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► To cite this version:

Stéphane Vassilopoulos, Sarah Oddoux, Séverine Groh, Marine Cacheux, Julien Fauré, et al.. Caveolin 3 is associated with the calcium release complex and is modified via in vivo triadin modification.: Association of calcium release complex and caveolin-3. *Biochemistry*, 2010, 49 (29), pp.6130-5. 10.1021/bi100796v . inserm-00567166

HAL Id: inserm-00567166

<https://inserm.hal.science/inserm-00567166>

Submitted on 10 Nov 2011

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Caveolin-3 is associated with the calcium release complex and is modified under in vivo triadin modification†

† This work was supported by grants from Association Française contre les Myopathies (AFM), from Groupement d'Intérêt Scientifique (GIS)-Maladies Rares, from Agence Nationale de la Recherche (ANR-Maladies Rares), from Société Française de Myologie (SFM) and from Fondation Ducoin.

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RECEIVED DATE :

TITLE RUNNING HEAD: Association of calcium release complex and caveolin-3

ABBREVIATIONS:

Cav-3, caveolin-3, CRC, calcium release complex, CSQ, Calsequestrin; DHPR, dihydropyridine receptor; EC coupling, excitation-contraction coupling; KO, knock out; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca²⁺ ATPase; T95, Trisk 95, T51, Trisk 51; WT, wild type

ABSTRACT. The triadin isoforms Trisk 95 and Trisk 51 are both components of the skeletal muscle calcium release complex. In order to investigate the specific role of Trisk 95 and Trisk 51 isoforms on muscle physiology, we overexpressed Trisk 95 or Trisk 51 using adenovirus-mediated gene transfer in skeletal muscle of newborn mice. Overexpression of either Trisk 95 or Trisk 51 alters the muscle fiber morphology, while leaving the expression of the ryanodine receptor, the dihydropyridine receptor and calsequestrin unchanged. We also observe an aberrant expression of caveolin 3 in both Trisk 95 and Trisk 51 overexpressing skeletal muscles. Using a biochemical approach, we demonstrate that caveolin 3 is associated with the calcium release complex in skeletal muscle. Taking advantage of muscle and non muscle cell culture models and triadin null mouse skeletal muscle, we further dissect the molecular organization of the caveolin 3 containing calcium release complex. Our data demonstrate that the association of caveolin 3 with the calcium release complex occurs via a direct interaction with the transmembrane domain of the ryanodine receptor. Taken together, these data suggest that caveolin 3 containing membrane domains and the calcium release complex are functionally linked, and that Trisk 95 and Trisk 51 are instrumental to regulate this interaction, the integrity of which may be crucial for muscle physiology.

KEYWORDS. Triadin, caveolin-3, ryanodine receptor, calcium release complex, mouse skeletal muscle, adenovirus.

In skeletal muscle, the excitation-contraction (EC) coupling process takes place at the triads where T-tubules and the reticulum sarcoplasmic terminal cisternae are in close contact. EC coupling requires the expression at the triads of a multimeric calcium release complex (CRC) that includes the T-tubule voltage-dependant calcium channel dihydropyridine receptor (DHPR) and several sarcoplasmic proteins, namely the calcium release channel ryanodine receptor (RyR) (1, 2), the sarcoluminal Ca^{2+} binding calsequestrin (CSQ), and two of the four triadin isoforms identified to date in skeletal muscle, Trisk 95 (T95) and Trisk 51 (T51) (3, 4). *In vitro* studies had identified a couple of regulatory RyR binding-domains on triadin, and these regions are common to both Trisk 95 and Trisk 51 (5, 6). Adenoviral-mediated overexpression of either Trisk 95 or Trisk 51 in primary cultures of skeletal muscle further demonstrated that only Trisk 95 plays a role in the regulation of depolarization-induced calcium release mechanism (4) suggesting a specific role of Trisk 95 in EC coupling. Interestingly, triadin null mice exhibit a structural myopathy (7) with impaired depolarization-induced calcium release (7, 8) indicating that the triadins are likely to be involved in the development of human myopathies for which a causative gene has not yet been identified. However, no modification has been yet identified in human triadins, both Trisk 95 and Trisk 51 being expressed in human skeletal muscle (9), and it is currently unknown whether overexpression of Trisk 95 and Trisk 51 would be detrimental for muscle function *in vivo*.

In the current study, we use adenovirus-mediated gene transfer to overexpress Trisk 95 or Trisk 51 in mouse skeletal muscle as an alternative approach to investigate the function of these triadin isoforms. Herein, we show that overexpression of either Trisk 95 or Trisk 51 alters the muscle fiber morphology while leaving the expression of RyR, DHPR and CSQ unchanged. We also observe that caveolin-3 (Cav-3), an essential structural component of caveolae involved in endocytosis and intracellular trafficking events (10) is aberrantly expressed in both Trisk 95 and Trisk 51 overexpressing skeletal muscles. We further demonstrate that Cav-3 is associated to the CRC via a direct interaction with the RyR and we propose that Trisk 95 and Trisk 51 level of expression is critical to regulate this interaction.

