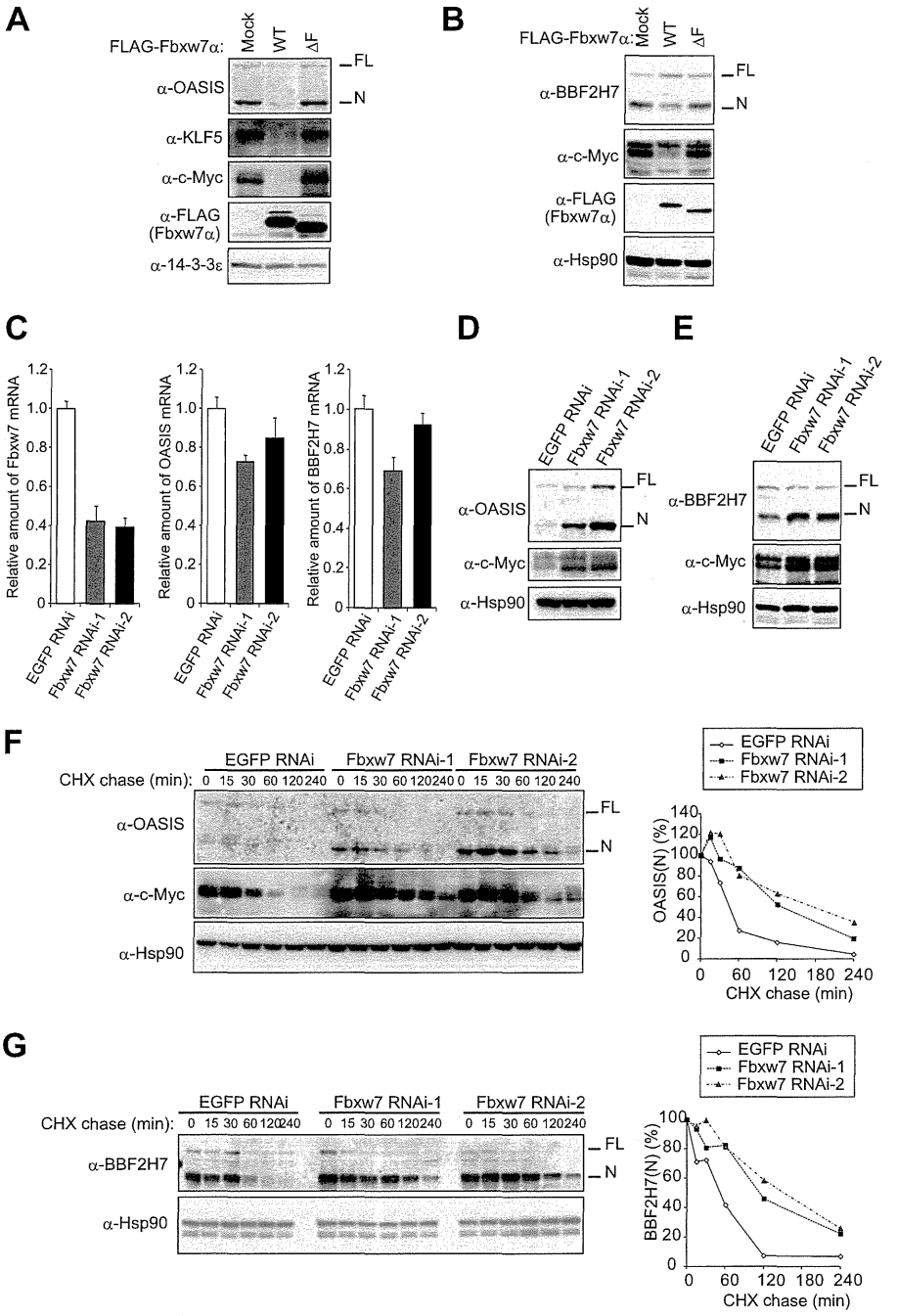


FIGURE 3. **Fbxw7 controls the stability of OASIS and BBF2H7.** *A* and *B*, immunoblot analysis of OASIS (*A*) and BBF2H7 (*B*) as well as of KLF5 and c-Myc in mCAT-HeLa cells stably infected with retroviruses encoding FLAG-tagged WT or ΔF mutant forms of Fbxw7α. *C*, RT and real-time PCR analysis of Fbxw7, OASIS, and BBF2H7 mRNAs in mCAT-HeLa cells stably infected with retroviruses encoding Fbxw7 or enhanced GFP (control) shRNAs. Normalized data are expressed relative to the corresponding value for control cells and are mean ± S.D. from three independent experiments. *D* and *E*, immunoblot analysis of OASIS (*D*) and BBF2H7 (*E*) as well as of c-Myc in mCAT-HeLa cells stably infected with retroviruses encoding Fbxw7 or enhanced GFP (control) shRNAs. *F* and *G*, cycloheximide (*CHX*) chase analysis of OASIS and c-Myc (*F*) and of BBF2H7 (*G*) in mCAT-HeLa cells depleted of Fbxw7. The percentage of endogenous OASIS(N) or BBF2H7(N) remaining after the various chase times was quantitated with ImageJ software.

introduced alanine mutations into this motif. Immunoprecipitation analysis revealed that a mutant (T207A/S211A) form of OASIS that contains two point mutations in the CPD (Fig. 5*A*) did not interact with Fbxw7α (Fig. 5*C*). Furthermore, cycloheximide chase analysis revealed that the N form of this mutant was more stable than that of WT OASIS in transfected HeLa cells (Fig. 5*E*). A similar mutant (T204A/S208A) of BBF2H7 also did not bind to Fbxw7α (Fig. 5*D*), and the N form of this mutant was more stable than that of the WT protein (Fig. 5*F*).

To verify that the CPD motifs of OASIS and BBF2H7 are phosphorylated, we investigated the phosphorylation status of FLAG-OASIS and FLAG-BBF2H7 in HeLa cells by LC-MS/MS analysis. Many peptides derived from FLAG-OASIS(FL) and FLAG-BBF2H7(FL) were highly phosphorylated (at least 22 phosphorylated sites in each protein) (Fig. 6, *A* and *B*, and supplemental Table S1). In particular, we detected phosphorylation of the CPD in BBF2H7, with Thr^{204} and either Ser^{207} or Ser^{208} being phosphorylated. We did not detect phosphoryla-

