

# Regulation of Cdh1–APC Function in Axon Growth by Cdh1 Phosphorylation

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The ubiquitin ligase Cdh1–anaphase promoting complex (Cdh1–APC) plays a key role in the control of axonal morphogenesis in the mammalian brain, but the mechanisms that regulate neuronal Cdh1–APC function remain incompletely understood. Here, we have characterized the effect of phosphorylation of Cdh1 at cyclin-dependent kinase (Cdk) sites on Cdh1–APC function in neurons. We replaced nine conserved sites of Cdk-induced Cdh1 phosphorylation with alanine (9A) or aspartate (9D) to mimic hypo- or hyperphosphorylation, respectively. We found that the 9A mutation triggered the proteasome-dependent degradation of Cdh1, and conversely the 9D mutation stabilized Cdh1 in neuronal cells. However, the phosphomimic 9D Cdh1 protein failed to associate with the APC core protein Cdc27. In addition, whereas wild-type and 9A Cdh1 predominantly localized to the nucleus, the 9D Cdh1 protein accumulated in the cytoplasm in neurons. Importantly, in contrast to wild-type and 9A Cdh1, the 9D Cdh1 mutant failed to inhibit axon growth in primary cerebellar granule neurons. Collectively, our results suggest that phosphorylation of neuronal Cdh1 at Cdk sites triggers the stabilization of an inactive form of Cdh1 that accumulates in the cytoplasm, leading to the inhibition of Cdh1–APC function in the control of axon growth. Thus, phosphorylation of Cdh1 may represent a critical mechanism regulating Cdh1–APC function in the nervous system.

## Introduction

The E3 ubiquitin ligase activity of the anaphase-promoting complex (APC) is critical for coordinating cell cycle transitions (Peters, 2006; Sullivan and Morgan, 2007). The association of the APC with one of two activating subunits, Cdh1 or Cdc20, enables this highly conserved, multi-subunit complex to target specific substrates for proteasomal degradation during distinct phases of the cell cycle (Peters, 2006; Sullivan and Morgan, 2007). Recent studies have defined new functions for the APC in postmitotic neurons (Stegmüller and Bonni, 2005; Kim and Bonni, 2007; Kim et al., 2009). In particular, Cdh1–APC controls axon growth and patterning in the mammalian brain (Konishi et al., 2004). Neuronal Cdh1–APC may also regulate synapse development and plasticity (Juo and Kaplan, 2004; van Roessel et al., 2004; Li et al., 2008) and neuronal survival (Almeida et al., 2005). Thus, Cdh1–APC appears to orchestrate diverse aspects of neuronal development and function.

The mechanisms by which Cdh1–APC controls axonal morphogenesis are beginning to be characterized. Structure–function analyses of Cdh1 have revealed that Cdh1–APC operates in the

nucleus in neurons to inhibit axon growth (Stegmüller et al., 2006). The identification of the transcriptional regulators SnoN and Id2 as physiologically relevant substrates of neuronal Cdh1–APC suggests that this ubiquitin ligase controls programs of gene expression that are dedicated to the growth of axons (Lasorella et al., 2006; Stegmüller et al., 2006; Ikeuchi et al., 2009).

How Cdh1–APC function is controlled in neurons remains to be elucidated. The TGF $\beta$ –Smad2 signaling pathway stimulates the ability of neuronal Cdh1–APC to trigger the degradation of SnoN and thereby inhibit axon growth (Stegmüller et al., 2008). Activated Smad2 may facilitate the assembly of a tripartite complex composed of Smad2, Cdh1, and SnoN, potentially promoting the interaction of Cdh1–APC with the substrate SnoN (Pot and Bonni, 2008). In addition to mechanisms of protein–protein interaction, neuronal Cdh1–APC function might also be regulated by phosphorylation of Cdh1 (Maestre et al., 2008). However, whether Cdh1 phosphorylation plays a role in Cdh1–APC regulation of axon growth remained to be addressed.

In mitotic cells, Cdh1–APC activity is tightly regulated by cyclin-dependent kinase (Cdk)-induced phosphorylation of Cdh1. The mitotic Cdk-induced phosphorylation of Cdh1 dissociates Cdh1 from the APC (Peters, 2006). Phosphorylation of Cdh1 may occur on as many as 11 sites in budding yeast and nine conserved residues in proliferating mammalian cells (Zachariae et al., 1998; Jaspersen et al., 1999; Kramer et al., 2000). Phosphorylation at four of these sites (serines 40, 151, and 163 and threonine 121) reportedly promotes the nuclear export of mammalian Cdh1 (Zhou et al., 2003). These observations led us to question the consequence of Cdh1 phosphorylation at Cdk sites on Cdh1–APC regulation of axon growth in mammalian neurons.

