THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 45, pp. 32766–32776, November 8, 2013

Substrate Binding Promotes Formation of the Skp1-Cul1-Fbxl3 (SCF^{Fbxl3}) Protein Complex*

Received for publication, August 16, 2013, and in revised form, September 25, 2013 Published, JBC Papers in Press, September 30, 2013, DOI 10.1074/jbc.M113.511303

Kanae Yumimoto, Tetsuya Muneoka, Tomohiro Tsuboi, and Keiichi I. Nakayama[1]

From the Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan and CREST, Japan Science and Technology Agency (JST), Kawaguchi, Saitama 332-0012, Japan

Background: The Skp1-Cul1-Fbxl3 (SCF^{Fbxl3}) complex regulates oscillation of the circadian clock by targeting Cry proteins for degradation.
Results: Fbxl3 interacts with Skp1 and Cul1 only in the presence of its substrate Cry1 *in vivo*.
Conclusion: Association of Cry1 with Fbxl3 is essential for SCF^{Fbxl3} complex formation *in vivo*.
Significance: Our findings provide important insight into the regulation of SCF ubiquitin ligase activity and circadian rhythmicity.

The Skp1–Cul1–F-box protein (SCF) complex is one of the most well characterized types of ubiquitin ligase (E3), with the E3 activity of the complex being regulated in part at the level of complex formation. Fbxl3 is an F-box protein that is responsible for the ubiquitylation and consequent degradation of cryptochromes (Crys) and thus regulates oscillation of the circadian clock. Here we show that formation of the SCF^{Fbxl3} complex is regulated by substrate binding *in vivo*. Fbxl3 did not associate with Skp1 and Cul1 to a substantial extent in transfected mammalian cells. Unexpectedly, however, formation of the SCF^{Fbxl3} complex was markedly promoted by forced expression of its substrate Cry1 in these cells. A mutant form of Fbxl3 that does not bind to Cry1 was unable to form an SCF complex, suggesting that interaction of Cry1 with Fbxl3 is essential for formation of SCF^{Fbxl3}. In contrast, recombinant Fbxl3 associated with recombinant Skp1 and Cul1 *in vitro* even in the absence of recombinant Cry1. Domain-swap analysis revealed that the COOH-terminal leucine-rich repeat domain of Fbxl3 attenuates the interaction of Skp1, suggesting that a yet unknown protein associated with the COOH-terminal domain of Fbxl3 and inhibited SCF complex formation. Our results thus provide important insight into the regulation of both SCF ubiquitin ligase activity and circadian rhythmicity.

The ubiquitin-proteasome system for protein degradation plays an essential role in various key biological processes, including cell cycle progression, gene transcription, and signal transduction. The ubiquitylation of specific substrates is mediated by three enzymes that act in concert: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (1, 2). E3 ubiquitin ligases confer specificity to ubiquitylation by recognizing target substrates and mediating transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to the substrate. The largest known class of E3s is the cullin-based subfamily of enzymes, which includes the Skp1–Cul1–F-box protein (SCF)[2] complex (3–5). Each SCF complex consists of four subunits: Skp1, Cul1, Rbx1 (also known as Roc1 or Hrt1), and an F-box protein. Cul1 serves as a scaffold that interacts via its COOH terminus with the RING finger protein Rbx1 to recruit an E2 and via its NH_2 terminus with Skp1. F-box proteins, which interact through the F-box motif with Skp1 and Cul1, are responsible for substrate recognition, with each such protein recognizing a different group of substrates. Approximately 70 F-box proteins have been identified in humans (6), and they fall into three categories based on the structure of the substrate-associated region: those with WD40 repeats (Fbxw), those with leucine-rich repeats (LRRs) (Fbxl), and those with other domains (Fbxo).

The activity of SCF-type ubiquitin ligases is controlled at the level of complex formation (7–9). CAND1, a cullin-binding protein, sequesters Cul1 by preventing the binding of Skp1 and F-box proteins, resulting in inactivation of E3 activity *in vitro* (10, 11). CAND1 was recently found to stimulate SCF activity *in vivo*, however, by enabling an F-box protein–Skp1 complex to access Cul1 that was previously occupied by a different F-box protein–Skp1 complex (12). Covalent attachment of the ubiquitin-like protein Nedd8 to a lysine residue in the COOH-terminal domain of Cul1 prevents the binding of CAND1 and thereby stimulates ubiquitylation activity (10, 11, 13, 14). Some F-box proteins form a homodimer through interaction of the conserved D-domain, which is required for the SCF complex to exert E3 ubiquitin ligase activity toward substrate (15–17). In addition, autoubiquitylation of F-box proteins by the corresponding SCF complex might serve as a homeostatic mechanism whereby bound substrate prevents an F-box protein from undergoing ubiquitylation, resulting in accumulation of the F-box protein only when its substrate is present (18, 19).

* This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
[1] To whom correspondence should be addressed: Dept. of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan. Tel.: 81-92-642-6815; Fax: 81-92-642-6819; E-mail: nakayak1@bioreg.kyushu-u.ac.jp.

[2] The abbreviations used are: SCF, Skp1–Cul1–F-box protein; Cry, cryptochrome; LRR, leucine-rich repeat; NAE, Nedd8-activating enzyme; Per, period.

Several F-box proteins regulate E3 activity in response to changes in the intracellular environment. For example, Fbxl5 senses the levels of cellular iron and oxygen through direct binding to these cellular components via its NH_2-terminal hemerythrin-like domain. Fbxl5 becomes labile when the supply of iron or oxygen is limited, whereas it is stabilized under iron-replete conditions that induce degradation of the iron-regulatory protein IRP2 by SCF^{Fbxl5} (20, 21). Fbxo31 is also stabilized as a result of phosphorylation by the kinase ATM in response to DNA damage. Stabilized Fbxo31 targets cyclin D1 for degradation by the proteasome, resulting in arrest of the cell cycle in G_1 phase (22). In plants, the F-box proteins TIR1 and COI1 are receptors of auxin and jasmonate, respectively (23, 24). These plant hormones fill a cavity in the substrate binding site of the corresponding F-box protein, thereby creating additional molecular surfaces that stabilize the binding of substrates.

The circadian clock of cells is tightly regulated by an internal transcription-translation-degradation cycle that is tuned to generate a circadian rhythm of ~24 h. Period (Per1, Per2, Per3) and cryptochrome (Cry1, Cry2) are core transcriptional factors of the mammalian clock that repress transcription of their own genes by interacting with the CLOCK-BMAL1 heterodimer, which constitutes the central machinery of circadian oscillation (25, 26). The levels of Per and Cry are also controlled by proteolysis mediated by the ubiquitin-proteasome system. β-TrCP (also known as Fbxw1 or Fbxw11) targets Per for degradation in a manner dependent on Per phosphorylation by casein kinase (27). Fbxl3 is thought to be responsible for the ubiquitin-dependent degradation of Cry (28–30). Forward-genetic mutagenesis screens revealed that mutant mice designated *overtime* and *afterhours* maintained a circadian period of ~26 and ~27 h in the dark, respectively (29, 30). Both mutations were mapped to the LRR region of Fbxl3 and were found to disrupt the interaction of Fbxl3 with Cry. Cry proteins are stabilized, and the repression of CLOCK-BMAL1 activity is enhanced in these mutants. These observations thus suggested that Fbxl3 is essential for directing Cry degradation during the circadian cycle.

Here we show that formation of an SCF complex by Fbxl3 is regulated by a novel mechanism. Fbxl3 was thus found to interact with Skp1 and Cul1 in a manner dependent on its association with substrate. A yet unknown protein associated with the COOH-terminal domain of Fbxl3 and inhibited SCF complex formation. This mechanism is unique to Fbxl3 among F-box proteins examined. Our data thus provide new insight into the regulation of SCF ubiquitin ligase activity and circadian rhythmicity.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa, HEK293T, and Neuro2A cells were cultured under an atmosphere of 5% CO_2 at 37 °C in DMEM (Invitrogen) supplemented with FBS (10%), sodium pyruvate (1 mM), L-glutamine (2 mM), 2-mercaptoethanol (50 μM), nonessential amino acids (10 ml/liter; Invitrogen), penicillin (100 units/ml), and streptomycin (100 mg/ml). Where indicated, the cells were treated with a Nedd8-activating enzyme (NAE) inhibitor (1 μM, Lifesensors) or the proteasome inhibitor MG132 (10 mM, Peptide Institute).

Construction of Expression Plasmids and Cell Transfection—Complementary DNAs encoding WT or mutant forms of FLAG epitope-tagged human Fbxl3, mouse Skp2, mouse Fbxw7α, or mouse Fbxo31; HA epitope-tagged human Cry1, human Cry2, mouse p27, or the intracellular domain of mouse Notch1; and Myc epitope-tagged mouse cyclin D1 were subcloned into the pcDNA3 vector (Invitrogen). Those encoding WT forms of mouse Fbxl3 or mouse Fbxl21 were subcloned into p3×FLAG (Sigma), whereas cDNAs encoding WT or mutant forms of HA-tagged mouse Cul1 were subcloned into pS-neo (31). The various vectors were introduced into HeLa cells with the use of the FuGENE HD transfection reagent (Roche Applied Science).

