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Altered properties of the CASQ1^{D244G} protein identified in patients with a Vacuolar Myopathy

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I. ABSTRACT

Calsequestrin 1 (CASQ1) is the main Ca^{2+} binding protein of the sarcoplasmic reticulum (SR) of skeletal muscle fibers. We identified in patients with a vacuolar myopathy characterized by distinctive inclusions in muscle fibers a mutation in the CASQ1 gene, which results in a substitution of a conserved aspartic acid in position 244 to a glycine (D244G). This mutation represents the first mutation in CASQ1 linked to an inherited human muscle disease. The affected aspartic acid is located in a high-affinity Ca^{2+} binding site, close to a region of the protein proposed to be relevant for interactions between adjacent monomers. Analysis of the predicted three-dimensional structure of the mutated CASQ1 revealed a conformational change in the affected Ca^{2+} binding site. Analysis of muscle biopsies from patients revealed, beside the presence of inclusions composed of SR proteins aggregates, altered Ca^{2+} release kinetics. Expression of the mutated protein in COS7 cells showed a reduced ability of CASQ1 in forming rod-shape polymers, whereas in cultured myotubes induced the formation of protein aggregates positive for CASQ1. *In-vivo* electroporation of mouse muscle fibers of the mutated CASQ1 resulted in formation of inclusions resembling those observed in biopsies from patients. In addition, transfection of COS7 cells with the mutated CASQ1 revealed a reduction of Ca^{2+} binding ability when compared with cells transfected with the wild type CASQ1 protein. Altogether, these data describe molecular and functional abnormalities of the mutated protein, which support the view that this mutation is responsible for the clinical phenotype observed in patients.

II. INTRODUCTION

i. Muscle tissue

Muscle tissue in the human body can be classified on the basis of the light microscopy appearance in two main sub-groups, striated and smooth muscle tissue.

Striated muscle tissue is characterized by the presence of a regular alternance of pale and dark bands along the main axis of fibers (Figure 1, left and center).

Two types of striated muscle tissue exist: skeletal and cardiac. The skeletal muscle tissue is voluntary, hence regulated by nerve pulses from the central nervous system, i.e. action potentials. The cardiac muscle tissue contracts beyond any voluntary stimulus, thanks to the presence of pacemaker cells, and is regulated by the autonomous nervous system which regulates frequency and contractility of the heart.

The smooth muscle tissue is characterized by the absence of striations and is innervated by the vegetative nervous system, so it contracts independently from voluntary control (Figure 1, right) (*Lowe and Anderson, 2015*).

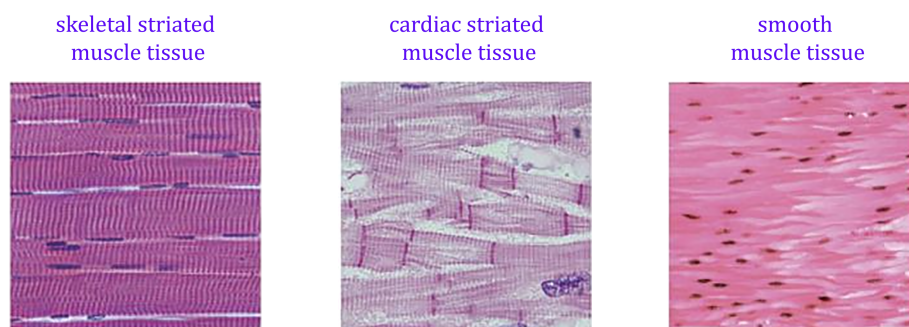


Figure 1_ Light microscopy images of different types of muscle tissue (Hematoxylin/Eosin staining), adapted from Mc Graw Hill Higher Education.

i.1 Skeletal muscle: general organization

Skeletal muscle tissue is composed of multinucleated, post-mitotic cells formed from the fusion of mononucleated myocytes during embryogenesis. The resulting syncytium at the end of differentiation, called myofiber or muscle fiber, is characterized by the presence of longitudinal cylindrical elements known as myofibrils; at the periphery of the fiber, external to the sarcolemma, are present quiescent myoblasts that confer a moderate regenerative ability to the muscle fiber, named satellite cells (Figure 2). The sarcolemma of each muscle fiber is enveloped by a layer of connective tissue, the endomysium. Group of fibers within a muscle are arranged in bundles and surrounded by another layer of connective tissue called perimysium. All these bundles associate in a muscle surrounded by a third layer of connective tissue known as epimysium, with tendons at its extremities (Figure 3) (Lowe and Anderson, 2015).

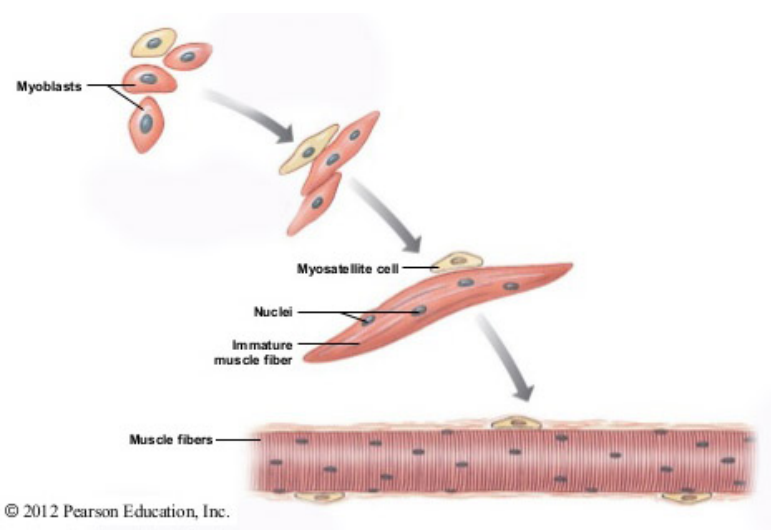


Figure 2_ Myofiber formation.

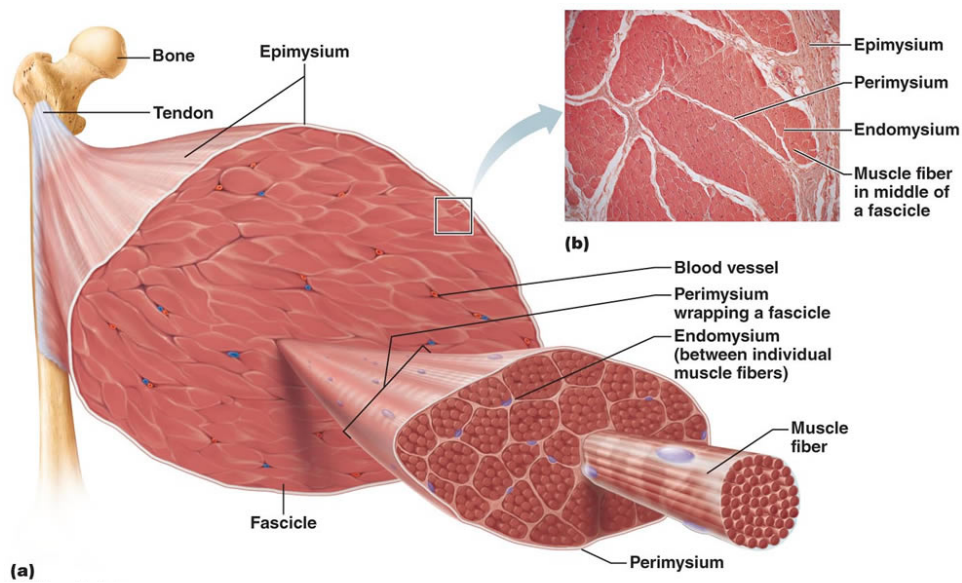


Figure 3_ Organization of a skeletal muscle attached to bone by tendons (a), histological appearance of a skeletal muscle transversal section (b). (copyright © 2013, Pearson Education inc.).

In humans, skeletal muscle usually comprises about half of total body weight and contains approximately 70 % of the whole body proteins (Frontera and Ochala, 2015). The striations of this tissue are due to the longitudinal alignment of thousands of repeated highly ordered contractile units, the sarcomeres, which form the myofibrils.

i.2 Sarcomeres

The sarcomeres are among the most regular proteic structures in animal cells, and are the result of the ordered arrangement of numerous thick and thin myofilaments in the muscle fiber sarcoplasm.

The thin filaments (which are about half the diameter of the thick filaments) are mainly composed of globular(G)-actin that polymerizes to form filamentous(F)-

actin, which in turn organizes itself in double helix structure. In every groove of the double helix, the actin filaments are associated in a head-to-tail manner with two polymers of the protein tropomyosin, a linear dimer constituted of two α -helical chains arranged in a coiled-coil structure (*Wegner, 1979*). A primary function of tropomyosin is to prevent the association of the myosin heads with the actin filament in the resting muscle (*Lehman et al., 2000*).

Tropomyosin binding to actin also has a function in stabilizing the thin filament, increasing its stiffness and hindering its fragmentation (*Wegner, 1982*).

Associated to the thin filament there is also troponin. Troponin is a complex protein constituted of three subunits called I, C and T (*Hitchcock and Lutter, 1975; Westfall and Metzger, 2001*). The I subunit (inhibitory of tropomyosin), is bound to actin (*Lehman et al., 2001*), the T subunit is associated with tropomyosin and anchors the other two subunits in the thin filament (*Jin and Chong, 2010*), while the C subunit is a Ca^{2+} binding protein (*Herzberg and James, 1988*). The binding of Ca^{2+} ions to the C subunit induces a conformational change in the Troponin, which in turn increases the distance between the subunits T and I. This conformational change moves the tropomyosin (associated with troponin T) exposing the actin myosin-binding sites (*Weber and Murray, 1973; Parry and Squire, 1973; Craig and Lehman 2001*).

Each sarcomere contains two sets of thin filaments at its extremities. One end of each thin filament is anchored to a network of interconnecting proteins (α -actinin is the most abundant protein present in this structure) called Z line or Z disk (from the German word *Zwischenscheibe*, middle disk). Two successive Z lines define the limits of one sarcomere, thus thin filaments from two adjacent sarcomeres are anchored to the two sides of each Z line (Figure 4 and Figure 5).

A light band, known as I band because is isotropic at polarized light, is dissected by the Z line. This band lies between the ends of the A bands of two adjacent sarcomeres and contains those portions of the thin filaments that do not overlap the thick filaments (*Adamo et al., 2012*). The assembly of actin filaments and the maintenance of their uniform length require a fine regulation of actin dynamics at each end of the filament, blocking the filament elongation and shortening. The giant protein nebulin (600-900 kDa) regulates the thin filament assembly and it is probably the molecular ruler for specifying the precise length of the filament (about 1 μ m) (*Kruger et al., 1991; McElhinny et al., 2003*). The C-terminal tail of this protein is partially inserted into the Z disk, while its N-terminal domain extends to the ends of the thin filaments (*Millevoi et al., 1998; McElhini et al., 2001*).

Nebulin has also shown the ability in regulating the actin-myosin interactions by inhibiting ATPase activity in a Ca²⁺ sensitive manner (*Root and Wang, 1994*).

The protein CapZ localizes to the Z line where it binds α -actinin, probably forming anchoring complex for the thin filaments and where is involved in nucleation and stabilization of the thin filaments by capping their ends (*Casella et al., 1987; Papa et al., 1999*).

CapZ is a heterodimer composed of the subunits α (36 kDa) and β (32 kDa), both of which are necessary for capping (*Hug et al. 1992; Casella and Torres, 1994*).

Another capping protein of about 40 kDa, named tropomodulin (Tmod), is responsible for thin filaments stabilization, principally by blocking the spontaneous dissociation of bound actin monomers (*Yamashiro et al., 2012*).

Tmod binds both actin with its C-terminal tail and tropomyosin with the N-

terminal region (*Babcock and Fowler, 1994; Fowler and Conley, 1999*); also an interaction with the protein nebulin has been reported (*McElhini et al., 2001*).

The thick filaments are composed almost entirely of the contractile protein myosin, which is composed of two large polypeptide heavy chains that possess an ATPase-mediated motor function, and four smaller light chains; these polypeptides combine to form a molecule consisting in a long tail with two globular heads that can bind to specific sites on the thin filament (*Rosati et al., 2006*).

After an increase of cytosolic Ca^{2+} concentration, the troponin subunit C on the thin filament binds the ion. This initiates the series of molecular events that displace the tropomyosin blocking the myosin binding sites on the actin filament and making them “active”. The space between overlapping thick and thin filaments is bridged by projections known as cross-bridges. These are portions of myosin tail and the globular heads that extend from the surface of the thick filaments toward the thin filaments. During muscle contraction, the cross-bridges make contact with the “active” sites of the actin filament and exert force on them thanks to ATP. The term contraction does not necessarily mean shortening, but refers to “activation” of the cross-bridges. When the force generation produces shortening, the overlapping thin and thick filaments move past each other, propelled by movements of the cross-bridges. This is known as the sliding-filament mechanism of muscle contraction (*Krans, 2010*) (Figure 4).

In the thick filaments two additional proteins that bind myosin are present: myosin binding protein C and H (MyBP-C, MyBP-H), respectively of about 140 kDa and 58 kDa (*Craig and Offer, 1976*). MyBP-C is critical for the correct myosin polymerization, while MyBP-H is necessary for the alignment of the thick

filaments; moreover both myosin-binding proteins are crucial for maintaining the precise length of the thick filaments (1,6 μm) (*Koretz 1979; Davis 1988*).

Thick filaments are located in the middle of each sarcomere producing a dark band known as the A band, because is anisotropic at polarized light (Figure 5).

Two additional bands are present in the A band region: the H zone is a narrow, light band in the center of the A band. It corresponds to the space between the opposing ends of the two sets of thin filaments in each sarcomere.

A narrow, dark band in the center of the H zone is named M line (from the German word Mittellinie, central line) (Figure 5) and corresponds to proteins (see below) that link together the central region of adjacent thick filaments (*Luther and Squire, 1978*). Filaments composed of the elastic protein titin extend from the Z line to the M line and are linked to both the M line proteins and the thick filaments (*Labeit and Kolmerer, 1995*). The canonical human titin protein consists of 34,350 amino acids and is the largest human protein known to date (about 3,7 MDa) (*Maruyama et al., 1977; Wang et al., 1979*). Titin is a key contributor to sarcomere integrity and stabilization (*Fukuda et al., 2008*). Titin is responsible for the passive elasticity of skeletal muscle; in the I band it functions as a molecular spring maintaining the overlap of the thin and thick filaments when myofibrils are stretched or shortened (*Krüger and Kötter, 2016*). This giant protein also serves as an adhesion template for the assembly of a functional contractile complex in muscle cells (*Kontogianni-Konstantopoulos et al., 2009*).

