

**Actin-related protein 5 functions as a novel modulator of MyoD and MyoG in skeletal muscle and in rhabdomyosarcoma**

Tsuyoshi Morita<sup>1, \*</sup> and Ken'ichiro Hayashi<sup>2</sup>

<sup>1</sup>Department of Biology, Wakayama Medical University, 580 Mikazura, Wakayama, 641-0011, Japan

<sup>2</sup>Department of RNA Biology and Neuroscience, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

\* Corresponding author: Tsuyoshi Morita

E-mail: [tsuyo@wakayama-med.ac.jp](mailto:tsuyo@wakayama-med.ac.jp)

Tel.: +81-73-447-2300

Fax.: +81-73-446-6720

## 1 **Abstract**

2 Myogenic regulatory factors (MRFs) are pivotal transcription factors in myogenic  
3 differentiation. MyoD commits cells to the skeletal muscle lineage by inducing  
4 myogenic genes through recruitment of chromatin remodelers to its target loci. This  
5 study showed that Actin-related protein 5 (Arp5) acts as an inhibitory regulator of  
6 MyoD and MyoG by binding to their cysteine-rich (CR) region, which overlaps with the  
7 region essential for their epigenetic functions. Arp5 expression was faint in skeletal  
8 muscle tissues. Excessive Arp5 in mouse hind limbs caused skeletal muscle fiber  
9 atrophy. Further, Arp5 overexpression in myoblasts inhibited myotube formation by  
10 diminishing myogenic gene expression, whereas Arp5 depletion augmented myogenic  
11 gene expression. Arp5 disturbed MyoD-mediated chromatin remodeling through  
12 competition with the three-amino-acid-loop-extension-class homeodomain transcription  
13 factors the Pbx1–Meis1 heterodimer for binding to the CR region. This antimyogenic  
14 function was independent of the INO80 chromatin remodeling complex, although Arp5  
15 is an important component of that. In rhabdomyosarcoma (RMS) cells, Arp5 expression  
16 was significantly higher than in normal myoblasts and skeletal muscle tissue, probably  
17 contributing to MyoD and MyoG activity dysregulation. Arp5 depletion in RMS  
18 partially restored myogenic properties while inhibiting tumorigenic properties. Thus,  
19 Arp5 is a novel modulator of MRFs in skeletal muscle differentiation.

20

## 21 **Introduction**

22 Actin-related protein 5 (Arp5), encoded by *Actr5*, is a nuclear-localized actin-like  
23 protein (Schafer and Schroer, 1999). Studies have investigated the role of Arp5 in the  
24 nucleus as one of the subunits of the ATPase-dependent chromatin remodeling complex

25 INO80 (Shen et al., 2000). INO80 regulates various DNA metabolic processes, such as  
26 gene expression, DNA replication, and DNA repair by nucleosome sliding (Polil et al.,  
27 2017). It contains  $\beta$ -actin and three Arp family members (Arp4, Arp5, and Arp8). Arp5  
28 forms the Arp5 module with Ies6 (encoded by *Ino80c*), which is necessary for ATP  
29 hydrolysis and nucleosome sliding by INO80 (Yao et al., 2015). However, a few  
30 features of Arp5 are unrelated to INO80. In *Arabidopsis*, Arp5 and Ino80, an essential  
31 ATPase component of INO80, have common and distinct features in plant growth and  
32 development (Kang et al., 2019). In addition, Arp5 plays an INO80-independent role in  
33 regulating the differentiation of vascular smooth muscle cells (SMCs) (Morita et al.,  
34 2014). In rat SMCs, Arp5 expression is strongly inhibited, although INO80 activity is  
35 generally necessary for cell growth and proliferation. Arp5 inhibits SMC differentiation  
36 by interacting with and inhibiting the SAP family transcription factor myocardin, which  
37 is a key regulator for the induction of SMC-specific contractile genes, indicating that  
38 low Arp5 expression in SMCs contributes to maintaining their differentiation status. In  
39 contrast, in cardiac and skeletal muscles, although Arp5 expression is low, its  
40 physiological significance is unclear.

41 Myogenic regulatory factors (MRFs), such as MYF5, MyoD, MyoG, and MRF4, are  
42 skeletal-muscle-specific basic helix–loop–helix (bHLH) transcription factors and master  
43 regulators of skeletal muscle development. They recognize a *cis*-regulatory element  
44 E-box, usually found in promoter and enhancer regions of muscle-specific genes, as a  
45 heterodimer with ubiquitous bHLH proteins of the E2A family (E12 and E47) (Funk et  
46 al., 1991). MRFs enhance the transcriptional activity of myogenic genes via chromatin  
47 remodeling by recruiting the switch/sucrose nonfermentable (SWI/SNF) complex to  
48 previously silent target loci (Roy et al., 2002; Ohkawa et al., 2007). This epigenetic

49 activity depends on a histidine- and cysteine-rich (H/C) region N-terminal to the bHLH  
50 domain in MRFs (Gerber et al., 1997). This region contains a CL-X-W motif, which is a  
51 binding site for the heterodimer of three-amino-acid-loop-extension (TALE)-class  
52 homeodomain transcription factors (Pbx1 and Meis/Prep1) (Knoepfler et al., 1999).  
53 Funk and Wright (1992) reported that E-box elements recognized by MyoG are  
54 occasionally flanked by a novel consensus motif TGATTGAC, which was also  
55 identified as a binding motif for the Pbx1–Meis/Prep1 heterodimer (Knoepfler et al.,  
56 1999). Thus, MRFs form a complex with the TALE heterodimer on DNA, leading to the  
57 recruitment of chromatin remodelers and an increase in the accessibility of their target  
58 loci.

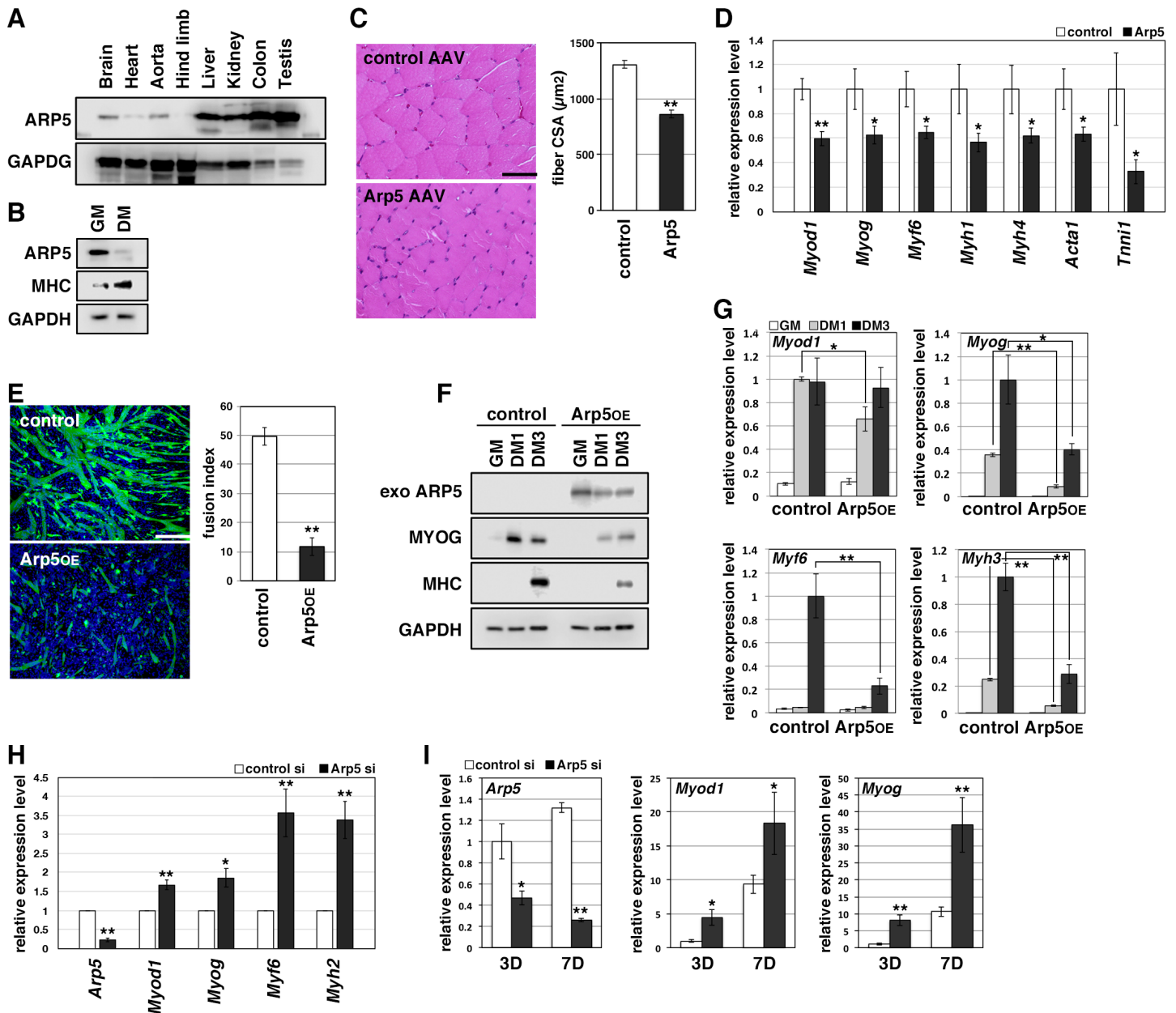
59 This study reported a novel role of Arp5 in myogenic differentiation of skeletal  
60 muscle cells. We identified Arp5 as an inhibitory binding protein for MyoD and MyoG  
61 in skeletal muscle and rhabdomyosarcoma (RMS) cells. Results showed that Arp5  
62 competes with the Pbx1–Meis1 heterodimer for interaction with the cysteine-rich (CR)  
63 region of MyoD and MyoG and consequently inhibits myogenic differentiation.

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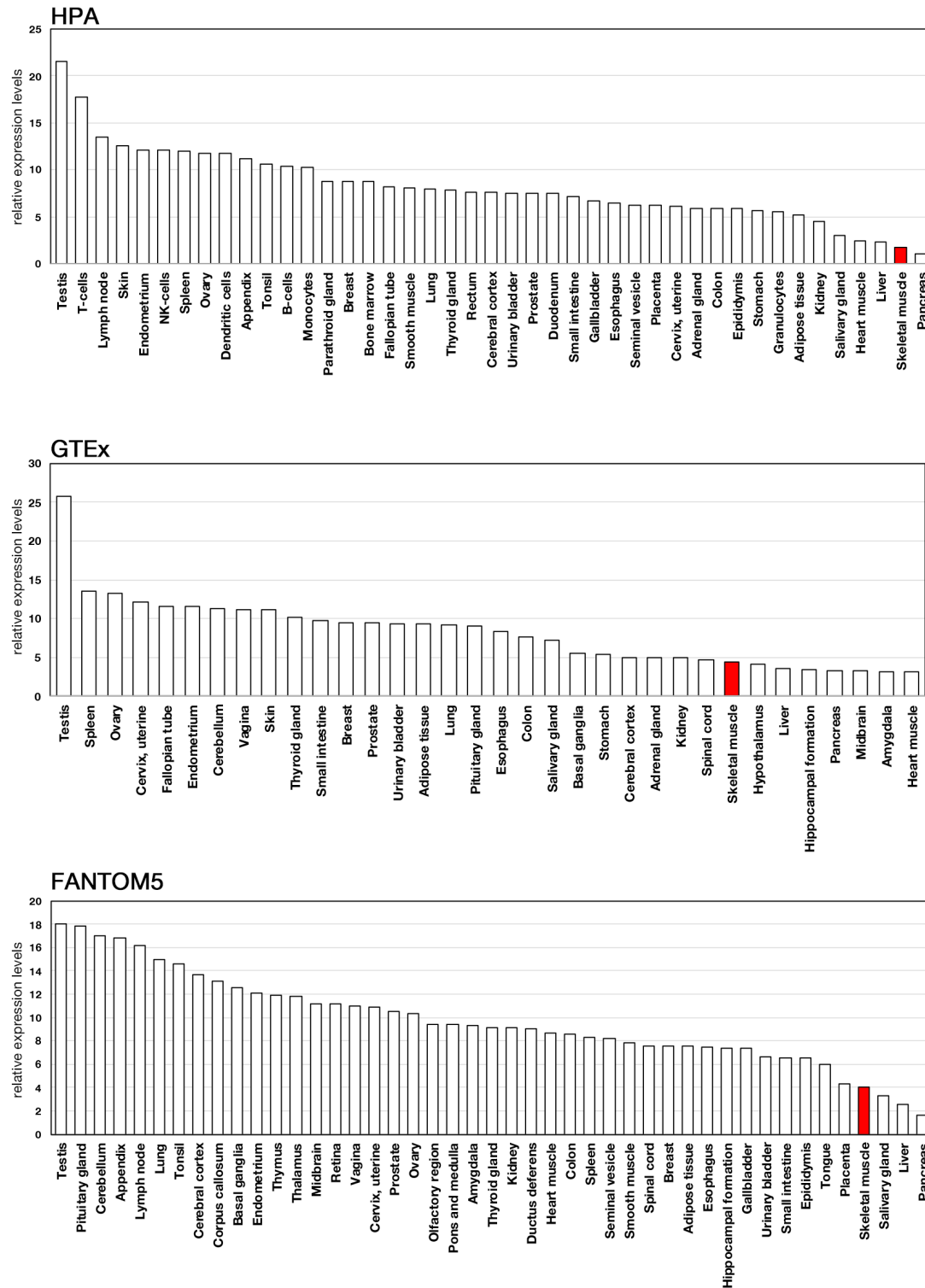
## 65 **Results**

