CUGBP1 and MBNL1 preferentially bind to 3’ UTRs and facilitate mRNA decay

Akio Masuda1, Henriette Skovgaard Andersen2*, Thomas Koed Doktor2*, Takaaki Okamoto1, Mikako Ito1, Brage Storstein Andresen9 & Kinji Ohno1

1Division of Neurogenetics, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan, 2Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense M, Denmark.

CUGBP1 and MBNL1 are developmentally regulated RNA-binding proteins that are causally associated with myotonic dystrophy type 1. We globally determined the in vivo RNA-binding sites of CUGBP1 and MBNL1. Interestingly, CUGBP1 and MBNL1 are both preferentially bound to 3’ UTRs. Analysis of CUGBP1- and MBNL1-bound 3’ UTRs demonstrated that both factors mediate accelerated mRNA decay and temporal profiles of expression arrays supported this. Role of CUGBP1 on accelerated mRNA decay has been previously reported, but the similar function of MBNL1 has not been reported to date. It is well established that CUGBP1 and MBNL1 regulate alternative splicing. Screening by exon array and validation by RT-PCR revealed position dependence of CUGBP1- and MBNL1-binding sites on the resulting alternative splicing pattern. This study suggests that regulation of CUGBP1 and MBNL1 is essential for accurate control of destabilization of a broad spectrum of mRNAs as well as of alternative splicing events.
High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) is a new method that enables global mapping of targets for specific RNA-binding proteins in living cells, thereby shedding light on their role in regulation of RNA processing of known and unknown targets.

In the present study, we performed HITS-CLIP analysis for CUGBP1 and MBNL1 on the mouse myoblast cell line C2C12 to extensively characterize their RNA-binding sites and functional roles in RNA processing. We identified position-dependence of CUGBP1/MBNL1-binding sites in regulating exon inclusion or skipping. Interestingly, we discovered that both CUGBP1 and MBNL1 preferentially bind to the 3’ UTR and destabilize target mRNAs. This points to a new important role of MBNL1 and suggests that binding to the 3’ UTRs and destabilization of mRNA are likely to be a fundamental function shared by CUGBP1 and MBNL1.

**Results**

**Genome-wide CUGBP1/MBNL1-RNA interaction maps.** In order to determine global CUGBP1/MBNL1-binding sites in vivo, we performed HITS-CLIP experiments using the mouse myoblast cell line, C2C12.

In C2C12 cells, CUGBP1 is constantly expressed throughout myoblast differentiation, whereas expression of MBNL1 is low in undifferentiated cells and gradually increases during differentiation (Supplementary Fig. S1), as previously described. We thus performed HITS-CLIP analysis of CUGBP1 and MBNL1 using undifferentiated and differentiated C2C12 cells, respectively. We also performed CLIP of MBNL1 using undifferentiated cells in three independent experiments, but this yielded an insufficient amount of RNA-protein complexes and failed to yield cDNA libraries suitable for high-throughput sequencing. In the HITS-CLIP analysis of CUGBP1, our first experiment yielded 34,733,815 CLIP tags of 32 nt, of which 29,545,067 (85.06%) were mapped to the mm9 genome allowing at most 2 mismatches and placing reads mapping to multiple locations to a single random site. A second CLIP experiment yielded 10,079,185 CLIP tags of 36 nt, of which 8,516,256 (84.49%) were mapped. In the first MBNL1 CLIP experiment, we obtained 13,218,685 CLIP tags, of which 11,044,152 (83.55%) were mapped, while the second CLIP experiment yielded 13,474,600 CLIP tags with 11,455,886 (85.02%) tags mapped to the mm9 genome. For the analysis of binding motif and binding region annotation, we selected only reads that were aligned uniquely in the genome and removed all potential PCR duplicates by collapsing reads with an identical 5’ start into a single read. This resulted in 177,013 and 130,828 CLIP tags from the two CUGBP1 CLIP experiments, while the two MBNL1 experiments yielded 59,156 and 583,841 CLIP tags respectively.

In an effort to confirm the specificity of our CLIP experiments, we performed CLIP analysis of polypyrimidine tract-binding protein (PTB), a multifunctional RNA-binding protein, using undifferentiated mouse C2C12 cells. We identified 12,841,778 CLIP tags of which 11,184,829 (87.10%) were mapped to the mouse mm9 genome. Removal of non-uniquely aligned reads and PCR duplicates yielded 307,995 unambiguous CLIP tags.

