Published in final edited form as:

Curr Opin Neurobiol. 2010 February ; 20(1): 92–99. doi:10.1016/j.conb.2009.12.004.

The Dynamic Ubiquitin Ligase Duo: Cdh1-APC and Cdc20-APC Regulate Neuronal Morphogenesis and Connectivity

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Abstract

The proper development and patterning of axons, dendrites, and synapses is essential for the establishment of accurate neuronal circuits in the brain. A major goal in neurobiology is to identify the mechanisms and principles that govern these fundamental developmental events of neuronal circuit formation. In recent years, exciting new studies have suggested that ubiquitin signaling pathways may play crucial roles in the control of neuronal connectivity. Among E3 ubiquitin ligases, Cdh1-anaphase promoting complex (Cdh1-APC) and Cdc20-APC have emerged as key regulators of diverse aspects of neuronal connectivity, from axon and dendrite morphogenesis to synapse differentiation and remodeling.

Introduction

The anaphase promoting complex (APC) is a large protein complex that is composed of at least 12 subunits [1, 2]. Among the subunits of the APC, APC2 represents a Cull1-related scaffold protein, and the RING finger protein APC11 encodes the catalytic E3 core subunit [1, 2]. The ubiquitin ligase activity of the APC is stimulated by interaction with one of two key co-activator subunits, Cdh1 or Cdc20, which also targets the APC to distinct substrates [1, 2] (Figure 1). Substrates of Cdc20-APC or Cdh1-APC contain a peptide sequence termed the destruction-box (D-box), which serves as the recognition motif for Cdc20 or Cdh1 [3]. Additional Cdh1 peptide recognition motifs, including the KEN box, A-box, and CRY box, have been identified within substrates of Cdh1-APC [4-6]. Although advances have been made in understanding the structure of the APC using electron microscopy studies [7-10], the precise molecular basis of APC-induced ubiquitination of substrates and the function of the numerous subunits in the complex remains a mystery.

The characterization of the APC in proliferating cells has provided invaluable clues for studies of the APC in postmitotic neurons. A major concept that has emerged from studies of the APC in proliferating cells is that Cdc20-APC and Cdh1-APC control distinct temporal phases of the cell cycle [1]. Cdh1-APC operates during mitotic exit and G1 phase of the cell cycle, while Cdc20-APC drives anaphase in early mitosis. Cdh1 and Cdc20 are dynamically

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controlled during distinct phases of the cell cycle by several modes of posttranslational modifications, including phosphorylation, ubiquitination, and interactions with APC inhibitors. An additional layer of regulation is provided by transcription of Cdc20 in proliferating cells. The functions and regulation of the APC during the cell cycle in dividing cells have been reviewed [1, 2, 11-14]. In this review, we will focus on studies implicating the two distinct APC ubiquitin ligase subtypes, Cdh1-APC and Cdc20-APC, in neuronal patterning and connectivity.

The APC orchestrates axon and dendrite morphogenesis

Nearly a decade after the APC was identified in cycling cells [15, 16], its function in postmitotic neurons first came into view in studies of neuronal morphogenesis [17••]. Earlier evidence had revealed that Cdh1 and the APC core subunits are expressed in mammalian brain neurons [18]. Later, Cdc20 was also found to be expressed in neurons in the developing brain [19••]. Functional analyses of Cdh1-APC and Cdc20-APC have uncovered critical roles for these two distinct APC complexes in the regulation of axon and dendrite morphogenesis, respectively [17••, 19••]. These studies suggest the key concept that the temporally distinct activities of Cdh1-APC and Cdc20-APC during the cell cycle appear to have been transposed to distinct subcellular compartments in postmitotic neurons to coordinate the growth and patterning of axons and dendrites.