### 66 **Arp5 prevents skeletal muscle development by inhibiting MRF expression**

67 Arp5 expression was significantly low in heart, aorta, and especially, hind limb muscle  
68 tissues (Figure 1A). Besides, Arp5 expression in primary mouse myoblasts significantly  
69 decreased with myotube differentiation (Figure 1B). The public human transcriptome  
70 databases (Human Protein Atlas [HPA], Genotype-Tissue Expression [GTEx], and  
71 Functional Annotation of the Mouse/Mammalian Genome 5 [FANTOM5]) showed low  
72 *ARP5* expression in skeletal muscle tissues (Figure 1—figure supplement 1). In this



**Figure 1. Actin-related protein 5 (Arp5) inhibits skeletal muscle differentiation.** (A) Arp5 expression in mouse tissues. (B) Arp5 and myosin heavy chain (MHC) expression in C2C12 cells cultured in growth medium (GM) or differentiation medium (DM). (C) Representative images of hematoxylin and eosin (H&E)-stained section of the hind limb muscle from mice injected with control or Arp5-AAV6 vector (left). Scale bar = 50  $\mu\text{m}$ . Muscle fiber cross-sectional area (CSA) measured in 190 fibers and statistically analyzed (right). (D) Myogenic gene expression in AAV6-vector-injected hind limb muscles. (E) Representative fluorescence images of differentiated C2C12 cells transfected with green fluorescent protein (GFP) alone (control) or together with Arp5 (Arp5<sup>OE</sup>) (left). Nuclei were visualized by Hoechst 33342. Scale bar = 100  $\mu\text{m}$ . The fusion index was measured on 22 images and statistically analyzed (right). (F) Myogenic protein expression in C2C12 cells transfected with control or Arp5 expression vector. The cells were cultured in GM or DM for 1 day (DM1) or 3 days (DM3) after transfection. (G) Myogenic gene expression in Arp5-transfected C2C12 cells. (H) Myogenic gene expression in mouse primary myoblasts transfected with control or Arp5 short interfering RNA (siRNA). (I) Myogenic gene expression in 10T1/2 cells treated with 5-azacytidine. The cells were transfected with control or Arp5 siRNA prior to 5-azacytidine treatment. All statistical data are presented as the mean  $\pm$  standard error of the mean (SEM). \* $P < 0.05$ , \*\* $P < 0.01$  (Student's  $t$ -test).



**Figure 1—figure supplement 1. Expression profiles of Actin-related protein 5 (ARP5) in human tissues.** Expression data of ARP5 were acquired from three public databases: Human Protein Atlas (HPA), Genotype-Tissue Expression (GTEx), and Functional Annotation of the Mouse/Mammalian Genome 5 (FANTOM5).

73 study, 5 weeks after injection of the Arp5-AAV6 vector, the muscle fiber thickness  
74 significantly reduced compared to the control group (Figure 1C). In these atrophic  
75 muscles, gene expression levels of MRFs (*Myod1*, *MyoG*, and *Myf6*) significantly  
76 decreased, accompanied by a decrease in other skeletal muscle markers, such as *Myh4*,  
77 *Acta1*, and *Tnni1* (Figure 1D).

78 In C2C12 cells, Arp5 overexpression significantly inhibited the fusion ability of  
79 myoblasts and the induction of MyoG and myosin heavy chain (MHC) under the  
80 conditions for myotube formation (Figure 1E,F). In addition, the induction of *Myod1*,  
81 *MyoG*, *Myf6*, and *Myh3* was also significantly inhibited (Figure 1G). Conversely, gene  
82 silencing of Arp5 by short interfering RNA (siRNA) (Arp5-si) increased MRF and  
83 *Myh2* expression (Figure 1H).

84 Long-term exposure to 5-azacytidine converts C3H 10T1/2 mouse embryo fibroblasts  
85 into differentiated skeletal muscle cells with the induction of endogenous MRF  
86 expression (Davis et al., 1987). There was significant induction of endogenous *Myod1*  
87 and *MyoG* 7 days after 5-azacytidine treatment, and Arp5 silencing significantly  
88 augmented *Myod1* and *MyoG* induction (Figure 1I). These findings show that Arp5  
89 plays an inhibitory role in skeletal muscle development via regulating MRF expression  
90 and activity.

91

## 92 **High Arp5 expression contributes to defective myogenic differentiation in** 93 **rhabdomyosarcoma**

94 RMS is a common soft-tissue sarcoma developed from skeletal muscle with defective  
95 myogenic differentiation. High MyoD and MyoG expression is used in the clinical  
96 diagnosis of RMS, but they are not fully active for the induction of their target genes

97 (Folpe 2002; Keller and Guttridge 2013). *ARP5* expression was significantly higher in  
98 human embryonal and alveolar RMS tissues compared with healthy and tumor-adjacent  
99 skeletal muscles (Figure 2A) and in RD cells compared with primary human myoblasts  
100 (Figure 2B), indicating that Arp5 overexpression contributes to MRF dysfunction in  
101 RMS.

102 Microarray analysis on Arp5-silencing RD cells showed that Arp5-si increased the  
103 expression of numerous genes involved in muscle function and development (Figure  
104 2C,D, Figure 2—Table supplement 1). The increased myogenic gene expression was  
105 also confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR)  
106 (Figure 2E). Unlike myoblasts, RD cells have little or no ability to form myotubes, even  
107 under serum-free culture conditions, but Arp5 depletion led RD cells to form numerous  
108 myotube-like structures with upregulation of myogenic marker proteins (Figure 2F).

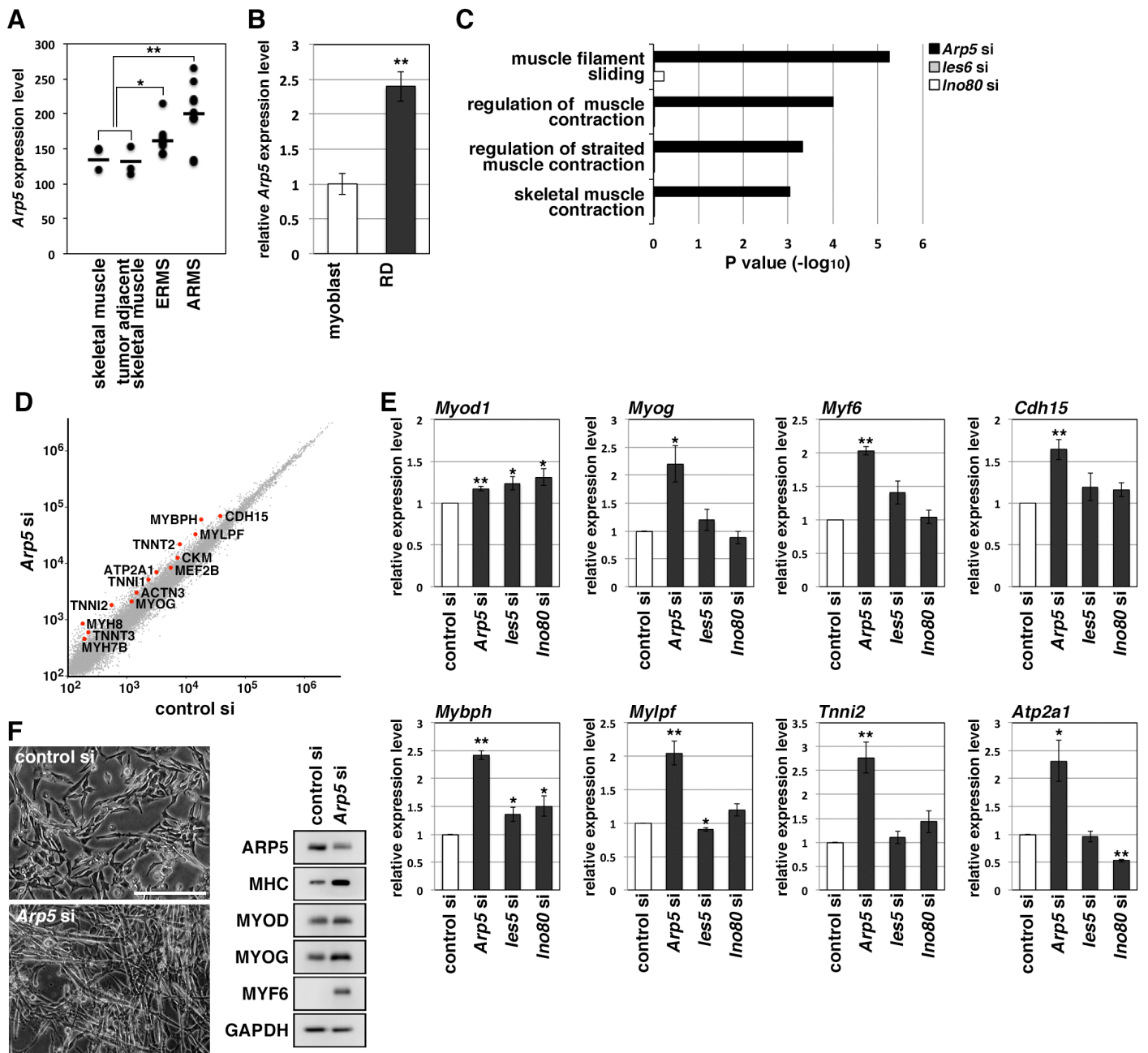
109 Arp5 is one of the subunits of INO80, so siRNA (*Ies6-si* and *Ino80-si*) also depleted  
110 the expression of other subunits (*Ies6* and *Ino80*). The genes altered by the silencing of  
111 each of these partly overlapped (Figure 2—figure supplement 1A,B); 39.2% and 32.4%  
112 of the genes altered by Arp5-si overlapped with those altered by *Ies6-si* and *Ino80-si*,  
113 respectively. Contrary to Arp5-si, however, *Ies6-si* and *Ino80-si* hardly increased the  
114 expression of muscle-related genes (Figure 2C,E, Figure 2—figure supplement 1C–E).  
115 These findings show that Arp5 overexpression inhibits the expression of muscle-related  
116 genes and, therefore, terminal myogenic differentiation in RMS in an  
117 INO80-independent manner.

