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Deficiency of selenoprotein S, an endoplasmic reticulum resident oxidoreductase, impairs the contractile function of fast-twitch hindlimb muscles

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Addinsall AB, Wright CR, Shaw CS, McRae NL, Forgan LG, Weng CH, Conlan XA, Francis PS, Smith ZM, Andrikopoulos S, Stupka N. Deficiency of selenoprotein S, an endoplasmic reticulum resident oxidoreductase, impairs the contractile function of fast-twitch hindlimb muscles. *Am J Physiol Regul Integr Comp Physiol* 315: R380–R396, 2018. First published April 18, 2018; doi:10.1152/ajpregu.00244.2017.—Selenoprotein S (Seps1) is an endoplasmic reticulum (ER) resident antioxidant implicated in ER stress and inflammation. In human vastus lateralis and mouse hindlimb muscles, Seps1 localization and expression were fiber-type specific. In male Seps1$^{+/-}$ heterozygous mice, spontaneous physical activity was reduced compared with wild-type littermates ($d = 1.10$, $P = 0.029$). A similar trend was also observed in Seps1$^{-/-}$ knockout mice ($d = 1.12$, $P = 0.051$). Whole body metabolism, body composition, extensor digitorum longus (EDL), and soleus mass and myofiber diameter were unaffected by genotype. However, in isolated fast EDL muscles from Seps1$^{-/-}$ knockout mice, the force frequency curve (FFC; 1–120 Hz) was shifted downward versus EDL muscles from wild-type littermates ($d = 0.55$, $P = 0.002$), suggestive of reduced strength. During 4 min of intermittent, submaximal (60 Hz) stimulation, the genetic deletion or reduction of Seps1 decreased EDL force production ($d = 0.52$, $P < 0.001$). Furthermore, at the start of the intermittent stimulation protocol, when compared with the 60-Hz stimulation of the FFC, EDL muscles from Seps1$^{-/-}$ knockout or Seps1$^{+/-}$ heterozygous mice produced 10% less force than those from wild-type littermates ($d = 0.31$, $P < 0.001$ and $d = 0.39$, $P = 0.015$). This functional impairment was associated with reduced mRNA transcript abundance of thioredoxin-1 (*Trx1*), thioredoxin interacting protein (*Txnip*), and the ER stress markers *Chop* and *Grp94*, whereas, in slow soleus muscles, Seps1 deletion did not compromise contractile function and *Trx1* ($d = 1.38$, $P = 0.012$) and *Txnip* ($d = 1.27$, $P = 0.025$) gene expression was increased. Seps1 is a novel regulator of contractile function and cellular stress responses in fast-twitch muscles.

endoplasmic reticulum stress; fast-twitch muscle; selenoprotein S; SEPS1; thioredoxin

INTRODUCTION

Selenoproteins are characterized by their inclusion of a selenocysteine (Sec) amino acid residue that accounts for their antioxidant function and bioactivity (75). In humans, there are 25 selenoproteins with glutathione peroxidases and thioredoxin reductases being the most highly characterized (38). These maintain the redox state of the glutathione and thioredoxin antioxidant pools and thereby regulate an array of stress responsive signaling pathways (29). There are seven selenoproteins localized to the endoplasmic reticulum (ER), the cellular mechanism of action of which is still poorly understood (reviewed in Ref. 75). Selenoproteins are important for skeletal muscle development and growth (20, 63), and their role in regulating contractile function is increasingly recognized (3, 49, 53). There is emerging interest in the ER resident selenoproteins, because of the importance of the ER in intracellular calcium (Ca^{2+}) signaling and homeostasis (1, 23). In skeletal muscle, the sarcoplasmic reticulum (SR), a specialized smooth ER, is responsible for the storage and release of Ca^{2+} during contractile activity. In various cells types, a role for Ca^{2+} regulation has been identified for selenoprotein T (28), selenoprotein K (SelK or *SELENOK*) (8), and selenoprotein N (SelN, *SEPN1*, or *SELENON*) (3, 49). Mutations in the *SEPN1* gene and the resultant loss of SELN expression increases skeletal muscle oxidative stress (3) and dysregulates SR Ca^{2+} release and/or reuptake (33, 49, 53), causing a myopathy associated with early onset axial and neck muscle weakness, spinal rigidity, and respiratory failure (4).

Like SELN, selenoprotein S (SEPS1, SelS, or *SELENOS*) is highly expressed in skeletal muscle, although its function is not well described. It is also one of few selenoproteins where mutations or gene variants have been linked to human disease (reviewed here (74)). Using family association studies, Curran et al. (18) showed that *SEPS1* gene variants, in particular the −105G>A polymorphism in the promotor region, were associated with increased plasma levels of proinflammatory cytokines. Since these initial studies, *SEPS1* gene polymorphisms have been associated with a range of conditions characterized by a heightened inflammatory state, including metabolic dis-

ease (2, 18, 59, 83, 89), cardiovascular disease (2, 17, 59, 70), preeclampsia (55), spontaneous preterm birth (90), cancer (30, 77), and Hashimoto's thyroiditis (41, 71).

SEPS1 is a single-pass transmembrane protein (75, 86). The incorporation of a Sec residue in the cytosolic COOH terminus (Sec188), in combination with two cysteine (Cys) residues localized to the transmembrane (Cys41) and cytosolic catalytic domains (Cys174), provides SEPS1 with its antioxidant activity (38, 75, 86). According to biochemical assays, SEPS1 can reduce disulfide bonds and has oxidoreductase activity against H_2O_2, primarily through its actions as a thioredoxin-dependent reductase (15, 46, 47). This is interesting, as thioredoxin regulates various stress-responsive signaling pathways including NF-κB (69) and NF-κB-regulated genes mediate cellular responses to inflammation and oxidative stress.

The promotor region of SEPS1 contains a functional ER stress element and two putative NF-κB binding sites, indicating the potential for transcriptional regulation of SEPS1 in response to ER, oxidative, and inflammatory stress (18, 26, 75). In vitro ER stress has been shown to increase SEPS1 gene and protein expression (26, 36). SEPS1 is part of an ER membrane-localized multiprotein complex, which includes glucose-regulated protein 78 (GRP78), Derlin1/2, P97 ATPase, and SelK. This complex is involved in the unfolded protein response (UPR) and allows for the retrograde translocation of misfolded proteins from the ER lumen to the cytosol for degradation by the ubiquitin proteasome pathway (75). SEPS1, specifically, is thought to facilitate the reduction of disulfide bonds as protein is translocated out of the ER (18, 44, 75, 98).

The physiological and functional significance of the regulation of oxidative and ER stress responses by SEPS1 is poorly understood. What is known is based predominantly on cell culture studies and limited to two in vivo studies linking reduced Seps1 expression with increased inflammation (31, 93). SEPS1 gene suppression in vitro is associated with increased oxidative and ER stress leading to reduced cell viability (18, 22, 24, 35, 36, 100), while overexpression is protective (25, 35, 36, 102). However, there is emerging evidence for a more complex role of SEPS1 in regulating cellular stress responses. The disordered region and a coil-coiled domain within its cytosolic tail provide SEPS1 with the capacity to bind various proteins (46), thus regulating disulfide bond reduction (15, 46, 47) and/or acting as an anchor for multiprotein complexes to the ER membrane beyond its well-described role in the UPR (86). A recent proteomics screen by Turanov et al. (86) in HEK-293T cells found SEPS1 to be associated with proteins important for Ca^{2+} homeostasis and signaling, in particular, sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a), which is essential for Ca^{2+} reuptake into the ER/SR. The redox state of the ER/SR needs to be tightly regulated, as alterations are detrimental to all aspects of organelle function (27). This includes Ca^{2+} signaling and homeostasis, as oxidation of SH residues on ER/SR resident proteins important for Ca^{2+} release or reuptake alters their function (49).

Given the intersect between oxidative and ER stress and Ca^{2+} signaling (27), we hypothesized that SEPS1 might be a novel selenoprotein capable of modulating skeletal muscle performance. Here, we characterized the expression and cellular localization of Seps1 in fast and slow twitch mouse hindlimb muscles and human vastus lateralis muscle using immunohistochemistry and Western blotting. Using adult global Seps1-deleted (GKO) mice, we investigated the effects of Seps1 reduction or deletion on the contractile function of isolated slow- and fast-twitch hindlimb muscles and gene markers of oxidative and ER stress. Body composition, whole body metabolism, and spontaneous physical activity were also assessed, given the association of Seps1 with metabolic disease (34, 89). Here, we established that Seps1 protein expression and localization was fiber type dependent and that the genetic reduction or deletion of Seps1 was associated with reduced spontaneous physical activity and compromised hindlimb contractile function in fast-twitch, but not slow-twitch, hindlimb muscles. This was associated with a fiber type-specific regulation of gene markers of the thioredoxin antioxidant system and ER stress. Despite epidemiological studies linking SEPS1 to metabolic disease, in mice the genetic reduction or deletion of Seps1 had no significant effect on whole body metabolism and body composition.

METHODS

Ethics approval. All animal studies were approved by the Animal Ethics Committees at The University of Melbourne (Austin Health) and Deakin University. Animal care, maintenance, and all procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Adult male BALB/c mice were used to assess SEPS1 localization and expression in extensor digitorum longus (EDL), soleus, and tibialis anterior (TA) muscles (see Figs. 1 and 4). Vastus lateralis muscle biopsies from healthy, recreationally active male subjects (age: 26.7 ± 2.8 yr; height: 183.3 ± 2.6 cm; weight: 81.5 ± 3.0 kg; and body mass index: 24.2 ± 0.6 kg/m²) were used to assess SEPS1 expression. These human studies adhered to the standards set by the latest revision of the Declaration of Helsinki and were approved by the Human Research Ethics Committee at Deakin University (DUHREC).

Global Seps1 knockout mice. Global Seps1 knockout mice were generated on a C57BL/6 background using PGK-Cre by A/Prof. Sofianos Andrikopoulos (Austin Health, The University of Melbourne). Mice were group housed in standard laboratory conditions of 22 ± 2°C, with relative humidity of 55 ± 8% and a 12-h light-dark cycle. Water and a standard chow diet were provided ad libitum. The following male mice were used in this study: $n = 9$ Seps1$^{-/-}$ knockout mice, $n = 10$ Seps1$^{+/-}$ heterozygous mice, and $n = 10$ wild-type littermates.

Whole body metabolism and body composition. At 10–11 wk of age and 48 h before testing, mice were acclimatized for 3 h to the metabolic cages (Accuscan Fusion v3.6; Columbus Instruments International). Mice were individually placed in metabolic cages for 24 h to determine oxygen consumption ($\dot{V}O_2$; ml/min), carbon dioxide production ($\dot{V}CO_2$; ml/min), and the respiratory exchange ratio (RER; $\dot{V}CO_2/\dot{V}O_2$). Resting daytime and nighttime energy expenditure was calculated according to the abbreviated Weir equation (66). Spontaneous physical activity of the mice was measured using infrared sensors inside the metabolic cages (Animal Activity Meter: Opto-Varimex-Mini; Columbus Instruments). After whole body metabolism was assessed, mice were returned to standard group housing conditions. Immediately before muscle function testing, conscious mice were placed in a rodent MRI (Body Composition Analyzer ESF-005; EchoMRI, Houston, TX) to determine body composition.

