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# Triadin (Trisk 95) overexpression blocks excitation-contraction coupling in rat skeletal myotubes

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## Abstract

In order to identify the function of triadin in skeletal muscle, adenovirus mediated over-expression of Trisk 95 or Trisk 51, the two major skeletal muscle isoforms, was induced in rat skeletal muscle primary cultures, and the physiological behaviour of the modified cells was analyzed. Over-expression did not modify the expression level of their protein partners: ryanodine receptor, dihydropyridine receptor, and the other triadin. Caffeine induced-calcium release was also unaffected by triadin over-expression. Nevertheless, in the absence of extracellular calcium, depolarization induced calcium release was almost abolished in Trisk 95 over-expressing myotubes (T95 myotubes), and not modified in Trisk 51 over-expressing myotubes (T51 myotubes). This was not due to a modification of dihydropyridine receptors as depolarization in presence of external calcium still induced a calcium release, and the activation curve of dihydropyridine receptor was unchanged, in both T95 and T51 myotubes. The calcium release complex was also maintained in T95 myotubes as Trisk 95, ryanodine receptor, dihydropyridine receptor and Trisk 51 were still co-localized. The effect of Trisk 95 over-expression on depolarization-induced calcium release was reversed by a simultaneous infection with an antisense Trisk 95 adenovirus, indicating the specificity of this effect. Thus, the level of Trisk 95 and not Trisk 51 is important on regulating the calcium release complex, and an excess of this protein can lead to an inhibition of the physiological function of the complex.

**MESH Keywords** Animals ; Calcium Channels, L-Type ; metabolism ; Calcium Signaling ; Carrier Proteins ; genetics ; physiology ; Cells, Cultured ; Electrophysiology ; Gene Expression ; Membrane Potentials ; Multiprotein Complexes ; Muscle Contraction ; physiology ; Muscle Fibers, Skeletal ; physiology ; Muscle Proteins ; genetics ; physiology ; Rats ; Ryanodine Receptor Calcium Release Channel ; metabolism ; Transfection

In skeletal muscle cells, excitation-contraction (E-C) coupling is the process by which depolarization of the plasma membrane produces a large transient release of Ca<sup>2+</sup> from the sarcoplasmic reticulum, which in turn triggers contraction. Dihydropyridine receptors (DHPRs), the L-type Ca<sup>2+</sup> channels localized in the T-tubule membrane, serve as the voltage sensors for E-C coupling [1, 2] and activate ryanodine receptors (RyRs), the intracellular Ca<sup>2+</sup> release channels of the sarcoplasmic reticulum membrane [3, 4]. In skeletal muscle, entry of Ca<sup>2+</sup> through DHPR is not required for E-C coupling [5]. Instead, a voltage-dependent conformational change in the II-III loop of the skeletal muscle DHPR  $\alpha$ 1 subunit (Cav1.1) activates the skeletal muscle ryanodine receptor, RyR1. Activation of RyR1 results in a release of Ca<sup>2+</sup> from the SR intracellular stores into the cytosol, which in turn activates the cellular contractile apparatus to initiate muscle contraction. In cardiac muscle, the entry of external calcium through DHPR is required to induce activation of RyR and release of internal Ca<sup>2+</sup> via RyR, a mechanism known as Calcium-Induced Calcium Release (CICR). CICR is not the initial mechanism responsible of Ca<sup>2+</sup> release in skeletal muscle, but it can contribute to the amplification of the signal, and both CICR and conformational coupling are involved in skeletal muscle contraction, to different extent [6].

Triadin is a transmembrane protein co-localized to the junctional SR membrane with RyR and was originally detected in rabbit skeletal muscle as a 95-kDa molecular weight protein [7–9]. Several isoforms of triadin are expressed in rat skeletal muscle, and we have previously cloned two of these isoforms [10], both expressed at equivalent amounts in rat skeletal muscle. One is the rat homolog of the 95-kDa triadin identified in rabbit skeletal muscle, named Trisk 95, and the second one, Trisk 51, is a truncated form of Trisk 95 (461 amino acids instead of 687 amino acids), almost identical for the major part of the protein but with a unique C-terminal end of 6 amino acids. In cardiac muscle, multiple isoforms of triadin have also been identified [11, 12], but the major if not only isoform expressed is CT1, a 32 kDa triadin isoform [13].

Trisk 95 is composed of a short NH<sub>2</sub>-terminal cytosolic domain, a single transmembrane domain, and a large C-terminal domain located in the lumen of the SR [9, 14]. The intraluminal domain, composed of a high density of positively and negatively charged amino acid residues, interacts with RyR and calsequestrin [15–17]. Trisk 51 shares this same structure but has a smaller intraluminal domain.

The function of triadins in skeletal muscle is not precisely established for the moment. In skeletal muscle, triadin has been shown to inhibit the calcium channel activity of purified RyR [18, 19]. These experiments were the first to identify a function of triadin in skeletal

muscle, and they led to the conclusion that triadin could regulate RyR function via inhibition of the channel. At that time, the presence of Trisk 51 in skeletal muscle had not been observed. In order to obtain new information concerning the respective function of Trisk 95 or Trisk 51 in skeletal muscle E-C coupling, we induced adenovirus mediated Trisk 95 or Trisk 51 over-expression in primary culture of skeletal muscle, and we then studied the behaviour of the calcium release complex in these triadin-modified cells. We investigated intracellular  $\text{Ca}^{2+}$  transients elicited by depolarization-induced calcium release ( $\text{K}^+$  depolarization), or by direct RyR calcium channel activation (caffeine or 4-chloro-m-cresol, 4-CmC). Here, we show that the depolarization-induced  $\text{Ca}^{2+}$  release was significantly decreased in the Trisk 95 over-expressing myotubes, and this effect was reversed by infection with an antisense virus. Trisk 51 over-expression has no effect, suggesting an isoform-specific action on E-C coupling. These results are discussed in terms of possible physiopathological consequences in some myopathies.

