

Cdo Interacts with APPL1 and Activates AKT in Myoblast Differentiation

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Cell–cell interactions between muscle precursors are required for myogenic differentiation; however, underlying mechanisms are largely unknown. Promyogenic cell surface protein Cdo functions as a component of multiprotein complexes containing other cell adhesion molecules, Boc, Neogenin and N-cadherin, and mediates some of signals triggered by cell–cell interactions between muscle precursors. Cdo activates p38MAPK via interaction with two scaffold proteins JLP and Bnip-2 to promote myogenesis. p38MAPK and Akt signaling are required for myogenic differentiation and activation of both signaling pathways is crucial for efficient myogenic differentiation. We report here that APPL1, an interacting partner of Akt, forms complexes with Cdo and Boc in differentiating myoblasts. Both Cdo and APPL1 are required for efficient Akt activation during myoblast differentiation. The defective differentiation of Cdo-depleted cells is fully rescued by overexpression of a constitutively active form of Akt, whereas overexpression of APPL1 fails to do so. Taken together, Cdo activates Akt through association with APPL1 during myoblast differentiation, and this complex likely mediates some of the promyogenic effect of cell–cell interaction. The promyogenic function of Cdo involves a coordinated activation of p38MAPK and Akt via association with scaffold proteins, JLP and Bnip-2 for p38MAPK and APPL1 for Akt.

INTRODUCTION

Differentiation of skeletal muscle progenitors is a multistep process that involves cell cycle withdrawal, expression of muscle-specific genes and formation of multinucleated myofibers by cell fusion (Molkentin and Olson, 1996). This process is coordinated by the myogenic basic helix-loop-helix factors (MyoD, Myf5, myogenin, and MRF4) and the myocyte enhancer factor 2 (MEF2) that form the core transcriptional network regulating myogenesis (Pownall *et al.*, 2002; Bergstrom *et al.*, 2002; Sartorelli and Caretti, 2005; Tapscott, 2005). These transcription factors auto- and cross-activate the expression of one another, resulting in a positive feedback network that amplifies and maintains the myogenic phenotype (Ludolph and Konieczny, 1995). Therefore activities of these transcription factors must be regulated tightly to ensure efficient cell differentiation and maintenance of the differentiated state of cells. However, the molecular mechanisms how these factors receive and interpret information from the extracellular environment are not well understood. Differentiation of skeletal muscle cells requires specific, but poorly understood, cell–cell recognition and adhesion. Cell adhesion molecules (CAMs), such as Cadherins and Immunoglobulin (Ig)/Fibronectin type III (FNIII) family proteins have been implicated in the determination and differentiation of myogenic precursors (Krauss *et al.*, 2005). Recently,

one of the odorant receptors MOR23 has been shown to be required for myotube formation and muscle regeneration, via regulation of muscle cell adhesion and migration (Griffin *et al.*, 2009).

Cdo (CAM-related/down-regulated by oncogenes), a member of the Ig/FNIII family of cell surface receptors, appears to be an integral component of a positive feedback network that controls myogenesis (Cole *et al.*, 2004). Cdo plays a promyogenic role in vivo and in vitro. Cdo deficient mice exhibit delayed skeletal muscle development, and *Cdo*^{-/-} primary myoblasts differentiate inefficiently in vitro (Cole *et al.*, 2004). Overexpression of Cdo in C2C12 myoblasts enhances differentiation, whereas knockdown of Cdo by RNAi decreases differentiation of these cells (Takaesu *et al.*, 2006). Cdo appears to function as a component of multiprotein complexes that include other cell adhesion molecules, Boc (brother of Cdo), Cadherins and Neogenin (Kang *et al.*, 2002; Kang *et al.*, 2003, 2004). Netrins have been shown to regulate multiple biological processes such as cell adhesion, migration, axon guidance, survival, and differentiation (Srinivasan *et al.*, 2003; Kang *et al.*, 2004; Cirulli and Yebra, 2007; Round and Stein, 2007; Fitamant *et al.*, 2008). Among Netrins, Netrin-3 is mainly expressed in C2C12 myoblasts and the recombinant chicken Netrin-2, related to the mouse Netrin-3, enhances myotube formation of C2C12 cells and activates focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) in primary myoblasts in a Neogenin/Cdo-dependent manner (Kang *et al.*, 2004, Bae *et al.*, 2009a). During myoblast differentiation, multiple signals emanate from Cdo multiprotein complexes, including activation of the p38MAPK (P38 mitogen-activated protein kinase) pathway via direct binding of the Cdo cytoplasmic tail to scaffold proteins, JLP (a scaffold protein for the p38MAPK pathway) and Bnip-2 (a scaffold-like protein for the small

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GTPase, Cdc42), and a nonreceptor tyrosine kinase Abl (Takaesu *et al.*, 2006; Kang *et al.*, 2008; Bae *et al.*, 2009b). Such signals positively regulate the activity of myogenic bHLH (basic helix loop helix) transcription factors, such as MyoD, at least in part via the phosphorylation of the ubiquitously expressed E-protein binding partners of the myogenic bHLH factors. Recently, we have shown that the p38MAPK pathway emanating from Cdo multiprotein complexes regulates neuronal differentiation of neural precursor cells (Oh *et al.*, 2009), suggesting a conserved signaling pathway initiated by Cdo to regulate differentiation of several different cell lineages. Therefore identifying additional components and regulators of Cdo-containing complexes is important to elucidate a mechanism of differentiation.

APPL (adaptor protein containing PH [pleckstrin homology] domain, PTB [phosphotyrosine binding] domain, and leucine zipper motif) was first identified as an interacting partner of Akt in a yeast two-hybrid screen (Mitsuuchi *et al.*, 1999) and later as an interacting protein of DCC (deleted in colorectal cancer; therefore APPL1 is also called DIP13). DCC is a member of Ig/FNIII superfamily receptors related to Neogenin and functions as a receptor for Netrins. APPL1 is required for apoptosis induced by DCC in the absence of ligands (Liu *et al.*, 2002). In addition, APPL1 has been reported to bind to various transmembrane receptors including TrkA (Lin *et al.*, 2006; Varsano *et al.*, 2006), AdipoR (adiponectin receptors; Mao *et al.*, 2006) and FSHR (follicle-stimulating hormone receptor; Nechamen *et al.*, 2007), mostly via its PTB domain and plays a key role in signaling pathways involving these receptors. One way APPL1 regulates signaling pathways triggered by TrkA and FSHR is by bridging the receptor and Akt as a scaffold protein and activating Akt. In addition, APPL1 has been shown to be required for nerve growth factor-mediated activation of ERK1/2, adiponectin-stimulated p38MAPK and AMP-activated protein kinase (AMPK; Lin *et al.*, 2006; Mao *et al.*, 2006; Varsano *et al.*, 2006). APPL1 is highly expressed in both differentiated C2C12 myotubes and mouse skeletal muscle, and interacts with Akt2 in mouse skeletal muscle (Mao *et al.*, 2006; Saito *et al.*, 2007). Moreover, APPL1 is required for the insulin-mediated Akt activation in C2C12 cells (Mao *et al.*, 2006).

Two key signaling pathways, p38MAPK and Akt, have been proposed to play essential roles in myogenesis (Tamir and Bengal, 2000; Gonzalez *et al.*, 2004; Simone *et al.*, 2004; Serra *et al.*, 2007). p38MAPK is required for the expression of muscle-specific genes and myoblast differentiation (Cuenda and Cohen, 1999; Puri *et al.*, 2000; Lluís *et al.*, 2006) via phosphorylation of MEF2 isoforms and the myogenic bHLH heterodimeric partner E47 (Wu *et al.*, 2000; de Angelis *et al.*, 2005; Lluís *et al.*, 2005; Perdiguero *et al.*, 2007). Several lines of evidence suggest that the Akt signaling pathway also plays important roles in myoblast differentiation (Fujio *et al.*, 2001; Vandromme *et al.*, 2001). Akt is activated during myoblast differentiation, and overexpression of Akt enhances myoblast differentiation, whereas inhibition of Akt by expression of a dominant-negative form of Akt blocks myotube formation. In addition, ectopic expression of a constitutively active form of Akt can rescue the suppression of myogenesis caused by inhibition of the PI3-kinase (Jiang *et al.*, 1999). Furthermore, activation of Akt is essential for cell survival in myogenesis mediated by insulin-like growth factor (IGF), a peptide growth factor that has been shown to be a major activator of the PI3-kinase/Akt pathway in myoblast differentiation (Lawlor and Rotwein, 2000a,b). It appears that both, Akt and p38MAPK pathways activate and reinforce each other's activity to induce efficient myogenic

differentiation (Li *et al.*, 2000; Wu *et al.*, 2000; Gonzalez *et al.*, 2004). The importance of a reciprocal reinforcement between these two signaling pathways is evident in rhabdomyosarcoma, an often aggressive soft tissue tumor occurring during childhood. Defects in both PI3-kinase/Akt and p38MAPK signaling pathways have been observed in rhabdomyosarcoma cell lines and these defects result in a failure of the exit from the cell cycle and terminal myogenic differentiation (Puri *et al.*, 2000; Xu and Wu, 2000).

