Control of the SCF^Skp2–Cks1 ubiquitin ligase by the APC/C^Cdh1 ubiquitin ligase

Tarig Bashir, N. Valerio Dorrello, Virginia Amador,
Daniele Guardavaccaro & Michele Pagano

Department of Pathology, MSB 599, New York University School of Medicine, and New York University Cancer Institute, 550 First Avenue, New York, New York 10016, USA

Skp2 and its cofactor Cks1 are the substrate-targeting subunits of the SCF^Skp2–Cks1 (Skp1/Cul1/F-box protein) ubiquitin ligase complex that regulates entry into S phase by inducing the degradation of the cyclin-dependent kinase inhibitors p21 and p27 (ref. 1). Skp2 is an oncoprotein that often shows increased expression in human cancers[2]; however, the mechanism that regulates its cellular abundance is not well understood. Here we show that both Skp2 and Cks1 proteins are unstable in G1 and that their degradation is mediated by the ubiquitin ligase APC/C^Cdh1 (anaphase-promoting complex/cyclosome and its activator Cdh1). Silencing of Cdh1 by RNA interference in G1 cells stabilizes Skp2 and Cks1, with a consequent increase in p21 and p27 proteolysis. Depletion of Cdh1 also increases the percentage of cells in S phase, whereas concomitant downregulation of Skp2 reverses this effect, showing that Skp2 is an essential

target of APC/C^Cdh1. Expression of a stable Skp2 mutant that cannot bind APC/C^Cdh1 induces premature entry into S phase. Thus, the induction of Skp2 and Cks1 degradation in G1 represents a principal mechanism by which APC/C^Cdh1 prevents the unscheduled degradation of SCF^Skp2–Cks1 substrates and maintains the G1 state.

We examined the cell-cycle regulation of Skp2 and Cks1 proteins and found that they decreased considerably in late M phase and G1, and were induced again as cells approached S phase (Fig. 1a). To assess whether the reduction in Skp2 and Cks1 was due to protein destabilization, we measured their half-lives in prometaphase cells and in cells released from a prometaphase block for 1 h. We found that both Skp2 and Cks1 are more stable in prometaphase than in late M/early G1 (Fig. 1b). Furthermore, in cells exiting the cell cycle, Cks1 was found to decrease in parallel with Skp2 (Fig. 1c), which has been previously reported to decrease in response to serum deprivation[3]. Treatment with the proteasome inhibitor ZLLL prevented the downregulation of both Skp2 and Cks1 (Fig. 1d). Thus, we conclude that Skp2 and Cks1 proteins are unstable in G0/G1.

