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# Kelch Repeat and BTB Domain Containing Protein 5 (Kbtbd5) Regulates Skeletal Muscle Myogenesis through the E2F1-DP1 Complex*[S]

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**Background:** Kbtbd5 is involved in skeletal muscle myogenesis, although the underlying mechanism is unclear.
**Results:** Kbtbd5 interacts with DP1 and regulates the activity of E2F1-DP1 in skeletal muscle myogenesis.
**Conclusion:** Kbtbd5 is an important regulator of skeletal muscle myogenesis through the regulation of E2F1-DP1 activity.
**Significance:** This is the first report to identify DP1 as a substrate of Kbtbd5.

**We have previously isolated a muscle-specific Kelch gene, Kelch repeat and BTB domain containing protein 5 (Kbtbd5)/Kelch-like protein 40 (Klhl40). In this report, we identified DP1 as a direct interacting factor for Kbtbd5 using a yeast two-hybrid screen and *in vitro* binding assays. Our studies demonstrate that Kbtbd5 interacts and regulates the cytoplasmic localization of DP1. GST pulldown assays demonstrate that the dimerization domain of DP1 interacts with all three of the Kbtbd5 domains. We further show that Kbtbd5 promotes the ubiquitination and degradation of DP1, thereby inhibiting E2F1-DP1 activity. To investigate the *in vivo* function of Kbtbd5, we used gene disruption technology and engineered *Kbtbd5* null mice. Targeted deletion of Kbtbd5 resulted in postnatal lethality. Histological studies reveal that the *Kbtbd5* null mice have smaller muscle fibers, a disorganized sarcomeric structure, increased extracellular matrix, and decreased numbers of mitochondria compared with wild-type controls. RNA sequencing and quantitative PCR analyses demonstrate the up-regulation of E2F1 target apoptotic genes (Bnip3 and p53inp1) in *Kbtbd5* null skeletal muscle. Consistent with these observations, the cellular apoptosis in *Kbtbd5* null mice was increased. Breeding of *Kbtbd5* null mouse into the *E2F1* null background rescues the lethal phenotype of the *Kbtbd5* null mice but not the growth defect. The expression of Bnip3 and p53inp1 in *Kbtbd5* mutant skeletal muscle are also restored to control levels in the *E2F1* null background. In summary, our studies demonstrate that Kbtbd5 regulates skeletal muscle myogenesis through the regulation of E2F1-DP1 activity.**

The first Kelch protein (*Kel*) was identified as a regulator of cytoplasmic flow between cells in the ring canal of *Drosophila* (1). Kelch proteins are usually composed of three domains, termed the BTB (BRC, ttk, and bab), BACK (BTB and C-terminal Kelch), and Kelch domains (2). The BTB domain functions as the protein-protein interaction domain for dimer formation and the interaction with a coactivator or corepressor or in the recruitment of the Cul3 complex (3). The BACK domain is named on the basis of its location between the BTB and Kelch domains. However, its function has not been defined (4). The Kelch domain usually consists of two to seven repeats, with each repeat forming the structure of $\beta$ strands and serving as the protein-interacting surface (5). A number of Kelch proteins have been identified (6, 7) and can be classified into three subfamilies on the basis of their domains: the Kelch-like (KLHL) subfamily, containing all of the BTB, BACK, and Kelch domains; the Kelch repeat and BTB domain containing protein (KBTBD) subfamily, containing BTB and Kelch domains (and, possibly, a BACK domain); and the Kelch domain containing protein (KLHDC) subfamily, which contains only the Kelch domain (7). Kelch proteins have diverse regulatory roles in cellular function, such as B cell signaling, cell morphology, cell cycle, oxidative and electrophilic stress response, and skeletal muscle myogenesis (5, 7, 8). Extensive biochemical studies have revealed that Kelch proteins function as the adaptors of Cul3 E3 ligase to recognize a broad range of substrates, thereby regulating multiple cellular processes (9, 10). For example, Klhl12 targets Dsh for ubiquitination and negatively regulates Wnt/$\beta$-catenin signaling (11), whereas, Klhdc5 modulates p60/Katanin protein stability and regulates cell cycle progression (12).

Activation of E2F transcription factors is the central event in cell cycle progression (13, 14). E2F factors regulate the expression of genes that are essential for cell division, such as cell cycle regulators (including cyclin E, cyclin A, Cdc2, Cdc25A, c-myc, pRb, and E2F1), enzymes involved in nuclear biosynthesis, and the main components of the DNA replication machinery (15, 16). The E2F complex is composed of the E2F factor and its sibling factor, DP[2] (17). There are eight members in the E2F subfamily and three members in the DP subfamily. E2F factors are further divided into two groups: the typical group that consists of activator E2Fs (E2F1, E2F2, and E2F3) and repressor E2Fs (E2F4, E2F5, and E2F6) and the atypical E2F7 and E2F8 (17, 18). The typical E2Fs (E2F1-E2F6) bind DNA by het-

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[2] The abbreviations used are: DP, dimerization protein; RNAseq, RNA sequencing; IP, immunoprecipitation; P, postnatal day; ARF, alternative reading frame.

erodimerizing with the DP subfamily, which is composed of DP1 and DP2 (18). E2F1 is localized in the nuclear compartment, whereas DP1 shuttles between the cytoplasmic and nuclear compartments and translocates into the nucleus when it binds E2F1 as a heterodimer (19, 20). Gene disruption of E2F1 results in dysregulation of T cell development because of a defect in thymocyte apoptosis, aberrant cell proliferation, and tumorigenesis (21, 22). Loss of DP1 results in an extraembryonic developmental defect and lethality but is not essential for embryonic development (23, 24). Sertad1 was originally identified as an antagonist of p16 and a coactivator of E2F1/DP1 (25, 26).

We have recently identified a muscle-specific Kelch gene, *Kbtbd5/Klhl40*, which is expressed in the myogenic lineage during embryogenesis (27). Kbtbd5 has been shown to be involved in protein ubiquitination (27, 28). Mutation of Kbtbd5 in the human is frequently associated with nemaline myopathy (29, 30). In this study, we identified DP1 as the substrate of Kbtbd5 and demonstrated that Kbtbd5 regulated E2F1-DP1 activity. In addition, gene targeting studies revealed that Kbtbd5 was essential for postnatal survival, and the lethality of the *Kbtbd5* null mouse was rescued in the *E2F1* null background.

## Experimental Procedures

*DNA and RNA Manipulation*—The following plasmids were purchased from Addgene: Ccne1 reporter (Addgene plasmid 8458 (31)), E2F1 (Addgene plasmid 10736 (32)), Cul3 (Addgene plasmid 19893 (33)), Roc1 (Addgene plasmid 19897 (33)), HA-Ub (Addgene plasmid 17608 (34)), and Keap1 (Addgene plasmid 21556 (35)). All other plasmids and their mutant constructs were cloned by PCR and then verified by DNA sequence analysis. The raw RNAseq data were mapped to the mouse genome (mm10) using TopHat (version 2.0.11) with default parameters (36), yielding an average of ~50 million mappable paired reads per sample. The expected counts of each gene were estimated by Cufflink pipeline, followed by differential expression analysis through edgeR (37, 38). cDNA synthesis and quantitative PCR were performed as described previously (39).

*Cell Transfection and Transcriptional Assays*—C2C12 myoblasts and NIH3T3 fibroblasts were maintained in complete DMEM supplemented with 10% FBS at 37 °C in the incubator with 5% $CO_2$ and transfected with Lipofectamine (Invitrogen) or FuGENE HD (Roche), respectively. The total amount of DNA was normalized with the vector DNA. Cells were harvested 24 h after transfection for luciferase assays as outlined in the manual of the manufacturer of the Dual-Luciferase system (Promega).