Antibodies—Antibodies to Skp1, to GAPDH (loading control), and to Hsp90 (loading control) were obtained from BD Biosciences. Those to Cul1 were from Invitrogen. Those to the HA epitope (HA11) were from Babco. Those to the Myc epitope (9E10) were from Roche Applied Science. Those to the FLAG epitope (M2) were from Sigma.

Immunoprecipitation and Immunoblot Analysis—Cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 10 μM NaF, 10 μM $Na_4P_2O_7$, 0.4 μM Na_3VO_4, 0.4 μM EDTA, leupeptin (20 μg/ml), aprotinin (10 μg/ml), and 1 mM PMSF. The lysates were centrifuged at 20,400 × *g* for 20 min at 4 °C to remove debris, and the resulting supernatants were incubated for 1 h at 4 °C with beads conjugated with M2 antibodies to FLAG (Sigma). The beads were isolated by centrifugation and washed three times with a wash buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100, after which the bead-bound proteins were eluted with the FLAG peptide (500 μg/ml), fractionated by SDS-PAGE, transferred to a Hybond P membrane (Amersham Biosciences), and subjected to immunoblot analysis. Immune complexes were detected with Supersignal West Pico or West Dura chemiluminescence reagents (Pierce) and were quantified with the use of ImageJ software (National Institutes of Health).

Gel Filtration Chromatography—FLAG-Fbxl3 immunoprecipitates prepared from HeLa cells as described above were fractionated on a Superose-6 HR 10/300 column (Amersham Biosciences) that had been equilibrated with the wash buffer described above. Fractions of 500 μl were collected for a total column volume and were subjected to immunoblot analysis. The column was routinely calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), and ovalbumin (43 kDa) as standards.

In Vitro Pulldown Assay—Recombinant His_6-Myc-tagged mouse Cul1 and mouse Skp1, His_6-FLAG-tagged human Fbxl3, and His_6-HA-tagged human Cry1 were expressed in and purified from Sf21 insect cells with the use of the Bac-to-Bac system (Invitrogen). Sf21 cells were infected with the recombinant viruses for 72 h, and each expressed protein was purified from the soluble fraction of cell lysates with nickel-nitrilotriacetic acid-agarose and subsequently eluted with 200 mM imidazole. His_6-FLAG-Fbxl3 (3 μg) was incubated for 1 h at 4 °C with 10 μl of bead-conjugated M2 antibodies to FLAG (Sigma) in the lysis buffer described above. Test proteins (1 μg) were then added, and the binding mixtures were incubated for 1 h at 4 °C. The beads were then isolated, washed three times with 1 ml of ice-cold wash buffer, resuspended in 20 μl of SDS sample buffer, and boiled before SDS-PAGE and immunoblot analysis.

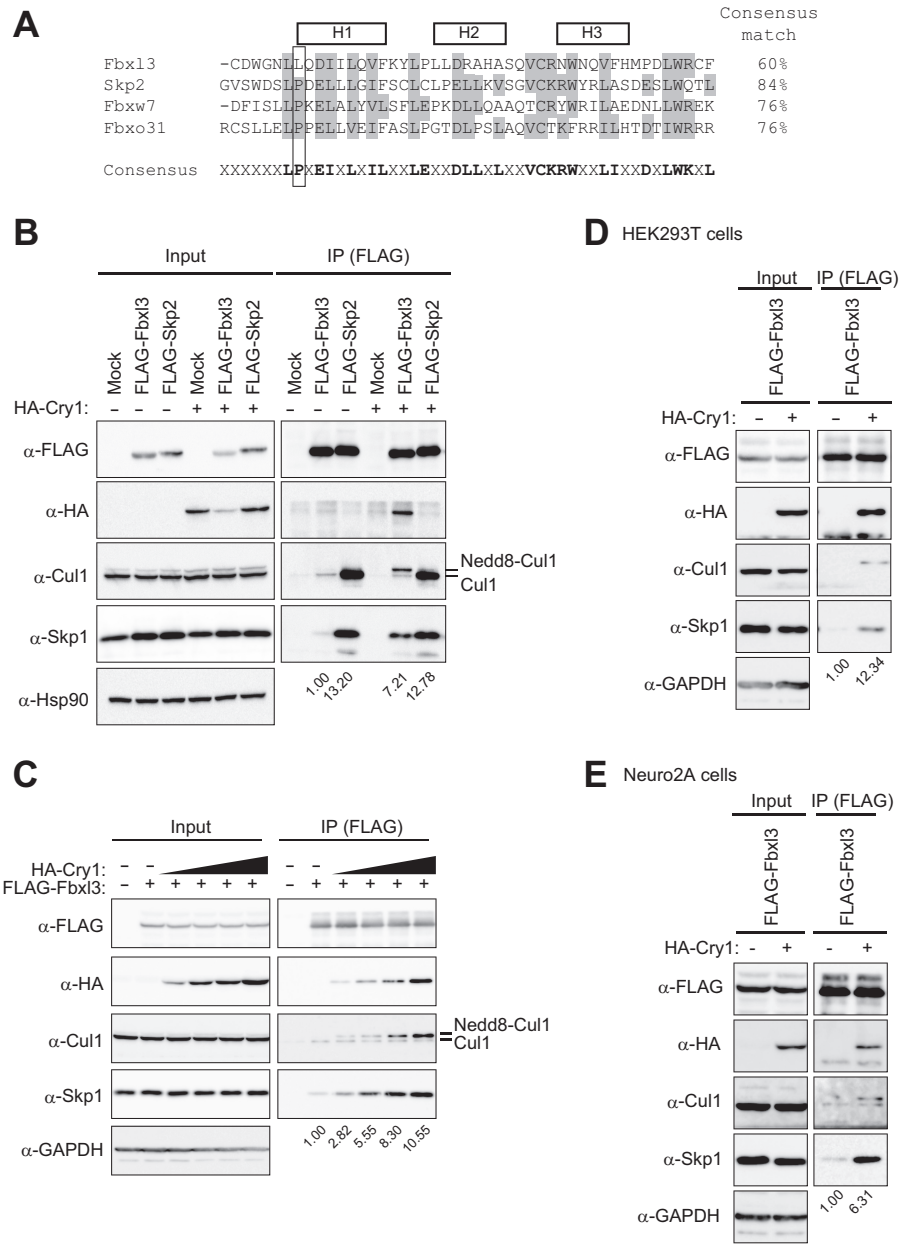

FIGURE 1. **Formation of the SCF^{Fbxl3} complex is dependent on the expression level of Cry1.** *A*, amino acid sequences of the F-box domains of human Fbxl3, Skp2, Fbxw7, and Fbxo31 are aligned. *Gray shading* highlights residues similar or identical to those of the consensus sequence. The general locations of helices (*H1–H3*) are also indicated. *B*, lysates of HeLa cells expressing FLAG-tagged forms of Fbxl3 or Skp2 as well as HA-tagged Cry1 were subjected to immunoprecipitation (*IP*) with anti-FLAG, and the resulting precipitates, as well as the original cell lysates (*Input*), were subjected to immunoblot analysis with antibodies to (α-) the indicated proteins. The relative intensities of Skp1 bands are indicated. *C*, lysates of HeLa cells expressing FLAG-tagged Fbxl3 in the absence or presence of various amounts (1×, 2×, 3×, 4×) of HA-tagged Cry1 were subjected to immunoprecipitation with anti-FLAG, and the resulting precipitates, as well as the original cell lysates, were subjected to immunoblot analysis with antibodies to the indicated proteins. *D* and *E*, lysates of HEK293T (*D*) or Neuro2A (*E*) cells expressing FLAG-tagged Fbxl3 as well as HA-tagged Cry1 were subjected to immunoprecipitation followed by immunoblot analysis as in *B*.

Cycloheximide Chase Analysis—Cycloheximide (100 μg/ml) was added to culture medium, and cells were harvested at the indicated times thereafter. The cells were lysed and subjected to immunoblot analysis as described above.

RESULTS

Fbxl3 Does Not Form an SCF Complex in the Absence of Substrate—Fbxl3 was not present in the list of proteins previously determined to interact with Cul1, whereas many other F-box proteins were included (32). Alignment of the amino acid sequences of the F-box domains of several F-box proteins revealed that certain key amino acids are not conserved in Fbxl3 (33) (Fig. 1*A*). In particular, a proline in the first helix that mediates the association with Cul1 and is conserved in Skp2, Fbxw7, and Fbxo31 is not conserved in Fbxl3. The overall similarity to the consensus sequence of the F-box domain is also lower for Fbxl3 (60% similarity) than for these other F-box proteins (76–84%). We therefore examined whether Fbxl3 is able to form a complex with Skp1 and Cul1. We transfected HeLa cells with an expression vector for FLAG-tagged Fbxl3 or FLAG-Skp2 and then subjected cell lysates to immunoprecipitation with antibodies to (anti-) FLAG followed by immunoblot analysis (Fig.

