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Junctophilins and Climp63: a novel interaction in skeletal muscle

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ABSTRACT

Contraction is the basis of skeletal muscle function. This is made possible by the excitation-contraction coupling (ECC) mechanism occurring at triads, membrane structures formed by the apposition of two terminal cisternae of the sarcoplasmic reticulum and one T-tubule, an invagination of the plasma membrane. At triads, many proteins contribute to ECC and in maintenance of this pivotal structure. Among them, Junctophilins (JPHs) play an essential role in triad formation and ECC regulation by acting as molecular bridges between T-tubules and the terminal cisternae of the SR and by interacting with many proteins of the Ca^{2+} release machinery. By using the Proximity-dependent labelling with BioID2 we identified Climp63 as a novel interactor of JPHs. Climp63 is a non-muscle specific protein, involved in endoplasmic reticulum shaping and interaction with the microtubular network. Interaction between JPHs and Climp63 has been confirmed by co-immunoprecipitation experiments showing that Climp63 is part of a multiprotein complex including JPHs, Triadin and Junctin. Preliminary experiments indicate that Climp63 specifically interacts with JPHs, but not with members of another protein family mediating the formation of membrane contact sites; this interaction is suggested to be mediated by the transmembrane regions of JPHs and Climp63. Analysis of the expression pattern of Climp63 during skeletal muscle differentiation showed that Climp63 is mainly expressed in the embryonic and first weeks of life, suggesting that it may play a role in the first steps of sarcoplasmic reticulum remodeling to form mature triads.

INTRODUCTION

SKELETAL MUSCLE TISSUE

The skeletal muscle is a major organ system representing almost 40% of total body weight and containing the majority (50-75%) of all proteins present in the body (Frontera & Ochala, 2015).

Its main function is to convert chemical energy into mechanical one, to allow the contraction necessary to create movements and maintaining body posture (Frontera & Ochala, 2015). In addition, it also significantly contributes to metabolic functions, by regulating blood glucose levels (Rose & Richter, 2005).

The skeletal muscle tissue is composed of bundles of long cylindrical shaped multinucleated cells, called myofibers, that develop from the fusion of multiple progenitor muscle cells named myoblasts (Sosa et al., 1994). A small population of stem cells remains even in the adulthood, with the name of satellite cells, positioned in the periphery of the muscle fibres as a reservoir for maintenance and regeneration of the tissue (Dumont et al., 2015).

The whole muscle is surrounded by a dense connective tissue layer called epimysium, while the bundles of fibers composing the muscle, also called fascicles, are surrounded by the perimysium. Each fiber is then connected to each other thanks to a layer of reticular connective tissue, the endomysium, filled by blood capillaries (**Figure 1**) (Exeter & Connell, 2010).

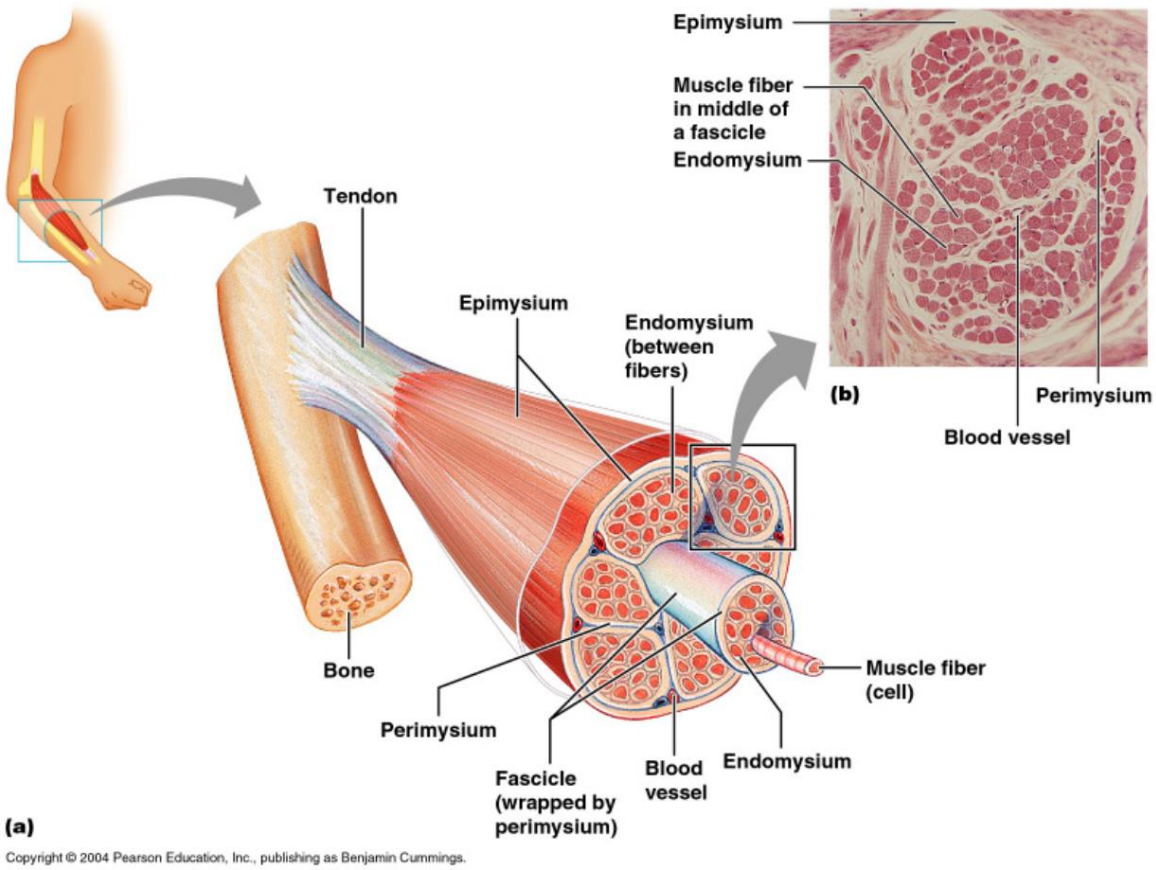


Figure 1 *(**a**) Schematic representation of a skeletal muscle attached to the bone by the tendon. The whole muscle is surrounded by the epimysium, encapsulating muscle fascicles wrapped by the perimysium. Each fascicle is composed by multiple muscle fibers, each surrounded by the endomysium. (**b**) Histological sample of skeletal muscle tissue showing a transversal section* (Marieb, 2004).

MICROSCOPIC ANATOMY OF SKELETAL MUSCLE FIBERS

Single fibers are delimited by the plasma membrane called sarcolemma and their cytoplasm is named sarcoplasm (Frontera & Ochala, 2015); here repeating bundles of myofilaments, called myofibrils, composed by actin, myosin, titin, and nebulin are present, that altogether give the muscle cells their striated appearance when observed under the light microscope (Clark et al., 2002).

Thin filaments are mainly composed by 2 strands of globular polymers of G-actin forming filamentous actin (F-actin) disposed in a helicoidal structure (Irving, 2017). Actin filaments are associated with 2 regulatory proteins: tropomyosin, a filamentous protein that covers the myosin binding sites on actin, and troponin formed by three subunits: Troponin T (TnT) which mediates the tropomyosin binding, Troponin C (TnC) able to bind Ca^{2+} and Troponin I (TnI) acting as the inhibitory subunit (Ebashi et al., 1969). Additional actin-binding proteins include CapZ and tropomodulin (Tmod family) that bind the minus and plus ends of actin filaments regulating their elongation and

shortening processes (Gokhin & Fowler, 2011); in addition, the filamentous protein nebulin interacts and stabilizes the thin filaments, regulating their assembly and function (Pappas et al., 2011).

Myosin, representing the main component of thick filaments, is the molecular engine of the contractile apparatus, being able to convert chemical energy from Adenosine Trisphosphate (ATP), into mechanical force thanks to the interaction with the thin filaments (Spudich et al., 1995). Class II myosin, the muscle specific isoform, is composed by two heavy chains (MHC, 223 kDa) and four light chains (MLC, 15-22kDa); each heavy chain is composed by a conserved globular head containing the ATPase activity alongside with the actin binding site, a neck domain acting as a hinge region, and a long tail capable of dimerization; the light chains (2 per head), bind the neck region of MHC and include MLC20, also known as the regulatory light chain and MLC17, also known as the essential light chain (Syamaladevi et al., 2012).

Different isoforms of MHC, differing in their contraction velocity and contractile properties are expressed in skeletal muscle fibers, allowing a categorization of muscle fibers into four subtypes, namely one slow and three fast subtypes. This classification partially overlaps with the metabolic properties of muscle fibers that can be categorized from glycolytic to oxidative (Schiaffino & Reggiani, 2011). Muscle fibers can change with age, neuromuscular activity, mechanical loading, and hormonal profile, allowing a sequential and reversible fiber type transition in response to different stimuli (Talbot & Maves, 2016).

Slow-twitch fibers are classified as type 1 (expressing MHC7), while fast-twitch fibers are classified as type 2, that are further subclassified into 3 types: 2A (expressing MHC2), 2X (expressing MHC1) and 2B (expressing myosin MHC4); fibers with hybrid MHC expression also exist, giving rise to further subtypes. Finally, additional MHCs isoforms are expressed only during muscle development (Schiaffino & Reggiani, 2011). As previously described, fiber types may also differ in their metabolic properties, with Type 1 and 2A fibers that primarily use oxidative metabolism, while type 2X and 2B fibers relying upon anaerobic glycolytic metabolism (**Figure 2)** (Pette & Staron, 2000; Schiaffino & Reggiani, 2011).

Mammals show a distinctive composition in fiber type for each muscle; for example, in mouse, *Soleus* muscles are composed predominantly by slow-twitch type 1 fibers, while *Extensor Digitourm Longus* (EDL) and *Tibialis anterior* (TA) are mainly composed by fast-twitch type 2 fibers; others, like *Gastrocnemius* and *Flexor Digitorum Brevis* (FDB) have a mixed composition with a slight predominance of fast-twitch type 2 fibers (Johnson et al., 1973).

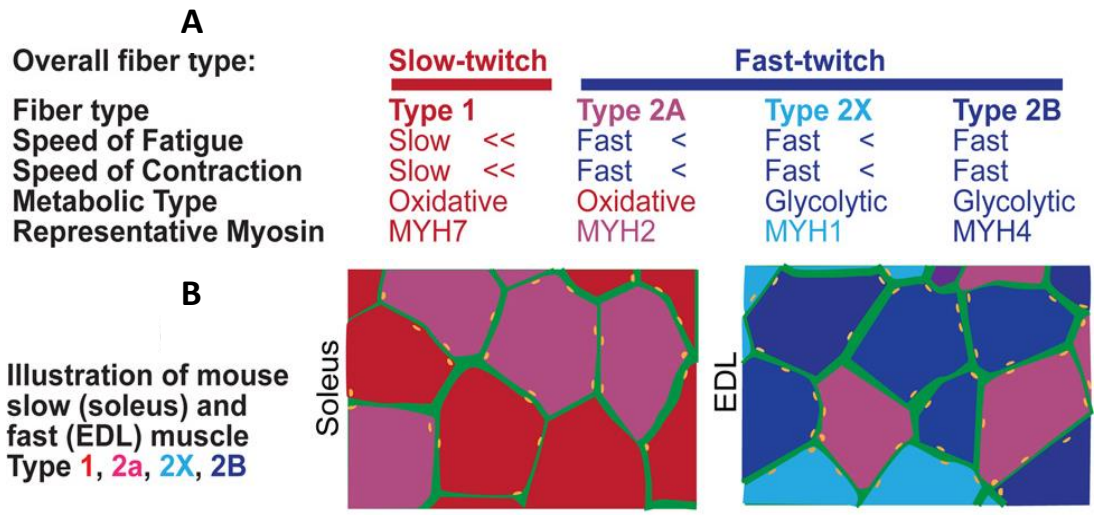


Figure 2 (A) *Summary scheme of slow-twitch and fast-twitch fiber subtypes, indicating the most representative MHC isotype and metabolic profile.* ***(B)*** *Representative images of a slow-twitch muscle, the soleus, and the EDL which is composed mainly by fast-twitch muscle fibers. Figure adapted from* Talbot & Maves, 2016 .

The relative arrangement of thick and thin filaments in a muscle fiber account for the striated pattern that can be observed under light microscopy, consisting of an alternation of light and dark bands. Light bands, also called I bands because they appear isotropic under polarized light, are mainly formed by the thin filaments, while dark bands, also called A bands because they appear anisotropic under polarized light, are composed by the overlap of thick and thin filaments. Analysis under the electron microscope reveals a more complex organization. Myofibrils are composed of sarcomeres, the functional and structural units of striated muscles, that repeat in series. Each sarcomere is defined as the segment of a myofibril included between two neighboring Z-disks; these are dark lines that dissect each I band in two symmetric halves and that anchor the actin myofilaments via the Z-disk protein α-actinin (Sheikh et al., 2007). Within the A-band, a lighter region called the H-zone, represents the zone where only thick filaments are present, with no overlap with thin filaments. Finally, within the H-zone, a thin M-line supports the anchorage of thick filaments at the center of the sarcomere, by binding to myomesin and C-protein (Lamber et al., 2022) (**Figure 3 and 4**).

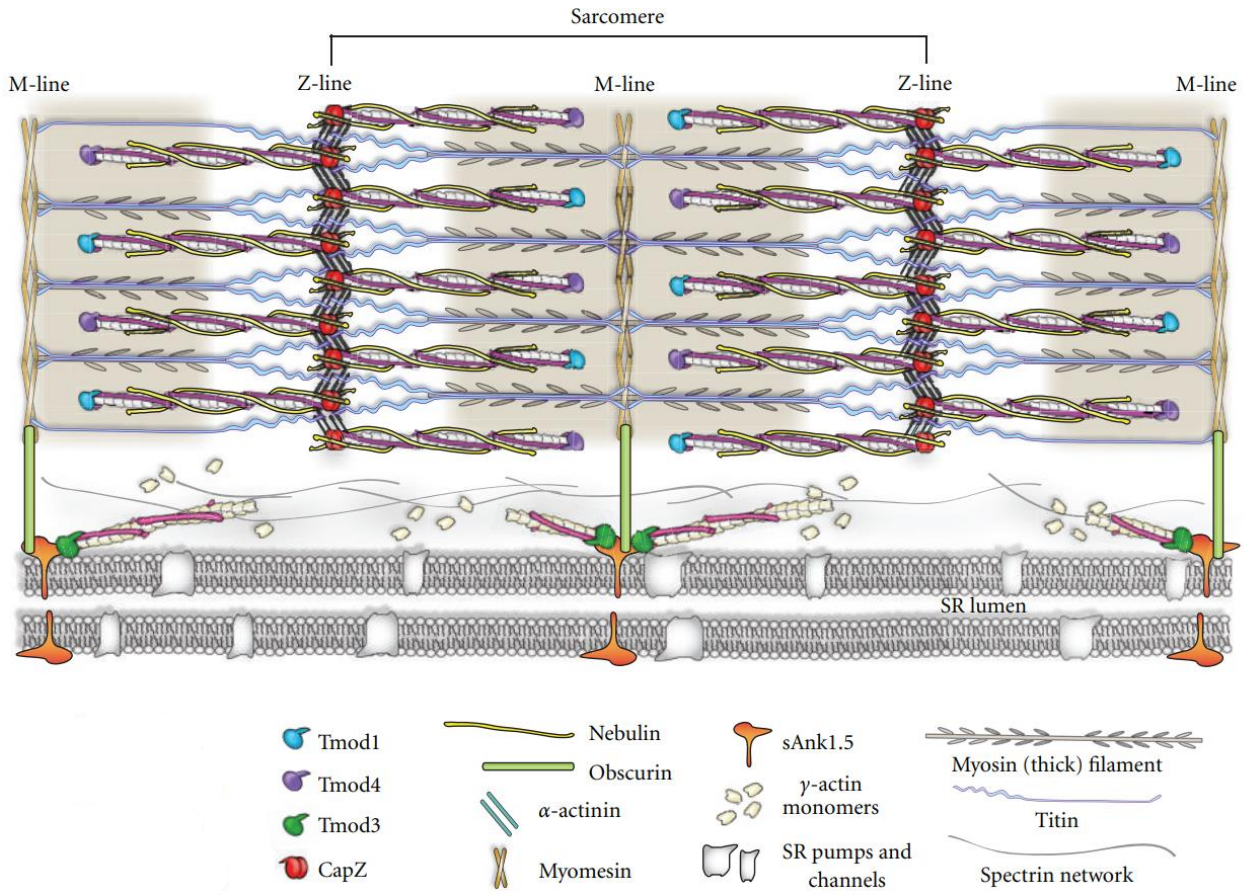


Figure 3 *Schematic representation of the contractile elements forming the muscle fiber. Thin filaments composed by actin and the accessory proteins tropomyosin and troponin are anchored to the Z disk, formed mainly by the rod-shaped actin-binding protein α-actinin. Myosin- formed, thick filaments are anchored at the level of the M line by the structural protein myomesin. Other fundamental proteins are titin, which connects, like a molecular spring, the thick filaments to the Z-disk, nebulin involved in the assembly of actin during muscle development and obscurin, which connects the contractile apparatus to the SR membrane, via its interaction with sAnk1.5. Image adapted from (Gokhin & Fowler, 2011).*

The giant protein titin extends from the Z-disk to the M-band, acting as a molecular spring granting the passive elasticity to the muscle structure, and providing guidance for the correct assembly of muscle filaments during development (Tskhovrebova & Trinick, 2003). Another giant protein is obscurin, which regulates myofibril assembly and the interaction between sarcomere and sarcoplasmic reticulum (Kontrogianni-Konstantopoulos & Bloch, 2006; Bagnato et al., 2003) (**Figure 3)**.

