

# Mechanism of Lysine 48-Linked Ubiquitin-Chain Synthesis by the Cullin-RING Ubiquitin-Ligase Complex SCF-Cdc34

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## SUMMARY

Ubiquitin chains linked via lysine 48 (K48) of ubiquitin mediate recognition of ubiquitinated proteins by the proteasome. However, the mechanisms underlying polymerization of this targeting signal on a substrate are unknown. Here we dissect this process using the cyclin-dependent kinase inhibitor Sic1 and its ubiquitination by the cullin-RING ubiquitin ligase SCF<sup>Cdc4</sup> and the ubiquitin-conjugating enzyme Cdc34. We show that Sic1 ubiquitination can be separated into two steps: attachment of the first ubiquitin, which is rate limiting, followed by rapid elongation of a K48-linked ubiquitin chain. Mutation of an acidic loop conserved among Cdc34 orthologs has no effect on attachment of the first ubiquitin onto Sic1 but compromises the processivity and linkage specificity of ubiquitin-chain synthesis. We propose that the acidic loop favorably positions K48 of a substrate-linked ubiquitin to attack SCF bound Cdc34~ubiquitin thioester and thereby enables processive synthesis of K48-linked ubiquitin chains by SCF-Cdc34.

## INTRODUCTION

Regulation of protein stability through the ubiquitin proteasome system (UPS) has emerged as a key mechanism that underlies many cellular and organismal processes. While the general paradigm of an enzymatic cascade involving a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) to catalyze the synthesis of ubiquitin chains onto a targeted protein has been

well established, only in the past several years have specific pathways with well-defined protein players been elucidated (reviewed in Pickart, 2004).

Two major types of E3s exist in eukaryotes, defined by the presence of either a HECT-domain or RING fold. The RING superfamily includes proteins with a similar fold that either bind zinc (RING-H2, RING-HC, RING-v, RING-D, RING-G, RING-S/T, RING-C2, PHD) or do not (U box). In contrast with HECT-domain enzymes, RING E3s do not form a covalent intermediate with ubiquitin but may instead activate the E2 to directly discharge ubiquitin thioesterified to its active-site cysteine onto the lysine of a substrate (Seol et al., 1999). Our poor understanding of how RING ligases work is cast in sharp relief by the sheer predominance of this class of ligase enzymes. RING proteins are encoded in all eukaryotic genomes analyzed to date, and the mouse genome alone encodes approximately 385 proteins with this fold (Semple, 2003). Although some of these proteins may not be ubiquitin ligases, most RING proteins studied so far appear to have ubiquitin-ligase activity in vitro.

The most intensively studied subclass of RING E3s are those of the cullin-RING ligase (CRL) superfamily (reviewed in Petroski and Deshaies, 2005). The highly conserved enzymatic core of CRLs comprises the C-terminal region of a cullin protein and one of two closely related RING proteins (Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999; Tan et al., 1999). The RING protein bound to the C-terminal domain of a cullin recruits the E2, whereas the N-terminal regions of cullins recruit receptors that in turn interact with specific substrates. To date, six different types of CRLs have been identified, each of which employs a distinct family of substrate receptors. Cul1 and Cul7 CRLs assemble with substrate receptors that contain an F box, whereas Cul2 and Cul5 recruit BC box proteins and Cul3 recruits BTB domain proteins. The human genome encodes ~80 F box, ~200 BTB domain, and ~40 SOCS/BC box proteins, suggesting that there may be 300 to 350 CRLs in addition to the ~400 potential ubiquitin ligases based on other RING-fold proteins—which would make this the largest class of enzymes in all of eukaryotic biology. However, the mechanism by which any CRL or RING protein promotes substrate ubiquitination remains unknown.

SCF, the prototypical CRL, consists of Skp1, Cul1 (known also as Cdc53 in budding yeast), an F box protein, and the RING protein. The minimal enzymatic core of SCF contains the C-terminal cullin homology domain of Cul1 and the RING protein, which serves as an E2 docking site. Skp1 binding to the N-terminal region of Cul1 connects this enzymatic core to the variable F box protein that recognizes substrates. For the vast majority of known cases, substrates are recruited to SCF by a posttranslational modification such as phosphorylation.

To analyze SCF-mediated protein ubiquitination, we have employed an *in vitro* system that faithfully recapitulates ubiquitination of the budding-yeast cyclin-dependent kinase (CDK) inhibitor Sic1 by SCF<sup>Cdc4</sup> and the E2 Cdc34 (Ubc3). At the G1-to-S transition of the cell cycle, multisite phosphorylation of Sic1 by G1 CDK results in its recognition by the F box protein Cdc4 (Nash et al., 2001; Verma et al., 1997). Through the activity of Cdc34, SCF<sup>Cdc4</sup> bound Sic1 is ubiquitinated. Next, it is rapidly degraded by the 26S proteasome, resulting in release of active S phase CDK and entry into S phase (Verma et al., 1997, 2001). These steps can all be recapitulated *in vitro* with highly purified components, allowing for detailed analyses of this pathway.

Despite the progress that has been made in biochemical reconstitution and structural analysis of SCF-E2 complexes, relatively little is known about how they actually work. Early reconstitution studies indicated that the Cul1-RING “catalytic core” of SCF activates ubiquitin discharge from Cdc34, but the underlying mechanism was not determined (Seol et al., 1999; Skowrya et al., 1999). Subsequent crystal structures of SCF<sup>Skp2</sup> (Zheng et al., 2002) as well as an F box-substrate complex ( $\beta$ -TRCP- $\beta$ -catenin peptide) (Wu et al., 2003) did not unveil the mechanism of ubiquitination but revealed a puzzling 50 Å gap between the bound substrate and the putative location of the ubiquitin thioester on an *in silico*-docked E2 enzyme. Activated Cdc34~ubiquitin thioesters may dissociate from SCF and diffuse across the gap toward the bound substrate (Deffenbaugh et al., 2003), but this proposed “hit-and-run” mechanism is controversial (Petroski and Deshaies, 2005). In this work, we set out to address a key unresolved question that lies at the heart of SCF action: what features of the SCF-Cdc34 ubiquitin-ligase complex enable the processive synthesis of a ubiquitin chain that is competent to target the substrate to the UPS for degradation? A chain of at least four ubiquitin molecules linked through isopeptide bonds that join lysine 48 (K48) of one ubiquitin to the C terminus of the next underlies the recognition and degradation of proteins by the 26S proteasome (Thrower et al., 2000). Although K48-linked ubiquitin chains are fundamental to the operation of the ubiquitin proteasome system, the mechanisms that underlie the synthesis of such chains remain unknown.

## RESULTS

### SCF Accelerates the Discharge of Ubiquitin from Cdc34

Assembly of a K48-linked ubiquitin chain on a substrate comprises multiple subreactions. To gain insight into this

process, we sought to strip it down to the simplest possible reaction—the discharge of a ubiquitin thioester (~Ub) from the active site of Cdc34 that accompanies formation of a ubiquitin conjugate. We devised an assay that allowed us to monitor a single round of discharge of ubiquitin from Cdc34 to eliminate confounding effects of the ubiquitin activating and charging steps. By first charging Cdc34 with ubiquitin in the presence of E1 and ATP and then treating the reactions with the alkylating agent N-ethylmaleimide (NEM) and EDTA, subsequent Cdc34 recharging was blocked (data not shown). Cdc34~Ub was then incubated under different reaction conditions, and we evaluated the loss of Cdc34~Ub by SDS-PAGE/Western blot analysis of Cdc34 under nonreducing conditions. We refer to these assays as “single discharge” because only one ubiquitin is turned over per Cdc34.

We first examined the effect of SCF (Figure 1A). Cdc34~Ub thioesters decayed rapidly in the presence of SCF (majority lost after <30 s) but were at least 5-fold more stable in the absence of SCF (comparable loss at >2.5 min). The activity of SCF in this assay was similar regardless of whether the Cdc53 subunit was conjugated with Nedd8 or not (see Figures S1B and S1C in the Supplemental Data available with this article online). Rapid SCF-dependent discharge required the lysines of ubiquitin because thioesters generated with a lysine-less mutant (K0 Ub) were slowly discharged even in the presence of SCF (right panel). This was a key result as it suggested that the major event monitored by our assay system was the attack of a Cdc34~Ub thioester by a lysine on a second ubiquitin molecule. The stability of Cdc34~K0 Ub, even in the presence of SCF, allowed us to systematically test the ability of different molecules to attack the thioester.

