

The role of the destruction box and its neighbouring lysine residues in cyclin B for anaphase ubiquitin-dependent proteolysis in fission yeast: defining the D-box receptor

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Programmed proteolysis of proteins such as mitotic cyclins and Cut2/Pds1p requires a 9-residue conserved motif known as the destruction box (D-box). Strong expression of protein fragments containing destruction boxes, such as the first 70 residues of Cdc13 (N70), inhibits the growth of *Schizosaccharomyces pombe* at metaphase. This inhibition can be overcome either by removal of all lysine residues from N70 using site-directed mutagenesis (K0-N70) or by raising the concentration of intracellular ubiquitin. Consistent with the idea that competition for ubiquitin accounts for some of its inhibitory effects, wild-type N70 not only stabilized D-box proteins, but also Rum1 and Cdc18, which are degraded by a different pathway. The K0-N70 construct was neither polyubiquitinated nor degraded *in vitro*, but it blocked the growth of strains of yeast in which anaphase-promoting complex/cyclosome (APC/C) function was compromised by mutation, and specifically inhibited proteolysis of APC/C substrates *in vivo*. Both K0-N70 and 20-residue D-box peptides blocked polyubiquitination of other D-box-containing substrates in a cell-free ubiquitination assay system. These data suggest the existence of a D-box receptor protein that recognizes D-boxes prior to ubiquitination.
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Introduction

Proteolysis by the ubiquitin–proteasome pathway is a fundamental mechanism for protein turnover, cell cycle control and signal transduction (Ciechanover, 1994; Hochstrasser, 1996; King *et al.*, 1996a; Hershko, 1997; Varshavsky, 1997; Elledge and Harper, 1998; Patton *et al.*, 1998b). In this process, ubiquitin, a highly conserved 76-residue protein, is first linked by a thioester linkage to a ubiquitin-activating enzyme (E1) in a reaction that uses ATP, then transferred to a small ubiquitin-carrier (E2). E2 acts alone or in conjunction with an E3 ubiquitin protein ligase to conjugate ubiquitin to the ϵ -amino group of lysine residues in substrate proteins to form a glycyl–lysine isopeptide bond (Hershko *et al.*, 1983). Multiple rounds of ubiquitin conjugation form polyubiquitinated substrates, which are then degraded to short peptides by the ATP-dependent 26S proteasome complex, and free ubiquitin is regenerated by the action of ubiquitin C-

terminal hydrolases or isopeptidases. In some cases, E3 enzymes themselves carry ubiquitin as a thioester (Huibregtse *et al.*, 1995; Hatakeyama *et al.*, 1997). In others, there is no evidence for the formation of such intermediates, in which cases E3 presumably serves as a kind of scaffold for simultaneous binding of E2 and substrate. In cell cycle control, two important E3s have been identified: the so-called SCF (Skp1/Cdc53-cullin/F-box protein) complex that promotes degradation of Sic1p, Cdc6p, Far1p and the Cln gene products at the G₁–S transition (Deshaies *et al.*, 1995; Lanker *et al.*, 1996; Willems *et al.*, 1996; Drury *et al.*, 1997; Henchoz *et al.*, 1997; Skowyra *et al.*, 1997; Verma *et al.*, 1997; Patton *et al.*, 1998a,b), and the anaphase-promoting complex/cyclosome (APC/C), which is required for the destruction of cyclins and other proteins that are degraded from the end of metaphase until the onset of S-phase (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995; Tugendreich *et al.*, 1995; Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996; Yamamoto *et al.*, 1996; Juang *et al.*, 1997; Shirayama *et al.*, 1998).

The APC/C is a 20S (1.5 MDa) multimeric complex that is required for the metaphase–anaphase transition and cyclin degradation *in vivo*, and for high-rate cyclin ubiquitination *in vitro* (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995). This E3-like complex appears to be regulated by mitotic phosphorylation, although it remains active during G₁ and indeed G₀ when the mitotic kinases have long been inactive (Amon *et al.*, 1994; Lahav-Baratz *et al.*, 1995; Brandeis and Hunt, 1996). Amon *et al.* (1994) have presented evidence that Cln/Cdc28p kinase is required to inactivate Clb proteolysis, and there is evidence for a similar role for cyclin E in *Drosophila* (Knoblich *et al.*, 1994). In addition, polo-like (Cdc5p) (Charles *et al.*, 1998; Shirayama *et al.*, 1998) and cAMP-dependent protein kinases (Grieco *et al.*, 1996; Yamashita *et al.*, 1996; Kotani *et al.*, 1998) have also been implicated in the control of APC/C activity.

