Cdo promotes neuronal differentiation *via* activation of the p38 mitogen-activated protein kinase pathway

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ABSTRACT **Neural basic helix-loop-helix transcription factors (bHLHs) control many aspects of neurogenesis, such as proliferation, fate determination, and differentiation. We have previously shown that the promyogenic cell surface receptor Cdo modulates the Cdc42 and p38 mitogen-activated protein kinase (MAPK) pathways *via* a direct association with two scaffold-type proteins, JLP and Bnip-2, to regulate activities of myogenic bHLH factors and myogenic differentiation. We report here that Cdo uses similar regulatory mechanisms to promote neuronal differentiation. Expression of JLP, a scaffold protein for p38MAPK, and Bnip-2, a regulator of Cdc42, is increased during differentiation of C17.2 neural precursor cells and P19 embryonal carcinoma cells. These molecules regulate Cdc42 and p38MAPK activities, which increase in a Cdo-dependent manner during neuronal differentiation of C17.2 cells and retinoic acid-treated P19 cells. Furthermore, enhancement or reduction of Cdc42 and p38MAPK activities enhances or reduces, respectively, neuronal differentiation of these cell lines. Cdc42 and p38MAPK activities also promote heterodimerization of neurogenin1 and E47, suggesting that one way they promote neurogenesis is *via* regulation of neural bHLH factor activities. These results imply that a conserved intracellular signaling mechanism initiated by Cdo regulates the activities of tissue-specific bHLH factors and therefore functions as a key regulator of differentiation of several different cell lineages.—Oh, J.-E., Bae, G.-U., Yang, Y.-J., Yi, M.-J., Lee, H.-J., Kim, B.-G., Krauss, R. S., Kang, J.-S. Cdo promotes neuronal differentiation *via* activation of the p38 mitogen-activated protein kinase pathway. *FASEB J.* 23, 2088–2099 (2009)**

Key Words: Cdc42 · myogenic differentiation · tissue-specific basic helix-loop-helix transcription factors · Bnip-2 · JLP · neurogenin1

Tissue-specific basic helix-loop-helix transcription factors (bHLHs) regulate many biological processes, including proliferation, cell fate commitment, and differentiation of multiple cell lineages during embryonic development. During development of the nervous system, the appropriate numbers and types of neurons and glial cells are generated to form a network that ensures intact brain function. Neural bHLHs, such as neurogenins, Mash1, and NeuroD, control many aspects of neurogenesis, such as fate determination and the transition from proliferation to terminal differentiation of neural progenitors and migration of neurons (1).

Neural bHLHs can promote neuronal differentiation of non-neural cells when expressed ectopically in the ectoderm of *Xenopus laevis* or zebrafish embryos and induce neuronal differentiation of uncommitted P19 mouse embryonal carcinoma cells (2, 3). Neural bHLHs share many common features with myogenic bHLHs, the regulators of myogenic differentiation. Neural bHLHs are transcriptional activators which, like the myogenic bHLHs, function as heterodimers with E proteins such as E12 and E47. These heterodimers bind to specific DNA sequences (E boxes) and subsequently promote the transcription of target genes involved in the development of the nervous system (4). However, the regulatory mechanisms and intracellular signaling pathways that control bHLH factor activity are not well understood.

p38MAPK is activated in response to environmental stresses and proinflammatory cytokines (5). In addition, p38MAPK is involved in fate specification, proliferation, survival, and differentiation of various cell types, including neuronal cells (6, 7). Sustained activation of p38MAPK has been shown to be important for neuronal differentiation and neurite outgrowth of PC12 cells induced by neurotrophic factors (8, 9). In addition, p38MAPK plays an antiapoptotic role *via* activation of MEF2C in neuronal differentiation of P19 embryonal carcinoma cells induced by retinoic acid (RA) (10). However, molecular mechanisms by which the p38MAPK pathway regulates neuronal differentiation are largely unknown.

Regulation of the dynamics of the actin cytoskeleton

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is one of the key events for morphological changes in neuronal differentiation and is modulated by the Rho family of the small GTPases (11). Cdc42, a member of this family, has been implicated in various aspects of neural development, including maintenance of neural progenitors, neurite extension, axon formation, axon guidance, and neuronal polarity *via* modulation of the actin cytoskeleton (12–14). Brain-specific Cdc42 conditional knockout mice display defects in the regulation of apical polarity and in the formation of axons in *in vivo*/*in vitro* experiments (14, 15). Like other small GTPases, Cdc42 cycles between the inactive, GDP-bound state and the active, GTP-bound state and is tightly controlled by a number of regulators under physiological conditions. Guanine nucleotide exchange factors activate GTPases by catalyzing the exchange of bound GDP for GTP, whereas GTPase-activating proteins (GAPs) act as negative regulators of GTPases by enhancing their intrinsic rate of GTP hydrolysis. The activity cycle of GTPases is also regulated by interactions with additional proteins (16). One candidate involved in the regulation of Cdc42, Bnip-2, binds to both Cdc42 and Cdc42GAP. Expression of Bnip-2 in several non-neuronal cell types induced cellular elongation and membrane protrusions in a manner dependent on its ability to bind Cdc42 and on the cellular activity of Cdc42 (17).

Cdo is a cell surface molecule that belongs to the Ig superfamily. It is highly expressed in developing muscles, the CNS, midface, and sensory organs and seems to play an important role in myogenesis and neurogenesis (18–21). Cdo-knockout mice show multiple defects in brain development, including holoprosencephaly, hydrocephalus, and reduced cortical thickness (20, 21). It appears that Cdo is involved in the control of proliferation and differentiation of neural progenitors, as suggested by the decrease of neuronal differentiation in cortices lacking Cdo expression. In addition, Cdo positively regulates differentiation of a neural progenitor cell line, most likely by enhancing heterodimerization of neurogenin1 and E47, leading to the stimulation of transcriptional activity of neurogenin1 (21).

Our recent data suggest that Cdo promotes myogenesis by modulating Cdc42 and p38MAPK activities (22, 23). The Cdo intracellular region binds scaffold proteins for Cdc42 and p38MAPK (Bnip-2 and JLP, respectively) and *via* these scaffold proteins Cdc42 and p38MAPK themselves; these interactions lead to Cdc42-dependent p38MAPK activity and myogenic differentiation (22, 23). Although the promyogenic role of Cdo and signaling pathways responsible for myogenic differentiation have been well established, it is as yet unclear how Cdo regulates the activities of neural bHLHs and, thereby, neurogenesis. Because tissue-specific bHLHs are key players in both neurogenesis and myogenesis and these transcription factors must heterodimerize with their ubiquitously expressed E protein partners to induce tissue-specific gene expression, we hypothesized that similar regulatory mechanisms might be used during both processes. In the present study, we analyzed the role of Cdo and the Cdc42 and p38MAPK pathways in the regulation of neuronal differentiation. We demonstrate that Cdc42 and p38MAPK activities are induced in a Cdo-dependent manner during neuronal differentiation of C17.2 neural progenitor cells and P19 embryonal carcinoma cells. Furthermore, Cdc42 and p38MAPK signaling enhances heterodimerization between neural bHLH and E proteins. Taken together, our observations reveal a conserved mechanism by which distinct cell lineages are regulated during cell differentiation.

MATERIALS AND METHODS

Cell culture and transfection

C17.2 cells, 10T1/2 cells, and neurosphere cultures were as described previously (21, 24). In brief, C17.2 cells were cultured in DMEM with 10% FBS and 5% horse serum (growth medium) and passaged at 50% confluence every 2 d. To induce differentiation, ~60% confluent cultures were transferred to DMEM containing 2% horse serum [differentiation medium (DM)]. 10T1/2 cells were cultured in DMEM plus 10% FBS. Transient transfection was performed with FuGene6 (Roche Diagnostics Corp., Indianapolis, IN, USA). Expression construct sequences are described in Zhang *et al.* (21). Primary neural progenitor cells were prepared from cortices of embryonic day (E) 13.5 embryos (21). Neurospheres were cultured in DMEM/F12 medium containing N2 supplements, B27, basic fibroblast growth factor (bFGF) (10 ng/ml), and epidermal growth factor (10 ng/ml). To analyze their differentiative capacity, cells were seeded onto plates coated with poly-L-ornithine (Sigma-Aldrich Corp., St. Louis, MO, USA) and laminin (Invitrogen, Carlsbad, CA, USA), and differentiation was induced by culturing these cells for 2–3 d in the absence of bFGF.