We report here that both, APPL1 and Cdo are expressed in developing premuscle cells and Cdo regulates Akt activation through indirect interaction with APPL1 during myoblast differentiation. Overexpression of APPL1 in C2C12 myoblasts enhances myoblast differentiation, whereas APPL1-depleted C2C12 cells by RNAi differentiate less efficiently. The cytoplasmic tail of Cdo appears to be required for complex formation with APPL1, and their association is induced by high cell density. Overexpression or knockdown of APPL1 increases or decreases Akt activation in C2C12 cells, respectively. Cells expressing a Cdo mutant deficient in complex formation with APPL1 exhibit a decrease in Akt activation and myoblast differentiation, when compared with the wild-type Cdo-expressing cells. *Cdo*^{-/-} primary myoblasts and Cdo-depleted C2C12 cells exhibit a decrease in Akt activation during differentiation. The defective differentiation of Cdo-depleted C2C12 cells are fully rescued by overexpression of AKT-DD (a constitutively active form of Akt), whereas overexpression of APPL1 fails to do so. Taken together, Cdo activates two key promyogenic kinases, p38MAPK and Akt via association with the scaffold-type of proteins, JLP and Bnip-2 for p38MAPK and with APPL1 for Akt during myogenic differentiation.

MATERIALS AND METHODS

Cell Culture and Expression Vectors

C2C12, 293T, Cos7 and primary myoblasts derived from *Cdo*^{+/+} and *Cdo*^{-/-} mice were cultured as previously described (Takaesu *et al.*, 2006). To induce differentiation of C2C12 myoblasts, cells at near confluence were switched to DMEM containing 2% horse serum (differentiation medium, DM) and myotube formation was observed normally at about 2–3 d of differentiation. The efficiency of the myotube formation in stable and transient differentiation assays was quantified as previously described (Bae *et al.*, 2009b). Student's *t* test was used for statistical analysis of myotube formation. To generate C2C12 cells that stably overexpress Cdo, APPL1, or RNAi for Cdo and APPL1, cells were transfected with the indicated expression vectors and FuGene6 (Roche Diagnostics, Indianapolis, IN) and cultures were selected in puromycin-containing medium.

The human APPL1 gene was amplified by RT-PCR of mRNAs purified from human embryonic kidney fibroblast cells. Full-length (aa1-709), BAR-PH (aa1-499), BAR (aa1-273) and PH-PTB (aa265-709) domains of APPL1 were inserted into mammalian expression vector pcDNA-hemagglutinin (HA) or pcDNA-SRT (spin-reorientation transitions; Lee *et al.*, 2001). Initially we tested four different APPL1 RNAi oligonucleotides, and two of them (5'-GGGAGGCAGCGUACAAAU-3' and 5'-AUUUGUACGCCUGCCUCCC-3'; 5'-GCGGAGAGAAGUGAAAAGUAA-3' and 5'-UUACUUUCACUUCUCCGC-3') displayed the effective knockdown in HeLa cells. Two mouse equivalent RNAi oligonucleotides were tested for their knockdown ability, and from these RNAi oligonucleotides we have inserted the following sequence into pSuper-puro vector: 5'-GGGAGGCAGGCGTACAAAT-3'. Cdo deletion mutants (Cdo Δ 986-1048, Cdo Δ 1035-1160, Cdo Δ 1160-1256), the glutathione S-transferase (GST)-Cdo intracellular construct, and the sequence for the pSuper/Cdo-RNAi construct were described previously (Bae *et al.*, 2009b). To generate a constitutively active mutant of AKT, we have mutated two active sites of human AKT, threonine³⁰⁸ and serine⁴⁷³, to aspartic acids (AKT-DD), as previously described (Flores *et al.*, 2008).

Western Blot Analysis, Antibodies, and Immunoprecipitation

Western blot analyses were carried out as previously described (Bae *et al.*, 2009b). Briefly, cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl/1% Triton X-100/1 mM EDTA) containing proteinase inhibitor (Roche Diagnostics), followed by SDS-PAGE. APPL1 rabbit polyclonal anti-

body was raised against C-terminal PTB domain of human APPL1. Sera were affinity-purified using Sulfolink beads (Pierce, Rockford, IL). Other antibodies used in this study were as follows: anti-Cdo (Zymed, South San Francisco, CA), anti-SRT (Lee *et al.*, 2001), anti-HA (Roche Diagnostics), anti-pan-cadherin (Sigma-Aldrich, St. Louis, MO), anti- β -tubulin (Zymed), anti-p38MAPK (Sigma-Aldrich), anti-pp38MAPK, anti-Akt, anti-pAkt (Cell Signaling, Beverly, MA), anti-MHC (MF20; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti-Troponin T (Sigma-Aldrich), anti-Boc (R&D Systems, Minneapolis, MN), anti-N-cadherin (Zymed), anti-Neogenin (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GFP (Zymed). To study formation of Cdo-APPL1 complexes, coimmunoprecipitation was performed as described previously (Bae *et al.*, 2009b).

For analysis of dissected hindlimbs by immunoblotting, hindlimb muscles from *Cdo*^{+/+} and *Cdo*^{-/-} mice at E15.5 (embryonic day 15.5) embryos and P1 (postnatal day 1), P3, and P5 mice were pulverized and solubilized with lysis buffer. Lysates were then analyzed by immunoblotting as described above.

Immunocytochemistry, Immunohistochemistry, and Microscopy

GFP-Cdo plasmids were transfected in C2C12 cells with FuGene6 (Roche Diagnostics). The following day, media was replaced with DM, and cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) after 48 h of transfection. After blocking, fixed cells were incubated with anti-APPL1 or anti-EEA1 (BD Bioscience, San Jose, CA) antibody. Fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used to detect primary antibody. Cells were examined under confocal laser scan microscope (LSM510 META, Zeiss, Thornwood, NY). For the results shown in Figure 6, C2C12/pSuper or C2C12/Cdo RNAi cells in 12-well plates were cotransfected with 100 ng of green fluorescent protein (GFP) expression vector and 900 ng of the indicated DNA constructs for 2 d and induced to differentiate by switching to DM. Cells were then fixed with 3% paraformaldehyde in PBS, followed by blocking with 5% goat serum in PBS. Cells were incubated with anti-MHC (Developmental Studies Hybridoma Bank) or anti-GFP (Zymed) antibody. FITC- or Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch) were used to detect primary antibody. Immunostaining for pp38MAPK and MHC was performed as described previously (Bae *et al.*, 2009b). Images were obtained on a Zeiss LSM-510 Meta confocal microscope. Quantification of the fluorescent signal for pp38MAPK was performed with ImageGauge software (Fuji Film, Tokyo, Japan). Immunohistochemistry was performed on 8- μ m paraffin sections that were rehydrated, treated with 10 μ g/ml proteinase K/50 mM Tris-HCl, pH 8.0 for 10 min, and incubated with anti-APPL1 antibody in 5% goat serum containing PBS. After extensive washing, sections were incubated with biotinylated goat anti-rabbit and streptavidin-horseradish peroxidase conjugates (Jackson ImmunoResearch).

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted using easy-Blue reagent (iNtRON Biotechnology, Sungnam, Korea). The cDNAs were reverse-transcribed from 1 μ g of total RNA using oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The PCR reactions were performed in 20 μ l using 5% of the reverse transcription (RT) reaction, 250 nM of each primer, and SYBR Premix Ex Taq (Takara, Tokyo, Japan). The PCR reactions were carried out on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Expression levels of Gapdh were used to normalize the expression levels of each sample. Primer sequences used for PCR were as follows: MHC: 5'-CCAAGGGCTGAATGAGGAG-3' and 5'-GCAAAGGCTCCAGGCTC-GAG-3'; Troponin T: 5'-TGCACTAAAAGACCGCATG-3' and 5'-TCTTCT-TGGCGTCATCCTCT-3'; Adiponectin: 5'-GCAGAGATGGCACTCCTGGA-3' and 5'-CCCTTCAGCTCCTGTGATTC-3'; and Gapdh: 5'-TGATGACAT-CAAGAAGGTGAAG-3' and 5'-TCCTTGGAGGCCATGTAGCCAT-3'.

TUNEL Assay

To monitor cell death, C2C12/pSuper or C2C12/APPL1 RNA interference (RNAi) cells that had been cultured in growth medium or DM were fixed, and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) was performed with a kit according to the manufacturer's instructions (Invitrogen) as previously described (Bae *et al.*, 2009a).

Yeast Two-Hybrid Assay

cDNA fragments encoding APPL1 were ligated to the Gal4 DNA-binding domain in the pGBT9 vector, and a cDNA encoding the Cdo intracellular domain was ligated to the Gal4 activation domain in the pGAD vector. Yeast strain AH109 (Clontech, Palo Alto, CA) was sequentially transformed with these vectors, and β -galactosidase (β -Gal) activity was detected by colony lift filter assay as previously described (Bae *et al.*, 2009b).

