



Staufen1 inhibits MyoD translation to actively maintain muscle stem cell quiescence

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Tissue regeneration depends on the timely activation of adult stem cells. In skeletal muscle, the adult stem cells maintain a quiescent state and proliferate upon injury. We show that muscle stem cells (MuSCs) use direct translational repression to maintain the quiescent state. High-resolution single-molecule and single-cell analyses demonstrate that quiescent MuSCs express high levels of Myogenic Differentiation 1 (MyoD) transcript in vivo, whereas MyoD protein is absent. RNA pulldowns and costainings show that MyoD mRNA interacts with Staufen1, a potent regulator of mRNA localization, translation, and stability. Staufen1 prevents MyoD translation through its interaction with the MyoD 3'-UTR. MuSCs from Staufen1 heterozygous (Staufen1^{+/-}) mice have increased MyoD protein expression, exit quiescence, and begin proliferating. Conversely, blocking MyoD translation maintains the quiescent phenotype. Collectively, our data show that MuSCs express MyoD mRNA and actively repress its translation to remain quiescent yet primed for activation.

Staufen1 | MyoD | quiescence | satellite cell | muscle stem cell

Adult stem cells exert a key role in the maintenance of organ homeostasis and in the regeneration of damaged tissues (1, 2). The latter property is well illustrated by stem cells residing in skeletal muscle, a tissue with relatively low turnover, but exhibiting a high regenerative potential (3, 4). Adult muscle stem cells (MuSCs) (also called “satellite cells”) are absolutely required for productive regeneration to occur as demonstrated by studies in which genetic ablation of MuSCs results in a dramatic impairment in muscle regeneration (5–7).

In undamaged adult muscle, MuSCs exist in a reversible state of prolonged exit from the cell cycle also known as quiescence (8). Upon muscle injury, these cells activate, enter the cell cycle, and expand rapidly to rebuild damaged muscle fibers (9). The control of quiescence is crucial to preserve the regenerative potential of skeletal muscle. Loss of quiescence can result in depletion of the stem cell pool, and this depletion, in turn, negatively affects tissue homeostasis and regenerative potential (10–12). The transition of a quiescent stem cell to an actively proliferating cell is tightly regulated and requires extensive metabolic and transcriptional activity (13, 14).

Recent evidence strongly suggests that stem cell quiescence is an actively maintained state and that activation of cells largely depends on the multiple levels of regulation (10, 11, 15, 16). A recent study showed that the phosphorylation state of the translation initiation factor eIF2 α controls the ability of MuSCs to activate (15). Evidence suggests that MuSCs may be actively poised in a quiescent state to allow for a rapid response to regenerative stimuli to occur (16). Specifically, recent studies showed that Myf5 mRNA is stored in mRNP granules in quiescent MuSCs. During activation, mRNP granules are dissociated, Myf5 mRNA becomes available for translation, Myf5 protein rapidly accumulates, and cells progress along the myogenic program (16).

Similar to Myf5, MyoD is a myogenic determination gene that has been implicated both in the myogenic commitment of muscle progenitors and in the process of MuSC activation. Indeed, the

appearance of MyoD protein has been functionally linked with MuSC activation and is controlled at the level of transcription and RNA degradation (17, 18). In this paper, we demonstrate that, contrary to prevailing models, quiescent MuSCs express MyoD transcript in vivo and actively block MyoD translation. When these cells activate, they increase protein translation by relieving this translational block. Therefore, MuSCs exist poised for activation by the regulation of translation of transcripts present in the quiescent state. Furthermore, we discovered that the RNA-binding protein Staufen1 plays a determinant role in this process and consequently in the control of stem cell quiescence.

Results

Quiescent MuSCs Express MyoD Transcript but Not MyoD Protein. It is well established that MyoD protein is undetectable in quiescent, nonactivated MuSCs but increases substantially during the first 24 h of activation, shown perhaps most convincingly by studies of MuSCs associated with single fiber explants (19). Intriguingly, we had previously found that the level of MyoD transcript, as assessed by microarray analysis, was as high in freshly isolated MuSCs from uninjured tissue as in cells isolated 3 days postinjury (20). Indeed, RNA-sequencing (RNA-seq) analysis of MuSCs isolated from uninjured and injured muscle showed comparable MyoD transcript levels (Fig. S14). We also observed MyoD transcript levels, measured by quantitative RT-PCR (qRT-PCR), to be essentially

Significance

This work addresses a fundamental mechanism for the translational control of a master regulator of myogenic differentiation, MyoD, by the RNA binding protein Staufen1. We show that muscle stem cells express the MyoD transcript in the quiescent state in vivo but block its translation through direct repression by Staufen1. Loss of this translational repression leads to MyoD translation and cell cycle entry, highlighting a novel role for MyoD in regulating the exit from quiescence. This mechanism of direct translational repression enables the cells to exist poised for activation and cell cycle entry. These data provide insight in the translational control of muscle stem cell quiescence.

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equivalent in freshly isolated MuSCs as in MuSCs cultured for 24 h (Fig. 1A), a time when MyoD protein is clearly detectable (Fig. 1B and C). Thus, there is clearly a discordance between the levels of MyoD transcript and MyoD protein during this critical transition of MuSCs out of the quiescent state and into the actively proliferating state.

A Majority of Isolated Quiescent MuSCs Express MyoD Transcript.

One explanation for the high level of MyoD transcript in the freshly isolated MuSC population, with MyoD protein being undetectable in nearly all cells, would be the presence of rare cells with high levels of transcript. To rule this out, we isolated MuSCs from uninjured muscles and analyzed gene expression by single-

cell qRT-PCR. Nearly all cells that were positive for Pax7 transcript were also positive for MyoD transcript (Fig. S1B). MuSCs heterogeneously express MyoD (Fig. 1D), reflecting approximately a 100-fold difference in expression levels. We also detected heterogeneous, but less variable, levels of MyoD transcript in activated MuSCs isolated 3.5 d after a muscle injury (Fig. 1E).

To detect RNA directly, we used single-molecule RNA fluorescence in situ hybridization (smFISH) (21). We found that nearly all MuSCs showed distinct and specific MyoD smFISH quanta (Fig. 1F and Fig. S1C). On average, MuSCs isolated from uninjured tissue contained 13 MyoD smFISH quanta versus 6 Pax7 smFISH quanta (Fig. 1G and H). We detected similar numbers of MyoD smFISH quanta on fiber explants (Fig. S1D

