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## Role of Serum response factor in cell stiffness and muscle cell fusion

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### ABSTRACT

Repair of muscle tissue and growth of multinucleated myofibers depend on adult muscle stem cells (muSCs) and their ability to fuse. In this study, we took advantage of two genetic models of fusion-defective muSCs (both deleted for Serum response factor (Srf) (Mut), and one overexpressing alpha-actin (Mut/Act+)) to decipher pathways and cellular properties that can restore fusion and gain insight into the mechanisms of mammalian muscle cell fusion. We first showed that Srf is involved in the fusion process after membrane mixing (hemifusion). Furthermore, Mut and Mut/Act+ muSCs were less rigid than control cells, and increasing apparent cell stiffness, presumably by increasing membrane and cortical tension, by hypotonic osmotic shock partially restored their fusion capacity, with a better efficacy when actin was overexpressed. These findings identify membrane and cortical tension/cell stiffness as a potential new player in myoblast fusion that may control Srf-dependent fusion in concert with the formation of actin structures at the fusion site.

**Category of the Short Note:** original study including positive and negative results

**Experimental approach:** *in vitro*

**Specie:** mouse

### KEYWORDS

Skeletal muscle, cell fusion, cell stiffness, Serum response factor



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## INTRODUCTION

Skeletal muscle tissue contains multinucleated post-mitotic myofibers and a small resident population of muscle stem cells (muSCs) that are maintained in a quiescent state in undamaged muscle. Upon injury or increased load, muSCs are activated and fuse to form new regenerated myofibers, or to supplement growing muscle fibers with new myonuclei. Thus, muSCs fusion is an essential step in both regeneration and hypertrophy, and is essential for the adaptability of adult muscle.

Fusion is a multi-step process that begins with membrane apposition, followed by lipid mixing of the membranes (hemifusion), pore formation, and pore expansion with the formation of actin structures to support this process (Wherley et al., 2024). By combining live imaging and metal-replica electron microscopy (EM) on unroofed muscle cells, our laboratory provided the first description of one of the initial steps of the fusion process in primary vertebrate myoblasts, involving asymmetrically distributed actin-based finger-like protrusions at the site of cell-cell fusion (Randrianarison-Huetz et al., 2018).

Srf (Serum response factor) is a ubiquitously expressed transcription factor whose target genes, including *Actin*, regulate the organisation of actin cytoskeleton and are involved in cell growth and migration (Pipes et al., 2006). Using an inducible and conditional deletion mouse model of *Srf* in muSCs, we have shown that Srf is not essential for muSC proliferation and differentiation, but is a key regulator of myoblast fusion required for both fusion partners (Randrianarison-Huetz et al., 2018).

One means by which Srf controls fusion is through the maintenance of the actin scaffold and the formation of actin-based finger-like protrusions at the fusion site, as demonstrated by the rescue of the fusion of Srf mutant muSCs with wild-type muSCs (heterotypic fusion) when the actin network was restored by alpha-actin overexpression (Randrianarison-Huetz et al., 2018). However, the homotypic fusion between two Srf mutant muSCs overexpressing actin (Mut/Act+) was not rescued, suggesting that additional target genes of Srf (other than those regulating the actin scaffold) are important in controlling fusion (see graphical abstract).

In this study, we took advantage of these unique genetic models of defective homotypic fusion (Srf mutant muSCs (Mut) and Srf mutant muSCs overexpressing actin (Mut/ Act+)) to dissect novel pathways and cell behaviours that can restore their fusion and provide insight into the mechanisms of mammalian muscle cell fusion.

## RESULTS AND DISCUSSION

We performed a series of experiments to understand the role of Srf in muscle cell fusion, focusing on its involvement in membrane mixing, cell stiffness, and fusion efficiency.

