Selenoprotein N is an endoplasmic reticulum calcium sensor that links luminal calcium levels to a redox activity

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SUMMARY

The Endoplasmic reticulum (ER) is the reservoir for calcium in cells. Luminal calcium levels are determined by calcium-sensing proteins that, in response to calcium fluctuations, trigger calcium dynamics. Here, we report that Selenoprotein N, SEPN1, is a type II transmembrane protein that senses ER calcium fluctuations by binding this ion through a luminal EF-hand domain. In vitro and in vivo experiments show that, via this domain, SEPN1 responds to diminished luminal calcium levels, dynamically changing its oligomeric state and enhancing its redox-dependent interaction with cellular partners, including the ER calcium pump SERCA. Importantly, single amino acid substitutions in the EF-hand domain of SEPN1 identified as clinical variations, are shown to impair its calcium-binding and calcium-dependent structural changes, suggesting a key role of the EF-hand domain in SEPN1 function. In conclusion, SEPN1 is a novel ER calcium sensor that responds to luminal calcium depletion, changing its oligomeric state and acting as a reductase to refill ER calcium stores.
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INTRODUCTION

Calcium is an important second messenger that mediates a plethora of functions ranging from muscle contraction to neurotransmitter release and egg fertilization (1). This ion has a steep concentration gradient across the plasma membrane and the different intracellular membranes, with a cytosolic concentration four orders of magnitude lower than that of the extracellular space or the lumen of the endoplasmic reticulum (ER). Noteworthy, the release of calcium from the intra organelle stores of the ER into the cytosol represents one of the most utilized signaling mechanisms of eukaryotic cells (2).

The ER and its specialized appendix in skeletal muscle, the sarcoplasmic reticulum, are the cellular reservoir for calcium, whose concentration ranges between 100 µM and 1mM (3) (4). Such high concentrations, compared to the low nanomolar concentration in the cytosol, are maintained by the activity of Sarcoplasmic reticulum calcium (SERCA) pumps, which are ER membrane proteins that force calcium entry into the ER by hydrolyzing ATP. Three differentially expressed genes encode at least five isoforms of the SERCA pump (SERCA1a, SERCA1b, SERCA2a, SERCA2 and SERCA3). Among these isoforms, SERCA2b interacts with the oxidoreductase ERp57 inhibiting calcium reuptake into the ER (5).

SERCA activity is opposed by Inositol triphosphate receptor (IP3R) and Ryanodine receptor (RYR), which instead determine calcium release from the ER (6) (7). Both SERCA pump activity and the activity of IP3R and RYR are regulated by redox, indicating a crosstalk between the redox state of calcium handling proteins and their activity in regulating luminal calcium levels (5, 8-10) (11).

Calcium levels in the ER are tightly regulated in order to maintain an environment that is suitable for protein folding and to maintain the steep gradient across the ER membrane required for rapid excitation-contraction coupling. To defend appropriate calcium levels, the ER is equipped with a calcium-sensing mechanism. One of the main components of this mechanism is the ER membrane protein STIM1. Through
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EF-hand domains it senses the reduced calcium luminal levels that occurs in skeletal muscle during excitation contraction coupling, and activates store-operated calcium entry (SOCE), consisting in uptake by the ER of calcium from the extracellular space (12) (13). While this phenomenon is key for the ER/SR to retrieve calcium from the extracellular space, cells, and in particular muscle cells, must also be able to rapidly restore basal low cytosolic calcium concentrations in order to allow the contractile apparatus to rapidly return to the resting state after contraction (14). However, no calcium sensor in the ER/SR, which could directly connect luminal calcium levels with the transfer of cytosolic calcium has been identified so far.

Previously, we reported that SEPN1 is a ubiquitously expressed protein (15), although its loss of function gives rise to SEPN1-related myopathy, activates SERCA2-mediated calcium uptake into the ER in a redox-dependent manner (16) (17). Here, we extend our earlier findings showing that SEPN1, a type II transmembrane protein, is a novel calcium sensor of the ER. The EF-hand domain in SEPN1 is localized in the ER lumen and leads this protein to bind calcium with an affinity constant in the range of the ER calcium concentration. In vivo and in vitro experiments show that low luminal calcium triggers a conformational change in SEPN1, activating it as a reductase. Single amino acid mutations in the EF-hand domain of SEPN1, indexed in the clinical genomic variation database ClinVar, affect SEPN1 calcium affinity and its conformational change. Our findings identify a feedback mechanism, through which SEPN1 senses luminal calcium levels and consequently regulates the ER redox poise, thereby modulating the downstream signal transduction and the SERCA-mediated replenishment of ER calcium stores.

RESULTS

SEPN1 is an ER type II membrane protein

Previous studies identified SEPN1 as an integral membrane protein of the endoplasmic reticulum (ER) (16). To determine the type of membrane insertion, we
first carried out a bioinformatics sequence analysis using the software tools for the prediction of a transmembrane domain, including SOSUI, SPLIT, PSIPRED and TMHMM. All the utilized algorithms produced a consensus output, revealing the presence of a single transmembrane domain in the N-terminal part of SEPN1 sequence and hence suggesting SEPN1 is a type II transmembrane protein. In order to experimentally verify SEPN1 topology, we first checked its N-glycosylation status. Examination of SEPN1 amino acid sequences using dedicated bio-informatics tools predicted the presence of four putative glycosylation sites in SEPN1 at positions Asn156, Asn449, Asn471 and Asn497 (Fig.1A). As the active site of the oligosaccharyl-transferase complex is located in the ER lumen, utilization of any one of these sites would demonstrate SEPN1 translocation across the ER membrane. To assess possible N-glycosylation of SEPN1, we analyzed FLAG-tagged SEPN1 by immunoblotting, after treatment of cell lysate with the enzyme endoglycosidase H (Endo H), which cleaves off N-linked oligosaccharides. As shown in Fig. 1A (lane 4), Endo H treatment caused a shift of the SEPN1 band to a faster migrating species, demonstrating the presence of N-glycosylation sites in the lumen. In agreement with a type II topology of SEPN1, Mass spectrometric (MS) analysis of human SEPN1 heterologously expressed in *Pichia pastoris* demonstrated the presence of high-mannose glycosylation at Asn156, Asn449 and Asn497 (Sup. Fig. 1A and B).

To confirm the type II topology, a protease protection assay was carried out on HeLa cells transfected with SEPN1 carrying either a C-terminal HIS tag or an N-terminal STREP tag (Fig. 1B). Membrane fractions prepared from these cells were digested with trypsin in the presence or absence of detergent. Protein regions residing outside the ER are accessible to protease, whereas luminal protein domains remain protected, but are degraded when the ER membrane is disrupted by detergent treatment. As shown in the Immunoblot of Fig. 1B (middle panel, lanes 1 and 2), the N-terminal STREP-tag was completely degraded by trypsin in the absence of
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detergent. Probing the same blot with an anti-SEPN1 antibody revealed that this
treatment caused degradation of the tag, but not of the majority of the polypeptide
(Fig. 1B, lower panel, lanes 1 and 2). In contrast, the C-terminal HIS tag was largely
protected from trypsin attack in the absence of detergent (upper panel). As expected,
the protected band almost completely disappeared under conditions of simultaneous
trypsin and detergent treatment (top panel, lane 3), and smaller fragments were
revealed by the anti-SEPN1 antibody under this condition (lower panels, lane 3).
Notably, only a slight shift in SEPN1 migration was observed after digestion (Fig. 1,
top panel, compare lanes 1 and 2), as expected if the exposed, cytosolic, N-terminal
domain is limited to the thirty amino acids preceding the predicted transmembrane
domain (amino acids 31 to 50). These results confirm the type II topology of SEPN1
predicted by software analysis tools and by the experimental verification of its
glycosylation.

Calcium-dependent conformational change of SEPN1

Having established the topology of SEPN1, we used bioinformatics tools to search
for known domains within the luminal sequence. This analysis predicted the presence
of a sequence resembling the well-characterized calcium-binding domain, known as
EF-hand, together with a thioredoxin (Trx) domain encompassing the selenocysteine
residue.

Canonical EF-hands usually are composed of an helix-loop-helix structure with two
well-defined alpha-helices that allow calcium ion coordination in a pentagonal
bipyramidal configuration (18). The lack of a solved 3D structure for SEPN1 makes it
impossible to evaluate the architecture of its EF hand directly, therefore we opted for
ab initio structure prediction using the QUARK algorithm
(https://zhanglab.ccmb.med.umich.edu/QUARK/) (19). Modelling of SEPN1 EF-hand
structure reveals a single alpha-helix followed by flexible loop and beta-sheet portion
instead of a second alpha-helix. Therefore, despite the high sequence similarity to an EF hand, the putative SEPN1 EF-hand may have an uncommon structure.