Myomesin is a 185 kDa protein located in the M line zone, where it arranges in an antiparallel staggered fashion (*Obermann et al. 1996*). This protein binds myosin and titin and it might be involved in the integration of thick filaments into assembling sarcomeres (*Ehler et al., 1999*).

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In the M line, associated to myomesin, a protein of 165 kDa is also present: the so-called myosin-binding protein or M-protein. M-protein is restricted to the M lines of fast fibers and its expression is developmentally regulated (Grove *et al.*, 1984; Noguchi *et al.*, 1992), where it likely anchors thick filament components to titin acting in conjunction with MyBP-C and myomesin (Nave *et al.* 1989; Clark *et al.* 2002).

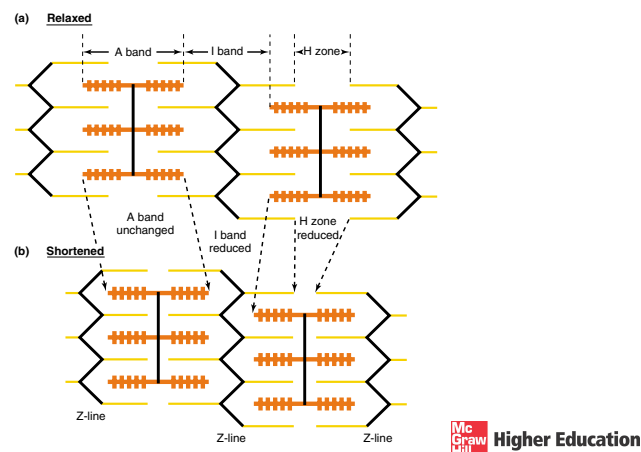


Figure 4_ The sliding of thick filaments overlapping thin filaments produces sarcomere shortening with no change in thick or thin filament length. The I band and H zone are reduced.

i.3 Sarcoplasmic reticulum

The endoplasmic reticulum (ER) of the skeletal muscle fiber is named sarcoplasmic reticulum (SR) and appears at the electron microscope as a network of tubules that forms a series of sleeve-like segments around each myofibril. This membrane network is well organized and precisely located in association with the sarcomeres (Porter and Palade, 1957)(Figure 5).

SR can be divided into two main domains, the so-called longitudinal SR and the junctional SR. Longitudinal SR is present in correspondance of A band, but also at

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I band where is more convoluted and formed by multiple layers. In the longitudinal SR at the A band, the tubules are interconnected with each other and disposed longitudinally with respect to the fiber central axis. In correspondence to the H band the interconnections increase, forming a peculiar structure named fenestrated region. At their ends (restricted to the level of the junctions between the A and the I bands), the longitudinal tubules coalesce into single dilated sacs called terminal cisternae. This portion of the SR is juxtaposed (but separated by a space of about 11-16 nm) with invaginations of the sarcolemma known as transverse tubules (T tubule), and is defined as junctional SR. Two terminal cisternae positioned on the opposite sides of one T tubule form a structure called triad (Franzini-Armstrong, 2004) (Figure 5).

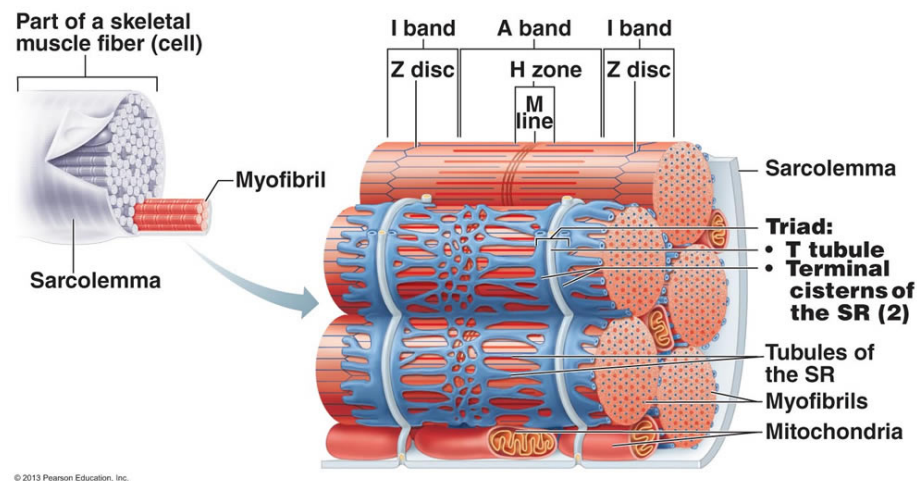


Figure 5_ Schematic representation of SR and myofilaments in a mammalian skeletal muscle fiber.

At triads, two integral membrane proteins are essential for EC-coupling (see chapter II. ii.). The L-type Ca^{2+} channel known as the dihydropyridine receptor (DHPR) (so named because of its affinity for the dihydropyridines drugs), whose

main role is to act as a voltage sensor on T-tubules and the ryanodine receptor 1 (RYR1) (so called because of its affinity for the plant alkaloid ryanodine), which is the skeletal muscle Ca^{2+} release channel located in the SR terminal cisternae (*Protasi et al., 1997*).

The SR starts to organize in close co-ordination with the contractile filaments very early during muscle fiber differentiation (*Takekura et al., 2001*). The specific molecules responsible for formation and maintenance of the triad are still to be defined. The two proteins, RYR1 and DHPR, do not contribute to junction formation, because triads are still formed in mice lacking both of them (*Felder et al., 2002*). However, interesting results about triad formation come from studies on members of the junctophilin protein family, JP1 and JP2. Junctophilins are anchored to the junctional SR by a trans-membrane domain in their C-terminal region and contact the plasma membrane through a number of MORN (Membrane Occupation and Recognition Nexus) motifs in their N-terminus. Junctophilins may thus provide a molecular bridge between the junctional SR and the T-tubule membranes at triads. Accordingly, mice knockout for JP1 and JP2 show altered triad formation (*Takeshima et al., 2000*).

Similarly, mice knocked out for the SR trans-membrane protein triadin (see chapter II. iii.) lost the transverse orientation of triads, suggesting that this protein might be important in the arrangement of triads within the fiber (*Shen et al., 2007*).

While the overall molecular complexes responsible for the interactions between SR and sarcomere are still to be revealed, two elements have been recognized in the muscle-specific protein ankirin 1.5 (ank1.5), and in obscurin, a large protein known to be associated with the myofibrils. Ank1.5 is a small protein (155 amino

acid), anchored to the SR membrane thanks to a trans-membrane domain followed by a short cytosolic tail (*Hopitzan et al., 2006*). In adult skeletal muscle, ank1.5 is located in the Z disk and the M band. Obscurin, is a muscle protein of about 800 kDa, at first identified for its interaction with titin (*Kontrogianni-Kostantinopoulos et al., 2003*).

It has been shown that the cytosolic tail of ank1.5 interacts with the C-terminal region of obscurin, thus contributing to form the molecular connection between the SR and the myofibrillar apparatus (*Bagnato et al., 2003; Armani et al., 2006*).

The importance of the interaction between these two proteins in maintaining the correct organization of the SR around the myofibrils during contraction has been further supported by studies on ank1.5 knockout mice. These mice showed a reduced contractile performance and a decreased resistance to fatigue, suggesting that ank1.5 may contribute to the regulation of muscle contraction (*Giacomello et al., 2015*). The special disposition of the SR relative to myofibrils is essential for its function, which is to control the level of Ca^{2+} in the sarcoplasm with respect to regulation of muscle contraction.

The longitudinal SR is responsible for Ca^{2+} uptake, thanks to the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) that pumps Ca^{2+} ions from the cytosol back into the lumen of the reticulum (*Apell, 2003*). The junctional SR is responsible for Ca^{2+} release into the sarcoplasm, in a mechanism known as excitation-contraction coupling that involves RYR1 and DHPR (*Franzini-Armstrong, 1999*).

ii. Excitation – contraction coupling

In a resting skeletal muscle fiber, the concentration of free Ca^{2+} in the cytosol surrounding the thick and thin filaments is very low, about 0,01-0,1 μM . At this low Ca^{2+} concentration, very few of the Ca^{2+} -binding sites on troponin are occupied, and thus cross-bridge activity is blocked by tropomyosin (*Szent-Györgyi, 1975*).

When an action potential (AP) from the nervous system spreads down the T-tubule, membrane depolarization causes voltage-driven conformational changes in the DHPR that trigger activation and opening of nearby associated RYR1 channels located in the terminal cisterna of the SR (*Zalk et al., 2007*).

Following this coupling between DHPR and RYR1, the opening of the channel causes a rapid increase in cytosolic Ca^{2+} concentration (about 1 μM or more), and Ca^{2+} binds to troponin, removing the blocking effect of tropomyosin and allowing cross-bridge cycling. The source of the increased cytosolic calcium ions concentration is the SR (Figure 6).

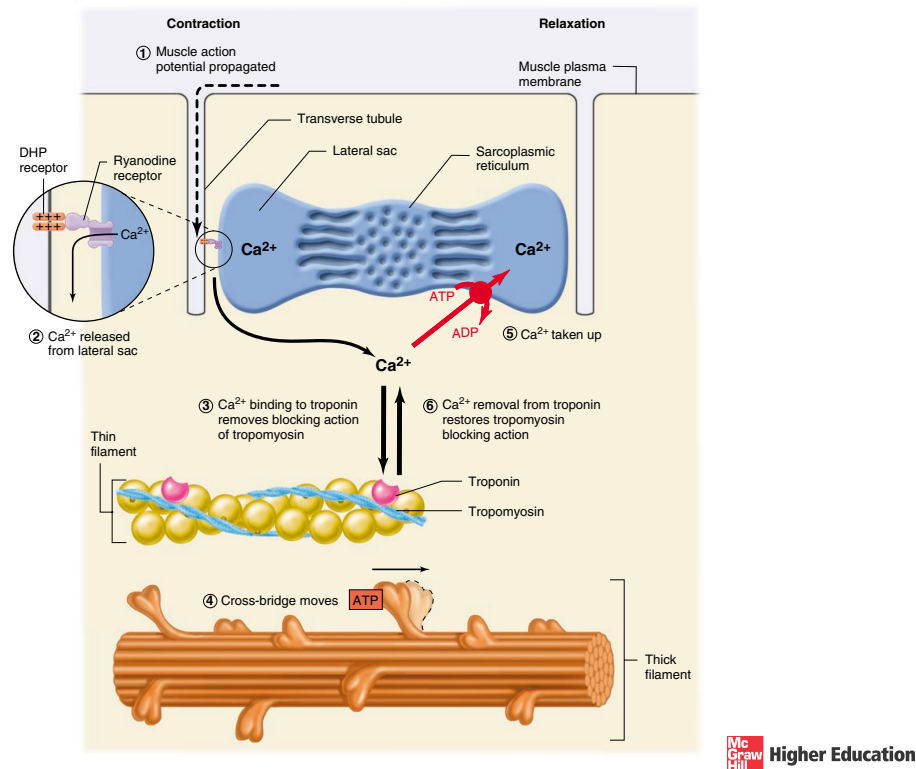


Figure 6_ Release and uptake of Ca²⁺ by the sarcoplasmic reticulum during contraction and relaxation of a skeletal muscle fiber.

This series of events that effectively translates the AP to the Ca²⁺ release from the SR that leads to contraction is named Excitation-Contraction (EC) Coupling.

After each cycle of contraction, Ca²⁺ is re-uptaken and stored again in the SR thanks to SERCA pumps, restoring low cytosolic levels of Ca²⁺ and allowing muscle relaxation (Figure 6) (Rossi and Dirksen, 2006; Rebbeck et al., 2014).

The SERCA pump is a single polypeptide of 110 kDa that utilizes the energy derived from ATP hydrolysis to transport Ca²⁺ across the SR membrane: two Ca²⁺ ions are transported for each molecule of ATP hydrolyzed (Periasamy and Kalyanasundaram, 2007). It is known that SERCA pumps activity is regulated in a tissue-specific manner by two small proteins, phospholamban and sarcolipin (Leberer et al., 1989; Asahi et al., 2003). The maintenance of the adequate

cytosolic Ca^{2+} concentration is crucial for sustained contractility of skeletal muscle fibers.

An AP, through stimulation of the DHPR, initiates full activation of RYR1 for constant, maximal Ca^{2+} release. However, stimuli subsequent to the initial AP release less Ca^{2+} than the first AP due to a complex mechanism of Ca^{2+} inactivation of Ca^{2+} release. Release is reduced or terminated before stores are fully depleted to keep constant the amount of Ca^{2+} released (*Posterino and Lamb, 2003*).

iii. Triadic proteins

Although the core components of the EC-Coupling machinery are DHPR and RYR1, a number of ancillary proteins have been shown to be essential for the structural formation of a multiprotein complex called Ca^{2+} release unit (CRU) (*Canato et al., 2015*) and for its regulation (Figure 7).

In this chapter the major components of the CRU and some other important triadic proteins will be described.

