Characterization of The Human SCF Ubiquitin Ligases – Structure, Function, and Regulation

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"So you've broken a wall with your head; how do you like it in another prison cell?"

-- Ezhi Lets

"Scientific curiosity can be satisfied much more easily by reading the publications of others than by working in the lab. It may take years to prove by experimentation what we can learn in the few minutes needed to read the published end result. So let us not fool ourselves; the driving force is hardly sheer curiosity."

-- Hans Selye, From dream to discovery (1964).

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Abstract

The SCF ubiquitin ligase complex of budding yeast triggers DNA replication by *catalyzing* ubiquitination of the S phase CDK inhibitor SIC1. SCF is composed of several evolutionarily conserved proteins, including ySKP1, CDC53 (Cullin), and the F-box protein CDC4. We isolated hSKP1 in a two-hybrid screen with hCUL1, the human homologue of CDC53. We showed that hCUL1 associates with hSKP1 *in vivo* and directly interacts with hSKP1 and the human F-box protein SKP2 *in vitro*, forming an SCF-like particle. Moreover, hCUL1 complements the growth defect of yeast CDC53^{ts} mutants, associates with ubiquitination-promoting activity in human cell extracts, and can assemble into functional, chimeric ubiquitin ligase complexes with yeast SCF components. These data demonstrated that hCUL1 functions as part of an SCF ubiquitin ligase complex in human cells. However, purified human SCF complexes consisting of CUL1, SKP1, and SKP2 are inactive *in vitro*, suggesting that additional factors are required.

Subsequently, mammalian SCF ubiquitin ligases were shown to regulate various physiological processes by targeting important cellular regulators, like IκBα, β-catenin, and p27, for ubiquitin-dependent proteolysis by the 26S proteasome. Little, however, is known about the regulation of various SCF complexes. By using sequential immunoaffinity purification and mass spectrometry, we identified proteins that interact with human SCF components SKP2 and CUL1 *in vivo*. Among them we identified two additional SCF subunits: HRT1, present in all SCF complexes, and CKS1, that binds to SKP2 and is likely to be a subunit of SCF^{SKP2} complexes. Subsequent work by others demonstrated that these proteins are essential for SCF activity. We also discovered that COP9 Signalosome (CSN), previously described in plants as a suppressor of photomorphogenesis, associates with CUL1 and other SCF subunits *in vivo*. This interaction is evolutionarily conserved and is also observed with other Cullins, suggesting that all Cullin based ubiquitin ligases are regulated by CSN. CSN regulates Cullin Neddylation presumably through

CSN5/JAB1, a stochiometric Signalosome subunit and a putative deneddylating enzyme. This work sheds light onto an intricate connection that exists between signal transduction pathways and protein degradation machinery inside the cell and sets stage for gaining further insights into regulation of protein degradation.

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Chapter 1. Introduction

The goal of this chapter is to provide a review of our current knowledge and understanding of the COP9 Signalosome and processes involved in ubiquitin-dependent degradation in the cell cycle, with emphasis on SCF ubiquitin ligase function.

Cell Cycle Regulation by Proteolysis

In an attempt to carry on fundamental processes that keep the flame of life burning, every single living cell faces two challenges: it has to precisely duplicate its genetic information once and only once before each cell division and then properly segregate it to daughter cells. To accomplish this, the cell goes though biochemically and temporarily distinct phases of the cell cycle. The cell replicates its DNA in S phase, then condenses its chromosomes, aligns them on a microtubular spindle, and segregates them to the opposite poles in Mitosis, or M phase. The gap between mitosis and the onset of DNA replication is called G1 phase, and the gap between S phase and mitosis – G2 phase. What drives cells from one phase into the other and what prevents them from entering the next stage before completing the prior one perplexed scientific minds for many years. It is now clear that the general strategy employed to insure proper cell cycle transitions is the use of stimulating factors and inhibitory barriers that must be overcome in order for the transition to occur. Early cell fusion experiments led to a discovery of an M-phase promoting factor (MPF) capable of driving early G2 cells into mitosis. Ensuing symbiosis of yeast genetics, frog biochemistry and mammalian tissue culture led rapidly to the current notion that DNA replication and mitosis are induced by the activation of S and M phase specific cyclin dependent protein kinases (CDKs). CDKs are activated by phosphorylation and binding to a variety of cyclins – key regulators of the cell cycle transitions whose abundance varies through

the cell cycle. CDK inhibitors (CDKi), on the other hand, function as negative regulators of the cell cycle by binding to Cyclin/CDK complexes and inhibiting their kinase activity. Within the logical framework of the cell cycle, the same molecules that are used to promote one transition often inhibit the subsequent transition. For example, in *Sacharomyces cerevisiae* CDK inhibitor SIC1 promotes exit from mitosis by inactivating mitotic CLB/CDC28 kinases and acts as a barrier to S phase entry by inhibiting S phase CDKs. Likewise, S phase cyclins promote initiation of DNA replication but prevent the reestablishement of new competent origins thus preventing rereplication, while mitotic cyclins promote entry into mitosis but inhibit mitotic exit. Coupling of positive and negative regulatory signals ensures establishment of a cell cycle phase where defined sets of events occur. Once the task is accomplished, the inhibitory barriers are overcome to allow the transition to the next phase.

Investigations into the nature of cyclin and CDKi periodicity led to a discovery of the involvement of ubiquitin-dependent proteolysis in cell cycle control. Ubiquitin dependent degradation of a subset of cell cycle regulators with dual positive/negative roles (e.g., SIC1, PDS1, mitotic cyclins), whose destruction is necessary for cell cycle progression, relieves the negative barriers they impose on cell division, while destruction of strictly positive cell cycle regulators (e.g., G1 cyclins, CDC6) is important for cellular homeostasis and resetting the balance of regulatory factors for the next cell cycle.

The G1/S transition in *S. cerevisiae* depends on degradation of the CLB/CDC28 inhibitor SIC1, which accumulates to high levels in G1, gets phosphorylated at multiple sites by CLN/CDC28 at the end of G1, then ubiquitinated and rapidly degraded at G1/S transition (Schwob et al., 1994). SIC1 ubiquitination is carried out by the SCF ubiquitin ligase and CDC34 ubiquitin conjugating enzyme (Verma et al., 1997). The same pathway is involved in the turnover of G1 cyclins (Willems et al., 1996), which is important for the adaptation of the rate of cell division to changing environmental conditions; and CDC6, a protein essential for the initiation of DNA replication (Elsasser et al., 1999). In the fission yeast *Schizosaccharomyces*

pombe, degradation of the CDC6 homologue, CDC18, may limit DNA replication to only once per cell cycle. Similarly, in higher eukaryotes, p27 CDK inhibitor accumulates at high levels in quiescent cells and is rapidly degraded though an SCF pathway when cells are stimulated by growth factors (Carrano et al., 1999; Pagano et al., 1995). Human G1 cyclins, E and D1, were also shown to be substrates of the SCF pathway (Singer et al., 1999; Yu et al., 1998).

Mitotic events are regulated by a different ubiquitin-dependent pathway, involving APC/Cyclosome. Sister chromatid separation at the onset of anaphase is triggered by degradation of an anaphase inhibitor PDS1 (Cohen-Fix et al., 1996), and exit from mitosis requires degradation of mitotic cyclins (Hershko, 1999). Inactivation of any one of at least ten APC subunits prevents cyclin destruction and sister chromatid separation (Irniger et al., 1995). Budding yeast ASE1, a protein localized in the midzone of the mitotic spindle and required for spindle elongation, is also an APC substrate (Juang et al., 1997).

The Ubiquitin System

Ubiquitin is one of the most conserved eukaryotic proteins. It is a small 76-amino acid protein involved in a variety of cellular functions, including regulation of intracellular protein degradation, antigen processing, cell cycle progression, receptor endocytosis, and signal transduction. Ubiquitin (Ub) is conjugated to other proteins inside the cell through a well-defined enzymatic pathway (Hershko and Ciechanover, 1998). It begins with an ATP-dependent activation of a Ub-activating enzyme, E1, which activates Ub through adenylation and subsequently forms a high-energy E1~Ub thiol ester. In yeast, UBA1 is essential for viability and encodes a 114 kDa activating enzyme required for ubiquitin conjugation (McGrath et al., 1991). Next, ubiquitin is transferred to a ubiquitin-conjugating enzyme, E2, which also forms a thiol ester bond with Ub. More than 30 E2 enzymes have currently been identified. Finally, Ub is transferred to a target protein through an isopeptide linkage between the conserved C-terminal Gly residue of Ub and ε–amino group of the Lys residue of the target protein. Sequential

conjugation of the internal Lys residue of Ub to a C-terminal Gly residue of a new Ub molecule results in a formation of polyubiquitin chains which target proteins for degradation by the 26S proteasome (Pickart and Rose, 1985). The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitinated proteins to small peptides (Voges et al., 1999). It is composed of two subcomplexes, a core catalytic 20S particle and a regulatory 19S particle. The 20S complex is barrel-shaped and consists of four stacked rings, two identical outer α rings and two identical inner β rings. The α and β rings are composed each of seven distinct subunits, giving the complex the general structure of $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The catalytic sites are localized in some of the β subunits. Each extremity of the 20S complex is capped by a 19S particle. The yeast 19S complex has been further resolved into two sub-complexes, the `base', which consists of six ATPases of the AAA family and three additional proteins, and an 8-subunit `lid' that is homologous to COP9 Signalosome (see below).

In many cases, the transfer of Ub from an E2 to a target protein requires the involvement of a ubiquitin-ligase, E3, that is discussed in detail in the next section.

SCF and Other Ubiquitin Ligases

E3 ubiquitin ligases cooperate with E2 ubiquitin conjugating enzymes to assemble polyubiquitin chains on substrate proteins. Generally, ubiquitin ligases, whether single proteins or multi-protein complexes, contain protein-protein interaction domains that provide substrate-binding activities, and catalytic domains that promote ubiquitin transfer from an E2 enzyme to a substrate molecule. Based on sequence homology and catalytic principles involved, all know ubiquitin ligases can be divided into two major classes: HECT domain and RING finger based ubiquitin ligases.

HECT domain proteins provide a simple and general model for an E3 function. They are found broadly in eukaryotes and are defined by a 350 amino acid C-terminal HECT homology

domain (Homologous to E6-AP C-Terminus), originally identified in E6-AP (Scheffner et al., 1993). E6-AP is the cellular ubiquitin ligase recruited by the human papilloma virus E6 oncoprotein to induce degradation of the p53 tumor suppressor. A conserved Cys residue within the catalytic HECT domain of E6-AP forms a thiol ester with ubiquitin molecule which is then transferred to p53 (Scheffner et al., 1995). Multiple HECT domain proteins have been identified, including NEDD4 in humans; RSP5, UFD4, and TOM1 in *S. cerevisiae*; and Pub1 in *S. pombe*. In all of them, it appears that the large N-terminal domains confer the ability to bind substrates and the C-terminal HECT domains serve to directly transfer ubiquitin to the substrates through a E1-E2-E3 ubiquitin thiol ester cascade.

The identification of RING finger domains in SCF complexes and other non-HECT domain ubiquitin ligases suggested that these domains are functionally significant in ubiquitination. RINGs are zinc-binding domains with a defined octet of Cys and His residues in a consensus sequence CX2CX(9-39)CX(1-3)HX(2-3)C/HX2CX(4-48)CX2C (Jackson et al., 2000; Saurin et al., 1996). RING fingers are subcategorized into RING-H2 (found in HRT1, HRT2, and APC11) and RING-HC (found in several other ligases) depending on whether a Cys or His occupies the fifth coordination site, respectively. Stuctures of RING finger proteins show two interleaved zinc binding sites, in contrast to the tandem arrangements of zinc binding sites of zinc fingers. In contrast to HECT domain ubiquitin ligases that form thiol esters with ubiquitin, there is no evidence for such intermediates involving RING finger E3s (Seol et al., 1999). Since RING finger proteins can directly bind to E2 enzymes (Zheng et al., 2000), it is likely that RING fingers mediate ubiquitination by recruiting E2 enzymes to the vicinity of proteins to be ubiquitinated, thus facilitating the direct transfer of Ub from E2s to target proteins. However, there is evidence that the role of the RING finger is more complex. For example, UBR1 binds its cognate E2, RAD6, predominantly through regions outside the RING, yet mutations in this region only weakly reduce E3 activity, while a mutation in the RING did not affect binding to E2 or substrate,

but abolished ubiquitination (Xie and Varshavsky, 1999). One possibily is that the RING may function as an allosteric activator of an E2.

RING finger E3s play prominent roles in diverse cellular processes, including cell cycle, signaling, transcription, apoptosis, proliferation, and DNA repair. In one of the well understood examples, CBL, an adapter protein containing both SH2 and RING-HC finger domains, functions as a ubiquitin ligase that recognizes phosphorylated tyrosine on receptor protein-tyrosine kinases (RPTKs) through its SH2 domain and terminates signaling by mediating active receptor ubiquitination and degradation (Joazeiro et al., 1999). Oncogenic versions of CBL contain deletions or point mutations in the RING domain, suggesting that CBL becomes transforming by losing the activity of the RING finger and presumably ubiquitin-ligase activity.

The SCF subfamily of RING finger E3s was originally discovered and extensively studied in budding yeast *S. cerevisiae*. SCF complexes consist of at least four subunits: CDC53/CUL1, HRT1/RBX1/ROC1, SKP1 and one of the F-box family of proteins (Feldman et al., 1997). All known SCF subunits are highly conserved throughout eukaryotes. Cullin and the RING finger protein HRT1 form a catalytic core of E3 that binds to and activates E2 CDC34 or UBCH5 (Seol et al., 1999). Cullin/CDC53 has three functional domains: an N-terminal domain that binds SKP1 (Michel and Xiong, 1998; Patton et al., 1998), an internal Cullin homology (CH) domain that recruits CDC34 and HRT1 (Ohta et al., 1999), and a short C-terminal domain which is the most conserved part of the protein that contains neddylation determinants (Lammer et al., 1998) and is probably involved in the regulation of SCF activity, as discussed in the next section. HRT1 is a small 121 amino acid RING finger protein that binds directly to Cullins and CDC34, likely tethering E2 to the CH domain. SKP1 binds to the CDC53/HRT1 core and mediates recruitment of various F-box adapter proteins, which contain a ~45 amino acid motif called an F-box and bind to substrates through protein-protein interaction domains, thus conferring substrate specificity to this family of E3s. Multiple F-box proteins have been shown to bind SKP1. This interaction requires an intact F-box and some outside sequences (Bai et al., 1996), and is direct

(Lyapina et al., 1998; Schulman et al., 2000). The N-terminal ~100 amino acid domain of SKP1 contains both a Cullin-binding site and an F-box binding determinant, although downstream sequences might stabilize association of SKP1 with different F-box proteins (Michel and Xiong, 1998; Ng et al., 1998). There are over 400 F-box proteins currently in the database, with 20 F-box proteins in *S. cerevisiae,* over 100 in *C elegans*, and ~50 described so far in vertebrates (Skowyra et al., 1997). All F-box proteins can be divided into three classes, based on the associated protein-protein interaction domains: Fbw, containing WD40 repeats, e.g., CDC4 and βTrCP; Fbl, containing leucine-rich repeats (LRR), e.g., GRR1 and SKP2; and Fbx, containing other repeats or unique sequences (Cenciarelli et al., 1999; Regan-Reimann et al., 1999; Winston et al., 1999).

SCF activity might be directly involved in regulating the abundance and repertoire of various SCF complexes. The F-box proteins CDC4, GRR1, and MET30, are unstable and are degraded in a proteasome- and SCF-dependent manner (Galan and Peter, 1999; Mathias et al., 1999; Zhou and Howley, 1998). It has been proposed that substrate binding to an F-box protein protects it from auto-ubiquitination, thus insuring that there is a direct correlation between the concentration of an F-box protein and the concentration of its targets, and that the core ubiquitin ligase is being effectively recycled between various SCF complexes (Deshaies, 1999).

Many proteins have been implicated as substrates of the SCF pathway. These proteins participate in a variety of cellular functions, including regulation of CDK activity, activation of transcription, signal transduction, and DNA replication (Deshaies, 1999). In all studied cases, phosphorylation of the substrates is required for recognition by the F-box proteins. In the SCF^{CDC4} complex, each of the known substrates, SIC1 (Feldman et al., 1997), CDC6 (Elsasser et al., 1999), and GCN4 (Kornitzer et al., 1994), must be phosphorylated before they can be recognized by the WD40 repeat β-propeller domain of CDC4 and ubiquitinated. Another WD40-repeat protein containing SCF complex, $SCF^{\beta TrCP}$, recognizes a specific phosphoserine motif

(DSG_XS) found in its substrates IkBα and β-catenin (Fuchs et al., 1999; Winston et al., 1999; Yaron et al., 1998). G1 cyclins (Deshaies et al., 1995) and p27 (Pagano et al., 1995), the known *substrates* of the LRR-containing F-box proteins GRR1 and SKP2, respectively, also follow the same rule. This mechanism allows for differential temporal regulation of SCF substrate stability in the presence of fully active SCF complexes.

SCF might serve as a prototype for other modular Cullin-based ubiquitin ligases. There are at least five other human Cullins, which, in contrast to CUL1, do not bind SKP1. Human CUL2 binds to the von Hippel-Lindau gene product (VHL) through elongin B and elongin C (which shares homology with the N-terminal region of SKP1) to form a complex (CUL2-VBC) which appears to have ubiquitin ligase activity (Iwai et al., 1999; Kamura et al., 1999; Lisztwan et al., 1999). VHL down-regulates hypoxia-inducible mRNAs, presumably by controlling proteolysis of hypoxia regulated transcription factors HIF1α and HIF2 α (Iliopoulos et al., 1996; Krek, 2000; Stebbins et al., 1999). VHL can be replaced in this complex by SOCS box-containing proteins, which, analogous to F-box proteins, might confer substrate specificity (Deshaies, 1999). Human CUL3 was recently shown to be involved in the ubiquitination of Cyclin E and control of S phase in mammalian cells (Singer et al., 1999). Human CUL4 associates with UV-damaged DNA binding protein and may play a role in DNA repair (Shiyanov et al., 1999). The RING finger protein HRT1/RBX1/ROC1, a core component of SCF ubiquitin ligases, has been shown to interact with all Cullins (Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999). Thus, Cullins may define a family of E3 ubiquitin ligases that have diverse biological functions.

Another prominent RING finger-containing E3 is the Anaphase Promoting Complex (APC/Cyclosome) which is required for the methaphase to anaphase transition and exit from mitosis (Cohen-Fix et al., 1996; King et al., 1995; Yeong et al., 2000) APC is a multicomponent ubiquitin ligase that consists of at least 12 essential subunits, including Cullin family member

APC2 and a RING-H2 protein APC11 (Gmachl et al., 2000; Leverson et al., 2000; Yu et al., 1998). Also, like SCF, APC associates with WD repeat-containing adapter proteins CDC20/Fizzy, CDH1/HCT1/Fizzy-related, and AMA1, that activate APC toward specific substrates (Cooper et al., 2000; Visintin et al., 1997). Whereas APC^{CDC20} is active at the beginning of anaphase, when it targets the anaphase entry inhibitor PDS1 for degradation, APC^{CDH1} is required for the degradation of Cyclin B at the end of mitosis. Both CDC20 and HCT1 are regulated: CDC20 – by binding to an inhibitory MAD2 checkpoint protein (Fang et al., 1998) and degradation though APC^{CDH1}, and CDH1 – by inhibitory phosphorylation by Cyclin B/CDK (Kramer et al., 2000; Zachariae et al., 1998). CDC20 binding to APC also requires phosphorylation of APC by a mitotic kinase (Kramer et al., 2000). APC substrates are targeted for ubiquitination and degradation through two destruction signals: the D (destruction) box and the KEN box (Pfleger and Kirschner, 2000). The 9 amino acid D box is found in all known APC^{CDC20} substrates, but also in some APC^{CDH1} substrates. The KEN box is a 7 amino acid motif that appears to target substrates to APC^{CDH1}.

Ubiquitin-like Proteins

The complexity of the ubiquitin pathway is further compounded by the existence of several ubiquitin-like proteins, namely, UCRP (ubiquitin cross-reactive protein), sentrins, APG12, URM1, and NEDD8 (see Table 1). UCRP is a type 1 interferon-inducible protein that contains two ubiquitin domains (Narasimhan et al., 1996). UCRP has been shown to conjugate to a large number of intracellular proteins, the identities of which have not been determined. Recent studies suggest that UCRP conjugation proceeds though an enzymatic pathway that is distinct from that of ubiquitin, at least with respect to the activation step. However, the identity of the UCRP activating enzyme remains unknown.

The sentrin family of ubiquitin-like proteins consists of three family members that have similar substrate specificity: sentrin 1, sentrin 2, and sentrin 3. These proteins are able to conjugate to RanGAP1, Ran BP2, PML, p53, IκBα, and yeast septins among others (Yeh et al., 2000). Sentrins and SMT3 (their yeast homologue) are 95-103 amino acid long proteins with almost 20% identity and 50% homology to ubiquitin. They are activated by an E1-like protein, UBA2, which encodes a 71 kDa protein that is similar to the C-terminus of E1 and bears a Cys residue at a position similar to the active-site Cys of UBA1 (Dohmen et al., 1995). UBA2 cooperates with another 40 kDa protein, termed AOS1, to conjugate sentrin (SMT3 in yeast) to target proteins (Johnson et al., 1997). Both human and yeast AOS1 and UBA2 share extensive homology with the N- and C-terminal halves of yeast UBA1. Both sentrins and SMT3 utilize UBC9 as the conjugating enzyme (Johnson et al., 1997), which forms thiol esters with all sentrin proteins, but not ubiquitin or NEDD8 (Gong et al., 1997). All components of the SMT3 conjugation pathway are essential in yeast.

Table 1. Ubiquitin-like proteins

MODIFIER	ACTIVATING ENZYME E1	CONJUGATING ENZYME E2	LIGASE E3	SUBSTRATE	FUNCTION
Ubiquitin	UBA1	UBC1-8,10,11,13	SCF, APC, etc.	p27, IκBα, Cyclins, etc.	Proteasome-dependent degradation Endocytosis
UCRP	?	?	?	?	?
Sentrin/SMT3/ SUMO1	AOS1/UBA2	UBC9	?	PML, IκBα,, RanGAP1, etc.	Targetting?
NEDD8/RUB1	ULA1/UBA3	UBC12	SCF, VBC, etc.	Cullins	Regulation?
APG12	APG7	APG10	None	APG5	Autophagy
APG8	APG7	?		Phosphatidyl-ethanolamine	Lipidation
URM1	UBA4	?	?	?	?

NEDD8 is a small protein of 81 amino acids, which is 60% identical and 80% homologous to ubiquitin. The yeast and plant homologues of NEDD8 are called RUB1 (related to ubiquitin 1) (del Pozo and Estelle, 1999; Lammer et al., 1998; Liakopoulos et al., 1998). *Unlike* ubiquitin, which is detectable equally in the nucleus and cytosol, NEDD8 is highly enriched in the nucleus (Kamitani et al., 1997). In contrast to sentrins, the overall structures of NEDD8 and RUB1 are more closely related to ubiquitin (Rao-Naik et al., 1998; Whitby et al., 1998). The activating enzyme complex for human NEDD8 is composed of two subunits: APP-BP1 (also known as AXR1 in plants and Ula1/Enr2 in yeast) and UBA3 (Ecr1 in plants), which are homologous to the N- and C-terminal halves of ubiquitin E1, respectively. UBA3 contains the active site Cys residue required for the formation of a thiol ester linkage with NEDD8 and binds preferentially to NEDD8, but not ubiquitin or sentrin (Gong and Yeh, 1999). UBC12 functions as an E2 for NEDD8 and RUB1 (Liakopoulos et al., 1998) and a dominant-negative form of UBC12 can abolish NEDD8 conjugation *in vivo* (Wada et al., 2000).

The RUB1 conjugation system in plants is involved in the normal developmental response to the hormone auxin (Pozo et al., 1998). Cullins are the major and only known targets of NEDD8 modification in yeast, plants, and mammals. Budding yeast CDC53/Cullin is required for G1/S progression and is a subunit of an SCF ubiquitin ligase that targets CDC28 inhibitor Sic1 for degradation, allowing cells to initiate DNA replication (Feldman et al., 1997; Verma et al., 1997). RUB1 is non-essential in budding yeast *S. cerevisiae*, but genetic evidence suggests that the inability to modify CDC53 by RUB1 renders cells sensitive to alterations in the levels of the SCF components CDC4, CDC34, and CDC53, suggesting that RUB1 conjugation to CDC53 is required for optimal function of SCF complexes. In fission yeast, however, the Nedd8 modification pathway plays an essential role in SCF function (Osaka et al., 2000). Disruption of the genes encoding Nedd8, UBA3, or Ubc12 is lethal. The Cullin 1 ortholog in *S. pombe*, Pcu1, is completely modified by Nedd8 when assembled in SCF complexes. A $Pcu1^{K713R}$ mutant that is defective for Nedd8 conjugation does not complement the lethality of *pcu1* deletion. Forced

expression of $Pcu1^{K713R}$ or depletion of Nedd8 results in impaired cell proliferation and stabilization of the CDK inhibitor Rum1, which is functionally homologous to budding yeast Sic1, and is a substrate of the SCF complex. Human Cullins, CUL1-5, are also modified by NEDD8 (Hori et al., 1999), and NEDD8 modification of the Cullins may play a crucial role in establishing their ligase activity. NEDD8 modified CUL1 and other SCF components are enriched in the centrosome, suggesting that NEDD8 modified SCF is involved in centrosome duplication (Freed et al., 1999). Accumulated evidence suggests that conjugation of NEDD8 to CUL1 greatly enhances SCF activity. Covalent linkage of NEDD8 to bacterially expressed CUL1 is both necessary and sufficient to enhance the ability of the SCF core ubiquitin ligase to promote polyubiquitin chain formation (Wu et al., 2000). Moreover, a K720R mutation in CUL1, which eliminates NEDD8 modification significantly reduces the ability of $SCF^{\beta TrCP}$ to support ubiquitination of phosphorylated IκBα, and β-catenin (Read et al., 2000). Similarly, p27 ubiquitination activity in cell extracts depends on the presence of the ubiquitin-like protein NEDD8 and enzymes that catalyze NEDD8 conjugation to proteins. Moreover, reconstitution of the p27 ubiquitination activity with recombinant SCF^{SKP2} also requires NEDD8 conjugation pathway components. Inactivation of the NEDD8 conjugation pathway by a dominant negative mutant of the NEDD8-conjugating enzyme UBC12 blocks the ubiquitination and degradation of p27 in cell extracts (Podust et al., 2000).

Unlike sentrins and NEDD8, three other recently discovered modifiers, APG12, APG8, and URM1, do not show apparent overall homology to ubiquitin and other related proteins, but nevertheless are components of protein conjugation pathways with striking resemblance to that of ubiquitin. URM1 is a 99 amino acid protein that possesses the C-terminal Gly-Gly motif, which is a common feature of ubiquitin and ubiquitin-related modifiers (Furukawa et al., 2000). A portion of UBA4 protein, which serves as an activating enzyme for URM1, has high similarity to regions in other E1-like enzymes, including an ATP-binding motif and a conserved Cys residue.

URM1 is covalently attached to at least one protein inside the cell, presumably through an isopeptide bond. The exact function of the Urm1 system and its other components are still to be determined.

A lot more is known about the APG12 and APG8 conjugation pathway that is conserved from yeast to humans and is required for autophagy, a process responsible for non-selective degradation of cytoplasmic components in the lysosome (Mizushima et al., 1998). APG7 shows significant homology with the E1 Ub-activating enzyme UBA1 and forms a thiolester bond between its active-site Cys residue and a carboxy-terminal Gly residue of a 186 amino acid protein APG12 (Tanida et al., 1999). The activated APG12 is transferred to APG5 through formation of an isopeptide bond between Lys residue of APG5 and the C-terminal Gly residue of APG12. Unlike the Ub-mediated conjugation system, APG5 appears to be the only target for activated APG12. APG12-APG5 conjugation depends on APG10 and, although it shows no homology to E2 Ub carrier enzymes, APG10 acts as a protein-conjugating enzyme (E2) for the APG12 modifier. Apg8 is also activated by an E1 protein, Apg7, and is transferred subsequently to the E2 enzyme Apg3 (Ichimura et al., 2000). Apg8 is then covalently conjugated to phosphatidylethanolamine through an amide bond between the C- terminal glycine and the amino group of phosphatidylethanolamine (Ichimura et al., 2000). This lipidation has an essential role in membrane dynamics during autophagy.

