Triadin and CLIMP-63 form a link between triads and microtubules in muscle cells

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ABSTRACT

In skeletal muscle, the triad is a structure comprising a transverse (T)-tubule and sarcoplasmic reticulum (SR) cisternae. Triads constitute the basis of excitation–contraction coupling as the cradle of the Ca2+ release complex. We have shown previously that triadin, a member of this complex, has shaping properties on reticulum membrane and is indirectly involved in a link between triads and microtubules. We have identified here that CLIMP-63 (also known as CKAP4), as the partner of triadin, is responsible for this association of triads and microtubules. Triadin and CLIMP-63 interact through their respective luminal domains and the shaping properties of triadin depend on the capacity of CLIMP-63 to bind microtubules with its cytosolic portion. In skeletal muscle, CLIMP-63 is localized in the SR, including triads, and is associated with the Ca2+ release complex through its interaction with triadin. Knockout of triadin in muscles results in the redelocalization of CLIMP-63 from triads, its dissociation from the Ca2+ release complex and a disorganization of the microtubule network. Our results suggest that the association of triadin and CLIMP-63 could be involved in the shaping of SR terminal cisternae and in the guidance of microtubules close to the triads.

KEY WORDS: Triad, Microtubule, Sarcoplasmic reticulum, Triadin, CLIMP-63, Ca2+ release complex

INTRODUCTION

Skeletal muscle contraction is initiated by a burst of Ca2+ out of its major store, the sarcoplasmic reticulum (SR). This rise in cytosolic Ca2+ concentration is triggered by an action potential that propagates along the plasma membrane and reaches invaginations, the transverse tubules (T-tubules). T-tubules make close and periodic contacts with the SR to form peculiar membrane structures called triads (Flucher and Franzini-Armstrong, 1996; Wagenknecht et al., 2002). Triads, defined as two terminal cisternae of SR surrounding a T-tubule, are highly organized membrane structures, precisely distributed in the muscle fiber. Excitation–contraction coupling, that is, the transformation of the action potential into a massive Ca2+ release, is performed at the triads by a macromolecular complex, the Ca2+ release complex (CRC) (Franzini-Armstrong and Jorgensen, 1994). The main players of CRC are the dihydropyridine receptor (DHPR), a L-type Ca2+ channel of the T-tubule membrane, and ryanodine receptor (RyR1), the SR Ca2+ channel. Although these channels are anchored in two different membranes, they are associated (Block et al., 1988; Marty et al., 1994). Thus, the correct function of the CRC relies on the precise alignment of these two membranes, and on the formation and maintenance of triads.

A number of proteins associate with either the DHPR or RyR1 and regulate their activity. Among them is the triadin. Triadin is a multi-protein family, with four isoforms, denoted Trisk 95, Trisk 51, Trisk 49 and Trisk 32, which arise from the alternative splicing of a unique gene, the TRDN gene (Thevenon et al., 2003). Triadins are specifically expressed in striated muscles, Trisk 95 and Trisk 51 being the major skeletal muscle isoforms and Trisk 32 the major cardiac isoform (Marty, 2015). Triadins are type II transmembrane proteins that are completely identical in their cytosolic and transmembrane domains. They diverge because of the length of their luminal domain and the sequence of their C-terminal end. They are localized in the SR membrane, and are associated to RyR1 at the skeletal muscle triad, or to RyR2 at the cardiac dyad (Marty, 2015). Mutations in the TRDN gene have been implicated in severe human cardiac arrhythmia with sudden death (Roux-Buisson et al., 2012).

We have previously shown that expression of triadin Trisk 95 in COS-7 cells induces deformation of the endoplasmic reticulum (ER) and of the microtubule network, creating a link between ER and microtubules (Fourest-Lieuvin et al., 2012). However, this link is indirect, as we observed that Trisk 95 domains implicated in its shaping properties are luminal (Fourest-Lieuvin et al., 2012). Therefore, in ER membrane there must be another transmembrane protein, on the one hand able to bind Trisk 95 luminal domains and, on the other hand, cytoplasmic microtubules. Because of its ability to shape ER and SR membrane (Fourest-Lieuvin et al., 2012), triadin could be involved in the overall structuration of SR cisternae at the triad. In addition, triadin knockout leads to deformations of part of the triads and to a reduction in excitation–contraction coupling and muscle strength (Oddoux et al., 2009; Shen et al., 2007). Therefore, the ER and SR membrane-shaping properties of triadin might favor the RyR1–DHPR coupling.

In the present study, using co-immunoprecipitation coupled to mass-spectrometry-based proteomics, we identified cytoskeleton-linking membrane protein of 63 kDa (CLIMP-63; also known as CKAP4) as a Trisk 95 partner. CLIMP-63 was discovered in the early 1990s (Mundy and Warren, 1992; Schweizer et al., 1993). It is a non-glycosylated type II transmembrane protein expressed in all tissues of higher eukaryotes. It is localized in the ER membrane, and more specifically concentrated in ER sheets where it would have the role of a luminal spacer (Klopfenstein et al., 2001; Schweizer et al., 1995; Shibata et al., 2010). The cytosolic domain of CLIMP-63 binds to microtubules and therefore creates a link between the ER and the microtubule network (Klopfenstein et al., 1998; Vedrenne and Hauri, 2006). After a detailed characterization of the molecular
interaction between Trisk 95 and CLIMP-63, we show that the protein pair Trisk-95–CLIMP-63 could be involved in the guidance of the microtubule network in the vicinity of the triads in muscle cells.

RESULTS
CLIMP-63 is a partner for the triadin isoform Trisk 95
To look for a protein able to mediate the link between Trisk 95 and the microtubules, we analyzed proteins associated with Trisk 95 using co-immunoprecipitation combined with mass-spectrometry-based proteomics. Trisk 95 coupled to GFP, or GFP alone as a control, were expressed in HEK293 cells and immunoprecipitated using anti-GFP antibodies. The semi-quantitative analysis of the proteins co-immunoprecipitated with Trisk-95–GFP and GFP allowed us to identify proteins enriched with Trisk 95 (Table S1). From these potential binding partners, we selected the ER proteins that could bind microtubules and focused our attention on CLIMP-63. CLIMP-63 is an ER membrane protein whose main function is to shape and anchor the ER onto the microtubule network. CLIMP-63 binds microtubules through its N-terminal cytosolic domain and exhibits a long intra-luminal coiled-coil domain (Klopfenstein et al., 2001; Vedrenne and Hauri, 2006). It is a ubiquitous protein expressed in cell lines and muscle cells (Razzaq et al., 2003; Schweizer et al., 1995). Furthermore, CLIMP-63 has been described to induce ER rope-like structures (RLSs) when overexpressed in proliferating cells (Klopfenstein et al., 1998; Vedrenne and Hauri, 2006), which is the same phenotype as that observed upon Trisk 95 overexpression (Fourest-Lieuvin et al., 2012). Therefore, we raised the hypothesis that CLIMP-63 could be the missing link between triadin and the microtubule network.