P19 cell culture, RA-induced neuronal differentiation, and transfection

P19 mouse embryonic carcinoma cells were cultured in α-minimum essential medium (α-MEM) supplemented with 10% FBS and 1% penicillin and streptomycin. We compared two established protocols, an aggregation based- and a monolayer based-differentiation protocol in serum-free medium. We did not observe any significant differences in the efficiency of neuronal differentiation or protein expression pattern between these protocols, except that the former required an extended differentiation culture time (data not shown). Therefore, we used the monolayer culture condition for further studies. P19 cells were seeded at a density of 3×10^5 cells/60-mm poly-L-lysine (PLL)-coated dish 1 d before treatment with RA. Cells were then treated with 5×10^{-7} M all-*trans*-RA (Sigma-Aldrich Corp.) in serum-free medium (DMEM/F12 medium supplemented with insulin-transferrin-selenium and antibiotics) for 2 d and switched into serum-free medium in the absence of RA with replacement of the medium every 2 d (25). Transient transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were seeded at a density of 3×10^5/10-cm cell culture plates in α-MEM containing 10% FBS and antibiotics, and on the next day transfection was performed with constructs indicated in the text. One day later, cells were trypsinized and replated in PLL-coated dishes

as described above according to neuronal differentiation methods.

Western blot and immunoprecipitation

Western blot analyses were performed as described previously (21). For Western blotting, cells were lysed in extraction buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, and Complete protease inhibitor cocktail (Roche Diagnostics Corp.), and SDS-PAGE was performed. Primary antibodies used for this study were the following: anti-Cdo (Zymed Laboratories, Burlingame, CA, USA), anti-nestin (R401; Hybridoma Bank, University of Iowa, Iowa City, IA, USA), anti-pancadherin (Sigma-Aldrich Corp.), anti-N-cadherin (Zymed), anti-β-tubulin (Zymed), anti-β-tubulin III (Sigma-Aldrich Corp.), anti-MAP2 (Zymed), anti-JLP (Abcam Inc., Cambridge, MA), anti-Bnip-2 (26), anti-glial fibrillary acidic protein (GFAP) (Sigma-Aldrich Corp.), anti-p38 (Sigma-Aldrich Corp.), anti-pp38 (Cell Signaling Technology Inc., Danvers, MA), anti-Cdc42 (Upstate Biotechnology, Lake Placid, NY), anti-Cdc42GAP (Abnova Corporation, Taipei City, Taiwan), anti-flag epitope (Sigma-Aldrich Corp.), anti-myc (9E10; Mount Sinai Hybridoma Core Facility, Annenberg, NY), and anti-HA (12CA5; Mount Sinai Hybridoma Core Facility). For coimmunoprecipitation, 10T1/2, C17.2, and P19 cells in 10-cm plates were cotransfected with 5 μg of myc-neurogenin1 and 5 μg of indicated combination of constructs for 2 d. Cell lysates were then coimmunoprecipitated with anti-E47 antibody (Santa Cruz Biotechnology, Inc.) and Western blotted with anti-myc (neurogenin1), anti-E47, anti-flag (Cdc42GAP), and HA (MKK6EE and Cdc42V12) antibodies.

Cdc42 activity assay

In C17.2 cells, levels of the GTP-bound activated form of Cdc42 were analyzed with the Cdc42 Activation Assay Kit (Upstate Biotechnology) with PAK1-PBD-agarose, according to the manufacturer's instruction.

Immunocytochemistry and microscopy

C17.2 cells in 12-well plates were cotransfected with 100 ng of enhanced green fluorescent protein (EGFP) expression vector and 900 ng of the indicated DNA constructs for 2–3 d and then fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 in PBS, blocked, and probed with anti-β-tubulin III (1:200; Sigma-Aldrich Corp.), anti-MAP2 (1:200; Zymed), and Alexa Fluor 568 goat anti-mouse antibody (1:400; Molecular Probes Inc., Eugene, OR). For P19 cells, cells were transiently cotransfected with 1 μg of EGFP and 9 μg of the indicated DNA constructs in 10-cm plates for 1 d. On the next day, cells were replated with a seeding density of 3×10^5 cells/60-mm PLL-coated dishes, and differentiation was induced according to the differentiation protocol described above. Zeiss LSM-510 Meta confocal and Axiophot2 fluorescence microscopes were used for imaging and measurement of neurite length. For nuclei staining, the cells were incubated in 4 μg/ml of 4′,6-diamidino-2-phenylidole in PBS for 20 min (BD Biosciences, Franklin Lakes, NJ, USA). To monitor cell proliferation, C17.2 and P19 cells were transiently cotransfected with 1 μg of EGFP and 9 μg of the indicated DNA constructs and labeled with 10–100 μM bromodeoxyuridine (BrdU) (Sigma-Aldrich Corp.) at 37°C. Ten to 20 min later, cells were fixed with 4% paraformaldehyde, incubated with 2 N HCl, and neutralized with 0.1 M borate buffer. Immunocytochemical analysis was performed with anti-GFP (1:200; Invitrogen), anti-BrdU (1:200; Chemicon International Inc., Temecula, CA, USA); secondary antibody was probed with Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 568 goat anti-mouse antibody (1:400; Molecular Probes).

Statistical analysis

Statistical analysis of the results are expressed as mean ± SE from at least three independent experiments. Error bars represent means + SE. For comparison between multiple groups, statistical significance was tested by a Mann-Whitney *U* test using SPSS (12.0 version; SPSS, Chicago, IL, USA).

RESULTS

Cdo, Bnip-2, and JLP are involved in neuronal differentiation

We have shown previously that $Cdo^{-/-}$ mice display thin cerebral cortices and reduced expression of the neuronal marker β-tubulin III, suggesting a defect in cortical development (21). To examine the role of Cdo in neuronal differentiation, neural progenitors were isolated as neurospheres from developing cortices of $Cdo^{+/+}$, $Cdo^{+/-}$, and two $Cdo^{-/-}$ E13.5 embryos of the same litter, and their differentiation capacity was assessed. Differentiation was analyzed by immunostaining for a pan-neuronal marker, β-tubulin III (green), and a glial marker, GFAP (red) (**Fig. 1*A*, *B***). Two independent $Cdo^{-/-}$ neural progenitor preparations showed a reduction in numbers of β-tubulin III-positive neurons without a significant difference in GFAP-positive glial cells, compared with control wild-type and heterozygous cells. In addition, the amount of β-tubulin III protein produced by $Cdo^{-/-}$ neurospheres was substantially lower than that produced by control neurospheres, whereas changes in GFAP expression were modest (Fig. 1*C*). These data indicate that neurospheres lacking Cdo do not differentiate efficiently into neurons.

Our previous studies suggested that, in myogenesis, one downstream recipient of Cdo-initiated signals is the ubiquitously expressed E proteins, the heterodimeric partner of the myogenic bHLHs (19, 23). Cdo activates p38MAPK signaling *via* Bnip-2, Cdc42, and JLP (22, 23). Furthermore, p38MAPK promotes myogenic differentiation in part through phosphorylation of E proteins, thereby inducing heterodimerization with MyoD (27). Cdo expression is up-regulated during neuronal differentiation of C17.2 neural precursor cells, and overexpression of Cdo promotes neuronal differentiation of these cells. Moreover, Cdo enhances the heterodimerization of neurogenin1 with E47 in transfected 10T1/2 and C17.2 cells (21). Therefore, we asked whether the Cdo/Cdc42/p38MAPK pathway is involved in neuronal differentiation *via* regulation of the core neural bHLHs.