Deubiquitinating and Deneddylating Enzymes

Because of its irreversibility, protein degradation requires precise timing and specific recognition of different substrates at appropriate cellular milieu. This is thought to be mediated by a combinatorial usage of different E2 and E3 enzymes and by a large family of deubiquitinating enzymes (Wilkinson and Hochstrasser, 1998). Deubiquitinating enzymes are ubiquitin-specific thiol proteases that cleave either linear ubiquitin precursor proteins or ubiquitin conjugates. Some deubiquitinating enzymes appear to perform an editing function, which controls the fidelity of the conjugation process, thus preventing inappropriate degradation of

cellular proteins (Kam et al., 1997). Two families of deubiquitinating enzymes have been identified on the basis of *in vitro* activities and/or sequence identity. The ubiquitin C-terminal hydrolases (UCHs) are small (approximately 25 kDa) thiol proteases that share amino acid sequence identity and cleave esters and amides from the C terminus of ubiquitin. Unrelated in sequence to the UCHs are the ubiquitin-specific proteases (UBPs), a large family of proteins differing greatly in length but characterized by sequence similarity in several regions: the Cys box, the His box, and six other blocks of amino acid sequence identity. The proteases specific for other ubiquitin-like proteins, Sentrin 1 and SMT3, are structurally different from the ubiquitin-specific UBPs and the UCHs. However, some of the UCHs and UBPs may have dual specificity for both ubiquitin and NEDD8. For example, UCH-L3, which was originally identified as a ubiquitin-specific hydrolase, was found to possess NEDD8 C-terminal hydrolase activity (Wada et al., 1998). Recently, a novel UBP, USP21, capable of deconjugating ubiquitin from ubiquitinated proteins, was shown to be capable of removing NEDD8 from NEDD8 conjugates, but not Sentrin 1 from Sentrin 1 conjugates (Gong et al., 2000). It is not known whether this overlap is limited to certain UBPs and UCHs, or is a generalized property of these enzymes. It is also possible that there is a family of NEDD8-specific proteases yet to be discovered that are structurally different from UBPs and UCHs.

Cop9 Signalosome

To grow and develop normally, all organisms need to perceive and process information from both their biotic and abiotic surroundings. Animal cells are influenced by the availability of nutrients and growth factors. In plants, a particularly important environmental cue is light, to which organisms respond in many different ways. Plants have developed highly sophisticated photosensory and transductory mechanisms to allow the modulation of development in response to light intensity, wavelength, duration, and direction. After germination, the young plant seedling must choose between two developmental programs, depending on the available light. In

the absence of light, seedlings undergo skotomorphogenesis, characterized by a long hypocotyl (primary stem), an apical hook, and unopened cotyledons (embryonic leaves) – features that allow the seedling to grow through a layer of soil and emerge in the light. Light induces the photomorphogenic program, a developmental process that optimizes the body plan of the seedling for efficient photosynthetic growth. During photomorphogenesis, a new gene expression program is induced, the rate of hypocotyl growth decreases, the apical hook opens, cotyledons expand, and chloroplasts develop.

COP9 Signalosome (CSN) was originally discovered in *Arabidopsis thaliana* as a suppressor of the photomorphogenic program. There are two types of photomorphogenic mutants in *A. thaliana*. The first group of mutants displays the dark grown characteristics when grown in specific light conditions and probably represent mutations in genes encoding downstream components of the light signaling cascade. The mutants in the second group, which include mutations in CSN subunits, exhibit light-grown seedling characteristics when grown in darkness (Wei et al., 1994).

Signalosome consists of eight subunits (CSN1-8) (Wei and Deng, 1998). Interestingly, subunits of the COP9 Signalosome posses significant sequence homologies with the lid subunits of the 19S regulator of the 26S proteasome (see Table 2) (Seeger et al., 1998; Wei et al., 1998) and with the translational initiation complex eIF3 (Glickman et al., 1998). The 19S regulator consists of two subcomplexes: the base and the lid (see above). Six subunits of the CSN, 19S lid, and several subunits of eIF3 contain PCI domains (for Proteasome, COP9, Initiation Factor 3) (Table 2). The PCI domain is a purely α-helical domain of ~200 amino acid residues, which is generally localized at the extreme C-terminus of the protein (Hofmann and Bucher, 1998). The PCI domain does not contain any invariant residues or any conserved pattern of charged residues that would suggest catalytic activity.

Table 2. COP9 Signalosome subunits

SUBUNIT	OTHER NAMES	MW (KDA)	19S LID COUNTERPART	% IDENTITY	DOMAIN
CSN1	GPS1	57	Rpn7	22	PCI
CSN2	Trip15	51	Rpn2	21	PCI
CSN3	SGN3	46	Rpn3	20	PCI
CSN4		45	Rpn5	19	PCI
CSN5	JAB1	38	Rpn11	28	MPN
CSN6	hVIP	36	Rpn8	22	MPN
CSN7a,b		30	Rpn9	15	PCI
CSN8	hCOP9	23	Rpn12	18	PCI

A second domain (MPN) is observed in two subunits of the CSN, 19S lid, and eIF3 complexes. The MPN domain is distinct from the PCI domain. It spans ~140 amino acid residues, typically is present at the N-terminus of proteins, and is predicted to have an α/β structure. All proteins containing PCI and MPN domains appear to be part of large multiprotein compexes.

The remarkable parallels between CSN and 19S lid suggest that perhaps they have evolved from a common ancestor and still exhibit similarities in structure and function. Electron microscopy studies suggest that while a common architectural plan for the two complexes could not be deduced, both COP9 Signalosome and 19S proteasome lid lack any symmetry in subunit arrangement and exhibit a central groove, possibly qualified for scaffolding functions (Kapelari et al., 2000). Cellular localization of CSN complex is similar to that of the 26S proteasome and is mostly nuclear and perinuclear (Chamovitz et al., 1996; Wei and Deng, 1998). The exact function of the CSN is likely to be different from the 19S lid complex and remains largely elusive. COP9 Signalosome is essential for development of *Drosophila melanogaster* (Freilich et al., 1999) and haploinsufficiency of CSN subunit 3 might be responsible for developmental disorders in the Smith-Mergenis syndrome (Elsea et al., 1999). Several lines of evidence suggest

that CSN has a role in signal tranduction processes. CSN1 was previously identified as GPS1, which was isolated as a suppressor of the lethality of an *S. cerevisiae* mutant lacking α subunit of a trimeric G protein (Spain et al., 1996). Overexpression of CSN1 in mammalian cells inhibits JNK and represses JUN-dependent promoter activity. CSN1 also contains canonical MAP kinase kinase (MAPKK) activation loop motifs (Seeger et al., 1998). The purified complex is associated with a kinase that phosphorylates transcriptional regulators such as c-Jun, IκBα, and p105 (Seeger et al., 1998). CSN subunits 2 and 7 are also phosphorylated by the associated kinase activity (Kapelari et al., 2000). CSN2 is also known as Trip15, which was identified in a two-hybrid screen as a protein that interacts with the thyroid hormone receptor and the retinoic acid receptor (Lee et al., 1995). CSN5 was described previously as JAB1 (JUN activation domain binding protein 1) and was shown to interact with JUN and coactivate JUN mediated gene expression (Claret et al., 1996). JAB1 interacts with p27 CDK inhibitor, causes its export into the cytosol, and promotes its degradation through an unknown mechanism (Tomoda et al., 1999). Interestingly, CSN5 is the only Signalosome subunit that is found in both complexed and monomeric form *in vivo* (Kwok et al., 1998). Monomeric CSN5 seems to be cytoplasmic, while CSN5 complexed with Signalosome is nuclear. Notably, CSN5 has the highest homology (28% identity) to its 19S lid counterpart than any other CSN subunit. It also contains a conserved Cys which is flanked by a highly conserved sequence with similarities to the active site "Cys box" seen in many deubiquitinating enzymes. CSN6 interacts with HIV-1 Vpr protein and is a potential host cell target responsible for the viral Vpr-induced cellular differentiation and growth arrest (Mahalingam et al., 1998). Interestingly, cells expressing antisense CSN6 were blocked at G2/M phase, suggesting that CSN might have a role in cell cycle progression. Similar to CSN1, CSN6 polypeptide has MAPKK activation loop motifs. While there does not seem to be a complete CSN complex in *S. cerevisiae*, CSN was described in *S. pombe* (Mundt et al., 1999), which indicates that fission yeast is evolutionarily much closer to metazoans than budding yeast.

Fission yeast cells that lack CSN1 (*Δcaa1* cells) experience significant problems in passing through S phase and are synthetically lethal with S/M checkpoint mutants, suggesting that *Δcaa1* cells require checkpoint function for viability.

More is known about CSN function in plant photomorphogenesis. A number of genes that regulate photomorphogenesis but are not part of COP9 Signalosome have been identified in *A. thaliana*. Two important components of this photomorphogenic pathway are Hy5 and COP1, which act antagonistically in regulation of photomorphogenic development (Ang et al., 1998). Hy5 is a bZIP transcription factor that binds directly to the light response elements in the promoters of the light-inducible genes and activates their transcription (Chattopadhyay et al., 1998; Oyama et al., 1997). Hy5 abundance inversely correlates with light intensity: Hy5 protein levels are highest in light-grown seedlings and rapidly decline in the dark due to 26S proteasome dependent degradation (Osterlund et al., 2000). Hy5 degradation in the darkness requires CSN and the product of COP1 gene. COP1 is a repressor of photomorphogenesis and is not a component of the CSN complex. In the dark, COP1 protein localizes to the nucleus and represses Hy5 activity (Osterlund et al., 1999). Light stimulus leads to COP1 relocalization into the cytoplasm. COP9 Signalosome positively regulates COP1 function and cellular localization by either promoting nuclear accumulation of COP1 or perhaps inhibiting its export from the nucleus (Chamovitz et al., 1996) (Fig.1).

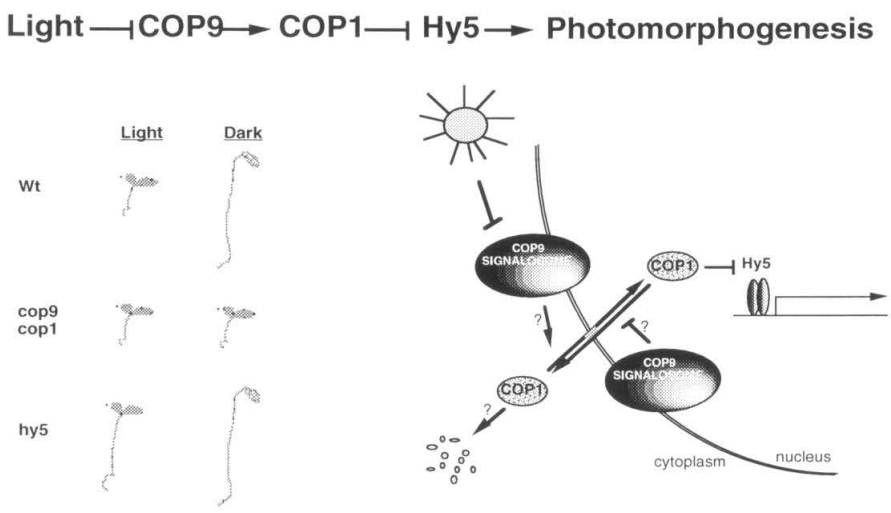

Figure 1. The model of plant photomorphogenesis

COP1 contains an N-terminal RING-HC finger domain, which interacts with a RING-H2 protein CIP8 (Torii et al., 1999), a coiled-coil motif, and a C-terminal WD40 repeat domain that directly binds to Hy5 protein (Torii et al., 1998). Given the striking parallels between COP1 domain structure and SCF ubiquitin ligases it is likely that COP1 is part of a yet to be described multi-domain ubiquitin ligase that regulates photomorphogenesis by targeting the critical transcription factors for degradation in the dark (Osterlund et al., 2000).

Future Directions

Recent discovery of Cullins and SCF ubiquitin ligases and some of their targets rekindled the interest in ubiquitin-mediated protein degradation within the scientific community. Although we now understand the general architecture of CUL1 and CUL2-based ubiquitin ligases, many more questions are brought to life with each new advance in the field. First, the components of other Cullin-based ubiquitin ligases remain a mystery. Recent recruitment of mass spectrometry to the arsenal of research tools available to molecular biologists and biochemists (Seol et al.,

1999) should aid considerably in the elucidation of other ubiquitin ligase components. Second, although a number of substrates of Cullin-based ubiquitin ligases have been discovered, this discovery was largely driven by educated guessing. Once this resource is exhausted, clever genetic, biochemical, or pharmacological screens would have to be devised to continue with the progress. Third, as the network of Cullin based ubiquitin ligases and their substrates emerges, understanding the regulation of this network and its connections to the signal transduction network will become an interesting intellectual challenge. The discovery of SCF regulation by COP9 Signalosome, described here, sets the stage for further research on the regulation of intracellular protein degradation. Major questions, emerging from this study, are:

- does CSN have other activities besides promoting deneddylation?
- is CSN activity regulated? If so, how?
- how does CSN influence Cullin ubiquitin ligase activity, apart from regulation of Cullin neddylation?
- are all neddylated proteins regulated by CSN deneddylating activity?
- what are other neddylated protein affected by CSN?
- are other ubiquitin ligases also regulated by CSN?
- how does CSN integrate various signal transduction pathways and relay them onto its substrates?
- does CSN associate with an ATPase, analogous to the 19S base particle?

As a rule, basic structural components of many biological pathways tend to be evolutionarily conserved, unlike the regulatory networks they organize into. Thus, although building materials of Cullin based ubiquitin ligases would likely be very similar across species, the regulation of their activity might differ significantly between species. The glimpses of these differences can be seen already. Whereas SCF components are evolutionarily conserved in budding yeast, fission yeast, plants, drosophila, and animal cells, COP9 Signalosome is not. CSN is absent in *S. cerevisiae*, except for JAB1 homologue, is present but not essential in *S. pombe*

(Mundt et al., 1999), *A. thaliana* (Wei and Deng, 1992), is essential in *D. melanogaster* (Freilich et al., 1999), and more than likely, animal cells. Thus, it seems that CSN function gained importance in parallel with evolving complexity of signal transduction pathways, and therefore became essential in multicellular organisms with intricate communication between cells within an organism. As reported here, CSN harbors a deconjugating activity towards RUB1/NEDD8. Interestingly, the RUB1/NEDD8 modification pathway is dispensable in *S. cerevisiae* (Lammer et al., 1998; Liakopoulos et al., 1998) and *A. thaliana* (Pozo et al., 1998), but is absolutely essential in *S. pombe* (Mundt et al., 1999), and, possibly, animal cells. Additionally, CSN seems to positively regulate COP1 putative ubiquitin ligase activity in plants (Osterlund et al., 2000), whereas our data suggest that CSN is a negative regulator of SCF in fission yeast. Thus, elucidation of the exact CSN function and its role in regulating various signal transduction and protein degradation pathways in different organisms will not be a trivial task to pursue.

Literature Cited

Ang, L., Chattopadhyay, S., Wei, N., Oyama, T., Okada, K., Batschauer, A., and Deng, X. (1998). Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of Arabidopsis development. Molecular Cell *1*, 213-222.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell *86*, 263-274.

Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nature Cell Biology *1*, 193-9.

Cenciarelli, C., Chiaur, D., Guardavaccaro, D., Parks, W., Vidal, M., and Pagano, M. (1999). Identification of a family of human F-box proteins. CURRENT BIOLOGY *9*, 1177-1179.

Chamovitz, D., Wei, N., Osterlund, M., vonArnim, A., Staub, J., Matsui, M., and Deng, X. (1996). The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. Cell *86*, 115-121.

Chattopadhyay, S., Ang, L., Puente, P., Deng, X., and Wei, N. (1998). Arabidopsis bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. Plant Cell *10*, 673-683.

Claret, F., Hibi, M., Dhut, S., Toda, T., and Karin, M. (1996). A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. Nature *383*, 453-457.

Cohen-Fix, O., Peters, J. M., Kirschner, M. W., and Koshland, D. (1996). Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes & Development *10*, 3081-93.

Cooper, K., Mallory, M., Egeland, D., Jarnik, M., and Strich, R. (2000). Ama1p is a meiosis-specific regulator of the anaphase promoting complex/cyclosome in yeast. Proceedings Of The National Academy Of Sciences Of The United States Of America *97*, 14548-14553.

del Pozo, J. C., and Estelle, M. (1999). The Arabidopsis cullin AtCUL1 is modified by the ubiquitin-related protein RUB1. Proceedings of the National Academy of Sciences of the United States of America *96*, 15342-7.

Deshaies, R. J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annual Review Cell and Developmental Biology *15*, 435-67.

Deshaies, R. J., Chau, V., and Kirschner, M. (1995). Ubiquitination of the G1 cyclin Cln2p by a CDC34p-dependent pathway. EMBO Journal *14*, 303-12.

Dohmen, R. J., Stappen, R., McGrath, J. P., Forrova, H., Kolarov, J., Goffeau, A., and Varshavsky, A. (1995). An essential yeast gene encoding a homolog of ubiquitin-activating enzyme. Journal of Biological Chemistry *270*, 18099-109.

Elsasser, S., Chi, Y., Yang, P., and Campbell, J. L. (1999). Phosphorylation controls timing of CDC6p destruction: A biochemical analysis. Molecular Biology of the Cell *10*, 3263-77.

Elsea, S., Mykytyn, K., Ferrell, K., Coulter, K., Das, P., Dubiel, W., Patel, P., and Metherall, J. (1999). Hemizygosity for the COP9 signalosome subunit gene, SGN3, in the Smith-Magenis syndrome. American Journal of Medical Genetics *87*, 342-348.

Fang, G., Yu, H., and Kirschner, M. W. (1998). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. Genes & Development *12*, 1871-83.

Feldman, R., Correll, C., Kaplan, K., and Deshaies, R. (1997). A complex of CDC4p, Skp1p, and CDC53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221-230.

Freed, E., Lacey, K., Huie, P., Lyapina, S., Deshaies, R., Stearns, T., and Jackson, P. (1999). Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. Genes & Development *13*, 2242-2257.

Freilich, S., Oron, E., Kapp, Y., Nevo-Caspi, Y., Orgad, S., Segal, D., and Chamovitz, D. (1999). The COP9 signalosome is essential for development of Drosophila melanogaster. Current Biology *9*, 1187-1190.

Fuchs, S., Chen, A., Xiong, Y., Pan, Z., and Ronai, Z. (1999). HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IκB and β-catenin. Oncogene *18*, 2039-2046.

Furukawa, K., Mizushima, N., Noda, T., and Ohsumi, Y. (2000). A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. Journal of Biological Chemistry *275*, 7462-5.

Galan, J., and Peter, M. (1999). Ubiquitin dependent degradation of multiple F-box proteins by an autocatalytic mechanism. Proceedings of the National Academy of Sciences of the United States of America *96*, 9124-9129.

Glickman, M., Rubin, D., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V., and Finley, D. (1998). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell *94*, 615-623.

Gmachl, M., Gieffers, C., Podtelejnikov, A., Mann, M., and Peters, J. (2000). The RING-H2 finger protein APC11 and the E2 enzyme UBC4 are sufficient to ubiquitinate substrates of the anaphase-promoting complex. Proceedings of the National Academy of Sciences of the United States of America *97*, 8973-8978.

Gong, L., Kamitani, T., Fujise, K., Caskey, L. S., and Yeh, E. T. (1997). Preferential interaction of sentrin with a ubiquitin-conjugating enzyme, Ubc9. Journal of Biological Chemistry *272*, 28198-201.

Gong, L., Kamitani, T., Millas, S., and Yeh, E. T. (2000). Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins. Journal of Biological Chemistry *275*, 14212-6.

Gong, L., and Yeh, E. T. (1999). Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway. Journal of Biological Chemistry *274*, 12036-42.

Hershko, A. (1999). Mechanisms and regulation of the degradation of cyclin B. Philosophical Transactions of The Royal Society of London Series B-Biological Sciences *354*, 1571-1575.

Hershko, A., and Ciechanover, A. (1998). The Ubiquitin System. Annual Review Biochemistry *67*, 425-479.

Hofmann, K., and Bucher, P. (1998). The PCI domain: a common theme in three multiprotein complexes. Trends in Biochemical Sciences *23*, 204-205.

Hori, T., Osaka, F., Chiba, T., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S., and Tanaka, K. (1999). Covalent modification of all members of human cullin family proteins by NEDD8. Oncogene *18*, 6829-6834.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000). A ubiquitin-like system mediates protein lipidation. Nature *408*, 488-492.

Iliopoulos, O., Levy, A. P., Jiang, C., Kaelin, W. G., Jr., and Goldberg, M. A. (1996). Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. Proceedings of the National Academy of Sciences of the United States of America *93*, 10595-9.

Irniger, S., Piatti, S., Michaelis, C., and Nasmyth, K. (1995). Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. Cell *81*, 269-277.

Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R., Canaway, J., Klausner, R., and Pause, A. (1999). Identification of the von Hippel-Lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. Proceedings Of The National Academy Of Sciences Of The United States Of America *96*, 12436-12441.

Jackson, P., Eldridge, A., Freed, E., Furstenthal, L., Hsu, J., Kaiser, B., and Reimann, J. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends in Cell Biology *10*, 429-439.

Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T., and Liu, Y. C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2- dependent ubiquitin-protein ligase. Science *286*, 309-12.

Johnson, E., Schwienhorst, I., Dohmen, R., and Blobel, G. (1997). The ubiquitin-like protein SMT3p is activated for conjugation to other proteins by an Aos1p/UBA2p heterodimer. EMBO Journal *16*, 5509-5519.

Juang, Y., Huang, J., Peters, J., McLaughlin, M., Tai, C., and Pellman, D. (1997). APC-mediated proteolysis of Ase 1 and the morphogenesis of the mitotic spindle. Science *275*, 1311-1314.

Kam, Y., Xu, W., DeMartino, G., and Cohen, R. (1997). Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature *385*, 737-740.

Kamitani, T., Kito, K., Nguyen, H. P., and Yeh, E. T. (1997). Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein. Journal of Biological Chemistry *272*, 28557-62.

Kamura, T., Conrad, M. N., Yan, Q., Conaway, R. C., and Conaway, J. W. (1999). The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates RUB1 modification of cullins CDC53 and Cul2. Genes & Development *13*, 2928-33.

Kamura, T., Koepp, D. M., Conrad, M. N., Skowyra, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Jr., Elledge, S. J., Conaway, R. C., Harper, J. W., and Conaway, J. W. (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657-61.

Kapelari, B., Bech-Otschir, D., Hegerl, R., Schade, R., Dumdey, R., and Dubiel, W. (2000). Electron microscopy and subunit-subunit interaction studies reveal a first architecture of COP9 signalosome. Journal of Molecular Biology *300*, 1169-1178.

King, R., Peters, J., Tugendreich, S., Rolfe, M., Hieter, P., And Kirschner, M. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the Mitosis-specific conjugation of ubiquitin to Cyclin B. Cell *81*, 279-288.

Kornitzer, D., Raboy, B., Kulka, R. G., and Fink, G. R. (1994). Regulated degradation of the transcription factor GCN4. European Molecular Biology Organization Journal *13*, 6021-6030.

Kramer, E., Scheuringer, N., Podtelejnikov, V., Mann, M., and Peters, J. (2000). Mitotic regulation of the APC activator proteins CDC20 and CDH1. Molecular Biology of the Cell *11*, 1555-1569.

Krek, W. (2000). VHL takes HIF's breath away. Nature Cell Biology *2*, E121-E123.

Kwok, S., Solano, R., Tsuge, T., Chamovitz, D., Ecker, J., Matsui, M., and Deng, X. (1998). Arabidopsis homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic cop/det/fus mutations. Plant Cell *10*, 1779-1790.

Lammer, D., Mathias, N., Laplaza, J. M., Jiang, W., Liu, Y., and al., e. (1998). Modification of yeast CDC53p by the ubiquitin-related protein RUB1p affects function of the SCFCDC4 complex. Genes & Development *12*, 914-926.

Lee, J., Choi, H., Gyuris, J., Brent, R., And Moore, D. (1995). 2 Classes of Proteins Dependent on Either the Presence or Absence of Thyroid-Hormone for Interaction with the Thyroid-Hormone Receptor. Molecular Endocrinology *9*, 243-254.

Leverson, J., Joazeiro, C., Page, A., Huang, H., Hieter, P., and Hunter, T. (2000). The APC11 RING-H2 finger mediates E2-dependent ubiquitination. Molecular Biology of the Cell *11*, 2315-2325.

Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998). A novel protein *modification* pathway related to the ubiquitin system. EMBO Journal *17*, 2208-14.

Lisztwan, J., Imbert, G., Wirbelauer, C., Gstaiger, M., and Krek, W. (1999). The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. Genes & Development *13*, 1822-1833.

Lyapina, S., Correll, C., Kipreos, E., and Deshaies, R. (1998). Human CUL1 forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein. Proceedings of the National Academy of Sciences of the United States of America *95*, 7451-7456.

Mahalingam, S., Ayyavoo, V., Patel, M., Kieber-Emmons, T., Kao, G., Muschel, R., and Weiner, D. (1998). HIV-1 Vpr interacts with a human 34-kDa mov34 homologue, a cellular factor linked to the G(2)/M phase transition of the mammalian cell cycle. Proceedings of the National Academy of Sciences of the United States of America *95*, 3419-3424.

Mathias, N., Johnson, S., Byers, B., and Goebl, M. (1999). The abundance of cell cycle regulatory protein CDC4p is controlled by interactions between its F box and Skp1p. Molecular and Cellular Biology *19*, 1759-67.

McGrath, J. P., Jentsch, S., and Varshavsky, A. (1991). UBA1: an essential yeast gene encoding ubiquitin-activating enzyme. European Molecular Biology Organization Journal *10*, 227-236.

Michel, J. J., and Xiong, Y. (1998). Human CUL-1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A. Cell Growth and Differentiation *9*, 435-49.

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M., and Ohsumi, Y. (1998). A protein conjugation system essential for autophagy. Nature *395*, 395-8.

Mundt, K., Porte, J., Murray, J., Brikos, C., Christensen, P., Caspari, T., Hagan, I., Millar, J., Simanis, V., Hofmann, K., and Carr, A. (1999). The COP9/signalosome complex is conserved in fission yeast and has a role in S phase. Current Biology *9*, 1427-1430.

Narasimhan, J., Potter, J., and Haas, A. (1996). Conjugation of the 15-kDa interferon-induced ubiquitin homolog is distinct from that of ubiquitin. Journal of Biological Chemistry *271*, 324-330.

Ng, R. W., Arooz, T., Yam, C. H., Chan, I. W., Lau, A. W., and Poon, R. Y. (1998). Characterization of the cullin and F-box protein partner Skp1. FEBS Lett *438*, 183-9.

Ohta, T., Michel, J. J., Schottelius, A. J., and Xiong, Y. (1999). ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Molecular Cell *3*, 535-41.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh-e, A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., and Kato, S. (2000). Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. EMBO Journal *19*, 3475-3484.

Osterlund, M., Ang, L., and Deng, X. (1999). The role of COP1 in repression of Arabidopsis photomorphogenic development. Trends in Cell Biology *9*, 113-118.

Osterlund, M., Hardtke, C., Wei, N., and Deng, X. (2000). Targeted destabilization of HY5 during light-regulated development of Arabidopsis. Nature *405*, 462-466.

Oyama, T., Shimura, Y., and Okada, K. (1997). The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes & Development *11*, 2983-2995.

Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G., and Rolfe, M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science *269*, 682-685.

Patton, E. E., Willems, A. R., Sa, D., Kuras, L., Thomas, D., Craig, K. L., and Tyers, M. (1998). CDC53 is a scaffold protein for multiple CDC34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. Genes & Development *12*, 692-705.

Pfleger, C., and Kirschner, M. (2000). The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. Genes & Development *14*, 655-665.

