The loop-less tmCdc34 E2 mutant defective in polyubiquitination in vitro and in vivo supports yeast growth in a manner dependent on Ubp14 and Cka2

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Abstract

Background: The S73/S97/loop motif is a hallmark of the Cdc34 family of E2 ubiquitin-conjugating enzymes that together with the SCF E3 ubiquitin ligases promote degradation of proteins involved in cell cycle and growth regulation. The inability of the loop-less Δ12Cdc34 mutant to support growth was linked to its inability to catalyze polyubiquitination. However, the loop-less triple mutant (tm) Cdc34, which not only lacks the loop but also contains the S73K and S97D substitutions typical of the K73/D97/no loop motif present in other E2s, supports growth. Whether tmCdc34 supports growth despite defective polyubiquitination, or the S73K and S97D substitutions, directly or indirectly, correct the defect caused by the loop absence, are unknown.

Results: tmCdc34 supports yeast viability with normal cell size and cell cycle profile despite producing fewer polyubiquitin conjugates in vivo and in vitro. The in vitro defect in Sic1 substrate polyubiquitination is similar to the defect observed in reactions with Δ12Cdc34 that cannot support growth. The synthesis of free polyubiquitin by tmCdc34 is activated only modestly and in a manner dependent on substrate recruitment to SCFCdc4. Phosphorylation of C-terminal serines in tmCdc34 by Cka2 kinase prevents the synthesis of free polyubiquitin chains, likely by promoting their attachment to substrate. Nevertheless, tmCDC34 yeast are sensitive to loss of the Ubp14 C-terminal ubiquitin hydrolase and DUBs other than Ubp14 inefficiently disassemble polyubiquitin chains produced in tmCDC34 yeast extracts, suggesting that the free chains, either synthesized de novo or recycled from substrates, have an altered structure.

Conclusions: The catalytic motif replacement compromises polyubiquitination activity of Cdc34 and alters its regulation in vitro and in vivo, but either motif can support Cdc34 function in yeast viability. Robust polyubiquitination mediated by the S73/S97/loop motif is thus not necessary for Cdc34 role in yeast viability, at least under typical laboratory conditions.

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Introduction

The covalent attachment of ubiquitin to other proteins often serves as the signal for their degradation by the 26 S proteasome [1]. Protein ubiquitination depends on a cascade of ubiquitin transfer reactions that begins with the formation of a high-energy thiolester between the C-terminus of ubiquitin and the catalytic site cysteine of the E1 ubiquitin-activating enzyme. The activated ubiquitin is transferred to the active site cysteine of one of many E2 ubiquitin-conjugating enzymes and then conjugated to specific substrates in a manner dependent upon specific E3 ubiquitin ligases. While the term “ubiquitin ligase” implies that all E3s are enzymes, only the HECT-type E3s contain a catalytic site cysteine that directly participates in the ubiquitin transfer. The RING-type E3s promote ubiquitination of specific substrates by the catalytic site of an E2. The significance of this difference is unclear, as both types of ubiquitin transfer cascades lead to formation of an isopeptide bond between the C-terminus of ubiquitin and a lysine residue on the substrate. Protein substrates can be modified with one or multiple ubiquitins and a chain of polyubiquitin can be synthesized when a lysine of ubiquitin serves as the isopeptide bond acceptor. A chain involving lysine 48 (K48) of ubiquitin and composed of at least 4 ubiquitins serves as the primary signal for substrate proteolysis [2,3].

The RING-type SCF E3s are the largest and arguably most extensively studied family of ubiquitin ligases, with members present in all eukaryotes [4]. The discovery of the SCF E3s was initiated by the observation that the S. cerevisiae CDC34 gene encodes an E2 [5] that together with the Cdc53, Skp1 and Cdc4 cell cycle regulatory proteins promote degradation of the Sic1 S-phase cyclin-dependent kinase inhibitor, thereby permitting entry into S phase [6,7]. A subsequent biochemical reconstruction [8-11] showed that Skp1, Cdc53, Rbx1 and Cdc4, an F-box protein, form the SCF<sup>Cdc4</sup> E3 complex. The large number of F-box proteins with different C-terminal protein-protein interaction motifs enables the family of SCF E3s to recruit many substrates for ubiquitination by Cdc34, usually in response to substrate phosphorylation.

Of the eleven E2 ubiquitin-conjugating enzymes in S. cerevisiae, only Cdc34 functions with SCF E3s <i>in vivo</i>. This specificity is attributed to the unique C-terminus of Cdc34 that includes a domain necessary for binding to SCF [12-14], because Cdc34 mutants that lack this C-terminal domain cannot support yeast viability and a fusion protein containing this domain and the catalytic core of Rad6 can replace Cdc34 function <i>in vivo</i>. Based on this evidence it has been proposed that the catalytic cores of the E2s are interchangeable [15,16].

On the other hand, three elements, S73, S97 and an acidic loop, distinguish the E2 cores of Cdc34 and Ubc7 from other ubiquitin conjugating enzymes (Figure 1A) [17]. The significance of these elements is best illustrated by the lethality associated with the substitution of residue S97 or deletion of the loop alone (Δ12) [17]. The loop-less Δ12Cdc34 monoubiquitinates the SCF<sup>Cdc4</sup>, dependent substrate Sic1 with a rate comparable to the rate of wild type Cdc34 (0.2 pmol/s) but synthesizes K48-type di-ubiquitin at a 10-fold slower rate (0.08 pmol/s vs. 0.8 pmol/s). Based on these findings it has been proposed that the loop plays a key role in the synthesis of K48-type polyubiquitin [18,19]. Similarly, the S97Δ replacement prevents self-association of Cdc34 molecules [20], a step implicated in the activation of polyubiquitination [21]. An E2 protein lacking both of these elements would be predicted to be even more defective and fail to support yeast growth. Contrary to this expectation, the S97Δ substitution and the loop deletion act as intragenic suppressors, and the suppression is best when combined with the S73K substitution, which by itself has no phenotype. The resulting triple mutant (tm) Cdc34 (S73K, S97Δ, Δ12) has the K73/Δ97/Δloop motif typical of other E2s, no longer displays the self-associating defect typical of the S97Δ substitution alone and supports the growth of cdc34-2ts mutant yeast at a non-permissive temperature. A recent genetic study shows that the <sup>ts</sup> CDC34 allele supports yeast growth even when expressed from the natural chromosomal location of <i>CDC34</i> [22]. However, this gene replacement causes numerous yet not fully understood changes in gene expression and evokes sensitivity to loss of several genes, including genes previously linked to function of the Cdc34/SCF pathway. Among these genes is CKA2, which encodes the catalytic subunit of casein kinase 2 that phosphorylates Cdc34 [23,24]; RPN10, which encodes the classic ubiquitin-binding receptor of the proteasome; and RAD23, which encodes the UBA-UBL type of a shuttle protein that genetically and biochemically interacts with RPN10 [25,26]. One of the key unanswered questions is whether <sup>ts</sup>Cdc34 supports cell growth despite compromised polyubiquitination, or the S73K and S97Δ substitutions, directly or indirectly, correct the defect in polyubiquitination associated with the loop absence.

We show that replacement of the catalytic motif compromises polyubiquitination activity of Cdc34 and alters its regulation <i>in vitro</i> and <i>in vivo</i>. Nevertheless, either motif can support Cdc34 function in cell growth and division, with normal cell size and cell cycle profile. These findings suggest that robust polyubiquitination catalyzed in a manner dependent on the S73/S97/loop motif is not necessary for Cdc34 function <i>in vivo</i>, at
least under typical laboratory conditions. The S73/S97/loop motif conservation could be associated with a yet unappreciated role of Cdc34, possibly under conditions of stress and/or compromised growth.

**Results**

Isogenic CDC34 and tmCDC34 yeast strains have similar growth properties, cell size and cell cycle profiles under typical laboratory conditions

Due to an essential role of Cdc34 in cell division [5], mutations that compromise Cdc34 function in vivo lead to cell cycle arrest. Monitoring growth and cell cycle phenotype is thus a common approach to assess the role of Cdc34 residues and/or motifs.

