Cell, Vol. 114, 611–622, September 5, 2003, Copyright ©2003 by Cell Press

Release of Ubiquitin-Charged Cdc34-S∼Ub from the RING Domain Is Essential for Ubiquitination of the SCF^{Cdc4}-Bound Substrate Sic1

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Summary

The *S. cerevisiae* SCF^{Cdc4} is a prototype of RING-type SCF E3s, which recruit substrates for polyubiquitination by the Cdc34 ubiquitin-conjugating enzyme. Current models propose that Cdc34 ubiquitinates the substrate while remaining bound to the RING domain. In contrast, we found that the formation of a ubiquitin thiol ester regulates the Cdc34/SCF^{Cdc4} binding equilibrium by increasing the dissociation rate constant, with only a minor effect on the association rate. By using a F72VCdc34 mutant with increased affinity for the RING domain, we demonstrate that release of ubiquitin-charged Cdc34-S∼Ub from the RING is essential for ubiquitination of the SCF^{Cdc4}-bound substrate Sic1. Release of ubiquitin-charged E2 from E3 prior to ubiquitin transfer is a previously unrecognized step in ubiquitination, which can explain both the modification of multiple lysines on the recruited substrate and the extension of polyubiquitin chains. We discuss implications of this finding for function of other ubiquitin ligases.

Introduction

Modification of proteins with polyubiquitin chains is the rate-limiting step in proteolysis by the 26S proteasome (reviewed in Glickman and Ciechanover, 2001). In the process of ubiquitination, the E1, called a ubiquitin-activating enzyme, first forms a thiol ester bond with a carboxyl group of ubiquitin, thus activating it for nucleophilic attack. The activated ubiquitin is next trans-esterified to one of several E2 ubiquitin-conjugating enzymes. A complex of an E2 and an E3 functions as the ubiquitin ligase that catalyzes the formation of an isopeptide bond with a substrate protein and extends polyubiquitin chains.

Although the chemistry of ubiquitin activation, transfer, and conjugation are relatively well understood, the mechanism by which the E2s and E3s select each other and collaborate in synthesis of polyubiquitin chains is only beginning to become clear (reviewed in Pickart, 2001). One striking distinction between the two main classes of ubiquitin ligases is the role of the E2. In the case of HECT (*h*omology to *E*6-AP *C t*erminus)-type E3s, the E2 transfers activated ubiquitin to a catalytic site of E3, and the E3 facilitates ubiquitination. In contrast, in the case of RING (really interesting new gene)-type E3s, which do not contain catalytic activity, the E2 is directly responsible for ubiquitination of the E3-bound substrate.

Degradation of *S. cerevisiae* CDK (cyclin-dependent kinase) inhibitor Sic1 is one of the best-characterized systems for studying ubiquitin-mediated proteolysis. Genetic (Schwob et al., 1994; Bai et al., 1996) and biochemical (Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997a) efforts to dissect the mechanism of Sic1 degradation led to the discovery of SCF^{Cdc4}, an E3 complex consisting of *S*kp1, *C*dc53, Rbx1, and *F*-box Cdc4 proteins. SCF^{Cdc4} is the prototype of a large class of RING-H2 type SCF E3s. Multiple phosphorylation sites set a threshold for recognition of Sic1 via a phospho-receptor in WD40 domain of the Cdc4 subunit, suggesting a binding equilibrium-based substrate recognition mechanism (Verma et al., 1997b, Nash et al., 2001; Orlicky et al., 2003). A separate domain of SCF^{Cdc4} consisting of Cdc53 (called Cullin1 in metazoans) and Rbx1 (also called Hrt1 and Roc1) recruits the E2. Rbx1 is a RING-H2 finger protein, the characterization of which established the RING domain as a hallmark of a large class of ubiquitin ligases, including SCF, the von Hippel Lindau tumor suppressor complex, and the anaphase-promoting complex, APC (Kamura et al., 1999a; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999; Tan et al., 1999).

In addition to ubiquitination of the Sic1 substrate, SCF^{Cdc4} triggers autoubiquitination of Cdc34 (reviewed in Deshaies, 1999). The Cdc53-Rbx1 core is sufficient to stimulate autoubiquitination of Cdc34, a phenomenon of yet unknown function observed with many RING-type ubiquitin ligases (reviewed in Joazeiro and Weissman 2000). Rbx1 also stimulates modification of Cdc53 with a ubiquitin-like protein Rub1 (called Nedd8 in metazoans) (Kamura et al., 1999b). While modification with Rub1 stimulates SCF-dependent ubiquitination, possibly at a step of Cdc34 recruitment (Kawakami et al., 2001, Wu et al., 2002), in *S. cerevisiae* Rub1 is not essential unless mutations compromise SCF function (Lammer et al., 1998).

Key questions regarding the mechanism by which the E2s and E3s collaborate in synthesis of polyubiquitin chains arise from recent crystal structures of SCF complexes. The possibility that the E3 has a role in positioning the E2 and the substrate for catalysis is suggested by modeling of the UbcH7/c-Cbl RING structure (Zheng et al., 2000) into the structures of SCF^{Skp2} (Schulman et al., 2000; Zheng et al., 2002), Skp1-βTRCP-β-catenin peptide (Wu et al., 2003), and Skp1-Cdc4-cyclin E peptide (Orlicky et al., 2003). In these models, the catalytic site of E2 bound to the RING domain faces the predicted substrate binding site, located on the opposite end on of a strikingly rigid, cradle-like structure. That the substrate also has to be positioned in a defined fashion is suggested by the sensitivity of Cul1 to insertion of a flexible

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linker (Zheng et al., 2002), and by the sensitivity of the α-helical linker of Cdc4 to translational and rotational rearrangements (Orlicky et al., 2003).

The large distance (50 Å) separating the E2 active site from the predicted substrate binding site in the rigid SCF E3 raises questions about the mechanism of catalysis. Assuming that catalysis requires close proximity of the E2 and the substrate, the question is what mechanism promotes E2-substrate contact and how is the position of the active site adjusted during synthesis of a polyubiquitin chain? This issue is additionally complicated by the observation that naturally unstable proteins, including Sic1 (Skowyra et al., 1997; Petroski and Deshaies, 2003), Cln2 (Skowyra et al., 1999), Gcn4 (Kornitzer et al., 1994), and p53 (Nakamura et al., 2000) are polyubiquitinated on multiple lysines. Finally, ubiquitination of a substrate is often accompanied by autoubiquitination of the E2, and there is currently no model explaining how these two processes are coordinated with each other.

To address these questions, we characterized the dynamics of the interaction between the Cdc34 E2 ubiquitin-conjugating enzyme and SCF^{Cdc4}, and examined its role in the mechanism of ubiquitination. Our results are inconsistent with a model in which an E2 functions in an E2/E3 complex. As we present below, the dynamic binding equilibrium between the ubiquitin-charged E2 and the E3 is a critical factor in the mechanism of ubiquitination and may explain the spatial flexibility of catalysis.

Results

Dissociation of Ubiquitin-Charged Cdc34-S∼Ub from SCF^{Cdc4}

Recruitment of Cdc34 to the RING domain of Rbx1 is essential for SCF^{Cdc4}-dependent ubiquitination. However, the interaction of Cdc34 with the RING domain is not characterized kinetically, and it is unknown whether Cdc34 remains in a constitutively active complex with SCF^{Cdc4}, or binds and dissociates during each round of catalysis. The following results indicate that the interaction of Cdc34 with SCF^{Cdc4} is dynamic in response to formation of Cdc34-ubiquitin thiol ester.

We first measured the stability of Cdc34/SCF^{Cdc4} complexes isolated from an in vitro ubiquitination assay with Cdc34Δ, a C-terminal deletion mutant that recapitulates Cdc34 function in vivo (Kolman et al., 1992; Silver et al., 1992; Ptak et al., 1994). Ten percent of Cdc34Δ dissociated from SCF^{Cdc4} within 90 min of incubation at 25°C (Figure 1A, lanes 9–12, top). This slow dissociation was specific because in the same reaction mixtures Sic1 remained bound to SCF^{Cdc4} (Figure 1A, lanes 1–4, bottom). Dissociation of Cdc34Δ was accelerated by the presence of the Uba1 ubiquitin-activating enzyme, ubiquitin and ATP (Ub^{mix}), because without resupplementing these components, less than 1% of Cdc34Δ dissociated (Figure 1A, lanes 13–16, top). Despite dependence on Ub^{mix}, neither SCF^{Cdc4}-bound nor released Cdc34Δ was autoubiquitinated (Figure 1A, lanes 1–12, top). This was not because of a lack of catalytic activity; in the same reactions SCF^{Cdc4}-bound Sic1 was modified with multiple ubiquitins (Figure 1A, lane 4, bottom) in a manner that correlated with dissociation of Cdc34Δ. We observed a similar rate of Cdc34Δ dissociation in the absence of Sic1 (Figure 1B, compare lanes 9–12 with 13–16), showing that the dissociation is independent of the presence of a substrate. In contrast, the catalytically inactive Cdc34ΔC95A mutant protein was released only at a very slow rate regardless of the presence or absence of Ub^{mix} (Figure 1C, lanes 9–16). Thus, accelerated dissociation of Cdc34Δ depends on formation of a ubiquitin thiol ester with Cdc34Δ, but not on substrate recruitment.

Remarkably, later addition of Ub^{mix} did not stimulate dissociation of Cdc34Δ prebound to SCF^{Cdc4} in the absence of Ub^{mix} (Figure 1D, lanes 9–16). This suggests that formation of a functional Cdc34-SCF^{Cdc4} complex requires that Cdc34Δ forms a thiol ester with ubiquitin prior to the interaction with SCF^{Cdc4}. Consistently, when supplemented with Ub^{mix}, Cdc34Δ prebound to SCF^{Cdc4} in the uncharged form remained inactive in Sic1 ubiquitination (Figure 1E, lane 2, top) and autoubiquitination (Figure 1E, lane 2, bottom). These activities were rescued by addition of 3 μM Cdc34Δ-S∼Ub (Figure 1E, lane 3), likely through replacement of uncharged Cdc34Δ from SCF^{Cdc4} (Figure 1F). We observed similar rescue of SCF^{Cdc4} prebound to a Cdc34ΔC95A active site mutant (Figure 1E, lanes 4 and 5), demonstrating that the catalytic activity of prebound Cdc34Δ was not required for rescue.