Pickart, C. M., and Rose, I. A. (1985). Functional heterogeneity of ubiquitin carrier proteins. Journal of Biological Chemistry *260*, 1573-1581.

Podust, V., Brownell, J., Gladysheva, T., Luo, R., Wang, C., Coggins, M., Pierce, J., Lightcap, E., and Chau, V. (2000). A Nedd8 conjugation pathway is essential for proteolytic targeting of p27(Kip1) by ubiquitination. Proceedings of the National Academy of Sciences of the United States of America *97*, 4579-4584.

Pozo, J. C., Timpte, C., Tan, S., Callis, J., and Estelle, M. (1998). The ubiquitin-related protein RUB1 and auxin response in Arabidopsis. Science *280*, 1760-3.

Rao-Naik, C., delaCruz, W., Laplaza, J. M., Tan, S., Callis, J., and Fisher, A. J. (1998). The rub family of ubiquitin-like proteins. Crystal structure of Arabidopsis RUB1 and expression of multiple rubs in Arabidopsis. Journal of Biological Chemistry *273*, 34976-82.

Read, M., Brownell, J., Gladysheva, T., Hottelet, M., Parent, L., Coggins, M., Pierce, J., Podust, V., Luo, R., Chau, V., and Palombella, V. (2000). Nedd8 modification of Cul-1 activates SCF beta(TrCp)-dependent ubiquitination of I kappa B alpha. Molecular and Cellular Biology *20*, 2326-2333.

Regan-Reimann, J., Duong, Q., and Jackson, P. (1999). Identification of novel F-box proteins in Xenopus laevis. Current Biology *9*, R762-R763.

Saurin, A. J., Borden, K. L., Boddy, M. N., and Freemont, P. S. (1996). Does this have a familiar RING? Trends in Biochemical Sciences *21*, 208-14.

Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell *75*, 495-505.

Scheffner, M., Nuber, U., and Huibregtse, J. M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature *373*, 81-3.

Schulman, B., Carrano, A., Jeffrey, P., Bowen, Z., Kinnucan, E., Finnin, M., Elledge, S., Harper, J., Pagano, M., and Pavietich, N. (2000). Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. Nature *408*, 381-386.

Schwob, E., Bohm, T., Mendenhall, M. D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae [published erratum appears in Cell 1996 Jan 12;84(1):following 174]. Cell *79*, 233-44.

Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., Gordon, C., Naumann, M., and Dubiel, W. (1998). A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. FASEB Journal *12*, 469-478.

Seol, J., Feldman, R., Zachariae, W., Shevchenko, A., Correll, C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Shevchenko, A., Nasmyth, K., and Deshaies, R. (1999). CDC53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme CDC34. Genes & Development *13*, 1614-1626.

Shiyanov, P., Nag, A., and Raychaudhuri, P. (1999). Cullin 4A associates with the UV-damaged DNA-binding protein DDB. Journal of Biological Chemistry *274*, 35309-35312.

Singer, J., Gurian-West, M., Clurman, B., and Roberts, J. (1999). Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. Genes & Development *13*, 2375-2387.

Skowyra, D., Craig, K., Tyers, M., Elledge, S., and Harper, J. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*, 209-219.

Spain, B., Bowdish, K., Pacal, A., Staub, S., Koo, D., Chang, C., and Colicelli, J. (1996). Two human cDNAs, including a homolog of Arabidopsis FUS6 (COP11), suppress G-protein- and mitogen-activated protein kinase-mediated signal transduction in yeast and mammalian cells. Molecular and Cellular Biology *16*, 6698-6706.

Stebbins, C., Kaelin, W., and Pavletich, N. (1999). Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. Science *284*, 455-461.

Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., and Kominami, E. (1999). Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy. Molecular Biology of the Cell *10*, 1367-79.

Tomoda, K., Kubota, Y., and Kato, J. (1999). Degradation of the cyclin-dependent-kinase inhibitor p27(Kip1) is instigated by Jab1. Nature *398*, 160-165.

Torii, K., McNellis, T., and Deng, X. (1998). Functional dissection of Arabidopsis COP1 reveals specific roles of its three structural modules in light control of seedling development. EMBO Journal *17*, 5577-5587.

Torii, K., Stoop-Myer, C., Okamoto, H., Coleman, J., Matsui, M., and Deng, X. (1999). The RING finger motif of photomorphogenic repressor COP1 specifically interacts with the RING-H2 motif of a novel Arabidopsis protein. Journal of Biological Chemistry *274*, 27674-27681.

Verma, R., Annan, R. S., Huddleston, M. J., Carr, S. A., Reynard, G., and Deshaies, R. J. (1997). Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. Science *278*, 455-60.

Verma, R., Feldman, R., and Deshaies, R. J. (1997). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. Molecular Biology of the Cell *8*, 1427-1437.

Visintin, R., Prinz, S., and Amon, A. (1997). CDC20 and CDH1: A family of substrate-specific activators of APC-dependent proteolysis. Science *278*, 460-463.

Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S Proteasome: A Molecular Machine Designed for Controlled Proteolysis. Annual Review Biochemistry *68*, 1015-1068.

Wada, H., Kito, K., Caskey, L. S., Yeh, E. T. H., and Kamitani, T. (1998). Cleavage of the C-terminus of NEDD8 by UCH-L3. Biochemical and Biophysical Research Communications *251*, 688-92.

Wada, H., Yeh, E. T., and Kamitani, T. (2000). A dominant-negative UBC12 mutant sequesters NEDD8 and inhibits NEDD8 conjugation in vivo. Journal of Biological Chemistry *275*, 17008-15.

Wei, N., Chamovitz, D., And Deng, X. (1994). Arabidopsis COP9 is a component of a novel signaling complex mediating light control of development. Cell *78*, 117-124.

Wei, N., and Deng, X. (1998). Characterization and purification of the mammalian COP9 complex, a conserved nuclear regulator initially identified as a repressor of photomorphogenesis in higher plants. Photochemistry and Photobiology *68*, 237-241.

Wei, N., and Deng, X. (1992). Cop9 - A New Genetic-Locus Involved in Light-Regulated Development and Gene-Expression in Arabidopsis. Plant Cell *4*, 1507-1518.

Wei, N., Tsuge, T., Serino, G., Dohmae, N., Takio, K., Matsui, M., and Deng, X. (1998). The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. Current Biology *8*, 919-922.

Whitby, F. G., Xia, G., Pickart, C. M., and Hill, C. P. (1998). Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes. Journal of Biological Chemistry *273*, 34983-91.

Wilkinson, K. D., and Hochstrasser, M. (1998). The Deubiquitinating Enzymes. In Ubiquitin and the Biology of the Cell, J. Peters, J. Harris and D. Finley, eds. (New York: Plenum Press), pp. 99-120.

Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Kobayashi, R., Wittenberg, C., and Tyers, M. (1996). CDC53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. Cell *86*, 453-463.

Winston, J., Koepp, D., Zhu, C., Elledge, S., and Harper, J. (1999). A family of mammalian F-box proteins. Current Biology *9*, 1180-1182.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999). The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro [published erratum appears in Genes Dev 1999 Apr 15;13(8):1050]. Genes & Development *13*, 270-83.

Wu, K., Chen, A., and Pan, Z. (2000). Conjugation of Nedd8 to CUL1 enhances the ability of the ROC1-CUL1 complex to promote ubiquitin polymerization. Journal of Biological Chemistry *275*, 32317-32324.

Xie, Y., and Varshavsky, A. (1999). The E2-E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. EMBO Journal *18*, 6832-6844.

Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998). Identification of the receptor component of the IκBα-ubiquitin ligase. Nature *396*, 590-4.

Yeh, E., Gong, L., and Kamitani, T. (2000). Ubiquitin-like proteins: new wines in new bottles. Gene *248 (2000)*, 1-14.

Yeong, F., Lim, H., Padmashree, C., and Surana, U. (2000). Exit from mitosis in budding yeast: Biphasic inactivation of the CDC28-Clb2 mitotic kinase and the role of CDC20. Molecular Cell *5*, 501-511.

Yu, H., Peters, J., King, R., Page, A., Hieter, P., and Kirschner, M. (1998). Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science *279*, 1219-1222.

Yu, Z.-K., Gervais, J., and Zhang, H. (1998). Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21CIP1/WAF1 and cyclin D proteins. Proceedings of the National Academy of Sciences of the United States of America *95*, 11324-29.

Zachariae, W., Schwab, M., Nasmyth, K., and Seufert, W. (1998). Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. Science *282*, 1721-1724.

Zheng, N., Wang, P., Jeffrey, P., and Pavletich, N. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell *102*, 533-539.

Zhou, P., and Howley, P. (1998). Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases. Molecular Cell *2*, 571-580.

Chapter 2. Human CUL1 forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein

The data presented in this chapter were generated in collaboration with C. Correll and E. Kipreos (39). This work has been previously published (Lyapina et al., 1998; see Appendix I).

Introduction

The irreversible nature of proteolysis makes it well-suited to serve as a regulatory switch for controlling unidirectional processes. This principle is clearly evident in the organization of the cell division cycle, where initiation of DNA replication, chromosome segregation, and exit from mitosis are triggered by the destruction of key regulatory proteins (1-3).

Proteins are typically marked for proteolytic degradation by attachment of multiubiquitin chains. This process is initiated by ubiquitin-activating enzyme (E1), which activates ubiquitin by adenylation and becomes linked to it via a thiolester bond. Ubiquitin is then transferred to a ubiquitin-conjugating enzyme, E2. Whereas E2s can directly attach ubiquitin to lysine residues in a substrate, most physiological ubiquitination reactions probably require a ubiquitin ligase, or E3 (4). E3s have been implicated in substrate recognition and, in one case, transfer of ubiquitin from E2 to a substrate via an E3~ubiquitin-thiolester intermediate (5). Once the substrate is multiubiquitinated, it is then recognized and degraded by the 26S proteasome.

A novel ubiquitination pathway has recently been discovered in budding yeast. Components of this pathway include the CDC53, CDC4, and ySKP1 gene products, which assemble into a ubiquitin ligase complex known as SCF (for SKP1, Cullin, F-box protein): since several of the yeast and human subunits have identical names, e.g., SKP1, we distinguish them with the letters y or h, respectively. SCF collaborates with the E2 enzyme yCDC34 to catalyze

ubiquitination of the CDK inhibitor SIC1. The specificity of SCF is thought to be governed by ySKP1 and the F-box-containing subunit CDC4, which together form a substrate receptor that tethers SIC1 to the complex. The assembly of this receptor is thought to be mediated by a direct interaction between ySKP1 and the F-box sequences of CDC4 (6, 7).

Whereas genetic analysis has revealed that SIC1 proteolysis requires CDC4, G1 cyclin proteolysis appears to depend upon a distinct F-box-containing protein known as GRR1 (8). Alternative SCF complexes (SCF^{GRR1}) assembled with GRR1 instead of CDC4 bind G1 cyclins but not SIC1, suggesting that there exist multiple SCF complexes in yeast whose substrate specificities are dictated by the identity of the F-box subunit (7).

Components of the SCF ubiquitination pathway have been highly conserved during evolution. Human homologues of yCDC34 and ySKP1 have been reported (9, 10), and F-box-containing proteins like CDC4 and GRR1 have been identified in many eukaryotes (11). Many of these F-box proteins also contain either WD-40 repeats (like CDC4) or leucine-rich repeats (like GRR1). A potential human counterpart of GRR1, SKP2, has been identified along with hSKP1 as a Cyclin A/CDK2-associated protein that is necessary for S-phase progression (10). Homologues of CDC53, which are known as Cullins, are also present in many eukaryotes, including humans and nematodes (12, 13).

Studies in budding yeast suggest that SCF substrates must be phosphorylated before they can be ubiquitinated (14, 15). Several human cell cycle regulators are targeted for ubiquitination following their phosphorylation by CDKs, implicating them as potential substrates of SCF pathway(s) in human cells. Among them is the CDK inhibitor p27, the abundance of which may be regulated by CDC34-dependent ubiquitination (16, 17). In addition, Cyclins E and D1 are degraded by a ubiquitin-dependent pathway following phosphorylation at a specific site (18-20). The observation that Cyclin A/CDK2 associates preferentially with hSKP1 and SKP2 in transformed cells to the exclusion of PCNA and p21 (10) raises the possibility that Cyclin A is

also a target of an SCF pathway. Alternatively, SCF-bound Cyclin A/CDK2 may phosphorylate SCF subunits or potential substrates such as E2F-1/DP-1, thereby activating SCF-dependent ubiquitination (21, 22).

Despite the conservation of SCF components from yeast to humans, several observations raise the question of whether the metazoan homologues are actually components of SCF-like ubiquitin ligases. First, whereas *S.cerevisiae CDC53*^{ts} mutants arrest at the G1/S transition, *C.elegans cul-1* mutants fail to exit the cell cycle, resulting in hyperplasia of most larval tissues (12). It is unclear whether this discrepancy arises because cul-1 and CDC53 have different functions, or because they are components of distinct ubiquitin ligase complexes with different substrate specificities. Second, the recent discovery of ubiquitin-like proteins (RUB1/NEDD8 and SMT3/SUMO1) that are conjugated to proteins by pathways that involve E1 and E2 homologues (23) suggests that some homologues of SCF components might function in these alternative pathways. Indeed, attachment of RUB1 to CDC53 fails to occur in *skp1* mutants, suggesting that ySKP1 may be directly involved in the "rubinylation" of CDC53 (24). Third, the best characterized human Cullin, CUL2, assembles with the von Hippel-Lindau tumor suppressor protein (VHL)/Elongin B/Elongin C complex that has been suggested to regulate mRNA transcript elongation and accumulation of hypoxia-inducible mRNAs (25, 26). Forth, yeast SKP1 assembles into a centromere-binding complex independent of its role in SCF, consistent with the possibility that vertebrate SCF subunits may serve as components of a variety of unrelated molecular machines.

To address whether SCF-like activities are present in animal cells, we sought hCUL1 binding partners, and we tested whether putative human SCF subunits can assemble together to yield complexes with ubiquitin ligase activity. We report here that *hCUL1* is a direct functional homologue of *CDC53* since it can suppress the temperature-sensitive growth of *CDC53* mutants, associate with ubiquitin-conjugation activity in human cell lysates, and substitute for CDC53 in

the reconstitution of SIC1 ubiquitination with purified components. Moreover, hCUL1 directly binds to the putative SCF subunits hSKP1 and SKP2. Taken together, these data provide strong evidence that an SCF-dependent ubiquitination pathway is conserved from yeast to mammals.

Results

Human CUL1-interacting proteins. To identify human proteins that interact with hCUL1 we performed a two-hybrid screen (30, 31). A full length *hCUL1* cDNA, fused to the LexA DNA-binding domain, was used as a bait to identify cDNAs from a HeLa library that encode hCUL1 interactors. This screen yielded clones encoding hSKP1, Protein Phosphatase 2A (PP2A) catalytic subunit, and the 20S proteasome subunit HsN3. None of these clones interacted with LexA-hCDK2 or LexA-Lamin C baits, suggesting that their interaction with LexA-hCUL1 was specific. Here we examine in detail the interaction of hCUL1 with hSKP1 (see below). The physiological significance of hCUL1's interaction with HsN3 and PP2A has not yet been evaluated.

Human CUL1 interacts with hSKP1 *in vivo*. The identification of hSKP1 as a hCUL1-interacting protein suggested that these proteins may be subunits of a complex in human cells that is similar to the SCF ubiquitin ligase of budding yeast. To test whether hCUL1 interacts with hSKP1 *in vivo*, we prepared affinity-purified rabbit polyclonal antibodies directed against the N- and C-termini of hCUL1. Fig. 1A shows specificity of the affinity purified antibodies. Both antibodies recognized one major polypeptide of ~80 kDa in transformed (HeLa S3) and non-transformed (WI-38) cell lines (Fig. 1A, lanes 1, 2, 6, 7). This species comigrated with hCUL1 produced in Hi5 cells infected with a baculovirus that contains full length *hCUL1* cDNA (Fig. 1A, lanes 3 and 8). A more rapidly migrating species of recombinant hCUL1 detected in Hi5 cells by the anti-C-terminal antibodies (lane 3) presumably represents a breakdown product or initiation of translation downstream of the normal start codon, as this species was not detected by

the anti-N-terminal antibodies. As expected, addition of a Polyoma antigen-hexahistidine tag to *hCUL1* (^{PHis6}hCUL1) yielded a more slowly migrating hCUL1 band (Fig. 1A, lanes 4 and 9).

Neither polyclonal antibody precipitated hCUL1 from crude human cell lysates, precluding analysis of hCUL1 complexes in non-transfected cells. Thus, to evaluate the potential interaction of hCUL1 with hSKP1 *in vivo*, we transfected HeLa S3 cells with ^{PHis6}hCUL1 and ^{HA}hSKP1 expression vectors. Lysates were prepared from these cells 24 hr post-transfection and immunoprecipitated using cross-linked anti-Polyoma or anti-HA antibody beads. Proteins bound to the beads were separated by SDS-PAGE and analyzed by immunoblotting with anti-hCUL1 and anti-HA antibodies (Fig. 1B). Consistent with the two-hybrid data, hCUL1 was specifically detected in hSKP1 immunoprecipitates and vice versa.

Human CUL1, hSKP1, and SKP2 assemble into an SCF-like complex that can associate with Cyclin A/CDK2 kinase. Human SKP1 was initially identified as a Cyclin A/CDK2-associated protein in transformed human cells (10). This association is mediated by SKP2, a human F-box protein with leucine-rich repeats, reminiscent of the GRR1 protein. CDC53/Cullin and ySKP1, together with the F-box protein GRR1, constitute a putative SCF ubiquitin ligase complex that targets G1 cyclins for degradation (6, 7). The homology of hSKP1, SKP2, and hCUL1 proteins with components of the ySCF complex suggests that the human proteins may form a similar complex. We addressed this possibility by immunoprecipitating ^{PHis6}hCUL1 from [^{35}S]-labeled insect cells infected with baculoviruses that express ^{PHis6}hCUL1, hSKP1, and SKP2 (Fig. 2A); and by testing whether hCUL1 can assemble with a previously-described complex containing cyclin A/CDK2, hSKP1 and SKP2 (Fig. 2B). The interaction of hCUL1 with the cyclin A/CDK2^{HA}/hSKP1/SKP2 complex was monitored by immunoprecipitating CDK2^{HA} from [^{35}S]-labeled insect cells infected with all five viruses in various combinations. As shown in Fig. 2A, ^{PHis6}hCUL1 efficiently assembled with hSKP1 and SKP2, suggesting that these proteins form a ternary complex similar to ySCF. Surprisingly,

hCUL1 interacted with cyclin A/CDK2^{HA} complexes in the absence of SKP2 or hSKP1 (Fig. 2B, lane 6; note that hSKP1 does not associate with Cyclin A/CDK2^{HA} complex in the absence of SKP2). This may be due to either a direct interaction between hCUL1 and cyclin A/CDK2^{HA} or the presence of a bridging protein in insect cells (e.g., see reference 6). Regardless, the ability of hCUL1 expressed in insect cells to assemble into complexes containing a cyclin-dependent kinase is likely to be physiologically significant, since ^{PHis6}hCUL1 immunoprecipitates prepared from HeLa S3 cells contained histone H1 kinase activity (data not shown).

Human CUL1 directly interacts with hSKP1 and SKP2. The results in Fig. 2 suggest that hCUL1, hSKP1, and SKP2 can assemble into an SCF-like particle when co-expressed in insect cells. Due to the strong conservation of SCF components, however, these interactions might be mediated by other proteins provided by the host cells (for an example see ref. 6). To test whether the observed interactions are direct, we produced GST-hSKP1, GST-SKP2, and MBP-hCUL1 in bacteria. The GST fusions (or unfused GST control) were mixed with MBP-hCUL1 or MBP and recovered by binding to GSH-Sepharose beads. Bound proteins were resolved by SDS-PAGE and visualized by Coomassie Blue staining (Fig. 3). MBP-hCUL1, but not MBP, bound specifically and efficiently to GST-hSKP1 and GST-SKP2, but not GST. This result demonstrates that hCUL1 can bind to both hSKP1 and SKP2 without the participation of other proteins.

Human CUL1 is functionally homologous to CDC53 and can form an active chimeric SCF complex with ySKP1 and CDC4. The above observations indicate that hCUL1, the closest human homologue of CDC53, can assemble with hSKP1 and the F-box protein SKP2 into a complex reminiscent of the yeast SCF^{GRR1} complex. We next tested whether this complex – in the presence of hCDC34, E1 enzyme and ubiquitin – was able to ubiquitinate proteins that either bind to it (cyclin A) (10), are known to be degraded in S phase (cyclin E, E2F-1) (18, 19, 21, 22), or have been implicated as substrates of hCDC34 (p27) (16, 17). These efforts were

unsuccessful, raising the question of whether SCF^{SKP2} complexes possess ubiquitin ligase activity (data not shown). Moreover, ubiquitin ligase activity of the analogous yeast SCF^{GRR1} complex has not been demonstrated yet, and might require additional unidentified components. However, we were able to address whether hCUL1 is a functional component of a ubiquitin ligase complex genetically and biochemically by taking advantage of the considerable knowledge of this pathway in yeast. First, we asked if *hCUL1* can complement the $CDC53^{ts}$ mutation. We introduced *hCUL1* and *CDC53* under the control of the *GAL1* promoter into a yeast strain carrying a temperature sensitive mutation in the *CDC53* gene. Individual transformants were spotted at different dilutions on glucose (non-inducing conditions, data not shown) and galactose (inducing conditions) media at permissive (24°C) and restrictive (33°C) temperatures (Fig. 4). Only transformants that expressed wild type CDC53 or hCUL1 proteins were able to grow at the restrictive temperature. However, *hCUL1* failed to complement a *CDC53* null strain (data not shown).

The ability of hCUL1 to complement the $CDC53^{ts}$ mutation implied that hCUL1 can assemble into functional SCF complexes with yeast proteins. To test this idea we examined whether hCUL1 can interact with the budding yeast SCF subunits ySKP1 and CDC4. All three proteins were co-expressed in [^{35}S]-methionine-radiolabeled insect cells in various combinations as indicated in Fig. 5. Human CUL1 specifically co-precipitated with $ySKP1^{HA}$ (Fig. 5A, lane 2) or ^{PHA}CDC4/ySKP1 (Fig. 5B, lane 4), indicating that it can form a chimeric SCF^{CDC4} complex with yeast proteins.

Our previous findings identified SCF^{CDC4} as a functional E3 that required the presence of all three subunits (CDC4, CDC53, and ySKP1) to catalyze ubiquitination of phosphorylated SIC1 (6, 7). Preceding its ubiquitination, phosphorylated SIC1 is recruited to SCF^{CDC4} by binding to the CDC4/ySKP1 substrate receptor (6, 7). Given that hCUL1 and hSKP1 assembled with CDC4 (Fig. 5B), we sought to test whether these hybrid SCF complexes were able to promote

ubiquitination of phosphorylated SIC1. Purified chimeric SCF complexes were incubated with MBP-SIC1^{MHis6} and purified ubiquitination components. In the presence of SCF^{CDC4} (Fig. 6A, lane 1), MBP-SIC1^{MHis6} was efficiently converted to high molecular weight forms. Omission of either CDC4, CDC53, or ySKP1 resulted in no activity (Fig. 6A, lanes 2-4). Replacement of CDC53^{PHA} with ^{PHis6}hCUL1 resulted in an SCF complex with modest ubiquitination activity that was dependent upon both CDC4 and ySKP1 (lanes 5, 6, and 9). Additionally, an SCF complex containing both ^{PHis6}hCUL1 and hSKP1 along with ^{PHA}CDC4 was also able to catalyze ubiquitination of MBP-SIC1^{MHis6} (lane 10). The conversion of MBP-SIC1 to high molecular weight forms by hybrid CDC4/hCUL1/hSKP1 complexes required both substrate phosphorylation (Fig. 6B, lane 5) and the presence of ubiquitin (Fig. 6B, lane 6). Interestingly, co-expression of ^{PHA}CDC4, CDC53^{PHA}, and hSKP1 did not result in a functional SCF complex (Fig. 6A, lane 7).

CDC53 was previously shown to interact with yCDC34 (32). Thus, we presumed that an SCF complex containing hCUL1 would prefer to use hCDC34 as an E2 as opposed to yCDC34. However, SCF^{CDC4} complexes containing ^{PHis6}hCUL1 with either ySKP1^{His6} or hSKP1 appeared to work much more efficiently with yCDC34 than with hCDC34 serving as the E2 (Fig. 6C, lanes 3, 4, 7, and 8). Although we do not understand the basis for this preference, it is possible that there exist additional human CDC34-like E2s that interact preferentially with hCUL1-containing complexes. Alternatively, the interaction between an F-box subunit and an E2 enzyme might also contribute to the specificity for a particular E2 (38).

Human CUL1 assembles with ubiquitination-promoting activities in human cell extracts. The data presented so far are consistent with hCUL1 functioning as a component of a ubiquitin ligase complex in human cells. Since we have failed so far to detect ubiquitination activity using recombinant hCUL1/hSKP1/SKP2 complexes, we sought to develop an assay that would allow us to identify either substrates or cofactors of a hCUL1-dependent ubiquitination

pathway. ^{PHis6}hCUL1 produced in insect cells in the presence or absence of hSKP1 plus SKP2 was bound to anti-Polyoma beads and incubated with crude HeLa S3 lysates to allow binding of other potential SCF components, regulators, and substrates. After washing away unbound proteins, E1, hCDC34, biotinylated ubiquitin, and an ATP-regenerating system were then added to the beads. Following an incubation, reactions were fractionated by SDS-PAGE, transferred to nitrocellulose, and blotted with streptavidin-HRP to detect ubiquitin conjugates. Whereas ^{Phis6}hCUL1 or ^{Phis}6hCUL1/hSKP1/SKP2 complexes isolated from insect cells exhibited little ubiquitination activity (Fig. 7A, lane 2 and Fig. 7B, lanes 3 and 4), a high molecular weight smear characteristic of ubiquitinated proteins appeared (Fig. 7A, lane 3 and Fig. 7B, lanes 6 and 7) when these same components were preincubated with HeLa S3 lysate prior to the assay. In contrast, no signal was detected when naked polyoma beads were preincubated with HeLa S3 lysate (Fig. 7A, lane 1). The appearance of slowly migrating biotinylated proteins depended on the addition of ubiquitin and ATP-regenerating system to the reaction (Fig. 7B, lanes 8 and 9), indicating that the high molecular weight smear was due to ubiquitination occurring during the in vitro incubation.

Discussion

Multiple homologues of the ySKP1, CDC53, and F-box subunits of the SCF ubiquitin ligase complex have been identified (10-13) and implicated in various cellular processes, including kinetochore function (33, 34), S-phase progression (10), exit from the cell cycle (12), transcript elongation, regulation of hypoxia-inducible genes, and suppression of tumorigenesis (25, 26). Based on the close homology between hCUL1 and CDC53, we sought to address whether hCUL1 functions as part of an SCF-like ubiquitin ligase complex in human cells. A two-hybrid screen to identify proteins that interact with hCUL1 yielded hSKP1, suggesting that hCUL1 does indeed assemble into SCF-like complexes in human cells. Several other observations reported here support this hypothesis. First, hCUL1 associates with hSKP1 in

transfected HeLa S3 cells. Second, hCUL1 assembles into complexes with both hSKP1 and the F-box protein SKP2 *in vitro*. Third, *hCUL1* complements the growth defect of a *CDC53*^{ts} mutant. Fourth, hCUL1 and hSKP1 can form chimeric SCF complexes with CDC4, and these complexes are able to ubiquitinate the SCF^{CDC4} substrate SIC1 *in vitro*. Fifth, hCUL1 associates with ubiquitination-promoting activity in HeLa S3 cell lysate. Taken together, these data strongly suggest that hCUL1 is a subunit of an SCF-like E3 complex in human cells.