*Yeast Two-hybrid Screen, Western Blot Analysis, Coimmunoprecipitation, and GST Pulldown Assays*—A pGBKT7 vector-Kbtbd5 construct was used to screen a skeletal muscle cDNA library as outlined in our previous report (40). Western blot analysis and coimmunoprecipitation (co-IP) assays were performed as described in our previous studies using the following antibodies: anti-HA (Santa Cruz Biotechnology), anti-Myc (Santa Cruz Biotechnology), anti-FLAG (Sigma Aldrich), anti-tubulin (Sigma Aldrich), and anti-ubiquitin (BD Biosciences) sera (41). Briefly, 5% of the total lysate as the input was utilized for Western blot analysis to examine protein overexpression. Then the total lysate was incubated with anti-HA or anti-Myc sera for immunoprecipitation. The total immunoprecipitation complex was analyzed in the SDS-PAGE gel and then immunoblotted with anti-Myc or anti-HA, respectively. *In vitro* protein synthesis was performed by TNT Quick systems (Promega) in the presence or absence of [$^{35}$S]methionine. The $^{35}$S-labeled Kbtbd5 deletional proteins were first examined with SDS-PAGE gel as the input. GST-DP1 (199–350) was overexpressed in *Escherichia coli* BL21 cells, extracted with B-PER bacterial protein extraction reagent (Pierce Biochemicals), and then purified with glutathione-Sepharose CL-4B (GE Healthcare). The *in vitro* synthesized $^{35}$S-labeled proteins were incubated with the GST-DP1 (199–350) fusion protein bound to Sepharose beads, washed, and resuspended in sample loading buffer. Note that the GST control was performed at the same time as the GST pulldown assays to evaluate the Kbtbd5 deletional proteins. The pulldown proteins were analyzed using a 4–20% polyacrylamide gel and imaged using Typhoon 2000 (41).

*Immunostaining, LacZ Staining, Histology, and TUNEL Assay*—Immunostaining, LacZ staining, and H&E staining were performed as described previously (39, 42). Neonatal skeletal muscle was fixed in 4% paraformaldehyde prior to sectioning. A TUNEL assay was performed with a DeadEnd colorimetric TUNEL system (Promega) according to the directions outlined in the user manual. Briefly, the tissue sections were deparaffinized, rehydrated, and permeabilized with protease K buffer before incubation with the TdT reaction mixture. The slides were treated with 2× SSC buffer, 0.3% hydrogen peroxide and then incubated with streptavidin HPR reaction buffer. The sections were imaged with an Zeiss Axio Imager M1 upright microscope equipped with Zen software (39).

*Ubiquitination Assay*—For DP1 ubiquitination *in vivo*, C2C12 cells were transfected with expression plasmids for HA-ubiquitin, Myc-DP1, and FLAG-Kbtbd5 or the vector as a control. Twenty-four hours after transfection, the cells were treated with 0.01 mM MG132 (Calbiochem) for 5 h before lysis with radioimmune precipitation assay buffer (0.15 M NaCl, 50 mM Tris (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplied with protease inhibitor (Roche) and 10 mM *N*-ethylmaleimide (EMD). The protein extract was incubated with a Myc antibody conjugated with agarose (Santa Cruz Biotechnology), followed by three washes with radioimmune precipitation assay buffer. The final immunoprecipitation complex was resuspended in 2× SDS sample buffer and analyzed using 4–15% gradient SDS-PAGE. DP1 ubiquitination was detected using a 3F10 antibody directed against the HA epitope (Roche).

*Generation of Kbtbd5 Knockout Mice and Animal Husbandry*—To construct the targeting vector, a 9.07-kb region of the *Kbtbd5* gene was subcloned from a BAC clone (RP23 103H3). The region was designed so that the short homologous arm extended 2.34 kb 3′ to exon 1. The long homologous arm ended at the 5′ side of exon 1 and was 5.58 kb long. The LacZ/Neo cassette was used to replace 1150 bp of the coding region for exon 1. The targeting vector was confirmed using restriction enzyme analysis and DNA sequence analysis. The targeting vector was linearized and electroporated into C57BL/

5 × 129/SvEv hybrid ES cells using standard techniques (43). After G418 antibiotic selection, the surviving clones were expanded and screened for correct integration by PCR. Clone 114 was expanded and microinjected into C57BL/6J mouse blastocysts. The 100% chimeras were bred with C57BL/6N wild-type female mice (The Jackson Laboratory), and the offspring was genotyped using a PCR strategy. Mice (#144 and #145) were confirmed as having a heterozygous genotype. Those heterozygous mice were then bred to EIIA-Cre to remove the floxed Neo cassette. The resulting chimeric mice were bred for germ line transmission. These heterozygous mice carrying the LacZ/Neo cassette were bred to EIIA-Cre mice to remove the neomycin cassette. All mice were maintained at the University of Minnesota using protocols approved by the Institutional Animal Care and Use Committee and Research Animal Resources.

*Statistics*—All data represent the mean ± S.D. of at least three replicates. Statistical significance analysis was carried out using Student's *t* test ($p < 0.05$).

## Results

*Identification of DP1 as a Kbtbd5-binding Protein*—Our previous study revealed that Kbtbd5 had an important role in skeletal muscle myogenesis because the knockdown of Kbtbd5 resulted in the perturbation of myogenic differentiation (27). To identify potential Kbtbd5-interacting proteins, we screened a skeletal muscle cDNA library using a yeast two-hybrid system (44). A number of clones were isolated from this screen, and these genes were classed into two major categories: cytoskeletal structure and transcriptional regulation (Fig. 1*A*). The high abundance of Nebulin-related anchoring protein (NRAP) and Myoz1 in the screen suggested that Kbtbd5 might be involved in the cytoskeletal regulation because both NRAP and Myoz1 have been reported in the regulation of cytoskeletal structure (45, 46). Two genes related to transcriptional regulation were Gatad2b and Sertad1 (Fig. 1*A*). Gatad2b has been reported as a component of the Mi-1·NuRD repression complex involved in chromatin remodeling (47). Previous studies have reported the functional role of Sertad1 in the regulation of the cell cycle, which led us to evaluate the protein-protein interaction between Kbtbd5 and Sertad1 because cell cycle regulation is coordinated with muscle differentiation (25, 26). Both Myc-Sertad1 and HA-Kbtbd5 were overexpressed in C2C12 cells. As shown in Fig. 1*B*, HA-Kbtbd5 was detected in the co-IP complex of Myc-Sertad1 using a Myc antibody, and Myc-Sertad1 was detected in the co-IP complex with HA-Kbtbd5 in the reciprocal experiment using a HA antibody. However, we could not detect their direct interaction using *in vitro* binding assays (Fig. 2, *A* and *B*). These results indicated that there was/were additional protein(s) that served as a bridge between Sertad1 and Kbtbd5. Both Cdk4 and DP1 have been reported as Sertad1-interacting proteins (25, 26). HA tagged proteins (Sertad1, Cdk4, E2F1, and DP1) were synthesized successfully *in vitro*, as revealed by [$^{35}$S]methionine incorporation (Fig. 2*A*). For the *in vitro* co-IP assay, Kbtbd5 was labeled with [$^{35}$S]methionine, whereas the HA-tagged proteins (Sertad1, Cdk4, E2F1, and DP1) were synthesized in the absence of [$^{35}$S]methionine. DP1 was identified as the only protein directly interacting with

**A**

| Class | Gene | Clone |
|---|---|---|
| Cytoskeletal structure | Acta1 | 1 |
| | Myoz1 | 24 |
| | Myoz3 | 1 |
| | NRAP | 44 |
| | Titin | 2 |
| | Tnni2 | 2 |
| | Tnnt1 | 1 |
| Transcriptional regulation | Gatad2b | 1 |
| | Sertad1 | 2 |

**B**

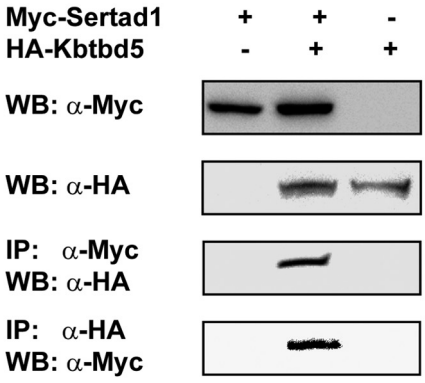


FIGURE 1. *A*, summary of the yeast two-hybrid screen. Positive clones were identified from the yeast two-hybrid screen. Sequence analysis revealed two major classes of genes: cytoskeletal structure genes and transcriptional regulation genes. *Clone*, the total number isolated from the screening. *B*, Myc-Sertad1 and HA-Kbtbd5 were overexpressed in C2C12 cells and detected using Western blot (*WB*) analysis (*first* and *second panels*). HA-Kbtbd5 could be coimmunoprecipitated with Myc-Sertad1 using a Myc antibody, and the reverse was also true (*third* and *fourth panels*).