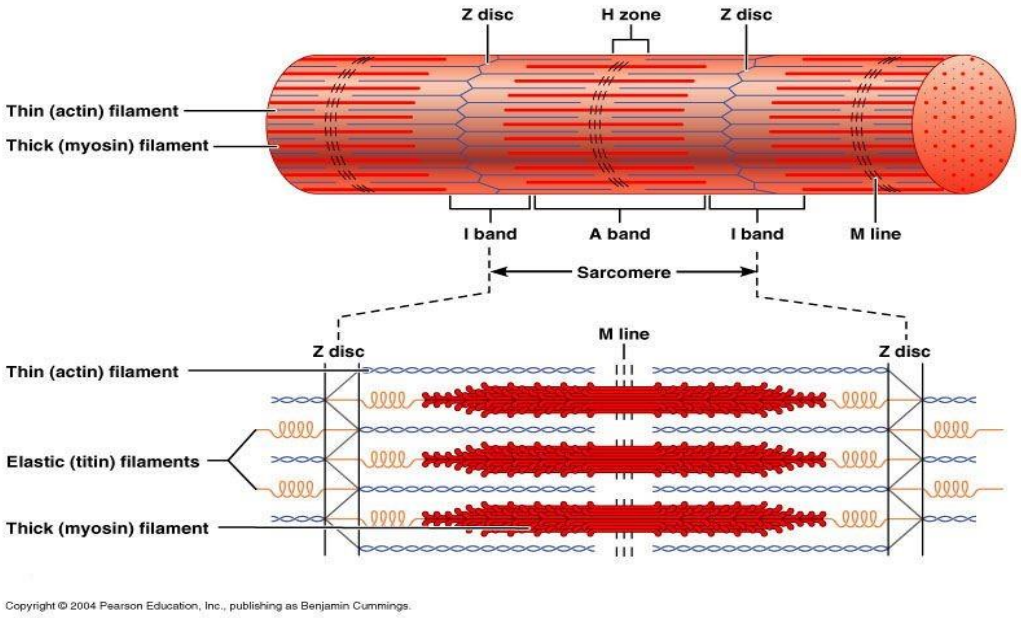


Figure 4 *Schematic representation of myofibril bands and sarcomere composition* (Pearson Education Inc., 2004).

THE SARCOPLASMIC RETICULUM, TRIADS AND THE EXCITATION-CONTRACTION COUPLING MECHANISM

The Sarcoplasmic Reticulum (SR) of skeletal muscles is a specialized form of endoplasmic reticulum dedicated to the Ca^{2+} homeostasis necessary for muscle contraction (Kaisto & Metsikkö, 2003). The first pioneering analysis of striated muscle cells using electron microscopy (Block & Franzini-Armstrong, 1988) disclosed the SR organization into numerous interconnected tubules forming a network of longitudinal elements surrounding the myofibrils. These structures, called longitudinal SR (l-SR), flow into collective terminal cisternae named junctional SR (j-SR), that maintain a precise spatial localization with reference to the underlying sarcomere. In skeletal muscles, the l-SR is localized around the A and I-bands, while the j-SR terminal cisternae are positioned in the regions at the borders between A and I-bands; here they establish contact with muscle cell specific infoldings of the sarcolemma, called T-Tubules (TT)(**Figure 5**) (Block and Franzini-Armstrong, 1988; Sorrentino, 2011). A TT interposed between two terminal cisternae of the j-SR forms a triad, a structure that mediates the process of Excitation-Contraction Coupling (ECC) in skeletal muscles (Dulhunty et al., 2002). ECC is the mechanism that allows muscle contraction thanks to the interaction of the dihydropyridine receptors (DHPR) (Rios & Brum, 1987), voltage-dependent Ca^{2+} channels located on the TT, that, in response to the sarcolemma depolarization induced by acetylcholine released at the neuromuscular junction (Hubbard, 1973; Katz, 1996), undergo a

conformational change that is transmitted (Ríos & Pizarro, 1991) to the nearing ryanodine receptors 1 (RyR1), Ca^{2+} channels located on the terminal cisternae of the j-SR. Activated RyR1 channels will trigger Ca^{2+} efflux from the SR into the myofiber's cytosol, leading to muscle contraction (Avila & Dirksen, 2000; Kuo & Ehrlich, 2015). The activity of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCA) pumps, embedded on the l-SR, allows Ca^{2+} re-uptake from the cytosol for the next contraction-relaxation cycle to take place (Bublitz et al., 2013).

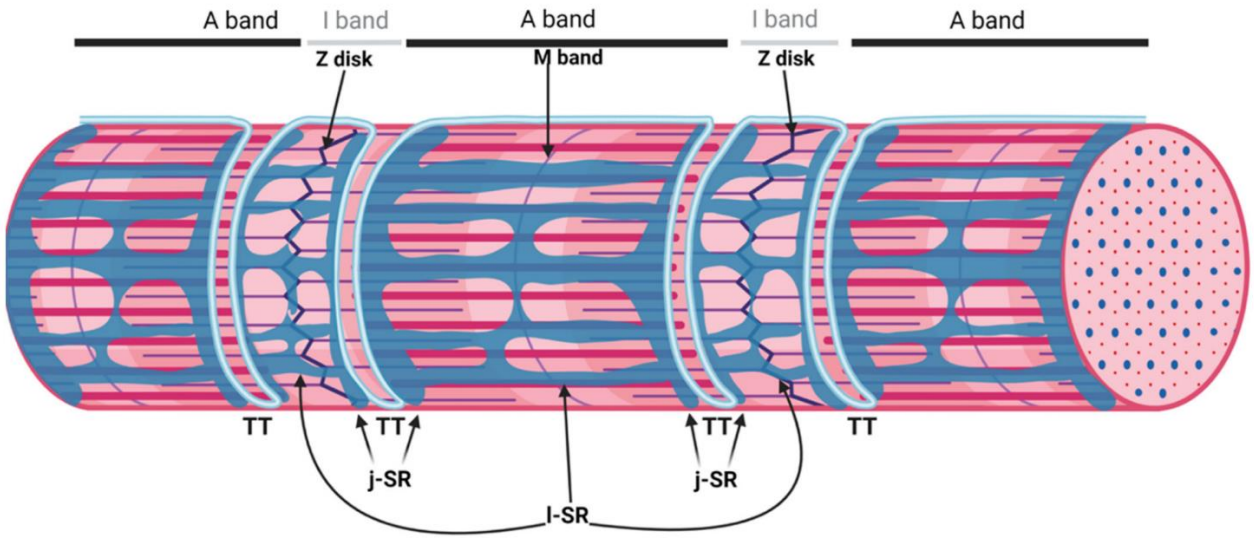


Figure 5 *Schematic representation of SR and myofilaments in a mammalian skeletal muscle fiber. The regular alternation of dark and light bands is depicted in the upper part of the image. Longitudinal SR (l-SR), Junctional SR (j-SR) and T tubules (TT) have a precise localization in relation to the underlaying myofibrils* (Rossi et al., 2022a).

TRIAD BIOGENESIS

Electron and confocal microscopy studies have characterized, from a morphological point of view, the events of TT maturation and triad formation, revealing that juxtaposition of the j-SR and TT begins in the embryonic period of development (E) and evolves through sequential steps in the postnatal-life (P) (Flucher et al., 1993; Takekura et al., 2001). In mammals, the first step is represented by the formation of tubular SR adjacent to myofibrils, that will subsequently develop into reticular structures surrounding myofibrils (Luff & Atwood, 1971). SR was detected with electron microscopy as early as day E14; by day E15 immunohistochemical analysis revealed the presence of RyR1 clusters positioned at the periphery of developing myotubes, and by day E16 punctate signals of RyR1 increase in number through all the fiber and start positioning at the A-I band borders. In the period from day E17 to birth, the j-SR starts to acquire a transversal orientation with RyR1 signals appearing at each side of Z disks. TT development starts shortly after the SR, when, at day E15, small

tubular invaginations can be observed at the periphery of developing muscle fibers. By day E19, these invaginations penetrate deeper into the fiber, with the majority remaining longitudinal, until late after birth; final maturation, with complete transverse orientation is achieved around 3 weeks of post-natal age (Franzini-Armstrong, 1991; Takekura et al., 2001).

The first associations between SR and sarcolemma membranes are present as early as E14-E15**,** mainly at the periphery of the fiber, and then, starting from E16, internal junction sites appear as dyads, formed by one TT and one j-SR cisterna. By E17-E18 most of j-SR/TT junctions are represented by triads, showing the presence of electron dense regions between SR-TT borders that are represented by RyR1 channels located in the SR membrane; inside the SR vesicles, dark signals represent Calsequestrin, the main Ca^{2+} binding protein of the SR (Jorgensen et al., 1985). Triad formation and maturation can be observed during in vitro differentiation, where the main steps of SR and TT development are recapitulated and can be followed using antibodies against RyR1, marking the j-SR (**Figure 6**) (Rossi, et al., 2022a).

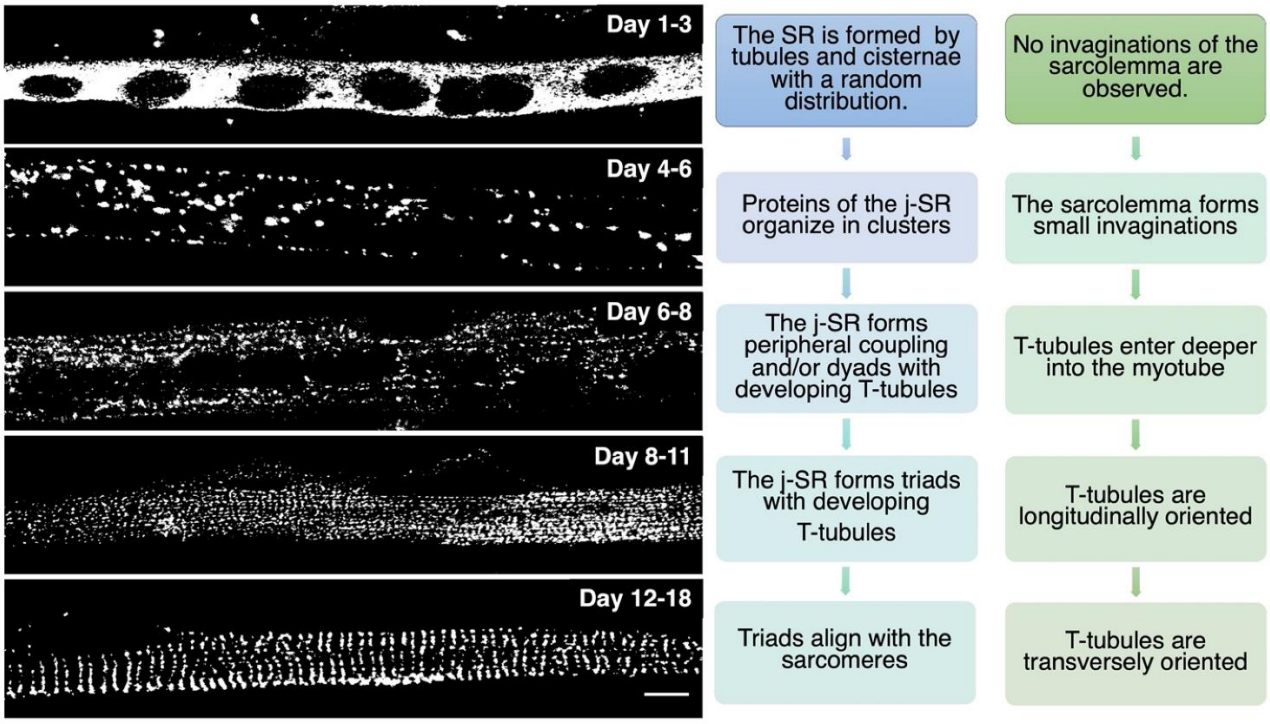


Figure 6 *Image depicting the main stages of triad maturation in primary rat myocytes differentiating in vitro. Cells were induced to differentiate from 1 – 18 days in vitro and were labelled with anti RyR1 antibodies marking the nascent j-SR component. In the first steps, RyR1 signal shows a diffuse distribution in the SR. At days 4-6 and 6-8 RyR1 clusters were observed, organized in peripheral couplings at the surface sarcolemma and as dyads with the nascent TTs inside the muscle cell. At day 8-11, triads are observed and by day 12-18 a full maturation with a precise transverse orientation is reached. Image from Rossi et al., 2022a.*

While the events that characterize TT maturation and triads formation are morphologically defined, the specific molecular mechanisms guiding these processes are more elusive. RyR1 and DHPR, although essential for Ca^{2+} release, are not required for the formation of the triadic structures, since studies using immunofluorescence and electron microscope have shown that triads still form in knockout (KO) models for either one or both proteins (Felder et al., 2002)**.** Several lipid binding proteins and phospholipids regulating enzymes have been described to participate in the TT and j-SR biogenesis and organization, like **Caveolin 3 (Cav3), amphiphysin2 / Bridging integrator-1 (BIN1), dysferlin (DYSF), Mitsugumins, Myotubularin (MTM1)** and **Junctophilins (JPHs).** Their role will be discussed in the following paragraphs (Al-Qusairi & Laporte, 2011).

Caveolins are structural proteins of caveolae, peripheral invaginations occurring in cholesterol-rich regions of the plasma membrane (Parton et al., 2006). Caveolin 3 (Cav3), the main skeletal muscle isoform (Tang et al., 1996), participates in the maturation of TTs (Parton et al., 1997). Mutations in this protein are associated with several myopathies characterized by TT structural alterations like Limb-girdle muscular dystrophy (LGMD1C) (Minetti et al., 1998), rippling muscle disease (Betz et al., 2001), familial hypertrophic cardiomyopathy (Hayashi et al., 2004) and long QT syndrome 9 (Vatta et al., 2006). Additional evidence of the importance of Cav3 in maintaining an appropriate TT architecture also comes from knockout animal models where muscle fibers show morphological abnormalities in the TT system with tubule dilation and loss of transversal orientation (Galbiati et al., 2001). Although its precise role remains unknown, Cav3 is often used as a marker for the formation and development of the TT (Ziman et al., 2010).

Amphiphysin 2, also called Bridging integrator-1 (BIN1), is part of a class of proteins able to shape the membrane thanks to the interaction with membrane lipids via their BAR (Bin/Amphiphysin/Rvs) domain (Lee et al., 2002). They are also postulated to participate in TT formation, acting either alone (Wechsler-Reya et al., 1998) or by recruiting another membrane tubulating protein, Dynamin2 (DNM2) (Bitoun et al., 2005; Takei et al., 1999). Compelling evidence of its involvement in this process comes from experiments involving Bin1 expression in fibroblast cells, where it can induce the appearance of TT-like structures (Lee et al., 2002). Bin1 mutations are associated with alteration in TT structure observed both in “in vitro” studies (Kojima et al., 2004) and in human muscles from patients affected by forms of centronuclear myopathies (Toussaint et al., 2011) or in murine null mutation models (Razzaq et al., 2001) .