The best studied substrates of ubiquitin- and APC/C-mediated proteolysis are the mitotic cyclins. Murray *et al.* (1989) showed that sea urchin cyclin B lacking its first 90 residues could not be degraded and prevented exit from mitosis. Analysis of the N-terminal region defined the sequence essential for cyclin proteolysis, the so called ‘destruction box’ or D-box (Glotzer *et al.*, 1991), and showed that constructs containing D-boxes were rapidly polyubiquitinated when cyclin destruction was switched on. The consensus for the motif in B-type cyclins is RXALGXIXN, in which arginine (R) at position 1 and leucine (L) at position 4 are highly conserved except for cyclin B3, which has phenylalanine (F) instead of L at position 4. Mutations in the D-box stabilize cyclins and severely reduce or abolish their ubiquitination (Glotzer *et al.*, 1991; Lorca *et al.*, 1992; Amon *et al.*, 1994; Stewart

et al., 1994). The cyclin B destruction box is portable, for in most cases, chimeras containing the N-terminus of cyclin B attached to other proteins are degraded as though they were cyclins (Glotzer *et al.*, 1991; Amon *et al.*, 1994; Brandeis and Hunt, 1996; Yamano *et al.*, 1996). In an extensive study of the destruction signal in cyclins A and B, King *et al.* (1996b) defined a minimal stretch of 27 residues that conferred programmed proteolysis on cyclin B, and made the important observation that although at least one lysine residue was required for proteolysis of the target, no particular one was essential or even preferred. They also confirmed our finding (Klotzbücher *et al.*, 1996) that the D-box of cyclin A does not behave as a transportable module; indeed, we concluded that cyclin A must be bound to Cdc2 or CDK2 in order to be degraded. None of these studies has revealed what recognizes the D-box, although its small size suggests that it should form a typical tight binding site for another protein, the hypothetical D-box receptor.

The regulation of destruction of D-box-containing proteins still presents puzzling features. For example, cyclin A and Pds1p are degraded before cyclin B, yet they all contain destruction boxes and require the APC/C for their proteolysis. Part of the explanation may come from the 'fizzy' family of proteins, which are required for D-box protein proteolysis (Matsumoto, 1997; Schwab *et al.*, 1997; Sigrist and Lehner, 1997; Visintin *et al.*, 1997; Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998; Lim *et al.*, 1998). These elements provide connections between spindle checkpoint control mechanisms (Hwang *et al.*, 1998; Kim *et al.*, 1998) and in *Saccharomyces cerevisiae*, the timing of proteolysis of different substrates seems to depend on different members of the family, Cdc20p being responsible for Pds1p and Hct1p/Cdh1p for Clb2p and Ase1p (Sigrist and Lehner, 1997; Visintin *et al.*, 1997). Their molecular mode of action remains to be clarified, and there is no evidence so far that any member of the fizzy family, or component(s) of the APC/C directly bind to D-boxes.

Holloway *et al.* (1993) have shown that large amounts of the N-terminus of sea urchin cyclin B (residues 13–110) causes metaphase arrest in a *Xenopus* cell-free system. We also found that strong expression of the N-terminal 70 residues of fission yeast cyclin B Cdc13 (N70) similarly arrested *Schizosaccharomyces pombe* cells at metaphase (Yamano *et al.*, 1996). We wanted to determine whether the metaphase arrest caused by high concentrations of D-box-containing fragments was due to competition for the hypothetical D-box receptor or if these constructs simply consumed all the cell's ubiquitin and/or other components of the proteolysis machinery. We show here that raising the intracellular ubiquitin concentration or removing all lysine residues from the N70 was sufficient to overcome growth arrest, and it seems that the major effect of N70 is to block all ubiquitin-mediated proteolysis, not just that of D-box proteins. The lysine-less version of N70 was a more selective inhibitor of the degradation of D-box-containing proteins, however, and did block cell growth when the APC/C was compromised by mutation. Thus, competition for D-box receptor(s) can be demonstrated *in vivo*. Cell-free biochemical assays demonstrate that polyubiquitination and proteolysis of cyclin require both the D-box and internal lysine residues.

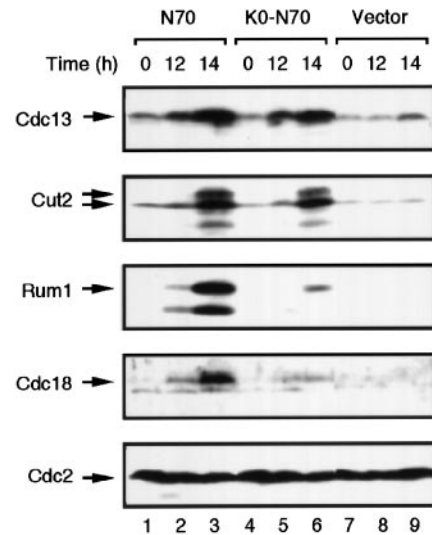


Fig. 1. Levels of ubiquitin-mediated proteolysis substrates after induction of the N-terminus of Cdc13 in *S.pombe*. Wild-type *S.pombe* was transformed with pREP1(N70), pREP1(KO-N70) or empty vector. Transformants were grown in minimal medium in the presence of thiamine, and then transferred to medium without thiamine to induce expression. Samples of the transformed cells expressing N70 or KO-N70 or empty vector were taken at the indicated times (h) after removal of thiamine from the medium and analysed by immunoblotting with anti-Cdc13, anti-Cut2, anti-Rum1, anti-Cdc18 or anti-Cdc2 antibodies, as indicated in the Figure.

Competition experiments show that the D-box of cyclin B must be recognized by a component that is present in anti-Cdc27 immunoprecipitates from *Xenopus* egg extracts.

