Cell

EXTENDED EXPERIMENTAL PROCEDURES

FRET Assay

Fluorimeter scans were performed on FluoroLog-3 (Jobin Yvon) in a buffer containing 30 mM Tris (pH 7.6), 100 mM NaCl, 0.5 mM DTT, and 1 mg/ml Ovalbumin (Sigma) in a volume of 250 μl. Mixtures were excited at 430 nm and the emissions were scanned from 450 nm to 650 nm. Stopped flow reactions were performed on a Kintek stopped flow machine in the same buffer as the fluorimeter scans.

Neddylation Assay

Cul1^{PKA}-Rbx1 was incubated with [γ-^{32}P] ATP (132 nM) and Protein Kinase A for 45 min at 30°C to make radiolabeled Cul1. Neddylation reactions contained ATP (2 mM), Nedd8 (15 μM), Nedd8 E1 (1 μM), Ubc12 (10 μM), and radiolabeled Cul1^{PKA}-Rbx1 (100 nM). Additional proteins were included as mentioned in the text. Reactions were performed and quenched in buffers previously described for ubiquitylation assays (Pierce et al., 2009). Reactions were analyzed by running on 10% gels, drying, and quantifying with a phosphor screen (Molecular Devices). Rapid kinetics were performed on a Kintek RQF-3 Rapid Quench Flow.

Ubiquitylation Assay

CycE was incubated with [γ-^{32}P] ATP (132 nM) and Protein Kinase A for 45 min at 30°C to make radiolabeled CycE. Ubiquitylation reactions contained ATP (2 mM), ubiquitin (60 μM), ubiquitin E1 (1 μM), Cdc34b (10 μM), Cul1-Rbx1 (150 nM), and Fbxw7-Skp1 (varying concentrations). Additional proteins were included as mentioned in the text. Reactions were performed and quenched in buffers previously described for ubiquitylation assays (Pierce et al., 2009). Reactions were analyzed by running on 16% gels, drying, and quantifying with a phosphor screen (Molecular Devices).

Cul1-Cand1 Dissociation Assay

Cul1 with N-terminal tagged GST-Rbx1 (0.1 μM) was preincubated with Cand1^{TAMRA} (0.1 μM) for 10 min and captured on glutathione sepharose 4B resin. Aliquots of resin were transferred to Micro Bio-Spin columns (Bio-Rad), resuspended in 1 μM of the indicated proteins, and incubated for 15 s or 5 min. Reactions were terminated by separation of beads and supernatant by centrifugation, and equivalent portions of each were fractionated by SDS-PAGE. Gels were scanned by a Typhoon scanner to quantify Cand1^{TAMRA}. To analyze the dissociation rate of the Cul1-Cand1 complex, at various time points following the addition of 1 μM Cand1 to 0.1 μM GST-Rbx1-Cul1-Cand1^{TAMRA}, an aliquot was withdrawn and incubated with glutathione sepharose 4B resin for 15 min and processed for SDS-PAGE analysis and fluorography. The gels were then stained by SilverQuest staining kit (invirogen) to detect the total Cand1 bands. The intensity of Cand1^{TAMRA} and total Cand1 band was measured with ImageJ (NIH), and the intensity of Cand1^{TAMRA} was normalized by the intensity of total Cand1 at each time point and was applied to single exponential fit in Prism.

Native PAGE Analysis

Protein samples containing 20 mM Tris (pH 8.0), 200 mM NaCl, 5 mM DTT, and 10% glycerol were fractionated on a native gel containing 0.1 M Tris (pH 8.8), 5% acrylamide, and 5% glycerol in running buffer containing 189 mM glycine and 25 mM Tris (pH 8.8) for 3 hr with 100 V power supplied. Gels were scanned by a Typhoon scanner to detect Cand1^{TAMRA}.