What are the candidate substrates for hCUL1-dependent ubiquitination in human cells? SIC1, CLN2, and FAR1 must be phosphorylated before they can be ubiquitinated by the budding yeast SCF/CDC34 pathway (6, 7, 35). The stability of many mammalian regulatory proteins - including IκBα, β-catenin, p27, Cyclin D, and Cyclin E - is known to be controlled by phosphorylation (16-20, 36, 37). Further work will be required to determine whether any of these proteins are substrates for human SCF complexes. SCF-associated Cyclin A might also be a substrate of the SCF^{SKP2} pathway. This is less likely, though, since cyclin A is thought to be primarily destroyed via the APC/cyclosome pathway, and both cyclin A and SKP2 activities are essential for entry into S phase (10). Instead, the tight association of cyclin A/CDK2 with SCF subunits both in vivo and in vitro might reflect an efficient coupling between substrate phosphorylation and ubiquitination in transformed cells.

A study that complements our findings was reported by Lisztwan et al. (38). These authors demonstrated that hCUL1, hSKP1, and SKP2 assemble into a complex both in unperturbed and transfected human cells. Moreover, SKP2 was also shown to bind the ubiquitin-conjugating enzyme hCDC34 in human cells, suggesting that SKP2 is part of an SCF-like ubiquitin ligase. SKP2 was also shown to associate with cyclin A/CDK2, and mutational analysis suggested that cyclin A/CDK2 binding might regulate SKP2/hSKP1 but not SKP2/hCUL1 interaction *in vivo*. Our data support these findings and extend them by establishing that hCUL1 interacts directly with hSKP1 and SKP2 without the participation of other eukaryotic proteins,

and hCUL1 and hSKP1 can assemble into active ubiquitin ligase complexes either in insect cells or in HeLa S3 cell lysates.

Further characterization of the SCF pathway in human cells will require the identification of functional F-box subunits and physiological substrates. The ability to stimulate the E3 activity of insect cell-derived hCUL1 with HeLa S3 cell lysate provides a novel strategy for identifying these proteins. Moreover, this assay can be readily adapted to test whether the related hCUL2-hCUL5 proteins also assemble into ubiquitin ligase complexes in human cells. Lastly, by converting either the chimeric SCF complex assay (Fig. 6) or the biotin-Ub-based assay (Fig. 7) to a microtiter plate format, it should be feasible to screen chemical libraries to identify compounds that modulate the activities of hSKP1 and hCUL1. Given its critical role in cell division in budding yeast, inhibitors of SCF might be valuable lead compounds for the development of novel anti-cancer chemotherapeutics.

Experimental Procedures

Yeast strains and reagents. Yeast strains, plasmids, and a HeLa cDNA library for the two-hybrid screen were a generous gift from R. Brent (Massachusetts General Hospital, Boston, MA). Wx131.2c *CDC53-2*^{ts} strain was obtained from M. Goebl (Indiana University, Indianapolis, IN). Baculoviruses expressing hCDK2^{HA}, hCyclin A (D. Morgan, UCSF, San Francisco, CA), SKP2 (H. Zhang, Yale, New Haven, CT), hSKP1 (P. Sorger, MIT, Cambridge, MA) and plasmids pGEX-KG-hSKP1, pGEX-KG-SKP2 (P. Jackson, Stanford, Palo Alto, CA), pCS2+nβgal, pCS2+SMC1 (S. Handeli, FHCRC, Seattle, WA) were kindly provided by the indicated investigators. Other baculoviruses were previously described (6). Ubiquitin and the Protein Biotinylation Kit were purchased from Sigma, and biotinylated ubiquitin was prepared according to the manufacturer's instructions. Ubiquitin aldehyde was a generous gift from R. Cohen (University of Iowa, Iowa City, Iowa).

Plasmid and baculovirus construction. Full length hCUL1 ORF was assembled from ESTs HE2AB96 and HSVAD74 and subcloned into pRS316 and pMALc (New England Biolabs). The same hCUL1 fragment was also subcloned into pVL1393 (PharMingen) to generate a hCUL1-expressing baculovirus. An N-terminal epitope-tagged version of hCUL1 was constructed by inserting a DNA cassette that contains two tandem repeats of the Polyoma epitope (MEYMPME) followed by six histidine residues (designated as PHis6) into pRS316-hCUL1. ^{PHis6}hCUL1 fragment was then subcloned into pFASTBAC1 (Gibco BRL) to generate a ^{PHis6}hCUL1 baculovirus, and pDNA3.1/Zeo (Invitrogen) to generate pcDNA3.1-PHis6-hCUL1. pCS2+HA-hSKP1 was generated by subcloning a hSKP1 fragment from pGEX-KG-hSKP1 into pCS2+HA.

Antibodies. Anti-hCUL1 antibodies were generated in rabbits immunized with either a fusion protein containing the first 41 residues of hCUL1 followed by GST (BAbCO), or a fusion protein containing GST followed by the last 86 residues of hCUL1 (Caltech antibody facility). Antibodies against hCUL1 and GST were affinity purified using MBP fusions of the corresponding peptides and GST, respectively, as described (27). Monoclonal anti-Polyoma antibodies were bound to protein A-Sepharose beads and cross-linked to protein A with dimethylpimilimadate (27) at a concentration of approximately 2 mg of antibodies per ml of protein A resin. Anti-HA resin was generated by coupling 1 ml of anti-HA ascites to 1 ml of CNBr activated agarose (Pharmacia Biotech) according to the manufacturer's protocol.

Expression and purification of proteins. Proteins expressed in bacteria or yeast were purified according to standard protocols and as described (6). For the expression and purification of chimeric SCF complexes, Hi5 insect cells were infected with baculoviruses expressing ^{PHA}CDC4

(PHA designates an epitope-tag consisting of two tandem repeats of the Polyoma epitope followed by three hemagglutinin epitopes), CDC53^{PHA}, ^{PHis6}hCUL1 (MOIs of 6), ySKP1^{His6}, or hSKP1 (MOIs of 4) as indicated in the results. Seventy-two hours post-infection, cells were collected and lysates were prepared as described (6). The Polyoma tagged proteins were affinity-purified from these lysates (6) to yield the various SCF complexes.

Cell cultures and transfections. WI-38 human lung fibroblasts were purchased from ATCC. HeLa S3 cells were a gift from S. Handeli (FHCRC, Seattle, WA). Cells were grown in DMEM-F12 (Gibco BRL) supplemented with 10% FBS (Gibco BRL) at 37^{0}C/5%CO_2. Cells were transfected in 100 mm dishes by the modified calcium phosphate method (28). 10 μg pCS2+HA-hSKP1 and 7.5 μg pcDNA3.1-PHis6-hCUL1 vectors were used per transfection plate. Transfection efficiency was monitored by co-transfection of 2.5 μg pCS2+nβgal plasmid per transfection plate followed by standard colorimetric βgal assays (29). Total DNA concentration was 20 μg/100 mm dish and was adjusted for every transfection plate by adding empty vectors. Cells were harvested and lysed 24 hr post-transfection.

Immunoprecipitations and Western blotting. Baculovirus-infected insect cells were harvested and lysed at 48 hr (for Sf9 cells) or 72 hr (for Hi5 cells) post-infection in 0.8 ml of lysis buffer per 100 mm plate (as described in ref. 6). Metabolic labeling was done by incubating insect cells for 3 hr in methionine-deficient medium plus 20 μCi/ml of Tran[^{35}S]-label prior to lysis. WI-38 and HeLa S3 cells were lysed in 0.4 ml of lysis buffer per 100 mm plate. Lysates were cleared by centrifugation at 14,000g for 15 min, adjusted to 10% glycerol, frozen in liquid nitrogen, and stored at -80^{0}C. Cell lysates (1 mg) were incubated with 50 μl of antibody-coupled beads (1:1 suspension in lysis buffer) for 2 hr at 4^{0}C. Precipitates were washed five times with 1 ml of lysis

buffer and analyzed by SDS-PAGE followed by Western blotting or autoradiography. Western blotting was performed as described (27). ^{PHis6}hCUL1 and ^{HA}hSKP1 were detected by rabbit polyclonal anti-hCUL1 and biotinylated anti-HA (12CA5) primary antibodies and visualized by incubation with goat anti-rabbit-HRP and streptavidin-HRP conjugates, followed by ECL detection (Amersham).

Ubiquitination reactions. Crude Sf9 cell lysates (500 µg) prepared from cells infected with ^{Phis6}hCUL1 baculovirus were incubated with 20 µl anti-Polyoma beads for 2 hr at 4^0C to allow ^{Phis6}hCUL1 binding. Beads were washed three times with lysis buffer and incubated with 1 mg of crude HeLa S3 lysate overnight at 4^0C. Beads were then washed three times with lysis buffer and supplemented with 6 µg biotinylated ubiquitin (BUb), 500 ng hCDC34, 25 ng ^{His6}yUBA1, 1 µl of 10X ATP-regenerating system (6), 1 µl of 10X reaction buffer (6), and 0.5 µM ubiquitin aldehyde. Reactions were adjusted to 10 µl by adding 20 mM HEPES [pH 7.6], 100 mM KOAc, 1 mM DTT, incubated for 90 min at 30^0C, and terminated by adding Laemmli sample buffer. Samples were analysed by Western blotting with streptavidin-HRP conjugate. All ubiquitination reactions with chimeric SCF complexes were performed as described (6).

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Figures

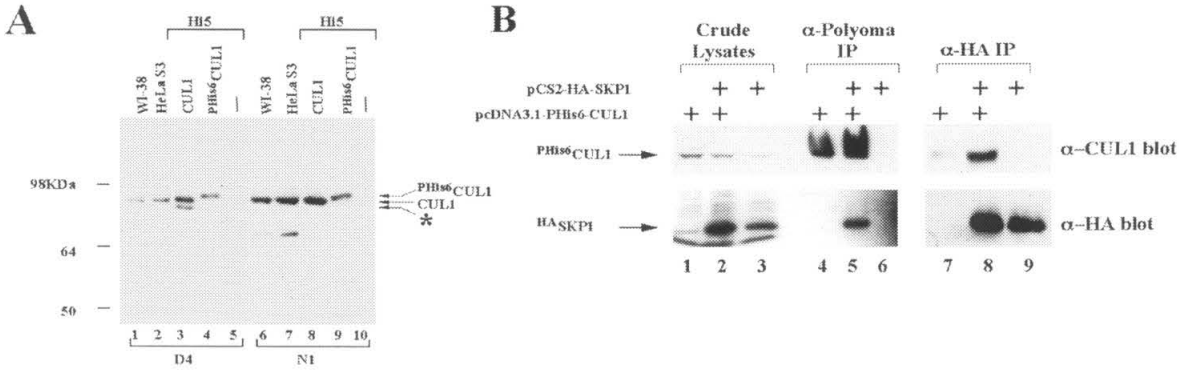


Figure 1. Human CUL1 and hSKP1 interact *in vivo*. (A) hCUL1 detection by affinity purified anti-hCUL1 antibodies. 50 μg of crude human cell lysates (lane 1, 2, 6, and 7) and 0.5 μg of crude lysates from Hi5 insect cells, uninfected (lanes 5 and 10) or infected with hCUL1 (lanes 3 and 8) or ^{PHis6}hCUL1 (lanes 4 and 9) viruses, were resolved on an 8% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-hCUL1 antibodies. D4: serum raised against C-terminal part of hCUL1; N1: serum raised against N-terminal part of hCUL1. Asterisk designates putative N-terminally truncated hCUL1 that is recognized by C-terminal antibody. (B) HeLa S3 cells were transfected with pcDNA3.1-PHis6-hCUL1 (lanes 1, 4, and 7), pCS2+HA-hSKP1 (lanes 3, 6, and 9), or both plasmids (lanes 2, 5, and 8). Lysates (1 mg) were prepared 24 hr post-transfection and immunoprecipitated with anti-Polyoma (lanes 4-6) or anti-HA (lanes 7-9) beads. Proteins retained on the beads were analyzed by Western blotting. Lanes 1-3 contained 20 μg of crude lysate.

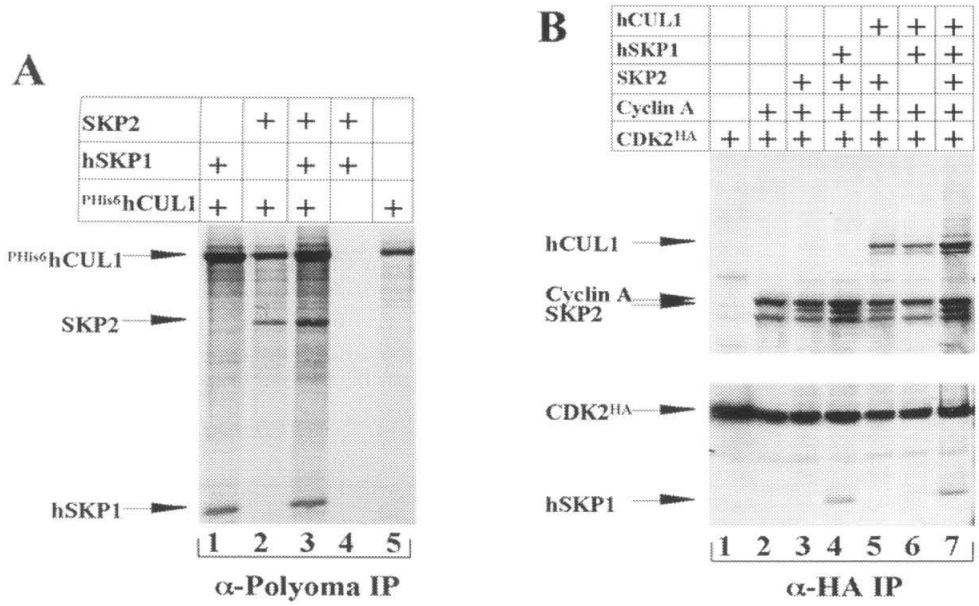


Figure 2. Human CUL1 can interact with human SKP1, SKP2, and Cyclin A/CDK2. Sf9 insect cells were infected with baculovirus constructs that express various human proteins as indicated. Cells were labeled with Tran[^{35}S]-label for 3 hr prior to harvesting. ^{PHis6}hCUL1 (panel A) and ^{HA}hCDK2 (panel B) together with associated proteins were immunoprecipitated by anti-Polyoma and anti-HA beads respectively. The composition of the protein complexes in the immunoprecipitates was analyzed by SDS-PAGE followed by autoradiography.

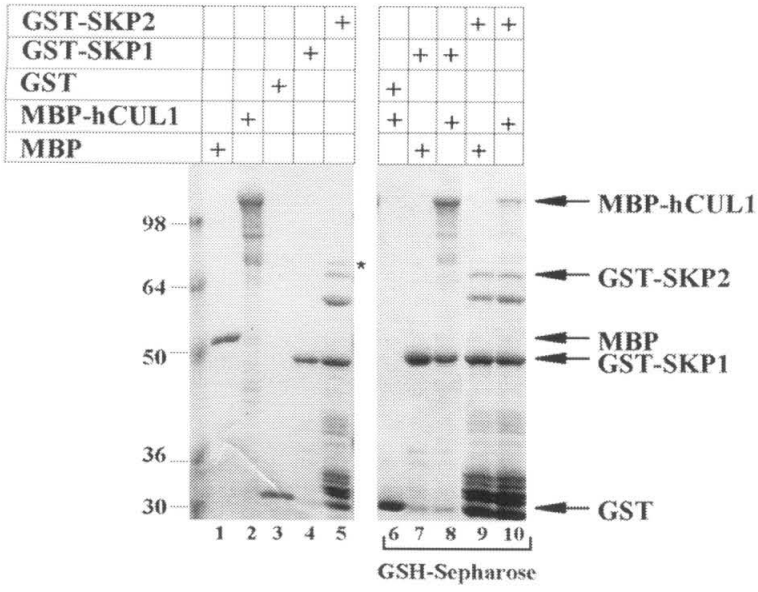


Figure 3. Human CUL1 binds directly to hSKP1 and SKP2. MBP, MBP-hCUL1, GST, GST-hSKP1, and GST-SKP2 were individually expressed in and purified from bacteria. Each protein was present in the binding reactions at 65 μg/ml. 4 μg of each protein were loaded in lanes 1-5, which represents 1/5 of the input for the binding reactions. Proteins were mixed as indicated (lanes 6-10) and incubated on ice for 1 hr. GST and GST fusions were collected on GSH-Sepharose for 1 hr at 4^0C. Proteins bound to GSH-Sepharose were resolved by SDS-PAGE and visualized by staining with Coomassie Blue. Positions of the full length fusion proteins are indicated by the arrows. An ~70 kDa band that copurified with GST-SKP2 from bacteria is marked by an asterisk.

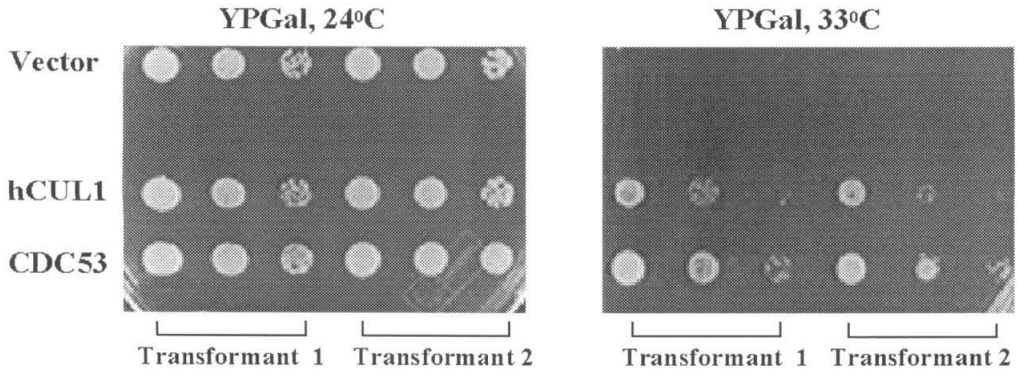


Figure 4. *hCUL1* complements the *CDC53*^{ts} mutant phenotype. A *CDC53-2*^{ts} mutant strain was transformed with pTS161-CDC53 and pTS161-hCUL1 plasmids that allow controlled expression of CDC53 and hCUL1 from the galactose-inducible *GAL1* promoter. Empty vector alone was used as a negative control. Ten-fold serial dilutions of the individual transformants were spotted on synthetic galactose medium and incubated for 5 days at restrictive (33^0C) and permissive (24^0C) temperatures.

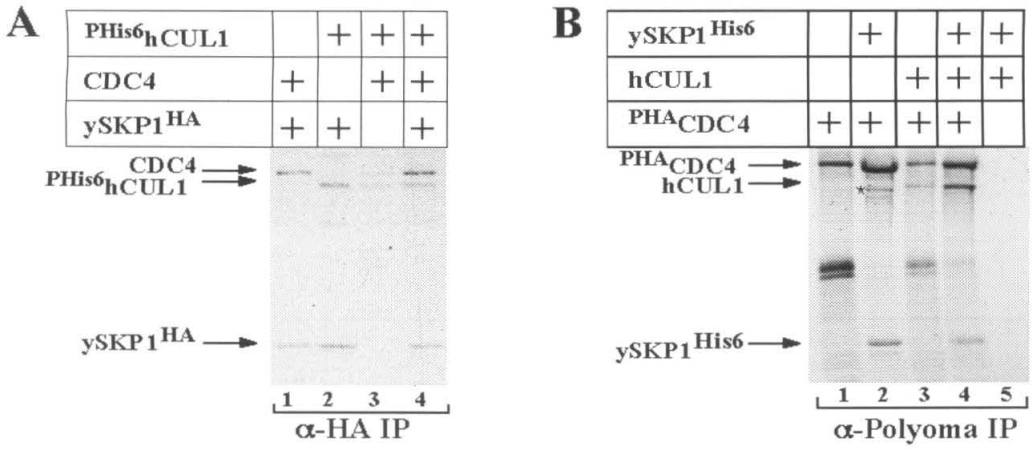
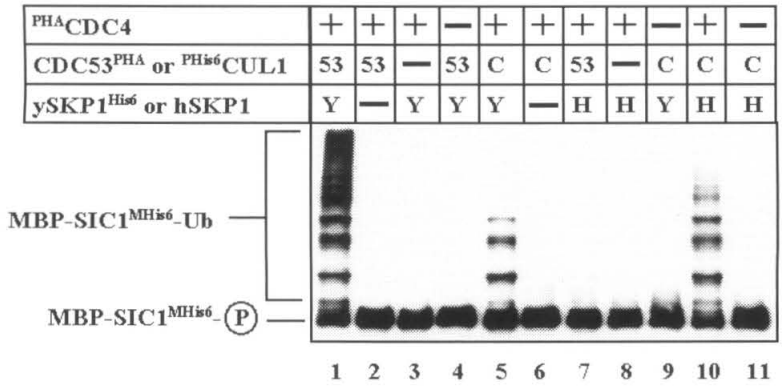
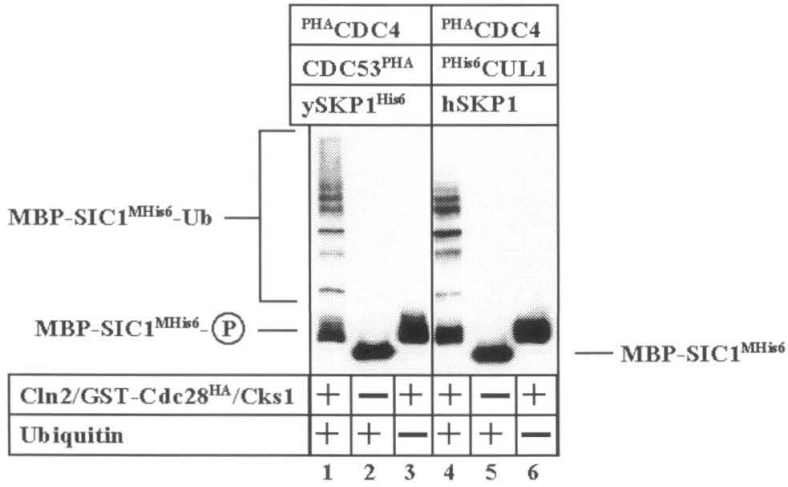


Figure 5. Human CUL1 can interact with yeast SCF components ySKP1 and CDC4. Hi5 insect cells were infected with baculovirus constructs expressing various proteins as indicated. Cells were labeled with Tran[^{35}S]-label for 3 hr prior to harvesting. Yeast SKP1^{HA} (panel A) and ^{PHA}CDC4 (panel B) together with associated proteins were immunoprecipitated by anti-HA and anti-Polyoma beads, respectively. The composition of the protein complexes in the immunoprecipitates was analyzed by SDS-PAGE followed by autoradiography. The asterisk marks an unidentified contaminant that migrates reproducibly faster than the authentic hCUL1.

A


PHACDC4 + + + — + + + + — + —
CDC53PHA or PHis6CUL1 53 53 — 53 C C 53 — C C C
ySKP1His6 or hSKP1 Y — Y Y Y — H H Y H H
MBP-SIC1MHis6-Ub
MBP-SIC1MHis6-(P)
1 2 3 4 5 6 7 8 9 10 11

B


PHACDC4 PHACDC4
CDC53PHA PHis6CUL1
ySKP1His6 hSKP1
MBP-SIC1MHis6-Ub
MBP-SIC1MHis6-(P)
MBP-SIC1MHis6
Cln2/GST-Cdc28HA/Cks1 + — + + — +
Ubiquitin + + — + + —
1 2 3 4 5 6

C

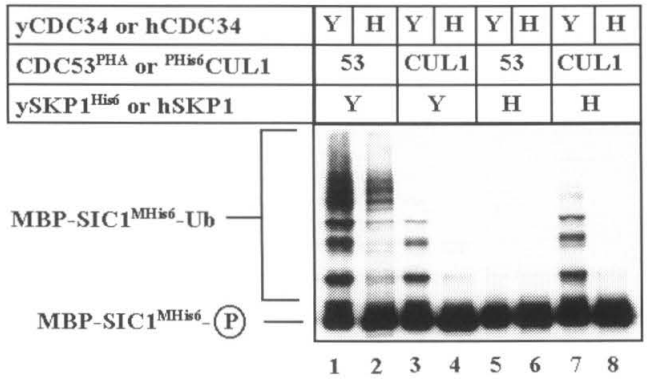

yCDC34 or hCDC34 Y H Y H Y H Y H
CDC53PHA or PHis6CUL1 53 CUL1 53 CUL1
ySKP1His6 or hSKP1 Y Y H H
MBP-SIC1MHis6-Ub
MBP-SIC1MHis6-(P)
1 2 3 4 5 6 7 8

Figure 6. SIC1 is ubiquitinated by chimeric SCF complexes. (A) Hi5 insect cells were infected with various baculoviruses expressing ^{PHA}CDC4, CDC53^{PHA} (53), ^{PHis6}hCUL1 (C), ySKP1^{His6} (Y), or hSKP1 (H) as indicated. At 72 hr post-infection, lysates were prepared and SCF complexes were affinity purified on an anti-Polyoma matrix, and eluted complexes were incubated for 2 hr at 25°C in the presence of MBP-SIC1^{MHis6} and purified ubiquitination components (see Materials and Methods). At the end of the incubation the samples were fractionated by SDS-PAGE and immunoblotted with anti-Myc antibodies to detect MBP-SIC1^{MHis6}. Bound antibodies were visualized by ECL. (B) The indicated SCF complexes were purified from baculovirus-infected insect cell lysates and incubated with the full set of ubiquitination components (lanes 1 and 4), or in the absence of CLN2/GST-CDC28^{HA}/CKS1 (lanes 2 and 5) or ubiquitin (lanes 3 and 6). (C) Purified SCF complexes containing CDC53^{PHA} (53), ^{PHis6}hCUL1 (CUL1), ySKP1^{His6} (Y), or hSKP1 (H) subunits were incubated with ubiquitination components containing either yCDC34 (Y) or hCDC34 (H).

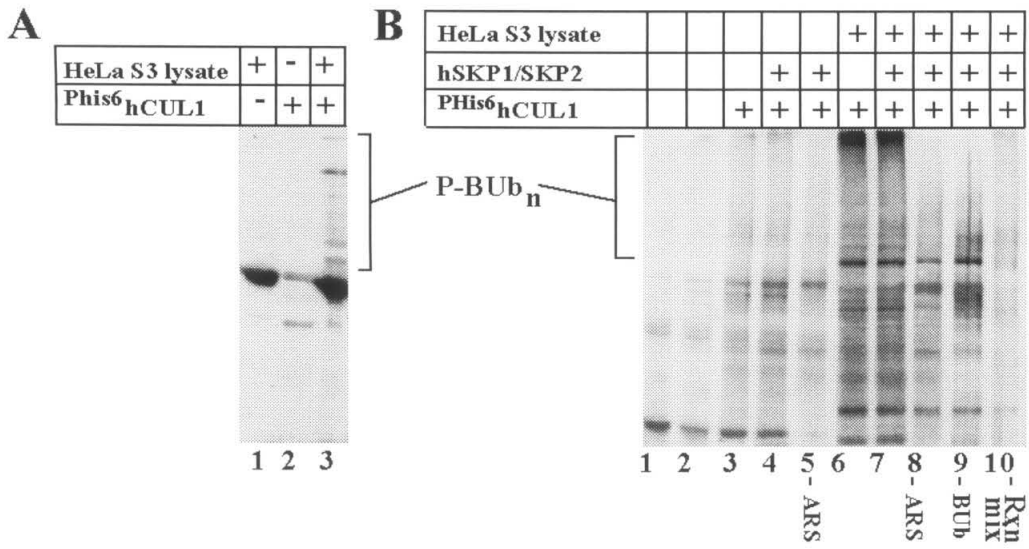


Figure 7. Human CUL1 associates with ubiquitination activity in HeLa S3 lysates. (A) Anti-Polyoma beads were incubated in the presence of ^{PHis6}hCUL1, HeLa S3 lysates, or both as indicated, washed five times with lysis buffer, and mixed with the ubiquitination reaction components (reaction mix): ^{His6}yUBA1, hCDC34, biotinylated ubiquitin (BUb), and ATP-regeneration system (ARS). Incorporation of biotinylated ubiquitin into proteins present in the reactions was monitored by probing with streptavidin-HRP conjugate followed by ECL detection. (B) ^{PHis6}hCUL1 alone, or together with hSKP1 and SKP2, was produced in insect cells and bound to anti-Polyoma beads that were then washed and incubated at 4^0C in the presence or absence of crude HeLa S3 lysates to allow bead 'activation'. 'Activated' beads were then treated as in panel A. Dependence on the presence of ATP and BUb in the reactions was determined by omitting these components from the reaction mix (lanes 5, 8 and 9, respectively). The entire reaction mix was omitted in lane 10. Lane 1 contained reaction mix only. P-BUb$_n$ designates a ladder of ubiquitinated proteins produced in the reaction.