To validate the association between CLIMP-63 and Trisk 95, Trisk 95 without the GFP moiety was expressed in HEK293 cells, and an immunoprecipitation using an anti-CLIMP-63 antibody was performed, which demonstrated the specific co-immunoprecipitation of Trisk 95 with CLIMP-63 (Fig. 1A).

We have shown previously that Trisk 95, when expressed in COS-7 cells, is located in the ER and can induce ER membrane deformation and microtubule network disorganization in 16% of transfected cells (Fourest-Lieuvin et al., 2012). We wondered whether CLIMP-63 could colocalize in the ER with Trisk 95 and be associated with the Trisk 95-induced phenotypes. To assess this possibility, COS-7 cells were transfected with Trisk 95 and immunolabeled for triadin, CLIMP-63 and tubulin. In control non-transfected cells, endogenous CLIMP-63 was mainly localized in ER sheets, and microtubules exhibited a normal radial pattern (Fig. 1B, cell 1). In every cell transfected with Trisk 95, CLIMP-63 colocalized with Trisk 95 in the ER (Fig. S1A). In the Trisk 95-transfected cell shown in Fig. 1B (cell 2), the ER shape was altered, with the formation of RLSs, and CLIMP-63 colocalized with Trisk 95 in these RLSs. In addition, microtubule arrays were disorganized and co-aligned with the ER RLSs (Fig. 1B, cell 2). Hence, Trisk 95 expression could induce CLIMP-63 and microtubule redistribution, suggesting a connection between the three.

Thus, co-immunoprecipitation and colocalization experiments show that CLIMP-63 and Trisk 95 are associated in cell lines. We next wondered which regions of both proteins were responsible for this interaction.

The luminal domains of Trisk 95 and CLIMP-63 are involved in their association
We have shown previously that it is the luminal domain of Trisk 95, and more specifically the so-called RLS domain comprising the coiled-coil region 306–341 with the two cysteine residues C270 and C649, which is involved in the formation of RLSs (Fourest-Lieuvin et al., 2012). We therefore hypothesized that the luminal domains of Trisk 95 and CLIMP-63 would be involved in the interaction. We investigated this by using different triadin mutants (Fig. 2C), either as previously described (Fourest-Lieuvin et al., 2012) or generated for this study: (1) a mutant without the cytosolic N-terminal domain (Acetyo-T95), (2) a mutant without the cytosolic and transmembrane domains but with ER targeting and retention sequences (T95-lumen), (3) a mutant deleted of most the luminal domain (T95-mini), and finally (4) a mutant in which the specific sequences responsible for the RLS phenotype are removed (T95-ΔRLS). Wild-type CLIMP-63 (CLIMP, Fig. 2C) was co-transfected with each of the above deletion mutants in COS-7 cells. As previously described (Klopfenstein et al., 1998), CLIMP-63 overexpression by itself induced ER deformation in 78% of the transfected cells (Figs S1B and S2). When CLIMP-63 was co-transfected with Trisk 95 (T95),

Fig. 1. Endogenous CLIMP-63 associates with transfected Trisk 95 in COS-7 and HEK293 cells. (A) Immunoprecipitation was performed with an antibody against CLIMP-63 on HEK293 cells that were either not transfected (T95 –) or transfected with the construct pcDNA3.1-Trisk 95 (T95 +). Western blot analysis demonstrated the presence of Trisk 95 (T95) among the immunoprecipitated proteins. Molecular masses in kDa are indicated on the left. (B) Trisk 95 was expressed in COS-7 cells, and the cells were stained with antibodies against CLIMP-63 (CLIMP, green), Trisk 95 (T95, red) and β-tubulin to label microtubules (tubulin, gray). The CLIMP-63 and T95 stainings are merged in the right panel. The area of boxes denoted 1 and 2 are magnified in lower panels for each image. The cells expressing Trisk 95 shown here exhibit an ER deformation called rope-like structures (RLSs) and a disorganization of microtubules (box 2), which is not the case in cells that do not express T95 (box 1). Endogenous CLIMP-63 always colocalizes with Trisk 95 in Trisk-95-expressing cells (merged panel and box 2). Scale bar: 20 μm.
Δcyto-T95 or T95-lumen, the cells exhibiting RLSs showed a perfect colocalization between CLIMP-63 and Trisk 95 proteins in these ER structures (Fig. 2Aa–Ac,B). In contrast, cells co-transfected with CLIMP-63 and T95 or Flag, T95, Δcyto-T95 and T95-lumen colocalize with CLIMP-63 in RLSs, whereas T95-mini and T95-ΔRLS do not. Scale bar: 10 µm. (B) Percentage of transfected cells with CLIMP-63 and triadin colocalized in the RLSs (for each condition, 500 transfected cells from three different experiments were examined). (C) Schematic representation of T95 (T95, in red), T95 fused to GFP (T95–GFP), and T95 deleted for the cytosolic domain (Δcyto-T95), for the cytosolic and transmembrane domains (T95-lumen), for the luminal domain (T95-mini and T95-mini-GFP) or for the RLS-specific sequences (T95-ΔRLS, coiled-coil domain removed and cysteines mutated). CLIMP-63 (CLIMP, in green) is also represented. The T95-lumen construct is fused to Flag and ER retention (KDEL) sequences. TM, transmembrane domain; CC, coiled-coil domain. Numbers indicate amino acids. (D) T95, Δcyto-T95 or T95-ΔRLS transfected in HEK293 cells were immunoprecipitated with an antibody to T95. Immunoblot (IB) analysis of co-immunoprecipitated (IP) proteins was performed with anti-T95 (upper) and anti-CLIMP-63 (lower panel) antibodies. (E) T95–GFP or T95-mini-GFP transfected in HEK293 cells were immuno-precipitated with the kit GFP trap (IP GFP). Immunoblot analysis of co-immunoprecipitated proteins was performed with anti-triadin (upper and middle) and anti-CLIMP-63 (lower panel) antibodies. In D and E, molecular masses in kDa are indicated on the left.

Triadin is a family with different members, differing only in their luminal domains (Fig. S3A). To see whether other triadin isoforms associate with CLIMP-63, HEK293 cells were transfected with different adenoviral vectors encoding the different isoforms and immunoprecipitations were performed with an antibody against the common N-terminal end of triadin. In each case, CLIMP-63 was co-immunoprecipitated, indicating that all the triadin isoforms are able to interact with CLIMP-63 (Fig. S3B).

