Effects of sarcolipin deletion on skeletal muscle adaptive responses to functional overload and unload

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Abstract

Overexpression of sarcolipin (SLN), a regulator of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs), stimulates calcineurin signaling to enhance skeletal muscle oxidative capacity. Some studies have shown that calcineurin may also control skeletal muscle mass and remodeling in response to functional overload and unload stimuli by increasing myofiber size and the proportion of slow fibers. To examine whether SLN might mediate these adaptive responses we performed soleus and gastrocnemius tenotomy in wild-type (WT) and \(Sln^{-/-}\) mice and examined the overloaded plantaris and unloaded/tenotomized soleus muscles. In the WT overloaded plantaris, we observed ectopic expression of SLN, myofiber hypertrophy, increased fiber number, and a fast-to-slow fiber type shift, which were associated with increased calcineurin signalling (NFAT dephosphorylation and increased stabilin-2 protein content) and reduced SERCA activity. In the WT tenotomized soleus, we observed a 14-fold increase in SLN protein, myofiber atrophy, decreased fiber number, and a slow-to-fast fiber type shift, which were also associated with increased calcineurin signalling and reduced SERCA activity. Genetic deletion of \(Sln\) altered these physiological outcomes, with the overloaded plantaris myofibers failing to hypertrophy, increase in fiber number, and transition towards the slow fiber type, while the unloaded soleus muscles exhibited greater myofiber atrophy, reductions in fiber number, and an accelerated slow-to-fast fiber type shift. In both the \(Sln^{-/-}\) overloaded and unloaded muscles, these findings were associated with elevated SERCA activity and blunted calcineurin signaling. Thus, SLN plays an important role in adaptive muscle remodeling potentially through calcineurin stimulation, which could have important implications for other muscle diseases and conditions.

**Keywords:** SERCA, calcineurin, NFAT, muscle growth, muscle atrophy, tenotomy, stabilin-2
**Introduction**

Skeletal muscle contractility, energy metabolism, and remodeling in response to altered activity and/or loading patterns are regulated by Ca$^{2+}$ (7). The sarcoplasmic reticulum (SR) with the SR Ca$^{2+}$ (SERCA) pump and other important SR proteins make up the membrane system that regulates calcium inside muscle cells. Sarcolipin (SLN) is a small (31 amino acids) integral SR protein that regulates SERCA pump function through physical interactions. When bound to SERCA, SLN inhibits the rate of SR Ca$^{2+}$ uptake by reducing the apparent Ca$^{2+}$ affinity of SERCA and/or reducing maximal SERCA activity (2, 26, 61, 62) and uncoupling SERCA-mediated Ca$^{2+}$ transport from ATP hydrolysis (10, 57). Studies examining the physiological role of SLN using Sln knockout (Sln$^{-/-}$) (5, 9, 47, 62) and Sln overexpressing mice (35, 58) have established that SLN is a crucial regulator of muscle thermogenesis and key modulator of skeletal muscle contractility and metabolism.

A recent study showed that skeletal muscle-specific Sln overexpression augments calcineurin activity (35). Calcineurin is a serine threonine phosphatase that is activated by sustained low-amplitude elevations in intracellular Ca$^{2+}$ [Ca$^{2+}$i], (15, 17), and it has been shown to promote a slow-oxidative fiber phenotype (15, 17, 60) and myofiber hypertrophy (19, 54). Park *et al.* (2016) have shown that calcineurin increases the expression of stabilin-2, a phosphatidylserine receptor that is critical for myoblast fusion, by dephosphorylating nuclear factor of activated T-cells c1 (NFATc1) (45), the most prominent NFAT isoform in skeletal muscle (59). Although there is considerable debate regarding calcineurin’s role in mediating myofiber hypertrophy (30, 42, 48, 51, 55), genetic deletion of stabilin-2 in mice reduces myofiber size (45), suggesting that
calcineurin may have a role in promoting myofiber hypertrophy through myoblast fusion. In support of this view, recent findings from our laboratory showed that genetic deletion of \textit{Sln} in a mouse model of centronuclear myopathy reduced the number of slow fibers and decreased muscle mass through reductions in both myofiber cross-sectional area (CSA) and total fiber count (22). Importantly, these findings were all associated with blunted calcineurin signaling as indicated by elevations in the phosphorylation status of NFATc1 and lowered stabilin-2 expression (22).