In the rescued reactions, only about 10% of uncharged Cdc34Δ was replaced with Cdc34Δ-S∼Ub (Figure 1F). This is similar to the maximal percentage of Cdc34Δ dissociated in the release assays (Figures 1A and 1B, lane 12), suggesting that the remaining Cdc34Δ was bound to SCF^{Cdc4} in a non-functional and possibly RING-independent manner. These resistant complexes were typically found with high concentrations of uncharged Cdc34Δ ($K_D \sim 3$ μM), and were observed even in ubiquitination assays, in which Cdc34Δ was not charged with a ubiquitin thiol ester prior to addition of SCF^{Cdc4}. Importantly, formation of these complexes could be prevented by preincubation of Cdc34Δ with Ub^{mix} (Figure 1G, lanes 1 and 2) which enriched the fraction of Cdc34Δ-ubiquitin thiol ester prior to SCF^{Cdc4} binding (Figure 1G, lanes 5 and 6). Thus, dynamic interaction of ubiquitin-charged Cdc34-S∼Ub and SCF^{Cdc4} appears to be solely responsible for the observed ubiquitination activity.

Formation of a Ubiquitin Thiol Ester with Cdc34 Regulates the Cdc34-SCF^{Cdc4} Binding Equilibrium Primarily by Increasing the Dissociation Rate Constant

To characterize the kinetics of Cdc34 binding to SCF^{Cdc4} we used a flow cytometry-based approach (Buranda et al., 1999, 2001). To avoid putative complications by autoubiquitination, we monitored only the initial rates of Cdc34Δ binding to $^{G}SCF^{Cdc4}$ (see Experimental Procedures), and used Cdc34Δ that was labeled with a Cy2 fluorescent probe randomly attached to one of the eight lysines of the fully functional 3KCdc34Δ mutant (K173R, K189R, or K197R). This mutant protein was created to prevent modification within unconserved sequence (residues 171–209) previously implicated in Cdc34 binding to SCF (Mathias et al., 1998).

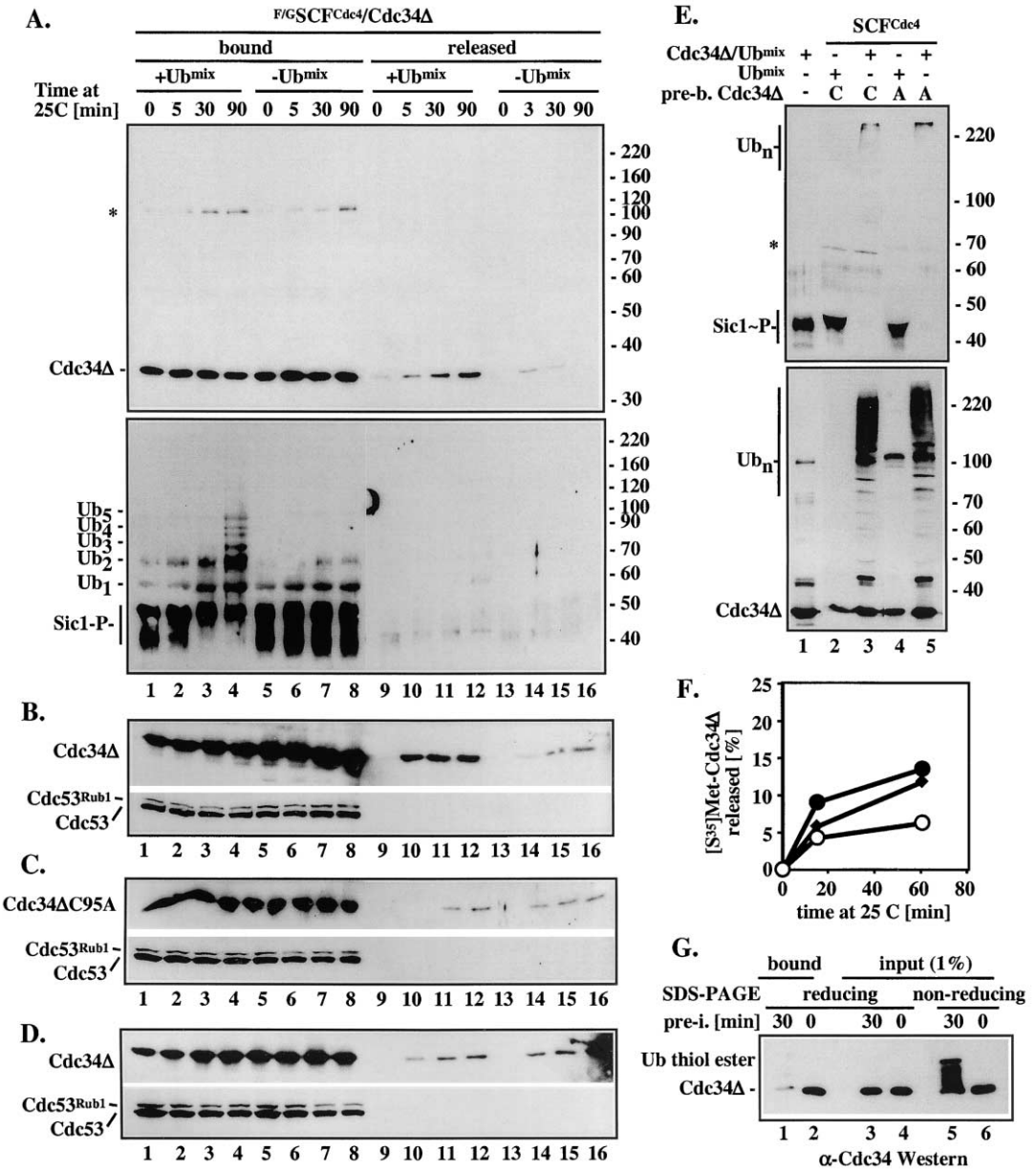

Figure 1. Implications for Binding Equilibrium between Cdc34-S~Ub and SCF^{Cdc4}

(A) Ubiquitination of Sic1 correlates with accelerated dissociation of Cdc34Δ from SCF^{Cdc4}. ^{F}SCF^{Cdc4}/Cdc34Δ complexes were isolated on α-FLAG agarose from a standard ubiquitination assay (3 μM Cdc34Δ) without Sic1 and resuspended in mixtures missing Cdc34Δ but containing Sic1 with or without Ub^{mix} (Uba1 E1, Ub, and ATP). After incubation at 25°C for the times indicated, the beads (lanes 1–8) were separated from the supernatants (lanes 9–16) followed by SDS-PAGE and Western for Cdc34 (top) and Sic1 (bottom).

(B) Ub^{mix} is sufficient to accelerate dissociation of Cdc34Δ from SCF^{Cdc4}. Assay like (A) but without Sic1 and with ^{G}SCF^{Cdc4} instead of ^{F}SCF^{Cdc4}.

(C) Ub^{mix} does not accelerate dissociation of the Cdc34Δ active site mutant. Assay like (B) except Cdc34ΔC95A was used instead of Cdc34Δ.

(D) Accelerated dissociation depends on formation of Cdc34Δ-ubiquitin thiol ester prior to SCF^{Cdc4} binding. Assay like (B), but Cdc34Δ was prebound to ^{G}SCF^{Cdc4} in the absence of Ub^{mix}.

(E) Binding of uncharged Cdc34Δ to SCF^{Cdc4} is nonfunctional. Cdc34Δ (labeled C for intact C95) or Cdc34ΔC95A (labeled A) bound to SCF^{Cdc4} in the absence of Ub^{mix} like in (D) were supplemented with Sic1 and incubated at 25°C for 1 hr with Ub^{mix} alone (lanes 2 and 4) or with Ub^{mix} and 3 μM Cdc34Δ (lanes 3 and 5) followed by SDS-PAGE/Western. Lane 1: reaction without SCF^{Cdc4}.

(F) Replacement of uncharged Cdc34Δ from SCF^{Cdc4}. Pre-formed Met[S^{35}]-Cdc34Δ/SCF^{Cdc4} complexes were incubated like in (E) with Ub^{mix} alone (open circles), or with 3 μM of nonradioactive Cdc34Δ in the presence (closed circles) or absence of Ub^{mix} (diamonds). At the times indicated the supernatants were removed and scored for radioactivity by liquid scintillation counting.

(G) Formation of ubiquitin-thiol ester prevents stable binding of Cdc34Δ to SCF^{Cdc4}. 3 μM Cdc34Δ was incubated (pre-i.) for 30 or 0 min at 25°C with Ub^{mix} prior to ^{G}SCF^{Cdc4} binding, followed by washing and analysis of total bound Cdc34Δ by SDS-PAGE/Western.