Previous analysis of growth phenotypes suggested that the triple mutant tmCdc34 in which the S73/S97/loop motif conserved among the Cdc34-like E2s is replaced with the K73/D97/no loop motif typical for other E2s (Figure 1A-D) supports yeast growth. This conclusion was based on complementation of the cdc34-2 temperature sensitive (ts) and cdc34Δ mutant yeast by over-expressed chimeric chCDC34/RAD6 [16] or triple mutant tmCDC34 [17]; an approach that could mask a functional defect due to overproduction of the complementing protein. However, we obtain similar results when cdc34-2ts yeast express the tmCDC34 allele from the GAL1 promoter under non-inducing conditions thereby ensuring only low level expression (Figure 1E). In contrast, growth is not supported by S97/CD34 that carries only the S97D replacement, or by Δ12CDC34 that lacks the loop alone. Disruption of the S73/S97/loop motif in Cdc34, but not its replacement with the alternative E2 core motif is thus lethal for yeast.

We next generated a yeast strain in which the wild-type ORF region of CDC34 is replaced by an ORF encoding tmCdc34, leaving the 5’ and 3’ regions intact [22]. The integration of tmCDC34 at the natural chromosomal locus of CDC34 supports yeast growth on plates (Figure 2A) and in liquid culture (Figure 2B). In liquid growth medium CDC34 (BL2) yeast have an estimated doubling time of 86 +/- 2 min and tmCDC34 (RC85) yeast double every 92 +/- 4 minutes. Apart from...
this modest difference, these strains have similar cell size and budding profiles, and accumulate to similar cell densities in stationary phase (Figure 2C). Analysis of DNA isolated from dividing \( \text{CDC34}^{\text{BL2}} \) and \( \text{tmCDC34}^{\text{RC85}} \) yeast verifies that they have similar cell cycle profiles under typical laboratory conditions (Figure 2D).

These findings verify that \( \text{tmCdc34} \) protein supports yeast growth despite lacking the catalytic core elements conserved among the Cdc34-like E2s. However, the steady-state level of \( \text{tmCdc34} \) protein in yeast extracts is approximately 4-fold higher than the level of Cdc34 (Figure 2E) even when extracts are prepared by boiling cells directly in SDS-PAGE loading buffer (data not shown). Since the increased steady-state level of \( \text{tmCdc34} \) is seen in a strain isogenic to the \( \text{CDC34} \) strain, the difference is likely due to a difference in post-translational regulation associated specifically with the replacement of the catalytic E2 motif.

**Purified \( \text{tmCdc34} \) forms an ubiquitin-thiolester and monoubiquitinates substrates, but is defective in substrate polyubiquitination**

To address how \( \text{tmCdc34} \) functions without the catalytic core elements conserved among the Cdc34-like E2s, we compared the various activities of purified \( \text{tmCdc34} \) to the activities of wild type Cdc34 and loop-less \( \Delta^{12}\text{Cdc34} \). The comparison of Cdc34 and \( \text{tmCdc34} \) was done with full-length proteins, but the comparison of all three catalytic cores (wt, \( \text{tm} \), \( \Delta^{12} \)) could be performed only with Cdc34 proteins terminated at residue 244 (Figure 1D) because full-length \( \Delta^{12}\text{Cdc34} \) is insoluble (this work).

The previous characterization of \( \Delta^{12}\text{Cdc34} \) was also done with a C-terminally truncated mutant (\( \Delta^{12}\text{Cdc34}^{270} \)) [18]. Ectopic expression of Cdc34\(^{244} \) rescues growth of \( \text{cdc34}^{\Delta} \) yeast [15,16,27] and supports yeast viability when expressed from the chromosomal location of \( \text{CDC34} \) [28], demonstrating that the C-terminal tails of Cdc34 proteins are functional, at least in the context of the wild type E2 core. All Cdc34 proteins used in this study elute as monomers in gel filtration chromatography (data not shown).

We first tested the ability of the purified Cdc34\(^{244} \), \( \Delta^{12}\text{Cdc34}^{244} \) and \( \text{tmCdc34}^{244} \) proteins to form ubiquitin-thiolester. Cdc34 molecules charged with an ubiquitin thiolester are stable only under conditions preventing ubiquitin discharge. Those conditions are ensured by the absence of SCF, substrate and several C-terminal lysines in Cdc34 that serve as intramolecular substrates.
The charging was performed for a short time (5 minutes) with 1 pmol of Uba1 E1 and 1-4 pmol of Cdc34\textsuperscript{244}, \textit{Δ}\textsubscript{12}Cdc34\textsuperscript{244} or \textit{tm}Cdc34\textsuperscript{244} followed by SDS-PAGE under either non-reducing conditions (to preserve the thiolester bond) or reducing conditions (to verify that the ubiquitin-thiolester is sensitive to reduction). The charging was not allowed to proceed to completion, thereby ensuring that we could detect an increase or decrease in the percentage of ubiquitin-charged E2 molecules. Under these assay conditions, similar fractions (10-50%) of total Cdc34\textsuperscript{244}, \textit{Δ}\textsubscript{12}Cdc34\textsuperscript{244} and \textit{tm}Cdc34\textsuperscript{244} molecules are converted into ubiquitin-thiolesters within a range of protein concentrations (Figure 3A). This result agrees with a previous finding that \textit{Δ}\textsubscript{12}Cdc34\textsuperscript{270} forms an ubiquitin-thiolester similarly to wild type Cdc34 [18]. Neither the loop deletion nor the triple mutant alteration of the E2 core thus compromises formation of the ubiquitin-thiolester.

We next tested the ability of full-length Cdc34 and \textit{tm}Cdc34 to autoubiquitinate one of their C-terminal lysines (Figure 1D) [28]. This reaction is inefficient and it is catalyzed only in the absence of SCF [28]. However, due to the intra-molecular nature [28], autoubiquitination provides unique insight into the intrinsic function of Cdc34. We find that autoubiquitination of full-length \textit{tm}Cdc34 is compromised (Figure 3B, α-Cdc34, compare lanes 6-9 and 1-5). The lack of high molecular weight species in reactions with wild type ubiquitin is consistent with a defect in polyubiquitination of monoubiquitinated \textit{tm}Cdc34, as it resembles the pattern observed with methylated ubiquitin that cannot be incorporated into polyubiquitin (Figure 3B, α-Cdc34, compare lanes 6-9, 11-14, and 15-18). However, longer reaction times are also needed to detect monoubiquitinated \textit{tm}Cdc34 in amounts similar to Cdc34 (Figure 3B, α-Cdc34, lanes 11-14 and 15-18). Furthermore, the intra-molecular nature of autoubiquitination [28] does not allow this assay to discriminate between catalytic defects or a change in the intra-molecular contacts between the E2 C-terminus and the E2 core.

**Figure 3** Purified \textit{tm}Cdc34 forms ubiquitin thiolester and monoubiquitinates substrates, but is defective in substrate polyubiquitination. All assays were performed at 25°C in 10-20 μL and included 1 pmol of Uba1 E1 and 1.3 nmol of ubiquitin or its derivative. (A). Formation of ubiquitin-thiolester. The indicated Cdc34\textsuperscript{244} proteins with wt, tm or \textit{Δ}\textsubscript{12} E2 core (1-4 pmol) were incubated for 5 minutes with Uba1 and ubiquitin followed by SDS-PAGE under reducing (+ βME) or non-reducing (- βME) conditions and α-Cdc34 WB. (B). Autoubiquitination of full length Cdc34 and \textit{tm}Cdc34. Full length Cdc34 or \textit{tm}Cdc34 (5 pmol) was incubated for the times indicated with Uba1 and ubiquitin or methylated ubiquitin followed by α-Cdc34 WB. (C). \textit{SCF}\textsuperscript{Cd4}-dependent polyubiquitination of Sic1. The indicated Cdc34\textsuperscript{244} proteins (5 pmol) were incubated for the times indicated with Uba1, the Sic1/Clb5/Clb2 substrate complex (2 pmol) and \textit{SCF}\textsuperscript{Cd4} (2 pmol) followed by 10% SDS-PAGE and α-Sic1 WB. Reactions shown in lanes 4, 8 and 12 do not have Sic1. (D). \textit{SCF}\textsuperscript{Methyl} dependent polyubiquitination of Met4. Tests were performed as described in C except that with the \textit{SCF}\textsuperscript{Methyl} E3 and \textit{Met}4 substrate. (E). \textit{SCF}\textsuperscript{Methyl} dependent monoubiquitination of \textit{Met}4. Tests as in D, but analyzed with shorter (1 instead of 5 minutes) western blot exposure time, which is necessary to visualize unubiquitinated and monoubiquitinated \textit{Met}4 as separate species due to similarities in their molecular weights.
The altered pattern of autoubiquitination nevertheless provides evidence of a profound intrinsic (independent of SCF) difference between the activities of \textsuperscript{tm}Cdc34 and Cdc34.