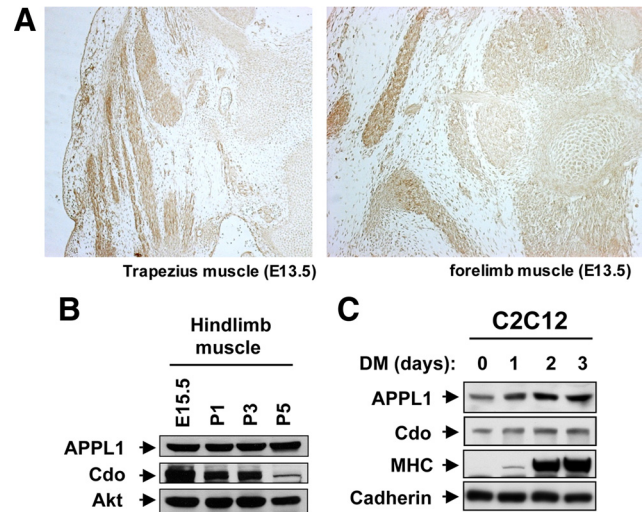


Figure 1. Expression of APPL1 in developing premuscles and cultured myoblasts. (A) Transverse sections of premuscle mass of trapezius and forelimb obtained from E13.5 embryos were stained with an antibody against APPL1. (B) Extracts of hindlimb muscles from E15.5 embryo and P1, P3 and P5 mice were probed with antibodies to APPL1 and Cdo, and Akt as a loading control. (C) Lysates of C2C12 cells that were at near confluence (0) or in DM for indicated times were immunoblotted with antibodies to APPL1, Cdo, and MHC and Cadherin as a loading control.

RESULTS

APPL1 Is Expressed in Developing Premuscles and Cultured Myoblasts

To investigate roles of APPL1 in myogenesis and the relationship between Cdo and APPL1 in this process, we analyzed the expression pattern of APPL1 in developing muscles. Paraffin sections of forelimb and pretrapezius premuscles from E13.5 embryos were subjected to immunohistochemistry with anti-APPL1 antibodies. As shown in Figure 1A, APPL1 was expressed ubiquitously as seen by the brown staining; however, the strongest immunoreactivity was observed in the developing forelimb and trapezius premuscles from E13.5 embryos. Cdo has previously been shown to be expressed in developing premuscles of E13.5 embryos (Mulieri *et al.*, 2000). APPL1 was ubiquitously expressed in extracts of dissected hindlimb muscles of E15.5 embryos and P1, P3, and P5 mice, whereas Cdo expression was altered during muscle development. It was expressed at highest levels in the developing hindlimb at E15.5 and drastically down-regulated in the P5 hindlimb muscle extracts (Figure 1B). This expression pattern of Cdo is consistent with its proposed role as a regulator of myogenesis. In addition, APPL1 and Cdo were also coexpressed in differentiating C2C12 myoblasts and APPL1 levels increased during differentiation (Figure 1C).

APPL1 Promotes Myoblast Differentiation

To explore a function of APPL1 in myogenesis, we modulated APPL1 levels by stable expression of the SRT-tagged APPL1 (APPL1 [SRT]) or APPL1 RNAi in C2C12 cells and analyzed effects of altered APPL1 levels on myoblast differentiation. Forced expression of the APPL1 (SRT) in C2C12 cells generally resulted in a modest twofold increase of APPL1 (Figure 2A). The control and APPL1 (SRT)-expressing C2C12 cells were induced to differentiate for 2 d in DM (D2), fixed, and immunostained with anti-myosin heavy

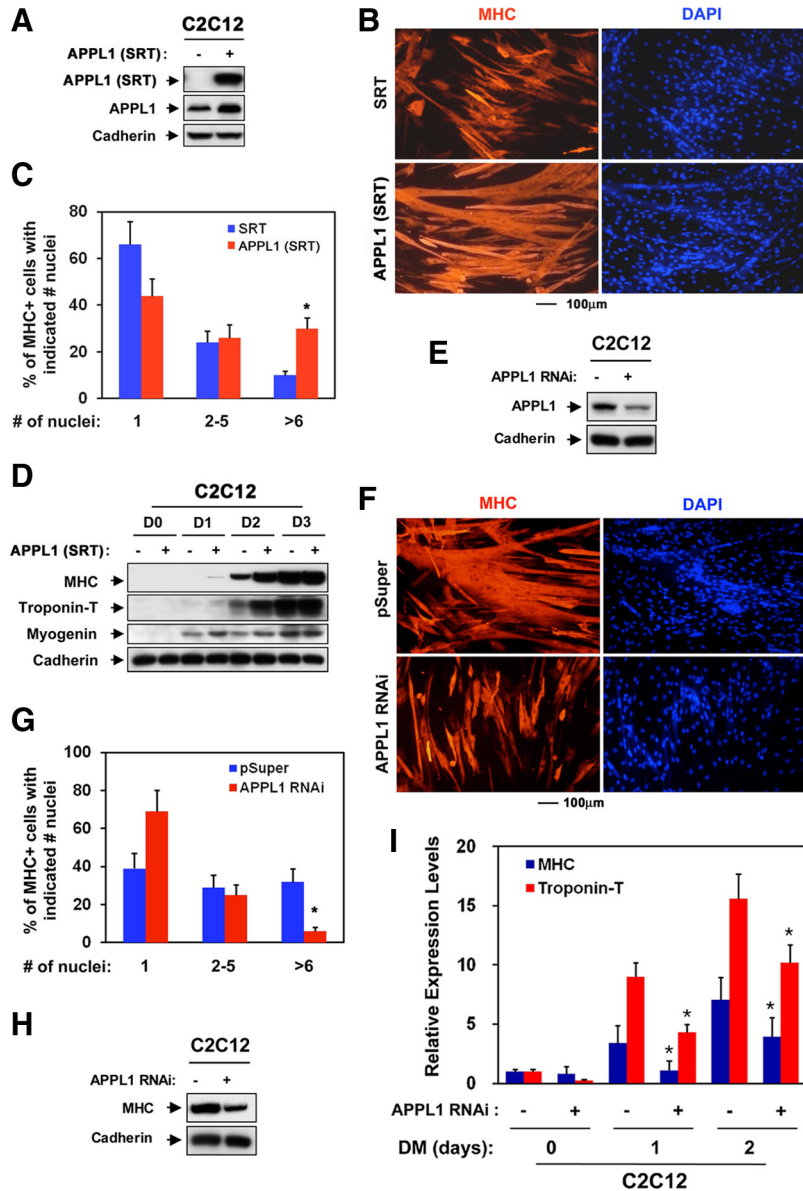


Figure 2. APPL1 promotes myoblast differentiation. (A) Lysates of cell lines shown in B were immunoblotted with antibodies to SRT, APPL1, and to Cadherin as a loading control. (B) Photomicrographs of C2C12 cells that stably express SRT-tagged APPL1 (APPL1 [SRT]) or control (pcDNA-SRT) vectors were cultured in DM for 2 d, fixed, and stained with an antibody to MHC. (C) Quantification of myotube formation shown in B. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results. Significant difference from control, * $p < 0.01$. (D) Lysates of cell lines shown in B were immunoblotted with antibodies to MHC, Troponin T, or Myogenin and to Cadherin as a loading control. (E) Lysates of cell lines shown in F were immunoblotted using antibodies recognizing APPL1 to reveal the level of RNAi-mediated depletion and Cadherin as a loading control. (F) Photomicrographs of C2C12 cells stably transfected with APPL1 RNAi or control (pSuper) vectors were cultured in DM for 3 d, fixed, and immunostained with an antibody to MHC. (G) Quantification of myotube formation by cell lines shown in F. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results. Significant difference from control, * $p < 0.01$. (H) Lysates of control and APPL1 RNAi cell lines shown in F were immunoblotted using antibodies recognizing MHC and Cadherin as a loading control. (I) RNAs of the control and APPL1 RNAi cell lines from the different differentiation time course were subjected to qRT-PCR for MHC and Troponin T expression. The expression level of the control cells at the D0 condition was set as 1, and the relative expression levels were plotted. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results. Significant difference from control, * $p < 0.01$.

chain (MHC) antibodies followed by DAPI staining. Photomicrographs of these cells revealed that overexpression of APPL1 resulted in the formation of larger myotubes with more nuclei per myotube, in comparison to control cells (Figure 2, B and C). In addition, APPL1 (SRT)-expressing C2C12 cells showed an enhanced expression of differentiation markers, such as myogenin, MHC, and Troponin T, in comparison to control cells (Figure 2D). These data suggest that the increase in APPL1 levels resulted in enhanced myoblast differentiation on a morphological as well as a biochemical level. In converse experiments, the stable expression of APPL1 RNAi in C2C12 cells resulted in the reduction of APPL1 protein levels to 30%, relative to the control cells (Figure 2E). These cells were further analyzed for their differentiation ability by inducing differentiation for 3 d. As shown in Figure 2, F and G, these cells formed fewer myotubes with fewer nuclei, compared with control vector transfected cells. The formation of big myotubes with more than six nuclei was specifically affected by the APPL1 knockdown (Figure 2G). APPL1 RNAi-expressing cells exhibit a signifi-

cant reduction of MHC levels at D3, compared with the control cells (Figure 2H). To further analyze the effect of APPL1 on expression of differentiation markers in early differentiation stage, RNAs of C2C12/APPL1 RNAi cells from the differentiation time course were analyzed for MHC and Troponin T by quantitative RT-PCR (qRT-PCR). As shown in Figure 2I, the expression of these differentiation markers during the early differentiation stage was significantly decreased. These data suggest that APPL1 appears to be required for both biochemical and morphological differentiation of myoblasts. However the myoblast fusion seems to be more affected by the APPL1 deficiency than the expression of muscle-specific proteins.

APPL1 Forms Complexes with Cdo

Because both Cdo and APPL1 were expressed in early developing muscles and C2C12 myoblasts and promoted myoblast differentiation, we next asked whether Cdo and APPL1 interact functionally. To test this possibility, we first examined if these proteins physically interact with each other.