### Lysine Residues of the SCF Bound Substrate or Lysine 48 of Ubiquitin Are Required for Rapid Discharge of Ubiquitin from Cdc34~Ub

As our reactions in Figure 1A did not contain an SCF substrate, we first sought to determine the effect of adding Sic1. Phosphorylated Sic1 (phospho-Sic1) facilitated the rapid discharge of Cdc34~K0 Ub thioesters in an SCF-dependent manner (Figure 1B, top panel). We had previously developed a mutant Sic1 (Sic1 K0) that cannot be ubiquitinated by SCF because it lacks lysines (Petroski and Deshaies, 2003). SCF loaded with phospho-Sic1 K0 did not potentially accelerate ubiquitin discharge from Cdc34 (Figure 1B, bottom panel, lanes 7–12; compare with lanes 19–24).

Since ubiquitin's lysines were required for rapid SCF-dependent turnover of Cdc34~Ub in the absence of added Sic1 substrate, we sought to address whether our assay recapitulates the known specificity of SCF-Cdc34 for synthesizing ubiquitin chains linked through degradation-competent K48 linkages (Feldman et al., 1997). Cdc34~K0 Ub was evaluated in single-discharge reactions supplemented with “empty” SCF (i.e., without bound substrate) plus either ubiquitin containing only lysine 48 (K48 Ub) or ubiquitin containing lysine 48 mutated to arginine (K48R Ub). Whereas K48 Ub promoted rapid discharge of K0 Ub from Cdc34,

K48R Ub did not (Figure 1C), even though it retains six lysines. Taken together, these results suggested that SCF activates the discharge of ubiquitin from Cdc34~Ub to either lysines of a bound substrate (Sic1) or the K48 residue of a free ubiquitin molecule.

### SCF Increases the $V_{\max}$ of Diubiquitin Synthesis

SCF could promote the turnover of Cdc34~Ub thioesters by increasing the affinity of Cdc34~Ub for the attacking nucleophile or by increasing the rate at which a bound nucleophile attacks the thioester to form an isopeptide bond. Although our immunoblotting assay provided important insights into the factors that promote discharge of ubiquitin from Cdc34~Ub, we now sought a more readily quantifiable assay to address the mechanism of SCF<sup>Cdc4</sup> action. Accordingly, we performed single-discharge experiments in which Cdc34 charged with radiolabeled K48R Ub was incubated with excess unlabeled wild-type “acceptor” ubiquitin ± SCF. As shown in Figure 1D and quantified in Figure 1E, SCF promoted the loss of labeled Cdc34~K48R Ub and concomitant appearance of diubiquitin. As expected, radiolabeled Cdc34~K48R Ub was sensitive to reducing agent (bottom panel), whereas radiolabeled diubiquitin was not.

We employed this assay to measure the initial rate of diubiquitin formation at different concentrations of cold ubiquitin acceptor. Plots of initial rate versus acceptor ubiquitin concentration (Figure 1F) revealed three important points. First, the estimated  $K_M$  for acceptor ubiquitin was extremely high, ~600  $\mu$ M. This has important implications, which will be considered in the Discussion. Second, the estimated  $K_M$  for ubiquitin was similar in the presence (600  $\mu$ M) or absence (625  $\mu$ M) of SCF, whereas the  $V_{\max}$  was at least 40-fold greater in the presence of 100 nM SCF (2.6 versus 0.06 pmol/s). Because SCF primarily affects  $V_{\max}$ , we infer that a constitutive binding site for acceptor ubiquitin resides on Cdc34~K48R Ub, and SCF catalyzes attack of thioesterified ubiquitin by noncovalently bound ubiquitin, yielding an isopeptide bond between the two molecules. Third, given the  $V_{\max}$  (2.6 pmol diubiquitin/s) and the relative amounts of Cdc34 (16 pmol) and SCF (2 pmol), it follows that the entire reaction cycle of Cdc34~Ub binding to SCF, isopeptide-bond formation, and dissociation of discharged Cdc34 from SCF occur with a  $t_{1/2}$  of ~0.5 s.

### Specific Residues in Ubiquitin and Cdc34 Are Required to form K48-Linked Diubiquitin

To build on the insights that emerged from kinetic analysis of the single-discharge assay, we sought to define residues in Cdc34 and ubiquitin that contribute to the formation of a ubiquitin chain. Because our chase assay monitors discharge of ubiquitin from Cdc34~Ub, we reasoned that we could identify residues of ubiquitin that are important for diubiquitin synthesis, regardless of whether they influence the rate of charging by E1 or E2.

Sixteen surface residues of ubiquitin are essential for vegetative growth of yeast (Sloper-Mould et al., 2001). We generated and assayed a series of ubiquitin derivatives mutated for these essential residues (Figure S2A). Whereas most of the mutant ubiquitin readily attacked Cdc34~K48R Ub thio-

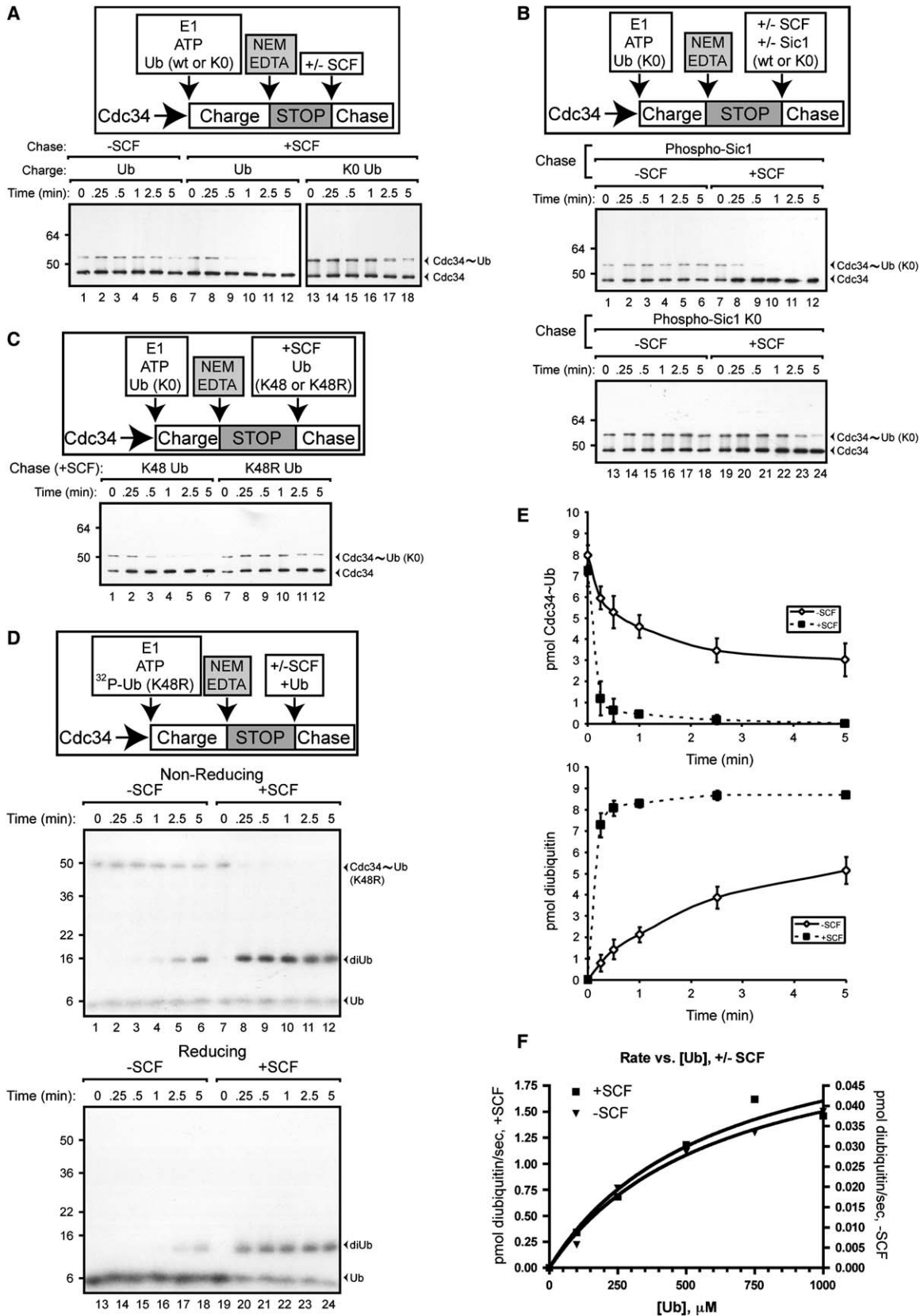
esters in the presence of SCF to form diubiquitin, several mutants were unable to efficiently support this process (Figure 2A). Besides K48, the C-terminal tail of ubiquitin and two residues in the hydrophobic patch adjacent to K48—144 and G47—had an important role in diubiquitin synthesis. By contrast, of the essential residues implicated in endocytosis (T12, F4, and Q2), only T12 contributed significantly to diubiquitin synthesis. Finally, Nedd8, which has K48 and is 80% homologous to ubiquitin, failed to accept ubiquitin from Cdc34~K48R Ub (Figures S2B and S2C).