Literature Cited

1. Schwob, E., Böhm, T., Mendenhall, M. & Nasmyth, K. (1994) *Cell* 79, 233-244.

2. Glotzer, M., Murray, A. W. & Kirschner, M. W. (1991) *Nature* 349, 132-138.

3. Cohen-Fix, O., Peters, J.-M., Kirschner, M. W. & Koshland, D. (1996) *Genes Dev.* 10, 3081-3093.

4. Hershko, A., Heller, H., Elias, S. & Ciechanover, A. (1983) *J. Biol. Chem.* 258, 8206-8214.

5. Scheffner, M., Nuber, U. & Huibregtse, J. M. (1995) *Nature* 373, 81-83.

6. Feldman, R. M. R., Correll, C. C., Kaplan, K. B. & Deshaies, R. J. (1997) *Cell* 91, 221-230.

7. Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J. & Harper, J. W. (1997) *Cell* 91, 209-219.

8. Barral, Y., Jentsch, S. & Mann, C. (1995) *Genes Dev.* 9, 399-409.

9. Plon, S. E., Leppig, K. A., Do, H. N. & Groudine, M. (1993) *Proc. Natl. Acad. Sci. USA* 90, 10484-10488.

10. Zhang, H., Kobayashi, R., Galaktionov, K. & Beach, D. (1995) *Cell* 82, 912-925.

11. Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W. & Elledge, S. J. (1996) *Cell* 86, 263-274.

12. Kipreos, E. T., Lander, L. E., Wing, J. P., He, W. W. & Hedgecock, E. M. (1996) *Cell* 85, 1-20.

13. Mathias, N., Johnson, S. L., Winey, M., Adams, A. E., Goetsch, L., Pringle, J. R., Byers, B. & Goebl, M. G. (1996) *Mol. Cell. Biol.* 16, 6634-6643.

14. Lanker, S., Valdivieso, M. H. & Wittenberg, C. (1996) *Science* 271, 1597-1601.

15. Verma, R., Annan, R., Huddleston, M., Carr, S., Reynard, G. & Deshaies, R. J. (1997) *Science* 278, 455-460.

16. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. & Rolfe, M. (1995) *Science* 269, 682-685.

17. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M. & Clurman, B. E. (1997) *Genes Dev.* 11, 1464-1478.

18. Won, K. A. & Reed, S. I. (1996) *EMBO J.* 15, 4182-4193.

19. Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M. & Roberts, J. M. (1996) *Genes Dev.* 10, 1979-1990.

20. Diehl, J. A., Zindy, F. & Sherr, C. J. (1997) *Genes Dev.* 11, 957-972.

21. Dynlacht, B. D., Flores, O., Lees, J. A. & Harlow, E. (1994) *Genes Dev.* 8, 1772-1786.

22. Hofmann, F., Martelli, F., Livingston, D. M. & Wang, Z. (1996) *Genes Dev.* 10, 2949-2959.

23. Hochstrasser, M. (1998) *Genes Dev.* 12, 908-913.

24. Lammer, D., Mathias, N., Laplaza, J.M., Jiang, W., Liu, Y., Callis, J., Goebl, M. & Estelle, M. (1998) *Genes Dev.* 12, 914-926.

25. Pause, A., Lee, S., Worrell, R. A., Chen, D. Y. T., Burgess, W. H., Linehan, W. M. & Klausner, R. D. (1997) *Proc. Natl. Acad. Sci. USA* 94, 2156-2161.

26. Lonergan, K.M., Iliopoulos, O., Ohh, M., Kamura, T., Conaway, R.C., Conaway, L. W. & Kaelin, W.G. (1998) *Mol. Cell. Biol.* 18(2), 732-741.

27. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

28. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* 7, 2745-2752.

29. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) *Molecular cloning: Assay for β-galactosidase in extracts of mammalian cells* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

30. Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. (1993) *Cell* 75, 791-803.

31. Fields, S. & Song, O. (1989) *Nature* 340, 245-246.

32. Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Kobayashi, R., Wittenberg, C. & Tyers, M. (1996) *Cell* 86, 453-463.

33. Connelly, C. & Hieter, P. (1996) *Cell* 86, 275-285.

34. Kaplan, K. B., Hyman, A. A. & Sorger, P. K. (1997) *Cell* 91, 491-500.

35. Henchoz, S., Chi, Y., Catarin, B., Herskowitz, I., Deshaies, R.J. & Peter, M. (1997) *Genes Dev.* 11(22), 3046-3060.

36. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D. & Miyamoto, S. (1995) *Genes Dev.* 22, 2723-2735.

37. Aberle, H., Baueer, A., Stappert, J., Kispert, A. & Kemler, R. (1997) *EMBO J.* 16, 3797-3804.

38. Lisztwan, J., Marti, A., Sutterluty, H., Gstaiger, M., Wirbelauer, C. & Krek, W. (1998) *EMBO J.* 17, 368-383.

39. Edward Kipreos provided unpublished CUL1 ESTs. Craig Correll generated the data presented in Fig. 6.

Chapter 3. COP9 Signalosome Binds Cullin Ubiquitin Ligases And Regulates Cullin Neddylation

The data presented in this chapter were generated in collaboration with G. Cope, A. Shevchenko, D. Wolf, and A. Shevchenko. This work has been submitted for publication.

Introduction

Human CUL1 is a member of the Cullin protein family (Kipreos et al., 1996). All known Cullins bind RING-H2 finger proteins HRT1 and HRT2 (Ohta et al., 1999), are modified by covalent attachment of a ubiquitin-like protein NEDD8/RUB1 (Hori et al., 1999), and are confirmed or likely components of multi-subunit ubiquitin ligases. CUL1-based SCF ubiquitin ligases consist of at least four subunits: CUL1 and HRT1, which harbor a core ubiquitin ligase activity, a variable F-box protein serving as a substrate receptor, and SKP1, linking the two modules together. The two best studied mammalian SCF complexes, SCF^{SKP2} and $SCF^{\beta TrCP}$ target p27 (Carrano et al., 1999) and IκBα, β-catenin (Winston et al., 1999) for ubiquitin-dependent degradation, respectively. All known SCF substrates need to be phosphorylated before they can be recognized by the F-box receptor subunit. Thus it is thought that ubiquitination of proteins by SCF is regulated on the level of substrate phosphorylation (Deshaies, 1999). Additionally, SCF activity is stimulated by attachment of the ubiquitin-like protein NEDD8 (i.e., neddylation) to CUL1 (Osaka et al., 2000). It is not known if there are other forms of SCF regulation at play, or if there are additional SCF subunits yet to be discovered.

Results

In order to address these questions we constructed retroviral vectors that expressed Myc_9-TEV-tagged forms of the human F-box protein SKP2 and C-terminally truncated human CUL1 (Δ692-752) and used them to infect mouse NIH 3T3 cells. SCF complexes were purified from these cells on anti-Myc beads, eluted with TEV protease (the tagged proteins were linked to Myc_9 by a recognition site for the Tobacco Etch Virus (TEV) protease; Seol et al. 2001), resolved by SDS-PAGE, and silver stained (Fig.1). Bands corresponding to specific interacting proteins were excised from silver stained gels and analyzed by MALDI and Nano electrospray tandem mass spectrometry as described previously (Seol et al., 1999; Shevchenko et al., 1996).

SKP2 eluates (Fig.1, lane 2) contained SCF subunits, CUL1 and SKP1, which were previously known to interact with SKP2 (Lyapina et al., 1998). We also identified a mouse homologue of yeast SGT1, previously reported to bind ySKP1 and have an essential but undefined role in kinetochore function and turnover of SCF substrates (Kitagawa et al., 1999). SGT1 is likely to be an SCF component or a regulator of SCF function. Surprisingly, we have also identified relatively high amounts of CKS1 in the SKP2 eluates. CKS1 binds and activates cyclin dependent kinases (Reynard et al., 2000). Although we did find CDC2 in SKP2 eluates, the small amount of the kinase present could not account for the high amount of CKS1 in the IP, thus CKS1 could only be brought down through binding to SKP1 or SKP2. Since there was no CKS1 in the CUL1 IP (Fig. 1, lane 3) which contained an equivalent amount of SKP1, it is most likely that CKS1 binds directly to SKP2 and is a bona fide stochiometric subunit of SCF^{SKP2} complexes.

Analysis of CUL1ΔC eluates (Fig.1, lane 3) also revealed specific binding of SCF subunits, SKP1, HRT1, and a number of F-box proteins, as expected. Elongation Factor 2 (EF-2) was also present in the IP, but the significance of this interaction is unclear. Unexpectedly, the other non-SCF proteins associated with CUL1ΔC turned out to be all eight subunits of the COP9

Signalosome (which has recently been renamed CSN (Deng et al., 2000)). CSN is an eight-subunit complex of about 450-500 kDa that was originally discovered in *Arabidopsis thaliana* as a suppressor of photomorphogenesis (Wei and Deng, 1998). CSN subunits have significant sequence homologies with components of the lid subunits of the 19S regulator of the 26S proteasome (Seeger et al., 1998) and with the translational initiation complex eIF3 (Glickman et al., 1998), but the biochemical function of CSN remains elusive.

In plants, CSN positively regulates COP1, a protein with an N-terminal RING-HC finger domain, a coiled-coil motif, and a C-terminal WD40 repeat domain that directly binds to the Hy5 transcription factor, responsible for the activation of light-inducible gene transcription (Chattopadhyay et al., 1998; Oyama et al., 1997). COP1 is thought to target Hy5 for degradation in the dark, and CSN regulates COP1 function and cellular localization by either promoting COP1 import into the nucleus or inhibiting its export from the nucleus (Chamovitz et al., 1996).

Since we identified COP9 Signalosome in the IP of a C-terminally truncated CUL1 that lacks the neddylation site, we first wanted to test if full length CUL1 can also interact with CSN. We transfected HeLa cells with constructs that expressed, Myc_9-tagged full length and truncated CUL1, immunopurified CUL1 from these cells using anti-Myc beads, and probed immunoprecipitates with anti-CSN8 antibodies and anti-SKP1 antibodies as a positive control (Fig. 2). Equal amounts of CSN8 and SKP1, which is known to bind to the N-terminus of CUL1 (Michel and Xiong, 1998), (Fig. 2, lanes 2 and 3) were associated with both forms of CUL1, suggesting that the C-terminus of Cullin and its neddylation are dispensable for binding to CSN.

Next, we tested if other SCF subunits and other Cullins can also bind to the CSN. We transfected Flag-tagged CSN1 into HeLa cells and purified CSN complex using anti-Flag beads (Sigma). CSN1 and associated proteins were eluted with Flag peptide and analyzed by SDS-PAGE and Western blotting (Fig. 3). As expected, CSN8, a subunit of Signalosome, was present in the eluate, confirming that we were pulling down CSN complexes and not a non-functional monomeric form of Flag-CSN1. Blotting with antibodies against human Cullins 1-3 revealed that

all of them bind CSN. Other data suggest that CUL4a and CUL5 can interact with CSN subunits as well (S. Schwartz, personal communication).

If CUL1 bound to CSN as part of SCF complexes, other SCF components should also be present in the Signalosome IPs. Indeed, we found HRT1, SKP1, and the F-box protein SKP2 in Flag-CSN1 eluates. We also found a slower migrating modified (perhaps, phosphorylated) form of an SCF substrate, IκBα, present in the CSN eluates, consistent with the notion that it was associated with CSN via its binding to SCF. Thus, all Cullins seem to associate with CSN in the context of respective ubiquitin ligase components and, possibly, substrates. What is the significance of this association? Since all Cullins bind to Signalosome, it is unlikely that CSN is regulated by associated Cullin ubiquitin ligases. Rather, since CSN was previously implicated in functioning downstream of various signal transduction cascades, Signalosome might be a global integrator of signaling pathways that radiates its outputs onto various associated ubiquitin ligases to activate or repress their activity.

Since we observed the presence of an SCF substrate in CSN1 IPs, we wanted to test if CSN perturbations have any effect on the stability of SCF substrates, particularly, IκBα. TNFα stimulation of HeLa cells leads to a rapid activation of IκBα kinase (IKK) and IκBα phosphorylation, which, in turn, triggers IκBα ubiquitination by $SCF^{\beta TrCP}$ and degradation by 26S proteasome (Fig. 4 (A), lanes 1 and 2). Treatment of these cells with a proteasome inhibitor LLnL prevents IκBα degradation and leads to its accumulation as a phosphorylated intermediate (Fig. 4 (A), lane 3). Overexpression of a dominant negative form of βTrCP, lacking the F-box (βTrCPΔF), similarly leads to IκBα accumulation in a phosphoshifted form (Fig. 4 (A), lane 6). Surprisingly, overexpression of CSN1 also prevented TNFα inducible degradation of IκBα; however, IκBα accumulated in a non-phosphorylated form in these cells (Fig. 4 (A), lane 8). This can be a result of either lower IKK activity or increased phosphatase activity/IκBα accessibility to the phosphatase in CSN1 overexpressing cells. Treatment with a

protein phosphatase inhibitor Calyculin A prevents IκBα dephosphorylation by PP2A phosphatase and mimics TNFα induced IκBα degradation due to low levels of active IKK in uninduced cells (Fig. 4 (A), lane 5). Treatment of CSN1 overexpressing cells with both TNFα and Calyculin A led to IκBα stabilization in a phosphorylated form, suggesting that CSN1 overexpresion does not prevent IKK activation in response to TNFα, but, instead, influences some other step in the IκBα degradation pathway (Fig. 5). Preliminary data suggest that IKK activity is similar in TNFα induced cells with or without CSN1 overexpression (data not shown). CSN might also function as a scaffold, bringing IKK, its substrates, and SCF in close proximity to each other, facilitating ubiquitin-mediated substrate proteolysis. We tested if IKK was associated with CSN (Fig. 4 (B)). We observed that IKK, as well as wt and phosphomutant forms of IκBα were able to bind CSN independent of each other (Fig. 4 (B), lanes 1-5), consistent with the above hypothesis.

Although previous studies suggested that CSN1 overexpression leads to a gain of function phenotype in *A. thaliana* (Kang et al., 2000), we could not conclusively determine whether CSN was acting as a positive or negative regulator of the SCF pathway in our transfection experiments. To find out in more detail how Signalosome might regulate SCF activity, we turned to a unicellular eukaryote *Schizosaccharomyces pombe*, where we could address the problem using genetic techniques. Unlike budding yeast *S. cerevisiae*, which doesn't appear to have the CSN complex, except for a CSN5(JAB1) homologue, fission yeast *S. pombe* appears to contain a full CSN complex, and a fission yeast CSN1 homologue, Caa1, is required for proper S phase progression (Mundt et al., 1999). First, we sought to confirm that the fission yeast CUL1 homologue, Pcu1, interacts with *S. pombe* Signalosome. Strains whose *pcu1*$^{+}$ and *caa1*$^{+}$ chromosomal loci were modified to encode proteins tagged with multimerized Myc epitopes were transformed with a plasmid that expressed HA-tagged CSN2 homologue ($Sgn2^{HA}$) from an inducible plasmid to test if we can detect $Pcu1^{Myc}$ interaction with $Sgn2^{HA}$. Anti-Myc

immunoprecipitates prepared from *caa1myc13*$^{+}$ cells (but not those from untagged strains) contained Sgn2^{HA} (Fig. 6 (A), lane 7), as expected. Similarly, Sgn2^{HA} was selectively recovered in anti-Myc precipitates from *pcu1myc13*$^{+}$ cells (Fig. 6 (A), lane 8), indicating that Cullin/CSN interaction is evolutionarily conserved. Interaction of Pcu1 with *S. pombe* CSN was evaluated further by probing Caa1^{Myc} and Sgn2^{HA} immunoprecipitates with anti-Pcu1 antibodies. As can be seen from Fig. 6 (B), endogenous Pcu1 could be detected in Sgn2^{HA} IPs (lane 6), confirming our earlier notion. Our failure to detect Pcu1 associated with Caa1 may stem from effects of the Myc epitope tag on Caa1. Taken together, these observations suggest that the interaction of CSN with SCF is conserved from yeast to human cells.

To investigate the role of CSN in SCF function we analyzed SCF components in *Δcaa1* cells (Mundt et al., 1999). Interestingly, endogenous Pcu1 accumulated in a modified form (Fig. 7 (A), lane 2) in *Δcaa1* strain, but not other mutants that, similar to *Δcaa1*, have a high proportion of cells accumulating in S phase (lanes 5-7), as well as F-box protein deletion mutants *Δpop1* and *Δpop2* (lanes 3, 4), suggesting that Pcu1 modification did not result from an abnormal cell cycle position of the *Δcaa1* mutant cells. *S. pombe* Pcu1 is modified on Lys 713 by covalent attachment of Nedd8 *in vivo* (Osaka et al., 2000). We reasoned that the altered migration we observed could result from increased neddylation. To test this hypothesis, we introduced plasmids that expressed wt and K713R Pcu1^{Myc} (Osaka et al., 2000) into *caa1*$^{+}$ and *Δcaa1* strains and analyzed extracts from these cells by Western blotting with anti-Myc antibodies (Fig. 7 (B)). The K713R mutation completely abolished Pcu1 modification observed in *Δcaa1* cells (compare lanes 3 and 4), suggesting that slower migrating Pcu1 forms are indeed neddylated Pcu1. We also immunopurified endogenous Pcu1 from wt and *Δcaa1* cells transformed with a plasmid that expressed HA_3-tagged *Brassica napus* Nedd8 (Fig. 7 (C)). Higher amounts of Nedd8^{HA3} were detected in Pcu1 IPs from *Δcaa1* cells than from wt cells (Fig. 7 (C), lanes 7 and 8, bottom

panel), confirming that Pcu1 neddylation is increased in *Δcaa1* cells. Clearly, functional Signalosome might be required for Pcu1 deneddylation or repression of Pcu1 neddylation.

How does increased neddylation of Pcu1 in *Δcaa1* cells affect SCF function? First, we tested the integrity of SCF complexes in *Δcaa1* strain. The protein levels of Pcu1, the SKP1 *homologue* Psh1 and the HRT1 homologue Pip1 (Fig. 8 (A and B)) were unaffected in *Δcaa1* cells. Psh1 and Pip1 binding to Pcu1 in wt and *Δcaa1* cells was indistinguishable as well (Fig. 8 (B)). Since it has been previously noted that neddylation activates SCF activity (Osaka et al., 2000), we tested if ubiquitin ligase activity of SCF complexes in *Δcaa1* cells was increased (Fig. 8 (C)). Two independent ubiquitin incorporation assays with SCF complexes from $Pcu1^{Myc13}$ tagged strains (lanes 1-3) or SCF complexes purified using anti-Pcu1 antibodies from untagged strains (lanes 4-6) revealed that SCF ubiquitin ligation activity is 2-4 fold higher in *Δcaa1* strain compared with the wt (lane 2 vs. 3, and lane 5 vs. 6). However, we did not observe any change in the half-life of the SCF substrate Rum1 in *Δcaa1* strains (data not shown), perhaps because substrate phosphorylation, and not SCF activity, is the limiting step that determines the kinetics of Rum1 degradation.

Because Nedd8 is strongly enriched in the nucleus (Kamitani et al., 1997), and CUL1 must be imported into the nucleus to be neddylated (Furukawa et al., 2000), increased Pcu1 modification might be an indirect result of increased nuclear localization of Pcu1. Additionally, increased neddylation might lead to a redistribution of Pcu1 antigen within the cell, as it has been reported that neddylated CUL1 is enriched at the kinetochores (Freed et al., 1999). We looked at $Pcu1^{Myc13}$ localization in wt and *Δcaa1* strains carrying chromosomally tagged Pcu1 by indirect immunofluorescence (Fig. 8 (D)), but we did not observe any noticeable changes in Pcu1 localization. We also looked at endogenous untagged Pcu1 localization by biochemical fractionation (Fig. 8 (E)). While cytoplasmic enzyme Pyc1 was found exclusively in the cytoplasm, and a nuclear protein Mcm5 was found exclusively in the nucleus, Pcu1 was found

both in the nucleus and cytoplasm in all strains tested, confirming our immunofluorescence localization data.

Because increased neddylation of Pcu1 was the only obvious effect of the *Δcaa1* mutation on SCF, we reasoned that CSN might govern the balance of neddylating and deneddylating activities in *S. pombe*. Neddylation is reminiscent of ubiquitination and is mediated by the two-subunit E1-like enzyme, Ula1/Uba3, and the E2-like enzyme Ubc12. All components of neddylation pathway are essential in *S.pombe* (Osaka et al., 2000). Although a variety of deubiquitinating enzymes has been described, no NEDD8-specific proteases have been discovered yet. However, UCH-L3, which was originally identified as a ubiquitin-specific hydrolase, was found to possess NEDD8 C-terminal hydrolase activity (Wada et al., 1998). Similarly, a novel UBP, USP21, capable of deconjugating ubiquitin from ubiquitinated proteins, was shown to be capable of removing NEDD8 from NEDD8 conjugates (Gong et al., 2000).

We reasoned that Pcu1 hyperneddylated state in *Δcaa1* cells could result from either increased neddylating activity or a decreased deneddylating activity in *Δcaa1* cells. To determine whether increased accumulation of Pcu1-Nedd8 conjugates in *Δcaa1* cells was due to global deregulation of neddylation, *caa1*$^{+}$ and *Δcaa1* cells were transformed with a plasmid that expressed ^{HA3}Nedd8, and anti-HA immunoprecipitates prepared from these cells were immunoblotted with anti-HA to evaluate the pattern of Nedd8-modified proteins (Fig. 7 (D)). Whereas little signal was detected in immunoprecipitates prepared from *caa1*$^{+}$ cells (lane 3), multiple ^{HA3}Nedd8-modified proteins were detected in immunoprecipitates from *Δcaa1* cells (lane 4), suggesting that loss of CSN function leads to enhanced neddylation of multiple substrates. Next we sought to determine whether the global hyperneddylation that occurred in *Δcaa1* cells was due to accumulation of an inhibitor of deneddylation present in *Δcaa1* extracts (a dominant activity with respect to wt cells) or a loss of deneddylating enzyme activity (a recessive activity). To discriminate between these possibilities, we prepared extracts from tagged *caa1*$^{+}$ *pcu1myc13*$^{+}$

and untagged *pcu1*$^{+}$ *Δcaa1* strains, incubated them in the absence of ATP (to prevent *de-novo* neddylation), and followed the neddylation state of the endogenous untagged Pcu1 from *Δcaa1* extracts by Western blotting (Fig. 10 (A, B)). Incubation with wt extracts led to an almost 100% conversion of fully neddylated Pcu1 form *Δcaa1* extracts into an unmodified form, suggesting that there is a deficit of deneddylating activity in *Δcaa1* cells (lanes 1 and 2).

We purified CSN from pig spleen to test its biochemical activity *in vitro* (Fig. 9 (A and B)). Remarkably, purified CSN (Fig. 10 (A)) could restore deneddlylating activity to *Δcaa1* extract (lane 3), suggesting that Signalosome either stimulated a cryptic deneddylating enzyme in *Δcaa1* cell extract or itself harbored a deneddylating activity. The latter possibility was strongly supported by the observation that purified pig spleen CSN deneddylated Pcu1^{Myc13} that was first immunopurified from *Δcaa1* extract (Fig. 10 (C)). Deneddylating activity might either be provided by one of the CSN subunits or by a tightly associated deneddylating enzyme. Interestingly, CSN5 (JAB1) orthologs contain an invariant cysteine which is flanked by a highly conserved sequence with similarities to the active site "Cys box" seen in many deubiquitinating enzymes (Glickman et al., 1998) (Fig. 10 (E)). Furthermore, the deneddylating activity of purified CSN was obliterated by preincubation with the sulfhydryl agent N-ethylmaleimide (Fig. 10 (B), lanes 4 and 5). We reasoned that CSN5/JAB1 and its homologues from other species might define a new class of deneddylating enzymes, potentially accounting for the deneddylating activity detected in the purified Signalosome preparations.

Discussion

Our findings provide the first clear insight into the biochemical function of the CSN. The best understanding of CSN function comes from its role in controlling photomorphogenesis in plants. In darkness, the putative ubiquitin ligase COP1 enables rapid turnover of the transcriptional regulator Hy5. In seedlings exposed to light or in CSN mutants, COP1

redistributes to the cytoplasm, allowing Hy5 to accumulate and promote transcription of photomorphogenetic genes. It has been proposed that CSN regulates photomorphogenesis by governing the nucleocytoplasmic distribution of COP1. We argue instead that CSN controls COP1-mediated turnover of Hy5 by modulating the neddylation of COP1, Hy5, or an affiliated protein, with redistribution of COP1 to the cytoplasm occurring in response to the accumulation of Nedd8 conjugates. It will be interesting to see how light impinges on the CSN-dependent activation of COP1, and whether a similar circuit involving CSN regulates light-entrained behaviors in animals.

Intriguingly, the catalytic CSN5/JAB1 subunit is the only CSN subunit that is found both in monomeric and complexed forms (Kwok et al., 1998). Moreover, it is the only subunit of CSN found in *S. cerevisiae*, and mutants that lacked this protein hyperaccumulated neddylated CDC53 Cullin (Fig. 10 (D)). Thus, the role of JAB1-like proteins as deneddylating enzymes is highly conserved. JAB1 was originally described as a JUN activation domain binding protein and was shown to interact with JUN and coactivate JUN mediated gene expression (Claret et al., 1996). Recently, mammalian JAB1 was reported to bind a number of other proteins: lutropin/choriogonadotropin receptor (LHR) (Li et al., 2000) and CDK inhibitor p27 (Tomoda et al., 1999), which are targeted for degradation by binding to JAB1; as well as LFA1 integrin (Bianchi et al., 2000) and MIF cytokine (Kleemann et al., 2000), which seem to regulate JAB1 activity through an unknown mechanism. The network of JAB1 interactors and its connection to Signalosome function remained enigmatic for several years now. Our data indicate that Signalosome binds SCF and, possibly, other Cullin-based ubiquitin ligases and negatively regulates their functional outputs by controlling Cullin deneddylation through JAB1. An alternative, though less likely, interpretation is that Signalosome activates SCF through some other yet to be discovered mechanism, and the observed hyperneddylation of Pcu1 results from a feedback loop mechanism that compensates for loss of SCF activity in the CSN mutant cells by hyperneddylating Pcu1. Our data suggests that JAB1 interacting proteins are either regulators of

JAB1 deneddylating activity or substrates of JAB1 deneddylating enzyme. Our observation that multiple Nedd8-modified species accumulate in *Δcaa1* cells suggests that additional neddylated cellular proteins and ubiquitin ligases are regulated by COP9 Signalosome and will be discovered in the near future.

Experimental Procedures

HeLa and NIH 3T3 cell transfections, lysate preparation, immunoprecipitations, and protein sequencing were performed as described previously (Lyapina et al., 1998), (Seol et al., 1999).

S. pombe manipulations and indirect immunofluorescence were performed according to protocols published in http://www.bio.uva.nl/pombe/handbook/

Subcellular fractionation. Strains were grown in YES media until OD_{595}=0.5, harvested and washed in ice cold 'stop' buffer (50 mM NaF, 10 mM TRIS 7.5, and 0.02% NaAzide) and re-suspended in 600 µL Buffer S (1.4 M Sorbitol, 40 mM HEPES (pH 7.2), 0.5 mM MgCl2). Spheroplasts were made by incubating the cell suspension at 30°C for 40 min in the presence of 100 µg/mL zymolyase (ICN). Cells were washed twice in 1 mL of ice cold Buffer F (20 mM HEPES (pH 7.2), 0.5 mM MgCl2) supplemented with protease inhibitor mix (PIM; 1 mM PMSF, 1 mM benzamidine, 0.25 µg/mL pepstatin, and 5 µg/mL each of leupeptin, aprotinin, and chymostatin) and 18% Ficoll 400 (w/v). Cells were gently lysed in 200 µL of the same buffer using a stainless steel pestle (VWR). To remove unlysed cells, the suspension was gently centrifuged (2000 Krpm). Intact nuclei were isolated by layering the supernatant on 100 µL Buffer F supplemented with PIM, 7% Ficoll 400 (w/v), and 20% glycerol, followed by gentle centrifugation. Nuclei were lysed in 200 µL of 20 mM HEPES (pH 7.2), 500 mM NaCl, 1%

triton X-100, 1 mM EDTA, plus PIM. Cytosolic and nuclear fractions were analyzed by SDS-PAGE followed by Western blotting. Cytoplasmic and nuclear fraction purity was confirmed using antibodies specific to Mcm5 (from S. Forsburg) and S. cerevisiae pyruvate kinase (from J. Thorner), respectively.

