Satellite cell loss and impaired muscle regeneration in selenoprotein N deficiency

Perrine Castets1,2,3,4,5, Anne T. Bertrand1,2,3,4, Maud Beuvin1,2,3,4, Arnaud Ferry1,2,3,4, Fabien Le Grand6,7, Marie Castets8, Guillaume Chazot8, Mathieu Rederstorff9, Alain Kro19, Alain Lesure8, Norma B. Romero1,2,3,4, Pascale Guicheney1,5 and Valérie Allamand1,2,3,4,*

1UPMC Univ Paris 06, IFR14, Paris F-75013, France, 2CNRS, UMR7215, Paris F-75013, France, 3Inserm, U974, Paris F-75013, France, 4Institut de Myologie, Paris F-75013, France, 5Inserm, U956, Paris F-75013, France, 6Institut Cochin, Université Paris Descartes, CNRS, UMR8104, Paris F-75014, France, 7Inserm, U1016, Paris F-75014, France, 8CNRS, UMR5238, Université de Lyon, Centre Léon Bérard, Lyon F-69008, France and 9Architecture et Réactivité de l’ARN, Université de Strasbourg, CNRS, IBMC, Strasbourg F-67084, France

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Selenoprotein N (SelN) deficiency causes a group of inherited neuromuscular disorders termed SEPN1-related myopathies (SEPN1-RM). Although the function of SelN remains unknown, recent data demonstrated that it is dispensable for mouse embryogenesis and suggested its involvement in the regulation of ryanodine receptors and/or cellular redox homeostasis. Here, we investigate the role of SelN in satellite cell (SC) function and muscle regeneration, using the Sepn1−/− mouse model. Following cardiotoxin-induced injury, SelN expression was strongly up-regulated in wild-type muscles and, for the first time, we detected its endogenous expression in a subset of mononucleated cells by immunohistochemistry. We show that SelN deficiency results in a reduced basal SC pool in adult skeletal muscles and in an imperfect muscle restoration following a single injury. A dramatic depletion of the SC pool was detected after the first round of degeneration and regeneration that totally prevented subsequent regeneration of Sepn1−/− muscles. We demonstrate that SelN deficiency affects SC dynamics on isolated single fibres and increases the proliferation of Sepn1−/− muscle precursors in vivo and in vitro. Most importantly, exhaustion of the SC population was specifically identified in muscle biopsies from patients with mutations in the SEPN1 gene. In conclusion, we describe for the first time a major physiological function of SelN in skeletal muscles, as a key regulator of SC function, which likely plays a central role in the pathophysiological mechanism leading to SEPN1-RM.

INTRODUCTION

Satellite cells (SCs) are essential for adult muscle homeostasis, growth and repair. They are maintained in a quiescent state under basal conditions, but upon injury, they are activated, enter the cell cycle and give rise to a population of muscle precursors that proliferate, differentiate and then fuse to form new fibres, allowing muscle architecture restoration (1). In parallel, a subset of SCs is able to return to its quiescent state, thereby replenishing the initial pool and allowing repeated repair of the tissue (2). Most SCs are identifiable by the expression of the paired box transcription factor Pax7, whereas expression of the myogenic regulatory factors Myf5, MyoD and Myogenin defines muscle precursors upon activation and as differentiation begins (1,3). Self-renewing cells are described as activated Pax7+/MyoD+ cells that maintain Pax7 expression, lose that of MyoD and exit from the cell cycle (4–6). Recently, the Pax7+/Myf5− satellite ‘stem-like’ cells were proposed to ensure the expansion of the SC population, whereas the Pax7+/Myf5+ SCs commit to the myogenesis differentiation programme (7). Several transcription factors and signalling pathways have been described to regulate SC activation and the balance between proliferation and differentiation (1), whereas the mechanisms controlling cell-cycle exit towards a quiescent state remain poorly understood.

