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SCF E3-Mediated Autoubiquitination Negatively Regulates Activity of Cdc34 E2 but Plays a Nonessential Role in the Catalytic Cycle In Vitro and In Vivo▽

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One of the several still unexplained aspects of the mechanism by which the Cdc34/SCF RING-type ubiquitin ligases work is the marked stimulation of Cdc34 autoubiquitination, a phenomenon of unknown mechanism and significance. In in vitro experiments with single-lysine-containing Cdc34 mutant proteins of *Saccharomyces cerevisiae*, we found that the SCF-mediated stimulation of autoubiquitination is limited to specific N-terminal lysines modified via an intermolecular mechanism. In a striking contrast, SCF quenches autoubiquitination of C-terminal lysines catalyzed in an intramolecular manner. Unlike autoubiquitination of the C-terminal lysines, which has no functional consequence, autoubiquitination of the N-terminal lysines inhibits Cdc34. This autoinhibitory mechanism plays a nonessential role in the catalytic cycle, as the lysineless ^{K0}Cdc34$^{\Delta C}$ is indistinguishable from Cdc34$^{\Delta C}$ in ubiquitination of the prototype SCF^{Cdc4} substrate Sic1 in vitro, and replacement of the *CDC34* gene with either the ^{K0}*cdc34*$^{\Delta C}$ or the *cdc34*$^{\Delta C}$ allele in yeast has no cell cycle phenotype. We discuss the implications of these findings for the mechanism of Cdc34 function with SCF.

Posttranslational modification of proteins with ubiquitin has emerged as a major regulatory mechanism in eukaryotic cells, with both proteolysis-related and non-proteolysis-related functions (14, 19, 34, 35). The variety of mechanisms of substrate recognition by specific ubiquitin ligases well reflects the large number of proteins targeted for ubiquitination. However, once a substrate is selected, its ubiquitination is catalyzed via a conserved cascade of ubiquitin transfer reactions.

The first two steps of the ubiquitin transfer cascade do not involve a specific substrate protein and appear to be facilitated in a constitutive manner. In the first step, E1, called a ubiquitin-activating enzyme, forms a ubiquitin adenylate and subsequently a high-energy thiol ester bond with a carboxyl group of ubiquitin, thus activating it for nucleophilic attack. In the second step, the activated ubiquitin is *trans*-esterified to the active-site cysteine of one of several E2 ubiquitin-conjugating enzymes. The diversity in the ubiquitin transfer cascade begins when the ubiquitin-charged E2 is recruited to the proximity of a substrate through an interaction with a specific E3, which can be, but is not always, an enzyme. In the case of HECT (*h*omology to *E*6-AP *C* *t*erminus)-type E3s, E2 transfers ubiquitin to the catalytic site of E3 and E3 catalyzes ubiquitination. In contrast, in the case of RING (*r*eally *i*nteresting *n*ew *g*ene)-type E3s, which do not have their own catalytic sites, E2 is directly responsible for ubiquitination of the E3-bound substrate. It is still unknown why such a difference evolved and why the enzymatic activity of HECT-type E3s is required if RING-type E3s can depend on the activity of E2 without having their own catalytic sites. In light of this paradox, however, the term "ubiquitin ligase" cannot be treated as equivalent to E3 alone. Rather, it represents a functional unit composed of an E2 and an E3, which together can catalyze an isopeptide bond between the C terminus of ubiquitin and a lysine or, in some instances, an N-terminal residue of a specific substrate protein.

Among the several still unexplained aspects of the mechanism by which specific ubiquitin ligases work is the phenomenon of autoubiquitination. On one hand, autoubiquitination is frequently used as a measure of putative RING-type ubiquitin ligase activity (21, 29), suggesting that the ability to autoubiquitinate is one of the most fundamental features of a ubiquitin ligase. On the other hand, autoubiquitination is rarely taken into consideration in the interpretation of experiments addressing the mechanism by which ubiquitin ligases work with their bona fide substrates, and in most cases, its mechanism and role are unknown.

The Cdc34/SCF (*S*kp1, *C*dc53/Cul1, *F*-box protein) ubiquitin ligase of *Saccharomyces cerevisiae* serves as a prototype for a large family of conserved RING-type ubiquitin ligases that, via an array of distinct F-box proteins, can recruit many substrates (33). Among these are key cell cycle regulatory proteins and signaling molecules, including the Sic1 inhibitor of S-phase cyclin-dependent kinase (CDK), G_1 cyclins, the pheromone response-dependent CDK inhibitor Far1, the transcription factors Gcn4 and Met4, the DNA replication factor Cdc6, the cell polarization and budding protein Gic2, and HO endonuclease. Cdc34/SCF also plays an essential role in mitosis, where its

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
YPH499	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	40
Y1171	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $cdc34^{\Delta C}$*::URA3*	This study
Y1741	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $^{K0}cdc34^{\Delta C}$*::URA3*	This study
Y1643	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rub1Δ::KANMX6*	This study
Y1644	*matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $cdc34^{\Delta C}$*::URA3 rub1Δ::KANMX6*	This study
Y1767	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $^{K0}cdc34^{\Delta C}$*::URA3 rub1Δ::KANMX6*	This study
Y1743	*mat**a** ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ63 leu2-Δ1 pB354 CEN TRP GAL-3myc-SIC1*	This study
Y1747	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $cdc34^{\Delta C}$*::URA3 pB354 CEN TRP GAL-3myc-SIC1*	This study
Y1750	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $^{K0}cdc34^{\Delta C}$*::URA3 pB354 CEN TRP GAL-3myc-SIC1*	This study
Y1758	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS414 CEN TRP GAL1*	This study
Y1759	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS414 CEN TRP GAL1::cdc34ΔC*	This study
Y1760	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS414 CEN TRP GAL1::*$^{K0}cdc34^{\Delta C}$	This study
Y1761	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $cdc34^{\Delta C}$*::URA3 pRS414 CEN TRP GAL1*	This study
Y1762	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $cdc34^{\Delta C}$*::URA3 pRS414 CEN TRP GAL1::*$cdc34^{\Delta C}$	This study
Y1763	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $^{K0}cdc34^{\Delta C}$*::i::URA3 pRS414 CEN TRP GAL1*	This study
Y1764	*mat**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* $^{K0}cdc34^{\Delta C}$*::URA3 pRS414 CEN TRP GAL1::*$^{K0}cdc34^{\Delta C}$	This study

substrates include the CDK-inhibitory kinase Swe1 and some still unidentified targets in the assembly of the kinetochore.

While Cdc34/SCF is one of the best-characterized ubiquitin ligases, as with other ligases, analysis of the mechanism by which te Cdc34 collaborates with SCF in ubiquitination of a specific substrate is complicated by autoubiquitination. Ubiquitinated forms of Cdc34 can be detected in vivo (15) and in vitro (3), where the protein autoubiquitinates with K-48-linked polyubiquitin chains in a manner stimulated by Rbx1/Cdc53, the Cdc34 recruitment domain of SCF (38, 42). Nevertheless, the steady-state levels of Cdc34 protein do not change during the cell cycle in yeast (16) and mammalian (37) cells, and the Cdc34 protein is stable in vivo (16), raising a question about the role of the autoubiquitination phenomenon.

In this study, we show that autoubiquitination of Cdc34 is a surprisingly precise process targeting distinct lysine residues in the absence and presence of SCF. Unlike the SCF-independent autoubiquitination of the C-terminal lysines, which is intramolecular and has no effect on Cdc34 function, autoubiquitination of the N-terminal lysines depends on interaction between Cdc34 molecules and inhibits Cdc34. This autoinhibitory mechanism plays a nonessential role in vitro and in vivo, at least in the context of the baking-yeast system.

MATERIALS AND METHODS

Antibodies. We used polyclonal rabbit anti-Cdc34, anti-Cdc4, anti-Cdc53 (30), anti-MBPSic1 (2), anti-Skp1 (41), anti-FLAG M2 agarose (Sigma), and anti-hemagglutinin (HA) agarose (Covance). Antibody detection was by ECL (Amersham).

Recombinant proteins. We used $Uba1^{His6}$ (10); ^{Gst}Ub and $^{Gst}Ub^{RA}$ (41); baculoviruses encoding yeast Cdc53, Rbx1, Cdc4, $^{Gst}Skp1$, and $^{Flag}Skp1$ (41, 42); a C-terminal six-His fusion in pET21+ (Novagen) of $^{His}Cdc34$ and $^{His}Cdc34^{\Delta C}$ terminated at amino acid (aa) 244 (7); and their K0 and single-lysine-containing RK mutant derivatives (this work). The molecular mass of octadimethyl ubiquitin was confirmed by mass spectrometry (AFFINITI Research Products Ltd., United Kingdom). Ubiquitin (bovine) was from Sigma.

