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HDAC4 promotes Pax7-dependent satellite cell activation and muscle regeneration

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Abstract

During muscle regeneration, the transcription factor Pax7 stimulates the differentiation of satellite cells (SCs) toward the muscle lineage but restricts adipogenesis. Here, we identify HDAC4 as a regulator of Pax7-dependent muscle regeneration. In HDAC4-deficient SCs, the expression of Pax7 and its target genes is reduced. We identify HDAC4-regulated *Lix1* as a Pax7 target gene required for SC proliferation. HDAC4 inactivation leads to defective SC proliferation, muscle regeneration, and aberrant lipid accumulation. Further, expression of the brown adipose master regulator Prdm16 and its inhibitory microRNA-133 are also deregulated. Thus, HDAC4 is a novel regulator of Pax7-dependent SC proliferation and potentially fate determination in regenerating muscle.

Keywords HDAC4; muscle regeneration; Pax7

Subject Categories Development & Differentiation; Chromatin, Epigenetics, Genomics & Functional Genomics

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Introduction

Skeletal muscle fulfills diverse functional demands and manages mechanical stress by modifying contractile and metabolic properties of myofibers and replacing those damaged via regenerative adult stem cells. The on-demand and activity-dependent muscle remodeling is achieved by elaborate reprogramming of muscle gene transcription [1]. In myofibers, histone deacetylase 4 (HDAC4) is a critical factor that connects neural activity to the muscle transcriptional programs. Inactivation of HDAC4 suppresses denervation-induced muscle atrophy and synaptic gene expression while increases re-innervation [2–5]. These findings highlight a central regulatory role of HDAC4 in activity-dependent muscle remodeling.

Muscle regeneration triggered by myofiber damage is another key adaptive program required for maintaining muscle integrity. Satellite cells (SCs), the principal muscle stem cells, are essential for damage-induced muscle regeneration [6,7]. The paired box transcription factor Pax7 is the master regulator of SCs [8]. Genetic ablation of Pax7 leads to a loss of SC pool and severely impaired muscle regeneration [9–11]. Regeneration failure in *Pax7* knockout (KO) mice is also accompanied by accumulation of lipids in muscle [9]. Intriguingly, SCs have been shown to potentially develop into non-muscle lineages [12]. Recent studies reveal a shared lineage of muscle and brown adipose tissue [13] and SCs have the potential to differentiate into brown adipocytes [14]. In this program, microRNA-133 (miR-133), which targets and inhibits the master regulator of brown adipose tissue Prdm16, restricts SCs toward the muscle lineage [14]. How Pax7 regulates SC activation and fate commitment during muscle regeneration remains incompletely understood. Here, we provide evidence that HDAC4 is a novel regulatory component in Pax7-dependent SC expansion and fate determination critical for adult muscle regeneration.

Results and Discussion

HDAC4 is required for satellite cell expansion in damaged muscle

We previously observed in muscle biopsy from an ALS patient that HDAC4 is localized not only to myofiber nuclei but also centrally located nuclei, a marker for newly regenerating fibers [15]. Moreover, expression of HDAC4, similar to Pax7, progressively increased in tibialis anterior (TA) muscles damaged by cardiotoxin (CTX) (Fig 1A and B). These observations indicate that HDAC4 might be involved in muscle regeneration. To investigate the possibility, we generated satellite cell (SC)-specific *HDAC4* knockout (KO) mice by crossing $HDAC4_{Lox}$ mice with a tamoxifen (Tmx)-inducible Cre^{ERT2} transgene that was placed at the endogenous Pax7 locus (Pax7-Cre^{ERT2}). $HDAC4_{Lox}$;Pax7-Cre^{ERT2} and control $HDAC4_{Lox}$ mice were treated with Tmx to activate Cre-mediated excision of HDAC4 in SCs [16], followed by CTX injection in TA and gastrocnemius (GA) muscles to activate SCs and muscle regeneration. The analyses of SCs purified by fluorescence-activated cell sorting (FACS) showed the efficiency of *HDAC4* KO by this method is approximately 60–80% (Supplementary Fig S1A–C).

During FACS analyses, we noted that the number of activated SCs was significantly reduced in *HDAC4* KO mice, whereas non-SC

