Poly(C)-binding protein 1 (Pcbp1) regulates skeletal muscle differentiation by modulating microRNA processing in myoblasts

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Regulation of gene expression during muscle development and disease remains incompletely understood. microRNAs are a class of small non-coding RNAs that regulate gene expression and function post-transcriptionally. The poly(C)-binding protein 1 (Pcbp1, hnRNP-E1, or eCP-1) is an RNA-binding protein that has been reported to bind the 3′-UTRs of target genes to regulate mRNA stability and protein translation. However, Pcbp1’s biological function and the general mechanism of action remain largely undetermined. Here, we report that Pcbp1 is a component of the miRNA-processing pathway that regulates miRNA biogenesis. siRNA-based inhibition of Pcbp1 in mouse skeletal muscle myoblasts led to dysregulated cellular proliferation and differentiation. We also found that Pcbp1 null mutant mice exhibit early embryonic lethality, indicating that Pcbp1 is indispensable for embryonic development. Interestingly, hypomorphic Pcbp1 mutant mice displayed defects in muscle growth due to defects in the proliferation and differentiation of myoblasts and muscle satellite cells, in addition to a slow to fast myofibril switch. Moreover, Pcbp1 modulated the processing of muscle-enriched miR-1, miR-133, and miR-206 by physically interacting with argonaute 2 (AGO2) and other miRNA pathway components. Our study, therefore, uncovers the important function of Pcbp1 in skeletal muscle and the microRNA pathway, signifying its potential as a therapeutic target for muscle disease.

Control of muscle cell proliferation and differentiation is essential to the process of muscle development, function, and regeneration. A variety of transcriptional and epigenetic regulators have been demonstrated key to this process (1, 2). Recent studies have linked miRNAs5 to muscle gene expression, muscle development, and disease (3–6). Muscle expressed miRNAs, miR-1, miR-133, and miR-206 in particular, have been demonstrated to play critical roles in myocyte proliferation and differentiation (4, 6). Most importantly, the expression and function of many miRNAs have been associated with muscle diseases (3). However, little is known about whether the miRNA biogenesis pathway, which is involved in the processing and maturation of miRNAs, also participate in the regulation of muscle gene expression and muscle function.

Ribo nucleoprotein (RNPs) particles are large heteromeric protein complexes bound to the new, actively transcribed RNA. RNA-binding particles are composed of several “core” and “minor” proteins termed heterogeneous nuclear ribonucleoproteins (hnRNPs) (7–9). However, protein abundance within the RNP complex does not imply hierarchy of function as exemplified in the latest classifications of RNA-binding proteins (9–12). hnRNPs comprise an extensive family of RNA-binding proteins (RBPs) characterized and classified by RNA-binding domain sequence and structure homology (8, 10, 11). The KH-RNA-binding domain family of RBPs is a component of the poly(C)-binding proteins (Pcbp1–4, also known as eCP1–4 or hnRNP-E1–4) along with hnRNP-K/J and is characterized by the evolutionary conservation of multiples copies of the ~70-amino acid KH RNA binding domain (8, 13–15). Pcbp1 is a gene that lacks introns, and its miRNA is highly homologous with that of Pcbp2. It has been rationalized that Pcbp1 originated from a Pcbp2 mRNA transposition event, a further mutation, and eventual positive selection (14, 16). Pcbp1 has been identified as a multilevel regulator of gene expression and protein function, acting as: 1) transcription factor, i.e. regulating expression of elf4eE or by activating the internal ribosome entry sites of certain genes, such as Bag-1 (17, 18); 2) translation factor, enhancing or suppressing protein expression by its specific binding to poly(C) regions occurring preferentially within the 3′-UTR of target mRNAs or by its interaction with second...