We next sought to analyze the activity of \textsuperscript{tm}Cdc34 in the presence of SCF E3. We obtained similar results with full-length \textsuperscript{tm}Cdc34 and C-terminally truncated \textsuperscript{tm}Cdc34\textsuperscript{244}. However, only in experiments with C-terminally truncated proteins terminated at residue 244 could we compare the activities of \textsuperscript{tm}Cdc34 and Cdc34 to the activity of \textsuperscript{Δ}Cdc34 due to the poor solubility of full-length \textsuperscript{Δ}Cdc34. In SCF\textsuperscript{Cdc4}\textsuperscript{244}-dependent assays with \textsuperscript{tm}Cdc34\textsuperscript{244}, high molecular weight species of the Sic1 substrate (indicative of its polyubiquitination; Sic1-\textsuperscript{Ub}_{n}) are visualized via western blot with α-Sic1 antibodies only after long incubation times, exceeding by at least 12-fold (> 60 vs. 5 min) the time necessary to detect Sic1 modified by Cdc34\textsuperscript{244} (Figure 3C, lanes 5-8, 1-4). \textsuperscript{tm}Cdc34\textsuperscript{244} is as defective as \textsuperscript{Δ}Cdc34\textsuperscript{244} in the formation of high molecular weight Sic1 species and both mutant E2s catalyze a similar accumulation of Sic1 monoubiquitinated on one (Sic1-\textsuperscript{Ub}_{1}) or two (Sic1-\textsuperscript{Ub}_{2}) lysines (Figure 3C, lanes 5-8, 9-12 and data not shown). Monoubiquitination of additional lysines on Sic1 can be observed in the presence of high Cdc34 concentrations (1-3 μM; [8,9,29,30]) that exceed by about 10-fold the estimated concentration of Cdc34 in cells [31]. We also tested polyubiquitination of \textsuperscript{F}Met4, the SCF\textsuperscript{Met30}-dependent substrate modified on a single lysine residue K163 \textit{in vivo} [32] and \textit{in vitro} [33]. Western blot with α-Flag antibodies visualizes polyubiquitinated forms of \textsuperscript{F}Met4 after 5 minutes of reaction with Cdc34\textsuperscript{244}, but 30 minutes are needed for similar polyubiquitination by \textsuperscript{tm}Cdc34\textsuperscript{244}, suggesting a rate at least 6-fold slower (Figure 3D; α-\textsuperscript{F}Met4, lanes 1-5 and 6-10, \textsuperscript{F}Met4-\textsuperscript{Ub}_{n}). In contrast, western blot with α-Flag antibodies does not detect polyubiquitinated \textsuperscript{F}Met4 even after 60 minutes of incubation with \textsuperscript{Δ}Cdc34\textsuperscript{244} (Figure 3D; α-\textsuperscript{F}Met4, lanes 11-15, \textsuperscript{F}Met4-\textsuperscript{Ub}_{n}), suggesting that \textsuperscript{Δ}Cdc34\textsuperscript{244} is less active than \textsuperscript{tm}Cdc34\textsuperscript{244}. This difference reflects a difference in the synthesis of polyubiquitin chain on monoubiquitinated \textsuperscript{F}Met4, as monoubiquitinated \textsuperscript{F}Met4 appears with a similar rate in reactions with \textsuperscript{tm}Cdc34\textsuperscript{244} and \textsuperscript{Δ}Cdc34\textsuperscript{244} (Figure 3E; due to similarities of the apparent molecular weights, unubiquitinated and monoubiquitinated forms of \textsuperscript{F}Met4 are detectable as separate species only under short western blot exposure times). This observation agrees with the finding that deletion of the loop does not affect substrate monoubiquitination by Cdc34 [18].

In summary, \textsuperscript{tm}Cdc34 is normally charged with ubiquitin thiolester and monoubiquitinates substrates in a manner similar to wild type Cdc34 and \textsuperscript{Δ}Cdc34. However, either the loop deletion or the motif replacement compromises substrate polyubiquitination by Cdc34.

Unlike in reactions with Cdc34, SCFCdc4\textsuperscript{Cdc4} stimulates the synthesis of free polyubiquitin chains by \textsuperscript{tm}Cdc34 only modestly and in a manner dependent on substrate recruitment

We next addressed how the defect in substrate polyubiquitination by \textsuperscript{tm}Cdc34 relates to the synthesis of free polyubiquitin chains \textit{in vitro}. The \textit{in vitro} synthesis of free polyubiquitin chains by wild type Cdc34 is an acknowledged aspect of its function [18,21,34-36].

We first tested the accumulation of free polyubiquitin chains under conditions of a standard ubiquitination assay with wild type Cdc34 and SCFCdc4. Western blot analysis performed with antibodies specific to ubiquitin detects large amounts of high molecular weight polyubiquitin conjugates in the absence but not presence of the Sic1 substrate (Figure 4A, α-\textsuperscript{Ub} WB, lanes 4 and 3). Only the more sensitive western blots with α-Sic1 and α-Cdc34 antibodies can visualize the low amounts of polyubiquitinatated Sic1 and Cdc34 present in the same reaction mixtures (Figure 4A, α-Sic and α-Cdc34 WBs). The polyubiquitin conjugates detectable with α-\textsuperscript{Ub} antibodies cannot represent polyubiquitinatated Sic1 because they appear in mixtures lacking Sic1 (Figure 4A, compare α-\textsuperscript{Ub} and α-Sic1 WB, lane 4). The polyubiquitin conjugates detectable with α-\textsuperscript{Ub} antibodies cannot represent polyubiquitinatated Cdc34, as similar amounts of polyubiquitinatated Cdc34 are detectable in the mixtures containing or lacking these conjugates (Figure 4A, compare α-\textsuperscript{Ub} and α-Cdc34 WB, lanes 3 and 4). To verify that these species represent free polyubiquitin chains, we tested whether they are sensitive to Isopeptidase T (IsoT), which disassembles polyubiquitin chains in a manner dependent on the free C-terminus [37]. Most of the conjugates that are detectable by western with α-\textsuperscript{Ub} antibodies disappear upon incubation with IsoT (Figure 4B; α-\textsuperscript{Ub} WB, lanes 1-4), verifying that they represent free polyubiquitin chains. Wild type Cdc34 thus synthesizes super-stoichiometric amounts of free polyubiquitin chains in the presence of SCFCdc4 and substrate recruitment prevents this process, presumably by re-directing the polyubiquitination activity to substrate.

In contrast, SCFCdc4\textsuperscript{Cdc4} stimulates the synthesis of free polyubiquitin by \textsuperscript{tm}Cdc34 only modestly and in a manner dependent on the Sic1 substrate (Figure 4A, α-\textsuperscript{Ub} WB, compare lane 7 and 8). The ubiquitin conjugates detectable with α-\textsuperscript{Ub} antibodies in reactions with \textsuperscript{tm}Cdc34 likely represent free polyubiquitin chains, because the same α-\textsuperscript{Ub} western blot conditions prevent detection of substrate-attached polyubiquitin chains in reactions even with the more active Cdc34 (described above). We did not verify that the high molecular weight ubiquitin conjugates visualized by α-\textsuperscript{Ub} western blot in \textsuperscript{tm}Cdc34-dependent reactions are sensitive to IsoT, because the
necessity for additional dilution in preparation for IsoT treatment (see Methods) prevents their detection.

In summary, Cdc34 synthesizes super-stoichiometric amounts of free polyubiquitin in the presence of SCF<sup>cdc4</sup> and substrate recruitment is sufficient to prevent this process. This regulatory mechanism is disrupted in reactions with tmCdc34, which synthesizes fewer free polyubiquitin chains and in a manner dependent on, not attenuated by, substrate recruitment.

