

Identification of a family of human F-box proteins

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and M. Pagano^{*}

F-box proteins are an expanding family of eukaryotic proteins characterized by an approximately 40 amino-acid motif, the F box (so named because cyclin F was one of the first proteins in which this motif was identified) [1]. Some F-box proteins have been shown to be critical for the controlled degradation of cellular regulatory proteins [2,3]. In fact, F-box proteins are one of the four subunits of ubiquitin protein ligases called SCFs. The other three subunits are the Skp1 protein; one of the cullin proteins (Cul1 in metazoans and Cdc53 or Cul A in the yeast *Saccharomyces cerevisiae*); and the recently identified Roc1 protein (also called Rbx1 or Hrt1). SCF ligases bring ubiquitin conjugating enzymes (either Ubc3 or Ubc4) to substrates that are specifically recruited by the different F-box proteins. The need for high substrate specificity and the large number of known F-box proteins in yeast and worms [2,4] suggest the existence of a large family of mammalian F-box proteins. Using Skp1 as a bait in a yeast two-hybrid screen and by searching DNA databases, we identified a family of 26 human F-box proteins, 25 of which were novel. Some of these proteins contained WD-40 domains or leucine-rich repeats; others contained either different protein-protein interaction modules or no recognizable motifs. We have named the F-box proteins that contain WD-40 domains Fbws, those containing leucine-rich repeats Fbls, and the remaining ones Fbxs. We have further characterized representative members of these three classes of F-box proteins.

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Results and discussion

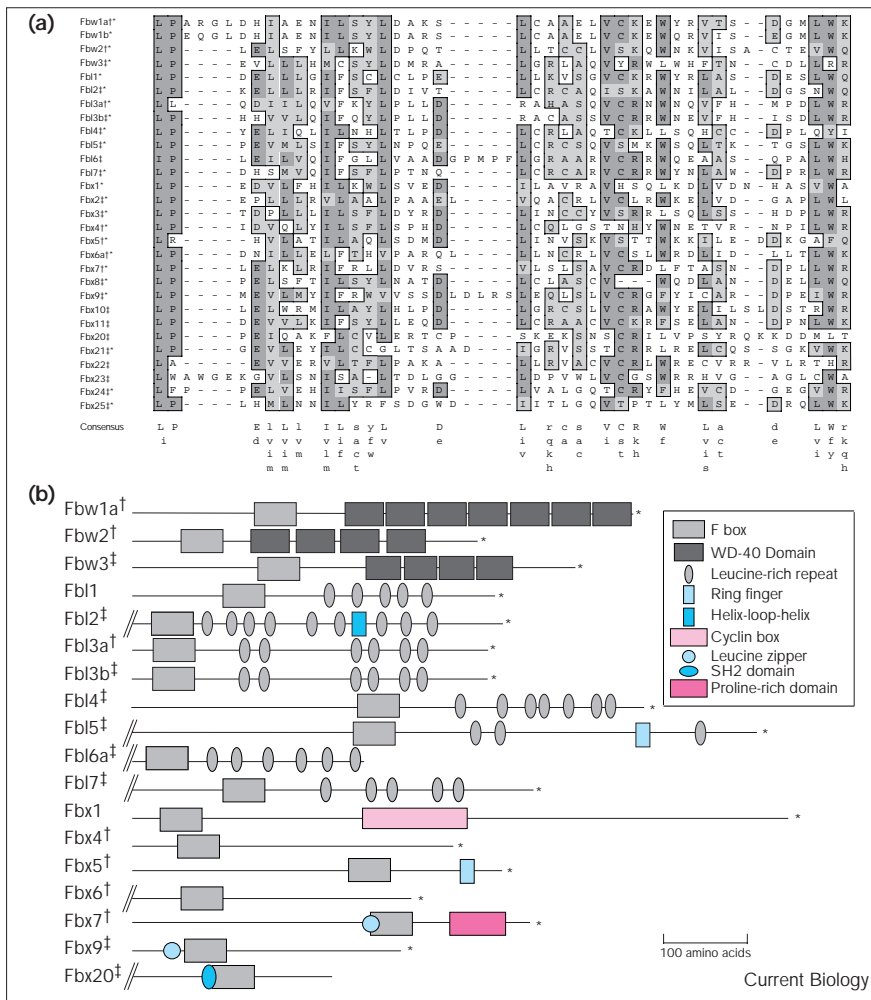
We used an improved version of the yeast two-hybrid system [5] and human Skp1 as bait to search for novel

