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APC/C^{Cdh1} controls the proteasome-mediated degradation of E2F3 during cell cycle exit

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Key words: cell cycle, ubiquitylation, ubiquitin ligase, E2F3, APC/C, Cdh1, Cdc20

Abbreviations: APC/C, anaphase promoting complex/cyclosome; D-box, destruction box; pRB, retinoblastoma tumor suppressor

E2F transcription factors regulate gene expression in concert with the retinoblastoma tumor suppressor family. These transcriptional complexes are master regulators of cell cycle progression and, in addition, control the expression of genes involved in DNA repair, G_2/M checkpoint and differentiation. E2F3 has recently attracted particular attention, because it is amplified in various human tumors. Here we show that E2F3 becomes unstable as cells exit the cell cycle. E2F3 degradation is mediated by the anaphase-promoting complex/cyclosome and its activator Cdh1 (APC/C^{Cdh1}). E2F3 interacts with Cdh1 but not Cdc20, the other APC/C activator. Enforced expression of Cdh1 results in proteasome-dependent degradation of E2F3, whereas the overexpression of Cdc20 has no effect on E2F3 turnover. Finally, silencing of Cdh1 by RNA interference stabilizes E2F3 in differentiating neuroblastoma cells. These findings indicate that the APC/C^{Cdh1} ubiquitin ligase targets E2F3 for proteasome-dependent degradation during cell cycle exit and neuronal differentiation.

Introduction

Upon differentiation, cells stop proliferating and withdraw from the cell cycle. Terminal cell cycle exit and differentiation are highly orchestrated processes regulated by a complex network of molecular pathways. Key regulators of these processes are the E2F transcription factors, which, in concert with members of the retinoblastoma family (pRb, p107 and p130), control the expression of genes involved in cell cycle progression. The E2F protein family is composed of eight members, E2F1 to E2F8, which are classified into activators and repressors based on their transcriptional regulatory activities.[1,2] E2F1 to E2F3 are considered as activators, whereas E2F4 to E2F8 are repressors. Generally, activating E2F transcription factors drive cell cycle progression by inducing the expression of pro-proliferating genes, while repressing E2F transcription factors block cell cycle progression by inhibiting the expression of these genes.

E2F3 is the only E2F family member whose locus (6p22.3) is amplified in human tumors. Amplification and overexpression of E2F3 is found in lung, bladder, prostate and ovarian cancers.[3-6] The E2F3 locus encodes two isoforms: E2F3A and E2F3B, which are distinguished exclusively by their N termini.[7-9] Two distinct promoters control the expression of alternative first exons that are spliced to a common second exon. As a result, the two E2F3 isoforms are identical at the regions encoding for their known functional parts. However, E2F3A and E2F3B do not have complete functional overlap. Indeed, during cell cycle, E2F3A shows a peak of expression in S phase, whereas E2F3B is expressed throughout the cell cycle and in G_0 cells.[8]

Cells employ numerous ways to tightly regulate the activity of E2F3, both at the transcriptional and post-transcriptional level, emphasizing the importance of accurately controlling the activity of E2F3. The regulation of E2F3 protein turnover is another way to control the activity of E2F3;[10] however, the molecular mechanisms regulating E2F3 degradation are currently unknown. Here, we report that the anaphase-promoting complex/cyclosome targets E2F3 for ubiquitin-mediated degradation in quiescent cells, thus identifying a novel way to control E2F3 activity in cells.

Results

Proteasome-dependent degradation of E2F3 in cells exiting the cell cycle. We examined the expression of both E2F3 isoforms (E2F3A and E2F3B) during cell cycle exit. Asynchronously growing T98G cells (revertants from T98 glioblastoma cells that acquired the property to accumulate in G_0/G_1 in low serum) were serum-deprived to allow cell cycle exit, and protein levels were analyzed by immunoblotting. We found that E2F3A levels steadily decreased; E2F3B and E2F4 remained unchanged, whereas, as expected, p27 levels increased (**Fig. 1**). Treatment with the proteasome inhibitor MG132 prevented E2F3A downregulation, suggesting that its decrease in response to serum deprivation is at least partially due to protein degradation. Surprisingly, MG132 also induced the accumulation of E2F3B.