Expression Constructs

To produce recombinant dimeric Fbxw7 protein from insect cells, we generated a pFastBac-GST-TEV construct in which the TEV sequence was immediately followed by a GlyAlaGlySer linker followed by Fbxw7 coding sequence, starting with proline 63 of the common region of Fbxw7. This construct contains the Fbxw7 dimerization domain and was selected based on structure prediction analyses. Gel filtration on Superdex200 confirmed that following cleavage with TEV, the Skp1-Fbxw7$^{69–707}$ complex migrated as an ~160 kD dimer. RDB 2726 (pVL1393 GST TEV site Fbxw7$^{69–707}$) The GST TEV Fbxw7$^{230–707}$ sequences were transferred from pFastBac to pVL1393 to generate RDB 2726. RDB 2727 (pVL1393 GST TEV site Fbxw7$^{230–707}$ Sortase tag) was cloned by appending the sequence encoding LPETGGHHHHHH to the extreme 3′ end of the coding region for Fbxw7$^{230–707}$ in RDB 2726 by PCR. RDB 2725 (pVL1393 human wild-type Skp1) was cloned from a plasmid (Brenda Schulman) into pVL1393 by standard methods. RDB 2396 (pAcyc184 ^{CFP}Cul1 NTD) was created by inserting CFP coding sequences into the extreme 5′ end of the region coding for Cul1 NTD in RDB 2080. RDB 2511 (pCool Cul1^{PKA} CTD-GST Thrombin site Rbx1) was cloned by fusing DNA sequences encoding RRGSL to the extreme 3′ end of the DNA coding for Cul1 in RDB 2081. RDB 2562 (pGEX4T-2 GST Thrombin site Cand1) was cloned from a plasmid (Ning Zheng) by PCR and inserted into pGEX4T-2 by standard cloning. The FLAG-Cry1 expression construct were generous gifts from the Pagano lab.

Protein Purification

Ubiquitin was purchased from Boston Biochem. CycE and GGGGK-TAMRA were purchased from New England Peptide. Protein Kinase A was purchased from New England Biolabs. Human Uba1, UbcH3b (Cdc34b), APPBP1-Uba3, Ubc12, Nedd8, Cul1-Rbx1, neddylated Cul1-Rbx1, Skp2-Skp1, and β-TrCP$^{139–569}$-Skp1ΔΔ were purified as described (Saha and Deshaies, 2008). Sortase SrtA ΔN59 was purified as previously described (Popp et al., 2009). ^{CFP}Cul1-Rbx1 was expressed by cotransforming

BL21 *E. coli* with RDB 2396 and RDB 2081 and inducing overnight at 16°C. ^{CFP}Cul1-Rbx1 was purified on glutathione resin followed by digestion with thrombin and chromatography on Mono S cation exchange and S200 resins. Neddylated ^{CFP}Cul1-Rbx1 was made as previously described for neddylated Cul1-Rbx1 (Saha and Deshaies, 2008). Cul1^{PKA}-Rbx1 was expressed by cotransforming BL21 *E. coli* with RDB 2080 and RDB 2511 and inducing overnight at 16°C. Cul1^{PKA}-Rbx1 was purified on glutathione resin, thrombin digest, Mono S cation exchange, and S200. Fbxw7-Skp1 was expressed in Hi5 cells from viruses made from RDB 2726 and RDB 2725 at an ROI of 1:2 for 48 hr. Fbxw7-Skp1 was purified on glutathione resin followed by digestion with TEV protease and chromatography on Mono Q anion exchange and S200 columns. Fbxw7-Skp1ΔΔ was expressed in Hi5 cells from a virus made from RDB 2726 and a previously described virus (Saha and Deshaies, 2008) and was purified as described for Fbxw7-Skp1. Fbxw7$^{Sortase\ Tag}$-Skp1 was expressed in Hi5 cells from viruses made from RDB 2727 and RDB 2725 at a ROI of 1:2 for 48 hr. Fbxw7$^{Sortase\ Tag}$-Skp1 was purified purified as described for Fbxw7-Skp1, omitting the S200 step. Fbxw7$^{Sortase\ Tag}$-Skp1 was incubated with 60 μM Sortase SrtA ΔN59 and 250 μM GGGGK-TAMRA at 30°C for 24 hr in a buffer containing 10 mM $CaCl_2$. Fbxw7^{TAMRA} was further purified on S200. β-TrCP$^{139–569}$-Skp1 was expressed in Hi5 cells from a virus made from RDB 2725 and a previously described virus (Saha and Deshaies, 2008). β-TrCP$^{139–569}$-Skp1 was purified on glutathione resin followed by digestion with thrombin and chromatography on Mono Q anion exchange and S200 resins. Cand1 was expressed by transforming BL21 *E. coli* with RDB 2562 and inducing overnight at 16°C. Cand1 was purified on glutathione resin, followed by digestion with thrombin and chromatography on Mono Q anion exchange and S200 resins.

