Fbx7 Functions in the SCF Complex Regulating Cdk1-Cyclin B-phosphorylated Hepatoma Up-regulated Protein (HURP) Proteolysis by a Proline-rich Region*[S]

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F-box proteins, components of SCF ubiquitin-ligase complexes, are believed to be responsible for substrate recognition and recruitment in SCF-mediated proteolysis. F-box proteins that have been identified to function in the SCF complexes to date mostly have substrate-binding motifs, such as WD repeats or leucine-rich repeats in their C termini. However, many F-box proteins lack recognizable substrate-binding modules; whether they can function in the SCF complexes remains unclear. We show here that Fbx7, an F-box protein without WD repeats and leucine-rich repeats, is required for the proteasome-mediated proteolysis of the hepatoma up-regulated protein (HURP). Depletion of Fbx7 by small interfering RNA leads to depression of HURP ubiquitination and accumulation of HURP abundance. In the SCFPbx7 complex, Fbx7 recruits HURP through its C-terminal proline-rich region in a Cdk1-cyclin B-phosphorylation dependent manner. Mutation of the multiple Cdk1-cyclin B phosphorylation sites on HURP or the proline-rich region of Fbx7 abolishes the association between Fbx7 and HURP. Thus, Fbx7 is a functional adaptor of the SCF complex with a proline-rich region as the substrate-binding module. In addition to Fbx7, data base analyses reveal two putative mammalian proline-rich region-containing F-box proteins, KIAA1783 and RIKEN cDNA 2410015K21. Taken together, these findings further expound the diverse substrate-recognition abilities of the SCF complexes.

The ubiquitin-proteasome pathway plays a central role in regulation of many biological processes, including cell cycle progression, transcription, and signal transduction. The formation of ubiquitin-protein conjugates requires three components: a ubiquitin-activating enzyme (E1),¹ a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Ubiquitinated proteins are rapidly degraded by the 26 S proteasome. In the ubiquitin-mediated proteolysis, E3s play a major role in providing specificity of substrate recognition and timing for ubiquitin-dependent proteolysis (1, 2).

The SCF (Skp1, Cdc53/Cullin1, F-box protein) complex, one of the E3s, is a highly diverse multicomponent complex consisting of common components, such as Skp1, Cullin1, and Rbx1, as well as variable components known as F-box proteins (3–5). By biochemical and topological studies, the SCF complex is known to utilize F-box protein to recruit phosphorylated substrate to its core (2, 4, 6, 7). Thus, the substrate specificity of the SCF complex is thought to depend on the F-box protein component. Each F-box protein contains an N-terminal motif of ~40 amino acids, known as the F-box, which links the F-box protein to other components of the SCF complex by binding Skp1 (5, 8). In general, F-box proteins have additional C-terminal protein-protein interaction motifs responsible for substrate binding. The two most common motifs are WD repeats (9) and leucine-rich repeats (10), both of which have been found to bind phosphorylated substrates and recruit them to the SCF complexes (11). Substrate recognition by the SCF complex is thought to require the same core components (i.e. Cullin1, Skp1, and Rbx1) but to utilize various F-box proteins. The ability of the same core to bind multiple F-box proteins increases the substrate repertoire.

Modern advances in genome sequencing and molecular cloning have led to the identification of numerous F-box proteins, which are classified into three subfamilies according to the C-terminal substrate-binding motifs that they contain (7, 12, 13). The Fbw and Fbl families represent the F-box proteins containing WD repeats and leucine-rich repeats, respectively, and have been shown to function in the SCF complexes and recruit substrates for ubiquitination. The Fbx family represents the F-box proteins without known substrate-binding motifs and includes the majority of F-box proteins. However, only limited numbers of Fbx family members, such as Fbx2, Fbx4, and Fbx6b (14–16), have been recently demonstrated to function in the SCF complexes. The structural and functional characterization of the rest of the Fbx family members still needs to be defined. Whether these novel Fbx family members function in the SCF complexes, as do those of the Fbw and Fbl families, is one of the central issues in current studies of ubiquitin-mediated proteolysis, especially because F-box proteins have also been found to be associated with various biological path-

zinedithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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ways other than SCF-mediated proteolysis (7, 17, 18). The gap causing slow progress in this field is due mainly to the lack of substrates. Research to identify the downstream targets of these novel Fbx family members is needed to fill this gap.

