$p27^{Kip1}$ ubiquitination and degradation is regulated by the SCF^{Skp2} complex through phosphorylated Thr187 in p27

Lyuben M. Tsvetkov, Kun-Huei Yeh, Soo-Jung Lee, Hong Sun and Hui Zhang

Many tumorigenic processes affect cell-cycle progression by their effects on the levels of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ [1,2]. The phosphorylation- and ubiquitination-dependent proteolysis of p27 is implicated in control of the G1–S transition in the cell cycle [3–6]. To determine the factors that control p27 stability, we established a cell-free extract assay that recapitulates the degradation of p27. Phosphorylation of p27 at Thr187 was essential for its degradation. Degradation was also dependent on SCF^{Skp2}, a protein complex implicated in targeting phosphorylated proteins for ubiquitination [7–10]. Immunodepletion of components of the complex — Cul-1, Skp1, or Skp2 — from the extract abolished p27 degradation, while addition of purified SCF^{Skp2} to Skp2-depleted extract restored the capacity to degrade p27. A specific association was observed between Skp2 and a p27 carboxy-terminal peptide containing phosphorylated Thr187, but not between Skp2 and the non-phosphorylated peptide. Skp2-dependent associations between Skp1 or Cul-1 and the p27 phosphopeptide were also detected. Isolated SCF^{Skp2} contained an E3 ubiquitin ligase activity towards p27. Our data thus suggest that SCF^{Skp2} specifically targets p27 for degradation during cell-cycle progression.

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Results and discussion

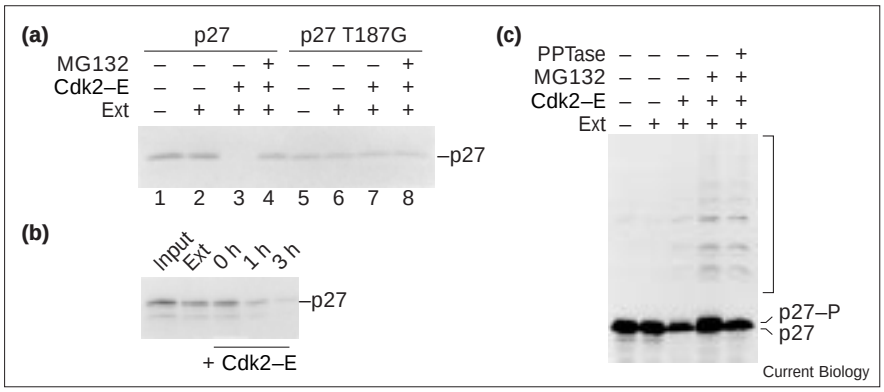
Selective p27 degradation in cell-free systems has been reported previously in synchronized S-phase cell extracts but not in G1 extracts [6,11]. To identify the proteins that control p27 stability, we prepared a cytosolic extract from asynchronized and exponentially growing HeLa cells. When recombinant ^{35}S-labeled p27 was incubated with this extract, p27 was quite stable, even upon prolonged incubation. Addition of an active Cdk2–cyclin E kinase to the extract led to the rapid degradation of p27 (Figure 1a,b and see Supplementary material published with this article on the internet). The requirement for Cdk2–cyclin E in p27 destruction is probably due to its ability to phosphorylate p27 at Thr187, which has been shown to trigger p27 degradation [4,5]. Indeed, conversion of Thr187 to glycine (T187G) stabilized p27 in the extract (Figure 1a). In the presence of Cdk2–cyclin E, a fraction of p27 slightly shifted its electrophoretic mobility. This fraction was sensitive to phosphatase (Figure 1c), suggesting that it contains phosphorylated forms of p27.

Another fraction of p27 was converted into multiple high-molecular-weight phosphatase-insensitive species in the presence of Cdk2–cyclin E (Figure 1c). Addition of specific inhibitors of the 26S proteasome, such as MG132, stabilized p27 and caused the accumulation of both the phosphorylated and the high-molecular-weight forms (Figure 1c). Modified ubiquitins such as methyl ubiquitin and ubiquitin aldehyde also caused accumulation of p27 ladders (Supplementary material, Figure S1b). This is likely to be due to their interference with the degradation rate of highly polyubiquitinated proteins and their inhibition of de-ubiquitination [12]. These observations indicate that the high-molecular-weight species of p27 are the polyubiquitinated forms. Thus our *in vitro* system faithfully recapitulated ubiquitin-dependent degradation of p27 in a Cdk2–cyclin E-dependent process that requires phosphorylation of the Thr187 residue in p27.