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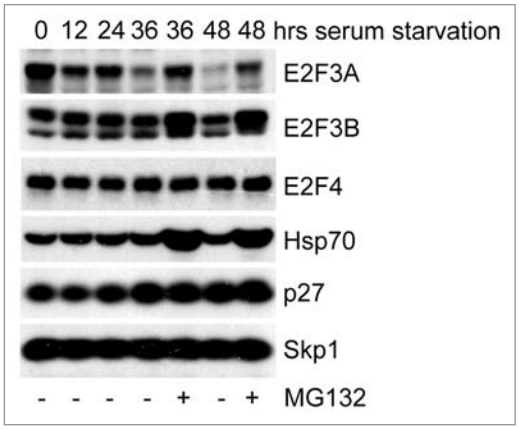

Figure 1. E2F3A and E2F3B are targeted for proteasome-dependent degradation in cells withdrawing from the cell cycle. Asynchronous T98G glioblastoma cells were deprived of serum for the indicated times. Where indicated, the proteasome inhibitor MG132 (10 μM) was added for the last 5 hours. Cells were collected and lysed. Whole-cell extracts were analyzed by immunoblotting with antibodies for the indicated proteins. Skp1 was blotted as a loading control.

Cdh1 targets E2F3 for proteasome-dependent degradation. The finding that E2F3A is degraded in cells undergoing quiescence suggests that the degradation of E2F3A may be mediated by the APC/C^{Cdh1}, a crucial regulator of the G_0 phase.[11] To test this hypothesis, we transfected asynchronous T98G cells with a construct expressing either HA-tagged E2F3A (**Fig. 2A**), HA-tagged E2F3B (**Fig. 2B**) or HA-tagged Skp2 (**Fig. 2C**), together with a construct expressing either FLAG-tagged Cdc20 or FLAG-tagged Cdh1. Ectopic expression of Cdh1, but not Cdc20, led to a decrease in the steady-state levels of both E2F3A and E2F3B. Similarly, Skp2, a known substrate of APC/C^{Cdh1},[12,13] was downregulated upon Cdh1 ectopic expression. To test if E2F3A and E2F3B downregulation was due to proteasome-dependent degradation, the proteasome inhibitor MG132 was added to cells overexpressing Cdh1. As shown in **Figure 3**, proteasomal inhibition prevents Cdh1-dependent downregulation of E2F3A and E2F3B. Altogether, these results demonstrate that APC/C^{Cdh1} targets both E2F3A and E2F3B for proteasome-dependent degradation.

E2F3A and E2F3B interact with Cdh1 but not with Cdc20. We next tested whether Cdh1 physically interacts with the two E2F3 isoforms. HEK293T cells were transfected with a construct expressing FLAG-tagged Cdc20 or FLAG-tagged Cdh1. FLAG immunoprecipitations were then performed to examine the binding of Cdh1 and Cdc20 to E2F3A and E2F3B. Endogenous E2F3A and E2F3B coimmunoprecipitated with FLAG-tagged Cdh1, but not with FLAG-tagged Cdc20 (**Fig. 4A**). These results demonstrate that both E2F3A and E2F3B interact with the APC/C activator Cdh1. Degradation of many APC/C substrates depends on a destruction motif (D-box) with the consensus sequence RxxLxxIxN;[14] however, many APC/C substrates contain only a minimal D-box (RxxL).[12,13] Analysis of the primary sequence of E2F3A (NCBI Reference Sequence NP_001940.1) revealed the presence of four RxxL motifs at amino acids 36–39 (D-box I), 109–112 (D-box II), 135–138 (D-box III) and 158–161 (D-box IV) that could potentially serve as D-boxes (**Fig. 4B**). E2F3B contains only the putative D-box III and D-box IV. To test whether any of these regions is required for the interaction of E2F3 with Cdh1, we generated D-box mutants of E2F3 in which the arginine and leucine amino acids were replaced by alanine. As shown in **Figure 4C**, all four E2F3A D-box mutants and the two D-box mutants of E2F3B are still able to interact with Cdh1, suggesting that none of these four RxxL motifs is required for the binding of E2F3 to Cdh1.

APC/C^{Cdh1} ubiquitylates E2F3 in vitro. To test whether E2F3 is ubiquitylated by APC/C^{Cdh1}, we reconstituted the ubiquitylation of E2F3 in vitro. As shown in **Figure 5**, in vitro-transcribed/translated E2F3A was ubiquitylated in the presence of E1, UBCH10, Cdh1 and ubiquitin, although some E2F3A ubiquitylation was present in the absence of Cdh1. This is likely due to the presence in the rabbit reticulocyte lysate of endogenous APC/C and Cdh1 that can contribute to the ubiquitylation of APC/C targets.[15] The same results were obtained when we used E2F3B as a substrate in the ubiquitin conjugation reaction (data not shown). Thus, in agreement with our findings in vivo, the in vitro results indicate that Cdh1 promotes E2F3 ubiquitylation.

