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Ankyrin Repeat Domain Protein 2 and Inhibitor of DNA Binding 3 Cooperatively Inhibit Myoblast Differentiation by Physical Interaction*

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Background: Dystrophic skeletal muscles overexpress ankyrin repeat domain protein 2 (ANKRD2), which inhibits myoblast differentiation.
Results: Skeletal muscles of the *mdm* mouse overexpress ANKRD2 and inhibitor of DNA binding 3 (ID3) proteins, which cooperatively inhibit myoblast differentiation by physical interaction.
Conclusion: Activation of SREBP-1/ANKRD2/ID3 pathway impairs, at least in part, skeletal muscle development in *mdm* mice.
Significance: We provide evidence revealing a novel mechanism by which expression of ANKRD2 inhibits myoblast differentiation.

Ankyrin repeat domain protein 2 (ANKRD2) translocates from the nucleus to the cytoplasm upon myogenic induction. Overexpression of ANKRD2 inhibits C2C12 myoblast differentiation. However, the mechanism by which ANKRD2 inhibits myoblast differentiation is unknown. We demonstrate that the primary myoblasts of *mdm* (muscular dystrophy with myositis) mice (pMB^{mdm}) overexpress ANKRD2 and ID3 (inhibitor of DNA binding 3) proteins and are unable to differentiate into myotubes upon myogenic induction. Although suppression of either ANKRD2 or ID3 induces myoblast differentiation in *mdm* mice, overexpression of ANKRD2 and inhibition of ID3 or vice versa is insufficient to inhibit myoblast differentiation in WT mice. We identified that ANKRD2 and ID3 cooperatively inhibit myoblast differentiation by physical interaction. Interestingly, although MyoD activates the *Ankrd2* promoter in the skeletal muscles of wild-type mice, SREBP-1 (sterol regulatory element binding protein-1) activates the same promoter in the skeletal muscles of *mdm* mice, suggesting the differential regulation of *Ankrd2*. Overall, we uncovered a novel pathway in which SREBP-1/ANKRD2/ID3 activation inhibits myoblast differentiation, and we propose that this pathway acts as a critical determinant of the skeletal muscle developmental program.

In general, the regeneration ability of normal postnatal skeletal muscle fibers relies on the successful activation of satellite cells into proliferating myoblasts, differentiation of myoblasts into myotubes, and fusion of myotubes with the adjacent muscle fibers. Occurrence of defects in any of these processes severely affects the formation of new muscle fibers. In line with this, studies have shown that impairment in the myogenic program hinders skeletal muscle development in many physiological and experimental conditions (1–4). Although protein catabolism can accelerate skeletal muscle wasting in cancer patients, an impaired myogenic program can also play an important role in muscle wasting in cancer cachexia (5, 6). However, the mechanism that inhibits the myogenic ability of muscle precursor cells is incomplete.

ANKRD2 (ankyrin repeat domain protein 2) is a member of the family of muscle ankyrin repeat proteins, expressed mostly in skeletal muscles. In proliferating myoblasts, ANKRD2 generally resides in the nucleus, whereas in differentiating myoblasts, ANKRD2 begins to accumulate in the cytoplasm (7). Enforced expression of ANKRD2 in C2C12 myoblasts inhibits differentiation (7, 8). However, the mechanism by which ANKRD2 inhibits myoblast differentiation is unknown. Moreover, data from microarray analyses have shown that inhibition of MyoD in myogenic C2C12 cells down-regulates ANKRD2 expression (9, 10). We have previously shown NF-κB-mediated regulation of ANKRD2 in mouse diaphragm muscle after a mechanical stimulus (11). These results suggest that both basal expression and differential regulation of ANKRD2 in skeletal muscles are a complex process with many unknown details.

ID3 (inhibitor of DNA binding 3) belongs to the family of ID proteins containing four distinct genes, *Id1* through *Id4*, in humans and mice. These proteins contain a helix-loop-helix (HLH)[2] structural motif that facilitates formation of heterodimers with the ubiquitous basic HLH transcription factors known as E-proteins (12, 13). Sequestration of E-proteins prevents them from forming transcriptionally active dimers with tissue-specific basic HLH proteins. ID proteins are transcriptional regulators that influence the proliferation and differentiation of many cell types including skeletal muscle cells (14, 15). Developing somites, activated satellite cells, and proliferating and terminally differentiated myoblasts express ID3 (16–19). These studies suggest that ID3 plays a key role in the skeletal

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[2] The abbreviations used are: HLH, helix-loop-helix; GM, growth medium; SREBP, sterol regulatory element binding protein; MB, myoblast; mdm, muscular dystrophy with myositis; qPCR, quantitative PCR.

TABLE 1
Primers used in PCR

Primer name	Sequence (5′–3′)	Underlined residues	Purpose
ANKRD2 sense (forward)	CCGGAATTCATGGAGGGTACCATGGAGGGG	EcoRI	Cloning
ANKNRD2 sense (reverse)	AACCCGGCTCGAGCGGCCGCGAGCAAAGCCAGCACTTTATTG	NotI	Cloning
ANKRD2 anti-sense (forward)	AACCCGGCTCGAGCGGCCGCATGGAGGGTACCATGGAGGG′	EcoRI	Cloning
ANKNRD2 anti-sense (reverse)	CCGGAATTCAGCAAAGCCAGCACTTTATTG′	NotI	Cloning
ID3 sense (forward)	CCGGAATTCTGTTTGCTGCTTTAGGTGTCTCTTTT	EcoRI	Cloning
ID3 sense (reverse)	CCCGGCTCGAGCGGCCGCATAAACCATTTTTGAACACTTTG	NotI	Cloning
ANKRD2 (forward)	GCCTCGGGGTTCAGAGTCCT		qPCR
ANKRD2 (reverse)	GATCTCACGTCGCAGGTCCA		qPCR
ID3 (forward)	ACCTGGAGCCCGAGAGAAGG		qPCR
ID3 (reverse)	AGGGTGGGGACAGAGTGACG		qPCR

muscle development especially in the activation and proliferation of muscle precursor cells. However, little is known about the role of ID3 and its interaction with other proteins in the skeletal muscle myogenic program.