Here, we find that Cdh1 phosphorylation may represent a key

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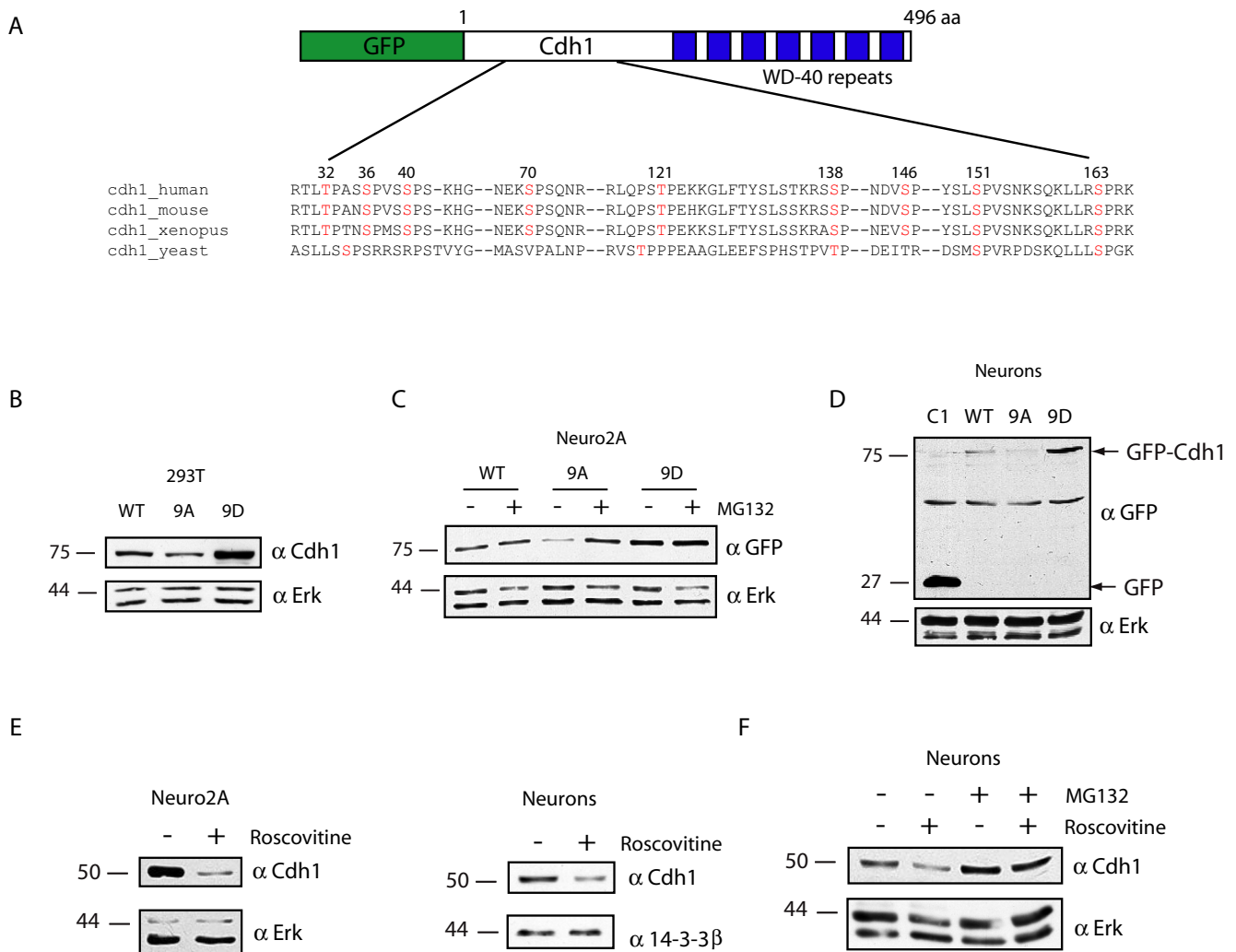
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**Figure 1.** Cdh1 phosphorylation at Cdk sites promotes Cdh1 stability. **A**, Schematic of nine conserved sites of potential Cdk phosphorylation in Cdh1 (S/T-P), numbered according to human Cdh1. **B**, Lysates of 293T cells transfected with GFP–Cdh1 WT, 9A, or 9D were immunoblotted using a polyclonal antibody to Cdh1 or Erk, the latter to serve as a loading control. Quantitation of Cdh1 levels normalized by Erk revealed that the levels of the 9D and 9A mutant proteins were, respectively, increased by 122% and reduced by 57% relative to wild-type Cdh1 in 293T cells (average of 4 experiments). **C**, Lysates of Neuro2A cells transfected with GFP–Cdh1 WT, 9A, or 9D and treated with MG132 (5  $\mu$ M) or the vehicle control DMSO were immunoblotted for GFP or Erk. The levels of the 9D and 9A mutant proteins were, respectively, increased by 150% and reduced by 37% relative to wild-type Cdh1 in Neuro2A cells (average of 2 experiments). MG132 treatment, respectively, increased WT, 9A, and 9D levels by 80, 134, and 9%. **D**, Lysates of granule neurons transfected with pEGFP–C1, GFP–Cdh1 WT, GFP–Cdh1 9A, or GFP–Cdh1 9D were immunoblotted for GFP or Erk. The 9D Cdh1 mutant is expressed at higher levels than wild-type Cdh1, which is higher than the 9A Cdh1 mutant in primary granule neurons. **E**, Lysates of Neuro2A cells or granule neurons treated with roscovitine (20  $\mu$ M) or DMSO were immunoblotted using a monoclonal antibody to Cdh1 and the Erk or 14-3-3 $\beta$  antibody. Roscovitine reduces Cdh1 levels by 60% in both Neuro2A and neurons (average of 2 experiments each). **F**, Lysates of granule neurons treated with MG132 (10  $\mu$ M), roscovitine (20  $\mu$ M), both MG132 and roscovitine, or DMSO were immunoblotted using the Erk antibody or the Cdh1 monoclonal antibody. Roscovitine decreased Cdh1 levels by 50%, whereas MG132 increased Cdh1 levels by almost twofold (average of 2 experiments). The combination of MG132 and roscovitine treatment restored Cdh1 levels to 1.3-fold above control Cdh1 levels.