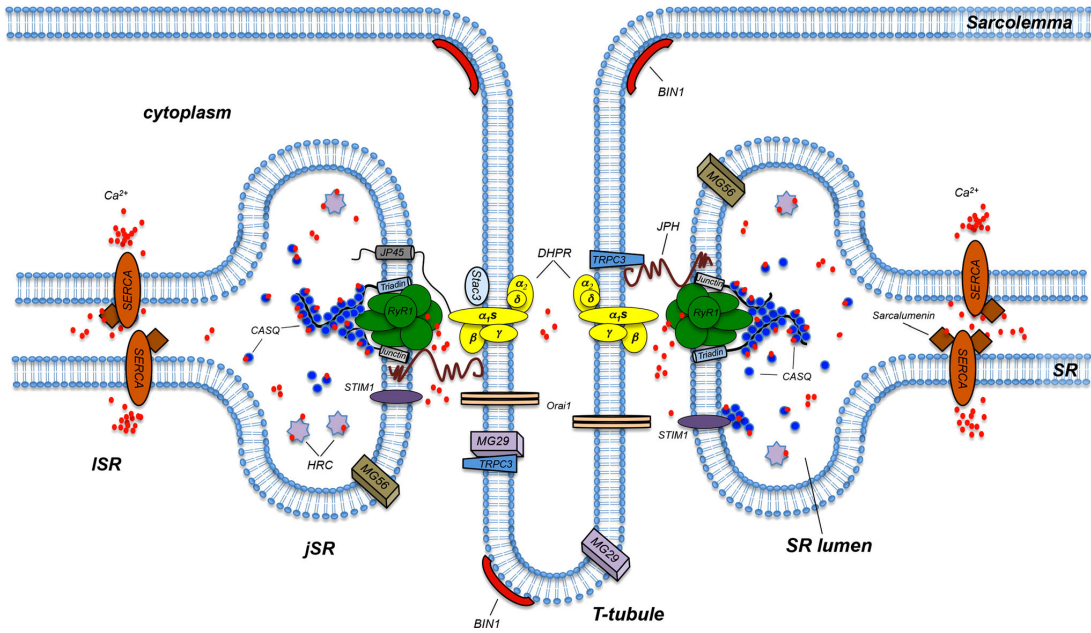


Figure 7_ Schematic model of the proteins organization in the triad of the skeletal muscle cell, from Barone et al., 2015.

The skeletal muscle DHPR is comprised of five different subunits: α_{1S} , β_{1a} , α_2 , δ and γ . The α_{1S} is the pore forming subunit, while β_{1a} , α_2 , δ and γ are auxiliary subunits (Catterall, 2000, 2011; Obermair et al., 2005).

The α_{1S} subunit acts as the voltage sensor (Rios and Pizarro 1991). The β_{1a} subunit is located on the cytoplasmic side and affects channel gating and trafficking of the α_{1S} subunit (Buraei and Yang 2010). Recently, the molecular architecture studies has provided structural insights into the direct interaction between DHPR and RYR1, involving the β_{1a} subunit of DHPR for the docking to RYR1, and the mobile II–III loop of the α_{1S} subunit for the transmission of the signal to RYR1 during EC-coupling (Rebbeck et al., 2011; Hu et al. 2015).

In mammals three different genes of RYR are present, coding for three different isoforms with distinct tissue distribution: RYR1 is primarily expressed in skeletal

muscle; RYR2 is the major isoform found in cardiac muscle; and RYR3 is found in small amounts in a wide variety of tissues, in a developmental-related manner (*Giannini and Sorrentino, 1995; Flucher et al., 1999*). In skeletal muscle fibers, RYR1 can be present alone or associated with RYR3. In adult muscles, the expression of RYR3 is restricted to soleus, diaphragm and abdominal muscles (*Conti et al., 1996*), where it localizes near the RYR1 clusters in a parajunctional position, without any interaction with T-tubules membrane proteins (*Felder and Franzini-Armstrong, 2002*). The expression of two RYR isoforms in these muscles, whose functional duties imply a continuous contractile activity either to keep posture or to power breathing, may suggest that the presence of RYR3 could confer a greater resistance to fatigue (*Sorrentino and Reggiani, 1999*).

RYR is a giant homotetrameric complex composed of four identical subunits each associated with FKBP12 proteins. In skeletal muscle, the binding of FKBP12 to RYR1 stabilizes the fully opened and closed states of the channel (*Jayaraman et al., 1992*), controls channel gating frequency (*Gaburjakova, 2001*), and modulates sensitivity to activation by Ca^{2+} (*Mayrleitner et al., 1994; Marx et al., 2000*). All the effects of FKBP12 on RYR1 profoundly influences Ca^{2+} release from the SR, enhancing the gain of EC-coupling (*Avila et al., 2003; Tang et al., 2004; Avila and Dirksen, 2005*).

As mentioned before, several proteins important for the EC-coupling mechanism localizes in the triad; among them the most studied are: triadin, junctin, juncate, JP-45 and calsequestrin.

Triadin is a single-transmembrane protein localized in the SR, composed of a short N-terminal cytoplasmic domain and a large C-terminal luminal domain (*Marty et al., 2009*). Several isoforms of triadin are known, arising from alternative splicing of the TRDN gene on human chromosome 6 (*Marty, 2015*). In skeletal muscle a triadin isoform of about 95 kDa also known as Trisk95 is expressed, where it self-aggregates in oligomers by the formation of disulphide bridges between two cysteine residues located in the C-terminal luminal domain (*Thevenon et al., 2003*). In the luminal portion of triadin, multiple KEKE motifs are necessary for the binding to RYR1 (*Lee et al., 2004; Wium et al., 2012*) and calsequestrin (see chapter II. iv.), but studies on the implication of triadin in EC-coupling revealed its ability to bind also to the cytosolic part of RYR1 (*Guo and Campbell, 1995; Groh et al., 1999*).

The role of triadin in modulating RYR1 is controversial. In lipid bilayer experiments, when applied to the cytosolic face of RYR1, Trisk95 inhibits the channel activity, while it promotes the opening when added to the luminal side (*Ohkura et al., 1998*). However, knockdown of triadin in C2C12 myotubes showed that this protein facilitates electrically induced Ca^{2+} release (*Wang et al., 2009*). Likewise, expression of RYR1 constructs lacking the triadin-binding region abolishes depolarization-induced Ca^{2+} release (*Goonasekera et al., 2007*). Moreover, studies on triadin null-mice confirmed that the absence of this protein significantly alters the EC-coupling mechanism (*Shen et al., 2007; Boncompagni et al., 2012a*).

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Two transmembrane proteins localized in the Junctional SR, Junctin and Junctate, have a structural homology with triadin and are coded by alternative splicing of the aparyl- β -hydroxylase gene (*Treves et al., 2009*).

Junctin has a N-terminal cytoplasmic domain able to bind RYR1 (Li et al., 2015), and a C-terminal highly charged domain in the lumen of the SR. Similarly to triadin, the C-terminus of junctin contains KEKE motifs thought to interact with the luminal side of RYR1, increasing the open probability of the channel (Zhang et al., 1997; *Wei et al., 2009*).

Junctate is a moderate affinity, high capacity Ca^{2+} -binding protein, whose localization is not restricted to the triad (*Treves et al., 2000*). Junctate is thought to be involved in the formation of SR/sarcolemma contact sites and in the SOCE mechanism (*Divet et al., 2007*).

Another junctional SR membrane protein is the junctional sarcoplasmic reticulum protein 1 (JP-45).

JP-45 is a 45 kDa protein with a single transmembrane portion followed by an luminal domain rich in positively charged amino acids, and a cytoplasmic domain that is able to bind the α_{1S} subunit of DHPR (*Anderson et al., 2003, 2006*).

Interestingly, JP-45 ablation in mice leads to a decrease of α_{1S} subunit expression and to a loss of skeletal muscle strength in young mice (*Delbono et al., 2007, 2012*).

Located in the SR and in T-tubules, there are also minor triadic proteins described and discussed in excellent reviews (*Treves et al., 2009; Beard et al.,*

2009; Sorrentino, 2011; Rebbeck et al., 2014; Barone et al., 2015; Seidel et al., 2015).

iv. Calsequestrin

Calsequestrin (CASQ) is a high-capacity (40-50 mol Ca^{2+} /mol CASQ), moderate affinity ($K_d=1\text{nM}$) Ca^{2+} -binding acid glycoprotein of about 44 kDa, first identified by MacLennan and Wong in 1971.

It was described by Clara Franzini-Armstrong in 1987 as an electron dense matrix present in the luminal face of the junctional SR and localized nearby RYRs clusters (MacLennan and Wong, 1971; Franzini-Armstrong et al., 1987).

Two isoforms of CASQ are known, coded by distinct genes: CASQ1 (MIM #114250) and CASQ2 (MIM #114251). The two isoforms have a sequence homology of about 65%.

In adult muscle tissue, CASQ1 is predominantly expressed in fast skeletal muscle fibers, while CASQ2 is present only in slow skeletal muscle fibers and cardiac muscle (Schiaffino and Reggiani, 2011).

In skeletal muscle, CASQ serves as the main Ca^{2+} buffer of the SR (Damiani et al., 1990); this ability directly influences the amount of Ca^{2+} that can be released during the electromechanical coupling by increasing the Ca^{2+} load that can be maintained within the SR (Launikonis et al., 2009). Crystallographic studies (PDB id: 3UOM) and biochemical and structural observations revealed that CASQ1 is composed of three nearly identical thioredoxin-like domains (simply named: I, II, III); the CASQ1 monomer holds eight high- and nineteen low-affinity Ca^{2+} -

binding sites and in order to increase its Ca^{2+} - binding capacity organizes itself in dimers by front-to-front interactions and consequently in polymers by back-to-back interactions, depending on Ca^{2+} concentrations (Figure 8) through C-terminal calcium-sensitive disordered motifs, consisting of repeated aspartic acid residues: the so called consecutive aspartate stretch (CAS). Each monomer of CASQ also contains several phosphorylation sites (Beard *et al.*, 2004). The CAS is also responsible for the ability of phosphorylated CASQ1 to inhibit the RYR1 in a $[\text{Ca}^{2+}]$ -dependent manner and in association with triadin and junctin (Sanchez *et al.*, 2012; Kumar *et al.*, 2013; Bal *et al.*, 2015; Beard and Dulhunty, 2015); on the other hand it has been demonstrated that when the protein is dephosphorylated interacts directly with the channel promoting its activation (Szegedi *et al.*, 1999; Herzog *et al.*, 2000) and its Ca^{2+} - binding capacity is lower (Beard *et al.*, 2008). These data are also supported by studies on CASQ1 knock out mice, where CASQ1 deficiency results in profound remodeling of the EC-coupling apparatus (Paolini *et al.*, 2007; Protasi *et al.*, 2011).

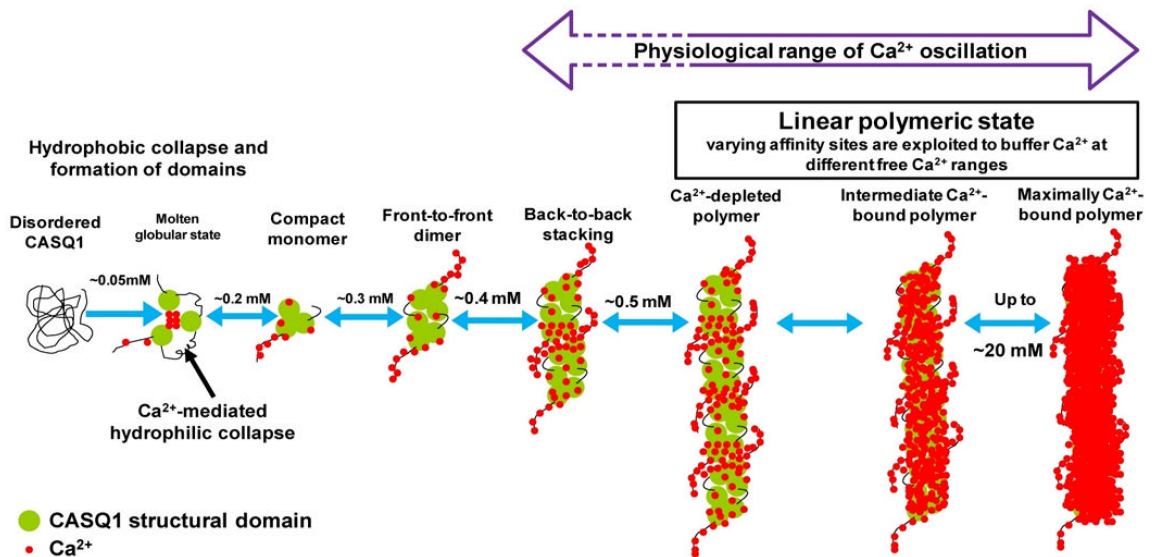


Figure 8_ Proposed model of Ca^{2+} - dependent CASQ1 polymerization and Ca^{2+} binding by Kumar *et al.*, 2013.

In the resting skeletal muscle more of the 70% of stored Ca^{2+} is bound to CASQ1. The buffer action of this protein prevent the ion to precipitate as phosphate, allowing its storage in a much higher quantity and even maintaining low the gradient that the SERCA pumps must oppose to re-introduce the calcium ions in the SR (*Murphy et al., 2009*).

It has been suggested that CASQ1 is also involved in the SOCE mechanism: reduction in CASQ1 content determines an increase SOCE activity (*Zhao et al., 2010*). More recently it has been demonstrated that CASQ1 might regulate SOCE mechanism by the binding to STIM1, with the result of a lower STIM1/Orai1 association (*Wang et al., 2015; Zhang et al., 2016*).

Finally, a link between CASQ1 and DHPR bypassing RYR1 and mediated by JP-45 has been also proposed (*Anderson et al., 2003, 2006*).

v. Diseases caused by alteration of Ca^{2+} entry

The primary mechanism that increases the intracytosolic Ca^{2+} in the skeletal muscle fiber is the EC-coupling, but like in other cell types, also a mechanism known as store-operated calcium entry (SOCE) is present. This mechanism contributes to maintain proper contractile function, by facilitating external Ca^{2+} influx when SR Ca^{2+} stores begin to be depleted (*Kurebayashi and Ogawa, 2001*). SOCE is regulated by stromal interaction molecule 1 (STIM1), a finely tuned sensor of Ca^{2+} levels in the interior of the SR. STIM1 is a single-pass transmembrane protein with a helix-loop-helix domain in the lumen of the SR able to sense changes in Ca^{2+} content, and a coiled-coil domain located in the

cytosolic side of the SR membrane. When the luminal domain of STIM1 senses diminished Ca^{2+} concentrations in the SR, conformational changes induce the cytosolic domain of the protein to interact with the Ca^{2+} release-activated Ca^{2+} channel protein 1 (Orai1), a tetrameric ion channel complex located in the sarcolemma. This interaction leads to trans-sarcolemmal Ca^{2+} influx and the consequent increase of intracellular Ca^{2+} levels (*Kiviluoto et al., 2011; Stiber et al., 2011*).

