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A Mitofusin 2 / Hif1α axis sets a maturation checkpoint in regenerating skeletal muscle.

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Abstract
A fundamental issue in regenerative medicine is whether there exist endogenous regulatory mechanisms that limit the speed and efficiency of the repair process. We report the existence of a maturation checkpoint during muscle regeneration which pauses myofibers at a neonatal stage. This checkpoint is regulated by the mitochondrial protein mitofusin 2 (Mfn2), whose expression is activated in response to muscle injury. Mfn2 is required for growth and maturation of regenerating myofibers; in the absence of Mfn2, new myofibers arrested at a neonatal stage, characterized by centrally nucleated myofibers and loss of H3K27me3 repressive marks at the neonatal myosin heavy chain gene. A similar arrest at the neonatal stage was observed in infantile cases of human centronuclear myopathy. Mechanistically, Mfn2 upregulation suppressed expression of Hypoxia-induced Factor 1α (Hif1α), which is induced in the setting of muscle damage. Sustained Hif1α signaling blocked maturation of new myofibers at the neonatal-to-adult fate transition, revealing the existence of a checkpoint that delays muscle regeneration. Correspondingly, inhibition of Hif1α allowed myofibers to bypass the checkpoint, thereby accelerating the repair process. We conclude that skeletal muscle contains a regenerative checkpoint which regulates the speed of myofiber maturation in response to Mitofusin 2 and Hif1α activity.
Introduction

In response to injury, skeletal muscle undergoes a synchronized sequence of events over several days, including clearance of muscle debris, revascularization of the damaged region, activation and proliferation of adult muscle stem cells (MuSCs), fusion of muscle stem cells to form new myofibers, and maturation of new myofibers into adult fiber types (1). Promoting and accelerating muscle repair following injury has been a clinical goal for many years, and several supplements and therapies have been evaluated (2-4), although the underlying mechanisms are not well-known. An understanding of how the individual steps in regeneration are coordinated would positively impact our ability to design therapies promoting efficient and rapid tissue regeneration in the setting of traumatic injury. To this end, a body of work has investigated requirements for efficient muscle repair, largely focusing on early events including maintenance of the MuSC population through asymmetric division and niche factors, activation of MuSCs via expression of the myogenic regulatory factors and miRNAs, and modulation/clearance of the immune infiltrate (5-8).

In contrast, the requirements for the growth and maturation of myofibers during the late stages of muscle repair are relatively understudied, and whether the maturation stage of regeneration can be accelerated is unknown. During this latter stage, myofibers grow in size and sequentially express distinct myosin heavy chain (Myh) genes, transitioning from embryonic (Myh3) to neonatal (Myh8) to adult (Myh1,2,4,7) myosin expression. These myosin genes are functionally distinct, and confer contractile properties to the myofiber (9). Concomitant with these fate specification events, changes in mitochondrial content and morphology are apparent, and constitute a hallmark of myofiber maturation.
into adult muscle fibers (10-12). Establishment of a large mitochondrial population is critical to the health of adult myofibers; however, it is not known if mitochondrial changes represent a passive characteristic of adult fiber specification, or if the organelle directly regulates the myofiber maturation process itself.

Here, we investigate role of mitochondria in impacting regeneration following muscle injury, focusing on the mitochondrial outer membrane protein Mitofusin 2 (Mfn2). We find that Mitofusin 2 is upregulated during MuSC activation, and is specifically required for the growth and adult fate specification in the latter stage of myofiber maturation. This finding is in contrast to the lack of a requirement for Mitofusin 2 in fully differentiated muscle fibers. Mechanistically, regenerating mfn2-knockout myofibers exhibited excess and sustained Hif1α activity, which results in epigenetic alterations at the myosin heavy chain locus, an accumulation of centrally nucleated growth-arrested myofibers, and a pause at the neonatal-to-adult fiber type transition. Interestingly, we observe a similar neonatal pause in pediatric cases of severe centronuclear myopathy. Further analysis in animal models revealed that elevated Hif1α signaling is sufficient to arrest regenerating myofibers at the neonatal-to-adult transition, thereby delaying the growth and maturation of regenerating muscle. During severe injury, we find that myofibers engage this regenerative checkpoint at the neonatal-to-adult fiber type transition that synchronizes the maturation of muscle fibers with the re-establishment of perfusion. Pharmacologic or genetic inhibition of Hif1α results in this checkpoint being bypassed, thereby accelerating the regenerative process. Together, these findings reveal a role for mitochondria and Hif1α in regulating late stages of regeneration, and inform on a strategy to accelerate muscle repair in response to traumatic injury.
Results

Induction of mitofusin 2 in activated muscle stem cells is required for growth of new myofibers.

