18. H. M. O. Leyser, F. B. Pickett, S. Dharmasiri, M. Estelle, *Plant J.* **10**, 403 (1996).
19. W. M. Gray, M. Estelle, *Trends Biochem. Sci.* **25**, 133 (2000).
20. C. K. Worley *et al.*, *Plant J.* **21**, 553 (2000).
21. S. Lyapina *et al.*, *Science* **292**, 1382 (2001).
22. J. C. del Pozo, C. Timpte, S. Tan, J. Callis, M. Estelle, *Science* **280**, 1760 (1998).
23. S.-J. Li, M. Hochstrasser, *Mol. Cell. Biol.* **20**, 2367 (2000).
24. K. Wu, A. Chen, Z.-Q. Pan, *J. Biol. Chem.* **275**, 32317 (2000).
25. A. G. v. Arnim, X.-W. Deng, *Cell* **79**, 1035 (1994).
26. R. J. Deshaies, *Annu. Rev. Cell Dev. Biol.* **15**, 435 (1999).
27. J. M. Staub, N. Wei, X.-W. Deng, *Plant Cell* **8**, 2047 (1996).
28. G. Serino *et al.*, *Plant Cell* **11**, 1967 (1999).
29. For root length measurements, seedlings were scanned and root length was measured with NIH Image software using the digital images. The average and standard error of 10 seedlings is shown for each experimental condition.
30. P. Gil *et al.*, *Plant Physiol.* **104**, 777 (1994).
31. Experimental details can be found as supplementary material at www.sciencemag.org/cgi/content/full/1059776/DC1
32. We thank the *Arabidopsis* Biological Resource Center at Ohio State University for expressed sequence tag clones. This work was supported by a NSF grant (MCD-0077217) to X.-W.D. C.S. is a postdoctoral fellow of the Deutsche Forschungsgemeinschaft (Schw751/1-1), G.S. was a fellow of the Istituto Pasteur-Fondazione Cenci-Bolognetti, and X.-W.D. is a NSF Presidential Faculty Fellow.

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Promotion of NEDD8-CUL1 Conjugate Cleavage by COP9 Signalosome

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SCF ubiquitin ligases control various processes by marking regulatory proteins for ubiquitin-dependent proteolysis. To illuminate how SCF complexes are regulated, we sought proteins that interact with the human SCF component CUL1. The COP9 signalosome (CSN), a suppressor of plant photomorphogenesis, associated with multiple cullins and promoted cleavage of the ubiquitin-like protein NEDD8 from *Schizosaccharomyces pombe* CUL1 in vivo and in vitro. Multiple NEDD8-modified proteins uniquely accumulated in CSN-deficient *S. pombe* cells. We propose that the broad spectrum of activities previously attributed to CSN subunits—including repression of photomorphogenesis, activation of JUN, and activation of p27 nuclear export—underscores the importance of dynamic cycles of NEDD8 attachment and removal in biological regulation.

SCF ubiquitin ligases consist of at least four subunits: CUL1 and HRT1 (also known as ROC1 or RBX1), which harbor a core ubiquitin ligase activity; a variable F-box protein that serves as a substrate receptor; and SKP1, which links the two modules together (*1*). SCF activity is stimulated in vitro by attachment of the ubiquitin-like protein NEDD8 (that is, "neddylation") to CUL1, but the physiological role and dynamics of this modification are poorly understood (*2–5*). It is not known whether there are other forms of SCF regulation at play in vivo, or whether there are additional SCF subunits yet to be discovered.

To address these questions, we expressed Myc_9-tagged forms of two human proteins, the F-box protein hSKP2 and COOH-terminally truncated hCUL1 (Δ692-752; hereafter called CUL1ΔC), from retroviral vectors in NIH 3T3 cells. SCF complexes were purified from these cells on anti-Myc beads (*6*) and evaluated by SDS–polyacrylamide gel electrophoresis (PAGE) and silver staining (Fig. 1A). Bands corresponding to specific interacting proteins were excised and analyzed by matrix-assisted laser desorption/ionization mass spectrometry and nanoelectrospray tandem mass spectrometry (*7–9*).