Dysferlin (DYSF), or dystrophy-associated fer-1-like protein, is an integral membrane protein able to bind membrane phospholipids in the presence of Ca^{2+} (Anderson et al., 1999). The most characterized role of DYSF is as a mediator of skeletal muscle membrane repair; studies using high-resolution imaging revealed its accumulation in the vicinity of membrane injury (Roostalu & Strähle, 2012). DYSF-defective fibers show a slower sarcolemma reseal time after damage compared to wild type controls and DYSF deficient mice present an abnormal accumulation of vesicles at the damaged site (Bansal et al., 2003; Klinge et al., 2010) . Interestingly, Dysferlin and Cav3 interact in skeletal muscle (Hernández-Deviez et al., 2006; Matsuda, 2001), and together with the triadic protein Mitsugumin-53, they play a crucial role in the membrane repair process (Cai et al., 2009a). Mutations associated with *DYSF* are associated with myopathies like limb girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy (MM) and distal anterior compartment myopathy (Bashir et al., 1998; Illa et al., 2001; Liu et al., 1998; Cai et al., 2009c).

Mitsugumin-53 (MG53), also known as tripartite motif 72 (TRIM72), is a skeletal and cardiac muscle-specific protein of 53 kDa, that contributes to the vesicle trafficking involved in membrane repair machinery (Cai et al.; 2009a; Cai et al., 2009b). MG53 increases during myogenesis and its contribution in the repair of damaged membranes is mediated by binding to phosphatidylserine at the damaged site, acting as a scaffold to recruit other "repair proteins" like Cav3, DYSF and annexin V (Cai et al., 2009a). MG53 knockout mice develop progressive myopathy, likely correlated with an impediment in the membrane repair mechanism (Cai et al., 2009a). Expression of a recombinant protein has been shown to ameliorate the muscle phenotype in the muscular dystrophy mdx mouse model and in cardiotoxin-injected mice (Weisleder et al., 2012; Zhang et al., 2017). Currently, pathogenic mutations have not been identified, but MG53 has been found to be upregulated in patients with muscular dystrophy (Waddell et al., 2011) .

Mitsugumin 29 (MG29) is a 29 kDa skeletal muscle transmembrane protein belonging to the synaptophysin family, which is involved in the process of synaptic vesicles and cell membrane fusion (Takeshima et al., 1998). This protein has an early expression during myogenesis, firstly associating to newly formed SR vesicles and then, once formed, to triads (Komazaki et al., 1999). MG29 knockout mice present with decreased muscle mass and general weakness, correlated with structural abnormalities in both SR and TT; they show swollen TT and less organized triads, suggesting a role for MG29 in the assembly of these structures (Nishi et al., 1999). Additional evidence comes from studies conducted in mouse model of heart failure, where transgenic

expression of MG29 induced a protective phenotype increasing the organization of TTs and preserving cardiac contraction and relaxation parameters (Correll et al., 2017).

Myotubularin (MTM1) is a lipid phosphatase that localizes at the SR cisternae of triadic junctions (Toussaint et al., 2011). The protein is ubiquitously expressed, but its muscle specific role is highlighted by the fact that mutations in this gene are associated with X-linked myotubular myopathy, a skeletal muscle disorder resulting in severe muscle weakness with abnormal nuclei positioning (Laporte et al., 1996). Mice knockout for MTM1 present with progressive centronuclear myopathy with SR and TT disorganization, that appears as a higher presence of longitudinal TT and complete absence of the invaginations in some triadic junctions (Al-Qusairi et al., 2009; Amoasii et al., 2013). The loss of the phosphatase activity of the protein has been proposed to lead to abnormal dephosphorylation of targeted PtdIns3P/PtdIns(3,5)P2 and subsequent abnormal endosomal vesicles trafficking and maturation (Robinson & Dixon, 2006). Amoasii et al., (2013) also proposed a model where MTM1 regulates SR membrane curvature by acting on the PtdIns3P membrane subdomains, which would in turn influence TT organization.

MTM1 colocalizes and interacts with BIN1, and this interaction results in increased membrane tubulation (Royer et al., 2013). It is likely that MTM1 plays a role in the late stage and/or maintenance of TT rather than in the early biogenesis, because its expression increases in postnatal life and MTM1 null mice show less altered TT structures at younger stages rather than at later ages (Al-Qusairi et al., 2009).

TRIADIC PROTEINS

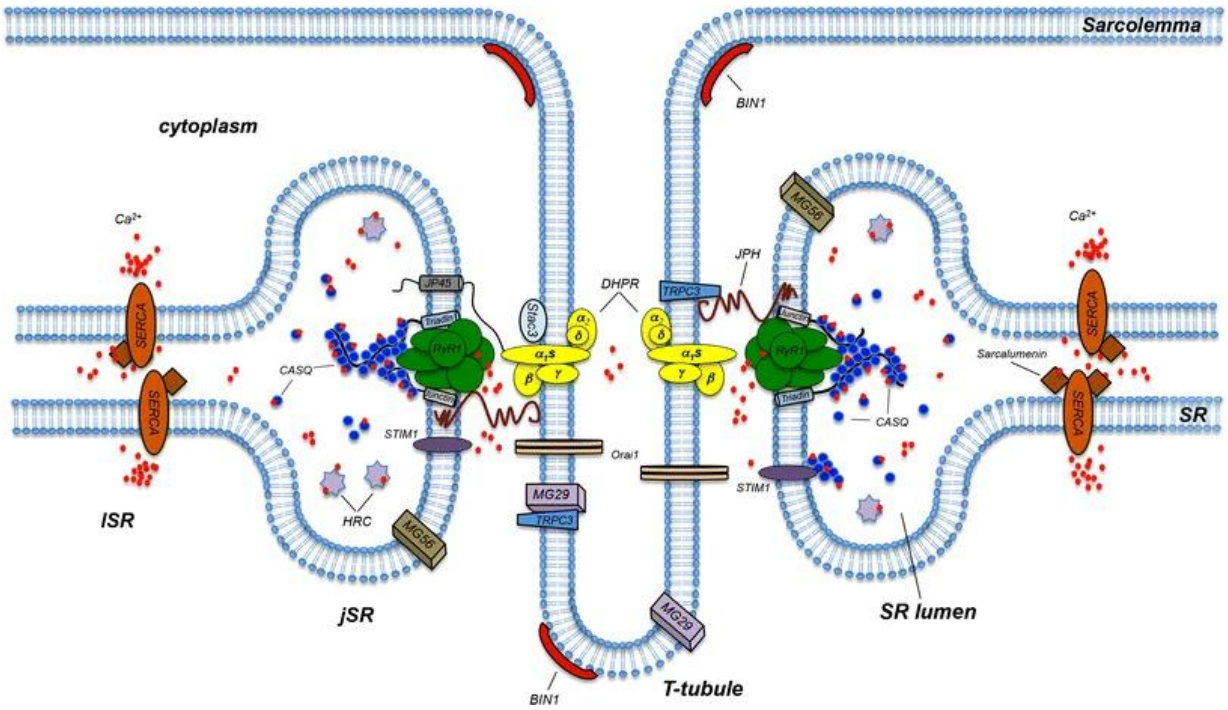


Figure 7 *Schematic representation of a triadic structure. Dihydropyridine receptors (DHPR) on the T-tubule (TT) are voltage gated Ca^{2+} channels composed by 5 subunits (α1s, α2, δ, β1α, and γ1). Following the motoneuron-junction induced depolarization of the sarcolemma, they undergo a conformational change that is transmitted to the adjacent Ryanodine receptors 1 (RyR1) on the junctional Sarcoplasmic reticulum (j-SR). RyR1 are homotetrameric Ca^{2+} channels that, when opened during the Excitation Contraction Coupling (ECC) mechanism, allow the ion efflux from the SR to the cell cytoplasm, where it will enable the contraction process. Following this mechanism, the sarcoplasmic/endoplasmic reticulum Ca^{2}+ ATPases (SERCA) will actively pump the ion back in the reticulum. Several other proteins participate in this process and in forming and maintaining the triadic architecture pivotal for the ECC mechanism to occur: STAC3 on the TT regulates the coupling of DHPR with RyR1; Junctophilins (JPH) have a structural role of maintaining the apposition between TT and J-SR; Triadin and Junctin are 2 transmembrane (TM) proteins of the j-SR that have regulatory roles on the ECC mechanism via their interaction with RyR1 and other proteins; JP-45 on the j-SR able to interact with Calsequestrin and the DHPR; Transient receptor potential canonical type 3 (TRPC3) on the TT are non-selective cation channels that allow Ca^{2+} entry . Inside the lumen of the SR, vhistidine-rich Ca^{2+}-binding protein (HRC) and Calsequestrin (CASQ) bind and buffer Ca^{2+}. Another mechanism to replenish the intraluminal ion concentration, other than the SERCA pumps, is represented by the Store Operated Calcium Entry (SOCE): in low intracellular Ca^{2+} conditions the j-SR TM proteins STIM aggregates and relocate in the proximity of the Orai1 channels on the plasma membrane, opening them and allowing the ion influx from the extracellular space inside the reticulum. Other important players in the formation of the triadic structure are: Bridging integrator-1 (Bin1) able to generate membrane curvatures pivotal to TT formation; Mitsugumin-29 (MG29) and Mitsugumin-53 (MG53) which have a role in the first steps of the assembly of the triadic structure. Image from* (Barone et al., 2015).

Ryanodine receptors (RyR) are a family of Ca^{2+} release channels found on the j-SR and represent the largest ion channels known to date. There are three RyR isoforms (RyR1, RyR2 and RyR3) encoded by three distinct genes that share a 70% sequence similarity; RyR1 is mainly expressed in skeletal muscle, RyR2 is mostly expressed in cardiac muscle, while RyR3 has a wider range of tissue expression, including skeletal muscle (Rossi & Sorrentino, 2002). The skeletal muscle specific RyR1 channels are made by 4 identical monomers of 565 kDa, forming a homotetramer of ~2.2 MDa (Lai et al., 1988); each subunit is built around an extended scaffold of α-solenoid repeats (Groves &

Barford, 1999) and consists of a small cytoplasmic C-terminal portion, a TM region and a big N-terminus located in the cytoplasm. The pore is structurally homologous to that of the six-transmembrane (6 TM) ion channel superfamily, with 6 TM helices per subunit surrounding the central pore (Zalk et al., 2015).

In skeletal muscles, RyR1 channels are mainly activated by direct interaction with the DHPRs situated on the TT. These channels act as voltage-sensors that open RyR1 and activate Ca^{2+} efflux from the SR into the cytosol, thus triggering muscle contraction according to the ECC mechanism and the sliding filaments model (Block et al., 1988; Schneider & Chandler, 1973).

Several sites for small molecules and auxiliary proteins can be found on RyR1, including a specific site for the drugs dantrolene and ryanodine, an alkaloid found in a poisonous plant, from which the receptor takes its name (Endo et al., 1970). RyR1 has positive and negative regulators, that bind both the cytoplasmic and SR luminal portions: examples of positive modulators are Homer1c, ATP, imperatoxin, caffeine, halothane, Protein Kinase A, Ca^{2+}/Calmodulin-dependent protein kinase II (CamKII) and pentachlorobiphenyl (PCB95); negative regulators include Fk506-binding proteins (FKBPs), dantrolene, natrin and Mg^{2+}, while molecules like ryanodine, calmodulin (CaM) or Ca^{2+} have different effects depending on Ca^{2+} concentration (van Petegem, 2012). Furthermore, RyRs have also shown to be modulated by post-translational modifications including oxidation, phosphorylation and nitrosylation (Santulli et al., 2018); these modifications mainly concern the cytoplasmic shell and are proposed to influence the gating mechanism mainly by interacting with other regulators (Gambardella et al., 2017; Zalk et al., 2015).

Triadin (Trisk) is a muscle specific protein, firstly identified and isolated in the triads of skeletal muscles (Brandt et al., 1990; Kim et al., 1990). Structurally it is a type II TM protein anchored in the SR membrane, with a short cytoplasmic segment and a longer luminal domain that interacts and modulates different components of the calcium release complex at triads and dyads (Marty, 2015). The Triadin gene (TRDN) generates, via alternative splicing, 4 different isoforms named according to their molecular weight: the skeletal muscle isoforms Trisk 95 and Trisk 51 (Marty et al., 2009; Perez, 2011) the main cardiac isoform, Trisk 32 (also known as CT1) (Kobayashi & Jones, 1999) and, finally, Trisk 49, which has a l-SR, rather than a j-SR, localization (Vassilopoulos et al., 2005). All Triadin isoforms share a similar structure with common cytoplasmic N-terminal and TM domains, while they differ in the length and composition of their luminal segments (Marty, 2015). Targeting and

retention of Trisk95 at the j-SR in mediated by three different regions: residues 18–47 in the cytoplasmic domain and amino acid regions 106–214 and 233–729 located in the luminal portion (Rossi et al., 2014a).

Known molecular interactors of Triadin include SR transmembrane proteins like RyRs (Groh et al., 1999), Junctin (Zhang et al., 1997) and intraluminal proteins like Calsequestrin (CSQ) (Kobayashi et al., 2000) and Histidine-rich Ca^{2+}-binding protein (HRC) (Lee et al., 2001). The CSQ1 binding site in Triadin includes stretches of basic amino acids that form the so-called KEKE motifs; interaction of CASQ1 with Triadin has been shown to anchor the intraluminal protein to the triads facilitating its interaction with RyR1 (Kobayashi et al., 2000; Zhang et al., 1997). Interaction with RyR1 occurs at both luminal and cytoplasmic regions; the cytoplasmic binding site is Ca^{2+}-dependent and has been proposed to have an inhibitory effect on the channel (Groh et al., 1999; Guo & Campbell, 1995), while binding to the intraluminal site results in channel activation (Ohkura et al., 1998). Functional studies have proven that Triadin is able to regulate RyR1 activity by itself in vitro (Groh et al., 1999; Ohkura et al., 1998), or via its interaction with Calsequestrin in vivo (Kučerová et al., 2012) .

Triadin also interacts with the microtubule (MT) network as observed in COS-7 cells, where expression of recombinant proteins results in ER and microtubule network deformation with the appearance of links between ER and MT called Rope like structures (RLS) (Fourest-Lieuvin et al., 2012). Mass-spectrometry-based proteomic and co-immunoprecipitation experiments revealed that interaction with the MT network is indirect, mediated by the ER membrane protein Climp63 (Osseni et al., 2016). The authors demonstrated that the intraluminal portion of Climp63 and Triadin, more specifically, the coiled-coil region from aa 306-341 of Triadin, are involved in association, and that the presence of the MT-binding domain located in the cytoplasmic region of Climp63 is necessary for the induction of the RLS.

Triadin knockout mice present with skeletal muscle defects correlated with the presence of abnormal triads orientation (Oddoux et al., 2009; Shen et al., 2007), ECC functional abnormalities, ER deformation, alteration of MT anchoring and reduced levels of CASQ at the triadic regions (Osseni et al., 2016).

Junctin and Junctate are two TM proteins localized on the SR deriving from the alternative splicing of the same gene, aspartyl-beta hydroxylase; they share the first 76 aa, coding for the cytoplasmic

N-terminal domain and the TM, but differ in their luminal domain (Treves et al., 2009). Junctate is a 33 kDa protein present at the j-SR of skeletal muscle and working as a moderate affinity, high capacity Ca^{2+} binding protein (Treves et al., 2009). This protein has also been recognized as a Ca^{2+}-sensing component of the Store Operated Calcium Entry (SOCE) mechanism that, upon intracellular Ca^{2+} store depletion, induces the ion influx from the extracellular space, through activation of the plasma membrane Calcium Release-Activated Calcium Channel Protein 1 (ORAI1) by the Stromal Interaction Molecule 1 (STIM1) located on the SR (Srikanth et al., 2012).

