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LETTERS

Degradation of Id2 by the anaphase-promoting complex couples cell cycle exit and axonal growth

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In the developing nervous system, Id2 (inhibitor of DNA binding 2, also known as inhibitor of differentiation 2) enhances cell proliferation, promotes tumour progression and inhibits the activity of neurogenic basic helix–loop–helix (bHLH) transcription factors[1,2]. The anaphase promoting complex/cyclosome and its activator Cdh1 (APC/C^{Cdh1}) restrains axonal growth but the targets of APC/C^{Cdh1} in neurons are unknown[3–5]. Id2 and other members of the Id family are very unstable proteins that are eliminated as cells enter the quiescent state, but how they are targeted for degradation has remained elusive[6,7]. Here we show that Id2 interacts with the core subunits of APC/C and Cdh1 in primary neurons. APC/C^{Cdh1} targets Id2 for degradation through a destruction box motif (D box) that is conserved in Id1 and Id4. Depletion of Cdh1 stabilizes Id proteins in neurons, whereas Id2 D-box mutants are impaired for Cdh1 binding and remain stable in cells that exit from the cell cycle and contain active APC/C^{Cdh1}. Mutants of the Id2 D box enhance axonal growth in cerebellar granule neurons *in vitro* and in the context of the cerebellar cortex, and overcome the myelin inhibitory signals for growth. Conversely, activation of bHLH transcription factors induces a cluster of genes with potent axonal inhibitory functions including the gene coding for the Nogo receptor, a key transducer of myelin inhibition. Degradation of Id2 in neurons permits the accumulation of the Nogo receptor, thereby linking APC/C^{Cdh1} activity with bHLH target genes for the inhibition of axonal growth. These findings indicate that deregulated Id activity might be useful to reprogramme quiescent neurons into the axonal growth mode.

To identify protein complexes of Id2 in human neuroblastoma cells, we used immunoaffinity chromatography followed by tandem mass spectrometry. New Id2 partners are the APC/C subunits Apc1, Apc5 and Apc8/Cdc23 (Supplementary Fig. 1a). The identification *in vivo* of complexes of Id2 and subunits of APC/C indicates that Id2 might be targeted for degradation by APC/C. The enzymatic E3 ubiquitin ligase activity of APC/C requires either the Cdc20 or Cdh1 co-activator[4]. Expression of Cdh1 but not that of Cdc20 led to a marked decrease in Id2 level; this effect was prevented by proteasomal inhibition (Fig. 1a). Moreover, Id2 was eliminated at a faster rate in the presence of Cdh1 (Fig. 1b). Expression of Cdh1 decreased endogenous Id2 in asynchronously growing U2OS cells without changing the cell cycle distribution, and also in cells synchronized in the S phase of the cell cycle with aphidicolin (Fig. 1c, d, and Supplementary Fig. 1b, c). Activation of APC/C^{Cdh1} also eliminated E47-bound Id2 and destabilized Id2 carrying a deletion of the amino-terminal 15 amino-acid residues, which have been shown to contribute to Id2 ubiquitination[7] (Supplementary Fig. 1d, e). Id2 associated with Cdh1 in the absence of proteasomal inhibition, but the interaction was more efficient in cells treated with MG-132 (Supplementary Fig. 1f, g). To examine whether Id2 becomes unstable in cells undergoing quiescence we monitored Id2 expression in response to serum deprivation. Id2 was downregulated and underwent accelerated degradation in NIH 3T3 fibroblasts deprived of serum mitogens (Supplementary Fig. 2a, b). We observed similar results in serum-starved U2OS-Id2 and SK-N-SH neuroblastoma cells undergoing arrest in G0/G1 after treatment with retinoic acid (Supplementary Fig. 3a–c)[8]. To examine whether APC/C^{Cdh1} regulates Id2 stability *in vivo* we studied the levels of Id2 after a decrease in APC/C activity through the depletion of Cdh1. Knockdown of Cdh1 by RNA interference led to elevation of the steady-state state levels of