## Experimental procedures

### Antibodies

Rabbit or guinea pig antibodies directed against the specific C-terminal end of Trisk 95 or Trisk 51 were described previously [10]. Polyclonal antibody directed against RyR purified from pig skeletal muscle was previously characterized [20]. Sheep anti- $\alpha 1$  subunit of DHPR was from Upstate Biotechnology.

### Western blot

The presence of RyR,  $\alpha 1$  subunit of DHPR, Trisk 95 or Trisk 51 in different samples was assayed by Western Blot [10], using a chemiluminescent reagent (Western lightning Chemiluminescence reagent plus, Perkin Elmer) after electrophoretic separation of the protein on a 5–15% acrylamide gel and electrotransferred to Immobilon P (Millipore). The secondary antibodies were labeled with HRP (Jackson ImmunoResearch laboratories).

### Cell culture

Primary cultures of neonatal rat skeletal muscle were obtained as described previously [10]. Cells were seeded on laminin-coated plates, in a proliferation medium composed of Ham's F-10 with glutamax-I (Invitrogen) supplemented with 20% fetal bovin serum (FBS) (Invitrogen), 2% Ultrosor (BIOSEPR) and 2% penicillin-streptomycin (Invitrogen) at 37°C and 5%  $\text{CO}_2$ . After 2–3 days, differentiation into myotubes was induced with DMEM with glutamax-I (Invitrogen) supplemented with 5 % horse serum (Invitrogen).

### Viruses and infection

In this study, we used adenoviral-mediated gene transfer to modify Trisk 95 or Trisk 51 expression in primary cultures of rat skeletal muscle. The viruses were engineered and produced by the Gene Vector Production Network, at Genethon III (Evry, France). Four 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 Trisk 95 [10]; AdV-AS-Trisk 95, an adenovirus with the nucleotides 1765–2064 of Trisk 95 in antisense orientation; and AdV-Trisk 51, an adenovirus with the full length sequence of rat skeletal muscle Trisk 51 [10]. All the transgenes were under the control of a CMV promoter.

Cells were infected by overnight incubation with the chosen virus, at a multiplicity of infection (MOI) 40, in proliferation medium, and then differentiation was induced by a change to differentiation medium. Control cells were infected with the control virus (AdV-DsRed). Trisk 95-over-expressing myotubes (T95-myotubes) consisted in cells infected with AdV-Trisk 95, and Trisk 51-overexpressing myotubes (T51-myotubes) with AdV-Trisk 51. For double infection, AdV-Trisk 95 (MOI 40) and AdV-ASTrisk 95 (MOI 60) were simultaneously added to the cell culture. Calcium imaging studies were performed after 2–3 days of infection/differentiation. Infection of COS-7 cells was performed in the same way, except that the cells were kept in proliferation medium before and after infection (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin).

### Immunofluorescence labeling

Myotubes (2 to 3 days differentiation) grown on plastic dishes coated with laminin were washed with PBS and fixed for 15 min at room temperature with 4% paraformaldehyde in PBS. After two washes with PBS for 5 min, the cells were permeabilized with 0.5% triton X-100 in PBS for 5 min then saturated in PBS supplemented with 0.1% Triton X-100, 0.5% bovine serum albumin, and 2% goat serum for 30 min at room temperature. Cells were incubated with primary antibodies at room temperature for 2 h. After three washes with PBS, cells were incubated for 30 min at room temperature with fluorescent secondary antibodies, then washed three times with PBS, and the cover slips were mounted on microscope slides with anti-fading medium (DakoCytomation fluorescent mounting medium, DakoCytomation). For double/triple labeling, the two/three primary antibodies (from different species) and later the two/three secondary antibodies were added at the same time. The secondary antibodies were either labeled with Alexa-488, Alexa-546 (both from Molecular Probes), or Cy5 (from Jackson ImmunoResearch laboratories). The samples were analyzed by confocal laser scanning microscopy using a Leica TCS-SP2 operating system. The objectives used were Leica x40 oil immersion (NA 1.25) or Leica x100 oil immersion (NA 1.96). Fluorochromes

were excited and collected sequentially (400 Hz line by line) by using the 488 nm line of an argon laser for Alexa-488, the 543 nm line of an helium-neon laser for Alexa-546, and the 633 nm line of an helium-neon laser for Cy5 excitation. Fluorescence emission was collected from 498 to 541 nm for Alexa-488, from 554 to 625 nm for Alexa-546, and from 640 nm to 730 nm for Cy5.

### **Immunoprecipitation**

Rat skeletal muscle microsomes were solubilized in presence of CHAPS, and immunoprecipitation was performed with the chosen antibody as described previously [20]. The immunoprecipitated proteins were then analyzed by Western blotting.