Ex vivo muscle function testing. At 12 wk of age, mice were anesthetized via intraperitoneal injection of medetomidine (0.6 mg/kg), midazolam (5 mg/kg), and fentanyl (0.05 mg/kg), such that they were unresponsive to tactile stimuli. Isometric contractile properties of isolated fast-twitch EDL and slow-twitch soleus hindlimb muscles were evaluated ex vivo, as described in detail previously (81). Briefly,

EDL and soleus muscles were tied at the proximal and distal tendons with braided surgical silk, surgically excised, and transferred to the 1300A Whole Mouse Test System (Aurora Scientific, ON, Canada) organ bath filled with Krebs-Ringer solution (in mM: 137 NaCl, 24 NaHCO$_3$, 11 D-glucose, 5 KCl, 2 CaCl$_2$, 1 NaH$_2$PO$_4$H$_2$O, 1 MgSO$_4$, and 0.025 D-tubocurarine chloride), bubbled with carbogen (5% CO$_2$ in O$_2$; BOC Gas, Geelong, Australia), and thermostatically maintained at 25°C. The distal tendon of the muscle was tied to an immobile pin, and the proximal tendon was attached to the lever arm of a dual mode force transducer (300-CLR; Aurora Scientific). EDL and soleus muscles were stimulated via two platinum electrodes that flanked the length of the muscle. All stimulation parameters and contractile responses were controlled and measured using Dynamic Muscle Control Software (DMC v5.415), with on board controller interfaced with the transducer control/feedback hardware (Aurora Scientific).

Optimal muscle length (L_o) was determined via micromanipulations of muscle length and a series of 1-Hz twitch contractions. After 4 min of rest, maximum isometric tetanic force (P_o) production was determined from the plateau of the force frequency curve (FFC), whereby the EDL was stimulated at 10, 30, 50, 60, 80, and 100 Hz and the soleus at 120 Hz and 10, 20, 30, 50, 60, 80, and 100 Hz with 2 min rest between stimulations. Muscles were again rested for 4 min. To assess tolerance to repeated contractile activity and as an indicator of fatigability, muscles were stimulated submaximally (60 Hz) once every 5 s for 4 min at optimal length (60, 68, 88). Recovery of P_o was determined by stimulating the muscles at 60 Hz at 2, 5, and 10 min postfatigue testing. The 60-Hz submaximal stimulation was chosen over maximal protocols due to improved physiological relevance.

Within minutes of completion of function testing, muscles were trimmed of tendons, weighed, and snap frozen in liquid nitrogen. EDL and soleus muscles excised from the contralateral hindlimb were embedded in optimal cutting temperature compound (TissueTek OCT Compound; ProSciTech, Kirwan, Australia) and frozen in thawing isopentane (Sigma-Aldrich, Castle Hill, Australia) for histological analysis. Tibialis anterior (TA) and gastrocnemius muscles were also excised and snap frozen in liquid nitrogen for biochemical analyses. Muscle cross-sectional area was determined by dividing the muscle mass by the product of optimum fiber length (L_f) and 1.06 mg/mm^3, the density of mammalian muscle. L_f was determined by multiplying L_o by previously determined L_o/L_f ratios; 0.44 for the EDL and 0.71 for the soleus (48). Since P_o is dependent on muscle size, P_o values were normalized for muscle cross-sectional area and expressed as specific force (sP_o; kN/m^2) for the FFC and fatigue and recovery data. The FFC data were also expressed as a percentage of P_o. To assess the rate of fatigue and force recovery, these data were normalized to the first 60-Hz contraction and expressed as a percentage.

Histology. Transverse 8-µm-thick frozen sections were cut from the midbelly region of EDL and soleus muscles. Hematoxylin and eosin (H&E; Sigma-Aldrich) staining was used for muscle morphometric analysis. Digital images of H&E-stained muscle were captured at ×200 magnification (DM1000 upright microscope; Leica). Fluorescently labeled wheat germ agglutinin (WGA) was used to measure muscle fiber cross-sectional area, as previously described (76). WGA-stained muscle cross sections were imaged using a Confocal Laser Scanning Microscope (Fluoview FV10i, Olympus, Notting Hill, Australia) at ×200 magnification. All image analysis was completed using Image-Pro Plus software (Media Cybernetics, Rockville, MD). A custom-built macro was set up to isolate cell membrane-specific-positive fluorescence using thresholding and to calculate minimum Feret's diameter, with 203 ± 46 muscle fibers quantified per cross section. Centrally nucleated fibers and areas of degeneration were also analyzed and normalized to total muscle cross-sectional area examined (1.14 ± 0.06 mm^2 per sample).

Immunohistochemistry. Myosin heavy chain (MyHC) antibodies were used to assess the fiber type-specific localization of Seps1 in mouse EDL and soleus muscles. Briefly, EDL and soleus muscle cross sections from wild-type BALB/c mice were reacted the anti-Seps1 antibody, as described above, and MyHC fiber typing was completed on sequential serial sections following the protocol described by Bloemberg and Quadrilatero (11), using anti-MyHC I [BA-F8; lot: 11515-43 µg/ml; Developmental Studies Hybridoma Bank (DSHB); diluted 1:20], anti-MyHC IIa (SC-71; lot: 81315-65 µg/ml; DSHB; diluted 1:50), and/or anti-MyHC IIb (BF-F3; DSHB; diluted 1:20) antibodies. After 1–2 h of incubation with the MyHC primary antibodies, sections were reacted with an Alexa Fluor 350 goat anti-mouse IgG2b (diluted 1:500; A21140; lot: 1717023; Thermo Fisher Scientific) for MyHC1, Alexa Fluor 488 goat anti-mouse IgG1 (diluted 1:500) for MyHC IIa, and/or Alexa Fluor 555 goat anti-mouse IgM (diluted 1:500; A21426; lot: 1708371; Thermo Fisher Scientific) for MyHC IIb for 1 h. Nuclei were counterstained with DAPI. Sections were imaged on fluorescent light imager (Zoe; Bio-Rad) at ×200 magnification.

To confirm the fiber type-specific localization of Seps1, the anti-Seps1 antibody was preincubated with a blocking peptide, a commercially available Seps1 antigen (5:1; APrEST71935; lot: PRL02206; Sigma-Aldrich), as per manufacturer's recommendations. EDL and soleus muscle cross sections from wild-type C57BL/6 mice were reacted with the anti-Seps1 antibody with or without blocking peptide, as described above. WGA (FITC) was used to stain the sarcolemma (37) and extracellular matrix constituents of blood vessels (6), and nuclei were labeled with DAPI. Sections were imaged on a benchtop confocal laser scanning microscope (Fluoview FV10i, Olympus) at ×200 magnification.

To determine whether the fiber type-specific localization of SEPS1 is also observed in human muscles, cross sections from frozen vastus lateralis muscle biopsies were coreacted with an anti-Seps1 antibody (diluted 1:200; HPA010025; lot: A101692; Sigma-Aldrich) and an anti-SERCA1 antibody (diluted 1:1,000; CaF2-5D2; lot: 123115-24 µg/ml; DSHB) or an anti-SERCA2 antibody (diluted 1:100; ab2861; lot: GR139308-5; Abcam). Briefly, muscle cross sections were fixed in 4% paraformaldehyde (PFA), permeabilized with Tris-buffered saline (TBS) containing 0.5% Triton X-100, blocked with 10% normal goat serum in TBS, and then incubated with the respective primary antibodies for 1–2 h. Following which muscle sections were incubated for 1 h with an Alexa Fluor 594 goat anti-rabbit secondary antibody (diluted 1:1,500; A11012; lot: 1652425; Thermo Fisher Scientific) to detect SEPS1 and an Alexa Fluor 488 IgG1 goat anti-mouse (diluted 1:1,000; A21121; lot: 1608647; Thermo Fisher Scientific) to detect SERCA1 or an Alexa Fluor 488 IgG2a goat anti-mouse (diluted 1:1,000; A21131; lot: 1696196; Thermo Fisher Scientific) to detect SERCA2. Nuclei were counterstained with DAPI. Sections were imaged on a benchtop confocal laser scanning microscope (Fluoview FV10i; Olympus) at ×200 magnification.

Real-time quantitative PCR. Whole EDL and soleus muscles from Seps1 GKO mice were homogenized in Tri-Reagent solution (Ambion; Thermo Fisher Scientific). Total cellular RNA was extracted and purified using an RNeasy Mini Kit (Qiagen, Mulgrave, Australia). An iScript cDNA synthesis kit (Bio-Rad) was used to reverse transcribe 0.25 µg of total RNA. Quantitative RT-PCR was performed using IQ SYBR Green Super mix (Bio-Rad) and oligonucleotide primers for the genes of interest (Table 1). cDNA concentrations were determined using Quant-iT OliGreen ssDNA reagent (Thermo Fisher Scientific), and Ct values were normalized to cDNA content.

Immunoblotting. Vastus lateralis biopsies, TA, EDL, and soleus muscles from BALB/c mice, and TA muscles from Seps1$^{-/+}$ heterozygous and Seps1$^{-/-}$ knockout mice and wild-type littermates (10–20 µg), as well as proliferating C2C12 myoblasts (positive control), were homogenized in RIPA buffer (Merck Millipore) with protease and phosphatase inhibitors (Thermo Fisher Scientific). Total protein content was determined using a BCA protein assay (Thermo Fisher Scientific). To confirm the genetic reduction or deletion of Seps1, ten 10-µm-thick EDL and soleus muscle cross sections from Seps1$^{-/+}$ heterozygous and Seps1$^{-/-}$ knockout mice and wild-type littermates (10–20 µg), as well as proliferating C2 homozygous mice

Table 1. *Mouse primers used for quantitative RT-PCR*

	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
Chop	GTCCCTAGCTTGGCTGACAGA	TGGAGAGCGAGGGCTTTG
Gpx1	CCATCAGTTCGGACACCAGGAG	TCACCATTCACTTCGCACTTCTC
Grp78	TTCCTGCGTCGGTGTATTCA	GCGGTTGCCCTGATCGT
Grp94	TCCATCTCTTCTCCCTCATCC	CGGCAACACTTCGGTCAG
Mcp-1	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCCTTCTTA
Txnip	ATCGTGGCGTGGCAAGAG	CGTAGATCAGCAAGGAGTATTCG
Txr1	CGTGGTGGACTTCTCTGCTACGTGGTG	GGTCGGTATGCATTTGACTTCACAGTC

and wild-type littermates were placed into 50 µl of 2× Laemmelli sample buffer (Bio-Rad) and subjected to two freeze-thaw cycles. For SDS-PAGE, 7.5 µg of unfractionated muscle homogenate or 15 µl of cyrosection lysates were separated on a 4–15% gradient TGX Stain-Free criterion gel (Bio-Rad) at 100 V. Following which the gel was activated and proteins were visualized using the Chemidoc XRS system (Bio-Rad). Proteins were then transferred to PVDF membranes using a Turbo Blot Transfer System (Bio-Rad) at 2.5 A and 25 V for 12 min. Membranes were blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBS-Tween), before being incubated for 1 h with the anti-Seps1 antibody (diluted 1:200 in 1% BSA in TBS-Tween). The blots were then incubated for 1 h with a goat anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (diluted 1:2,500 in 1% BSA in TBS-Tween; cat. no. 111 035 003; lot: 120104; Jackson Laboratories). Blots were imaged using ECL chemiluminescence. Band densitometry was performed on the Western blots and the stain-free gels to confirm even loading, using Quantity One software (Bio-Rad). Seps1 protein expression was normalized to the optical density of the total protein on the TGX-stain free protein gel (54, 56, 57).

Citrate synthase enzyme activity. Gastrocnemius muscles (15 µg) were homogenized on ice in buffer containing 0.175 M KCl and 2 mM EDTA, before undergoing two freeze thaw cycles and centrifugation at 10,000 g for 60 s, and then citrate synthase activity was measured in the supernatant, as previously described and normalized to per gram of total protein (50). The gastrocnemius, a mixed muscle, was used for the citrate synthase activity, thiobarbituric acid reactive substances (TBARS), and the reduced and oxidized glutathione analyses due to sample limitations in EDL and soleus muscles.

TBARS. To assess the effects of Seps1 reduction or deletion on lipid peroxidation in skeletal muscles, a commercially available TBARS assay kit was used (10009055; Cayman Chemical). Briefly, gastrocnemius muscles (~29 mg) were homogenized on ice in RIPA buffer containing protease inhibitors, followed by centrifugation at 1,600 g for 10 min at 4°C. The supernatant was collected for analysis. Standards and samples were then prepared as per manufacturer's instructions, and TBARS concentrations were assessed using spectrometer (Bio Rad X mark microplate spectrometer) and normalized to protein (µg/µl) following BCA assay.

