

Evaluation of a Diffusion-Driven Mechanism for Substrate Ubiquitination by the SCF-Cdc34 Ubiquitin Ligase Complex

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Summary

Release of ubiquitin-charged Cdc34 from the SCF ubiquitin ligase followed by diffusion-driven collision with substrate has been proposed to underlie ubiquitination of the canonical SCF substrate Sic1. Cdc34 F72V, reported to be defective in dissociation from SCF, served as key validation. Here, we test predictions of this “hit-and-run” hypothesis. We find that Cdc34 F72V is generally defective in SCF-mediated activation but, contrary to expectation, does not compete with wild-type Cdc34 *in vitro* or *in vivo* and can fulfill the physiological role of Cdc34 with only moderate delay in Sic1 turnover. Whereas a hit-and-run mechanism might explain how Cdc34 can transfer ubiquitin to the ends of growing ubiquitin chains on SCF-bound substrates, molecular modeling suggests that an E2 docked to SCF can do so without dissociating. We propose that interactions between Cdc34~Ub and SCF directly activate ubiquitin transfer within a substrate-SCF-Cdc34~Ub ternary complex.

Introduction

The ubiquitin system is controlled by a hierarchical enzymatic cascade in which a single activating enzyme (E1) transfers ubiquitin onto the active-site cysteine of several conjugating enzymes (E2s) in a labile thioester linkage (Pickart, 2001). Ubiquitin ligases (E3s) bind these charged E2s and facilitate ubiquitin transfer onto lysine residues of specifically bound substrate proteins. More than 500 distinct E3s may exist in humans (Yang et al., 2005), consistent with their broad involvement in cellular homeostasis.

E3s can be pigeonholed into two major functional categories, dependent upon the presence of a HECT domain or RING motif (Pickart, 2001). HECT ligases, such as E6AP, form a catalytic intermediate with ubiquitin on a conserved cysteine residue prior to covalent modification of substrate. RING ligases and structurally related PHD and U box ligases, in contrast, facilitate direct transfer of ubiquitin from E2 onto substrate.

Protein ubiquitination has largely become synonymous with degradation by the 26S proteasome. However, it is evident that the extent of ubiquitination and type of ubiquitin-ubiquitin linkage can confer distinct

fates. Monoubiquitination regulates a variety of processes including endocytosis (Hicke et al., 2005), whereas a ubiquitin chain of at least four ubiquitin molecules linked together via the lysine 48 residue is required for efficient proteasome targeting (Thrower et al., 2000). In contrast, lysine 63-linked ubiquitin chains on a protein may regulate intracellular signal transduction pathways (Chen, 2005). However, recent observations suggest that ubiquitin signaling may be more complex than currently envisioned (Kim and Rao, 2006).

In spite of our knowledge about E3 diversity and distinct modes of ubiquitin signaling, we know surprisingly little about the biochemical mechanisms underlying this posttranslational protein modification. The SCF family of RING ligases has emerged as an important paradigm for studying the mechanisms of protein ubiquitination (Petroski and Deshaies, 2005a). X-ray crystallography, molecular modeling, and mutagenesis studies suggest that SCF serves as a rigid scaffold that properly positions the E2 charged with ubiquitin (E2~Ub) relative to the substrate to be modified (Orlicky et al., 2003; Zheng et al., 2002). A perplexing observation arising from these studies is the existence of an ~50–60 Å gap that separates the substrate-binding site on the F box subunit from the thioester bond of E2~Ub docked onto SCF. As the primary function of RING ligases may be to bring E2~Ub into sufficiently close proximity to the ε amino group of a substrate’s lysine residue to facilitate nucleophilic attack (Passmore and Barford, 2004), it is unclear how SCF facilitates substrate ubiquitination across this gap. Another challenge is to understand how E2 and E3 catalyze the assembly of long ubiquitin chains given that the position of the distal end of the chain changes with respect to the position of the active site within the E2-E3 complex as the chain elongates.

A recent study proposed a mechanism to reconcile both of these conundrums (Deffenbaugh et al., 2003). The “hit-and-run” model posits that E2~Ub transiently associates with SCF and is released prior to collision with substrate. An appealing aspect of this hypothesis is that it enables interaction of E2~Ub with substrate regardless of the substrate’s exact position in three-dimensional space. The linchpin of this hypothesis was the identification of a critical residue within SCF’s E2, Cdc34, proposed to be a key determinant of its release—phenylalanine 72 (F72). Mutagenesis of F72 to valine (F72V) slows dissociation of Cdc34~Ub from SCF, which was deemed responsible for the severe defect of this mutant in substrate ubiquitination. To further understand the mechanism of ubiquitin transfer by Cdc34 through SCF, we sought to test predictions of the hit-and-run model.

Results

Uncharged Cdc34 Does Not Sequester SCF

The hit-and-run model was inspired by two observations. First, ~10% of Cdc34 molecules that coprecipitate with SCF dissociate during the course of a 90 min ubiquitination reaction (Figure 1A of Deffenbaugh et al. [2003]),

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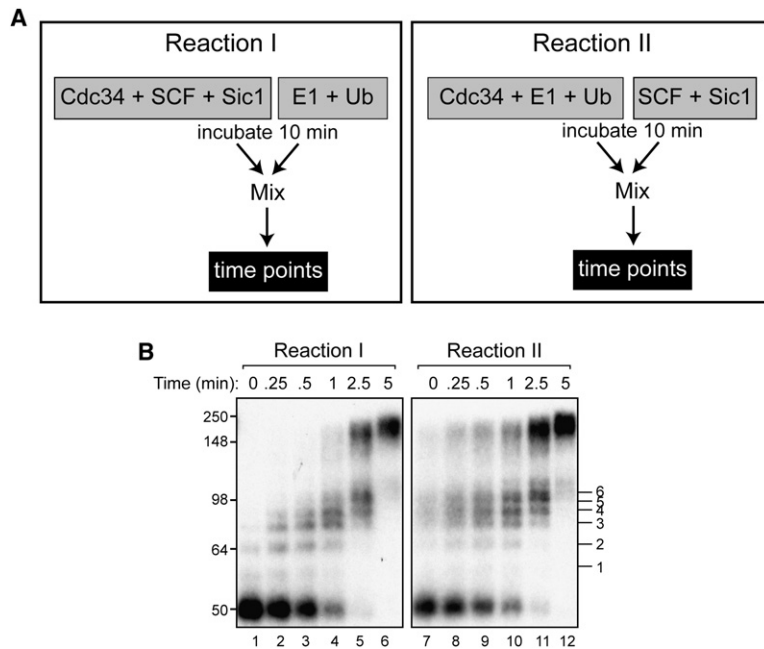


Figure 1. Preformation of Cdc34~Ub Prior to SCF Addition Has Little Effect on the Rate of Sic1 Ubiquitination

Order-of-addition experiments were performed as schematically shown in (A) to test the effect of preincubating SCF with uncharged Cdc34 on the rate of Sic1 ubiquitination. (B) The rate of Sic1 ubiquitination was examined in experiments performed as described in (A).

and the amount of dissociated Cdc34 correlates with the extent of Sic1 ubiquitination. Second, Cdc34~Ub has a 40-fold lower affinity for SCF than uncharged Cdc34, largely due to an increase in off rate (Deffenbaugh et al., 2003). These observations suggested that interaction of Cdc34~Ub with SCF is highly dynamic. Moreover, the slow dissociation of uncharged Cdc34 from SCF implies that Cdc34~Ub must dissociate *before* it transfers ubiquitin onto substrate, lest SCF become clogged with slowly dissociating molecules of spent Cdc34.