Alteration of this mechanism could lead to a particular form of myopathy called tubular aggregate (TA) myopathy, which is a disorder that primarily affects the skeletal muscles. TAs in ageing mice do not contain RYRs, but accumulate SERCA and CASQ (*Boncompagni et al., 2012b*). This mild, slowly progressive muscle condition causes muscle pain, cramping, or weakness that begins in childhood and worsens over time (*Jain et al., 2008*).

The muscle fatigue may also lead to an unusual walking style (gait). Some people with this pathology develop joint deformities (contractures) in the arms and legs. TA myopathy (TAM) is also a component of a life-tethering condition called Stormorken syndrome, which, in addition to tubular aggregates, is characterized also by hematopoietic abnormalities.

The disorder is diagnosed by the presence in muscle biopsy of aggregates of hexagonal tubules at the electron microscopy analysis. Both tubular aggregate myopathy and Stormorken syndrome are due to mutations in either STIM1 or ORAI1 (*Bohm et al., 2013; Misceo et al., 2014*). All mutations responsible of TAM identified till now are affecting the stromal interaction molecule 1 (STIM1) and the Calcium Release-Activated Calcium Modulator 1 (ORAI1) proteins and cause

alterations of intracellular Ca^{2+} homeostasis in muscle cells (Böhm et al., 2013; Endo et al., 2015; Okama et al., 2016).

The TAs, which are the pathognomonic histopathologic aberration of the disease, are likely composed of altered SR-derived membranes (*Morgan-Hughes, 1998*).

vi. Diseases due to mutations in triadic proteins

Thanks to the increased knowledge of the genetic basis of human skeletal myopathies, it has become evident that many muscle diseases primarily involve alterations of the three main substructures which the muscle utilizes to generate force and control movement: the neuromuscular junction, the triad and the sarcomere. In an attempt to organize and classify the muscle diseases that affect the EC-coupling and considering this substructural categorization, Dowling et al. (2014) define the disorders that affect triad function as “triadopathies”, dividing them in: primary triadopathies (abnormalities triad structure and function due to mutations on triadic proteins), and secondary triadopathies (alterations in triad function and usually not in its structure). More recently, Ríos and colleagues proposed a more strictly classification, which only includes diseases that affect one or more components of the couplon as much to alter its function: “the couplonopathies” (*Ríos et al., 2015*). The couplon is defined as the functional unit of Ca^{2+} release in the EC-coupling of both cardiac and skeletal muscle and comprise RYR, DHPR and all the ancillary proteins associated with their Ca^{2+} release function (*Stern et al., 1997; Stern et al., 1999*).

II. INTRODUCTION

Mutations in the RYR1 gene are responsible for more than 60% of the cases of a pathological condition called malignant hyperthermia susceptibility (MHS), which causes a predisposition to eventually fatal hypermetabolic crises as a result of an aberrant Ca^{2+} release from the SR. The crises are triggered by the administration of some volatile anesthetics and/or muscle relaxants or are a consequence of excessive heat or over-exercising.

Mutations in RYR1 gene are present in three hotspots: hotspot 1 in the N-terminal region controlling RYR1 activity, hotspot 2 in the central regulatory region that hosts different regulatory sites for proteins and kinases, and finally hotspot 3 in the C-terminal region that contains the channel pore (*Dirksen and Avila, 2004*).

The massive metabolic reaction involves increased carbon dioxide production, metabolic and respiratory acidosis, accelerated oxygen consumption, confusion, abnormally rapid or irregular heartbeat, dramatic and dangerous increase in body temperature, activation of the sympathetic nervous system, hyperkalemia, disseminated intravascular coagulation and multiple organ dysfunction, concurrent muscle contraction and stiffening (*Kozack and MacIntyre, 2001*).

Mutations in RYR1 have been associated also to central core disease (CCD) (*Durham et al., 2007*). The symptoms of the disease usually involve decreased muscle tone at birth, delayed motor development, weakness of the facial muscles, and skeletal abnormalities such as scoliosis and hip dislocation (*Quinlivan et al., 2003*). The mutated channel results to be poorly functioning with reduced Ca^{2+} conductance, but there are mutations that lead to a phenotype affected by both CCD and MHS. In contrast, MHS + CCD diseases show a

pathophysiology of excessive RYR1 channel pore opening (*Murayama et al., 2016*).

Skeletal muscle diseases caused by mutations in triadin are very rare as well as no junctin mutations have been associated to cardiac or skeletal muscle diseases. Considering triadin-null mice, lacking all cardiac and skeletal triadin, a combined phenotype of CPVT and skeletal muscle weakness has been observed (Rios et al., 2015). Finally, it has also been shown that ablation of CASQ1 gives an MH-like Phenotype, only in male mice (*Dainese et al., 2009*).

A myopathy linked to a mutation in CASQ1

In this thesis I will narrow the focus on a particular form of myopathy due to a mutation in the sixth exon of human CASQ1 gene, which besides altering the Ca^{2+} release function of the couplon, causes the accumulation of SR proteins aggregates (*Rossi et al., 2014*).

In 2006 Tomelleri and colleagues described a group of patients affected by an undefined mild form of myopathy characterized by weakness, fatigue and episodes of muscle cramping. Rossi et al. in 2014 described a group of subjects with the same symptoms of those observed by Tomelleri. Both group of patients showed also elevated plasma creatine kinase levels and light microscopy of muscle biopsies revealed the presence of vacuoles that did not stain by routine histochemical analysis. Electron microscopy analysis of the muscle biopsies showed two type of inclusions in the vacuoles: type-1 rectangular-shaped inclusions (dark-stained for toluidine blue) filled of electron-dense material resembling CASQ accumulation in the SR (*Paolini et al., 2011*) and type-2

II. INTRODUCTION

rounded inclusions (faint-stained for toluidine blue) rich in glycogen, various cytoplasmic debris and abnormal mitochondria (Figure 9), and also other morphological alterations.

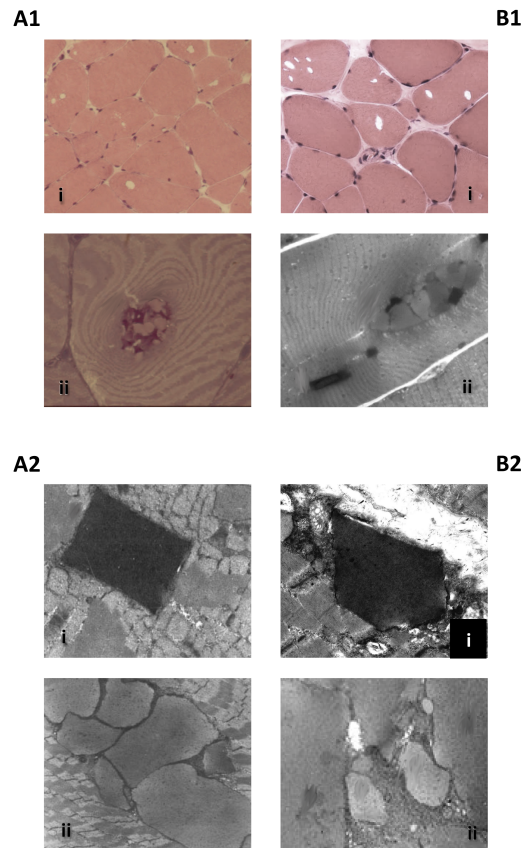


Figure 9_ Comparison between biopsies analysis adapted from Tomelleri et al., 2006 (A1-A2) and from Rossi et al., 2014 (B1-B2). Panels A1/B1, histological analysis of CASQ1 D244G mutated muscle biopsies stained for: hematoxylin/eosin (i), and toluidine blue (ii). Panels A2/B2, Electron microscopy: type-1 inclusions (i) and type-2 inclusions (ii).

Sequence analysis of the CASQ1 gene in the patients described in Rossi et al., 2014, revealed a mutation (c.731A>G) (Fig 10_A) responsible for an amino acid change from aspartic acid to glycine in position 244 (D244G), which is conserved

from snakes to human (Fig 10_B). The mutation segregated with the vacuolar myopathy in four families analyzed by Rossi and colleagues (2014).

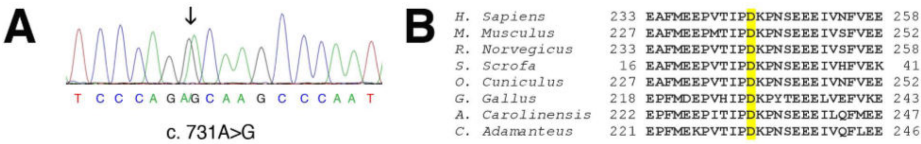


Figure 10_ Adapted from Rossi et al., 2014: (A) Electropherogram of the CASQ1 gene sequence of one patient heterozygous for the c.731A>G mutation (arrow); (B) Phylogenetic alignment of the CASQ1 orthologs with the conserved aspartic acid (in position 244 in human CASQ1) highlighted in yellow.

III. AIM AND RATIONALE

In the SR lumen of skeletal muscle fibers, CASQ1 is the most abundant Ca^{2+} buffering protein and indirectly regulates RYR1 ability to release Ca^{2+} . Recently a mutation in the CASQ1 gene, which results in the substitution of a conserved aspartic acid in position 244 to a glycine was found in a group of patients affected by a form of myopathy characterized by weakness, fatigue and the presence of large vacuoles inside muscle fibers (*Rossi et al. 2014*).

This thesis was focused on the characterization of structural and functional alterations caused by the D244G mutation in CASQ1 and on their correlation to myopathic phenotype. In particular we wanted to verify eventual conformational changes in the mutated protein structure and its ability to form aggregates. We wanted also to assess whether the mechanisms of Ca^{2+} storage and release were altered in cells expressing the mutated protein.

To this end, we isolated skeletal muscle fibers from patients carrying the D244G mutation and performed immunofluorescence analysis and Ca^{2+} release measurements to better define the effect of the mutation. In addition, we generated either GFP-tagged or untagged expression vectors of CASQ1^{WT} or CASQ1^{D244G} recombinant proteins to study the effect of mutated CASQ1 in muscular or non-muscular systems. Bioinformatics and three-dimensional structure prediction tools were also adopted to study possible conformational changes in CASQ1^{D244G}.

IV. MATERIALS AND METHODS

i. Patients and ethics statement

In this study have been involved eight patients (six males and two females) affected by a mild myopathy. Age range of subjects is between 23 and 58 years. Written informed consent and ethics committee approval and were obtained for all patients. This study complies with the ethical standards laid down in the 1964 Declaration of Helsinki. All experiments that required animals followed the official guidelines laid down by the European Community Council (directive 86/609/EEC) incorporated into Italian Government Legislation. Experiments have been performed with the approval by the Local Ethical Committee and Ministero della Salute, Rome, Italy.

ii. Protein structure and tridimensional prediction

A three-dimensional structure of CASQ1 p.Asp244Gly was predicted using the automated protein homology modeling server SWISS-MODEL (<http://swissmodel.expasy.org/>). Chain A of human CASQ1 protein (PDB: 3UOM) was used as template.

The comparison between wild type CASQ1 and the p.Asp244Gly CASQ1 mutated protein was performed using the UCSF Chimera 1.9 software (University of California).

iii. Generation of expression vectors

Human CASQ1 cDNA (NM 001231.4) was amplified from total RNA extracted from human skeletal muscle tissue using specific primers. The cDNA coding for the Asp244Gly CASQ1 mutant was generated by PCR using primers designed to introduce the adenosine to guanosine nucleotide change into the wild type CASQ1 cDNA (forward primer: 5' -gtg acc atc cca gGc aag ccc aat agc-3' ; reverse primer: 5' -gct att ggg ctt gCc tgg gat ggt cac-3') and sequenced using an ABI Prism 310 apparatus (Applied Biosystems). The amplified sequences were cloned into the pEGFP-N3 vector (Clontech Laboratories, Mountain View, CA) using the EcoRI-BamHI sites to generate vectors expressing the wild type or the mutated protein in frame with GFP (respectively CASQ1^{WT}-GFP and CASQ1^{D244G}-GFP).

Both cDNAs were also inserted into the pCDNA3 vector (Invitrogen, Carlsbad, CA) using the HindIII-BamHI sites to generate expression vectors without the GFP tag (CASQ1^{WT} and CASQ1^{D244G}).

iv. Cell culture and transfections

African green monkey kidney-derived COS-7 cells were cultured in Dulbecco's modified eagle medium containing 2 mM L-glutamine (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma- Aldrich), 100 U/ml penicillin (Sigma-Aldrich), 1 mM sodium pyruvate (Lonza Group, Basel, Switzerland), supplemented with

IV. MATERIALS AND METHODS

10% heat-inactivated fetal bovine serum (Sigma–Aldrich). Cells were grown at 37°C at 5% CO₂. COS-7 cells were transiently transfected one day after seeding with X-tremeGENE HP DNA transfection Reagent (Roche Applied Science, Penzberg, Germany) according to manufacturer’s instructions and analyzed one day after transfection in all the experiments performed.

Myoblasts were obtained from hind limb muscles of 2-days-old rats (Sprague–Dawley; Harlan Laboratories, Indianapolis, IN). Briefly, minced muscles were enzymatically dissociated by treatment with 0.125% trypsin and cells were resuspended in Dulbecco’s modified eagle medium containing 2 mM L-glutamine (Sigma–Aldrich), 100 µg/ml streptomycin (Sigma–Aldrich), 100 U/ml penicillin (Sigma–Aldrich), 1 mM sodium pyruvate (Lonza Group, Basel, Switzerland), supplemented with 10% heat-inactivated fetal bovine serum (Sigma–Aldrich). Cell suspension was seeded on 0.025% laminin (Sigma–Aldrich) coated glass coverslips. Cells were grown at 37°C at 5% CO₂. After 2 days, cells were transiently transfected with the Lipofectamine-Plus method (Life Technologies) following manufacturer’s instructions. Myoblasts were induced to differentiate with minimum essential medium alpha containing 2 mM L-glutamine (Sigma–Aldrich), 100 µg/ml streptomycin (Sigma–Aldrich), 100 U/ml penicillin (Sigma–Aldrich), 1 mM sodium pyruvate (Lonza), 1 mM dexamethasone (Sigma–Aldrich), 0.05 mM hydrocortisone, supplemented with 10% heat-inactivated fetal bovine serum (Sigma–Aldrich) and 5% heat-inactivated horse serum (Sigma–Aldrich).

v. Immunofluorescence

All patients underwent an open biopsy of vastus lateralis muscle and single fibers were isolated from the biopsies and fixed with 3% (v/v) paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) and permeabilized in HEPES–triton buffer (20 mM HEPES pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100). Next, pools of single fibers were stained over night in wet chamber at 4°C, using the following primary antibodies: anti-CASQ1 mouse monoclonal antibody diluted 1:300 (clone MA3-913; Thermo Scientific), anti-RyR mouse monoclonal antibody at a final concentration of 1 µg/ml (clone 34C; Thermo Scientific, Waltham, MA), anti-RyR1 rabbit polyclonal antibody produced in our laboratory and anti-SERCA monoclonal antibody diluted 1:1 (clone Y/IF4 kindly provided by Prof. M. East). Cy3- or Cy2-conjugated antimouse or antirabbit secondary antibodies (Jackson Laboratories, Bar Harbor, ME) were used for immunofluorescence detection, with an incubation of 1 h at room temperature in a wet chamber.

