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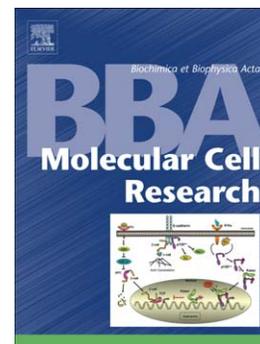
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**TRPC1 and TRPC4 channels functionally interact with STIM1L to promote myogenesis and maintain fast repetitive Ca<sup>2+</sup> release in human myotubes**

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Running title: STIM1L and TRPC interaction

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## ABSTRACT

STIM1 and Orai1 are essential players of store-operated  $\text{Ca}^{2+}$  entry (SOCE) in human skeletal muscle cells and are required for adult muscle differentiation. Besides these two proteins, TRPC (transient receptor potential canonical) channels and STIM1L (a longer STIM1 isoform) are also present on muscle cells. In the present study, we assessed the role of TRPC1, TRPC4 and STIM1L in SOCE, in the maintenance of repetitive  $\text{Ca}^{2+}$  transients and in muscle differentiation. Knockdown of TRPC1 and TRPC4 reduced SOCE by about 50% and significantly delayed the onset of  $\text{Ca}^{2+}$  entry, both effects similar to STIM1L invalidation. Upon store depletion, TRPC1 and TRPC4 appeared to interact preferentially with STIM1L compared to STIM1. STIM1L invalidation affected myoblast differentiation, with the formation of smaller myotubes, an effect similar to what we reported for TRPC1 and TRPC4 knockdown. On the contrary, the overexpression of STIM1L leads to the formation of larger myotubes. All together, these data strongly suggest that STIM1L and TRPC1/4 are working together in myotubes to ensure efficient store refilling and a proper differentiation program.

**Keywords:** skeletal muscle, STIM1L, TRPC, calcium channel, SOCE

## 1. INTRODUCTION

SOCE (Store-Operated  $\text{Ca}^{2+}$  Entry) is a ubiquitous phenomenon activated by internal  $\text{Ca}^{2+}$  store depletion. It involves the Stromal Interaction Molecule 1 (STIM1), which is a single-pass transmembrane protein that senses the ER/SR  $\text{Ca}^{2+}$  concentration [1 2 3], and a  $\text{Ca}^{2+}$  channel of the Orai family [4, 5, 6]. STIM1 has a homolog STIM2, while the Orai family comprises 3 channels, Orai1, Orai2 and Orai3. In skeletal muscle,  $\text{Ca}^{2+}$  signals are playing fundamental roles, both in mature fibers and for the proper skeletal muscle differentiation. In particular  $\text{Ca}^{2+}$  entering through the SOCE pathway was reported to be required at different stages of the differentiation process. In previous studies, we showed that STIM1 and Orai1

are the major SOCE molecules in myoblasts, and that in absence of STIM1 or Orai1, the human differentiation process was severely affected [7, 8]. In mouse models where STIM1 or Orai1 were knockdown, the animals have a reduced muscle mass and force, and are more prone to fatigue [9, 10]. In human harboring mutations in either STIM1 or Orai1, muscle growth and function are also altered in addition of the severe combined immunodeficiency, highlighting the important role of these two molecules for the development and physiology of skeletal muscle (review in [11]). In 2011, we identified a new splice variant of STIM1 that has an extra 106aa sequence in the C-terminal part of the protein [8]. We called this isoform STIM1L (long), and for clarity we will name the “classical” STIM1 isoform, STIM1S (short). STIM1L is expressed during the differentiation process and is abundantly present in human and mouse skeletal muscle. We also found this isoform in the heart and brain of mouse [8], while others detected STIM1L mRNA only in human skeletal muscle [12]. In human myotubes, STIM1S and STIM1L are expressed at a similar level, and invalidation of each isoform reduces SOCE by about 50% [8]. Interestingly, invalidation of STIM1L (while not STIM1S), significantly delayed the onset of SOCE in myotubes [8], suggesting a role of STIM1L in the fast  $\text{Ca}^{2+}$  entry observed in skeletal muscles [13, 8]. Overexpression of STIM1L in cell lines such as MEF (mouse embryonic fibroblasts) or HEK (human embryonic kidney) cells also induced a robust Orai1-dependent SOCE [14, 12]. Interestingly, Horinouchi reported in addition an interaction between STIM1L and TRPC3 and TRPC6 in HEK cells [12].

TRPC belong to the large family of TRP (Transient Receptor Potential) channels, comprised seven members (TRPC1-7) that are all activated downstream of the PLC/IP<sub>3</sub>/DAG pathway, and are non-selective cation channels with moderate  $\text{Ca}^{2+}$  selectivity. Furthermore, it was reported that STIM1S directly or indirectly interacts with TRPC (all except TRPC7) conferring them a SOCE function [15, 16, 17]; review in [18]). In human and mouse skeletal

muscle TRPC1 and TRPC3-6 are expressed [19]. The most studied TRPC1 is an important regulator of myogenesis [20, 21, 22] and recent paper showed that this channel is strongly implicated in muscle regrowth after immobilization [23]. We also recently demonstrated that TRPC1 and TRPC4 expression is transiently increased during human myogenesis and that these channels participated in SOCE of myoblasts [24]. Silencing TRPC1 or TRPC4 led to the formation of smaller myotubes, pointing to a role during post-natal human muscle differentiation [24].

In the present study, we investigated in human myotubes the role of TRPC1, TRPC4 and STIM1L in SOCE. We showed that all three proteins are part of SOCE, that they appear to work together and to contribute to the fast activation of skeletal muscle SOCE associated with sustained  $[Ca^{2+}]_i$  transients during repetitive membrane depolarization. In addition, these molecular players of SOCE are also required for the formation of normal sized myotubes.

## 2. MATERIALS and METHODS

### 2.1 Materials

Thapsigargin was obtained from Sigma. Acetoxymethyl ester form of Fura-2 (Fura-2/AM) and Pluronic F-127 were from Life Technologies (Carlsbad, CA). YFP-STIM1 was a gift from Dr. A. B. Parekh (University of Oxford, United Kingdom). YFP-STIM1L was constructed as previously described [8]. Ha-hTRPC4<sup>EE647-648KK</sup> [17] and Ha-hTRPC1<sup>F562A</sup> [25] were from the laboratory of Prof. S. Muallem.