Hepatoma up-regulated protein (HURP) was initially identified through bioinformatics analysis that was performed to identify cell cycle-regulated gene with an elevated expression in the G2/M phase, followed by a sharp decline in early to middle G1 phase. Immunofluorescence studies reveal that HURP localizes to the spindle poles during mitosis. Overexpression of HURP in 293T cells results in an enhanced cell growth at low serum levels and at polyhema-based, anchorage-independent growth conditions (19). Furthermore, elevated gene expression of HURP and its related truncated form KIAA0008 is highly associated with human hepatocellular carcinoma, colon cancer, breast cancer and transitional cell carcinoma (19–22), suggesting that HURP may have a role in carcinogenesis.

Here, we demonstrate that HURP undergoes proteasome-mediated proteolysis and the SCF\(\text{Fbx7}\) complex is the corresponding upstream E3 ligase. Fbx7 interacts with HURP through its C-terminal proline-rich region (PRR), suggesting that PRR is a new substrate-binding module of F-box proteins other than WD repeats and leucine-rich repeats. By data base search, we further identify two putative PRR-containing F-box proteins, KIAA1783 and RIKEN cDNA 2410015K21. Thus, these PRR-containing molecules might constitute a new subfamily of mammalian F-box proteins.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies—**Protease inhibitors: leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and pepstatin; proteasome inhibitors: MG132 (N-CBZ-Leu-Leu-Leucinal), proteasome inhibitor I, and lactacystin; calpain inhibitors: ALLN (calpain inhibitor I, MG 101) and A-LLM (calpain inhibitor II); and Cdk1-specific inhibitors: roscovitine and olomoucine, were purchased from Sigma and Calbiochem. Cdk1-cyclin B and phosphatase were purchased from New England Biolabs. Anti-HURP polyclonal antibody was generated against whole molecule of recombinant HURP protein (19). Anti-Cdk1 monoclonal antibody was purchased from CHEMICON. Anti-Skp1 and anti-Cul1 monoclonal antibodies were purchased from BD Transduction. Anti-HA monoclonal antibody (3F10) was purchased from Roche Diagnostics. Anti-FLAG monoclonal antibody (M5) was purchased from Sigma. Anti-Fbx7 antibody was purchased from Zymed. Anti-ubiquitin antibody was purchased from Santa Cruz Biotechnology and MPM2 antibody was purchased from Upstate. Alkaline phosphatase-conjugated horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody were purchased from PerkinElmer Life Sciences and used in 1:2000–1:5000 dilutions.

**Cell Culture, Cell-cycle Synchronization, Transfection, and Immunofluorescence Analysis—**293T cells were cultured in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum and 2 mM glutamine (Invitrogen) and maintained in a humidified incubator at 37 °C in the presence of 5% CO\(_2\). To synchronize 293T cells in the G0/M phase, exponentially growing cells were incubated with 100 ng/ml nocodazole (Sigma) for 16 h. Cells were released from the G0/M block by removing the nocodazole. Continuous time point samples were collected for various assays. Transfection of cells was performed with LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Analysis of the subcellular localization of various proteins by indirect immunofluorescence was carried out as previously described (19).

**Construction of Expression Vectors and Site-directed Mutagenesis—**Human full-length HURP cDNA (designated as HURP-WT) and Cdk1 cDNA were subcloned into FLAG-CMV2 (Kodak) or a modified version of pcDNA3.0 (Invitrogen), with an HA epitope tag for mammalian expression, or T7 promoter-driven vectors for the in vitro coupled transfection reaction. The HURP phosphorylation site mutant (HURP-PM) and various Fbx7-truncated mutants (Fbx7-ΔN, Fbx7-ΔP, Fbx7-ΔC, and Fbx7-ΔNP) were generated by PCR-based mutagenesis (QuickChange™ Site-directed mutagenesis kit, Stratagene). FLAG-tagged F-box protein constructs used in this study were kindly provided by Dr. Michele Pagano (New York University School of Medicine).

**Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting Analysis—**To prepare cell-free lysates, cells were harvested, washed with phosphate-buffered saline, and lysed in extraction buffer, which was composed of 50% lysis buffer (20 mM PIPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, 1 mM Na3VO4, and 10 μg/ml of leupeptin, aprotinin, chymostatin, and pepstatin) and 50% IP washing buffer (10 mM HEPES, pH 7.6, 2 mM MgCl\(_2\), 50 mM NaCl, 5 mM EGTA, 0.1% Triton X-100, and 50 mM β-glycerophosphate). After incubation at 4 °C for 30 min, cellular debris was removed by centrifugation at 13,000 × g for 30 min. Protein concentrations were determined using BCA Protein Assay reagents (Pierce). In general, 1 mg of total cell lysate was incubated with antibodies against target epitopes and Protein A/G-agarose beads (Oncogene Research Product) to immunoprecipitate the target proteins. The immunoprecipitation products were resolved by 8–15% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Millipore) and detected with various antibodies conjugated to alkaline phosphatase or horseradish peroxidase, and developed using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate or the Western Lighting system (PerkinElmer Life Sciences).