**Figure 4** The synthesis of free polyubiquitin chains in vitro and in vivo. (A) Regulation of free polyubiquitin chain synthesis by substrate recruitment to SCF<sup>cdc4</sup>. Standard ubiquitination reactions with Cdc34 or tmCdc34 (5 pmol) were analyzed by western blots with α-Ub, α-Sic1 or α-Cdc34 antibodies. (B) IsoT sensitivity. Reactions as in A lanes 4 and 8 were analyzed for IsoT sensitivity (Methods). (C) Levels of ubiquitin and ubiquitin conjugates in vivo. Boiled cell extracts (Methods) were analyzed by western blot with α-Ub (Covance) or α-Rpn10 antibodies. Lane 4: 200 ng of free polyubiquitin chains Ub<sub>1-6</sub> purchased from Enzo. (D) Growth of tmCDC34 but not CDC34 yeast is sensitive to loss of UBP14. Haploids with the indicated genotypes were selected on haploid selection media with G418 and nourseothricin at 30°C for three days. (E) Overexpression of Ubp14 but not Ubp15<sup>C354A</sup> supports growth of tmCDC34 ubp14<sup>Δ</sup> yeast. Heterozygous diploids (RC171 and RC172) were transformed with the indicated plasmids that overexpress Ubp14 (pUBP14) or Ubp14<sup>C354A</sup> (pUBP14-C354A), patched onto sporulation media and incubated at 26°C for five days. Haploids with the indicated genotypes were selected as in D. (F) Accumulation of ubiquitin conjugates in yeast extracts enriched with Uba1, ubiquitin, ATP and MgCl<sub>2</sub>. Extracts with active ubiquitin-proteasome system (10 μg of total proteins; see Methods) were enriched with Uba1 (10 pmol), ubiquitin (1.3 nmol) ATP (2 mM) and MgCl<sub>2</sub> (2 mM), incubated at 25°C for the times indicated and analyzed by 10% or 18% SDS-PAGE followed by α-Ub (Sigma) or α-Rpn10 WB.

tmCDC34 cells have fewer polyubiquitin conjugates in vivo, but their disassembly depends on the Ubp14 C-terminal ubiquitin hydrolase

To test whether tmCdc34 protein is defective in ubiquitin conjugation in vivo, we first performed western blot analysis with α-ubiquitin antibodies of whole cell extracts prepared by direct boiling of tmCDC34 cells in SDS-PAGE loading buffer. This procedure leads to rapid denaturation of proteins, including proteasome and...
ubiquitin proteases, and is therefore likely to extract intact ubiquitin conjugates. In these experiments, \textsuperscript{tm}CDC34 cells have fewer ubiquitin conjugates of a broad molecular weight than CDC34 cells (Figure 4C, 10% PAGE, lanes 1 and 2; Ub\textsubscript{2}-Ub\textsubscript{b}). This difference is not accompanied by an accumulation of short polyubiquitin chains and the levels of ubiquitin are similar in both extracts (Figure 4C, 18% PAGE, lanes 1 and 2; Ub\textsubscript{2,4}; Ub\textsubscript{1}). The control western blot performed with \textalpha-Rpn10 antibodies verifies that equal amounts of total proteins were analyzed (Figure 4C, \textalpha-Rpn10). A reduction in ubiquitin conjugates but not ubiquitin levels is thus detected by analysis of the total ubiquitin conjugates in \textsuperscript{tm}CDC34 yeast.

The patterns of ubiquitin conjugates detectable in CDC34 and, to a lesser degree, in \textsuperscript{tm}CDC34 cells are similar and involve species of various molecular weights (Figure 4C, 10% PAGE, lanes 1 and 2; Ub\textsubscript{2}-Ub\textsubscript{b}). To assess whether these species represent ubiquitin conjugated to substrates or free polyubiquitin, we sought to test how their pattern changes upon deletion of the UBP14 gene, which encodes the only yeast ubiquitin hydrolase that disassembles polyubiquitin in a manner dependent on the free C-terminus [37,38]. In the absence of UBP14, free polyubiquitin species would be expected to accumulate in the form of short chains composed of 2-6 ubiquitins, as only longer chains are efficiently disassembled by other DUBs, while the pattern of ubiquitin conjugated to substrates should not change unless the levels of free ubiquitin drop [38]. The high molecular weight ubiquitin conjugates (Ub\textsubscript{n}) detectable in extracts prepared from CDC34 yeast are poorly detectable in extracts prepared from CDC34 upb14\Delta yeast (Figure 4C, 10% SDS-PAGE, Ub\textsubscript{n}, lanes 1 and 3). The loss of ubiquitin conjugates correlates with an appearance of short Ub\textsubscript{2,6} chains but not a change in ubiquitin level (Figure 4C, 18% SDS-PAGE, Ub\textsubscript{2,6}; Ub\textsubscript{1}, lanes 1 and 3). Free polyubiquitin chains thus represent a prominent fraction of the total ubiquitin conjugates in wild type yeast, at least during vigorous growth. A more accurate estimation is not possible based on these data alone, because western blot detection is not quantitative and the available \textalpha-ubiquitin antibodies have different affinities to short and long polyubiquitin chains.

If a significant fraction of the total ubiquitin conjugates detectable in CDC34 yeast extracts are free polyubiquitin chains, the lower level of similarly sized ubiquitin conjugates in \textsuperscript{tm}CDC34 yeast (Figure 4C, 10% SDS-PAGE, lanes 1 and 2) could indicate that \textsuperscript{tm}CDC34 yeast have fewer free polyubiquitin chains of similar lengths. In this case, fewer short Ub\textsubscript{2-6} conjugates should be detectable in \textsuperscript{tm}CDC34 cells lacking UBP14.

Unexpectedly, this simple prediction could not be tested, because unlike wild type yeast, which are viable in the absence of UBP14, the \textsuperscript{tm}CDC34 upb14\Delta double mutant yeast are inviable (Figure 4D, \textsuperscript{tm}CDC34 upb14\Delta and CDC34 upb14\Delta). This lethality is corrected by ectopic expression of Ubp14 but not an inactive Ubp14 C354A mutant (Figure 4E), verifying the requirement for Ubp14 function. Thus, \textsuperscript{tm}CDC34 cells have fewer polyubiquitin conjugates in vivo, but these conjugates can be disassembled only by Ubp14.

To get an insight into the Ubp14 dependence, we prepared cell extracts in a manner retaining activity of the ubiquitin-proteasome system (Methods). This procedure is more time consuming than extraction by cell boiling and does not include protease inhibitors. The extracts therefore do not retain the ubiquitin conjugates that were present in cells prior to lysis, leading to similarly low backgrounds in the isogenic CDC34 and \textsuperscript{tm}CDC34 yeast, and in the isogenic UBP14 and upb14\Delta yeast (data not shown). Instead of inhibiting DUBs, we then attempted to overwhelm their activity by supplementing the extracts with purified Uba1, Ub, ATP and MgCl\textsubscript{2}, which should maximize the ubiquitin conjugation activities of E2s and E3s in the extracts. As expected, the supplementing components alone are insufficient to conjugate ubiquitin (Figure 4F, \textalpha-Ub, lanes 5, 10, 15, 20), but stimulate an accumulation of similar amounts of ubiquitin conjugates in the control extracts prepared from CDC34 yeast, or the isogenic UBP14 or upb14\Delta yeast (Figure 4F, 10% PAGE, \textalpha-Ub, lanes 1-5, 11-15 and 16-20). We have thus created conditions under which the lack of Ubp14 activity plays no major role in the accumulation of high molecular weight ubiquitin conjugates. Under these conditions, more of high molecular weight ubiquitin conjugates accumulate in \textsuperscript{tm}CDC34 yeast extract (Figure 4F, \textalpha-Ub WB, 10% PAGE, Ub\textsubscript{n}, lanes 1-5, 6-10, 11-15 and 16-20). This effect is repetitive and the control western blot with \textalpha-Rpn10 antibodies verifies that equal amounts of total proteins were analyzed (Figure 4F, \textalpha-Rpn10).

