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CONTROL OF CELL CYCLE EXIT AND NEURONAL DIFFERENTIATION BY SCF AND APC/C UBIQUITIN LIGASES

BIO/11

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ABSTRACT

We have developed several assays based on immunopurification strategies followed by mass spectrometry analysis to systematically identify novel substrates of APC/C and SCF ubiquitin ligases. Using these assays we have identified a number of novel substrates of the $SCF^{\beta TrCP}$ and the APC/C^{Cdh1} ubiquitin ligases. We have made the decision to focus on three transcriptional regulators, namely TFAP4, targeted for degradation by $SCF^{\beta TrCP}$, and E2F3 and E2F6, targeted for degradation by APC/C^{Cdh1}. We have found that proteasome-dependent degradation of these three transcriptional regulators occurs during cell cycle exit and neuronal differentiation. The major aims of this work are to biochemically characterize the proteasome-dependent degradation of TFAP4, mediated by $SCF^{\beta TrCP}$, and E2F3 and E2F6, mediated by APC/C^{Cdh1}, and to investigate the biological function of their degradation during cell cycle exit and neuronal differentiation.

INTRODUCTION

Cells employ ubiquitin-mediated degradation to erase a pre-existing network of regulatory transcription factors so that a new system of regulators can establish a different phenotypic state. Indeed, these phenotypic changes require dismantling an existing transcriptional program to set up a new gene expression program. Such a mechanism is utilized during a number of diverse developmental transitions, ranging from the mating-type determination and switching system of the yeast Saccharomyces cerevisiae to the establishment of neuronal identity in mammals (Becker and Bonni, 2005; Geng et al., 2012; Laney and Hochstrasser, 2004; Muratani and Tansey, 2003). In the embryonic brain, the precursors of neurons, known as neuroblasts, divide in specific proliferative zones from where they undergo multiple rounds of cell division and migrate relatively long distances, generating clusters of neuronal precursor cells (Becker and Bonni, 2005; Lui et al., 2011). At specific sites and times, neuroblasts stop dividing and differentiate into mature neurons. The post-mitotic cells extend axonal processes which form synapses essential for neuronal physiology.

Ubiquitin-mediated degradation of transcriptional regulators (both activators and repressors) has been shown to be required for timely cell cycle exit and neuronal differentiation. The ubiquitin-proteasome system involves two discrete and sequential processes: the tagging of substrates by covalent attachment of multiple ubiquitin molecules, and the degradation of poly-ubiquitylated proteins

by the 26S proteasome (Figure 1) (Hershko, 1996, 1997; Hershko and Ciechanover, 1992, 1998; Hershko et al., 2000). Ubiquitin is transferred and covalently attached to substrates through an enzymatic cascade involving ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). E3 ubiquitin ligases represent the essential regulators of ubiquitylation because they physically interact with target substrates, linking them to E2 ubiquitin-conjugating enzymes (Nakayama and Nakayama, 2006).

It is estimated that 650 E3 ubiquitin ligases are encoded by the human genome (Figure 2). There are two major types of E3s, the RING ubiquitin ligases and the HECT ubiquitin ligases defined by the presence of either a RING or a HECT domain (Deshaies and Joazeiro, 2009). Cullin-based E3s are multi-subunit RING ubiquitin ligases that employ the RING proteins Rbx1 or Rbx2 to bind E2s and various cullin-associated specificity factors to bind substrates (Deshaies, 1999; Petroski and Deshaies, 2005). Human cells express seven different cullins (CUL1, 2, 3, 4A, 4B, 5 and 7). In addition, at least two other proteins (the APC2 subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C) and the p53 cytoplasmic anchor protein (PARC) contain a ‘cullin-homology domain’.

The best understood cullin-based E3 is the SCF ubiquitin ligase, which employs F-box proteins as substrate specific receptors and Cul1 as a scaffold (Jin et al., 2004; Kipreos and Pagano, 2000; Nakayama and Nakayama, 2005). Other cullins interact with an analogous set of specificity factors: SOCS-box proteins for

Cul2 and Cul5, BTB proteins for Cul3, and DCAF proteins for Cul4. There are ~300 such cullin adaptors in humans. Moreover, there may be as many as 300 non-cullin-based RING-E3s in humans. By comparison, the number of HECT ubiquitin ligases is much smaller (~50).

Acting in opposition to E3s are deubiqutinating enzymes, Dubs, which remove ubiquitin and ubiquitin-like proteins from substrates. While Dubs were initially linked to ubiquitin recycling and processing, it is now clear that they play key regulatory roles in fundamental biological processes by binding unstable proteins and removing ubiquitin chains.

Two ubiquitin ligases previously implicated in the control of cell cycle exit and neuronal differentiation are the APC/C^{Cdh1} and the $SCF^{\beta TrCP}$.

The $SCF^{\beta TrCP}$ ubiquitin ligase. $SCF^{\beta TrCP}$ is a multi-subunit RING ubiquitin ligase composed of Cul1, which simultaneously interacts with the RING subunit Rbx1 and the adaptor protein Skp1 (Figure 3). Skp1 in turn binds the F-box protein βTrCP (β-transducin repeat-containing protein), the substrate receptor subunit that recruits specific substrate proteins. Via its WD40 β-propeller structure, βTrCP recognizes a di-phosphorylated motif with the consensus DpSGXX(X)pS in which the serine residues are phosphorylated by specific kinases to allow interaction with βTrCP. βTrCP1 and βTrCP2, two paralogous F-box proteins that (to date) share identical biochemical properties and substrates, have been implicated in the regulation of at least two different signal transduction pathways,

the Wnt/βcatenin and NFκB signaling, by mediating the ubiquitylation and degradation of the transcriptional coactivator β-catenin and the NF-κB inhibitor IκB, respectively (Figure 4) (Frescas and Pagano, 2008). More recently, βTrCP has been implicated in control of the cell division cycle (Busino et al., 2003; Guardavaccaro et al., 2008; Guardavaccaro et al., 2003; Guardavaccaro and Pagano, 2006; Peschiaroli et al., 2006). During S and G2, this ubiquitin ligase keeps Cdk1 inactive by inducing the degradation of its activating phosphatase Cdc25A. At G2/M, it induces the degradation of Wee1, contributing to Cdk1 activation. In mitosis $SCF^{\beta TrCP}$ promotes the degradation of Emi1, an inhibitor of APC/C, thereby attenuating Cdk1 activity via the APC/C-mediated degradation of two activating cyclin subunits (Cyclin A and Cyclin B).

βTrCP is highly expressed in postmitotic neurons where induces the degradation of Emi1, which in turn inhibits APC/C. APC/C controls axonal growth and patterning in the mammalian brain (Konishi et al., 2004; Lasorella et al., 2006). Another substrate of βTrCP is REST, a transcriptional repressor of neuronal genes containing a 23 bp conserved motif, known as RE1 (repressor element 1) (Ballas et al., 2001; Ballas et al., 2005; Ballas and Mandel, 2005). Several lines of evidence now point to REST as a key protein for regulating the large network of genes essential for neuronal function. In addition, REST behaves as an oncoprotein in neuronal cells. REST is ubiquitylated and targeted for degradation by βTrCP during the G2 phase of the cell cycle to allow the transcriptional

derepression of Mad2 (mitotic arrest deficient 2) (Guardavaccaro et al., 2008), an essential component of the spindle assembly checkpoint. Expression of a stable REST mutant inhibits Mad2 expression in G2 and results in a phenotype that is consistent with faulty activation of the spindle checkpoint. Importantly, an indistinguishable phenotype was observed by expressing REST-FS, an oncogenic frameshift mutant identified in colorectal cancer (Westbrook et al., 2005) that lacks the βTrCP-binding domain. Moreover, another study shows that βTrCP-mediated degradation of REST is required for proper neural differentiation and that non-degradable REST mutants attenuate differentiation (Westbrook et al., 2008). Together, these two studies indicate that levels of REST must be accurately controlled to avoid putting neuronal tissues at risk of cancer. Indeed, increased levels of REST due to overproduction (Fuller et al., 2005; Lawinger et al., 2000; Su et al., 2006) and/or C-terminal truncations, as observed in human medulloblastomas and neuroblastomas (unpublished observations), would inhibit differentiation and generate chromosomal instability, two fundamental mechanisms that contribute to tumorigenesis. Interestingly, REST variants (which lack the βTrCP-binding motif) are also observed in non-neuronal tumors, but the implication of these findings is not yet understood. It is possible that such truncated variants contribute to cell transformation by promoting aneuploidy and genetic instability in both neuronal and non-neuronal tissues.

The APC/C^{Cdh1} ubiquitin ligase. The anaphase promoting complex/cyclosome (APC/C) plays a central role in the mechanism that drives mitotic exit (Peters, 2002). It is composed of a number of invariable core components and a variable subunit, either Cdc20 or Cdh1, which provides specificity by interacting directly with the substrate (Figure 5) (Skaar and Pagano, 2008). Cdh1 contains a C-terminal WD40 domain that is believed to recognize its substrates by interacting with specific recognition elements in these substrates such as destruction box (D-box) and KEN-box (Pfleger *et al*., 2000). APC/C^{Cdh1} triggers the destruction of cyclins and other regulators of cell division, thereby initiating the mitotic exit (Figure 6). It has been recently shown that APC/C^{Cdh1} not only drives the degradation of mitotic regulators but also of inhibitors of differentiation (Kim and Bonni, 2007; Konishi et al., 2004; Lasorella et al., 2006). Indeed, in neuroblasts during the final cell division, cyclins and other mitotic factors are marked for degradation by APC/C^{Cdh1}. Moreover, in the following post-mitotic period, the same ubiqutin ligase targets for proteasome-dependent degradation Id2 (inhibitor of DNA binding 2, also known as inhibitor of differentiation-2) a key regulator of gene expression. Id2 inhibits a protein complex composed of E12 and E47, two basic helix-loop-helix (bHLH) transcription factors that trigger the expression of neuron-specific genes. The resulting expression of key gene products control axon growth and later events in neuronal maturation.

The E2F transcription factors. E2F transcription factors are key regulators of the cell division cycle (Dyson, 1998; Trimarchi and Lees, 2002). This family of transcription factors comprises 8 members that can be divided into distinct subclasses that have antithetical functions in promoting either cell proliferation or cell cycle exit and terminal differentiation. E2F1, 2 and 3 are activating transcription factors that stimulate cell cycle progression by inducing the expression of pro-proliferative genes, whereas E2F4 to E2F8 are transcriptional repressors that inhibit cell cycle progression by repressing these genes.

E2F3 is an important member of the activating E2F transcription factors subclass. It regulates the expression of target genes important for S-phase entry and DNA replication, cell cycle progression, mitotic events, DNA repair, cell cycle checkpoints and apoptosis (Humbert et al., 2000; Trimarchi and Lees, 2002). E2F3 has two isoforms: E2F3A and E2F3B. These two isoforms are transcribed from different exons: E2F3A is transcribed from exon1a, whereas E2F3B is transcribed from a previously unrecognized promoter within exon1b. The protein products are identical at the region encoding for their known functional parts. However, E2F3B lacks the N-terminal domain, which might be responsible for a function specific for E2F3A.

E2F6 belongs to the “repressive” E2F subclass. It contains the DNA binding and dimerizations domains (present in E2F1-5), however it lacks the C-terminal domains required for pocket-protein binding and transactivation (Trimarchi and

Lees, 2002). E2F6 acts mainly as a transcriptional repressor blocking the expression of E2F target genes. It has been shown that E2F6 represses gene expression by recruiting polycomb group proteins-containing complexes to target DNA sequences (Attwooll et al., 2005; Trimarchi et al., 2001).

TFAP4 (transcription factor AP4) is a ubiquitously expressed basic helix-loop-helix leucine-zipper (bHLH-LZ) transcription factor that binds the consensus E-box sequence 5'-CAGCTG-3' (Jung and Hermeking, 2009). TFAP4, which exclusively forms homodimers, regulates the expression of genes involved in cell proliferation and apoptosis. It has been shown that the proto-oncogene c-MYC directly induces the expression of TFAP4 by binding the CACGTG box in the first intron of the TFAP4 gene. In turn, TFAP4 directly represses the cyclin-dependent kinase inhibitor p21 and interferes with p21 up-regulation and cell cycle arrest during monoblast differentiation.