118

### 119 **Arp5 knockout leads to loss of tumorigenicity of RD cells**

120 In Arp5-knockout RD (Arp5-KO) cells, a few nucleotides downstream of the start

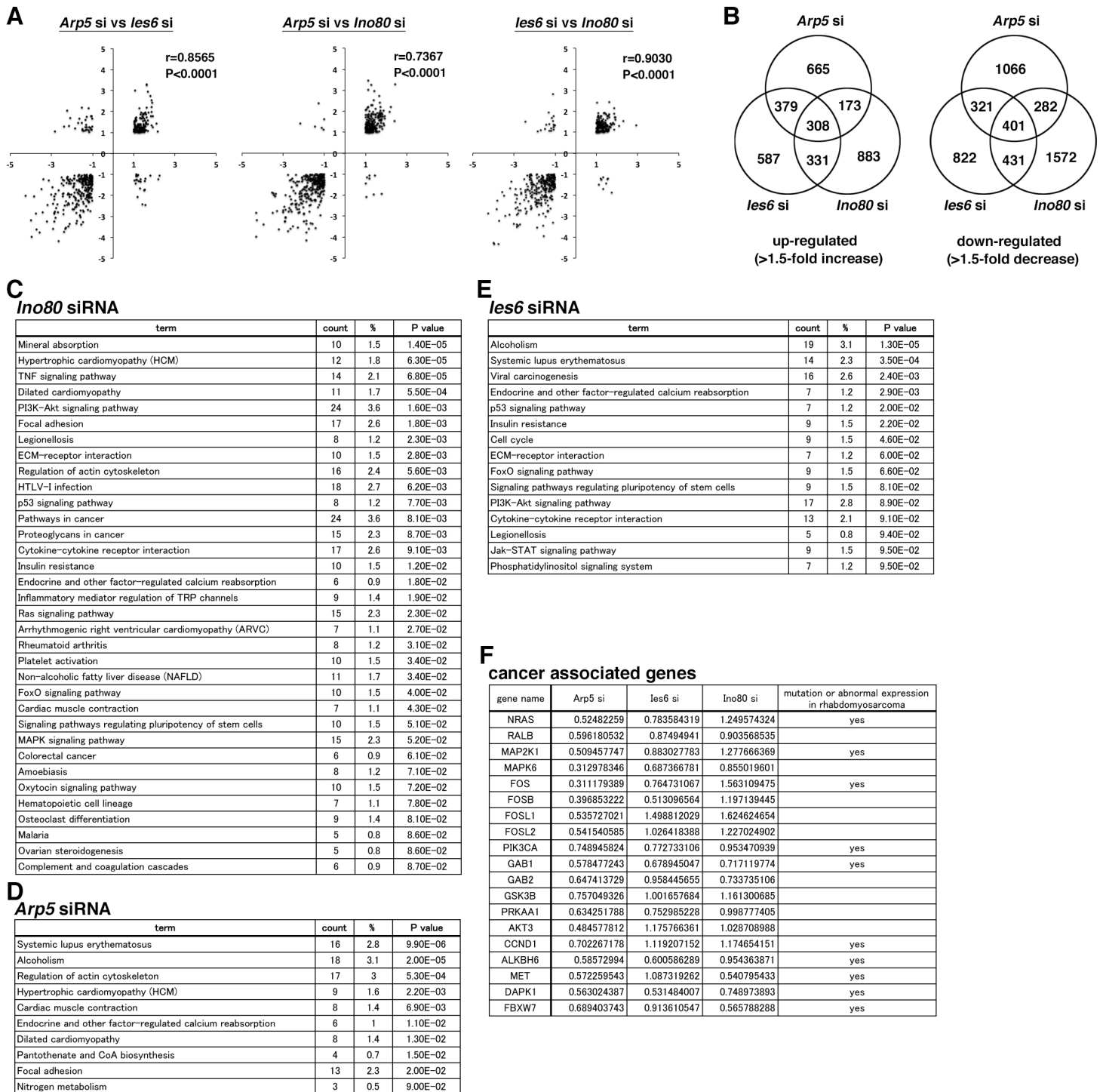




**Figure 2. Actin-related protein 5 (Arp5) knockdown increases myogenic gene expression in rhabdomyosarcoma (RMS) cells.** (A) *Arp5* expression in normal skeletal muscle, tumor-adjacent skeletal muscle, embryonal RMS (ERMS), and aveolar RMS (ARMS). Bars indicate average expression levels. (B) *ARP5* expression in human primary myoblasts and RD cells. (C) Enrichment analysis of muscle-related Gene Ontology terms from DNA microarray data on comparison between control-si and *Arp5*-, *les6*-, or *Ino80*-si samples. (D) Scatter plot of gene expression level in control-si and *Arp5*-si RD cells. (E) Myogenic gene expression in control-, *Arp5*-, *les6*-, and *Ino80*-si RD cells. (F) Representative phase-contrast images of control-si and *Arp5*-si RD cells (left). Scale bar = 100  $\mu$ m. Myogenic protein expression in these cells (right). All statistical data are presented as the mean  $\pm$  standard error of the mean (SEM). \* $P < 0.05$ , \*\* $P < 0.01$  (Student's *t*-test).

gene name	relative expression level (vs control siRNA)		
	Arp5 siRNA	les siRNA	Ino80 siRNA
ACTN3	2.076003774	0.687842617	0.938970893
ATP2A1	2.212933663	0.64311357	0.418973526
CDH15	1.835053382	1.238002233	1.384500699
CKM	1.747237254	0.743827933	0.996854717
MEF2B	1.519753751	1.037254304	1.03429799
MYBPH	3.366623852	1.222955635	1.993441588
MYH7B	4.734458903	0.719439084	0.806704257
MYH8	2.644230776	1.950191916	0.185822279
MYLPF	2.279431645	0.962704219	1.355377816
MYOG	1.743530176	0.955793102	0.734200156
TNNI2	3.297109791	1.233338111	1.630513745
TNNT2	2.811110999	0.606930828	1.855443232
TNNT3	2.376308547	1.227166077	1.549523985

**Figure 2—Table supplement 1. List of the myogenic genes whose expression level is upregulated by Arp5 knockdown in RD cells**



**Figure 2—figure supplement 1. Expression profiles of genes altered by Actin-related protein 5 (Arp5), Ies6, and Ino80 knockdown.** (A) Scatter plot of fold changes (log<sub>2</sub>) in the expression level of genes altered by Arp5, Ies6, and Ino80 knockdown. The dataset was filtered for genes with more than twofold increase or decrease. The Pearson's correlation coefficient (r) was calculated. (B) Venn diagrams of the number of genes whose expression level increased (left) or decreased (right) by more than 1.5-fold by Arp5, Ies6, and Ino80 knockdown. (C) Enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway from the DNA microarray data on Arp5 knockdown in RD cells. (D) Enrichment analysis of the KEGG pathway from the DNA microarray data on Ies6 knockdown in RD cells. (E) Enrichment analysis of the KEGG pathway from the DNA microarray data on Ino80 knockdown in RD cells. (F) List of cancer-associated genes whose expression level was downregulated by Arp5 knockdown in RD cells.

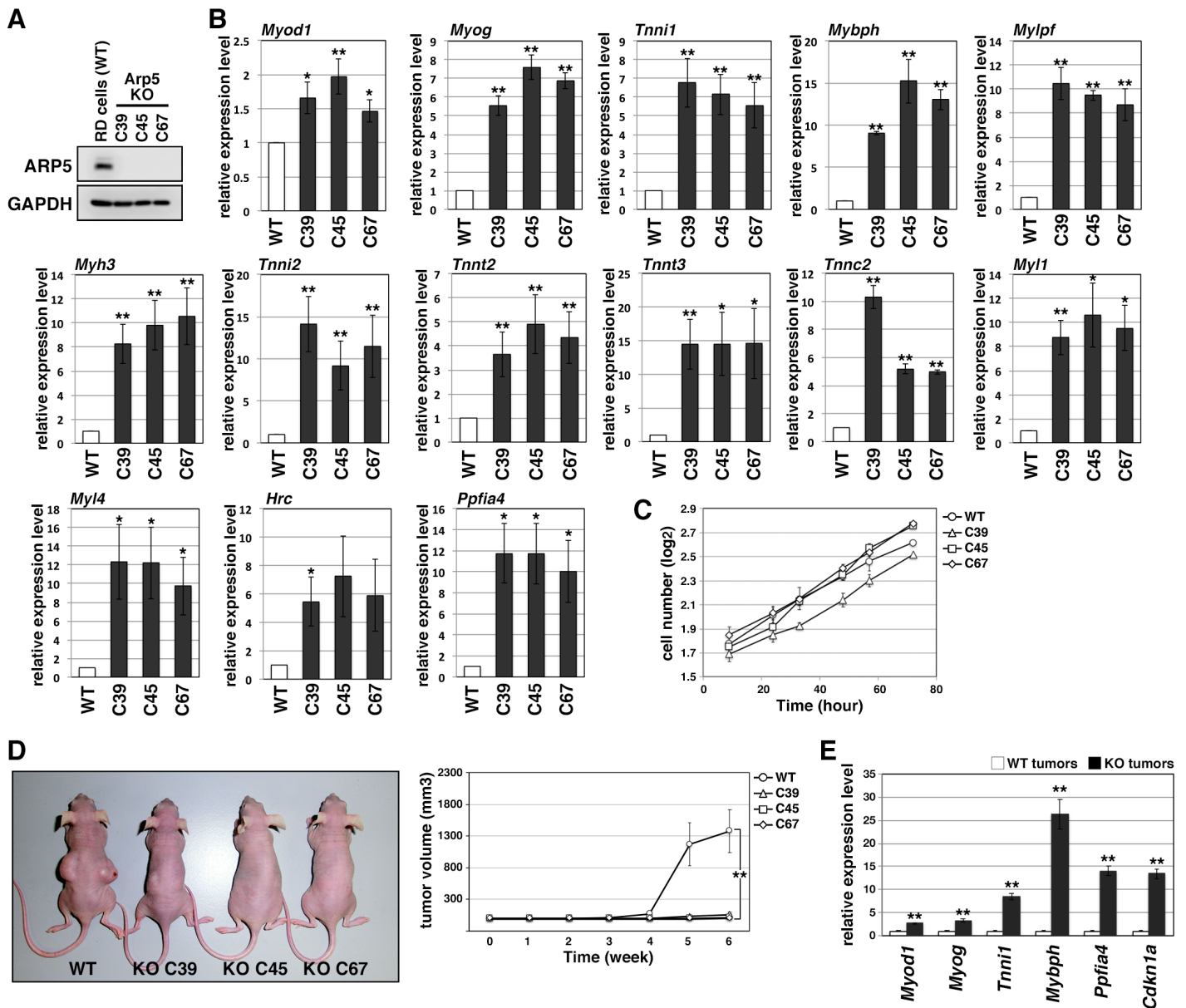
121 codon of *Arp5* were deleted by CRISPR-Cas9 genome editing, causing *Arp5* deletion  
122 (Figure 3A). *Arp5*-KO cells showed significant upregulation of many kinds of skeletal  
123 muscle-related genes (Figure 3B).

124 Under culture conditions, *Arp5*-KO cells proliferated as well as parental RD cells  
125 (doubling time of wild-type [WT] RD cells [ $t_2$ (WT)] = 29.7 h,  $t_2$ (C39) = 29.8 h,  $t_2$ (C45)  
126 = 23.8 h,  $t_2$ (C67) = 26.7 h; Figure 3C). However, when transplanted subcutaneously into  
127 nude mice, they rarely formed tumor nodules differently from parental cells (Figure 3D).  
128 These rare tumor nodules showed higher expression of muscle-related genes and  
129 *Cdkn1a*, which encodes a cyclin-dependent kinase inhibitor p21<sup>WAF1/CIP1</sup> controlling cell  
130 cycle arrest and myogenic differentiation during muscle development and regeneration  
131 (Halevy et al., 1995; Figure 3E). Thus, *Arp5* deletion partially restores the impaired  
132 myogenic differentiation potential of RD cells and inhibits their tumorigenesis in vivo.

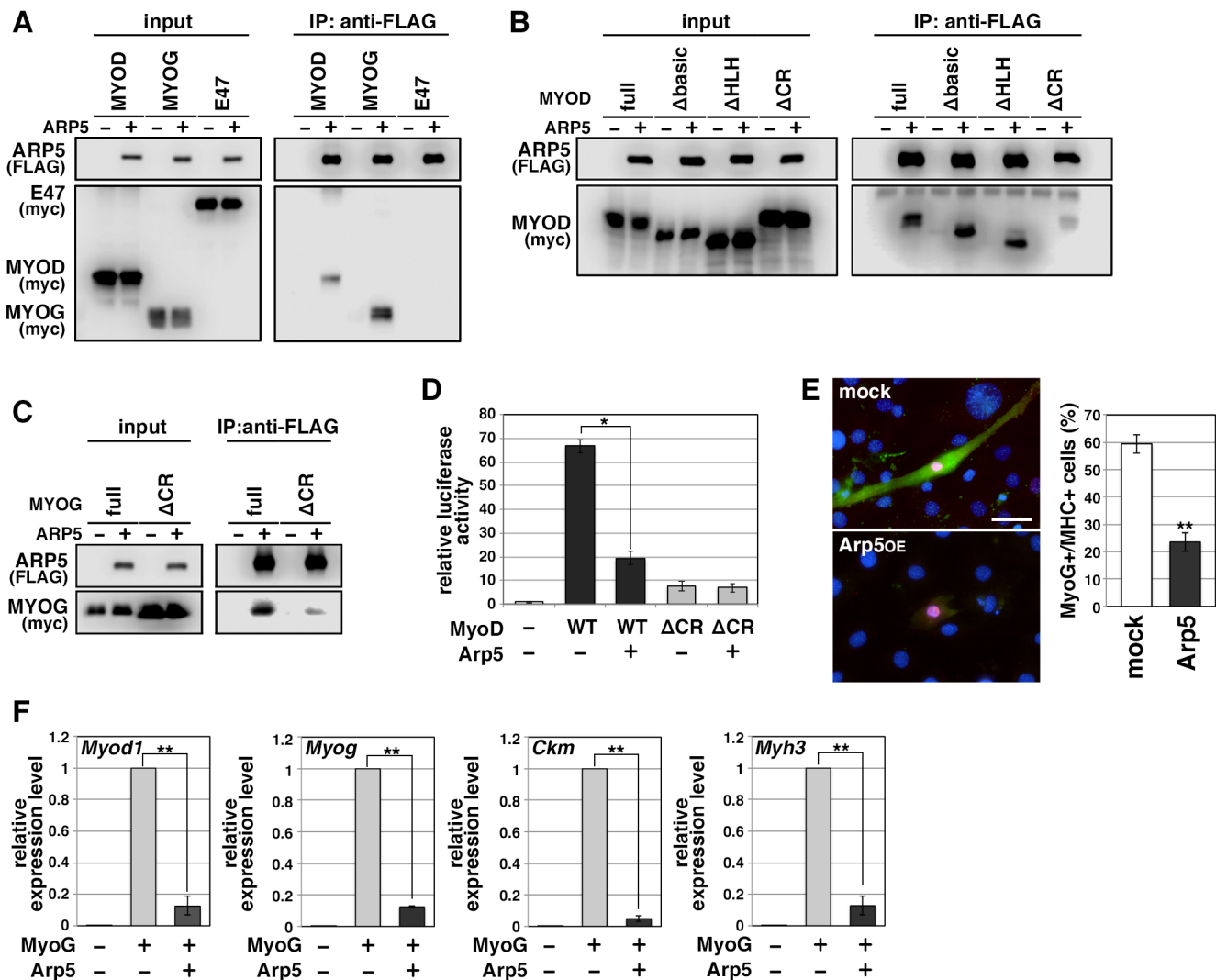
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### 134 ***Arp5* inhibits MyoD and MyoG activity through binding to their cysteine-rich** 135 **region**

136 MRF transcription is regulated by positive autoregulation (Tapscott SJ, 2005). To  
137 examine whether *Arp5* inhibits the activity or transcription of MRF,  
138 immunoprecipitation and reporter promoter analysis were performed.  
139 Immunoprecipitation showed that *Arp5* binds to both MyoD and MyoG but not to the  
140 ubiquitous bHLH protein E47 (Figure 4A). MyoG was precipitated more efficiently  
141 than MyoD with *Arp5*. The amino acid sequences of basic and HLH regions were  
142 highly conserved between MyoD and MyoG (Figure 4—figure supplement 1), but these  
143 domains were not necessary for their interaction (Figure 4B). The CR region, a part of  
144 the H/C region, was also highly conserved between them (Figure 4—figure supplement



**Figure 3. Actin-related protein 5-knockout (Arp5-KO) RD clones show increased expression of myogenic genes and decreased tumorigenicity.** (A) Arp5 expression in three individual clones of Arp5-KO cells (C39, C45, and C67) and their parental RD cells (wild-type [WT]). (B) Myogenic gene expression in WT and Arp5-KO cells. (C) Growth curve of WT and Arp5-KO cells. (D) Xenograft model of WT and Arp5-KO cells in nude mice (left). Tumor volumes measured every week after inoculation and statistically analyzed (right). (E) Myogenic gene expression in xenograft tumors. All statistical data are presented as the mean  $\pm$  standard error of the mean (SEM). \* $P < 0.05$ , \*\* $P < 0.01$  (Student's  $t$ -test).