human F-box proteins. The MaV103 yeast strain harboring a fusion between the Gal4 DNA-binding domain and Skp1 was transformed with an activated T-cell cDNA library expressed as fusion proteins with the Gal4 activation domain. After the initial selection and re-transformation steps, three different reporter assays were used to select 13 clones that specifically interacted with human Skp1. After sequence analysis, the 13 rescued cDNAs were found to be derived from seven different open reading frames, all encoding F-box proteins, six of which were novel (Figure 1). One of these, β -Trecp, was also identified by others while our screen was in progress [6]. To identify additional human members of this family, we searched various databases using the amino-acid sequences corresponding to the conserved residues of the F-box motif of human F-box proteins. This analysis revealed the presence of human expressed sequence tags (ESTs) encoding nineteen additional, as yet unidentified, human F-box proteins (Figure 1a).

Computer analysis of the newly identified human F-box proteins revealed several interesting features (Figure 1b). Three F-box proteins contained WD-40 domains; seven contained leucine-rich repeats, and six contained other potential protein-protein interaction modules not yet identified in F-box proteins, such as leucine zippers, ring fingers, helix-loop-helix domains, proline-rich motifs and Src homology 2 (SH2) domains. Outside these domains, human F-box proteins, like those of other organisms, shared very limited homology.

A partially overlapping group of vertebrate F-box proteins has been identified by the laboratories of W. Harper and S. Elledge (mammalian F-box proteins) [7] and P. Jackson (*Xenopus* F-box proteins) [8]. To avoid future confusion over nomenclature, we have agreed on a consistent naming and numbering scheme for this very large family of proteins. Three subdivisions have been created within the F-box protein family that recognize the additional motifs in the protein sequences. Thus, we have named the F-box proteins that contain WD-40 domains Fbws, those containing leucine-rich repeats Fbls, and the remaining ones Fbxs. All other previously identified human F-box proteins — β -Trecp (Fbw1a), β -Trecp2 (Fbw1b), Skp2 (Fbl1) and cyclin F (Fbx1) — are the first members of the respective three classes; the other members have been numbered in the order of their discovery. A compilation of mammalian F-box sequences from this paper and Winston *et al.* [7] is shown in the Supplementary material of the latter paper.

Figure 1



The family of human F-box proteins and its three classes, Fbw, Fbl and Fbx. A single dagger denotes F-box proteins identified through a two-hybrid screen; a double dagger denotes those identified by database searching. **(a)** Alignment of the F boxes of F-box proteins identified through a two-hybrid screen or BLAST searches and those of the four previously known F-box proteins (Fbw1a/ β -Trcp, Fbw1b/ β -Trcp2, Fbl1/Skp2 and Fbx1/cyclin F). The alignment was performed using the Clustal W method (MacVector) followed by manual re-adjustment. An asterisk indicates the presence of a stop codon; sequences without asterisks are incomplete at the 3' end. Dark grey, identical residues in at least 13 F boxes; light grey, residues that are similar. The consensus sequence is indicated below; capital letters indicate identical residues and lower case letters indicate similar residues. The cDNA sequences corresponding to the F-box proteins reported in this figure have been deposited in the GenBank database (accession numbers AF129530–AF129537 and AF174589–AF174606). **(b)** Schematic representation of the human F-box proteins containing putative protein–protein interaction domains. Double slashes, cDNAs that were incomplete at the 5' end; asterisks, stop codons.

As representative examples of all three classes of F-box proteins, we characterized the full-length members (Figure 2 and Supplementary material). We determined whether Fbw2, Fbl3a, Fbx4 and Fbx7, like Fbw1a/ β -Trcp and Fbl1/Skp2, interact with human Skp1 and Cul1 *in vivo*. FLAG epitope-tagged versions of wild-type Fbw1a/ β -Trcp, Fbw2, Fbl3a, Fbx4 and Fbx7, and mutant versions of Fbw1a, Fbw2 and Fbl3a lacking the F box (Δ F), were expressed in HeLa cells, and the cell extracts subjected to immunoprecipitation with an anti-FLAG antibody. As detected in immunoblots, F-box proteins coprecipitated with Cul1 and Skp1, but not with Cul2 (another human cullin), and only using extracts from cells expressing wild-type F-box proteins (Figure 2a and data not shown).