*To whom correspondence should be addressed at: Inserm, U974, Institut de Myologie, Groupe Hospitalier Pitié-Salpêtrière, 47, bd de l’Hôpital, 75651 Paris Cedex 13, France. Tel: +33 142165707; Fax: +33 142165700; Email: v.allamand@institut-myologie.org

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Selenoproteins are defined by a specific selenocysteine (Sec) residue inserted in their peptidic sequence via a recoded UGA codon (8). Most of them are involved in cell redox homeostasis, owing to the great reactivity of the Sec amino acid (9). Among them, selenoprotein N (SelN) is the only selenoprotein known to be responsible for a human genetic disorder. Indeed, SelN deficiency, due to mutations in the SEPN1 gene, causes SEPN1-related myopathies (SEPN1-RM), characterized by a generalized early-onset muscle atrophy, myotendinous contractures and muscle weakness, mostly affecting axial muscles and leading to severe scoliosis, spine rigidity and respiratory insufficiency (10–13). Although some data have been recently obtained regarding the function of SelN, the mechanisms underlying the pathology remain largely unknown. A higher oxidant activity and susceptibility to oxidative stress have been detected in vitro in SelN-deficient human cells (14). In addition, physical and functional interactions between SelN and ryanodine receptors (RyR) suggest that SelN may regulate the latter by modulating the redox state of their reactive cysteines (15). However, SelN most likely has additional functions as its expression pattern is ubiquitous and predominant in developing tissues, contrasting with that of RyR in mammals (16). Although SelN appeared essential for zebrafish development (15,17), SelN-deficient mice displayed normal embryogenesis (16) and postnatal growth (Rederstorff et al., in preparation).

Here, we established that SelN plays a major role in SC function, including the generation and/or the maintenance of the SC pool in skeletal muscles. In the mouse, SelN deficiency leads to a reduced SC pool in adult muscles, impaired regeneration and SC exhaustion during this first round of regeneration, hence preventing further restoration of the tissue. Most relevant to the human pathology, we show that muscle biopsies from SEPN1-RM patients specifically display a drastic reduction in the number of SCs. Taken together, these results point towards a novel SelN-dependent mechanism in muscles, most likely central in the pathomechanism of SEPN1-RM.

**RESULTS**

**Adult SelN-deficient muscles display a reduced SC pool**

To gain insights into the role of SelN in SC function, we first characterized the size of the SC pool in SelN-deficient muscles, in 15–21-day, 4- and 10-month-old mice. At these ages, Sepn1<sup>-/-</sup> mice were indistinguishable from wild-type (WT) littermates (Rederstorff et al., in preparation). Pax7 immunolabelling on Sepn1<sup>-/-</sup> tibialis anterior (TA) cryosections revealed that the total number of SCs was unaltered in the younger mice, whereas it was significantly reduced compared with WT at 4 and 10 months (Fig. 1A). Accordingly, at 4 months of age, Pax7 transcript expression was decreased by 41% in Sepn1<sup>-/-</sup> TA compared with WT (Fig. 1B). Likewise, on freshly isolated extensor digitorum longus (EDL) and TA fibres, the number of SCs per fibre was reduced by 40% in 4-month-old Sepn1<sup>-/-</sup> mice (Fig. 1C). Altogether, these findings demonstrate that skeletal muscles from adult Sepn1<sup>-/-</sup> mice exhibit a reduced SC pool.

**SelN is highly expressed during cardiotoxin-induced muscle regeneration**

Following cardiotoxin (CTX) injury in hindlimb compartments of adult WT mice, Sepn1 expression was increased by 3- and 6-fold at 24 h and 3 days, respectively, the latter corresponding to the maximal expression detected, with a progressive decrease observed thereafter (Fig. 2A). These results were reinforced by western blotting, which demonstrated an important increase in SelN expression between days 3 and 10 after injury (Fig. 2B). As increased oxidative constraints are known to occur during muscle regeneration (18), the expression of other selenoproteins was also quantified after injury. A major up-regulation was detected only for SelP transcripts, which peaked at 5 days after injection (Supplementary Material, Fig. S1). We also observed a decrease in the expression of SelW transcripts from 3 to 7 days after injury, probably related to the loss of mature myofibres, and an increase in Sep15 and SelS transcript levels at 3 days that may be linked with their role in inflammatory cells (19) (Supplementary Material, Fig. S1).

