Stac3 Is a Novel Regulator of Skeletal Muscle Development in Mice

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Abstract

The goal of this study was to identify novel factors that mediate skeletal muscle development or function. We began the study by searching the gene expression databases for genes that have no known functions but are preferentially expressed in skeletal muscle. This search led to the identification of the Src homology three (SH3) domain and cysteine rich (C1) domain 3 (Stac3) gene. We experimentally confirmed that Stac3 mRNA was predominantly expressed in skeletal muscle. We determined if Stac3 plays a role in skeletal muscle development or function by generating Stac3 knockout mice. All Stac3 homozygous mutant mice were found dead at birth, were never seen move, and had a curved body and drooping forelimbs. These mice had marked abnormalities in skeletal muscles throughout the body, including central location of myonuclei, decreased number but increased cross-sectional area of myofibers, decreased number and size of myofibrils, disarrayed myofibrils, and streaming Z-lines. These phenotypes demonstrate that the Stac3 gene plays a critical role in skeletal muscle development and function in mice.

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Introduction

Skeletal muscle is essential to human health and well-being and is at the core of the animal production industry. It is responsible for approximately 40 percent of body mass in mammals and provides power for respiration, locomotion, and structural support essential for survival. The basic unit of skeletal muscle is the myofiber, a multinucleated cell created by the fusion of single-nucleated muscle progenitor cells, myoblasts. Formation of myofibers from myoblasts (i.e., myogenesis) is chiefly regulated by the myogenic regulatory factors (MRFs), including MyoD, Myf5, myogenin, and MRF4 [1–5]. MyoD and Myf5 are required for myogenic lineage determination [5,6], whereas myogenin and MRF4 regulate differentiation of myoblasts into functional myofibers [5,7–9].

Myogenesis is a complex process. Despite the identification of the aforementioned MRFs, this process is likely controlled by additional factors. To identify novel factors that regulate myogenesis, we searched the gene expression databases for genes that are preferentially expressed in skeletal muscle using the GeneNote program [10]. This search led to the identification of the Src homology three (SH3) domain and cysteine rich (C1) domain 3 (Stac3) gene. The Stac3 gene is the third member of the Stac gene family that also includes Stac1 and Stac2 [11]. Unlike Stac3, Stac1 and Stac2 are expressed specifically in neurons [12]. Prior to the present study, no function had been associated with any member of the Stac family.

Because it is expressed predominantly in skeletal muscle and because it is predicted to encode two important functional domains, we hypothesized that Stac3 might play an important role in skeletal muscle development and function. We tested this hypothesis by disrupting Stac3 gene transcription in mice. Our data show that Stac3 is essential for development of functional skeletal muscle and viable mice, and that Stac3 mutant mouse is a novel mammalian model for myopathies.

Materials and Methods

RT-PCR and real-time PCR

Total RNA from mouse tissue samples was isolated with an RNasy Mini Kit from QIAGEN (Valencia, CA), following the manufacturer’s instructions. cDNA was transcribed from total RNA using random primers and the ImProm-II™ reverse transcriptase [Promega, Madison, WI], according to the manufacturer’s instructions. PCR and real-time PCR were performed using the PCR Master Mix (Promega) and the SyberGreen PCR Master Mix (Applied Biosystems Inc., Foster City, CA), respectively. In these PCRs, Gapdh mRNA or 18s rRNA was amplified as an internal control. The Stac3, Gapdh, and 18s cDNAs were amplified using the following PCR primers: 5’-TACAGCGACGGAACATTCG-3’ and reverse 5’-TCTGCAATGTTTCCATCCGT-3’; 5’-ACCCAGAAA-GACTGTGGATGG-3’ and 5’-GGATGCAGGGAT-GATGTGCT-3’; and 5’-CTATAGGAGGCCGCCGGGGG-3’ and 5’-CTCTGGTCGCCCTTGGGGCGG C-3’, respectively. All PCR products were verified by DNA sequencing.