To further address the mechanism and specificity of ubiquitin-chain synthesis by SCF-Cdc34, we next sought to identify features of Cdc34 that are required for diubiquitin production. Evaluation of various Cdc34 mutants focused our attention on an acidic loop (amino acids 103 to 114) that, among *S. cerevisiae* E2s, is unique to Cdc34 and Ubc7 and is located near the catalytic cysteine. A mutant in which the acidic residues are converted to alanine does not complement *S. cerevisiae* *cdc34* $\Delta$  unless it is vastly overexpressed from the *GAL1* promoter (Liu et al., 1995; Pitluk et al., 1995). However, the role of the acidic loop in Cdc34 function is unknown.

We generated mutants of Cdc34 containing deletions of the entire loop (Cdc34  $\Delta$ 103–114) or a portion of it (Cdc34  $\Delta$ 108–114) as well as mutants with acidic residues changed to alanine (Cdc34 1D 2E: E109A, D111A, E113A and Cdc34 3D 2E: D104A, D108A, E109A, D111A, E113A). These mutant proteins were charged with K48R Ub and tested in our thioester chase assay in the presence of SCF and ubiquitin to determine if they could sustain the formation of diubiquitin. As shown in Figure 2B and quantified in Figure 2C, all of the Cdc34 proteins containing alterations within this loop region were severely impaired in the synthesis of diubiquitin, even though they were equivalently charged with [<sup>32</sup>P]ubiquitin (wt initial rate of 0.8 pmol diubiquitin/s; mutants were all less than 0.08 pmol/s, with 1 mM ubiquitin in the chase mix). As shown below, we provide strong evidence that the defect of these mutants was very specific and not due to a general loss of function.

### The Rate of Sic1 Ubiquitination by Cdc34-SCF Is Independent of the Number of Ubiquitin-Accepting Sites

Our analysis of diubiquitin synthesis suggested that specific, acidic-loop-dependent catalysis of isopeptide-bond formation might underlie the SCF-dependent synthesis of a ubiquitin chain upon substrate. However, to establish that the acidic loop has a physiological role in ubiquitin-chain synthesis, it was essential to evaluate ubiquitination of an authentic SCF substrate. To better define the reaction under study, we employed in addition to wild-type Sic1 a mutant of Sic1 that contains only the six N-terminal lysines that are the physiological targets of ubiquitination in vivo (KOC) or a Sic1 mutant that contains a single ubiquitin-accepting lysine residue (K32 only) that is sufficient to sustain turnover in vivo (Petroski and Deshaies, 2003). The significance of the K32 Sic1 substrate is that it immediately distinguishes between the synthesis of a ubiquitin chain versus monoubiquitination of multiple lysine residues of the substrate. This is a relevant issue because, in



some reconstitution systems, multiple monoubiquitination events predominate over chain synthesis (Carroll and Morgan, 2002).

As shown in Figure 3, both K0C and K32 Sic1 were converted to ubiquitinated forms with similar kinetics (0.17 to 0.2 pmol/s), regardless of whether the reaction was carried out with wild-type Ub (Figures 3A and 3B) or K0 Ub (Figures 3C and 3D). Similar rates of ubiquitination were obtained for other “single-lysine” Sic1 substrates (data not shown). As expected, K32 Sic1 was modified by only a single K0 Ub attachment, whereas K0C Sic1 was modified with up to six K0 Ub molecules (Figure 3C). Importantly, at every single time point, the pattern of conjugates on K0C Sic1 obtained with ubiquitin (Figure 3A) differed greatly from that observed with K0 Ub (Figure 3C). This suggests that the primary reaction products were Sic1 molecules bearing a ubiquitin chain, as opposed to Sic1 ubiquitinated on multiple lysine residues. This result implies that SCF-Cdc34 preferred to polymerize a ubiquitin chain rather than to conjugate single ubiquitin molecules onto multiple lysine residues in Sic1.

#### The Attachment of the First Ubiquitin Is Rate Limiting

To test directly the implication that the first ubiquitin attachment to Sic1 is rate limiting, we generated Sic1 containing a single ubiquitin molecule conjugated to lysine 36 (Sic1-Ub1; see Experimental Procedures and Figure S3) and compared its rate of ubiquitination to unmodified Sic1 generated under identical conditions (Figure 4A). At 800 nM Cdc34, Sic1-Ub1 was ubiquitinated 4-fold faster than unmodified Sic1 (Figure 4B; Sic1-Ub1 conversion is 0.4 units/min, while Sic1 is 0.1 units/min). This rate difference was enhanced at low (80 nM) E2 concentrations (0.25 units/min for Sic1-Ub1, 0.03 units/min for Sic1). These results provide direct evidence that the first attachment of ubiquitin onto Sic1 is slower than the rate of subsequent ubiquitin-ubiquitin attachments.

Why does attachment of a single ubiquitin to Sic1 accelerate the rate of subsequent ubiquitin attachments? Attachment of ubiquitin to Sic1 may simply increase the relative proximity of acceptor sites for Cdc34~Ub because ubiquitin may partially span the proposed ~50 Å gap between the

SCF bound substrate and Cdc34's catalytic cysteine (Zheng et al., 2002). Alternatively, Cdc34 may have an intrinsic (but not absolute) preference for lysine 48 of ubiquitin (as demonstrated in Figures 1 and 2). To test the latter possibility, we prepared Sic1-Ub1 lacking a free amino group at amino acid 48 of ubiquitin and compared its rate of ubiquitination to Sic1. Strikingly, the rate enhancement obtained by conjugating a single ubiquitin onto Sic1 (Figure 4A) was vitiated by selective modification of lysine 48 (Figure 4C; 0.09 units/min for both Sic1 and Sic1-Ub1). Whereas this result by itself does not address the potential contribution of increased proximity, it does establish that there is something special about lysine 48.

#### The Acidic Loop of Cdc34 Is Required for Processive Synthesis of Lysine 48-Linked Ubiquitin Chains on Sic1

Taken together, the results presented so far suggest that the transfer of ubiquitin from Cdc34~Ub to SCF<sup>Cdc34</sup> bound Sic1 is the rate-limiting step in substrate ubiquitination. Once ubiquitin is attached to Sic1, transfer of subsequent ubiquitin to form a K48-linked chain occurs at a more rapid rate because SCF-Cdc34~Ub prefers to transfer its Ub to lysine 48 of a substrate-conjugated ubiquitin. The diubiquitin synthesis assays suggested that the acidic loop of Cdc34 might be required for rapid polymerization of a ubiquitin chain upon substrate. We now sought to test this possibility directly. Paradoxically, whereas the loop mutants were defective in the thioester chase assay (Figure 2B), they supported rapid ubiquitination of K32 Sic1 by K0 Ub (Figure 5A). In fact, loop mutants were consistently more active than wt Cdc34 in attaching ubiquitin onto Sic1. This is a key result because it indicates that the diubiquitin synthesis defect of the loop mutants (shown in Figures 2B and 2C) was very specific.

We next evaluated the pattern of conjugate formation on Sic1 using wt (Figure 5B) or K48-only ubiquitin (Figure 5C). Whereas the overall rate of conversion of unmodified Sic1 to an ubiquitinated form was essentially identical when wt Cdc34 and various loop mutants were compared, the pattern of ubiquitin conjugation was strikingly different. The product profile seen with each loop mutant was suggestive

#### Figure 1. SCF Accelerates the Discharge of Ubiquitin from Cdc34 onto Lysine Residues of Sic1 or Ubiquitin Itself

(A) Cdc34 was charged with ubiquitin (Ub) or lysine-less ubiquitin (K0 Ub) in the presence of E1 and ATP, treated with NEM/EDTA to prevent subsequent rounds of Cdc34 recharging, and added to “chase” reactions conducted in either the presence (+SCF) or absence (–SCF) of SCF. At the indicated times, reaction aliquots were removed and added to nonreducing sample buffer prior to analysis by SDS-PAGE and Western blotting with anti-Cdc34 antisera. Uncharged Cdc34 (Cdc34) and Cdc34 thioesterified (~) with Ub or K0 Ub are indicated.