**Preparation of Recombinant Protein, in Vitro Kinase Reaction, and Phosphorylation Site Determination—**Human HURP recombinant proteins were prepared using a bacterial expression system. HURP cDNA was subcloned into expression vector pET28a (Novagen) and expressed as His-tagged fusion protein. The proteins were expressed and trapped in inclusion bodies and solubilized with 2 M urea. The solubilized proteins were partially purified by nickel-agarose (Qiagen) under denaturing conditions. The purified proteins were then dialyzed to remove the denaturant as described in the manufacturer’s manual. The purified recombinant HURP proteins were incubated with bacloovirus-expressed human Cdk1-cyclin B complex (New England Biolabs) in kinase buffer (50 mM Tris-HCl, 10 mM MgCl\(_2\), 1 mM EGTA, 2 mM dithiothreitol, and 0.01% Brij 35, pH 7.5) containing [γ-\(^{32}\)P]ATP. After incubation at 30 °C for 30 min, samples were subjected to SDS-PAGE and phosphorylated bands were visualized by autoradiography. For phosphorylation site determination, Echerichia coli expressed recombinant HURP proteins were incubated with Cdk1-cyclin B in kinase buffer containing ATP. After reaction for 30 min, samples were subjected to SDS-PAGE and phosphorylated bands were visualized by autoradiography. The phosphorylation sites were determined by LC/MS/MS analysis as previously described (23).

**Ubiquitination Assay—**293T cells were transfected with expression vectors encoding HA-tagged HURP or Myc-tagged ubiquitin separately or together. 48 h after transfection, cells were harvested and lysed. Equal amounts of total lysates were subjected to immunoprecipitation with anti-HA antibody. The immunoprecipitation products were analyzed by Western blotting with anti-HA or anti-ubiquitin antibodies.

**Cycloheximide Inhibition Assay—**The turnover rates of proteins were determined using cycloheximide inhibition of protein synthesis (24, 25). Cycloheximide blocks protein synthesis through interaction with the translase enzyme in eukaryotic cells. Cells were treated with 50 μg/ml cycloheximide (Sigma) and harvested at different stages. Equal amounts of cell lysates were subjected to SDS-PAGE and analyzed by Western blotting.

**Data Bases Used in This Study—**The data bases used in this study includes BLAST and PROSITE.

**Small Interference RNA (siRNA)—**The following target sequence was used for Fbx7 siRNA: CCCACACCAUUCUCAAUCUA. To generate functional siRNA, we designed the gene-specific insert such that it specifies a 19-nucleotide sequence corresponding to nucleotides 1,136–1,154 (CCACACACCTTACCCTTACTTA) of Fbx7, separated by a 9-nucleotide non-complementary spacer (TTCAAGAGA) from the reverse complement of the same 19-nucleotide sequence. This insert was then subcloned in a vector system, named pSUPER as described (26) to direct the synthesis of siRNA in mammalian cells.
RESULTS