The SKP2 (*6*) and CUL1ΔC eluates contained a rich harvest of associated proteins. Analysis of CUL1ΔC eluates (Fig. 1A) yielded SKP1, HRT1, and a number of F-box proteins, as expected. Unexpectedly, all eight subunits (CSN1 to CSN8) of the COP9 signalosome (CSN) (*10*) were also found associated with CUL1ΔC. The ~500-kD CSN was originally discovered in *Arabidopsis thaliana* as a suppressor of photomorphogenesis (*11*). CSN subunits have significant sequence homologies to components of both the lid subcomplex of the 26*S* proteasome and the translation initiation complex eIF3 (*12–14*), but the biochemical function of CSN remains elusive.

To address the specificity of CSN-CUL1ΔC interaction, we tested other SCF subunits and other cullins to see whether they also bound CSN. Flag-tagged CSN1 was transfected into HeLa cells, purified on anti-Flag beads, and analyzed by SDS-PAGE and Western blotting (Fig. 1B). CSN8 associated with Flag-CSN1, as did HRT1, SKP1, and SKP2. Although CSN complex was purified in association with CUL1ΔC, Flag-CSN1 also bound full-length endogenous CUL1. Moreover, a direct comparison revealed that CUL1ΔC and CUL1 bound CSN1 with equal efficiency (*6*). CUL2 and CUL3 were retrieved in Flag-CSN1 eluates (Fig. 1B), and preliminary data suggest that CUL4 and CUL5 also bound CSN (*15*). Because CSN binds all cullins, it might be a global signal integrator that radiates its output onto various associated ubiquitin ligases to modulate their activity.

To further define the SCF-CSN interaction, we performed a comprehensive two-hybrid analysis of SCF and CSN subunits (*6*). Whereas SKP1 did not interact with any CSN subunit, CUL1 interacted strongly with CSN2 and weakly with CSN6. Conversely, HRT1 interacted weakly with the NH_2-terminal domain of CSN1 and moderately with CSN6. Thus, COP9 signalosome interacts with the conserved catalytic core of SCF primarily via the CSN2 and CSN6 subunits.

To probe the functional relation between CSN and SCF, we turned to *Schizosaccharomyces pombe*; *S. pombe* contains a high molecular weight CSN-like complex, and the fission yeast CSN1 homolog Caa1 (*16*) is required for proper S phase progression (*17*). First, we sought to confirm that the fission yeast CUL1 homolog Pcu1 interacts with *S. pombe* CSN. Strains whose $pcu1^+$ and $caa1^+$ chromosomal loci were modified to encode proteins tagged with multimerized Myc epitopes were transformed with a plasmid

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that expressed hemagglutinin (HA)–tagged CSN2 homolog Sgn2^{HA}. Sgn2^{HA} was selectively recovered in anti-Myc precipitates from both *caa1 myc13*$^{+}$ and *pcu1 myc13*$^{+}$ cells (Fig. 1C). Moreover, endogenous Pcu1 was selectively detected in Sgn2^{HA} immunoprecipitates (*6*). In addition to Pcu1, *S. pombe* Pcu3 was also coimmunoprecipitated with CSN (*18*). Thus, interaction of CSN with multiple cullin-based ubiquitin ligases is conserved from fission yeast to human cells.

