Targeting β1-integrin signaling enhances regeneration in aged and dystrophic muscle in mice

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Interactions between stem cells and their microenvironment, or niche, are essential for stem cell maintenance and function. Our knowledge of the niche for the skeletal muscle stem cell, i.e., the satellite cell (SC), is incomplete. Here we show that β1-integrin is an essential niche molecule that maintains SC homeostasis, and sustains the expansion and self-renewal of this stem cell pool during regeneration. We further show that β1-integrin cooperates with fibroblast growth factor 2 (Fgf2), a potent growth factor for SCs, to synergistically activate their common downstream effectors, the mitogen-activated protein (MAP) kinase Erk and protein kinase B (Akt). Notably, SCs in aged mice show altered β1-integrin activity and insensitivity to Fgf2. Augmenting β1-integrin activity with a monoclonal antibody restores Fgf2 sensitivity and improves regeneration after experimentally induced muscle injury. The same treatment also enhances regeneration and function of dystrophic muscles in mdx mice, a model for Duchenne muscular dystrophy. Therefore, β1-integrin senses the SC niche to maintain responsiveness to Fgf2, and this integrin represents a potential therapeutic target for pathological conditions of the muscle in which the stem cell niche is compromised.

Sarcopenia, the slow progressive loss of skeletal muscle mass concomitant with advancing age, affects elderly people. Age-related muscle wasting is characterized by loss of muscle quantity and quality due to changes in muscle metabolism, function and regeneration. The poor regeneration of aged muscle is not attributed solely to the loss of stem cells (i.e., satellite cells (SCs)), although SC numbers decline during aging in mice and humans. Instead, the impaired regeneration is linked to age-related changes in both extrinsic systemic and local environments, as well as to intrinsic defects.

Fgf2, a well-studied niche signal, has important roles in driving SC proliferation. SCs are maintained in a quiescent state by repressing Fgf2 signals. Aging increases the level of Fgf2 in skeletal muscle and decreases the level of the FGF signaling inhibitor Spry1 in SCs, which results in loss of a portion of the SC pool owing to breaking quiescence. Paradoxically, aged SCs are nonresponsive to Fgf2 during their proliferation and self-renewal. Inhibiting FGF signaling in aged SCs rescues the quiescent phenotype at the expense of regeneration, whereas ectopically activating Fgf receptor 1 (Fgfr1) augments Fgf2 sensitivity and increases SC proliferation and renewal after injury. We further demonstrate that activating β1-integrin signaling restores FGF sensitivity in aged SCs and improves muscle regeneration. Activating β1-integrin in the mdx mouse can also promote SC expansion and improve function. We propose that β1-integrin is a potential therapeutic target of pathological conditions in which the SC niche is compromised.

RESULTS

SCs without β1-integrin cannot maintain quiescence

To define the function of β1-integrin in adult SCs, we used a Pax7Cre-ERT2(CE) driver6, in which the first exon of Pax7 was replaced by Cre-ERT2, for tamoxifen (tmx)-inducible gene inactivation of an Itgb1 conditional allele (Itgb1f)9 in SCs. The ROSA26 reporter alleles (R26RFP or R26RLacZ), which express either yellow fluorescent protein (YFP) or β-galactosidase (LacZ), were used for lineage marking and for assessing recombination efficiency (Supplementary Fig. 1a–c). Three days after administering the tmx regimen, YFP+ conditional-mutant (Itgb1f−/−) SCs no longer had detectable β1-integrin (Supplementary Fig. 1d,e) but maintained Pax7 expression, as compared to control SCs (Fig. 1a). To assess whether
β1-integrin loss affected SC niche occupancy, we followed lineage-marked cells up to 180 d (Fig. 1b–d). The number of Itgb1−/− SCs was unchanged 7 d after administering the tmx regimen, as compared to that in control cells, but it was reduced to about half of that in the control cells at 21 d after tmx treatment. The number of Itgb1−/− SCs continued to decline over time, but the SCs were not completely lost at 180 d after tmx treatment. We did not detect programmed cell death (PCD) of Itgb1−/− SCs at these time points (Supplementary Fig. 1f). Instead, experiments with lineage-labeled myofibers suggested that the number of mutant SCs declined due to differentiation and incorporation into existing muscle fibers (Fig. 1b,c).

To test whether the slow loss in the numbers of SCs was indeed due to spurious activation and differentiation, we performed continuous administration of 5-bromo-2′-deoxyuridine (BrdU) for 30 d. A significant portion of Itgb1−/− SCs incorporated BrdU during this period, revealing aberrant cell cycle entry (Fig 1e). A greater fraction of cells that express MyoD, a major regulator of muscle differentiation, was also detected in the mutant SCs than in the control SCs (Fig. 1f), supporting the hypothesis of spurious differentiation. Given that there was no increase in the number of SCs, those that became activated probably had limited proliferative capacity. Furthermore, the cell polarity protein cadherin 15 (Cdh15; also known as m-cadherin) and par-3 family cell polarity regulator (Pard3) were not localized appropriately in Itgb1−/− SCs (Supplementary Fig. 2), suggesting that loss of mutant SCs is associated with polarity defects. We conclude that β1-integrin senses the quiescent SC niche to maintain polarity and to prevent spurious activation and differentiation.