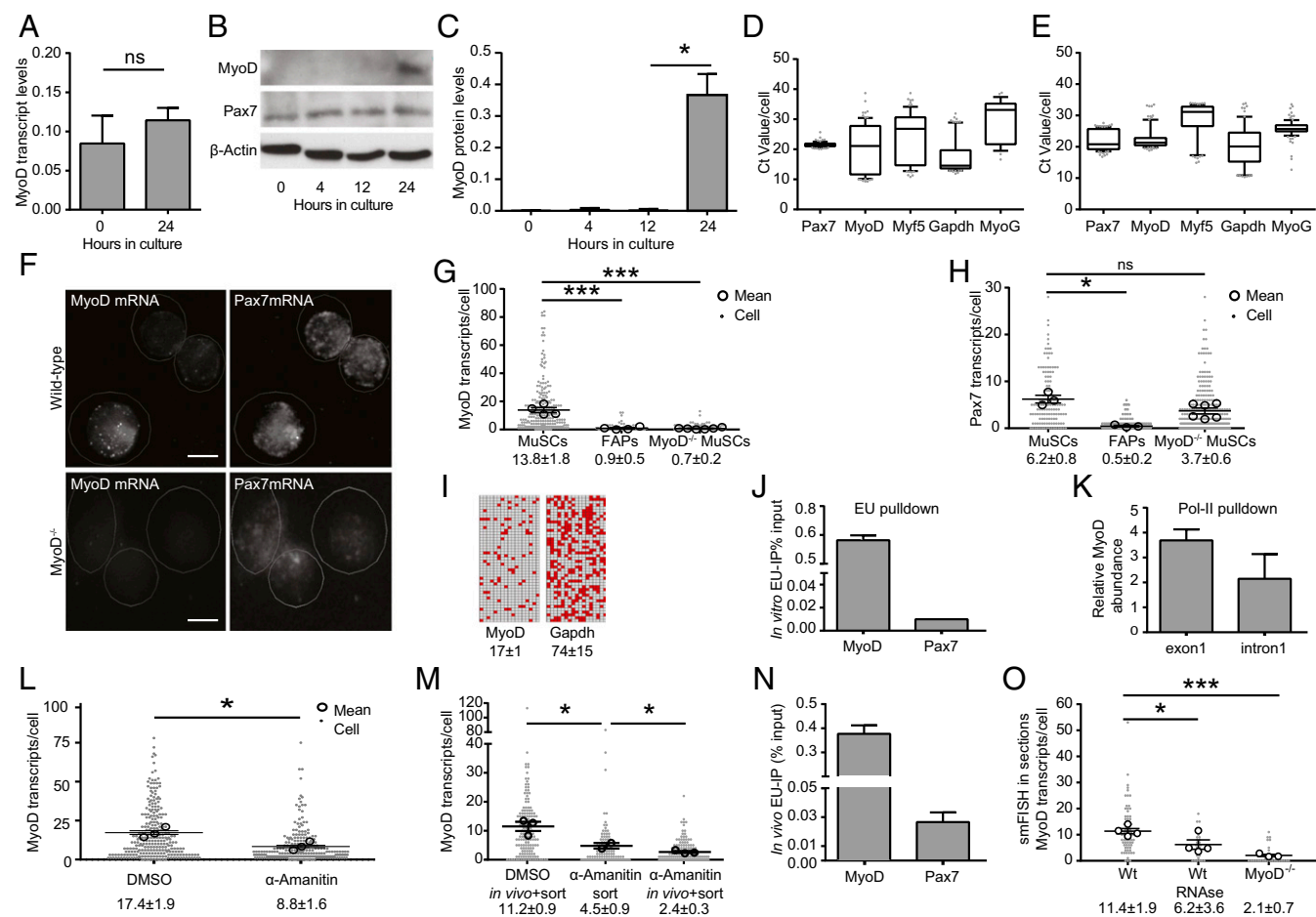


Fig. 1. Active MyoD transcription in MuSCs in vivo. (A) qRT-PCR analysis of the MyoD transcript relative to GAPDH in freshly sorted MuSCs and after 24 h in culture. (B and C) Western blot analysis of MyoD, Pax7, and β -actin protein expression in MuSCs at specified time points after sorting. Representative image (B) and quantification of MyoD level corrected for β -actin (C). (D and E) Gene expression of Pax7, MyoD, Myf5, Gapdh, and MyoG was analyzed by qRT-PCR in single cells from uninjured (D) or injured (E) mice. Raw Ct values are plotted. In D the cells with MyoG levels below the limit of detection (57 of 96) were omitted from the graph. (F) Freshly isolated MuSCs from wild-type and MyoD^{-/-} mice were fixed and simultaneously hybridized with two differentially labeled probe libraries directed against Pax7 and MyoD and counterstained with DAPI. Single transcripts appear as spots under a fluorescent microscope. “MyoD” and “Pax7” show processed images for those specific channels rendered in grayscale. (Scale bar, 5 μ m.) (G and H) Quantification of smFISH for MyoD (G) and Pax7 (H) on freshly isolated MuSCs and fibroadipogenic progenitors (FAPs, negative control) isolated from wild-type mice, as well as MuSCs isolated from MyoD^{-/-} mice. Dots denote single cells and circles denote the average per animal. (I) Digital PCR chip images with reaction-positive wells shown in red. (J) Total and EU-labeled nascent RNA was prepared from MuSCs that were exposed to EU during the isolation procedure. Levels of nascent MyoD and Pax7 transcripts were determined as percentage of total RNA by means of qRT-PCR. (K) Association of the active form of RNA polymerase II with the MyoD1 locus was assessed by CHIP-PCR, using primer pairs that detect exon1 or intron1 of the MyoD1 gene. Signal was normalized to IgG control. (L) RNA transcription during the isolation procedure of MuSCs was inhibited using α -amanitin, and the number of MyoD smFISH quanta per cell was determined. Dots denote single cells and circles denote the average per animal. (M) RNA transcription in vivo for 4 h and the number of MyoD smFISH quanta was determined in quiescent MuSCs. Dots denote single cells and circles denote the average per animal. (N) EU was injected 24 h before isolation of MuSCs from hindlimb muscles and total and EU-labeled nascent RNA was prepared. The levels of nascent MyoD and Pax7 transcripts were determined as percentages of total RNA. (O) Quantification of smFISH for MyoD on muscle cryosections from wild-type and MyoD^{-/-} mice, as well as wild-type cryosections pretreated with RNase. Data are reported as mean \pm SEM. * P < 0.05, *** P < 0.001; ns, not significant.

and *E*). The MyoD levels were confirmed using digital PCR, which detected on average 17 MyoD transcripts per cell (Fig. 1J).

Two independent assays to measure transcript levels yielded highly comparable results (Fig. S1B). We conclude that MuSCs isolated from uninjured skeletal muscle tissue contain, on average, 13–18 molecules of MyoD transcript and that these levels remain relatively constant during MuSC activation, even as the expression levels of MyoD protein increase dramatically.

Isolated Quiescent MuSCs Contain Mature MyoD Messenger RNA. For messenger RNA to be translated, it needs to be fully mature, with a 5'-CAP and a 3'-poly(A) tail. The qRT-PCR analyses made use of intron-spanning primers and suggested that the transcripts are fully spliced and mature (Fig. 1A). Immunoprecipitation (IP) of 5'-capped mRNA and IP of 3'-poly(A) mRNA further confirmed the presence of mature MyoD transcripts (Fig. S1F and G). Moreover, *in vitro* translation assays showed that RNA from quiescent MuSCs from wild-type mice, but not from MyoD knockout mice, resulted in detectable MyoD protein (Fig. S1H). Together, these results show that the MyoD RNA in freshly isolated quiescent MuSCs is capped, spliced, and polyadenylated and corresponds to mature messenger RNA that can be translated into protein.

Quiescent MuSCs Actively Transcribe MyoD During the Cell Isolation Procedure. As freshly isolated MuSCs are still quiescent but in the earliest stages of activation, we determined whether any of the MyoD transcript detected in freshly isolated cells could have been transcribed during the isolation procedure. Labeling of nascent transcripts with the nucleotide analog 5-ethynyluridine (EU) indeed showed evidence of *de novo* MyoD transcription during the cell isolation procedure (Fig. 1J). Active MyoD transcription in freshly isolated MuSCs was confirmed by chromatin IPs for the active form of RNA polymerase II (serine 5 phosphorylated CTD subunit) showing that it is bound to the MyoD locus (Fig. 1K).

To assess the relative amount of MyoD mRNA that was present *in vivo* before the isolation procedure and any *de novo* synthesis of MyoD transcript, we isolated MuSCs in the presence of the RNA polymerase II inhibitor α -amanitin (22). As expected, the number of EU-positive cells decreased to ~20% of that observed without inhibitor (Fig. S1I). Approximately 50% of the MyoD transcript detected by smFISH in freshly isolated MuSCs was present in the MuSCs *in vivo* without any change in the number of cells expressing the transcript (Fig. 1L). These data suggest that although active transcription occurs during the cell purification procedure, a substantial proportion of the transcript is already present in the cells in the quiescent state *in vivo*.

