MUSCLE REPAIR

JMJD3 activated hyaluronan synthesis drives muscle regeneration in an inflammatory environment

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Muscle stem cells (MuSCs) reside in a specialized niche that ensures their regenerative capacity. Although we know that innate immune cells infiltrate the niche in response to injury, it remains unclear how MuSCs adapt to this altered environment for initiating repair. Here, we demonstrate that inflammatory cytokine signaling from the regenerative niche impairs the ability of quiescent MuSCs to reenter the cell cycle. The histone H3 lysine 27 (H3K27) demethylase JMJD3, but not UTX, allowed MuSCs to overcome inhibitory inflammation signaling by removing trimethylated H3K27 (H3K27me3) marks at the *Has2* locus to initiate production of hyaluronic acid, which in turn established an extracellular matrix competent for integrating signals that direct MuSCs to exit quiescence. Thus, JMJD3-driven hyaluronic acid synthesis plays a proregenerative role that allows MuSC adaptation to inflammation and the initiation of muscle repair.

Muscle stem cells (MuSCs) provide skeletal muscle with an efficient mode for regeneration of damaged tissue (*1*). After injury, the regenerative process is initiated by necrosis of damaged myofibers and the release of myokines that instruct recruitment of various tissue-resident and infiltrating cell types that coordinate muscle repair (*2*). Tight control of signal integration from the regenerative milieu promotes the expansion of muscle progenitors to mediate both myofiber repair and stem cell–niche repopulation (*3*, *4*). Recent work identified a population of infiltrating macrophages that establish a transient niche necessary for quiescent MuSCs to reenter the cell cycle (*5*). However, it remains unknown how MuSCs adapt to this modified niche to initiate regeneration.

In response to injury, MuSCs undergo a stress response that is accompanied by alteration of the epigenetic landscape (*6*). Among the different epigenetic changes induced by injury in MuSCs, global levels of trimethylated histone H3 lysine 27 (H3K27me3) are reduced as stem cells transition from a quiescent to a proliferative state (*7*). Removal of H3K27me3 marks is mediated by the KDM6 family of H3K27 demethylases, which includes JMJD3 and UTX proteins (*8*, *9*). Although H3K27me3 marks are tightly linked to transcriptional repression (*10*, *11*), the importance of active H3K27me3 removal to tissue development and repair has been called into question because mouse embryos that lack both JMJD3 and UTX survive to term (*12*). However, regeneration after injury is linked to inflammation, which differs from tissue development. Here, we examined the importance of H3K27 demethylation by JMJD3 and UTX for MuSC adaptation to the regenerative niche of injured muscle.

The *Utx* and *Jmjd3* genes are ubiquitously expressed, and we observed that both proteins are present in quiescent MuSCs (fig. S1). To determine how each H3K27 demethylase contributes to regeneration, we generated tamoxifen-inducible MuSC-specific knockouts of *Utx* (UTX^{scKO}) or *Jmjd3* ($JMJD3^{scKO}$) in mice (fig. S2). Upon acute cardiotoxin (CTX) injury of the tibialis anterior (TA) muscle, both UTX^{scKO} and $JMJD3^{scKO}$ mice showed impaired myofiber regeneration, demonstrating that KDM6 proteins cannot compensate for each other in MuSC-mediated regeneration (Fig. 1A and fig. S3). To investigate the specific roles for UTX and JMJD3, single-cell RNA sequencing (scRNA-seq) was performed on purified MuSCs that were isolated 40 hours after injury. Pseudotime trajectory analysis showed that $JMJD3^{scKO}$ MuSCs were enriched in clusters that expressed an immediate-early MuSC activation gene signature, whereas UTX^{scKO} cells proceeded to proliferate (Fig. 1, B and C; and fig. S4). These findings suggested that JMJD3 might be required for efficient transitioning of MuSCs from a quiescent to a proliferating state. Indeed, using the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) to identify cells undergoing DNA replication, we observed that MuSCs that lacked JMJD3 were impaired in their progression into the cell cycle (Fig. 1D). Thus, JMJD3, but not UTX, is required for efficient activation of MuSCs. At the same time, we observed that UTX, but not JMJD3, is required for MuSCs to exit the cell cycle and undergo terminal differentiation (fig. S5) (*13*). Thus, JMJD3 and UTX play nonredundant roles in MuSC-mediated regeneration.