**SCs without β1-integrin cannot sustain proliferation after injury**

To assess whether Itgb1−/− SCs could support regeneration, we conducted muscle-injury experiments, using cardiotoxin (CTX), at 3 d after tmx treatment (Supplementary Fig. 3a). We performed these and all subsequent assays at this time point because the mutant SC number had not yet decreased, allowing us to appropriately analyze the separate role of β1-integrin in SC-driven muscle regeneration. Severely defective regeneration was observed at 5, 10 and 30 d after CTX-mediated injury (Fig. 1g–i and Supplementary Fig. 3b–d). Consistent with studies in embryos16, adult Itgb1−/− myoblasts migrated and fused poorly in vitro (Supplementary Fig. 3e–g), which explains the reduced diameter of the regenerated fibers (Fig. 1j).

The drastic reduction in the number of regenerated fibers (Fig. 1h) in the mutant muscle suggested a defect in the ability of the SCs to expand. To test this, we pulsed proliferating cells with...
5-ethynyl-2'-deoxyuridine (EdU) from day 2 to day 5 daily after treatment with CTX. I bg1−/− SCs proliferated normally at day 2, but they did not sustain the same proliferative rates as control SCs over time (Fig. 2a,b). Although PCD did not appear to be a contributor to the reduced number of mutant cells (Supplementary Fig. 1f), we cannot exclude other cell-elimination processes. To ascertain the molecular changes in I bg1−/− SCs, we performed cell cycle analyses of FACS-isolated YFP+ control and mutant SCs that were cultured for 72 h. Similar results were obtained in three separate experiments. (d-f) Representative FACS-aided cell cycle analyses using DNA content (as determined by propidium iodide (PI) staining) of control and mutant SCs at 72 h (d), pie charts summarizing cell fractions in the G1, S and G2/M phases of the cell cycle at 24 h (left), 48 h (middle) and 72 h (right) of culture (e), and the percentage deviation plot of mutant versus control cells in the cell cycle phases at the stipulated time points (f) (n = 3). Data are expressed as mean ± s.d. *P < 0.05; **P < 0.01; n.s., non-significant; by Student’s t-test. Scale bar, 50 μm.

(c) Representative western blot analysis of FACS-isolated control and I bg1−/− YFP+ SCs that were cultured for 72 h. Similar results were obtained in three separate experiments. (d-f) Representative FACS-aided cell cycle analyses using DNA content (as determined by propidium iodide (PI) staining) of control and mutant SCs at 72 h (d), pie charts summarizing cell fractions in the G1, S and G2/M phases of the cell cycle at 24 h (left), 48 h (middle) and 72 h (right) of culture (e), and the percentage deviation plot of mutant versus control cells in the cell cycle phases at the stipulated time points (f) (n = 3). Data are expressed as mean ± s.d. *P < 0.05 by Student’s t-test. Scale bar, 50 μm.

We next assessed the relevance of the cooperativity between integrin and FGF signaling in the SCs. By using the single-myofiber assay9 to examine SC fates, we determined that the majority of control SCs maintained Pax7 expression (Pax7+MyoD− and Pax7+MyoD+). The
addition of Fgf2 increased the population of expanding Pax7\(^+\)MyoD\(^+\) cells but did not alter the fraction of self-renewed Pax7\(^+\)MyoD\(^−\) cells (Fig. 3e,f). In contrast, the majority of mutant SCs expressed only MyoD (Pax7\(^−\)MyoD\(^+\)) and was committed to differentiation, at the expense of Pax7\(^+\)MyoD\(^+\) and Pax7\(^−\)MyoD\(^−\) fractions. The fractions of the latter two populations were restored to amounts similar to those in the control by Fgf2 addition, again suggesting compensation by other integrins. Because SCs express almost all of the integrin subtypes\(^26\), we tested whether the RGD peptide, a broad-spectrum integrin-binding competitor that interferes with ECM engagement\(^14\), was sufficient to disrupt the FGF response. SCs that were treated with the RGD peptide had a reduced fraction of Pax7\(^+\)MyoD\(^−\) cells, which could not be rescued by Fgf2 treatment (Supplementary Fig. 5c–e). Thus, RGD-binding integrins in the SCs, including those containing β1-integrin, cooperate with Fgf2. SC self-renewal has been associated with FGF stimulation of asymmetric phosphorylation of the α\(^1\)- and β\(^1\)-subunits (p38-α and p38-β) of p38 MAP kinase\(^9,27\). Fewer Itgb1\(^−/−\) SCs showed polarized pp38, relative to that in control cells, and Fgf2 treatment could increase the fraction of mutant SCs with polarized pp38 (Fig. 3g,h). Taken together, we have uncovered a mechanism for β1-integrin in sustaining SC expansion and self-renewal.