The predicted difference between the simulated structure of the EF-hand in SEPN1 and those of canonical EF-hands raised the question as to whether this domain is functional and able to bind calcium. To solve this conundrum, we generated synthetic peptides of 36 amino acids corresponding to a wild-type SEPN1 EF-hand (WT) and three mutants with single amino acid substitution: D80A, bearing a mutation of the most important calcium coordinating residue in canonical EF-hands; and two mutants M85V and Y86C reported in ClinVar database as genomic variations possibly associated with SEPN1-related myopathy (https://www.ncbi.nlm.nih.gov/clinvar/variation/195363/ and https://www.ncbi.nlm.nih.gov/clinvar/variation/461631/) (Fig. 2A). These peptides were subjected to calcium titration (0 to 5 mM), during which peptide conformational changes were monitored by measuring molecular ellipticity with circular dichroism (CD). The CD spectra for WT revealed significant calcium-dependent conformational changes. In contrast, CD spectra for D80A, M85V and Y86C did not show any conformational change even at calcium concentrations as high as 5mM (Fig. 2B).

The Kd of WT for calcium, calculated from four independent experiments with different peptide preparations, was estimated at 242±50 µM (mean ± SEM) (Fig. 2C and D), a value in line with luminal calcium concentration (20). A similar Kd value (193±11µM) of WT for calcium was obtained by measuring intrinsic tyrosine fluorescence of the peptide (Sup. Fig. 2A-D) and by isothermal calorimetry (129±4µM) (Sup. Fig. 2 E-F). Isothermal calorimetry also led to establishing that the stoichiometry between SEPN1 and Ca^{2+} is 1:1 and to confirm the unresponsiveness of D80A to calcium (Sup. Fig. 2 E-F). In addition, a deconvolution of secondary structure from the CD data by DichroWeb K2D algorithm (http://dichroweb.cryst.bbk.ac.uk/) (21) confirmed that WT peptide was completely
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unstructured before calcium addition (100% random coil), but adopted a more structured form during the calcium titration including an alpha-helix content raising up to 28%, and up to 32% for beta-sheets after addition of 300 µM calcium, which recalls the best predicted structures by QUARK (Fig. 2E).

Taken together, these data suggest that the WT peptide binds calcium with an affinity constant that is in the concentration range of ER calcium and undergoes marked conformational changes upon calcium binding, differently from the three mutants that do not respond to calcium (up to 5 mM of calcium).

**Calcium-dependent oligomerization of SEPN1**

To investigate the effect of calcium concentration on SEPN1 structure and activity in cells, we created a panel of FLAG-tagged SEPN1 mutants and expressed them in mammalian cells (Fig. 3A). By non-reducing Immunoblot SEPN1 appeared in three major bands, corresponding to a predominant form (apparent Mw around 76 kDa), compatible with a glycosylated monomer, a slow migrating form (apparent Mw around 195 kDa, labelled “oligomer”), as well as a shorter form (apparent Mw around 54 kDa), presumably resulting from incomplete translation of SEPN1 mRNA (the selenocysteine-encoding UGA codon can be also read as a stop codon by the translation machinery); indeed this band was absent from the SEPN1U428C mutant, in which the selenocysteine was replaced with the cysteine (Fig.3B, lane 2).

Furthermore, multiple bands close to SEPN1 oligomers were noted, which most likely arise from the association of SEPN1 with its truncated form since SEPN1U428C mutant, without any truncated forms, displayed only one major SEPN1 oligomer band.

Importantly, the capability of SEPN1 to form oligomers was abolished in the EF-hand mutant SEPN1D80A (Fig.3B, lane 7).

As shown in the right-hand part of the blot of Fig. 3A, all the higher Mw forms disappeared upon DTT treatment, suggesting the involvement of disulfide bonds in
the oligomerization. As the cysteine mutant of the Trx domain of SEPN1 (C427S, U/C428S) is still able to form SEPN1 oligomers, we hypothesized that the three cysteines residues upstream to this domain in the SEPN1 sequence (C49, C108, and C243) might be involved in disulfide bridge formation. In order to test their involvement in SEPN1 oligomerization, we mutagenized each of these residues with the redox inert amino acid, serine, and analyzed these mutants under reducing and non-reducing conditions (Fig.3A, lane 4-6). Only SEPN1^C108S lost the ability to oligomerize, indicating the involvement of this cysteine in disulfide bond formation (Fig.3A, lane 5).

Because of the problem of the generation of the truncated form of SEPN1 carrying the selenocysteine UGA codon, all subsequent experiments were carried out with the SEPN1^U428C mutant; when lacking other mutations, we refer to this form simply as SEPN1, and specify only additional engineered mutations.

To investigate whether SEPN1 self-association underlies the generation of high molecular species (oligomers), two differently tagged SEPN1 forms (MYC and FLAG) were co-expressed, and the ability of the anti-FLAG antibody to pull down the MYC-tagged SEPN1 was tested. The results of this co-immunoprecipitation experiment demonstrated indeed the capacity of SEPN1 to self-associate suggesting that the high molecular weight band contains two copies of the SEPN1 monomers (Fig. 3B). However, given its apparent Mw (around 195 kDa), this band could represent either a SEPN1 homodimer with anomalous SDS-PAGE migration, or contain additional components. Keeping in mind this uncertainty, we refer to it as an oligomer.

The absence of the oligomer in the D80A mutant (Fig. 3A, lane 7) suggested that the oligomeric state of SEPN1 might be regulated by calcium. To test this hypothesis calcium was depleted from the ER compartment by, a short (two-hour) exposure of cells transfected with FLAG-tagged SEPN1 or with MYC-tagged SEPN1 to the irreversible SERCA inhibitor, thapsigargin or the reversible one, cyclopiazonic acid.
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(CPA). Both inhibitors incubation led to a shift of the ratio monomer/oligomer of SEPN1 in favour of the monomer (Sup. Fig. 3A and 3B).

To analyze the effect of calcium on the oligomerization of SEPN1 by an alternative method, we compared the sucrose gradient sedimentation profiles of FLAG-tagged SEPN1 and FLAG-tagged SEPN1\textsuperscript{D80A} (EF-hand mutant) in lysates from cells treated or not with thapsigargin. In qualitative agreement with the results of the non-reducing immunoblot (Sup. Fig. 3C) after thapsigargin treatment, FLAG-tagged SEPN1 was recovered mainly as a protein with a molecular weight corresponding to a SEPN1 monomer (61.8% of the total) under thapsigargin conditions, in a region overlapping the bovine serum albumin (BSA) (Mw 66.5 kDa) marker. In the absence of thapsigargin treatment (DMSO-treated cells) SEPN1 was instead recovered nearly completely in its oligomeric form (96.2% of the total) sedimenting slightly ahead of the IgG marker (Mw 150 kDa) (Fig. 3C). Importantly, and in accordance with the results of non-reducing immunoblot, the aggregation pattern of FLAG-tagged SEPN1\textsuperscript{D80A}-transfected cells did not change under the two conditions of thapsigargin (78.4 of the total monomer and 21.5% oligomer) and DMSO (79.7% of the total monomer and 21.5% oligomer); SEPN1\textsuperscript{D80A} was almost exclusively present as monomer in both conditions confirming that calcium binding to SEPN1 is a prerequisite for its monomer to oligomer conformational change (Fig. 3C and 3D).

The sucrose gradient analysis showed that a much higher proportion of SEPN1 is in an oligomeric state under basal ER luminal calcium concentrations than revealed by the non-reducing gel analysis. This suggests that oligomer formation may be mediated by non-covalent bonds, and that only a portion of the oligomer is further stabilized by disulfide bonding. We confirmed this hypothesis by analyzing the peak monomer (fractions 4-5) and oligomer (fractions 7-8) fractions from the sucrose gradients by non-reducing SDS-PAGE-immunoblot. While SEPN1 after thapsigargin treatment and its EF-hand mutant (D80A) treated or not with thapsigargin showed only a monomer band, the oligomer peak fractions showed both monomer and
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oligomer, indicating dissociation of some of the oligomer under denaturing conditions (Fig. 3D).

Thus, both the sucrose gradient and the immunoblot analyses indicate that luminal calcium levels and calcium binding by the EF-hand regulate the oligomeric state of SEPN1.

The attacking amino acid of SEPN1 is in a reduced form

We previously showed that the mutant of SEPN1, SEPN1\textsuperscript{C427S, U428C}, was able to trap interactors in a redox-dependent manner (17). Therefore, we investigated whether the attacking amino acid cysteine 428 (C428) is in a reduced form, that is compatible with the redox state of an attacking amino acid, in both monomeric and oligomeric forms of SEPN1 and in conditions of calcium depletion.

