The transcription factor Slug represses p16\(^{\text{ink4a}}\) and regulates murine muscle stem cell aging

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Activation of the p16\(^{\text{ink4a}}\)-associated senescence pathway during aging breaks muscle homeostasis and causes degenerative muscle disease by irreversibly dampening satellite cell (SC) self-renewal capacity. Here, we report that the zinc-finger transcription factor Slug is highly expressed in quiescent SCs of mice and functions as a direct transcriptional repressor of p16\(^{\text{ink4a}}\). Loss of Slug promotes derepression of p16\(^{\text{ink4a}}\) in SCs and accelerates the entry of SCs into a fully senescent state upon damage-induced stress. p16\(^{\text{ink4a}}\) depletion partially rescues defects in Slug-deficient SCs. Furthermore, reduced Slug expression is accompanied by p16\(^{\text{ink4a}}\) accumulation in aged SCs. Slug overexpression ameliorates aged muscle regeneration by enhancing SC self-renewal through active repression of p16\(^{\text{ink4a}}\) transcription. Our results identify a cell-autonomous mechanism underlying functional defects of SCs at advanced age. As p16\(^{\text{ink4a}}\) dysregulation is the chief cause for regenerative defects of human geriatric SCs, these findings highlight Slug as a potential therapeutic target for aging-associated degenerative muscle disease.
Skeletal muscle is a homeostatic tissue that is capable of making up turnover caused by daily wear-and-tear as well as regenerating upon damage. However, such homeostasis declines with aging. Sarcopenia, featured with progressive loss of muscle mass and strength, is one of the most common health issues for individuals at advanced age. Therefore, determining the mechanisms that underlie skeletal muscle aging becomes essential for clinical therapy for the degenerative muscle disease of this kind.

To date, the key factors determining aging-associated decline of muscle regenerative capacity has remained open to debate. It is believed that proper muscle regeneration necessitates optimal extrinsic environmental supplies. Direct evidence supporting this viewpoint came from a whole muscle grafting experiment demonstrating that the regenerative ability of young and old muscles depended mainly on the age of the host. More recent findings of increasing fibroblast growth factor (FGF) signaling in aged muscle fiber, as well as the circulating protein growth differentiation factor 11 as a rejuvenating factor for aged skeletal muscle further highlighted the importance of local and systemic environment in muscle aging, respectively. In contrast, muscle stem cells (MuSCs) purified from aged mice show a substantial decline in the number of MuSCs that engraft and regenerate in recipient muscle, shedding light on the role of stem cell intrinsic changes in regulation of muscle aging. Indeed, restoring the disordered signaling pathway, such as p38 MAPK, in aged MuSCs rejuvenate MuSC function and muscle regeneration in old mice.

Regardless of extrinsic environment or cell-autonomous mechanisms, aging-associated changes lead to stem cell exhaustion in the elderly. Pax7-expressing satellite cells (SCs) function as a necessary stem cell population responsible for growth, maintenance, and regeneration of skeletal muscle. Cellular senescence is an important cause of stem cell exhaustion with aging in multiple tissues. Telomere shortening, nontelomeric DNA damage and derepression of the INK4/ARF locus are all able to induce senescence. Notably, derepression of p16Ink4a switches geriatric SCs from reversible quiescence into senescence, leading to incompetency of activation on muscle injury even in a youthful environment. However, those cell intrinsic components regulating p16Ink4a expression in SCs remain largely unknown.

In this study, we establish Slug, a member of zinc-finger transcription factor in the Slug/Snail superfamily, as a transcriptional repressor of p16Ink4a in SCs. Akin to mice with aging, loss of Slug endows adult SCs features of pre-senescence by largely inducing p16Ink4a expression, triggering apparent regenerative defects during serial muscle damage. Importantly, reduced Slug and elevated p16Ink4a expressions in SCs simultaneously occur with chronological aging. Restoration of Slug expression is capable of rejuvenating aged SC functions. Our results highlight Slug as a key target for aging-associated degenerative muscle disease.

**Results**

**Slug deletion causes a defect in muscle regeneration.** The transcription factor Slug is expressed in a variety of normal tissues in the adult mouse, indicating its important roles in development. In agreement with this notion, Slug knockout mice showed numerous abnormalities such as smaller body size and weight. Since skeletal muscle accounts for ~40% of adult body weight, we examined if the reduced body weight in Slug-deficient mice is due to a reduction of muscle mass. Hindlimb muscles of adult Slug mutant mice were reduced by 21–36% in weight. To some extent, the lost muscle mass was caused by reduction in myofiber size in Slug-deficient mice. However, when normalized to body weight, none of the relative weights of these muscles were affected by the absence of Slug. Changes in muscle mass may be resulted from changes in protein or cell turnover. The latter reflects the balance between myonuclear accretion and loss. Proliferation and fusion of SCs increases the number of myonuclei within the muscle fibers. Therefore, we determined the effect of Slug deficiency on MuSC maintenance. Unexpectedly, Slug-deficient mice had even slightly higher fraction of SCs in non-lineage cell sub-population than wild-types after a single injury. The total yield of SCs calculated as per milligram of harvested hindlimb muscle was also increased in Slug knockout mice. Such Slug ablation-induced increases in MuSC frequency and number were further confirmed by staining of Pax7+ nuclei on freshly prepared TA muscle cryosections.

SCs is essential for the maintenance and regeneration of skeletal muscle. Thus, we determined how Slug null-induced increase of SCs affects muscle regeneration upon injury. H&E stained showing that Slug-deficient muscles regenerated as well as the wild-types after a single injury. Strikingly, upon second injury, Slug-deficient muscles exhibited severely impaired regeneration when compared with wild-type counterparts. Collectively, these data demonstrated that Slug is essential for efficient muscle repair during continuous muscle regeneration, suggesting a key role of Slug in regulation of SC function.

To determine whether the impaired muscle regeneration in global Slug knockout mice is a SC-driven defect, we generated SC-specific Slug knockout mouse line using the Cre/loxP system. Unlike the global Slug knockout mice, Slugfl/fl:Pax7Cre+/+ (designated as SlugKO) mice showed no apparent differences with the control animals (Slugfl/fl:Pax7Cre+/+) in body size and weight. Furthermore, SCs from Ctrl but not SlugKO mice displayed positive-staining for Slug protein, indicating that Slug is efficiently deleted in SCs in SlugKO mice.