MATERIALS AND METHODS

Animals

Wild type mice (C57BL/6) were bred at the University of Iowa, IA, USA, from stocks originally obtained from Jackson Laboratories (Bar Harbor, ME). Triadin null mice have been described previously (7) and were bred and maintained on C57BL/6 background at the University Joseph Fourier, France. Animal care and procedures were approved and performed in accordance with the standards set forth by the Institutional Ethics Committee, the National Research Council Guide for the care and use of laboratory animals, the National Institute of Health and the Animal Care Use and Review Committee of the University of Iowa.

Antibodies and microsomes preparation

MAbs against CSQ (clone VIIID12, Affinity BioReagents) and Cav-3 (BD Transduction Laboratories) were used as described in the company datasheet. Sheep anti-DHPR α 1 subunit was obtained from Upstate Biotechnology. Polyclonal antibodies against the RyR, the common N-terminal end of tradins, Trisk 95 and Trisk 51 were described previously (2, 3, 11). Guinea pig anti-Ca²⁺-ATPase was a gift from Dr. A. M. Lompré (12). Crude microsomes were prepared from 1 month old mouse gastrocnemius muscle as previously described (13).

Viruses

The viruses were engineered and produced by the Gene Vector Production Network, at Genethon III (Evry, France). Three type 5 adenoviruses were used in this study, a control virus (AdV-DsRed) with the cDNA of the red fluorescent protein (DsRed), AdV-Trisk 95, an adenovirus with the full-length sequence of rat skeletal muscle T95 (EMBL AJ243304, 687 aa), and AdV-Trisk 51, an adenovirus with

the full-length sequence of rat skeletal muscle T51 (EMBL AJ243303, 461 aa). All the transgenes were under the control of a CMV promoter.

In vivo infection

Adenovirus injections into 3- to 4-day-old wild type pups were performed as previously described (14), with some modification: the hamstring, quadriceps, calf and tibialis anterior muscles of one leg were each injected percutaneously with 10^{10} p.f.u. diluted in 10 μ l of saline; the same muscles of the contralateral leg were each injected with an equal volume of saline. Pups were reintroduced to the mother and kept in quarantine for 5 days. All pups survived after injections. Muscles were harvested 30 days after injection, snap frozen in liquid nitrogen cooled isopentane and stored at -80°C until use.

Cell Culture and cell infection

Rat myogenic L6 cells (clone C5) or COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin. L6 cells were infected at a multiplicity of infection (MOI) 200 with the adenoviruses encoding T95 (AdV-Trisk 95) or encoding T51 (AdV-Trisk 51). The cells were collected 48h after infection.

Plasmids and transfection

The transmembrane domain of human RyR1 (accession number J05200) corresponding to amino acids 4457-5038 was cloned in pEGFP-C1 (Clontech), to produce a 93kDa fusion protein corresponding to the GFP fused with the channel part of RyR1. Human caveolin-3 (accession number AF036365) was cloned in pCDNA3.1 (Invitrogen). COS-7 cells were transfected with the two plasmids (2.5 μ g of each

plasmid for 8.10^5 cells) using ExGen 500 (Euromedex), and the cells were collected 24h after transfection.

Immunoprecipitation

Five hundred μg of microsomes from rat skeletal muscle or 300 μg cells were solubilised at 2mg/ml in presence of 1.6% CHAPS, 0.9M NaCl, 0.1% phospholipids, 100 μM CaCl_2 , 50 μM EGTA, 20 mM Pipes (pH 7.1) and protease inhibitors (1 mM diisopropyl fluorophosphates, 100 μM phenylmethanesulfonyl fluoride), and immunoprecipitation was performed with antibodies against rat Trisk 95, rat Trisk 51 or non immune serum as described previously (2), using 10mg protein A immobilized on sepharose 4B. All the immunoprecipitated proteins were then analyzed by Western blotting.

Western Blot

The presence of RyR, DHPR, Trisk 95, Trisk 51, calsequestrin, Ca^{2+} -ATPase SERCA or caveolin-3 in different samples was assayed by Western blot, using a chemiluminescent reagent (Western lightning Chemiluminescence reagent plus, PerkinElmer Life Science). After electrophoretic separation on a 5–15% acrylamide gel, the proteins were electrotransferred to Immobilon P (Millipore) as previously described (3). The secondary antibodies were labelled with horseradish peroxidase (Jackson ImmunoResearch laboratories). Quantitative analysis was performed using a Chemidoc XRS and the Quantity One Software (Biorad), and the amount of each protein was normalized to the amount in AdV-DsRed infected muscles, after loading correction by Coomassie blue staining of myosin, as described previously (15).

