

SYMPOSIUM REVIEW

Triadin regulation of the ryanodine receptor complex

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Abstract The calcium release complex is the major player in excitation–contraction coupling, both in cardiac and skeletal muscle. The core of the complex is the ryanodine receptor, and triadin is a regulating protein. Nevertheless, the precise function of triadin is only partially understood. Besides its function in the anchoring of calsequestrin at the triad/dyad, our recent results allow us to propose hypotheses on new triadin scaffolding functions, based on the studies performed using different models, from triadin knockout mice to human patients, and expression in non-muscle cells, taking into account the presence of multiple triadin isoforms.

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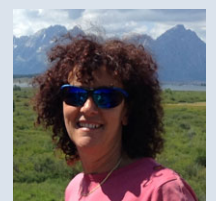
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Abbreviations CPVT, catecholaminergic polymorphic ventricular tachycardia; CRC, calcium release complex; CSQ, calsequestrin; DHPR, dihydropyridine receptor; EC, excitation–contraction; ER, endoplasmic reticulum; FKBP, FK506 binding protein; KO, knockout; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

Upon stimulation of the skeletal muscle at the neuromuscular junction, a membrane depolarization propagates along the plasma membrane up to the T-tubules, invaginations of the plasma membrane deep into the muscle fibre. This depolarization is then transformed into a large intracellular calcium release from the sarcoplasmic reticulum (SR), which in turn induces the muscle contraction. This step, called excitation–contraction (EC) coupling, is performed by the calcium release complex (CRC), localized exclusively in a specific region of skeletal muscle, the triad, composed of the close apposition of two terminal cisternae of sarcoplasmic reticulum on both sides of a T-tubule. The core of this complex is composed of two calcium channels, the dihydropyridine receptor

(DHPR), a voltage-activated calcium channel in the T-tubule membrane, and the ryanodine receptor (RyR), the sarcoplasmic reticulum calcium channel (Lanner, 2012). A number of associated proteins are organized around these two channels (calsequestrin, triadin, junctin, ...), and an efficient muscle contraction relies on the synchronous work of all these proteins. In skeletal muscle, both channels, even anchored in two different membranes, are physically associated (Marty *et al.* 1994): the membrane depolarization activates DHPR, which by a conformational change directly activates RyR and induces the calcium release from the SR (a mechanism called ‘voltage-induced calcium release’). The function of the calcium release complex is therefore based upon the precise organization of the triad and the implantation of

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the complex in this specific structure. In cardiac cells, a similar calcium release complex but with a slightly different activation, anchored in the cardiac dyad, is responsible for cardiac excitation–contraction coupling: membrane depolarization activates the cardiac DHPR, which induces an influx of the external calcium into the cell, and this calcium in turn activates RyR and induces a huge calcium release, a mechanism called ‘calcium-induced calcium release’ (CICR).

This extremely precise organization has to be maintained in the muscle fibre, a cell which undergoes large deformations during each contraction, and triadin could possibly be involved in this function. Triadin was first identified as a 95 kDa protein, specifically localized in the triad of the skeletal muscle (Brandt *et al.* 1990; Kim *et al.* 1990). Since its identification in 1990, the specific function of triadin has been the focus of numerous studies, in cardiac as well as in skeletal muscle, the two muscles in which triadin expression has been demonstrated. Because of its specific triad localization (at the origin of the name of the protein), and its association with RyR in the triads, involvement of triadin in excitation–contraction coupling has been presumed (Caswell *et al.* 1991; Knudson *et al.* 1993). Protein interaction studies have shown that molecular partners of triadin are RyR (Liu *et al.* 1994; Caswell *et al.* 1999; Groh *et al.* 1999), calsequestrin (CSQ), the protein which traps calcium inside the sarcoplasmic reticulum (Guo & Campbell, 1995; Kobayashi *et al.* 2000, Shin *et al.* 2000), and junctin (Zang *et al.* 1997). Functional studies have shown that triadin is able by itself to regulate the activity of the RyR calcium channel *in vitro* (Ohkura *et al.* 1998; Groh *et al.* 1999), or *in vivo* via its interaction with CSQ (Terentyev *et al.* 2007; Kucerova *et al.* 2012). The identification of different triadin isoforms raised the question of their ability to all perform the same functions.