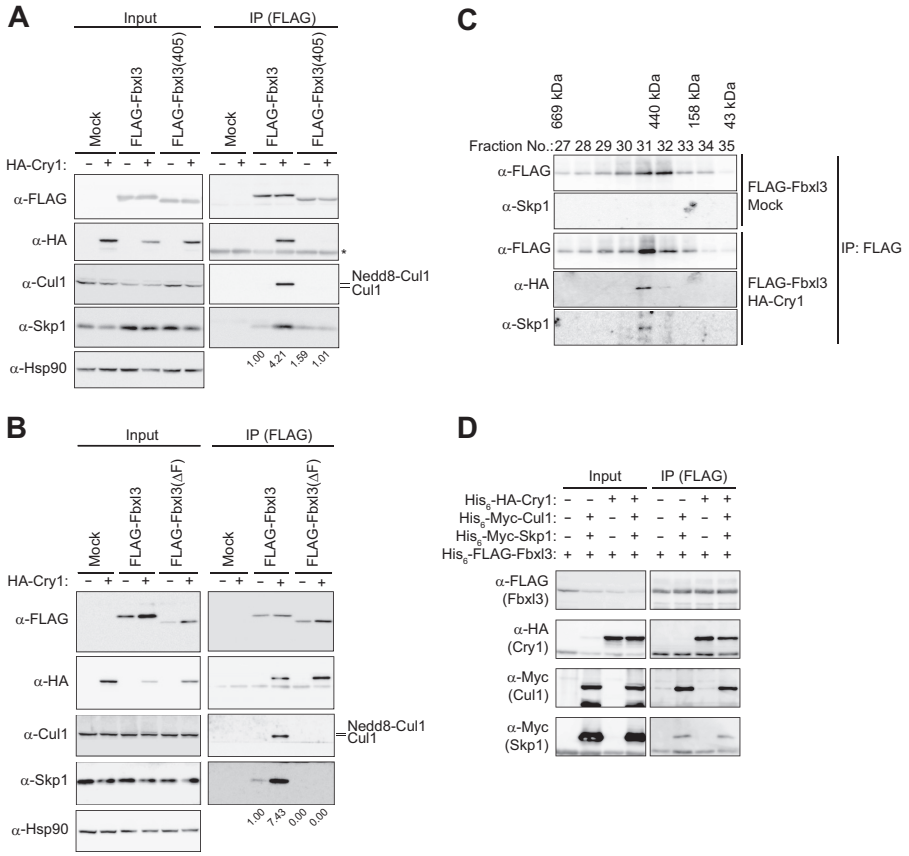

FIGURE 2. **The interaction of Cry1 with Fbxl3 is required for formation of the SCF^{Fbxl3} complex.** *A*, lysates of HeLa cells expressing FLAG-tagged forms of Fbxl3 or Fbxl3(405) as well as HA-tagged Cry1 were subjected to immunoprecipitation (*IP*) with anti-FLAG, and the resulting precipitates, as well as the original cell lysates (*Input*), were subjected to immunoblot analysis with antibodies to the indicated proteins. The *asterisk* indicates a nonspecific band. *B*, lysates of HeLa cells expressing FLAG-tagged forms of Fbxl3 or Fbxl3(ΔF) as well as HA-tagged Cry1 were subjected to immunoprecipitation with anti-FLAG, and the resulting precipitates, as well as the original cell lysates, were subjected to immunoblot analysis with antibodies to the indicated proteins. *C*, lysates of HeLa cells expressing FLAG-tagged Fbxl3 in the absence or presence of HA-tagged Cry1 were subjected to immunoprecipitation with anti-FLAG, and the resulting precipitates were fractionated by gel filtration chromatography. Column fractions were subjected to immunoblot analysis with antibodies to the indicated proteins. The elution positions of molecular size standards are indicated in kilodaltons. *D*, recombinant Cul1 or Skp1 with an NH_2-terminal His_6-Myc tag, recombinant Cry1 with an NH_2-terminal His_6-HA tag, and recombinant Fbxl3 with an NH_2-terminal His_6-FLAG tag were expressed separately in Sf21 cells and affinity-purified with nickel-nitrilotriacetic acid beads. The recombinant proteins were mixed as indicated and subjected to immunoprecipitation with anti-FLAG. The resulting precipitates, as well as the original cell lysates, were subjected to immunoblot analysis with antibodies to the indicated proteins.

1*B*). Whereas FLAG-Skp2 bound to endogenous Skp1 and Cul1, FLAG-Fbxl3 showed little, if any, association with these proteins. These results suggested that Fbxl3 contains an atypical F-box domain and has low affinity for Skp1 and Cul1. Consistent with previous observations (28), HA-tagged Cry1 interacted with FLAG-Fbxl3, whereas it did not associate with FLAG-Skp2, in transfected HeLa cells. Unexpectedly, however, the interaction of Fbxl3 with Skp1 and Cul1 was markedly enhanced in the presence of Cry1. A major proportion of Cul1 associated with Fbxl3 appeared to be neddylated.

To examine whether the extent of SCF complex formation was dependent on the expression level of substrate, we transfected cells with various amounts of the vector for HA-tagged Cry1 (Fig. 1*C*). The amounts of Skp1 and Cul1 (mostly neddylated) associated with Fbxl3 increased as the expression level of Cry1 increased, suggesting that formation of the SCF^{Fbxl3} complex is indeed dependent on the concentration of the substrate Cry1. We also examined the association of Fbxl3 with Cul1 and Skp1 in the absence or presence of Cry1 in HEK293T (Fig. 1*D*) and Neuro2A (Fig. 1*E*) cells. As observed in HeLa cells, Fbxl3 interacted with Skp1 and Cul1 only when Cry1 was expressed in HEK293T and Neuro2A cells, suggesting that SCF^{Fbxl3} complex formation dependent on the binding of substrates to Fbxl3 is not specific to HeLa cells but rather observed generally in any mammalian cell types tested.

Substrate Binding to Fbxl3 Is Essential for Formation of an SCF Complex—To investigate whether the binding of Fbxl3 to Cry1 is required for formation of an SCF complex, we tested the mutant Fbxl3(405), which lacks the 23 COOH-terminal amino acids of the full-length protein, for association with Cry1 and SCF complex formation (Fig. 2*A*). The mutant protein did not interact with Cry1 or with Skp1 or Cul1 even in the presence of exogenous Cry1 in transfected HeLa cells, suggesting that the association of Fbxl3 with Cry1 is essential for formation of the SCF^{Fbxl3} complex. To exclude the possibility that Fbxl3 associates indirectly with Skp1 and Cul1 via interaction with Cry1, we examined whether a mutant of Fbxl3 (ΔF) that lacks the F-box domain associates with Skp1 and Cul1 in HeLa cells (Fig. 2*B*). The Fbxl3(ΔF) mutant did not bind to endogenous Skp1 or Cul1 even when it was associated with exogenous Cry1.

We also performed gel filtration chromatography of FLAG-Fbxl3 immunoprecipitates prepared from transfected HeLa

