Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex

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Cell-cycle transitions are driven by waves of ubiquitin-dependent degradation of key cell-cycle regulators. SCF (Skp1/Cullin/F-box protein) complexes and anaphase-promoting complexes (APC) represent two major classes of ubiquitin ligases whose activities are thought to regulate primarily the G1/S and metaphase/anaphase cell-cycle transitions, respectively[1,2]. The major target of the Skp1/Cul1/Skp2 (SCF^{SKP2}) complex is thought to be the Cdk inhibitor p27 during S phase, whereas the principal targets for the APC are thought to be involved in chromatid separation (securin) and exit from mitosis (cyclin B). Although the role of the APC in mitosis is relatively clear, there is mounting evidence that APCs containing Cdh1 (APC^{CDH1}) also have a function in the G1 phase of the cell cycle[2,3]. Here, we show that the F-box protein Skp2 is polyubiquitinated, and hence earmarked for destruction, by APC^{CDH1}. As a result, accumulation of SCF^{SKP2} requires prior inactivation of APC^{CDH1}. These findings provide an insight into the orchestration of SCF and APC activities during cell-cycle progression, and into the involvement of the APC in G1.

Both transcriptional and post-transcriptional changes are thought to underlie cell-cycle-dependent changes in Skp2 (refs 4–6). Skp2 levels are lowest during the G1 phase of the cell cycle, a period when the APC^{CDH1} ubiquitin ligase complex is active. We noted that Skp2 contains a canonical D-box motif (R-X-X-L-X-X-X-X-N/D/E)[7], suggesting that it might be a direct substrate of APC^{CDH1} (Fig. 1a). In support of these clues, we found evidence of Skp2 bound to Cdh1 in co-immunoprecipitation and glutathione *S*-transferase (GST) pull-down assays (Supplementary Figs 1–3). This interaction seemed to be specific because it was abolished by deletion of the first 90 amino acid residues from the Skp2 amino terminus, which contains the D-box motif, or by removal of the Cdh1 WD-40 domain. A Skp2 mutant in which the D box alone was deleted (Skp2Δdb) showed some binding to Cdh1 in these assays. However, it is known that the D-box motif in another APC substrate, cyclin B, is important for its polyubiquitination by APC, but not for APC binding[8]. Although Skp2 lacks a recognizable KEN-box motif, there are many examples of APC^{CDH1} substrates that contain a D box without a KEN box[3].

As a direct test of the role of the D-box motif in APC-mediated degradation, we examined the cell-cycle dependence of Skp2 degradation. We found that Skp2, but not Skp2Δdb, was rapidly degraded by cell extracts prepared from synchronized G1 cells but not by extracts from cells at the S phase, at which point APC^{CDH1} is inactive (Fig. 1b, c). Moreover, degradation of Skp2 was inhibited in the presence of a recombinant polypeptide corresponding to the N terminus of cyclin B1, which is a very specific competitive APC inhibitor[9] (Fig. 2a). Earlier studies showed that *Xenopus* interphase cell extracts can support APC^{CDH1}-dependent polyubiquitination and degradation if supplemented with recombinant Cdh1 (ref. 10). Skp2, but not Skp2Δdb, was efficiently degraded by such extracts (Fig. 2b) in a Cdh1-dependent manner. By contrast, cyclin B, but not Skp2, was destroyed by *Xenopus* extracts under conditions where APC^{CDC20}, but not APC^{CDH1}, was active (data not shown). Finally, Skp2, but not Skp2Δdb, was polyubiquitinated *in vitro* by immunoaffinity-purified APC complexes derived from mammalian (Fig. 2c) or *Xenopus* (Fig. 2d) cell extracts. As expected, polyubiquitination was abolished when methyl-ubiquitin was substituted