MATERIALS AND METHODS

Cell lines, synchronization method and drug treatments

Human embryonic kidney HEK293T and human glioma T98G cells obtained from ATCC were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% v/v fetal calf serum (FCS), 100 U/ml Penicillin/Streptomycin and 2 mM L-Glutamine. Human neuroblastoma SK-N-SH cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 with 10% (v/v) fetal bovine serum (FBS). All cells were maintained in a humidified 37°C incubator with 5% CO_2. To inhibit the degradation of substrate proteins, the proteasome inhibitor MG132 (10 μM) was added to HEK293T and T98G cells 5 hours prior to 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 hours. Subsequently, the culture medium was replaced with serum starvation release media (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 retinoic acid (RA, 10 μM). To measure protein half-lives, cells were incubated with 100 μg/ml cycloheximide.

Cell extract preparation, immunoprecipitations and western blot analysis

Cell extracts were prepared as follows: monolayers were washed twice with room temperature PBS and harvested. Cells were transferred into Eppendorf tubes and spun down for 30 seconds at 14,000 rpm. Cells were lysed in Triton Lysis Buffer (0.1% Triton-X100, 50 mM Tris-HCl pH 7.4, 0.25 M NaCl, 1 mM EDTA, 50 mM NaF) supplied with: 0.1 mM phenylmethyl sulfonylflouride (PMSF), 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate (NaV), tosyl phenylalanyl chloromethyl ketone (TPCK), 10 μg ml^{-1} tosyl lys chloromethyl ketone (TLCK), and a mixture of 1 μg ml^{-1} leupeptin, 10 μg ml^{-1} soybean trypsin inhibitor and 1 μg ml^{-1} aprotinin (PIN). After incubation on ice for 30 minutes, samples were subsequently centrifuged at 14,000 rpm for 20 minutes at 4°C. Supernatants were transferred into new Eppendorf tubes. Samples were assayed for protein concentration by DC Protein Assay (Lowry - Bio-Rad). For immunoprecipitations, HEK293T cells from one 15-cm plate were lysed in Triton Lysis Buffer as described above. Cell extracts (protein concentration 7-8 mg ml^{-1}) were precleared by adding 30 μl of either Protein-A or Protein-G Sepharose (Invitrogen) and incubated for 1 hour. Precleared cell extracts were then incubated with 4 μg of the appropriate antibody and 30 μl of either Protein-A or Protein-G Sepharose (Invitrogen) for 3 hours. Immunoprecipitates were washed with 1 ml of Triton Lysis Buffer for 4 times. After the last wash, immunoprecipitates were resuspended in 13 μl of 2x Laemmli sample buffer, and

boiled for 4 minutes at 90°C for denaturation. For western blot analysis, protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a pre-activated PVDF membrane (Millipore) at 22 V (for either 2 hours at room temperature or overnight at 4°C). After transfer, the PVDF membrane was stained with Ponceau-Red solution (Sigma-Aldrich) to check for transfer efficiency and even 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 hour 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 hours at room temperature. Antibodies to HA (Covance), FLAG (Sigma-Aldrich), p27 (BD Transduction Laboratories), and Actin (Santa Cruz Biotechnology or Bethyl Laboratories) were all used at 1:1000 dilution. Other antibodies were used at different dilutions: E2F3 at 1:500 (Santa Cruz Biotechnology), Cdh1 at 1:250 (Nalgene), Cdc20 at 1:500 (Santa Cruz Biotechnology), and Cyclin A at 1:2000 (Santa Cruz Biotechnology). After three washes for a total of 20-30 minutes in PBS-T, the membranes were incubated with horseradish peroxidase-linked secondary antibodies (GE Healthcare) at a 1:2000 dilution in 5% w/v low-fat dried milk dissolved in PBS-T for 1 hour, and then washed three times in PBS-T as described above. The immuno complexes were visualized by an enhanced

chemiluminescence detection system (Thermo Scientific) according to the manufacturer's instructions. If necessary, membranes were stripped by incubation at 65°C for 20-30 minutes with gentle agitation in stripping buffer (2% v/v SDS, 65 mM Tris pH 6.8, 1% v/v 2-Mercaptoethanol) and subsequently washed for 3 times in PBS-T for a total of 15 – 30 minutes.

Mammalian expression plasmids and transfection

All cDNAs were cloned in the pcDNA3.1 plasmid. Plasmids were purified by Maxi-Prep kits (Qiagen) according to the manufacturer's instructions. 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. Plasmids were diluted in 250 mM $CaCl_2$, incubated for 5 minutes at room temperature, and mixed with 2xBBS {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 additional minutes. Transfection mixtures were then added dropwise to the cells. Culture media was replaced with fresh DMEM 5 hours after transfection. The proteasome inhibitor MG132 (10 µM) was added to the transfected cells 5 hours prior collection. Cells were collected and lysed as described above 48 hours after transfection. For transfection by Metafectene, T98G cells were seeded at a confluency of 90-

100% in 6-well plates with antibiotic-free DMEM. The following day, 4.1 µg of plasmid DNA was diluted in 100 µl of serum-free and antibiotic-free DMEM, and 8 µL of Metafectene (Biontex Laboratories GmbH) was diluted in 100 µl of serum-free and antibiotic-free DMEM. The DNA/lipid complexes were gently mixed and incubated at room temperature for 20 minutes, then added dropwise to the cells. Culture media was replaced with fresh DMEM 4 hours after transfection. When indicated, 10 µM MG132 was added to inhibit proteasome-dependent degradation. Cells were collected 38-40 hours after transfection.

RNA interference

Exponentially growing cells were seeded at a confluency of 30-50% in 6-well plates with antibiotic-free DMEM. The transfection complex contained 4 µl siRNA (Dharmacon) of either lacZ, Cdh1 or Cdc20 (20 µM stock), and 4 µl Metafectene. Transfection was performed as described above. Each sample was transfected twice: a second transfection was performed 48 hours after the first transfection. The siRNA oligonucleotide sequences were 5'- AATGAGAAGTCTCCCAGTCAG -3' for Cdh1, and 5' - AAACCTGGCGGTGACCGCTAT - 3' for Cdc20. For βTrCP silencing, we used an siRNA oligomer corresponding to both nt 515–535 of human βTrCP1 and nt 262–282 of human βTrCP2 (oligo 1/2, GUG GAA UUU GUG GAA CAU CdTdT).

Ubiquitylation assay

In vitro ubiquitylation assays are performed in a 10 µl reaction containing 50 mM Tris pH 7.6, 5 mM MgCl2, 0.6 mM DTT, 2 mM ATP, 1.5 ng/µl E1 (Boston Biochem), 10 ng/µl Ubc3, 10 ng/µl Ubc5, 2.5 µg/µl ubiquitin (Sigma), 1 µM ubiquitin aldehyde, 2 µl of unlabeled in vitro transcribed/translated βTrCP or Cdh1 and 1 µl of [^{35}S] labeled in vitro transcribed/translated substrate. The reactions are incubated at 30°C for the indicated times.

Retrovirus-mediated gene transfer

For retrovirus production, GP-293 packaging cells (Clontech) were transfected with the FuGENE-6 reagent according to the manufacturer's instructions. Forty-eight hours after transfection, the virus-containing medium was collected and supplemented with 8 µg/ml polybrene (Sigma). Cells were then infected by replacing the cell culture medium with the viral supernatant for six hours.

GST fusion proteins and pull-down assay

GST-Cdh1 was expressed in E.coli (BL-21) using the pGEX 4T2 vector (Amersham). For protein purification, bacteria were grown to an optical density of 600 nm in Luria-Bertani medium, induced at 37°C with 0.1 mM isopropyl-1-thio-Dgalactopyranoside, and cultivated for 2 hours. Bacteria were then pelleted, resuspended in 3 NETN-buffer [100mM NaCl, 1mM EDTA, 50 mM Tris·HCl (pH

7.4), 0.5 % Nonidet P-40, 1mM phenylmethylsulfonyl fluoride, 5mM benzamidine], and sonicated. Insoluble material was removed by centrifugation. Thirty microliters of Glutathione-S-Sepharose 4b beads (Amersham) were added to the cleared lysate, incubated for 30 min at 4°C, and washed 3 times with NETN-buffer. GST pull-down assays were performed as follows: wild type E2F6 was in vitro transcribed/translated and ^{35}S-radiolabeled using the TNT system (Promega). GST fusion proteins were added and incubated for one hour at 4 °C. Subsequently, protein complexes were washed thoroughly with NETN buffer, subjected to SDS-PAGE, and visualized by autoradiography.

RESULTS I

$SCF^{\beta TrCP}$-MEDIATED DEGRADATION OF TFAP4 DURING NEURONAL DIFFERENTIATION.

To identify interactors of the $SCF^{\beta TrCP}$ ubiquitin ligase, we developed an immunopurification method that, unlike a traditional tandem affinity purification (TAP), which detects all binding proteins, enriches for ubiquitylated substrates, using the ability of SCF complexes to promote the *in vitro* ubiquitylation of co-purified substrates (approach #1, Figure 7). The conditions we have used are as follows. HA-βTrCP was overexpressed in HEK293T cells together with Skp1 (since the F-box domain is extremely hydrophobic, the binding to Skp1 increases its solubility). Cells were harvested 48 hours after transfection. The proteasome inhibitor MG132 was added to the medium 8 hours before collecting the cells to block the degradation of the substrate. For a single immunoprecipitation we have used 480 mg of cell extract obtained from approximately sixty 150-mm Petri dishes. We have immunoprecipitated HA-βTrCP with anti-HA affinity gel, washed and incubated the immunocomplexes at 30°C for 2 hours with gentle agitation in the presence of the following components: FLAG-Ubiquitin, His-E1, His-E2s (Ubc3 and Ubc5) and ATP. HA-βTrCP and interacting proteins (substrates are not released from the ligase after ubiquitylation) were eluted twice by competition with an HA peptide (1 mg/ml, 40 minutes at R/T on a rotating wheel). The FLAG-polyubiquitylated products generated in this reaction

were subsequently subjected to a second immunoprecipitation with anti-FLAG M2 affinity gel and then eluted by competition with a FLAG-peptide (250 μg/ml, 40 min at R/T on a wheel). The eluate was digested and directly subjected to liquid chromatography-tandem mass spectrometry (LC/MS/MS) at the proteomics facility of the Netherlands Proteomics Centre. Alternatively, traditional tandem affinity purification was carried out (approach #2, Figure 8). Cells were infected with retroviruses encoding 2xFLAG-2xHA-βTrCP and treated with either MG132 to inhibit proteasome-dependent degradation or DMSO vehicle alone. Purifications were performed sequentially for the FLAG and HA tags prior to analysis of the entire sample by mass spectrometry. Notably, this technique utilizes the low expression of the exogenous protein driven by the retroviral LTR promoter, minimizing overexpression artifacts. Finally, we have used a third approach in which the βTrCP1(R474A), a mutant that is unable to interact with its substrate, was used as a negative control (approach #3, Figure 9).

Using all three immunopurification methods, we recovered peptides corresponding to the basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor TFAP4. To confirm the binding between βTrCP and TFAP4, we overexpressed different FLAG-tagged F-box proteins and the APC/C activators Cdh1 and Cdc20 in HEK293T cells. We then carried out FLAG immunoprecipitations to examine the interaction with endogenous TFAP4. We

found that βTrCP1 and its paralog βTrCP2 were the only proteins able to coimmunoprecipitate with TFAP4 (Figure 10A).

Structural studies have shown that arginine 474 in the WD40 β-propeller of βTrCP contacts the destruction motif of the βTrCP substrate βcatenin. We compared the ability of wild type βTrCP2 and the βTrCP2(R474A) mutant to pull down endogenous TFAP4. Whereas wild type βTrCP2 immunoprecipitated TFAP4, the βTrCP2(R474A) mutant did not (Figure 10B) suggesting that TFAP4 is a substrate of $SCF^{\beta TrCP}$.

To explore if $SCF^{\beta TrCP}$ was directly responsible for TFAP4 ubiquitylation, we reconstituted TFAP4 ubiquitylation *in vitro*. Immunopurified βTrCP1, but not an inactive βTrCP1(ΔF box) mutant, induced the ubiquitylation of TFAP4 *in vitro* (Figure 10C).

To test whether $SCF^{\beta TrCP}$ regulates TFAP4 stability, we analyzed TFAP4 turnover upon depletion of βTrCP. Knockdown of βTrCP by RNA interference using a double-stranded RNA (dsRNA) oligonucleotide that efficiently silences both βTrCP1 and βTrCP2 led to an increase of the TFAP4 half-life (Figure 11).