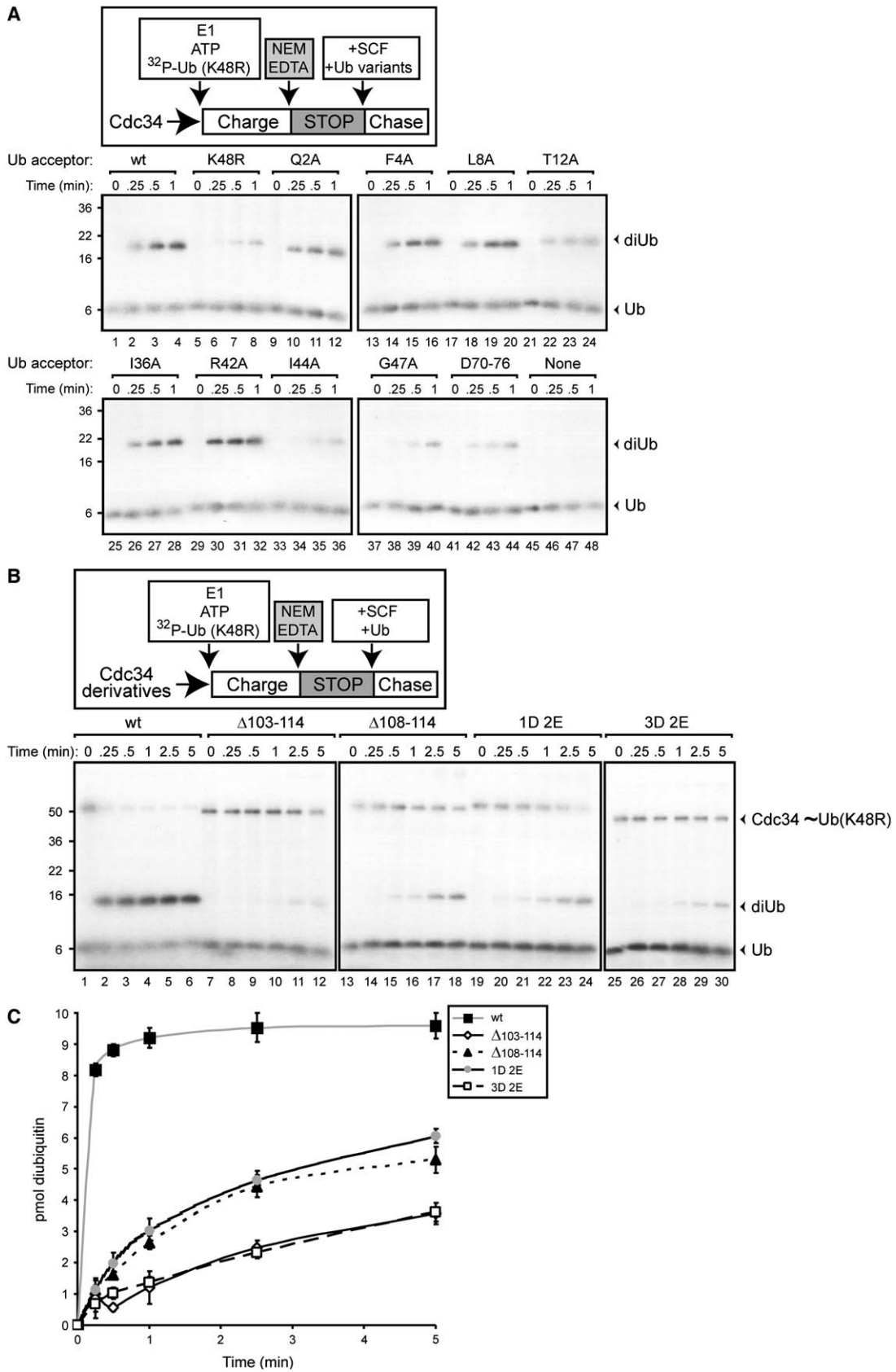
(B) Same as (A), except that Cdc34 was charged with K0 Ub and added to reactions containing 2.5 μM of either phosphorylated Sic1 (Phospho-Sic1) or phosphorylated Sic1 with all lysine residues mutated to arginine (Phospho-Sic1 K0) in the absence or presence of SCF.

(C) Same as (A), except that Cdc34 thioesterified with K0 ubiquitin was chased in the presence of SCF and either ubiquitin containing only lysine 48 with all other lysine residues mutated to arginine (K48 Ub) or ubiquitin containing lysine 48 mutated to arginine (K48R ubiquitin), both at 500 μM.

(D) Same as (A), except that Cdc34 was charged with <sup>32</sup>P-labeled K48R ubiquitin and added to reactions containing 1 mM unlabeled ubiquitin in the presence or absence of SCF. Aliquots were removed at the indicated times and evaluated by SDS-PAGE and phosphorimaging under conditions that preserved (nonreducing, top panel) or disrupted (reducing, bottom panel) Cdc34~Ub thioesters.

(E) The results of experiments (n = 3) as shown in (D) were quantified for the kinetics of loss of ubiquitin-charged Cdc34 (top panel) or for the formation of diubiquitin (bottom panel) in either the presence or absence of SCF. Error bars represent the standard deviation of the mean.

(F) SCF influences the rate but not the mechanism of diubiquitin synthesis. The rate of diubiquitin synthesis with respect to ubiquitin concentration was determined using a fixed amount of Cdc34 charged with <sup>32</sup>P-labeled K48R ubiquitin under single-discharge conditions in the presence of variable amounts of cold ubiquitin (100 μM to 1 mM) with 100 nM SCF (left y axis) or without (right y axis). Initial rates were extrapolated from experiments (n = 3) and plotted as a function of ubiquitin concentration added in the chase reactions.



of a more distributive reaction mechanism regardless of whether wt (Figure 5B) or K48-only (Figure 5C) ubiquitin was used, which is expected if attachment of the first ubiquitin occurred normally but failed to accelerate the rate of subsequent ubiquitin transfers. To quantify this difference, the average ubiquitin-chain length on Sic1 was measured for Cdc34 and the various mutants at the initial phase of the reaction (0.25 min). Whereas Cdc34 transferred on average ~3.6 ubiquitins per modified Sic1, the loop mutants transferred fewer, ranging from 2.1 to 2.4 ubiquitins per Sic1 (Figure 5E). Finally, we examined the linkage specificity of Cdc34 3D 2E by evaluating the products formed in reactions that contained K32 Sic1 and K48R Ub. Cdc34 efficiently promoted the attachment of a single K48R ubiquitin to K32 Sic1 and only poorly sustained the formation of non-K48 linkages to yield diubiquitinated Sic1 (Figure 5D). By contrast, Cdc34 3D 2E catalyzed the formation of K32 Sic1 species with di-, tri-, and even tetraubiquitin chains.

To address the mechanism by which the 3D 2E mutation exerts its effects, we performed a kinetic analysis of diubiquitin synthesis in the presence of SCF, similar to in Figure 1F. The extrapolated kinetic parameters for synthesis of diubiquitin from free ubiquitin and wt Cdc34~K48R Ub thioesters were a  $K_M$  of 533  $\mu$ M (for free ubiquitin) and  $V_{max}$  of 2.8 pmol diubiquitin synthesized/s (Figure 5F). By contrast, with 3D 2E Cdc34~Ub thioesters, the  $K_M$  for ubiquitin was estimated to be 880  $\mu$ M, and the  $V_{max}$  was estimated as 0.38 pmol diubiquitin/s. Thus, like SCF, the acidic loop promotes diubiquitin synthesis primarily by enhancing a chemical step in the formation of an isopeptide bond between two ubiquitin molecules.

## DISCUSSION

### A Model for How CRL-Cdc34 Complexes Catalyze Processive Synthesis of Ubiquitin Chains

Our analysis of Sic1 ubiquitination leads us to propose a model for how SCF CRLs work with Cdc34 to catalyze processive synthesis of a degradation-competent K48-linked ubiquitin chain on a substrate (Figure 6). Assembly of an ubiquitin chain on Sic1 can be thought of as two separate reactions that have distinct properties. Sic1 that binds to SCF is first modified with ubiquitin on any lysine that can attack the SCF bound Cdc34~ubiquitin thioester. This reaction, which is relatively slow, is immediately followed by the more rapid attachment of successive ubiquitin molecules to the “initiator” ubiquitin to form a ubiquitin chain. The switch from the slow initial attachment of ubiquitin to the rapid polymerization of a chain is governed by a highly conserved acidic loop that is located near the active site of Cdc34. Previous genetic studies attributed an essential physiological role for the acidic loop in Cdc34 function (Liu

et al., 1995; Pitluk et al., 1995), and our work now reveals its key role in substrate ubiquitination. Whereas the acidic loop is dispensable for covalent linkage of a “chain-priming” ubiquitin to Sic1, it is critical for the accelerated, processive addition of successive ubiquitins to form a ubiquitin chain. We postulate that SCF, upon binding Cdc34~Ub, acts in part through the acidic loop to stabilize a conformation of Cdc34~ubiquitin that favors formation of K48-linked diubiquitin. Our model, the observations that form the basis of it, and its implications for the mechanism of protein ubiquitination by SCF-Cdc34 are discussed in more detail below.