Overloading and unloading skeletal muscle are two classical models employed in the examination of muscle remodeling, and calcineurin plays a vital role in each. For example, functionally overloaded plantaris muscles exhibit myofiber hypertrophy, and a fast-to-slow fiber type shift in response to greater and longer durations of load-bearing activity (23, 25, 31); however, these adaptations are attenuated if calcineurin signaling is inhibited (19, 38). Conversely, unloading skeletal muscle, in general, causes myofiber atrophy and a slow-to-fast fiber type shift (16, 37, 49). These effects of skeletal muscle unloading can be countered by calcineurin activation (36, 56). To further examine SLN’s role in enhancing calcineurin signaling and adaptive muscle remodeling, we overloaded and unloaded the plantaris and soleus muscles, respectively in wild-type (WT) and \textit{Sln}-null (\textit{Sln}\textsuperscript{-/-}) mice. Based on our previous findings (22), we hypothesized that the genetic deletion of SLN would result in smaller myofiber sizes, lower fiber number counts, and exhibit a faster fiber phenotype in overloaded and unloaded muscles. These hypothesized findings would be associated with altered SERCA function and blunted calcineurin signaling.
Materials and Methods

Mice. *Sln*−/− mice were described previously (3) and were a kind gift from Dr. Muthu Periasamy. The *Sln*−/− mice (34.9 ± 1.0 g) and their WT littermates (33.3 ± 1.0 g) used in this study were adult mice ranging from 3-6 months of age. Animals were housed in an environmentally controlled room with a standard 12:12-hour light-dark cycle and allowed access to food and water *ad libitum*. All animal procedures were reviewed and approved by the Animal Care Committee of the University of Waterloo and are consistent with the guidelines established by the Canadian Council on Animal Care.

Simultaneous mechanical plantaris overloading and soleus unloading surgery. To mechanically overload the plantaris muscles while simultaneously unloading the soleus muscle, WT and *Sln*−/− mice (3-4 months) were first anaesthetized with 2% isofluorane in a precision vaporizer. Next, the soleus and gastrocnemius tendons were transected as previously described (31) and mice were left for two weeks in individually housed cages to adapt. Mice were injected subcutaneously with Metacam analgesic (1 mg/kg body weight) once daily for 3 days post-surgery. After two-weeks, the mice were sacrificed by cervical dislocation and the plantaris (sham and overload) and soleus (sham and unloaded) muscles were isolated and either homogenized in homogenizing buffer (250 mM sucrose, 5 mM HEPES, 0.2 mM PMSF, 0.2% [w/v] NaN₃) or embedded in O.C.T. compound (Tissue-Tek) frozen in liquid nitrogen-cooled isopentane and then stored at -80°C until further analysis.
**Western blotting.** Western blotting was performed to determine expression levels of SLN, phosphorylated-NFATc1 (p-NFATc1), total NFATc1, and stabilin-2. Standard glycine-based SDS-PAGE on 7.5% total acrylamide gels was performed for p-NFATc1, total NFATc1, and stabilin-2, whereas tricine-based SDS-PAGE on 13% total acrylamide gels was performed for SLN. Primary antibodies against p-NFATc1 (PA5-38301) and total NFATc1 (MA3-024) were obtained from Pierce Antibodies. The primary antibody directed against SLN was generated by Lampire Biological Laboratories (21). The primary antibody for stabilin-2 was obtained from Biorbyt (orb158499). Quantitation of optical densities was performed using GeneTools (Syngene, MD, USA) and values were normalized to the densitometric sum of all bands visualized through ponceau staining or actin, and expressed relative to sham in order to compare the responses to the overload and unload stimuli between genotypes. There were no significant differences in the absolute values between WT sham and Sln^−/− sham soleus or plantaris muscles (data not shown).

