Minor sarcoplasmic reticulum membrane components that modulate excitation-contraction coupling in striated muscles

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- **Abstract**

Alterations of Ca^{2+} signaling have been linked to several genetic neuromuscular diseases, thus it is important to uncover the toolkit components and intracellular pathways involved in Ca^{2+} signaling under normal and pathological conditions.

Calcium homeostasis in striated muscles

Over the decades, the role(s) played by Ca^{2+} in skeletal muscle have been unveiled and it is now clearly established that it is the key molecule underlying muscle contraction. Its importance is given by the fact that movement of the contractile proteins is dependent on the Ca^{2+} released from the sarcoplasmic reticulum (SR), an organelle constituting approximately 10% of the cell's volume and fully dedicated to uptake and release of Ca^{2+} (Peachey, 1965; Volpe, 1991). The SR can be structurally divided into two distinct portions: the terminal cisternae, which face the transverse tubules (an invagination of the plasma membrane) and the longitudinal sarcoplasmic reticulum, connecting two terminal cisternae. The terminal cisternae can be further divided into junctional face membrane (the domain facing the transverse tubules) and non junctional membrane (Saito, 1984; Costello, 1986).

Depolarization of the plasma membrane of skeletal muscle leads to release of Ca^{2+} from the SR resulting in muscle contraction, by a process known as excitation-contraction coupling (Schneider, 1972; Melzer, 1995; Berchtold, 2000). Excitation-contraction coupling (EC coupling) occurs at the triad, a structure composed of the two membrane compartments, transverse tubules containing the voltage sensing dihydropyridine receptor (DHPR, an L-type Ca^{2+} channel) and terminal cisternae on which ryanodine receptor (RyR) Ca^{2+} release channels are localized (Mitchell, 1983; Rios 1991). The disposition of DHPRs and RyRs on their respective membranes is highly ordered and each RyR tetramer faces alternative rows of DHPRs (figure 1) (Block, 1988; Franzini-Armstrong, 1994; Paolini, 2004). These two Ca^{2+} channels are the basic unit underlying excitation-contraction coupling but they do not function alone, but rather can be thought of as macromolecular structures, composed of a number of accessory proteins involved in their fine regulation.

Major and minor protein components of the sarcoplasmic reticulum

One of the major advances in the field of excitation-contraction coupling was the development of reproducible procedures enabling the fractionation of SR membranes enriched in proteins involved in calcium handling (Campbell, 1980; Meissner, 1973; Saito, 1984). This revealed that protein components of the longitudinal SR (LSR) and terminal cisternae are different, reflecting the functional subspecialization of these membrane fractions, which are respectively, Ca^{2+} uptake and Ca^{2+} release. Figure 2 shows a 5-15**%** SDS PAG stained with Coomassie Brilliant Blue of the protein components of LSR, terminal cisternae and junctional face membrane. The major component of the LSR constituting approximately 80% of the total proteins present, is the 110 kDa CaATPase (SERCA), i.e. the pump responsible for pumping the Ca^{2+} released by the RyR1s back into the SR. The 22kDa protein band present in the longitudinal SR fraction is phospholamban, a protein involved in regulating the activity of the SERCA pump in heart and slow twitch muscle fibers. When phospholamban is de-phosphorylated, it inhibits the activity of SERCA, whereas in its phosphorylated state, inhibition is relieved (Slack, 1997; Liu, 1997; MacLennan, 2003). Ablation of phospholamban causes a significant (25%) decrease in the time to half relaxation of isolated solei with no change in the contraction time (Jay, 1997), supporting its important role in regulating of SERCA activity.

Two glycoproteins of 160 kDa (sarcalumenin) and 53 kDa (53 kDa glycoprotein) represent minor protein constituents of the LSR membrane fraction and are generated by alternative splicing of the same transcript, which is expressed both in heart and skeletal muscle. The large transcript (sarcalumenin) is a low affinity (k_D= 0.6 mM) high capacity (35 mol/mol protein) Ca^{2+} binding protein, while the shorter product of 53 kDa, lacks the NH_2 and thus the Ca^{2+} binding domain. Sarcalumenin is involved in the maintenance of the SERCA protein as illustrated by the fact that sarcalumenin KO animals exhibit significant decreased SERCA activity and SERCA protein content (Leberer, 1990; Yoshida, 2005).