Junctin is a 26 kDa j-SR TM protein that, similarly to Triadin, can interact via its intraluminal C-terminus domain with Triadin, CASQ and RYR1, modulating the Ca^{2+} channel activity (Zhang et al., 1997). Recently, it has been established that multiple regions within the luminal portion of junctin can interact with Triadin and CASQ (aa 49–140, 118–154 and 155–210), while one region is responsible for the interaction with RyR1 (aa 155–210) (Rossi et al., 2022b).

Dihydropyridine receptor (DHPR) is a L-type voltage-gated Ca^{2+} channel located on TTs of striated muscle cells (Yu et al., 2005). The skeletal muscle specific isoform is composed by a heteromultimeric complex that includes α1s (212 kDa), β1α (58 kDa), α2-δ1 (125 kDa), and γ1 (25 kDa) subunits (Heiny J. & Meissner G., 2012). The α1s subunit (also referred to as CaV1.1) is an integral membrane protein containing 4 motifs each with 6 TM domains, acting as the pore-forming and the voltage-sensing unit (Schneider & Chandler, 1973). The β1a subunit is a cytoplasmic protein that associates with the cytosolic linkers I–II of the α1s subunit mediating its trafficking and affecting the functionality of the channel (Buraei & Yang, 2010; van Petegem et al., 2004). The physical coupling between DHPR and RyR1 seems to require both the β1a subunit docking RyR1 and the cytosolic linker between domains II–III of the α1s subunit (Hu et al., 2015). The auxiliary α2-δ1 subunit is an extracellular protein postulated to act as an anchoring point to the membrane through a glycosylphosphatidylinositol (GPI) lipid modification (Davies et al., 2010) The γ subunit is an integral membrane protein with 4-TM domains and may play a regulatory role by interacting with the voltage-sensing domain of the α1s subunit (Heiny J. & Meissner G., 2012).

DHPR-RyR1 interaction and trafficking of the α1s subunit to the plasma membrane is supported by the SH3 and cysteine-rich domain 3 (STAC3) protein (Rufenach & van Petegem, 2021; Polster et al., 2015, 2016). Accordingly, STAC3 co-immunoprecipitates with both RyR1 and the α1s subunit, hinting at a possible direct interaction (Horstick et al., 2013). The proposed region of interaction

between STAC3 and DHPR is in the cytosolic linker II –III of the α1s subunit; indeed, disruption of this region negatively affects ECC and causes a congenital Native American myopathy (NAM) (Nelson et al., 2013; Wong et al., 2017). STAC3 knockout mice are completely paralyzed and die shortly after birth with presence of defects in muscle development, mass, and morphology (Nelson et al., 2013).

Junctophilins (JPHs) are a family of proteins expressed in all excitable cells like neurons, cardiac and skeletal muscle that mediate the apposition between ER/SR and plasma membrane (Takeshima, 2000). In mammals there are 4 isoforms expressed in a tissue preferential manner: Junctophilin 1 (JPH1) the skeletal muscle isoform, Junctophilin 2 (JPH2), expressed in skeletal muscle and in cardiac muscle, Junctophilin 3 (JPH3) and Junctophilin 4 (JPH4) preferentially expressed in the neuronal tissue (Garbino et al., 2009). All isoforms have a similar structure with the presence, at the N-terminus, of 8 domains named membrane occupation and recognition nexus (MORN) motifs: these are organized in two groups of six and two motifs, respectively, separated by a joining region. In the central part of the protein, an α-helical and a divergent domain are present; a TM domain is localized at the C-terminus of the protein (Landstrom et al., 2014). Each MORN motif is formed by a sequence of 14 amino acids with hydrophobic proprieties that are highly conserved in all isoforms and across different species (Garbino et al., 2009). MORN motifs recognize and bind membrane phospholipids like phosphatidic acid, phosphatidylinositol (PI)4P,PI(4,5)P2 and PtdIns (3,4,5)P3 (Ma et al., 2006; Rossi et al., 2019). The α-helix domain is also highly conserved (Garbino et al., 2009) and is postulated to provide mechanical elasticity to the protein (Nishi et al., 2000; van Oort et al., 2011). The divergent region shows the lowest similarity score among isoforms, although there is a certain degree of conservation among the same isoforms of different species; its function remains unclear (Garbino et al., 2009). The short TM domain at the C-terminus anchors the protein to the ER/SR membranes; together with MORN motifs that bind phospholipids at the plasma membrane and the central α-helical domain that provides elasticity, the C-terminal TM domain allows JPHs to play the critical role of supporting the formation of contact sites between ER/SR and plasma membrane spaced consistently at 12-15 nm in depth (Takeshima et al., 2015) **Figure 8**.

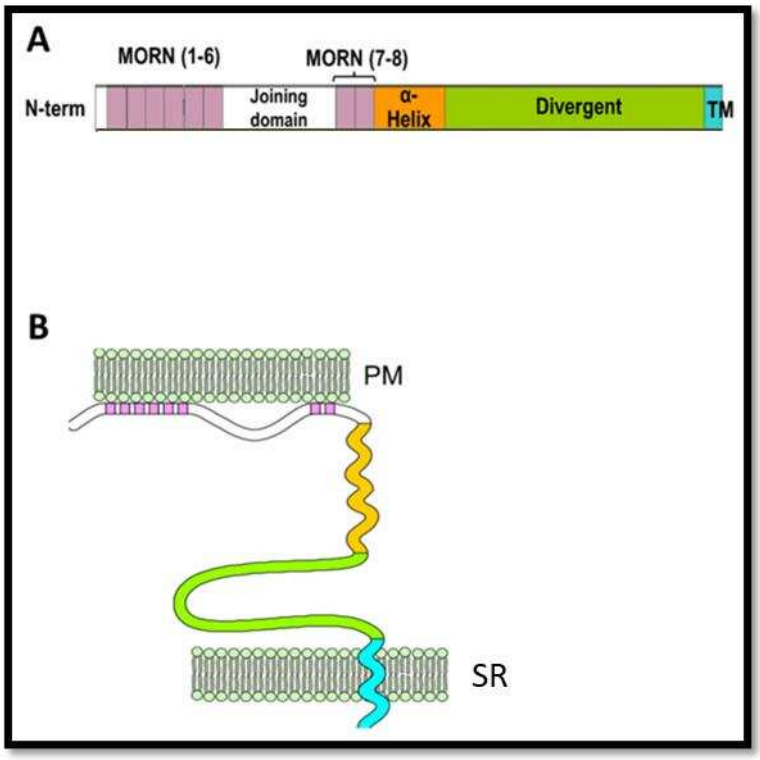


Figure 8 *(**A**) Linear sequence of JPHs proteins with, at the N-terminus, the two sets of MORN domains that are able to bind membrane phospholipids, in the middle of these domains there is the Joining region, while in the central region, the α-Helix domain provides mechanical elasticity to the protein. The divergent region and the TM domains represent the last two domains of the protein.* ***(B)*** *Schematic representation of JPHs mediating the contact sites between the PM and the SR. Image adapted from* (Perni, 2022).

In skeletal muscle, JPH1 and JPH2 are expressed at triads, where they are essential in maintaining the correct spacing between TT and j-SR membranes, as demonstrated by experiments of JPHs silencing resulting in a significant reduction in the number of triads and appearance of highly deformed structures, a phenotype also accompanied by defects in Ca^{2+} handling due to alteration in RyR1 and DHPR clustering (Hirata et al., 2006; van Oort et al., 2011; Golini et al., 2011).

Additional evidence of the structural role of JPHs comes from studies on knockout mice where JPH1 knockout mice die shortly after birth presenting disruption of the SR ultrastructure and impaired ECC (Ito et al., 2001; Komazaki et al., 2002) and JPH2 knockout mice showing similar abnormalities accompanied by embryonic mortality (Takeshima, 2000). The JPH2 isoform, in addition to dyads and triads was also demonstrated to form and maintain contact points between the SR and the sarcolemma, named peripheral couplings (Ito et al., 2001); these structures are abundant in the early stages of muscle development and reported only in low number in some adult vertebrate skeletal muscles (Spray et al., 1974)**.**

JPH2 is more expressed during embryonic life, while JPH1 becomes the most abundant isoform in the postnatal period. Accordingly, during embryonic life, absence of JPH1 in knockout mice result in no significant differences in muscle development compared to wild type animals, with a regular formation of dyads and peripheral couplings. On the contrary, absence of JPH2 in the heart is accompanied by severe structural abnormalities including the complete absence of peripheral couplings (Takeshima, 2000). According to these studies, JPH2 is postulated to mediate formation of peripheral couplings and dyads during the first steps of skeletal muscle development, while JPH1 plays an essential role in the conversion from dyads to triads, triad maintenance and the efficiency of ECC (Komazaki et al., 2002; Ito et al., 2001) (**Figure 9**).

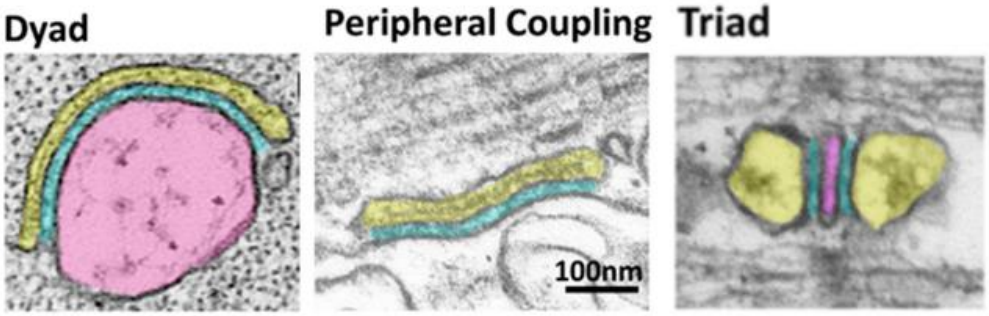


Figure 9 *Electron micrographs depicting the different contact sites that the SR and the sarcolemma can establish. Dyads with one SR and one TT; Peripheral couplings between the SR and the sarcolemma; and triads with one TT flanked by two j-SR. TTs are pseudo-colored in pink; SR in yellow and the space between the two in blue. Image adapted form* Perni, 2022.

In addition to its structural role, JPHs are also involved in regulating Ca^{2+} homeostasis by binding and regulating various proteins involved in ECC (Landstrom et al., 2014). JPH1 has been reported to interact and modulate gating of RyR1 channels thanks to three cysteine residues in JPHs that are able to stabilize the closed or open conformation of the channel in response to conformational changes induced by physiological ligands (Phimister et al., 2007). A similar interaction was reported between JPH2 and RyR2, while JPH2 was not found to be able in regulating RyR1 (Minamisawa et al., 2004; van Oort et al., 2011). Co-immunoprecipitation experiments demonstrated that both JPH1 and JPH2 can form homo- and heterodimers and bind the skeletal muscle DHPR channel (Rossi et al., 2019; Golini et al., 2011). This link is mediated by amino acids 230–369 and amino acids 216–399 in JPH1 and JPH2, respectively. This interaction was found to be pivotal for the assembly of DHPR with other proteins and for regulation of the ECC mechanism. Accordingly, JPHs depletion in

cultured myotubes resulted in lower density of L-type Ca^{2+} current and intramembrane charge movement with no concurrent alteration of SR Ca^{2+} release (Golini et al., 2011). Another interaction occurs between JPHs and the scaffolding protein Cav3 (Golini et al., 2011); a model is proposed in which lipid rafts containing Cav3 are used by JPHs as anchoring points necessary to form junctional membrane complex (Poulet et al., 2021) .

Other known interactions include Triadin, CASQ (Phimister et al., 2007), but also Orai1 and STIM1, the two main proteins involved in activation of SOCE (Li et al., 2010), as well as the Ca^{2+}-permeant channel TRPC3 (Woo et al., 2009). Based on these reports, a model in which JPHs play an essential structural role in forming and maintaining membrane junctional contact points and in providing a scaffold like structure in which junctional proteins can assemble within the calcium release complex, was proposed.

According to this model, studies in the literature demonstrated that several cardiomyopathies present with disruption in TT distribution and morphology and a decrease in JPH2 expression, suggesting that JPH2 downregulation could be a common mechanism at the basis of disruption of TT (Minamisawa et al., 2004; Wei et al., 2010; Zhang et al., 2013). Indeed, studies performed in cultured cardiomyocytes demonstrated that JPH2 overexpression has a stabilizing effect on TTs, preventing their disruption and maintaining a higher number of functional DHPR channels at the plasma membrane (Poulet et al., 2021). An additional role as a transcriptional regulator was proposed for JPH2; following heart stress, JPH2 is cleaved in a calpain-mediated way, liberating a N-terminus fragment able to translocate to the nucleus and influence expression of an array of genes involved in a self-protective mechanism in cardiomyocytes, that result in attenuated hypertrophic response and milder heart failure development in mice (Guo et al., 2018). A similar observation was performed in skeletal muscle damaged by extreme contraction: a decrease in JPH1 expression accompanied by ECC dysfunction and increased cytosolic Ca^{2+} concentration was reported (Corona et al., 2010); JPH1 decrease was proposed to be mediated by a Ca^{2+}- dependent calpain mediated proteolysis (Murphy et al., 2013; Tammineni et al., 2023).

Calsequestrin (CASQ) is a Ca^{2+} binding protein located in the SR lumen of skeletal muscle, where it participates in the regulation of Ca^{2+} homeostasis, by buffering the free ion; there are 2 isoforms of Calsequestrin, CASQ1 expressed in fast- and slow-twitch fibers, and CASQ 2 present in slow-twitch fibers and in cardiac muscle (Biral et al., 1992) The two isoforms have a conserved homology,

differing in the Ca^{2+}-binding C-terminal tail that is longer in CASQ2; this last domain can also interact with Junctin, Triadin and RyR1, forming an ECC modulating complex (Witherspoon & Meilleur, 2016). CASQs can also be defined as a low affinity Ca^{2+} binding protein, that is present as a monomer when the intraluminal ion concentration is low and is able to polymerize with increasing Ca^{2+} concentration (Beard et al., 2009). CASQs have also a role in regulating SOCE by binding STIM1 and reducing its clustering and thus its interaction with ORAI 1 (Zhang et al., 2016). Mutations in CASQ2 are correlated with autosomal recessive forms of catecholaminergic polymorphic ventricular tachycardia (CPVT) (Lahat et al., 2001) while mutations in CASQ1 are correlated with vacuolar myopathy and tubular aggregate myopathy (Rossi, et al., 2014b; Barone et al., 2017)

THE MICROTUBULE BINDING PROTEIN CLIMP63 AND TRIADS

Climp63 (cytoskeleton-linking membrane protein 63) or CKAP4 (cytoskeleton-associated protein 4) is a non-glycosylated protein abundant in endoplasmic reticulum (Mundy & Warren, 1992). More specifically, it is a type 2 transmembrane protein ubiquitously expressed, composed of 602 amino acids in humans and 575 aa in mouse. The N-terminal cytoplasmic tail of Climp63 (aa 1-105 in humans) contains a ER-localization domain crucial for its correct cellular positioning (aa 2-23 in humans) (Schweizer et al., 1994), a proline rich segment (aa 36-59 in humans) likely involved in protein-protein interactions (Li, 2005), a microtubule binding domain (aa 62-101 in humans), followed by a short TM domain (22 aa in humans, aa 106-127) anchoring it to the ER. At the C-terminus, a long intraluminal segment containing three coiled-coil domains is present (475 aa in humans, aa 128-602); between the first and the second coiled-coil domain (from aa 192 to 258 in humans) an interacting segment for the endonuclease dicer is present (Pépin et al., 2012; Klopfenstein et al., 2001; Sandoz & van der Goot, 2015) (**Figure 10**).