Using the *in vitro* p27 degradation system, we examined the involvement of the candidate ubiquitin E3 ligase the SCF complex — Skp1/Cdc53(Cullins)/F-box proteins — in p27 degradation. SCF complexes represent a conserved family of protein complexes that target phosphorylated proteins for ubiquitin-dependent proteolysis [13,14]. We first examined whether Cul-1, a human Cdc53 homolog, is required for p27 degradation [8–10]. Cul-1 was depleted from the HeLa cell extracts by passage through an affinity-purified anti-Cul-1 antibody column. Depletion of Cul-1 abolished the ability of the extracts to degrade p27, whereas parallel mock depletion using purified IgG from pre-immune serum had no effect (Figure 2a). Western blotting confirmed the removal of Cul-1 from the extracts (Figure 2d). Immunodepletion of another component of the SCF complex, Skp1 [7,15], also inhibited p27 degradation (Figure 2b,d).

The requirement for Cul-1 and Skp1 in p27 degradation implies that an F-box protein is also involved. The F-box protein is a component of the SCF complex that interacts directly with the phosphorylated substrate, thus defining

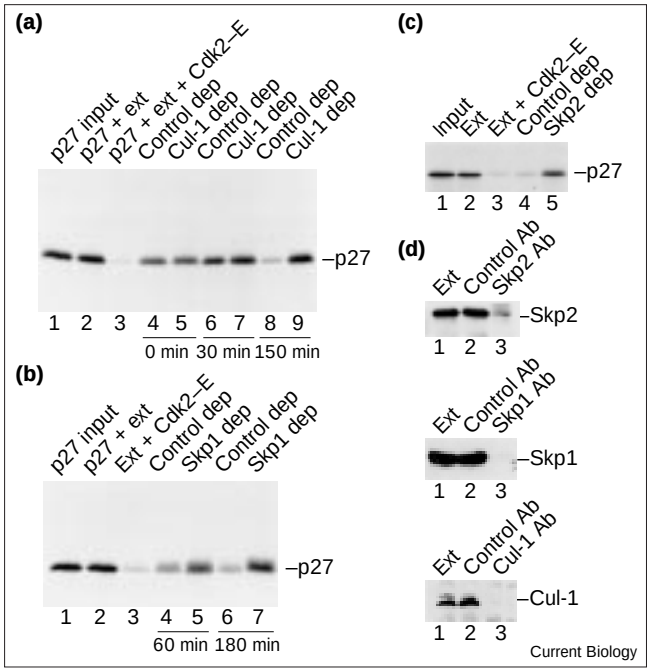
Figure 1



Phosphorylation-dependent p27 degradation in HeLa extracts. (a) ^{35}S-labeled p27 or p27 T187G mutant was incubated with HeLa extract (Ext) for 3 h at 30°C. Cdk2–cyclin E (Cdk2–E) or MG132 (20 μM) were added as indicated. The p27 reaction products were isolated by immunoprecipitation. (b) Time course of p27 degradation in the extracts. (c) p27 was incubated with HeLa extracts in the absence or presence of Cdk2–cyclin E and MG132. The reaction products were treated with lambda phosphatase (PPTase). The phosphorylated and high-molecular-weight p27 species (in brackets) are indicated.

the substrate specificity for ubiquitination [14]. To identify the F-box protein(s) that specifically binds to p27, ^{35}S-labeled HeLa cell extracts were incubated with GST–p27 fusion protein either with or without prior phosphorylation by Cdk2–cyclin E. Examination of the labeled proteins specifically associated with the phosphorylated GST–p27 beads revealed the presence of a protein of molecular weight 45 kDa, similar to that of the F-box protein Skp2 (data not shown). To determine whether Skp2 is involved in p27 degradation, HeLa extracts were depleted of Skp2 using an affinity-purified anti-Skp2 antibody column. Degradation of p27 was inhibited in the immunodepleted extracts (Figure 2c,d).

