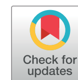

Muscle development and regeneration controlled by AUF1-mediated stage-specific degradation of fate-determining checkpoint mRNAs

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AUF1 promotes rapid decay of mRNAs containing 3′ untranslated region (3′UTR) AU-rich elements (AREs). AUF1 depletion in mice accelerates muscle loss and causes limb girdle muscular dystrophy. Here, we demonstrate that the selective, targeted degradation by AUF1 of key muscle stem cell fate-determining checkpoint mRNAs regulates each stage of muscle development and regeneration by reprogramming each myogenic stage. Skeletal muscle stem (satellite) cell explants show that *Auf1* transcription is activated with satellite cell activation by stem cell regulatory factor CTCF. AUF1 then targets checkpoint ARE-mRNAs for degradation, progressively reprogramming the transcriptome through each stage of myogenesis. Transition steps in myogenesis, from stem cell proliferation to differentiation to muscle fiber development, are each controlled by fate-determining checkpoint mRNAs, which, surprisingly, were found to be controlled in their expression by AUF1-targeted mRNA decay. Checkpoint mRNAs targeted by AUF1 include *Twist1*, decay of which promotes myoblast development; *CyclinD1*, decay of which blocks myoblast proliferation and initiates differentiation; and *RGS5*, decay of which activates Sonic Hedgehog (SHH) pathway-mediated differentiation of mature myotubes. AUF1 therefore orchestrates muscle stem cell proliferation, self-renewal, myoblast differentiation, and ultimately formation of muscle fibers through targeted, staged mRNA decay.

AUF1 | satellite cells | mRNA decay | AU-rich elements | muscle regeneration

Roughly 5% of mRNAs have 3′ untranslated regions (3′UTRs) containing AU-rich elements (AREs) that target these mRNAs for regulated rapid decay, an essential mechanism that controls gene expression. AREs are typically defined as adjacent repeated AUUUA sequences (1), recognized by any of several regulatory proteins known as AU-rich binding proteins (AUBPs). AUBPs have multiple roles in mRNA posttranscriptional control including splicing, transport, stability, and translation (2). AUF1 (HNRNPD) binds certain ARE-mRNAs and promotes their rapid degradation.

AUF1 consists of four related protein isoforms identified by their molecular weights (p37, p40, p42, p45), derived by differential exon splicing of a common pre-mRNA (3). We previously developed an *Auf1*$^{-/-}$ germ-line homozygous knockout (KO) mouse to better understand its physiological roles (4). In muscle, AUF1 is only expressed in activated skeletal muscle stem (satellite) cells and their proliferating progenitors known as myoblasts (5, 6). Mice deleted in the AUF1 gene undergo accelerated skeletal muscle wasting with age known as sarcopenia (6, 7), are severely impaired in skeletal muscle regeneration following injury (6), and display all of the hallmarks of a distinct form of skeletal myopathy that affects the limbs and upper chest known as limb girdle muscular dystrophy (LGMD). In humans, LGMD type 1G disease is associated with mutations in the *Auf1* gene (6, 8).

We lack a mechanistic molecular understanding of AUF1 regulation of myogenesis. Loss or inactivation of satellite cells and myoblasts with age, traumatic muscle injury, or myopathic (muscle weakness) diseases impairs muscle regeneration. Satellite cells become activated upon muscle fiber (myofiber) injury, which activates a staged program of differentiation that includes development of proliferating myoblasts, inhibition of myoblast proliferation, differentiation to myocytes, fusion into myotubes, and ultimately development of terminally differentiated myofibers (*SI Appendix*, Fig. S1). The process of myogenesis is orchestrated by genes that regulate myogenic regulatory factors (MRFs), many of which are checkpoint transcription factors that must be inhibited to reprogram progressive stages of myogenesis (9). Both satellite cells and myoblasts require expression of AUF1 for myogenesis (6, 10). We therefore sought to understand the molecular role of AUF1 in the programming of myogenesis. We show that AUF1 controls each temporally staged phase of myogenesis, including the differentiation of satellite cells to myoblasts, myoblast proliferation, their exit from the cell cycle, differentiation into myocytes, and ultimately development of terminally mature, differentiated myotubes, by reprogramming each phase through the selective, targeted decay of stage-specific MRF mRNAs.

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Results

AUF1 Deletion Results in Hyperproliferative and Undifferentiated Satellite Cells. The process of myogenesis can be tracked by the rapid expression and subsequent clearance of certain MRFs (11) (*SI Appendix*, Fig. S1). Myf5 and MyoD expression can be used

Significance

Muscle-wasting diseases are due in part to improper regulation of mRNA stability. Mutations in the mRNA decay protein AUF1 are linked to a specific form of muscle-wasting disease known as limb girdle muscular dystrophy that affects the muscles of the limbs and torso. How AUF1 is involved in regulating or promoting muscle regeneration is largely unknown. Here, we demonstrate that AUF1 controls all of the major stages of muscle development and regeneration, from control of the muscle stem (satellite) cell differentiation program through the development of mature muscle fibers, by selectively targeting for rapid degradation the major differentiation checkpoint mRNAs that block entry into each next phase of muscle development, thereby promoting each stage of muscle development.

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to identify activated proliferating myoblasts. Myf5 expression ceases when myoblasts differentiate to elongated multinucleate myocytes, concurrent with MyoD and Myogenin expression. MyoD, Myogenin, and Myosin Heavy Chain (MHC) are expressed in nascent myotubes, which are partially differentiated and multinucleate. Nascent myotubes terminally differentiate into mature myotubes (and myofibers if in animals), which involves the gradual loss of MyoD and Myogenin but not MHC expression.

Whole hindlimb skeletal muscles of WT and AUF1 KO mice were surgically excised from 4-mo-old mice and myofiber-satellite cell complexes placed in culture. Satellite cells remain viable and attached to myofibers but become activated. While some activated satellite cells divide asymmetrically to restore themselves, most differentiate into proliferating myoblasts, cease replication, and further differentiate to form de novo myotubes or repair existing myotubes through fusion (12). WT myofiber–satellite cell complexes underwent typical differentiation to multinucleated nascent myotubes after 10 d, demonstrating elongated multinucleate structures with inhibition of MyoD and expression of Myogenin (Fig. 1 *A* and *B*) (13). In contrast, AUF1 KO preparations showed continued satellite cell proliferation, poor ability to differentiate, and then only to early proliferating myoblasts and some myocytes, with coexpression of both MyoD and Myogenin (Fig. 1 *A* and *B*).