**Immunofluorescent staining.** Soleus and plantaris muscles embedded in O.C.T. compound were cut into 10 µm thick cryosections with a cryostat (Thermo Electronic) maintained at -20°C. Immunofluorescence analysis of MHC expression was performed as previously described (8) with primary antibodies against MHCI, MHCIIa, and MHCIIb to assess fiber type distribution. Fibers that were not positively stained with MHCI, IIa, or IIb antibodies were considered as type IIX fibers. Slides were visualized with an Axio Observer Z1 fluorescent microscope equipped with standard red, green,
blue filters, an AxioCam HRm camera, and AxioVision software (Carl Ziess). AxioVision software was also used to stitch all images in order to capture the whole muscle cryosection, and quantification of fiber distribution, total fiber number, and cross-sectional area (CSA) was performed using imageJ software.

**SERCA activity assay.** SERCA activity was assessed in soleus and plantaris muscle homogenates over Ca$^{2+}$ concentrations ranging from $p$Ca 7.1 to 4.8 in the presence of the Ca$^{2+}$ ionophore A23187 (Sigma C7522) using a Ca$^{2+}$-dependent, enzyme-linked spectrophotometric plate reader assay that has been described previously (18). Maximal SERCA activity was taken from the raw data and SERCA’s apparent affinity for Ca$^{2+}$ was measured with the $p$Ca$_{50}$. The $p$Ca$_{50}$ is the [Ca$^{2+}$] required to elicit half-maximal SERCA activity, and was obtained after SERCA activity-$p$Ca curves were generated with GraphPad Prism™ by non-linear regression curve fitting using an equation for a general cooperative model for substrate activation.

**Statistics.** All values are presented as means ± standard error (SEM). Statistical significance was set to $P \leq 0.05$. Most comparisons were made using a two-factor mixed model ANOVA with surgery (sham vs. overload or unload) analyzed as a ‘within-subjects’ factor and genotype (WT vs. $Sln^{-/-}$) analyzed as a ‘between-subjects’ factor. We also made planned comparisons on the effects of overload and unload (vs. sham) separately in WT mice and $Sln^{-/-}$ mice using a Fisher’s LSD test. A Student’s t-test was used to compare sham soleus muscle weights between genotypes. All statistical analyses were done using GraphPad Prism™.
Results

Functionally overloaded plantaris muscles display ectopic SLN expression that is required for fast-to-slow fiber type transitions, and myofiber hypertrophy and hyperplasia. Plantaris muscles from WT and Sln−/− mice were subjected to functional overload for two weeks by performing soleus and gastrocnemius tenotomy (31). Murine adult plantaris muscles do not typically express SLN protein; however, induction of ectopic SLN expression was observed in overloaded plantaris muscles from WT mice (Figure 1A). As expected, after two weeks of functional overload, the WT plantaris myofibers transitioned towards the slower fiber type with significant increases in %type I ($P = 0.0002$) and IIA fibers ($P = 0.004$), a significant decrease in %type IIB fibers ($P = 0.05$, Figure 1B and C) and no change ($P = 0.57$) in %type IIX fibers. Compared with sham, the Sln−/− overloaded plantaris exhibited a significant reduction in %type IIB fibers ($P = 0.05$, Figure 1C), but unlike WT mice, %type IIX fibers were significantly increased ($P = 0.03$; Figure 1C) and there were no changes in %type I ($P = 0.79$) or IIA fibers ($P = 0.64$).

With respect to myofiber hypertrophy, a significant interaction ($P = 0.004$) indicates that the average plantaris myofiber CSA increases in WT mice but not Sln−/− mice in response to muscle overloading stimuli (Figure 1D). In addition to myofiber size, it has been shown that functionally overloading a plantaris muscle results in an increase in fiber number (23, 25). With respect to total fiber number, there was no significant interaction ($P = 0.13$); however, planned comparisons indicate that total fiber number was increased significantly ($P = 0.03$) in WT mice but not Sln−/− mice ($P = 0.75$, Figure 1E).
Interestingly, both WT and $Sln^{-/-}$ exhibited significant increases in absolute and relative plantaris muscle weight (Figure 1F) in response to functional overload.