mode of regulation of Cdh1–APC function in neurons. Our results suggest that phosphorylation of Cdh1 at nine conserved Cdk sites disrupts the association of Cdh1 with the APC, leading to the stabilization of an inactive form of Cdh1 in the cytoplasm and consequent inhibition of Cdh1–APC function in limiting the growth of axons.

## Materials and Methods

**Reagents.** The 9A and 9D mutations in green fluorescent protein (GFP)–Cdh1 (Stegmüller et al., 2006) were generated using Quik-Change site-directed mutagenesis (Stratagene) to mutate the Cdh1 sites shown in Figure 1A. The GFP (Invitrogen), Erk 1/2 (Cell Signaling Technology), monoclonal Cdh1 (Genetex), polyclonal Cdh1 (Zymed), Cdc27 (Santa Cruz Biotechnology), Discosoma red (DsRed) (Clontech), and  $\beta$ -galactosidase ( $\beta$ -gal) (Promega) antibodies were purchased. MG132

(carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) (Sigma) and roscovitine (Calbiochem) were purchased.

**Immunoprecipitations and immunoblotting analyses.** Immunoprecipitation and immunoblotting analyses were done as described previously (Kim et al., 2009).

**Glycerol density gradient.** Using a protocol modified from the study of Zachariae et al. (1996), lysates of granule neurons were prepared in buffer A (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and 100 mM Tris, pH 7.2), layered on 15–35% glycerol density gradients in buffer A, and centrifuged for 18 h at 28,000 rpm in a Beckman Coulter SW60 rotor. Fractions were analyzed by SDS-PAGE and immunoblotting for Cdc27 and Cdh1.

**Primary neuron cultures and transfection.** Granule neurons were prepared from postnatal day 6 Long–Evans rat pups and transfected either 8 h or 2 d *in vitro* after plating using a modified calcium phosphate

method as described previously (Konishi et al., 2004) with indicated plasmids together with either DsRed or  $\beta$ -gal expression plasmid to visualize transfected neurons. To rule out the possibility that the effects of RNA interference (RNAi) or protein expression on axonal length were attributable to any effect of these manipulations on cell survival, the anti-apoptotic protein Bcl-x<sub>L</sub> was coexpressed in the morphology experiments. The expression of Bcl-x<sub>L</sub> has little or no effect on axonal length (Konishi et al., 2004). Neurons were subjected to immunocytochemistry using a GFP, DsRed, and/or  $\beta$ -gal antibody.

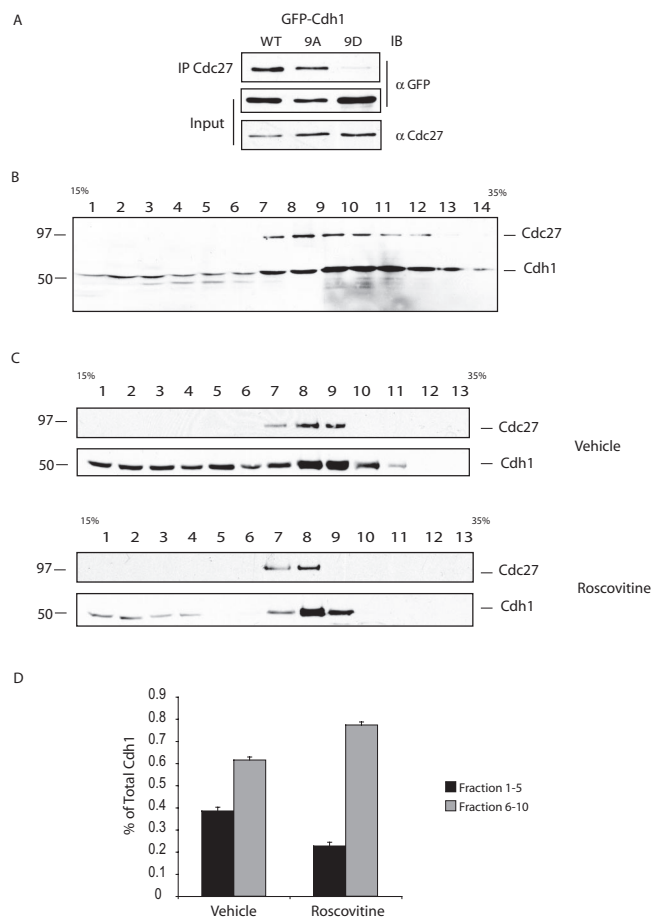
**Axon growth assay and morphometry.** Axon length morphometry was done as described previously (Gaudilliere et al., 2004; Konishi et al., 2004). Briefly, images of transfected DsRed-positive neurons were captured in a blinded manner using a Nikon Eclipse TE2000 epifluorescence microscope. Axon length was measured using SPOT imaging software. Cellular localization was quantified by counting the percentage of transfected  $\beta$ -gal-positive neurons harboring exclusively nuclear or nuclear and strong cytoplasmic GFP staining.