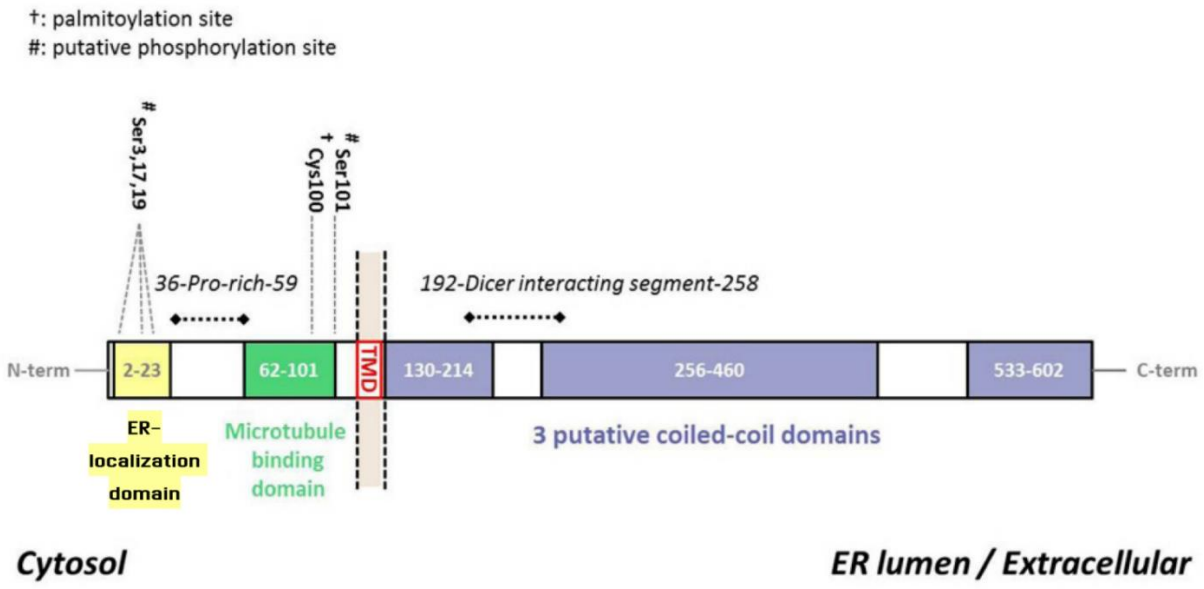


Figure 10 *Linear representation of human Climp63 protein: At the N-terminus it has a cytosolic segment with an ER targeting sequence, a proline rich segment, a microtubule binding domain and palmitoylation and phosphorylation site; followed by a short TM domain anchoring it in the ER or at the plasma membrane; a long luminal/extracellular domain at the C-terminus with three coiled-coil domains able to form oligomers that act as ER luminal spacers and a segment able to interact with the endonuclease dicer are present (Sandoz & van der Goot, 2015).*

Climp63 is expressed predominantly in the rough ER (RER) where it functions as a luminal spacer within ER cisternae maintaining a defined width of about 50 nm (Shibata et al., 2010). The function of Climp63 as luminal spacer is mainly mediated by the three coiled-coil domains that promote formation of oligomers among neighboring proteins located either on the same side of the cisterna or on opposite membranes (Shen et al., 2019). Climp63 dimerization is also supported by a cysteine residue (Cys126) located in the TM region, that can form disulphide bridges (Kikuchi et al., 2017). According to this model, depletion of Climp63 causes the ER cisternae to collapse (Shibata et al., 2010), while expression of mutant Climp63 with longer luminal domains correlates with an increase in ER luminal width (Shen et al., 2019). In eukaryotic cells, the ER is a dynamic organelle organized into a perinuclear domain and a peripheral domain; this last one is further divided into sheets, that are continuous with the nuclear envelope and are shaped as flat cisternae, and tubular ER, which branches towards the periphery of the cell (Voeltz et al., 2002). Tubular ER is defined by extreme membrane curvatures that are formed and maintained by Reticulons (RTN) and DP1/Yop1 protein family (Voeltz et al., 2006), integral membrane proteins characterized by TM segments forming double harpins (Hu et al., 2008). On the contrary, the flat sheet-shaped cisternae, are maintained by Climp63, P180 and Kinectins **(Figure 11)** (Lin et al., 2012; Shibata et al., 2010). The function of Climp63 is regulated by the ER luminal protein Calumenin-1 (Calu1), belonging to the CREC (Cab45,

reticulocalbin, ERC-45, and calumenin) family involved in apoptosis, cell migration, protein folding, and Ca^{2+} signaling (Chen et al., 2016; Deng et al., 2018; Honoré, 2009; Wang et al., 2015). Calu1 was shown to directly interact and modulate ER sheet distribution in a Climp63 dependent manner. Overexpression of Calu1 has a negative effect on the width of the ER, while Calu1 knockout leads to a slight increase in lumen width, an effect that was postulated to be ascribed to conformational changes in Climp63 that interfere with self-association and microtubule binding (Shen et al., 2019).

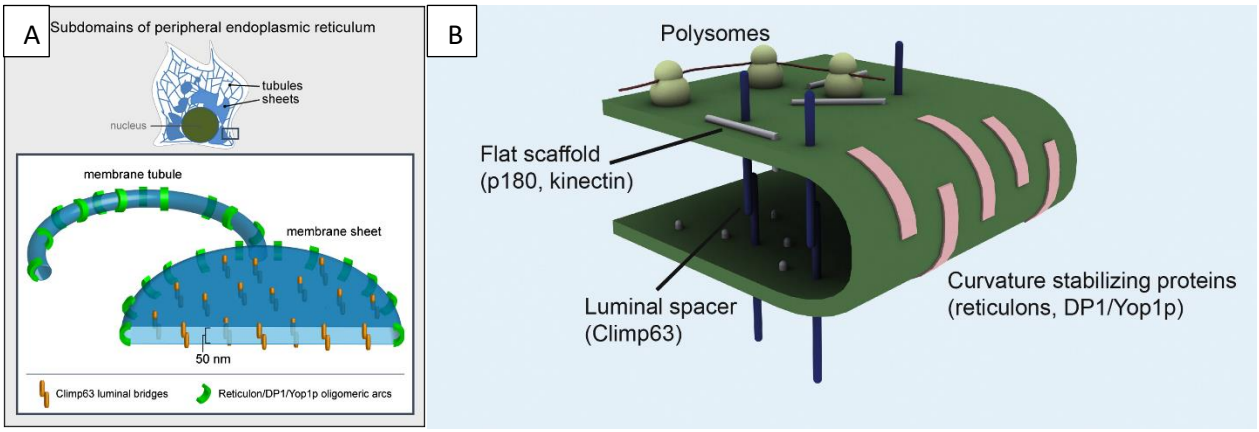


Figure 11 (A) *Representation of peripheral ER sheets and tubules, with Climp63 maintaining the luminal width and reticulons and DP1/Yop1p stabilizing the extreme curvatures.* ***(B)*** *Schematic representation of ER sheets with reticulons and DP1/Yop1p positioned at the edges of the sheets stabilizing this extreme membrane curvature. While at the core, Climp63 acts as a luminal spacer maintaining the distance between the two apposed sides of the membrane, and p180 and kinectin act as scaffold to maintain these structures flat. Knocking down all three proteins, decreases ER luminal width but does not prevent the ER sheet formation, alluding to additional factors contributing to this process ; other components like polysomes positioned on the surface of the membrane are postulated to have a role in ER sheet formation and maintenance* (Puhka et al., 2007)*. Figure B from* (Lin et al., 2012)*, Figure A from* (Shibata et al., 2010)*.*

The role of Climp63 in the ER is not limited to its function as a luminal spacer; many studies demonstrate that Climp63 can also induce the rearrangement of the ER through its interaction with the microtubular cytoskeleton (Klopfenstein et al., 1998; Vedrenne et al., 2005). The microtubular network is actually closely linked to the ER, with microtubules co-aligned with the ramifications of the ER. Accordingly, MT-depolymerization induces changes in the ER (Terasaki et al., 1986). Binding to MT is regulated by protein kinase C (PKC) dependent phosphorylation of residues Ser 3, 17 and 19 of Climp63; phosphorylation of these residues abolishes binding to MT causing the ER to collapse around the nucleus, in a mechanism that was theorized to be a rapid way for the cell to remodel the ER at the beginning of mitosis (Vedrenne et al., 2005). Overexpression of Climp63 in COS7 cells is associated with the formation of RLS, where ER membranes and MT are bundled together, while the expression of a MT-binding deficient form of Climp63 results in absence of RLS and collapse of

the ER near the nucleus indicating its inability to interact with and to be guided by the cytoskeleton (Vedrenne & Hauri, 2006; Klopfenstein et al., 1998). Similarly, Zheng and collaborators (2022) reported that silencing of Climp63 induces a rearrangement of the ER, that is restored only following transfection with a MT-binding competent Climp63 (Zheng et al., 2022).

Finally, the ER shaping properties of Climp63 are also directly involved in the capability of the cell to repositioning the nucleus during cell migration. Janota and collaborators showed the ER accumulates at the perinuclear region in a Climp63 dependent manner; this ER arrangement favors formation of asymmetric contact points between the proteins present at the nuclear lamina and the actin cytoskeleton, that are crucial for nuclear repositioning (Janota et al., 2022).

CLIMP63 NON-CONVENTIONAL ROLES

Besides the ER compartment, Cimp63 can also be present at the plasma membrane where it functions as a receptor for the antiproliferative factor (APF)(Conrads et al., 2006), tissue plasminogen activator (tPA) (Razzaq et al., 2003), surfactant protein A (SP-A)(Gupta et al., 2006), alginate exopolysaccharides (Barbier et al., 2012), and Dickkopfs (DKKs) protein (Barbier et al., 2012; Kimura et al., 2016; Li et al., 2021). Translocation of Climp63 to the plasma membrane is mediated by reversible palmitoylation of Cys100 (in the human sequence) (Zacharias et al., 2012). Following plasma membrane translocation, Climp63 interacts with the antiproliferative factor (APF) and further translocate to the nucleus, where it acts as a transcription factor that inhibits cell proliferation (Conrads et al., 2006; Planey et al., 2009; Zacharias et al., 2012).

CLIMP63 IN THE MUSCLE

Climp63 is also expressed in skeletal muscle cells, where it localizes at the SR. Interestingly, it is present at both the longitudinal and the junctional SR. Using co-immunoprecipitation and immunofluorescence experiments, Osseni and collaborators showed that the luminal domain of Climp63 interacts with the luminal portion of Triadin, suggesting that this association may support the formation of a link between triads and microtubules (Osseni et al., 2016). Moreover, depletion of Triadin in knockout mice was found to impair Climp63 triadic localization and to induce disorganization of the MT network; a similar microtubular network disruption was also observed in

muscles overexpressing a Climp63 mutant retaining its ability to interact with Triadin but unable to bind MTs (Osseni et al., 2016).

Another article reports the interaction of Climp63 with RyR2 in cardiac muscle and the increase in Climp63 expression in cardiac muscle from CASQ2 knockout mice, suggesting a general role of Climp63 in striated muscles (Bongianino et al., 2020).

AIM

Triads are skeletal muscle specific structures where the ECC mechanism takes place. At this site, multiple proteins participate in this process and in the formation and maintenance of the triadic structure. Among them, JPHs play a pivotal role in the assembly and preservation of triads. Considering their central roles, we investigated novel potential JPHs interactors at the triadic region using the Proximity-dependent labelling with BioID2. Among potential candidates, we characterized Climp63 as a novel interactor of JPHs at triads and analyzed its expression pattern in skeletal and cardiac muscles at different stages of muscle development in mice.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

Experiments were performed on the immortalized cell line derived from human epithelial kidney HEK293T. Cells were cultured at 37°C in humidified 5% CO_2 atmosphere using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-Glutamine, 100U/ml streptomycin-penicillin, 1 mM sodium pyruvate. Unless specified otherwise, all reagents used for cell culture were supplied by Sigma-Aldrich.

Transfection of cells was performed by Lipofectamine™ 3000 Transfection Reagent and PLUS Reagent ™ (Invitrogen) following the manufacturer's instructions. In short, cells were seeded in naked, or laminin coated plates (0.025 mg/ml, Corning®) in growth medium. Plasmid were complexed with the Plus Reagent diluted in Opti-Mem (Gibco) without serum and incubated for 15 minutes at room temperature (rt), Lipofectamine was diluted in Opti-Mem, and at the end of the incubation, the two solutions were mixed and further incubated for 15 minutes at rt. Plasmid DNAs complexed with Plus and Lipofectamine reagents were added to the cells and incubated for 24-48 hours at 37°C in humidified 5% CO_2 atmosphere in culture medium.

EXPRESSION VECTORS

For the Proximity dependent labelling with BioID2 experiments, 3xmycBioIDJPH1 (previously generated in the laboratory) (**Figure 12)** and pcc3xmycBioID2-JPH2 (VectorBuilder) expression vectors were used. Co-Immunoprecipitation experiments were performed using the plasmids: Climp63-mCherry (VectorBuilder), mCherry-Climp63Δcytosol (VectorBuilder), Climp63Δlumen-mCherry (VectorBuilder), pEGFP (Clontech), CASQ1-GFP, Triadin-GFP, Junctin-GFP, GFP-JPH1, GFP-JPH2, GFP-TMJPH1, GFP-TMJPH2 (all available in the laboratory), GFP-Esyt1 and GFP-Esyt2, kindly provided by Prof. De Camilli (Yale University, USA).

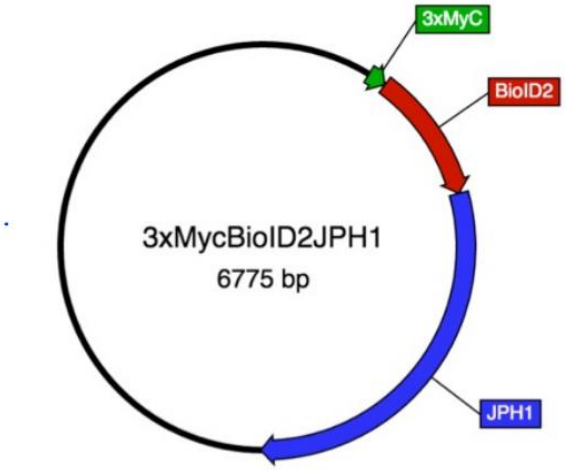


Figure 12: *Schematic representation of the 3xmycBioID2-JPH1 expression vector. In blue the coding sequence of the JPH1 protein. In red the BIoID2 enzyme and in green the 3xmyc tag.*

ANIMAL CARE

In vivo experiments were performed using the C57BL/6 mice strain. Animals were placed inside ventilated cages with controlled temperature and humidity and were provided free access to food and water. All the handling of the mice were done in accordance with the Italian law 26/2014 and were pre-approved by the "Organismo Preposto al Benessere degli Animali" (OPBA) of the University of Siena.

PROXIMITY DEPENDENT LABELLING WITH BIOID2

Proximity dependent labelling with BioID2 was performed in cultured HEK293T cells and in mouse FDB muscle. 877.000 HEK293T cells were plated in a petri dish of 60 mm of diameter (SARSTEDT) and the next day were transfected with 10 µg of either 3xmycBioIDJPH1 or pcc3xmycBioID2-JPH2 expression vectors in the presence or in the absence of 50 mM biotin (Thermo Scientific). Cells were incubated overnight (o.n.) at 37°C in humidified 5% CO_2 atmosphere. After 24 hours, cells were lysed in Cell lysis buffer (Cell Signaling Technology, Inc) supplemented with 0,1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). Protein quantification was performed using the Bradford assay.

Mouse FDB muscles were transfected with 15 µg of either 3xmycBioIDJPH1 or pcc3xmycBioID2-JPH2 plasmids via electroporation method. Mice were anesthetized using isoflurane gas and injected in

the subcutaneous region of the posterior paws with 15 µl of 2 mg/ml hyaluronidase (Sigma-Aldrich). The solution was allowed to act for 40 minutes. After the incubation the animal was anesthetized again and the paw injected with 15 µg of the plasmid of interest and immediately electroporated by positioning subcutaneously two needle electrodes at the ends of the paw and running 20 impulses of 1 Hz, 120 V with a peak length of 20 ms (Electroporator ECM® 830). After transfection, mice were subjected to 7 days of daily intraperitoneal biotin injection at the final concentration of 24 mg/kg after which they were sacrificed by carbon dioxide (CO_2) inhalation followed by cervical dislocation.