To determine whether JMJD3 was acting through its enzymatic activity, a mutant mouse was generated that expresses an enzyme-dead JMJD3 protein ($JMJD3^{scDD}$) (fig. S6, A and B). Similar to $JMJD3^{scKO}$ cells, MuSCs that expressed mutant JMJD3 were inefficient at exiting quiescence (Fig. 1D) and, as a result, showed impaired regeneration after injury (fig. S6, C and D). This demonstrates that JMJD3 functions through H3K27 demethylation to facilitate the exit of quiescence for MuSCs in injured muscle.

Next, we used ex vivo experiments to explore the mechanism through which JMJD3 contributes to the activation process. Using MuSCs purified from uninjured mice, we were surprised to find that $JMJD3^{scKO}$ MuSCs reentered the cell cycle to the same extent as wild-type MuSCs (Fig. 2A and fig. S7). Thus, in the absence of muscle injury, JMJD3 was not necessary for cell cycle reentry. This indicated that JMJD3-mediated removal of H3K27me3 may be required for MuSCs to integrate signals from the regenerative environment. To test this, we performed TA muscle injury in one leg of $JMJD3^{scKO}$ mice and then waited 36 hours before measuring cell cycle reentry of MuSCs isolated from the contralateral leg. MuSCs lacking JMJD3 were impaired in their ability to reenter the cell cycle when recovering from a distal injury (Fig. 2A and fig. S8). Thus, circulating factors that originate in the injured muscle must be responsible for preventing MuSCs that lack JMJD3 from reentering the cell cycle. A soluble extract prepared from an injured wild-type muscle (dMusEx) could also inhibit cell cycle reentry of purified MuSCs that lack JMJD3 (Fig. 2B and fig. S9), which shows that the circulating factors that inhibit MuSC activation are released from injured muscles independent of JMJD3. Thus, JMJD3 facilitates MuSC activation in a non–cell autonomous manner by overcoming an inhibition signal that originates in the regenerative niche. Using an extract prepared from mice with dystrophic muscle (MdxMusEx), we also found that JMJD3 is required for MuSC activation in conditions of chronic degeneration (Fig. 2B), highlighting the importance of JMJD3 for mediating activation of MuSCs in muscular dystrophy.

To identify JMJD3 target genes in MuSCs, we used a combination of genomics and transcriptomics (Fig. 3, figs. S10 and S11, table S1, and supplementary text). We defined JMJD3 direct targets based on three properties: (i) decreased expression in $JMJD3^{scKO}$ MuSCs,

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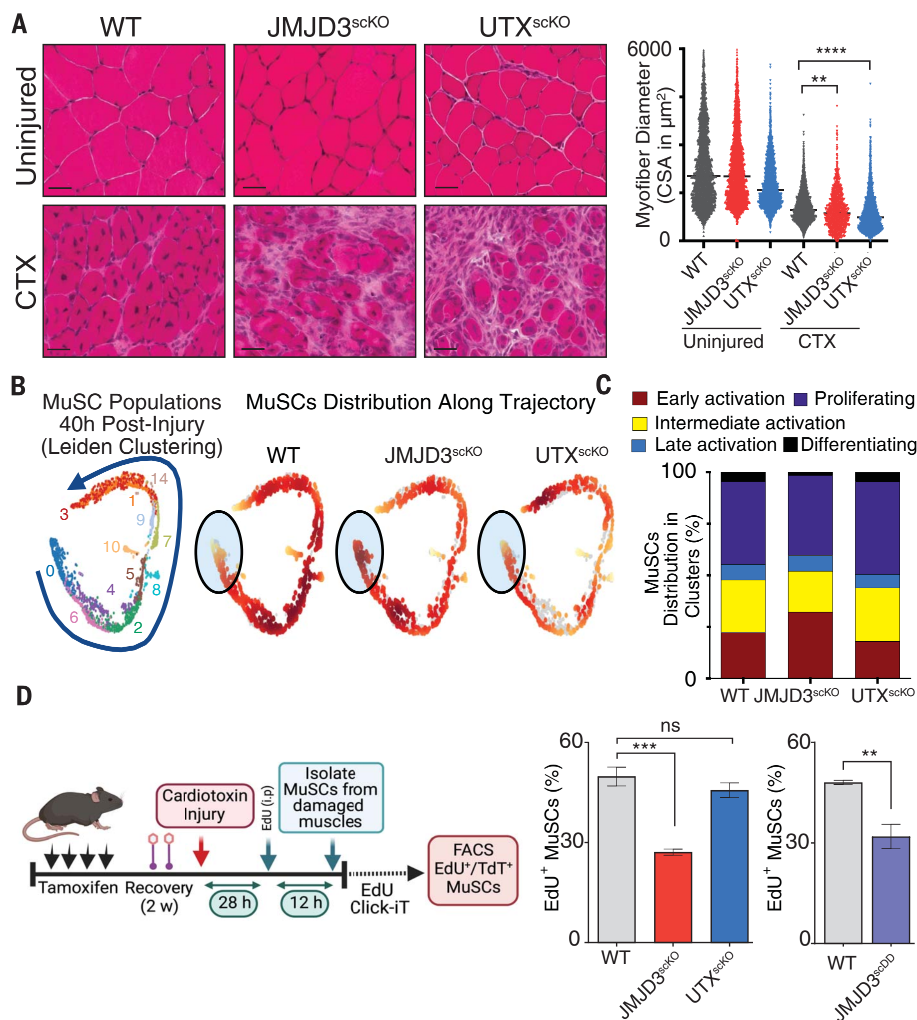