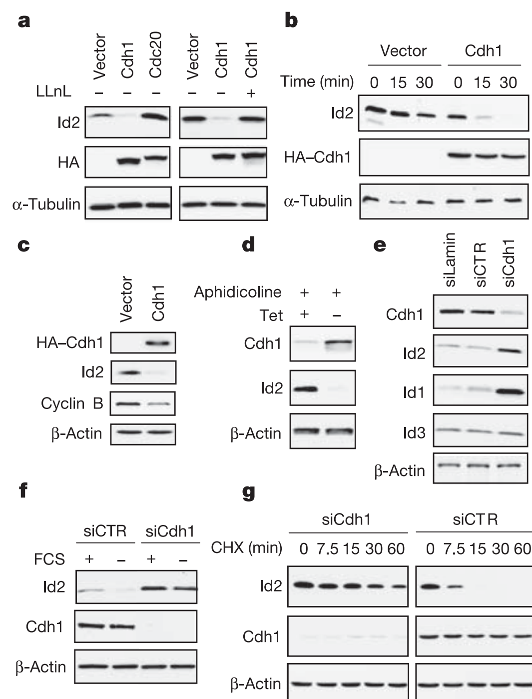

Figure 1 | Id2 is a substrate of APC/C^{Cdh1}. **a**, HeLa cells were transfected with Id2, haemagglutinin (HA)-conjugated Cdh1 or HA–Cdc20 and treated with N-Acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL). **b**, Transfected HeLa cells were treated with cycloheximide. **c**, Endogenous Id2 in U2OS cells transfected with HA–Cdh1. **d**, U2OS-tet-Cdh1 cells were treated with aphidicoline before the removal of tetracycline. **e**, **f**, LAN-1 neuroblastoma cells (**e**) and U2OS-Id2 cells (**f**) transfected with siRNA oligonucleotides. **g**, LAN-1 cells were transfected with siRNA; quiescence was induced by retinoic acid and cells were treated with cycloheximide (CHX).

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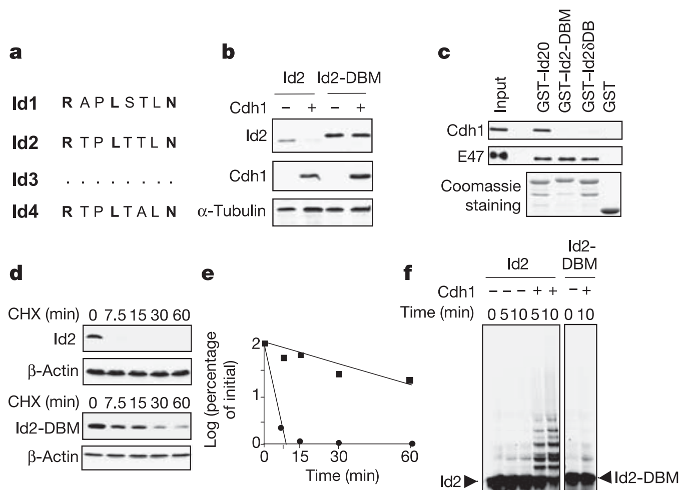

Figure 2 | D-box-dependent degradation of Id proteins by APC/C^{Cdh1}. **a**, Alignment of D boxes in Id proteins. **b**, HeLa cells were transfected with Id2 or Id2-DBM and HA–Cdh1 and analysed by western blotting. **c**, Pull-down assay with GST fusion proteins and Cdh1 and E47 translated *in vitro*. **d**, HeLa cells were transfected with Id2 or Id2-DBM and treated with cycloheximide (CHX). **e**, Quantification of Id2 (circles) and Id2-DBM (squares) from **d**. **f**, *In vitro* ubiquitination assay of Id2 and Id2-DBM by immunopurified APC/C in the presence or absence of Cdh1 translated *in vitro*. Arrowheads, unmodified Id2/Id2-DBM.

