.

Konopka, C. A., J. B. Schleede, et al. (2006). "Dynamin and cytokinesis." Traffic **7**(3): 239-47.

Kranias, E. G. (1985). "Regulation of Ca2+ transport by cyclic 3',5'-AMP-dependent and calcium-calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum." Biochim Biophys Acta **844**(2): 193-9.

Kristiansen, M., G. P. Knudsen, et al. (2003). "X-inactivation patterns in carriers of X-linked myotubular myopathy." Neuromuscul Disord **13**(6): 468-71.

Kulyte, A., R. Dryselius, et al. (2005). "Gene selective suppression of nonsense termination using antisense agents." Biochim Biophys Acta **1730**(3): 165-72.

Laporte, J., F. Bedez, et al. (2003). "Myotubularins, a large disease-associated family of cooperating catalytically active and inactive phosphoinositides phosphatases." Hum Mol Genet **12 Spec No 2**: R285-92.

Laporte, J., V. Biancalana, et al. (2000). "MTM1 mutations in X-linked myotubular myopathy." Hum Mutat **15**(5): 393-409.

Laporte, J., F. Blondeau, et al. (1998). "Characterization of the myotubularin dual specificity phosphatase gene family from yeast to human." Hum Mol Genet **7**(11): 1703-12.

Laporte, J., F. Blondeau, et al. (2002). "The PtdIns3P phosphatase myotubularin is a cytoplasmic protein that also localizes to Rac1-inducible plasma membrane ruffles." J Cell Sci **115**(Pt 15): 3105-17.

Laporte, J., L. J. Hu, et al. (1996). "A gene mutated in X-linked myotubular myopathy defines a new putative tyrosine phosphatase family conserved in yeast." Nat Genet **13**(2): 175-82.

Lee, E., M. Marcucci, et al. (2002). "Amphiphysin 2 (Bin1) and T-tubule biogenesis in muscle." Science **297**(5584): 1193-6.
Lee, S. J. and A. C. McPherron (1999). "Myostatin and the control of skeletal muscle mass." Curr Opin Genet Dev **9**(5): 604-7.
Lemmon, M. A. and K. M. Ferguson (2000). "Signal-dependent membrane targeting by pleckstrin homology (PH) domains." Biochem J **350 Pt 1**: 1-18.
Li, S., L. Tiab, et al. (2005). "Mutations in PIP5K3 are associated with Francois-Neetens mouchetee fleck corneal dystrophy." Am J Hum Genet **77**(1): 54-63.
Lichte, B., R. W. Veh, et al. (1992). "Amphiphysin, a novel protein associated with synaptic vesicles." Embo J **11**(7): 2521-30.
Linde, L. and B. Kerem (2008). "Introducing sense into nonsense in treatments of human genetic diseases." Trends Genet **24**(11): 552-63.
Lindmo, K. and H. Stenmark (2006). "Regulation of membrane traffic by phosphoinositide 3-kinases." J Cell Sci **119**(Pt 4): 605-14.
Lisanti, M. P., P. E. Scherer, et al. (1994). "Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis." Trends Cell Biol **4**(7): 231-5.
Liu, Y. W., M. C. Surka, et al. (2008). "Isoform and splice-variant specific functions of dynamin-2 revealed by analysis of conditional knock-out cells." Mol Biol Cell **19**(12): 5347-59.
Look, A. T. (1997). "Oncogenic transcription factors in the human acute leukemias." Science **278**(5340): 1059-64.
Lyfenko, A. D., S. Ducreux, et al. (2007). "Two central core disease (CCD) deletions in the C-terminal region of RYR1 alter muscle excitation-contraction (EC) coupling by distinct mechanisms." Hum Mutat **28**(1): 61-8.
Lynch, G. S. and J. G. Ryall (2008). "Role of beta-adrenoceptor signaling in skeletal muscle: implications for muscle wasting and disease." Physiol Rev **88**(2): 729-67.
MacLennan, D. H., M. Asahi, et al. (2003). "The regulation of SERCA-type pumps by phospholamban and sarcolipin." Ann N Y Acad Sci **986**: 472-80.
MacLennan, D. H., C. J. Brandl, et al. (1985). "Amino-acid sequence of a Ca2+ + Mg2+-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence." Nature **316**(6030): 696-700.
MacLennan, D. H., C. Duff, et al. (1990). "Ryanodine receptor gene is a candidate for predisposition to malignant hyperthermia." Nature **343**(6258): 559-61.
MacLennan, D. H., W. J. Rice, et al. (1997). "The mechanism of Ca2+ transport by sarco(endo)plasmic reticulum Ca2+-ATPases." J Biol Chem **272**(46): 28815-8.
MacLennan, D. H. and P. T. Wong (1971). "Isolation of a calcium-sequestering protein from sarcoplasmic reticulum." Proc Natl Acad Sci U S A **68**(6): 1231-5.
Maguire, A. M., K. A. High, et al. (2009). "Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial." Lancet.
Maguire, J., T. Santoro, et al. (1994). "Gem: an induced, immediate early protein belonging to the Ras family." Science **265**(5169): 241-4.
Mammucari, C., G. Milan, et al. (2007). "FoxO3 controls autophagy in skeletal muscle in vivo." Cell Metab **6**(6): 458-71.
Manuvakhova, M., K. Keeling, et al. (2000). "Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system." Rna **6**(7): 1044-55.
Margreth, A., E. Damiani, et al. (1993). "Ratio of dihydropyridine to ryanodine receptors in mammalian and frog twitch muscles in relation to the mechanical hypothesis of excitation-contraction coupling." Biochem Biophys Res Commun **197**(3): 1303-11.