HeLa cells transfected with FLAG-SEPN1\textsuperscript{U428C} and exposed to thapsigargin or its diluent DMSO were harvested and the free thiols were N-ethylmaleimide (NEM) alkylated (Fig.4A); the protein lysates were then immunoprecipitated with FLAG M2 antibody and run on a non-reducing SDS-PAGE. As expected, Coomassie staining of the gel revealed two main bands of FLAG-SEPN1\textsuperscript{U428C} corresponding to the monomer and oligomer. These two bands were excised from the gels, dithiothreitol (DTT)-reduced, and then alkylated with iodoacetamide (IAA), a treatment which led to the labelling of the cysteines involved in disulphide bonds. The samples were then subjected to nLC-ESI-MS/MS sequence analysis (Fig.4A). The mass spectra of the fragmented peptides showed that, irrespective of thapsigargin treatment, both the monomeric and the oligomeric species of SEPN1 contain the C428 alkylated by NEM, hence, showing that they are present in a reduced form compatible with that of a redox-attacking amino acid (Fig. 4B and C).

A redox active SEPN1 trapping mutant displays more interactors upon luminal calcium depletion
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To test whether in conditions of low luminal calcium SEPN1 has a stronger trapping potential, we exploited the two mutants FLAG-SEPN1<sup>C427S, U428C</sup> and FLAG-SEPN1<sup>C427S, U428S</sup>. Previously, these two mutants led to the identification of SERCA2 as a redox-dependent interactor of SEPN1 and to confirm the interaction with SERCA 2a and 2b isoforms by co-immunoprecipitation experiments (17). The first of these mutants remains covalently bound to its substrate via Cys 428, while the second one, in which the attacking cysteine is mutated to a serine residue, serves as a control. We first asked whether the interaction between SEPN1 and SERCA2 was improved under conditions of luminal calcium depletion. HeLa cells were transfected with FLAG-SEPN1<sup>C427S, U428C</sup> and FLAG-SEPN1<sup>C427S, U428S</sup> and exposed to thapsigargin or its vehicle DMSO. After the treatments, cells were harvested and lysate subjected to the immunoprecipitation with FLAG M2 antibody. Immunoblot analysis showed that the co-immunoprecipitation between FLAG-SEPN1<sup>C427S, U428C</sup> and SERCA2 was enhanced under condition of calcium depletion (Fig.5A, lanes 2 and 3) and did not change between FLAG-SEPN1<sup>C427S, U428S</sup> and SERCA2 (Fig.5A, lanes 4 and 5) (17).

To test whether the FLAG-SEPN1<sup>C427S, U428C</sup> has a strong trapping potential in general under condition of calcium depletion, protein lysates were immunoprecipitated with Flag M2 antibody, run on a non-reducing SDS-PAGE and stained with Coomassie. This staining revealed for both FLAG-SEPN1<sup>C427S, U428C</sup> and FLAG-SEPN1<sup>C427S, U428S</sup> two main bands corresponding to monomer and oligomer of SEPN1 and a series of other bands throughout the lanes. Each lane was cut into three slices (1-3) that underwent in-gel tryptic digestion; the eluted peptides were subjected to nLC-ESI-MS/MS sequence analysis, leading to the identification of the peptides belonging to SEPN1 interactors (Fig.5B). The interactors were clustered into two heat maps, obtained by comparing the LFQ intensity of each single protein normalized to the SEPN1 signal (Fig. 5C). The heatmaps obtained from FLAG-SEPN1<sup>C427S, U428C</sup> and
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FLAG-SEPN1$_{C427S,\ U428S}$ in DMSO-treated cells showed no important difference in terms of abundance of the interactors; instead the abundance of interactors was strikingly in favor FLAG-SEPN1$_{C427S,\ U428C}$ when the cells had been exposed to thapsigargin. Thus, the redox capability of SEPN1 is more pronounced in conditions of luminal calcium depletion.

Furthermore, the pathway analysis of SEPN1 interactors indicated enrichment in proteins of the response to endoplasmic reticulum stress (Sup. Fig. 4) highlighting an important role of SEPN1 during ER stress response as already hypothesized in (22) (23).

**The reductive shift in the ER redox poise triggered by calcium depletion is absent in SEPN1 KO cells**

To investigate the role of endogenous SEPN1 we generated SEPN1 knock out (KO) HeLa cells, using the CRISPR/CAS9 technology. The resulting SEPN1 KO cells had undetectable SEPN1, as revealed by immunoblot with a specific SEPN1 antibody (Sup. Fig. 5 A).

To track changes in ER redox poise, we took advantage of an ER-localized roGFP2, a redox biosensor that was shown to be a PDI client, and transfected it into WT and SEPN1 KO cells (24) (25). The roGFP2 co-localized with luminal ER protein disulphide isomerase (PDI), confirming its ER localization in WT and SEPN1 KO cells (Fig. 6A). Next, we measured the redox changes of this sensor in live cells after exposure to DTT or thapsigargin by comparing sensor emission intensity at 525 nm when excited at 405 (Ex$_{405}$Em$_{525}$) and 488 (Ex$_{488}$Em$_{525}$) nm. The baseline redox-signal of the ratio between Ex$_{405}$Em$_{525}$ and Ex$_{488}$Em$_{525}$ was set at 1; thus, a signal > 1 or <1 after DTT or thapsigargin indicated respectively the oxidation or the reduction of the sensor compared to baseline.

As expected, ER roGFP2 was rapidly reduced after DTT-treatment and oxidized to baseline values after the washout of the reductant both in WT and SEPN1 KO cells.
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Moreover, WT and SEPN1 KO cells showed similar redox profile of roGFP2 during the DTT treatment and its washout (Fig. 6B). A clear difference between WT and SEPN1 KO cells was instead observed in response to thapsigargin treatment. As previously reported (25), in WT cells, addition of thapsigargin caused a progressive reduction of the redox sensor over a timespan of about twenty minutes, after which the probe remained in a reduced state. Differently from WT cells, the redox profile of roGFP2 in SEPN1 KO cells was not responsive to thapsigargin (Fig. 6C and Sup. Fig. 5B), suggesting that SEPN1 is an important player involved in the increased reduced poise of the ER lumen after calcium depletion, and indicating that it plays a key role in adjusting the lumen’s redox poise to calcium levels.

**DISCUSSION**

SEPN1-related myopathy (SEPN1-RM) is a congenital disorder arising from loss-of-function mutations in the SEPN1 gene. It presents in infancy with a heterogeneous clinical manifestation spanning from a mild myopathy to severe muscle weakness that can lead to death due to respiratory failure (26).

Functional analyses on a SEPN1 knock-out mouse model suggested that the lack of SEPN1 leads to redox and calcium store impairment, thereby sensitizing skeletal muscle to oxidative insult and leading to chronic ER stress, which is part of the pathogenic mechanism of SEPN1-related myopathy (27) (22, 23, 28). However, the impact on the muscle phenotype of single SEPN1 mutations, which span the whole gene sequence in humans, has not been investigated so far and could explain the clinical heterogeneity in SEPN1-RM.

A major obstacle to this lack of genotype/phenotype correlation is the paucity of data on SEPN1 function, partly due to the difficulty in obtaining a pure and functionally active SEPN1, as it is a membrane and a selenocysteine-containing protein. As an alternative to the full-length protein, we used peptides containing the putative EF-hand domain of SEPN1 to probe for calcium-induced conformational changes, and
Selenoprotein N is an ER calcium sensor expressed the selenocysteine to cysteine variant of SEPN1 in cells to test whether the protein is activated as a reductase after ER calcium depletion. In this study, we were able to show that SEPN1 senses luminal calcium and, when the level of this ion is above 300 µM, a conformational change in a more organized structure is induced in the protein; furthermore, upon luminal calcium depletion, a SEPN1 oligomer, that is prevalent under basal calcium concentrations, dissociates to generate the monomeric polypeptide. The self-association between SEPN1 protomers is mediated by non-covalent interactions, and also by a disulphide bridge involving C108, which is immediately downstream to the EF-hand. Interestingly, this cysteine is not evolutionary conserved, as it appears only in the Homo lineage and is reported as a single nucleotide polymorphism (SNP) in the respective sequence databases, with a low allele frequency in all populations 0.16 (0.30 in highest population) according to 1000 Genomes data (http://www.ensembl.org/Homo_sapiens/Variation/Explore?db=core;r=1:25804663-25805663;v=rs7349185;vdb=variation;vf=502242574). This low frequency might suggest a potential detrimental effect of this cysteine variant, perhaps related to a dampened oligomer-monomer transition upon calcium depletion. In addition, we show that in conditions of low calcium, SEPN1 is more present as a monomer and presumably with the thioredoxin domain more accessible to targets. Accordingly, we observed an enhanced redox trapping potential of SEPN1, not only versus its target SERCA2, but in general versus many other interactors, indicating an enhanced activity as a reductase and a contribution to the ER stress response pathway as suggested by the interaction with proteins belonging to this pathway.