Kinetic Analysis

Regressions were performed in Matlab.

Construction of Stable Cell Lines

Targeted integration of ^{FLAG}Cul1 coding sequence into the Flip-In T-REx 293 cells was carried out as described previously (Lee et al., 2011). To construct a cell line depleted of Cand1, Hek-293T cells in a 100 mm cell culture dish were cotransfected with four plasmids to produce ecotropic retroviruses: 0.5 μg pCAGG-HIVgpco (gagpol), 0.5 μg pCAG4-RTR2 (rev-tat), 0.5 μg pHDM.G (vesicular stomatitis virus glycoprotein G, VSV-G), and 3.5 μg pGIPZ vector encoding shCand1 sequences (Open Biosystems, Cat #: RHS4531-NM_018448). Exponentially growing Tet-inducible ^{FLAG}Cul1 cells (2×10^5 cells in 100 mm diameter culture dish) were infected with 2 ml ecotropic virus-containing supernatant five times at 4 hr intervals. Infected cells were selected with 1 μg/ml puromycin, and a cell line that displayed the most profound Cand1 depletion was used for further experiments. ''Heavy'' and ''light''-labeled cultures of the stable cell lines described above were grown as described previously (Lee et al., 2011).

Immunoblotting

Cells were lysed with 50 mM HEPES, pH 7.5, 5 mM $Mg(OAc)_2$, 70 mM KOAc, 0.2% n-dodecyl-D-maltoside) containing protease inhibitors (Roche) Cleared lysates were immunoprecipitated for 1 hr with M2-agarose anti-FLAG beads (Sigma), and the beads were washed four times with lysis buffer. Recovered proteins were eluted from the beads by boiling in 2 × Laemmli sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membrane (Millipore). Proteins were detected using antibodies to Cul1 (Invitrogen, Cat#: 322400), Cand1 (Santa Cruz Biotechnology, Cat#: sc-10672), Skp2 (Invitrogen, Cat#: 323300), Rbx1 (Invitrogen, Cat#: 342500), Csn1 (Biomol, Cat#: PW8285), Csn4 (Biomol, Cat#: PW8360), Nedd8 (Chemicon International, Cat#: AB3870), and Skp1(Santa Cruz Biotech, Cat#: sc-1568). Antibodies against Fbxo11 and Fbxl3, were generous gifts from the Pagano lab. Serum against Fbxo9 was from the Bassermann lab.

Targeted Mass Spectrometric Analyses, PseudoMRM

Peptides diagnostic for specific F box proteins were selected for pseudoMRM based on both uniqueness to the protein of interest and previous mass spectrometric observations (either in public spectral libraries or our prior experiments). To quantify the targeted peptides, we prepared and fractionated whole-cell lysates on a 160 min gradient as described previously (Wiśniewski et al., 2009). The Orbitrap Elite (Thermo Fisher Scientific, San Jose, CA) was set to acquire continuously MS/MS data of predetermined sets of peptide ions listed in Supplemental Data. Per analysis, two segments (75 and 84 min) targeting ~30 precursor ions were used. At least two precursor masses were selected for each protein quantified. The isolation width was 1 Da, and normalized collision energy was 35%. The target value for automatic gain control was set to 5×10^3 with a maximal ion injection time of 10 ms and 1 microscan per MS/MS scan. A typical duty cycle was 3–4 s.

