Neurogenic muscle atrophy is the loss of skeletal muscle mass and function that occurs with nerve injury and in denervating diseases such as amyotrophic lateral sclerosis. Aside from prompt restoration of innervation and exercise where feasible, there are currently no effective strategies for maintaining skeletal muscle mass in the setting of denervation. We conducted a longitudinal analysis of gene expression changes occurring in atrophying skeletal muscle, and identified \textit{Gadd45a} as a gene that shows one of the earliest and most sustained increases in expression in skeletal muscle after denervation. We evaluated the role of this induction using genetic mouse models and found that mice lacking GADD45A show accelerated and exacerbated neurogenic muscle atrophy, as well as loss of fiber type identity. Our genetic analyses demonstrate that, rather than directly contributing to muscle atrophy as proposed in earlier studies, GADD45A induction likely represents a protective negative feedback response to denervation. Establishing the downstream effectors that mediate this protective effect and the pathways they participate in may yield new opportunities to modify the course of muscle atrophy.
GADD45A is a protective modifier of neurogenic skeletal muscle atrophy
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Abstract

Neurogenic muscle atrophy is the loss of skeletal muscle mass and function that occurs with nerve injury and in denervating diseases such as amyotrophic lateral sclerosis. Aside from prompt restoration of innervation and exercise where feasible, there are currently no effective strategies for maintaining skeletal muscle mass in the setting of denervation. We conducted a longitudinal analysis of gene expression changes occurring in atrophying skeletal muscle, and identified Gadd45a as a gene that shows one of the earliest and most sustained increases in expression in skeletal muscle after denervation. We evaluated the role of this induction using genetic mouse models and found that mice lacking GADD45A show accelerated and exacerbated neurogenic muscle atrophy, as well as loss of fiber type identity. Our genetic analyses demonstrate that, rather than directly contributing to muscle atrophy as proposed in earlier studies, GADD45A induction likely represents a protective negative feedback response to denervation. Establishing the downstream effectors that mediate this protective effect and the pathways they participate in may yield new opportunities to modify the course of muscle atrophy.

Brief summary
GADD45A is induced after muscle denervation and provides protective negative feedback response. Strategies to augment this induction may offer therapeutic targets to prevent denervation atrophy.

Keywords
Skeletal muscle, denervation, neurogenic muscle atrophy, myofiber type, GADD45A
Introduction

Skeletal muscle atrophy is the decline of muscle mass and function resulting from degradation of contractile proteins and corresponding reduction in individual myofiber size. Muscle atrophy can result from many causes, including disuse, glucocorticoid use, cancer cachexia, or denervation.

The decrease in myofiber size and contractile function observed in muscle atrophy reflects the active degradation of contractile proteins such as myosin and actin, which occurs largely through induction of muscle-specific ubiquitin ligases and targeted proteasomal degradation (1, 2). Lysosomal proteolysis is also thought to contribute (3). Activation of members of the Forkhead Box O (FOXO) and NF-κB transcription factor families are thought to coordinate these processes, although the proximal events triggered by denervation that lead to these degradative events are not well characterized (4, 5). Muscle atrophy has substantial consequences, reflected in the relationship between functional capacity and quality of life in mild or moderate denervating disease, in the relationship between body mass index and survival in the severe denervation of ALS, and in the impaired capacity for chronically denervated and severely atrophied muscle to be functionally reinnervated (6, 7).

Growth Arrest and DNA Damage-inducible A (GADD45A) was first identified in a Chinese Hamster Ovary (CHO)-based screen for early response genes involved in the DNA damage response to ultraviolet (UV) irradiation (8). It is now known to be a member of a family of small, highly conserved, stress-inducible acidic nuclear proteins with ubiquitously low or absent expression that show cell type-specific induction in response to cellular stress. GADD45A and its related family members (GADD45B and GADD45C) do not appear to have enzymatic activity, but exert their effects in response to cell stress by modulating the function of a wide variety of binding partners, with pleiotropic effects related to cellular senescence, apoptosis, cell cycle arrest, DNA repair, and epigenetic modification (9).

We performed a longitudinal analysis of gene expression changes in denervated, atrophying gastrocnemius muscle using a mouse model of tibial nerve transection and identified Gadd45a as the most significantly differentially expressed transcript at 1 day post-denervation, with significantly sustained induction through at least 90 days post-denervation. Using conventional and conditional mouse models of targeted Gadd45a gene deletion, we found that absence of Gadd45a induction in the setting of denervation is associated with significantly accelerated and exacerbated skeletal muscle atrophy compared to wild type or heterozygous littermates. These observations indicate that Gadd45a induction after denervation mediates a protective effect during muscle atrophy, and suggest that identification of effectors of this response may reveal new targets to modify the course of atrophic disease.

Results

Gadd45a induction is an acute and sustained response to denervation injury

Several transcription factors and proteins involved in coordinating the degradative events of muscle atrophy have been defined, but how these events are triggered by denervation, how they
interact and intersect, and how they change over time in relation to the phenotype of chronically atrophied muscle is not well understood. We used RNA-Seq to characterize changes in mRNA expression occurring in mouse gastrocnemius following tibial nerve transection. We performed a longitudinal analysis capturing transcriptional changes occurring acutely after denervation (1 day post-denervation), early changes during the window of most rapid atrophy (3, 7, and 14 days post-denervation), and changes associated with chronic denervation (30 and 90 days post-denervation) (10).

We observed that Gadd45a shows one of the earliest, largest in magnitude, and most sustained changes in expression following denervation, as determined using a limma-voom paired analysis of gene expression changes among denervated and contralateral intact gastrocnemii (Figure 1A). Gadd45a was the top most significantly differentially expressed transcript in denervated gastrocnemius at 1 day post-denervation (mean log₂FC = 2.94, FDR-corrected P = 3.21e-16), and remained the most significantly differentially expressed transcript throughout the first 14 days post-denervation (mean log₂FC, FDR-corrected P at 3 days: 4.98, 1.05e-23; 7 days: 5.43, 9.81e-27; 14 days: 5.67, 1.57e-27). Gadd45a levels showed a modest but statistically significant decline at 90 days post-denervation (mean log₂FC = 4.22, FDR-corrected P = 2.56e-23). Time-series analysis revealed that Gadd45a was among the top 5 most differentially expressed genes detected in atrophying muscle across the entire 90-day interval post-denervation (Hotelling’s T² statistic = 4903.43, distance = 5.99) (Figure 1B). We validated this pattern of expression by qPCR in gastrocnemius muscle from an independent C57BL/6J mouse tibial nerve denervation cohort (Figure 1C), and confirmed a corresponding increase in GADD45A expression at the protein level by Western analysis at 14 days post-denervation (Figure 1D-E).