The *Auf1*$^{-/-}$ satellite cell/myoblast hyperproliferation phenotype was found to be a cell-autonomous defect due to loss of AUF1 expression. Hyperproliferative AUF1 KO satellite cell–myofiber complexes were transduced with lentivirus vectors expressing Flag-tagged cDNAs of each individual AUF1 isoform (p37, p40, p42, p45) or the empty vector. All AUF1 protein isoforms except p37 were well expressed in both satellite cells and myofibers (Fig. 1*C*). The p40, p42, or p45 AUF1 isoforms, which were well expressed, down-regulated satellite cell proliferation to that of WT, quantified by the number of nuclei per mm^2 (Fig. 1*D*). AUF1 expression itself therefore halts satellite cell and myoblast proliferation and induces the differentiation program of myogenesis.

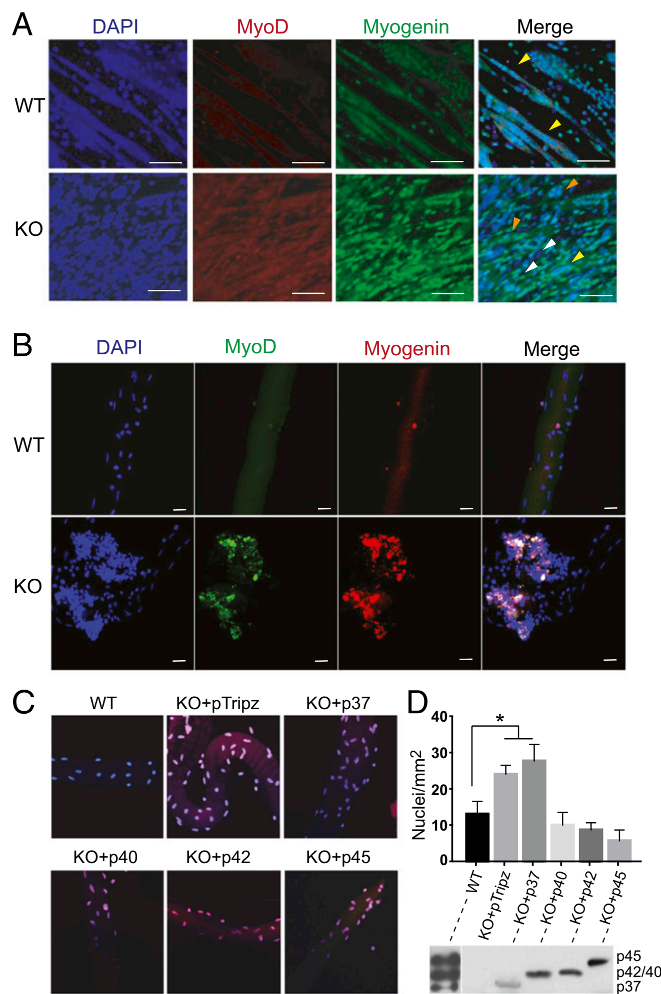

Fig. 1. *Auf1*$^{-/-}$ satellite cells show aberrant terminal differentiation. (*A*) Representative cultured mass preparation of hindlimb skeletal muscles harvested from 4-mo-old mice, 10 d in culture. $n = 3$. Proliferating myoblasts (MyoD), white arrows; elongated myocytes (Myogenin), orange arrows; myofibers, yellow arrows. Nuclei stained with DAPI. (*B*) Representative IF staining of MyoD (green) and Myogenin (red) in WT and AUF1 KO-isolated myofibers, cultured for 72 h. Ten myofibers analyzed per mouse, $n = 3$. (*C*) Representative IF staining of Flag-AUF1 (red) and nuclei (DAPI, blue) in WT and AUF1 KO myofibers. Myofibers from mass preparations of the TA muscle as in *A* transduced with lentivirus vectors expressing AUF1 cDNAs for 72 h. Ten myofibers per group analyzed, $n = 3$. (*D*) Quantification of nuclei number and AUF1 in myofibers transduced with individual AUF1 isoforms, as in *C*, $n = 3$. (Scale bars: 100 μm.)

AUF1 Expression Is Activated by CTCF Stem Cell Factor and Promotes Myoblast Differentiation. AUF1 is only expressed with activation and differentiation of satellite cells to myoblasts (6). We therefore characterized the expression of the major AUBPs (HuR, TTP, AUF1) known to be involved in myogenesis (10, 14–16). Progression from replicating myoblasts to differentiated myotubes can be replicated in C2C12 myoblasts, which proliferate under high serum but are induced to differentiate under low serum and form multinucleated myotubes. Of the AUBPs, only *Auf1* mRNA and protein were strongly increased during C2C12 cell differentiation (Fig. 2*A* and *SI Appendix*, Fig. S2*A*). HuR reportedly opposes AUF1-mediated mRNA decay in myogenesis, stabilizing *MyoD*, *p21*, and *Myogenin* mRNAs during terminal myotube differentiation (15). We found that AUF1 expression is reduced at this time, consistent with these data. We therefore investigated the mechanism of increased AUF1 expression with satellite cell/myoblast differentiation.

An siRNA-based loss-of-function screen was carried out to identify transcription factors that activate AUF1 expression. A list of 200 transcription factor candidates was generated based on potential consensus target sequences in AUF1/HNRNPD transcriptional regulatory regions, which were filtered against those expressed in activated satellite cells using our RNA-sequencing (RNA-seq) data (6). Candidate mRNAs were identified and silenced in differentiating C2C12 myoblasts to test their effect on AUF1 expression (*SI Appendix*, Fig. S2*B*). AUF1 expression was strongly reduced with silencing of BCL6, CTCF, and FOXJ2, all of which regulate myoblast differentiation (17–19) (*SI Appendix*, Fig. S2*C*). We focused on CTCF because analysis of the *Auf1* promoter region indicated a clear consensus binding site (encodeproject.org) compared with the other factors (*SI Appendix*, Fig. S2*D*) and because CTCF acts as a master regulator of stem cell gene expression (20).