5 The abbreviations used are: miRNA, microRNA; Pcbp1, poly(C)-binding protein 1; siPcbp1, siRNA-Pcbp1; RNP, ribonucleoprotein particle; hnRNP, heterogeneous nuclear ribonucleoprotein; RBP, RNA-binding protein; KH, KH homology; AGO2, argonaute 2; co-IP, co-immunoprecipitation; MHC, myosin heavy chain; qPCR, quantitative PCR; EDL, extensor digitorum longus; ES, embryonic stem; TA, tibialis anterior; DHSB, developmental Studies Hybridoma Bank; RISC, RNA-induced silencing complex.
ary structures in the 5′-UTR or 3′-UTR of the target mRNAs (BAT and DICE regions) (8, 13, 19–23); 3) post-transcriptional regulator, as a modulator of alternative splicing to direct differential gene expression (24, 25); 4) molecular chaperone and stress responder by interacting with other protein partners to induce or inhibit certain cell functions such as iron chelation and hypoxia-response blockade through inhibition of hypoxia-induced factors (HIFs) expression (26).

Pcbp1 is ubiquitously expressed (15, 16); however, Pcbp1 protein expression has been recently identified to be enriched in certain specific organ niches. Indeed, Pcbp1 protein is expressed specifically in chief cells of the gastric mucosa, suggesting that its expression and function are cell context-dependent and related to growth, apoptosis, and proliferation or differentiation (27, 28). Moreover, Pcbp1 expression has been reported to be enhanced in many cancers including prostate cancer and colorectal cancer. Pcbp1 may have a function in epithelial-mesenchymal transformation and metastases, as reports have indicated it acts as a checkpoint by modulating protein translation of the PRL-3 phosphatase mRNA and, thus, activation of AKT (23). Despite the recent reports and accumulation of information on Pcbp1 functions, the overall molecular mechanisms of Pcbp1 function are still not clear. Here, we report a novel function of Pcbp1 and its involvement in the miRNA maturation process that results in the modulation of skeletal muscle proliferation, development, and differentiation.

Results

Pcbp1 interacts with components of the miRNA pathway

We initially identified Pcbp1 from a screen of potential myocardin-interacting proteins in a yeast two-hybrid assay using myocardin as bait (29, 30). However, surprisingly, Pcbp1 and myocardin did not appear to directly interact, although we have observed that Pcbp1 was capable of modulating the transcriptional activity of myocardin with an unknown mechanism. Next, we applied an unbiased approach to identify Pcbp1-interacting proteins. We performed immunoprecipitation and mass spectrometry using specific antibodies against Pcbp1 protein in heart and skeletal muscle tissues of different stages of development (postnatal day 1 and 2-month-old adults). Mass spectrometry results showed that Pcbp1 interacts with argonaute 2 (AGO2), the core member of the RNA-induced silencing complex (RISC) and miRNA-processing machinery (31). This result is in accordance with previous reports that indicated that Pcbp1 and AGO2 localize to the P-body (32, 33). Given that Pcbp1 is a RNA-binding protein known to bind to 3′-UTRs of its target mRNAs, we hypothesized that Pcbp1 might interact with the machinery of the miRNA biogenesis and/or target inhibition, which also associate with the 3′-UTRs of mRNAs targeted by miRNAs.

To confirm the interaction of Pcbp1 with AGO2 and other members of the miRNA pathway, we performed co-immunoprecipitation (co-IP) assays using FLAG-tagged Pcbp1 and MYC-tagged candidate proteins. We included the miRNA microprocessor (Dgcr8), the endoribonuclease Dicer, and the RNA-binding protein Trbp as well as members of the RISC machinery (AGO1 and others). Indeed, the co-IP experiments confirmed the interaction between Pcbp1 and AGO2 (Fig. 1). Interestingly, we also found that Pcbp1 interacted preferentially with Trbp, a Dicer co-factor and key modulator of heart development and function (34, 35). Pcbp1 also interacted with AGO1 and Ddx3x (DEAD box helicase 3, X-linked) (36). However, Pcbp1 did not appear to directly interact with Dgcr8 or Dicer (Fig. 1, A and B). Next, we asked whether the interaction of Pcbp1 and its partner proteins is RNA-dependent. We treated the cell extracts with RNase A and H before immunoprecipitation and found that depletion of RNAs did not abolish the interaction between Pcbp1 and AGO2, Ddx3x, and Trbp (Fig. 1, C and D), indicating that these protein-protein interactions are RNA-independent.