Results

N70 arrests cells at metaphase partly by sequestering ubiquitin, and partly by competition for D-box receptor protein(s)

We previously showed that strong expression of the N-terminal 70 residues of Cdc13 (N70) arrested the growth of *S.pombe* at metaphase, presumably by competition with authentic substrates of the ubiquitin–APC/C pathway of programmed proteolysis. As expected, the arrested cells accumulated Cdc13 (Yamano *et al.*, 1996). To test what other unstable proteins were protected from degradation by N70, we measured the levels of Cut2 (Funabiki *et al.*, 1996), whose destruction is required for the onset of anaphase, and included two other cell cycle proteins, Rum1 and Cdc18, which are normally very unstable during mitosis (Jallepalli *et al.*, 1997; Benito *et al.*, 1998), but which are degraded by a ubiquitin-dependent pathway that does not require the APC/C (Kominami and Toda, 1997). Figure 1 shows that strong expression of N70 stabilized Cut2 as well as Cdc13, which probably accounts for the arrest at metaphase (high levels of Cdc13 arrest cells in anaphase). Surprisingly, however, high levels of N70 also caused Rum1 and Cdc18 to accumulate in the metaphase-arrested cells. N70 that had a mutated D-box did not arrest the cells, and did not cause accumulation of any of these proteins (data not shown).

We originally assumed that N70 protected D-box-containing proteins by competition for a hypothetical

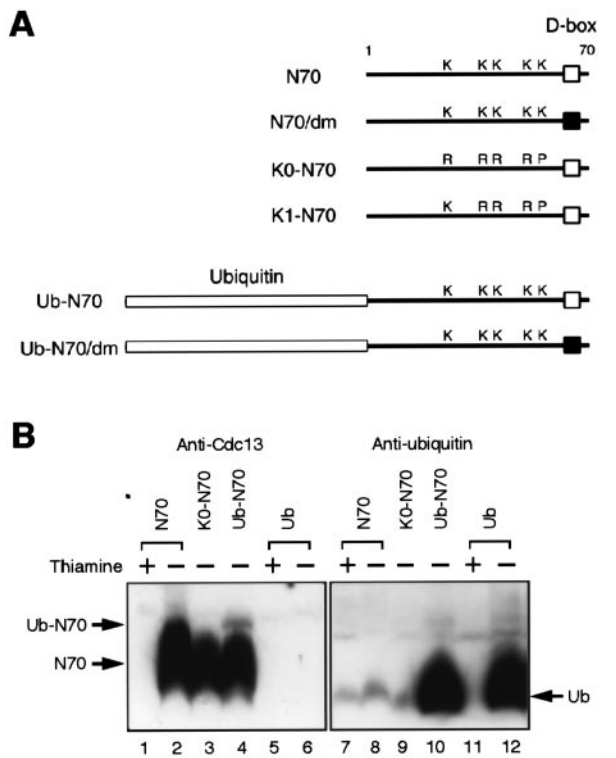


Fig. 2. Schematic diagram of N70-derived constructs. (A) The position of the five lysine residues (K32, K46, K47, K57 and K58) and the D-box (spanning residues 59–67) in N70 are shown. N70/dm is the D-box mutant (R59A; L62A), and K0-N70 is the lysine-less version of N70. K1-N70 is the same construct as K0-N70 except that lysine 32 is not mutated. Ub-N70 consists of ubiquitin fused to the N-terminus of the N70 construct. This fusion protein is efficiently processed to ubiquitin and N70 *in vivo*. (B) Immunoblots of yeast cell extracts expressing N70 (lanes 1, 2, 7 and 8), K0-N70 (lanes 3 and 9), Ub-N70 (lanes 4 and 10) or Ub alone (lanes 5, 6, 11 and 12) with anti-Cdc13 (lanes 1–6) or anti-ubiquitin (lanes 7–12). Thiamine (+) = off; thiamine (–) = on.

D-box recognition element. The stabilization of substrates that neither contain D-boxes nor require the APC/C for their proteolysis suggested the possibility that when N70 becomes a prime target for ubiquitination at the metaphase–anaphase transition, it sequesters ubiquitin or other components of the proteolytic machinery, which results in stabilization of other ubiquitin–proteasome substrates. To test this possibility, we made a construct with ubiquitin fused to the N-terminus of N70 (Ub-N70) (Figure 2A), which should increase the concentration of ubiquitin in parallel with N70 as a result of the ubiquitin-processing protease. Figure 2B shows that the protein encoded by this construct was efficiently processed to free ubiquitin and N70 (lanes 4 and 10), and the level of the unprocessed protein was very low. It is also clear that the concentration of free ubiquitin in the yeast was massively increased when Ub-N70 was expressed (compare lanes 7–9 with lane 10). Figure 2B, lane 12, shows that expressing Ub alone, without N70 attached, gave a similar high level of Ub expression. The Ub-N70 construct did not inhibit colony formation by *S.pombe*, unlike N70 itself, which was extremely toxic (Figure 3A). It thus appears that simply raising the intracellular ubiquitin concentration is sufficient to overcome the inhibition imposed by high levels of N70.