A nuclear Cdh1-APC ubiquitin signaling pathway regulates axon growth and patterning

A series of investigations have led to the identification of a Cdh1-APC ubiquitin signaling pathway that restricts the growth of axons and controls their patterning in the mammalian brain [17••, 20, 21, 22•, 23-25, 26•]. Using granule neurons of the rat cerebellar cortex as a model system for studies of neuronal morphogenesis [27-30], Konishi et al. discovered that knockdown of Cdh1 in neurons specifically stimulates the growth of axons but not dendrites [17••] (Figure 2). Granule neurons expressing the APC inhibitor Emi1 or a dominant interfering form of APC11 display longer axons than control neurons. These results suggest that the ubiquitin ligase activity of Cdh1-APC inhibits axon growth.

Knockdown experiments in cerebellar slices and in postnatal rat pups *in vivo* revealed that beyond the control of axon growth, Cdh1-APC controls the layer-specific pattern of axon morphogenesis in the cerebellar cortex [17••]. Loss of Cdh1 also endows granule neurons with the ability to overcome the axon growth-inhibitory signals imposed by myelin, raising the prospect that inhibition of Cdh1-APC may represent a novel therapeutic strategy to stimulate axon regeneration following injury and disease.

Recent studies have explored the two major questions of how neuronal Cdh1-APC is regulated in neurons and the mechanisms by which Cdh1-APC controls axon morphogenesis [22•, 23-25, 26•]. An important clue relevant to these studies is the finding that neuronal Cdh1-APC remarkably operates in the nucleus to control the growth of axons [22•]. Regulation of the subcellular localization of Cdh1 by phosphorylation appears to bear important consequences on Cdh1-APC control of axon growth [23]. These studies suggest that Cdk-induced phosphorylation of Cdh1 at nine conserved sites stabilizes an inactive form of Cdh1 that accumulates in the cytoplasm, which fails to inhibit axon growth [23].

Extrinsic cues that may regulate Cdh1-APC activity in neurons are just beginning to be characterized (Figure 2). TGF β enhances Cdh1-APC activity in proliferating cells by inducing the translocation of the signaling protein Smad2 or Smad3 to the nucleus, where Smad2/3 binds to Cdh1 and stimulates Cdh1-APC activity [31, 32]. In agreement with this model, inhibition of TGF β /Smad2 signaling stimulates axon growth in granule neurons [24].

The nuclear site of action of Cdh1-APC in neurons has also provided important insight in the study of the mechanism by which Cdh1-APC restrains axon growth [22•]. The transcription modulator SnoN has been identified as a substrate of neuronal Cdh1-APC in the control of axon growth (Figure 2). Knockdown of SnoN in granule neurons inhibits axon growth in primary granule neurons and profoundly impairs the development of parallel fiber axons *in vivo*. Importantly, SnoN knockdown suppresses the ability of Cdh1 knockdown to stimulate axon growth [22•]. These results define Cdh1-APC and SnoN as components of a nuclear ubiquitin signaling pathway that orchestrates axon growth in the mammalian brain.

SnoN is widely believed to repress transcription [33, 34]. Surprisingly, the landscape of gene expression changes triggered by SnoN knockdown in gene profiling experiments suggests an important transcriptional activating role for SnoN in neurons [25]. Consistent with this conclusion, the transcriptional coactivator p300 interacts with SnoN, and p300 is required for the axon growth-promoting function of SnoN in neurons [25]. These results support the concept that SnoN may activate or repress transcription in a cell- and gene-specific manner [34-36]. Among SnoN targets in neurons, the gene encoding the signaling protein Ccd1 represents a physiologically relevant target gene in the control of axon growth. Ccd1 is enriched in axon growth terminals and appears to be necessary for SnoN-dependent axon growth [25]. Thus, Ccd1 links the nuclear Cdh1-APC/SnoN ubiquitin pathway to the local control of axon growth.