First, we analyzed the expression of JLP and Bnip-2, components of the Cdo signaling pathway, during

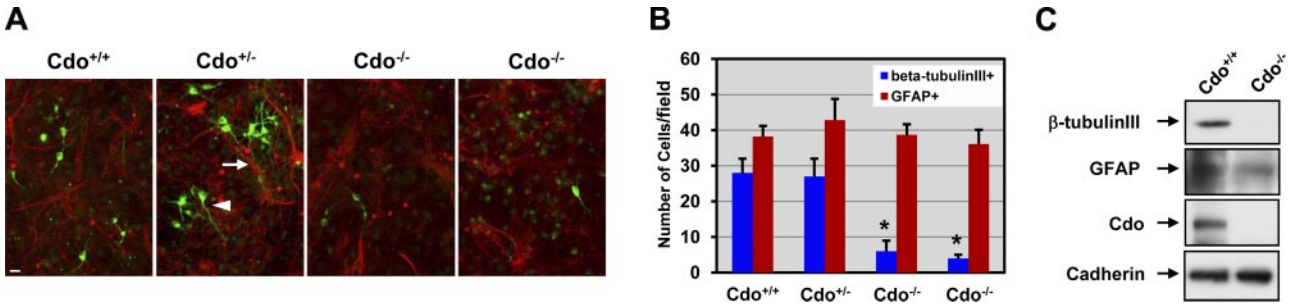

Figure 1. Impaired neuronal differentiation in $Cdo^{-/-}$ neurosphere cultures. *A*) Primary neural progenitors isolated from cortices of two control ($Cdo^{+/+}$ and $Cdo^{+/-}$) and two $Cdo^{-/-}$ E13.5 embryos were induced to differentiate for 2 d and then were fixed and immunostained for the pan-neuronal marker, β-tubulin III (green) and the glial differentiation marker, GFAP (red). Scale bar = 20 μm. *B*) Quantification of four different fields of the representative experiment shown in *A*. At least 100 cells/field were counted. The *y* axis represents the number of the β-tubulin III- and GFAP-positive cells per field. Arrowhead and arrow indicate β-tubulin III (green)- and GFAP (red)-positive cells, respectively. Data are expressed as means ± SE (n=3). *$P < 0.01$ *vs.* control. *C*) Lysates of $Cdo^{+/+}$ and $Cdo^{-/-}$ neural progenitors were Western blotted with antibodies to β-tubulin III, GFAP, or Cdo and as a loading control with pancadherin antibody.

neuronal differentiation of C17.2 cells. C17.2 cells were induced to differentiate by transfer to low-serum medium (DM) and were harvested at various time points of differentiation. The expression of nestin, a neural progenitor marker gradually decreased, whereas the expression of β-tubulin III increased during differentiation. On differentiation, the level of Bnip2 proteins increased dramatically, whereas the expression of JLP was not changed throughout the differentiation time course of C17.2 cells (**Fig. 2*A***). Next, we asked whether these proteins can promote neuronal differentiation of C17.2 cells. To do so, C17.2 cells were transiently cotransfected with an EGFP expression vector and expression vectors for Cdo, Bnip-2, or JLP (Fig. 2*B*). The transfected cells were recognized by EGFP expression. Two days after transfection, cells were subjected to immunostaining for β-tubulin III and MAP2 expression. Overexpression of Cdo, Bnip-2, or JLP increased the number of β-tubulin III- as well as MAP2-positive cells by ~3.5-fold, compared with the control vector transfected cells (Fig. 2*C, D*).

To assess whether endogenous Cdo, JLP, and Bnip-2 are required for neuronal differentiation of C17.2 cells, cells were cotransfected with EGFP and small interfering (si) RNAs against Cdo, Bnip-2, or JLP and 1 d later transferred to DM for 2 d. siRNA-mediated reduction of Cdo, Bnip-2, or JLP substantially decreased expression of β-tubulin III (Fig. 2*E*). In addition, these cells were subjected to immunostaining for β-tubulin III and MAP2 expression. The C17.2 transfectants with vectors for Cdo, JLP, or Bnip-2 siRNA exhibited a reduction in immunoreactivity to antibodies for β-tubulin III and MAP2, compared with the control cells (Fig. 2*F, G*). These data indicate that Cdo, JLP, and Bnip-2 are required for neuronal differentiation of C17.2 cells.

Cdo-dependent p38MAPK and Cdc42 signaling pathways are activated in neuronal differentiation

The above data suggest the possibilities that Bnip-2 and JLP regulate Cdc42 and p38MAPK signaling pathways during differentiation of C17.2 cells. To address this question, we asked whether there are changes in Cdc42 and p38MAPK activities during neuronal differentiation of C17.2 cells. As shown in **Fig. 3*A***, levels of the active phosphorylated-form of p38MAPK (pp38) were increased during neuronal differentiation of C17.2 cells, whereas the total amount of p38MAPK was not changed. The level of active GTP-bound Cdc42 (Cdc42*) was also increased without a change in total Cdc42 expression during neuronal differentiation of C17.2 cells (Fig. 3*A*). To examine the functional involvement of Cdo in p38MAPK and Cdc42 activation in the neuronal differentiation of C17.2 cells, cells were transiently transfected with either control or Cdo-siRNA expression vectors. The levels of pp38MAPK and active Cdc42 were reduced significantly in the Cdo-siRNA-expressing cells without alteration of the total expression levels of these proteins (Fig. 3*B, C*), suggesting that Cdo is required for the efficient activation of p38MAPK and Cdc42 during neuronal differentiation. Bnip-2 regulates activation of Cdc42, and the Bnip-2/Cdc42 pathway enhances p38MAPK activation during myoblast differentiation (22). Therefore, we asked whether p38MAPK activation in C17.2 cells is also Bnip-2 dependent. Overexpression of Bnip-2 enhanced p38MAPK activation in C17.2 cells, whereas siRNA-mediated knockdown of Bnip-2 decreased it (Fig. 3*D, E*). To confirm the relationship between Cdc42 and p38MAPK activation, we inhibited Cdc42 by overexpressing Cdc42GAP, a negative regulator of Cdc42 in C17.2 cells. As expected, the level of active Cdc42 was decreased in Cdc42GAP-overexpressing C17.2 cells; moreover, the levels of pp38MAPK were reduced in these cells (Fig. 3*F, G*). Conversely, knockdown of Cdc42GAP by siRNA expression enhanced the activity of Cdc42, as well as the production of pp38MAPK (Fig. 3*H, I*), suggesting that Bnip-2/Cdc42 activity is linked to p38MAPK signaling in C17.2 cells.

To assess functional roles of p38MAPK and Cdc42 in neuronal differentiation, MKK6EE, a constitutively active upstream kinase of p38MAPK, Cdc42V12, or