HURP Is a Phosphoprotein Regulated by the Ubiquitin-Proteasome Pathway—Previous studies have shown that in HeLa cells the HURP transcript is low in G1 and S phases, but is predominantly expressed in the G2/M phase (19). We showed here that HURP protein expression levels also underwent periodic changes along with cell cycle progression. 293T cells were synchronized in the G2/M phase by nocodazole treatment for 16 h and subsequently released into cell cycle progression by removing nocodazole. At the indicated time points, cells were harvested and analyzed by Western blotting (WB) with antibodies against HURP, cyclin B, and actin. Exponentially growing cells (EG) were used as control. The positions of native (HURP) and phosphorylated (p-HURP) HURP are indicated. B, the mobility up-shifts of endogenous HURP in G2/M-arrested 293T cells were sensitive to phosphatase treatment. Cell lysates were prepared from exponentially growing 293T cells (EG) or nocodazole-arrested 293T cells (Noc). Equal amounts of lysates were incubated at 37 °C for 1 h in phosphatase buffer in the presence or absence of 100 units of λ phosphatase. The reaction mixtures were subjected to SDS-PAGE and analyzed by Western blotting with antibodies against HURP and actin. The positions of native (HURP) and phosphorylated (p-HURP) HURP are indicated. C, the mobility up-shifts of [35S]methionine-labeled HURP in G2/M-arrested 293T lysates were sensitive to phosphatase treatment. In vitro translated [35S]methionine-labeled HURP was incubated with equal amounts of lysates (50 μg of protein/reaction) prepared from exponentially growing 293T cells (EG) or nocodazole-arrested 293T cells (Noc) at 37 °C for 30 min in phosphatase buffer in the presence or absence of 100 units of λ phosphatase. The reaction mixtures were subjected to SDS-PAGE and analyzed by autoradiography. The positions of native (HURP) and phosphorylated (p-HURP) HURP are indicated. D, proteasome inhibitors stabilized HURP during exit from the G2/M block. 293T cells were synchronized in the G2/M phase by nocodazole treatment for 16 h (lane 2). Subsequently, synchronized cells were released into cell cycle progression in the presence of either proteasome inhibitors (25 μM, lanes 4–6) or calpain inhibitors (25 μM, lanes 7 and 8) for 9 h. Cells were harvested and analyzed by Western blotting with antibodies against HURP and actin. Exponentially growing cells (lane 1) were used for comparison with nocodazole-arrested cells (lane 2). Me2SO was used as solvent control (lane 3). E, in vivo ubiquitination of HURP. 293T cells were transfected with expression vectors encoding HA-tagged HURP or Myc-tagged ubiquitin (Ub) separately or together as indicated at the top of each lane. After 48 h, cells were harvested and equal amounts of lysates were immunoprecipitated (IP) with anti-HA antibody followed by Western blotting with anti-HA and anti-ubiquitin antibodies. High molecular weight immunoprecipitation products (HA-HURP-Ubn) accumulated in cells cotransfected with HURP and ubiquitin but not in cells transfected with ubiquitin alone.
phoretic mobility shifts on SDS-PAGE as cells were synchronized in the G2/M phase (Fig. 1, A, lane 2; B, lane 3). These mobility up-shifts were abolished when H9261 phosphatase was added to lysates, indicating they were because of protein phosphorylation (Fig. 1 B, lane 4). To further support this finding, [35S]methionine-labeled HURP was incubated with nocodazole-arrested 293T lysates, which resulted in a reminiscent mobility up-shift pattern (Fig. 1 C). The mobility up-shifts of [35S]methionine-labeled HURP were also sensitive to H9261 phosphatase treatment (Fig. 1 C), characteristic of protein phosphorylation. Moreover, simultaneous decreases in the protein quantity and in the degree of phosphorylation of HURP were observed as G2/M-arrested cells were released into the cell cycle progression by removing nocodazole (Fig. 1 A). Taken together, these results demonstrate that HURP is a mitotic phosphoprotein that exhibits periodic variations both in the phosphorylation state and in protein quantity as cells progress through the cell cycle.

To determine the molecular mechanism involved in the degradation of HURP, 293T cells were transfected with three kinds of conventional proteasome inhibitors (lactacystin, pro
teesome inhibitor I, and MG132) to block HURP from degradation taking place as G2/M-arrested 293T cells were released into cell cycle progression. Because MG132 exhibits additional inhibitory activities against calpain, two calpain inhibitors (ALLM and ALLN) were used as controls. Treatment with proteasome inhibitors, but not calpain inhibitors, could block HURP degradation, implying the involvement of proteasomes in HURP degradation (Fig. 1 D). Because ubiquitination is the signal for effective recognition and degradation of proteins by the proteasome, we then investigated whether HURP is a substrate of ubiquitination. 293T cells transfected with Fbx7 siRNA. 293T cells were transfected with Fbx7 siRNA. After 48 h, equal amounts of extracts were prepared and analyzed by Western blotting with anti-HURP and anti-Fbx7 antibodies.