**Consensus motifs.** To determine RNA-binding motifs associated with CUGBP1/MBNL1 in vivo, we used the motif-finding algorithm, Multiple EM for Motif Elicitation (MEME). We used SeqMonk to identify likely binding regions, and identified 1,841 CUGBP1-binding regions and 302 MBNL1-binding regions. Comparison of SeqMonk’s maximum depth scores between samples indicates that binding regions in each replicated experiment are highly overlapping, while PTB binding regions did not overlap with those of the other four CLIP experiments (Supplementary Fig. S2). The lower number of identified MBNL1 regions supported by two independent experiments (Supplementary Fig. S2b) was likely due to the large difference in the number of CLIP tags in the two MBNL1 experiments. The regions demonstrate enrichment of GU-rich motifs for CUGBP1 and YGCY-containing motifs for MBNL1 (Fig. 1).

Our in vivo binding motifs are in accordance with previously suggested binding motifs for CUGBP125-32 and MBNL121,33-35. We identified 1,824 PTB binding regions in the mouse genome and detected a CU-rich motif, which is essentially identical to the motif for PTB recently identified by HITS-CLIP analysis of a human cell line.

We also analyzed the CUGBP1 and MBNL1 motifs enriched in regions containing reads with multiple potential mapping locations (Supplementary Fig. S3), and compared them with the motifs with unique mapping (Fig. 1). Following removal of potential PCR duplicates, we observed 699,382 tags that were non-uniquely aligned in the 1st CUGBP1 CLIP experiment, 219,128 tags in the 2nd CUGBP1 CLIP experiment, 105,432 and 216,882 tags in the two MBNL1 CLIP experiments respectively and finally 851,324 tags in the PTB CLIP experiment. We observed that enriched motifs in these regions (Supplementary Fig. S3) are very similar to the CUGBP1 and MBNL1 motifs enriched in the binding regions containing uniquely aligned reads (Fig. 1), suggesting that these regions share the same properties as the uniquely aligned regions and that they may contain functional binding sites.

**HITS-CLIP analysis of splicing targets.** We next studied the effects of CUGBP1/MBNL1 binding on alternative splicing. CUGBP1 tags are clustered in intronic regions flanking alternative rather than...
constitutive exons (Fig. 2a). MBNL1 tags are similarly clustered in intronic regions flanking alternative exons, and are also enriched in alternative and constitutive exons. In order to investigate if and how CUGBP1/MBNL1 binding around splice sites regulate alternative splicing, we knocked down these factors by siRNA in undifferentiated C2C12 cells (Supplementary Fig. S4a). We analyzed alterations of splicing globally using the Affymetrix Mouse Exon 1.0 ST Array (GEO accession number, GSE29990) and identified 8 CUGBP1-responsive and 24 MBNL1-responsive exons (Supplementary Table 1, Figs. S5 and S6abc). We also analyzed 29 CUGBP1-tagged and 51 MBNL1-tagged exons/introns known to be alternatively spliced according to the ENSEMBL version 61, and identified 16 CUGBP1-responsive and 21 MBNL1-responsive exons by RT-PCR (Supplementary Figs. S5 and S6abc). We made the compiled dataset C, which is comprised of the 24 CUGBP1-regulated exons (15 skipped and 9 included), as well as the compiled dataset M consisting of the 45 MBNL1-regulated exons (25 skipped and 20 included). The datasets include 1 and 9 previously identified target exons of CUGBP1 and MBNL1, respectively (Supplementary Fig. S5). In addition, 9 exons are shared between datasets C and M. Mbnl1 siRNA sufficiently suppressed MBNL1 expression up to day 3 after differentiation (Supplementary Fig. S4b), and we observed that as many as 44 of the 45 MBNL1-regulated exons in dataset M respond similarly to MBNL1 knockdown in both differentiated and undifferentiated cells (Supplementary Figs. S4 and S5).

We also made dataset M2 that includes 26 additional MBNL1-dependent cassette exons (15 skipped and 11 included) that were previously identified in skeletal muscle of MBNL1 knockout mice.
(Supplementary Table S2), and found that 18 exons are similarly regulated by Mbnl1 knockdown in undifferentiated C2C12 cells (Supplementary Fig. S6d and Table S2).