Pcbp1 is dynamically expressed in skeletal muscle

Previous studies have reported that Pcbp1 mRNA is ubiquitously expressed, yet enriched in cardiac and skeletal muscle (15). We examined Pcbp1 protein expression in mouse skeletal muscle at several stages of development including embryonic (E16.5), postnatal (P0-P7), juvenile (P10-P21), and 2 months old (2m) using Western blot analysis. We found that Pcbp1 protein is dynamically expressed in the skeletal muscle with higher expression levels in embryonic and immature stages of development, whereas its expression is lower in adult muscle (Fig. 2A). Interestingly, we observed that the anti-Pcbp1 antibodies also detected a second, slightly higher molecular weight band that shows increased expression pattern according to age. This could be an isoform of the Pcbp1 protein or a post-translationally modified Pcbp1 protein.

We next examined Pcbp1 protein expression in the mouse skeletal myoblast cell line C2C12. We either maintained the C2C12 cells in growth medium to keep them as undifferentiated myoblasts or induced them to form multinucleated myotubes under differentiation conditions (6). Unexpectedly, we found that the expression levels of Pcbp1 proteins were not drastically altered during myoblast differentiation (Fig. 2B). As a positive control, the expression of MyHC, a muscle terminal differentiation marker, was substantially induced when C2C12 cells were differentiated into myotubes (Fig. 2B).

Inhibition of Pcbp1 enhanced skeletal muscle myoblast differentiation

Next, we investigated the role of Pcbp1 during myoblast differentiation. C2C12 myoblasts were seeded at similar confluence, transfected with siRNA for Pcbp1 (siPcbp1) or control siRNA (siCNTRL). We used two independent siRNAs to knock down Pcbp1. We confirmed that each siRNA or the combination of both siRNAs substantially knocked down the expression of endogenous Pcbp1 (Fig. 3A). Substantial inhibition of endogenous Pcbp1 could be observed even 4 days after siRNA transfection (Fig. 3B). After transfection, C2C12 myoblasts were cultured for 24 h in growth medium; subsequently, they were switched into differentiation medium for 1, 3, and 5 days, respectively. Whereas control myoblasts differentiated into rod-shaped myotubes upon differentiation stimulation, knock-down of Pcbp1 further enhanced the differentiation process (Fig. 3C). Immunostaining using the MF20 antibody, which recognizes myosin heavy chain (MHC), showed a substantial...
increase of MHC positive multinuclei myotubes (Fig. 3D), supporting the view that inhibition of Pcbp1 enhances myogenic differentiation. Together, these results suggest that Pcbp1 inhibits the skeletal muscle differentiation process in vitro.

Pcbp1 is required for animal development

To define the in vivo function of Pcbp1 in skeletal muscle, we generated Pcbp1 mutant mice. The whole coding sequence of the Pcbp1 genomic locus was flanked by the loxP sequences to generate the Pcbp1 floxed allele \( Pcbp1^{f/f} \) (Fig. 4A; “Experimental Procedures”). PCR assays and Southern blot analyses were applied to confirm correct gene targeting events (Fig. 4, B and C). Heterozygous mice (\( Pcbp1^{f/+} \)), which are phenotypically normal, were intercrossed to obtain homozygous Pcbp1 floxed allele (\( Pcbp1^{f/f} \)) mice. Unexpectedly, genotyping of progeny at birth and 4 weeks postnatally from the intercrossing of \( Pcbp1^{f/+} \) mice showed that \( Pcbp1^{f/f} \) mice were underrepresented, indicating premature loss of \( Pcbp1^{f/f} \) mice (Table 1). This was a surprising result because these \( Pcbp1^{f/f} \) mice were not crossed with a Cre mouse line to delete the Pcbp1 genomic sequences. These results suggest that non-deleted Pcbp1 homozygous floxed mice are hypomorphic.