### **Intracellular calcium measurements**

Changes in intracellular calcium were measured using the calcium-dependent fluorescent dye Fluo 4-AM (Molecular Probes). Myotubes were incubated for 1 h at room temperature with Fluo-4-AM 10  $\mu$ M, in Krebs buffer (NaCl 136 mM, KCl 5 mM, CaCl<sub>2</sub> 2 mM, MgCl<sub>2</sub> 1mM, HEPES 10 mM pH 7.4). Uptake of the dye was facilitated by the addition of 0.02% pluronic F-127 acid (SIGMA). After loading, myotubes were washed for 1 h at 37°C to allow de-esterification of the dye. Calcium imaging was performed in Krebs buffer. To obtain a calcium-free Krebs solution, CaCl<sub>2</sub> was left out, while 1 mM EGTA and 50  $\mu$ M lanthanum were added. In the depolarization solution, NaCl was replaced by KCl (140 mM final concentration). The other agonists, caffeine and 4-CmC, were prepared as stock solutions in Krebs buffer and used at final concentrations of 10 mM and 500  $\mu$ M respectively.

Fluorescence was measured by confocal laser scanning microscopy, using a Leica TCS-SP2 operating system, in the xyt mode. Fluo 4 was excited by using the 488 nm wavelength and its fluorescence was collected from 500 to 570 nm. The fluorescence images were collected every 1.6 s, during 2 to 4 min, and then analyzed frame by frame. Fluorescence curves are expressed as a function of time, as  $\Delta F/F$ , where F represents the baseline fluorescence immediately prior to depolarization and  $\Delta F$  represents the fluorescence changes from baseline. Data are given as mean  $\pm$  SEM, and n represents the number of myotubes in each condition.

### **Patch clamp**

Whole-cell currents through voltage-gated calcium channels were recorded by means of the patch-clamp technique [21]. After removal of the culture medium, the cells were perfused with a medium containing (in mM): TEACl 145, CaCl<sub>2</sub> 2, MgCl<sub>2</sub>, HEPES 10, pH 7.4 (TEAOH). Patch-pipettes were pulled from thick wall borosilicate glass capillaries (1.5 mm o.d  $\times$  0.86 mm i.d., Clark Electromedical Instruments, U.K.). When filled with the following intracellular solution (in mM): CsCl 120, EGTA 10, MgCl<sub>2</sub> 2, Na<sub>2</sub>-ATP 3, HEPES 10, pH 7.2 (CsOH), they had a resistance of about 2–5 M $\Omega$ . Electrical signals were stimulated and recorded by means of the pClamp software (version 6.0, Axon Instruments). Voltage-gated calcium channel currents were filtered at 1–2 kHz and sampled at 5–10 kHz on an Axoclamp 200B amplifier (Axon Instruments). Myotubes were stimulated at a frequency of 0.2 Hz from a holding potential of -60 mV. Electrophysiological recordings were done at room temperature. Data are expressed as mean  $\pm$  SEM, with n being the number of myotubes tested.

## **RESULTS**

### **Trisk 95 and Trisk 51 are associated with RyR**

We have previously shown that two triadin isoforms are expressed in rat skeletal muscle: Trisk 95, the 95 kDa isoform, and Trisk 51, the 51 kDa isoform [10]. In order to determine their association with RyR, immunoprecipitations were performed. These immunoprecipitations were done on solubilized rat skeletal muscle microsomes using antibodies specific for Trisk 51 (Figure 1 - lane 2) or Trisk 95 (Figure 1 - lane 3), and the immunoprecipitated proteins were analyzed by Western blot with anti-RyR antibodies. The presence of RyR was observed among the proteins immunoprecipitated by both antibodies, whereas it is absent from the proteins immunoprecipitated with non immune serum (lane 4). This indicates that both triadin isoforms are involved in the calcium release complex, and are, directly or not, associated with RyR. Thus, both isoforms could regulate RyR function.

### **Trisk 95 or Trisk 51 over-expression doesn't modify expression of the other partners in the complex**

Trisk 95 or Trisk 51 over-expression was induced by adenoviral infection. Satellite cells were infected and induced to differentiate, and after 3 days of infection/differentiation, myotubes were formed and the cells were collected. The expression of both triadin isoforms in differentiated myotubes has been previously described [10]. The infection of satellite cells with AdV-T95 induced 15 fold Trisk 95 over-expression (figure 2, panel A), whereas Trisk 51 expression was not significantly modified (figure 2, panel B). In a similar way, the infection of satellites cells with AdV-T51 induced 40 fold over-expression of Trisk 51 (figure 2, panel B), whereas Trisk 95 expression was not significantly modified (figure 2, panel A). The expression level of RyR and DHPR was evaluated in control or in triadin over-expressing myotubes. Neither Trisk 95 over-expression nor Trisk 51 over-expression modified the expression level of RyR or DHPR (figure 2, panels C and D). Thus Trisk 95 or Trisk 51 over-expression did not induce modifications in the expression level of the other proteins of the calcium release complex.

## RyR calcium channel function in triadin modified cells

In order to determine if RyR calcium channel function is preserved in T95-myotubes and in T51-myotubes, we performed Fluo-4 confocal microscopy calcium imaging. We measured the calcium release induced by RyR activators: 10 mM caffeine [22] or 500  $\mu$ M 4-CmC [23]. The addition of caffeine (figure 3) or 4-CmC on cultured myotubes induced a massive calcium release from the internal stores in all the myotubes. Comparison of the intensity of calcium signals induced by caffeine (figure 3) showed no significant differences between control myotubes, T95-myotubes or T51-myotubes. Caffeine induced an increase in the relative fluorescence of  $2.07 \pm 0.15$  in control myotubes, whereas the increase in the relative fluorescence was of  $1.92 \pm 0.12$  in T95-myotubes, and  $2.02 \pm 0.19$  in T51-myotubes, indicating a similar RyR functionality (non significantly different, Student T test). Thus Trisk 95 or Trisk 51 over-expression didn't modify the function of RyR as a calcium channel.

