The Stat3-Fam3a axis promotes muscle stem cell myogenic lineage progression by inducing mitochondrial respiration

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Metabolic reprogramming is an active regulator of stem cell fate choices, and successful stem cell differentiation in different compartments requires the induction of oxidative phosphorylation. However, the mechanisms that promote mitochondrial respiration during stem cell differentiation are poorly understood. Here we demonstrate that Stat3 promotes muscle stem cell myogenic lineage progression by stimulating mitochondrial respiration in mice. We identify Fam3a, a cytokine-like protein, as a major Stat3 downstream effector in muscle stem cells. We demonstrate that Fam3a is required for muscle stem cell commitment and skeletal muscle development. We show that myogenic cells secrete Fam3a, and exposure of Stat3-ablated muscle stem cells to recombinant Fam3a in vitro and in vivo rescues their defects in mitochondrial respiration and myogenic commitment. Together, these findings indicate that Fam3a is a Stat3-regulated secreted factor that promotes muscle stem cell oxidative metabolism and differentiation, and suggests that Fam3a is a potential tool to modulate cell fate choices.
Accumulating evidence indicates that metabolism is not only a consequence of the stem cell functional status but rather an active player that regulates stem cell fate choices. Proliferative stem cells rely on glycolysis to obtain the energy they require, as well as to generate the necessary metabolic intermediates to sustain their growth. Next, the switch from self-renewal towards commitment and differentiation requires the induction of oxidative metabolism. Stimulation of mitochondrial respiration during cell differentiation is a common feature shared by a wide range of stem cell compartments, including hematopoietic stem cells, neuronal stem cells, mesenchymal stem cells, embryonic stem cells, and muscle stem cells. However, the mechanisms that induce mitochondrial function for successful stem cell differentiation are still poorly understood.

MuSCs are responsible for skeletal muscle formation during development, as well as for the maintenance of tissue homeostasis and repair during adulthood. MuSC transition between different functional stem cell states can be identified by the expression of myogenic markers, which are transcription factors dynamically expressed during MuSC myogenic lineage progression. This makes MuSCs a powerful tool for studying the mechanisms that regulate stem cell fate choices. MuSCs reside in a quiescent state in adult skeletal muscle. Upon injury, MuSCs become activated, proliferate, and differentiate to form new myofibers and repair the tissue. Skeletal muscle regeneration is a very efficient process, and temporally coordinated changes in metabolism play a central role in regulating proper MuSC myogenic lineage progression to ensure successful muscle regeneration. Quiescent MuSCs display a low metabolic rate that mainly relies on fatty acid oxidation. MuSC activation initially induces their glycolytic pathway in order to sustain cell proliferation, and then a switch to oxidative metabolism is required to allow further MuSC commitment to the myogenic lineage and differentiation. Several pathological conditions and aging can compromise MuSC function, alter the balance between self-renewal and differentiation, and result in the reduction of skeletal muscle regenerative capacity that contributes to the muscle loss associated with these diseases. Thus identifying the factors that regulate MuSC metabolism and therefore cell fate choices is highly relevant for therapeutic purposes.

We and others recently provided evidence that signal transducer and activator of transcription factor 3 (Stat3) regulates MuSC fate choices during skeletal muscle regeneration. Stat3 is a transcription factor that mediates the intracellular signaling of several cytokines, including interleukin (IL)-6, leukemia inhibitory factor, and Oncostatin M. We demonstrated that Stat3 promotes MuSC progression into committed myogenic progenitors and differentiation. However, the mechanism by which Stat3 regulates MuSC myogenic commitment is currently poorly understood. Stat3 regulates a wide range of biological processes in different cell types, including proliferation, migration, survival, and metabolism. From the metabolic point of view, Stat3 is able to promote glycolysis or mitochondrial respiration depending on the cellular context. However, little is currently known about the role of Stat3 in regulating MuSC metabolism.

In our study, we demonstrate that Stat3 promotes mitochondrial respiration during MuSC myogenic lineage progression. We further identify Fam3a as a regulator of MuSC function that acts as a major direct downstream effector of Stat3. Fam3a is a cytokine-like protein shown to increase ATP production and promote mitochondrial respiration in vascular smooth muscle cells, neuronal cells, and hepatocytes. We demonstrate that Fam3a is required for proper MuSC myogenic lineage progression and skeletal muscle development in vivo. We further show that Fam3a is secreted by myogenic cells and that treatment with recombinant Fam3a rescues the reduced mitochondrial respiration and defective myogenic commitment of Stat3-ablated MuSCs both in vitro and in vivo during adult skeletal muscle repair. Overall, this work positions the Stat3–Fam3a axis as a driver of mitochondrial respiration during MuSC commitment and differentiation and suggests that therapeutic interventions targeting this axis could be utilized to promote MuSC-mediated tissue repair.