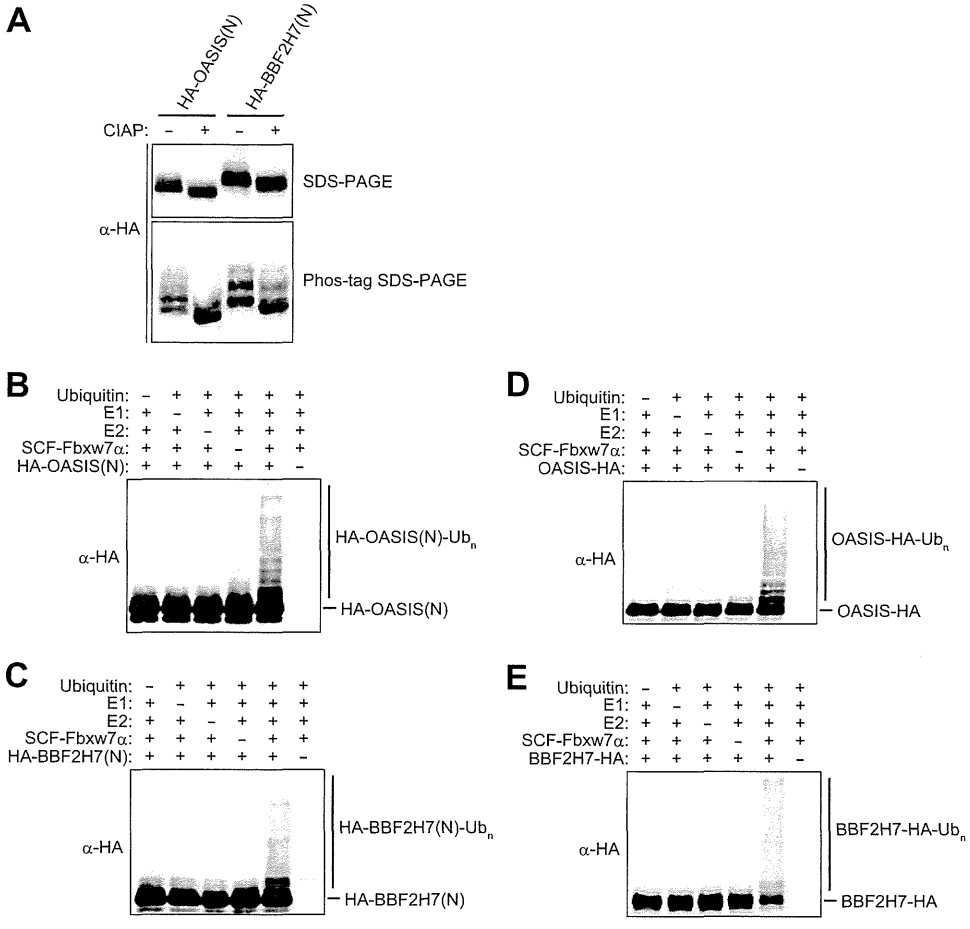


FIGURE 4. **Fbxw7 mediates the ubiquitylation of OASIS and BBF2H7.** *A*, HA-tagged forms of OASIS(N) or BBF2H7(N) purified from Sf21 insect cells were incubated with or without calf intestinal alkaline phosphatase (*CIAP*, 1 units/μl) for 3 h at 37 °C before conventional or Phos-tag SDS-PAGE followed by immunoblot analysis with antibodies to HA. *B* and *C*, HA-tagged forms of OASIS(N) (*B*) or BBF2H7(N) (*C*) were subjected to an *in vitro* ubiquitylation assay with immunopurified $SCF^{Fbxw7\alpha}$ as well as with E1, E2, ATP, and ubiquitin, as indicated. Reaction mixtures were subjected to immunoblot analysis with antibodies to HA. The positions of unmodified and polyubiquitylated (Ub_n) forms of OASIS(N) and BBF2H7(N) are indicated. *D* and *E*, HA-tagged full-length forms of OASIS (*D*) or BBF2H7 (*E*) were subjected to an *in vitro* ubiquitylation assay as in *B* and *C*.

tion of the CPD of OASIS, however. Therefore, we generated a phosphomimetic mutant of OASIS by replacing Thr^{207} and Ser^{211} with Glu (T207E/S211E) and examined its stability in cells depleted of endogenous Fbxw7 by RNAi (Fig. 6*C*). We detected a small but reproducible increase in the rate of degradation for the N form of the HA-tagged mutant compared with that for the N form of the HA-tagged WT protein. Together, these observations suggested that phosphorylation of the CPD of OASIS and BBF2H7 is required for the interaction of these proteins with Fbxw7 and their consequent degradation.

Fbxw7 Inhibits Osteogenesis by Mediating the Degradation of OASIS—OASIS and BBF2H7 participate in mesenchymal differentiation, being essential for osteogenesis and chondrogenesis, respectively. OASIS controls osteoblast differentiation through activation of Col1A1 gene transcription (37). We examined changes in the abundance of Fbxw7, OASIS, and Col1A1 mRNAs during the osteogenic differentiation of mouse C2C12 mesenchymal cells induced by treatment with BMP2 (Fig. 7*A*). The amount of Fbxw7 mRNA increased slightly during the first 2 days of the differentiation process and then declined. In contrast, the abundance of OASIS and Col1A1 mRNAs was increased substantially after 2 days and remained at this high level until the end of the 6-day culture period.

We next examined the effect of Fbxw7 depletion on the differentiation of C2C12 cells into osteoblasts. RNAi-mediated depletion of Fbxw7 resulted in the accumulation of OASIS in C2C12 cells and markedly increased both collagen deposition and Col1A1 gene expression (Fig. 7, *B–D*). Additional depletion of OASIS greatly attenuated these latter effects of Fbxw7 depletion, suggesting that they were mediated by up-regulation of OASIS expression. Conversely, overexpression of Fbxw7α in C2C12 cells resulted in a decrease in the abundance of Col1A1 mRNA, whereas forced expression of Fbxw7α(ΔF) had no such effect (Fig. 7*E*). Thus, these data suggest that Fbxw7 inhibits osteogenesis by promoting the degradation of OASIS and the consequent attenuation of collagen deposition.

Fbxw7 Controls Chondrogenesis by Targeting Notch and BBF2H7 for Degradation—Similar to the role of OASIS in osteogenesis, BBF2H7 controls chondrogenesis through transcriptional activation of the Sec23a gene. We examined changes in the abundance of Fbxw7, BBF2H7, and Sec23a mRNAs during chondrogenesis in micromass cultures of mesenchymal cells isolated from mouse embryos (Fig. 8*A*). The amount of Fbxw7 mRNA was increased after 2 days of culture, but it had returned to the original level at day 4 and had declined slightly below this level at day 6. The abundance of BBF2H7 and

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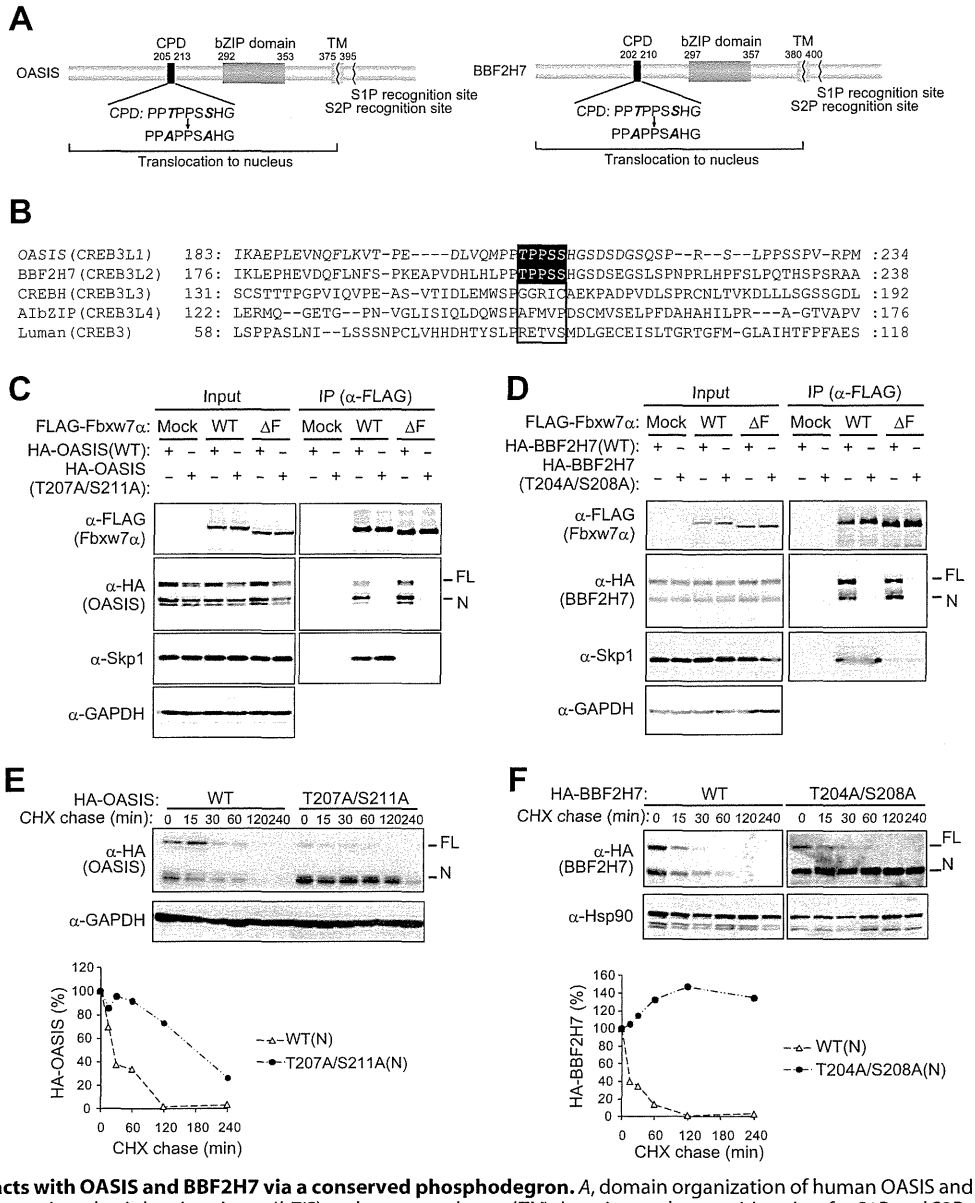


FIGURE 5. **Fbxw7 interacts with OASIS and BBF2H7 via a conserved phosphodegron.** *A*, domain organization of human OASIS and BBF2H7. Potential CPD sequences, introduced mutations, basic leucine zipper (*bZIP*) and transmembrane (*TM*) domains, and recognition sites for S1P and S2P are shown. *B*, alignment of amino acid sequences of human CREB3 and CREB3L family members. Potential CPDs and their corresponding sequences are *boxed*. *C* and *D*, effects of CPD mutation on the binding of OASIS or BBF2H7 to Fbxw7α. Lysates of mCAT-HeLa cells expressing the HA-tagged forms of OASIS (*C*) or BBF2H7 (*D*), or of their CPD mutants, together with the FLAG-tagged WT or ΔF mutant forms of Fbxw7α were subjected to immunoprecipitation (*IP*) with antibodies to FLAG, and the resulting precipitates were subjected to immunoblot analysis with the indicated antibodies. *E* and *F*, cycloheximide (*CHX*) chase analysis of HA-tagged forms of OASIS (*E*) or BBF2H7 (*F*), or of their CPD mutants, in mCAT-HeLa cells. The percentages of HA-tagged OASIS(N) and BBF2H7(N) proteins remaining after the various chase times were quantitated with ImageJ software.