293T cells were transiently transfected with Cdo and APPL1 (SRT) constructs, and lysates were subjected to immunoprecipitation analysis with SRT antibodies followed by immunoblotting with antibodies recognizing SRT or Cdo. As shown in Figure 3A, Cdo coprecipitates with APPL1 (SRT). To assess whether Cdo and APPL1 interact endogenously in myoblasts, cell lysates of proliferating as well as differentiating C2C12 myoblasts from a 3-d time course were immunoprecipitated with anti-Cdo antibodies and analyzed by immunoblotting with antibodies to Cdo or APPL1. In proliferating C2C12 cells cultured in growth medium (G), APPL1 was poorly coprecipitated with Cdo (Figure 3B). When cells were confluent (DM, day 0), the expression of APPL1 and its binding to Cdo was drastically increased and the interaction stayed constant during the 3 d-time course of differentiation (Figure 3B). These results suggest that Cdo and APPL1 interact with each other in myoblasts and cell density appears to be an important factor for the expression of APPL1 and the interaction between Cdo and APPL1. APPL1 has been shown to be associated with a distinct endosomal compartment involved in the regulation of multiple signaling pathways, and APPL1 endosomes mature into EEA1-positive early endosomes (Miaczynska *et al.*, 2004). Therefore we assessed whether Cdo colocalized with APPL1 and/or EEA1 in endosomal compartments of C2C12 cells. Because no available antibodies to Cdo can be used for immunocytochemistry of the endogenous Cdo protein, C2C12 cells were transiently transfected with GFP-tagged Cdo, and 1 d later, these cells were switched into DM. At differentiation day 1 (D1), cells were fixed and immunostained with APPL1 or EEA1 antibodies followed by rhodamine-conjugated secondary antibodies. The confocal images were taken close to the basal plane of the cells. Cdo-GFP was detected along the plasma membrane and in the cytoplasm as a punctuated pattern. A fraction of APPL1 colocalized with Cdo-GFP mainly in the vicinity of the plasma membrane and most likely at the contact site of two cells (marked with arrows in the middle panel, Figure 3C). However Cdo-GFP did not appear to colocalize significantly with the EEA1-positive endosomal compartments (Figure 3C, bottom panel).

To assess the structural requirement for association between Cdo and APPL1, we have transiently transfected three Cdo intracellular mutants that harbor deletions in the cytoplasmic tail (Figure 3D) and analyzed the ability of these mutants to coprecipitate with APPL1. All three Cdo mutants showed a reduction in APPL1 binding; however, the Cdo mutant lacking the midportion (Cdo Δ 1035-1160) of the cytoplasmic tail precipitated with APPL1 most inefficiently (Figure 3E). Previously we have shown that this Cdo mutant (Cdo Δ 1035-1160) behaved as a loss-of-function mutant in myoblast differentiation (Takaesu *et al.*, 2006), suggesting APPL1 may be a regulator of the Cdo-mediated myoblast differentiation via association with this protein. Next we asked whether Cdo and APPL1 interacted directly by utilizing a yeast two-hybrid system. Although the positive control proteins, p53 and SV40 large T antigen (T)-expressing yeast formed colonies on the selective plate, the yeast expressing both APPL1 and the Cdo intracellular region failed to grow, suggesting that Cdo and APPL1 most likely interact indirectly (Figure 3F). We have further analyzed a potential interaction between APPL1 and Neogenin, Boc, or N-cadherin that are components of Cdo multiprotein complexes (Kang *et al.*, 2002, 2003, 2004). APPL1 was coimmunoprecipitated endogenously with Boc in 293T as well as in C2C12 cells at D0 and D2 (Figure 3G). Interestingly, the interaction between APPL1 and Boc decreased in differentiating C2C12

cells, even though the level of APPL1 increased. To further ask whether Neogenin and N-cadherin interacted with APPL1, we transiently transfected 293T cells with APPL1 (SRT) and N-cadherin or Neogenin. The lysates were subjected to immunoprecipitation with antibodies to N-cadherin or Neogenin, followed by Western blotting. As shown in Figure 3, H and I, N-cadherin and Neogenin did not interact with either the endogenous or the transfected APPL1, suggesting that these proteins are not interacting with APPL1. Because the interaction between Cdo and APPL1 appeared to be indirect, we then asked whether Boc and APPL1 interacted directly by using a yeast two-hybrid system. However we failed to detect any interaction between APPL1 and the Boc intracellular region in yeasts (data not shown). Taken together, these data suggest that APPL1, Cdo, and Boc may form complexes, and this interaction is mediated by a yet-to-be-identified protein.

Complex Formation of Cdo and APPL1 Is Mediated by the Region Encompassing Amino Acids 273-486 of APPL1

To investigate the role of Cdo and APPL1 complexes, we have generated HA-tagged APPL1 (APPL1 [HA]) deletion mutants according to its domain structure (Figure 4A). 293T cells were transiently cotransfected with the Cdo expression vector and the APPL1 (HA) construct of either the full-length or the deletion mutants and 2 d later, lysates were subjected to coimmunoprecipitation with anti-Cdo antibodies, followed by immunoblot analysis. As shown in Figure 4B, the APPL1 mutant 1-499 encompassing the BAR and PH domain and the mutant 265-709 containing the PH and PTB domains were coimmunoprecipitated with Cdo. However the mutant 1-273 containing the BAR domain alone did not precipitate with Cdo, suggesting that the 274-485 region containing the PH domain and the surrounding regions of APPL1 is required for complex formation with Cdo (Figure 4B). To explore the functional consequence of these deletion mutants, C2C12 cells stably expressing the full-length APPL1 protein or deletion mutants were induced to differentiate for 3 d and were assessed for their differentiation ability. Cells expressing APPL1 mutants 1-499 (which can bind to Cdo, but lacks PTB domain) and 265-709 differentiated as efficiently as the full-length APPL1-expressing cells, as seen by the formation of bigger myotubes and an enhanced expression of MHC, compared with the control cells (Figure 4, C–E). However expression of the mutant 265-709 containing both complex forming region with Cdo and the PTB domain displayed more efficient differentiation with larger myotubes that contained six or more nuclei and elevated levels of MHC, compared with the full-length APPL1-expressing cells. In addition, overexpression of the APPL1 mutant 1-273 did not show any effect on myotube formation as well as MHC expression, compared with the vector-expressing cells (Figure 4, C–E). These data suggest that the complex formation of Cdo with APPL1 is required for Cdo-mediated efficient myoblast differentiation.

Cdo and APPL1 Are Required for Akt Activation in Myoblast Differentiation

Because APPL1 promotes myogenic differentiation and forms complexes with Cdo, whose promyogenic properties are exerted mainly through p38MAPK, we examined the possibility that APPL1 may be involved in regulation of p38MAPK activity in myoblast differentiation. C2C12 cells were transfected stably with APPL1 RNAi, and lysates after 2 d of differentiation were subjected to immunoblot analysis with antibodies to APPL1, phosphorylated-p38MAPK (pp38), p38MAPK, Cdo and JLP (a scaffold protein interacting with

