Bcl-2-associated autophagy regulator Naf-1 required for maintenance of skeletal muscle

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Received November 28, 2011; Revised January 23, 2012; Accepted February 10, 2012

Nutrient-deprivation autophagy factor-1 (NAF-1) was identified as an endoplasmic reticulum (ER) BCL-2-interacting protein, which functions to mediate the ability of ER BCL-2 to antagonize Beclin 1-dependent autophagy and depress ER calcium stores. In humans, a point mutation in Naf-1 (synonyms: Cisd2, Eris, Miner1 and Noxp70) is responsible for the neurodegenerative disorder Wolfram Syndrome 2. Here, we describe the generation and characterization of the Naf-1 gene deletion in mice. Naf-1 null mice display discernable clinical signs of degeneration at 2–3 months of age, with early evidence of significant defects in the structure and performance of skeletal muscle. Skeletal muscles from Naf-1 knockout mice demonstrate a significant shift towards slow-twitch (type I) fibers and greater resistance to muscle fatigue. Force-generating capacity is dramatically reduced in Naf-1−/− muscle. Consistent with its role in ER BCL-2-mediated regulation of autophagy and calcium flux, these physiological deficiencies were accompanied by augmented autophagy and dysregulated calcium homeostasis. In contrast, this also included adaptive enlargement of mitochondria with extensive cristae structures. Thus, NAF-1, a BCL-2-associated autophagy regulator, is required for homeostatic maintenance of skeletal muscle. Our findings uncover a novel pathway that is required for normal muscle maintenance, which may ultimately provide a novel therapeutic target for treating certain muscle pathologies.

INTRODUCTION

At the endoplasmic reticulum (ER), roles for BCL-2 include influences on cellular calcium (Ca2+) homeostasis (1–3) as well as regulation of both apoptosis (4) and the cell survival macroautophagy pathway (5–7). Such controls of apoptosis and autophagy by Bcl-2 are multi-faceted, but in part are likely linked to its influence on ER Ca2+ flux. Autophagy is a catabolic process whereby cells degrade their own cellular constituents through the formation of autophagosomes, double membrane vesicles that fuse with lysosomes to break down autophagosomal content and allow for the recycling of macromolecular nutrients (8). Autophagy is dependent on the haploinsufficient tumor suppressor protein Beclin 1, an essential member of a complex that includes hVps34, a class III phosphatidylinositol 3-kinase, in order to generate the phosphatidylinositol 3-phosphate required for autophagosome membrane formation (9,10). BCL-2 at the ER membrane negatively regulates this process by physical association with Beclin 1 to interfere with formation of the Beclin 1/hVps34 complex (5). We recently identified the small 15 kDa ER transmembrane (TM) protein, nutrient-deprivation autophagy factor-1 (NAF-1, synonyms: Cisd2, ERIS, Miner1 and Noxp70), as a BCL-2/BCL-XL-associated co-factor that is required for BCL-2 to functionally antagonize Beclin 1-dependent autophagy at the ER (11). Since NAF-1 does not influence the ability of BCL-2 at the ER to regulate caspase-dependent apoptosis, it appears to be responsible for partitioning ER BCL-2 to the autophagy pathway. Moreover, like BCL-2, NAF-1 physically associates with the ER inositol 1,4,5-triphosphate (IP3) Ca2+ leak channel and is required for BCL-2-dependent regulation of the channel. In order to extend these cell-based and...
biochemical findings to a physiological setting, we have studied the consequences of functional Naf-1 gene deletion in the mouse. A previous study of Naf-1/Cisd2 in the mouse suggested a potential contribution to aging, but no mechanistic function was ascribed to the protein and contributions to muscle physiology were not determined (12).