Kbtbd5. Sertad1, Cdk4, or E2F1 did not bind to Kbtbd5 under the same conditions (Fig. 2*B*). We also observed that Kbtbd5 could be coimmunoprecipitated with HA-Sertad1 in the presence of DP1 (Fig. 2*C*), which supported the original hypothesis that DP1 serves as an adaptor protein between Kbtbd5 and Sertad1. We further confirmed the interaction between Kbtbd5 and DP1 in C2C12 cells (Fig. 2*D*). Previous studies have established that the DP1 protein shuttles between the cytoplasm and nucleus and translocates into the nuclear compartment upon heterodimerization with E2F1 (19, 20). Using immunohistochemical techniques, we observed the nuclear localization of DP1 when E2F1 was coexpressed (Fig. 2, *E* and *F*). To define the regulation of DP1 subcellular localization by Kbtbd5, we overexpressed FLAG-Kbtbd5, Myc-DP1, and HA-E2F1 together in C2C12 cells (Fig. 2, *G* and *H*). As shown in Fig. 2*H*, Kbtbd5 was localized to the cytoplasmic compartment (*left panel*), and DP1 translocated from the nuclear compartment to the cytoplasm (*center panel*), where they were colocalized (*right panel*), which further supported the notion that Kbtbd5 and DP1 interact.

*Characterization of the Kbtbd5- and DP1-interacting Domains*—To define the protein-protein interacting domains between Kbtbd5 and DP1, we performed *in vitro* binding assays

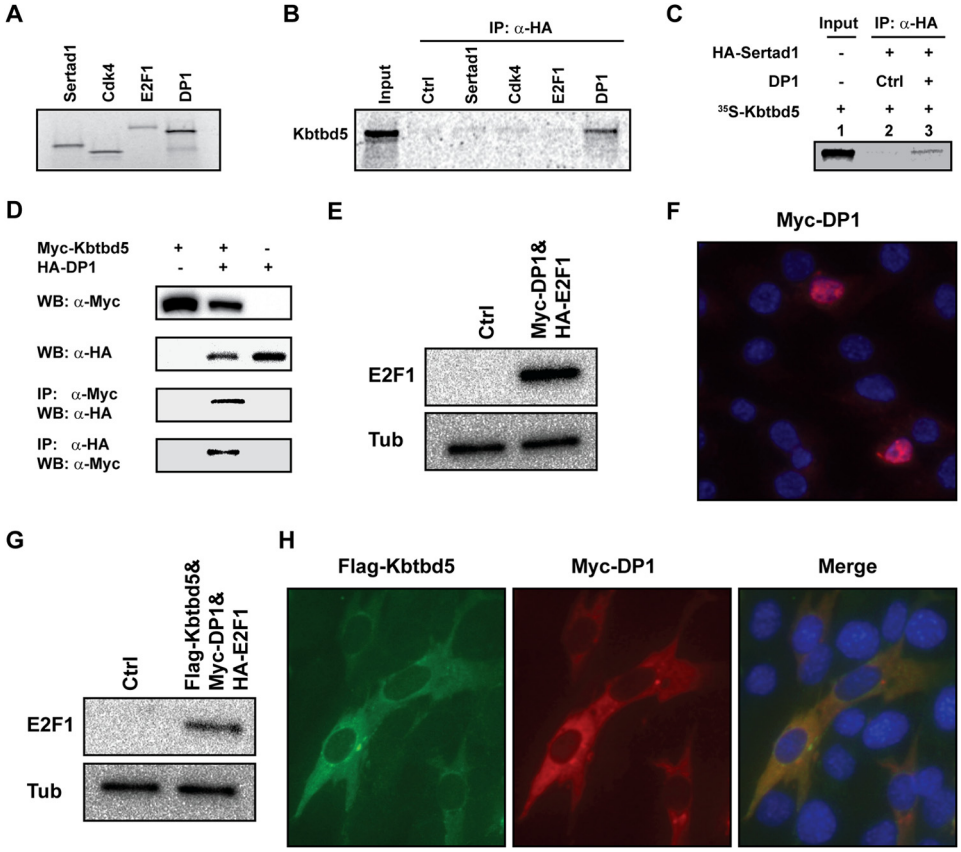


FIGURE 2. **Identification of DP1 as the binding candidate of Kbtbd5.** *A*, HA-tagged Sertad1, Cdk4, E2F1, and DP1 proteins were synthesized *in vitro* in the presence of [$^{35}$S]methionine. These $^{35}$S-labeled proteins were analyzed by SDS-PAGE gel and exposed to PhosphorImager, which was then scanned with Typhoon 2000. *B*, HA-tagged Sertad1, Cdk4, E2F1, and DP1 proteins were synthesized in the absence of [$^{35}$S]methionine for the coimmunoprecipitation assays. $^{35}$S-labeled Kbtbd5 was coimmunoprecipitated specifically with HA-tagged DP1 but not Sertad1, Cdk4, or E2F1. *Ctrl*, protein synthesis with the vector control. *C*, $^{35}$S-Kbtbd5 was coimmunoprecipitated with HA-Sertad1 in the presence of DP1 *in vitro*. *D*, Myc-Kbtbd5 and HA-DP1 were cotransfected into C2C12 myoblasts. DP1 was coimmunoprecipitated with Kbtbd5 using an anti-Myc serum (*WB*, anti-HA serum), and Kbtbd5 was also present in the DP1 immunoprecipitation complex (*WB*, anti-Myc serum). *E*, coexpression of Myc-DP1 and HA-E2F1. Expression of HA-E2F1 was confirmed by Western blot analysis. *Ctrl*, cell lysate without HA-E2F1 overexpression; *Tub*, tubulin. *F*, immunostaining revealed that DP1 was localized in the nuclear compartment (*red*) when cotransfected with E2F1 into C2C12 cells. The nuclei were marked with DAPI (*blue*). *G*, Myc-DP1, HA-E2F1 and FLAG-Kbtbd5 were cotransfected into C2C12 cells. Expression of HA-E2F1 was confirmed by Western blot analysis. *Ctrl*, cell lysate without HA-E2F1 overexpression. *H*, immunostaining revealed that Kbtbd5 was localized in the cytoplasmic compartment (*green*, *left panel*), that DP1 was sequestered in the cytoplasmic compartment (*red*, *center panel*), and that DP1 was colocalized with Kbtbd5 (*yellow*, *right panel*) when Myc-DP1, HA-E2F1, and FLAG-Kbtbd5 were co-overexpressed. Nuclei were stained with DAPI (*blue*).

using Kbtbd5 and DP1 deletional constructs and co-IP and GST pulldown assays. The DP1 deletional constructs and their interactions with DP1 are summarized in Fig. 3*A*. HA-tagged DP1 deletional proteins were synthesized *in vitro* with [$^{35}$S]methionine, as shown in Fig. 3*B*, which verified the expression of each deletional protein. Each deletional protein was then synthesized without [$^{35}$S]methionine, incubated with the $^{35}$S-labeled Kbtbd5, and immunoprecipitated using a HA antibody. The deletional constructs harboring the DP domain (199–355) were able to bind to Kbtbd5 but not the other constructs (Fig. 3, *A* and *C*). We then utilized GST-DP1 (199–350) to define the Kbtbd5 interaction domain, which is summarized in Fig. 3*D*. $^{35}$S-labeled Kbtbd5 deletional proteins were translated successfully *in vitro* and then utilized for the GST pulldown assay (Fig. 3*E*, *top panel*). As shown in Fig. 3*E*, the full-length (1–621) and the Kbtbd5 deletional protein (240–621) were pulled down by GST-DP1 (199–350) (*bottom panel*) but not with the GST-only control (*center panel*). We also observed that the pulldown of Kbtbd5 deletions (23–128) and (129–239) by GST-DP1 (199–350) was weaker.