**Aged SCs are defective in integrin activity**

The characteristics of Itgb1\(^−/−\) SCs are similar to those of aged SCs; both are gradually lost from the niche\(^2\), cannot sustain proliferation\(^28\), are committed to differentiation\(^5\) and are defective in self-renewal\(^9\). Given that ECM composition\(^29\) and stiffness\(^30\) change in aged muscles, we wondered whether the environment has an effect on β1-integrin or overall integrin activity in aged cells, thereby desensitizing aged SCs to Fgf2 (ref. 9). To probe for changes in integrin activity in aged SCs, we first used an antibody (9E8G7) that recognizes the ‘high-affinity’ ligand-bound active β1-integrin\(^31,32\) on young and aged SCs. The majority of young myofiber-associated SCs showed well-aligned basal-membrane-bound β1-integrin (compare 1-integrin) on 1-integrin in its active subtypes\(^26\), we tested whether the RGD peptide, a broad-spectrum integrin-binding competitor that interferes with ECM engagement\(^14\), was sufficient to disrupt the FGF response. SCs that were treated with the RGD peptide had a reduced fraction of Pax7\(^+\)MyoD\(^−\) cells, which could not be rescued by Fgf2 treatment (Supplementary Fig. 5c–e). Thus, RGD-binding integrins in the SCs, including those containing β1-integrin, cooperate with Fgf2. SC self-renewal has been associated with FGF stimulation of asymmetric phosphorylation of the α\(^1\)- and β\(^1\)-subunits (p38-α and p38-β) of p38 MAP kinase\(^9,27\). Fewer Itgb1\(^−/−\) SCs showed polarized pp38, relative to that in control cells, and Fgf2 treatment could increase the fraction of mutant SCs with polarized pp38 (Fig. 3g,h). Taken together, we have uncovered a mechanism for β1-integrin in sustaining SC expansion and self-renewal.

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Figure 4  Activating β1-integrin in aged SCs can rescue aged-associated SC defects. (a,b) Representative images of myofiber-associated young and aged SCs stained for Pax7 (green) and activated β1-integrin (act; β1; red), or with DAPI (blue) 1 h after isolation (a) and quantification of activated β1-integrin patterns in Pax7+ cells that have been scored as basal (open arrowhead in a) or non-basal (unevenly or nondetectable in a) (b) (n = 3 mice per condition; 20 myofibers per mouse). Numerical data are expressed as mean ± s.d. *P < 0.05 by Student's t-test. In a, the basal surface is indicated by the dashed line, and all images were taken at the same exposure. Scale bar, 10 μm. (c) Representative images of young (top) and aged (bottom) Pax7+ (green) SCs stained for ILK, parvin, paxillin and vinculin (all in red), or stained with DAPI (blue), 1 h after isolation. Dashed line indicates the basal surface (n = 3). Scale bar, 5 μm. All images were taken at the same time. (d) Schematic for β1-integrin activation in young (3-month-old) or aged (18-month-old) muscles after needle-track (NT) injury. 2 d after injury, IgG vehicle (10 μg/ml; V), TS2/16 activating antibody (10 μg/ml; A) or RGD peptide inhibitor (10 μg/ml; I) was injected into the injury site. Muscles were harvested 3 d later. Rleg, right leg; Lleg, left leg. (e,f) Representative images of muscle sections stained with H&E (top) or embryonic myosin heavy chain (eMyHC, bottom) for regenerated myofibers (e) and average number of eMyHC+ fibers in injured areas (f) of inhibitor (I)-, TS2/16 antibody (A)- or vehicle (V)-treated young (YI, YA or YY, respectively), or vehicle- or TS2/16-treated aged (AV and AA, respectively), mice (n = 3; ten sections per animal). Data represent mean ± s.d. **P < 0.01; n.s., not significant; by two-way ANOVA. Scale bar, 150 μm.

or β1-integrin that was undetectable (Fig. 4a,b). To ascertain age-associated changes in overall integrin activity, we monitored spatial patterns of their common effectors—integrin-linked kinase (ILK), parvin and paxillin (Fig. 4c). ILK, parvin and paxillin were localized to the laminar side in young SCs as expected. In contrast, aged SCs showed disorganized distribution patterns of these effectors. Vinculin, which binds to actin but not directly to integrin33, encircled young SCs, and this pattern was slightly disorganized in aged SCs.