To further investigate the implication of SelN in muscle regeneration, immunohistochemical analyses were performed on regenerating muscle sections with a previously characterized SelN antibody (20). No signal was detected in adult uninjured WT muscles, notably in mature fibres and quiescent Pax7<sup>+</sup> cells (Supplementary Material, Fig. S2A). At 3 and 5 days after injury, SelN expression was observed in numerous mononucleated cells surrounding the regenerating fibres, with no signal in newly formed eMHC<sup>+</sup> fibres (Fig. 2C). Similar results were obtained on late embryonic (E18) muscle sections: abundant mononucleated cells were detected around muscle groups and surrounding embryonic fibres (Supplementary Material, Fig. S2B). In order to determine which cell types were expressing SelN in regenerating muscles, immunolabelling with different specific cell markers was performed. Co-immunostaining revealed that SelN was expressed in some Pax7<sup>+</sup> cells, with variable signal intensity (Fig. 2C). A faint signal was also detected in MyoD<sup>+</sup> and Myogenin<sup>+</sup> cells, indicating that SelN was expressed in muscle precursors (Supplementary Material, Fig. S2A). Consistently, by quantitative reverse transcription polymerase chain reaction (qRT-PCR), Sepn1 transcripts were almost undetectable in quiescent murine SCs, using cells freshly purified by Fluorescence Activated Cell Sorting (FACS) (α7integrin<sup>+</sup>/CD31<sup>+</sup> / Sca1<sup>+</sup>/CD45<sup>-</sup>) previously described in (21), whereas a strong expression was detected upon activation (SC-derived myoblasts) (Fig. 2D).

Among the SelN<sup>+</sup> cells detected in regenerating muscles, some of them were CD45<sup>-</sup> at 3 days, with only a few remaining CD45<sup>+</sup> at 5 days (Supplementary Material, Fig. S2A). Using the F4/80 antibody, we further demonstrated that SelN was expressed in some macrophages at 3 days after injury, whereas no co-expression was observed at day 5 (Fig. 2C). These results indicate that hematopoietic cells may express SelN, depending on the cell type and regeneration timing (22). Lastly, only some SelN<sup>+</sup> cells were shown to be BrdU<sup>+</sup>, thereby indicating no correlation between SelN expression and cell proliferation (Supplementary Material, Fig. S2A).

At 7 days after injury, SelN expression was still detected in numerous mononucleated cells, most of which were located...
SelN deficiency leads to massive muscle atrophy by impairing its ability to sustain successive regeneration cycles

The regenerative capacity of adult Sepn1−/− skeletal muscles was evaluated following single (SI) and double (DI) injuries in the hindlimb muscle compartments. Uninjured TA muscles from 4-month-old WT and mutant mice displayed identical mass, contractile properties and morphology (Table 1 and Supplementary Material, Fig. S3A–C). Similarly, in mutant and WT muscles injured only once (SI7, SI15 and SI30), no difference was observed in the injured-to-contralateral TA mass ratio, tetanic forces and muscle section area (Table 1). The number of regenerative fibres per mm², their size distribution and the number of myonuclei per fibre were comparable in WT and Sepn1−/− mice from 7 to 30 days after injury (Fig. 3A and B and Supplementary Material, Fig. S3A–F). However, 15 days after injury, necrotic and calcified fibres were present only in Sepn1−/− muscles (Fig. 3A and B). Furthermore, fat deposition was observed in mutant mice as early as 5 days, with significant 3- and 6-fold increases in its area at 7 and 30 days, respectively, compared with WT (Fig. 3C). These data indicate that SelN deficiency leads to imperfect muscle regeneration: although restoration of the fibres is observed, calcification and adipogenesis indicate a defect in regeneration.

SCs are lost in SelN-deficient muscles during regeneration

As SC renewal during muscle regeneration is essential for efficient repeated repair of the tissue (2), we investigated the behaviour of the SC population during the first round of regeneration. The number of Pax7+ nuclei was first quantified on injured TA sections by immunostaining and expressed per muscle unit area (mm²) or as a percentage of total nuclei located under the basal lamina (n = 3, *P < 0.03). Scale bar, 50 μm. (B) qRT-PCR analysis of Pax7 transcripts in 4-month-old TA muscles (n = 3, *P < 0.04). (C) Quiescent SCs were quantified on single fibres isolated from 4-month-old WT and mutant EDL and TA muscles (n ≥ 5 mice, >330 cells, *P < 0.03). All data are represented as mean ± SEM.