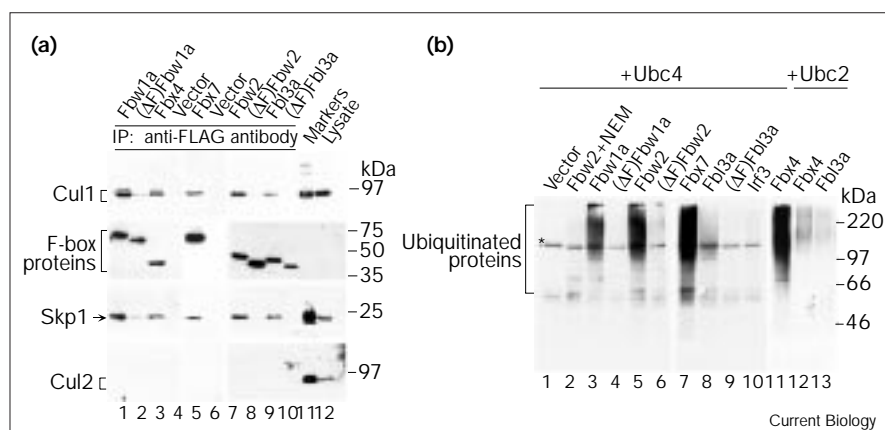
The binding of F-box proteins to the Skp1–Cul1 complex is consistent with the possibility that F-box proteins associate with a ubiquitin ligation activity. To test this possibility, we expressed FLAG-tagged F-box proteins together with human Skp1 and Cul1 in HeLa cells. Cell extracts were

subjected to immunoprecipitation with an anti-FLAG antibody and assayed for ubiquitin ligase activity in the presence of the human ubiquitin-activating enzyme (E1) and a human Ubc. All of the wild-type F-box proteins tested, but not F-box protein mutants, associated with a ubiquitin ligase activity that produced a high molecular weight smear characteristic of ubiquitinated proteins (Figure 2b). The ligase activity was sensitive to N-ethylmaleimide (NEM; lane 2) and required the presence of both Ubc4 and E1 (data not shown). Results similar to those with Ubc4 were obtained using human Ubc3 (data not shown), whereas Ubc2 was unable to sustain the ubiquitin ligase activity of these SCFs (Figure 2b, lanes 12,13 and data not shown). This is the first demonstration that, in addition to Fbws and Fbls, Fbxs can associate with a ubiquitin ligase activity.

In humans, two SCF complexes have been identified and characterized, one of which contains Fbw1a/ β -Trcp and the other Fbl1/Skp2. We and others have recently shown that SCF^{Fbl1/Skp2} is required for ubiquitination of the

Figure 2

All three classes of human F-box proteins associate with a ubiquitin ligase activity *in vivo*. (a) HeLa cells were transfected with plasmids encoding FLAG-tagged versions of Fbw1a/ β -Trcp (lane 1), (Δ F)Fbw1a (lane 2), Fbx4 (lane 3), Fbx7 (lane 5), Fbw2 (lane 7), (Δ F)Fbw2 (lane 8), Fbl3a (lane 9), (Δ F)Fbl3a (lane 10), or with empty vector (lanes 4,6). Cells were lysed and extracts subjected to immunoprecipitation (IP) with a rabbit anti-FLAG antibody (lanes 1–10). Immunoprecipitates were then immunoblotted with an anti-FLAG antibody (to detect F-box proteins) or antibodies against the indicated proteins. Lane 11, 10 ng purified recombinant Cul1, Skp1 and Cul2 proteins used as markers; lane 12, lysate from non-transfected HeLa cells (25 μ g protein). (b) HeLa cells were transfected with plasmids encoding human Skp1, Cul1 and FLAG-tagged versions of Fbw1a/ β -Trcp (lane 3), (Δ F)Fbw1a (lane 4), Fbw2 (lanes 2,5), (Δ F)Fbw2 (lane 6), Fbx7 (lane 7), Fbl3a (lanes 8,13), (Δ F)Fbl3a (lane 9), an irrelevant FLAG-tagged protein (Irf3, lane 10), Fbx4 (lanes 11,12) or with an