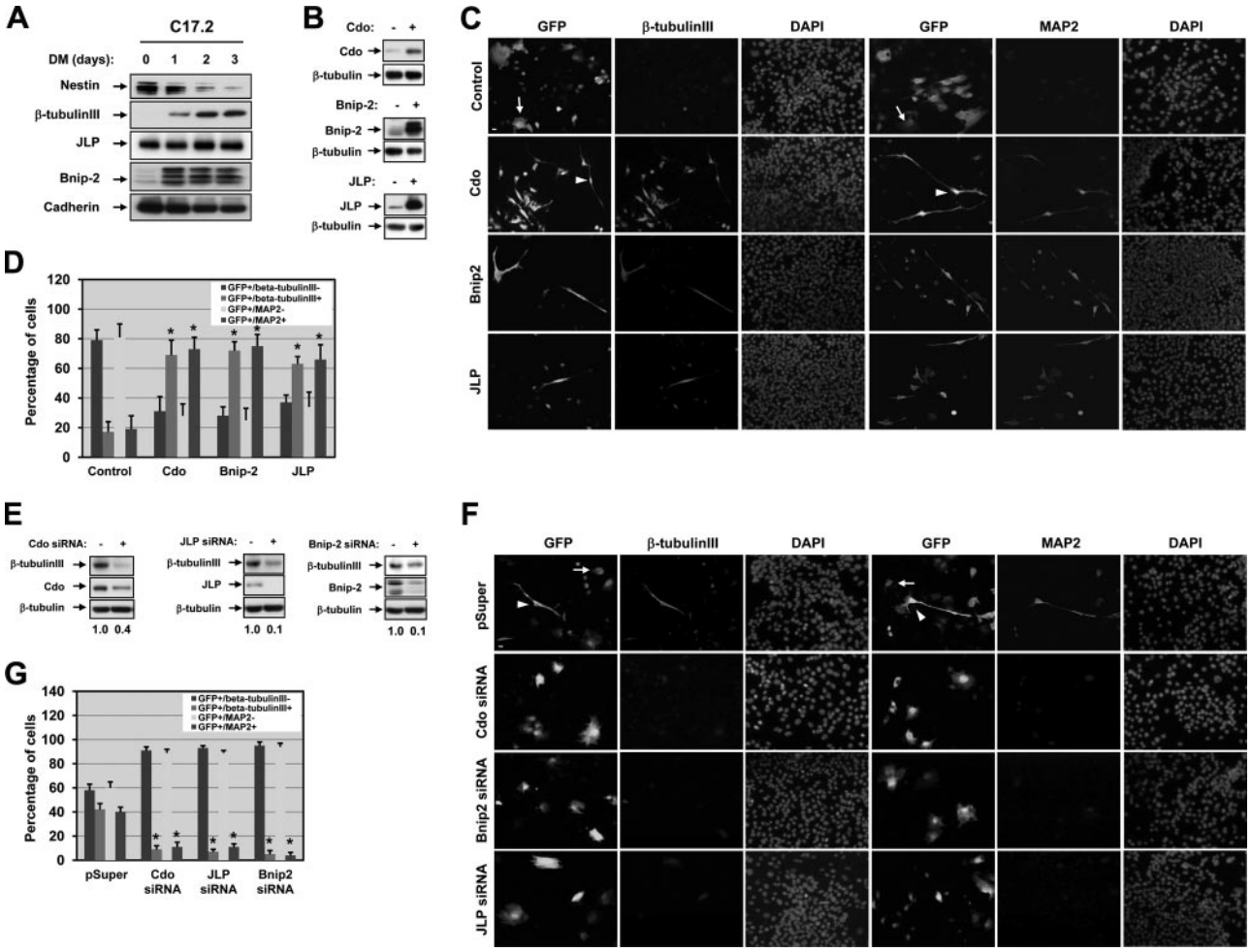

Figure 2. Effects of overexpression or knockdown of Cdo, Bnip-2, and JLP on neuronal differentiation of C17.2 cells. *A*) Lysates of C17.2 cells cultured in growth medium to ~60% confluence and then transferred to DM for the indicated times were subjected to Western blot analysis with the indicated antibodies. *B*) Lysates of C17.2 cells transiently transfected either with control, Cdo, Bnip-2, or JLP expression vectors were subjected to Western blot analysis with antibodies to Cdo, Bnip-2, or JLP and as a loading control with β-tubulin antibody. Transfection efficiency was 60–70% for each experiment. *C*) C17.2 cells were cotransfected with EGFP and either control or the indicated expression vectors for Cdo, Bnip-2, or JLP. Two days later, cells were fixed and immunostained with β-tubulin III or MAP2 antibody. Arrowhead marks GFP-positive and β-tubulin III/MAP2-positive cells; arrow marks GFP-positive and β-tubulin III/MAP2-negative cells. *D*) Quantification of the representative experiment shown in *C*. Number of transfected cells that expressed β-tubulin III or MAP2 was counted and plotted as percentage of total number of transfected cells. At least 50 cells/field were counted. Data are means ± SE (n=4). *E*) Lysates of C17.2 cells transiently transfected with either control or siRNA constructs for Cdo, Bnip-2, or JLP were Western blotted with antibodies to Cdo, Bnip2, or JLP and as a loading control with β-tubulin antibody. Knockdown protein and β-tubulin loading control signals were quantified by densitometry; ratio reported under each lane in arbitrary units with control transfectants was set to 1. *F*) C17.2 cells were transiently transfected with EGFP expression vector with either control, Cdo, Bnip-2, or JLP siRNA expression vectors. One day after transfection, cells were transferred to DM for 2 d, fixed, and immunostained with β-tubulin III or MAP2 antibody (red). Arrowhead marks GFP-positive and β-tubulin III/MAP2-positive cells; arrow marks GFP-positive and β-tubulin III/MAP2-negative cells. *G*) Quantification of the representative experiment shown in *F*. Among cells transfected with indicated siRNA and EGFP, number of cells that expressed β-tubulin III or MAP2 was counted and plotted as in *D*. Values represent means ± SE of triplicate determinations (n=3). *$P < 0.01$. DAPI, 4′,6-diamidino-2-phenylidole. Scale bars = 20 μm.

Cdc42GAP was cotransfected with EGFP into C17.2 cells, and 2 d later, cells were immunostained with antibody to β-tubulin III or MAP2. Overexpression of MKK6EE or Cdc42V12 increased the number of β-tubulin III- and MAP2-positive cells by ~3-fold, compared with the control C17.2 cells. In contrast, Cdc42GAP-transfected cells did not alter in β-tubulin III and MAP2 expression, compared with the control cells (**Fig. 4*A–C***). To determine whether p38MAPK activation is required for neuronal differentiation, C17.2 cells were treated with the p38MAPK inhibitor, SB203580. Because treatment of these cells with the typically used concentrations (5–10 μM) of SB203580 induced apoptosis in differentiating cultures (data not shown), they were treated with a lower dose of this compound (1 μM) that did not induce apoptosis. Immunostaining and Western blot analyses for β-tubulin III expression revealed a reduction in the number of β-tubulin III-positive cells and overall β-tubulin III levels when C17.2 cells were treated with SB203580 compared with the

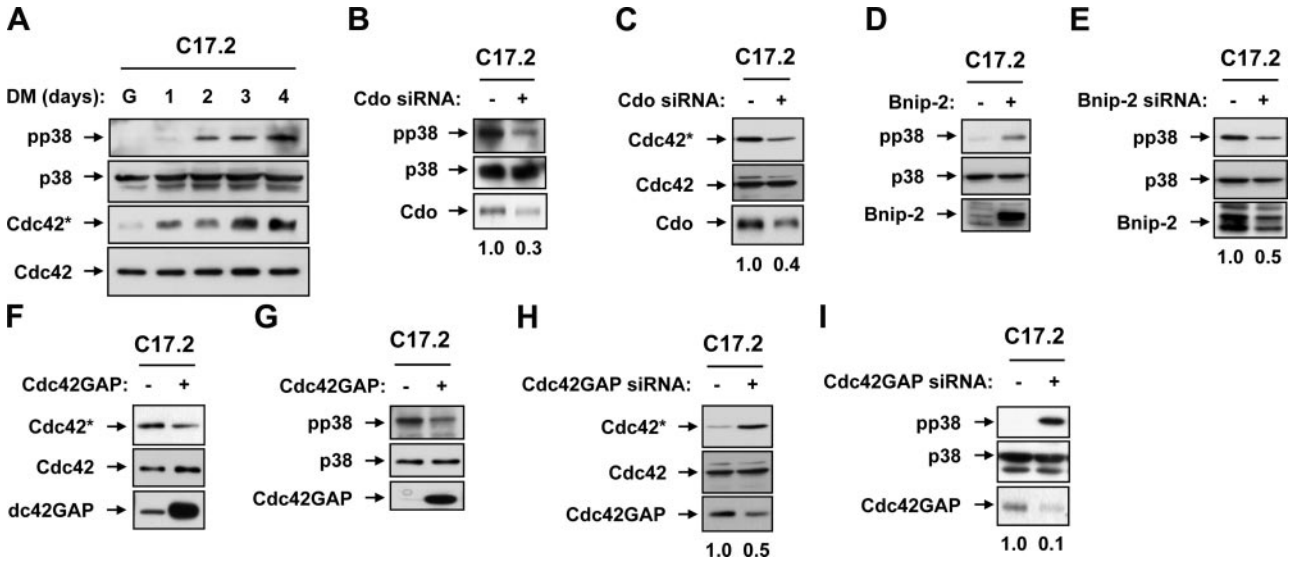

Figure 3. Activation of p38MAPK and Cdc42 in neuronal differentiation of C17.2 cells. *A*) Lysates of C17.2 cells cultured in growth medium to ~60% confluence and then transferred to DM for indicated times were subjected to Western blot analysis with anti-phospho-p38MAPK (pp38) or anti-p38MAPK (p38) antibodies. Activity of Cdc42 was analyzed by using GST-PAK1-PBD beads, a substrate for Cdc42 that only binds to the active, GTP-bound form of Cdc42. Pulldown assays with GST-PAK1-PBD beads and Western blot analysis were performed to assess level of GTP-bound Cdc42 (Cdc42*) with lysates described above. *B, C*) Lysates of C17.2 cells expressing a control or Cdo siRNA expression vector in DM for 2 d were assessed for levels of pp38 (*B*) and GTP-bound Cdc42 (Cdc42*) (*C*). Cell lysates were also Western blotted with antibodies for p38, Cdc42, or Cdo. *D*) Lysates of C17.2 cells transfected with a control or Bnip-2 expression vector in DM for 2 d were Western blotted with antibodies for pp38, p38, or Bnip-2. *E*) Lysates of C17.2 cells transfected with a control or Bnip-2 siRNA expression vector in DM for 2 d were Western blotted with antibodies for pp38, p38, or Bnip-2. *F, G*) Levels of Cdc42* (*F*) and pp38 (*G*)were assessed in C17.2 cells transfected with control or Cdc42GAP expression vector in DM for 2 d. Cell lysates were also Western blotted with antibodies for Cdc42, p38, or Cdc42GAP antibodies. *H, I*) Levels of Cdc42* (*H*) and pp38 (*I*) in C17.2 cells expressing a control or Cdc42GAP siRNA vector in DM for 2 d were assessed. Cell lysates were also Western blotted with antibodies for total Cdc42 expression, p38 expression, and expression of Cdc42GAP. Knockdown protein and β-tubulin loading control signals were quantified by densitometry; ratio is reported under each lane in arbitrary units, with control transfectants set to 1.

control cells (Fig. 4*D, E*), suggesting that activation of p38MAPK is required for neuronal differentiation of C17.2 cells. Consistent with the notion that p38MAPK activity is regulated by Cdo (Fig. 3*B*) and important for neuronal differentiation (Fig. 4*D*), expression of MKK6EE in E13.5 $Cdo^{-/-}$ neural progenitor cells rescued their defective neuronal differentiation phenotype (Supplemental Fig. S1).