In addition to transcription, RNA degradation might also be occurring during the isolation procedure, leading to an underestimation of the amount of MyoD transcript present in the quiescent MuSCs *in vivo*. To estimate RNA degradation rates, mice were treated with α -amanitin 4 h before isolation of MuSCs from the diaphragm, a muscle that would be expected to have been exposed to the highest concentrations of the inhibitor. Following continuous treatment with the inhibitor during the isolation procedure, the number of smFISH quanta was ~25% of that without continuous treatment and ~50% of that from cells treated with inhibitor only during the isolation procedure (i.e., no treatment *in vivo*) (Fig. 1M). These data suggest a half-life for MyoD transcript of approximately 4 h both *in vivo* and *ex vivo* (Fig. S1J, see *Methods* for calculations), similar to the MyoD transcript half-life reported in differentiated C2C12 myoblasts (23). This result further supports the conclusion that quiescent MuSCs actively transcribe the *MyoD1* gene *in vivo*.

Quiescent MuSCs Express MyoD Transcript *In Vivo*. To directly test for MyoD transcription *in vivo*, we pulsed mice with a systemic injection of EU and isolated MuSCs after a 24-h chase. Again, we could detect evidence of active MyoD transcription in quiescent

MuSCs *in vivo* (Fig. 1N). To definitively determine that most MuSCs *in vivo* express MyoD messenger RNA, we analyzed muscle cryosections for MyoD smFISH quanta. Because Pax7 antibodies were incompatible with the smFISH protocol, we used a YFP lineage tracer in combination with a Pax7CreER driver to specifically label the MuSCs with YFP (24). Isolated MuSCs of this genetic background express MyoD RNA in an RNA polymerase II-dependent manner, similar to wild-type cells and with a similar half-life (Fig. S1K and L). We could detect MyoD smFISH quanta in 90% of YFP-positive cells and found that MuSCs *in vivo* contain ~11 MyoD transcripts (Fig. 1O and Fig. S1M). This shows that MuSCs *in vivo* contain levels of MyoD RNA that are comparable to those observed in isolated MuSCs, without the confounding synthesis during isolation. In contrast to prevailing models, we conclude that quiescent MuSCs *in vivo* express MyoD mRNA.

Translational Regulation of the MyoD Transcript. The findings of active MyoD transcription without detectable MyoD protein translation in quiescent MuSCs *in vivo*, combined with the lack of increased MyoD transcription during *ex vivo* activation when protein levels increase dramatically, raise the question as to the mechanism of translational repression at play in the quiescent state. Thus, we sought to examine what mechanisms might be active in MuSCs to control the translation of the MyoD transcript. We did not observe any colocalization of MyoD transcript and the RNA granule marker Ddx6 (Fig. S1N and O), which prevents Myf5 translation in quiescent MuSCs (16). We therefore looked for other candidate regulators in our transcriptome data (20). We focused on the genes that are most highly expressed in quiescent MuSCs (median expression plus three SDs yielded a list of 2,195 genes). From among these, we selected those that decreased by more than 30% by 36 h after injury, yielding 247 unique gene symbols, including known quiescence genes *Pax7*, *Notch3*, *Hes1*, *CalcR*, and *Spry1*. Gene ontology (GO) analyses revealed that 20 of the 247 gene symbols are annotated as RNA binding proteins and therefore potential candidate repressors of MyoD translation (Fig. S2A). Among these 20 candidates, only the RNA binding protein Staufen1, which can control mRNA localization, translation, and degradation (25), has a reported function in myogenic cells (26). Gene expression analysis by qRT-PCR confirmed Staufen1 to be highly expressed in quiescent MuSCs compared with activated MuSCs (Fig. 2A). Using C2C12 myoblasts as a model, we observed an enrichment of MyoD transcript after IP of endogenous Staufen1 protein (Fig. 2B), confirming that Staufen1 can interact with MyoD mRNA.

Previous reports showed that Staufen1 preferentially binds double-stranded RNA structures in the 3'-UTR of its targets (27, 28). To test whether Staufen1 might also bind to MyoD transcript at its 3'-UTR, we created luciferase reporters for the protein coding sequence or the 3'-UTR of MyoD. IP of Staufen1 from C2C12 cells expressing these reporters showed that Staufen1 interacts with the 3'-UTR-containing reporters but not the reporters containing only the ORF (Fig. S2B). Next, we tested whether Staufen1 binds to a secondary structure in the 3'-UTR of MyoD. After treatment of freshly isolated MuSCs with dimethyl sulfate (29), MyoD 3'-UTR amplicons remained detectable, suggesting that they stem from paired, protected nucleotides. In contrast, Pax7 3'-UTR amplicons disappeared from the dimethyl sulfate-treated samples, indicating those are exposed, unpaired nucleotides (Fig. S2C). We conclude that Staufen1 protein binds to secondary structures in the MyoD 3'-UTR.

Next, we tested whether Staufen1 binds to MyoD transcript in MuSCs. Following IP of Staufen1 protein from freshly isolated MuSCs, we indeed could detect enrichment for MyoD transcript (Fig. 2C). Interestingly, in addition to the mature, spliced MyoD transcript, we observed intron-retaining MyoD transcripts to co-IP with Staufen1, although the total levels of intron-retaining transcripts were much less than those of mature transcript (Fig. 2D).