Results
Stat3 promotes mitochondrial function in MuSCs. In order to analyze the impact of Stat3 on MuSC gene expression during activation, we performed RNA-seq whole-transcriptome analysis in control and Stat3 genetically ablated MuSCs (Stat3 knockout (KO)). To this aim, we took advantage of the Pax7-CreER driver, as Pax7 is a transcription factor specifically expressed in MuSCs in skeletal muscle. We induced Cre-mediated Stat3 ablation in 3-month-old Pax7-CreER<sup>T</sup>T<sub>2</sub> male mice and control littermates (Pax7-CreER<sup>T</sup>T<sub>2</sub>;Stat3<sup>−/−</sup>) by tamoxifen (Tmx) treatment, as previously reported. At least 2 weeks after treatment, tibialis anterior muscles were injured by intramuscular injection of barium chloride (BaCl<sub>2</sub>) to induce MuSC activation. MuSCs were isolated from uninjured or 3 days post-injury (3 dpi) mice by fluorescence-activated cell sorting (FACS), based on α7-integrin and CD34 surface marker expression, as previously reported. Comparison between control uninjured and control activated MuSCs (3 dpi) showed major changes in their transcriptome (Fig. 1b). Pathway analysis (using Gene Set Enrichment Analysis (GSEA)) revealed that the top enriched pathways during activation of control (Ct) MuSCs were related to cell cycle, protein synthesis, and mitochondrial metabolism, consistent with previous reports. Activation of Stat3 KO MuSCs also caused extensive changes in their transcriptome (activated—3 dpi vs uninjured Stat3 KO MuSCs) (Fig. 1b). GSEA showed that pathways associated with cell cycle and protein synthesis were among the top 20 enriched pathways in activated Stat3 KO MuSCs compared to uninjured Stat3 KO MuSCs (Fig. 1c). GSEA showed that pathways associated with cell cycle and protein synthesis were among the top 20 enriched pathways in activated Stat3 KO MuSCs were associated with mitochondrial oxidative phosphorylation (Fig. 1c). In contrast to what we observed during activation of control MuSCs, none of the top 20 enriched pathways in activated Stat3 KO MuSCs were associated with mitochondrial oxidative phosphorylation (Fig. 1c). We further interrogated the data by performing pathway analysis using the differentially expressed transcripts between activated control and activated Stat3 KO MuSCs. GSEA identified previously described Stat3-regulated pathways such as integrin signaling, cytokine–cytokine receptor interaction, and Janus-activated kinase–Stat signaling among the top enriched pathways (Fig. 1d and Supplementary Data 3). The respiratory electron transport pathway appeared in the top 10 differentially enriched pathways, further suggesting that Stat3 promotes mitochondrial function during the activation process (Fig. 1d and Supplementary Data 3). Thus we analyzed the mitochondrial respiration of MuSCs lacking Stat3 after 3 days in culture in growth conditions. Consistent with our pathway analysis, Stat3-ablated MuSCs exhibited a decreased mitochondrial respiration assessed as reduced basal and maximal oxygen consumption rate (OCR) (Fig. 1e, f). Stat3 ablation did not affect the extracellular acidification rate (ECAR), readout for glycolysis (Fig. 1g). Overall, this data indicates that Stat3 promotes mitochondrial respiration during MuSC commitment to the myogenic lineage without having a major impact on glycolysis.
Fig. 1 Stat3 promotes mitochondrial function in muscle stem cells (MuSCs) during activation. a Scheme of the experimental design to obtain the samples for RNA-seq. b Heat map of the differentially expressed genes among the different comparisons. Listed genes were differentially expressed in at least one of the comparisons. Normalized gene RPKM (reads per kilobase of transcript per million) values were averaged within groups for the generation of the heat map (n = 3 animals). c Pie charts showing the top 20 differentially enriched pathways (using Gene Set Enrichment Analysis (GSEA)) during the activation of control (Pax7-CreERT2;Stat3f/f) or Stat3 KO MuSCs (Pax7-CreERT2;Stat3f/f) mice grouped according to their functional category. d Top 12 pathways enriched in freshly isolated MuSCs from 3 dpi Pax7-CreERT2;Stat3f/f mice compared to 3 dpi MuSCs isolated from control littermates using GSEA. The red line indicates p value = 0.05 based on the pathway analysis (GSEA). e-g Measurement of the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of control and Stat3 KO MuSCs cultured in growth conditions for 3 days (n = 4 independent experiments). The Cell Mito Stress Test was performed in a Seahorse Xfp Analyzer. Data are represented as mean ± SEM (Student’s t test or two-way analysis of variance; *p < 0.05, ***p < 0.001, ****p < 0.0001).

Stat3 has been shown to promote mitochondrial respiration by translocating into the mitochondria22–24. In order to assess a potential role of mitochondrial Stat3 during MuSC activation, we performed immunofluorescence analysis of both total and S727 phosphorylated Stat3 in MuSCs cultured in vitro for 3 days. Stat3 S727 phosphorylation enhances its transnational activity in the nucleus, and it is also required to induce the translocation of Stat3 into the mitochondria24,30,31. Both analyses showed a predominant nuclear localization for Stat3 (Supplementary Fig. 1c, d), suggesting that nuclear Stat3 plays a major role in regulating mitochondrial respiration during MuSC activation.

Previous studies demonstrated that mitochondrial respiration actively promotes myogenic differentiation in cultured myoblasts32–36. Consistently, mitochondrial biogenesis and mitochondrial respiration are increased during MuSC myogenic commitment and differentiation29–31,37. Thus, to further validate that mitochondrial respiration is an important factor that promotes myogenic progression in MuSCs, we cultured MuSCs in vitro in growth media (GM) or differentiation media (DM) and treated them with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (or dimethyl sulfoxide, DMSO, as vehicle), an inhibitor of oxidative phosphorylation, for 24 h. In both contexts, the percentage of myogenin-positive cells was reduced in CCCP-treated MuSCs (Supplementary Fig. 1e, f), indicating that mitochondrial respiration is required for proper myogenic commitment and differentiation in MuSCs.

Overall, our findings indicate that Stat3-dependent regulation of mitochondrial respiration is a major mechanism that promotes MuSC commitment and differentiation.