Recent results from in vitro cultured C2C12 myoblasts and myotubes suggested that mitochondrial genes may be under control of myogenic regulatory factors (13). We therefore performed ChIP-seq analysis to investigate whether mitochondrial genes are regulated by the master myogenic regulatory factors (MyoG and MyoD) during in vivo muscle regeneration. We injured tibialis anterior (TA) muscles of wild-type mice containing the muscle satellite cell-specific Pax7-CreERT2 allele (14) driving conditional expression of a fluorescent and mitochondrially localized Dendra2 protein (15), which allows the facile detection and isolation of muscle satellite cells (MuSCs) and their progeny by FACS (Figure 1A). The TA muscle is routinely used in experimental studies of muscle regeneration, and exhibits robust activation of MuSCs, formation of new myofibers, and complete functional recovery in response to muscle injury (16, 17). In non-injured vehicle-injected muscle, quiescent Dendra2+ MuSCs (QSCs) were largely CD34+, a marker of quiescence in this lineage; while in 2 day post injury (dpi) muscle, Dendra2+ MuSCs lost CD34 expression, indicating an activated state (ASCs) (18) (Figure 1A). ChIP-seq experiments from these two populations allowed the identification of differentially bound peaks in QSCs vs. ASCs; this analysis revealed binding of MyoG and MyoD to candidate regulatory elements for 42 and 867 genes (respectively) specific to activated stem cells (Table S1, FDR<0.05), including previously validated targets (Figure 1B). We compared our identified MyoD and MyoG targets in ASCs against a list of known regulators of mitochondrial biology (Table S1; 696 genes). MyoG was not bound to any
mitochondrial genes, and MyoD was bound to 24 mitochondrial genes (Table S1, Figure 1C), including the master mitochondrial regulator Pgc-1β. MyoD bound in vivo to 3 discrete genomic regions within the Pgc-1β gene, including peaks in the proximity of the promoter and intron 1 (Figure 1D). We did not detect binding of MyoD or MyoG to the related family member, Pgc-1α (Figure 1E), and overexpression of MyoD in mouse 3T3 fibroblasts was sufficient to induce expression of Pgc-1β (Figure 1F). Correspondingly, we found that Pgc-1β transcripts were significantly induced in vivo in ASCs vs. QSCs, while Pgc-1α transcripts were unchanged (Figure 1G).

Pgc-1β has been established to promote transcription of genes involved in numerous mitochondrial processes, including biogenesis, oxidative phosphorylation and mitochondrial dynamics (19). In particular, Pgc-1β promotes transcription of the mitofusin genes, which are localized to the mitochondrial outer membrane and responsible for initiation of mitochondrial fusion, as well as calcium homeostasis (20-22). Indeed, exogenous Pgc-1β overexpression in vitro is selectively associated with Mfn2 (but not Mfn1) accumulation (Figure 1F). We found that Mfn2, but not Mfn1, was selectively induced in ASCs vs. QSCs (Figure 2A,B), suggesting a specific role for Mfn2 in muscle regeneration. We therefore made use of conditional knockout alleles for Mfn1 or Mfn2 (23), combined with the Pax7-CreERT2 driver, to deplete Mfn1 or Mfn2 levels in MuSCs (Figure S1A,B), and assess their role in muscle regeneration. Following tamoxifen-induced depletion and muscle injury (Figure 2C), animals with Mfn2-deleted MuSCs (hereafter, mfn2−/−) were able to generate MyoD+ and MyoG+ ASCs at 2 dpi (Figure 2D,E).

At 5 dpi, mfn2−/− animals form a large number of de novo myofibers, identified by expression of the embryonic myosin heavy chain (Myh3) and prominent centralized
nuclei; however the mutant myofibers were significantly diminished in size, and the injured muscle area retained a significant amount of interstitial tissue (Figure 2D,F). At 14 and 42 dpi, new myofibers (marked by centralized nuclei) from wild-type animals have continued to grow in size; however, myofibers and muscle in mfn2−/− animals remain significantly smaller with retained centralized nuclei (Figure 2D,F,G). In contrast, animals with Mfn1-deleted MuSCs (hereafter mfn1−/−) displayed no defects in stem cell activation, myofiber formation, fiber growth or muscle growth (Figure 2D-G).

The above results indicate that Mfn2 is not required in vivo for MuSC activation and fusion to form new myofibers, but is necessary for myofiber growth post-fusion. We investigated the requirement for Mfn2 in vitro, making use of primary MuSCs purified from tamoxifen-treated animals. mfn2−/− MuSCs exhibited no defects in their ability to generate a membrane potential in response to mitochondrial substrates (Figure S2A,B). Additionally, we did not observe elevated mitochondrial superoxide levels (Figure S2C,D) or defects in oxygen consumption rates (Figure S2E). Proliferation of MuSCs was unaffected by Mfn2 loss (Figure S2F), and mfn2−/− MuSCs were able to fuse and form myotubes at similar rates to wild-type MuSCs (Figure S2G,H). Thus, Mfn2 is dispensable for myoblast proliferation and differentiation in vitro, similar to our observations in vivo.

Mfn2 mutations in humans are a common cause of Charcot-Marie Tooth disease (type 2A), an autosomal dominant axonal neuropathy with associated muscle atrophy (24, 25). We tested the effect of the disease-associated T105M mutation, making use of a Cre-inducible Mfn2T105M allele inserted into the Rosa26 locus (26). Similar to mfn2−/− animals, expression of the dominant negative Mfn2T105M did not inhibit MuSC activation
or myofiber formation, but severely restricted the growth of new myofibers (Figure 3A,B, Figure S1C-E).