The oscillations of Skp2 and Cks1 roughly paralleled those of cyclin A and cyclin B (Fig. 1a), which suggested that they might be degraded through a similar mechanism. We therefore investigated the involvement of the ubiquitin ligase APC/C[4] in controlling the degradation of Skp2 and Cks1. Because APC/C is regulated by its association with one of two activator proteins, Cdh1 and Cdc20, we transfected 293T cells with a construct expressing either Skp2 (Fig. 1e) or Cks1 (Fig. 1f), along with a construct expressing Cdh1 or Cdc20. Enforced expression of Cdh1 resulted in a considerable
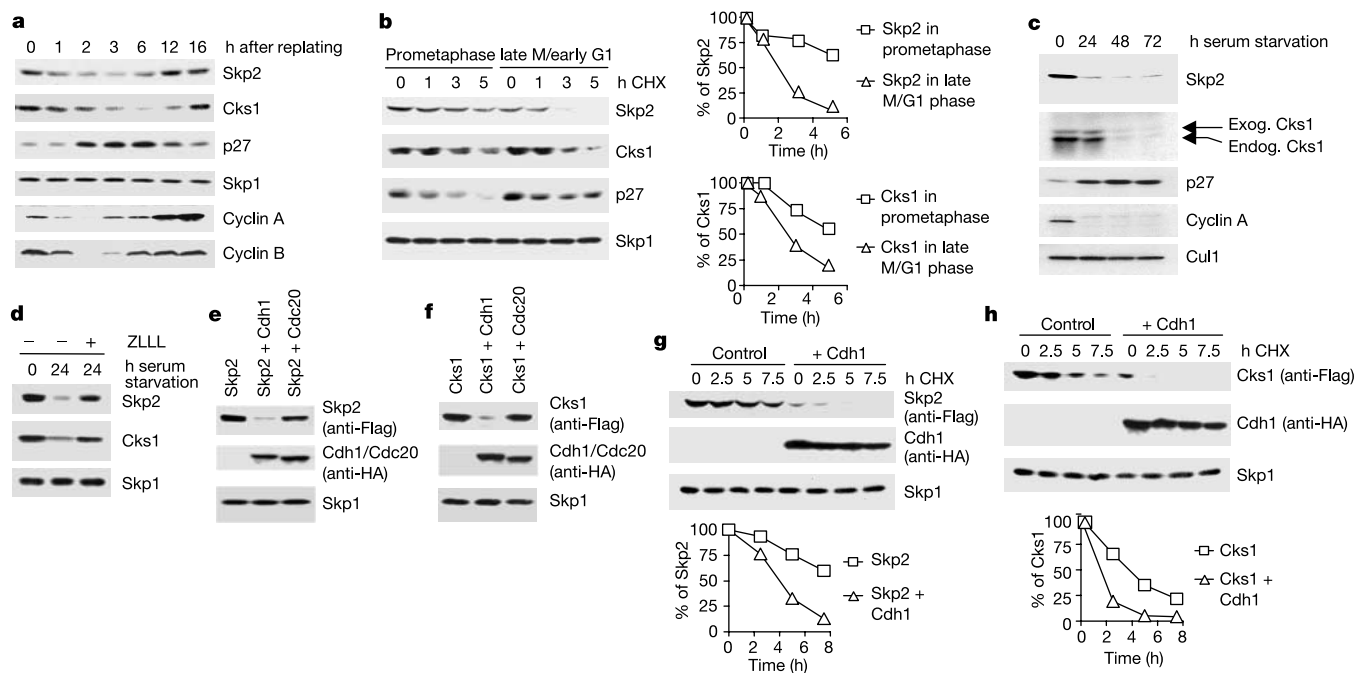

Figure 1 *In vivo* degradation of Skp2 and Cks1 is stimulated by Cdh1. **a–h**, Immunoblots. **a**, HeLa cells were released from nocodazole-induced prometaphase arrest and collected at the indicated times. **b**, Prometaphase cells were replated for 1 h in the presence (prometaphase) or absence (late M/early G1) of nocodazole. Cycloheximide (CHX) was then added and cell extracts were prepared at the indicated times. The results are quantified in the graphs on the right. **c**, Rat-1 cells infected with a retrovirus expressing Flag-tagged Cks1 were serum-deprived and extracts were prepared at the indicated times. **d**, Rat-1 cells were serum-deprived for 24 h and the proteasome inhibitor ZLLL (+)

or vehicle alone (−) was added for the last 6 h. **e**, Coexpression of Flag–Skp2 and either HA–Cdh1 or HA–Cdc20 in 293T cells. **f**, Coexpression of Flag–Cks1 and either HA–Cdh1 or HA–Cdc20 in 293T cells. **g**, HeLa cells were transfected with Flag–Skp2 with or without HA–Cdh1. Twenty-four hours after transfection, cells were treated with CHX. Skp2 expression is quantified in the graph below. **h**, HeLa cells were transfected with Flag–Cks1 with or without HA–Cdh1. The half-life of Cks1 was analysed as in **g**.

decrease in the steady-state amounts of both proteins, whereas overexpression of Cdc20 had no effect. Notably, the decrease in Skp2 and Cks1 expression correlated with their destabilization (Fig. 1g, h).