In addition to SnoN, the transcriptional regulatory protein inhibitor of DNA binding 2 (Id2) has been identified as a critical substrate of Cdh1-APC in the regulation of axon growth [26•]. Like SnoN, Id2 contains a conserved D-box. Cdh1-APC stimulates the ubiquitination of Id2 in a D-box-dependent manner, leading to Id2 degradation in neurons. Expression of an Id2 mutant protein in which the D-box is mutated (Id2-DBM) promotes axon growth in granule neurons. Id2 regulates transcription by inhibiting the function of basic helix-loop-helix (bHLH) transcription factors [37]. Expression of the bHLH transcription factor E47 suppresses axon growth and is epistatic to both Cdh1 knockdown and Id2-DBM expression in axon growth assays [26•]. Microarray analyses reveal that the transcriptional targets of E47 include ligands and receptors important for the control of axon guidance and growth including Sema3F, Unc5a, and Nogo receptor (NgR) [26•]. Consistent with these results, expression of Id2-DBM suppresses the ability of myelin to inhibit axon growth. Together, these results define a nuclear Cdh1-APC/Id2 ubiquitin pathway in neurons that regulates axon growth. Whether the Cdh1-APC/SnoN and Cdh1-APC/Id2 signaling pathways act in parallel or converge to regulate axon growth remains to be determined.

A centrosomal Cdc20-APC pathway drives dendrite growth and elaboration

The identification of Cdh1-APC function in the regulation of axon growth and patterning led to the intriguing question of whether the APC co-activator Cdc20 might also play a role in neuronal morphogenesis. Kim et al. found that Cdc20 is expressed in postmitotic neurons in the developing brain, with protein levels increasing with neuronal maturation [19••]. Remarkably, the authors discovered that in contrast to knockdown of Cdh1, knockdown of Cdc20 in granule neurons profoundly impairs dendrite growth and arborization in primary granule neurons and in the cerebellar cortex *in vivo* but has little or no effect on axon growth.

The subcellular compartment in which Cdc20 operates to control dendrite growth is distinct from the nuclear locus of Cdh1's axon regulatory function [19••]. Immunocytochemical and biochemical analyses suggest that a substantial pool of Cdc20 resides at the centrosome in granule neurons. Strikingly, structure-function analyses suggest that the centrosomal localization of Cdc20 is critical for Cdc20-dependent dendrite growth and elaboration.

These findings suggest that Cdc20-APC operates at the centrosome to drive dendrite growth and elaboration.

A mechanism of Cdc20-APC regulation by the centrosomally-localized histone deacetylase HDAC6 has been reported in neurons (Figure 2). HDAC6 interacts directly with Cdc20 and promotes dendrite growth in a Cdc20-dependent manner [19••]. Surprisingly, HDAC6 regulates Cdc20-APC independently of its deacetylase activity. Rather, HDAC6, through its ubiquitin binding domain ZnF UBP, promotes the polyubiquitination of Cdc20 and thereby stimulates the ubiquitin ligase activity of Cdc20-APC activity. The polyubiquitination of Cdc20 activates Cdc20-APC activity during mitosis [38, 39]. Knockdown of the major Cdc20-specific deubiquitinase USP44 in neurons augments the ubiquitination status of Cdc20 and substantially increases the length and complexity of dendrite arbors, suggesting that the dynamic modulation of Cdc20 polyubiquitination *per se* is critical for dendrite morphogenesis.

Since the ubiquitin ligase activity of Cdc20-APC is critical for its function, Cdc20-APC is anticipated to promote dendrite growth by inducing the ubiquitination and consequent degradation of a centrosomal protein that inhibits dendrite development. Using this rationale, the centrosomally localized protein Id1 has been identified as a substrate of neuronal Cdc20-APC [19••]. Knockdown of Id1 stimulates dendrite growth, and epistasis experiments suggest that Id1 operates downstream of Cdc20 [19••]. It will be important in future studies to explore the mechanism by which Id1 inhibits the growth and elaboration of dendrites. While Id1 is localized at the centrosome, Id1 is also established to inhibit bHLH-dependent transcription in the nucleus [37]. Therefore, it will be interesting to determine if Id1 controls dendrite morphogenesis by regulating transcription or by a local mechanism at the centrosome.