SREBPs (sterol regulatory element binding proteins) belong to the basic helix-loop-helix leucine zipper family of DNA-binding proteins such as MyoD. In mammals, two distinct genes encode SREBP isoforms, SREBP-1 and SREBP-2, each with distinct structural, regulatory, and functional features (20). The SREBP-1 gene encodes SREBP-1a and SREBP-1c proteins via promoters. Like liver and adipose tissues (21, 22), skeletal muscles express high levels of SREBP-1 proteins (23–25). Whereas SREBP-1 regulates genes that are associated with lipid metabolism, recent studies have shown the role of SREBP-1 in skeletal muscle development. For example, microarray analysis of human myotubes over expressing SREBP-1a or SREBP-1c identified many potential targets of SREBP-1 proteins, including a number of muscle-specific genes and markers of muscle differentiation (26). Whereas SREBP-1 regulates genes that are associated with lipid metabolism, recent studies have shown a role for SREBP-1 in skeletal muscle development. Interestingly, enforced expression of SREBP-1 proteins in human myoblasts inhibit their differentiation, and expression of SREBP-1 proteins in mature myotubes *in vitro* and in mouse skeletal muscle *in vivo* can induce muscle atrophy (27). These studies suggest the negative role of SREBP-1 in skeletal muscle development. However, how SREBP-1 proteins inhibit skeletal muscle development is yet to be uncovered.

To explore the mechanism of ANKRD2 regulation and its role in skeletal muscle pathogenicity, we have used the skeletal muscles of *mdm* (muscular dystrophy with myositis) mice. We show that the skeletal muscles of *mdm* mice overexpress ANKRD2 and ID3 proteins, which cooperatively inhibit myoblast differentiation by physical interaction. Although MyoD regulates ANKRD2 in the skeletal muscle of WT mice, we show that SREBP-1 regulates ANKRD2 in *mdm* skeletal muscle, suggesting that differential regulation of ANKRD2 significantly affects muscle regeneration in this model of muscular dystrophy.

EXPERIMENTAL PROCEDURES

Primary Myoblast Isolation—The *mdm* (B6.B6C3Fe-Ttn$^{mdm\text{-}J}$/Cx) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The animal protocol for our experiments was approved by the Animal Care and Use Committee of the Baylor College of Medicine. Heterozygous (Ttn$^{mdm/+}$) breeder pairs were used to generate homozygous WT (Ttn$^{+/+}$) and *mdm* (Ttn$^{mdm/mdm}$) mice (28). Gastrocnemius, tibialis anterior, and soleus muscles were excised from the hind limb muscle of 4-week-old wild-type and *mdm* mice. The excised skeletal muscles were weighed and enzymatically dissociated by incubating 30 min in a solution containing 1.5 units/ml collagenase D, 2.4 units/ml dispase II, and 2.5 mM $CaCl_2$ per gram tissue at 37 °C with 75 rpm in a rotation incubator. The resulting suspension was filtered through 50-μm nylon mesh, centrifuged for 5 min at 350 × *g*, and resuspended in F-12-based primary myoblast growth medium (GM; 80% Ham's F-12, 20% fetal calf serum, 0.025% basic fibroblast growth factor in 0.5% BSA, 100 units/ml penicillin, and 100 μg/ml streptomycin). After cell counting, 4000–10,000 cells were cultured in a 60-mm collagen-coated culture dish containing GM at 37 °C and 5% CO_2. At 70–80% confluents, cells were dislodged in PBS with no trypsin or EDTA by rocking the dish firmly. (This treatment facilitates the myoblasts to come off freely and leave most of the fibroblasts in the dish.) The myoblasts were plated in a new dish and cultured in F-12 GM. This step was repeated for the first week of culture expansion or until most of the fibroblasts were gone from the culture. After the fibroblasts were no longer visible in the culture, the medium was changed to F-12/DMEM-based growth medium (50% F-12 growth medium and 50% DMEM). The existence of myoblasts was confirmed by immunofluorescent staining for desmin.

Construction of Expression Plasmids—Sense and antisense *Ankrd2* (1100 bp) and sense *Id3* (960 bp) cDNAs were synthesized and cloned into pcDNA 3.1D/V5-His-TOPO vector (Invitrogen) according to the manufacturer's instructions. PCRs were performed to synthesis inserts with AccuPrime Pfx DNA polymerase according to the manufacturer's instructions (Invitrogen). Primer information is detailed in Table 1. Constructs were sequenced by the DNA Sequence Core Facility of Baylor College of Medicine to verify insert identities.

ChIP Assays—ChIP assays were performed in the skeletal muscles of WT and *mdm* mice as described previously (11).

Transfection and Luciferase Assay—Cells were grown in Opti-MEM I medium (Invitrogen) for 24 h before transfection. Cells were transfected with 2.2 μg of pcDNA expression vector bearing *Ankrd2* or *Id3* using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For siRNA-mediated knockdown studies, cells were transfected with 500 pmol of siRNA specific for mouse ID3, MyoD, SREBP-1, or nonspecific siRNA (Santa Cruz Biotechnology). RNA transfection studies were performed with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's

instructions. After 8 h, the transfection medium was replaced with the growth medium. Subsequent assays were made after 24 to 48 h of transfection.

Immunocytochemistry—Primary myoblasts were cultured on sterile glass coverslips. At 60–70% confluence, the cells were gently washed twice in PBS, fixed, and permeabilized with methanol-acetone (1:1) at −20 °C for 5 min. After washing twice in PBS, the cells were blocked in 1% normal goat serum for 1 h at room temperature. The cells were incubated in primary antibody (rabbit anti-mouse tubulin) for 2 h at room temperature. After washing in PBS, the cells were incubated in secondary antibody (goat anti-rabbit) conjugated with Alexa Fluor 547 for 2 h at room temperature followed by washing in PBS. The cells then were mounted with DAPI (nuclear stain) based mounting media for fluorescent microscope analysis.

RT-PCR—Real-time RT-PCRs were performed as described previously (11). The amount of amplified transcripts ($2^{-\Delta CT}$) was estimated by the comparative C_T (Δ^{CT}) method and normalized to an endogenous reference (GAPDH) relative to a calibrator. All PCR products were verified on agarose gel stained with ethidium bromide to discriminate between the correct amplification products and the potential primer dimers.

Western Blot—Western blots were performed as described previously (11). Anti-ANKRD2 (sc-138111), anti-MyoD (sc-377186), anti-Myf5 (sc-302), anti-myogenin (sc-52903), anti-ID3 (sc-490), anti-SREBP-1 (sc-8984), or anti-tubulin (sc-53646) antibodies were used to detect respective proteins. All of the antibodies were purchased from Santa Cruz Biotechnology.

In Vitro Pulldown Assay—After transfection, cells were collected by gentle scraping and lysed in radioimmune precipitation assay buffer containing PMSF and protease inhibitor mixture. After centrifugation, supernatants were precleared with blocked protein G beads (Sigma) for 1 h at 4 °C. Target protein-protein complexes were immunoprecipitated using either 10 μg of anti-ANKRD2, anti-His, or anti-ID3 antibody (Santa Cruz Biotechnology) overnight at 4 °C with rotation. Immunocomplexes were captured by incubating with blocked protein G-agarose/Sepharose bead (Sigma) and enhanced by adding a bridging antibody to the other samples (Pierce Biotechnology). Agarose/Sepharose beads were collected by centrifugation and immunoprecipitated antibody-protein-protein complexes eluted with sample buffer and dissociated by boiling. Supernatants were then transferred to fresh micro centrifuge tubes and used in appropriate Western blotting.