Naf-1 is a member of a unique 3-membered family of proteins, each harboring a signature 39 amino acid-long CDGSH domain, which binds a two iron—two sulphur (2Fe—2S) cluster (13,14). A homozygous mutation was identified in the gene encoding NAF-1, located on human chromosome 4q24, that is causative for Wolfram syndrome type 2 (WFS2) (15). Wolfram syndromes are rare autosomal recessive neurodegenerative diseases that result in a number of clinical manifestations, namely diabetes insipidus, diabetes mellitus, optic atrophy and deafness (referred to as DIDMOAD). Other symptoms include psychiatric illnesses, renal-tract abnormalities and gonadal disorders. To date, two genes have been associated with Wolfram syndrome, WFS1 and WFS2 (NAF-1), resulting in Wolfram syndrome types 1 and 2, respectively. WFS1 was mapped to chromosome 4p16.3 and encodes for wolframin, an ER TM glycoprotein; like NAF-1, WFS-1 plays a role in regulating intracellular Ca2+ homeostasis (16—20). Multiple Wolfram syndrome type 1 (WFS1) causative mutations have been identified within the WFS1 gene, while only a single mutation has been identified in WFS2/NAF-1. A missense mutation (G to C at nucleotide 109) results in aberrant splicing of the NAF-1 mRNA, leading to the theoretical production of a severely truncated NAF-1 protein (15). It is not known whether the predicted open reading frame encoding just 42 amino acids is expressed but ectopic expression of this fragment in cultured cells had no observable manifestations (11). Individuals with WFS2 have been reported to develop symptoms additional to those found in WFS1 patients, including bleeding diathesis and defective platelet aggregation (21).

Here, we describe the generation and characterization of the Naf-1 gene deletion in mice. Naf-1-deficient mice demonstrate clinical signs of progressive degeneration beginning at 2—3 months of age, resulting in a need to euthanize at about 1 year. The most striking early onset phenotypic difference between wild-type and Naf-1 knockout mice was observed in skeletal muscle as evidenced by electron microscopy analysis. Skeletal muscles from 2—3-month-old Naf-1 null mice exhibit decreased force-generating capacity and a shift towards the slow-twitch fiber phenotype. The observed impairment in skeletal muscle function is accompanied by enhanced autophagy, especially in the diaphragm, and is associated with enlarged mitochondria containing abundant cristae, which in other studies has been described as a survival response to exacerbated cytoplasmic autophagy (22,23). Augmented autophagy was also observed in primary myoblasts derived from Naf-1−/− mice compared with the wild-type. Moreover, and consistent with our earlier findings in epithelial cell cultures (11), we show that the loss of Naf-1 results in dysregulated intracellular Ca2+ homeostasis in both Naf-1−/− primary myoblast and myotube cultures. Thus, NAF-1, which is required for BCL-2-associated suppression of autophagy and Ca2+ flux at the ER, plays a critical role in homeostatic maintenance of skeletal muscle.

**RESULTS**

**Characterization of Naf-1 gene contribution**

The causative defect in WFS2 is a single missense mutation on human chromosome 4 within the Naf-1 gene (15). The gene product, NAF-1, is evolutionarily conserved across a wide range of species (11). Alignment of the NAF-1 human and mouse amino acid sequences revealed that the two sequences are 96% identical (Fig. 1A), suggesting that NAF-1 serves similar functions in both species. In order to study the physiological contribution of the NAF-1 gene, we generated Naf-1 knockout mice (Fig. 1B). The Naf-1 gene trap ES cell line was obtained from the Sanger International Gene Trap Center (24) and congenic C57BL/6 Naf-1+/− mice were generated as described in Materials and Methods. In this study, comparisons were made between wild-type and Naf-1−/− litter mates derived from breedings between heterozygotes.

Analysis of protein expression in mouse spleen tissue extracted from Naf-1 wild-type and knockouts confirmed the absence of Naf-1 protein expression in Naf-1−/− mice by immunoblot (Fig. 1C). A minor cross-reacting or non-specific immunoreactive band migrating slightly faster than NAF-1 was detected in both wild-type and knockout tissue. As NAF-1 functions to mediate BCL-2 regulation of Beclin 1-dependent autophagy (11), we examined protein expression levels and overall tissue distribution of BCL-2 and Beclin 1 from various tissues obtained from Naf-1+/+ and Naf-1−/− mice. We found that gene deletion of Naf-1 did not affect BCL-2 or Beclin 1 protein expression in the tissues examined (Fig. 1D).