Substrates of the $SCF^{\beta TrCP}$ ubiquitin ligase share a conserved DSGXXS degron that is bound by βTrCP. We found that TFAP4 has a modified motif in which the first serine is replaced by glutamic acid similarly to other known substrates of βTrCP (Figure 12A). We generated a mutant TFAP4 protein in which the

glutamic acid 135 and serine 139 residues were substituted with alanine. This TFAP4 mutant was not able to pull down endogenous βTrCP in immunoprecipitation experiments (Figure 12B) and was markedly stabilized as shown by half-life experiments conducted in several cell lines (Figure 12C).

It has been shown that TFAP4 is down-regulated during a temporal window from embryonic to adult brain (Jung et al., 2008). In addition, TFAP4 was shown to form a transcriptional complex that represses the expression of neuron-specific genes in non-neuronal cells and participates in regulating the developmental expression of neuron-specific genes in the brain. To test if TFAP4 is down-regulated during neuronal differentiation, we used two established in vitro models of neuronal differentiation. Firstly, we employed SK-N-SH neuroblastoma cells, which are known to undergo arrest in G0/G1 and differentiate into the neural lineage after treatment with retinoic acid. We observed that TFAP4 is down-regulated (Figure 13A) and undergo accelerated degradation (Figure 13B) in SK-N-SH cells treated with retinoic acid. Furthermore, we employed mouse embryonic stem cells (ES) plated on gelatin-coated tissue culture dishes in a serum-free medium supporting neural differentiation (N2-B27). These cells commit synchronously along the neural fate and eventually undergo terminal neuronal differentiation. Similarly to what observed in SK-N-SH cells, TFAP4 is down-regulated in ES cells during neuronal differentiation (Figure 13C).

Next, to study the biological significance of βTrCP-mediated degradation of TFAP4 during neuronal differentiation, we expressed the TFAP4 stable mutant (in which the glutamic acid 135 and serine 139 residues were substituted with alanine) in SK-N-SH neuroblastoma cells. We then asked whether degradation of TFAP4 is required for neuronal differentiation. We observed that persistent levels of TFAP4 impair neuronal differentiation as indicated by the levels of neurofilament 68, a marker of neuronal differentiation (Figure 14).

RESULTS II

CDH1-MEDIATED DEGRADATION OF E2F6.

To identify novel substrates of APC/C^{Cdh1} we expressed 2xFLAG-2xHA epitope-tagged Cdh1, the substrate recognition subunit of the APC/C ubiquitin ligase, in HEK293T cells. We analyzed proteins that co-purify with FLAG-HA-epitope-tagged Cdh1 by LC/MS/MS after sequential FLAG-HA immunoprecipitations and peptide elution (see approach #2) and found that Cdh1 immunocomplexes contain two members of the E2F transcription factors family, namely E2F3 (next chapter) and E2F6. To confirm that Cdh1 physically interacts with E2F6, we overexpressed FLAG-tagged Cdh1 or the other APC/C activator Cdc20 in HEK293T cells. After FLAG immunoprecipitation we observed that Cdh1, but not Cdc20, interacts with endogenous E2F6 (Figure 15A). Conversely, when we pulled down HA-tagged E2F6 we coimmunoprecipitated endogenous Cdh1 (Figure 15B). A direct E2F6-Cdh1 physical interaction was confirmed by a pull-down assay with GST-Cdh1 fusion protein and E2F6 translated in vitro (Figure 15C). Altogether these results indicate that Cdh1 interacts with E2F6.

Before analyzing a possible role of Cdh1 in targeting E2F6 for degradation, we sought to find the physiological conditions required to degrade E2F6 in cells. We synchronized human glioblastoma T98G cells in G0 by serum starvation and released them from quiescence by serum addition. As shown in figure 16A, E2F6 levels were low in G0 and then increased in S phase paralleling Cyclin A levels

and inversely correlating with p27 levels. To detect E2F6, we used two different antibodies, which we first characterized (Figure 16B). Both antibodies were able to detect by immunoblotting endogenous E2F6 in asynchronous HEK293T cell lysates and in vitro translated HA-tagged E2F6, but not mock in vitro translated samples.

The results shown in figure 16A indicate that levels of E2F6 are low in G0 cells. To test if E2F6 down-regulation in G0 is due to proteasome-dependent degradation, asynchronous T98G cells were serum-deprived and the proteasome inhibitor MG132 was added for the last 6 hours (Figure 16C). Treatment with MG132 blocked the down-regulation of E2F6. Similar results were obtained in IMR90 primary fibroblasts (Figure 16D).

To investigate whether Cdh1 targets E2F6 for degradation in cells exiting the cell cycle, we transfected T98G cells with a double-strand RNA oligonucleotide that silences Cdh1. T98G cells were then serum-deprived to allow them to exit the cell cycle. Cdh1 knockdown inhibited the degradation of E2F6 in G0 (Figure 17A). Similarly, the degradation of Skp2, an established substrate of APC/C^{Cdh1}, was blocked in cells in which Cdh1 was knocked down. The effects seen on E2F6 levels in Cdh1-silenced cells were not due to a change in their cell cycle progression, as indicated by the cell cycle profile determined by FACS analysis (data not shown).

Next, we tested if Cdh1 is able to ubiquitylate E2F6 in vitro. In vitro translated E2F6 was ubiquitylated in the presence of E1, UbcH10, APC/C^{Cdh1}, ubiquitin and ATP (Figure 17B), however we were unable to detect stimulation of E2F6 ubiquitylation by Cdh1, likely because the reticulocyte lysate contains high levels of endogenous Cdh1 that contributes to the ubiquitylation of APC/C substrates (Crane et al., 2004).

We observed that E2F6 contains two minimal D-box motifs (RxxL) and one KEN-box motif at residues 41-44, 54-57 and 175-177, respectively (Figure 18A-B). These motifs are conserved in different species (Figure 18C). To test if these motifs are required for Cdh1-dependent degradation of E2F6, we generated HA-tagged E2F6 mutants in which the arginine and the leucine in the D-boxes and the lysine, glutamic acid and the asparagine in the KEN-box were replaced by alanine. We expressed these mutants in T98G cells by retroviral transduction. T98G cells were then serum starved to analyze the G0 specific degradation of the E2F6 mutants (Figure 18D). Surprisingly, the E2F6 mutants were degraded similarly to wild type E2F6, suggesting that the D-boxes and the KEN-box have no role in targeting E2F6 for Cdh1-dependent degradation.

It has been shown that APC/C^{Cdh1}-mediated degradation couples cell cycle exit and neuronal differentiation (Huynh et al., 2009; Konishi et al., 2004; Stegmuller and Bonni, 2005; Yang et al., 2010; Zhao et al., 2008). To test if E2F6 is down-regulated during neuronal differentiation, we used two established in vitro models

of neuronal differentiation (as above - RESULTS I). We analyzed the expression of E2F6 in SK-N-SH neuroblastoma cells induced to differentiate by retinoic acid and in mouse embryonic stem cells induced to commit and differentiate into the neural lineage. In both systems E2F6 was down-regulated and underwent accelerated degradation during neuronal differentiation (Figure 19A-B and data not shown).

Finally, to examine whether APC/C^{Cdh1} controls E2F6 expression in SK-N-SH cells exiting the cell cycle, we analyzed E2F6 levels upon Cdh1 silencing in SK-N-SH cells after retinoic acid addition. Cdh1 knockdown by RNA interference lead to E2F6 stabilization (Figure 19C).

RESULTS III

CDH1-MEDIATED DEGRADATION OF E2F3.

Immunoaffinity chromatography followed by mass spectrometry lead to the identification of E2F3 in Cdh1 complexes (see above - RESULTS II). To confirm the Cdh1-E2F3 interaction, the two co-activators of the APC/C, Cdh1 and Cdc20, were screened for their ability to bind to E2F3A and E2F3B. HEK293T cells were transfected with either Cdh1 or Cdc20 mammalian expression plasmids. Both E2F3A and E2F3B were co-immunoprecipitated with FLAG-tagged Cdh1, but not with FLAG-tagged Cdc20 (Figure 20).

To test whether Cdh1 is involved in the degradation of E2F3, we transfected T98G cells with either HA-tagged E2F3A, E2F3B or Skp2, along with either FLAG-tagged Cdc20 or FLAG-tagged Cdh1 (Figure 21). Ectopic expression of Cdh1 lead to decreased levels of both E2F3A and E2F3B, whereas over-expression of Cdc20 had no effect. As expected, Cdh1 over-expression caused a decrease in the levels of Skp2, a known substrate of APC/C^{Cdh1} (Bashir et al., 2004).

To examine whether the decrease of E2F3 protein levels upon Cdh1 overexpression is due to proteasome-dependent degradation, T98G cells were transfected with a construct expressing either HA-tagged E2F3A or E2F3B, along with a construct expressing FLAG-tagged Cdh1. The proteasome inhibitor MG132 was added 7 hours before collecting the cells (Figure 22). The decrease

in the amount of both E2F3A and E2F3B induced by Cdh1 was prevented by the addition of MG132, indicating that APC/C^{Cdh1} targets both E2F3A and E2F3B for proteasome-dependent degradation. Next we asked whether APC/C^{Cdh1} ubiquitylates E2F3A in vitro. Cdh1 was immunopurified and incubated with in vitro translated E2F3 in the presence of ubiquitin, UBCH10 and E1. As shown in figure 23, E2F3A is ubiquitylated in vitro by APC/C^{Cdh1}.

Next, we examined the abundance of E2F3A and E2F3B during neuronal differentiation. We employed SK-N-SH cells induced to differentiate upon retinoic acid treatment (Figure 24A). As shown in figure 24B, E2F3A levels decreased steadily during neuronal differentiation, whereas E2F3B did not change. As expected, levels of Cyclin A decreased and levels of p27 increased as cells exit cell cycle and differentiate.

To test whether Cdh1 is required for the degradation of E2F3 during neuronal differentiation, we used RNAi to reduce the endogenous expression of Cdh1 or Cdc20 in SK-N-SH neuroblastoma cells. Seven hours after siRNA transfection, retinoic acid was added to induce neuronal differentiation. Cells were collected at 0, 2, 3 and 4 days after RA treatment and analyzed by western blotting.

As shown in figure 25, silencing of Cdh1 (but not Cdc20) reduced the degradation of E2F3A and induced accumulation of E2F3B.

DISCUSSION

In this study, we have used immunoaffinity chromatography followed by mass spectrometry analysis to identify novel substrates of $SCF^{\beta TrCP}$ and APC/C^{Cdh1}, two ubiquitin ligases involved in cell cycle exit and neuronal differentiation.

Historically, it has been much easier to identify the ubiquitin ligase targeting a specific substrate (using candidate-based approaches and/or a binding screen with different ubiquitin ligases) than vice versa (i.e., find substrates of specific ubiquitin ligases). For example, despite the large number of E3 ligases and the large number of labs studying E3s, as yet only a few studies have successfully employed the yeast 2-hybrid system to identify a substrate using a ubiquitin ligase as a bait. Failure is likely due to the fact that the interaction with the substrate is transient and in the case of F-box proteins phosphorylation-dependent. Our approach has not only led to the identification of substrates of βTrCP and Cdh1, but it has also provided a potentially powerful method for the identification of many E3 substrates.

Using this approach we have found that $SCF^{\beta TrCP}$ targets for proteasome-dependent degradation TFAP4 as cells enter the quiescent state and activate a program that triggers neuronal differentiation. TFAP4 is a basic helix-loop-helix transcription factor, which has been shown to act both as an activator and a repressor for the expression of different target genes. We have found that (i) βTrCP interacts with and ubiquitylates TFAP4, (ii) βTrCP controls TFAP4

degradation, (iii) a conserved phosphodegron is required for the degradation of TFAP4 and its interaction with βTrCP, (iv) TFAP4 is destabilized during neuronal differentiation, (v) βTrCP-dependent degradation of TFAP4 is necessary for proper neuronal differentiation of neuroblastoma cells. Additional studies are needed to understand the biological function of TFAP4 degradation mediated by βTrCP. A key point would be to investigate the function of TFAP4 degradation in the developing brain *in vivo*. For instance, what is the effect of expressing the degradation-resistant TFAP4 mutant in the developing brain in mice? Expressing the non-degradable TFAP4 mutant by in utero electroporation or generating TFAP4 knock-in mice in which the murine wild-type TFAP4 gene is replaced by one that encodes a non-degradable form of the substrate would be key approaches to study TFAP4 degradation *in vivo*.