Marks, A. R., P. Tempst, et al. (1989). "Molecular cloning and characterization of the ryanodine receptor/junctional channel complex cDNA from skeletal muscle sarcoplasmic reticulum." Proc Natl Acad Sci U S A **86**(22): 8683-7.

Martin, V., R. Bredoux, et al. (2002). "Three novel sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) 3 isoforms. Expression, regulation, and function of the membranes of the SERCA3 family." J Biol Chem **277**(27): 24442-52.

Marx, S. O., K. Ondrias, et al. (1998). "Coupled gating between individual skeletal muscle Ca2+ release channels (ryanodine receptors)." Science **281**(5378): 818-21.

Mayran, N., R. G. Parton, et al. (2003). "Annexin II regulates multivesicular endosome biogenesis in the degradation pathway of animal cells." Embo J **22**(13): 3242-53.

McCarthy, T. V., K. A. Quane, et al. (2000). "Ryanodine receptor mutations in malignant hyperthermia and central core disease." Hum Mutat **15**(5): 410-7.

McMahon, H. T., P. Wigge, et al. (1997). "Clathrin interacts specifically with amphiphysin and is displaced by dynamin." FEBS Lett **413**(2): 319-22.

Mikami, A., K. Imoto, et al. (1989). "Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel." Nature **340**(6230): 230-3.

Minetti, C., F. Sotgia, et al. (1998). "Mutations in the caveolin-3 gene cause autosomal dominant limb-girdle muscular dystrophy." Nat Genet **18**(4): 365-8.

Mochizuki, Y. and P. W. Majerus (2003). "Characterization of myotubularin-related protein 7 and its binding partner, myotubularin-related protein 9." Proc Natl Acad Sci U S A **100**(17): 9768-73.

Monnier, N. and J. Lunardi (2000). "[Biology of malignant hyperthermia: a disease of the calcium channels of the skeletal muscle]." Ann Biol Clin (Paris) **58**(2): 147-56.

Monnier, N., I. Marty, et al. (2008). "Null mutations causing depletion of the type 1 ryanodine receptor (RYR1) are commonly associated with recessive structural congenital myopathies with cores." Hum Mutat **29**(5): 670-8.

Monnier, N., V. Procaccio, et al. (1997). "Malignant-hyperthermia susceptibility is associated with a mutation of the alpha 1-subunit of the human dihydropyridine-sensitive L-type voltage-dependent calcium-channel receptor in skeletal muscle." Am J Hum Genet **60**(6): 1316-25.

Morrison, B. M., J. L. Lachey, et al. (2009). "A soluble activin type IIB receptor improves function in a mouse model of amyotrophic lateral sclerosis." Exp Neurol **217**(2): 258-68.

Mouly, V., A. Aamiri, et al. (2005). "Myoblast transfer therapy: is there any light at the end of the tunnel?" Acta Myol **24**(2): 128-33.

Muhlberg, A. B., D. E. Warnock, et al. (1997). "Domain structure and intramolecular regulation of dynamin GTPase." Embo J **16**(22): 6676-83.