Figure 5  Activating β1-integrin in aged SCs enhances FGF signaling to promote SC expansion. (a-c) Representative images of myofiber-associated aged YFP+ SCs that were cultured with IgG or with TS2/16 (10 μg/ml) in the presence of Fgf2 (10 ng/ml), for 72 h, and stained for Pax7 and MyoD (a), and quantification of Pax7+MyoD+ versus MyoD+ cells (n = 3 mice per condition; 15 myofibers per mouse) (b) and average number of YFP+ cells per myofiber (n = 3 mice per condition; 15 myofibers per mouse) (c) in myofibers that were cultured with IgG or with TS2/16 (10 μg/ml), in the presence or absence of Fgf2 (10 ng/ml). In a, asterisks indicate Pax7+MyoD+ cells, and open arrowheads indicate MyoD+ cells. DAPI was used to stain nuclei. Scale bar, 25 μm. In b, c, data are mean ± s.d. *P < 0.05; **P < 0.01; by two-way ANOVA for TS2/16 + Fgf2 versus others; n.s., not significant between the other groups. (d,e) Representative images of myofiber-associated aged SCs that were cultured for 24 h with IgG or TS2/16 and stained for Pax7 and Fgfr1 (n = 20) (d) and quantification of Fgfr1+ cells within the Pax7+ population (n = 3 experiments; 20 myofibers per condition) (e). Data are mean ± s.d. *P < 0.05 and ***P < 0.001 by Student's t-test. Scale bar, 10 μm. (f,g) Representative images of aged SCs that were cultured with IgG or TS2/16, with (or without; not shown) Fgf2, and were stained for phospho–FGF receptors (pFGFR) and Pax7 (n = 20 images of each group) (f) and percentage of pFGFR+ cells within the Pax7+ SC population (n = 3 experiments; 20 myofibers scored per condition) (g). Data are mean ± s.d. *P < 0.05 and ***P < 0.001 by Student's t-test. Scale bar, 10 μm. (h) Reciprocal co-immunoprecipitation (co-IP) analysis, as determined by western blotting, of HA-tagged Itgb1 (left) and Flag-tagged Fgfr1 (right) in HEK293T cells, with IgG or TS2/16 added. Input lysates are in the top two rows, and immunoprecipitated fractions are in the bottom two rows. Arrowheads indicate co-immunoprecipitated Flag-Fgfr1 (left) and HA-Itgb1 (right). Similar results were obtained in three separate experiments.
Thus, aged SCs show abnormal localization of, and disparate changes in, common integrin effectors, reflecting a dysregulation of overall integrin activity.

If dysregulated integrin signaling underlies the dysfunction of aged SCs, then activating β1-integrin alone may be sufficient for rescue. To test this, we injected injured muscles with a β1-integrin-activating antibody TS2/16 (ref. 34) and observed robust muscle regeneration in control young mice that were injected with vehicle (YV) (Fig. 4d–f). Treatment with the RGD peptide repressed muscle regeneration in control young mice (Y1). Although treatment with TS2/16 did not enhance the already robust levels of regeneration in young animals (YA), it did improve regeneration in aged mice (AA) to a level comparable to that in the young mice. As controls, we showed that TS2/16 treatment did not rescue regeneration of mdx/−/− muscle, but it could activate β1-integrin-dependent signaling (Supplementary Fig. 7a–d). Regenerated muscles still had measurable improvement 30 d after injection with a single dose of TS2/16 (Supplementary Fig. 7e–g).

To determine whether TS2/16 treatment improves SC function, we applied it to myofiber-associated aged SCs and assessed their expansion and Pax7 expression. Treatment with the IgG control, or that with TS2/16 or Fgfl2 alone, did not show an effect. Treatment with TS2/16 and Fgfl2 together increased the fraction of Pax7+ cells, the number of fiber-associated myogenic cells and the fraction of SCs showing polarized pp38 (Fig. 5a–c and Supplementary Fig. 8). Because treatment with TS2/16 alone is sufficient to improve aged muscle regeneration in vivo, we surmise that muscle damage releases a sufficient amount of Fgf2 to cooperate with TS2/16-activated β1-integrin in aged SCs. Mechanistically, we found that TS2/16 treatment increased the fraction of aged SCs on myofibers with detectable Fgfr1, relative to that after treatment with control IgG (Fig. 5d,e). Although it not sufficient to enhance expansion, treatment with TS2/16 alone increased the proportion of aged SCs with detectable phosphorylated FGF receptors (pFGFR). Treatment with TS2/16 and Fgfl2 together stimulated pFGFR in almost all of the aged SCs (Fig. 5f,g), consistent with their dual requirement for enhancing expansion of aged SCs. Associations between FGF receptors and either α-integrins or αβ3-integrins were suggested to underlie FGF–integrin cooperativity. We show here that Fgfr1 can associate with β1-integrin and that TS2/16 enhances their association (Fig. 5h). These data indicate that TS2/16 operates at multiple layers to enhance FGF signaling and restore the responsiveness in aged SCs.
Activating β1-integrin improves dystrophic muscles

The positive effect of TS2/16 on regeneration in the environment of aged cells led us to consider whether it might be beneficial in another context of impaired muscle regeneration, namely muscular dystrophy. For this, we used the mdx mouse model, in which mice lack dystrophin expression due to a nonsense mutation in the Dmd gene15. Because muscle in the mdx mouse contains disorganized ECM, we anticipated that more Pax7+ SCs would reside outside of the myofiber lamina, relative to those in the control mice (C57BL/10; Supplementary Fig. 9a–c). Additionally, mdx SCs that were associated with myofibers showed abnormal patterns of active β1-integrin (Supplementary Fig. 6b,c). We administered TS2/16 into the tibialis anterior (TA) muscle of mdx mice and found that a single dose was sufficient to promote the expansion of myogenic cells, as shown by increased EdU incorporation 3 d after treatment (Fig. 6a,b). We extended TS2/16 treatment to four weekly injections (Fig. 6c) and found that the cross-sectional area and muscle fiber diameter were increased, relative to those in mice that were treated with control IgG (Fig. 6c–e). Also, the percentage of Pax7+ cells outside of the myofiber lamina was reduced, reflecting an improvement in SC–niche interaction (Supplementary Fig. 9c–e). Because β7/1-integrin binds to laminin, the ECM component that engages with the dystrophin complex, activating β1-integrin probably also enhances the integrity of muscle fibers in mdx mice via improving their connection to the ECM.