CTCF silencing in C2C12 myoblasts strongly reduced AUF1 and MyoD expression during differentiation (Fig. 2*C*), whereas ectopic overexpression of CTCF in HEK 293 cells promoted AUF1 expression (Fig. 2*D*). Silencing AUF1 in C2C12 myoblasts did not impact CTCF expression (*SI Appendix*, Fig. S2*E*), indicating that AUF1 is a downstream target of CTCF but CTCF expression does not require AUF1. CTCF bound the *Auf1* proximal promoter region, shown by immunoprecipitation-qPCR analysis compared with a positive control (Igf2/hH19 promoter), which contains three CTCF-binding sites (21) and a negative control intronic region (*SI Appendix*, Fig. S2*F*). Moreover, the 200-bp genomic DNA fragment of the *Auf1* promoter was sufficient for CTCF transcriptional responsiveness using a luciferase

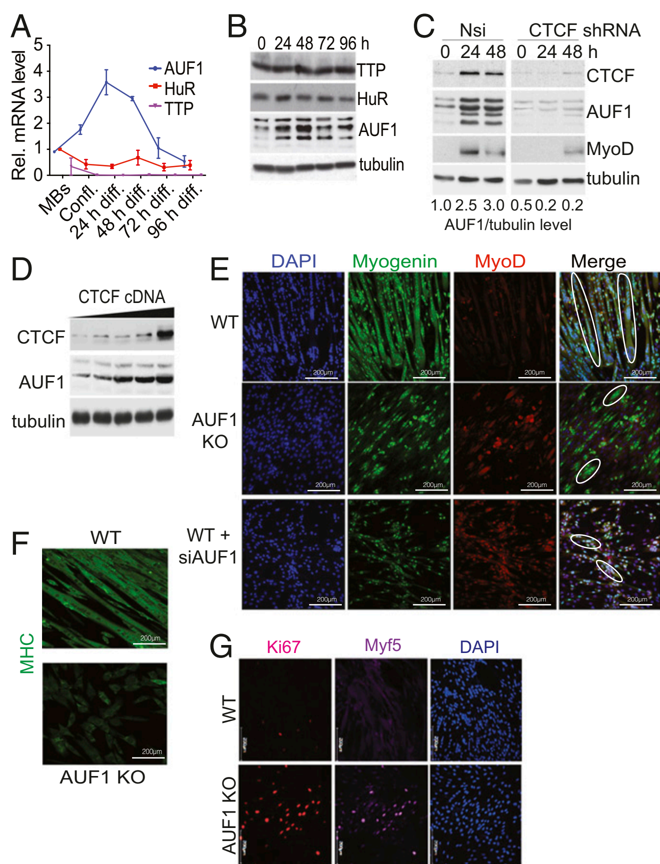

Fig. 2. AUF1 expression is essential for myoblast differentiation and myotube formation. (*A*) Expression of AUBP mRNAs during differentiation at 96 h determined by qRT-PCR ($n = 3$), normalized to invariant *GAPDH* mRNA. (*B*) Expression of AUBP proteins during differentiation corresponding to *A*, $n = 4$. Numbers below immunoblot: Twist1 fold increase normalized to WT cells. (*C*) Immunoblot of CTCF, AUF1, MyoD, and β-tubulin control during C2C12 myoblast differentiation. CTCF was silenced in C2C12 cells by lentiviral-mediated shRNA. Numbers under immunoblot correspond to AUF1 fold change relative to β-tubulin levels, normalized to 0-h Nsi time point. $n = 4$. (*D*) Immunoblot of CTCF and AUF1 with increasing transfection of a CTCF cDNA expression plasmid in HEK 293 cells. Overexpression of CTCF was performed using pMy-CTCF (Addgene), $n = 3$. CTCF Chromatin-IP (ChIP) analysis using *Auf1* promoter in C2C12 cells at 48 h after induction of differentiation. DNA enrichment in fragmented ChIP assay with anti-CTCF antibody relative to anti-rabbit IgG IP control, normalized to intron signal measured by qRT-PCR. $n = 3$. $^{*}P < 0.05$ by *t* test. *H19* promoter with three CTCF binding sites is a positive control. (*E*) Representative IF staining of Myogenin (green) and MyoD (red) in WT and AUF1 KO C2C12 cells, or WT C2C12 cells transfected with siAUF1. Images taken at 96 h after induction of differentiation. Note: light green stain in WT sample is nonspecific. White circles indicate examples of myotube formation. (*F*) Representative IF staining of MHC at 96 h after induction of differentiation in WT and AUF1 KO myoblasts, $^{*}P < 0.05$ by *t* test, $n = 5$. (*G*) Representative IF staining of Myf5 (purple) and Ki67 (red) in cultured AUF1 WT and KO C2C12 myoblasts postdifferentiation. Nuclei stained by DAPI. (Scale bars: 200 μm.)

reporter (*SI Appendix*, Fig. S2*G*). CTCF is therefore important for *Auf1* transcriptional activation.

CTCF was shown to regulate myoblast differentiation through recruitment of MyoD to activate muscle-specific genes (18, 22). We therefore asked whether CTCF activation controls myoblast differentiation through activation of AUF1 expression. CTCF-silenced cells were stained for Myogenin 96 h after induction of differentiation. Immunofluorescence (IF) analysis indicated much less differentiation of myoblasts to myotubes in CTCF-silenced cells compared with control cells (*SI Appendix*, Fig. S3*A*). We therefore tested whether restoring AUF1 expression rescues myogenesis despite CTCF silencing. FLAG-tagged AUF1 p42 and p45 cDNAs were introduced into CTCF-silenced C2C12 cells by retroviral infection. With ectopic AUF1 expression, staining for Myogenin and MHC, a marker of terminally differentiated myotubes, indicated strong recovery of differentiated myotubes even in the absence of CTCF expression (*SI Appendix*, Fig. S3*A*). Taken together, our data indicate that AUF1 is a critical CTCF target gene, which lies downstream of CTCF in myogenesis.

CRISPR Cas 9 deletion of the *Auf1* gene resulted in 90% fewer myotubes and the inability of myoblasts to differentiate compared with WT C2C12 cells, similar to *Auf1* silencing (Fig. 2*E* and *SI Appendix*, Fig. S3 *B* and *C*). Thus, attenuated myogenesis with loss of AUF1 expression is not an off-target effect of shRNA silencing. AUF1 KO myoblasts expressed higher levels of MyoD (Fig. 2*E*) only very weakly expressed MHC, a marker of terminally differentiated myotubes (Fig. 2*F* and *SI Appendix*, Fig. S3 *D* and *E*) and Myf5, and were highly proliferative, shown by up-regulation of Ki67 (Fig. 2*G*). The block to differentiation in AUF1 KO C2C12 cells could be rescued by ectopic expression of cDNAs for p40, p42, or p45 AUF1, which promoted normal differentiation to myotubes and reexpression of MHC (*SI Appendix*, Fig. S3 *E* and *F*). Loss of AUF1 expression is therefore responsible for the hyperproliferative phenotype of satellite cells and myoblasts, and their inability to differentiate and program myogenesis.