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Generating Stac3 mutant mice

Stac3 mutant mice were generated by the Knockout Mouse Project (KOMP; University of California at Davis) using the knock-out-first strategy [13]. Two Stac3-targeted (Stac3\textsuperscript{tm1a(-SA-geo-pA)-KOMP\textsuperscript{Wtsi}}) embryonic stem (ES) clones (EPD0101_1_A09, EPD0101_1_A10) on a C57BL/6N background were injected into blastocysts from C57BL/6N Albino mice. In both clones, the Stac3 gene is inserted with the "SA-\textsuperscript{geo}-pA" trapping cassette between exons 1 and 2, and this insertion is expected to disrupt the generation of Stac3 mRNA and Stac3 protein. Seven male offspring from injecting the A09 ES cells and one male from injecting the A10 cells were found to be chimeric (30–95%). Two males with 95% and 85% chimera were selected to breed with C57BL/6N Albino females. The 95% male chimera successfully transmitted the targeted Stac3 locus to its offspring as evidenced by the coat color (black) and by genotyping (see below). Pairs of mice heterozygous for the targeted Stac3 allele (Stac3\textsuperscript{+/-}) were mated to generate Stac3 homozygous mutant (Stac3\textsuperscript{-/-}) mice. Mice were housed at designated facility on a timed 12 h light/dark schedule with free access to standard rodent diet and water. All procedures involving animals were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Genotyping

Genomic DNA was isolated from ear notches or tail clips using a DNeasy Blood & Tissue Kit from Qiagen. Mouse genotypes were determined by PCR using two pairs of primers. One pair of primers (forward 5'-CTCTCATAGCTCTACGCCAGTC-3' and reverse 5’-CTCTGCTTGTGAGTGTGGA-3') was designed to flank the third loxP site in the trapping cassette. This pair of primers was expected to generate a 344 bp PCR product from the targeted Stac3 allele and a 317 bp product from the wild-type Stac3 allele. The second pair of primers (forward 5’-TGGTGAGGCTTTTGACTCTC-3' and reverse 5’-TGGATACCTTGTTGATGTGG-3') was designed to amplify a 468 bp intron region of the wild-type Stac3 allele. These PCR reactions were set up using the PCR Master Mix from Promega. Sequences of all PCR products were confirmed by DNA sequencing.

Histology

Whole mouse fetuses or dissected hind limbs were fixed in 10% neutral buffered formalin for 48 h, embedded in paraffin, and sectioned on a microtome according to standard procedures. Serial 6 μm sagittal sections were cut from the whole body. Serial 6 μm longitudinal and cross sections were cut from the limbs. For limbs, sections were collected until representative sections from the widest girth of the limb were captured. Sections were mounted on Superfrost Plus micro slides (VWR International, Radnor, PA) and were deparaffinized using standard paraffin clearing and dehydration methods. Slides were stained with hematoxylin and eosin (H&E) following standard procedures. Stained sections were photographed using a Nikon Eclipse E600 microscope connected to a QColor3 OLYMPUS digital camera.

Electron microscopy

Transmission Electron Microscopy (TEM) was used to evaluate the ultrastructure of skeletal muscle. Lower leg samples from newborn mice were fixed in 3% glutaraldehyde dissolved in phosphate buffered saline (PBS). The remaining steps were performed by the Morphology Service Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine (Blacksburg, VA), following standard protocols. Both thin (60–90 nm) and thick (3 μm) sections were cut. The thick sections were stained with H&E to verify the location of the extensor digitorum longus (EDL) muscle, and the thin sections were stained with uranyl acetate and lead citrate for electron microscopy. Electron micrographs were captured using a Zeiss 10 CA electron microscope at a voltage of 60 KV (Zeiss Electron Microscopy, Thornwood, NY) on an AMT 4.10 & PCDIG side mount camera using the AMT V601 Image Capture Engine software (Advanced Microscope Technologies, Woburn, MA).

Whole-mount β-galactosidase staining

This was done as described previously [14]. Briefly, mouse embryos were fixed in 4% glutaraldehyde dissolved in PBS supplemented with 5 mM EGTA and 2 mM MgCl\textsubscript{2} for 30 min. Fixed embryos were washed in PBS supplemented with 5 mM EGTA, 2 mM MgCl\textsubscript{2}, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40. To stain for β-galactosidase, embryos were incubated in 0.1% X-gal in PBS supplemented with 2 mM MgCl\textsubscript{2}, 5 mM EGTA, 5 mM K\textsubscript{2}Fe(CN)\textsubscript{6}, 5 mM K\textsubscript{3}Fe(CN)\textsubscript{6}, and 0.01% Nonidet P-40. All steps were performed at room temperature. Pictures of stained embryos were taken with a S2-CTV OLYMPUS anatomical microscope or a Nikon Eclipse 80i microscope connected to a QColor3 OLYMPUS digital camera.