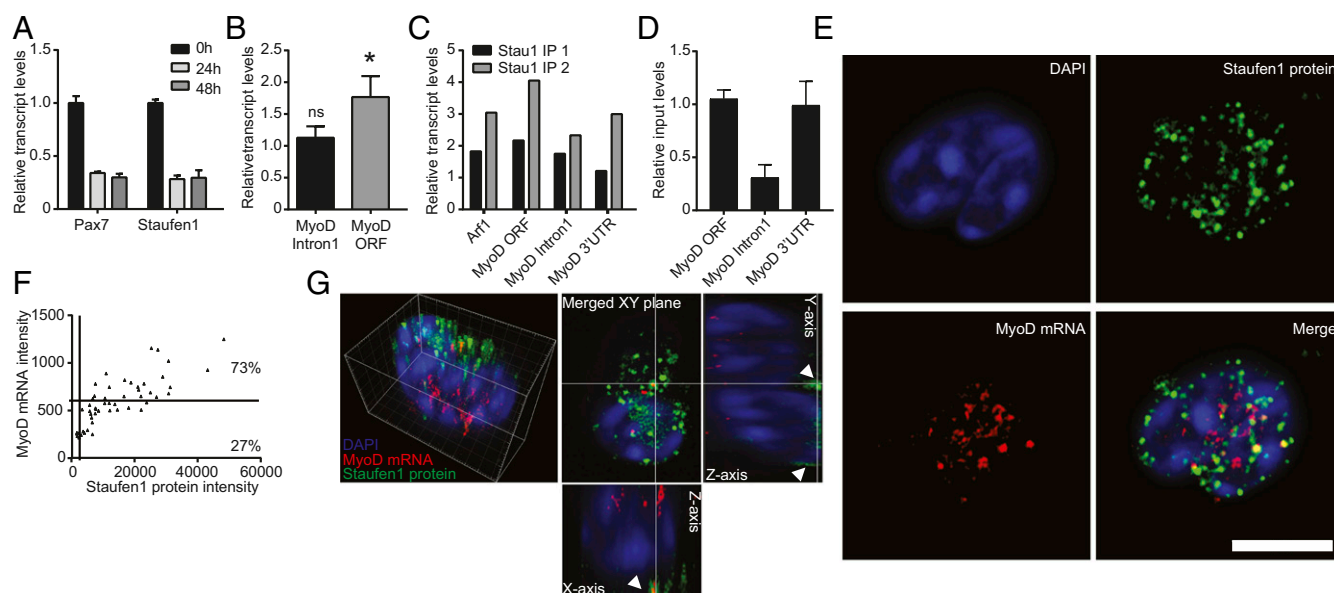


Fig. 2. Localization of MyoD transcript to Staufen1 foci. (A) qRT-PCR analysis of the Staufen1 (Stau1) transcript relative to Gapdh in freshly sorted MuSCs and after 24 and 48 h in culture. Pax7 was used as a control. (B) RNA-IP using an anti-Staufen1 antibody or IgG control was carried out using C2C12 cells. RNA immunoprecipitates and input lysate RNA were reverse transcribed, amplified using PCR with primer pairs for intron1 or the ORF in MyoD, and plotted relative to IgG control. (C) RNA-IP using an anti-Staufen1 antibody was carried out on freshly isolated quiescent MuSCs. RNA immunoprecipitates and input lysate RNA were reverse transcribed and amplified using PCR with primer pairs for intron1, the ORF, or the 3'-UTR in MyoD. Arf1 was used as a positive control. Values were normalized to IgG control and plotted relative to Gapdh. (D) qRT-PCR on input material for the Staufen1 IPs with primer pairs for intron1, the ORF, or the 3'-UTR in MyoD. Values are standardized to Gapdh. (E–G) Staufen1 protein and MyoD mRNA localization was visualized in quiescent MuSCs by combining smFISH for MyoD (red) with immunofluorescence for Staufen1 (green). Cells were counterstained with DAPI (blue) and imaged by confocal microscopy. (E) Representative photographs of colocalization analysis of Staufen1 protein and MyoD1 transcripts. (Scale bar, 5 μ m.) (F) Quantification of MyoD mRNA smFISH staining at Staufen1 foci. Each triangle represents a Staufen1 focus for which the MyoD and Staufen1 staining intensities are plotted. Solid lines represent thresholds that were determined by staining knockout cells (MyoD) or by using secondary antibodies only (Staufen1). The number of foci above or below the MyoD threshold are given as a percentage. (G) A 3D confocal image (*Left*) rendered as a 2D image in the XY plane (*Top Middle*) with orthogonal views of the XZ (*Bottom*) and ZY (*Right*) planes. White lines denote the location of the 2D image in the orthogonal planes. White arrowheads denote the localization of Staufen1 and MyoD mRNA outside the nucleus. Data are reported as mean \pm SEM. * P < 0.05; ns, not significant.

As a final proof, we costained freshly isolated MuSCs with a Staufen1 antibody and MyoD smFISH probes. We observed a strong correlation between Staufen1 protein immunofluorescence signal and MyoD mRNA staining in the cytosol, with 73% of Staufen1 foci costaining with MyoD transcript, whereas Staufen1 does not colocalize Pax7 mRNA (Fig. 2 E–G and Fig. S2 D and E).

Next we sought to determine whether that interaction between Staufen1 and MyoD results in translational suppression. In vitro translation of MyoD decreased in a dose-dependent manner in response to increasing levels of recombinant Staufen1 protein (Fig. 3 A–C). Moreover, coexpression of the luciferase-MyoD reporters described above with Staufen1 in C2C12 myoblasts led to a decrease in luciferase activity for reporters containing the MyoD 3'-UTR, whereas it had no effect on luciferase-MyoD-ORF reporters in C2C12 myoblasts (Fig. 3 D). We conclude that Staufen1 limits MyoD by suppressing translation and via the interaction of Staufen1 with the 3'-UTR of the MyoD transcript.

We next tested whether Staufen1 can regulate the translation of endogenous MyoD transcripts in quiescent MuSCs. To this end, we overexpressed recombinant GFP-Staufen1 in freshly isolated MuSCs and measured MyoD protein levels after 24 h. In the presence of recombinant Staufen1, MyoD protein levels were significantly reduced (Fig. 3 E and F). Furthermore, we found that, in control cells, there is a clear positive correlation between the amount of MyoD transcript and the amount of MyoD protein (Fig. S2 F). Conversely, in GFP-Staufen1 transfected cells, this correlation was lost and higher levels of MyoD transcript did not lead to higher levels of MyoD protein (Fig. 3 G). These data suggest that Staufen1 can directly limit MyoD translation in quiescent MuSCs.

Next, we analyzed the effect of reduced endogenous Staufen1 protein on MyoD protein levels. As predicted, MuSCs from Staufen1^{+/-} mice had reduced Staufen1 protein levels and higher levels of MyoD protein both in vitro and in vivo (Fig. 3 H–K and Fig. S2 G and H). Because Staufen1 has been reported to control mRNA degradation and translation of the same targets, we tested the effect of Staufen1 on MyoD mRNA levels. Recombinant Staufen1 resulted in a modest reduction in MyoD transcript levels (Fig. S2 I). Staufen1 itself lacks RNase activity and recruits the RNase Upf1 to degrade transcripts (30). Indeed, we observed Upf1 up-regulation in activating MuSCs and colocalization of MyoD mRNA and Upf1 protein, but not of MyoD mRNA and the Staufen1-independent RNase Upf2 (Fig. S2 J–N). Furthermore, knockdown of Upf1 but not Upf2 could rescue the Staufen1-induced reduction in MyoD transcript levels (Fig. S2 O and P), indicating that Staufen1 can indirectly also control MyoD transcript levels via Upf1. However, MuSCs from Stau1^{+/-} mice express modestly reduced, rather than increased, levels of MyoD mRNA (Fig. S2 Q). Moreover, in vitro and in vivo transcription inhibition experiments showed that MyoD mRNA turnover occurs at rates comparable to those seen in wild-type cells (Fig. S2 R and S compare with Fig. 1 N and O). These data suggest that mRNA degradation plays at most only a minimal role in how Staufen1 controls MyoD protein levels in the quiescent MuSCs. Indeed, in MuSCs from Stau1^{+/-} mice compared with MuSCs from controls, the MyoD protein level is higher relative to the amount of MyoD transcript per cell (Fig. 3 L). Conversely, after expression of recombinant Staufen1, the ratio of MyoD protein to MyoD transcript decreased compared with control cells (Fig. S2 T). We conclude that Staufen1 blocks MyoD translation in quiescent MuSCs.

