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28. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Supporting Online Material

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Notch-Mediated Restoration of Regenerative Potential to Aged Muscle

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A hallmark of aging is diminished regenerative potential of tissues, but the mechanism of this decline is unknown. Analysis of injured muscle revealed that, with age, resident precursor cells (satellite cells) had a markedly impaired propensity to proliferate and to produce myoblasts necessary for muscle regeneration. This was due to insufficient up-regulation of the Notch ligand Delta and, thus, diminished activation of Notch in aged, regenerating muscle. Inhibition of Notch impaired regeneration of young muscle, whereas forced activation of Notch restored regenerative potential to old muscle. Thus, Notch signaling is a key determinant of muscle regenerative potential that declines with age.

Quiescent skeletal muscle precursor cells, or satellite cells, are positioned between the basal lamina and the plasma membrane of muscle fibers (*1*, *2*). In response to injury, these cells undergo activation, a process defined as the break from quiescence and the initiation of cell proliferation. They then progress along a myogenic lineage pathway to generate myoblasts, which ultimately fuse to each other or to injured myofibers to promote repair and regeneration (*1*). Satellite cells represent the endogenous source of muscle precursor cells and account for more than 99% of the regenerative potential of adult muscle (*3*, *4*). The efficacy of skeletal muscle regeneration is markedly impaired with age (*5*, *6*), but the role of age-related changes in satellite cell activation in this decline is unknown. We recently showed that satellite cell activation, proliferation, and cell lineage determination are regulated by the Notch signaling pathway (*7*), a pathway well characterized for its role in myogenesis (*8*, *9*) and in tissue formation during embryogenesis (*10*–*12*). Although alterations in Notch signaling have been associated with developmental abnormalities and diseases (*11*, *13*), changes in Notch signaling have not been implicated in the aging process. These studies demonstrate that the age-related decline of muscle regenerative potential is due to a decline in Notch signaling and can be reversed by Notch activation.

The diminished regenerative potential of aged muscle was evident when injured hindlimb muscles of young (2 to 3 months), adult (5 to 7 months), and aged (23 to 24 months) mice were analyzed for activated, proliferating satellite cells associated with the myofibers (Fig. 1A; fig. S1A). Accordingly, many fewer myoblasts were generated in myofiber explant cultures (*7*) from

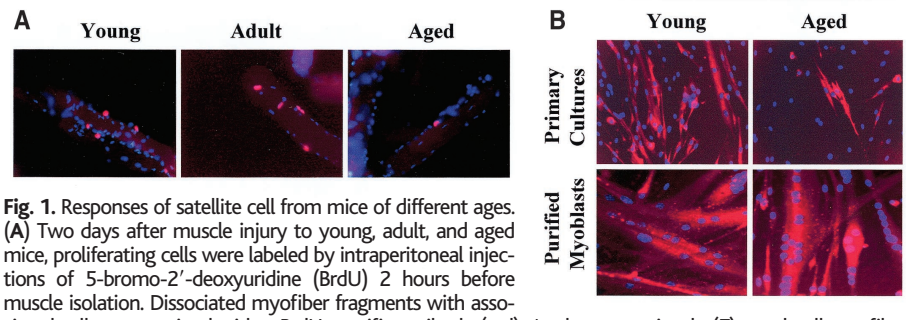

Fig. 1. Responses of satellite cell from mice of different ages. (**A**) Two days after muscle injury to young, adult, and aged mice, proliferating cells were labeled by intraperitoneal injections of 5-bromo-2′-deoxyuridine (BrdU) 2 hours before muscle isolation. Dissociated myofiber fragments with associated cells were stained with a BrdU-specific antibody (red). As shown previously (*7*), nearly all myofiber-associated, $BrdU^+$ cells express myogenic markers such as M-cadherin or desmin, which confirms that they are derived from satellite cells. Hoechst costain (blue) labels nuclei. There were one-fourth to one-fifth as many $BrdU^+$ satellite cell–derived myoblasts associated with myofibers from old mice as from young or adult mice (fig. S1A; $n \geq 3$). (**B**) (Top) After 4 days ex vivo, cells from young and aged explant cultures were switched to differentiation medium to promote fusion and then stained with an antibody to embryonic myosin heavy chain (red) and with Hoechst dye. A marked decrease in the number of myotubes reflected the decreased myoblast production from old muscle. Results from adult cultures were similar to those shown for young cultures (see fig. S1B). (Bottom) Young and aged myoblasts were adherence-purified, expanded, plated at equal numbers, and then cultured in differentiation medium. Under these conditions, myoblasts from aged mice fused to form myotubes as readily as did myoblasts from young mice ($n \geq 3$).