*Kbtbd5 Regulates the Ubiquitination of DP1 and the Activity of the E2F1-DP1 Complex*—Previous studies have reported the ubiquitination and proteasomal degradation of E2F1 during the cell cycle (48–51). DP1 may also be ubiquitinated in the cytoplasm when it is not dimerized with E2F1 (52). The role of Kelch proteins as the Cul3 adaptor in the substrate ubiquitination has been well established (53). For example, Nrf2 has been reported to be the substrate of Keap1-Cul3-dependent ubiquitination during oxygen stress (54). On the basis of these reports and our protein-protein interaction studies, we hypothesized that DP1 was the substrate of Kbtbd5 in Cul3-dependent ubiquitination. As shown in Fig. 4*A*, the DP1 protein was stabilized when C2C12 cells were exposed to MG132 (a proteasome inhibitor) for just 1 h, and the DP1 protein level was increased further upon longer exposure (5 h). These results indicated that the stability of DP1 protein was regulated by ubiquitination in C2C12 myogenic cells. To investigate the functional role of Kbtbd5 as the adaptor of DP1 ubiquitination, we overexpressed Myc-tagged DP1, FLAG-tagged Kbtbd5, and HA-tagged ubiquitin in C2C12 cells and then treated the cells with MG132 to

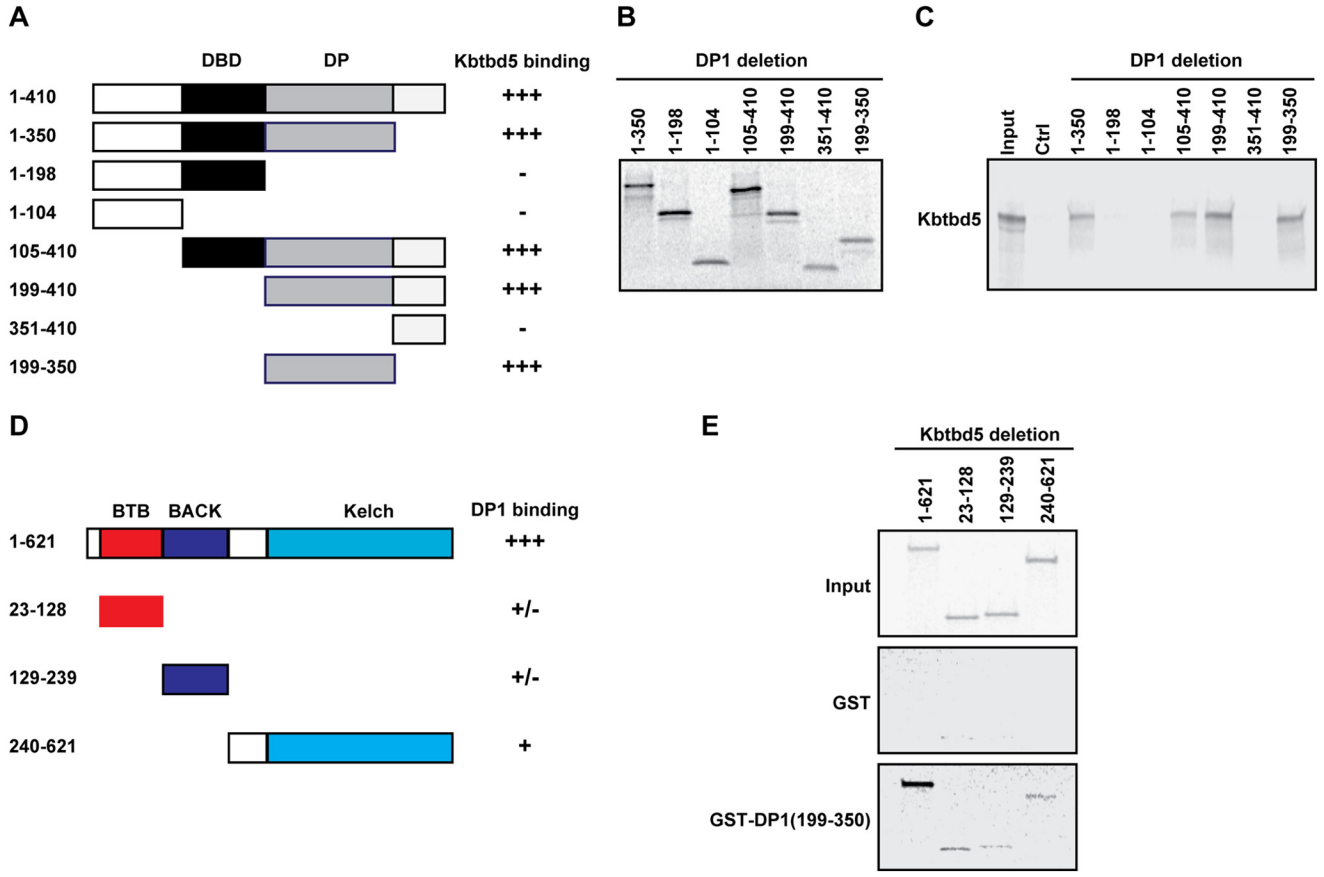


FIGURE 3. **Kbtbd5 and DP1 protein-protein interaction domain mapping.** *A*, schematic of the interaction between DP1 deletional constructs and Kbtbd5. The DP domain of DP1 interacted with Kbtbd5 (+++, strong interaction; −, no interaction). *DBD*, DNA-binding domain. *B*, all of the HA-tagged DP1 deletional constructs were utilized to synthesize proteins *in vitro*, as revealed with [$^{35}$S]methionine incorporation. *C*, $^{35}$S-labeled Kbtbd5 was coimmunoprecipitated by DP1 mutant proteins harboring the DP domain (199–350) but not the mutant proteins lacking the DP domain. *D*, schematic of the interaction between Kbtbd5 deletions and DP1 (+++, strong interaction; +, interaction; +/−, weak interaction). *E*, Kbtbd5 deletional proteins were synthesized *in vitro* with [$^{35}$S]methionine to a similar level as shown in the input (*top panel*). Full-length and deletion (240–621) Kbtbd5 proteins were pulled down by GST-DP1 (199–350) but not the GST-only control (*center* and *bottom panels*). Also note that full-length Kbtbd5 protein was pulled down with higher efficiency than any of the deletional proteins (*bottom panel*).

prevent DP1 degradation. DP1 expression was maintained to a similar level with Kbtbd5 or the vector control upon MG132 treatment (Fig. 4*B*, *center panel*). The ubiquitination of DP1 was determined using immunoprecipitation with a Myc antibody followed by Western blot analysis with anti-HA serum. As shown in Fig. 4*B*, the ubiquitination of DP1 was relatively low in the control but increased dramatically upon coexpression with Kbtbd5 (Fig. 4*B*, top panel). These studies demonstrated that Kbtbd5 could serve as an adaptor and, therefore, promoted DP1 ubiquitination. To evaluate the functional significance of this regulation, we utilized an E2F1 reporter (a Ccne1 promoter fused to luciferase) and transcriptional assays (31, 32). As shown in Fig. 4*C*, the E2F1-DP1 complex transactivated the *Ccne1-Luc* reporter more than 30-fold, and the transactivation was attenuated to 16-fold in a dose-dependent fashion by increasing the amount of Kbtbd5. Overexpression of all of these genes was confirmed, as shown in Fig. 4*D*. In contrast, another Kelch protein, Keap1, did not repress E2F1-DP1 activity (Fig. 4*D*). In addition, E2F1 activity was also repressed from 15-fold to 6-fold in a dose-dependent fashion by increasing Kbtbd5 (Fig. 4, *E* and *F*). We reason that Kbtbd5 may interact with the endogenous DP1 protein, thereby reducing the pool of DP1 protein available for the E2F1-DP1 transactivation complex.