Statistical Analysis—All experiments were repeated at least three times. In addition, assays producing quantitative data were run in triplicate. Statistical significance was determined by one-way analysis of variance followed by Bonferroni test or the unpaired Student's *t* test, as appropriate. The criterion for significance (α) was set at 0.05.

RESULTS

Myoblasts from the Skeletal Muscles of mdm Mice Display Impaired Differentiation Program—Studies have shown that the myogenic ability of muscle precursor cells becomes progressively reduced in muscular dystrophies (1, 2). Because *mdm* mice have severe muscular dystrophy, we sought to determine whether the primary myoblasts of *mdm* mice (pMB^{mdm}) exhibit

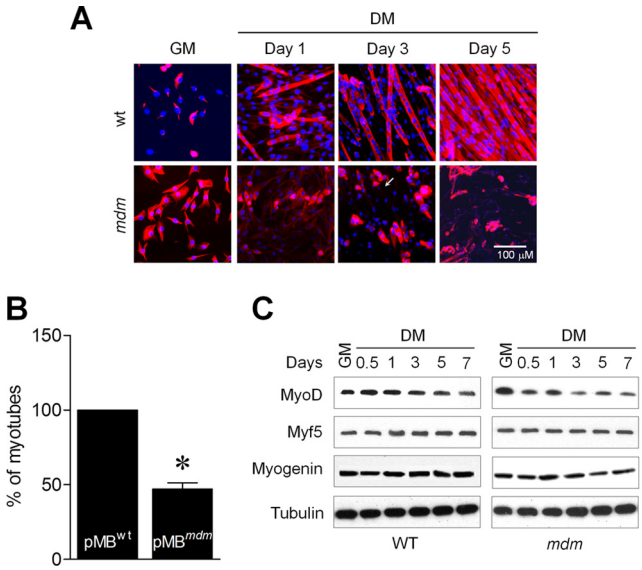

FIGURE 1. **Myoblasts of *mdm* mice show impaired-differentiation program.** *A*, pMB^{WT} and pMB^{mdm} were cultured in DM for 5 days. Formation of myotubes was determined by immunocytochemistry. *B*, the number of myotube formations were counted on day 3 in pMB^{WT} and pMB^{mdm} cultured in DM. *C*, Western blot analyses showed MyoD, myogenin, and Myf5 expressions between pMB^{WT} and pMB^{mdm} during differentiation. Gel and immunocytochemistry pictures are representative of three separate experiments. Each *error bar* indicates mean ± S.E. ($n = 3$). *, $p < 0.05$.

a normal differentiation program. Surprisingly, culture of pMB^{mdm} in differentiation media (DM) were unable to generate large multinucleated myotubes (Fig. 1*A*) and showed a significant decrease in total myotube numbers (Fig. 1*B*) compared with similar culture of wild-type (pMB^{WT}) myoblasts. These results suggest that the severe skeletal muscle wasting and muscular dystrophy in *mdm* mice could be due to, at least in part, the impaired differentiation program of the myoblasts. The coordinated expressions and functions of the myogenic regulators MyoD, myogenin, and Myf5 are crucial for the normal differentiation program (29–32). We therefore examined the expression levels of myogenic regulators in myogenic-induced pMB^{WT} and pMB^{mdm}. Although there were no apparent changes in the expression levels of myogenin and Myf5 between pMB^{WT} and pMB^{mdm}, the MyoD expression levels were markedly altered in pMB^{mdm} (Fig. 1*C*). However, the MyoD levels were still detectable in pMB^{mdm}. These results indicate that the impaired differentiation program in the pMB^{mdm} may be due to dysregulation of proteins other than the myogenic regulators.

Overexpression of ANKRD2 Inhibits Differentiation of pMB^{mdm}—Our earlier study has shown up-regulation of ANKRD2 in the diaphragm muscle of *mdm* mice (11). ANKRD2 negatively regulates myoblast differentiation (7, 8) and is under the control of MyoD (9). These results suggest the possibility that the impaired differentiation program in pMB^{mdm} may be due to the overexpression of ANKRD2. To explore this, we first determined the kinetics of ANKRD2 expression in the skeletal muscles of WT and *mdm* mice at different ages. We excised different hind limb skeletal muscles from *mdm* mice and their WT littermates at 1–8 weeks of age. The data show that ANKRD2 mRNA and protein levels in skeletal muscles were not significantly different at one week of age, but levels increased to 3-fold higher at 2 weeks and 4.5-fold higher at 4

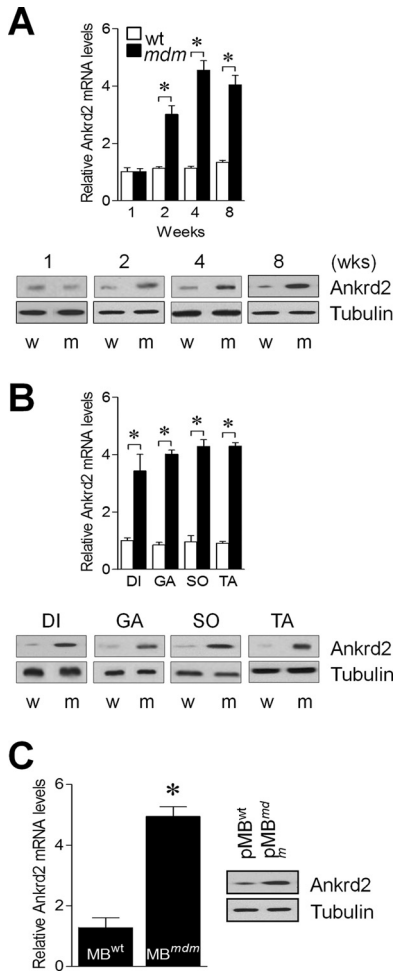

FIGURE 2. **Skeletal muscles of *mdm* mice over express ANKRD2.** Hind limb skeletal muscles were excised from WT and *mdm* mice, and ANKRD2 expression was determined by real-time RT-PCR and Western blot methods (*A*). diaphragm (*DI*), gastrocnemius (*GA*), soleus (*SO*), tibialis anterior (*TA*), and primary myoblasts (*pMB*) were isolated from the hind limb skeletal muscles of WT and *mdm* mice, and ANKRD2 expression was determined by real-time RT-PCR and Western blot (*B* and *C*). *w* and *m* indicate wild-type and *mdm*, respectively. Gel images are representative of three separate experiments. Each *error bar* indicates mean ± S.E. ($n = 3$). *, $p < 0.05$. *Wks.*, weeks.