Data Analysis of PseudoMRM

Raw files were processed in Skyline (v. 1.3.0.3871) (MacLean et al., 2010). All peaks were manually curated. Peak areas were extracted in a report from Skyline. The protein ratio was based on the mean of the overall peptide ratios. The overall peptide ratios were based on the mean of the peptide ratios in individual experiments. The peptide ratios within an experiment were based on the mean of the ratios within each fractionation. To estimate the standard error of the protein ratio, a bootstrap analysis similar to that performed for the pull-down experiments was performed, where a pooled variance was calculated for SILAC pairs of fragment ions within the same MS analysis and each hierarchical level was sampled with replacement. For the analysis to determine the effect

of Cand1 depletion on total F box levels, three peptides each from Beta-actin, Alpha-tubulin 3, and Glyceraldehyde-3-phosphate dehydrogenase were used as loading controls to normalize the SILAC ratios in each experiment.

SUPPLEMENTAL REFERENCES

MacLean, B., Tomazela, D.M., Shulman, N., Chambers, M., Finney, G.L., Frewen, B., Kern, R., Tabb, D.L., Liebler, D.C., and MacCoss, M.J. (2010). Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics *26*, 966–968.

Wiśniewski, J.R., Zougman, A., and Mann, M. (2009). Combination of FASP and StageTip-based fractionation allows in-depth analysis of the hippocampal membrane proteome. J. Proteome Res. *8*, 5674–5678.

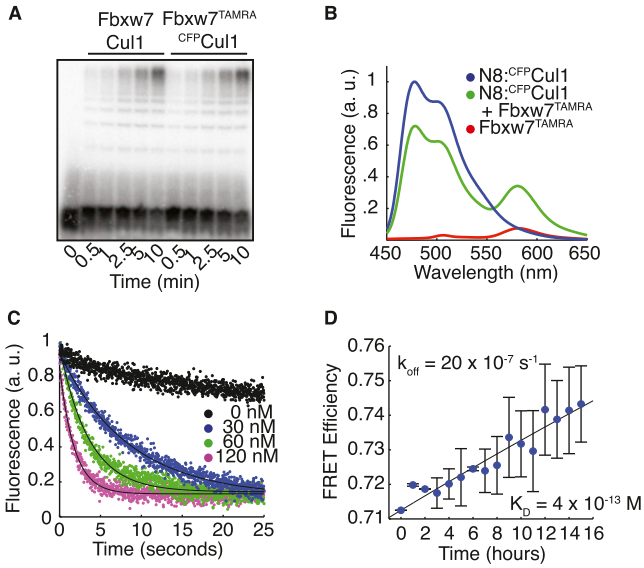

Figure S1. Neddylation Does Affect Dyamics of SCF^{Fbxw7} Assembly, Related to Figure 1

(A) 150 nM Cul1-Rbx1 or ^{CFP}Cul1-Rbx1was preincubated with 150 nM Fbxw7-Skp1 or Fbxw7^{TAMRA}-Skp1 and 600 nM radiolabeled CycE for 5 min. 2 mM ATP, 60 μM ubiquitin, 1 μM ubiquitin E1, and 10 μM Cdc34b were preincubated for 2 min and added to the mix. Samples were quenched at the indicated times and analyzed by SDS-PAGE followed by phosphorimaging.

(B) As in Figure 1A except with neddylated Cul1.

(C) As in Figure 1B except with neddylated Cul1.

(D) As in Figure 1E except with neddylated Cul1. Error bars, ±SD, n = 3.