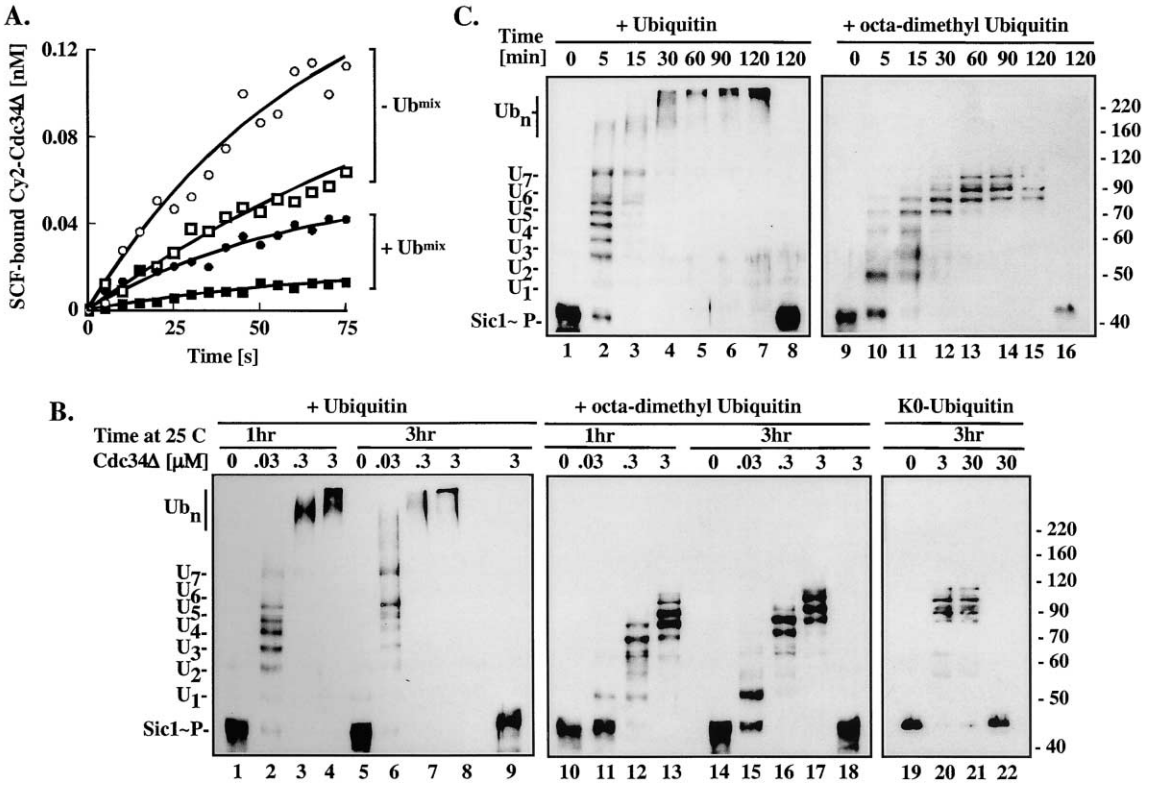

Figure 2. Binding Kinetics of Cdc34Δ to SCF^{Cdc4} versus Kinetics and Lysine Specificity of Sic1 Ubiquitination

(A) Initial rates of binding of Cy2-3KCdc34Δ to SCF^{Cdc4} were measured in real time by flow cytometry and analyzed as described in Experimental Procedures. Reactions were performed with Ub^{mix} (filled symbols) and without Ub^{mix} (open symbols), using 50 nM (squares) and 100 nM Cy2-3KCdc34Δ (circles).

(B) Concentration of Cdc34Δ controls the rate and lysine specificity of Sic1 ubiquitination. One hour (lanes 1–4) and three hour (lanes 5–8) Sic1 ubiquitination assays were preformed with 0.03, 0.3, 3, and 30 μM Cdc34Δ as indicated, using ubiquitin (lanes 1–9), octa-dimethyl ubiquitin (lanes 10–18), or K0-ubiquitin (lanes 19–22). Lanes 9,18, 22: controls without SCF^{Cdc4}.

(C) Kinetics of Sic1 ubiquitination with 3 μM Cdc34Δ. Sic1 ubiquitination was monitored in time as indicated using ubiquitin (lanes 1–8) or octa-dimethyl ubiquitin (lanes 9–16) followed by SDS-PAGE and α-Sic1 Western. Lanes 8 and 16: assays without Cdc34Δ.

We performed the measurements at 50 nM and 100 nM of Cy2-3KCdc34Δ (Figure 2A), and both concentrations gave rate constants that agreed within an experimental error. We determined that uncharged Cdc34Δ bound SCF^{Cdc4} with an average K_D of 7.0 nM, an average association rate constant $k_{on} = 1.34 \times 10^5\ M^{-1}s^{-1}$, and an average dissociation rate constant $k_{off} = 0.001\ s^{-1}$. In contrast, ubiquitin-charged Cdc34Δ-S∼Ub interacted with SCF^{Cdc4} with an average $K_D = 290.0$ nM, $k_{on} = 4.1 \times 10^4\ M^{-1}s^{-1}$, and $k_{off} = 0.010s^{-1}$. Thus, formation of the ubiquitin-thiol ester causes 30- to 40-fold increase in the K_D, primarily by increasing the dissociation rate constant (10-fold increase) and a smaller effect on the association rate (3-fold decrease).

Release of Ubiquitin-Charged Cdc34-S∼Ub from the RING Domain Is Essential for Ubiquitination of SCF^{Cdc4}-Bound Substrate Sic1

To address significance of Cdc34-S∼Ub release from the RING domain, we sought to generate Cdc34 mutant with increased affinity to the RING. A good candidate for mutagenesis was residue Phe72, which by analogy to the UbcH7/c-Cbl RING crystal structure (Zheng et al., 2000; reviewed in VanDemark and Hill 2002) should interact exclusively with a hydrophobic groove of the RING domain. Because of its position, substitutions of Phe72 were unlikely to interfere with formation of a ubiquitin-thiol ester and with SCF^{Cdc4}-independent autoubiquitination of Cdc34 on its C terminus (Banerjee et al., 1993).

We found as predicted that residue F72 is important for Cdc34 to function together with SCF^{Cdc4}. Three distinct nonconservative substitutions (F72V, F72D, and F72L) lowered ubiquitination of Sic1 by at least 20-fold (Figure 3A, α-Sic1, lanes 8–13, and data not shown), while a conservative F72Y substitution had no effect (Figure 3A, α-Sic1, lanes 5–7). The defect in Sic1 ubiquitination resulted from the inability of Cdc34 to function with SCF^{Cdc4}, because F72 mutants were active in SCF^{Cdc4}-independent autoubiquitination (Figure 3A, α-Cdc34, lanes 10 and 13) and in the formation of a ubiquitin-thiol ester (data not shown). The F72Cdc34 mutant proteins were undistinguishable from Cdc34 in gel filtration chromatography (data not shown), indicating monomeric structure (Ptak et al., 1994).

Importantly, the Cdc34 F72V mutant protein was defective in ubiquitin-mediated release from SCF^{Cdc4} (Figure 3B, compare lanes 9–12 with 13–16). In flow cytome-

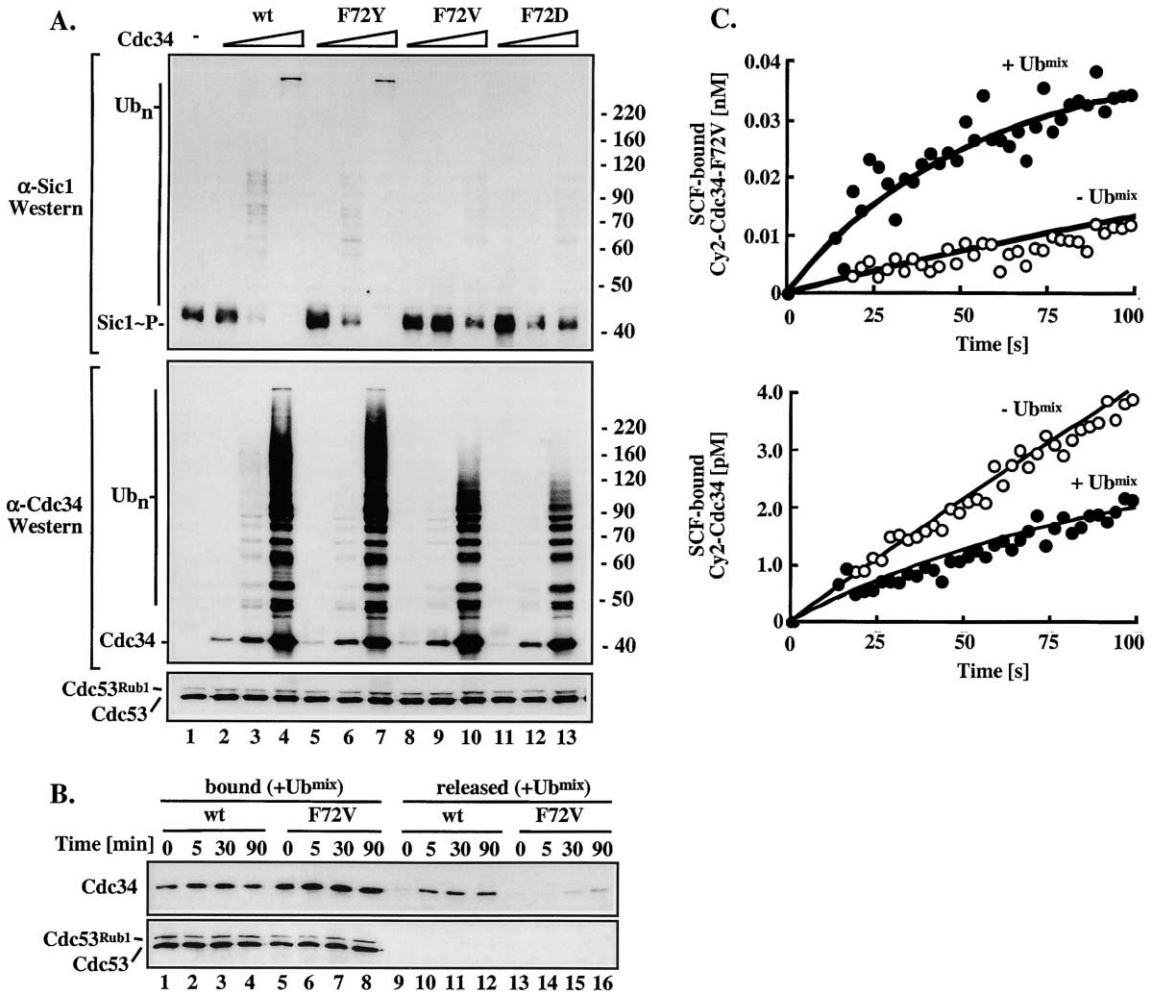

Figure 3. Stabilization of Cdc34-S∼Ub Binding to the RING Domain Inactivates SCF^{Cdc4}-Dependent Ubiquitination

(A) Residue F72 of Cdc34 is important for SCF^{Cdc4}-dependent ubiquitination. Full-length Cdc34 and Cdc34 F72Y/V/D mutants (0.01, 0.1, and 1 μM) were tested in Sic1 ubiquitination (top) and autoubiquitination (bottom).

(B) F72 has a key role in release of Cdc34-S∼Ub from SCF^{Cdc4}. Top: Cdc34 or Cdc34F72V (3 μM) were prebound to SCF^{Cdc4} in the presence of Ub^{mix} like in Figure 1B, followed by wash and monitoring Cdc34 dissociation in the presence of Ub^{mix}.

