DHPR $\alpha 1S$ subunit controls skeletal muscle mass and morphogenesis

France Piétri-Rouxel^{1,*}, Christel Gentil¹,
Stéphane Vassilopoulos², Dominique
Baas³, Etienne Mouisel¹, Arnaud Ferry^{1,4},
Alban Vignaud¹, Christophe Hourdé¹,
Isabelle Marty⁵, Laurent Schaeffer³,
Thomas Voit¹ and Luis Garcia¹

¹UMR 7215, CNRS, UMR S 974 Inserm, Institut de Myologie, Université Pierre et Marie Curie, Paris, France, ²Department of Bioengineering and Therapeutic Sciences, School of Pharmacy, University of California, San Francisco, CA, USA, ³UMR 5239, CNRS, ENS, Université Lyon 1 46, allée d'Italie Lyon cedex 07, France, ⁴Université Paris Descartes, Paris, France and ⁵Inserm U836, Grenoble Institut des Neurosciences, Université Joseph Fourier, Grenoble, France

The $\alpha 1S$ subunit has a dual function in skeletal muscle: it forms the L-type Ca^{2+} channel in T-tubules and is the voltage sensor of excitation–contraction coupling at the level of triads. It has been proposed that L-type Ca^{2+} channels might also be voltage-gated sensors linked to transcriptional activity controlling differentiation. By using the U7-exon skipping strategy, we have achieved long-lasting downregulation of $\alpha 1S$ in adult skeletal muscle. Treated muscles underwent massive atrophy while still displaying significant amounts of $\alpha 1S$ in the tubular system and being not paralysed. This atrophy implicated the autophagy pathway, which was triggered by neuronal nitric oxide synthase redistribution, activation of FoxO3A, upregulation of autophagy-related genes and autophagosome formation. Subcellular investigations showed that this atrophy was correlated with the disappearance of a minor fraction of $\alpha 1S$ located throughout the sarcolemma. Our results reveal for the first time that this sarcolemmal fraction could have a role in a signalling pathway determining muscle anabolic or catabolic state and might act as a molecular sensor of muscle activity.

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Introduction

Skeletal muscle growth is dependent on innervation and exercise. Prolonged periods of muscle inactivity because of bed rest, hindlimb unloading, immobilization, microgravity

or denervation result in a significant muscle atrophy (Borisov *et al*, 2001; Adams *et al*, 2003; Dedkov *et al*, 2003; De-Doncker *et al*, 2005). Direct electrical stimulations can, to a large extent, substitute for innervation and preserve or even restore almost normal properties in denervated muscle fibres (Kern *et al*, 2004; Squecco *et al*, 2008). Nevertheless, mechanisms implicated in muscle homeostasis under such circumstances are poorly understood. These molecular mechanisms are likely triggered by action potentials or prompted by the contractile function itself. In neurons, L-type Ca^{2+} channels have been shown to act as voltage-gated sensors linked to transcriptional activity controlling differentiation (Wheeler *et al*, 2008). In skeletal muscle, L-type Ca^{2+} channels are made of five subunits ($\alpha 1S$, $\alpha 2$, β , γ , δ). The $\alpha 1S$, known as the dihydropyridine receptor (DHPR), is a four-repeat transmembrane protein, which contains the basic functional elements of the channel, including the Ca^{2+} selective pore and the S4 'voltage-sensing' transmembrane segment (Ahern *et al*, 2001). It is predominantly located in T-tubules, although a minor fraction of DHPR has been found on the sarcolemma (Jorgensen *et al*, 1989). $\alpha 1S$ is also located in triads where it interacts with the type 1 ryanodine sensitive Ca^{2+} release channel (RyR1) and forms the voltage sensor of the excitation–contraction (EC) coupling.

We hypothesize that subtle modification of $\alpha 1S$ contents in adult muscle could allow to decipher unexpected functions for this multi-functional protein. Indeed, complete knockout of the $\alpha 1S$ subunit during development is lethal as observed in the *mdg/mdg* dysgenic mouse model (Pai, 1965; Banker, 1977; Chaudhari, 1992). To create a viable model, which would overcome this problem, we have chosen a strategy for long-lasting RNA interference based on the use of U7 snRNA chimaeras. The U7 system has been successfully used in several paradigms for rescuing mutated mRNAs by either prompting exon skipping or exon inclusion to restore protein synthesis (Goyenvallé *et al*, 2004; Marquis *et al*, 2007). Here, we used this system to decline amounts of $\alpha 1S$ subunit by forced skipping of an exon affecting its mRNA reading frame. To guarantee long-lasting effects, the U7 snRNA chimaeras were delivered intramuscularly by using adenovirus-associated viral vectors (AAV). After 6 months, treated muscles showed a significant atrophy even though muscle fibres were not EC uncoupled and were still capable to contract when stimulated. We also show that decreasing $\alpha 1S$ induced redistribution of the neuronal nitric oxide synthase (nNOS), activation of FoxO3A, increased expression of key autophagy-related genes and initiated autophagosome formation. Finally, confocal investigations allowed to identify which subcellular compartment displayed the decrease of $\alpha 1S$ after exon skipping treatment. We found that atrophy and ultrastructural disorganization of muscle fibres correlated with $\alpha 1S$ disappearance from the sarcolemma while $\alpha 1S$ remained present in T-tubules and triads. All together, these results suggest that the minor sarcolemmal fraction of $\alpha 1S$ has a role

*Corresponding author. UMR 7215, CNRS, UMR S 974 Inserm, Institut de Myologie, Université Pierre et Marie Curie, 105 boulevard de l'Hôpital, Paris 75013, France. Tel.: +33 01 4077 9635; Fax: +33 01 5360 0802; E-mail: france.pietri-rouxel@upmc.fr

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in a signalling pathway controlling muscle maintenance. At that level, $\alpha 1S$ might act as a sensor of muscle activity in charge of controlling muscle mass and morphogenesis.