In our typical reactions (Petroski and Deshaies, 2003, 2005c), two premixes containing SCF + Sic1 and Cdc34 + E1 + ubiquitin are combined. Thus, preformation of Cdc34~Ub may underlie the rapid rates of ubiquitination we observed. On the other hand, if SCF first encounters Cdc34 instead of Cdc34~Ub, Sic1 ubiquitination should be greatly reduced because the $t_{1/2}$ for dissociation of uncharged Cdc34 was reported by Deffenbaugh et al. to be >11 min—more than twice the duration of our reactions. We sought to test this prediction by setting up reactions according to the protocol outlined in Figure 1A. For reaction I, Cdc34 was incubated with SCF + Sic1 prior to addition of E1 and ubiquitin. In contrast, for reaction II, Cdc34 was incubated with E1 and ubiquitin to form Cdc34~Ub prior to addition of SCF + Sic1. As shown in Figure 1B, the initial rate of reaction I was slightly slower than that of reaction II, which is not surprising given the absence of Cdc34~Ub at the start of this reaction. However, after the first minute the two protocols exhibit identical kinetics. This result is inconsistent with the idea that Cdc34~Ub must dissociate from SCF prior to discharging ubiquitin to prevent SCF from becoming clogged with uncharged Cdc34.

Cdc34 F72V Is Impaired in Conjugating Ubiquitin to Substrate Lysines and Polymerizing Ubiquitin Chains in the Presence of SCF

Whereas the dynamic association of Cdc34~Ub with SCF was the initial impetus for the hit-and-run hypothe-

sis, it was the identification of the F72V mutation in Cdc34, which impairs its release from SCF, that provided critical support for this idea. Given that our data are inconsistent with slow dissociation of uncharged Cdc34 from SCF, we sought to further characterize Cdc34 F72V by examining the rate of Sic1 ubiquitination.

These experiments employed either ubiquitin (Ub) or lysine-less ubiquitin (K0 Ub) to allow us to distinguish between initial modification (K0 Ub) or ubiquitin-chain synthesis (Ub) on Sic1. Sic1 containing only the six lysine residues that are individually sufficient (and collectively necessary) for its *in vivo* destruction (Petroski and Deshaies, 2003) was used to eliminate nonproductive conjugation events (i.e., those that do not support 26S proteasome degradation).

In comparing rates of consumption of unmodified Sic1 in reactions using K0 Ub (Figure 2A), F72V was 5.7-fold slower than wild-type (WT). Furthermore, the overall pattern of Sic1 modification by F72V was severely altered—it occurred in a step-like manner characteristic of a distributive reaction, in contrast to the more processive behavior seen with WT.

To evaluate the effects of the F72V mutation on chain synthesis, we performed similar experiments with unmodified Ub (Figure 2C). Again, WT Cdc34 was ~5.5-fold more rapid than F72V at converting Sic1 to modified species (Figure 2D). Whereas the rapid and simultaneous appearance of one to six distinct Sic1-Ub species was observed with WT (e.g., even at “0” min), reactions employing F72V yielded considerably fewer modifications that appeared more distributive over time. Even after 60 min, the majority of Sic1 molecules modified by F72V accumulated only four to six attachments (Figure 2C, lane 12). Further experiments utilizing K48-only ubiquitin or Sic1 containing only a single lysine residue yielded similar results to Figure 2D, and experiments with K48R ubiquitin yielded results similar to Figure 2A (data not shown). These observations suggest that F72V is defective for SCF-dependent substrate

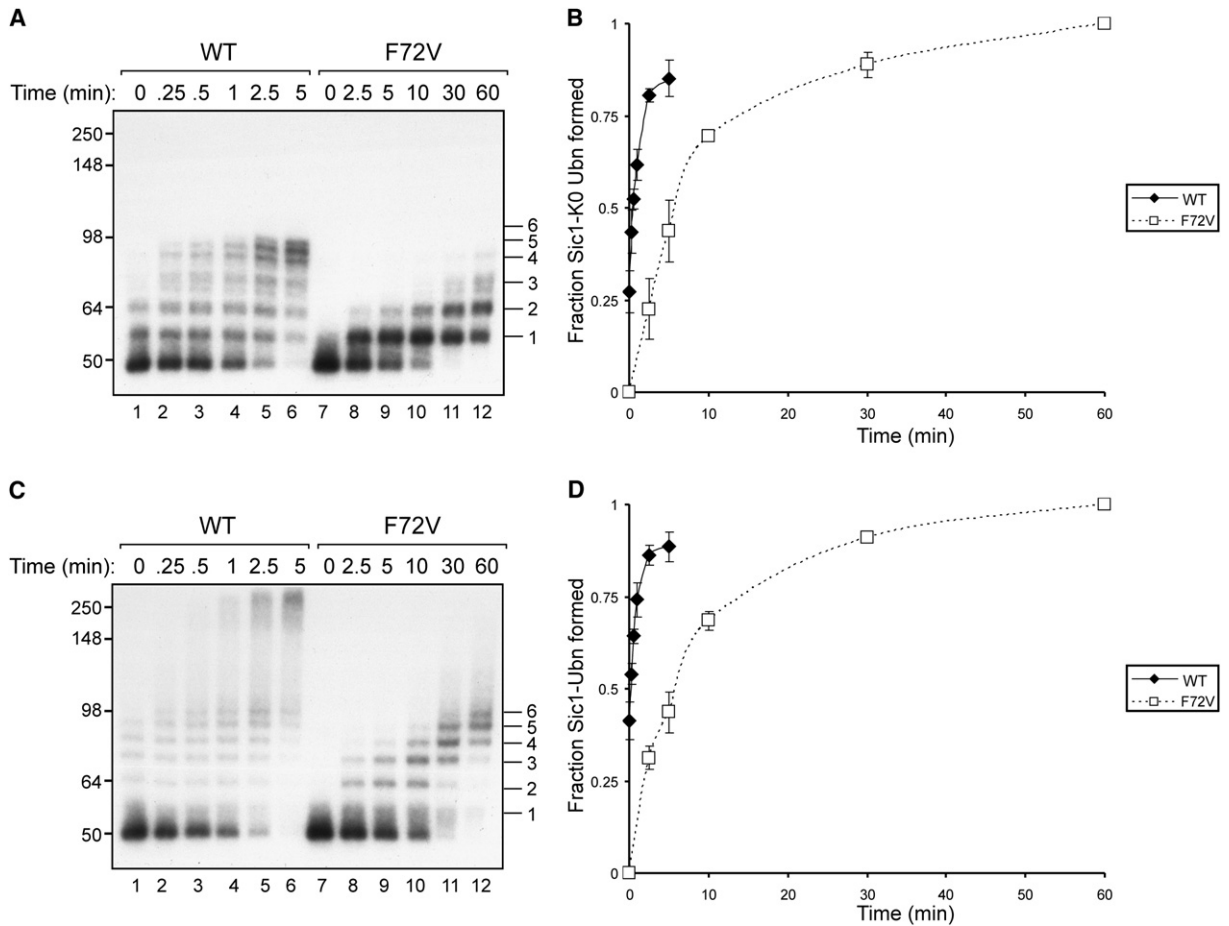


Figure 2. Cdc34 F72V Is Defective in SCF-Dependent Substrate Ubiquitination

The ability of Cdc34 F72V to modify Sic1 lysine residues with K0 ubiquitin (A) and synthesize ubiquitin chains on Sic1 with unmodified ubiquitin (C) in the presence of SCF was determined at the times indicated. The number of ubiquitin molecules on Sic1 is indicated. Quantification of the fraction of ³²P-labeled Sic1 modified with K0 ubiquitin (B) and ubiquitin (D) was measured by phosphor screen image analysis. Error bars represent the standard deviation of the mean (n = 3).

ubiquitination, yet can still synthesize K48-linked ubiquitin chains, albeit at a much slower rate.

Cdc34 F72V Does Not Inhibit Cdc34-SCF-Dependent Sic1 Ubiquitination In Vitro

If Cdc34 F72V is defective in ubiquitination of Sic1 because its ubiquitin-charged form cannot be efficiently released from SCF (Deffenbaugh et al., 2003), it should interfere with the activity of WT Cdc34 in our in vitro system. To test this hypothesis, competition experiments were performed in which increasing amounts of F72V were pre-mixed with SCF and phosphorylated Sic1 for 15 min prior to the addition of a fixed amount of Cdc34, E1, and ubiquitin (Figure 3A). The experiment was designed such that the final concentration of WT Cdc34 in the ubiquitination reactions was 200 nM—the concentration where the overall rate of Sic1 consumption was approximately half-maximal (see Figure S1 in the Supplemental Data available with this article online)—which should allow for the rate of Sic1 ubiquitination to be sensitive to the presence of competing F72V molecules. Identical reactions containing Cdc34 C95S L99S, known to be a dominant-negative inhibitor of

Cdc34 activity (Banerjee et al., 1995), were performed in parallel.