*Kbtbd5 Is Essential for Skeletal Muscle Myogenesis*—To examine the *in vivo* function for Kbtbd5, we engineered a targeting construct by inserting a LacZ reporter and a Neo cassette into exon 1 of the *Kbtbd5* gene to trace endogenous *Kbtbd5* gene expression and, ultimately, pursue a gene disruption strategy (Fig. 5*A*). Successful targeting of the Kbtbd5 locus was achieved as outlined in Fig. 5, *B* and *C*. Heterozygous mice were mated with the *EIIA-Cre* mice to remove the floxed Neo cassette and then backcrossed with C57BL/6J wild-type mice to remove the EIIA-Cre transgene to generate *Kbtbd5* heterozygous (+/−) mice (55). Using RT-PCR and Western blot analysis, we confirmed that Kbtbd5 mRNA and protein were absent in *Kbtbd5* null mice (Fig. 5, *D* and *E*). LacZ staining of the embryos confirmed the specificity of *Kbtbd5* gene expression in the muscle lineage, as outlined in our previous report (Fig. 5, *F* and *G*) (27). The postnatal *Kbtbd5* null neonates were normal in size at birth, with the expected Mendelian inheritance (Fig. 6*A*). However, the *Kbtbd5* null mice failed to gain weight compared with their wild-type littermates and did not survive to weaning (*i.e.* 21 days old) (Fig. 6, *B*–*E*). To examine the muscle structure of *Kbtbd5* null skeletal muscle, we used light and ultrastructural morphological techniques. Histological analysis revealed that the myofibers (normalized to body weight) in the *Kbtbd5* null

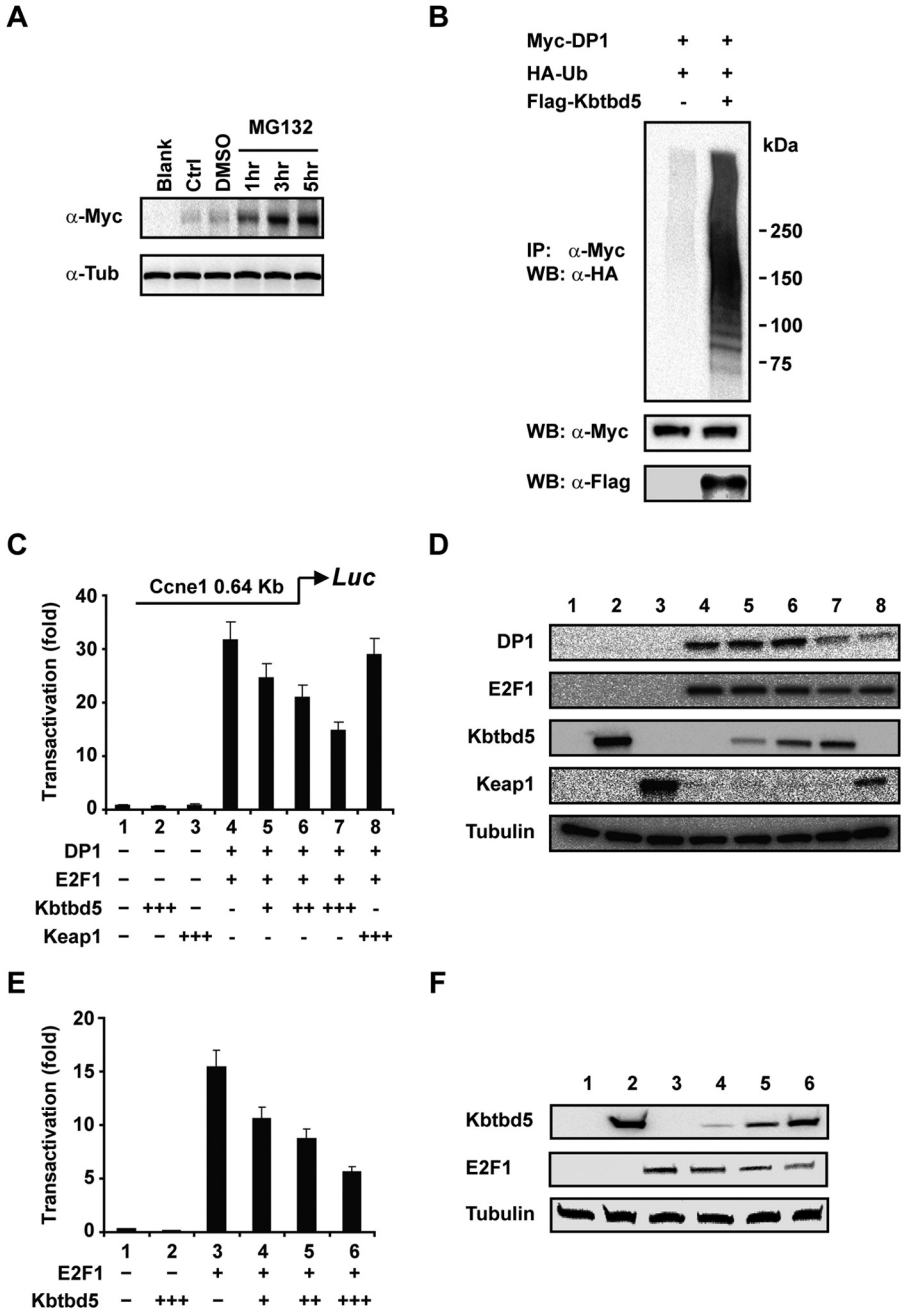


FIGURE 4. **Regulation of DP1 stability and activity by Kbtbd5.** *A*, C2C12 myoblasts were transfected with a Myc-DP1 expression plasmid and then treated with dimethyl sulfoxide or MG132 for the indicated times. DP1 protein was stabilized by MG132 treatment. *Blank*, no transfection; *Ctrl*, no treatment; *Tub*, tubulin. *B*, 293T cells were transfected with Myc-DP1, HA-Ub, and FLAG-Kbtbd5. Protein expressions of Myc-DP1 and FLAG-Kbtbd5 were confirmed using Western blot (*WB*) analysis (*center* and *bottom panels*). Myc-DP1 was immunoprecipitated by the Myc antibody, and the ubiquitination of DP1 was detected using a HA antibody. The ubiquitination of DP1 was enhanced dramatically in the presence of Kbtbd5. *C*, the effect of Kbtbd5 on DP1 activity was evaluated using transcriptional assays with the Ccne1-Luc reporter. The E2F1-DP1 complex potently transactivated the reporter (32-fold). Kbtbd5 repressed E2F1/DP1 transcriptional activity in a dose-dependent manner to 16-fold. However, another BBK gene, Keap1, did not repress E2F1/DP1 activity at all. *D*, the overexpression of each protein in *C* was confirmed by Western blot analysis. *E*, E2F1 transactivated the reporter 16-fold. Kbtbd5 repressed E2F1 transcriptional activity from 16-fold to 5-fold. *F*, Western blot analysis verified the overexpression of each protein in *E*.

skeletal muscle were significantly smaller compared with the wild-type controls (Fig. 7, *A* and *B*). Further examination by EM revealed that the sarcomeric structure in the *Kbtbd5* null skeletal muscle was perturbed, with fewer mitochondria and increased extracellular matrix (Fig. 7*C*). We performed an RNAseq analysis and examined the pathways of differentially expressed genes ($q$ value $< 0.05$) using ToppGene suites (56) to examine the molecular profiles in *Kbtbd5* knockout mice (supplemental Table S1). These dysregulated genes were involved in six major pathways: apoptosis, cellular proliferation, epithelio-genesis, immune response, metabolism, and myogenesis. E2F1 has been reported previously as an important regulator of cellular apoptosis (57). Among the genes involved in apoptosis, both Bnip3 and Trp53inp1 have been reported as important E2F1 target genes (57–60). To further confirm the RNAseq data, we performed quantitative PCR. As shown in Fig. 7*D*,