Lysis Method. Strains were grown in YES (fission yeast) or YPD (budding yeast) media to OD_{595}=0.5, harvested, and washed in ice cold 'stop' buffer (50 mM NaF, 1 0mM Tris 7.5, and 0.02% Na azide). Cells were resuspended in an equal volume of Lysis Buffer (20 mM HEPES (pH 7.2), 150 mM NaCl, 0.2% triton X-100, 10 mM EDTA, 50 mM NaF, mM DTT) supplemented with PIM. An equal volume of glass beads was added (0.5 µm Sigma). Cells were vortexed at maximum speed 6 times for 30 seconds. Lysates were cleared by centrifugation. Protein concentrations were determined using Bio-Rad protein assay. Lysates were used in deneddylation assays as indicated. For immunoprecipitations, 1mg of extract was used with 0.5 µg of the indicated antibody.

CSN purification was performed as described (Wei and Deng, 1998).

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Figures

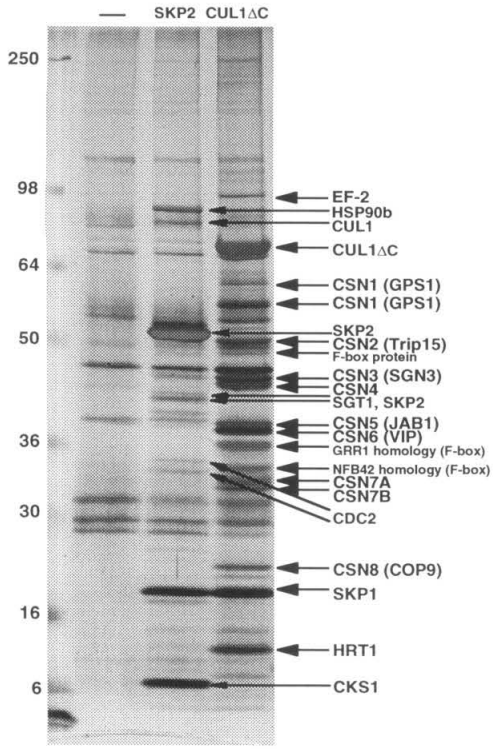


Figure 1. Identification of SCF interacting proteins by mass spectrometry.

Mouse NIH 3T3 cells were infected with retroviruses that expressed Myc_9-TEV-tagged hSKP2 or hCUL1ΔC. Cell extracts prepared from uninfected (lane 1) cells and cells infected with SKP2 (lanes 2) or CUL1ΔC (lane 3) retroviruses were adsorbed to anti-Myc beads, eluted with TEV protease (Gibco BRL), and analyzed by SDS-PAGE. Specific bands were excised from the gel and protein identity was determined by mass spectrometry as described (Shevchenko et al., 1996).

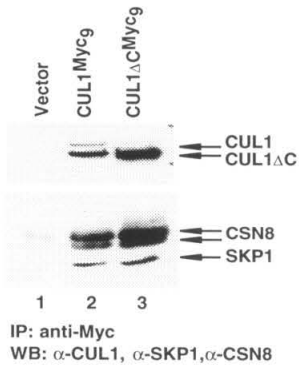


Figure 2. COP9 Signalosome association with hCUL1 does not depend on CUL1 neddylation. HeLa cells were transiently transfected with pCEP4 vectors that expressed full length (lane 2) or C-terminally truncated (lacking the NEDD8 modification site) forms of Myc_9-hCUL1 (lane 3). The Cullins and associated proteins were retrieved on anti-Myc beads, separated by SDS-PAGE, and visualized by Western blotting with anti-CUL1, anti-SKP1, and anti-CSN8 antibodies.

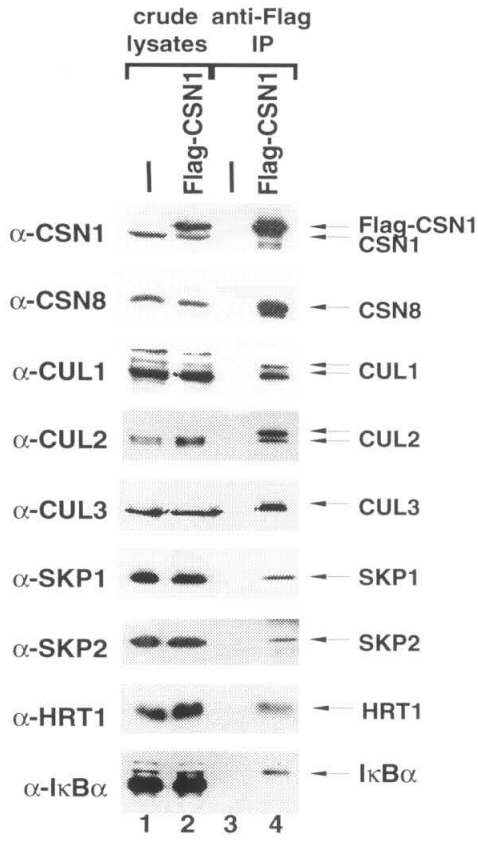


Figure 3. Association of Cullins and SCF components with COP9 Signalosome.

HeLa cells were transiently transfected with vector alone (lanes 1, 3) or with a vector that expressed Flag-tagged CSN1 subunit of COP9 Signalosome (lanes 2, 4). CSN1 complexes (lanes 3, 4) were purified on anti-Flag beads, eluted with Flag peptide, resolved by SDS-PAGE, and probed for associated endogenous proteins with various antibodies as indicated. For comparison, unfractionated cell lysates were evaluated in parallel (lanes 1, 2)

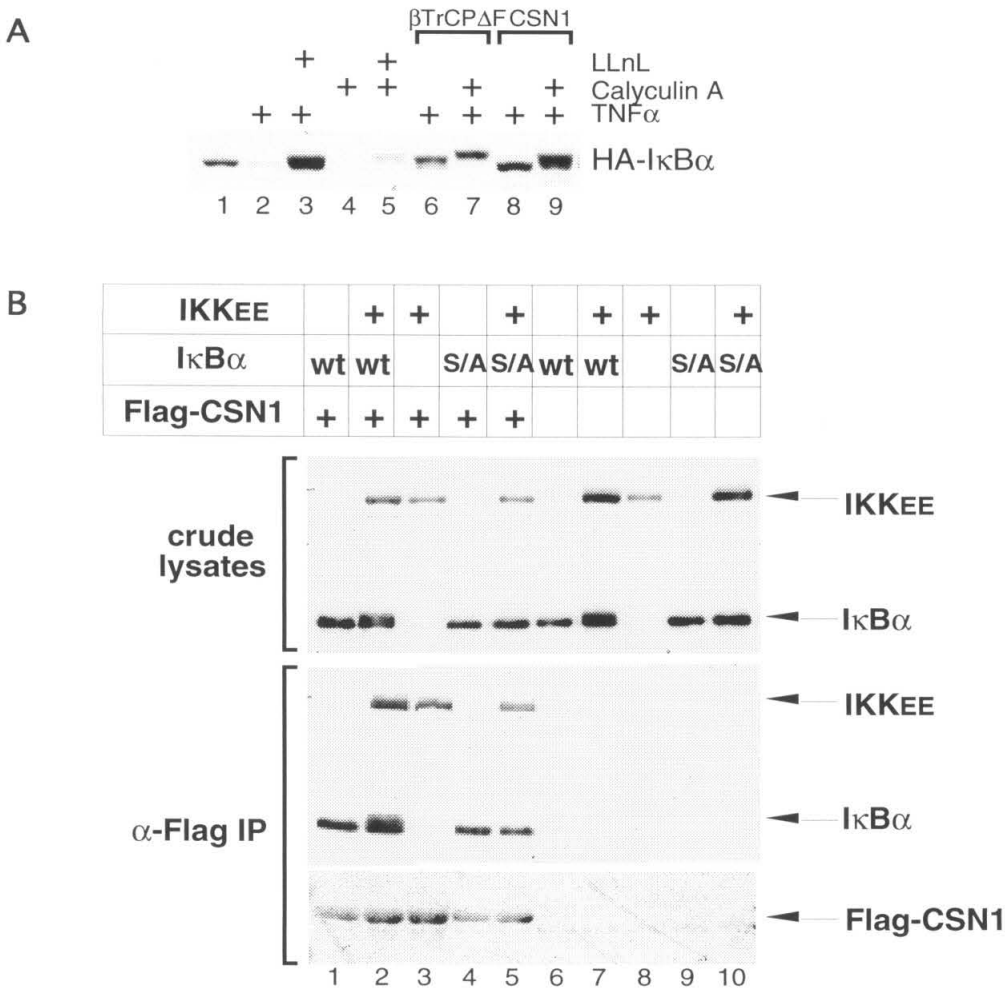
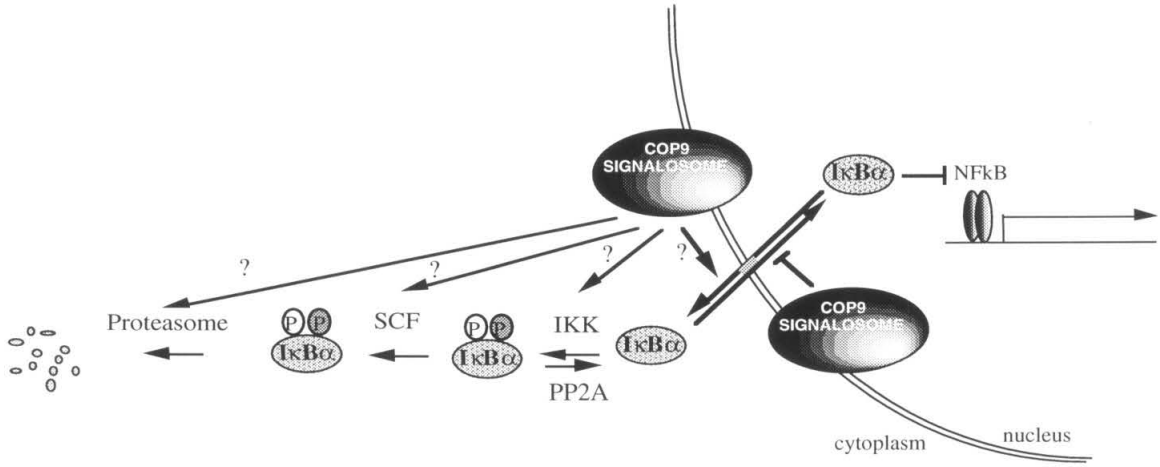


Figure 4. CSN overexpression in HeLa cells prevents IκBα degradation in response to TNFα. **A.** HeLa cells were transiently transfected with pCMV4-HA-IκBα and pCS2+β-Galactosidase vectors with or without either βTrCPΔF (F-box deletion mutant of βTrCP) or Flag-CSN1 expression vectors. 36 hrs post-transfection cells were treated with 100 μM proteasome inhibitor LLnL for 6 hrs, 1 μM of protein phosphatase inhibitor Calyculin A for 3 hrs, and 10 nM TNFα for 15 min, as indicated. Cells lysates were prepared, normalized for β-Galactosidase activity, and analyzed by SDS-PAGE followed by Western blotting with anti-HA antibodies. Co-

expression of either βTrCPΔF or Flag-CSN1 prevented HA-IκBα degradation in responce to TNFα.

B. 293T cells were transiently transfected with a mixture of plasmids expressing Flag-CSN1, HA-IKK^{EE}, and wt or S32,36A mutant of HA-IκBα, as indicated. Cells lysates prepared 36 hrs post-transfection were incubated with anti-Flag beads for 1 hr at +4°C. The beads were washed three times with lysis buffer, boiled in SDS-PAGE loading buffer, and associated proteins were resloved by SDS-PAGE and visualized by Western blotting with anti-HA and anti-CSN1 antibodies. Both wt and S32,36A mutant of HA-IκBα, as well as HA-IKK^{EE}, were found in CSN1 IPs.



1. Substrate localization
2. Kinase/phosphatase activity/localization ==> IκBα phosphorylation
3. SCF activity/localization ==> IκBα ubiquitination
4. Proteasome targeting ==> IκBα degradation

Figure 5. Regulation of IκBα degradation by COP9 Signalosome.

CSN can potentially impact IκBα degradation at each step involved in this process. Since IκBα is known to shuttle in and out of the nucleus, CSN might be involved in IκBα and/or SCF localization. Alternatively, CSN might regulate IKK activation/IκBα phosphorylation, recognition of phosphorylated IκBα and its ubiquitination by SCF, targeting of ubiquitinated IκBα to the proteasome, or any combination of these processes.

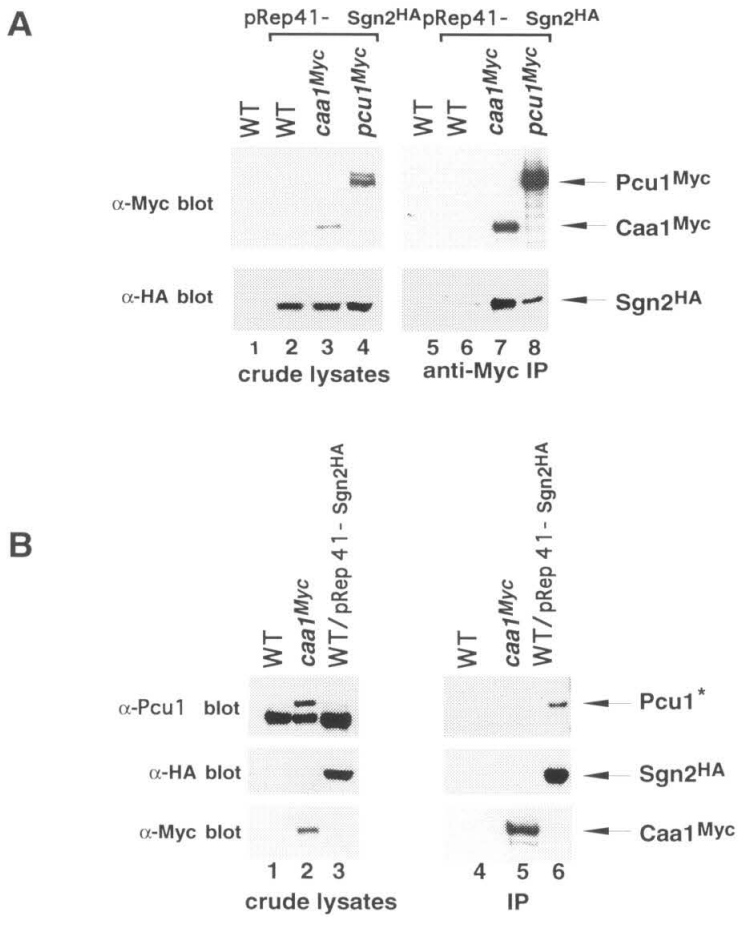


Figure 6. *S. Pombe* Cullin 1 (Pcu1) associates with COP9 Signalosome.

A. Untagged (lanes 2 and 6), *caa1*^{Myc} (lanes 3 and 7), and *pcu1*^{Myc} (lanes 4 and 8) strains were transformed with a pRep41-based plasmid that expressed HA-tagged CSN subunit Sgn2^{HA}. Extracts prepared from these strains were either evaluated directly (left panels), or were first immunoprecipitated with anti-Myc antibodies (right panels) prior to SDS-PAGE followed by Western blotting with anti-Myc and anti-HA antibodies, as indicated. **B.** Same as panel A, except association of endogenous Pcu1 with Caa1^{Myc} (lane 5) or Sgn2^{HA} (lane 6) was evaluated by anti-Pcu1 Western blot of anti-Myc and anti-HA immunoprecipitates (lanes 4-6), respectively.

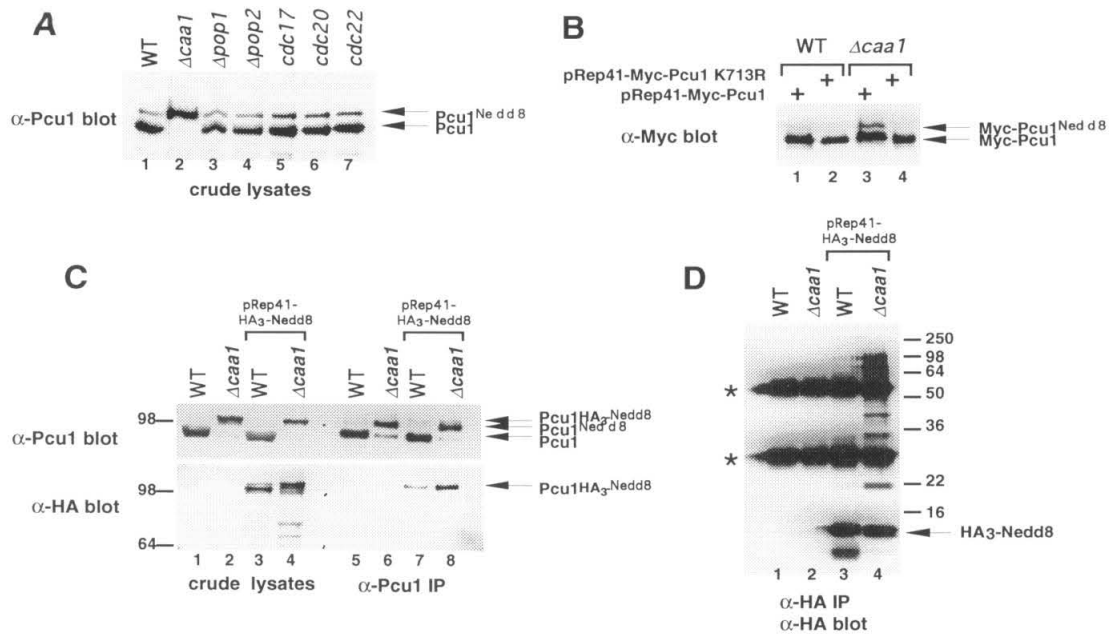


Figure 7. Pcu1 hyperaccumulates as a neddylated species in *Δcaa1* cells.

A. Extracts from wt and various deletion and point mutant strains were probed with anti-Pcu1 antibodies to detect endogenous Pcu1. **B.** Wt and *Δcaa1* mutant cells were transformed with pRep41 plasmids that expressed Myc-tagged wt or K713R mutant Pcu1 that lacks the neddylation site. Crude extracts from these strains were probed with α-Myc antibodies to detect Pcu1. **C.** Wt and *Δcaa1* strains were transformed with a plasmid that expressed HA_3-tagged *Brassica napus* Nedd8 from the inducible nmt1 promoter. Crude lysates (lanes 1-4) and anti-Pcu1 immunoprecipitates (lanes 5-8) from wt and *Δcaa1* strains (12 hrs post induction) with (lanes 1,2,5,6) or without (lanes 3,4,7,8) the plasmid were immunoblotted with anti-Pcu1 (top panel) or anti-HA (bottom panel) antibodies to detect Pcu1-^{HA3}Nedd8 conjugates. **D.** The strains described in panel C were harvested 12 hrs after induction of the nmt promoter to minimize overexpression of ^{HA3}Nedd8, and lysates were sequentially immunoprecipitated and immunoblotted with anti-HA antibodies to detect ^{HA3}Nedd8 conjugation to cellular proteins. * indicates the position of the heavy and light antibody chains.

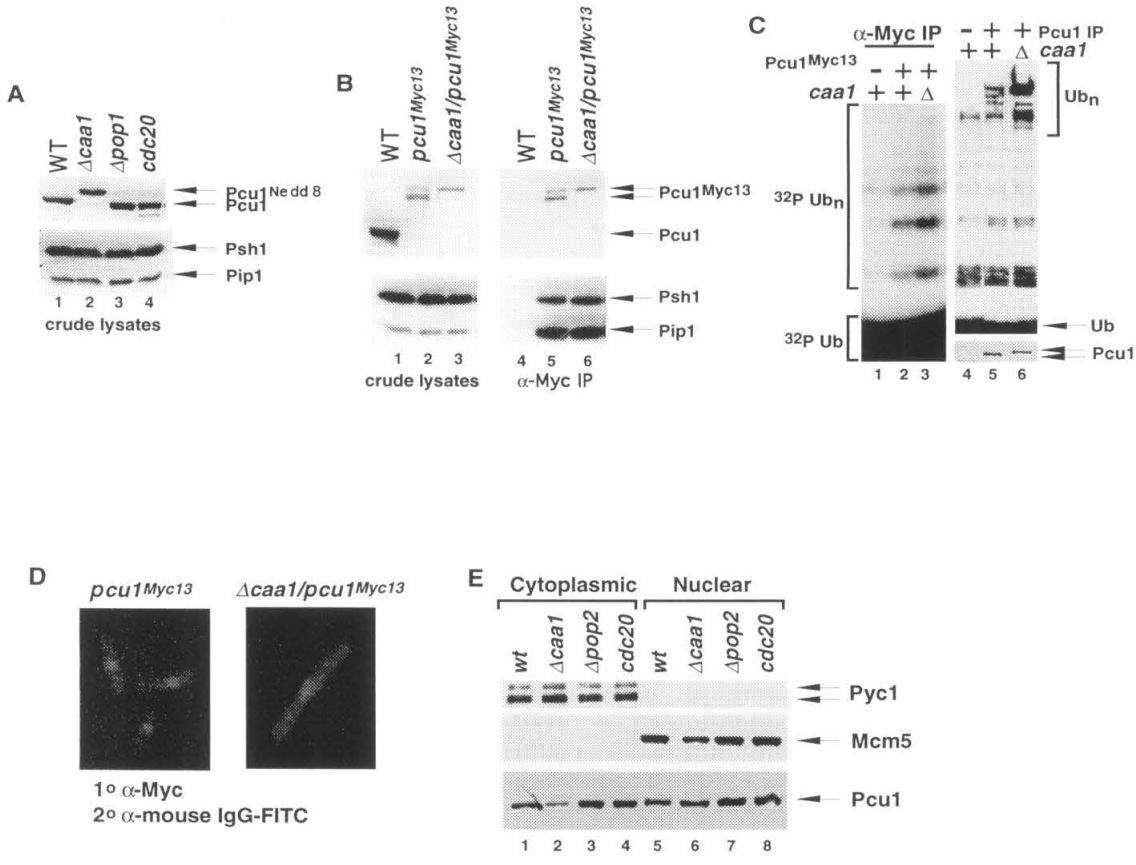


Figure 8. SCF composition is unperturbed and SCF activity is slightly increased in *Δcaa1* mutant cells.

A. Western blot analysis of SCF subunits in various mutant strains. Extracts (30 μg) from wt, *Δcaa1, Δpop1,* and *CDC20* mutant strains were resolved by SDS-PAGE and analyzed by western blotting with α-Pcu1, α-Psh1, and α-Pip1 antibodies. **B.** SCF assembly in wt and *Δcaa1* strains. SCF subunits were evaluated in crude lysates (lanes 1-3) or in anti-Myc immunoprecipitates (lanes 4-6) from *pcu1myc13*$^+$ or *Δcaa1 pcu1myc13*$^+$ strains. Wt untagged stain was used as a negative control (lanes 1 and 4). Psh1 and Pip1 binding to Pcu1 was analyzed by Western blotting with the respective antibodies. **C.** Analysis of SCF activity in wt and *Δcaa1* strains.

Lanes 1-3: SCF complexes purified from wt and *Δcaa1* strains via the Myc epitope on endogenous Pcu1^{Myc13} were washed and incubated in the presence of ^{32}P-Ub, ATP, E1, and hCDC34 for 1 hr at 30°C. Formation of ^{32}P-Ub conjugates (^{32}P-Ub$_n$) was analyzed by SDS-PAGE followed by autoradiography. An untagged *pcu1*$^+$ strain was used as a negative control (lane 1). Lanes 4-6: SCF complexes immunopurified from wt and *Δcaa1* strains using anti-Pcu1 antibodies were washed and incubated in the presence of Ub, ATP, E1, and hCDC34 for 1 hr at 30°C. Formation of Ub conjugates (Ub$_n$) was analyzed by SDS-PAGE followed by Western blotting with anti-Ub antibodies (Chemicon). As a negative control, Pcu1 antibody was omitted from the immunoprecipitation (lane 4). The amount of Pcu1 in the reactions was monitored by reblotting the same nitrocellulose with anti-Pcu1 antibodies (lanes 4-6, bottom panel). **D.** Analysis of Pcu1 localization by immunofluorescence. Pcu1 localization was visualized in *pcu1myc13*$^+$ and *Δcaa1 pcu1myc13*$^+$ strains by immunostaining with mouse anti-Myc antibodies followed by anti-mouse IgG-FITC antibodies and confocal microscopy. **E.** Analysis of Pcu1 localization by subcellular fractionation. Proteins in the nuclear (lanes 5-8) and cytoplasmic (lanes 1-4) factions derived from homogenized spheroplasts were resolved by SDS-PAGE and analyzed by Western blotting with indicated antibodies. Antibodies against known cytoplasmic (pyruvate kinase) and nuclear (Mcm5) proteins were used to monitor the efficiency of the fractionation procedure.

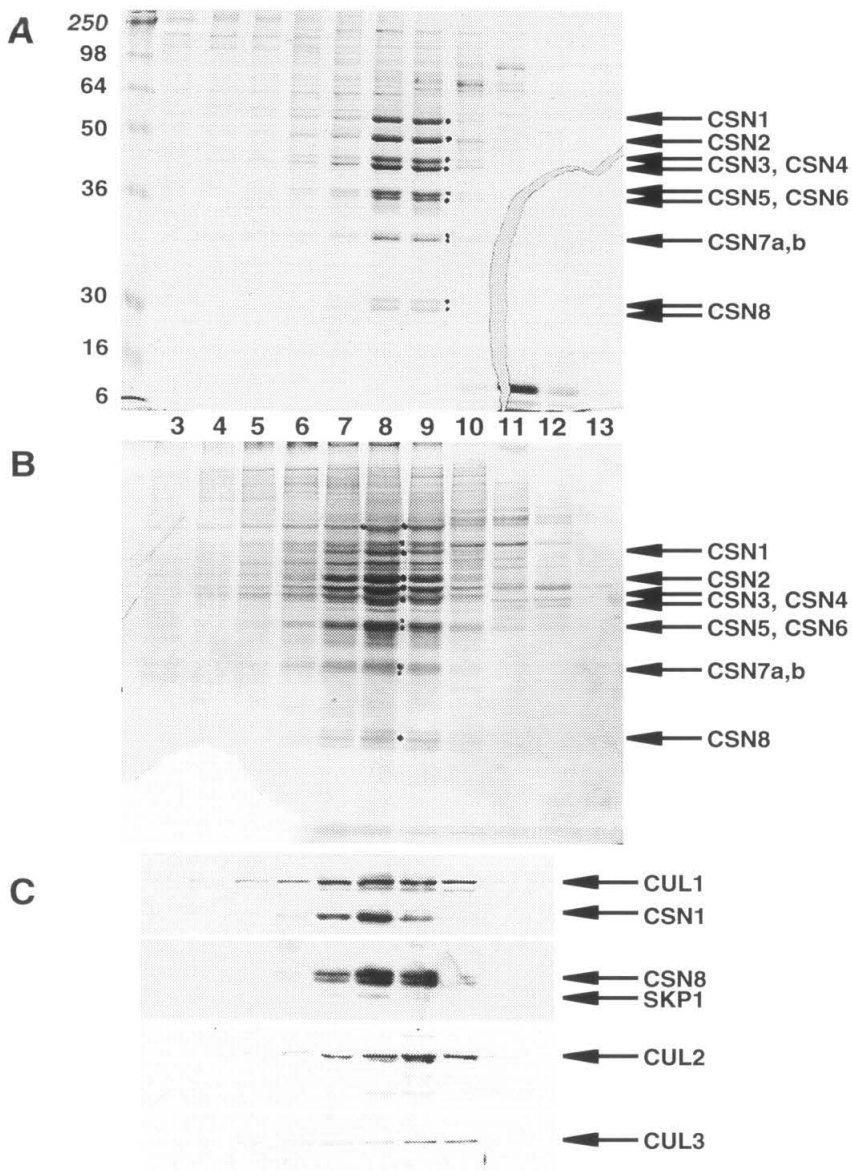


Figure 9. Co-purification of Cullins and SKP1 with COP9 Signalosome.