empty vector (lane 1). Cells were lysed and extracts were immunoprecipitated with a rabbit anti-FLAG antibody. Immunoprecipitates were incubated in the presence of purified recombinant E1 and Ubc4 (lanes 1–11) or Ubc2 (lanes 12,13) and biotinylated ubiquitin. The reaction in lane 2 also contained NEM. Ubiquitinated

proteins were visualized by blotting with horseradish peroxidase (HRP)-conjugated streptavidin. The bracket on the left highlights the smear of ubiquitinated proteins produced in the reaction, the asterisk indicates ubiquitin conjugated with E1 that was resistant to boiling.

cyclin-dependent kinase inhibitor p27^{Kip1}, both *in vivo* [9,10] and *in vitro* [9,11] and that SCF^{Fbw1a/ β Trcp} recruits β -catenin and I κ B α for ubiquitin-mediated degradation [12,13]. Degradation of many other mammalian cellular regulatory proteins (for example, cyclins, c-Myc, c-Jun, E2F and p300) is controlled by the ubiquitin pathway [14]. Yet, the specific enzymes involved in the degradation of these regulatory proteins are not known. This description of a large family of human F-box proteins will allow a better understanding of the role and the specificity of SCF complexes in the regulation of ubiquitin-mediated proteolysis.

Supplementary material

Supplementary material including additional methodological details and three figures showing specific interaction of F-box proteins with Skp1 through the F box, subcellular localization of F-box proteins, expression in human tissues and a compilation of F-box sequences from this paper and [7] is available at <http://current-biology.com/supmat/supmatin.htm>.

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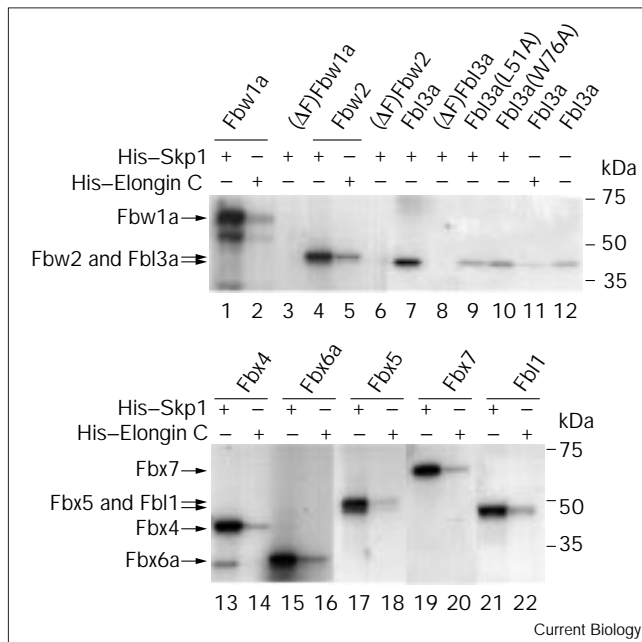
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Figure S1