**Figure 4. Actin-related protein 5 (Arp5) inhibits the activity of MyoD and MyoG through direct interaction.** (A) Co-immunoprecipitation assay between Arp5 and basic helix–loop–helix (bHLH) transcription factors MyoD, MyoG, and E47. (B) Co-immunoprecipitation assay between Arp5 and truncated series of MyoD. (C) Co-immunoprecipitation assay between Arp5 and the CR-region-deleted MyoG. (D) MyoG promoter-controlled luciferase reporter assay in C2C12 cells. (E) Representative fluorescence images of 10T/2 cells transfected with MyoG and Arp5 (left). The cells were immunostained with anti-MyoG (red) and anti-myosin heavy chain (MHC, green) antibodies. Nuclei were visualized by Hoechst 33342 (blue). Scale bar = 50  $\mu$ m. The percentage of MyoG+ MHC+ double-positive cells in MyoG+-positive cells was calculated in 786 cells and statistically analyzed (right). (F) Endogenous myogenic gene expression in 10T1/2 cells transfected with MyoG and Arp5. All statistical data are presented as the mean  $\pm$  standard error of the mean (SEM). \*P < 0.05, \*\*P < 0.01 (Student's t-test).

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MyoD      MELLSPPLRDIDLTPGPDGSLCSFETADDFYDDPCFDSPLRFFEDLDPRLVHVGALLKPE 60
Myogenin  -----MELYETSPYFYQEP-----HFYDGENYLPVHLOGFEPPG 34
          **   **   *
                                     CR          basic
MyoD      EHAHFSTAVHPGPGAREDEHVRAPSGHHQAGRCLLWACKACRKRKTTNADRRKAATMRERR 120
Myogenin  YERTELSLSPEARGPLEEKGLGTP--EHCPGQCLPWACKVCKRKSVSVDRRRAATLREKR 92
          *   *           *   *   *   *   *   *   *   *   *   *   *
                                     HLH
MyoD      RLSKVNEAFETLKRCTSSNPQRLPKVEILRNAIQYIEGLQALLRDQDAAPPGAAAFYAP 180
Myogenin  RLKKNVNEAFEALKRSTLLNPQRLPKVEILRSIQYIERLQALLSSLNQEERDLRYRGGG 152
          ** ***** ** * ***** ** * ** *****
MyoD      GPLPPGRGSEHYSGDSDASSPRSNCSGMDYSGPPSGPRRQNGYDTAYYSEAVRESRPG 240
Myogenin  GPQP-----MVPSECNSHSASCSP-----EWGNALEFGPNPGDHLAA 190
          ** *           *   *   **
KSAAVSSLDCLSSIVERISTDSPAAPALLLADAPPESPPGPPEGASLSDTEOGTQTPSPD 300
Myogenin  DPTDAHNLHSLTSIVDSITVED-----MSVAFPDETMPN----- 224
          *   * *** *           *   *   *
MyoD      AAPQCPAGSNPNAIYQVL 318
Myogenin  -----

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**Figure 4—figure supplement 1. Sequence alignment between human MyoD and MyoG proteins.** The CR (red), basic (green), and helix–loop–helix (HLH) regions are highlighted. Asterisks indicate conserved amino acid residues between them.

145 1), and CR region deletion mostly abolished the Arp5-binding ability of both MyoD and  
146 MyoG (Figure 4B,C). MyoG promoter-controlled luciferase reporter assay  
147 demonstrated that MyoD strongly enhances MyoG promoter activity, while Arp5  
148 significantly inhibits it (Figure 4D). MyoD  $\Delta$ CR, in which the CR region was deleted,  
149 also increased MyoG promoter activity but to a lesser extent, and this activation was  
150 completely unaffected by Arp5 (Figure 4D), indicating that the direct interaction with  
151 MyoD via the CR region is necessary for Arp5 to inhibit MyoD activity.

152 Ectopic MyoG expression differentiated 10T1/2 cells into MHC-positive myogenic  
153 cells with induction of endogenous myogenic marker genes such as *Myod1*, *MyoG*, *Ckm*,  
154 and *Myh3* (Figure 4E,F). Co-expression of Arp5 with MyoG, however, significantly  
155 decreased the frequency of MHC-positive cells and significantly inhibited the induction  
156 of myogenic genes (Figure 4E,F). Thus, Arp5 inhibits MyoD/MyoG activity through  
157 direct interaction via their CR region.

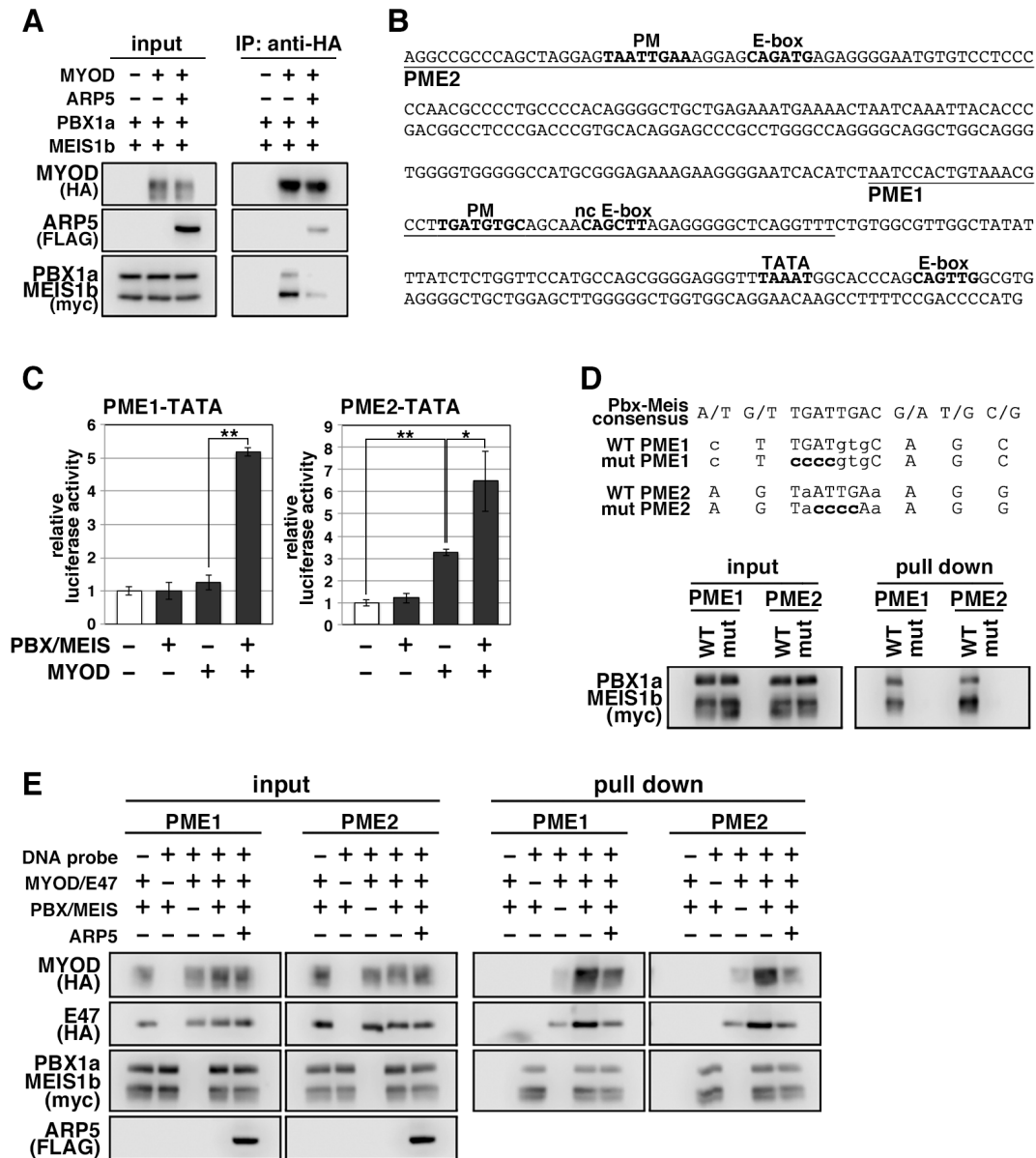
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### 159 **Arp5 competes with Pbx1–Meis1 for binding to the cysteine-rich region of** 160 **MyoD/MyoG**

161 The H/C region of MyoD is an essential region for its chromatin remodeling activity  
162 and effective induction of target genes (Gerber et al., 1997). Pbx1 and Meis1 homeobox  
163 proteins are promising candidates as mediators between MyoD and chromatin  
164 remodelers because MyoD binds to Pbx1 and Meis1 via the H/C region (Knoepfler et al.,  
165 1999). MyoD and the Pbx1–Meis1 heterodimer interacted in the absence of the  
166 intermediary DNA; furthermore, this interaction was interrupted by Arp5 (Figure 5A).

167 The *MyoG* locus has been most analyzed for MyoD-mediated chromatin remodeling.  
168 The proximal promoter region of *MyoG* reportedly contains two flanking sequences,