(C) F72V substitution stabilizes interaction of Cdc34-S∼Ub with SCF^{Cdc4}. Initial rates of binding to ^{G}SCF^{Cdc4} were monitored by flow cytometry, using 50 nM Cy-Cdc34F72 mutant protein (top) or Cy-Cdc34 (bottom), in the presence (black circles) or absence (open circles) of Ub^{mix}, and analyzed as described in experimental procedures.

try experiments (Figure 3C, top), we determined that F72VCdc34 protein interacted with SCF^{Cdc4} with a high affinity (K_D 18.9 nM −Ub^{mix}), which was further increased by formation of ubiquitin-thiol ester (K_D 12.5 nM +Ub^{mix}). The increased affinity of ubiquitin-charged F72VCdc34-S∼Ub was primarily a result of an increased association rate constant (1.6×10^5 $M^{-1}s^{-1}$ +Ub^{mix} and 3.7×10^4 $M^{-1}s^{-1}$ −Ub^{mix}), in combination with a very small increase in the dissociation rate constant (0.002 s^{-1} + Ub^{mix} and 0.001 s^{-1} −Ub^{mix}). In the same conditions, Cdc34 bound SCF^{Cdc4} with a K_D of 273 nM, and the formation of Cdc34-ubiquitin thiol ester increased the K_D to 1.25 μM (Figure 3C, bottom). Although these values are distinct from those established for Cdc34Δ (Figure 2A), suggesting a role for the C terminus of Cdc34 in SCF^{Cdc4} binding, formation of a ubiquitin-thiol ester lowered the affinity for SCF^{Cdc4} in both cases. This was not true for the F72VCdc34 mutant. Thus, stabilization of the interaction between ubiquitin-charged Cdc34 and SCF^{Cdc4} by F72V replacement in Cdc34 inactivates the ability of Cdc34 to ubiquitinate Sic1 in SCF^{Cdc4}-dependent fashion.

Properties of the Cdc34-S∼Ub/SCF^{Cdc4} Binding Equilibrium versus Properties of Sic1 Ubiquitination

The binding and dissociation rates of the interaction between Cdc34Δ-S∼Ub and SCF^{Cdc4} suggested that Cdc34Δ will not be limiting in the micromolar range of Cdc34Δ concentrations and under these conditions the rate of Sic1 ubiquitination should be maximal. In contrast, when the concentration of Cdc34Δ is in the low nanomolar range, Sic1 ubiquitination should be limited. To test these predictions we performed Sic1 ubiquitination with 30 nM, 300 nM, and 3 μM Cdc34Δ (Figure 2B). Comparing the 1 and 3 hr reaction times, it can be seen that Sic1 is polyubiquitinated very slowly at 30 nM

Cdc34Δ (Figure 2B, lanes 2 and 6). As predicted, an increase of Cdc34Δ concentration to 300 nM and 3 μM stimulated the rate of Sic1 ubiquitination (Figure 2B, lanes 3 and 4 and 7 and 8).

Remarkably, in experiments performed with octa-dimethylated ubiquitin, we observed that Cdc34Δ concentration controls not only the rate but also the lysine specificity of Sic1 ubiquitination. Octa-dimethylated ubiquitin is unable to form ubiquitin chains via linkages with other ubiquitin molecules, but can be transferred to lysine residues within a substrate protein to give mono-ubiquitinated products. At 30 nM Cdc34Δ (1/10 of K_D value), Sic1 was modified primarily on a single lysine (Figure 2B, lanes 11 and 15). In contrast, at 3 μM Cdc34Δ (10-fold above the K_D), up to seven lysines on Sic1 were ubiquitinated (Figure 2B, lanes 13 and 17). We confirmed these results using K-0 ubiquitin, a lysine-less ubiquitin mutant (Figure 2B, lane 20). Further increase of Cdc34Δ concentration to 30 μM (100-fold above K_D) did not increase the number of lysines modified on Sic1 (Figure 2B, lane 21). In these experiments, none of the accompanying proteins were ubiquitinated, except for Cdc34Δ (data not shown).

Modification of seven lysines on Sic1 is consistent with some flexibility in target selection by Cdc34-S∼Ub, but ubiquitination of a single lysine at 30nM Cdc34Δ demonstrated specificity higher than expected from a diffusion-based reaction. Moreover, even under conditions of rapid catalysis, not all the modified lysines were equal targets. Kinetics of Sic1 ubiquitination with octa-dimethylated ubiquitin at 3 μM Cdc34Δ showed that initially only one of the available lysines was favored (Figure 2C, lane 10). This lysine was likely modified with a single chain consisting of 5–7 ubiquitins, because in assays with regular ubiquitin of a comparable activity at least seven bands accumulated at the same time (Figure 2C, lane 2). Only after this initial target lysine was modified did additional lysines become ubiquitinated (Figure 2C, lanes 11–15 and 3–7). Thus, while the rate of Sic1 ubiquitination at different Cdc34 concentration is consistent with predictions from the Cdc34-S∼Ub/SCF^{Cdc4} binding constant, the reactions were more selective than expected from a diffusion-based process, suggesting that additional factors may be involved.

Autoubiquitination Is a Byproduct of a High-Rate of Cdc34 Collisions Typical for Concentrations of Cdc34 that Exceed the K_D for Its Interaction with SCF^{Cdc4}

In addition to ubiquitination of Sic1, SCF^{Cdc4} triggers autoubiquitination of Cdc34 (Seol et al., 1999; Skowyra et al., 1999). However, the molecular basis for autoubiquitination, and how this process is coordinated with ubiquitination of the SCF^{Cdc4}-bound substrate are unknown (reviewed in Deshaies 1999).

Surprisingly, while characterizing the binding equilibrium between Cdc34Δ-S∼Ub and SCF^{Cdc4}, we observed that autoubiquitination occurs at higher concentrations of Cdc34Δ than are required for ubiquitination of Sic1. In Sic1 ubiquitination assays, autoubiquitination of Cdc34Δ began to be detectable at 3 μM Cdc34Δ (Figure 4A, lanes 4 and 8), which is 10-fold higher than the K_D for Cdc34-S∼Ub binding to SCF^{Cdc4}. Increasing the Cdc34Δ concentration to 30 μM led to a further increase in the autoubiquitination rate (Figure 4B, lane 4). Interestingly, at 30 μM Cdc34Δ, a similar pattern of autoubiquitination began to be detectable in reactions without SCF^{Cdc4} (Figure 4B, lane 9). This suggests that SCF^{Cdc4} stimulates autoubiquitination by lowering the concentration requirement for Cdc34Δ.

The high concentration requirement for Cdc34Δ in autoubiquitination suggested that the reaction depends either on a transient interaction between Cdc34Δ molecules or on oligomerization of Cdc34Δ. If binding to SCF^{Cdc4} facilitated oligomerization of Cdc34Δ, however, autoubiquitination should be detectable in the range of Cdc34Δ concentrations required for Sic1 ubiquitination. Instead, efficient autoubiquitination was observed only at Cdc34Δ concentrations exceeding the K_D value for Cdc34-S∼Ub binding to SCF^{Cdc4}. Moreover, the Cdc34Δ autoubiquitinated in SCF^{Cdc4}-dependent reactions accumulated in the SCF^{Cdc4}-free form (Figure 4C, top). In the same assays Sic1 remained bound to SCF^{Cdc4} (Figure 4C, middle), and we did not observe dissociation of SCF^{Cdc4} subunits (Figure 4C, bottom).

These observations suggest two possible models for autoubiquitination. In the first model, the Cdc34Δ-S∼Ub released from SCF^{Cdc4} is more active and can more efficiently ubiquitinate other Cdc34Δ molecules in solution, providing that they are sufficiently concentrated to allow intermolecular collisions. In the second, SCF^{Cdc4} has no effect on Cdc34 activity but increases the local concentration of Cdc34Δ by an unknown mechanism.

Endogenous Cdc34 and SCF Are Functionally Equivalent to Recombinant Proteins

An important question was whether the mechanism that we identified using recombinant proteins can be applied to the function of endogenous Cdc34 and SCF. This is important for two reasons. First, Cdc34 is phosphorylated in vivo (Goebl et al., 1994), which is unlikely for a recombinant protein expressed in *E. coli*. Second, Cdc53 is more efficiently modified with the Rub1 ubiquitin-like protein in yeast cells than by the Rub1 homolog in the insect cells used for production of recombinant SCF^{Cdc4} complexes.

We found that like the ubiquitin-charged recombinant Cdc34-S∼Ub, endogenous Cdc34 does not form stable complexes with SCF. In gel filtration chromatography of crude yeast extracts containing ubiquitin, ATP, and the Uba1 E1 (see Experimental Procedures) Cdc34 eluted as 70 kDa protein (Figure 5A, crude extracts GF, Fr. 16 and 17), similar to recombinant $Cdc34^{His}$ purified from *E. coli* (Figure 5A, top). We obtained the same results with and without phosphatase inhibitors, suggesting that phosphorylation is not required for Cdc34 recruitment by SCF (data not shown). In contrast, ^{HA}Cdc53 was found in large complexes of 670 kDa (Figure 5A, crude extracts GF, Fr. 8 and 9). We did not detect Cdc34 in this size range, even when Cdc53 was immunoprecipitated (Figure 5A, crude extracts GF/IP). Our Western blotting conditions allowed detection of as little as 1% of the total Cdc34 in the extracts (Figure 5A, bottom right), demonstrating that at least 99% of the endogenous Cdc34 is not bound to SCF.