To study domains of CLIMP-63 involved in the interaction, two mutant forms of CLIMP-63 were used (Fig. 3A): one with the luminal part replaced by GFP (CLIMP-Δlumen) and one with the cytoplasmic
The RLS phenotype induced by Trisk 95 depends on the capacity of CLIMP-63 to bind microtubules

When expressed in cell lines, both CLIMP-63 and Trisk 95 induce ER deformations, the RLSs, which are concomitant with microtubule array disorganization (see Fig. 1 and Fig. S1). Because it seems from the above results that Trisk 95 binds to microtubules through CLIMP-63, we hypothesized that the capacity of Trisk 95 to form RLSs was due to its ability to bind and recruit CLIMP-63. To test this, endogenous CLIMP-63 was knocked down by RNA interference (RNAi) in COS-7 cells before transfection of the cells with Trisk 95, and the extent of Trisk 95-induced RLSs was evaluated. Unfortunately, the knockdown of CLIMP-63 was not complete (Fig. S4A–C) and Trisk 95 was still able to induce RLSs in cells (Fig. S4D,E). A similar situation has already been described in neurons where the knockdown of CLIMP-63 is incomplete (Cui-Wang et al., 2012).

We then used a mutant form of CLIMP-63, referred to as CLIMP-MT(−) because of its inability to bind to microtubules (Vedrenne et al., 2005). This mutant is a phospho-mimetic form of CLIMP-63, with four serine residues of the cytosolic domain mutated to glutamic acid residues (Fig. 4A). As a control, a phospho-deficient mutant of CLIMP-63 was used. This mutant, referred to as CLIMP-MT(+), is always bound to microtubules and has three phospho-sites mutated to alanine residues (Fig. 4A) (Vedrenne et al., 2005). Trisk 95 was co-expressed in COS-7 cells with either CLIMP-MT(−) or CLIMP-MT(+) and the formation of RLSs was quantified. All of the cells co-transfected with CLIMP-MT(−) and Trisk 95 exhibited RLSs (Fig. 4B, upper panel, 4C). By contrast, cells co-expressing CLIMP-MT(−) and Trisk 95 never exhibited RLSs (Fig. 4B, lower panel, 4C). Rather, these cells presented a fenestrated ER that did not resemble RLSs, and microtubule arrays in these cells appeared normal. This absence of RLSs was not due to the incapacity of Trisk 95 to bind to the CLIMP-MT(−) mutant: indeed, CLIMP-MT(−) was still able to co-immunoprecipitate Trisk 95 (Fig. 4D, lane 5). Hence, CLIMP-MT(−), being unable to bind microtubules, acted as a dominant-negative mutant and abolished the capacity of Trisk 95 to induce RLSs. These experiments show that the Trisk 95–CLIMP-63 association is not dependent on the CLIMP-63–microtubule interaction but that the ER-remodeling induced by Trisk 95 is dependent on the ability of CLIMP-63 to bind microtubules.

In muscle fibers, CLIMP-63 is a partner for Trisk 95 at triads

After the detailed characterization of the interaction between Trisk 95 and CLIMP-63 in cell lines, we examined the situation in skeletal muscle. Immunoprecipitation was performed on mouse muscle homogenates (SK, Fig. 5A, lane 1) with an antibody against Trisk 95. As shown in Fig. 5A, lane 2, CLIMP-63 was co-immunoprecipitated by Trisk 95 in these muscle extracts. We further studied the localization of CLIMP-63 using immunofluorescence labeling of CLIMP-63 and Trisk 95 on isolated mouse extensor digitorum longus (EDL) muscle fibers (Fig. 5B). Triads labeled by the anti-Trisk 95 antibody showed characteristic double rows of dots (Fig. 5B, red). CLIMP-63 also appeared as dots with a striated pattern (Fig. 5B, green). A partial overlap between the stainings of Trisk 95 and CLIMP-63 could be detected on the merged image as yellow dots (Fig. 5B). A colocalization analysis was performed with ImageJ software: the binary mask images represent the colocalized points between the green and the red stainings (Fig. 5B, right panel) and quantification from these binary images showed that 35.7±1.2% (mean±s.e.m.) of CLIMP-63 colocalized with Trisk 95 in WT muscle fibers.
muscles. Thus CLIMP-63, as a reticulum protein present in the ER and SR, partially colocalizes with the triad-specific protein Trisk 95. In this SR subdomain, the triad, these proteins might interact as shown by co-immunoprecipitation experiments.

**CLIMP-63 localization at triads is altered in triadin KO muscles**

CLIMP-63 might be specifically localized at the triad through its interaction with Trisk 95. Therefore, the localization of CLIMP-63 was studied in EDL fibers of triadin-knockout (KO) mice, devoid of all isoforms of triadin. Triads were stained using an anti-RyR1 antibody. In triadin KO EDLs, RyR1 staining (Fig. 6A, red) revealed double rows of dots and was unmodified compared to wild type (WT) EDLs, as observed previously (Oddoux et al., 2009). In contrast, CLIMP-63 staining (Fig. 6A, green) was less striated in KO fibers (Fig. 6A, lower panel), less concentrated at the I-band and less well colocalized with triads (see line scans in Fig. 6B). Quantification of the relative amount of CLIMP-63 colocalized with RyR1 in WT and triadin KO muscles showed a significant reduction from 37.5±1.3% in WT to 13.8±0.8% (mean±s.e.m.) in KO muscles (Fig. 6C, quantified from binary mask images as shown in Fig. 6A). Quantitative western blots performed on muscle homogenates from WT and triadin KO mice demonstrated that the total amount of CLIMP-63 was not altered by triadin depletion (Fig. 6D). Rather, in triadin KO muscles, CLIMP-63 seems to redistribute in the whole ER–SR.

![Fig. 4. Trisk 95 phenotype is abolished by a mutant of CLIMP-63 that does not bind to microtubules.](image)

(A) Schematic representation of CLIMP-63 in which phospho-regulated serine residues in the cytosolic portion were mutated either to alanine residues [mutant CLIMP-MT(+)]) or to glutamic acid residues [mutant CLIMP-MT(−)]. CLIMP-MT(+) can bind microtubules while CLIMP-MT(−) cannot. (B) COS-7 cells were co-transfected with Trisk 95 (T95) and either CLIMP-MT(+) or CLIMP-MT(−) and labeled with antibodies against CLIMP-63 (CLIMP, green), T95 (red) and β-tubulin (gray). All cells co-expressing T95 and CLIMP-MT(+) present RLSs in which both proteins are colocalized (merged panel and zooms), and their microtubules are disorganized. In cells co-expressing T95 and CLIMP-MT(−), both proteins are colocalized in the ER (merged panel and zooms) but the ER never exhibit RLSs and the microtubule network appears normal. Scale bar: 20 µm. (C) Quantification of transfected cells exhibiting RLSs. Cells were either transfected with T95, CLIMP-MT(+) or CLIMP-MT(−) separately, or co-transfected as indicated. Quantifications were also performed on non-transfected cells as a control (first column). Values are percentages of cells exhibiting RLSs among transfected cells (for each condition, 500 transfected cells from three different experiments were examined). (D) Co-immunoprecipitation experiments performed on extracts of HEK293 cells expressing the indicated proteins. CLIMP-MT(+) or CLIMP-MT(−) were fused to EGFP in this experiment and were immunoprecipitated with the kit anti-GFP trap (IP GFP). Immunoblot (IB) analysis of co-immunoprecipitated proteins was performed with anti-Trisk 95 (upper) and anti-GFP (lower panel) antibodies. Molecular masses in kDa are indicated on the left.
Immunoprecipitations were performed using anti-RyR1 antibody on WT and triadin KO muscle homogenates. Whereas in WT muscles, immunoprecipitation of RyR1 resulted in the co-immunoprecipitation of both Trisk 95 and CLIMP-63, both proteins were absent in the immunoprecipitation made from triadin KO muscles (Fig. 6E). Therefore, in the absence of triadin, CLIMP-63 localization at triads is reduced and CLIMP-63 is not associated with the Ca\(^{2+}\) release complex, highlighting the triadin–CLIMP-63 link in muscles.