Using denervated human skeletal muscle samples obtained from patients with traumatic nerve injuries or acute flaccid myelitis, we confirmed that GADD45A expression was significantly increased in human denervated muscle compared to intact control muscle (Figure 1F-G, mean fold-change = 22.5 (95% CI 13.7, 37.1), t = 12.714, df = 31.629, P = 5.664e-14), even when expression levels of TRIM63 and FBXO32, the ubiquitin E3 ligases classically associated with the proteolytic degradation intrinsic to muscle atrophy, were low. This is consistent with observations in our tibial nerve denervation mouse model of neurogenic atrophy, wherein Trim63 and Fbxo32 show significant induction during the first 7 or 14 days post-denervation, respectively, but then return to baseline or below baseline levels (Figure 1A). The severity of nerve injury and loss of muscle function in the included cases were consistent with extensive denervation, which could explain the generally high level of GADD45A induction observed. These cases represent acute denervation injuries, with subsequent denervation durations ranging from 2 - 48 months, and evaluation of individual ΔCt values demonstrate that GADD45A expression remains sustained at remarkably high levels in a wide distribution of muscle groups even long after initial denervation compared to normal skeletal muscle (Figure 1H).

Gadd45a-null mice show significantly accelerated and more severe neurogenic atrophy

Based on earlier studies suggesting that viral overexpression of GADD45A is sufficient to induce muscle atrophy, we obtained a conventional genetic knockout mouse model of Gadd45a (11) to evaluate whether preventing Gadd45a induction can ameliorate neurogenic atrophy. We
did not detect any difference among genotypes (wild type, heterozygous, and knockout) in baseline (intact, non-denervated) gastrocnemius mass (n = 16-28 for each genotype; two-way ANOVA with genotype and gender as covariates, P < 0.001 for gender, P = 0.78 for genotype, P = 0.71 for genotype-gender interaction) (Figure 2A). However, multiple linear regression with genotype, time, and genotype-time interaction as predictor variables indicated that, compared to wild type littermates, Gadd45a-null muscle had a 25 ± 6% greater loss of mass by 14 days after denervation (P < 0.001), which was sustained to 29 ± 7% greater loss of mass at 90 days after denervation (P < 0.001) (mean ± SEM, n = 3-8 for each genotype at each time point) (Figure 2B-C). Heterozygous mice did not differ significantly from wild type mice [wild type vs. heterozygous gastrocnemius mass (genotype-time interaction): P = 0.90 at 7 days, P = 0.55 at 14 days, P = 0.96 at 72 days] (Figure 2B). Gender did not appear to significantly modify the relationship between mass and denervation duration or mass and genotype. The rate of mass loss differed significantly among knockouts vs. wild type mice during the first 14 days post-denervation (P < 0.001), but did not differ from 14-30 days (P = 0.50) or 30-72 days (P = 0.44) post-denervation (Figure 2B).

This accelerated atrophy in the absence of GADD45A was evident at the level of individual myofibers (Figure 2D-M). Type IIA and type IIB myofibers differed in size at baseline in intact gastrocnemius (P = 0.002), but there was no difference in relative size of these two fiber types among wild type versus Gadd45a knockout littermates (Figure 2D,I,N, mean minimum Feret diameter, type IIA, 28.5 ± 7.4 μm for wild type and 31.4 ± 8.1 μm for knockout; type IIB, 42.0 ± 8.5 μm for wild type and 42.6 ± 8.6 μm for knockout, P = 0.28 for overall difference between genotypes, P = 0.42 for genotype-fiber type interaction, two-way ANOVA with correction for intra-replicate correlation, n = 3 for each genotype). However, at 14 days post-denervation both type IIA and IIB myofibers were significantly more atrophied in Gadd45a-null gastrocnemius muscle compared to wild type muscle (Figure 2F,K,O, type IIA, 26.0 ± 7.5 μm for wild type and 17.3 ± 9.0 μm for knockout, P = 0.02; type IIB, 22.5 ± 5.8 μm for wild type and 19.3 ± 7.5 μm for knockout, P < 0.001, two-way ANOVA with correction for intra-replicate correlation, n = 3 for each genotype).

Altogether, these findings suggest that Gadd45a induction after skeletal muscle denervation confers a protective effect against atrophy, as might be predicted for a stress-induced transcript.

Denervation induces Gadd45a expression specifically in skeletal muscle myocytes

We were unable to identify an antibody that specifically and reliably detects endogenous GADD45A in skeletal muscle cryosections. We therefore generated a V5-epitope tagged Gadd45a allele also capable of conditional deletion, using CRISPR-Cas9 technology (Supplemental Figure 1A). We confirmed the expected genomic modifications by Sanger sequencing (Supplemental Figure 1B-C), and detected expression of V5-tagged protein at the expected molecular weight exclusively in denervated gastrocnemius muscle from Gadd45a<sup>flox</sup> mice, but not in intact (non-denervated) muscle or wild type muscle (Supplemental Figure 1D).

Denervation of mice carrying V5-tagged conditional Gadd45a revealed that the Gadd45a induction associated with denervation injury results exclusively from expression in skeletal
muscle myocytes. We selectively excised the first three coding exons of Gadd45a from myonuclei by breeding Gadd45a\textsuperscript{flox} mice with a transgenic mouse line carrying Cre-recombinase expression directed by the human skeletal actin (HSA) promoter (HSA-Cre79; B6.Cg-Tg(ACTA1-cre)79Jme/J). Mice with skeletal muscle-specific deletion of Gadd45a (HSA-Cre:Gadd45a\textsuperscript{flox}) showed minimal induction of Gadd45a mRNA in denervated gastrocnemius compared to contralateral intact muscle at 14 days post-denervation (Figure 3A-B, t(2) = -4.7547, P = 0.0413, n = 3). In contrast, Gadd45a\textsuperscript{flox} and wild type mice showed significantly increased Gadd45a mRNA levels in denervated gastrocnemius at 14 days post-denervation (Figure 3A-B, t(2) = -12.8978, P = 0.006 and t(2) = -39.8712, P = 0.0006, respectively, n=3 of each genotype). The magnitude of Gadd45a induction appeared to differ between Gadd45a\textsuperscript{flox} and wild type mice, although this did not achieve statistical significance (one-way ANOVA, F(2,6) = 95.54, P < 0.0001; Gadd45a\textsuperscript{flox} vs wild type P = 0.054). The minimal degree of induction observed in HSA-Cre:Gadd45a\textsuperscript{flox} muscle differed significantly from both of the other genotypes (one-way ANOVA, F(2,6) = 95.54, P < 0.0001, HSA-Cre:Gadd45a\textsuperscript{flox} vs. Gadd45a\textsuperscript{flox} P < 0.001, HSA-Cre:Gadd45a\textsuperscript{flox} vs. wild type P < 0.001).