BIOID PULLDOWN ASSAY

HEK293T cells or FDB muscle fibers were processed for the BioID2 pulldown assay, as described by Roux et al., 2013. Cell lysis was performed with Cell lysis buffer (Cell Signaling Technology, Inc) + 0,1 mM PMSF (Sigma-Aldrich). Cells were completely detached with a scraper and transferred to a 15 ml tube added with 2% Triton X-100 in Phosphate Buffer Saline (PBS) (Sigma-Aldrich). Two sessions of 30 pulses of sonication at 30% of power (Bandelin Sonopuls HD 2200) were applied. Cells were kept in ice; after 30 pulses, proteins were resuspended in 50 mM Tris-Cl pH 7.4, followed by a final session of sonication. Samples were centrifuged (Eppendorf MicroCentrifuge 5452) at 4°C for 11 minutes at 16.000 g. The pellet was discarded, and the supernatant used for purification. Biotinylated proteins were purified using Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen™). 150 µl of Streptavidin Magnetic Beads were equilibrated in cell lysis buffer and 50 mM Tris-Cl pH 7.4 in 1:1 proportion. The tubes were placed in MagneSphere Technology Magnetic Separation Stand for beads separation (Promega). The protein samples were transferred to the tube containing the magnetic beads and incubated overnight at 4 °C in agitation. At the end of the incubation, the tubes were placed on the magnetic separation stand and the supernatant, containing unbound proteins, was removed. Beads were washed 3 times with Washing Buffer 1 (2% SDS), and once with Washing Buffer 2 (0.1 % deoxycholic acid, 1% Triton X-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES, pH 7.5) and Washing Buffer 3 (0.5 % deoxycholic acid, 0.5% NP-40, 1 mM EDTA, 250 mM LiCl, 10 mM Tris-Cl, pH 7.4). Each washing step lasted 8 minutes in agitation. Finally, 50 mM Tris-Cl pH 7.4 was added to remove detergent residues, and beads were pelleted by centrifugation at 2000 g for 5 minutes at rt. The supernatant was discarded, and the beads were resuspended in 100 µl SDS-PAGE sample buffer (62.5 mM Tris HCl pH 6.8, 2% SDS, 10% Glycerin, 5% β-mercaptoethanol, 0.004% Bromophenol Blue) and incubated in boiling water for 5 minutes to break the streptavidin-biotin

bonds and release the purified proteins. Samples were stored at -20°C or used immediately for Western Blot analysis.

FDB MUSCLE FIBER ISOLATION AND CULTURE

FDB muscles were dissected surgically and placed in myorelaxant solution (0.1 M KCl, 5 mM EGTA, 5 mM $MgCl_2$, 3 mM BDM (2,3-butanedione monoxime), 0.25 mM DTT, 10 mM Histidine) in ice for 10 minutes. Muscles were enzymatically digested with 0.2 % Collagenase type 1 solution (Sigma-Aldrich) diluted in Tyrode (134 mM NaCl, 2.68 mM KCl, 1.80 mM CaCl2, 1.05 mM $MgCl_2$, 417 µM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.56 mM Glucose, pH 7.4) + 10% heat inactivated FBS; muscles were incubated for 40-60 minutes in a humified incubator at 37°C/ 5% CO_2 with gentle manual swing every 10 minutes. Muscle bundles were washed for 10 minutes at 37 °C/5% CO_2 with the following solutions: Tyrode; Tyrode + 10% FBS; and Tyrode + 10% FBS + 100 U/ml Penicillin/Streptomycin (Sigma-Aldrich). Single fibers were obtained via disaggregation of the muscle bundles with mechanical action by pipetting up and down with serological pipettes of decreasing diameter. Single fibers were cultured into glass slides Labtek (Sarstedt 8-well on cover glass II) previously coated with 0.025 mg/ml laminin (Sigma-Aldrich) and incubated overnight in a humified incubator at 37°C/ 5% CO_2.

PROTEIN EXTRACTION FROM MUSCLE TISSUES

FDB, EDL, Tibialis Anterior, Gastrocnemius, Soleus and heart muscles were dissected from mice starting from embryonic day 15 to 12 months old and processed independently. Samples were homogenized using a Tissue Rupture (QIAGEN) in RIPA Buffer (Cell Signaling Technology, Inc) added with 0.1 mM PMSF (Sigma-Aldrich). Lysates were centrifuged at 10.000 rotations per minutes (rpm) at 4°C (Eppendorf), and supernatant, containing the proteins were transferred in a new tube and immediately analyzed or conserved at -80°C. Protein concentration was determined by Bradford assay and the absorbance at 595 nm was measured with Ultrospec 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience). The protein concentrations were determined according to a standard curve of known concentrations of Bovine Serum Albumin (BSA).

CO-IMMUNOPRECIPITATION EXPERIMENTS

Co-immunoprecipitation (CO-IP) was performed using GFP-TRAP Dynabeads (Chromotek) following manufacturer's instructions. Briefly, transfected HEK293T cells were mechanically detached from the plate using a cell scraper and lysed using a lysis buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5 % Nonidet™ P40, 1 mM PMSF). Lysates were centrifuged at 4°C for 11 minutes at 16.000 g. The pellet was discharged, and the supernatant used for GFP-Trap Dynabeads purification. Slurry beads were equilibrated via 2 washes with Wash buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Nonidet™ P40, 0.5 mM EDTA) separating the beads from the supernatant using a magnetic stand. 500 µg of extracted proteins were diluted in Dilution buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF) at the final concentration of 1 µg/µl and incubated with 25 µl of beads for 1 hour at 4°C in end-over-end rotation. Tubes were placed in the magnetic stand, the supernatant was discarded, and the beads were washed with wash buffer for 2 times. Finally, the purified proteins were detached from the beads via incubation with Elution buffer (120 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.04% bromophenol blue, 10% β-mercaptoethanol) for 5 minutes at 95°C and analyzed via SDS-PAGE and Western Blot.

SDS-PAGE AND WESTERN BLOT

Total protein lysates were diluted in Sample Buffer 4X (62.5 mM Tris HCl, 2% SDS, 10% Glycerin, 5%, β-mercaptoethanol, 0.004% Bromophenol blue, pH 6.8) heated for 5 minutes at 95° C and processed by SDS-PAGE electrophoresis on gradient polyacrylamide gels 4-15% (Bio-Rad). Gel electrophoresis was performed in a Bio-Rad Chamber with the Tris-Glycine-SDS (TGS) buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS). Page Ruler Plus Prestained Protein Ladder (Thermo Fisher Scientific) was used to estimate the size of the separated proteins. Proteins were transferred to Nitrocellulose Blotting Membrane (GE Healthcare, Life science, Germany) using the Trans-Blot Turbo Transfer System (Bio-Rad) in combination with the paper stacks in transfer buffer (Bio-Rad) at predefined running conditions (30 minutes, up to 1.0 A, 25 V).

For immunoblot, membranes were incubated in blocking buffer (50 mM Tris HCl pH 7.4 , 150 mM NaCl , 5% non-fat milk , 0.2% Tween 20) for 1 h, and then incubated o.n. in agitation at 4°C with the primary antibodies diluted in blocking buffer. The following antibodies were used: monoclonal anti-myc antibody (1:1000 Sigma-Aldrich), monoclonal anti-GFP antibody (1.1000 Sigma-Aldrich), polyclonal anti-Climp63 antibody (1:500 Bethyl Laboratories), polyclonal anti-JPH1 antibody (1:1000

Thermo Fisher Scientific); polyclonal anti-JPH2 antibody (1:500 Thermo Fisher Scientific), polyclonal anti-Triadin antibody (1:10.000, kindly provided by Prof. Isabelle Marty, INSERM, Grenoble, France). Membranes were washed 3 times for 10 minutes with washing solution (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0,5% milk, 0,2% Tween) and incubated for 1 hr at room temperature with either anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (GE Healthcare), diluted in washing solution without Tween-20. After 3 steps of washing, chemiluminescence signals were acquired under the ChemiDoc XRS+ imaging system (Bio-Rad) using enhanced chemiluminescence solution.

STATISTICAL ANALYSIS

Statistical and graphical analysis were performed with Graph Prism 6.0 software. Protein quantification experiment was performed 2-3 times with lysates from different animals (from 2 - 4). The graph shows the mean and the standard error of the results. P values: ≤ 0.05, 0.01 and 0.001 are indicated by *, ** and *** respectively.

IMMUNOFLUORESCENCE

Immunofluorescence analysis was performed on FDB muscle fibers. Isolated fibers were fixed in PBS containing 3% paraformaldehyde supplemented with 2% sucrose for 7 minutes at rt. The solution was removed and 3 washing steps with PBS + 0.2% bovine serum albumin (BSA) (Fraction V) were performed at rt. Fibers were permeabilized with an incubation of 3 minutes at rt in Hepes TritonX-100 buffer (20 mM HEPES pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% TRITON X-100), followed by 3 washing steps. Fibers were placed in a blocking solution composed of PBS + 5% Goat serum (Sigma-Aldrich) + 2% BSA + 0.1% Triton X-100 (Sigma-Aldrich) for 1 hour at rt. The blocking solution was discarded, and the fibers were incubated overnight with primary antibodies (**Table 1**) diluted in PBS + 2% BSA in a humidified chamber at 4°C. The following day, primary antibody was discarded, and the wells washed for 3 times at rt for 10 minutes, followed by 1 hour incubation with secondary antibodies (Invitrogen) diluted in PBS + 0.2% BSA. The secondary antibodies were discarded, and three final washing steps were performed before analyzing the fibers under the confocal microscope (Zeiss LSM/510 META).

Antibody	Dilution
Climp63 (Bethyl-Laboratories): polyclonal	1.500
α-actinin (Sigma-Aldrich): monoclonal	1:1000
JPH1 (Thermo Fisher Scientific): polyclonal	1:1000
Goat anti-Rabbit IgG Alexa Fluor Plus 488 (Invitrogen)	1:2000
Goat anti-mouse IgG Alexa Fluor 555 IgG (Invitrogen)	1:2000

Table 1 *Primary and secondary antibodies used for the immunofluorescence experiments*.

Results

CLIMP63 IS LOCALIZED IN PROXIMITY OF JPH1 AND JPH2 IN HEK293T CELLS AND IN *FLEXOR DIGITORUM BREVIS (*FDB) MUSCLE FIBERS AT TRIADS

Our laboratory has been interested in studying the mechanisms regulating triad assembly and maintenance for many years. Recently, we set up a Proximity-dependent biotin identification (BioID2) approach to search for novel interactors of JPHs in skeletal muscles. To this aim, expression vectors for chimeric JPH1 or JPH2 proteins fused to the biotin ligase (BirA*) enzyme were developed. In the presence of biotin, BirA* is able to covalently attach this small molecule to all nearing proteins in the radius of 10 nm, thus allowing to identify JPHs potential interactors. Previous studies performed in our laboratory using N-terminal BirA*-JPH1 and -JPH2 fusion proteins in HEK293T cells resulted in the identification of Climp63 as a novel JPHs interactor (**Figure 13**).

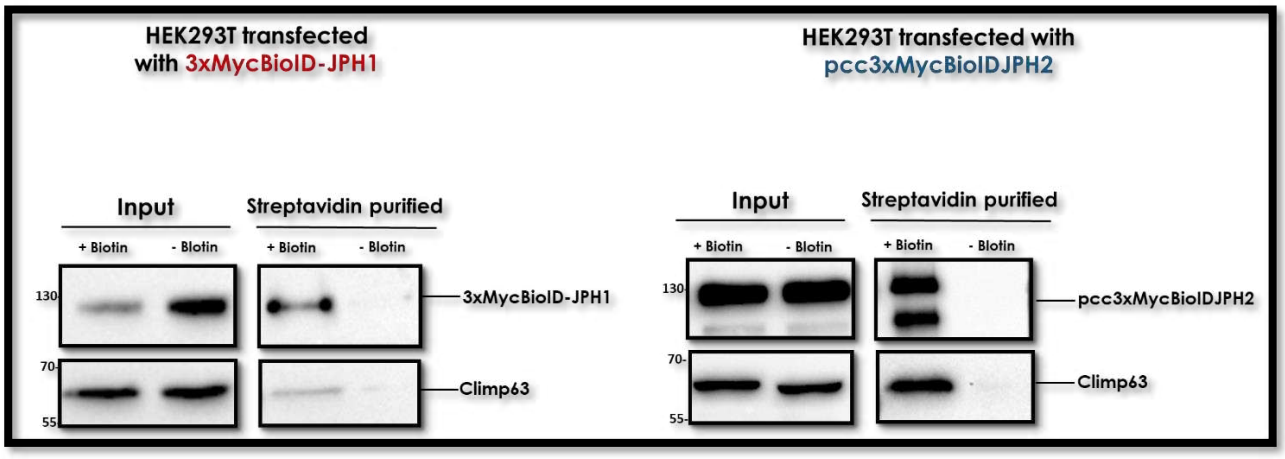


Figure 13 BioID2 Pulldown on HEK293T cells expressing BirA*-JPH1 (3xMycBioID-JPH1) or -JPH2 (pcc3xMycBioIDJPH2) chimeric proteins. *HEK293T cells were transfected with 3xMycBioID-JPH1 or pcc3xMycBioIDJPH2 and analyzed in the presence or absence of biotin. Total protein lysates (Input) and streptavidin-purified fraction were separated by SDS-PAGE and analyzed by Western Blot using anti-myc or anti Climp63 specific antibodies.*

To further confirm these data, we expressed N-terminal BirA*-JPH1 and -JPH2 fusion proteins in FDB muscle fibers; mice were treated with biotin and proteins were analyzed following streptavidin-purification. As shown in **Figure 14**, Climp63 was also found to be biotinylated in skeletal muscle, supporting the results obtained in HEK293T cells and indicating that it is localized at triads, in proximity of JPHs.

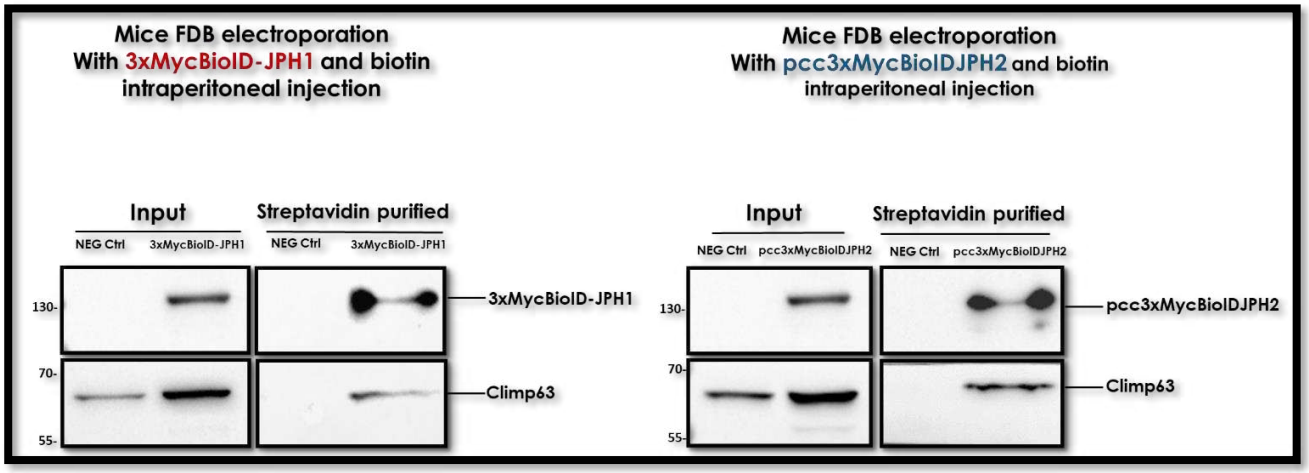


Figure 14 ***BioID2 pulldown on mouse FDB muscle transfected with 3xMycBioID-JPH1 or pcc3xMycBioIDJPH2.*** *FDB muscles were transfected by electroporation with 3xMycBioID-JPH1 or pcc3xMycBioIDJPH2 plasmids. Biotin was administered by intraperitoneal injection. Total protein lysates were obtained (Input) and streptavidin-purified fractions were separated by SDS-PAGE and analyzed by Western Blot using anti-myc or anti-Climp63 specific antibodies. The contralateral non-transfected FDB muscle was used as negative control (NEG Ctrl).*

Accordingly, immunofluorescence analysis on isolated FDB muscle fibers revealed that Climp63 has a partial localization consistent with that of a triadic protein, with a double row of dots flanking the Z-disk, labelled by α-actinin (**Figure 15A**). It must be underlined, however, that most of the fibers analyzed showed low to undetectable immunofluorescence signals for Climp63, likely due to a low level of expression of the protein. To improve the analysis of Climp63 localization, we expressed an mCherry-tagged Climp63 recombinant protein in FDB muscles. Co-staining with anti α-actinin antibody revealed that Climp63-mCherry localizes in a region of the SR consistent with the j-SR; (**Figure 15B**). Accordingly, a partial co-localization with JPH1 at triads could be observed (**Figure 15C**).