Immunofluorescence and histological analyses

Seven μm cryosections were prepared and analyzed by immunofluorescence or H&E staining as previously described (16). For immunofluorescent staining sections were washed with phosphate-buffered saline (PBS), blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature and incubated with a primary antibody in PBS-1% BSA overnight at 4°C. After 1h incubation at room temperature with an Alexa fluor 488- (Invitrogen) or Cy3-conjugated secondary antibody in PBS-1% BSA, the samples were mounted with PermaFluor (Beckman Coulter). Sections were observed under an MRC-600 laser scanning confocal microscope (Bio-Rad). Digitized images were captured under identical conditions. Images of H&E staining were photographed using a Leica DM RXA microscope equipped with an Olympus DP70 digital camera.

RESULTS

Effect of Trisk 95 and Trisk 51 overexpression on the expression of the CRC components

Trisk 95 and Trisk 51, the two major skeletal muscle triadin isoforms were *in vivo* overexpressed using adenovirus injection in new born mice. As a control, the muscles were injected with an adenovirus encoding DsRed. In order to determine the effect of Trisk 95 or Trisk 51 overexpression on the expression of their calcium release complex's partners, immunoblot analysis of crude muscle extracts was carried on 30 days after gene transfer (figure 1A and B). Protein expression levels were normalized to the amounts measured in control muscles infected with AdV-DsRed (figure 1A, lane 3). Both Trisk 95 and Trisk 51 were overexpressed by 60% (figure 1A, lanes 1 and 2, and figure 1B) but the expression levels of RyR, alpha 1 subunit of DHPR, CSQ and SERCA were not changed (figure 1B). This experiment has been done on muscles from different animals, with similar results (no modification of expression level of the proteins assayed, except for the overexpressed triadin). Nevertheless, the level of overexpression depends on the infection level, which is not identical between all the animals. Using Western blot on muscle homogenate, the infected fibers are mixed with non infected fibers, which constitute the majority of the fibers. To discriminate between infected and non-infected fibers, and to evaluate more precisely the modification induced on the other proteins, we chose to analyze the expression of the CRC components in Trisk 95 and Trisk 51 overexpressing muscles by immunofluorescence. While the stainings for RyR, DHPR and CSQ were unchanged in Trisk 95 infected fibers, we could observe a reduction in Trisk 51 expression in Trisk 95 infected fibers (figure 1C), an effect which was not observed in Western blot. Likewise, only Trisk 95 expression was reduced in Trisk 51 infected fibers (figure 1D).

Effect of Trisk 95 and Trisk 51 overexpression on the muscle histology

Histological analysis revealed no sign of muscle damage in control skeletal muscles injected with either saline (data not shown) or AdV-DsRed (figure 2A, panel a). However, Trisk 95 and Trisk 51 infected

skeletal muscles were characterized by the presence of fibrotic tissue, atrophic fibers, and regenerating fibers (figure 2A, panels b & c). The muscle damage was greater in T51-infected muscles than in T95-infected muscle, as assessed by the increased fibrosis and the presence of necrotic fibers. To specifically characterize Trisk 95 and Trisk 51 infected fibers, muscle sections were analyzed after immunostaining with caveolin-3 (Cav-3) as a plasma membrane marker (figure 2B). In contrast to muscles infected with AdV-DsRed (figure 2B, a-c and g-i), membrane staining revealed that the size of fibers infected by AdV-Trisk 95 and AdV-Trisk 51 was abnormal. Trisk 95 infected fibers were smaller than non infected fibers (figure 2B, panels d-f) and Trisk 51 infected fibers had a round shape (figure 2B, panels j-l) and lost the contact between them. Contrasting with the control AdV-DsRed infected fibers (Figure 2B, insets), the expression pattern of Cav-3 was altered in fibers over-expressing Trisk 95 and Trisk 51. In these later, Cav-3 was shown to accumulate intracellularly and to co-localize with the triadins. As Cav-3 is expressed during the differentiation of the satellite cells in myotubes (17), it could be hypothesized that the aberrant staining of Cav-3 specifically occurs in regenerating fibers. However, nuclei staining indicated that none of the cells displaying an intracellular staining of Cav-3 were regenerating cells (data not shown). Immunoblot analysis showed a similar amount of Cav-3 in crude extract of infected muscle as compared to control samples from DsRed infected muscles (figure 2C and D) suggesting most probably a relocalization of Cav-3 upon triadin overexpression.

Association of Cav-3 with component of the skeletal muscle CRC

In vivo modification of triadin expression results in modification of Cav-3, indicating a functional link between the two proteins. We studied their possible association by immunoprecipitation in rat skeletal muscle (figure 3A). Using isoform specific antibodies, both Trisk 95 and Trisk 51 co-immunoprecipitated Cav-3 (figure 3A, lanes 2 and 3). Trisk 95 and Trisk 51 also co-immunoprecipitated each other and the RyR, which indicates that Cav-3 is associated with the CRC.

An association between DHPR and Cav-3 was proposed in skeletal muscle (18) and shown in cardiac muscle (19). Thus, it is possible that the molecular complex between the triadins and Cav-3 requires a direct interaction of Cav-3 with DHPR. To test the direct interaction between DHPR and Cav-3, we analyzed the co-immunoprecipitation in rat skeletal muscle of Cav-3 with DHPR or the triadins (figure 3A). DHPR efficiently co-immunoprecipitated Cav-3 and RyR (figure 3A- lane 5), but not the triadins. Conversely, both Trisk 95 and Trisk 51 co-immunoprecipitated Cav-3 but not DHPR (figure 3A – lanes 2 and 3), suggesting that the association of triadin and Cav-3 is not DHPR-dependent.