Importantly, the in vitro ubiquitination activity of en-

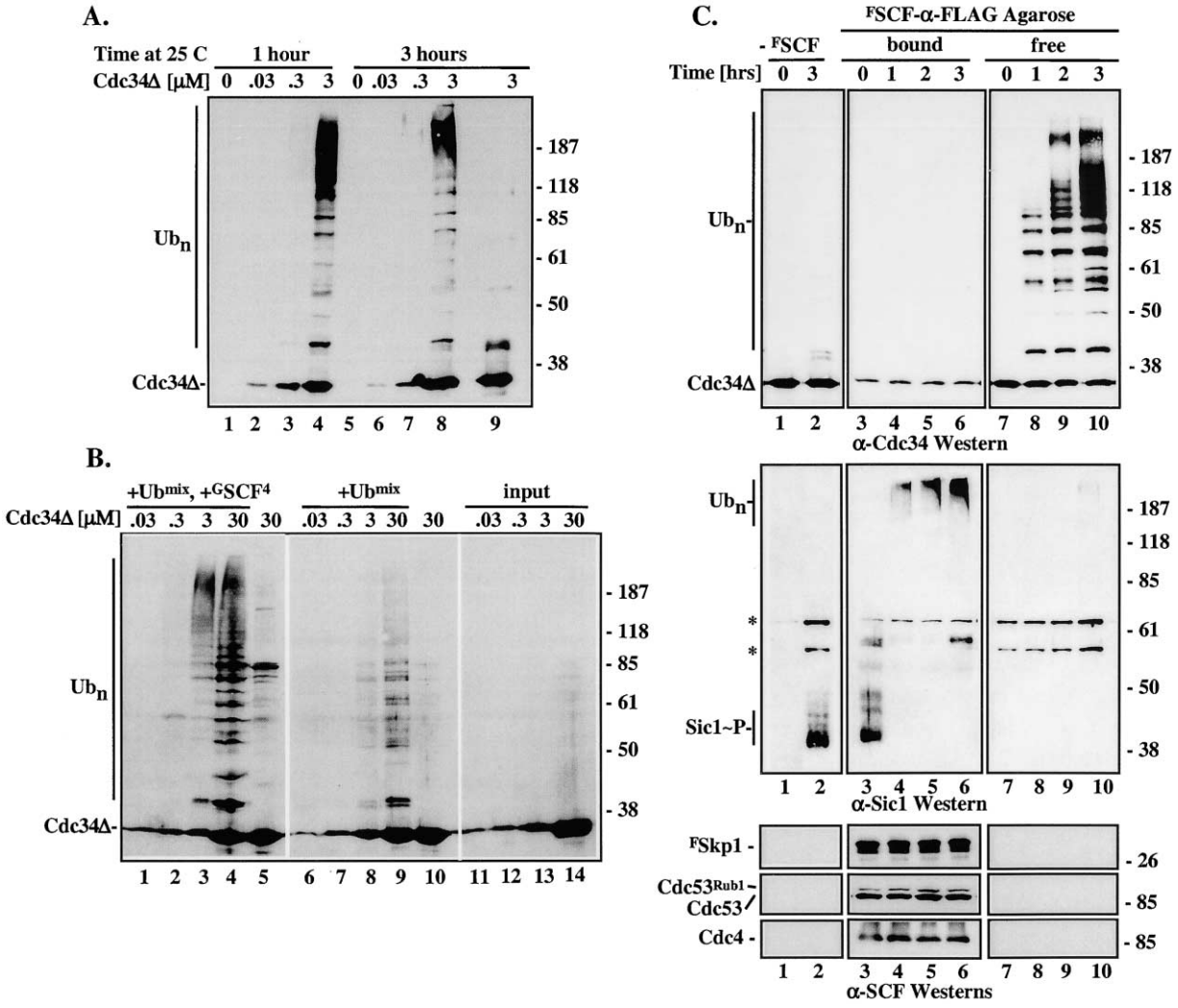

Figure 4. Autoubiquitination Is a Byproduct of Cdc34-S∼Ub Collisions

(A) High concentration requirement for Cdc34Δ in autoubiquitination. Autobiquitination assays were performed in the presence of Sic1, using increasing concentrations of Cdc34Δ, as indicated. Lane 9: control without SCF^{Cdc4}.

(B) SCF^{Cdc4} lowers concentration requirement for Cdc34Δ in autoubiquitination. Autoubiquitination in the absence of Sic1 was performed with (lanes 1–4) and without (lanes 6–9) SCF^{Cdc4}, using increased concentrations of Cdc34Δ, as indicated. Lane 5: control −SCF^{Cdc4}. Lane 10: control −Ub^{mix}.

(C) Cdc34Δ autoubiquitinated in SCF^{Cdc4}-dependent reactions accumulates in SCF^{Cdc4}-free form. Sic1 ubiquitination was performed with immobilized $^{F}SCF^{Cdc4}$ complexes and 3 μM Cdc34Δ. At the times indicated the beads (lanes 3–6) were separated from the supernatants (lanes 7–10) and analyzed by SDS-PAGE/Western for Cdc34 (top), Sic1 (middle), and SCF^{Cdc4} (bottom). Lanes 1 and 2: controls without $^{F}SCF^{Cdc4}$.

dogenous Cdc34 and Cdc53 immune complexes were similar to those of the recombinant proteins. ^{HA}Cdc53 immune complexes stimulated autoubiquitination of Cdc34Δ (Figure 5B, lanes 2–4) in the same concentration range as recombinant $^{F}SCF^{Cdc4}$ (Figure 4A, lanes 2–4). Purified endogenous Cdc34 together with recombinant $^{G}SCF^{Cdc4}$ ubiquitinated Sic1 (Figure 5C, lane 2). Remarkably, when purified endogenous Cdc34 and ^{HA}Cdc53 immune complexes were tested together for their interaction in the in vitro ubiquitination assays, we could detect a small amount of stable Cdc34/^{HA}Cdc53 complexes forming at high concentration of Cdc34 (Figure 5D, lane 8). In this experiment, the autoubiquitinated Cdc34 was detectable only in SCF-free form (Figure 5D, lane 12), fully recapitulating our findings from the recombinant in vitro system. Thus, in crude yeast extracts, Cdc34 protein exists in SCF-free form, most likely as a ubiquitin thiol ester, which can functionally interact with SCF. This suggests that the dynamic binding equilibrium between Cdc34-S∼Ub and SCF can account for function of both recombinant and endogenous proteins.

Discussion

We characterized the dynamics of the interaction between Cdc34 ubiquitin-conjugating enzyme and SCF^{Cdc4} and examined its role in the mechanism of ubiquitination. Our results are inconsistent with a model in which an E2 functions in an E2/E3 complex. In contrast, a dynamic binding equilibrium between ubiquitin-charged

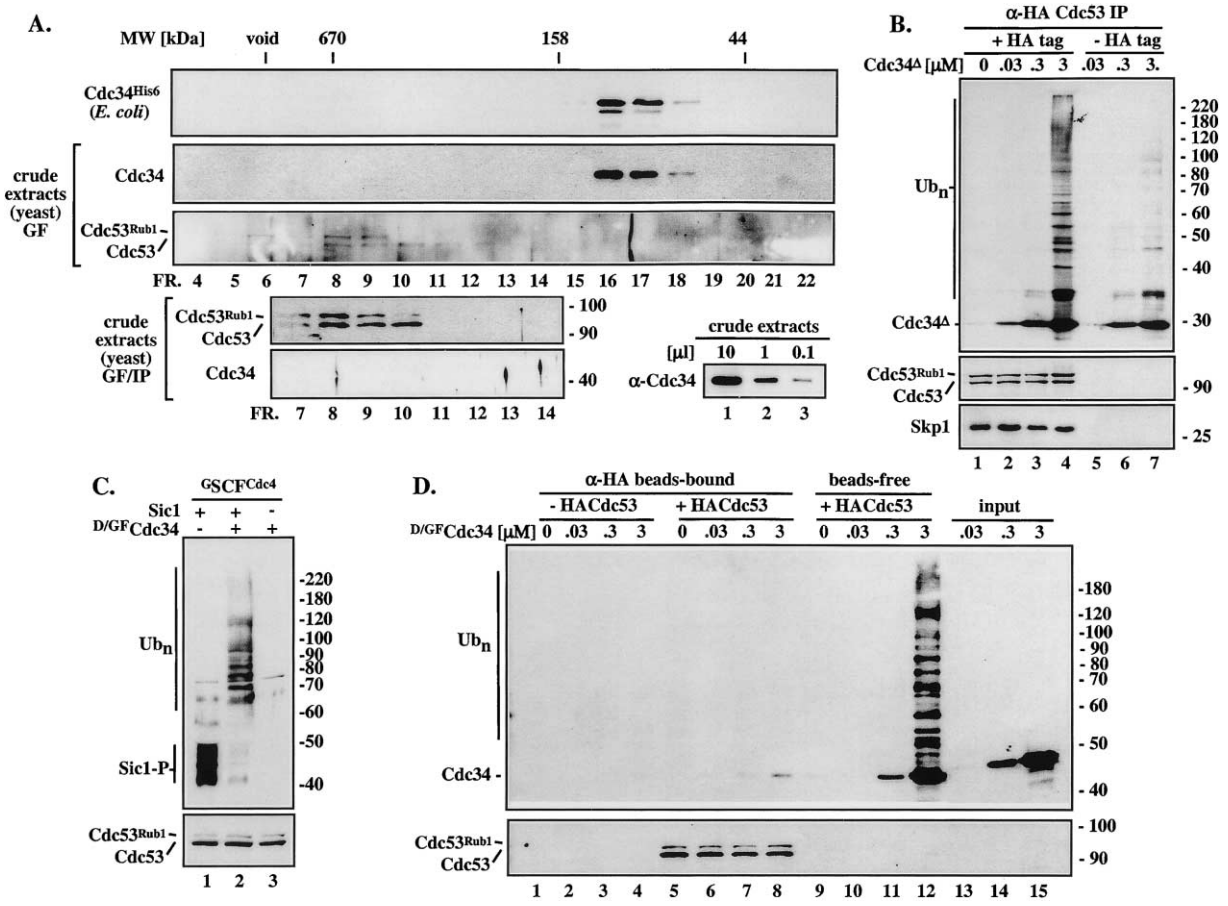

Figure 5. Endogenous Cdc34 and SCF Are Equivalent to Recombinant Proteins

(A) In crude yeast extracts Cdc34 does not form stable complexes with SCF. Gel filtration (GF) chromatography was performed at 25°C and followed by SDS-PAGE/Western either directly (fractions 4–22) or after α-HA Cdc53 immunoprecipitation (GF/IP of fractions 7–14). Top: GF of Cdc34^{His6} purified from *E. coli* (2 μg). Bottom right: sensitivity of Cdc34 Western blot in crude yeast extracts.