## Results

The Cdk-induced phosphorylation of Cdh1 has been characterized in yeast and proliferating non-neural mammalian cells (Peters, 2006; Sullivan and Morgan, 2007). Recent evidence suggests that Cdh1 is also phosphorylated in mammalian neurons (Maestre et al., 2008). However, the role of Cdh1 phosphorylation in Cdh1–APC function in axon growth remained unknown. To investigate the role of Cdh1 phosphorylation in neuronal cells, we generated plasmids encoding a GFP–Cdh1 fusion protein mutated to alanine (9A) or aspartate (9D) at nine sites of Cdk phosphorylation conserved from *Xenopus* to human to mimic hypo- or hyper-phosphorylation, respectively (Fig. 1A).

We first expressed the wild-type (WT) and phosphorylation mutants of Cdh1 in 293T cells and mouse Neuro2A neuroblastoma cells. Remarkably, we found a clear and consistent difference in the protein levels of hypo- versus hyper-phosphorylation mutant Cdh1 in these cells (Fig. 1B,C). The phosphomimic GFP–Cdh1 9D was expressed at higher levels than GFP–Cdh1 WT, which in turn was expressed at higher levels than GFP–Cdh1 9A in both 293T cells and neuronal cells (Fig. 1B,C). To determine the basis for the difference in the level of expression of wild-type and phosphorylation mutants of Cdh1 protein, we assessed the effect of treatment of cells with the proteasome inhibitor MG132 on the levels of the Cdh1 proteins. The levels of wild-type and both mutant Cdh1 proteins increased in 293T cells upon exposure to MG132, suggesting that Cdh1 levels are exquisitely regulated by proteasome-dependent degradation (data not shown). Notably, we also found that the levels of GFP–Cdh1 9A, and to a lesser extent GFP–Cdh1 WT, were elevated in Neuro2A cells upon exposure to MG132 (Fig. 1C). In contrast, the levels of GFP–Cdh1 9D, which were basally high, were not further elevated in Neuro2A cells treated with MG132 (Fig. 1C). These results suggest that the phosphorylation of Cdh1 at Cdk sites stabilizes Cdh1 protein, whereas dephosphorylation destabilizes Cdh1 in neuronal cells. A caveat that cannot be ruled out is that mutation of Cdh1 at Cdk sites might trigger conformational changes in Cdh1 protein that account for the observed changes in Cdh1 stability.