Next, we investigated the effect of SC-specific Slug deletion on the maintenance and regenerative capacity of SCs. Consistently, both the overall frequency and total yield of SCs calculated as per milligram of muscles were increased in SlugKO mice. Although individual myofiber diameters of the intact TA and cross-sectional area of TA on day 10 post single BaCl2 injury were not different between Ctrl and mutant littermates, more severely compromised muscle regeneration with a large increase of both necrotic fibers and fibrotic tissue was observed in SlugKO mice administered with double and triple muscle damages. The impaired secondary but not primary muscle regeneration was also detected in Slugfl/fl:Pax7CreER mice, in which Slug was deleted specifically in adult SCs by administering tamoxifen prior to muscle injury. Taken together, these results demonstrated that the presence of Slug in SCs is essential for SC-specific Slug loss impairs skeletal muscle regeneration.
for SC-driven skeletal muscle regeneration, and Slug regulates muscle regeneration via its SC-specific function.

**Slug-null SCs fail to replenish SC pool after activation.** Maintaining SC pool size is crucial for constant muscle turnover/injuries and ongoing repair. We showed that muscle regeneration after first injury was normal, indicating that Slug-deleted SCs in intact muscles were functional in terms of activation and differentiation upon injury. Indeed, primary Slug−/− SCs showed robust potential for differentiation upon **in vitro** induction and **in vivo** transplantation (Supplementary Fig. 4). However, the consequent regeneration became severely compromised in the absence of Slug (Figs. 1e, 2i and Supplementary Fig. 3e). These results prompted the question whether Slug-deficient SCs were capable of self-renewing and replenishing the stem cell pool after activation. SlugKO displayed a sharp decrease in SC number of regenerated TA muscle when compared with control mice (Fig. 3b, c). Injection of tractable adult SCs into pre-injured adult muscle followed with fluorescence-activated cell sorting (FACS) analysis provides a quantitative assay for SC self-renewal. By this assay, we analyzed the stem cell repopulation of donor-derived SCs (GFP+) as a fraction of the total SC population from the primary recipients (Fig. 3d). Because a small portion of SCs remain cycling 1 month after transplantation, we analyzed SCs with distinct immunophenotype (CD31−CD45−Sca1−Vcam 1+), which consist of both quiescent and activated MuSCs. Via this analysis, we found that the frequency of Slug−/− SCs was about three-fold lower than that of Slug+/+ SCs (Fig. 3e, f). Since the compromised self-renewing capability of SCs from Slug−/−GFP mice may be due to embryonic absence of Slug, we induced an acute Slug knockout in SCs from Slugfl/fl/Pax7-zsgreen mice by infecting with retrovirus expressing Cre recombinase (Supplementary Fig. 5a, b). Cre-expressing SCs yielded about five-fold less GFP+ fraction in the total SC population from the recipients compared to that of Ctrl virus-infected SCs (Supplementary Fig. 5c, d). Consistently, by zsgreen staining we identified considerably less Cre retrovirus-infected SCs in the SC niche, beneath the basa lamina and atop myofibers (p < 0.01 by student’s t-test (n.s., not significant). All these experiments were independently repeated three times with similar results. Data are presented as mean ± SEM. Also see Supplementary Fig. 1. Source data are provided as a Source Data file.
Fig. 2 SC-specific Loss of Slug Impairs Muscle Regeneration. **a** Slug expression in different muscle resident cells. Left, representative flow cytometric gating of SCs (CD31−CD45−Sca1+), pan-lymphocytes (LCs, CD45+), epithelial cells (ECs, CD31+), and fibro-adipogenic progenitors (FAPs, CD31−CD45−Sca1+) from freshly prepared skeletal muscle cells. Right, qPCR analysis of Slug expression. \(^{*}p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001\) by student’s \(t\)-test. **b** Quantification of Slug expression in undifferentiated and differentiated SCs. Left, representative images of SCs and myotubes. Scale bar, 100 \(\mu\)m. Right, qPCR analysis of Slug expression. \(^{*}p < 0.05, ^{***}p < 0.001\) by student’s \(t\)-test. QSC, quiescent satellite cell; ASC, activated satellite cells upon culture in growth medium for 3 days; MT, myotube. **c** Gene targeting strategy for generation of SC-specific Slug knockout mice. **d** Diagram of Slug-specific primer design for genotyping PCR. M, DNA marker; Ng, negative control for PCR. **e** Comparison of adult Slug\(^{fl/fl}\)Pax7\(^{Cre/+}\) (Slug\(^{cKO}\)) and Slug\(^{fl/+}\)Pax7\(^{Cre/+}\) (Ctrl). **f** Immunofluorescence staining of Slug in SCs of Slug\(^{cKO}\) and Ctrl mice (n = 3 mice). Scale bar, 100 \(\mu\)m. **g** Yield of SCs per mg of muscle from Ctrl and Slug\(^{cKO}\) mice (n = 3 mice for each genotype). \(^{*}p < 0.05\) by student’s \(t\)-test. **h** H&E staining of intact and injured TA muscles in Slug\(^{cKO}\) and Ctrl mice (n = 5 mice per group). For single injury, TA muscles were harvested at day 10 after BaCl\(_2\) injection. For consecutive injury, mice with primary injury were recovered for 1 month followed by a second BaCl\(_2\) injection at the same sites. TA muscles were harvested 10 days after each injury. Scale bar, 100 \(\mu\)m. The experiment was repeated independently for three times with similar results. **i** Quantiﬁcation of the myoﬁber CSA (\(\mu\)m\(^2\)) shown in h. \(^{*}p < 0.05\) by student’s \(t\)-test (n.s., not signiﬁcant). Data are shown as mean ± SEM of three independent replicates. Also see Supplementary Fig. 2 and 3. Source data are provided as a Source Data file.
that a small population of AraC-resistant, myofiber-associated Pax7+ cells arose following the first SC division in culture by self-renewing and behaved as quiescent SCs6,21. We treated myofibers with AraC from day 3–5 after isolation (Fig. 3i) and detected a lower number of surviving Pax7+Ki67− SC daughters from SlugcKO mice compared to those from Ctrl mice (Fig. 3j, k). Together, these results provide convincing evidence that Slug-deficient SCs have an intrinsic defect in self-renewal following muscle regeneration.