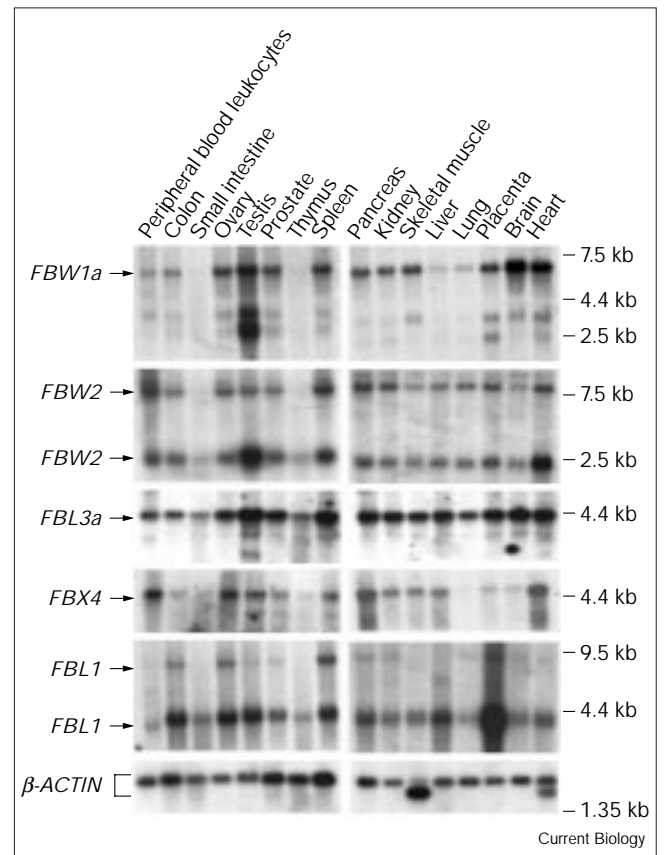
F-box proteins interact specifically with Skp1 through their F box. The various wild-type and mutant F-box protein cDNAs were transcribed and translated *in vitro* in the presence of [³⁵S]methionine. Similar amounts of *in vitro* translated proteins (indicated at the top of each lane) were subjected to a His tag pull-down assay using nickel-agarose beads to which similar amounts of either His-tagged Skp1 (lanes 1,3,4,6–10,13,15,17,19,21), His-tagged elongin C (lanes 2,5,11,14,16,18,20,22), or His-tagged p27 (lane 12) were pre-bound. Human elongin C, which is related to Skp1, was used as a negative control. Immunoblotting with an antibody against the His tag showed that similar amounts of His-tagged proteins were used (data not shown). Bound *in vitro* translated proteins were analyzed by SDS-PAGE and autoradiography. The arrows on the left point to the indicated F-box proteins. The apparent molecular weights of the protein standards are indicated on the right.

Supplementary materials and methods

Plasmid construction for the yeast two-hybrid system

The pPC97-CYH2 and the pPC86 plasmids [S1,S2] contain *LEU2* and *TRP1* as selectable markers and encode, respectively, the DNA-binding domain (DB, amino acids 1–147) and the transcriptional activation domain (AD, amino acids 768–881) of yeast Gal4. The in-frame DB fusion with full-length human Skp1 was obtained by homologous recombination of the PCR product amplified with the following two oligonucleotides: 5' primer, 5'-AGTAGTAAACAAAGGTCAAAGACAGTTGACTGTATCGTCGAGGATGCCTTCAATTAAGTT-3'; 3' primer, 5'-GCGGTTACTTACTTAGAGCTCGACGCTTACTTACTTAGCTCACTTCTTTCACACCA-3'. The 5' primer corresponds to a DB sequence present in the pPC97-CYH2 plasmid (italicized) flanked by the 5' sequence of the *SKP1* gene. The 3' primer corresponds to a sequence within the polylinker of the pPC97-CYH2

Figure S2



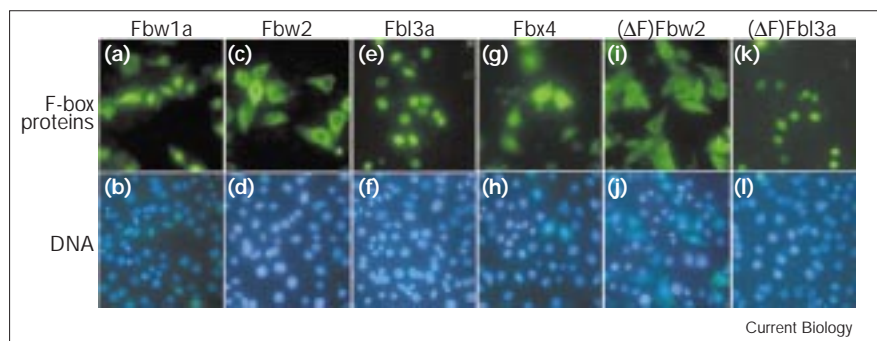
Expression of transcripts encoding F-box proteins in human tissues. Membranes containing electrophoretically fractionated poly(A)⁺ mRNA from different human tissues were hybridized with specific probes prepared from *FBW1a/β-TRCP*, *FBW2*, *FBL3a*, *FBX4*, *FBL1/SKP2* and *β-ACTIN* cDNAs. The arrows on the left point to the major transcripts.

plasmid (italicized) flanked by the 3' sequence of the *SKP1* gene. The PCR product (100 ng) was transformed into yeast cells (MaV103 strain [S1,S2]) with or without 100 ng pPC97-CYH2 plasmid previously restricted with *SalI* and *BglI*. As a result of the homologous recombination, only yeast cells containing the pPC97-CYH2 plasmid homologously recombined with *SKP1* cDNA grew efficiently in the absence of leucine.