## Depolarization-induced calcium release in triadin modified cells

Depolarization of the plasma membrane induces activation of the DHPR and results both in i) activation of RyR and internal calcium release, and ii) influx of external calcium via DHPR if external calcium is present [24, 25]. Depolarization was induced in the absence of external calcium (figure 4, panel A), on control, on T95-myotubes or on T51-myotubes, by addition of 140 mM KCl. It resulted, as expected [26–28], in an increase in intracellular calcium concentration followed by a return to the basal level in control myotubes, as well as in T51-myotubes. Nevertheless, in T95-myotubes, this effect was almost abolished, and depolarization induced only a minor calcium release (figure 4A). The relative increase in fluorescence was significantly reduced ( $p < 0.0001$  – Student T test) from  $1.54 \pm 0.21$  in control myotubes to  $0.30 \pm 0.06$  in T95-myotubes. The fluorescence response was not different in T51-myotubes ( $1.60 \pm 0.18$ ) compared to control myotubes ( $1.54 \pm 0.21$ ).

In order to test the functionality of DHPR and to check that Trisk 95 over-expression hadn't led to an inhibition of DHPR, the same depolarization experiment was performed, but this time in the presence of external calcium (figure 4, panel B). As expected, in control myotubes, as well as in T51-myotubes, the depolarization induced a larger calcium transient ( $2.08 \pm 0.22$  in presence of external calcium instead of  $1.54 \pm 0.21$  in the absence of calcium for control myotubes, and  $2.06 \pm 0.18$  in presence of calcium compared to  $1.60 \pm 0.18$  in the absence of calcium, for T51-myotubes). The same effect was observed in T95-myotubes, and depolarization induced a larger calcium transient than in the absence of extracellular calcium, but the relative increase in fluorescence remained significantly lower than in control myotubes ( $1.01 \pm 0.10$  in T95 myotubes and  $2.08 \pm 0.22$  in control myotubes –  $p < 0.0001$ , Student T test). These results suggest that i) the DHPR still functions as a permeating  $\text{Ca}^{2+}$  channel in Trisk 95 over-expressing myotubes ii) the depolarization-induced calcium release was absent in these myotubes, and only the calcium induced calcium release component is observed. Thus, over-expression of Trisk 95 abolishes only the “skeletal type” excitation-contraction coupling (the direct coupling between DHPR and RyR), and the calcium-induced calcium release is not affected. Over-expression of Trisk 51 did not induce any modification in the depolarization-induced calcium release.

## Electrophysiological analysis of DHPR in T95- myotubes and T51-myotubes

As the depolarization in absence of external calcium (skeletal type E-C coupling) was abolished when Trisk 95 was over-expressed, we wanted to check if Trisk 95-over-expression could have shifted the maximal activation potential of the DHPR. If so, then the addition of 140 mM KCl which results in a depolarization to about 0 mV may not be adapted to DHPR's optimal activation. Whole-cell patch-clamp analysis was performed on control, T95-myotubes and T51-myotubes. Depolarizing steps were delivered to the myotube membrane via the patch pipette, and the resulting DHPR calcium current was recorded. The relationship between the depolarization and the maximal intensity of the recorded current is represented in figure 5. The curve corresponding to T95-over-expressing myotubes is superimposed with the curves obtained in control myotubes and in T51-myotubes. There is no significant modification of DHPR activation in T95-overexpressing myotubes compared to control myotubes or T51-myotubes. Thus, the absence of  $\text{Ca}^{2+}$  release in T95-overexpressing myotubes upon membrane depolarization (figure 4A) is not the result of a shift in the activation potential of DHPR, with a subsequent reduced activation of DHPR upon membrane depolarization.

## Co-localization of the members of the calcium release complex

Over-expression of Trisk 95 did not modify the expression of the other members of the calcium release complex (RyR, DHPR, Trisk 51), nor modify the function of RyR and of DHPR independently. We then hypothesized that Trisk 95 over-expression might induce a dissociation of the members of the calcium release complex, and so we used immunostaining to check the co-localization of RyR, DHPR, Trisk 95 and Trisk 51. Double or triple immunofluorescent labeling was performed on T95-over-expressing myotubes, with antibodies directed against RyR, DHPR, T95 or T51 (figure 6). In T95-myotubes, DHPR, T95 and RyR presented a similar punctuated pattern (figure 6, panels a, b, c), as observed for normal myotubes [29, 30]. Most of the dots co-localized for the 3 antibodies, indicating that the co-localization was preserved in T95-myotubes. The over-expressed Trisk 95 was normally targeted to the calcium release complex. In figure 6, panels d and e, double immunofluorescent labeling was performed with antibodies against DHPR and Trisk 51 on T95-myotubes, and both proteins were still co-localized (panel f). Thus, not only is Trisk 95 correctly targeted in T95-myotubes, but also its over-expression does not disrupt the calcium release complex.