The organization of transversal microtubules is disrupted both in triadin KO muscles and in muscles overexpressing CLIMP-MT(−)

We have previously shown that in skeletal muscle some microtubule bundles come into close proximity to triads (Fourest-Lieuvin et al., 2012). Because the protein pair triadin–CLIMP-63 is most probably involved in the link between triads and microtubules, we compared the organization of microtubules in isolated EDL muscle fibers from WT and triadin KO mice. In WT EDLs, the microtubule network appears as an orthogonal grid with longitudinal and transversal microtubule bundles, as previously described (Oddoux et al., 2013; Ralston et al., 1999, 2001). Although the organization of transversal microtubules is not as precise as that of the triads, these microtubules presented a striated pattern with the same 2-µm step as triads and they were usually localized within the double rows of RyR dots (Fig. 7A, WT panel). These transversal microtubules apparently make periodic contacts with triads, as suggested by a 34.7±1.4% (mean±s.e.m.) colocalization between tubulin and RyR1 stainings (quantified from binary mask images in Fig. 7A, WT panel). In triadin KO EDLs, transversal microtubules appeared less organized and less bundled, and often single oblique microtubules escaped from the double row of triads (Fig. 7A, KO panel). The percentage of tubulin staining colocalized with RyR1 was significantly reduced from 34.7±1.4% in WT animals to 26.4±1.4% in triadin KO mice (quantified from binary mask images in Fig. 7A). To assess the overall microtubule disorganization in triadin KO EDLs, a blind test (see Materials and Methods) was performed by two different persons to sort randomized images from WT and KO fibers and classify them in three categories: organized (MT org +), less organized (MT org +/-) and disorganized microtubules (MT org –), representative images in Fig. 7B). Results showed that WT fibers were mostly sorted out as ‘organized’ (65%) while KO fibers were evenly distributed in the three categories, the main pattern (38%) being ‘MT org +/-’ (Fig. 7B). Hence, transversal microtubules were less organized and less bundled between rows of triads in triadin KO muscles. The absence of the triadin–CLIMP-63 pair in triadin KO muscles seems to induce a reduction in the co-alignment between triads and microtubules.

In order to examine further the influence of CLIMP-63 on microtubule organization in muscle cells, the dominant negative CLIMP-MT(−) mutant was expressed in flexor digitorum brevis (FDB) muscles. GFP alone (as a control) or GFP–CLIMP-MT(−) was electroporated in FDBs of WT mice, and microtubules visualized by immunofluorescent staining on isolated fibers. The microtubule network in FDBs is slightly different from that in EDLs, being less bundled and less regularly organized in orthogonal grid. Nevertheless, FDB transversal microtubules are usually localized within the double rows of RyR dots as in EDL fibers (Fig. 8A, control panel). In FDBs expressing CLIMP-MT(−), a disorganization of the microtubule network could be observed, with a reduced localization of transverse microtubules between RyR dots and with many single microtubules running obliquely or longitudinally [Fig. 8A, CLIMP-MT(−) panel]. The overall microtubule organization was tested as in experiments on EDLs, using a blind test. We found that control FDB fibers expressing GFP were mostly sorted out as ‘organized’ (MT org +, 47%) whereas fibers expressing GFP–CLIMP-MT(−) were mainly classified as ‘disorganized’ (MT org –, 45%, Fig. 8B). Therefore, as observed for triadin KO muscles, expression of the microtubule-binding deficient mutant CLIMP-MT(−) alters muscle microtubule arrays.

**DISCUSSION**

In this study, we identify CLIMP-63 as a partner of triadin in muscle cells, and show that it is involved in the link between triads and the microtubule network. This protein pair, triadin and CLIMP-
63, might play an important role in the shaping of triads. The ER–SR in muscle cells forms a highly structured 3D network surrounding the myofibrils and is organized in subdomains, the longitudinal ER–SR and the junctional SR (the terminal cisternae) (Franzini-Armstrong, 1980; Ogata and Yamasaki, 1997). The terminal cisternae in association with the T-tubule form the triads, which are the sites of Ca²⁺ release and play a major role in excitation-contraction coupling. The triads are exclusively localized at the interface of A and I bands in mammalian skeletal muscles (Ogata and Yamasaki, 1997). The mechanisms leading to the formation and maintenance of these subdomains are unknown, as well as the mechanisms leading to the exclusive localization of proteins of the CRC at the triads.

CLIMP-63 is an ER transmembrane protein that has been mainly studied in proliferating mono-nucleated cells (Vedrenne and Hauri, 2006). CLIMP-63 is concentrated in ER sheets where it appears to have a role in the shaping of these ER subdomains (Klopfenstein et al., 2001; Shibata et al., 2010). Our study is the first description of CLIMP-63 in mature muscle fibers, where CLIMP-63 staining appears as bright dots localized in the I-bands, resembling the