### **Srf is not required for muSCs hemifusion**

We first asked why Srf-deleted muSCs (Mut) failed to fuse and which step of the fusion process is controlled by Srf. Differentiation of mononucleated cycling muSCs (myoblasts) into multinucleated myotubes can be induced *in vitro* by culturing dense myoblast cultures in growth factor-deprived medium (DM) for 3 days. Myocytes represent MuSCs engaged into the differentiation process, one day after DM switch. In a previous transcriptomic study, we identified the genes differentially expressed between myoblasts (D0), myocytes (D1) and myotubes (D3 after the onset of differentiation) expressing or not Srf (Randrianarison-Huetz et al, 2018). Analysis of the potential biological functions of these genes using a gene ontology programme (Ingenuity) revealed an overrepresentation of genes involved in cholesterol biosynthesis and metabolism at D1 and D3 (Figure 1A). Interestingly, cholesterol has been shown to be important for the regulation of lipid mixing (hemifusion), which initiates membrane fusion (Bu et al., 2018). Therefore, we performed membrane mixing experiments using lipid probes coupled to different dyes, and scored hemifusion events as cells exhibiting both lipid probes (Figures 1B-C). No difference was observed between control and Srf mutant cells (Figure 1D), indicating that Srf is not required for membrane mixing of muSCs.

Regulation of the actin cytoskeleton has previously been identified as one of the key biological functions affected by Srf deletion in muscle cells (Randrianarison-Huetz et al., 2018). Indeed, Srf-deleted muscle cells have less total actin and F-actin, and impaired actin architecture. This suggests that lipid membrane bending to form fusion intermediates or hemifusion is not dependent on the actin cytoskeleton, as mutant cells exhibited hemifusion even with disrupted actin network.

### **Srf is required for muSC cell stiffness**

Furthermore, the impaired actin cytoskeleton of Mut muSCs is accompanied by an absence of actin-based protrusions at the fusion site and a loss of their actin ring composed of F-actin at the cell cortex, which could affect the behaviour of the “attacking” and the “receiving” cells during fusion (Randrianarison-Huetz et al., 2018). Indeed, in *Drosophila*, actin remodelling has been shown to be important for fusion, in particular for the generation of opposing forces at the fusogenic synapse: actin protrusions to attack cells and a submembrane actomyosin network to resist at the invasive forces (Kim et al., 2015). Furthermore, the regulation of cell surface tension by actin cortex architecture has been demonstrated in the literature (Chugh et al., 2017). Given that Mut and Mut/ Act<sup>+</sup> muSCs had less F-actin at their cortex, we wondered whether this might reduce their cortical tension, and thus affect their global apparent stiffness. To test this hypothesis, we measured at the viscoelastic modulus of isolated muSCs using a single-cell rheometer. Under these conditions, the modulus is dominated by elasticity (cell stiffness) which has been suggested to rely mainly on cortical tension (Alibert et al., 2021) (Desprat et al., 2006) (Figure 2A). We showed a decrease in the apparent viscoelastic modulus in muSCs lacking Srf, regardless of whether they overexpressed actin or not (Figure 2B), highlighting the importance of Srf in controlling cellular stiffness in muscle cells. Notably, the overexpression of alpha-actin was not sufficient to restore stiffness in Srf mutant muSCs.