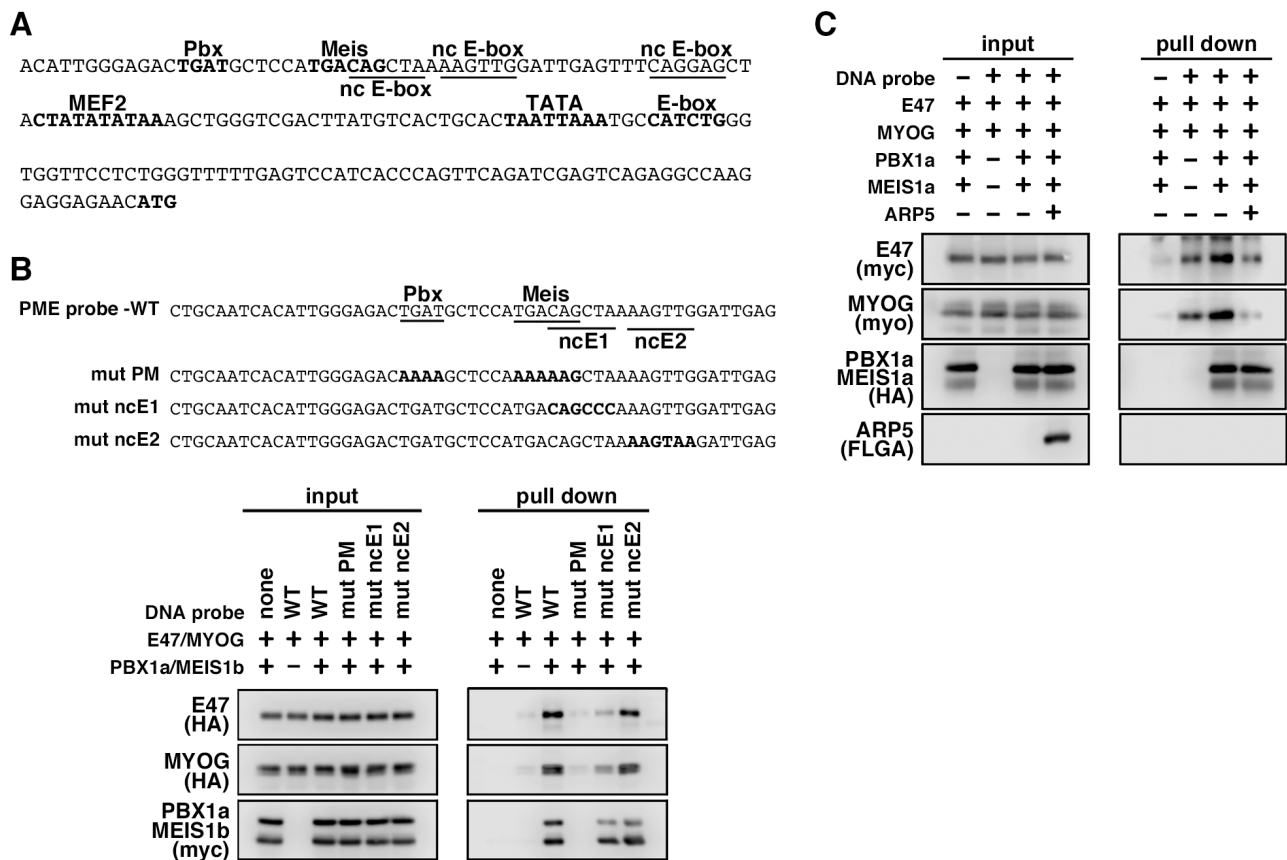


**Figure 5. Actin-related protein 5 (Arp5) disturbs the interaction between MyoD and the Pbx1–Meis1 heterodimer.** (A) Co-immunoprecipitation assay between MyoD and Pbx1a–Meis1b. Co-incubation with Arp5 protein (lanes 3 and 6) diminished their interaction. (B) Sequence of the proximal promoter region of human MYOG. The core sequences of the indicated cis-regulatory elements are highlighted in bold. Pbx1–Meis1 heterodimer-binding motif/noncanonical E-box (PM/ncE)-containing regions (PME1 and PME2) are underlined. (C) PME1/2-TATA-controlled luciferase reporter assay in C2C12 cells. (D) Pull-down assay of PME1/2 DNA probes with Pbx1a–Meis1b protein. The consensus sequence of the PM motif is presented. The mutated nucleotides in the mut PME1/2 probes are highlighted in bold. (E) Pull-down assay of PME1/2 DNA probes with MyoD, E47, Pbx1a–Meis1b, and Arp5 proteins.

169 Pbx1–Meis1 heterodimer-binding motif (PM) and noncanonical E-box (ncE) (Berkes et  
170 al., 2004; Figure 5B). When this regulatory region (PM/ncE-containing region 1  
171 [PME1]) was fused to the TATA minimal promoter, the PME1–TATA construct was  
172 activated by the combination of Pbx1, Meis1, and MyoD (Figure 5C, left). Upstream of  
173 PME1, a novel predicted regulatory region containing PM and E-box motifs (PME2)  
174 was identified (Figure 5B). A pull-down assay using DNA-probe-conjugated beads of  
175 the PMEs demonstrated that the Pbx1–Meis1 heterodimer recognizes the PM motif of  
176 both PME1 and PME2 and that this interaction completely disappears by disruption of  
177 the PM motif via mutagenesis (Figure 5D). The PME2–TATA construct was also  
178 synergically activated by MyoD and the Pbx1–Meis1 heterodimer (Figure 5C, right).  
179 Thus, both PME1 and PME2 are functional *cis*-regulatory elements for MyoD and the  
180 Pbx1–Meis1 heterodimer.

181 The DNA–protein pull-down assay also demonstrated that the MyoD–E47  
182 heterodimer weakly binds to PME1 and PME2 DNA probes, which is significantly  
183 augmented by co-incubation with the Pbx1–Meis1 heterodimer (Figure 5E). The Pbx1–  
184 Meis1 heterodimer was constantly associated with the PMEs regardless of whether  
185 MyoD–E47 was present. Arp5 interrupted the augmented interaction of MyoD–E47 with  
186 the PMEs but did not affect the interaction of the Pbx1–Meis1 heterodimer with the  
187 PMEs (Figure 5E).

188 We also identified the predicted PME element in the proximal promoter region of  
189 *Myf6*, which contains Pbx- and Meis-binding motifs separated by six nucleotides  
190 (acTGATgctccaTGACag) close to noncanonical E-boxes (Figure 6A). Jacobs et al.  
191 (1999) reported that this type of gapped PM site is also functional for the Pbx1–  
192 Meis1-binding site, and we observed significant interaction between the Pbx1–Meis1



**Figure 6. MyoG and the Pbx1–Meis1 heterodimer recognize the proximal promoter region of human myogenic regulatory factor 6 (MRF6).** (A) Sequence of the proximal promoter region of human *MRF6*. The core sequences of the indicated cis-regulatory elements are highlighted in bold. The core sequences of putative noncanonical E-box motif (nc E-box) are underlined. (B) Pull-down assay of Mrf6 Pbx1–Meis1 heterodimer-binding motif/noncanonical E-box (PM/ncE)-containing region (PME) probes with MyoG, E47, and Pbx1a–Meis1b proteins. The mutated nucleotides in the mut PME probes (mut PM, mut ncE1, and mut ncE2) are highlighted in bold (top). The Pbx1–Meis1 heterodimer recognized the gapped Pbx–Meis-binding motif, while MyoG–E47 bound to the ncE1 site with the Pbx–Meis complex (bottom). (C) Pull-down assay of Mrf6 PME probes with MyoG, E47, Pbx1a–Meis1b, and Arp5 proteins.

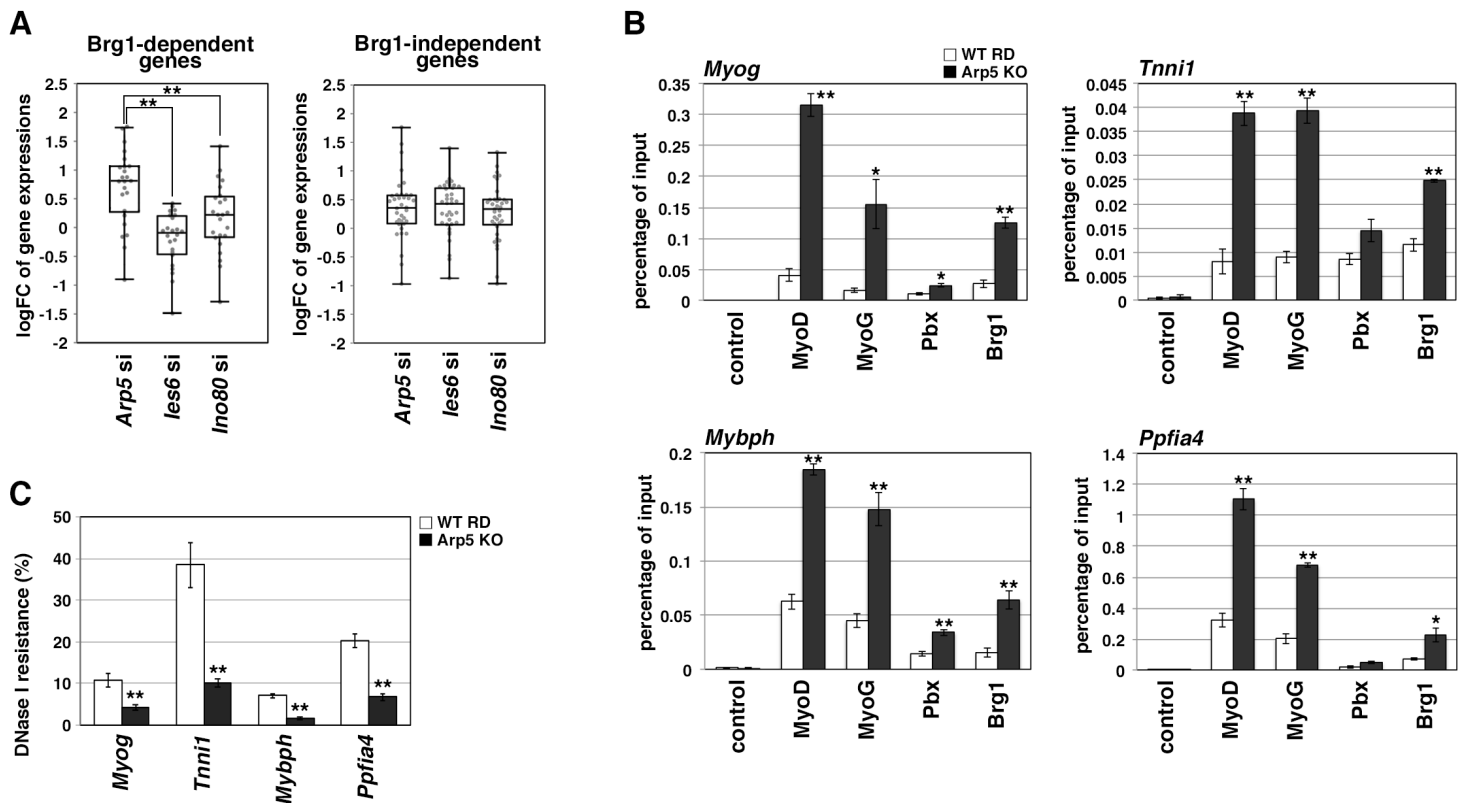
193 heterodimer and *Myf6*'s PM motif (Figure 6B). Similar to PME in the *MyoG* promoter,  
194 MyoG bound to *Myf6*'s PME synergically with the Pbx1–Meis1 heterodimer, while  
195 Arp5 interrupted this binding (Figure 6C). These findings show that Arp5 attenuates  
196 MyoD/MyoG recruitment to PME-containing myogenic enhancer regions by disturbing  
197 the interaction between MyoD/MyoG and the Pbx1–Meis1 heterodimer.

198

### 199 **Arp5 prevents Brg1–SWI/SNF complex recruitment to MyoD/MyoG target loci**

200 During MyoD-mediated chromatin remodeling, the Pbx1–Meis1 heterodimer is  
201 believed to be involved in the recruitment of Brg1-based SWI/SNF chromatin  
202 remodeling complex to MyoD target loci as a pioneering factor (de la Serna et al., 2001).  
203 Serna et al. (2005) reported that the induction of approximately one-third of MyoD  
204 target genes depends on the Brg1–SWI/SNF complex. Therefore, we investigated the  
205 change in MyoD target gene expression by Arp5-si in terms of Brg1 dependency.  
206 Arp5-si upregulated Brg1-dependent genes to a larger extent compared to  
207 Brg1-independent genes (1.77-fold vs. 1.38-fold,  $p = 0.02$ ; Figure 7A). This Brg1  
208 dependency was seen more clearly by comparing the effect of Arp5-si versus Ies6-si and  
209 Ino80-si: Arp5-si more effectively increased Brg1-dependent gene expression compared  
210 with Ies6-si and Ino80-si, whereas Brg1-independent gene alteration was comparable  
211 between them (Figure 7A). Thus, Arp5, but not Ies6 and Ino80, affects the expression of  
212 MyoD target genes in a Brg1-dependent manner.

213 To determine the role of Arp5 in regulating Brg1 recruitment to MyoD target loci, we  
214 performed chromatin immunoprecipitation (ChIP) assay using Arp5-KO RD cells.  
215 MyoD, MyoG, Pbx, and Brg1 were recruited to the proximal promoter region of *Myog*.  
216 MyoD, MyoG, and Brg1 significantly accumulated in Arp5-KO cells compared with



**Figure 7. Actin-related protein 5 (Arp5) inhibits the recruitment of MyoD, MyoG, and Brg1-based switch/sucrose nonfermentable (SWI/SNF) to the enhancer region of myogenic genes.** (A) Box-and-whisker plot of fold changes (log<sub>2</sub>) in the expression level of Brg1-dependent and Brg1-independent myogenic genes by Arp5, Ies6, and Ino80 knockdown. (B) Chromatin immunoprecipitation (ChIP) analysis using antibodies against MyoD, MyoG, Pbx, and Brg1 in wild-type (WT) and Arp5-knockout (KO) cells. Enrichment efficiency of DNA fragments of MyoG, Tnni1, Mybph, and Ppfi4 enhancer loci was quantified by real-time polymerase chain reaction (PCR). (C) DNase I sensitivity assay of the enhancer loci in WT and Arp5-KO RD cells. All statistical data are presented as the mean ± standard error of the mean (SEM). \*P < 0.05, \*\*P < 0.01 (Student's t-test).

217 parental RD cells (Figure 7B). Arp5 KO increased Pbx1 recruitment to a lesser extent.  
218 ChIP assay against the enhancer regions of Brg1-dependent myogenic genes, *Tnni1*,  
219 *Mybph*, and *ppfia4*, using public ChIP-Seq and DNase I hypersensitive site (DHS)-Seq  
220 databases identified Pbx1- and MyoD-binding regions in their promoter and intronic  
221 regions; these regions were DNase I hypersensitive and positive for H3K27Ac, which  
222 are markers for the identification of active enhancer regions (Figure 7—figure  
223 supplement 1). Pbx–Meis-binding motifs actually exist close to canonical and  
224 noncanonical E-box motifs in the focused areas (Figure 7—figure supplement 1). ChIP  
225 data showed that all the proteins of interest were recruited to the predicted enhancer  
226 regions and that Arp5 KO significantly augmented MyoD, MyoG, and Brg1  
227 accumulation in these regions (Figure 7B).