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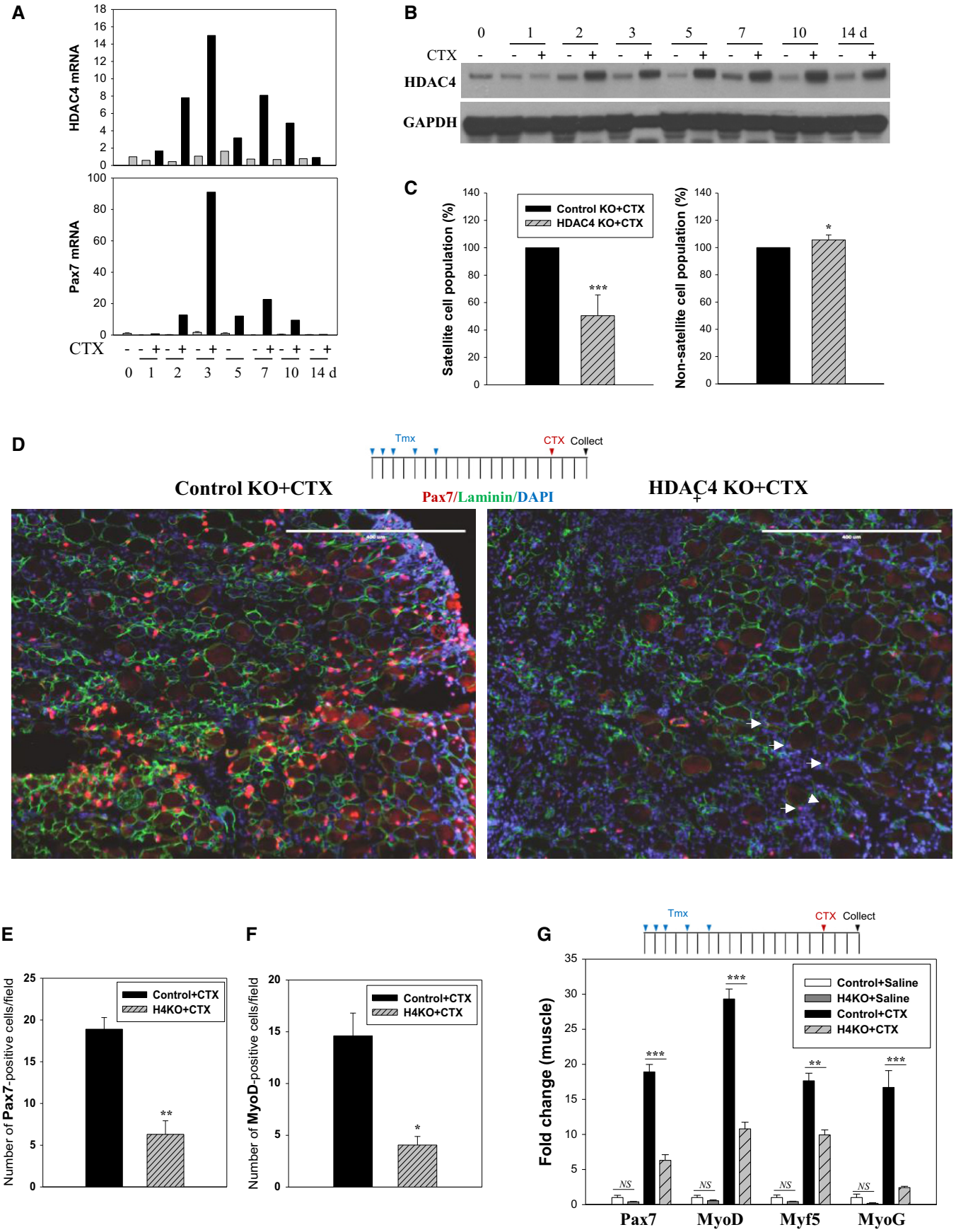

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Figure 1. HDAC4 is required for SC expansion in damaged muscle.

A Expression of *HDAC4* mRNA at different time points in damaged muscle. TA muscles in 8-week-old C57BL/6 male mice (eight mice) were untreated (0 day), treated with saline (left leg) or cardiotoxin (CTX) (right leg) and muscles were harvested at indicated time points. Columns, mean of PCR duplicates. $n = 1$ for each time point.

B Expression of HDAC4 protein in damaged muscle. TA muscles in 8-week-old C57BL/6 male mice (8 mice) were untreated (0 day), treated with saline (left leg) or CTX (right leg) and muscles were harvested at indicated time points. $n = 1$ for each time point.

C Effect of SC-specific *HDAC4* KO on populations of activated SCs by FACS analysis. TA and GA muscles in mice treated with tamoxifen (Tmx) were injured by CTX treatment for 2–3 days. Columns, mean; bars, SD of nine (SCs) or four (non-zs) independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ versus control KO+CTX (unpaired Student's *t*-test).

D–F Effects of *HDAC4* KO on the number of activated SCs expressing Pax7 or MyoD. TA muscles in 7-week-old male mice were stained with indicated antibodies. Note that SCs expressing low levels of Pax7 were also counted as positive (white arrow). Field means a grid (250 × 250 μm). Approximately 40 grids per muscle were counted. Scale bar: 400 μm. Columns, mean; bars, SEM. $n = 3$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control KO+CTX (unpaired Student's *t*-test).