Additional points to be addressed are: to identify the kinase that triggers TFAP4 phosphorylation on Ser139 and to determine the TFAP4 target genes whose expression is affected by TFAP4 degradation. To this aim, the identification of the full range of TFAP4 target genes would be required. We believe that an unbiased approach of chromatin immunoprecipitation (ChIP) in combination with sequencing (ChIP-seq) would be the method of choice.

An immunopurification method similar to the one used for βTrCP has been used to identify novel substrates of the APC/C^{Cdh1} ubiquitin ligase. This approach has led to the identification of two E2F transcription factors, namely E2F3 and E2F6,

as novel targets of APC/C^{Cdh1}. Both transcription factors interact with Cdh1 and are targeted for proteasome-dependent degradation in T98G cells that shift from proliferation to assume their quiescent state. Similarly, in differentiating neuroblastoma cells, both E2F3A and E2F6 are degraded via a Cdh1-mediated mechanism, as Cdh1 silencing prevents E2F3 and E2F6 destabilization. However, the specific domains in E2F3 and E2F6 responsible for the interaction with Cdh1 remain to be determined. The identification of these regions would enable us to clone E2F3 and E2F6 degradation-resistant mutants and analyze the effects of defective E2F3 and E2F6 degradation during cell cycle exit and differentiation in both in vitro and in vivo models.

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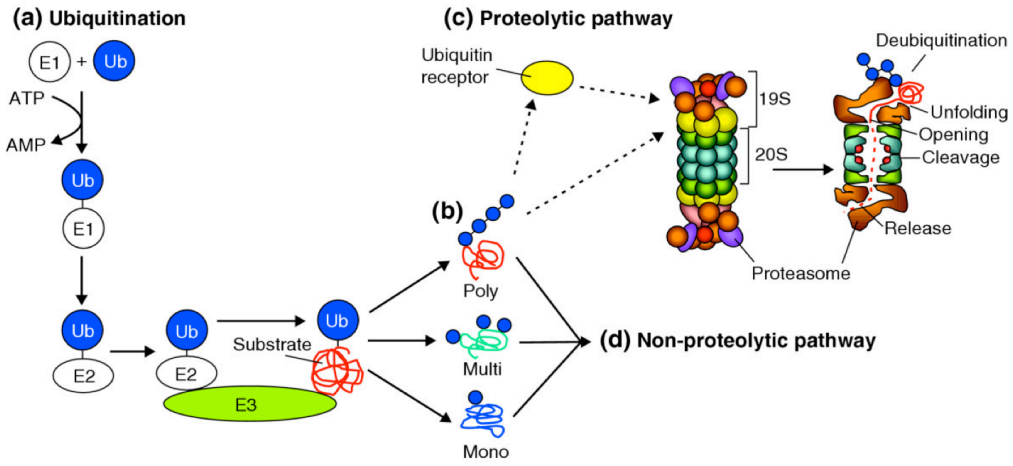


Figure 1. The ubiquitin-proteasome system (UPS).

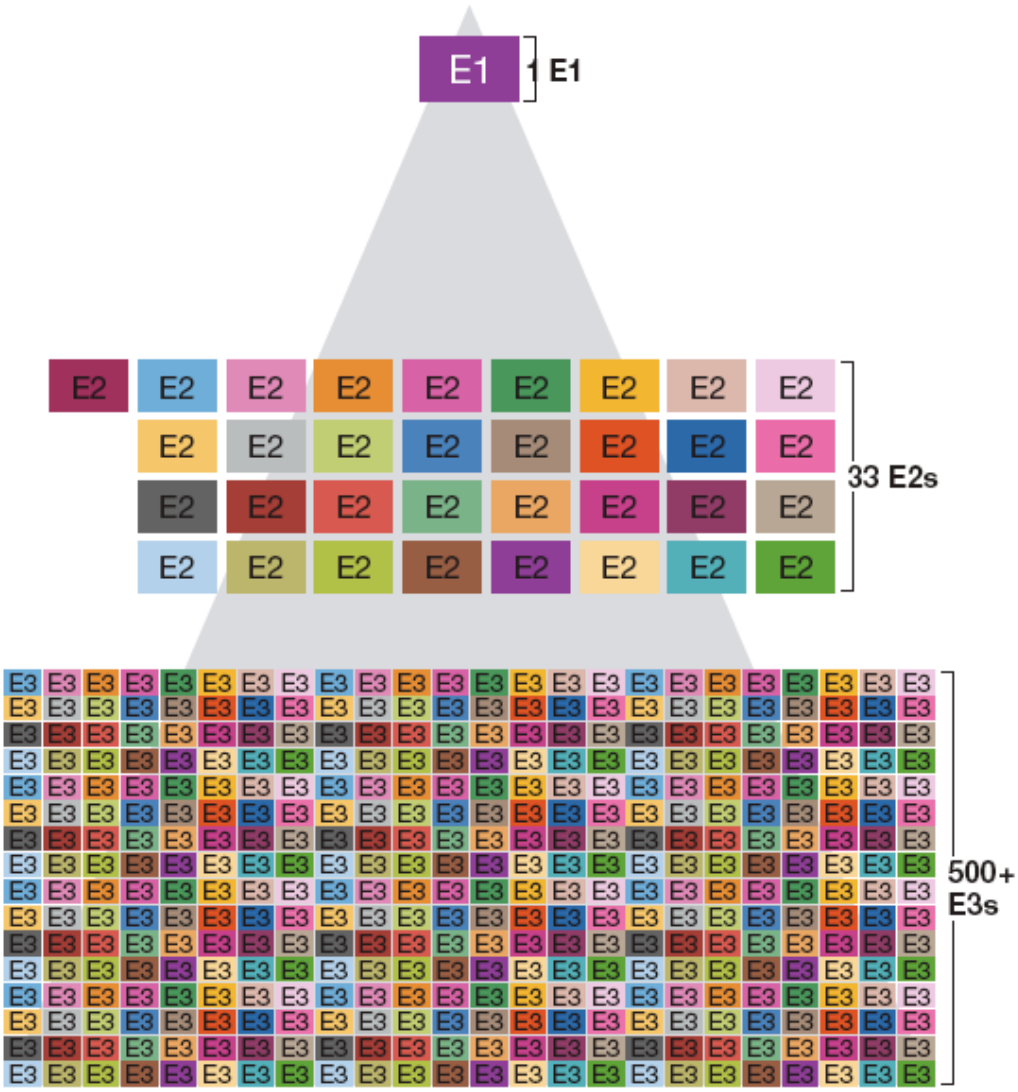


Figure 2. The hierarchical structure of the UPS (courtesy of Matt Petroski).

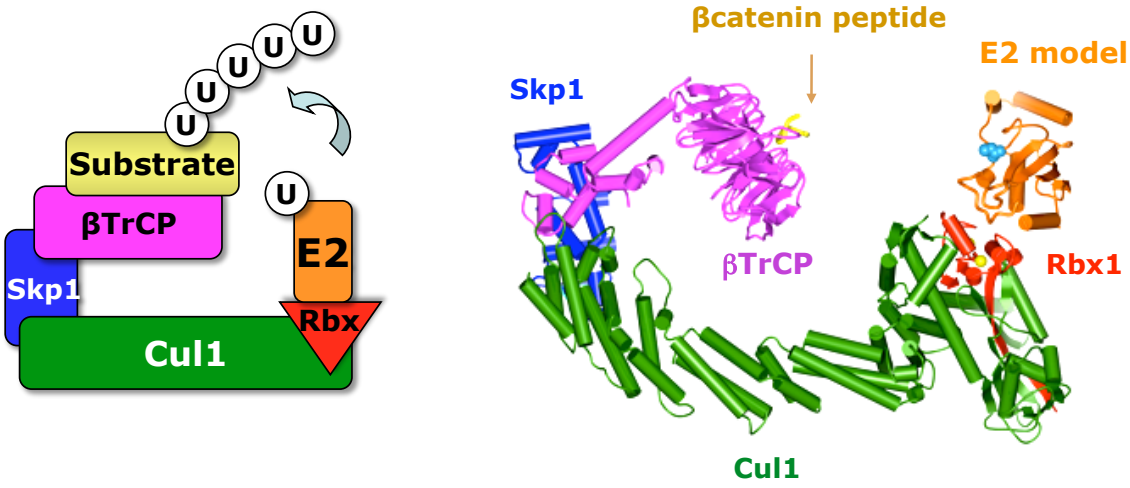


Figure 3. Structure of the $SCF^{\beta TrCP}$ ubiquitin ligase.

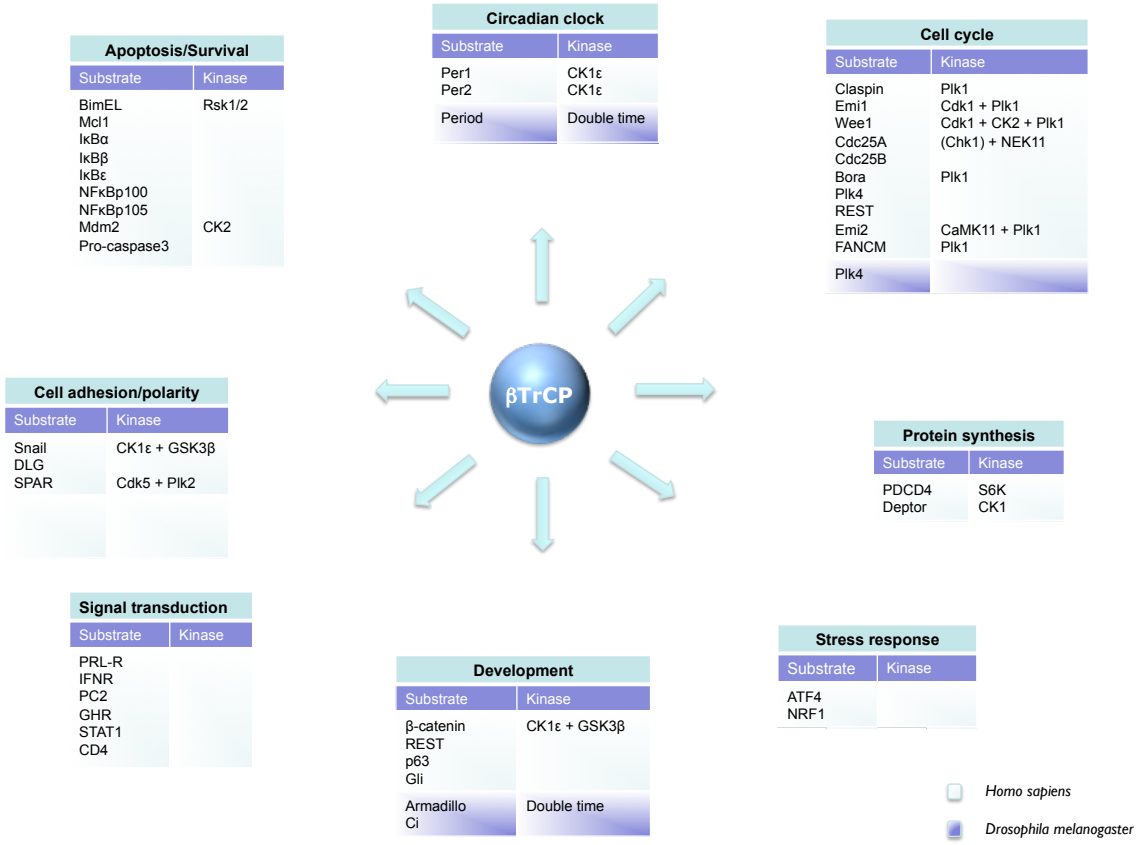


Figure 4. Biological processes in which the SCF$^{\beta TrCP}$ ubiquitin ligase is involved.