The \( Pcbp1^{f/f} \) mice are smaller in size at birth (Fig. 4D), and this difference is maintained throughout adulthood (Fig. 4E). The body weight of \( Pcbp1^{f/f} \) homozygous mice was measured and showed \( \sim 20–30\% \) reduction when compared with their wild type (WT) littermate controls (Fig. 4F). We dissected the tibialis anterior (TA), gastrocnemius (Gast) and quadriceps (Quad) muscles from \( Pcbp1^{f/f} \) mice and compared them to those of their WT littermate controls and found that skeletal muscles are smaller in \( Pcbp1^{f/f} \) mice (Fig. 4G). Weight measurements further confirmed the above observation. However, the TA weight to tibia length ratio is unchanged, indicating that
this growth deficiency is proportional to the whole body and not specific to the muscle groups (Fig. 4H).

Despite their smaller size, Pcbp1floxed mice are viable, albeit with a lower fertility rate. The smaller size and lower weight of Pcbp1floxed led us to investigate if the expression of the Pcbp1 gene was affected by the targeting strategy. Indeed, Quantitative PCR (qPCR) and Western blot results show that the expression of the Pcbp1 gene is reduced up to 85% in Pcbp1floxed mice (Fig. 4, I and J). These results indicate that the targeting strategy (flanking the Pcbp1 locus with the loxP sequences) has impaired expression of the Pcbp1 gene and that down-regulation of Pcbp1 affects the development of skeletal muscle and whole body growth in general. Consistent with our observations, a recent report showed that homozygote Pcbp1 mutation leads to embryonic lethality, whereas heterozygote mice exhibit haploinsufficiency; these mice were also smaller in size (37). These results, therefore, suggest that the Pcbp1floxed allele is hypomorphic, leading to the reduced expression of the Pcbp1 gene.

**Pcbp1 is required for the growth of skeletal muscle and the development of slow-twitch myofibers**

The embryonic lethality of Pcbp1 null mice prevented us from investigating the function of this gene in postnatal and adult mice. Given that the floxed allele of Pcbp1 (Pcbp1floxed) reduced the expression of Pcbp1 protein and resulted in smaller animals, we decided to study putative skeletal muscle defects in these hypomorphic Pcbp1floxed mice. We performed histological sections on tibialis anterior (TA), gastrocnemius (GAS), and quadriceps (Quad) muscles, and our results revealed that transverse section fiber size in Pcbp1floxed skeletal muscles is skewed toward a smaller type. Fibers per cross-section are more numerous, and the distribution of fiber size shifts to the accumulation of smaller fibers compared with that of WT muscles (Fig. 5, A and B).

Skeletal muscle myocyte physiological requirements are strongly related to the metabolic state of the muscle fibers. Thus, higher metabolic state correlates with a higher speed of contraction (fast twitch) and vice versa (slow twitch). We, therefore, asked whether the fiber type composition was changed in Pcbp1floxed skeletal muscles. We performed immunohistochemical analysis for slow (anti-MHCα and -β) and fast-twitch (anti-MHC-IIb) myofiber types in Pcbp1floxed and control skeletal muscles. We observed a decrease in the slow-twitch myofiber type and an induction in the fast-twitch myofiber type in the soleus and TA muscle bundles of Pcbp1floxed mice when compared with WT controls (Fig. 5, C and D). These results indicate that Pcbp1 regulates muscle fiber formation and consequently induces a slow- to fast-twitch myofiber shift in the affected muscle.
Role of Pcbp1 in skeletal muscle

A

WT allele

Pcbp1 targeting vector

Pcbp1 allele

B

5’

3’

5’-LoxP

C

M

10Kbp

8Kbp

6Kbp

5Kbp

D

P1

Pcbp+/−

+/+

E

1m

Pcbp−/−

+/+

F

Body weight (P1)

Body weight (2m)

+/+

f/f

G

Quad

TA

Gast/Sol

WT

Pcbp+/−

H

Quadriceps weight (g)

Tibialis Anterior weight (g)

Gastrocnemius weight (g)

N=7

N=6

N=7

N=6

N=7

N=6

I

TA

Gas/Sol

WT

Pcbp+/−

J

TA

Gas/Sol

WT

Pcbp+/−
neonatal satellite cells, we isolated satellite cells from skeletal muscles of mice, cultured, and fixed. Myofiber-associated satellite cells were visualized by immunostaining for Pax7, a satellite cell marker.