As shown in figure 2, the protein composition of the JFM is far more complex than that of the LSR (Costello, 1986); aside the most abundant components, including the ryanodine receptor (RyR), calsequestrin (CsQ), histidine rich Ca^{2+} binding protein and

traidin(s) (TRISK) which will not be discussed in this review, there are a number of other minor protein components whose function has recently been unraveled or that still await functional characterization. Much work has focused on the identification of the full set of protein constituents of the junctional face membrane (JFM) and on understanding their functional role in excitation-contraction coupling. Two main approaches have been used to characterize the minor membrane-protein components at the molecular, cellular and functional level: (i) the immuno-proteomic approach utilized by Takeshima's lab, which combines production of monoclonal Abs against membrane proteins selected on the base of specific triadic immunostaining of muscle sections, cDNA cloning, expression and biochemical analysis of identified proteins to gene knock out techniques (Weisleder, 2008). (ii) Junctional face membrane purification or heparin agarose chromatography and identification of proteins co-eluting with the RyR, combined with Western blotting, Mass Spec analysis and peptide sequencing (Divet, 2005). These approaches have been relatively successful and at least 5 minor membrane protein components that will be described in the next section, have been identified and characterized.

Mitsugumin-29:

This is a 29 kDa membrane protein related to the synaptophysin-family, originally identified in the SR of skeletal muscle and in the endoplasmic reticulum of kidney renal tubules (Shimuta, 1998). Analysis of its primary sequence, as well as biochemical and ultrastructural evidence suggest that mitsugumin-29 contains 4 transmembrane spanning domains and that it is localized in the transverse tubules of mature skeletal muscles where it self-associates as hexamers (Shimuta, 1998; Brandt, 2001). Though mitsugumin-29 does not tightly associate with other proteins, experimental evidence suggests that it can functionally interact with the RyR1, whereby it increases RyR1 open probability without affecting channel current amplitude (Pan, 2004). Muscles isolated from mitsugumin-29 KO mice exhibit swollen transverse tubules, vacuolated SR and misaligned triadic structures. These ultrastructural changes are accompanied by dysfunctional Ca^{2+} handling; specifically, the intracellular Ca^{2+} stores of myotubes from mitsugumin-29 KO mice deplete more rapidly and refill more slowly after depolarization than myotubes from control mice (Pan, 2002). Such alterations lead to "global" functional changes, so that

muscles from mitsugumin-29 KO mice fatigue more rapidly than their wild type counterpart (Nagaraj, 2000). Taken together these results suggest that mitsugumin-29 functions as a tethering structure, forcing the transverse tubules into a conformation, which favors the formation of triadic structures. Lack of integral triads then leads to altered Ca^{2+} handling and defective SOC-dependent Ca^{2+} influx.

Junctophilin-1:

Junctophilins are membrane spanning proteins with a large cytoplasmic region containing a 14 amino acid repeat motif (MORN motif) with selective binding affinity for the plasma membrane and a carboxy-terminal transmembrane segment spanning the ER/SR (Takeshima, 2000). At least three isoforms encoded by distinct genes, exist: junctophilin-1 is specifically expressed in skeletal muscle, junctophilin-2 is expressed in the heart, in skeletal muscles and in smooth muscles and junctophilin-3 is expressed in the brain (Nishi, 2000). In skeletal muscle junctophilin-1 (72 kDa protein) is involved in physically linking the transverse tubules to the SR membrane. Protein overlay and surface plasmon assays suggest that it achieves this by interacting with phospholipids, especially with sphingomyelin and phosphatidylchloine, rather than through protein-protein interactions (Weisler, 2008). Ablation of junctophilin-1 severely affects muscle function leading homozygous KO mice to premature death within 20 h after birth. Ultrastructural examination of the skeletal muscles of junctophilin-1 KO mice shows morphological abnormalities, including incomplete formation of the junctional complexes between transverse tubules and the SR, swollen terminal cisternae and reduced numbers of triads. As a consequence muscles develop less contractile force after electrical stimulation and show abnormal sensitivity to extracellular Ca^{2+} (Ito, 2001; Komazaki, 2002).