Naf-1-deficient mice (Fig. 1B) appear to develop normally but begin to manifest clinical signs between 2 and 3 months of age. These include low body weight, ruffled fur, tremors, lethargy and general weakness of the animal.

**Naf-1−/− skeletal muscle electron microscopy**

Among the earliest manifestations noted in the knockout mice were defects in the structure and performance of skeletal muscle. Analysis of diaphragm muscle from Naf-1+/+ and Naf-1−/− 3.5-month-old mice by electron microscopy, for example, revealed significant skeletal muscle structural disarray in Naf-1 deleted animals (Fig. 1E). This included disintegration of the sarcomeric structure and increased intermyofibrillar space in many areas, as well as the striking presence of numerous vacuolar structures suggestive of dilated sarcoplasmic reticulum (Fig. 1Ec and d). Of note, the mitochondria observed in Naf-1−/− diaphragm were significantly enlarged compared with the wild-type and contained extensive and compact cristae. A similar phenotype was observed in electron micrographs of tibialis anterior (TA) muscle (data not shown). In contrast, no differences were observed in the overall structure of cardiac muscle in Naf-1 knockout mice compared with the wild-type (data not shown). It is noteworthy that, unlike skeletal muscle in which BCL-2 is readily detected by immunoblot (see below, Fig. 4A), BCL-2 in cardiac muscle was undetectable (Fig. 1D).
Figure 1. Naf-1 gene deletion. (A) Alignment of NAF-1 human and mouse amino acid sequences. Hyphens (−) represent amino acid residues which are identical in both sequences. (B) Naf-1+/+ and Naf-1−/− 5.5-month-old mice. (C) Spleen tissue obtained from Naf-1+/+ and Naf-1−/− mice were analyzed for expression of Naf-1 by immunoblot analysis. (D) Tissues collected from Naf-1+/+ and Naf-1−/− mice were homogenized and lysed. Lysates were resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subjected to immunoblot analysis with the indicated antibodies. (E) Representative electron micrographs of diaphragm muscle dissected from 3.5-month-old Naf-1+/+ (a, b, c, and d) and Naf-1−/− (c and d) mice. Scale bars represent 1 μm. (F) Primary myoblasts isolated from diaphragm muscles of 2-month-old Naf-1+/+ and Naf-1−/− mice were fixed and stained with anti-TOM20 antibody. Representative images are shown.
**Naf-1**<sup>−/−</sup> skeletal muscle fiber type and size

Immunohistochemical analysis of diaphragm sections reacted with antibody against slow type I myosin heavy chain revealed that **Naf-1**<sup>−/−</sup> diaphragm exhibited a higher proportion of slow-twitch fibers (Fig. 2A). The conversion to a slow-twitch fiber phenotype of **Naf-1**<sup>−/−</sup> muscle was confirmed in larger numbers of mice, in which there was an approximate doubling of the percentage of slow-twitch fibers in the **Naf-1**<sup>−/−</sup> group compared with wild-type litter mates (Fig. 2B). This phenotypic transformation was not accompanied by a significant change in overall mean fiber size (1540 versus 1643 μm<sup>2</sup> in **Naf-1**+/<sup>+</sup> and **Naf-1**<sup>−/−</sup>, respectively), although there were mild alterations in the fiber size distribution curves of the two groups (Fig. 2C).

**Naf-1**<sup>−/−</sup> skeletal muscle contractile properties

In order to determine whether the above changes in muscle structure and myosin heavy chain phenotype in **Naf-1**<sup>−/−</sup> mice led to changes in the physiologic function of diaphragm myofibers, *in vitro* contractile properties were assessed. In the **Naf-1**<sup>−/−</sup> group, there was a dramatic decrease in diaphragmatic force-generating capacity during brief tetanic (Fig. 3A), single twitch (Fig. 3B) and repetitive submaximal (Fig. 3C) electrical stimulation protocols. However, in keeping with the increased percentage of slow-twitch fibers, which are inherently more resistant to fatigue, the **Naf-1**<sup>−/−</sup> diaphragms were better able to maintain their initial force level during repetitive stimulations (Fig. 3D).