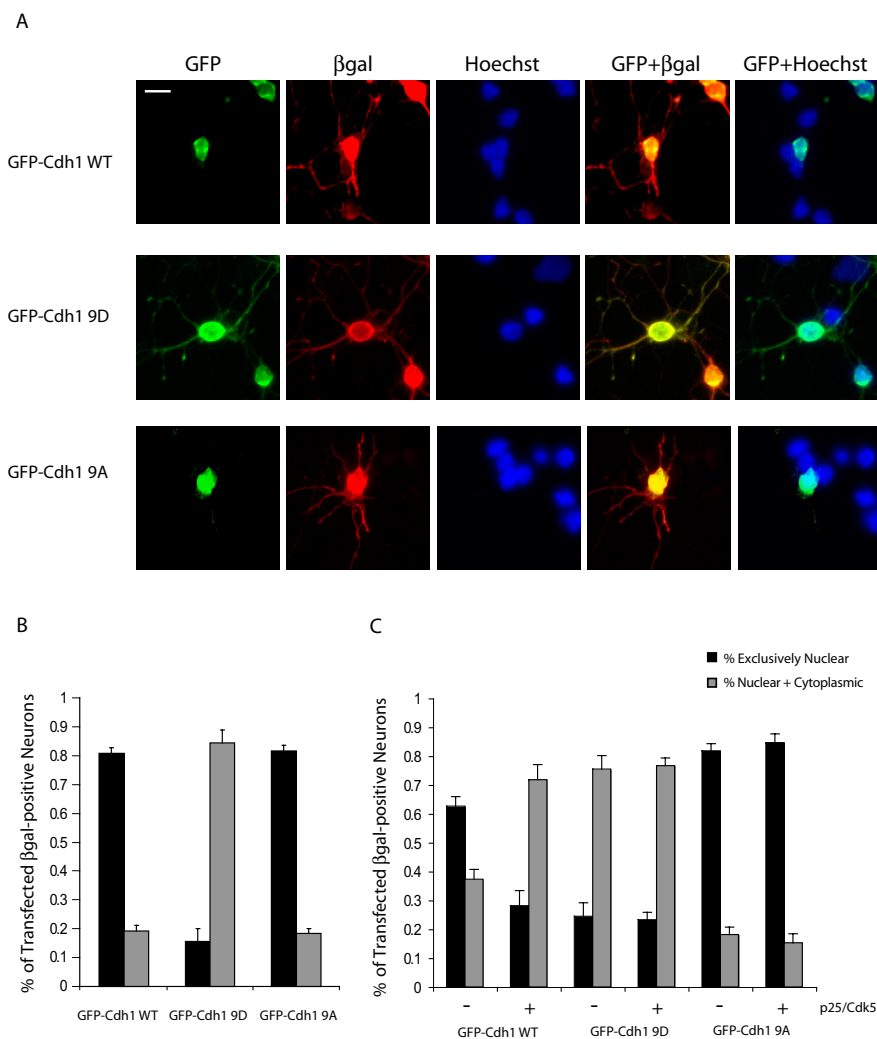
We next determined the effect of Cdh1 phosphorylation at Cdk sites on the stability of Cdh1 in primary neurons. In agreement with results in 293T and Neuro2A cells, the 9D Cdh1 mutant was expressed at higher levels, whereas the 9A Cdh1 mutant was expressed at lower levels, than wild-type Cdh1 upon expression in primary rat cerebellar granule neurons (Fig. 1D). In addition, we found that MG132 treatment was sufficient to elevate endogenous Cdh1 protein levels in primary neurons (Fig. 1F). To



**Figure 2.** Cdh1 phosphorylation at Cdk sites disrupts the association of Cdh1 with the APC core protein Cdc27. **A**, The APC core protein Cdc27 was immunoprecipitated (IP) from lysates of 293T cells expressing GFP–Cdh1 WT, 9A, or 9D followed by immunoblotting with the GFP or Cdc27 antibody. The 9D mutation, but not the 9A mutation, disrupts the ability of Cdh1 to form a complex with the APC core protein Cdc27. **B**, Glycerol density gradient (15–35%) centrifugation of extracts was performed on cerebellar granule neurons, and fractions were immunoblotted using a Cdc27 antibody and a monoclonal antibody to Cdh1. Endogenous Cdh1 is found in two pools in neurons: a pool that comigrates with Cdc27 and a pool that is free of Cdc27. **C**, **D**, Glycerol density gradient (15–35%) centrifugation of extracts was performed on cerebellar granule neurons treated for 11 h with MG132 (10  $\mu$ M) or either roscovitine (20  $\mu$ M) or the vehicle control DMSO. Roscovitine treatment increased the pool of Cdh1 cofractionating with Cdc27 from 62 to 77% but decreased the free pool of Cdh1 from 38 to 23% ( $n = 3$ ; ANOVA with Fisher's PLSD *post hoc* test,  $p < 0.0001$ ).

test the hypothesis that Cdk phosphorylation stabilizes Cdh1 protein, we assessed the effect of inhibiting endogenous Cdk activity on the levels of endogenous Cdh1 in neuronal cells, including primary granule neurons. Exposure of Neuro2A cells and primary granule neurons to the Cdk inhibitor roscovitine robustly reduced the levels of endogenous Cdh1 (Fig. 1E,F). The roscovitine-induced reduction in Cdh1 levels was reversed upon exposure of neurons to MG132, suggesting that Cdk inhibition triggers the proteasomal degradation of Cdh1 in neurons (Fig. 1F). Together, these results suggest that phosphorylation of Cdh1 at Cdk sites inhibits the degradation of Cdh1 by the ubiquitin-proteasome pathway in neurons.

