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RESEARCH ARTICLE

Ccndbp1 is a new positive regulator of skeletal myogenesis

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ABSTRACT

Skeletal myogenesis is a multistep process in which basic helix-loop-helix (bHLH) transcription factors, such as MyoD (also known as MyoD1), bind to E-boxes and activate downstream genes. Ccndbp1 is a HLH protein that lacks a DNA-binding region, and its function in skeletal myogenesis is currently unknown. We generated Ccndbp1-null mice by using CRISPR–Cas9. Notably, in Ccndbp1-null mice, the cross sectional area of the skeletal tibialis anterior muscle was smaller, and muscle regeneration ability and grip strength were impaired, compared with those of wild type. This phenotype resembled that of myofiber hypotrophy in some human myopathies or amyoplasia. Ccndbp1 expression was upregulated during C2C12 myogenesis. Ccndbp1 overexpression promoted myogenesis, whereas knockdown of Ccndbp1 inhibited myogenic differentiation. Co-transfection of Ccndbp1 with MyoD and/or E47 (encoded by *TCF3*) significantly enhanced E-box-dependent transcription. Furthermore, Ccndbp1 physically associated with MyoD but not E47. These data suggest that Ccndbp1 regulates muscle differentiation by interacting with MyoD and enhancing its binding to target genes. Our study newly identifies Ccndbp1 as a positive modulator of skeletal myogenic differentiation *in vivo* and *in vitro*, providing new insights in order to decipher the complex network involved in skeletal myogenic development and related diseases.

KEY WORDS: Ccndbp1, MyoD, Myogenesis, Skeletal muscle regeneration

INTRODUCTION

Members of the basic helix-loop-helix (bHLH) family of transcription factors play key roles in cell proliferation, cell survival, cell lineage commitment and cell fate determination through homo- or hetero-dimerization, and association with sequence-specific DNA elements called E-box sequences (Massari and Murre, 2000). The development of skeletal muscle, also called skeletal myogenesis, is a well-delineated multistep process that depends on linage commitment, cell cycle withdrawal and the activities of two groups of key bHLH transcription factors. One group of bHLH members, the E-proteins such as E12 and E47 (isoforms encoded by *TCF3*, which is also known as *E2A*), form either homo- or hetero-dimers, and are ubiquitously expressed in many tissues (Londhe and Davie, 2011). The DNA-binding specificity of E-proteins is dependent on the E-box consensus sequence CANNTG (Cordle et al., 1991; Voronova and Lee, 1994). The other group of bHLH members, the MyoD family, is specifically expressed in muscle. Proteins of this group are also known as myogenic regulatory factors (MRFs), which include MyoD (also known as MyoD1) (Tapscott et al., 1988), myogenin (Wright et al., 1989), Myf5 (Braun et al., 1989) and MRF4 (Rhodes and Konieczny, 1989). Comprising a basic region and two amphipathic helices, the bHLH domain in the MRFs and E-proteins is required for DNA binding and protein dimerization (Murre et al., 1989). MRF–E-protein heterodimers bind to the E-box consensus DNA sequence (CANNTG) in the promoter and enhancer region of muscle-specific genes and initiate the muscle gene activation (Lassar et al., 1989). The MRF expression pattern has been extensively described in cultured myogenic cells and the developing muscles. Each MRF has a unique expression pattern, suggesting that MRFs vary in their *in vivo* functions (Montarras et al., 1991; Dedieu et al., 2002; Chanoine et al., 2004). MyoD and Myf5 might be required for myoblast determination and cell cycle regulation (Rudnicki et al., 1992; Tajbakhsh et al., 1996; Lindon et al., 1998). Myogenin might be crucial for promoting myotube formation (Wright et al., 1989; Hasty et al., 1993; Nabeshima et al., 1993), whereas MRF4 might be required in myofibrillogenesis, the late stage of muscle differentiation (Rhodes and Konieczny, 1989; Patapoutian et al., 1995). Mutation or loss of function of these key factors and other related proteins, such as dystrophin, could cause congenital myopathy or amyoplasia. However, the mechanisms of muscle diseases vary and are largely unknown, and there are few efficient therapies (Sarnat, 1994).

MRF and E-proteins play positive roles in myogenesis, and several bHLH proteins negatively regulate myogenesis. The Id family of HLH proteins efficiently heterodimerize with E-proteins; however, lack of a basic region before the HLH domain means that they form 'non-functional' Id–E-protein heterodimers that compete with MRFs to heterodimerize with E12 and E47, and thus quench the E-protein activity and inhibit myogenic differentiation (Jen et al., 1992). Twist is also a bHLH protein that represses muscle-specific gene transcription by blocking MyoD binding to DNA and inhibiting myocyte enhancer factor 2 (MEF2) transactivity (Spicer et al., 1996). Mist1 is one member of the bHLH family that is expressed in myogenic stem cells. As a dominant-negative regulator of MRFs, Mist1 heterodimerizes with MyoD to form transcriptionally inactive DNA-bound complexes (Lemercier et al., 1998). Another muscle-restricted bHLH transcription factor, MyoR, heterodimerizes with E-proteins to prevent the binding of MRFs, represses the activation of E-box-dependent muscle genes and blocks myogenesis (Lu et al., 1999).

Ccndbp1 was initially identified by us and other laboratories as cyclin-D- and Grap2-interacting protein (DIP1, GCIP), and human homolog of murine maternal Id-like molecule (HHM) (Terai et al., 2000; Xia et al., 2000; Yao et al., 2000). Containing a

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HLH domain without a basic region, Ccndbp1 is similar to members of the Id family. Additionally, similar to the Myc family, Ccndbp1 contains putative leucine-zip domains. Previously, a northern blot assay has been used to detect expression of Ccndbp1 in all human tissues examined, with the highest expression in muscle, heart, leukocytes and brain (Xia et al., 2000). Mouse Ccndbp1, also called maternal Id-like molecule (Maid), has been isolated previously from a cDNA library that had been enriched for maternal transcripts from a mouse two-cell-stage embryo. During the transition from gamete to embryo, Ccndbp1 might be involved in the transcriptional and translational regulation of gene expression (Hwang et al., 1997). Our previous data indicates that the tumor suppressor Ccndbp1 is highly expressed in terminally differentiated tissues, and its expression is reduced in proliferating cells (Xia et al., 2000; Xiang et al., 2002). However, the role of Ccndbp1 in skeletal myogenesis has not been explored yet.

Here, we generated Ccndbp1-knockout (KO) mice by using CRISPR–Cas9 and found that the cross sectional area of the tibialis anterior muscle in Ccndbp1-KO mice was smaller than that in wild-type (WT) mice. The skeletal muscle regeneration efficiency of Ccndbp1-KO mice was impaired significantly compared to that in WT mice. Consistently, impaired forelimb grip strength was observed in KO mice. This hypotrophic myofiber phenotype resembles myopathy and amyoplasia in humans to some extent. Furthermore, we found that Ccndbp1 was upregulated during C2C12 myogenesis. Overexpression of Ccndbp1 promoted C2C12 myogenesis, whereas knocking down Ccndbp1 impaired myogenesis. A luciferase assay indicated that Ccndbp1 cooperates with MyoD–E47 heterodimers to activate E-box-dependent reporter gene transcription. A coimmunoprecipitation assay showed that Ccndbp1 interacts specifically with MyoD but not E47. The N-terminus (called here the N-bundle) and HLH domain are required for Ccndbp1–MyoD association. Chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assay (EMSA) indicated that Ccndbp1 overexpression enhances the binding efficiency of MyoD to its target promoters or enhancers. These results suggest that Ccndbp1 regulates muscle differentiation by interacting with MyoD and enhancing its binding to target genes. Our study identified Ccndbp1 as a new positive modulator of muscle differentiation *in vivo* and *in vitro*, providing a more detailed mechanism that can be used to decipher the complex network involved in skeletal muscle development and related diseases.

RESULTS

Myofiber hypotrophy in Ccndbp1-KO mice

To study the function of Ccndbp1 *in vivo*, we generated knockout mice using the CRISPR–Cas9 technique. Two different guide RNAs targeting two different regions of the *Ccndbp1* locus were injected into the zygote with Cas9, and two lines of Ccndbp1$^{-/-}$ (KO) mice were generated with a stop codon at amino acid 237 (Line 1, KO1) or a frameshift at amino acid 45 (Line 2, KO2) (Fig. 1A; Fig. S1). WT1 and WT2 are wild-type littermates of the KO1 and KO2 homozygotes, respectively. First, for the adult (6-weeks postnatal) mouse in both KO lines (Line 1 and Line 2), the body weight was not significantly different between WT, Ccndbp1$^{+/-}$ (Hetero lines) and Ccndbp1$^{-/-}$ (KO) mice (Fig. 1B). The Ccndbp1$^{+/-}$ and Ccndbp1$^{-/-}$ mice were normal in morphology and mortality, consistent with previous reports (Sonnenberg-Riethmacher et al., 2007). However, by hematoxylin and eosin (H&E) staining in a skeletal tibialis anterior muscle section, we found that the cross sectional area of myofiber in Ccndbp1 homozygous KO Line 1 mice was smaller compared to that of WT1 and Ccndbp1 heterozygous KO Line 1 mice (Fig. 1C). The frequency distribution of myofiber area in Ccndbp1-KO Line 1 mice was shifted to the left (lower) compared with values in WT mice (Fig. 1D). This phenotype of a smaller cross sectional area was also confirmed in Ccndbp1-KO Line 2 mice (Fig. 1E,F). We next performed a strength functional assay. By measuring the forelimb grip strength of 6-week-old mice, we found that the loss of Ccndbp1 significantly reduced the forelimb strength in both male and female mice (Fig. 1G). These *in vivo* data suggest that the skeletal muscle myogenesis efficiency and function is impaired in Ccndbp1-KO mice, and this result phenocopies some human skeletal muscle diseases, such as myofiber hypotrophy.