### Transfer of the First Ubiquitin

Substrate bound to SCF becomes covalently modified by attachment of a single ubiquitin molecule in a stochastic process. This reaction competes with polymerization of unanchored, K48-linked ubiquitin chains (Ohta et al., 1999; Tan et al., 1999) that form when the Cdc34~ubiquitin thioester is attacked by free ubiquitin instead of a substrate lysine. However, the high local concentration of bound substrate (~3 mM in a sphere of 50 Å radius) relative to cellular ubiquitin (10–20  $\mu$ M; Haas, 1988) should bias the reaction toward substrate ubiquitination.

A large body of published data indicates that attachment of the first ubiquitin onto substrate by CRLs is not sequence specific. For example, SCF ubiquitin ligases have diverse substrates, which are modified on lysines that are not embedded within obvious sequence motifs. This is in striking contrast to what is seen for other covalent modification systems, including attachment of the ubiquitin-like protein SUMO by Ubc9. However, the site at which a substrate is initially ubiquitinated is governed by the relative position of lysines within the substrate-SCF complex (Wu et al., 2003) and may be influenced by other factors such as the polypeptide fold or associated proteins (Petroski and Deshaies, 2003).

### Transfer of Subsequent Ubiquitin Molecules

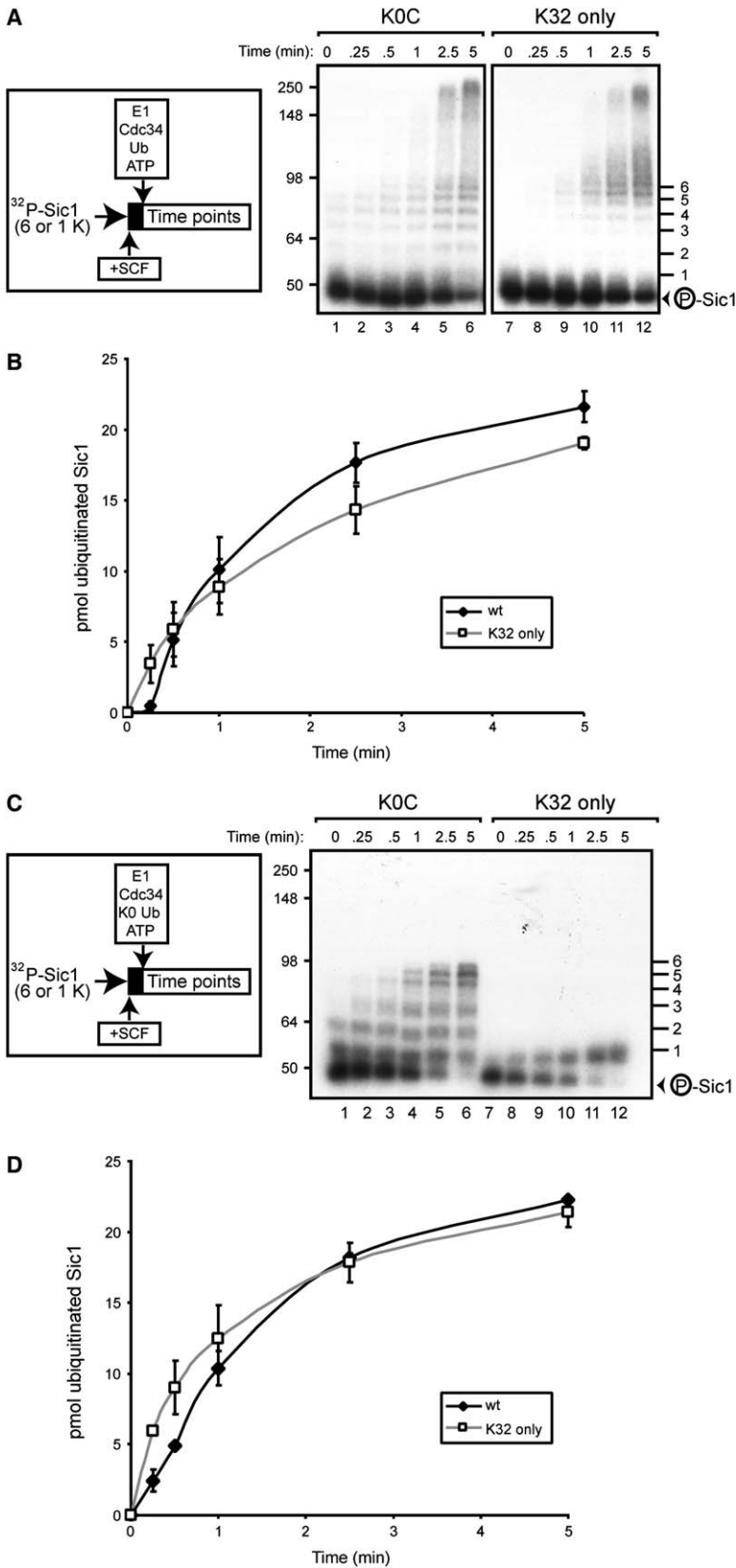
Once the first ubiquitin is attached to substrate, the properties of the ubiquitination reaction change dramatically. Whereas the first ubiquitin transfer is slow and relatively non-specific, subsequent transfers are fast and sequence specific. We suggest that this occurs because of the presence of a “privileged” site on Cdc34~Ub that presents substrate-conjugated ubiquitin in an environment conducive for nucleophilic attack of the thioester bond. The estimated  $K_M$  for this site is ~600  $\mu$ M in the presence or absence of SCF, pointing to a constitutive ubiquitin binding site wholly contained within Cdc34~Ub. Although SCF does not change the  $K_M$  for the attacking ubiquitin, it enhances  $V_{max}$  for diubiquitin synthesis by >40-fold, suggesting that SCF brings about a conformational change in Cdc34~Ub—most likely involving the acidic loop—that accelerates

### Figure 2. Specific Residues of Ubiquitin and Cdc34 Are Required for Ubiquitin Discharge from Cdc34

(A) Cdc34 charged with radiolabeled K48R ubiquitin prior to treatment with NEM and EDTA was added to reaction mixes containing SCF and the various ubiquitin derivatives indicated (400  $\mu$ M). At various times postaddition, reaction aliquots were removed and added to nonreducing sample buffer prior to SDS-PAGE and autoradiography. The relative position of diubiquitin (diUb) and free labeled ubiquitin (Ub) are indicated.

(B) The ability of Cdc34 containing mutations and deletions within amino acids 103 to 114 to support discharge of  $^{32}$ P-labeled K48R ubiquitin from the active-site cysteine onto free ubiquitin (1 mM) was assessed in the presence of SCF.