Triadin, a multiprotein family with different localizations and partners

Triadin is in fact a multiple protein family, with different isoforms produced by the alternative splicing of a single *TRDN* gene (Thevenon *et al.* 2003). We have shown that at least three triadin isoforms are expressed in rat skeletal muscle (Marty *et al.* 2000; Vassilopoulos *et al.* 2005). We named these proteins Trisk 95, Trisk 51 and Trisk 32, according to their molecular weight (Fig. 1). They have a common structure, and differ only by the length of their luminal segment and their unique C-terminal end. The smallest triadin, Trisk 32, is a minor skeletal muscle isoform, but it is the main, if not the only, cardiac triadin, also called CT1 (Kobayashi & Jones, 1999). In skeletal muscle it is localized not only in the triad but in the whole sarcoplasmic reticulum, and it is associated both

with RyR1 in the triad and with the inositol trisphosphate (IP₃) receptor in longitudinal SR, which it is able to regulate (Olah *et al.* 2011). In cardiac muscle Trisk 32 is colocalized with and associated to both RyR2 and the IP₃ receptor. The two major skeletal muscle isoforms, Trisk 95 and Trisk 51, are exclusively expressed in skeletal muscle, in approximately equivalent amounts depending on the species (Marty *et al.* 2009). They are only localized in the triads of skeletal muscle, associated to RyR1 and CSQ1, and therefore are integral members of the calcium release complex (Marty *et al.* 2000).

Function of triadin: development of knockout mouse models

In order to identify the function of triadin, two mouse models have been developed: one in the USA by the team of C. F. Perez and P. D. Allen (Shen *et al.* 2007), and

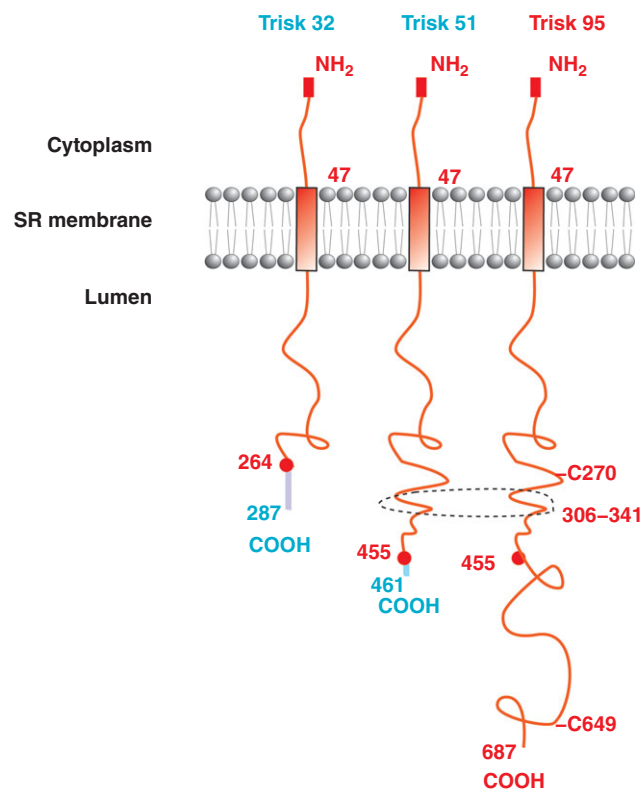


Figure 1. Structure of the main rat triadin isoforms

The structures of the triadin isoforms in the sarcoplasmic reticulum are identical: a common N-terminal domain in the cytoplasm, a transmembrane helix, and a luminal part of variable length with a unique C-terminal end. The common parts are in red and the specific segments, starting at the red circles corresponding to the last common amino acids, are in a different colour. The domains involved in membrane deformation and indirect interaction with the microtubules are shown: the two cysteines C270 and C649, and the coiled-coil segment 306–341. The numbers refer to the rat sequence.