To test whether Cav-3 and the triadins are able to associate directly, we studied their association in the L6 rat muscle cell line that do not express the known CRC proteins but express Cav-3. L6 cells were infected with AdV-Trisk 95 or AdV-Trisk 51 in order to induce the expression of each triadin isoform (figure 3B, lanes 2-4). The absence of RyR and the presence of Cav-3 were confirmed in these cells (figure 3B, lanes 2-4). Direct association of Cav-3 with either triadin was studied by co-immunoprecipitation with the anti-T95 and the anti-T51 antibodies respectively. Although Trisk 95 and Trisk 51 were efficiently immunoprecipitated, Cav-3 was co-immunoprecipitated with none (figure 3B, lanes 5-7), indicating that Cav-3 is not engaged in a direct interaction with any of the triadins. As both triadin are linked in skeletal muscle, as demonstrated by their co-immunoprecipitation (figure 3A), it is possible that the simultaneous presence of Trisk 95 and Trisk 51 is required for association with Cav-3. To test this hypothesis, L6 cells were co-infected with Adv-Trisk 95 and AdV-Trisk 51. However, Cav-3 was not co-immunoprecipitated with either anti-triadin isoform antibody (data not shown) suggesting that the presence of the two isoforms is not sufficient for the association with Cav-3

RyR1 is associated with Cav-3.

An association between RyR2 and Cav-3 was shown in cardiac muscle (20). To test whether Cav-3 directly interacts with RyR1 in skeletal muscle, we took advantage of the triadin null mouse model (7) to analyze the co-immunoprecipitation of Cav-3 with RyR1 in the absence of triadin. As shown in figure

4A, Cav-3 was efficiently co-immunoprecipitated with RyR in both WT (figure 4A, lane 1) and triadin null skeletal muscle microsomes (figure 4A, lane 3), demonstrating that triadin was not necessary for this association, and that the RyR-Cav-3 interaction could be direct. In order to confirm the direct interaction of RyR and Cav-3, immunoprecipitation experiments were performed in a non muscle cell line. COS 7 cells were co-transfected either with Cav-3 and GFP-RyR transmembrane domain (last 582 amino acids fused in frame with GFP on its N-terminal part), or Cav-3 and GFP alone. Anti-GFP antibody efficiently co-immunoprecipitated RyR and Cav-3 (figure 4B, lane 6) but failed to co-immunoprecipitate Cav-3 in the presence of GFP alone (figure 4B, lane 5), confirming the direct interaction between Cav-3 and RyR1 transmembrane domain.

DISCUSSION

In vivo overexpression of the triadin isoforms Trisk 95 or Trisk 51 was induced by adenoviral-mediated gene transfer in the hindlimb muscles of newborn mice. Overexpression of one triadin isoform induced a reduction in the expression level of the second one. The different triadin isoforms are issued of the alternative splicing of the same gene (9), and even if the mechanisms driving the specific expression of each isoform are unknown, it is clear that the two triadins Trisk 95 and Trisk 51 are not only always associated together, as seen in the co-immunoprecipitation experiments, they also mutually regulate their expression, in order to keep the total amount of triadin in the skeletal muscle almost constant. The expression of the main components of the calcium release complex, i.e., RyR, DHPR, the Ca²⁺-ATPase SERCA and CSQ, was not affected. However, an interesting finding of our study was the alteration of Cav-3 expression in triadin infected fibers. Using a biochemical approach, we demonstrated the existence of a molecular complex including the triadins and Cav-3. Using differential immunoprecipitations in WT and triadin null skeletal muscle, as well as in muscle and non-muscle cells, we further demonstrated that Cav-3 was associated with the CRC via a direct interaction with the transmembrane domain of RyR1. Our data also suggest that two different populations of CRC exist in the skeletal muscle triads, one involving DHPR- RyR-Cav-3, and one involving Trisk 95-Trisk 51-RyR-Cav-3, as shown in figure 3. This could be related to the early electronic microscopy study demonstrating that only half of the RyR were coupled to DHPR (1), and one could imagine that DHPR and triadin are mutually excluding each other in the CRC, the RyR uncoupled to DHPR being in interaction with triadin.