**Regenerating mfn2−/− myofibers arrest at the neonatal stage.**

During regenerative growth, new myofibers proceed from an embryonic to neonatal to adult fate, classified by sequential expression of myosin heavy chain genes localized at the Myh locus (27-29). By 14 dpi, wild-type myofibers have taken on adult fates indicated by a mixture of adult type I (Myh7), type IIa (Myh2), type IIx (Myh1) and type IIb (Myh4) fiber types, and a lack of embryonic (Myh3) and neonatal (Myh8) fiber types (Figure 4A,B). The growth-arrested mfn2−/− myofibers do not adopt an adult fate, as evidenced by a lack of adult Myh expression; instead the vast majority of new fibers adopt a neonatal Myh8+ fate (Figure 4A,B). The mechanisms underlying sequential expression of Myh genes are not currently understood, but potentially rely on epigenetic histone marks, which have been previously implicated in MuSC maintenance (30-32). Western blot analysis of 14 dpi regenerating myofibers revealed upregulation of a subset of KDM (lysine demethylase) family members, including KDM4 and KDM6 family members (Figure 4C). As these enzymes exhibit H3K9 and H3K27 demethylase activity, respectively, we therefore examined H3K9me3 and H3K27me3 deposition in 14 dpi wild-type and mfn2−/− myofibers. Global levels of H3K9me3 and H3K27me3 were not significantly affected (Figure 4C). To more precisely examine genome-wide deposition, we performed ChIP-seq analysis in 14 dpi wild-type and mfn2−/− regenerating myofibers. H3K9me3 deposition was observed at expected loci based on previous reports (33, 34) (Figure S3A), and k-means clustering revealed similar deposition patterns between wild-
type and \( mfn2^{-/-} \) regenerating myofibers (Figure 4D, Table S2). H3K27me3 is deposited by Polycomb Gene (PcG) complexes, and evaluation of H3K27me3 deposition revealed similar patterns at known PcG-binding genes (35) between wild-type and \( mfn2^{-/-} \) myofibers (Figure S3B,C). However, k-means analysis of genome-wide H3K27me3 peaks revealed a cluster with significantly decreased deposition in \( mfn2^{-/-} \) myofibers (Figure 4E), which included peaks in the Myh8 locus (Table S2). Examination of the Myh locus revealed significant deposition of H3K27me3 marks in an intragenic region of the Myh8 locus in wild-type 14 dpi myofibers (Figure 4F,G). Intragenic deposition of H3K27me3 is highly associated with repressed transcription (36, 37). Interestingly, the H3K27me3 peak at the Myh8 locus was significantly reduced in \( mfn2^{-/-} \) regenerating myofibers (Figure 4F,G). This identified region spanned multiple introns, and was verified by ChIP-qPCR in independent experiments (Figure S3D,E). No significant deposition of H3K27me3 was noted at other Myh genes, and no deposition of H3K9me3 was observed within the Myh locus (Figure 4F). We also did not observe differential deposition or enrichment of H3K9me3 or H3K27me3 in MyoD or MyoG targeted regions (Figure S3F). Thus, the observed maturation arrest at the neonatal (Myh8+) stage observed in \( mfn2^{-/-} \) regenerating myofibers correlates with loss of repressive H3K27me3 marks at the Myh8 locus.

**Sustained Hif1α signaling underlies the regenerative arrest in \( mfn2^{-/-} \) myofibers.**

To examine the underlying mechanisms regarding regulation of the neonatal-to-adult transition in \( mfn2^{-/-} \) animals, we employed RNAseq to compare wild-type and \( mfn2^{-/-} \)-activated stem cells (Figure S4A, Table S3). Gene ontology analysis of down-regulated
genes revealed enrichment in developmental pathways, consistent with the observed effects on myofiber development (Figure S4B, Table S3). In contrast, genes upregulated in mfn2−/− ASCs were enriched for metabolic pathways, which included the transcriptional upregulation of Hif1α (Figure S4A,C, Table S3). Indeed, gene set enrichment analysis indicated that Hif1α targets were significantly enriched among upregulated genes in mfn2−/− ASCs (Figure 5A). At 14 dpi, mfn2−/− and Mfn2T105M regenerating myofibers exhibited continued upregulation of Hif1α transcripts and protein, as well as increased levels of Hif1α targets (including the histone demethylases KDM4B, KDM4C, KDM6B), increased phosphorylation of PDH, and impaired mitochondrial biogenesis (Figure 4C, Figure 5B-D); these effects are consistent with known roles of Hif1α in regulation of mitochondrial biology (38). To further investigate consequences of these arrested myofibers, we performed steady-state metabolomics measurements in wild-type and mfn2−/− regenerating myofibers at 5 and 14 dpi. Unsupervised hierarchical clustering separated mature 14dpi wild-type myofibers; however, the metabolic profiles of 14 dpi mfn2−/− myofibers were interspersed with the 5 dpi immature myofibers (Figure S5A). We identified a number of metabolites altered in mfn2−/− myofibers at 5 and 14 dpi (Figure S5B, Table S4). Overall, we did not observe changes in most TCA cycle metabolites (Figure S5C). Interestingly, metabolites related to ketone and amino acid oxidation (acetoacetate, β-hydroxybutyrate, glutaryl carnitine) were significantly upregulated in mfn2−/− myofibers at both 5 and 14 dpi (Figure S5C).

We examined Hif1α protein levels in vivo during muscle regeneration in wild-type, mfn2−/− and Mfn2T105M animals. Consistent with previous reports (39), wild-type animals exhibited a significant increase in Hif1α protein levels 2 days post-injury within the
damaged region of muscle (Figure 5E). By 5 dpi, Hif1α levels have largely diminished to pre-injury levels, indicating that the injury-induced rise in Hif1α is transient in wild-type animals (Figure 5E). Injured mfn2<sup>−/−</sup> and Mfn2<sup>T105M</sup> animals also display a significant upregulation of Hif1α at 2 dpi, and Hif1α levels remain high and present in the nuclei of new myofibers at 5 and 14 dpi (Figure 5E). This was not due to impaired vascularization of the regenerating region, based on CD31 staining for capillaries at 14 dpi (Figure S4D). Thus, Mfn2 is required post-injury to lower Hif1α levels in later stages of regeneration.