AUF1 Initiates Myoblast Differentiation by Destabilizing the Twist1 ARE-mRNA. RNA-seq data from WT and AUF1 KO satellite cells and from AUF1-silenced C2C12 mouse myoblasts (6) were used to identify ARE-mRNAs that are targets of AUF1 and could be involved in the hyperproliferation phenotype by virtue of overexpression due to increased mRNA stabilization in the absence of AUF1 expression. We identified several key mRNAs that fit this profile: *Twist1*, *Bmp4*, and *Sox8*. TWIST1 is a transcription factor known to inhibit myogenesis by blocking the differentiation-inducing function of key myogenic factors MyoD and MEF2, and to maintain myoblast proliferation by activating expression of the *CyclinD1* gene (23). BMP4 (bone morphogenic protein 4) prevents myogenic differentiation of satellite cells by inhibition of certain myogenic genes (24). SOX8 is a transcription factor that also inhibits myogenesis and blocks expression of MyoD and Myogenin (24). Of these genes, we found neither *Bmp4* nor *Sox8* to be highly increased in mRNA levels in siAUF1-treated C2C12 cells (*SI Appendix*, Fig. S4 *A* and *B*), indicating that they are not direct targets of AUF1. However, TWIST1 lies upstream of these factors in the myogenic differentiation pathway, and its mRNA was increased ~15-fold in AUF1 KO C2C12 cells compared with WT, as was TWIST1 protein (Fig. 3 *A* and *B*). *Twist1* mRNA and protein levels were also strongly increased in the tibialis anterior (TA) muscle of AUF1 KO mice compared with WT animals (*SI Appendix*, Fig. S4 *C* and *D*).

The mouse *Twist1* mRNA has two 3′UTR ARE motifs (10) that could serve as potential AUF1-binding sites. We therefore immunoprecipitated AUF1 from nondifferentiating WT C2C12 cells and at 48 h after differentiation, followed by qRT-PCR to quantify *Twist1* mRNA. AUF1 showed a 10-fold increase in association with the *Twist1* mRNA in differentiating C2C12 cells (Fig. 3*C*), consistent with increased expression levels at this time (Fig. 2*B*), and possibly phosphorylation-mediated binding activation (25). *Twist1* mRNA half-life was determined in WT and AUF1 KO C2C12 cells treated with actinomycin D to block new transcription (Fig. 3*D*). In the absence of AUF1, *Twist1* mRNA stability was increased ~fivefold, consistent with increased mRNA and protein levels.

Silencing *Twist1* and *CyclinD1* mRNAs Only Partially Restores Myogenic Differentiation in AUF1 KO Myoblasts. TWIST1 inhibits myogenesis by blocking the functions of myogenic regulatory factors such as MyoD and MEF2 (26). AUF1 KO myoblasts

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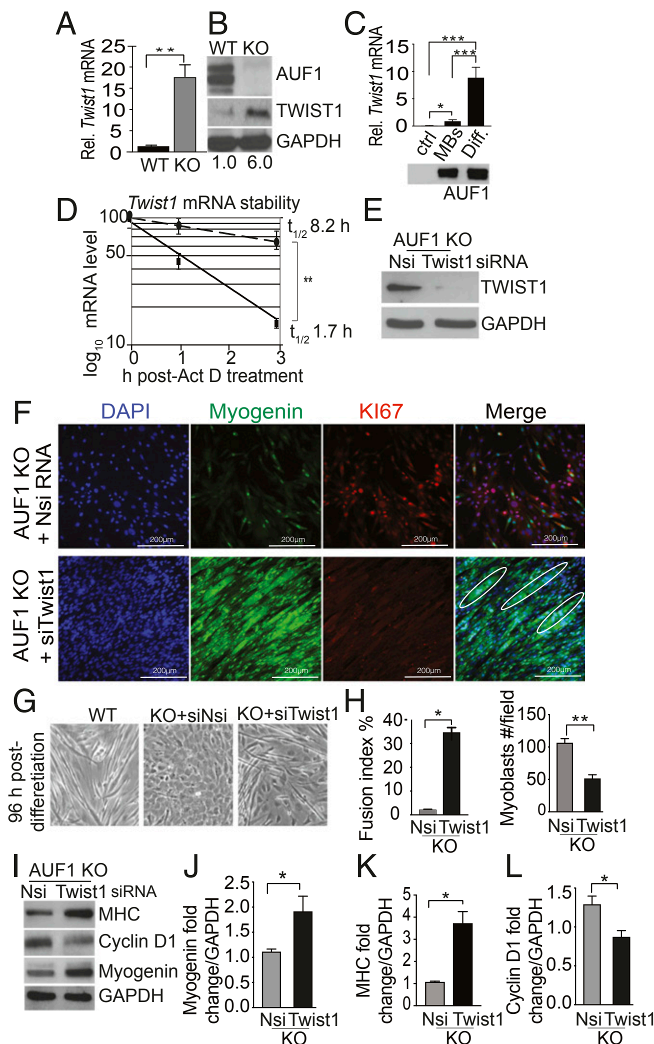