Fig. 1. JMJD3 and UTX play nonredundant roles in muscle regeneration where JMJD3 is required for injury-induced activation of satellite cells. (**A**) Hematoxylin and eosin staining of TA muscle cross sections from wild-type (WT), JMJD3^{scKO}, and UTX^{scKO} mice at 7 days after injury (CTX). Regeneration was quantified by measuring myofiber diameter. Data are means ± SD, and $N = 3$. *****p* < 0.0001, and ***p* < 0.01 [by analysis of variance (ANOVA)]. Scale bars are 60 μm. CSA, cross-sectional area. (**B**) Clustering and trajectory analysis of combined scRNA-seq data from lineage marked MuSCs [isolated based on TdTomato expression (TdT$^+$)] from the WT, JMJD3^{scKO}, and UTX^{scKO} mice isolated 40 hours after injury. Numbers indicate distinct MuSC clusters identified. The ovals highlight the enrichment of cells in the immediate-early activated MuSC state. (**C**) Distribution of MuSCs in clusters representative of different stages of the regenerative process. (**D**) Activation of MuSCs was measured by using in vivo EdU incorporation to measure the first passage of cells through the S phase of the cell cycle between 24 and 40 hours after injury. Fluorescence-activated cell sorting (FACS) analysis identified MuSCs (TdT$^+$) that were positive for EdU. Data are means ± SD, and $N = 3$; ****p* < 0.001, ***p* < 0.01, and ns is not significant (by Student's *t* test). i.p., intraperitoneally.

(ii) JMJD3 binding within 2500 base pairs of the promoter, and (iii) accumulation of H3K27me3 marks near the gene upon ablation of JMJD3. Of the 41 genes that met these criteria (table S1), we focused on those coding for extracellular matrix (ECM)–related proteins because they may facilitate communication with the regenerative milieu. *Has2* (Fig. 3) was of particular interest because it encodes a key enzyme involved in the synthesis of hyaluronic acid (HA), a glycosaminoglycan polymer that incorporates into the ECM to alter rigidity (*14*) while acting as a rheostat for various signaling pathways (*15*). By reanalyzing published datasets (*16*, *17*), we found that *Has2* is not expressed in quiescent MuSCs (fig. S12, A and B) but is up-regulated in injured muscle as MuSCs reenter the cell cycle. In regenerating muscle 40 hours after injury, HA enveloped the activated MuSCs of wild-type or dystrophic mice while also contributing to the ECM of the necrotic myofibers (Fig. 4A and fig. S13, A to D). MuSCs that lacked JMJD3 were devoid of cell-enveloping HA, even though HA was present in the ECM of necrotic myofibers (Fig. 4A and fig. S13). This dynamic incorporation of HA into the ECM of MuSCs suggested that it may play an important role in regeneration of healthy muscle. To test this, we used the chemical inhibitor 4-methylumbelliferone (4-MU) to inhibit HA production (*18*) in wild-type MuSCs. Whereas purified wild-type MuSCs (from uninjured mice) were able to efficiently exit quiescence in the presence of 4-MU (Fig. 4B and fig. S14), the same MuSCs incubated with an extract from damaged muscle showed impaired cell cycle reentry upon 4-MU treatment (Fig. 4B and supplementary text). Thus, HA synthesis is required for wild-type MuSCs to repair muscle after injury. Addition of exogenous HA to cultures was sufficient for JMJD3^{scKO} MuSCs to overcome an inhibition to cell cycle reentry (Fig. 4C and figs. S15 to S17), further emphasizing the importance of HA incorporation into the ECM of MuSCs to initiate population expansion in response to signals from the regenerative niche.