The APC regulates synaptic connectivity

Although the functions of the APC in the regulation of axon and dendrite morphogenesis are expected to profoundly impact neuronal circuitry, APC functions have also been more directly linked to the control of synapse development and hence neuronal connectivity. The picture that is coming into view from these studies is that the APC plays a critical role in distinct aspects of synapse formation, though we are still in the early days of our understanding of the role and mechanisms of APC regulation of synapse differentiation and remodeling in the brain.

Cdh1-APC controls synaptic differentiation and transmission in the invertebrate nervous system

The critical role of Cdh1-APC in synapse differentiation was first recognized in invertebrate model systems [40••, 41••] (Figure 3). Core components of the APC in *Drosophila* are expressed in motor neurons and muscle cells that communicate at the neuromuscular junction (NMJ). Loss-of-function APC2 mutant flies exhibit an increase in the number of presynaptic boutons at the NMJ [40••]. Specific knockdown of APC2 in motor neurons, but not in muscle cells, recapitulated the increase in bouton density. Consideration of the potential targets of the APC in motor neurons led the investigators to examine the protein Liprin α , which harbors a D-box motif and augments synapse size in *Drosophila* [42]. Motor neurons in APC2 mutant flies have increased Liprin α expression compared to neurons in wild type controls [40••]. Importantly, in the background of Liprin α loss-of-function mutant flies, loss of APC2 fails to increase presynaptic bouton number, suggesting that Liprin α is genetically downstream of APC2 in the regulation of synapse size. The authors surmised that Cdh1-APC is the form of the APC that restrains presynaptic bouton number, because immunohistochemical analyses suggest that Cdh1 and APC2 are expressed at presynaptic

sites at the NMJ [40••]. In view of the recent discovery that Cdc20-APC regulates presynaptic differentiation in mammalian brain neurons (see below), it will be interesting to also explore the role of Cdc20-APC in presynaptic differentiation at the fly NMJ.

Evidence for a postsynaptic function of the APC in invertebrate nervous systems has also been identified. In *C. elegans*, loss-of-function mutants of several core APC subunits exhibit an increase in the abundance of the AMPA receptor subunit GLR-1 in ventral nerve cord neurons [41••]. Expression of APC subunits driven by the *glr-1* promoter restored the normal width of GLR-1 synaptic puncta in APC mutant worms, suggesting that Cdh1-APC controls AMPA receptor levels in a cell-autonomous manner [41••]. However, GLR-1 lacks an obvious APC recognition motif, and GLR-1 ubiquitination appeared to be unchanged in APC subunit mutants compared to that in wild type worms. A postsynaptic locus of APC action may also regulate the fly NMJ [40••]. Just as in nematodes, however, the mechanism by which the APC regulates postsynaptic glutamate receptors at the fly NMJ remains elusive.

A Cdc20-APC ubiquitin signaling pathway orchestrates presynaptic differentiation in the brain

While Cdh1-APC has been implicated in synapse development in invertebrate systems, until recently the role of the APC in synapse differentiation in mammalian brain neurons remained to be identified. Yang et al. recently established assays that facilitate the study of presynaptic differentiation in the robust model system of cerebellar granule neurons [43••] (Figure 4). Using this system, knockdown of Cdh1 surprisingly does not appear to alter the number of presynaptic sites. Remarkably, the authors discovered that Cdc20-APC drives presynaptic differentiation in granule neurons [43••]. Knockdown of Cdc20 reduces the density of the synaptic vesicle and active zone proteins in granule neuron axons. Further, Cdc20 knockdown reduces the density of synapsin co-clusters with the postsynaptic protein PSD-95 and with sites of uptake of the dye FM4-64, suggesting that Cdc20-APC promotes the formation of functional synapses. Cdc20 knockdown also impairs the differentiation of presynaptic sites in the cerebellar cortex in postnatal rat pups *in vivo*.