What is the consequence of phosphorylation-induced stabilization of Cdh1 on Cdh1–APC function? To address this question, we first determined the effect of Cdh1 phosphorylation on its ability to associate with the APC. We expressed wild-type and phosphorylation mutants of GFP–Cdh1 in cells and assessed their ability to form a physical complex with the APC core protein



**Figure 3.** Cdh1 phosphorylation at Cdk sites promotes the cytoplasmic accumulation of Cdh1 in granule neurons. **A**, Granule neurons transfected with an expression plasmid encoding GFP–Cdh1 WT, 9D, or 9A, together with the  $\beta$ -gal expression plasmid were subjected to immunocytochemistry with an antibody to  $\beta$ -gal or GFP and the DNA dye Hoechst. Scale bar, 10  $\mu$ m. **B**, Wild-type and the 9A Cdh1 mutant were exclusively nuclear in  $\sim$ 80% of neurons. However, the 9D mutation significantly reduced the number of neurons displaying exclusively nuclear localization (ANOVA with Fisher's PLSD *post hoc* test,  $p < 0.0001$ ). A total of 788 neurons were analyzed. **C**, Neurons transfected as in **A** together with expression plasmids encoding p25 and Cdk5 or their vector control pCMV5 were analyzed as in **A** and **B**. The percentage of neurons in which Cdh1 appeared in the cytoplasm increased from  $<40$  to  $>70\%$  upon p25/Cdk5 expression (ANOVA with Fisher's PLSD *post hoc* test,  $p < 0.0001$ ). In contrast, 9A Cdh1 and 9D Cdh1 remained predominantly nuclear or nuclear and cytoplasmic, respectively, upon p25/Cdk5 expression. A total of 2500 neurons were analyzed.

Cdc27 by immunoblotting Cdc27 immunoprecipitates with the GFP antibody. The 9A Cdh1 mutant associated with Cdc27 as effectively as wild-type Cdh1 (Fig. 2A). In contrast, the phosphomimetic 9D Cdh1 mutant failed to associate effectively with Cdc27 (Fig. 2A). These results suggest that phosphorylation of Cdh1 stabilizes an inactive form of Cdh1 that is unable to complex with the APC. Consistent with these results, Cdk phosphorylation of Cdh1 in yeast and *Xenopus* extracts triggers the dissociation of Cdh1 from the APC (Zachariae et al., 1998; Jaspersen et al., 1999; Kramer et al., 2000). Thus, phosphorylation of Cdh1 at Cdk sites appears to represent a highly evolutionarily conserved mechanism that regulates the association of Cdh1 with the APC.

If the association of Cdh1 with the APC represents a mode of regulation in mammalian neurons, one would predict that Cdh1 is present in two pools, a pool that is bound to the APC and a pool of Cdh1 that is not in complex with the APC. To test this predic-

tion, we separated granule neuron lysates on a glycerol gradient and subjected the different fractions to immunoblotting with antibodies to Cdh1 and Cdc27. We found that endogenous Cdh1 appears in two peaks, one low-percentage and the other high-percentage glycerol fractions. The APC core protein Cdc27 was found in a similar peak as the high-percentage glycerol Cdh1 peak but not in the low-percentage glycerol Cdh1 peak (Fig. 2B). These results suggest that, within neurons, endogenous Cdh1 is indeed found in two forms, a major portion that is complexed with the APC and another portion that is not complexed with APC. In other experiments, we found that exposure of granule neurons to the Cdk inhibitor roscovitine significantly increased the proportion of Cdh1 cofractionating with Cdc27 in neurons and decreased the portion of “free” Cdh1 not in complex with the APC (Fig. 2C,D). Together, these results support the conclusion that phosphorylation of Cdh1 at Cdk sites disrupts the association of Cdh1 with the APC in neurons.

We next assessed the effect of the Cdh1 phosphorylation on the subcellular localization of Cdh1 in neurons. We expressed the wild-type and phosphorylation mutant GFP–Cdh1 proteins together with  $\beta$ -galactosidase in granule neurons. We subjected transfected neurons to immunocytochemistry with GFP and  $\beta$ -galactosidase antibodies, using the latter to visualize transfected granule neurons. We also used the DNA dye bisbenzamide (Hoechst 33258) to visualize the nucleus in neurons. In agreement with our previous findings (Konishi et al., 2004; Stegmüller et al., 2006), wild-type Cdh1 was found predominantly in the nucleus (Fig. 3A). In  $\sim$ 80% of granule neurons, Cdh1 was found exclusively in the nucleus, whereas in the remaining neurons, Cdh1 was present in both the nucleus and cytoplasm (Fig. 3B). The 9A mutation had little effect

on the subcellular localization of Cdh1 (Fig. 3A,B). In contrast, the 9D Cdh1 mutant was strikingly localized in both the cytoplasm and nucleus rather than exclusively in the nucleus (Fig. 3A,B). In other experiments, we assessed the effect of expression of the neuron-specific kinase Cdk5 together with its activating partner p25 on the subcellular localization of Cdh1 in neurons (Fig. 3C). Although wild-type Cdh1 was predominantly found exclusively in the nucleus in control neurons, Cdh1 was found in both the nucleus and cytoplasm in the majority of neurons in which p25/Cdk5 was expressed. Notably, p25/Cdk5 expression had little or no effect on the subcellular localization of the 9A and 9D Cdh1 mutant proteins in neurons. Together, these results suggest that phosphorylation of Cdh1 at Cdk sites triggers the dissociation of Cdh1 from the APC, leading to the accumulation of Cdh1 in the cytoplasm in neurons.