Silencing of Cdh1 by RNA interference induces stabilization of E2F3 during neuronal differentiation. The finding that E2F3A and E2F3B become unstable in cell undergoing quiescence prompted us to test whether E2F3 is targeted for degradation during neuronal differentiation, a process regulated by APC/C^{Cdh1}.[16-18] To this aim, we employed human neuroblastoma SK-N-SH cells induced to differentiate with retinoic acid. We found that in differentiating neuroblastoma cells, the levels of E2F3A decreased steadily, whereas the levels of E2F3B did not change (**Fig. 6A and B**). As expected, levels of cyclin A decreased and levels of p27 increased as cells exit the cell cycle and differentiate. To test if Cdh1 plays a role in the degradation of E2F3A during neuronal differentiation, we silenced the expression of Cdh1 in SK-N-SH cells using a previously validated siRNA oligonucleotide.[12,19,20] SK-N-SH cells were then treated with retinoic acid, and the expression levels of E2F3A and E2F3B were examined at various time points thereafter. As shown in **Figure 6C**, Cdh1 knockdown reduced the degradation of E2F3A and induced accumulation of E2F3B in differentiating neuroblastoma cells. In contrast, Cdc20 siRNA did not have any effect on the abundance of E2F3A and E2F3B. Together, these results suggest a role for APC/C^{Cdh1} in mediating the degradation of E2F3A and E2F3B in differentiating neuroblastoma cells.

Discussion

APC/C^{Cdh1} governs the G_1 and G_0 phases of the cell cycle by targeting a number of key substrate proteins for degradation.[11,21] In the present report, we show that APC/C^{Cdh1} controls the stability of the E2F3 transcription factor during cell cycle exit. We demonstrate that E2F3 physically interacts with Cdh1 but not with the related APC/C activator Cdc20. We also show that APC/C^{Cdh1} is able to ubiquitylate E2F3A in vitro, and that the degradation of E2F3A is stimulated by Cdh1, but not by Cdc20. Finally, we demonstrate that Cdh1 knockdown stabilizes E2F3 during