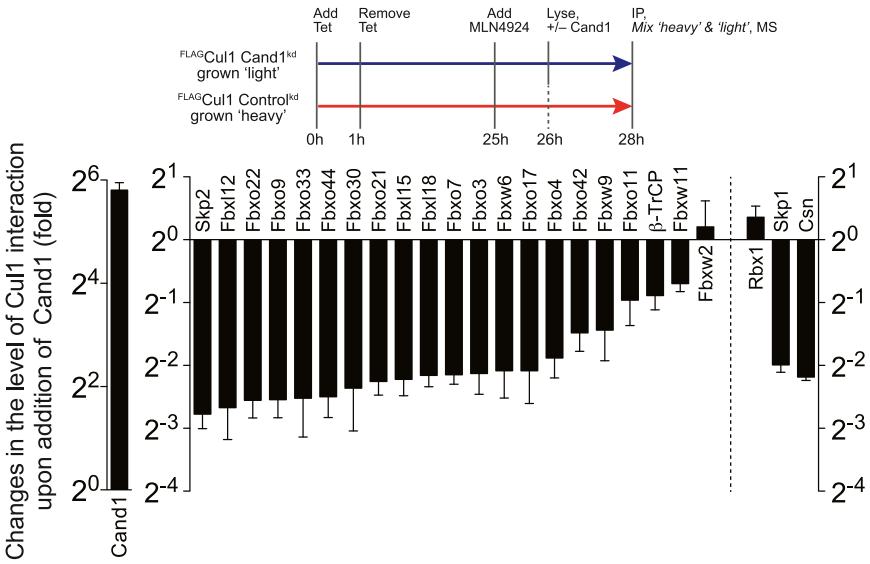

Figure S2. Addition of Recombinant CAND1 to CAND1-Depleted Lysates Reduces F Boxes Bound to Cul1, Related to Figure 2

Tet-^{FLAG}Cul1 Cand1^{kd} cell cultures were grown in medium formulated with isotopically "heavy" or "light" lysine and arginine. The cells were simultaneously treated with 1 μg/ml tetracycline for 1 hr to induce a pulse of ^{FLAG}Cul1 synthesis, and then returned to tetracycline-free medium for a further 24 hr. Both cultures were then treated with 1 μM MLN4924 for 1 hr. The idea behind this manipulation was to minimize neddylation, which should maximize the sensitivity of SCF complexes to disassembly by Cand1. Cells were then lysed and the lysate from light-labeled cells was supplemented with 9.6 μg Cand1 per mg lysate, whereas the "heavy" lysate was mock treated. After 2 hr at 23°C, ^{FLAG}Cul1 was immunoprecipitated and the "light" and "heavy" samples were combined for analysis by mass spectrometry as described in Methods. The isotopic ratio for Cul1 (1.04) was set to 1.0 and all other proteins were normalized to it. The normalized median values for light/heavy ratios for all sequenced peptides of a given protein are shown. All proteins shown were represented by a minimum of 2 quantified peptides. For CSN, all eight subunits were identified by at least 7 peptides, and the average ratio for all identifications is presented. The ratio for Cand1 was 52, consistent with recruitment of a large amount of recombinant Cand1 relative to the endogenous Cand1 present in ^{FLAG}Cul1 immunoprecipitates from Cand1-depleted cells. Error bars represent the standard errors of the overall protein group ratios, calculated from the two biological replicates using bootstrap analysis. In the first biological replicate the labeling was done as indicated, and in the second the labeling (and ratio calculation) was reversed. See also Table S3.