G mRNA expression of genes in regenerating muscle. TA muscles in 7-week-old male mice were used. Columns, mean; bars, SEM. $n = 4$ for each group. $^{**}P < 0.01$, $^{***}P < 0.001$; *NS*, not significant, $P > 0.05$ (unpaired Student's *t*-test).

cells were slightly increased (Fig 1C), suggesting that SC expansion might be impaired. To determine whether HDAC4 is required for injury-stimulated SC activation, we quantified Pax7-positive SCs in TA muscles treated with CTX. Immunostaining showed that the frequency of Pax7-positive cells as well as the staining intensity of Pax7 was much lower in *HDAC4* KO compared to CTX-treated control muscles (Fig 1D and E, and Supplementary Fig S1D). MyoD-positive activated SCs were similarly reduced in *HDAC4* KO muscle (Fig 1F). In contrast, numbers of quiescent SCs in uninjured muscle were not significantly different in control and *HDAC4* KO muscle, indicating that HDAC4 regulates SC expansion during regeneration (Supplementary Fig S1E and F). Supporting this conclusion, RNA analyses revealed that expression of genes required for SC activation and muscle differentiation, including Pax7, MyoD, Myf5, and Myogenin (MyoG), were all markedly reduced in damaged *HDAC4* KO muscles (Fig 1G). A modest reduction of these genes was detected in saline-treated *HDAC4* KO muscle, likely due to mild muscle injury caused by the injection procedure, which generates local activation of SCs. Further, intraperitoneal injection of Tmx alone did not significantly reduce the expression of Pax7 and myogenic factors in uninjured muscle of *HDAC4* KO mice (Supplementary Fig S2A). Together, these results show that HDAC4 inactivation impairs SC expansion induced by injury.

HDAC4 regulates the expression of Pax7 and its target genes in activated SCs

We next investigated whether HDAC4 regulates gene transcription program in SCs. SCs were isolated from CTX-injured control and *HDAC4* SC KO muscles, cultured in growth medium (GM) and subjected to RNA analysis. We found that the expression of the SC-master transcription factor, Pax7, was significantly reduced in *HDAC4* KO SCs (Fig 2A). Immunoblot analysis confirmed its reduction in *HDAC4* KO SCs (Fig 2B). Supporting a Pax7 deficiency, the expression of Myf5, a well-established Pax7 target, was also reduced (Fig 2A). This reduction of Pax7 and Myf5 is specifically caused by the loss of HDAC4, as their expression was not changed in activated SCs or damaged TA muscles isolated from *HDAC4*$^{lox/lox}$ and *HDAC4*$^{lox/lox}$; *Pax7-Cre*^{ERT2} mice without Tmx treatment (Supplementary Fig S2B and C). Importantly, the expression of MyoD was not affected in *HDAC4* KO SCs (Fig 2A), consistent with a previous report that MyoD is not regulated by Pax7 [17]. MyoG, a downstream effector of muscle differentiation, was modestly decreased in *HDAC4* KO SCs. To further determine the role of HDAC4 in Pax7-dependent gene transcription, we assessed a set of Pax7 target genes previously identified [17,18]. As shown in Fig 2A, Pax7 target genes including *Lix1, Mest, PlagL1,* and *Cipar1* were all reduced in *HDAC4* KO SCs. In contrast, ID2 and ID3 were unchanged in *HDAC4* KO SCs. Conversely, *Igfbp2,* a gene negatively regulated by Pax7 [17], was increased in *HDAC4* KO SCs. We further analyzed Pax7 target gene expression in regenerative TA muscle. In addition to *Myf5* (Fig 1G), *Lix1* and *Mest* were significantly reduced in regenerative *HDAC4* KO muscle, whereas other Pax7 targets were unaffected (Fig 2C). Together, these results show that HDAC4 positively regulates Pax7 and a subset of Pax7 target genes in activated SCs.

HDAC4 regulates SC proliferation

The reduction of Pax7-positive cells in injured *HDAC4* KO muscle indicates a defect in SC expansion. To assess whether HDAC4 regulates SC proliferation, SCs were purified from CTX-treated control and *HDAC4* SC KO muscle, cultured in GM, and pulse-labeled with BrdU. *HDAC4* KO SCs showed greater than twofold reduction in proliferation (Fig 3A and B). Apoptosis, as indicated by active caspase-3, was similar between control and *HDAC4* KO SCs (Fig 3C). To determine whether HDAC4 regulates differentiation, purified SCs were cultured in differentiation medium (DM) and assessed by the expression of myosin heavy chain. Both control and KO SCs underwent efficient differentiation although quantification revealed a modest but statistically significant reduction in *HDAC4* KO SCs (Supplementary Fig S3A and B). Collectively, these results indicate that HDAC4 mainly regulates SC proliferation.