Figure 2



Inhibition of p27 degradation by depletion of SCF^{Skp2}. HeLa extracts (ext) were passed through affinity-purified (a) anti-Cul-1, (b) anti-Skp1 or (c) anti-Skp2 antibody columns. Control depletions were conducted using a purified immunoglobulin G (IgG) column. The control and Cul-1-depleted extracts were assayed for p27 degradation activity in the presence of Cdk2–cyclin E (Cdk2–E) for 3 h or for various times as indicated. Lanes 1–3 show results with p27 alone and undepleted HeLa extracts as indicated. Reaction products were treated with lambda phosphatase except in (b). Dep, depleted. (d) Specific removal of Skp2, Skp1 and Cul-1 by immunodepletion. Untreated HeLa extract, control depleted, and Skp2-, Skp1-, or Cul-1-depleted extracts were western blotted by anti-Skp2 (top), anti-Skp1 (middle) or anti-Cul-1 (bottom) antibodies. Ab, antibody used for depletion.

To directly examine the specific binding of Skp2 to phosphorylated p27, we synthesized a pair of peptides corresponding to the carboxy-terminal end of p27 (amino acids 175–198). One (p27CP) contains phosphorylated Thr187 and the other (p27C) unphosphorylated Thr187 (Figure 3a). The peptides were each coupled to Sulfo-Link agarose beads, which were then used as affinity resins for binding analysis of F-box proteins. We initially tested whether these peptides could interact with several known F-box proteins, including Skp2, β-TrCP and MD6, as well as with a number of unpublished ones that we identified by searching expressed sequence tag (EST) databases. F-box proteins that had been translated and ^{35}S-labeled *in vitro* were incubated with the p27 peptide beads. Analysis of the F-box proteins associated with p27 peptide beads revealed a specific interaction between Skp2 and p27CP but not p27C (Figure 3b). We did not detect specific associations between p27CP and other available F-box proteins (Figure 3b and data not shown). These results suggest that Skp2 interacts selectively and specifically with p27CP.

To determine whether endogenous Skp2 in the HeLa extract can also interact with p27CP, the peptide beads were incubated with the extracts. Western blot analysis detected a strong and specific interaction between endogenous Skp2 and p27CP (Figure 3c). p27C did not

interact with Skp2 significantly. As a control for Skp2 binding, we also assayed for the binding of the F-box protein β-TrCP [14] to the p27 peptides, and found no interaction (Figure 3c).

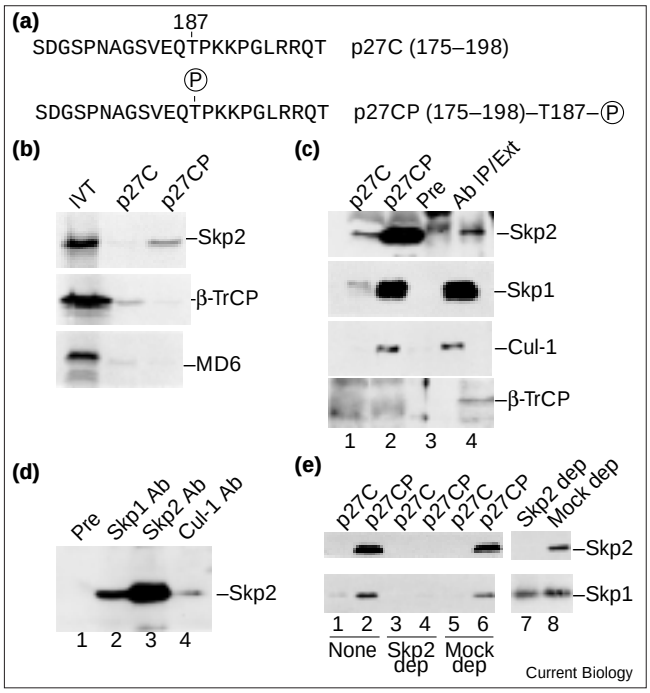
Previous studies indicated that Skp2 interacts with Skp1 and Cul-1 *in vivo* [8–10]. In the HeLa extract, such interactions were also detected (Figure 3d). Using the peptide bead pull-down assays, we also detected specific interaction of Skp1 or Cul-1 with p27CP beads but not with p27C beads (Figure 3c). We then carried out assays for Skp1 or Cul-1 binding to p27 peptides in Skp2-depleted extracts. Depletion of Skp2 greatly reduced the binding of Skp1 (Figure 3e) or Cul-1 (data not shown) to p27CP beads, although the total levels of Skp1 and Cul-1 in the Skp2-depleted extract were not substantially altered (Figure 3e and data not shown). These studies suggest that Skp2 is the SCF component that binds to the phosphorylated Thr187 of p27. Upon binding to phosphorylated p27, Skp2 brings in its associated Skp1 and Cul-1 which targets p27 for degradation through the proteasome.