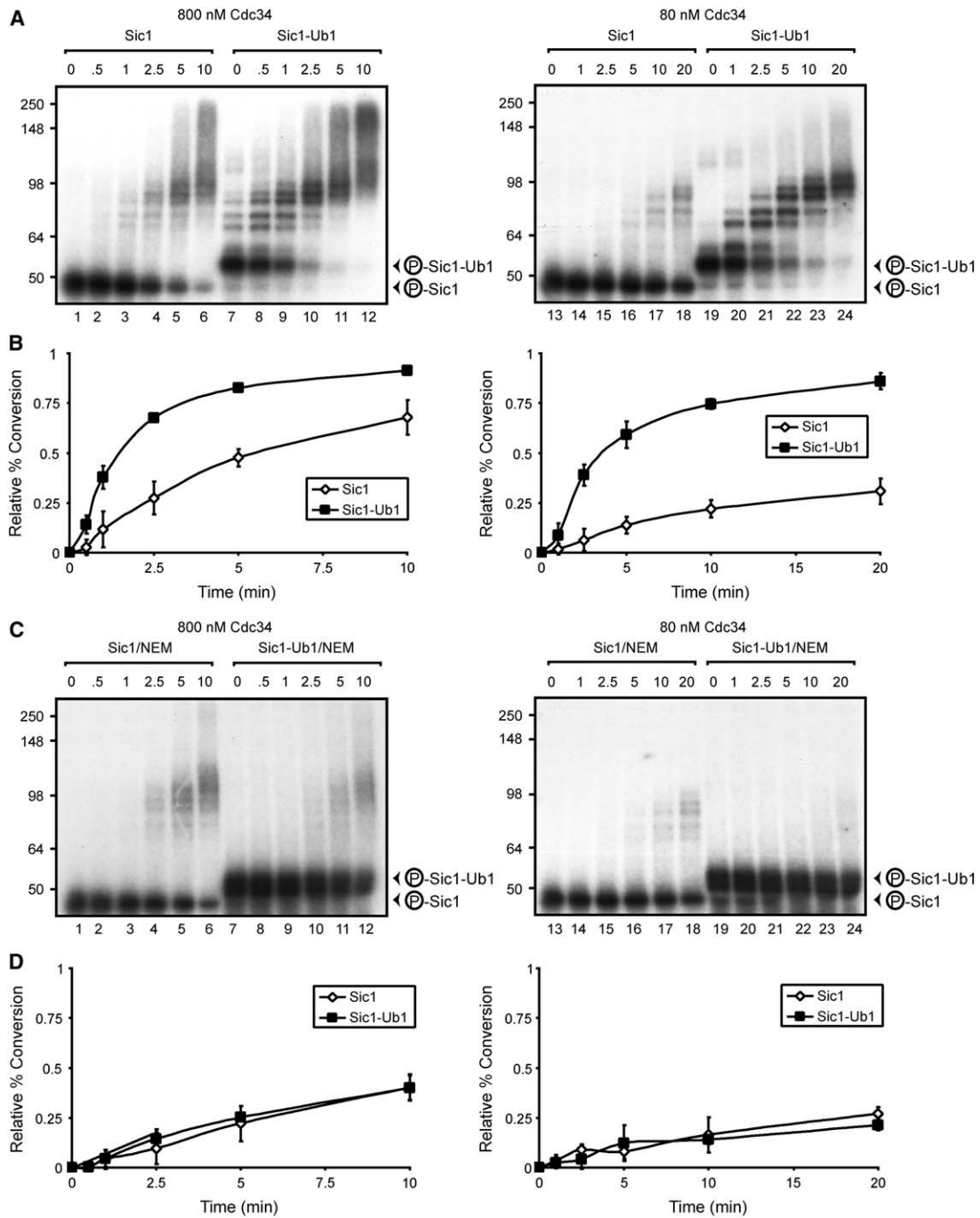
(C) Quantification of the results shown in (E). Error bars represent standard deviation ( $n = 3$ ).



**Figure 3. The Rate of Sic1 Ubiquitination Is Independent of the Number of Lysine Residues**

(A and C) Sic1 (1.25  $\mu$ M) containing the six most N-terminal lysine residues (K0C) or a mutant that contains only a single lysine (K32 only) were phosphorylated by G1-CDK in the presence of [ $\gamma$ - $^{32}$ P]ATP and added to ubiquitination reactions containing 100 nM SCF and 800 nM Cdc34. Reactions shown in (A) contained ubiquitin, whereas (C) was used K0 Ub. Reaction aliquots quenched at the times indicated were evaluated by SDS-PAGE and autoradiography.

(B and D) Phosphorimager quantification of the results shown in (A) and (C), respectively. Error bars represent standard deviation ( $n = 3$ ).



**Figure 4. The Attachment of the First Ubiquitin onto Sic1 Is Rate Limiting**

(A) Ubiquitination of  $^{32}\text{P}$ -labeled, phosphorylated Sic1 or phosphorylated Sic1 containing a single ubiquitin molecule conjugated to lysine 36 (Sic1-Ub1) was evaluated in the presence of 800 nM Cdc34 (left panel) and 80 nM Cdc34 (right panel).

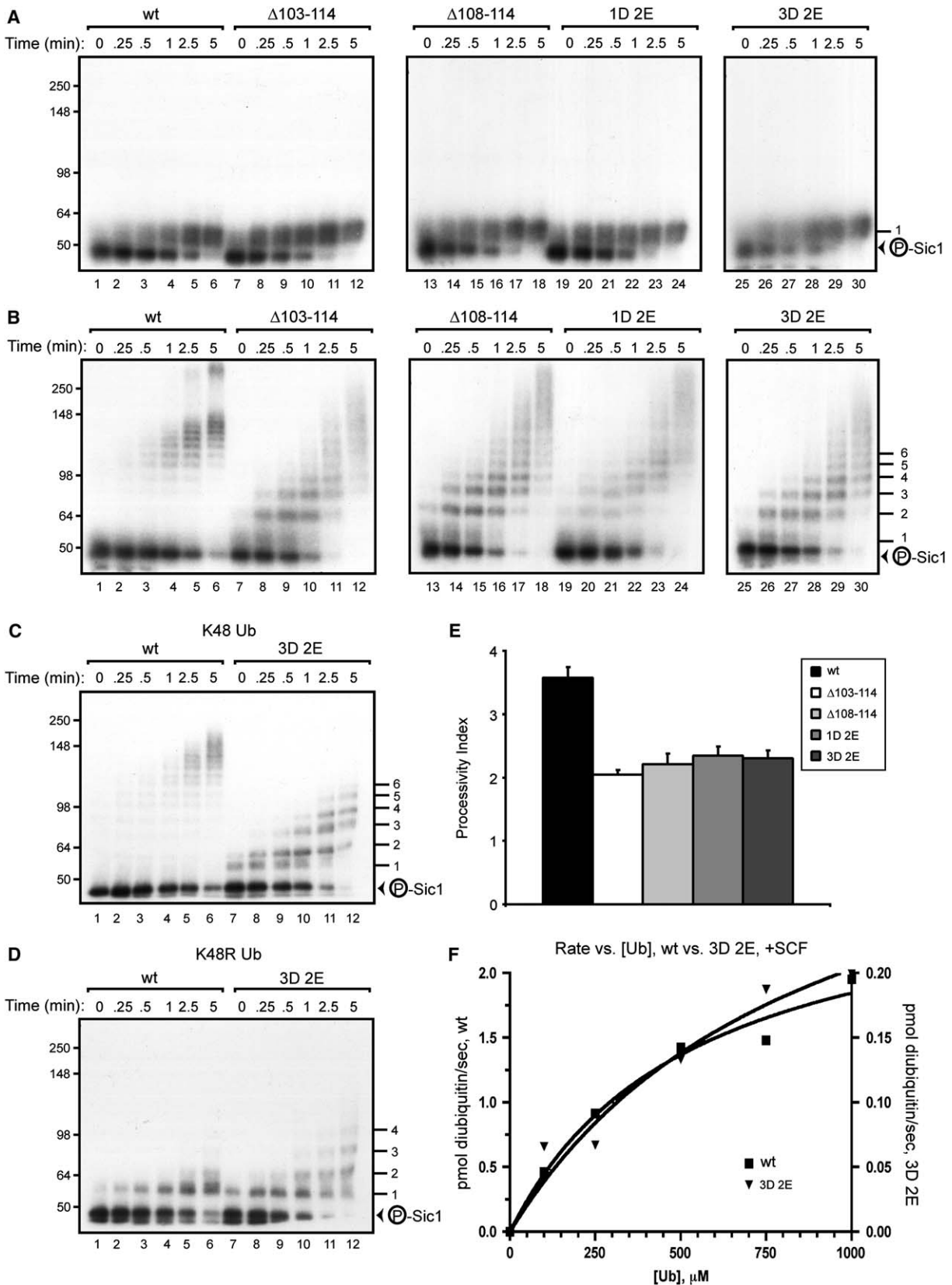
(B) Phosphorimager quantification of the results shown in (A). The y axis represents the degree of conversion of input substrate into higher-MW forms. Error bars represent standard deviation ( $n = 3$ ).

(C) Lysine 48 of ubiquitin is required for the increased rate of ubiquitination of Sic1-Ub1 relative to Sic1. Same as (A), except that the ubiquitin on Sic1-Ub1 lacked an amino group at amino acid 48.

(D) The results in (C) were quantified as in (B).

isopeptide-bond formation. At concentrations of free ubiquitin and Cdc34~Ub approaching saturation, SCF catalyzes formation of a minimum of 1.3 diubiquitins per molecule

per second. Thus, once the first ubiquitin transfer occurs, a degradation-competent tetraubiquitin chain is formed in seconds, which is consistent with our detection of Sic1



species that are conjugated with chains of up to five ubiquitins long within the few seconds that it takes to remove a 0-time reaction aliquot.