Sec23a mRNAs was increased after days 2 and 4, respectively, but had declined substantially at days 6 and 8, respectively, consistent with previous observations (38).

We next investigated the effect of Fbxw7 deficiency on the formation of cartilage by the micromass cultures. Conditional ablation of floxed (F) *Fbxw7* alleles (45) by treatment of *CAG-CreERT2;Fbxw7*$^{F/F}$ cells with tamoxifen at the start of the culture resulted in attenuation of chondrogenesis, as revealed by staining with Alcian blue after culture for 8 days (Fig. 8*B*). Immunoblot analysis of Fbxw7-depleted chondrocytes after culture for 4 days revealed accumulation of the phosphorylated form of the intracellular domain of Notch1 (*NICD1*), another target of Fbxw7, but not that of BBF2H7 (Fig. 8*C*). Notch suppresses the differentiation and proliferation of early chondrogenic cells (46), suggesting that the accumulation of Notch1 induced by depletion of Fbxw7 might inhibit chondrocyte differentiation.

To eliminate any effect of Notch1 accumulation induced by *Fbxw7* ablation during early chondrogenesis, we depleted Fbxw7 2 days after the onset of chondrocyte differentiation and exposed the cells from day 0 to DAPT, an inhibitor of γ-secretase activity that is necessary for Notch activation. Under these conditions, ablation of *Fbxw7* resulted in a slight enhancement of chondrogenesis compared with that apparent in control cells (Fig. 8*D*), and it triggered a substantial increase in the abundance of BBF2H7(N) (*E*). We next examined the effect of

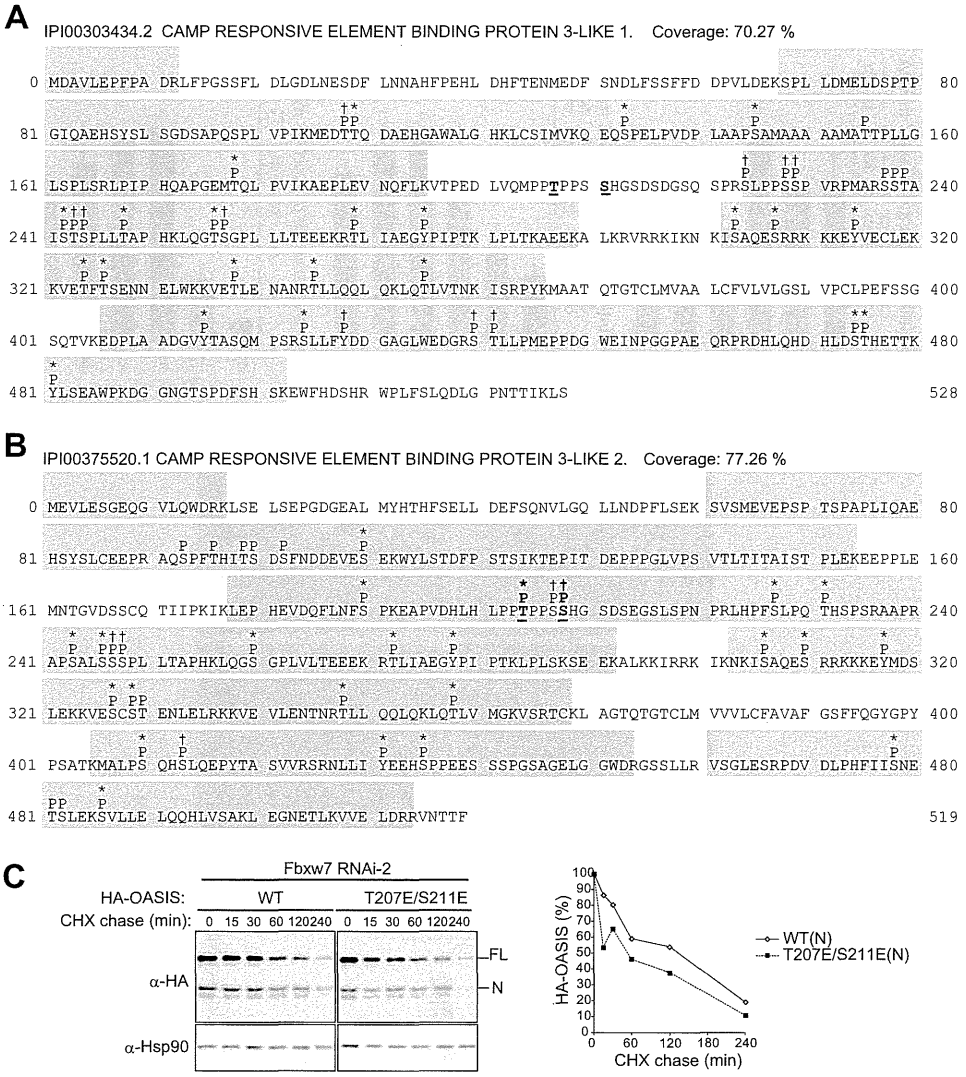


FIGURE 6. **Phosphorylation of OASIS and BBF2H7 expressed in mCAT-HeLa cells.** *A* and *B*, phosphorylation sites of OASIS (*A*) and BBF2H7 (*B*) identified by LC-MS/MS analysis of FLAG-tagged forms of the human proteins expressed in and purified from mCAT-HeLa cells. Potential phosphorylated residues (*P*) are indicated above the amino acid sequences. *Asterisks* and † indicate phosphorylation probabilities of > 99% and 45–99%, respectively. Phosphorylation sites within the CPD of OASIS or BBF2H7 are shown in boldface and *underlined*. *C*, cycloheximide (*CHX*) chase analysis of the HA-tagged WT and phosphomimetic mutant (T207E/S211E) forms of OASIS in mCAT-HeLa cells depleted of Fbxw7. The percentages of HA-tagged OASIS(N) proteins remaining after the various chase times were quantitated with ImageJ software.

Fbxw7α overexpression on chondrogenesis in murine chondrogenic ATDC5 cells. Forced expression of Fbxw7α resulted in marked inhibition of chondrogenesis in ATDC5 cells (Fig. 8, *F* and *G*), an effect that was accompanied by down-regulation of BBF2H7(N) (*H*). Expression of Fbxw7α(ΔF) had no such effects, suggesting that they were attributable to the ubiquitin ligase activity of Fbxw7α. Together, these results indicate that Fbxw7 regulates chondrogenesis both positively and negatively by promoting the degradation of Notch and BBF2H7, respectively.

DISCUSSION

With the use of DiPIUS, an unbiased approach to the identification of substrates for specific ubiquitin ligases, we found that OASIS and BBF2H7 are substrates of Fbxw7. Validation analyses confirmed that OASIS and BBF2H7 are *bona fide* substrates of Fbxw7, and we further found that Fbxw7 plays key roles in bone and cartilage formation by controlling the levels of OASIS and BBF2H7, respectively.

Our analysis of changes in the abundance of Fbxw7 mRNA during osteogenic and chondrocytic differentiation suggested that Fbxw7 might antagonize osteogenesis and chondrogenesis by promoting the degradation of OASIS and BBF2H7, respectively, predominantly during the early phase of such differentiation. Subsequent down-regulation of Fbxw7 during the later stages of differentiation might allow the up-regulation of Col1A1 and Sec23a and, thereby, promote the production of bone and cartilage matrix proteins by osteoblasts and chondrocytes, respectively. The accumulation of these matrix proteins in the ER may induce ER stress and, thereby, trigger the cleavage and activation of OASIS and BBF2H7. Such a sequence of events would constitute a positive feedback loop, suppression of which by Fbxw7 might impede the premature activation of the Col1A1 and Sec23a genes and, thereby, prevent excessive accumulation of matrix proteins before cell maturation.