Thus, our studies support not only that SEPN1 is one of the long-sought reductases of the ER, but also that its redox activity is regulated by calcium levels (29). Previously, we characterized SERCA2 isoforms as SEPN1 interactor in human cultured cells and we showed a longer relaxation time after electrical stimulation in
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SEPN1-depleted *flexor digitorum brevis* (FDB) muscle fibers, indicating reduced calcium entry in the sarcoplasmic reticulum, which is in good agreement with reduced SERCA activity also in SEPN1 KO muscle. Thus, we can hypothesize an effect of SEPN1 on muscle and non-muscle SERCA isoforms (22). Therefore, considering that SERCA2 is activated to pump calcium into the ER after reduction of the two luminal cysteines, our results suggest that SEPN1 acts as an intermediary between ER calcium handling and redox regulation to refill the ER/SR calcium store in skeletal muscle (17) (5, 30) (22) (Fig.7).

Our findings are particularly relevant to the pathogenic mechanism of SEPN1-RM, as the three single-amino acid mutants of the EF-hand domain of SEPN1 that we analyzed all displayed impaired calcium affinity and calcium–dependent conformational change, potentially affecting SEPN1 redox function. Similarly to the SEPN1 mutants investigated here, mutations in the EF-hand of STIM1 affect calcium-binding and lead a pathogenic phenotype due to constitutive STIM activation (31). Further analysis will be important to determine whether the mutations in the EF-hand of SEPN1 similarly lead to constitutive reductase activation.

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**Author Contribution**
AC, EV, SC, SF, AC, AC, MB, MB, LK conducted the experiments, AC, AB, MS, NB, AL, AB, GM EZ designed and analysed the experiments, AC, AL, NB and EZ wrote the paper.

**Declaration of Interests**

The authors declare no competing interests.
MATERIALS AND METHODS

Expression constructs

Expression plasmids encoding FLAG-tagged human SEPN1 (WT protein and active site mutants C427S/U428C and C427S/U428S) in pSelExpress vector were previously described (17). Cells transfected with pSelExpress-SEPN1 WT were cultured in the presence of 500 nM sodium selenite to facilitate the expression of full-length SEPN1. Same WT sequence was also subcloned into pcDNA3.1 vector (Invitrogen) and further used for mutagenesis. Single amino acid substitutions in SEPN1 sequence (C49S, D80A, M85V, Y86C, C108S, C243S, U428C) in either pcDNA3.1 or pSelExpress expression vectors were made using Agilent QuikChange II XL Site-Directed Mutagenesis Kit according to manufacturer’s instructions. The mutagenic oligonucleotide primer pairs for each substitution can be found in Supplementary Methods. All resulting constructs were verified by Sanger sequencing.

For creation of His-tagged SEPN1 construct, the sequence coding for a U428C mutant of human protein was PCR amplified and cloned into the pQE-TriSystem vector (Qiagen), resulting in SEPN1 construct with 8xHis-tag at C-terminus. In parallel, primers that introduced a StrepII-tag at the N-terminus were used to amplify the same sequence, and the PCR fragment was cloned into the eukaryotic expression vector pXJ41 (15).

The pcDNA3 roGFP2 plasmid encoding SS_FLAG_roGFP2 (where SS is an artificial signal sequence) under control of CMV promoter was a gift from David Ron.

Cell culture and transfection

SEPN1 KO HeLa cells were generated by using CRISPR/Cas9 technology (Origene) following manufacturer’s guidelines. pCas-Guide constructs encoding Cas9 and custom guide RNA sequence (GAACTGGCGCTGAAGACCCT) targeting exon 2 of
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human SEPN1 gene were ordered from Origene. 293TN and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 2 mM glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37°C. The cells were transfected at 20-50% confluence with FuGENE HD transfection reagent (Promega) using optimized conditions. Briefly, 3 ul of reagent were used for every 1 ug of DNA, and the transfection mix was prepared in Opti-MEM (Gibco). For experiment with His- and Strep-tagged SEPN1, cells were grown in 6-well plates and transiently transfected at 80% confluency with Nanofectine (PAA, GE Healthcare) using manufacturer’s recommendations. Cell-based assays were performed in triplicate.

**SEPN1 protease protection assay**

Forty-eight hours post-transfection HeLa cells were washed with PBS and scraped in 100 µl PBS, followed by centrifugation at 600 g for 5 min at 4°C to pellet cells. The cells were lysed in 100 µl of buffer A (0.25 M sucrose, 5 mM HEPES pH 7.4). Protein concentration was then determined using standard Bradford protocol.

Trypsin treatment and microsome enrichment: 50 µg of total protein were incubated with 10U of DNAsel, RNAse free (Fermentas) in a final volume of 100 µl of buffer A for 30 min at 30°C. Then, 5 µg trypsin (T8003, Sigma-Aldrich) were added and protein digestion was conducted for 30 min at room temperature. The volume was then adjusted to 1 ml with cold buffer A containing protease inhibitors (1 mM AEBSF and 1 mM benzamidine), and samples were centrifuged at 100,000 g for 1 h at 4°C in order to generate a microsome-enriched fraction. Microsomes were resuspended in 30 µL buffer A and half of the sample was loaded on an analytical gel. For control treatment on membrane disrupted microsomes: 50µg of total protein fraction were adjusted to a volume of 100 µl with buffer A and incubated with 10U of DNAsel for 30
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min at 30°C and centrifugation at 12,500 g for 20 min at 4°C. The membrane pellet was resuspended in 30 µl of buffer A supplemented with 0.2% Triton-X100, with or without trypsin (0.1 µg trypsin/µg protein) and incubated at room temperature for 30 min.

**Western blotting**

Cells were lysed in cold buffer containing 150 mM NaCl, 20 mM HEPES pH 7.5, 10 mM EDTA and 1% Triton X100, and supplemented with protease inhibitors cocktail (Roche) and 20 mM NEM. Protein concentration was determined by standard BCA assay (Pierce). Samples with equal protein concentration were mixed with non-reducing Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 0.01% bromophenol blue) and heated for 5 min at 95°C. For reducing SDS-PAGE, samples were supplemented with 100 mM DTT. Protein samples separated by either reducing or non-reducing SDS-PAGE were then transferred to Protran nitrocellulose membrane (Merck) and probed with the following antibodies: mouse pan-actin from Sigma Aldrich (clone C4, 1:5000), Selenoprotein N (A-11, 1:1000) and SERCA2 (F-1, 1:1000) from Santa Cruz Biotechnology, anti-His (1:4000) from Pierce, or mouse anti-Strep (1:2000) from IBA BioTAGnology.

**EndoH treatment**

293TN cells transfected with pSelExpress-SEPN1U428C were lysed in standard lysis buffer described above. Buffer exchange was performed on PD-10 column (GE Healthcare) by using gravity protocol as described in manual, and samples were eluted with PBS. A portion of the eluate (36 ul) containing 50 ug of total protein was supplemented with 4 ul of 10x glycoprotein denaturating buffer (New England Biolabs) and heated at 100°C for 10 minutes. A half of the resulting sample was then treated with recombinant endoglycosidase H (EndoH, New England Biolabs) with 1000 units of enzyme in 30 ul total reaction volume, supplemented with 10x
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GlycoBuffer 3 (New England Biolabs), for 1 hr at 37°C. A parallel control reaction was performed under the same conditions but without EndoH. The samples were then mixed with 4x Laemmli buffer and analyzed by Western blotting. Two experimental replicates were performed for this assay.

Bioinformatic analysis

SEPN1 topology was analysed by several secondary structure prediction algorithms, including SOSUI (version 1.11, http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html), SPLIT (version 4.0, http://splitbioinf.pmfst.hr/split/4/), PSIPRED (version 4.0, http://bioinf.cs.ucl.ac.uk/psipred/) and TMHMM (version 2.0, http://www.cbs.dtu.dk/services/TMHMM/). A FASTA sequence corresponding to human SEPN1 isoform 2 from UNIPROT database (https://www.uniprot.org/uniprot/Q9NZV5-2.fasta) was used as an input.

Peptide synthesis

Peptides corresponding to SEPN1 sequence (residues from T67 to S102) were synthesized by solid-phase chemistry using FMOC (fluorenylmethyloxycarbonyl) group protected amino acid with Syro-I peptide synthesizer (Biotage) at 0.1 mM scale. Solutions containing the peptides purified by reverse phase HPLC (>95% purity) were freeze-dried and the powder stored at -80°C until use.