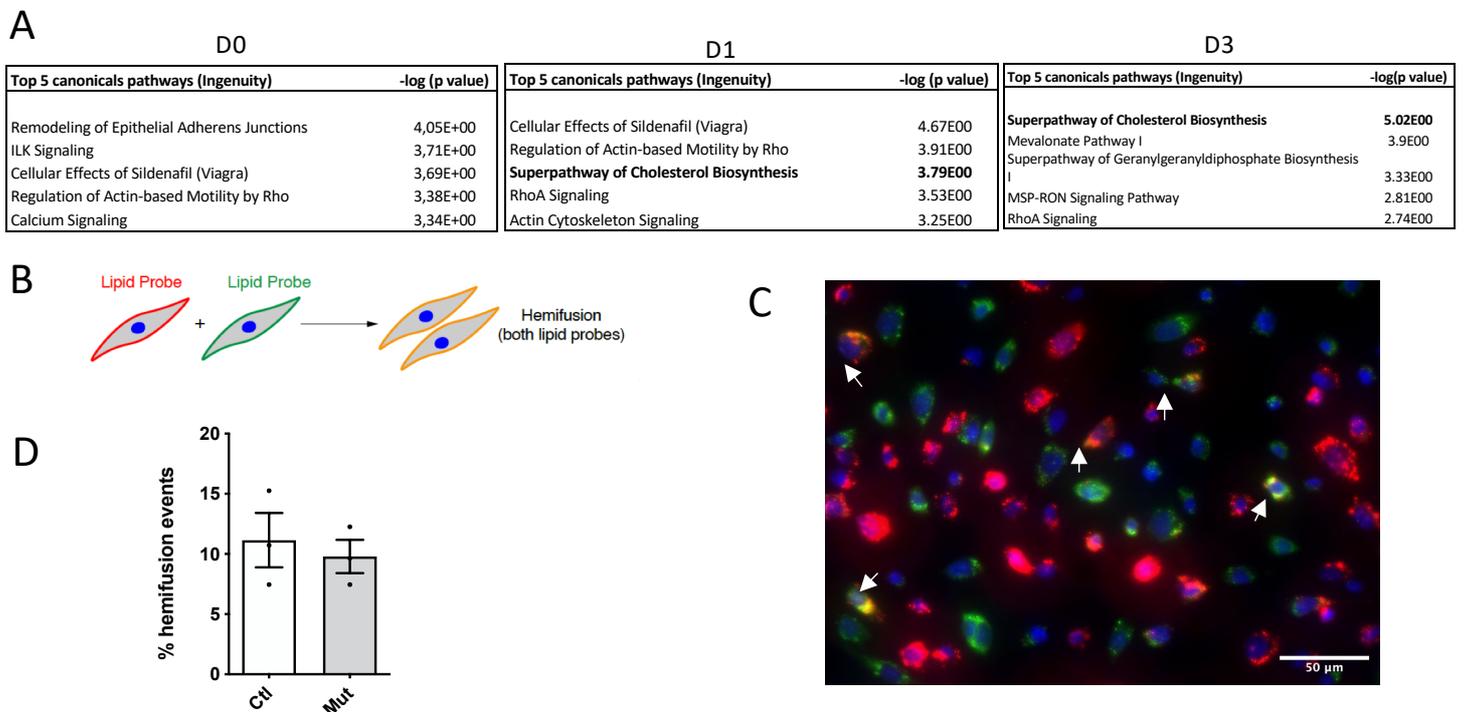
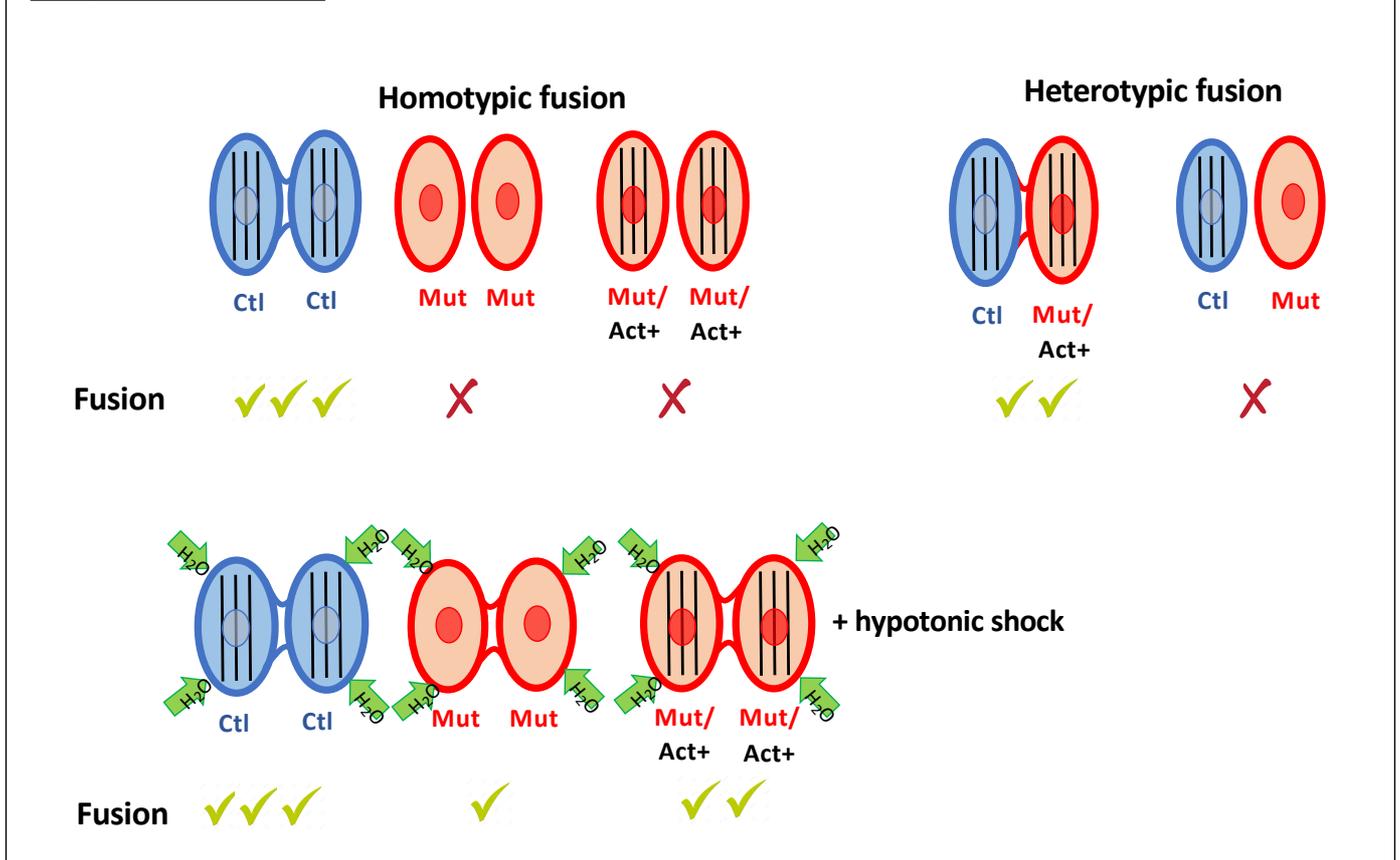
### **Fusion of Srf-deficient muSCs is partially restored by hypotonic osmotic shock**