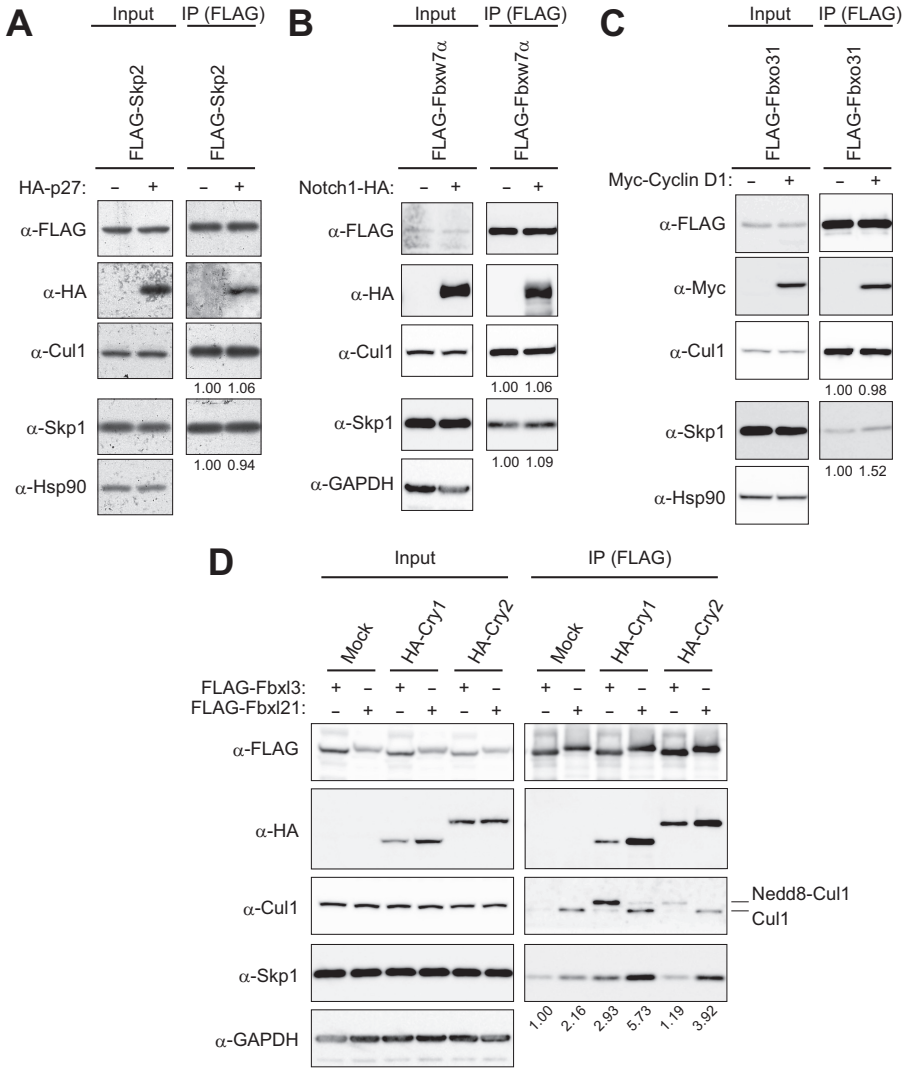

FIGURE 3. **Substrate-dependent formation of an SCF complex by Fbxl3 is specific to this F-box protein.** *A–C*, lysates of HeLa cells expressing FLAG-tagged Skp2 and HA-tagged p27 (*A*), FLAG-tagged Fbxw7α and the HA-tagged intracellular domain of Notch1 (*B*), or FLAG-tagged Fbxo31 and Myc epitope-tagged cyclin D1 (*C*) were subjected to immunoprecipitation (*IP*) with anti-FLAG, and the resulting precipitates and original cell lysates (*Input*) were subjected to immunoblot analysis with antibodies to the indicated proteins. The relative intensities of Cul1 or Skp1 bands are indicated. *D*, lysates of HeLa cells expressing FLAG-tagged forms of Fbxl3 or Fbxl21 as well as HA-tagged forms of Cry1 or Cry2 were subjected to immunoprecipitation with anti-FLAG, and the resulting precipitates and original cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins.

cells (Fig. 2*C*). Co-expression of HA-Cry1 in the cells resulted in a decrease in the amount of FLAG-Fbxl3 in the monomer fraction (fraction 34) and an increase in the amount in a fraction of higher molecular weight (fraction 31) in which both HA-Cry1 and endogenous Skp1 were associated with FLAG-Fbxl3. These results suggested that expression of HA-Cry1 facilitated the assembly of Fbxl3 with the core components of the SCF complex. Although a portion of FLAG-Fbxl3 was detected in fractions of even higher molecular weight (fractions 27–30), we did not detect a substantial difference in the intensity of the FLAG-Fbxl3 bands in these fractions between cells expressing or not expressing HA-Cry1. The expression of HA-Cry1 thus appeared to promote the incorporation of monomeric Fbxl3 into the SCF complex rather than to affect potential protein aggregation.

To examine further whether the direct interaction between Fbxl3 and Cry1 is necessary for formation of an SCF complex, we performed an *in vitro* pulldown assay. Recombinant His_6-FLAG-Fbxl3, His_6-Myc-Cul1, His_6-Myc-Skp1, and His_6-HA-Cry1 were separately expressed in and purified from Sf21 insect cells for the assay. In contrast to the *in vivo* results obtained with HeLa cells, recombinant Fbxl3 was found to interact with recombinant Skp1 and Cul1 *in vitro* even in the absence of recombinant Cry1 (Fig. 2*D*). This finding thus suggested that Fbxl3 might associate with an as yet unknown protein that interferes with the interaction of Fbxl3 with Skp1 and Cul1 in HeLa cells. Together, our data thus suggested that the association with Cry1 via the LRR domain is required for Fbxl3 to be able to interact with Skp1 and Cul1 via the F-box domain.

Substrate-dependent Formation of an SCF Complex Is Specific to Fbxl3—We examined whether F-box proteins with a more typical F-box domain also associate with Skp1 and Cul1 only in the presence of substrate. Ectopic expression of p27, a well characterized substrate of Skp2, had no effect on the interaction of Skp2 with Cul1 and Skp1 (Fig. 3*A*). Likewise, the efficiency of SCF complex formation by Fbxw7α or Fbxo31 was not

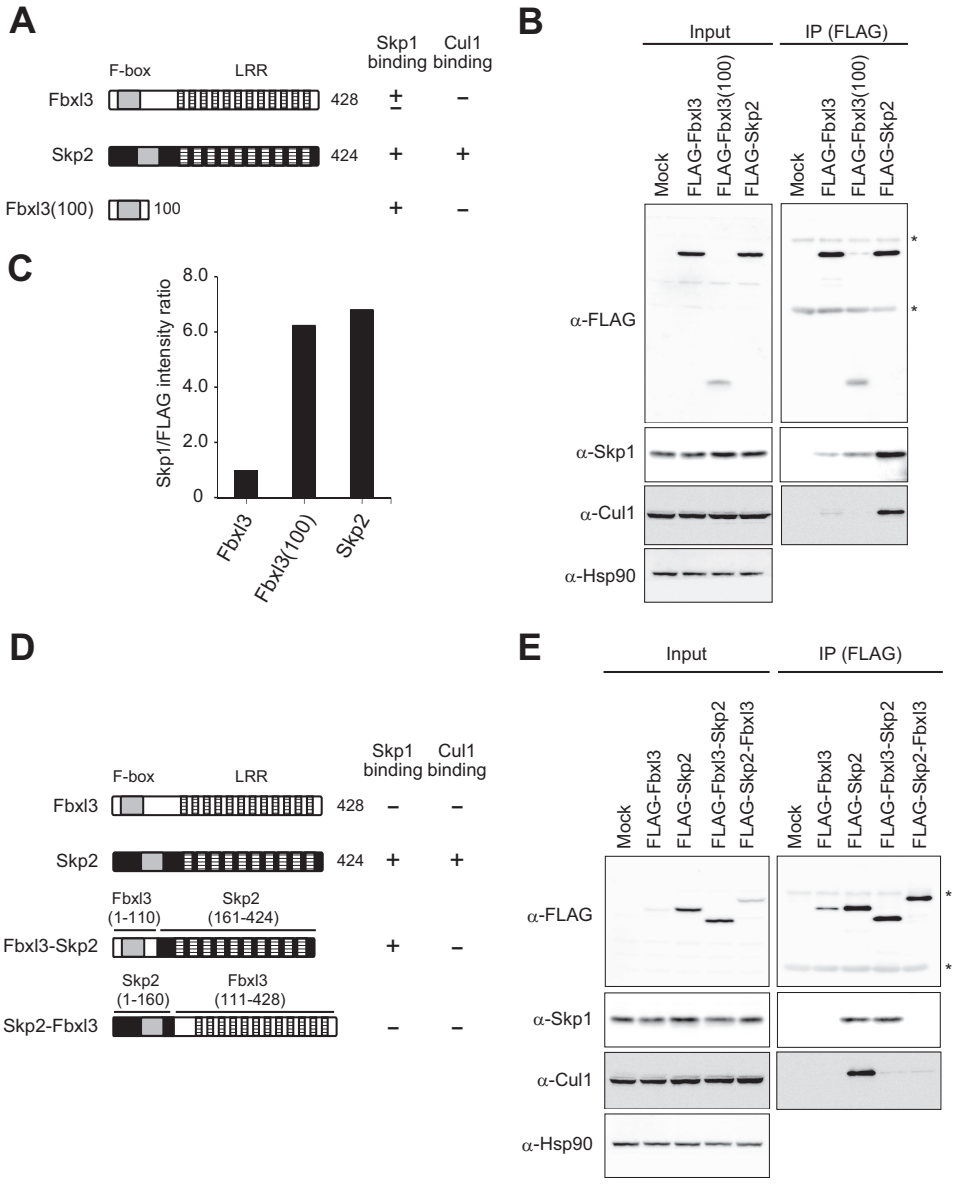

FIGURE 4. **The COOH-terminal LRR domain of Fbxl3 inhibits interaction with Skp1.** *A*, schematic represents human Fbxl3, Skp2, and the Fbxl3(100) deletion mutant and presents a summary of their abilities to bind to Skp1 and Cul1 as determined in *B* and *C*. *B* and *C*, lysates of HeLa cells expressing FLAG-tagged forms of Fbxl3, Fbxl3(100), or Skp2 were subjected to immunoprecipitation (*IP*) with anti-FLAG, and the resulting precipitates, as well as the original cell lysates (*Input*), were subjected to immunoblot analysis with antibodies to the indicated proteins (*B*). *Asterisks* indicate nonspecific bands. The normalized efficiency of interaction of each exogenous protein with endogenous Skp1 was quantified (*C*). *D*, schematic represents human Fbxl3, Skp2, and their domain-swap mutants as well as a summary of their abilities to bind to Skp1 and Cul1 as determined in *E*. *E*, lysates of HeLa cells expressing FLAG-tagged forms of Fbxl3, Skp2, or their domain-swap mutants were subjected to immunoprecipitation with anti-FLAG, and the resulting precipitates and original cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins. *Asterisks* indicate nonspecific bands.

affected by forced expression of their corresponding substrates Notch1 and cyclin D1 (Fig. 3, *B* and *C*).