Muller, A. J., J. F. Baker, et al. (2003). "Targeted disruption of the murine Bin1/Amphiphysin II gene does not disable endocytosis but results in embryonic cardiomyopathy with aberrant myofibril formation." Mol Cell Biol **23**(12): 4295-306.

Musaro, A., K. McCullagh, et al. (2001). "Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle." Nat Genet **27**(2): 195-200.

Musaro, A., K. J. McCullagh, et al. (1999). "IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1." Nature **400**(6744): 581-5.

Nakai, J., T. Ogura, et al. (1997). "Functional nonequality of the cardiac and skeletal ryanodine receptors." Proc Natl Acad Sci U S A **94**(3): 1019-22.

Nakata, T., A. Iwamoto, et al. (1991). "Predominant and developmentally regulated expression of dynamin in neurons." Neuron **7**(3): 461-9.

Nakata, T., R. Takemura, et al. (1993). "A novel member of the dynamin family of GTP-binding proteins is expressed specifically in the testis." J Cell Sci **105 (Pt 1)**: 1-5.

Nandurkar, H. H., M. Layton, et al. (2003). "Identification of myotubularin as the lipid phosphatase catalytic subunit associated with the 3-phosphatase adapter protein, 3-PAP." Proc Natl Acad Sci U S A **100**(15): 8660-5.

Nicot, A. S. and J. Laporte (2008). "Endosomal phosphoinositides and human diseases." Traffic **9**(8): 1240-9.

Nicot, A. S., A. Toussaint, et al. (2007). "Mutations in amphiphysin 2 (BIN1) disrupt interaction with dynamin 2 and cause autosomal recessive centronuclear myopathy." Nat Genet **39**(9): 1134-9.

Nishi, M., S. Komazaki, et al. (1999). "Abnormal features in skeletal muscle from mice lacking mitsugumin29." J Cell Biol **147**(7): 1473-80.

Noda, M., S. Shimizu, et al. (1984). "Primary structure of Electrophorus electricus sodium channel deduced from cDNA sequence." Nature **312**(5990): 121-7.

Noda, Y., T. Nakata, et al. (1993). "Localization of dynamin: widespread distribution in mature neurons and association with membranous organelles." Neuroscience **55**(1): 113-27.

Noguchi, S., M. Fujita, et al. (2005). "Gene expression analyses in X-linked myotubular myopathy." Neurology **65**(5): 732-7.

Odermatt, A., P. E. Taschner, et al. (1996). "Mutations in the gene-encoding SERCA1, the fast-twitch skeletal muscle sarcoplasmic reticulum Ca2+ ATPase, are associated with Brody disease." Nat Genet **14**(2): 191-4.

Odermatt, A., P. E. Taschner, et al. (1997). "Characterization of the gene encoding human sarcolipin (SLN), a proteolipid associated with SERCA1: absence of structural mutations in five patients with Brody disease." Genomics **45**(3): 541-53.

Ohkura, M., K. Furukawa, et al. (1998). "Dual regulation of the skeletal muscle ryanodine receptor by triadin and calsequestrin." Biochemistry **37**(37): 12987-93.

Oshikawa, J., K. Otsu, et al. (2004). "Insulin resistance in skeletal muscles of caveolin-3-null mice." Proc Natl Acad Sci U S A **101**(34): 12670-5.

Ottenheijm, C. A., C. Fong, et al. (2008). "Sarcoplasmic reticulum calcium uptake and speed of relaxation are depressed in nebulin-free skeletal muscle." Faseb J **22**(8): 2912-9.

Pan, Y., E. Zvaritch, et al. (2003). "Targeted disruption of the ATP2A1 gene encoding the sarco(endo)plasmic reticulum Ca2+ ATPase isoform 1 (SERCA1) impairs diaphragm function and is lethal in neonatal mice." J Biol Chem **278**(15): 13367-75.

Paolini, C., M. Quarta, et al. (2007). "Reorganized stores and impaired calcium handling in skeletal muscle of mice lacking calsequestrin-1." J Physiol **583**(Pt 2): 767-84.

Parton, R. G., M. Way, et al. (1997). "Caveolin-3 associates with developing T-tubules during muscle differentiation." J Cell Biol **136**(1): 137-54.