Cdc34 proteins were expressed either in yeast (from pRS414 plasmid) (39) or *Escherichia coli* [BL21(DE3) LysS] and purified on Ni^{+}-nitrilotriacetic acid resin (QIAGEN), followed by DEAE and high-performance liquid chromatography (HPLC) gel filtration chromatography on Superdex 200 (Amersham) in U buffer (50 mM Tris, pH 7.5, 50 mM KCl, 0.2 mM dithiothreitol). [^{35}S]Met-labeled Cdc34Δ (570 cpm/pmol) was labeled in vivo by expressing $Cdc34\Delta^{His6}$ in *E. coli* grown in synthetic medium with a 30-min pulse of 8 mCi Tran^{35}S label (ICN) after 3 h of induction with IPTG (isopropyl-β-D-thiogalactopyranoside).

$^{FLAG}SCF^{Cdc4}$, $^{GST}SCF^{Cdc4}$, Sic1/Clb5/$^{GST}Cdc28^{HA}$, and Cln2/CKS/$^{GST}Cdc28^{HA}$ complexes were assembled by coinfecting SF9 insect cells (Invitrogen) for 40 h with the appropriate baculoviruses, followed by cell lysis and immunoprecipitation (41). Sic1 was phosphorylated as described before (41).

Polyubiquitination of Sic1. Ubiquitination was performed for 1 h at 30°C in 20-μl reaction mixtures containing 2 pmol of purified Sic1/Clb5/^{GST}Cdc28 substrate complex, 2 pmol of $^{F}SCF^{Cdc4}$ or $^{GST}SCF^{Cdc4}$, 70 pmol of $Cdc34^{\Delta C}$, 1 pmol of Uba1, and 13 pmol of ubiquitin (7).

Yeast extracts. Yeast extracts (5 to 10 mg/ml protein) were prepared by grinding cells harvested at the logarithmic phase of growth on synthetic medium and blast frozen in a 1:0.7 ratio of E buffer (50 mM Tris, pH 7.5, 20 mM $MgCl_2$, 5 mM ATP or ATPγS, 0.2 mM dithiothreitol, 10% glycerol), followed by thawing and centrifugation.

HPLC. One hundred microliters of a sample containing 2 μg of purified protein or 100 μg of crude extracts was separated at 25°C on a Superdex 200 HR 10/30 column (Amersham) in buffer U with a flow rate of 0.5 ml/min and a fraction size of 500 μl.

α-Factor synchronization and fluorescence-activated cell sorter analysis. Yeast cells were grown at 30°C to a density of 2×10^6 to 4×10^6 cells/ml, followed by incubation with α-factor for 2 h. To release them from the arrest, the cells were centrifuged for 5 min at 3, 000 × *g*, washed twice, suspended in fresh medium, and incubated at 30°C for the times indicated. For each time point, 10^7 cells were collected and fixed with 1 ml of 70% alcohol in 0.2 M Tris-HCl, pH 7.5, at 4°C overnight, followed by separation from alcohol, resuspension in 200 μl of 1-mg/ml RNase in 0.2 M Tris-HCl, pH 7.5, and incubation at 37°C for 2 h. The cells were then treated with 10 μl of 20-mg/ml proteinase K at 50°C and stained with 1 ml of 3-μg/ml propidium iodide in 0.2 M Tris-HCl, pH 7.5, at 4°C overnight. The total DNA content of the cells was measured using a flow cytometer.

Yeast strains. Yeast strains are listed in Table 1.

RESULTS

SCF quenches autoubiquitination of C-terminal lysines and stimulates autoubiquitination of selected N-terminal lysines in Cdc34. To address the mechanism and the role of Cdc34 autoubiquitination, we prepared a collection of Cdc34 mutants, including ^{K0}Cdc34, a lysineless protein with arginines instead of lysines, and $^{K0}Cdc34^{RK}$ mutants, in which a single lysine is reintroduced at its original location within the lysineless ^{K0}Cdc34 protein (see Fig. 2A for lysine localization).

In experiments with the $^{K0}Cdc34^{R293K}$ mutant protein, we found that autoubiquitination of the C-terminal lysine K293, one of the four C-terminal targets for SCF-independent reac-