We further investigated whether addition of SCF^{Skp2} to the Skp2-depleted extract could restore p27 degradation activity. Recombinant SCF^{Skp2} was expressed and assembled using the baculovirus expression system and purified. When recombinant SCF^{Skp2} was added back to the Skp2-depleted extract, p27 degradation capacity was restored (Figure 4a). This restoration depended on the presence of Skp2 in the complex; complexes assembled in the absence of Skp2 could not rescue the Skp2 deficiency in the extract. Addition of purified Skp2 alone could partially rescue p27 degradation in the Skp2-depleted extract (data not shown), but the assembled SCF^{Skp2} complex consistently produced better restoration, indicating that the complex is required for p27 degradation.

p27 ubiquitination was also assayed directly using recombinant SCF^{Skp2}. In a purified system containing recombinant SCF^{Skp2}, Cdk2–cyclin E, ubiquitin-activating enzyme E1 and ATP, a fraction of p27 was converted into multiple high-molecular-weight species (Figure 4b). The formation of high-molecular-weight p27 depended on the presence of ubiquitin and Cdc34, a conserved E2 conjugating enzyme that is implicated in SCF-mediated ubiquitination [9,13]. These data suggest that SCF^{Skp2} can ubiquitinate p27 in the presence of E1 and E2. However, p27 ubiquitination using the purified proteins was not very efficient. It is possible that SCF^{Skp2} may require additional modifications or activities for efficient p27 ubiquitination.

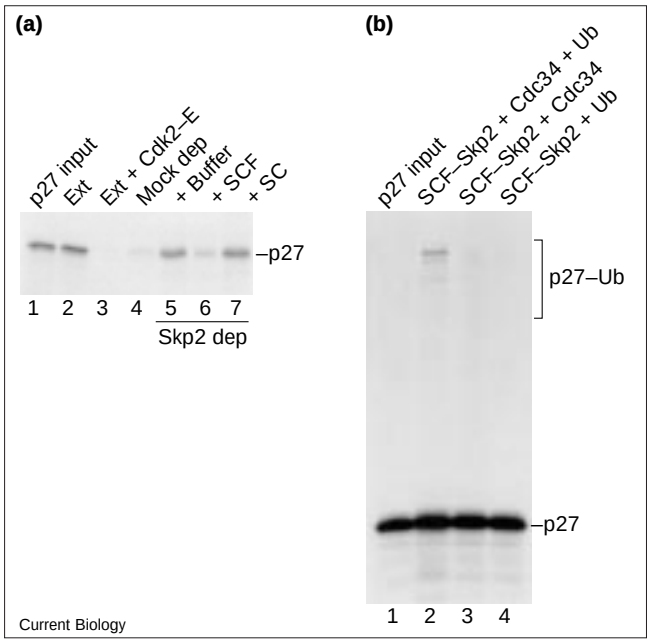
Our results strongly suggest that SCF^{Skp2} specifically binds to the Thr187-phosphorylated form of p27 and targets it for degradation through a ubiquitin-dependent process. These results are consistent with our previous observation that Skp2 regulates a critical event during the G1–S transition [7]. Skp2 level is elevated during late G1 and S phase [7,9], suggesting that Skp2 is probably rate-limiting for the assembly and activation of SCF^{Skp2}. However, p27 regulation could occur at several levels *in vivo*, including cellular compartmentalization, alternative degradation pathway(s), and sequestration of p27 by cyclin D complexes [1,16,17]. Some of these controls may be