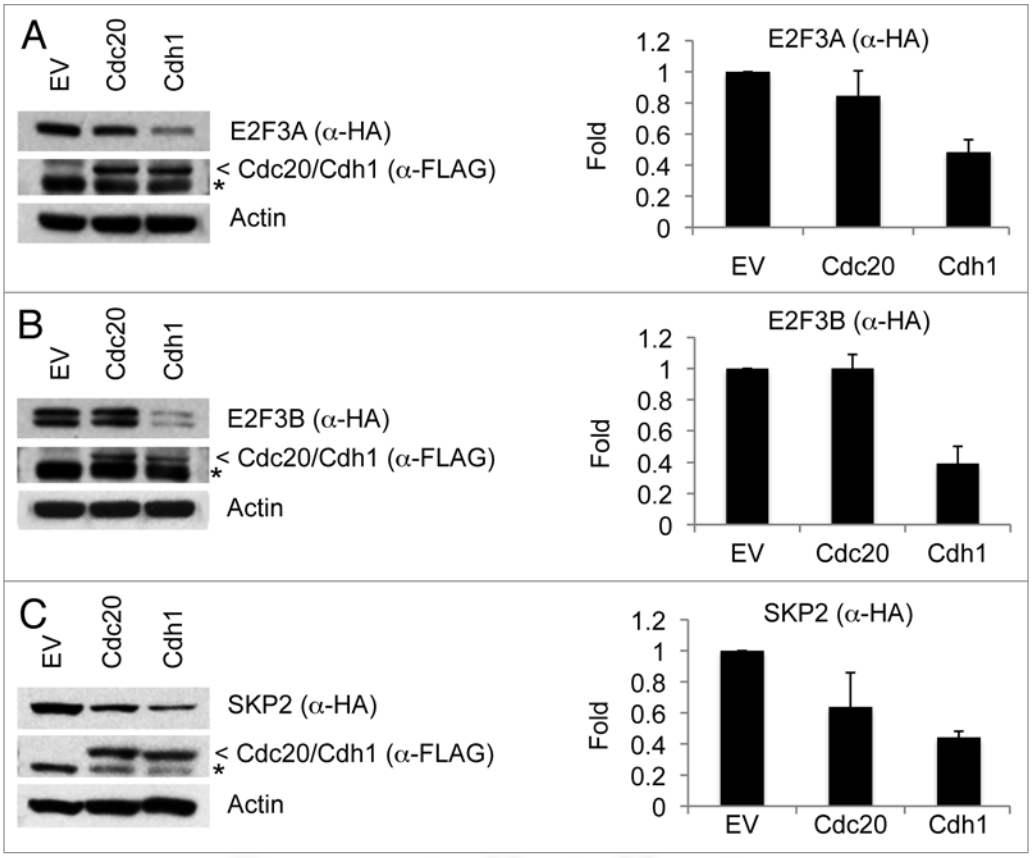

Figure 2. Degradation of E2F3A and E2F3B is stimulated by Cdh1, but not by Cdc20. T98G cells were co-transfected with plasmids expressing HA-tagged E2F3A, HA-tagged E2F3B or HA-tagged Skp2, along with FLAG-tagged Cdc20, FLAG-tagged Cdh1 or an empty vector (EV). A plasmid expressing EGFP was co-transfected as a marker of transfection. Cells were lysed, and E2F3A (A), E2F3B (B), Skp2 (C) together with Cdh1 or Cdc20 were detected by immunoblotting using anti-HA and anti-FLAG antibodies, respectively. Actin was blotted as a loading control. The asterisks (*) indicate nonspecific bands. Graphs on the right show quantification E2F3A (A), E2F3B (B) and Skp2 (C) expression. The value given for the amount of protein present in the control sample (EV) was set as 1 (n = 3).

cell cycle exit and neuronal differentiation of human neuroblastoma cells.

Additional studies will be required to identify the region of E2F3 that mediates the binding between E2F3 and Cdh1. We have shown that the RxxL motifs present in E2F3 are not required for E2F3 binding to Cdh1. In the accompanying paper, Budhavarapu et al. report that E2F1 interacts directly with Cdh1 via its C-terminal region. Due to the structural and functional similarities between E2F1 and E2F3, it is interesting to speculate that a yet-to-be-identified region at the C terminus of E2F3 might be required for the degradation of both E2F3A and E2F3B.

The finding that APC/C^{Cdh1} regulates the stability of the E2F3B isoform in quiescent cells is somehow surprising. Indeed, our data indicate that during cell cycle exit, E2F3A levels steadily decrease, while E2F3B levels remain unchanged. This is in agreement with other studies[8,22] showing that E2F3A is mostly expressed in growing cells, with enrichment in S phase, whereas E2F3B is expressed both in proliferating and in quiescent cells. Furthermore, it is well established that while E2F3A induces the expression of genes involved in cell cycle progression, E2F3B controls genes involved in differentiation and development. The reason for the ubiquitin-mediated degradation of E2F3B in absence of an effect on its steady-state level in quiescent cells is currently unknown. In conclusion, Cdh1-mediated degradation of E2F3 during cell cycle exit represents a novel mechanism to control E2F3 abundance.

Experimental Procedures

Cell lines, synchronization method and drug treatments. All cells were obtained from ATCC. Human embryonic kidney HEK293T and human glioblastoma T98G cells were cultured in Dulbecco's modified Eagle's medium (DMEM); human neuroblastoma SK-N-SH cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12). Both cell culture media were supplemented with 10% v/v Fetal Calf Serum (FCS), 100 U/ml Penicillin/Streptomycin and 2 mM L-Glutamine. All cells were maintained in a humidified 37°C incubator with 5% CO_2. The proteasome inhibitor MG132 (10 μM) was added to HEK293T and T98G cells 5 h prior to cell collection. T98G cells were synchronized by serum starvation. Cells were washed three times with PBS and supplied with serum starvation medium (DMEM supplied with 0.02% v/v FCS, 2 mM L-glutamine) and incubated for 48 h. Subsequently, the culture medium was replaced