To rescue cell stiffness, we artificially increased membrane and cortical tension by inducing a hypotonic osmotic shock in myocytes and we observed the effects on cell fusion efficiency (Figures 2C-2D). Cell fusion capacity was assessed by normalizing the percentage of fusion index after osmotic shock to that of cells of the same genotype that did not receive osmotic shock treatment. The fusion index scores the percentage of nuclei within differentiated cells that express the sarcomeric Myosin Heavy Chain protein (MyHC<sup>+</sup>) that are found in cells with 2 or more nuclei. This normalized representation allows the evaluation of the extent of rescue. In control cells, hypotonic shock had no effect, but in Mut muSCs, it increased cell fusion by approximately 1.5-fold (Figure 2E). However, it is important to note that without normalisation, the increase in fusion capacity of Srf mutant cells did not reach the level of wild-type cells (Figure 2F). Hypotonic osmotic shock is an osmotic change that imposes a mechanical stress on the membrane and may also favour the appearance of membrane pores. Thus, the beneficial effect of the osmotic shock observed in Srf mutant muSCs is likely due to a combination of different physical factors that improve fusion.

We also wondered whether the overexpression of actin in Srf mutant cells could further ameliorate cell fusion after osmotic shock. We observed a 2-fold increase in Mut/Act<sup>+</sup> muSC fusion after shock, which was significantly higher than that observed without actin overexpression (Figure 2D, E). Taken together, our results show that the rescue of Srf mutant muSC stiffness partially restores homotypic fusion and that this restoration is greater in Srf mutant cells with a preserved actin scaffold.

Of note, the cortical stiffness of Mut muSCs, as assessed by measuring the apparent viscoelastic modulus, was not rescued by ectopic alpha-actin expression (Figure 2B). These results seem to indicate that, in this later model, actin is not involved in maintaining cellular stiffness, even if alpha-actin overexpression is important for protrusion formation, as shown in our previous study (Randrianarison-Huetz et al., 2018). The better fusion rescue of Mut/Act<sup>+</sup> muSCs subjected to transient osmotic shock may be due to a combination of different factors that enhance fusion: transient turgescence of the cells, which alters stiffness and membrane properties, and restoration of actin-based protrusions at the fusion site.