Pearen, M. A., J. G. Ryall, et al. (2009). "Expression profiling of skeletal muscle following acute and chronic beta2-adrenergic stimulation: implications for hypertrophy, metabolism and circadian rhythm." BMC Genomics **10**: 448.

Pelissier, A., J. P. Chauvin, et al. (2003). "Trafficking through Rab11 endosomes is required for cellularization during Drosophila embryogenesis." Curr Biol **13**(21): 1848-57.

Perez-Reyes, E., A. Castellano, et al. (1992). "Cloning and expression of a cardiac/brain beta subunit of the L-type calcium channel." J Biol Chem **267**(3): 1792-7.

Peter, B. J., H. M. Kent, et al. (2004). "BAR domains as sensors of membrane curvature: the amphiphysin BAR structure." Science **303**(5657): 495-9.

Peterson, B. Z., C. D. DeMaria, et al. (1999). "Calmodulin is the Ca2+ sensor for Ca2+ -dependent inactivation of L-type calcium channels." Neuron **22**(3): 549-58.

Pierson, C. R., K. Tomczak, et al. (2005). "X-linked myotubular and centronuclear myopathies." J Neuropathol Exp Neurol **64**(7): 555-64.

Politano, L., G. Nigro, et al. (2003). "Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results." Acta Myol **22**(1): 15-21.

Poodry, C. A. and L. Edgar (1979). "Reversible alteration in the neuromuscular junctions of Drosophila melanogaster bearing a temperature-sensitive mutation, shibire." J Cell Biol **81**(3): 520-7.

Powers, P. A., S. Liu, et al. (1992). "Skeletal muscle and brain isoforms of a beta-subunit of human voltage-dependent calcium channels are encoded by a single gene." J Biol Chem **267**(32): 22967-72.

Praefcke, G. J. and H. T. McMahon (2004). "The dynamin superfamily: universal membrane tubulation and fission molecules?" Nat Rev Mol Cell Biol **5**(2): 133-47.

Prather, I. D., D. E. Brown, et al. (1995). "Clenbuterol: a substitute for anabolic steroids?" Med Sci Sports Exerc **27**(8): 1118-21.

Previtali, S. C., B. Zerega, et al. (2003). "Myotubularin-related 2 protein phosphatase and neurofilament light chain protein, both mutated in CMT neuropathies, interact in peripheral nerve." Hum Mol Genet **12**(14): 1713-23.

Qualmann, B., J. Roos, et al. (1999). "Syndapin I, a synaptic dynamin-binding protein that associates with the neural Wiskott-Aldrich syndrome protein." Mol Biol Cell **10**(2): 501-13.

Radermacher, M., V. Rao, et al. (1994). "Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle." J Cell Biol **127**(2): 411-23.

Ramjaun, A. R. and P. S. McPherson (1998). "Multiple amphiphysin II splice variants display differential clathrin binding: identification of two distinct clathrin-binding sites." J Neurochem **70**(6): 2369-76.

Razzaq, A., I. M. Robinson, et al. (2001). "Amphiphysin is necessary for organization of the excitation-contraction coupling machinery of muscles, but not for synaptic vesicle endocytosis in Drosophila." Genes Dev **15**(22): 2967-79.

Reiken, S., A. Lacampagne, et al. (2003). "PKA phosphorylation activates the calcium release channel (ryanodine receptor) in skeletal muscle: defective regulation in heart failure." J Cell Biol **160**(6): 919-28.

Reynet, C. and C. R. Kahn (1993). "Rad: a member of the Ras family overexpressed in muscle of type II diabetic humans." Science **262**(5138): 1441-4.

Richter, M., L. Schleithoff, et al. (1997). "Functional characterization of a distinct ryanodine receptor mutation in human malignant hyperthermia-susceptible muscle." J Biol Chem **272**(8): 5256-60.

Rios, E. and G. Brum (1987). "Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle." Nature **325**(6106): 717-20.

Robinson, F. L. and J. E. Dixon (2005). "The phosphoinositide-3-phosphatase MTMR2 associates with MTMR13, a membrane-associated pseudophosphatase also mutated in type 4B Charcot-Marie-Tooth disease." J Biol Chem **280**(36): 31699-707.

Roggo, L., V. Bernard, et al. (2002). "Membrane transport in Caenorhabditis elegans: an essential role for VPS34 at the nuclear membrane." Embo J **21**(7): 1673-83.