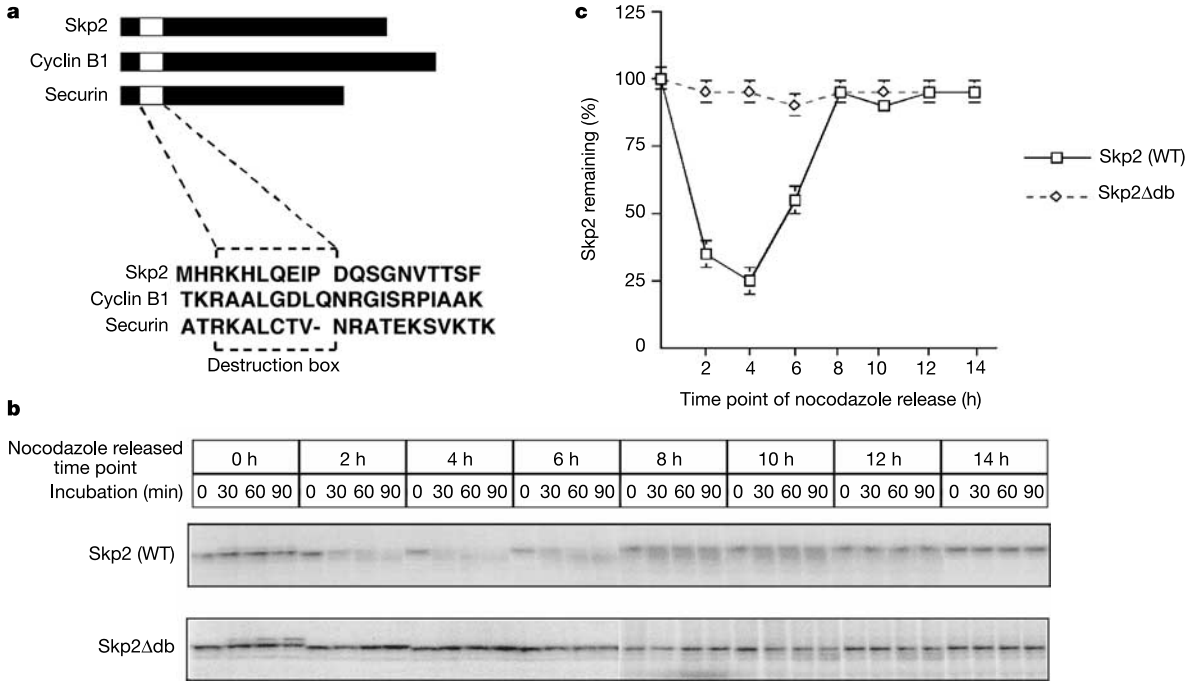

Figure 1 D-box-dependent degradation of Skp2 in G1 extracts. **a**, Alignment of D boxes in Skp2, cyclin B1 and securin. **b**, Autoradiograms of ^{35}S-labelled Skp2 after incubation for 0–90 min in synchronous HeLa cell extracts prepared at the indicated times after nocodazole release. WT, wild type. **c**, Quantification of band intensities in **b**.

for ubiquitin in these reactions (Fig. 2c). Collectively, these data indicate that APC^{CDH1} can polyubiquitinate Skp2 in a D-box-dependent manner.

To investigate whether APC^{CDH1} is a major regulator of Skp2 *in vivo*, we monitored Skp2 levels after experimental reduction of APC activity through depletion of Cdh1. Transfection of HeLa cells with three different short interfering (si)RNAs (out of a total of five siRNAs tested) decreased the steady-state levels of Cdh1 in asynchronously growing HeLa cells (Fig. 3a). Notably, transfection of cells with these three siRNAs increased Skp2 levels compared with the ineffective Cdh1 siRNAs or various control siRNAs. The small fluctuations in Skp2 levels in the absence of Cdh1 downregulation are probably due to siRNA off-target effects.