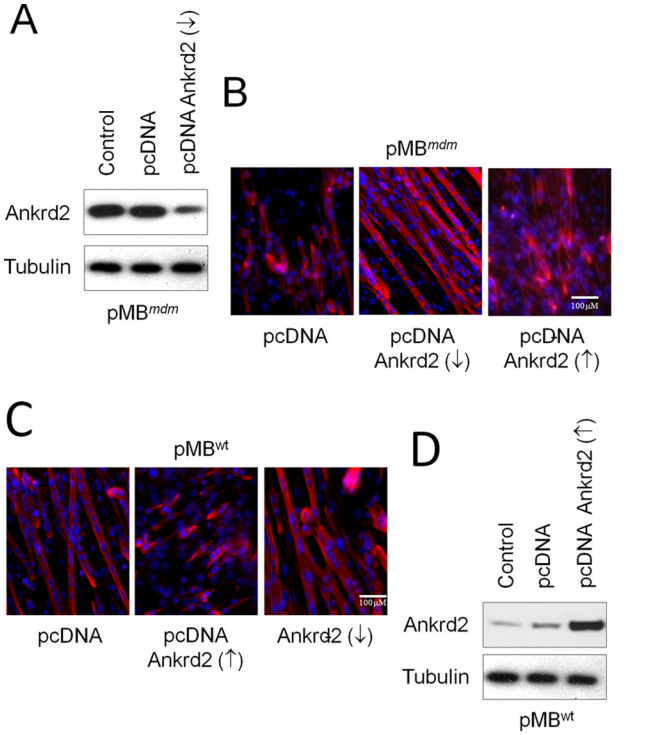

FIGURE 3. **ANKRD2 negatively regulates myoblast differentiation program.** pMB^{WT} and pMB^{mdm} cultured in GM were transfected with either pcDNA, pcDNA-ANKRD2-sense or pcDNA-ANKRD2-antisense vector for 48 h. *A,* knockdown of ANKRD2 protein was confirmed by Western blot in $pMB^{mdm/Ankrd2\downarrow}$. *B,* knockdown of ANKRD2 promotes differentiation of $pMB^{mdm/Ankrd2\downarrow}$, and co-transfection of ANKRD2-sense vector in $pMB^{mdm/Ankrd2\downarrow}$ voids the antisense ANKRD2-induced differentiation program. *C,* overexpression of ANKRD2 inhibits differentiation of $pMB^{WT/Ankrd2\uparrow}$, and co-transfection of ANKRD2-antisense vector in $pMB^{WT/Ankrd2\uparrow}$ voids the sense Ankrd2-induced differentiation program. *D,* overexpression of ANKRD2 protein was confirmed by Western blot in $pMB^{mdm/Ankrd2\uparrow}$. Gel and immunocytochemistry pictures are representative of three separate experiments. ↑, overexpression; ↓, down-regulation.

weeks in *mdm* mice compared with their WT littermates (Fig. 2*A*). Interestingly, the elevated ANKRD2 levels in *mdm* mice were similar in all skeletal muscles that were analyzed (Fig. 2*B*) and were also elevated in cultured pMB^{mdm} compared with pMB^{WT} (Fig. 2*C*). These results indicate that the skeletal muscles of *mdm* mice begin to overexpress ANKRD2 from 2 weeks of age and that overexpression is consistent in all skeletal muscles.

Second, we generated transgenic myoblasts stably overexpressing an antisense ANKRD2 mRNA ($pMB^{mdm/ANKRD2\downarrow}$) that effectively decreased ANKRD2 protein levels (Fig. 3*A*). Interestingly, inhibition of ANKRD2 by antisense ANKRD2 induced differentiation of $pMB^{mdm/Ankrd2\downarrow}$, and co-transfection of ANKRD2-sense vector in $pMB^{mdm/Ankrd2\downarrow}$ voided the antisense Ankrd2-induced differentiation program (Fig. 3*B*). To further study whether ANKRD2 indeed inhibits myoblast differentiation, we generated ANKRD2 overexpressing WT transgenic myoblasts ($pMB^{WT/ANKRD2\uparrow}$). Culture of $pMB^{WT/ANKRD2\uparrow}$ in DM was unable to differentiate into myotubes and inhibition of ANKRD2 in these myoblasts by antisense ANKRD2 reinstated the differentiation program (Fig. 3*C*). The levels of ANKRD2 expression were confirmed in $pMB^{WT/ANKRD2\uparrow}$ (Fig. 3*D*). These results suggest that ANKRD2 is a negative regulator of the myoblast differentiation program and that ANKRD2 up-regulation in the skeletal muscles of *mdm* mice could be a critical factor causing skeletal muscle growth deficiency through inhibition of myoblast differentiation.

Overexpression of ID3 in pMB^{mdm} Inhibits Differentiation Program—Studies have shown that overexpression of ID3 in C2C12 myoblasts inhibits the differentiation program (12, 33). Thus, we tested whether ID3, similar to ANKRD2, inhibits the differentiation of pMB^{mdm}. We show that the skeletal muscles of *mdm* mice overexpressed *Id3* mRNA and protein (Fig. 4*A*). The level of *Id3* in the skeletal muscles of *mdm* mice was ~3.5-fold higher than that in the skeletal muscles of WT mice as determined by quantitative PCR (qPCR) (Fig. 4*B*). Furthermore, the myogenic pMB^{WT} showed a rapid decline in the levels of ID3 at 12 h, which were barely detectable after 24 h. In contrast, pMB^{mdm} in DM showed no significant decline in the levels of ID3. To study whether knockdown of ID3 in pMB^{mdm} could induce differentiation, we applied an siRNA-based strategy to inhibit ID3 protein expression. Interestingly, inhibition of ID3 by *Id3* siRNA was capable of inducing pMB^{mdm} differentiation (Fig. 4*C*), and overexpression of ID3 in these myo-