### 2.2 Cell culture and transfection

Muscle samples, cell dissociation, and clonal culture from satellite cells were prepared as previously described [26; 27]. Human muscle samples were obtained from children (operated

for clubfoot and less than 4 years old) without any known neuromuscular disease. All work on human subjects was carried out in accordance with the Declaration of Helsinki. Human samples were obtained with informed consent, as approved by the University Hospital of Geneva Research Committee on the use of humans as experimental subjects (Protocol 05-078). The differentiation was induced by switching from a serum-rich medium (GM, growth medium) to a serum-low medium (DM, differentiation medium). Myoblasts were transfected in suspension by incubating  $4 \times 10^5$  cells in a solution containing 500  $\mu$ l of Opti-MEM, 3  $\mu$ l of Lipofectamine RNAiMax (Invitrogen), and 100 nM of a specific siRNA (Invitrogen) according to manufacturer protocols (Invitrogen). The transfection efficiency assessed by Block-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen) measurements was  $\sim 90\%$ . Myoblasts were incubated with siRNA for 3 h in growth medium and then changed to differentiation medium. Around 60 h afterward, multinucleated myotubes were formed, and  $\text{Ca}^{2+}$  measurements were performed.

Stim1<sup>-/-</sup>/Stim2<sup>-/-</sup> MEFs (DKO cells) generated by targeted gene disruption [28] were a kind gift from Dr Masatsugu Oh-Hora (Tokyo Medical and Dental University, Tokyo, Japan). The cells were maintained at 37°C in 5% CO<sub>2</sub> in Dulbecco's modified eagle medium (22320-022, Life Technologies), 10% FCS, 5 mg/ml streptomycin and 5 units/ml penicillin. DKO cells were seeded on 25-mm diameter glass coverslips and transfected at 50% confluence with Lipofectamine 2000 (Life Technologies) by adding 2  $\mu$ g of plasmid/coverslip.

Sequences of the different siRNA are as followed (sense strand siRNA): siSTIM1L 5'-GCCGGGUAUCUCUGCGGCGtt-3' (Ambion); 5' -siTRPC1 CGGACUUCUAAAUAUGCUAtt-3' (Qiagen) and 5'-siTRPC4 UGUCUAUGUUGGAGAUGCUCUAUUA-3' (Invitrogen). The siRNA AllStar from Qiagen was used as a negative control.

### ***2.3 Cytosolic calcium measurements***

For Ca<sup>2+</sup> imaging, myotubes cells were plated on 30 mm glass coverslips. The changes in cytosolic Ca<sup>2+</sup> concentration were measured with Fura-2. Cells were loaded with 2 μM Fura-2/AM and 1 μM pluronic acid for 30 min in the dark at room temperature in a medium containing (in mM): 135 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 Hepes, 10 glucose, pH adjusted at 7.45 with NaOH. Myotubes were washed twice and equilibrated for 10–15 min in the same buffer to allow de-esterification. Ratiometric images of Ca<sup>2+</sup> signals were obtained using a microscope (Axio Observer, Zeiss) equipped with a Lambda DG4 illumination system (Sutter Instrument Company, Novato, CA, USA), which rapidly changed the excitation wavelengths between 340 nm (340AF15; Omega Optical) and 380 nm (380AF15; Omega Optical). Emission was collected through a 415DCLP dichroic mirror, and a 510WB40 filter (Omega Optical), by a cooled, 12-bit CCD camera (CoolSnap HQ, Roper Scientific, Trenton, NJ, USA). For Mn<sup>2+</sup> quench experiments, cells were excited at 360 nm (360BP10; Omega optical). Image acquisition and analysis were performed with the Metafluor 6.3 software (Universal Imaging, West Chester, PA, USA).

### ***2.4 Western blots***

Western blots were performed as previously described [29]. Briefly, myotubes were lysed using modified NP40 cell lysis buffer (Invitrogen), 50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub> and 1% Nonidet P40. Total proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated in T-TBS (0.1% Tween 20, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl) and 5% nonfat milk. Blots were incubated with primary antibodies diluted in T-TBS and nonfat milk as follows: mouse anti-TRPC1 polyclonal antibody (1:500, Santa Cruz Biotechnology), rabbit anti-TRPC4 polyclonal antibody (1:200, Alomone), STIM1, Orail (1:1000, Sigma Aldrich), and mouse

monoclonal antibody against  $\alpha$ -tubulin (clone DM1A, Sigma) 1:10,000. Blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse diluted 1:10,000 (BioRad) or with HRP-conjugated goat anti-rabbit diluted 1:10,000 (BioRad), respectively. Antibodies were revealed using ECL reagents and hyperfilm ECL (Amersham Biosciences). Image-J Software was used to quantify the level of protein expression.

### ***2.5 Co-Immunoprecipitation experiments***

Experiments were done as previously described [30]. Briefly, after lysis, 2 $\mu$ g of antibody was added to a volume of lysates containing 300 $\mu$ g of myotube proteins diluted with 600  $\mu$ l of NET solubilization buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 5mM EDTA, 0.05% Nonidet P-40 (v/v)) and incubated at 4°C overnight with constant mixing. Then, the protein-antibody complex was incubated for 1 h at 4°C with constant mixing with 40  $\mu$ l of Protein A-Sepharose (PAS) (Amersham Biosciences). The immune complexes were collected by centrifugation and washed three times in NET. After denaturing, samples were subjected to SDS-PAGE. The negative control was performed using lysates with beads (PAS) without any antibody.

### ***2.6 Statistics***

All analysis was performed with GraphPad Prism 6 software. All values are reported as mean  $\pm$  S.E.M. For all experiments, the difference between two groups was assessed with two-tailed unpaired Student's *t* test and among at least three groups was assessed with one-way or two-way analysis of variance completed by Fisher's least significant difference post hoc test for multiple comparisons. The statistical significance was defined by a value of  $p \leq 0.05$ .