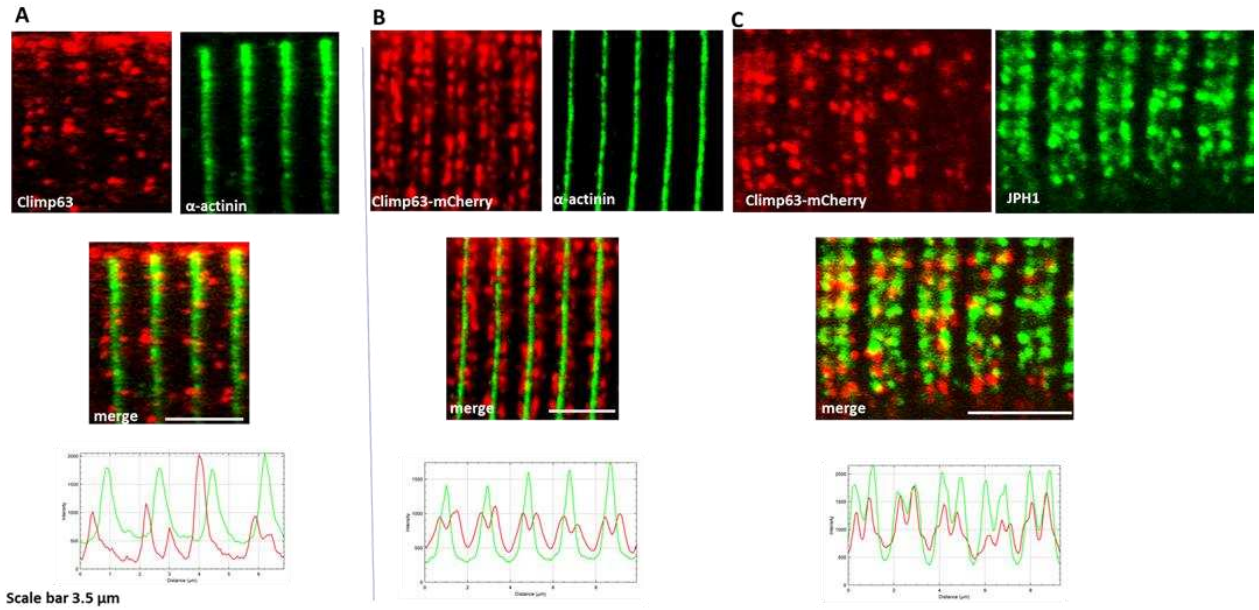


Figure 15 ***Immunofluorescence images of isolated FDB muscle fibers.*** *(A) Confocal images of adult mouse FDB muscle fibers stained with a primary polyclonal antibody against Climp63, and a secondary anti rabbit antibody conjugated with AlexaFluor 555; to detect Z-disks, fibers were counterstained with a primary monoclonal antibody against α-actinin, and a secondary anti mouse antibody conjugated with AlexaFluor 488. (B ,C) Confocal images of adult mouse FDB muscle fibers expressing plasmids encoding Climp63-mCherry (B and C). Fibers were counterstained with primary monoclonal antibodies against α-actinin (B) or polyclonal antibodies against JPH1 (C), both revealed with AlexaFluor488-conjugated secondary antibodies. Bottom panels: Fluorescence intensity plots with red peaks corresponding to the Climp63 signal, and green peaks corresponding to α-actinin (A and B) or JPH1 signals (C).*

Climp63 co-immunoprecipitates with GFP-tagged triadic proteins in FDB muscle fibers and HEK293T cells

Based on the results obtained with the BIoID technique and immunofluorescence analysis, we performed co-immunoprecipitation experiments on FDB muscles expressing GFP-tagged JPH1 and JPH2 proteins, to verify whether Climp63 interacts with JPHs at triads. Western blot analysis of immunocomplexes obtained with anti-GFP antibody conjugated magnetic beads revealed that JPHs can co-immunoprecipitate Climp63, supporting the previously obtained results (**Figure 16A**). We extended our analysis to other components of triads and, interestingly, we found that Climp63 can be co-immunoprecipitated by Triadin and Junctin (**Figure 16B and 16C**), but not by the intraluminal protein Calsequestrin-1 or by GFP alone (**Figure 16D and 16E**).

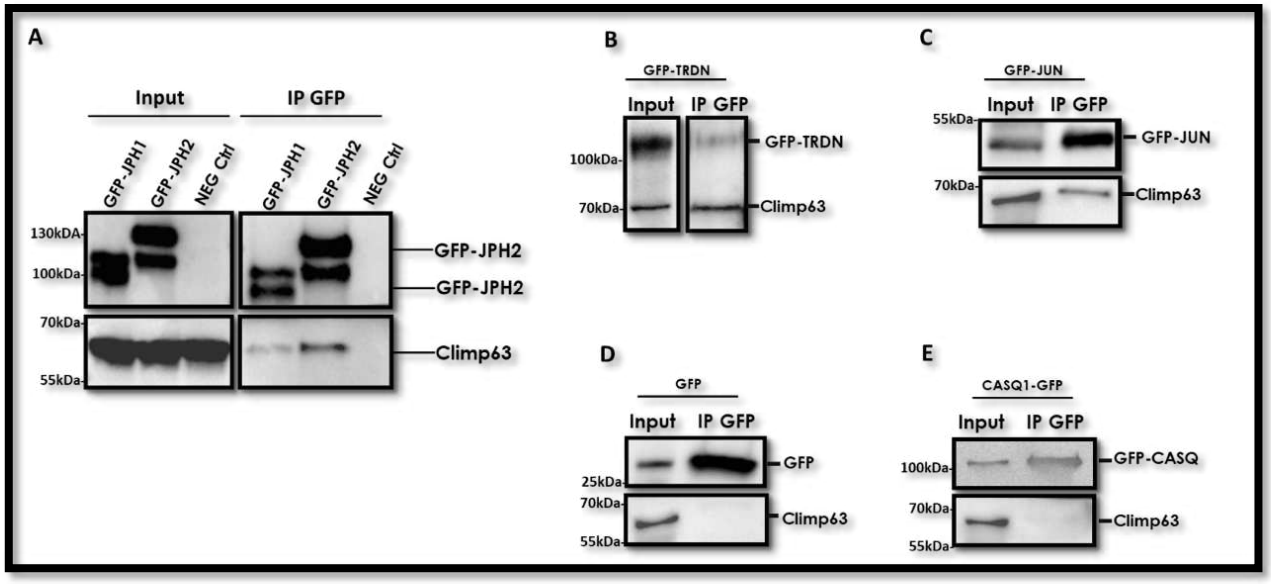


Figure 16 ***Co-immunoprecipitation on FDB muscle fibers transfected with GFP tagged proteins:*** *Total lysates from FBD expressing GFP-JPH1, GFP-JPH2 (A), Triadin-GFP (TRDN-GFP)(B), Junctin-GFP (GFP-JUN)(C), GFP (D) or Calsequestrin-GFP (CASQ1-GFP)(E) were immunoprecipitated with anti-GFP conjugated magnetic beads. Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by mouse monoclonal anti-GFP antibodies and rabbit polyclonal anti-Climp63 antibodies. Neg Ctrl: non transfected FDB muscle.*

To assess whether interaction between Climp63 and JPHs requires the presence of muscle-specific proteins, we performed co-immunoprecipitation experiments using HEK293T cells expressing GFP-JPH1 or GFP-JPH2 (**Figure 17**). The results obtained showed that interaction between Climp63 and JPHs also occurs in HEK293T cells, indicating that it does not require the presence of muscle specific proteins. In addition to JPHs, ER/PM contact sites can be mediated by protein belonging to the Extended Synaptotagmin family (E-Syt2 and E-Syt3); these proteins are not expressed in skeletal muscle and do not share amino-acid sequence similarity with JPHs. We thus tested whether E-Syts can also interact with Climp63. Interestingly, co-immunoprecipitation experiments performed in HEK293T cells expressing GFP-tagged E-Syt2 or E-Syt3 proteins showed that Climp63 does not bind to E-Syts, indicating a specific interaction between JPHs and Climp63 (**Figure 17**).

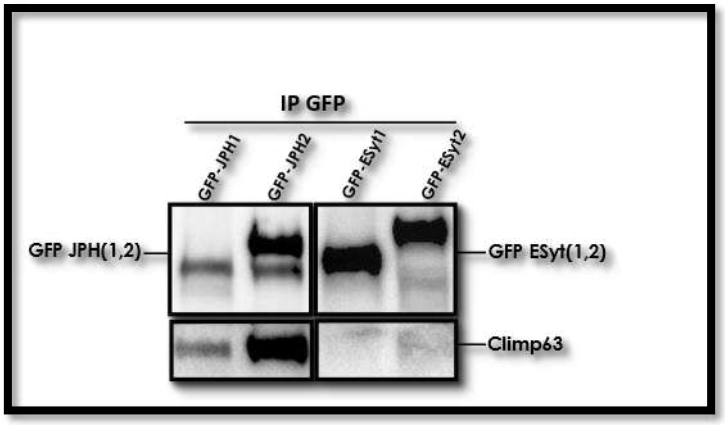


Figure 17 Co-immunoprecipitation on HEK293T cells transfected with GFP tagged proteins: *Total lysates from HEK293T cells expressing GFP-JPH1, GFP-JPH2, GFP-E-Syt1 or GFP-E-Syt2 were immunoprecipitated with anti-GFP conjugated magnetic beads. Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by mouse monoclonal anti-GFP antibodies and rabbit polyclonal anti-Climp63 antibodies.*

To further identify which domains in JPH1 and Climp63 mediate this interaction, we performed a preliminary characterization using mutant proteins deleted of the cytosolic or the luminal domain of Climp63 (mCherry-Climp63Δcytosol and Climp63Δlumen-mCherry) or GFP-JPH1 and GFP-JPH2 mutant proteins containing only the transmembrane domain (**Figure 18A).** Co-immunoprecipitation experiments revealed that all mutant forms of Climp63 were able to interact with JPHs, despite the deletion of the cytosolic or luminal domain (**Figure 18B**). Furthermore, the transmembrane domain of JPH1 and JPH2 were also able to co-immunoprecipitate both full-length and deleted forms of Climp63 (**Figure 18C**).

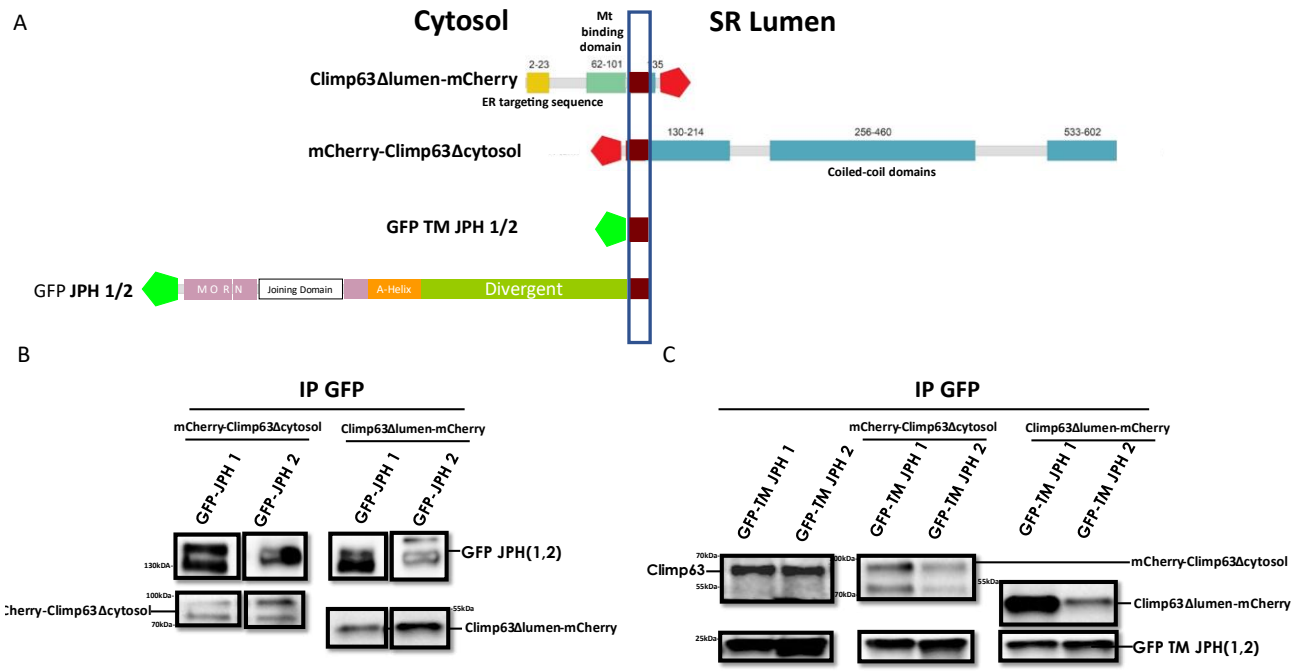
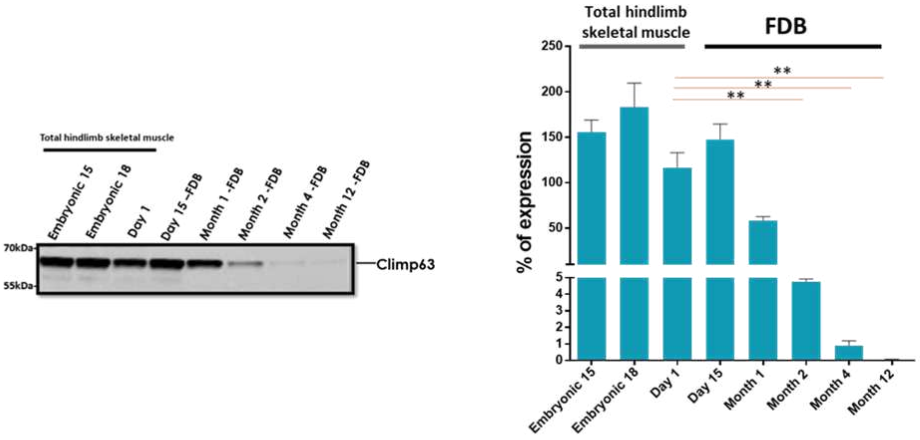


Figure 18 ***Co-immunoprecipitation on HEK293T cells using truncated forms of Climp63 and JPHs****: (A) schematic representation of the Climp63 and JPHs constructs used for the Co-Immunoprecipitation experiments, with the green GFP and red mCherry fluorescent proteins. (**B**) Total lysates from HEK293T cells co-expressing GFP-JPH1 or GFP-JPH2 with mCherryClimp63Dcytosol or Climo63Dlumen, were immunoprecipitated with anti-GFP conjugated magnetic beads. Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by mouse monoclonal anti-GFP antibodies. (C) Total lysates from HEK293T cells co-expressing GFP-TM JPH1 or GFP-TM JPH2 with mCherryClimp63Dcytosol or Climo63Dlumen, were immunoprecipitated with anti-GFP conjugated magnetic beads. Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by mouse monoclonal anti-GFP antibodies and rabbit polyclonal anti-Climp63 antibodies.*

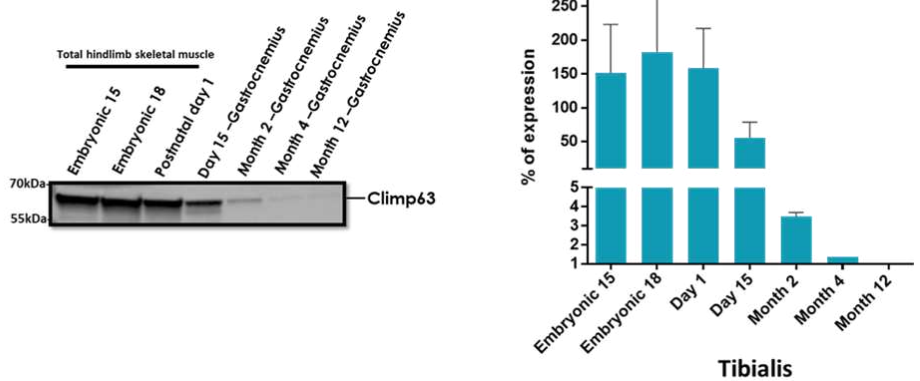
Expression of Climp63 during muscle development

Muscle differentiation is associated with SR remodeling in a multistep process that begins during the embryonic life and achieves maturation after birth. On this line, we investigated the expression of Climp63 during embryonic and post-natal development of mouse muscles. To this aim, total proteins from hind limb muscles at embryonic day 15, 18 and post-natal day 1 were prepared; single skeletal muscles, namely FDB, EDL, Gastrocnemius, Tibialis Anterior and Soleus, as well as cardiac muscle were isolated. Western blot analysis revealed that Climp63 is highly expressed in striated muscles during the embryonic life, while its expression begins to decline starting from postnatal day 15, to be barely detectable from 2 month on. Notably, expression of Climp63 in FDB muscles started to decline at later ages; in addition, the levels of expression of Climp63 at postnatal day 15 were apparently higher in FDB compared to the other muscles analyzed (**Figure 19**).