Fig. 6. Absence of triadin results in delocalization and dissociation of CLIMP-63 from the triads. (A) Isolated EDL muscle fibers from WT or triadin KO mice were double-stained with antibodies against CLIMP-63 (CLIMP, green) and RyR1 to label triads (RyR, red). Each image represents one single confocal plane and a merged image is shown. Scale bar: 4 µm. The binary image (mask, right panel) represents in black the CLIMP-63 staining area that colocalizes with the RyR1 staining. Such binary images were used for quantification of colocalization shown in C. (B) The fluorescence intensity of each staining was plotted as a function of distance for four successive triads in fibers of WT or triadin KO mice. The green curve corresponds to CLIMP-63 staining and the red curve to RyR1 staining. (C) Percentage of CLIMP-63 staining area colocalizing with RyR labeling, calculated with image binarization as in A. Values are mean±s.e.m., n=27 (WT) and 26 (KO) quantified fibers from 2 different dissections. ****P<0.0001, Mann–Whitney two-tailed test. (D) Representative western blot for an analysis of the amount of CLIMP-63 in WT and triadin KO muscle homogenates. A CLIMP-63:actin ratio of 101±5% was measured in KO muscles compared to WT muscles set at 100% (mean±s.e.m., n=6, not significant by Wilcoxon test comparison). Antibodies used are indicated on the right. (E) Co-immunoprecipitation (IP) experiments performed on WT or triadin KO mouse muscle homogenates with an antibody to RyR1 (IP RyR). Immunoblot (IB) analysis of co-immunoprecipitated proteins was performed with anti-RyR (upper), anti-Trisk 95 (middle) and anti-CLIMP-63 (lower panel) antibodies. (D, E) Molecular masses in kDa are indicated on the left.
staining described in muscles for rough ER markers like BiP or PDI (Kaisto and Metsikko, 2003). However, in addition, a portion of the dots of CLIMP-63 (~40%) colocalize with Trisk 95 at the triads where the ryanodine receptor RyR1 is also present. This partial colocalization of CLIMP-63 and triadin reflects the fact that CLIMP-63 is a protein present in the whole ER–SR of muscle cells whereas Trisk 95 is specific to the junctional SR at the triads. CLIMP-63 is probably able to diffuse more freely than Trisk 95 in the different ER–SR subdomains, and it might be maintained in the junctional SR by Trisk 95. Therefore, in the absence of Trisk 95, CLIMP-63 is partly delocalized (Fig. 6). Based on the observations in COS-7 cells, we can speculate on the functional impact of the triadin–CLIMP-63 interaction at the triad. We show in COS-7 cells that triadin and CLIMP-63 interact through their luminal domain, most probably through their respective luminal coiled-coil domains. Besides, both proteins are known to multimerize in the ER and SR membrane to form oligomers (Fourest-Lieuvin et al., 2012; Schweizer et al., 1994). Thus, CLIMP-63 and Trisk 95 could form hetero-oligomers in the SR cisternae membrane and these hetero-oligomers could shape the SR membrane. Indeed, in WT muscles, the SR terminal cisternae in triads appear as small rounded bags in electron microscopy images. In triadin KO mouse muscles, the SR terminal cisternae are flattened, an observation which has been attributed to calsequestrin depletion, but could also reflect the depletion of both triadin and CLIMP-63 (Oddoux et al., 2009). Along this line, Rapoport and co-workers describe CLIMP-63 as an ER luminal spacer and have found that CLIMP-63 depletion by RNAi in COS-7 cells results in a collapse of the ER sheet luminal width from 55±9 nm to 28±4 nm (mean±s.e.m.) (Shibata et al., 2010). Consistent with this, in triadin KO muscle, the luminal size
of the SR terminal cisternae is reduced from 68±3 nm to 26±1 nm (mean±s.e.m.) (Oddoux et al., 2009). Thus, it is likely that the formation of triadin–CLIMP-63 hetero-oligomers does not compete with the CLIMP-63 properties as a luminal spacer, but rather maintains the SR cisternae structure.

CLIMP-63 is also a microtubule-binding protein. Its microtubule-binding domain has been mapped to amino acids 62–101 in the cytosolic N-terminal part of the protein (Klopfenstein et al., 1998; Vedrenne et al., 2005). When overexpressed, CLIMP-63 has a strong effect on the microtubule network, inducing bundles of stabilized microtubules (Klopfenstein et al., 1998; Vedrenne and Hauri, 2006; Vedrenne et al., 2005). In our work, we observed that the ability of Trisk 95 to cause a reorganization of the microtubule network in COS-7 cells is mediated by CLIMP-63. Indeed, the pseudo-phosphorylated CLIMP-MT(−) mutant, which cannot bind microtubules (Vedrenne et al., 2005), is still able to bind Trisk 95 but abolishes the effects of Trisk 95 on the microtubule network. It is likely that Trisk 95 expressed in COS-7 cells oligomerizes with endogenous CLIMP-63 and induces its aggregation or re-localization. These Trisk 95–CLIMP-63 aggregates would induce the formation of ER RLSs and bundled arrays of microtubules, a mechanism already suggested by the group of Hauri (Vedrenne and Hauri, 2006).

The roles of microtubules in skeletal muscle have not been extensively studied. In muscle cells, microtubules are organized in bundles exhibiting a transversal and longitudinal orientation and forming a grid-like network (Oddoux et al., 2013; Ralston et al., 1999, 2001). This network is dynamic, with microtubules polymerizing and depolymerizing continuously along a bundled framework (Oddoux et al., 2013). Microtubule bundles in muscles are involved in the stretch-activated production of reactive oxygen species (ROS), and alteration in this microtubule-dependent ROS production is a major defect in Duchenne muscular dystrophy (Kerr et al., 2015; Khairallah et al., 2012). Dynamic microtubule bundles might also be necessary for the morphogenesis of organelle subdomains such as the ER or SR subdomains (Cui-Wang et al., 2012; Vedrenne and Hauri, 2006). In neurons, local zones of increased ER complexity are observed underneath dendritic spines, which could favor the growth of spines as well as the local synthesis and export of synaptic-specific proteins. The shaping of these complex ER zones appears to rely on CLIMP-63 and its interconnection with dendritic microtubule bundles (Cui-Wang et al., 2012). In muscle cells, our work shows that microtubule arrays are partly disorganized in the absence of triadin or after overexpression of the microtubule-binding-deficient CLIMP-MT(−) mutant. Hence, triadin–CLIMP-63 hetero-oligomers at triads could favor the organization of at least some of the transversal microtubules in muscle cells, and could guide these microtubules close to the terminal SR cisternae. This precise microtubule
localization could be of major importance for the targeting of CRC proteins to the triads as has been shown for the targeting of the DHPR to cardiac T-tubules (Hong et al., 2010). The triadin–CLIMP-63 protein pair is most probably not the only microtubule organizer in muscle fibers, and the creation of orthogonal grid of microtubules would be expected to require numerous other proteins. For example, dystrophin as well as the complex ankyrin-B–dynactin-4 have been proposed as being responsible for microtubule anchoring at costameres (Ayalon et al., 2008; Belanto et al., 2014; Prins et al., 2009).

In conclusion, absence of triadin observed in human patients (Roux-Buisine et al., 2012) and triadin KO mice (Ouddx et al., 2009) could have wider consequences than the dysregulation of RyR1 and alteration of Ca2+ release: it could also affect the overall microtubule organization in muscle fibers.

MATERIALS AND METHODS

Ethics statement

All procedures using animals were approved by the Institutional ethics committee and followed the guidelines of the National Research Council Guide for the care and use of laboratory animals.