### 3 RESULTS

#### *3.1 TRPC1 and TRPC4 channels contribute to Store-Operated Ca<sup>2+</sup> Entry (SOCE) in human myotubes.*

To investigate the role of TRPC1 and TRPC4 channels in SOCE in human myotubes, we reduced the expression of these channels by siRNA and evaluated maximum SOCE. This was achieved by the application of 1  $\mu$ M Tg (thapsigargin, an irreversible SERCA pump inhibitor) in Ca<sup>2+</sup>-free solution to deplete the internal Ca<sup>2+</sup> stores, followed by the re-addition of 2 mM external Ca<sup>2+</sup> to elicit Ca<sup>2+</sup> entry. The response to Tg, evaluated as the area under the curve, was slightly decreased upon siTRPC1, and significantly reduced after siTRPC4, suggesting a lower SR Ca<sup>2+</sup> content (data not shown). The quantification of SOCE was done by measuring the maximal Ca<sup>2+</sup> elevation after Ca<sup>2+</sup> re-addition, and revealed a 50% SOCE reduction after TRPC1 or TRPC4 silencing (Figure 1A-B). A similar reduction was obtained after STIM1L silencing (Figure 1A-B and as shown in [8]). The siRNA against TRPC1 or TRPC4 channels reduced TRPC1 and TRPC4 expression by 50% and 60%, respectively (Fig 1C-D). The siRNA against STIM1L reduced the expression of STIM1L by about 90% without affecting the expression level of the other isoform, STIM1S (Figure 1E). We also verified that TRPC1 or TRPC4 invalidation did not impact on the expression of STIM1S or STIM1L (Supplemental Figure 1).

In physiological conditions, SOCE is activated following store depletion consecutive of excitation-contraction coupling. To assess the involvement of TRPC1 and TRPC4 channels in this process, we stimulated myotubes by repetitive applications of 65 mM KCl pushes to induce membrane depolarizations. In control conditions, myotubes exhibited similar cytosolic Ca<sup>2+</sup> elevations, after an initial slight decline of the amplitude (Figure 2A), as previously reported [31, 8]. Upon silencing of TRPC1 and TRPC4, the first Ca<sup>2+</sup> transient was reduced after KCl stimulation, in line with the reduced SR Ca<sup>2+</sup> content. Moreover, TRPC1 and

TRPC4-silenced myotubes did not sustain  $[Ca^{2+}]_i$  transients, with a decrease by 42% and 25% of the amplitude, respectively (Figure 2B-D). Interestingly, these reductions are very similar to those observed after STIM1L silencing [8], and suggested thus a similar phenotype after either TRPC1/4 or STIM1L invalidation. Furthermore, these data support the idea that TRPC-dependent SOCE is important to maintain  $[Ca^{2+}]_i$  transients in human myotubes.

### ***3.2 TRPC1 and TRPC4 channels interact with STIM1L after $Ca^{2+}$ store depletion and participate to the fast $Ca^{2+}$ entry.***

It is well known that SOCE in skeletal muscle is significantly faster than SOCE in other cell types [13], and we showed that this property is partially due to the presence of STIM1L [8]. We thus investigated the effect of siTRPCs on the kinetic of SOCE activation, using  $Mn^{2+}$  quench assay.  $Mn^{2+}$  entering in cell quenches the Fura-2 fluorescence, and we can measure the delay between store depletion and the fura-2 quench, which reflects the kinetic of SOCE activation after Tg addition. We first confirmed that the knockdown of STIM1L in human myotubes delays activation of  $Ca^{2+}$  influx by about 50 seconds compared to siControl transfected myotubes (10 sec). Likewise, invalidation of either TRPC1 or TRPC4 delayed the onset of  $Mn^{2+}$  quench, although TRPC4 silencing had a weaker effect than TRPC1 or STIM1L invalidation (Figure 3A-B). These results suggested that TRPC1 and TRPC4 are involved, together with STIM1L, in the fast  $Ca^{2+}$  entry.

Our data suggest that STIM1L is functionally coupled to TRPC1 and TRPC4 in myotubes, and recently Horinouchi et al showed, using overexpression system, a stronger interaction between STIM1L and TRPC3 or TRPC6, than between STIM1S and TRPCs [12]. Hence, to determine whether STIM1L and TRPC1/4 physically interact with each other, we performed co-immunoprecipitation (Co-IP) experiments on native myotubes. No interaction was

observed before store depletion, while after 10 minutes of 1  $\mu$ M Tg treatment, STIM1L interacted with TRPC1 and TRPC4, an interaction that appeared much stronger between TRPCs and STIM1L than between TRPCs and STIM1S (Fig. 3C). No interaction was observed between TRPC1 and TRPC4 in absence or presence of Tg (data not shown).

We then used the MEF (mouse embryonic fibroblasts) cells ablated for *stim1* and *stim2* genes (MEF DKO cells), to investigate whether the ability of STIM1L to open TRPCs is cell type dependent. MEF DKO cells have a clean genetic background that excludes STIM1S and STIM2 as possible interference players. Using the classical  $\text{Ca}^{2+}$  re-addition protocol after Tg stimulation, we confirmed that STIM1L overexpression in MEF DKO cells induced a robust SOCE, which is even more potent than SOCE elicited in STIM1S overexpressing DKO cells (Supplemental Fig 2A-B; [14]). To assess the role of TRPCs in SOCE, we overexpressed dominant-negative mutants for TRPC1 or TRPC4 channels (DN-TRPC1 and DN-TRPC4, respectively). Interestingly, in STIM1L overexpressing MEF DKO cells, SOCE was significantly reduced by 45% in presence of DN-TRPC1 or DN-TRPC4 (Supplemental Fig. 2C-D), while DN-TRPC1 or DN-TRPC4 co-expression had no consequence on SOCE-induced by STIM1S overexpression (Supplemental Fig. 2E-F). These data confirmed the involvement of TRPC1 and TRPC4 in STIM1L-dependent SOCE.