To exclude the possibility that the observed changes in Skp2 were due to changes in the distribution of the population of cells in the cell cycle, we measured the cell-cycle state by fluorescence-activated cell sorting (FACS) and by immunoblotting for cell-cycle markers. In these experiments, HeLa cells were treated with Cdh1 or control siRNA, synchronized at mitosis with nocodazole, and released into G1. In cells treated with control siRNA, Skp2 levels fell after release and remained depressed until approximately 12 h later, at which point cells began to enter S phase (Fig. 3b, c). p27 levels rose during G1 coincident with the fall in Skp2 levels. By contrast, in cells that were depleted of Cdh1 by siRNA, Skp2 remained at high levels; other APC^{CDH1} substrates, such as Plk and Cdc20, also avoided downregulation. In the continued presence of high levels of Skp2, accumulation of p27 was markedly attenuated. The turnover of some other APC substrates, such as cyclin A, geminin and cyclin B, was minimally affected, perhaps because inhibition of Cdh1 allows Cdc20 to persist—APC^{CDC20} can substitute for APC^{CDH1} for these substrates[11] (Fig. 3b; see also Supplementary Fig. 4). These behaviours were clearly observed within 6 h after nocodazole release, before any changes in cell-cycle distribution were apparent by FACS (Fig. 3c). Cells treated with Cdh1 siRNA (but not control siRNA) entered S phase approximately 3 h earlier than the untreated cells, in keeping with previous results obtained with a neutralizing Cdh1 antibody[12]. These kinetic studies suggest that persistence of high levels of Skp2 produced by downregulation of Cdh1 is a cause, rather than a consequence, of accelerated cell-cycle progression. In support of this conclusion, accelerated cell-cycle progression after Cdh1 removal was abrogated by Skp2 siRNA (Fig. 3d, e). Therefore Skp2 is epistatic to Cdh1 in this assay.

In a reciprocal set of experiments, we induced Cdh1 expression in asynchronously growing U2OS cells and this led to marked downregulation of Skp2, in keeping with an earlier report[12]. As expected, increased levels of Cdh1 also led to decreased levels of other APC substrates (Fig. 3f). Downregulation of Skp2 in this setting was attenuated by proteasome inhibitors (Fig. 3g). Finally, as a test of the specificity of Skp2 for APC, we found that Skp2Δdb (as with a Skp2 truncation mutant that cannot bind to Cdh1) was degraded much more slowly relative to wild-type Skp2 in HeLa cells entering G1 after nocodazole-induced mitotic arrest (Fig. 4a–c; see also Supplementary Fig. 2b). In these experiments, the amount of Skp2 introduced into the cells was kept low so that proteins required for Skp2 degradation would not become limiting. Under these conditions, exogenous Skp2 does not alter cell-cycle kinetics (Fig. 4d). We conclude that the disappearance of Skp2 in G1 is largely due to polyubiquitination by APC^{CDH1} in a D-box-specific manner.

Skp2 is the substrate recognition module of the SCF^{SKP2} ubiquitin ligase complex and is an important regulator of S-phase entry. As with most key regulators its abundance must be tightly controlled. Loss of Skp2 leads to inappropriate accumulation of p27, c-Myc and cyclin E, and it is thought that this contributes to the associated polyploidy and centrosomal duplication[13–15]. Conversely, forced expression of Skp2 leads to proliferation and transformation *in vitro* and tumour formation *in vivo*[16–19]. In keeping with these findings, increased Skp2 levels have been linked to poor prognosis of human cancer patients[20]. Our findings shed new light on the cellular pathways in G1 for regulating Skp2, and on the importance of APC in G1 control. They also imply that under some circumstances APC^{CDH1} might possess tumour suppressor activity through its inhibition of the Skp2 oncoprotein. In support of these ideas, an accompanying paper reports that ectopic expression of Skp2Δdb in $Skp2^{+/+}$ HeLa cells promotes S-phase entry[21], and we have observed that ectopic expression of Skp2Δdb, but not wild-type Skp2, blocks neuronal differentiation *in vitro* (N.G.A. and M.W.K., unpublished data).