(B) Endogenous ^{HA}Cdc53 complexes stimulate autoubiquitination of recombinant Cdc34Δ. Varying concentrations of Cdc34Δ^{His6} were tested with α-HA Cdc53 immune complexes (lanes 1–4), or with control α-HA beads (lanes 5–7), followed by SDS-PAGE/Western.

(C) Endogenous Cdc34 is functional in Sic1 ubiquitination with recombinant ^{G}SCF^{Cdc4}. $^{D/GF}$Cdc34 (0.5 μM) indicates Cdc34 purified from yeast extracts on DEAE Sephacryl followed by gel filtration on Superdex 200.

(D) $^{D/GF}$Cdc34 autoubiquitinated in SCF-dependent reactions accumulates in the SCF-free form. $^{D/GF}$Cdc34 (0.3–3 μM) was incubated in a ubiquitination assay with α-HA ^{HA}Cdc53 immune complexes (lanes 5–8) or with control α-HA beads (lanes 1–4), followed by removal of the supernatants (lanes 9–12) and bead wash prior to SDS-PAGE/Western. Cdc34 levels in the control binding reactions were identical to those in lanes 9–12 (data not shown).

Cdc34-S∼Ub and SCF^{Cdc4} is a critical factor in ubiquitination. The role of a ubiquitin-thiol ester in the regulation of the Cdc34-SCF^{Cdc4} binding equilibrium and the release of ubiquitin-charged Cdc34-S∼Ub from its primary recruitment site on the RING domain are two previously unrecognized aspects of the mechanism of ubiquitination. They provide experimental basis for the "Hit and Run" model of Cdc34 function with SCF^{Cdc4} proposed on Figure 6.

The key aspect of the dynamic binding equilibrium between Cdc34-S∼Ub and SCF^{Cdc4} is that the ubiquitin-charged Cdc34 is recruited to the RING domain and released in close proximity of the substrate. Significance of this release is suggested by the observation that the ubiquitin-charged F72VCdc34 mutant protein, which binds SCF^{Cdc4} with about 100-fold higher affinity than Cdc34-S∼Ub (K_D of 12.5 nM versus K_D of 1.25 μM, respectively), remains inactive in the ubiquitination of Sic1. The key to interpretation of this experiment is the demonstration that the F72VCdc34 protein is fully functional in reactions independent of SCF^{Cdc4}. This includes formation of a ubiquitin thiol ester and autoubiquitination of the C terminus of Cdc34 (Banerjee et al., 1993). Moreover, F72VCdc34 binds SCF^{Cdc4} with a high affinity (K_D 18.9 nM), which is further increased by formation of a ubiquitin-thiol ester (K_D 12.5 nM), suggesting specific interaction through the RING domain. Although only crystal structure could define details of the interaction between F72VCdc34 and SCF^{Cdc4}, the position of the equivalent residue in structure of UbcH7/c-Clb RING (Zheng et al., 2000) suggests that the F72V substitution locks otherwise functional Cdc34-S∼Ub molecule in stable interaction with the RING domain.

What advantage does a release of ubiquitin-charged Cdc34-S∼Ub from the RING domain bring to the mechanism of ubiquitination? In our opinion, the one most

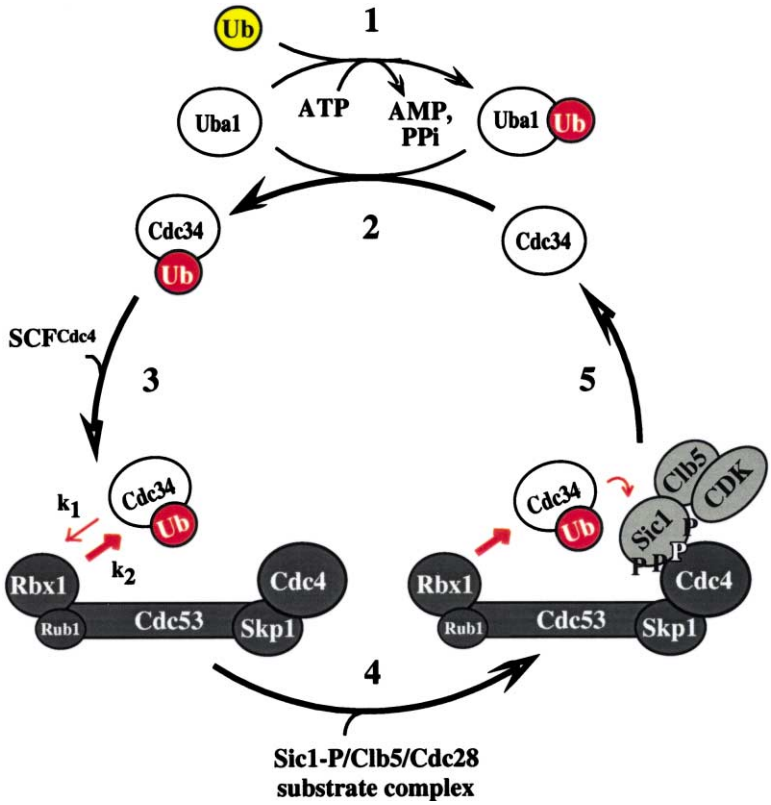

Figure 6. The "Hit and Run" Model for Cdc34 Function with SCF^{Cdc4}

Functional interaction between Cdc34 and SCF^{Cdc4} depends on formation of Cdc34-ubiquitin thiol ester prior to SCF^{Cdc4} binding (1 and 2). Unlike Cdc34, the ubiquitin-charged Cdc34-S∼Ub remains in a dynamic binding equilibrium with SCF^{Cdc4}, characterized by decreased association rate constant k_1 and increased dissociation rate constant k_2 (3). Sic1 recruitment triggers ubiquitination, which depends on release of the ubiquitin-charged Cdc34-S∼Ub from the RING domain (4). After ubiquitin transfer, Cdc34 is recruited into another cycle of interaction with SCF^{Cdc4} by being charged with activated ubiquitin (2). Autoubiquitination is a byproduct of a high rate of collisions between Cdc34-S∼Ub molecules (not shown). Ubiquitin is marked in yellow while activated ubiquitin in the form of a thiol ester is marked in red.

obvious advantage is to ensure a certain degree of flexibility in selection of target lysines. Importantly, a demand for such flexibility stems from the nature of polyubiquitination, which must accommodate multiple lysines, at least one of which is on the substrate protein, and one on each of the substrate-attached ubiquitins. Considering that ubiquitin is an 8 kDa protein, synthesis of a single chain of 6 ubiquitins would move the target lysine 80 Å (Cook et al., 1994), and chains synthesized in vitro are even longer. Although required for efficient ubiquitination, the E3 could hardly be expected to ensure direct positioning of the E2 by the E3 to each of the individual target lysines. Moreover, naturally unstable proteins are frequently modified on multiple lysines, as demonstrated for Sic1 by a recent elegant analysis with lysine mutants (Petroski and Deshaies, 2003). According to this work, any of the six N-terminal lysines of Sic1 can be modified with a chain of 6–20 ubiquitins while Sic1 is in a complex with Clb5/Cdc28, like in our studies, and even more lysines can become substrates when Sic1 is not assembled with Clb5/Cdc28. Importantly, modification of a single lysine is sufficient for degradation of Sic1 (Petroski and Deshaies, 2003). Thus, it is possible that the modification of multiple lysines is simply a side effect associated with a mechanism that, above all, must ensure a certain degree of spatial flexibility necessary for extension of a polyubiquitin chain.

If flexibility of target selection is essential for polyubiquitination, what ensures efficiency of Cdc34-S∼Ub collisions with the substrate (the Cdc34-S∼Ub dissociation rate constant of 0.6 min^{-1} is comparable with the rate of Sic1 ubiquitination) and what prevents ubiquitination of the substrate-associated Clb5/Cdc28 subunits or SCF^{Cdc4}? It appears that one key issue here is the proximity of the SCF^{Cdc4}-bound substrate, less than 50 Å from the Cdc34 recruitment site on the RING (Orlicky et al., 2003). Strikingly, our experiments demonstrate that the lysine specificity of Sic1 ubiquitination is higher than would be expected from random, diffusion-based interactions with Cdc34-S∼Ub. If ubiquitination resulted from random contacts, the end product would always be ubiquitination of multiple residues. Instead, we observed that only one of the available lysines was modified when the Cdc34 concentration was low (10-fold lower than the K_D), and during the first few minutes when the Cdc34 concentration was high (10-fold higher than the K_D). These results are consistent with the results of a recent analysis performed with β-catenin degron peptide, which demonstrated that moving the primary target lysine 4–8 residues away from its original position makes it less favorable for ubiquitination (Wu et al., 2003). Both these and our findings support the previously proposed role of substrate presentation in the mechanism of ubiquitination. Whether and how a dynamic interaction between the substrate and SCF^{Cdc4} (Orlicky et al., 2003) contributes to ubiquitination remains to be determined. Moreover, it needs to be tested how SCF subunits escape ubiquitination. The possibility that the core SCF subunits evolved to be resistant to ubiquitination is suggested by the observation that Sgt1 protein, which was identified as subunit of SCF with unknown function (Kitagawa et al., 1999) is efficiently ubiquitinated when assembled with SCF through Skp1 (D.S., unpublished data).

An interesting question raised by our experiments is why the functional interaction between Cdc34 and

SCF^{Cdc4} depends on formation of Cdc34-ubiquitin thiol ester prior to Cdc34 recruitment to the RING. One possibility is that the SCF^{Cdc4}-bound Cdc34 cannot properly engage in the interaction with activated ubiquitin. An important clue to how formation of a ubiquitin thiol ester changes the properties of E2 comes from a recent NMR spectra-based reconstruction of Ubc1-S~Ub thiol ester intermediate (Hamilton et al., 2001). This analysis demonstrates that the interaction engages a large area of approximately 1823 A, with the C terminus of ubiquitin wrapping around the E2 through both polar and nonpolar interactions and terminating in the thiol ester. Moreover, formation of a ubiquitin-thiol ester, but not the interaction with SCF, has been recently demonstrated to facilitate self-association of Cdc34 (Varelas et al., 2003). Both reports suggest that a ubiquitin thiol ester triggers major changes in Cdc34, which perhaps cannot be facilitated when Cdc34 is bound to SCF^{Cdc4}.