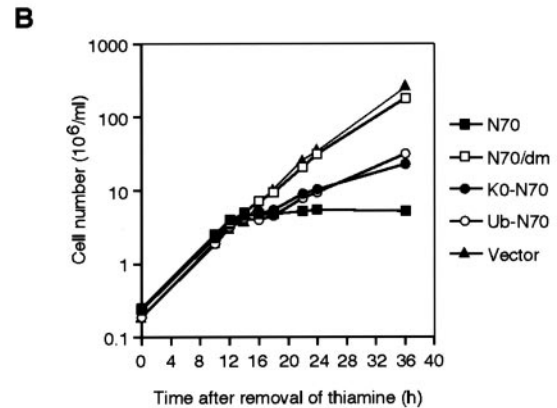
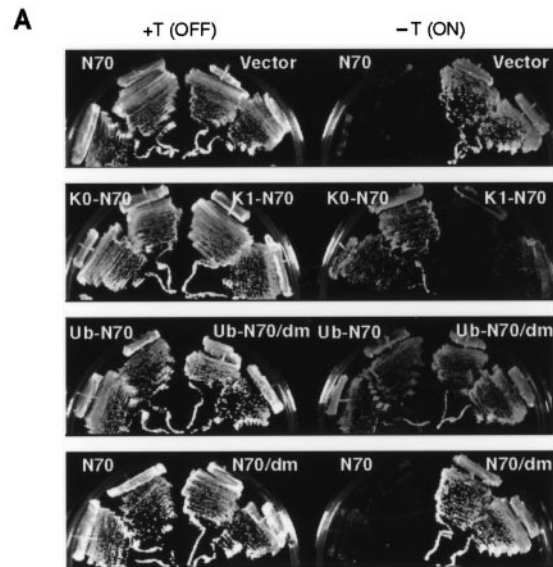


Fig. 3. Phenotype of cells expressing N70 and its variants. (A) Wild-type *S.pombe* was transformed with N70, K0-N70, K1-N70, Ub-N70, Ub-N70/dm, N70/dm or empty vector, and the transformants streaked on minimal plates in the presence (+T = off, left panel) or absence (–T = on, right panel) of thiamine, and incubated at 32°C. (B) Growth curve of cells harbouring the indicated constructs in minimal liquid media. Protein induction usually starts about 12 h after removal of thiamine from the medium.

A slightly different test of the hypothesis that N70 caused a relatively non-specific inhibition of ubiquitin-dependent proteolysis was to prevent the polyubiquitination of N70 by making a version of it in which all lysines were removed by site-directed mutagenesis (Figure 2A). This lysine-less N70 (K0-N70) can only be ubiquitinated at its N-terminus, which, as shown above, would yield a product that is rapidly processed to Ub and N70. Figure 3A shows that removal of lysines from N70 largely suppressed its toxicity and permitted colony formation. A version of N70 containing one remaining lysine residue (K1-N70) inhibited colony formation almost as strongly as N70 itself. Not surprisingly, versions of these constructs with mutated destruction boxes (N70/dm and Ub-N70/dm) had no effect on colony formation.

We examined the growth curves of cells expressing these constructs after induction of the *nmt1* promoter. N70 itself (filled squares, Figure 3B) rapidly and completely arrested growth as the promoter became active at about 12–14 h after removal of thiamine from the medium. In

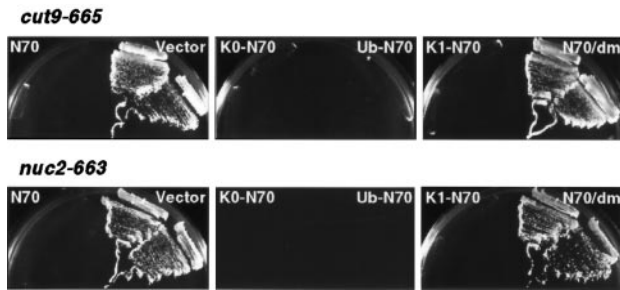


Fig. 4. Expression of K0-N70 and Ub-N70 are synthetically lethal with APC/C subunit mutants. Each of the two APC/C ts mutant strains *cut9-665* (CDC16 homologue) and *nuc2-663* (CDC27 homologue) was transformed with the indicated constructs, and the transformants were streaked on minimal plates in the absence of thiamine (*mtl1* promoter active) and incubated at 29°C.

the case of K0-N70 and Ub-N70, there was a transient inhibition of growth when induction started, and we measured the levels of the various ubiquitin-mediated proteolytic substrates in these cells at 14 h after induction. Figure 1 shows that the APC/C substrates Cdc13 and Cut2, but not Rum1 or Cdc18, were stabilized by K0-N70 (lanes 4–6). At 14 h after induction, ~40% of the cells expressing K0-N70 displayed three condensed chromosomes typical of metaphase arrest, but some kind of adaptation must occur later to allow the cells to resume growth (Figure 3B, circles).