Loss of Ccndbp1 delays the regeneration of tibialis anterior muscle *in vivo*

To further explore the function of Ccndbp1 in muscle differentiation *in vivo*, we injected WT and Ccndbp1-KO mice with cardiotoxin (CTX). H&E staining was performed to monitor the muscle regeneration progress at various time points. Three days after CTX injection, WT1 and Ccndbp1-KO Line 1 mice exhibited extensive myofiber degeneration, suggesting that the injury causes severe myofiber degradation (Fig. 2A, upper panel). Seven days after CTX injection, the majority of damaged myofibers in WT1 mice had been replaced by newly formed myofibers with centralized nuclei. In contrast, in Ccndbp1-KO Line1 mice, damaged myofibers were still present 7 days after CTX injection (Fig. 2A, middle panel). Fourteen days after CTX injection, muscle architecture had been largely reconstructed in both WT1 and Ccndbp1-KO Line1 mice, indicating that the absence of Ccndbp1 delays regeneration but does not completely prevent this process (Fig. 2A, lower panel). Consistently, 7 days after injury, the tibialis anterior muscle weight was significantly decreased in Ccndbp1-KO Line1 mice compared to that in WT1 mice (Fig. 2B). We also quantified the average cross sectional area in regenerated myofibers in WT1 and Ccndbp1-KO Line 1 mice 14 days after injury. The average cross sectional area of new myofibers in Ccndbp1-KO mice was significantly smaller than in WT 14 days after injury (Fig. 2C). Central nucleation is a hallmark of muscle regeneration. At day 14 post injury, tibialis anterior muscle regeneration efficiency was quantified as the percentage of central nuclei, which is the percentage of myofibers with centrally located nuclei. The percentage of central nuclei in Ccndbp1-KO Line 1 mice was significantly decreased compared to that of WT mice (Fig. 2D). H&E staining of the regenerated myofibers in Ccndbp1-KO Line 2 mice at 14 days after injury also confirmed that the loss of Ccndbp1 decreased the area of newly regenerated myofibers and the percentage of central nuclei (Fig. 2E–G). These results demonstrate that the absence of Ccndbp1 delays the skeletal muscle regeneration process and decreases the regenerated myofiber area, indicating that the loss of Ccndbp1 delays skeletal muscle regeneration *in vivo*.

Ccndbp1 is upregulated during myogenesis

To investigate the expression profile of Ccndbp1 during myogenesis, we examined the expression pattern of Ccndbp1 during the C2C12 cell differentiation process. C2C12 cells are multipotent mesenchymal-derived murine cells that are capable of differentiating into myocytes (Bains et al., 1984; Ohyama et al., 2002; Akimoto et al., 2005). As shown by real-time quantitative reverse transcription PCR (qRT-PCR), northern blot and western blot results, when C2C12 cells were cultured in growth medium, both Ccndbp1 mRNA and protein were expressed at relatively lower levels, whereas they were upregulated after C2C12 cells were

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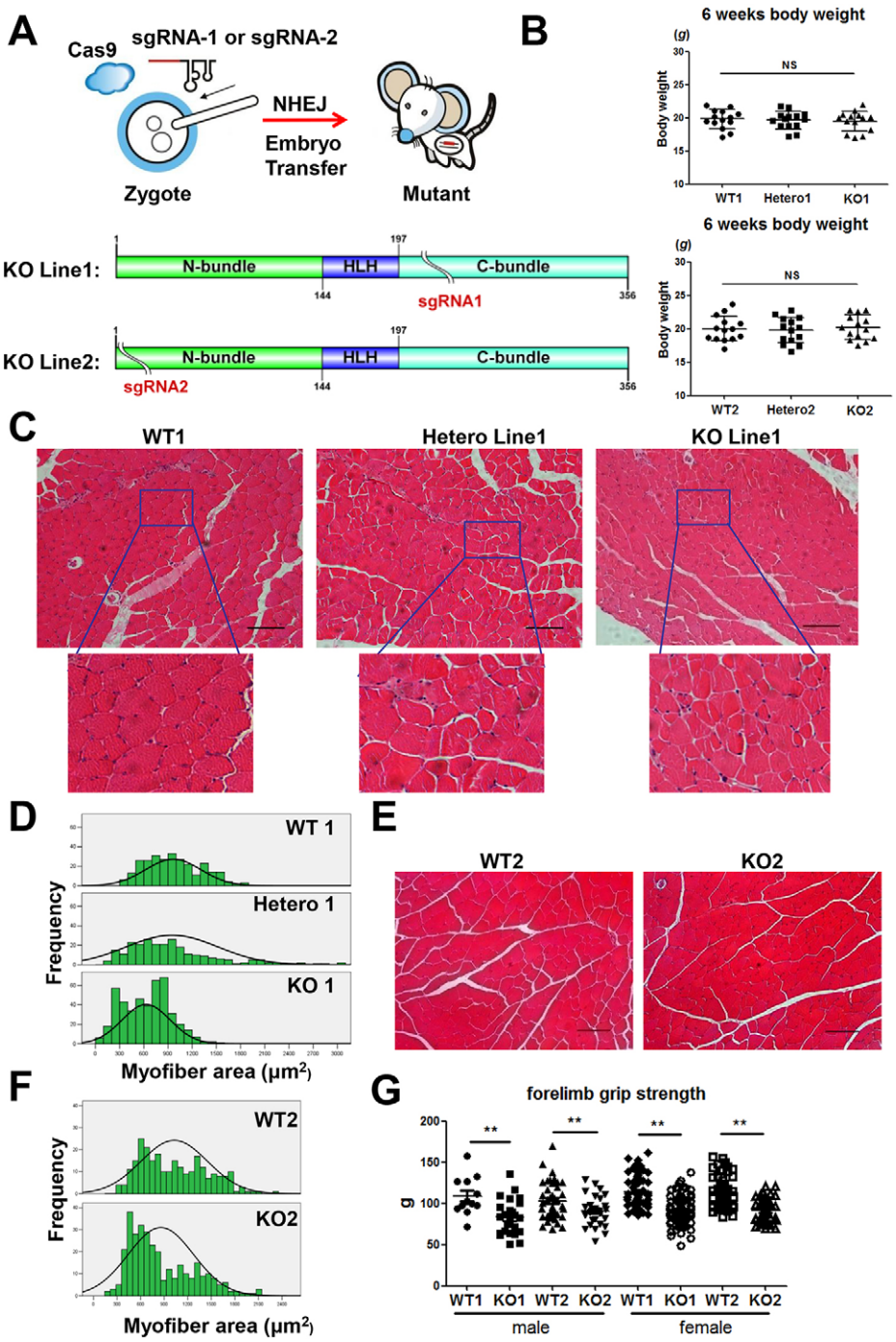

Fig. 1. Ccndbp1-KO mice show a phenotype of myofiber hypotrophy in tibialis anterior muscle. (A) Schematic diagram of CRISPR–Cas9-generated Ccndbp1-KO mice. Two gRNAs that targeted different loci were each injected into a zygote, finally generating two lines of Ccndbp1-KO mice. The DNA sequences were aligned using the Align X function and showed the frameshift in Ccndbp1-KO mice compared with WT mice. (B) Body weights of 6-week-old male control (WT), heterozygous (Hetero) and homozygous (KO) mice (Line 1 mice shown in upper panel and Line 2 mice shown in lower panel). Data are mean±s.d. NS, no significant difference (Student's *t*-test). (C) H&E-stained sections of tibialis anterior muscle from control (WT), heterozygous and KO mice (Line 1) at 6 weeks of age. Magnified images (×25) of the indicated regions are shown. Scale bars: 100 µm. (D) Statistical analysis of the myofiber cross sectional area distribution in WT, heterozygous and KO mice (Line 1). (E) H&E-stained sections of tibialis anterior muscle from WT and KO Line 2 mice at 6 weeks of age. Scale bars: 100 µm. (F) Statistical analysis of the myofiber cross sectional area distribution of WT and KO mice (Line 2). (G) Forelimb grip strength of both male and female WT and KO Line1 and Line 2 mice. Data are mean±s.d. Two kinds of WT controls were littermates of the respective KO lines (WT1 male, *n*=2; KO1 male, *n*=4; WT2 male, *n*=7; KO2 male, *n*=6; WT1 female, *n*=8; KO1 female, *n*=11; WT2 female, *n*=5; KO2 female, *n*=4; six measurements were taken from each mouse). ***P*<0.01 (Student's *t*-test).

transferred into differentiation medium (Fig. 3A–C). Myogenin, a myogenic differentiation marker, was upregulated after C2C12 cells were transferred into differentiation medium, demonstrating that myogenic differentiation occurred in our experiments (Fig. 3C). These results demonstrate that Ccndbp1 is upregulated during C2C12 myogenesis, indicating that it could play a role in the muscle differentiation process.