Reduced and oxidized glutathione. To assess muscle redox state, gastrocnemius muscles (40–50 µg) were homogenized on ice in 0.1% formic acid, and then centrifuged at 8,000 g for 15 min. The supernatants were collected and analyzed for reduced (GSH) and oxidized (GSSG) glutathione content, as previously described (51, 79). The concentration of GSH or GSSG was normalized to the wet weight of the gastrocnemius muscle sample.

Statistical analyses. All results are presented as means ± SE. One-way general linear model ANOVA was used to assess differences in Seps1 protein expression in fast- and slow-twitch mouse hindlimb muscles. For experiments characterizing the Seps1 global knockout mice, differences between Seps1$^{-/-}$ knockout mice and wild-type littermates or Seps1$^{+/-}$ heterozygous mice and wild-type littermates were investigated using two-tailed unpaired *t*-tests or two-way general linear model ANOVA with Tukey post hoc analyses, as appropriate. Effect sizes were calculated using Cohen's formula *(d)*

(82). All statistical analyses were performed using Minitab statistical software v17 (Sydney, Australia), with $P < 0.05$ being statistically significant.

RESULTS

Differential localization and expression of SEPS1 in fast and slow-twitch muscle fibers. In mouse soleus muscle cross sections, Seps1 immunoreactivity was greater in fast MyHC IIa-positive fibers compared with slow MyHC I-positive fibers (Fig. 1, *A* and *B*). Whereas in EDL muscle cross sections, where the proportion of MyHC I-positive fibers is less than 1% (5, 11), SEPS1 staining was relatively uniform with immunoreactivity observed in MyHC IIa- and MyHC IIb-positive fibers (Fig. 1, *C* and *D*). The specificity of greater Seps1 immunoreactivity in fast-twitch fibers was confirmed by preincubating the anti-Seps1 primary antibody with a commer-
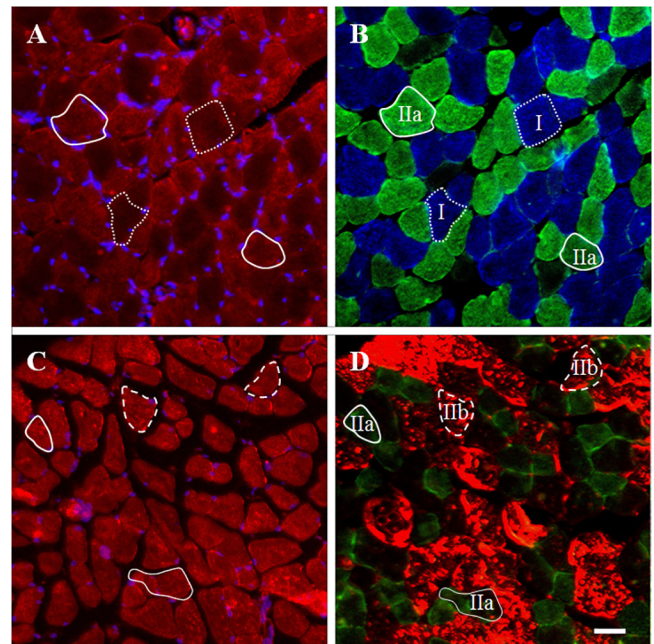

Fig. 1. Selenoprotein S (Seps1) immunoreactivity in mouse hindlimb muscles is fiber-type specific. *A*: Seps1 (red) is highly expressed in slow soleus muscles from wild-type BALB/c mice, with variable immunoreactivity between individual muscle fibers. *B*: serial soleus muscle cross sections were probed for myosin heavy chain (MyHC) IIa (green) and MyHC 1 (blue) antibodies. As indicated by the solid and dotted muscle fiber outline, respectively, Seps1 immunoreactivity appeared to be greater in fast MyHC IIa-positive fibers compared with slow MyHC I-positive fibers. *C*: Seps1 (red) is also highly expressed in fast extensor digitorum longus (EDL) muscles. *D*: serial EDL muscle cross sections were probed for MyHC IIa (green) and MyHC IIb (red). As indicated by the solid and dashed muscle fiber outline, respectively, Seps1 is expressed in both IIa and IIb fibers. Scale bar = 100 µm, n = 3.

cially available blocking peptide (Fig. 2, *D* and *J*). In muscle fiber cross sections, Seps1 expression was predominantly intracellular (Fig. 2, *A* and *G*). A lack of costaining with WGA indicated limited Seps1 localization to the sarcolemma (Fig. 2, *C*, *F*, *I*, and *L*). This is in line with in vitro observations in HepG2 or U251 cells using similar techniques (14). Seps1 was
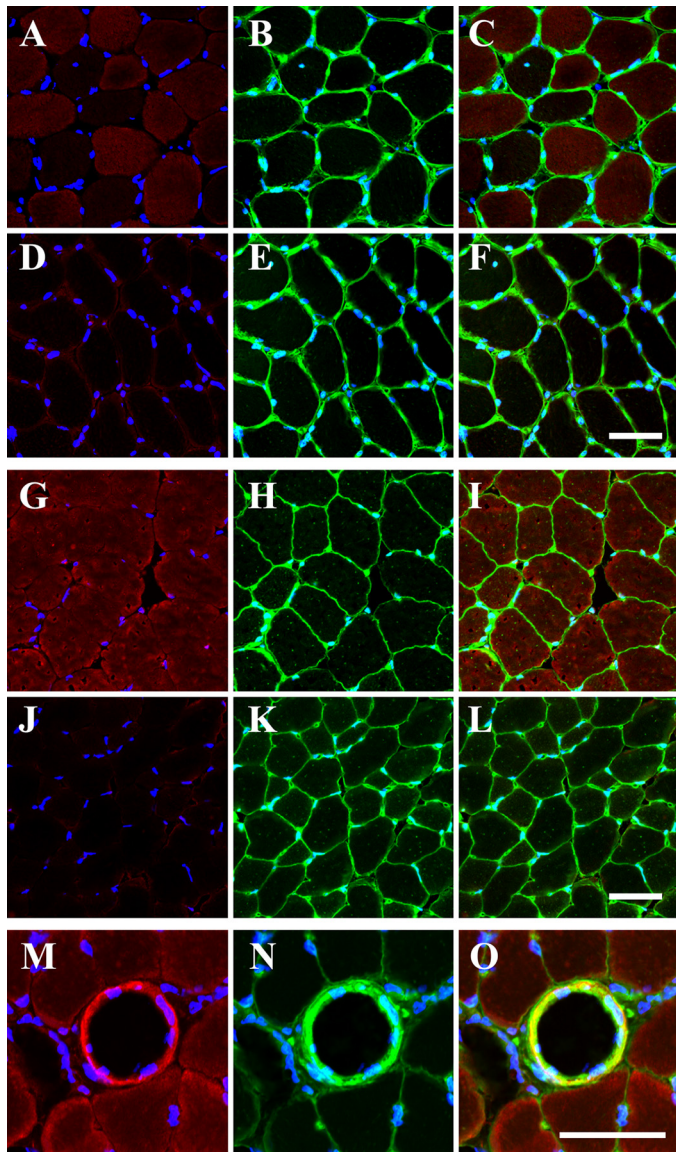

Fig. 2. Selenoprotein S (Seps1) immunoreactivity in mouse soleus and extensor digitorum longus (EDL) muscles. To confirm greater Seps1 immunoreactivity in type II compared with type I fibers, the anti-Seps1 antibody was preincubated with a blocking peptide (a commercially available Seps1 antigen). Soleus and EDL muscles were reacted with the anti-Seps1 antibody (*A* and *C*; *G* and *I*) or the anti-Seps1 antibody plus blocking peptide (*D* and *F*; *J* and *L*) and wheat germ agglutinin (green) was used to stain glycoproteins in the sarcolemma and extracellular matrix (ECM) (*B* and *E*; *H* and *K*). In soleus muscles, Seps1 immunoreactivity was variability between individual fibers (*A* and *C*), and no sarcolemmal colocalization observed (*C*). In EDL muscles, Seps1 was expressed in all myofiber (*G* and *I*), and again no sarcolemmal colocalization was observed (*I*). When soleus (*D* and *F*) and EDL (*J* and *L*) muscles were reacted with the anti-Seps1 antibody preincubated with a blocking peptide no intra-myofiber staining was observed. *M*: in muscle cross sections, Seps1 is especially highly expressed in vascular smooth muscle of arteries and is colocalized with the blood vessel ECM (*N* and *O*). Nuclei are counterstained with DAPI (blue). Scale bar = 50 μm; *n* = 3.

also highly expressed in blood vessels, and this includes vascular smooth muscle cells (Fig. 2*M*) (97). These differences in SEPS1 immunoreactivity between fast- and slow-twitch fibers were also observed in human vastus lateralis muscle cross sections (Fig. 3, *A* and *E*), with more prominent SEPS1 staining in muscle fibers expressing the fast SERCA1 isoform (Fig. 3*G*) versus the slow SERCA2 isoform (Fig. 3*C*).

Fiber-type differences in Seps1 expression were further investigated using Western blotting to assess total protein content in whole muscle lysates, irrespective of cellular localization. SEPS1 was readily detected in vastus lateralis whole muscle lysates (Fig. 4*A*). In mice, total Seps1 protein expression was greater in the slower soleus compared with fast-twitch EDL and TA hindlimb muscles ($d = 5.79$, $P < 0.001$; Fig. 4*B*). The observations of higher Seps1 protein levels in soleus compared with EDL or TA muscles and the preferential Seps1 immunoreactivity in type II fibers are not irreconcilable, given the high proportion of type II fibers, up to 60 to 70%, in mouse soleus muscles (5, 11, 91). Furthermore, Seps1 is highly expressed in blood vessels (Fig. 2, *M* and *O*), specifically vascular smooth muscle cells (97, 99). The density of microvascular network, which includes arterioles and venules, is far greater in soleus compared with EDL muscles. This will also contribute to the higher levels of Seps1 protein expression in whole tissue lysates from soleus muscles (92).

Characterization of Seps1 GKO mice: body composition, spontaneous physical activity, and whole body metabolism. Indicative of the successful genetic deletion or reduction, Seps1 protein expression was reduced in TA muscles from Seps1$^{-/-}$ knockout and Seps1$^{-/+}$ heterozygous mice by ~99% and 50%, respectively, when compared with wild-type littermates ($d = 2.13$, $P = 0.01$ and $d = 1.20$, $P = 0.046$, respectively; Fig. 5*A*), with similar observations made in EDL and soleus muscles (Fig. 5, *B* and *C*).

In 12-wk-old male mice, the genetic reduction or deletion of Seps1 had no significant effect on body weight and body composition, with similar fat mass and lean mass observed in Seps1$^{-/-}$ knockout, Seps1$^{-/+}$ heterozygous, and wild-type mice. EDL and soleus muscle weights were also not significantly different between genotypes (Table 2).