## Restoration of skeletal excitation-contraction coupling by Trisk 95-antisense virus

An antisense-Trisk 95 adenovirus (AdV-AS-T95) has been developed, to block Trisk 95 expression. The ability of this antisense virus to block the over-expression of Trisk 95 induced by infection with AdV-T95 was first checked in COS-7 cells. The presence of Trisk 95 was analyzed by Western blot either in AdV-T95 infected cells or in AdVT95 + AdV-AS-T95 infected cells. The result is presented in figure 7, panel A. Simultaneous infection of cells with AdV-AS-T95 and AdV-T95 led to a complete block of Trisk 95 expression. This indicates that AS-T95 virus has indeed the ability to block Trisk 95.

Rat skeletal muscle primary cultures were infected simultaneously with AdV-T95 and AdV-AS-T95 viruses, and calcium imaging analysis was performed. The same KCl induced-depolarization was realized on AdV-T95 + AdV-AS-T95 infected cells, in absence of external calcium, and is presented on figure 7 (panel B), with the previous experiments in gray (shadowed). This double infection in myotubes restored the depolarization induced calcium release. The relative fluorescence increase induced by KCl depolarization shifted from  $0.30 \pm 0.06$  in T95-myotubes to  $1.19 \pm 0.14$  in double infected myotubes (significantly different,  $p < 0.0001$ , Student T test). The depolarization had a similar effect in control myotubes and in T95 + AS-T95 myotubes ( $1.54 \pm 0.21$  compared to  $1.19 \pm 0.14$ , not significantly different,  $p = 0.17$ , Student T test). This indicates that the reduction in skeletal E-C coupling is specific to Trisk 95 expression.

## DISCUSSION

In order to study the respective function of Trisk 95 and Trisk 51 in the calcium release complex, Trisk 95 or Trisk 51 have been over-expressed in rat skeletal myotubes via recombinant adenoviruses. The over-expressions are specific to the targeted protein (Trisk 95 or Trisk 51), and the expression level of other proteins in the calcium release complex is unchanged (DHPR, RyR, Trisk 51 or Trisk 95). This situation, due to an acute modification of a single protein, has already been observed with adenoviral mediated over-expression of FKBP12.6 in cardiomyocytes [31] which did not induce any modification in RyR2 expression level. This contrasts with transgenic mice over-expressing single proteins of the cardiac calcium release complex, which results in a compensatory modification of the other proteins of the complex, as observed for cardiac calsequestrin over-expression [32], cardiac junctin over-expression [33], and cardiac triadin CT1 over-expression [34]. In the case of transgenic mice with overall modifications of different proteins in addition to the targeted one, the attribution of a function to a single protein is more difficult to achieve.

Trisk 95 or Trisk 51 over-expression had no effect on direct stimulation of RyR (caffeine or 4-CmC induced calcium release). Therefore RyR function was not modified by Trisk 95 or Trisk 51 over-expression, and in addition, the amount of releasable calcium was equivalent with or without T95/T51 over-expression. On the contrary, when  $\text{Ca}^{2+}$  release was induced by depolarizing the plasma membrane, control and T51-myotubes were different from Trisk 95 over-expressing myotubes. In the absence of external calcium, a situation in which only the skeletal E-C coupling was assayed, the ability of plasma membrane depolarization to induce  $\text{Ca}^{2+}$  release was largely altered in T95 over-expressing myotubes compared to control myotubes or T51 over-expressing myotubes. In the presence of external calcium, a situation in which both skeletal and cardiac E-C coupling were challenged, plasma membrane depolarization induced a smaller  $\text{Ca}^{2+}$  release in T95 myotubes than in control or T51 myotubes. Calcium induced calcium release (CICR) was still functioning in T95-myotubes, indicating that depolarization activated DHPR, induced an influx of calcium into the cell which activated RyR and resulted in internal calcium release. Nevertheless, as the skeletal E-C coupling was blocked, the resulting effect of depolarization in presence of external calcium was reduced compared to control myotubes.

Another technique, patch-clamp, was used to control the functionality of DHPR. Calcium currents via the DHPR were measured at different membrane potentials. The maximum current for each membrane depolarization (I-V curve) showed no difference between control, T95-myotubes and T51-myotubes. Thus, we concluded that neither Trisk 95 nor Trisk 51 over-expression induced any major modification in DHPR function.

As both DHPR and RyR are functional, but no longer functionally coupled, we hypothesized that T95 over-expression could have induced a dissociation of both channels, which we have previously shown to be associated in skeletal muscle [20], or that T95 over-expression could have induced a dissociation of Trisk 51 from the calcium release complex, and therefore the observed effect should be attributed to a depletion of the complex in Trisk 51. Thus double or triple immunolabeling was performed, and we observed that the over-expressed Trisk 95 was correctly targeted and remained perfectly co-localized with RyR, DHPR and Trisk 51. The amount of Trisk 95 associated with RyR is not known, but it can be increased 15 fold without dissociation of Trisk 95 from the calcium release complex.

Therefore, we imagine that both proteins RyR and DHPR are still co-localized and associated, but by steric hindrance, the overexpressed Trisk 95 should block the conformational change induced in RyR by DHPR upon membrane depolarisation.

All the triadin isoforms are issued of an alternative splicing of the same triadin gene [35], nevertheless the expression pattern of triadin is different in cardiac and skeletal muscle. Trisk 95 and Trisk 51 are mainly, if not exclusively, expressed in skeletal muscle. Therefore the

lack of effect of these two skeletal isoforms on the “cardiac part” of E-C coupling is probably to be related to the facts that they are not expressed in cardiac muscle and they act only in a skeletal environment. What is more surprising, is the major effect of Trisk 95 over-expression on skeletal E-C coupling compared to the lack of effect related to Trisk 51 over-expression. Both proteins are present in equivalent amount in rat skeletal muscle [10], both are associated with RyR, and they both have the same important RyR-interaction domains previously identified: the N-terminal cytoplasmic domain [19], and the KEKE region [36]. Nevertheless, as these two proteins do not have the same function, this may suggest that the functional regulation of RyR by triadin doesn't involve these interaction domains. The KEKE is probably necessary for the interaction RyR/triadin, but it is probably not sufficient for functional regulation. The regulation of E-C coupling via Trisk 95 should be related to a specific part of Trisk 95, and should somehow involve amino acids 455–687.