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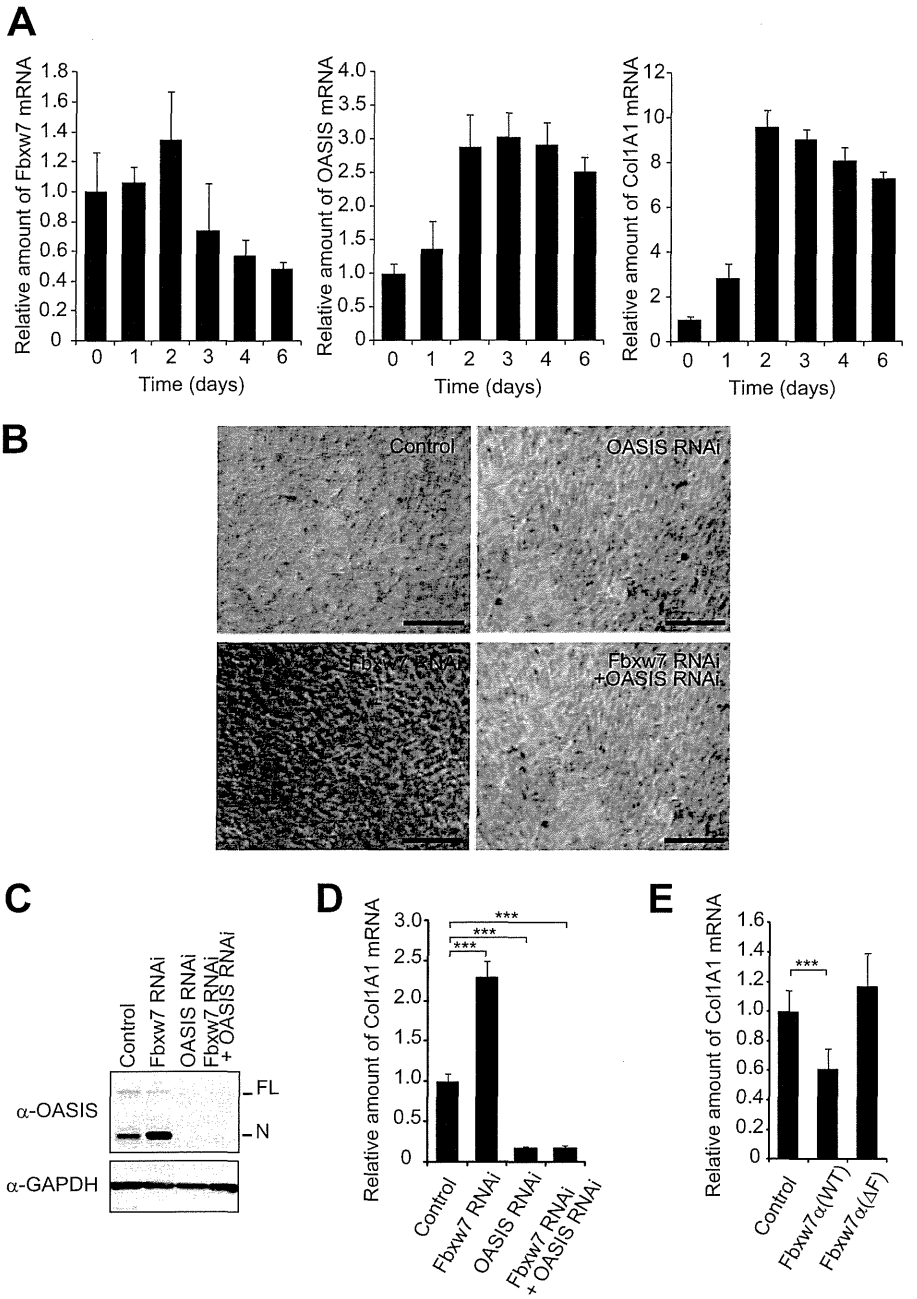


FIGURE 7. **Fbxw7 inhibits osteogenesis by promoting the degradation of OASIS.** *A*, RT and real-time PCR analysis of Fbxw7, OASIS, and Col1A1 mRNAs in C2C12 cells at the indicated times after induction of differentiation into osteoblasts. *B–D*, C2C12 cells depleted of Fbxw7, OASIS, or both proteins by RNAi were induced to differentiate into osteoblasts and then either stained with Sirius red to visualize collagen accumulation (*B*), subjected to immunoblot analysis with antibodies to OASIS (*C*), or assayed for the relative amount of Col1A1 mRNA (*D*). *Scale bars* = 100 μm (*B*). *E*, relative abundance of Col1A1 mRNA in C2C12 cells expressing ectopic WT or ΔF mutant forms of Fbxw7α and induced to differentiate into osteoblasts. Data in *A*, *D*, and *E* are mean ± S.D. from three independent experiments. ***, $p < 0.001$ (Tukey-Kramer multiple-comparison test).

In addition to osteogenesis and chondrogenesis, Fbxw7 controls adipogenesis by promoting the degradation of KLF5, SREBPs, and c/EBPα (29–32). Inactivation of Fbxw7 in mouse preadipocytes or adult human mesenchymal stem cells promotes their differentiation into mature adipocytes (31, 32). Genetic ablation or RNAi-mediated depletion of Fbxw7 in mouse liver induced fatty liver, reminiscent of that associated with nonalcoholic steatohepatitis in humans (29, 30). Fbxw7 also targets peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) for degradation (47), although the relationship between Fbxw7 and PGC-1α in adipogenesis or lipogenesis remains to be determined. Therefore, we therefore propose that Fbxw7 inhibits mesenchymal differentiation and maturation by concomitant targeting of multiple transcriptional factors for degradation and that it may, therefore, contribute to the maintenance of mesenchymal stem cells (Fig. 9). The biological relevance of Fbxw7 in each differentiation pathway appears to be determined by the expression of corresponding substrates and by the expression and activation of protein kinases that phosphorylate the CPDs of these proteins.