endogenous Id2 and Id1 in LAN1 neuroblastoma cells and exogenous Id2 in U2OS cells (Fig. 1e, and Supplementary Fig. 3d). It also prevented the downregulation of Id2 in quiescent U2OS cells and led to a tenfold increase in the Id2 half-life in LAN1 cells undergoing cell cycle arrest after treatment with retinoic acid (Fig. 1f, g, and Supplementary Fig. 3e, f). However, Id3 was unchanged in Cdh1-depleted cells (Fig. 1e). In support of a role of APC/C^{Cdh1} in Id2 stability, expression of Emi1, a specific inhibitor of APC/C (ref. 9), led to the accumulation of Id2 (Supplementary Fig. 3g).

We noted that Id2 contains a canonical D-box motif (RxxLxxxN)[10] at residues 100–107, indicating that Id2 might be a direct substrate of APC/C^{Cdh1}. The D-box motif is conserved in Id1 and Id4 but not in Id3 (Fig. 2a). We generated mutant Id2 proteins in which the key arginine and leucine residues were changed into glycine and valine, respectively (Id2-DBM) and the D box was deleted (Id2δDB). Id2-DBM and Id2δDB mutants were expressed at levels higher than wild-type Id2 and were resistant to Cdh1-mediated destabilization (Fig. 2b, and Supplementary Fig. 4a). Conversely, APC/C^{Cdh1} eliminated the Id2 mutant that lacks the HLH region (Id2δHLH) and also wild-type Id1 and Id4 but not Id3 (Supplementary Fig. 4b–e). Next we investigated whether the D box is essential for the recognition of Id2 by APC/C^{Cdh1}. Glutathione *S*-transferase (GST)–Id2 but not GST–Id2-DBM or GST–Id2δDB captured *in vitro* translated Cdh1 and Cdh1 from HeLa cell extract, whereas all GST–Id2 fusion proteins captured Apc1 and Cdc27 (Fig. 2c, and Supplementary Fig. 5a). Similarly, both Flag–Id2 and Flag–Id2-DBM associated with APC/C core subunits, but Flag–Id2-DBM precipitated much less