**Increased basal autophagy in skeletal muscle of **Naf-1**-deficient mice**

The autophagy-lysosome pathway is required for effective removal and elimination of toxic protein aggregates or damaged organelles, which is of particular importance in non-proliferating muscle cells. Emerging evidence demonstrates that autophagy plays an important role in controlling muscle mass during catabolic conditions as well as in basal myofiber homeostasis, and that defects in autophagy or autophagosome clearance lead to various muscle pathologies (extensively reviewed in 25). We previously demonstrated that knockdown of NAF-1 expression by small-hairpin RNA (shRNA) or small-interfering RNA in H1299 human lung carcinoma cells in culture results in enhanced autophagy in response to a starvation stimulus (11). To determine whether or not **Naf-1** influences basal autophagy in skeletal muscle, TA and diaphragm muscles from **Naf-1**+/<sup>+</sup> and **Naf-1**<sup>−/−</sup> mice were assayed by immunoblotting for the autophagy marker, microtubule-associated protein 1 light chain 3 (LC3). During autophagy, cytosolic LC3 (LC3 I) is conjugated to phosphatidyl-ethanolamine (LC3 II) and becomes integrated

![Figure 2.](https://example.com/figure2.png)
into the lipid membrane of autophagosomes (26,27). Due to their differential mobility by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the processing of LC3 I to LC3 II can be assessed by immunoblotting with anti-LC3 antibody and is commonly used to assay for autophagy. The ratio of LC3 II to LC3 I was significantly higher in tissues obtained from Naf-1−/− mice compared with the wild-type, particularly in diaphragm muscle (Fig. 4A and B). p62, a ubiquitin-binding scaffold protein that associates with protein aggregates and interacts with LC3 (28,29), is another common marker for autophagy. Steady-state levels of p62 were enhanced both in TA and diaphragm of Naf-1−/− mice (Fig. 4A and B). Additionally, expression of FoxO3a, a transcription factor known to stimulate autophagy in muscle (30), was significantly enhanced in Naf-1−/− diaphragm muscle. Consistent with the contribution of NAF-1 only to the autophagic arm of BCL-2 regulation, no evidence of apoptosis was detected in Naf-1−/− TA or diaphragm muscles as assayed by immunoblotting for cleavage of executor caspase 7 and cleavage of the caspase substrate PARP (Fig. 4C).

Since it is not known how whole animal physiological stress might combine with the loss of Naf-1 to enhance autophagy, we elected to examine protein expression levels of autophagy markers LC3 and p62 in primary myoblasts isolated from diaphragm muscle of Naf-1+/+ and Naf-1−/− mice, under nutrient replete conditions. Similar to our observation in diaphragm tissue, Naf-1−/− myoblasts demonstrated a higher basal LC3 II to LC3 I ratio and increased expression of p62 (Fig. 4D and E), suggesting a contribution of Naf-1 to the regulation of basal autophagy in this setting as well. Moreover, treatment of these cells with bafilomycin A1, which blocks fusion of autophagosomes with lysosomes, caused an increase in LC3 and p62 in both wild-type and Naf-1−/− myoblasts, but this was more pronounced in Naf-1−/− myoblasts (Fig. 4D). This was particularly true for p62, possibly reflective of its otherwise rapid degradation once the autophagosome fuses with the lysosomes.

Figure 3. Naf-1−/− skeletal muscles demonstrate diminished force-generating capacity and greater resistance to muscle fatigue. In vitro contractile properties of diaphragm muscle dissected from 3.5-month-old Naf-1+/+ and Naf-1−/− mice were assessed by brief tetanic (A), single twitch (B) and repetitive submaximal (C) electrical stimulation protocols (see Materials and Methods). (D) Resistance to fatigue is presented as the ability to maintain the initial level of force during repetitive stimulations (percentage of initial). All graphs represent the average ± SD of independent experiments (n = 4 or 5 Naf-1+/+ and n = 6 Naf-1−/−), **P < 0.005.
Collectively, the results are consistent with elevated autophagy in the \( \text{Naf-1}^{-/-} \) myoblasts.