**Slug directly represses p16Ink4a transcription in SCs.** To explore intrinsic factors responsible for losing self-renewal capacity of activated SCs in Slug-deficient mice, we initially retrieved and interrogated the global gene expression data (GEO accession: GSM38236) generated from Slug-silenced primary myoblasts22. Gene ontology enrichment analysis of biological processes (GOBP) identified that Slug silencing derepressed sets of genes related to transcription regulation, cell proliferation, and skeletal muscle differentiation processes (Fig. 4a, left panel). Notably, the genes downregulated upon Slug silencing identified enrichment of genes among various categories of cellular defense responses (Fig. 4a, right panel). Interestingly, p16Ink4a was listed in the most upregulated genes ranked in negative regulation of cell proliferation. It was previously reported that self-renewal capacity of aged stem cells (hematopoietic stem cells23, intestinal stem cells24, and skeletal MuSCs5) was attenuated. Therefore, we postulated p16Ink4a as a potential mediator for SC self-renewing defect caused by Slug deficiency.
We then performed microarrays analysis to compare the genome-wide gene expression profiles of wild-type and Slug-deficient SCs. Unsupervised hierarchical clustering analysis separated the samples into their respective genotypes (Fig. 4b). We identified 168 differentially expressed genes, of which 108 genes were upregulated (fold change > 2, p value < 0.05) and 60 genes were downregulated (fold change < -2, p value < 0.05) in Slug−/− SCs compared to control cells (Fig. 4b). Slug deletion enriched GO categories related to mitochondria metabolism and cell cycle regulators (Fig. 4c). To identify pathways enriched in Slug−/− SCs, gene set enrichment analysis (GSEA) was performed. Notably, signaling pathways involved in cell metabolism including glycolysis, PI3K-AKT-MTOR, oxidative phosphorylation, and reactive oxygen species were induced in quiescent Slug−/− SCs (Supplementary Fig. 7a,b), suggesting a switched metabolic reprogramming with relatively higher energy-consuming status in Slug null SCs. Meanwhile, E2F targets and G2M checkpoints signaling signatures were also enriched upon Slug deletion (Supplementary Fig. 7c,d). These gene sets enrichment data indicated that loss of Slug in SCs might disturb cell cycle progression and the balance between self-renewal and differentiation after activation.

Next, we examined expression of p16Ink4a and p19Arf, the two gene products of the INK4A/ARF locus, by qPCR in SCs of adult wild-type and Slug−/− mice, respectively. As shown in Fig. 4d, p16Ink4a knocked out was about two-fold higher in Slug-deficient SCs when compared with the wild-type counterparts, indicating a derepression of p16Ink4a in the absence ofSlug in resting SCs in vivo. In contrast, there was no apparent difference in p19Arf expression between the two types of SCs. Furthermore, we examined the proximal promoter region of p16Ink4a and identified potential Slug-binding sites (E-box) in ex vivo cultured myoblasts (Fig. 4e). To facilitate assessing the occupancy of endogenous Slug at the promoter region of p16Ink4a by chromatin immunoprecipitation (ChIP) assay, we performed Slug affinity tagging at its C-terminus in mouse myoblasts by CRISPR/Cas9-mediated gene tagging (Supplementary Fig. 8). ChIP-qPCR analysis displayed enriched binding in anti-Flag antibody immunoprecipitated gDNA fragments but not in anti-IgG control (Fig. 4f), indicating that Slug occupies the promoter region of p16Ink4a in vivo. Such direct regulation of p16Ink4a promoter by Slug was further confirmed by p16Ink4a-driven luciferase reporter assay in SC-derived myoblasts (Supplementary Fig. 9a,b). Importantly, this E-box element is also present in human p16INK4A promoter (Supplementary Fig. 9c). Knockout or overexpression of Slug in primary human myoblasts significantly derepressed or suppressed p16INK4A transcript (p < 0.001 by student’s t-test) (Supplementary Fig. 9d-g), respectively, indicating a highly conserved role of Slug in regulating p16INK4A.

Of note, loss of Slug caused a more robust dysregulation of p16INK4A in ex vivo cultured myoblasts compared to resting SCs in vivo. Normally, p16INK4A expression is elevated during aging and replicative senescence. Myoblasts are a type of proliferating myogenic progenitor cells derived from quiescent SCs under stimulating conditions. Therefore, we asked whether derepression of p16INK4A in Slug-deficient SCs would be exacerbated under stress conditions, including ex vivo culture and muscle damage. Strikingly, p16INK4A expression increased by over 8-fold in ex vivo cultured Slug−/− SCs and five-fold in in vivo injury-activated Slug−/− SCs, respectively, when compared with their corresponding wild-type controls (Fig. 4g) Notably, derepression of p16INK4A in cultured myoblasts was accompanied with gradual decline of Slug expression (Supplementary Fig. 10).

In spite of the increased p16INK4A transcription, primary muscle regeneration was normal in Slug-deficient mice. This is a departure from what was reported in geriatric mice model, i.e., that resting SCs fail to activate and expand to regenerate the muscle on injury due to derepression of p16INK4A. We suspected that p16INK4A mRNA but not protein was increased in resting Slug-deficient SCs, since p16INK4A mRNA could be induced to decay by an RNA-binding protein in early passage of fibroblasts but accumulated at protein level during late-passage of culture. Indeed, p16INK4A protein was undetectable in undamaged muscle cryosections from both Ctrl and SlugKO mice (Supplementary Fig. 11). Instead, there was an apparent co-expression of p16INK4A with Pax7 in muscle tissue harvested from SlugKO mice 30 days post injury (Fig 4h, i). Taken as whole, these results suggested that Slug deficiency leads to an increase in p16INK4A transcription in SCs, and replicative stress signaling triggered by SC activation and proliferation concurrently increases the stability of p16INK4A protein.

**Slug loss promotes senescence in SCs during proliferation.** In terms of the elevated p16INK4A protein in activated SCs in regenerating skeletal muscles of Slug-deficient mice, we hypothesized that Slug-ablated SCs acquired features of cellular senescence at late stages of regeneration during the transition of SCs from activation to quiescence or differentiation. To test this notion, we first used an in vitro reserve cell culture system. As shown in Fig. 5a, reserve cells from wild-type mice robustly proliferated while cells from Slug−/− mice were only sporadically distributed by day 7 of subculture (Fig. 5a). Compared to their wild-type control cells, significantly higher proportion of progeny from