In contrast, 7 and 30 days following the second injury (DI7 and DI30), a significant reduction of the injured-to-contralateral muscle mass ratio was observed in Sepn1−/− mice (Table 1). In addition, a 38% reduction in absolute force was detected in Sepn1−/− muscles at 30 days, whereas specific forces were similar to that of WT (Table 1). This indicates that the reduction in the absolute force of Sepn1−/− muscles was due to its atrophy rather than to a decrease in the contractile properties of the regenerated fibres. The section area of Sepn1−/− TA was 1.6-fold smaller than WT, at 7 and 30 days after the second injury (Table 1 and Fig. 3A). At the cellular level, rare regenerating fibres were detected at 7 days after injury in mutant muscles, the major part of the tissue being occupied by fibrosis, adipocytes and necrotic fibres (Fig. 3B and C). At 30 days, necrosis was no longer observed in Sepn1−/− mice, but fat deposition and fibrosis were still strikingly increased compared with WT muscles that exhibited an almost complete regeneration (Fig. 3).

Collectively, these results indicate that SelN deficiency affects the efficiency of muscle restoration following one injury and totally prevents further regeneration of the tissue.
of the resident pool. In contrast, in Sepn1−/− mice, the number of SCs was strikingly reduced compared with uninjured mutant muscles and injured WT muscles (Fig. 4A). It is worth noting that the few remaining SCs exhibited a correct localization, under the basal lamina. These findings were also observed in regenerating soleus muscles and were further confirmed using an antibody against M-cadherin, another marker of SCs (data not shown). These data indicate that a massive loss of SCs occurs between days 5 and 7 after injury in Sepn1−/− mice.

To further investigate this precocious depletion of SCs, we quantified Pax7 transcript expression from 3 to 30 days after
injury. Although Pax7 expression was similarly increased at 3 days in control and mutant muscles, the extent of Pax7 up-regulation was lower in Sepn1−/− TA from 5 days after injury. At 15 and 30 days, the expression levels detected in mutant muscles were reduced by 5-fold compared with WT and by 2-fold in comparison to unjured Sepn1−/− muscles (Fig. 4B). Similarly, we showed that Myf5 expression was significantly reduced in mutant muscles as early as 5 days and until 30 days after injury (Fig. 4C). These results point to a precocious loss of Pax7/Myf5 transcript expression in Sepn1−/− muscles, preceding the depletion of SCs detected by immunostaining. In contrast, MyoD and Myogenin expression, which reflect the extent of commitment and differentiation of the precursor populations, were unaltered in mutant muscles, at all stages of regeneration studied (Supplementary Material, Fig. S3G and H).

Altogether, these data indicate that the basal defect in the SCs in uninjured Sepn1−/− muscles worsens drastically during muscle regeneration, leading to exhaustion of the SC pool in regenerated Sepn1−/− muscles.

SelN deficiency affects SC function in vitro and increases the proliferation of muscle precursors

The cause of the drastic SC depletion observed in regenerating SelN-deficient muscles was then investigated. Cell death by apoptosis was ruled out by Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling staining and quantitative measurement of the caspase-3 activity in muscles following the first injury (Supplementary Material, Fig. S4).

We then analysed the cell fate decision of SCs in the absence of SelN, by isolating EDL fibres that were cultured for 24, 48 and 72 h. According to Zammit et al. (5), immunostaining was performed to distinguish between non-committed (Pax7+/only), activated (Pax7+/MyoD+) and committed (MyoD+/-only) cells (Fig. 5A). Almost all SCs were activated at 24 and 48 h on both mutant and WT fibres (data not shown). After 72 h in culture, a significant 63% reduction in the Pax7+/MyoD− cell population was observed in mutant compared with WT (Fig. 5A and B). These findings suggest that SelN deficiency affects the cell fate choice of SCs in the context of isolated fibres.

In light of the comparable pool of progenitors detected at 5 days after injury in mutant and WT, which contrasts with the basal defect in the SC population of Sepn1−/− muscles, we hypothesized that SelN deficiency may lead to an enhanced proliferation of muscle precursors. By performing BrdU/Desmin immunostaining in vitro, we demonstrated that the proliferation rate of Sepn1−/− primary myoblasts cultured in growth medium was increased by 60% (Fig. 5C). In contrast, when cultured in low serum-containing medium, mutant cells exhibited a reduction in their proliferation rate and a fusion index similar to that of WT, indicating an efficient differentiation (Fig. 5C and D).