To determine whether the above-mentioned long-term regimen resulted in functional rescue, we compared the contractile properties of IgG- and TS2/16-treated mdx TA muscles in situ; wild-type (WT) and untreated mdx TA muscles were analyzed in parallel as references (Fig. 6f–j). TS2/16-treated mdx muscles had reduced cross-sectional areas, as compared to mdx and IgG-treated mdx muscles, consistent with a reversal from hypertrophic pathology. Of note, TS2/16-treated muscles showed improvements in strength, using a variety of measurements including single-twitch force, maximum isometric tetanic force, frequency–force relationship, time to fatigue and fatigue index. Activating β1-integrin may therefore be a viable therapeutic means to improving muscle repair and function in disease conditions.

DISCUSSION

Our results frame a model in which β1-integrin functions as a sensor of the SC niche, which declines in function during aging (Supplementary Fig. 10). Because Igfbp1+/− cells show compromised pErk induction after Fgf2 treatment, the exit from their quiescence is more likely due to cell-polarity defects than to overt FGF–Erk signaling2. In the context of regeneration, β1-integrin has a distinct role in cooperating with Fgf2 to drive SC proliferation and renewal. In aged muscle, changes in ECM29,30 impose physiologically relevant alterations in integrin activity37, which presumably contribute to the decline of SC sensitivity to Fgf2. The current view regarding FGF and aging SCs is puzzling. Aging SCs are sensitive to increased FGF–Erk signaling, causing quiescence break and stem cell pool depletion2, and yet aged SCs are insensitive to Fgf2 for proliferation in vitro9. Our study provides a potential explanation—a fraction of aging SCs with sufficient integrin activity cooperates with increasing amounts of Fgf2 to break quiescence and becomes lost, whereas aging SCs with dysregulated integrin activity that is insufficient to support FGF signaling remain. As such these remaining aged SCs cannot support robust regeneration after injury, unless integrin activity is re-established to restore FGF signaling, for example, by administration of TS2/16.

Although activating β1-integrin alone can improve regeneration in both aged and dystrophic muscles, it may prove more effective to target all of the integrins that are relevant to SC expansion and FGF sensitivity, such as the RGD-binding integrins. There are at least eight integrins (three of which contain β1-integrin) that bind to the RGD motif, which is the integrin-binding site of many ECM molecules, including fibronectin14. Although β1-integrin and fibronectin cooperate with Fgf2, we cannot exclude contributions by other RGD-containing ECM components. Conversely, because SCs express almost all of the α- and β-integrins26, defining the contribution of each integrin in this context may involve considerable effort. Despite the complexity, our findings help to explain why ECM implantation can enhance muscle regeneration38. Future investigation into how the integrin–FGF axis intersects with other pathways, such as that involving the transcription factor signal transducer and activator of transcription 3 (Stat3), to regulate SC function is important. Although Stat3 expression is not changed in Igfbp1+/− SCs, Stat3 is upregulated in activated SCs39, and inhibiting Stat3 enhances regeneration in the contexts of aging and dystrophies16,11. We would be remiss in not mentioning that, other than Fgf2, integrins can cooperate with many growth factors12 that may also contribute to SC function. We provide here a proof-of-principle study that may be broadly applicable to muscle diseases that involve SC niche dysfunction, but further refinement is needed for this method to become a viable treatment. Given the role of integrins in other stem cell populations19,40, the knowledge derived from our study should, in general, have broader implications for aging and the decline of stem cell function.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA); all sequencing data can be found under accession code SRP070128.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.R. and C.-M.F. conceptualized the study; M.R. performed experimental analysis for the mutant mice, as well as for the FACS and RNA-seq data, and demonstrated the utility of TS2/16; L.L. performed mechanistic experiments to investigate integrin–FGF synergy and conducted in situ muscle force measurements. C.-M.F. initiated and supervised the project. All three authors wrote, discussed and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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online methods

Mouse studies. Mouse experiments in this study were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Carnegie Institution of Washington (Permit number A3861-01). The Pax7CreERT2 (also known as Pax7Cre) allele (B6;129-Pax7tm1(creERT2)Fan/J) has been described15. The Itgb1 allele (B6;129-Itgb1tm1(espj)j3) and the R26R2flox (B6;129S4-Gr(ROSA)26Sor1(cre)1Vad/J) and R26RFP (B6;129X1-Cgr(ROSA)26X SYT1FP10/J) reporter mice were obtained from the Jackson Laboratory. The experimental mice used in this study were PaxCyclCe+Jtg100, R26RFP;AzacLt;PaxCreCe+Jtg100;R26RFP;YFP, or PaxCyclCe+Jtg100 (referred to as Itgb1+/-). Reporter alleles were chosen based on the assay: YFP is preferable for immunofluorescence, and necessary for live-imaging and FACS sorting, whereas LacZ is useful for histological analyses. Controls used were PaxCyclCe+R26RFP;AzacLt;PaxCreCe+R26RFP;YFP, or PaxCyclCe+.