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**Fig. 3** SCs deficient in Slug exhibit intrinsic defects in self-renewal in vivo. a Ratio of SCs between SlugKO and Ctrl mice under indicated conditions. **p < 0.001 by student’s t-test.** b IHC for Pax7+ SCs in TA of SlugKO and Ctrl mice (n = 3 mice per group) at day 30 post single injury. Laminin indicates the boundaries of myofibers. Scale bar, 100 μm. Three independent experiments were performed with similar results. c Quantification of Pax7+ SC numbers in b **p < 0.01 by student’s t-test.** d Scheme of SC transplantation. 3000 SCs of indicated genotype were transplanted into each side of pre-injured TA muscles of recipient (n = 6), respectively. Donor-derived SCs (GFP+) in total recipient MuSCs (CD45− CD31− Scal− Vcam1−) were analyzed 4 weeks after transplantation. e Representative flow cytometric analysis of the frequency of donor-derived SCs (GFP+) within total recipient MuSCs. Similar results were seen in three independent transplantation experiments. **p < 0.05 by student’s t-test.** f Determination of relative ratio of repopulated MuSCs. Left, representative flow cytometric plotting of GFP+ cells from total recipient MuSCs. Right, PCR determining the ratio of SCs before and after transplantation. M DNA ladder, MBT mixed SCs before transplantation, MAT mixed SCs after transplantation, Ng negative control. i Scheme of AraC treatment indicating self-renewal. j Pax7 and Ki67 immunostaining in cultured myoblasts as treated in i Scale bar, 50 μm. Similar results were seen from three independent experiments. **p < 0.001 by student’s t-test.** Data are shown as mean ± SEM of three independent experiments. Unprocessed gel blots are provided in the Supplementary Fig. 1. Also see Supplementary Fig. 4-6. Source data are provided as a Source Data file.
**Slug**/− reserve cells was positive for **p16**Ink4a staining ($p < 0.001$ by student’s t-test) (Supplementary Fig. 12). SA-β-Gal staining showed that over 50% of **Slug**-deficient reserve cells-expanded cells was SA-β-Gal+, a marker of senescence, whereas few SA-β-Gal+ cells were detected in the control group (Fig. 5a, b).

Population doubling level (PDL) is an intrinsic measurement of the age of the particular culture of a cell line. In culture, an untransformed cell line has a finite life span expressed in the number of cumulative population doublings that can be achieved. To assess the accelerated senescence in **Slug**-deficient SCs under...
proliferative pressure in vitro, we determined the relative growth rates of control and Slug-mutant SCs by calculating the cumulative PDL. As shown in Fig. 5c, the growth rate of Slug-deficient SCs was clearly retarded at early passage 3 when wild-type SCs remained for exponential growth. SA-β-Gal staining demonstrated that about 40% of Slug-ablated myoblasts show strong positive SA-β-Gal staining while less than 5% of control cells were SA-β-Gal+ on passage 3 (Fig. 5d, e), indicating the existence of replicative senescence and growth retardation in early-passaged Slug-deficient myoblasts.

Next, we investigated whether cellular senescence in SCs would occur when Slug is specifically deleted in Pax7+ SCs during regeneration in vivo. Young adult mice were used in this study to exclude the process of regeneration from aging in geriatric mice6. We performed SA-β-Gal staining on cryosections of TA muscles from Ctrl and SlugcKO mice on day 10 post first injury when necrotic fibers were replaced by central-nucleated regenerated myofibers (Fig. 5f). Notably, despite the sporadic SA-β-Gal+ cells in control TA muscle sections, there was a four-fold increase in the number of SA-β-Gal+ cells in SlugcKO TA (Fig. 5f, g). Most of the SA-β-Gal+ cells were located beneath basal lamina and outside myofiber plasma membrane, a classical SC anatomical location (Fig. 5f). IHC results further demonstrated that these SA-β-Gal+ cells were also Pax7-positive. As expected, SA-β-Gal+/Pax7+ cells were not in cycling (Ki67−), suggesting the status of cellular senescence. In contrast, SCs with negative SA-β-Gal staining (SA-β-Gal−/Pax7+) were on the occasion of proliferating (Ki67+). In agreement with this notion, the majority of SCs in SlugcKO failed to re-activate as indicated by markedly lowered percentage of Ki67+ SCs by 2.5-day post second BaCl2 injury compared to Ctrl mice (Fig. 5i, j). Together, using multiple in vitro and in vivo assays we proved that lack of Slug facilitated entry of SCs into cellular senescence under proliferative pressure.

### loss restores impaired self-renewal of Slug−/− SCs

We demonstrated elevated p16Ink4a protein and acquired senescence feature in activated Slug-deficient SCs. At this point, a key question was whether p16Ink4a is causally involved in Slug loss-induced defects in SC self-renewing capacity and muscle regeneration. To answer this question, we assessed muscle regeneration in Slug+/+ p16+/+, Slug−/−p16+/+, and Slug−/−p16−/− mice following serial damages. By H&E staining, we showed that although muscle regeneration was severely compromised in TAs of Slug−/−p16+/+ mice following double or triple injuries, muscle repair was greatly improved in Slug−/−p16−/− mice after injuries (Fig. 6a, b).

Next, we assessed how removal of p16Ink4a would affect the self-renewing ability of Slug-ablated SCs by the quantitative repopulating assay (Fig. 6c). As expected, the frequency of Slug−/−p16+/+ donor-derived SCs was low (~1.96%) (Fig. 6d, e). However, the frequency of Slug−/−p16−/− donor-derived SCs was increased by two-fold in the recipients (Fig. 6d, e). Mechanistically, enhanced repopulation of Slug−/−p16−/− SC in recipient muscles after deletion of p16Ink4a might be partially ascribed to reduced senescence under proliferative stress, as Slug−/−p16−/− SC-derived myoblasts displayed little or no SA-β-Gal staining at ex vivo passage 3 when majority of Slug−/− cells were SA-β-Gal positive (Fig. 6f, g). In summary, p16Ink4a is a crucial mediator for the defects in SC self-renewal and muscle regeneration caused by Slug deficiency.
address whether forced expression of Slug could restore the intrinsic regenerative and self-renewing capacities of the aged SCs (Fig. 7e). Similar to what found in the geriatric SCs (Fig. 7c), a sharp increase in p16Ink4a expression was found in cultured SCs (Fig. 7f). Forced expression of Slug significantly suppressed stress-induced p16Ink4a (p < 0.001 by student’s t-test) (Fig. 7f). Furthermore, we demonstrated that Slug-overexpressing myoblasts gave rise to a substantially larger fraction of the total MuSC population in mdx recipients than myoblasts transduced with the control retrovirus (Fig. 7g, h). In addition to the self-renewal capacity, Slug overexpression also largely restored regenerative capability of SCs that underwent passaging and subculturing for weeks, as evidenced by an increase in the number of dystrophin-positive myofibers in mdx recipient (Fig. 7i, j).
Fig. 6  Removal of p16Ink4a partially rescues regeneration and self-renewal of Slug-deficient SCs.  

a  H&E staining of the double and triple BaCl2-injured TA muscles from Slug+/+p16+/+, Slug−/−p16+/+, and Slug−/−p16−/− mice (n = 3–6 mice per group). Left panel, the diagram for consecutive injury. Right panel, representative images of H&E staining of muscle tissue sections from injured mice. Scale bar, 100 μm.