## Graphical abstract



### Figure 1. Srf loss within muSCs does not impair hemifusion

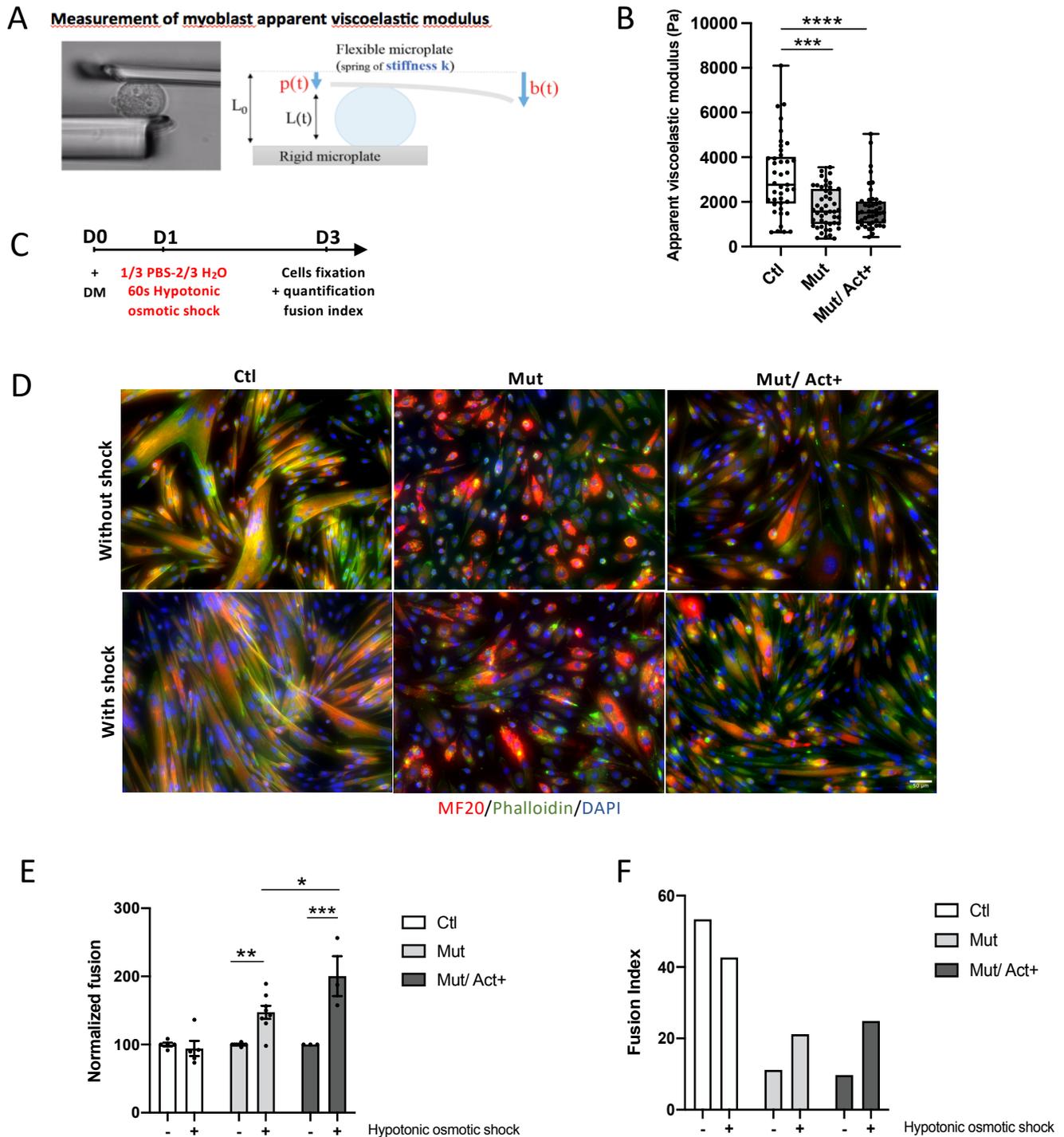
(A) Top five canonical pathways identified by gene ontology analysis using Ingenuity of genes differentially regulated by Srf in MBs (D0), myocytes at onset of differentiation (D1), and myotubes (D3).