To investigate the role of CSN in SCF function, we analyzed SCF components in cells lacking *caa1*$^{+}$ (Δ*caa1*) (*17*). Endogenous Pcu1 accumulated in a modified form in Δ*caa1* cells but not in other mutants that linger in S phase, nor in the F-box protein mutants Δ*pop1* and Δ*pop2* (Fig. 2A). *Schizosaccharomyces pombe* Pcu1 is modified on Lys^{713} by covalent attachment of Nedd8 (*19*), so we reasoned that the altered migration we observed for Pcu1 in Δ*caa1* cells might result from increased neddylation. To test this hypothesis, we introduced plasmids that expressed wild-type and K713R Pcu1^{Myc} (*19*) into *caa1*$^{+}$ and Δ*caa1* strains and analyzed extracts from these cells by Western blotting with antibodies to Myc. The K713R (Lys^{713} → Arg) mutation completely abolished the Pcu1 hypermodification observed in Δ*caa1* cells (*20*) (Fig. 2B). In addition, an anti-HA immunoblot of Pcu1 isolated from wild-type and Δ*caa1* cells transformed with a plasmid that expressed HA$_3$-tagged *Brassica napus* Nedd8 (HA$_3$-Nedd8) confirmed that elevated amounts of neddylated Pcu1 were present in Δ*caa1* cells (Fig. 2C).

How does increased neddylation of Pcu1 in Δ*caa1* cells affect SCF function? The levels of Pcu1, the SKP1 homolog Psh1, and the HRT1 homolog Pip1 (*6*), as well as their assembly together into complexes (Fig. 3A), were normal in Δ*caa1* cells. In addition, no large change in Pcu1 localization was observed in Δ*caa1* cells by subcellular fractionation (Fig. 3B) or by immunofluorescence microscopy (*6*). Rel-

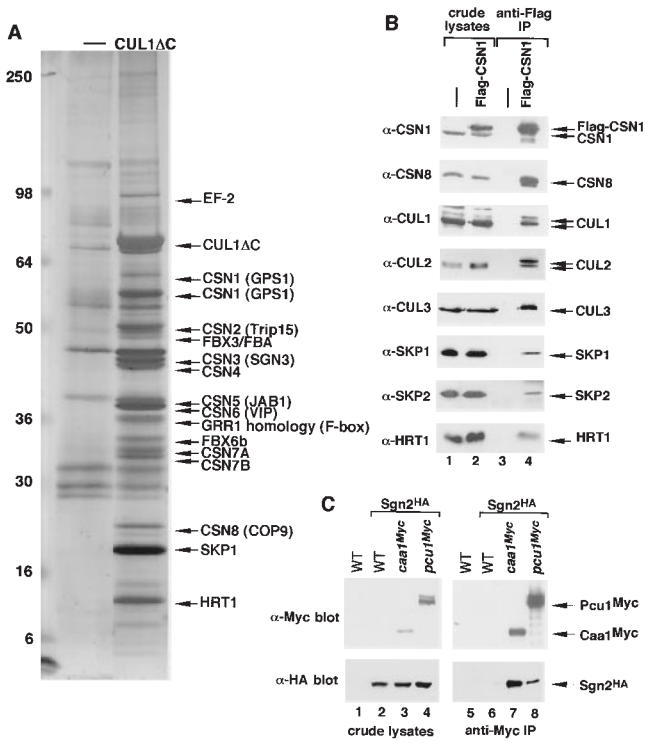


Fig. 1. COP9 signalosome (CSN) binds SCF. (**A**) Identification of SCF-interacting proteins by mass spectrometry. Mouse NIH 3T3 cells were infected with retroviruses that expressed Myc$_9$-TEV–tagged hCUL1ΔC. Cell extracts (500 mg) prepared from uninfected (left lane) cells and cells infected with CUL1ΔC (right lane) retroviruses were adsorbed to anti-Myc beads, eluted with TEV protease (Gibco BRL), and analyzed by SDS-PAGE (*6, 8*). Specific bands were excised from the gel, and protein identities were determined by mass spectrometry (*7*). (**B**) Association of cullins and SCF components with COP9 signalosome. HeLa cells were transiently transfected with vector alone (lanes 1 and 3) or with a vector that expressed Flag-tagged CSN1 subunit of COP9 signalosome (lanes 2 and 4). CSN1 (lanes 3 and 4) was 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 (30 μg) were evaluated in parallel (lanes 1 and 2). (**C**) 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} (*31*). Extracts prepared from these strains (*6*) were either evaluated directly (left panels) or were first immunoprecipitated (IP) with antibodies to Myc (right panels) before SDS-PAGE followed by Western blotting with antibodies to Myc and HA, as indicated.