Rohacs, T., C. M. Lopes, et al. (2005). "PI(4,5)P2 regulates the activation and desensitization of TRPM8 channels through the TRP domain." Nat Neurosci **8**(5): 626-34.

Rosado, J. A. and S. O. Sage (2000). "Regulation of plasma membrane Ca2+-ATPase by small GTPases and phosphoinositides in human platelets." J Biol Chem **275**(26): 19529-35.

Rosemblatt, M., C. Hidalgo, et al. (1981). "Immunological and biochemical properties of transverse tubule membranes isolated from rabbit skeletal muscle." J Biol Chem **256**(15): 8140-8.

Sacchetto, R., P. Volpe, et al. (1993). "Postnatal development of rabbit fast-twitch skeletal muscle: accumulation, isoform transition and fibre distribution of calsequestrin." J Muscle Res Cell Motil **14**(6): 646-53.

Sacheck, J. M., A. Ohtsuka, et al. (2004). "IGF-I stimulates muscle growth by suppressing protein breakdown and expression of atrophy-related ubiquitin ligases, atrogin-1 and MuRF1." Am J Physiol Endocrinol Metab **287**(4): E591-601.

Sandri, M., C. Sandri, et al. (2004). "Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy." Cell **117**(3): 399-412.

Sanoudou, D., J. N. Haslett, et al. (2003). "Expression profiling reveals altered satellite cell numbers and glycolytic enzyme transcription in nemaline myopathy muscle." Proc Natl Acad Sci U S A **100**(8): 4666-71.

Schaletzky, J., S. K. Dove, et al. (2003). "Phosphatidylinositol-5-phosphate activation and conserved substrate specificity of the myotubularin phosphatidylinositol 3-phosphatases." Curr Biol **13**(6): 504-9.

Schessl, J., L. Medne, et al. (2007). "MRI in DNM2-related centronuclear myopathy: evidence for highly selective muscle involvement." Neuromuscul Disord **17**(1): 28-32.

Schredelseker, J., A. Dayal, et al. (2009). "Proper restoration of excitation-contraction coupling in the dihydropyridine receptor beta1-null zebrafish relaxed is an exclusive function of the beta1a subunit." J Biol Chem **284**(2): 1242-51.

Schredelseker, J., V. Di Biase, et al. (2005). "The beta 1a subunit is essential for the assembly of dihydropyridine-receptor arrays in skeletal muscle." Proc Natl Acad Sci U S A **102**(47): 17219-24.

Scott, B. T., H. K. Simmerman, et al. (1988). "Complete amino acid sequence of canine cardiac calsequestrin deduced by cDNA cloning." J Biol Chem **263**(18): 8958-64.

Senderek, J., C. Bergmann, et al. (2003). "Mutation of the SBF2 gene, encoding a novel member of the myotubularin family, in Charcot-Marie-Tooth neuropathy type 4B2/11p15." Hum Mol Genet **12**(3): 349-56.

Sewry, C. A., C. Jimenez-Mallebrera, et al. (2008). "Congenital myopathies." Curr Opin Neurol **21**(5): 569-75.

Shen, J., W. M. Yu, et al. (2009). "Deficiency of MIP/MTMR14 phosphatase induces a muscle disorder by disrupting Ca(2+) homeostasis." Nat Cell Biol **11**(6): 769-76.

Sheridan, D. C., H. Takekura, et al. (2006). "Bidirectional signaling between calcium channels of skeletal muscle requires multiple direct and indirect interactions." Proc Natl Acad Sci U S A **103**(52): 19760-5.

Shou, W., B. Aghdasi, et al. (1998). "Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12." Nature **391**(6666): 489-92.

Shupliakov, O., P. Low, et al. (1997). "Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions." Science **276**(5310): 259-63.

Sipos, I., K. Jurkat-Rott, et al. (1995). "Skeletal muscle DHP receptor mutations alter calcium currents in human hypokalaemic periodic paralysis myotubes." J Physiol **483 (Pt 2)**: 299-306.

Slepnev, V. I., G. C. Ochoa, et al. (1998). "Role of phosphorylation in regulation of the assembly of endocytic coat complexes." Science **281**(5378): 821-4.

Smart, E. J., G. A. Graf, et al. (1999). "Caveolins, liquid-ordered domains, and signal transduction." Mol Cell Biol **19**(11): 7289-304.