To analyze whether overexpression of Cdo, MKK6EE, Cdc42V12, or Cdc42GAP alters proliferation, these vectors were cotransfected with EGFP, and 2 d later, these cells were subjected to BrdU incorporation analysis. Overexpression of Cdo, MKK6EE, and Cdc42V12 decreased in numbers of BrdU-positive cells, whereas overexpression of Cdc42GAP had no effect on proliferation of C17.2 cells (Supplemental Fig. S2*A–D*). Taken together, these results demonstrate that the Bnip-2/Cdc42 signaling pathway is interconnected with the p38MAPK pathway in a Cdo-dependent manner, promoting neuronal differentiation of C17.2 cells.

Cdo is required for the efficient neuronal differentiation of P19 embryonal carcinoma cells

The pluripotent embryonal carcinoma cell line P19 is a widely used *in vitro* model system for studying differentiation along several cell lineages (28). P19 cells can be induced to differentiate into a neuronal lineage in a process very similar to that of neuronal differentiation in the mammalian CNS *in vivo* by treatment with RA in serum-free culture conditions (25, 29). Therefore, we have used the P19 cell system to further verify the role of Cdo and Cdo-mediated Cdc42/p38MAPK signaling in neuronal differentiation. P19 cells were treated with 500 nM RA in serum-free medium for 2 d and then further cultured in serum-free medium without RA (IT). Three days after the end of RA treatment, P19 cells expressed a high level of β-tubulin III, suggesting neuronal differentiation of these cells (**Fig. 5*A***). Interestingly, the expression of Cdo was significantly up-regulated in these cells after treatment with RA (Fig. 5*A*). Moreover, a concomitant up-regulation of N-cadherin, which interacts with Cdo (30), was also detected during RA-induced neuronal differentiation of P19 cells. In addition, Bnip-2 and JLP proteins were expressed and slightly up-regulated during differentiation of these cells (Fig. 5*A*).

P19 cells were transfected with an efficiency of 70–80%; therefore, the next studies were performed with transient transfections. First, we assessed the effects of Cdo overexpression on the neuronal differentiation of P19 cells. To label the transfected cells, P19 cells were

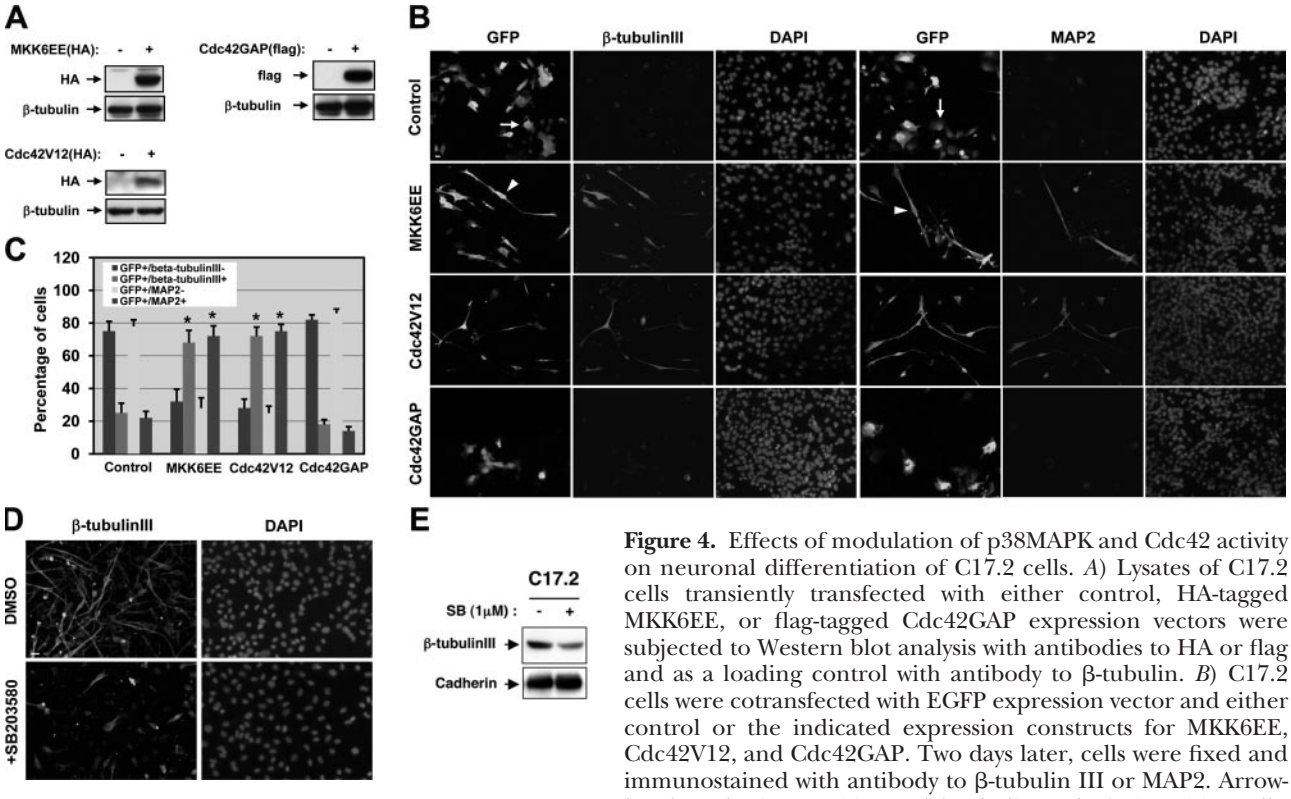

Figure 4. Effects of modulation of p38MAPK and Cdc42 activity on neuronal differentiation of C17.2 cells. *A*) Lysates of C17.2 cells transiently transfected with either control, HA-tagged MKK6EE, or flag-tagged Cdc42GAP expression vectors were subjected to Western blot analysis with antibodies to HA or flag and as a loading control with antibody to β-tubulin. *B*) C17.2 cells were cotransfected with EGFP expression vector and either control or the indicated expression constructs for MKK6EE, Cdc42V12, and Cdc42GAP. Two days later, cells were fixed and immunostained with antibody to β-tubulin III or MAP2. Arrowhead marks GFP-positive and β-tubulin III/MAP2-positive cells, arrow marks GFP-positive and β-tubulin III/MAP2-negative cells. *C*) Quantification of *B*. Transfected cells positive for β-tubulin III or MAP2 were counted and plotted as described for Fig. 2*D*. Data are means ± SE (n=4). *$P < 0.05$. *D*) C17.2 cells were treated with either DMSO or vehicle control, with or without 1 μM SB203580 in DM for 2 d, followed by immunostaining with antibody to β-tubulin III. *E*) Lysates of C17.2 cells treated with either DMSO or vehicle control, with or without 1 μM SB203580 in DM for 2 d, were subjected to Western blot analysis for β-tubulin III and pancadherin expression, as a loading control. Scale bars = 20 μm.

transiently cotransfected with EGFP and either control or Cdo expression vectors and induced to differentiate by RA treatment. At IT1, cells were subjected to immunostaining with antibody to β-tubulin III. Overexpression of Cdo caused a 2.5-fold increase in β-tubulin III and long neurite-positive P19 colonies after the RA induction (Fig. 5*B–D*). Next we asked whether Cdo is required for neuronal differentiation of P19 cells. To do so, P19 cells were cotransfected with EGFP and either control or Cdo-siRNA expressing vectors, and differentiation was stimulated by RA treatment. At IT2, cells were subjected to immunostaining with antibody to β-tubulin III. The knockdown of Cdo caused a reduction in expression of the neuronal marker β-tubulin III (Fig. 5*E*). Moreover, compared with the long neurite-like processes seen in the control β-tubulin III-positive colonies, Cdo-knockdown colonies exhibited a marked reduction in such processes, suggesting that Cdo is required for efficient neuronal differentiation of P19 cells (Fig. 5*F, G*). However, overexpression and knockdown of Cdo in P19 cells had no effect on proliferation of P19 cells (Supplemental Fig. S2*E, F*).