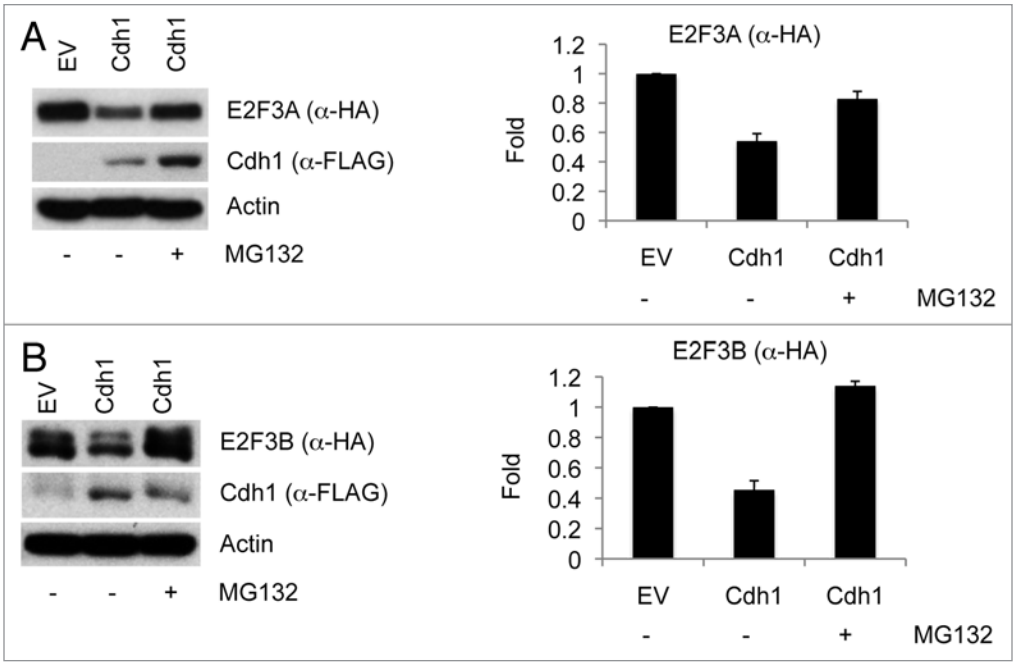

Figure 3. Cdh1-dependent degradation of E2F3 is prevented by proteasomal inhibition. T98G cells were co-transfected with plasmids expressing either HA-tagged E2F3A or HA-tagged E2F3B, along with either FLAG-tagged Cdh1 or an empty vector (EV). A plasmid expressing EGFP was co-transfected as a marker of transfection. Where indicated, cells were treated with 10 μM MG132 for 5 hours before harvesting. Cells were lysed, and E2F3A (A) and E2F3B (B), together with Cdh1, were detected by immunoblotting using anti-HA or anti-FLAG antibodies, respectively. Actin was blotted as a loading control. Graphs on the right show quantification of E2F3A (A), E2F3B (B) expression. The value given for the amount of protein present in the control sample (EV) was set as 1 (n = 3).

with DMEM supplied with 20% v/v FCS, 2 mM L-Glutamine, and cells were collected at the indicated time points. Neuronal differentiation was induced by treating SK-N-SH cells with 10 μM retinoic acid (Sigma-Aldrich).

Biochemical methods. Cell extracts were prepared as follows: cell monolayers were washed twice with room temperature PBS and harvested. Cells were lysed in Triton Lysis Buffer (0.1% Triton-X100, 50 mM TRIS-HCl pH 7.4, 0.25 M NaCI, 1 mM EDTA, 50 mM NaF) supplied with the following: 0.1 mM phenylmethyl sulfonylflouride (PMSF), 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate (NaV), 10 μg/ml tosyl phenylalanyl chloromethyl ketone (TPCK), 10 μg/ml tosyl lys chloromethyl ketone (TLCK) and a mixture of 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 1 μg/ml aprotinin.

For immunoprecipitations, HEK293T cells from one 15-cm plate were lysed in TLB as described above. Cell extracts were precleared by adding 40 μl Protein-G Sepharose (Invitrogen) and incubated for 1 h. Precleared cell extracts were then incubated with FLAG resin (Sigma-Aldrich) for 3 h. Immunoprecipitates were washed with 1 ml of TLB four times. After the last wash, immunoprecipitates were resuspended in Laemmli sample buffer and boiled for 4 min at 90°C for denaturation. For western blot analysis, protein samples were separated by SDS-PAGE. Following SDS-PAGE, separated proteins were transferred onto pre-activated PVDF membrane (Millipore). After transfer, the PVDF membrane was stained with Ponceau Red Solution (Sigma-Aldrich) to confirm transfer efficiency and equal loading. The PVDF membrane was washed in phosphate-buffered saline containing 0.1% v/v Tween 20 (PBS-T) to remove the Ponceau red stain, blocked for 1 h at room temperature in 5% w/v low-fat dried milk dissolved in PBS-T and probed with various antibodies. Incubation with the appropriate primary antibody was done in 5% w/v low-fat dried milk dissolved in PBS-T for 2 h at room temperature. Mouse monoclonal antibodies were from Invitrogen (Cul1), Covance (anti-HA), Sigma-Aldrich (anti-FLAG and anti-Cdc27), BD Transduction Laboratories (anti-p27), Thermo Scientific (anti-Cdh1) and Santa Cruz Biotechnology or Bethyl Laboratories (anti-Actin). Rabbit polyclonal antibodies were from Santa Cruz Biotechnology (anti-cyclin A, anti-E2F3, anti-Skp1 and anti-Cdc20). After three washes in PBS-T, the membranes were incubated with horseradish peroxidase-linked secondary antibodies (GE Healthcare) in 5% w/v low-fat dried milk dissolved in PBS-T and then washed three times in PBS-T. Proteins were visualized by an enhanced chemiluminescence detection system (Thermo Scientific) according to the manufacturer's instructions.