Somsel Rodman, J. and A. Wandinger-Ness (2000). "Rab GTPases coordinate endocytosis." J Cell Sci **113 Pt 2**: 183-92.

Sorrentino, V. and P. Volpe (1993). "Ryanodine receptors: how many, where and why?" Trends Pharmacol Sci **14**(3): 98-103.

Spiro, A. J., G. M. Shy, et al. (1966). "Myotubular myopathy. Persistence of fetal muscle in an adolescent boy." Arch Neurol **14**(1): 1-14.

Srivastava, S., Z. Li, et al. (2005). "The phosphatidylinositol 3-phosphate phosphatase myotubularin- related protein 6 (MTMR6) is a negative regulator of the Ca2+-activated K+ channel KCa3.1." Mol Cell Biol **25**(9): 3630-8.

Sweitzer, S. M. and J. E. Hinshaw (1998). "Dynamin undergoes a GTP-dependent conformational change causing vesiculation." Cell **93**(6): 1021-9.

Takahashi, M., M. J. Seagar, et al. (1987). "Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle." Proc Natl Acad Sci U S A **84**(15): 5478-82.

Takegawa, K., D. B. DeWald, et al. (1995). "Schizosaccharomyces pombe Vps34p, a phosphatidylinositol-specific PI 3-kinase essential for normal cell growth and vacuole morphology." J Cell Sci **108 (Pt 12)**: 3745-56.

Takei, K., V. I. Slepnev, et al. (1999). "Functional partnership between amphiphysin and dynamin in clathrin-mediated endocytosis." Nat Cell Biol **1**(1): 33-9.

Takekura, H., B. E. Flucher, et al. (2001). "Sequential docking, molecular differentiation, and positioning of T-Tubule/SR junctions in developing mouse skeletal muscle." Dev Biol **239**(2): 204-14.

Takeshima, H., S. Komazaki, et al. (1998). "Embryonic lethality and abnormal cardiac myocytes in mice lacking ryanodine receptor type 2." Embo J **17**(12): 3309-16.

Takeshima, H., S. Komazaki, et al. (2000). "Junctophilins: a novel family of junctional membrane complex proteins." Mol Cell **6**(1): 11-22.

Takeshima, H., T. Yamazawa, et al. (1995). "Ca(2+)-induced Ca2+ release in myocytes from dyspedic mice lacking the type-1 ryanodine receptor." Embo J **14**(13): 2999-3006.

Tanabe, T., K. G. Beam, et al. (1990). "Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling." Nature **346**(6284): 567-9.

Tanabe, T., H. Takeshima, et al. (1987). "Primary structure of the receptor for calcium channel blockers from skeletal muscle." Nature **328**(6128): 313-8.

Thompson, H. M., H. Cao, et al. (2004). "Dynamin 2 binds gamma-tubulin and participates in centrosome cohesion." Nat Cell Biol **6**(4): 335-42.

Torrente, Y., M. Belicchi, et al. (2007). "Autologous transplantation of muscle-derived CD133+ stem cells in Duchenne muscle patients." Cell Transplant **16**(6): 563-77.

Tosch, V., H. M. Rohde, et al. (2006). "A novel PtdIns3P and PtdIns(3,5)P2 phosphatase with an inactivating variant in centronuclear myopathy." Hum Mol Genet **15**(21): 3098-106.

Tripathy, A., L. Xu, et al. (1995). "Calmodulin activation and inhibition of skeletal muscle Ca2+ release channel (ryanodine receptor)." Biophys J **69**(1): 106-19.

Tsujita, K., T. Itoh, et al. (2004). "Myotubularin regulates the function of the late endosome through the gram domain-phosphatidylinositol 3,5-bisphosphate interaction." J Biol Chem **279**(14): 13817-24.

Tunwell, R. E., C. Wickenden, et al. (1996). "The human cardiac muscle ryanodine receptor-calcium release channel: identification, primary structure and topological analysis." Biochem J **318 (Pt 2)**: 477-87.

Tupling, A. R., M. Asahi, et al. (2002). "Sarcolipin overexpression in rat slow twitch muscle inhibits sarcoplasmic reticulum Ca2+ uptake and impairs contractile function." J Biol Chem **277**(47): 44740-6.