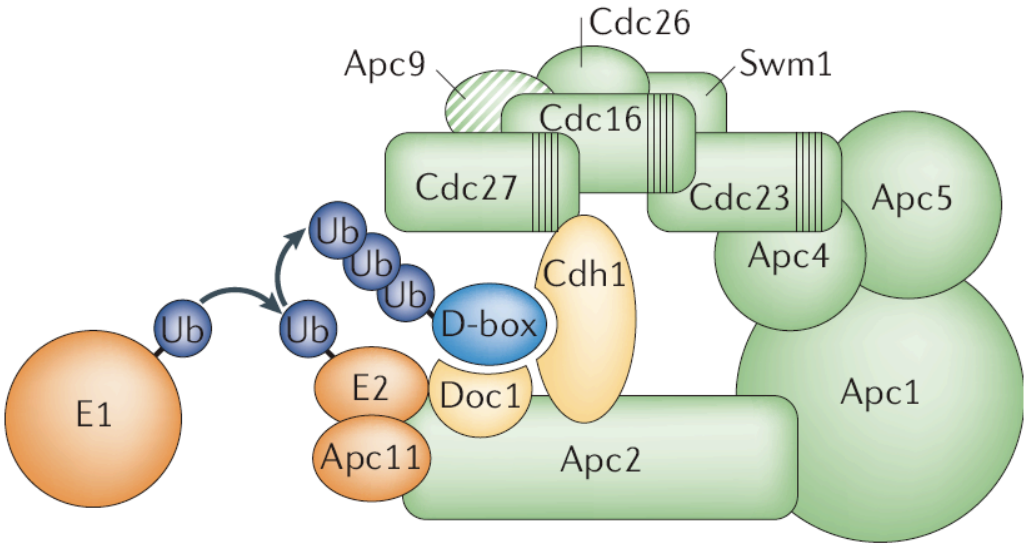


Figure 5. Structure of the APC/C^{Cdh1} ubiquitin ligase.

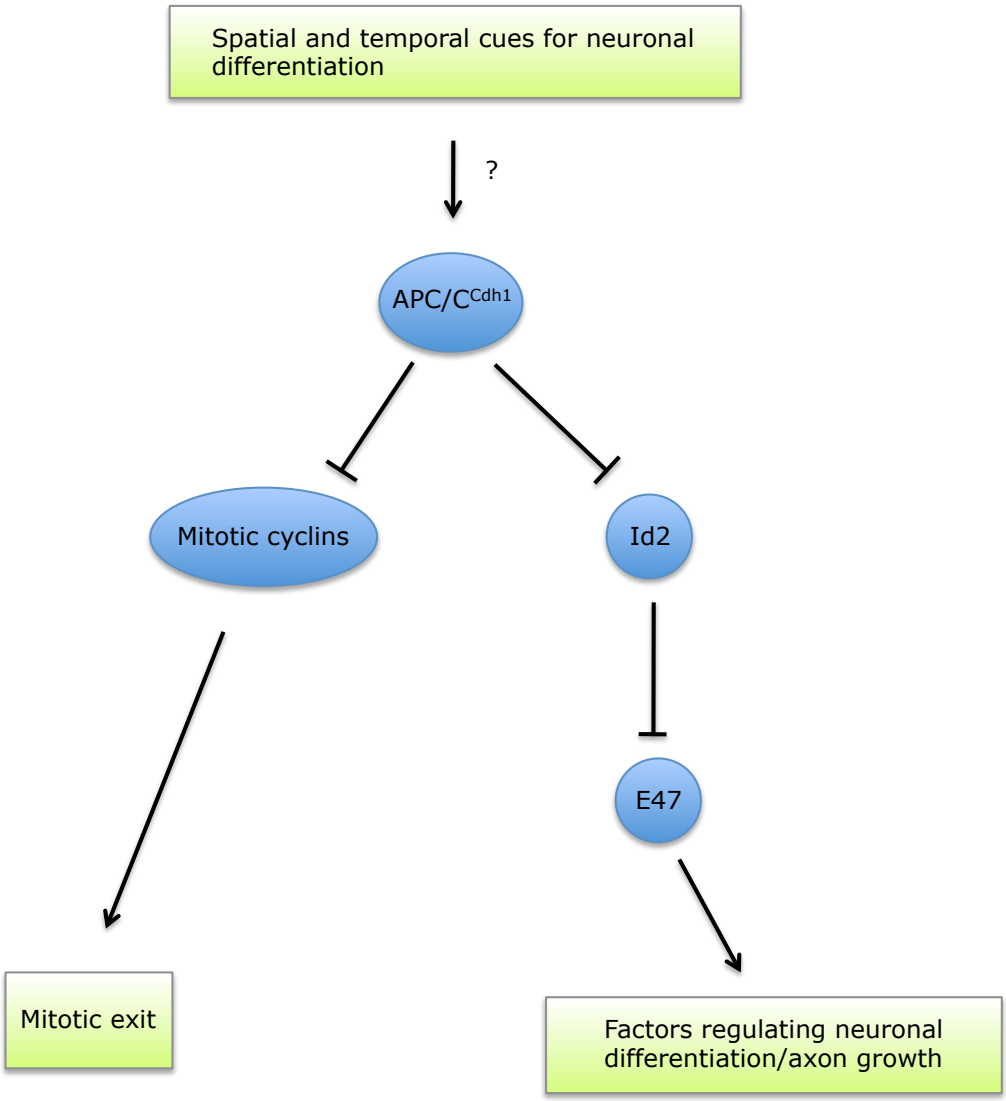


Figure 6. Functions of the APC/C^{Cdh1} ubiquitin ligase.

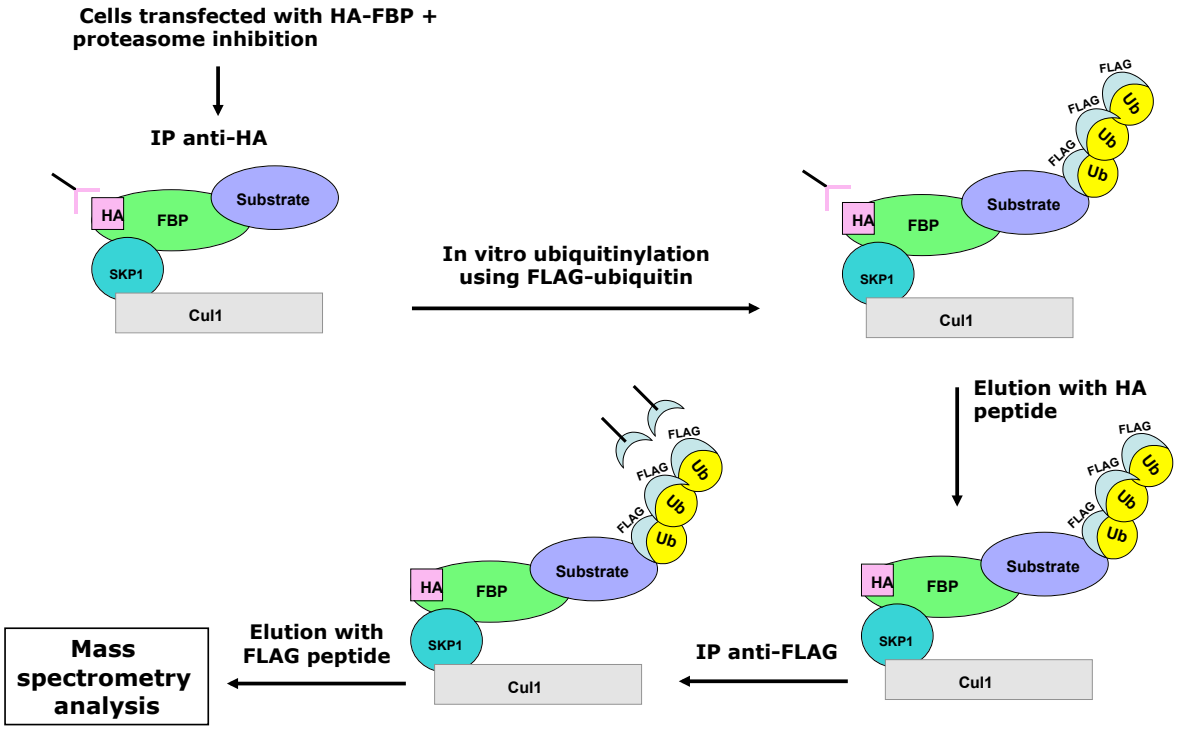
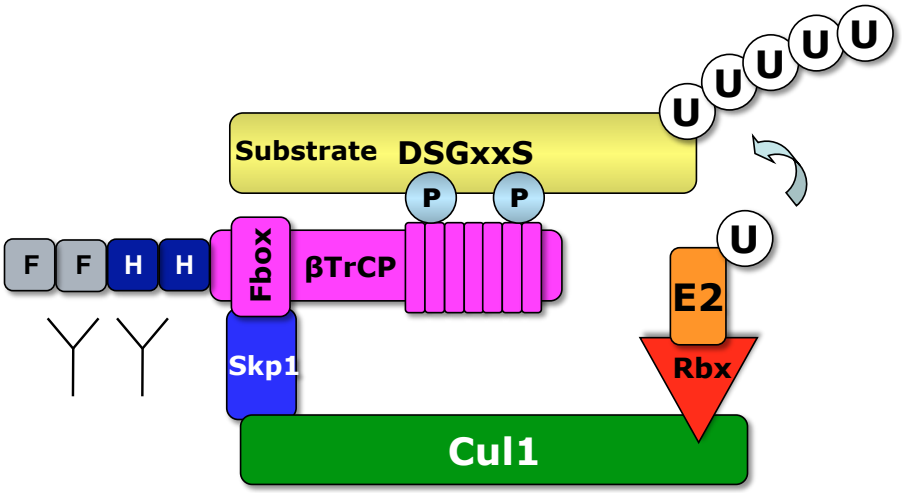
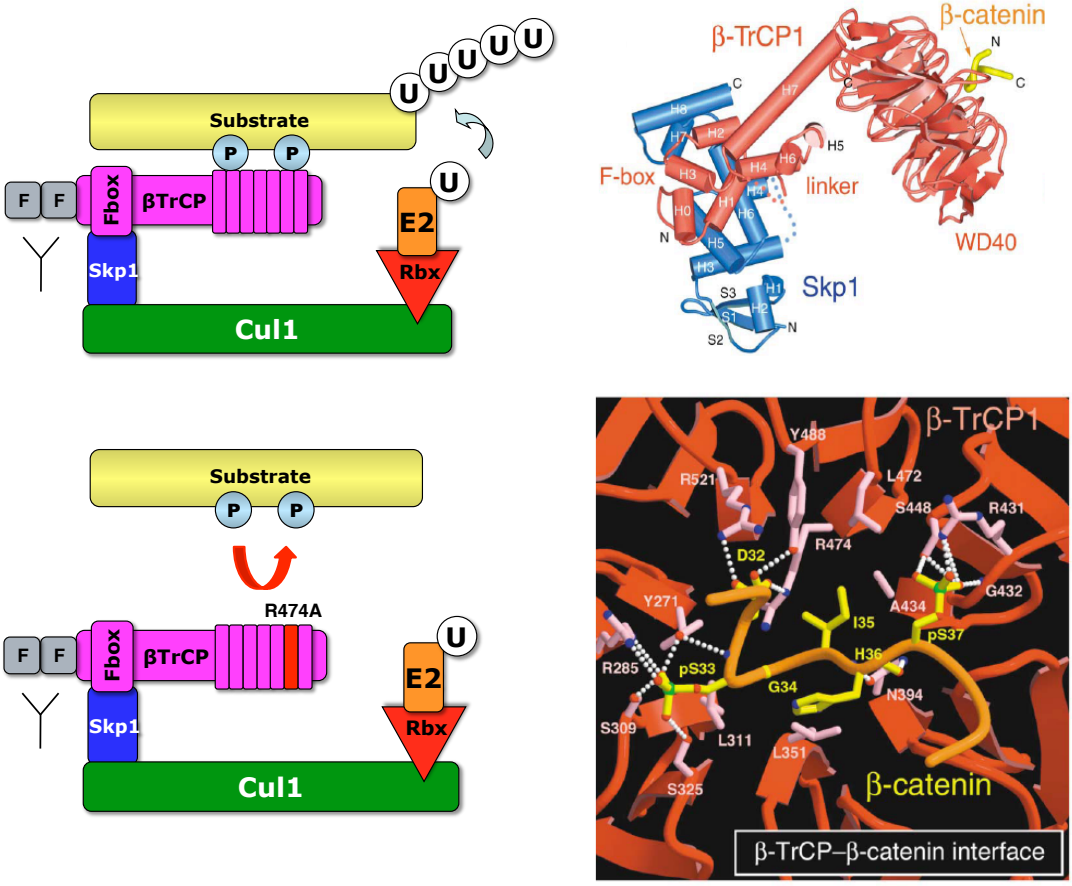


Figure 7. Identification of substrates of E3 ubiquitin ligases (approach 1).



1. Proteasome inhibition (MG132)
2. FLAG IP
3. FLAG elution
4. HA IP
5. HA elution
6. Mass spectrometry analysis

Figure 8. Identification of substrates of E3 ubiquitin ligases (approach 2).



1. Proteasome inhibition (MG132)
2. FLAG IP
3. FLAG elution
4. Mass spectrometry analysis

Figure 9. Identification of substrates of E3 ubiquitin ligases (approach 3).