As shown in Figure 3A, the rate of Sic1 conversion to its ubiquitinated form was slowed ~5-fold by inclusion of 2.5-fold molar excess of C95S L99S, but was not affected by equivalent excess of F72V. Even at 5-fold molar excess, F72V had little effect on the extent of Sic1 modification (the fold inhibition of 5× F72V versus no competitor is 1.2, Figure 3B). In contrast, however, 5-fold molar excess of C95S L99S suppressed formation of higher-molecular-weight ubiquitin conjugates on Sic1 by 7.6-fold (compare lanes 5 and 6 to 35 and 36 and 41 and 42, Figure S2 and Figure 3B). The inability of F72V to impair Sic1 ubiquitination by WT Cdc34 was surprising in light of our kinetic analysis (Figure 2) and F72V ~ Ub's reported 100-fold higher affinity for SCF relative to WT Cdc34 ~ Ub (Deffenbaugh et al., 2003).

The failure of F72V to competitively inhibit WT Cdc34 in vitro argues that its true molecular defect does not result from an abnormally high affinity for SCF. A further issue that arises from this experiment is that the titration data for Cdc34 (Figure S1) are inconsistent with the equilibrium dissociation constant of 1250 nM for SCF-Cdc34 ~ Ub that was reported by Deffenbaugh et al. (2003).

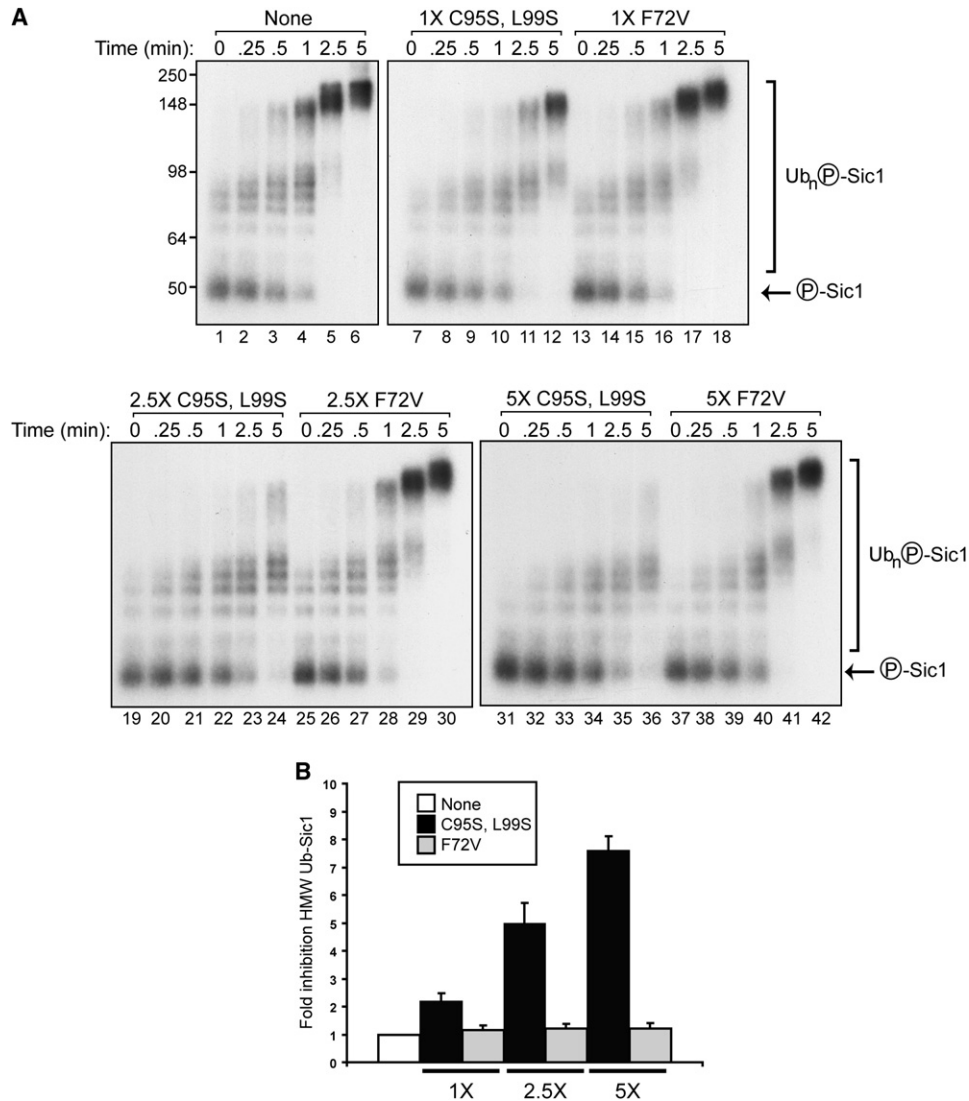


Figure 3. Cdc34 F72V Does Not Inhibit SCF-Dependent Substrate Ubiquitination by Cdc34

(A) The indicated molar ratio of either Cdc34 C95S L99S or Cdc34 F72V was preincubated with SCF/phosphorylated Sic1 for 10 min prior to the addition of E1, ubiquitin, and Cdc34 (final reaction concentration of 200 nM). Reaction aliquots were removed for analysis by SDS-PAGE and autoradiography.

(B) To measure the effects of increasing amounts of either C95S L99S or F72V on Sic1 ubiquitination reactions, the high-molecular-weight species (>98 kDa) was measured as a ratio of the total amount of Sic1 with respect to time. Initial rates were extrapolated ($n = 3$, see Figure S2), and ratios of rates of competitor to no competitor are shown. Error bars represent ratio of rates with no competitor above 1 standard deviation from the mean to the rates of various competitors at 1 standard deviation below the mean.

Cdc34 F72V Is Not Defective in SCF-Independent Functions In Vitro

To further test the effect of F72V on known Cdc34 functions, we first examined its ability to be charged with ubiquitin by E1 and ATP (Figure 4A). As reported by Deffenbaugh et al. (2003), both WT and F72V molecules rapidly formed thioesters with ubiquitin, suggesting that the defect conferred by F72V is downstream of charging by E1.

To evaluate SCF-independent functions of F72V, we prepared full-length, untagged Cdc34. The form of Cdc34 we generally use in our in vitro studies, Cdc34 Δ C-His6, lacks the C-terminal 25 amino acids, which are dispensable for Cdc34 function in vitro and in vivo (Seol et al., 1999). Whereas Cdc34 Δ C only ubiq-

uitinates itself in the presence of SCF (Seol et al., 1999; Skowyra et al., 1999) (see also Figure S3), full-length Cdc34 is modified even in the absence of SCF (Banerjee et al., 1993). Although the physiological significance of this "autoubiquitination" is unknown, it occurs in vivo and the attached ubiquitin chains are lysine 48 linked (Goebel et al., 1994; Seol et al., 1999; Verma et al., 2001). Reactions employing full-length Cdc34, ATP, E1, and ubiquitin were analyzed by gel electrophoresis and Coomassie staining (Figure 4B). The rate of ubiquitin-chain synthesis was identical between WT and F72V, with equal amounts of Ub₂, Ub₃, and Ub₄ formed at 2 hr. Likewise, the rate and extent of Cdc34 autoubiquitination were similar (compare lanes 5 and 10). Taken together, these results suggest, in agreement with

