The Human COP9 Signalosome Protects Ubiquitin-conjugating Enzyme 3 (UBC3/Cdc34) from β-Transducin Repeat-containing Protein (βTrCP)-mediated Degradation

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The COP9 signalosome (CSN) is an essential multisubunit complex that regulates the activity of cullin-RING ubiquitin ligases by removing the ubiquitin-like peptide NEDD8 from cullins. Here, we demonstrate that the CSN can affect other components of the ubiquitination cascade. Down-regulation of human CSN4 or CSN5 induced proteasome-mediated degradation of the ubiquitin-conjugating enzyme UBC3/Cdc34. UBC3 was targeted for ubiquitination by the cullin-RING ubiquitin ligase SCFβTrCP. This interaction required the acidic C-terminal extension of UBC3, which is absent in ubiquitin-conjugating enzymes of the UBCH5 family. Conversely, the UBC3 acidic domain was sufficient to impart sensitivity to SCFβTrCP-mediated ubiquitination to UBCH5 enzymes. Our work indicates that the CSN is necessary to ensure the stability of selected ubiquitin-conjugating enzymes and uncovers a novel pathway of regulation of ubiquitination processes.

Ubiquitin-mediated proteolysis regulates a wide range of cellular processes by controlling the stability of short-lived proteins, such as cell cycle factors, transcriptional regulators, and signal transducers. Protein ubiquitination requires coordinated activation of different enzymes: an E1 (ubiquitin-activating), an E2 (ubiquitin-conjugating), and an E3 (ubiquitin ligase) that is responsible for substrate recognition (1). E3 enzymes of the cullin-RING ubiquitin ligase (CRL) family contain a cullin and a RING domain protein that helps recruit E2 to the CRL complex (2). One of the most extensively studied CRLs is SCF, which consists of Skp1, Cul1, Rbx/Hrt1, and one of several F-box domain-containing proteins. Substrate specificity is dictated by the F-box protein: SCFSkp2 binds a number of cell cycle regulators (cyclins D1 and E and the cell cycle inhibitors p27 and p21) through the adaptor Skp2. Conversely, the adaptor β-transducin repeat-containing protein (βTrCP) targets β-catenin and IκB and is involved in the regulation of these transcriptional pathways (3). Together with the E2 UBC3/Cdc34, SCF plays an essential role in cell proliferation (4–6). In addition to UBC3, other E2 enzymes may be recruited by SCF: in humans, three E2 enzymes (UBCH5A, UBCH5B, and UBCH5C) homologous to Saccharomyces cerevisiae Ubc4 have been identified and shown to ubiquitinate several substrates in the context of SCF (7). The activity of CRLs is regulated by reversible conjugation of cullins with the ubiquitin-like protein NEDD8. Cullin neddylation is essential for CRL function, as demonstrated by its severe impairment upon disruption of the neddylation pathway (8–10). NEDD8 is thought to stimulate CRL activity through multiple mechanisms, which include displacement of the inhibitor CAND1/TIP120 and cullin conformational alterations that bring E2 in closer proximity to the substrate (11, 12). Cul1 neddylation also favors recruitment of E2 enzymes, possibly by providing a direct interaction surface for E2 (13, 14).

CRLs can be deneddylated by the COP9 signalosome (CSN), a highly conserved complex of eight subunits (CSN1–CSN8). The CSN was first characterized in Arabidopsis thaliana as a repressor of light-dependent development. It was subsequently identified in all eukaryotes analyzed and shown to participate in the regulation of multiple cellular pathways, such as cell proliferation, DNA repair, and developmental processes (15). The pleiotropic properties of CSN may be explained in part by its ability to control ubiquitin-dependent protein degradation through several mechanisms. The CSN can affect protein ubiquitination through association with a deubiquitinating enzyme of the cysteine protease family, called Ubp12 in Schizosaccharomyces pombe (16) or USP15 in mammalian cells (17). An additional ubiquitin isopeptidase activity, consisting in cleavage of monoubiquitin from Cul4, has been ascribed to CSN5 (18). The CSN may also affect protein degradation indirectly through the recruitment of protein kinases (inositol-1,3,4-triphosphate 5/6-kinase, protein kinase D, and casein II) (19, 20) and possibly of some of their substrates, such as c-Jun and the p53 tumor suppressor protein (21), affecting protein phosphorylation and stability. Perhaps the best characterized biochemical property of the CSN is its deneddylyase activity, which requires a zinc protease motif in CSN5, as well as the integrity of the whole complex (22, 23). In vitro studies have suggested that CSN-mediated deneddylation inhibits CRL activity (18, 24). However, CSN inactivation in vivo, while inducing cullin