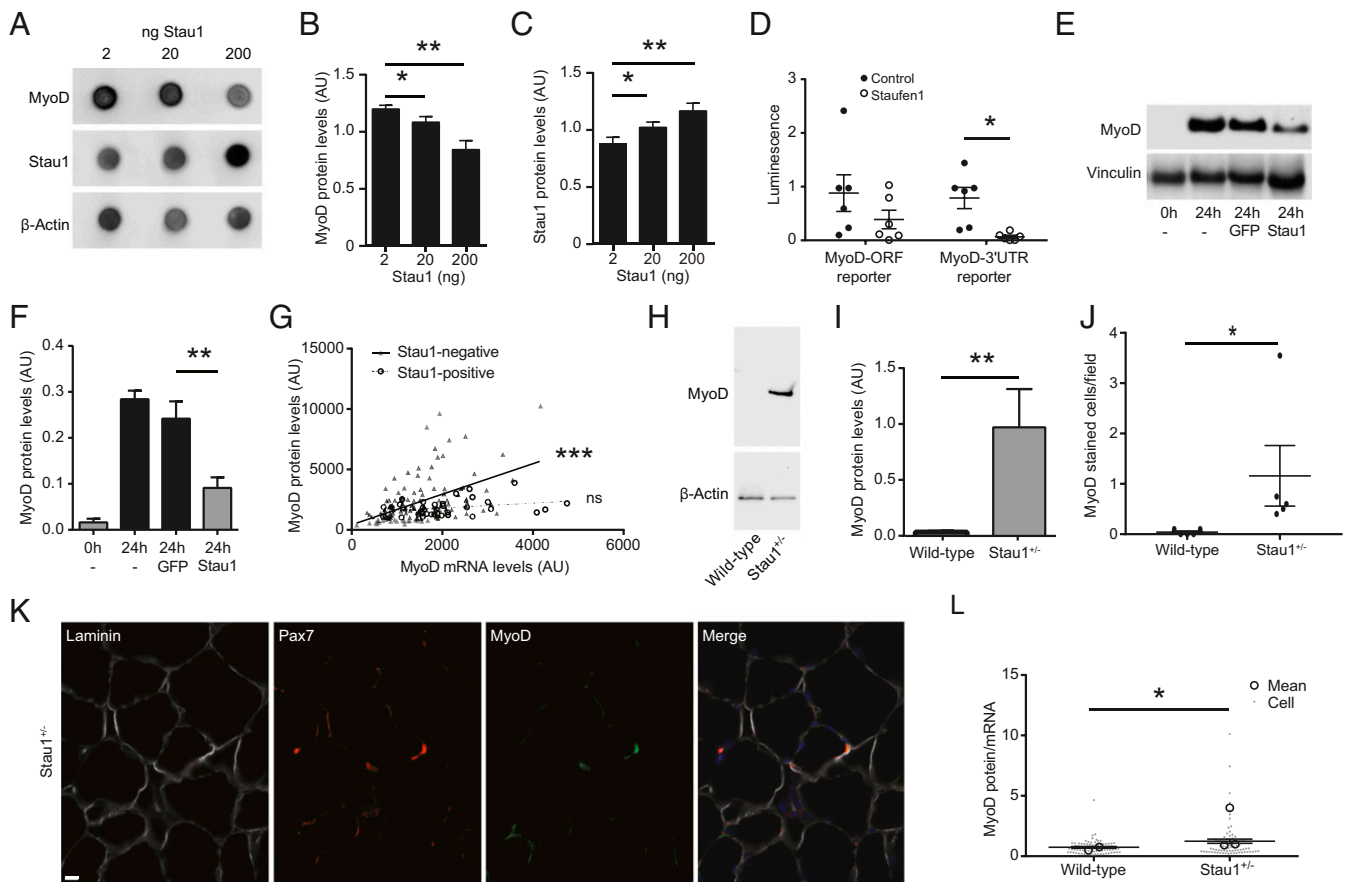


Fig. 3. Staufen1 controls MyoD protein levels in quiescent MuSCs. (A–C) Dot blots of *in vitro* translation assays. MuSC RNA was used as input with increasing amounts of recombinant Staufen1. Shown are representative dot blots (A) and quantifications of MyoD (B) and Staufen1 (C) levels relative to β -actin. (D) Luciferase assay in C2C12 cells expressing MyoD Firefly luciferase reporter constructs (Fig. S2B) and either GFP or recombinant Staufen1. Transfection levels were controlled using Renilla luciferase. (E and F) Western blot analysis of MuSCs transfected with Staufen1 for 24 h *in vitro*. (E) Representative Western blot. (F) Quantification of MyoD protein levels corrected for Vinculin. (G) Quantification of immunofluorescence levels for MyoD protein and smFISH levels for MyoD mRNA after Staufen1 overexpression. Plotted are cells that were Staufen1-positive and Staufen1-negative. Linear regression lines were modeled through the data points to calculate the correlation and statistical significance between mRNA and protein levels. (H and I) Western blot analysis of Staufen1 protein in MuSCs isolated from wild-type and *Staufen1*^{+/-} mice. β -Actin was used as a control. Representative image is shown in H and quantification of Staufen1 levels relative to β -actin is shown in I. (J) The number of MyoD-positive cells was scored in cryosections of tibialis anterior (TA) muscles from wild-type and *Staufen1*^{+/-} mice. (K) Representative cryosections from TA muscles from *Staufen1*^{+/-} mice stained for Laminin (white), Pax7 (red), and MyoD (green). (Scale bar, 10 μ m.) (L) Quantification of immunofluorescence levels for MyoD protein and smFISH levels for MyoD mRNA in MuSCs freshly isolated from wild-type and *Staufen1*^{+/-} mice. Plotted is the ratio of protein/mRNA for single cells. Dots denote cells and circles denote the average per animal. Data are reported as mean \pm SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Staufen1 Maintains MuSC Quiescence *In Vitro* and *In Vivo*. Having established that Staufen1 can interact with MyoD transcript in quiescent MuSCs and limit its translation, we asked whether this process has any impact on MuSC function *in vivo*. Analysis of MyoD knockout mice showed that MuSCs without MyoD are slower to divide compared with wild-type cells (31, 32). We therefore asked whether the Staufen1-MyoD axis impacts the propensity of MuSCs to break quiescence and begin proliferating. Nearly twice as many *Staufen1*^{+/-} MuSCs were 5-ethynyldeoxyuridine (EdU)-positive after 24 hours in culture compared with wild-type cells (Fig. 4A). Similar results were obtained with fiber explants and by knocking down Staufen1 *in vitro* with siRNAs or by knocking down Staufen1 *in vivo* with antisense oligonucleotide morpholinos (Fig. 4B and Fig. S3A–C). The increased EdU uptake in *Staufen1*^{+/-} MuSCs could be mitigated by siRNA-induced reduction of MyoD (Fig. 4A). We next asked whether Staufen1 similarly regulates MuSC quiescence *in vivo*. Following 3 days of systemic EdU injections, ~2% of the cells from wild-type animals showed EdU uptake, consistent with these cells being in a quiescent state, whereas, 7% of the cells from *Staufen1*^{+/-} mice

readily incorporate EdU (Fig. 4C). A similar increase in EdU incorporation was observed after *in vivo* knockdown of Staufen1 (Fig. S3D). To test whether MyoD levels could impact this effect, we pulsed *Staufen1*^{+/-}:MyoD^{+/-} mice with systemic injections of EdU. Cells isolated from these mice incorporated EdU at levels similar to those of wild-type MuSCs (Fig. 4C). These data suggest that loss of Staufen1 increases the propensity of a MuSC to break quiescence and start proliferating, an effect that is dependent upon the presence of MyoD protein. Consistently, the EdU-positive *Staufen1*^{+/-} MuSCs expressed higher levels of MyoD protein (Fig. S3E). To test directly for a causal link between Staufen1 and MyoD in regulating MuSC activation, we coexpressed GFP or GFP-Staufen1 with recombinant MyoD, with or without its 3'-UTR, in MuSCs from MyoD null mice. Strikingly, coexpression of GFP-Staufen1 with the recombinant MyoD without its 3'-UTR had no effect; only when we used a MyoD construct that included the 3'-UTR was Staufen1 able to prevent the increase in EdU incorporation (Fig. 4D and Fig. S3F and G). To conclusively demonstrate that the *Staufen1*^{+/-} phenotype depends on MyoD translation, we blocked MyoD translation *in vivo* using antisense *vivo*-morpholino oligonucleotides that