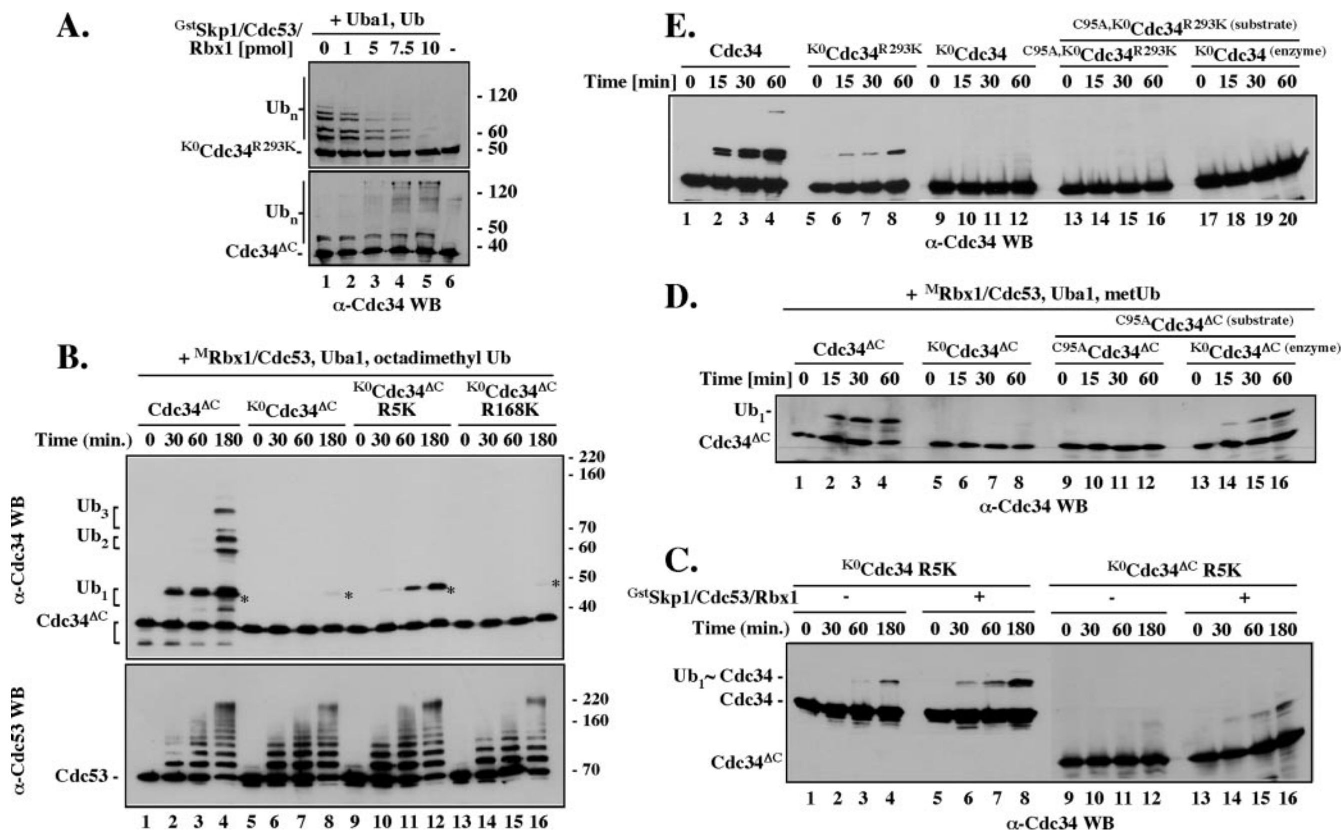

FIG. 1. An F-box protein-free SCF core quenches autoubiquitination of C-terminal lysines and promotes autoubiquitination of specific lysines on the N terminus of Cdc34. (A) SCF quenches autoubiquitination of the C-terminal lysine, K293, and stimulates autoubiquitination of lysines within Cdc34$^{\Delta C}$. Anti-Cdc34 (α-Cdc34) WB of autoubiquitination assays was performed with picomolar amounts of ^{Gst}Skp1/Cdc53/Rbx1 core, as indicated. Reaction mixtures were incubated at 25°C for 1 h with Uba1, ubiquitin (Ub), and either (top) a single C-terminal lysine K293-containing Cdc34 mutant (Cdc34$^{K0,\ R293K}$) or (bottom) the 1-to-244 fragment of Cdc34 missing the C terminus but containing intact N-terminal lysines (Cdc34$^{\Delta C}$) (Fig. 2A). (B) Kinetics of ubiquitination with single-lysine-containing Cdc34$^{\Delta C}$ mutant proteins. All reactions were performed at 25°C for 1 h in the presence of octadimethyl-ubiquitin and 10 pmol of ^{Myc}Rbx1/Cdc53 immobilized on anti-Myc beads. (Top) Anti-Cdc34 WB. Reactions with ^{K0}Cdc34$^{\Delta C}$ R5K (lanes 9 to 12) represent reaction rates typical for mutants containing a K5, K151, or K61 single lysine; reactions with ^{K0}Cdc34$^{\Delta C}$ R168K (lanes 13 to 16) represent reaction rates typical for mutants containing a K23, K24, K157, K168, or K189 single lysine; and lanes 1 to 4 show control with Cdc34$^{\Delta C}$ containing intact lysines. The asterisks indicate the only modifications detectable in reactions with ^{K0}Cdc34$^{\Delta C}$ that differ from a lysine ubiquitination by slightly higher electrophoretic mobility and could represent ubiquitination of the N terminus (5). (Bottom) Anti-Cdc53 WB. Shown is ubiquitination of Cdc53 in the same reaction mixtures. (C) An F-box protein-free SCF stimulates autoubiquitination of lysine K5 in the context of either Cdc34 or Cdc34$^{\Delta C}$. Shown is anti-Cdc34 WB of ubiquitination reactions with ^{Myc}Rbx1/Cdc53 complex immobilized on anti-Myc beads, Ub^{mix} (ubiquitin, Uba1, ATP, and $MgCl_2$), and the single lysine K5 containing full-length Cdc34 or Cdc34$^{\Delta C}$ mutant proteins, as indicated. (D) Autoubiquitination of lysine K5 is facilitated by an intermolecular mechanism in *trans*. Shown is anti-Cdc34 WB of ubiquitination reactions with ^{Myc}Rbx1/Cdc53 complex immobilized on anti-Myc beads, Ub^{mix} (ubiquitin, Uba1, and ATP), and either Cdc34$^{\Delta C}$, the lysineless ^{K0}Cdc34$^{\Delta C}$, the catalytically inactive ^{C95A}Cdc34$^{\Delta C}$ mutant with intact lysines, or a combination of these, as indicated (see the text for details). (E) Autoubiquitination of the C-terminal lysine K293 cannot be facilitated in *trans*, suggesting an intramolecular mechanism in *cis*. The experiment was performed as for panel D, but with the full-length Cdc34 constructs, as indicated.

tions (3), is quenched in the presence of ^{Gst}Skp1/Cdc53/Rbx1 complex (an F-box protein-free SCF core) (Fig. 1A, top, lanes 1 to 5). The quenching is specific to autoubiquitination of the C-terminal lysine, because under the same conditions, SCF core stimulates autoubiquitination of lysines within the Cdc34$^{\Delta C}$ protein (Fig. 1A, bottom, lanes 1 to 5). Cdc34$^{\Delta C}$ is a C-terminal deletion mutant terminated at amino acid 244 (Fig. 2A) that complements deletion of the *CDC34* gene in vivo (25, 36, 40), indicating that it recapitulates the essential function of Cdc34.

Analysis of the autoubiquitination rates with the single-lysine-containing Cdc34$^{\Delta C}$ mutant proteins revealed that lysines K5, K61, and K151 are the main targets for SCF core-dependent autoubiquitination, all undergoing autoubiquitination at the representative rate illustrated by the example of K5 (Fig. 1B, anti-Cdc34 WB, lanes 9 to 12). In contrast, lysines K23, K24, K157, K168, and K189 are not autoubiquitinated (Fig. 1B, anti-Cdc34WB, lanes 13 to 16). The differences in autoubiquitination rates reflect differences associated specifically with the target lysine residues on Cdc34, because all Cdc34 mutant proteins have similar activities in the SCF core-mediated ubiquitination of Cdc53 (Fig. 1B, anti-Cdc53 WB) and in the ^{Gst}SCF^{Cdc4}-mediated ubiquitination of Sic1 (data not shown), the prototype substrate of Cdc34/SCF^{Cdc4} (10, 41).

SCF core stimulates autoubiquitination of lysine K5 in the context of either Cdc34$^{\Delta C}$ or the full-length Cdc34 protein (Fig. 1C, lanes 13 to 16 and 5 to 8), suggesting that the N-

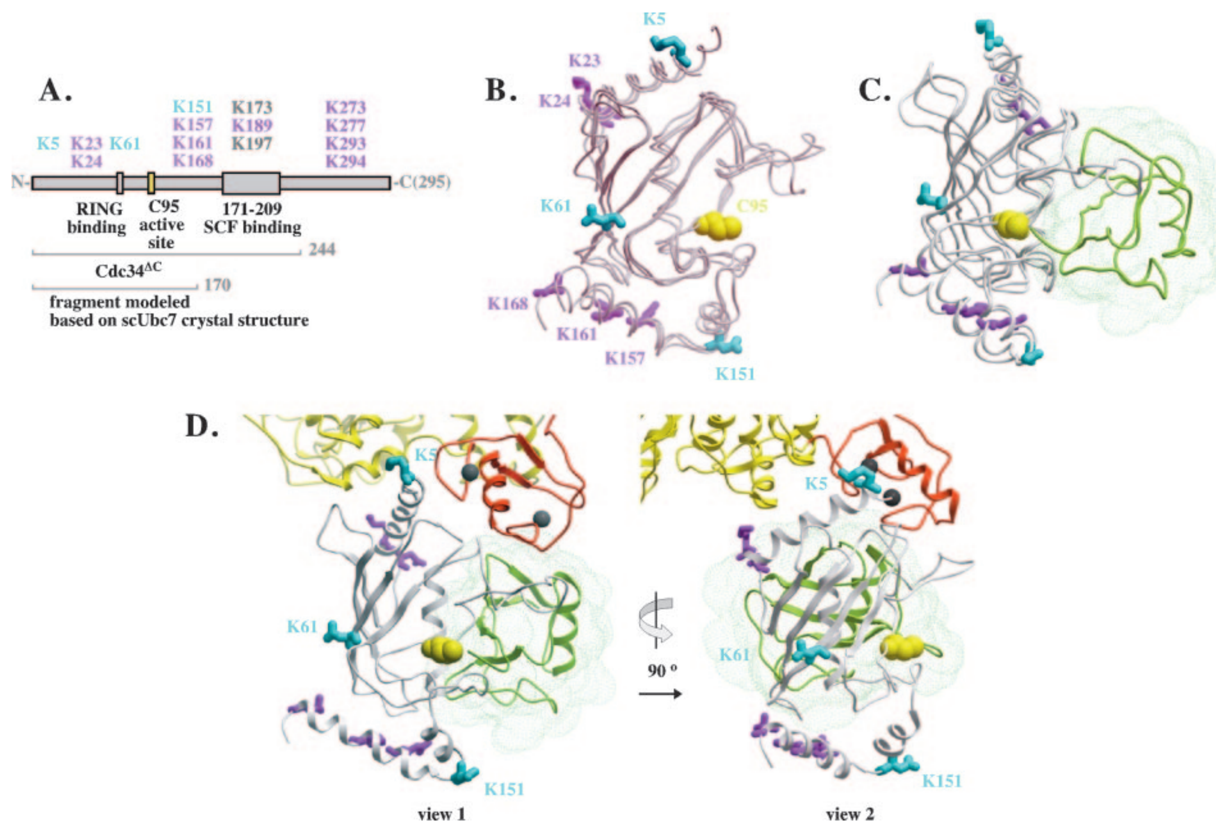

FIG. 2. Localization of lysines in the Cdc34 structure. (A) Scheme of Cdc34 protein. Lysines are marked in a color code as follows: turquoise, lysines whose autoubiquitination is stimulated by SCF; magenta, lysines that are not autoubiquitinated in the presence of SCF; gray, lysines that were not tested because the appropriate single-lysine mutant proteins were insoluble. Residue 244 represents the C-terminal end in the Cdc34$^{\Delta C}$ mutant that includes all determinants essential for Cdc34 function. Among these are the conserved core (aa 1 to 170) with the active-site Cys95 and the E3 RING domain-interacting fragment and the fragment of 39 amino acids (aa 171 to 209) unique to Cdc34 and implicated in binding to SCF (32) and oligomerization (36). (B) Mapping of the residues corresponding to Cdc34 lysines in the structure of ubiquitin-free scUbc7 monomer. Residues corresponding to Cdc34 lysines in the crystal structure of scUbc7 fragment (6) are shown in stick representation, using the same color code as in panel A. The active-site cysteine in Cdc34 is marked by yellow CPK representation. An ~33-Å space separates the position of K5 and the active site (not shown). To demonstrate similarity of the overall E2 fold, the structure of scUbc7 (6) was superimposed on the structure of human Ubc7 (hUbc7) crystallized either alone (1) or in complex with the c-Cbl RING domain (48). (C) Charging with activated ubiquitin does not have a major effect on the position of lysines. The structure of scUbc7 (6) with lysines marked as in panel B was superimposed on the structure of Ubc1~ubiquitin thiol ester (Protein Data Bank [PDB] identifier, 1FXT) (17). The ubiquitin molecule is shown by a green surface representation. (D) Localization of lysines in the context of RING domain-bound, ubiquitin-charged scUbc7. The structure of scUbc7~ubiquitin (gray; modeled as in panel B) was superimposed on the structure of the hUbc7/c-Cbl RING domain (PDB identifier, 1FBV) (48) by overlaying the E2 structures. Red indicates the RING domain. Views 1 and 2 show alternative orientations of the same structure. The superposition and figures were done with the ICM program (4).

terminal autoubiquitination of Cdc34$^{\Delta C}$ and Cdc34 are catalyzed by similar mechanisms.