Triadin (Trisk 95 or Trisk 51) overexpression results in an increased intracellular Cav-3 staining, which is indicative of an *in vivo* functional link between the two proteins. As we cannot demonstrate a Cav-3 overexpression equivalent to the triadin overexpression, this increased intracellular Cav-3 labelling is most probably due to Cav-3 relocalization. Our finding that Cav-3 is functionally linked to the proteins of the CRC raises the questions of the physiological relevance of the association between the caveolae and the SR. Cav-3 was proposed to play a role in calcium homeostasis (21-23) and previous studies

showed that the IP₃R and SERCA, both involved in calcium homeostasis (24) were detected within the caveolae of several different cell types, including muscle cells (21, 22). More recently Li *et al.* (24) showed the presence of microdomains enriched in cholesterol, sphingolipids and Cav-3 in the SR membrane. Assuming that the caveolae-SR contacts are partly responsible for the control of the cytoplasmic free Ca²⁺ concentrations in very precisely defined spaces (25), the CRC involving triadins could be located at those sites. Previous studies showed that caveolae are sites of extracellular Ca²⁺ entry (26) and suggested that caveolae are involved in re-filling depleted intracellular calcium stores, a mechanism called store operated calcium entry (SOCE). Caveolae could then act as intermediates between the SR and the plasma membrane. We have previously shown that Trisk 95 can modulate the mechanism coupling the depletion of intracellular Ca²⁺ stores to extracellular Ca²⁺ entry. The over-expression of Trisk 95 decreases this Ca²⁺ entry in myotubes (27). It would therefore be possible that the over-expression of Trisk 95 disturbs the SOCE by its effect on Cav-3. The re-localization of Cav-3 from the plasma membrane to intracellular compartments following the over-expression of Trisk 95 would directly disturb the Ca²⁺ entry by caveolae and would therefore reduce store operated Ca²⁺ entry.

Both Cav-3 null mice and transgenic mice overexpressing Cav-3 display a dystrophic phenotype (17). A likely explanation for Cav-3 null mice phenotype is the alteration in the intracellular trafficking and targeting of membrane proteins and protein complexes, such as dysferlin (2, 28) and the dystrophin glycoprotein complex (17). Noteworthy, Cav-3 null skeletal muscle also exhibit abnormal DHPR and RyR staining, suggesting that Cav-3 is involved in triad formation during muscle development (29, 30). Trisk 95 and Trisk 51 overexpressing muscles exhibited obvious signs of pathology that included central nucleated fibers and heterogeneous fiber sizes that resemble the ones of dystrophic muscles. Given that Trisk 95 and Trisk 51 seem to mutually regulate their expression level and given that triadin null mice exhibit muscle weakness (7), it is tempting to speculate that a precise expression level of the triadins is crucial for normal muscle function (31); an excess of triadin could be as deleterious as a lack of triadin on the muscle physiology. However, while Trisk 95 overexpression was shown to block EC coupling in skeletal muscle (4), Trisk 51 overexpression has no effect on EC coupling (4). Therefore, it is unlikely

that the muscle pathology observed in triadin overexpressing muscle results entirely from EC coupling dysfunction. In this context, one could speculate that Trisk 95 or Trisk 51 over-expression perturbs the traffic of proteins forming the Ca^{2+} -release machinery by Cav-3 trapping, thus resulting in the abnormal muscle phenotype observed here. A mechanism of Cav-3 trapping has been proposed for the mutant form of Cav-3, P104L, responsible for some case of myopathy (LGMD1C) (32, 33), therefore this mechanism has already been shown to be responsible of a pathology. Although no disease-causing mutation has been identified yet in triadin gene, it is possible that modification of the triadin expression level underlie a new pathophysiological mechanism for human myopathies, possibly involving Cav-3 alteration.

Acknowledgments

We thank Genethon (Evry- France) for the production of the adenoviruses used in this study.

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FIGURES LEGEND

Figure 1: Western blot and immunofluorescent analysis of the proteins of the calcium release complex in infected muscles

The experiment presented in panel A and B correspond to one representative experiment, which have been performed on 3 different infected muscles. The values have not been averaged between all the experiments as the infection level is not identical between all the infected muscles.

A: Expression of the components of the calcium release complex in skeletal muscle microsomes (20 μ g) in Trisk 95 infected muscle (lane 1), Trisk 51 infected muscle (lane 2), or control DsRed infected muscle (lane 3) as determined by Western blotting.

B: Level of proteins expressed as fold of control DsRed infected muscles, after loading correction using myosin quantity estimated by Coomassie blue staining. The quantification was performed on the Western blot presented on part A.

C: Effect of Trisk 95 over-expression on proteins of the calcium release complex. Double immunofluorescent labelling was performed on transversal sections of muscle infected with AdV-T95 for 30 days, using antibodies directed against T95 and the α_{1s} subunit of the DHPR (a-c), Trisk 95 and RyR (d-f), T95 and CSQ (g-i), or T95 and T51 (j-l). Scale bar: 15 μ m.

D: Effect of Trisk 51 over-expression on proteins of the calcium release complex. Double immunofluorescent labelling was performed on transversal sections of muscle infected with AdV-T51 for 30 days using antibodies directed against T51 and the α_{1s} subunit of the DHPR (a-c), Trisk 51 and RyR (d-f), T51 and CSQ (g-i), or T51 and T95 (j-l). Scale bar: 15 μ m.

Figure 2: Alteration of muscle morphology in infected muscles.