We combined datasets C and M into a single composite pre-mRNA and made integrated RNA maps from our HITS-CLIP reads mapped to the corresponding genomic regions as previously described for Nova and PTB. This showed that CUGBP1 binding to upstream intronic regions facilitates exon skipping, whereas CUGBP1 binding to downstream intronic regions promotes exon inclusion (closed arrows in Fig. 2b and Supplementary Fig. S7a). Results of the 2nd experiments are shown in Fig. 2 and those of the 1st experiments are in Supplementary Fig. S7. In contrast, although the binding sites of MBNL1 are more diffusely distributed and less abundant in regions flanking splice sites (Fig. 2c), MBNL1 binding close to the 3’ end of the downstream intron induces exon skipping (closed arrow in Fig. 2c and Supplementary Fig. S7b). The presence of a similar peak in dataset M2 (closed arrow in Supplementary Fig. S7c) further supports this observation.

We next analyzed the interaction between CUGBP1 and MBNL1 in splicing regulation. We made an RNA map of CUGBP1-binding sites in MBNL1-regulated exons from datasets M and M2 (Fig. 2d and Supplementary Fig. S7e), as well as an RNA map of MBNL1-binding sites in CUGBP1-regulated exons from dataset C (Fig. 2e and Supplementary Fig. S7f). Both RNA maps demonstrate the presence of CUGBP1 clusters in MBNL1-responsive exons and vice versa, which suggests that CUGBP1 and MBNL1 are likely to regulate alternative splicing of some of the same exons.

**MBNL1 and CUGBP1 both preferentially bind to the 3’ UTR.** MBNL1 has so far solely been categorized as an exon/intron-binding splicing regulatory protein, but to our surprise we found that the majority (55%) of MBNL1-binding regions are located in 3’ UTRs (Fig. 3a). The same pattern with preferential binding (53%) in 3’ UTRs is observed for CUGBP1, while only 2% of PTB binding regions are located in 3’ UTRs (Fig. 3a). Similarly, when HITS-CLIP tags are mapped to the size-normalized positions of all the genes in the mouse genome, CUGBP1 and MBNL1 CLIP tags, but not PTB CLIP-tags, are enriched close to the 3’ ends of genes (Fig. 3b). Additionally, 610 3’ UTRs, which constitutes 28.7% of the CUGBP1-tagged 3’ UTRs and 17.4% of the MBNL1-tagged 3’ UTRs, are shared

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**Figure 3 | Enrichment of CUGBP1 and MBNL1 CLIP-tags in the 3’ UTR.** (a) Distributions of CUGBP1, MBNL1, and PTB binding regions. Binding regions are mapped to CDS (coding sequence), 5’ and 3’ UTRs, introns, intergenic regions (incl. tRNA and rRNA genes), or antisense within genes according to the UCSC knownGene annotation of the NCBI Build 37.1 mouse genome (mm9). Pie-charts show ratios of binding regions mapped to the indicated regions. (b) Distributions of CUGBP1, MBNL1, and PTB CLIP-tags mapped to the relative positions of all the mouse genes. The relative positions of the genes are shown in percentages of the gene length in abscissa. The broken lines represent 15,638 genes with constitutive transcriptional start and end sites (Cons), and the solid lines represent 7,477 genes with alternative transcriptional start or end site (Alt). (c) Venn diagram of the numbers of genes with CUGBP1-, MBNL1-, and PTB-binding regions within the 3’ UTR. Binding regions were identified using the SeqMonk software.
between these two proteins (Fig. 3c). All these data document that both CUGBP1 and MBNL1 preferentially bind to 3' UTRs, indicating that this is a key function of both proteins in RNA processing. This suggests that the functional repertoire of MBNL1 should be expanded and that MBNL1, from being primarily regarded as regulator of alternative splicing, should also be considered as an important regulator of 3' UTR-mediated processes, such as mRNA stability/degradation.