228 Finally, we investigated the involvement of Arp5 in chromatin structure alteration of  
229 the MyoD/MyoG target loci. DNase I accessibility to the above-mentioned regions was  
230 compared between Arp5-KO and parental RD cells (Figure. 7C). The accessibility of all  
231 the target regions was significantly higher in Arp5-KO RD cells. The findings illustrate  
232 that Arp5 binds to MyoD and MyoG competitively with the Pbx1–Meis1 heterodimer  
233 and prevents the recruitment of the Brg1–SWI/SNF complex to their target loci,  
234 resulting in chromatin accessibility attenuation and, therefore, transcriptional  
235 suppression of myogenic genes.

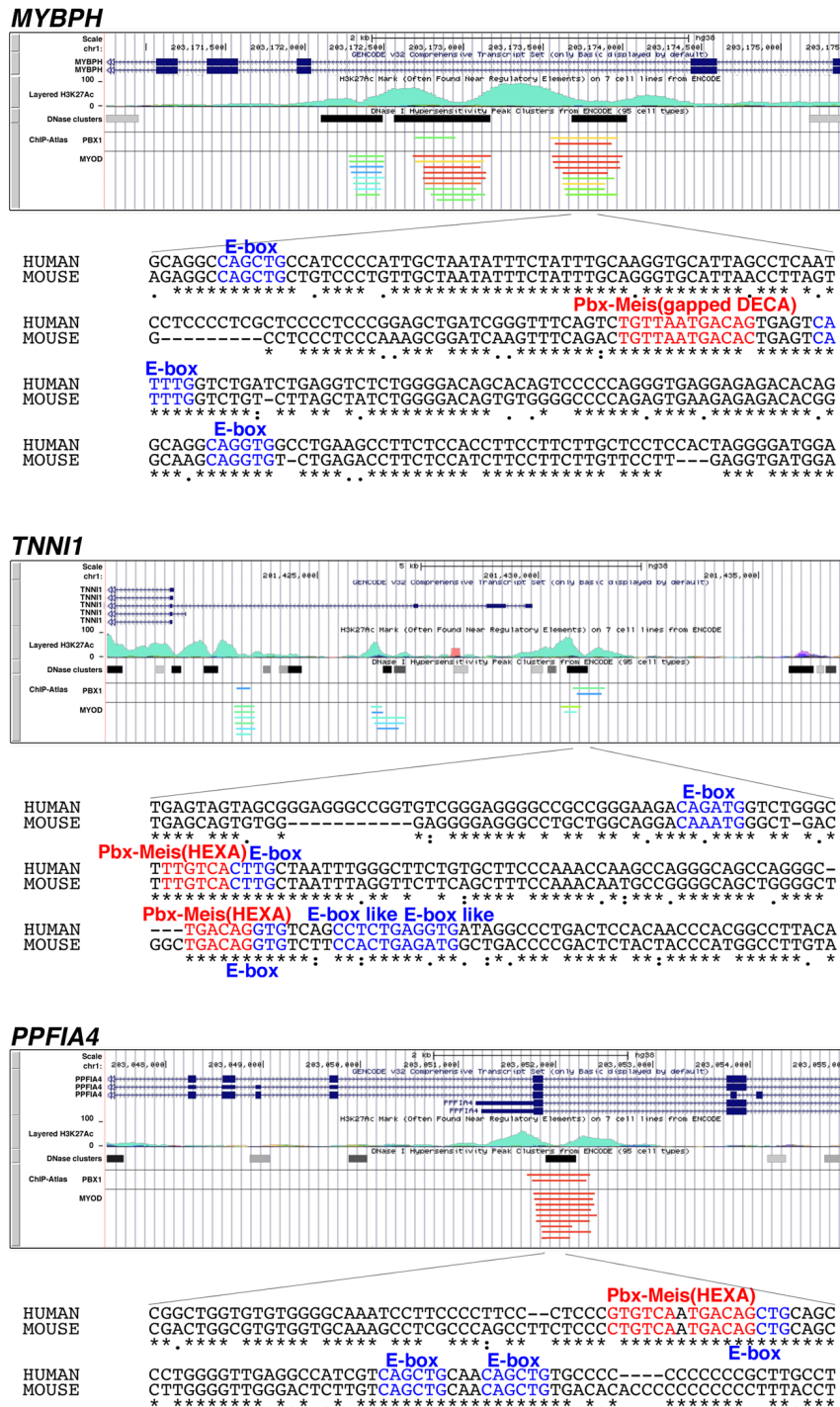
236

## 237 **Discussion**

238 In this study, we demonstrated a novel role of Arp5 in skeletal muscle differentiation.  
239 The H/C region of MyoD is critical for its chromatin remodeling activity. MyoD  
240 interacts with some epigenetic regulators, such as SWI/SNF, histone deacetylase

241 (HDAC), p300 histone acetyltransferase (HAT), and p300/CBP-associated factor  
242 (PCAF) (Yuan et al., 1996; Puri et al., 1997; Lu et al., 2000; Serna et al., 2001), but how  
243 the H/C region contributes to epigenetic regulation is unclear. The Pbx1–Meis1  
244 heterodimer is so far the only identified partner of MyoD-binding directly to this region.  
245 Here we reported Arp5 as another binding partner, which competes with the Pbx1–  
246 Meis1 heterodimer for binding to the H/C region. The Pbx1–Meis1 heterodimer is  
247 believed to recognize the PM motif in the enhancer region of myogenic genes and acts  
248 as a pioneering factor by marking this locus for the recruitment of MyoD and its  
249 co-factors, such as epigenetic regulators (Cho et al., 2015). Our findings clearly show  
250 that the Pbx1–Meis1 heterodimer directly binds to the PM motifs in the promoter region  
251 of *MyoG* and *Myf6* and augments the MyoD and MyoG binding to the canonical and  
252 noncanonical E-box close to the PM motifs. The ChIP-Seq database and ChIP analysis  
253 reveal Pbx1 recruitment to the enhancer regions of some myogenic genes with MyoD.  
254 Arp5 inhibits the recruitment of MyoD and MyoG to Pbx1–Meis1-marked loci and,  
255 consequently, attenuates the recruitment of chromatin remodelers, such as Brg1–  
256 SWI/SNF. Therefore, low Arp5 expression in skeletal muscle tissues contributes to an  
257 increase in the chromatin accessibility of MyoD/MyoG target loci and the maintenance  
258 of high transcriptional activity of myogenic genes.

259 Arp5 is a well-known subunit of INO80 and plays an essential role in its ATPase  
260 activity and nucleosome sliding (Yao et al., 2015). In HeLa cells knocked down for each  
261 subunit of INO80, such as Ino80, Arp8, Ies2, and Ies6, the expression of many genes  
262 was altered, and these genes were enriched in several functional pathways such as the  
263 p53 signaling pathway, cell cycle, focal adhesion, and extracellular matrix–receptor  
264 interaction (Cao et al., 2015). These profiling data are in good agreement with our



**Figure 7—figure supplement 1. Putative enhancer regions recognized by Pbx1 and MyoD/MyoG in human MYBPH, TNNI1, and PPFIA4.** Data on the enrichment of H3K27Ac histone markers and DNase I hypersensitivity clusters were acquired from the Encyclopedia of DNA Elements (ENCODE) public database, version 3 (<https://www.encodeproject.org>). Data on the chromatin immunoprecipitation (ChIP) sequence were acquired from the ChIP-Atlas public database (<https://chip-atlas.org>). Nucleotide sequences of putative enhancer regions are presented with highlighted Pbx–Meis-binding motif (red) and E-box motif (blue). Decameric (DECA, TGATTGACAG) and hexameric (HEXA, TGACAG) motifs are reported as a consensus sequence of the Pbx–Meis-binding site.



265 results using Ino80-si RD cells (Figure 2—figure supplement 1A–E). In osteogenic  
266 differentiation of mesenchymal stem cells, INO80 interacts with the WD repeat domain  
267 5 protein and regulates osteogenic marker expression (Zhou et al., 2016). When 11  
268 kinds of INO80 subunits are knocked down in mesenchymal stem cells, all the  
269 knockdown cells show a reduction in calcium deposition. Thus, each subunit seems  
270 indispensable for the function of INO80. In contrast, we observed many muscle-related  
271 genes to be upregulated only in Arp5-si cells but not in Ies6- and Ino80-si cells. Besides,  
272 Arp5 alone interrupted MyoD/MyoG–Pbx1–Meis1 complex formation, thereby  
273 inhibiting their activities. These results suggest that Arp5 regulates the activities of  
274 MyoD and MyoG independently of INO80. We previously demonstrated the  
275 INO80-independent role of Arp5 in regulating the phenotypic plasticity of SMCs  
276 through direct interaction with myocardin (Morita et al., 2014). Yao et al. (2016)  
277 reported that the total abundance of Arp5 in cells is approximately eightfold of that as a  
278 component of INO80. Thus, Arp5 seems to have multiple functions besides being a  
279 subunit of INO80.

280 RMS cells are useful for analyzing the antimyogenic function of Arp5 because they  
281 highly express Arp5 and MRFs simultaneously. MyoD and MyoG are potent markers of  
282 RMS and can induce terminal myogenic differentiation, while their activities are  
283 post-transcriptionally dysregulated in RMS (Keller and Guttridge, 2013). Our results  
284 provide insight into the post-transcriptional regulation of MyoD and MyoG: abundant  
285 Arp5 in RMS induces MyoD and MyoG dysfunction via direct interaction. Arp5  
286 deletion in RD cells leads to a loss of their tumorigenicity in nude mice due to the  
287 enhancement of myogenic differentiation. Arp5 is also identified as a T-cell acute  
288 lymphocytic leukemia and sarcoma antigen (Lee et al., 2003), although its contribution

289 to carcinogenesis and sarcomagenesis is unclear. In Arp5-si RD cells, the expression of  
290 several cancer-associated genes decreased, including those reported to be mutated and  
291 abnormally expressed in RMS (Figure 2—figure supplement 1F). Survival data from  
292 The Cancer Genome Atlas reveal that high Arp5 expression is unfavorable for the  
293 prognosis of liver and renal carcinoma (data not shown). Thus, Arp5 likely has  
294 oncogenic properties, in addition to its inhibitory role in myogenesis.

295 Here we reported that Arp5 expression is remarkably low in both human and mouse  
296 skeletal muscle. In contrast, RMS shows increased Arp5 expression with dysregulation  
297 of myogenic differentiation compared with tumor-adjacent skeletal muscles. Haldar et  
298 al. (2007) reported a synovial sarcoma mouse model in which the ectopic expression of  
299 a chimeric SYT-SSX fusion gene was driven from the *Myf5* promoter. In the tumors,  
300 Arp5 expression was approximately fivefold upregulated, while several myogenic  
301 markers were downregulated. Thus, Arp5 expression seems to be regulated according to  
302 the differentiation stage of skeletal muscle lineage cells. Alternative splicing coupled to  
303 nonsense-mediated messenger RNA (mRNA) decay (AS-NMD) is important for  
304 keeping the expression of Arp5 low in differentiated SMCs (Morita et al., 2014).  
305 AS-NMD contributes to post-transcriptional fine-tuning of broad gene expression (Nasif  
306 et al., 2018); furthermore, depletion of the splicing factor U2AF35 increased *Arp5*  
307 mRNA levels in Hek293 cells (Kralovicova et al., 2015). Tissue-specific alternative  
308 splicing is most frequently observed in skeletal muscle, and these splicing events are  
309 critical for proper skeletal muscle development and function (Nakka et al., 2018). These  
310 facts raise the possibility that AS-NMD contributes to the regulation of Arp5 expression  
311 in skeletal muscle and RMS.

312 This study reported a novel function of Arp5 in myogenic differentiation and

313 tumorigenesis. Arp5 is a novel modulator of MRFs in skeletal muscle differentiation.  
314 Our data also cultivated a better understanding of chromatin remodeling events  
315 mediated by MRFs and Pbx–Meis in myogenic gene expression. The major limitations  
316 of this study are the lack of data on the change in Arp5 expression and its relevance to  
317 MRF activation during skeletal muscle development in vivo. Although in vivo  
318 experiments were performed using the AAV6 vector, further studies are needed to fully  
319 elucidate the mechanism and significance of low Arp5 expression in muscle tissues. It  
320 will be also interesting to investigate whether transcriptional and post-transcriptional  
321 regulation of Arp5 contributes to physiological and pathological skeletal muscle  
322 dysfunction.