Our data show that autoubiquitination of Cdc34 depends on a high rate of collisions between SCF^{Cdc4}-free Cdc34-S~Ub molecules and that the collisions become efficient only when the concentration of Cdc34-S~Ub exceeds the K_D of Cdc34-S~Ub interaction with SCF^{Cdc4}. This collision-based mechanism of autoubiquitination explains why in conditions permissive for ubiquitination of Sic1 the Cdc34 is autoubiquitinated with a low efficiency. The observation that SCF^{Cdc4} lowers the concentration requirement for Cdc34 in autoubiquitination suggests two possible models for how SCF acts on Cdc34. In the first model, SCF-mediated autoubiquitination reflects transient activation of Cdc34, similarly to what was proposed previously (Seol et al., 1999; Skowyra et al., 1999). An alternative model is that SCF^{Cdc4} stimulates increase of a local concentration of Cdc34 by an unknown mechanism. Which of these mechanisms is responsible for autoubiquitination needs to be determined by direct kinetic characterization of the Cdc34-ubiquitin thiol ester in the presence and absence of SCF^{Cdc4}. Importantly, the collision-based mechanism of autoubiquitination is a good candidate mechanism for self-control of Cdc34 levels in vivo. Although Cdc34 was reported to be a stable protein (Goebl et al., 1994), under normal conditions only a small fraction of Cdc34 would be autoubiquitinated, and therefore, only a small fraction would be unstable.

There are two reasons to consider dynamic binding equilibrium as an E2-recruitment mechanism for all ubiquitin ligases. First, charging with activated ubiquitin is a common feature of all ubiquitin-conjugating enzymes. Second, according to the crystal structures of UbcH7/c-Cbl RING and UbcH7/E6-AP HECT domains, the mechanism of the E2-E3 interaction is structurally conserved (reviewed in VanDemark and Hill, 2002). The emerging view is that specific E2-E3 pairs involve subtle variations of a fundamentally similar interaction interface, and that this is the base for functional specificity of the individual pairs (Ptak et al., 2001; Ulrich, 2003). Our experiments with residue F72 of Cdc34, which is located in the conserved E2/RING domain interface, suggest that the structural conservation could be necessary to maintain a dynamic E2-E3 binding equilibrium. This conclusion is further supported by two recent two-hybrid studies with Rad6/Ubr1 (Xie and Varshavsky, 1999) and Ubc13-Mms2/Rad5 (Ulrich, 2003), which indirectly suggest a binding equilibrium as a mechanism for E2 recruitment to the RING.

How does our model change the view on HECT-type ubiquitin ligases? In principle, the dynamic E2-E3 binding equilibrium could facilitate the E2-substrate contact in reactions with HECT-type E3s, in which case the conserved active site cysteine of HECT E3 could either catalyze initial reactions or have a structural role. A more conservative possibility is that the dynamic E2-E3 interaction is essential for ubiquitin transfer from the E2 to the E3 active site. The mechanism of this transfer is currently a puzzle because of the distance separating the E2 and E3 active sites in HECT domain structures, even after modeling of possible conformational rearrangements (Huang et al., 1999; Verdecia et al., 2003).

Experimental Procedures

Antibodies

We used polyclonal rabbit α-Cdc34, α-Cdc4, α-Cdc53 (M. Goebl, Indiana University School of Medicine), α-Sic1 (R. Deshaies, California Institute of Technology), α-Skp1 (Skowyra et al., 1997), α-FLAG M2 agarose (Sigma), and α-HA agarose (Covance). Antibody detection was by ECL (Amersham).

Recombinant Proteins and Protein Complexes

We used $Uba1^{His6}$ (Feldman et al., 1997) and baculoviruses encoding yeast Cdc53, Rbx1, Cdc4, ^{FLAG}Skp1, ^{GST}Skp1, $^{GST}Cdc28^{HA}$, Clb5, Sic1, $Cln2^{HA}$, CKS, and CIV (Skowyra et al., 1997, 1999). C-terminal His6 fusion in pET21+ (Novagen) of Cdc34, Cdc34Δ (terminated at aa 244), 3KCdc34Δ (K173R, K189R, and K197R) triple mutant, and Cdc34 point mutants C95A, F72Y, F72V, F72D, and F72L were created for this work. K0-ubiquitin (You et al., 1999) was confirmed by mass spectrometry to lack lysines (gift from C. Pickart, Johns Hopkins University). The molecular mass of octa-dimethyl ubiquitin was confirmed by mass spectrometry (AFFINITI Research Products Ltd, UK). Ubiquitin (bovine) was from Sigma.

All Cdc34 proteins were expressed in *E. coli* (BL21 DE3 LysS) and purified on Ni^+-NTA resin (Qiagen) followed by DEAE and Superdex 200 chromatography (Amersham) in U buffer (50 mM Tris [pH 7.5], 50 mM KCl, and 0.2 mM DTT). Met [S^{35}]-Cdc34Δ (570 cpm/pmol) was labeled in vivo by expressing $Cdc34\Delta^{His6}$ in cells grown on synthetic medium with a 30 min pulse of 8 mCi Tran^{35}S-label (ICN) after 3 hr of induction with isopropylthio-β-galactoside (IPTG).

$^{FLAG}SCF^{Cdc4}$, $^{GST}SCF^{Cdc4}$, Sic1/Clb5/$^{GST}Cdc28^{HA}$, and Cln2/CKS/$^{GST}Cdc28^{HA}$ complexes were assembled by coinfecting 5×10^7 SF9 insect cells (Invitrogen) for 40 hr with the appropriate baculoviruses, followed by cell lysis and immunoprecipitation (Skowyra et al., 1997). Sic1 was phosphorylated as described before (Skowyra et al., 1997).

Preparation of Yeast Extracts and Endogenous Proteins

Yeast extracts were prepared by grinding yeast MTY1243 (Patton et al., 1998) that were "blast-frozen" in a 1:0.7 ratio in U buffer supplemented with 2 mM ATP, 10 mM $MgCl_2$, and protease inhibitors, followed by thawing and centrifugation (12,000 rpm, 30 min, 4°C). The extracts typically contained 10 mg/ml protein and all relevant components of the ubiquitination system, including Cdc34, SCF, ubiquitin, Uba1, and 26S proteasome. Cdc34 was purified on DEAE Sepharose (300 mM KCl elution), followed by Superdex 200 chromatography (Amersham). ^{HA}Cdc53 complexes were immunopurified like $^{F}SCF^{Cdc4}$, except that α-HA agarose was used instead of α-FLAG agarose.

Ubiquitination Assays

Ubiquitination was typically performed for 1 hr at 25°C in 20 μl mixtures containing buffer U, Ub^{mix} (2 mM ATP, 10 mM $MgCl_2$, 1 pmol $Uba1^{His6}$, and 1.3 nmols ubiquitin), ~2 pmols of SCF^{Cdc4} (calculated for a fully assembled complex composed of monomeric subunits), 2 pmols of Sic1-P/Clb5/$^{GST}Cdc28^{HA}$ (where indicated), and $Cdc34^{His6}$ (typically 70 pmols or as indicated). Reactions were stopped by boiling with SDS-Laemmli buffer, followed by separation of proteins

by SDS-PAGE and visualization by Western blotting with α-Sic1 or α-Cdc34 antibodies.

SCF^{Cdc4}/Cdc34 Binding and Release Assays

Purified $Cdc34^{His6}$ (3 μM, 70 pmols) was incubated at 25°C with 2 pmols of $^{GST}SCF^{Cdc4}$ immobilized on 10 μl G^{SH}-Sepharose beads, in 20 μl of mixtures containing or missing Ub^{mix}. After one hour, the beads were washed with NETN buffer (3 × 1 ml) and U buffer (3 × 1 ml), and then suspended in 20 μl of fresh ubiquitination mixtures without Cdc34 or as indicated. At the times indicated, the supernatants were separated from the beads or analyzed as unseparated fractions by SDS-PAGE/Western or by liquid scintillation counting (in assays with radioactive Cdc34).

Western Blots

Western blots were performed with sequential probing with α-Sic1, α-Cdc34, α-Cdc53, α-Skp1, and α-Cdc4 (Skowyra et al., 1997). Asterisks indicate positions of irrelevant polypeptides cross-reacting with the α-Sic1 and α-Cdc34 antibodies.

Flow Cytometry Measurements of Binding Kinetics

Cy2-3KCdc34Δ was prepared by modification of purified 3KCdc 34ΔHis6 with bisfunctional NHS-ester FluoroLink Cy2 reactive dye to 0.6 dye:protein ratio followed by gel filtration, as recommended by the manufacturer (Amersham). Similar modification was performed with full-length Cdc34 and Cdc34F72V mutant proteins. For the flow cytometry measurements, Cy2-3KCdc34 was preincubated with or without Ub^{mix} in 100 μl of U buffer, followed by addition of 50,000 $^{G}SCF^{Cdc4}$-carrying $Beadlyte^{R}$ G^{SH}-beads (Upstate, New York) and immediate measurement of the bead-associated Cy2 fluorescence in real time using Becton-Dickinson FACSCalibur, as previously described (Buranda et al., 1999, 2001). Because binding was terminated in 75–100 s we expect the kinetics to be characteristic of initial rates. Maximum binding was determined in 1 hr reaction. Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled 3KCdc34Δ.

The raw data were converted to ASCII format, suitable for kinetic fitting, using FCSQuery software developed by Bruce Edwards (University of New Mexico). The fluorescence data were then converted to surface coverage units in terms of bound proteins per bead as previously described, using Quantum Simply Cellular Calibration beads with fluorescein (Bangs Labs, Fishers, IN). The data were corrected for the difference in emission quantum yield between Cy2-3KCdc34Δ and fluorescein. The kinetic data were analyzed using a model based on the law of mass action and fit by nonlinear regression methods using a software package from Scientist Micromath Scientific Software, Salt Lake City UT.