Over a 24-h period, Seps1$^{+/-}$ heterozygous mice were 22% less active than their wild-type littermates ($d = 1.10$, $P = 0.029$; Fig. 6*A*). Similarly, in Seps1$^{-/-}$ knockout mice spontaneous physical activity was reduced by 16% ($d = 1.12$, $P = 0.051$; Fig. 6*A*). Corresponding to the nocturnal increase in activity, resting energy expenditure was higher during the 12-h night period than the 12-h day period ($d = 0.31$, $P < 0.0001$; Fig. 6*B*). Despite differences in spontaneous physical activity, the genetic reduction or deletion of Seps1 had no significant effect on day or night resting energy expenditure, as estimated by the Weir equation (Fig. 6*B*). Substrate utilization, as reflected by RER, was also not significantly different between Seps1$^{-/-}$ knockout or Seps1$^{+/-}$ heterozygous mice and their wild-type littermates (Fig. 6*C*). In concordance with the whole body metabolism data, the genetic reduction or deletion of Seps1 had no significant effect on muscle citrate synthase activity in gastrocnemius muscles (Fig. 6*D*). Despite being linked to metabolic disease in human populations (34) and obesity and insulin resistance in Israeli sand rats (*Psammomys obesus*) (89), the genetic reduction or deletion of Seps1 did not
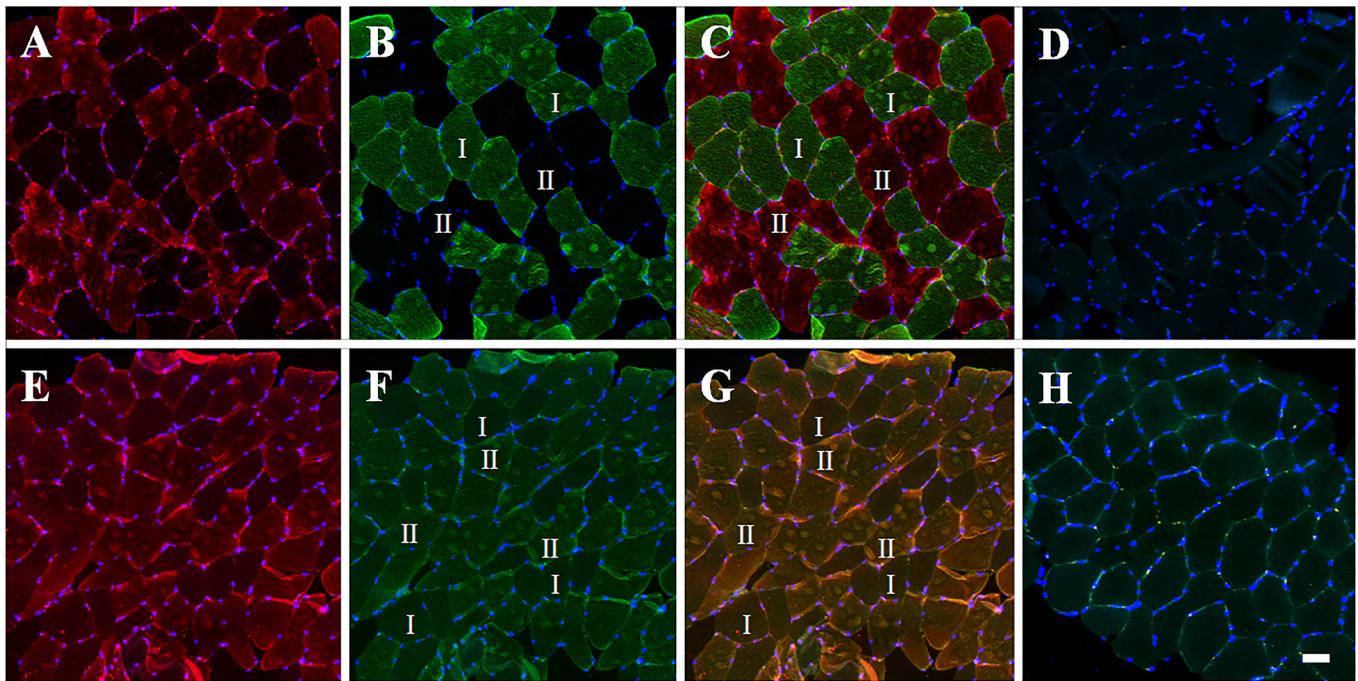
Fig. 3. Selenoprotein S (SEPS1) immunoreactivity in human skeletal muscle is fiber-type specific. *A* and *E*: SEPS1 (red) is highly expressed in human vastus lateralis muscle cross sections, with greater immunoreactivity in fast type II muscle fibers. *B*: slow type I myofiber were stained with a sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) antibody (green). *C*: overlay of SEPS1 and SERCA2 shows greater SEPS1 staining in type II fibers with no SERCA2 immunoreactivity. *D*: negative control muscle cross section reacted with the Alexa Fluor 594 goat anti-rabbit and the Alexa Fluor 488 IgG2a goat anti-mouse secondary antibodies. *F*: fast type II myofibers were stained with a SERCA1 antibody (green). *G*: overlay of SEPS1 and SERCA1 shows preferential immunoreactivity of SEPS1 in type II fibers (orange). *H*: negative control muscle cross section reacted with the Alexa Fluor 594 goat anti-rabbit and the Alexa Fluor 488 IgG1 goat anti-mouse secondary antibodies. Nuclei are counterstained with DAPI (blue). Scale bar = 50 μm. *n* = 3.

alter any measured parameters of whole body and muscle metabolism.

Contractile properties of isolated EDL and soleus muscles. Ex vivo contractile function testing was undertaken to assess
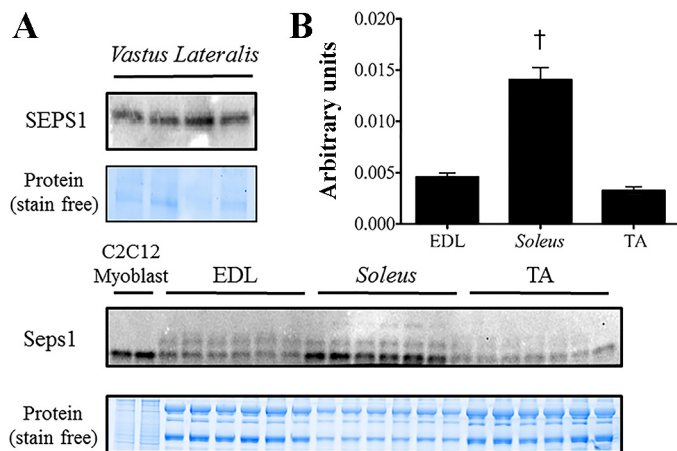

Fig. 4. Total selenoprotein S (Seps1) protein levels are higher in slow compared with fast-twitch hindlimb muscles. *A*: SEPS1 protein expression in vastus lateralis whole muscle homogenates. *B*: Seps1 protein expression in mouse extensor digitorum longus (EDL) and soleus muscles and tibialis anterior (TA) whole muscle homogenates. Proliferating C2C12 myoblast lysates were included as a positive control due to high levels of Seps1 expression. Seps1 protein levels were >2-fold higher in whole muscle homogenates from soleus compared with extensor digitorum longus (EDL) and TA muscles. †$P < 0.001$, as determined by one-way general linear model ANOVA [vastus lateralis biopsy samples (*n* = 4), BALB/c mouse hindlimb muscles (*n* = 6), and C2C12 myoblast lysates (*n* = 2 biological replicates)].

whether the genetic reduction or deletion of Seps1 would affect muscle performance. The twitch force (P_t), time to peak tension (TPT), one-half relaxation time (½RT), and L_o of EDL and soleus muscle were similar in Seps1$^{-/-}$ knockout, Seps1$^{+/-}$ heterozygous, and wild-type mice (Table 3). TPT is indicative of myosin heavy chain composition and Ca^{2+} sensitivity (62, 73, 101), while ½RT is reflective of muscle relaxation and Ca^{2+} reuptake and depends on SERCA isoform and function (72). The rate of force development (Dx/Dt) in response to a 1-Hz stimulation was also not significantly altered by Seps1 reduction or deletion in either EDL or soleus muscles.

When stimulated with increasing frequency (1–120 Hz) to assess maximal, isometric force production, EDL muscles from Seps1$^{-/-}$ knockout mice presented with a downward shift in the specific (sP_o) FFCs when compared with wild-type littermates ($d = 0.55$, $P = 0.004$; Fig. 7A), indicating that the loss of Seps1 is associated with decreased muscle strength. The FFCs of EDL muscles from Seps1$^{+/-}$ heterozygous mice and wild-type littermates did not significantly different. When force frequency data were expressed as percentage of maximum P_o, to determine whether the genetic reduction or deletion of Seps1 affects muscle force output in response to changes in stimulation frequency, EDL muscles from Seps1$^{-/-}$ knockout mice, but not Seps1$^{+/-}$ heterozygous mice, produced less force at submaximal stimulation frequencies when compared with EDL muscles from wild-type littermates ($d = 0.54$, $P < 0.05$; Fig. 7B).

As an indicator of endurance and fatigability, EDL muscles underwent 4 min of intermittent, submaximal (60 Hz) stimu-
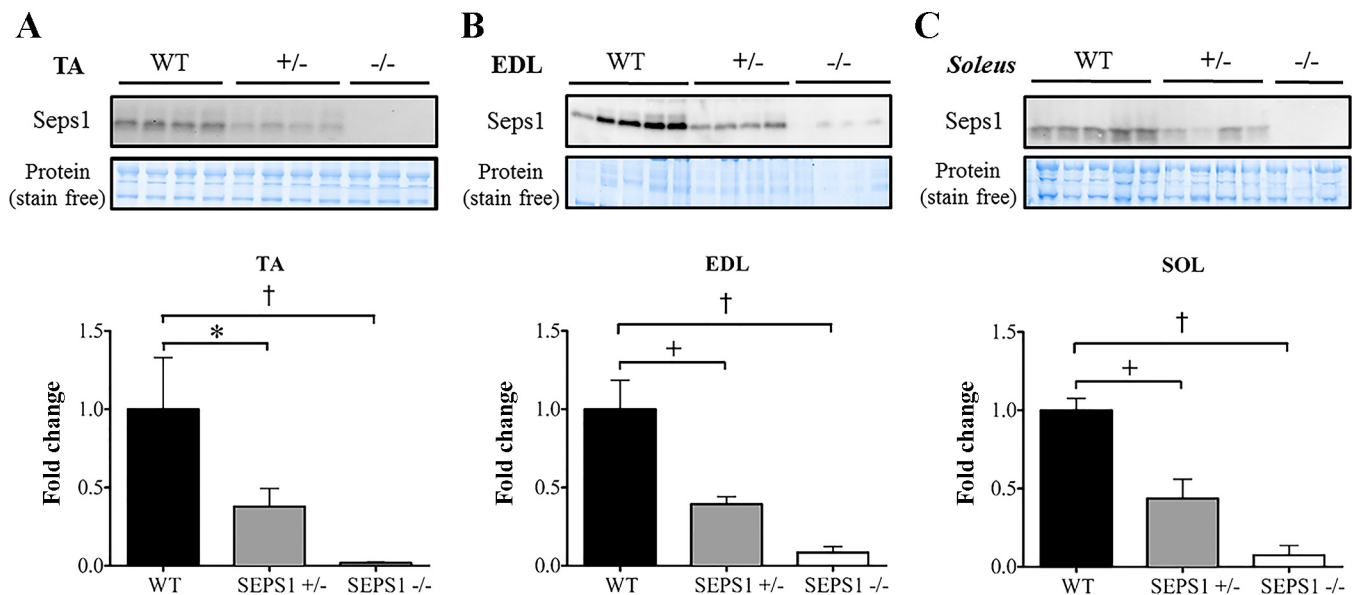
Fig. 5. The genetic reduction or deletion of selenoprotein S (Seps1) protein in fast- and slow-twitch mouse hindlimb muscles. *A*: in tibialis anterior (TA) whole muscle homogenate Seps1 protein expression was reduced by ~60% in $Seps1^{+/-}$ heterozygous mice and almost completely abolished in the $Seps1^{-/-}$ knockout mice when compared with wild-type (WT) littermates. These observations were reduced in cryosection lysates from *B*: extensor digitorum longus (EDL) and *C*: soleus muscle from $Seps1^{-/-}$ knockout, $Seps1^{+/-}$ heterozygous and wild-type mice. *$P < 0.05$ for $Seps1^{+/-}$ heterozygous vs. wild-type mice; $+P < 0.01$ for $Seps1^{+/-}$ heterozygous vs. wild-type mice; $†P < 0.01$ for $Seps1^{-/-}$ knockout vs. wild-type mice, as determined by an unpaired t-test [$Seps1^{-/-}$ knockout mice ($n = 9$), $Seps1^{+/-}$ heterozygous mice ($n = 10$), and wild-type littermates ($n = 10$)].

lation, with force recovery assessed at 2, 5, and 10 min postfatigue. During the intermittent, submaximal stimulation protocol and in the recovery period, EDL muscles from $Seps1^{-/-}$ knockout mice or $Seps1^{+/-}$ heterozygous mice produced ~20% less force than muscles from wild-type littermates (P_o: $d = 0.62$, $P < 0.001$; and $d = 0.43$, $P = 0.002$, respectively; data not shown). When this force output was expressed as percentage of initial force, fatiguability was not significantly affected by the genetic reduction or deletion of Seps1. These data, in addition to similar TPT and ½RT values and gastrocnemius muscle citrate synthase activity, suggest that the genetic reduction or deletion Seps1 was not associated with a fiber type shift. However, after the 4 min of intermittent, submaximal stimulation, percent force recovery was compromised in the $Seps1^{-/-}$ knockout mice when compared with wild-type littermates ($d = 0.37$, $P = 0.11$; Fig. 7*C*), perhaps indicative of contraction-induced damage or longer-lasting fatigue in the $Seps1^{-/-}$ knockout mice. The percent force recovery was also compromised in the $Seps1^{-/+}$ heterozygous mice ($d = 0.18$, $P = 0.11$; Fig. 7*C*).