Fam3a is a downstream target of Stat3 and MyoD in MuSCs. To identify relevant downstream targets of Stat3 that promote MuSC mitochondrial respiration and myogenic lineage progression, we performed multiple comparisons between all the transcriptomes obtained from the RNA-seq analysis (Fig. 2a). We focused our attention on genes that were differentially expressed during the process of activation in control MuSCs (Ct 3 dpi vs Ct),
that did not respond during the activation of Stat3 KO MuSCs (KO 3 dpi vs KO), and that were differentially expressed between activated Stat3 KO MuSCs and activated control MuSCs (KO 3 dpi vs Ct 3 dpi). This analysis identified 63 genes (Fig. 2a). Stat3 is primarily a transcriptional coactivator, thus we concentrated on the top 10 genes downregulated in activated Stat3 KO MuSCs (KO 3 dpi) compared to activated control cells (Ct 3 dpi). Among the significantly downregulated genes (Fig. 2b), we focused on...
Fam3a, a cytokine-like protein, as it was the only one that had been reported to be involved in the regulation of mitochondrial metabolism^{25–27}.

We validated Fam3a downregulation in activated Stat3 KO MuSCs compared to activated controls in samples different from the RNA-seq (Fig. 2c). We further observed upregulation of Fam3a at the mRNA level in MuSCs during myogenic differentiation in vitro, mirroring the expression pattern of Stat3 (Fig. 2d). To investigate whether Fam3a is a direct transcriptional target of Stat3, we performed Fam3a promoter analysis using JASPAR^{39} and identified one putative Stat3-binding site 2869 bp upstream of the transcription start site (TSS; Fig. 2e). Chromatin immunoprecipitation (ChIP) assay in C2C12 myoblasts showed that Stat3 is recruited to this site upon IL-6 stimulation, which promotes Stat3 activation and translocation into the nucleus (Fig. 2e). IL-6 treatment also caused enrichment of H3K27Ac, a marker of active transcription, in this region (Fig. 2e). Together, these findings indicate that Fam3a is a direct transcriptional target of Stat3.

Further analysis of the Fam3a promoter revealed the existence of putative MyoD-binding sites. MyoD is a transcription factor essential for MuSC commitment to the myogenic lineage and differentiation^{13}, and recent work demonstrated that MyoD regulates a set of genes responsible to sustain oxidative metabolism in C2C12 myotubes and adult skeletal muscle^{10}. By analyzing previously published ChIP-seq data^{40}, we observed MyoD binding to the Fam3a promoter in proximity to the TSS in C2C12 myotubes (Fig. 2f). Similarly, ChIP-seq analysis using myogenic conversion of human IMR90 fibroblasts to the myogenic lineage by the induction of ectopic MyoD expression showed the recruitment of MyoD to the Fam3a promoter (Fig. 2g). This MyoD recruitment was further increased by the induction of differentiation in myogenically converted IMR90 fibroblasts (Fig. 2g), suggesting that MyoD regulation of Fam3a is conserved between mouse and human species. Consistent with ChIP-seq data, MuSCs isolated from MyoD KO mice^{41,42} showed reduced Fam3a mRNA levels when cultured for 3 days in vitro (Fig. 2h).

Finally, to further validate that Stat3 and MyoD regulate Fam3a expression, we performed reporter assays using a construct containing the luciferase reporter gene under the control of the Fam3a promoter. HEK293 cells were transiently transfected with the reporter plasmid and a Renilla encoding plasmid (to monitor transfection efficiency), together with plasmids encoding for Stat3 and/or MyoD (Fig. 2i). Stat3 overexpression significantly increased the transcriptional activity of the reporter compared to control conditions, and MyoD overexpression induced a much higher transcriptional activation of the reporter (Fig. 2i). However, we did not observe an additive effect when transfecting together Stat3 and MyoD coding plasmids (Fig. 2i).

Altogether, our data indicate that both Stat3 and MyoD directly promote Fam3a expression by binding to the respective regulatory regions on the Fam3a promoter.

Fam3a promotes MuSC myogenic lineage progression in vitro. To analyze the function of Fam3a in MuSCs, we performed acute loss-of-function studies in vitro by utilizing lentiviruses expressing short hairpin RNA (shRNA) against Fam3a (shFam3a) or shRNA control (shCt). Freshly isolated MuSCs from 3-month-old C57BL/6J male mice were placed in culture in growth conditions, infected with lentiviruses, and after 72 h we analyzed myogenic lineage progression. We assessed the expression levels of the myogenic regulators Pax7 (marker of quiescent and proliferating MuSCs), MyoD (early marker of committed progenitors), and myogenin (late marker of committed progenitors). Fam3a mRNA levels were efficiently reduced with shRNA treatment, and this resulted in a reduced in myogenin mRNA (Fig. 3a). Immunofluorescence analysis showed that Fam3a knockdown increased the percentage of Pax7^{−}/MyoD^{−} and Pax7^{−}/MyoD^{+} populations and reduced the percentage of the Pax7^{−}/MyoD^{+} population (Fig. 3b). We also observed a reduction in the percentage of myogenin^{+} cells, consistent with our mRNA data, indicating a reduced rate of commitment into the myogenic lineage upon Fam3a repression (Fig. 3c). No differences in proliferation were observed (Supplementary Fig. 2), suggesting that Fam3a specifically affects MuSC commitment. Upon induction of terminal differentiation, MuSCs knocked down for Fam3a exhibited impaired ability to differentiate into myosin heavy chain-positive (MyHC^{+}) cells, as shown by a reduced differentiation index (Fig. 3d). Together, these findings demonstrate that Fam3a is required for proper myogenic lineage progression in vitro and its repression recapitulates the phenotype previously described for Stat3 gene deletion/inhibition in MuSCs^{43}, suggesting that Fam3a is a relevant downstream effector of Stat3 in MuSCs.