Results

Knockdown of the $\alpha 1S$ subunit

The $\alpha 1S$ subunit is encoded by the *Cacna1s* gene, made of 46 exons and located, in mouse, on chromosome 1 (Drouet *et al*, 1993). Exon 16 encodes an amino acid sequence forming a cytoplasmic loop (II–III loop) connecting trans-membrane repeats II and III of the $\alpha 1S$ subunit, which interacts directly with RyR1 and is crucial for skeletal muscle EC coupling (Tanabe *et al*, 1990; Casarotto *et al*, 2006; Cui *et al*, 2009). To knock down the $\alpha 1S$ subunit we have designed U7 snRNA chimaeras interfering specifically with its pre-mRNA splicing. We have chosen to target exon splicing enhancers (ESE) and the acceptor splice site (SA) of exon 16 (U7-ESE, U7-SA). A U7-Ctrl, not affecting $\alpha 1S$ splicing, was used as mock control (Figure 1A). Engineered U7 genes were cloned into AAV2 vector backbones, which were type 1 pseudotyped for intramuscular delivery: AAV2/1 (U7-ESE), AAV2/1 (U7-SA), AAV2/1 (U7-Ctrl). To improve skipping of exon 16, both AAV2/1 (U7-ESE) and AAV2/1 (U7-SA) vectors were co-injected into tibialis anterior muscles (TA^{ADHPR}). Contralateral muscles were used as controls (TA^{Ctrl}) and received mock AAV2/1 (U7-Ctrl). To show that these genomes were still active and produced the interfering U7 transcripts at 6 months post injection, total RNA from TA^{ADHPR} muscles was extracted, reverse transcribed into DNA and PCR amplified using a forward primer in the antisense sequence (ESE) and a reverse primer in the U7 stem loop. Results confirmed that vector expression was persistent up to 6 months post injection (Supplementary Figure S1).

Skipping exon 16 would allow abortion of $\alpha 1S$ synthesis by provoking a shift of its mRNA reading frame creating a premature stop codon in position 33 of exon 17 (Figure 1A). Skipping efficiency was assessed by real-time quantitative RT-PCR (qRT-PCR) on total mRNA (Supplementary Table S1). Such analysis showed that levels of the unskipped $\alpha 1S$ represented $18 \pm 6\%$ ($P \leq 0.001$, $n = 19$) and $10 \pm 7\%$ ($P \leq 0.001$, $n = 19$) of the total $\alpha 1S$ mRNAs at 2 and 6 months post injection, respectively (Figure 1B). Western blotting revealed a significant decrease of $\alpha 1S$. Six months post injection, $\alpha 1S$ levels in TA^{ADHPR} were about $56 \pm 13\%$ ($P \leq 0.001$, $n = 5$) compared with the contralateral TA^{Ctrl} (Figure 1C). It is unlikely that skipped mRNAs could still be translated into a truncated protein. Indeed, such a truncated protein should display a theoretical molecular weight of about 80 kDa. Western blots using polyclonal antibodies made against the N-terminal protein never detected such a band (Supplementary Figure S2). Levels of $\alpha 1S$ were also assessed by immuno-fluorescence staining on TA^{ADHPR} muscle sections. At 6 months, the majority of fibres showed a decrease in staining intensity (Figure 1D).

Atrophy, fibrosis and reduced maximal force after $\alpha 1S$ knockdown

A significant atrophy was observed in injected TA^{ADHPR}. Six months after injection, TA^{ADHPR} showed a loss of $41 \pm 3\%$ of muscle mass compared to contralateral TA^{Ctrl} ($P \leq 0.001$,

$n = 8$) (Figure 2A; Table I). Morphometric analysis showed that there was no change in the number of fibres per muscle. Atrophy was due to a decrease of the size of muscle fibres (Table I) as shown by the distribution of cross-section diameters, which was shifted towards lower values (Figure 2B). Average fibre diameter was $24 \pm 2 \mu\text{m}$ in injected TA^{ADHPR} compared to $43 \pm 4 \mu\text{m}$ in contralateral TA^{Ctrl} (Table I; Figure 2C). This atrophy was not accompanied by a modification of muscle fibre phenotype. Typically, the TA was almost entirely composed of fast-twitch fibres, expressing type 2b and 2x myosin heavy chains (MHC) isoforms and a very small number of muscle fibres expressing the MHC-2a isoform (Raffaello *et al*, 2006). No significant modifications were observed in the number of fibres expressing MHC-2x and -2b showing that the major muscle phenotype was maintained in treated muscle compared to contralateral (Table I). However, there was a slight decrease in the number of fibres expressing MHC-2a in TA^{ADHPR} compared to TA^{Ctrl} (Table I). Still, TA^{ADHPR} muscles showed striking morphological abnormalities without evidence of necrosis or core formation. There was no sign of infiltration by scavenger cells, which is a hallmark of necrosis, nor positive immunolabelling for neonatal and embryonic MHCs (data not shown), a more sensitive marker of regeneration given that this isoform is expressed during muscle development and particularly the formation of new fibres from satellite cells (Ecob-Prince *et al*, 1986; Mouly *et al*, 1993). In treated muscles (TA^{ADHPR}), subsequent atrophy was associated with fibrosis (Figure 2C) as shown by morphometric analysis using Sirius red for staining collagen deposition (Figure 2D). Injected TA^{ADHPR} muscles displayed a four-fold increase in fibrosis compared with the contralateral muscles (Figure 2E). Muscle function was assessed by the measurement of *in situ* muscle force in response to nerve stimulation, as described earlier (Vignaud *et al*, 2005a). Compared with values measured in the contralateral limb, absolute and specific maximal isometric tetanic force measured in injected TA^{ADHPR} were shown to be reduced by 62% ($40 \pm 11 \text{ g}$ compared to $106 \pm 20 \text{ g}$, $P \leq 0.001$, $n = 8$) and 34% ($1.1 \pm 0.3 \text{ g/mg}$ compared to $1.6 \pm 0.2 \text{ g/mg}$, $P \leq 0.05$, $n = 8$), respectively (Table I).