Unlike autoubiquitination of the C-terminal lysines, autoubiquitination of the N-terminal lysines is intermolecular. Structural similarity between N-terminal fragments of E2 ubiquitin-conjugating enzymes allowed us to analyze the possible localization of lysines in Cdc34 in the context of a ubiquitin-free (Fig. 2B) or ubiquitin-charged (Fig. 2C) monomeric structure and in the context of a monomer bound to the RING domain of E3 (Fig. 2D). None of the N-terminal lysines identified as targets for the SCF-dependent autoubiquitination (Fig. 2) is located in proximity to the active site, and one lysine, K5, is located in the E2/E3 RING interaction interface, arguing against an intramolecular autoubiquitination mechanism.

Indeed, we found that in the presence of ^{Myc}Rbx1/Cdc53, the catalytically active ^{K0}Cdc34$^{\Delta C}$ mutant protein that could not autoubiquitinate itself because of the lack of lysines (Fig. 1D, lanes 5 to 8) could ubiquitinate the ^{C95A}Cdc34$^{\Delta C}$ mutant protein (Fig. 1D, lanes 13 to 16), which, due to the lack of the catalytic site, could not autoubiquitinate its own lysine residues (Fig. 1D, lanes 9 to 12).

In contrast, the SCF-independent autoubiquitination of the C-terminal residue K293, detectable in the context of the catalytically active ^{K0}Cdc34^{R293K} mutant (Fig. 1E, lanes 5 to 8) but not the catalytically inactive ^{C95A,K0}Cdc34^{R293K} (Fig. 1E, lanes 13 to 16), could not be reconstituted via *trans* interactions with the catalytically active but lysineless ^{K0}Cdc34 (Fig. 1E, lanes 17 to 20).

Thus, unlike autoubiquitination of the N-terminal lysines, which is an intermolecular reaction in *trans* stimulated by SCF, autoubiquitination of C-terminal lysines is catalyzed

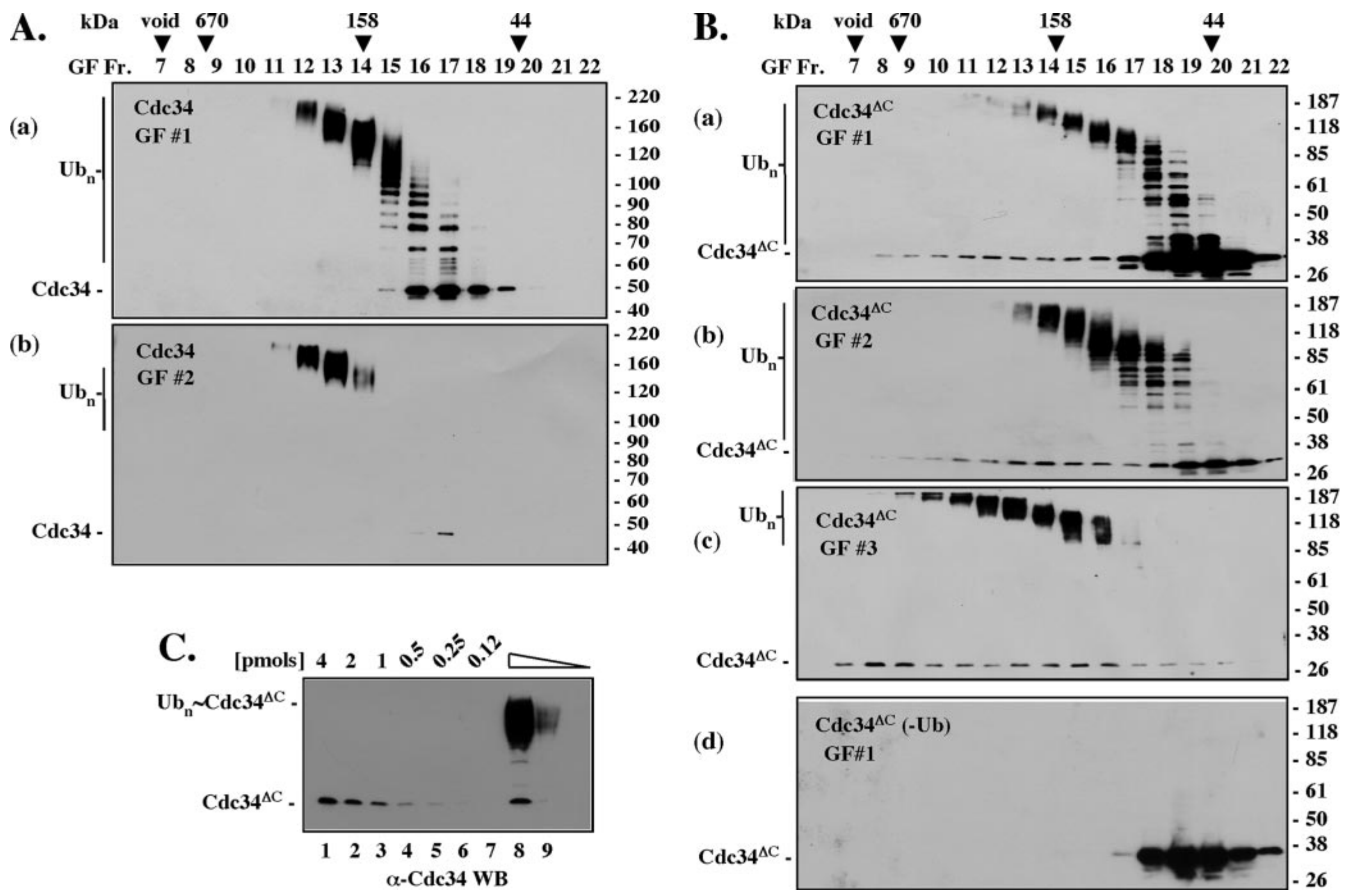

FIG. 3. Preparation of quantitatively polyubiquitinated Cdc34. (A) Cdc34 is autoubiquitinated in an SCF-independent manner. ^{His}Cdc34 protein was autoubiquitinated in the absence of SCF, followed by HPLC on a Superdex 200 column (GF no. 1), pooling of fractions (Fr.) 11 to 14, ~4-fold reconcentration on Centricon 10, and a second round of HPLC on a Superdex 200 (GF no. 2). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis/WB was performed with 3% of each fraction (15 out of 500 μl). Ub, ubiquitin. GF, gel filtration. (B) Cdc34$^{\Delta C}$ is autoubiquitinated in an SCF-dependent manner. The experiment was performed as for panel A, except that purified ^{His}Cdc34$^{\Delta C}$ protein was autoubiquitinated in the presence of anti-Myc beads bearing an ^{Myc}Rbx1/Cdc53 protein complex prior to HPLC of the bead-free supernatants and that different GF fractions, 12 to 16, were polled. Image d shows elution of control Cdc34$^{\Delta C}$ protein incubated in the absence of Uba1 and ubiquitin. (C) Representative quantitation of polyubiquitinated Cdc34$^{\Delta C}$. (Left) Anti-Cdc34 WB with picomolar amounts of nonubiquitinated Cdc34$^{\Delta C}$ (lanes 1 to 7), as indicated, and 3 μl (lane 8) or 0.3 μl (lane 9) of polyubiquitinated Ub$_n$~Cdc34$^{\Delta C}$ (GF. no. 3, fractions 12 to 16, concentrated). WB intensities were quantitated with ImageQuant and plotted as a function of a known amount of [^{35}S]methionine-labeled Cdc34$^{\Delta C}$ protein (570 cpm/pmol) (data not shown). Once calibrated, Image Quant WB intensities were used to estimate the amounts of polyubiquitinated Cdc34 and/or Cdc34$^{\Delta C}$ without radiolabeling (see Materials and Methods).

via an intramolecular mechanism that is quenched in the presence of SCF.