We next used small interfering RNAs (siRNAs) to reduce the expression of Cdh1. After transfection with siRNA, prometaphase HeLa cells were collected and replated in fresh medium. Cells transfected with siRNA targeting Cdh1 showed no G1-specific reduction in Skp2 and Cks1, a much less pronounced upregulation of p21 and p27 as compared with control cells (Fig. 2a), and an increase in the half-life of both Skp2 and Cks1 (Fig. 2b).

We also silenced Cdh1 expression in T98G cells exiting the cell cycle (as shown by the accumulation of p27; Fig. 2c). Silencing of Cdh1 prevented the degradation of Skp2 and Cks1 for up to 36 h after serum removal, when the effect of the siRNA started to wane, as judged by the recovery of Cdh1. It has been suggested that Skp2 downregulation in G0/G1 is due to Cul1-dependent auto-ubiquitination[3]; however, depletion of Cul1 did not prevent the degradation of Skp2 and Cks1 (Fig. 2c).

Downregulation of Cdh1 resulted in an increase in the number of HeLa cells incorporating 5-bromodeoxyuridine (BrdU; Fig. 2d). Notably, co-depletion of Skp2 reversed the stimulatory effect

induced by Cdh1 inhibition. Furthermore, single-cell immuno-fluorescence analysis (Fig. 2d, bottom) showed that Cdh1-depleted cells progressed faster through S phase, as $78.2 \pm 1.4\%$ of these cells showed a diffuse BrdU staining typical of advanced S phase, rather than the distinct foci of BrdU incorporation characteristic of initial DNA replication. By contrast, diffuse BrdU staining was present in only $65.7 \pm 2.1\%$ of control cells, $37.8 \pm 0.5\%$ of Skp2-depleted cells and $35 \pm 2.1\%$ of cells depleted in both Cdh1 and Skp2. Taken together, these results show that in G0/G1 the stability of Skp2 and Cks1 is regulated by APC/C^{Cdh1} and that Skp2 is an essential target of Cdh1, because in Skp2-depleted cells Cdh1 downregulation is insufficient to promote DNA replication.