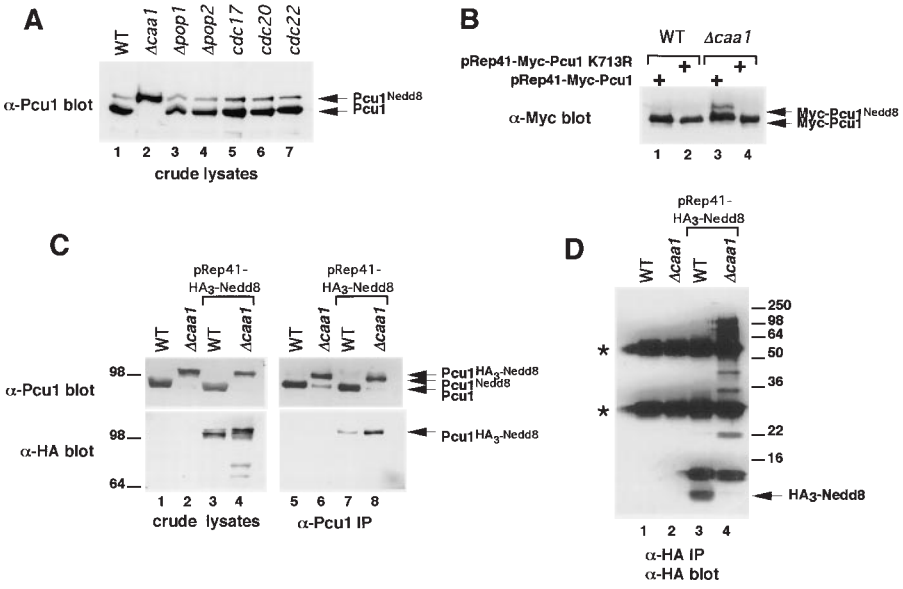
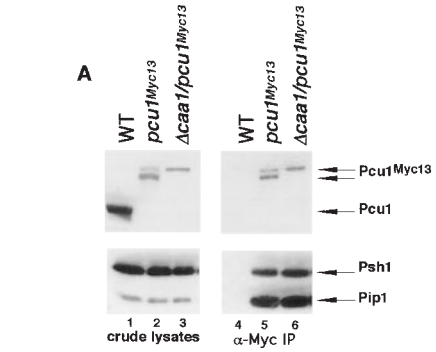


Fig. 2. Increased protein neddylation in Δ*caa1* cells. (**A**) A modified form of Pcu1 accumulates in Δ*caa1* cells. Extracts (*6*) from the indicated strains were probed with antibodies to Pcu1 to detect endogenous Pcu1. (**B**) Accumulation of modified Pcu1 in Δ*caa1* requires the Lys^{713} neddylation site. Wild-type and Δ*caa1* mutant cells were transformed with pRep41 plasmids (*31*) that expressed Myc-tagged wild-type or K713R mutant Pcu1 that lacks the neddylation site. Crude extracts from these strains (*6*) were probed with antibodies to Myc to detect Pcu1. (**C**) Δ*caa1* cells accumulate neddylated Pcu1. Wild-type and Δ*caa1* strains were transformed with a plasmid that expressed *Brassica napus* HA$_3$-Nedd8 from the inducible *nmt1*$^{+}$ promoter. HA$_3$-Nedd8 expression was induced for 12 hours (*31*). Crude lysates (lanes 1 to 4) and anti-Pcu1 immunoprecipitates (lanes 5 to 8) from wild-type and Δ*caa1* strains with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, and 6) the plasmid were immunoblotted with antibodies to Pcu1 (top panel) or HA (bottom panel) to detect Pcu1–HA$_3$-Nedd8 conjugates. (**D**) Accumulation of multiple Nedd8-modified species in Δ*caa1* cells. The strains described in (C) were harvested 12 hours after induction of the *nmt1*$^{+}$ promoter to minimize overexpression of HA$_3$-Nedd8, and lysates were sequentially immunoprecipitated and immunoblotted with antibodies to HA to detect HA$_3$-Nedd8 conjugation to cellular proteins. An asterisk indicates the position of the heavy and light antibody chains.