Mfn2 has been previously shown to regulate localization of NFATC2 (NFAT1), a calcium-dependent transcription factor (40). At 5 dpi and 14 dpi, mfn2<sup>−/−</sup> and Mfn2<sup>T105M</sup> regenerating myofibers displayed significant increases in nuclear localization of NFATC2 (Figure S4E). Transcription of Hif1α has been previously suggested to be induced by altered calcium levels (41), and we found that NFATC2 overexpression was sufficient to induce Hif1α protein levels in hypoxia-treated cells in vitro (Figure S4F). We also observed NFATC2 nuclear localization and stimulated transcription of Hif1α in mfn2<sup>−/−</sup> myotubes in vitro (Figure S2I,J). We therefore performed in vivo ChIP-seq analysis of NFATC2 binding regions, which indicated significantly increased occupancy of NFATC2 at the Hif1α promoter in 14 dpi mfn2<sup>−/−</sup> myofibers (Figure S4G). We did not observe changes in methylation marks at either Hif1α or NFATC2 loci (Figure S6A,B). These data support a model whereby upregulation of Mfn2 during muscle regeneration negatively regulates NFATC2 activity in order to suppress Hif1α transcription induced by muscle injury; however, we cannot rule out the possibility of alternative mechanisms for Hif1α stabilization.
Hif1α signaling has been previously linked to alterations in histone methylation, via the induction of demethylases or regulation of the PRC2 complex (42-44). We therefore tested if excess Hif1α signaling mediates the neonatal (Myh8+) arrest observed in regenerating Mfn2-mutant myofibers. We first arrested mfn2−/− or Mfn2T105M myofibers in the Myh8+ state at 14 dpi, and then treated animals for an additional 2 weeks with vehicle or PX-478, a compound known to reduce Hif1α levels (45, 46) (Figure 6A). PX-478 treatment for 14 days was sufficient to lower Hif1α levels in both mfn2−/− and Mfn2T105M myofibers (Figure 6B). ChIP-qPCR analysis targeting the Myh8 allele revealed that PX-478 treatment restored H3K27me3 deposition at the Myh8 locus (Figure 6C). Strikingly, we observed that PX-478 (but not vehicle) treatment was sufficient to release mfn2−/− and Mfn2T105M myofibers from their neonatal arrested state, allowing them to now adopt adult fates (Figure 6F). This was accompanied by a partial rescue of fiber and muscle size (Figure 6D,E) by 28 dpi. Thus, inhibition of Hif1α signaling is sufficient to release myofibers from the maturation arrest in these Mfn2 mutant animal models. To test the role of H3K27me3 demethylases, we repeated these experiments, but instead treated animals with GSK-J4, a potent inhibitor of KDM6A and KDM6B demethylases (47). Two week treatment starting at 14 dpi (Figure S7A) was sufficient to restore H3K27me3 deposition at the MyH8 locus (Figure S7B). We observed that GSK-J4 treated animals were able to proceed through the neonatal stage and express adult myosins (Figure S7C), accompanied by increased fiber size and muscle growth (Figure S7D,E). Thus, inhibition of H3K27 demethylases regulates procession through the neonatal stage during muscle regeneration in our Mfn2-mutant animal models.
*Excess Hif1α is sufficient to arrest regenerating myofibers at the neonatal – adult transition.*

Loss of Mfn2 is predicted to have a myriad of cellular and organellar effects in regenerating myofibers. However, our above results with the PX-478 compound suggest that elevated Hif1α signaling represents a key feature governing regenerative defects in our animal model. To definitively test the relevance of excess Hif1α signaling in mfn2−/− regenerating myofibers, we made use of a conditional knockout allele to deplete Hif1α levels in MuSCs (Figure S8A). Consistent with a previous report (39), Hif1α deletion alone in MuSCs did not impair regeneration of myofibers in response to injury, including the activation of MuSCs, and the formation and maturation of new myofibers (Figure 7A-D, Figure S8B,C). In the background of Mfn2 deletion, Hif1α removal largely rescued myofiber maturation defects, including significant improvements in myofiber and muscle size, mitochondrial content, as well as robust differentiation into adult fiber types (Figure 7A-D, Figure S8D). Thus, elevated Hif1α signaling is a key functional mechanism by which Mfn2 governs muscle regeneration.

These data suggest the possibility of a Hif1α-mediated regeneration checkpoint which regulates the transition between neonatal and adult fiber types. While loss of Hif1α has been previously studied in muscle stem cells (39), the effects of excess Hif1α in regenerating myofibers has yet to be examined. We therefore made use of conditional alleles targeting Hif1α stability, combined with the Pax7-CreERT2 driver. In these experiments, we prevented degradation of Hif1α by either conditional removal of VHL (the substrate recognition module for E3 ligase-degradation of Hif1α), or conditional expression of a Proline->Alanine mutant of Hif1α (HA-Hif1dPA) which prevents
recognition by Vhl (Figure S8E,F) (48, 49). In both models, we observed significant
gains in Hif1α levels post tamoxifen and during regeneration (Figure 8A). Both
genetic models largely recapitulated key features of the mfn2−/− model, including normal
activation of MuSCs, decreased fiber size and muscle weight, arrested fibers at the
neonatal Myh8+ stage, and loss of H3K27me3 deposition at the Myh8 locus (Figure 8A-
D, Figure S8G,H). Thus, elevated Hif1α signaling during muscle regeneration is sufficient
to inhibit myofiber maturation, including a specific blockade of the neonatal-to-adult fiber
type transition.