Additional key observations could be derived from the siRNA experiments with regard to the cell cycle in the presence of reduced amounts of Cdh1. First, cells still exited mitosis with normal kinetics, as judged by the degradation of cyclin A and cyclin B and the disappearance of phosphorylation on Ser 10 of histone H3 (Fig. 2a). By contrast, silencing of Cdc20 delayed mitotic exit by about 1 h (data not shown). Second, cyclin A and cyclin B are normally downregulated in mitosis, but cyclin A re-accumulated at G1/S slightly earlier in Cdh1-silenced cells than in control cells (Fig. 2a, time points 4 and 6). This effect was even more evident in
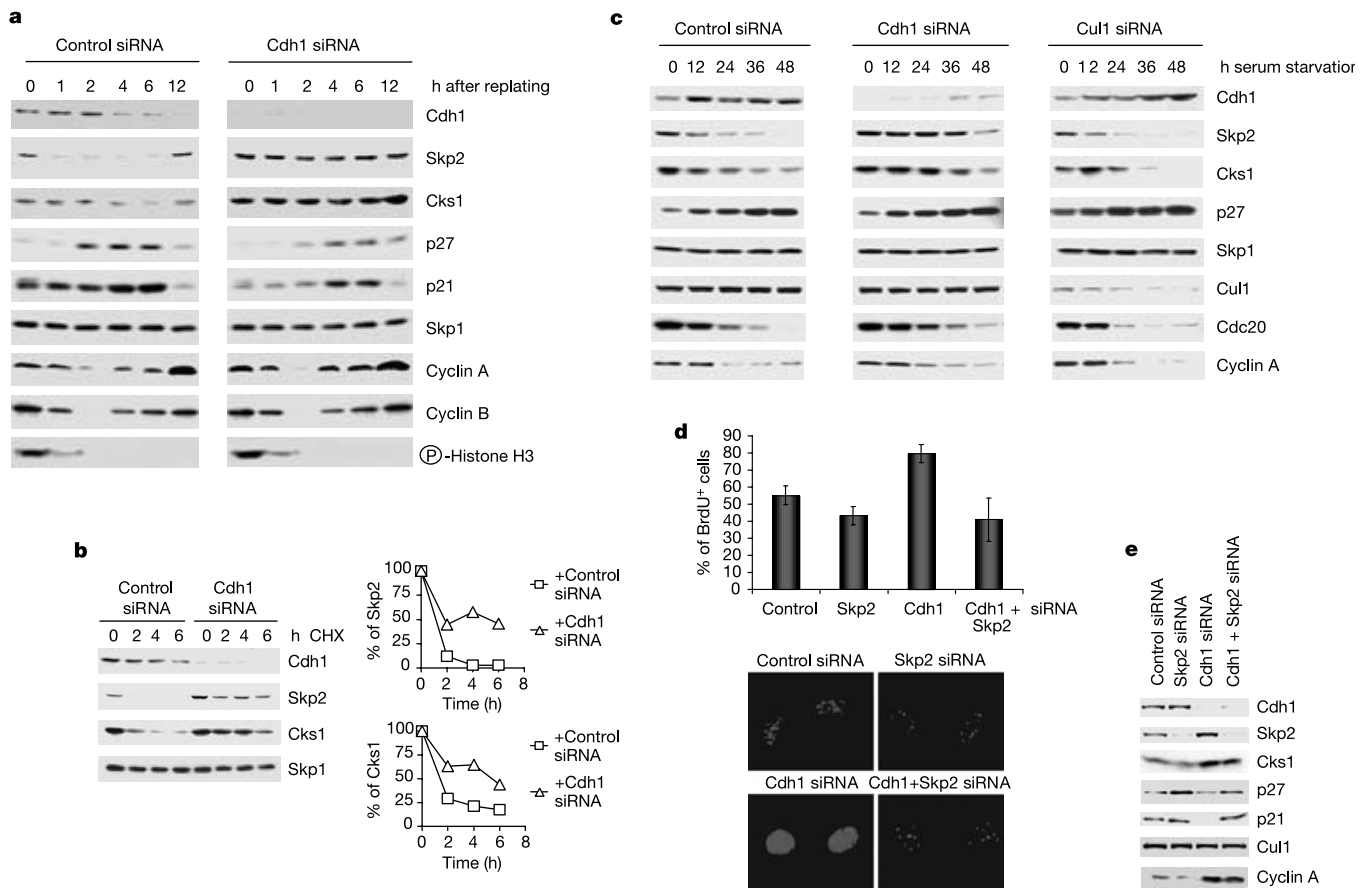