Lastly, immunostaining for MyoD and Ki67 performed on TA sections at 3 days after injury revealed a 55% increase in the proportion of cycling muscle precursors (Ki67+/MyoD−) in injured Sepn1−/− muscles compared with WT (Fig. 5E). Moreover, 5 days after injury, the proportion of Pax7+ cells that were cycling (Ki67+/Pax7+) or proliferating (BrdU+/Pax7+) was significantly increased in mutant muscles (Fig. 5F and G).

Taken together, these data demonstrate that SelN deficiency leads to an enhanced proliferation of muscle precursors, whereas their commitment towards differentiation appears unaltered.

Muscle biopsies from SEPN1-RM exhibit a striking SC loss

To investigate whether the loss of SCs also occurred in human SelN-deficient muscles, Pax7 immunostaining was performed

![Figure 3. SelN deficiency abolishes the repeated regenerative capacity of skeletal muscles. (A, B) TA muscles were stained with HE following an SI (SI7/15: 7 and 15 days after injury) or a DI (DI7/30: 7 or 30 days after second injection). White arrowhead indicates calcified fibres, also shown with Alizarin Red coloration (enlarged inset); black arrowheads, necrotic fibres; arrows, adipocytes. (C) Fat deposition was detected by Oil Red O staining and quantified relative to the total muscle area, in injured TA muscles at SI5, SI7, SI15, SI30, DI7 and DI30 (n ≥ 3, *P < 0.05, **P < 0.008). Scale bar: 500 μm (A, C); 100 μm (B).](https://academic.oup.com/hmg/article-abstract/20/4/694/587112/698_Human-Molecular-Genetics--Vol-20-No-4?highres=true)
on 8 genetically confirmed SEPN1-RM, 14 pathological control and 7 age-matched control muscle biopsies (Supplementary Material, Table S1).

In the control cohort, the number of SCs decreased with age, ranging from 18 to 8 SCs per 100 myonuclei in muscle biopsies from 3–10- and 30–40-year-old individuals, respectively (Fig. 6A and B). By comparison, Pax7 staining on all SEPN1-RM muscle biopsies revealed a drastically reduced SC number at all ages. Moreover, we observed that this defect worsened with age, with 1.5-, 2- and 10-fold reductions detected in muscle biopsies from 3-, 11–23- and 35-year-old patients, respectively, and a complete exhaustion of the SC pool in the biopsy from the oldest patient (Fig. 6A and B). Conversely, patients with confirmed mutations in the RYR1 gene, as well as other pathological controls, exhibited a proportion of SCs very similar to that of control individuals (Fig. 6B).

These striking observations strongly suggest that the early and progressive depletion of the SC population is a specific feature of SEPN1-RM.

**DISCUSSION**

Skeletal muscles possess a high regenerative potential dependent on the resident quiescent muscle progenitors (SCs) that are able both to reconstruct fibres and to self-renew, allowing the maintenance of the initial pool of SCs (2). In this study, we investigated whether SelN, deficient in SEPN1-RM, may play a critical role in muscle regeneration and SC function, taking advantage of the Sepn1<sup>−/−</sup> murine model ((16); Rederstorff et al., in preparation). The results we have obtained point to a decisive role of SelN in SC maintenance and consequently in the regenerative capacity of skeletal muscles.

A reduced basal number of SCs was detected in Sepn1<sup>−/−</sup> adult muscles, suggesting that SelN is involved in SC homeostasis independently from the regeneration context. This may reflect the fact that SelN deficiency impairs the generation of the uncommitted cell population entering into quiescence during the perinatal period or leads to a progressive loss of the quiescent SCs from 1 to 4 months of age (4,23).
Following an SI, we demonstrated that in the absence of SelN, muscle regeneration was imperfect, although the number and size of the regenerated fibres seemed unperturbed. Alteration of the local tissue environment in which the regeneration process occurs could be responsible for the calcification, residual necrotic fibres and fat deposition observed in knock-out (KO) muscles (24). These defects could also reflect a perturbation in the behaviour of the committing SC population that would be compatible with the observed muscle regeneration (25–27). It is possible that fat deposition may also be due to an initial imbalance between muscle and adipocyte progenitors (28,29).