Circular dichroism (CD)

Lyophilized synthetic peptides were dissolved in calcium-free buffer (Tris/acetate 2 mM, pH 7.5) and adjusted to 33 uM concentration. Far-UV CD spectra were recorded on Jasco J-815 CD spectropolarimeter at 25°C in the wavelengths range 190 – 260 nm, in a quartz cuvette with 1 mm optical pathway (Hellma 110-QS). The following
conditions were utilized: resolution, 0.1 nm; bandwidth, 1.0 nm; sensitivity, 100 mdeg; response, 16 s; speed, 20 nm/min; and accumulation, 1. All CD measurements were made at least in triplicate using three different batches of synthetic peptides. Buffer signal was subtracted and the resulting spectra were normalized at 260 nm. Calcium titration experiments were performed with increasing calcium concentrations (0; 0.01; 0.03; 0.1; 0.3; 1; 3; 5 mM). The dissociation constant of the EF-hand peptide complex with calcium (Kd[Ca$^{2+}$]) was calculated by using Hill equation (Hill constant equal to 1). The equation was solved for CD values at 222 nm by estimating initial values of molar ellipticity $[\theta]_{\text{max}}$ and $[\theta]_{\text{min}}$ and finding the best fits using non-linear least squares curve fitting method. To deconvolute the secondary structural types present in the peptides’ spectra, CD data were analyzed using the DichroWeb server (http://dichroweb.cryst.bbk.ac.uk/html/process.shtml) with the K2D algorithm (32).

**Immunoprecipitation**

Cells were lysed in cold lysis buffer supplemented with protease inhibitors cocktail (Roche) and 20 mM NEM. Samples containing 1-2 mg of total protein were precleared using SureBeads protein G magnetic beads (Bio-Rad Laboratories) for 1 hr and incubated with 20-30 ul of EZview Red anti-FLAG M2 affinity gel (Sigma-Aldrich) for 16 hrs at 4°C. Beads were then washed 4 times with lysis buffer, and immunoprecipitated proteins were detached from beads by heating to 70°C for 5 min in 2x non-reducing Laemmli buffer. All immunoprecipitation-based assays were performed in triplicate.

**Protein digestion and mass spectrometry**

Proteins were FLAG-immunoprecipitated after transfection of plasmids in SEPN1 KO HeLa cells, which were previously treated with either thapsigargin or DMSO. The proteins were resolved on a non-reducing 10% SDS-PAGE gel and stained by
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Coomassie. The whole lane was divided in three slices that were excised, reduced by 10 mM DTT, alkylated by 55 mM NEM and digested overnight by trypsin. Acidified peptide mixtures were desalted and concentrated on StageTipC18 (33) and injected as technical replicates on a nLC-ESI-MS/MS quadrupole Orbitrap QExactive-HF mass spectrometer (Thermo Fisher Scientific). Two injections per sample were performed as technical replicates.

For the analysis of cysteine modifications in SEPN1^{U428C}, bands corresponding to monomer and oligomer of the protein were excised and first treated with 55 mM NEM to alkylate the reduced cysteines, then disulfide bonds were reduced by 10 mM DTT and alkylated by 55 mM iodoacetamide (IAA). Protein was double digested first by trypsin overnight and then by Asp-N; acidified peptides mix was treated as described above. Proteins were identified and quantified processing raw files with MaxQuant. Raw files of the proteomic data together with all peptides identified and parameters used for the analysis were deposited into Peptide Atlas repository (PASS01535).

Immunofluorescence

Cells grown in ibidi µ-Slide 8 well were fixed in a complete growth medium supplemented with 3.7% formaldehyde for 15 min at 37°C. After several washes with PBS, cells were permeabilized by 0.2% Triton X100 in PBS, 10 min at room temperature (RT). Blocking of non-specific binding was performed by incubation with 20% normal goat serum (Vector Laboratories), 30 min RT. Primary antibody (mouse anti-PDI, clone 1D3, Enzo) was diluted at 1:200 in blocking solution. Secondary antibody (goat anti-Mouse IgG Alexa Fluor 546, ThermoFisher) were used at 1:500 dilution. Samples were incubated sequentially with primary and secondary antibodies for 1 hr at RT, with several washes with PBS / PBS + 0.5% Tween 20. Nuclei were stained with Hoechst 33342 (ThermoFisher) diluted to 1 µg/ml in PBS, 5 min RT.
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Images were acquired by confocal microscopy (Nikon A1) as previously described (23). Immunofluorescence assay was performed in two experimental replicates.

Sucrose gradient

Subconfluent HeLa cells were transfected and after 40 hrs were treated with cycloheximide (CHX, Alfa Aesar) at 50 ug/ml in complete growth medium for 6 hrs. CHX was used as an inhibitor of protein synthesis in order to decrease the amount of monomeric/oligomeric SEPN1 species preexisting to the following treatment. After CHX washout, cells were incubated with 1 uM thapsigargin (Tg) or DMSO as mock for 2 hours under standard growth conditions, then gently washed in PBS and collected by scraping in cold PBS. Cells were pelleted at 1200 g for 3 min (4°C) and subsequently incubated with PBS, NEM 20 mM (5 min on ice) and then washed twice with cold PBS and once with PBS supplemented with protease inhibitors cocktail. Pelleted cells were lysed in a buffer containing 20 mM NaCl, 25 mM TrisHCl pH 7.4 and 1% Triton X100 and supplemented with protease inhibitors and subsequently the unsoluble material pelleted at 16000 g for 10 minutes. Supernatant was collected after centrifugation and loaded on top of linear sucrose gradient (5-20% sucrose in a buffer containing 20 mM NaCl, 25 mM TrisHCl pH 7.4 and 0.2% Triton X100) prepared in 2-chamber gradient mixer. Centrifugation was performed in Beckman Optima 90 centrifuge equipped with SW-41 rotor, at 37,000 rpm for 17 hrs (4°C). After centrifugation, 19 individual fractions for each sample were collected by peristaltic pump. Each fraction was precipitated by TCA and redissolved in a small volume of PBS. Samples were then mixed with Laemmli buffer and loaded on SDS-PAGE. Fluorescent Western blotting was used to visualize FLAG-tagged proteins, with anti-FLAG M2 (1:1000) as primary antibody and IRDye 680RD goat anti-Mouse IgG (Li-Cor, 1:15000) as secondary antibody. Fluorescent signal from dried membranes was acquired on Li-Cor Odyssey instrument and quantified by Li-Cor Image Studio software. Two experimental replicates were analyzed.
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Confocal microscopy and ratiometric image analysis

Confocal ratiometric microscopy was done on a Nikon A1 confocal scan unit with a 40x objective at 1.49 zoom, managed by NIS elements software. Images at 512x512 pixels were obtained using laser excitation of 405 or 488 nm and emission light collected with a 525/50 nm filter, with a sequential scanning mode to avoid bleed-through effects. Random fields of view with 7-10 cells per condition were acquired longitudinally and analyzed as follows. Image analysis was done using the Ratio Plus ImageJ plugin. Briefly, we manually traced the cells to define quantification regions of interest. For each channel, namely Ex<sub>405</sub>Em<sub>525</sub> and Ex<sub>488</sub>Em<sub>525</sub>, we normalized background noise and applied a correction factor so to have an intensity ratio Ex<sub>405</sub>Em<sub>525</sub>/Ex<sub>488</sub>Em<sub>525</sub> approximately to 1 at baseline. The same correction factor was applied to calculate channel intensity ratio after background normalization at subsequent time points. Five experimental replicates were analyzed.

Statistics

All data were analysed using Prism 7 software (GraphPad).
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Figure Legends:

Figure 1. SEPN1 is a type II ER membrane protein

A) Schematic representation of the recombinant SEPN1 construct exploited in the experiments related to this figure and indicating the four glycosylation sites (Asn156, Asn449, Asn471 and Asn497). FLAG Immunoblot representing FLAG-SEPN1 from transfected cells from which proteins were extracted with lysis buffer, the buffer was exchanged by PD-10 desalting column and the proteins digested by the endoglycosidase Endo H. Ponceau S is shown as protein loading control.

B) Schematic representation of the two recombinant SEPN1 constructs exploited in the experiments related to this figure. The human SEPN1 was fused either to a His 6-tag at its C-terminal (SEPN1-HIS) or to a Strep-tag at its N-terminal end (STREP-SEPN1). TM domain stands for a predicted transmembrane domain between amino acids 31 to 50, as predicted by TMHMM Server v2.0; the U428C mutation is indicated. HeLa cells expressing SEPN1-HIS, STREP-SEPN1 or untransfected were subjected to anti-STRP, anti-HIS and anti-SEPN1 antibodies detection. Membrane enriched protein fractions were treated with detergent Triton-x100, and followed by limited trypsin digestion. Microsome-enriched fractions were subjected to trypsin digestion in absence of the detergent, to maintain membrane integrity. Both anti-tags and anti-SEPN1 detection indicated the expression of an intact SEPN1 of around 75 kDa. Trypsin treatment applied to the native membrane fractions remove the N-terminal Strep-tag whereas preserved the His-tag. Both tags were digested in the detergent-dissociated membrane fractions treated with trypsin and short-degraded forms of SEPN1 were detected.