It cannot be excluded that the 4-fold higher levels of \textsuperscript{tm}Cdc34 protein (Figure 2E) plays a role in the abnormal accumulation of high molecular weight ubiquitin conjugates in \textsuperscript{tm}CDC34 yeast extract. However, this interpretation would be inconsistent with the defective function of \textsuperscript{tm}Cdc34 in vivo (Figure 4C, lanes 1, 2). A more likely explanation is that the polyubiquitin conjugates accumulate due to a delay in their disassembly by DUBs other than Ubp14. If a similar delay characterizes the polyubiquitin conjugates synthesized in \textsuperscript{tm}CDC34 cells, their disassembly could be primarily dependent on C-terminal hydrolysis, explaining the essential role of Ubp14.
Phosphorylation of C-terminal serines in \( \text{tmCdc34} \) by Cka2 kinase eliminates the synthesis of free polyubiquitin chains, likely by stimulating their attachment to substrate. Among the genes recently identified as essential for growth of \( \text{tmCDC34} \) but not \( \text{CDC34} \) yeast is \( \text{CKA2} \) [22] that encodes the catalytic subunit of casein kinase 2 implicated in Cdc34 function [23,24]. To address how Cka2 affects \( \text{tmCdc34} \) activity, we first tested whether \( \text{GstCka2} \), or its homologue \( \text{GstCka1} \), purified from yeast can phosphorylate Cdc34 and \( \text{tmCdc34} \) in vitro, and whether the in vitro phosphorylation of Cdc34 or \( \text{tmCdc34} \) is limited to the most C-terminal serines [23] (Figure 5A, red), or also targets the catalytic E2 core [24] (Figure 5A, bold black).

Deletion of the C-terminus (Figure 5B, lanes 1-4), or replacement of the six C-terminal serines with alanines (Figure 5B, 6SA; lanes 5-10) prevents phosphorylation of Cdc34 by \( \text{GstCka1} \). Extended autoradiogram exposure times are required to detect phosphorylation of the Cdc346SA mutant and this level of phosphorylation is comparable to the control phosphorylation of BSA (Figure 5B, lanes 5-10, 4 and 17 days). Only the six most C-terminal serines are thus phosphorylated within full length Cdc34. Charging Cdc34 with an ubiquitin thiolester under conditions that prevent discharge also does not trigger phosphorylation of the E2 core (data not shown).

Similarly, \( \text{GstCka2} \) phosphorylates the six most C-terminal serines in Cdc34 (data not shown), or the two most C-terminal serines S207 and S216 in Cdc34424 over a range of concentrations Figure 5C, lanes 1-8). \( \text{GstCka2} \) phosphorylates Cdc34424 and \( \text{tmCdc34} \) with similar kinetics (Figure 5C, lanes 9-16), suggesting that the phosphorylation is not affected by the motif replacement. We do not observe phosphorylation of the control Rad6 E2 even under conditions of 50-fold greater sensitivity of \( [32P] \) detection (Figure 5C, lane 21), verifying that \( \text{GstCka2} \) function is specific to Cdc34. Only under conditions of such sensitivity, we could detect a low level of phosphorylation of Cdc34424 or \( \text{tmCdc34} \) carrying the S207A and S216A substitutions (Figure 5C, 43 h, lanes 18, 20). This signal represents ~1% of that associated with phosphorylation of S207 and S216 (Figure 5C, lanes 17, 19 and graph), does not account for quantitative phosphorylation of any other two serines, and was not observed with full-length Cdc34424 protein (Figure 5B, lanes 5-10).

Phosphorylation of \( \text{tmCdc34} \) by \( \text{GstCka2} \) kinase modestly stimulates the SCF\( ^{Cdc4} \)-dependent ubiquitination of conditions that prevent discharge also does not trigger phosphorylation of the E2 core (data not shown).

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**Figure 5** Yeast \( \text{GstCka1} \) or \( \text{GstCka2} \) kinases phosphorylate only the most C-terminal serines in Cdc34 and \( \text{tmCdc34} \). (A) Scheme of Cdc34. Red: the six most C-terminal serines. Residue 244 represents the C-terminal end in Cdc34424 that includes the E2 catalytic core (a.a. 1 to 170) with the active site cysteine C95, the E3 RING domain-interacting fragment, and the 39 a.a. C-terminal fragment (a.a. 171-209) also implicated in binding to SCF. (B) In a physiological range of protein concentrations, yeast \( \text{GstCka1} \) phosphorylates only the six most C-terminal serines within Cdc34. 1 hour assays included 10 \( \mu \text{M ATP} \) with 0.1 \( \mu \text{Ci of } [\gamma-32P] \)ATP (4500 Ci/mmol), 1 pmol of the indicated Cdc34 constructs and 0.5 or 2.5 pmol of \( \text{GstCka1} \). (C) \( \text{GstCka2} \) phosphorylates the S207 and S216 most C-terminal serines in Cdc34424 and \( \text{tmCdc34} \) Assays were performed for 1 hour (or as indicated), with 5 pmols (or as indicated) of the indicated constructs, 1 pmol of \( \text{GstCka1} \) kinase and 10 \( \mu \text{M ATP} \) with 5 \( \mu \text{Ci } [32P] \)ATP (4500Ci/mmol), leading to ~50-fold higher sensitivity of \( [32P] \) detection than in B. Graph: quantitation of the \( [32P] \) signal of the proteins at different autoradiogram exposure times. CB: Coomassie blue.
the Sic1 substrate (Figure 6A α-Sic1 WB, compare lanes 1-4 and 5-8). This effect leads to a nearly normal accumulation of polyubiquitinated Sic1 species in 60 minutes, which is a long reaction time (Figure 6A, α-Sic1 WB, lanes 7, 11 and 15). However, even in the presence of GstCka2 fewer polyubiquitinated Sic1 species accumulate in short reactions with tmCdc34 than with Cdc34 (Figure 6A, α-Sic1 WB, compare lanes 6, 10 and 14). The short reaction times are critical because they represent the time in which substrate degradation needs to be catalyzed in vitro. Cka2 thus stimulates, but does not fully rescue, the defective polyubiquitination activity of tmCdc34. This observation agrees with the finding that tmCDC34 yeast, which have active Cka2, accumulate fewer polyubiquitin conjugates than CDC34 yeast (Figure 4C, lanes 1 and 2).

The stimulation of Sic1 substrate polyubiquitination by GstCka2 correlates with a loss of free polyubiquitin chains in the same reaction mixtures (Figure 6A, α-Ub WB, compare lanes 3 and 7, arrows; see Figure 4A and 4B for verification that under the conditions used α-Ub western blot visualizes only free polyubiquitin chains). Phosphorylation of C-terminal serines in tmCdc34 thus may eliminate the synthesis of free polyubiquitin chains by promoting their attachment to substrate. To assess which of these effects plays a role in vivo, we tested whether overexpression of the Ubp14 C-terminal hydrolase rescues viability of tmCDC34 cka2Δ yeast. Overexpression of Ubp14 or its catalytically inactive mutant does not support growth of tmCDC34 cka2Δ yeast (Figure 6B), suggesting that the requirement for Cka2 function is not associated with its role in preventing the free polyubiquitin chain synthesis. Rather, it reflects the role in the stimulation of substrate polyubiquitination.

In control reactions with wild type Cdc34, GstCka2 has no effect on the SCF-Cdc4-dependent polyubiquitination of Sic1 substrate (Figure 6A, α–Sic1 WB, compare lanes 9-11 and 13-15). However, GstCka2 prevents the synthesis of free polyubiquitin chains in substrate-free reactions with SCF-Cdc4 (Figure 6A, α-Ub WB, lanes 12 and 16, green arrows). This effect is detectable only in substrate-free reactions, excluding the possibility that it results from redirecting the synthesis of polyubiquitin chains to substrate. Rather, it reflects a mechanism aimed at inhibiting the unproductive ubiquitin conjugation to free polyubiquitin chains typical of Cdc34 function with substrate-free SCFs. Under conditions of in vitro assays performed with low concentration of ubiquitin, or with an access of SCF-Cdc4 over substrate, this regulatory effect could indirectly promote substrate polyubiquitination by preventing rapid depletion of free ubiquitin. These possibilities could explain why under some experimental conditions the phosphorylation of wild type Cdc34 could stimulate Sic1 polyubiquitination [23].