Figure 3



Specific interactions between SCF^{Skp2} and the Thr187-phosphorylated p27 peptide (p27CP). (a) Sequences of p27 carboxy-terminal peptides (residues175–198) with (p27CP) or without (p27C) Thr187 phosphorylation. (b) Skp2 specifically binds to p27CP. ^{35}S-labeled F-box proteins, Skp2, β-TrCP, and MD6 (10 μl each) were incubated for 1 h with p27C beads or p27CP beads and proteins associated with the beads were purified and analyzed. IVT, *in vitro* translated input protein. (c) Selective binding of Skp2, Skp1 and Cul-1 to p27CP in HeLa extracts. HeLa extracts (400 μg) were incubated with p27CP beads or p27C beads for 1 h. The proteins associated with the beads were analyzed by western blotting with either anti-Skp2, anti-Skp1, anti-Cul-1 or anti-β-TrCP antibodies, as indicated. Immunoprecipitated Skp2, Skp1 and Cul-1 (IP) are included as controls (lane 4), as are pre-immune samples (pre; lane 3). For lane 4 of β-TrCP, HeLa extracts (25 μg) were directly loaded without immunoprecipitation. The amounts of HeLa extract for Skp2 immunoprecipitation were 25 μg and 100 μg for Skp1 and Cul-1, respectively. (d) Association between Skp1, Skp2 and Cul-1 in HeLa extracts. Skp1, Skp2 and Cul-1 complex were immunoprecipitated by specific antibodies as indicated. Skp2 was detected among the immunoprecipitated proteins by western blotting with anti-Skp2 antibodies. (e) Association between Skp1 and p27CP depends on the presence of Skp2. Undepleted HeLa extracts (none), mock-depleted or Skp2-depleted extracts (100 μg each) were incubated with either p27C beads or p27CP beads. Skp1 (bottom) or Skp2 (top) associated with the peptide beads was detected by western blotting. Skp2 and Skp1 in the mock- and Skp2-depleted extracts (25 μg each) were also detected by direct western blotting (lanes 7,8).

Figure 4



SCF^{Skp2} contains an E3 ubiquitination activity that acts on p27. (a) Restoration of p27 degradation activity in Skp2-depleted extracts by recombinant SCF^{Skp2}. Insect SF9 cells were co-infected with baculoviruses encoding GST–Skp1 and Cul-1, either in the presence or absence of Skp2-recombinant baculoviruses. The SCF^{Skp2} (SCF) and SC (lacking Skp2) complexes formed were isolated by glutathione–Sepharose. Recombinant SCF^{Skp2}, SC (1 μg each in 2 μl), or buffer alone (2 μl) was added to the Skp2-depleted extracts (200 μg) as indicated, and p27 degradation was assayed in the presence of Cdk2–cyclin E. Undepleted HeLa extract (ext) was included as the control. (b) Ubiquitination of p27 using recombinant proteins. p27 was incubated with recombinant SCF^{Skp2} in the presence of purified Cdk2–cyclin E and Cdk2–cyclin A kinases, human E1 ubiquitin-activating enzyme, ATP, ubiquitin and recombinant Cdc34, an E2 ubiquitin-conjugating enzyme (lane 2) as described in Supplementary material. The reactions in lanes 3 and 4 were carried out in the absence of ubiquitin or Cdc34, respectively.

cell-type specific [1,2]. This may in part explain why we have previously failed to detect a change in total p27 levels in several cancer cell lines using antisense oligonucleotides against the components of SCF [8]. Our present finding that SCF^{Skp2} is involved in targeting p27 for degradation might help to explain why Skp2 is highly induced in many transformed cells [7] and may provide a mechanism for the enhanced p27 degradation activity or altered p27 levels associated with many cancers [1,2].

Supplementary material

Additional methodological details and figures are published with this paper on the internet.