Altogether, these data show that $\alpha 1S$ knockdown in normal muscle resulted in important morphological changes involving atrophy and fibrosis. These alterations were not caused by muscle paralysis although fibre dismantling ultimately affected muscle function.

$\alpha 1S$ knockdown induces nNOS redistribution, FoxO3A nuclear translocation and upregulation of autophagy-related gene expression

It has been proposed that nNOS, a molecular partner of the dystrophin–glycoprotein complex, has a role in the mechanism of atrophy in denervation-induced and hindlimb unloading models (Suzuki *et al*, 2007). In TA^{ADHPR} muscles, levels of nNOS mRNA were found to be reduced by $41 \pm 1\%$ ($P \leq 0.001$, $n = 9$) (Figure 3A), a significant decrease that was confirmed at the protein level by western blot analysis (Supplementary Figure S3A). Immuno-histochemical analysis revealed that nNOS staining shifted from the sarcolemma to the cytoplasm (Figure 3B), a redistribution that was not due to the disruption of the dystrophin–glycoprotein complex, which was not affected in TA^{ADHPR} muscles (see stainings for dystrophin and

Figure 1 Generation of a local knockdown of $\alpha 1S$ subunit. **(A)** Schema of exonic chaining of pre-mRNA, exons 15, 16 and 17 are represented by blue boxes. Sequences of exon acceptor site SA (in blue) and exon splicing enhancer ESE (in black) of exon 16 were targeted by antisense sequences introduced in U7smOPT cassettes. **(B)** qRT-PCR analysis using primers overlapping exons 15 and 16 permitted to quantify the non-skipped $\alpha 1S$ form: $18 \pm 6\%$ ($**P \leq 0.001$, $n = 19$) and $10 \pm 7\%$ ($**P \leq 0.001$, $n = 19$) (black bars) of the total $\alpha 1S$ mRNA (grey bars) at 2 and 6 months post injection in TA of AAV1-(U7-ESE) and AAV1-(U7-SA): Δ DHPR or AAV1-(U7-Ctrl): c as a control, respectively. **(C)** Six months post injection, lysates from TA $^{\Delta$ DHPR (Δ DHPR) and TA Ctrl (c) were immuno-blotted for $\alpha 1S$ or α -actin for four mice. Graph depicts mean $\pm$ s.e.m. of relative expression of $\alpha 1S$ subunit determined by densitometry and normalized to the α -actin expression for each muscle. Results were expressed in protein levels of $\alpha 1S$ subunit in TA $^{\Delta$ DHPR normalized to TA Ctrl for each mice, $**P \leq 0.001$, $n = 4$. **(D)** Longitudinal cryo-sections from TA $^{\Delta$ DHPR (Δ DHPR) and TA Ctrl (c) were stained with anti- $\alpha 1S$ subunit (red) and anti-laminin (green) antibodies, nuclei were visualized by Dapi (blue) and imaged by confocal microscopy. Bars represent 20 μ m.

associated α , β and γ sarcoglycans in Supplementary Figure S4). We evaluated NOS activity by using NADPH-d histochemical analysis (Chen *et al*, 2008). As expected, NOS activity was redistributed towards the cytoplasm in TA $^{\Delta$ DHPR atrophic fibres (Supplementary Figure S3B).

It has been shown in tail suspension experimental models that nNOS redistribution induced muscle atrophy through regulation of FoxO3A (Suzuki *et al*, 2007), a pivotal transcription factor involved in muscle mass regulation (Mammucari *et al*, 2007). Phosphorylated forms of FoxO3A are found in the cytoplasmic compartment, whereas dephosphorylated forms translocate to the nucleus to activate transcription of target genes (Cao *et al*, 2005). Western blot analysis of TA $^{\Delta$ DHPR muscles using an antibody recognizing the phosphorylated form showed that FoxO3A was mainly

dephosphorylated (Figure 3C). Concurrently, immunostaining of TA $^{\Delta$ DHPR muscle sections with an antibody recognizing the non-phosphorylated form of FoxO3A showed its accumulation in myonuclei (Figure 3D), thus confirming its translocation after activation.