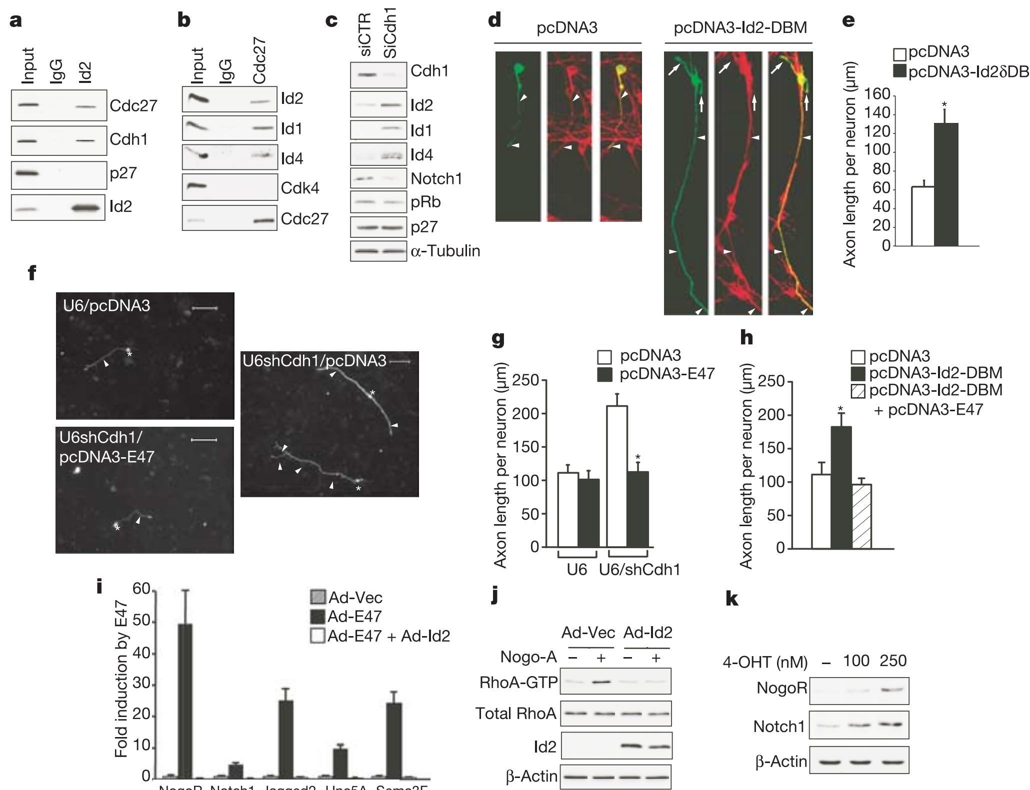

Figure 3 | An APC/C^{Cdh1}–Id–bHLH pathway controls axonal growth. **a**, **b**, Immunoprecipitation of Id2 (**a**) and Cdc27 (**b**) from CGNs. **c**, CGNs transfected with siRNA oligonucleotides and analysed by western blotting. Notch1 is the Val 1744-cleaved intracellular fragment. **d**, GFP (green) and Tau (red) staining in CGNs transfected with vector and Id2-DBM. **e**, Axonal length measured in CGNs transfected with vector or Id2δDB together with GFP ($n = 119$; asterisk, $P \leq 0.0001$). Results are means ± s.e.m. **f**, CGNs transfected with E47 and U6-CTR or U6-shCdh1 together with GFP. **g**, Quantification of axonal length from **f** ($n = 300$; asterisk, $P \leq 0.001$). Results are means ± s.e.m. **h**, Axonal length measured from CGNs transfected with Id2-DBM in the absence or presence of E47 ($n = 76$; asterisk, $P \leq 0.01$). Results are means ± s.e.m. **i**, Expression of E47 target genes in SK-N-SH neuroblastoma cells infected with adeno-vector, adeno-E47 and adeno-E47 plus adeno-Id2. NogoR, Nogo receptor. Results are means ± s.d. ($n = 3$). **j**, RhoA activation assay in adenovirus-infected SK-N-SH cells treated with Nogo-A peptide. **k**, SF210-E47-ER cells treated with the indicated concentrations of 4-OHT.

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Cdh1 than Flag–Id2 (Supplementary Fig. 5b). To determine the importance of the D-box-mediated degradation of Id2 we monitored the rate of degradation of Id2 and Id2-DBM. In transiently transfected HeLa cells the half-life of Id2-DBM was extended more than tenfold compared with wild-type Id2 (Fig. 2d, e). In addition, treatment of U2OS cells with the antimitogenic cytokine transforming growth factor-β efficiently depleted Id2 but did not decrease Id2-DBM (Supplementary Fig. 5c). Accordingly, wild-type Id2 but not Id2-DBM was polyubiquitinated *in vitro* by APC/C in the presence, but not in the absence, of Cdh1 (Fig. 2f, and Supplementary Fig. 5d). Together, these data indicate that APC/C^{Cdh1} can polyubiquitinate Id2 in a D-box-dependent manner. In contrast, Id2 did not affect the integrity of the APC/C complex or impair the ability of APC/C^{Cdh1} to degrade other substrates (Supplementary Fig. 6).