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hypersmallon, results in the accumulation of some CRL substrates, indicating that CRL function may be impaired when CSN activity is deficient (25). To explain this apparent contradiction, we have proposed that the CSN is required to transiently inactivate CRLs (perhaps affecting E2 recruitment) to protect selected substrate adaptors, such as Skp2, from autocatalytic ubiquitination and destruction (26–28). In this work, we investigated how the loss of the CSN affects the activity of the E2 enzymes associated with SCF. We found that human UBC3, but not UBC4/5, is a target for SCFβTrCP-mediated ubiquitination and that the CSN is required to protect UBC3 from proteasome-dependent degradation.

EXPERIMENTAL PROCEDURES

Plasmids—The pCMVΔ8.9 and vesicular stomatitis virus G lentiviral vectors were provided by P. Charneau (Institut Pasteur, Paris). The short hairpin RNA (shRNA) plasmids (pTCN.control, pTCN.iCSN4, and pTCN.iCSN5) have been described previously (28). The expression vectors pCS2+/HAhUBC3 (wild type), pCS2+/HAhUBC3–5PT, and pCS2+/HAhUBC3–(1–200) were a generous gift of Dr. P. R. Yew (University of Texas Health Science Center, San Antonio, TX). The pCS2+/HAhUBC3–(1–229) plasmid was generated by amplifying wild-type UBCH5A with oligonucleotides that introduce a stop codon at amino acid 229. The pcDNAI-HAUBCH5A and pcDNAI-HAUBCH5B plasmids were obtained by PCR amplification. pcDNAI-HAUBCH5A +3tail and pcDNAI-HAUBCH5B +3tail were similarly generated by PCR amplification: the C-terminal fragment (amino acids 199–236) of UBCH3 was subcloned in-frame at the 3’-end of UBCH5A or UBCH5B.

Cell Culture and Transient Transfection—HEK293T and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (HyClone). Expression plasmids were transfected into HEK293T and HeLa cells with the jetPEITM transfection reagent (Polyplus Transfection) according to the manufacturer’s protocol. Analysis of transfected cells was performed 48 h after transfection.

Lentivirus Production and Transduction—2 × 10⁶ HEK293T cells were plated on 100-mm plates. The following day, the cells were cotransfected by calcium phosphate precipitation with 10 μg of the pCMVΔ8.9 packaging vector, 5 μg of the vesicular stomatitis virus G envelope vector, and 10 μg of the shRNA vector pTCN (pTCN.control, pTCN.iCSN4, or pTCN.iCSN5). The next day, the medium was substituted with Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum and 100 μg/mL of antihuman tumor necrosis factor (HyClone) for 24 h. Supernatants were collected, centrifuged at 2500 rpm for 5 min, and concentrated with Amicon Ultra filter devices (Millipore) at 3300 rpm for 10 min. 5 × 10⁵ HEK293T or HeLa cells seeded in a 6-well plate were incubated with the viral supernatant. To evaluate the efficiency of transduction, it has been proposed that the CSN is required to transiently inactivate CRLs (perhaps affecting E2 recruitment) to protect selected substrate adaptors, such as Skp2, from autocatalytic ubiquitination and destruction (26–28). In this work, we investigated how the loss of the CSN affects the activity of the E2 enzymes associated with SCF. We found that human UBC3, but not UBC4/5, is a target for SCFβTrCP-mediated ubiquitination and that the CSN is required to protect UBC3 from proteasome-dependent degradation.