To minimize contractile dysfunction and allow for full recovery of force producing capacity, isolated EDL and soleus muscles are rested for 2 min between each contraction of the FFC and for 4 min between the end of the FFC and the start of the fatigue protocol. Indeed, in EDL muscles from wild-type mice, the force produced at 60-Hz stimulation during the FFC was not significantly different from the force produced in response to the first 60-Hz contraction of the fatigue protocol. Unexpectedly, in EDL muscles from $Seps1^{-/-}$ knockout and $Seps1^{+/-}$ heterozygous mice, the force produced at 60-Hz stimulation at the start of the fatigue protocol was reduced by 10% compared with the force produced during the FFC ($d = 0.31$, $P < 0.002$ and $d = 0.39$, $P = 0.02$, respectively; Fig. 7*D*), indicative of a reduced tolerance to high-frequency contractions in fast-twitch EDL muscles with Seps1 reduction or deletion, perhaps due to damage to or oxidation of proteins involved in excitation-contraction coupling.

Unlike in EDL muscles, the genetic reduction or deletion of Seps1 did not affect soleus muscle strength (Fig. 8*A*) nor did it affect submaximal force production (Fig. 8*B*). As such, the absolute (P_o) force output during 4 min of intermittent, submaximal (60 Hz) stimulation or at 2, 5, and 10 min of recovery was similar in soleus muscles irrespective of genotype (data not shown). The genetic reduction or deletion of Seps1 also did not significantly affect the rate of fatiguability and force recovery (Fig. 8*C*). Finally, the force produced by soleus muscles from $Seps1^{-/-}$ knockout mice, $Seps1^{+/-}$ heterozygous mice, and wild-type littermates did not significantly differ from the 60-Hz stimulation of the FFC and the first 60-Hz stimulation of the fatigue protocol (Fig. 8*D*).

Table 2. *Body composition and muscle weight*

Genotype	Body Weight, g	Fat Mass, g	Lean Mass, g	EDL Weight, mg	SOL Weight, mg
WT	26.65 ± 0.41	1.98 ± 0.19	22.60 ± 0.46	10.95 ± 0.38	8.10 ± 0.32
$Seps1^{+/-}$	26.36 ± 0.80	2.34 ± 0.24	22.85 ± 0.66	10.79 ± 0.51	9.14 ± 0.63
$Seps1^{-/-}$	27.52 ± 1.09	2.55 ± 0.42	22.71 ± 0.82	11.14 ± 0.60	8.68 ± 0.59

Values shown are means ± SE [$Seps1^{-/-}$ knockout mice ($n = 9$), $Seps1^{+/-}$ heterozygous mice ($n = 10$), and wild-type (WT) littermates ($n = 10$)]. EDL, extensor digitorum longus; SOL, soleus; Seps1, selenoprotein S.
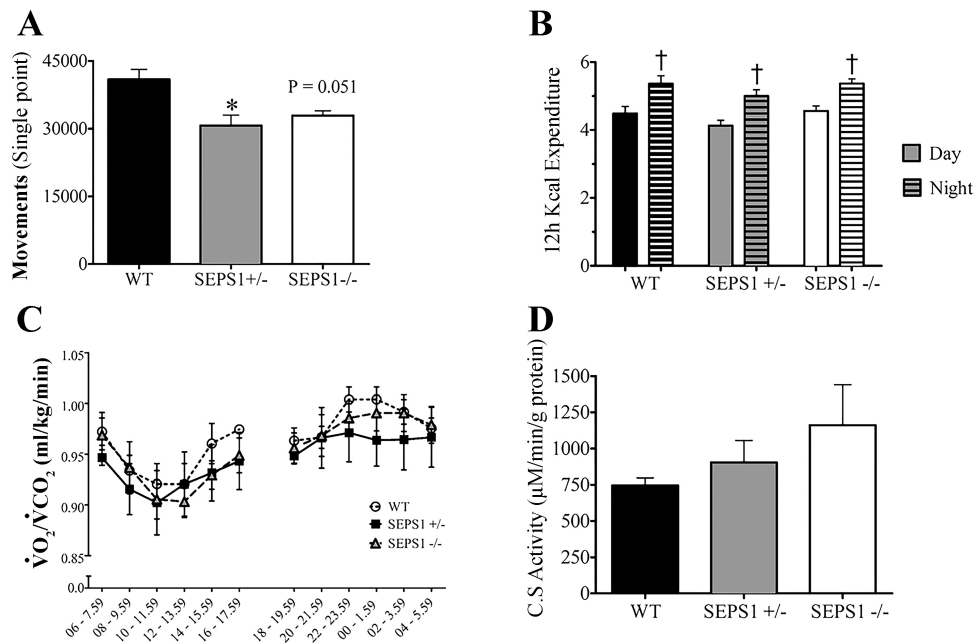
Fig. 6. Analysis of spontaneous physical activity and whole body metabolism in selenoprotein S (Seps1) global Seps1-deleted (GKO) mice. *A*: during 24 h in metabolic cages, the total spontaneous physical activity of Seps1$^{-/+}$ heterozygous mice was reduced by 22% compared with their wild-type (WT) littermates. A trend toward reduced physical activity was also observed in the Seps1$^{-/-}$ knockout mice ($P = 0.051$). *B*: resting energy expenditure, during the day (6 AM to 6 PM) and at night (6 PM to 6 AM), was similar between Seps1$^{-/-}$ knockout or Seps1$^{-/+}$ heterozygous mice and their WT littermates. It should be noted that energy expenditure at night was greater than during the day. *C*: the genetic reduction or deletion of Seps1 did not significantly affect whole body substrate metabolism as indicated by a similar respiratory exchange ratio ($\dot{V}O_2/\dot{V}CO_2$) during the day or night. *D*: citrate synthase (C.S.) activity was also not significantly different in gastrocnemius muscles from Seps1$^{-/-}$ knockout or Seps1$^{-/+}$ heterozygous mice and their WT littermates. *$P < 0.05$, for Seps1$^{+/-}$ heterozygous vs. WT mice, as determined by an unpaired *t*-test. †$P < 0.0001$, for night vs. day, as determined by an unpaired *t*-test [Seps1$^{-/-}$ knockout mice ($n = 9$), Seps1$^{+/-}$ heterozygous mice ($n = 9$), and WT littermates ($n = 9$)].

Muscle structure of EDL and soleus muscles. Given the effects of Seps1 reduction or deletion on EDL muscle contractile function and the fact that mutations in SELN, another ER resident selenoprotein, cause a muscle myopathy characterized by variability in fiber size, degeneration, focal increases in connective tissue, and centrally nucleated fibers (3, 84), a morphometric analysis of muscle cross sections was undertaken. Muscle fiber cross-sectional area (Fig. 9, *A* and *B*), the proportion of centrally nucleated fibers (a marker of recent damage and repair; Fig. 9, *C* and *D*), and percentage of connective tissue and mononuclear infiltration were similar in EDL and soleus muscles from Seps1$^{-/-}$ knockout or Seps1$^{+/-}$ heterozygous mice and their wild-type littermates (Fig. 9, *E* and *F*). In line with the normal muscle morphology of EDL and soleus muscles from Seps1$^{-/-}$ knockout or Seps1$^{+/-}$ heterozygous mice, the mRNA transcript abundance of *Mcp-1*,

a proinflammatory cytokine implicated in monocyte and macrophage recruitment, was not significantly altered by the genetic reduction or deletion of Seps1 (Fig. 9, *G* and *H*).

Gene markers of cellular stress and glutathione redox state. Cell culture studies have implicated Seps1 in regulating cellular stress responses (26, 36, 46, 100). As such, the effects of Seps1 reduction or deletion on the gene expression of the thioredoxin antioxidant system and ER stress markers in EDL and soleus muscles were assessed. In the fast-twitch EDL muscles from Seps1$^{-/-}$ knockout mice, the mRNA transcript abundance of thioredoxin inhibitor protein (*Txnip*) and thioredoxin-1 (*Trx1*) was reduced when compared with wild-type littermates ($d = 1.27$, $P = 0.025$ and $d = 1.38$, $P = 0.012$, respectively; Fig. 10, *A* and *B*). In soleus muscles, the genetic deletion of Seps1 was associated with increased *Txnip* and *Trx-1* mRNA transcript abundance ($d = 1.05$, $P = 0.045$ and

Table 3. *Twitch contractile properties of the fast EDL and slow soleus muscles*

Muscle/Genotype	P_t, mN	TPT, s	½RT, s	Dx/Dt, mN/s	L_o, mm
EDL					
WT	119.9 ± 8.9	0.200 ± 0.022	0.023 ± 0.003	22,312 ± 7,149	5.595 ± 0.17
Seps1$^{+/-}$	107.3 ± 6.1	0.220 ± 0.001	0.022 ± 0.003	13,082 ± 2,758	5.553 ± 0.121
Seps1$^{-/-}$	105.0 ± 10.3	0.220 ± 0.001	0.019 ± 0.001	9,002 ± 2,126	5.589 ± 0.145
SOL					
WT	38.8 ± 2.7	0.236 ± 0.003	0.061 ± 0.010	20,400 ± 6,892	6.542 ± 0.787
Seps1$^{+/-}$	37.6 ± 3.1	0.234 ± 0.003	0.048 ± 0.005	12,788 ± 4,688	7.773 ± 0.104
Seps1$^{-/-}$	35.5 ± 3.1	0.233 ± 0.002	0.052 ± 0.007	6,763 ± 3,958	7.825 ± 0.082