Apart from the regulation of the cell cycle, the expression of APC/C^{Cdh1} in postmitotic neurons has been puzzling[11]. Previous studies indicated that APC/C^{Cdh1} directs cell-cycle-independent functions in postmitotic neurons, including the negative control of axonal growth. At embryonic day 16 (E16), Id2 was physically associated with brain APC/C (Supplementary Fig. 7a). We therefore examined whether a complex containing Id2 and APC/C^{Cdh1} exists in differentiated neurons, and whether APC/C^{Cdh1} directs the degradation of Id2 to control axonal growth. We found that lysates from primary cerebellar granule neurons (CGNs) and differentiated neuroblastoma cells contained Id2–core APC/C and Id2–Cdh1 complexes (Fig. 3a, b, and Supplementary Fig. 7d, e). Furthermore, postmitotic neurons transfected with Cdh1 short interfering RNA (siRNA) accumulated Id2, Id1 and Id4 in the absence of cell cycle re-entry as indicated by the lack of bromodeoxyuridine incorporation, retinoblastoma gene product phosphorylation or decrease in the cyclin-dependent kinase inhibitor p27 (Fig. 3c, and Supplementary Fig. 7f, g)[3]. Next we determined whether expression of the Id2 mutants that are resistant to APC/C^{Cdh1}-mediated degradation stimulated axonal elongation. CGN axons are identified on the basis of morphology, positive staining for Tau, and negative staining for MAP-2 (refs 3, 12). In the presence of green fluorescent protein (GFP) expression to mark transfected cells, expression of Id2-DBM and Id2δDB but not wild-type Id2 significantly increased axonal length in CGNs, cortical neurons and differentiated SK-N-SH cells (Fig. 3d, e, Supplementary Figs 7h and 8a, b, and data not shown). The same effect was evident in postnatal day 6 (P6) CGNs transfected with Id2-DBM and cultured on top of organotypic cerebellar slices from P9 rat pups[3] (Supplementary Fig. 8c, d).

Having shown that, resembling Cdh1 silencing, the expression of undegradable Id2 leads to unrestrained axonal growth, we investigated whether restoring bHLH activity to neurons depleted of Cdh1 or expressing degradation-resistant Id2 rescues the deregulated axon growth. As expected, knockdown of Cdh1 in CGNs promoted axonal growth. Ectopic expression of the bHLH transcription factor E47 abolished the stimulation of axonal growth by Cdh1 knockdown and Id2-DBM (Fig. 3f–h). These results indicate that downstream targets of bHLH transcription factors mediate the axonal inhibitory functions and activation of these targets requires the control of Id protein accumulation by APC/C^{Cdh1}. To identify the relevant targets recruited by the Cdh1–Id–bHLH pathway for the control of axonogenesis, we infected SK-N-SH cells with an adenovirus expressing E47 (Ad-E47) and determined the gene expression profile. E47 induced the expression of genes coding for inhibitors of axonal growth including secreted molecules (Sema3F), ligands (Jagged-2) and receptors (Nogo receptor, Unc5A, Notch1) of multiple inhibitory and repellant signals for axons[13–15] (Supplementary Fig. 9a). Prominent among them, the Nogo receptor is one of the best-known inhibitors of axonal growth on which all myelin-inhibitory signals (Nogo-A, myelin associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein) converge[16,17]. We confirmed the microarray results with quantitative real time polymerase chain reaction from cells transduced with Ad-E47 and found that coinfection with Id2 adenovirus (Ad-Id2) prevented the E47-mediated induction of anti-axonal genes (Fig. 3i, and Supplementary Fig. 9b). The main intracellular transducer of axonal inhibitory signals driven by Nogo receptor is the small GTP-binding protein RhoA[17]. Ad-Id2 abolished the activation of RhoA by Nogo-A inhibitory peptide (Fig. 3j). To investigate whether bHLH transcription induces anti-axonal genes in non-neuronal cells, we generated SF210 glioma cells expressing conditionally active E47 fused to the ligand-binding domain of oestrogen receptor (E47-ER). Activation of bHLH transcription by 4-hydroxytamoxifen (4-OHT) increased Nogo receptor protein and the Val 1744-cleaved active fragment of Notch1 (Fig. 3k). Conversely, knockdown of Cdh1 decreased E47-mediated transcriptional activation of an E-box-luciferase plasmid and inhibited the basal and E47-induced expression of the antiaxonal signature and the Val 1744-cleaved intracellular fragment of Notch1 (Fig. 3c, and Supplementary Fig. 9c–e). Together, these results underscore the functional relevance of the Cdh1–Id–bHLH pathway for axonal growth.