Figure 2 Cdh1 silencing stabilizes Skp2 and Cks1 in G1 and G0. **a–c**, Immunoblots. **a**, HeLa cells were transfected with siRNA corresponding to a non-relevant mRNA (control siRNA) or to Cdh1 mRNA. Twelve hours after the last transfection, nocodazole was added for 16 h. Prometaphase cells were collected and replated in fresh medium for the indicated times. **b**, Silencing of Cdh1 and synchronization were done as in **a**. Prometaphase cells were replated in drug-free medium for 1 h before CHX was added for the indicated times. The results are quantified in the graphs on the right. **c**, Silencing of Cdh1 and Cul1 was done as in **a**, except that T98G cells were used. Twenty-four hours after the last transfection, cells were deprived of serum for the indicated times. **d**, Top, HeLa cells were transfected with the indicated siRNAs and labelled with BrdU for 60 min, and the percentage of BrdU-positive cells was determined by immunofluorescence; bottom, representative cells visualized by immunofluorescence using an antibody against BrdU. **e**, Cells treated as in **d** were analysed by immunoblotting.
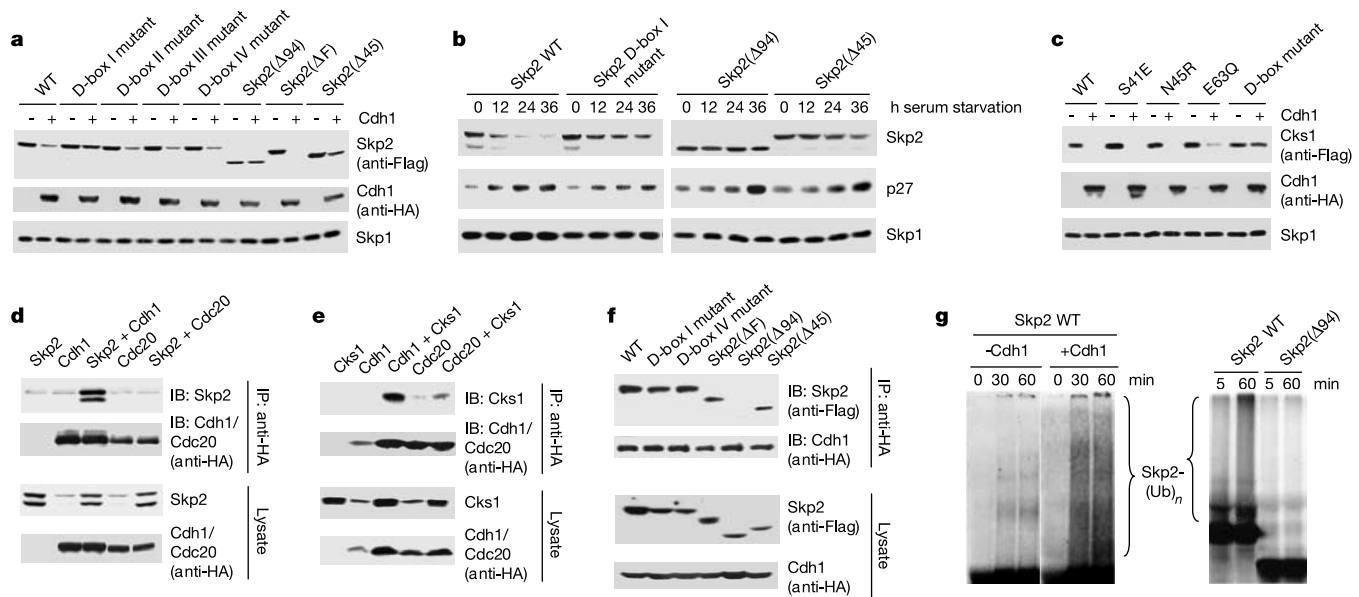
Figure 3 Requirements for APC/C^Cdh1-mediated degradation of Skp2 and Cks1. **a**–**f**, Immunoblots. **a**, Expression of Flag-tagged wild-type Skp2 or the indicated Skp2 mutants with or without HA–Cdh1 in 293T cells. **b**, Rat-1 cells were infected with retroviruses expressing human wild-type Skp2 or the indicated Skp2 mutants, and deprived of serum for the indicated times. **c**, Expression of Flag-tagged wild-type Cks1 or the indicated Cks1 mutants with or without HA–Cdh1 in 293T cells. **d**, Skp2 and either HA–Cdh1 or HA–Cdc20 were expressed in 293T cells, and ZLLL was added 6 h before the

cells were collected. Cell extracts were immunoprecipitated (IP) with an antibody against HA and analysed by immunoblotting (IB). **e**, As **d**, except that Cks1 rather than Skp2 was expressed. **f**, As **d**, but with the indicated Skp2 mutants. **g**, Immunopurified APC/C supplemented with recombinant Cdh1 ubiquitinates *in vitro* translated wild-type Skp2 but not Skp2(Δ94). The brackets mark a ladder of bands of relative molecular mass >45,000 corresponding to polyubiquitinated Skp2.

asynchronous cells, in which the silencing of Cdh1 induced a marked increase in cyclin A (Fig. 2e). Third, the accumulation of p27 and p21 was markedly reduced in cells re-entering G1 from mitosis (Fig. 2a) and in asynchronous cells (Fig. 2e), but not in serum-starved cells (Fig. 2c and data not shown). Thus, Cdh1 seems to be rate limiting for the accumulation of SCF^Skp2–Cks1 substrates in cycling cells, but not in cells withdrawing from the cell cycle.