Ccndbp1 overexpression enhances C2C12 myogenesis

To examine the function of Ccndbp1 in myogenic differentiation, C2C12 myoblasts were stably transfected with plasmids encoding mouse Ccndbp1 and vector control. The expression level of exogenous Ccndbp1 was approximately tenfold greater than the endogenous level (Fig. 4A). Equivalent numbers of stable cells were pooled and induced to differentiate. After 5 days in differentiation medium, Ccndbp1-overexpressing C2C12 cells exhibited differential morphology and had more elongated myofibers compared to control cells (Fig. 4B). Myosin heavy chain (MHC) is a well-known terminal marker highly upregulated after myogenic differentiation. After 5 days in differentiation medium, cultured C2C12 cells that had been stably transfected with Ccndbp1 or vector were fixed and stained with an MHC-specific antibody (clone A4.1025, recognizing all MHC isoforms). As shown in Fig. 4C, MHC-positive myotubes were more fused and longer in Ccndbp1-overexpressing cells than in control cells. The ratios of MHC-positive cells and multinucleated myotubes in

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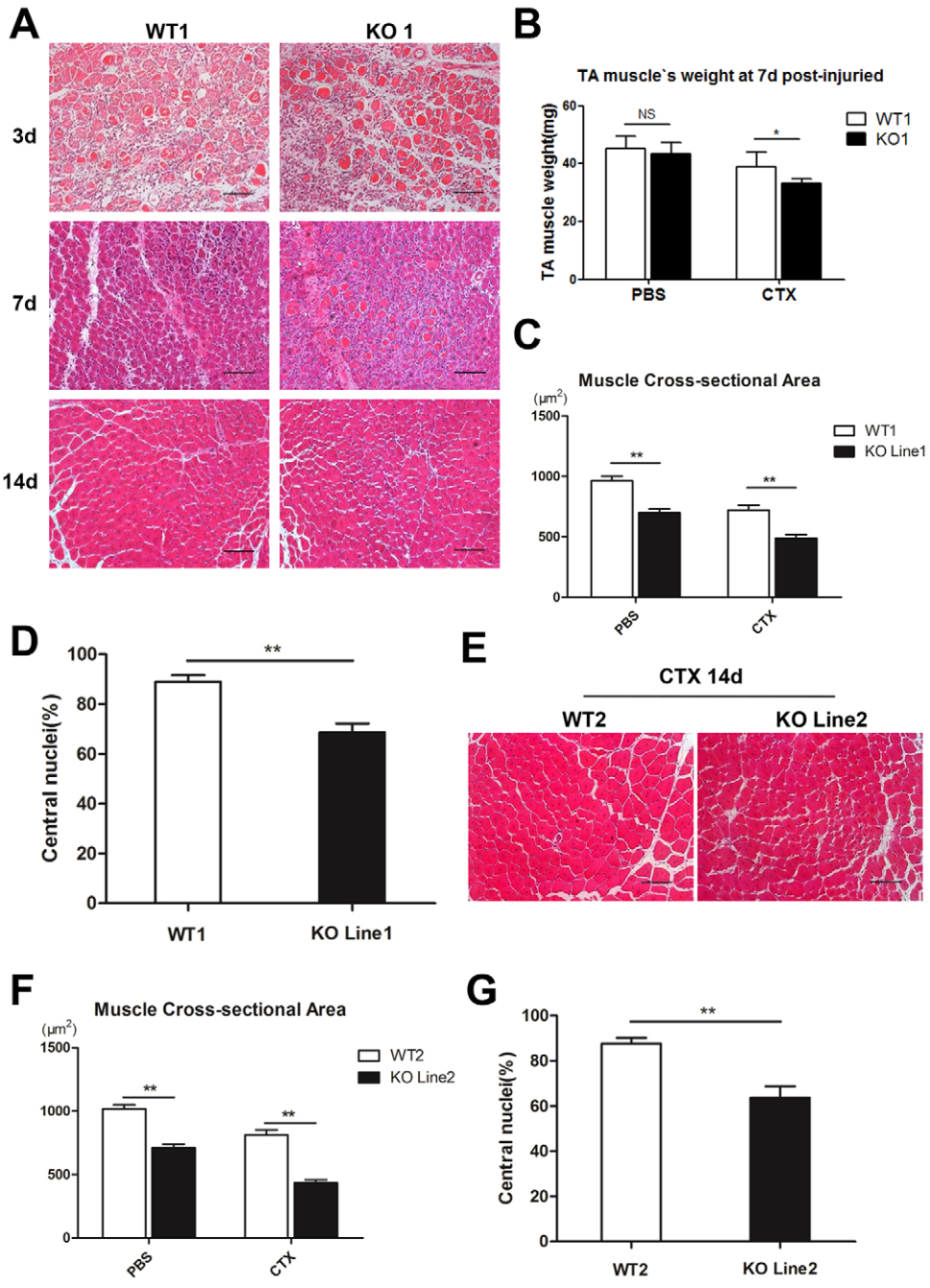

Fig. 2. Delayed skeletal muscle regeneration in Ccndbp1-KO mice after CTX injury. (A) Representative sections of tibialis anterior muscles from WT and Ccndbp1-KO mice (Line 1) stained with H&E at 3, 7 and 14 days after CTX injury. Scale bars: 100 µm. (B) The tibialis anterior muscles were weighed from Ccndbp1-KO mice compared with those from WT mice at 7 days post injury (n=6 in each group).The right leg tibialis anterior muscle of WT or Ccndbp1-KO mice was injected with CTX, whereas the left tibialis anterior muscle was injected with PBS as control. (C) Average cross sectional areas of tibialis anterior muscles in WT (n=3) and Ccndbp1-KO mice (n=3) (Line 1) 14 days after PBS or CTX treatment. The graph is based on the average myofiber cross section area distribution from three independent experiments. (D) The regenerating efficiency of muscles in WT and Ccndbp1-KO mice (Line 1) was calculated as the percentage of central nuclei, which means the percentage of myofibers that had centrally located nuclei. (E) Representative cross sections of tibialis anterior muscles from WT and Ccndbp1-KO mice (Line 2) stained with H&E at 14 days after CTX injury. Scale bars: 100 µm. (F) Statistical analysis of the KO Line 2 myofiber cross sectional area at day 14 after PBS or CTX injection (n=3). (G) The regenerating efficiency of WT and Ccndbp1-KO mice (Line 2) was calculated as the percentage of central nuclei. Error bars indicate the s.e.m. Student's t-test, $^{**}P$<0.01.

Ccndbp1-overexpressing and control cells are shown as the differentiation index and fusion index, respectively, confirming the pro-differentiation effect of Ccndbp1 overexpression in C2C12 cells (Fig. 4D,E). The ability of Ccndbp1 to enhance myogenesis was also confirmed by qRT-PCR validation of the expression of MHC (*Myh1*) and another myogenesis marker, myogenin, whereas the expression of MyoD did not change significantly during differentiation (Fig. 4F–H). These data demonstrate that Ccndbp1-overexpressing C2C12 cells differentiate with a substantially higher efficiency than control cells *in vitro*.

Ccndbp1 knockdown impairs C2C12 myogenesis

To further examine the requirement of Ccndbp1 for myogenic differentiation, C2C12 myoblasts were stably infected with retrovirus expressing small hairpin (sh)RNAs C1 and C2 that target Ccndbp1, as well as a control shRNA. The knockdown efficiency of C1 and C2 targeting endogenous Ccndbp1 was greater than 70%, and the C2 shRNA was more effective than the C1 shRNA (Fig. 5A). Control and Ccndbp1 stable-knockdown cells were induced to differentiate. After 5 days in differentiation medium, Ccndbp1-knockdown cells exhibited a different morphology and appeared to have fewer elongated myofibers compared to control cells (Fig. 5B). Immunofluorescence staining of MHC and statistical analysis of the differentiation index and fusion index confirmed the impaired differentiation effect of Ccndbp1 knockdown in C2C12 cells (Fig. 5C–E). qRT-PCR detection of MHC (*Myh1*) also suggested that the differentiation efficiency is lower in Ccndbp1-knockdown cells compared to that in control cells (Fig. 5F). These data demonstrate that Ccndbp1 is required for the differentiation of C2C12 myoblasts *in vitro*.

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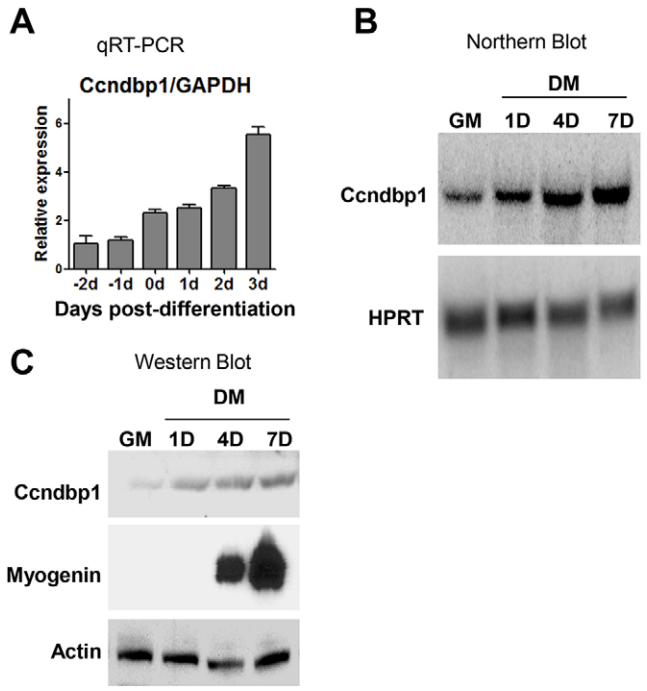

Fig. 3. Ccndbp1 is upregulated during C2C12 myogenesis. (A) qRT-PCR analysis was performed of Ccndbp1 mRNA from C2C12 cells at different days post differentiation; when the cells reached growth arrest, it was defined as day 0 and growth medium (GM) was changed for differentiation medium (DM). GAPDH was used as internal control. Data are presented as mean±s.d. (B) Northern blot analysis of Ccndbp1 mRNA transcripts was performed in murine C2C12 cells in growth medium, as well as at days 1, 4 and 7 in differentiation medium with the ^{32}P-labeled mouse Ccndbp1 cDNA probe. Transcripts of hypoxanthine-guanine phosphoribosyltransferase (HPRT) were measured as an equal loading control. (C) Western blot analysis of Ccndbp1 protein expression was performed in C2C12 cells grown in growth medium, as well as at days 1, 4 and 7 (1D, 4D and 7D, respectively) in differentiation medium. Western blotting for the myogenic marker myogenin was performed to ensure that myogenic differentiation occurred properly. Actin was used as loading control.