DNA constructs and siRNAs

Full-length rat triadin Trisk 95 (accession number #AJ243304), Trisk 51 (#AJ243303), Trisk 49 (#AJ812275), Trisk 32 (#AJ812276) and several Trisk 95 mutants inserted into the expression vectors pcDNA3.1 (Life Technologies) or pEGFP-N1 (Clontech) are as previously described (Fourest-Lieuvin et al., 2012; Marty et al., 2000; Vassilopoulos et al., 2005). To construct T95-mini–GFP, the stop codon of T95-mini in pcDNA3.1 was removed by a PCR-based method, and the amplified sequence was inserted into pcEGFP-N1. For the construction of T95-lumen, the plasmid pDsRed2-ER (Clontech) was digested by AgeI and BglII to remove DsRed2. The luminal domain of Trisk 95 (amino acids 113–687) was amplified by PCR with Flag and KDEL sequences inserted at the end of the PCR fragment, which was then inserted into an AgeI- and BglIII-digested plasmid. T95-lumen encoded by this construct is thus composed of the calreticulin ER-targeting peptide, the luminal domain of Trisk 95, and Flag and KDEL peptides, and is targeted into the lumen of the ER. Plasmids coding for full-length human CLIMP-63, GFP-fusion constructs of CLIMP-63 [CLIMP-Δlumen and Δcyto–CLIMP; respectively named Δlumen-GFP and GFP-Δcytoplasmic in Klopfenstein et al. (2001)], and CLIMP-MT(−) or CLIMP-MT(+ ) mutants of CLIMP-63 were kindly provided by Hessel Farhan and Hans-Peter Hauri (Klopfenstein et al., 2001; Vedrenne et al., 2005). The CLIMP-MT(−) mutant is phospho-mimetic in the cytosolic domain of the protein, with serine residues at positions 3, 17, 19 and 101 mutated into glutamic acid residues. The CLIMP-MT(+) mutant is phospho-deficient, with serine residues at positions 3, 17 and 19 mutated into alanine residues (Vedrenne et al., 2005).

For knockdown of CLIMP-63 in COS-7 cells, a siRNA(KD) was designed according to Nikovov et al. (2007) based on human sequence of CLIMP-63: 5′-CCAAUCCAUCAACGACAAAT-3′ corresponding to nucleotide position 734–752 in CLIMP-63 sequence. Scramble RNA (Sc) was obtained from scrambled sequence of siRNA(KD): 5′-CUCC-AAAACUGACGAAAT-3′. Both RNAs were synthesized by Invitrogen.

Viruses and transduction

In this study, we used adenoviral-mediated gene transfer to express Trisk 95, Trisk 51, Trisk 49 or Trisk 32 in HEK293 cells. These viruses were engineered and produced either by the Gene Vector Production Unit at Genethon III (Evry, France) or by the Atlantic Gene Therapies (Nantes, France). Lentiviral vectors carrying either the rat Trisk 95 cDNA, the human CLIMP-63 cDNA or the cDNAs of the mutants CLIMP-MT(+) or CLIMP-MT(−) were produced in the laboratory by a triple transfection of pWXPXld, pSXPX and pCMV-VSV-G (Addgene, Cambridge, MA) into HEK293T cells, as described previously (Bovia et al., 2003; Zufferey et al., 1997). Titers of viruses in cell culture supernatants were determined as previously described (Nguyen et al., 2005). For all transduction experiments, cells were transduced by overnight incubation with the chosen virus at a multiplicity of infection of 40 for adenoviruses and 5 for lentiviruses. Control cells were infected with the control viruses (adenovirus ΔDsRed or lentivirus-GFP).

Antibodies

The primary antibodies used in this study are presented in Table S2. Secondary antibodies used for immunofluorescence studies were coupled to Alexa Fluor 488 or Alexa Fluor 546 (Molecular Probes); or to Cy3 or Cy5 (Jackson ImmunoResearch Laboratories). Secondary antibodies used for western blotting were horseradish peroxidase (HRP)-coupled anti-rabbit-IgG polyclonal antibodies (Jackson ImmunoResearch Laboratories). For western blotting after immunoprecipitation, TrueBlot® HRP-coupled anti-rabbit-IgG polyclonal antibodies were used (Rockland).

Proteomic analyses

Proteomic experiments were performed as previously described (Milbradt et al., 2014). Briefly, proteins were loaded on a 4–12% NuPAGE gel (Invitrogen) before R-250 Coomassie Blue (BioRad) staining and in-gel digestion using modified trypsin (Promega, sequencing grade). Resulting peptides were analyzed by online nanoliquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS with Ultimate 3000 and LTQ-Orbitrap Velos pro, Thermo Scientific) using a 120-min gradient. Peptides and proteins were identified through concomitant searches against the Uniprot databank (September 2014 version, Homo sapiens taxonomy, 138,560 sequences) and a classical contaminants database (in-house, 260 sequences) and the corresponding reversed databases using Mascot (version 2.5). The IRMA software, version 1.31.1 (Dupierris et al., 2009), was used to filter the results: conservation of rank one peptides, peptide identification with a false discovery rate (FDR) <1% (as calculated on peptide scores by employing the reverse database strategy), and minimum of one specific peptide per identified protein group. Filtered results were uploaded into a relational mass spectrometry identification database before performing a compilation, grouping and comparison of the protein groups from the different samples using a in-house tool (hEIDI). Differential analysis of GFP and Trisk 95-GFP co-immunoprecipitation eluates was performed using extracted spectral counts and specific spectral counts (SSC). A protein was considered as a potential binding partner if it was identified with a minimum of 10 SSC and enriched at least fourfold in the Trisk 95–GFP sample compared to GFP one.

Cell culture and transfection

COS-7 and HEK293 cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies). Transient transfections were carried out using Exgen 500 reagent (Euromedex), according to the manufacturer’s protocol. After 28 h of transfection, cells were treated for either immunoprecipitation or immunofluorescence. For immunoprecipitation, cells grown in 60-mm diameter plates were collected by trypsinization, washed with PBS and stored at −20°C until used. For immunofluorescence, cells grown on coverslips were fixed for 6 min in methanol at −20°C.

Electroporation and preparation of muscle fibers

EDL muscle fibers were manually dissociated as described in Prins et al. (2009). Electroporations of FDB muscles were performed according to DiFranco et al. (2009). 10 µl of a 2 mg/ml hyaluronidase (Sigma) solution was injected subcutaneously in each footpad of the anesthetized mice, followed by a 20 µl injection of the endotoxin-free pEGFP-N1 or pEGFP-CLIMP-MT(−) (3–5 mg/ml) plasmids. Electroporation pulses (20×20 ms at 100 V) were delivered with acupuncture needles and a BTX ECM830 electroporator. Five days after electroporation, FDB muscles were dissected, dissociated enzymatically for 45 min at 37°C in 1.5 mg/ml collagenase (Sigma) and subjected to mechanical dissociation.
Single EDL and FDB fibers were fixed for 45–60 min at room temperature in PBS containing 4% paraformaldehyde and permeabilized for 90 min in PBS with 1% Triton X-100 at 30°C before being assessed for immunofluorescence as described below.