**SLN is upregulated in response to muscle unloading caused by tenotomy and its deletion exacerbates the slow-to-fast fiber type shift and muscle atrophy.** Using the same WT and $Sln^{-/-}$ mice that were used in the functional overload experiments; we also examined the tenotomized soleus to determine the role of SLN in adaptive muscle remodeling that occurs in response to unloading or disuse. Figure 2A shows that the SLN protein was increased 14-fold in unloaded/tenotomized WT soleus compared with sham, which is consistent with other mouse models of disuse atrophy (11). As expected with tenotomy (37) and despite no change ($P = 0.29$) in %type I fibers, a significant decrease and increase in %type IIA ($P = 0.005$) and %type IIX ($P = 0.0003$) fibers, respectively, was observed in WT soleus (Figure 2B and C). This result is consistent with a slow-to-fast fiber type shift. In $Sln^{-/-}$ mice, our planned comparisons revealed an accelerated slow-to-fast fiber type shift in response to tenotomy with significant reductions in both %type I ($P = 0.02$) and %type IIA ($P = 0.003$) fibers and a more pronounced increase in %type IIX fibers, with a mean difference of $14 \pm 1.8\%$ ($P < 0.0001$), compared to the mean difference observed in WT mice ($8.6 \pm 1.8\%$) (Figure 2C).

There was no significant interaction between genotype and surgery for myofiber CSA, but our planned comparisons show that tenotomy caused a significant reduction in average myofiber CSA in $Sln^{-/-}$ mice ($P = 0.008$) but not WT mice ($P = 0.11$, Figure 2D). Furthermore, the myofibers from the $Sln^{-/-}$ sham soleus appeared to be smaller than the WT sham soleus, but a Student’s t-test indicated that this was not statistically significant
With respect to total fiber number, no significant interaction ($P = 0.18$, Figure 2E) was observed as both $Sln^{-/-}$ and WT mice displayed significant hypoplasia following tenotomy, which is consistent with the extensive degeneration that occurs in this model (4, 37). However, the effect was more pronounced in $Sln^{-/-}$ mice (-369 ± 78, $P = 0.0008$) than WT mice (-213 ± 78, $P = 0.02$) (Figure 2E). A significant interaction was found for both the absolute ($P = 0.003$) and relative ($P = 0.01$) soleus muscle weights, suggesting that the $Sln^{-/-}$ mice experienced more pronounced atrophy in response to the unloading stimuli (Figure 2F).

**Increases in calcineurin signaling in the overloaded plantaris and unloaded soleus**

**are blunted in $Sln^{-/-}$ mice.** To determine whether differences in adaptive muscle remodeling between WT and $Sln^{-/-}$ mice were associated with altered calcineurin signaling, we examined the phosphorylation status of its well-known substrate, NFATc1. Analyses of total NFATc1 and p-NFATc1 protein content and the ratio of p-NFATc1/NFATc1 did not reveal any significant interaction effects. However, our planned comparisons show that WT plantaris muscles displayed a trending 29% decrease in p-NFATc1 ($P = 0.08$) in response to the overload stimulus, whereas no reduction was seen in overloaded $Sln^{-/-}$ muscles ($P = 0.80$) (Figure 3A). Similarly, in response to functional overload, we found a significant increase in total NFATc1 in the WT mice ($P = 0.01$), but not in the $Sln^{-/-}$ mice ($P = 0.16$) (Figure 3A). When expressed as a ratio, p-NFATc1/NFATc1 was significantly reduced in WT mice (-61%, $P = 0.01$) but not in $Sln^{-/-}$ mice (-22%, $P = 0.40$) (Figure 3A). In addition to the phosphorylation status of NFATc1, we examined the expression of stabilin-2 as another surrogate marker of
calcineurin signaling. In the overload experiments, we found a significant interaction ($P = 0.02$) with only the WT mice displaying a significant 2.0-fold increase in stabilin-2 expression ($P = 0.01$) (Figure 3B).