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CSN Prevents βTrCP-mediated Degradation of UBC3

In Vivo UBC3 Ubiquitination—48 h after transfection, HEK293T cells were treated with 20 μM MG132 for 5 h and lysed in 1% SDS, 50 mM Tris (pH 8.5), 5 mM EDTA, 1 mM AEBSF, and 10 mM N-ethylmaleimide. The lysates were incubated at 95 °C for 5 min, diluted up to a final volume of 1 ml with nondenaturing buffer (1% Triton X-100, 50 mM Tris (pH 8.0), 300 mM NaCl, 1 mM AEBSF, and 10 mM N-ethylmaleimide), subjected to mechanical shearing, and cleared by incubation with 60 μl of Pansorbin® cells (Calbiochem) for 30 min at 4 °C. Homogenates were spun at 14,000 rpm for 20 min, and supernatants were measured by the Bradford assay (Bio-Rad). Equal amounts of protein were incubated with 1 μg of anti-HA antibody for 2 h at 4 °C, followed by incubation with protein G-Sepharose™ 4 Fast Flow (GE Healthcare). After six washes with nondenaturing buffer, bound proteins were eluted with sample buffer and analyzed by SDS-PAGE and Western blotting.

Co-immunoprecipitation Assays—Transfected HEK293T cells were lysed in Triton X-100 buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 0.1% Triton X-100, 15% glycerol, 1 mM AEBSF,
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20 mM NaF, and 1 mM Na$_2$VO$_3$). After centrifugation at 14,000 rpm for 30 min, equal amounts of protein were precleared with 15 μl of protein A-Sepharose$^\text{TM}$ 4 Fast Flow (GE Healthcare) and subsequently incubated with 20 μl of anti-FLAG$^\text{®}$ M2-agarose (Sigma) for 1 h at 4°C. The FLAG beads were washed five times with Triton X-100 buffer, and bound proteins were eluted with FLAG$^\text{®}$ peptide (Sigma) and analyzed by SDS-PAGE and Western blotting. For co-immunoprecipitation of endogenous proteins, cells were lysed in 20 mM Tris (pH 8.0), 100 mM NaCl, 0.1% digitonin, 15% glycerol, 1 mM AEBSF, 20 mM NaF, and 1 mM Na$_2$VO$_3$, and immunoprecipitates were washed as described above. Monoclonal antibody 90.12 was used for UBC3 immunoprecipitation.

RNA Extraction and Quantitative Real-time PCR—RNA was extracted with the NucleoSpin RNA II kit (Macherey-Naghel) following the manufacturer’s instructions and quantified with a NanoDrop ND-1000 spectrophotometer. 1 μg of total RNA was reverse-transcribed with SuperScript$^\text{TM}$ II (Invitrogen) using anchored oligo(dT)25V (MP Biomedicals) or p(dN)$_n$ random primers (Roche Applied Science). Real-time fluorogenic reverse transcription-PCR (Applied Biosystems) was performed according to the manufacturer’s protocol: 20 ng of cDNA was amplified with 4 μl of the “Assays-on-Demand” oligonucleotide in a final volume of 25 μl (Applied Biosystems TaqMan® gene expression assays COPS4, Hs00275045_m1; COP5S, Hs00272789_m1; CUL1, Hs00269187_m1; CUL4A, Hs00757716_m1; SKP2, Hs00180634_m1; BTRC, Hs00182707_m1; CDC34, Hs00366152_m1; UBE2D2, Hs00362082_m1; UBE2D3, Hs00180634_m1; BTRC, Hs00704312_m1; and CTNNB1, Hs00170025_m1). Samples were analyzed with the ABI Prism 7300 real-time PCR machine. Relative mRNA levels were normalized using 18 S rRNA/VICT$^\text{TM}$-MGB. As a control, normalization to human glyceraldehyde-3-phosphate dehydrogenase/VICT$^\text{TM}$-MGB or human hypoxanthine-guanine phosphoribosyltransferase/VICT$^\text{TM}$-MGB (Applied Biosystems) was performed and gave similar results.

Transient Transfection of Small Interfering RNA Oligonucleotides—7 × 10$^4$ HEK293T or HeLa cells were seeded in a 12-well plate. 24 h later, the cells were transiently transfected with a small interfering RNA (siRNA) oligonucleotide (5 nm) using INTERFERin$^\text{TM}$ (Polyplus Transfection). After 48 h, the cells were lysed with radioimmune precipitation assay buffer and analyzed by Western blotting. The siRNA oligonucleotides used were control (1022076), Cul1.5 (SI02225657), Cul1.6 (SI02225664), Cul4A.1 (SI00356937), Cul4A.2 (SI00356944), Skp2.5 (SI00287919), Skp2.8 (SI02665962), bTrCP.A (SI00058065), bTrCP.B (SI02632322), and bTrCP (09-3342-3/3) (MWG Biotech) and USP15 (s19338 and s19339, Applied Biosystems).