**Discussion**

Robust polyubiquitination activity is thought to be key to the function of the Cdc34 ubiquitin-conjugating enzyme that, together with the SCF ubiquitin ligases, promotes degradation of proteins involved in cell cycle and growth regulation. However, we find that the tmCdc34 protein in which the S73/S97/no loop motif is replaced with the K73/D97/no loop motif typical of other E2s supports yeast growth with normal cell size and cell cycle profile despite producing fewer polyubiquitin conjugates in vitro and in vivo. The significance of this finding comes from the observation that the in vitro defect in Sic1 substrate polyubiquitination by tmCdc34 is similar to the defect of α12Cdc34 protein that cannot support growth, and that tmCDC34 yeast contain fewer polyubiquitin conjugates in vivo than CDC34 yeast, despite 4-fold higher steady-state levels of tmCdc34 protein. While several cofactors are implicated in the function of tmCdc34 in vivo [22], these cofactors thus do not ensure normal ubiquitin conjugation in tmCDC34 yeast.

If robust polyubiquitination is not necessary for Cdc34 function in vivo, a yet undefined aspect of the SCF-proteasome pathway would be expected to promote substrate proteolysis under conditions of compromised polyubiquitination. In support of this possibility, tmCDC34 yeast are sensitive to loss of the RPN10 and RAD23 genes [22] that encode ubiquitin-binding receptors of the proteasome implicated in Sic1 degradation [25,26]. If SCFCdc34 indeed directly interacts with the proteasome, as one study suggests [39], this interaction could localize tmCdc34 and substrate to the proximity of the Rpn10 and/or Rad23, thereby compensating for the lack of robust polyubiquitination. This model is outlined in Figure 7. However, it cannot be excluded that the defect in tmCdc34 is corrected at the proteasome: for example, in a manner dependent on the ubiquitin-interacting motif (UIM) of Rpn10 recently implicated in Sic1 degradation [40,41]. An insight into which of these models reflects tmCdc34 function in tmCDC34 yeast could come from analysis of the kinetic properties (K_m and k_cat) of tmCdc34 protein in vitro, and from testing how Rpn10, and Rad23, individually or as part of the proteasome, affect these properties.

The observation that the catalytic E2 core motifs define distinct mechanisms of function and regulation is consistent with their conservation. However, their analysis in the context of a single E2 that collaborates with only one class of E3s provides the first direct evidence that the E2 motifs control the robustness and regulation
**Figure 6** Cka2 inhibits synthesis of free polyubiquitin chains by Cdc34 constructs

(A) Effects of Cka2 on Sic1 ubiquitination and synthesis of free polyubiquitin chains by Cdc34 proteins. 5 pmol of Cdc34 or tmCdc34 were phosphorylated at 25°C for 1 hour, as in 5B. The assays were then supplemented with 1 pmol E1, 660 pmol of ubiquitin, 2 pmol of FlagSCFCdc4 and 2 pmol of Sic1/Cln5/GstCdc28, where indicated, and incubated at 25°C for the times indicated, followed by α-Sic1 and α-Ub WBs.

(B) Ectopic expression of Ubp14 or Ubp15C354A does not rescue growth of tmCDC34 cka2Δ yeast. The heterozygous diploid RC174 was transformed with the indicated plasmids that overexpress Ubp14 (pUBP14) or catalytically inactive Ubp14 (pUBP14-C354A) from the ADH1 promoter. These transformed diploids were patched onto sporulation media and incubated at 26°C for five days. Haploids with the indicated genotypes were selected by streaking the heterozygous diploids on haploid selection media with G418 and nourseothricin. Plates were incubated at 30°C for three days.
of polyubiquitination. What predictions could be made based on this finding for the role and regulation of other E2s? The most obvious prediction is that the alternative motif would support well the function of the E2s that catalyze monoubiquitination, a process that is not related to protein degradation but yet regulates several aspects of cell biology, including endocytosis [42], chromatin remodeling [43] and DNA damage response [44,45]. An example of such an E2 is Rad6, which monoubiquitinates histones and PCNA. The second prediction is that the alternative motif has the potential to support not only monoubiquitination, but also polyubiquitination, at least in reactions with some E3 partners. However, the mechanism of polyubiquitination catalyzed in a manner dependent on each motif would be different. In support of this possibility, two major self-oligomerization schemes linked to an activation of polyubiquitination emerge from analyses of the E2s that differ in their motifs. The Cdc34/Ubc7-like Ube2g2 E2 self-oligomerizes via a RING-independent interaction with its gp78 E3 partner [46]. In contrast, the Ubc5-like E2s, which carry the K73/D97/no loop motif, self-oligomerize in a way dependent on the RING domain and non-covalent binding between ubiquitin and several residues located opposite the active site [47-50]. Finally, Cdc34 has a high preference for lysine K48 that is independent of its SCF E3 partner [51-53], but other E2s have lower intrinsic fidelity in lysine selection and frequently collaborate with E3s that define the lysine specificity [53,54]. The low intrinsic fidelity in lysine selection could thus be an evolutionary adaptation that is associated with the K73/D97/no loop motif and that allows the synthesis of different linkages depending on the E3 context. In this view, the alternative motif could have evolved to provide a basis for the diversity of polyubiquitin linkages and signaling, while the original motif would have been selected based on the robustness of K48-type of polyubiquitination.

If the motif replacement in Cdc34 lowers the fidelity of lysine selection during polyubiquitin chain synthesis and SCF<sup>Cdc4</sup> did not evolve to correct for this change, then polyubiquitin chains synthesized by<sup>tm</sup>Cdc34 could have altered and/or branched linkages instead of, or in addition to, the K48 type of polyubiquitin typical for Cdc34. This model could explain why<sup>tm</sup>CDC34 yeast are sensitive to loss of Ubp14, which is the only DUB that cleaves polyubiquitin in a manner independent of the Ub-Ub linkage [55,56], and why DUBs other than Ubp14 disassemble polyubiquitin conjugates less efficiently when they are produced in<sup>tm</sup>CDC34 cell
extracts. A similar delay could cause ubiquitin depletion and/or create conditions under which abnormal polyubiquitin chains potently block the proteasome even without massive accumulation [53]. Ubp14 function is not required in yeast ([38], this work), but an inhibition of ubiquitin receptors at the proteasome by an excess of free polyubiquitin [57] is thought to be responsible for the developmental defects associated with Ubp14 absence in multi-cellular organisms, including D. discoideum [58] and A. thaliana [59].

What linkages are present in polyubiquitin chains synthesized by \( ^{tm}\text{Cdc34} \) protein \textit{in vitro} and \textit{in vivo}, and whether the same linkages are present in substrate-attached and free polyubiquitin are yet unknown. These questions would best be addressed by quantitative mass spectrometry, as ubiquitin mutants could additionally affect the already fragile \( ^{tm}\text{Cdc34}/SCF \) interaction interface (Figure 1B and 1C), which is sensitive to loss of phosphorylation within the C-terminus of \( ^{tm}\text{Cdc34} \) and does not properly respond to the regulation by substrate recruitment. What is the basis of the functional communication between the seemingly independent E2 and substrate-interacting domains of SCF[\( ^{Cdc34} \)] is yet unknown, but a similar communication is suggested by the observation that substrate recruitment promotes modification of the Cul1 homologue of Cdc53 with the Nedd8 ubiquitin-like protein, leading to E2 activation [60]. Phosphorylation would potentiate the acidic nature of the C-terminus, but how the C-terminus of Cdc34 or \( ^{tm}\text{Cdc34} \) participates in the mechanism of polyubiquitination is also unclear, as the C-terminus is absent from all known E2 structures (Figure 1C, gray arrow). Why is Cka2, and not Cka1, essential in \( ^{tm}\text{CDC34} \) yeast if each kinase phosphorylates the six most C-terminal serines in \( ^{tm}\text{Cdc34} \) \textit{in vitro}? Cka1 and Cka2 could differ in their times or levels of expression, or localizations, or an undefined feature could give Cka2 a regulatory advantage in \( ^{tm}\text{CDC34} \) cells.