The role of p38MAPK in neuronal differentiation of P19 cells was also analyzed. Levels of the active form of p38MAPK increased dramatically at d 2 of RA treatment and then decreased in fully differentiated cultures that had high expression of β-tubulin III (**Fig. 6*A***). Interestingly, the activation of p38MAPK was closely correlated with the induction of Cdo expression (Fig. 5*A*), suggesting a possible causal connection. Indeed, Cdo-siRNA transfection significantly blunted the response to RA treatment compared with that in control vector-transfected cells; Cdo knockdown also resulted in a reduction in β-tubulin III expression (Fig. 6*B, C*).

To investigate the role of p38 activation in neurite extension, P19 cells were induced to differentiate by RA treatment in the presence of 2.5 μM SB203580 or DMSO vehicle control, followed by immunostaining with the antibody to β-tubulin III. The control P19 cells showed extensive neurite growth, whereas cells treated with SB203580 exhibited a dramatic reduction in expression of β-tubulin III as well as the neurite extension (Fig. 6*D*). Furthermore, the control cells expressed high levels of β-tubulin III, whereas the SB203580-treated P19 cells showed a marked reduction in the β-tubulin III expression, indicating that p38MAPK activation is required for neuronal induction and differentiation by RA treatment (Fig. 6*E*). Finally, P19 cells were transiently cotransfected with EGFP and either control, MKK6EE, or Cdc42V12 expression vectors and immunostained for β-tubulin III expression. Colonies expressing MKK6EE or Cdc42V12 exhibited an ~3-fold increase in β-tubulin III and neurite-positive P19 cells

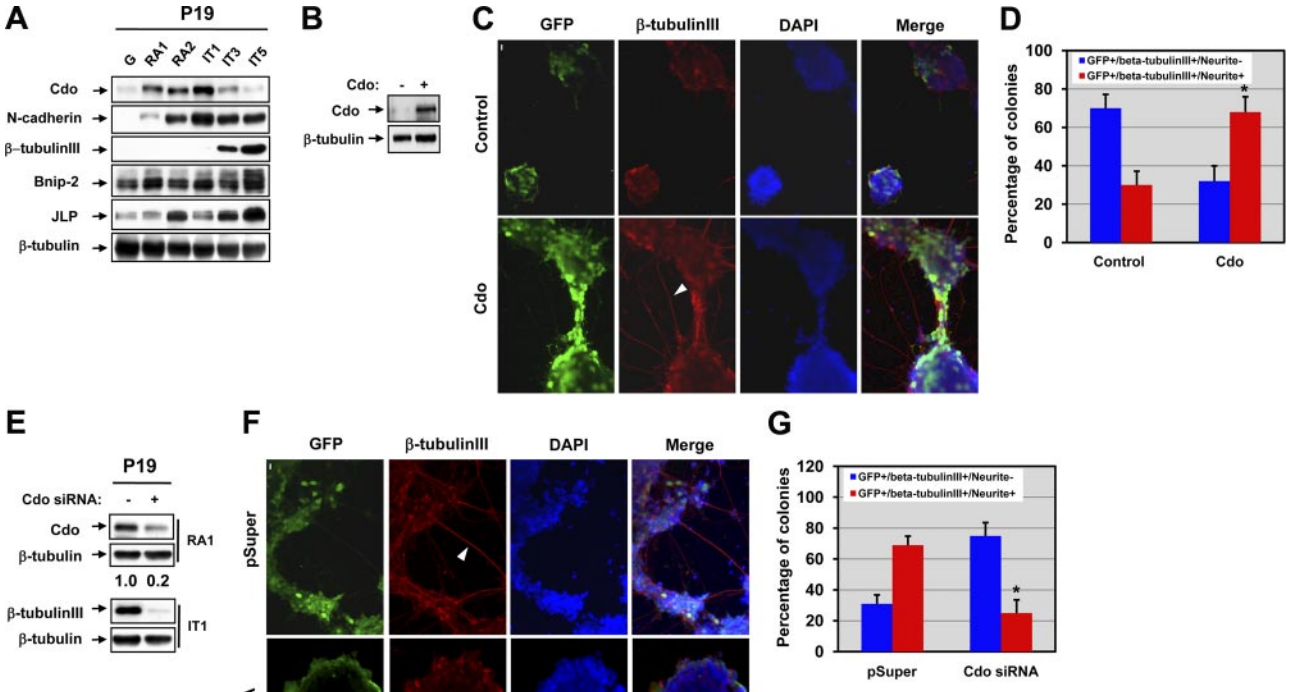

Figure 5. P19 cells express Cdo, Bnip-2, and JLP, and Cdo is required for neuronal differentiation of these cells induced by RA. *A*) P19 embryonal carcinoma cells were induced to differentiate into neurons by treatment with 500 nM RA in serum-free medium for 2 d and then cultured further in serum-free conditions without RA for 5 d (IT). Lysates of P19 cells harvested at indicated time points were Western blotted with antibodies to Cdo, pancadherin, β-tubulin III, Bnip-2, or JLP and as a loading control with β-tubulin antibody. Exponentially proliferating cells without RA treatment (*G*) were used as control for differentiation. *B*) P19 cells were transiently transfected with either control or Cdo expression vectors, collected on first day of RA treatment (RA1), and lysates were subjected to Western blotting for expression of Cdo and as a control β-tubulin. *C*) P19 cells transiently cotransfected with EGFP and either control or Cdo expression vectors were analyzed for β-tubulin III expression and neurite extension at IT1. Arrowhead indicates neurites from a colony. *D*) Quantification of the representative experiment shown in *C*. Transfected P19 colonies that possess neurites were counted and plotted as percentage of total transfected colonies. More than 6 fields and at least 10 colonies/field were counted. Data are means ± SE (n=4). *$P < 0.05$. *E*) P19 cells were transiently transfected with either control or Cdo siRNA expression vectors, collected on the first day of RA treatment (RA1) or the first day of the IT condition (IT1), and lysates were subjected to Western blot analysis for expression of Cdo, β-tubulin III, or β-tubulin, as a loading control. Knockdown protein and β-tubulin loading control signals were quantified by densitometry; ratio is reported under each lane in arbitrary units, with control transfectants set to 1. *F*) P19 cells were transiently cotransfected with EGFP expression vector and either control (pSuper) or Cdo siRNA expression vectors and fixed at IT2, followed by immunostaining with antibody to β-tubulin III. EGFP-positive colonies were photographed. Arrowhead indicates neurites from a colony. *G*) Quantification of the representative experiment shown in *F*. Colonies that possess neurites were selected and counted as in *D*. Data are means ± SE (n=3). *$P < 0.01$. Scale bars = 20 μm.

compared with control cells (Fig. 6*F–H*). These data suggest that activation of p38MAPK is required in neuronal differentiation and neurite extension in P19 cells.