Autoubiquitination of N-terminal lysines blocks charging of the Cdc34 active site with ubiquitin and inhibits Cdc34 function with SCF^{Cdc4}. To test how autoubiquitination affects the activity of Cdc34, we first sought to prepare Cdc34 samples quantitatively autoubiquitinated on either the C-terminal or N-terminal lysine. Two rounds of gel filtration chromatography on a Superdex 200 HPLC column separated C-terminally polyubiquitinated Cdc34 from unmodified Cdc34 (Fig. 3A). The separation protocol was more complicated in the case of Cdc34$^{\Delta C}$. Cdc34$^{\Delta C}$ autoubiquitinated in an SCF-dependent manner accumulates in solution as an SCF-free protein (7). Cdc34$^{\Delta C}$ could thus be autoubiquitinated in the presence of bead-immobilized ^{Myc}Rbx1/Cdc53 and separated from the SCF components by centrifugation. However, unlike full-length Cdc34, autoubiquitinated Cdc34$^{\Delta C}$ could not be fully separated from unmodified Cdc34$^{\Delta C}$ by gel filtration chromatography (Fig. 3B, a to c). In mixtures lacking ubiquitin, Cdc34$^{\Delta C}$ does not elute in the high-molecular-weight range (Fig. 3B, d), suggesting that the coelution of nonubiquitinated and polyubiquitinated Cdc34$^{\Delta C}$ is associated with an aspect of autoubiquitination. Based on quantitative Western blot (WB) analysis (Fig. 3C) verified by autoradiography of [^{35}S]methionine-labeled Cdc34$^{\Delta C}$ (data not shown), we estimate that even the best of the final preparations of polyubiquitinated Cdc34$^{\Delta C}$ contain ~1% unmodified Cdc34$^{\Delta C}$.

Strikingly, we find that while C-terminally polyubiquitinated Ub$_n$~Cdc34 is comparable to unmodified Cdc34 in the ubiquitination of Sic1, the SCF^{Cdc4}-dependent substrate for Cdc34 (Fig. 4A, anti-Sic1 WB; cf. anti-Cdc34 WB), the activity of Ub$_n$~Cdc34$^{\Delta C}$ preparation is severely reduced (Fig. 4B, a, compare lanes 1 to 5 with 11 to 15). The loss of Ub$_n$~Cdc34$^{\Delta C}$ activity is striking, even compared with an ~10-fold-smaller

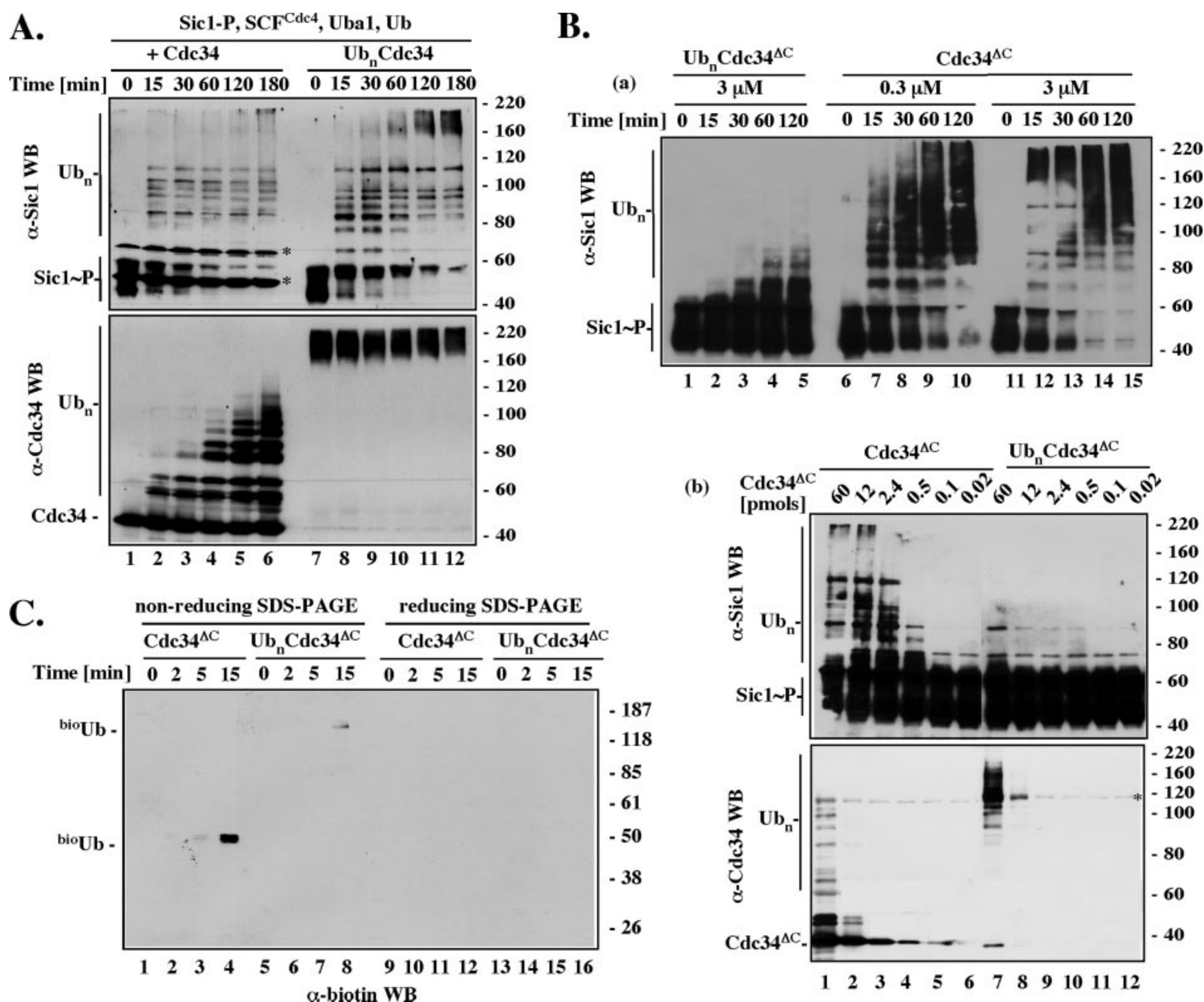

FIG. 4. Autoubiquitination of N-terminal, but not C-terminal, lysines inhibits Cdc34 function. (A) Cdc34 preautoubiquitinated on C-terminal lysines is active in the SCF^{Cdc4}-dependent ubiquitination of Sic1. SCF^{Cdc4}-dependent ubiquitination of phosphorylated Sic1 protein (Sic1-P) was performed as described in Materials and Methods, using 3 μM of Cdc34 (lanes 1 to 6) or Ub$_n$~Cdc34 polyubiquitinated on C-terminal lysines (lanes 7 to 12). Polyubiquitinated Cdc34 was prepared as described in the legend to Fig. 3A and quantitated as described in the legend to Fig. 3C. The asterisks indicate the positions of unmodified Cdc34 cross-reacting with anti-^{GstMyc}Sic1 antibodies. Ub, ubiquitin. (B) Cdc34$^{\Delta C}$ preautoubiquitinated on N-terminal lysines is defective in the SCF^{Cdc4}-dependent ubiquitination of Sic1. (a) The experiment was performed as for panel A, except that we used either 0.3 μM (lanes 6 to 10) or 3 μM (lanes 11 to 15) of nonubiquitinated Cdc34$^{\Delta C}$ as a reference for the activity of 3 μM of Ub$_n$~Cdc34$^{\Delta C}$ polyubiquitinated on N-terminal lysines (lanes 1 to 5). Polyubiquitinated Cdc34$^{\Delta C}$ was prepared as described in the legend to Fig. 3B and quantitated as described in the legend to Fig. 3C. (b) Sic1 ubiquitination with various amounts of either nonubiquitinated (lanes 1 to 6) or preautoubiquitinated (lanes 7 to 12) Cdc34$^{\Delta C}$, as indicated. (C) Autoubiquitination of N-terminal lysines compromises charging of the Cdc34 active site. Cdc34$^{\Delta C}$ autoubiquitinated on N-terminal lysines (as in panel B) was incubated with purified Uba1 and biotinylated ubiquitin for the times indicated, followed by analysis in nonreducing and reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as indicated, and anti-biotin WB.

amount of unmodified Cdc34$^{\Delta C}$ (Fig. 4B, a, compare lanes 1 to 5 with lanes 6 to 10). The residual activity observed in these preparations could be accounted for by the fraction of nonubiquitinated Cdc34$^{\Delta C}$, as a similar ubiquitination of Sic1 can be observed with a comparable amount of unmodified Cdc34$^{\Delta C}$ (Fig. 4B, b, lanes 4 and 7). Further, preparations of Cdc34$^{\Delta C}$ autoubiquitinated on the N-terminal lysines are defective in formation of the ubiquitin-thiol ester (Fig. 4C).

Thus, the SCF-independent polyubiquitination of C-terminal lysines has no effect on Cdc34 function. In contrast, Cdc34 autoubiquitinated on N-terminal lysines is not functional.