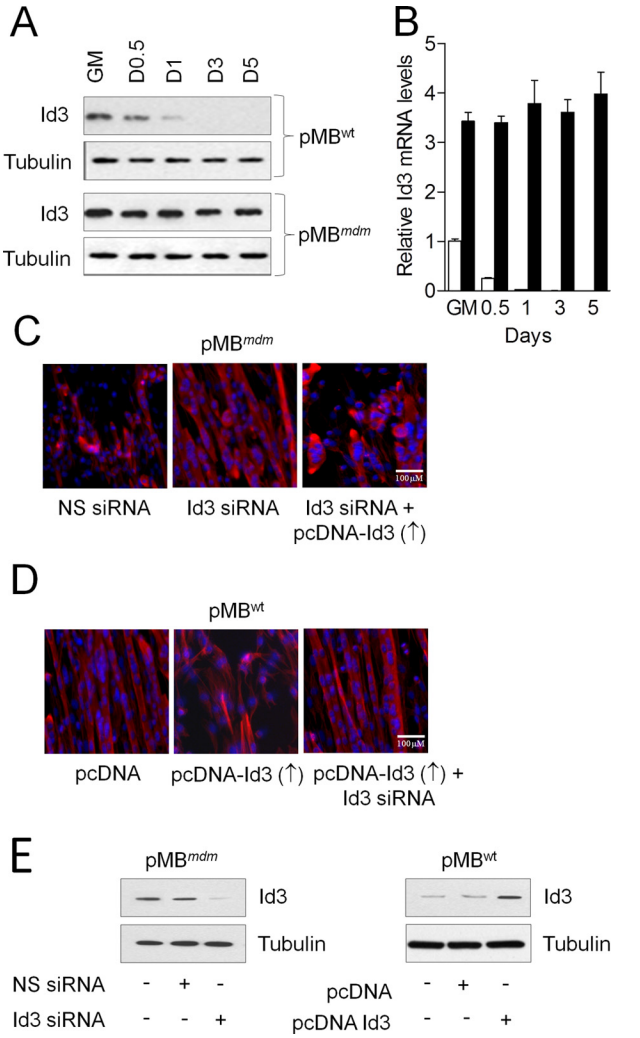

FIGURE 4. **Up-regulation of ID3 in the skeletal muscle of *mdm* mice inhibits its myoblast differentiation program.** *A* and *B*, total RNA and protein were isolated from proliferating and myogenic pMB^{WT} and pMB^{mdm} to determine ID3 mRNA and protein expressions. *C*, pMB^{mdm} were transfected with nonspecific (*NS*) or ID3 siRNA with or without pcDNA-ID3 construct. *D*, pMB^{WT} were transfected with either pcDNA or pcDNA-ID3 with or without ID3 siRNA. After 24 h, the GM was replaced with DM, and the myogenic program was determined by ICH. Overexpression or knockdown of ID3 was determined by Western blot (*E*). Gel and immunocytochemistry images are representative of three separate experiments. Each *error bar* indicates mean ± S.E. ($n = 3$). *, $p < 0.05$. ↑, overexpression; ↓, down-regulation.

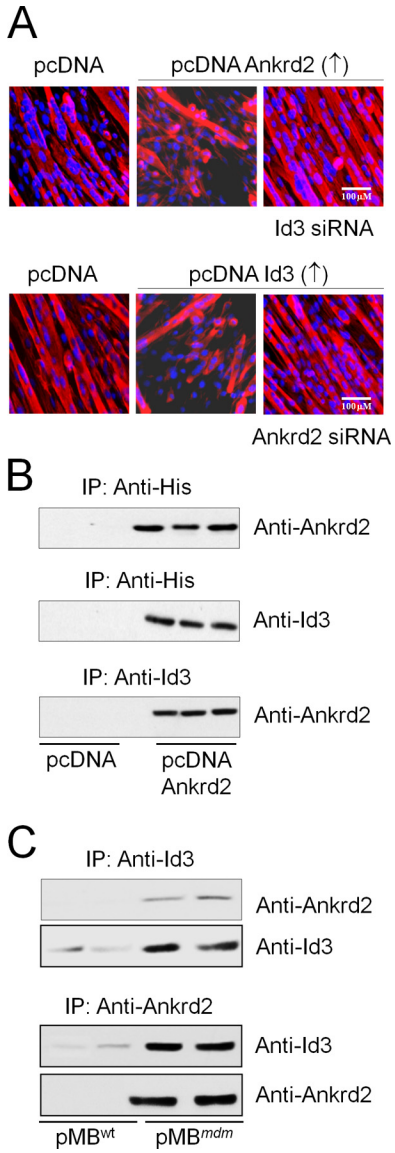

FIGURE 5. **ANKRD2 and ID3 physically interact with each other and cooperatively inhibit myoblast differentiation program.** *A*, pMB$^{WT/ANKRD2\uparrow}$ were transfected with or without ID3 siRNA. After 24 h, the GM was replaced with DM and differentiation was determined by ICH. *B*, total cell lysate was isolated from pMB$^{WT/ANKRD2}$ and immunoprecipitated with anti-His antibody, and the pellets were resolved on SDS-PAGE and subjected to immunoblot analysis with anti-ANKRD2 or anti-ID3 antibody. The cell lysate was also immunoprecipitated with anti-ID3 antibody followed by resolving on SDS-PAGE and subjected to immunoblot analysis with anti-ANKRD2 antibody. *C*, total cell lysate was isolated from pMB^{mdm} and immunoprecipitated with either anti-ANKRD2 or anti-ID3 antibody, and the pellets were resolved on SDS-PAGE and subjected to immunoblot analysis with anti-ANKRD2 or anti-ID3 antibody. Gel and immunocytochemistry images are representative of three separate experiments. ↑, overexpression; ↓, down-regulation.

blasts inhibited differentiation. To determine whether this was a unique feature caused by the *mdm* mutation, we overexpressed ID3 in pMB^{WT}. Enforced expression of ID3 in pMB^{WT} hindered the serum withdrawal-induced myoblast differentiation (Fig. 4*D*). These results indicate that similar to ANKRD2, overexpression of ID3 in pMB^{WT} and pMB^{mdm} inhibits differentiation program. We also determined ID3 levels in pMB^{mdm} after *Id3* siRNA transfection and confirmed that it significantly reduced the endogenous ID3 levels. Similarly, the enforced-expression of ID3 in pMB^{WT} significantly increased the endogenous ID3 levels, suggesting the specificity of the siRNA and overexpression constructs (Fig. 4*E*).