Immunohistochemistry

Antigen retrieval was performed by boiling slides in 10 mM citrate buffer adjusted to pH 6 at 95°C for 30 min and then cooling them at room temperature for 20 min. Nonspecific binding was blocked by incubating slides with 5% goat serum (Sigma-Aldrich, St. Louis, MO) diluted in PBS for 1 h at room temperature. Myofibers were detected by incubating slides with 1:200 diluted sarcomeric myosin heavy chain antibody MF20 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and then with 1:200 diluted DyLight 594-conjugated goat anti-mouse secondary antibody (Fisher Scientific, Pittsburgh, PA). Cell membranes were stained with 1:400 diluted fluorescein-conjugated wheat germ agglutinin (Invitrogen, Grand Island, NY).
Figure 2. Generation and analyses of Stac3 mutant mice. A, Schematic representation of the targeted Stac3 allele. The wild-type (WT) Stac3 allele has 12 exons, with exon 3 containing the translation start codon. The targeted allele is inserted with a trapping cassette between exons 1 and 2, and this insertion is expected to disrupt the generation of normal Stac3 mRNA. Arrows indicate locations of PCR primers for genotyping. B, Genotypes and life of newborn offspring from intercrossing mice heterozygous for the targeted Stac3 allele. “+/+”, “+/-”, and “-/-” indicate wild-type mice, and mice heterozygous and homozygous for the targeted Stac3 allele, respectively. A total of 10 litters were included in this analysis. C, Images of a Stac3 homozygous mutant mouse and a wild-type littermate. Note the curved body shape and dropping forelimbs in the mutant. D, Genotyping by PCR. See panel A for the locations of two pairs of primers. E, Validation of diminished expression of Stac3 mRNA in Stac3-/- mice by standard RT-PCR. Shown is a representative image. F, Real-time RT-PCR validation of diminished expression of Stac3 mRNA in Stac3-/- mice. Data are expressed as mean ± SEM (n = 4). ** P<0.01 vs. “+/+”. G, LacZ staining of E13 wild-type and Stac3-/- embryos. Shown are representative images. The image on the right highlights the stained somites in an E13 Stac3-/- embryo.

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Quantitative analyses of myofibers
The EDL muscle was used to determine muscle fiber characteristics due to its uniformity and easy recognition. Total number and average cross sectional area of myofibers in an EDL muscle were determined by counting and averaging all myosin heavy chain (MyHC) positive fibers on three serial cross sections at the widest girth of the EDL muscle using the NIS-Elements AR Ver3.1 software.

Statistical analyses
Data were analyzed using ANOVA followed by Tukey HSD multiple comparison of means in the JMP software (SAS, Cary, NC). A difference was considered statistically significant when $P<0.05$. Data are presented as means ± standard error of the mean (SEM).

Results
Stac3 mRNA was predominantly expressed in skeletal muscle
Searching the gene expression database at GeneNote with “skeletal muscle” [10], the program generated a list of human genes that were predicted to be specifically expressed in skeletal muscle. This list contained Stac3. We became more interested in this gene than the other genes on the list because it had no known function but was predicted to encode a protein containing a SH3 domain and a C1 domain, two classical functional domains [15,16]. We examined the expression profile of Stac3 mRNA in adult mice by quantitative RT-PCR. As shown in Fig. 1, Stac3 mRNA was abundantly expressed in skeletal muscle of four different locations, but was not expressed or expressed at very low levels in any of non-skeletal muscle tissues or organs examined, including brain, heart, and the smooth muscle-containing stomach.

Stac3 mutant mice died at birth
Mice heterozygous for the targeted Stac3 allele (Stac3<sup>+/−</sup>) were generated by injecting blastocysts with the Stac3-targeted ES cells (Fig. 2A). Both male and female Stac3<sup>+/−</sup> mice were viable and fertile and grew normally compared to their wild-type littermates. Breeding between Stac3<sup>+/−</sup> mice resulted in normal-sized litters with a near Mendelian representation of genotypes, but all of the mice homozygous for the targeted Stac3 allele (Stac3<sup>−/−</sup>) were found dead at birth (Fig. 2B). All Stac3<sup>−/−</sup> fetuses were never seen move and did not respond to touch, but they had heart beating when dissected out of the uterus. Genotypes of Stac3<sup>−/−</sup> or Stac3<sup>+/−</sup> fetuses were confirmed by PCR (Fig. 2D). At E18.5, Stac3<sup>−/−</sup> fetuses weighed approximately 11% and 14% less than their heterozygous and wild-type littermates, respectively ($P<0.05$).