These findings were confirmed at the protein level using an anti-V5 antibody to detect epitope-tagged GADD45A (Figure 3C). No target was detected in denervated or control muscle from C57BL/6J mice lacking the V5 epitope. However, a ~20 kDa protein corresponding to the expected molecular weight of V5 epitope-tagged GADD45A was detected in denervated gastrocnemius, but not contralateral intact gastrocnemius, from Gadd45a\textsuperscript{flox} mice. Importantly, this signal was completely abolished in mice with skeletal muscle-specific deletion of Gadd45a (HSA-Cre:Gadd45a\textsuperscript{flox}). Altogether, these findings confirm that the GADD45A induction observed in denervated muscle occurs predominantly, and likely exclusively, in skeletal muscle myocytes.

**Gadd45a induction protects against neurogenic skeletal muscle atrophy**

Baseline masses of the gastrocnemius and soleus muscles from age-matched and littermate wild type, heterozygous, Gadd45a\textsuperscript{flox}, and HSA-Cre:Gadd45a\textsuperscript{flox} mice did not differ among genotypes (Figure 3N, one-way ANOVA, gastrocnemius: F(3,20) = 2.05, P = 0.1391; soleus: F(3,20) = 2.08, P = 0.1344; N = 4-7 per genotype). At 14 days post-denervation, however, we observed a statistically significant difference in relative mass (denervated/contralateral intact) among genotypes for both gastrocnemius and soleus (Figure 3O, one-way ANOVA, gastrocnemius: F(3,20) = 3.46, P = 0.0359; soleus: F(3,20) = 16.26, P = 1.37e-05, N = 4-7 per genotype). Post-hoc analysis showed that the relative loss of gastrocnemius and soleus mass among wild type, HSA-Cre:Gadd45a\textsuperscript{em+}, and Gadd45a\textsuperscript{flox} genotypes was similar (gastrocnemius: wild type 0.50 ± 0.04, Gadd45a\textsuperscript{flox} 0.46 ± 0.06, HSA-Cre:Gadd45a\textsuperscript{em+} 0.45 ± 0.07, P = 1.0 wild type vs. HSA-Cre:Gadd45a\textsuperscript{em+}; P = 1.0 wild type vs Gadd45a\textsuperscript{flox}, soleus: wild type 0.73 ± 0.09, Gadd45a\textsuperscript{flox} 0.59 ± 0.16, HSA-Cre:Gadd45a\textsuperscript{em+} 0.73 ± 0.08, P = 0.176 wild type vs. Gadd45a\textsuperscript{flox}, P = 1.0 wild type vs HSA-Cre:Gadd45a\textsuperscript{em+}). However, denervated gastrocnemius and soleus muscle from HSA-Cre:Gadd45a\textsuperscript{flox} mice showed significantly larger loss of mass compared to wild type (gastrocnemius: 0.41 ± 0.04, P = 0.029; soleus: 0.38 ± 0.06, P < 0.001). These data further support the conclusion that GADD45A induction specifically in skeletal muscle myocytes confers a protective effect against neurogenic atrophy.
We further generated mice with ubiquitous deletion of Gadd45a using B6.C-Tg(CMV-cre)1Cgn/J mice. ~50% of female CMV-Cre:Gadd45a<sup>flox</sup> mice demonstrated dystocia with their first pregnancy, resulting in death, as previously described in a separate mouse model with ubiquitous targeted deletion of Gadd45a (11). The relative loss of gastrocnemius and soleus muscle mass among CMV-Cre:Gadd45a<sup>flox</sup> mice did not differ significantly from HSA-Cre:Gadd45a<sup>flox</sup> mice (Figure 3O).

Absence of Gadd45a induction in skeletal muscle myocytes results in atrophy of multiple fiber types and apparent loss of fiber type identity

We performed myofiber morphometry analysis of intact soleus muscle and soleus muscle at 14 days post-tibial nerve transection among congenic age-matched wild type, heterozygous, Gadd45a<sup>flox</sup>, HSA-Cre:Gadd45a<sup>flox</sup>, and CMV-Cre:Gadd45a<sup>flox</sup> mice, which enabled us to evaluate each transverse muscle section in its entirety. Minimum Feret diameters of type I, type Ia, and non-type I/Iia myofibers did not differ among any of the genotypes within intact, uninjured soleus muscle (Figure 4A-E, K). At 14 days post-denervation, however, all three fiber types showed significantly reduced minimum Feret diameters in HSA-Cre:Gadd45a<sup>flox</sup> and CMV-Cre:Gadd45a<sup>flox</sup> soleus muscle compared to denervated wild type, heterozygous, and Gadd45a<sup>flox</sup> soleus (Figure 4F-J, L; HSA-Cre:Gadd45a<sup>flox</sup>, type I: 18.9 ± 5.2 μm, P = 0.04 (vs. wild type); type Ia: 17.5 ± 6.2 μm, P = 0.02; non-type I/Iia: 15.3 ± 5.1 μm, P = 0.02; CMV-Cre:Gadd45a<sup>flox</sup>, type I: 14.7 ± 4.8 μm, P = 0.002; type Ia: 15.1 ± 6.1 μm, P = 0.002; non-type I/Iia: 11.4 ± 4.1 μm, P < 0.001; wild type, type I: 26.4 ± 5.1 μm; type Ia: 29.8 ± 5.5 μm; non-type I/Iia: 26.0 ± 5.3 μm). Myofiber size for each respective myofiber type did not differ between denervated HSA-Cre:Gadd45a<sup>flox</sup> and CMV-Cre:Gadd45a<sup>flox</sup> soleus (P = 0.1, P = 0.36, and P = 0.12 for type I, type Ia, and non-type I/Iia, respectively), further suggesting that the entire effect of GADD45A induction is conferred through its activity in skeletal muscle myocytes. Type Iib myofibers are rare in the mouse soleus and were not quantified.