Mammalian expression plasmids and transfection. E2F3A, E2F3B, Skp2, Cdh1 and Cdc20 cDNAs were cloned in pcDNA3.1. E2F3A, E2F3B and Skp2 were HA-tagged, whereas Cdh1 and Cdc20 were FLAG-tagged. Transfection by calcium phosphate was performed as follows. Exponentially growing HEK293T cells were plated in 15-cm plates one day before transfection, so that cells were 70–80% confluent by the time of transfection. Cells were transfected with 25 μg of plasmid and 1 μg of EGFP (as a transfection control). Plasmids were

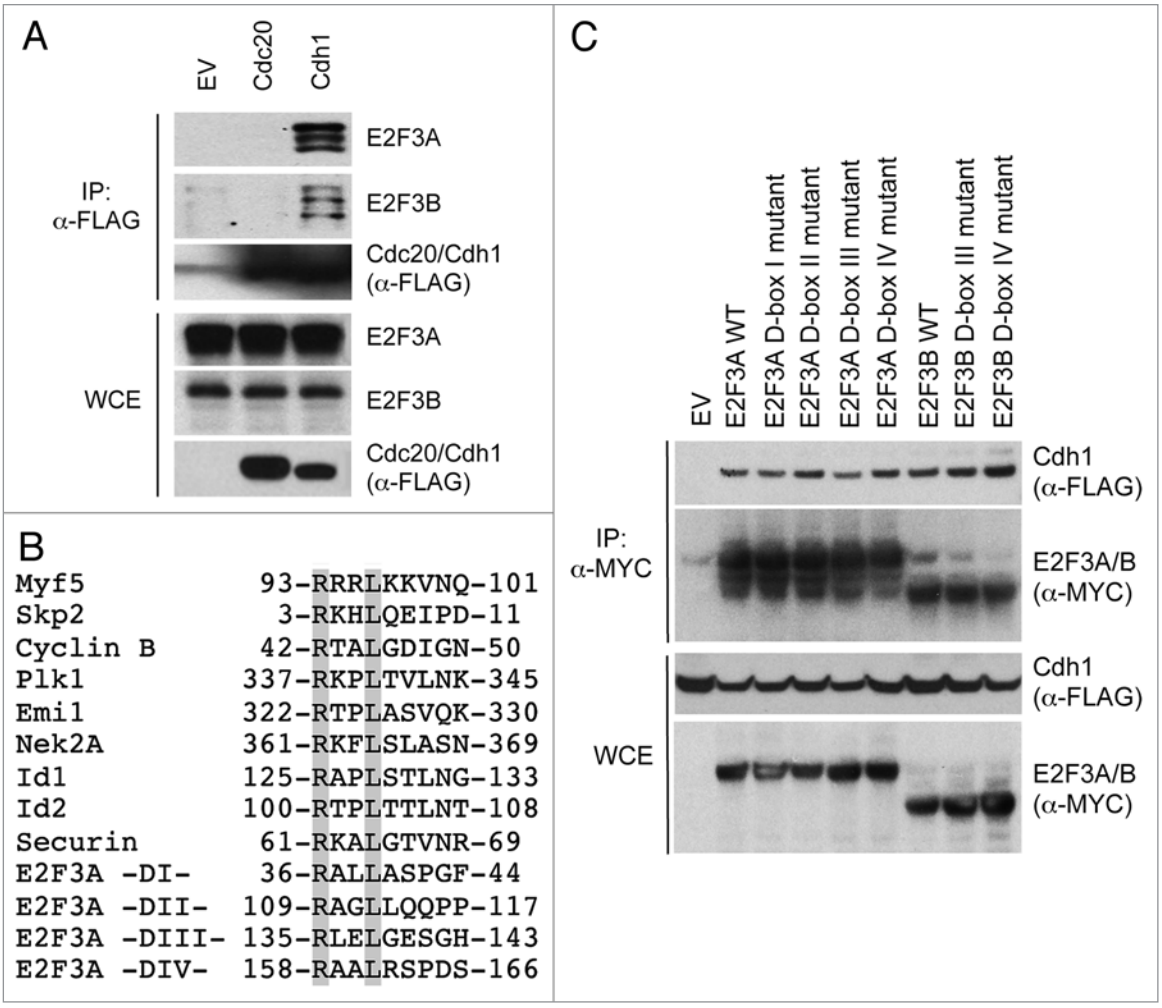

Figure 4. Cdh1, but not Cdc20, interacts with E2F3A and E2F3B. (A) HEK293T cells were transfected with either empty vector (EV), FLAG-tagged Cdc20 or FLAG-tagged Cdh1. The proteasome inhibitor MG132 (10 μM) was added 5 hours before harvesting. Cells were lysed and whole-cell extracts (WCE) were subjected either to immunoblotting or immunoprecipitation (IP) with anti-FLAG resin and subsequent immunoblotting for the indicated proteins. (B) Alignment of amino acid regions of putative destruction box motifs in E2F3A with the D-box motifs of known substrates of APC/C^{Cdh1}. (C) HEK293T cells were transfected with FLAG-tagged Cdh1 along with the indicated Myc-tagged E2F3A and E2F3B mutants. Cells were lysed, and whole-cell extracts were subjected either to immunoblotting or immunoprecipitation (IP) with anti-Myc resin and subsequent immunoblotting for the indicated proteins.