**Immunofluorescence microscopy**

Cells and muscle fibers were saturated in PBS supplemented with 0.1% Triton X-100, 0.5% bovine serum albumin and 2% goat serum for 30 min. They were incubated with primary antibodies in PBS with 0.1% Tween 20 either at room temperature for 1 h (COS-7 cells) or at 4°C overnight (muscle fibers). After incubation for 1–3 h at room temperature with fluorescent secondary antibodies, coverslips were mounted on microscope slides with FluorSave™ reagent (Calbiochem). Images were captured on a Zeiss LSM710 confocal microscope or Leica SPE confocal microscope.

**Preparation of muscle homogenates**

Quadriceps muscles were collected from adult mouse hindlimbs. Crude homogenates were prepared by a 2–20 s homogenization of 50–100 mg of muscle in 0.5 ml of buffer composed of 200 mM sucrose, 20 mM HEPES pH 7.4, 0.4 mM CaCl₂, 100 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM diisopropyl fluorophosphate (DFP) using a Minilys homogenizer (Bertin Technologies).

**Immunoprecipitation, SDS-PAGE and western blotting**

Mouse skeletal muscle homogenates or homogenized transfected HEK293 cells were solubilized for 30 min at 4°C in a buffer composed of 0.9 M NaCl, 1.6% CHAPS, 0.1% phospholipids (Avanti Polar Lipids), 20 mM Pipes (pH 7.1), 200 µM PMSF and 1 mM DFP. Immunoprecipitations were then performed as described previously (Marty et al., 1994) with the chosen antibody and Dynabeads® Protein G (Life Technologies). All Triadin or CLIMP-63 constructs fused with GFP were immunoprecipitated from transfected HEK293 cell extracts using GFP-Trap® (Chromotek), according to the manufacturer’s protocol. The immunoprecipitated proteins were then analyzed by SDS-PAGE (5–15% gradient gels, Bio-Rad Laboratories) and western blotting performed with antibodies described above and with ECL substrate reagent (Thermo Scientific). Acquisitions were done using a ChemiDoc XRS apparatus (Bio-Rad) and quantification was performed using Quantity One software.

**Colocalization analyses and quantification of microtubule disorganization on immun-fluorescence images of EDL muscle cells**

To quantify CLIMP-63 colocalization with Trisk 95 or RyR1, or microtubule colocalization with RyR1, a binary mask corresponding to the overlap between the green and the red stainings was generated with a house ImageJ macro (available from the corresponding author on request). This mask corresponded to the surface of colocalization on one confocal plane. A ratio was calculated between the surface of colocalization and the total green surface (corresponding either to CLIMP-63 or microtubules). To quantify microtubule disorganization, A.O. and M.S. first captured images with the confocal microscope, either on EDLs from WT and KO mice in the same focal plane. A ratio was calculated between the surface of colocalization and the total green surface (corresponding either to CLIMP-63 or microtubules).

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: A.F.-L., I.M., J.F.; Methodology and Investigation: A.O., M.S., O.S., M.B., Y.C.; Proteomic analysis: M.B., Y.C.; Formal analysis: A.O., I.M., A.F.-L.; Writing—original draft preparation: A.F.-L., I.M.; Writing—review and editing: A.F.-L., I.M., J.F., Y.C., A.O., M.S.; Funding acquisition: I.M., Y.C.; Supervision: I.M., A.F.-L.

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**Supplementary information**

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.188862.supplemental

**References**

Ayalon, G., Davis, J. Q., Scotland, P. B. and Bennett, V. (2008). An ankyrin-based mechanism for functional organization of dystrophin and dystroglycan. Cell 135, 1189-1200.

Belanto, J. J., Mader, T. L., Eckhoff, M. D., Strandjord, D. M., Banks, G. B., Gardner, M. K., Lowe, D. A. and Ervasti, J. M. (2014). Microtubule binding distinguishes dystrophin from utrophin. Proc. Natl. Acad. Sci. USA 111, 5723-5728.

Block, B. A., Imagawa, T., Campbell, K. P. and Franzini-Armstrong, C. (1988). Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. J. Cell Biol. 107, 2587-2600.

Bovia, F., Salmon, P., Matthes, T., Kvell, K., Nguyen, T. H., Werner-Favre, C., Barnet, M., Nagy, M., Leuba, F., Arrighi, J.-F. et al. (2003). Efficient transduction of primary human B lymphocytes and nondividing myeloma B cells with HIV-1 derived lentiviral vectors. Blood 110, 1727-1733.

Cui-Wang, T., Hanus, C., Cui, T., Heiton, T., Bourne, J., Watson, D., Harris, K. M. and Eilhers, M. D. (2012). Local zones of endoplasmic reticulum complexity confine cargo in neuronal dendrites. Cell 148, 309-321.

DiFranco, M., Quinez, M., Capote, J. and Vergara, J. (2009). DNA transfection of mammalian skeletal muscles using in vivo electroploration. J. Vis. Exp. 32, e1520.

Duprières, V., Masselson, C., Court, M., Kieffer-Jaquindod, N. and Bruley, C. (2009). A toolbox for validation of mass spectrometry peptides identification and generation of database: IRMa. Bioinformatics 25, 1980-1981.

Flucher, B. E. and Franzini-Armstrong, C. (1996). Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle. Proc. Natl. Acad. Sci. USA 93, 8101-8105.

Fourest-Lieuvin, A., Rendu, J., Osseni, A., Pernet-Gallay, K., Rossi, D., Oudoux, S., Brocard, J., Sorrentino, V., Marty, I. and Faure, J. (2012). Role of triadin in the organization of reticulum membrane at the muscle triad. J. Cell Sci. 125, 3443-3453.

Franzini-Armstrong, C. (1980). Structure of sarcoplasmic reticulum. Fed. Proc. 39, 2403-2409.

Franzini-Armstrong, C. and Jorgensen, A. O. (1994). Structure and development of E-C coupling units in skeletal muscle. Annu. Rev. Physiol. 56, 509-534.

Hong, T.-T., Smyth, J. W., Gao, D., Chu, K. Y., Vogan, J. M., Fong, T. S., Jensen, B. C., Colecraft, H. M. and Shaw, R. M. (2010). BIN1 localizes the L-type calcium channel to cardiac T-tubules. PLoS Biol. 8, e1000312.

Kaiso, T. and Metsikko, K. (2003). Distribution of the endoplasmic reticulum and its relationship with the sarcoplasmic reticulum in skeletal myofibrils. Exp. Cell Res. 287-288, 7-57.

Kerr, J. P., Robison, P., Shi, G., Bogush, A. I., Kemptema, A. M., Hexum, J. K., Becerra, N., Racki, D. A., Martin, S. S., Raiteri, R. et al. (2015). Detyrosinated microtubules modulate mechanotransduction in heart and skeletal muscle. Nat. Commun. 6, 8526.

Khaustov, R. J., Shi, G., Sbrana, F., Prosser, B. L., Borroto, C., Mazaitis, M. J., Hoffman, E. P., Mahurkar, A., Sachs, F., Sun, Y. et al. (2012). Microtubules underlie dysfunction in Duchenne muscular dystrophy. Sci. Signal. 5, ra56.