Values shown are means ± SE [Seps1$^{-/-}$ knockout mice ($n = 9$), Seps1$^{+/-}$ heterozygous mice ($n = 10$), and wild-type (WT) littermates ($n = 10$)]. EDL, extensor digitorum longus; SOL, soleus; P_t, maximum twitch force; TPT, time to peak twitch; ½RT, one-half relaxation time; Dx/Dt, maximal rate of contraction; L_o, fiber length.
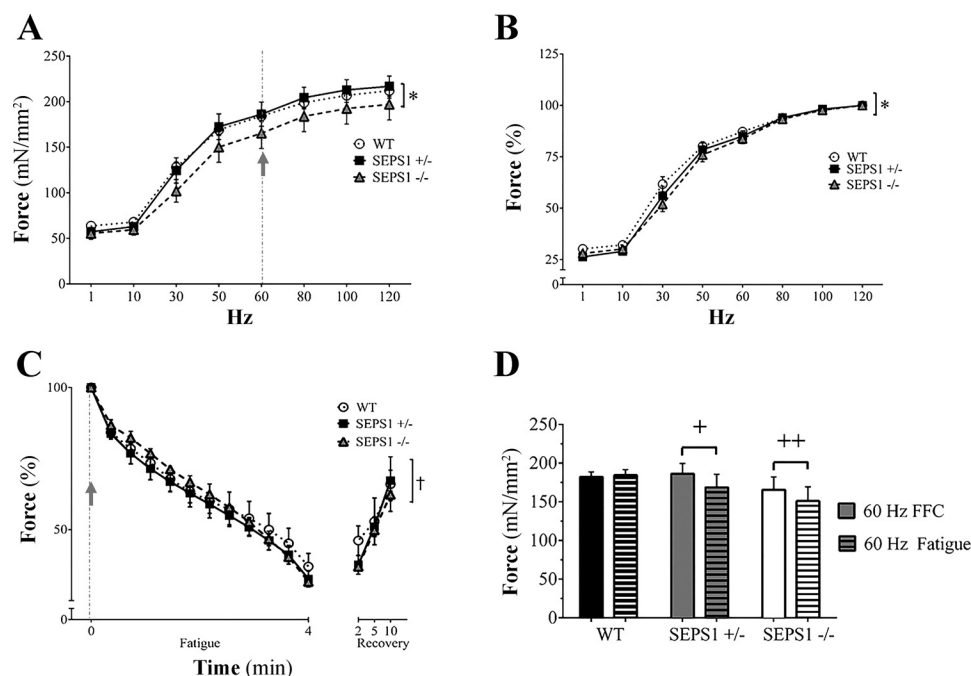
Fig. 7. Genetic reduction or deletion of selenoprotein S (Seps1) compromises force production in isolated fast-twitch extensor digitorum longus (EDL) muscles *A*: when force output was normalized to muscle cross-sectional area (specific force; sP$_o$), the genetic deletion of Seps1 still reduced muscle strength, as indicated by a downward shift of the force frequency curve (FFC) in EDL muscles of Seps1$^{-/-}$ knockout mice when compared with wild-type (WT) littermates. The force frequency curves of EDL muscles from Seps1$^{+/-}$ heterozygous and WT mice were not significantly different. *B*: the force frequency data was also expressed as %maximum P$_o$, to determine how the genetic reduction or deletion of Seps1 affects muscle force output in response to changes in stimulation frequency. In EDL muscles from Seps1$^{-/-}$ knockout mice, but not Seps1$^{-/+}$ heterozygous mice, the genetic deletion of Seps1 reduced force production at submaximal stimulation frequencies when compared with EDL muscles from WT littermates. *C*: fatigability, when expressed as % initial force, was similar between genotypes; however, the genetic reduction and or deletion of Seps1 did modulate % force recovery when compared with WT littermates. *D*: the specific force produced at 60-Hz stimulation from the FFC (dashed line in *A*) was compared with the specific force produced by the first contraction of the intermittent, submaximal (60 Hz) stimulation fatigue protocol (dashed line in *B*). sP$_o$ output was maintained in EDL muscles from WT mice, while a significant decrease was observed in EDL muscles from Seps1$^{-/-}$ knockout and Seps1$^{-/+}$ heterozygous mice, indicating a reduced tolerance to higher frequency stimulation between 80 and 120 Hz. *$P < 0.05$, main effect for genotype in Seps1$^{-/-}$ knockout vs. WT mice, as determined by two-way repeated measures ANOVA. †$P < 0.001$, main effect for genotypes in Seps1$^{-/-}$ knockout or Seps1$^{-/+}$ heterozygous mice vs. WT mice, as determined by two-way repeated measures ANOVA). +$P < 0.05$ and ++$P < 0.01$, for the difference in force output between 60-Hz stimulation from the FFC and the first contraction of the 60-Hz fatigue protocol, as determined by a paired *t*-test [Seps1$^{-/-}$ knockout mice ($n = 9$), Seps1$^{+/-}$ heterozygous mice ($n = 10$), and WT littermates ($n = 10$)].

$d = 1.50$, $P = 0.009$, respectively; Fig. 10, *F* and *G*). The genetic reduction or deletion of Seps1 had no significant effect on ER stress chaperone *Grp78* in EDL or soleus muscles (Fig. 10, *C* and *H*). However, in EDL muscles from Seps1$^{-/-}$ knockout mice *Grp94* ($d = 1.11$, $P = 0.02$; Fig. 10*D*) and Seps1$^{+/-}$ heterozygous mice, *Chop* ($d = 0.99$, $P = 0.047$; Fig. 10*E*) mRNA transcript abundance was reduced when compared with wild-type littermates, whereas no such effect was observed in the soleus muscles. These findings are interesting given that *Grp94*, in addition to regulating protein folding, is a Ca^{2+} binding protein linked to the regulation of Ca^{2+} homeostasis in the ER (10). Altogether, the genetic reduction or deletion of Seps1 has distinct effects on the gene markers of ER stress and the thioredoxin antioxidant system in EDL compared with soleus muscles.

As additional markers of oxidative stress, TBARS concentrations (a marker of lipid peroxidation) and the redox state the glutathione antioxidant system were quantified in gastrocnemius muscles from Seps1$^{-/-}$ knockout, Seps1$^{-/+}$ heterozygous and wild-type mice. The genetic reduction and deletion of Seps1 did not significantly alter TBARS concentrations in gastrocnemius muscle lysates (Fig. 11*A*). In concordance with the lipid peroxidation data, oxidized glutathione (GSSG) content and the reduced-to-oxidized glutathione ratio (GSH:

GSSG) also did not differ between genotypes (Fig. 11, *C* and *D*), although a very modest increase in reduced glutathione content was observed in Seps1$^{-/-}$ knockout mice compared with wild-type littermates ($d = 1.00$, $P = 0.045$; Fig. 11*B*). The mRNA transcript abundance of glutathione peroxidase 1 (*Gpx1*) was similar in EDL and soleus muscles from Seps1$^{-/-}$ knockout, Seps1$^{-/+}$ heterozygous and wild-type littermates (Fig. 11, *E* and *F*).

DISCUSSION

In skeletal muscle, the careful regulation of oxidative and ER stress responses is necessary for optimal metabolic and contractile function. A transient increase in reactive oxygen species can improve muscle performance (39, 78) and stimulate adaptation to exercise (9, 65). Similarly, the unfolded protein response is activated in response to unaccustomed contractile activity (58) and also promotes adaptation to exercise (94). ER resident selenoproteins can regulate ER redox state, the UPR, and Ca^{2+} homeostasis; this highlights their physiological relevance to skeletal muscle contractile function and adaptation. Indeed, mutations in SELN lead to a myopathy where a hyperoxidized ER/SR disrupts Ca^{2+} release (33) and/or reuptake leading to muscle weakness and wasting (49).
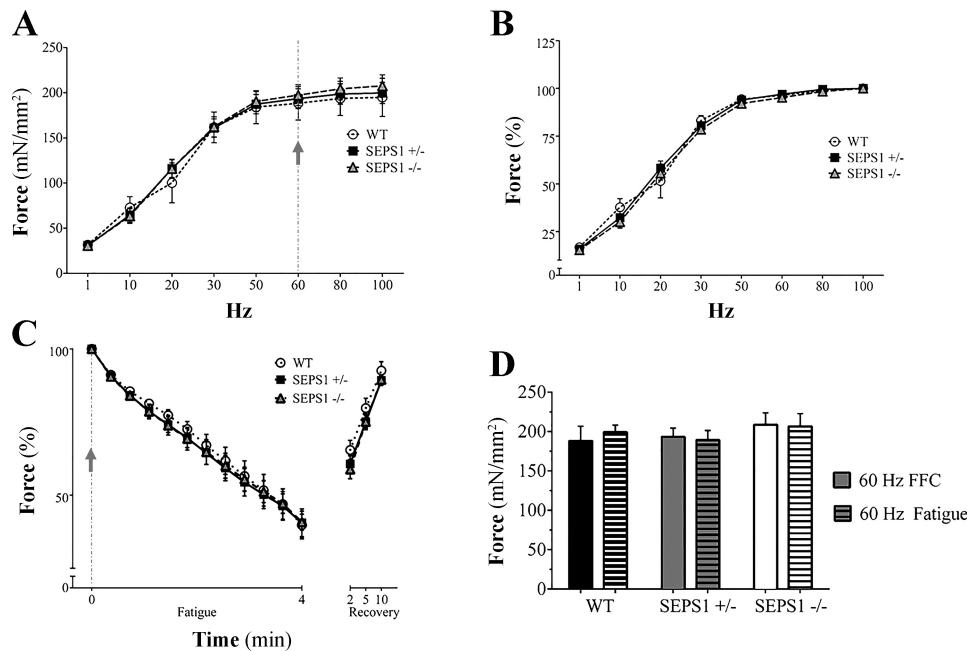
Fig. 8. Genetic reduction or deletion of selenoprotein S (Seps1) does not alter the performance of isolated slow-twitch soleus muscles. *A*: muscle strength was similar in soleus muscles from $Seps1^{-/-}$ knockout or $Seps1^{+/-}$ heterozygous compared with wild-type (WT) littermates, as indicated by similar force frequency curves (FFCs) normalized to muscle cross-sectional area. *B*: the genetic reduction or deletion of Seps1 also had no significant effect on force production at submaximal stimulation frequencies. *C*: the genetic reduction or deletion of Seps1 did not affect the rate of fatigue nor did it affect force recovery. *D*: the specific force produced at 60-Hz stimulation from the force frequency curve (dashed line in *A*) was compared with the force produced by the first contraction of the intermittent, submaximal (60 Hz) stimulation fatigue protocol (dashed line in *B*). Specific output was maintained in soleus muscles from $Seps1^{-/-}$ knockout, $Seps1^{+/-}$ heterozygous and WT mice [$Seps1^{-/-}$ knockout mice (*n* = 9), $Seps1^{+/-}$ heterozygous mice (*n* = 10) and WT littermates (*n* = 10)].

SEPS1 can regulate ER and oxidative stress responses in various cell lines, although its function in vivo has not been well characterized. Here, we report for the first time that Seps1 expression and cellular localization is fiber type dependent, and when Seps1 is genetically reduced or deleted, the contractile performance of fast-twitch, but not slow-twitch, muscles is compromised. Thus identifying Seps1 as a novel ER resident selenoprotein with the potential to modulate skeletal muscle contractile function.

In human vastus lateralis and mouse EDL and soleus muscles, fast type II muscle fibers demonstrated greater intramyofiber immunoreactivity to the anti-Seps1 antibody than slow type I muscle fibers. Seps1 sarcolemmal staining was limited, and this is in concordance with Bubenik et al. (14), who using immunohistochemical techniques, found Seps1 to be predominantly localized to the ER. Given its localization to the ER membrane, SR localization of Seps1 is likely in skeletal muscle (43, 75). Seps1 was also highly expressed in blood vessels, and vascular smooth muscle cells are known to synthesize Seps1 (97, 99). Skeletal muscle is a heterogeneous tissue composed of muscle fibers, blood vessels, nerves, and extracellular matrix. While immunohistochemistry provides valuable insight into cellular localization, whole muscle lysates and Western blotting are needed to assess total Seps1 protein levels. Interestingly, Seps1 protein levels were greater in whole muscle lysates from soleus compared with EDL and TA muscles. We propose that fiber type differences in microvasculature, as well as the high proportion (60–70%) of type IIa fibers (5, 11, 91), contribute to the higher levels of Seps1 expression in soleus muscles.

In global Seps1 knockout mice, the genetic reduction or deletion of Seps1 decreased spontaneous physical activity. This reduction was unlikely the result of an altered metabolic profile, as resting energy expenditure, substrate utilization (as reflected by RER), and gastrocnemius muscle citrate synthase activity were not affected by the genetic deletion or reduction of Seps1. Given the global knockout mouse model and that

Seps1 is highly expressed in the brain (e.g., cerebrum and cerebellum) (97), central nervous system effects of Seps1 reduction or deletion on physical activity cannot be excluded. With regard to body composition, the genetic reduction or deletion of Seps1 had no significant effect on lean and fat mass. This lack of effect on fat mass in mice fed a standard chow diet ad libitum is in contrast to 3T3-L1 preadipocytes cell culture studies identifying Seps1 as an antiadipogenic selenoprotein, whose degradation (and thus reduced expression) promotes adipocyte differentiation (35). This highlights the importance of in vivo studies to elucidate selenoprotein function. The lack of effect of Seps1 on whole body metabolism was also somewhat intriguing, given the associations among SEPS1, glucose metabolism, and type 2 diabetes reported in human subjects (21, 34, 99) and the initial observation of dysregulated hepatic Seps1 expression in fed and fasted diabetic Israeli sand rats (89).