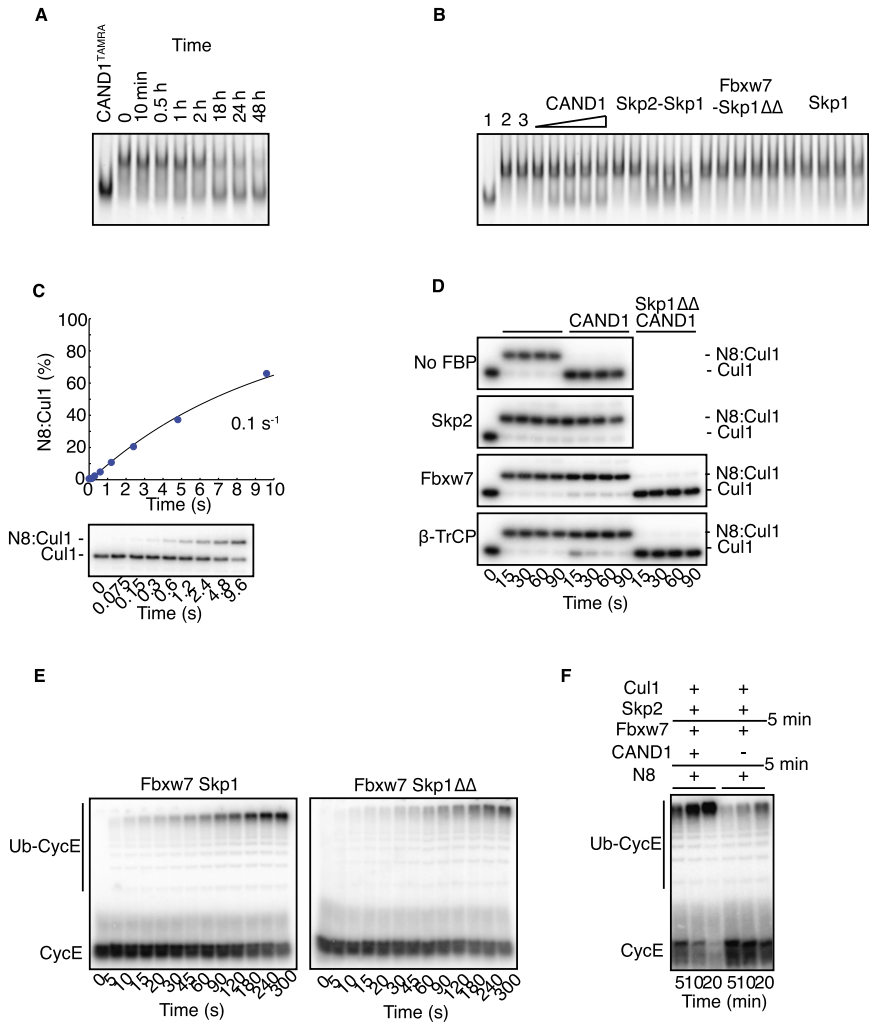

Figure S3. Gel Shift and Neddylation Assays Reveal that Fboxes Can Remove CAND1 from Cul1, Related to Figure 3

(A) 100 nM Rbx1-Cul1-Cand1^{TAMRA} was supplemented with 1 μM Cand1 for indicated time periods and was fractionated by native PAGE. The first lane contains 100 nM Cand1^{TAMRA} standard.

(B) 100 nM Rbx1-Cul1-Cand1^{TAMRA} was supplemented with indicated proteins at concentrations of 50 nM, 100 nM, 200 nM, 300 nM, 400 nM, respectively, and samples were immediately fractionated by native PAGE. Lane 1: 100 nM Cand1^{TAMRA}. Lane 2: 100 nM Rbx1-Cul1-Cand1^{TAMRA}. Lane 3: 100 nM Rbx1-Cul1-Cand1^{TAMRA} supplemented with buffer.

(C) 100 nM radiolabeled Cul1^{PKA}-Rbx1 was supplemented with a preincubated (2 min) mixture containing 2 mM ATP, 15 μM Nedd8, 300 nM F-box-Skp1, 1 μM Nedd8 E1, and 10 μM Ubc12. At the indicated times aliquots were withdrawn and quenched for subsequent evaluation by SDS-PAGE and phosphorimaging.