Lysines are not required for Cdc34 function with SCF in vitro. The negative regulatory role of SCF-mediated autoubiquitination raised the possibility that autoubiquitination is part of the catalytic cycle, contradicting our previous conclusion that autoubiquitination is a by-product of Cdc34 function with SCF typical for conditions of high Cdc34 concentration (7). To test whether autoubiquitination is essential for Cdc34 function, we generated a lysineless ^{K0}Cdc34$^{\Delta C}$ mutant protein that, due to the lack of lysines, could not be autoubiquitinated (Fig. 1B, lanes 5 to 8).

Recombinant ^{K0}Cdc34$^{\Delta C}$ protein purified from *E. coli* is

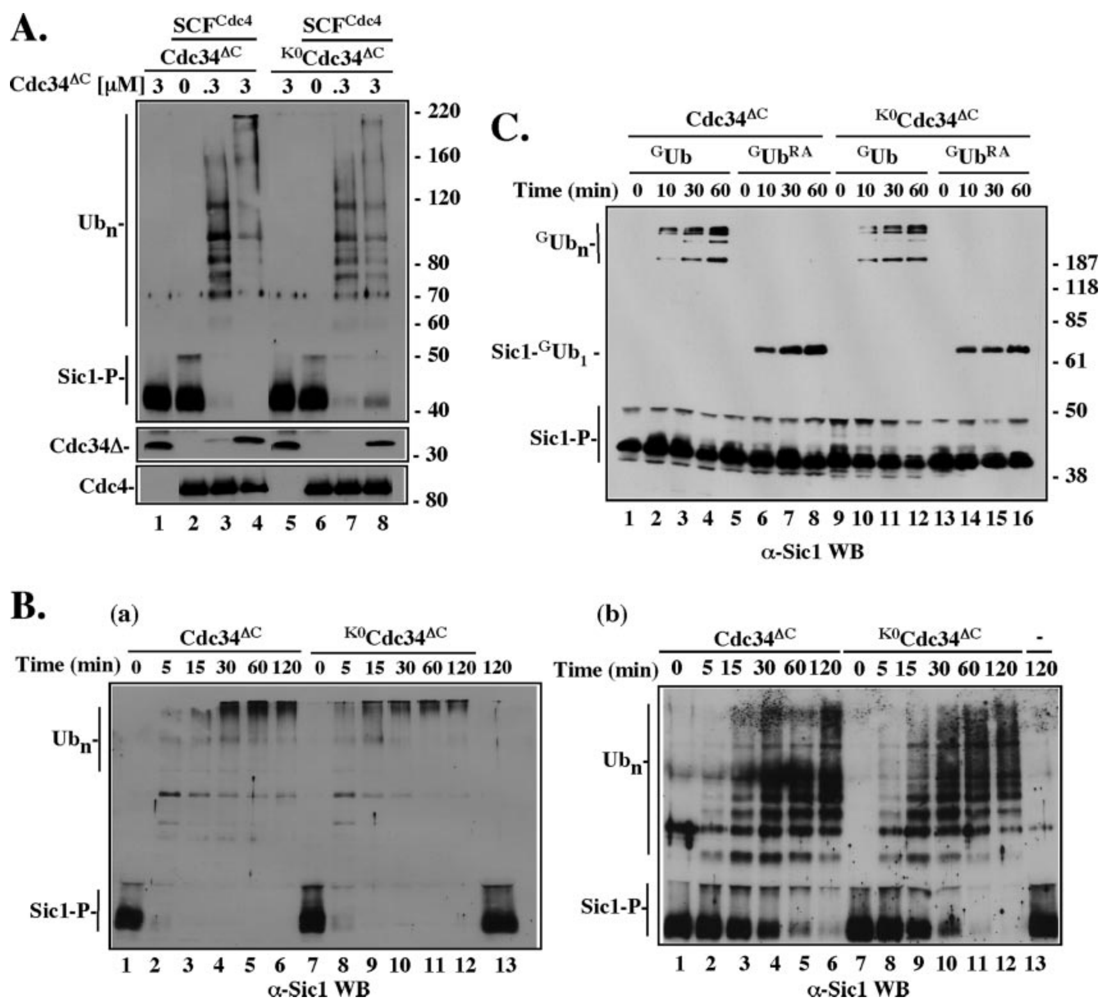

FIG. 5. Recombinant ^{K0}Cdc34$^{\Delta C}$ purified from *E. coli* is fully functional in vitro. (A) Similar concentrations of Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ proteins are required for Sic1 ubiquitination. Shown is anti-Sic1 WB (top) of Sic1 ubiquitination assays performed as described in Materials and Methods, with different amounts of Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$, as indicated. Cdc4 represents one of the ^{GST}SCF^{Cdc4} subunits and is shown as a control. Ub, ubiquitin. (B) Similar kinetics of Sic1 ubiquitination. The experiment was performed as for panel A, but with different reaction times with either a high, 3 μM (a), or low, 0.3 μM (b), concentration of Cdc34 or ^{K0}Cdc34$^{\Delta C}$, as indicated. (C) Like Cdc34 and Cdc34$^{\Delta C}$, ^{K0}Cdc34$^{\Delta C}$ modifies Sic1 exclusively with a K48 type of polyubiquitin. The assay was performed as for panel B, a, but with a ^{GST}Ub or ^{GST}UbRA mutant that could not form K48-type linkage, as indicated.

comparable to Cdc34$^{\Delta C}$ under several experimental conditions. We observed similar concentration requirements for Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ in the SCF^{Cdc4}-dependent ubiquitination of Sic1 (Fig. 5A) and similar kinetics of Sic1 ubiquitination either at a high, 3 μM (Fig. 5B, a), or at a low, 0.3 μM (Fig. 5B, b), concentration of Cdc34$^{\Delta C}$ or ^{K0}Cdc34$^{\Delta C}$. Like Cdc34$^{\Delta C}$, ^{K0}Cdc34$^{\Delta C}$ synthesizes primarily K48-type polyubiquitin, as in both cases the polymerization reaction is blocked in assays with ^{Gst}Ub^{RA} mutant protein (Fig. 5C, lanes 5 to 8 and 13 to 16; compare this with reactions performed with ^{Gst}Ub, lanes 1 to 4 and 9 to 12), which cannot form K48-based linkages. Direct mass spectrometric analysis of the type of isopeptide linkage formed in reactions with wild-type ubiquitin confirmed this observation (24).

Based on these data, we concluded that lysines are not required for Cdc34$^{\Delta C}$ function in vitro.

Lysines are not required for Cdc34 function in vivo. To test whether ^{K0}Cdc34 protein is functional in vivo, we replaced the chromosomal copy of the *CDC34* gene in yeast with either the *cdc34*$^{\Delta C}$::*URA* allele, recreating the viable strain characterized previously (25, 36, 40), or with the lysineless-protein-encoding mutant allele ^{K0}*cdc34*$^{\Delta C}$::*URA*.

Yeasts carrying either the *cdc34*$^{\Delta C}$::*URA* or the ^{K0}*cdc34*$^{\Delta C}$::*URA* allele instead of *CDC34* are viable at a wide range of temperatures (data not shown) and have no obvious growth phenotype on yeast-peptone-dextrose (YPD), synthetic dextrose (SD), or SD/URA drop-out plates (Fig. 6A, c and d), even when combined with a *RUB1* deletion (Fig. 6A, e and f) known to sensitize growth to defects in Cdc34 and/or SCF (26). A similar conclusion can be drawn based on fluorescence-activated cell sorter analysis monitoring the timing of cell cycle progression in cells released from α-factor arrest in early G_1 phase (data not shown). These observations are in striking contrast to the classic growth arrest phenotype typical for several *cdc34* and *scf* mutants defective in degradation of Sic1, the key substrate of Cdc34/SCF^{Cdc4} at the G_1/S phase transition.