Finally, we explored the nature of the niche-derived signals that are responsible for impeding cell cycle reentry of MuSCs that lack JMJD3. Gene set enrichment analysis (GSEA) of RNA sequencing (RNA-seq) data identified the interferon-γ (IFN-γ), interleukin-6 (IL-6), and tumor necrosis factor–α (TNF-α) cellular responses as being up-regulated in MuSCs from JMJD3^{scKO} mice (fig. S18). Using recombinant cytokines, addition of exogenous IFN-γ or IL-6 [but not TNF-α, transforming growth factor–β (TGF-β), or IL-4] to the culture medium was sufficient to impair cell cycle reentry of JMJD3^{scKO} MuSCs (Fig. 4C, figs. S19 and S20, and supplementary text). Macrophages (Ly6C$^+$ cells) that are present within the regenerative niche at 24 hours after injury express both IFN-γ and IL-6 (fig. S21) and likely contribute to the signaling that impairs activation of MuSCs. This proinflammatory signaling is likely reinforced by neutrophils that are known to secrete IFN-γ and IL-6 in regenerating muscle (*19*). We propose that proinflammatory cytokines (including IFN-γ and IL-6)

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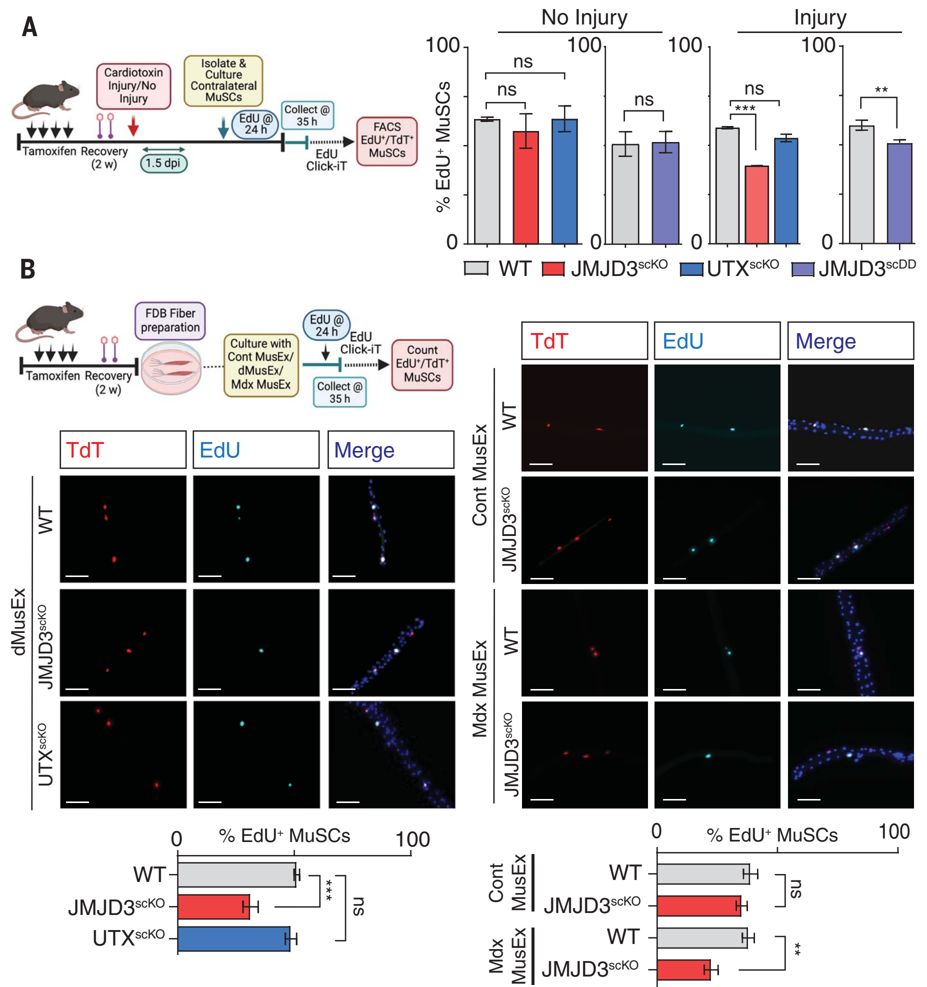