COS-7 cells grown on glass coverslips and transfected with CASQ1^{D244G}–GFP or CASQ1^{WT}–GFP (see Generation of CASQ1 Expression Vectors) were fixed for 7 min at room temperature, after 24 h from transfection with 3% (v/v) paraformaldehyde in PBS and permeabilized in HEPES–triton buffer for 3 min at room temperature; anti-CASQ1 mouse monoclonal antibody diluted 1:300 (clone MA3-913; Thermo Scientific) was used as primary antibody with an over night incubation in wet chamber at 4°C and Cy3-conjugated antimouse antibody

IV. MATERIALS AND METHODS

(Jackson Laboratories, Bar Harbor, ME) was used as secondary antibodies for 1 h at room temperature in wet chamber.

Myotubes expressing CASQ1^{D244G} or CASQ1^{WT} were fixed after 12 days of differentiation with 3% (v/v) paraformaldehyde in PBS for 7 min at room temperature and permeabilized in HEPES-triton buffer for 3 min at room temperature. Next, myotubes were stained over night in wet chamber at 4°C, using the following primary antibodies: anti-CASQ1 mouse monoclonal antibody diluted 1:300 (clone MA3-913; Thermo Scientific), anti-RyR1 rabbit polyclonal antibody produced in our laboratory. Cy3- or Cy2-conjugated antimouse or antirabbit secondary antibodies (Jackson Laboratories, Bar Harbor, ME) were used for immunofluorescence detection, with an incubation of 1 h at room temperature in a wet chamber.

Images were acquired using a Zeiss 510 meta confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

vi. Electron microscopy

Mice were anesthetized with 1.25% 2,2,2-tribromoethanol (Avertin solution; Roche Applied Science) and hind flexor digitorum brevis (FDB) muscles injected with 10 µg of DNA, after 45 min of hyaluronidase administration. Then, two electrodes were placed at each side of the muscle (one in the fingers side and one in the heel side) and twenty electric pulses of 120 V with fixed duration of 20 msec and interval of 1 sec were delivered using an electric pulse generator

(Electro Square Porator ECM830; BTX-Genetronics, San Diego, CA-Genetronics, Inc.).

Muscle specimens were excised after 10 days and fixed in 3.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4, room temperature) for 2 h followed by buffer rinse and post fixation for 1 h in 2% osmium tetroxide. The specimens were rapidly dehydrated in graded ethanol and acetone, infiltrated with EPON–acetone (1:1) mixture for 2 h, and embedded in EPON (*Tomasi et al., 2012*). Ultrathin sections (40– 50 nm) were cut and, after staining with 4% uranyl acetate and lead citrate, examined with a Morgagni Series 268D electron microscope (FEI Company, Brno, Czech Republic), equipped with a Megaview III digital camera (*Boncompagni et al., 2006; Tomelleri et al., 2006*).

vii. Time lapse confocal acquisition

COS7 cells (15000 cells/well) were seeded in coverglass bottomed 4-well tissue culture chambers (Sarstedt AG & Co, Nümbrecht, Germany) and transfected the day after with CASQ1^{WT}–GFP or CASQ1^{D244G}–GFP expression vectors as described in IV.v. Next, the cells were placed on a Zeiss LSM 510 Meta confocal microscope. During the experiment the cells were maintained in modified Krebs buffer (135 mM NaCl, 6.2 mM KCl, 1.2 mM MgCl₂, 12 mM HEPES, pH 7.3, 11.5 mM glucose and 2 mM CaCl₂) and images were acquired every minute for 10 min with a 63X 1.4 N.A. plan-apochromat oil immersion objective (Zeiss) used with a pinhole aperture of 4.96 airy units. After the second acquisition the medium was substituted with a Krebs modified buffer without Ca²⁺ supplemented with 0,5

mM EGTA and 1 μ M thapsigargin. GFP was excited at 488 nm with an argon laser with low laser power (0.5%) and emitted fluorescence collected with a long-pass 505 emission filter.

viii. Fluorescence recovery after photo-bleaching

For FRAP analyses, 15000 cells/well were seeded in coverglass bottomed 4-well tissue culture chambers (Sarstedt AG & Co, Nümbrecht, Germany) and transfected the day after as described in IV.v. FRAP experiments were performed by using a Zeiss LSM 510 Meta confocal microscope. Cells were imaged in coverglass bottomed chambers and culture medium was substituted with modified Krebs buffer (135 mM NaCl, 6.2 mM KCl, 1.2 mM MgCl₂, 12 mM HEPES, pH 7.3, 11.5 mM glucose and 2 mM CaCl₂) before starting the experiment. A 63X 1.4 N.A. plan-apochromat oil immersion objective (Zeiss) was used with a pinhole aperture of 4.96 airy units. GFP was excited at 488 nm with an argon laser with low laser power (0.5%) and emitted fluorescence collected with a long-pass 505 emission filter. After acquisition of 10 prebleach images, photobleaching of GFP in a circular area 1.08 μ m in diameter was performed by using the argon laser lines 458, 477, and 488 nm at 50% laser power and 50% transmission. Recovery of fluorescence in the bleached region was recorded every 50 ms over a period of 1–10 min until fluorescence level reached a plateau. Fluorescence intensities were acquired for the bleached region (I_{frap}), the whole cell (I_{whole}), and a background region (I_{base}). The data were low-pass 3 X 3 filtered with the Zeiss LSM 510 software for image noise reduction, and data analysis

was performed by using macros designed in IgorPro software (WaveMetrics Inc.). The average fluorescence intensity within the bleached region was normalized to prebleach intensity for each time point and corrected for loss of fluorescence during acquisition. Fluorescence loss during acquisition was basically no more than 10%. The normalized data were fitted by the exponential equation: $I_{\text{frap-est}}(t) = A (1 - e^{-\tau_1 t})$ (Armani *et al.*, 2006), where A represents the mobile fraction. Fitting quality was evaluated by the probability Q value and accepted when $Q > 0.01$. The diffusion constant was calculated by using the Soumpasis diffusion fitting (Soumpasis, 1983). Statistical analyses were performed by using InStat Software (Prism 6 GraphPad). Data were initially evaluated for normal distribution with the Kolmogorov–Smirnov test. Not normally distributed data were compared by Mann–Whitney test. Comparisons between 2 groups with normally distributed variables were analyzed by Student’s unpaired t-test.

ix. Single fiber Ca^{2+} release and force measurement

Small fragments of biopsy specimens were immersed in glycerol-containing skinning solution (Salviati and Volpe, 1988) for at least 24 h. Single fibers were manually dissected and segments of approximately 1 mm length were cut and mounted with small aluminum clips (T clips) at both ends. The experimental setup was composed of a force transducer (AE 801; Sensoror, Horten, Norway), an electromagnetic puller to control fiber length and an aluminum plate, where seven small chambers (100 μl) were milled (Doria *et al.*, 2011). Fibers were

mounted between the force transducer and the electromagnetic puller in the first chamber filled with relaxing solution. Next, the SR was loaded by moving the fiber to a chamber containing solution at pCa 6.45 in the presence of 5 mM ATP. After the fibers were washed to remove excess EGTA, Ca^{2+} release was induced by transferring the fibers to a release solution with low EGTA (0.1 mM) and low free Mg^{2+} -containing caffeine in concentrations variable from 0.1 to 20 mM. The fibers were finally transferred to activating solution to measure their ability to develop force during a maximal activation (pCa 4.7) and quickly brought back to the relaxing solution to start a new cycle of loading and release. The release of Ca^{2+} was detected by the transient tension development and quantified by measuring the tension-time area according to a method first developed by Endo and Iino (1988) and widely used. The transient is determined by the combination of the Ca^{2+} release from the SR with the Ca^{2+} removal due to diffusion into the medium, buffering by EGTA, and reuptake in the SR (*Makabe et al., 1996*). Experimental and model analyses suggest that the area gives the best indication on the amount of Ca^{2+} release, whereas other parameters such as the rate of tension rise is more related to the rates of Ca^{2+} release and of tension generation by cross-bridges. The tension-time area was normalized to the tension developed during maximal activation (pCa 4.7) to account for the ability of the myofibrillar apparatus to develop force and thus expressed in seconds.

x. Ca^{2+} imaging

COS7 (15000 cells/well) were seeded in coverglass bottomed 4-well tissue culture chambers (Sarstedt AG & Co, Nümbrecht, Germany) and transfected the day after with CASQ1WT or CASQ1D244G or the GFP empty vectors as described in IV.v. Then, the cells were loaded 2 days after transfection, at room temperature, using Fura-2-AM (1 μM ; Biotium, Hayward, CA, USA) in modified Krebs buffer (135 mM NaCl, 6.2 mM KCl, 1.2 mM MgCl_2 , 12 mM HEPES, pH 7.3, 11.5 mM glucose and 2 mM CaCl_2) supplemented with 1% BSA. After 30 min, the cells were washed three in the same buffer without BSA and de-esterification was allowed to occur for 30 min at room temperature. Fura-2-loaded cells were placed on a fluorescence image micro- scope and before starting the $[\text{Ca}^{2+}]$ measurements, GFP-positive cells were selected. The digital fluorescence-imaging microscopy system was mounted on a Nikon Diaphot 300 (Nikon, Tokyo, Japan) inverted microscope. Fluorescence images were collected through a Nikon oil immersion 40X/13 numerical aperture objective and acquired by a cooled charge-coupled device camera (Photometrics, Roper Scientific, USA) and a MetaFluor imaging system (Universal Imaging, Downingtown, PA, USA). During the experiment, the medium was substituted with the same medium above described, but depleted of Ca^{2+} and supplemented with 0,5 mM EGTA to avoid extracellular Ca^{2+} influx. Thapsigargin (Alomone Labs, Jerusalem, Israel) responses were measured. For calibration, minimal and maximal Fura-2 responses were subsequently determined using 2 μM of ionomycin (Enzo Life Sciences, Farmingdale, NY, USA) supplemented with 0,5 mM EGTA or 5 mM

CaCl₂, respectively, in modified Krebs buffer. The cytosolic [Ca²⁺] ([Ca²⁺]_i) was calculated using $[Ca^{2+}] \text{ (nM)} = K_d \times (F_{380\text{max}}/F_{380\text{min}}) \times (R - R_{\text{min}}) / (R_{\text{max}} - R)$, where K_d is the dissociation constant of Fura-2 for Ca²⁺ at room temperature (135 nM). In each experiment, 18–25 GFP-positive cells were measured, which was repeated on at least three different days. Replicate experiments within the same day were also averaged and used for obtaining final averages for all days. Area under curve (AUC) values were calculated by integrating the averaged curve from the time point when thapsigargin was added, until calibration was started 5 min later.

xi. Western blot

COS-7 cells were lysed in a buffer containing 25 mM Hepes, pH 7.5, 1% Triton X-100, 10% glycerol, 0.3 M NaCl, 1.5 mM MgCl₂, 1 mM DTT, 2 mM EDTA, 2 mM EGTA and protease inhibitor cocktail (Sigma–Aldrich). The protein concentration of samples was determined by Bradford assay (Bio-Rad) using bovine serum albumin (BSA) as standard. Proteins (10 µg) were separated by Criterion™ TGX™ precast 4–15% polyacrylamide gels using TGS buffer () and transferred onto a nitrocellulose blotting membrane (Amersham™ Protran™ 0.45 µm NC, GE Healthcare Life Science). After blocking with TBS containing 0.1% Tween and 5% non-fat dry milk powder, the membrane was incubated with the primary antibody overnight at 4°C. Anti-CASQ1 mouse monoclonal antibody (clone MA3-913; Thermo Scientific) diluted 1:1000 in TBS containing 0.1% Tween and 1% non-fat dry milk powder was used as primary antibody. Next, membranes were

IV. MATERIALS AND METHODS

incubated for 1 h at room temperature with a ECL™ anti-mouse IgG, horseradish peroxidase linked whole antibody (from sheep) (Amersham™ , GE Healthcare Life Science) (dilution 1:2000 in 0.1% Tween/TBS and 1% non-fat dry milk powder). Protein detection was performed with SuperSignal^R West Dura Extended Duration Substrate (Thermo Scientific) and acquired with Bio-Rad Chemidoc™ imaging system. Bands quantification was done with Image Lab™ Software (Bio-Rad).

V. RESULTS

i. Fibers from patients carrying D244G mutation present aggregates containing SR proteins

In congenital myopathies, protein deposits might be initiated by the aggregation of one mutated protein that may recruit other additional proteins (*e.g. Goebel and Blascheck, 2011*). The presence of vacuoles containing inclusions that stain positive for CASQ1 has been reported in muscle biopsies from subjects affected by an uncharacterized form of myopathy (*Tomelleri et al., 2006*). We found a group of patients with a mild myopathy characterized by the same symptoms and histology described by Tomelleri et al. in 2006. Interestingly, all of the patients analyzed carry the D244G mutation (*Rossi et al., 2014*).

Altogether, these observations prompted us to further investigate the inclusions found in the patients' biopsies. We verified if aggregates contained CASQ1 and/or others SR proteins.

Skeletal muscle biopsies from patients were fixed in paraformaldehyde and single fibers were mechanically isolated at the stereomicroscope. The isolated fibers were stained with antibodies against CASQ1, SERCA and RYR1.