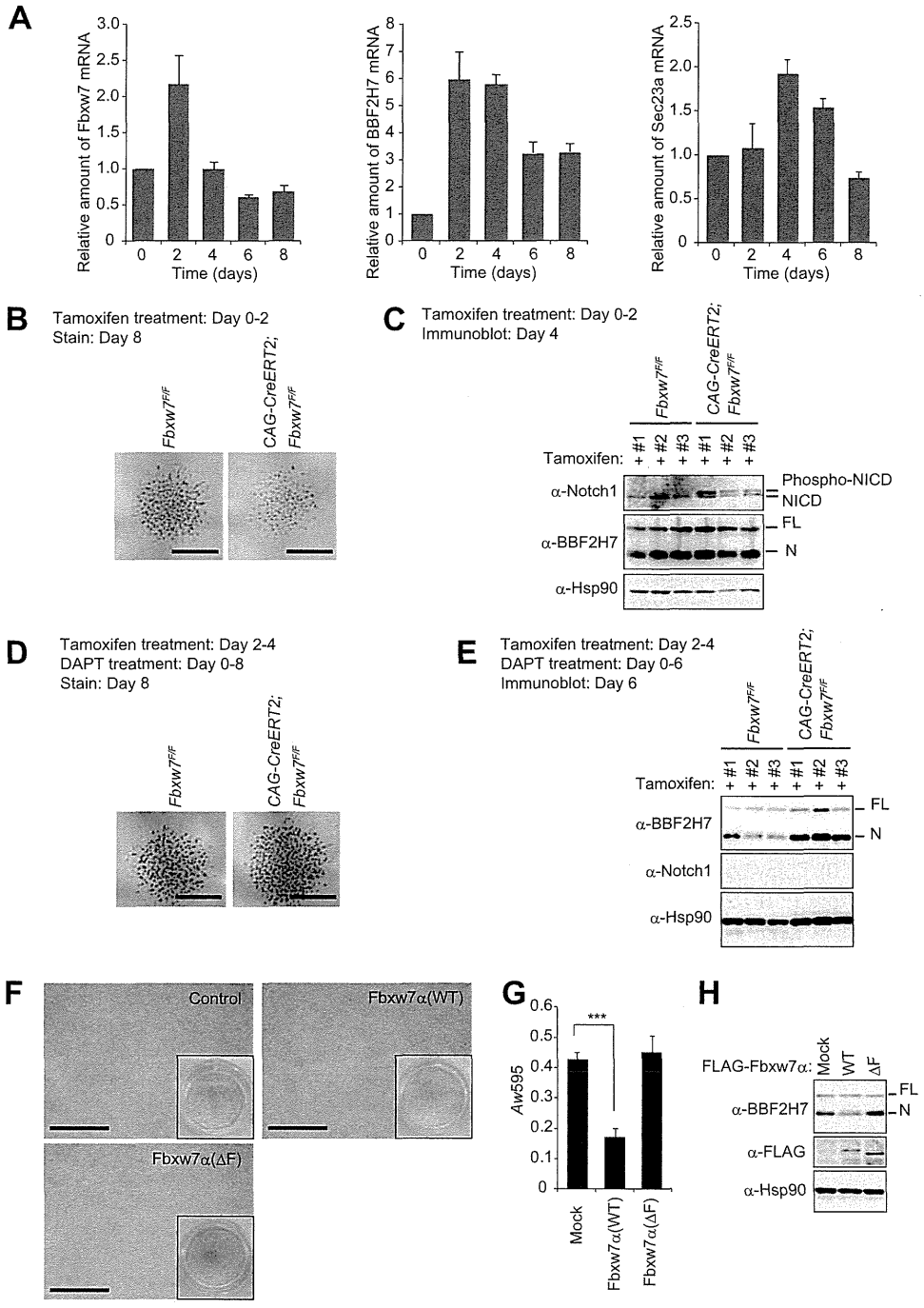


FIGURE 8. **Fbxw7 controls chondrogenesis by targeting Notch and BBF2H7 for degradation.** *A*, RT and real-time PCR analysis of Fbxw7, BBF2H7, and Sec23a mRNAs in mesenchymal cells isolated from mouse embryos and maintained as micromass cultures for the indicated times. Data are mean ± S.D. from three independent experiments. *B* and *C*, mesenchymal cells isolated from *CAG-CreERT2;Fbxw7*$^{F/F}$ or control (*Fbxw7*$^{F/F}$) mouse embryos and maintained as micromass cultures were exposed to tamoxifen for the initial 2 days of culture and subjected to staining with Alcian blue after 8 days (*B*) or to immunoblot analysis of Notch1 and BBF2H7 after 4 days (*C*). Immunoblot data are shown for cultures derived from three different embryos. *D* and *E*, mesenchymal cells isolated from *CAG-CreERT2; Fbxw7*$^{F/F}$ or control (*Fbxw7*$^{F/F}$) mice and maintained as micromass cultures in the presence of 1 μM DAPT were exposed to tamoxifen for 2 days, beginning 2 days after the onset of culture. The cells were stained with Alcian blue after 8 days (*D*) or subjected to immunoblot analysis of Notch1 and BBF2H7 after 6 days (*E*). *F–H*, ATDC5 cells expressing the FLAG-tagged WT or ΔF mutant forms of Fbxw7α were induced to differentiate into chondrocytes by exposure to 1 μM DAPT and were then either stained with Alcian blue for visualization (*F*) or absorbance-based measurement (*G*) of the accumulation of acid mucosubstances or subjected to immunoblot analysis with antibodies to BBF2H7 or to FLAG (*H*). The *insets* in *F* show low-magnification images of the culture plates. Quantitative data (*G*) are means ± S.D. from three independent experiments. ***, $p < 0.001$ (Tukey-Kramer multiple-comparison test). *Scale bars* = 1 mm (*B* and *D*) or 100 μm (*F*).

The regulation of certain transcriptional factors in response to specific conditions depends on their localization. Under normoxic conditions, hypoxia-inducible factor 1α (HIF1α) is localized to the cytoplasm, where it undergoes oxygen-dependent proline hydroxylation followed by von Hippel-Lindau protein-dependent proteasomal degradation. During hypoxia, however, the proline hydroxylases are inactive, and HIF1α is able to translocate to the nucleus and induce the transcription of genes related to oxygen homeostasis and metabolism (48). Notch proteins are transcriptional factors that contain both EGF-like

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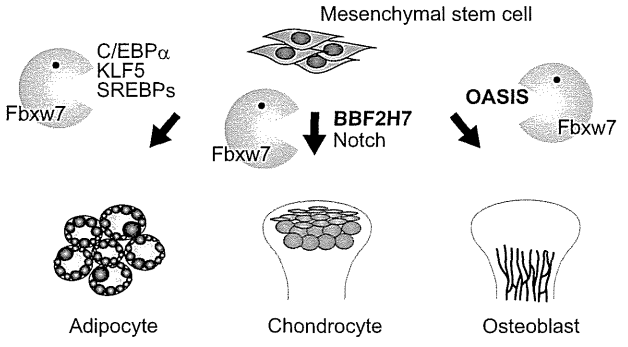


FIGURE 9. **Fbxw7 controls mesenchymal differentiation.** Fbxw7 mediates ubiquitin-dependent degradation of substrates that contribute to adipogenesis, chondrogenesis, and osteogenesis. Thus, it inhibits adipogenesis by targeting C/EBPα, KLF5, and SREBPs for degradation. We show that Fbxw7 also regulates chondrogenesis and osteogenesis by promoting the degradation of Notch, BBF2H7, and OASIS, respectively.

extracellular domains and cytoplasmic regions. The binding of a Notch ligand to the EGF-like extracellular domain triggers Notch cleavage both outside and within the transmembrane domain, resulting in the nuclear translocation of the cleaved Notch intracellular domain and its association with the constitutive DNA-binding protein RBP-Jκ (49). Finally, SREBPs are localized at the ER membrane under normal conditions. In response to a reduction in the intracellular level of cholesterol, however, they move to the Golgi complex, where they are cleaved sequentially by two proteases. These cleavage steps release the mature form of SREBPs, which enters the nucleus and activates the transcription of genes related to cholesterol and fatty acid metabolism (50). Fbxw7 targets nuclear forms of all of these transcriptional factors (HIF1α, Notch, and SREBPs) as well as those of OASIS and BBF2H7 for degradation (5, 12, 13, 31, 51, 52). Fbxw7 might, therefore, function to turn off the transactivation activity of these factors in the nucleus, thereby contributing to the strict regulation of transcription in response to various stimuli or conditions.

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

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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).

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

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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.

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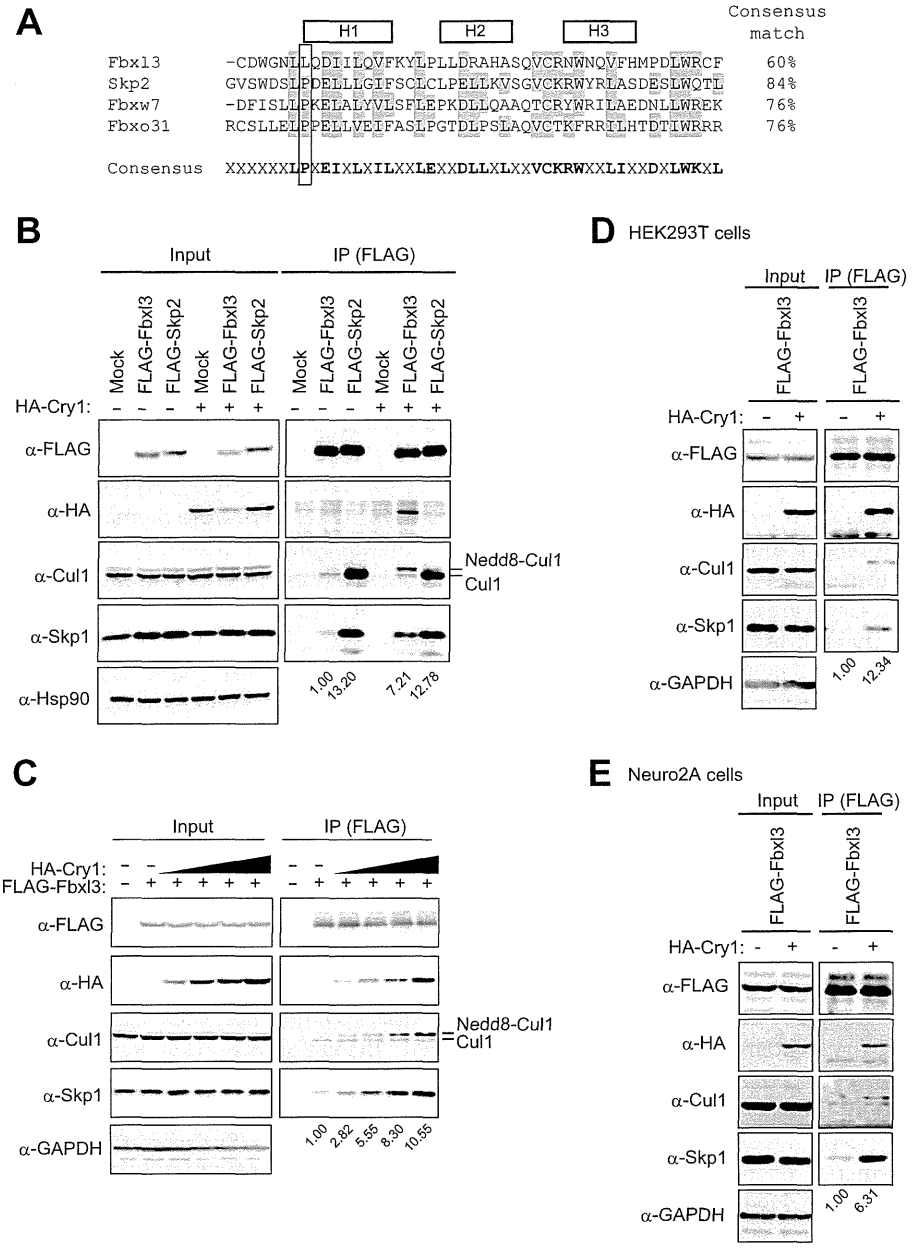