one in France by our team (Oddoux *et al.* 2009). These mice have exactly the same phenotype: they are viable and fertile, and they have cardiac and skeletal muscle defects. From a structural point of view, the muscles of these mice are characterized by the presence of triads in an abnormal orientation, being longitudinal or oblique instead of the normal transversal orientation. Interestingly, in the two mouse models, the amount of triads

in abnormal orientation is identical (30%). A moderate but clear muscle weakness, associated with reduction in the amplitude of calcium releases, was observed in the skeletal muscle of these mice (Shen *et al.* 2007; Oddoux *et al.* 2009). These mice also presented cardiac arrhythmia induced by isoproterenol (isoprenaline) injection, which mimics β -adrenergic stress (Chopra *et al.* 2009).

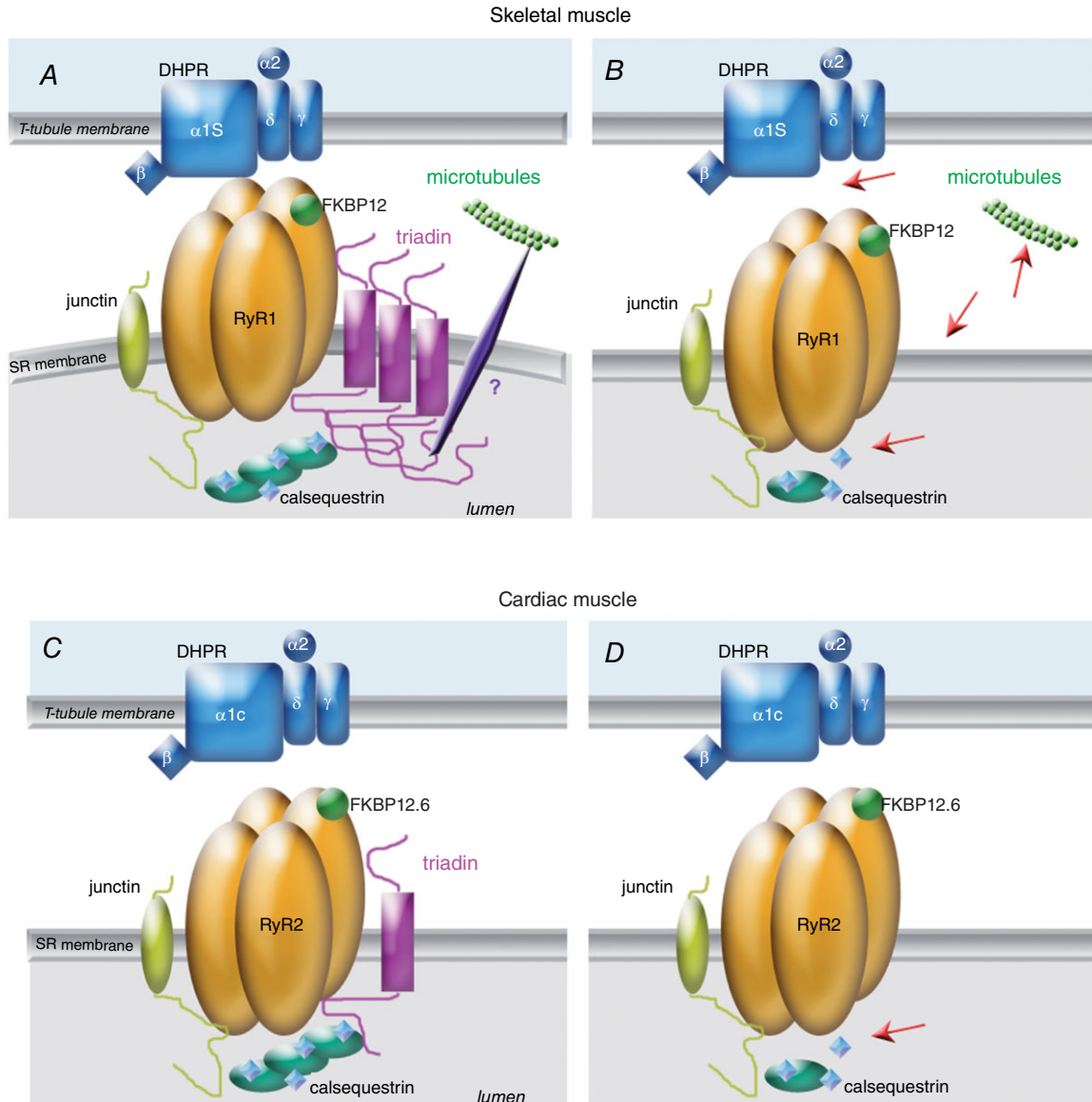


Figure 2. Function of triadin in skeletal and cardiac muscle, and alteration in triadin KO models

A, in skeletal muscle of wild-type animals, the triadin isoform Trisk 95 forms polymers which induce membrane deformation, improving the DHPR–RyR contact and the EC coupling. Triadin anchors CSQ at the triad and via an indirect interaction allows the anchoring of the triad/CRC to the microtubules network. *B*, in the absence of triadin, the red arrows point to the major skeletal muscle defects: in the absence of membrane deformation, the EC coupling is altered, the amount of CSQ at the triad is reduced, the anchoring to the microtubules is altered, and some triads are not maintained in the normal transversal orientation. Association of the shorter skeletal muscle isoform Trisk 51 to Trisk 95 stops the elongation of these large Trisk 95 polymers. *C*, in cardiac muscle, the main function of the cardiac triadin Trisk 32 is the anchoring of CSQ in the vicinity of RyR2. *D*, this function is altered in triadin KO heart (red arrow). FKBP, FK506 binding protein.

Triadin and human diseases