For young versus aged comparisons, mice were used at 3–6 months of age (young) or 18–24 months of age (aged). For non-lineage-marked SC studies, aged C57BL/6J mice were used (JAX and NIH). Sex was mixed. For dystrophic muscle studies, C57BL/10-background control and mdx male mice (JAX) were used at 3–4 months of age.

Mice were given tamoxifen (txm; 20 mg/ml in corn oil (Sigma)) at 3 mg per 40 g body weight per intraperitoneal injection, once a day consecutively for 5 d. All experiments, except where noted, were conducted 3 d after the final injection. For injury, mice were anesthetized using 2,2,2-tribromoethanol (Sigma), which was dissolved in 2-methyl-2-butanol (Sigma) as a 100% stock solution, diluted 1:80 in PBS, and injected intraperitoneally (wt/vol).

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For dystrophic muscle studies, C57BL/10-background control and mdx male mice (JAX) were used at 3–4 months of age.

Mice were given tamoxifen (txm; 20 mg/ml in corn oil (Sigma)) at 3 mg per 40 g body weight per intraperitoneal injection, once a day consecutively for 5 d. All experiments, except where noted, were conducted 3 d after the final injection. For injury, mice were anesthetized using 2,2,2-tribromoethanol (Sigma), which was dissolved in 2-methyl-2-butanol (Sigma) as a 100% stock solution, diluted 1:80 in PBS, and injected intraperitoneally (wt/vol).

For dystrophic muscle studies, C57BL/10-background control and mdx male mice (JAX) were used at 3–4 months of age.

Muscle sample processing. TA muscles were harvested, fixed for 8 min in ice-cold 4% paraformaldehyde (EM) in phosphate-buffered saline (PBS), incubated sequentially in 10% and 20% sucrose in PBS overnight, frozen in isopentane (Sigma) and liquid nitrogen, and stored in a −80 °C freezer until cryosectioning. Cross-sections (10 μm) were stained with hematoxylin and eosin (H&E; Surgipath), or Gomori’s one-step trichrome staining kit (Polysciences), or they were subjected to X-gal (Sigma) reactions as described previously43 or used for immunostaining and EdU reactions (see below).

Satellite cell isolation and myoblast culture. YFP-marked cells were isolated as described previously17. Briefly, for SC preparation, muscles were dissected and incubated in 0.2% collagenase type I (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) at 37 °C with gentle shaking for 1.5 h. Muscle was then triturated in 10% FBS in DMEM, washed with PBS, and incubated in 0.2% dispase (Gibco) in DMEM at 37 °C with gentle shaking for 30 min. Cells were filtered through a 70-μm cell strainer (VWR) and subjected to cell sorting using the BD FACS ARIA III, gating first for cell size using forward and side scatter, and then for YFP fluorescence. FACS Diva (for cell isolation) software was used. Cells were then used for downstream analyses. For cell cycle analysis, migration, and programmed cell death assays, as well as for protein extracts for western blotting (see below), or for RNA isolation for RNA-seq analysis (see below) over a time course, cells were cultured in ‘minimal’ growth medium (10% horse serum (HS), 1% penicillin–streptomycin, 1% Glutamax (GIBCO) on Matrigel-coated (BD biosciences) tissue culture dishes. For differentiation and fusion, cells were cultured in medium containing 2% HS on Matrigel. For Erk and Akt signaling assays (see below), when considerably more cells were needed, cells were expanded as myoblast cultures in ‘enriched’ growth medium (20% FBS, 5% HS, 1% penicillin–streptomycin, 1% Glutamax (Gibco), 0.1% chick embryo extract (MP Biomedicals), and 10 ng/ml FGF (R&D systems)) on Matrigel, until sufficient cell numbers were reached, typically in 5–7 d. All cell cultures were placed in 37 °C tissue culture incubators with 5% CO2.

Myofibers with associated SCs were isolated from extensor digitorum longus (EDL) muscles by 1.5-h digestion in 0.2% collagenase type I in DMEM at 37 °C. The digested muscle was then transferred to tissue culture dishes containing DMEM, 1% penicillin–streptomycin, and 1% Glutamax. Live myofibers were isolated with a fire-polished glass pipette. Isolation of individual myofibers by pipette was repeated to remove dead myofibers and cellular debris. They were either immediately fixed for assays (for example, probe for activated β1-integrin) or placed in DMEM, 10% HS, 1% penicillin–streptomycin, and 1% Glutamax with daily medium and reagent changes. Please note that this minimal growth medium contains no additional additives. Depending on the assays, myofibers were cultured for different amounts of time before fixation. For Par3, p38, Fgfr1, and pFgfl analysis, myofibers were cultured for 36 h. For renewal assays with Pax7 and MyoD expression, 48- to 96-h cultures were used (specified in the text or figure legend). They were either cultured with or without Fgf2 (10 ng/ml), and with or without IgG or Ts216 (10 μg/ml). Phosphatase Inhibitor Cocktail Set II (Calbiochem) was used as directed during fixation.