### **3.3 STIM1L participates in myotube differentiation**

We previously showed that the knockdown of TRPC1 or TRPC4 had a negative impact on myoblast differentiation, leading to the formation of smaller myotubes compared to control conditions [24]. As we reported in the present study a privileged interaction between STIM1L and TRPC channels in myotubes, we decided to investigate the effect of siSTIM1L on myotube differentiation. We first confirmed the results of Darbellay et al [8] that the expression of STIM1L increases during the differentiation process (Figure 4A-B).

Simultaneously, there was a slight reduction of STIM1S expression after 48 hours of differentiation. Then, to assess its role during differentiation, we transfected myoblasts with siSTIM1L and, after two days, we initiated differentiation and let the cells two additional days in differentiation medium. Silencing STIM1L did not significantly impair the percentage of MEF2 positive nuclei (assessed by immunostaining), an early marker of myogenesis (Figure 4C-D). However, the size of the myotubes was reduced, as shown by the decreased number of nuclei per myotubes (Fig. 4C, E). To note, the MEF2 antibody used for immunostaining experiments is not specific for a MEF2 isoform (MEF2A-D). As reported in previous studies, MEF2C expression increases during human myoblast differentiation [32, 33, 34]. We thus analyzed specifically the expression of the MEF2C isoform by Western blot. As shown in Figure 4F, MEF2C expression was significantly reduced by about 50% in STIM1L deficient condition. Overall, the impact of STIM1L silencing is comparable to the one of TRPCs silencing [24] which is in favor of these three molecules being part of a common signaling pathway.

Since the differentiation was impaired in STIM1L deficient condition, we further evaluated the impact of STIM1L overexpression on the differentiation process. Figure 4G-H shows that, in STIM1L overexpressing cells, the number of nuclei per myotube significantly increased compared to pcDNA3 overexpressing condition. These data confirm the importance of STIM1L-dependent SOCE in the differentiation process.

#### 4. DISCUSSION

In the present study, we show that TRPC1 and TRPC4 are involved in human myotube SOCE. These two channels appear to work together with STIM1L, a splice variant of STIM1 that is abundantly expressed in skeletal muscles. Invalidation of either STIM1L or TRPC1/TRPC4 gave rise to a similar outcome/phenotype in terms of a decreased SOCE

amplitude and delayed SOCE onset, muscle differentiation, as well as the inability of myotubes to sustain  $[Ca^{2+}]_i$  transients in response to repetitive membrane depolarizations.

In a previous study, we showed that TRPC1 and TRPC4 are important during myoblast differentiation to allow the formation of normal size myotubes, as in the absence of these channels, the formed myotubes are smaller in size, a defect associated with a reduced SOCE in myoblasts [24]. Using our *in vitro* model of human myotube differentiation, we wanted to assess the involvement of TRPCs in myotube SOCE as well as the role of STIM1L in differentiated myotubes. Indeed, STIM1L is weakly expressed in myoblasts but is present to a similar extent as STIM1S in myotubes. So far very little is known about STIM1L and its putative partners in myotubes. We have shown, using either myoblasts or MEF DKO cells, that overexpression of Orai1 together with STIM1L significantly enhanced SOCE, compared to STIM1L alone, strongly suggesting that STIM1L gates Orai1 [14]. In our previous paper, we reported that STIM1S and STIM1L participate to SOCE, each accounting for about 50% of the  $Ca^{2+}$  entry in myotubes. Furthermore, we showed that removing STIM1L but not STIM1S significantly delayed the onset of  $Ca^{2+}$  entry upon store depletion [8]. Indeed, a particular feature of SOCE in skeletal muscle is its rapid activation kinetic compared to most cells where SOCE is playing an important role [13, 35, 36]. The precise requirement for this fast  $Ca^{2+}$  entry is not known, but STIM1L localization (under the PM even before store depletion), had been proposed to play an important role in this process [8]. It is important to note that in non-muscle tissues, the overexpression of STIM1L does not accelerate the onset of SOCE [14], suggesting that other molecules, probably only expressed in muscle tissue, are determinant for the fast SOCE in skeletal muscles. Interestingly, silencing either TRPC1 or TRPC4 in myotubes also led to a delayed  $Ca^{2+}$  entry, even if the effect was not as pronounced as after STIM1L silencing.

Physiologically,  $\text{Ca}^{2+}$  signals in skeletal muscle mainly result from membrane depolarization coupled to RyR1 opening. The consequent decrease in SR  $\text{Ca}^{2+}$  content activates SOCE to replenish the SR. In the present paper we showed that both siTRPC1 and siTRPC4 impaired sustained  $[\text{Ca}^{2+}]_i$  transients after repetitive KCl-induced membrane depolarization. Interestingly, the same result was obtained with siRNA against STIM1L, but not against STIM1S [8]. This further suggests that STIM1L and TRPC1/4 are part of a same  $\text{Ca}^{2+}$  entry pathway. For a long time, it was assumed that  $\text{Ca}^{2+}$  entry was of minimal importance for skeletal muscle contraction, as contraction can occur in  $\text{Ca}^{2+}$  free medium at least for some time (review in [37]). Now, and in particular after the discovery of the molecules implicated in SOCE, STIM and Orai, it is clear that the lack of SOCE leads to altered muscle differentiation, as well as defective function of adult tissue. It is thus of importance to determine which molecules besides STIM and Orai participate to SOCE in skeletal muscle. Historically, TRPC channels were the first to be proposed as SOCE channels in different cellular systems [38]. TRPC expression was assessed in 2002 by the group of Gailly on mouse skeletal muscle [39], and most TRPCs except TRPC2 and 7, were further detected on muscle (rev [19]). Their role however is far from being fully elucidated. Most papers dealing with TRPC in skeletal muscle focused on TRPC1. Dysregulation of TRPC1 was reported to be involved in myopathies [39; 40], or to be important for muscle regrowth after disuse [23]. TRPC1 was also reported to be part of SOCE in myotubes [41; 42], although Berbey found an intracellular localization of TRPC1, pointing to a possible role as  $\text{Ca}^{2+}$  release channel [43]. Furthermore, TRPC1<sup>-/-</sup> mice developed muscle fatigue but without alteration of SOCE [44]. The same group reported a role for TRPC1 in SOCE myoblasts and its importance during myoblast migration and differentiation [20]. Hence, and as proposed by Gailly, it could be that TRPC1 functions as a store-operated channel in myoblasts and is differently activated in myotubes and/or adult fibers. Our present data point to a role of TRPC1 as SOCE channels

while also being involved in sustained  $[Ca^{2+}]_i$  transients in human myotubes, which could be linked to its involvement in preventing muscle fatigue. This however would need to be confirmed in adult human muscle fibers.