Figure 2. Ca$^{2+}$ binding and Ca$^{2+}$-dependent conformational change in SEPN1

A) Alignment of SEPN1 predicted EF-hand sequence to the EF-Hand HMM Logo plot (obtained from PFAM https://pfam.xfam.org/family/PF00036#tabview=tab4). The
important conserved amino acid D80 within SEPN1 sequence, depicted in red, was mutated to A. Pathogenic mutants identified within this sequence are highlighted by a star and correspond to the mutants M85V and Y86C. Residue numbering is based on UniProt entry Q9NZV5.

B) Circular dichroism spectra of the indicated peptides at different calcium concentrations (0-5 mM).

C) CD (mdeg) values at 220 nm of the WT peptide at different calcium concentrations.

D) KdCa^{2+} of WT calculated from CD values at 220 nm in four different experiments with different peptide preparations.

E) Table indicating analysis of the secondary structure of the WT by K2D algorithm (http://dichroweb.crytb.bbk.ac.uk/).

**Figure 3. Ca^{2+}-dependent oligomeric change of SEPN1 in cells**

A) Scheme of FLAG-SEPN1 protein and its mutants. The EF-hand domain, the thioredoxin (TRX) domain and all the amino acid mutants are noted. Below, non-reducing and reducing FLAG-Immunoblot of the indicated FLAG-SEPN1 and its mutants. The truncated, monomeric and oligomeric SEPN1 is indicated. Asterisk indicates an additional band that is visible for the selenocysteine-containing form and whose origin we have not analyzed. Membrane staining with Ponceau S is shown as protein loading control.

B) Scheme of FLAG-SEPN1 and MYC-SEPN1 protein. Immunoblot of MYC-tagged SEPN1 immunopurified with FLAG-M2 antibody from lysate of HeLa cells that were untransfected or transfected with expression plasmids of the indicated proteins. The lower two panels represent the 5% of the total input protein lysate immunopurified. The proteins were resolved on reducing SDS-PAGE.
C) Scheme of FLAG-SEPN1 and FLAG-SEPN1<sup>D80A</sup>. Sucrose gradient analysis of FLAG-SEPN1 and FLAG-SEPN1<sup>D80A</sup> in HeLa cells treated for two hours with DMSO or thapsigargin. Samples were analyzed on 4-20% sucrose gradients. Equal aliquots of the 19 fractions were analyzed by FLAG Immunoblot. The positions and the molecular weight of size markers (BSA, IgG and catalase) are established from Coomassie-stained gel. On the right, tables indicating the percentage of the monomer and the oligomer.

D) Non-reducing FLAG Immunoblot of the peak fractions of BSA (which has similar size to SEPN1 monomer) and IgG (which has similar size to the oligomer) indicating that SEPN1 protomers are kept together to form the oligomer not only by a disulphide bridge (running as an oligomer) but also by weak interactions (running as a monomer).

**Figure 4. The attacking amino acid of SEPN1 in TRX domain is present in a reduced form**

A) Schematic representation of a SEPN1 construct exploited in the experiments related to this figure. Coomassie stained non-reducing SDS-PAGE of FLAG-immunopurified SEPN1 after treatment with thapsigargin (Tg) or DMSO indicating the bands of the monomeric and oligomeric form that were cut and the redox state of cysteines analyzed by nLC-ESI-MS/MS sequence analysis. Lanes 1: transfected SEPN1<sup>U428C</sup> and treated with DMSO, 2: empty, 3: transfected with SEPN1<sup>U428C</sup> and treated with Tg.

B) Representative MS/MS spectrum for the peptide (423-432) derived from the monomer of SEPN1 and bearing NEM-alkylation of the two cysteines 427 and 428.

C) The table reports the redox state of Cysteine 427 and 428 in SEPN1 after treatment with Tg or DMSO. Alkylation is reported as + NEM if the cysteines are alkylated by NEM and present in a reduced form; or as + Carbamidomethyl if the
cysteines are alkylated by IAA and present in an oxidized form; the probability of different alkylation types (derived from MaxQuant analysis) is reported in parenthesis.

**Figure 5. SEPN1 trapping mutant in conditions of low luminal calcium engages a higher number of interactors**

A) Schematic representation of SEPN1 constructs exploited in the experiments of this figure. FLAG and SERCA2 Immunoblot of FLAG-tagged SEPN1 immunopurified with FLAG-M2 antibody from lysate of cells that were untransfected or transfected with expression plasmids of the indicated proteins and treated with Tg or DMSO. The lower two panels represent the 5% of the total input protein lysate immunopurified. The proteins were resolved on reducing SDS-PAGE. On the right, a graph indicating the relative levels of SERCA2 associated to its bait, FLAG-SEPN1 (set to 1) in arbitrary units in three different experiments (N=3, unpaired t-test, P<0.05).

B) Coomassie stained non-reducing SDS-PAGE of FLAG-immunopurified SEPN1 from cells after treatment with Tg or DMSO indicating the bands of the monomeric and oligomeric form. Each lane was divided into three slices (1-3) that were cut and analyzed for protein identification by mass-spectrometry. Lanes 1: transfected with empty expression vector, 2: empty, 3: transfected with SEPN1\textsuperscript{C427S, U428C} and treated with Tg, 4: empty, 5: transfected with SEPN1\textsuperscript{C427S, U428C} and treated with DMSO, 6: empty, 7: transfected with SEPN1\textsuperscript{C427S, U428S} and treated with Tg, 8: empty, 9: transfected with SEPN1\textsuperscript{C427S, U428S} and treated with DMSO.

C) Unsupervised Hierarchical Clustering heat maps show the comparison between SEPN1\textsuperscript{C427S, U428C} versus SEPN1\textsuperscript{C427S, U428S} and SEPN1\textsuperscript{C427S, U428C} versus SEPN1\textsuperscript{C427S, U428S} treated with Tg. Numbers 1-3 indicates the interactors identified in the slice 1-3 of the Coomassie stained non-reducing SDS-PAGE in B.
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Figure 6. A reducing shift is lacking in SEPN1 KO cells after luminal calcium depletion

A) Fluorescent photomicrographs of WT and SEPN1 KO cells transiently expressing an ER-localized roGFP2, immunostained for PDI proteins as an ER marker. The merge panels with orthogonal views show an overlap of the roGFP2 signal with PDI (scale bar 20 µm).

B) Traces of time-dependent changes in the fluorescence excitation ratio of roGFP2, reflecting the alterations in the redox state of roGFP2 localized in the ER of WT and SEPN1 KO cells. Cells were exposed to a DTT pulse of 20 minutes followed by a washout of the reductant.

C) Cells were exposed to the irreversible SERCA inhibitor Tg for two hours. Each data point represents the mean ± SEM of the fluorescence excitation ratio of roGFP2. The experiment was reproduced five times with similar results (P<0.001, two-way ANOVA, see Sup. Fig. 5 B).

Figure 7. SEPN1 working model

Within the ER/SR, SEPN1 senses calcium levels by binding this ion through an EF-hand domain. When luminal calcium concentration gets low (under 300 µM), SEPN1 conformation changes and causes a shift towards a monomeric form, which is redox-active versus its partner (among others) SERCA. Activation of SERCA then leads to calcium entry into the ER and refilling of calcium stores.
Significance Statement (120 word)

SEPN1 is a type II transmembrane protein of the endoplasmic reticulum (ER) which senses luminal calcium through an EF-hand domain. Upon calcium depletion, a SEPN1 oligomer, prevalent under basal calcium concentration, dissociates, to generate a monomeric polypeptide which has an enhanced redox trapping potential not only for its target the calcium pump, SERCA2 but in general for many other interactors indicating an enhanced activity as a reductase. Thus, our studies support not only that SEPN1 is one of the long-sought reductases of the ER but also identify a feedback mechanism, through which SEPN1 senses luminal calcium level to modulate the downstream signal transduction and the SERCA-mediated replenishment of ER calcium stores, a crucial mechanism for excitation-contraction coupling in skeletal muscle.
Figure 1

A

B

Table:  

| Treatment          | 1 | 2 | 3 | 4 | 5 | 6 |
|--------------------|---|---|---|---|---|---|
| SEPN1-Tag:         | + | + | + | - | - | - |
| Triton:            | - | - | + | + | - | + |
| Trypsin:           | - | + | + | - | + | + |