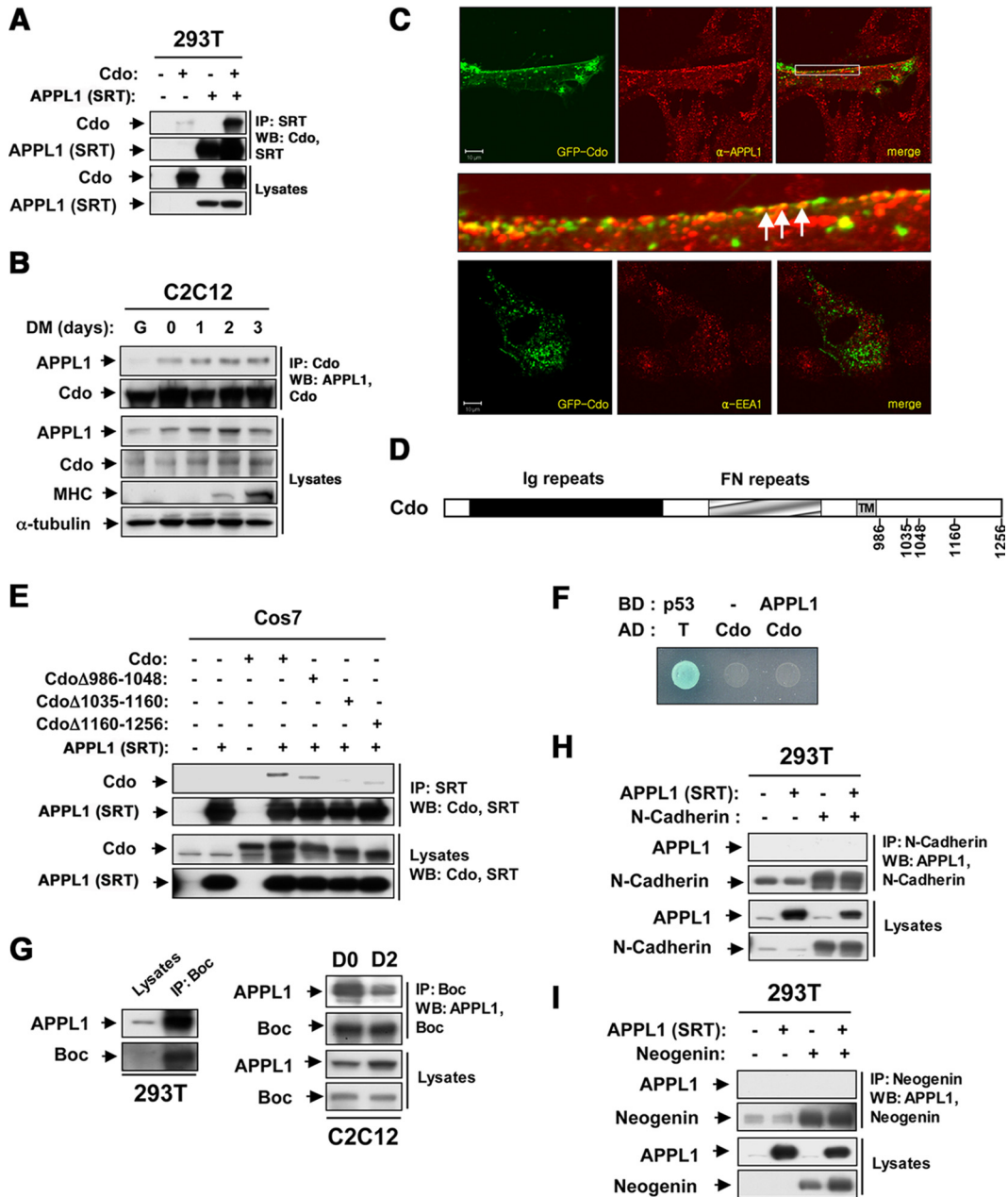


Figure 3. APPL1 forms complexes with Cdo and Boc, but not with Neogenin and N-cadherin. (A) Lysates of 293T cells transiently transfected with Cdo, APPL1 (SRT), or control (–) expression vectors as indicated were immunoprecipitated (IP) with an antibody to SRT. The precipitates and total lysates were immunoblotted (WB) with antibodies against Cdo and SRT (APPL1). (B) Lysates of C2C12 cells that were proliferating in growth medium (G) at near confluence (0) or in DM for the indicated times were immunoprecipitated with antibodies to Cdo and immunoblotted with antibodies to APPL1 or Cdo. Total lysates were also immunoblotted with antibodies to APPL1, Cdo, or MHC to monitor differentiation, and to α -tubulin as a loading control. (C) C2C12 cells were transiently transfected with Cdo-GFP and fixed after 48 h and immunostained with antibodies to APPL1 (top and middle panels) or EEA1 (bottom panel). The enlarged image of the boxed area in the top panel is shown in the middle panel. Size bar, 10 μ m. (D) Schematic representation of rat Cdo, the amino acid numbers indicating the deletion constructs. (E) Lysates of Cos7 cells transiently transfected with Cdo, Cdo deletion mutants, APPL1 (SRT), or control (–) expression vectors as indicated were immunoprecipitated with SRT antibodies and then immunoblotted with antibodies to Cdo or SRT. Total lysates were also immunoblotted with Cdo or SRT antibodies. (F) Yeast cells transformed with the indicated vectors were tested for expression of β -Gal, indicative of interaction. AD, Gal4 activation domain; BD, Gal4 DNA-binding domain. The set of p53 and SV40 large T antigen (T) served as a positive control. (G) Lysates of 293T and C2C12 cells at D1 and D2 were subjected to immunoprecipitation with Boc antibodies and immunoblotted with antibodies to Boc and APPL1. (H) 293T cells were transiently transfected with APPL1 (SRT) and N-cadherin, and lysates were immunoprecipitated with N-cadherin antibodies, followed by immunoblotting with N-cadherin and APPL1. (I) The transiently transfected 293T cells with Neogenin and APPL1 (SRT) were lysed and subjected to immunoprecipitation with Neogenin antibodies, followed by immunoblotting with Neogenin and APPL1 antibodies.

Cdo and p38MAPK). As shown in Figure 5A, APPL1 knock-down did not alter the level of pp38 and Cdo as well as JLP,

suggesting APPL1 is not required for Cdo-mediated p38MAPK activation. Because APPL1 is implicated in Akt

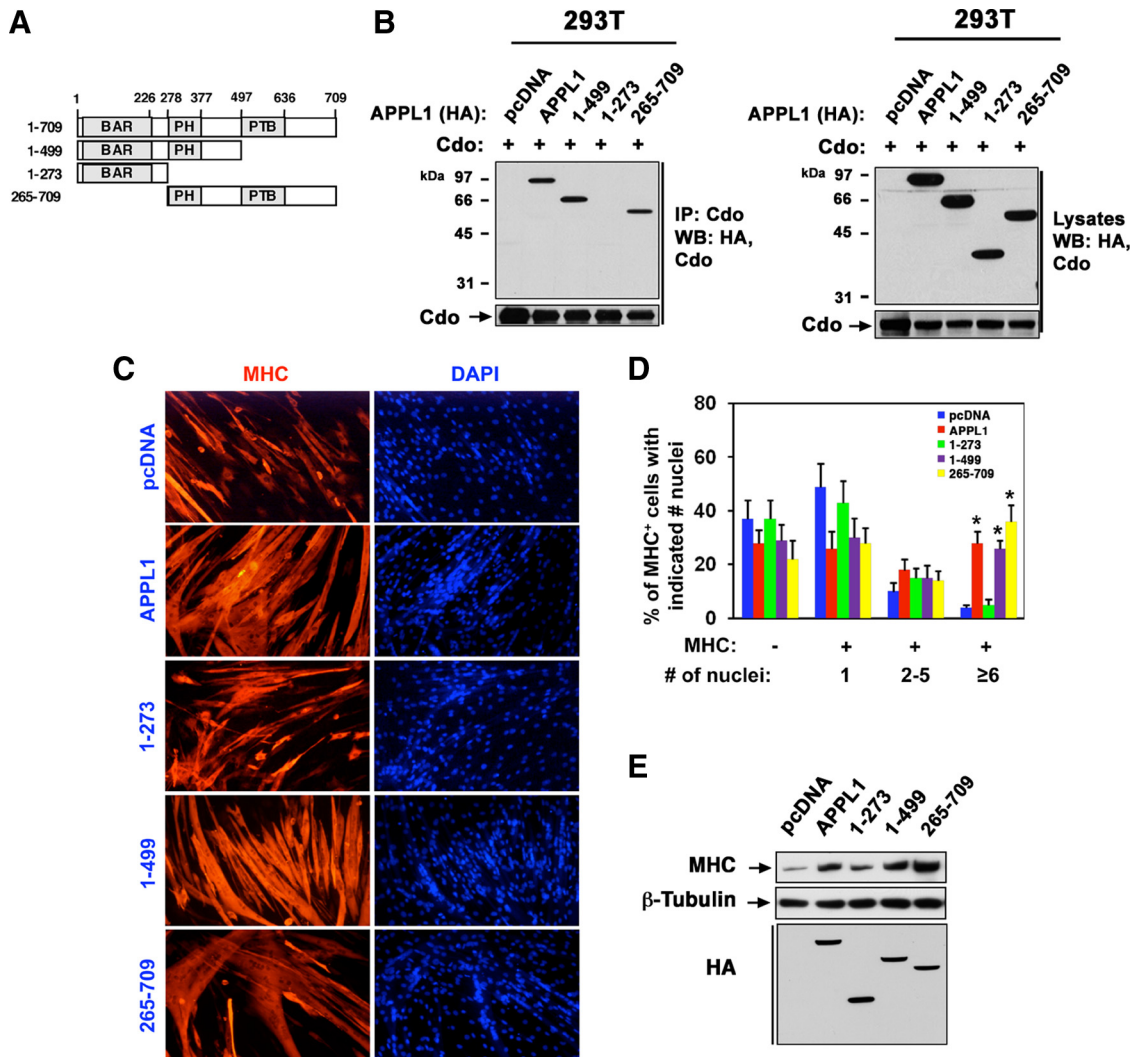
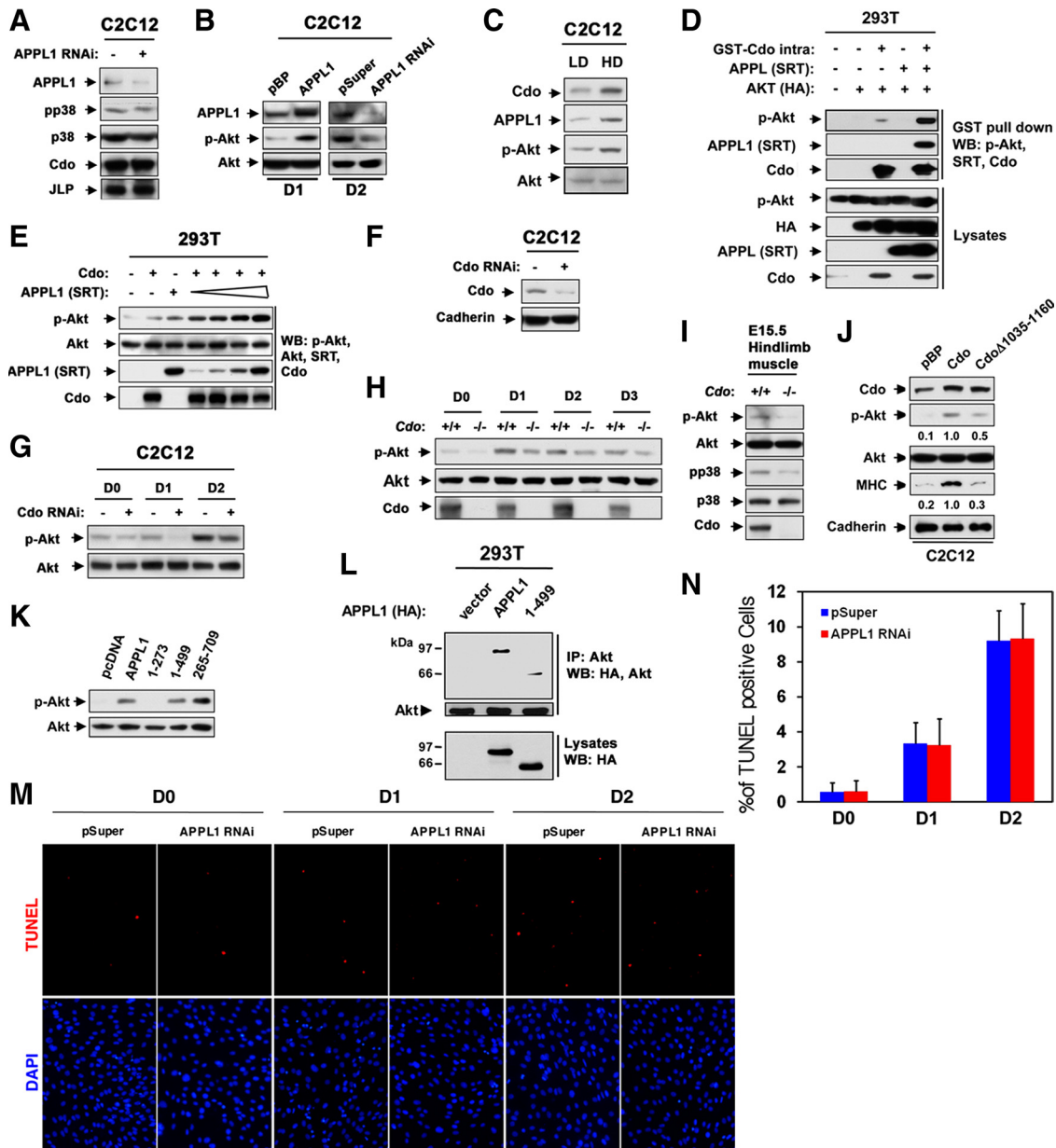