FIGURE 1. **Formation of the SCF^{Fbxl3} complex is dependent on the expression level of Cry1.** *A*, amino acid sequences of the F-box domains of human Fbxl3, Skp2, Fbxw7, and Fbxo31 are aligned. *Gray shading* highlights residues similar or identical to those of the consensus sequence. The general 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.

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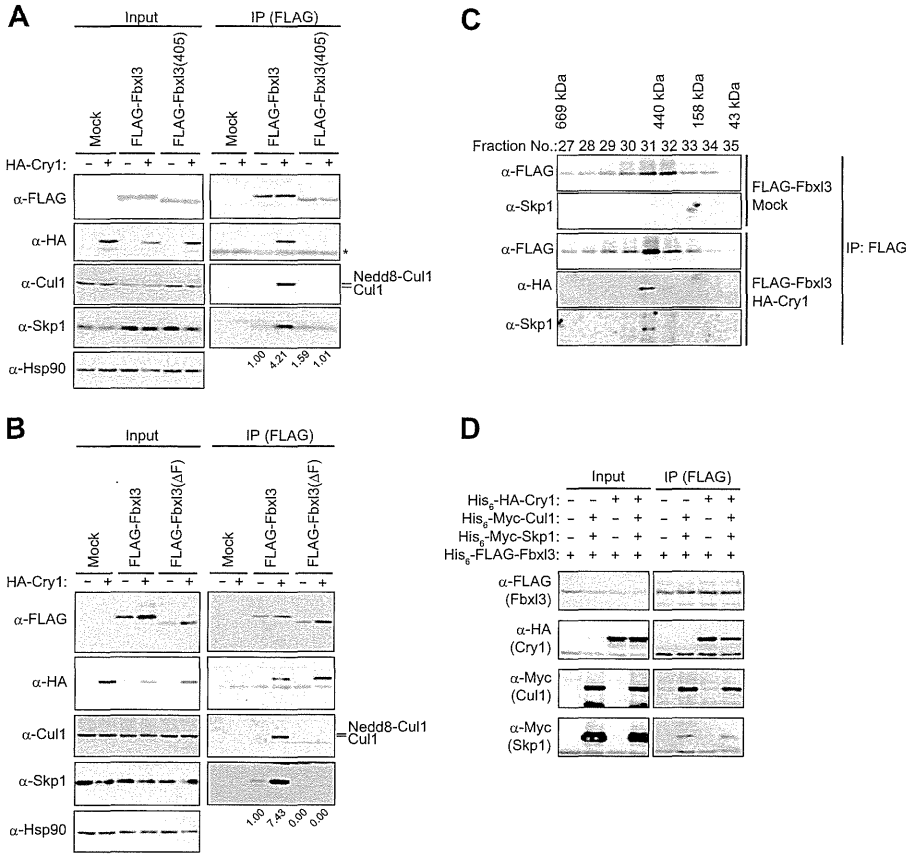


FIGURE 2. **The interaction of Cry1 with Fbxl3 is required for formation of the SCF^{Fbxl3} complex.** *A*, lysates of HeLa cells expressing FLAG-tagged forms of Fbxl3 or Fbxl3(405) as well as HA-tagged Cry1 were subjected to immunoprecipitation (*IP*) with anti-FLAG, and the resulting precipitates, 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

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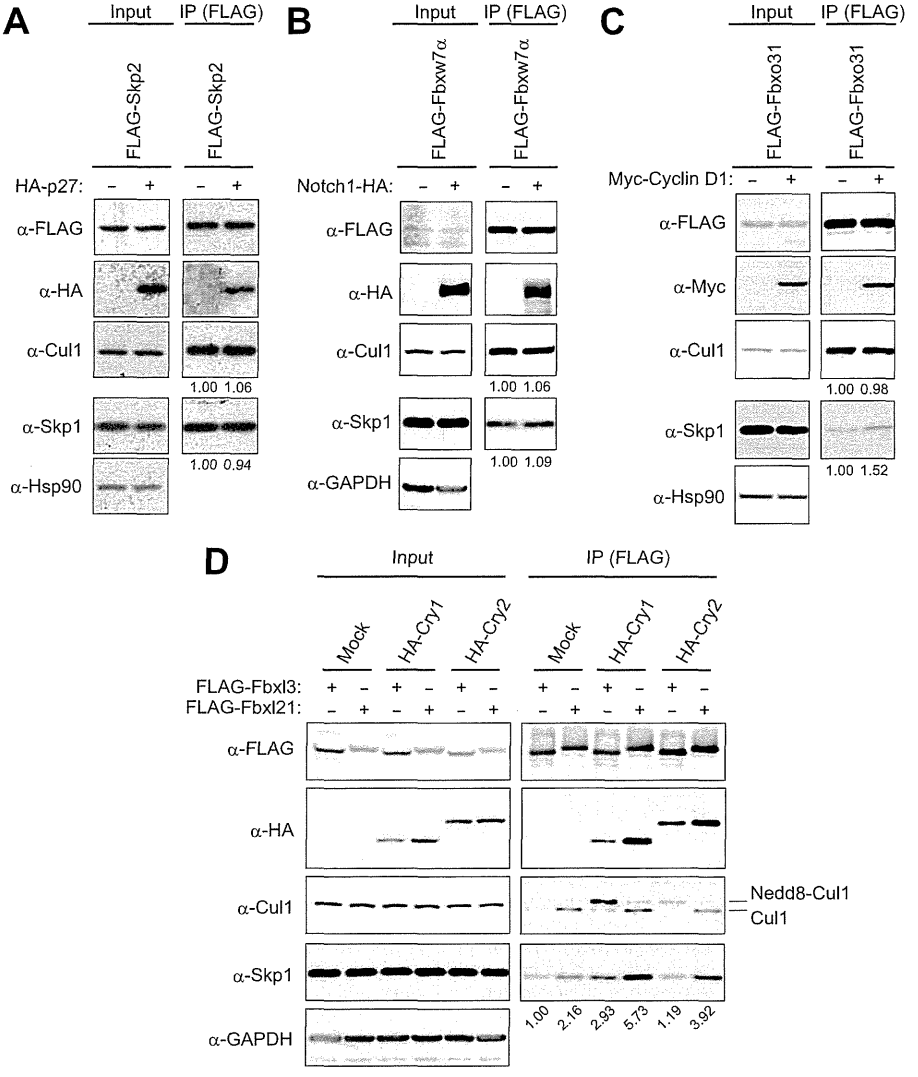


FIGURE 3. **Substrate-dependent formation of an SCF complex by Fbxl3 is specific to this F-box protein.** *A–C*, lysates of HeLa cells expressing FLAG-tagged Skp2 and HA-tagged p27 (*A*), FLAG-tagged Fbxw7α and the HA-tagged intracellular domain of Notch1 (*B*), or FLAG-tagged Fbxo31 and Myc epitope-tagged 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