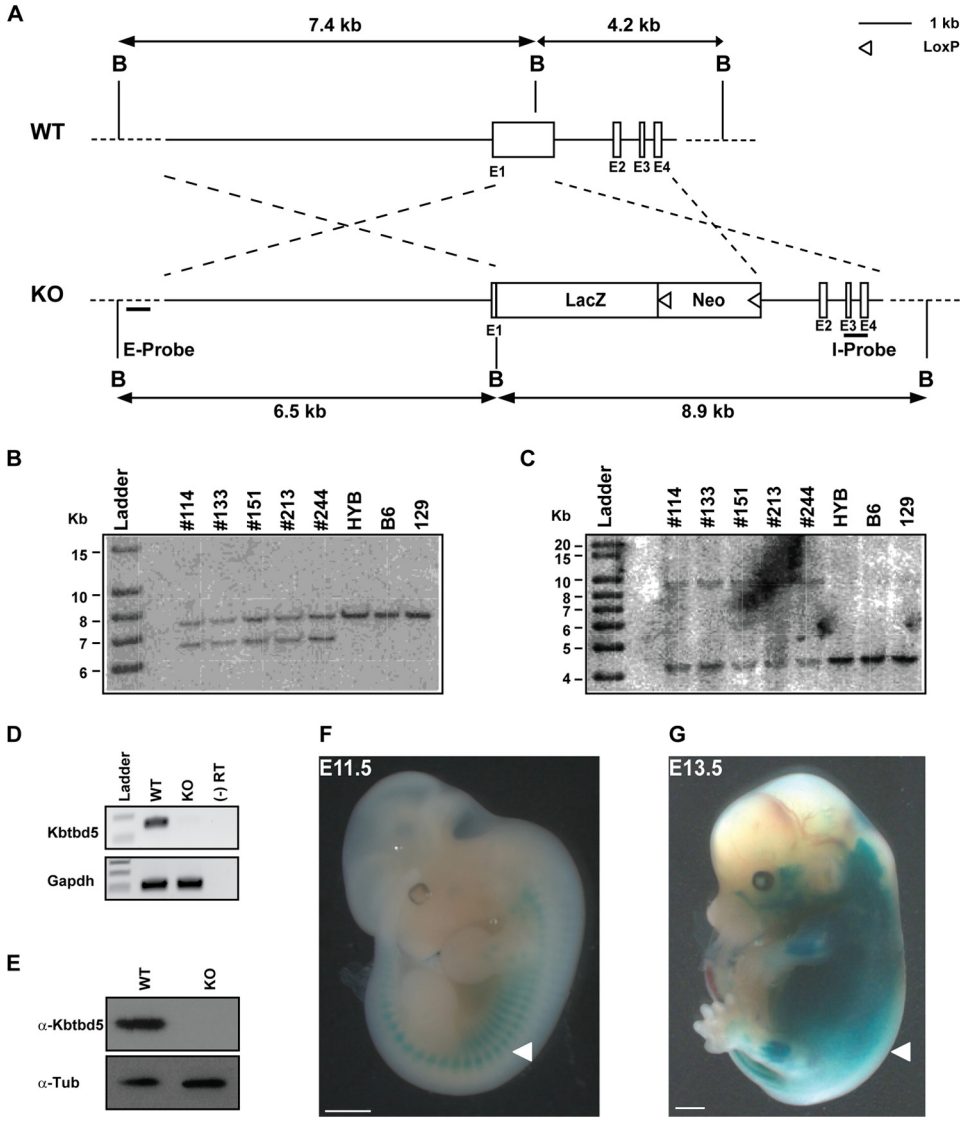


FIGURE 5. *A*, gene targeting strategy of Kbtbd5. LacZ and Neo selection cassettes were inserted into the Kbtbd5 exon 1 coding region. The Neo cassette was floxed with LoxP sites. *E1-E4*, exons 1–4; *B*, BamHI; *E-Probe*, external probe; *I-Probe*, internal probe. *B* and *C*, ES cells were screened by PCR and Southern blot analyses to verify the correctly targeted ES clones using an external probe (*B*) or internal probe (*C*). Wild-type control DNA samples were from HYB (C57Bl/6 X129/SvEv), B6 (C57Bl/6), and 129 (129/SvEv) mouse strains. ES cell clone #114 was expanded and injected into the blastocysts to produce chimeric mice. *D*, absence of Kbtbd5 mRNA in the neonatal hind limb muscle of *Kbtbd5* knockout (*KO*) mice. *(−)RT*, reverse transcription product is not included in the reaction. *E*, Kbtbd5 protein is absent in the neonatal hind limb muscle of *Kbtbd5* KO mice. *F* and *G*, LacZ staining of *Kbtbd5*$^{+/-}$ embryos at embryonic day (*E*) 11.5 (*F*) and 13.5 (*G*) stages during embryogenesis. Somites and differentiated muscle are marked by *arrowheads*. *Scale bars* = 500 $\mu$m.

Bnip3 and p53inp1 were up-regulated in the absence of Kbtbd5 but not other E2F1 target apoptotic genes (57, 59). To investigate the cellular effect on the skeletal muscle, we performed TUNEL assays. As shown in Fig. 7*E*, we observed increased cellular apoptosis in the *Kbtbd5* null skeletal muscle, which was further quantified in Fig. 7*F*.

*Partial Rescue of the Kbtbd5 Null Phenotype*—The knockout of *E2F1* resulted in perturbed cellular apoptosis pathways in T cell development and increased tumor growth (21, 22). Previous studies have reported apoptosis in the somite of *E2F7/E2F8* double null mice, which was rescued in the *E2F1* null background (61). The results of our studies prompted us to examine the physiological rescue of the *Kbtbd5* knockout phenotype in the *E2F1* null background. The *Kbtbd5* heterozygotes were bred into the *E2F1* null background to produce mice lacking both *Kbtbd5* and *E2F1*. Our studies revealed that the double knockout mice were viable at birth, with the expected Mendelian inheritance, and survived to weaning (Fig. 8*A*). However, these double knockout mice were still smaller compared with the controls in the *E2F1* null background (Fig. 8, *B* and *C*). Quantitative RT-PCR revealed that Bnip3 and p53inp1 gene expression was restored to control levels (Fig. 8*D*). These data indicated that enhanced cellular apoptosis in the skeletal muscle was the major cause of lethality during postnatal development. In summary, our results support the following model (Fig. 9). Kbtbd5 regulates the subcellular localization of DP1 through direct protein-protein interaction and promotes the protein ubiquitination of DP1 in a proteasome-dependent manner, thereby repressing the E2F1-DP1 target genes (Bnip3 and p53inp1).

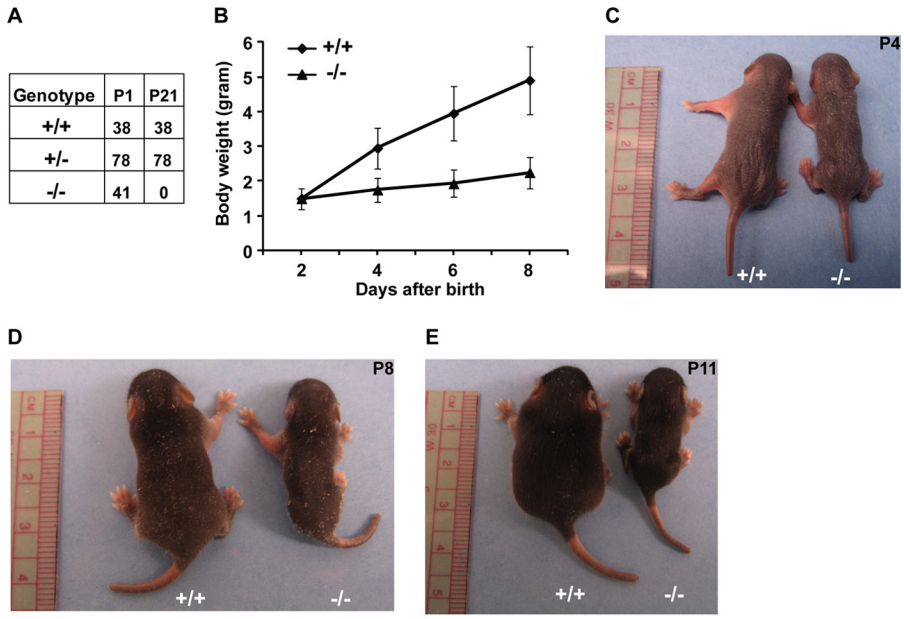


FIGURE 6. **Kbtbd5 is essential for neonatal survival.** *A*, genotyping of the progeny from intercrossed *Kbtbd5* heterozygotes. *Kbtbd5* null mice were viable at birth (*P1*). Most of the null progeny were lethal by ~2 weeks of age, and none survived to weaning (*P21*). *B*, the body weight of *Kbtbd5* wild-type (+/+) and null (−/−) mice during postnatal development. The null mice experienced minimal growth compared with the wild-type littermate controls. *C*—*E*, representative picture of the *Kbtbd5* null and wild-type littermates at P4 (*C*), P8 (*D*), and P11 (*E*).