(D) 100 nM radiolabeled Cul1^{PKA}-Rbx1 was preincubated with 300 nM Cand1. Reactions were started with addition of 2 mM ATP, 15 μM Nedd8, 300 nM Fboxes-Skp1, 1 μM Nedd8 E1, and 10 μM Ubc12. Reactions were evaluated as in (C).

(E) As in Figure S1A except with either 150 nM Fbxw7-Skp1 or 150 nM Fbxw7-Skp1ΔΔ.

(F) 150 nM Cul1-Rbx1 was preincubated with 150 nM Skp2-Skp1 for 5 min then mixed with 150 nM Fbxw7-Skp1, 600 nM radiolabeled CycE and either buffer or 200 nM Cand1. After 5 min, 2 mM ATP, 15 μM Nedd8, 1 μM Nedd8 E1, and 10 μM Ubc12 were added to the mix. After 5 min, preincubated 2 mM ATP, 60 μM ubiquitin, 1 μM ubiquitin E1, and 10 μM Cdc34b were added to the mix.

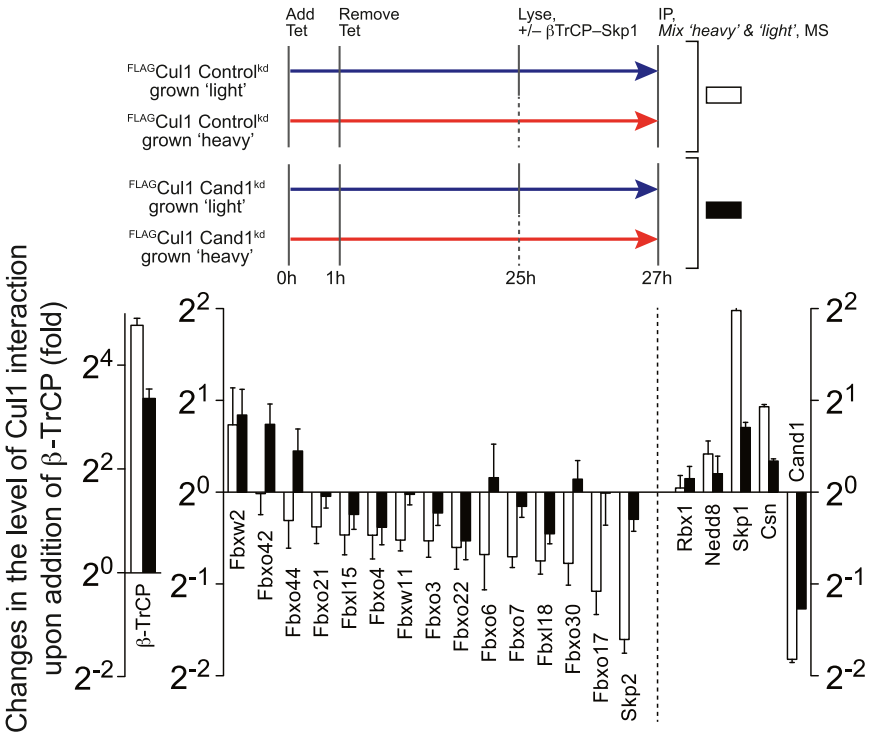

Figure S4. CAND1 Acts as an F box Exchange Factor in the Presence of Many SCF Complexes, Related to Figure 4