Figure 4. The complex formation of Cdo and APPL1 is mediated by the region encompassing amino acids 273-486 of APPL1. (A) Schematic representation of the domain structure of APPL1 and the deletion constructs of APPL1. (B) 293T cells were transiently transfected with the HA-tagged full-length or deletion mutants of APPL1 and Cdo or control (-) expression vectors as indicated. Cells were harvested after 48 h, immunoprecipitated using an antibody to Cdo, and analyzed by immunoblotting with antibodies to HA or Cdo. Total lysates were also immunoblotted with antibodies to HA or Cdo. (C) C2C12 cells were stably transfected with HA-tagged APPL1 (APPL1 [HA]) and APPL1 mutants, or control (pcDNA) expression vectors. These cell lines were cultured in DM for 2 d, fixed, and immunostained with an antibody to MHC followed by DAPI stain. (D) Quantification of myotube formation by cell lines shown in C. Values represent means of triplicate determinations \pm 1 SD. The experiment was repeated three times with similar results. Significant difference from control, * $p < 0.01$. (E) Lysates of cell lines shown in C were immunoblotted with antibodies to HA to reveal the level of ectopic expression of APPL1 and APPL1 mutants. In addition, lysates were immunoblotted with antibodies to MHC and β -tubulin as a loading control.

activation in other cell systems and Akt plays an essential role in myoblast differentiation, we generated stable C2C12 cells with either increased or reduced levels of APPL1 and analyzed the phosphorylation of Akt by immunoblotting. The antibody to a phosphorylated form of Akt (p-Akt) reveals higher or lower immunoreactivity in response to either the increased or reduced APPL1 protein level, respectively (Figure 5B). Because Cdo and APPL1 associate efficiently in confluent C2C12 cells, we analyzed whether cell density regulates Akt activities. C2C12 cells were harvested from either 50% (LD) or 100% (HD) confluent cultures and analyzed with antibodies recognizing Cdo, APPL1, p-Akt, or Akt. As shown in Figure 5C, cells at high cell density express enhanced levels of Cdo, APPL1, and p-Akt. Next we explored whether Cdo forms a complex with APPL1 and Akt by using a GST-Cdo intracellular region fusion construct

(GST-Cdo_{intra}) in GST pulldown experiments. 293T cells were transiently cotransfected with GST-Cdo_{intra}, Akt, and APPL1 or the control vector, and lysates were subjected to precipitation with glutathione beads. As shown in Figure 5D, p-Akt was coprecipitated with GST-Cdo_{intra} most likely mediated via the endogenous APPL1, and this association was strongly increased when APPL1 was overexpressed. We then asked whether Cdo and APPL1 collaborate in the regulation of Akt activities by cotransfecting 2.5 μ g of Cdo expression vector with an increasing amount of APPL1 (0.25, 0.5, 1, or 2.5 μ g). As a control, 2.5 μ g of either Cdo or APPL1 were cotransfected with the control vector. Two days later, cells were harvested and analyzed by immunoblotting with antibodies recognizing p-Akt, Akt, APPL1, or Cdo. Ectopic expression of Cdo or APPL1 alone increased phosphorylation of endogenous Akt. The p-Akt levels were further in-



creased by cotransfection of APPL1 and Cdo in a dose-dependent manner (Figure 5E). These data suggest that Cdo and APPL1 collaborate in the activation of Akt. To confirm Cdo's role in Akt activation during myoblast differentiation, C2C12 cells stably expressing control or Cdo RNAi (Figure 5F) as well as primary myoblasts isolated from *Cdo*^{+/+} and *Cdo*^{-/-} mice were induced to differentiate and analyzed for Akt activation by immunoblotting. C2C12 cells that expressed Cdo RNAi displayed lower levels of p-Akt after 1 d in DM than did control cells (Figure 5G). Similarly, a reduction in Akt phosphorylation was observed in *Cdo*^{-/-} primary myoblasts after 1 d in DM, relative to the control *Cdo*^{+/+} cells (Figure 5H). To investigate the *in vivo* role of Cdo in Akt activation, we analyzed extracts of dissected E15.5 hindlimb muscles by immunoblotting with antibodies against p-Akt, Akt, pp38, p38, or Cdo. Cdo was easily detected in extracts from *Cdo*^{+/+} hindlimb muscles (Figure 5I). As expected, pp38 levels were decreased in extracts from *Cdo*^{-/-} hindlimb muscle extracts showed a reduction in p-Akt levels, relative to the *Cdo*^{+/+} extracts (Figure 5I). Next, we examined whether Cdo and APPL1-containing complex regulates Akt phosphorylation in myogenic differentiation. C2C12 cells stably expressing the full-length or a Cdo mutant (*Cdo*Δ1035-1160, lacking the ability to associate with APPL1) were analyzed for their ability to activate Akt. Overexpression of Cdo resulted in enhanced levels of p-Akt as well as MHC expression, relative to the control vector-expressing cells. Even though *Cdo*Δ1035-1160-expressing cells failed to enhance MHC expression, Akt activation was partially reduced, compared with the full-length Cdo-expressing cells, but it was higher, in relation to the control vector-transfected cells (Figure 5J). These data suggest that this domain might be required for full activation of Akt by Cdo, which is required for efficient myogenic differentiation. Because the deletion of this domain in Cdo did not abrogate Akt activation, there might be other pathways for Cdo to activate Akt besides association with APPL1 through this domain. C2C12 cells stably expressing the full-length or deletion mutants of APPL1 shown in Figure 4E were also analyzed for p-Akt levels. Akt activation correlated well with their ability to induce differentiation. Like the full-length APPL1, the APPL1 mutants, 1-499 and 265-709, showed a strong induction of Akt activation (Figure 5K). Interestingly, APPL1 mutant 265-709-expressing cells resulted in higher Akt activation, compared with the full-length APPL1-expressing cells. However the 1-273 APPL1 mutant failed to activate Akt phosphorylation, compared with the control full-length APPL1 (Figure 5K). Because it has been reported that APPL1 can interact with Akt via its PTB domain, we further analyzed whether APPL1 mutant 1-499 can interact with Akt, thereby activating Akt, when it is complexed with Cdo. We have transfected 293T cells with the full-length or the 1-499 mutant APPL1, followed by immunoprecipitation with an Akt antibody and analyzed interaction of Akt with APPL1. As shown in Figure 5L, both APPL1 proteins were detected in the precipitate, suggesting the 1-499 mutant APPL1 can form a complex with Akt. Because APPL1 has been shown to homodimerize via the N-terminal BAR domain (Li *et al.*, 2007), it is possible that the PTB domain mutant form complexes with the endogenous APPL1 thereby with Akt. However the level of the 1-499 mutant in the precipitate was decreased, compared with that of the full-length APPL1, suggesting the PTB domain of APPL1 is required for the efficient interaction between APPL1 and Akt. Taken together, these data suggest that indirect association of APPL1 with Cdo is required for APPL1 to activate Akt in myoblast

differentiation. Because Akt has been proposed to regulate cell survival and cell survival is required for the efficient myoblast differentiation, we asked whether the impaired myoblast differentiation of APPL1 knockdown cells was due to a decrease in cell survival. The control and APPL1-deficient C2C12 cells were induced to differentiate and fixed at various time points of differentiation, and cell death was analyzed by TUNEL and DAPI staining. As shown in Figure 5, M and N, these cells did not display any difference in cell death. Taken together, Cdo/APPL1/Akt pathway may be specifically involved in myoblast differentiation rather than regulating cell survival.