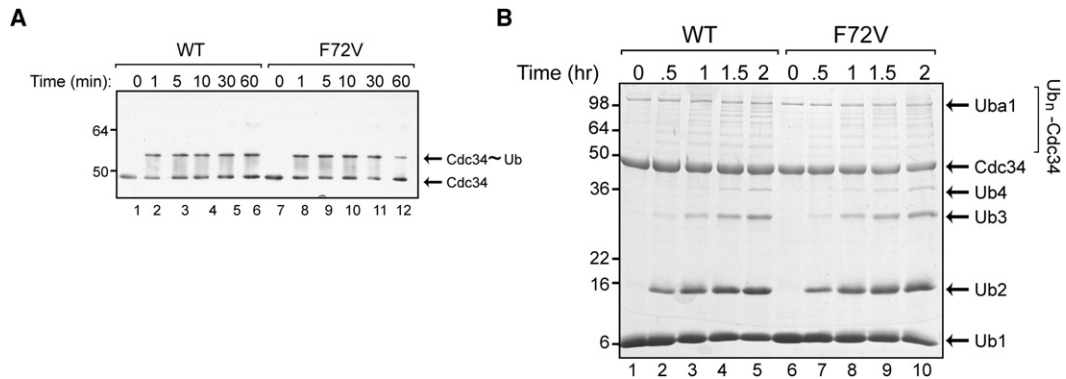


Figure 4. Cdc34 (F72V) Functions in E3-Independent Activities

(A) Cdc34 (1.6 μ M, WT or F72V) was added to ATP, ubiquitin (500 μ M), and E1 (100 nM). Reactions were analyzed by SDS-PAGE and western blotting. (B) SCF-independent activity of full-length WT and F72V Cdc34 is similar. Cdc34 (WT or F72V, 5 μ M) was added to E1 (Uba1), ATP, and ubiquitin. Reaction aliquots taken at the times indicated were analyzed by Coomassie staining after SDS-PAGE. Ubiquitin and unanchored ubiquitin chains (Ub1, Ub2, etc.), Cdc34 autoubiquitination (Ubn-Cdc34), and E1 (Uba1) are indicated.

Deffenbaugh et al. (2003), that F72V functions equivalently to WT in the absence of SCF.

Cdc34 F72V Is Defective in SCF-Dependent Thioester Discharge

Relatively slow E3-independent ubiquitination is presumably based on stochastic collision between nucleophile (K48 of the attacking ubiquitin or a C-terminal lysine of Cdc34) and the thioester bond of Cdc34~Ub, resulting in isopeptide bond formation. We have previously developed an assay to directly examine a single round of ubiquitin discharge from Cdc34 by treating reactions with the alkylating agent N-ethylmaleimide (NEM) and EDTA after preincubation with E1, ubiquitin, and ATP (Petroski and Deshaies, 2005c). These studies demonstrated that SCF accelerates the loss of ubiquitin from Cdc34~Ub that requires lysine residues of SCF-bound Sic1 or K48 of ubiquitin.

First, we examined the charged state of WT or F72V in the absence or presence of NEM/EDTA treatment (Figure 5A). NEM/EDTA treatment of WT resulted in the almost-instantaneous loss of Cdc34~Ub upon SCF addition (estimated >90% loss after 1 min, compare lane 7 to lane 8). By comparison, thioester discharge from F72V occurred slowly (estimated >90% loss only after 30 min, compare lane 19 to lane 23). Moreover, SCF-dependent autoubiquitination of WT (using Cdc34 Δ C) was observed in the absence of NEM/EDTA treatment (lanes 4–6). However, we did not observe autoubiquitination of F72V in the absence of NEM/EDTA, even after 60 min (lane 18, compare to lane 6, also see Figure S3). Note that in the long exposures (bottom panels) a small fraction of WT Cdc34~Ub is refractory to discharge. We do not know the basis of this.

To quantify differences in Cdc34~Ub stability in the presence of SCF, we performed thioester chase experiments with Cdc34 charged with radiolabeled K48R ubiquitin. Whereas Cdc34~K48R Ub thioesters are stable, addition of unlabeled WT ubiquitin stimulates formation of K48-linked diubiquitin and concomitant loss of Cdc34~Ub (Petroski and Deshaies, 2005c). This reaction is accelerated 40-fold by SCF via an increase in the apparent V_{max} ; SCF has no effect on the apparent K_M for the attacking ubiquitin. These results led us to

conclude that SCF primes Cdc34~Ub for attack by ubiquitin, but SCF does not provide a binding site for the attacking ubiquitin. Whereas SCF potentially stimulated the loss of WT Cdc34~Ub and the appearance of diubiquitin (Petroski and Deshaies, 2005c and Figure 5B, left panel), there was no effect of SCF on F72V~Ub (Figure 5B, right panel and measured in Figure 5C). Taken together, these results suggest that F72V cannot be activated by SCF. Importantly, because the attacking ubiquitin is diffusing freely in solution, there should be no requirement for F72V to dissociate from SCF to sustain this reaction.

Cdc34 F72V Expression Delays Sic1 Turnover In Vivo

Although Cdc34 F72V is defective for SCF-dependent activation in vitro, it is unknown how this mutation affects Cdc34 function in vivo. To address this, we generated haploid yeast strains in which the chromosomal *CDC34* gene is deleted and the essential function of Cdc34 is provided on a *URA3* plasmid containing the *CDC34* open reading frame driven by the *CDC34* promoter. Various *HIS3* plasmids carrying different *CDC34* alleles were individually cotransformed into this strain, allowing for growth and viability to be assessed under selective conditions. Expression of Cdc34-Myc13 F72V, either with WT Cdc34 coexpression (–Ura or –His) or in its absence (5-FOA), supported growth identical to Cdc34-Myc13 (Figures 6A and 6B). Identical results were obtained upon depletion of Cdc34 expressed from a tetracycline-suppressible genomic copy of *CDC34* in a strain that coexpressed plasmid-encoded Cdc34-Myc13 F72V (data not shown).

We examined Sic1 turnover in strains containing either WT or F72V Cdc34-Myc13 as the only source of Cdc34 (Figure 6B, right panel). Sic1 is normally degraded in a Cdc34-dependent manner as cells progress from G1 phase of the cell cycle to S phase (Schwob et al., 1994). Whereas expression of WT resulted in the destruction of Sic1 within 45 min of release from G1, Sic1 degradation was delayed in *cdc34* F72V cells by 15–30 min (Figure 6C). Taken together, these observations suggest that the defect conferred by the F72V mutation results in a delay of substrate turnover but does not eliminate its essential function.