It is known that the ubiquitin-proteasomal pathway is markedly increased in atrophying muscle because of transcriptional activation of ubiquitin, several proteasomal subunit genes and the two muscle-specific ubiquitin ligases atrogin-1/MAFbx and MuRF1 (Cao *et al*, 2005). In TA $^{\Delta$ DHPR muscle extracts, qRT-PCR revealed that atrogin-1/MAFbx and MuRF1 were not over-expressed at 6 month post injection (Figure 3E). Moreover, atrogin-1/MAFbx and MuRF1 were not even transiently upregulated before this time point (expression of these genes was measured 1, 2, and 4 months

Figure 2 Consequence of $\alpha 1S$ subunit knockdown. (A) Six months post injection, TAs from eight mice were dissected and weighed. (B) The internal diameters (shortest diameter) from all fibres throughout the total muscle section were recorded and analysed. Muscles from five different animals were examined. The bar graph presents mean $\pm$ s.e.m. of the number of myofibres by fibre diameter class for TA^{ΔDHPR} (black) and TA^{Ctrl} (grey). (C) Transversal sections of TA^{ΔDHPR} (ΔDHPR) and TA^{Ctrl} (c) were stained with haematoxylin and eosin, bars represent 100 μ m. (D) To quantify fibrosis, transversal sections of total muscle were stained with Red Sirius and quantified using Histolab Software (marked in blue), data were normalized with total surface of each muscle (orange line surrounding the sections), bars represent 500 μ m. (E) Quantification is presented in bar graph and showed 4.1 $\pm$ 0.1 fold increase of fibrosis in TA^{ΔDHPR} (ΔDHPR) compared to TA^{Ctrl} (c) (** P < 0.001, n = 4).

post injection) suggesting that the ubiquitin-proteasome pathway was not involved in TA^{ΔDHPR} induced atrophy. We then inquired whether the induction of atrophy was accompanied by an upregulation of autophagy-related genes, *LC3*, *Gabap11*, *Atg4b* and *Atg12*; the genes involved in regulating autophagy *Vps34* (a class III PI3K), *Bnip* and finally the lysosomal proteinase *cathepsinL* gene (Kelekar, 2008). By using qRT-PCR, we showed that *Bnip3*, *LC3*, *PI3KIII* and *CathepsinL* expression was significantly increased in TA^{ΔDHPR} compared to contralateral TA^{Ctrl} (Figure 3E).

These results indicate that knockdown of $\alpha 1S$ levels induced atrophy through the autophagy pathway that was most probably triggered by nNOS redistribution, FoxO3A translocation to myonuclei and activation of autophagy-related gene expression.

Fall of $\alpha 1S$ induces autophagosome formation and alters morphology of the sarcoplasmic reticulum

Autophagic activity and presence of autophagic vacuoles were revealed at the ultrastructural level by using electron microscopy (EM). Characteristic double-membrane-limited structures corresponding to autophagosomes were observed in atrophic TA^{ΔDHPR} muscle fibres (Figure 4A). Autolysosome structures, characterized by highly electron dense signals and corresponding to the fusion of autophagosomes and lysosomes, were frequently observed. The occurrence of autophagosomes was also assessed on tissue sections and isolated fibres from TA^{ΔDHPR} muscles by using LC3 immunostaining, a specific marker for autophagosome formation (Mizushima *et al*, 2004; Mizushima and Kuma, 2008). We found that LC3 staining on TA^{ΔDHPR} transverse sections

Table 1 Morphometric and functional characteristic of TA^{ADHPR}

	TA ^{Ctrl}	TA ^{ADHPR}
Mass (mg)	65 ± 8	39 ± 3**
Total number of fibres	3405 ± 161	3249 ± 443
Mean of CSA (mm)	43 ± 4	25 ± 2**
<i>MHC composition (%)</i>		
MHC-1	0.09 ± 0.03	0.03 ± 0.03
MHC-1/2a	0.00	0.02 ± 0.01
MHC-2a	1.5 ± 0.7	0.3 ± 0.06*
MHC-2a/2x	2.7 ± 0.4	1.08 ± 0.50
MHC-2x	38.90 ± 1.60	33.50 ± 3.50
MHC-2x/2b	16.40 ± 3.90	16.50 ± 1.30
MHC-2b	40.40 ± 3.50	47.80 ± 4.80
Absolute MTI force (g)	106 ± 20	40 ± 11**
Specific MTI force (g/mg)	1.6 ± 0.2	1.1 ± 0.3*

Investigations 6 months post injection of AAV1 (U7-ESE) and AAV1 (U7-SA) into the TA (TA^{ADHPR}), or AAV1 (U7-Ctrl) (TA^{Ctrl}) were carried out; $n = 8$ for mass, total number of fibres, mean of cross-section area (CSA), absolute and maximal tetanic isometric (MTI) force investigations; and $n = 3$ for MHC composition analyses. Values are mean ± s.e.m. and were significantly different for TA^{ADHPR} from TA^{Ctrl}, ** $P \leq 0.001$; * $P \leq 0.05$.

labelled small vacuolar structures surrounded by dystrophin (Figure 4B). We then carried out double staining on isolated fibres by using antibodies against LC3 and the polyubiquitin-binding protein p62 (also known as sequestosome SQSTM1), which serves as an adaptor molecule mediating the degradation of polyubiquitinated proteins by the autophagic pathway (Raben *et al*, 2008). Confocal analysis showed widespread rising of colocalized LC3 and p62 staining all along Δ DHPR atrophied fibres, whereas control fibres did not displayed such a phenomenon (Figure 4C).

EM analysis of TA^{ADHPR} muscles also revealed that sarcomeres were smaller and less organized than controls, although Z and M lines were still properly aligned. Triads were correctly located and displayed electron dense structures enclosing the EC coupling machinery, but most of the terminal SR cisternae were unusually dilated and the longitudinal SR displayed unusual circumvolutions and invaginations (Figure 5A). Confocal analysis by using antibodies against SERCA, a marker of longitudinal SR, and RyR1, a marker of triads, confirmed this SR disorganization at the level of whole isolated fibres (Figure 5B). Western blot analysis showed that levels of RyR1 were only slightly reduced in TA^{ADHPR} muscles (Figure 6A). Triadin, a protein associated to the EC complex, was also not significantly affected (data not shown). Confocal double stainings showed that $\alpha 1S$ and RyR1 were still co-localized in atrophic TA^{ADHPR} fibres (Figure 6B), a result in agreement with EM observations and the fact that atrophic TA^{ADHPR} fibres were still EC coupled.