ative to Pcu1 isolated from wild-type cells, Pcu1 from Δ*caa1* cells exhibited two to four times as much ubiquitin ligase activity, consistent with its increased neddylation (Fig. 3C). However, no change in the half-life of the SCF substrate Rum1 was seen in Δ*caa1* cells (*21*). Substrate phosphorylation, rather than changes in SCF activity, may govern the kinetics of Rum1 degradation in *S. pombe*.



B

	WT	Δcaa1	Δpop2	cdc20
Cytoplasmic	31%	18%	42%	51%
Nuclear	69%	82%	58%	49%

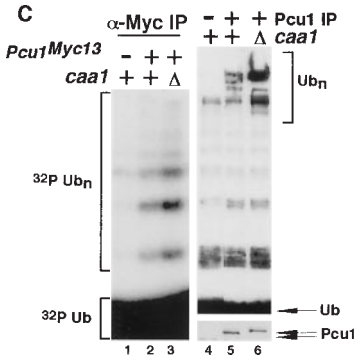


Fig. 3. SCF composition is unperturbed and SCF activity is slightly increased in Δ*caa1* mutant cells. (**A**) Steady-state levels of SCF subunits and assembly of SCF are normal in Δ*caa1* strains. SCF subunits were evaluated in crude lysates (lanes 1 to 3) or in anti-Myc immunoprecipitates (lanes 4 to 6) from *pcu1*$^{+}$, *pcu1 myc13*$^{+}$, or Δ*caa1 pcu1 myc13*$^{+}$ strains. Recovery of Pcu1^{Myc13} (top panel) and Psh1 plus Pip1 (bottom panel) in the immunoprecipitates was analyzed by Western blotting with the respective antibodies. (**B**) Pcu1 localization is normal in Δ*caa1* cells. Nuclear and cytoplasmic fractions (15 μg) of extracts derived from homogenized spheroplasts (*6*) were resolved by SDS-PAGE and analyzed by Western blotting with antibodies to Pcu1. Immunoblot analysis with specific antibodies confirmed that Mcm5 and pyruvate kinase were localized exclusively to the nuclear and cytoplasmic fractions, respectively. (**C**) Elevated Pcu1-associated ubiquitin ligase activity in Δ*caa1* strains. To measure the intrinsic ubiquitin ligase activity of Pcu1, we used an "IP-ubiquitination assay" (*32*). Lanes 1 to 3: SCF complexes were purified from wild-type and Δ*caa1* strains via the Myc epitope on endogenous Pcu1^{Myc} (*6*), washed, and incubated in the presence of ^{32}P-labeled ubiquitin (^{32}P-Ub), ATP, E1, and hCdc34 for 1 hour at 30°C (*32*). 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 to 6: SCF complexes immunopurified from wild-type and Δ*caa1* strains using antibodies to Pcu1 were processed as described above, except that conjugates of unlabeled ubiquitin (Ub$_n$) were detected by immunoblotting with anti-ubiquitin (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 antibodies to Pcu1 (bottom panel).

Because increased neddylation of Pcu1 was the only obvious effect of the Δ*caa1* mutation on SCF, we reasoned that CSN might directly govern the balance of activities that attach and remove (i.e., deneddylate) Nedd8 from cullins. To determine whether increased accumulation of Pcu1-Nedd8 conjugates in Δ*caa1* cells was accompanied by global deregulation of neddylation, we transformed *caa1*$^{+}$ and Δ*caa1* cells with a plasmid that expressed *B. napus* HA$_3$-Nedd8; anti-HA immunoprecipitates prepared from these cells were then immunoblotted with anti-HA (Fig. 2D). Little signal was detected in immunoprecipitates prepared from *caa1*$^{+}$ cells, whereas multiple HA$_3$-Nedd8–containing species accumulated in Δ*caa1* cells (Fig. 2D) (*22*). Among these is Pcu3, which accumulated as a neddylated species in multiple CSN-deficient mutants (*18*).