The cardiac phenotype of triadin knockout (KO) mice is quite similar to the phenotype of human patients affected by catecholaminergic polymorphic ventricular tachycardia (CPVT). CPVT is a rare genetic cardiac disease (Liu *et al.* 2008; Cerrone *et al.* 2009) characterized by stress-induced syncope or heart arrest, with an initial onset before the age of 10. Sixty to seventy per cent of affected patients have mutations in the cardiac ryanodine receptor gene *RYR2* or in the cardiac calsequestrin gene *CASQ2*. Therefore CPVT has been proposed as a genetic disease of the cardiac calcium release complex. Based on the phenotype of triadin KO mice, and as triadin constitutes a link between RyR2 and CSQ2, we hypothesized that triadin mutation could be responsible for CPVT in humans. Therefore we searched for mutation in the triadin gene *TRDN* in a large cohort of 97 CPVT patients, in which no mutation had been identified in *RYR2* or in *CASQ2*. We identified three triadin mutations in two families (Roux-Buisson *et al.* 2012). In the first family, the affected child was homozygous for a mutation (D18Afs*13) resulting in a STOP codon before the transmembrane segment. In the second family, the two affected brothers had two mutations, one on each allele: one amino acid substitution in position 59, in the transmembrane domain (T59R), and one STOP codon in position 205 (Q205*). The two premature STOP codons most probably resulted in the absence of protein, and we studied the effect of the third mutation, the T59R, in more detail. We induced the *in vivo* expression of the wild-type cardiac triadin Trisk 32, or the mutant Trisk 32-T59R, in the triadin KO mice, using systemic injection of recombinant adeno-associated virus (Roux-Buisson *et al.* 2012). One month after injection, the cardiomyocytes of these mice were isolated and we studied the expression level of the re-expressed triadin, its localization and its function. Whereas the wild-type Trisk 32 is perfectly re-expressed, colocalized with RyR2 in the cardiac dyad, the mutant Trisk 32-T59R can only be detected at the mRNA level, but not at the protein level. Expressing the wild-type Trisk 32 or the mutant Trisk 32-T59R in the model cell line COS-7 we observed that the mutant protein is degraded by the proteasome. Therefore, the three mutations identified in the patients resulted in the absence of protein, the patients being natural triadin KO, and CPVT in humans can be the result of the absence of triadin.