Having characterized the effect of Cdh1 phosphorylation at

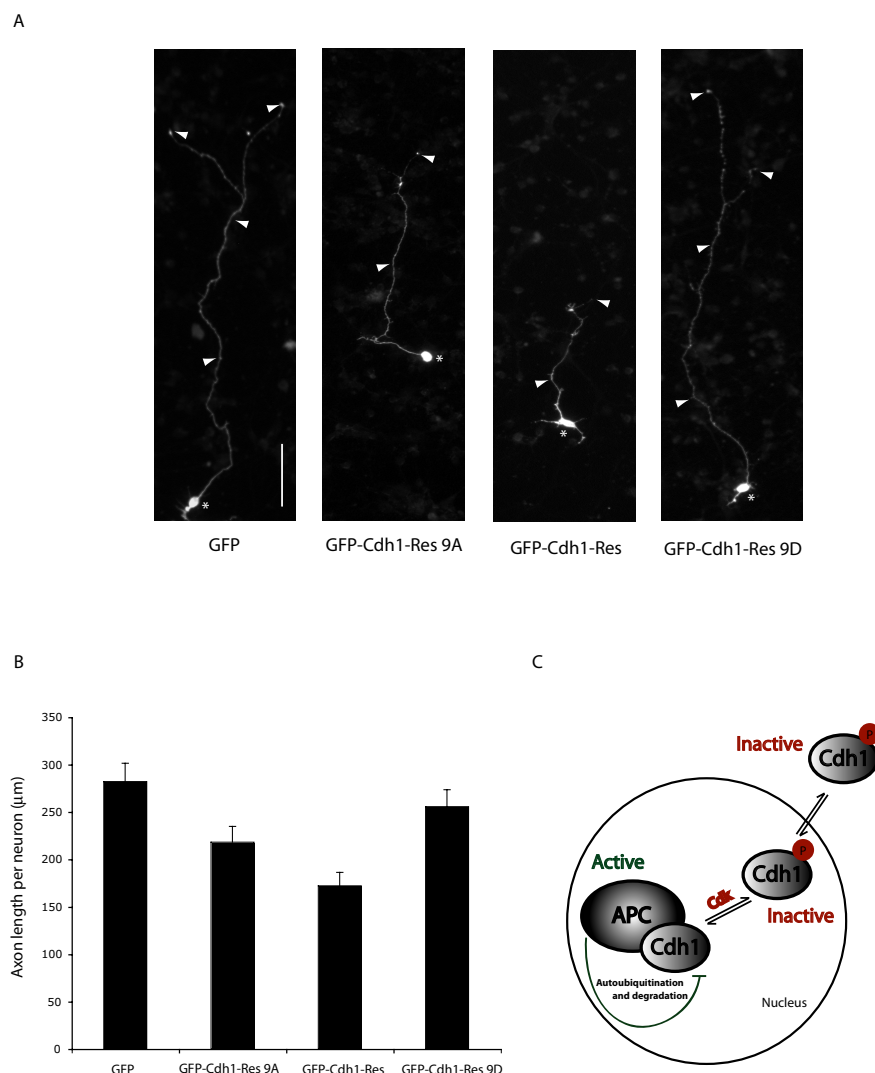


Cdk sites on the activity of Cdh1 and subcellular localization, we next determined the effect of Cdh1 phosphorylation on its function in the control of axon growth in primary rat cerebellar granule neurons. Although the overexpression of Cdh1 on its own does not lead to a gain of function in neurons, the expression of an RNAi-resistant form of Cdh1 (Cdh1–Res) inhibits axon growth in the background of Cdh1 knockdown (Stegmüller et al., 2006). We therefore performed structure–function analyses of Cdh1–Res in this background and tested the effect of the 9A and 9D phosphorylation mutations in Cdh1 on its ability to inhibit axon growth. We found that both Cdh1–Res and Cdh1–Res 9A significantly inhibited axon growth in the background of Cdh1 RNAi (Fig. 4). Although the 9A mutant protein might have been anticipated to be more effective than wild-type Cdh1–Res at limiting axon growth because of the enhanced association of the 9A mutant with the APC, this was not observed (Fig. 4). We attribute the inability of the 9A mutant protein to further inhibit axon growth to its reduced stability, countering the positive effect of the 9A mutation on the interaction of Cdh1 with the APC, with a net result that the 9A mutant is similar or even somewhat less effective than wild-type Cdh1 in limiting axon growth. In contrast to Cdh1–Res and Cdh1–Res 9A, we found that the 9D phosphomimic Cdh1 mutant failed to inhibit axon growth (Fig. 4). Together, our results suggest that phosphorylation of Cdh1 at Cdk sites suppresses the ability of Cdh1–APC to limit axon growth in primary neurons.