## Discussion

We have reported previously that Kbtbd5 is a direct downstream target gene of *MyoD* and regulates myogenic differentiation. In this study, we made three major findings: the identification of DP1 as the substrate of Kbtbd5, the definition of the regulation of E2F1-DP1 activity by Kbtbd5, and the definition of the functional role of Kbtbd5 using gene disruption technology. These data defined a novel pathway in the regulation of skeletal muscle myogenesis, as summarized in Fig. 9.

Our first discovery was the identification of DP1 as the substrate of Kbtbd5. Initially, we isolated Sertad1 as the binding candidate of Kbtbd5 from the yeast two-hybrid screen and confirmed the protein-protein interaction between Kbtbd5 and Sertad1 in mammalian cells. However, *in vitro* binding assays did not support the direct interaction between Kbtbd5 and Sertad1. By screening the known Sertad1 binding proteins, we identified DP1 as the direct binding protein of Kbtbd5 but not Cdk4 or E2F1, other Sertad1 interacting factors. Sertad1 was initially isolated as a p16INK4-binding candidate in a yeast two-hybrid screen and, ultimately, characterized as a genuine binding target of Cdk4. It was further predicted that Cdc28 might be the molecular bridge between Sertad1 and p16INK4 (25, 26). These indirect protein-binding candidates, isolated using the yeast two-hybrid screen, may be due to the homology of yeast and mammalian proteins, such as Cdc28 as the yeast homolog protein of CDK4. In this study, we predicted that these evolutionarily conserved proteins as well as the yeast DP1 homolog served as the bridging protein between Sertad1 and Kbtbd5. Two conserved domains have been identified in DP1 and DP2: the DNA-binding domain and the DP domain. Outside of these domains, there is little conservation between DP1 and DP2 (17). The DP domain of DP1 has been reported to interact with the DP of E2F1 to form the E2F1-DP1 heterodimer complex, although there is no conservation between these two DP domains (62). Our domain mapping studies defined that Kbtbd5 binds to the DP domain of DP1 but not that of E2F1. These findings support an interesting model where Kbtbdt5 might compete with E2F1 for the interaction with the DP domain of DP1, thereby disrupting the E2F1-DP1 complex. Previous studies have shown that the BTB domain can form a heterodimer or recruit the Cul3 complex, whereas the Kelch domain interacts with substrates. In this report, we demonstrated that the DP domain of DP1 could bind to all three Kbtbd5 domains. Each Kbtbd5 domain contributes to the interaction with the DP domain of DP1, which might reflect the higher organization of the Kbtbd5 protein domains.

Our second discovery was the definition of the regulation of E2F1-DP1 activity by Kbtbd5. E2F1 protein is regulated by ubiquitination through the F-box-containing protein p45SKP ligase and also by RING of Cullins (ROC)-Cullin ligase (51, 63). The tumor repressor alternative reading frame (ARF) also regulates E2F1 activity at multiple levels, including the regulation of E2F1 translocation, the inhibition of E2F1 transcription, and the proteolysis of E2F1 (64, 65). Coexpression of DP1 with E2F1 could stabilize E2F1 protein from degradation and be resistant to the interaction with ARF. This study shows that Kbtbd5 interacts with DP1 and promotes the ubiquitination of DP1. DP1 was shuttled between the cytoplasm and the nucleus and was localized in the nucleus as a heterodimer with E2F1 (19, 20). DP1 becomes ubiquitinated when deprived of the heterodimerization, although the adaptor for DP1 ubiquitination remains undefined (52). SOSC3 regulates the subcellular localization of DP1, and overexpression of SOSC3 resulted in cell cycle arrest (66). Our data revealed that DP1 was sequestered in the cytoplasm and coexpressed with E2F1 in the presence of Kbtbd5. The disruption of the E2F1-DP1 heterodimer may be due to the high-affinity protein-protein interaction between Kbtbd5 and DP1. We also demonstrated that Kbtbd5 presented

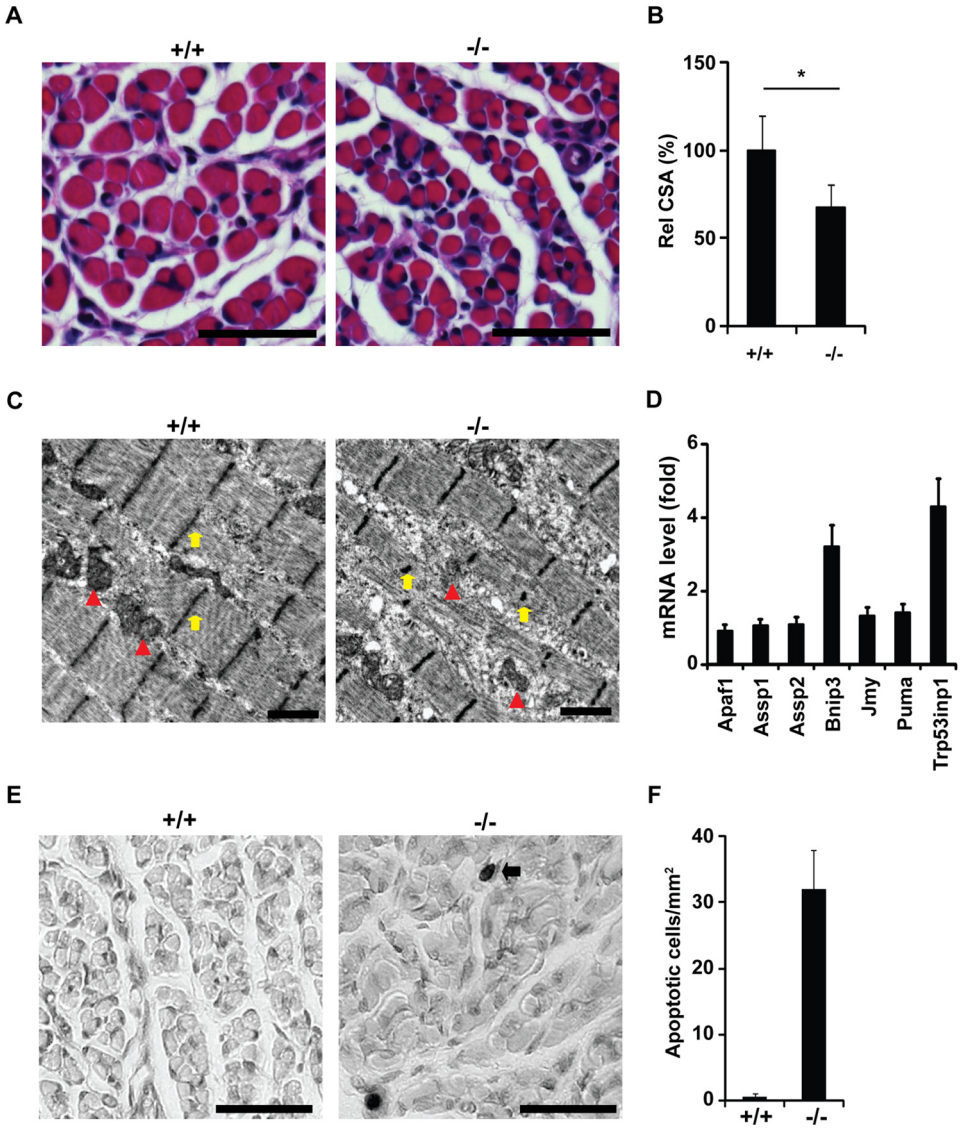


FIGURE 7. **Perturbed skeletal muscle myogenesis in *Kbtbd5* null mice.** *A*, hematoxylin and eosin staining of the gastrocnemius muscle of Kbtbd5 wild-type (+/+) and null (−/−) neonatal mice. We observed that the myofibers in the null mice were significantly smaller than those in the wild-type control at high magnification. *Scale bars* = 50 μm. *B*, quantification of the cross-sectional area (*CSA*) of the myofibers/body weight presented in *A*, with the wild-type controls normalized to 100%. *Rel*, relative. *, $p < 0.05$. *C*, ultrastructure of the skeletal muscle in the wild-type neonate on day 1 reveals a normal sarcomeric structure (*yellow arrows*) with normal mitochondria (*red arrowheads*) (*left panel*, *scale bars* = 1 μm). The ultrastructure of the skeletal muscle in the *Kbtbd5* null neonate on day 1 reveals a disorganized sarcomere (*yellow arrows*), increased extracellular matrix, and decreased numbers of mitochondria (*red arrowheads*) (*right panel*, *scale bars* = 1 μm). *D*, E2F1 downstream target genes (Bnip3 and p53) are associated with apoptosis and up-regulated in the skeletal muscle of *Kbtbd5* null (P1) neonates. The gene expression is first normalized to Gapdh level. The fold of mRNA level refers to the ratio of the gene expression in the *Kbtbd5* null mice to that in *Kbtbd5* wild-type mice. *E*, apoptotic cells are increased dramatically in the skeletal muscle of the *Kbtbd5* null (P4) neonates, as shown by TUNEL assay (*arrow*). Scale bar = 50 μm. *F*, quantification of apoptotic cells in the gastrocnemius muscle per square millimeter.