SRP-27/TRIC-A:

Mitsugumin-33 or TRIC-A (trimeric intracellular cation-selective channel) (Yazawa, 2007) also known as SRP-27 (sarcoplasmic reticulum protein of 27 kDa) (Bleunven, 2008) is expressed in excitable tissues and is particularly enriched in fast twitch skeletal muscles, where its expression level peaks after 2 months of post-natal development. Mice lacking TRIC/SRP-27 are viable and display no overt phenotype.

Double-labeling immunocytochemistry experiments of mouse muscle fibers indicate that SRP-27 is localized in the perinuclear endoplasmic reticulum as well as in a SR subcompartment, which is adjacent to, but distinct from, that containing the RyR1 and SERCA (Bleunven, 2008). Interestingly, SRP-27/TRIC-A could be pulled-down by beads coated with maurocalcine and RyR1, but not with maurocalcine alone, raising the possibility that SRP-27/TRIC-A is part of the RyR1 macromolecular complex (Bleunven, 2008). Hydrophobicity plots and biochemical analysis also revealed that TRIC-A/SRP-27 is an integral ER/SR protein containing up to three membrane spanning domains, whose amino-terminus is located in the lumen of the ER/SR and whose carboxy terminus is exposed to the cytoplasm. Sequence comparison also predicts the presence of an ion-conducting pore between the first and second transmembrane domains and cross-linking experiments demonstrate that TRIC-A/SRP-27 tends to form homo-oligomers (dimers and trimers). Three dimensional reconstruction studies of the native protein suggest that it acquires a pyramidal elongated structure, similar to that of bacterial porin channels (Yazawa, 2007). Interestingly, reconstitution in lipid bilayers, suggest that TRIC-A/SRP-27 is a cation channel, with a selectivity of K^+ over Na^+ (permeability ratio P_K/P_{Na} =1.5) (Yazawa, 2007). To gain more insight into the function of this channel, Yazawa (2007) followed changes in the membrane potential of isolated muscle fibers from control and TRIC-A/SRP-27 KO mice. The lack of TRIC-A/SRP-27 reduced the K^+ permeability accompanying thapsigargin-induced Ca^{2+} efflux, without affecting Ca^{2+} permeability, suggesting that this protein may act as a monovalent-cation channel. Such a channel could be activated physiologically during RyR1-mediated Ca^{2+} release to counter-balance the charge movement due to efflux of Ca^{2+} (Somlyo, 1981), which would otherwise leave the SR lumen with a negative charge. However, the role of TRIC-A/SRP-27 as a monovalent-cation contercurrent channel during Ca^{2+} release has been challenged by data of Gillespie and Fill (Gillespie, 2008) which indicates that the RyR1 channel mediates its own potassium countercurrent during SR Ca^{2+} release. This would obviate the need of an additional countercurrent carrier during SR Ca^{2+} release, leaving the exact functional role of TRIC-A/SRP-27 controversial.

JP-45 is a 45 kDa polypeptide containing a single transmembrane segment, which is highly enriched in skeletal muscle junctional face membrane where its expression is developmentally regulated, reaching maximal levels during the second month of post-natal development (Anderson, 2003). Originally JP-45 was identified as a protein weakly phosphorylated by cAMP dependent protein kinase and co-eluting with the RyR1 and DHPR from a heparin-agarose column (Zorzato, 2000). Surprisingly however, co-immunoprecipitation experiments revealed that JP-45 is not part of the RyR1 macromolecular complex, but rather it interacts with calsequestrin via its luminal carboxy –terminal domain and with $Ca_v1.1$, through its cytoplasmic amino terminus (Anderson, 2003). Extensive pulldown and co-immunoprecipitation experiments, revealed that JP-45 binds to different regions on the $Ca_v1.1$, namely to its carboxy- terminus and to a region within the I-II loop referred to as AID, where the ß1a subunit also binds (Anderson, 2006). The interaction between $Ca_v1.1$ and ß1a is thought to be essential for targeting or stabilizing $Ca_v1.1$ on the plasma membrane (Flucher, 2002).