Following the second injury, muscle regeneration was completely abolished in mutant muscles, leading to drastic muscle atrophy, with only rare newly formed fibres and massive fat deposition. We established that this inability of the mutant muscles to undergo repeated cycles of regeneration was associated with the drastic depletion of the SC pool occurring during the first regeneration event. Failure to reconstitute the quiescent SC population during regeneration could be due to (i) cell death, (ii) the inability to generate a sufficient pool of muscle progenitors during the regenerative process, (iii) a perturbed fate determination of these cells from a self-renewing potential to myogenic differentiation or transdifferentiation and lastly (iv) a defect in the niche repopulation. We first ruled out, in vivo, increased cell death as a mechanism for SC depletion in Sepn1−/− regenerating muscles, as well as in 15–21-day-old mice (data not shown). Second, despite an initial defect in the SC number in the muscles of mutant mice, a comparable pool of Pax7+ cells was observed at 5 days after injury in WT and mutant muscles, which was related to an enhanced proliferation of Sepn1−/− muscle precursors. This indicates that following injury, SCs were able to fully expand the pool of muscle progenitors in the absence of SelN. Accordingly, one could hypothesize that SelN may limit the cycling of cells and that the early hyper-proliferation of SCs in SelN mutants is associated with their inability to enter into quiescence (Supplementary Material, Fig. S5). Indeed, we established that Sepn1−/− muscle precursors exhibit enhanced cycling and proliferation rates that could be interpreted as a compensatory mechanism with regard to the initial reduced SC number, but also as a causative process because this was also detected in cultured primary myoblasts. Interestingly, cell differentiation appeared unaltered both in vivo and in vitro, suggesting that SelN plays a key role in the reversible cell-cycle exit towards quiescence but not in the permanent cell-cycle exit towards differentiation. Overall, SelN deficiency may alter the cell fate decision of the pool of activated SCs, as defined by Zammit et al. (5) and as suggested by the results obtained on isolated

Figure 5. SelN deficiency impairs SC dynamics and enhances proliferation of muscle precursors. (A) EDL-isolated single fibres were cultured for 72 h and immunostained for MyoD and Pax7. Nuclei are detected by DAPI staining (blue). Arrows, black and white arrowheads indicate Pax7+–only, Pax7+/MyoD+ and MyoD+–only cells, respectively. Scale bar, 10 μm. (B) The number of Pax7 and/or MyoD-positive cells was quantified relative to the total number of myogenic cells on single fibres at 72 h (n ≥ 3 mice, >1400 cells, *P < 0.02 from WT). (C, D) Primary myoblast cultures were obtained from isolated EDL fibres and cultured in growth (GM) or differentiation (DM) medium. (C) Proportion of BrdU+ cells in the total number of Desmin+ cells in GM and DM (n ≥ 3, *P < 0.03). (D) Fusion index quantified in control and mutant cultures at 24, 48 or 72 h in DM (n ≥ 3). (E) The proportion of cycling muscle precursors (MyoD+/Ki67+) in injured muscles 3 days after injury was quantified after immunostaining on cryosections, with regard to the total number of MyoD+ cells (n = 3, *P < 0.02). The proportion of cycling (Pax7+/Ki67+, F) or proliferating (Pax7+/BrdU+, G) Pax7+ cells was quantified after immunostaining performed on TA sections, 5 days after injury (n = 3, *P < 0.05).
fibres. Hence, almost all *Sepn1*−/− muscle progenitors may commit towards differentiation, with only rare cells maintaining Pax7 expression and exiting from cell cycle to return into quiescence and renew the SC pool (Fig. S5). Based on the model of Kuang *et al.* (7) and as suggested by the quantification of Pax7 and Myf5 transcript expression, SelN deficiency could alter the balance between the ‘stem-like’ cell population and the committed pool, and/or their dynamics during regeneration, notably by affecting the symmetric versus asymmetric division processes. Lastly, as SC dysfunction also occurs in regeneration-independent contexts, we hypothesize that a defect in the niche repopulation is unlikely to be the major process leading to SC loss in the regenerating mutant muscles, but this remains to be demonstrated.