Ccndbp1 interacts with MyoD and might enhance its binding to target genes

Previous studies have shown that the myogenic bHLH E-proteins and MyoD work cooperatively to form a heterodimer and activate muscle-specific gene expression. Because Ccndbp1 is also a HLH protein that is highly expressed in muscle, we investigated whether Ccndbp1 can interfere with heterodimer-mediated muscle-specific gene activation. Plasmids expressing Ccndbp1, E47 and MyoD were co-transfected with a 4Rtk–luc reporter plasmid into 293 T cells in various combinations, as indicated in Fig. 6A. Containing four E-box sites fused with a minimal thymidine kinase promoter, 4Rtk–luc is a MyoD-responsive reporter, as previously reported (Weintraub et al., 1990). As expected, co-transfection of MyoD and E47 activated 4Rtk–luc transcription more efficiently than transfection of either alone (Fig. 6A, bar 7 compared to bars 3 and 5). Interestingly, Ccndbp1 overexpression significantly enhanced MyoD- or E47-mediated transactivation of the 4Rtk–luc reporter compared to vector controls (Fig. 6A, bars 4 and 6 compared to bars 3 and 5, respectively). Furthermore, the co-transfection of Ccndbp1 with both E47 and MyoD further activated 4Rtk–luc reporter gene transcription to a higher level than in combination with E47 or MyoD alone (Fig. 6A, bars 7 and 8 compared to others). Consistently, the co-transfection results of Ccndbp1, MyoD and E47 with another E-box-containing reporter plasmid, MCK4800, which contains a 4800-bp muscle creatine kinase (MCK; also known as CKM) promoter, showed that Ccndbp1 overexpression could activate the MyoD–E47 complex transactivity on the MCK4800 promoter (Fig. 6B). These results suggest that Ccndbp1 can activate myogenesis synergistically with MyoD–E47 heterodimers.

To obtain more detail about the relationship between Ccndbp1 and MyoD–E47, 293 T cells were transfected with E47-, MyoD- and Ccndbp1-encoding plasmids, and then subjected to coimmunoprecipitation with GST beads. As previously reported, MyoD interacted with E47, indicating that the system is effective and reliable (Fig. 6C, lane 1). Interestingly, Flag-tagged Ccndbp1 bound to GST-tagged MyoD but not to E47 (Fig. 6C, lane 6 compared to lane 7). Myf5 is another MRF that could have redundant functions with MyoD (Kablar et al., 1997). We performed a coimmunoprecipitation assay and found that there was no association between Myf5 and Ccndbp1, indicating the specificity of the MyoD–Ccndbp1 interaction (Fig. S2). We also mapped the interaction domain of Ccndbp1 and MyoD. Five constructs containing the Ccndbp1 N-terminal (N-bundle), HLH and C-terminal (C-bundle) domains were used to perform coimmunoprecipitation assays. A region containing the N-bundle and HLH domain is required for Ccndbp1–MyoD association (Fig. S3). In order to determine whether Ccndbp1 blocks the MyoD–E47 interaction, we examined their interaction by performing coimmunoprecipitation assays. GST–E47 could interact with MyoD–SFB and pull down Ccndbp1 as well, suggesting that formation of a MyoD–E47–Ccdndp1 complex is possible (Fig. 6D). Next, we performed a ChIP assay in 2-day-differentiated Ccndbp1-overexpressing C2C12 cells and found that MyoD binding to several target promoters or enhancers, including those of myogenin, Tcap and Lmod2, was enhanced in Ccndbp1-overexpressing C2C12 cells compared to control cells (Fig. 6E). Furthermore, we performed an EMSA assay by using purified proteins and C2C12 nuclear extracts with different probes containing the E-Box sequence. No inhibitory effects of Ccndbp1 on the formation of E47–MyoD–E-box complexes was observed. Adding Ccndbp1 might enhance formation of the complex and retard migration of the complex in the gel (Fig. S4, lane 4 compared to lane 3, and lane 8 compared to lane 7). The results of the C2C12 nuclear extract EMSA indicate an enhancement of endogenous complex formation in Ccndbp1-overexpressing C2C12 nuclear extracts (Fig. S4). These results suggest that Ccndbp1 could promote myogenesis by associating with MyoD and enhancing the formation of associated complexes and their activity (Fig. 6F).

DISCUSSION

MRFs, a family of bHLH transcription factors that includes MyoD and Myf5, play key roles in muscle development by heterodimerizing with E-proteins and binding to E-box DNA elements with consensus CANNTG sequences in the promoter or enhancer region of muscle-specific genes (Lassar et al., 1989). Previous studies have shown that many other HLH and bHLH proteins, such as Id-family proteins Mist1, Twist and MyoR, negatively regulate myogenesis (Benezra et al., 1990; Spicer et al., 1996; Lemercier et al., 1998; Lu et al., 1999). Previously, we have identified Ccndbp1 as a HLH protein that is associated with Grap2 and cyclin D1. Ccndbp1 is expressed in all the human tissues that have been examined, with the highest levels in muscle, heart, brain and leukocytes (Xia et al., 2000). At the same time, Ccndbp1 has been confirmed to bind to E12 in yeast two-hybrid and *in vitro* co-

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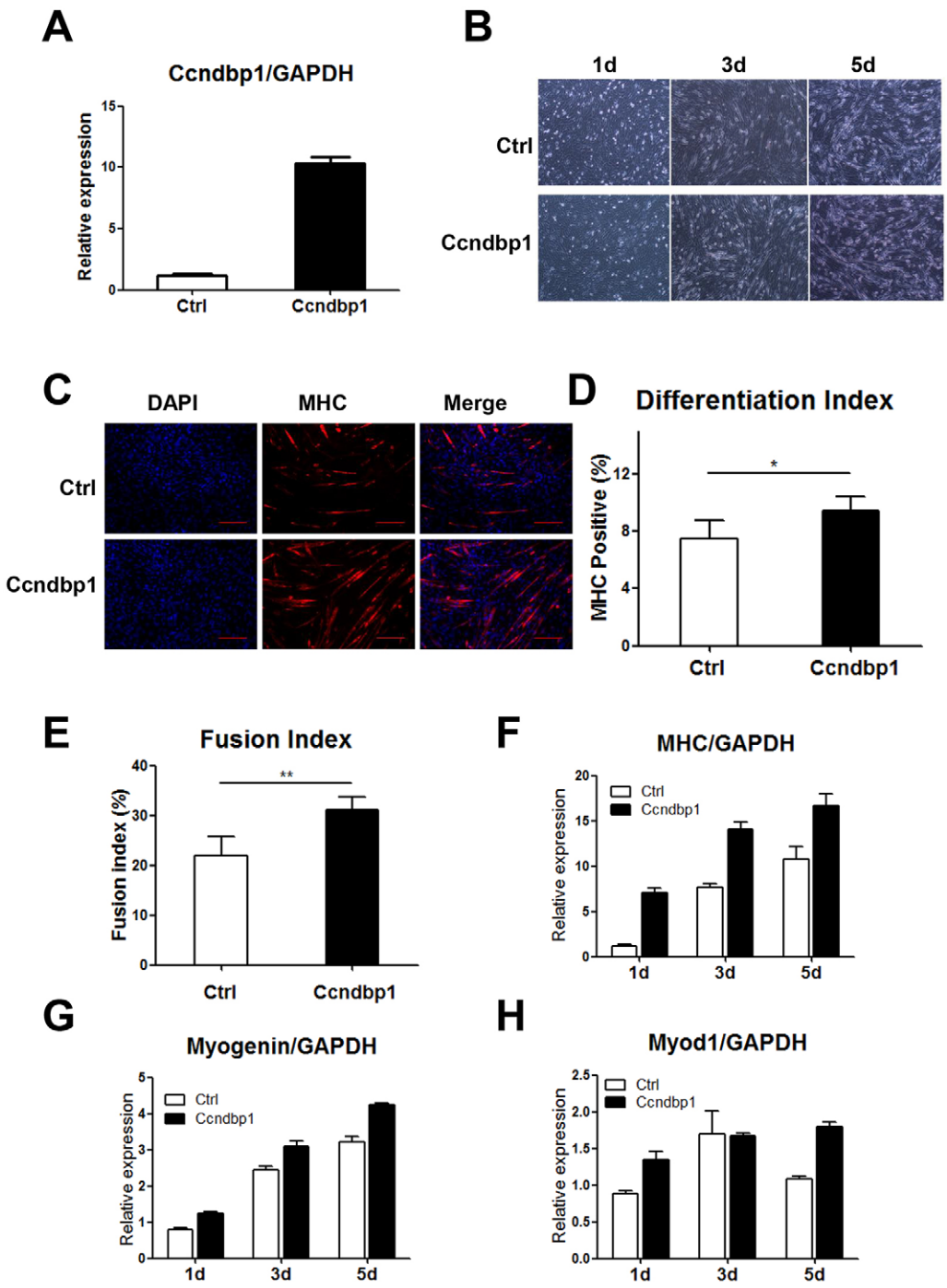

Fig. 4. Ccndbp1 overexpression promotes myogenesis in C2C12 cells. (A) C2C12 cells were stably transfected with either a vector (Ctrl) or Ccndbp1-encoding plasmids and screened for expression with 1 µg/ml puromycin. The Ccndbp1 expression level was detected by performing qRT-PCR; GAPDH was used as internal control. (B) C2C12 cells stably transfected with either control vector or Ccndbp1-encoding plasmids were subjected to differentiation conditions. Photos were taken at days 1, 3 and 5 of differentiation. (C) Control vector and Ccndbp1 stably transfected C2C12 myoblasts were induced to differentiate in differentiation medium and immunostained for myosin heavy chain (MHC, all isoforms, green) and DAPI (blue) at 5 days after differentiation. (D) Graph illustrating percentage of MHC-positive cells per field of vision in Fig. 2C as the differentiation index. The data are mean±s.d. of at least three independent experiments. (E) Graph illustrating the fusion index, representing the percentage of multi-nucleated myotubes per field of vision after differentiation for 5 days. (F–H) qRT-PCR analysis was performed to detect the levels of different myogenic markers MHC, myogenin and MyoD (MyoD1) at days 1, 3 and 5 post-differentiation. GAPDH was used as internal control. **P*<0.05; ***P*<0.01 (Student's *t*-test).

precipitation assays (Terai et al., 2000). Therefore, we hypothesized that Ccndbp1 might act as a HLH transcriptional regulatory protein and that might regulate myogenic differentiation.