As it can be seen in figure 1, RYR1 and CASQ1 showed a typical localization in striated pattern corresponding to the Junctional SR, indicating that the overall SR structure did not significantly differs from normal individuals' fibers. Nevertheless, staining with antibodies against RYR1 and CASQ1 also revealed the presence of protein aggregates of variable size along the entire muscle fiber. In addition staining with antibodies against SERCA showed that these aggregates

also contained this protein, indicating that these aggregates likely represent local accumulation of SR proteins from both longitudinal or junctional SR.

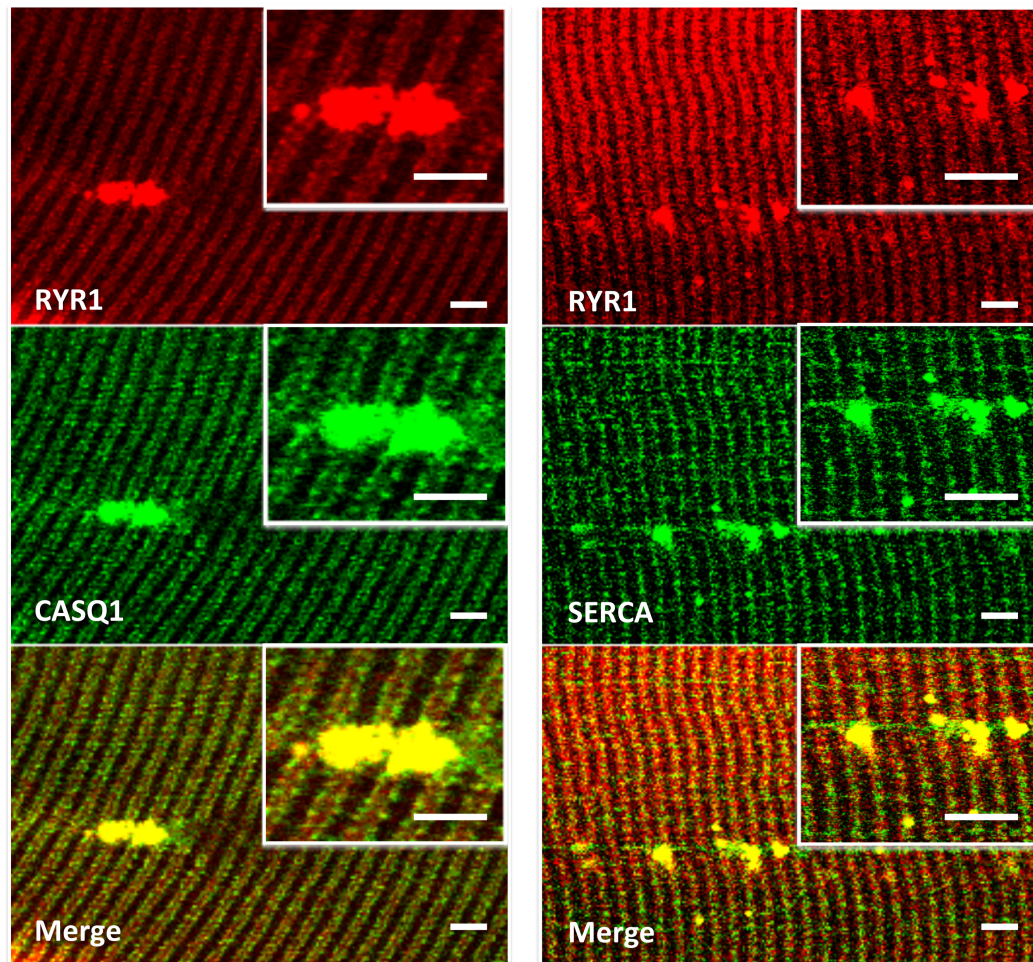


Figure 1_ Immunofluorescence analysis of single isolated fibers from one patient carrying D244G mutation, stained for different SR proteins. Merged images show colocalization of SERCA and CASQ1 with RYR1 in the aggregates. Scale bar 5 μ m.

ii. Conformational changes in CASQ1^{D244G} mutated protein

CASQ1 can bind about 80 Ca²⁺ ions thanks to high- and low- affinity Ca²⁺ binding sites (*Kumar et al., 2013*). The aspartic acid 244 is located in one of the high affinity Ca²⁺ binding site in CASQ1 (*Sanchez et al., 2012*). We thus wanted to investigate whether the substitution of the aspartic acid in position 244 to glycine induced a conformational change in the correspondent Ca²⁺ binding region.

Thanks to crystallographic studies, the human CASQ1 protein have been characterized and a three-dimensional model is available (PDB:3UOM). We predicted the three-dimensional structure of the D244G mutated CASQ1 thanks to the protein homology-modeling server SWISS-MODEL, which is a fully automated online software that uses suitable templates to predict one or more accurate model for three-dimensional protein structures. We used as template the chain A of human CASQ1 protein, from the PDB:3UOM (Figure 2, left panel).

To compare the predicted three-dimensional structure of CASQ1^{D244G} with the wild type protein, we used the UCSF Chimera 1.9 software. No significant changes in the secondary structure, visualized as ribbon representation, were observed in the mutated protein, but right panel of figure 2 shows a surface representation of the high-affinity Ca²⁺ binding site; where the mutation is predicted to induce abnormalities in the local molecular environment surrounding the binding task for Ca²⁺, resulting in the partial loss of the molecular groove.

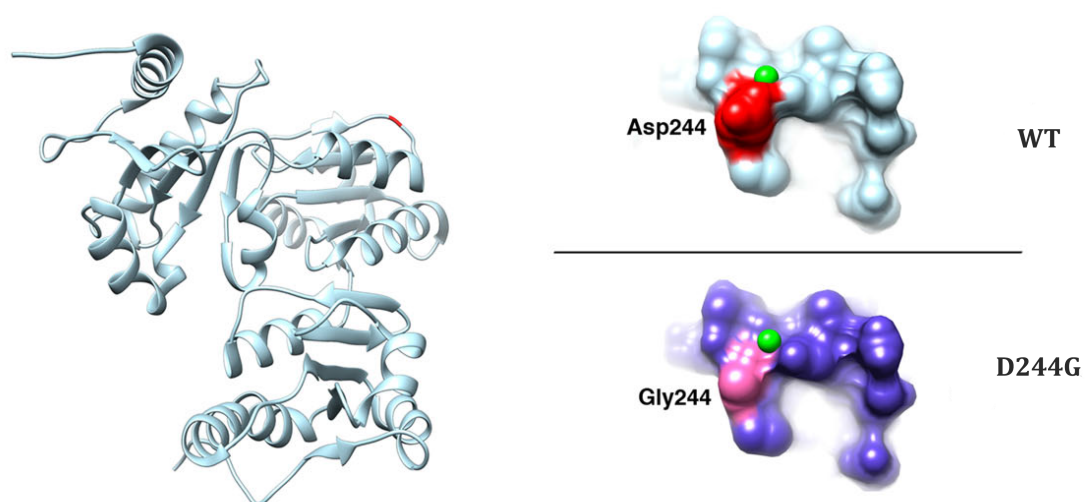


Figure 2_ Three – dimensional ribbon representation of chain A of the model of human CASQ1 (PDB: 3UOM), aspartic acid 244 highlighted in red on the left. On the right, details of predicted surface structures of the local molecular environment surrounding the amino acid in position 244 on CASQ1 protein sequence (green spheres: Ca^{2+} ion).

iii. Altered Ca^{2+} release in fibers from patients carrying D244G mutation

In skeletal muscle, CASQ1 participates in the regulation of the SR Ca^{2+} storage and also contributes to modulate the activity of RYR1 channel, hence partially determining the amount of Ca^{2+} released during EC-coupling (*Launikonis et al., 2009; Beard and Dulhunty, 2015*).

Based on results obtained by conformational studies, we wanted to verify whether the D244G mutation may also affect the function of the protein. We thus isolated single muscle fibers from patients, and compared them with fibers isolated from healthy subjects, in order to study the capacity to release Ca^{2+} from SR.

A caffeine dose-response protocol was applied on isolated muscle fibers, and tension-time area was adopted as a bona fide indicator of the quantity of Ca^{2+} released (*Endo and Iino, 1988; Salviati and Volpe, 1988; Lamb et al., 2001*).

We analyzed a balanced pool of fast and slow fibers from two different patients, and a pool of healthy control fibers was used as control. All these experiments were performed in collaboration with Prof. Carlo Reggiani's group of research (University of Padova, Italy).

Figure 3 represents the graphic of the average dose-response curves of the pools of fibers analyzed. The extrapolated maximal Ca^{2+} release was not significantly different between healthy and patients' fibers. On the contrary, there is a significant difference in the EC50 points of the curves, suggesting that fibers from patients requires larger caffeine load to release as much Ca^{2+} as the healthy

fibers. This may reflect a deregulation of RYR1 activity in the fibers carrying the D244G mutation.

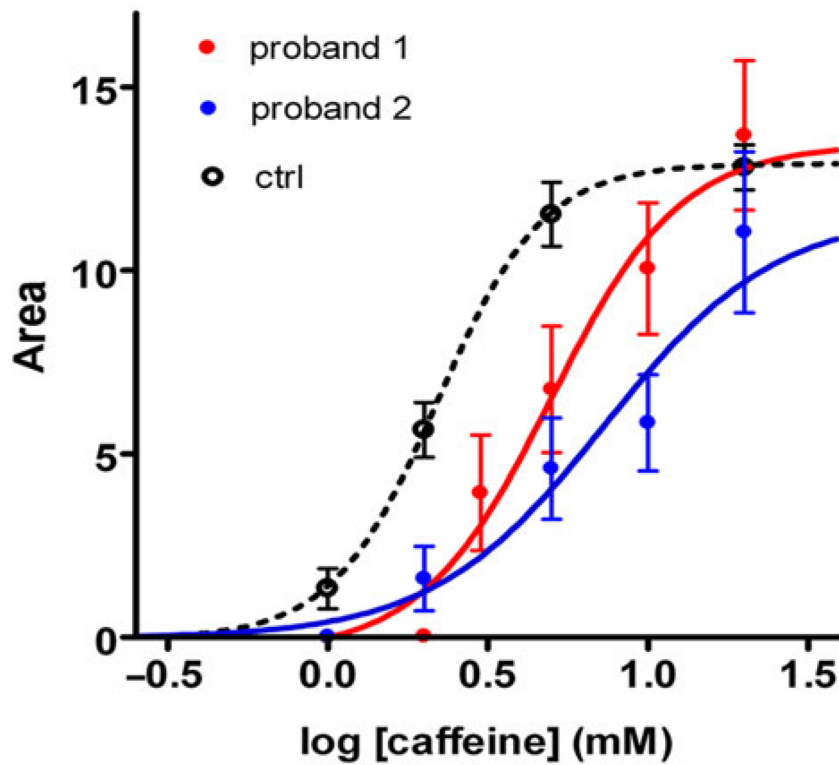


Figure 3_ Graph representing caffeine dose-response curves of permeabilized skeletal muscle fibers from a healthy patient (ctrl) and two subjects carrying the D244G mutation (proband 1 and proband 2). Curves are interpolated by a sigmoidal Hill equation: $Y = B + \{ (A_{max} - B) / (1 + 10^{([log EC_{50} - X] * n))} \}$.

iv. Expression of Recombinant CASQ1^{D244G} Protein Causes Aggregates

The presence of CASQ1 aggregates in fibers from patients prompted us to investigate whether CASQ1^{D244G} possesses an intrinsic ability to form aggregates. In order to verify this hypothesis we expressed recombinant CASQ1^{WT} and CASQ1^{D244G} in cultured primary myocytes isolated from neonatal rat lower limbs. The myocytes were induced to fuse and form myotubes by differentiation medium. Following 12 days in myogenic medium, myotubes were double stained with a monoclonal antibody against CASQ1 and a polyclonal antibody against RYR1. As shown in figure 4_A, both recombinant proteins colocalized with RYR1 at the Junctional SR. However, CASQ1 overexpression induced protein deposits. The CASQ1^{D244G} expression resulted in appearance of CASQ1 positive large aggregates, more frequent and in a higher number with respect to the WT protein overexpression. These aggregates resemble those found in patients' biopsies, even if they did not stain positive for RYR1. Likely, the deposit of other SR proteins was not initiated after the 12 days of differentiation.

In addition, vectors expressing recombinant CASQ1^{WT} and CASQ1^{D244G} were electroporated *in vivo* in Flexor Digitorum Brevis (FDB) muscle of CD1 mice.

After 10 days, the FDB transfected muscles were excised, fixed in glutaraldehyde and prepared for electron microscopy analysis.

As it can be seen in figure 4_B, the muscles transfected with the mutated construct present inclusions similar to those observed in subject affected by the

vacuolar myopathy. Similar aggregates were not found in muscles transfected with the plasmid expressing the CASQ1^{WT} protein.

Electron microscopy was performed in collaboration with Dr. Cecilia Paolini and Prof. Feliciano Protasi (University Gabriele D'Annunzio, Chieti, Italy).