At first glance, the poor affinity of the noncovalent binding site on Cdc34~Ub for free ubiquitin might seem odd given that the intracellular concentration of ubiquitin is 10–20  $\mu\text{M}$  (Haas, 1988). However, this strongly biases polymerization in favor of substrate-linked ubiquitin chains because a ubiquitin conjugated to SCF bound substrate will be present at millimolar concentrations in the vicinity of SCF bound Cdc34~Ub and thus can saturate the noncovalent binding site despite its weak affinity.

#### Role of the Acidic Loop in Ubiquitin-Chain Synthesis

We propose that the rate of transfer of ubiquitin between Cdc34~Ub and substrate molecules bound to SCF increases following conjugation of the first ubiquitin to substrate because of a conserved acidic loop within Cdc34 that channels the K48 residue of a substrate-linked ubiquitin to attack the thioester in such a way as to favor the reaction coordinate that leads to the transition state. This proposed role for the acidic loop accounts for two remarkable properties of Cdc34 mutants that have deletions or point mutations in this region. (1) Acidic-loop mutants are defective in formation of K48-linked diubiquitin and exhibit a modest (1.6-fold) increase in the  $K_M$  for ubiquitin and a substantial (7.4-fold) decrease in  $V_{\max}$ . (2) Acidic-loop mutants are fully proficient in catalyzing attachment of ubiquitin directly to Sic1 but are defective in subsequent processive extension of a ubiquitin chain on Sic1. In addition to facilitating a chemical step in isopeptide-bond formation between two ubiquitins, the acidic loop appears to restrict the available lines of attack because acidic-loop mutants have loosened specificity and can assemble ubiquitin chains linked by lysines other than K48. Indeed, computer modeling based on the three-dimensional structures of Ubc7 and ubiquitin suggests that the acidic loop may constrain the directions from which the incoming ubiquitin can attack the thioester (G. Kleiger, personal communication).

Given that both SCF and the acidic loop have a substantial effect on  $V_{\max}$  for diubiquitin synthesis but a relatively modest effect on the  $K_M$  for the attacking ubiquitin, we propose that SCF works at least in part through the acidic loop to stabilize the transition state intermediate in diubiquitin formation. This proposal is consistent with two observations: the stimulatory effect of SCF on diubiquitin synthesis is largely dependent upon the acidic loop, and, conversely, the stimulatory effect

of the loop is largely dependent upon SCF (M.D.P., unpublished data). Docking of the loop-containing Cdc34 homolog Ubc7 (PDB ID code 2UCZ) onto SCF (using the same approach used by Zheng et al. [2002] to dock UbcH7 onto SCF) reveals that the acidic loop closely abuts the surface of the RING subunit (G. Kleiger, personal communication). Determining the exact mechanism by which the SCF acts through the acidic loop to catalyze selectively the formation of ubiquitin-ubiquitin isopeptide linkages awaits crystal structures of SCF-Cdc34~Ub complexes.

#### Generality of the Model

We have established our model based on analysis of Sic1 ubiquitination by SCF–Cdc34. Does this hypothesis account for the ubiquitination of substrates by other ubiquitin-conjugating enzymes and other CRLs? In addition to Cdc34, budding-yeast Ubc7 and the related human proteins contain an acidic insertion loop near the catalytic site, and thus we propose that these proteins and their partner ligases operate by a similar mechanism. What about other E2s? Ubc4/5 family members are by far the most commonly used ubiquitin-conjugating enzymes in reconstitution studies. These enzymes do not possess an acidic insertion loop, and the mechanism by which they promote substrate ubiquitination is not known. Evaluation of published data suggests that, although these enzymes can act processively, they typically do not form high-molecular-weight ubiquitin conjugates upon substrate with the speed and efficiency observed for Cdc34 in our system (Carroll et al., 2005; Carroll and Morgan, 2002; Passmore et al., 2003)—a possible exception being the ubiquitination of Hsl1 by anaphase-promoting complex (APC) (Carroll et al., 2005). Moreover, the modest processivity exhibited by APC-Ubc4 in ubiquitination of cyclin B is achieved primarily by monoubiquitination of multiple lysines (Carroll and Morgan, 2002). Even in the polymerization of free ubiquitin chains activated by Cul1-Roc1, reactions carried out with Ubc5C contain a higher proportion of low-MW conjugates than those carried out with Cdc34 (Wu et al., 2002). Thus, Ubc4/5 enzymes are intrinsically less processive than Cdc34 in the synthesis of multiubiquitin chains. In addition, those chains that are synthesized by Ubc4/5 exhibit greater linkage plasticity. Significant K48-independent ubiquitination by Ubc4/5 has been reported (Wu et al., 2002), and direct mapping of the topology of ubiquitin chains reveals that Ubc4, when used in conjunction with APC, forms a high proportion of other linkages on cyclin B (R. King,

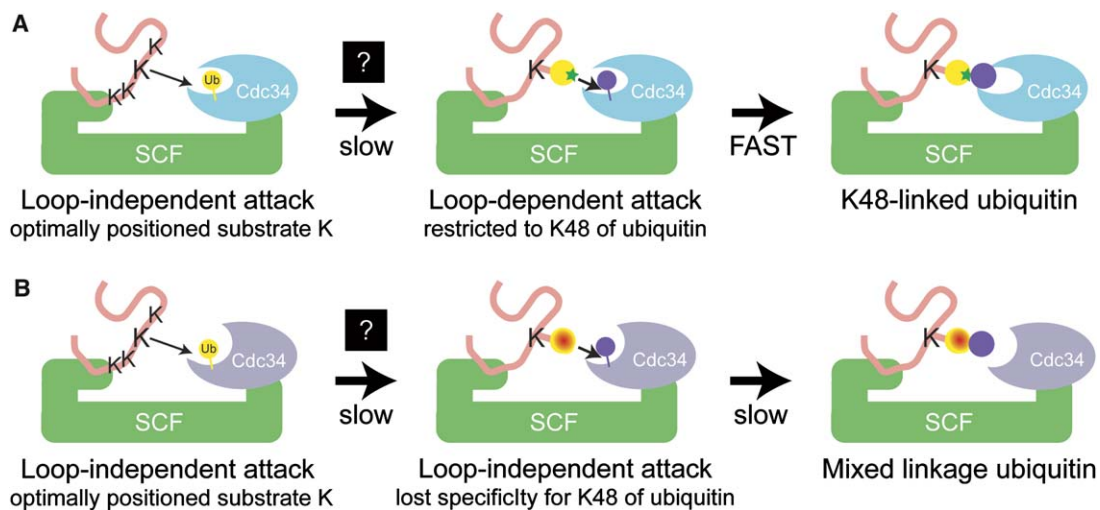
#### Figure 5. The Acidic Loop of Cdc34 Is Required for Processive Ubiquitin-Chain Synthesis and Specificity for Lysine 48-Linked Ubiquitin-Chain Synthesis

(A and B) The ability of Cdc34 containing various acidic-loop mutations (800 nM) to transfer either K0 ubiquitin (A) or ubiquitin (B) onto  $^{32}\text{P}$ -labeled K32-only Sic1 (2.5  $\mu\text{M}$ ) in the presence of SCF was assessed. At the indicated times, reaction aliquots were quenched prior to analysis by SDS-PAGE and autoradiography. The relative positions of phospho-Sic1 species that are unmodified or are conjugated with one to six ubiquitin molecules are indicated.

(C and D) Same as (A) and (B), except that K48-only ubiquitin (K48 Ub; [C]) or K48R ubiquitin (K48R Ub; [D]) were employed.

(E) A processivity index (a weighted average of the number of ubiquitin molecules transferred per molecule of ubiquitinated Sic1) was measured at 0.25 min for experiments ( $n = 3$ ) shown in (B). A fully distributive reaction by this calculation should have a processivity-index measurement of  $\sim 1$ . Error bars represent standard deviation.

(F) The acidic loop of Cdc34 influences the rate and mechanism of diubiquitin synthesis. Experiments similar to those in Figure 1D were performed to determine the rate of diubiquitin synthesis with respect to ubiquitin concentration in the presence or absence of the acidic loop of Cdc34 with 100 nM SCF. Initial rates were extrapolated for reactions ( $n = 3$ ) performed with various concentrations of cold ubiquitin and plotted as a function of ubiquitin concentration added in chase reactions.



**Figure 6. Model for the Processive Synthesis of Lysine 48-Linked Ubiquitin Chains onto SCF Bound Sic1 by Cdc34**

(A) Attachment of the first ubiquitin to substrate is slow, whereas subsequent attachments to form a K48-linked (green star) ubiquitin chain are fast, due to the contribution of the acidic loop.