ANKRD2 and ID3 Cooperatively Inhibit pMB differentiation by Physical Interaction—Because ANKRD2 and ID3 play similar roles in the myoblast differentiation program, we sought to determine whether these two proteins act cooperatively or independently. We used transgenic myoblasts expressing either ANKRD2 (pMB$^{WT/ANKRD2\uparrow}$) or ID3 (pMB$^{WT/ID3\uparrow}$). Although pMB$^{WT/ANKRD2\uparrow}$ or pMB$^{WT/ID3\uparrow}$ were unable to differentiate into myotubes, knockdown of ID3 in pMB$^{WT/ANKRD2\uparrow}$ using ID3 siRNA or ANKRD2 in pMB$^{WT/ID3\uparrow}$ using the ANKRD2-antisense construct effectively induced myoblast differentiation (Fig. 5*A*). These results suggest that both ANKRD2 and ID3 inhibit myoblast differentiation in a cooperative manner. To determine whether the inhibition of myoblast differentiation by ANKRD2 and ID3 could be caused by a physical

interaction between these proteins, we conducted a series of co-immunoprecipitation experiments. First, we isolated cell lysate from $pMB^{WT/ANKRD2\uparrow}$ or $pMB^{WT/pcDNA}$ and immunoprecipitated with either anti-His or anti-ID3. Cell lysates from $pMB^{WT/pcDNA}$ showed no co-precipitation of ANKRD2 with ID3, whereas cell lysate from $pMB^{WT/ANKRD2\uparrow}$ immunoprecipitated with anti-His showed co-precipitation of ID3. Similarly, myoblasts immunoprecipitated with anti-ID3 showed co-precipitation of ANKRD2 (Fig. 5*B*). These results indicate that there is likely a physical interaction between exogenously expressed ANKRD2 and endogenous ID3. To further support this result and rule out artifacts due to overexpression, we attempted to detect interactions between endogenous ANKRD2 and ID3. We used pMB^{mdm}, which overexpresses both ANKRD2 and ID3 proteins. Immunoprecipitation of cell lysate of pMB^{mdm} with anti-ANKRD2 antibody showed co-precipitation of ID3. Similarly, immunoprecipitation of cell lysate of pMB^{mdm} with anti-ID3 antibody showed co-precipitation of ANKRD2. These results corroborate the existence of a physical interaction between the endogenous ANKRD2 and ID3 proteins and further support the hypothesis that their combined actions cooperatively inhibit myoblast differentiation.

WT and mdm Mice Skeletal Muscles Differentially Regulate ANKRD2—Data from microarray analyses show that MyoD-silenced C2C12 myoblasts down-regulate *Ankrd2* gene expression (9). We have shown that mechanical stretch can up-regulate ANKRD2 expression through NF-κB in WT and *mdm* mouse diaphragm muscles (11). These results suggest a differential regulation of *Ankrd2* in skeletal muscle. It has been shown that SREBP-1 overexpression can inhibit myoblast differentiation and promotes skeletal muscle atrophy (19). However, the mechanism of regulation of the basal level *ANKRD2* expression in skeletal muscle is unknown. To end this, we analyzed the *Ankrd2* promoter region bioinformatically, focusing on MyoD, NF-κB, and SREBP-1 transcription factors. A scan of the 1.7-kb genomic sequence located upstream of the ATG of the *Ankrd2* gene identified two putative MyoD (−865 and −1449; *green*), five NF-κB (−271, −1036, −1207, −1531, and −1656; *red*), and two SREBP-1 (−929 and −1451; *blue*) binding sites (Fig. 6*A*), which led us to consider whether *ANKRD2* is a transcriptional target of any of these regulators. Using ChIP assays, we identified that MyoD but not NF-κB and SREBP-1 bound the *Ankrd2* promoter in skeletal muscles of WT mice as evidenced by qPCR and the visualization of PCR products on 1% agarose gel (Fig. 6*B*). In contrast, SREBP-1 but not MyoD and NF-κB bound the *Ankrd2* promoter in skeletal muscles of *mdm* mice. To further confirm this, we generated a 1500-bp (−200 to −1700) *Ankrd2* promoter construct, encompassing MyoD, NF-κB, and SREBP-1 binding sites, named pGL-WT (Fig. 6*C*) and a mutant *Ankrd2* promoter construct with mutated-MyoD or mutated-SREBP-1 binding site, named pGL-mtMyoD or pGL-mtSREBP-1 (Fig. 6*C*). Transfection of pGL-WT construct into pMB^{WT} and pMB^{mdm} showed higher luciferase activities than those cells transfected with pGL vector alone (Fig. 6*D*). In contrast, transfection of pMB^{WT} with pGL-mtMyoD but not pGL-mtSREBP-1 or transfection of pMB^{mdm} with pGL-mtSREBP-1 but not pGL-mtMyoD showed less luciferase activity. Finally, we tested whether the endogenous MyoD and SREBP-1 is able to influence the promoter activity of *Ankrd2* in pMB^{WT} and pMB^{mdm}, respectively. To achieve this, we knocked down MyoD in pMB^{WT} and SREBP-1 in pMB^{mdm} using siRNA-based strategy, which decreased the endogenous levels of MyoD in pMB^{WT} and SREBP-1 in pMB^{mdm} (Fig. 6*E*). Transfection of pMB^{WT} with pGL-WT construct increased the luciferase activity, but knockdown of MyoD by siRNA abolished the luciferase activity (Fig. 6*F*). Similarly, transfection of pMB^{mdm} with pGL-WT construct increased the luciferase activity, but knockdown of SREBP-1 by siRNA abolished the luciferase activity (Fig. 6*F*). Taken together, these data indicate that the *Ankrd2* promoter is a direct transcriptional target of both MyoD and SREBP-1 in the skeletal muscles of WT and *mdm* mice, respectively.

DISCUSSION

In the present study, we demonstrated that primary myoblasts and skeletal muscles of *mdm* mice overexpress ANKRD2 and ID3 proteins. The myoblasts of *mdm* mice were incompetent to differentiate into myotubes upon myogenic induction. We found that an ANKRD2-ID3 complex inhibits myoblast differentiation by physical interaction. Although MyoD activates the *Ankrd2* promoter in skeletal muscles of WT mice, SREBP-1 activates this promoter in the skeletal muscles of *mdm* mice. Overall, we provide the first experimental evidence demonstrating that the ANKRD2/ID3 pathway is critical for the developmental program of skeletal muscles.

Impaired muscle development in skeletal muscle diseases such as muscular dystrophies is associated with an impaired myogenic program (1–4). Studies have shown that many different genetic muscular dystrophies display overexpression of ANKRD2 (34), which is known to negatively regulate the differentiation program in C2C12 myoblasts (7, 8). However, it is not known whether overexpression of ANKRD2 in dystrophic skeletal muscles directly leads to disease pathology disruption of the myogenic program or is a consequence of the disease process. To address this issue, we used primary myoblasts and skeletal muscles of *mdm* mice, a genetic model of muscular dystrophy caused by a small in-frame deletion within the titin gene. Skeletal muscles of *mdm* mice began to uniformly overexpress ANKRD2 from 2 weeks of age uniformly in all skeletal muscles analyzed, suggesting that ANKRD2 is an early player in *mdm* skeletal muscle pathology. This observation is in agreement with our previous study showing that the *mdm* mouse diaphragm begins to display muscle weakness and histopathological signs of muscular dystrophy, including centrally located nuclei, increased variation in myofiber diameter, hypertrophy, and fatty or connective tissue infiltration at 2 weeks of age (28). To explore the role of ANKRD2 more precisely in *mdm* skeletal muscle, we isolated primary myoblasts from 2-week-old *mdm* mice (pMB^{mdm}) and their wild-type littermates (pMB^{WT}). Down-regulation of ANKRD2 or ID3 in *mdm* myoblasts restored the ability to differentiate. Overexpressing either ANKRD2 or ID3 similarly disrupted differentiation of WT myoblasts. This result suggests that both proteins are critical to the disease process and that they function independently of the titin mutation. In the present study, we did not notice any impairment in cell proliferation between pMB^{mdm} and