Fbxl21 is the most closely related paralog of Fbxl3, and it also interacts with Cry1 and controls the circadian pacemaker (34). Ubiquitylation of Cry proteins by SCF^{Fbxl21} was recently shown to increase their stability (35, 36). We found that Fbxl21 bound to Cry1 and Cry2 more efficiently than did Fbxl3 in transfected HeLa cells (Fig. 3*D*). In the absence of exogenous Cry1 and Cry2, the efficiency of Fbxl21 binding to Skp1 and Cul1 was higher than that for Fbxl3 binding to these proteins. Of note, Fbxl21 interacted with the nonneddylated form of Cul1, whereas Fbxl3 preferentially bound to the neddylated form. In the presence of exogenous Cry1, the efficiency of association of Fbxl21 with Skp1 was increased, whereas the interaction with Cul1 was not affected, suggesting that formation of an SCF complex by Fbxl21 occurs via a distinct mechanism. Interaction of Cry2 with Fbxl3 was less effective than was that of Cry1 in promoting formation of the SCF complex, suggesting that the E3 activity of Fbxl3 toward Cry1 is higher than that toward Cry2. Collectively, our results suggested that the substrate-dependent regulation of SCF complex formation by Fbxl3 is specific to this F-box protein.

The COOH-terminal LRR Domain of Fbxl3 Inhibits the Interaction of Fbxl3 with Cul1 and Skp1 through Its F-box Domain—Given that the association with substrate via its COOH-terminal LRR domain affects the interaction of Fbxl3 with Skp1 and

Cul1 via its NH_2-terminal F-box domain, we hypothesized that the LRR domain interferes with formation of the SCF complex. To test this hypothesis, we constructed the Fbxl3(100) mutant, which contains the F-box domain of Fbxl3 but lacks the LRR domain (Fig. 4*A*). This mutant associated with Skp1 in transfected HeLa cells to an extent similar to that observed for Skp2 (Fig. 4, *B* and *C*), suggesting that the LRR domain of Fbxl3 prevents association with Skp1. However, Cul1 did not interact with the Fbxl3(100) mutant. To confirm the inhibitory effect of the LRR domain of Fbxl3 on formation of the SCF complex, we constructed both an Fbxl3 mutant (Fbxl3-Skp2) in which the LRR domain is replaced with the corresponding domain of Skp2 as well as a reciprocal Skp2 mutant (Skp2-Fbxl3) in which the LRR domain is replaced with that of Fbxl3 (Fig. 4*D*). The Fbxl3-Skp2 mutant interacted with Skp1 but not Cul1 (Fig. 4*E*), consistent with our results obtained with the Fbxl3(100) mutant (Fig. 4, *B* and *C*). Although a Skp2 mutant that lacks the LRR domain retained the ability to associate with both Skp1 and Cul1 (data not shown), the Skp2-Fbxl3 mutant did not interact with either Skp1 or Cul1 (Fig. 4*E*), suggesting that the LRR domain of Fbxl3 actively interferes with binding to Skp1 and Cul1 and thereby blocks formation of the SCF complex.

Neddylation of Cul1 Is Not Required for Interaction with Fbxl3—Nedd8 is a posttranslational modifier that increases the ubiquitin ligase activity of the SCF complex (37–39) by promoting E2 recruitment (40, 41). Given that Fbxl3 appeared to interact preferentially with the neddylated form of Cul1, we examined whether neddylation of Cul1 is required for the interaction with Fbxl3. Fbxl3 interacted with both neddylated and nonneddylated forms of Cul1 in HeLa cells expressing exogenous Cul1 and Cry1 (Fig. 5*A*). A Cul1 mutant (K720R) that is not neddylated also interacted with Fbxl3 in the presence of Cry1, suggesting that the neddylation of Cul1 is dispensable for formation of the SCF^{Fbxl3} complex triggered by the association of Cry1 with Fbxl3. To confirm this observation, we treated cells with an inhibitor of NAE. Interaction of Fbxl3 with the nonneddylated form of endogenous Cul1 was detected in cells expressing exogenous Cry1 and treated with the NAE inhibitor (Fig. 5*B*). Together, our data thus indicated that neddylation of Cul1 is not essential for formation of the SCF^{Fbxl3} complex.

Fbxl3 Is More Stable Than Other F-box Proteins—We expected that Fbxl3 might be more stable than typical F-box proteins, given that Fbxl3 might not undergo autoubiquitylation in the absence of its substrate Cry1. To test this hypothesis, we examined the stability of four F-box proteins, Fbxl3, Skp2, Fbxw7α, and Fbxo31, in transfected HeLa cells with a cycloheximide chase assay. Fbxl3 was relatively stable with a half-life ($t_{1/2}$) of 8.0 h (Fig. 6*A*) compared with Skp2 ($t_{1/2}$ = 2.3 h) (Fig. 6*B*), Fbxw7α ($t_{1/2}$ = 4.3 h) (Fig. 6*C*), and Fbxo31 ($t_{1/2}$ = 0.85 h) (Fig. 6*D*). The stability of all four F-box proteins was greatly increased by treatment of the cells with the proteasome inhibitor MG132, suggesting that all four proteins undergo proteasomal degradation. However, only Fbxl3 was insensitive to the NAE inhibitor (which blocks the neddylation of Cul1), with the other three F-box proteins being stabilized by treatment of cells with this agent. These results suggested that the stability of Skp2, Fbxw7α, and Fbxo31 is regulated by the SCF complex (likely by self-ubiquitylation), whereas that of Fbxl3 is regulated by a mechanism that is independent of the SCF complex. Given that formation of SCF^{Fbxl3} is also not dependent on Cul1 neddylation (Fig. 5*B*), regulation of the stability of and complex formation by Fbxl3 appears unique to this F-box protein.

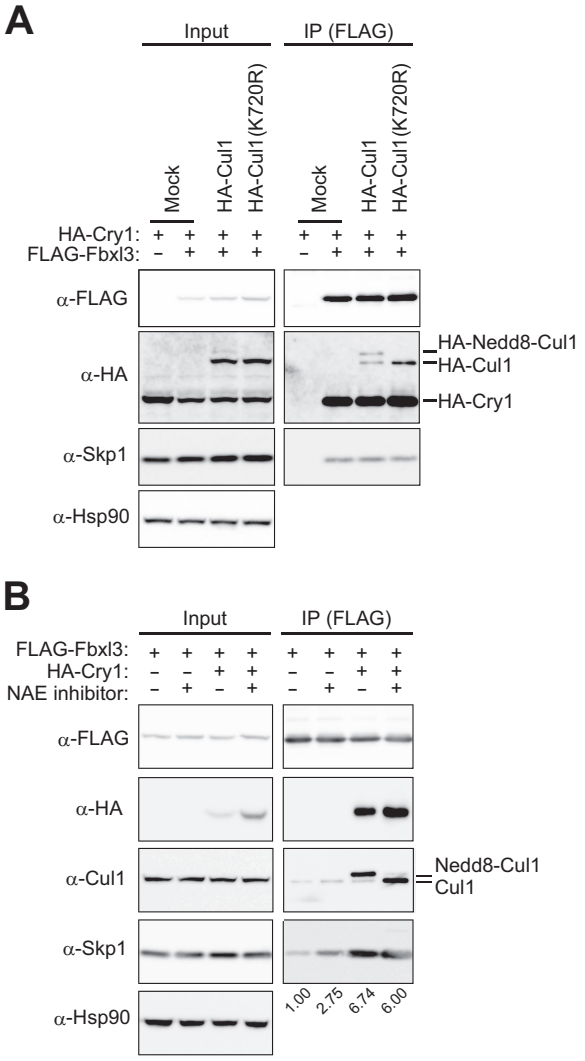

FIGURE 5. **Neddylation of Cul1 is not required for assembly of SCF^{Fbxl3}.** *A*, lysates of HeLa cells expressing FLAG-tagged Fbxl3 as well as HA-tagged forms of Cry1, Cul1, or Cul1(K720R) were subjected to immunoprecipitation (*IP*) with anti-FLAG, and the resulting precipitates, as well as the original cell lysates (*Input*), were subjected to immunoblot analysis with antibodies to the indicated proteins. *B*, HeLa cells expressing FLAG-tagged Fbxl3 and HA-tagged Cry1 were treated with 1 μM NAE inhibitor for 24 h, lysed, and subjected to immunoprecipitation with anti-FLAG. The resulting precipitates and original cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins.