When comparing the defects observed in the absence of SelN during the first cycle of regeneration, both the SC loss and the cellular hypotheses described above could be related to an intrinsic alteration of the SC homeostasis (suggesting a cell-autonomous mechanism) and/or to perturbations of the environmental conditions (including the inflammatory response). Both the basal defect in the SC pool and the modified *in vitro* SC dynamics suggest a mechanism independent of the injury context. However, due to the fact that complete SC exhaustion occurs following one injury, the involvement of the necrosis/regeneration conditions in the aggravation of this SC loss should not be excluded. This could reflect either an increased sensitivity of SelN-deficient SCs to the ‘hostile’ environment of the regenerating muscles or abnormal acute conditions in the absence of SelN. This latter point is supported by the up-regulation of SelN expression during regeneration, which was associated with myogenic, but also non-myogenic (including macrophages) cells, in accordance with a cell non-autonomous mechanism. Indeed, the role of pro- and anti-inflammatory macrophages in regulating both myogenesis and regeneration has been described and supports the hypothesis that SC depletion could be due to extrinsic SelN deficiency (22). Similarly, it has recently been established that fibro/adipogenic progenitors and endothelial cells are also involved in muscle progenitor dynamics and, although we could not demonstrate that SelN is expressed in these cells, they could be associated with the SC defect observed in the absence of SelN (28,30,31).

Recent data have suggested that SelN participates in the regulation of RyR in mature fibres (15). RyR and the IP3 receptor, another calcium channel, have been described to be expressed in SCs (32–34), but no data have yet been obtained regarding their physiological role in SCs. Nevertheless, involvement of calcium-associated pathways in SC dynamics has been suggested (35–37), thus providing a possible link between the proposed role of SelN in Ca2⁺ homeostasis and SC maintenance. As SelN localizes to the endoplasmic reticulum (ER) (20), its suggested reductase activity (38) may modify other proteins, either secreted or located at the plasma membrane or in the ER, notably by modulating their oxidative state. Effectors of pathways involved in SC self-renewal or in the regulation of the inflammatory response are therefore interesting candidates to be considered.

Most importantly, we established that muscles from *SEPN1*-RM patients display a reduced SC population, already detectable in a 3-year-old patient. This defect was specifically identified in *SEPN1*-RM muscles and notably was not observed in Central Core Disease biopsies. In light of the results obtained in the mouse, we hypothesize that this defect is related to an abnormal generation and/or maintenance of the quiescent SC population. Thereby, one could suppose that early SC exhaustion in humans is most likely a central part of the pathomechanism leading to *SEPN1*-RM. In particular, we suggest that the premature depletion of the pool of muscle progenitors that participate in muscle growth could limit both the size and number of developing fibres, a key feature of muscle atrophy. We also propose that this early SC loss could play a role in the midlife decline of the muscular function observed in patients, due to the limited turn-over capacity of SelN-deficient muscle tissues. Based on the clinical variability observed within *SEPN1*-RM, we believe that several other factors may be involved in the
pathomechanism and modulate the consequences of the SC dysfunction described herein.

MATERIALS AND METHODS

Animals

To induce complete necrosis of both TA and soleus muscles, 6.7 and 13 μg of CTX (Latoxan, Valence, France) were injected into the hindlimb anterior and posterior compartments of 4-month-old mice, respectively. Contralateral limbs were sham-injected with physiological serum. Mice were euthanized 6 h, 24 h, 3, 5, 7, 10, 15 or 30 days following injection and muscles were harvested. For double regeneration experiments, CTX was injected as described above, 30 days after the first injection, and muscles were dissected 7 or 30 days later. In single- and double-injection protocols, BrdU (BD Pharmingen, Le Pont-De-Claix, France) was injected intraperitoneally 1 h before sacrifice. All animal studies were performed in accordance with the European Union Guidelines for Animal Care.