IB: anti-SEPN1
IB: anti-STREP
IB: anti-HIS
IB: anti-FLAG
IB: anti-FRAG
Figure 2

**TLGTDGLFLFSSLDTDGMYISPEEFKPIAELTGS**

A

B

C

D

E

| [Ca\(^{2+}\)] mM | Alpha helix | Beta sheet | Random coil |
|-----------------|-------------|------------|-------------|
| 0               | 0.00        | 0.00       | 1.00        |
| 0.01            | 0.00        | 0.00       | 1.00        |
| 0.03            | 0.00        | 0.01       | 0.99        |
| 0.1             | 0.27        | 0.32       | 0.41        |
| 0.3             | 0.28        | 0.32       | 0.40        |
| 1               | 0.28        | 0.32       | 0.40        |
| 3               | 0.28        | 0.32       | 0.40        |
| 5               | 0.28        | 0.31       | 0.41        |
**Figure 3**

**Panel A**

- SEPN1: WT U428C C49S C108S C243S D80A - WT C49S C108S C243S D80A
- DTT: - - - - - - + + + + + +

**Panel B**

- SEPN1: NH2 FLAG EF-hand Trx COOH
- NH2 MYC EF-hand Trx COOH

**Panel C**

- IB: anti-FLAG
- IB: anti-MYC
- Input 5%

**Panel D**

- IP: anti-FLAG
- IB: anti-FLAG
- IB: anti-MYC
- Fraction: 4 5 7 8 4 5 7 8
- Tg: - - - - + + + +
Figure 5

A

B

C

IP: anti-FLAG

Input 5%

- -
- -- - -
-
-- - -
- -
- -
no Tg
Tg
0
1
2
3
SERCA2  
bound to SEPN1C427S, U428C 
*
Figure 6

(A) ro-GFP2, PDI, merge

(B) DTT, DTT washout

(C) Tg

ro-GFP2 Ratio Ex405/Em525 vs. Ex488/Em525

WT

SEPN1 KO
Figure 7

Cytoplasm

ER lumen

Ca\(^{2+}\)

Ca\(^{2+}\) depletion

SERCA

Ca\(^{2+}\)

Ca\(^{2+}\)
Significance Statement (120 word)

SEPN1 is a type II transmembrane protein of the endoplasmic reticulum (ER) which senses luminal calcium through an EF-hand domain. Upon calcium depletion, a SEPN1 oligomer, prevalent under basal calcium concentration, dissociates, to generate a monomeric polypeptide which has an enhanced redox trapping potential not only for its target the calcium pump, SERCA2 but in general for many other interactors indicating an enhanced activity as a reductase. Thus, our studies support not only that SEPN1 is one of the long-sought reductases of the ER but also identify a feedback mechanism, through which SEPN1 senses luminal calcium level to modulate the downstream signal transduction and the SERCA-mediated replenishment of ER calcium stores, a crucial mechanism for excitation-contraction coupling in skeletal muscle.
Selenoprotein N is an endoplasmic reticulum calcium sensor that links luminal calcium levels to a redox activity

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Supplementary Results

Results related to Supplementary Figure 1

We confirmed the glycosylation pattern of SEPN1 by heterologous expression of human (hSEPN1) and zebrafish (zSEPN1) SEPN1 in Pichia pastoris followed by mass-spectrometric analysis. Both zSEPN1 and hSEPN1 recombinant proteins were successfully expressed and secreted in the supernatant after induction with methanol, and purified by immobilized metal ion affinity chromatography (IMAC). The produced SEPN1 proteins appeared as a major band or as a doublet on SDS–PAGE migrating, around 72 kDa (Supp Fig. 1A), higher than the expected mass for the recombinant proteins (56 and 57kDa for zSEPN1 and hSEPN1, respectively), indicating the presence of post-translational modifications.

Bio-informatics analysis predicted the presence of two NxS/T consensus sites for N-linked glycosylation in zSEPN1 at positions Asn451 and Asn499, and four putative glycosylation sites in hSEPN1 at positions Asn156, Asn449, Asn471 and Asn497. Studies of nLC-MS/MS spectrometry allowed the peptides assignment to the entire zSEPN1 sequence except two peptides flanking the two predicted glycosylation sites. Analyses using mannose and N-acetylglucosamine (GlcNac) as diagnostic ions confirmed the glycosylation at both sites. The glycosylation of the high mannose type was characterized as two GlcNac and ten mannoses on Asn451 and two GlcNac and fourteen mannoses on Asn499 (Supp Fig. 1B). The MS/MS spectra obtained on the human protein revealed the modification of Asn156 with two GlcNac and eleven mannoses and the probable modifications of the two conserved Asn449 and Asn497 as the corresponding tryptic peptides were not detected at their expected unmodified mass (data not shown). Finally, the second non-conserved site in hSEPN1 Asn471 was unambiguously identified to be not modified. Altogether, the presence of N-glycosylations on the Asn of SEPN1 is in agreement with its intraluminal localization.
Figure legends

**Supplementary Figure 1**

A) Expression of zebrafish (zSEPN1) and human (hSEPN1) proteins in the yeast *Pichia pastoris* expression system. Selenocysteine to cysteine mutants of zSEPN and hSEPN sequences were cloned into the pPICZαA vector (Invitrogen™). In these constructs, the SEPN1 N-terminal sequence, including the transmembrane domain, was substituted by the yeast α-factor signal sequence, that allowed for efficient secretion in the medium of the recombinant protein. The two constructs were transformed into yeast *Pichia pastoris* GS115. The culture media containing the secreted recombinant zSEPN1 (upper panel) and hSEPN1 (lower panel) proteins and the nickel-affinity chromatography purified fractions were analyzed by SDS-PAGE. The expressed proteins were abundantly represented in the culture media (lanes 1). Both proteins were retained on the column, as a band around 72 kDa absent in the flow-through fractions (lanes 2). After two washes, zSEPN1 or hSEPN1 were eluted from the column using imidazole (lanes 5 to 8). A single band was obtained for the purified zSEPN1, while purified hSEPN1 appeared as a doublet.

B) LC-MS/MS analysis of the modified peptides from purified zSEPN1. The two peptides, which sequences are depicted on the top, correspond to tryptic peptides including residues Asn451 (upper panel) or Asn499 (lower panel) of zSEPN1. Both peptides are glycosylated according to their intact mass (not shown) and the MS/MS fragmentation pattern from the precursor peptide at m/z=1111.64 (8+, eluted at 43.52min) and at m/z= 1161.80 (4+, eluted at 59.57min) respectively. We can confidently deduce that these peptides are modified by a high-mannose N-glycosylation due to (i) the sequence of 11 and 12 consecutive amino acids matching only on SEPN1 protein, (ii) the presence of a single consensus site NxS/T in each tryptic peptide, and (iii) the detection of four diagnostic glycan ions in the two patterns (immonium ions displayed on the spectra according to the international nomenclature: circle = Hex, square = HexNAc).

**Supplementary Figure 2**
Tyrosine fluorescence spectra of the WT (A) and D80A (B) peptides recorded at different calcium concentrations showed a clear change in emission for the WT peptide, but no significant change for the mutant of the EF-hand motif. C) Plot of calcium concentrations vs changes in fluorescence intensity at single wavelength (310 nm) displayed a typical saturation curve. D) KdCa2+ of WT calculated from tyrosine fluorescence values at 310 nm in three different experiments. E) ITC

**Supplementary Figure 3**

A) Non-reducing and reducing immunoblot of cells transfected with FLAG-SEPN1 or with B) MYC-SEPN1 and exposed to the irreversible (thapsigargin) or the reversible (CPA) SERCA inhibitors. The two tables on the right indicate the monomer/oligomer ratio in arbitrary units.

C) Reducing and non-reducing Immunoblot of the input protein lysate of the sucrose gradients in Figure 3C.

**Supplementary Figure 4**

Pathway annotation of proteins interacting with SEPN1C427S, U428C after ER calcium depletion (Tg) by Gene Ontology (GO) program searching for biological process and below, related table with the adjusted p-values. Proteins belonging to the ER stress response pathway stand out as indicated by the lowest p-value in the table.

**Supplementary Figure 5**

A) Non-reducing and reducing immunoblot of endogenous SEPN1 in lysates of WT and SEPN1 KO HeLa cells with the indicated treatments. An anti Actin blot serves as the loading control.

B) Traces of time-dependent changes in the fluorescence excitation ratio of roGFP2, reflecting the alterations in the redox state of roGFP2 localized in the ER of WT and SEPN1 KO cells exposed to Tg. The trace represents data from 5 different experiments (N=50 cells, two-way ANOVA, P<0.001).