DISCUSSION

The catalytic activity of SCF-type ubiquitin ligases is regulated by several mechanisms (7–9). The SCF complex is activated by conjugation of the ubiquitin-like protein Nedd8 to the Cul1 subunit, and neddylated Cul1 is resistant to CAND1-induced dissociation that inhibits ubiquitin transfer from an associated E2 to substrate (40, 41). Several F-box proteins control the assembly and activity of the SCF complex in response to changes in the intracellular environment. We have now revealed a new mode of regulation of SCF complex forma-

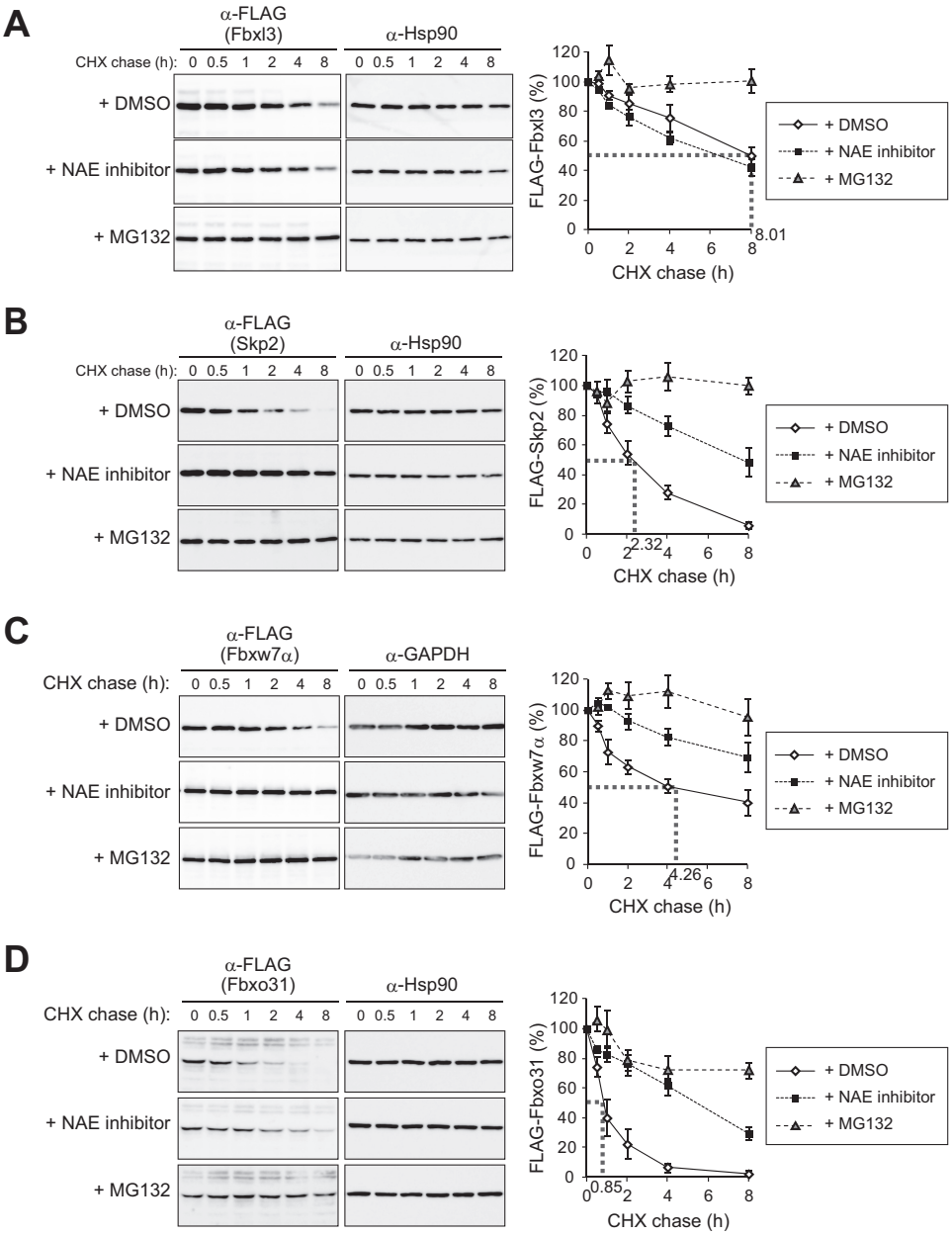

FIGURE 6. **Fbxl3 is more stable than other F-box proteins.** HeLa cells expressing FLAG-tagged forms of Fbxl3 (*A*), Skp2 (*B*), Fbxw7α (*C*), or Fbxo31 (*D*) were exposed to cycloheximide (*CHX*) for the indicated times in the presence of NAE inhibitor, MG132, or dimethyl sulfoxide (*DMSO*) vehicle, after which cell lysates were subjected to immunoblot analysis with anti-FLAG (*left panels*). The percentage of each FLAG-tagged F-box protein remaining after the various chase times was determined (*right panels*, $n = 3$, means ± S.D. (*error bars*)). The half-life ($t_{1/2}$) of each F-box protein is indicated.

tion mediated at the level of substrate binding. Fbxl3 does not form an SCF complex in the absence of its substrate Cry1, likely because it associates with an as yet unknown protein that interferes with its interaction with Skp1-Cul1. We speculate that the binding of Cry proteins to the LRR domain of Fbxl3 induces dissociation of this unknown protein and thereby promotes formation of the SCF^{Fbxl3} complex (Fig. 7).

Many F-box proteins are unstable, with autoubiquitylation of these proteins within the corresponding SCF complex underlying their turnover (42, 43). However, we have now found that Fbxl3 is more stable than more typical F-box proteins, likely because autoubiquitylation of Fbxl3 does not occur in the absence of its substrate Cry1. In most instances, binding of an F-box protein to its substrate is dependent on posttranslational modification such as phosphorylation of the substrate, and the binding is usually irreversible (44). Recent determination of the three-dimensional structures of mouse Cry1 and Cry2 has suggested that Per proteins and FAD mask the binding domain of Cry proteins for Fbxl3 (45). Dissociation of Per and FAD from Cry1 and Cry2 thus appears to be a prerequisite for the association of these Cry proteins with Fbxl3. The liberated Cry proteins may need to be immediately captured by Fbxl3 for degradation before they can reassociate with Per and FAD. The relatively high stability of Fbxl3 compared with other F-box proteins might be required for such immediate interaction with the free form of Cry1 or Cry2 (Fig. 7).

We have also found that Skp1 and Cul1 interact in different manners with the F-box domain of Fbxl3. Immunoprecipita-

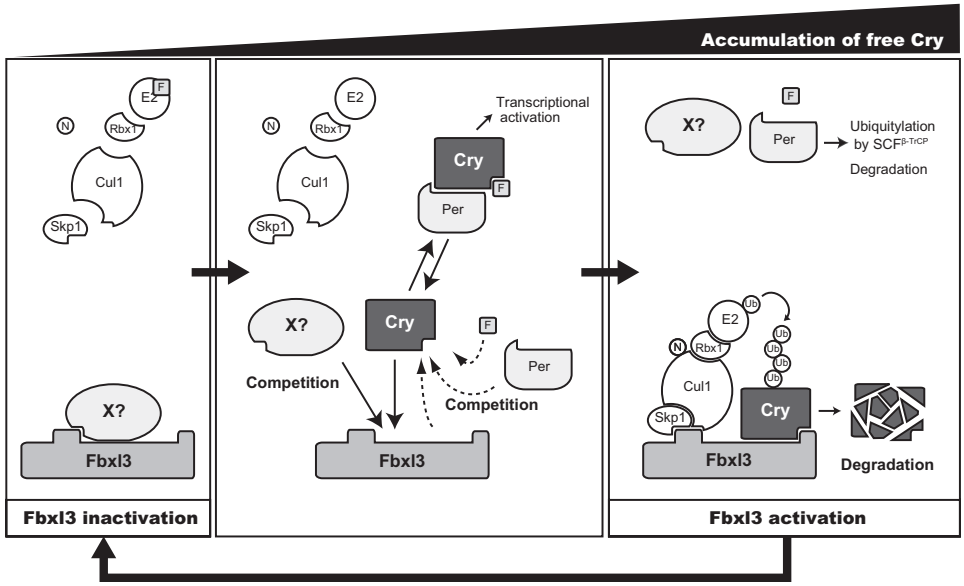

FIGURE 7. **Model for regulation of SCF^{Fbxl3} complex formation.** Fbxl3 essentially does not form an SCF complex in the absence of its association with Cry1 (*left*), given that it appears to associate with an as yet unknown protein (*X*) that interferes with its interaction with Skp1-Cul1 in mammalian cells. As Cry1 accumulates, a subset of Cry1 dissociates from Per and FAD (*center*), and the binding of the free form of Cry1 to the LRR domain of Fbxl3 induces the dissociation of this unknown protein and thereby promotes formation of the SCF^{Fbxl3} complex (*right*). *N*, Nedd8; *F*, FAD; *Ub*, ubiquitin. See "Discussion" for further details.

tion analysis with the Fbxl3(100) and Fbxl3-Skp2 mutants indicated that Skp1 was able to associate with only the F-box domain of Fbxl3, whereas Cul1 was not. The linker region between the F-box domain and the substrate recognition (LRR) domain may thus also be required for the interaction of Fbxl3 with Cul1. A deletion mutant of Fbxw8 that lacks the corresponding linker region also did not interact with Cul1 (46).