Fam3a is required for skeletal muscle development in vivo. To investigate the role of Fam3a in skeletal muscle in vivo, we generated a Fam3a KO mouse model by utilizing CRISPR/Cas9 genome-editing strategies (Fig. 4a). We designed a guide to specifically target exon 2 and generate frame-shift mutations that disrupt the open reading frame (Supplementary Fig. 3a). Fam3a contains an N-terminal secretion signal peptide (SP) followed by the sequence corresponding to the mature protein. With this strategy, we generated frame-shift mutations in the region encoding for the SP and therefore disrupted the protein before any part of the mature and active Fam3a protein is produced (Supplementary Fig. 3a).
We injected the guide and Cas9 mRNAs into zygotes and assessed skeletal muscle development in E15 embryos and P0 pups (Fig. 4a). As Fam3a is an X chromosome-encoded gene, we focused our studies on males. DNA sequencing of the 5 Fam3a KO embryos analyzed in this study showed a frame-shift mutation in exon 2 (Supplementary Fig. 3b). Fam3a KO embryos exhibited significantly smaller hind limb muscles, shown by the reduced embryonic myosin heavy chain (eMyHC) area (Fig. 4b), as well as a reduction in the number of myogenin+ nuclei (Fig. 4c, d), compared to controls. We further analyzed Fam3a KO male mice at birth (P0). The three Fam3a KO pups used in this study displayed a frame-shift mutation in exon 2 (Supplementary Fig. 3b). Also in this context, we observed a reduction in eMyHC area and an accumulation of uncommitted MuSCs (assessed as increased number of Pax7+ cells) (Fig. 4e, f). Additionally, laminin was also more disrupted in Fam3a KO pups compared to controls (Fig. 4e, f). These findings are consistent with a role of Fam3a in promoting myogenic commitment and muscle differentiation during development, with its ablation leading to a deficient MuSC progression into the myogenic lineage in vivo and compromised skeletal muscle formation.

To assess whether the reduction in muscle mass was maintained in adulthood, we analyzed body and tissue weights from 3-month-old wild-type (WT) and Fam3a KO male mice. Indeed, Fam3a deletion resulted in decreased body weight, together with a significant reduction in the gastrocnemius, quadriceps, and heart weights (Supplementary Fig. 4). We did not observe differences in the weight of other tissues such as liver, epididymal white adipose tissue, spleen, or kidneys (Supplementary Fig. 4). Together, this data indicates that Fam3a ablation causes a reduction in skeletal muscle mass during development that is maintained in adulthood.

Fam3a is known to induce mitochondrial respiration in several cell types25–27. Thus we assessed whether Fam3a also promotes mitochondrial function in MuSCs. To this aim, we measured the mitochondrial respiration in WT and Fam3a KO MuSCs cultured in growth conditions for 72 h (n = 4 independent experiments). We did not observe differences in the weight of other tissues such as liver, epididymal white adipose tissue, spleen, or kidneys (Supplementary Fig. 4). Together, this data indicates that Fam3a ablation causes a reduction in skeletal muscle mass during development that is maintained in adulthood.

**Fig. 3** Fam3a promotes muscle stem cell (MuSC) myogenic lineage progression in vitro. a Gene expression analysis of MuSCs infected with lentiviruses coding for short hairpin RNA (shRNA) Control (shCt) or shRNA against Fam3a (shFam3a) and cultured in growth conditions for 72 h (n = 4 independent experiments). b, c Immunofluorescence analysis and quantification of myogenic markers of MuSCs infected with shCt or shFam3a coding lentiviruses and cultured in growth conditions for 72 h (n = 3 independent experiments). Scale bars 50 μm. d Immunofluorescence analysis of myogenic differentiation of MuSCs infected with shCt or shFam3a coding lentiviruses and cultured in differentiation conditions for 72 h (n = 3 independent experiments). Scale bar 100 μm. Data are represented as mean ± SEM (Student’s t test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)
Fig. 4 Fam3a ablation compromises skeletal muscle development in vivo. **a** Scheme of the generation of the Fam3a knockout (KO) mouse model and the experimental design. **b–d** Immunofluorescence analysis of transversal sections of the hind limbs of wild-type (WT) and Fam3a KO E15 embryos. Quantification of the embryonic myosin heavy chain (eMyHC) area and the number of Pax7- and Myogenin-positive cells (*n* = 5 embryos). Scale bars 300 and 30 μm. **e, f** Immunofluorescence analysis of transversal sections of the hind limbs of WT and Fam3a KO P0 pups. Quantification of the eMyHC area and the number of Pax7-positive cells (*n* = 3–4 pups). Scale bars 100 and 25 μm. Data are represented as mean ± SEM (Student’s *t* test; *p* < 0.05, **p** < 0.01).
withouth affecting glycolysis (Supplementary Fig. 5a–c). This reduction in mitochondrial oxygen consumption was not due to reduced mitochondrial content or changes in the expression of Pgc1a or different subunits of the electron transport chain in MuSCs (Supplementary Fig. 5d–f). Consistent with this data, Fam3a overexpression in C2C12 myoblasts did not affect the expression of Pgc1a or different subunits of the electron transport chain at the mRNA or protein level (Supplementary Fig. 5g–j). Together, this data demonstrates that Fam3a does not promote mitochondrial respiration by regulating mitochondrial content, biogenesis, or the expression of the electron transport chain complexes in myogenic cells, suggesting that alternative molecular mechanisms are taking place.

Overall, our data indicate that Fam3a is required for proper skeletal muscle development by stimulating mitochondrial respiration in MuSCs and promoting their myogenic lineage progression.