RESULTS

Down-regulation of CSN4 or CSN5 Destabilizes UBC3—To investigate whether the CSN affects E2 recruitment to SCF$^\text{βTrCP}$, we down-regulated the expression of CSN4 or CSN5 by lentivirus-mediated RNA interference. As shown previously (28), down-regulation of either subunit was sufficient to disrupt the CSN complex and induce cullin hyperneddylation and degradation of the F-box protein Skp2. In CSN-deficient cells (CSN$^\text{kd}$ cells), we observed a marked decrease in UBC3 protein levels, without a concomitant decrease in mRNA levels, pointing to a post-transcriptional mechanism for UBC3 loss (Fig. 1A). UBC3 decrease was observed following CSN down-regulation in transformed cell lines (293T and HeLa), as well as in primary cultures of normal human fibroblasts and T lymphocytes (Fig. 1, A and B). CSN down-regulation did not affect the levels of UBC4/5 proteins or of the small ubiquitin-like modifier E2 (Fig. 1A). Treatment of CSN$^\text{kd}$ cells with the proteasome inhibitor MG132 recovered UBC3 protein levels without increasing UBC3 mRNA (Fig. 1C), indicating that UBC3 loss in these cells results from proteasome-dependent degradation. To confirm that the loss of UBC3 in CSN$^\text{kd}$ cells was due to increased turnover, we analyzed UBC3 levels in total lysates from control and CSN4$^\text{kd}$ cells at different time points after the addition of the protein synthesis inhibitor CHX. UBC3 turnover was accelerated in CSN4$^\text{kd}$ cells compared with control cells (Fig. 1D), indicating that loss of the CSN destabilizes UBC3. Similar results were obtained by down-regulation of CSN5 (data not shown).

CUL1 Is Involved in UBC3 Degradation—Ubiquitinated forms of UBC3/Cdc34 have been detected in yeast cells (4) and in S. cerevisiae Cdc3 ubiquitination is enhanced by recruitment to SCF (6). We therefore asked whether the degradation of human UBC3 observed in CSN$^\text{kd}$ cells was also dependent on CUL1. Down-regulation of CUL1 using siRNA recovered UBC3 protein levels in CSN$^\text{kd}$ cells without affecting UBC3 levels in control cells (Fig. 2A and supplemental Fig. S1A). This effect is specific for Cul1 because inhibition of Cul4 did not influence UBC3 levels (supplemental Fig. S1B).

UBC3 Is a Substrate of SCF$^\text{βTrCP}$—Mammalian UBC3 presents at its C terminus a consensus sequence for binding to the adaptor βTrCP (amino acids 230–236, DSGTTL) (supplemental Fig. S2). Recruitment of substrates to βTrCP through the classical consensus sequence (DpSGXXpXp) depends on phosphorylation of the serine residues, and amino acids 231, 233, and 236 of UBC3 have been shown to be phosphorylated in vivo (31). We therefore explored the possibility that UBC3 is targeted for degradation through an SCF$^\text{βTrCP}$-dependent pathway. Endogenous UBC3 and βTrCP interacted in co-immunoprecipitation assays using either anti-UBC3 (middle panel) or anti-βTrCP (right panel) antibody for the immunoprecipitation (Fig. 2B). HA-UBC3 co-immunoprecipitated both with full-length βTrCP and with a deletion construct (βTrCPΔBox) that lacks the F-box domain and does not bind to SCF (Fig. 2C), indicating that the interaction between UBC3 and βTrCP does not require binding to CUL1. We then investigated whether the observed UBC3/βTrCP interaction resulted in UBC3 ubiquitination in vivo. Low levels of UBC3-ubiquitin conjugates were visible in UBC3 immunoprecipitates in the presence of transfected Myc-ubiquitin. Overexpression of βTrCP alone, and to a lesser extent, βTrCP2 increased the amount of ubiquitinated UBC3, whereas overexpression of an unrelated F-box protein (SKP2) did not induce UBC3 ubiquitination (Fig. 2D). Consistent with the role of βTrCP in promoting UBC3 ubiquitination, down-regulation of endogenous βTrCP levels by RNA interference caused an accumulation of the endogenous UBC3 protein, as well as increased levels of a...
known βTrCP target, β-catenin (Fig. 2E), demonstrating that βTrCP plays a role in regulating UBC3 levels in vivo. Down-regulation of SKP2 did not affect UBC3 levels, as predicted by Skp2 inability to promote UBC3 ubiquitination (Fig. 2F).