Activation of Cdc42/p38MAPK signaling enhances heterodimerization of neurogenin with E47

We have shown that one way Cdo regulates neuronal differentiation is by modulating the activity of neurogenin1, a neurogenic bHLH transcription factor, possibly *via* a mechanism similar to that described for Cdo in myogenic differentiation, *i.e.,* enhanced heterodimerization of neurogenin1 with E proteins (21). To analyze whether heterodimerization of neurogenin1 with E proteins is enhanced on differentiation, C17.2 cells were transfected with neurogenin(myc) vector and harvested in growth and differentiation conditions. These cells were then subjected to immunoprecipitation with E47 antibodies. The association between neurogenin and E47 was substantially enhanced in differentiating C17.2 cells (**Fig. 7*A***). Next, we tested whether Cdc42 and p38MAPK are also involved in this activity. Neurogenin1 was cotransfected either with control or MKK6EE vectors into 10T1/2 fibroblasts, as well as C17.2 and P19 cells, and subjected to coimmunoprecipitation assays with E47 antibodies. In the presence of MKK6EE, a substantial increase in association of neurogenin1 with endogenous E47 was observed in all three cell types (Fig. 7*B, C*). Finally, neurogenin(myc) was expressed with either Cdc42GAP(flag) or Cdc42V12(HA) in 10T1/2 cells and subjected to coimmunoprecipita-

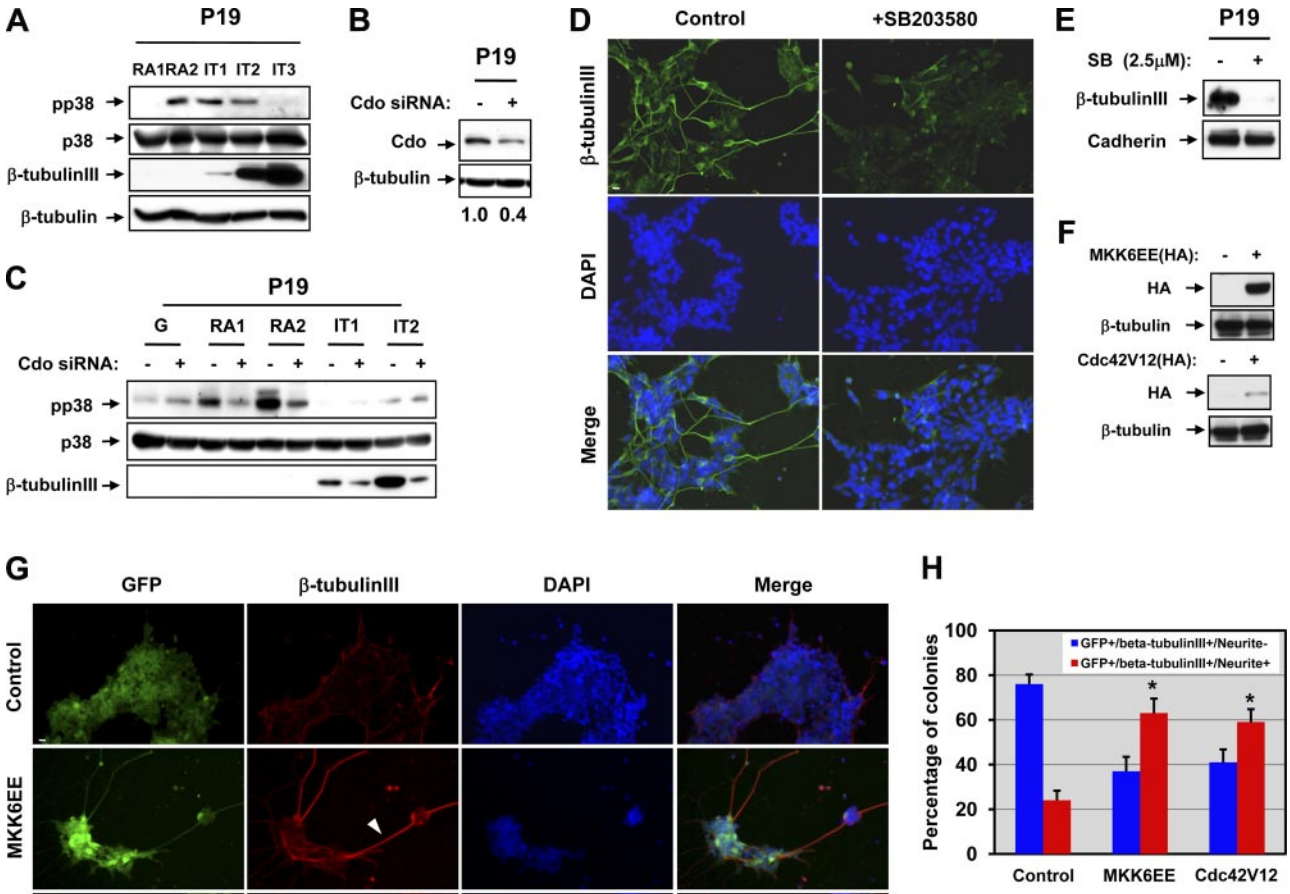

Figure 6. Activation of p38MAPK during neuronal differentiation of P19 cells. *A*) Lysates of P19 cells induced to differentiate were Western blotted with antibodies to pp38, p38, or β-tubulin III and as a loading control with β-tubulin antibody. *B*) P19 cells transiently transfected with either control (−) or Cdo siRNA (+) expression vectors were harvested on first day of the RA treatment, and lysates were Western blotted with Cdo antibody or β-tubulin antibody as a loading control. Knockdown protein and β-tubulin loading control signals were quantified by densitometry; ratio is reported under each lane in arbitrary units, with the control transfectants set to 1. *C*) P19 cells transiently transfected with either control (−) or Cdo siRNA (+) expression vectors were harvested at indicated time points, and lysates were Western blotted with antibodies to pp38, p38, or β-tubulin III. *D*) EGFP-transfected P19 cells were treated with either DMSO or SB203580 on RA treatment, and cells were subjected to immunostaining with antibody to β-tubulin III at IT2. *E*) P19 cells treated either with DMSO or SB203580 under differentiation conditions were collected at IT2 and Western blotted with β-tubulin III antibody and as a control with pancadherin antibody. *F*) P19 cells were transfected either with control, HA-tagged MKK6EE, or HA-tagged Cdc42V12 vectors, and lysates were subjected to Western blot analysis with antibodies for expression of MKK6EE, Cdc42V12, and β-tubulin as a loading control. *G*) P19 cells were transiently cotransfected with EGFP and either control, MKK6EE(HA), or Cdc42V12(HA) expression vectors, and EGFP-positive colonies were photographed at IT1. Arrowhead indicates neurites from a colony. *H*) Quantification of the representative experiment shown in *G*. Colonies that possessed neurites were selected and counted as described for Fig. 5*D*. Data are means ± SE (n=4). *$P < 0.05$. Scale bars = 20 μm.

tion with E47 antibodies. Cdc42GAP decreased association of neurogenin1 with E47, whereas Cdc42V12 enhanced complex formation of neurogenin1 with E47 (Fig. 7*C*). These data indicate that heterodimerization between neurogenin1 and E47 was stimulated by Cdc42 and p38MAPK.

DISCUSSION

There is substantial similarity between vertebrate myogenesis and neurogenesis in their reliance on tissue-specific bHLH transcription factors (7, 31). We have previously reported a regulatory mechanism in myogenic differentiation in which the cell surface receptor Cdo binds the scaffold proteins, Bnip-2 and JLP, which in turn regulate Cdc42 and p38MAPK signaling pathways, respectively. Cdc42 bound to Cdo *via* Bnip-2 appears to trigger signals that result in activation of p38MAPK bound to Cdo *via* JLP, and p38MAPK activated in this manner contributes to myoblast differentiation (22, 23). Recipients of Cdo-initiated signals include E proteins, the heterodimeric partners of myo-