The effect of Trisk 95 over-expression on E-C coupling could be reversed by simultaneous infection with an antisense virus which blocked T95 over-expression. Thus this effect is indeed correlated to the amount of Trisk 95, and one could imagine that a similar effect could be obtained in pathological situations. Either the total amount of Trisk 95 is an important factor, or most probably the relative amount of Trisk 95 compared to RyR is the key factor of a functional calcium release complex. Indeed, we have recently observed in a pathological situation a drastic reduction of RyR expression in a patient affected with Multi-mini Core Disease [37]. In fact, reduction of RyR compared to triadin could be equivalent to increase in triadin level compared to RyR. Triadin expression levels should be tested in this patient for any modification, which could provide insight as to the physiological importance of the RyR/triadin stoichiometry.

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## Abbreviations list

CICR: Calcium induced calcium release

4-CmC: 4-chloro-m-cresol

Cy5: Indocarbocyanine 5

DHPR: dihydropyridine receptor

E-C coupling: excitation-contraction coupling

RyR: ryanodine receptor

T95: Trisk 95

T51: Trisk 51

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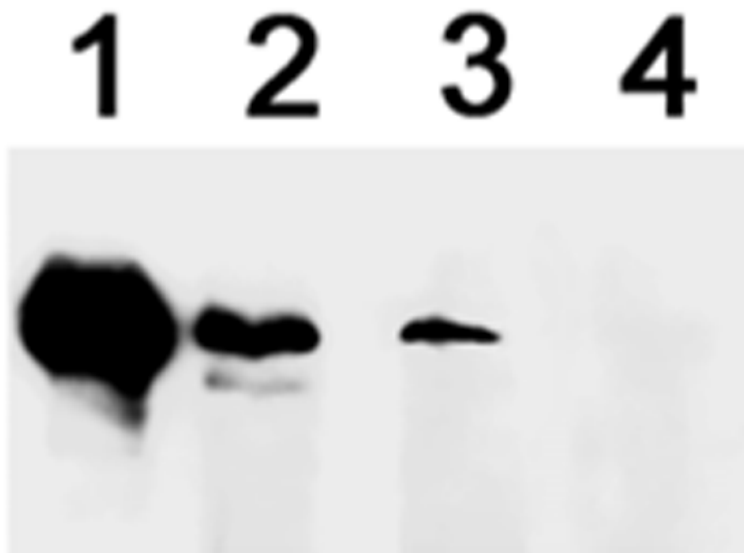


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**Figure 1**

Association of RyR with Trisk 51 and Trisk 95

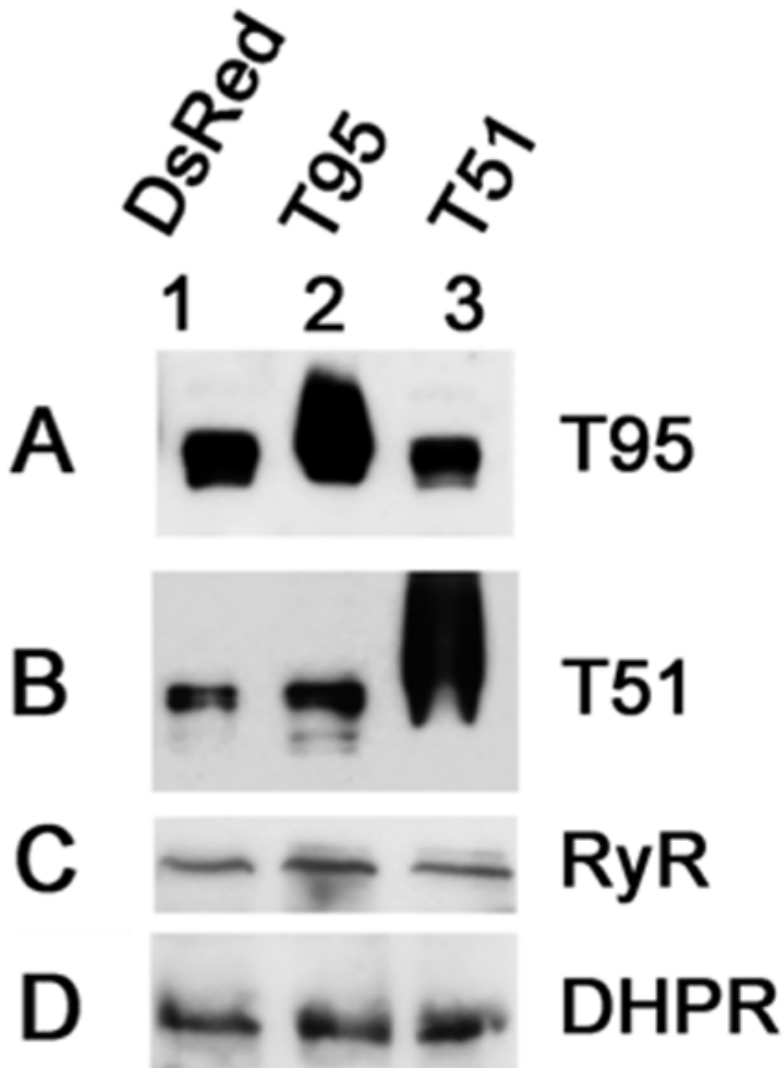
Immunoprecipitations were performed on solubilized rat skeletal muscle microsomes with anti-Trisk 51 (lane 2), anti-Trisk 95 (lane 3) or non immune serum (lane 4), and the sample were analyzed by Western blot with anti-RyR antibodies. Lane 1: 5  $\mu$ g of rat skeletal muscle microsomes.