b  Ratio of myofiber CSA in the intact and BaCl2-injured TA muscles between Slug+/+p16+/+, Slug−/−p16+/+, and Slug−/−p16−/− mice. *p < 0.05, ***p < 0.001 by one-way ANOVA; n.s. not significant.

c  Scheme of SC transplantation. SCs isolated from Slug−/−p16+/+ and Slug−/−p16−/− mice were infected with GFP-expressing retroviruses, and injected into either side of the BaCl2-pre-injured TA muscle of mdx recipient, respectively. Total mononucleated muscle cells were isolated separately from either TA muscle 4 weeks after transplantation, and subjected to flow cytometric analysis for the fraction of donor-derived SCs (GFP+) in MuSC (CD45−CD31−Sca1−Vcam I+) subpopulation of recipient mice.

d  Representative flow cytometry plots showing the frequency of donor-derived SCs (GFP+) within the total recipient MuSC (CD45−CD31−Sca1−Vcam I+) subpopulation in mononucleated TA muscle cells. e  Percent of donor-derived cells (GFP+) in total MuSC (CD45−CD31−Sca1−Vcam I+) subpopulation of mononucleated TA muscle cells from mdx recipients (n = 4–6). ***p < 0.001 by student’s t-test.

f  Representative SA-β-Gal staining of cultured primary SC-derived myoblasts. 10^4 SCs of indicated genotype were plated in Matrigel-coated wells of 24-well plate, and passaged weekly. Bright-field imaging and SA-β-Gal staining were performed in cells on day 7 of culture at passage 3. Scale bar, 100 μm.

g  Percentage of the SA-β-Gal+ cells in serially passaged myoblasts on day 7 of culture at passage 3. ***p < 0.01; by student’s t-test. n.s. not significant.

Data are shown as mean ± SEM of three independent replicates. Source data are provided as a Source Data file.
Taken as a whole, these findings demonstrated that lack of sufficient Slug expression is an important factor for derepression of p16<sup>Ink4a</sup> in aged SCs, and that restoring Slug expression improves p16<sup>Ink4a</sup>-caused regenerative and self-renewing defects in SCs.

**Discussion**

Slug is a well-established regulatory transcriptional factor essential for epithelial-mesenchymal transition (EMT) during embryogenesis<sup>34,35</sup>, tumor metastasis<sup>36,37</sup>, adult stem cells<sup>38-41</sup>, and cellular reprogramming<sup>12,43</sup>. Slug is ubiquitously expressed in majority of normal tissues in the adult<sup>13</sup>. Slug knockout mice exhibit several severe abnormalities, including postnatal growth retardation<sup>44</sup>, attenuated acute cutaneous inflammatory response<sup>45</sup>, thinner epidermis<sup>46</sup>, and delayed hair follicle development<sup>47</sup>. These defects are typical phenotypes found in mice at advanced age, reminiscent of the essential role of Slug in aging.

Here, we described an undiscovered function of Slug in MuSC aging. Our results illustrated that Slug is highly expressed in SCs, and acts as a transcriptional repressor of p16<sup>Ink4a</sup>. Lack of Slug

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**Figure Legends:**

(a) Graph showing the expression level of Slug in young vs aged SCs.

(b) Flow cytometry analysis of integrin expression in young and aged MuSCs.

(c) Western blot analysis of p16<sup>Ink4a</sup> and HPRT expression in young and aged SCs.

(d) Graph depicting the expression level of p16<sup>Ink4a</sup> in young vs aged SCs.

(e) Schematic diagram of the experimental setup for MuSC transplantation.

(f) Graph showing the frequency of p16<sup>Ink4a</sup>-positive myofibers in fresh SCs vs cultured SCs.

(g) Flow cytometry analysis of donor-derived fraction in primary recipient and cultured SCs.

(h) Graph showing the frequency of Dystrophin-positive myofibers in pMIG-R1 vs pMIG-Slug SCs.

(i) Immunofluorescence staining of Dystrophin in pMIG-R1 vs pMIG-Slug SCs.

(j) Graph showing the frequency of Dystrophin-positive myofibers in pMIG-R1 vs pMIG-Slug SCs.
triggers SCs into pre-senescent state by accumulating p16Ink4a expression and compromising SC self-renewing capacity after activation, leading to MuSC exhaustion and muscle regeneration defect. This study revealed a cell-autonomous mechanism of SCs losing their intrinsic regenerative and self-renewal capacities during chronological aging.

The role of Slug in skeletal muscle regeneration was firstly reported by the Hoffman group and they showed that Slug is a downstream target of MyoD and its expression was markedly increased at the whole muscle tissue level on day 4 and 10 during muscle regeneration. Of note, they demonstrated that Slug knockout mice were defective for muscle regeneration after the first round of injury by cardiotoxin, which is different from our current findings in the global and conditional Slug knockout mouse models. Here, we demonstrated that Slug is highly expressed in quiescent SCs and its expression is greatly reduced in differentiated myotubes. This is consistent with the more recent work showing gradually diminished Slug protein in differentiating myoblasts in vitro. Our current findings do not support that Slug is a downstream target of MyoD, since Slug expression is abundant in MyoD-negative quiescent SCs. As revealed by Soleimani et al., Snail1/Slug compete with MyoD and prevent its occupancy on differentiation-specific regulatory elements in undifferentiated primary myoblasts. Therefore, lack of competitive Slug binding should facilitate easier access of MyoD to those differentiation-regulating elements on its first appearance after injury and initiate myogenic program. Indeed, Slug-deficient mice were able to regenerate their muscle upon first round of muscle injury. In our current studies, we further proved that the failure of muscle repair in Slug-deficient mice after multiple injuries was partially due to the exhaustion of SC pool that was caused by their SC self-renewing defect. However, in view of the abundant expression of Slug in myoblasts, it is possible that Slug is playing a role outside of self-renewal, which could affect muscle regeneration and transplantation potential, by biasing SC proliferation versus differentiation.