For FGF and fibronectin stimulation, we modified the condition described previously for other cell types18,21. On the day of the experiment, control and Itgb1+/- myoblasts were detached by treatment with 2 mM EDTA in serum-free base medium (SFM; high-glucose DMEM, 0.5% BSA, penicillin–streptomycin), washed twice, and cultured in SFM on Petri dishes as cell suspensions for 2 h to minimize the residual effect of growth factors and the contact-dependent signaling from prior culture conditions. 50,000 cells were then transferred to each well of a 12-well dish containing SFBM with or without fibronectin (10 μg/ml) for 20 min. The wells were either coated with Sigma-cote (to prevent attachment) or pre-coated with fibronectin (10 μg/ml overnight). Specifically, concentrations of Fgf2 were then added, and cells were harvested 10 min later for western blots (see below for detailed procedure). Blots were first probed with anti-pErk1/2 and anti-pAkt, followed by HRP-conjugated secondary antibodies, and binding was then detected with ECL (Amersham). Blots were then stripped and reprobed with anti-Erk1/2 and anti-Akt. Fold of stimulation is presented as pErk/Akt and pAkt/Akt ratios relative to pErk/Akt and pAkt/Akt ratios, respectively.
cells in SFBM. The pErk/Erk and pAkt/Akt ratios of control cells in SFBM were used as the normalization denominator and were set at an arbitrary unit of 1.

**Immunostaining.** Cells or muscle sections were fixed for 10 min in 4% paraformaldehyde, permeabilized with 0.1% Triton-X 100 (Sigma) in PBS for 15 min at room temperature (RT), rinsed with wash buffer (0.05% Triton-X 100 in PBS), and treated with blocking buffer (10% normal goat serum (NGS) (Genetex) and 1% blocking powder (Perkin Elmer) in wash buffer) for 1–2 h before overnight incubation at 4 °C with primary antibodies diluted in blocking buffer. Primary antibodies against the following antigens were diluted as follows: activated β1-integrin, 1:200; β1-integrin, 1:200; BrdU, 1:200; cleaved caspase-3, 1:400; eMyHC, 1:200; GFP (used to detect YFP), 1:500 (rabbit) or 1:200 (chick); ILK, 1:50; laminin, 1:2,000; MHC, 1:20; MyoD, 1:50; parvin, 1:400; Pax7, 1:20; Paxillin, 1:250; pp38, 1:50; vinculin, 1:400. Cells or muscle sections were washed with wash buffer and incubated with the appropriate Alexa-Fluor-conjugated secondary antibodies (1:1,000, Invitrogen), which could be detected at different fluorescent wavelengths, in blocking buffer for 1 h at RT. After washing the slides and following an incubation with DAPI (1 μg/ml for 5 min), the slides were mounted with Fluoromount-G (SouthernBiotech) and coverslips (VWR). For BrdU detection, slides were treated with antigen-unmasking solution (Vector) by boiling for 10 min before blocking and adding the primary antibody. For EdU detection, the Click-iT reaction kit (Invitrogen) was used before the samples were incubated with DAPI.

**Western blot.** For protein extraction, cells were lysed in T-Pers Tissue Protein Extraction Reagent (Thermo Fisher Scientific), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), 1× Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific), and Complete Protease Inhibitor Tablets (Roche). Total protein extract was resolved by SDS–PAGE on precast gels (BioRad) and was then transferred to Immuno–blot PVDF membranes (BioRad) using a BioRad mini-Protein II Transfer system. Membranes were rinsed in 0.1% Tween (Sigma) in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl), blocked for 1 h at RT in 5% low-fat milk (Carnation) in 0.1% Tween–TBS (blocking buffer) and were then incubated with primary antibodies in blocking buffer overnight at 4 °C. Primary antibodies against the following antigens were used at specified dilutions: β1-integrin, 1:1,000 (Millipore); cyclin A, 1:200; cyclin B1, 1:200; cyclin D1, 1:200; cyclin D2, 1:200; cyclin D3, 1:200; cyclin E, 1:200; Gapdh, 1:5,000; pErk, pAkt, Akt, Fak, and pFak, all at 1:1,000. After washing in 0.1% Tween–TBS, appropriate secondary antibodies (Amersham, Invitrogen, and BioRad) were diluted 1:10,000 in blocking buffer, added to the blots and incubated for 1 h at RT.

**Co-immunoprecipitation.** HEK293T cells (Clontech; tested negative for mycoplasma contamination by MycoProbe Mycoplasma Detection Kit (R&D Systems)) were plated on fibronectin-coated 6-well dishes and transfected with pcDNA3-FLAG-1-Flag and/or pcDNA3-Bbg1-HA by Lipofectamine2000 (Invitrogen) overnight, according to the manufacturer’s manual. The next day they were incubated in medium containing 5 μg/ml IgG or TS2/16 for 1 h at 4 °C. Cells were washed with cold PBS and lysed in 1% NP40 (50 mM HEPES, 150 mM NaCl, 10% glycerol, protease inhibitors (Thermo Fisher Scientific)) for 25 min at 4 °C. Lysates were clarified by centrifugation and subjected to immunoprecipitation with anti-HA affinity matrix (Pierce) or anti-Flag affinity matrix (Sigma) for 2 h at 4 °C. The affinity matrix was washed and the proteins were eluted in 2× SDS–PAGE sample buffer for western blotting, using anti-Flag and anti-HA antibodies (Sigma).