Ursu, D., S. Sebille, et al. (2001). "Excitation-contraction coupling in skeletal muscle of a mouse lacking the dihydropyridine receptor subunit gamma1." J Physiol **533**(Pt 2): 367-77.

Vaessen, S. F., R. J. Veldman, et al. (2009). "AAV gene therapy as a means to increase apolipoprotein (Apo) A-I and high-density lipoprotein-cholesterol levels: correction of murine ApoA-I deficiency." J Gene Med **11**(8): 697-707.

van Wijngaarden, G. K., P. Fleury, et al. (1969). "Familial "myotubular" myopathy." Neurology **19**(9): 901-8.

Vangheluwe, P., M. Schuermans, et al. (2005). "Sarcolipin and phospholamban mRNA and protein expression in cardiac and skeletal muscle of different species." Biochem J **389**(Pt 1): 151-9.

Varsanyi, M., H. G. Tolle, et al. (1983). "Activation of sarcoplasmic reticular Ca2+ transport ATPase by phosphorylation of an associated phosphatidylinositol." Embo J **2**(9): 1543-8.

Vergne, I., E. Roberts, et al. (2009). "Control of autophagy initiation by phosphoinositide 3-phosphatase jumpy." Embo J **28**(15): 2244-2258.

Vicinanza, M., G. D'Angelo, et al. (2008). "Function and dysfunction of the PI system in membrane trafficking." Embo J **27**(19): 2457-70.

Wagenknecht, T., R. Grassucci, et al. (1989). "Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum." Nature **338**(6211): 167-70.

Wagenknecht, T., M. Radermacher, et al. (1997). "Locations of calmodulin and FK506-binding protein on the three-dimensional architecture of the skeletal muscle ryanodine receptor." J Biol Chem **272**(51): 32463-71.

Wallgren-Pettersson, C. (2000). "72nd ENMC International Workshop: myotubular myopathy 1-3 October 1999, Hilversum, The Netherlands." Neuromuscul Disord **10**(7): 525-9.

Wallgren-Pettersson, C., A. Clarke, et al. (1995). "The myotubular myopathies: differential diagnosis of the X linked recessive, autosomal dominant, and autosomal recessive forms and present state of DNA studies." J Med Genet **32**(9): 673-9.

Wang, X., C. Blagden, et al. (2005). "Runx1 prevents wasting, myofibrillar disorganization, and autophagy of skeletal muscle." Genes Dev **19**(14): 1715-22.

Wang, X. and C. G. Proud (2006). "The mTOR pathway in the control of protein synthesis." Physiology (Bethesda) **21**: 362-9.

Warnock, D. E., J. E. Hinshaw, et al. (1996). "Dynamin self-assembly stimulates its GTPase activity." J Biol Chem **271**(37): 22310-4.

Weaver, A. M., A. V. Karginov, et al. (2001). "Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation." Curr Biol **11**(5): 370-4.

Wechsler-Reya, R. J., K. J. Elliott, et al. (1998). "A role for the putative tumor suppressor Bin1 in muscle cell differentiation." Mol Cell Biol **18**(1): 566-75.

Weed, S. A., A. V. Karginov, et al. (2000). "Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex." J Cell Biol **151**(1): 29-40.

Wei, L., E. M. Gallant, et al. (2009). "Junctin and triadin each activate skeletal ryanodine receptors but junctin alone mediates functional interactions with calsequestrin." Int J Biochem Cell Biol.

Weiss, R. G., K. M. O'Connell, et al. (2004). "Functional analysis of the R1086H malignant hyperthermia mutation in the DHPR reveals an unexpected influence of the III-IV loop on skeletal muscle EC coupling." Am J Physiol Cell Physiol **287**(4): C1094-102.

Welch, E. M., E. R. Barton, et al. (2007). "PTC124 targets genetic disorders caused by nonsense mutations." Nature **447**(7140): 87-91.

Wildey, G. M. and P. H. Howe (2009). "Runx1 is a co-activator with FOXO3 to mediate transforming growth factor beta (TGFbeta)-induced Bim transcription in hepatic cells." J Biol Chem **284**(30): 20227-39.

Wissenbach, U., E. Bosse-Doenecke, et al. (1998). "The structure of the murine calcium channel gamma-subunit gene and protein." Biol Chem **379**(1): 45-50.

Witke, W., A. V. Podtelejnikov, et al. (1998). "In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly." Embo J **17**(4): 967-76.