In this report, we generated two lines of Ccndbp1-KO mice and found that the average myofiber size in skeletal tibialis anterior muscle sections was significantly smaller in Ccndbp1-KO mice compared to in WT mice. Importantly, the grip strength was decreased in Ccndbp1-KO mice, confirming that Ccndbp1 is required for skeletal muscle function (Fig. 1). This phenotype resembles the myofiber hypotrophy in human myopathies or amyoplasia to some extent. The loss of Ccndbp1 delays the regeneration rate in CTX-injured skeletal tibialis anterior muscle (Fig. 2). These results demonstrate that Ccndbp1 promotes myogenesis *in vivo*. Next, we found that both Ccndbp1 mRNA and protein levels were upregulated during the myogenic differentiation process in C2C12 cells (Fig. 3). To further assess the function of Ccndbp1 in myogenesis, Ccndbp1 was stably overexpressed and knocked down in C2C12 cells. The *in vitro* differentiation results showed that Ccndbp1 overexpression promotes myogenesis, whereas RNA interference (RNAi)-mediated knockdown of Ccndbp1 impaired myogenesis in C2C12 cells, which was confirmed by assessing myotube formation and myogenic differentiation biomarkers, including MHC and myogenin (Figs 4 and 5). As a HLH protein that is predicted to lack a DNA-binding region, Ccndbp1 was once suspected to be an Id-like molecule (Hwang et al., 1997). Id-family proteins negatively regulate myogenesis by forming inactive

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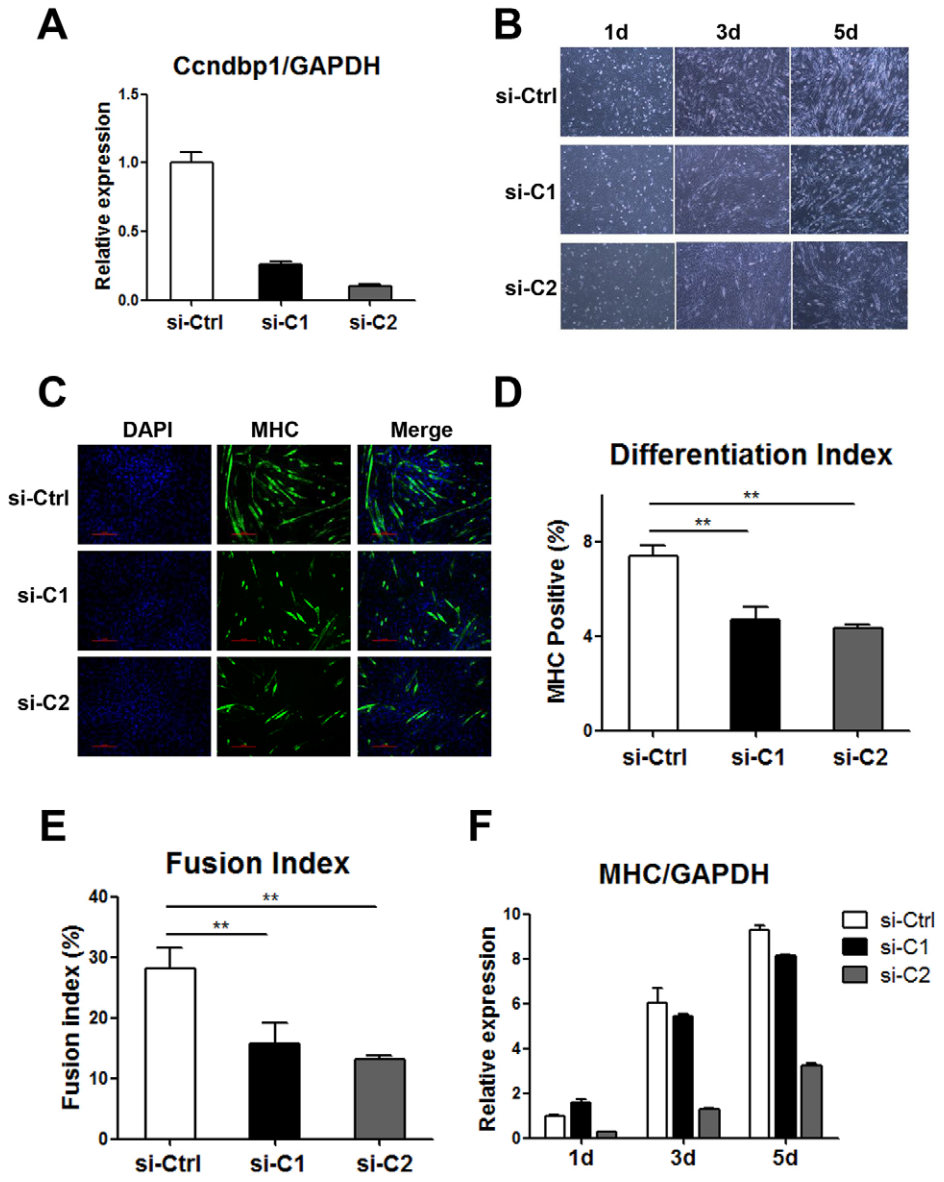

Fig. 5. Ccndbp1 knockdown inhibits myogenic differentiation in C2C12 cells. (A) C2C12 myoblasts were infected with control shRNA (si-Ctrl) or two shRNAs against Ccndbp1 (si-C1 and si-C2), and screened with 1 µg/ml puromycin for 1 week to form stable cell lines. Knockdown efficiency was detected by performing qRT-PCR with GAPDH as internal control. (B) Control and two shRNA-Ccndbp1 stable C2C12 cell lines were subjected to differentiation conditions, and photos were taken at days 1, 3 and 5 of differentiation to compare cell morphology changes. (C) Control and two shRNA-Ccndbp1 stable C2C12 myoblast lines were induced to differentiate in differentiation medium, and were immunostained for MHC (all isoforms, green) and DAPI (blue) at 5 days post-differentiation. (D) Graph illustrating the percentage of MHC-positive cells per field of vision in Fig. 3C as the differentiation index. The data are mean±s.d. of at least three independent experiments. (E) Graph illustrating the fusion index in control and two shRNA-Ccndbp1 stable C2C12 myoblasts, representing the percentage of multi-nucleated myotubes per field of vision after differentiation for 5 days. (F) qRT-PCR analysis was performed to detect the level of MHC at days 1, 3 and 5 post-differentiation. GAPDH was used as internal control. **P*<0.05; ***P*<0.01 (Student's *t*-test).

heterodimers with E-proteins (Jen et al., 1992; Langlands et al., 1997). Interestingly, our data reveal that the effect of Ccndbp1 on C2C12 myogenesis is the opposite to that of Id proteins. We demonstrated that Ccndbp1 overexpression could promote the transactivation of MyoD–E47 on E-box reporter genes. The coimmunoprecipitation assay revealed that Ccndbp1 specifically interacts with the key myogenic transcription factor MyoD but not E47. Furthermore, the ChIP and EMSA analyses showed that Ccndbp1 overexpression enhanced the binding efficiency of MyoD or MyoD–E47 to its target gene promoters or enhancers (Fig. 6). Collectively, we have identified Ccndbp1 as a new positive regulator of skeletal myogenesis both *in vivo* and *in vitro*. We propose the model that during the differentiation process, Ccndbp1 improves myogenesis by interacting with MyoD, promoting formation and activity of MyoD-containing complexes (Fig. 6F).