Although fiber type-transition is known to occur in skeletal muscle as a result of chronic denervation and reinnervation, we unexpectedly observed that denervated soleus muscle from HSA-Cre:Gadd45a<sup>flox</sup> and CMV-Cre:Gadd45a<sup>flox</sup> mice showed a significant increase in the proportion of myofibers lacking expression of either myosin I or Ia compared to wild type and heterozygous mice (48.1% and 53.3% compared to 9.6% and 6.9%, respectively; Figure 4M). These non-I/Iia fibers showed the most severe atrophy and were accompanied by significant loss of myofibers with type Ila identity in particular (3.0% and 1.8% type Ila myofibers remaining in denervated HSA-Cre:Gadd45a<sup>flox</sup> and CMV-Cre:Gadd45a<sup>flox</sup> soleus compared to 56.5% and 59.7% in denervated wild type and heterozygous soleus, respectively). Consistent with observations of gastrocnemius/soleus mass and myofiber dimensions in the soleus, denervated Gadd45a<sup>flox</sup> mice showed a proportion of non-I/Iia myofibers and relative loss of type Ila myofibers intermediate between that of wild type/heterozygous mice and HSA-Cre:Gadd45a<sup>flox</sup>/CMV-Cre:Gadd45a<sup>flox</sup> mice (26.0% non-I/Iia myofibers and 37.6% type Ila fibers). The relative proportions of each myofiber type were similar among all genotypes in intact, uninjured soleus.
Discussion

A variety of stimuli are known to induce GADD45A expression, and the consequences of this induction appear to be cell type-specific. Earlier studies detected increased expression of Gadd45a in denervated soleus, gastrocnemius, and/or triceps muscle from the SOD1<sup>G86R</sup> and SOD1<sup>G93A</sup> mouse models of ALS, as well as in soleus and extensor digitorum (EDL) muscles from rodent models of upper or lower motor neuron transection (12). Gadd45a induction has also been observed in muscle from older individuals subjected to 5 days of bed rest (13). Increased GADD45A expression has been described in gene expression microarray analyses of deltoid (14, 15) and quadriceps (15) muscles from individuals with ALS. Higher levels of GADD45A expression observed at later stages of disease correlate with more severe and widespread muscle atrophy (14).

Congruent with these observations, we detected significantly increased GADD45A expression in human skeletal muscle samples from patients with traumatic nerve injury or acute flaccid myelitis with denervation durations ranging from 2 – 48 months (Figure 1F-H), consistent with earlier suggestions that GADD45A induction in skeletal muscle is a direct response to denervation. Previous studies using virus-mediated overexpression of GADD45A in non-denervated muscle from C57BL/6J mice suggested that the expression of GADD45A alone was sufficient to trigger muscle atrophy (16-19). Our findings using a series of genetic models suggest GADD45A induction in denervated skeletal muscle delays the rate of atrophy and myofiber type transition, potentially even serving to preserve myofiber identity during chronic denervation.

Gadd45a induction has been observed in a variety of additional settings associated with muscle atrophy, including aging, muscle disuse, starvation, chronic obstructive pulmonary disease (COPD), and critical illness (20), and has been suggested to be required for muscle atrophy in starvation, immobilization, and denervation (18). In an immobilization model of muscle atrophy, this has been proposed to occur through GADD45A-mediated activation of MAP3K4 (MEKK4) via conformational release of an autoinhibitory domain from the MAP3K4 kinase domain, leading to phosphorylation and activation of downstream MAP kinase kinases and as yet unknown downstream effectors (17). These studies used electroporation of mouse tibialis anterior (TA) muscles with plasmids encoding siRNA targeting Gadd45a or GADD45A overexpression constructs, and the effects were studied at 1 week post-immobilization (17). Our studies using three genetic mouse models (ubiquitous targeted ablation, Cre-mediated skeletal muscle-specific deletion, and Cre-mediated ubiquitous deletion) in the otherwise normal physiologic context of atrophying denervated muscle strongly support a model wherein Gadd45a induction is a protective modifier of myofiber atrophy and fate. This interpretation is further supported by the observation that Gadd45a<sup>lox</sup> mice carrying a V5-epitope, which unintentionally appears to have yielded a modestly hypomorphic allele, show an atrophy phenotype intermediate to that of Gadd45a knockout and Gadd45a heterozygous or wild type mice. The V5 epitope comprises 14 amino acids while GADD45A itself is only 165 amino acids, and this apparent hypomorphic feature may relate to altered mRNA stability or altered interaction with effector proteins (e.g., the GADD45A N-terminus is known to interact with TET1, described below).
*Gadd45a* induction has also been observed in several models of neuronal injury including ischemia, spinal cord transection, and sciatic nerve crush, and findings from all of these studies suggest that its upregulation has a protective role after neuronal insult (21-26). Robust *Gadd45a* induction was observed in rat dorsal root ganglion neurons (DRGs) within 1 day after spinal nerve ligation, and persisted as long as the injured nerves were prevented from regenerating (27). shRNA-mediated knockdown of *Gadd45a* in DRGs via intrathecal infusion was associated with increased death of DRG neurons following spinal cord ligation, whereas transduction of cultured neonatal DRGs with HSV expressing human GADD45A significantly enhanced survival (27). *Gadd45a* has been found to be upregulated in DRGs after sciatic nerve transection as well, where it has been suggested to participate in p53 signaling, apoptosis, cellular senescence, and/or MAPK signaling (28).

Beyond the neuromuscular system, GADD45A has also been shown to have a protective role in mouse models of ventilator-induced lung injury as well as radiation- and bleomycin-induced lung injury (29-31). *Gadd45a* expression is induced in endothelial cells in these models of lung injury, and *Gadd45a*-null mice (11) show reduced AKT signaling and more severe susceptibility to these forms of lung injury. In these mouse models, GADD45A deficiency was proposed to result in differential ubiquitination of AKT via reduced expression of the deubiquitinase UCHL1, resulting in both increased proteasomal degradation of AKT and decreased AKT phosphorylation/activation in response to mechanical stress (30, 31). Considering the known role of AKT signaling in mediating skeletal muscle hypertrophy, it will be of interest to evaluate whether the protective effect of GADD45A we observed in denervated muscle is also mediated at least in part through modulation of AKT signaling. *Gadd45a*-null mice also show more severe fibrosis in a mouse model of non-alcoholic steatohepatitis (NASH), a form of hepatic scarring that can lead to liver failure, suggesting a protective role in this condition as well (32). Finally, *Gadd45a* is downregulated in a variety of malignancies, including chronic myelocytic leukemia (33). Its induction in endothelial cells has been shown to suppress tumor angiogenesis via reduction of mTOR-mediated phosphorylation and activation of STAT3 and resulting VEGFa expression (34, 35).