Fig. 2. JMJD3 mediates MuSC activation in a non–cell autonomous manner. (**A**) Purified MuSCs (TdT$^+$) from injured (CTX injury to contralateral leg 36 hours before MuSC isolation from uninjured muscles) or uninjured mice were assayed in vitro using EdU for cell cycle reentry between 24 and 40 hours after injury. FACS analysis identified MuSCs (TdT$^+$) that were positive for EdU. Data are means ± SD, and $N = 3$; ***$p < 0.001$, **$p < 0.01$, and ns is not significant (by Student's t test). dpi, days postinjury. (**B**) Ex vivo activation assay where flexor digitorum brevis (FDB) myofiber–associated MuSCs were treated with soluble extracts prepared from uninjured (ContMusEx), injured (dMusEx), or dystrophic (MdxMusEx) muscle. Immunofluorescence analysis identified MuSCs (TdT$^+$) that were positive for EdU. Data are means ± SD, and $N = 3$; ***$p < 0.001$, **$p < 0.01$, and ns is not significant (by Student's t test). Scale bars are 50 μm.

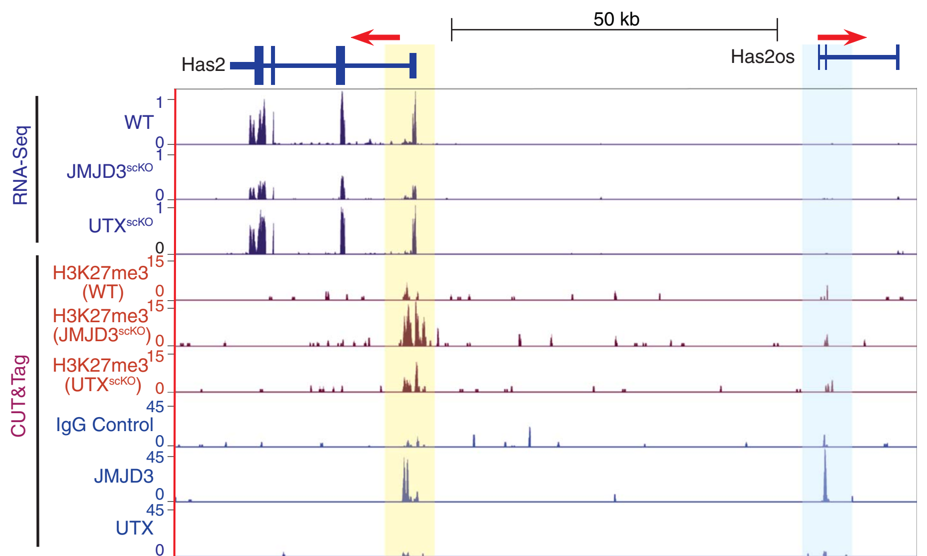

Fig. 3. *Has2* is a direct transcriptional target of JMJD3. UCSC (University of California Santa Cruz) browser track showing the *Has2* locus in MuSCs at 30 hours after CTX injury. Plots include RNA-seq data for WT, JMJD3^{scKO}, and UTX^{scKO} mice; CUT&Tag analysis shows enrichment of JMJD3, UTX, and H3K27me3.

produced by immune cells from the regenerative environment play a critical role in preventing MuSCs from undergoing an untimely exit of quiescence. To overcome the cytokine-mediated block to stem cell function, MuSCs initiate *Has2* expression in a JMJD3-dependent manner, where incorporation of HA into the remodeled ECM renders the stem cells competent to receive proregenerative signals. Thus, HA has both anti-inflammatory effects on injured tissues (*20*) and proregenerative activity that stimulates MuSC-mediated regeneration.