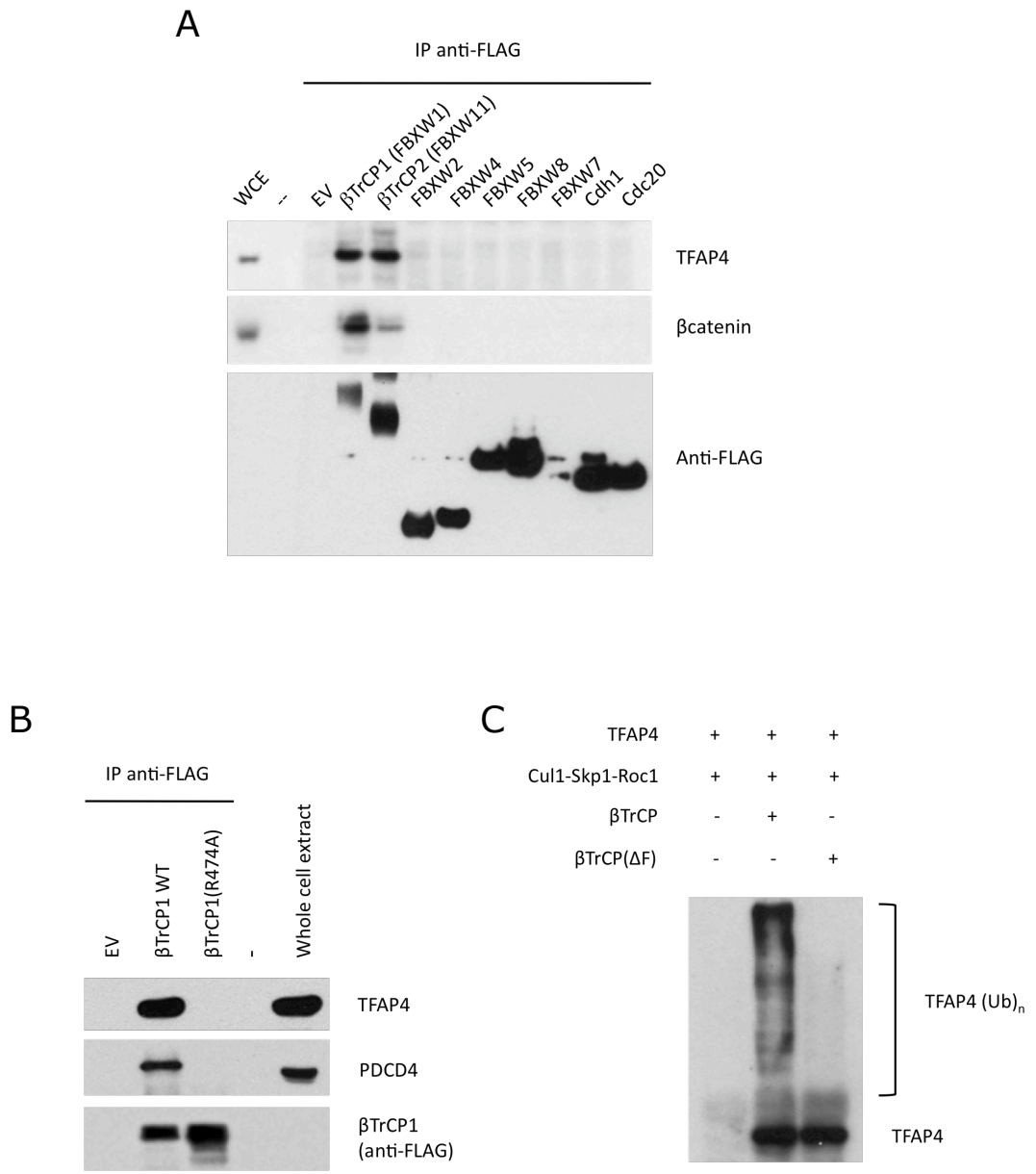


Figure 10. βTrCP interacts with and ubiquitylates TFAP4.

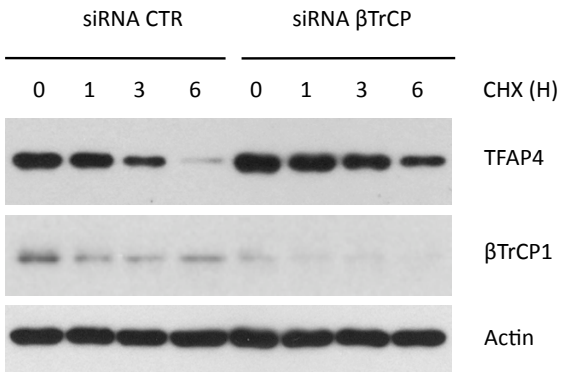
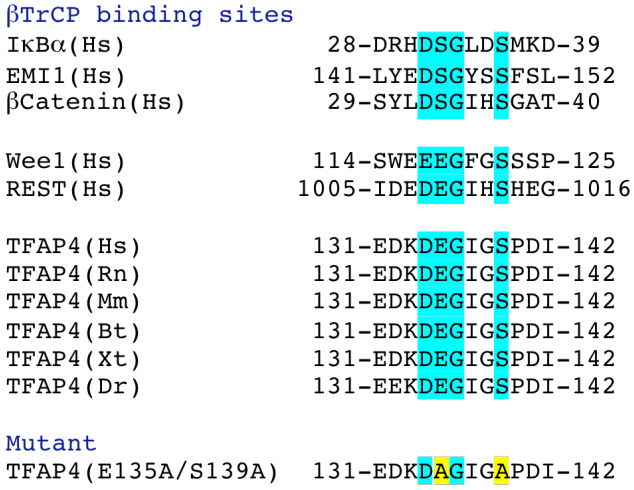
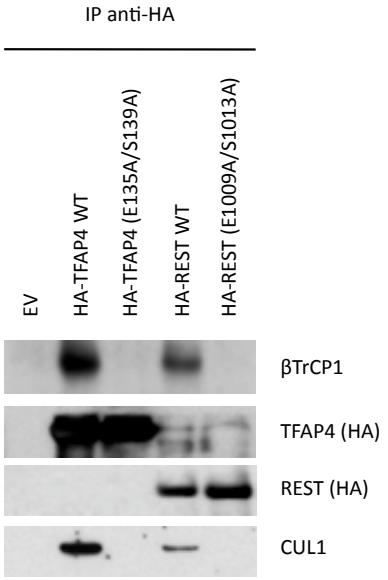


Figure 11. βTrCP controls TFAP4 degradation.

A



B



C

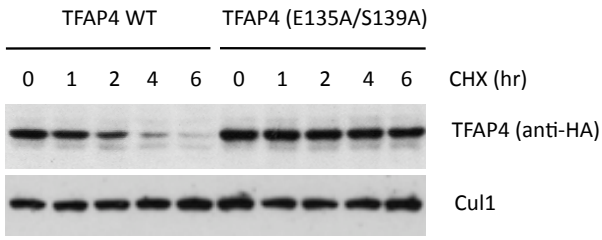


Figure 12. The interaction of TFAP4 with βTrCP depends on a conserved phospho-degron.

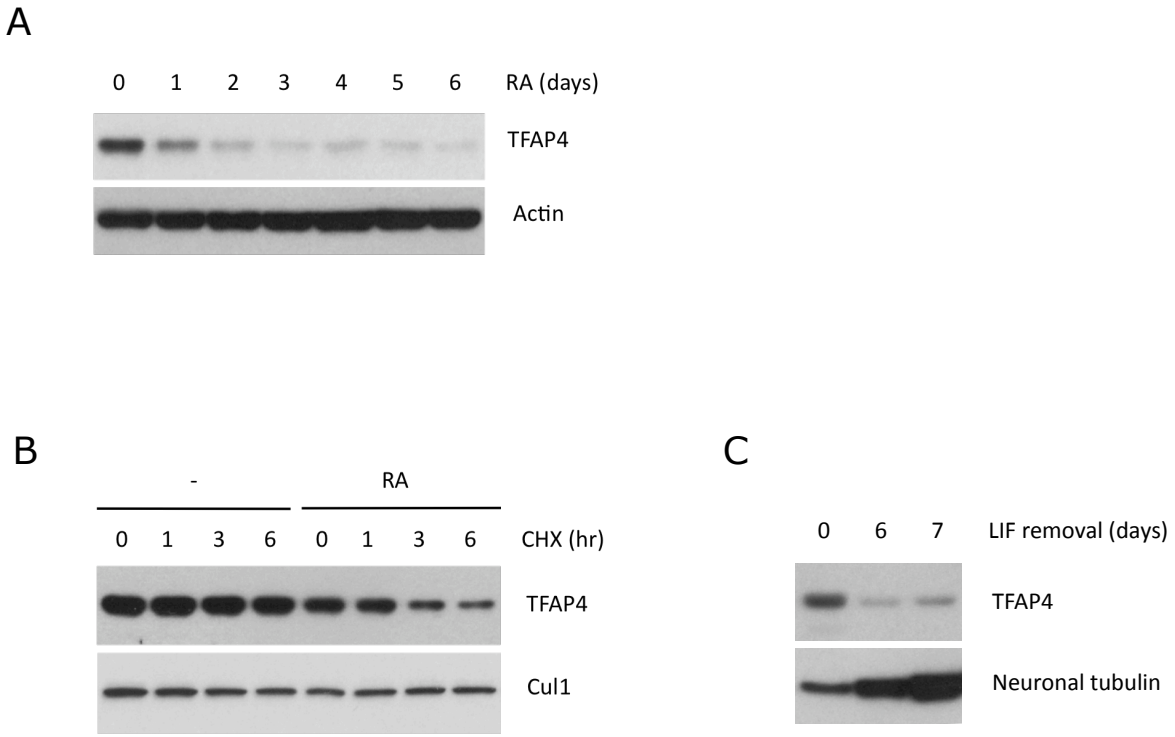


Figure 13. TFAP4 is degraded during neuronal differentiation.

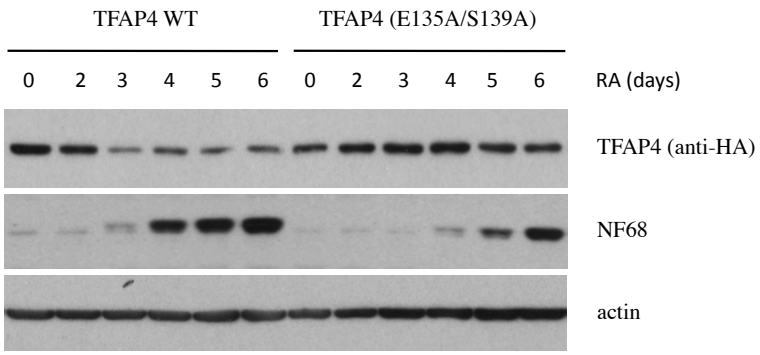


Figure 14. Defective degradation of TFAP4 delays neuronal differentiation.