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Supplementary material

$p27^{Kip1}$ ubiquitination and degradation is regulated by the SCF^{Skp2} complex through phosphorylated Thr187 in p27

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Supplementary materials and methods

Recombinant proteins

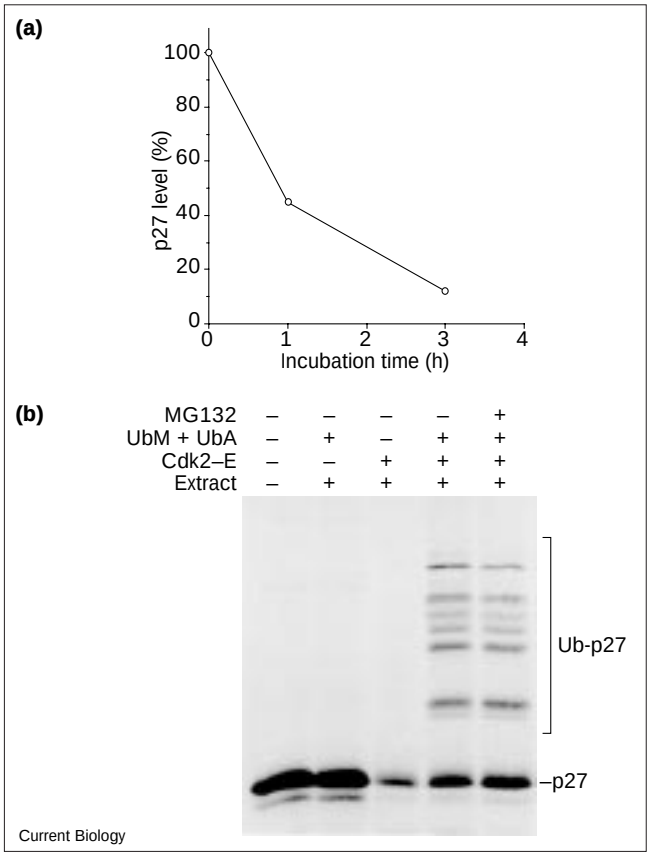
Cyclin E, p27, Skp1 and Skp2 were each cloned into pVL1392 (PharMingen) vector as glutathione-*S*-transferase (GST) fusion proteins. Human Cul-1, Skp2, and Skp1 cDNAs were also cloned directly into baculovirus pVL1392 or pVL1393 expression vectors. The construction of these baculoviruses was according to [S1]. The baculoviruses for Cdk2 and GST–cyclin A were kindly provided by David Morgan (UCSF) and Helen Piwnica-Worms (University of Washington, St Louis, USA) and have been described previously [S1]. The cDNA clone encoding human E1 ubiquitin-activating enzyme was kindly provided by Arthur Haas (Medical College of Wisconsin) and was cloned into the baculovirus expression vector, pAcSG–His–NT (PharMingen), as a His_6-tagged protein. The E1 protein was expressed in the baculovirus expression system and purified by ubiquitin affinity chromatography [S2]. The purification was monitored by protein staining and the E1 activity was assayed by covalent conjugation of biotinylated ubiquitin [S3]. For ^{35}S-labeled p27, SF9 cells were infected with baculoviruses encoding GST–p27. 40 h after infection, cells were labeled with ^{35}S-methionine for 3 h as described [S1]. The labeled GST–p27 protein was isolated by glutathione–Sepharose beads and the p27 portion was released from the beads by thrombin treatment for 30 min at room temperature (Calbiochem) [S4]. Thrombin was subsequently inactivated by 1 mM phenylmethylsulfonyl fluoride (PMSF). The purified p27 is monitored by autoradiography and quantified by protein staining and western blot analysis.

To assemble Cdk2–cyclin E, GST–cyclin E and Cdk2 baculoviruses were individually expressed in SF9 cells. The lysates were made in hypotonic buffer (20 mM Hepes pH 7.2, 5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT)). The lysates containing GST-cyclin E and Cdk2 were mixed and incubated in the presence of 10 mM ATP at 30°C for 1 h to assemble the active Cdk2–cyclin E kinase. The kinase was then affinity purified using the glutathione beads and quantified by protein staining and western blotting. The activity of purified kinase was monitored by the histone H1 assay [S1]. To produce SCF^{Skp2}, baculoviruses encoding GST–Skp1 and Cul-1, in the presence or absence of baculoviruses encoding Skp2, were co-infected into insect SF9 cells and were affinity purified using glutathione–Sepharose columns. The successful assembly of the complex was monitored and quantified by protein staining and western blotting. The *in vitro* translated proteins were produced and labeled with [^{35}S]methionine in TNT rabbit reticulocyte lysates according to the manufacturer's instructions (Promega). The human Cdc34 cDNA clone was kindly provided by Michele Pagano (New York University Medical Center) and was cloned into pGEXKG as a GST fusion protein and expressed in *Escherichia coli* BL21. GST–Cdc34 was isolated by glutathione column and the GST portion was removed by thrombin. The Cdc34 protein was further purified by Mono Q column and monitored by protein staining. The methyl ubiquitin and ubiquitin aldehyde were purchased from BostonBiochem.