In our current study, Slug deletion caused only a small increase of $p16^{INK4a}$ mRNA in quiescent SCs and did not prevent their activation and differentiation following the first muscle damage. We infer that the compensatory regulation of Snail1, another member of the Snail/Slug superfamily of zinc-finger transcription factor, was probably also crucial to keep $p16^{INK4a}$ transcription under control. This conjecture is supported by the fact that both Snail1 and Slug recognize and bind to the identical E-box motifs in myoblasts. Interestingly, the Gridley group have reported a compensatory regulation of the Snail1 and Snail2 (Slug) during chondrogenesis. Such compensation from Snail expression might also facilitate muscle regeneration as observed in Slug-deficient mice after first round of muscle injury as Snail1 can indirectly influence key myogenic transcription factors like Myf5 to activate myogenesis. Of note, both Snail1 and Slug proteins decrease during myoblast differentiation. Therefore, we reasoned that the large increase in $p16^{INK4a}$ mRNA expression in cultured Slug-deficient myoblasts and accumulated $p16^{INK4a}$ protein in activated SCs were due to the loss of the dual regulation conferred by both Slug and Snail1. Future studies using $Snail2$ and Slug double knockout mice will be essential in testing this hypothesis. In addition, an increase in $p16^{INK4a}$ mRNA but not protein expression level in quiescent Slug−/− SCs suggests a post-translational regulation mechanism. It is possible that $p16^{INK4a}$ protein is unstable in resting SCs. Indeed, we reported that $p16^{INK4a}$ translation is suppressed by miR-2450, which is highly expressed in quiescent SCs but greatly downregulated in activated SCs. These findings might explain our finding that $p16^{INK4a}$ mRNA but not protein accumulated in quiescent Slug−/− SCs.

Quiescent SCs rely on fatty acid and pyruvate oxidation to maintain a low metabolic rate in quiescence. The energy supply switches to glycolysis during early activation, while both mitochondrial density and oxidative phosphorylation activity are increased following differentiation. It was previously demonstrated that the mitochondrial-associated metabolism pathway is more silent in Pax7Hi SCs being of higher level of stemness and responsible for self-renewal. A more recent study of in vitro culture of SCs in conditioned medium favoring either glycolysis or oxidative phosphorylation showed that a shift from glycolysis to oxidative phosphorylation negatively affects the return to quiescence of activated SCs. Although SCs remain in quiescence in the absence of Slug, a batch of genes in glycolysis, oxidative phosphorylation, and nutrient-sensitive PI3K-AKT-mTOR pathways were upregulated therein. Such metabolic gene expression profiling is consistent with our observation of the attenuated self-renewal and accelerated senescence in Slug null SCs.

An aging-associated reduction of Slug/Snai2 expression was observed in mouse SCs. Mechanistically, Slug expression was reported to be under control of a number of signaling pathways such as FGF, Wnt, TGFβ, Notch, stem cell factor (SCF), integrins, and estrogens etc. Several of these signaling molecules including FGF3 and Notch are known as inducers of Slug and were reported to decline with age in mouse SCs. Therefore, we speculate that these impaired upstream signaling pathways might account for Slug insufficiency in aged SCs. Interestingly, active Notch and Notch ligand Delta are also decreased in old human muscle when compared to young...
muscle\textsuperscript{41}. Indeed, we identified the potential Slug-binding consensus sequence (E-box) in the promoter region of human \textit{p}16\textsuperscript{Ink4a} gene as well (Supplementary Fig. 9c). Gain and loss of function of SLUG in primary human myoblasts causes down and upregulation of \textit{p}16\textsuperscript{Ink4a} expression, respectively (Supplementary Fig. 9d-g). Function of Slug in SCs might therefore be highly conserved in mice and human. Future studies exploring small molecules that are able to induce Slug expression in aged mice are warranted to test the improvement of aging-associated muscle stem cell defects in vivo.

In conclusion, although \textit{p}16\textsuperscript{Ink4a} has been well recognized as a key factor for stem cell aging, upstream regulators for causing its dysregulation remain unknown. Our studies demonstrated that Slug plays an important role in repressing \textit{p}16\textsuperscript{Ink4a} transcription in skeletal MuSCs. Loss of Slug gives rise to severe regenerative defects during continuous muscle injury by accelerating entry of SC senescence. Restoration of Slug expression during chronological aging rejuvenates the function of aged SCs by suppressing \textit{p}16\textsuperscript{Ink4a} expression. These results offer a promising therapeutic target for aging-associated degenerative muscle disease.

**Methods**

**Mice.** C57BL/6, C57BL/6-Tg(CAG-EGFP)10sb/J, Pax7tm1(cre)Mrc, B6.Cg-Pax7tm1(cre)Mrc/J (referred to as Pax7\textsuperscript{loxP}—2FRT\textsuperscript{−}/2FRT\textsuperscript{−}mice) were purchased from Jackson Laboratory (Bar Harbor, ME). Pax7\textsuperscript{loxP}—2FRT\textsuperscript{−}mice were mated with the NCI Mouse Repository. Pax7\textsuperscript{loxP}—2FRT\textsuperscript{−}/2FRT\textsuperscript{−} transgenic mice were a kind gift from Dr. M. Kyba (University of Minnesota). Slug conditional knockout mouse line was generated in the current study. All compound genetically-engineered mice were a result of crosses of two separate lines, where one or both of the compound lines carried neomycin (neo) for positive selection and HSV-tk marker for negative selection.

**Gene targeting and generation of a Slug-flxed allele.** The Slug targeting construct was generated by PCR using 129X1/svJ mouse genomic DNA. Primers for generating the targeting construct are shown in Supplementary Table 1. First, a ~3 Kb genomic sequence containing Slug promoter region was PCR-amplified as the left arm with SlugKO-P1 and SlugKO-P2 primers and cloned into NotI and Sall sites of pLoXP-2FRT vector (designated as pLoXP-2FRT-SlugN). Second, a ~4 Kb genomic sequence covering the genomic region from the immediate downstream of SlugKO-P2 binding site to the intron 3 was PCR-amplified as the right arm with SlugKO-P3 and SlugKO-P4 primers, following by cloning into BamH1 and Kpn1 sites of pBS vector (designated as pBS_SlugC). Third, a LoXP linker was cloned into EcorI site of pBS_SlugC and the resultant plasmid was named as pBS_SlugC-LoXP. Lastly, the Slug_C-LoXP fragment was isolated from pBS_SlugC-LoXP and Kpn1 and BamH1 sites and then cloned into pLoXP-2FRT_SlugN. The resulting construct was designated as pLoXP-2FRT_Slug, which carries neomycin (neo) for positive selection and HSV-\textit{tk} marker for negative selection. The linearized targeting construct was nucleofected into R1 ES cells (129/x1/svJ/Sc-CF1) by Nucleofector II (Lonza, Allendale, NJ), and followed by positive selection with G418 and negative selection with FIAU. Correctly targeted ES cells were first screened for the presence of the DNA sequence containing LoxP and FRT sites by PCR with SlugKO-P1 and Neo-R1 primers, then screened for the left arm by long-PCR with SlugKO-P10 and Neo-R1 and confirmed by DNA sequencing. Two positive ES cell clones were expanded and microinjected into C57BL/6 blastocysts to generate chimeric Slug\textsuperscript{fl/fl} founder mice at the Mouse Transgenic & Gene Targeting Core of Maine Medical Center Research Institute. The neo cassette was deleted from the germline of the resulting founder mice by using Flp-expressing transgenic mice (ACTB:FLPe B6; SJL, The Jackson Laboratory), Flp

**Slug gene was performed using a multiplex PCR method according to the provided protocol. To delete Slug in adult SCs, tamoxifen (Sigma) was administered intraperitoneally (100 mg kg\textsuperscript{−1} body weight per day) for 5 consecutive days.