Several groups have shown that neddylated SCF complexes are more active than their nonneddylated counterparts regardless of the nature of the substrate (37–39, 41, 47, 48). Crystal structure data indicate that neddylation induces a pronounced conformational change in the COOH-terminal region of cullins that prevents interaction with the RING domain of Rbx1 (13). Cullin neddylation results in positioning of Rbx1 and its bound activated E2 in close proximity to the acceptor lysine of the substrate and thereby promotes the transfer of ubiquitin. Neddylation and the associated conformational change in the COOH-terminal domain of Cul1 are thought not to affect the binding interface of the NH_2-terminal domain and interaction with Skp1 and the F-box protein, consistent with our present observation that neddylation of Cul1 is not required for interaction with Fbxl3. A model was recently proposed in which substrate induces displacement of COP9-signalosome and thereby promotes formation of a neddylated, active SCF complex (49). In agreement with this model, we found that most SCF^{Fbxl3} complexes are neddylated in transfected HeLa cells in the presence of HA-Cry1, suggesting that deneddylation of Cul1 is strongly inhibited in SCF^{Fbxl3} compared with other SCF complexes for which F-box proteins are able to form such a complex independently of substrate binding. In addition, Fbxl3 itself might suppress the deneddylation activity mediated by COP9-signalosome.

Large scale proteomic analyses have recently provided insight into the network of Cul1-interacting proteins and the cellular composition of SCF complexes (32, 50). These studies identified 42 and 26 different F-box proteins, respectively, that interact with Cul1 in HEK293(T) cells, suggesting that ~40% of F-box proteins might not be expressed or might be unable to form an SCF complex under the chosen experimental conditions. We propose two other possibilities for the absence of certain F-box proteins in Cul1 complexes: (i) a subset of F-box proteins such as Fbxl3 might associate with Cul1 only in the presence of their substrates; and (ii) another subset of F-box proteins might not interact with Cul1 at all. With regard to this second possibility, we previously showed that Fbxo45 is unable to form an SCF complex as a result of an amino acid substitution in the consensus sequence of the F-box domain for Cul1 binding (51). Instead, Fbxo45 interacts with the RING finger-type ubiquitin ligase PAM through its SPRY domain.

Mammals possess two homologous *Cry* genes, *Cry1* and *Cry2*. Whereas mice lacking both *Cry1* and *Cry2* exhibit arrhythmic running activity in constant darkness (52), those deficient in one of the two *Cry* genes show distinct phenotypes. Mice lacking Cry1 or Cry2 thus manifest a free-running period ~1 h shorter or longer than that of WT mice, respectively (52, 53). At the cellular level, whereas dissociated Cry1-deficient neurons of the suprachiasmatic nucleus or fibroblasts are largely arrhythmic, the corresponding Cry2-deficient neurons and fibroblasts exhibit robust rhythmicity (54). These observations suggest that Cry1 and Cry2 make distinct contributions to the clock, even though their amino acid sequences are highly similar. Although Fbxl3 mediates proteasomal degradation of both Cry1 and Cry2, we found that Cry2 had only a small effect on formation of the SCF^{Fbxl3} complex *in vivo*. Consistent with our data, the half-life of Cry2 is prolonged to a lesser extent compared with that of Cry1 in the Fbxl3 mutant mouse *afterhours* (30). The Cry2-specific degradation pathway was shown to depend on phosphorylation by glycogen synthase kinase-3β and DYRK1A at serine residues that are unique to Cry2 (55, 56). Collectively, these observations suggest that the degradation of

Cry1 and that of Cry2 might be controlled by distinct mechanisms, possibly accounting for the opposite phenotypes of Cry1- and Cry2-deficient mice.

Acknowledgments—We thank N. Nishimura, K. Tsunematsu, and other laboratory members for technical assistance and A. Ohta for help with preparation of the manuscript.

REFERENCES

1. Pickart, C. M. (2001) Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* **70,** 503–533
2. Pickart, C. M. (2004) Back to the future with ubiquitin. *Cell* **116,** 181–190
3. Nakayama, K. I., and Nakayama, K. (2006) Ubiquitin ligases: cell-cycle control and cancer. *Nat. Rev. Cancer* **6,** 369–381
4. Frescas, D., and Pagano, M. (2008) Deregulated proteolysis by the F-box proteins SKP2 and β-TrCP: tipping the scales of cancer. *Nat. Rev. Cancer* **8,** 438–449
5. Welcker, M., and Clurman, B. E. (2008) FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat. Rev. Cancer* **8,** 83–93
6. Jin, J., Cardozo, T., Lovering, R. C., Elledge, S. J., Pagano, M., and Harper, J. W. (2004) Systematic analysis and nomenclature of mammalian F-box proteins. *Genes Dev.* **18,** 2573–2580
7. Bosu, D. R., and Kipreos, E. T. (2008) Cullin-RING ubiquitin ligases: global regulation and activation cycles. *Cell Div.* **3,** 7
8. Deshaies, R. J., and Joazeiro, C. A. (2009) RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem.* **78,** 399–434
9. Merlet, J., Burger, J., Gomes, J. E., and Pintard, L. (2009) Regulation of cullin-RING E3 ubiquitin-ligases by neddylation and dimerization. *Cell. Mol. Life Sci.* **66,** 1924–1938
10. Liu, J., Furukawa, M., Matsumoto, T., and Xiong, Y. (2002) NEDD8 modification of CUL1 dissociates $p120^{CAND1}$, an inhibitor of CUL1-SKP1 binding and SCF ligases. *Mol. Cell* **10,** 1511–1518
11. Zheng, J., Yang, X., Harrell, J. M., Ryzhikov, S., Shim, E. H., Lykke-Andersen, K., Wei, N., Sun, H., Kobayashi, R., and Zhang, H. (2002) CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. *Mol. Cell* **10,** 1519–1526
12. Pierce, N. W., Lee, J. E., Liu, X., Sweredoski, M. J., Graham, R. L., Larimore, E. A., Rome, M., Zheng, N., Clurman, B. E., Hess, S., Shan, S. O., and Deshaies, R. J. (2013) Cand1 promotes assembly of new SCF complexes through dynamic exchange of F box proteins. *Cell* **153,** 206–215
13. Duda, D. M., Borg, L. A., Scott, D. C., Hunt, H. W., Hammel, M., and Schulman, B. A. (2008) Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation. *Cell* **134,** 995–1006
14. Saha, A., and Deshaies, R. J. (2008) Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation. *Mol. Cell* **32,** 21–31
15. Barbash, O., Zamfirova, P., Lin, D. I., Chen, X., Yang, K., Nakagawa, H., Lu, F., Rustgi, A. K., and Diehl, J. A. (2008) Mutations in Fbx4 inhibit dimerization of the SCF^{Fbx4} ligase and contribute to cyclin D1 overexpression in human cancer. *Cancer Cell* **14,** 68–78
16. Tang, X., Orlicky, S., Lin, Z., Willems, A., Neculai, D., Ceccarelli, D., Mercurio, F., Shilton, B. H., Sicheri, F., and Tyers, M. (2007) Suprafacial orientation of the SCFCdc4 dimer accommodates multiple geometries for substrate ubiquitination. *Cell* **129,** 1165–1176
17. Welcker, M., and Clurman, B. E. (2007) Fbw7/hCDC4 dimerization regulates its substrate interactions. *Cell Div.* **2,** 7
18. Deshaies, R. J. (1999) SCF and cullin/RING H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.* **15,** 435–467
19. Li, Y., Gazdoiu, S., Pan, Z. Q., and Fuchs, S. Y. (2004) Stability of homologue of Slimb F-box protein is regulated by availability of its substrate. *J. Biol. Chem.* **279,** 11074–11080
20. Salahudeen, A. A., Thompson, J. W., Ruiz, J. C., Ma, H. W., Kinch, L. N., Li, Q., Grishin, N. V., and Bruick, R. K. (2009) An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. *Science* **326,** 722–726
21. Vashisht, A. A., Zumbrennen, K. B., Huang, X., Powers, D. N., Durazo, A., Sun, D., Bhaskaran, N., Persson, A., Uhlen, M., Sangfelt, O., Spruck, C., Leibold, E. A., and Wohlschlegel, J. A. (2009) Control of iron homeostasis by an iron-regulated ubiquitin ligase. *Science* **326,** 718–721
22. Santra, M. K., Wajapeyee, N., and Green, M. R. (2009) F-box protein FBXO31 mediates cyclin D1 degradation to induce G_1 arrest after DNA damage. *Nature* **459,** 722–725
23. Tan, X., Calderon-Villalobos, L. I., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M., and Zheng, N. (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446,** 640–645
24. Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S. Y., Howe, G. A., and Browse, J. (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. *Nature* **448,** 661–665
25. Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., and Weitz, C. J. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280,** 1564–1569
26. Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* **103,** 1009–1017
27. Shirogane, T., Jin, J., Ang, X. L., and Harper, J. W. (2005) SCFβ-TRCP controls clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein. *J. Biol. Chem.* **280,** 26863–26872
28. Busino, L., Bassermann, F., Maiolica, A., Lee, C., Nolan, P. M., Godinho, S. I., Draetta, G. F., and Pagano, M. (2007) SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science* **316,** 900–904
29. Siepka, S. M., Yoo, S. H., Park, J., Song, W., Kumar, V., Hu, Y., Lee, C., and Takahashi, J. S. (2007) Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. *Cell* **129,** 1011–1023
30. Godinho, S. I., Maywood, E. S., Shaw, L., Tucci, V., Barnard, A. R., Busino, L., Pagano, M., Kendall, R., Quwailid, M. M., Romero, M. R., O'neill, J., Chesham, J. E., Brooker, D., Lalanne, Z., Hastings, M. H., and Nolan, P. M. (2007) The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. *Science* **316,** 897–900
31. Hatakeyama, S., Jensen, J. P., and Weissman, A. M. (1997) Subcellular localization and ubiquitin-conjugating enzyme (E2) interactions of mammalian HECT family ubiquitin protein ligases. *J. Biol. Chem.* **272,** 15085–15092
32. Bennett, E. J., Rush, J., Gygi, S. P., and Harper, J. W. (2010) Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. *Cell* **143,** 951–965
33. Schulman, B. A., Carrano, A. C., Jeffrey, P. D., Bowen, Z., Kinnucan, E. R., Finnin, M. S., Elledge, S. J., Harper, J. W., Pagano, M., and Pavletich, N. P. (2000) Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. *Nature* **408,** 381–386
34. Dardente, H., Mendoza, J., Fustin, J. M., Challet, E., and Hazlerigg, D. G. (2008) Implication of the F-box protein FBXL21 in circadian pacemaker function in mammals. *PLoS One* **3,** e3530
35. Hirano, A., Yumimoto, K., Tsunematsu, R., Matsumoto, M., Oyama, M., Kozuka-Hata, H., Nakagawa, T., Lanjakornsiripan, D., Nakayama, K. I., and Fukada, Y. (2013) FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. *Cell* **152,** 1106–1118
36. Yoo, S. H., Mohawk, J. A., Siepka, S. M., Shan, Y., Huh, S. K., Hong, H. K., Kornblum, I., Kumar, V., Koike, N., Xu, M., Nussbaum, J., Liu, X., Chen, Z., Chen, Z. J., Green, C. B., and Takahashi, J. S. (2013) Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm. *Cell* **152,** 1091–1105
37. Read, M. A., Brownell, J. E., Gladysheva, T. B., Hottelet, M., Parent, L. A., Coggins, M. B., Pierce, J. W., Podust, V. N., Luo, R. S., Chau, V., and Palombella, V. J. (2000) Nedd8 modification of cul-1 activates SCF(β(TrCP))-dependent ubiquitination of IκBα. *Mol. Cell. Biol.* **20,** 2326–2333
38. Wu, K., Chen, A., and Pan, Z. Q. (2000) Conjugation of Nedd8 to CUL1