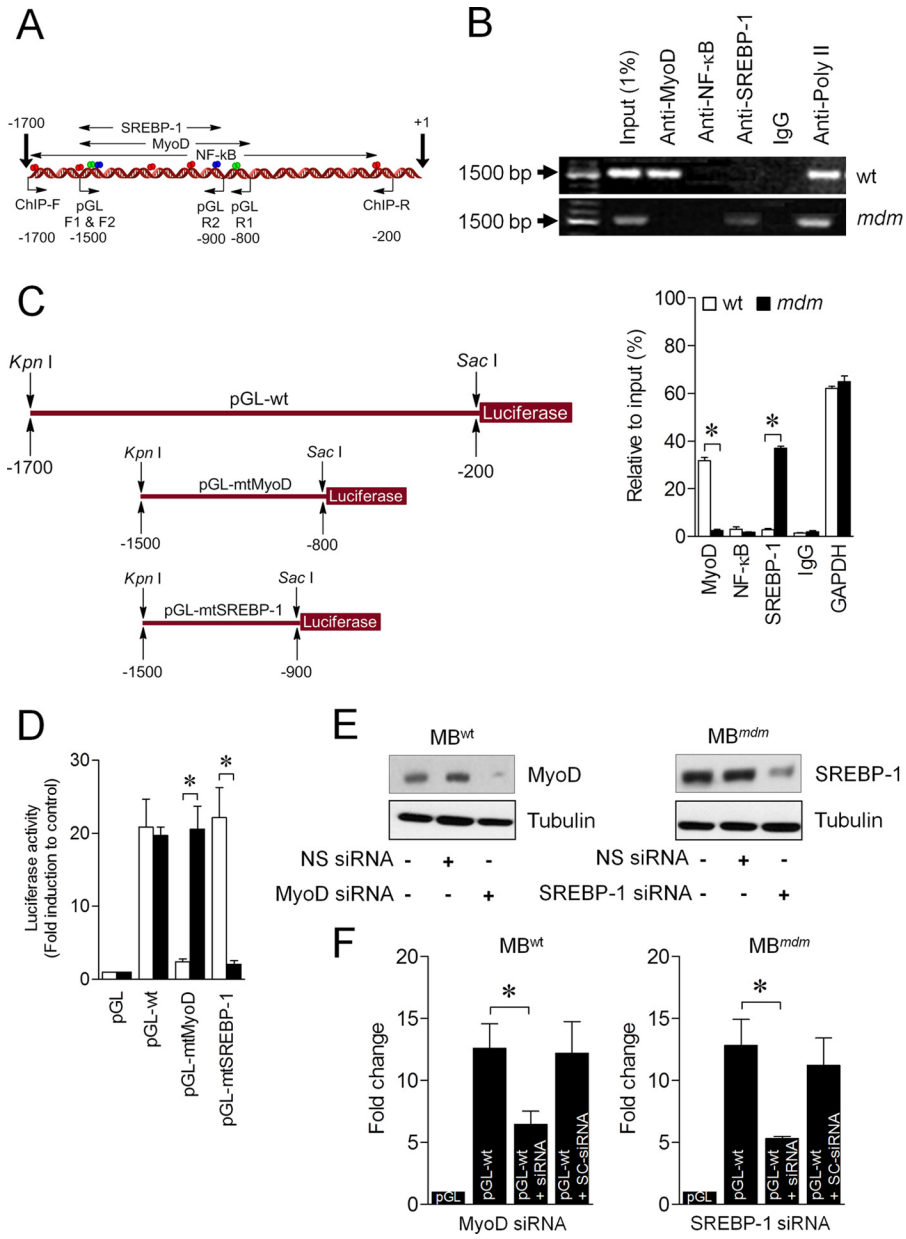

FIGURE 6. **MyoD and SREBP-1 regulate ANKRD2 expression.** *A*, schematic representation of Ankrd2 promoter. The region between −1700 and +1 bp contains putative binding elements for MyoD (*green*), NF-κB (*red*), and SREBP-1 (*pink*). *B*, chromatin was isolated from the skeletal muscles of WT and *mdm* mice and precipitated with anti-c-MyoD, anti-NF-κB, anti-SREBP-1, anti-RNA poly II, or nonspecific IgG. qPCRs were performed with three sets of primers, specific for ANKRD2 promoter to identify the specific transcription factor and its region of binding to the ANKRD2 promoter and resolved in 1% agarose gel. *C*, 1500-bp pGL-WT, 700-bp (pGL-mtMyoD), or 600-bp (pGL-SREBP-1) promoter regions were synthesized and linked to luciferase (*Luc*) reporter gene. *D*, pMB^{WT} and pMB^{mdm} were transfected with empty vector, pGL-WT, pGL-mtMyoD, or pGL-SREBP-1 vector. Forty-eight h after transfection, firefly luciferase activities were estimated and normalized to *Renilla* luciferase activities. *E* and *F*, MB^{WT} were transfected with MyoD siRNA or nonspecific siRNA (*NS* siRNA) followed by transfection of either pGL or pGL-WT. MB^{mdm} were transfected with SREBP-1 siRNA or nonspecific siRNA followed by transfection of either pGL or pGL-WT. After 48 h, total protein was isolated, and MyoD and SREBP-1 protein expression was determined by Western blot (*E*). Forty-eight h after transfection, firefly luciferase activities were estimated and normalized to *Renilla* luciferase activities (*F*). Gel pictures are representative of three separate experiments. Each *error bar* indicates mean ± S.E. ($n = 3$). *, $p < 0.05$.

$pMB^{WT/ANKRD2\uparrow}$, suggesting that ANKRD2 does not perturb cell proliferation. This result is in agreement with an earlier study showing that proliferating myoblasts and injured skeletal muscles accumulate ANKRD2 in the nucleus (35).