**Myofiber culture and AraC treatment.** EDL muscles were dissected and digested for 1 h in DMEM with 0.2% Collagenase type I. Pre-digested EDLs were then transferred to horse serum-precoated dish containing prewarmed DMEM, and gently flushed with a fire polished large bore glass pipette to release myofibers. Live and healthy single myofibers were obtained by three consecutive transfers of individual myofibers with small bore glass pipette into new horse serum-precoated dishes containing prewarmed DMEM. Live myofibers were cultured in uncoated plastic 24-well-plates at density of 1 fiber per well with DMEM supplemented with 15% biotinylated antibodies reactive to CD45, CD11b, CD31, and Sca1 in the anterior compartment, experiment, myofibers were cultured for 72 h and then incubated with or without 100\(\mu\)M AraC (C1768, Sigma, USA) for 48 h (from day 3–5) and fixed (day 5) for immunostaining.

**Skeletal muscle cell culture and generation.** Mouse skeletal MuSCs were cultured using the MuSC growth medium [DMEM/F10 (1:1) with 20% FBS, 2.5 ng/ml bFGF (PROSPEC, East Brunswick, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, USA)] for 5 days. For all the experiments, cells were switched from growth medium into the fusion medium (DMEM supplemented with 5% horse serum) for 5 days.

**Comparison of proliferation potential.** Cumulative population doubling level (CPDL) was calculated using the formula \(\text{CPDL} = \frac{\log_{10}(N_{\text{fi}}) - \log_{10}(N_{\text{fi}\text{+1}})}{\log_{10}(2)}\) in which \(N_{\text{fi}}\) is the inoculum cell number and \(N_{\text{fi}\text{+1}}\) is the cell harvest number. To determine cumulative doubled muscle cell population doubling level for each passage was calculated and then added to the levels of the previous passages. Cumulative doubling number was first calculated from passage 1.

**Histology and immunohistochemistry in muscle cryosections.** Fresh TA muscles were embedded in Tissue-Tek\textsuperscript{O.C.T.} compound (Fisher Scientific, Hampton, USA), frozen in liquid nitrogen-cooled isopentane, and stored at −80 °C until analysis. Frozen muscles were cross-sectioned (10 μm) using a Leica CM1850 cryostat. For histology, the sections were dried at room temperature for over 3 h before staining. Sections were then rehydrated in PBS for 5 min and fixed in 10% formalin for 15 min, and proceeded with routine haematoxylin and eosin (H&E) staining. For immunohistochemistry (IHC) study, air-dried muscle sections were fixed with 4% paraformaldehyde (PFA) and permeabilized in 0.2% Triton X-100. Tissue sections were then washed with phosphate buffered saline (PBS) and incubated with 1% goat serum, 2% bovine serum albumin, and 1% Tween-20 for 1h, and then followed by incubation with anti-Pax-7 (DSHB, Iowa, USA, 1:10), anti-Laminin (clone A5, Invitrogen, Carlshad, USA, 1:100 dilution), anti-p-65 (M-156, Santa Cruz Bio technology, USA), anti-Kit (Clone SolA15, eBioscience, USA, 1:100), and/or anti-dystrophin (Clone MANDYS8, Sigma, USA, 1:100) primary antibodies overnight at 4°C. After washing with PBS, sections were then incubated with Alexa-conjugated secondary antibodies (Invitrogen, Carlshad, USA, 1:200 dilution).

**Immunofluorescence staining.** Freshly isolated SCs were cytopsinated onto slides and fixed with 4% PFA. After being permeabilized by 0.2% Triton X-100, cells were then blocked in PBS with 1% bovine serum albumin (BSA) for 1 h at room temperature followed by staining with anti-Pax7 (DSHB, Iowa, USA, 1:10) and anti-Slug (Clone C102G7, Cell Signaling, USA, 1:400) primary antibodies overnight at 4°C. Cells were washed three times with PBS and incubated with appropriate secondary antibody for 1 h at room temperature.
SA-β-galactosidase staining. Cellular senescence was evaluated by β-galactosidase activity using MarkGenetM™ Cellular Senescence Assay Kit (MarkGene, Eugene OR, USA). Briefly, cells or tissue sections were fixed for 4 and 20 min, respectively. Fixed samples were then washed in PBS (pH 7.0) twice for 10 min and incubated with X-gal containing staining buffer at 37 °C overnight for cells and 48 h for tissue sections. For tissue sections, X-gal substrate was changed after 24 h. Samples were washed in PBS, and post-fixed in 1% PFA 5 min for cells and 30 min for sections. After being washed three times for 10 min in PBS, samples were mounted in PBS, 20% glycerol or proceeded for IHC.

Muscle injury and SC transplantation. Experimental mice were anesthetized by intraperitoneal injection of ketamine (100 mg kg⁻¹ body weight) and xylazine (10 mg kg⁻¹ body weight). For single muscle injury, TA muscles were injected with 50 µl of 1.2% BaCl₂ (B0750, Sigma, USA) at multiple sites and harvested 10 days post injury. For double or triple injuries, mice were allowed to recover for 1 month after last BaCl₂ injection, and then injured again with 50 µl of 1.2% BaCl₂. For in vivo cellular senescence detection, TA muscles were injured with 50 µl cardiotoxin (Naja pallida, 10 µM; Calbiochem, USA). For SC transplantation, recipient mdx mice were anesthetized by intraperitoneal injection of ketamine (100 mg kg⁻¹ body weight) and xylazine (10 mg kg⁻¹ body weight). TA muscles were injected with 50 µl of 1.2% BaCl₂ 1 day before transplantation. Next day, SCs (or myoblasts) were re-suspended in 15 µl of PBS and then injected into pre-injured TA muscle. TA muscles were harvested from recipient mice 4 weeks after transplantation and analyzed by cryosectioning and microscopy.