**Figure 2**

Western blot analysis of the proteins of the calcium release complex in normal or triadins over-expressing myotubes

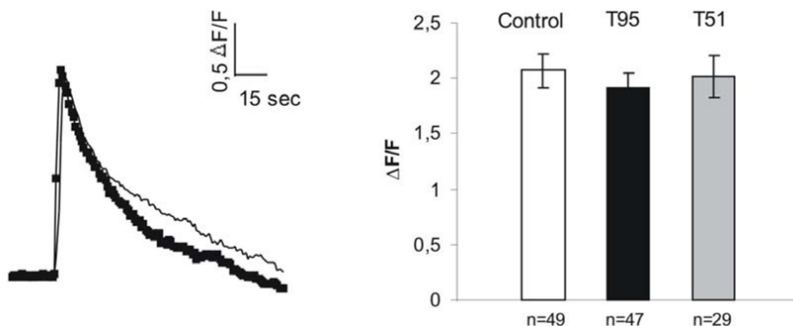
The expression level of the proteins involved in the calcium release complex was analyzed by Western blot with antibodies against Trisk 95 (panel A), Trisk 51 (panel B), RyR (panel C), DHPR (panel D). Panel A: Thirty  $\mu\text{g}$  of control and T51 myotubes were loaded in lanes 1 and 3, and 5  $\mu\text{g}$  of T95 myotubes were loaded in lane 2. Panel B: Thirty  $\mu\text{g}$  of control and T95 myotubes were loaded in lanes 1 and 2, and 2  $\mu\text{g}$  of T51 myotubes were loaded in lane 3. Panels C and D: One hundred micrograms of control myotubes (lanes 1), T95-myotubes (lanes 2) or T51-myotubes were loaded in each lane, for RyR and DHPR analysis.



**Figure 3**

Caffeine-induced calcium release on normal or triadin over-expressing myotubes

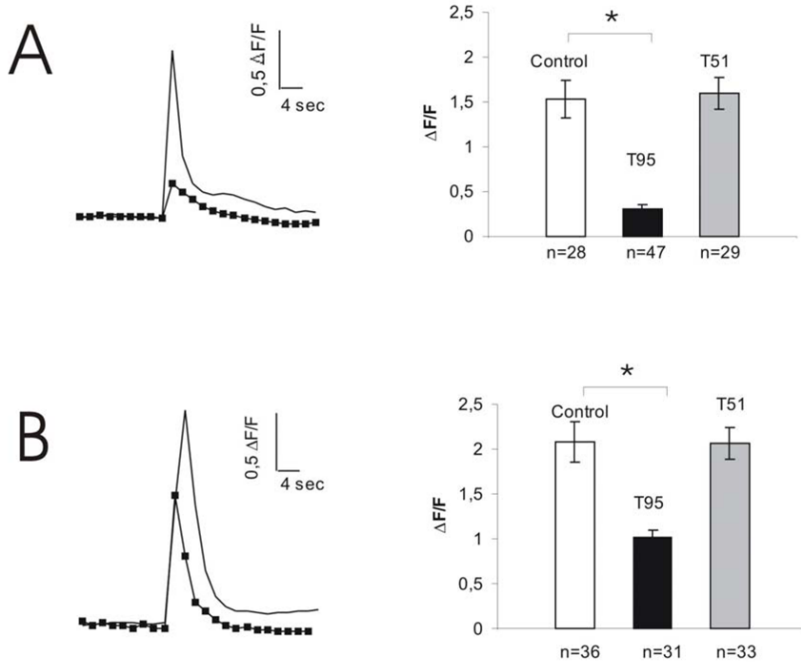
Application of caffeine (10 mM) induces a massive calcium release in the myotubes. Left panel: fluorescence variation typical curves in control myotubes (no symbol) or in T95 over-expressing myotubes (black squares). The return to basal calcium level is not significantly different. Right panel: histograms of the fluorescence variation in control myotubes ( $2.07 \pm 0.15$ ), Trisk 95-overexpressing myotubes ( $1.92 \pm 0.12$ ), Trisk 51-overexpressing myotubes ( $2.02 \pm 0.19$ ), expressed as mean  $\pm$  SEM, with n being the number of myotubes tested.