Stac3 mRNA expression was markedly diminished in Stac3 mutant mice
Insertion of the trapping cassette between exon 1 and exon 2 of the Stac3 allele was expected to disrupt the generation of normal Stac3 transcript (Fig. 2A). This was confirmed by the fact that Stac3 mRNA was barely detectable in any tissues examined, including skeletal muscle, in E18.5 Stac3<sup>−/−</sup> fetuses (Fig. 2E and 2F). As in adult wild-type mice (Fig. 1), Stac3 mRNA was preferentially expressed in skeletal muscle or skeletal muscle-containing organs such as the tongue in E18.5 wild-type fetuses (Fig. 2E and 2F). The trapping cassette contained a β-galactosidase gene, and this gene was expected to be expressed under the control of the original Stac3 promoter (Fig. 2A). LacZ staining of E13 embryos confirmed...
the expected activity of β-galactosidase in somites and limb muscles in Stac3+/− but not in wild-type embryos (Fig. 2G).

Skeletal muscle of Stac3 mutant mice had abnormal morphology

Based on gross dissection and H&E staining, Stac3−/− mice had no obvious structural aberrations in viable organs such as heart, liver, kidney, and the gastrointestinal tract, and had no edema or necrosis, disorders that are typically associated with in utero death. The diaphragms of newborn Stac3−/− mice were slightly thinner than those of their wild-type littersmates (Fig. 3A and 3B). In newborn wild-type mice, most myofibers had peripherally located nuclei (Fig. 3A, 3C, 3E, 3G, and 3I) and were polygonally shaped on cross sections (Fig. 3A, 3C, and 3I). However, in their Stac3−/− littermates, the majority of myofibers had centrally located nuclei (Fig. 3B, 3D, 3F, 3H, and 3J) and appeared round on cross sections (Fig. 3B, 3D, and 3J). Quantitative analyses of the EDL muscle revealed that more than 70% of myosin heavy chain-positive cells in newborn Stac3−/− mice had centrally located nuclei, whereas that number was only 11% in wild-type or heterozygous littermates (P<0.01) (Fig. 4A).

Skeletal muscle of Stac3 mutant mice had fewer but larger myofibers

The total number of myofibers in the EDL muscle of newborn Stac3−/− mice was 26% or 18% less than that of wild-type or heterozygous littersmates, respectively (P<0.05, Fig. 4B). However, the average cross-sectional area (CSA) of myofibers in the EDL muscle of Stac3−/− mice was 20% greater than that of wild-type or Stac3+/− mice (P<0.01, Fig. 4C). The myofibers in the EDL muscle of newborn wild-type or Stac3+/− mice had a normal distribution of CSA (Fig. 4D). Those in Stac3−/− mice, however, had a skewed-to-the right distribution (Fig. 4D). Despite these differences, the average size of the EDL muscle was not different between Stac3−/− mice and their wild-type or heterozygous littermates (P>0.1).

Myofibers of Stac3 mutant mice had fewer, smaller, and disorganized myofibrils

We examined the ultrastructure of skeletal muscle with transmission electron microscopy (TEM). Myofibers in the EDL muscle of newborn wild-type mice had abundant, well-aligned, and near-uniform myofibrils and sarcomeres (Fig. 5A and 5C). In contrast, those of newborn Stac3−/− littersmates had fewer, smaller, disarrayed, and sometimes fragmented myofibrils (Fig. 5B and 5D). Compared to sarcomeres in the EDL muscle of wild-type mice (Fig. 5C), those of Stac3−/− mice had streaming Z-lines (Fig. 5D). The TEM also confirmed the central location of myonuclei in newborn Stac3−/− mice (Fig. 5B) and the peripheral location of myonuclei in newborn wild-type mice (Fig. 5A).
Discussion

In this study, we showed that the functionally unknown Stac3 gene was specifically expressed in skeletal muscle, and we investigated the potential role of this gene in skeletal muscle development and function by disrupting its transcription in mice. All Stac3\(^{-/-}\) mice were found dead at birth. This demonstrates that Stac3 is essential to the development of viable mice. Whether at birth or dissected from the uterus, the Stac3\(^{-/-}\) mice had an abnormal body curvature and dropping forelimbs, and did not respond to prodding. These phenotypes are similar to those caused by mutation of the myogenin gene [7,8], the ryanodine receptor gene [17], and the dihydropyridine receptor gene [18], genes that are known to play a critical role in either the development of functional skeletal muscle or contraction of skeletal muscle. When a mouse is born, it becomes dependent on respiration for oxygen intake, which requires contraction of diaphragm and intercostal muscles [19,20]. Therefore, it is likely that Stac3 mutant mice died at birth because their dysfunctional diaphragm and intercostal muscles could not support breathing. Because Stac3 is not expressed in the nervous system, it is unlikely that Stac3 mutant mice die from dysfunctional neural control.