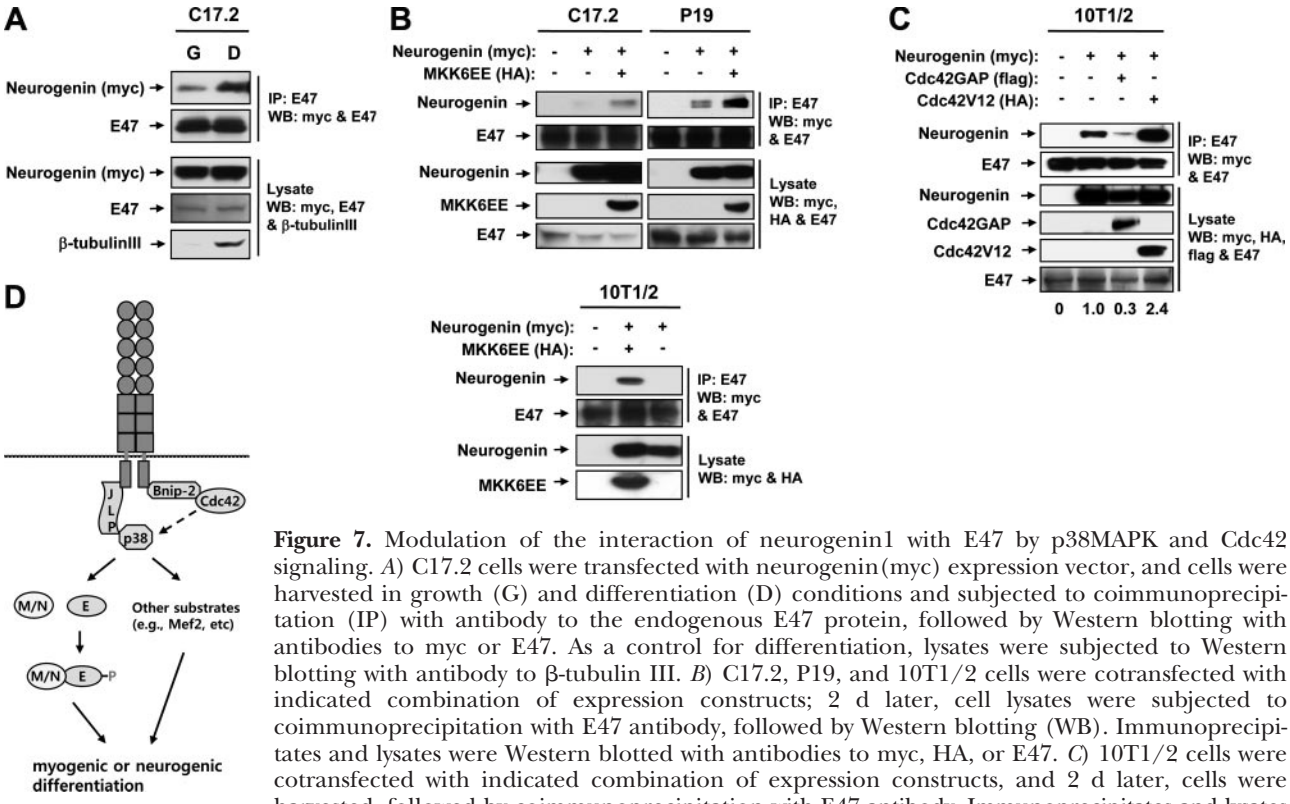

Figure 7. Modulation of the interaction of neurogenin1 with E47 by p38MAPK and Cdc42 signaling. *A*) C17.2 cells were transfected with neurogenin(myc) expression vector, and cells were harvested in growth (G) and differentiation (D) conditions and subjected to coimmunoprecipitation (IP) with antibody to the endogenous E47 protein, followed by Western blotting with antibodies to myc or E47. As a control for differentiation, lysates were subjected to Western blotting with antibody to β-tubulin III. *B*) C17.2, P19, and 10T1/2 cells were cotransfected with indicated combination of expression constructs; 2 d later, cell lysates were subjected to coimmunoprecipitation with E47 antibody, followed by Western blotting (WB). Immunoprecipitates and lysates were Western blotted with antibodies to myc, HA, or E47. *C*) 10T1/2 cells were cotransfected with indicated combination of expression constructs, and 2 d later, cells were harvested, followed by coimmunoprecipitation with E47 antibody. Immunoprecipitates and lysates were Western blotted for myc, E47, flag, or HA. Because expression levels of transfected proteins were uneven, signals of immunoprecipitates and lysates were quantified by densitometry; immunoprecipitate/lysate ratio is reported under each lane in arbitrary units, with control transfectants with neurogenin(myc) alone set to 1. *D*) Model of Cdo-mediated p38MAPK activation during myogenic and neurogenic differentiation. Cdo interacts with itself and is shown as a dimer. Cytoplasmic tail of Cdo forms a complex with JLP and p38MAPK. Cdo also binds to Bnip-2 and, *via* Bnip-2, to Cdc42, leading to activation of Cdc42, which in turn triggers activation of p38MAPK *via* a yet to be identified mechanism. p38MAPK phosphorylates E proteins, leading to enhancement of heterodimerization with myogenic or neurogenic bHLH factors (M/N). These heterodimers can induce gene expression required for myogenic and neurogenic differentiation. In addition, p38MAPK can potentially regulate activities of other factors, such as Mef2, to induce myogenic and neurogenic differentiation.

genic bHLHs (19). Because p38MAPK phosphorylates E proteins to enhance their heterodimerization (27) and E proteins are ubiquitously expressed, this regulatory mechanism can be generalized, yet results in tissue-specific transcriptional responses. Cdo is expressed at high levels in neuronal precursor cells and specific CNS structures, including the developing cortex. *Cdo*$^{-/-}$ embryos display defects in brain development, including thinning of the cortex, reduced differentiation, and hydrocephalus (21).

In this study, we have tested the hypothesis that myogenesis and neurogenesis may be regulated by similar Cdo-dependent signaling mechanisms. p38MAPK and Cdc42 activities are induced during neuronal differentiation of C17.2 and P19 cells. Reduction of Cdo, JLP, or Bnip-2 expression levels caused a decrease of p38MAPK activation and impaired neuronal differentiation. Ectopic expression of the p38MAPK activator MKK6EE enhanced neuronal differentiation, neurite extension, and heterodimerization of neurogenin1 with E47. Moreover, inhibition of p38MAPK activity by the specific inhibitor, SB203580, blocked neuronal differentiation in C17.2 neuronal progenitors and RA-induced P19 cells. Modulation of Cdc42 activities by expression of the negative regulator Cdc42GAP or a constitutively active form of Cdc42, Cdc42V12, influenced neuronal differentiation in a fashion similar to modulation of p38MAPK activities.

The role of p38MAPK in neuronal differentiation is not fully understood. Neurite extension in nerve growth factor (NGF)-treated PC12 cells is blocked by SB203580, and p38MAPK functioned in concert with ERK in NGF-induced phosphorylation of the transcription factor cAMP response element-binding protein (CREB) (8, 32). In addition, p38MAPK activity is induced in PC12 cell differentiation by other neurotrophic factors and bone morphogenetic protein-2 (9, 33). The p38MAPK pathway promotes neuronal differentiation of adult hippocampal neural progenitors while inhibiting glial differentiation of these cells (34). p38MAPK has also been suggested to play an important role in survival of differentiated neurons derived from RA-induced P19 cells *via* activation of transcription factor MEF2C (10). This study is, to our current knowledge, the first to link a signaling pathway initiated at the cell surface that functions *via* p38MAPK to promote neuronal differentiation and that can regulate neural bHLH factor activity. Whether Cdo-mediated p38MAPK activation also regulates neuronal dif-

ferentiation *via* modulation of activities of CREB or MEF2C remains to be addressed.

Neuronal development and differentiation require multiple coordinated events, including cell migration, process outgrowth, adoption of a cell type-specific transcriptional program, and the formation of connections with appropriate targets. Cdc42 has been shown to play an important role in these processes, especially in neuronal migration and neurite extension *via* the regulation of the actin cytoskeleton. Modulation of Cdc42 activity by overexpression of Cdc42GAP or expression of Cdc42V12 affected both neurite extension and the interaction of neurogenin1 with E47. Therefore, it is possible that Cdc42 may regulate neuronal differentiation *via* multiple mechanisms: regulation of actin cytoskeleton dynamics in migratory and morphological events and transducing signals through p38MAPK to enhance the transcriptional activities of neural bHLHs. Interestingly, modulation of p38MAPK activity *via* overexpression of JLP and MKK6EE or treatment with the SB203580 inhibitor also affected neurite extension. This finding suggests that morphological and transcriptional events in neuronal differentiation are likely to be coordinated and not easily separable, a phenomenon that also occurs in myogenic differentiation (22). Despite the similarities between myogenic and neuronal differentiation, however, obvious differences exist. For example, differentiated myofibers are formed by fusion of muscle precursor cells into multinucleated syncytia, whereas neurons are mononucleated cells that elaborate axonal and dendritic processes. Furthermore, a relatively limited number of myofibers are bundled into muscles, whereas neurons often make connections with thousands of other cells. These distinctions must arise from both distinct genetic programs and differential cell behavior during development.

Cdo is a component of a multiprotein complex in myoblasts that contains the related Ig superfamily members, Boc and neogenin, and the cell adhesion molecule, N-cadherin (35). This latter interaction between Cdo and N-cadherin has been shown to be important for the promyogenic role of Cdo (36, 37). The mechanism by which Cdo itself is activated to signal through Cdc42 and p38MAPK is not known; however, it might be linked with N-cadherin. N-cadherin has been shown to be required for neuronal differentiation of P19 cells (38), and N-cadherin-initiated signals regulate the activities of Rho family GTPases and thereby the dynamics of the actin cytoskeleton which in turn regulates neurite outgrowth (30, 39). Therefore, it is tempting to speculate that Cdo functions with N-cadherin to coordinate neuronal differentiation, and future studies will address this question. In summary, we have shown that a conserved Cdo/Cdc42/p38MAPK pathway regulates neuronal differentiation and can activate neural bHLH factors, revealing a common mechanism between myogenesis and neurogenesis. FJ

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