Few papers investigated the role of TRPC4 channel in skeletal muscle. One study reported that it forms heteromers with TRPC1 and the complex function as store-operated channels [41], while another paper could not detect TRPC4 expression in microsomal membrane protein fractions prepared from adult mice skeletal muscle [45]. On the other hand, Harisseh et al showed a sarcolemmal localization of TRPC1 and TRPC4 in human control and Duchenne muscular dystrophy myotubes [46]. Hence, it could be that the expression level of TRPC4 decreases during the time course of differentiation, and/or that human and mice do not express the same TRPC channels. Our data are in favor of TRPC1 and TRPC4 functioning as SOCE channels together with STIM1L, while we did not detect heteromers between both channels (data not shown). On the contrary, in several cell types like endothelial and mesangial cells, rat cardiac cells and mouse myotubes, it was reported that TRPC1 and TRPC4 form heteromers [47]; [48]; [41]; [49]. What we observe is a physical interaction between STIM1L and TRPC1 or TRPC4, and a functional interaction between these molecules. This leads us to postulate that the new STIM1L isoform, abundantly expressed in skeletal muscle, is critical for TRPC1/4 activation, while in other cellular systems including myoblasts, STIM1S is carrying this function. We do not know yet whether STIM1L directly gate these channels or is indirectly involved in their activation.

## 5. CONCLUSIONS

Our data highlighted the essential role of STIM1L during human myogenesis whose function is likely due to the activation of  $Ca^{2+}$  influx partially mediated by TRPC1 and TRPC4 channels. Undoubtedly, the key SOCE player Orai1 is also pivotal for human myogenesis, as

already reported [7]. Hence, in addition to the STIM1S/Orai1-induced  $\text{Ca}^{2+}$  entry, our results strengthen the hypothesis that the three molecules, STIM1L, TRPC1 and TRPC4 are working together to allow  $\text{Ca}^{2+}$  entry that is required for muscle differentiation, but also for the fast SOCE that is likely required to sustain repetitive  $[\text{Ca}^{2+}]_i$  transients, and also to prevent muscle fatigue. Whether this molecular assembly holds true in adult human skeletal muscle remains to be determined.

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### **Figure legends**

**Figure 1: TRPC1 and TRPC4 contribute to SOCE in human myotubes.** A) Myotubes were loaded with 2  $\mu\text{M}$  Fura-2 and imaged to monitor cytosolic  $\text{Ca}^{2+}$  changes. Cells were stimulated with 1  $\mu\text{M}$  thapsigargin to passively deplete the stores, followed by the addition of 2 mM  $\text{Ca}^{2+}$ . Only the re-addition phase is presented. Cells were transiently transfected with control siRNA, or siRNA against TRPC, TRPC4 and STIM1L.  $\text{Ca}^{2+}$  changes were evaluated 4h after transfection. Each trace represents the mean of a representative coverslip. B)

Quantification of the effect of the different siRNA on SOCE amplitude (n = 3-10 different clones). C-E) Myotubes were transfected with siRNA against TRPC1 (C), TRPC4 (D) or STIM1L (E), and the knockdown efficiency was assessed by Western blots,  $\alpha$ -Tubulin was used as loading control. The quantification of the blots is shown for each condition (three different clones for each).

**Figure 2: Repetitive  $[Ca^{2+}]_i$  transients require TRPC1 and TRPC4 function.** Cytoplasmic  $Ca^{2+}$  responses generated by successive KCl (65 mM) applications were assessed using Fura-2, 60h after siControl (A) siTRPC1 (B) and siTRPC4 (C) transfection and differentiation induction. Measures were performed on large multinucleated myotubes. siAllstar siRNA was used as control. D) Amplitude of the second KCl-induced  $Ca^{2+}$  peak divided by the amplitude of the tenth KCl-induced  $Ca^{2+}$  peak. (n = 4-5 different experiments).

**Figure 3: Kinetics of SOCE activation following store depletion.** Representative  $Mn^{2+}$  quench recordings (A) and quantification (B) of myotubes transfected with siControl, siSTIM1L, siTRPC1 and siTRPC4 (n=3-9 different clones). Cells were exposed to 500  $\mu$ M  $Mn^{2+}$  prior the addition of 1  $\mu$ M Tg, and the quench of fura-2 fluorescence was measured at 360 nm excitation wavelength. C) Left panel: Representative co-immunoprecipitation experiments in human myotubes. Lysates (input lane) from human myotubes treated or not with 1 $\mu$ M Tg for 10 min were incubated with antibodies against TRPC1, TRPC4 and STIM1 (IP). Western blots of the immunoprecipitated proteins were probed with antibodies against STIM1. Right panel: quantification after Tg treatment, of the ratio between STIM1L and STIM1S after immunoprecipitation with TRPC1 and TRPC4 antibodies. The input corresponds to the control (n=3-4 separated experiments).

**Figure 4: STIM1L contributes to the regulation of human myoblast differentiation.** A) Western blot analysis of STIM1L expression during the first 48 h after initiation of differentiation.  $\alpha$ -Tubulin was used as a loading control. B) Quantification of A (4-5 different clones). C) Human myoblasts were transfected with siSTIM1L, kept for 2 days in growth medium (GM), and 2 more days in differentiation medium (DM). MEF2 and myotubes were stained using an antibody against MEF2 (red) and myosin heavy chain (MF20 antibody, green), and nuclei in blue (DAPI). Scale bars: 20  $\mu$ m. (D, E) Quantification of MEF2 positive nuclei (D) and the number of nuclei per myotubes (E) after siControl and siSTIM1L treatment (n=4 clones for each condition). F) Western blot analysis of MEF2C expression after 2 days of differentiation in control and siSTIM1L condition.  $\alpha$ -Tubulin was used as a loading control (left panel). Quantification of the blot is shown on the right panel (n= 3 different clones). G) STIM1L overexpression increased the size of human myotubes. Myoblasts were transfected with pcDNA3 or STIM1L, kept for 2 days in proliferation medium, and transferred into differentiation medium. Myosin heavy chain (MF20) expression is stained in green. Scale bars: 20  $\mu$ m. H) Number of nuclei per myotube in the same conditions as in G (n=3 different clones in each condition). The number of nuclei per myotube obtained in control condition was normalized to 1.