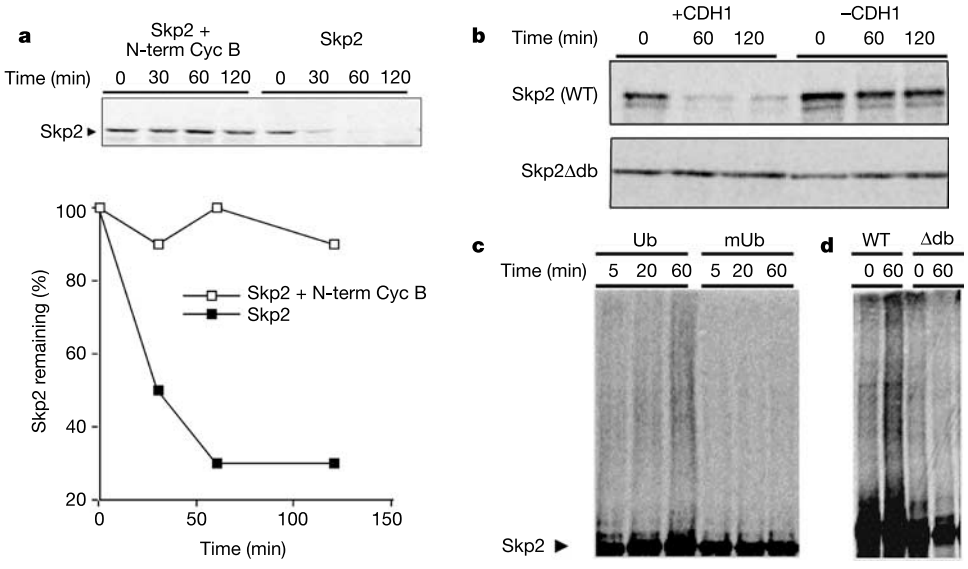

Figure 2 Polyubiquitination and destruction of Skp2 by APC *in vitro*. **a**, **b**, Autoradiograms of ^{35}S-labelled Skp2 after incubation for 0–120 min in HeLa cell extract prepared from G1 cells (**a**) or *Xenopus* interphase extracts that were or were not supplemented with Cdh1 (**b**). An N-terminal fragment of cyclin B1 was added in the reactions in **a** where indicated. **c**, **d**, Autoradiogram of ^{35}S-labelled Skp2 after *in vitro* ubiquitination by anti-APC immunoprecipitates derived from HeLa cells (**c**) or *Xenopus* interphase extracts supplemented with Cdh1 (**d**) in the presence of recombinant wild-type ubiquitin (Ub) or methyl-ubiquitin (mUb). All the reactions in **d** contained ubiquitin.