Our study has revealed a role for the epigenetic enzyme JMJD3 in directing MuSC

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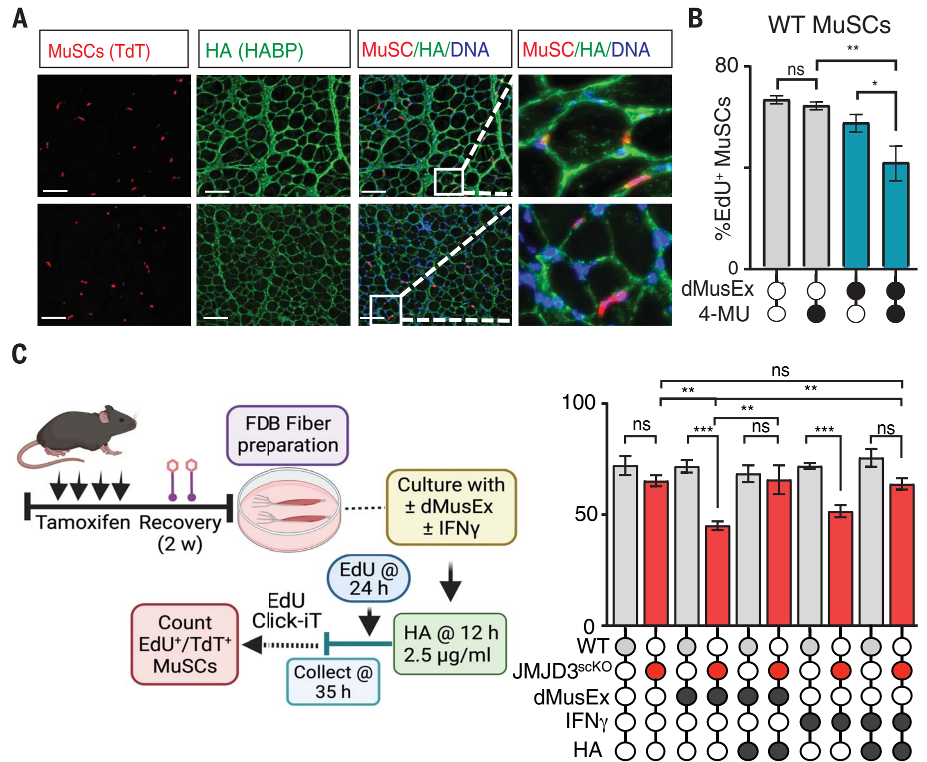

Fig. 4. HA incorporation into the ECM of MuSCs allows exit of quiescence. (**A**) Immunofluorescence analysis of TA muscle cross sections at 72 hours after CTX injury from WT and JMJD3^{scKO} mice. MuSCs (TdT^{+}), HA [hyaluronic acid binding protein (HABP)], and nuclear DNA [4′,6-diamidino-2-phenylindole (DAPI)] are shown. Scale bars are 75 μm. (**B**) Activation of MuSCs from WT mice was measured in the presence of 4-MU by in vitro EdU incorporation. An extract from damaged WT mouse muscle (dMusEx) was added as indicated. FACS analysis identified MuSCs (TdT^{+}) that were positive for EdU. Open circles indicate that the variable was not included in the experimental samples of this condition; filled circles indicate that the variable was included in the experimental samples of this condition. Data are means ± SD, and $N = 3$; $^{**}p < 0.01$, $^{*}p < 0.05$, and ns is not significant (by Student's t test). (**C**) Ex vivo activation assay of myofiber-associated MuSCs. FDB myofibers isolated from uninjured mice were incubated with either a damaged muscle extract (dMusEx) or recombinant IFN-γ protein. HA was added to cultures where indicated. Immunofluorescence analysis identified MuSCs (TdT^{+}) that were positive for EdU. Open circles indicate that the variable was not included in the experimental samples of this condition; filled circles indicate that the variable was included in the experimental samples of this condition. Data are means ± SD, and $N = 3$; $^{***}p < 0.001$, $^{**}p < 0.01$, and ns is not significant (by Student's t test).

adaption to the regenerative niche by facilitating expression of JMJD3 target gene *Has2*. The resulting production of HA allows MuSCs to integrate proregenerative signaling from the local environment to facilitate the repair of injured muscle.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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JMJD3 primes stem cells for inflammation
After muscle injury, muscle stem cells must coordinate with immune cells in the inflamed tissue to ensure efficient repair. Nakka *et al*. identified an essential role for the epigenetic enzyme KDM6B/JMJD3 in establishing the communication between muscle stem cells and infiltrating immune cells during muscle repair (see the Perspective by Gabellini). They found that, in response to injury, removal of the transcriptionally repressive histone H3K27me modification by KDM6B/JMJD3 allows muscle stem cells to produce hyaluronic acid that is then incorporated into the extracellular matrix. This remodeling of the extracellular matrix allows the muscle stem cell to receive signals from the infiltrating immune cells that initiate regeneration. —SMH and BAP

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