Fig. 3. AUF1 targeted decay of *Twist1* mRNA partially restores myogenesis. Relative expression of TWIST1 mRNA (*A*) and protein levels in WT C2C12 myoblasts and AUF1 KO C2C12 myoblasts (*B*), $n = 3$. Numbers under blot refer to fold increase in AUF1 normalized to GAPDH protein. $^{**}P < 0.01$ by unpaired Mann–Whitney *U* test. (*C*) *Twist1* mRNA associated with endogenous AUF1 protein in myoblasts 48 h after induction of differentiation in WT C2C12 cells. Representative AUF1 IP per immunoblot. $n = 5$. $^{*}P < 0.05$, $^{***}P < 0.001$ by unpaired Mann–Whitney *U* test. (*D*) *Twist1* mRNA decay rate in WT C2C12 cells (solid line) or AUF1 KO C2C12 cells (dotted line) in nondifferentiation medium. Transcription inhibited with actinomycin D (ActD), samples collected as shown. $^{**}P < 0.01$ by unpaired Mann–Whitney *U* test. $n = 3$ with SEM shown. (*E*) Representative immunoblot of TWIST1 levels in AUF1 KO C2C12 myoblasts transfected with nonsilencing control (Nsi) or *Twist1* siRNAs. GAPDH, loading control. $n = 3$. (*F*) Representative IF staining of Myogenin (green) and Ki67 (red) in cultured AUF1 KO C2C12 myoblasts transfected with nonsilencing control (siNsi) or *Twist1* siRNAs at 96 h after induction of differentiation. Nuclei were stained by DAPI (blue). (Scale bar: 200 μm.) White ovals indicate examples of myotube formation. (*G*) Representative phase contrast microscopic images of WT and AUF1 KO C2C12 cells transfected with nonsilencing (Nsi) siRNA or si*Twist1* RNA. (*H*) Fusion index and myoblast number calculated from five independent experiments. Fusion index calculated as the percentage nuclei within multinucleated myotubes compared with total number of nuclei from AUF1 KO myoblasts transfected with Nsi or *Twist1* siRNAs. Myoblast number calculated as number of isolated myoblasts per field, scoring at least five fields in each experiment, mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$ by unpaired Mann–Whitney *U* test. (*I*) Representative immunoblot of Cyclin D1, MHC, and Myogenin in AUF1 KO C2C12 cells transfected with Nsi or *Twist1* siRNAs. GAPDH, loading control. (*J*–*L*) Myogenin, MHC, and Cyclin D1 quantification in AUF1 KO C2C12 cells transfected with Nsi or Twist1 siRNAs, $n = 3$, ±SEM, $^{*}P < 0.05$ by unpaired Mann–Whitney *U* test.

when transferred to differentiation media continued to proliferate and failed to form mature myofibers, with significant overexpression of TWIST1 protein (*SI Appendix*, Fig. S4*E*). However, with silencing of TWIST1 in AUF1 KO myoblasts, mature myotubes start to form (Fig. 3 *E*–*G*), and increased sevenfold, measured as myoblast fusion index, but with a large number of uncommitted myoblasts remaining (Fig. 3*H*). TWIST1-silenced AUF1 KO myoblasts also expressed increased levels of Myogenin and MHC, and reduced levels of cyclin D1 protein compared with control AUF1 KO myoblasts (Fig. 3 *I*–*L*). In summary, silencing TWIST1 in AUF1 KO myoblasts partially rescues the differentiation phenotype, indicating that while AUF1 promotes myoblast differentiation by destabilizing *Twist1* mRNA, there must be other downstream ARE-mRNA targets.

Cyclin D1 is a downstream target of TWIST1 that promotes cell proliferation (27). Cyclin D1 programs a brief burst of satellite cell and myoblast proliferation, required for self-renewal, but must be down-regulated to allow exit from the cell cycle, which enables myoblast differentiation (28). We determined whether AUF1 is required to destabilize *CyclinD1* mRNA and protein levels to block myoblast proliferation after induction of differentiation initiated by AUF1 decay of *Twist1* mRNA. Inhibition of Cyclin D1 expression enables myoblasts to exit the cell cycle and initiate differentiation. Cyclin D1 protein level was 10- to 15-fold higher in AUF1 KO myoblasts compared with WT myoblasts (*SI Appendix*, Fig. S4 *F* and *G*). Moreover, *CyclinD1* mRNA stability was increased ∼3.5-fold in AUF1 KO C2C12 cells compared with WT (*SI Appendix*, Fig. S4*H*). However, while simultaneously silencing *Twist1* and *CyclinD1* mRNAs increased myogenic differentiation, it was still only partially restored (*SI Appendix*, Fig. S4 *I* and *J*), shown by a small increase in myotube differentiation and maturation comparing silencing of *Twist1* alone to *Twist1* with *CyclinD1*. These data indicate that AUF1 is required to destabilize both *Twist1* and *CyclinD1* mRNAs to enable myoblast differentiation, but silencing both is still not sufficient to enable complete myogenic maturation.

AUF1 Activates the SHH Pathway To Promote Terminal Myogenesis Through Degradation of RGS5 mRNA. In addition to Twist1 and Cyclin D1, analysis of our RNA-seq data for AUF1 KO myoblasts and satellite cells showed that the Sonic Hedgehog (SHH) pathway inhibitor RGS5 mRNA is strongly elevated in AUF1 KO cells and might represent a later-stage myogenesis checkpoint target of AUF1. The SHH pathway plays a complex but essential role in myogenesis. RGS5 is a GTPase that inhibits SHH signaling (29, 30). SHH expression in satellite cells and myoblasts maintains a self-renewing but controlled proliferation phenotype and reprograms the cell to differentiate (29). Physiologically, inhibition of SHH results in incomplete skeletal muscle repair and muscle weakness (29). SHH has been shown to increase in satellite cells and myoblasts with differentiation, bind its receptor patched (PTCH1), causing the activation of smoothened (SMO), a G protein-coupled receptor (GPCR) GTPase, and the GLI transcription factors, which promote myogenesis (29, 30). While the SHH pathway promotes satellite cell and myoblast proliferation, it is critical for late steps including myotube maturation during myogenesis (28–30). We therefore investigated whether the SHH pathway is the next downstream checkpoint targeted by AUF1 in myogenesis progression.

We found that *RGS5* protein and mRNA are strongly increased in AUF1 KO C2C12 cells compared with WT (Fig. 4*A* and *SI Appendix*, S5A). The *RGS5* 3′UTR has five canonical adjacent AREs (6). mRNA half-life analysis carried out in WT and AUF1 KO C2C12 myoblasts 48 h after transfer to differentiation medium showed a 12-fold increased stability in the absence of AUF1 (Fig. 4*B*). As with Twist1, AUF1 shows strongly increased association with *RGS5* mRNA in differentiating C2C12 cells (*SI Appendix*, Fig. S5*B*). In addition, immunohistochemical (IHC) staining of the TA muscle from WT and AUF1 KO mice showed a strong increase in RGS5 protein levels