Expression of K0-N70 or Ub-N70 is synthetically lethal with APC/C deficient mutants

Since K0-N70 specifically stabilized APC/C substrates, we decided to investigate the effect of expression of K0-N70 when the normal function of the 20S APC/C was reduced. We used temperature-sensitive (ts) *cut9-665* (a homologue of *CDC16/APC6* in *S.cerevisiae*) or *nuc2-663* (*CDC27/APC3* in *S.cerevisiae*) mutant strains which were transformed with pREP1 vectors expressing N70, K0-N70, Ub-N70, K1-N70 or N70/dm. The empty pREP1 vector served as a control. The transformants were streaked on minimal plates without thiamine to induce expression from the *mtl1* promoter. Figure 4 shows that strong expression of K0-N70 or Ub-N70 was synthetically lethal with *cut9-665* or *nuc2-663* mutants at the semi-permissive temperature of 29°C, although wild-type cells formed colonies under the same conditions (Figure 3A). In contrast, neither N70/dm nor empty vector prevented colony formation in these APC/C temperature-sensitive strains. Even at the permissive temperature (25°C) of the *cut9* or *nuc2* mutant cells, expression of K0-N70 or Ub-N70 strongly reduced colony formation, whereas if the expression was repressed by addition of thiamine, colonies were well formed at both temperatures (data not shown).

Proteolysis of amino terminus of cyclin depends on the proteasome and requires both the D-box and lysine residues

To explore the mechanism of the recognition and proteolysis of cyclin, we turned to the cell-free destruction assay derived from *Xenopus* egg extracts (Murray *et al.*, 1989; Félix *et al.*, 1990; Yamano *et al.*, 1996). In these metaphase-arrested extracts, addition of 0.4 mM CaCl₂ triggers rapid and specific cyclin proteolysis. N70 itself was

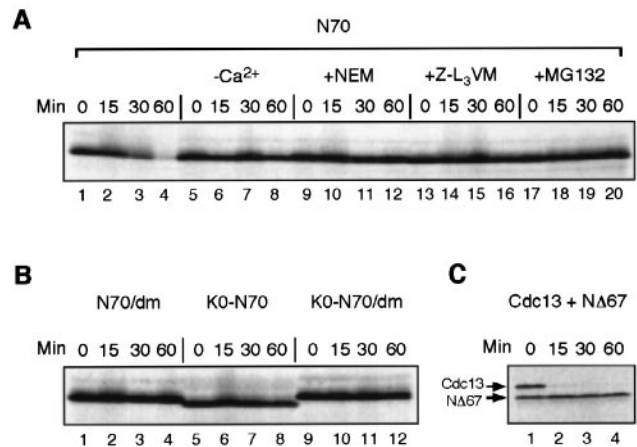


Fig. 5. Destruction of the N-terminus of cyclin in a *Xenopus* egg cell-free system. (A) Cell-free destruction assay of N70 in *Xenopus* egg extracts. CaCl₂ (0.4 mM) was added at time zero to all reactions except the controls (lanes 5–8). NEM (5 mM) or 100 μM proteasome inhibitors (Z-L₃VM or MG132) were added to the indicated reactions at time zero. (B) Destruction of N70/dm (lanes 1–4), K0-N70 (lanes 5–8) and K0-N70/dm (lanes 9–12) in *Xenopus* egg extracts, all with added Ca²⁺. (C) Quality control; Cdc13 was well destroyed, whereas Cdc13 NA67 was stable. The sampling times (min) are indicated above the lanes.

destroyed in a Ca²⁺-dependent manner in this assay system (Figure 5A). Its proteolysis was blocked by *N*-ethylmaleimide (NEM) and proteasome inhibitors such as MG132 or Z-L₃VM (Bogyo *et al.*, 1997). These data indicate that proteolysis of N70 in a *Xenopus* cell-free system is due to the standard ubiquitin–proteasome pathway, and not by some alternative system (Glas *et al.*, 1998).

We also checked the requirement for the D-box and adjacent lysine residues for the proteolysis of N70. We found that N70/dm and the lysine-less construct K0-N70 were stable in the cell-free destruction assay, indicating that the proteolysis of N70 requires an intact D-box and lysine residues in the same polypeptide (Figure 5B). Full-length Cdc13 and Cdc13NA67, which lacks the D-box, served as positive and negative controls in this assay (Figure 5C).

Definition of the minimum sequence necessary for destruction

To define the minimum length required for recognition by the hypothetical D-box receptor, we fused N-terminally truncated versions of N70 to the Gal4 DNA-binding domain (Gal4bd), and tested their destructibility in *Xenopus* egg extracts. Figures 6A and B show that as few as 20 residues (51–70) of Cdc13 fused to the Gal4bd protein destabilized it [Gal4bd itself is a very stable protein in this system (Yamano *et al.*, 1996)]. Residues 59–70 of Cdc13, essentially the D-box itself, worked poorly (Figure 6A, lanes 17–20, and 6B). Consistent with these data, a truncated version of Cdc13 lacking the first 50 residues (Cdc13 NA50) was reasonably unstable in the *Xenopus* cell-free system, whereas Cdc13 NA58, which starts just before the D-box, was hardly degraded, and Cdc13 NA67 was stable (Figure 6C).