Lix1 is required for Pax7-dependent SC proliferation

Although ID3 was proposed as a Pax7 target required for SC proliferation [19], its expression in SCs or regenerative muscle was not affected by HDAC4 inactivation (Fig 2A and C). Our analysis of a limited set of Pax7 target genes has identified *Lix1* (limb expression 1) and *Mest* (mesoderm-specific transcript) as two genes whose expressions were strongly suppressed in both *HDAC4* KO SCs and muscles (Fig 2A and C). To test the possibility that they might be the factors that promote SC proliferation, we knocked down *Lix1* or *Mest* in SCs isolated from CTX-activated muscle. These siRNAs effectively suppressed *Lix1* or *Mest* expression (Supplementary Fig S3C and D). We found that *Lix1* KD significantly reduced SC proliferation without

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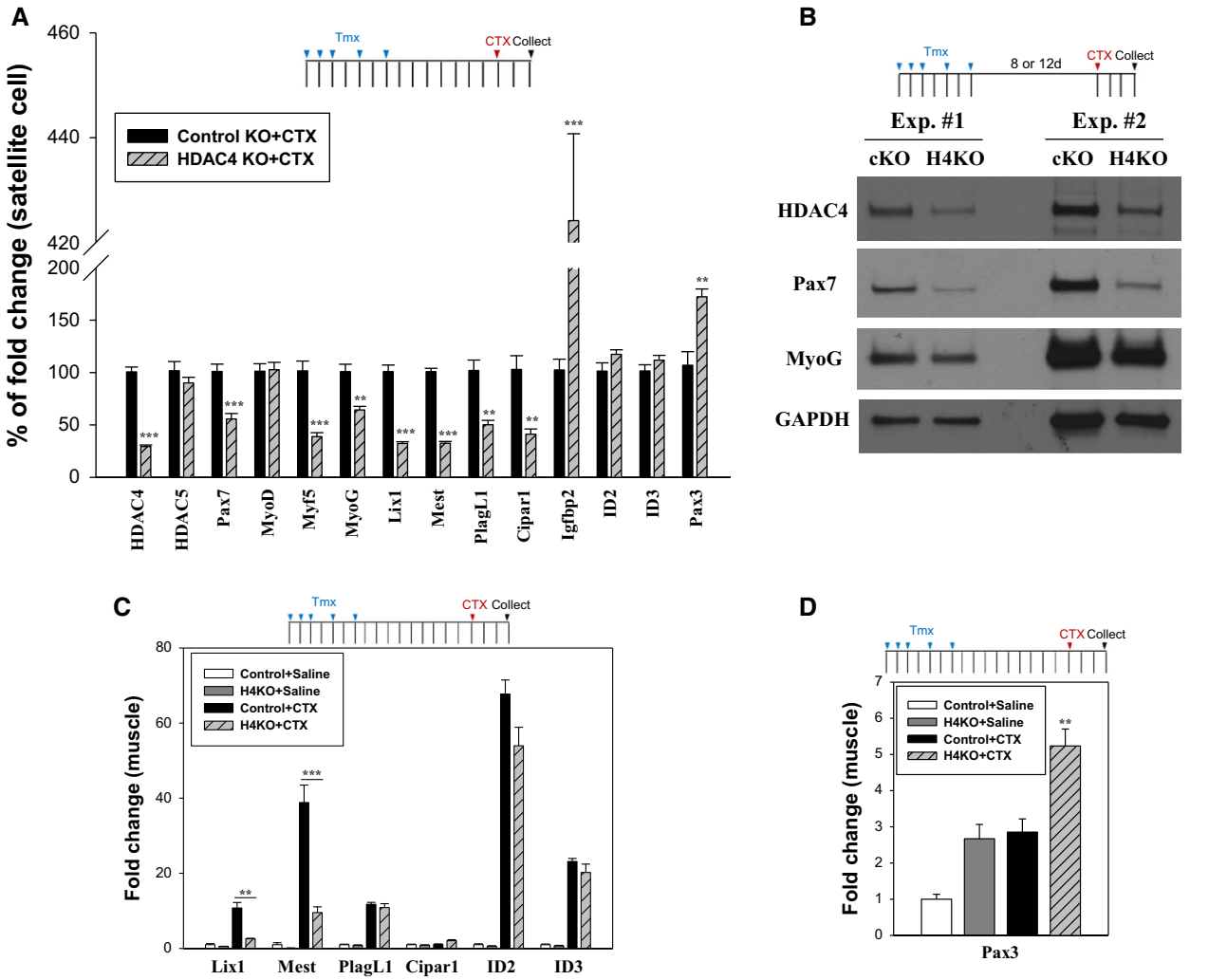

Figure 2. HDAC4 regulates the expression of *Pax7* and associated genes in SCs.

A mRNA expression of genes in SCs. Activated SCs isolated from 15-week-old female mice were cultured for 3 days in growth medium (GM). Medium was changed at 2 days after plating. Columns, mean; bars, SEM. $n = 5$ for each group. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control KO+CTX (unpaired Student's *t*-test).

B Protein expression of HDAC4, Pax7, and MyoG in SCs. Activated SCs isolated from muscles in 13-week-old female (Exp. no. 1) or 10-week-old male (Exp. no. 2) mice were cultured for 2 days in GM.