With respect to the soleus unloading experiments, we did not find any significant interaction effects; however, our planned comparisons show that only the $Sln^{-/-}$ mice exhibited a significant increase in p-NFATc1 ($P = 0.03$) (Figure 3C). Total NFATc1 was significantly increased in both WT ($P = 0.0004$) and $Sln^{-/-}$ ($P = 0.0011$) unloaded soleus muscles compared with sham (Figure 3C). Thus, compared with sham, there was a significant reduction (-57%) in p-NFATc1/NFATc1 in the unloaded WT soleus ($P = 0.01$), whereas the 32% reduction in the unloaded soleus from $Sln^{-/-}$ mice was not significant ($P = 0.18$) (Figure 3C). When examining stabilin-2 expression, no significant interaction effects were found ($P = 0.69$), but our planned comparisons revealed an increase in stabilin-2 expression in tenotomized soleus muscles that was statistically significant for WT mice (4.3-fold, $P = 0.02$) but only trending for $Sln^{-/-}$ mice (3.6-fold, $P = 0.06$) (Figure 3D).

**The effects of functional overload and tenotomy on SERCA function are different between WT and $Sln^{-/-}$ mice.** Next, we assessed SERCA function across the overloaded and unloaded conditions relative to sham. We did not find a significant interaction ($P = 0.35$) between genotype and surgery in the overloaded condition. However, our planned comparisons show that maximal SERCA activity was reduced significantly ($P = 0.004$) in WT mice, whereas only a trend was observed in $Sln^{-/-}$ mice ($P = 0.06$, Figure 4A). In contrast, a significant interaction ($P = 0.0006$) was observed with tenotomy, whereby
maximal SERCA activity in soleus homogenates was decreased in WT mice \((P = 0.0015)\) and actually increased in the \(Sln^{-/-}\) mice \((P = 0.02)\) (Figure 4B). SERCA’s apparent affinity for \(Ca^{2+}\) was similar across all conditions and genotypes (Table 1).

Discussion

The \(Sln\) gene is highly responsive to upregulation leading to large increases in SLN mRNA/protein in skeletal muscle with excessive caloric intake (5, 9) and many myopathic conditions (1, 11, 12, 20, 24, 34, 41, 43, 44, 52). Collectively, these studies likely indicate that SLN underpins many important adaptations. Recently, we discovered that genetic deletion of \(Sln\) in a mouse model of centronuclear myopathy caused reductions in myofiber CSA, total fiber number, and increased the proportion of fast glycolytic fibers. Furthermore, these physiological changes were associated with markers of blunted calcineurin signaling (22). In the present study, we examined the role of SLN in adaptive skeletal muscle remodeling in the functionally overloaded plantaris and unloaded/tenotomized soleus. In both conditions, genetic deletion of \(Sln\) resulted in smaller myofiber CSA and a greater proportion of faster fibers, which were associated with blunted calcineurin signaling and increased SERCA activity. Collectively, these results demonstrate that SLN plays a key role in adaptive muscle remodeling possibly through its ability to inhibit SERCA and thereby alter calcineurin signaling.

Nearly 20 years ago, Chin et al. demonstrated that fiber-type-specific gene expression in skeletal muscles is controlled, in part, by the calcineurin-NFAT signaling pathway (15). This finding was consistent with the ability of calcineurin to discriminate between calcium signals with different amplitudes and durations (17, 60), which were
known to differ across myofiber types (for review see (50)). It is believed that functional overload of a muscle imposes a tonic activity pattern as the muscle takes on a postural role, which results in changes in the intracellular Ca$^{2+}$ signal that activates the calcineurin-NFAT axis and promotes adaptive muscle remodeling (39). In contrast to an overloaded muscle, the absence of constant motor unit activation seen with muscle unloading or disuse causes myofibers to revert to their default fast fiber type (15, 38). Paradoxically, muscle unloading leads to increased resting intracellular Ca$^{2+}$ and nuclear NFATc1 content, which apparently counteracts the slow-fast fiber type shift because inhibition of calcineurin during muscle unloading decreases further the expression of slow muscle myosin (MHC I) (for review see (56)).