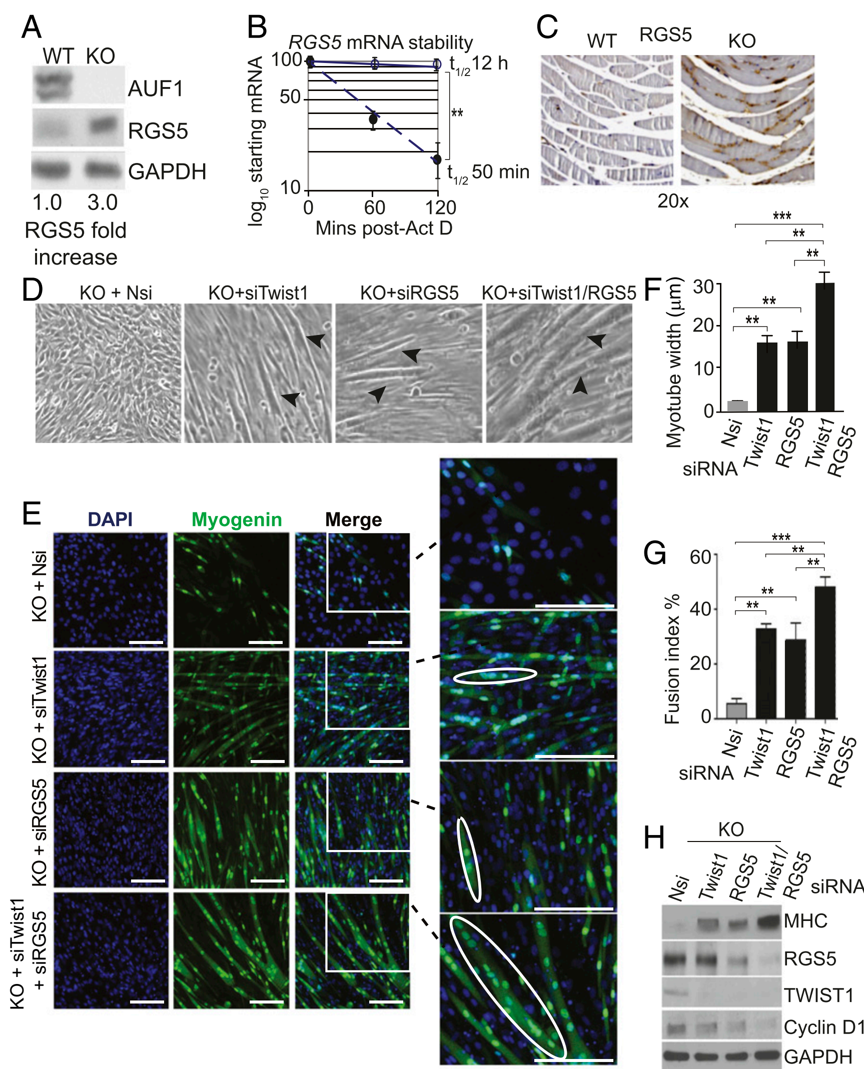

Fig. 4. Targeted decay of *RGS5* and *Twist1* mRNAs restores myotube differentiation and maturation in the absence of AUF1. (*A*) Immunoblot of AUF1 and RGS5 and RGS5 quantification relative to GAPDH in WT and (*Left*) AUF1 KO (*Right*) C2C12 myoblasts 96 h after differentiation, $n = 3$. (*B*) *RGS5* mRNA decay rate. WT C2C12 cells, dotted line; AUF1 KO C2C12 cells, solid line. $n = 3$, ±SEM, $^{**}P < 0.01$ by unpaired Mann–Whitney *U* test. (*C*) IHC staining of RGS5 in WT and AUF1 KO mouse TA muscle. (Magnification: 20×.) (*D*) Phase contrast microscopic images of AUF1 KO C2C12 cells transfected with Nsi, si*Twist1*, si*RGS5*, or both si*Twist1* and si*RGS5* at 96 h after induction of differentiation. Arrows, differentiated myotubes. Representative of $n = 5$. (*E*) IF stain of Myogenin in AUF1 KO C2C12 cells transfected with Nsi, si*Twist1*, si*RGS5*, or both si*Twist1* and si*RGS5*. Nuclei, DAPI (blue) at 96 h after induction of differentiation. (Scale bar: 200 µm.) (*F*) Myotube width in AUF1 KO C2C12 cells transfected with Nsi, si*Twist1*, si*RGS5*, or both si*Twist1* and si*RGS5* at 96 h after induction of differentiation. $n = 5$, ±SEM by Kruskal–Wallis test. (*G*) Fusion index in AUF1 KO C2C12 cells transfected with Nsi, si*Twist1*, si*RGS5*, or both si*Twist1* and si*RGS5* 96 h after induction of differentiation. $n = 5$. (*H*) Immunoblot of MHC, RGS5, TWIST1, and Cyclin D1 in AUF1 KO C2C12 cells transfected with Nsi, siTWIST1, siRGS5, or both siTWIST1 and siRGS5 at 96 h after induction of differentiation. $n = 3$.

only in KO mice (Fig. 4*C*). To determine whether the increased stability of *RGS5* mRNA resulting from loss of AUF1 prevents myogenic terminal differentiation, AUF1 KO C2C12 myoblasts were silenced for *RGS5* alone, or *RGS5* and *Twist1*, and transferred to differentiation medium. Silencing *Twist1* or *RGS5* alone only partially rescued myotube differentiation but, when combined, resulted in development of large, mature wide myotubes (Fig. 4 *D*–*F*) with a high fusion index (Fig. 4*G*), hallmarks of terminally differentiated myotubes. The level of MHC protein was also higher in AUF1 KO myoblasts when silenced in *Twist1* and *RGS5* compared with silencing either alone (Fig. 4*H* and *SI Appendix*, Fig. S5*C*). Dual TWIST1/RGS5 silencing also down-regulated Cyclin D1 expression commensurate with myotube differentiation (Fig. 4*H*). Cosilencing *Twist1* and *RGS5* mRNAs strongly increased the number of mature myotubes compared with silencing either alone, quantified by scoring the number of centro-nuclei per fiber (*SI Appendix*, Fig. S5*D*). Thus, silencing *Twist1* and *RGS5*, which also down-regulates *CyclinD1* expression, allows myoblasts to exit the cell cycle, differentiate, and fuse to form mature myotubes despite the absence of AUF1. Importantly, we also found that the loss of AUF1 modulates TWIST1 and RGS5 during postinjury TA muscle regeneration in mice. Fifteen days after injection of $BaCl_2$ to induce muscle damage, TWIST1 and RGS5 were increased in AUF1 KO mice compared with WT, which was associated with impaired myofiber regeneration (*SI Appendix*, Fig. S5 *E*–*G*).