323

## 324 **Materials and Methods**

### 325 **Cell cultures, treatment, and transfections**

326 Human RMS RD cells (supplied by the Japanese Collection of Research Bioresources  
327 cell bank), human skeletal myoblasts (Thermo Fisher Scientific), and mouse C3H  
328 muscle myoblasts (C2C12 cells; ATCC) were cultured in Dulbecco's Modified Eagle's  
329 Medium (DMEM) supplemented with 20% FCS. Mouse primary fibroblasts were  
330 isolated from the hind limbs of 3-week-old C57BL/6j mice as follows: The extracted  
331 hind limb muscle tissues were minced and incubated in a digestion solution (260 U type  
332 I collagenase, 3000 PU dispase II, and 10 mM CaCl<sub>2</sub> in 1 mL of Hanks' Balanced Salt  
333 solution [HBSS]) for 60 min at 37°C. The dispersed cells were washed twice with  
334 HBSS and plated on a type I collagen-coated culture dish with DMEM. After 30 min of  
335 incubation, the culture medium was removed, and the attached cells were cultured with

336 DMEM supplemented with 20% FCS. The differentiated myoblasts were incubated in  
337 differentiation medium (DMEM supplemented with 2% house serum) for 1–3 days.

338 For myogenic transdifferentiation of the mouse embryo cell line, 10T1/2 cells  
339 (ATCC) were treated with 3  $\mu$ M 5-azacitidine for 24 h and then cultured in DMEM  
340 supplemented with 10% FCS for 3–7 days.

341 To establish Arp5-KO cell lines, RD cells were transfected with the all-in-one  
342 Cas9/gRNA plasmid pSpCas9 BB-2A-GFP (PX458; Addgene; gRNA target sequence,  
343 ccgttcgcgcagcggcgtgccgc). One day after transfection, green fluorescent protein  
344 (GFP)-positive cells were sorted and individually cultured. Gene KO in the isolated  
345 clones was validated by DNA sequencing and Western blotting.

346 In knockdown experiments, cells were transfected with predesigned siRNAs using  
347 Lipofectamine RNAiMAX (Thermo Fisher Scientific). The siRNA sequences are listed  
348 in Table 1. In overexpression experiments, cells were transfected with pCAGGS  
349 expression vectors using Lipofectamine 3000 (Thermo Fisher Scientific). The coding  
350 sequences of human *ARP5*, *MYOD1*, *MYOG*, *E47*, *Pbx1a*, and *MEIS1b* were inserted  
351 into vectors with FLAG-, HA-, and Myc-tag sequences. For the intramuscular  
352 expression of exogenous Arp5, the adeno-associated virus pseudotype 6 (AAV6)  
353 expression vector encoding *ARP5* was constructed using AAVpro Helper Free System  
354 (AAV6) (TAKARA BIO). Finally, titration of AAV6 particles was performed using the  
355 AAVpro titration kit (TAKARA BIO).

356

### 357 **Animal studies**

358 All animal experiments were conducted in accordance with the guidelines for animal  
359 experiments specified by the Wakayama Medical University, Japan, and Osaka

Table 1. List of PCR primer and siRNA sequences used in this study

real time RT-PCR

gene name	species	forward primer sequence (5'→3')	anti-sense primer sequence (5'→3')
18srRNA	mouse/human	gtaaccggtgaacccatt	cccatccaatcggtagtagcg
28srRNA	mouse/human	gcgacctagatcagacgtg	gggtcttcgtagccacat
rpl13a	mouse/human	tgccgaagatggcggagg	cacagcgtacgaccaccact
actr5	mouse/human	agcaagccagagaccctga	agccttgggtacctgtccag
myod1	mouse	ggatggtgtccctggtcttc	cctctggaagaacggctctg
myog	mouse	ggcaatggcactggagttcg	gcacaccagcctgacagac
myf6	mouse	ccagtgccaagtgttctg	cgctgaagactgctggagg
myh1	mouse	gtcaacaagctgagggtgaa	ggtcacttctgcaactggatc
myh2	mouse	ggctgtccgatgctgtg	cacacaggcgcagtgccaa
myh3	mouse	ctccagcagcgtagagagcg	ctagttagcactcagctcacc
myh4	mouse	gtgaagagccgagaggttcacac	ctctgtcaccttcaacagaaagatg
acta1	mouse	gcactgctgtgctgctc	cctgaaccacagcagcatt
tnni1	mouse	cgacctcccagtagaggtggc	gaaagataggtagtggggctgg
myd1	human	cccgcgctccaactgctc	cggtggagatgctcctccac
myog	human	ggcagtgccactggagttcag	gtgatgctgtccagatgga
myf6	human	gattcctgctgcaactgca	cgaaggctactcagggctgacg
cdh15	human	ctggacatcgcgcactcatc	gagggtgtgtcgttaaggcg
mybph	human	aggcatctgtgactgccg	cctcatcacagcctcctcc
mylpf	human	gaggatgtatcacggagcc	tggtcagcagctcctcagg
tnni1	human	ccatgtctgcatggaaggc	aggagctcagagcgcagcac
tnni2	human	ggacacagagaaggagcggg	cgagtggcctaggactcggactc
tnnt2	human	gcaggagaaagtcaagcagcaga	ccggtgactttagccttcccg
tnnt3	human	agaccctgcaccagctggag	cggtcctgagcgtgtgat
tnnc2	human	gacggcgacaagaacaacga	ggtagaggcgaactgccaactcc
myh3	human	cgagacttcactccagcagg	ctgtctgctccagaagggc
myl1	human	acatcatgtctatcgaatggagctctc	ctggagagttgtcatgggtgtg
myl4	human	catcatgtcagggtaagcagagtc	catctcagctcaccagccg
hrc	human	cggtctgctcctcaggaag	ccagcatgtctgcccaggcc
atp2a1	human	tgcaagtcstgggctcga	ctgtgacaaggctcagagatg
ppfia4	human	gccaagaagatcagcctgaag	gcatggctgctccgggaag
cdkn1a	human	gctctgctgcaggggacag	gaaatctgtcatgctgtctgcc

ChIP assay & DNase I sensitivity assay

gene name	species	forward primer sequence (5'→3')	anti-sense primer sequence (5'→3')
myog	human	ggccatgctgggagaagaag	cgctggcatggaaccagag
tnni1	human	gggaagacagatggtctggc	ggcctatcacctcagaggtctg
mybph	human	cggagctgctcgggttcag	gctcaggccacctgctct
ppfia4	human	gctgtgctgctcaggttac	cagggtgctcagctgtcattg

knockdown assay

gene name	species	target sequence (5'→3')
control	-	MISSION siRNA Universal Negative control (Sigma)
arp5	mouse	acagatggaccagttcac
arp5	human	cctggcatgaaagccagaa
ies6	human	actgctgctcagcaccatt
ino80	human	agcagctgcctcagggca

360 University School of Medicine, Japan.

361 For Arp5 overexpression in mouse skeletal muscle tissues, we injected  $1 \times 10^9$  gv/ $\mu$ L  
362 of control-AAV6 (empty vector) or Arp5-AAV6 vector injected into the hind limbs of  
363 5-day-old C57BL/6j mice. Five weeks after injection, the hind limb muscles were  
364 extracted, fixed in 10% formalin, embedded in paraffin, and then cut into 5- $\mu$ m-thick  
365 sections. The sections were stained with hematoxylin and eosin and observed.

366 In xenograft experiments,  $2.5 \times 10^7$  cells were suspended in 1 mL of an EHS-gel  
367 basement membrane matrix (FUJIFILM Wako Pure Chemical Corporation) diluted to  
368 1:1 with phosphate-buffered saline (PBS), and 200  $\mu$ L of the suspension was  
369 subcutaneously inoculated into both sides of the flank of 3-week-old nude mice. The  
370 tumor size was measured every week using a pair of calipers, and the tumor volume was  
371 estimated as volume =  $1/2(\text{length} \times \text{width}^2)$ . After 6 weeks of inoculation, the mice were  
372 sacrificed and xenograft tumors were extracted to obtain total RNA.

373

#### 374 **Immunocytochemistry**

375 Cells were cultured on coverslips and fixed with 10% formaldehyde solution. Fixed  
376 cells were incubated in blocking solution (0.1% Triton X-100, 0.2% bovine serum  
377 albumin [BSA], and 10% normal goat serum in PBS) for 30 min at 37°C and further  
378 incubated in a primary antibody solution (1:100 dilution of antibodies in Can Get Signal  
379 immunostaining reagent [TOYOBO]) for 2 h. Next, the cells were washed twice with  
380 PBS and incubated in a secondary antibody solution (1:400 dilution of Alexa 488–  
381 conjugated secondary antibody [Thermo Fisher Scientific] and 1:1000 dilution of  
382 Hoechst 33342 [Thermo Fisher Scientific]) in the blocking solution] for 1 h. The cells  
383 were again washed twice, mounted on glass slides with Fluoromount (Diagnostic

384 BioSystems), and observed under an all-in-one fluorescence microscope (BZ-9000;  
385 Keyence).

386

### 387 **Western blotting**

388 Western blotting was performed, as previously described (Morita et al., 2018). Briefly,  
389 total proteins were extracted from cells with 2% sodium dodecyl sulfate (SDS) sample  
390 buffer. The proteins were electrophoretically separated using 10% polyacrylamide gels  
391 and then transferred to polyvinylidene difluoride membranes. For Western blotting,  
392 anti-Arp5 (Proteintech), anti-MyoG (Santa Cruz Biotechnology, Inc.), anti-MyoD  
393 (Santa Cruz Biotechnology, Inc.), anti-MYF6 (Santa Cruz Biotechnology, Inc.),  
394 anti-MHC (Developmental Studies Hybridoma Bank), anti-glyceraldehyde 3-phosphate  
395 dehydrogenase (GAPDH) (Thermo Fisher Scientific), anti-FLAG (Sigma-Aldrich),  
396 anti-HA (Roche Applied Science), and anti-Myc (Santa Cruz Biotechnology, Inc.)  
397 antibodies were used as primary antibodies.

398

### 399 **Real-time RT-PCR**

400 Total RNA was isolated and then reverse-transcribed using RNAiso Plus (TAKARA  
401 BIO) and the PrimeScript RT Reagent Kit with gDNA Eraser (TAKARA BIO).  
402 Real-time RT-PCR was performed using the THUNDERBIRD SYBR qPCT Mix  
403 (TOYOBO) on the Mic real-time PCR cycler (Bio Molecular System). Nucleotide  
404 sequences of the primer sets used in this study are listed in Table 1.

405

### 406 **Reporter promoter analysis**

407 To examine the promoter activity of human *MYOG*, the proximal promoter region

408 (−299/−1) of *MyoG* was isolated by PCR and inserted into the pGL3-basic vector  
409 (Promega). The DNA fragments of PME1 and PME2 in the *MyoG* promoter region were  
410 also amplified and inserted into the pGL3- $\gamma$ -actin-TATA vector. These reporter vectors  
411 were transfected into cells, together with the pSV- $\beta$ Gal vector (Promega) and the  
412 indicated gene expression vectors. After 2 days of transfection, luciferase activity was  
413 measured using the Luciferase Assay System (Promega), which was normalized to  
414  $\beta$ -galactosidase activity.

415

#### 416 **Co-immunoprecipitation assay**

417 HEK293T cells were transfected with expression vectors to synthesize recombinant  
418 proteins. The next day, cells were lysed with 0.5% NP-40, 10% glycerol, and protease  
419 inhibitor cocktail (Nacalai Tesque) in PBS and gently sonicated. The lysate was  
420 centrifuged to remove cell debris and then incubated with Protein G Sepharose Fast  
421 Flow (Sigma-Aldrich) for 1 h at 4°C to remove nonspecifically bound proteins. After  
422 centrifugation, the cell lysate proteins were mixed in the indicated combinations and  
423 incubated with the ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich) or the Anti-HA  
424 Affinity Matrix from rat immunoglobulin G (IgG)<sub>1</sub> (Sigma-Aldrich) for 2 h at 4°C. The  
425 beads were washed thrice with 0.5% NP-40 in PBS, and binding proteins were eluted  
426 using SDS sample buffer.

427

#### 428 **Protein–DNA pull-down assay**

429 DNA probes for the protein–DNA pull-down assay were generated by PCR using  
430 5'-biotinylated primers and then conjugated to Dynabeads M-280 Streptavidin (Thermo  
431 Fisher Scientific). The recombinant proteins synthesized in HEK293T cells were



432 extracted and incubated with the DNA-probe-conjugated beads in 0.5% NP-40, 10%  
433 glycerol, and protease inhibitor cocktail (Nacalai Tesque) in PBS for 2 h at 4°C. The  
434 beads were washed thrice with 0.5% NP-40 in PBS, and binding proteins were eluted  
435 using SDS sample buffer.

436

#### 437 **ChIP assay**

438 ChIP assays were performed in WT and Arp5-KO RD cells using the SimpleChIP Plus  
439 Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the  
440 manufacturer's instructions. Anti-MyoD, anti-MyoG, anti-Pbx1/2/3/4, and anti-Brg1  
441 antibodies (all from Santa Cruz Biotechnology, Inc.) were used for  
442 immunoprecipitation.