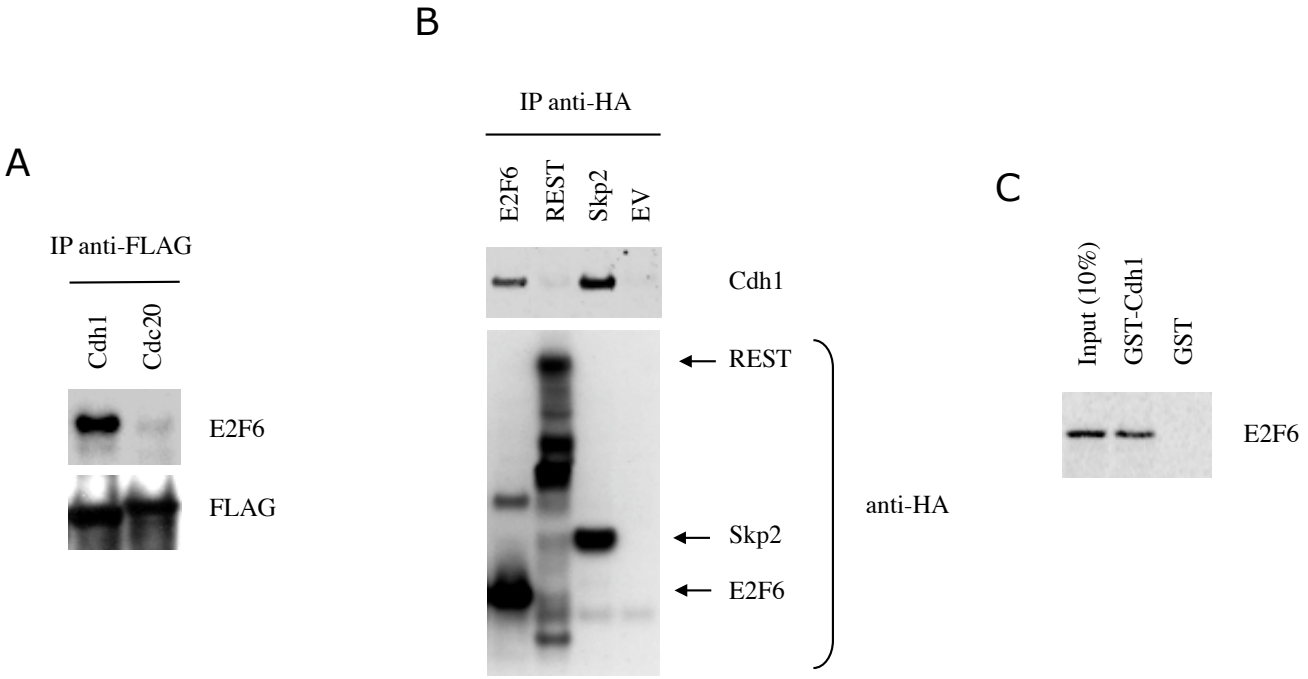


Figure 15. E2F6 binds Cdh1 in vivo and in vitro

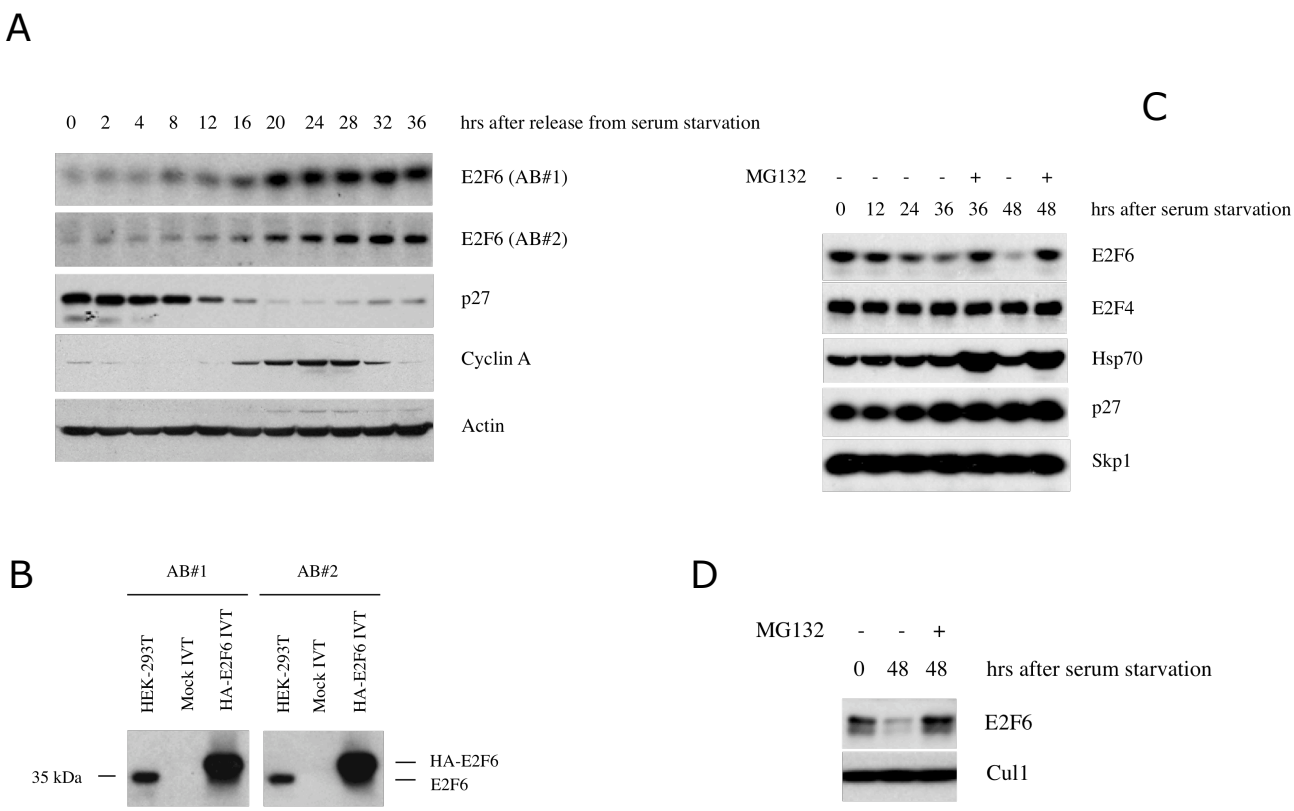


Figure 16. E2F6 is degraded in G0.

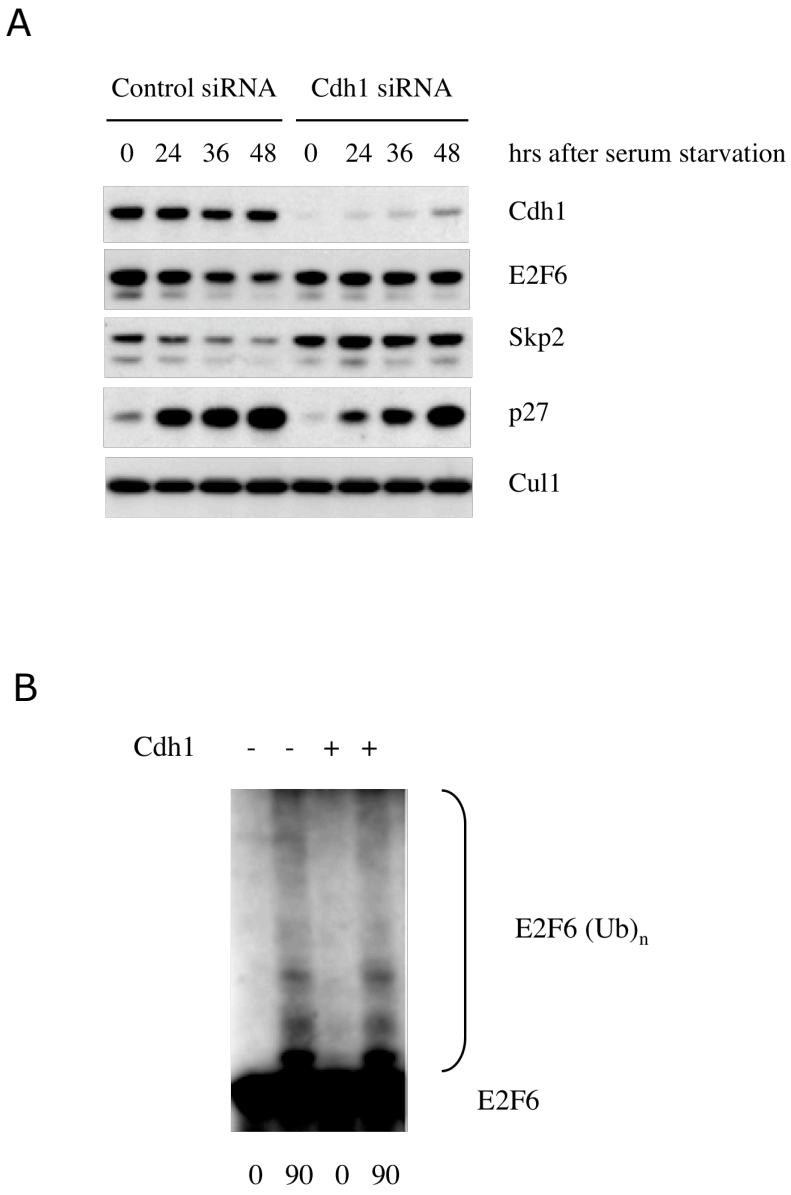


Figure 17. Cdh1-mediated degradation of E2F6 in G0.

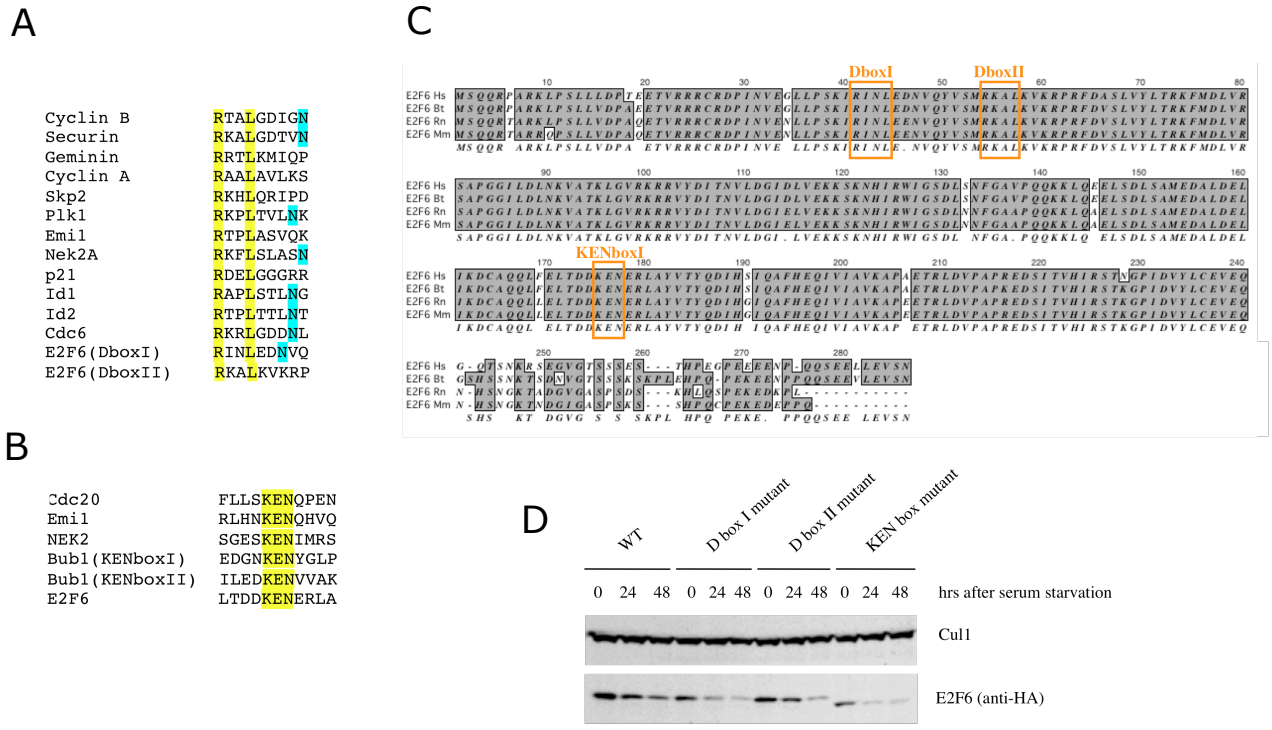


Figure 18. The D-box and the KEN-box motifs in E2F6 are not required for its degradation.