How GADD45A confers a protective effect during neurogenic atrophy is presently unknown. GADD45A interacts with numerous diverse proteins and as a result is proposed to have many diverse functions. Recent studies suggest that p38α signaling positively regulates skeletal muscle atrophy, and mice with skeletal muscle-specific ablation of p38α or heterozygous inactivating mutations in p38 show reduced atrophy of TA and/or gastrocnemius muscle in sciatic nerve resection models of denervation atrophy (36, 37). GADD45A has been shown to bind directly to p38α, although this interaction appears to result in tissue-specific and opposing functions on p38 activity (38). A potentiating effect on p38 kinase activity has been observed in some studies, as suggested by impaired p38-mediated MK2 activation in cells lacking GADD45A (39) and a requirement for GADD45A in H-ras-mediated activation of p38 (40). GADD45A also indirectly regulates p38 signaling by binding and activating MEKK4 (41), leading to phosphorylation and activation of MKK3/MKK6 which leads to activation of p38 (42). However, GADD45A is not required for activation of p38 in all settings of cellular stress, as no reduction in JNK or p38 kinase activation was observed in *Gadd45a* knockout MEFs subjected to UV irradiation compared to wild type MEFs (43). Furthermore, constitutive activation of T-cell p38 has been
observed in the absence of GADD45A, demonstrating that GADD45A can serve as a negative regulator of p38 activity as well (44).

Although the major degradative events of muscle atrophy have been classically associated with proteasomal degradation, in particular the E3 ubiquitin ligases TRIM63 and FBXO32, lysosomal degradation and autophagy also contribute. GADD45A is a negative regulator of autophagy, binding directly to BECN1 and thereby inhibiting its interaction with PIK3CK (45). GADD45A has also been shown to induce cell cycle arrest at the G2/M phase through interaction with cyclin B1 and induces cellular senescence in human fibroblasts (46-50). This could be pertinent as skeletal muscle myocytes are terminally differentiated cells, but experimentally forced cell cycle re-entry (e.g., through overexpression of the skeletal muscle transcription factor MyoD, as occurs in denervated muscle) is associated with loss of skeletal muscle-specific gene expression (51).

Finally, GADD45A has a unique role in regulating methylation-dependent expression of specific target genes. This role emerged from an unbiased expression screen for DNA demethylases, and GADD45A overexpression was subsequently shown to promote demethylation and activation of methylation-silenced reporter genes. Premethylated CpG islands undergo active demethylation when transfected into ES cells; however, after Gadd45a knockdown in ES cells, these sequences retained substantial degrees of methylation (52). Deletion of three GADD45 family members (Gadd45a, Gadd45b, and Gadd45c), all of which function in demethylation, in embryonic stem cells was associated with locus-specific hypermethylation, primarily found in intronic and intergenic regions (53). Deletion of Gadd45a/b in mouse ES cells leads to hypermethylation at sites mostly overlapping with sites known to be dependent on TDG for demethylation.

GADD45A is now known to interact with key enzymes involved in DNA demethylation, including TET methylcytosine dioxygenase 1 (TET1) and thymine DNA glycosylase (TDG) (54-57). Active demethylation involves TET1-mediated 5mC oxidations followed by base-excision or nucleotide-excision repair conversions of oxidized intermediates to cytosines (58, 59). GADD45A is thought to promote TET1 activity and/or recruit key components of DNA repair at specific genomic loci, leading to cytosine demethylation within targeted CpG islands and activation of gene expression (56, 57, 60-62). GADD45A has also been described to bind directly to R-loops at CpG islands, leading to recruitment of TET1 and associated demethylation machinery to mediate local DNA demethylation (63). Gadd45a deficiency has been shown to be associated with hypermethylation of only a small number of previously mapped 5hmC- and 5fC-enriched regions in ES cells, suggesting that GADD45A may contribute to the regulation of only a small subset of TET-TDG regulated genomic targets (57). Altogether, these observations highlight the possibility that the effects of GADD45A deficiency on skeletal muscle atrophy observed in our murine genetic series may result from epigenetic-dependent effectors.

Fiber type transition is well-known to occur in denervated muscle, particularly in conditions where chronic denervation and reinnervation processes are present, and this transition typically reflects fiber type identity driven by the activity pattern of the reinnervating axon. We observed that Gadd45a deficiency also results in loss of myofiber identity during denervation. Whether this reflects enhanced degradation of sarcomeric components related to atrophy or authentically failed preservation of myofiber identity warrants further exploration, and could have implications for the processes that seemingly permanently alter myofiber phenotype during chronic denervation and that lead to impaired reinnervation capacity and functional restoration.
In summary, our work shows that a naturally occurring negative feedback mechanism exists in denervated skeletal muscle with the potential to modify the severity of atrophy and fate of individual denervated myofibers. Ongoing studies are underway to define the upstream and downstream signaling context within which GADD45A confers protection, with the intention of identifying targets that may be further harnessed to protect muscle from atrophy. This work could offer insight into pathways that may be modulated to successfully limit skeletal muscle atrophy, potentially preserving the capacity for muscle function and lengthening the window during which functional reinnervation may be achieved.

Methods

Animal Husbandry

Gadd45a-null mice were previously described (11). Genotyping was performed with genomic DNA extracted from tail biopsies using the REDExtract-N-Amp tissue lysis and PCR kit (Sigma-Aldrich, St. Louis, MO, cat. #XNAT) using the following primers: Gadd45a-mutant: 5’-AGA ACG AGA TCA GCA GCC TCT-3’; Gadd45a-same: 5’-GAA GAC CTA GAC AGC ACG GTT-3’; Gadd45a-WT: 5’-CCT CGT CTT ACC TCT GCA CAA-3’. Thermocycling conditions were: 94°C 3 min, 35 cycles of 94°C 30 sec, 54°C 30 sec, 72°C 1 min, and final extension at 72°C for 10 min. Expected product sizes were 324 bp (wild type) and 211 bp (knockout). B6.Cg-Tg(Acta1-cre)79Jme/J mice (HSA-Cre79, JAX# 006149) and B6.C-Tg(CMV-cre)1Cgn/J mice (CMV-Cre, JAX #006054) were obtained from the Jackson Laboratory (Bar Harbor, ME). Cre genotyping was performed using the following primers: Cre-F: 5’-ATT GGT GTC ACT TGG TCG TGG C-3’; Cre-R: 5’-GGA AAA TGC TCC TTC TGT TCG GGC CAA-3’. Actin-F: 5’-GAC GAT ATC GCT GCG CTG GTC GTC G-3’; Actin-R: 5’-GCC TGT GGT ACG ACC AGA GGC ATA CAG-3’. Thermocycling conditions were: 94°C 2 min, 30 cycles of 94°C 30 sec, 60°C 30 sec, 72°C 1 min, and final extension at 72°C for 10 min. Expected product sizes were 207 bp (cre) and 1000 bp (actin). All oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Animal subjects were housed in a controlled environment with a 12:12-h light-dark cycle with ad libitum access to water and food (Envigo 2018 SX).