Witt, C. C., C. Burkart, et al. (2006). "Nebulin regulates thin filament length, contractility, and Z-disk structure in vivo." Embo J **25**(16): 3843-55.

Wu, X., J. Wang, et al. (2007). "The effect of insulin on expression of genes and biochemical pathways in human skeletal muscle." Endocrine **31**(1): 5-17.

Xue, Y., H. Fares, et al. (2003). "Genetic analysis of the myotubularin family of phosphatases in Caenorhabditis elegans." J Biol Chem **278**(36): 34380-6.

Yamamoto, M., Y. Toya, et al. (1998). "Caveolin is an activator of insulin receptor signaling." J Biol Chem **273**(41): 26962-8.

Yeates, T. O. (2002). "Structures of SET domain proteins: protein lysine methyltransferases make their mark." Cell **111**(1): 5-7.

Yin, H. L. and P. A. Janmey (2003). "Phosphoinositide regulation of the actin cytoskeleton." Annu Rev Physiol **65**: 761-89.

Yuan, S. H., W. Arnold, et al. (1991). "Biogenesis of transverse tubules and triads: immunolocalization of the 1,4-dihydropyridine receptor, TS28, and the ryanodine receptor in rabbit skeletal muscle developing in situ." J Cell Biol **112**(2): 289-301.

Zanoteli, E., A. S. Guimaraes, et al. (2000). "Temporomandibular joint involvement in a patient with centronuclear myopathy." Oral Surg Oral Med Oral Pathol Oral Radiol Endod **90**(1): 118-21.

Zanoteli, E., J. Laporte, et al. (2005). "Deletion of both MTM1 and MTMR1 genes in a boy with myotubular myopathy." Am J Med Genet A **134**(3): 338-40.

Zanoteli, E., A. S. Oliveira, et al. (1998). "Centronuclear myopathy: clinical aspects of ten Brazilian patients with childhood onset." J Neurol Sci **158**(1): 76-82.

Zanoteli, E., N. Vergani, et al. (2009). "Mitochondrial alterations in dynamin 2-related centronuclear myopathy." Arq Neuropsiquiatr **67**(1): 102-4.

Zarain-Herzberg, A., D. H. MacLennan, et al. (1990). "Characterization of rabbit cardiac sarco(endo)plasmic reticulum Ca2(+)-ATPase gene." J Biol Chem **265**(8): 4670-7.

Zhang, C., H. Y. Feng, et al. (2005). "[Therapy of Duchenne muscular dystrophy with umbilical cord blood stem cell transplantation]." Zhonghua Yi Xue Yi Chuan Xue Za Zhi **22**(4): 399-405.

Zhang, L., J. Kelley, et al. (1997). "Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane." J Biol Chem **272**(37): 23389-97.

Zhang, P. and J. E. Hinshaw (2001). "Three-dimensional reconstruction of dynamin in the constricted state." Nat Cell Biol **3**(10): 922-6.

Zhang, Y., H. S. Chen, et al. (1993). "A mutation in the human ryanodine receptor gene associated with central core disease." Nat Genet **5**(1): 46-50.

Zhu, J., P. J. Bilan, et al. (1996). "Rad, a novel Ras-related GTPase, interacts with skeletal muscle beta-tropomyosin." J Biol Chem **271**(2): 768-73.

Zhu, X., J. E. Yeadon, et al. (1994). "AML1 is expressed in skeletal muscle and is regulated by innervation." Mol Cell Biol **14**(12): 8051-7.

Zimmermann, P. (2006). "The prevalence and significance of PDZ domain-phosphoinositide interactions." Biochim Biophys Acta **1761**(8): 947-56.

Zorzato, F., J. Fujii, et al. (1990). "Molecular cloning of cDNA encoding human and rabbit forms of the Ca2+ release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum." J Biol Chem **265**(4): 2244-56.

Zou, W., Q. Lu, et al. (2009). "Caenorhabditis elegans myotubularin MTM-1 negatively regulates the engulfment of apoptotic cells." PLoS Genet **5**(10): e1000679.

Zuchner, S., M. Noureddine, et al. (2005). "Mutations in the pleckstrin homology domain of dynamin 2 cause dominant intermediate Charcot-Marie-Tooth disease." Nat Genet **37**(3): 289-94.