To investigate whether impaired skeletal muscle performance contributed to the decrease in physical activity observed with Seps1 deletion or reduction, the isometric contractile function of EDL and soleus muscles was assessed ex vivo. The genetic reduction or deletion of Seps1 did not appear to cause a fiber type shift in EDL or soleus muscles, as indicated by similar ½RT and TPT values in response to a 1-Hz (twitch) stimulation. However, it did compromise muscle strength and tolerance of high-frequency and intermittent, submaximal frequency stimulation in fast-twitch EDL muscles, as indicated by a downward shift in the FFC and a reduction in the submaximal force production of EDL muscles from $Seps1^{-/-}$ knockout mice, and, furthermore, a decrease in force output between the 60-Hz stimulation of the FFC and the first 60-Hz stimulation of the fatigue protocol in both the $Seps1^{+/-}$ heterozygous and $Seps1^{-/-}$ knockout mice. Between 60- and 120-Hz stimulation, the isometric force produced by EDL muscles increased by ~15%, a rather modest increase in force given the doubling in stimulation frequency. High-frequency stimulations, especially in isolated whole muscles, can lead to inadequate oxygen
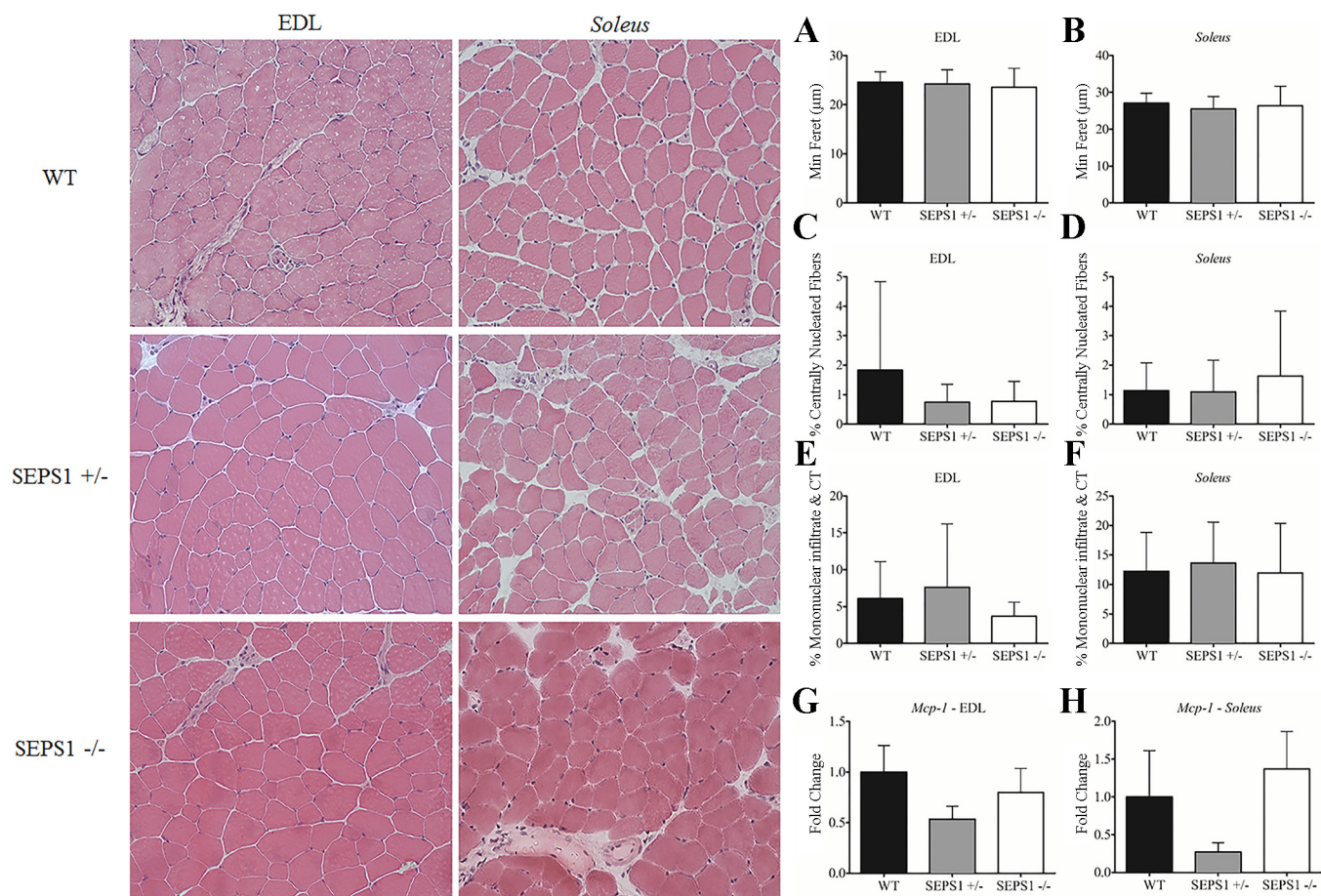
Fig. 9. Muscle morphology and markers of degeneration and inflammation in extensor digitorum longus (EDL) and soleus muscles from selenoprotein S (Seps1) global Seps1-deleted (GKO) mice. In EDL and soleus muscles, the genetic reduction or deletion of Seps1 did not alter the muscle fiber cross-sectional area (as measured by the minimal feret diameter) nor was it associated with muscle pathology (*A* and *B*), as indicated by a similar proportion of centrally nucleated fibers (*C* and *D*), percentage of mononuclear infiltrate and connective tissue (CT) in muscle cross sections (*E* and *F*), and similar *Mcp-1* mRNA transcript abundance (*G* and *H*) from in EDL or soleus muscles from Seps1$^{-/-}$ knockout, Seps1$^{+/-}$ heterozygous and wild-type (WT) mice [Seps1$^{-/-}$ knockout mice ($n = 9$), Seps1$^{+/-}$ heterozygous mice ($n = 10$), and WT littermates ($n = 10$)].

diffusion and hypoxia-induced cellular stress (7) and thus can compromise contractile performance. This could contribute the impairment in force recovery in EDL muscles from Seps1$^{-/-}$ knockout and Seps1$^{-/+}$ heterozygous mice following 4 min of intermitted, submaximal stimulation. It also needs to be acknowledged that the Ringer solution is bubbled with 95% O$_2$ (carbogen), which in itself can be a source of damaging oxidants. Unlike in EDL muscles, the genetic deletion or reduction of Seps1 did not affect soleus muscle strength and tolerance of high-frequency and intermittent, submaximal frequency stimulation. A higher resting metabolic rate and greater depletion of high-energy phosphates during ex vivo contractile function testing (7) may make EDL muscles more vulnerable to dysfunction than soleus muscles. In addition, soleus muscles may be better protected by their greater antioxidant capacity (32, 40) and more oxidative phenotype (72). Thus the reduction in physical activity in Seps1$^{-/+}$ heterozygous and Seps1$^{-/-}$ knockout mice may be due to impaired contractile function of fast-twitch muscles, as these are the predominant fiber type in mouse hindlimb muscles (11, 72). It should be noted that the impairment in EDL contractile function associated with Seps1 reduction or deletion did not appear to be the consequence of altered muscle morphology or myopathy. As

EDL and soleus muscle cross sections from Seps1$^{-/-}$ knockout and Seps1$^{+/-}$ heterozygous were structurally similar to those from wild-type mice, with no significant differences in muscle fiber size and markers of degeneration and regeneration observed.

The impairment in contractile function following Seps1 reduction or deletion was associated with a differential regulation of gene markers of oxidative and ER stress in EDL compared with soleus muscles. *Trx-1* mRNA transcript abundance was decreased with Seps1 deletion in EDL muscles, whereas in soleus muscles it was increased. Based on biochemical assays, the oxidoreductase activity of Seps1 against H$_2$O$_2$ is thought to be due to its interactions with thioredoxin (46), and here Seps1 is shown to be associated with the thioredoxin antioxidant system in vivo for the first time. *Txnip* gene expression was similarly affected by SEPS1 deletion, with decreased expression in EDL muscles and increased expression in soleus muscles. As well as being an inhibitor of thioredoxin, TXNIP can be upregulated in response to ER stress and is thought to link ER stress to inflammation (45). Seps1 reduction or deletion may have greater effects on thioredoxin antioxidant system, as *Gpx1* mRNA transcript abundance in EDL and soleus muscles and the GSH-to-GSSG ratio in gastrocnemius

muscles did not differ between Seps1$^{-/-}$ knockout or Seps1$^{-/-}$ heterozygous mice and their wild-type mice.