The brain-enriched transcription factor NeuroD2, which contains a D-box motif, has been uncovered as a physiologically relevant substrate of Cdc20-APC in the control of presynaptic differentiation [43••] (Figure 4). Cdc20 interacts with NeuroD2 in a D-box-dependent manner and NeuroD2 is regulated by the ubiquitin-proteasome system in neurons. Knockdown of Cdc20 increases the levels of endogenous NeuroD2 protein in primary granule neurons. Depletion of NeuroD2 increases the density of presynaptic sites in primary granule neurons and in the cerebellar cortex *in vivo* in rat postnatal pups. Epistasis analyses show that NeuroD2 lies downstream of Cdc20 in the control of presynaptic number. Together, these results suggest that the Cdc20-APC-induced degradation of NeuroD2 drives presynaptic differentiation.

A NeuroD2 target gene has been identified that mediates the NeuroD2-dependent suppression of presynaptic differentiation [43••]. Among NeuroD2 targets, Complexin II (Cplx2) regulates synaptic vesicle membrane fusion and has been implicated in the control of presynaptic number in *Drosophila* [44, 45]. Expression of exogenous Cplx2 in granule neurons inhibits the increase in presynaptic site density elicited by NeuroD2 knockdown [43••]. In addition, Cplx2 knockdown increases the number of functional presynaptic sites both in primary granule neurons and in the cerebellar cortex *in vivo* in rat postnatal rat pups. Knockdown of Cplx2 also reverses the Cdc20 knockdown-triggered decrease in presynaptic density, suggesting that Cplx2 lies downstream of Cdc20. Collectively, these findings define a novel Cdc20-APC ubiquitin signaling pathway that orchestrates presynaptic differentiation in the mammalian brain.

Perspectives

With the rapid progress in our understanding of APC function in neuronal development, common themes have emerged. The recruitment of the two APC coactivators, Cdh1 and Cdc20, in different subcellular compartments allows for the specific targeting and degradation of APC substrates in distinct aspects of neuronal morphogenesis. Whereas Cdh1-APC controls axon growth from the nucleus [17•, 22•, 26•], Cdc20-APC operates at the centrosome to drive dendrite growth and arborization [19•]. In a similar vein, the two APC subtypes may regulate distinct temporal phases of neuronal maturation. While Cdh1-APC controls the first phases of axon development [17•, 22•, 26•], i.e. axon growth and patterning, Cdc20-APC coordinates the later phases of axon morphogenesis intimately linked to neuronal connectivity, i.e. presynaptic differentiation [43•].

An important question for future studies is the function of Cdh1-APC in postsynaptic differentiation in mammalian brain neurons. Inhibition of Cdh1-APC in rat hippocampal neurons has been reported to increase the levels of Liprin α in dendrites [46]. However, whether Cdh1-APC inhibition leads to alterations of dendritic spines or synapses in these neurons remains unexplored. In other studies, abnormalities in learning and memory and late phase of long-term potentiation (L-LTP) at the CA1 synapse in hippocampal slices have been observed in mice heterozygous for the Cdh1 gene [47]. However, whether and how impairment of synapse differentiation contributes to these phenotypes remains to be investigated. Together, these studies raise the interesting possibility that Cdh1-APC might control postsynaptic differentiation in the mammalian brain, though the nature of this function and its underlying mechanism are unknown.

Beyond the control of neuronal morphogenesis and connectivity, additional roles of the APC have been characterized in other aspects of neuronal development, from the control of neural stem cell proliferation to neuronal cell death to metabolic control of glycolysis in neurons [48-54]. These studies suggest that the APC may have pleiotropic roles in the brain, from development to maturity.