**Fig. 2.** Fbx7 functions as an adaptor linking the SCFP\(^{\text{Fbx7}}\) complex with HURP. A, HURP associated with Fbx7. 293T cells were transfected with various FLAG-tagged F-box proteins with or without HA-HURP. Cell lysates were immunoprecipitated with anti-HA antibody followed by Western blotting (WB) with anti-FLAG or anti-HA antibody. Lane 1 shows F-box protein expression levels. Lane 2 shows HA-HURP immunoprecipitation results of F-box proteins. Lane 3 shows immunoprecipitation results of F-box proteins, B, HURP formed a complex with SCFP\(^{\text{Fbx7}}\). 293T cells were transfected with HA-HURP or FLAG-Fbx7 separately or together as indicated at the top of each lane. 1 mg of cell lysates were immunoprecipitated by anti-HA antibody followed by Western blotting with antibodies against HA, FLAG, Skp1, and Cullin1 (lower). 50 \(\mu\)g of the input lysates were also analyzed by Western blotting with anti-HA and anti-Fbx7 antibodies to show the expression levels of HA-HURP and FLAG-Fbx7 (upper). C, HURP ubiquitination was depressed in 293T cells transfected with Fbx7 siRNA. 293T cells were transfected with HA-HURP, Myc-Ub, and Fbx7 siRNA as indicated at the top of each lane. After 48 h, equal amounts of extracts were prepared for immunoprecipitation with anti-HA antibody. Immunoprecipitated products were immunoblotted with anti-HA to reveal HURP ubiquitination. D, accumulation of HURP in 293T cells transfected with Fbx7 siRNA. 293T cells were transfected with Fbx7 siRNA. After 48 h, equal amounts of extracts were prepared and analyzed by Western blotting with anti-HURP and anti-Fbx7 antibodies.
Fbx7 Serves as an Adaptor Linking the SCF Complex with HURP—To identify the upstream E3 ligase responsible for HURP ubiquitination, we collected a set of F-box protein expression constructs and assayed for their association with HURP by immunoprecipitation. 293T cells were transfected with expression vectors encoding HA-tagged HURP or FLAG-tagged F-box proteins (Fbx1A, Fbx2, Fbx3A, Fbx4, and Fbx7) separately or together followed by immunoprecipitation and Western blotting analysis. The results showed that Fbx7, but not any of the other F-box proteins tested, physically associated with HURP (Fig. 2A).

To determine whether Fbx7 serves as an adaptor and recruits HURP to a SCF complex, we expressed HA-tagged HURP (HA-HURP) and FLAG-tagged Fbx7 (FLAG-Fbx7) separately or together in 293T cells and immunoprecipitated the cell lysates with anti-FLAG antibody. Then, we examined the existence of other SCF components in the immunoprecipitates by Western blotting analysis. Endogenous Skp1 and Cullin1 were detected in the FLAG-Fbx7 immunoprecipitate (Fig. 2B, lane 2). This data, in agreement with previous reports, represented the reconstitution of the SCFPbx7 complex (12). Moreover, co-expression of HA-HURP and FLAG-Fbx7 followed by immunoprecipitation with anti-FLAG antibody resulted in the complex formation of HURP, Fbx7, Skp1, and Cullin1 (Fig. 2B, lane 3).

To further illustrate the role of Fbx7 in this complex and test the significance of Fbx7 in the stability of its putative substrate, HURP, we used the siRNA to reduce the expression of Fbx7 and then assessed its effect on HURP ubiquitination. Results showed that HURP ubiquitination was depressed in cells transcontracted with plasmid-based double-stranded RNA against Fbx7 (Fig. 2C). Furthermore, we assessed its effect on HURP abundance. HURP accumulated in siRNA-inhibited 293T cells when compared with cells transfected with a control empty vector (Fig. 2D). Collectively, these data indicate that Fbx7 is involved in the regulation of HURP stability.

Fbx7 Associates with Cdk1-Cyclin B-phosphorylated HURP—There is compelling evidence that protein phosphorylation is a regulatory factor in the interactions between the substrate of the SCF complex and the F-box protein (11, 27, 28). As shown in Fig. 1A, HURP is a mitotic phosphoprotein, suggesting that protein phosphorylation may provide a mode of regulating the interaction between Fbx7 and HURP. In fact, MMP2 antibody, an antibody specifically recognizing a subset of mitosis-specific phosphoproteins (29, 30), could recognize the immunoprecipitated HURP from nocardazole-arrested cells (Fig. 3A), suggesting that HURP might be the downstream substrate of Cdk1-cyclin B, which has been shown to be able to generate MMP2 reactivity (31, 32). Therefore, we next addressed whether HURP is a downstream substrate of Cdk1-cyclin B. First, active Cdk1-cyclin B could phosphorylate HURP in vitro. When recombinant HURP protein was added to the kinase reaction in the presence of recombinant Cdk1-cyclin B and [γ-32P]ATP, an [γ-32P]ATP-incorporated band was observed at the 10-min point in the Cdk1-cyclin B-mediated kinase reaction, but not in the control lanes (Fig. 3B). Second, Cdk1-specific inhibitors, roscovitine (33) and olomoucine (34), partially abolished the mobility up-shifts of HURP as [32P]methionine-labeled HURP was incubated with nocardazole-arrested 293T lysates (Fig. 3C), suggesting that HURP is a potential substrate of Cdk1-cyclin B in the G/M phase. Third, overexpression of FLAG-tagged HURP and HA-tagged Cdk1 in 293T cells followed by immunoprecipitation with anti-FLAG showed that HURP was communoprecipitated with Cdk1, indicating that HURP can associate with Cdk1 in culture cells (Fig. 3D). Fourth, immunofluorescence analysis also demonstrated that endogenous Cdk1 and HURP colocalized to the mitotic spindles in the metaphase (Fig. 3E). Taken together, these results indicate that HURP is a potential substrate of Cdk1-cyclin B.