MBNL1 destabilize mRNAs. To analyze the function of CUGBP1/MBNL1 binding to 3' UTRs, we made luciferase reporter constructs harboring CUGBP1/MBNL1-binding sites in the 3' UTR. Since no CLIP tags were observed in the 3' UTR of Gapdh (Supplementary Fig. S8), we made a luciferase-Gapdh 3' UTR expression vector, and then inserted 12 repeats of GT and 7 repeats of CTG immediately after the stop codon of luciferase to introduce a CUGBP1-binding site (GU rich motif) and an MBNL1-binding site (YGCY motif), respectively (Fig. 4a). We also inserted 12 AC repeats as a control. Due to the high expression level of CUGBP1 in C2C12 cells we used HEK293 cells for transient transfection of these reporter constructs along with CUGBP1/MBNL1 expression vectors. For the constructs with Gapdh 3' UTR alone or with AC repeats inserted, overexpression of CUGBP1 or MBNL1 had no effect on luciferase activity (Fig. 4b). For the GT repeat construct, overexpression of CUGBP1 decreased the luciferase activity, but MBNL1 had no effect. For the CTG repeat construct overexpression of MBNL1 dramatically decreased the luciferase activity, and also overexpression of CUGBP1 significantly reduced luciferase activity (Fig. 4b). In order to shed light on the mechanism underlying the effect of CUGBP1 or MBNL1 on decay, we studied the relationship between change in mRNA half-life and coverage of gene ontology (Fig. 3a). We found that genes displaying prolongation of half-lives in response to CUGBP1/MBNL1 knockdown harbors more CUGBP1-tags in their 3' UTRs, compared to those displaying shortening of half-lives (Fig. 5c). Similarly, genes that display prolongation of their half-lives in response to MBNL1 knockdown have more MBNL1-tags in their 3' UTRs (Fig. 5c).

Gene Ontology analysis of CUGBP1/MBNL1-bound 3' UTRs revealed that the terms 'cytoskeletal protein binding,' 'transcription factor binding' and 'RNA binding' are significantly overrepresented for CUGBP1- and MBNL1-bound genes (Table 1).

Figure 4 | Decay of luciferase mRNA by overexpression of CUGBP1/MBNL1. (a) Schemes of luciferase reporter plasmids harboring Gapdh 3' UTR. Each construct was made carrying either SV40 or tet-responsive promoter. (b) Luciferase activity after overexpression of CUGBP1/MBNL1. HEK293 cells were transfected with the indicated SV40-driven luciferase reporter constructs. Luciferase activity is normalized for the transfection efficiency using co-transfection of pRL/SV40. (c) Decay of luciferase mRNA after overexpression of CUGBP1/MBNL1. HEK293 cells were transfected with the indicated tet-responsive promoter-driven luciferase reporter constructs. Doxycycline was added to the medium to stop transcription at time 0. Temporal profiles of luciferase mRNA decay were quantified by real time RT-PCR and are normalized for Gapdh mRNA levels. All experiments were triplicated, and the mean and s.d. are indicated (* p < 0.05; ** p < 0.01).

Figure 4 | Decay of luciferase mRNA by overexpression of CUGBP1/MBNL1. (a) Schemes of luciferase reporter plasmids harboring Gapdh 3' UTR. Each construct was made carrying either SV40 or tet-responsive promoter. (b) Luciferase activity after overexpression of CUGBP1/MBNL1. HEK293 cells were transfected with the indicated SV40-driven luciferase reporter constructs. Luciferase activity is normalized for the transfection efficiency using co-transfection of pRL/SV40. (c) Decay of luciferase mRNA after overexpression of CUGBP1/MBNL1. HEK293 Tet-off cells were transfected with the indicated tet-responsive promoter-driven luciferase reporter constructs. Doxycycline was added to the medium to stop transcription at time 0. Temporal profiles of luciferase mRNA decay were quantified by real time RT-PCR and are normalized for Gapdh mRNA levels. All experiments were triplicated, and the mean and s.d. are indicated (* p < 0.05; ** p < 0.01).
Figure 5 | Global analysis of mRNA decay by expression array of C2C12 cells treated with CUGBP1/MBNL1 siRNA. (a) Half-lives of mRNAs in C2C12 cells with the indicated siRNAs. Red lines represent means and 95% confidence intervals. ** p < 0.01 and *** p < 0.001. (b) Real-time RT-PCR analysis of the stability of four representative endogenous mRNAs, which were detected by expression arrays. CLIP-tag distributions are shown above each gene structure. C2C12 cells were treated with either control (ctl), CUGBP1 (Cug), or MBNL1 (Mb) siRNA. Actinomycin D was added to the medium to stop transcription at time 0. Temporal profiles of decay of the indicated genes were analyzed by real-time RT-PCR and are normalized for Gapdh mRNA levels. All experiments were triplicated, and the mean and s.d. are indicated (* p < 0.05 and ** p < 0.01). (c) Tag counts in the 3' UTR of each gene are plotted in two categories of prolonging (up) and shortening (down) of half-lives after MBNL1 and CUGBP1 siRNAs. Red lines represent means and 95% confidence intervals. ** p < 0.01. Tag counts were normalized by the gene expression level at 0 h of cells treated with control siRNA.