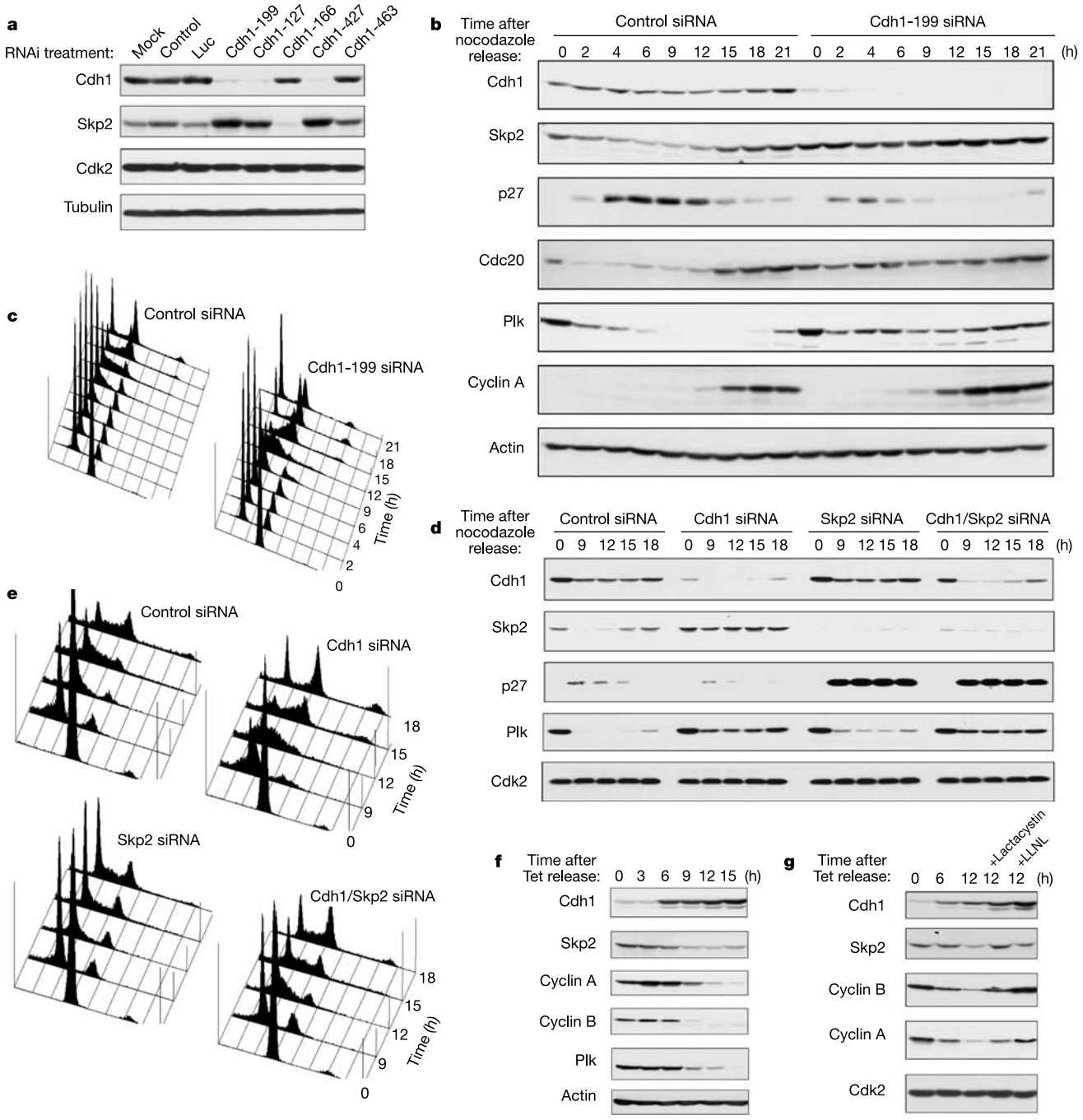

Figure 3 Cdh1 is necessary and sufficient for downregulation of Skp2 in G1. **a**, Immunoblot analysis of HeLa cells transfected with the indicated siRNA. The control lane is scrambled cyclin A siRNA; Luc, siRNA directed against firefly Luciferase. siRNA, short interfering RNA. **b**–**e**, Immunoblot (**b**, **d**) and FACS analysis (**c**, **e**) of HeLa cells transfected with the indicated siRNA, synchronized by growth in nocodazole, and then released for the indicated periods of time. In **c**, **e**, the *y* axis indicates cell number and the *x* axis indicates DNA content as determined by propidium iodide staining. **f**, **g**, Immunoblot analysis of U2OS cells that were engineered to produce Cdh1 after removal of tetracycline (Tet)[12]. In **e** the proteasomal inhibitors lactacystin or LLNL were added where indicated.

Although pointing to APC as the key regulator of the stability of Skp2 in G1, our data are not necessarily incompatible with earlier indications that Skp2 may be regulated in quiescent cells by a different ubiquitin protein ligase containing the Skp2-bound SCF components Skp1, Cul1 and Roc1 (also called Rbx1 or Hrt1)[6]. Our study involved cells that had not entered a quiescent period and were proceeding through the cell cycle. Notably, we found that a Skp2 F-box mutant that cannot form SCF complexes is a better APC^{CDH1} substrate than wild-type Skp2 *in vivo*, possibly owing to competition between APC^{CDH1} and *apo*-SCF^{SKP2} (data not shown).