**Fam3a is secreted by myogenic cells.** Fam3a protein sequence analysis using LocTree3 predicted that Fam3a is a secreted protein (Supplementary Fig. 6a). However, previous reports indicated a localization of Fam3a in the mitochondria. To strengthen our prediction, we used two additional softwares that predict protein localization: TargetP and MitoFates. TargetP is a software that predicts protein localization by analyzing the presence of N-terminal presequences containing mitochondrial targeting peptides or secretory SPs. TargetP analysis of the Fam3a protein sequence also predicted that Fam3a is a secreted protein, while it correctly predicted that the protein Citrate Synthase was mitochondrial (Supplementary Fig. 6a). Finally, we used MitoFates as software that analyzes the presence of N-terminal mitochondrial targeting signals and their cleavage sites. Consistent with the previous predictions, Fam3a did not contain any mitochondrial localization presequence while it was identified in Citrate Synthase (Supplementary Fig. 6b). Overall, the analysis of the Fam3a protein sequence using three different softwares indicated that Fam3a is a secreted protein that lacks a mitochondrial localization signal.

To directly investigate the subcellular localization of Fam3a in muscle cells, we performed colocalization studies in transiently transfected C2C12 myoblasts with a construct expressing Fam3a-Myc-Flag. In order to label Golgi, endoplasmic reticulum (ER), and mitochondria, we used the markers GM-130, KDEL, and Tomm20, respectively. In accordance with previously published studies, these markers were specific for each compartment as they presented no or minimal colocalization among them (Supplementary Fig. 6c, d). Consistent with our Fam3a localization predictions, we did not detect colocalization between exogenous Fam3a and mitochondria 48 h after transfection (Supplementary Fig. 6d, e). Instead, exogenous Fam3a colocalized with GM-130, marker of cis-Golgi (Fig. 5a and Supplementary Fig. 6d). Upon treatment with the secretion inhibitor monensin, Fam3a-Myc-Flag strongly colocalized with the ER, as shown by colocalization with KDEL (Fig. 5b and Supplementary Fig. 6d). Western blot analysis detected accumulation of Fam3a-Myc-Flag within C2C12 myoblasts upon monensin treatment (Fig. 5c, d). Finally, we observed the presence of Fam3a-Myc-Flag in the media of transfected cells (Fig. 5e). Together, these findings demonstrate that Fam3a is secreted by myogenic cells.

Fam3a is a ubiquitously expressed cytokine-like protein, suggesting that it could also be produced by other muscle-resident cell types known to promote MuSC myogenic lineage progression, such as fibroadipogenic progenitors (FAPs) or macrophages (MPs). To address this question, we performed quantitative real-time PCR (qPCR) analysis in muscle-resident cell types isolated from two highly myogenic contexts: postnatal muscle growth and adult tissue regeneration (Supplementary Fig. 7). During postnatal growth, we isolated mononucleated cells (MuSCs, MPs, and FAPs) and myofibers from P15 pups and 3-month-old C57BL6/j mice (Fig. 5f and Supplementary Fig. 7). Our results show that myofibers contain much higher levels of Fam3a transcript than mononucleated cells (Fig. 5f), suggesting that myofibers are the main producers of Fam3a in the skeletal muscle during postnatal growth and also in the adult skeletal muscle.

To assess Fam3a expression during adult skeletal muscle regeneration, we isolated mononucleated cells from 3-month-old male mice at three different time points: uninjured, 3 dpi, and 7 dpi (Fig. 5g and Supplementary Fig. 7). Three days after injury, both MPs and FAPs are the highest Fam3a-expressing cells at the population level (Fig. 5g). Seven days after injury, FAPs are the main Fam3a-expressing cells in the tissue (Fig. 5g), suggesting that FAPs may be a relevant source of Fam3a during skeletal muscle regeneration. Overall, our data suggest that Fam3a is a secreted factor that may mediate the coordination of the different muscle-resident cell types during myogenesis.

**Fam3a rescues myogenic commitment of Stat3 KO MuSCs.** To evaluate whether the secretion of Fam3a is relevant for its role on MuSC function, we performed rescue studies by adding Fam3a recombinant protein into the culture media. First, we used shRNA-mediated Fam3a loss of function (shFam3a). Addition of recombinant Fam3a increased the percentage of myogenin+ cells in shFam3a MuSCs cultured in both GM and DM (Fig. 6a and Supplementary Fig. 8a). Treatment did not affect the percentage of myogenin+ cells in control MuSCs (shCt) (Fig. 6a and Supplementary Fig. 8a), suggesting that control cells produce sufficient Fam3a levels to sustain their myogenic lineage progression and differentiation in culture. In a second set of experiments, we asked whether incubation with recombinant Fam3a was sufficient to rescue the defects in myogenic lineage progression and differentiation of Stat3 KO MuSCs in vitro. Indeed, addition of Fam3a protein into the media of Stat3 KO MuSCs rescued the percentage of myogenin+ cells up to the levels of control cells when cultured in GM (Fig. 6b), validating that it is a relevant Stat3 downstream effector. Treatment with recombinant Fam3a was not sufficient to rescue the deficit in myogenic differentiation of Stat3 KO MuSCs, suggesting that other downstream targets of Stat3 also play a relevant role in differentiation conditions (Supplementary Fig. 8b). Treatment did not affect the percentage of myogenin+ cells in control MuSCs (Fig. 6b and Supplementary Fig. 8b).