C, D mRNA expression of potential Pax7-target genes and *Pax3* in damaged muscle. cDNAs used in Fig 1G were used. Columns, mean; bars, SEM. $n = 4$ for each group. $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired Student's *t*-test).

inducing apoptosis (Fig 3D–F), whereas *Mest* KD had no effect (Supplementary Fig S3E). Expression of Pax7 and myogenic factors were unaffected by *Lix1* KD (Supplementary Fig S3C). These results indicate that *Lix1* is a Pax7 target gene required for efficient SC proliferation.

HDAC4 is required for efficient damage-induced muscle regeneration

We next determined whether muscle regeneration is impaired in *HDAC4* SC KO mice. At 8 days post-CTX treatment, control muscles underwent proper regeneration as illustrated by relatively uniform myofibers that are positive for embryonic myosin (eMyHC) and centrally localized nuclei (Fig 4A). In *HDAC4* SC KO muscle, the regenerating fibers appeared to be smaller and interspersed with infiltrated cells. Quantification using eMyHC staining confirmed a reduction of regenerative fibers in the *HDAC4* KO mice (Fig 4B). At 5 weeks post-CTX treatment, *HDAC4* KO muscles showed reduced muscle size, whereas the overall muscle structure appeared to be normal (Supplementary Fig S4A), indicating delayed muscle regeneration. Supporting these results, regeneration-associated muscle hypertrophy was significantly inhibited in *HDAC4* KO TA muscle (Fig 4C). In *Pax7* SC-specific KO animals, the muscle regeneration defect becomes more apparent when Cre-mediated recombination (driven by Tmx-inducible Cre) was sustained by continuous administration of Tmx and muscle was injured by repeated cycles of CTX injection [9,10]. Adapting this experimental condition, we found that muscle regeneration was indeed more profoundly impaired in *HDAC4* SC KO muscles, as indicated by a significant reduction in muscle size (Fig 4D). In SC-specific *Pax7* KO mice, regeneration

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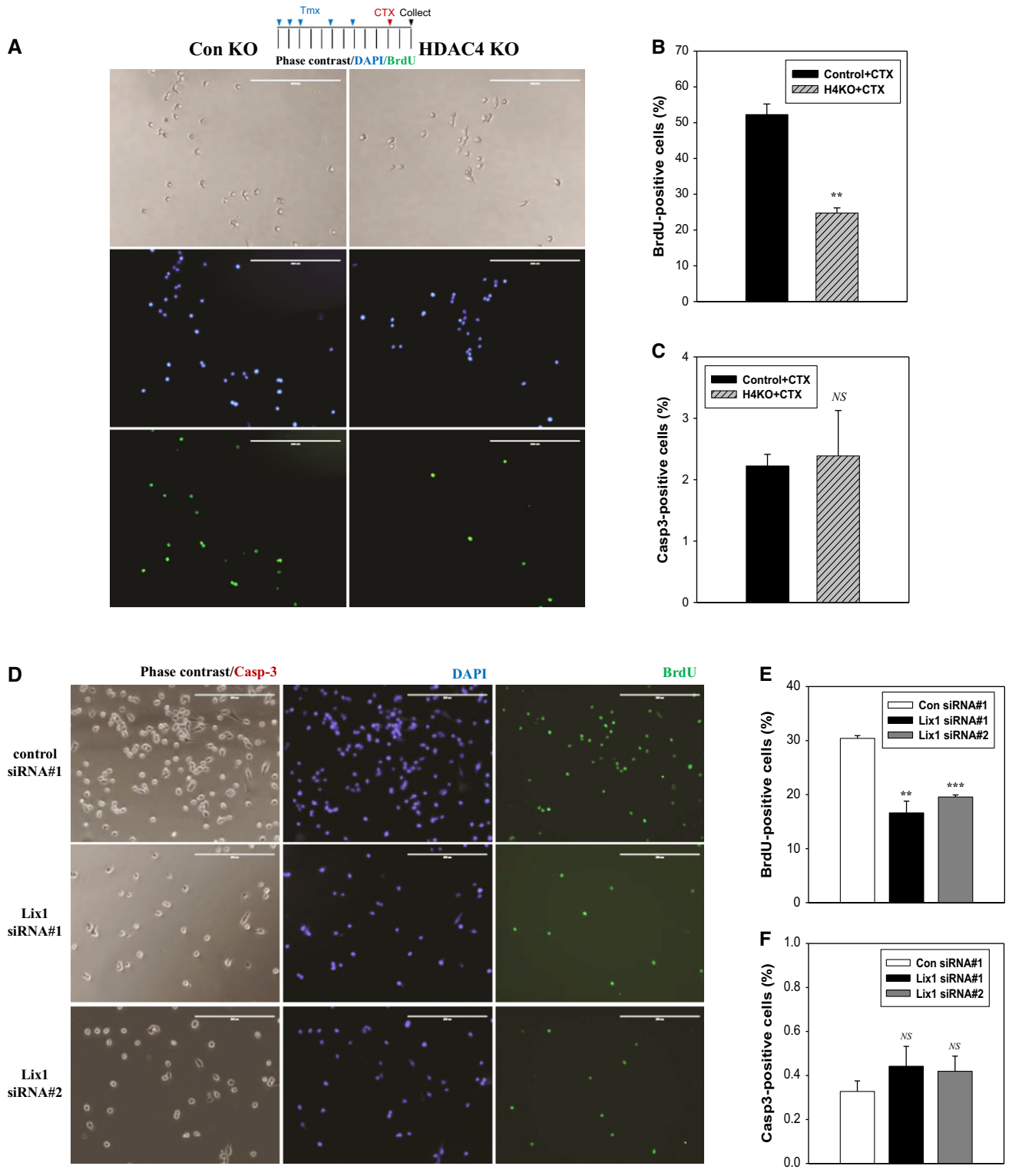