Although the cell cycle often seems to be a linear progression of events, once compared to a row of dominoes[22], in fact its successful orchestration requires events in one phase to set the tempo or to monitor events in the other phase. The tempo is insured not only by mutually inhibitory phosphorylation events but also by mutually inhibitory degradation events. Thus, in addition to a kinase cycle we

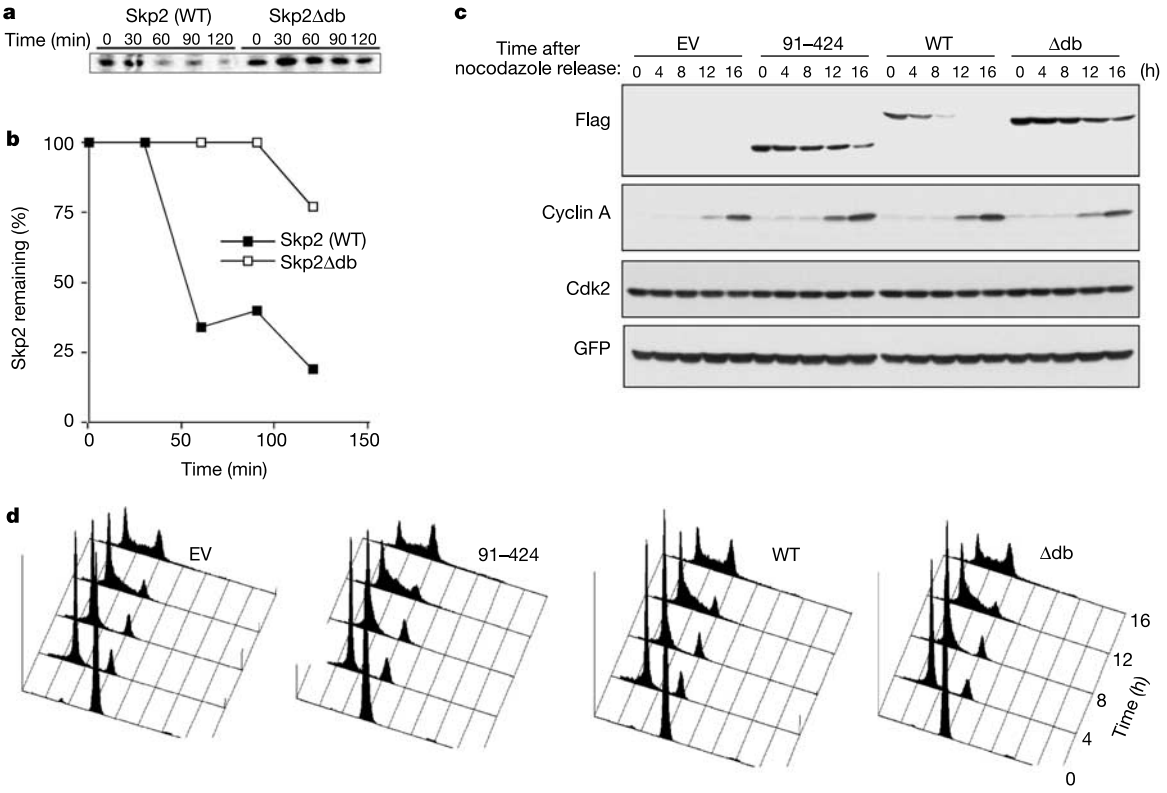

Figure 4 D-box-dependent turnover of Skp2 in G1. **a**, **b**, Pulse–chase analysis of HeLa cells transfected to produce HA–Skp2 (wild type or Δdb). Cells were synchronized in M phase with a thymidine block followed by growth in nocodazole. Nocodazole was absent during the chase. HA–Skp2 was recovered by anti-HA immunoprecipitation and was detected by autoradiography (**a**). **b**, Quantification of band intensities in **a**. **c**, **d**, Anti-Flag immunoblot (**c**) and FACS analysis (**d**) of HeLa cells transfected to produce limiting amounts of Flag–Skp2 (wild type, Δdb or 91–424), along with a green fluorescent protein (GFP) marker plasmid, synchronized in M phase with nocodazole, and then released for the indicated time periods. See Fig. 3c, e legend for definition of axes in **d**. Transfection efficiency was >50% by GFP. EV, empty vector.