Table 1 | The five most frequent Gene Ontology terms of mRNAs that are bound by CUGBP1 and MBNL1 to the 3' UTR

| CLIP data | GO ID    | Term                           | P Value  |
|-----------|----------|--------------------------------|----------|
| CUGBP1    | GO:0008092| cytoskeletal protein binding    | 1.58E-06 |
|           | GO:0003723| RNA binding                     | 1.40E-04 |
|           | GO:0008134| transcription factor binding     | 9.65E-04 |
|           | GO:0051082| unfolded protein binding         | 0.003184 |
|           | GO:0019904| protein domain specific binding  | 0.006603 |
| MBNL1     | GO:0008092| cytoskeletal protein binding     | 7.31E-20 |
|           | GO:0008134| transcription factor binding      | 2.20E-08 |
|           | GO:0003723| RNA binding                      | 0.001893 |
|           | GO:0019899| enzyme binding                   | 0.002046 |
|           | GO:0032553| ribonucleotide binding           | 0.004210 |

We utilized the mRNAs that have more than 8-fold coverage of CLIP tags in their 3' UTR for the analysis by DAVID53,54.
PITX2 is a homeobox transcription factor that regulates left-right asymmetric morphogenesis and it is also deeply implicated in myogenesis during mouse embryonic development. We found that the decay of Pitx2 mRNA is prolonged by knocking down MBNL1, but not CUGBP1 in undifferentiated C2C12 cells (Fig. 6b and c). This is consistent with the fact that Pitx2 harbors a much higher number of MBNL1-CLIP tags than that of CUGBP1-CLIP tags in the 3′ UTR (Fig. 6a). We also observed that down regulation of both CUGBP1 and MBNL1 decreases the decay of Myod1 and Mbnl2 mRNA, but not that of Gapdh mRNA (Supplementary Fig. S8). Similarly, down regulation of CUGBP1 decreases the decay of other myogenic transcription factors such as Myog and Mef2a mRNAs, and also of Cugbp2 (Supplementary Fig. S9). Furthermore, knockdown of CUGBP1 and MBNL1 prolongs decay of Mbnl1 and Cugbp1 mRNAs, respectively, suggesting a mechanism for cross-regulation of expression of MBNL1, CUGBP1, and their family proteins (Supplementary Fig. S8).

To analyze more directly the role of MBNL1 binding to the 3′ UTR in regulation of mRNA decay, we examined the mRNA stability of firefly luciferase fused with the 3′ UTR of Pitx2 (Fig. 6a). There are 11 YGCCY motifs in the 3′ UTR of Pitx2, and 4 of the 11 motifs have MBNL1-CLIP tags. We introduced artificial mutations in these 4 motifs to prevent binding of MBNL1 (Fig. 6a). Consistent with the proposed role for MBNL1 in mRNA decay, we observe that disruption of the MBNL1-binding motifs in the Pitx2-3′ UTR abolished responsiveness to MBNL1 knockdown (Fig. 6d). Furthermore, immunoblots demonstrated that MBNL1 knockdown enhanced expression of endogenous PITX2 in C2C12 cells (Fig. 6e). These data suggest that MBNL1 promotes decay of Pitx2 mRNA and thereby represses expression of the PITX2 protein.

Taken together, all of our data are consistent with a model where CUGBP1 and MBNL1 facilitate mRNA decay through binding to the 3′ UTR of target genes.

**Discussion**

CUGBP1 and MBNL1 are developmentally regulated RNA-binding proteins that are causally associated with myotonic dystrophy type 1. In this study, we show that both CUGBP1 and MBNL1 preferentially bind to 3′ UTRs and destabilize the bound mRNAs. In particular, we show that CUGBP1 and MBNL1 destabilize myogenic differentiation factors and RNA-binding proteins. In addition, our results confirm and significantly expand the current knowledge of the splicing-regulatory effects of CUGBP1 and MBNL1. Taken together, the data from the present study indicates that CUGBP1 and MBNL1 are closely related and cross regulate alternative splicing and mRNA decay.

MBNL1 binding to 3′ UTRs has not been previously reported. We show for the first time that MBNL1 binds to 3′ UTRs and promotes mRNA decay in both artificial constructs and in endogenous genes. We also demonstrate by expression arrays that both CUGBP1 and MBNL1 facilitate mRNA decay by binding to 3′ UTRs. The present study demonstrates global in vivo interactions between CUGBP1 and 3′ UTRs and reveals that CUGBP1 also preferentially binds to 3′ UTR rather than exons/introns. We provide in vivo evidence that CUGBP1 facilitates mRNA decay of a broad spectrum of genes in addition to the previously reported genes.