**Adaptive response of mitochondria to \( \text{Naf-1} \) gene deletion**

Strikingly, we saw no evidence for enhanced mitophagy in electron micrographs of \( \text{Naf-1}^{-/-} \) muscle (Fig. 1E). Rather, mitochondria were significantly enlarged and contained densely packed cristae. Steady-state mitochondrial mass, on the other hand, did not appear to be influenced by \( \text{Naf-1} \) in this tissue, as judged by immunoblots of mitochondrial proteins (outer membrane VDAC and matrix Hsp60) relative to cytoplasmic loading control (Hsp90) (data not shown). Additionally, immunofluorescence analysis of primary diaphragm myoblasts derived from \( \text{Naf-1}^{+/+} \) and \( \text{Naf-1}^{-/-} \) mice with a mitochondrial marker (TOM20) revealed mitochondria that were significantly more elongated in the absence of \( \text{Naf-1} \) (Fig. 1F). Recently, such dramatic changes in mitochondrial
morphology (Fig. 1E and F) have been recognized as an adaptive response to maintain cell viability and adenosine-5'-triphosphate (ATP) generation in the face of enhanced cytoplasmic autophagy (22,23). Such adaptation may allow skeletal muscle in relatively young Naf-1−/− animals to maintain a minimal level of performance and therefore to resist unsustainable degeneration until a later age. Other investigators have concluded that the deletion of Naf-1/Cisd2 results in mitochondrial damage in certain tissues (12), which we potentially attribute to prolonged dysregulation of Ca2+ homeostasis (11).

**Naf-1 is required for regulation of ER Ca2+ stores in skeletal muscle cells**

We previously reported that expression of ER-targeted BCL-2 (BCL-2b5) in epithelial cells results in a depression of ER Ca2+ stores, and that the reduction in ER Ca2+ stores by BCL-2b5 requires its interacting partner, NAF-1 (11). BCL-2b5 is BCL-2 where the TM domain of BCL-2 is replaced with the ER-selective insertion sequence of cytochrome b5 (31). In the previous study, ER Ca2+ stores were measured indirectly by assays for increases in cytosolic 

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\text{[Ca}^{2+}\text{]}_\text{ER}
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response to thapsigargin, a selective inhibitor of the sarcoplasmic reticulum calcium ATPase (SERCA) pump (11). To extend and confirm these important findings, we employed the Ca2+-sensitive ER-targeted aequorin (ER-Aeq) photoprotein to directly measure changes in ER luminal Ca2+ concentrations ([Ca2+]ER). H1299 Neo and BCL-2b5 cells treated with either control or NAF-1 shRNA were transfected with plasmid encoding ER-Aeq and aequorin measurements were obtained as described in Materials and Methods. No difference in [Ca2+]ER was observed between H1299 Neo cells treated with either control (shCTRL) or NAF-1 (shNAF) shRNA (Fig. 5Aa,c and B). H1299 BCL-2b5 shCTRL cells maintained a lower [Ca2+]ER compared with Neo cells (Fig. 5Ab and B), while knock-down of NAF-1 reverted [Ca2+]ER in these cells almost to the level of non-BCL-2 control (Fig. 5Ad and B). These results confirm our previous findings utilizing the Ca2+-sensitive fluorescent indicator Fura2-AM, and reaffirm that BCL-2b5 requires NAF-1 in order to reduce ER Ca2+ stores.

Since the aequorin results were fully consistent with the findings using Fura2-AM, it indicates that the Fura2-AM assay can be used to extend these findings for Naf-1 to skeletal muscle, employing primary myoblasts derived from diaphragms of Naf-1+/+ and Naf-1−/− mice. In contrast to control H1299 cells, which express low levels of endogenous BCL-2, diaphragm muscle expresses easily detectable levels of the protein (Fig. 4A) and therefore might respond directly to the loss of NAF-1. Primary myoblasts were loaded with Fura2-AM, and ER Ca2+ levels were determined using the thapsigargin release protocol. Consistent with the findings by NAF-1 knock-down in H1299 BCL-2b5 cells, Naf-1−/− myoblasts demonstrated a higher ER Ca2+ content compared with its wild-type counterpart (Fig. 5C).