COP9 Signalosome (CSN) was purified by conventional chromatography using five different resins as described (Wei and Deng, 1998). Protein fractions coming off the last, gel filtration (Superose 6), column were collected and analysed by SDS-PAGE followed by Coomassie

staining (panel A). Arrows indicate CSN subunit positions in the gels. Superose 6 fractions form a different purification were analyzed by SDS-PAGE and silver staining (panel B) and Western blotting with indicated antibodies (panel C). Cullins 1, 2, and 3, as well as SKP1, were co-fractionating with CSN through all purification steps.

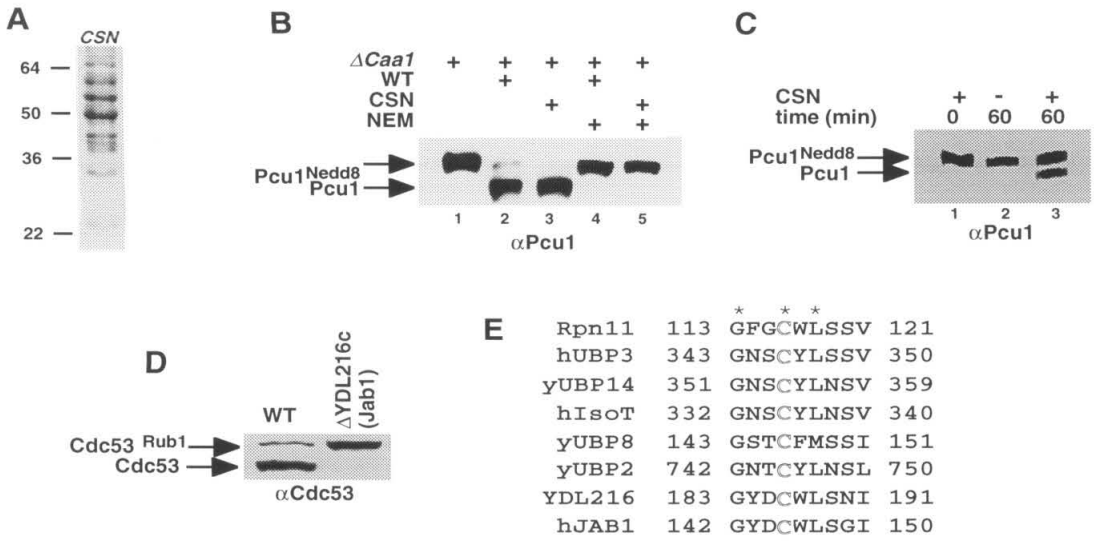


Figure 10. Cop9 Signalosome has deneddylating activity.

A. Coomassie stain of CSN purified from pig spleen. **B.** Wild type *S. pombe* extracts and purified pig spleen CSN possess deneddylating activity. Extract (40 µg) from *Δcaa1* cells was incubated separately, or in the presence of purified pig CSN (3 µg) or p*cu1myc13*$^{+}$ cell extract (40 µg) for 30 minutes at 30°C in the presence or absence of 10mM NEM, as indicated. Pcu1 neddylation was evaluated by SDS-PAGE followed by western blotting with anti-Pcu1 antibodies. **C.** Purified Pcu1^{Myc13} is deneddylated by purified pig CSN. Pcu1^{Myc13} was immunoprecipitated from *Δcaa1* extracts using anti-Myc antibodies. Antibody beads were washed five times with lysis buffer and incubated for the indicated times at 30°C in the presence or absence of 3 µg of purified CSN. **D.** Deletion of the Jab1 homologue in *S. cerevisiae* (YDL216c) results in the hyperaccumulation of a modified form of CDC53. Total cell lysate (30 µg) from wt and *YDL216Δ* strains was separated by SDS-PAGE and evaluated by western blotting with anti-CDC53 antibodies.

Literature Cited

Bianchi, E., Denti, S., Granata, A., Bossi, G., Geginat, J., Villa, A., Rogge, L., and Pardi, R. (2000). Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity. Nature *404*, 617-+.

Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nature Cell Biology *1*, 193-9.

Chamovitz, D., Wei, N., Osterlund, M., vonArnim, A., Staub, J., Matsui, M., and Deng, X. (1996). The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. Cell *86*, 115-121.

Chattopadhyay, S., Ang, L., Puente, P., Deng, X., and Wei, N. (1998). Arabidopsis bZIP protein HY5 directly interacts with light- responsive promoters in mediating light control of gene expression. Plant Cell *10*, 673-683.

Claret, F., Hibi, M., Dhut, S., Toda, T., and Karin, M. (1996). A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. Nature *383*, 453-457.

Deng, X., Dubiel, W., Wei, N., Hofmann, K., Mundt, K., Colicelli, L., Kato, J., Naumann, M., Segal, D., Seeger, M., Glickman, M., Chamovitz, D., and Carr, A. (2000). Unified nomenclature for the COP9 signalosome and its subunits: an essential regulator of development. Trends in Genetics *16*, 202-203.

Deshaies, R. J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annual Review Cell and Developmental Biology *15*, 435-67.

Freed, E., Lacey, K., Huie, P., Lyapina, S., Deshaies, R., Stearns, T., and Jackson, P. (1999). Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. Genes & Development *13*, 2242-2257.

Furukawa, M., Zhang, Y., McCarville, J., Ohta, T., and Xiong, Y. (2000). The CUL1 C-terminal sequence and ROC1 are required for efficient nuclear accumulation, NEDD8 modification, and ubiquitin ligase activity of CUL1. Molecular and Cellular Biology *20*, 8185-8197.

Glickman, M., Rubin, D., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V., and Finley, D. (1998). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell *94*, 615-623.

Gong, L., Kamitani, T., Millas, S., and Yeh, E. T. (2000). Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins. Journal of Biological Chemistry *275*, 14212-6.

Hori, T., Osaka, F., Chiba, T., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S., and Tanaka, K. (1999). Covalent modification of all members of human cullin family proteins by NEDD8. Oncogene *18*, 6829-6834.

Kamitani, T., Kito, K., Nguyen, H. P., and Yeh, E. T. (1997). Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein. Journal of Biological Chemistry *272*, 28557-62.

Kang, D., Wang, X., Cao, K., Sun, C., Deng, X., and Wei, N. (2000). A gain-of-function phenotype conferred by over-expression of functional subunits of the COP9 signalosome in Arabidopsis. Plant Journal *23*, 597-608.

Kipreos, E. T., Lander, L. E., Wing, J. P., He, W. W., and Hedgecock, E. M. (1996). cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. Cell *85*, 1-20.

Kitagawa, K., Skowyra, D., Elledge, S. J., Harper, J. W., and Hieter, P. (1999). SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. Molecular Cell *4*, 21-33.

Kleemann, R., Hausser, A., Geiger, G., Mischke, R., Burger-Kentischer, A., Flieger, O., Johannes, F., Roger, T., Calandra, T., Kapurniotu, A., Grell, M., Finkelmeier, D., Brunner, H., and Bernhagen, J. (2000). Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. Nature *408*, 211-216.

Kwok, S., Solano, R., Tsuge, T., Chamovitz, D., Ecker, J., Matsui, M., and Deng, X. (1998). Arabidopsis homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic cop/det/fus mutations. Plant Cell *10*, 1779-1790.

Li, S., Liu, X., and Ascoli, M. (2000). p38(JAB1) binds to the intracellular precursor of the lutropin/choriogonadotropin receptor and promotes its degradation. Journal of Biological Chemistry *275*, 13386-13393.

Lyapina, S., Correll, C., Kipreos, E., and Deshaies, R. (1998). Human CUL1 forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein. Proc. Natl. Acad. Sci. USA *95*, 7451-7456.

Michel, J. J., and Xiong, Y. (1998). Human CUL-1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A. Cell Growth and Differentiation *9*, 435-49.

Mundt, K., Porte, J., Murray, J., Brikos, C., Christensen, P., Caspari, T., Hagan, I., Millar, J., Simanis, V., Hofmann, K., and Carr, A. (1999). The COP9/signalosome complex is conserved in fission yeast and has a role in S phase. Current Biology *9*, 1427-1430.

Ohta, T., Michel, J. J., Schottelius, A. J., and Xiong, Y. (1999). ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Molecular Cell *3*, 535-41.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh-e, A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., and Kato, S. (2000). Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. EMBO Journal *19*, 3475-3484.

Oyama, T., Shimura, Y., and Okada, K. (1997). The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes & Development *11*, 2983-2995.

Reynard, G. J., Reynolds, W., Verma, R., and Deshaies, R. J. (2000). Cks1 is required for G(1) cyclin-cyclin-dependent kinase activity in budding yeast. Molecular and Cellular Biology *20*, 5858-64.

Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., Gordon, C., Naumann, M., and Dubiel, W. (1998). A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. FASEB Journal *12*, 469-478.

Seol, J., Feldman, R., Zachariae, W., Shevchenko, A., Correll, C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Shevchenko, A., Nasmyth, K., and Deshaies, R. (1999). CDC53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme CDC34. Genes & Development *13*, 1614-1626.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. Analytical Chemistry *68*, 850-858.

Tomoda, K., Kubota, Y., and Kato, J. (1999). Degradation of the cyclin-dependent-kinase inhibitor p27(Kip1) is instigated by Jab1. Nature *398*, 160-165.

Wada, H., Kito, K., Caskey, L. S., Yeh, E. T. H., and Kamitani, T. (1998). Cleavage of the C-terminus of NEDD8 by UCH-L3. Biochemical and Biophysical Research Communications *251*, 688-92.

Wei, N., and Deng, X. (1998). Characterization and purification of the mammalian COP9 complex, a conserved nuclear regulator initially identified as a repressor of photomorphogenesis in higher plants. Photochemistry and Photobiology *68*, 237-241.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999). The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro [published erratum appears in Genes & Development 1999 Apr 15;13(8):1050]. Genes & Development *13*, 270-83.

Mass spectrometry was performed by Anna and Andrej Shevchenko. Greg Cope generated the data presented in Fig. 8E and Fig. 10. Dieter Wolf provided unpublished S. pombe reagents.

Appendix I.

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Cell Biology

Human *CUL1* forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein

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ABSTRACT **The SCF ubiquitin ligase complex of budding yeast triggers DNA replication by catalyzing ubiquitination of the S phase cyclin-dependent kinase inhibitor SIC1. SCF is composed of three proteins—ySKP1, CDC53 (Cullin), and the F-box protein CDC4—that are conserved from yeast to humans. As part of an effort to identify components and substrates of a putative human SCF complex, we isolated *hSKP1* in a two-hybrid screen with *hCUL1*, the closest human homologue of *CDC53*. Here, we show that hCUL1 associates with hSKP1 *in vivo* and directly interacts with both hSKP1 and the human F-box protein SKP2 *in vitro*, forming an SCF-like particle. Moreover, *hCUL1* complements the growth defect of yeast *cdc53*^{ts} mutants, associates with ubiquitination-promoting activity in human cell extracts, and can assemble into functional, chimeric ubiquitin ligase complexes with yeast SCF components. Taken together, these data suggest that hCUL1 functions as part of an SCF ubiquitin ligase complex in human cells. Further application of biochemical assays similar to those described here can now be used to identify regulators/components of hCUL1-based SCF complexes, to determine whether the hCUL2–hCUL5 proteins also are components of ubiquitin ligase complexes in human cells, and to screen for chemical compounds that modulate the activities of the hSKP1 and hCUL1 proteins.**

The irreversible nature of proteolysis makes it well suited to serve as a regulatory switch for controlling unidirectional processes. This principle is clearly evident in the organization of the cell division cycle, where initiation of DNA replication, chromosome segregation, and exit from mitosis are triggered by the destruction of key regulatory proteins (1–3).

Proteins typically are marked for proteolytic degradation by attachment of multiubiquitin chains. This process is initiated by a ubiquitin-activating enzyme (E1), which activates ubiquitin by adenylation and becomes linked to it via a thiolester bond. Ubiquitin then is transferred to a ubiquitin-conjugating enzyme, E2. Whereas E2s can attach ubiquitin directly to lysine residues in a substrate, most physiological ubiquitination reactions probably require a ubiquitin ligase, or E3 (4). E3s have been implicated in substrate recognition and, in one case, transfer of ubiquitin from E2 to a substrate via an E3–ubiquitin-thiolester intermediate (5). Once the substrate is multiubiquitinated, it then is recognized and degraded by the 26S proteasome.

A ubiquitination pathway recently has been discovered in budding yeast (1, 6, 7). Components of this pathway include the CDC53, CDC4, and ySKP1 gene products, which assemble into a ubiquitin ligase complex known as SCF^{CDC4} (for SKP1, Cullin, F-box protein CDC4); because several of the yeast and human subunits have identical names—e.g., SKP1—we distinguish them with the letters y or h, respectively. SCF^{CDC4} collaborates with the E2 enzyme yCDC34 to catalyze ubiquitination of the cyclin-dependent kinase (CDK) inhibitor SIC1. The specificity of SCF^{CDC4} is thought to be governed by ySKP1 and the F-box-containing subunit CDC4, which together form a substrate receptor that tethers SIC1 to the complex. The assembly of this receptor is thought to be mediated by a direct interaction between ySKP1 and the F-box domain of CDC4 (6, 7).

Whereas genetic analysis has revealed that SIC1 proteolysis requires CDC4, G_1 cyclin proteolysis appears to depend on a distinct F-box-containing protein known as GRR1 (8). Alternative SCF complexes (SCF^{GRR1}) assembled with GRR1 instead of CDC4 bind G_1 cyclins but not SIC1, suggesting that there exist multiple SCF complexes in yeast whose substrate specificities are dictated by the identity of the F-box subunit (7).

Components of the SCF ubiquitination pathway have been highly conserved during evolution. Human homologues of yCDC34 and ySKP1 have been reported (9, 10), and F-box-containing proteins like CDC4 and GRR1 have been identified in many eukaryotes (11). Many of these F-box proteins also contain either WD-40 repeats (such as CDC4) or leucine-rich repeats (such as GRR1). A potential human counterpart of GRR1, SKP2, has been identified along with hSKP1 as a Cyclin A/CDK2-associated protein that is necessary for S-phase progression (10). Homologues of CDC53, which are known as Cullins, also are present in many eukaryotes, including humans and nematodes (12, 13).

Studies in budding yeast suggest that SCF substrates must be phosphorylated before they can be ubiquitinated (14, 15). Several human cell cycle regulators are targeted for ubiquitination after their phosphorylation by CDKs, implicating them as potential substrates of an SCF pathway(s) in human cells. Among them is the CDK inhibitor p27, the abundance of which may be regulated by CDC34-dependent ubiquitination (16, 17). In addition, Cyclins E and D1 are degraded by a ubiquitin-dependent pathway after phosphorylation at a specific site (18–20). The observation that Cyclin A/CDK2 associates preferentially with hSKP1 and SKP2 in transformed cells to the exclusion of a proliferating cell nuclear antigen and p21 (10) raises the possibility that Cyclin A is also a target of an SCF pathway. Alternatively, SCF-bound Cyclin A/CDK2 may phosphorylate SCF subunits or potential substrates such as E2F-1/DP-1, thereby activating SCF-dependent ubiquitination (21, 22).

Despite the conservation of SCF components from yeast to humans, several observations raise the question of whether the metazoan homologues are actually components of SCF-like ubiquitin ligases. First, whereas *Saccharomyces cerevisiae cdc53*^{ts} mutants arrest at the G_1/S transition, *Caenorhabditis elegans cul-1* mutants fail to exit the cell cycle, resulting in hyperplasia of most

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: CDK, cyclin-dependent kinase; GST, glutathione *S*-transferase; MBP, maltose binding protein; BUb, biotinylated ubiquitin; HRP, horseradish peroxidase.

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larval tissues (12). It is unclear whether this discrepancy arises because cul-1 and CDC53 have different functions or because they are components of distinct ubiquitin ligase complexes with different substrate specificities. Second, the recent discovery of ubiquitin-like proteins (RUB1/NEDD8 and SMT3/SUMO1) that are conjugated to proteins by pathways that involve E1 and E2 homologs (23) suggests that some homologs of SCF components might function in these alternative pathways. Indeed, attachment of RUB1 to CDC53 fails to occur in *skp1* mutants, suggesting that ySKP1 may be involved directly in the "rubinylation" of CDC53 (24). Third, the best characterized human Cullin, CUL2, assembles with the von Hippel–Lindau tumor suppressor protein/Elongin B/Elongin C complex that has been suggested to regulate mRNA transcript elongation and accumulation of hypoxia-inducible mRNAs (25, 26). Fourth, ySKP1 is a subunit of the centromere-binding CBF3 complex, suggesting that vertebrate SCF subunits may serve as components of a variety of unrelated molecular machines (34).

To address whether SCF-like activities are present in animal cells, we sought hCUL1 binding partners, and we tested whether putative human SCF subunits can assemble together to yield complexes with ubiquitin ligase activity. We report here that *hCUL1* is a direct functional homologue of *CDC53* because it can suppress the temperature-sensitive growth of *cdc53* mutants, can associate with ubiquitin-conjugation activity in human cell lysates, and can substitute for CDC53 in the reconstitution of SIC1 ubiquitination with purified components. Moreover, hCUL1 directly binds to the putative SCF subunits hSKP1 and SKP2. Taken together, these data provide strong evidence that an SCF-dependent ubiquitination pathway is conserved from yeast to mammals.

MATERIALS AND METHODS

Yeast Strains and Reagents. Yeast strains, plasmids, and a HeLa cDNA library for the two-hybrid screen were a generous gift from R. Brent (Massachusetts General Hospital). Wx131.2c *cdc53–2*^{ts} strain was obtained from M. Goebl (Indiana University). Baculoviruses expressing hCDK2^{HA}, hCyclin A (D. Morgan, University of California, San Francisco), SKP2 (H. Zhang, Yale University), hSKP1 (P. Sorger, Massachusetts Institute of Technology) and plasmids pGEX-KG-hSKP1 and pGEX-KG-SKP2 (P. Jackson, Stanford University) and pCS2+nβgal and pCS2+HA-SMC1 (S. Handeli, Fred Hutchinson Cancer Research Center) were kindly provided by the indicated investigators. Other baculoviruses have been described (6). Ubiquitin and the Protein Biotinylation Kit were purchased from Sigma, and biotinylated ubiquitin (BUb) was prepared according to the manufacturer's instructions. Ubiquitin aldehyde was a generous gift from R. Cohen (University of Iowa).

Plasmid and Baculovirus Construction. Full-length hCUL1 ORF was assembled from expressed sequence tags HE2AB96 and HSVAD74 and subcloned into pRS316 and pMALc (New England Biolabs). The same hCUL1 fragment also was subcloned into pVL1393 (PharMingen) to generate a hCUL1-expressing baculovirus. An N-terminal epitope-tagged version of hCUL1 was constructed by inserting a DNA cassette that contains two tandem repeats of the Polyoma epitope (MEYMPME) followed by six histidine residues (designated as PHis6) into pRS316-hCUL1. ^{PHis6}hCUL1 fragment then was subcloned into pFASTBAC1 (GIBCO/BRL) to generate a ^{PHis6}hCUL1 baculovirus and was subcloned into pDNA3.1/Zeo (Invitrogen) to generate pcDNA3.1-PHis6-hCUL1. pCS2+HA-hSKP1 was generated by subcloning a hSKP1 fragment from pGEX-KG-hSKP1 into pCS2+HA-SMC1.

Antibodies. Anti-hCUL1 antibodies were generated in rabbits immunized with either a fusion protein containing the first 41 residues of hCUL1 followed by glutathione *S*-transferase (GST) (Babco, Richmond, CA) or a fusion protein containing GST followed by the last 86 residues of hCUL1 (California Institute of Technology antibody facility). Antibodies against hCUL1 and GST were affinity purified by using maltose binding protein (MBP) fusions of the corresponding peptides and GST, respectively, as described (27). Monoclonal anti-Polyoma antibodies were bound to protein A-Sepharose beads and crosslinked to protein A with dimethylpimilimadate (27) at a concentration of $\approx$2 mg of antibodies per ml of protein A resin. Anti-HA resin was generated by coupling 1 ml of anti-HA ascites to 1 ml of CNBr activated agarose (Pharmacia) according to the manufacturer's protocol.

Expression and Purification of Proteins. Proteins expressed in bacteria or yeast were purified according to standard protocols and as described (6). For the expression and purification of chimeric SCF complexes, Hi5 insect cells were infected with baculoviruses expressing ^{PHA}CDC4 (PHA designates an epitope-tag consisting of two tandem repeats of the Polyoma epitope followed by three hemagglutinin epitopes), CDC53^{PHA}, ^{PHis6}hCUL1 (multiplicities of infection of 6), ySKP1^{His6}, or hSKP1 (multiplicities of infection of 4). Cells were collected 72 hr post-infection, and lysates were prepared as described (6). The Polyoma tagged proteins were affinity purified from these lysates (6) to yield the various SCF complexes.

Cell Cultures and Transfections. WI-38 human lung fibroblasts were purchased from ATCC. HeLa S3 cells were a gift from S. Handeli. Cells were grown in DMEM-F12 (GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL) at 37°C/5%CO_2. Cells were transfected in 100-mm dishes by the modified calcium phosphate method (28). 10 μg pCS2+HA-hSKP1 and 7.5 μg pcDNA3.1-PHis6-hCUL1 vectors were used per transfection plate. Transfection efficiency was monitored by cotransfection of 2.5 μg pCS2+nβgal plasmid per transfection plate followed by standard colorimetric βgal assays (29). Total DNA concentration was 20 μg/100-mm dish and was adjusted for every transfection plate by adding empty vectors. Cells were harvested and lysed 24 hr post-transfection.

Immunoprecipitations and Western Blotting. Baculovirus-infected insect cells were harvested and lysed at 48 hr (for Sf9 cells) or 72 hr (for Hi5 cells) post-infection in 0.8 ml of lysis buffer per 100-mm plate (as described in ref. 6). Metabolic labeling was done by incubating insect cells for 3 hr in methionine-deficient medium plus 20 μCi/ml of Tran[^{35}S]-label before lysis. WI-38 and HeLa S3 cells were lysed in 0.4 ml of lysis buffer per 100-mm plate. Lysates were cleared by centrifugation at 14,000 $\times$ *g* for 15 min, were adjusted to 10% glycerol, were frozen in liquid nitrogen, and were stored at $-$80°C. Cell lysates (1 mg) were incubated with 50 μl of antibody-coupled beads (1:1 suspension in lysis buffer) for 2 hr at 4°C. Precipitates were washed five times with 1 ml of lysis buffer and were analyzed by SDS/PAGE followed by Western blotting or autoradiography. Western blotting was performed as described (27). ^{PHis6}hCUL1 and ^{HA}hSKP1 were detected by rabbit polyclonal anti-hCUL1 and biotinylated anti-HA (12CA5) primary antibodies and were visualized by incubation with goat anti-rabbit-horseradish peroxidase (HRP) and streptavidin-HRP conjugates followed by ECL detection (Amersham).

Ubiquitination Reactions. Crude Sf9 cell lysates (500 μg) prepared from cells infected with ^{Phis6}hCUL1 baculovirus were incubated with 20 μl anti-Polyoma beads for 2 hr at 4°C to allow ^{Phis6}hCUL1 binding. Beads were washed three times with lysis buffer and were incubated with 1 mg of crude HeLa S3 lysate overnight at 4°C. Beads then were washed three times with lysis buffer and were supplemented with 6 μg BUb, 500 ng hCDC34, 25 ng ^{His6}yUBA1, 1 μl of 10$\times$ ATP-regenerating system (6), 1 μl of 10$\times$ reaction buffer (6), and 0.5 μM ubiquitin aldehyde. Reactions were adjusted to 10 μl by adding 20 mM Hepes (pH 7.6), 100 mM potassium acetate, 1 mM DTT, were incubated for 90 min at 30°C, and were terminated by adding Laemmli sample buffer. Samples were analyzed by Western blotting with streptavidin–HRP conjugate. All ubiquitination reactions with chimeric SCF complexes were performed as described (6).

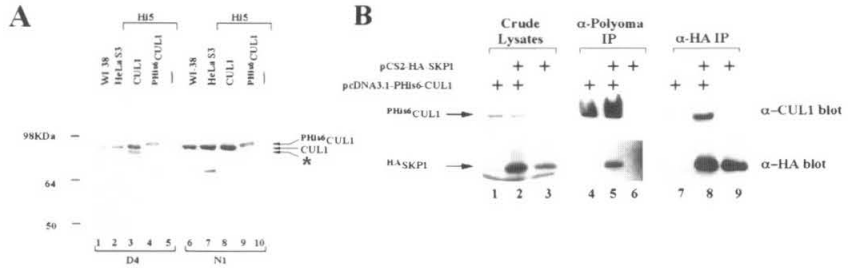

FIG. 1. hCUL1 and hSKP1 interact *in vivo*. (*A*) hCUL1 detection by affinity-purified anti-hCUL1 antibodies. Crude human cell lysates (50 μg) (lanes 1, 2, 6, and 7) and 0.5 μg of crude lysates from Hi5 insect cells, uninfected (lanes 5 and 10) or infected with hCUL1 (lanes 3 and 8) or ^{PHis6}hCUL1 (lanes 4 and 9) viruses, were resolved on an 8% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-hCUL1 antibodies. D4, serum raised against C-terminal part of hCUL1; N1, serum raised against N-terminal part of hCUL1. Asterisk designates putative N-terminally truncated hCUL1 that is recognized by C-terminal antibody. (*B*) HeLa S3 cells were transfected with pcDNA3.1-PHis6-hCUL1 (lanes 1, 4, and 7), pCS2+HA-hSKP1 (lanes 3, 6, and 9), or both plasmids (lanes 2, 5, and 8). Lysates (1 mg) were prepared 24 hr post-transfection and were immunoprecipitated with anti-Polyoma (lanes 4–6) or anti-HA (lanes 7–9) beads. Proteins retained on the beads were analyzed by Western blotting. Lanes 1–3 contained 20 μg of crude lysates.

RESULTS

Human CUL1-Interacting Proteins. To identify human proteins that interact with hCUL1, we performed a two-hybrid screen (30, 31). A full-length *hCUL1* cDNA, fused to the LexA DNA-binding domain, was used as a bait to identify cDNAs from a HeLa library that encode hCUL1 interactors. This screen yielded clones encoding hSKP1, protein phosphatase 2A catalytic subunit, and the 20S proteasome subunit HsN3. None of these clones interacted with LexA-hCDK2 or LexA-Lamin C baits, suggesting that their interaction with LexA-hCUL1 was specific. Here, we examine in detail the interaction of hCUL1 with hSKP1 (see below). The physiological significance of the interaction of hCUL1 with HsN3 or protein phosphatase 2A has not been evaluated yet.

Human CUL1 Interacts with hSKP1 *in Vivo*. The identification of *hSKP1* as a hCUL1-interacting protein suggested that these proteins may be subunits of a complex in human cells that is similar to the SCF ubiquitin ligase of budding yeast. To test whether hCUL1 interacts with hSKP1 *in vivo*, we prepared affinity-purified rabbit polyclonal antibodies directed against the N and C termini of hCUL1. Fig. 1*A* shows specificity of the affinity-purified antibodies. Both antibodies recognized one major polypeptide of ≈80 kDa in transformed (HeLa S3) and nontransformed (WI-38) cell lines (Fig. 1*A*, lanes 1, 2, 6, and 7). This species comigrated with hCUL1 produced in Hi5 cells infected with a baculovirus that contains full length *hCUL1* cDNA (Fig. 1*A*, lanes 3 and 8). A more rapidly migrating species of recombinant hCUL1 detected in Hi5 cells by the anti-C-terminal antibodies (Fig. 1*A*, lane 3) presumably represents a breakdown product or initiation of translation downstream of the normal start codon because this species was not detected by the anti-N-terminal antibodies. As expected, addition of a Polyoma antigen-hexahistidine tag to hCUL1 (^{PHis6}hCUL1) yielded a more slowly migrating hCUL1 band (Fig. 1*A*, lanes 4 and 9).