A: Histological analysis by Hematoxylin-eosin staining of gastrocnemius infected for 30 days with AdV-DsRed (CTRL – panel a), AdV-Trisk 95 (inf T95 –panel b) or AdV-Trisk 51 (inf T51 – panel c). Only in muscle infected with T95 or T51, the presence of fibrotic tissue is observed, as well as a few

atrophic fibers (asterisks), necrotic fibers stained in pink (inf T51 only), and a few regenerating fibers, characterised by their centrally located nuclei (arrows). Scale bar: 50 μ m.

B: Double immunofluorescent labelling with anti-Cav-3 and anti-T95 antibodies (a-f) or anti-Cav-3 and anti-T51 (g-l) antibodies was performed on muscle sections from CTRL DsRed infected muscle (a-c and g-i), T95 infected muscle (d-f) or T51 infected muscle (j-l). Cav-3 and T95 (inset a-c and d-f) or Cav-3 and T51 (inset g-i and j-l) intracellular staining was compared by confocal microscopy.

C: The expression level of Cav-3 was analysed by Western-blot on T95 infected muscle (lane 1), T51 infected muscle (lane 2) or DsRed infected muscle (lane 3). Five micrograms of mouse skeletal microsomes were loaded in each lane.

D: The quantification of the amount of caveolin-3 in each lane of panel C was performed and compared to the amount in DsRed infected muscle (CTRL), after correction of loading by myosin quantification.

Figure 3: Association of Cav-3 with the CRC and the triadins.

A: Co-immunoprecipitation on rat skeletal muscle

Immunoprecipitation was performed on 500 μ g rat skeletal muscle with antibodies against Trisk 95 (lane 2), with antibodies against Trisk 51 (lane 3), with pre-immune serum (lane 4) or with antibodies against DHPR (lane 5). The immunoprecipitated proteins were then analyzed by Western blot with antibodies against RyR, against DHPR, against the N-terminal end of triadin or against Cav-3. Lane 1 : 5 μ g of rat skeletal muscle microsomes as a control.

B: Co-immunoprecipitation in L6 cells.

Immunoprecipitation was performed on 300 μ g Trisk 95 infected cells (lane 2), Trisk 51 infected cells (lane 3) or control cells (lane 4), with antibodies specific of T95 or of T51. The immunoprecipitated proteins were then analyzed by Western blot (lanes 5 to 8) with anti-RyR, anti-Nter triadin or anti-Cav 3. Lane 1: 5 μ g of rat skeletal muscle microsomes as a control. RyR is absent from L6 cells (infected and control, lanes 2-4), and is not co-immunoprecipitated with triadins isoforms (lanes 5-8).

Figure 4: Co-immunoprecipitation of Cav-3 with RyR.

A: Immunoprecipitation was performed on mouse skeletal muscle from WT or triadin KO mouse with antibodies against RyR (lanes 1 and 3), or with pre-immune serum (lanes 2 and 4). The immunoprecipitated proteins were then analyzed by Western blot with antibodies against RyR or Cav-3.

B: Immunoprecipitation was performed with anti-GFP antibodies on COS 7 cells co-transfected with GFP and Cav-3 (lane 5) or GFP-RyR and Cav-3 (lane 6). The immunoprecipitated proteins were then analyzed by Western blot with antibodies against GFP or Cav-3.