(B) Schematic representation of the assay to evaluate hemifusion, where one set of myoblasts is labelled with DiI lipid dye (red) and another set is labelled with DiD lipid dye (green). Hemifusion events are scored as mononucleated cells that contain both lipid dyes (Leikina et al, 2018)

(C) Representative image from primary myoblasts. Arrows show double-labelled mononucleated cells. Scale bar 50  $\mu$ m.

(D) Quantification of mononucleated double-labelled cells as a percentage of total cells in control and mutant cells (n=3).

Data are mean  $\pm$  SEM.



**Figure 2. Srf loss within muSCs impaired cellular rigidity and hypotonic osmotic shock partially restores their fusion**

(A) Principle of an experiment with the single cell rheometer. A myoblast is caught between two microplates, a flexible one (top, stiffness  $k$ ) and the rigid one (bottom, much stiffer than cells). At the beginning of the experiment, the flexible microplate is displaced by a distance  $b$ , held constant over time by a feedback loop applying a displacement  $\Delta L(t) = L(t) - L_0$  to the rigid microplate. The cell strain is retrieved from its elongation  $\Delta L(t)$ .

(B) Boxplots of the values of elastoviscosity obtained with single cell rheometer from Ctl, Mut and Mut/ Act+ MBs ( $n=41$  to 47 cells).

(C) Cell fusion assay to evaluate the restoration of fusion by osmotic shock. After 1 day of differentiation, cells were treated with a hypotonic buffer (1/3 PBS, 2/3 H<sub>2</sub>O) during 60 seconds. Then, they were grown in differentiation medium for two additional days and fixed.

(D) Immunostaining for myosin heavy chain with MF20, nuclear staining with DAPI and F-actin staining with phalloidin on Ctl, Mut and Mut/ Act+ myoblasts 3 days after differentiation induction. Scale bar 50  $\mu\text{m}$ .

(E) Quantification of normalized fusion as a percentage of the fusion index of the same genotype that did not receive a hypotonic shock ( ) in Ctl, Mut and Mut/ Act+ cells after 3 days of differentiation ( $n=3-8$ ).

(F) A representative experiment of osmotic shock experiment. Complete fusion was quantified as a proportion of nuclei within multinucleated cells (fusion index) in Ctl, Mut and Mut/ Act+ cells after 3 days of differentiation ( $n=1$ ).

Data are mean  $\pm$  SEM. \*p value  $<0.05$ , \*\*p value  $<0.01$ , \*\*\*p value  $<0.001$ , \*\*\*\*p value  $<0.0001$ .

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## AUTHOR CONTRIBUTIONS

KK, DP and AA designed and carried out experiments, analysed results, and wrote the short Note. VRH provided expertise. PM provided expertise and financial support. AS designed experiments, analyses results, wrote the Short Note, and provided financial support.

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## MATERIALS AND METHODS

### Mouse models

*Srf<sup>flox/flox</sup>* mice are homozygous for *Srf* floxed alleles harboring LoxP sites flanking exon 2 of endogenous *Srf* gene (Parlakian et al., 2004). *Pax7<sup>CreERT2/+</sup>* knock-in mice express Cre-ERT2 recombinase from the endogenous muscle stem cell specific *Pax7* locus (provided by C.M. Fan, Carnegie Institution for Science, Washington, DC; Lepper et al., 2009), and Tg:*Pax7-nGFP* transgenic mice express nuclear localized EGFP under the *Pax7* promoter (provided by S. Tajbakhsh, Pasteur Institute, Paris, France; BAC containing 55-kbp upstream *Pax7* initiator ATG; Sambasivan et al., 2009).