Cells and extracts

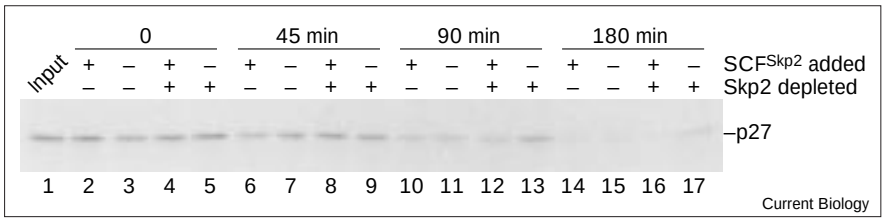
HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum at 37°C as previously described [S1]. For extract preparation, a suspension of HeLa cells was grown to $0.5–1 \times 10^6$ cells per ml (log phase) and extracts were made according to Brandeis and Hunt [S5] with modification. Cell pellets were washed twice with PBS and then with hypotonic buffer (see above). The cells were resuspended in two volumes of hypotonic buffer. They were lysed by Dounce homogenization using a loose pestle. The cytosolic extracts were prepared by centrifugation at 15,000 rpm using a Sorvall SS34 rotor. Aliquots of the extracts were immediately frozen in liquid nitrogen and kept at –80°C until use. For a typical p27 degradation reaction, 200 μg cytosolic extract was used in a total volume of 50 μl, with no more than 20% dilution of the extract. The reaction mixture also contained 2 mM ATP, 20 mM creatine phosphate, 50 μg/ml creatine kinase, 20 mM Hepes pH 7.2, 1 mM DTT and 10 mM $MgCl_2$. The reactions were initiated by adding ^{35}S-labeled p27 (2 μl of *in vitro* translated or 0.25–0.5 μg baculovirus-produced) and Cdk2–cyclin E (1–3 μg) and incubation at 30°C for 1–3 h. The requirement for Cdk2–cyclin E was usually titrated batchwise for different extract preparations beforehand and we found that a threshold level of Cdk2–cyclin E was required for p27 degradation. The

Figure S1



Ubiquitination of p27 in HeLa cytosolic extracts. (a) Time course of p27 degradation in extracts. The reaction products of p27 in Figure 1b were quantified and plotted. (b) Accumulation of ubiquitinated p27 in the presence of modified ubiquitins. p27 was incubated with HeLa extract, Cdk2–cyclin E, and methyl ubiquitin (UbM, 0.5 μg/ml) and ubiquitin aldehyde (UbA, 1 μM) as indicated. The ubiquitinated p27 ladders accumulated because methylated ubiquitin shortens the polyubiquitinated chain and thus slows down the rate of degradation while ubiquitin aldehyde inhibits de-ubiquitination of ubiquitinated proteins by isopeptidases. The reactions were carried out as described in Figure 1a.

Figure S2



Time course of SCF^{Skp2}-mediated restoration of p27 degradation in Skp2-depleted extracts. Mock- or Skp2-depleted HeLa extracts (200 μg) were incubated with recombinant SCF^{Skp2} (2 μg) and labeled p27 substrate (0.25 μg) in the presence of Cdk2–cyclin E (2 μg). The reactions were stopped at various time points as indicated. The reaction products were separated by SDS–PAGE and visualized by autoradiography.