Klopfenstein, D. R. C., Kappeler, F. and Hauri, H.-P. (1998). A novel direct interaction of endoplasmic reticulum with microtubules. EMBO J. 17, 6168-6177.

Klopfenstein, D. R. C., Klumperman, J., Lustig, A., Kammerer, R. A., Oorschot, V. and Hauri, H.-P. (2001). Subdomain-specific localization of CLIMP-63 (p63) in the endoplasmic reticulum is mediated by its luminal α-helical segment. J. Cell Biol. 153, 1287-1300.

Marty, I. (2015). Triadin regulation of the ryanodine receptor complex. J. Physiol. 593, 3261-3266.

Marty, I., Robert, M., Villaz, M., De Jongh, K., Lai, Y., Catterall, W. A. and Ronjat, M. (1994). Biochemical evidence for a complex involving dityrosine peptide receptor...
and ryanodine receptor in triad junctions of skeletal muscle. Proc. Natl. Acad. Sci. USA 91, 2270-2274.

Marty, I., Thevenon, D., Scotto, C., Groh, S., Sainnier, S., Robert, M., Grunwald, D. and Villaz, M. (2000). Cloning and characterisation of a new isoform of skeletal muscle triadin. J. Biol. Chem. 275, 8206-8212.

Milbradt, J., Kraut, A., Hutterer, C., Sonntag, E., Schmeiser, C., Ferro, M., Wagner, S., Lenac, T., Claus, C., Pinkert, S. et al. (2014). Proteomic analysis of the multicentric nuclear egress complex of human cytomegalovirus. Mol. Cell. Proteomics. 13, 2132-2146.

Mundy, D. I. and Warren, G. (1992). Mitosis and inhibition of intracellular transport stimulate palmitoylation of a 62-kD protein. J. Cell Biol. 116, 135-146.

Nguyen, T. H., Bellodi-Privato, M., Aubert, D., Pichard, V., Myara, A., Trono, D. and Ferry, N. (2005). Therapeutic lentivirus-mediated neonatal in vivo gene therapy in hyperbilirubinemic Gunn rats. Mol. Ther. 12, 852-859.

Nikonov, A. V., Hauri, H.-P., Lauring, B. and Kreibich, G. (2005). Climp-63-mediated binding of microtubules to the ER affects the lateral mobility of translocon complexes. J. Cell Sci. 120, 2248-2258.

Oddoux, S., Brocard, J., Schweitzer, A., Szentesi, P., Giannesini, B., Faure, J., Pernet-Gallay, K., Bendahan, D., Lunardi, J., Csernoch, L. et al. (2009). Triadin deletion induces impaired skeletal muscle function. J. Biol. Chem. 284, 34918-34929.

Oddoux, S., Zaal, K. J., Tate, V., Kenea, A., Nandkeolyar, S. A., Reid, E., Liu, W. and Ralston, E. (2013). Microtubules that form the stationary lattice of muscle fibers are dynamic and nucleated at Golgi elements. J. Cell Biol. 203, 205-213.

Ogata, T. and Yamasaki, Y. (1997). Ultra-high-resolution scanning electron microscopy of mitochondria and sarcoplasmic reticulum arrangement in human red, white, and intermediate muscle fibers. J. Cell Biol. 120, 2248-2258.

Ogata, T. and Yamasaki, Y. (1997). Ultra-high-resolution scanning electron microscopy of mitochondria and sarcoplasmic reticulum arrangement in human red, white, and intermediate muscle fibers. J. Cell Biol. 120, 2248-2258.

Ralphsson, N., Cacheux, M., Fourest-Lieuvin, A., Fauchet, P., Kermer, P., Sainsbury, P., Debacker, J., Denjoy, I., Durand, P., Guicheney, P., Knyzd, F., Leenhardt, A. et al. (2012). Absence of triadin, a protein of the calcium release complex, is responsible for cardiac arrhythmia with sudden death in human. Hum. Mol. Genet. 21, 2759-2767.

Schweizer, A., Ericsson, M., Bachi, T., Griffiths, G. and Hauri, H. P. (1993). Characterization of a novel 63 kDa membrane protein. Implications for the organization of the ER-to-Golgi pathway. J. Cell Sci. 104, 671-683.

Schweizer, A., Rohrer, J., Hauri, H. P. and Kornfeld, S. (1994). Retention of p63 in an ER-Golgi intermediate compartment depends on the presence of all three of its domains and on its ability to form oligomers. J. Cell. Biol. 125, 25-39.

Schweizer, A., Rohrer, J., Slot, J. W., Geuze, H. J. and Kornfeld, S. (1995). Reassessment of the subcellular localization of p63. J. Cell Sci. 108, 2477-2485.

Shen, X., Franzini-Armstrong, C., Lopez, J. R., Jones, L. R., Kobayashi, Y. M., Wang, Y., Kerrick, W. G. L., Caswell, A. H., Potter, J. D., Miller, T. et al. (2007). Triadins modulate intracellular Ca2+ homeostasis but are not essential for excitation-contraction coupling in skeletal muscle. J. Biol. Chem. 282, 37864-37874.

Shibata, Y., Shemesh, T., Prinz, W. A., Palazzo, A. F., Kozlov, M. M. and Rapoport, T. A. (2010). Mechanisms determining the morphology of the peripheral ER. Cell 143, 774-788.

Thevenon, D., Smida-Rezgui, S., Chevessier, F., Groh, S., Henry-Berger, J., Beatriz Romero, N., Villaz, M., DeWaard, M. and Marty, I. (2003). Human skeletal muscle triadin: gene organization and cloning of the major isoform, Trisk. 51. Biochem. Biophys. Res. Commun. 303, 669-675.

Vassilopoulos, S., Thevenon, D., Rezgui, S. S., Brocard, J., Chapel, A., Lacampagne, A., Lunardi, J., DeWaard, M. and Marty, I. (2005). Triadins are not triad-specific proteins: two new skeletal muscle triadins possibly involved in the architecture of sarcoplasmic reticulum. J. Cell Biol. 280, 28601-28609.

Vedrenne, C. and Hauri, H.-P. (2006). Morphogenesis of the endoplasmic reticulum: beyond active membrane expansion. Traffic 7, 639-646.

Vedrenne, C., Klopfenstein, D. R. and Hauri, H.-P. (2005). Phosphorylation controls CLIMP-63-mediated anchoring of the endoplasmic reticulum to microtubules. Mol. Biol. Cell 16, 1928-1937.

Wagenknecht, T., Hsieh, C.-S., Rath, B. K., Fleischer, S. and Marko, M. (2002). Electron tomography of frozen-hydrated isolated triad junctions. Biophys. J. 83, 2491-2501.

Zufferey, R., Nagy, D., Mandel, J. R., Naldini, L. and Trono, D. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat. Biotechnol. 15, 871-875.