Inhibition of Pcbp1 reduced numbers of satellite cells and enhanced the premature differentiation of primary myoblasts

Satellite cells, the skeletal muscle stem cells, are responsible for muscle regeneration and normal postnatal muscle growth. We asked whether Pcbp1 directly regulates the proliferation and differentiation of satellite cells. Extensor digitorum longus (EDL) muscle fibers were dissociated from Pcbp1f/f and control mice, cultured, and fixed. Myofiber-associated satellite cells were visualized by immunostaining for Pax7, a satellite cell marker. Pcbp1f/f myofibers overall showed a reduced number of Pax7+ cells per fiber. Quantification revealed a reduction of total numbers of nuclei per myofiber in Pcbp1f/f muscle compared with that of controls (Fig. 6A). Further analysis indicates that the ratio of Pax7+ cells per total number of nuclei per myofiber is reduced by ~25% in Pcbp1f/f mice when compared with WT controls (Fig. 6B).

To confirm the above observation and better understand how Pcbp1 regulates the proliferation and differentiation of satellite cells, we isolated satellite cells from skeletal muscles of neonatal Pcbp1f/f or control WT mice and cultured them in vitro. We found that reduction of Pcbp1 in isolated Pcbp1f/f satellite cells, often referred to as activated primary myoblasts, substantially inhibits cell proliferation as measured by direct cell counting (Fig. 6C). In contrast, satellite cells isolated from Pcbp1f/f mice differentiate into multinuclear myofibers much more robustly when compared with those of wild type controls, suggesting that Pcbp1 represses myoblast differentiation (Fig. 6D). These data are consistent with the results obtained from C2C12 myoblasts where knockdown of Pcbp1 by siPcbp1 resulted in an enhanced myoblast differentiation. Taken together, our results suggest that Pcbp1 regulates the proliferation and differentiation of skeletal muscle satellite cells.

Pcbp1 modulates miRNA maturation in skeletal muscle

We hypothesize that Pcbp1 regulates myoblast proliferation and differentiation by modulating the maturation process of miRNAs. To test this hypothesis, we knocked down Pcbp1 in C2C12 cells with Pcbp1 siRNAs and examined the expression of muscle-enriched miRNAs. Northern blotting demonstrates that the expression levels for mature miR-1, miR-133, and miR-206, known for their roles in skeletal muscle proliferation and differentiation program, are significantly down-regulated when the endogenous Pcbp1 level was inhibited (Fig. 7A). Quantification of the changes of miRNA expression indicated that the decreased expression of these miRNAs was statistically significant (Fig. 7B). Finally, we examined the expression of these miRNAs in skeletal muscles of Pcbp1f/f and control mice. Consistent with the results of C2C12 myoblasts, we found that the expression of these miRNAs decreased in skeletal muscle of Pcbp1f/f mice (Fig. 7C). These results indicate that Pcbp1 is required for the biogenesis of miRNAs in skeletal muscles.

Discussion

In this study we reported that the RNA-binding protein Pcbp1 plays an important role in myoblast proliferation, differentiation, and myofiber type specification. We showed that inhibition of Pcbp1 affects myoblast differentiation in vitro. Reduction of Pcbp1 expression by genetic alteration of the Pcbp1 locus leads to muscle defects, apparently resulted from precocious myocyte differentiation and reduction of their proliferation. Mechanistically, our results link the function of Pcbp1 to miRNA processing; Pcbp1 physically interacts with AGO2 and its associated proteins. Inhibition of Pcbp1 results in reduction of muscle miRNAs, suggesting that these miRNAs may mediate Pcbp1 function in skeletal muscle.