**SUPPLEMENTARY METHODS**
Mutagenesis

Single amino acid substitutions in SEPN1 sequence (C49S, D80A, M85V, Y86C, C108S, C243S, U428C) in either pcDNA3.1 or pSelExpress expression vectors were made using Agilent QuikChange II XL Site-Directed Mutagenesis Kit according to manufacturer's instructions. The mutagenic oligonucleotide primer pairs for each substitution are given in a table below (all sequences in 5’-3’ order). All resulting constructs were verified by Sanger sequencing.

| Mutant | Forward primer | Reverse primer |
|--------|----------------|----------------|
| C49S   | Tgcctagcggagactctcacggtgca | Tgcagccgtgagagtctccgctaggca |
| D80A   | Catgtctccatcggtggcaggagctaacag | Ctggttagctcccgtgccacccagagatggagacatg |
| M85V   | Tcaggagagaagctacgcttccatcgggtctcc | Ggacacccagagagctggtacatcttcttctga |
| Y86C   | Attcctcaggagagatgcaggtctccatcgggt | Cacgcgatggagacatgtgcatcttctcctgaat |
| C108S  | Ttctcttctcagagaagcagagggtcggggttcc | Ccacacacgctgagccatccttactgaggaagagaa |
| C243S  | Tgacggtcagggatggcagacacagcggggtgctgg | Ggcggtgtgcacccctgtggagcagctta |
| U428C  | Agttcgtccggatcgcagcatgactgacgctg | Cgacgcagctgatcgccggatcgcggacgaact |

Cell culture and transfection

SEPN1 KO HeLa cells were generated by using CRISPR/Cas9 technology (Origene) following manufacturer's guidelines. pCas-Guide constructs encoding Cas9 and custom guide RNA sequence (GAACCTGGCCGCTGAAGACCCT) targeting exon 2 of human SEPN1 gene were ordered from Origene. Subconfluent HeLa cells were transfected in 6cm Petri dishes with 5 ug of pCas-Guide DNA using OptiMEM medium (Gibco) and FuGENE HD transfection reagent (Promega) at 1:3 DNA:reagent ratio according to manufacturer's manual. Transfection medium was substituted with normal growth medium 16 hours post transfection. 72 hours post transfection cells were collected, lysed and analyzed by Western blotting for Cas9 expression. Expression of SEPN1 was analyzed by RT-PCR. Individual clones were isolated by diluting the transfected cellular pool and
seeding in 96-well plate at a density of <1 cell per well. Clones were grown for 1 week before further analysis. Genomic DNA was isolated from clones in 6-well plates. PCR using genomic DNA as a template was performed with oligonucleotide primers specific for introns surrounding exon 2 of SEPN1 gene (forward: 5’-ctcaggaagatggtggaga-3’; reverse: 5’-ctcagtgaagacaccgttg-3’). Introduction of InDels destroying the correct SEPN1 sequence was verified by sequencing of purified PCR products.

293TN and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 2 mM glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37°C.

The cells were transfected at 20-50% confluence with FuGENE HD transfection reagent (Promega) using optimized conditions. Briefly, 3 ul of reagent were used for every 1 ug of DNA, and the transfection mix was prepared in Opti-MEM (Gibco). 16 hours post transfection the medium was replaced with complete DMEM.

For experiment with His- and Strep-tagged SEPN1, cells were grown in 6-well plates and transiently transfected at 80% confluency with Nanofectine (PAA, GE Healthcare) using modified manufacturer’s recommendations. Notably, 3 µl of Nanofectine were used for 2 µg total vector DNA per well of a 6-well plate. Twenty-four hours post-transfection cells were washed with phosphate buffered saline (PBS) and cultured as described above.

**Peptide synthesis**

Peptides corresponding to SEPN1 sequence (residues from T67 to S102) were synthesized by solid-phase chemistry using FMOC (fluorenlymethyloxycarbonyl) group protected amino acid with Syro-I peptide synthesizer (Biotage) at 0.1 mM scale. Merck Nova Syn TGA resin (0.26 mmol/g) was used. The FMOC group was automatically removed using 22% of piperidine in N-methylpyrrolidone solution and amino acids activated with TBTU (O-(benzotriazol-1-yl)-N,N,N,N-tetramethyluronium-tetrafluoroborate) and N,N-diisopropylethylamine. After the last coupling cycle of each amino acid, a capping step with acetic anhydride was performed. Peptides were then cleaved from the resin with a cocktail of TFA (trifluoroacetic acid) and TIS (triisopropylsilane) (95:5
vol/vol) for 3 hours, precipitated and washed with cold diethyl ether 3 times. Subsequently the peptides were purified by reverse phase HPLC on a semi-preparative C18 column with mobile phases of 0.1% TFA in water (eluent A) and 0.08% TFA in acetonitrile (eluent B), using a linear gradient from 5 up to 100% of eluent B in 60 min. The peaks were collected and accurate masses of peptides were recorded by MALDI-TOF mass spectrometer (ABI 4800, Applied Biosystems) operating in reflector mode. The solutions containing the peptides with a purity greater than 95% were finally freeze-dried and the powder stored at -80°C until use.

Intrinsic tyrosine fluorescence

Lyophilized synthetic peptides were dissolved in calcium-free buffer (Tris/acetate 2 mM, pH 7.5) and adjusted to 33 uM concentration. Fluorescent spectra were recorded on Perkin Elmer LS-50B spectrophotometer with FL WinLab software, at room temperature in a quartz cuvette with 10 x 4 mm optical pathway (Hellma 108-QS). The following conditions were used: emission wavelengths range, 285 – 400 nm; excitation wavelength, 274 nm; and scan speed, 60 nm/min. All measurements were made at least in triplicate using two different batches of synthetic peptides. Buffer signal was subtracted from the resulting spectra. Calcium titration experiments were performed with increasing calcium concentrations (0; 0.05; 0.25; 0.5; 1; 2.5 mM). The dissociation constant of the EF-hand peptide complex with calcium (Kd[Ca^{2+}]) was calculated by using Hill equation (Hill constant equal to 1). The equation was solved for fluorescence intensity values at 310 nm by finding the best fits using non-linear least squares curve fitting method.

ITC

Mass spectrometry analysis

Peptides were separated on a linear gradient from 95% solvent A (2% ACN, 0.1% formic acid) to 50% solvent B (80% acetonitrile, 0.1% formic acid) over 36 min and from 50 to 100% solvent B in 2 min at a flow rate of 0.25 µl/min on UHPLC Easy-nLC 1000 (Thermo Scientific) connected to a 25-cm fused-silica emitter of 75 µm inner diameter (New Objective, Inc. Woburn, MA, USA), packed in-house with ReproSil-Pur C18-AQ 1.9 µm beads (Dr Maisch Gmbh, Ammerbuch, Germany) using a high-pressure bomb loader (Proxeon, Odense, Denmark).
Peptides deriving from the monomeric and oligomeric form of SEPN1\textsuperscript{U428C} were separated on a linear gradient from 95% solvent A (2% ACN, 0.1% formic acid) to 50% solvent B (80% acetonitrile, 0.1% formic acid) over 23 min and from 50 to 100% solvent B in 2 min at a flow rate of 0.25 µl/min

MS data were acquired using a data-dependent top 15 method for HCD fragmentation. Survey full scan MS spectra (300–1650 Th) were acquired in the Orbitrap with 60000 resolution, AGC target 3\textsuperscript{e6}, IT 20 ms. For HCD spectra, resolution was set to 15000 at m/z 200, AGC target 1\textsuperscript{e5}, IT 80 ms; NCE 28%, isolation width 1.2 m/z and a dynamic exclusion of 20 sec.

To improve the detection of SEPN1\textsuperscript{U428C} peptides containing Cys +NEM and +IAA was applied an inclusion mass list with m/z of 2+ and 3+ for tryptic and Asp-N predicted peptides.

Proteins were identified and quantified processing raw files with MaxQuant ver. 1.5.2.8 searching against the database uniprot_cp_human_2015_03, trypsin specificity (or trypsin and AspN for SEPN1\textsuperscript{U428C}) and up to two missed cleavages; N-ethylmaleimide and N-ethylmaleimide + water of cysteine, oxidation of methionine were set as variable modifications. Mass deviation for MS/MS peaks was set at 20 ppm. The peptides and protein false discovery rates (FDR) were set to 0.01; the minimal length required for a peptide was six amino acids; a minimum of two peptides and at least one unique peptide were required for high-confidence protein identification.

Reverse and know contaminants hits were eliminated; LFQ Intensities of identified proteins were normalized by the corresponding SEPN1 intensity and z-score was calculated with Perseus ver. 1.5.0.31. For analysis of the modification on cysteines of SEPN1\textsuperscript{U428C}, raw files were processed with MaxQuant ver. 1.5.2.8 searching against the database uniprot_cp_human + SEPN1\textsuperscript{U428C} sequence.