Our data demonstrate that both Stat3 and Fam3a promote mitochondrial function during MuSC activation. Thus we assessed whether treatment with Fam3a could also rescue the reduced mitochondrial respiration observed in Stat3 KO MuSCs. Incubation of Stat3 KO MuSCs with Fam3a recombinant protein for 72 h in growth conditions increased their basal and maximal mitochondrial respiration to levels similar to control cells (Fig. 6c, d) without affecting the ECAR (Fig. 6e). Consistent with our previous data, treatment with recombinant Fam3a did not affect mitochondrial respiration in control MuSCs (Supplementary Fig. 8c–e).

Finally, to demonstrate that Fam3a secretion also plays a relevant role during adult skeletal muscle regeneration in vivo, we performed rescue studies using Tnmx-treated 4-month-old Pax7-CreERT2 allele male mice and control littermates. Tibialis anterior muscles of these mice were injured with barium chloride, and 1 day after injury we delivered Fam3a recombinant protein (or...
Vehicle as control) by intramuscular injection (Fig. 6f). Vehicle-treated muscles from Pax7-CreER;Stat3f/f mice displayed a higher number of Pax7<sup>+</sup> cells 6 days after the initial injury compared to vehicle-treated muscles from control animals, as previously shown (Fig. 6g).<sup>20</sup> Strikingly, treatment with recombinant Fam3a of Pax7-CreER;Stat3f/f injured muscles normalized the number of Pax7<sup>+</sup> cells to the levels of control mice (Fig. 6g). Moreover, treatment with recombinant Fam3a significantly increased the number of myogenin<sup>+</sup> cells in injured Pax7-CreER;Stat3f/f muscles (Fig. 6h). Overall, this data indicates that Fam3a is a...
major downstream effector of Stat3 in MuSCs that promotes myogenic commitment in vivo.

**Discussion**

Understanding the mechanisms that regulate stem cell fate choices is one of the goals of regenerative medicine in order to develop efficient therapeutic approaches, and stem cell metabolic reprogramming is emerging as a strategy with the potential to improve tissue repair1,2. Common to several stem cell compartments, MuSC commitment and differentiation requires the induction of oxidative metabolism 2,3,9–12. For instance, the induction of mitochondrial function is required for neuronal...
Fam3a rescues impaired commitment of Stat3 knockout (KO) muscle stem cells (MuSCs) in vitro and in vivo. a Immunofluorescence analysis and quantification of myogenin expression in MuSCs infected with shCt or shFam3a coding lentiviruses and cultured in growth conditions for 72 h in the presence or absence of recombinant Fam3a (1000 ng/ml) (n = 3 independent experiments). Scale bar 25 μm. b Immunofluorescence analysis and quantification of myogenin expression in control and Stat3 KO MuSCs cultured in growth conditions for 72 h in the presence or absence of recombinant Fam3a (1000 ng/ml) (n = 5 independent experiments). Scale bar 25 μm. c-d Measurement of the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of Stat3 KO MuSCs cultured in growth conditions for 3 days in the presence or absence of recombinant Fam3a (1000 ng/ml) (n = 5 independent experiments). The Cell Mito Stress Test was performed using the Seahorse XFp Analyzer. f Scheme of the experimental design for the intramuscular Fam3a treatment in vivo. g, h Immunofluorescence analysis and quantification of Pax7- and myogenin-positive cells in transversal sections of the tibialis anterior muscles of Pax7-CreERWT;Stat3f/f and Pax7-CreER;Stat3f/f male mice 6 days after barium chloride injury. Vehicle or 1 μg of Fam3a recombinant protein was injected intramuscularly 1 day after inducing injury (n = 3–4 animals). Scale bar 25 μm. Data are represented as mean ± SEM (Student’s t test or two-way analysis of variance; *p < 0.05, **p < 0.01, ***p < 0.001).
purposes as they can be utilized to modulate the balance between MuSC self-renewal and differentiation, improve tissue repair in diseased conditions with deficient skeletal muscle regenerative capacity, and ameliorate muscle wasting.

Methods

Animal procedures. All protocols were approved by the Sanford Burnham Prebys Medical Discovery Institute Animal Care and Use Committee and by the Italian Ministry of Health, the National Institute of Health (INS), the Santa Lucia Foundation (Rome) Animal Care and Use Committee. The study is compliant with all relevant ethical regulations regarding animal research. Mice were housed according to institutional guidelines, in a controlled environment at a temperature of 22 °C ± 1 °C, under a 12-h dark-light period and provided with standard chow diet and water ad libitum. Male Pax7-CreERT2Stat3mice and control Pax7-CreERT2Stat3mice litters (between 3- and 7-month-old) were used. Pax7-CreERT2Stat3mice were maintained in C57BL/6jbackground, Fam3a KO mice were generated in a C57BL/6j background, and F0 generation E15 male embryos and P0 male pups were analyzed. Adult 3-month-old Fam3a KO mice were also used in this study. MyoD−/− mice were purchased from Jackson Laboratories. Some of the implanted females were sacrificed 15 days after re-implantation for E15 embryos analysis. Tail DNA was collected for genotyping. Injected embryos were then re-implanted into recipient pseudo-pregnant ICR female mice. Some of the implanted females were sacrificed 15 days after at least 2 weeks from the last Tmx injection (mice were between 4- and 7-month-old). The Illumina machine built-in software provided read quality scores (Q > 30). The filtered read regions (FastQ file) were then added to the cells together with the secondary sequencing reads (FastQ file) for 72 h when indicated.