Single isolated fibres and primary myoblast cultures

Individual fibres were isolated from EDL and TA of 4-month-old mice, as described in (38), and either directly fixed in 4% paraformaldehyde (PFA) or grown for 1–3 days in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Cergy Pontoise, France) supplemented with 10% foetal bovine serum (FBS; Invitrogen, Cergy Pontoise, France) and 0.5% chicken embryo extract (MP Biomedicals, Illkirch, France), on horse serum-coated culture dishes. For primary myoblast cultures, isolated fibres were obtained from EDL of 15–21-day-old mice and plated on matrigel-coated culture dishes. Myoblasts were grown in F10-media (Invitrogen, Cergy Pontoise, France) supplemented with 20% FBS and 5 ng/ml basic fibroblast growth factor, and differentiated in DMEM 4% horse serum (VWR International, Fontenay-sous-Bois, France).

Quantitative real-time RT-PCR

Total RNA was extracted from muscle cryosections using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Courtaboeuf, France). RNA was also isolated from α7integrin+/CD31−/Sca1+/CD45− FACS-sorted quiescent SCs described in (21) and from these cells maintained in culture for 3 days. Quantitative PCR was performed on DNase-treated RNA, reverse transcribed to cDNA using the SuperScript II First-Strand Synthesis System (Invitrogen), amplified using primers listed in Supplementary Material, Table S2, with the LightCycler480 SYBR Green I Master mix (Roche, Meylan, France) and analysed with the LightCycler480 analysis software (Roche, Meylan, France), as described (16). Data were normalized to the expression levels of the 18s rRNA.

Western blot analysis

Western blot analysis was performed as previously described (16). Membranes were stained with Ponceau Red for loading control and immunoprobred with SelN (ab137; (20)) and pan-Actin (Sigma, Lyon, France, A2066) primary antibodies.

Contractility measurement

The in situ isometric contractile properties of TA muscles were studied as previously described (39). The absolute maximal tetanic force (P0) was recorded and analysed with the PowerLab system (4SP; ADInstruments) and software (Chart 4; ADInstruments). The specific force (P0/m) was calculated as follows: P0/m = P0 (N)/muscle mass (g).

Immunohistochemistry

The following antibodies were used on 5–8 μm muscle cryosections or single isolated fibres: Pax7 (DSHB, IA, USA), MyoD (Santa Cruz, Heidelberg, Germany, sc-304), BrdU (Abcam, Cambridge, UK, ab6326), Ki67 (Dako, Tappes, France, TEC-3), SelN (ab137 (20)), F4/80 (Abcam, Cambridge, UK, ab6640), embryonic MHC (DSHB, IA, USA, F1.652) and laminin (Abcam, Cambridge, UK, ab11575). Following microwave oven antigen retrieval (40), sections were blocked with 3% IgG free bovine serum albumin and ChromoPure Mouse IgG, Fab Fragments (Jackson ImmunoResearch, Suffolk, UK). Individual fibres were fixed with 4% PFA, permeated with PBS, 0.5% Triton-X100 and blocked in PBS, 0.35% carrageenan, 10% goat serum and 10% horse serum. Sections and fibres were incubated with primary antibodies and with appropriate Alexa Fluor secondary antibodies (Invitrogen, Cergy Pontoise, France) or biotin-conjugated antibodies (Jackson ImmunoResearch, Suffolk, France) successively. They were mounted in VectaShield DAPI (Vectorlabs, Peterborough, UK) and observed with an Axioskop microscope (Zeiss, Munich, Germany). Images were captured using the MetaView software (Ropper Scientific, Trenton, NJ).

Histological coloration

Eight-micrometre muscle muscle cryosections were fixed with 4% PFA, stained with haematoxylin/eosin, and dehydrated in ethanol and xylene before mounting in Eukitt medium. For lipid coloration, sections were fixed in 4% formaldehyde, 2% CaCl2 at 4 °C during 30 min, stained with 0.5% Oil Red O during 15 min at 37 °C, counterstained with haematoxylin and mounted in gelatine.

Images and statistical analyses

The MetaMorph (Molecular Devices, CA, USA) and NIS (Nikon) software were used for cell counting and area measurement. Quantifications were performed at 25 × magnification (three section levels for mouse muscles with a minimum of eight fields and a minimum of six fields for human biopsies). Results are presented as mean ± SEM of independent samples or animals; n represents the number of individual experiment (n ≥ 3). Comparisons between groups were performed using the Student’s t-test with a 0.05 level of confidence accepted for statistical significance.
SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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