Substrates of APC/C are often characterized by the presence of a 'destruction box' (D-box), which was first identified in cyclin B as a stretch of nine amino acids (RxxLxxIxN) that when mutated stabilizes the protein[4]. However, many substrates contain only the minimal RxxL motif in their D-boxes, which together with additional regions in the substrate regulate their degradation by APC/C. We identified five RxxL motifs in human Skp2 that potentially might be D-boxes (amino acids 3–6, 84–87, 234–237, 294–297 and 415–418), but no other known putative Cdh1 recognition motifs (such as the KEN-box or A-box). One potential D-box (amino acids 234–237) is located in a leucine-rich repeat, which is part of the putative substrate-binding motif and therefore less likely to be also a recognition site for Cdh1.

We mutated the arginine and leucine residues in the other four motifs to alanine and determined the stabilities of these D-box mutants and three Skp2 deletion mutants in 293T cells overexpressing Cdh1. Of these Skp2 mutants, one with a mutation at the very amino terminus (D-box I) and two N-terminal deletion mutants, Skp2(Δ45) and Skp2(Δ94), were resistant to the effect of overexpressed Cdh1 (Fig. 3a). Notably, a Skp2 mutant lacking the F-box, Skp2(ΔF), was efficiently downregulated by Cdh1, showing that Skp2 does not need to be part of an SCF complex to be a substrate of APC/C. We also investigated the stabilities of the D-box I and deletion mutants in Rat-1 fibroblasts exiting the cell cycle. Skp2 was monitored by immunoblotting with an antibody against human Skp2 that does not recognize endogenous rat Skp2

(ref. 5). All three Skp2 mutants were clearly stabilized under conditions that promoted rapid degradation of wild-type Skp2 (Fig. 3b), confirming that the degradation of Skp2 is dependent on D-box I.

We next investigated the requirements for Cdh1-mediated degradation of Cks1. There is one RxxL motif at the carboxy terminus of Cks1 (amino acids 70–73), which when mutated prevented Cdh1-induced degradation (Fig. 3c), suggesting that this region represents a bona fide D-box. In addition, a Cks1 mutant that cannot bind cyclin-dependent kinases (CDKs), Glu63Gln, and two mutants that cannot bind Skp2, Ser41Glu and Asn45Arg (ref. 6), were still downregulated when Cdh1 was coexpressed (Fig. 3c), showing that stable binding to either CDKs or Skp2 is not a prerequisite for APC/C^Cdh1-mediated degradation of Cks1.

Furthermore, we found that Skp2 physically interacts with Cdh1 but not with Cdc20 (Fig. 3d), confirming the specificity of Cdh1 for this substrate. Similarly, Cks1 co-immunoprecipitated with Cdh1 and to a lesser extent with Cdc20 (Fig. 3e), which is not surprising because Cks1 binds to APC/C^Cdc20 to promote its phosphorylation[7]. Notably, wild-type Skp2, but not Skp2(Δ94), was detected in anti-Cdh1 immunoprecipitates (Fig. 3f). Although the Skp2 D-box I mutant and Skp2(Δ45) were more stable than the wild-type protein (Fig. 3a, b), they still bound Cdh1 (Fig. 3f). These results show that a region of Skp2 between amino acids 46 and 94 mediates the binding between Skp2 and Cdh1, in line with the notion that the RxxL motif is an APC/C recognition motif but does not necessarily mediate a stable interaction with this ligase. The importance of the Skp2 N-terminal region was further shown by the ability of APC/C^Cdh1 to ubiquitinate *in vitro* wild-type Skp2 but not Skp2(Δ94) (Fig. 3g). In contrast to Skp2, we were unable to *in vitro* ubiquitinate Cks1 using APC/C^Cdh1, suggesting that an essential factor was missing or that Cks1, although downstream of APC/C^Cdh1, might not be its direct substrate.