Cell culture procedures. All cells were cultured in incubators at 37 °C and 5% CO2. Freshly isolated MuSCs were plated on tissue culture plates coated with laminin (cat.#11243217001, Roche) and maintained in GM (45% DMEM, 40% F10, 5% fetal bovine serum (FBS)). Myogenic differentiation was induced with DMEM and 2% horse serum for 2 or 3 days. MuSC proliferation was measured by 5-ethynyl-2'-deoxyuridine (EdU) incorporation (5 μg/ml, cat.A10044, Life Technologies) for 4 h at the end of the culture. MuSCs were treated with CCF2/DCM (12.5 μM) or ethynylated in GM and cultured in DM (cat.#2279, Sigma) for 24 h when indicated. MuSCs were treated with recombinant FAM3A (1000 ng/ml, cat.#TP303495, OriGene) for 72 h when indicated.

RNA sequencing. MuSCs were isolated from Pax7-CreERT2Stat3mice and Pax7-CreERT2Stat3mice. Total RNA was isolated according to the manufacturer’s instructions with DNAase digestion (cat.#217084, Qiagen). RNA quality was verified by Bioanalyzer (Agilent 2100 Bioanalyzer) and RNA-seq was performed using the Illumina HiSeq 2500 instrument by the Analytical Genomics and Bioinformatics Core Facility (SBP, Lake Nona). Up to 1 μg of total RNA was used to prepare standard Illumina single read TrueSeq libraries and library quality was controlled by using Agilent Bioanalyzer, Qubit, and KAPA qPCR. The KAPA primers target Illumina P5 and P7-region in the genome and reads with MAPQ score <10 were removed.

Generation of Fam3a KO mice. CRISPR/Cas9 gene editing to generate Fam3a mutant embryos was performed. A single guide RNA (sgRNA) to target inside exon 2 was designed using GuideSeq.com to ensure maximum specificity and cutting efficiency (CTAGCACCTGCTCCTAG). DNA template for the sgRNA was generated by PCR amplification (Q5 DNA Polymerase; cat.#M0491S, New England BioLabs) of sdRNA ultramer oligonucleotide (Integrated DNA Technologies); sgRNA was transcribed from this template using the HiScribe T7 High Yield RNA Synthesis Kit (cat.#E2040S, New England Biolabs), treated with DNase I (cat.#M0303S, New England Biolabs), and purified using the Megaclear Kit (cat.#AM1908, Invitrogen). For mouse zygote injections, 50 ng/μl Cas9 mRNA (Life Technologies) and 20 ng/μl sgRNA were combined in IDTE buffer (IDT). Fertilized oocytes were collected from 3- to 4-week-old superovulated C57BL/6j females (prepared by injecting 5 IU each of pregnant mare serum gonadotropin and human chorionic gonadotropin (Sigma)), then transferred into M2 medium (Millipore), and injected with the Cas9 mRNA/sgRNA solution into the cytoplasm. Injected embryos were then re-implanted into recipient pseudo-pregnant ICR female mice. Some of the implanted females were sacrificed 15 days after re-implantation for E15 embryos analysis. Tail DNA was collected for genotyping by PCR (cat.#Bio-21126, LifeMyTag Extract Kit) (see Supplementary Table 1 for primer sequences) followed by Sanger sequencing to assess for mutations. Fam3a KO mice that displayed in-frame indels were excluded from the study. Mutations were confirmed using the National Institute of Health (San Antonio, TX) and the Santa Lucia Foundation (Rome) Animal Care and Use Committee. The study is compliant with all relevant ethical regulations regarding animal research. Mice were housed according to institutional guidelines, in a controlled environment at a temperature of 22 °C ± 1 °C, under a 12-h dark-light period and provided with standard chow diet and water ad libitum. Male Pax7-CreERT2Stat3mice and control Pax7-CreERT2Stat3mice litters (between 3- and 7-month-old) were used. Pax7-CreERT2Stat3mice were maintained in C57BL/6jbackground, Fam3a KO mice were generated in a C57BL/6j background, and F0 generation E15 male embryos and P0 male pups were analyzed. Adult 3-month-old Fam3a KO mice were also used in this study. MyoD−/− mice were purchased from Jackson Laboratories. Some of the implanted females were sacrificed 15 days after re-implantation for E15 embryos analysis. Tail DNA was collected for genotyping. Injected embryos were then re-implanted into recipient pseudo-pregnant ICR female mice. Some of the implanted females were sacrificed 15 days after inducing barium chloride injury. Histological analysis was performed 6 days after injections.

RNA sequencing. MuSCs were isolated from Pax7-CreERT2Stat3mice and Pax7-CreERT2Stat3mice. Total RNA was isolated according to the manufacturer’s instructions with DNAase digestion (cat.#217084, Qiagen). RNA quality was verified by Bioanalyzer (Agilent 2100 Bioanalyzer) and RNA-seq was performed using the Illumina HiSeq 2500 instrument by the Analytical Genomics and Bioinformatics Core Facility (SBP, Lake Nona). Up to 1 μg of total RNA was used to prepare standard Illumina single read TrueSeq libraries and library quality was controlled by using Agilent Bioanalyzer, Qubit, and KAPA qPCR. The KAPA primers target Illumina P5 and P7-region in the genome and reads with MAPQ score <10 were removed three times with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% horse serum to remove debris and interstitial cells. Single myofibers were then pooled and lysed with QIAzol lysis reagent (QIAGEN cat.#79306) for subsequent RNA extraction and qPCR analysis.

Single myofiber isolation. Single myofibers were isolated from the gastrocnemius/soleus muscles from adult and P15 mice. In brief, muscles were harvested and subjected to enzymatic dissociation (700 μml collagenase type II, Gibco cat. #17101-015) for 60 min at 37 °C in a shaking water bath. Disassociated single myofibers were manually collected under a dissection microscope and analyzed three times with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% horse serum to remove debris and interstitial cells. Single myofibers were then pooled and lysed with QIAzol lysis reagent (QIAGEN cat.#79306) for subsequent RNA extraction and qPCR analysis.