Compared with the MRF family of bHLH transcription factors, whose roles in myogenesis have been extensively studied, the function of the widely expressed *E2A*-encoded bHLH proteins (E47 and E12) is poorly defined (Roberts et al., 1993). E-proteins can heterodimerize with other tissue-specific bHLH proteins and are also involved in cardiogenic, neurogenic and hematopoietic differentiation through heterodimerization with the cardiogenic family of proteins, such as eHand and dHand; the neurogenic family of proteins, such as neurogenin and NeuroD; and the hematopoietic family of bHLH proteins, such as TAL1 (also known as SCL) and Lyl1 (Hsu et al., 1991; Kageyama et al., 1995; Bounpheng et al., 2000; Ik Tsen Heng and Tan, 2003). The heterodimers of E47 and other HLH proteins activate E-box-dependent transcription, and E-box elements are present in the promoter and enhancer regions of many tissue-specific genes that regulate muscle, neuron and hematopoietic gene expression. Ccndbp1 was also broadly expressed at the highest level in muscle, brain and peripheral blood leucocytes (Xia et al., 2000). Our previous data reveals that Ccndbp1 interacts with Grap2, an important leukocyte-specific adaptor protein in immune cell signaling. Ccndbp1 might be involved in differentiation processes other than myogenesis, especially leukocyte differentiation. A recent study found that T cells and cytokines are required for muscle stem cell proliferation *in vivo* (Fu et al., 2015). Our protein network analysis also indicates that Ccndbp1 interacts with many immune-response-related factors

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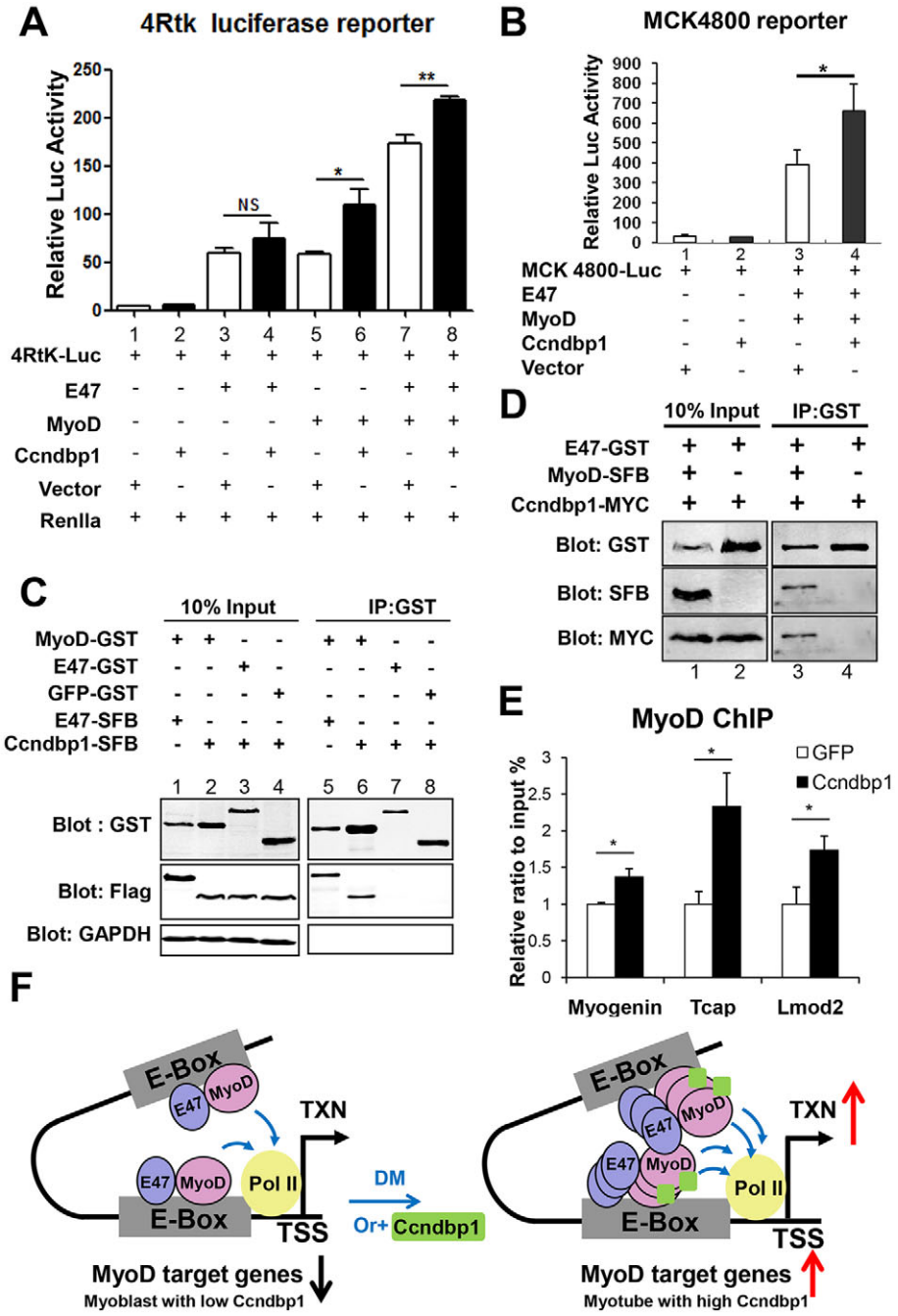

Fig. 6. Ccndbp1 promotes MyoD–E47-dependent transactivation of E-box genes and physically interacts with MyoD. (A) 293 T cells were transiently transfected with 4Rtk–luc and expression vectors encoding the indicated proteins. Cells were harvested 48 h post transfection with 1×passive buffer (Promega). Firefly and *Renilla* luciferase activity was measured using the Dual-Luciferase Reporter Assay System kit (Promega). The data are presented as the normalized ratio of firefly luciferase activity to the *Renilla* luciferase activity. Results are mean±s.d. of triplicate transfections, each conducted in duplicate. (B) 293 T cells were transiently transfected with MCK4800–luc with expression vectors encoding the indicated proteins, and the luciferase assay was performed as described in A. (C) Coimmunoprecipitation assay. 293 T cells were transfected with the indicated combinations of MyoD–GST, E47–GST, GFP–GST, E47–SFB and Ccndbp1–SFB. At 48 h post transfection, the cell lysate was subjected to coimmunoprecipitation, and western blot analysis was performed with the indicated antibodies. GST–MyoD and SFB–E47 were coimmunoprecipitated as positive controls. GST–GFP and SFB–Ccndbp1 were used as negative controls. Input represents 10% of the amount of cell lysate used in the coimmunoprecipitation experiment. GAPDH was used as loading control. IP, immunoprecipitation. (D) Coimmunoprecipitation assay of E47, MyoD and Ccndbp1. 293 T cells were transfected with the indicated combinations of E47–GST, MyoD–SFB and Ccndbp1–Myc. At 48 h post transfection, the cell lysate was subjected to coimmunoprecipitation, and western blot analysis was performed with the indicated antibodies. (E) ChIP assay of MyoD binding to target gene promoters or enhancers (myogenin, Tcap and Tmod2) in 2-day-differentiated C2C12 cells overexpressing Ccndbp1 or control vector (GFP). Data are normalized as a percentage of the input and are presented as mean±s.d.; **P*<0.05 (Student's *t*-test); NS, not significant. (F) A model for the role of Ccndbp1 in promoting skeletal myogenesis through binding to MyoD and enhancing its binding to target genes. Left, myoblasts express low levels of Ccndbp1; thus, the MyoD transactivity is low, and MyoD-targeted genes are expressed to a low level in order to maintain an undifferentiated state. Right, after differentiation or overexpression of Ccndbp1, high levels of Ccndbp1 result in binding to MyoD, stimulating formation of complexes and their activity. MyoD-targeted genes are expressed to a high level, promoting the myoblast switch to myotubes. Pol II, RNA polymerase II; TXN, transcription; TSS, transcriptional start site.

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(data not shown). The function of Ccndbp1 in T-cell function and cytokine secretion remains unknown. Further studies on the effect of Ccndbp1 on hematopoietic differentiation will help to elucidate other functions of Ccndbp1 in the near future.

Another interesting issue is that both E47 and E12 are expressed in C2C12 cells, but which of them Ccndbp1–MyoD acts through is unknown. Both E12 and E47 are spliced products from the *E2A* gene and contain two different transcriptional activation domains in the N-terminus (Aronheim et al., 1993). Thus, E47 is distinguished from E12 through differences in dimerization and DNA-binding domains. E47 is reported to bind to DNA to a greater extent than E12 and to stimulate transcription with a much higher efficiency than E12, which could account for the different effects of E12 and E47 on the activation of E-box-dependent gene transcription when co-transfected with Ccndbp1 (Choi et al., 1996; Sigvardsson, 2000; Frasca et al., 2003). During myogenesis, E47 might have an important function. Consistently, we found that Ccndbp1 is unable to activate the transactivation of 4Rtk–luc upon co-transfection of MyoD with E12 (data not shown). We speculate that E47 and E12 have different functions in muscle differentiation and that Ccndbp1 might play a positive role in myogenesis through MyoD–E47 heterodimer-mediated transactivation.

In this work, we found that Ccndbp1 could enhance myogenesis by interacting with MyoD, promoting its binding to target genes. Moreover, other possible mechanisms cannot be completely ruled out. Ccndbp1 is reported to be a negative cell cycle regulator, although this mechanism has not been demonstrated previously in skeletal muscle differentiation. MyoD has also been found to regulate cell cycle exit and thus to promote myogenesis (Kitzmann and Fernandez, 2001; Rajabi et al., 2014). We found in our work that Ccndbp1 overexpression could inhibit the G1/S transition during C2C12 differentiation (data not shown), and it is known that MyoD regulates myogenesis through cell cycle regulation, so Ccndbp1 might also cooperate with MyoD to regulate the cell cycle in muscle cells. How Ccndbp1 associates with MyoD and promotes its binding to target genes and crosstalk with transcriptional cascades or other signal transduction pathways remain to be uncovered in the near future. The interaction of Ccndbp1 with MyoD could explain, at least in part, the muscle-differentiation-promoting effect of Ccndbp1 *in vivo* and *in vitro*. In summary, our work identifies Ccndbp1 as a new myogenic regulator of skeletal muscle, providing new insight into the complex network involved in skeletal muscle development and related diseases.