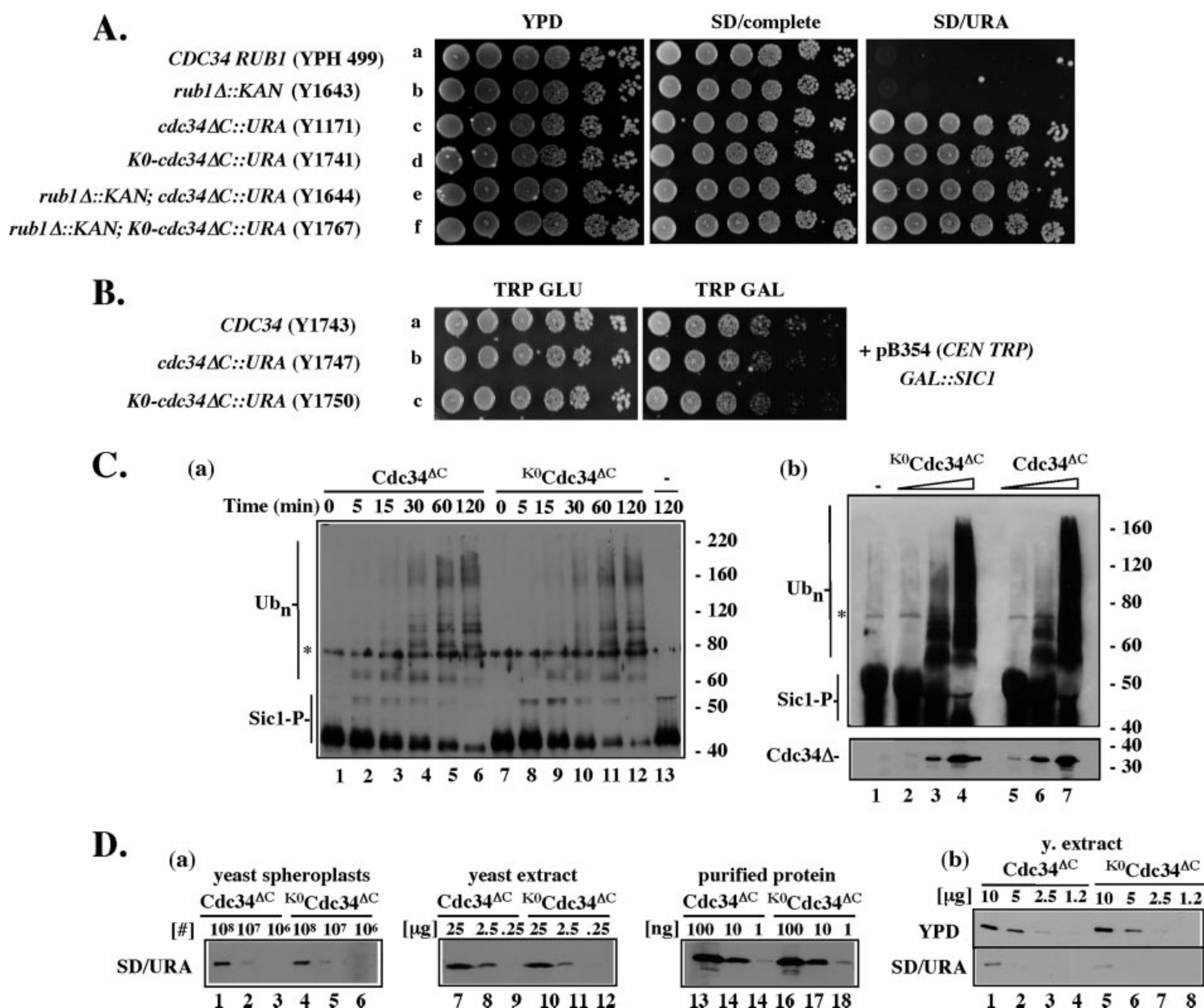

FIG. 6. Lysines are not essential for Cdc34 function in vivo. (A) Growth of yeast expressing *CDC34*, *cdc34*$^{\Delta C}$, or ^{K0}*cdc34*$^{\Delta C}$ from the chromosomal locus. Growth assays were done at 30°C on plates as indicated using yeast with chromosomal copies of *CDC34* and *RUB1* (YPH499); *rub1*Δ (Y1643); *cdc34*$^{\Delta C}$ (YKK1171); ^{K0}*cdc34*$^{\Delta C}$ (YKK1741); *rub1*Δ *cdc34*$^{\Delta C}$ (Y1644); and *rub1*Δ ^{K0}*cdc34*$^{\Delta C}$ (Y1767), as indicated. (B) Effect of Sic1 overexpression. Growth was tested at 30°C on synthetic TRP plates with glucose or galactose, using the indicated strains transformed with pB354 (CEN TRP) *GAL*::*SIC1* plasmid. (C) In vitro Sic1 ubiquitination assay with Cdc34$^{\Delta C}$ or ^{K0}Cdc34$^{\Delta C}$ protein purified from yeast. (a) Anti-Sic1 WB of the kinetics of Sic1 ubiquitination with ~3 μM Cdc34$^{\Delta C}$ or ^{K0}Cdc34$^{\Delta C}$, as indicated. (b) Sic1 ubiquitination assay with various amounts (10-fold dilutions) of Cdc34$^{\Delta C}$ or ^{K0}Cdc34$^{\Delta C}$. (Top) Anti-Sic1 WB. (Bottom) Anti-Cdc34 WB. (D) Quantitative WB analysis of the steady-state levels of Cdc34$^{\Delta C}$ and ^{K0}Cdc34ΔC proteins expressed from the chromosomal locus in yeast. Y1171 (Cdc34$^{\Delta C}$) and Y1741 (^{K0}Cdc34$^{\Delta C}$) strains were grown on SD/URA (a) or YPD (b) growth medium. WBs were performed using known numbers of yeast spheroplasts or total proteins in crude extract, with reference to purified recombinant proteins, as indicated. The conditions of WB in b were adjusted in such a way that the larger amounts of Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ proteins expressed during growth on YPD could be detected in a linear range (top). Under those conditions, the levels of Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ expression during growth in SD/URA medium were barely detectable (bottom).

Overproduction of Sic1, expected to sensitize the growth assay to a possible difference in the activities of Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$, also did not reveal a difference in the growth phenotypes of *CDC34*, *cdc34*$^{\Delta C}$, or ^{K0}*cdc34*$^{\Delta C}$ yeast (Fig. 6B, a to c). Finally, the activities of endogenous ^{K0}Cdc34$^{\Delta C}$ and Cdc34$^{\Delta C}$ proteins purified from yeast are comparable in the SCF^{Cdc4}-dependent ubiquitination of Sic1 in vitro (Fig. 6C, a and b).

Quantitative WB analysis performed with yeast spheroplasts (Fig. 6D, a, lanes 1 to 6) or crude extracts (Fig. 6D, a, lanes 7 to 12) revealed similar steady-state levels of Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ proteins in a range of detection suitable to visualize 1 to 10 ng of Cdc34 (equivalent to 30 to 300 fmol of Cdc34 monomers) (Fig. 6D, a, lanes 13 to 18). Growth on the rich YPD medium led to about a fourfold increase in the steady-state levels of both Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ (Fig. 6D, b, compare top and bottom), eliminating the possibility that the lack of a growth phenotype results from a difference in the levels of Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$. The Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ proteins accumulated to similar levels when overexpressed from the *GAL1* promoter located on the pRS414 *TRP CEN*-based vector (Fig. 7B). Overexpression of Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ had similar growth-inhibitory effects regardless of whether it was performed in yeast with chromosomal *CDC34* (Fig. 7A, a to c), *cdc34*$^{\Delta C}$ (Fig. 7A, d and e), or ^{K0}*cdcd34*$^{\Delta C}$ (Fig. 7A, f and g).

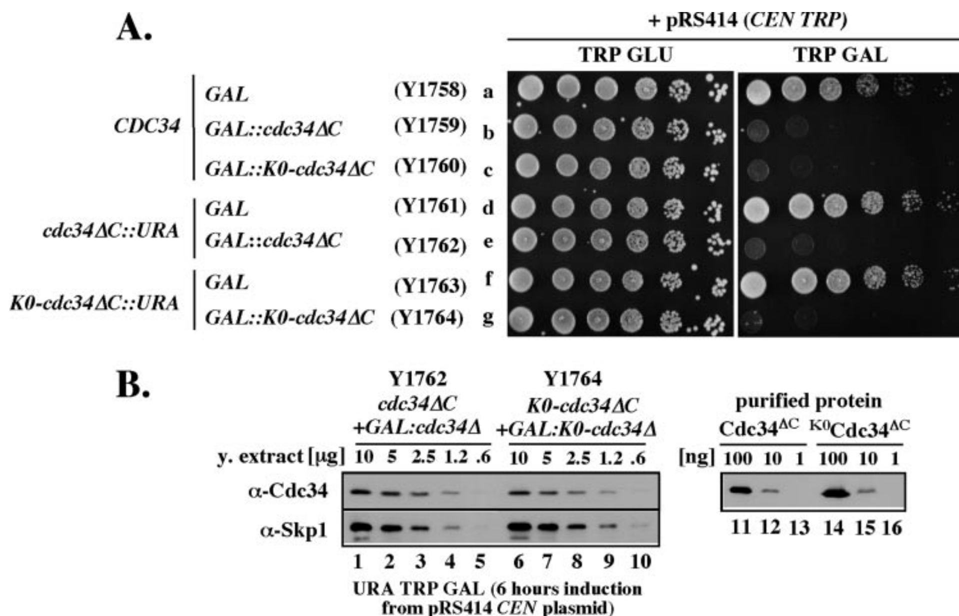

FIG. 7. Overexpression from the GAL1 promoter leads to accumulation of similar steady-state levels of ^{K0}Cdc34$^{\Delta C}$ and Cdc34$^{\Delta C}$ and has comparable growth-inhibitory effects. (A) Growth effects. Growth assays were done at 30°C on plates as indicated using yeast with chromosomal copies of *CDC34* (YPH499), *cdc34*$^{\Delta C}$ (YKK1171), or ^{K0}*cdc34*$^{\Delta C}$ (YKK1741) transformed with pRS414 (*CEN TRP*) plasmids carrying no extra open reading frame (a, d, and f), *cdc34*$^{\Delta C}$ (b and e), or ^{K0}*cdc34*$^{\Delta C}$ (c and g), as indicated. (B) Steady-state levels of the overproduced Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ proteins. Shown is quantitative anti-Cdc34 (α-Cdc34) WB analysis of Cdc34$^{\Delta C}$ and ^{K0}Cdc34$^{\Delta C}$ proteins in crude extracts prepared from yeast Y1762 and Y1764 grown in URA TRP GAL medium for 6 h. A control anti-Cdc34 WB, on the right, indicated that the proteins were overproduced by at least 10-fold (compare with Fig. 6D).