To evaluate the significance of deregulated Id2 in neurons whose axonal growth had been arrested by the inhibitory myelin components that require Nogo receptor for signalling, we transfected CGNs plated on MAG-Fc inhibitory substrate with Id2-DBM or empty plasmid. Id2-DBM stimulated axonal growth in the absence of the MAG-Fc inhibitor and rescued axonal elongation in the presence of the myelin inhibitor. Conversely, MAG-Fc markedly inhibited axonal growth in neurons transfected with the empty plasmid (Fig. 4a, b). Next we determined whether Id2 stability differs between neurons that are actively growing axons and on reaching synaptogenesis when growth is terminated. Cortical neurons undergo axonal elongation *in vitro* that culminates with extensive synaptogenesis at DIV (days

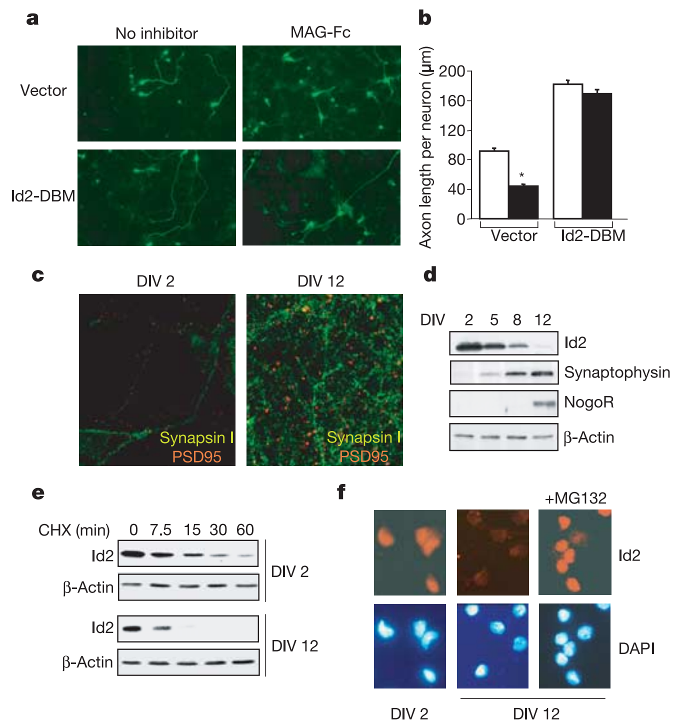

Figure 4 | Significance of APC/C^{Cdh1}-mediated degradation of Id2 for axonal growth. a, Axonal outgrowth of Id2-DBM and vector-transfected CGNs in the presence and absence of MAG-Fc. **b**, Quantification of axonal length from **a**. Open bars, no inhibitor; filled bars, MAG-Fc. Results are means ± s.e.m. ($n = 150$; asterisk, $P \leq 0.0001$). **c**, Co-staining for presynaptic synapsin-I (green) and postsynaptic PSD95 (red) in DIV 2 and DIV 12 cortical neurons. **d**, Id2 expression in cortical neurons undergoing synaptogenesis. NogoR, Nogo receptor. **e**, DIV 2 and DIV 12 cortical neurons treated with cycloheximide (CHX) for the indicated durations. **f**, Id2 (red) and nuclei (4,6-diamidino-2-phenylindole (DAPI), blue) staining in DIV 2 and DIV 12 cortical neurons in the presence and absence of MG132.