Cdk1-cyclin B is known to phosphorylate numerous proteins and regulate their stability (35–37). To examine whether Cdk1-cyclin B-mediated HURP phosphorylation is the key step in the interaction between HURP and Fbx7, we determined the in vitro Cdk1-cyclin B phosphorylation sites on HURP by LC MS/MS analysis. Nine sites (Ser67, Thr329, Thr401, Thr402, Ser406, Thr415, Ser438, Thr442, and Ser450) were identified with 82% amino acid sequence coverage, indicating that Cdk1-cyclin B can phosphorylate HURP at multiple sites in vitro (Fig. 4A). Initially, we replaced each phosphorylation site individually with alanine and some combination of two phosphorylation sites mutations on HURP. These mutants were assayed for their association with Fbx7; however, none of these mutants tested had a significant difference in terms of association with Fbx7 (data not shown), raising the possibility that multisite phosphorylation of HURP may be required for the association with Fbx7. Next, we constructed a HURP mutant in which all nine phosphorylation sites were replaced with alanines (designated as HURP-PM). HURP-PM exhibited a more depressed phosphorylation state than HURP-WT, both as incubating with nocardazole-arrested cell lysates (Fig. 4B) and as expressing in mitotic cells (Fig. 4C), suggesting that Cdk1-cyclin B-mediated HURP phosphorylation could recapitulate in the in vivo situation. Subsequently, we examined whether a complex of Fbx7 and HURP-PM could be detected in 293T cells. The results showed that mutation of all nine Cdk1-cyclin B phosphorylation sites on HURP abolished the association between Fbx7 and HURP (Fig. 4D). In addition, comparisons of ubiquitination and turnover rates between HURP-WT and HURP-PM showed that HURP-PM was less susceptible to ubiquitination and had a more extended half-life than HURP-WT in 293T cells (Fig. 4, E and F). These data demonstrate that Cdk1-cyclin B phosphorylation is a prerequisite for the recruitment of HURP by the SCFPbx7 complex.

Fbx7 Recruits Substrates through Its C-terminal PRR—Previous results have shown that Fbx7 functions as an adaptor linking the SCFPbx7 complex and HURP. To further identify the protein binding modules responsible for such linkage, we constructed three Fbx7 truncated derivatives in which amino acid residues corresponding to the N-terminal region upstream to F-box (amino acids 1–294), F-box (amino acids 295–333) (12, 13), or the C-terminal region downstream to the F-box (amino acids 334–482) were deleted and designed as Fbx7-ΔN, Fbx7-ΔF, or Fbx7-ΔC, respectively (Fig. 5A). 293T cells were transfected with expression vectors encoding HA-tagged HURP or FLAG-tagged Fbx7 derivatives separately or together followed by immunoprecipitation with anti-FLAG antibody. Then, we examined the existence of HURP and Skp1 in the immunoprecipitates of various Fbx7 derivatives by Western blotting analysis (Fig. 5B).

In the part of HURP binding, results showed that only deletion of the C-terminal region of Fbx7 abolished the association between Fbx7 and HURP, suggesting that this region includes the substrate-binding modules of Fbx7 (Fig. 5B, lanes 2–5). As discussed earlier, Fbx7 lacks WD repeats or leucine-rich repeats in its C-terminal region (amino acids 334–482) and primary sequence analyses showed that this region contains a PRR (amino acids 383–474), in which prolines occupy approximate 30% for the amino acid residue composition (Fig. 5A) (12). Because proline-rich regions have been defined as protein-protein interaction modules in many cellular processes (38), we subsequently deleted the proline-rich region of Fbx7 (designated...
as Fbx7-ΔP; Fig. 5A) and assayed for the interaction between Fbx7-ΔP and HURP by the same procedure. As shown in Fig. 5B, lane 6, deletion of the proline-rich region abolished the association between Fbx7 and HURP, indicating that the proline-rich region is the substrate-binding module of Fbx7.

In the part of Skp1 binding, results showed that Fbx7 wild type (Fbx7-WT), Fbx7-ΔN and Fbx7-ΔP associated with Skp1 (Fig. 5B, lanes 2, 3, and 6), whereas Fbx7-ΔF and Fbx7-ΔC did not exhibit such association (Fig. 5B, lanes 4 and 5). Our finding is consistent with previous studies, which suggest that the amino acid 295–333 region of Fbx7 is responsible for Skp1 binding (12, 13). However, by comparing the results of Fbx7-ΔC and Fbx7-ΔP, the amino acid 333–383 region in Fbx7 might also be involved in Skp1 binding. As the F-box is a degenerate motif without strict consensus and borders, our results suggest that the amino acid region corresponding to the F-box of Fbx7 might be broader.