Several approaches have been exploited to unravel the function of JP-45 in skeletal muscle: (i) acute over-expression and depletion of JP-45 in differentiated C2C12 myotubes (Anderson, 2006; Gouadon, 2006) and (ii) chronic depletion in JP-45 knock out mice (Delbono, 2007). Interestingly both over-expression and ablation of JP-45 result in a decrease of voltage-dependent Ca^{2+} release. This effect could be due to a decrease of functional expression of the $Ca_v1.1$ on the transverse tubules (Anderson, 2006; Delbono, 2007). Alternatively, the effect on Ca^{2+} release may be linked to alterations of the interaction of JP-45 with calsequestrin. Gouadon (2006) showed that low levels of JP-45 over-expression affect the permeability of the Ca^{2+} release unit by altering excitation-contraction coupling transfer function (Gouadon, 2006). Given that the lumenal carboxy–terminus of JP-45 binds to calsequestrin, it is possible that JP-45 constitutes a key protein for a signalling pathway between calsequestrin and $Ca_v1.1$. Over-expression of JP-45 may result in the accumulation of JP-45 molecules which are not associated with calsequestrin, and this in turn may send an inhibitory signal to the $Ca_v1.1$

Interestingly the skeletal muscle phenotype of young JP-45 KO mice, is reminiscent of that of aged mice. During mouse ageing, the membrane density of the voltage sensor ($Ca_v1.1$), the SR membrane content of JP-45 and the Ca^{2+} currents of muscle membranes

are significantly lower compared to those of young animals (Anderson, 2006; Delbono, 1995; Gonzales, 2003; Renganathan 1997, 1998). These data suggest that both $Ca_v1.1$ and JP-45 may be important for the maintenance of muscle strength, and indicate that JP-45 KO mice may be a useful experimental model to investigate alteration of excitation contraction coupling linked to ageing.

Junctate/ humbug

Junctate is a 33 kDa protein with a single ER/SR membrane spanning domain, expressed in a variety of excitable and non-excitable tissues (Treves, 2000; Dinchuk, 2000; Hong, 2001). Figure 3A shows how transcripts deriving from the same gene (*BAH* or AßH-J-J locus) located on human chromosome 8q12.1 can give rise to four distinct classes of proteins via a complex pattern of alternative splicing. Though the complexity of AßH-J-J locus is more the exception than the rule, it illustrates how important it is to precisely establish the number and type of gene products when deciding to create knock out animal models of protein(s) encoded by the *BAH* gene.

Figure 3B illustrates schematically how the different polypeptides are assembled to yield: (i) junctin, a structural calsequestrin binding protein present in cardiac and skeletal muscle SR that forms a quaternary complex with triadin, RyR1 and calsequestrin (Jones, 1995; Kagari, 1996; Zhang, 1997). (ii) Aspartyl-ß-hydroxylase an enzyme catalyzing posttranslational hydroxylation of aspartate and asparagine residues within epidermal growth factor-like domains present in receptors and receptor ligands involved in cell growth and differentiation, and extracellular matrix molecules (Stenflo, 1989; Gronke 1989; Monkovic, 1992). (iii) Junctate, a moderate affinity (k_D 217 µM), high capacity (21 moles Ca^{2+}/mole protein) Ca^{2+} binding protein (Treves, 2000). (iv) Humbug, a truncated version of aspartyl-ß-hydroxylase, lacking its catalytic domain, which shares with junctate the high capacity moderate affinity Ca^{2+} binding domain (Dinchuk, 2000; Treves, 2000; Hong, 2001). Analysis of the genomic organization of the AßH-J-J locus has revealed the presence of two distinct promoters P1 and P2 (Feriotto, 2006) that are regulated by specific transcription factors, giving rise to polypeptides with distinct amino-termini and tissue distribution. Transcripts starting from exon 1 (green box in fig.3A), which is under the control of the P1 promoter, are expressed in most tissues and