Atrophy in Δ DHPR muscle fibres correlates with the disappearance of the sarcolemmal fraction of $\alpha 1S$

As some $\alpha 1S$ expression remained preserved in exon skipping-treated muscles undergoing atrophy, we wondered in which compartment $\alpha 1S$ was really decreased. To address this point, we have investigated more deeply $\alpha 1S$ localization in treated muscles, particularly when atrophy was clearly established. This study was carried out by 3D confocal analysis on isolated flexor digitorum brevis (FDB) muscle fibres (Figure 7; Supplementary Movies S5A and S5B). In

control fibres, $\alpha 1S$ was chiefly found in the inner compartment encompassing T-tubules and triads. However, 3D-projection analysis revealed that a minor fraction of $\alpha 1S$ was present on the sarcolemma as already shown by using EM techniques and suggested by subcellular fractionation (Jorgensen *et al*, 1989; Munoz *et al*, 1995; Vassilopoulos *et al*, 2009). In exon skipping-treated fibres undergoing atrophy, $\alpha 1S$ was still present in the inner compartments and displayed a quite preserved striated distribution. The major effect concerned the sarcolemmal $\alpha 1S$ fraction, which was missing in treated fibres.

Discussion

$\alpha 1S$ knockdown in skeletal muscle induces atrophy

In skeletal muscle, it is well known that $\alpha 1S$ has a dual function: it forms the L-type Ca^{2+} channel in T-tubules and is the voltage sensor of EC coupling at the level of triads. Muscular dysgenesis (*mdg/mdg*), a null mutation on the *Cacnals* gene encoding $\alpha 1S$, has been described in the mouse (Pai, 1965; Platzer and Gluecksohn-Waelsch, 1972; Chaudhari, 1992). In *mdg/mdg*, skeletal muscles are paralysed because of a complete lack of EC coupling and muscle fibres never fully differentiate: and show a poor index of fusion, reduced basal lamina, sarcomere disorganization and triad misfolding (Bournaud and Mallart, 1987; Rieger *et al*, 1987; Tanabe *et al*, 1988). As animals died at birth, it was impossible to discriminate additional roles for this protein, particularly in adult healthy muscle, and subsequent studies mainly focused on the role of $\alpha 1S$ during skeletal muscle development (Tanaka *et al*, 2000). Here, by using an exon skipping approach (Goyenvalle *et al*, 2004), we could, for the first time, achieve loco-regional long-lasting downregulation of $\alpha 1S$ in a normal well established adult muscle without impairing the quality of life of treated animals. This strategy allowed silencing $\alpha 1S$ mRNAs by about 80% at 2 months after intramuscular injection of AAV2/1 vectors harbouring U7 chimaeras designed to disrupt its reading frame. Levels of U7 chimaeras were stable over time, thereby maintaining and improving $\alpha 1S$ silencing. Surprisingly, no significant decay in $\alpha 1S$ protein was observed until 6 months of treatment (its expression was tested by western blot at 2 and 4 months post injection) suggesting that $\alpha 1S$ has a natural turnover roughly between 2 and 4 months. At 6 months, treated fibres displayed about a 56% loss of $\alpha 1S$ protein. Interestingly, this fall did not abrogate either the absolute or the specific tetanic force, which was only slightly decreased, indicating that EC coupling was still effective. Despite these results, the foremost surprising finding was that treated fibres underwent massive atrophy. The fact that $\alpha 1S$ knockdown was far from being complete confirmed that subsequent effects were not trivial consequences of a major defect affecting primary functions of muscle fibres such as action potential genesis, EC coupling, contraction or even innervation.

It is known that atrophy caused by experimental denervation induces modifications of fibre MHC phenotype in both fast and slow type muscles. In denervated rat soleus and TA, both slow and fast fibres undergo a rapid atrophy (Dedkov *et al*, 2003), which is associated with a marked transformation towards a fast phenotype (Huey and Bodine, 1998; Loughna and Morgan, 1999). In our model, Δ DHPR-induced atrophy was not associated with a variation of the total

Figure 3 nNOS membrane dissociation, FoxO3A accumulation in myonuclei and upregulation of autophagy-related genes as consequences of $\alpha 1S$ subunit loss. **(A)** mRNA from TA^{ADHPR} and TA^{Ctrl} tissues were extracted and nNOS expression was quantified by qRT-PCR. Results are expressed as mean $\pm$ s.e.m., ** $P < 0.001$, $n = 4$. **(B)** Transversal cryo-sections of TA^{ADHPR} (Δ DHPR) and TA^{Ctrl} (c) were stained with anti-nNOS (red), anti-laminin (green) antibodies, nuclei with Dapi (blue) and imaged by confocal microscopy. Bars represent 20 μ m. **(C)** The tissues from extracts were analysed by western blot with FoxO3a-P antibody and normalized with α -actin antibody. Graph depicts mean $\pm$ s.e.m. of relative expression of FoxO3a-P determined by densitometry and normalized to the α -actin expression for each muscle, * $P \leq 0.005$, $n = 3$. **(D)** Longitudinal cryo-sections of TA^{ADHPR} (Δ DHPR) and TA^{Ctrl} (c) were stained with anti-FoxO3a (red), anti-laminin (green) antibodies, nuclei with Dapi (blue) and imaged by confocal microscopy. Bars represent 20 μ m. **(E)** mRNA from TA^{ADHPR} and TA^{Ctrl} tissues were extracted and regulation of autophagy genes expression was followed by qRT-PCR. Bnip, CathepsinL, LC3 and PI3KIII expression (noted in red) were significantly increased in TA^{ADHPR} compared with the controlateral TA^{Ctrl}, ** $P < 0.001$, $n = 4$.

number of fibres. Rather, morphometric analysis indicated that all fibres shifted towards a smaller diameter and no modification of MHC-2x and -2b distribution was observed.