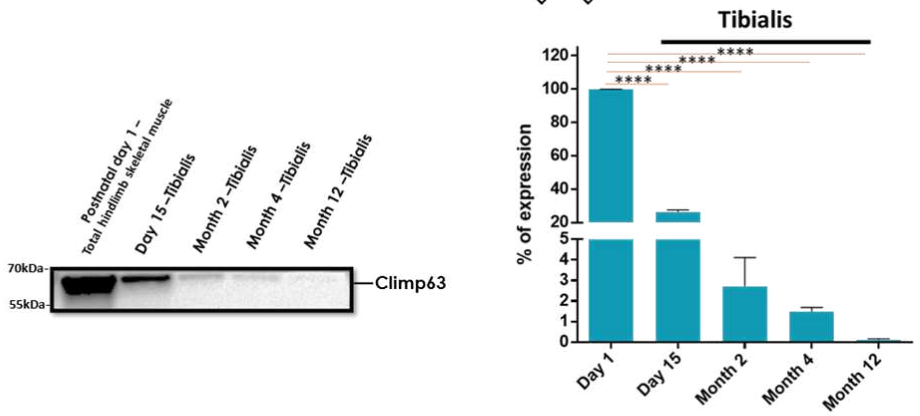
A


Total hindlimb skeletal muscle
Embryonic 15
Embryonic 18
Day 1
Day 15 -FDB
Month 1 -FDB
Month 2 -FDB
Month 4 -FDB
Month 12 -FDB
70kDa
55kDa
Climp63
Total hindlimb skeletal muscle
FDB
% of expression
Embryonic 15
Embryonic 18
Day 1
Day 15
Month 1
Month 2
Month 4
Month 12

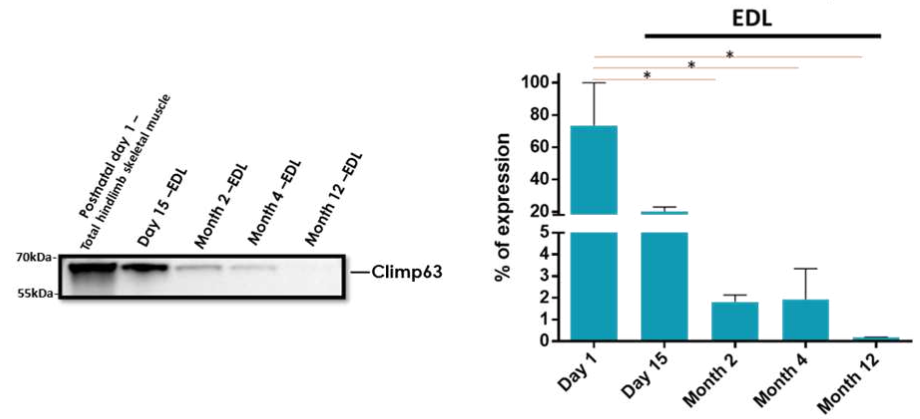
B


Total hindlimb skeletal muscle
Embryonic 15
Embryonic 18
Postnatal day 1
Day 15 -Gastrocnemius
Month 2 -Gastrocnemius
Month 4 -Gastrocnemius
Month 12 -Gastrocnemius
70kDa
55kDa
Climp63
Gastrocnemius
% of expression
Embryonic 15
Embryonic 18
Day 1
Day 15
Month 2
Month 4
Month 12

C


Postnatal day 1 – Total hindlimb skeletal muscle
Day 15 -Tibialis
Month 2 -Tibialis
Month 4 -Tibialis
Month 12 -Tibialis
70kDa
55kDa
Climp63
Tibialis
% of expression
Day 1
Day 15
Month 2
Month 4
Month 12

D


Postnatal day 1 – Total hindlimb skeletal muscle
Day 15 -EDL
Month 2 -EDL
Month 4 -EDL
Month 12 -EDL
70kDa
55kDa
Climp63
EDL
% of expression
Day 1
Day 15
Month 2
Month 4
Month 12

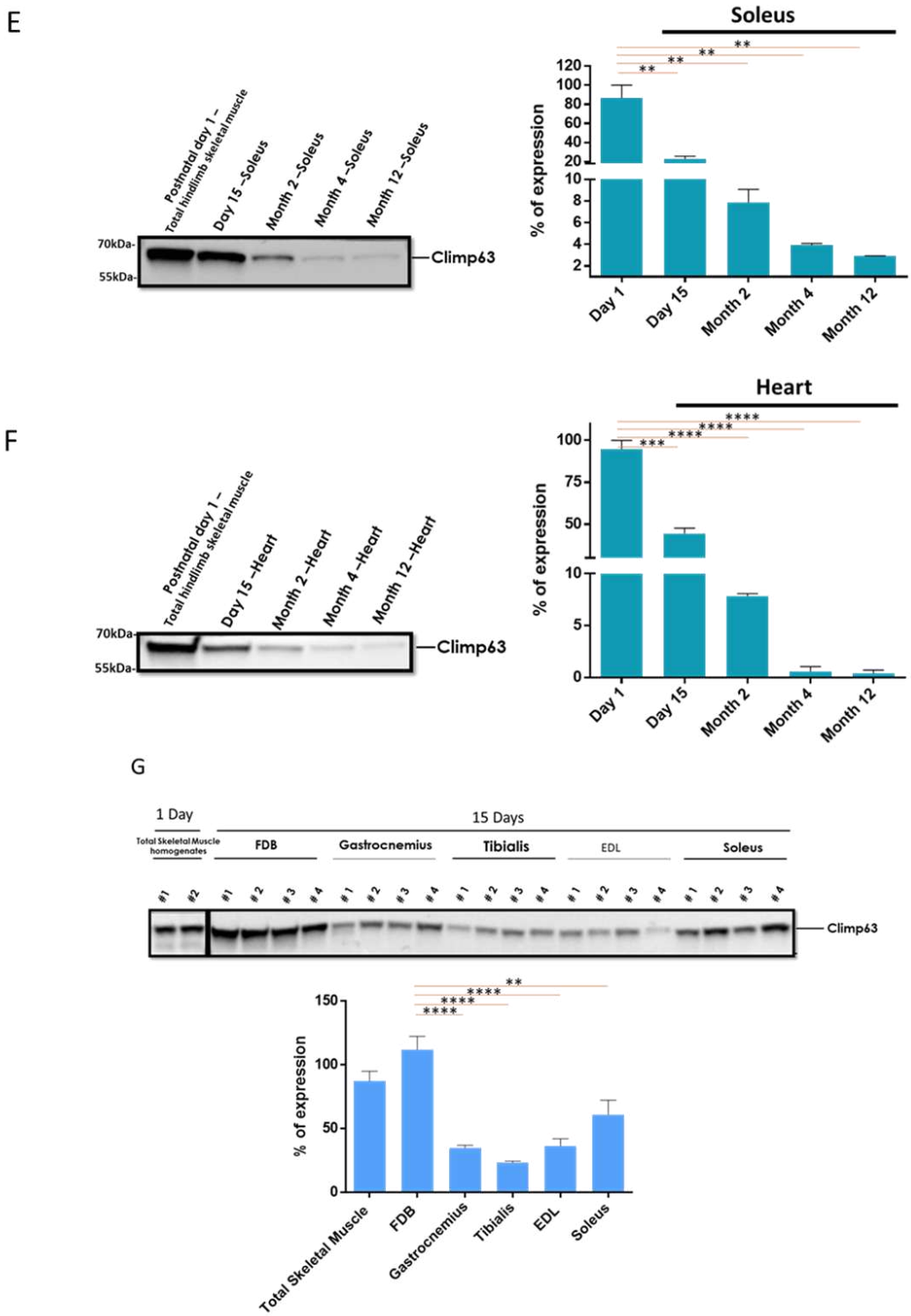


Figure 19 ***Western Blot analysis of Cimp63 expression in skeletal and cardiac muscles at different stages of development***. *Chemiluminescence signals and densitometric analysis of Climp63 expression in total hindlimb skeletal muscles and in FDB (A), Gastrocnemius (B), Tibialis Anterior (C), EDL (D), Soleus (E) and Heart (F) from Embryonic day 15, 18, post-natal day 1 and 15, and post-natal month 2 ,4 and 12 (also 1 month for the FDB in A). Histograms indicate mean expression values from 2-3 independently processed protein lysates ± standard error. P values ≤0.05, 0.01 and 0.001 are indicated by *, ** and*** respectively compared to the total skeletal muscle lysates from 1 day post-natal. G) Chemiluminescence signals and densitometric analysis of Climp63 expression in FDB, Gastrocnemius, Tibialis Anterior, EDL and Soleus muscles at post-natal day 15. Histograms indicate mean expression values from 4 independently processed protein lysates ± standard error. P values ≤0.05, 0.01 and 0.001 are indicated by , ** and*** respectively compared to FDB protein level at post-natal day 15.*

Conclusion and Discussion

Triads, made by the apposition of the membranes of TTs, invaginations of the muscle plasma membranes, and j-SR, the Ca^{2+} reservoir of the cell, are pivotal for the ECC (Dulhunty et al., 2002). Many proteins present at this region contribute to ECC by modulating DHPR and RyR1 channels and by contributing to biogenesis and maintenance of the triadic structure. Among them, JPHs, play a regulatory role in the Ca^{2+} release machinery and a morphological role in the development and maintenance of these structures, acting as molecular bridges between TTs and the terminal cisternae of the j-SR (Takeshima, 2000). In addition, JPHs interact with different proteins present at triads, including Triadin, Calsequestrin, Cav-3, RyR1 and DHPR, forming a molecular scaffold for the assembly of the calcium release complex (Golini et al., 2011; Minamisawa et al., 2004; Phimister et al., 2007; Rossi et al., 2022a). Considering the role of JPHs in both regulatory and morphological aspects of triads, characterizing known and new interactions of JPHs within this region is of interest of our laboratory since many years.

Using the Proximity-dependent labelling with BioID2, we identified Climp63 as a novel interactor of JPHs at triads. Interaction between JPHs and Climp63 was further confirmed by co-immunoprecipitation experiments, both in HEK293T cells and in mouse skeletal muscles. Interestingly, this interaction is specific for JPHs, since Climp63 is not co-immunoprecipitated by members of the Extended Synatptotagmin family, non-muscle specific proteins that, like JPHs, form PM-ER contact sites in eukaryotic cells (Giordano et al., 2013).

Previous studies by Osseni and collaborators also showed that Climp63 is partially localized at triads, where it interacts with the luminal domain of Triadin (Osseni et al., 2016). Ablation of Triadin in knockout mice results in Climp63 mis-localization, triad alteration and flattering of terminal cisternae, indicating that Climp63 supports the shaping of the SR and maintains the correct width of terminal cisternae. In addition, expression of dominant negative Climp63 mutants, unable to bind microtubules, resulted in disorganization of the microtubule network in FDB muscles. According to these studies, we found that Climp63 co-immunoprecipitates with JPH1, JPH2 and Triadin. Moreover, we also found that it co-immunoprecipitates with Junctin, a protein that shares with Triadin a high degree of sequence homology in its luminal domain, further supporting the hypothesis that Climp63, Triadin and Junctin forms luminal hetero-oligomers that contribute to maintain the

architecture of SR cisternae. This luminal assembly, however, does not, apparently, include Calsequestrin that we found to not co-immunoprecipitate with Climp63.

Given that JPHs are tail-anchored proteins, interaction with Climp63 is unlikely to occur in the SR lumen. Co-immunoprecipitation experiments using deletion mutants of either Climp63 or JPHs, however, did not allow us to identify the regions of interaction in the two proteins. Indeed, Climp63 mutants deleted in their luminal or cytosolic domains still interact with JPH1 and JPH2, suggesting that neither domain is essential for this interaction. Moreover, experiments performed with GFP-fusion proteins containing only the TM domain of JPHs also resulted in Climp63 co-immunoprecipitation, suggesting that the TM region alone may be involved in protein interaction, although further experiments are needed to confirm these results.

Analysis of the expression profile of Climp63 during skeletal muscle development revealed that it is predominately expressed in the embryonic and first days after birth, declining drastically starting from post-natal day 15 and maintaining basal levels of protein expression until 12 months of age. The only exception was observed in the FDB muscles where, at 15 days of age, the expression level of Climp63 remains elevated and started to decline only at later ages. Studies by Zuurveld and collaborators showed that, in rats, post-natal differentiation of FDB muscles occurs later than in Soleus and EDL muscles (Zuurveld et al., 1985) suggesting that the higher levels of expression of Climp63 observed in mouse FDB muscle at post-natal day 15 may be due to its intrinsic slower maturation compared to the other muscles analyzed. In any case, in most of mouse skeletal muscles, post-natal day 15 represents an important stage in muscle development since, around this age, TTs and triads complete their maturation, passing from a longitudinal to a transversal orientation with respect to the major axis of the fiber. Considering the expression pattern of Climp63, it could be postulated, that this protein may play a major role during the first steps of SR remodeling, while in mature muscles, its role may be limited to contribute to maintain the overall structure of the SR cisternae.

Based on data from Osseni and collaborators, Climp63 may also play a role as microtubule organizer in skeletal muscles; interaction among Climp63, JPHs and the microtubular network has not been investigated in this thesis, nor literature data are available on the role of microtubule in transition of longitudinal to transversal orientation of triads. Studies performed in skeletal muscle indicate that alteration in the microtubular network is associated with muscle diseases, including Duchenne muscle dystrophy (Prins et al., 2016). Different studies performed on failing hearts and in ventricular

cardiomyopathies showed that TTs are disorganized and JPH2 is mis-localized, with a significant alteration of ECC (Zhang et al., 2014). Interestingly, in these conditions, the microtubular cytoskeleton was found to be excessively densified and treatment with nocodazole was able to rescue the disease phenotype, TT organization and JPH2 localization (Prins et al., 2017). Research in other cell types, such as neurons, suggests that microtubules play an important role in mediating the remodeling of the endoplasmic reticulum during differentiation processes. For example, in mature neurons, the ER is distributed along the entire axon with a tubular arrangement and this organization appears to be regulated by ER-microtubule interaction (Wu & Akhmanova, 2017). In neurons, Climp63 is preferentially localized in somato-dendritic regions and is excluded from axons, confirming that this protein is directly involved with the formation of the cisternae of the reticulum. The peculiar distribution of Climp63 in defined regions of the reticulum and its interaction with the microtubules may therefore indicate an involvement of this protein in cisternae formation and consolidation of ER organization in polarized cells, and skeletal muscle fibers.

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