**Figure 4**

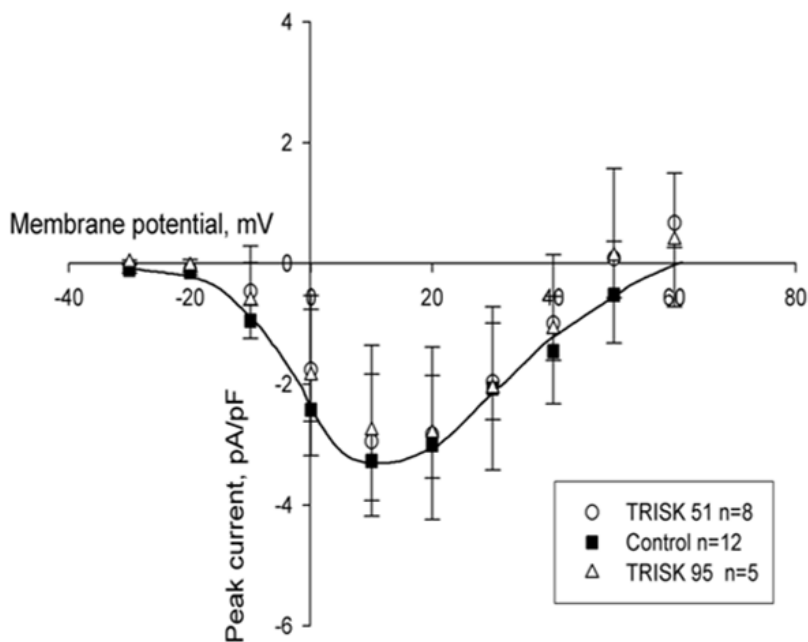
Depolarization-induced calcium release in control or triadin over-expressing myotubes

Panel A: KCl depolarization (140 mM) was induced in absence of external calcium. The left curves represent the fluorescence variation in control (no symbol) or Trisk 95 over-expressing myotubes (black squares). The histograms on the right present the mean  $\pm$  SEM of fluorescence variation in control myotubes ( $1.54 \pm 0.21$ ), in T95 myotubes ( $0.30 \pm 0.06$ ) or in T51 myotubes ( $1.6 \pm 0.18$ ), with n being the number of myotubes tested. The asterisk (\*) indicates statistically significant difference ( $p < 0.0001$ , Student T test). Panel B: the same KCl depolarization was realized in presence of 2 mM external calcium. In this condition, the mean fluorescence variation was  $2.08 \pm 0.22$  in control myotubes,  $1.01 \pm 0.10$  in T95 myotubes and  $2.06 \pm 0.18$  in T51 myotubes. The asterisk (\*) indicates statistically significant difference ( $p < 0.0001$  Student T test).

**Figure 5**

I-V relationship of DHPR in control or triadin over-expressing myotubes

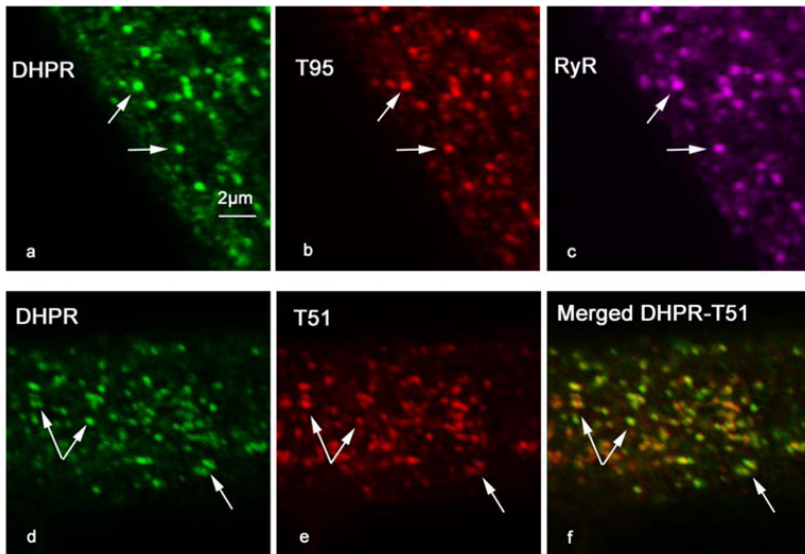
Voltage changes from an holding potential of  $-60$  mV were imposed to myotubes through the patch pipette, and the resulting calcium current was recorded. The maximum amplitude of this current was plotted as a function of membrane depolarization. Data presented are mean  $\pm$  SEM, with n being the number of myotubes tested.



**Figure 6**

Immunofluorescent labeling of Trisk 95 over-expressing myotubes

Trisk-95 over-expressing myotubes were fixed and triple immunofluorescent labeling was performed with anti-DHPR (panel a), anti Trisk 95 (panel b) and anti-RyR (panel c) antibodies, or double immunofluorescent labeling with anti-DHPR (panel d) and anti-Trisk 51 (panel e). Panels a, b and c were obtained on the same myotube, and most of the dots are co-localized (arrows on some co-localized dots). Panels d and e are from the same myotube, and most of the dots are co-localized (arrows and merged image in panel f).

**Figure 7**

Antisens-T95 virus reverses the effect of T95-virus

Panel A: Western blot analysis of COS cells infected with T95 virus alone or doubly infected with T95 and AS-T95 virus. This double infection leads to extinction of T95 over-expression. Panel B: Fluo-4 calcium imaging on doubly infected myotubes. Calcium release induced by KCl depolarization (140 mM) in absence of external calcium. Left curves represent typical fluorescence variations in control myotubes, T95 myotubes (gray squares) (same curves as figure 4B, presented in gray), and T95+AS-T95 myotubes (open circle). The histograms on the right show the means  $\pm$  SEM of fluorescence variation in control myotubes ( $1.54 \pm 0.21$ ), in T95-myotubes ( $0.30 \pm 0.06$ ) or in T95 + AST95 ( $1.19 \pm 0.14$ ). An asterisk (\*) indicates statistically significant difference ( $p < 0.0001$ , Student T test).