(B) In the absence of the acidic loop, the bias toward K48 is reduced, and ubiquitin chains that contain other lysine linkages (fuzzy orange circle) are synthesized at an overall slower rate.

personal communication). In sum, the mechanism by which Ubc4/5 work remains poorly understood and demands further study. Since Ubc4/5 family members do not possess an acidic loop, they may employ a different strategy to achieve processivity and linkage specificity. For example, the topology of K63-linked ubiquitin chains is determined by specific, noncovalent binding of free ubiquitin to the Mms2 subunit of Mms2-Ubc13 heterodimeric E2 enzyme, such that its K63 residue is positioned to attack the Ubc13~ubiquitin thioester (McKenna et al., 2003; VanDemark et al., 2001).

The relevance of the mechanism described here to other CRLs is a thorny question. Many reconstitution studies with CRLs have employed Ubc4/5 family members. However, we wish to draw attention to two points. First, in budding-yeast cells, Cdc34 is the only E2 known to promote turnover of substrates by SCF ubiquitin ligases *in vivo* (see Schwob et al., 1994 for example), even though Ubc4/5 have been shown to work with yeast SCFs *in vitro* (Kus et al., 2004). Second, the ability of human Cul1-RING to activate ubiquitin-chain synthesis by human Cdc34 is stimulated dramatically (10-fold) by attachment of Nedd8 to Cul1, whereas polymerization observed with Ubc4/5 is indifferent to Cul1 neddylation (Wu et al., 2002). However, inactivation of the Nedd8 E1 enzyme *in vivo* leads to accumulation of CRL substrates that can be ubiquitinated by Ubc4/5 *in vitro*, including I $\kappa$ B $\alpha$ , HIF-1 $\alpha$ , and p27 (Oh et al., 2002). Taken together, these observations raise a legitimate question as to whether Ubc4/5 family members comprise a major pathway for ubiquitination of CRL substrates *in vivo*.

## EXPERIMENTAL PROCEDURES

### Recombinant Protein Expression and Purification

Descriptions of plasmids used in this study are provided in Table S1.

### Cdc34

All experiments used *S. cerevisiae* Cdc34 $\Delta$ C-His6 expressed and purified from bacteria (Seol et al., 1999). This form of Cdc34 lacks the C-terminal 25 amino acids. Mutagenesis of sequences encoding the acid loop of Cdc34 was performed with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All mutations were confirmed by DNA sequencing.

### Ubiquitin and Ubiquitin Derivatives

Ubiquitin (25 mg/ml; 3.1 mM) and its K48-only, K48R, and K0 derivatives (each at 5 mg/ml; 625  $\mu$ M) were purchased from Boston Biochem and dissolved in water.

A plasmid coding for K48C ubiquitin was a generous gift from Cecile Pickart. K48C ubiquitin was purified from BL21-RIL codon optimized bacteria (Stratagene) as described (Piotrowski et al., 1997), dialyzed against 50 mM Tris-HCl (pH 8.0), and adjusted to 10 mg/ml (1.25 mM).

The ubiquitin mutants used in Figure 2 were expressed in BL21-RIL bacteria as GST fusion proteins containing a protein kinase A phosphorylation site sandwiched between an upstream tobacco etch virus protease (TEV) cleavage site and ubiquitin. All ubiquitin point mutants were confirmed by sequencing. For radiolabeled K48R ubiquitin, GST-TEV-K48R Ub (300  $\mu$ M) purified on glutathione (GSH) Sepharose was incubated in the presence of 200 U of cAMP-dependent protein kinase (New England Biolabs) and 100  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP for 30 min at room temperature. After desalting by gel filtration, TEV cleavage was performed with 3  $\mu$ M GST-TEV protease in 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 1 mM DTT for 2 hr at 30°C. The resulting material was boiled for 10 min and cooled on ice, and precipitated material was removed by centrifugation. Other ubiquitin derivatives used in Figure 2 were prepared identically, except the kinase step was omitted.

### Sic1-Ub1

Radiolabeled phosphorylated K36-only Sic1 was ubiquitinated with K48C ubiquitin exactly as described below, except that reactions were scaled up 50-fold to 1 ml. After 15 min at room temperature, the reactions were treated for 30 min at 30°C with either ethylamine (2% v/v) to convert cysteine 48 to a lysine mimetic (Piotrowski et al., 1997) or *n*-ethylmaleimide (NEM, 10 mM) to alkylate the cysteine prior to SDS-PAGE on a 10% gel. The ratio of Sic1:Sic1-Ub1 generated was approximately

1:1. Sic1 and Sic1-Ub1 were identified by autoradiography; electroeluted from gel slices in 25 mM Tris base, 192 mM glycine; concentrated by acetone precipitation in the presence of 10 µg/ml BSA; and resuspended in kinase assay buffer 8.0 (KAB 8.0, 20 mM HEPES [pH 8.0], 200 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM EDTA, 10% glycerol) in the presence of 3 M guanidine HCl. After dialysis against KAB 8.0, the proteins were evaluated by SDS-PAGE and autoradiography (Figure S3) and used in Sic1 ubiquitination assays.

## Ubiquitination Assays

### Sic1 Ubiquitination

Yeast G1-CDK, E1 (Uba1), SCF<sup>Cdc4</sup>, and the various Sic1 substrates were prepared as described (Petroski and Deshaies, 2003). Sic1 (25 µM) was phosphorylated by GSH Sepharose bound G1-CDK from baculovirus-infected insect cells (10 µl of GSH Sepharose per 500 µl insect-cell lysates, one-fourth of a single 10 cm<sup>2</sup> plate) in the presence of 20 µCi [ $\gamma$ -<sup>32</sup>P]ATP and 100 µM ATP for 1 hr at room temperature, followed by another 1 hr incubation in the presence of 2 mM ATP. Reactions were initiated by combining the SCF-plus-substrate mixture with a mixture of E1, E2, and ubiquitin. The 0-time sample was withdrawn ~5 s afterwards. Final concentrations of components were Cdc34, 80 nM or 800 nM as noted; Uba1, 150 nM; ubiquitin, 77.5 µM or K0 ubiquitin, 38.8 µM; Sic1, 1.25 µM; SCF, 100 nM; Tris-HCl [pH 7.6]; 30 mM; MgCl<sub>2</sub>, 5 mM; NaCl, 100 mM; DTT, 1 mM; and ATP, 2 mM. After brief mixing, aliquots were removed at the various times indicated, added to an equal volume of 2× SDS-PAGE sample buffer, and analyzed by SDS-PAGE and autoradiography. The rates of Sic1 ubiquitination—measured as the loss of unmodified Sic1—were quantified by phosphorimager analysis (n = 3).

### Thioester Discharge Assays

Cdc34 (1.6 µM) was incubated in the presence of Uba1 (300 nM) and either ubiquitin (77.5 µM), K0 ubiquitin (38.8 µM), or [<sup>32</sup>P]K48R ubiquitin (16 µM) in 30 mM Tris-HCl (pH 7.6), 5 mM MgCl<sub>2</sub>, 2 mM ATP for 15 min at room temperature. The charging reaction was treated with 10 mM N-ethylmaleimide (NEM) and 50 mM EDTA for 15 min at room temperature (added as 1/10 volume to charging reaction). This reaction was diluted into an equal volume of chase mixes containing a final concentration of 30 mM Tris-HCl (pH 7.6), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT in the presence or absence of SCF (100 nM final concentration) and ubiquitin or ubiquitin derivatives (variable concentration; see figure legends) or phosphorylated Sic1 derivatives (2.5 µM final concentration). The 0-time sample was withdrawn ~5 s afterward. Samples taken at the indicated times were added to nonreducing sample buffer containing 4 M urea and analyzed by SDS-PAGE followed by immunoblotting with antisera against Cdc34 or autoradiography for experiments utilizing <sup>32</sup>P-labeled K48R ubiquitin. For reducing conditions, sample buffer lacking 4 M urea but containing 150 µM 2-mercaptoethanol was used, and samples were heated to 100°C for 5 min prior to loading. For experiments using <sup>32</sup>P-labeled K48R ubiquitin, the rate of appearance of diubiquitin was measured by phosphor screen analysis. Due to the very rapid rate of SCF-catalyzed reactions, initial rates for Figure 1E were estimated by fitting experimental data to a rectangular hyperbola using GraphPad and may yield underestimates of the true K<sub>M</sub> for ubiquitin and V<sub>max</sub> for diubiquitin formation.

### Supplemental Data

Supplemental Data include Supplemental References, one table, and three figures and can be found with this article online at <http://www.cell.com/cgi/content/full/123/6/1107/DC1/>.

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