Acknowledgments

We thank C. Pickart for the generous gift of K0-Ubiquitin, M. Goebl and R. Deshaies for generous gifts of antibodies, C. Hill and A. VanDemark for excellent suggestions regarding F72, R. Deshaies and W. Harper for communicating their manuscripts in press, T. Heyduk, A. Waheed, and J. Eissenberg for discussions, and D. Andrews, D. Dorsett and Sue Wickner for critical comments on the manuscript. This work was supported by National Institutes of Health grants GM65267 (D.S.), GM60799/EB00264 (L.S.), CA88339 (L.S.), American Cancer Society grant RGS CSM-102954 (D.S.), a grant from the Edward Mallinckrodt, Jr. Foundation (D.S.), and National Science Foundation grant MCB-9907611 (T.B.).

Received: March 20, 2003
Revised: July 29, 2003
Accepted: July 30, 2003
Published: September 4, 2003

References

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell *86*, 263–274.

Banerjee, A., Gregori, L., Xu, Y., and Chau, V. (1993). The bacterially expressed yeast Cdc34 gene product can undergo autoubiquitination to form a multiubiquitin chain-linked protein. J. Biol. Chem. *268*, 5668–5675.

Buranda, T., Jones, G.M., Nolan, J.P., Keij, J., Lopez, G.P., and Sklar, L.A. (1999). Ligand receptor dynamics at Steptavidin-coated particle surfaces: a flow cytometric and spectrofluorometric study. J. Phys. Chem. *103*, 3399–3410.

Buranda, T., Lopez, G., Simons, P., Pastuszyn, A., and Sklar, L. (2001). Detection of epitope-tagged proteins in flow cytometry: Fluorescence resonance energy transfer-based assays on beads with femtomole resolution. Anal. Biochem. *298*, 151–162.

Cook, L., Jeffrey, L.C., Kasperek, E., and Pickart, C. (1994). Structure of tertaubiquitin shows how multiubiquitin chains can be formed. J. Mol. Biol. *236*, 601–609.

Deshaies, R.J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cel. Dev. Biol. *15*, 435–467.

Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/Cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1. Cell *91*, 149–151.

Glickman, M.H., and Ciechanover, A. (2001). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. *82*, 373–428.

Goebl, M., Goetsch, L., and Byers, B. (1994). The Ubc3 (Cdc34) ubiquitin-conjugating enzyme is ubiquitinated and phosphorylated in vivo. Mol. Cell. Biol. *14*, 3022–3029.

Hamilton, K.S., Ellison, M.J., Barber, K.R., Williams, R.S., Huzil, J.T., McKenna, S., Ptak, C., Glover, M., and Shaw, G.S. (2001). Structure of a conjugating enzyme-ubiquitin thiolester intermediate reveals a novel role for the ubiquitin tail. Structure *9*, 897–904.

Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P.M., Huibregtse, J.M., and Pavletich, N.P. (1999). Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2–E3 enzyme cascade. Science *286*, 1321–1326.

Joazeiro, C.A., and Weissman, A.M. (2000). Ring finger proteins: mediators of ubiquitin ligase activity. Cell *102*, 549–552.

Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin, W.G., Jr., Elledge, S.J., Conaway, R.C., et al. (1999a). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657–661.

Kamura, T., Conrad, M.N., Yan, Q., Conaway, R.C., and Conaway, J.W. (1999b). The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of Cullins Cdc53 and Cul2. Genes Dev. *13*, 2928–2933.

Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., et al. (2001). NEDD8 recruits E2-ubiquitin to SCF E3 ligase. EMBO J. *20*, 4003–4012.

Kitagawa, K., Skowyra, D., Elledge, S.J., Harper, J.W., and Hieter, P. (1999). SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. Mol. Cell *4*, 21–33.

Kolman, C.J., Toth, J., and Gonda, D.K. (1992). Identification of a portable determinant of cell cycle function within the carboxyl-terminal domain of the yeast CDC34 (UBC3) ubiquitin conjugating (E2) enzyme. EMBO J. *11*, 3081–3090.

Kornitzer, D., Raboy, B., Kulka, R.G., and Fink, G.R. (1994). Regulated degradation of the transcription factor Gcn4. EMBO J. *13*, 6021–6030.

Lammer, D., Mathias, N., Laplaza, J.M., Jiang, W., Liu, Y., Callis, J., Goebl, M., and Estelle, M. (1998). Modification of yeast Cdc53p by the ubiquitin-related protein Rub1p affects function of the SCFCdc4 complex. Genes Dev. *12*, 914–926.

Mathias, N., Steussy, C.N., and Goebl, M.G. (1998). An essential domain within Cdc34p is required for binding to a complex containing Cdc4p and Cdc53p in Saccharomyces cerevisiae. J. Biol. Chem. *273*, 4040–4045.

Nakamura, S., Roth, J.A., and Mukhopadhyay, T. (2000). Multiple

lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. Mol. Cell. Biol. *20*, 9391–9398.

Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F.B., Mendenhall, M.D., Sicheri, F., Pawson, T., and Tyers, M. (2001). Multiple phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. Nature *414*, 514–521.

Ohta, T., Michael, J.J., Schottelius, S.J., and Xiong, Y. (1999). ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Mol. Cell *3*, 535–541.

Orlicky, S., Tang, X., Willems, A., Tyers, M., and Sicheri, F. (2003). Structural basis for phospho-dependent substrate selection and orientation by the SCF^{Cdc4} ubiquitin ligase. Cell *112*, 243–256.

Patton, E.E., Willems, A.R., Sa, D., Kuras, L., Thomas, D., Craig, L.L., and Tyers, M. (1998). Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. Genes Dev. *12*, 692–705.

Petroski, M.D., and Deshaies, R.J. (2003). Context of multiubiquitin chain attachment influences the rate of Sic1 degradation. Mol. Cell *11*, 1435–1444.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annu. Rev. Biochem. *70*, 503–533.

Ptak, C., Prendergast, J.A., Hodgins, R., Kay, C.M., Chau, V., and Ellison, M.J. (1994). Functional and physical characterization of the cell cycle ubiquitin-conjugating enzyme Cdc34 (Ubc3). J. Biol. Chem. *269*, 26539–26545.

Ptak, C., Gwozd, C., Huzil, T.J., Gwozd, T.J., Garen, G., and Ellison, M.J. (2001). Creation of a pluripotent ubiquitin-conjugating enzyme. Mol. Cell. Biol. *21*, 6537–6548.

Schulman, B.A., Carrano, A.C., Jeffrey, P.D., Bowen, Z., Kinnucan, E.R., Finnin, M.S., Elledge, S.J., Harper, J.W., Pagano, M., and Pavletich, N.P. (2000). Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. Nature *408*, 381–386.

Schwob, M., Bohm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40Sic1 controls the G1/S transition in S. cerevisiae. Cell *79*, 233–244.

Seol, J.H., Feldman, R.M., Zachariae, W., Shevchenko, A., Correll, C.C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., et al. (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. *13*, 1614–1626.

Silver, E.T., Gwozd, T.J., Ptak, C., Goebl, M., and Ellison, M.J. (1992). A chimeric ubiquitin conjugating enzyme that combines the cell cycle properties of CDC34 (UBC3) and the DNA repair properties of RAD6 (UBC2): implications for the structure, function and evolution of the E2s. EMBO J. *11*, 3091–3098.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*, 209–219.

Skowyra, D., Koepp, D.M., Kamura, T., Conrad, M.N., Conaway, R.C., Conaway, J.W., Elledge, S.J., and Harper, J.W. (1999). Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1. Science *284*, 662–665.

Tan, P., Fuchs, S.Y., Chen, A., Wu, K., Gomez, C., Ronai, Z., and Pan, Z. (1999). Recruitment of a ROC1-Cul1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of IkBa. Mol. Cell *3*, 527–533.

Ulrich, H.D. (2003). Protein-protein interactions within an E2-RING finger complex. Implications for ubiquitin-dependent DNA damage repair. J. Biol. Chem. *278*, 7051–7058.

VanDemark, A., and Hill, C.P. (2002). Structural basis of ubiquitylation. Curr. Opin. Struct. Biol. *12*, 822–830.

Varelas, X., Ptak, C., and Ellison, M.J. (2003). Cdc34 self-association is facilitated by ubiquitin thiol ester formation and is required for its catalytic activity. Mol. Cell. Biol. *23*, 5388–5400.

Verdecia, M., Joazeiro, C.A.P., Wells, N.J., Ferrer, J.-L., Bowman, M.E., Hunter, T., and Noel, J.P. (2003). Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. Mol. Cell *11*, 249–259.

Verma, R., Feldman, R., and Deshaies, R.J. (1997a). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. Mol. Biol. Cell *8*, 1427–1437.

Verma, R., Annan, R.S., Huddleston, S.A., Carr, S.A., Reynard, G., and Deshaies, R.J. (1997b). Phosphorylation of Sic1p by G1 Cdk is required for its degradation and entry into S phase. Science *278*, 455–460.

Wu, K., Chen, A., Tan, P., and Pan, Z.-Q. (2002). The Nedd8-conjugated ROC1–CUL1 core ubiquitin ligase utilizes Nedd8 charged surface residues for efficient polyubiquitinatin chain assembly catalyzed by Cdc34. J. Biol. Chem. *277*, 516–527.

Wu, G., Guozhou, X., Schulman, B.A., Harper, J.W., and Pavletich, N.P. (2003). Structure of β-TRCP-Skp1-βcatenin complex: destruction-motif binding and lysine specificity of the SCF ubiquitin ligase. Mol. Cell *11*, 1445–1456.

Xie, Y., and Varshavsky, A. (1999). The E2–E3 interaction in the N-rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. EMBO J. *18*, 6832–6844.

You, J., Cohen, R.E., and Pickart, C.M. (1999). Construct for high level expression and low misincorporation of lysine for arginine during expression of pET-encoded eukaryotic proteins in E. coli. Biotechniques *27*, 950–954.

Zheng, N., Wang, P., Jeffrey, P.D., and Pavletich, N.P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell *102*, 533–539.

Zheng, N., Schulman, B.A., Song, L., Miller, J.J., Jeffrey, P.D., Wang, P., Chu, C., Koepp, D.M., Elledge, S.J., Pagano, M., et al. (2002). Structure of the Cul1-Rbx1-Skp1-F box Skp2 SCF ubiquitin ligase complex. Nature *416*, 703–708.