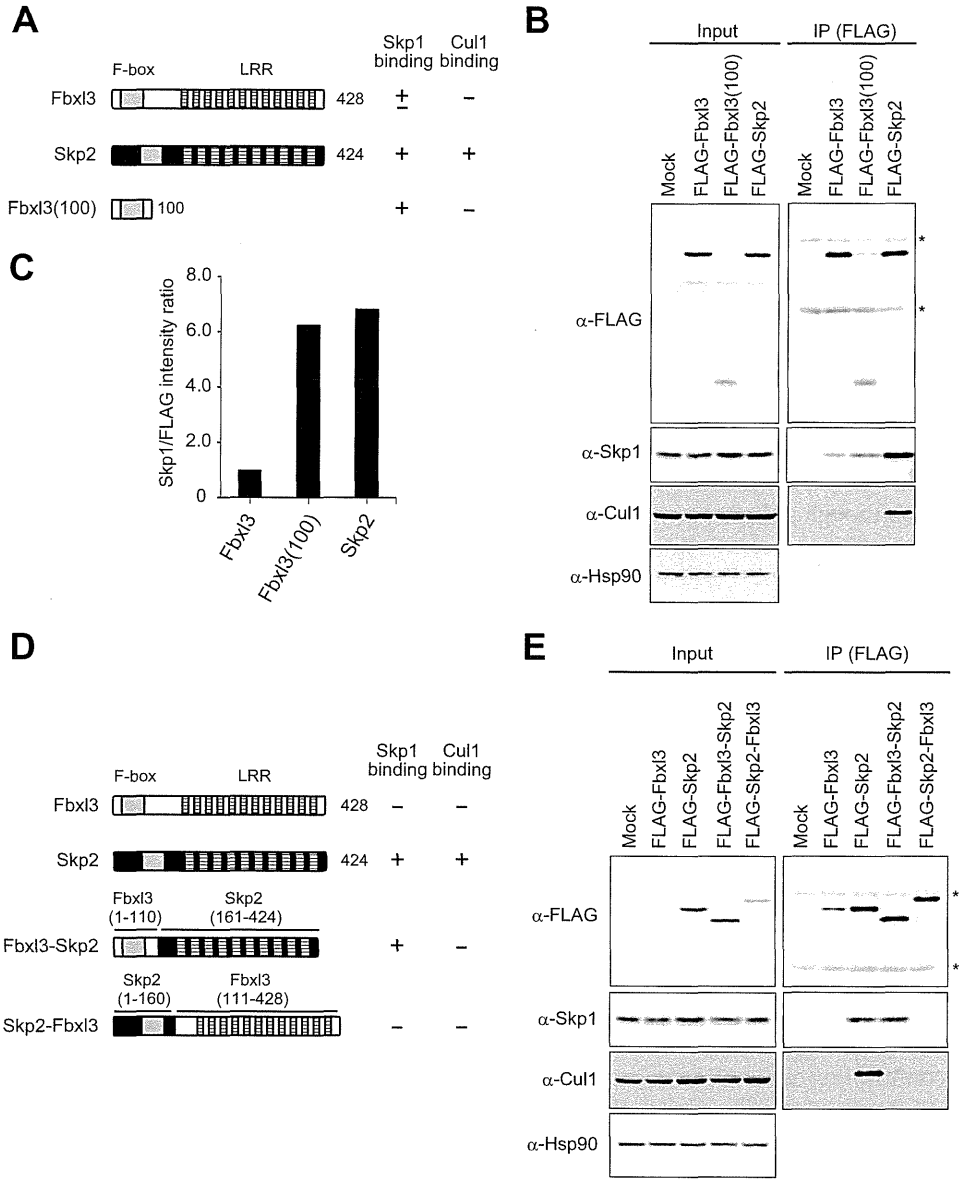


FIGURE 4. **The COOH-terminal LRR domain of Fbxl3 inhibits interaction with Skp1.** *A*, schematic represents human Fbxl3, Skp2, and the Fbxl3(100) deletion mutant and presents a summary of their abilities to bind to Skp1 and Cul1 as determined in *B* and *C*. *B* and *C*, lysates of HeLa cells expressing 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

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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.

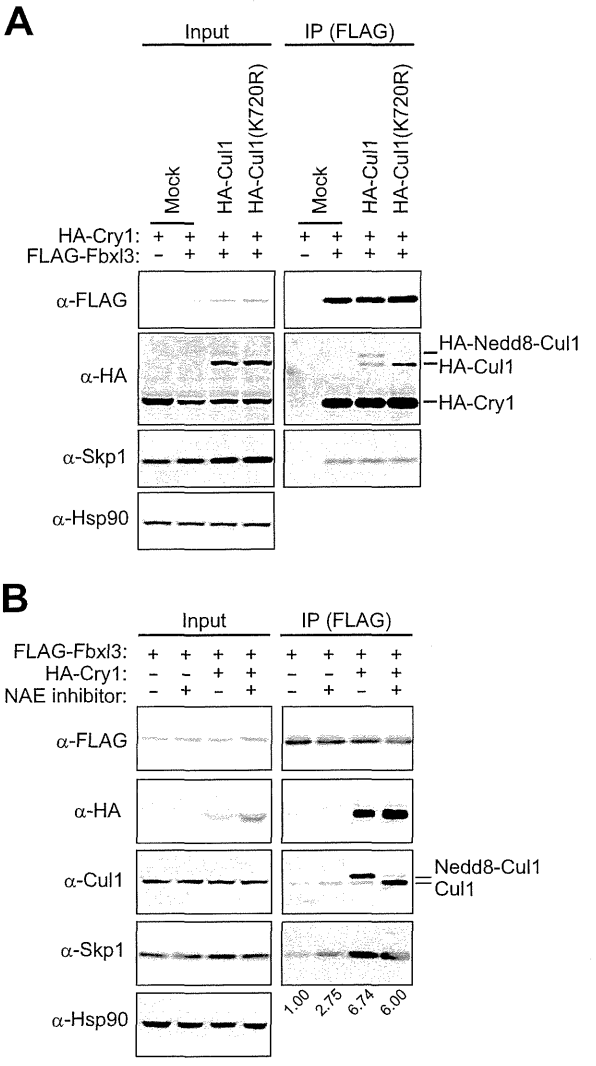


FIGURE 5. **Neddylation of Cul1 is not required for assembly of SCF^{Fbxl3}.** *A*, lysates of HeLa cells expressing FLAG-tagged Fbxl3 as well as HA-tagged forms of Cry1, Cul1, or Cul1(K720R) were subjected to immunoprecipitation (*IP*) with anti-FLAG, and the resulting precipitates, as well as the original 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-

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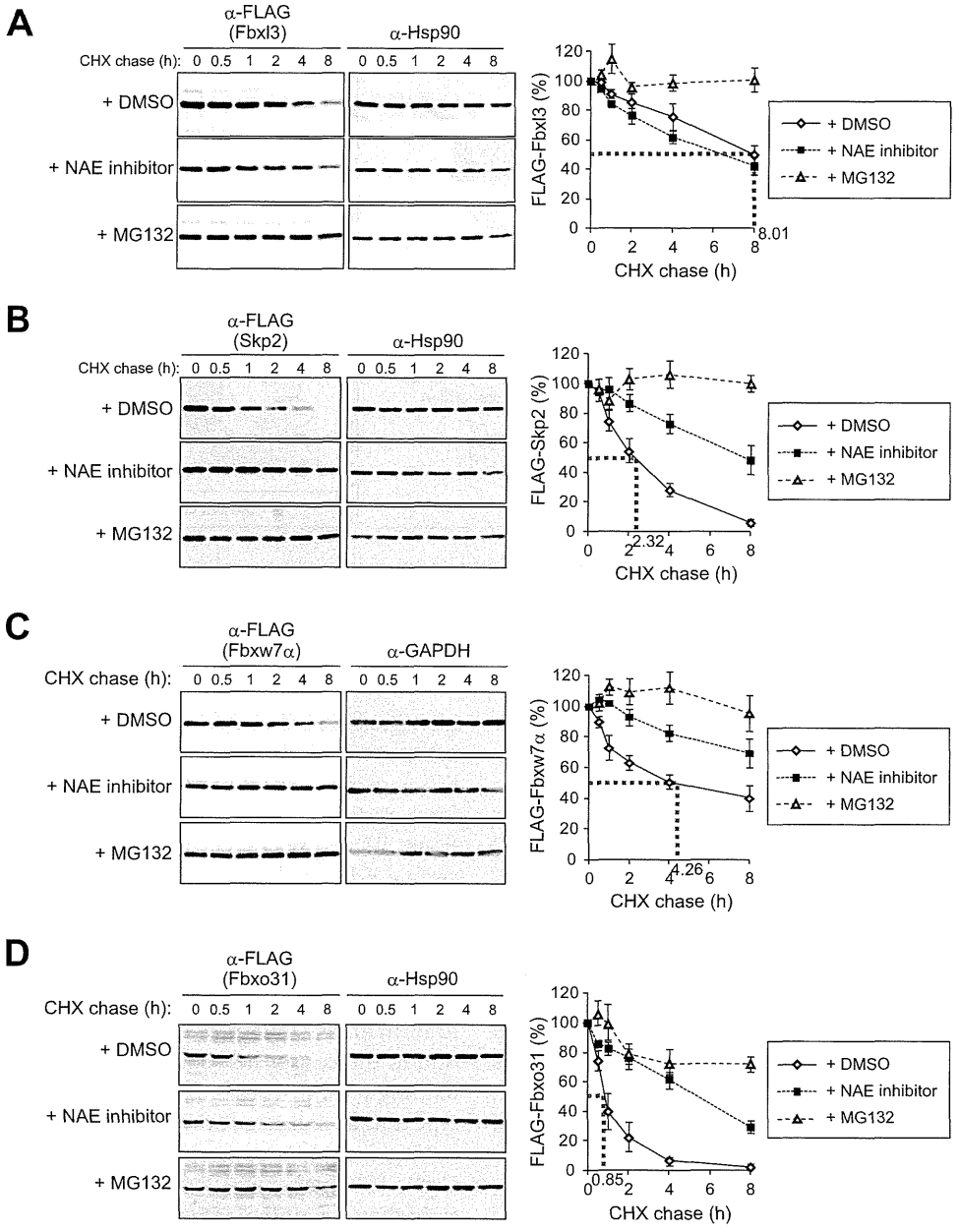