RNA extraction and qPCR. Total RNA was extracted from samples (SCs or myoblasts) using Quick-RNA™ MicroPrep kit (ZYMO Research, USA). First strand cDNA synthesis was performed using RT™ Master Mix (LAMDA BIO-TECH). qPCR was performed using GoTag® Green Master Mix (Promega Madison, USA) on Bio-Rad Thermal Cycler. qPCR was performed using Bulseye EvaGreen Qpcr MasterMix (MIDSCI, USA). The primer sequences for PCR are listed in Supplementary Table 1.

cDNA microarray and data analysis. Total RNAs extracted using Quick RNA™ MicroPrep kit (ZYMO Research, Irvine, USA) were first analyzed by Bioanalyzer (Agilent Technologies, Santa, Clara, USA). A triplicate of RNA samples with an RNA integrity number (RIN) >9.0 were used for subsequent labeling and hybridization with Mouse Gene 2.0 ST Arrays (Affymetrix, USA). Expression data were processed using Gene Expression Console software (Affymetrix, Santa Clara, USA). The significance of differentially expressed genes was determined using Transcriptome Analysis Console software (Affymetrix, Santa Clara, USA). GSEA version 3.0 (http://software.broadinstitute.org/gsea) and MSigDB gene sets version 6.1 were used for functional analysis. Nodes in the Enrichment Map represent the common nodes. The edge thickness is proportional to the overlap of two gene sets. The significance >0.5. Nodes in the Enrichment Map represent the common nodes. The edge thickness is proportional to the overlap of two gene sets. The node colors map enrichment significance: blue/downregulated, red/upregulated.

Luciferase reporter assay. Myoblast cells were seeded in 48-well plates and cultured in myoblast growth medium. A total of 0.5 μg of DNA containing 0.1 μg of PG3-p1b luciferase reporter plasmid, 0.4 μg of expression plasmid (pMIG-R1 or pMIG-Slug) was transfected using PEI. A co-transfected 0.025μg CMV-lacZ plasmid was used to normalize transfection efficiency. Cells were lysed 24 h using Glo Lysis Buffer (E266A, Promega) after transfection, and the luciferase activity of each extract was assayed using Bright-Glo™ Luciferase Assay System (E2620, Promega). One microtiter cell lysate was added in 399-μl z buffer. One hundred microliter ONPG (o-nitrophenyl beta-D-galactopyranoside; Sigma #N41127) with concentration of 4 mg/ml was added and mixed well. When the sample become yellow, 500 μl 1M carbonate was added to stop the reaction and the activity of beta-galactosidase was measured at A420 to normalize the luciferase activity.

Western blot analysis. Total protein was lysed using Celllytic™ MT Cell Lysis reagent (#C3223, Sigma, USA). Proteins were determined using a bicinchoninic acid assay (Pierce) with β-actin (#4970, Cell Signaling Technology, USA) as loading control. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk TBST for 2 h at room temperature and washed 3 times in TBST for 15 min. First antibody was added followed by biotinylated secondary antibody, Washing and electroblotted onto polyvinylidene difluoride membranes. Immunoblotting was performed with anti-Flag (F4058, Sigma, USA), anti-HA (5B1D10, Cell Signaling Technology, USA), or anti-IgG (Millipore, USA) control antibodies. Bound antibodies were detected using Clarity Max™ Western ECL substrate (#6862, Bio-Rad, USA). Uncropped blots can be found in Supplementary Fig 14 and the Source data file.

Data availability

Data supporting the findings of this manuscript are available within the article and its supplementary information files or from the corresponding author upon reasonable request. All data from DNA microarray experiments have been deposited in Gene Expression Omnibus database under accession code GSE188507. The source data underlying Fig. 1–7 and Supplementary Figs 1, 3–5, 8–10 and 12 are provided as a Source Data file.

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References

1. Frontera, W. R., Hughes, V. A., Lutz, K. J. & Evans, W. J. A cross-sectional study of muscle strength and mass in 45- to 78-yr-old men and women. J. Appl. Physiol. (1985) 71, 644–650 (1991).
2. Carlson, B. M. & Faulkner, J. A. Muscle transplantation between young and old rats: age of host determines recovery. Am. J. Physiol. 256, C1266–C1267 (1989).
3. Athukudahalakal, J. V., Jones, K. M., Basson, M. A. & Brack, A. S. The aged niche disrupts muscle stem cell quiescence. Nature 490, 355–360 (2012).
4. Sinha, M. et al. Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle. Science 344, 649–652 (2014).
5. Sousa-Victor, P. et al. Geriatric muscle stem cells switch reversible quiescence into senescence. Nature 506, 316–321 (2014).
6. Bernet, J. D. et al. p38 MAPK signaling underlies a cell-autonomous loss of stem cell self-renewal in skeletal muscle of aged mice. Nat. Med. 20, 265–271 (2014).
7. Cosgrove, B. D. et al. Rejuvenation of the muscle stem cell population restores strength to injured aged muscles. Nat. Med. 20, 255–264 (2014).

8. Montara, D. E. et al. Direct isolation of satellite cells for skeletal muscle regeneration. Science 309, 2064–2067 (2005).

9. Relais, F. & Zammit, P. S. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Development 139, 2845–2856 (2012).

10. Lopez-Otin, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. Cell 153, 1194–1217 (2013).

11. Sharpless, N. E. & Sherr, C. J. Forging a signature of in vivo senescence. Nature 439, 202–208 (2006).

12. Sherr, C. J. Ink4–Arf locus in cancer and aging. Cell 114, 209–218 (2003).

13. Parent, A. E., Choi, C., Caudy, K., Gridley, T. & Kusewitt, D. F. The developmental transcription factor slug is widely expressed in tissues of adult mice. J. Histochem. Cytochem. 52, 959–965 (2004).

14. Pallafacchina, G., Blaauw, B. & Schiaf...
65. Merico, D., Isserlin, R., Stueker, O., Emili, A. & Bader, G. D. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS ONE* 5, e13984 (2010).

66. Isserlin, R., Merico, D., Voisin, V. & Bader, G. D. Enrichment Map—a Cytoscape app to visualize and explore OMICs pathway enrichment results. *F1000Res* 3, 141 (2014).

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**Author contributions**

Conceptualization, P.Z. and W.S.W; Methodology, P.Z., C.P., Y.G., F.W. and Y.Z.; Investigation, P.Z., C.P., Y.G., F.W. and Y.Z.; Formal Analysis, P.Z., C.P., Y.G., F.W. and Y.Z.; Writing, P.Z. and W.S.W.; Resources, P.Z., Y.G., F.W. and Y.Z.; Funding Acquisition, W.S.W; Supervision, W.S.W.

**Additional information**

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