CRISPR-Cas9-mediated generation of conditional/epitope-tagged Gadd45a mouse

Guide RNAs targeting intronic regions flanking the first three coding exons of murine Gadd45a (NCBI Reference Sequence NC_000072.6:c67039407-67033096, Mus musculus strain C57BL/6J) were designed using the web-based CRISPR design tool of MIT (crispr.mit.edu). Two of the top gRNAs (score ≥ 90) were selected for use as follows: Gadd45a-gRNA-5’: 5’-CAG CAC CAC CTT CGT CC^CGT-3’; Gadd45a-gRNA-3’: 5’-CGG AGC GTG TCT AAG CT^CGT-3’. Alt-R CRISPR-Cas9 crRNA oligos and a 1.5 kb single-stranded donor DNA megamer containing the desired 5’ loxP, 3’ loxP, a V5 epitope tag introduced directly after the start codon, and 180 nucleotide flanking regions were synthesized by IDT (Coralville, IA). The V5 epitope sequence was as follows: 5’-GGT AAG CCT ATC CCT AAC CTC CTC TCT GGT CTC GAT TCT AGC-3’. Pronuclear injection of one-cell C57BL/6J embryos (Jackson Laboratory) was performed by the JHU Transgenic Core using standard microinjection techniques (64) using a mix of Cas9 protein (30 ng/ul, PNABio), tracrRNA (0.6 µM, Dharmacon), crRNA (0.6 µM, IDT), and ssDNA oligo (5 ng/ul, IDT) diluted in RNase-free
injection buffer (10 mM Tris-HCl, pH 7.4, 0.25 mM EDTA). Injected embryos were transferred into the oviducts of pseudopregnant ICR females (Envigo) using the technique described in Nagy et al. (64). Founders were screened for 5’ loxP and V5 epitope insertion using the following primers: Gadd45a-loxP-F3: 5’-AGG ACA CTT GAA CCA CTG CAA-3’ and Gadd45a-V5-R: 5’-CAG GCA CAC TTA CCT TTC GGT-3’; expected product sizes were 510 bp (unmodified) and 586 (modified). 3’ loxP insertion was screened using the following primers: Gadd45a-loxP-F1: 5’-TGC TGC TAC TGG AGA ACG AC-3’ and Gadd45a-loxP-R2: 5’-AAT TAG CCA CGC GAG GTT GT-3’; expected product sizes were 285 bp (unmodified) and 319 bp (modified). PCR fragments from founding lines were purified using AMPure XP beads (Beckman Coulter) and verified by Sanger sequencing. A homozygous founder carrying both modified sites was back-crossed 3 generations to C57BL/6J mice. This line has been deposited with the Mutant Mouse Resource & Research Centers (MMRRC) at the Jackson Laboratory and is available as stock #050634-JAX (C57BL/6J-Gadd45a<sup>em1Ahoke</sup>/Mmjax, abbreviated as Gadd45a<sup>flox</sup>). Genotyping was performed using the REDExtract-N-Amp tissue lysis and PCR kit (Sigma-Aldrich, cat. #XNAT) and the following thermal cycling conditions: 94°C 3 min; 35 cycles of 94°C 30 sec, 54°C 30 sec, 72°C 1 min, and final extension at 72°C for 10 min.

**RNA-Seq library preparation, sequencing, and bioinformatics analysis**
The murine denervation dataset was described previously (10). RNA-sequencing was carried out using TrueSeq RiboZero gold (stranded) kit (Illumina, catalogue #20020597). Libraries were indexed and sequenced on 18 lanes using HiSeq4000 (Illumina) with 69-bp paired end reads. Quality control (QC) was performed on base qualities and nucleotide composition of sequences using FastQC version 0.11.5. Paired-end reads were aligned to the most recent *Mus musculus* mm10 reference genome (GRCm38.75) using the STAR spliced read aligner (version 2.4.0) (65). Total counts of read-fragments aligned to known gene regions within the mouse (mm10) refSeq (refFlat version 07.24.14) reference annotation were used as the basis for quantification of gene expression. Fragment counts were derived using HTSeq (version 0.6.0) and the mm10 refSeq transcript model (66). Low count transcripts were filtered, and count data were normalized using the method of trimmed mean of M-values (TMM) (67) followed by removing unwanted variation using Bioconductor package RUVseq (68) with k value of 1. Differentially expressed genes (FDR < 0.1) were then identified using the Bioconductor package limma with voom function to estimate mean-variance relationship, followed by empirical Bayes moderation (69-71). Pairwise comparisons between denervated and contralateral intact muscle at each timepoint were used as the basis for model contrasts.

**Human Skeletal Muscle Specimens**
Denervated human skeletal muscles were obtained from specimens discarded during elective nerve repair surgeries at Johns Hopkins Hospital. Histologically normal control skeletal muscle specimens were obtained from archived samples available from the Johns Hopkins Neuromuscular Pathology Lab, and were age- and site-matched to denervated samples to the extent possible. Specimens were frozen in liquid nitrogen-cooled isopentane and stored at -80°C until processed for RNA isolation.

**Tibial nerve denervation surgery**
Mice were anesthetized with 1.5% isoflurane/2% oxygen using a VetEquip inhalation system (Livermore, CA). The left hindlimb was shaved and sterilized, and a 1 cm incision was
introduced in the skin overlying the dorsal thigh. Myofascial planes were gently separated to reveal the sciatic nerve. The tibial nerve branch was identified at its distal branch point and gently separated from the sciatic and peroneal nerves, then ligated proximally and distally using a 10-0 polyamide monofilament suture. The tibial nerve was then transected, the nerve length between ligatures carefully resected, and the proximal stump sutured to the biceps femoris muscle to prevent distal reinnervation. The incision was then closed using stainless steel wound clips. Mice were monitored for recovery from anesthesia and then returned to their home cages.

**RNA Isolation**

Skeletal muscle was homogenized in TRIzol (Ambion, cat. #15596018) using RNase-free stainless steel beads (Next Advance, cat. #SSB02-RNA). Homogenates were centrifuged at 10,000 rpm for 10 min to pellet debris, and RNA was purified from the TRIzol supernatant using a Direct-Zol RNA mini purification kit with on-column DNase digestion (Zymo Research, cat. #R2072). RNA integrity (RIN) was assayed using an Agilent 2100 Bioanalyzer. RIN for RNA isolated from fresh frozen human skeletal muscle was 7.3 ± 0.8 (normal muscle, mean ± S.D.) and 7.7 ± 1.1 (denervated muscle, mean ± S.D.), with no significant difference in RIN between denervated and control samples (Welch’s t-test, t(24.60) = -1.25, P = 0.22). RIN for RNA isolated from mouse skeletal muscle was not regularly assayed, but was found to routinely be ~8.0 in previous experiments using identical methods, with no significant difference in RIN by denervation status (10).