Next, we sought to determine whether Δ*caa1* cells accumulated an inhibitor of deneddylation or were deficient in deneddylating enzyme activity. To discriminate between these possibilities, we prepared extracts from *caa1*$^{+}$ *pcu1 myc13*$^{+}$ and Δ*caa1 pcu1*$^{+}$ strains, incubated them in the absence of adenosine triphosphate (ATP) to prevent neddylation, and monitored Pcu1 by Western blotting (Fig. 4B). Whereas neddylated Pcu1 was stable in Δ*caa1* extract, it was efficiently deneddylated upon mixing with *caa1*$^{+}$ extract, which suggests a deficit of deneddylating activity in Δ*caa1* cells. Remarkably, CSN purified from pig spleen (Fig. 4A) restored deneddylating activity to Δ*caa1* extract (Fig. 4B), suggesting that CSN either stimulated a cryptic deneddylating enzyme in Δ*caa1* cell extract or was tightly associated with a deneddylating activity. The latter possibility was supported by the observation that purified pig spleen CSN cleaved immunopurified Nedd8-Pcu1^{Myc13} conjugates (Fig. 4C). Pcu1 deneddylation promoted by CSN was sensitive to the alkylating agent *N*-ethylmaleimide (NEM) (Fig. 4B) (*23*), suggesting that cleavage of Nedd8 conjugates, like cleavage of ubiquitin and SUMO conjugates, is carried out by a cysteine protease. Further work will be required to identify the enzymatic subunit that mediates deneddylation.

CSN5/JAB1 is the only CSN ortholog found in *Saccharomyces cerevisiae*, and mutants that lacked this protein, which we call Rri1 (encoded by *YDL216c*), accumulated neddylated Cdc53 cullin (Fig. 4D). Thus, the role of JAB1-like proteins in deneddylation is highly conserved. JAB1 was originally described as a JUN activa-

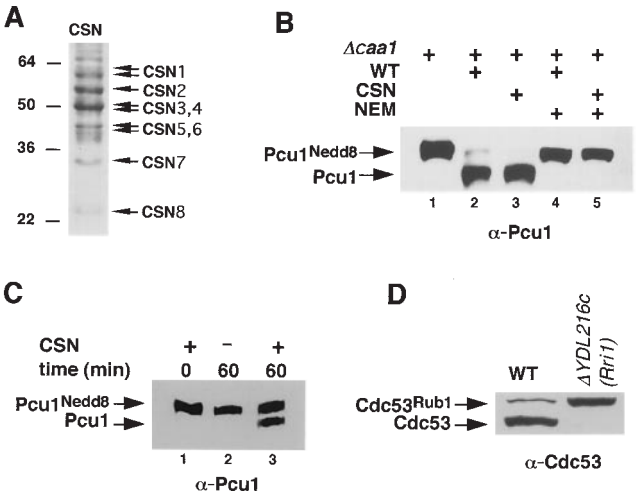


Fig. 4. COP9 signalosome has deneddylating activity. (**A**) Coomassie stain of CSN purified from pig spleen. CSN was purified as described (*33*). (**B**) *Schizosaccharomyces pombe* Δ*caa1* extract lacks deneddylating activity, but is complemented by purified pig CSN. Extract (40 μg) from Δ*caa1* cells (*6*) was incubated separately or in the presence of purified pig CSN (3 μg) or *pcu1 myc13*$^{+}$ cell extract (40 μg) for 30 min at 30°C in the presence or absence of 10 mM NEM, as indicated. Pcu1 neddylation was evaluated by SDS-PAGE followed by Western blotting with antibodies to Pcu1. (**C**) Purified Pcu1^{Myc13} is deneddylated by purified pig CSN. Pcu1^{Myc13} was immunoprecipitated from Δ*caa1* extracts using antibodies to Myc (*6*). 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* homolog *Rri1* in *S. cerevisiae* results in accumulation of a modified form of Cdc53. Total cell lysate from wild-type and *rri1*Δ strains was separated by SDS-PAGE and evaluated by Western blotting with antibodies to Cdc53.