Expression of a Constitutively Active Akt Rescues the Defective Differentiation of Cdo-depleted C2C12 Cells

We next investigated whether the defective differentiation response displayed by Cdo depleted myoblasts by RNAi was caused by defects in Akt activation. To do so, the control or Cdo RNAi-expressing C2C12 cells were transiently transfected with APPL1 or AKT-DD (a constitutively active form of AKT) expression vector or a control vector, plus a GFP expression vector to mark transfectants. The defect in the differentiation response of Cdo-depleted C2C12 cells were rescued by overexpression of AKT-DD. About 73% of pcDNA-transfected C2C12/pSuper cells were MHC-positive (32% mononucleate; ~22% containing 2–5 nuclei; ~18% containing six or more nuclei; Figure 6, A and B). Overexpression of AKT-DD in control cells increased slightly the percentage of MHC-positive cells to 76%; however, it enhanced specifically the formation of larger myotubes with more than six nuclei to 27%. pcDNA-transfected C2C12/Cdo RNAi cells exhibited a reduction in differentiation, the percentage of MHC-positive cells decreasing to ~61%, with ~40% mononucleate, ~16% containing two-to-five nuclei, and ~5% containing six or more nuclei. APPL1-transfected C2C12/Cdo RNAi cells showed only a slight increase in MHC-positive myotubes, the percentage of MHC-positive cells increasing to 65%, with 18% containing 2–5 nuclei and ~9% containing six or more nuclei, whereas AKT-DD-transfected C2C12/Cdo RNAi cells resulted in a full rescue of differentiation, the percentage of MHC-positive cells increasing to 72% and distribution of transfectants displaying one (26%), 2–5 (18%), and six or more nuclei (23%) being nearly identical between these cultures and the double control-vector cultures (Figure 6, A and B). These data suggest that Akt promotes myotube formation and the defect in efficient activation of Akt may be the cause of the defective differentiation of Cdo-deficient C2C12 cells.

Because activities of both p38MAPK and Akt are required for the efficient myoblast differentiation and Cdo-depleted C2C12 myoblasts exhibited defects in activation of both p38MAPK and Akt, we next examined the possibility that overexpression of AKT-DD in Cdo RNAi-expressing C2C12 cells restores p38MAPK activation, thereby rescuing differentiation of these cells. The control or Cdo RNAi-expressing C2C12 cells were transiently transfected with an AKT-DD expression vector or a control vector, plus a GFP expression vector to mark transfectants. Cells from a near-confluent condition (D0) and cultures in DM for 48 h (D2) were subjected to analysis for production of pp38 by immunocytochemistry with anti-pp38 antibodies. Under the D0 culture condition, ~68% of C2C12/pSuper/pcDNA cells were positive for pp38 signals, and AKT-DD overexpression in C2C12/pSuper cells resulted in an increase in pp38 expression to ~90%. In contrast, only ~10% of C2C12/Cdo-RNAi/pcDNA cells were positive for pp38. Overexpression of AKT-DD in C2C12/Cdo-RNAi cells resulted in an increase

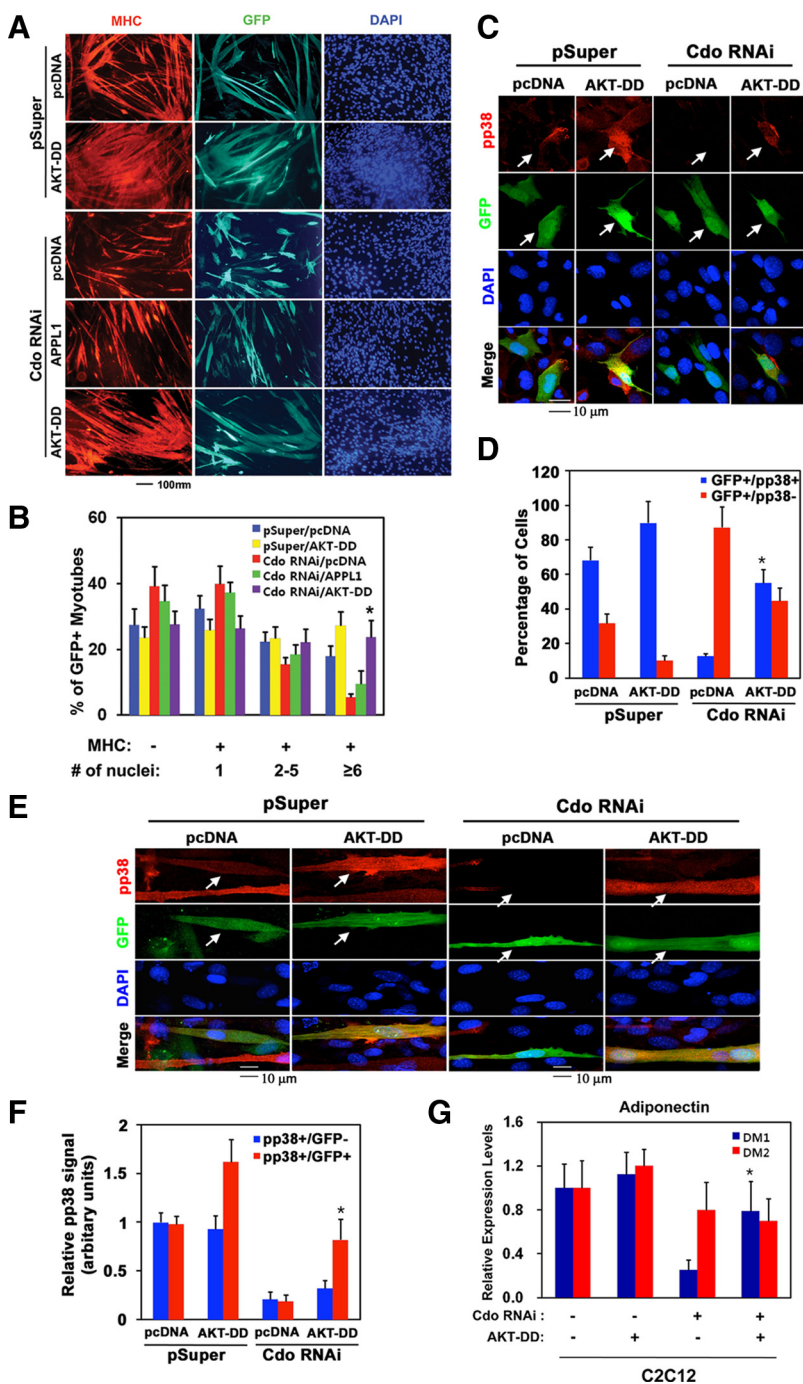


Figure 6. The full rescue of the defective differentiation of Cdo-depleted C2C12 cells by overexpression of AKT-DD, but not APPL1. (A) C2C12/pSuper and C2C12/Cdo RNAi cells were transiently transfected with pcDNA, APPL1, or AKT-DD expression vector, plus GFP expression vector, plus GFP expression vector to mark transfected cells. These cell lines were induced to differentiate for 3 d, fixed, and immunostained with an MHC antibody, followed by DAPI staining. (B) Quantification of myotube formation by cell lines shown in A. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated with similar results. Significant difference from control, $*p < 0.01$. (C) C2C12/pSuper and C2C12/Cdo RNAi cells were transiently transfected with pcDNA or AKT-DD expression vector, plus GFP expression vector to mark transfected cells. Confluent cultures were then fixed and stained with antibodies to pp38 (red) and visualized for GFP expression (green). Cell nuclei were visualized by staining with DAPI (blue). White arrows indicate transfected cells. (D) Quantification of cultures shown in C. GFP+ cells were scored as positive or negative for pp38 staining. Values represent of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results. Significant difference from control, $*p < 0.01$. (E) C2C12/pSuper and C2C12/Cdo RNAi cells were transiently transfected with pcDNA or AKT-DD expression vector, plus GFP expression vector to mark transfected cells. Cultures were incubated in DM for 48 h and then fixed and stained with antibodies to pp38 (red) and visualized for GFP expression (green). Nuclei were visualized by DAPI staining (blue). White arrows indicate transfected cells. (F) Quantification of cultures shown in E. The intensity of the immunofluorescent pp38 signals in GFP+ and GFP- cells was quantified, with the values obtained from control vector-transfected C2C12/pSuper cultures set to 1.0. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results. Significant difference from control, $*p < 0.01$. (G) RNAs of the control and AKT-DD-expressing C2C12 cells from differentiation day 1 (DM1) and DM2 were subjected to qRT-PCR for Adiponectin expression. The expression level of the control cells at DM1 was set as 1 and the relative expression levels are plotted. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated twice with similar results. Significant difference from control, $*p < 0.01$.