Library screen for Skp1 interactors and cloning of full-length F-box proteins

MaV103 strain [S1] harboring the DB-Skp1 fusion was transformed with an activated T-cell cDNA library (Alala 2, [S3]) cloned in pPC86. Transformation was performed using the standard lithium acetate method. Approximately 3×10^6 yeast transformants were first plated onto synthetic complete plates lacking both leucine and tryptophan (Sc-Leu-Trp) and incubated for 60 h. The resulting transformants were replica-plated onto Sc-Leu-Trp plates containing 20 mM 3-aminotriazole (3-AT). Growing yeast colonies were picked after an additional four-day

Figure S3



Subcellular localization of F-box proteins. HeLa cells were transfected with mammalian expression plasmids encoding FLAG-tagged versions of (a,b) Fbw1a/β-Trcp, (c,d) Fbw2, (e,f) Fbl3a, (g,h) Fbx4, (i,j) (ΔF)Fbw2, or (k,l) (ΔF)Fbl3a. After 24 h, (a,c,e,g,i,k) F-box proteins were stained with a rabbit anti-FLAG antibody and (b,d,f,h,j,l) DNA with bisbenzamide. Fbw1a/β-Trcp and Fbx4 were found distributed both in the cytoplasm and in the nucleus, Fbw2 was detected mainly in the cytoplasm and Fbl3a mainly in the nucleus. The identical localization of wild-type and mutant F-box proteins demonstrates that the presence of the F box and the F-box-dependent binding to Skp1 do not determine the subcellular localization of F-box proteins.

incubation and further tested in the MaV103 'three reporter assays': first, growth on Sc-Leu-Trp plates supplemented with 20 mM 3-AT to assay for *GAL1::HIS3*; second, expression of β-galactosidase activity to assay for *GAL1::lacZ*; and third, no growth on Sc-Leu-Trp plates supplemented with 0.2% 5-fluoro-orotic acid to assay for *SPAL10::URA3*. The corresponding AD plasmids were transformed back into cells expressing either the DB-Skp1 bait fusion or DB alone as a negative control. Thirteen AD plasmids that repeatedly tested positive in at least two reporter assays were rescued in *Escherichia coli* and sequenced. Two of the clones encoding Fbx4 and Fbx5 appeared to be full length; full-length clones of four other cDNAs encoding Fbw1a/β-Trcp, Fbw2, Fbl3a and Fbx7 were obtained by rapid amplification of cDNA ends (RACE) using Marathon-Ready cDNA libraries (Clontech) according to the manufacturer's instructions. A full-length clone encoding Fbx6 was not obtained. Criteria for full-length clones included at least two of the following: identification of an open reading frame (ORF) yielding a sequence related to known F-box proteins; presence of a consensus Kozak translation initiation sequence at a putative initiator methionine codon; identification of a stop codon in the same reading frame but upstream of the putative initiation codon; inability to further increase the size of the clone by RACE using three different cDNA libraries. Note that all the F-box proteins obtained with the two-hybrid screen (Fbw1a/β-Trcp, Fbw2, Fbl3a, Fbx4, Fbx5, Fbx6 and Fbx7) were used as negative controls in experiments published very recently in [S4]. In that study, they were indicated as Fbp1, Fbp2, Fbp3a, Fbp4, Fbp5, Fbp6 and Fbp7, respectively.

DNA database searches and analysis of protein motifs

The National Center for Biotechnology Information and The Institute for Genomic Research which encompass the major database of ESTs were searched for sequences with homology to F-box protein genes by using BLAST, PSI-BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and TGI Sequence Search (http://www.tigr.org/cgi-bin/BlastSearch/blast_tgi.cgi). ESTs that overlapped more than 95% in at least 100 bp were assembled into novel contiguous ORFs using Sequencher 3.0. Protein domains were identified using the ProfileScan Server (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html), BLOCKS Searcher (http://www.blocks.fhcr.org/blocks_search.html) and IMB Jena (<http://genome.imb-jena.de/cgi-bin/GDEWWW/menu.cgi>).