MATERIALS AND METHODS

Generation of Ccndbp1-knockout mice with CRISPR–Cas9

For *in vitro* Cas9 mRNA transcription, the pT7-3XFlag-hCas9 plasmid was linearized with restriction enzyme *Pme*I and *in vitro* transcribed using the mMESSAGE mMACHINE T7 ULTRA transcription kit (Thermo Fisher). The guide (g)RNA sequences were cloned into the pDR274 vector and were *in vitro* transcribed using the MEGAshortscript T7 transcription kit (Thermo Fisher). Subsequently the Cas9 mRNA and the gRNAs were purified using the MEGAclear kit (Thermo Fisher), dissolved in RNase-free water and quantified using NanoDrop 1000 (Thermo Fisher). The target sequences of gRNAs were as follows: Line1 (KO1), 5'-CCAGTATGAG-TCCCGATTGC-3'; Line1 (KO2), 5'-CCCCTTCCTTGCTCCGCCTC-3'. Cas9 mRNA, together with gRNA, were injected into 0.5-day zygotes of C57BL/6J mice. Two hours after injection, these zygotes were transplanted into the oviduct of 0.5-day pseudopregnant recipient female mice. And 20 days later, the founder mice were born. All animal experiments were approved by the Institutional Animal Care and Use Committees of Sun Yat-sen University. Forelimb grip strength was measured at 6 weeks postnatally. For each mouse, the assay was performed six times and each time before a strength assay, a 5 min break was added. The strength was quantified, and the mean was calculated.

Tibialis anterior muscle injury assay

Muscle injury was induced (6–8-week-old male mice) through injection of CTX (Sigma-Aldrich), as previously described (Fu et al., 2015). Briefly, 20 µl of 10 µM CTX was injected into the right tibialis anterior muscle using 31-gauge needles (BD Biosciences), three injections were performed for one right leg tibialis anterior muscle; the left tibialis anterior muscle was injected with PBS as negative control. After 3, 7 and 14 days, the tibialis anterior muscles were harvested, and H&E staining was performed as previously reported (Ma et al., 2007). The myofiber average cross sectional area was quantified by using Image pro plus6 software, and the percentage of fibers with central nuclei was calculated. For each sample, at least 900 fibers from three independent visual fields were analyzed. The average cross sectional areas and the grip strength are presented as means±s.e.m. All the other statistical data are presented as means±s.d. The statistical significance of differences between each mean was calculated using Student's *t*-test; significance was taken as $P<0.05$ (*) or $P<0.01$ (**).

Cell culture and C2C12 cell differentiation

C2C12 murine myoblasts and 293 T cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone) supplemented with 10% fetal bovine serum (FBS; Hyclone). All the cells were maintained in the incubator under 5% CO_2. Before differentiation, C2C12 cells were grown to 100% confluence in growth medium, comprising 90% DMEM supplemented with 10% FBS. Differentiation was induced by replacing growth medium with differentiation medium, comprising 98% DMEM supplemented with 2% horse serum (Gibco). Differentiation medium was replaced every 24 h. For overexpression, the Ccndbp1-pENTR vector was cloned into a HA-Flag-DEST-Retrovirus vector using an LR recombination reaction. The HA-Flag-DEST-Ccndbp1 and control vectors were transfected into C2C12 cells using Lipofectamine 2000 (Thermo Fisher) and stably selected in 1 µg/ml puromycin (Sigma-Aldrich) for 1 week. For RNAi, two shRNAs were designed using the Whitehead Institute siRNA designing tool, verified by BLAST searches to ensure specificity, annealed and ligated into a pSiren-RetroQ vector. Retroviruses were generated by co-transfecting recombinant pSiren-RetroQ plasmids with pCL10A1 helper plasmid into 293 T cells using Lipofectamine 2000 (Thermo Fisher). Cell culture supernatants containing retroviruses were harvested 48 h and 72 h later, and were passed through 0.45-µm filters. C2C12 cells were passaged onto 6-well plates 24 h before infection. Virus-containing supernatants were supplemented with 20 µg/ml polybrene and added to the cells with centrifugation at 1200 ***g*** at 30°C for 1.5 h. Twenty-four hours post spin infection, C2C12 cells were selected with 1 µg/ml puromycin.

Northern blot analyses

Total RNA was isolated from C2C12 cells by using TRIZOL reagent (Thermo Fisher), separated on a 1% agarose-formaldehyde gel and then transferred by capillary blotting overnight onto Hybond$^+$ membrane (Amersham-Pharmacia Biotech) in 10× saline sodium citrate (SSC). The Ccndbp1 probe was labelled with [α-^{32}P]dCTP (Amersham) using the Random Prime DNA labelling system (Thermo Fisher) and hybridized according to the manufacturer's protocol. Pre-hybridization and hybridization were performed in Rapid-Hyb buffer (Amersham) at 68°C for 1 h and overnight, respectively. The membrane was washed twice in 2× SSC with 0.1% SDS at room temperature for 30 min, followed by two washes in 0.1× SSC with 0.1% SDS at 68°C for 30 min. For the internal control, the membrane was stripped and re-hybridized with a hypoxanthine guanine phosphoribosyl transferase (HPRT) probe using the same protocol. Blots were exposed overnight onto a BioRad Phosphorimager screen.

Coimmunoprecipitation and western blot analyses

For coimmunoprecipitation experiments, 4 µg of GST-tagged plasmids and 2 µg of SFB-tagged plasmids (S tag, Flag epitope tag and streptavidin-binding peptide tag) were co-transfected into 6-cm-dish-plated 293 T cells (90% confluence) with PEI (Sigma-Aldrich). After 48 h, the cells were

washed and lysed in 1 ml NETN buffer with freshly added proteinase inhibitors from Roche. After centrifugation at 15,000 ***g*** for 5 min, the supernatant was added to 20 µl of equilibrated anti-GST beads (GE Healthcare) and incubated at 4°C. After 2 h, the beads were washed four times with NETN buffer. The beads were boiled in SDS loading buffer and western blotted with the indicated antibodies.

Cell extracts of equal total protein (20 µg each) were separated by performing SDS-PAGE, and the proteins were transferred to Nylon membrane (Merck Millipore). The membranes were blocked with 5% nonfat milk in TBST (10 mM Tris pH 8.0, 150 mM NaCl with 0.5% Tween-20) washing buffer for 1 h at room temperature and then incubated at 4°C overnight with primary antibodies. After washing the membranes were labelled with HRP-conjugated secondary antibodies and detected with ECL (PIERCE) (for antibodies against Ccndbp1, myogenin and actin), or labelled with fluorescence-conjugated secondary antibodies and detected with Odyssey Imagers (for GST, Flag, Myc and GAPDH).

The antibodies used were: rabbit polyclonal anti-Flag (1:5000; F7425, Sigma), mouse monoclonal anti-GST (1:5000; M20007, Abmart), mouse monoclonal anti-GAPDH (1:5000; M20006M, Abmart), goat polyclonal anti-actin (1:1000; sc-1616, Santa Cruz), mouse monoclonal anti-myogenin (1:1000; sc-12732, Santa Cruz). The anti-rabbit Ccndbp1 antibody was an in-house-generated polyclonal rabbit sera directed against purified human Ccndbp1.

Immunofluorescence

For staining of MHC, cells growing on glass coverslips were rinsed with PBS, fixed in 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100 and blocked for 1 h in PBS containing 2% BSA. After incubation with the anti-MHC monoclonal antibody (1:500; 05-716, Millipore) overnight at 4°C and washed three times with PBS; fluorescein-conjugated goat anti-mouse secondary antibodies (Jackson Laboratory) were diluted in PBS containing 2% BSA and applied to the cells for 1 h at room temperature. After three washes with PBS, cells were stained with DAPI for 1 min, and the coverslips were mounted with mounting medium (DAKO). Coverslips were visualized at 200× on a CCD camera mounted on a Nikon Ti-E microscope using imaging software.

Luciferase reporter assays

293 T cells were transfected with 0.1 µg 4Rtk or MCK4800 plasmids and an expression vector encoding the indicated proteins in the well of a 24-well plate. A plasmid encoding *Renilla* luciferase was used as internal control, and an empty expression vector was used to normalized the total transfection amount. Either 0.1 µg of E47 or MyoD, or a combination of both expression plasmids, were transfected into 293 T cells together with 0.1 µg Ccndbp1 or control vectors. 48 h after transfection, the cells were lysed and harvested in 200 µl reporter lysis buffer (Promega). The luciferase assay was performed using dual luciferase assay kit (Promega) and PerkinElmer Vector X5 Multilabel Plate Reader. Each extract was assayed three times, and the relative luciferase activity was calculated as the relative light units relative to *Renilla* luciferase luminescence and normalized.

Electrophoretic mobility shift assays

Nuclear extracts were prepared from C2C12 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). GST–E47, GST–MyoD and GST–Ccndbp1 were expressed and purified from *Escherichia coli*, as previously reported (Lluís et al., 2005). EMSAs were performed using LightShift® Chemiluminescent EMSA Kit (Thermo Scientific) according to the instruction manual. Probes used were synthesized with a 5′ biotin modification and annealed as double-stranded DNA. The sequences of the sense strands of the oligonucleotide used in EMSAs was as follows: Probe A, MEF2c enhancer, 5′-GAGTG-ACATGAACAGGTGCACCCTGGCCTG-3′; Probe B, quail fast skeletal TNI gene enhancer, 5′-CGTCTGAGGAGACAGCTGCAGCTCC-3′; Probe C, MCK promoter, 5′-CCCCAACACCTGCCTGCCTGA-3′.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.H. performed the overexpression analyses, EMSA assays, differentiation assays and quantification, and generated RNAi C2C12 lines. B.C. performed luciferase, H&E staining and CTX injury assays in mice. M.Y. performed western blotting and protein purification. P.L. constructed the knockout mice. Y.Z. performed the qPCR assay. M.L., J.H. and Z.S. helped to analyze data. Y.H. and W.M. designed experiments and wrote the manuscript.

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Supplementary information

Supplementary information available online at
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References

Akimoto, T., Pohnert, S. C., Li, P., Zhang, M., Gumbs, C., Rosenberg, P. B., Williams, R. S. and Yan, Z. (2005). Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. *J. Biol. Chem.* **280**, 19587-19593.