Perhaps the most interesting question raised by our study is why the Cdc34/Ubc7-specific motif evolved at all if its replacement in Cdc34 has only a minimal effect on cell growth and division? It is possible that either motif can support Cdc34 function under typical laboratory conditions, but the mechanism defined by the original motif is better for survival under sub-optimal and/or stress conditions. As the only E2 essential for yeast viability and an E2 that has the largest number of substrates known, Cdc34 would likely be required to function under a variety of growth conditions. However, we cannot eliminate the possibility that the motif conservation reflects its ability to undergo post-transcriptional regulation that controls Cdc34 activity and/or levels. This possibility is suggested by the presence of serine residues in the \( S73/\ S97/\text{loop} \) motif and by the finding that the steady-state level of \( ^{tm}\text{Cdc34} \) is increased by about four-fold in a strain isogenic to CDC34 yeast. This change is significant, as the steady-state levels of Cdc34 do not change during the cell cycle [5] and are unaffected by replacement of all lysines to arginines that prevents autoubiquitination [28]. Furthermore, even a five-fold enrichment 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 [61,62]; and a similar 4-fold accumulation of Cdc34 has been linked to the development of pediatric T-cell acute lymphoblastic leukemia [63]. If such a modest increase in Cdc34 levels disregulates cell growth and division, a role of the \( S73/\ S97/\text{loop} \) motif in the control of Cdc34 activity and/or levels could play a role in its conservation. It will be of great interest to determine what aspect of the catalytic E2 motif function and/or regulation is responsible for its conservation.

Conclusions

Replacement of the catalytic \( S73/\ S97/\text{loop} \) motif that is conserved among all members of the Cdc34/Ubc7 family with the \( K73/D97/\text{no loop} \) motif present in other E2s compromises the polyubiquitination activity of Cdc34 \textit{in vitro} and \textit{in vivo}, and alters its regulation. However, either motif can support the essential function of Cdc34 in cell growth and division, at least under typical laboratory conditions, raising the question of what is the basis for the motif conservation. We discuss the possibility that growth under suboptimal and/or stress conditions, or a requirement for stringent regulation of Cdc34 levels via the serine residues present in the motif could explain the motif conservation. We predict that future analysis of the cellular pathways that are sensitive to the motif replacement will answer the question of the motif conservation and may define new roles and/or regulatory aspects of Cdc34. The essential role of Cka2, Rpn10 and Rad23 in \( ^{tm}\text{CDC34} \) yeast [22] also opens the possibility to define the still enigmatic role of these cofactors in the SCF-proteasome pathway.

Materials and methods

Yeast Strains and their construction

All strains and their full genotypes are listed in Table 1. Standard methods were used for strain construction [64]. Strains RC171, RC172, RC173, and RC174 were constructed as described in [22].

Plasmids

see Table 2.

Antibodies

We used \( \alpha/\text{FLAG} \) M2 and \( \alpha/\text{Ub} \) (Sigma), \( \alpha/\text{Ub} \) and \( \alpha/\text{HA} \) (Covance), \( \alpha/\text{Cdc34} \) [28], \( \alpha/_{\text{MBP}}\text{Sic1} \), \( \alpha/_{\text{Gst}}\text{Rpn10} \)
Table 1 Yeast strains used in this study

| Strain     | Genotype                                      | Reference |
|------------|-----------------------------------------------|-----------|
| B62        | MATα thr2·Kan· his3Δ1 leu2·200 met15·0 ura3·0 | [73]      |
| 3195       | MATα ubp14·Kan· his3Δ1 leu2·200 met15·0 ura3·0 | [73]      |
| 13195      | MATα ubp14·Kan· his3Δ1 leu2·200 met15·0 ura3·0 | [73]      |
| BL2        | MATα                                          | [22]      |
| EJ758      | MATα his3·200 leu2·3,112 ura3·52 pep4·His3 pYEX4AT+·rec·CKA1 | [74]      |
| EJ758 (YOR061w) | MATα his3·200 leu2·3,112 ura3·52 pep4·His3 pYEX4AT+·rec·CKA2 | [74]      |
| FY24       | MATα ura3·52 trp1·Δ63 leu2·Δ1                | F. Winston|
| FY78       | MATα his3Δ200                                 | F. Winston|
| KS418      | MATα CDC34Δura3 leu2·201 lys2·ade2·ade3      | [22]      |
| MT1901     | MATα mfa1ΔpmFA1·His3 can1·Δ3 ura3·0 leu2·0 his3·0 lys2·0 | M. Tyers |
| RC6        | MATα CDC34Δ(NAT1) ura3 leu2·201 ade2·ade3   | [22]      |
| RC21       | MATα/α CDC34Δ(NAT1)/CDC34 ura3·ura3 leu2·201 trp1·TRP1 lys2·LYS2 ade2·ADE2 ade3·ADE3 his3·His3 MFA1/mfa1·Δ·pmFA1-HIS3 can1·CAN1 | [22]      |
| RC29       | MATα/α cdc34tm(NAT1)/cdc34Δura3·Δ3 leu2·Δ1 can1·Δ  | [22]      |
| RC94       | MATα/cdc34(NAT1) mfa1·Δ·pmFA1·His3 his3·Δ1 leu2·Δ0 can1·Δ | [22]      |
| RC85       | MATα/α CDC34Δ(NAT1)                          | [22]      |
| RC171      | MATα/α CDC34Δ(NAT1)/CDC34 ubp14·Kan·ΔUBP14 mfa1·Δ·pmFA1·His3·MFA1 his3·Δ1 his3·Δ3 ura3·Δ3 leu2·200 can1·CAN1 met15·0·MET15 | This study |
| RC172      | MATα/α CDC34Δ(NAT1)/CDC34 ubp14·Kan·ΔUBP14 mfa1·Δ·pmFA1·His3·MFA1 his3·Δ1 his3·Δ3 ura3·Δ3 leu2·200 can1·CAN1 met15·0·MET15 | This study |
| RC173      | MATα/α CDC34Δ(NAT1)/CDC34 cka2·Kan·ΔCKA2 mfa1·Δ·pmFA1·His3·MFA1 his3·Δ1 his3·Δ3 ura3·Δ3 leu2·200 can1·CAN1 met15·0·MET15 | This study |
| RC174      | MATα/α CDC34Δ(NAT1)/CDC34 cka2·Kan·ΔCKA2 mfa1·Δ·pmFA1·His3·MFA1 his3·Δ1 his3·Δ3 ura3·Δ3 leu2·200 can1·CAN1 met15·0·MET15 | This study |
| RC175      | MATα/α CDC34Δ(NAT1)/CDC34 pps7·Kan·ΔRFS78 mfa1·Δ·pmFA1·His3·MFA1 his3·Δ1 his3·Δ3 ura3·Δ3 leu2·200 can1·CAN1 met15·0·MET15 | [22]      |
| RC176      | MATα/α CDC34Δ(NAT1)/CDC34 pps7·Kan·ΔRFS78 mfa1·Δ·pmFA1·His3·MFA1 his3·Δ1 his3·Δ3 ura3·Δ3 leu2·200 can1·CAN1 met15·0·MET15 | [22]      |
| YL10-1     | MATα cdc34·2 leu2·Δ1 ura3·52 trp1·Δ63 his3·Δ3 Gal+ | [17]      |

and α-Gst·Skp1 [65]. Antibody detection was by ECL (Amersham).

Recombinant proteins and protein complexes
We used: apyrase, ubiquitin and methylated ubiquitin (Sigma); Lys48-linked multibuquitin chains and Isopetidase T (Enzo Research); yeast Uba1Hae [9]; baculoviruses expressing yeast Sic1, Clb5, Cln2ΔHA, Gst·Cdc28, Cdc53, Rbx1, Cdc4, Gsk2, Skp1, Flag·Skp1 [8,10], and HA·Met30 [33]; C-terminal His6 fusion (pET21+; Novagen) of full length Cdc34 [29], tmCdc34 (triple mutant with the K73/D97/Δ12 instead of the S73/S97/loop motif, this work), Cdc34Δ53A (with S207, 216, 263, 268, 282 and 292A replacements) [23]; Cdc34Δ244 terminated at residue 244 [29], tmCdc34Δ244 and Δ12Cdc34Δ244 (this work); S207,216A Cdc34Δ244, tm,S207,216 Cdc34Δ244, C95A Cdc34, S97FCdc34, Cdc34Δ70, Rad6 (this work).