enhances the ability of the ROC1-CUL1 complex to promote ubiquitin polymerization. *J. Biol. Chem.* **275,** 32317–32324
39. Podust, V. N., Brownell, J. E., Gladysheva, T. B., Luo, R. S., Wang, C., Coggins, M. B., Pierce, J. W., Lightcap, E. S., and Chau, V. (2000) A Nedd8 conjugation pathway is essential for proteolytic targeting of $p27^{Kip1}$ by ubiquitination. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 4579–4584
40. Sakata, E., Yamaguchi, Y., Miyauchi, Y., Iwai, K., Chiba, T., Saeki, Y., Matsuda, N., Tanaka, K., and Kato, K. (2007) Direct interactions between NEDD8 and ubiquitin E2-conjugating enzymes up-regulate cullin-based E3 ligase activity. *Nat. Struct. Mol. Biol.* **14,** 167–168
41. Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., Omata, M., and Tanaka, K. (2001) NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J.* **20,** 4003–4012
42. Zhou, P., and Howley, P. M. (1998) Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases. *Mol. Cell* **2,** 571–580
43. Galan, J. M., and Peter, M. (1999) Ubiquitin-dependent degradation of multiple F-box proteins by an autocatalytic mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 9124–9129
44. Skaar, J. R., Pagan, J. K., and Pagano, M. (2013) Mechanisms and function of substrate recruitment by F-box proteins. *Nat. Rev. Mol. Cell Biol.* **14,** 369–381
45. Xing, W., Busino, L., Hinds, T. R., Marionni, S. T., Saifee, N. H., Bush, M. F., Pagano, M., and Zheng, N. (2013) SCF^{FBXL3} ubiquitin ligase targets cryptochromes at their cofactor pocket. *Nature* **496,** 64–68
46. Tsunematsu, R., Nishiyama, M., Kotoshiba, S., Saiga, T., Kamura, T., and Nakayama, K. I. (2006) Fbxw8 is essential for Cul1-Cul7 complex formation and for placental development. *Mol. Cell. Biol.* **26,** 6157–6169
47. Morimoto, M., Nishida, T., Honda, R., and Yasuda, H. (2000) Modification of cullin-1 by ubiquitin-like protein Nedd8 enhances the activity of SCF^{skp2} toward $p27^{kip1}$. *Biochem. Biophys. Res. Commun.* **270,** 1093–1096
48. Ohh, M., Kim, W. Y., Moslehi, J. J., Chen, Y., Chau, V., Read, M. A., and Kaelin, W. G., Jr. (2002) An intact NEDD8 pathway is required for cullin-dependent ubiquitylation in mammalian cells. *EMBO Rep.* **3,** 177–182
49. Emberley, E. D., Mosadeghi, R., and Deshaies, R. J. (2012) Deconjugation of Nedd8 from Cul1 is directly regulated by Skp1-F-box and substrate, and the COP9 signalosome inhibits deneddylated SCF by a noncatalytic mechanism. *J. Biol. Chem.* **287,** 29679–29689
50. Lee, J. E., Sweredoski, M. J., Graham, R. L., Kolawa, N. J., Smith, G. T., Hess, S., and Deshaies, R. J. (2011) The steady-state repertoire of human SCF ubiquitin ligase complexes does not require ongoing Nedd8 conjugation. *Mol. Cell. Proteomics* **10,** M110.006460
51. Saiga, T., Fukuda, T., Matsumoto, M., Tada, H., Okano, H. J., Okano, H., and Nakayama, K. I. (2009) Fbxo45 forms a novel ubiquitin ligase complex and is required for neuronal development. *Mol. Cell. Biol.* **29,** 3529–3543
52. van der Horst, G. T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A. P., van Leenen, D., Buijs, R., Bootsma, D., Hoeijmakers, J. H., and Yasui, A. (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* **398,** 627–630
53. Vitaterna, M. H., Selby, C. P., Todo, T., Niwa, H., Thompson, C., Fruechte, E. M., Hitomi, K., Thresher, R. J., Ishikawa, T., Miyazaki, J., Takahashi, J. S., and Sancar, A. (1999) Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 12114–12119
54. Liu, A. C., Welsh, D. K., Ko, C. H., Tran, H. G., Zhang, E. E., Priest, A. A., Buhr, E. D., Singer, O., Meeker, K., Verma, I. M., Doyle, F. J., 3rd, Takahashi, J. S., and Kay, S. A. (2007) Intercellular coupling confers robustness against mutations in the SCN circadian clock network. *Cell* **129,** 605–616
55. Harada, Y., Sakai, M., Kurabayashi, N., Hirota, T., and Fukada, Y. (2005) Ser-557-phosphorylated mCRY2 is degraded upon synergistic phosphorylation by glycogen synthase kinase-3β. *J. Biol. Chem.* **280,** 31714–31721
56. Kurabayashi, N., Hirota, T., Sakai, M., Sanada, K., and Fukada, Y. (2010) DYRK1A and glycogen synthase kinase 3β, a dual-kinase mechanism directing proteasomal degradation of CRY2 for circadian timekeeping. *Mol. Cell. Biol.* **30,** 1757–1768