Cell culture procedures. All cells were cultured in incubators at 37 °C and 5% CO2. Freshly isolated MuSCs were plated on tissue culture plates coated with laminin (cat.#11243217001, Roche) and maintained in GM (45% DMEM, 40% F10, 5% fetal bovine serum (FBS)). Myogenic differentiation was induced with DMEM and 2% horse serum for 2 or 3 days. MuSC proliferation was measured by 5-ethynyl-2'-deoxyuridine (EdU) incorporation (5 μg/ml, cat.A10044, Life Technologies) for 4 h at the end of the culture. MuSCs were treated with CCF2/DCM (12.5 μM) or ethynylated in GM and cultured in DM (cat.#2279, Sigma) for 24 h when indicated. MuSCs were treated with recombinant FAM3A (1000 ng/ml, cat.#TP303495, OriGene) for 72 h when indicated.
C2C12 myogenic cells were obtained from ATCC (cat.-1772) and we did not perform further authentication. C2C12 cells were grown in High Glucose DMEM supplemented with 10% FBS (10%). Myogenin knock-down was induced with High Glucose DMEM and 2% horse serum for 5 days. C2C12 myogenic cells were treated with recombinant IL-6 (100 ng/ml, cat.# 216-16, Peprotech) for 24 h when indicated. C2C12 cells were treated with 200 μM Momenin (cat.#M5273, Sigma) for 2 h when indicated.

HEK-293 cells were obtained from ATCC (cat.-1573) and grown in High Glucose DMEM supplemented with 10% FBS. We did not perform further authentication.

Luciferase reporter assay. The Fam3a regulatory region (~3000 to +100 bp) was cloned into a pGL3 Luciferase plasmid (Fam3a-Luc). The following vectors were used: Fam3a-Luc, pRL Renilla Luciferase Control Reporter Vector (Promega), pcDNA3-Stat3, and pcDNA3-myc-MyoD. HEK293 cells were transfected with jetPRIME (cat.#114-07, Polyplus transfection) following the manufacturer’s instructions. Luciferase and Renilla activities were measured 48 h after transfection using the Dual Luciferase Reporter Assay System (cat.#E1910 Promega) following the manufacturer’s instructions.

Gene expression analysis. RNA was extracted from cells using the Qiagen miNeasy Micro Kit (cat.#271084, Qiagen) following the manufacturer’s protocol. Total RNA was quantified with a Qubit TM 3.0 Fluorometer (cat.#Q33216, Thermo Scientific). First-strand cDNA was synthesized from total RNA using the SuperScript® VILO® cDNA Synthesis Kit (cat.#11754050, Invitrogen) following the manufacturer’s instructions. cDNA was used in qPCR reactions consisted of Power SYBR® Green PCR Master Mix (cat.# 4367659, Life Technologies). 250 nM forward and reverse primers, and 0.5 ng of cDNA. All measurements were normalized to RpGPD expression using the 2−ΔΔCT method. The sequences of the primers used are in Supplementary Table 1.

Western blotting. Total protein extracts for western blot analysis were obtained by homogenizing C2C12 cells in RIPA buffer (50 mM Tris-HCl pH = 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) supplemented with protease inhibitors (cat.#11836153001, Sigma) and phosphatase inhibitors (cat.#4906837001, Sigma) cocktails. Cell debris was removed by centrifugation at 700 × g for 10 min and 4 °C and the supernatant was collected. Then the supernatant was placed in the −20 °C for 24 h. Next, cells were lysed in a 10X loading buffer with 8 ng/ml polybrene (cat.#TR-1003-6, Millipore) O/N at 37 °C and 5% CO2. Western blot images included in this manuscript are available in Supplementary Fig. 9.

Unprocessed images for western blots included in this manuscript are available in Supplementary Table 1.
Quantification and statistical analysis. Data are represented as mean ± SEM. The investigators were not blinded to allocation during experiments or to mouse genotype. Sample size was chosen based on literature and variability observed in previous experience in the laboratory. Comparisons between groups used the Student’s t test assuming two-tailed distributions with an alpha level of 0.05. For ChIP studies, comparisons between groups used the Student’s t test assuming one-tailed distributions with an alpha level of 0.05. Comparisons between the Pearson’s correlation coefficients were performed using one-way analysis of variance (ANOVA). Comparisons between the OCR curves were performed using ordinary two-way ANOVA. Statistical tests were performed using GraphPad Prism 7 or Microsoft Excel for Macintosh.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. FASTQ files from the RNA-seq performed on freshly isolated MuSCs from Pax7-CreER;Stat3fl/fl mice described in this paper have been deposited in the Sequence Read Archive (SRA) database under accession code PRJNA510443. Public MyoD ChIP-seq data from C2C12 myotubes were downloaded from GEO under the accession code GSE141785. Raw data files for the MyoD ChIP-seq from IMR90 human cells have been deposited in the SRA database under accession code PRJNA26256. Processed data for the MyoD ChIP-seq from IMR90 human cells have been deposited in GEO under the accession code GSE128527. A reporting summary for this article is available as a Supplementary Information file.

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Author contributions

Conceptualization, D.S. and A.S.; methodology, D.S., T.J.C., M.J.S., U.E., C.N., A.D., L.L., and A.S.; formal analysis, D.S.; investigation, D.S., T.J.C., M.J.S., U.E., C.N., and L.L.; writing—review and editing, D.S., T.J.C., M.J.S., U.E., C.N., P.L.P., G.D., L.L., and A.S.; supervision, A.S.; funding acquisition, D.S., P.L.P., G.D., L.L., and A.S.;
Additional information

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