FIGURE 6. **Fbxl3 is more stable than other F-box proteins.** HeLa cells expressing FLAG-tagged forms of Fbxl3 (*A*), Skp2 (*B*), Fbxw7α (*C*), or Fbxo31 (*D*) were exposed to cycloheximide (*CHX*) for the indicated times in the presence of NAE inhibitor, MG132, or dimethyl sulfoxide (*DMSO*) vehicle, 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-

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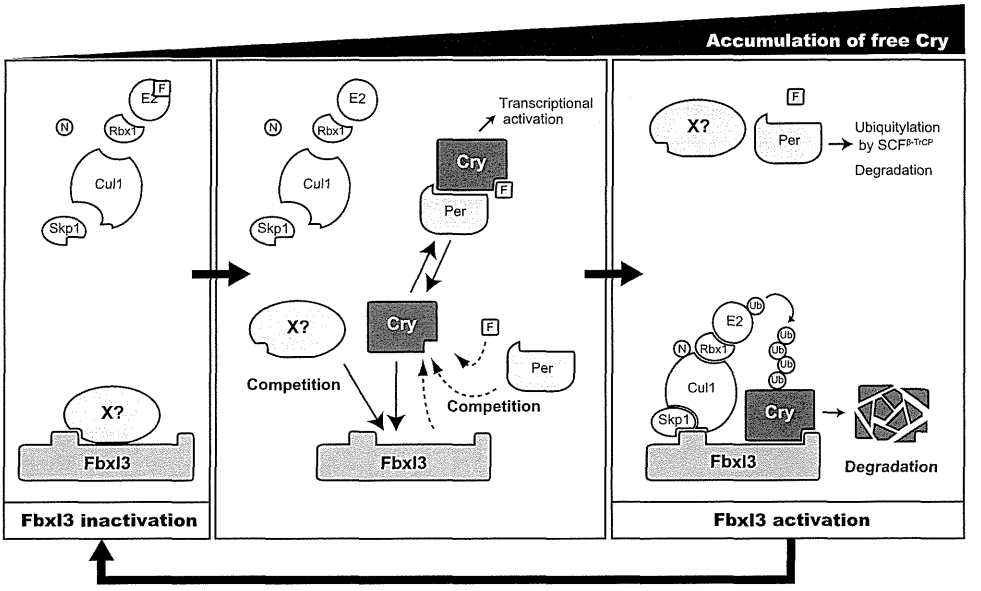


FIGURE 7. **Model for regulation of SCF^{Fbxl3} complex formation.** Fbxl3 essentially does not form an SCF complex in the absence of its association with Cry1 (*left*), given that it appears to associate with an as yet unknown protein (*X*) that interferes with its interaction with Skp1-Cul1 in mammalian 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

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

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Regular Article

LYMPHOID NEOPLASIA

p57 regulates T cell development and prevents lymphomagenesis by balancing p53 activity and pre-TCR signaling

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Key Points

- Ablation of p57 in T cells blocks differentiation at an early developmental stage as a result of excessive activation of E2F.
- Additional ablation of E2F1 or p53 normalizes p57-deficiency phenotypes, but loss of both p57 and p53 eventually results in thymic lymphoma.

T cells are key components of the immune system, playing a central role in cell-mediated immunity. The sequential differentiation of T cells is associated with strict regulation of the cell cycle at each developmental stage. A balance between p53 activity and pre–T cell receptor (TCR) signaling regulates proliferation and differentiation decisions made by these cells. The relation between maintenance of this balance and the function of cell cycle regulators has remained largely unknown, however. We now show that mice with T cell–specific deficiency of the cyclin-dependent kinase inhibitor p57 manifest a differentiation block at the early stage of T cell maturation. Further genetic analysis showed that this defect is attributable to an imbalance between p53 activity and pre-TCR signaling caused by hyperactivation of the E2F-p53 pathway. Moreover, ablation of both p57 and p53 in T cells led to the development of aggressive thymic lymphomas with a reduced latency compared with that apparent for p53-deficient mice, whereas ablation of p57 alone did not confer susceptibility to this hematologic malignancy. Our results thus show that the p57-E2F-p53 axis plays a pivotal role in the proper development of T cells as well as in the prevention of lymphomagenesis. (*Blood.* 2014;00(00):1-11)

Introduction

Development and maturation of T lymphocytes in the thymus are essential for establishment of the peripheral immune system. Most immature T cells in the thymus initially neither express CD4 nor CD8, and they are therefore referred to as double-negative (DN) cells. This DN population can be further divided into 4 subsets (DN1 to DN4) defined by the sequential pattern of expression of CD44 and CD25: $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3), and $CD44^-CD25^-$ (DN4).[1] This maturation of T cells requires a sequence of events that is closely associated with rearrangement of the T cell receptor (TCR) genes and signaling through this receptor. Activation of DN3 cells by pre-TCR–dependent signaling, referred to as the β-selection checkpoint, results in the generation of DN4 cells, which, in turn, differentiate into $CD4^+CD8^+$ (double-positive, or DP) cells via a transient $CD8^+$ (immature single-positive, or ISP) stage.[2] Subsequent downregulation of either CD4 or CD8 in DP cells gives rise to mature single-positive (SP) cells.[3] Differentiation of DN3/DN4 cells into DP cells is blocked by a defect in pre-TCR signaling resulting from genetic ablation of RAG1 or RAG2, DNA-dependent protein kinase (DNA-PK), or the CD3γ chain, and this block is prevented by concurrent loss of p53, suggesting that the β-selection checkpoint at the DN3 stage depends on a balance between pre-TCR signaling and p53 activation.[4-6] The precise mechanism of such p53 activation remains largely unclear, however.

Progression of the cell cycle is controlled by pairs of cyclins and cyclin-dependent kinases (CDKs).[7] CDKs normally inactivate the retinoblastoma protein (Rb) by hyperphosphorylation and thereby activate E2F transcription factors[8] that play a key role in cell cycle progression by regulating the transcription of genes that contribute to this process.[9] However, excessive E2F activity induces p53-dependent or p53-independent apoptosis in a manner dependent on cellular context.[10-13]

The activity of cyclin-CDK pairs is under the control of negative cell cycle regulators that belong to the Cip/Kip family of CDK inhibitors. Members of the Cip/Kip family, including $p21^{Cip1}$ (p21), $p27^{Kip1}$ (p27), and $p57^{Kip2}$ (p57), mainly target CDK2 and CDK4 (and CDK1 in some situations) for inhibition.[14] Among these members, p21 is dispensable for T cell development.[15] Loss of p27 in mice results in an increase in overall animal growth as well as the development of pituitary tumors and hyperplasia of multiple organs including the thymus.[16-18] Unlike p21- or p27-deficient mice, mice lacking p57 die immediately after birth, manifesting severe developmental defects,[12,19,20] which has hindered the functional characterization of p57-deficient T cells.

In the present study, we established mice in which the p57 gene is conditionally disrupted in T lymphocytes. We found that acute inactivation of p57 results in a differentiation block at the transition from DN3 to DN4 cells. Further genetic analysis showed that this

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