Histological analyses revealed that the skeletal muscle of newborn Stac3\(^{-/-}\) mice had several abnormalities compared to that of wild-type mice, including: 1) most of their nuclei remained in the center of myofibers; 2) they had fewer yet larger myofibers; 3) they had a disproportional number of myofibers with large cross-sectional areas; 4) their myofibers had fewer, smaller, and less organized myofibrils. With these abnormalities, a skeletal muscle is unlikely able to function. Among these abnormalities, the most striking was that most of the myonuclei were located in the center of myofibers at a developmental stage (i.e. birth) when they should have been relocated to the periphery of myofibers [21]. While having centrally located nuclei is typically indicative of regeneration in adult skeletal muscle [5,22], it is unlikely that skeletal muscle in fetal Stac3\(^{-/-}\) mice was undergoing regeneration because every skeletal muscle examined had centrally located nuclei. Translocation of myonuclei from the center to the periphery is the hallmark of myofiber maturation during late embryonic development [21]. Therefore, it is possible that Stac3

Figure 5. Representative electron micrographs of extensor digitorum longus (EDL) muscle of newborn Stac3\(^{-/-}\) mice and Stac3\(^{+/+}\) littermates. Note the differences in the location of myonuclei and the size and arrangement of myofibrils between Stac3 homozygous mutant and wild-type muscles. Arrows point to myonuclei (micrographs A and B), and arrowheads indicate Z-lines (micrographs C and D). Scale bars = 10 \(\mu\)m for micrographs A and B; Scale bars = 500 nm for micrographs C and D.
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gene mutation disrupted myofiber maturation. This possibility is also supported by the observation that Stac3−/− myofibers had fewer and smaller myofibrils than wild-type myofibers at the same developmental stage. It is also possible that Stac3 disruption disrupted the ill-defined mechanism that controls the translocation of myonuclei during skeletal muscle development.

Myofibers of Stac3−/− mice had greater cross-sectional areas than those of wild-type or Stac3+/− littermates. This difference was probably not due to myofiber hypertrophy in Stac3−/− mice because myofibers in those mice indeed had fewer and smaller myofibrils than in wild-type or Stac3+/−. The difference was more likely caused by increased fusion of myoblasts into myotubes in Stac3 mutant mouse embryos. The relative numbers of proliferating myoblasts versus differentiating myoblasts during muscle development can determine the total number of myofibers formed in a mature muscle [23,24]. If myoblasts withdraw from the cell cycle and begin differentiation before an adequate number of founder myoblasts have developed, this would decrease the overall number of myofibers formed. Therefore, an increase in myoblast fusion could also explain why a Stac3−/− skeletal muscle had fewer myofibers than a wild-type or a Stac3+/− skeletal muscle. Myogenesis is a highly orchestrated program, and premature myoblast fusion could lead to the formation of dysfunctional muscle [25,26]. Therefore, increased fusion of myoblasts may also explain why skeletal muscle in Stac3 mutant mice was unable to contract.

Unlike the master regulators of skeletal muscle development, the MRFs (MyoD, myogenin, MRF4, Myf5), Stac3 is not a transcription factor and does not appear to have direct DNA-binding ability. The Stac proteins are believed to be confined to the cytosolic domain of the cell with no evidence for enzymatic activity [15,27]. However, Stac3 does possess two domains (SH3 and C1) that are frequent components of signaling proteins [16,28].

In summary, we have demonstrated that the Stac3 gene is exclusively expressed in skeletal muscle and that it is essential for development of functional skeletal muscle and mice viable at birth. The domain structures of Stac3 protein suggest that it is a signaling protein, and further investigation into the molecular and cellular cause of abnormalities in Stac3 mutant skeletal muscle may reveal novel signaling mechanisms that regulate skeletal muscle development or function. The centrally located myonuclei and lack of muscle contraction phenotypes of Stac3−/− mutant mice are hallmarks of centronuclear/myotubular myopathies in humans [31,32]. Therefore, the Stac3 mutant mouse may represent a useful animal model for understanding the pathophysiology of this muscle disease. While this manuscript was being prepared, a study published in JBC indicates that Stac3 is essential for myotube formation in zebrafish [33]. Our study, however, shows that Stac3 gene inactivation does not prevent myotube formation in mice (Fig. 3, Fig. 5). The reason for this difference is not clear, but it may be related to the use of different model systems between the two studies.

Author Contributions
Conceived and designed the experiments: HJ. Performed the experiments: BMR XG XC. Analyzed the data: BMR XG XC DEG HJ. Wrote the paper: BMR XG DEG HJ.

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