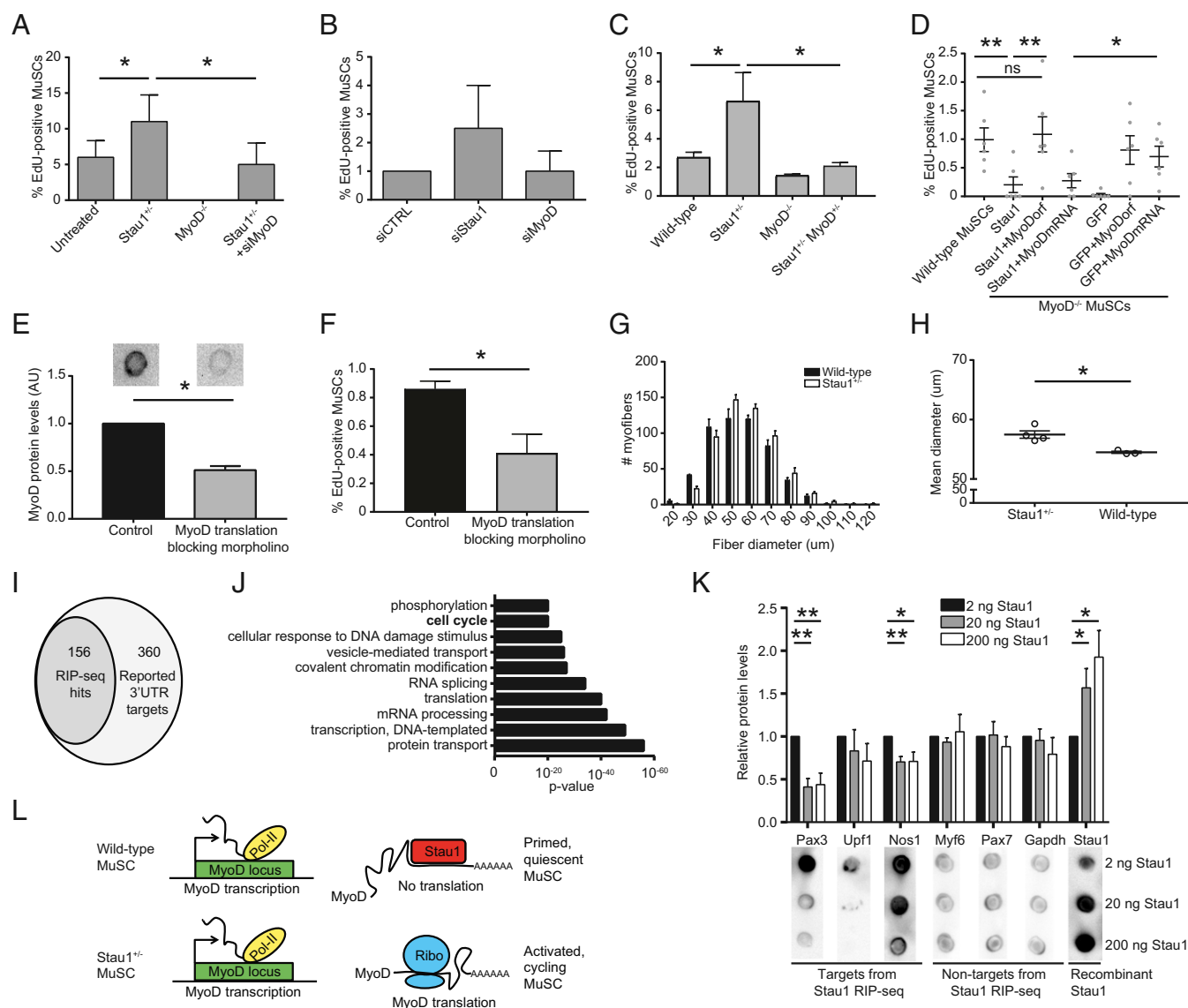


Fig. 4. Stau1 controls MuSC quiescence in vitro. (A) EdU incorporation in MuSCs in vitro after a 24-h pulse. Cells were isolated from wild-type, MyoD^{-/-}, and Stau1^{+/+} mice. The *Rightmost* bar represents Stau1^{+/+} cells transfected with siRNA against MyoD. (B) Wild-type MuSCs were treated with control siRNA, siRNA to MyoD, or siRNA to Stau1 and pulsed with EdU for 24 h, after which EdU incorporation was measured. (C) Wild-type, MyoD^{-/-}, Stau1^{+/+}, and Stau1^{+/+}MyoD^{-/-} mice were injected with a dose of EdU every 12 h for 72 h before isolation of MuSCs and quantification of in vivo EdU incorporation. (D) MuSCs from MyoD^{-/-} mice were transfected with Stau1 or GFP expression plasmids, and with plasmids containing either the MyoD ORF ("MyoDorf") or the MyoD ORF plus untranslated regions ("MyoDmRNA"). MuSCs from wild-type mice were used as controls. Cells were pulsed with EdU for 24 h in vitro and EdU incorporation was quantified. (E) Control morpholinos or antisense morpholinos complementary to the translation initiation sequence in MyoD were tested in an in vitro translation assay, and MyoD protein was quantified by dot blot. Representative dot blots are depicted *Above* each bar. (F) Stau1^{+/+} mice were injected with control morpholinos or antisense vivo-morpholinos to block MyoD translation in vivo and pulsed for 3 d with EdU. Cells were isolated and analyzed for in vivo EdU incorporation. (G) Histogram of fiber diameters from cryosections of TA muscles of control and Stau1^{+/+} mice. Sections were stained for laminin and the numbers of myofibers were graphed according to fiber diameter. $P < 0.05$, χ^2 test. (H) Quantitative analysis of mean fiber size for uninjured TA muscles in wild-type and Stau1^{+/+} mice. (I) Venn diagram depicting 156 (30%) of 516 reported 3'-UTR-bound Stau1 targets confirmed by Stau1 RIP-seq analysis of wild-type MuSCs. (J) GO-term analysis of Stau1 RNA-IP sequencing hits. The top 10 most significantly enriched GO terms are shown. (K) Dot blot analysis of in vitro translation assays with MuSC RNA input and increasing concentrations of recombinant Stau1 protein. Pax3, Upf1, and Nos1 were enriched in Stau1 RNA-IP sequencing experiments, whereas Myf6, Pax7, and Gapdh were not. Representative dot blots are shown *Below* each set of bars. (L) Model of Stau1-mediated regulation of MyoD. Data are reported as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$.

blocked MyoD translation in in vitro translation assays (Fig. 4E). We injected these vivo-morpholinos, or controls, into Stau1^{+/+} animals. After blocking MyoD translation in vivo, fewer cells incorporated EdU both in vivo (Fig. 4F) and ex vivo (Fig. S3H). These results demonstrate that MyoD translation is downstream of Stau1 in regulating MuSC quiescence in vivo. We conclude that Stau1 prevents exit of quiescence in vivo by suppressing the accumulation of MyoD protein.

We asked whether the loss of Stau1-mediated repression of MyoD translation in MuSCs would impact muscle homeostasis and repair. There was a significant increase in fiber diameter in uninjured muscles from Stau1^{+/+} mice compared with wild-type control mice (Fig. 4G and H), demonstrating that a change in MuSC quiescence impacts muscle homeostasis. To assess the effects of Stau1 deletion on muscle repair, we injured muscles of Stau1^{+/+} and control mice. Five days after injury, regenerating

myofibers from *Staufen1*^{+/-} mice were significantly larger than those of control mice (Fig. S3 I and J). Moreover, this increase was partially reversed in *Staufen1*^{+/-}:*MyoD*^{+/-} mice. Nevertheless, there was no discernible difference in *in vivo* EdU incorporation in the proliferating MuSCs, suggesting that *Staufen1*-*MyoD* regulation does not alter the proliferative response of activated MuSCs (Fig. S3K).

Here we provide evidence for direct translational repression governing the quiescent state of MuSCs and show that the regulation of quiescence is mediated by the translational repression of *MyoD*. We asked whether the *Staufen1*-dependent translational repression extends beyond *MyoD*. Using RNA IP sequencing (RIP-seq), we identified over 3,000 enriched transcripts, including 30% of previously reported 3'-UTR-bound *Staufen1* targets (27), in freshly isolated MuSCs (Fig. 4I). We performed gene ontology analyses on the *Staufen1* target genes and found "cell cycle" among the most enriched categories (Fig. 4J), consistent with our findings that *Staufen1* controls MuSC quiescence and cell cycle entry. We selected three of the most highly enriched transcripts, *Nos1*, *Pax3*, and *Upf1*, as well as three transcripts that are expressed in quiescent MuSCs but not enriched in the RIP-seq datasets, *Myf6*, *Pax7*, and *Gapdh*. *Pax3* was previously shown to be a target of *Staufen1* in proliferating C2C12 myoblasts (30). Using *in vitro* translation assays with quiescent MuSC RNA as input, we show that *Staufen1* could repress translation of the three targets, but not the three nontargets, demonstrating that the mechanism of translational repression extends beyond *MyoD* (Fig. 4K).