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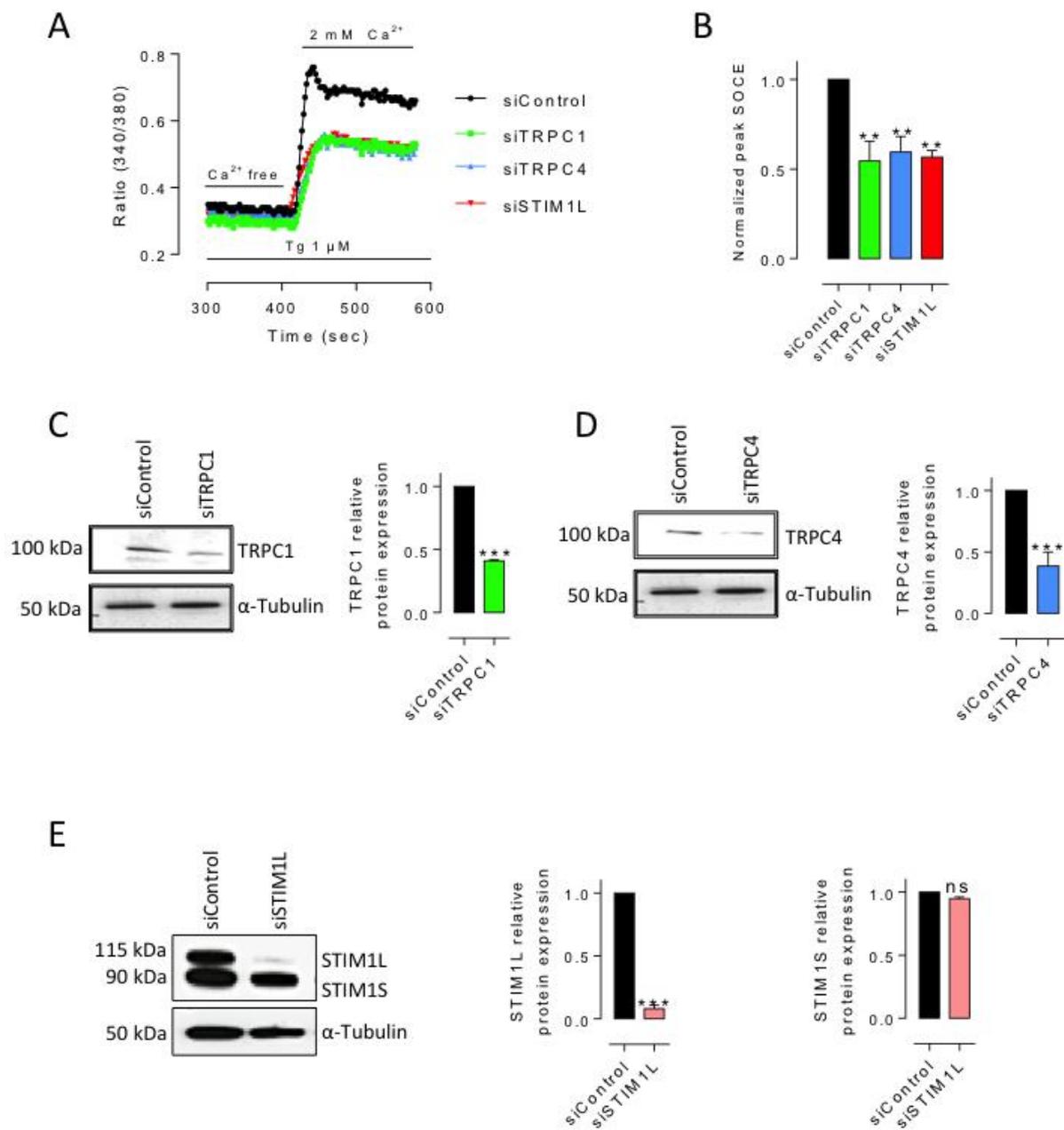