Triadin expression in non-muscle cells

Since the initial studies on triadin by the groups of K. P. Campbell and L. R. Jones (Guo & Campbell, 1995; Zang *et al.* 1997), triadin has been proposed to serve as an anchor for calsequestrin at the triad/dyad. But this cannot account for all the defects observed in triadin KO models.

To get an insight into the additional functions of triadin, we studied the intrinsic properties of triadin in a system devoid of its usual muscle partners. We expressed triadin in a non-muscle cell line, the COS-7 cells, and analysed both the endoplasmic reticulum (ER) structure and the microtubule network, using specific antibodies (Fourest-Lieuvain *et al.* 2012). We observed that triadin induced huge modifications of the ER structure, forming rope-like ER structures, associated with a massive reorganization of the microtubule network, the microtubules being bundled at the periphery of the cell. Expression of triadin was also associated to constriction of the ER, which forms narrow tubes in the presence of triadin. These different experiments thus point to a new function of triadin. Triadin induces deformation of the reticulum, acts as a protein connecting the reticulum (or the calcium release complex) to the microtubule network, and allows the anchoring of triads to the microtubules network. We dissected the triadin's sequences involved in this function, and we identified two domains: the two cysteines of Trisk 95, in positions 270 and 649, and a coiled-coil domain in position 306–341. These domains are partially present in Trisk 51, which has only one cysteine, and they are absent from Trisk 32 (Fig. 1).

Conclusion: the possible functions of triadin

The studies performed on triadin allow us to propose multiple functions for triadin in skeletal and cardiac muscle. Trisk 95 is able to form large polymers because of its two cysteines (Froemming *et al.* 1999). These polymers induce deformation of reticulum membrane, improving the contact between RyR1 and DHPR and therefore the skeletal muscle excitation–contraction coupling. All the triadin isoforms are able to anchor CSQ, a soluble protein of the reticulum, at the cardiac dyad or skeletal muscle triad. Trisk 95 interacts indirectly with the microtubules via interaction with another protein of the reticulum

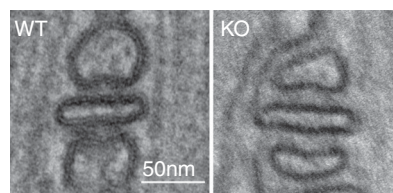


Figure 3. Triad alteration in skeletal muscle of triadin KO mouse

In addition to the abnormal triad orientation observed in 30% of the triads, some alterations in the structure of all the triads can be visualized using electron microscopy on extensor digitorum longus muscle of triadin KO mice (Oddoux *et al.* 2009): a flattening of sarcoplasmic reticulum terminal cisternae resulting from the reduction in the amount of CSQ, and an increase in the distance between T-tubules and terminal cisternae.

membrane. This interaction allows the anchoring of the CRC and of the triad to the microtubules, and is involved in the formation or maintenance of triads during muscle development or contraction. Trisk 51 has only one cysteine, therefore its association with Trisk 95 induces a reduction in the size of triadin polymers, and probably a reduction in the membrane deformation. In the absence of triadin (KO mouse model), these functions are altered, as presented in Fig. 2A and B for skeletal muscle defects, and Fig. 2C and D for cardiac defects. In cardiac muscle, as the only triadin isoform, Trisk 32, is not able to induce membrane deformation, the main result of Trisk 32 deletion is the reduction in amount of calsequestrin in the dyads, which most probably explains the major part of the observed cardiac abnormalities. The interaction of Trisk 32 with microtubules has not been established yet. Some of these hypotheses are still speculative, and additional studies are necessary to confirm them. The schemes of Fig. 2 should therefore be considered as a working hypothesis for the future studies, but some of the above defects can be observed in electron microscopy images of wild-type (WT) or triadin KO triads (Fig. 3): the flattening of terminal cisternae resulting from CSQ reduction, and the increased distance between T-tubule and terminal cisternae.

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Additional information

Competing interests

None declared.

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