Seps1 deletion did not increase the expression of gene markers of ER stress in mouse hindlimb muscles. Rather, *Grp94* and *Chop* mRNA transcript abundance was significantly decreased in EDL muscles from Seps1$^{-/-}$ knockout mice compared with wild-type littermates, whereas in soleus muscle SEPS1 deletion had no significant effect on the gene expression of *Grp78*, *Grp94*, and *Chop*. Seps1 has been shown to be protective against ER stress by facilitating the retrograde translocation of misfolded proteins out of the ER, this function may be context and cell-type dependent. In various intestinal cell
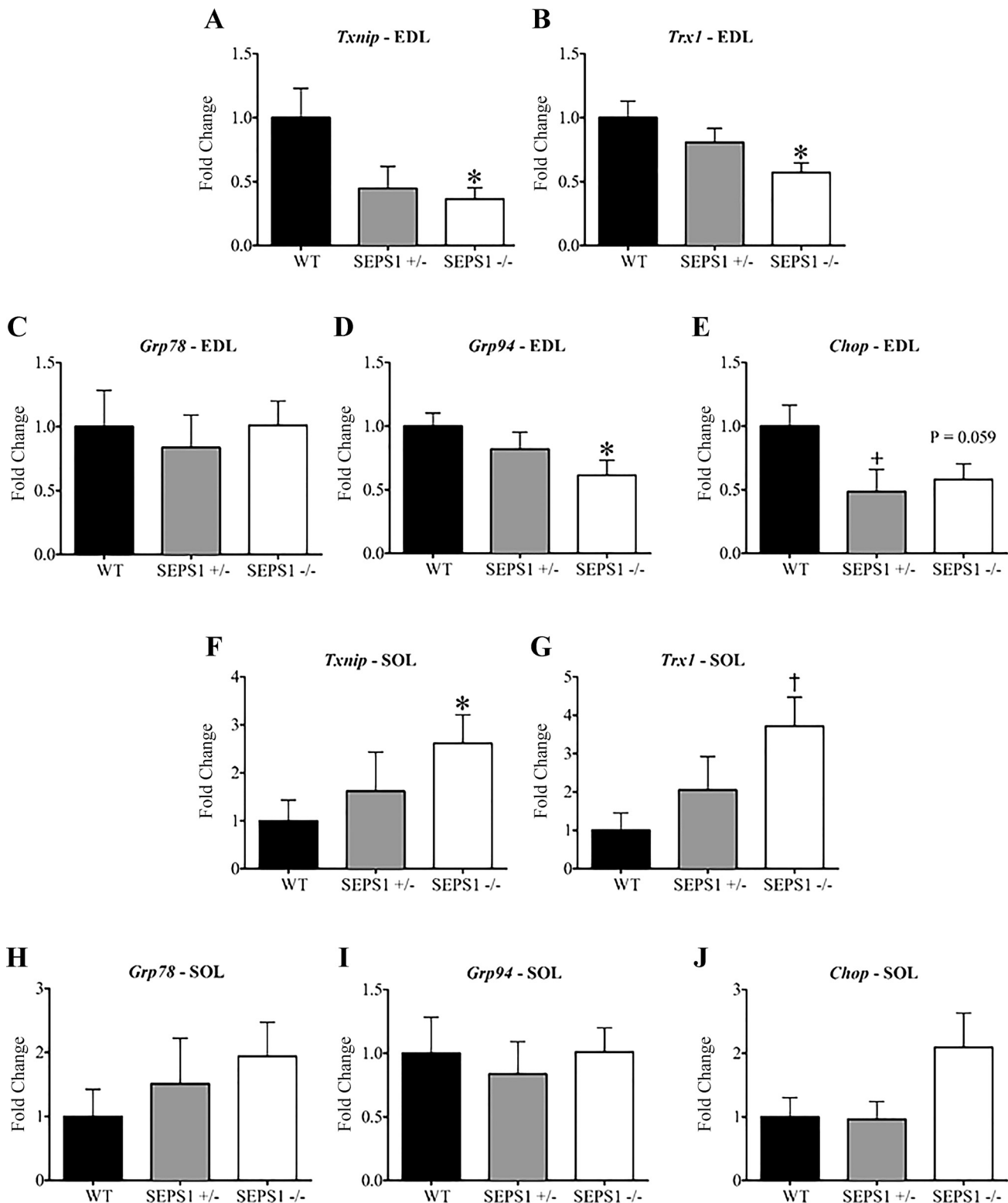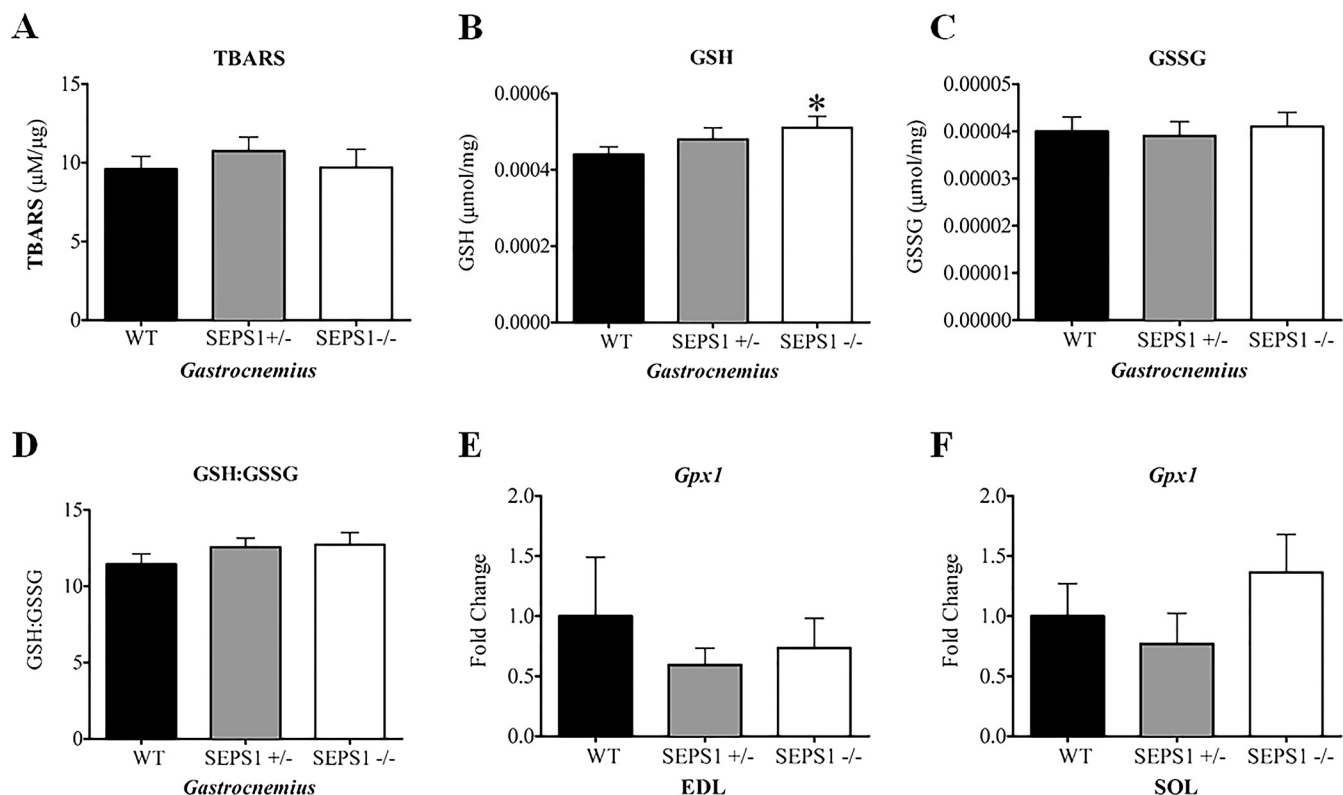
Fig. 11. Effects of selenoprotein S (Seps1) reduction or deletion on lipid peroxidation and the glutathione antioxidant pool in hindlimb muscles. *A*: in gastrocnemius muscles the genetic reduction or deletion of Seps1 had no significant effect on thiobarbituric acid reactive substances (TBARS) concentrations, a maker of lipid peroxidation and oxidative stress. *B*. reduced glutathione (GSH) levels were higher in gastrocnemius muscles from Seps1$^{-/-}$ knockout mice, but not Seps1$^{-/+}$ heterozygous, when compared with wild-type (WT) littermates. *C*: the genetic reduction or deletion of Seps1 had no effect on oxidized glutathione (GSSG) levels. *D*: the GSH-to-GSSG ratio also did not differ between gastrocnemius muscles from WT and Seps1$^{-/+}$ heterozygous or Seps1$^{-/-}$ knockout mice. *E* and *F*: in EDL (*E*) and soleus muscles (*F*) the genetic reduction of Seps1 had no significant effect on glutathione peroxidase 1 (*Gpx1*) mRNA transcripts. *$P < 0.05$, for Seps1$^{-/-}$ knockout vs. WT mice, as determined by an unpaired *t*-test [Seps1$^{-/-}$ knockout mice ($n = 9$), Seps1$^{+/-}$ heterozygous mice ($n = 10$), and WT littermates ($n = 10$)].

lines, SEPS1 gene knockdown did not exacerbate the UPR in the basal state or in response to pharmacologically induced ER stress (80).

Here, we report that Seps1 is preferentially localized to type II fibers in mouse hindlimb and human vastus lateralis muscles and may be a novel antioxidant regulator of fast-twitch muscle contractile function. These findings of a fiber type-specific effect of Seps1 deletion or reduction align well with our observations in dystrophic *mdx* mice, the murine model of Duchenne muscular dystrophy characterized by chronic contraction induced injury, inflammation, and oxidative stress (85, 95). In *mdx* mice, the genetic reduction of Seps1 exacerbated the inflammatory profile of EDL, but not soleus, muscles (93). In EDL muscles only, the genetic reduction of Seps1 increased

the expression of gene markers of inflammation (proinflammatory cytokines *Tgfb1* and *Mcp1* and the macrophage marker *F4/80*), and this was associated with a reduction in myofiber size (93). Overall, fiber type differences in selenoprotein expression and the function are not well described in the literature. In humans, SELN-related muscle myopathy is associated with atrophy of slow type I fibers (16). In mice, SelN protein expression is higher in EDL than soleus muscles (12), whereas glutathione peroxidase enzyme activity is higher in soleus muscles than in the deep and superficial vastus lateralis muscles (32, 40).

Further research is required to investigate the differential association between Seps1 levels and *Trx-1* and *Txnip* gene expression in fast- and slow-twitch hindlimb muscles. The

Fig. 10. mRNA transcript abundance of oxidative and endoplasmic reticulum (ER) stress markers in extensor digitorum longus (EDL) and soleus muscles from selenoprotein S (Seps1) global Seps1-deleted (GKO) mice. *A* and *B*: in EDL muscles, from Seps1$^{-/-}$ knockout mice, but not Seps1$^{+/-}$ heterozygous mice, thioredoxin inhibitor protein (*Txnip*; *A*) and thioredoxin-1 (*Trx1*; *B*) gene expression was reduced when compared with wild-type (WT) littermates. *C*: glucose-regulated protein 78 (*Grp78*) mRNA transcript abundance was not altered by Seps1 reduction or deletion. *D*: *Grp94* gene expression was reduced in Seps1$^{-/-}$ knockout mice when compared with WT littermates. *E*: *Chop* gene expression was significantly reduced in Seps1$^{+/-}$ heterozygous mice when compared with WT littermates. Reduced *Chop* gene expression also tended to be reduced in Seps1$^{-/-}$ knockout mice ($P = 0.059$). *F* and *G*: in soleus muscles, *Txnip* (*F*) and *Trx1* (*G*) gene expression was increased in Seps1$^{-/-}$ knockout mice, but not Seps1$^{+/-}$ heterozygous mice, when compared with WT littermates. *H–J*: the mRNA transcript abundance of *Grp78* (*H*) or *Grp94* (*I*) and *Chop* (*J*) mRNA levels did not differ between genotypes. *$P < 0.05$, for Seps1$^{-/-}$ knockout vs. WT mice, as determined by an unpaired *t*-test. +$P < 0.05$, for Seps1$^{+/-}$ heterozygous vs. WT mice, as determined by an unpaired *t*-test. †$P < 0.01$, for Seps1$^{-/-}$ knockout vs. WT mice, as determined by an unpaired *t*-test [Seps1$^{-/-}$ knockout mice ($n = 9$), Seps1$^{+/-}$ heterozygous mice ($n = 10$), and WT littermates ($n = 10$)].

thioredoxin antioxidant system is an important regulator of oxidative, ER and inflammatory stress responses (29, 61). All of which, depending on the biological context, mediate skeletal muscle adaptation to exercise (64) or dysfunction in chronic disease (19, 85). The underlying mechanism leading to the impairment in force production following the genetic reduction or deletion of Seps1 remains to be elucidated. The cytosolic Sec residue of Seps1, either directly or through interactions with binding partners, may regulate the redox state of the contractile apparatus, SERCA pumps and/or the ryanodine receptor, all of which can affect the cross-bridge cycling and Ca^{2+} release and reuptake and ultimately force output (39). Whether this impairment in ex vivo force production in the $Seps1^{-/+}$ heterozygous and $Seps1^{-/-}$ knockout mice modulates exercise capacity and adaptation to exercise training remains to be determined.

Perspectives and Significance

Adequate dietary intake of selenium is necessary for skeletal muscle health (87). In certain contexts, selenium supplementation can improve contractile function, presumably through increased selenoprotein synthesis (12). However, the mechanisms by which selenoproteins modulate skeletal muscle health and function appear to be protein specific and complex. For example, deficiency in selenoprotein P, which functions as the key selenium transport protein in circulation and is produced mainly by the liver, renders obese mice more responsive to endurance training due to enhanced reactive oxygen species signaling (52). In contrast, deficiency of the ER resident selenoproteins Seps1 and SelN (33, 49, 53) is deleterious for skeletal muscle performance. In concordance with our previous observations in dystrophic hindlimb muscles from *mdx* mice (93), the genetic reduction (e.g., a 50% decrease in protein Seps1 levels) or deletion of Seps1 had deleterious effects on fast-, but not slow-, twitch hindlimb muscles. It is the fast-twitch muscle fibers that are preferentially vulnerable to contractile dysfunction with aging and chronic disease (13), when cellular and inflammatory stressors are increased (42) and micronutrient intake, including selenium, is suboptimal (67, 96).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.A. and N.S. conceived and designed research. A.B.A., C.R.W., C.S.S., N.L.M., L.G.F., C.-H.W., X.A.C., P.S.F., Z.M.S., and N.S. performed experiments; A.B.A., X.A.C., P.S.F., Z.M.S., and N.S. analyzed data; A.B.A. and N.S. interpreted results of experiments; A.B.A. prepared figures; A.B.A. and N.S. drafted manuscript; A.B.A., C.R.W., N.L.M., S.A., and N.S. edited and revised manuscript; A.B.A., C.R.W., C.S.S., N.L.M., L.G.F., C.-H.W., X.A.C., P.S.F., Z.M.S., S.A., and N.S. approved final version of manuscript.

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