In each of the defined functions of Cdh1-APC and Cdc20-APC in neuronal connectivity, from axon and dendrite morphogenesis to presynaptic differentiation, several important questions remain to be addressed. It will be important to determine the regulatory mechanisms that control these pathways and to identify extrinsic cues that might interact with the cell-intrinsic functions of the APC in neuronal morphogenesis and connectivity. With advances in our understanding of the local cytoskeletal mechanisms that control neuronal morphology and development, it will be interesting to understand how the APC and its targets interface with regulators of cytoskeletal dynamics [55-59]. Identification of additional substrates of neuronal Cdh1-APC and Cdc20-APC should shed new light on the mechanisms of defined APC function as well as lead to uncovering novel functions of the APC in neurons. Finally, a major line of future research should elucidate the functions of the APC in the development and function of the brain using conditional knockout strategies. Such studies should unravel the range of behaviors that are controlled by the APC. This approach will also facilitate the determination of the role of the APC in the pathogenesis of neurological and psychiatric diseases. In view of the profound effects of the APC on neuronal connectivity, it is conceivable that manipulation of APC components and associated signaling pathways might have therapeutic implications for diverse neurological and psychiatric disorders, from developmental cognitive disorders of mental retardation and autism spectrum disorders to neurodegenerative diseases.

Acknowledgments

We thank members of the Bonni laboratory for helpful discussions. Supported by NIH grants NS051255 and NS041021 (A.B.); a NSF fellowship, the Lefler fellowship, and the Ryan foundation (Y.Y.); a Ruth L. Kirschstein National Research Service Award fellowship, National Cancer Institute, and Brain Science Foundation grant (A.H.K.).

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Figure 1. The structure of the APC

The APC is composed of at least 12 core subunits including the Cul1-related scaffold protein APC2 and the RING finger protein APC11 shown in red. The APC associates with one of two co-activators, Cdh1 or Cdc20 (shown in yellow), which confer substrate specificity and stimulate the ubiquitin ligase activity of the APC. The tetratricopeptide repeat (TPR) containing core subunits Cdc27, Cdc23, and Cdc16 (shown in blue) act as a scaffold to promote the interaction of Cdh1 and Cdc20 with the APC.

Figure 2. Cdh1-APC and Cdc20-APC govern the spatially distinct processes of axon and dendrite morphogenesis

Cdh1-APC operates in the nucleus to regulate axon growth and patterning. Cdh1-APC restricts axon growth by targeting the transcriptional regulators SnoN and Id2 for degradation. SnoN and Id2 regulate the transcription of target genes enriched at the axon growth cone including Ccd1 and NgR. The TGFβ/Smad2 and Cdk signaling pathways converge upon Cdh1-APC to regulate axon morphogenesis. Upon activation by TGFβ, Smad2 interacts with Cdh1 and induces Cdh1-APC-dependent ubiquitination of SnoN. Conversely, Cdk inhibits Cdh1-APC activity by phosphorylating Cdh1 at nine conserved sites, leading to the accumulation of an inactive form of Cdh1 in the cytoplasm. Cdc20-APC operates at the centrosome to drive dendrite growth and elaboration. Polyubiquitination of Cdc20 enhances Cdc20-APC activity and dendrite growth. HDAC6 promotes the polyubiquitination of Cdc20, while USP44 deubiquitinates Cdc20. The centrosomally-localized protein Id1 represents a substrate of neuronal Cdc20-APC that inhibits dendrite growth.

Figure 3. Cdh1-APC regulates synapse size and glutamatergic neurotransmission in invertebrates

Cdh1 and core subunits of the APC are localized at the presynaptic bouton at the *Drosophila* NMJ. Presynaptic APC controls the abundance of the active zone protein Liprin α and restricts the number of presynaptic boutons contacting the postsynaptic muscle cell. On the postsynaptic side, Cdh1-APC limits the expression of glutamate receptor subunits in worms and flies. The mechanism of APC regulation of glutamate receptors awaits further study.

Figure 4. Cdc20-APC drives presynaptic differentiation in mammalian brain neurons

Top: during early axon differentiation, NeuroD2-dependent transcription of the target gene Cplx2 suppresses presynaptic differentiation. *Bottom:* As Cdc20 expression increases with neuronal maturation, Cdc20-APC targets NeuroD2 protein for degradation and thereby promotes the formation of functional presynaptic sites. Figure adapted from [43••].