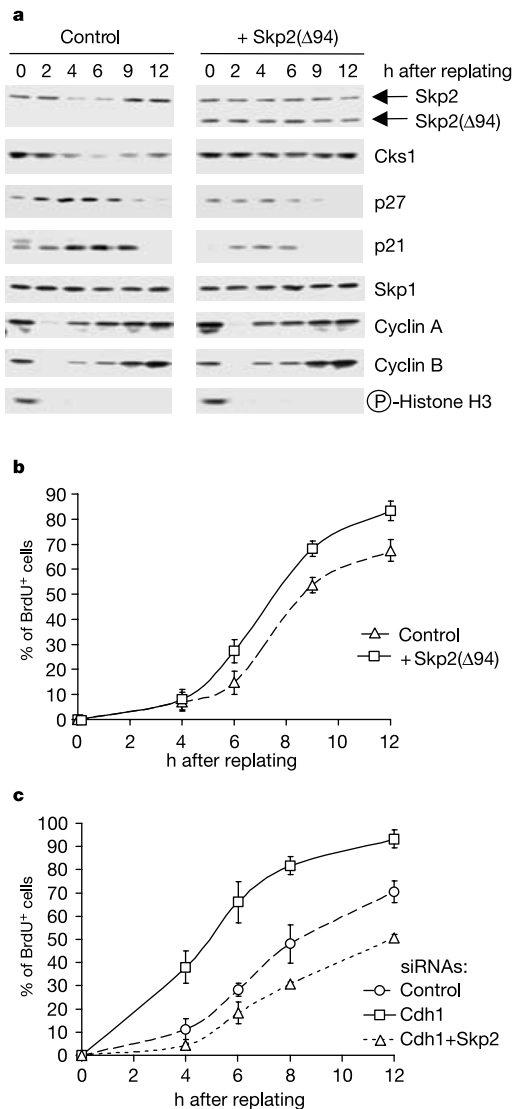To examine further the biological significance of Skp2 destruc-

Figure 4 Biological relevance of Skp2 degradation. **a**, HeLa cells were either mock infected or infected with a retrovirus expressing Skp2(Δ94). After nocodazole treatment, prometaphase cells were replated in fresh medium for the indicated times. Cells were collected and analysed by immunoblotting. **b**, The percentage of BrdU-positive cells was determined in three independent experiments done as in **a**. **c**, The percentage of BrdU-positive cells was determined in three independent experiments done as in Fig. 2d, with the exception that cells were synchronized as in Fig. 2a and released from prometaphase for the indicated times before BrdU was added for 30 min.

tion, we evaluated the effect of expressing the stable Skp2(Δ94) mutant in synchronized cells. As compared with control cells, cells expressing Skp2(Δ94) showed accelerated degradation of p21 and p27, and stabilization of endogenous Skp2 and Cks1 in G1 (Fig. 4a). Notably, these cells progressed considerably faster into S phase (Fig. 4b), although not to the same extent as Cdh1-depleted cells (Fig. 4c). This difference is probably due to the fact that Skp2 is an essential, but not the only, substrate that is degraded through APC/C^{Cdh1} to prevent premature entry into S phase. Extensive overexpression of Skp2 achieved with adenoviral vectors accelerates S-phase entry[8], whereas physiological amounts of Skp2 expressed from retroviral vectors are subject to regulated proteolysis and

are unable to force cells into S phase (refs 3, 5, and Fig. 3b). By contrast, the expression of physiological amounts of a stable Skp2 mutant in G1 induced premature S-phase entry. These results show that Skp2 destruction is an essential event for proper cell-cycle control.