Interestingly, we find that MBNL1 promotes decay of Cugbp1 mRNA and that CUGBP1 promotes decay of Mbnl1 mRNA, and that this is associated with corresponding changes at the protein level during differentiation of C2C12 cells (Supplementary Fig. S4b). This may suggest that expression of CUGBP1 and MBNL1 are mutually regulated in myogenic differentiation. Kuyumcu-Martinez and colleagues report that expanded CUG repeats of DMPK through an unknown mechanism leads to phosphorylation and thereby to stabilization of CUGBP1 in DM1 myoblasts. Our studies additionally suggest that loss of MBNL1 in DM1 could lead to decreased decay of CUGBP1 mRNA and hence to further increase of CUGBP1 activity. Although CUGBP1 is not upregulated in adult MBNL1-knockout mice, this mechanism could lead to increased misregulation of splicing and decay of the mRNAs of target genes in embryonic development that culminates in the DM1 phenotype.

We find that binding sites for CUGBP1 and MBNL1 are enriched around alternative cassette exons (Fig. 2a). The binding sites for CUGBP1 are prominent in adjacent intronic regions flanking alternative exons. Our functional analysis reveals that binding of CUGBP1 to the upstream intron facilitates exon skipping, whereas...
binding to the downstream intron enhances exon inclusion (Fig. 2b). Interestingly, similar regulation of alternative splicing has been observed for NOVA, FOXP2 and PTB20,29,46, indicating the presence of a common underlying mechanism shared by these proteins.

In contrast to CUGBP1, MBNL1 tags are also enriched in coding exons. Until now, splicing cis-elements of MBNL1 have been mapped exclusively to introns, and no exonic cis-element has been reported to our knowledge23,29,37,46. Although MBNL1 preferentially binds to exons, MBNL1 binding to introns is enriched at alternative rather than constitutive splice sites (Fig. 2a). This enrichment is diffusely distributed throughout regions harboring 500 nt upstream or downstream of alternative exons, in contrast to the prominent intronic peaks observed for CUGBP1 tags. This could suggest that MBNL1 needs to bind simultaneously to the target exon and adjacent introns to regulate splicing. Functional analysis of MBNL1 reveals that binding of MBNL1 close to the 3′ end of the downstream intron facilitates exon skipping, whereas no characteristic binding pattern is observed for exons included in response to MBNL1 (Fig. 2c). PTB has also been reported to regulate alternative splicing through binding close to the 3′ end of the downstream intron26. In contrast to MBNL1, however, binding of PTB to this region promotes exon inclusion. We similarly find binding of PTB to this region in our HTS-CLIP data in MBNL1-regulated exons (Supplementary Fig. S7d). Interestingly, the MBNL1-binding motif is enriched in PTB-regulated exons29. MBNL1 may thus compete for binding with other splicing factors like PTB and regulate alternative splicing events.

Post-transcriptional gene expression regulation is crucial to achieve precise developmental and tissue-specific control of cellular processes. Our studies reveal that CUGBP1 and MBNL1 preferentially bind to the 3′ UTRs of mRNAs encoding RNA-binding proteins and transcription factors, which can regulate cell development. During development of murine skeletal muscles, the nuclear level of MBNL1 increases, while that of CUGBP1 decreases41,29. Genes with mRNAs that can be bound both by CUGBP1 and MBNL1 are likely to be down-regulated by CUGBP1 in undifferentiated cells. If these genes need to be tightly down-regulated also in differentiated cells, MBNL1 can substitute for CUGBP1 in order to achieve continued destabilization of the target mRNA. We conclude that finely-tuned expression of CUGBP1 and MBNL1 may be important regulators of myogenic differentiation through precise regulation of both alternative splicing and mRNA stability.

Methods

Antibodies. Antibodies to CUGBP1 (3B1), MHC (H300), myogenin (M225) and PTB (N20) were purchased from Santa Cruz Biotechnology. Anti-GAPDH pAb was purchased from Sigma. Anti-PITX2 pAb was purchased from Abcam. Anti-MBNL1 rabbit serum (A2764) was a kind gift of Dr. Charles A. Thornton at University of Rochester. The specificity of antibodies against CUG BP1 and MBNL1 is supported by the data in previous reports 23 and also by our siRNA experiments (Supplementary Fig. S1).