*Neonatal Myh8+ fibers are characteristic in severe centronuclear myopathy.*

The histological deficits present in Mfn2−/− regenerating myofibers are reminiscent of a
centronuclear myopathy (CNM) phenotype. CNM comprises a group of rare genetic
muscle disorders with variable severity, ranging from life-threatening infantile
presentations to milder adult-onset forms (50). Histological findings include centrally
placed nuclei within muscle fibers, sometimes accompanied by peri-nuclear
mitochondria. A number of genes have been implicated in CNM, including Mtm1, Dnm2,
Bin1 and Ryr1; however, the precise disease pathophysiology is still under investigation.
We therefore investigated myofiber fate and HIF1α status in muscle biopsies from
genetically confirmed CNM patients and age/sex-matched controls (Table S5). Two
patients with infantile CNM displayed a significant number of central nuclei myofibers with
peri-nuclear mitochondrial localization (Figure 9A, Figure S9A). Interestingly, CNM
myofibers stained strongly positive for the neonatal MYH8 marker (Figure 9A). We
investigated HIF1α status in the severely, affected patients, making use of staining for
CA3 (carbonic anhydrase 3), a HIF1α target and marker of hypoxia in clinical specimens (51, 52). From this analysis, we observed elevated CA3 levels in the severe CNM samples as compared with controls (Figure 9A). We also observed increased nuclear localization of NFATC2 in CNM patients, particularly the patient with a Dnm2 mutation (CNM1; Figure 9A). Thus, our findings suggest that affected fibers in severe CNM mimic results from our Mfn2-mutant animal models, including a maturation arrest at the neonatal MyH8 stage and elevated HIF1α activity, although CNM disease is not associated with MFN2 deficiencies (Figure S9A).

Regenerating myofibers following ischemic injury engage a Hif1α-dependent checkpoint at the neonatal-adult fate transition.

The above results reveal that genetic modifications associated with excess Hif1α levels are sufficient to arrest regenerating myofibers at the neonatal (Myh8+) stage following chemically-induced injury. In principle, arrested myofibers may delay muscle recovery by inhibiting tissue maturation and growth. We therefore investigated if wild-type animals engage a similar arrest during recovery from severe muscle injury. We implemented an ischemic injury model, making use of a femoral artery ligation protocol that robustly limits blood flow to the affect limb and is associated with severe muscle injury (53, 54). We assessed wild-type animals at 5 to 14 days post-ligation (dpl), and noted that Hif1α levels declined at 7-9 dpl in the TA muscle (Figure 10A, Figure S10A), which correlates with the timing of reperfusion of the limb by peripheral arteries (based on previous studies (54)). Wild-type regenerating myofibers remained arrested in the neonatal Myh8+ state until day 9, at which point Myh8 levels began declining and were
undetectable by day 12 (Figure 10B, Figure S10A). Concomitantly, we observed the increasing deposition of H3K27me3 marks at the Myh8 locus starting at day 9 (Figure 11D), as well as the appearance and growth of adult fiber types starting at day 10 (Figure 11A, Figure S10A). Thus, in response to ischemic injury, regenerating myofibers remain arrested in a Myh8+ state for several days, and transition from a neonatal to adult fate coincident with reperfusion, lowering of Hif1α levels, and H3K27me3 deposition at the Myh8 locus.

To assess the role of Hif1α in this process, we performed femoral artery ligations in the setting of conditional removal of Hif1α. In these animals, myofiber maturation was significantly accelerated (Figure 11B,C). In particular, Myh8+ fibers had completely differentiated by day 8, approximately 4 days earlier as compared with wild-type animals (Figure 10B, 11B, Figure S10A). These events correlated with an earlier deposition of H3K27me3 marks at the Myh8 locus, and an earlier appearance and growth of adult fiber types (Figure 11A,C,D). Thus, regenerating myofibers arrest at a Myh8+ state in the setting of severe injury in a Hif1α-dependent manner. In the setting of Hif1α removal, this arrest is bypassed, resulting in accelerated growth and maturation of muscle tissue.
Discussion

The ability of Hif1α to regulate fate specification of regenerating myofibers constitutes a new physiological role for its already versatile signaling pathway. In regenerating muscle, Hif1α activity regulates myofiber fate specification and epigenetic control of the neonatal Myh8 locus, where repressive H3K27me3 marks appear necessary to suppress expression of developmental myosins and allow fibers to adopt adult fates. These data support a model in which muscle regeneration contains a checkpoint that prevents adult fiber type specification in the presence of Hif1α signaling. Importantly, inhibition of Hif1α allows myofibers to pass this checkpoint, thereby accelerating the regeneration process. We note that a limitation of our study in that our experiments exclusively focused on the tibialis anterior muscle of mice which predominantly consists of fast-twitch (type IIX and IIB) fibers. It is possible that other muscles with alternative fiber type compositions, including human muscles, may respond differently during the regenerative process.

The involvement for Hif1α suggests that ischemia and reperfusion of the injured area play important roles in regulating myofiber specification during muscle repair. Indeed, a large number of studies have previously assessed the role of both hypoxia and hyperoxia in MuSC activation, in both in vitro and in vivo contexts (55). Although some of these studies have provided mixed results, hyperbaric oxygen therapy has been shown in animal models to increase the size of myofibers following muscle injury, and is commonly used in athletes to promote muscle recovery (56-58). The precise mechanisms relating hyperoxia to muscle regeneration are unknown, but have largely focused on proliferation of activated MuSCs. A previous study (39) indicated that removal of Hif1α in
MuSCs allows for an increased number of activated stem cells which promoted increased size of regenerated myofibers. Our results complement these findings, by showing that loss of Hif1α also promotes muscle regeneration by accelerating transition of myofibers into an adult fate.