Interestingly, we observed that reduced Pcbp1 expression in mice resulted in a switch of fast- to slow-twitch myofibers. Skeletal muscle contractility is closely correlated with fast-twitch and slow-twitch myofibrils (38). Several important molecular pathways, such as the AMP-activated protein kinase (AMPK), the peroxisome proliferator-activated receptor γ co-activator 1-α (PGC-1α), and the calcineurin and protein kinase C, have been implicated in the regulation of myofiber switch (39–45). Additionally, the function of the transcription factor Sox6 has been linked to the regulation of fast- and slow-myofiber switch (46). We speculate some of these above regulators are miRNA targets in a manner that genetic mutation of Pcbp1 results in alteration of miRNA levels, leading to the change of these target genes.

Pcbp1 (and Pcbp2) was identified as a member(s) of the P-body mRNA degradation complex, which comprises other multifunctional protein molecules, such as AGO2, the core member of the RISC (33, 47). These analyses suggest that Pcbp1 could be involved in the processing and function of miRNAs. However, thus far there was no direct evidence to link the function of this protein to the miRNA pathway. Our study demonstrated that Pcbp1 physically interacts with components of the miRNA-processing machinery to modulate the expression/process- ing of muscle miRNAs. However, it remains unclear about how Pcbp1 specifically regulates the processing of a subset of...
miRNAs. Additional experiment evidence, such as miRNA-de-
pendent rescue, is needed to establish that the function of
Pcbp1 is mediated by miRNAs, not other targets, in skeletal
muscle. By using a loss-of-function approach, our genetic stud-
ies demonstrated that Pcbp1 plays a key role in the regulation of
muscle gene expression, skeletal muscle differentiation, and
muscle stem cell-mediated muscle regeneration. Importantly,
Pcbp1 was reported as an RNA-binding protein, which could
also target the poly(C) regions in the 3'-UTR region of several
genes including p21, the human androgen receptor, and the
human α-globin among others (19, 21, 48, 49). Clearly, it
remains to be determined if these Pcbp1 targets contribute to
its function in regulating skeletal muscle. Given Pcbp1 is highly
conserved in human, it will be important to define whether
human Pcbp1 gene is involved in muscular dystrophy or muscle
degeneration related muscle disorders.

**Experimental procedures**

**Cell culture, siRNA transfection, and viral infection**

The mouse myoblast cell line C2C12 was maintained in a
proliferative state utilizing growth medium (DMEM supple-
mented with 10% FBS and 1% penicillin/streptomycin antibiot-
ics) and incubated at 37 °C and 5% CO₂. DMEM supplemented
with 2% horse serum and 1% antibiotics was utilized for in-
duction of differentiation. Cells were maintained in growing con-
ditions and split when reaching 50% confluence to avoid sponta-
nous differentiation. C2C12 cells were seeded (0.3 × 10⁶

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**Figure 5. Pcbp1 regulates myocyte size and myofiber type.**

A, transverse sections of Quadriceps (Quad), gastrocnemius (GAS), and tibialis anterior (TA) from 2-month-old Pcbp1f/f and control mice were stained for laminin. B, cross-sectional areas were measured and the distribution of myocyte size in the indicated muscle types is shown. C, immunostaining to detect fast- and slow-twitch myofibers in TA and soleus muscles. D, quantification of fast- and slow-twitch myofibers in Pcbp1f/f and control mice. Scale bar = 100 μm. *, p < 0.05.
cells) in 6-well plates at ~80% confluence the night before treatment. Control siRNA (MISSION® siRNA Universal Negative Control #1 (catalog no. SIC001-10NMOL) and siRNA against mouse Pcbp1 (MISSION® SASI_Mm01_00177173, MISSION® SASI_Mm01_00177174) were purchased from Sigma. siRNA transfection was performed by using Lipofectamine RNAi Max following the manufacturer’s instructions. Adenovirus infection was performed by incubating cells with Ad-EGFP or Ad-Pcbp1 at a 10 multiplicity of infection for 6 h.