We next designed synthetic 20-mer peptides corresponding to the minimum D-box peptides and tested their ability to inhibit destruction of Cdc13 in the cell-free system (Figure 6D). Without added peptide, Cdc13 was destroyed

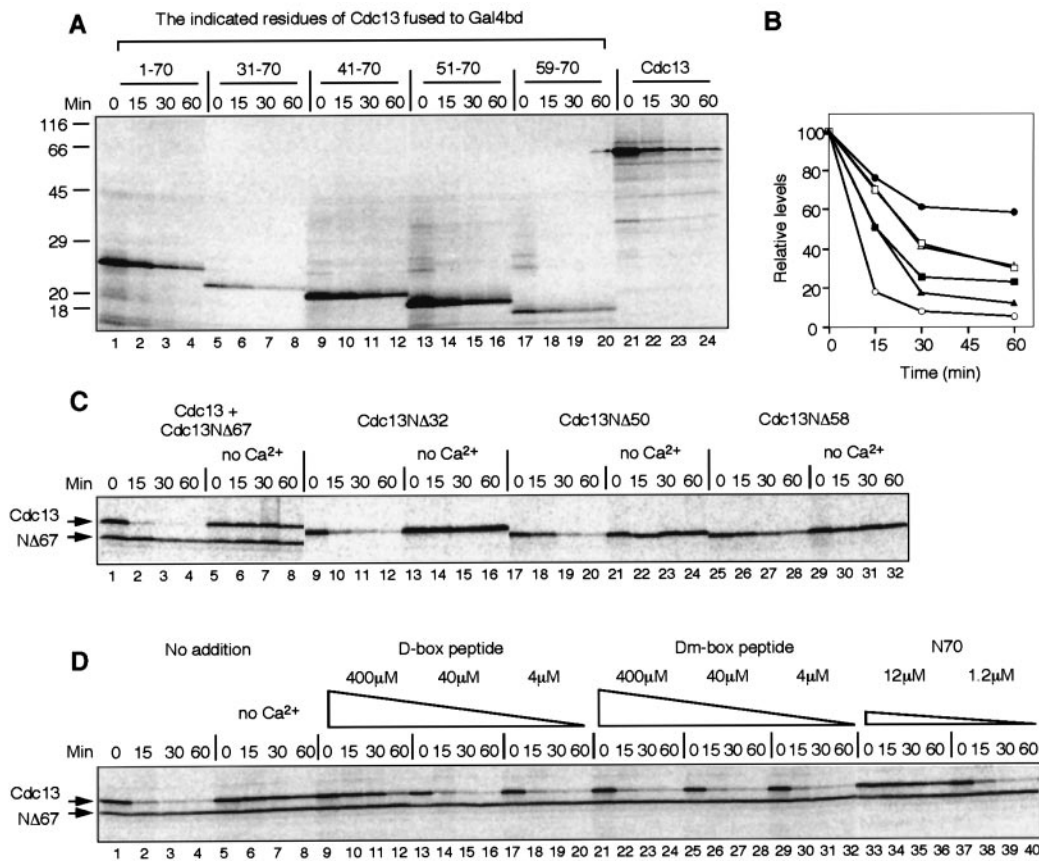


Fig. 6. Definition of a minimal destruction module (A) A series of N-terminal fragments of Cdc13 fused to Gal4bd as indicated were used as substrates. Cdc13 served as a positive control (lanes 21–24). (B) Quantitative data of intensities (A): Cdc13, ○; 1–70, ▲; 31–70, □; 41–70, △; 51–70, ■; 59–70, ○. (C) Destruction assay of Cdc13 and Cdc13 NA67 (lanes 1–8); Cdc13 NA32 (lanes 9–16); Cdc13 NA50 (lanes 17–24) and Cdc13 NA58 (lanes 25–32). CaCl₂ was added in lanes 1–4, 9–12, 17–20 and 25–28. (D) Destruction of Cdc13 in the presence of D-box competitor peptides. Cdc13 and Cdc13 NA67 were translated together to provide destructible and indestructible substrates to controls for non-specific proteolysis. No additions (lanes 1–8); D-box peptide (lanes 9–20); mutant D-box (Dm) peptide (lanes 21–32) and full-length N70 (lanes 33–40) added at the indicated concentrations. CaCl₂ was added to all reactions except lanes 5–8.

in a Ca²⁺-dependent manner (lanes 1–8). A 20-residue peptide with an intact D-box (52–70; D-box peptide) inhibited proteolysis of Cdc13 when added at 0.4 mM (lanes 9–12), whereas the same peptide with a mutated D-box (Dm-box peptide) had no effect on the proteolysis of Cdc13 (lanes 21–24). N70 protein inhibited proteolysis of Cdc13 at 12 μM (lanes 33–36). On a molar basis, therefore, N70 was about 30 times more effective than the D-box peptide at inhibiting the degradation of Cdc13 in these extracts. Nevertheless, these results indicate that short peptides spanning residues 52–70 can be recognized by the D-box receptor. Complete inhibition required a millimolar concentration of competitor peptide, whereas the cyclins are typically present in *Xenopus* extracts at 10–100 nM (Kobayashi *et al.*, 1994), essentially all of which can be rapidly degraded, implying very tight binding by the recognition element(s).