Figure 1

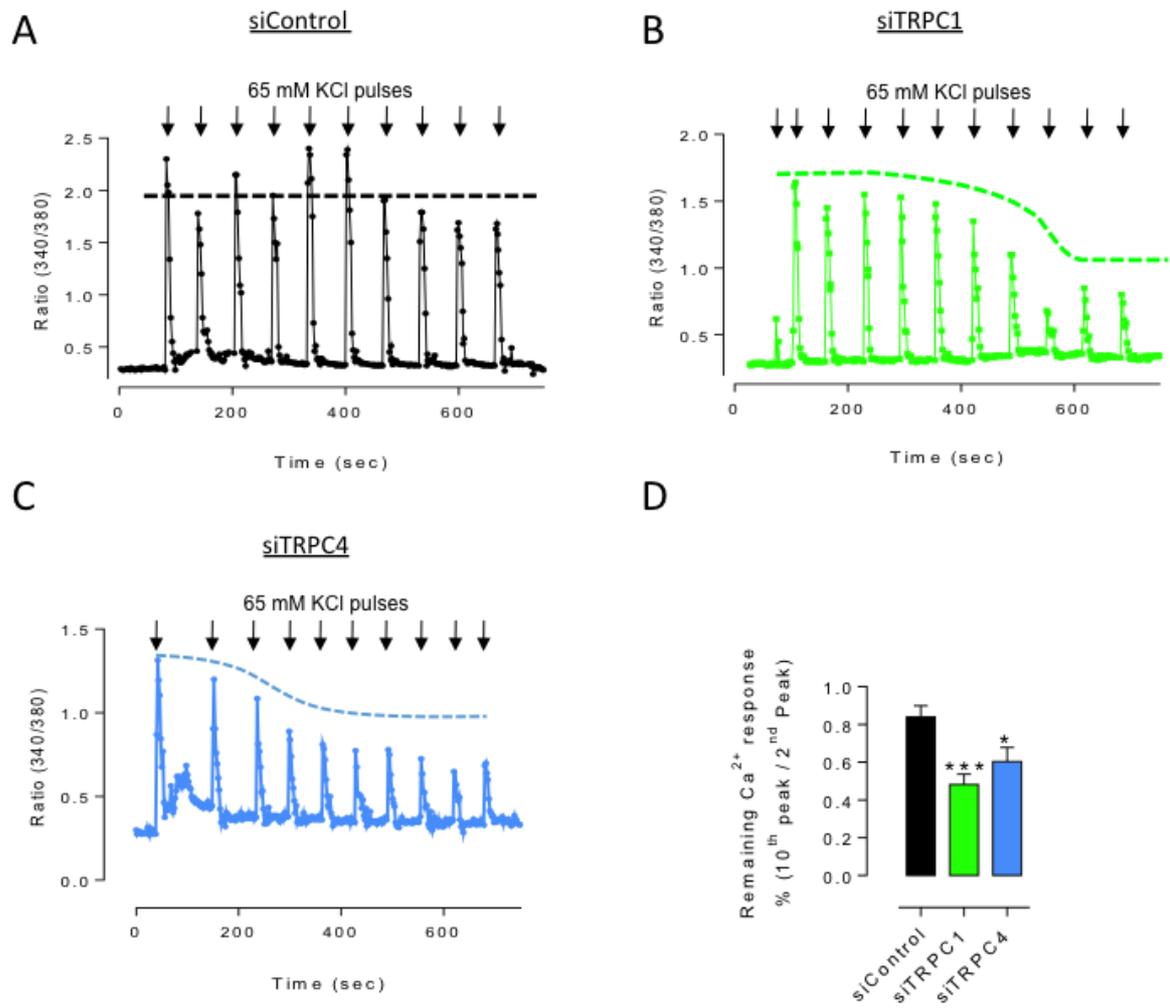


Figure 2

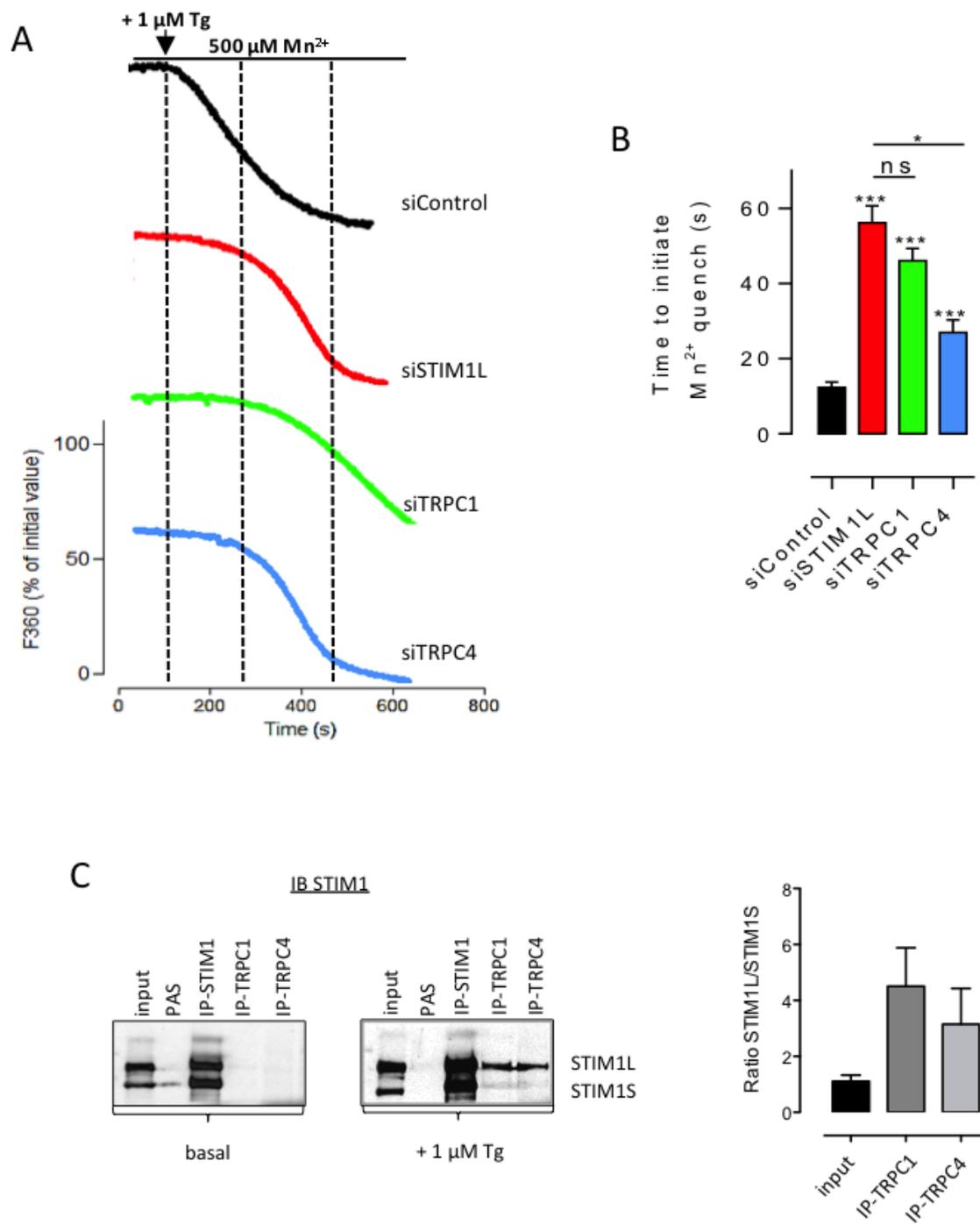
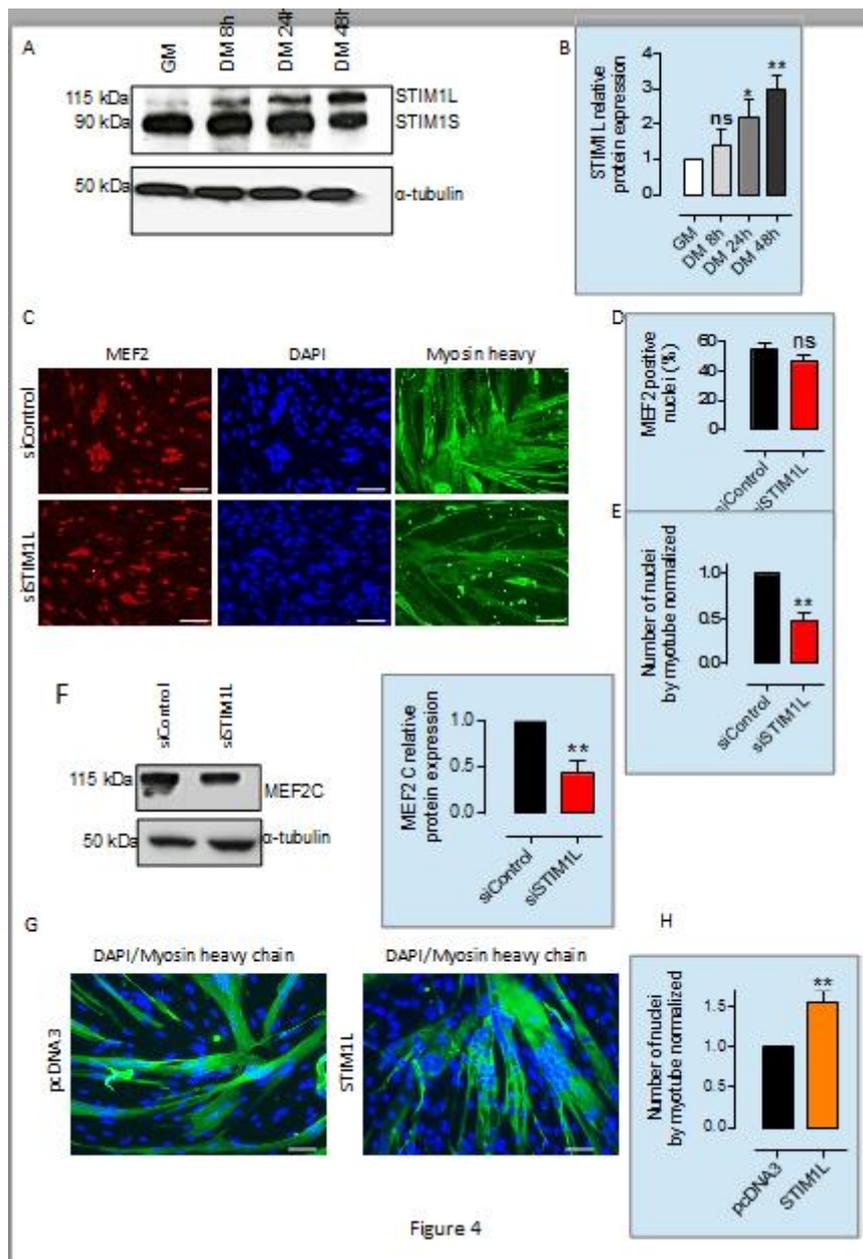
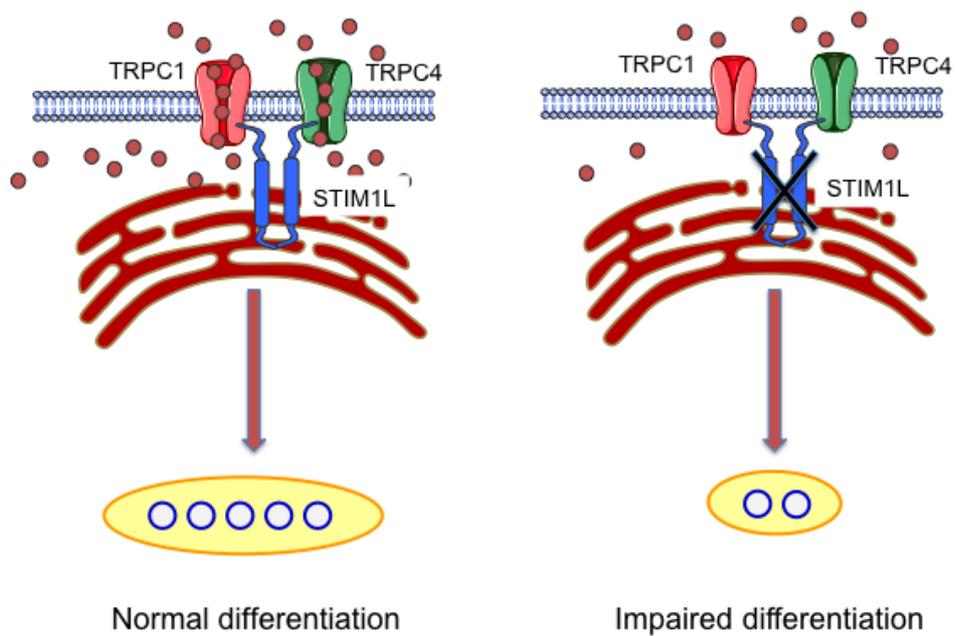


Figure 3





Graphical abstract

ACCEP

### Highlights

This study investigates the role of TRPC and STIM1L in human myotubes

TRPC1 and TRPC4 channels are part of store-operated  $\text{Ca}^{2+}$  entry in human myotubes

TRPC1 and TRPC4 channels functionally interact with STIM1L

STIM1L and TRPC1/4 are necessary to sustain repetitive  $\text{Ca}^{2+}$  transients

STIM1L and TRPC1/4 are required for a proper skeletal muscle differentiation

ACCEPTED MANUSCRIPT