in pp38-positive cells to 55% (Figure 6, C and D). Under the D2 culture condition, C2C12/pSuper/pcDNA cells formed small, multinucleated myotubes and displayed a robust pp38 signal, whereas C2C12/Cdo RNAi/pcDNA cells were mainly mononucleate and had evidently weaker pp38 signal. Overexpression of AKT-DD in the control C2C12/pSuper cells appeared to enhance pp38 signal. In addition, AKT-DD-transfected C2C12/Cdo RNAi cells formed small, multinucleated myotubes and these GFP-positive myotubes displayed a robust pp38 signal (Figure 6E). To quantify this, we scored the intensity of the immunofluorescent pp38 signal in untransfected (GFP-) versus transfected (GFP+) cells on the same coverslips, with the average pp38 signal in

untransfected cells set to 1.0. Control vector-transfected cells also had a pp38 signal of ~ 1.0 , whereas AKT-DD-expressing control cells displayed an increase in a pp38 signal to 1.6. Both untransfected or control vector-transfected C2C12/Cdo RNAi cells had a pp38 signal of ~ 0.2 , whereas the signal for AKT-DD-transfected C2C12/Cdo RNAi was ~ 0.8 (Figure 6F). In these cells, we did not see any alteration in the p38MAPK signal (data not shown). Taken together, these data suggest that forced activation of Akt by overexpression of AKT-DD in Cdo-deficient C2C12 cells rescues defective differentiation of these cells and restores activation of p38MAPK in these cells. To address how p38MAPK was activated in AKT-DD-expressing C2C12/

Cdo RNAi cells, we analyzed the expression of well-known p38MAPK activators, such as Adiponectin, Insulin, and their receptors in these cells. RNAs of the AKT-DD-expressing or the control C2C12/Cdo RNAi cells at D1 or D2 were subjected to qRT-PCR for the expression of Adiponectin, Insulin, and their receptors. We did not observe any changes in expression of Insulin, Insulin receptor, and Adiponectin receptor 1 (data not shown). Interestingly control C2C12/Cdo RNAi cells displayed a transient reduction in Adiponectin expression at D1, whereas AKT-DD-expressing C2C12/Cdo RNAi cells showed a comparable expression of Adiponectin to the control cells. These data suggest that the Cdo/Akt pathway may be required for expression of Adiponectin in the early stage of myoblast differentiation.

DISCUSSION

Skeletal myogenesis is an excellent model system to study molecular mechanisms of cell differentiation, involving cell cycle exit, cell survival, and tissue-specific gene expression (Pownall *et al.*, 2002; Tapscott, 2005; Krauss *et al.*, 2005). p38MAPK and Akt signaling pathways have been shown to play crucial roles in myogenesis via regulation of activities of myogenic bHLH transcription factors (Wu *et al.*, 2000; Gonzalez *et al.*, 2004). These signaling pathways are regulated by a variety of extracellular promyogenic signals, including IGFs and cell-cell interactions (Rommel *et al.*, 2001; Charge and Rudnicki, 2004; Krauss *et al.*, 2005). Cell-cell interactions of precursors are also essential for myoblast differentiation and myotube formation (Krauss *et al.*, 2005; Griffin *et al.*, 2009). In this study, we show that Cdo activates Akt through an indirect interaction with APPL1 in C2C12 myoblasts. Cdo and APPL1 association appears to be strongly increased in the confluent culture condition. This association persists in differentiating myoblasts cultured in DM over several days, suggesting that their complex formation is initiated by cell-cell contact. It is noteworthy that both APPL1 and Cdo expression and complex formation between Cdo and APPL1 are each increased in cells cultured in high cell density conditions, regardless of the presence of high serum concentration in the culture medium. These results suggest further that cell-cell contact may be a stimulus for these processes. We have previously shown that Cdo forms higher-order complexes with the related Ig superfamily proteins, Boc and Neogenin, and cell adhesion molecule Cadherin/ β -Catenin complexes at sites of cell-cell contact (Krauss *et al.*, 2005), and this complex regulates some of the cell contact-mediated signaling. Because APPL1 forms complexes with both Cdo and Boc in C2C12 myoblasts it may be involved in the cell contact-mediated Akt activation. Consistent with this notion, the high cell density condition enhances Akt activation in C2C12 cells. However this interaction between Cdo and Boc with APPL1 appears to be indirect, and the mediator of this interaction is currently unknown.

Although mutant Cdo Δ 1035-1160 exhibited the most severe defect in its association capacity to APPL1, the binding affinity of all deletion mutants was affected, suggesting that any changes in the cytoplasmic tail of Cdo decreased their association with APPL1. It also has been shown that changes in the Cdo's cytoplasmic tail affected Cdo's binding to another scaffold protein JLP (Takaesu *et al.*, 2006). It is possible that multiple regions of the Cdo cytoplasmic domain are required to provide the structural integrity sufficient for complex formation with APPL1 or that the Cdo deletion mutants may not be localized to an appropriate subcellular compartment for association. Interestingly, APPL1 has been

implicated in regulation of endocytosis via association with the small GTPase Rab5, a key regulator of transport from the plasma membrane to the early endosomes. It has been proposed that endosomes bearing APPL1 are particularly adapted for signaling and that these endosomes mature into EEA1-positive early endosomes (Miaczynska *et al.*, 2004). It is possible that APPL1 forms complexes with Cdo in a specific compartment that might regulate downstream signaling of Cdo to activate Akt. Consistent with this notion, it appears that transiently transfected GFP-tagged Cdo colocalized with a fraction of the APPL1-positive endosomal compartment in the vicinity of cell membrane, but no significant levels of Cdo-GFP were found in the EEA1-positive early endosome compartment. Further studies will examine the role of the subcellular localization of Cdo in regulation of downstream signaling during myogenic differentiation. APPL1 has been previously shown to translocate into nucleus and to interact with components of the nucleosome remodeling and histone deacetylase complex NuRD/MeCP1 to induce gene expression required for EGF-induced proliferation (Miaczynska *et al.*, 2004). It is possible that APPL1 may be activated by Cdo-mediated signaling and regulates directly expression of muscle-specific genes via its function in nucleus. However, we did not observe any significant nuclear localization of APPL1 in differentiating myoblasts. Additional work will be required to examine whether a nuclear function of APPL1 is required for its promyogenic role. However, the study with the BAR domain-deleted APPL1 mutant, which has been shown to localize exclusively in the cytoplasm (Miaczynska *et al.*, 2004), displayed enhanced activities in induction of differentiation and Akt activation, compared with the wild-type APPL1. This result suggests that its function in the nucleus may be dispensable in myoblast differentiation.

Because components of Cdo multiprotein complexes including Boc, Neogenin, and N-cadherin are coexpressed in a variety of cell types undergoing lineage specification and differentiation, this multiprotein complex may regulate differentiation of multiple cell lineages mediated by cell-cell interaction during development. Interaction through these molecules enhances differentiation by activation of multiple cytoplasmic-signaling molecules. Cdo activates p38MAPK via binding to multiple scaffold proteins. Cdo binds directly to Bnip-2 and JLP, scaffold proteins for Cdc42 and p38MAPK, respectively, and the formation of these complexes results in Bnip-2/Cdc42-dependent activation of p38MAPK bound to JLP. In addition, we have recently shown that nonreceptor tyrosine kinase Abl binds both to the intracellular region of Cdo and to JLP during myogenesis and that Abl regulates p38MAPK activity during differentiation (Takaesu *et al.*, 2006; Kang *et al.*, 2008; Bae *et al.*, 2009b). In addition to p38MAPK, Akt pathways are also required for efficient differentiation, and APPL1 is regarded to be a scaffold protein for Akt activation. It appears that Cdo/APPL1/Akt might form different pools of signaling complexes of Cdo/JLP/p38MAPK. The evidences are two-fold: first, we failed to observe any interaction between JLP and APPL1 (data not shown), and second, overexpression and knockdown of APPL1 in myoblasts did not affect p38MAPK activities, whereas Cdo-depleted myoblasts exhibited both defective p38MAPK and Akt activation during differentiation. These data suggest that Cdo signals through p38MAPK and Akt via interaction with scaffold proteins, JLP and APPL1, to induce efficient terminal differentiation, which may be defective in rhabdomyosarcomas. In accordance with this hypothesis, we have previously shown that stable overexpression of Cdo in a rhabdomyosarcoma cell

line RD enhanced myogenic differentiation of these cells (Wegorzewska *et al.*, 2003). Interestingly, the defective differentiation and p38MAPK activation of Cdo-deficient myoblasts (Takaesu *et al.*, 2006; Kang *et al.*, 2008) can be rescued by overexpression of AKT-DD, suggesting a cross-regulation between these pathways. The exact mechanism of this cross-regulation is currently unknown. Adiponectin may be one of the candidate linking p38MAPK and Akt signaling pathways mediated by Cdo/APPL1 complexes. Adiponectin expression in early stages of differentiation appears to be dependent on Cdo, which is rescued by expression of AKT-DD in Cdo-depleted cells. Currently it is unclear how Cdo regulates adiponectin expression. Recently, it has been shown that p38MAPK activates IGF2 expression, which in turn activates Akt to promote myogenesis (Gonzalez *et al.*, 2004). It is tempting to speculate that Cdo-mediated activation of p38MAPK and Akt may induce IGF2 and Adiponectin, respectively, to stimulate each other thereby constituting autoregulatory networks to induce efficient myogenic differentiation. It is yet unknown whether Cdo/p38MAPK pathway induces IGF2 expression and further study will characterize the detailed mechanism of this cross-talk. Although both Cdo and APPL1 are required for Akt activation, it is also clear that not all Akt pathway activities during myoblast differentiation are Cdo- or APPL1-dependent, as residual Akt activities are detected in cells depleted for Cdo or APPL1. Potential additional mechanisms for activation of Akt in differentiating myoblasts may include signaling by IGFs.

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