## Discussion

In this study, we have characterized a Cdh1 phosphorylation-dependent mechanism that regulates Cdh1–APC function in the control of axon growth in mammalian neurons (see model in Fig. 4C). We found that phosphorylation of Cdh1 at Cdk sites stabilizes an inactive form of Cdh1 that fails to associate with the APC. Accordingly, phosphorylation of Cdh1 at Cdk sites promotes the accumulation of Cdh1 in the cytoplasm in neurons and suppresses the ability of Cdh1–APC to limit the growth of axons. These findings suggest that phosphorylation of Cdh1 may represent a critical mode of Cdh1–APC regulation in the mammalian brain.

Our findings have several important ramifications. The finding that phosphorylation of Cdh1 at Cdk sites stabilizes Cdh1 proteins suggests that phosphorylation inhibits the degradation of Cdh1 by the ubiquitin–proteasome pathway. Intriguingly, Cdh1 is reported to undergo autoubiquitination and consequent degradation, and remarkably the nine conserved sites of Cdk-induced phosphorylation reside within a 180 amino acid region of the N-terminal domain of Cdh1 that is required for Cdh1 autoubiquitination (Listovsky et al., 2004). Together, these ob-



**Figure 4.** Phosphorylation of Cdh1 inhibits its ability to limit axon growth. **A**, Granule neurons transfected with an expression plasmid encoding GFP–Cdh1–Res, GFP–Cdh1–Res 9A, GFP–Cdh1–Res 9D, or the control vector, together with the U6/Cdh1 RNAi plasmid and DsRed expression plasmid were subjected to immunocytochemistry using a DsRed antibody. Images of transfected neurons with asterisks indicating cell bodies and arrowheads indicating axons. Scale bar, 50  $\mu$ m. **B**, Axon length was significantly shorter in GFP–Cdh1–Res 9A- and GFP–Cdh1–Res-expressing neurons compared with GFP-expressing neurons in the background of U6/Cdh1 (ANOVA with Fisher’s PLSD *post hoc* test,  $p < 0.01$ ). The 9D Cdh1 mutant failed to inhibit axon growth in the background of Cdh1 RNAi. A total of 584 neurons were measured. **C**, A schematic of regulation of Cdh1–APC function by Cdk-induced Cdh1 phosphorylation in neurons. Active and inactive Cdh1 refer to its function in limiting axon growth.

servations support the conclusion that phosphorylation of Cdh1 at Cdk sites may inhibit the ability of Cdh1–APC to trigger Cdh1 autoubiquitination, leading to its stabilization.

The paradoxical ability of Cdk inhibition to promote Cdh1 association with the APC even as it promotes Cdh1 self-degradation underscores the importance of gaining additional insights into the ability of Cdk to regulate Cdh1–APC activity. In future studies, it will be important to identify the Cdk that phosphorylates Cdh1 in neurons. Recent evidence suggests that Cdk5 may phosphorylate Cdh1 in cortical neurons (Maestre et al., 2008). In agreement with this finding, we also found that expression of p25/Cdk5 alters the subcellular localization in neurons. Intriguingly, Cdk5 has been implicated in neurite outgrowth (Nikolic et al., 1996; Hahn et al., 2005), making it a promising candidate for the regulation of Cdh1–APC activity in the control of axon growth. Beyond the potential role of Cdh1 phosphorylation in the developmental regulation of axon growth, the ability of

Cdh1 phosphorylation to inhibit Cdh1–APC activity may allow harnessing of the therapeutic potential of this pathway in axonal regeneration after injury.

The abundant number of Cdk sites within Cdh1 raises the interesting possibility that processive phosphorylation of Cdh1 might regulate Cdh1 localization and stability. Although the 9D mutation altered significantly the subcellular localization of Cdh1 from exclusively nuclear to both nuclear and cytoplasmic, the 9D phosphomimic did not display an entirely cytoplasmic localization in neurons. These results suggest that phosphorylation of Cdh1 at Cdk sites may inhibit Cdh1–APC activity primarily by disrupting the association of Cdh1 with the APC. It will be interesting to determine whether a subset of Cdk phosphorylation sites specifically alters Cdh1 localization, stability, or activity or whether these phenomena are an inseparable part of the auto-regulation of Cdh1–APC activity.

Our findings suggest that, just as in dividing cells, in which multiple mechanisms regulate Cdh1 activity, Cdh1 might be regulated by several mechanisms in postmitotic neurons. We have identified TGF $\beta$ –Smad2 signaling and Cdh1 phosphorylation as two opposing modes of regulation, the former activating and the latter inhibiting Cdh1–APC function (Stegmüller et al., 2008 and this study). It will be important to determine in future studies whether and how these mechanisms might be coordinated in neurons. As parallels are established for the regulation of Cdh1 in dividing cells and postmitotic neurons, the intricate regulation of Cdh1–APC activity in proliferating cells may prove equally exquisite in the mammalian nervous system.

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