Δ DHPR atrophy is mediated by autophagy

Hindlimb unloading is widely used for studying muscle atrophy because of muscle inactivity while innervation is

preserved (De-Doncker *et al*, 2005; Suzuki *et al*, 2007). In this model, redistribution of nNOS has been described to mediate muscle atrophy through regulation of FoxO3A (Suzuki *et al*, 2007). In our model of Δ DHPR-induced atrophy, nNOS was also redistributed to the cytoplasm and activated FoxO3A was found translocated into muscle nuclei, suggesting a similar atrophic pathway. Activation of FoxO3A has been shown to

Figure 4 Formation of autophagosomes in $TA^{\Delta DHPR}$. (A) Ultrathin sections were imaged by electron microscopy, M, mitochondria; C, collagen fibres; T, T-tubule. Arrows show double-membrane vesicles called autophagosomes. (B) Transversal cryo-sections were stained with anti-LC3b (red), anti-dystrophin (green) antibodies, nuclei with Dapi (blue) and imaged by confocal microscopy. Upper panel: TA^{Ctrl} (Ctrl) and lower panel: $TA^{\Delta DHPR}$ ($\Delta DHPR$). Bars represent 20 μm . (C) Myofibres isolated from control (Ctrl) or 6 months post-injected FDB ($\Delta DHPR$) muscles were processed for immuno-fluorescent labelling for P62 (green) and LC3b (red) and imaged by confocal microscopy. Scale bars, 10 μm .

be essential for fibre atrophy on denervation and fasting (Sandri *et al*, 2004, 2006) through the ubiquitin/proteasome pathway by upregulating two crucial ubiquitin ligase genes encoding atrogin-1 and MuRF1 (Sandri *et al*, 2004). FoxO3A can also control atrophy independently of the ubiquitin-proteasomal pathway through the autophagic/lysosomal pathway. Indeed, it has been shown that constitutive over-expression of active FoxO3A stimulated the autophagic process selectively (Zhao *et al*, 2008). Here, we did not observe atrogin-1 or MuRF1 over-expression. Therefore, it was unlikely that $\alpha 1S$ -knockdown muscle atrophy involved the canonical ubiquitin-proteasomal pathway. Rather, it

involved the autophagic pathway as confirmed by the upregulation of several autophagy-related genes such as *Bnip3*, *LC3*, *PI3KIII* and *CathepsinL* (Zhao *et al*, 2007). Moreover, LC3 protein was observed in large vacuolar structures expressing dystrophin, a hallmark of a select group of autophagic vacuolar myopathies including Danon disease and X-linked myopathy with excessive autophagy (Malicdan *et al*, 2008). The precise relevance of these structures and the mechanism by which they are formed remain to be clarified. Autophagy is the engulfment of cytosol and organelles by double-membrane vesicles termed autophagosomes. Autophagosome formation is known to occur near

Figure 5 Impact of the $\alpha 1S$ subunit knockdown on maintenance of muscle ultrastructure. (A) Ultrathin sections of TA^{ΔDHPR} (Δ DHPR) and TA^{Ctrl} (c) were imaged by electron microscopy. Cis, terminal cisternae; T, tubule; SR, sarcoplasmic reticulum. Bars: 500 nm. (B) Myofibres isolated from Ctrl or Δ DHPR FDB muscles 6 months post injection were processed for immuno-fluorescent labelling for RyR1 (green), SERCA (red), Dapi (blue) and imaged by confocal microscopy. Scale bars, 10 μ m.

the endoplasmic reticulum (ER). In live imaging experiments, a vesicular punctuate compartment is in dynamic equilibrium with the ER and could provide a membrane platform for accumulation of autophagosomal proteins, expansion of autophagosomal membranes and emergence of fully formed autophagosomes (Xie and Klionsky, 2007). At the ultrastructural level, Δ DHPR fibres displayed many double-membrane-limited structures characteristic of autophagosomes in addition to disorganization of the SR, which displayed abnormal circumvolutions and invaginations. This disorganization could suggest that the SR might also be a donor for membranes required to form autophagosomes.

Underlying mechanism for muscle atrophy induced by $\alpha 1S$ knockdown

The $\alpha 1S$ subunit has a dual function depending on its localization. It is the voltage sensor for EC coupling in triads whereas it forms the L-type Ca^{2+} channel along T-tubules (Romey *et al*, 1988). The biological significance of the L-type Ca^{2+} channel is still unknown, although Bannister *et al* (2009) recently proposed it could contribute to calcium entry involved in the maintenance of myoplasmic calcium levels during either sustained depolarization or during trains of repetitive brief stimuli. It has also been reported in neurons that L-type Ca^{2+} channels could modulate transcrip-