DP1 as the substrate to the Cul3 complex for ubiquitination *in vivo*. The E2F1-DP1 activity was regulated by Kbtbd5 at multiple levels. The DNA-binding affinity of E2F1 was decreased because the E2F1 DNA-binding was dependent on the E2F1-DP1 heterodimer. E2F1 became associated with and regulated by ARF because of the absence of DP1. E2F1 was also ubiquitinated through p45SKP or ROC-Cullin ligase and then degraded by the proteasome. In summary, Kbtbd5 negatively governed E2F1 activity through the regulation of DP1 localization and stability. Previous studies have reported that E2F1 negatively regulates myogenic differentiation by inhibiting the activity of MyoD (67). We have previously reported that Kbtbd5 was a direct downstream target gene of MyoD (27). Taken together, these studies support the notion that MyoD transactivates *Kbtbd5* gene expression, which, in turn, represses E2F1 in a negative feedback loop mechanism.

Our third finding was the definition of the *in vivo* functional role of Kbtbd5. The functional roles of Kelch proteins in skeletal muscle myogenesis and myopathy have received intense interest (7). Mutation of Klhl9 has been reported to result in distal myopathies, and mutation of Kbtbd5 (Klhl40), Klhl41, and Kbtbd13 have important roles in the development of nemaline myopathy (29, 68–70). All of these proteins have been shown to serve as the Cul3 adaptor, although the substrates remain unclear. Our previous studies have shown that knockdown of Kbtbd5, using a lentiviral shRNA infection strategy,

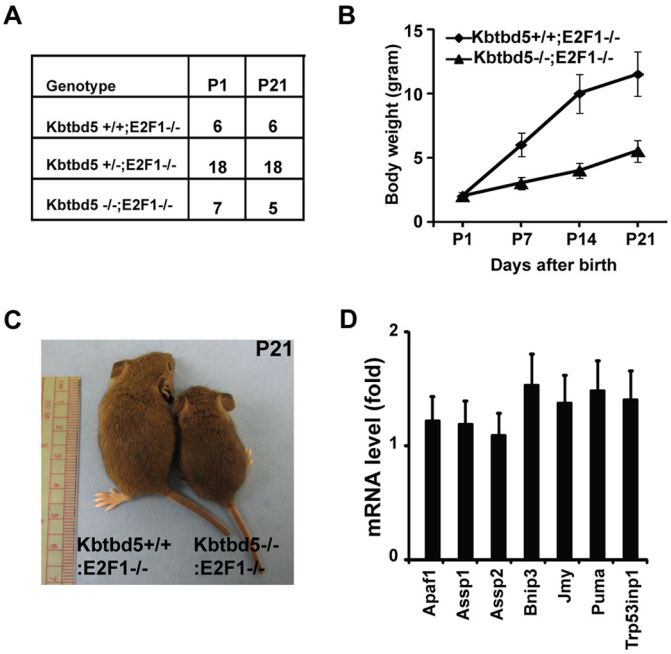


FIGURE 8. **Retarded growth of *Kbtbd5;E2F1* double knockout mice.** *A*, progeny of *Kbtbd5*$^{+/-}$:*E2F1*$^{-/-}$ mice. Most of the *Kbtbd5:E2F1* double-null mice were viable at the time of weaning. *B*, the growth curve of the progeny revealed a partial rescue in the double mutant mice (*Kbtbd5*$^{-/-}$:*E2F1*$^{-/-}$) compared with *Kbtbd5*$^{+/+}$:*E2F1*$^{-/-}$ control mice. *C*, representative image of the *Kbtbd5*$^{+/+}$:*E2f1*$^{-/-}$ and *Kbtbd5*$^{-/-}$:*E2f1*$^{-/-}$ littermates on P21. *D*, the dysregulated gene expression of Bnip3 and Trp53inp1 in *Kbtbd5* null mice was rescued to the normal level in the skeletal muscle of the *Kbtbd5:E2F1* double-null neonates (P1) and comparable with the levels in control *Kbtbd5*$^{+/+}$:*E2F1*$^{-/-}$ mice. The gene expression was first normalized to Gapdh level. The fold of mRNA level refers to the ratio of gene expression in *Kbtbd5*$^{-/-}$:*E2F1*$^{-/-}$ mice to that in *Kbtbd5*$^{+/+}$:*E2F1*$^{-/-}$ mice.

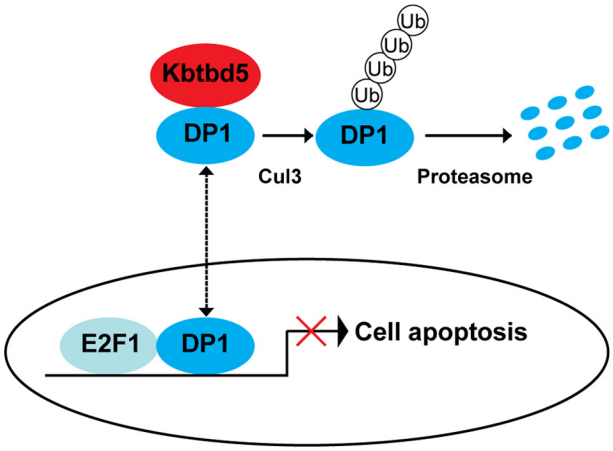


FIGURE 9. **Summary of the overall hypothesis.** Kbtbd5 interacts with DP1, sequesters it in the cytoplasmic compartment, presents it to the Cullin 3 ubiquitination complex, and promotes its degradation in a proteasome-dependent pathway. The cellular apoptosis in the skeletal muscle regulated by E2F1/DP1 is attenuated upon reduction of DP1 by Kbtbd5.

could perturb myogenic differentiation (27). In this work, we utilized a gene-targeting strategy and demonstrated that Kbtbd5 was essential for skeletal muscle myogenesis. Our studies have revealed multiple defects in *Kbtbd5* null skeletal muscle, including smaller muscle fibers, reduced numbers of mitochondria, disorganized sacromeric structure, and perturbed extracellular matrices. Dysregulation of mitochondria is a marker of cellular apoptosis, which is consistent with the TUNEL assays. Our biochemical studies identified DP1 as one of the Kbtbd5 substrates, and Kbtbd5 represses the activity of the E2F1-DP1 complex. Consistent with these findings, RNAseq analysis and quantitative PCR revealed that E2F1 downstream apoptotic genes (Bnip3 and Trp53inp1) were up-regulated in *Kbtbd5* null mice. In *Kbtbd5* null mice, the activity of E2F1-DP1 was increased because of the derepression by Kbtbd5, resulting in enhanced cellular apoptosis. Interestingly, we did not observe dysregulation of other E2F1 apoptotic target genes, including Apaf1, Assp1, Assp2, Jmy, or Puma. One possible explanation for this finding is that the apoptotic program regulated by E2F1 is tissue-specific, so only these skeletal muscle-specific target genes were dysregulated in *Kbtbd5* null mice. Although the phenotype of the *Kbtbd5* null mouse was rescued partially in the *E2F1* null background, the growth defect was not restored. These findings indicate that Kbtbd5 regulates skeletal muscle myogenesis through additional mechanisms, such as cellular proliferation and metabolism regulation. The defect in skeletal muscle is similar to the phenotype observed in patients carrying Kbtbd5 mutations. Further illustration of the underlying mechanisms regulated by Kbtbd5 will provide a platform to examine nemaline myopathy in skeletal muscle myogenesis.

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