The C-terminal Acidic Domain of UBC3 Is Necessary for Targeting to βTrCP and Is Sufficient to Destabilize UBC3—To investigate the role of the DSGTEES motif in targeting UBC3 to βTrCP, we tested the ability of βTrCP1 to interact with a deletion mutant of UBC3 lacking this motif (UBC3-(1–229)) (Fig. 3A). Deletion of amino acids 230–236 markedly decreased the interaction of UBC3 with endogenous βTrCP (Fig. 3B) or βTrCPΔFbox (supplemental Fig. S3A), although some residual βTrCP was still able to co-immunoprecipitate with UBC3-(1–229) in both cases. Consistently, βTrCP overexpression still promoted some ubiquitination of UBC3-(1–229), although less efficiently compared with wild-type UBC3 (Fig. 3C). Interaction with endogenous βTrCP and βTrCPΔFbox was also impaired in the absence of phosphorylation of the UBC3 C-terminal domain, as shown by the analysis of a phosphorylation-defective construct (UBC3–5PT) (supplemental Fig. S3) (31). These findings indicate that the DSGTEES motif plays a major role in UBC3 binding to βTrCP1. However, in the absence of the DSGTEES motif, additional elements may come into play in UBC3 interaction with βTrCP. Several βTrCP substrates that contain variant or non-canonical recognition motifs have been identified recently (3). Notably, acidic residues have been reported to substitute for the phosphorylated residues of the classical consensus sequence (32). UBC3/Cdc34 is characterized by a C-terminal extension that contains an acidic domain essential for yeast Cdc34 cell cycle functions (supplemental Fig. S2) (4, 33). Deletion of the UBC3 acidic domain (UBC3-(1–200)) (Fig. 3A) completely abolished the interaction between UBC3 and endogenous βTrCP (Fig. 3B) or βTrCPΔFbox (supplemental Fig. S3A), as well as βTrCP-induced ubiquitination (Fig. 3C), suggesting that multiple sequences in the C-terminal extension can interact with βTrCP. As a consequence of the above findings, we predicted UBC3-(1–200) to be more stable than wild-type UBC3. To assess the relative stability of these proteins, we coexpressed them with GFP as an internal control in cells treated or not with MG132. Western blot analysis and quantification of the UBC3/GFP ratio showed that MG132 treatment increased the levels of wild-type UBC3, but not of UBC3-(1–200), indicating that the latter is less sensitive to proteasome-dependent degradation (Fig. 3D).

Enzymes of the UBC4/5 family, which were stable in CSNdeficient cells (Fig. 1A), lack the acidic domain present in UBC3 (supplemental Fig. S2). We therefore asked whether this domain could confer instability to UBC4/5 proteins. We constructed chimeric UBCH5A and UBCH5B proteins containing UBC3 amino acids 200–236 at their C termini (Fig. 4A). Turnover of the UBCH5A+3tail chimera in CHX-treated cells was enhanced compared with turnover of wild-type UBCH5A (Fig. 4B), indicating that the acidic domain has a destabilizing effect on the protein. Similarly, proteasome inhibition did not affect the levels of wild-type UBCH5B but caused accumulation of UBCH5B+3tail (Fig. 4C), showing that amino acids 200–236 of UBC3 are sufficient to confer proteasomal sensitivity to UBCH5B. The acidic domain also conferred to the chimeric protein the ability to bind βTrCPΔFbox in co-immunoprecipitation assays (Fig. 4D). As a consequence, UBCH5B+3tail was more efficiently ubiquitinated in vivo in a βTrCP-dependent manner (Fig. 4E). Taken together, these data demonstrate that UBC3 is a ubiquitination substrate of SCLβTrCP and that targeting to βTrCP requires the UBC3 acidic C-terminal extension.