Baseline levels of total intracellular Ca2+ were also determined by loading primary myotubes derived from Naf-1+/+ and Naf-1−/− myoblasts with Fura2-AM. Accordingly, resting levels of cytoplasmic Ca2+ were consistently elevated in Naf-1−/− myotubes compared with the wild-type (Fig. 5D).

**DISCUSSION**

In humans, the loss of NAF-1 function is responsible for the human neurodegenerative disorder WFS2. The ER protein NAF-1 associates with BCL-2/BCL-XL and the IP3 receptor, and is required to partition BCL-2 at the ER toward antagonizing the autophagy pathway and not the apoptosis pathway. As well, it is required for BCL-2 at the ER to regulate Ca2+ stores (11). Here, we demonstrate that results from Naf-1 gene deletion in the mouse are consistent with this model, as judged by our focus on skeletal muscle. These tissues, especially diaphragm, which expresses easily detectable levels of BCL-2, are among the earliest tissues in knockout animals that manifest overt degeneration with evidence of enhanced autophagy but no obvious apoptosis. Ablation of Naf-1 results in dysregulated intracellular Ca2+ homeostasis in both Naf-1−/− primary myoblast and myotube cultures. The specialized ER, the sarcoplasmic reticulum, in this Naf-1−/− tissue exhibits features associated with dilation and vacuolization, potentially associated with the changes that we observed in Ca2+ homeostasis.

Functional studies revealed physiological defects in the contractile properties of diaphragm muscle of Naf-1−/− mice, and an augmented shift toward slow-twitch fibers in the knockouts. Of note, whereas Bcl-2-null mice are overwhelmingly influenced by defects in apoptosis, they do manifest a shift to slow-twitch fibers as demonstrated here for Naf-1 gene deletion, in which neonatal skeletal muscle of Bcl-2-null mice had only two-thirds as many fast muscle fibers as muscles in wild-type mice (32).

Moreover, it is intriguing that, in addition to enhanced basal autophagy, skeletal muscle of Naf-1−/− animals contains enlarged and elongated mitochondria with dense cristae, recently described as an adaptive response to maintain organelle integrity, ATP production and cell viability in the face of potentially damaging nutrient withdrawal (22,23). It would appear therefore that disruption of NAF-1 function mimics nutrient deprivation in this regard, consistent with NAF-1’s proposed role in the core BCL-2/Beclin 1 mechanism for responding to nutrient stress (11).