**Generation of Pcbp1f/f allele and mouse model**

The Pcbp1 coding sequence followed by ~350 bp of the 3′-UTR was amplified by PCR using genomic DNA (C57BL/6J x 129/SvJae) as a template and cloned into the pGEM-T easy vector containing the thymidine kinase negative selector to create the “P-TK” vector. LoxP sequences were cloned in the same direction into the 5′- and 3′-ends of the Pcbp1–3′-UTR sequence to create the “PLoxP-TK” vector. An frt-flanked Neo-mycin resistance gene was cloned into the 3′-end of the PLoxP-Tk vector to create the PLoxP-Neo-TK vector. Similarly a 2.2-kb fragment 5′-upstream of the Pcbp1 ATG codon sequence and a 4.7-kb 3′-downstream fragment were amplified by PCR and cloned into the PLoxP-Neo-TK vector to create the final “2.2K-PLoxP-Neo-4.7K-TK” vector, which was named Pcbp1f targeting construct (Fig. 4A). Next, 30 μg of the final Pcbp1f targeting vector was linearized with the restriction endonuclease Ndel, purified with phenol:chloroform:isoamyl alcohol (25:24:1) and used for embryonic stem (ES) cell electroporation. Targeted ES cells were screened by PCR and Southern blot, and four clones were identified to possess the correct homologous recombination. One clone was utilized for blastocyst injection, and five male and one female high percentage chimeric mice were obtained. The resulting chimeric mice were bred to C57BL/6 mice to obtain germ-line transmission. The neomycin cassette was subsequently excised by breeding with β-actin-Flp mice.

**Virus production**

The Pcbp1 coding sequence was amplified by PCR and cloned into the pIRES2-EGFP vector; subsequently the Pcbp1-IRES2-EGFP sequence was excised and cloned into the pENTR3c shuttle vector. Finally, recombination into the pAd-CMV/V5-DEST vector was achieved following the manufacturer’s instructions (Invitrogen). The final adenoviral construct was linearized with PacI restriction endonuclease, purified with phenol:chloroform:isoamyl alcohol (25:24:1), resuspended in sterile distilled water, and diluted to a concentration of 1 μg/μl. For adenovirus production human epithelial kidney cells (HEK-293AD) were seeded in a 6-well plate, transfected with 1 μg of linearized plasmid, and maintained following the manufacturer’s instructions (Invitrogen) as well as standard adenovirus production protocols (50).

**RNA isolation, RT-PCR and qPCR**

RNA isolation was achieved by isopropyl alcohol precipitation after TRIzol (Life Technologies) or Tri-PURE (Roche Applied Science) following manufacturer’s instructions. Retrotranscriptase reactions (M-MLV, Life Technologies) and real-time PCR were performed following manufacturer’s instructions (Affimetrix). PCR primers for mouse Pcbp1 were:
forward, GAGAGTCATGACCATCCCGTA; reverse, GCGGAGAAATGGTGTGTTGT.

**Western blot and co-immunoprecipitation**

Cells or tissues were homogenized in lysis buffer (Tris-HCl pH 7.5, 300 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% Triton X-100 supplemented with 1× proteinase inhibitor mixture, Roche Applied Science). Lysates were precipitated by centrifugation at 4 °C and 10,000 rpm for 10 min. Equal amounts of total protein were loaded on an SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and blotted with various antibodies: Pcbp1 (MBL RN024P, 1:4000), MF-20 (DHSB, 1:2000), myogenin (DHSB F5D 1:500), MyoD (DHSB D7F2, 1:1000).

Human embryonic kidney cells (HEK-293T) transected with various plasmids were lysed with protein lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and 1× proteinase inhibitor mixture). Lysates were cleared by centrifugation at 10,000 rpm, and protein content was measured by the BCA method (Bio-Rad). For co-immunoprecipitation assays lysates were incubated with 2 µg of antibody against a Myc or FLAG tag (Sigma) overnight at 4 °C followed by incubation with protein A/G-agarose beads for 2 h at 4 °C.