βTrCP Suppression Restores UBC3 Levels in CSN4−/− Cells—The decrease in UBC3 observed in CSN4−/− cells depended on the...
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FIGURE 2. UBC3 is a target of SCFβTrCP. A, UBC3 protein levels in CSN knockdown cells are rescued by CUL1 suppression. shRNA-transduced 293T cells were transfected with a control oligonucleotide or with two different siRNAs targeting CUL1. UBC3 content in total lysates was analyzed by Western blotting (upper panel). Cullin mRNA levels were analyzed by quantitative reverse transcription-PCR (lower panel). B, endogenous UBC3 and βTrCP interact. UBC3 (middle panel) and βTrCP (right panel) were immunoprecipitated (IP) from HEK293T cell lysates with specific antibodies. The control used was the irrelevant mouse (left panel) or rabbit (right panel) IgG antibody. WB, Western blot. C, the F-box is not required for βTrCP interaction with UBC3. 293T cells were transfected with the indicated vectors (full-length βTrCP, βTrCP-FL) and the F-box deletion mutant βTrCPΔFbox, and βTrCP was immunoprecipitated with anti-FLAG antibodies. UBC3 wt, wild-type UBC3. D, βTrCP overexpression promotes UBC3 ubiquitination. 293T cells were transfected as indicated. HA-UBC3 was immunoprecipitated with anti-HA antibody, and ubiquitinated forms of UBC3 were detected by anti-Myc antibody. E, down-regulation of βTrCP increases UBC3 protein levels. 293T cells were transfected with the indicated vectors (FLAG-βTrCP, FLAG-βTrCPΔFbox). F, down-regulation of SKP2 has no effect on UBC3 protein levels. 293T cells were transfected with two siRNA oligonucleotides targeting SKP2.

DISCUSSION

In this work, we have shown that ubiquitin-dependent degradation controls the levels of the ubiquitin-conjugating enzyme UBC3 and that the CSN is necessary to maintain UBC3 stability. In the absence of CSN activity, UBC3 is degraded through a proteasome-dependent pathway that involves CUL1. Furthermore, we found that βTrCP suppression recovers UBC3 levels, indicating that direct targeting by SCFβTrCP plays an important role in UBC3 loss in CSN-deficient cells.

Several βTrCP substrates present the consensus binding motif DpSGX_{2–3}S, which is also found at the C terminus of human UBC3. Deletion of the βTrCP consensus motif of UBC3 strongly reduced but did not completely abolish binding to βTrCP or βTrCP-dependent ubiquitination. These findings suggested that additional sequences in UBC3 could still mediate binding to βTrCP. In fact, further deletion of adjacent sequences in the acidic tail of UBC3 completely abolished binding to βTrCP and stabilized the molecule against ubiquitin-dependent degradation. These results are in line with recent reports demonstrating that variant or noncanonical recognition motifs can bind to βTrCP (3). In particular,
acids residues have been shown to substitute for the negative charges of phosphorylated residues present in the classical βTrCP-binding site. *Xenopus* Cdc25 and human CDC25B have been described to interact with βTrCP through a non-phosphorylated DDG motif; the acidic context in which the DDG motif is found also affects the interaction, as shown by increased binding upon phosphorylation of adjacent sequences (32). In some instances (such as in the case of Wee1 or Cdc25), increased binding upon phosphorylation of adjacent sequences is required for interaction with βTrCP (32, 37), possibly allowing cooperative binding of suboptimal sites, as seen for Sic1 binding to the C terminus deleted mutants of UBC3 (1–208) are still capable of interaction with TrCP and UBC3 ubiquitination. In fact, truncated UBC3-(1–194) and human UBC3 (UBC3-(1–194) and UBC3-(1–208)) are still capable of autoubiquitination in vitro, but with efficiencies comparable or superior to those of the wild-type protein. It remains to be determined whether autoubiquitination of human UBC3 on N-terminal sequences occurs in vivo. However, autoubiquitination does not seem to be determinant for the loss of UBC3 observed upon CSN knockdown, which may rather be explained by βTrCP-mediated ubiquitination. In fact, truncation of the C terminus (amino acids 200–236) was sufficient to stabilize UBC3 equally in control and CSNkd cells (supplemental Fig. S4). This stabilizing deletion abolished ubiquitination by βTrCP (Fig. 3C) while not impairing recruitment to SCF (43) or autoubiquitination (42). Furthermore, down-regulation of βTrCP in CSN-deficient cells efficiently recovered UBC3 levels (7-fold increase in CSN5kd cells and 14-fold increase in CSN4kd cells versus 1.6-fold increase in control cells) (Fig. 5A), suggesting that SCFβTrCP is targeting UBC3 more actively in cells deficient in CSN activity. An implication of these findings is that SCFβTrCP is competent for ubiquitination in cells with impaired CSN activity, in contrast with previous results demonstrating that CSN down-regulation is accompanied by an accumulation of substrates of another SCF complex, namely SCFSkp2 (25, 28). The different behavior of the two SCF complexes in response to CSN loss may be due to intrinsic properties of their F-box subunits: whereas SKP2 is strongly destabilized in CSN-deficient cells (27, 28, 44), our study found that βTrCP levels were largely maintained and possibly sufficient to preserve SCFβTrCP function. The biochemical
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**FIGURE 4.** The acidic UBC3 C-terminal extension is a destabilizing element. A, shown is a schematic representation of the UBC5 constructs. UBC3 WT, wild-type UBC3. B, the addition of the UBC3 C-terminal extension reduces UBC5A half-life. 293T cells were transfected with HA wild-type UBC5A or HA-UBCH5A+3tail and treated with CHX for the indicated times. Lysates were analyzed by Western blotting. UBC5A/actin ratios are represented relative to time 0. C, the UBC3 C-terminal extension destabilizes UBC5B. 293T cells were transfected with the indicated UBC5 constructs and a GFP vector. Cells were treated and analyzed as described in the legend to Fig. 3D. D, the UBC3 C-terminal extension promotes interaction with βTrCP. 293T cells were transfected with the indicated constructs. βTrCP was immunoprecipitated (IP) with anti-FLAG antibodies. Total lysates (lower panel) and immunoprecipitated samples (upper panels) were analyzed by Western blotting. Lower molecular weight bands in the fourth lane represent proteolytic products of UBC5B + 3tail. E, the UBC3 C-terminal extension directs βTrCP-dependent ubiquitination of UBC5B. 293T cells were transfected with the indicated constructs. UBC5B was immunoprecipitated with anti-HA antibody.