tion domain binding protein and was shown to coactivate JUN-mediated gene expression (*24*). Recently, mammalian JAB1 was reported to bind a number of other proteins: The lutropin/choriogonadotropin receptor (*25*) and CDK inhibitor p27 (*26*) are targeted for degradation by JAB1, whereas signals propagated by the LFA1 integrin (*27*) and MIF cytokine (*28*) modulate JAB1 by an unknown mechanism(s). Our work suggests that a key function of the JAB1-containing CSN is to promote cleavage of NEDD8 from cullins and other NEDD8-modified proteins, and that the defects of CSN mutants arise from a failure to cleave NEDD8 from specific substrates. For example, failure to remove NEDD8 from COP1, HY5, or an affiliated protein may constitutively activate light-dependent gene expression in *A. thaliana* by blocking COP1-dependent turnover of the photomorphogenetic regulator HY5 (*29*).

The functional impact of CSN on its substrates is difficult to predict. NEDD8 attachment is essential in vivo (*19*) and promotes $SCF^{\beta\text{-}TRCP}$ activity in vitro (*3*), but conversely, diminished CSN function inhibits turnover of an SCF^{TIR1} target in *A. thaliana* (*30*). Thus, NEDD8 may need to be cyclically attached to and cleaved from CUL1 for optimal SCF function. Cycles of neddylation, like cycles of protein phosphorylation, may serve different functions in different contexts. Nonetheless, given the potentially large number of cullin-based ubiquitin ligases and the observed accumulation of additional NEDD8-modified proteins in Δ*caa1* cells, CSN is likely to modulate a broad range of biological processes.

References and Notes

1. R. J. Deshaies, *Annu. Rev. Cell Dev. Biol.* **15**, 435 (1999).
2. V. Podust *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4579 (2000).
3. M. Read *et al.*, *Mol. Cell. Biol.* **20**, 2326 (2000).
4. K. Wu, A. Chen, Z. Pan, *J. Biol. Chem.* **275**, 32317 (2000).
5. M. Morimoto, T. Nishida, R. Honda, H. Yasuda, *Biochem. Biophys. Res. Commun.* **270**, 1093 (2000).
6. Supplementary information is available at *Science* Online (www.sciencemag.org/cgi/content/full/1059780/DC1).
7. A. Shevchenko, M. Wilm, O. Vorm, M. Mann, *Anal. Chem.* **68**, 850 (1996).
8. J. H. Seol, A. Shevchenko, A. Shevchenko, R. J. Deshaies, *Nature Cell Biol.* **3**, 384 (2001).
9. A. Shevchenko, A. Loboda, W. Ens, K. G. Standing, *Anal. Chem.* **72**, 2132 (2000).
10. X. Deng *et al.*, *Trends Genet.* **16**, 202 (2000).
11. N. Wei, X. Deng, *Trends Genet.* **15**, 98 (1999).
12. N. Wei *et al.*, *Curr. Biol.* **8**, 919 (1998).
13. M. Seeger *et al.*, *FASEB J.* **12**, 469 (1998).
14. M. Glickman *et al.*, *Cell* **94**, 615 (1998).
15. S. E. Schwarz, unpublished data.
16. Animal and *A. thaliana* proteins are referred to as ABC (e.g., CUL1, NEDD8) and yeast and *B. napus* proteins are referred to as Abc (e.g., Pcu1, Nedd8) to conform to existing nomenclature and make it easier to discern the origin of proteins referred to in the text.
17. K. Mundt *et al.*, *Curr. Biol.* **9**, 1427 (1999).
18. C. Zhou, D. A. Wolf, unpublished data.
19. F. Osaka *et al.*, *EMBO J.* **19**, 3475 (2000).
20. The reduced level of neddylation observed for wild-type Pcu1 in Fig. 2B, as compared to Fig. 2A, most likely results from saturation of the neddylation pathway by overproduction of Pcu1 from the *nmt1*$^+$ promoter.
21. R. Verma, unpublished data.
22. We propose, on the basis of mononeddylation of cullins, that each band represents a discrete polypeptide (as opposed to multineddylated forms of a single protein).
23. G. A. Cope, R. J. Deshaies, unpublished data.
24. F. Claret, M. Hibi, S. Dhut, T. Toda, M. Karin, *Nature* **383**, 453 (1996).
25. S. Li, X. Liu, M. Ascoli, *J. Biol. Chem.* **275**, 13386 (2000).
26. K. Tomoda, Y. Kubota, J. Kato, *Nature* **398**, 160 (1999).
27. E. Bianchi *et al.*, *Nature* **404**, 617 (2000).
28. R. Kleemann *et al.*, *Nature* **408**, 211 (2000).
29. M. Osterlund, C. Hardtke, N. Wei, X. Deng, *Nature* **405**, 462 (2000).
30. C. Schwechheimer *et al.*, *Science* **291**, 1379 (2001).
31. Manipulations of *S. pombe* and indirect immunofluorescence were performed according to protocols published at www.bio.uva.nl/pombe/handbook.
32. S. Lyapina, C. Correll, E. Kipreos, R. Deshaies, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7451 (1998).
33. N. Wei, X. Deng, *Photochem. Photobiol.* **68**, 237 (1998).