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in vitro) 12 as shown by the accumulation of synaptophysin and maturation of the postsynaptic marker PSD-95 into large clusters juxtaposed to presynaptic synapsin-I puncta (Fig. 4c, d)[18]. The steady-state levels of Id2 decreased progressively after DIV 2 and became almost undetectable at DIV 12, when the Nogo receptor protein was markedly upregulated (Fig. 4d). The reduction of the Id2 steady state between DIV 2 and DIV 12 coincided with an increased rate of Id2 protein degradation (Fig. 4e). APC/C^{Cdh1} inhibition by silencing of Cdh1 led to an increase in Id1 and Id2 levels also in cortical neurons (Supplementary Fig. 10). APC/C^{Cdh1} activity is localized in the nucleus[19]. Consistent with this notion is the observation that MG132-mediated inhibition of the proteasome in DIV 12 neurons led to the accumulation of Id2 in the nucleus at levels comparable to those detected at DIV 2 (Fig. 4f).

We show that APC/C^{Cdh1} primes Id2 for degradation through a D box as a recognition site for the Cdh1 coactivator. The activity of Id proteins, which are invariably depleted in cells undergoing quiescence, corresponds to the consequences of inactivation of APC/C^{Cdh1} in postmitotic cells. Indeed, in a manner consistent with reactivation of the cell cycle in cells carrying inactive APC/C (ref. 20), deregulated expression of Id2 prevents cell cycle arrest by a wide range of antiproliferative signals[21–23]. Under certain experimental conditions, ectopic Id2 is able to override the quiescent state and drive terminally differentiated cells back into the cell cycle[24]. The observation that the most aggressive tumours frequently contain the largest amounts of Id proteins raises the possibility that inactivation of the control of Id protein stability by APC/C^{Cdh1} might also contribute to Id accumulation in cancer.

By setting the timing of Id protein degradation in the nervous system, APC/C^{Cdh1} initiates a chain of events that terminates with the activation of downstream targets of bHLH transcription factors. The dual activity of the brain APC/C^{Cdh1} as an inducer of the postmitotic state and an inhibitor of axonal growth can be viewed as an integrated process that restrains Id proteins during distinct stages of neural development and, as a consequence, sets the timing of bHLH-dependent transcription. The general implication of our findings is that neurons in active axonal growth should be viewed as relatively 'undifferentiated' compared with neurons that have reached their targets and are unable to resume neurite outgrowth. We suggest that, by disabling the APC/C^{Cdh1}–Id–bHLH axis, degradation-resistant forms of Id proteins might provide beneficial effects for axonal regeneration of damaged neurons.

METHODS

Transfections and siRNA. Cell transfection was performed with Lipofectamine 2000 (Invitrogen). CGNs, cortical neurons and SK-N-SH cells differentiated with retinoic acid and brain-derived neurotrophic factor[25] were transfected three times with 100 nM siRNA oligonucleotides and the Gene Silencer siRNA Transfection Reagent (Gene Therapy Systems)[26]. siRNAs were Cdh1 Smart Pool from Dharmacon (5′-CGACAGAUCAUCAUCCAAA-3′, 5′-CAACUGGAGC GUAAACUUC-3′, 5′-CGCCAGAGCUUCAGGACGA-3′, 5′-AAGGGUCUCU UUACGUAUU-3′) and U6shCdh1 (5′-GGCUUCGUGCAGAUCUGGGAA-3′). Lamin and firefly luciferase Smart Pools (Dharmacon) served as negative controls.
RhoA assay. SK-N-SH cells were infected with Ad-vector or Ad-Id2 at a multiplicity of infection of 50. After 24 h, cells were stimulated by the addition of the Nogo-A peptide Nogo-P4 (8 μM; Alpha Diagnostic) for 30 min. GTP-RhoA was precipitated by using beads with GST–Rho-binding domain (RBD) of rhotekin, and detected with anti-RhoA antibodies in accordance with the manufacturer's guidelines (Cytoskeleton). Additional methods are described in Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The microarray data have been deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/query/entry) under accession number E-MEXP-413. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to A.I. (ai2102@columbia.edu).

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