share their NH_2 terminus with aspartyl-ß-hydroxylase (Treves, 2000; Dinchuk, 2000; Feriotto, 2007). Exon 1a (blue box in fig. 3A) is approximately 8 kb downstream from exon 1 and is under the control of the P2 promoter whose induction is controlled by the muscle specific transcription factor MEF-2 (Feriotto, 2005). Transcripts starting from this exon are expressed in striated muscles and share their NH_2 termini with junctin/junctate (Treves, 2000; Dinchuk, 2000). Exon 2 encodes the transmembrane domain (yellow box in fig.3A) and together with exon 3, is shared by all family members deriving from the AßH-J-J locus. The carboxy-terminal portion of the proteins depends on which exons are transcribed: junctin results from transcription of exons 4a and 5a, while all other products are generated via transcription of exons 4-24 and result in a variety of products of different sizes. The longer transcripts give rise to the enzyme aspartyl-ß-hydroxylase with an apparent molecular mass of approximately 120 kDa. The shorter transcripts generate proteins with molecular masses ranging from 40 to 53 kDa which share the acidic Ca^{2+} binding domain (Treves, 2000; Dinchuk, 2000; Hong, 2001). Heart expresses junctin and junctate, as well as humbug and aspartyl-ß-hydroxylase (Treves, 2000; Dinchuk, 2000; Hong, 2001). Interestingly, humbug is also highly expressed in a variety of invasive human tumors and its level of expression has been suggested to be useful as a prognostic marker for cancer progression (Ince, 2000; Palumbo, 2002; Maeda, 2004; Xian, 2006; Wang, 2007; Lee, 2008).

In the rest of this section only the functional properties of junctate will be discussed, and the reader is referred to other articles describing the function of aspartyl-ß-hydroxylase and junctin (Gronke, 1989; Wang, 2007; Jones, 1995; Kagari, 1996; Zhang, 1997). Because of its calcium binding properties, a number of approaches were undertaken to define the potential role of junctate in Ca^{2+} homeostasis: (i) acute over-expression and depletion junctate from cultured cells (Treves, 2000; Treves, 2004) and (ii) chronic over-expression of junctate in skeletal muscles (Divet, 2007) and heart (Hong, 2008). Acute over-expression of junctate in COS-7 and HEK293 cells is accompanied by significant functional and structural changes of the ER membrane. Specifically, in HEK cells over-expressing TRPC3 channels (HEKT3) over-expression of junctate induces extensive proliferation of the ER resulting in significantly larger and more frequent couplings between the ER and the plasma membrane (Treves, 2004). The

induction of ER plasma membrane couplings by junctate is in agreement with co-immunoprecipitation data showing that junctate forms a supramolecular complex with InsP3R and TRPC channels. These structural changes are paralleled by alterations of Ca^{2+} homeostasis, specifically by increased peak Ca^{2+} release and store-depletion activated Ca^{2+} influx; on the other hand, knocking down junctate results in diminished agonist induced peak $[Ca^{2+}]_i$ transients and store depletion activated Ca^{2+} influx (Treves, 2000, 2004). The increase of store depletion-activated Ca^{2+} influx is mediated by the short cytoplasmic NH_2-terminal domain of the protein, while the luminal carboxy-terminus Ca^{2+}binding domain of junctate (and thus also of humbug) increases the Ca^{2+} content of ER/SR stores and affects calcium transients evoked by SERCA inhibitors.

As to the effect of chronic over-expression of junctate, some discrepancies have arisen from the transgenic mouse models. Over-expression in skeletal muscles does not lead to an overt phenotype but is accompanied by a small increase in Ca^{2+} loading and Ca^{2+} storage of the SR resulting in a significant increase in RyR1 mediated Ca^{2+} release and an increased Ca^{2+} influx following depletion of intracellular Ca^{2+} stores (Divet, 2007). These changes were attributed to the over-expression of junctate's Ca^{2+} binding sites since the expression levels of other SR Ca^{2+} handling proteins such as SERCA, calsequestrin or sarcalumenin were not changed. Interestingly, the increased Ca^{2+} cycling across the SR membrane was accompanied by an adaptive increase in the number of mitochondria in fast fibres of EDL (but not soleus) muscles, which was not due to fast-to slow fibre type transition (Divet, 2007). Junctate over-expression in the heart on the other hand, leads to severe cardiac hypertrophy, bradycardia and arrythmias as well alterations in the expression level of the SR proteins SERCA2, calsequestrin-2 and calreticulin. This decreased SR content of Ca^{2+} handling proteins is accompanied by an up-regulation of the Na^+/Ca^{2+} exchanger and plasmalemma calcium pump, two component of the cardiac sarcolemma involved in extrusion of Ca^{2+} from the cytoplasm. The decrease of the major calcium binding proteins of cardiac SR is paralleled by a decrease in caffeine induced Ca^{2+} release indicating a lower cardiac Ca^{2+} SR loading. At the moment the reasons for these apparent opposite effects of junctate over-expression in the heart and in skeletal muscle have not yet been elucidated.