34. We thank C. Lois, J Roberts, T. Caspari, A. Carr, S. Forsburg, J. Thorner, T. Toda, F. Osaka, Y. Xiong, and P. Nurse for generously providing retroviral vectors, *S. pombe* strains, expression plasmids, and antibodies; M. Petroski for ^{32}P-labeled ubiquitin; and R. Verma for experimental input, helpful discussions, and pointing out JAB1 homology to ubiquitin-specific proteases. We also thank P. Jackson and members of his laboratory for hosting this project in its early days. Supported by an Amgen fellowship (S.L.) and by the W. M. Keck Foundation and HHMI.

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Genetic Analysis of Digestive Physiology Using Fluorescent Phospholipid Reporters

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Zebrafish are a valuable model for mammalian lipid metabolism; larvae process lipids similarly through the intestine and hepatobiliary system and respond to drugs that block cholesterol synthesis in humans. After ingestion of fluorescently quenched phospholipids, endogenous lipase activity and rapid transport of cleavage products results in intense gall bladder fluorescence. Genetic screening identifies zebrafish mutants, such as *fat free*, that show normal digestive organ morphology but severely reduced phospholipid and cholesterol processing. Thus, fluorescent lipids provide a sensitive readout of lipid metabolism and are a powerful tool for identifying genes that mediate vertebrate digestive physiology.

To assay lipid metabolism in living larvae, we synthesized fluorescently tagged or quenched phospholipids (Figs. 1A and 2A). Such modified lipids are effective substrates for phospholipase A_2 (PLA_2) cleavage and sensitive reporters of enzymatic activity in vivo (*1, 2*). PLA_2 was targeted because of its importance in the generation of lipid signaling molecules, host defenses, lipid absorption, and cancer (*3–5*). Given the shared features of lipid processing in mammals and teleosts (*6, 7*), zebrafish mutagenesis screens using lipid reporters should identify genes relevant to human lipid metabolism and disease.

Reporters were constructed by covalently linking fluorescent moieties to sites adjoining the cleavage site of phospholipids. Dye-dye or dye-quencher interactions modify fluorescence without impeding enzyme-substrate interactions (*8*). PLA_2 cleavage results in immediate unquenching and detectable fluorescence. Quenched phospholipids [*N*-((6-(2,4-dinitro-

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