Finally, while we have focused on the early onset degeneration of skeletal muscle resulting from Naf-1 gene deletion in the mouse, continued and severe deterioration of multiple tissues in these animals was the norm, as previously described for Cisd2 deletion (12), requiring euthanization of the animals at about 1 year of age. Also of note is the dysregulated Ca2+ homeostasis that was observed here in the mouse and which also extends to man, in which lymphoblastoid cells from WFS2 individuals exhibit defects in intracellular Ca2+ when treated with thapsigargin, compared with controls (15). The observed Ca2+ defects in Naf-1-deficient mice and lymphoblasts of WFS2 patients correlate to previous studies demonstrating a role for the WFS1 protein in modulating ER Ca2+ load (16). Such defects in Ca2+ homeostasis in multiple tissues would be expected to lead to progressive deterioration in physiological status as individual’s age.
Figure 5. Loss of Naf-1 results in a rise in ER Ca\(^{2+}\) content. (A) H1299 Neo and BCL-2b5 cells treated with either CTRL (a and c) or Naf-1 (b and d) shRNA were transiently transfected with plasmid encoding ER-Aeq. Twenty-four hours post-transfection, aequorin was reconstituted with coelenterazine and cells were depleted of ER Ca\(^{2+}\) (see Materials and Methods). [Ca\(^{2+}\)]\(_{ER}\) measurements were performed in a luminometer chamber with constant perfusion of saline buffer containing 1 mM CaCl\(_2\) to induce ER Ca\(^{2+}\) uptake. After reaching a steady state, cells were stimulated with 50 \(\mu\)M His and 50 \(\mu\)M adenosine-5\'-triphosphate. Shown are representative traces of [Ca\(^{2+}\)]\(_{ER}\) aequorin measurements. (B) Graph depicts [Ca\(^{2+}\)]\(_{ER}\) as the average of six independent experiments as described in (A). (C) Primary myoblasts were isolated as in Figure 4C. Cells were loaded with Fura-2AM in Ca\(^{2+}\)-free buffer, and ER Ca\(^{2+}\) stores were measured as the difference in cytoplasmic Ca\(^{2+}\) concentration before and after addition of thapsigargin (TG, 2 \(\mu\)M). Graph depicts TG-sensitive ER Ca\(^{2+}\) stores as the average of four independent experiments. (D) Baseline intracellular levels of Ca\(^{2+}\) in primary myotubes derived from Naf-1\(^{+/+}\) and Naf-1\(^{-/-}\) myoblasts were determined using Fura-2AM. Scatter plot depicts [Ca\(^{2+}\)] from individual experiments (n = 7 Naf-1\(^{+/+}\) and n = 8 Naf-1\(^{-/-}\)).
MATERIALS AND METHODS

Generation of Naf-1-deficient mice

The Naf-1 gene trap embryonic stem cell line XS0445 was obtained from the Sanger International Gene Trap Center. The identity of the trapped gene was confirmed by DNA sequencing at the Mutant Mouse Regional Resource Center at the University of California Davis. This construct generates exon 1 (encoding 34 amino acids and lacking the Naf-1 ER-targeting TM segment) fused to β-Gal. Injection of the 129SV ES cells into C57BL/6 blastocysts and transfer of the embryos to foster mothers were performed by the McGill Transgenic Facility. The chimeras were backcrossed with C57BL/6 to five generations to create the congenic strain. The identified heterozygous mice were crossed and the F2 offspring were screened for homozygosity by immunoprecipitation of blood samples with anti-NAF-1 antibody. Mice were bred and maintained by the McGill Transgenic Facility.

Cell culture

H1299 Neo and BCL-2b5 cells were cultured as described earlier (33). Generation of primary myoblast cultures from the mouse diaphragm from single living muscle fibers and differentiation into myotubes were performed as previously described (34).

Immunoblot analysis

Cell or tissue lysates were resolved by 4%–16% gradient SDS–PAGE. For immunoblot analysis of LC3 processing, gels were transferred in CAPS transfer buffer as described (35). The following antibodies were used in this study: mouse anti-actin (ICN), rabbit anti-Bcl-2 (Santa Cruz), rabbit anti-Caspase 7 (Cell Signaling), rabbit anti-FoxO3a (Cell Signaling), rabbit anti-LC3B (Cell Signaling), rabbit anti-NAF-1 (11), anti-PARP (Biomol), guinea pig anti-p62 (PROGEN) and mouse anti-tubulin (Sigma).

Electron microscopy

Muscles from euthanized Naf-1+/+ and Naf-1−/− mice were dissected and subsequently fixed in 0.1 M phosphate buffer pH 7.4 containing 4% paraformaldehyde and 2.5% glutaraldehyde. Samples were washed with 0.1 M sodium cacodylate buffer (pH 7.4) containing 7% sucrose, postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide and embedded in epon. Ultrathin sections were cut and stained with uranyl acetate and lead citrate before analysis using a Philips CM120 electron microscope.