Figure 3. HDAC4 and HDAC4-regulated Lix1 regulate SC proliferation.

A–C Effects of *HDAC4* KO on proliferation and apoptosis in SCs. Isolated SCs from 10-week-old male mice were cultured for 2 days in GM. Proliferation and apoptosis were monitored by staining cells with BrdU and caspase-3 antibodies, respectively. Approximately, 300 nuclei were measured per muscle. Scale bar: 200 μm. Columns, mean; bars, SEM. $n = 3$ for each group. $^{**}P < 0.01$; *NS*, not significant, $P > 0.05$ (unpaired Student's *t*-test).

D–F Effects of *Lix1* knockdown (KD) on proliferation and apoptosis in SCs. Pooled SCs from muscles treated with CTX for 2 days in 8-week-old C57BL/6 male mice (10 mice) were transfected with Lix1 siRNAs and cultured for 2 days in GM. Medium was changed at 1 day after plating. Approximately 1,500 nuclei were counted per well. Scale bar: 200 μm. Columns, mean; bars, SEM. $n = 3$ for triplicate wells. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control siRNA no. 1 (unpaired Student's *t*-test).

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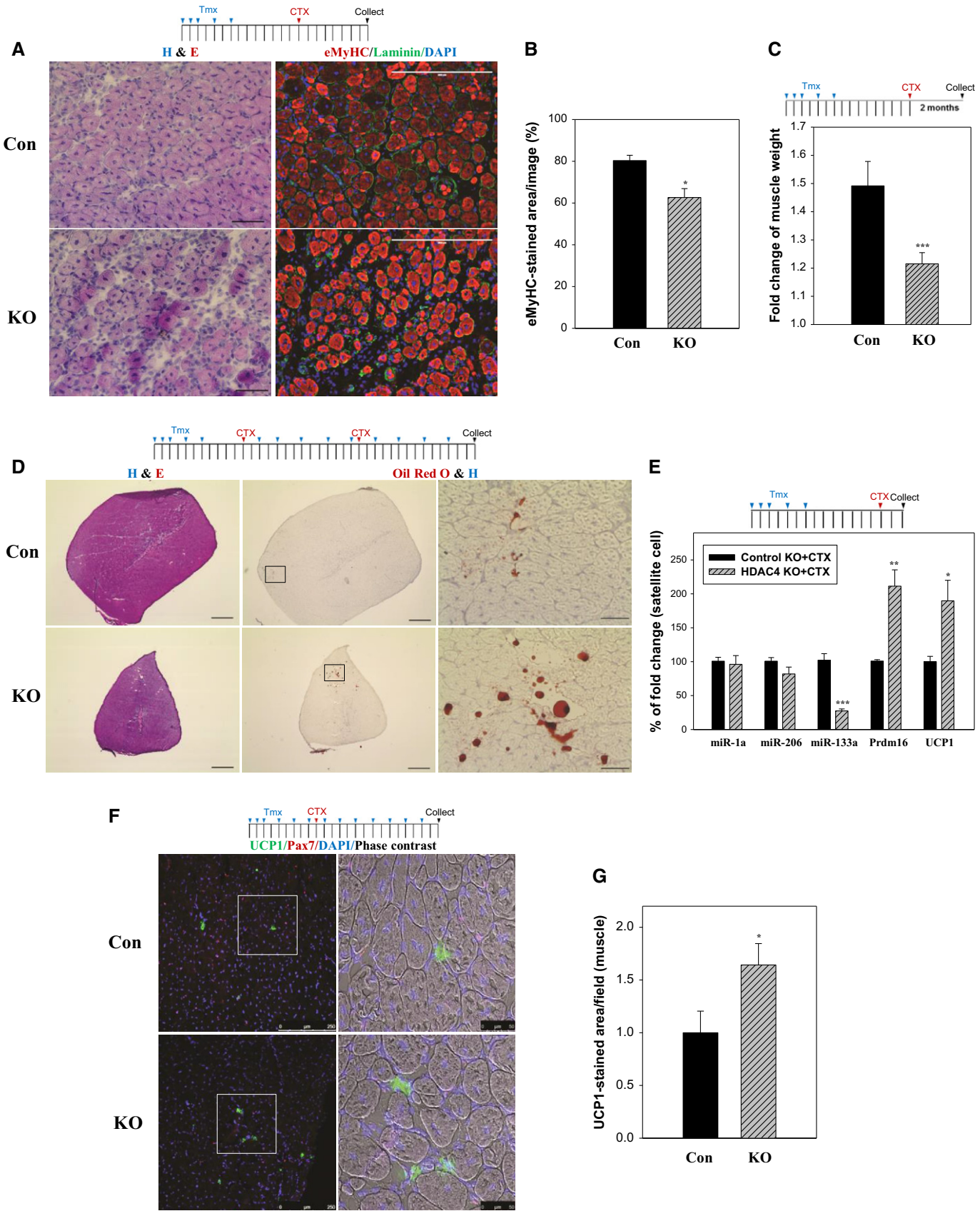