Experiments in cultured cells and transgenic mice have shown that Skp2 is the product of a proto-oncogene[2]. In human carcinomas and lymphomas, p27 destabilization is predictive of poor prognosis and correlates with high concentrations of Skp2 (ref. 9). We have shown here that the degradation of Skp2 and Cks1 is controlled by APC/C^{Cdh1}, which represents a principal mechanism to maintain the G1 state and to avoid premature entry into S phase, a potential cause of genetic instability. Deregulation of this mechanism might contribute to overexpression of Skp2 and Cks1 and thereby promote uncontrolled proliferation in cancer cells. □

Methods

Cell culture and biochemistry

Cell culture and synchronization of HeLa, Rat-1, 293T and T98G cells were done as described[3,5,10]. Antibodies against Skp2, Skp1, p27, p21, cyclin A, cyclin B, Cul1, phospho-histone H3, BrdU, haemagglutinin (HA) and Flag have been described[5,10]. Antibodies against Cdh1 and Cdc20 were a gift from J. Lukas. An antibody against human Cks1 (which does not crossreact with Cks2) was generated in collaboration with Zymed. Immunoblot analysis, immunoprecipitations and immunofluorescence were done as described[10]. We carried out *in vitro* ubiquitination as described[11], except that we used extracts from serum-deprived T98G cells.

Plasmids, transfections, infections and siRNAs

Skp2 and Cks1 mutants were generated using the QuickChange Site-directed Mutagenesis kit (Stratagene). Cell transfections and retroviral infections were done as described[10,12]. The siRNA oligonucleotide sequences were 5′-AATGAGAAGTCT CCCAGTCAGdTdT-3′ (Cdh1), 5′-AAGGGAGUGACAAAGACUUUGdTdT-3′ or 5′-AAUCUAAGCCUGGAA GGCCUGdTdT-3′ (Skp2) and 5′-UAGACAUUGGGUU CGCCGUdTdT-3′ (Cul1).

1. Reed, S. Ratchets and clocks: the cell cycle, ubiquitylation and protein turnover. *Nature Rev. Mol. Cell. Biol.* **4,** 855–864 (2003).
2. Pagano, M. & Benmaamar, R. When protein destruction runs amok, malignancy is on the loose. *Cancer Cell* **4,** 251–256 (2003).
3. Wirbelauer, C. *et al.* The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. *EMBO J.* **19,** 5362–5375 (2000).
4. Peters, J. M. The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol. Cell* **9,** 931–943 (2002).
5. Carrano, A. C. & Pagano, M. Role of the F-box protein Skp2 in adhesion-dependent cell cycle progression. *J. Cell Biol.* **153,** 1381–1389 (2001).
6. Sitry, D. *et al.* Three different binding sites of Cks1 are required for p27–ubiquitin ligation. *J. Biol. Chem.* **277,** 42233–42240 (2002).
7. Sudakin, V., Shteinberg, M., Ganoth, D., Hershko, J. & Hershko, A. Binding of activated cyclosome to p13^{Suc1}. Use for affinity purification. *J. Biol. Chem.* **272,** 18051–18059 (1997).
8. Sutterluty, H. *et al.* p45^{Skp2} promotes p27^{Kip1} degradation and induces S phase in quiescent cells. *Nature Cell Biol.* **1,** 207–214 (1999).
9. Bloom, J. & Pagano, M. Deregulated degradation of the CDK inhibitor p27 and malignant transformation. *Semin. Cancer Biol.* **13,** 41–47 (2003).
10. Guardavaccaro, D. *et al.* Control of meiotic and mitotic progression by the F box protein β-Trcp1 *in vivo. Dev. Cell* **4,** 799–812 (2003).
11. Fang, G., Yu, H. & Kirschner, M. W. Direct binding of Cdc20 protein family members activates the anaphase-promoting complex in mitosis and G1. *Mol. Cell* **2,** 163–171 (1998).
12. Bloom, J., Amador, V., Bartolini, F., DeMartino, G. & Pagano, M. Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation. *Cell* **115,** 71–82 (2003).