Polyubiquitination of N70 requires both the D-box and internal lysine residues

We set up an *in vitro* ubiquitination assay using bacterially expressed recombinant mouse E1, *Xenopus* UBCx (E2), anti-Cdc27 immunoprecipitates (IP) from *Xenopus* anaphase extracts and ¹²⁵I-labelled ubiquitin (Figure 7A) (King *et al.*, 1995; Fang *et al.*, 1998). Bacterially expressed N70 and its variants were used as substrates. Polyubiquitin-

ation of wild-type N70 was observed when E1, UBCx, anti-Cdc27IP and the substrate were included in the reaction (Figure 7A, lane 5), whereas no polyubiquitination occurred if any one of the components was omitted (Figure 7A, lanes 1–4). The polyubiquitination was abolished by *N*-ethylmaleimide (Figure 7A, lane 7). Mutating the D-box (Figure 7A, lane 6) or eliminating lysine residues from N70 (Figure 7A, lane 8) completely abolished the polyubiquitination. A low level of monoubiquitination of lysine-less N70 was observed (Figure 7A, lane 8) that was abolished by mutation of the D-box (Figure 7A, lane 9). Thus, polyubiquitination of N70 requires both the D-box and its internal lysine residues.

The D-box is recognized before ubiquitin is conjugated to cyclin

Finally, we asked whether the *in vitro* ubiquitination of destruction-box-containing substrates could be inhibited by the lysine-less construct (K0-N70) which, though it cannot be ubiquitinated, should compete with other substrates for the hypothetical D-box receptor. As a substrate for ubiquitination in the experiment shown in Figure 7B, we used the first 100 residues of *Xenopus* cyclin B1 fused to glutathione *S*-transferase (N100-GST). As competitors, we used either K0-N70, K0-N70/dm or the synthetic peptides corresponding to residues 52–70 of

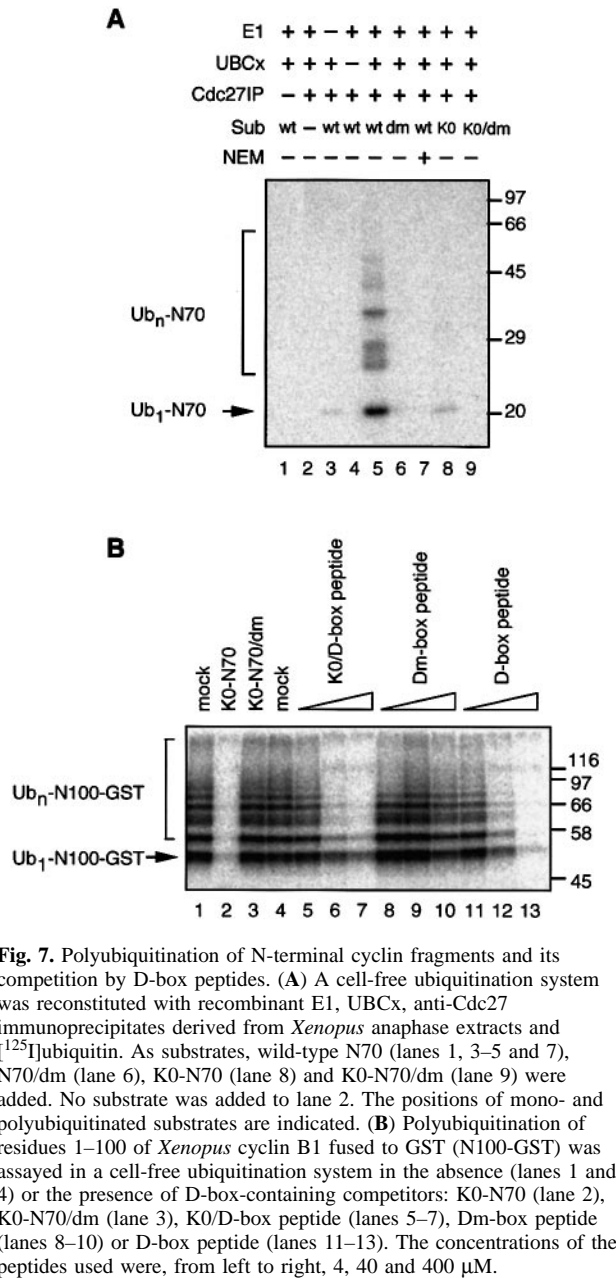


Fig. 7. Polyubiquitination of N-terminal cyclin fragments and its competition by D-box peptides. **(A)** A cell-free ubiquitination system was reconstituted with recombinant E1, UBCx, anti-Cdc27 immunoprecipitates derived from *Xenopus* anaphase extracts and [¹²⁵I]ubiquitin. As substrates, wild-type N70 (lanes 1, 3–5 and 7), N70/dm (lane 6), K0-N70 (lane 8) and K0-N70/dm (lane 9) were added. No substrate was added to lane 2. The positions of mono- and polyubiquitinated substrates are indicated. **(B)** Polyubiquitination of residues 1–100 of *Xenopus* cyclin B1 fused to GST (N100-GST) was assayed in a cell-free ubiquitination system in the absence (lanes 1 and 4) or the presence of D-box-containing competitors: K0-N70 (lane 2), K0-N70/dm (lane 3), K0/D-box peptide (lanes 5–7), Dm-box peptide (lanes 8–10) or D-box peptide (lanes 11–13). The concentrations of the peptides used were, from left to right, 4, 40 and 400 μM.