**FIGURE 5.** Down-regulation of βTrCP, but not of USP15, recovers UBC3 levels in CSN-deficient cells. A, shRNA-transduced cells were transfected with control siRNA oligonucleotides or oligonucleotides targeting βTrCP. Cell lysates were analyzed by Western blotting. B, βTrCP is maintained in CSN-deficient cells. Lysates of shRNA-transduced cells were analyzed by Western blotting. C, HeLa cells were transfected in duplicates with control RNA oligonucleotides or oligonucleotides targeting USP15. Total lysates were analyzed by Western blotting with the indicated antibodies.

β-catenin response to CSN suppression have not been uniform, with some describing an increase in β-catenin levels after CSN down-regulation and others indicating no changes (36, 47–49). At present, the cause of these discrepancies remains unclear, but it could reflect the different cellular contexts analyzed or the different timing of the analysis after CSN suppression.

Taken together, our data suggest that, in response to CSN down-regulation, UBC3 is degraded through increased processing by the SCF^{βTrCP} complex. Further work is necessary to identify the stimuli that induce βTrCP-dependent ubiquitination of UBC3. No variations in the levels of UBC3 protein during cell cycle progression have been described (4, 50), although serum deprivation can alter UBC3 levels in human cells (data not shown) (50). Given the importance of UBC3 for cell cycle progression, we are currently testing the hypothesis that UBC3 degradation may represent a “checkpoint” in response to DNA damage or other stress stimuli (growth factor deprivation, oxidative signals). The levels of βTrCP mRNA and protein are increased in response to cellular stress (42), and several known SCF^{βTrCP} substrates are degraded under conditions of cellular stress (51–54). It is possible that, in our experimental system, inhibition of CSN activity and the consequent disruption of cell cycle progression and intracellular signaling pathways may constitute such a stress signal. It should also be noted that, under our culture conditions and in the cell lines examined, UBC3 seems, to a certain extent, to be targeted by βTrCP in the absence of additional stimulation because down-regulation of βTrCP in HeLa and 293T cells caused an increase in UBC3 levels (Fig. 2E). In conclusion, this work uncovers a further layer of regulation of ubiquitination processes by demonstrating that UBC3 is a target itself of βTrCP-mediated ubiquitination and that the CSN is required to protect selected ubiquitin-conjugating enzymes from proteasome-dependent degradation.

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