Discussion

In the current study, our data reveal high levels of *MyoD* transcripts in quiescent MuSCs *in vivo* in the absence of any detectable *MyoD* protein. The resulting pool of transcripts is prevented from being translated into protein by *Staufen1*, which directly interacts with structures in the *MyoD* 3'-UTR to suppress translation. MuSCs that lack one *Staufen1* allele have increased levels of *MyoD* protein, break quiescence, and enter the cell cycle more rapidly than wild-type cells. Loss of *MyoD* prevents cell cycle entry of quiescent *Staufen1*^{+/-} MuSCs. Accordingly, we propose that *Staufen1* limits *MyoD* translation in quiescent MuSCs to maintain quiescence while priming the cells for rapid activation (Fig. 4N).

Prior studies showed that *MyoD* protein is below the detection level but increases dramatically during MuSC activation (19, 33, 34). Whereas there is general consensus that *MyoD* protein is undetectable in quiescent MuSCs, contrasting observations have been reported regarding the presence of *MyoD* transcripts. Initial single-cell studies showed the *MyoD* transcript to be detectable in a very small number of cells isolated from uninjured tissue and to increase substantially when the cells activate (35, 36). In line with these observations, use of mice in which *Cre* had been knocked into the *MyoD* locus (*MyoD*-iCre) revealed that only 10% of MuSCs on freshly isolated fibers expressed *Cre* (37). In contrast, transcriptional profiling identified levels of *MyoD* transcript in freshly isolated MuSCs that were comparable to levels observed in activated MuSCs and therefore difficult to ascribe to a small subpopulation of *MyoD*-expressing cells (20). Our data reported here clearly show that nearly all MuSCs express *MyoD* transcript to some extent and, in line with previous reports, do not express the *MyoD* protein. Importantly, our data revealed a high variability in transcript levels between individual *MyoD*-expressing MuSCs (Fig. 1E), perhaps accounting for divergent findings as to the percentage of quiescent MuSCs expressing the *MyoD* transcript. Intriguingly, lineage tracing and genetic ablation experiments with the *MyoD*-iCre mice showed that most MuSCs have transited through a *MyoD*-positive state during development (37, 38). Although this observation has been attributed to a well-documented expression of *MyoD* in the developmental precursors of MuSCs, it possibly highlights a dynamic activity of the *MyoD* locus in the MuSC lineage.

Various posttranscriptional regulatory mechanisms have been shown to be involved in maintaining the quiescent state of MuSCs. Two miRNAs were shown to control transcript fate to maintain quiescence: miR31 sequesters *Myf5* into mRNP granules (16) and miR489 suppresses the expression of the oncogene *DEK* (11). In both cases, suppression of protein expression contributes to maintenance of stem cell quiescence.

Two recent studies found that unchecked *MyoD* transcription can lead to accumulation of *MyoD* protein and spontaneous differentiation. In the early stages of activation, Tristetraprolin (TTP), a zinc-finger protein involved in mRNA degradation, targets excess *MyoD* transcript to mRNA decay pathways to prevent its accumulation (18). Loss of TTP induces a threefold increase in *MyoD*-positive cells, suggesting that the MuSCs spontaneously activate, possibly due to increased *MyoD* expression (18). This effect is less pronounced than that observed in *Staufen1*^{+/-} mice. Our data indicate that control by TTP alone is insufficient, since loss of *Staufen1* leads to the accumulation of enough *MyoD* protein to drive MuSCs to activate and enter the cell cycle. A second study demonstrated that the *MyoD* locus is repressed by the lysine methyltransferase *Suv4-20h1* (17). Knockout of *Suv4-20h1* resulted in strong up-regulation of *MyoD* transcript and protein levels. These cells spontaneously differentiated in noninjured tissue while concurrent genetic ablation of *MyoD* rescued the phenotype, suggesting that *MyoD* expression was driving the differentiation. Importantly, we find that *MyoD* translation is inhibited by *Staufen1* and that a loss-of-function allele for *Staufen1* causes increased *MyoD* protein expression in MuSCs, which spontaneously break quiescence. Altogether, these observations suggest that *MyoD* can drive MuSCs into the cell cycle and trigger their differentiation program. Consistently, *MyoD* null cells are slower to enter the cell cycle and have a differentiation defect *in vitro* (31, 32).

Staufen1 is a ubiquitously expressed, double-stranded RNA binding protein that associates with secondary structures. It has been shown to be involved in mRNA transport, splicing, translation, and decay and, as such, plays a key role in the posttranscriptional regulation of gene expression (39–41). *Staufen1* regulates terminal differentiation of the human keratinocytes in the epidermis, guides adipogenesis, and plays a role in the early stages of murine embryonic stem cell differentiation (42–44). Only a limited number of studies have investigated the role of *Staufen1* in the skeletal muscle, and all of them focused on the control of the differentiation program (26, 30, 45). In multinucleated myotubes, *Staufen1* targets several inhibitors of differentiation, including *Pax3*, for degradation, while in undifferentiated C2C12 myoblasts, it acts as a transcript stabilizer regulating the expression of *Dvl2*, a repressor of myoblast differentiation (30, 45). Intriguingly, recent experiments with differentiating myoblasts *in vitro* showed that recombinant *Staufen1* reduced *MyoD* protein expression (26). Although they were unable to identify the molecular mechanism behind this process, these findings are consistent with our data and interpretations.

Our results indicate that *Staufen1* regulates the expression of *MyoD* protein at the translational level in quiescent MuSCs. The fact that quiescent MuSCs have the capacity to regulate *MyoD* translation through a *Staufen1*-dependent mechanism points to a cell that is poised to activate. This contrasts with MuSCs at later stages in the myogenic process, which utilize different functions of *Staufen1* to regulate the process of terminal differentiation. Previous work showed that *Staufen1* controls transcript turnover in proliferating C2C12 myoblasts and that *Staufen1* protein levels decrease during differentiation (30). Recent studies using mass cytometry showed *MyoD* expression to peak early in regeneration after muscle injury and to decrease 6 days after injury (46). It is possible that *MyoD* expression is tightly regulated and that *Staufen1* expression increases when the cells reach the myoblasts stage to control *MyoD* protein levels. Intriguingly, such a

washed in 2× SSC buffer and treated with secondary antibody together with DAPI counterstain. Samples were mounted in 2× SSC buffer. Images were deconvolved with Velocity software (PerkinElmer) and transcripts quantified with FISH-quant (55). For protein and RNA costains, cells were imaged on a Zeiss LSM880 Airyscan confocal microscope. Images were subsequently processed and deconvolved using ImageJ.

Single-Cell PCR. Single-cell PCR was performed according to Fluidigm protocols, as previously described (56). Single cells were resorted into PCR mix, preamplified for 20 cycles, and diluted. Diluted preamplified mix was loaded on 96 × 96 chips and analyzed with a BioMark HD.

Digital PCR. Digital PCR was performed as described (57) and according to Fluidigm protocols. Five hundred cells were resorted into PCR mix, preamplified for one cycle, diluted for digital PCR on 12 × 12 chips, and analyzed with a BioMark HD. Calculations were performed with dPCR software (Fluidigm) and using the corrections provided as described (57).

DNA and siRNA Transfection. Reverse transfections with DNA plasmid and siRNA were performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions and transfected cells were analyzed 24 h after plating. For ChIP experiments, cells were transfected with X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's protocols, with the following modifications: for one 10-cm plate, 10 μg plasmid DNA (5 μg reporter and 5 μg Staufen1-GFP) was used with a 2.2:1 ratio of reagent to DNA. Cells were harvested 48 h posttransfection.