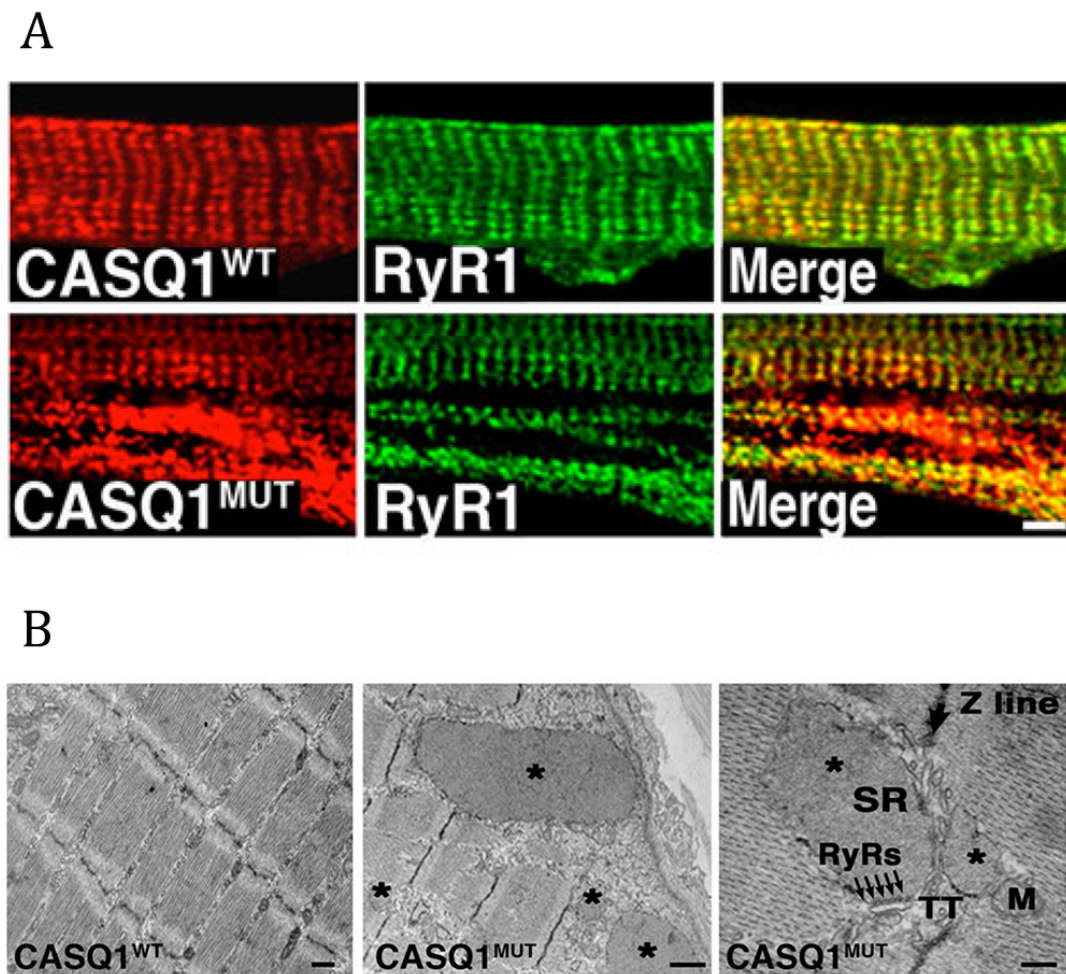


Figure 4_ Immunofluorescence of 12 days differentiated rat myotubes expressing recombinant CASQ1-WT or CASQ1-D244G; immunostained for RYR1 and CASQ1 (A); scale bars 7 μ m. EM images of FDB mouse fibers expressing recombinant CASQ1-WT or CASQ1-D244G (B); vacuoles filled of electron-dense material are marked with asterisks; scale bars from the left 1–0,5–0,1 μ m.

v. Altered Ca^{2+} - dependent organization of mutated CASQ1

In general, CASQ1 molecule exists as either a monomer or polymeric structures in dependence of the environmental Ca^{2+} concentration. Polymerization is mediated by front-to-front and back-to-back interactions between CASQ1 monomers and it is promoted by increasing levels of Ca^{2+} (*Park et al., 2003; Kumar et al., 2013*). In skeletal muscle fibers, expression of CASQ1^{D244G} induces large aggregates containing SR proteins (*Rossi et al., 2014*).

To verify whether the D244G mutation could alter the protein molecular organization, vectors expressing the CASQ1^{WT} or the CASQ1^{D244G} protein in frame with the green fluorescence protein (CASQ1^{WT}-GFP and CASQ1^{D244G}-GFP respectively) were transfected in COS7 cells. The transfected cells were analyzed at confocal microscope with a time-lapse protocol, acquiring one image per minute and in order to study Ca^{2+} -dependent organization of CASQ1 we depleted the intracellular Ca^{2+} stores of COS7 ER. At the start of the time-lapse imaging, the cells were maintained in Krebs' buffer with 1,5 mM Ca^{2+} . As shown in figure 5, at physiological Ca^{2+} concentrations the regular rod-shape structures observed in cells expressing CASQ1^{WT}-GFP, appeared to reflect the assembly of large polymers. After 1 minute, the depletion of the Ca^{2+} stores was induced by thapsigargin administration in modified Krebs' buffer without Ca^{2+} (to avoid influx of external Ca^{2+}). Thapsigargin blocks SERCA pumps activity, leading to the complete depletion of the endoplasmic reticulum Ca^{2+} content.

The observed linear structures showed a high sensibility to Ca^{2+} levels. After thapsigargin-induced Ca^{2+} stores depletion, CASQ1 polymers lost the initial rod-

shape arrangement, which starts to change in a dot-shape organization already after one minute of treatment (Figure 5_A).

Of note, the same linear structures were not observed in cells expressing the CASQ1^{D244G}-GFP protein, and no additional effects were observed following thapsigargin administration (Figure 5_B), suggesting that the mutation may affect Ca²⁺-dependent organization of CASQ1 polymers.

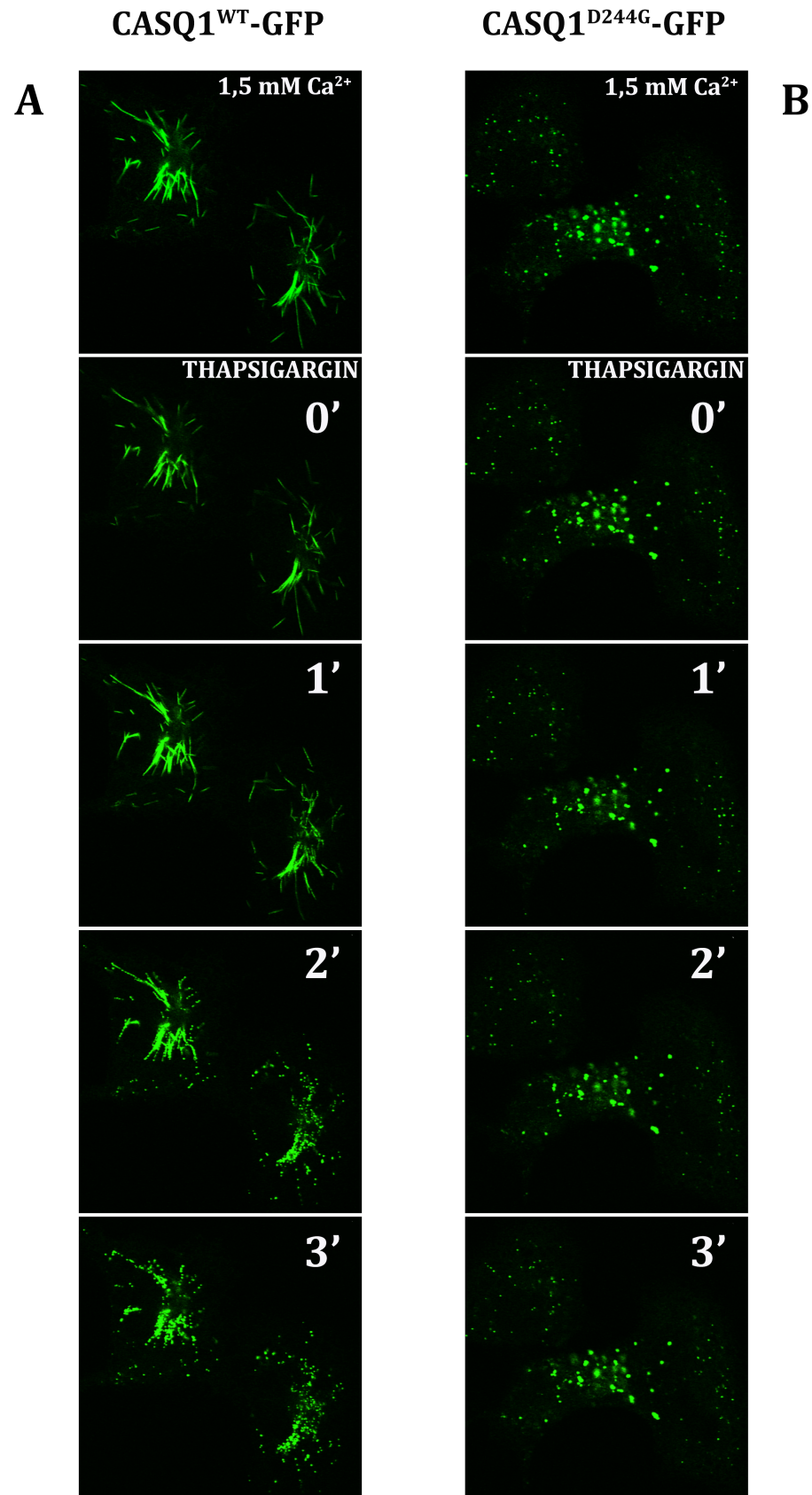


Figure 5_ Confocal time lapse images series of COS7 cells expressing recombinant CASQ1-WT (A) or CASQ1-D244G (B), before (1,5 mM Ca²⁺ in the medium) and after thapsigargin administration (EGTA in the medium).

vi. D244G mutation does not affect mobility of CASQ1 protein

Fluorescent recovery after photobleaching (FRAP) is a technique where fluorescent molecules in a selected region of a cell are irreversibly bleached using a high-powered laser beam and subsequent movement of surrounding non-bleached fluorescent molecules is recorded at lower laser power. GFP fusion proteins are ideal for use in FRAP analysis, because their bleaching does not induce detectable damage to the cell (*Lippincott-Schwartz et al., 1999*). A kinetic parameter that can be discerned from FRAP studies is the mobile fraction, which is the fraction of fluorescent molecules that can diffuse into the bleached region during the time lapse of an experiment (*White and Stelzer, 1999*). When mobile fraction increases, likely the protein has been released from either a restricted compartment or a fixed macromolecular complex. We observed a different molecular organization between CASQ1^{WT} and the mutated protein. As it can be seen in figure 5, at physiological Ca²⁺ concentrations the appearance as large linear aggregates of the WT protein seems to reflect the ability in forming polymers. This ability appears to be lost in the D244G mutated protein, where rod-shape aggregates were never detected. To further investigate aggregation of mutated and WT CASQ1 recombinant proteins we performed FRAP analysis on COS7 cells expressing CASQ1^{WT}-GFP or CASQ1^{D244G}-GFP recombinant proteins. As shown in figure 6, the recovery of fluorescence after bleaching the GFP signal of the tagged proteins was barely detected and similar for both the recombinant proteins analyzed, thus resulting in nearly overlapping FRAP curves (Figure 6_B). Accordingly, no significant differences were detected either in the mobile

fraction or in the half max time values that were calculated from the FRAP curves of both CASQ1^{WT}-GFP and CASQ1^{D244G}-GFP (Figure 6_C-D). The similarity of mobile fraction values between the two recombinant proteins indicates that the substitution of aspartic acid in position 244 to a glycine does not alter CASQ1 protein mobility.

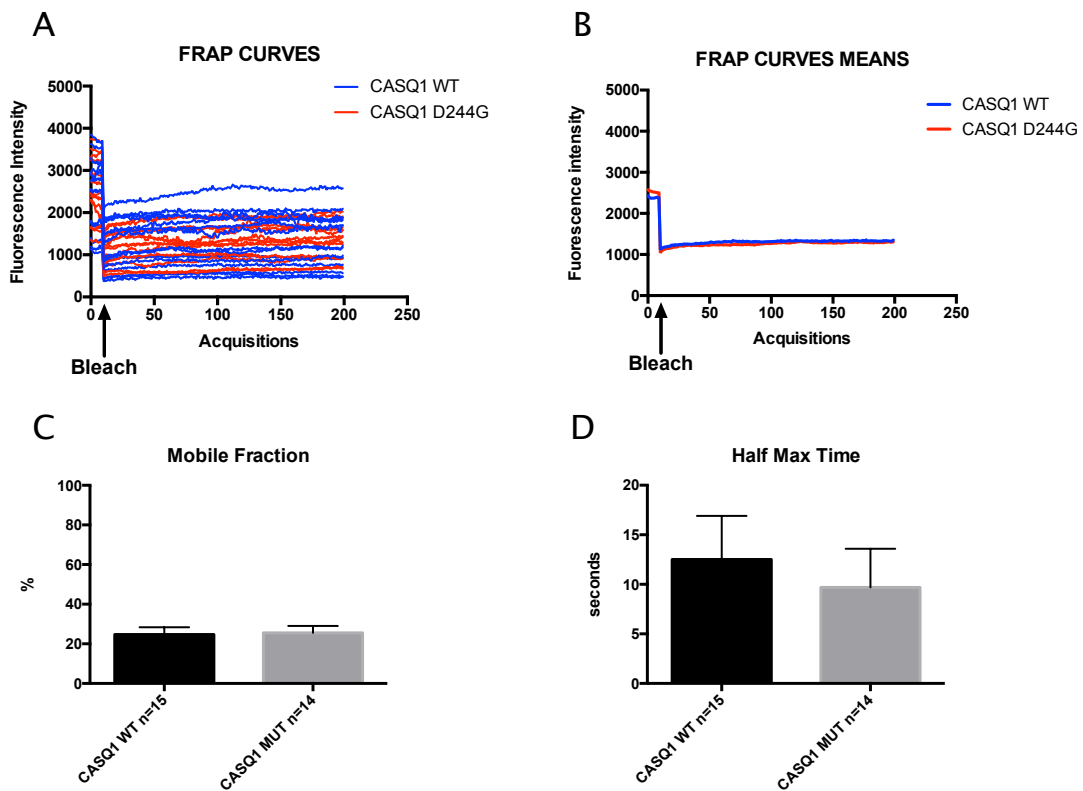


Figure 6_ FRAP analysis of COS7 cells expressing the recombinant CASQ1-WT or CASQ1-D244G (MUT) in frame with GFP. Graph representations of *FRAP curves of all the cells analyzed (A)* and *FRAP curves means (B)*; *Histograms of the means of the percentage of mobile fractions (C)* and of *the half time max (D)*.

vii. D244G mutation alters Ca²⁺- buffering ability of CASQ1

The D244G mutation occurs in a part of the sequence of CASQ1 involved in the formation of a high-affinity Ca²⁺ binding site. Moreover, isolated muscle fibers from subjects carrying this mutation showed altered Ca²⁺ release kinetics from the SR (*Rossi et al., 2014*). We wanted to assess whether the alterations in Ca²⁺ handling described in patients' fibers (see chapter V. iii.), was directly associated with an effect of the mutation on CASQ1 ability to increase Ca²⁺ storage.