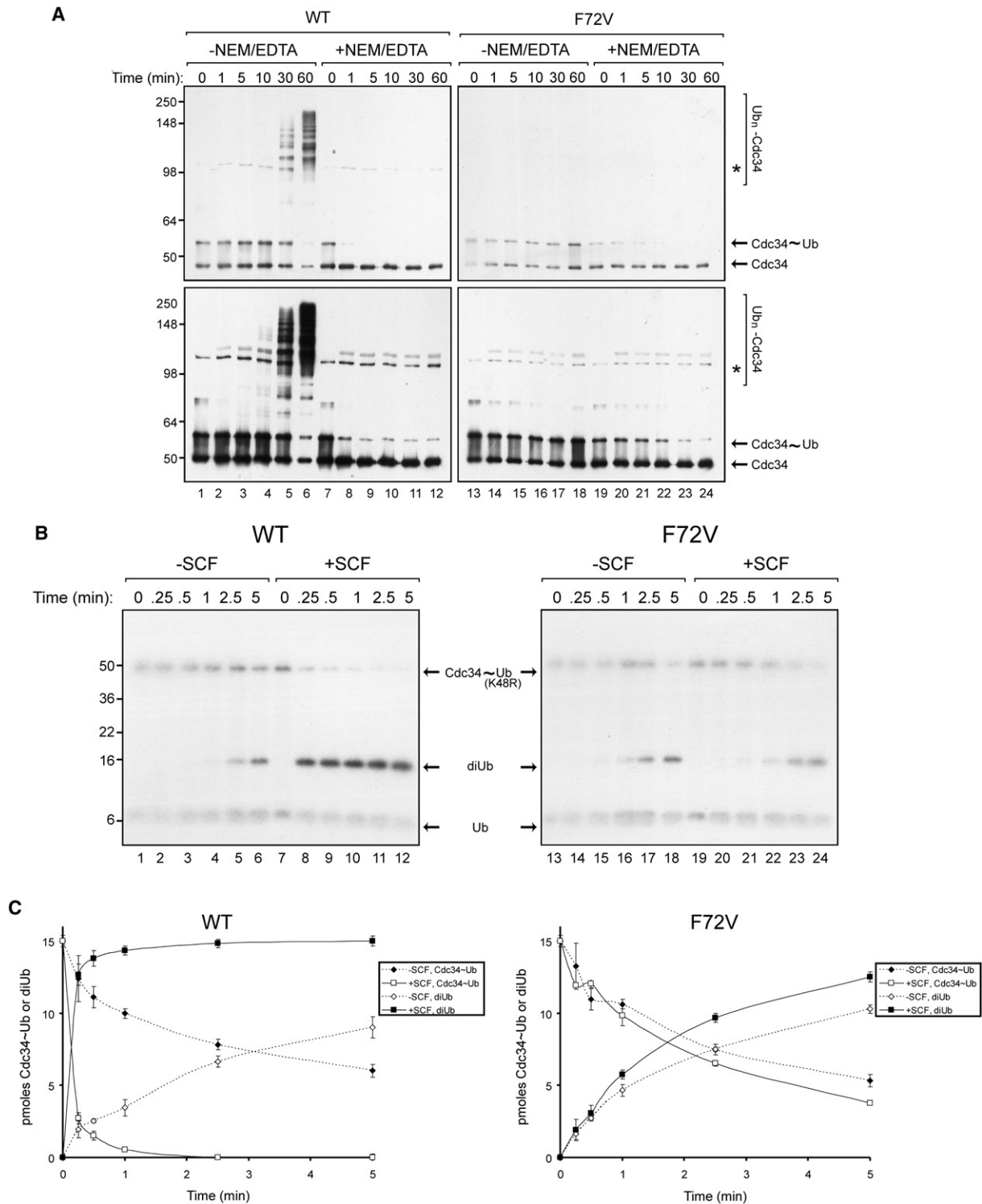


Figure 5. Cdc34 F72V Is Defective in SCF-Dependent Activation

(A) SCF-dependent autoubiquitination and thioester discharge assays were performed (WT, left panels and F72V, right panels). Cdc34 charged with ubiquitin was added to reactions containing SCF (-NEM/EDTA) or pretreated with NEM/EDTA before addition to SCF (+NEM/EDTA). Reactions were analyzed by western blotting with anti-Cdc34 antisera. The bottom panels are longer exposures of top panels.

(B) Diubiquitin synthesis assays were performed in which Cdc34 (WT, left panel and F72V, right panel) was charged with radiolabeled K48R ubiquitin and then treated with NEM/EDTA prior to addition to chase reactions containing 1 mM unlabeled ubiquitin in the presence or absence of SCF. At indicated times, reaction aliquots were removed and added to nonreducing sample buffer prior to analysis.

(C) The experiment shown in (B) was quantified for WT (left panel) or F72V (right panel) for the picomoles of Cdc34~Ub remaining and diubiquitin formed in the presence or absence of SCF. Data points represent the mean, with error bars representing the standard deviation ($n = 3$).

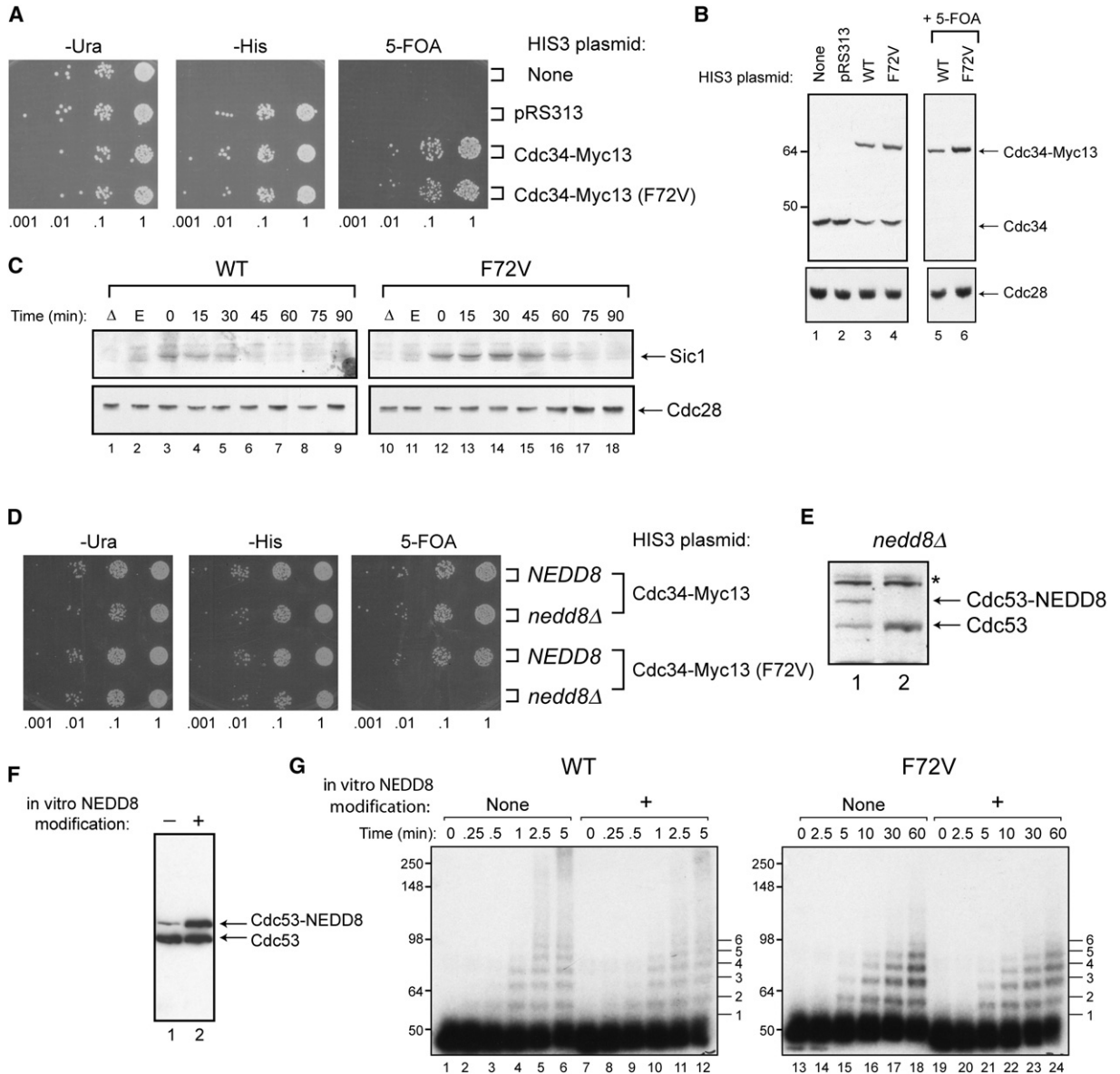


Figure 6. Cdc34 F72V Is Sufficient as the Only Source of Cdc34 In Vivo

(A) Haploid yeast strains with the genomic *CDC34* deleted and carrying *URA3* plasmids with WT *CDC34* plus *HIS3* plasmids with the indicated inserts were serially diluted onto synthetic media lacking uracil (–Ura) or histidine (–His) or containing 5-fluoroorotic acid (5-FOA). The presence of 5-FOA selects for loss of the *URA3* plasmid bearing WT *CDC34*.

(B) Lysates derived from the strains shown in (A) were immunoblotted with antisera against Cdc34. An immunoblot using anti-Cdc28 antisera was used to verify equivalent protein loading.

(C) Yeast strains expressing either WT or F72V Cdc34-Myc13 as the only source of Cdc34 were synchronized in G1 of the cell cycle. At the indicated times after release, cell extracts were prepared and analyzed by immunoblotting with anti-Sic1 antisera. “Δ” is lysate derived from yeast lacking Sic1, and “E” is exponentially growing yeast. An anti-Cdc28 immunoblot is shown as a loading control.