Aronheim, A., Shiran, R., Rosen, A. and Walker, M. D. (1993). The E2A gene product contains two separable and functionally distinct transcription activation domains. *Proc. Natl. Acad. Sci. USA* **90**, 8063-8067.

Bains, W., Ponte, P., Blau, H. and Kedes, L. (1984). Cardiac actin is the major actin gene product in skeletal muscle cell differentiation in vitro. *Mol. Cell. Biol.* **4**, 1449-1453.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L. and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61**, 49-59.

Bounpheng, M. A., Morrish, T. A., Dodds, S. G. and Christy, B. A. (2000). Negative regulation of selected bHLH proteins by eHAND. *Exp. Cell Res.* **257**, 320-331.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H. H. (1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J.* **8**, 701-709.

Chanoine, C., Della Gaspera, B. and Charbonnier, F. (2004). Myogenic regulatory factors: redundant or specific functions? Lessons from Xenopus. *Dev. Dyn.* **231**, 662-670.

Choi, J. K., Shen, C. P., Radomska, H. S., Eckhardt, L. A. and Kadesch, T. (1996). E47 activates the Ig-heavy chain and TdT loci in non-B cells. *EMBO J.* **15**, 5014-5021.

Cordle, S. R., Henderson, E., Masuoka, H., Weil, P. A. and Stein, R. (1991). Pancreatic beta-cell-type-specific transcription of the insulin gene is mediated by basic helix-loop-helix DNA-binding proteins. *Mol. Cell. Biol.* **11**, 1734-1738.

Dedieu, S., Mazeres, G., Cottin, P. and Brustis, J. J. (2002). Involvement of myogenic regulator factors during fusion in the cell line C2C12. *Int. J. Dev. Biol.* **46**, 235-241.

Frasca, D., Nguyen, D., Riley, R. L. and Blomberg, B. B. (2003). Decreased E12 and/or E47 transcription factor activity in the bone marrow as well as in the spleen of aged mice. *J. Immunol.* **170**, 719-726.

Fu, X., Xiao, J., Wei, Y., Li, S., Liu, Y., Yin, J., Sun, K., Sun, H., Wang, H., Zhang, Z. et al. (2015). Combination of inflammation-related cytokines promotes long-term muscle stem cell expansion. *Cell Res.* **25**, 655-673.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501-506.

Hsu, H. L., Cheng, J. T., Chen, Q. and Baer, R. (1991). Enhancer-binding activity of the tal-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. *Mol. Cell. Biol.* **11**, 3037-3042.

Hwang, S.-Y., Oh, B., Füchtbauer, A., Füchtbauer, E.-M., Johnson, K. R., Solter, D. and Knowles, B. B. (1997). Maid: a maternally transcribed novel gene encoding a potential negative regulator of bHLH proteins in the mouse egg and zygote. *Dev. Dyn.* **209**, 217-226.

Ik Tsen Heng, J. and Tan, S. S. (2003). The role of class I HLH genes in neural development–have they been overlooked? *Bioessays* **25**, 709-716.

Jen, Y., Weintraub, H. and Benezra, R. (1992). Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. *Genes Dev.* **6**, 1466-1479.

Kablar, B., Krastel, K., Ying, C., Asakura, A., Tapscott, S. J. and Rudnicki, M. A. (1997). MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. *Development* **124**, 4729-4738.

Kageyama, R., Sasai, Y., Akazawa, C., Ishibashi, M., Takebayashi, K., Shimizu, C., Tomita, K. and Nakanishi, S. (1995). Regulation of mammalian neural development by helix-loop-helix transcription factors. *Crit. Rev. Neurobiol.* **9**, 177-188.

Kitzmann, M. and Fernandez, A. (2001). Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts. *Cell. Mol. Life Sci.* **58**, 571-579.

Langlands, K., Yin, X., Anand, G. and Prochownik, E. V. (1997). Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. *J. Biol. Chem.* **272**, 19785-19793.

Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D. and Weintraub, H. (1989). MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell* **58**, 823-831.

Lemercier, C., To, R. Q., Carrasco, R. A. and Konieczny, S. F. (1998). The basic helix-loop-helix transcription factor Mist1 functions as a transcriptional repressor of myoD. *EMBO J.* **17**, 1412-1422.

Lindon, C., Montarras, D. and Pinset, C. (1998). Cell cycle–regulated expression of the muscle determination factor Myf5 in proliferating myoblasts. *J. Cell Biol.* **140**, 111-118.

Lluís, F., Ballestar, E., Suelves, M., Esteller, M. and Muñoz-Cánoves, P. (2005). E47 phosphorylation by p38 MAPK promotes MyoD/E47 association and muscle-specific gene transcription. *EMBO J.* **24**, 974-984.

Londhe, P. and Davie, J. K. (2011). Sequential association of myogenic regulatory factors and E proteins at muscle-specific genes. *Skelet. Muscle* **1**, 14.

Lu, J., Webb, R., Richardson, J. A. and Olson, E. N. (1999). MyoR: a muscle-restricted basic helix-loop-helix transcription factor that antagonizes the actions of MyoD. *Proc. Natl. Acad. Sci. USA* **96**, 552-557.

Ma, W., Stafford, L. J., Li, D., Luo, J., Li, X., Ning, G. and Liu, M. (2007). GCIP/CCNDBP1, a helix–loop–helix protein, suppresses tumorigenesis. *J. Cell Biochem.* **100**, 1376-1386.

Massari, M. E. and Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol. Cell. Biol.* **20**, 429-440.

Montarras, D., Chelly, J., Bober, E., Arnold, H., Ott, M. O., Gros, F. and Pinset, C. (1991). Developmental patterns in the expression of Myf5, MyoD, myogenin, and MRF4 during myogenesis. *New Biol.* **3**, 592-600.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B. et al. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**, 537-544.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esuml, E., Li, S., Nonaka, I. and Nabeshima, Y.-i. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**, 532-535.

Ohyama, M., Suzuki, N., Yamaguchi, Y., Maeno, M., Otsuka, K. and Ito, K. (2002). Effect of enamel matrix derivative on the differentiation of C2C12 cells. *J. Periodontol.* **73**, 543-550.

Patapoutian, A., Yoon, J. K., Miner, J. H., Wang, S., Stark, K. and Wold, B. (1995). Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* **121**, 3347-3358.

Rajabi, H. N., Takahashi, C. and Ewen, M. E. (2014). Retinoblastoma protein and MyoD function together to effect the repression of Fra-1 and in turn cyclin D1 during terminal cell cycle arrest associated with myogenesis. *J. Biol. Chem.* **289**, 23417-23427.

Rhodes, S. J. and Konieczny, S. F. (1989). Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev.* **3**, 2050-2061.

Roberts, V. J., Steenbergen, R. and Murre, C. (1993). Localization of E2A mRNA expression in developing and adult rat tissues. *Proc. Natl. Acad. Sci. USA* **90**, 7583-7587.

Rudnicki, M. A., Braun, T., Hinuma, S. and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* **71**, 383-390.

Sarnat, H. B. (1994). New insights into the pathogenesis of congenital myopathies. *J. Child Neurol.* **9**, 193-201.

Sigvardsson, M. (2000). Overlapping expression of early B-cell factor and basic helix-loop-helix proteins as a mechanism to dictate B-lineage-specific activity of the lambda5 promoter. *Mol. Cell. Biol.* **20**, 3640-3654.

Sonnenberg-Riethmacher, E., Wüstefeld, T., Miehe, M., Trautwein, C. and Riethmacher, D. (2007). Maid (GCIP) is involved in cell cycle control of hepatocytes. *Hepatology* **45**, 404-411.

Spicer, D. B., Rhee, J., Cheung, W. L. and Lassar, A. B. (1996). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. *Science* **272**, 1476-1480.

Tajbakhsh, S., Rocancourt, D. and Buckingham, M. (1996). Muscle progenitor cells failing to respond to positional cues adopt non-myogenic fates in myf-5 null mice. *Nature* **384**, 266-270.

Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P. F., Weintraub, H. and Lassar, A. B. (1988). MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science* **242**, 405-411.

Terai, S., Aoki, H., Ashida, K. and Thorgeirsson, S. S. (2000). Human homologue of maid: A dominant inhibitory helix-loop-helix protein associated with liver-specific gene expression. *Hepatology* **32**, 357-366.

Voronova, A. F. and Lee, F. (1994). The E2A and tal-1 helix-loop-helix proteins associate in vivo and are modulated by Id proteins during interleukin 6-induced myeloid differentiation. *Proc. Natl. Acad. Sci. USA* **91**, 5952-5956.

Weintraub, H., Davis, R., Lockshon, D. and Lassar, A. (1990). MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. *Proc. Natl. Acad. Sci. USA* **87**, 5623-5627.

Wright, W. E., Sassoon, D. A. and Lin, V. K. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* **56**, 607-617.

Xia, C., Bao, Z., Tabassam, F., Ma, W., Qiu, M., Hua, S.-b. and Liu, M. (2000). GCIP, a novel human grap2 and cyclin D interacting protein, regulates E2F-mediated transcriptional activity. *J. Biol. Chem.* **275**, 20942-20948.

Xiang, H., Wang, J., Mao, Y., Liu, M., Reddy, V. N. and Li, D. W.-C. (2002). Human telomerase accelerates growth of lens epithelial cells through regulation of the genes mediating RB/E2F pathway. *Oncogene* **21**, 3784-3791.

Yao, Y., Doki, Y., Jiang, W., Imoto, M., Venkatraj, V. S., Warburton, D., Santella, R. M., Lu, B., Yan, L., Sun, X.-H. et al. (2000). Cloning and characterization of DIP1, a novel protein that is related to the Id family of proteins. *Exp. Cell Res.* **257**, 22-32.