**miRNA Northern blot**

30 µg of total RNA was electrophoresed into a 15% acrylamide-Tris borate-EDTA gel, transferred to a nitrocellulose mem-

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**Figure 7. Pcbp1 regulates muscle miRNA maturation.**

A, miRNA Northern blots showing expression of miR-1, miR-133, and miR-206 in untreated (unt) and siRNA-control (siCNTL)- and siRNA-Pcbp1 (siPcbp1)-treated C2C12 cells at one day of differentiation. U6 served as a loading control. B, quantification of miRNA expression relative to U6 expression. *, p < 0.05. C, qPCR analyses of miRNA expression in skeletal muscles (quadriceps) of Pcbp1 flox/flox and control mice.
brane, UV-light-fixed, probed with radioactive probes against various miRNAs in hybridization buffer (0.5 mM Na₂HPO₄, 7% SDS) at 37 °C overnight, washed, and film-exposed for various times at −80 °C. Oligonucleotides were diluted at a concentration of 1 mM and used in a reaction containing [γ-³²P]ATP (PerkinElmer Life Sciences) and polynucleotide kinase following the manufacturer’s instructions (New England BioLabs). Radioactive oligonucleotides were purified using Sephadex G-25 columns (Roche Applied Science).

**Skeletal muscle fiber isolation**

EDL and soleus muscles were obtained by excision from hind-limb muscle and further digested using 0.2% collagenase type I (w/v). Fiber dissociation was performed under microscope in DMEM (with pyruvate) in 10-min intervals until necessary. Fibers were washed extensively in DMEM (with pyruvate), collected by centrifugation at 50 × g for 1 h and cultured in 0.2% collagen-coated culture dishes in medium containing DMEM (with pyruvate), 5% horse serum, and 2% FBS. Fixed fibers were fixed with 4% paraformaldehyde in PBS and stained, and the number of satellite cells was quantified on a per fiber basis.

**Skeletal muscle primary myoblast isolation**

Isolation of primary myoblast was performed following standard protocols as found elsewhere (51, 52). Briefly, mouse pups were sacrificed within 2 days after birth. Pups were dipped in alcohol for sterilization, the head was severed, internal organs were discarded, and the carcasses were then washed with sterile PBS. An incision on the dorsal side was performed, and skin was removed from the carcass. Skeletal muscles were exposed and excised from the carcass. Samples were finely minced in PBS, transferred to a 15-ml tube, and washed twice. Mincing samples were incubated in a collagenase/dispose enzyme mixture for 30 min at 37 °C followed by pipetting-trituration. Triturating and enzymatic digestion was repeated twice more. Cells were accumulated by centrifugation and cultured in medium containing DMEM, 20% FBS (Invitrogen), 1% chick embryo extract, 10 ng/ml bovine FGF, and 0.5% penicillin-streptomycin. Induction of differentiation was performed in similar fashion to C2C12 cell treatment.

**Immunocytochemistry/histochemistry**

Skeletal muscle samples were isolated from sacrificed mice at different ages, immersed in cold (using a dry ice or liquid nitrogen bath) isopentene, immersed in optimal cutting temperature (OCT) compound, and stored at −80 °C. Samples were cryo-sectioned, air-dried for 5 min at room temperature, fixed in 4% paraformaldehyde at room temperature for 5 min, washed 3X with PBS, permeabilized with 0.5% Triton X-100 in PBS, washed again 3x with PBS, and blocked with 5% normal serum in PBS at room temperature for 1 h. Incubation with primary antibodies against slow anti-MHC-α,β (DHSB, BA-D5, 1:200), fast anti-MHC-IIb (DHSB, F-18, 1:200), Pax7 (DHSB, 1:200), and myogenin (DHSB, 1:200) was performed overnight at 4 °C. Samples were washed 3X with PBS and incubation with the respective secondary antibodies was performed at room temperature for 2 h. Finally, samples were stained with DAPI or Hoeschst dye, washed with PBS, and mounted with fluorescent stable mounting medium. Staining for the identification of membranes was performed with wheat germ agglutinin following a similar methodology to secondary antibody treatment.

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