Figure 6 $\alpha 1S$ subunit knockdown and RyR1 expression. **(A)** Lysates from TA^{ΔDHPR} (Δ DHPR) and TA^{Ctrl} (c) were immuno-blotted for RyR1 or α -actin. ($n = 4$). Graph depicts mean $\pm$ s.e.m. of relative expression of RyR1 determined by densitometry and normalized to the α -actin expression for each muscle. Results were expressed in protein levels of RyR1 in TA^{ΔDHPR} normalized to TA^{Ctrl} for each mice, $*P \leq 0.005$, $n = 4$. **(B)** Longitudinal cryo-sections of TA^{ΔDHPR} (Δ DHPR) and TA^{Ctrl} (c) were stained with anti- $\alpha 1S$ subunit (red), anti-RyR1 (green) antibodies and imaged by confocal microscopy. Bars = 20 μ m.

tional activity through EC coupling (Wheeler *et al*, 2008). It is likely that this class of channels could have a similar role in skeletal muscle by mediating the effect of muscle electrical activity on gene expression. Our data strongly suggest that a minor fraction of $\alpha 1S$ located on the sarcolemma could have this role and be involved in muscle differentiation.

Obviously, substantial knockdown of any important protein for muscle function would cause muscle atrophy. Our

results advocate for the involvement of $\alpha 1S$ in a pathway controlling muscle mass through a specific mechanism. Indeed, although denervated muscles also undergo an atrophic process, they never show upregulation of autophagy-related genes and autophagosome formation (Supplementary Figure S6). In addition, the mechanism involved in atrophy after $\alpha 1S$ downregulation cannot be explained by a simple loss of muscle contractility as treated muscles are still EC coupled. Moreover, quantitative defect of RyR1 has been reported in families presenting a recessive form of congenital myopathies with cores (Monnier *et al*, 2008). In some patients, the measurement of protein expression showed a decrease of about 50% of the RyR1 while these patients did not display atrophy of skeletal muscle. These data strongly suggest that a fall of any important protein for muscle function would not systematically result in the same physiological and molecular consequences.

Hence, our results support a new role for the sarcolemmal subpopulation of $\alpha 1S$, which might act as a voltage sensor of a still unknown molecular machinery located on the sarcolemma, in the close vicinity of muscle nuclei, in charge of controlling transcriptional activity of genes involved in the muscle anabolism/catabolism balance.

Materials and methods

Antibodies

For western blot, primary antibodies used were $\alpha 1S$ subunit, phosphorylated FoxO3A and nNOS (Abcam); for immuno-staining $\alpha 1S$ subunit (Chemicon), FoxO3 and LC3b (Cell Signaling), laminin and α -actin (Sigma-Aldrich), P62 (Progen), SERCA and nNOS (Abcam). For MHC isoform analysis primary antibodies were MHC-1 (hybridoma#BA-D5, Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ), MHC-2a (hybridoma#SC-71, DSMZ), MHC-2x (hybridoma#6H1, Developmental Studies Hybridoma Bank) and MHC-2b (hybridoma#BF-F3, DSMZ). RyR1 antibody was a gift from Dr I Marty, secondary antibodies were from Invitrogen and Jackson ImmunoResearch.

$\alpha 1S$ subunit targeting vector construction

Antisense sequences (Supplementary Table S1) were introduced into the U7smOPT gene as described earlier (Goyenvalle *et al*, 2004): U7-c was a non-functional construction used as negative control. U7-SA, U7-ESE and U7-c sequences were introduced separately or at the XbaI site of the pSMD2 AAV2 vector. AAV2/1 pseudotyped vectors were prepared by transfection in 293 cells as described earlier (Riviere *et al*, 2006) and vector particles were purified on cesium chloride gradients from cell lysates obtained 72 h after transfection and titered by qPCR. Titres for U7-SA and U7-ESE were 2.4×10^{13} and 3.0×10^{13} vector genomes (vg) ml^{-1} , respectively.

In vivo gene transfer

All procedures were in accordance with the institutional and European agreement for human treatment of animals. Experiments were performed on adult C57/Black6 mice. Anaesthesia was achieved with a mix of 100 mg/kg ketamine and 10 mg/kg xylazine. Two intramuscular injections in 24 h of a mixture (50 μ l/TA) containing AAV (U7-SA) and AAV (U7-ESE) were carried out in TA of the right hindlimb; the contralateral muscles were injected in the same procedure with vector AAV (U7-Ctrl) and used as control.

Gene expression analysis

Total RNA was prepared from 600 μ m of TA using Quiagen total RNA isolation kit. Complementary DNA generated with Invitrogen Superscript II Plus reverse transcriptase (Invitrogen) was analysed by real-time qPCR performed on Opticon2 (Bio-Rad) using iTaq SyberGreen Supermix with ROX (Bio-Rad). We therefore quantified in all samples transcripts of the PO gene encoding human acidic ribosomal phosphoprotein ubiquitously expressed as the endogenous

Figure 7 Localization of $\alpha 1$ S subunit. Whole skeletal muscle fibres enzymatically isolated from Ctrl (A–D) or Δ DHPR (E–H) FDB muscles 6 months post injection were processed for immuno-fluorescent labelling for $\alpha 1$ S subunit (red) and laminin (green). Scale bars, 10 μ m; Arrows indicate $\alpha 1$ S subunit expression on sarcolemma; antibody labelling was visualized by serial confocal microscopy and represented as movies of the optical sections (Supplementary Movies S5A and S5B). (D, H) Present projections of confocal Z-series (step between each frame is 1 μ m) along XZ and YZ planes as indicated.

RNA control and each sample was normalized on the basis of its PO content. Primers used are listed in Supplementary Table S1.