(D) Serial dilutions similar to (A) were performed in the indicated yeast strains that either contain (*NEDD8*) or have a deletion (*nedd8Δ*) of the *RUB1* gene, which encodes NEDD8 in *S. cerevisiae*.

(E) Immunoblot analysis with anti-Cdc53 antiserum of lysates derived from yeast strains from (D) that express NEDD8 (lane 1) or lack NEDD8 (lane 2). The “*” indicates a nonspecific band recognized by anti-Cdc53 antisera that was used to verify normalized loading.

(F) In vitro neddylation reactions with recombinant SCF complexes were performed in the presence of NEDD8 and the NEDD8 E1 and E2 enzymes. The reactions were analyzed by western blotting with anti-Cdc53 antiserum.

(G) Sic1 ubiquitination assays were performed with Cdc34 (WT, left panel or F72V, right panel) in the presence of SCF isolated from insect cells that has either been used as is (–NEDD8) or treated with a NEDD8 conjugation system (+NEDD8). In the presence of conjugating enzymes, the proportion of NEDD8-conjugated Cdc53 increases from ~5% to ~50% (see [F]). Reaction aliquots were analyzed by autoradiography for labeled Sic1. The number of ubiquitin modifications of Sic1 is indicated.

Cdc34 F72V Requires an Intact NEDD8-Conjugation Pathway for In Vivo Function

All cullins are modified by the ubiquitin-like protein NEDD8, and this modification promotes the activity of SCF-Cdc34 (Pan et al., 2004). It has been suggested that NEDD8 stimulates activity because it stabilizes the association of E2 and SCF (Kawakami et al., 2001). If the modest Sic1 turnover defect of F72V arises because F72V associates too tightly with SCF (Deffenbaugh et al., 2003), this phenotype may be suppressed by deletion of *RUB1*, which encodes NEDD8 in *S. cerevisiae*. Although NEDD8 is not essential in budding yeast, its deletion is synthetically lethal with temperature-sensitive mutations in other SCF pathway components (Lammer et al., 1998). As shown in Figure 6D, coexpression of Cdc34 with Cdc34-Myc13 F72V supported similar growth in the presence or absence of NEDD8. In contrast to the rationale underlying this experiment, however, cells sustained by Cdc34-Myc13 F72V required the presence of NEDD8 for viability.

Based on the synthetic lethality observed between *cdc34 F72V* and *nedd8 Δ* , the impairment of F72V observed in our various in vitro E3-dependent assays might be exacerbated because only a low proportion (~5%) of the Cdc53 (Cul1) in our SCF preparations purified from baculovirus-infected insect cells was neddylated (Figure 6F, lane 1). We increased Cdc53 neddylation ~10-fold by incubating SCF with NEDD8 and purified neddylation enzymes and compared the activity of these preparations in Sic1 ubiquitination reactions with either WT or F72V Cdc34 (Figure 6G). We did not detect an increase in F72V activity with increased amounts of Cdc53 neddylation.

A Diffusion-Based Mechanism of E2 Action Is Not Required to Enable Progressive Ubiquitin-Chain Extension

An attractive feature of hit and run is that it provides a simple explanation for how E2~Ub can “reach” the terminal-most ubiquitin as a substrate-linked ubiquitin chain elongates (Deffenbaugh et al., 2003). This problem becomes acute when one considers that, during polymerization of a hexa-ubiquitin chain, the position of the target lysine (i.e., K48 of the distal-most ubiquitin) can move up to 80 Å. The proposal that a diffusion-based mechanism enables ubiquitin-chain synthesis must rest on the assumption that the chain is rigid (Deffenbaugh et al., 2003). However, recent NMR data suggest significant interdomain motion in a K48-linked diubiquitin molecule (Ryabov and Fushman, 2006). This motion can be explained by rotations related to any K48 side-chain torsion angles and/or backbone dihedrals of ubiquitin’s gly-gly C terminus.

To address whether linkage flexibility may allow SCF to accommodate a ubiquitin chain, we used the crystal structure of the F box protein β -TrCP with bound β -catenin phosphopeptide (Wu et al., 2003) to generate a model of SCF with E2 docked on the RING domain. Our goal was to generate a K48-linked ubiquitin chain covalently attached to the β -catenin phosphopeptide and place the ϵ -NH₂ of the K48 residue of the distal-most ubiquitin within at least 2 Å of the E2 active-site cysteine without modifying the rigid SCF scaffold. This was accomplished by modest changes in dihedral an-

gles of the linker region between ubiquitin molecules, where flexibility is expected. Our effort revealed numerous potential conformations of the ubiquitin chain that can accommodate the spatial constraints posed by the SCF-E2 complex. Representative structures of SCF with a diubiquitin or heptaubiquitin chain positioned to accept additional ubiquitin from the docked E2 are shown in Figure 7. Similar manipulations to those shown allow SCF to accommodate a K48-linked ubiquitin chain of any length. We conclude that flexibility of ubiquitin chains makes it unnecessary to postulate a diffusion-based mechanism to sustain their polymerization.

Discussion

The hit-and-run model for protein ubiquitination proposes that release of Cdc34~Ub from SCF is an essential step preceding transfer of ubiquitin to substrate (Deffenbaugh et al., 2003). Our observations do not support a diffusion-based mechanism of ubiquitin transfer. Instead, we favor the hypothesis that SCF activates discharge of ubiquitin from Cdc34~Ub bound to SCF.

Relationship of SCF-Cdc34~Ub Dynamics to the Hit-and-Run Mechanism

The conception of the hit-and-run model rested on two observations. First, it was reported that ~10% of Cdc34 molecules that coprecipitate with SCF dissociate during a 90 min ubiquitination reaction, and this dissociation correlates with Sic1 ubiquitination (Figure 1A of Deffenbaugh et al.). However, it is evident that each of these dissociated Cdc34 molecules must have transferred multiple ubiquitins to Sic1 because inspection of their data reveals that the number of ubiquitins transferred to Sic1 exceeds the number of Cdc34 molecules that dissociated from SCF. The absence of a simple one-to-one relationship between Cdc34 dissociation and Sic1 modification does not support a simple interpretation. Competent Cdc34 molecules may simply be establishing a new equilibrium with SCF during the 90 min incubation. Moreover, spent Cdc34 needs to dissociate from SCF on a time scale of seconds—not tens of minutes—to sustain the high rates that have been observed for ubiquitination of SCF substrates in vitro (Petroski and Deshaies, 2005c) and their degradation in vivo (Chi et al., 2001).

The second pillar of hit and run was the observation that Cdc34~Ub has a 40-fold-lower affinity (290 nM) for SCF than uncharged Cdc34 (7 nM), largely due to a 10-fold-slower off rate for the latter (Deffenbaugh et al., 2003). Implicit in this is the idea that Cdc34~Ub must dissociate from SCF before it discharges its ubiquitin cargo onto substrate, because otherwise SCF would become clogged with slowly dissociating Cdc34. The marked difference in dissociation rates is unexpected given that enzymes normally have higher affinity for their substrates than products, lest their activity be limited by product inhibition. Indeed, Ubc2~Ub has an ~8-fold higher affinity for its ligase Ubr1/E3 α than the uncharged species (Siepmann et al., 2003). A direct prediction of this observation is that reactions in which SCF is preincubated with uncharged Cdc34 should proceed much more slowly than reactions initiated with preformed Cdc34~Ub, because in the former case the