**Quantitative PCR**

0.45 - 1 ug total RNA was used for cDNA synthesis using the high capacity cDNA reverse transcription kit containing random primers (ThermoFisher Scientific cat. #4368814), with the following cycling conditions: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min. Quantitative PCR was performed using TaqMan gene expression assays (ThermoFisher Scientific cat. #4331182) with TaqMan Fast gene expression master mix (ThermoFisher Scientific cat. #4444554) and 10-50 ng cDNA equivalents of template DNA. The following FAM-MGB-labeled TaqMan primer/probe sets were used: *Gadd45a*, Mm00432802_m1; *Trim63*, Mm01185221_m1; *Fbxo32*, Mm00499523_m1; *GADD45A*, Hs00169255_m1; *TRIM63*, Hs00822397; *FBXO32*, Hs01041408_m1. Real-time PCR was performed using a StepOnePlus real-time PCR system (Applied Biosystems). We determined relative quantities using the comparative Ct (ΔΔCt) method (72, 73), using VIC-MGB-labeled *TBP* (human, cat. #4448489, Hs00427620_m1) or VIC-MGB-labeled *Tbp* (mouse, cat. #4448489, Mm01277042_m1) as endogenous controls for normalization (74). All reactions were performed in technical triplicates with the number of biological replicates as indicated in the text/figures.

**Immunofluorescence and myofiber morphometry**

Gastrocnemii were frozen in O.C.T. in liquid nitrogen-cooled isopentane (75), then sectioned at 10 μm. Mid-belly transverse sections were blocked with M.O.M. in PBS (1:40 dilution, Vector Laboratories, catalogue #MKB-2213) at room temperature for 1 h, then incubated overnight at 4 °C with a mixture of BA-D5 supernatant (1:100, myosin heavy chain type I), SC-71 supernatant (1:100, myosin heavy chain type IIa), BF-F3 concentrate (1:100, myosin heavy chain type IIb) [all from the Developmental Studies Hybridoma Bank (DSHB)], and rat-anti-laminin (1:1000, Sigma, catalogue #L0663) in 1% BSA/PBS. Sections were then washed 3 × 5 min in PBS and incubated with a mixture of the following secondary antibodies (all at 1:500) for 2 h at
room temperature: goat-anti-mouse IgG2b-DyLight-405, IgG1-Alexa Fluor-488, IgM-Alexa Fluor-594 (all from Jackson ImmunoResearch, catalogue numbers 115-475-207, 115-545-205, and 115-585-075, respectively), and goat anti-rat-IgG-Alexa Fluor-647 (Thermo Fisher Scientific, catalogue #A-21247), diluted in 1% BSA/PBS. Sections were washed 3 × 5 min in PBS and coverslipped using Prolong Gold antifade (Thermo Fisher Scientific, catalogue #P36930). Images were collected using an Airyscan 800 confocal microscope (Zeiss). For myofiber morphometry, transverse gastrocnemius and soleus sections and soleus sections were imaged in their entirety using a BZ-X700 fluorescence microscope (Keyence). Myofiber minimum Feret diameters were determined using QuantiMus (76). For QuantiMus analysis, > 2000 myofibers each fiber type were measured from each of 3-4 biological replicates for each genotype, with the exception of denervated HSA-Cre:Gadd45a<sup>fl</sup> and CMV-Cre:Gadd45a<sup>fl</sup> soleus muscle which showed a significant reduction in the population of type I myofibers.

**Western analysis**

Skeletal muscle was homogenized using steel beads (Next Advance, cat. #SSB02-RNA) in ice-cold T-PER buffer (ThermoFisher Scientific, cat. #78510) containing protease inhibitors (Roche, cat. #11697498001). Lysates were centrifuged and protein concentration measured by BCA assay (ThermoFisher Scientific, cat. #23227). 50 ug protein lysates denatured in SDS buffer were boiled for 5 min, electrophoresed through 4-20% polyacrylamide gels in Tris-Glycine SDS running buffer (ThermoFisher Scientific, cat. #LC2675), then transferred to nitrocellulose membranes in transfer buffer containing 20% methanol for 30 minutes at 100 V. Protein loading and transfer was evaluated by Ponceau S staining (Cell Signaling, cat. #59803) (77), and membranes were then blocked with 5% nonfat milk in TBST for 1 h at room temperature. Membranes were incubated overnight at 4°C in 5% nonfat milk-TBST containing goat-anti-V5 antibody (1:1000, Abcam, cat. #ab9137, lot #GR172741) or rabbit-anti-GADD45A (1:1000, Abcam, cat. # ab180768, lot #GR3218627). Membranes were washed in TBST and incubated for 1 h at room temperature with 5% nonfat milk-TBST containing HRP-conjugated rabbit-anti-goat secondary antibody (1:2500, ThermoFisher Scientific, cat. #R-21459) or goat-anti-rabbit secondary antibody (1:3000, Amersham, cat. #NA934). Membranes were then incubated with ECL (GE, cat. #GERPN3243) or Femto (ThermoFisher Scientific, cat. #34095) reagents, visualized using Hyperfilm ECL (Amersham, cat. #GE28-9068-38), and scanned.

**Statistics**

Two-group comparisons were made using Welch’s t-test. One-way ANOVA with post hoc Bonferroni correction was used for comparisons having more than two groups. Skeletal muscle masses were compared using a linear regression model with genotype, gender, and time as covariates and an interaction term between genotype and time. Skeletal muscle myofiber minimum Feret diameters were compared using a linear regression model with genotype and myofiber type as covariates and an interaction term between genotype and myofiber type, with correction for intra-animal correlation. Myofiber proportions were compared using Fisher’s exact χ² test. Statistical analyses were performed using R version 3.5.1 (78) and/or Stata version 11.2 (College Station, TX) (79). Data are expressed as means ± S.D., means ± S.E.M., or means with 95% C.I. as indicated. P < 0.05 is considered statistically significant.

**Study approval**
Human skeletal muscle specimen collection was approved by the Johns Hopkins School of Medicine Institutional Review Board (IRB #00081649) and all samples were obtained with written informed consent from the patient or guardian. All mouse experiments were carried out under protocols approved by the JHU Animal Care and Use Committee (#MO17M98, #MO20M28).

Author Contributions
J.T.E. and A.H. designed the study; J.T.E. and D.K. conducted experiments; J.T.E., R.K., and D.N. analyzed data; J.T.E. wrote the manuscript; J.T.E. and A.H. edited the manuscript.

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Declaration of Interests
The authors declare no competing interests.