Discussion

We demonstrated that the myogenic differentiation program is regulated by AUF1 in satellite cells and myoblasts through selective mRNA decay, successively reprogramming myogenesis, first by exiting the cell cycle, then initiating differentiation, and orchestrating the terminal differentiation program. A number of studies have demonstrated that the complex and temporally ordered process of myogenesis involves a tightly regulated network of targeted, rapid mRNA decay that serves to reprogram the different phases of muscle regeneration (6, 10, 14, 16, 31, 32). A role for TTP in constitutive maintenance of the myogenic stem cell state was previously described (31), although TTP knockout mice do not develop myopathic disease (2, 6). From our work and others, AUF1 has emerged as having crucial roles in regulating, reprogramming, and staging the complex and temporally organized gene expression network of muscle development (33, 34).

Of the AUBPs involved in myogenesis, HuR has been the best studied, and may oppose some of the actions of AUF1, and possibly cooperate in others. HuR typically binds to AREs in the 3′UTR and prevents rapid decay mediated by AUF1 and TTP, thereby increasing translation (35). HuR has been shown to stabilize mRNAs encoding MyoD and Myogenin (not direct

targets of AUF1), and the cell cycle inhibitor p21 (a target of AUF1) during the late stage of myogenesis, facilitating development of terminally differentiated myotubes (36). It was shown that HuR and the mRNA decay factor KSRP interact to specifically promote late-stage myogenesis by down-regulating the levels of *Nucleophosmin* mRNA by binding to its AU-rich 3′UTR sequences (15). Nucleophosmin blocks entry into myogenesis. How the combined activities of HuR, AUF1, TTP, and possibly KSRP function collectively in myogenesis is clearly complicated and needs to be much better described, but as a first step requires a greater understanding of each AUBP's independent activity.

Although increased expression of AUF1 is observed in many tissues, the mechanisms involved in regulating AUF1 transcription have been largely unknown. That CTCF regulates AUF1 transcription during myoblast differentiation is important in several respects. It is consistent with previous findings showing that CTCF is a primary activator of myogenesis by regulating muscle-specific gene expression (18). It is also consistent with the reported interaction between CTCF and MyoD, which forms a CTCF/MyoD complex that activates muscle-specific gene expression. Our data indicate that in addition, CTCF functions through the activation of AUF1 expression. As AUF1 silencing does not affect MyoD expression, it is likely that a CTCF/MyoD complex might control the expression of AUF1. Our studies therefore show that AUF1 is a critical player in myogenesis, from the maintenance of adult muscle stem cells to muscle regeneration.

Materials and Methods

Immunofluorescence. All studies were approved by the New York University Institutional Animal Care and Use Committee under protocol #160706. Male and female mice had their TA muscles removed, frozen in OCT (Tissue-Tek), fixed in 4% paraformaldehyde, and blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline. C2C12 cells were fixed in 4% paraformaldehyde and blocked in 3% BSA in phosphate-buffered saline. Samples were immunostained overnight with antibodies: rabbit anti-MyoD (Santa Cruz Biotechnology, SC-760), mouse anti-Myogenin (Santa Cruz Biotechnology, SC-12732), rabbit anti–KI-67 (Abcam, ab15550), mouse anti-Myosin (R&D Systems, MAB4470), mouse anti-Twist1 (Novus, NBP2-37364) and mouse-RGS5 (Santa Cruz Biotechnology, sc-390245). Alexa Fluor donkey 488, 555, and 647 secondary antibodies were used at 1:300 and incubated for 1 h at room temperature. Slides were sealed with Vectashield with DAPI (Vector).

Myofiber Preparation. Myofibers were harvested from WT and KO mice at 4 mo of age. In brief, the extensor digitorum longus was dissected from the hindlimb and digested in 1.5 U/mL Collagenase Type I (Worthington) for 1.5 h at 37 °C. Individual fibers were released through repetitive flushing by a pasteur pipette. Myofibers were cultured for 72 h in F12 media (Corning) supplemented with 15% horse serum (Gibco), 1% penicillin streptomycin (Life Technologies), and 2.5 μg/μL FGF (Sigma) at 37 °C in a 5% CO_2 tissue culture incubator (6).

Mass Preparations. Whole hindlimb skeletal muscles were harvested from 4-mo-old WT and AUF1 KO mice, digested in 1.5 U/mL Collagenase Type I (Worthington) for 1 h in a 37° C water bath. Mass preparations were cultured for 10 d in F12 media (Corning) supplemented with 15% horse serum (Gibco), 1% penicillin streptomycin (Life Technologies), and 2.5 μg/μL FGF (Sigma) at 37° C in a 5% CO_2 tissue culture incubator. Myofiber-satellite cell complexes were transduced in optiMEM with lentivirus encoding one AUF1 isoform (p37, p40, p42, or p45) for 72 h. Three to five mice per genotype were studied as indicated.

$BaCl_2$ Hindlimb Injury. Male and female mice 3 mo of age were injected with 50 μL of filtered 1.2% $BaCl_2$ in saline solution into the left TA muscle. The right TA muscle remained uninjured as a control. Mice were killed at 15 d after injection, and TA muscles were frozen in OCT (Tissue-Tek). Three mice per genotype were studied.

Quantification and Statistical Analysis. Nonparametric Kruskal–Wallis tests were used to analyze groups. Individual tests are noted in figure legends. Statistical analyses used GraphPad Prism.