Force production

Muscle performance was evaluated by the measurement of *in situ* muscle contraction in response to nerve stimulation, as described earlier (Vignaud *et al*, 2005a,b). Animals were anaesthetised (i.p., pentobarbital sodium, 50 mg $\times$ kg⁻¹). The distal tendon of the TAs was attached to an isometric transducer (Harvard Bioscience) using a silk ligature. All data provided by the isometric transducer were recorded and analysed on a microcomputer. The sciatic nerves were distally stimulated by a bipolar silver electrode. Responses to tetanic stimulation (pulse frequency 50–143 Hz) were successively recorded. Absolute maximal tetanic forces were determined. Muscle masses were measured to calculate specific tensions. After contractile measurements, the animals were killed with an overdose of pentobarbital.

Morphometric analysis

For muscle analysis, tissues were sectioned at 10 μ m on a cryostat (Leica CM3050S). The internal diameters (shortest diameter) of all fibres in the total transversal muscle section were recorded and analysed with Elix software (MDS Analytical Technologies).

Dissociated fibre cultures

Myofibres were isolated from the dissected FDB muscle of 6 months post-injection mice by digestion with collagenase 1a (SIGMA) and mechanical dissociation (Kaisto *et al*, 1999). Isolated fibres were cultured (37°C, 5% CO₂) in Dulbecco's Modified Eagle medium with high glucose, 1% horse serum (GIBCO) and penicillin/streptomycin on coverslips coated with Matrigel (Becton Dickinson Labware, Bedford, MA). Myofibres were used for experiments after overnight culture.

Histology and immuno-fluorescence analysis

Muscle sections were stained with haematoxylin and eosin or processed for immuno-histochemistry. Fibrosis was labelled by Red Sirius (3%) then dehydrated with ethanol and cleared in xylene as already described (Dooley *et al*, 2003). Analyses of labelled zone were carried out on Histolab Software (MDS Analytical Technologies). For immuno-staining procedures, 10 μ m sections were cut on a Tissue-Tek II cryostat and fixed on glass slides. Slides were rehydrated in PBS, fixed with 4% PAF for 10 min or not (LC3b and dystrophin), blocked in 4% BSA (Sigma-Aldrich) for 1 h, incubated with primary antibodies for overnight at 4°C, washed in PBS, incubated for 1 h with secondary antibodies, thoroughly washed in PBS, incubated with DAPI for nuclear staining for 5 min and mounted in Fluoromount (Southern Biotech) and finally imaged by confocal laser scanning microscope (LEICA SPE DM2500), the brightness/contrast adjustments done with Photoshop CS version 9.0.2 (Adobe) in control and test samples were identical. For myofibres isolated from FDB, immuno-labelling was performed

directly in the 24-well culture plate. Cells were fixed (15 min, 4% paraformaldehyde in PBS, RT) washed (2 $\times$, 5 min, PBS), permeabilized (10 min. PBS with 0.5% Triton X-100), and immuno-labelled using the same procedure described for muscle sections. Myofibres were analysed by confocal laser scanning microscopy using a LEICA SP2 operating system using an ApoPlan $\times$ 63, 1.40 NA oil lens. DAPI, Alexa-488, Alexa-568 or Cy3 fluorescence were sequentially excited using lasers with wavelengths of 405 nm (DAPI), 488 nm (Alexa-488) and 561 nm (Alexa-568, Cy3). Z-series from the top to the bottom of fibres were sequentially collected for each channel with a step of 1 μ m between each frame. For MHC analysis, frozen unfixed 10 μ m sections were blocked 1 h in PBS plus 1% BSA, 1% sheep serum, 0.01% Triton X-100 and 0.001% sodium azide. Sections were then incubated overnight with primary antibodies against laminin and MHC isoforms. After washes in PBS, sections were incubated 1 h with secondary antibodies. After washes in PBS, slides were finally mounted in Fluoromount (Southern Biotech). Morphometric analyses were made on two serial sections of whole 6 months post-injection TA muscles. Images were captured using a digital camera (Hamamatsu ORCA-AG) attached to a motorized fluorescence microscope (Zeiss AxioImager.Z1), and morphometric analyses were made using the software MetaMorph 7.5 (Molecular Devices).

Western blotting

For $\alpha 1$ S subunit and RyR1, muscle tissues were lysed in a buffer containing 200 mM sucrose, 20 mM Hepes pH 7.4, 0.4 mM CaCl₂ with a mix of protease inhibitors (Roche). Samples were quickly micro centrifuged. Laemmli buffer was added and samples were denatured at RT. Soluble proteins were resolved by SDS-PAGE (3–8% Invitrogen) and transferred to nitrocellulose membranes. For FoxO3A and nNOS, muscles were lysed in RIPA buffer containing 150 mM NaCl, 50 mM Hepes, 1% NP-40, 0.5% sodium deoxyolate, 0.1% SDS, 5 mM EDTA with 1 mM PMSF and a mix of protease inhibitors (Roche). Soluble proteins were resolved by SDS-PAGE and western blotting. Western blots were revealed with enhanced chemiluminescence (Thermo Scientific), films were scanned and band signal intensities were determined using Image Processing and Analysis in Java (version 1.37v).

Ultrastructure analysis

For EM, muscles were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h. After post fixation for 1 h at 4°C in 2% osmium tetroxide and acetone dehydration, the samples were embedded in polybed 812 resin. Ultrathin sections were contrasted with uranyl acetate/methanol (7%) and lead citrate and observed with a Philips CM120 electron microscope.

Statistical analysis

Statistical analysis of differences between mouse cohorts was performed using the Student's *t*-test. Excel (Microsoft) was used for calculations, and results were expressed as mean $\pm$ s.e.m.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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