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References


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**Figure 1.** *Gadd45a* induction is an acute and sustained response to denervation injury. *Gadd45a* is the most significantly differentially expressed transcript in acutely denervated mouse gastrocnemius, and shows sustained induction up to 3 months post-denervation (A-D, *** *P* < 0.001). GADD45A is similarly induced in a diverse range of denervated human skeletal muscle from individuals with acute denervation injury or acute flaccid myelitis, and remains elevated up to 4 years after denervation (F-H). All of the normalized *GADD45A* threshold detection cycles (ΔCt) from denervated skeletal muscle are substantially lower than controls, indicating higher mean expression levels (H). There was no difference in mean age or gender distribution among denervated human muscle samples and controls within the study cohort (age, Welch’s t-test (t(28.52)) = 0.9323, *P* = 0.36; gender, Fisher’s exact test, *P* = 0.67).
Figure 2. Gadd45a-null mice show significantly accelerated and exacerbated neurogenic atrophy. Conventional Gadd45a-null mice showed no difference in baseline gastrocnemius mass compared to wild type and heterozygous littermates (A). However, denervated gastrocnemius from Gadd45a-null mice showed significantly accelerated and exacerbated atrophy compared to both wild type and heterozygous littermates (B-C, ** $P < 0.01$, *** $P < 0.001$, scale bar 5 mm). This was reflected at the level of individual myofibers, with both type IIa and type IIb myofibers from Gadd45a-null mice showing significantly greater reduction in mean minimum Feret diameter compared to wild type littermates (D-O, scale bar 100 µm).
**Figure 3.** Denervation induces *Gadd45a* expression specifically in skeletal muscle myocytes. Skeletal muscle-specific deletion of *Gadd45a* (HSA-Cre:*Gadd45a*^flox^) essentially abolished its induction in denervated gastrocnemius compared to *Gadd45a*^flox^ and wild type littermates (A, B). Accordingly, a target corresponding to endogenous V5 epitope-tagged GADD45A is detected in denervated gastrocnemius from *Gadd45a*^flox^ mice (but not contralateral intact muscle), but is abolished in denervated HSA-Cre:*Gadd45a*^flox^ gastrocnemius. Baseline gastrocnemius and soleus muscle mass from wild type, heterozygous, *Gadd45a*^flox^, HSA-Cre:*Gadd45a*^flox^ (muscle-specific *Gadd45a* deletion), and CMV-Cre:*Gadd45a*^flox^ (ubiquitous *Gadd45a* deletion) does not significantly differ (D-H, N; soleus muscle shown, scale bar 300 µm). However, at 14 days post-denervation, gastrocnemius and soleus muscle from HSA-Cre:*Gadd45a*^flox^ mice shows significantly greater loss of mass compared to wild type, heterozygous, and *Gadd45a*^flox^ genotypes (I-M, O). The degree of gastrocnemius and soleus mass loss in CMV-Cre:*Gadd45a*^flox^ mice does not significantly differ from HSA-Cre:*Gadd45a*^flox^ mice, consistent with the inference that the protective effect of *Gadd45a* induction is mediated entirely through its expression in skeletal muscle myocytes.
**Contralateral Intact**

- **A**: HSA-Cre:Gadd45a<sup>ALL</sup>
- **B**: Gadd45a<sup>ALL</sup> (HSA-Cre positive)
- **C**: Gadd45a<sup>ALL</sup> (HSA-Cre negative)
- **D**: CMV-Cre:Gadd45a<sup>ALL</sup>

**Denervated (14 days)**

- **E**: HSA-Cre:Gadd45a<sup>ALL</sup>
- **F**: Gadd45a<sup>ALL</sup> (HSA-Cre positive)
- **G**: Gadd45a<sup>ALL</sup> (HSA-Cre negative)
- **H**: CMV-Cre:Gadd45a<sup>ALL</sup>

**K**

- **Contralateral Intact**
  - **type Ila**
  - **type I**
  - **non-Vila**

**L**

- **Denervated (14 days)**
  - **type Ila**
  - **type I**
  - **non-Vila**

**M**

- **Contralateral Intact**
  - **type Ila**
  - **type I**
  - **non-Vila**

**Denervated (14 days)**

- **type Ila**
- **type I**
- **non-type Ila**

**Minimum Fiber Diameter (μm)**

- **Contralateral Intact**
  - **Gadd45a<sup>ALL</sup>**
  - **HSA-Cre:Gadd45a<sup>ALL</sup>**
  - **Gadd45a<sup>ALL</sup>**

- **Denervated (14 days)**
  - **Gadd45a<sup>ALL</sup>**
  - **HSA-Cre:Gadd45a<sup>ALL</sup>**
  - **Gadd45a<sup>ALL</sup>**

**Proportion of total myofibers**
Figure 4. Absence of Gadd45a induction in skeletal muscle myocytes results in increased atrophy of multiple fiber types and loss of fiber type identity. Baseline minimum Feret diameters of type I, type IIa, and non-I/IIa myofibers do not differ among wild type, heterozygous, Gadd45a\textsuperscript{flox}, HSA-Cre:Gadd45a\textsuperscript{flox} (muscle-specific Gadd45a deletion), and CMV-Cre:Gadd45a\textsuperscript{flox} (ubiquitous Gadd45a deletion) genotypes (A-E, K, scale bar 100 µm). Type I, type IIa, and non-I/IIa myofibers from denervated soleus of HSA-Cre:Gadd45a\textsuperscript{flox} and CMV-Cre:Gadd45a\textsuperscript{flox} mice all show significantly reduced minimum Feret diameters by 14 days post-denervation compared to all other genotypes (F-J, L). In addition, denervated soleus from HSA-Cre:Gadd45a\textsuperscript{flox} and CMV-Cre:Gadd45a\textsuperscript{flox} genotypes shows a significant increase in the proportion of non-I/IIa myofibers, with prominent loss of Myh2-expressing fibers (type IIa), suggesting accelerated loss of myofiber identity in the absence of GADD45A expression.
Supplementary Figure. Generation of a conditional, V5 epitope-tagged Gadd45a allele. We used CRISPR-Cas9 technology to introduce loxP sites around the first three coding exons of murine Gadd45a, in addition to a N-terminal V5 epitope tag to monitor GADD45A expression prior to Cre recombination (A). The expected genomic modifications were confirmed in founder lines by PCR and Sanger sequencing (B, C). A V5 epitope-containing target of the expected molecular weight was detected in denervated gastrocnemius from the founder lines, but not in contralateral intact gastrocnemius or wild type denervated and intact gastrocnemius, suggesting appropriate induction of V5 epitope-tagged GADD45A in Gadd45a\textsuperscript{flo} lines (D).