diluted in 250 mM $CaCl_2$, incubated for 5 min at room temperature and mixed with 2x BBS (50 mM BES pH 6.96 [N-N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid], 280 mM NaCl, 1.5 mM Na_2HPO_4) and incubated for 20 min. Transfection mixtures were then added dropwise to the cells. Culture media was replaced with fresh DMEM 5 h after transfection. Cells were collected and lysed 48 h after transfection. T98G cells were transfected by Metafectene (Biontex Laboratories GmbH) according to the manufacturer's instructions. When indicated, 10 μM MG132 was added to inhibit proteasome-dependent degradation.

Gene silencing by small interfering RNA. The sequences of the siRNA oligonucleotides targeting Cdh1 and Cdc20 were: 5'-AAT GAG AAG TCT CCC AGT CAG-3' and 5'-AAA CCT GGC GGT GAC CGC TAT-3', respectively. SK-N-SH cells were transfected with the oligos twice, at 24 and 48 h after plating using Metafectene (Biontex Laboratories GmbH) according to the manufacturer's instructions.

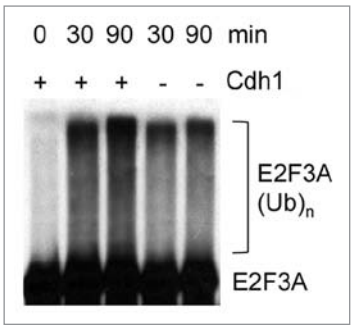

Figure 5. E2F3 is ubiquitylated by APC/C^{Cdh1}. In vitro ubiquitylation assay of ^{35}S-labeled, in vitro translated E2F3A was performed with immunopurified APC/C, E1, UBCH10 and ubiquitin in the presence or absence of Cdh1 translated in vitro. Samples were incubated at 30°C for the indicated times and analyzed by SDS-PAGE followed by autoradiography. The bracket on the right side marks a ladder of bands corresponding to polyubiquitylated E2F3A.