requirement for Cdk2–cyclin E is also dependent on the amount of exogenously added p27, reflecting the fact that p27 serves both as an inhibitor and a substrate for the kinase. A substantial amount of endogenous p27 is present in the extract which is also degraded by addition of Cdk2–cyclin E. The reactions were stopped by adding 0.1% SDS, followed by addition of 1 ml NP-40 lysis buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40, and in the presence of protease inhibitors (5 μg/ml leupeptide, soybean trypsin inhibitor, aprotinin, and 100 mM benzamidine, all from Sigma). The reaction products were immunoprecipitated by p27 antibodies, fractionated in SDS–PAGE, and visualized by autoradiography. Degradation of endogenous p27 in the extracts was monitored by directly loading onto an SDS–PAGE in the SDS sample buffer, followed by western blotting with anti-p27 antibodies. For the ubiquitination by recombinant proteins in Figure 4b, purified and labeled p27 (0.2 μg) was incubated with recombinant SCF^{Skp2} (0.5 μg) in the presence of purified cyclin E and Cdk2–cyclin A kinases (1 μg each), recombinant human E1 ubiquitin-activating enzyme (0.5 μg), and recombinant Cdc34 (1 μg) in a reaction containing 20 mM Hepes pH 7.2, 10 mM $MgCl_2$, 2 mM ATP and 6 μM ubiquitin at 30°C for 3 h. The reaction products were isolated by immunoprecipitation with anti-p27 antibodies.

Antibodies and cDNA clones

Full-length GST–p27 was purified from bacteria and used as the antigen for immunization of rabbits. The Skp1, Skp2, and Cul-1 antibodies were described previously [S1,S6]. Other relevant antibodies were purchased from commercial sources (Santa Cruz Biotechnology and PharMingen). The rat anti-human β-TrCP antibody was kindly provided by Tian Xu (Yale University). The cDNA clones encoding F-box proteins such as MD6 and β-TrCP were obtained from Research Genetics and sequenced. Immunoprecipitation and western blot analysis were conducted as described previously [S1].

Depletion of the SCF complexes from HeLa extracts

Cul-1, Skp1 and Skp2 antibodies were affinity purified using recombinant proteins as antigens as described previously [S1]. Pre-immune IgG was purified by Sepharose–protein A beads (Pharmacia). For depletion of Skp2, Cul-1 or Skp1 proteins from HeLa extracts, 4 mg of affinity-purified antibodies or IgG were coupled to 1 ml Sepharose–protein A column. HeLa extracts (5–10 ml) were passed through the antibody–protein A column three times at 4°C. The flowthrough fractions of the columns were collected and examined for the efficiency of depletion using western blot analysis. They were then used as depleted extracts. For restoration of p27 degradation activity in Skp2-depleted extract, 2 μg purified Skp2, SCF^{Skp2} or SC (no Skp2) complexes were added to Skp2-depleted extracts and the degradation of p27 was monitored as described above.

Peptide synthesis

Peptides containing the carboxy-terminal end of p27 (residues 175–198, 24mer) – either CSDGSPNAGSVEQTPKKPGLRRRQT, or the same peptide phosphorylated on Thr187 (*T), CSDGSPNAGSVEQ*TPKKPGLRRRQT, were synthesized using the peptide synthesis facility at the HHMI Biopolymer/W. M. Keck Biotechnology Resource Laboratory at the Yale University School of Medicine. The Thr187-phosphorylated and unphosphorylated forms of the p27 carboxy-terminal peptides were conjugated to SulfoLink beads (Pierce) through the cysteine residue added at the amino terminus of the peptides according to the manufacturer's instruction (Pierce). For coupling reactions, 0.5 mg peptide was conjugated onto 2 ml of Sulfolink beads for 30 min and the residual sites on the beads were blocked by 20 mM cysteine for 2 h at room temperature. The beads were washed extensively with PBS and then hypotonic buffer. They were stored at 4°C. For F-box-protein-binding assays, 10 μl of the *in vitro* translated F-box proteins, including Skp2, β-TrCP, and MD6, were mixed with 20 μl peptide beads in 250 μl NP-40 lysis buffer containing protease inhibitors (5 μg/ml leupeptide, trypsin inhibitor, aprotinin and 100 μM benzamidine) and 100 mM NaF. Binding assays were performed at 4°C for 1 h with end-to-end rotation. The beads were washed four times with NP-40 buffer and the proteins associated with the beads were analyzed. Similar procedures were used for the extract binding except that 100–400 μg of HeLa extract was used as the source of SCF complexes, replacing the *in vitro* translated F-box proteins.

Supplementary references

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