Cell culture. Detailed methods are included in the Supplementary Information.

HTS-CLIP. C2C12 cells were UV-irradiated at 400 mJ and CLIP was performed as previously described46. High-throughput 36-bp single-end and 40-bp single-end sequencing was performed using an Illumina Genome Analyzer II. All HTS-CLIP data were registered in ArrayExpress with an accession number E-MTAB-414 and in ERA with an accession number ERP006789. Detailed information is provided in the Supplementary Information.

Bioinformatics analysis. Illumina reads were first prepared by removing the 4-bp tag and filtering sequences composed primarily of Illumina adapter. The resulting reads were mapped to the mouse genome (NCBI Build 37.1/mm9) with default parameters using the BWA20 mapping software. To extract consensus motifs from the mapped reads, we considered only uniquely aligned reads and first removed duplicate reads to avoid potential PCR-mediated deviations in addition to bias from very highly expressed transcripts. We then extended the reads to 110 nt, the expected mean of the CLIP fragments and used the SeqMonk software (www.bioinformatics.bbsrc.ac.uk/projects/sequonk) to identify binding regions by using the program’s built-in peak detection algorithm. Peaks were scored using both a reads per peak scoring scheme and a maximum depth scoring scheme (effectively the height of the peak) in order to filter out peaks. For the identification of CUGBP1- and MBNL1-binding regions, we used PTB as a negative control and removed peaks present in the PTB dataset as well. We then selected CUGBP1 peaks that were present in the two independent CUGBP1 CLIP experiments and MBNL1 peaks that were similarly corroborated by the two MBNL1 experiments. PTB binding regions were identified by removing peaks that were present in either of the four CUGBP1 and MBNL1 experiments. Finally, we restricted the set of binding regions to only those spanning 70–150 bp since this was the fragment length used in the CLIP experiments. We analyzed each dataset using a motif analysis tool, MEME41, using a background Markov model based on the entire mouse genome.

We analyzed the mapped illumina reads and binding regions and mapped them to UCSC knownGene annotations42 of the mouse genome (NCBI Build 37.1/mm9) by running and writing Perl and Excel VBA programs, as well as by running BEDTools utilities43. Normalized complexity maps of CUGBP1/MBNL1/PTB RNA interactions were generated as previously described44. For the control, normalized complexity map was similarly generated by analyzing 100 sets of 15 to 50 constitutive exons that were randomly selected from 118,969 constitutive exons in mm9. To identify enriched Gene Ontology terms, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.745,46.

Construction of plasmids. To construct luciferase reporter vectors with the 3′ UTR of Gapdh and Pitx2, 3′ UTRs of these genes were amplified by PCR. Amplified DNA was ligated into the XbaI and BamHI sites of the pGL3-promoter vector (Promega) to substitute for the 3′ UTR of the luciferase gene. DNA fragments harboring GT and CTG repeats were amplified by self-priming PCR using primers terminating in an XbaI site, and ligated into the XbaI site to make the pGL3-Gapdh-3′ UTR. To construct tet-responsive luciferase constructs, the tet-responsive promoter region was excised from pTRE-tet vector (Clontech) to make a 1,500-bp site and cloned into the Xhol-HindIII site of the pGL3-promoter vector with the 3′ UTR of Gapdh and Pitx2. To introduce mutations in 3′ UTR of Pitx2 in the luciferase construct, we used the QuikChange site-directed mutagenesis kit (Stratagene).

To construct expression vectors for MBNL1 and CUGBP1, the human MBNL1 cDNA and human CUGBP1 cDNA (Open Biosystems) were subcloned into the mammalian bidirectional expression vector pBI-CMV2 (Clontech), which should constitutionally express the insert and AcGFP1.

RNA interference and transfection. The siRNA duplexes against CUGBP1 and MBNL1 were synthesized by Sigma. The sense sequences of the siRNAs were as follows: Cugbp1-1, 5′-GGCUU-UGGGUAUUGAGUAUUA-3′; Cugbp1-2, 5′-GCUU-AAAGUGCAAGCUCUAAA-3′; Mbnl1-1, 5′-CACUGAAGGAAUUGAGAA-3′; and Mbnl1-2, 5′-GCACAAUGAUGAAUUAC-3′. We purchased the AllStar Negative Control siRNA (1027281) from Qiagen. C2C12 cells were seeded on 24-well plates, and transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Tet-off advanced HEK293 cells were seeded on 96-well plates, and were transfected with luciferase reporter gene constructs using FuGENE 6 (Roche) according to the manufacturer’s instructions. At 48 hrs after transfection, cells were either harvested for RNA extraction or processed for isolation of total proteins or nuclear extracts.