In addition to protein interaction analysis, we assessed the roles of these protein domains by a dominant-negative approach. If one protein domain is critical for Fbx7 function as an adaptor, overexpression of the deleted mutant of that domain should lead to stabilize HURP. 293T cells were cotransfected with HURP and various Fbx7 derivatives followed by cycloheximide inhibition assay. Results showed that HURP was more
stable in the presence of Fbx7-ΔF and Fbx7-ΔP (Fig. 5C), further supporting the roles of F-box and PRR in Fbx7.

In addition to Fbx7, we identified two putative mammalian PRR-containing F-box proteins, KIAA1783 and RIKEN cDNA 2410015K21, by data base search (Fig. 6). KIAA1783, originally cloned from human brain, contains three proline-rich
regions in the C-terminal region downstream to its putative F-box. RIKEN cDNA 2410015K21 is a mouse gene encoding a 523-amino acid protein with a proline-rich region downstream to its putative F-box. Furthermore, RIKEN cDNA 2410015K21 shares 66% identity with human Fbx7 at the amino acid level, suggesting that it might be the mouse homologue of Fbx7.

![Diagram of Fbx7 structure and truncated mutants](image)

**A** Fbx7 associates with HURP through its C-terminal PRR. A, schematic overview of the Fbx7 structure and truncated mutants. The abilities of each Fbx7 mutant to bind Skp1 or HURP are summarized. Positive interaction, +; no interaction, −. B, identification of the protein-binding modules of Fbx7. 293T cells were transfected with HA-HURP-WT or various FLAG-tagged Fbx7 mutants as indicated at the top of each lane. 1 mg of lysates was immunoprecipitated with anti-FLAG antibody followed by Western blotting (WB) with anti-HA, anti-FLAG, and anti-Skp1 antibodies (lower). 50 µg of the input lysates were also analyzed by Western blotting with anti-HA, anti-FLAG, and anti-Skp1 antibodies as input control (upper).

**B** C, HURP is more stable in cells overproducing Fbx7ΔF or Fbx7ΔP. 293T cells were transfected with HA-HURP-WT or various FLAG-tagged Fbx7 mutants. After 24 h, the influences of various Fbx7 mutants on HURP-WT turnover were compared by the cycloheximide inhibition assay.

![Western blots of HURP and Skp1](image)
These findings hint that the proline-rich region might represent a general substrate-binding module of F-box proteins.

**DISCUSSION**

Identification of downstream substrates of F-box proteins, particularly with those novel Fbx family members, is an essential step to provide better understanding of their unexplored functions. For example, Fbx2 was recently reported to function in the SCF complex and was demonstrated to interact with its substrates in response to specific glycosylation via a novel FBA domain (14). In contrast, Fbx3 and Fbx8 were proposed to contain putative protein interaction domains similar to bacterial ApaG protein and yeast Sec7p protein (39). Further identification of the downstream substrates of Fbx3 and Fbx8 is needed to validate the authenticity of the proposed interaction domains and reveal the
roles of these novel Fbx members. The finding of a physical association between Fbx7 and HURP offers an opportunity to examine the biological functions of Fbx7. In this study, we demonstrated: 1) Fbx7 functions as an adaptor linking the core SCF complex with its substrate, HURP; 2) Cdk1-cyclin B-mediated multiple site phosphorylation on HURP is a prerequisite for the association between Fbx7 and HURP; 3) the PRR within the C-terminal region of Fbx7 is the substrate-binding module responsible for recruiting substrate (Fig. 7). Our observations reveal that Fbx7 is a functional substrate-binding adaptor in the SCF complex and the proline-rich region is a new substrate-binding module of F-box proteins.

Previous study has shown that overexpression of FLAG-Fbx7 in cells followed by immunoprecipitation with anti-FLAG antibody resulted in the isolation of active Fbx7-Skp1-Cullin1 complex (12). In our study, co-expression of HA-HURP and FLAG-Fbx7 followed by immunoprecipitation with anti-FLAG antibody resulted in the formation of the HURP-Fbx7-Skp1-Cullin1 complex (Fig. 2B). In contrast, as we mapped the protein interaction domains for Fbx7-Skp1 binding or Fbx7-HURP binding by deletion mutation, we could only disrupt the association of Fbx7-Skp1 or Fbx7-HURP, but not the whole HURP-Fbx7-Skp1 complex (Fig. 5). These results indicate that Fbx7 functions as an adaptor linking the core SCF complex with its substrate, HURP. HURP is degraded by proteosome (Fig. 1D). Both ablation of Fbx7 by siRNA and disruption of the association between Fbx7 and HURP can stabilize HURP (Figs. 2, 4, and 5). These data further illustrate the biological significance of Fbx7 in the HURP-Fbx7-Skp1-Cullin1 complex.