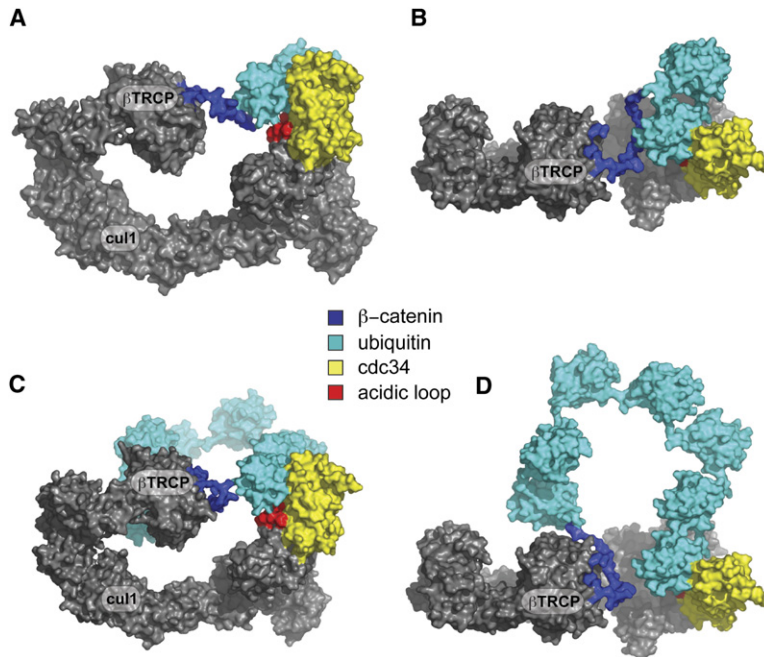


Figure 7. Molecular Models of SCF-Cdc34 with Ubiquitinated Substrate

The SCF complex (Hrt1, Cul1, Skp1, and β -TrCP) is gray, the β -catenin phosphopeptide substrate is dark blue, ubiquitin chains are cyan, and Cdc34 is yellow. The acidic loop on Cdc34, shown to be important for processive synthesis of K48-linked ubiquitin chains, is red. The models in (A) and (B) contain a diubiquitin chain. The models in (C) and (D) contain a ubiquitin chain of length seven. The models in (B) and (D) have been rotated 90° relative to (A) and (C), respectively. This figure was generated using PyMol (DeLano, 2002). See supplemental text.

uncharged Cdc34 would clog SCF, and ubiquitination of Sic1 bound to SCF could not proceed until uncharged Cdc34 dissociates. However, we found that, following a brief interval in which reactions initiated with pre-charged Cdc34 enjoyed a modest advantage (most likely attributable to the time it takes to charge Cdc34 with ubiquitin), there was little effect of precharging on the overall rate of conversion of Sic1 to ubiquitin-conjugated species. We conclude that the slowly dissociating Cdc34 observed by Deffenbaugh et al. must not be bound to a functionally relevant site of SCF. Thus, the slow dissociation they reported does not provide a compelling rationale for a hit-and-run mechanism.

Cdc34 F72V Cannot Be Activated by SCF

In addition to the correlative arguments discussed above, key validation of the hit-and-run hypothesis was provided by a *cis* mutation in Cdc34 (phenylalanine 72 to valine). The F72V mutation was proposed to inhibit release of Cdc34 ~ Ub from SCF, resulting in a “stalled” Sic1-SCF-Cdc34 ~ Ub complex. If this interpretation is correct, we reasoned that Cdc34 F72V should dominantly inhibit the activity of WT Cdc34 in vitro and in vivo. It should also not fulfill essential physiological roles of Cdc34. Neither prediction was fulfilled. A 5-fold molar excess of Cdc34 F72V over a rate-limiting amount of WT Cdc34 did not significantly impair the rate or extent of Sic1 ubiquitination (Figure 3A). Moreover, Cdc34 F72V did not inhibit the essential role of WT Cdc34 upon their coexpression in vivo (Figure 6A) and was sufficient to sustain cell proliferation (Figure 6C).

To identify the molecular defect of Cdc34 F72V, we examined SCF-independent and SCF-dependent functions of Cdc34. We did not detect differences between Cdc34 and Cdc34 F72V in ubiquitin charging by E1 or SCF-independent autoubiquitination (both results confirming data reported by Deffenbaugh et al. [2003]) and SCF-independent ubiquitin-chain synthesis, but all

SCF-dependent activities were compromised by the mutation. The delayed substrate ubiquitination we observed with this mutant (Figure 2) manifested as a failure of SCF to activate ubiquitin discharge from F72V Cdc34 ~ Ub (Figure 5A). Of particular note, SCF was unable to activate ubiquitin discharge from F72V Cdc34 ~ Ub onto ubiquitin to form diubiquitin (Figure 5B). Given the freely diffusible nature of the attacking ubiquitin, it is unlikely that a mutant whose primary defect is in SCF dissociation would be defective in this assay. These results are instead more consistent with a defect that specifically renders Cdc34 F72V insensitive to activation by SCF. The inability of F72V to compete with WT Cdc34 indicates that F72 is required for docking of Cdc34 to a site on SCF that mediates E2 activation.

It is striking that F72V is so strongly defective in vitro but is able to sustain life. Other compensatory mechanisms may exist in vivo to ameliorate the decreased activity of Cdc34 F72V, or its ability to assemble a competent targeting signal on its substrates may barely exceed a minimum threshold required for viability, rendering the system sensitive to other perturbations such as elimination of NEDD8.

Other Evidence against the Hit-and-Run Hypothesis

Several results in the literature strongly support the hypothesis that E2s transfer ubiquitin to substrate in the context of a ternary complex with E3. First, Wu et al. constructed a series of peptides in which the position of the sole modifiable lysine was moved relative to the phosphoserine that tethers the peptide to the F box subunit of the ubiquitin ligase SCF $^{\beta$ -TrCP. When the lysine was moved from ten residues upstream of the phosphoserine to six residues upstream, the rate of peptide ubiquitination decreased by >10-fold (Wu et al., 2003). Although this dramatic effect is readily understood in the context of classical enzyme action, it is difficult to understand how a diffusion-based mechanism would

be sensitive to moving the lysine acceptor site by such a small distance. Second, the observation that insertion of a flexible linker in Cul1 destroys SCF activity (Zheng et al., 2002) is easily understood from a traditional perspective that SCF holds its reactants in apposition, but is difficult to square with the idea that Cdc34~Ub randomly collides with substrate upon release from SCF. Third, SCF strongly activates transfer of ubiquitin from Cdc34~Ub to a freely diffusible substrate (Petroski and Deshaies, 2005c). This is most easily understood as SCF inducing in bound Cdc34~Ub a conformational change that favors transfer of ubiquitin. It is expected that Cdc34~Ub would return to the less-active ground state upon dissociation from SCF, and thus freely diffusing Cdc34~Ub should be relatively unreactive. Fourth, the existence of a stable complex that contains the SUMO E2 Ubc9 simultaneously bound to both SUMO-modified RanGAP1 substrate and RanBP2 E3 suggests that transfer of SUMO from Ubc9~SUMO to RanGAP1 occurs in the context of a ternary complex with the E3 (Reverter and Lima, 2005).

The Mechanism of Action of SCF-Cdc34 and Its Relationship to Structure

Taking into consideration our data as well as other published data discussed above, we favor the view that SCF is an enzyme that operates by a sequential bisubstrate mechanism, with Sic1 plus Cdc34~Ub being the two substrates and Sic1-Ub plus Cdc34 the products. We propose that, as for other bisubstrate enzymes, the ternary complex (Sic1-SCF-Cdc34~Ub) is in equilibrium with the reactants (free Sic1 and Cdc34~Ub), and dissociation of either reactant limits the rate of the forward reaction. Ultimately, the rate of reaction is determined by the relative rate at which reactants combine on the enzyme surface to yield products less the rate at which they dissociate from the enzyme.

Although we argue that Cdc34~Ub transfers its cargo to substrate within the context of the substrate-SCF-Cdc34~Ub ternary complex, we note two caveats. First, dissociation of spent Cdc34 must occur to allow subsequent cycles of ubiquitin transfer. This is because the E1-binding site on E2 overlaps with the E3-binding site (Huang et al., 2005). Thus, it is presumed that E2 would need to dissociate from E3 so that it can be recharged with ubiquitin, and in fact this has been shown in an elegantly conceived experiment (Eletr et al., 2005). However, unlike the sequence proposed in hit and run, we predict that during a normal reaction cycle Cdc34 dissociates from SCF *after* it transfers ubiquitin onto bound substrate, not *before*. Second, it is impossible to exclude the possibility that ubiquitin transfer by a hit-and-run mechanism never occurs. Due to the normal dynamics of the system, Cdc34~Ub will sometimes dissociate before discharging ubiquitin, and a small fraction of these dissociated molecules may collide productively with SCF-bound substrate and transfer ubiquitin. However, we consider it likely that these events, if they occur, would account for only a minor fraction of the Cdc34~Ub molecules fluxing through SCF.