To investigate the effect of muSC-specific *Srf* deletion in adult muscle, we used the mouse strain *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* (Randrianarison-Huetz et al., 2018). In all experiments, 2-mo-old *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* mice were given five i.p. injections of tamoxifen (TMX, 1 mg/d; MP Biomedicals) to induce *Srf* deletion and were referred to as mutant mice (Mut). Non-injected *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP* mice were used as control mice (Ctl).

To assay whether the overexpression of  $\alpha$ -actin could rescue some of the outcomes of the *Srf* loss, the mouse strain *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP:CMV-flx-CAT-flx-Actc1* was generated (Randrianarison-Huetz et al., 2018). When injected with TMX, *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP:CMV-flx-CAT-flx-Actc1* mice lost *Srf* and the CAT cassette allowing the concomitant overexpression of *Actc1* in SCs. TMX-injected *Pax7<sup>CreERT2/+</sup>·Srf<sup>flox/flox</sup>·Pax7-nGFP:CMV-flx-CAT-flx-Actc1* were referred to as Mut/Act+ mice.

Mice were genotyped by PCR using the following primers: Gfp-F, 5'-CGACGTAAACGGCCACAAGTTC-3'; Gfp-R, 5'-GACGTT GTGGCTGTTGTAGTTG-3'; Cre-F, 5'-CCTGGAAATGCTTCTGT CCG-3'; Cre-R, 5'-CAGGGTGTATAAGCAATCCC-3'; ActTg-F, 5'-CGAGGGACCTAATAACTTCG-3'; ActTg-R, 5'-GCCGATAAAAC TTGTGCTT-3'; Srflox-F, 5'-TTCGGAAGTCCCGGGCACTAAA-3'; and Srflox-R, 5'-CTGTAAGGGATGGAAGCAGA-3'.

### Primary muscle cell culture

Primary cultures were derived from hindlimb muscles of control, mutant, and Mut/Act+ mice all harbouring the *Pax7-nGFP* transgene allowing the prospective selection by flow cytometry (fluorescence Activated Cell Sorting or FACS) of muSCs as previously described in (Randrianarison-Huetz et al., 2018). In standard conditions, myoblasts were grown at 4,000 cells/cm<sup>2</sup> in proliferation medium (DMEM/F12, 2% Ultrosor G (PALL Life Sciences), 20% Fetal Calf Serum, 2 % penicillin/streptomycin) on plastic dishes coated with 0.02% Gelatin. For differentiation, myoblasts were seeded at 20,000 cells/cm<sup>2</sup> in Matrigel (1/10e)-coated dishes and cultivated in differentiation medium (DM) (DMEM/F12, 2% Horse Serum, 2 % penicillin/streptomycin) at 37°C in 5% CO<sub>2</sub>.

### Lipid mixing assays

To detect hemifusion events cells were labelled with lipid probes. Primary cells cultured in growth medium on 0.02% gelatin-coated dishes were trypsinized and resuspended in differentiation media and divided in two sets of 500 000 cells. One set was labelled for 40 min at 37°C under agitation with fluorescent lipid Vybrant™ Dil (5 $\mu$ L per mL of cell suspension, ThermoFisher Scientific, #V22885) and the other with fluorescent lipid Vybrant™ DiD (5 $\mu$ L per mL of cell suspension, ThermoFisher Scientific, #V22887) dyes, following the manufacturer instructions. Cells were then washed twice with PBS to remove excess dye.

For the hemifusion assay, labeled cells were mixed at a 1:1 ratio and plated in growth medium at approximately 70% confluence per well in in Matrigel (1/10e)-coated 12-well plate dishes, ensuring that the cells could contact each other without fusing. Cells were allowed to adhere for at least 2 hours at 37°C and then, cells were switched to differentiation medium. After 24h hemifusion events were quantified by counting the number of double-labeled cells using fluorescence microscopy.

### **Whole single-cell microrheology**

Primary myoblasts were cultured in growth medium on 0.02% gelatin-coated dishes at 4,000 cells/cm<sup>2</sup>. Twenty-four hours after, cells were trypsinized, washed 2 times in PBS and resuspended in growth medium. Isolated cells were caught between one rigid and one flexible glass microplates, which were previously coated with a 2% pluronic F127 solution (Sigma-Aldrich) for 45 min. Oscillations in the frequency range of 0.1–6.4 Hz were applied to the flexible plate by a computer-controlled piezo electric stage.