Conclusions

The past three decades have seen major advancements in our understanding the role of skeletal muscle SR proteins in excitation-contraction coupling. The use of genetically modified animal models has also taught us that few of these proteins, namely the ryanodine receptor, Cav1.1 and junctophyllin, are essential for EC coupling. On the other hand, the minor protein components seem to be important for the regulation of the EC coupling machinery. Mouse and cellular models have also shown that acute and/or chronic over-expression/depletion of a variety of minor components do not result in lethal phenotypes and/or in severe damage of to the EC coupling machinery, suggesting that its fine regulation is provided by functionally redundant minor components. The comprehension of EC coupling and its involvement in the pathophysiology of neuromuscular disorders awaits the identification and functional characterisation of the complete array of proteins of the transverse tubule and junctional face membrane compartments.

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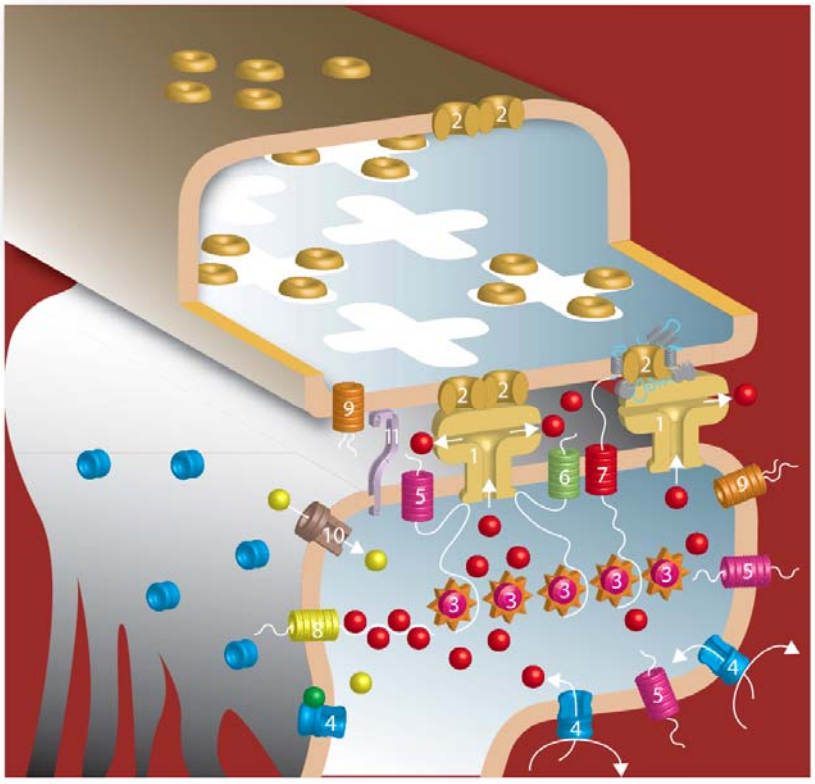
FIGURE LEGENDS:

Figure 1: **Schematic representation of the protein components of skeletal muscle sarcoplasmic reticulum.**

Figure 2: Coomassie Brilliant Blue stained gradient (5-15%) SDS PAGE of protein components present in the longitudinal sarcoplasmic reticulum (LSR), in terminal cisternae (TC) and in the junctional face membrane (JFM) fractions obtained from rabbit SR. (reproduced from figure 2, Zorzato 1986).