Studies on the mechanism of ubiquitin transfer have not yet been able to reconcile the chemistry of ubiquitination with structures of RING ligases containing bound or computationally docked E2s. Whereas the chemistry

of ubiquitin transfer requires that the nucleophile (lysine of the substrate or ubiquitin) be in close proximity to the thioester bond linking ubiquitin to the active-site cysteine of the E2, all RING E3 structures suggest a large gap between the reactants. A major question is how is this gap bridged during catalysis? One possibility is that large conformational changes occur in SCF during catalysis. Whereas this may be the case for HECT domain ligases (Verdecia et al., 2003), it seems unlikely for SCF as there are no obvious hinge regions or rotatable domains evident in the crystal structure (Zheng et al., 2002). Moreover, rigidity of the Cul1 scaffold appears to be required, as insertion of a flexible linker totally abrogates activity.

The diffusion-based hit-and-run model was seen as a solution to the conundrum posed by the ~50 Å gap between the substrate-binding site and thioester bond of E2 docked onto SCF *in silico*. While there may be rare instances in which the initial ubiquitination of substrate is mediated by dissociated Cdc34~Ub, there are other ways in which this gap may be spanned. First, it is possible that in natural substrates the lysine residue or residues that are ubiquitinated are typically situated far enough from the ligase-binding motif that conformational dynamics of the polypeptide chain allow the target lysine to sample the region in vicinity of SCF-bound Cdc34~Ub. Second, emerging evidence suggests that protein oligomerization may be a key aspect of understanding how ubiquitin transfer occurs. For example, yeast Cdc34 and human UbcH5c self-associate when charged with ubiquitin (Brzovic et al., 2006; Varelas et al., 2003). Furthermore, a related ligase—the APC—has recently been shown to multimerize (Passmore et al., 2005) and F box proteins (the SCF substrate receptor) can oligomerize (Seibert et al., 2002; Suzuki et al., 2000; Wolf et al., 1999). Such multimerization could bring substrate and E2~Ub into closer proximity. It is worth noting that a region of Cdc4 (immediately upstream of the F box) that may mediate multimerization of SCF^{Cdc4} (C. Correll and R.J.D., unpublished data) was deleted from the proteins used to determine three-dimensional structure of Cdc4 and β-TrCP.

A second structural challenge that was suggested to be resolved by the hit-and-run hypothesis is how ubiquitin chains of varying lengths can be accommodated within the same ~50 Å gap referred to above. Structural data (Phillips et al., 2001; Ryabov and Fushman, 2006) indicate that ubiquitin chains are not rigid rods but in fact are flexible, and thus it should be feasible to spool a chain outwards as it grows, such that the proximal (attached to substrate) and distal (attacks Cdc34~Ub) ends can occupy and repetitively sample fixed locations, respectively. Indeed, our molecular modeling based on structural data for SCF and ubiquitin chains supports this contention (Figure 7).

Experimental Procedures

Protein Expression and Purification *Cdc34*

Except where noted, experiments utilized yeast Cdc34ΔC-His6 (Seol et al., 1999), which lacks 25 C-terminal amino acids that are SCF-independent sites of ubiquitination. Point mutations in the Cdc34 open reading frame were generated by standard mutagenesis protocols.

Full-length untagged Cdc34 (Figure 4B) was expressed in *E. coli* and purified by DEAE and monoQ chromatography.

Ubiquitin and Derivatives

Ubiquitin, K0 ubiquitin, and NEDD8 purchased from Boston Biochem were resuspended in water. Radiolabeled K48R ubiquitin was prepared as described (Petroski and Deshaies, 2005c).

Ubiquitination and Neddylation Reaction Components

Yeast E1, SCF^{Cdc4}, and the various Sic1 substrates were prepared as described (Petroski and Deshaies, 2003, 2005b). The NEDD8 E1 (Ula1-His6/Uba3) and E2 (His6-Ubc12) were expressed and prepared as described (Petroski and Deshaies, 2005c).

Ubiquitination Assays

Sic1 Ubiquitination

Sic1 was prephosphorylated by G1-CDK complex in the presence of [γ -³²P]ATP and used at 0.6 μ M per ubiquitination reaction. Reactions (20 μ l) were performed at room temperature in 30 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 100 mM NaCl, 1 mM DTT, and 2 mM ATP with 150 nM E1, 100 nM SCF, 800 nM Cdc34 proteins, and 77.5 μ M ubiquitin or 38.8 μ M K0 ubiquitin. For E2 competition experiments (Figure 3), competitors were premixed with SCF/Sic1 prior to addition of Cdc34, E1, and ubiquitin.

E3-Independent Ubiquitin-Chain Synthesis

Full-length, untagged Cdc34 (WT or F72V, 10 μ M) was incubated at room temperature with 150 nM E1 and 500 μ M ubiquitin in 30 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM DTT, and 2 mM ATP with 1 \times ATP regenerating system (Verma et al., 2000) and analyzed by SDS-PAGE/Coomassie staining.

Thioester Formation and Discharge Assays

To analyze thioester formation, Cdc34 (1.6 μ M), Uba1 (150 nM), ubiquitin (77.5 μ M) or ³²P K48R ubiquitin (16 μ M), and ATP (2 mM) in 30 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, and 100 mM NaCl was incubated for 15 min at room temperature and subsequently added to an equal volume of nonreducing sample buffer (with 8 M urea). Samples were analyzed by SDS-PAGE and immunoblotting using anti-Cdc34 antibodies (Petroski and Deshaies, 2005c).

For thioester discharge experiments (Petroski and Deshaies, 2005c), Cdc34 charged with ubiquitin was treated with 10 mM NEM and 2.5 mM EDTA for 15 min at room temperature. Cdc34 was diluted 1:2 into chase mixes that contained a final concentration of 30 mM Tris HCl (pH 7.6), 100 mM NaCl, 5 mM MgCl₂, and 1 mM DTT in the presence or absence of SCF (100 nM) and ubiquitin (500 μ M). Samples were added to nonreducing sample buffer and analyzed by SDS-PAGE followed by immunoblotting (unlabeled ubiquitin) or autoradiography (³²P-labeled ubiquitin).

Yeast Strains

The heterozygous diploid strain YSC1021-669597 (derived from BY4743) containing a single copy of *CDC34* (*CDC34/cdc34 Δ*) and the haploid *sic1 Δ* strain (YSC1021-551353) were from Open Biosystems. After transformation with a *URA3* plasmid containing the *CDC34* open reading frame driven by the *CDC34* promoter, YSC1021-669597 was sporulated and tetrads were dissected. A haploid strain that contained the *CDC34* plasmid as the only source of Cdc34 was identified by replica plating on 5-fluoroorotic acid (5-FOA) medium. Various *CDC34* mutants carried on *HIS3* plasmids were transformed into this strain. Plasmid and strain descriptions are shown in Tables S1 and S2.

Stability of Sic1 In Vivo

Yeast strains were grown to 0.5 OD₆₀₀ in 1% yeast extract, 2% peptone, and 2% dextrose (YPD) medium at 30°C. Cells were arrested in G1 phase with α factor (20 μ g/ml) for 3 hr at 25°C and released by washing with fresh YPD. Samples were taken at indicated times postrelease. Cell lysates were analyzed by western blotting with anti-Sic1 and anti-Cdc28 antiserum.

Supplemental Data

Supplemental Data include four figures, two tables, Supplemental References, and supplemental text and can be found with this article online at <http://www.molecule.org/cgi/content/full/24/4/523/DC1/>.

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