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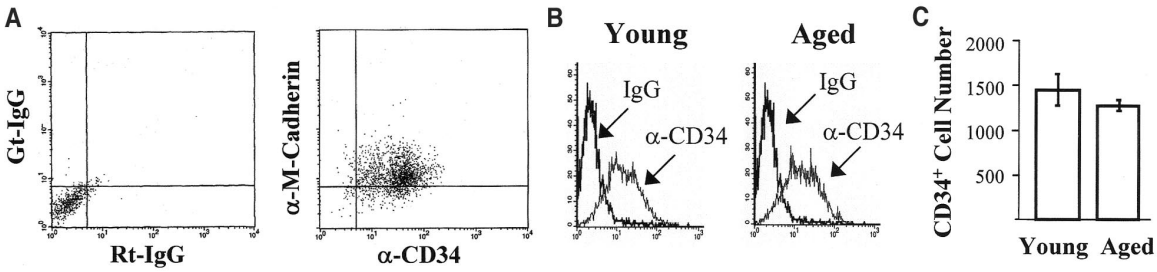

Fig. 2. Satellite cell numbers from mice of different ages. (**A**) Purified populations of satellite cells were derived from adult muscle (see Methods). Cells were costained with antibodies to CD34 and M-cadherin and subjected to flow cytometry. (Left) Negative controls; (right) more than 95% of cells were $CD34^+$/M-$cadherin^+$. Similar results were obtained from muscles of all ages ($n \geq 5$). (**B**) Satellite cells were purified from young and aged muscles, stained with a CD34-specific or control antibody, and analyzed by FACS. The analysis shows cell count on the *y* axis and the immunofluorescence of either the control (IgG) or CD34-specific antibody on the *x* axis. (**C**) The numbers of $CD34^+$ satellite cells from young and aged skeletal muscle were determined in multiple independent experiments. Data represent means ± S.D ($n = 4$).