RT-PCR for splicing analysis. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized using an oligo-dT primer and ReverTra Ace (Toyobo), and PCR amplifications were performed using GoTaq (Promega) for 30–35 cycles. Sequences of the primers used for PCR are listed in Supplementary Table S4. The intensities of PCR-amplified bands were quantified with the ImageJ 1.42q software (NIH). We then calculated a percentage of exon inclusion (% inclusion) as the ratio of the intensity of the upper band divided by the sum of intensities of all the bands.

Real-time RT-PCR for RNA stability analysis. Total RNA was extracted using RNeasy mini kit (Qiagen) or CellAmp Direct RNA Prep Kit (Takara) according to the manufacturer’s instructions. cDNA was synthesized as described above and real-time PCR was performed using the Mx3005P QPCR System (Stratagene) and the SYBR Premix Ex Taq II (Takara). Sequences of the primers used for PCR are listed in Supplementary Table S4.

Microarray analysis. Total RNA was extracted using the RNeasy mini kit according to the manufacturer’s instructions. We synthesized and labeled cDNA fragments from 100 ng of total RNA using the GeneChip WT cDNA Synthesis Kit (Ambion). The labeled cDNAs were hybridized to the Affymetrix Mouse Exon 1.0 ST Arrays for splicing analysis or the Affymetrix Mouse Gene 1.0 ST Arrays for analyzing temporal profiles of expression of CUGBP1/MBNL1-targeted genes following the manufacturer’s protocols. The robust multichip analysis (RMA) algorithm was used to normalize the array signals across chips with the Affymetrix Expression Console software 1.1.2. All microarray data were uploaded to the Gene Expression Omnibus database (accession numbers, GSE29990 on exon arrays and GSE27583 for expression arrays).

Western blotting. For preparation of total cell lysates, cells were lysed in buffer A (10 mM HEPES pH 7.8, 1 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2 μg/ml Aprotinin, 0.5% NP-40) and incubate on ice for 20 min. After sonication, samples were centrifuged (15,000 rpm, 5 min) and the supernatants were stored at −80°C for further experiments. For preparation of nuclear cell lysates, cells were suspended in 400 μl of buffer A. Nuclei were pelleted, and the cytoplasmic
proteins were carefully removed. The nuclei were then resuspended in buffer C (50 mM HEPES pH 7.8, 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 2% Glycerol, 1 mM DTT, 2 µg/ml Aprotinin, and 0.5 mM PMSF). After vortexing and stirring for 20 min at 4°C, the samples were centrifuged, and the supernatants were stored at −80°C. Samples were analyzed on a 10% SDS polyacrylamide gel, and the proteins were transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 1% BSA in Tris-buffered saline containing 0.05% Tween20 (TBST) for 1 hr, incubated for 1 hr with primary antibodies in TBST, washed three times with TBST, and incubated for 1 hr with horseradish peroxidase-conjugated anti-mouse or -rabbit immunoglobulin (GE) diluted 1: 5,000 in TBST. After three washes in TBST, the blot was developed with the enhanced chemiluminescence system (GE) according to the manufacturer’s instructions.

Luciferase assay. HEK293 cells seeded on a 96 well plate were transfected with 10 ng of GGL3-Gapdh-3' UTR with or without GT and CTG repeats, 5 ng of pRL/SV40 (Promega), and 40 ng of pBII-CMV2-based CUGBP1 or MBNL1 expression vector using FuGENE 6. At 48 hrs after the transfection, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

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Author contributions
A.M., H.S.A, T.O., and M.I. performed the experiments. A.M., T.K.D., B.S.A., and K.O. analyzed the data. A.M., T.K.D., B.S.A. and K.O. prepared the manuscript. All authors reviewed the manuscript.
Additional information

Accession codes: All HITS-CLIP data were registered in ArrayExpress with an accession number E-MTAB-414 and in ENA with an accession number ERP000789.

All microarray data were uploaded to the Gene Expression Omnibus database with accession numbers, GSE39990 for exon arrays and GSE27583 for expression arrays.

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