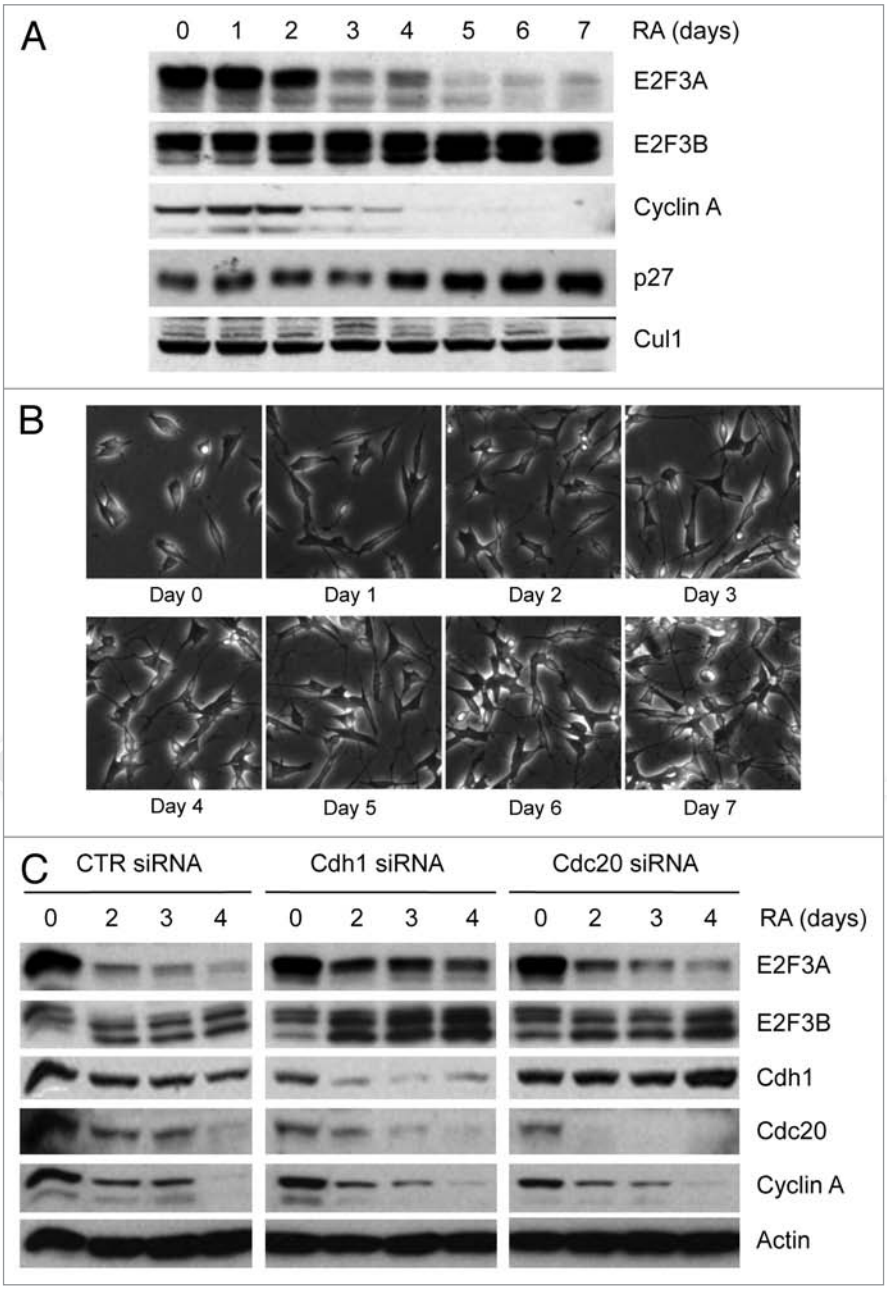

Figure 6. Cdh1 silencing stabilizes E2F3 during neuronal differentiation. (A) SK-N-SH neuroblastoma cells were treated with 10 μM retinoic acid (RA) to induce neuronal differentiation. At the indicated times, cells were collected and lysed. Whole-cell extracts were analyzed by immunoblotting with antibodies for the indicated proteins. Phase contrast images of all samples were taken at 10x magnification before harvesting (B). (C) Cdh1 silencing stabilizes E2F3A and E2F3B during neuronal differentiation. SK-N-SH cells were transfected twice with siRNA targeting a non-relevant mRNA (control siRNA), Cdh1 mRNA or Cdc20 mRNA. After transfection, 10 μM retinoic acid was added to induce neuronal differentiation. Cells were collected at the indicated time points and analyzed by immunoblotting with antibodies for the indicated proteins. Actin was blotted as a loading control.

In vitro ubiquitylation assay. Ubiquitylation assays were performed as previously described.[12] Briefly, an anti-Cdc27 antibody was added to cell extracts and incubated for approximately 3 h at 4°C. Protein G-agarose was then added and incubated for 45 min at 4°C on a rotating wheel. The beads were washed four times in Triton buffer and four times in QA buffer (10 mM TRIS-HCl pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT). The resulting beads were used for two reactions of in vitro ubiquitylation. Ubiquitylation assays were performed in a volume of 10 μl containing 50 mM Tris pH 7.6, 5 mM $MgCl_2$, 0.6 mM DTT, 2 mM ATP, 2 μl in vitro transcribed/translated unlabeled Cdh1, 50 ng/μl E1 (Boston Biochem), 100 ng/μl Ubc1, 100 ng/μl Ubc10, 2.5 μg/μl ubiquitin (Sigma), 1 μM ubiquitin aldehyde and 1 μl ^{35}S-methionine-labeled in vitro transcribed/translated E2F3. The reactions were incubated at 30°C for the indicated times and analyzed by SDS-PAGE and autoradiography.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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