Figure 3: Genomic organization, splicing pattern and main protein products deriving from of the A-ß-J-J locus. Panel A: (reproduced from figure 4, Dinchunk, 2000). Coloured boxes represent different exons. Products deriving from exon 1 (green box) give rise to ß-aspartyl-hydroxylase/humbug; products deriving from exon 1b (light blue) give rise to junctin/junctate. Yellow box encodes the transmembrane domain. Panel B: schematic representation of the 4 main proteins (junctin, junctate, aspartyl-ß-hydroxylase and humbug) derived by assembling the different exons (colors of the protein domains match those of the exons from which they are derived).

Figure 1



1 RyR	4 SERCA	7 JP-45	10 SRP-27/TRIC
2 DHPR	5 TRISC	8 Junctate	11 junctophilin
3 CSQ	6 junctin	9 MG-29	

Figure 2

Protein composition of skeletal muscle sarcotubular membranes

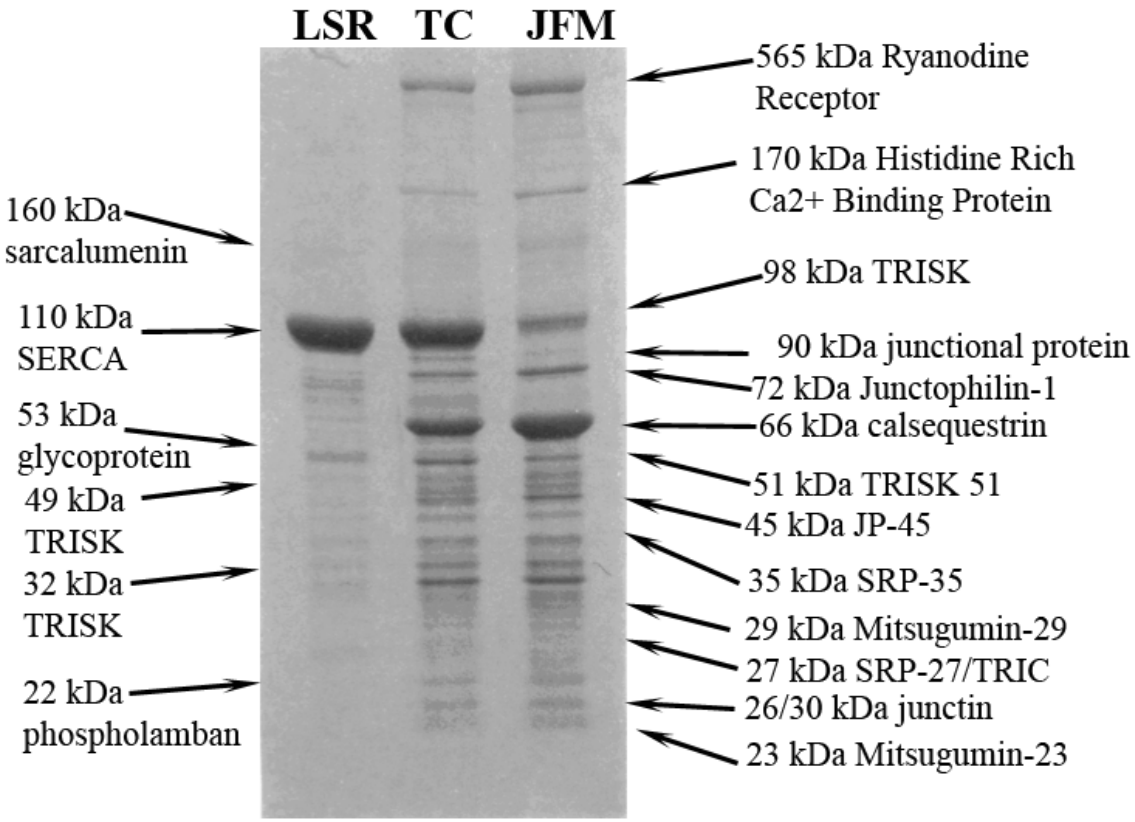
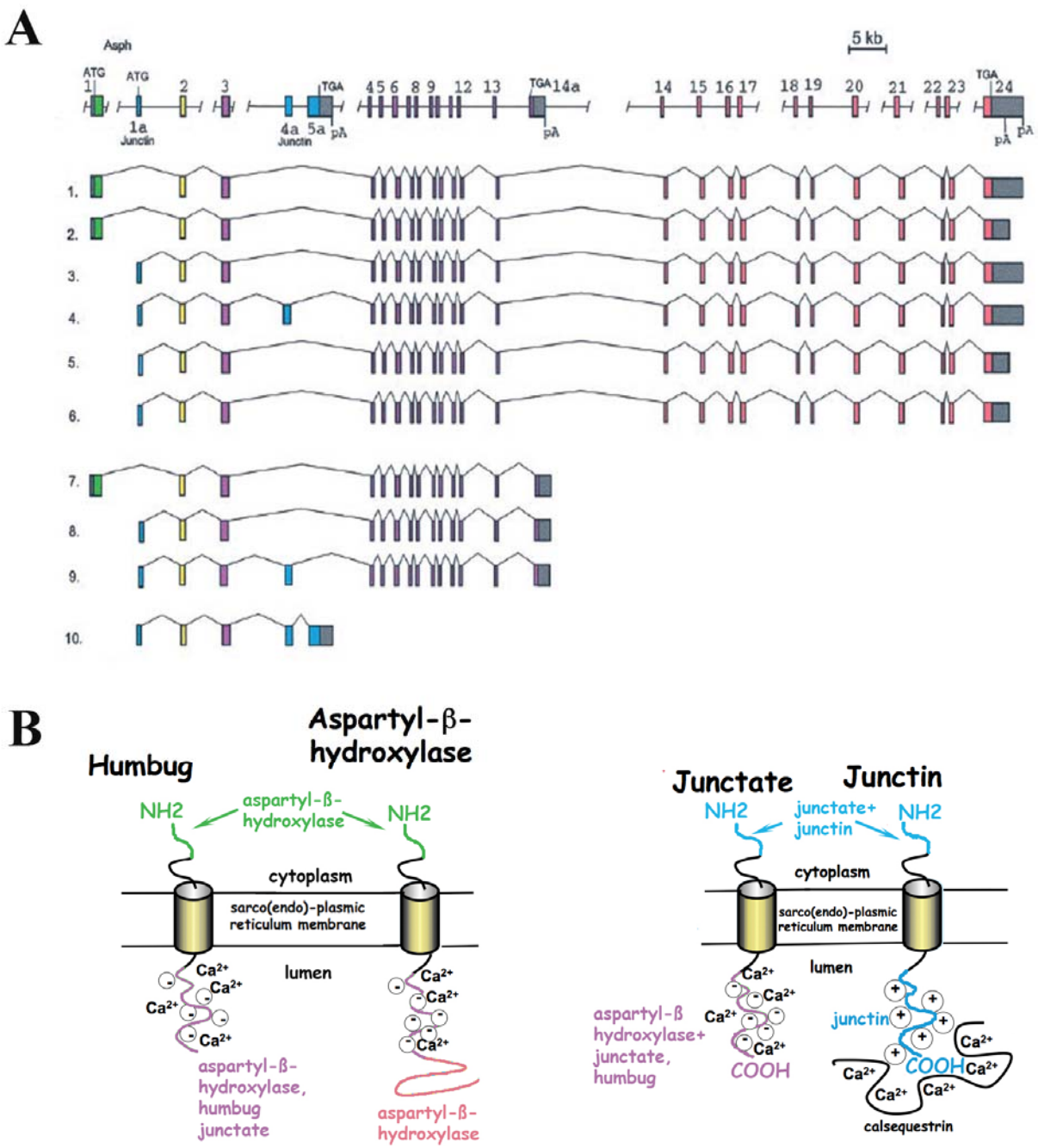


Figure 3


A
Asph
5 kb
ATG
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1a
Junctin
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Junctin
pA
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
B
Humbug
Aspartyl-β-hydroxylase
NH2
aspartyl-ß-hydroxylase
cytoplasm
sarco(endo)-plasmic reticulum membrane
lumen
Ca2+
aspartyl-ß-hydroxylase, humbug junctate
aspartyl-ß-hydroxylase
Junctate
Junctin
junctate+ junctin
aspartyl-ß hydroxylase+ junctate, humbug
COOH
junctin
calsequestrin