Accession numbers

The 26 sequences reported in this paper have been deposited in the GenBank database (accession numbers AF129530–AF129537 and AF174589–AF174606).

Construction of F-box mutants

(ΔF)Fbw1a (residues 32–179), (ΔF)Fbw2 (residues 60–101), (ΔF)Fbl3a (residues 40–76), (ΔF)Fbx4 (residues 55–98) were obtained by deletion

with the appropriate restriction enzymes with conservation of the reading frame. Fbl3a mutants in which the Leu51 residue was mutated to Ala (L51A), or the Trp76 residue mutated to Ala (W76A) were generated by oligonucleotide-directed mutagenesis using the polymerase chain reaction of the QuikChange site-directed mutagenesis kit (Stratagene). All mutants were sequenced in their entirety.

Recombinant proteins

The cDNA fragments encoding the entire coding region of human Skp1, and Elongin C with a hexahistidine (His) tag at the amino terminus were inserted into the baculovirus expression vector pBacpak-8 (Clontech) and cotransfected into Sf9 cells with linearized baculovirus DNA using the BaculoGold transfection kit (Pharmingen). Recombinant viruses were used to infect 5B cells and assayed for expression of their encoded protein by immunoblotting as described [S5]. His-tagged proteins were purified with Nickel–agarose (Invitrogen) according to the manufacturer's instructions.

Northern blot analysis

Northern blots were performed using human multiple-tissue mRNAs from Clontech. Probes were radiolabeled with [α - 32 P]dCTP (Amersham) using a random primer DNA labeling kit (Gibco BRL; 2×10^6 cpm/ml). Washes were performed with $0.2 \times$ SSC, 0.1% SDS, at 55–60°C. *FBW1a/β-TRCP* and *FBL3a* probes were two *HindIII* restriction fragments (nucleotides 1–571 and 1–450, respectively), *FBW2*, *FBX4* and *FBL1/SKP2* probes were their respective full-length cDNAs, and the β -*ACTIN* probe was from Clontech.

Antibodies, transient transfections, immunoprecipitation and immunoblotting

Polyclonal antibodies to Cul1, Cul2 and Skp1, as well as mouse monoclonal antibodies to Cul1, were generated and characterized as described [S6]. Mouse anti-FLAG antibody was from Kodak, mouse anti-histidine antibody from Clontech and rabbit anti-FLAG antibody from Zymed. The cDNAs encoding the human proteins Fbw1a, (ΔF)Fbw1a, Fbw2, (ΔF)Fbw2, Fbl3a, (ΔF)Fbl3a, Fbl3a(L51A), Fbl3a(W76A), Fbx4, (ΔF)Fbx4 were inserted into the mammalian expression vector pCDNA3 (Invitrogen) in frame with sequences encoding a FLAG tag at the 5' end. Cells were transfected with FuGENE transfection reagent (Boehringer) according to the manufacturer's instructions. Conditions for protein extraction, immunoblotting, and immunoprecipitation have been previously described [S4,S5].

Ubiquitination assay

Immuno-beads containing SCF complexes immunoprecipitated with an anti-FLAG antibody were added together with purified recombinant human E1 and Ubc enzymes (Ubc2, Ubc3 or Ubc4) to a reaction mix

containing biotinylated ubiquitin. Samples were then analyzed by immunoblotting with HRP–streptavidin. E1, Ubc enzymes and biotinylated ubiquitin were produced as described [S7]. Polyubiquitinated species generated in this assay may be formed by cellular substrates that co-immunoprecipitated with the ligase, as proposed for similar experiments performed with Fbl1/Skp2 [S8]; or unanchored ubiquitin polymers as proposed in [S9]; or auto-ubiquitinated components of the Ubc–SCF complex.

Immunofluorescence

Transfected cell monolayers growing on glass coverslips were rinsed in PBS and fixed with 4% paraformaldehyde in PBS for 10 min at 4°C followed by permeabilization for 10 min with 0.25% Triton X-100 in PBS. Other fixation protocols gave comparable results. Immunofluorescence stainings were performed using 1 µg/ml rabbit anti-FLAG antibody as described [S10].

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