have a degradation cycle, with APC active in M and early G1, and SCF active in late G1, S and early G2 (ref. 1). As APC levels drop in the G1 phase, kinase levels rise; these set in motion the next phase of the cell cycle. The potential importance of events in the M phase that reach into G1 is suggested by our finding that APC prevents accumulation of the SCF component Skp2 in G1 and this allows levels of p27 to rise. Yet another intercycle interaction reaching from G1 into the next mitotic cycle involves another F-box protein, TOME-1, which is also degraded by APC^{CDH1} (ref. 23). Degradation of TOME-1 allows the accumulation of Wee1, which has a role in mitotic entry during the next cycle. As proteolysis via both SCF and APC seems to be central to progression through G1, it will be important to understand the key features of this proteolysis engine, such as when APC activity is terminated in G1 and whether active SCF affects the accumulation of active APC. □

Methods

Plasmids

Myc-tagged tagged Cdh1 plasmids[24] were a gift of J. Lukas. Skp2 N-terminal and carboxy-terminal deletion mutants were made by polymerase chain reaction (PCR) amplification of the desired regions of a human Skp2 complementary DNA with primers that introduced 5′ *Hind*III sites and 3′ *Sal*I sites followed by subcloning into pCMV2–Flag cut with these two enzymes. Haemagglutinin (HA)-tagged Skp2 cDNAs were also cloned as *Cla*I–*Asc*I fragments into pGEX4T (Pharmacia) and as *Eco*RI–*Xho*I fragments into pCS2.

Antibodies

Anti-Cdh1 antibody (CC43) was from Oncogene. Anti-Skp2 antibody (SC-7164), anti-Skp1 antibody (SC-7163), anti-cyclin A antibody (SC-751), anti-cyclin B antibody (SC-245), anti-Cdc20 antibody (SC-8358), anti-geminin antibody (SC-13015), anti-Plk1 antibody (SC-17783), anti-Cdk2 antibody (SC-163), anti-p27 antibody (SC-528), anti-Myc antibody (SC-40), anti-Cdc27 antibody (AF3.1), polyclonal anti-actin antibody (SC-1615), polyclonal anti-HA antibody (SC-805) and peroxidase-conjugated anti-goat secondary antibody (SC-2056) were from Santa Cruz. Anti-tubulin antibody (T-5168), polyclonal anti-Flag antibody (F7425), monoclonal anti-Flag antibody (F-3165), peroxidase-conjugated anti-mouse secondary antibody (A4416) and peroxidase-conjugated anti-rabbit secondary antibody (A4914) were from Sigma. Monoclonal anti-HA antibody (MMS-101P) was from Covance.

Cell synchronization

For Figs 3b, d and 4b, adherent HeLa cells were arrested in M phase by growth in 330 nM nocodazole for 18 h, washed, and re-seeded into fresh media. For Fig. 4a, adherent HeLa cells were grown in the presence of thymidine (2 mM) for 18 h and then released into thymidine-free media for 3 h. The cells were then grown in 330 nM nocodazole for 12 h before release into nocodazole-free media.

Skp2 binding assays

Cells were lysed in EBC buffer (50 mM Tris pH 8.0, 120 mM NaCl, 0.5% Nonidet-P40) 48 h after transfection. One milligram of clarified cell extract was incubated with 1–2 μg of the indicated antibody overnight at 4 °C in a final volume of 750 μl. Monoclonal anti-actin or rabbit preimmune IgG were used as negative controls. Immune complexes were recovered on protein A Sepharose, washed five times with NETN buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet-P40), and eluted by boiling in SDS–containing sample buffer. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), wet-transferred to a PVDF membrane and immunoblotted with indicated antibodies. Binding to immobilized GST fusion proteins was as described previously[25].

Skp2 *in vitro* degradation assays

In vitro degradation assays were as described previously[26,27]. Briefly, ^{35}S-labelled Skp2 was made by *in vitro* translation using a rabbit reticulocyte lysate (TNT, Promega) and incubated with either HeLa cell extract[26] or *Xenopus* interphase egg extract[27] at room temperature supplemented with ubiquitin (0.1 μg ml^{-1}), cyclohexamide (1 μg ml^{-1}) and an energy-regenerating system[28]. Where indicated, recombinant-cyclin B residues 1–100 (final concentration 100 μM)[9] produced in *Escherichia coli* or recombinant Cdh1 (ref. 10) (final concentration 0.4 nM) produced in SF9 cells were added. Reactions were stopped by adding SDS sample buffer.