Our data adds to the proposed techniques to enhance muscle regeneration by suggesting a therapeutic intervention targeted to the latter stages of muscle repair, when newly developed myofibers are growing and transitioning into adult fiber types. Previous work on enhancing muscle growth during recovery has largely focused on nutrient supplementation, physical therapy and mechanical scaffolds (59). We show above that there exists a Hif1α-dependent pause at the neonatal-adult transition, which is targetable and offers an opportunity to accelerate muscle repair through the use of orally available Hif1α or KDM6 inhibitors. Hif1α loss in skeletal muscle is well-tolerated in animal studies (60), indicating that bypassing this checkpoint does not significantly impair tissue health. The KDM6 inhibitor used here (GSK-J4) has been reported to inhibit the expression of myogenin during early stages of muscle regeneration (61), suggesting that the beneficial effects of demethylase inhibition are restricted to the latter stage of muscle regeneration.

In future work, it will be interesting to test if these inhibitors synergize with therapies targeted to earlier stages of muscle regeneration (MuSC activation, immune cell clearance). In addition, developing muscle fibers in the embryo and neonatal stages go through a parallel fate specification pattern (9), and we find that pediatric CNM patients with severe disease exhibit histological and fate specification characteristics similar to our Mfn2−/− regenerating myofibers. It will be interesting to explore if a similar Hif1α-checkpoint
regulates the timing of adult fiber specification and growth during development, and impacts the development of CNM pathology.

These results also highlight a role for mitochondria in muscle stem cell function beyond their function as ATP generators. Here, we find that a key role for Mitofusin 2 in regenerating myofibers relates to its ability to negatively regulate Hif1α signaling. Removal of Mitofusin 2 alone is largely dispensable in adult muscle fibers (23, 62, 63), but is predicted to have a myriad of cellular and organellar effects. Our results instead highlight a context-specific requirement for Mfn2 in regenerating myofibers, that is functionally mediated by alterations in Hif1α signaling. Interestingly, we observed similar phenotypes with overexpression of the CMT2A disease-associated $Mfn2^{T105M}$ allele, suggesting that delayed myofiber regeneration may contribute to the disease pathophysiology in associated patients. Muscle biopsies are not routinely performed on CMT2A patients, and thus it will be important to assess in a future prospective study if these patients suffer from impaired tissue regeneration.
Methods

Detailed method information, including information on statistical analyses, is included in Supplemental Methods.

Data and material availability

Raw and processed data for RNA sequencing and ChIP-seq experiments are available at the NCBI GEO website under accession number GSE185106. Other data and materials are provided within the manuscript and supplementary materials, or available upon reasonable request.

Study Approval

All animal studies were approved by the University of Texas Southwestern Medical Center Institutional Animal Care & Use Committee. Human studies were approved as a retrospective study on archived excess patient tissue by the University of Texas Southwestern Medical Center Institutional Review Board.

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**Conflict of Interest Statement:** The authors have declared that no conflict of interest exists.
References