We performed immunofluorescence assays on COS7 cells expressing CASQ1^{WT}-GFP or CASQ1^{D244G}-GFP recombinant proteins, stained with a monoclonal antibody against CASQ1 to verify that the GFP signal was effectively related with CASQ1 recombinant tagged proteins. In figure 7_A-B a colocalization between the green signal of the GFP expressed in frame with the recombinant protein and the red signal of the anti-CASQ1 antibody can be clearly seen for both the recombinant protein expressed.

In addition, to evaluate the expression levels of the two recombinant proteins, we performed Western Blotting analysis with total lysates of COS7 cells expressing CASQ1^{WT} or CASQ1^{D244G}. As shown in figure 7_C-D, no significant differences of expression levels were observed between the two proteins analyzed.

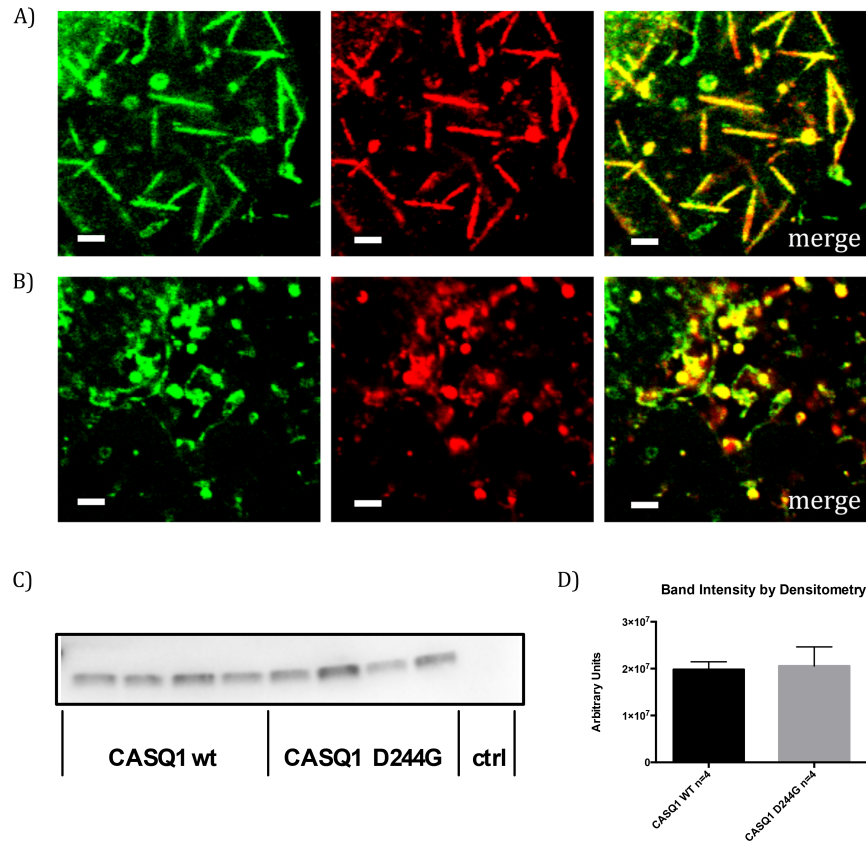


Figure 7_ Immunofluorescence analysis of COS7 cells expressing (A) CASQ1^{WT}-GFP or (B) CASQ1^{D244G}-GFP recombinant proteins in green the GFP signal, in red the signal of monoclonal antibody against CASQ1; scale bars 2 μ m. (C) Western Blot analysis of lysate from cells expressing CASQ1 recombinant proteins (4 different transfections per protein, plus 1 control expressing the empty vector); (D) densitometric quantification of band intensity (mean of the 4 bands); statistical analysis made with student's *t* test.

CASQ1^{WT}-GFP or CASQ1^{D244G}-GFP or the empty vector (as control) were expressed in COS7 cells. The transfected cells were analyzed in a Ca²⁺-imaging assay using FURA2-AM as cytosolic Ca²⁺ indicator. The Ca²⁺ stores were indirectly measured thanks to a thapsigargin-induced Ca²⁺ release (in modified Krebs' buffer without Ca²⁺ to avoid influx of external Ca²⁺). As mentioned before, thapsigargin blocks SERCA pumps activity, leading to the complete depletion of the endoplasmic reticulum Ca²⁺ content. Thapsigargin administration let all the Ca²⁺ contained in the ER to be released in the cytoplasm, where it is bound by FURA2, thus we are able to indirectly measure the quantity of Ca²⁺ stored inside the ER. In figure 8_A, the graphs show Ca²⁺ concentration-time curves. After thapsigargin administration, the Ca²⁺ release in cells transfected with CASQ1^{WT} is much higher with respect to the cells transfected with CASQ1^{D244G} or the GFP empty vector as control.

Results showed that the expression of CASQ1^{WT}-GFP increased the Ca²⁺ stores in the endoplasmic reticulum of COS7 cells, when compared to the control. On the contrary, no significant increase in Ca²⁺ storage was observed when CASQ1^{D244G}-GFP was expressed (Figure 8_B), suggesting that the mutation affects the Ca²⁺ binding ability of the protein.

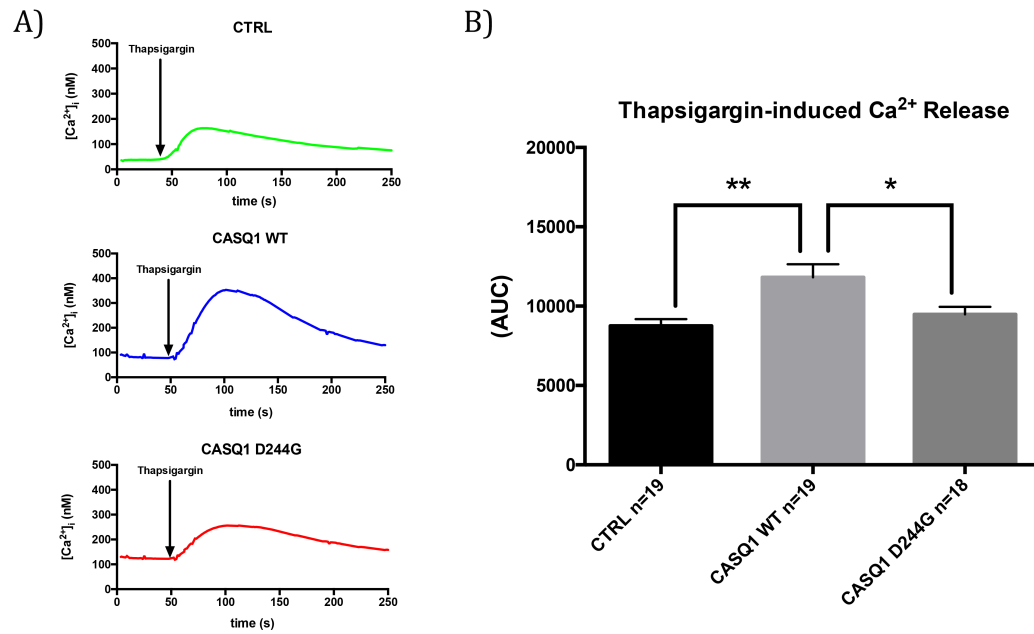


Figure 8_ The graphs report (A) representative thapsigargin – induced Ca^{2+} release curves on COS7 cells expressing $CASQ1^{WT}$ -GFP or $CASQ1^{D244G}$ -GFP recombinant proteins or the GFP alone (CTRL) and (B) the relative histogram of calculated AUCs of a pool of experiments. Statistical analysis made with tukey's multiple comparison test.

VI. DISCUSSION

CASQ1 is the most abundant Ca^{2+} binding protein in the SR of skeletal muscle fibers that participates to the maintenance of the SR Ca^{2+} storage (*MacLennan and Wong in 1971; Damiani et al., 1990; Launikonis et al., 2009*). Recently, we identified the first human mutation of CASQ1 (CASQ1^{D244G}) in a group of patients affected by an inherited form of myopathy characterized by the presence of large intracellular vacuoles in muscle fibers (*Rossi et al., 2014*). CASQ1 shares about 60% of its aminoacidic sequence with CASQ2. Mutations in the CASQ2 gene have been associated to an autosomal-recessive form of catecholaminergic polymorphic ventricular tachycardia (CPVT) (*Lahat et al., 2001*). In particular, the p.Arg33Gln mutation in CASQ2 appears to affect a high-affinity Ca^{2+} -binding site and to interfere with the assembly of longitudinal polymers, resulting in increased aggregates formation and in reduced Ca^{2+} -binding ability (*Kim et al., 2007*). In agreement with this data, the CASQ1^{D244G} mutation affects the aspartic acid in position 244 of CASQ1 protein that is located in a high-affinity Ca^{2+} -binding site and is also close to a region of the protein proposed to be essential for the interaction between adjacent monomers (*Park et al., 2003; Kumar et al., 2013*). Of note, we found that the vacuoles present in the fibers from patients contain several SR protein aggregates. Moreover, we demonstrated that the overexpression of the CASQ1^{D244G} recombinant protein in 12-days differentiated rat myotubes and in skeletal muscle fibers of mice results in a higher frequency of aggregates with respect to the overexpression of CASQ1^{WT}. However, the aggregates found in patients' fibers stained positive for different SR proteins, while in 12-days differentiated myotubes the aggregates resulted positive only for CASQ1. This suggests that probably the aggregates are composed only by CASQ1 at the initial phase and that 12 days are not a sufficient period of time for

the recruitment of the other SR proteins. These findings suggest that the abnormal aggregation of SR proteins in fibers of patients carrying the CASQ1^{D244G} mutation is likely initiated by the deposit of the CASQ1 mutated protein, which might form the seed for the aggregation of the other SR proteins detected in the inclusions.

The crystal structure of CASQ1 shows two different polymerization contacts, front-to-front and back-to-back. These contacts form the basis of linear and highly ordered polymers in dependence of Ca²⁺ levels (*Kumar et al., 2013*). Also in the cardiac isoform of CASQ a similar polymerization behavior was observed and missense mutations in CASQ2 sequence were reported to alter the typical linear aggregates formation (*Park et al., 2004; Kim et al., 2007*). These observations prompted us to investigate whether the aberrant deposits of SR proteins observed in fibers from patients carrying the CASQ1^{D244G} mutation could be a consequence of an altered polymerization of the mutated protein. Indeed, the results presented on this thesis showed that when expressed in COS7 cells, CASQ1^{D244G} loses the ability to form linear polymers in dependence of Ca²⁺ concentration. We also analyzed the protein dynamics of the mutated CASQ1^{D244G} and of the CASQ1^{WT} with confocal microscope. In FRAP analysis performed on transfected COS7 cells, no differences between the CASQ1^{WT} and CASQ1^{D244G} mobile fractions inside the aggregates were observed. This result further supports the hypothesis that the formation of abnormal aggregates and the anomalous polymerization behavior of the CASQ1^{D244G} may be due to an altered interaction capability between CASQ1 monomers. These data are consistent to what observed by Lewis and colleagues (2015), which showed by turbidity assay that the CASQ1^{D244G} mutation induces alterations in the protein polymerization .

It is known that mutations in the CASQ genes usually result in an altered Ca^{2+} binding. Furthermore, as mentioned before, the substitution of the aspartic acid in position 244 in CASQ1 with a glycine affects a high affinity Ca^{2+} -binding (Kim *et al.*, 2007; Sanchez *et al.*, 2012).

Accordingly, we detected altered Ca^{2+} kinetics in fibers from biopsies of patients carrying the CASQ1^{D244G} mutation. This might reflect a reduced Ca^{2+} -binding ability of the CASQ1^{D244G} protein or alterations in its regulatory function on Ca^{2+} release and uptake. Results presented here show that the overexpression of recombinant CASQ1^{WT} in COS7 cells results in an increase of the ER Ca^{2+} -stores that is not detectable following the overexpression of the CASQ1^{D244G} protein, suggesting that the mutation reduces the Ca^{2+} -storage ability of the protein. These results agree with the evidence from the predicted three-dimensional structure of the mutated CASQ1, which showed how the Ca^{2+} binding site affected by the CASQ1^{D244G} mutation loses its typical groove conformation. Also these data are consistent to what observed by Lewis and colleagues (2015). They additionally demonstrated by crystallographic studies that the Ca^{2+} -binding site affected by the mutation loses the ability to bind the ion at low Ca^{2+} levels and that the general structure surrounding the substituted amino acid is altered.

We observed that in COS7 cells transfected with the CASQ1^{D244G} the thapsigargin-induced Ca^{2+} release was significantly lower with respect to the cells transfected with CASQ1^{WT}, reflecting a reduced ability of CASQ1^{D244G} to efficiently store Ca^{2+} in the ER. In fibers from patients carrying the CASQ1^{D244G} mutation the general responsiveness to increasing concentrations of caffeine was significantly reduced in comparison to healthy control fibers. However, after stimulation with 20mM caffeine the maximal Ca^{2+} -release was not significantly different between

healthy and patients' fibers. These results suggest that the mutation might affect a regulatory ability of CASQ1 on RYR1 opening. In agreement with these data, a direct interaction between CASQ2 and RYR2 has been recently proposed by Handhale and colleagues (2016), who demonstrated that CASQ2 binds to the first luminal loop of RYR2. On the other hand, it has been reported that CASQ1 does not directly interact with RYR1, but it rather forms a complex with junctin and triadin. The interaction is made possible thanks to the binding of junctin and triadin on both CASQ1 and RYR1, acting as a molecular bridge between them (Rossi *et al.*, 2014-*b*). The protein complex formed by these three proteins finally regulates the opening of the channel in a Ca^{2+} -dependent manner (Bal *et al.*, 2015; Beard and Dulhunty, 2015).

Therefore, it will be interesting to perform future experiments in order to study possible modifications in the binding between CASQ1^{D244G}, junctin and triadin and the eventual alterations in the regulatory ability on RYR1 channel of the complex formed by these three proteins.

In conclusion, this thesis demonstrates that the CASQ1^{D244G} mutation alters the structure of CASQ1 inducing an abnormal polymerization behavior, the propensity to form disordered aggregates and altering its Ca^{2+} -binding ability. These modifications, together with the overall structural alterations causing vacuolar aggregates observed in muscle fibers carrying CASQ1^{D244G} mutation appear to establish the basis of the resulting myopathy in these patients.

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VIII. ACKNOWLEDGEMENTS

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