SCF, Sic1 and Cln2 complexes were assembled by co-infecting SF9 insect cells (Invitrogen) for 40 hours with

Table 2 Plasmids used in this study

| Plasmid | Description                                      | Reference |
|---------|--------------------------------------------------|-----------|
| pYL150  | 2μm, LEU2, Amp', GAL10 promoter +CDC34          | [17]      |
| pYL19   | pYL150 with S97D mutation in CDC34              | [17]      |
| pYL27   | pYL150 with 12-residue deletion of G-103 to T-114 in CDC34 | [17]      |
| pYL29   | pYL150 with 12-residue deletion of G-103 to T-114 and S73K and S97D mutations in CDC34 | [17]      |
| pUBP14  | ADH1 promoter and terminator elements + UBPI4 in pVT102-U | [38]      |
| pUBP14-Ala354 | ADH1 promoter and terminator elements +catalytically inactive UBPI4(C354A) mutant in pVT102-U | [38]      |
the appropriate baculoviruses, followed by cell lysis and immunoprecipitation [8]. Sic1 was phosphorylated as described previously [8].

All His-tagged proteins were expressed in *E. coli* (BL21 DE3 LysS) and purified on NTA resin (Qiagen) followed by DEAE and HPLC gel filtration chromatography on Superdex 200 (Amersham) in U buffer (50 mM Tris, pH 7.5, 50 mM KCl, 0.2 mM DTT).

GSTCka1 and GSTCka2 kinases were purified from yeast extracts prepared from cells harvested after 3 hours of treatment with 1 mM CuSO₄ during logarithmic growth on SD URA medium and blast-frozen in a 1:0.7 ratio of buffer U with 10% glycerol, protease and phosphatase inhibitors. 1 mg of total proteins was diluted to 1 ml in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Igepal, 0.1 mM DTT with protease and phosphatase inhibitors), incubated at 4°C with 20 μl of GST Sepharose (Sigma) for 30 minutes, followed by bead wash, equilibration with U buffer and elution with 40 mM glutathion in buffer U, leading to ~10 ng/μl of GSTCka1 or GSTCka2.

**Ubiquitination and phosphorylation in vitro**

Ubiquitination was performed at 25°C for 1 hour or as indicated in 10 μl containing buffer U with 1 mM ATP, 1 mM MgCl₂, 1 pmol of Uba1, 660 pmols of Ub, 2 pmol of FlagSCFcdc4 or GSTSCFMet30 (where indicated), 2 pmol of Sic1/Cln5/GSTCdc28 or fMet4 substrate (where indicated), and 1-5 pmol of the indicated Cdc34 proteins as specified in figures. For phosphorylation the assays included buffer U with 10 μM ATP and [³²P] ATP (ICN) as indicated, the indicated E2 protein constructs, and GSTCka1 or GSTCka2. The reactions were analyzed by WB or autoradiography.

**Preparation of yeast extracts with active ubiquitin-proteasome system**

Yeast were grown at 30°C in 2 liters of YPD medium [64] starting from OD 0.05 and harvested in the logarithmic phase of growth. Yeast extracts (5-10 mg/ml proteins) were prepared by grinding cells blast-frozen in a 1:0.7 ratio of cells to buffer U (50 mM Tris, pH 7.5; 50 mM KCl; 0.2 mM DTT). The aliquots of extract were frozen in liquid nitrogen and used immediately after thawing. Note that this method does not preserve polyubiquitin conjugates that have been made in cells prior to lysis, because upon cell lysis in the absence of protease inhibitors the polyubiquitin conjugates are either degraded by the proteasome or disassembled by ubiquitin proteases.

**Detection of ubiquitin conjugates in yeast cells**

To assess the steady-state levels of ubiquitin conjugates in cells, yeast cells (2.5 × 10⁸) were boiled directly in (150 μl) SDS-PAGE loading buffer with β-mercaptoethanol for 4 minutes at 100°C. Under such conditions, most proteins, including the proteasome and ubiquitin proteases (DUBs) are rapidly inactivated, therefore preserving polyubiquitin conjugates that were present in cells prior to lysis.

**Western blot detection of super-stoichiometric amounts of free polyubiquitin chains**

The sensitivity of Western blot detection is restricted by the concentration of the antibodies and the time of their incubation with the antigen-bearing membrane. Western blot performed with high concentrations of α-Ub antibodies (1:100 dilution) and/or for long incubation times (> 2 hr) allows detection of even small amounts of ubiquitin (~100 pmol). Western blot performed with low concentration of α-Ub antibodies (1:5000) and short incubation times (~30 minutes) can detect only large amounts (~μmol) of the same antigen. By varying these parameters, we established western blot conditions that allow detection of polyubiquitin chains only in amounts exceeding the amount of polyubiquitinated Sic1 (2 pmol), Cdc34 (a fraction of 4 pmol) and/or SCF subunits (a fraction of 2 pmol) in the reaction mixtures that were independently verified to contain polyubiquitinated Sic1, Cdc34 and/or SCF subunits via western blots performed with the more sensitive α-Sic1, α-Cdc34, α-Cdc4 or α-Cdc53 antibodies. The inability of western blots with α-Ub antibodies to detect polyubiquitinated Sic1, Cdc34 and/or SCF therefore serves as an internal control, which verifies that the α-Ub Western blots allow detection of only super-stoichiometric amounts of polyubiquitin conjugates. The free nature of these polyubiquitin conjugates is independently verified by their sensitivity to IsoT (see IsoT sensitivity assays).

**Isopeptidase T sensitivity assays**

Ubiquitination reactions were performed for 1 hour in the presence of ATP/MgCl₂, Uba1, Ub, full length Cdc34 and SCFcdc4 but not Sic1. 50% of the total ubiquitination mixtures were then incubated at 25°C for 10 minutes with apyrase (0.5 unit), which by hydrolyzing ATP prevents function of the Uba1 E1 and stops ubiquitin conjugation, followed by enriched with DTT (10 mM final) and incubation with Isopeptidase T (0.5 mg) for additional 0-30 minutes. The reactions were stopped by boiling with SDS-PAGE loading buffer, separated by SDS-PAGE and analyzed by western blot with α-Ub antibodies.

**Structural modeling**

Structural models of the E2 core (a.a. 1-170) of Cdc34 and ScRad6 (gray ribbon representation; PDB ID 1AYZ; [66])
and scUb7 fragment (gray ribbon representation; PDB ID 2UCZ; [67]). To model the ubiquitin-charged Cdc34 (a.a. 1-170) bound to the RING domain of Rbx1, scUb7 structure (dark blue; PDB ID 2UCZ; [67]) was first superimposed with the structure of Ub1 (light blue) charged with ubiquitin (green; PDB ID 1FXT; [68]) and with the structure of HUb7 (not shown) in complex with the RING of c-Cbl (not shown; PDB ID 1FBV; [69]). The RING domain of c-Cbl was then used to position the RING domain Rbx1 in complex with Cul1 (red and yellow, respectively; PDB ID 1LDK; [70]). Note that the E2 core domain (a.a. 1-170) used for modeling does not include the 39 a.a. (a.a. 171-209) C-terminal fragment unique to Cdc34 that would extend from the alpha helix marked with a gray arrow. The amino acid numbers refer to the position of the residues in Cdc34. Models were done with the ICM program [71].

Cell cycle distributions

Yeast cells were analyzed by flow cytometry for their DNA content using propidium iodide staining [72].

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Authors’ contributions

AL cloned and purified most of the mutant proteins used in this study, performed most of the in vitro tests, and contributed to the conceptual and experimental design of experiments. RC carried the construction and experimental design of experiments, prepared figures and wrote the manuscript. DS analyzed the IsoT sensitivity assays, performed in vitro rapid E2-E3 assembly and disassembly enable processive ubiquitylation of cullin-RING ubiquitin ligase substrates, Cell 2009, 139:957-968.

Competing interests

The authors declare that they have no competing interests.

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