Contractile function

The diaphragm muscle was surgically excised for in vitro contractility measurements as previously described in detail (36). After removal, the diaphragm was immediately placed into a chilled (4°C) and equilibrated (95% O2–5%CO2 pH 7.38) Krebs solution with the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1 KH2PO4, 25 NaHCO3 and 11 glucose. A muscle strip ~2 mm wide was dissected free, taking care to leave the central tendon and adjacent rib cage margins intact. The excised diaphragm strip was mounted into a jacketed tissue bath chamber filled with equilibrated Krebs solution, using a custom-built muscle holder containing two stimulation electrodes located on either side. A thermoequilibration period of 15 min was observed prior to initiating contractile measurements at 25°C. After placing the diaphragm strip at optimal length, the force–frequency relationship was determined by sequential supramaximal stimulation for 1 s at 10, 30, 50, 100 and 150 Hz, with 2 min between each stimulation train. After a 15 min rest period, the fatiguability of the muscle was assessed by measuring the loss of force in response to repeated stimulations (30 Hz, 0.33 duty cycle, 90 trains/min). All force data were acquired to computer at a sampling rate of 1000 Hz for later analysis. The muscle strip was then removed from the bath and its length from rib to central tendon was measured under a dissecting microscope with a microcaliper accurate to 0.1 mm. Diaphragm strip cross-sectional area was approximated by dividing muscle mass by its length and density; this allowed specific force (force/cross-sectional area) to be calculated, which was expressed as N/cm². A minimum of four up to six replicates were analysed. All data are presented as mean values ± SD. Group mean differences were determined by Student’s t-test (GraphPad Prism software). Statistical difference was defined as P < 0.05.

Immunocytochemical analysis of fiber type and size

Diaphragm muscle was embedded in mounting medium and serial sections (6 μm thick) midway between central tendon and rib cage were cut with a cryostat at −20°C. After air-drying, the sections were treated with a primary antibody (NOQ7.5.4D, Sigma) directed against the slow type I isoform of myosin heavy chain and then revealed with HRP-conjugated secondary antibody. The images were captured to computer, and the number of positively staining fibers was determined from randomly selected fields (minimum of 200 fibers per muscle); individual fiber cross-sectional areas were also directly measured from the calibrated computer image. These measurements allowed determination of the percentage of slow-twitch fibers and the cross-sectional area of all fibers within the microscopic fields examined.

Calcium measurements

For aequorin measurements, H1299 Neo and BCL-2b5 cells treated with either CTRL of NAF-1 lentiviral shRNA were described previously (11). Cells were seeded onto glass cover slips and transiently transfected with plasmid encoding ER-Aequorin (ER-Aeq; described in 1). Twenty-four hours post-transfection, cells were incubated in saline buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM MgSO4, 0.4 mM KH2PO4, 5.5 mM glucose and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) supplemented with 5 μM n-coelenterazine, 4 μM ionomycin and 600 μM ethyleneglycol tetraacetic acid (EGTA) at 37°.
for 40 min. Cells were transferred to a perfusion chamber within a purpose-built luminometer, and washed extensively with saline buffer containing 100 μM EGTA and 2% bovine serum albumin. Cells were then perfused with 1 mM CaCl2 for calibration of the aequorin signal, cells were lysed with saline solution to induce ER Ca2+ uptake until a steady state was reached, subsequently ER Ca2+ release was stimulated by treatment with 50 μM histamine (His) and 50 μM ATP. Finally, to release the remaining aequorin pool and to allow calibration into [Ca2+] values as described (37). Thapsigargin-sensitive ER Ca2+ stores in primary myoblast cultures were determined using Fura-2AM as described previously (33). Baseline intracellular [Ca2+] in myotube cultures was determined using Fura-2AM as described (38).

ACKNOWLEDGEMENTS

We are grateful for excellent technical help from Marilene Paquet for pathology analysis; Johanne Ouellette and Peter Rippstein for electron microscopy; and Eva Migon for mouse breeding. We thank Heidi McBride for critical reading of the manuscript and Haouaria Balghi, Erika Zecchini Rippstein for electron microscopy; and Eva Migon for helpful discussions.

Conflict of Interest statement. None declared.

FUNDING

N.C.C. is a recipient of the Canadian Institutes of Health Research Canada Graduate Scholarships Doctoral award. This work was supported by grants from the Canadian Institutes of Health Research.

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