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Figure 4. HDAC4 inactivation impairs muscle regeneration and increases adipocytes in regenerating muscle.

A, B Defective regeneration in *HDAC4* SC KO damaged muscle. TA muscles in 10-week-old male mice were stained with H&E for histology or with embryonic myosin (eMyHC) to mark regenerating fibers. Scale bar: 50 (left) and 200 μm (right). Columns, mean; bars, SEM. $n = 3$ for each group. $^{*}P < 0.05$, $^{***}P < 0.001$ versus control KO (unpaired Student's *t*-test).

C Reduced hypertrophy in *HDAC4* SC KO muscles. TA muscles in 9- to 10-week-old mice were used. Fold change is expressed as CTX-treated muscle/saline-treated contralateral muscle in each mouse. Columns, mean; bars, SD. $n = 4$ for control KO and $n = 5$ for *HDAC4* KO. $^{***}P < 0.001$ versus control KO (unpaired Student's *t*-test).

D Delayed muscle regeneration and increased lipid accumulation in SC-specific *HDAC4* KO muscle. TA muscles in 8-week-old male mice were stained with H&E or with Oil Red O and H to mark lipid and nuclei, respectively. Scale bar: 500 (left and middle) and 50 μm (right).

E RNA expression of miR-1a, miR-133a, miR-206, *Prdm16* and *UCP1* in *HDAC4* KO SCs. Total RNAs prepared in Fig 2A were used. Columns, mean; bars, SEM. $n = 5$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control KO+CTX (unpaired Student's *t*-test).

F, G Increased brown adipogenesis in *HDAC4* SC KO regenerating muscle. TA muscles in 12-week-old male mice were stained with indicated antibodies. Scale bar: 250 (left) and 50 μm (right). Columns, mean; bars, SEM. $n = 4$ for control KO and $n = 3$ for HDAC4 KO. $^{*}P < 0.05$ versus control KO+CTX (unpaired Student's *t*-test).

failure is accompanied by accumulation of lipids in muscle [9]. Analysis of *HDAC4* SC KO muscles also revealed aberrant increase in neutral lipid stained by Oil Red O (Fig 4D and Supplementary Fig S4B). Further analysis showed that Oil Red O-stained cells were located between regenerating myofibers (Supplementary Fig S4C), indicating an expansion of interstitial adipocytes in *HDAC4* SC KO muscle. Collectively, these data show that *HDAC4* SC KO impairs muscle regeneration.

HDAC4 inactivation increases brown adipocytes in regenerative muscle and deregulates miR-133

SC has the potential to develop into brown adipose tissue, a fate that is regulated by miR-133 [14]. miR-133 targets and inhibits Prdm16, the master transcription factor for brown fat adipogenesis [14,20,21]. Inhibition of miR-133 can reprogram SCs toward the brown adipose lineage [14]. To investigate whether HDAC4 regulates miR-133 expression, we assessed mature miR-133a and two other muscle microRNAs, miR-1a and miR-206, both of which repress Pax7 expression and SC proliferation [22]. As shown in Fig 4E, expression of miR-133a, but not miR-1a or miR-206, was reduced in *HDAC4* KO SCs. Consistent with a reduction in miR-133a, a significant induction of Prdm16 and a brown fat marker, UCP1, were observed in *HDAC4* KO SCs. Supporting these findings, UCP1-positive cells were increased in *HDAC4* KO muscle (Fig 4F and G). Collectively, these results indicate that HDAC4 restricts the expression of the miR-133–Prdm16 axis important for the brown adipocyte lineage determination.

In this study, we provide evidence that HDAC4 is required for efficient muscle regeneration induced by injury. The characterization of *HDAC4* KO has revealed a significant reduction in Pax7 expression in SCs (Fig 2A and B). Indeed, subsets of Pax7 target genes were significantly mis-regulated in *HDAC4* KO SCs (Fig 2A). Surprisingly, siRNA knockdown of *HDAC4* in purified and activated SCs *in vitro* did not cause a reduction in Pax7 expression (Supplementary Fig S5A). These results indicate that regulation of Pax7 by HDAC4 is indirect and requires an intact muscle microenvironment *in vivo*. Further supporting an indirect mechanism, *HDAC4* knockdown in C2C12 myoblasts did not affect acetyl-histone H4 (K8) levels in the promoter regions of *Pax7* or *Lix1* (Supplementary Fig S5B). It is of interest to note that HDAC4 can regulate cytokine production [2], which might influence the inflammatory program important for muscle regeneration [23]. How HDAC4 in SCs communicates with other components of the muscle regeneration program to affect Pax7 expression awaits further studies.