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Figure 1. MyoD promotes expression of Pgc1β and mitochondrial genes in activated MuSCs. (A) Schematic of muscle injury experiments and FACS isolation of MuSCs. Tamoxifen is administered for 5 consecutive days to induce recombination, followed by BaCl₂ (or vehicle) administration to induce muscle injury. Dendra²⁺ DAPI⁻ MuSCs are collected at 2 dpi. In vehicle-treated muscle, quiescent (CD34⁺) MuSCs (QSCs) are collected. In injured muscle, activated (CD34⁻) MuSCs (ASCs) are collected. (B) Representative snapshots of MyoD binding at the MyoG and Mef2a genes in 2 dpi QSCs and ASCs. Identified peaks in the proximity of the transcriptional start site are
indicated by red boxes. (C) Venn diagram of MyoG and MyoD bound genes in 2 dpi ASCs. Genes were compared with a list of known mitochondrial regulators (Table S1). (D) Representative snapshots of MyoD binding at the PGC-1β gene in 2 dpi QSCs and ASCs. (E) Representative snapshots of MyoD and MyoG binding at the PGC-1α gene in 2 dpi QSCs and ASCs. (F) Mouse 3T3-L1 fibroblasts were transfected with empty vector (pQC-empty) or MyoD-expressing vector (pQC-MyoD) and assessed by western blot at 48 hours post-transfection for the indicated targets. Histone 2B (H2B) is shown as a loading control. Molecular weight markers (in kDa) are indicated. (G) PGC-1α and PGC-1β mRNA levels (relative to β2-microglobulin; normalized) assessed by qRT-PCR in wild-type QSCs and ASCs at 2 dpi. Statistical significance was assessed using t-tests (G) with adjustments for multiple comparisons. For each ChIP-seq dataset, 3 biological replicates were analyzed. Box plots indicate median and interquartile ranges from the indicated number of biological replicates; whiskers are plotted using the Tukey method.
Figure 2. Mitofusin 2 is required for growth of regenerating myofibers. (A) Mfn1 and Mfn2 transcript levels (relative to β2-microglobulin; normalized) in wild-type QSCs and 2 dpi ASCs. (B) Western blots for Mfn1 and Mfn2 in wild-type QSCs and 2 dpi ASCs, as assessed by western blot. Histone 2B (H2B) is displayed as a loading control; molecular weight (kDa) is indicated. (C) Schematic of muscle injury experiments. Tamoxifen (TMX) is given for 5 consecutive days to induce recombination, followed by BaCl2-mediated muscle injury. Muscles are analyzed at the indicated timepoints. (D) Representative histology (H&E) and immunofluorescence images of muscle cross-sections of the indicated genotypes, timepoints. Staining for nuclei (DAPI, blue), myofiber boundaries (WGA (green) or Laminin (green)), and MyoD, MyoG or Myh3 (red) is presented. Scale bar, 50 μm. (E) MyoD+ and MyoG+ cell numbers from 2 dpi muscles, normalized to cross-sectional area. (F) Cross-sectional area of regenerating fibers from muscles at 5, 14 and 42 dpi. n=100-300 myofibers were analyzed from 6-11 mice per group. (G) Tibialis anterior (TA) muscle weight (normalized to body weight) from mice at the indicated timepoints. Statistical significance was assessed using one-way ANOVA (E,G), two tailed t-test (A), or Kruskal-Wallis (F) tests with adjustments for multiple comparisons. p-values reflect comparison with the wild-type group. Box plots indicate median and interquartile ranges from the indicated number of biological replicates; whiskers are plotted using the Tukey method.
Figure 3. The CMT2A Mfn2<sup>T105M</sup> allele inhibits growth of regenerating myofibers. (A) Representative histology (H&E) and immunofluorescence images of muscle cross-sections from Mfn2<sup>T105M</sup> mice at 5 and 14 dpi. Myh3 (red), Laminin (green), and DAPI (blue) staining are presented. Scale bar, 50 μm. (B) Cross-sectional area of regenerating fibers from muscles at 5 and 14 dpi. n=100-300 myofibers were analyzed from 6-10 mice per group. Statistical significance was assessed using Kolmogorov-Smirnov (B) tests. p-values reflect comparison with the wild-type group. Box plots indicate median and interquartile ranges from the indicated number of biological replicates; whiskers are plotted using the Tukey method.
Figure 4. *mfn2*−/− regenerating myofibers are arrested at a neonatal fate. (A) Representative immunofluorescence images of muscle cross-sections from mice of the indicated genotype at 14 dpi. Sections were stained with antibodies targeting fiber-type specific myosin heavy chains, including Myh7 (type I; purple), Myh2 (type IIa; red), Myh4 (type IIb; blue), Myh1 (type IIx; red), Myh3 (embryonic; red), and Myh8 (neonatal; red). Myofiber borders were visualized with laminin staining (green). Scale bar, 50 μm. (B) Quantitation of fiber types (as a percentage of total regenerating fibers) in wild-type and *mfn2*−/− animals at 14 and 42 dpi. (C) Levels of H3K9me3, H3K27me3, and a number of
KDM family members in 14 dpi myofibers. Molecular weight markers (in kDa) are indicated. Histone 2B (H2B) and Histone 3 (H3) are shown as loading controls. (D) Heatmaps representing normalized H3K9me3 ChIP-seq intensities of identified genome-wide peaks in 14 dpi myofibers of the indicated genotype, after k-means clustering. Peaks were ranked according to their ChIP-seq intensity in wild-type samples. n=3 mice per group. (E) Heatmaps representing normalized H3K27me3 ChIP-seq intensities of identified genome-wide peaks in 14 dpi myofibers of the indicated genotype, after k-means clustering. Peaks were ranked according to their ChIP-seq intensity in wild-type samples. n=3 mice per group. (F) Representative snapshots for H3K9me3 and H3K27me3 ChIP-seq analyses performed in 14 dpi myofibers of the indicated genotype, focusing on the myosin heavy chain locus. Increased deposition of H3K27me3 at the Myh8 gene is highlighted (red box). (G) Representative snapshots of H3K9me3 and H3K27me3 deposition at the Myh8 gene in 14 dpi myofibers of the indicated genotype. For each ChIP-seq dataset, 3 biological replicates were analyzed. Box plots indicate median and interquartile ranges from the indicated number of biological replicates; whiskers are plotted using the Tukey method.
Figure 5. Mfn2-mutant regenerating myofibers exhibit sustained Hif1α signaling.

(A) Gene set enrichment analysis of Hif1α target genes in mfn2−/− vs. wt ASCs. NES: normalized enrichment score. (B) Hif1α mRNA (relative to β2-microglobulin, normalized)
in 14 dpi myofibers. (C) Western blot analysis of the indicated proteins in 14 dpi myofibers. Molecular weights (kDa) are indicated. (D) Mitochondrial genome (mtDNA) content, normalized to nuclear genome content (nDNA) in 14 dpi myofibers. (E) Representative immunofluorescence images of Hif1α (red), nuclei (DAPI, blue) and myofiber boundaries (WGA, green) in muscle cross-sections at indicated timepoints. Scale bar, 50 μm.

Statistical significance was assessed using one-way ANOVA (B,D) with adjustments for multiple comparisons. Box plots indicate median and interquartile ranges from the indicated number of biological replicates; whiskers are plotted using the Tukey method.
Figure 6. Hif1α inhibition enables maturation of Mfn2-mutant regenerating myofibers. (A) Schematic of PX-478 experiment. Tamoxifen (TMX) administration (5 consecutive days) was followed by BaCl2 muscle injury. At 14-28 dpi, mice were treated with PX-478 (or vehicle). (B) Representative immunofluorescence images of Hif1α (red), nuclei (DAPI, blue) and myofiber boundaries (WGA, green) in 28 dpi muscle cross-sections of the indicated genotype, treatment. Scale bar, 50 μm. (C) Enrichment (% of input) from H3K27me3 ChIP-qPCR experiments targeting Myh8 in 28 dpi myofibers. (D) Cross-sectional area of 28 dpi myofibers of the indicated genotype, treatment. n=300 myofibers were analyzed from 6 mice per group. (E) TA muscle weight (normalized to body weight) of the indicated genotype, treatment at 28 dpi. (F) Representative immunofluorescence images of 28 dpi muscle cross-sections of the indicated genotype, treatment. Sections were stained with antibodies targeting fiber-type specific myosin heavy chains: Myh7 (type I; purple), Myh2 (type Ila; red), Myh4 (type IIb; blue), Myh1 (type IIX; red), Myh8 (neonatal; red), and myofiber boundaries (laminin, green). Scale bar, 50 μm. Statistical significance was assessed using two-way ANOVA (C,E), or Kruskal-Wallis (D) tests with adjustments for multiple comparisons. Box plots indicate median and interquartile ranges from the indicated number of biological replicates; whiskers are plotted using the Tukey method.
Figure 7. *Hif1α* deletion enables maturation in *mfn2*−/− regenerating myofibers. (A)