Here, we found that NFATc1 dephosphorylation and the subsequent fast-to-slow fiber type transition were blunted in overloaded plantaris muscles that do not express SLN. Similarly, using a model of soleus unloading caused by tenotomy, we observed an exaggerated slow-to-fast fiber type shift in $Slh^{-/-}$ mice compared with WT, which corresponded with lower calcineurin signaling (i.e. greater NFATc1 phosphorylation) in $Slh^{-/-}$ mice. Thus, SLN ablation mimics the effects of pharmacological inhibition of calcineurin on muscle remodeling caused by functional overload and unload stimuli, suggesting that upregulation of SLN protein is an adaptive response that is required for calcineurin activation to maintain the slow fiber phenotype. We propose that SLN’s inhibitory action on SERCA could increase [Ca$^{2+}$], and amplify calcineurin signaling to promote adaptive muscle remodeling, which are blunted in the absence of SLN. Consistent with this view, overload and tenotomy caused a significant reduction in maximal SERCA activity in WT muscles, whereas in $Slh^{-/-}$ mice, there was a trend for
reduced maximal SERCA activity in response to overload and it was actually increased significantly in response to tenotomy. However, measuring \([\text{Ca}^{2+}]_i\), directly would strengthen our conclusions.

Despite considerable debate (30, 42, 48, 51, 55), calcineurin has also been implicated in myofiber growth (19, 39, 40, 54), which may explain why we observed smaller myofibers in both the overloaded plantaris and unloaded soleus from \(Sln^{-/-}\) mice. It should be noted that adaptive increases in absolute and relative plantaris muscle weights were similar in WT and \(Sln^{-/-}\) mice, which likely reflects the difficulty in isolating plantaris muscles at consistent surgical ‘cut’ points. Our findings in the unloaded soleus are in agreement with our previous study whereby \(Sln\) deletion exacerbated soleus muscle atrophy observed in a mouse model of centronuclear myopathy (22). Overall, these results are consistent with our hypotheses suggesting that SLN upregulation is important for regulating adaptive changes in myofiber size.

Although the exact mechanisms are not yet fully established, calcineurin signaling may repress the expression of myostatin (39), a potent negative regulator of skeletal muscle growth (46). In addition, activation of the NFAT family of proteins induces muscle growth through myoblast fusion (27), by increasing the expression of various cell-cell interaction factors and/or proteins secreted for recruitment of myoblasts (28, 29, 45). Recent evidence suggests that calcineurin may increase myofiber CSA through myoblast fusion by increasing stabilin-2 content (45). In agreement with that study, we have found, both in this study and in our previous study (22), that adaptations in myofiber size and total fiber number in response to altered muscle loading are associated with increased calcineurin signaling and stabilin-2 expression, and require SLN. Our findings...
suggesting that SLN plays an important role in adaptive responses in muscle mass could have important implications in other muscle wasting conditions such as aging and cachexia. Of note, previous studies have shown an inability of plantaris muscles from aged mice to hypertrophy in response to functional overload (6, 32). Future studies should examine the role of SLN in age-related muscle loss and determine whether a failure to recruit SLN during functional overload could account for blunted adaptive muscle remodeling with age.

One limitation of our study is that we do not provide a direct measure of calcineurin phosphatase activity, as our measures of NFATc1 phosphorylation, stabilin-2 content, myofiber size, and fiber type distribution collectively represent the downstream effects of calcineurin signaling. Other Ca\(^{2+}\)-dependent enzymes that are known to alter fiber type and size, such as the calmodulin-dependent kinases (14), could also be involved in the phenotypic differences observed in this study. Moreover, it is possible that protein synthesis pathways, such as mTOR signaling, and protein degradation pathways, including calpains, caspases, cathepsins and the ubiquitin proteasomal degradation system, could all be involved in the changes in myofiber size seen after muscle overloading and unloading. Here, we chose to focus on calcineurin signaling in light of recent findings from our laboratory and others demonstrating that SLN may stimulate calcineurin (22, 35); however, future studies should examine the role of other signaling pathways in this model. Finally, future studies that attempt to improve calcineurin activation in the Sln\(^{-/-}\) mice to potentially restore normal physiological adaptations in response to overloading and unloading stimuli would examine directly our proposed mechanisms.
In summary, our results demonstrate a critical role for SLN in mediating adaptive muscle remodeling in response to functional overload and unload stimuli. We propose that SLN is upregulated to stimulate calcineurin-mediated adaptive responses, which are important for maintaining muscle mass and the slow-fiber phenotype. Since the promotion of muscle mass and the slow-oxidative fiber type are promising strategies in the treatment of other conditions such as muscular dystrophy (13, 53) and sarcopenia (33, 63), future studies should examine SLN as a potential therapeutic target.