We also found that the primary myoblasts and the skeletal muscle of *mdm* mice overexpressed ID3 protein (DNA-binding protein inhibitor 3). ID3 belongs to the ID family of proteins, encoded by four distinct genes, *Id1* through *Id4*, which are transcriptional regulators that influence the proliferation and differentiation of many cell types, including skeletal muscle cells (14, 15). Satellite cells express ID3 under the direct transcriptional control of Pax7 (36) and skeletal muscle markedly upregulates ID3 within 24 h of injury (37). Although the proliferating C2C12 myoblasts actively express ID3, differentiating myoblasts show reduced levels of ID3 (38). Enforced expression of ID3 in C2C12 myoblasts effectively inhibits the myogenic program (12, 33). These studies suggest that ANKRD2 and ID3 may have similar roles in skeletal muscle development. In

agreement with this hypothesis, we found that ID3 expression levels were detectable in proliferating pMB^{WT} and drastically reduced during differentiation. In contrast, ID3 overexpression in pMB^{mdm} did not change upon myogenic induction. Similar to ANKRD2, inhibition of ID3 in pMB^{mdm} induced differentiation, whereas enforced expression of ID3 in pMB^{WT} inhibited differentiation, suggesting that ANKRD2 and ID3 negatively regulate myoblast differentiation. We further expanded our study to identify the potential mechanism by which ANKRD2 and ID3 impair myoblast differentiation. ID proteins form heterodimers with the ubiquitous transcription factors known as E-proteins and that interaction prevents them from forming transcriptionally active dimers (12, 13). It has been shown that ID3 inhibits the myogenic program through physical interaction with E class basic HLH proteins to form inactive heterodimers (39, 40). In addition, ANKRD2 physically interacts with the structural proteins titin (41), myopalladin, and telethonin (42), and with the regulatory nuclear proteins promyelocytic leukemia protein, p53, and YB-1 (42). These studies provide a strong rationale to explore whether ANKRD2 and ID3 cooperatively inhibit myoblast differentiation by physical interaction. Although knockdown of either ANKRD2 or ID3 in pMB^{mdm} was adequate to induce differentiation program, ANKRD2 overexpression combined with ID3 knockdown or *vice versa* in pMB^{WT} does not inhibit myoblast differentiation. Furthermore, our data from co-immunoprecipitation assays revealed the existence of a strong physical interaction between ANKRD2 and ID3 proteins. These data indicate that ANKRD2 and ID3 cooperatively inhibit myoblast differentiation by physical interaction. We have previously shown the Akt-mediated up-regulation of ANKRD2 in diaphragm muscle (11). In agreement with this, other studies have shown that phosphorylation of ANKRD2 by Akt2 inhibited C2C12 myoblast differentiation (8), and myoblasts lacking Akt2 have a normal differentiation program (43, 44), suggesting that ANKRD2 is a downstream substrate of Akt2. In the present study, we have shown ID3 as a new substrate of ANKRD2 in the differentiation program.

Our earlier study shows that NF-κB binds the *Ankrd2* promoter and up-regulates *Aknrd2* expression in diaphragm muscle after mechanical stimuli (11). A microarray study shows that MyoD-silenced myogenic C2C12 myoblasts down-regulate ANKRD2 expression (9). These studies suggest that there are multiple key regulators of ANKRD2 expression. In this study, we have provided new insight to the regulation of ANKRD2 in skeletal muscles in WT and *mdm* mice. We show that MyoD bound to and transactivated the *Ankrd2* promoter in skeletal muscles of WT mice, as demonstrated by ChIP and luciferase assays. In contrast, activation of the *Ankrd2* promoter in the skeletal muscle of *mdm* mice was completely dependent on SREBP-1-binding sites and independent of MyoD and NF-κB binding sites, suggesting that SREBP-1 inhibits the differentiation of pMB^{mdm} through ANKRD2 up-regulation. The SREBP-1 gene produces SREBP-1a and SREBP-1c proteins through alternate promoters. Like liver and adipose tissues, which highly express SREBP-1 proteins (21, 22), skeletal muscles express significant levels of SREBP-1 proteins (23–25). For example, microarray analysis of human myotubes over expressing SREBP-1a or SREBP-1c identified many potential targets of

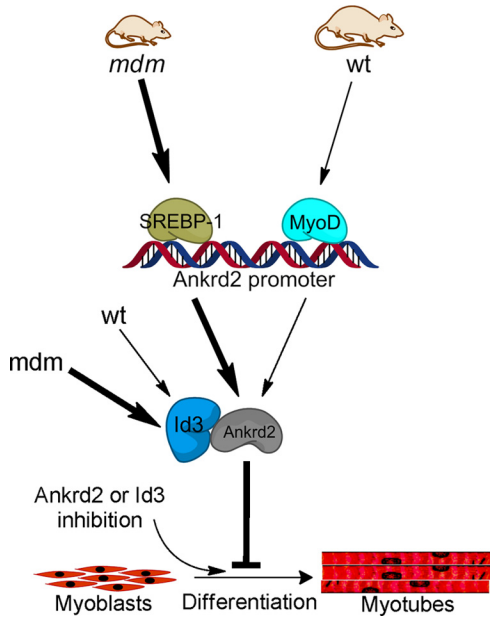

FIGURE 7. **Proposed pathway illustrating the mechanism by which ANKRD2 inhibits skeletal differentiation program.** The skeletal muscle of *mdm* mice up-regulates ANKRD2 and ID3 proteins, which cooperatively inhibit pMB^{mdm} differentiation program through physical interaction. Although MyoD regulates the *Ankrd2* gene in the skeletal muscle of WT mice, SREBP-1 regulates *Ankrd2* in the skeletal muscle of *mdm* mice. Inhibition of ANKRD2 or ID3 is sufficient to recover the myogenic program. *Arrows* in *lightface* and *boldface type* indicate up-regulation and basal-level regulation, respectively.

SREBP-1 proteins, including a number of muscle-specific genes and markers of muscle differentiation (26). In line with this result, a previous study demonstrated that enforced-expression of SREBP-1 proteins inhibit differentiation in human myoblasts and induce atrophy in mouse myotubes *in vitro* and skeletal muscle *in vivo* (27). Moreover, our site-directed promoter mutagenesis and knockdown of MyoD and SREBP-1 confirmed the differential regulation of ANKRD2 between the skeletal muscles of WT and *mdm* mice. These data provide novel evidence demonstrating that aberrant regulation of SREBP-1 in *mdm* skeletal muscle inhibits myoblast differentiation through ANKRD2 up-regulation.

In summary, we have shown that ANKRD2 up-regulation by SREBP-1 negatively regulates myoblast differentiation by interacting with ID3 in *mdm* skeletal muscle. A schematic summarizing our data is depicted in Fig. 7. These results prompt the speculation that ANKRD2 inhibition could be utilized in the context of skeletal muscle wasting to enhance new muscle fiber formation.

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