The differential requirement of Pax7 target genes on HDAC4 could reflect the up-regulation of Pax3 in *HDAC4* KO SCs and regenerating TA muscle (Fig 2A and D). This finding indicates that HDAC4 selectively regulates Pax7, but not Pax3, expression in SCs. Pax3 is highly related to Pax7 and can regulate similar sets of genes [17,18]. Thus, the elevated Pax3 might partially compensate for Pax7 and maintain the expression of common targets. The differential dependence of Pax7 target genes on HDAC4 led us to focus on *Lix1* and *Mest*, which are down-regulated in both *HDAC4* KO SCs and damaged muscles, as candidates that mediate Pax7-dependent SC proliferation. We found that knockdown of *Lix1*, but not *Mest*, suppressed the proliferation of activated SCs (Fig 3E and Supplementary Fig S3E). This finding suggests that Pax7-dependent SC proliferation involves Lix1. The biochemical function of Lix1 is largely unknown. Its *Drosophila* orthologous, Lowfat (Lft), is a component of the FAT-signaling pathway that regulates planar cell polarity (PCP) [24]. Interestingly, PCP plays an important role in SC expansion [25]. Whether Lix1 and HDAC4 regulate SC proliferation via the PCP pathway requires further investigation.

Supporting its role as a positive regulator of Pax7, *HDAC4* KO led to defects in SC proliferation and muscle regeneration. Similar to *Pax7* KO mice, regenerative muscles in *HDAC4* KO mice also showed aberrant lipid accumulation (Fig 4D). Interestingly, we found that *HDAC4* KO in SCs reduces expression of miR-133a, a negative regulator of the brown fat transcription factor Prdm16. Consistent with this finding, the expression of Prdm16 and a marker for brown adipocytes, UCP1, were both elevated in *HDAC4* KO SCs (Fig 4E). An increase in UCP1-positive cells was also observed in *HDAC4* KO regenerating muscle (Fig 4F and G). These findings are consistent with a previous report that miR-133 suppresses SC differentiation to brown adipocytes [14]. Our study therefore suggests that HDAC4 is required for maintaining miR-133a in SCs and thereby inhibits SC differentiation toward the brown adipose lineage. Interestingly, miR-133 over-expression can increase Pax7 levels in primary preadipocytes [14], indicating that miR-133 positively regulates Pax7 expression. Accordingly, HDAC4 could increase Pax7 expression in SCs via miR-133. Given the instructive role of miR-133 in brown adipose determination [14,20,21], this scenario implies a regulatory role of HDAC4 in SC fate determination. The physiological relevance of SC plasticity is not known. By directing some activated SCs toward an adipose lineage, this arrangement could potentially provide metabolic advantages to regenerative muscle. Since HDAC4 is regulated by multiple physiological and pathological conditions, its activity in SCs might influence the composition of muscle fibers and adipose tissue in regenerative muscle. Further

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studies are required to determine how HDAC4 regulates miR-133 expression and its impact on SC fate determination in response to patho-physiological conditions.

Materials and Methods

Mouse procedures

$HDAC4_{Lox}$ mice were provided by Dr. E. Olson [5]. $Pax7^{CreERT2}$ mice were purchased from Jackson Laboratory (B6;129-*Pax7*$^{tm2.1(cre/ERT2)Fan}$/J). Wild-type C57BL/6 mice were also from Jackson Laboratory. Conditional strains contain *HDAC4*^{Lox}; *Pax7-Cre*^{ERT2} (KO) or $HDAC4_{Lox}$ (control). Tamoxifen (Tmx; Sigma; T5648) treatment for Cre recombinase activation was performed by intraperitoneal injection to both control and *HDAC4* KO mice (200 μl of 10 mg/ml in corn oil). Mouse gender, age, numbers, and Tmx treatment are indicated in each figure legend. To induce muscle degeneration/regeneration, mice were anesthetized and injected with 50 μl of 10 μM cardiotoxin (Sigma; C9759) in one site of TA muscle or in two sites of GA muscle. All mice were housed at the Duke University mouse facilities in accordance with the IACUC.

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions
MCC designed and performed experiments and wrote the manuscript; SR conducted mouse genotyping, immunostaining, and quantification of data. RH performed ChIP experiments and analyzed data. BW helped with the animal works. MK provided technical assistances and edited the manuscript. CMF provided critical reagents, discussions and edited the manuscript. TPY supervised the project and edited the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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