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1. C. Y. Chen, A. B. Shyu, AU-rich elements: Characterization and importance in mRNA degradation. *Trends Biochem. Sci.* **20**, 465–470 (1995).
2. A. E. Moore, D. M. Chenette, L. C. Larkin, R. J. Schneider, Physiological networks and disease functions of RNA-binding protein AUF1. *Wiley Interdiscip. Rev. RNA* **5**, 549–564 (2014).
3. B. J. Wagner, C. T. DeMaria, Y. Sun, G. M. Wilson, G. Brewer, Structure and genomic organization of the human AUF1 gene: Alternative pre-mRNA splicing generates four protein isoforms. *Genomics* **48**, 195–202 (1998).
4. J. Y. Lu, N. Sadri, R. J. Schneider, Endotoxic shock in AUF1 knockout mice mediated by failure to degrade proinflammatory cytokine mRNAs. *Genes Dev.* **20**, 3174–3184 (2006).
5. T. Beiter *et al.*, Exercise, skeletal muscle and inflammation: ARE-binding proteins as key regulators in inflammatory and adaptive networks. *Exerc. Immunol. Rev.* **21**, 42–57 (2015).
6. D. M. Chenette *et al.*, Targeted mRNA decay by RNA binding protein AUF1 regulates adult muscle stem cell fate, promoting skeletal muscle integrity. *Cell Rep.* **16**, 1379–1390 (2016).
7. A. R. Pont, N. Sadri, S. J. Hsiao, S. Smith, R. J. Schneider, mRNA decay factor AUF1 maintains normal aging, telomere maintenance, and suppression of senescence by activation of telomerase transcription. *Mol. Cell* **47**, 5–15 (2012).
8. N. M. Vieira *et al.*, A defect in the RNA-processing protein HNRPDL causes limb-girdle muscular dystrophy 1G (LGMD1G). *Hum. Mol. Genet.* **23**, 4103–4110 (2014).
9. G. F. Mok, D. Sweetman, Many routes to the same destination: Lessons from skeletal muscle development. *Reproduction* **141**, 301–312 (2011).
10. A. C. Panda *et al.*, RNA-binding protein AUF1 promotes myogenesis by regulating MEF2C expression levels. *Mol. Cell Biol.* **34**, 3106–3119 (2014).
11. P. S. Zammit, T. A. Partridge, Z. Yablonka-Reuveni, The skeletal muscle satellite cell: The stem cell that came in from the cold. *J. Histochem. Cytochem.* **54**, 1177–1191 (2006).
12. B. Pawlikowski, C. Pulliam, N. D. Betta, G. Kardon, B. B. Olwin, Pervasive satellite cell contribution to uninjured adult muscle fibers. *Skelet. Muscle* **5**, 42 (2015).
13. J. D. Bernet *et al.*, p38 MAPK signaling underlies a cell-autonomous loss of stem cell self-renewal in skeletal muscle of aged mice. *Nat. Med.* **20**, 265–271 (2014).
14. A. Figueroa *et al.*, Role of HuR in skeletal myogenesis through coordinate regulation of muscle differentiation genes. *Mol. Cell Biol.* **23**, 4991–5004 (2003).
15. A. Cammas *et al.*, Destabilization of nucleophosmin mRNA by the HuR/KSRP complex is required for muscle fibre formation. *Nat. Commun.* **5**, 4190 (2014).
16. V. Dormoy-Raclet *et al.*, HuR and miR-1192 regulate myogenesis by modulating the translation of HMGB1 mRNA. *Nat. Commun.* **4**, 2388 (2013).
17. T. Kumagai *et al.*, The proto-oncogene Bc16 inhibits apoptotic cell death in differentiation-induced mouse myogenic cells. *Oncogene* **18**, 467–475 (1999).
18. P. Delgado-Olguín *et al.*, CTCF promotes muscle differentiation by modulating the activity of myogenic regulatory factors. *J. Biol. Chem.* **286**, 12483–12494 (2011).
19. M. S. Alexander *et al.*, Foxj3 transcriptionally activates Mef2c and regulates adult skeletal muscle fiber type identity. *Dev. Biol.* **337**, 396–404 (2010).
20. E. D. Rubio *et al.*, CTCF physically links cohesin to chromatin. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 8309–8314 (2008).
21. A. T. Hark *et al.*, CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* **405**, 486–489 (2000).
22. C. Battistelli, A. Busanello, R. Maione, Functional interplay between MyoD and CTCF in regulating long-range chromatin interactions during differentiation. *J. Cell Sci.* **127**, 3757–3767 (2014).
23. N. P. Mastroyiannopoulos, A. A. Antoniou, A. Koutsoulidou, J. B. Uney, L. A. Phylactou, Twist reverses muscle cell differentiation through transcriptional down-regulation of myogenin. *Biosci. Rep.* **33**, 903–911 (2013).
24. C. F. Bentzinger, Y. X. Wang, M. A. Rudnicki Building muscle: Molecular regulation of myogenesis. *Cold Spring Harb Perspect Biol* **4**:a008342 (2012).
25. G. M. Wilson *et al.*, Phosphorylation of p40AUF1 regulates binding to A + U-rich mRNA-destabilizing elements and protein-induced changes in ribonucleoprotein structure. *J. Biol. Chem.* **278**, 33039–33048 (2003).
26. D. B. Spicer, J. Rhee, W. L. Cheung, A. B. Lassar, Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. *Science* **272**, 1476–1480 (1996).
27. G. Q. Luo *et al.*, Effect and mechanism of the Twist gene on invasion and metastasis of gastric carcinoma cells. *World J. Gastroenterol.* **14**, 2487–2493 (2008).
28. N. A. Dumont, Y. X. Wang, M. A. Rudnicki, Intrinsic and extrinsic mechanisms regulating satellite cell function. *Development* **142**, 1572–1581 (2015).
29. M. Koleva *et al.*, Pleiotropic effects of sonic hedgehog on muscle satellite cells. *Cell. Mol. Life Sci.* **62**, 1863–1870 (2005).
30. G. Straface *et al.*, Sonic hedgehog regulates angiogenesis and myogenesis during post-natal skeletal muscle regeneration. *J. Cell Mol. Med.* **13**(8B), 2424–2435 (2009).
31. M. A. Hausburg *et al.*, Post-transcriptional regulation of satellite cell quiescence by TTP-mediated mRNA decay. *eLife* **4**, e03390 (2015).
32. R. K. Singh *et al.*, Rbfox2-coordinated alternative splicing of Mef2d and Rock2 controls myoblast fusion during myogenesis. *Mol. Cell* **55**, 592–603 (2014).
33. L. H. Apponi, A. H. Corbett, G. K. Pavlath, RNA-binding proteins and gene regulation in myogenesis. *Trends Pharmacol. Sci.* **32**, 652–658 (2011).
34. C. von Roretz, P. Beauchamp, S. Di Marco, I. E. Gallouzi, HuR and myogenesis: Being in the right place at the right time. *Biochim. Biophys. Acta* **1813**, 1663–1667 (2011).
35. I. Grammatikakis, K. Abdelmohsen, M. Gorospe, Posttranslational control of HuR function. *Wiley Interdiscip. Rev. RNA* **8**, e1372 (2017).
36. K. van der Giessen, S. Di-Marco, E. Clair, I. E. Gallouzi, RNAi-mediated HuR depletion leads to the inhibition of muscle cell differentiation. *J. Biol. Chem.* **278**, 47119–47128 (2003).