The deflection of the flexible plate (of calibrated stiffness) was monitored by an optical sensor, allowing us to measure the stress  $\sigma(t)$  applied to the cell and the resulting strain  $\epsilon(t)$ . The compression modulus  $E(\omega)$  is defined from the ratio  $\sigma(t)/\epsilon(t)$  and can be expressed as  $E(\omega) = E'(\omega) + iE''(\omega)$ , where  $E'(\omega)$  and  $E''(\omega)$  are the storage and the loss moduli. Knowing the amplitude and the phase of the two signals  $\sigma(t)$  and  $\epsilon(t)$ , we can deduce the values of  $E'$  and  $E''$  for each frequency. In this frequency range, cells were shown to exhibit a power-law behavior  $E(\omega) = E_0 \omega^\alpha$ . The shear modulus  $G$  can be obtained from the elongation modulus  $E$  using  $G = E / 2(1+\nu)$  where  $\nu$  is the Poisson ratio. Following [29], we took  $\nu = 0.33$ . The averaged values of  $G$  shown in Fig. 2B were calculated at  $\omega = 1$  Hz.

### **Application of hypotonic osmotic shock**

Primary myoblasts were seeded at 20,000 cells/cm<sup>2</sup> in Matrigel (1/10e)-coated dishes and cultured in differentiation medium (DM) (DMEM/F12, 2% Horse Serum, 2 % antibiotic/antimycotic) at 37°C in 5% CO<sub>2</sub>. After 24 h, to assess the ability of osmotic stress to improve fusion, differentiating cells were treated with PBS diluted with H<sub>2</sub>O (1:3) for 60 seconds as described (Leikina et al., 2018). The cells were then cultured in differentiation medium for 48 hours.

### **Cell immunostaining and fusion index counting**

Muscle cells cultured in dishes were fixed for 10 min in 4% PFA and then permeabilized and blocked in PBS with 0.1% Triton X-100 and 5% horse serum for 1h at RT. Cells were incubated overnight at 4°C with the following primary antibodies: mouse anti-MF20/MyHC (1/130; DSHB) diluted in the same buffer. After incubation for 1h at RT with fluorescent secondary antibodies anti-mouse IgG2b Alexa 546 (1/1000; Life Technologies, A21143), cells were stained with DAPI (for nuclei) and phalloidin Alexa Fluor 488 (1/500; Thermo Fisher Scientific, A12379; for F-actin) and mounted in Fluorescent Mounting Medium (Dako, S3023).

Fusion index corresponds to the proportion of nuclei in MyHC+ cells within multinucleated cells in control, Mut and Mut/Act+ cells 3 days after differentiation, with or without hypotonic shock.

### **Image acquisition**

Fluorescence images were acquired using an Olympus BX63F microscope with 20x objectives (UPLSAPO, NA 0.75) coupled with an ORCA-Flash4.0 LT camera (Hamamatsu), or using a Zeiss Axiovert 200M microscope with 20x objectives (LD PLANNEOFLUAR, NA 0.4), coupled with a CoolSnap-HQ camera (Photometrics), or using a Spinning Disk confocal microscope (Yokogawa X1) with a 100x oil-immersion objective (HCX PL APO, NA 1.47), coupled with a CoolSnap-HQ<sup>2</sup> camera (Photometrics) and Cell sens 7.7.5 (Molecular Devices). Images were merged and edited in ImageJ. Background was reduced using brightness and contrast adjustments applied to the whole image.

### **Statistical analysis**

Quantitative data sets were analyzed using an unpaired-test with Welch correction when two groups were analysed (Figure 1D), Kruskal-Wallis test with Dunn correction when three groups or more were analyzed (Figure 2B) and two-way ANOVA with Sidak's multiple comparison test when two categorical variables were analyzed (Figure 2E), using GraphPad Prism 8.0 software. Statistical significance was set at a p value <0,05.