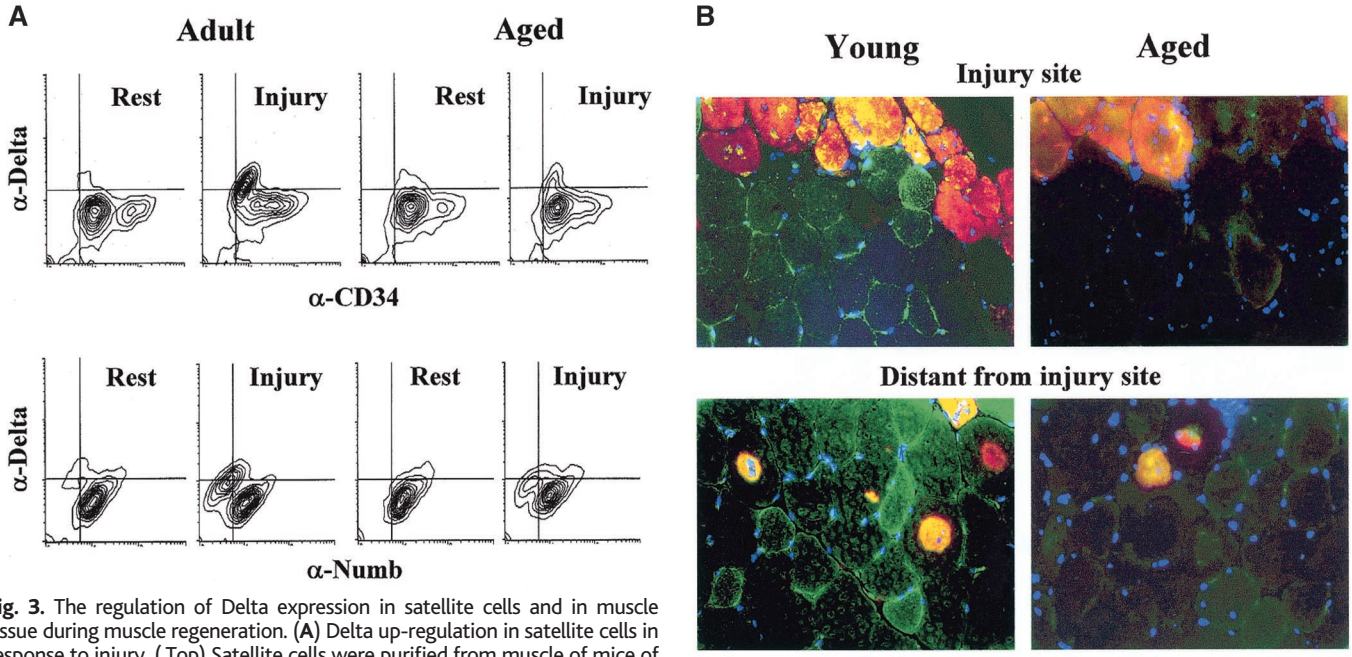

Fig. 3. The regulation of Delta expression in satellite cells and in muscle tissue during muscle regeneration. (**A**) Delta up-regulation in satellite cells in response to injury. (Top) Satellite cells were purified from muscle of mice of different ages, either before injury (rest) or 1 day after injury; costained with antibodies to CD34 and Delta; and analyzed by FACS. Injury induced the expression of Delta in a large percentage (~35%) of the cells from adult muscle but in a small percentage (~7%) of cells from aged muscle ($n = 4$). (Bottom) Satellite cells were also analyzed for the simultaneous expression of Delta and Numb at rest or in response to injury and revealed a coordinate increase in Delta and decrease in Numb in activated satellite cells from young or adult muscle. Similar results were obtained in three independent experiments. (**B**) Muscles from young and aged mice were subjected to freeze injury. The mice were injected with Evans blue dye 12 hours later and killed after 24 hours. Muscle cryosections were analyzed by immunofluorescence using a Delta-specific antibody (green) and Hoechst dye (blue). Evans blue dye, which is taken up only by injured fibers, appears red. Two cross-sectional areas are shown for each age. The top panels are through the injury site at the border of the injury. The bottom panels are cross sections ~300 μm caudal to the injuries. Results were similar in four independent experiments. Delta expression in adult muscle was similar to that shown for young muscle (and see fig. S2C).

aged mice than from young or adult mice (fig. S1B). This difference was dramatically apparent when the cells were induced to differentiate, even though there was no major defect in the propensity of aged myoblasts to fuse (Fig. 1B). This age-related decline in myoblast generation is consistent with results from single fiber preparations (*14*).

Previous studies, using various methodologies to study different muscles from different species, have diverged in their conclusions as to whether there is a decline, no change, or even a relative increase in satellite cell density with age (*15*–*18*). To determine whether a decrease in satellite cell number with age might account for the diminished myoblast production that we observed, we quantified purified satellite cells isolated from mouse hindlimb muscles over this age range. Fluorescence-activated cell sorting (FACS) analysis demonstrated that nearly 100% of the purified cells expressed both CD34 and M-cadherin (Fig. 2A), and less than 2% of the cells expressed the endothelial cell marker PECAM (fig. S1C), which confirmed that these were indeed satellite cells (*19*). There was no significant difference in satellite cell number between young and old muscle (Fig. 2, B and C). This was confirmed by Western blot analysis of CD34 expression in myofiber explants (fig. S1D). Therefore, the dramatic age-related decline in myoblast generation in response to injury is due to an impairment of activation rather than a decline in number of satellite cells.

Because Notch signaling plays a critical role in satellite cell activation and adult muscle regeneration (*7*), we compared satellite cells from adult and aged muscle for the expression of Notch-1, its ligand Delta-1, and its inhibitor Numb (*11*, *20*). In satellite cells from resting muscle of all ages, there was negligible expression of Delta-1 and high levels of Numb expression (Fig. 3A; fig. S2, B and C), consistent with previous results (*7*), and the levels of full-length Notch-1 were very similar (fig. S2A). In response to injury, satellite cells