Thus, ^{K0}Cdc34$^{\Delta C}$ appears to be fully active in vitro and in vivo, demonstrating that lysines play no critical role in Cdc34 function.

DISCUSSION

In this study, we addressed the mechanism and the role of autoubiquitination of the yeast Cdc34 E2 ubiquitin-conjugating enzyme that functions with the RING-type SCF E3s. Our analysis revealed three new aspects of the autoubiquitination phenomenon. First, the SCF-dependent reactions are associated with a change in the mechanism of autoubiquitination (intramolecular versus intermolecular) and in the selection of target lysines (C-terminal versus N-terminal lysines). Second, unlike the SCF-independent autoubiquitination of the C-terminal lysines, which has no effect on Cdc34 function, autoubiquitination of the N-terminal lysines inhibits Cdc34. Third the autoubiquitination-based inhibitory mechanism plays a nonessential role in Cdc34 function with SCF in vitro and in vivo, at least in the context of the baking-yeast system.

The observation that SCF changes the mechanism of autoubiquitination from intra- to intermolecular in a manner linked to a change in the selection of target lysines is perhaps the most striking finding in this report, with no precedent in the literature. Our data suggest that this change is associated with a rearrangement in the position of the Cdc34 C terminus. To serve as targets for intramolecular reactions in the absence of SCF, the C-terminal lysines of Cdc34 would have to be located in proximity to the active site of the same molecule. In contrast, in the presence of SCF, the C-terminal lysines are not accessible to modifications via either an intra- or intermolecular mechanism, suggesting that the C terminus is shielded.

While the shielding effect indicates an SCF-dependent change within the C terminus of Cdc34, the most distant part of the C terminus, including the C-terminal lysines (aa 244 to 295), is unlikely to play a role in Cdc34 activation by SCF. Indeed, autoubiquitination of lysine K5 is stimulated by SCF in similar manners in the contexts of Cdc34 and Cdc34$^{\Delta C}$ (this work). However, in addition to being shielded, the C terminus would also have to be moved away from its original location near the active site, as in the presence of SCF, the active site of Cdc34 can autoubiquitinate N-terminal lysines on other Cdc34 molecules. This rearrangement could result from direct binding of SCF to the essential part of the C terminus of Cdc34 (aa 171 to 209) (32) and could play a role in Cdc34 activation.

Whether the presumed SCF-dependent conformational change activates Cdc34 directly, by promoting a subtle conformational change near the active site, or indirectly, by promoting Cdc34 oligomerization, has yet to be established. On the one hand, the C-terminal fragment of Cdc34 implicated in SCF binding (32) has also been shown to play a role in Cdc34 oligomerization (36). Dimerization of human Cdc34 via fusion to glutathione *S*-transferase or FK506-binding protein mimics the requirement for SCF in Cdc34 activation in vitro (13), suggesting that SCF could activate Cdc34 by promoting its oligomerization. A further link between Cdc34 oligomerization and function is suggested by the finding that charging of the Cdc34 active site with ubiquitin, a prerequisite for a functional interaction with SCF (7), allows detection of the Cdc34-Cdc34 interaction in yeast extracts (45). On the other hand, Cdc34

oligomerization has been proposed to be independent of SCF, based on the observation that the Cdc34-Cdc34 interaction could be detected in SCF mutant strains defective in Cdc34 binding (45). While this interpretation is valid, it has since been shown that reduced binding between E3 and E2 does not necessarily indicate a nonfunctional interaction (7, 47). Further, coimmunoprecipitation experiments cannot discriminate between the possibility that charging with activated ubiquitin is sufficient to promote Cdc34 dimerization and that SCF either stimulates this process or facilitates formation of higher-order oligomers. In light of the observation that purified SCF stimulates oligomerization of the ubiquitin-charged Cdc34 in vitro (M. Scaglione and D. Skowyra, unpublished data), further analysis of this phenomenon in the in vitro system is likely to shed light on its role and mechanism.

Our analysis demonstrates that, unlike autoubiquitination of the C-terminal lysines, which has no direct effect on Cdc34 function, autoubiquitination of the N-terminal lysines inhibits Cdc34. The lack of a direct functional effect associated with autoubiquitination of the C-terminal lysines does not eliminate the possibility that this phenomenon protects other proteins, including other Cdc34 molecules, from random modification out of the SCF context. Indeed, both in yeast extracts and in a typical in vitro assay, the majority of total Cdc34 exists as an SCF-free protein (7). The fact that in the context of reactions with SCF the activity of Cdc34 is also guarded by an intrinsic autoinhibitory mechanism suggests that the function of the SCF-activated Cdc34 is not restricted to a specific, SCF binding-controlled context. Indeed, while autoubiquitination is typically catalyzed at a lower rate than ubiquitination of a bona fide SCF substrate (7), several reports link instability of the F-box protein subunits of SCF (12, 18, 31, 46), and even the Cul1 subunit of SCF (18), to autocatalytic mechanisms.

A possible explanation of how autoubiquitination of specific N-terminal lysines blocks Cdc34 function with SCF is suggested by the observation that autoubiquitinated Cdc34$^{\Delta C}$ is defective in charging with activated ubiquitin and that at least one of the N-terminal lysine residues, K5, is located in the domain of Cdc34 predicted to be a key contact in binding to both SCF (reference 48 and this work) and Uba1 (8, 20). Autoubiquitination could thus effectively eliminate Cdc34 from the ubiquitin transfer cascade. On the other hand, the localization of lysine K5 in the Cdc34-RING interface suggests that this residue is shielded from autoubiquitination when Cdc34 is bound to the RING domain. Assuming that the binding with RING represents a functional contact, the only mechanism by which SCF could stimulate modification of lysine K5 would be in *trans*, as revealed by our analysis. Whether the SCF-bound Cdc34 modifies another Cdc34 molecule not associated with SCF or the modification is a result of an interaction between activated Cdc34 diffusing from SCF (7) remains to be determined.

It is surprising that such an elaborate and effective autoinhibitory mechanism would be preserved in the course of evolution without playing an essential role. One possible explanation is that autoubiquitination is not essential in the context of *S. cerevisiae* but plays an essential role in more advanced organisms. Indeed, modification of the Cdc53 subunit of SCF with the ubiquitin-like protein Rub1/Nedd8, which activates SCF and is the subject of elaborate control by the COP9 signalosome (46, 49), is essential in *Schizosaccharomyces pombe* and higher organisms but not in *S. cerevisiae* (26; reviewed in reference 33). Another possibility is that the autoinhibitory effect, which depends on interaction between Cdc34 molecules, is enhanced under conditions of Cdc34 accumulation. While the steady-state levels of Cdc34 remain unchanged during the cell cycle (16), a local accumulation of SCF components could lead to a local accumulation of Cdc34, and such a change, even when transient, may need to be self-guarded by the autoinhibitory mechanism. Indeed, both Cdc34 and SCF components can localize to specific compartments, including centrosomes (11, 37) that organize the mitotic spindle. Even a fivefold enrichment of the total cellular pool of Cdc34 in mammalian cells is sufficient to inhibit an association of CENP-E with kinetochores and to either delay or block metaphase alignment of chromosomes (11, 28, 44). A similar four- to fivefold accumulation of Cdc34 has been linked to the development of pediatric T-cell acute lymphoblastic leukemia (9), suggesting that even a modest accumulation of Cdc34 could play a role in the development of disease.

Regardless of the possible scenario in which Cdc34 autoubiquitination could play a role, due to its inhibitory nature, this process would have to be reversible, implying an additional, still unexplored layer of regulation in Cdc34 function. This possibility is strengthened by the observation that autoubiquitination of Cdc34$^{\Delta C}$ is undetectable in vivo (reference 15 and this work) and that deubiquitinating enzymes have emerged as players in the functions of several ubiquitination systems, including Rsp5 (22, 23), Mdm2 (43), VHL (27), and even SCF, at least in the context of regulation by the COP9 signalosome (46, 49). Whether the same or distinct deubiquitinating enzymes are involved in the regulation of substrate ubiquitination and autoubiquitination and how their functions are coordinated with the catalytic cycle remain to be determined.

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