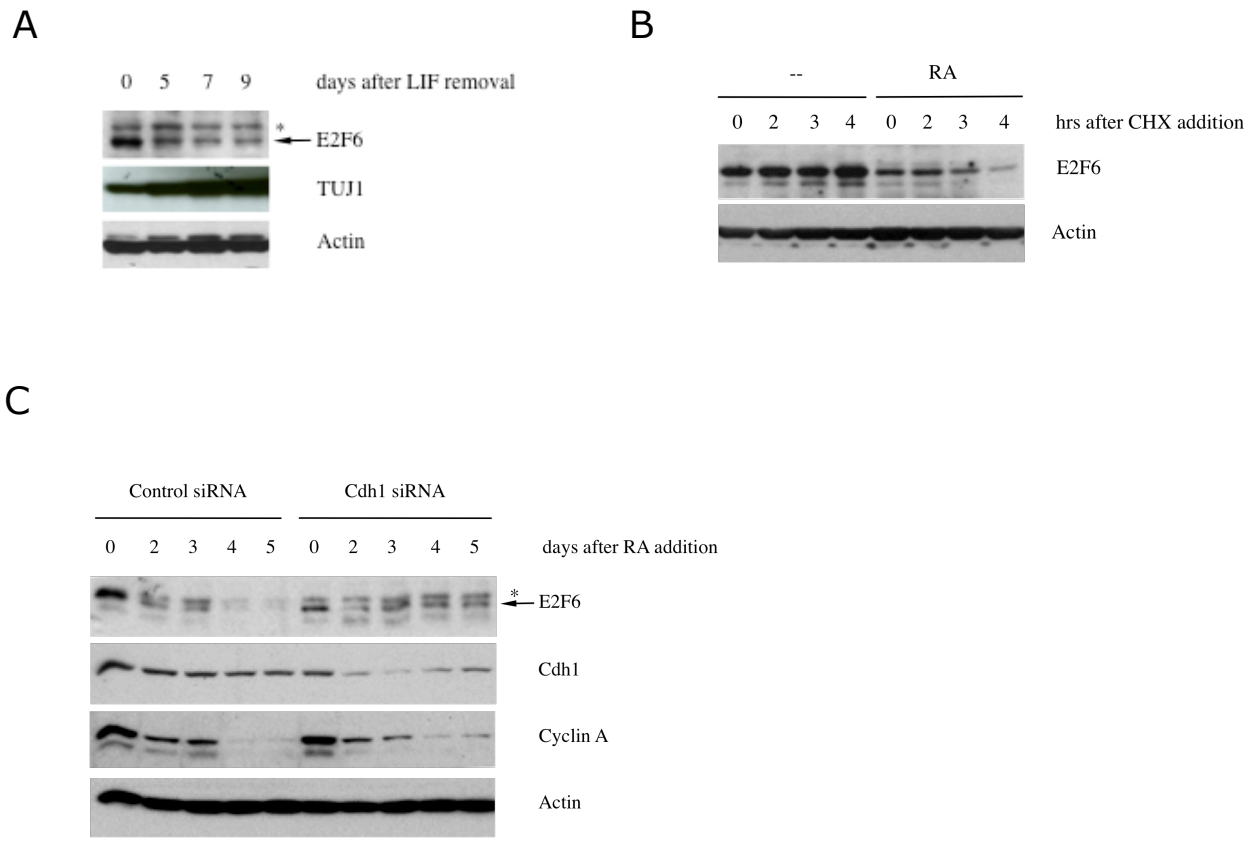


Figure 19. Cdh1 targets E2F6 for degradation during neuronal differentiation.

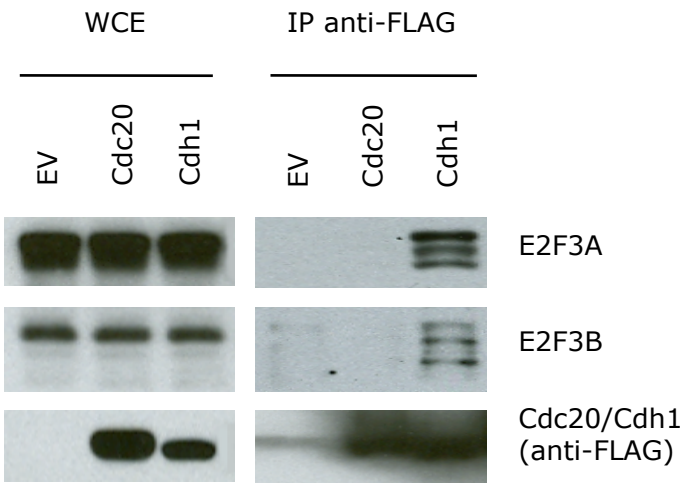


Figure 20. E2F3 interacts with Cdh1 and not with Cdc20.

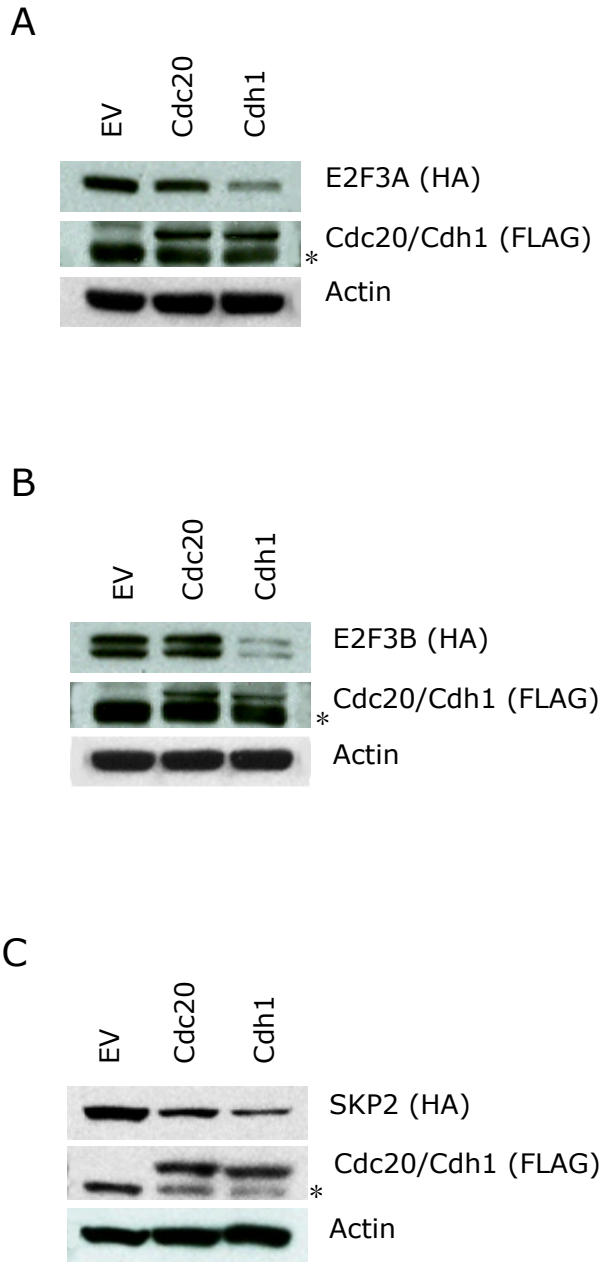


Figure 21. Cdh1 overexpression lead to a decrease in E2F3 protein levels.

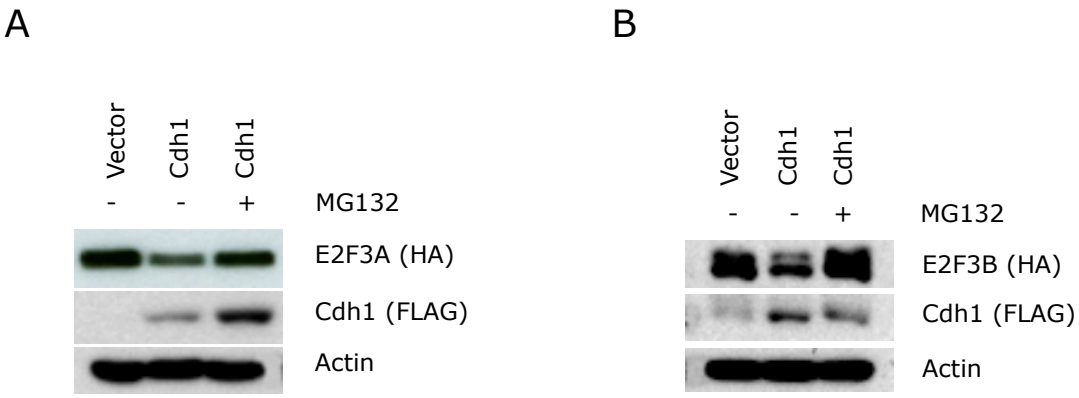


Figure 22. Cdh1-dependent degradation of E2F3 is prevented by proteasomal inhibition.

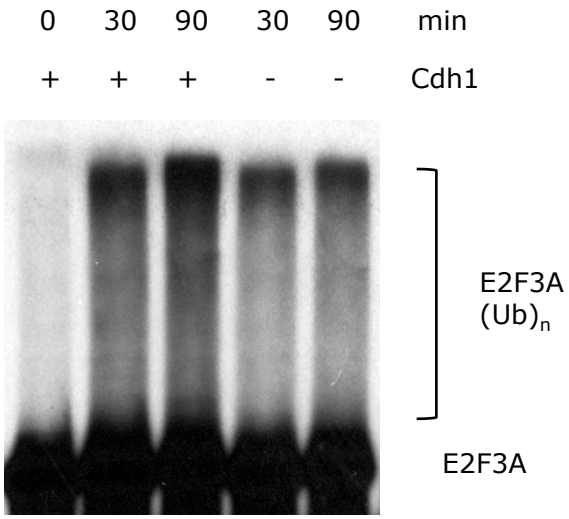
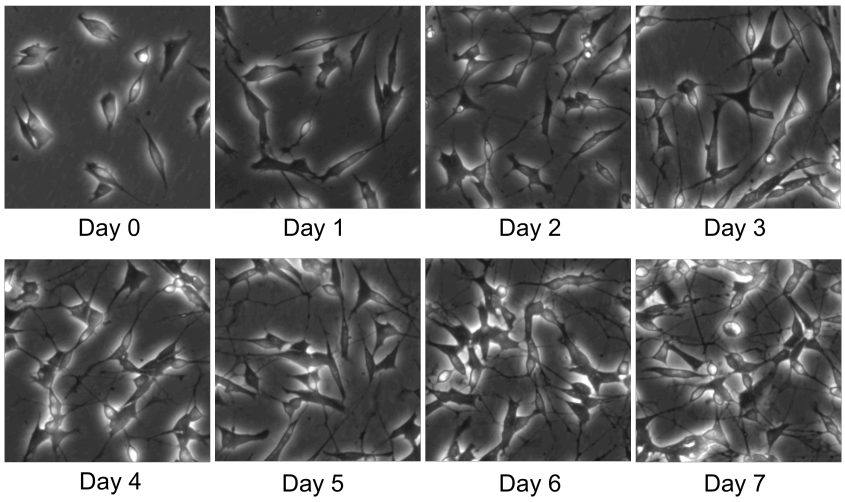


Figure 23. Cdh1 ubiquitylates E2F3 in vitro.

A



B

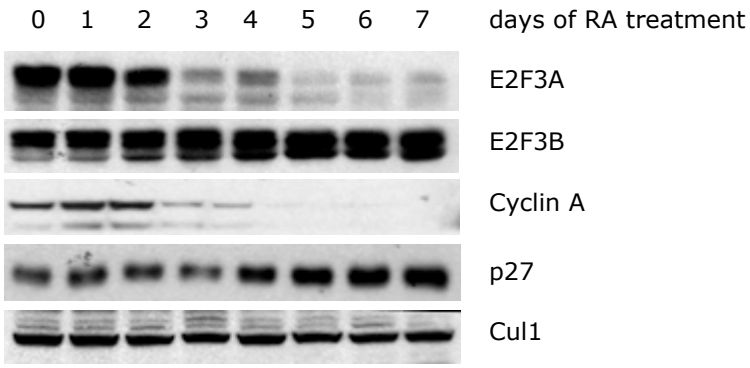


Figure 24. Levels of E2F3A decrease during neuronal differentiation.

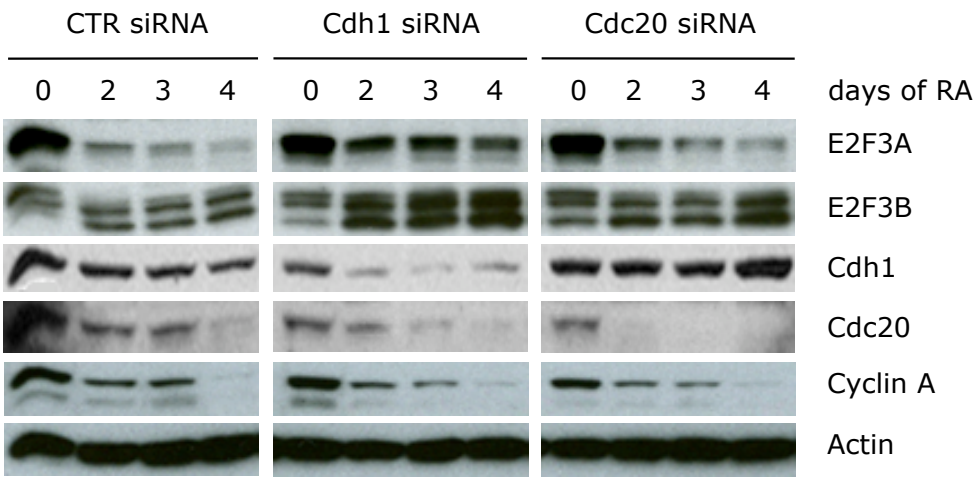


Figure 25. Cdh1 controls E2F3 degradation during neuronal differentiation.