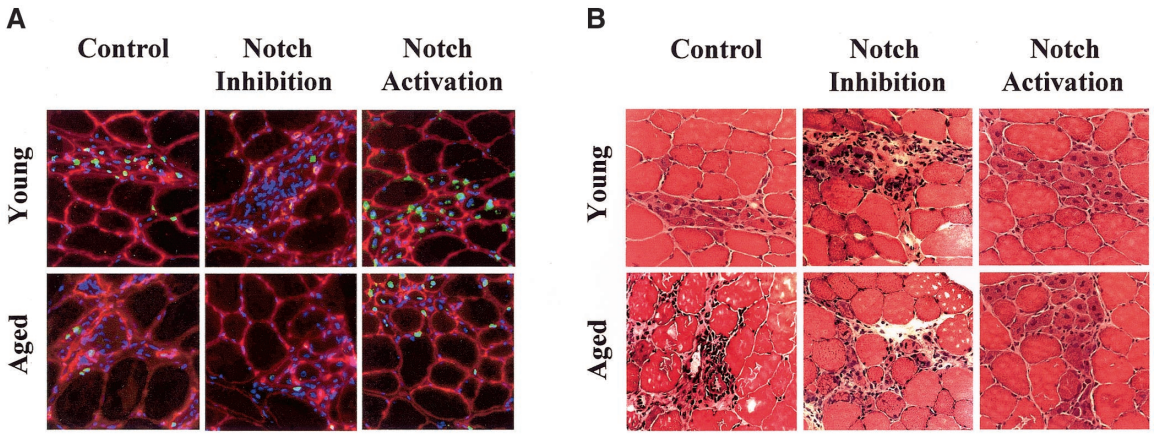

Fig. 4. Experimental modulation of Notch signaling dramatically affects muscle regeneration in young and aged muscle. (**A**) Muscles of young or aged mice were injected with an inhibitor of Notch signaling (Jagged-Fc), an activator of Notch signaling (Notch-1–specific antibody), or respective controls (see fig. S3, A to C). Two days later, BrdU was injected intraperitoneally. Muscles were removed 5 days after injury. Immunofluorescence was performed on sections using antibodies to BrdU (green) and laminin (red). Laminin staining delineates boundaries of nascent and mature myofibers. The inhibition of Notch by Jagged-Fc reduced BrdU incorporation into nascent myotubes and reduced myotube formation in young muscle, whereas activation of Notch by antibody against Notch-1 promoted BrdU incorporation and regeneration in old muscle. (**B**) Hematoxylin and eosin staining confirms the dramatic inhibitory effect of Jagged-Fc on regeneration of young muscle and the dramatic positive effect of Notch-1–specific antibody on regeneration of old muscle. At least three mice of each age were used for the quantitative analysis for each condition (fig. S3D).

from young and adult muscle up-regulated Delta-1, whereas old satellite cells failed to do so (Fig. 3A; fig. S2C). Delta up-regulation was associated with cell proliferation as demonstrated by PCNA staining (fig. S2B). Interestingly, increased Delta expression was associated with decreased Numb expression (Fig. 3A). Analysis of multiple experiments demonstrated that there were about one-fourth as many activated satellite cells ($CD34^+$/M-cad^+/$Delta^+$/$Numb^-$) in old muscle as in young or adult muscle in response to injury.

We used immunoblot analysis to test whether the reduced Delta up-regulation after injury was associated with lower levels of activated Notch in old cells. The levels of transmembrane Notch-1 were similar in cultures of all ages (fig. S2D). However, there were consistently lower levels of activated Notch in old satellite cells activated ex vivo. The biphasic pattern of Numb expression during activation of aged satellite cells was similar to that previously described in young cells (*7*).

We also analyzed the expression of Delta after muscle injury in vivo in young and aged mice. In young mice, Delta was induced adjacent to the injury site not only in satellite cells, but also prominently at myofiber membranes and possibly in interstitial cells in this region (Fig. 3B). This pattern was also observed at a distance from the injury site, which suggested a diffusible signal for Delta up-regulation. By contrast, there was almost no up-regulation of Delta in old muscle after injury, a finding confirmed by Western analysis, which included both satellite cells and myofibers (fig. S2C).

To further test the significance of Notch signaling for regeneration of young and aged muscle, we introduced specific inhibitors and activators of Notch at sites of injury and analyzed their effects on muscle repair. To inhibit Notch activation we used a soluble Jagged-Fc fusion protein (*21*), which in vitro blocked Notch activation, decreased cell proliferation, and enhanced differentiation of myogenic cells (fig. S3, A to C). When Jagged-Fc was introduced at sites of injury, there was a marked inhibition of young muscle regeneration (Fig. 4). Whereas regenerating young muscle was essentially devoid of inflammatory cells and nascent scar formation, the presence of the Notch inhibitor led to ineffective regeneration, similar to that seen in old muscle (Fig. 4). Analysis of multiple experiments showed a dramatic and consistent reduction in the number of regenerating myotubes in young muscle treated with Jagged-Fc compared with control treatment (fig. S3D).

We then tested whether forced activation of Notch could improve regeneration of aged muscle. We activated Notch directly, using an antibody to its extracellular domain (fig. S3A), which resulted in an increase in cell proliferation and an inhibition of myogenic differentiation in vitro (fig. S3, B and C). Strikingly, acute activation of Notch signaling in vivo markedly improved the regeneration of old muscle, rendering it similar to young muscle (Fig. 4, A and B). Analysis of multiple experiments showed that Notch activation at the time of injury reproducibly and significantly enhanced the formation of regenerating myotubes in aged, injured muscle (fig. S3D). Therefore, Notch activation is not only necessary for regeneration of young muscle but also sufficient to promote effective regeneration of aged muscle.

The results of this study demonstrate that inadequate activation of Notch-1 by Delta contributes to the loss of regenerative potential in old skeletal muscle. We propose that a decline in Notch signaling necessary for cell proliferation and cell fate determination may also occur in progenitor cells and stem cells in other tissues with age and may be a general mechanism underlying the diminished regenerative properties of aged tissues.

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