How E3s accurately target protein substrates is an open question. F-box proteins often include C-terminal motifs capable of substrate binding. The PRR within the C-terminal region of Fbx7 is the substrate-binding module responsible for recruiting HURP. Proline-rich regions are well known protein-protein interaction modules and have been shown to play a critical role in the assembly and regulation of many protein complexes involved in many cellular processes such as cell growth, cytoskeletal rearrangements, transcription, and posttranscriptional signaling (38). Here, we show that this versatile module also provides the specificity of substrate recognition in the ubiquitin-mediated proteolysis. Much effort has been dedicated to understanding the protein-protein interaction specificity of proline-rich regions. Several corresponding protein recognition domains that interact with proline-rich regions have been identified in recent years, including SH3, WW, EVH1/WH1, and GYP domains (38). However, HURP lacks these recognizable proline recognition domains, suggesting that Fbx7-HURP association may through a pattern out of our knowledge. In addition to Fbx7, data base searches reveal two putative mammalian PRR-containing F-box proteins, KIAA1783 and RIKEN cDNA 2410015K21. Thus, these PRR-containing molecules might constitute a new subfamily of F-box proteins and further explain how the SCF complexes can recognize various substrates specifically.

In addition to specific substrate binding modules, accumulative evidence suggests that proper post-translational modification of target substrates is a prerequisite for their recognition by E3s. The types of modification discovered so far include phosphorylation, hydroxylation, and N-glycosylation (2, 4, 7, 14, 16, 40, 41). HURP is a mitotic phosphoprotein. In vitro and in vivo analyses reveal that Cdk1-cyclin B is one of the upstream kinases of HURP. Mutation of the Cdk1-cyclin B phosphorylation sites on HURP disrupts the association between HURP and Fbx7 partially abolishes the turnover of HURP, implying that Cdk1-cyclin B phosphorylation on HURP is a prerequisite for targeting HURP by Fbx7. According to the current studies, in some cases, single (or simple) phosphorylation is sufficient for substrate recognition and the binding interaction seems to be based on the phosphorylation motif. For example, SCF^{Skp2} regulates p27 ubiquitination through phosphorylation Thr^{187} in p27 (42), and SCF^{Skp2}-mediated processing of NF-kB p105 requires phosphorylation of p105 serines 927 and 932 by IκB kinase (43). On the other hand, ubiquitination of Cdk inhibitor Sic1 by SCF^{F-box} requires phosphorylation of Sic1 at multiple, widely spaced sites and the Cdc4-Sic1 interaction seems to be based on modules other than phosphorylation motifs (44–47). The interaction between Cdc4 and a single module is so weak that multiple phosphorylated modules are required for effective Cdc4-Sic1 interaction. Therefore, this requirement for multisite phosphorylation imposes a threshold for programmed degradation control. In our study, the presence of Cdk1-cyclin B-phosphorylated HURP at multiple sites and multi, rather than single site phosphorylation was essential for Fbx7-HURP association and HURP stability. These findings raise a possibility that Fbx7-HURP association might be regulated in a multisite phosphorylation manner. Further study may help answer these basic questions posed by the complexity of signal transduction mechanisms.

In addition to Cdk1-cyclin B, from the results of Figs. 3C and 4, B and C, we can infer that HURP may be phosphorylated by other kinases. Actually, we also identified Aurora-A as a potential upstream kinase of HURP (data not shown). Aurora-A is a serine-threonine kinase implicated in the assembly and maintenance of the mitotic spindle. However, Aurora-A-mediated HURP phosphorylation is not discovered to be correlated with HURP-Fbx7 interaction so far. Cumulative evidence has demonstrated that HURP is highly associated with carcinogenesis. As a negative regulator of HURP, the role of Fbx7 in carcinogenesis will merit further investigation.

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Fig. 7. A schematic diagram represents the role of Fbx7 in the SCF-mediated proteolysis. Through the interaction between F-box and Skp1, Fbx7 works in coordination with Skp1, Cullin1, and Rbx1 to form the SCF^{F-box} complex. In the complex, Fbx7 serves as an adaptor specifically recruiting Cdk1-cyclin B-phosphorylated HURP for ubiquitination through its PRR.
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