Skp2 *in vitro* ubiquitination assays

Xenopus interphase extract[28] (1 ml) supplemented with recombinant Cdh1 (ref. 10) (final

Cdh1 concentration 0.4 nM) or HeLa cell extract (2 ml)[26] was incubated with 4 μg anti-Cdc27 antibody for 4 h at 4 °C. Immune complexes were captured on protein A Sepharose (Biorad) (50 μl), washed four times in XB buffer (20 mM HEPES pH 7.8, 100 mM KCl, 5 mM $MgCl_2$) supplemented with 1% Triton-X100, washed once with XB buffer with 300 mM NaCl, and once with XB. A total of 5 μl of washed beads were incubated with 1 μl ^{35}S-labelled Skp2, UbCH-10 (2.8 μM) (Boston Biochem), E1 (1 μM) (Boston Biochem) and an ATP-regenerating system[28] in a final volume of 7.5 μl at room temperature with shaking. Ubiquitin (Boston Biochem) or methyl-ubiquitin (Boston Biochem) (10 mg ml^{-1}) was also included, as indicated. Aliquots of 1 μl of the ubiquitination reactions were removed, boiled in SDS-containing sample buffer and resolved by SDS–PAGE.

siRNA

siRNAs were purchased from Dharmacon and transfected into subconfluent cells using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Cdh1 siRNAs were named according to the first Cdh1 codon in their recognition sites and corresponded to Cdh1 nucleotide sequences 266–286 (Cdh1-127), 305–325 (Cdh1-166), 338–358 (Cdh1-199), 566–586 (Cdh1-427) and 602–622 (Cdh1-463). The Cdh1-199 siRNA[29] and Skp2 siRNA[30] were described previously. An siRNA corresponding to a 'scrambled' cyclin A sequence (sense strand 5′-AACAACCUGCCCGCGACGGAA-3′) and a firefly luciferase siRNA served as negative controls.

Pulse–chase analysis

After transfection and synchronization, cells were grown in cysteine/methionine-free media (Invitrogen) for 20 min and labelled in media supplemented with trans-label (50 μl per 100-mm plate, 14 mCi ml^{-1}, ICN) for an additional 20 min. Cells were washed twice with PBS and chased with DMEM supplemented with 10% fetal bovine serum, 2 mM cysteine and 2 mM methionine. At various time points thereafter cell extracts were prepared in PBS containing 1% Triton-X100 and incubated with anti-HA antibody coupled to protein A sepharose (Santa Cruz) for 24 h at 4 °C. Immunoprecipitates were washed four times in PBS 1% Triton-X100, once in RIPA buffer, and analysed by SDS–PAGE.

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Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain

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Microtubules are cytoskeletal polymers of tubulin involved in many cellular functions. Their dynamic instability is controlled by numerous compounds and proteins, including colchicine[1] and stathmin family proteins[2,3]. The way in which microtubule instability is regulated at the molecular level has remained elusive, mainly because of the lack of appropriate structural data. Here, we present the structure, at 3.5 Å resolution, of tubulin in complex with colchicine and with the stathmin-like domain (SLD) of RB3. It shows the interaction of RB3-SLD with two tubulin heterodimers in a curved complex capped by the SLD amino-terminal domain, which prevents the incorporation of the complexed tubulin into microtubules. A comparison with the structure of tubulin in protofilaments[4] shows changes in the subunits of tubulin as it switches from its straight conformation to a curved one. These changes correlate with the loss of lateral contacts and provide a rationale for the rapid microtubule depolymerization characteristic of dynamic instability. Moreover, the tubulin–colchicine complex sheds light on the mechanism of colchicine's activity: we show that colchicine binds at a location where it prevents curved tubulin from adopting a straight structure, which inhibits assembly.

Tubulin, an αβ heterodimer initially identified as the cellular colchicine-binding protein[5,6], is the target of numerous small-molecule ligands that interfere with microtubule dynamics, several