Neither polyclonal antibody precipitated hCUL1 from crude human cell lysates, precluding analysis of hCUL1 complexes in nontransfected cells. Thus, to evaluate the potential interaction of hCUL1 with hSKP1 *in vivo*, we transfected HeLa S3 cells with ^{PHis6}hCUL1 and ^{HA}hSKP1 expression vectors. Lysates were prepared from these cells 24 hr post-transfection and were immunoprecipitated by using crosslinked anti-Polyoma or anti-HA antibody beads. Proteins bound to the beads were separated by SDS/PAGE and were analyzed by immunoblotting with anti-hCUL1 and anti-HA antibodies (Fig. 1*B*). Consistent with the two-hybrid data, hCUL1 was detected specifically in hSKP1 immunoprecipitates and vice versa.

Human CUL1, hSKP1, and SKP2 Assemble into an SCF-like Complex that Can Associate with Cyclin A/CDK2 Kinase. Human SKP1 was initially identified as a Cyclin A/CDK2-associated protein in transformed human cells (10). This association is mediated by SKP2, a human F-box protein with leucine-rich repeats, reminiscent of the GRR1 protein. CDC53 and ySKP1, together with the F-box protein GRR1, constitute a putative SCF^{GRR1} ubiquitin ligase complex that targets G_1 cyclins for degradation (6, 7). The homology of hSKP1, SKP2, and hCUL1 proteins with components of the ySCF complex suggests that the human proteins may form a similar complex. We addressed this possibility by immunoprecipitating ^{PHis6}hCUL1 from [^{35}S]-labeled insect cells infected with baculoviruses that express ^{PHis6}hCUL1, hSKP1, and SKP2 (Fig. 2*A*) and by testing whether hCUL1 can assemble with a previously described complex containing Cyclin A/CDK2, hSKP1, and SKP2 (Fig. 2*B*). The interaction of hCUL1 with the Cyclin A/$CDK2^{HA}$/hSKP1/SKP2 complex was monitored by immunoprecipitating $CDK2^{HA}$ from [^{35}S]-labeled insect cells infected with all five viruses in various combinations. As shown in Fig. 2*A*, ^{PHis6}hCUL1 efficiently assembled with hSKP1 and SKP2, suggesting that these proteins form a ternary complex similar to ySCF. Surprisingly, hCUL1 interacted with Cyclin A/$CDK2^{HA}$ complexes in the absence of SKP2 or hSKP1 (Fig. 2*B*, lane 6; note that hSKP1 does not associate with Cyclin A/$CDK2^{HA}$ complex in the absence of SKP2). This interaction may be caused by either a direct interaction between hCUL1 and Cyclin A/$CDK2^{HA}$ or the presence of a bridging protein in insect cells (e.g., see ref. 6). Regardless, the ability of hCUL1 expressed in insect cells to assemble into complexes containing a cyclin-dependent kinase is likely to be physiologically significant because ^{PHis6}hCUL1 immunoprecipitates prepared from HeLa S3 cells contained histone H1 kinase activity (data not shown).

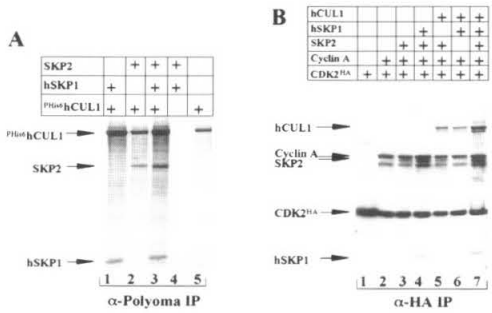

FIG. 2. Human CUL1 can interact with human SKP1, SKP2, and Cyclin A/CDK2. Sf9 insect cells were infected with baculovirus constructs that express various human proteins as indicated. Cells were labeled with Tran[^{35}S]-label for 3 hr before harvesting. ^{PHis6}hCUL1 (*A*) and $CDK2^{HA}$ (*B*) together with associated proteins were immunoprecipitated by anti-Polyoma and anti-HA beads, respectively. The composition of the protein complexes in the immunoprecipitates was analyzed by SDS/PAGE followed by autoradiography.

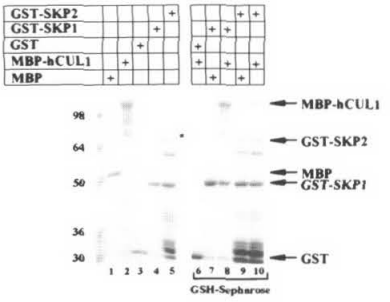

FIG. 3. Human CUL1 binds directly to hSKP1 and SKP2. MBP, MBP-hCUL1, GST, GST-hSKP1, and GST-SKP2 were expressed individually in and purified from bacteria. Each protein was present in the binding reactions at 65 μg/ml. Proteins (4 μg of each) were loaded in lanes 1–5, which represents 1/5 of the input for the binding reactions. Proteins were mixed as indicated (lanes 6–10) and incubated on ice for 1 hr. GST and GST fusions were collected on glutathione-Sepharose (GSH-Sepharose) for 1 hr at 4°C, and the beads then were washed three times with 20 mM Hepes (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 5 mM EDTA, and 2 mM DTT. Proteins bound to glutathione-Sepharose were resolved by SDS/PAGE and were visualized by staining with Coomassie blue. Positions of the full-length fusion proteins are indicated by the arrows. An ≈70-kDa band that copurified with GST-SKP2 from bacteria is marked by an asterisk.

Human CUL1 Directly Interacts with hSKP1 and SKP2. The results in Fig. 2 suggest that hCUL1, hSKP1, and SKP2 can assemble into an SCF-like particle when coexpressed in insect cells. Because of the strong conservation of SCF components, however, these interactions might be mediated by other proteins provided by the host cells (for an example, see ref. 6). To test whether the observed interactions are direct, we produced GST-hSKP1, GST-SKP2, and MBP-hCUL1 in bacteria. The GST fusions (or unfused GST control) were mixed with MBP-hCUL1 or MBP and were recovered by binding to glutathione-Sepharose beads. Bound proteins were resolved by SDS/PAGE and were visualized by Coomassie blue staining (Fig. 3). MBP-hCUL1 but not MBP bound specifically and efficiently to GST-hSKP1 and GST-SKP2 but not GST. This result demonstrates that hCUL1 can bind to both hSKP1 and SKP2 without the participation of other proteins.

Human CUL1 Is Functionally Homologous to CDC53 and Can Form an Active Chimeric SCF Complex with ySKP1 and CDC4. The above observations indicate that hCUL1, the closest human homologue of CDC53, can assemble with hSKP1 and the F-box protein SKP2 into a complex reminiscent of the yeast SCF^{GRR1} complex. We next tested whether this complex—in the presence of hCDC34, E1 enzyme, and ubiquitin—was able to ubiquitinate proteins that either bind to it (Cyclin A; ref. 10), are known to be degraded in S phase (Cyclin E, E2F-1; refs. 18, 19, 21, and 22), or

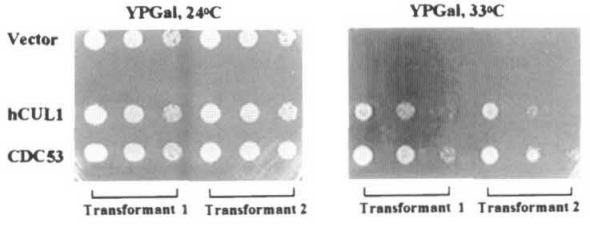

FIG. 4. hCUL1 complements the $cdc53^{ts}$ mutant phenotype. A $cdc53\text{–}2^{ts}$ mutant strain was transformed with pTS161-CDC53 and pTS161-hCUL1 plasmids that allow controlled expression of CDC53 and hCUL1 from the galactose-inducible *GAL1* promoter. The empty vector alone was used as a negative control. Serial dilutions 1/10 of the individual transformants were spotted on synthetic galactose medium and were incubated for 5 days at restrictive (33°C) and permissive (24°C) temperatures.

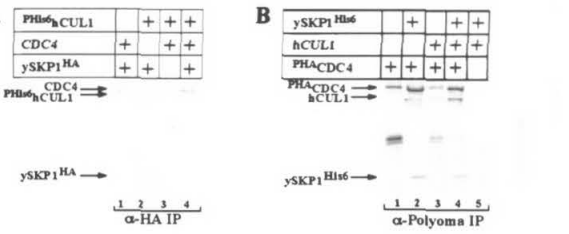

FIG. 5. Human CUL1 can interact with the yeast SCF components ySKP1 and CDC4. Hi5 insect cells were infected with baculovirus constructs expressing various proteins as indicated. Cells were labeled with Tran[^{35}S]-label for 3 hr before harvesting. Yeast $SKP1^{HA}$ (*A*) and ^{PHA}CDC4 (*B*) together with associated proteins were immunoprecipitated by anti-HA and anti-Polyoma beads, respectively. The composition of the protein complexes in the immunoprecipitates was analyzed by SDS/PAGE followed by autoradiography. The asterisk marks an unidentified contaminant that migrates reproducibly faster than the authentic hCUL1.

have been implicated as substrates of hCDC34 (p27; refs. 16, 17). These efforts were unsuccessful (data not shown), raising the question of whether SCF^{SKP2} complexes possess ubiquitin ligase activity. Moreover, ubiquitin ligase activity of the analogous yeast SCF^{GRR1} complex has not been demonstrated yet and might require additional unidentified components. However, we were able to address whether hCUL1 is a functional component of a ubiquitin ligase complex genetically and biochemically by taking advantage of the considerable knowledge of this pathway in yeast. First, we asked whether *hCUL1* can complement the $cdc53^{ts}$ mutation. We introduced *hCUL1* and *CDC53* under the control of the *GAL1* promoter into a yeast strain carrying a temperature-sensitive mutation in the *CDC53* gene. Individual transformants were spotted at different dilutions on glucose (noninducing conditions, data not shown) and galactose (inducing conditions) media at permissive (24°C) and restrictive (33°C) temperatures (Fig. 4). Only transformants that expressed wild-type CDC53 or hCUL1 proteins were able to grow at the restrictive temperature. However, *hCUL1* failed to complement a *cdc53* null strain (data not shown).

The ability of hCUL1 to complement the $cdc53^{ts}$ mutation implied that hCUL1 can assemble into functional SCF complexes with yeast proteins. To test this idea, we examined whether hCUL1 can interact with the budding yeast SCF subunits ySKP1 and CDC4. All three proteins were coexpressed in [^{35}S]-methionine-radiolabeled insect cells in various combinations, as indicated in Fig. 5. Human CUL1 specifically coimmunoprecipitated with $ySKP1^{HA}$ (Fig. 5*A*, lane 2) or ^{PHA}CDC4/ySKP1 (Fig. 5*B*, lane 4), indicating that it can form a chimeric SCF^{CDC4} complex with yeast proteins.

Our previous findings (6) identified SCF^{CDC4} as a functional E3 that required the presence of all three subunits (CDC4, CDC53, and ySKP1) to catalyze ubiquitination of phosphorylated SIC1 (6, 7). Preceding its ubiquitination, phosphorylated SIC1 is recruited to SCF^{CDC4} by binding to the CDC4/ySKP1 substrate receptor (6, 7). Given that hCUL1 and hSKP1 assembled with CDC4 (Fig. 5*B*), we sought to test whether these hybrid SCF complexes were able to promote ubiquitination of phosphorylated SIC1. Purified chimeric SCF complexes were incubated with $MBP\text{-}SIC1^{MHis6}$ and purified ubiquitination components. In the presence of SCF^{CDC4} (Fig. 6*A*, lane 1), $MBP\text{-}SIC1^{MHis6}$ was converted efficiently to high molecular weight forms. Omission of either CDC4, CDC53, or ySKP1 resulted in no activity (Fig. 6*A*, lanes 2–4). Replacement of $CDC53^{PHA}$ with ^{PHis6}hCUL1 resulted in an SCF complex with modest ubiquitination activity that depends on both CDC4 and ySKP1 (Fig. 6*A*, lanes 5, 6, and 9). Additionally, an SCF complex containing both ^{PHis6}hCUL1 and hSKP1 along with ^{PHA}CDC4 also was able to catalyze ubiquitination of $MBP\text{-}SIC1^{MHis6}$ (Fig. 6*A*, lane 10). The conversion of $MBP\text{-}SIC1^{MHis6}$ to high molecular weight forms by hybrid CDC4/hCUL1/hSKP1

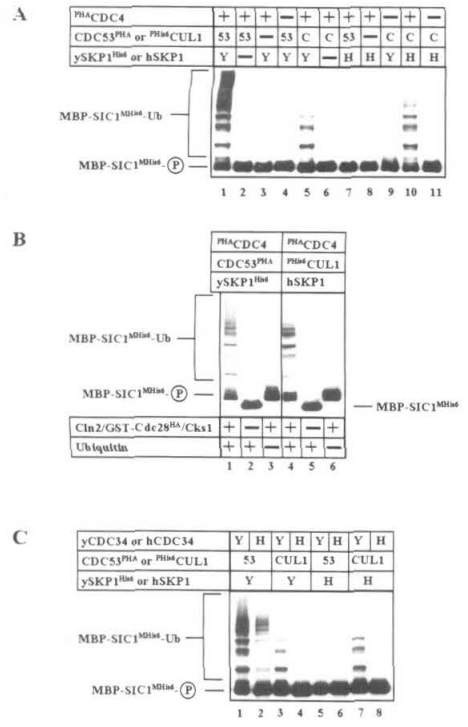

FIG. 6. SIC1 is ubiquitinated by chimeric SCF complexes. (*A*) Hi5 insect cells were infected with various baculoviruses expressing ^{PHA}CDC4, CDC53^{PHA} (53), ^{PHis6}hCUL1 (C), ySKP1^{His6} (Y), or hSKP1 (H) as indicated. At 72 hr post-infection, lysates were prepared and SCF complexes were affinity purified on an anti-Polyoma matrix, and eluted complexes were incubated for 2 hr at 25°C with MBP-SIC1^{MHis6} in the presence of ^{His6}yUBA1 (E1), yCDC34 (E2), purified CLN2/GST-CDC28^{HA}/CKS1, ubiquitin, and an ATP-regenerating system. At the end of the incubation, the samples were fractionated by SDS/PAGE and were immunoblotted with anti-myc antibodies to detect MBP-SIC1^{MHis6}. Bound antibodies were visualized by ECL. (*B*) The indicated SCF complexes were purified from baculovirus-infected insect cell lysates and were incubated with the full set of ubiquitination components (lanes 1 and 4) or in the absence of CLN2/GST-CDC28^{HA}/CKS1 (lanes 2 and 5) or ubiquitin (lanes 3 and 6). (*C*) Purified SCF complexes containing CDC53^{PHA} (53), ^{PHis6}hCUL1 (CUL1), ySKP1^{His6} (Y), or hSKP1 (H) subunits were incubated with ubiquitination components containing either yCDC34 (Y) or hCDC34 (H).

complexes required both substrate phosphorylation (Fig. 6*B*, lane 5) and the presence of ubiquitin (Fig. 6*B*, lane 6). Interestingly, coexpression of ^{PHA}CDC4, CDC53^{PHA}, and hSKP1 did not result in a functional SCF complex (Fig. 6*A*, lane 7).

CDC53 was shown to interact with yCDC34 (32). Thus, we presumed that an SCF complex containing hCUL1 would prefer to use hCDC34 as an E2 as opposed to yCDC34. However, SCF^{CDC4} complexes containing ^{PHis6}hCUL1 with either ySKP1^{His6} or hSKP1 appeared to work much more efficiently with yCDC34 than with hCDC34 serving as the E2 (Fig. 6*C*, lanes 3, 4, 7, and 8). Although we do not understand the basis for this preference, it is possible that there exist additional human CDC34-like E2s that interact preferentially with hCUL1-containing complexes. Alternatively, the interaction between an F-box subunit and an E2 enzyme might also contribute to the specificity for a particular E2 (38).

Human CUL1 Assembles with Ubiquitination-Promoting Activities in Human Cell Extracts. The data presented so far are consistent with hCUL1 functioning as a component of a ubiquitin ligase complex in human cells. Because we have failed so far to detect ubiquitination activity by using recombinant hCUL1/hSKP1/SKP2 complexes, we sought to develop an assay that

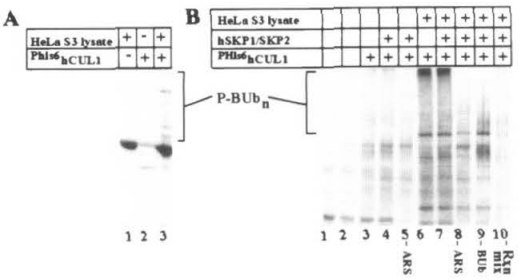

FIG. 7. Human CUL1 associates with ubiquitination activity in HeLa S3 lysates. (*A*) Anti-Polyoma beads were incubated in the presence of ^{PHis6}hCUL1, HeLa S3 lysates, or both as indicated, were washed five times with lysis buffer, and were mixed with the ubiquitination reaction components (Rxn mix) ^{His6}yUBA1, hCDC34, BUb, and ATP-regeneration system. Incorporation of BUb into proteins present in the reactions was monitored by probing with streptavidin-HRP conjugate followed by ECL detection. (*B*) ^{PHis6}hCUL1 alone or together with hSKP1 and SKP2 was produced in insect cells and was bound to anti-Polyoma beads that then were washed and incubated at 4°C in the presence or absence of crude HeLa S3 lysates to allow bead "activation". Activated beads then were treated as in *A*. Dependence on the presence of ATP and BUb in the reactions was determined by omitting these components from the reaction mix (lanes 5, 8, and 9, respectively). The entire reaction mix was omitted in lane 10. Lane 1 contained reaction mix only. P-BUb$_n$ designates a ladder of ubiquitinated proteins produced in the reaction.

would allow us to identify either substrates or cofactors of a hCUL1-dependent ubiquitination pathway. ^{PHis6}hCUL1 produced in insect cells in the presence or absence of hSKP1 plus SKP2 was bound to anti-Polyoma beads and incubated with crude HeLa S3 lysates to allow binding of other potential SCF components, regulators, and substrates. After washing away unbound proteins, E1, hCDC34, BUb, and an ATP-regenerating system then were added to the beads. After an incubation, reactions were fractionated by SDS/PAGE, were transferred to nitrocellulose, and were blotted with streptavidin-HRP to detect ubiquitin conjugates. Whereas ^{Phis6}hCUL1 or ^{Phis6}hCUL1/hSKP1/SKP2 complexes isolated from insect cells exhibited little ubiquitination activity (Fig. 7*A*, lane 2 and Fig. 7*B*, lanes 3 and 4), a high molecular weight smear characteristic of ubiquitinated proteins appeared (Fig. 7*A*, lane 3 and Fig. 7*B*, lanes 6 and 7) when these same components were preincubated with HeLa S3 lysate before the assay. In contrast, no signal was detected when naked polyoma beads were preincubated with HeLa S3 lysate (Fig. 7*A*, lane 1). The appearance of slowly migrating biotinylated proteins depended on the addition of ubiquitin and ATP-regenerating system to the reaction (Fig. 7*B*, lanes 8 and 9), indicating that the high molecular weight smear was caused by ubiquitination occurring during the *in vitro* incubation.

DISCUSSION

Multiple homologues of the ySKP1, CDC53, and F-box subunits of the SCF ubiquitin ligase complex have been identified (10–13) and implicated in various cellular processes, including kinetochore function (33, 34), S-phase progression (10), exit from the cell cycle (12), transcript elongation, regulation of hypoxia-inducible genes, and suppression of tumorigenesis (25, 26). Based on the close homology between hCUL1 and CDC53, we sought to address whether hCUL1 functions as part of an SCF-like ubiquitin ligase complex in human cells. A two-hybrid screen to identify proteins that interact with hCUL1 yielded hSKP1, suggesting that hCUL1 does indeed assemble into SCF-like complexes in human cells. Several other observations reported here support this hypothesis. First, hCUL1 associates with hSKP1 in transfected HeLa S3 cells. Second, hCUL1 assembles into complexes with both hSKP1 and the F-box protein SKP2 *in vitro*. Third, *hCUL1* complements the growth defect of a *cdc53*^{ts}

mutant. Fourth, hCUL1 and hSKP1 can form chimeric SCF complexes with CDC4, and these complexes are able to ubiquitinate the SCF^{CDC4} substrate SIC1 *in vitro*. Fifth, hCUL1 associates with ubiquitination-promoting activity in HeLa S3 cell lysate. Taken together, these data strongly suggest that hCUL1 is a subunit of an SCF-like E3 complex in human cells.

What are the candidate substrates for hCUL1-dependent ubiquitination in human cells? SIC1, CLN2, and FAR1 must be phosphorylated before they can be ubiquitinated by the budding yeast SCF/CDC34 pathway (6, 7, 35). The stability of many mammalian regulatory proteins—including IκB, β-catenin, p27, Cyclin D, and Cyclin E—is known to be controlled by phosphorylation (16–20, 36, 37). Further work will be required to determine whether any of these proteins are substrates for human SCF complexes. SCF-associated Cyclin A might also be a substrate of the SCF^{SKP2} pathway. This is less likely, though, because Cyclin A is thought to be primarily destroyed via the antigen-presenting cell/cyclosome pathway, and both Cyclin A and SKP2 activities are essential for entry into S phase (10). Instead, the tight association of Cyclin A/CDK2 with SCF subunits both *in vivo* and *in vitro* might reflect an efficient coupling between substrate phosphorylation and ubiquitination in transformed cells.

While this manuscript was in preparation, a study that complements our findings was reported by Lisztwan *et al.* (38). These authors demonstrated that hCUL1, hSKP1, and SKP2 assemble into a complex both in unperturbed and transfected human cells. Moreover, SKP2 also was shown to bind the ubiquitin-conjugating enzyme hCDC34 in human cells, suggesting that SKP2 is part of an SCF-like ubiquitin ligase. SKP2 also was shown to associate with Cyclin A/CDK2, and mutational analysis suggested that Cyclin A/CDK2 binding might regulate SKP2/hSKP1 but not SKP2/hCUL1 interaction *in vivo*. Our data support these findings and extend them by establishing that hCUL1 interacts directly with hSKP1 and SKP2 without the participation of other eukaryotic proteins, and hCUL1 and hSKP1 can assemble into active ubiquitin ligase complexes either in insect cells or in HeLa S3 cell lysates.

Further characterization of the SCF pathway in human cells will require the identification of functional F-box subunits and physiological substrates. The ability to stimulate the E3 activity of insect cell-derived hCUL1 with HeLa S3 cell lysate provides a strategy for identifying these proteins. Moreover, this assay can be adapted readily to test whether the related hCUL2-hCUL5 proteins also assemble into ubiquitin ligase complexes in human cells. Lastly, by converting either the chimeric SCF complex assay (Fig. 6) or the biotin-ubiquitin-based assay (Fig. 7) to a microtiter plate format, it should be feasible to screen chemical libraries to identify compounds that modulate the activities of hSKP1 and hCUL1. Given its critical role in cell division in budding yeast, inhibitors of human SCF might be valuable lead compounds for the development of novel anti-cancer chemotherapeutics.

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1. Schwob, E., Böhm, T., Mendenhall, M. & Nasmyth, K. (1994) *Cell* **79,** 233–244.
2. Glotzer, M., Murray, A. W. & Kirschner, M. W. (1991) *Nature (London)* **349,** 132–138.
3. Cohen-Fix, O., Peters, J.-M., Kirschner, M. W. & Koshland, D. (1996) *Genes Dev.* **10,** 3081–3093.
4. Hershko, A., Heller, H., Elias, S. & Ciechanover, A. (1983) *J. Biol. Chem.* **258,** 8206–8214.
5. Scheffner, M., Nuber, U. & Huibregtse, J. M. (1995) *Nature (London)* **373,** 81–83.
6. Feldman, R. M. R., Correll, C. C., Kaplan, K. B. & Deshaies, R. J. (1997) *Cell* **91,** 221–230.
7. Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J. & Harper, J. W. (1997) *Cell* **91,** 209–219.
8. Barral, Y., Jentsch, S. & Mann, C. (1995) *Genes Dev.* **9,** 399–409.
9. Plon, S. E., Leppig, K. A., Do, H. N. & Groudine, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10484–10488.
10. Zhang, H., Kobayashi, R., Galaktionov, K. & Beach, D. (1995) *Cell* **82,** 912–925.
11. Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W. & Elledge, S. J. (1996) *Cell* **86,** 263–274.
12. Kipreos, E. T., Lander, L. E., Wing, J. P., He, W. W. & Hedgecock, E. M. (1996) *Cell* **85,** 1–20.
13. Mathias, N., Johnson, S. L., Winey, M., Adams, A. E., Goetsch, L., Pringle, J. R., Byers, B. & Goebl, M. G. (1996) *Mol. Cell. Biol.* **16,** 6634–6643.
14. Lanker, S., Valdivieso, M. H. & Wittenberg, C. (1996) *Science* **271,** 1597–1601.
15. Verma, R., Annan, R., Huddleston, M., Carr, S., Reynard, G. & Deshaies, R. J. (1997) *Science* **278,** 455–460.
16. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. & Rolfe, M. (1995) *Science* **269,** 682–685.
17. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M. & Clurman, B. E. (1997) *Genes Dev.* **11,** 1464–1478.
18. Won, K. A. & Reed, S. I. (1996) *EMBO J.* **15,** 4182–4193.
19. Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M. & Roberts, J. M. (1996) *Genes Dev.* **10,** 1979–1990.
20. Diehl, J. A., Zindy, F. & Sherr, C. J. (1997) *Genes Dev.* **11,** 957–972.
21. Dynlacht, B. D., Flores, O., Lees, J. A. & Harlow, E. (1994) *Genes Dev.* **8,** 1772–1786.
22. Hofmann, F., Martelli, F., Livingston, D. M. & Wang, Z. (1996) *Genes Dev.* **10,** 2949–2959.
23. Hochstrasser, M. (1998) *Genes Dev.* **12,** 908–913.
24. Lammer, D., Mathias, N., Laplaza, J. M., Jiang, W., Liu, Y., Callis, J., Goebl, M. & Estelle, M. (1998) *Genes Dev.* **12,** 914–926.
25. Pause, A., Lee, S., Worrell, R. A., Chen, D. Y. T., Burgess, W. H., Linehan, W. M. & Klausner, R. D. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2156–2161.
26. Lonergan, K. M., Iliopoulos, O., Ohh, M., Kamura, T., Conaway, R. C., Conaway, L. W. & Kaelin, W. G. (1998) *Mol. Cell. Biol.* **18,** 732–741.
27. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
28. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752.
29. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) *Molecular Cloning: Assay for β-galactosidase in Extracts of Mammalian Cells* (Cold Spring Harbor Lab. Press, Plainview, NY).
30. Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. (1993) *Cell* **75,** 791–803.
31. Fields, S. & Song, O. (1989) *Nature (London)* **340,** 245–246.
32. Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Kobayashi, R., Wittenberg, C. & Tyers, M. (1996) *Cell* **86,** 453–463.
33. Connelly, C. & Hieter, P. (1996) *Cell* **86,** 275–285.
34. Kaplan, K. B., Hyman, A. A. & Sorger, P. K. (1997) *Cell* **91,** 491–500.
35. Henchoz, S., Chi, Y., Catarin, B., Herskowitz, I., Deshaies, R. J. & Peter, M. (1997) *Genes Dev.* **11,** 3046–3060.
36. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D. & Miyamoto, S. (1995) *Genes Dev.* **22,** 2723–2735.
37. Aberle, H., Baueer, A., Stappert, J., Kispert, A. & Kemler, R. (1997) *EMBO J.* **16,** 3797–3804.
38. Lisztwan, J., Marti, A., Sutterluty, H., Gstaiger, M., Wirbelauer, C. & Krek, W. (1998) *EMBO J.* **17,** 368–383.