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Disclosure
The authors declare no conflict of interest.
References


**Figure legends**

**Figure 1.** Overloaded plantaris muscles fail to undergo skeletal muscle remodeling in the absence of SLN. (A) Western blot image illustrating ectopic expression of SLN in the overloaded (OVL) plantaris. Ponceau stain demonstrates equal loading (75 µg of plantaris homogenate protein loaded). (B) Representative immunofluorescent fiber type staining. Type I, blue; type IIA, green; type IIX, unstained; type IIB, red. (C) Plantaris fiber type distribution in response to OVL stimuli in WT and Sln<sup>−/−</sup> mice (n = 6-8 per group). (D) Plantaris average myofiber CSA in response to OVL stimuli in WT and Sln<sup>−/−</sup> mice (n = 6-8 per group, with 600-800 fibers analyzed per muscle). (E) Plantaris total fiber count in response to OVL stimuli in WT and Sln<sup>−/−</sup> mice (n = 11-13 per group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P ≤ 0.0001. All values are means ± SEM. All scale bars are set to 100 µm.

**Figure 2.** Sln deletion exacerbates the physiological adaptations that occur with soleus unloading induced by tenotomy (Tny). (A) Western blot image depicting SLN content in WT sham and Tny soleus muscles (n = 5 per group). 25 µg of soleus homogenate protein was loaded for each lane and equal loading was ensured with ponceau stain. (B) Representative immunofluorescent fiber type staining. Type I, blue; type IIA, green; type IIX, unstained. (C) Soleus fiber type distribution in response to Tny in WT and Sln<sup>−/−</sup> mice (n = 8 per group). (D) Soleus average myofiber CSA in response to Tny in WT and Sln<sup>−/−</sup> mice (n = 8 per group with 600 fibers analyzed per muscle). (E) Soleus total fiber count in response to Tny in WT and Sln<sup>−/−</sup> mice (n = 11-13 per group). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. All values are means ± SEM. All scale bars are set to 100 µm.

**Figure 3.** Calcineurin signalling indicated by NFATc1 phosphorylation status and stabilin-2 expression in response to overload (OVL) and unload (Tny) stimuli in WT and Sln<sup>−/−</sup> mice. (A) Alterations in p-NFATc1, total NFATc1, and the ratio of p-NFATc1/NFATc1 in sham and overloaded plantaris muscles from WT and Sln<sup>−/−</sup> mice (n = 4-5 per group). (B) Stabilin-2 expression in sham and overloaded plantaris muscles from WT and Sln<sup>−/−</sup> mice (n = 6 per group). (C) Changes in p-NFATc1, total NFATc1, the ratio of p-NFATc1/NFATc1 in sham and unloaded soleus muscles from WT and Sln<sup>−/−</sup> mice (n = 5-6 per group). (D) Stabilin-2 expression in sham and unloaded soleus muscles from WT and Sln<sup>−/−</sup> mice (n = 6 per group). *P < 0.05, **P < 0.01, ***P < 0.001. All values are means ± SEM.

**Figure 4.** Maximal SERCA activity in response to functional overloaded plantaris (OVL, A) and unloaded soleus (Tny, B) in WT and Sln<sup>−/−</sup> mice compared with sham (n = 5-6 per group). *P < 0.05, **P < 0.01, ***P < 0.001. All values are means ± SEM.
Table 1. $p\text{Ca}_{50}$ values measured in plantaris and soleus muscles from WT and $Sln^{-/-}$ mice in response to functional overload (OVL) and unload (Tny).

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<th>Sham plantaris</th>
<th>OVL plantaris</th>
<th>Sham soleus</th>
<th>Tny soleus</th>
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<td>WT</td>
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<td>$Sln^{-/-}$</td>
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<td>6.13 ± 0.07</td>
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All values are means ± SEM. $p\text{Ca}_{50}$ is the $[\text{Ca}^{2+}]$ required to elicit half-maximal ATPase activity. A paired t-test between sham vs. OVL and sham vs. Tny within a genotype revealed no significant differences.