Representative histology (H&E) and immunofluorescence images of muscle cross-sections (5 dpi). Muscle cross-sections were stained for Myh3 (red), nuclei (DAPI, blue),
and myofiber boundaries (laminin, green). Scale bar, 50 μm. (B) Cross-sectional area of regenerating myofibers at 5 and 14 dpi. 100-300 myofibers were analyzed from n=6 mice per group. (C) Tibialis anterior (TA) muscle weight (normalized to body weight) from mice of the indicated genotype and treatment condition at 14 dpi. (D) Representative H&E and immunofluorescence images of muscle cross-sections at 14 dpi. Sections were stained with antibodies targeting fiber-type specific myosin heavy chains, including Myh7 (type I; purple), Myh2 (type IIa; red), Myh4 (type IIb; blue), Myh1 (type IIx; red), and Myh8 (neonatal; red), and myofiber boundaries (laminin, green). Scale bar, 50 μm. Statistical significance was assessed using one-way ANOVA (B,C), or Kruskal-Wallis (B) tests with adjustments for multiple comparisons. Box plots indicate median and interquartile ranges from the indicated number of biological replicates; whiskers are plotted using the Tukey method.
**Figure 8. Increased Hif1α signaling inhibits maturation in regenerating myofibers.**

(A) Representative H&E and immunofluorescence images of muscle cross-sections at 5 and 14 dpi. Sections were stained with antibodies targeting Myh3 (red), Hif1α (red), or Myh8 (red), as well as nuclei (DAPI, blue), and myofiber boundaries (WGA (green) or laminin (green)). Scale bar, 50 μm. (B) Cross-sectional area of regenerating fibers from muscles at 5 and 14 dpi. 300 myofibers were analyzed from n=6 mice per group. p-values reflect comparison with wild-type group. (C) TA muscle weight (normalized to body weight) from mice of the indicated genotype and treatment condition at 14 dpi. p-values reflect comparison with wild-type group. (D) Representative snapshots of H3K27me3 deposition at the Myh8 gene in 14 dpi myofibers of the indicated genotype. For each ChIP-seq dataset, 3 biological replicates were analyzed. Statistical significance was assessed using one-way ANOVA (B,C), or Kruskal-Wallis (B) tests with adjustments for multiple comparisons. Box plots indicate median and interquartile ranges from the indicated number of biological replicates; whiskers are plotted using the Tukey method.
Figure 9: Myh8+ fibers are characteristic of severe centronuclear myopathy. (A)
Representative images of histology (H&E) and immunofluorescence for MYH8, CA3 and
NFATC2 from a patient with infantile CNM due to a Dnm2 mutation (CNM1) and an
age/sex-matched control (Control 1), and a patient with infantile CNM due to a Mtm1
mutation (CNM2) and an age/sex-matched control (Control 2). Myofiber boundaries are
visualized with WGA staining, and nuclei are visualized with DAPI staining. Scale bar, 20 μm.
Figure 10. Regenerating myofibers pause at the neonatal-adult transition in response to ischemic injury. (A) Representative immunofluorescence images of muscle cross-sections from wild-type and hif1α−/− mice at the indicated time points (days post ligation). Muscle cross-sections were stained with antibodies targeting Hif1α (red). Nuclei were visualized with DAPI, and myofiber boundaries were visualized with Laminin staining (green) or WGA (green). Scale bar: 50 μm. dpl, days post ligation. (B) Same as (a), except cross-sections were stained with antibodies targeting Myh8 (neonatal myosin heavy chain; red). Myofiber boundaries were visualized with Laminin staining (green). Scale bar: 50 μm.
Figure 11. *Hif1α* deletion accelerates myofiber maturation during muscle regeneration in response to ischemic injury. (A) Representative immunofluorescence
images of muscle cross-sections from wild-type and hif1α−/− mice at the indicated time points (days post ligation). Muscle cross-sections were stained with antibodies targeting fiber-type specific myosin heavy chains, including Myh7 (type I; purple), Myh2 (type IIa; red), Myh4 (type IIb; blue), and Myh1 (type IIx; red). Myofiber boundaries were visualized with Laminin staining (green). Scale bar: 50 μm. dpl, days post ligation. (B) Quantitation of the percentage of regenerating myofibers positive for Myh8 staining in the indicate genotypes. (C) Quantitation of the percentage of regenerative myofibers of the indicated adult fiber type, in the indicated genotypes. (E) Quantitation of enrichment (% of input) from H3K27me3 ChIP-qPCR experiments targeting the Myh8 gene. Experiments were performed in regenerating myofibers from animals of the indicated genotype and timepoint. n=1 animal per genotype per timepoint. Statistical significance was assessed using two-way ANOVA (B,D).