Similar to Figure S2, except that four parallel cultures were grown: two cultures of Tet-^{FLAG}Cul1 Cand1^{kd} with one labeled heavy and the other light, and two cultures of Tet-^{FLAG}Cul1 control^{kd} cells, one labeled heavy and the other light. The cells were not treated with MLN4924 prior to lysis. Light-labeled lysates from control and Cand1^{kd} cells were supplemented with 5.0 μg β-TrCP-Skp1 per mg lysate, whereas heavy-labeled lysates were mock-supplemented. Following a 2 hr incubation at 23°C, ^{FLAG}Cul1 was immunoprecipitated from each sample and the Cand1^{kd} samples were mixed together and processed for mass spectrometry and data analysis as in (A), and the same was done for the control^{kd} samples. The prenormalization isotopic ratios for Cul1 were 1.0 for the Cand1^{kd} samples and 0.98 for control^{kd} samples. The results for β-TrCP were plotted on a different scale because of the magnitude of signal, most of which is likely to be due to recruitment of "light" recombinant protein. F boxes are shown to the left of the dotted line and other Cul1 interactors are shown to the right. Error bars and replicates are as described in Figure S2. See also Table S4.

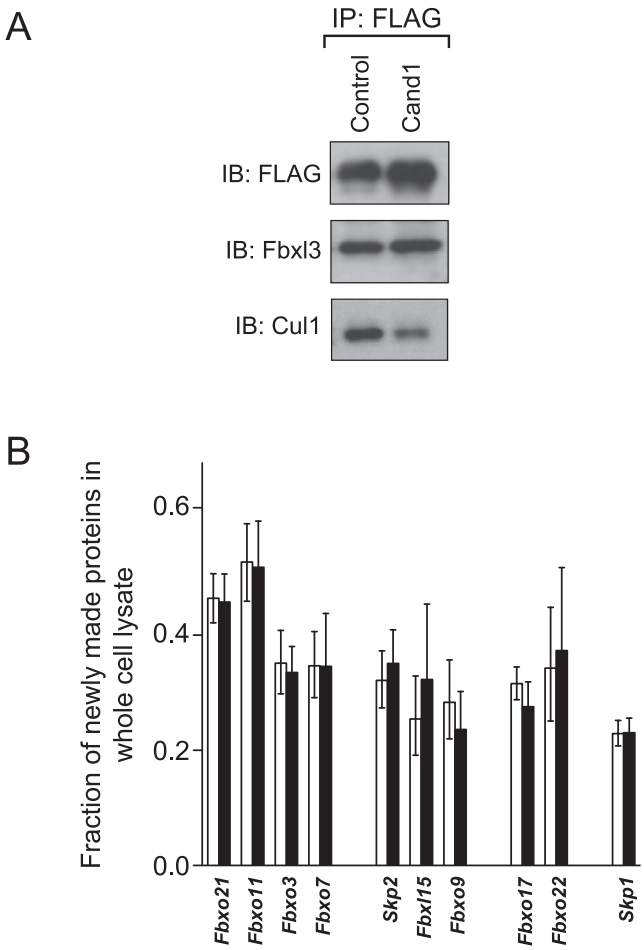

Figure S5. Newly Made Proteins in Whole-Cell Lysate, Related to Figure 5

(A) The lysates from cells infected with lentivirus containing control or Cand1 shRNA that were used for the experiment in Figure 5C were immunoprecipitated with anti-FLAG and then immunoblotted with antibodies against FLAG, Fbxl3, and Cul1. Note that the amount of Cul1 in the IP from Cand1-depleted cells was reduced, even though the amount of Cry1 was slightly greater.

(B) The same cells used in Figure 5D were grown in isotopically light lysine plus arginine, induced with 1 μg/ml tetracycline for 1 hr at t = 0 hr, treated with 5 μM epoxomicin at t = 48 hr, shifted to isotopically heavy lysine plus arginine at t = 49 hr, and lysed at t = 61 hr in 1 μM MLN4924 and 2 mM o-phenanthroline. PseudoMRM was used to measure the fraction of heavy-labeled species for 9 observable F box proteins in total cell lysate from Cand1-depleted (black bars) and control (white bars) cells. To estimate the standard error of the protein ratio, a bootstrap analysis was performed, where a pooled variance was calculated for SILAC pairs of fragment ions within the same MS analysis and each hierarchical level was sampled with replacement. See also Table S5.