443

#### 444 **DNase I sensitivity assay**

445 DNase I sensitivity assays were performed, as previously reported with slight  
446 modifications (Gerber et al., 1997). Briefly, WT and Arp5-KO RD cells were suspended  
447 in 0.5% NP-40, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, and Tris-HCl at pH 7.4 for 10 min at 4°C  
448 to isolate the nuclei. After centrifugation, the pelleted nuclei were resuspended in DNase  
449 I buffer (10 mM NaCl, 6 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 40 mM Tris-HCl at pH 8.0) and  
450 then treated with 1.5 U/50 mL of DNase I (Thermo Fisher Scientific) for 10 min at 37°C.  
451 The reaction was stopped by adding an equal volume of 40 mM  
452 ethylenediaminetetraacetic acid (EDTA). The digested nuclei were collected by  
453 centrifugation, suspended in 50 mM NaOH, and heated for 10 min at 100°C to extract  
454 DNA. The resulting solution was diluted 100 times with TE (1 mM EDTA, 10 mM  
455 Tris-HCl at pH 8.0). The digestion efficiency of the focused enhancer loci of myogenic

456 genes was calculated by comparing the amount of intact enhancer fragments between  
457 DNase-I-treated and DNase-I-untreated samples using real-time PCR.

458

#### 459 **DNA microarray**

460 Total RNAs were isolated from RD cells transfected with control, Arp5, Ies6, and Ino80  
461 siRNA for 2 days, using NucleoSpin RNA Plus (TAKARA BIO). mRNAs were  
462 reverse-transcribed, and Cy3-labeled complementary RNAs (cRNAs) were synthesized  
463 using the Low Input Quick Amp Labeling Kit (Agilent Technologies). The cRNAs were  
464 hybridized on a SurePrint G3 Mouse Gene Expression 8 × 60K Microarray (Agilent  
465 Technologies), and fluorescence signals were detected using the SureScan Microarray  
466 Scanner (Agilent Technologies). The fluorescence intensity was quantified using  
467 Feature Extraction Software (Agilent Technologies).

468

#### 469 **Statistics and reproducibility**

470 All statistical data were generated from experiments independently repeated at least  
471 three times, and values were expressed as the mean ± standard error of the mean (SEM).  
472 Data were assessed using Student's *t*-test, and \**P* < 0.05 and \*\**P* < 0.01 were  
473 considered statistically significant.

474

#### 475 **Data statement**

476 DNA microarray data have been deposited in the Gene Expression Omnibus (GEO)  
477 database (accession no. GSE169681). The data of Arp5 expression profiles in Figure 2A  
478 were obtained from the GEO dataset GSE28511.

479

480 **Competing Interest Statement**

481 The authors declare no conflicts of interest associated with this manuscript.

482

483 **Acknowledgments**

484 We thank all the staff in the Laboratory Animal Center, Wakayama Medical University  
485 and the Center for Medical Research and Education of Osaka University Graduate  
486 School of Medicine for their technical assistance. We also thank Enago ([www.enago.jp](http://www.enago.jp))  
487 for the English language review. This work was supported by JSPS KAKENHI Grant  
488 Number 15K07076 (to T. Morita), 18K06913 (to T. Morita) and 19K07351 (to K.  
489 Hayashi).

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491 **References**

492 Berkes CA, Bergstrom DA, Penn BH, Seaver KJ, Knoepfler PS, Tapscott SJ (2004) **Pbx**  
493 **marks genes for activation by MyoD indicating a role for a homeodomain**  
494 **protein in establishing myogenic potential.** *Mol Cell* **14**: 465–477. doi:  
495 10.1016/s1097-2765(04)00260-6.

496 Cao L, Ding J, Dong L, Zhao J, Su J, Wang L, Sui Y, Zhao T, Wang F, Jin J, Cai Y  
497 (2015) **Negative Regulation of p21Waf1/Cip1 by Human INO80 Chromatin**  
498 **Remodeling Complex Is Implicated in Cell Cycle Phase G2/M Arrest and**  
499 **Abnormal Chromosome Stability.** *PLoS One* **10**: e0137411. doi:  
500 10.1371/journal.pone.0137411.

501 Cho OH, Mallappa C, Hernández-Hernández JM, Rivera-Pérez JA, Imbalzano AN

- 502       **(2015) Contrasting roles for MyoD in organizing myogenic promoter structures**  
503       **during embryonic skeletal muscle development.** *Dev Dyn* **244**: 43–55. doi:  
504       10.1002/dvdy.24217.
- 505       Davis RL, Weintraub H, Lassar AB (1987) **Expression of a single transfected cDNA**  
506       **converts fibroblasts to myoblasts.** *Cell* **51**: 987–1000. doi:  
507       10.1016/0092-8674(87)90585-x.
- 508       de la Serna IL, Carlson KA, Imbalzano AN (2001) **Mammalian SWI/SNF complexes**  
509       **promote MyoD-mediated muscle differentiation.** *Nat Genet* **27**: 187–190. doi:  
510       10.1038/84826.
- 511       de la Serna IL, Ohkawa Y, Berkes CA, Bergstrom DA, Dacwag CS, Tapscott SJ,  
512       Imbalzano AN (2005) **MyoD targets chromatin remodeling complexes to the**  
513       **myogenin locus prior to forming a stable DNA-bound complex.** *Mol Cell Biol* **25**:  
514       3997–4009. doi: 10.1128/MCB.25.10.3997-4009.2005.
- 515       Folpe AL (2002) **MyoD1 and myogenin expression in human neoplasia: a review**  
516       **and update.** *Adv Anat Pathol* **9**: 198–203. doi:  
517       10.1097/00125480-200205000-00003.
- 518       Funk WD, Ouellette M, Wright WE (1991) **Molecular biology of myogenic regulatory**  
519       **factors.** *Mol Biol Med* **8**: 185–195.
- 520       Funk WD, Wright WE (1992) **Cyclic amplification and selection of targets for**  
521       **multicomponent complexes: myogenin interacts with factors recognizing**  
522       **binding sites for basic helix-loop-helix, nuclear factor 1, myocyte-specific**  
523       **enhancer-binding factor 2, and COMP1 factor.** *Proc Natl Acad Sci U S A* **89**:  
524       9484–9488. doi: 10.1073/pnas.89.20.9484.
- 525       Gerber AN, Klesert TR, Bergstrom DA, Tapscott SJ (1997) **Two domains of MyoD**

- 526 **mediate transcriptional activation of genes in repressive chromatin: a**  
527 **mechanism for lineage determination in myogenesis.** *Genes Dev* **11**: 436–450. doi:  
528 10.1101/gad.11.4.436.
- 529 Haldar M, Hancock JD, Coffin CM, Lessnick SL, Capecchi MR (2007) **A conditional**  
530 **mouse model of synovial sarcoma: insights into a myogenic origin.** *Cancer Cell*  
531 **11**: 375–388. doi: 10.1016/j.ccr.2007.01.016.
- 532 Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D, Lassar  
533 AB (1995) **Correlation of terminal cell cycle arrest of skeletal muscle with**  
534 **induction of p21 by MyoD.** *Science* **267**: 1018–1021. doi: 10.1126/science.7863327.
- 535 Jacobs Y, Schnabel CA, Cleary ML (1999) **Trimeric association of Hox and TALE**  
536 **homeodomain proteins mediates Hoxb2 hindbrain enhancer activity.** *Mol Cell*  
537 *Biol* **19**: 5134–5142. doi: 10.1128/mcb.19.7.5134.
- 538 Kang H, Zhang C, An Z, Shen WH, Zhu Y (2019) **AtINO80 and AtARP5 physically**  
539 **interact and play common as well as distinct roles in regulating plant growth**  
540 **and development.** *New Phytol* **223**: 336–353. doi: 10.1111/nph.15780.
- 541 Keller C, Guttridge DC (2013) **Mechanisms of impaired differentiation in**  
542 **rhabdomyosarcoma.** *FEBS J* **280**: 4323–4334. doi: 10.1111/febs.12421.
- 543 Knoepfler PS, Bergstrom DA, Uetsuki T, Dac-Korytko I, Sun YH, Wright WE, Tapscott  
544 SJ, Kamps MP (1999) **A conserved motif N-terminal to the DNA-binding**  
545 **domains of myogenic bHLH transcription factors mediates cooperative DNA**  
546 **binding with pbx-Meis1/Prep1.** *Nucleic Acids Res* **27**: 3752–3761. doi:  
547 10.1093/nar/27.18.3752.
- 548 Kralovicova J, Knut M, Cross NC, Vorechovsky I (2015) **Identification of**  
549 **U2AF(35)-dependent exons by RNA-Seq reveals a link between 3' splice-site**

- 550        **organization and activity of U2AF-related proteins.** *Nucleic Acids Res* **43**: 3747–  
551        3763. doi: 10.1093/nar/gkv194.
- 552        Lee SY, Obata Y, Yoshida M, Stockert E, Williamson B, Jungbluth AA, Chen YT, Old  
553        LJ, Scanlan MJ (2003) **Immunomic analysis of human sarcoma.** *Proc Natl Acad*  
554        *Sci U S A* **100**: 2651–2656. doi: 10.1073/pnas.0437972100.
- 555        Lu J, McKinsey TA, Zhang CL, Olson EN (2000) **Regulation of skeletal myogenesis**  
556        **by association of the MEF2 transcription factor with class II histone**  
557        **deacetylases.** *Mol Cell* **6**: 233–244. doi: 10.1016/s1097-2765(00)00025-3.
- 558        Morita T, Hayashi K (2014) **Arp5 is a key regulator of myocardin in smooth muscle**  
559        **cells.** *J Cell Biol* **204**: 683–696. doi: 10.1083/jcb.201307158.
- 560        Nakka K, Ghigna C, Gabellini D, Dilworth FJ (2018) **Diversification of the muscle**  
561        **proteome through alternative splicing.** *Skelet Muscle* **8**: 8. doi:  
562        10.1186/s13395-018-0152-3.
- 563        Nasif S, Contu L, Mühlemann O (2018) **Beyond quality control: The role of**  
564        **nonsense-mediated mRNA decay (NMD) in regulating gene expression.** *Semin*  
565        *Cell Dev Biol* **75**: 78–87. doi: 10.1016/j.semcd.2017.08.053.
- 566        Ohkawa Y, Yoshimura S, Higashi C, Marfella CG, Dacwag CS, Tachibana T, Imbalzano  
567        AN (2007) **Myogenin and the SWI/SNF ATPase Brg1 maintain myogenic gene**  
568        **expression at different stages of skeletal myogenesis.** *J Biol Chem* **282**: 6564–6570.  
569        doi: 10.1074/jbc.M608898200.
- 570        Poli J, Gasser SM, Papamichos-Chronakis M (2017) **The INO80 remodeller in**  
571        **transcription, replication and repair.** *Philos Trans R Soc Lond B Biol Sci* **372**:  
572        20160290. doi: 10.1098/rstb.2016.0290.
- 573        Puri PL, Sartorelli V, Yang XJ, Hamamori Y, Ogryzko VV, Howard BH, Kedes L, Wang

- 574 JY, Graessmann A, Nakatani Y, Levrero M (1997) **Differential roles of p300 and**  
575 **PCAF acetyltransferases in muscle differentiation.** *Mol Cell* **1**: 35–45. doi:  
576 10.1016/s1097-2765(00)80005-2.
- 577 Roy K, de la Serna IL, Imbalzano AN (2002) **The myogenic basic helix-loop-helix**  
578 **family of transcription factors shows similar requirements for SWI/SNF**  
579 **chromatin remodeling enzymes during muscle differentiation in culture.** *J Biol*  
580 *Chem* **277**: 33818–33824. doi: 10.1074/jbc.M205159200.
- 581 Schroer TA (1999) **Actin-related proteins.** *Annu Rev Cell Dev Biol* **15**: 341–363.
- 582 Shen X, Mizuguchi G, Hamiche A, Wu C (2000) **A chromatin remodelling complex**  
583 **involved in transcription and DNA processing.** *Nature* **406**: 541–544. doi:  
584 10.1038/35020123.
- 585 Tapscott SJ (2005) **The circuitry of a master switch: Myod and the regulation of**  
586 **skeletal muscle gene transcription.** *Development* **132**: 2685–2695. doi:  
587 10.1242/dev.01874.
- 588 Yao W, Beckwith SL, Zheng T, Young T, Dinh VT, Ranjan A, Morrison AJ (2015)  
589 **Assembly of the Arp5 (Actin-related Protein) Subunit Involved in Distinct**  
590 **INO80 Chromatin Remodeling Activities.** *J Biol Chem* **290**: 25700–25709. doi:  
591 10.1074/jbc.M115.674887.
- 592 Yao W, King DA, Beckwith SL, Gowans GJ, Yen K, Zhou C, Morrison AJ (2016) **The**  
593 **INO80 Complex Requires the Arp5-Ies6 Subcomplex for Chromatin**  
594 **Remodeling and Metabolic Regulation.** *Mol Cell Biol* **36**: 979–991. doi:  
595 10.1128/MCB.00801-15.
- 596 Yuan W, Condorelli G, Caruso M, Felsani A, Giordano A (1996) **Human p300 protein**  
597 **is a coactivator for the transcription factor MyoD.** *J Biol Chem* **271**: 9009–9013.

598 doi: 10.1074/jbc.271.15.9009.

599 Zhou C, Zou J, Zou S, Li X (2016) **INO80 is Required for Osteogenic Differentiation**  
600 **of Human Mesenchymal Stem Cells.** *Sci Rep* **6**: 35924. doi: 10.1038/srep35924.