Figure 1

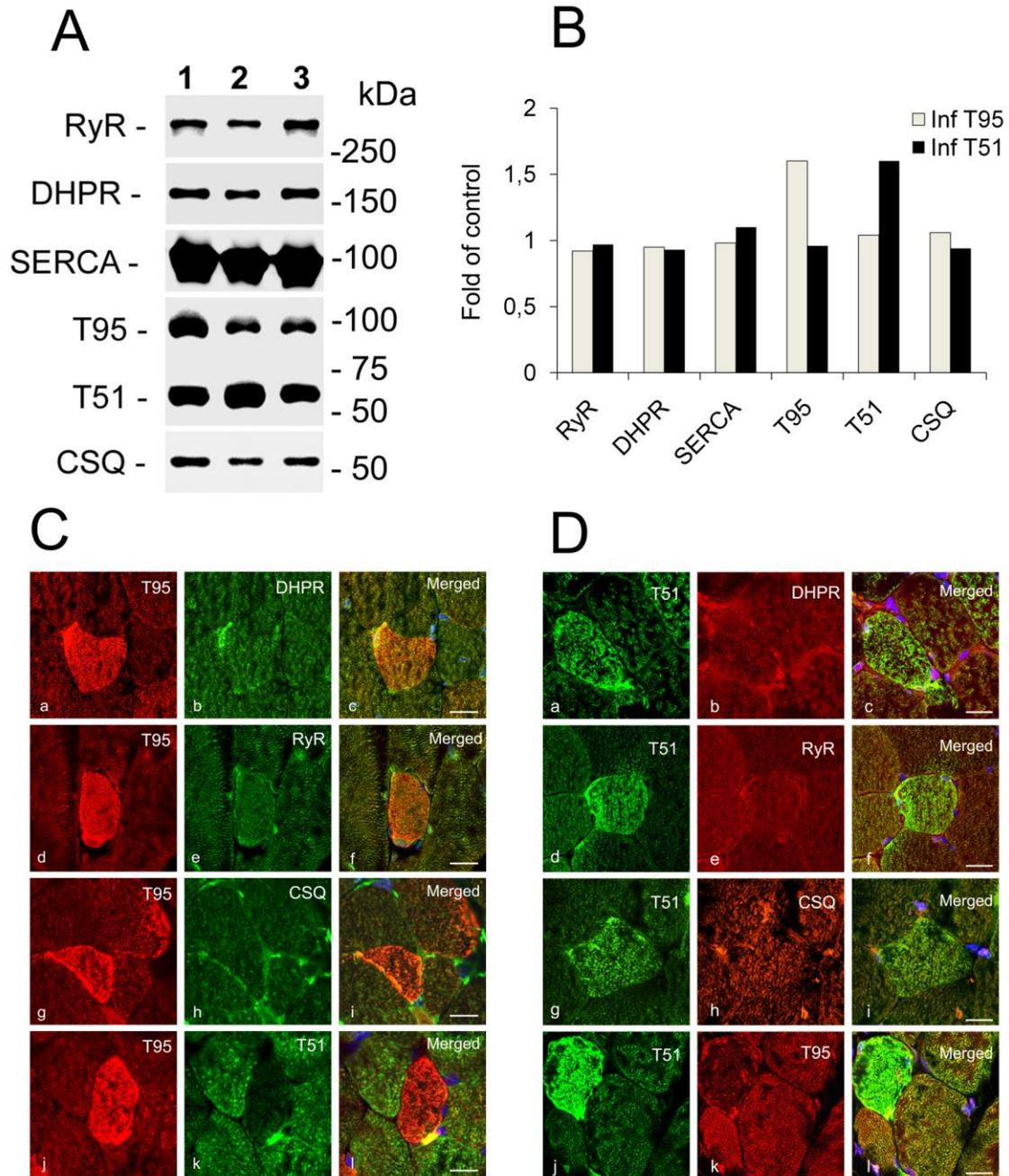


Figure 2

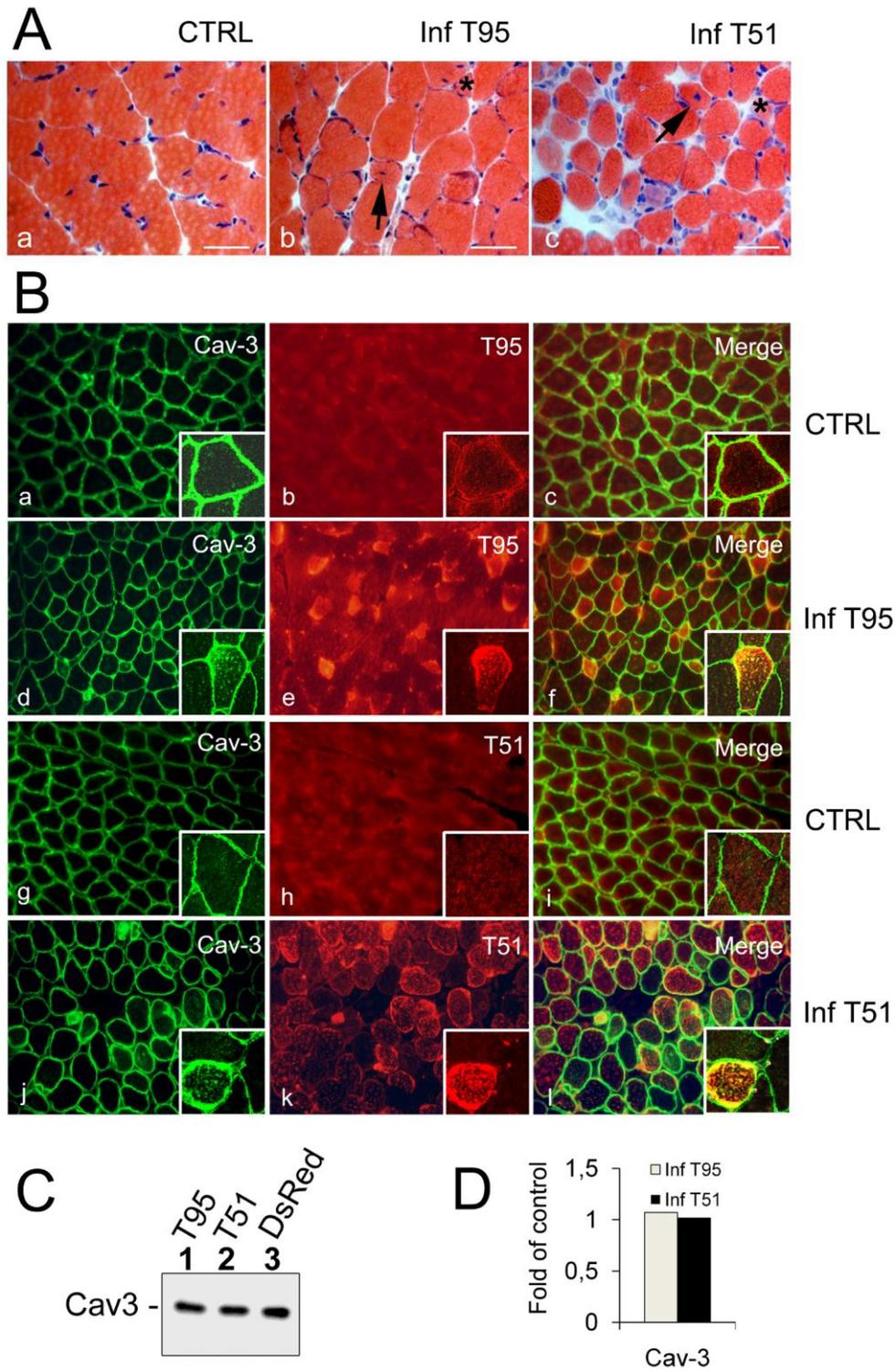


Figure 3

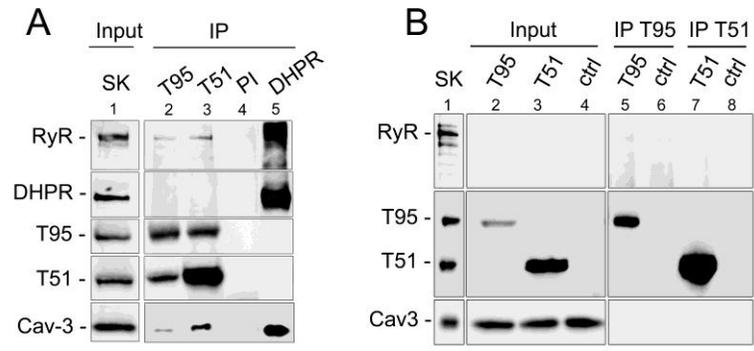


Figure 4

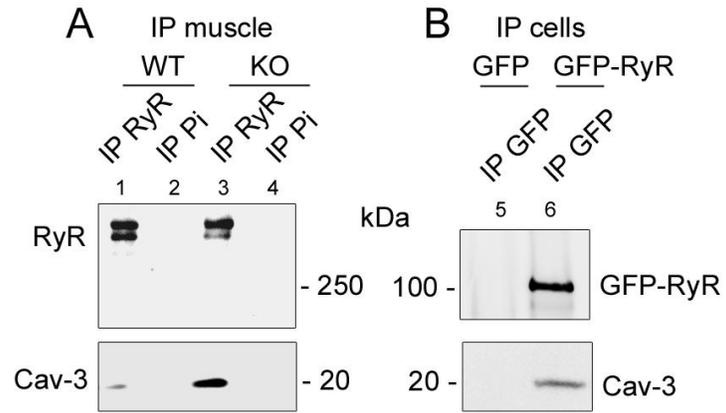
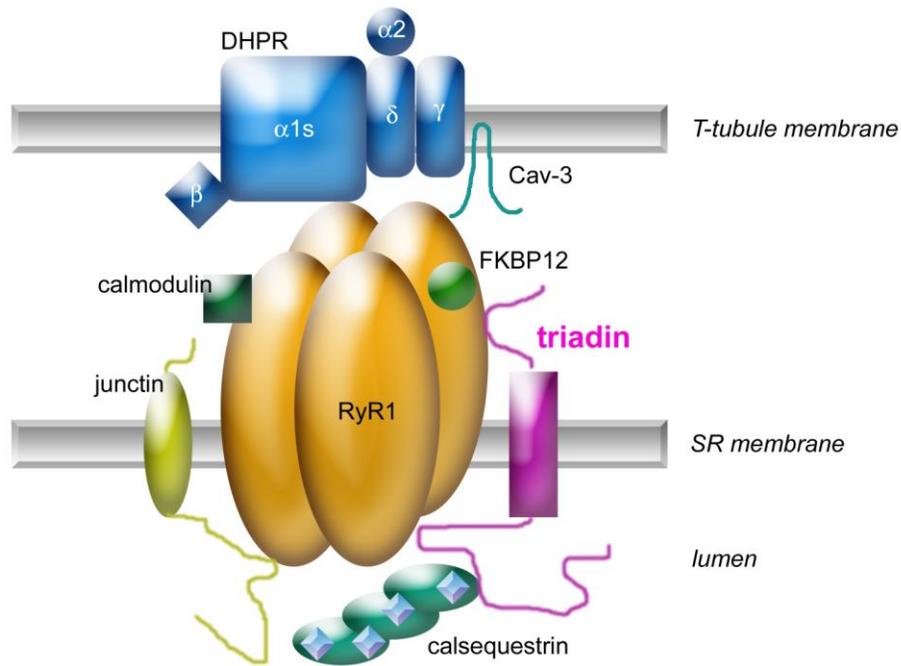


Table of contents graphic



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"Caveolin-3 is associated with the calcium release complex and is modified under in vivo triadin modification".

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