**Force measurements and fatigue analysis.** In situ force measurements of TA muscles were conducted as done previously44,45, and the data were analyzed using the 1300A Whole-Animal System (Aurora Scientific). Mice were anesthetized with isoflurane and placed on an isothermal stage. Intact TA muscles were dissected and constantly immersed in homemade Ringer’s solution (according to recipe in ref. 44). Single-twitch or tetanic contractions were elicited with electrical stimulations applied by two electrodes placed on either side of the muscle. In all experiments we used 0.2-ms pulses at 10 V supramaximal voltage. Muscle optimal length (L0) that allows a maximum isometric twitch force (P0) was determined by a series of twitch contractions with small variations of the muscle tension. To obtain maximum isometric tetanic force (Pmax), muscles were stretched for 300 ms at different frequencies from 50 to 200 Hz. A 1-min recovery period was allowed between stimulations. The muscles were then fatigued at 150 Hz with one contraction per second for 180 s. Muscle wet weight and L0 were used to calculate the cross-sectional area (CSA) of the TA muscle for normalization, to obtain specific isometric twitch force sP0 (kN/mm2) and sPmax (kN/mm2).

**Microscopy and image processing.** Images of H&E-stained muscle sections were captured from a Nikon 800 microscope with 10× or 20× Plan Apo objectives and a Canon EOS T3 camera using EOS Utility image-acquisition software. Fluorescence images of muscle sections and single myotubes were either captured with a Leica SP5 confocal microscope equipped with 40×/0.125 Plan Apo oil objectives using Leica image acquisition software or with a Zeiss Axioscope equipped with a 40×/0.5 Plan Apo oil objective and an Axioscam camera using Zeiss image acquisition software. Identical exposure times were used, and images were processed and scored with blinding using ImageJ64 (NIH). Where necessary, brightness and contrast were adjusted for an entire experimental image set. For quantification of polarized cell markers (Par3, m-cadherin, pp38), all authors individually scored each set of images with blinding, using ImageJ64; only those images that were agreed upon by all of the authors were included. For the remaining images, Imaris (Bitplane) was used for three-dimensional rendering of fluorescence data for quantification of polarization. A subset was also randomly selected for quantitative analysis via Imaris to confirm the authors’ scoring. Cell number, fiber diameter, fiber number, and fiber cross-sectional area were determined using ImageJ64 or Fiji, using images of a micrometer (VWR) taken under the same magnifications as the sample images as references for imaging-field sizes.

**Fluorescence-activated cell sorting (FACS) analysis of DNA content.** Cells were collected at staggered time points post activation (24, 48, 72 h). Cells were centrifuged (2500g, 4 °C, 10 min) and washed twice in cold PBS. Cell pellets were resuspended in solution of 70% cold ethanol and fixed overnight at 4 °C. After washing twice with cold PBS, the cells were resuspended in 1 ml of a 1:1 PI solution (0.1 mg/ml propidium iodide in 0.6% Triton-X in PBS (Sigma; P4170)); RNase solution (2 mg/ml in milli-Q H2O (Sigma; R5125)) and stained in the dark for 45 min. Cells were passed through meshed capped Falcon tubes, to avoid clumping, before running them on a FACS machine. FACS analysis was carried out with a BD FACs ARIA III machine and FACS Diva software, gating first for cell size, and using forward and side scatter and then gating for FYP+ cells using the 488 channel. ModFit LT V2.2.11 was used to analyze percentages of each phase of the cell cycle (G1, S, and G2/M). All data were determined to have ‘good’ reduced chi-square (RCS), measurement of fit.

**RNA-seq analysis.** For RNA-seq analysis, FACS-isolated SCs were cultured in minimal growth medium on Matrigel-coated plates. At 24, 48, and 72 h, RNA was isolated using the Arcturus PicoPure RNA isolation kit (Applied Biosystems). The Ovation RNA-seq System (NuGEN) was used to prepare amplified cDNA. Libraries for single-end sequencing were prepared using Illumina’s TrueSeqDNA sample prep kit LT. Sequencing was carried out on an Illumina HiSeq2000 to generate single-end 100–bp reads, which were aligned to the mouse genome (mm9) using TopHat (v2.0.7). cufflinks (v2.1.1) was used for differential expression analysis. Only genes that displayed ‘yes’ significance, as determined by Cuffdiff2, were analyzed further, if the P value of the observed change in FPKM was greater than the false discovery rate (FDR) after Benjamini–Hochberg correction for multiple-testing46.

**Statistical analyses.** Quantitative values are expressed as the mean ± s.d. or mean ± s.e.m. Statistical differences between groups were determined by using an Excel spreadsheet and by using a t-test for two-tailed paired comparisons. When comparing more than two sets, one-way or two-way ANOVA with Tukey’s post hoc test was performed using GraphPad Prism 6. P < 0.05 was determined to be significant for all experiments. All experiments requiring the use of animals or animals to derive cells were subject to randomization based on litter. No animal or samples were excluded from the study. Sample size was predetermined based on the variability observed in prior experiments and on preliminary data. Investigators were not blinded to outcome assessment.
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