Cloning. To create Luciferase-MyoD reporters, MyoD sequences were amplified by phusion polymerase (NEB) and cloned into pMIR-report using SacI and SpeI. Primers were MyoD-3'utrFW: tataACTAGTgagatcgactgcagcagcag and MyoD-3'utrRV: ttaaGAGCTCttgtataaattagcgtctttatttcc; and MyoDorfFW: aaggatACTAGTatggagctctatcgcccca and MyoDorfRV: aaggatGAGCTCtaaagcactgataaatcgatt. For ectopic expression, the whole MyoD transcript was amplified and cloned into pCDNA3.1-A using BamHI and EcoRI. Primers were MyoDFW: aaggatGGATCCagggccaggagcggccaggaca and MyoDRV: aaggatGAATTCaaattagcgtctttatttccaacact. To create GFP-Staufen1 vector, Staufen1 ORF was amplified and cloned into pEGFP-C1 using XhoI and BamHI. Primers were Stau1FW: aattCTCGAGtgtaagccctggaccct and Stau1RV: atatGGATCCcagcactcccgcagctg.

ChIP. Anti-Pol II ChIP experiments were performed according to previous protocols (20) with the following modifications. For each ChIP experiment, about 5 × 10⁶ cells were used, which were resuspended in 300 μL lysis buffer and sonicated with a Covaris S2 ultrasonicator to obtain DNA fragments between ~200 and 300 bp. Cell lysates were precleared with protein A+G Dynabeads (100-04D, Invitrogen) at 4 °C with agitation for at least 3 h. For each ChIP experiment, 5 μg Pol II antibody or normal rabbit IgG antibody (2729, Cell Signaling) was added into the precleared cell lysate. Following incubation at 4 °C with agitation overnight, cell lysate-antibody complexes were centrifuged (18,407 × g) for 10 min at 4 °C and the top 90% supernatant was transferred into a new tube before wash, elution, reverse cross-linking, and purification. Purified ChIP DNAs were quantified using the LightCycler 480 (Roche).

RIP. RNA IP was performed using 4 × 10⁵ MuSCs from wild-type or Staufen^{-/-} mice. Cells were homogenized in 50 mM Tris, pH 7.5, 100 mM KCl, 12 mM MgCl₂, 1% Nonidet P-40, 1 mM DTT, 200 units/mL Promega RNasin, 1 mg/mL heparin, and Sigma protease inhibitor mixture. Samples were centrifuged at 10,000 × g for 10 min. Staufen1 antibody was added to the supernatant and the mixture was rotated for 4 h at 4 °C, after which Protein G magnetic beads were added and the samples were rotated overnight at 4 °C. The following day, samples were placed in a magnet on ice and the pellets were washed three times for 5 min in high-salt buffer (50 mM Tris, pH 7.5, 300 mM KCl, 12 mM MgCl₂, 1% Nonidet P-40, 1 mM DTT) and taken up in 300 μL of Qiagen RLT buffer. Total RNA was prepared using the RNeasy Micro kit (Qiagen) according to manufacturer's instructions and quantified using the Qubit RNA HS assay kit and the Qubit Fluorometer (Molecular Probes). For RIP-PCR, immunoprecipitated RNA samples were converted to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), and qRT-PCR was performed with the LightCycler 480 Probe Master Kit (Roche) in the LightCycler480 II System (Roche). IP samples were analyzed alongside input material and IgG controls. The final results were analyzed by ddCt between the Stau1-IP and IgG-IP samples. Values were then set relative to the nontarget transcript Gapdh.

Samples for RIP-seq were processed as follows: synthesis and amplification of cDNA were done using the SMART-Seq v4 Ultra Low Input RNA Kit for

sequencing (634893, Clontech). The cDNA was sheared under the following conditions: 5 min with 10% duty cycle, peak power 175 W and 200 cycles per burst in the frequency-sweeping mode (S220 machine, Covaris). The sheared cDNA was purified with 2.2× AMPure XP SPRI beads (A63880, BeckmanCoulter). RNA-seq libraries were generated using the Ovation Ultralow system (0347, NuGEN). A small aliquot from each RNA sample processed was run on a Bioanalyzer High-Sensitivity DNA chip (Agilent) and used for Qubit quantification. High-throughput sequencing was performed on a HiSeq4000 platform, and the samples were mixed at equal concentrations in a single lane. The sequencing quality of all samples was checked using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequenced reads were aligned against the mouse reference genome version mm10 (Grcm38) using STAR 2.4.0j (58). The number of reads aligning to genes was counted with featureCounts 1.4.6 tool from the Subread package (59). The quality of the countdata was examined by density distribution plotting. Transcripts with zero or very low expression level were filtered out to minimize interference in downstream analysis. Enriched genes were identified using EdgeR version 3.12 (60). Gene ontology analysis was performed using ReViGo (61) and DAVID (62).

Half-Life Measurements. MuSCs were isolated in the continued presence of α-amanitin at 1 μg/mL. Cells isolated in the presence of the DMSO solvent or the α-amanitin were fixed and analyzed by smFISH. The difference in transcript counts was divided by the time of the isolation procedure to establish the half-life (the time in which half of the molecules have been lost) of MyoD transcripts.

Western Blot. Western blot analysis was performed on whole cell extracts of 1 × 10⁵ MuSCs that were counted, washed, and lysed in sample buffer immediately after FACS purification. Lysates were subjected to SDS/PAGE and transferred to PVDF membrane (Millipore). Membranes were incubated in blocking buffer before overnight incubation with primary antibodies, followed by peroxidase-labeled secondary antibodies, and developed using WesternBright ECL reagents (Advanta). Vinculin or β-actin was used as loading control.

Luciferase Assay. Cells were transfected with luciferase reporter constructs and incubated for 48 h. Cells were lysed and prepared with Promega luciferase kit. Luminescence was calculated as relative value of Firefly and Renilla luciferase.

In Vitro Translation Assay. Analysis of the role of Staufen1 in translation of MyoD1 was performed using the Retic Lysate IVT kit (Ambion) according to manufacturer's instructions. Bacterially expressed recombinant Stau1⁵⁵Δ2-his₆ protein (kindly provided by Luc DesGrosseillers, McGill University, Montreal, Canada) was added to the reactions. The reaction products were separated by SDS/PAGE and treated as described above.

Recombinant Protein Production and Purification. Plasmid for Stau1⁵⁵Δ2-his₆ was transformed into DH5a bacteria. Single colonies were grown overnight in 5-mL cultures and diluted 100× the next morning. When the cell suspension reached OD₆₀₀, cells were stimulated with 1 mM IPTG for 3 h. Cells were spun down and resuspended in lysis buffer [PBS, protease inhibitor tablet, phenylmethylsulfonyl fluoride (PMSF)] and sonicated with 10-s pulses for 3 min. Debris was spun down and the soluble fraction was incubated with prewashed nickel beads for 1 h at 4 °C. Beads were washed five times and eluted with 100 mM imidazole in PBS. Purity was confirmed by SDS/PAGE and Coomassie Blue staining and concentration was measured using bicinchoninic acid (Pierce) on the Nanodrop 200 Spectrophotometer. Freshly purified protein was used for the assays.

RNA Structure PCR. Dimethyl sulfate is highly reactive with solvent-accessible, unpaired residues but unreactive with bases engaged in Watson-Crick interactions. Nucleotides that are strongly protected or reactive to dimethyl sulfate can be inferred to be base paired or unpaired, respectively (29). Single-stranded RNA is damaged by dimethyl sulfate, preventing primer annealing, and does not show up in the PCR. DMSO was used as a control. RNA from wild-type MuSCs was treated with either dimethyl sulfate of DMSO, transcribed into cDNA, and detected by qRT-PCR with primers pairs detecting the 3'-UTRs of MyoD and Pax7.

Proliferation Assay. MuSCs (2–5 × 10⁵ cells per chamber) were cultured on ECM (Sigma)-coated eight-well chamber slides (BD Biosciences) in Ham's F-10 medium with 10% horse serum. EdU was added to cell cultures at a concentration of 10 μM and refreshed every 12 h. Incorporation was detected using the Click-iT EdU Imaging Kit (Life Technologies).