Cdc13. In the absence of competitor, polyubiquitination of N100-GST was detected in the *in vitro* ubiquitinating system (Figure 7B, lanes 1 and 4). Addition of K0-N70 completely abolished the polyubiquitination of N100-GST, whereas K0-N70 containing a mutated D-box (K0-N70/dm) had no effect (Figure 7B, lanes 2 and 3). Moreover, the 20-residue peptide with an intact D-box (K0/D-box or D-box peptides) blocked the polyubiquitination of N100-GST (Figure 7B, lanes 5–7 and lanes 11–13), whereas the D-box mutant peptide (Dm-box peptide) had little effect (Figure 7B, lanes 8–10). This suggests that the D-box is recognized before the process of ubiquitin conjugation by a component that is present in anti-Cdc27 immunoprecipitates. When anti-Cdc27 immunoprecipitates were omitted from the cell-free ubiquitination system, the UBCx-ubiquitin thioester formed to the same extent in all reactions (data not shown), showing that the added peptides did not affect ubiquitin transfer from E1 to E2 (UBCx).

Discussion

The results of the present study show that internal lysine residues and the D-box of Cdc13 are required for its polyubiquitination and proteolysis during anaphase. Indeed, according to cell-free ubiquitination assays, a 20-residue peptide containing the D-box is sufficient to be recognized by the APC/C. Such peptides also inhibit cyclin proteolysis in the *Xenopus* cell-free system. *In vivo*, we found that strong expression of a non-ubiquitinatable D-box polypeptide (K0-N70) specifically stabilized APC/C substrates 14 h after induction from the strong *nmt1* promoter, and expression of K0-N70 showed synthetic lethality with APC/C mutants. We propose that this is caused by sequestration of the hypothetical D-box receptor, resulting in impaired proteolysis of endogenous APC/C substrates. Thus, we provide *in vivo* and *in vitro* evidence that these N-terminal sequences containing the D-box are recognized by the ubiquitin-proteasome pathway.

We previously reported that strong expression of the N-terminus of Cdc13 (N70) blocked cell cycle progression at metaphase, whereas the same construct with a mutated D-box had no effect on cell growth (Yamano *et al.*, 1996). The results presented in this paper suggest that N70 becomes an excellent target for the APC/C at some point during metaphase, leading to a general inhibition of ubiquitin-dependent proteolysis as well as sequestration of the hypothetical D-box receptor. This stabilizes both Cut2, whose destruction is required for sister chromatid separation, and Cdc13, which needs to be degraded for cells to exit mitosis. Expression of indestructible Cut2 by itself does not result in metaphase arrest, but rather leads to a 'cut' phenotype, in which septum formation and cytokinesis occur without sister chromatid separation (Hirano *et al.*, 1986; Funabiki *et al.*, 1996). In contrast, expression of indestructible cyclin B causes an anaphase arrest (Holloway *et al.*, 1993; Surana *et al.*, 1993; Rimmington *et al.*, 1994; Sigrist *et al.*, 1995; Yamano *et al.*, 1996). It is noteworthy that strains of budding and fission yeast with mutations in 26S proteasome subunits arrest in metaphase at the non-permissive temperature (Ghisai *et al.*, 1993; Gordon *et al.*, 1993), so this point in the yeast cell cycle is probably particularly sensitive to inhibition of ubiquitin-mediated proteolysis. The stabilization of Cdc18 by strong expression of N70 probably accounts for the observation that G₁-arrested fission yeast cells entered S phase in the presence of N70 (Yamano *et al.*, 1996).

Despite this general inhibition of proteolysis by N70, we were able to demonstrate more specific effects of competition for recognition of the destruction box by the use of N70 lacking lysine residues (K0-N70), and hence non-ubiquitinatable. This polypeptide proved to be a more specific inhibitor of the degradation of APC/C substrates *in vivo* and of ubiquitination of D-box containing substrates *in vitro*. In fact, the APC/C activity of anti-Cdc27 immunoprecipitates was inhibited by synthetic peptides as short as 20 residues, provided that they contained an intact D-box, even though these peptides could not be ubiquitinated. This suggests that a component present in the anti-Cdc27 immunoprecipitate recognizes the D-box prior to conjugation of the first ubiquitin. The observation that expression of lysine-less N70 (K0-N70) is synthetically

lethal with *nuc2* or *cut9* mutants also suggests that the APC/C is involved, directly or indirectly, in the recognition of the D-box. Specificity in protein ubiquitination is thought to derive from the E3 ubiquitin–protein ligase, which binds substrates close to an E2-binding site. For example, in budding yeast, the SCF complex has substrate-specific adapter subunits called F-box proteins (Cdc4, Grr1 and Met30) that recruit various substrates to a core ubiquitination complex (Willems *et al.*, 1996; Skowrya *et al.*, 1997; Verma *et al.*, 1997; Patton *et al.*, 1998b). Similarly, a protein or complex of proteins in the APC/C must serve as a D-box recognition element. Immunopurified APCs from *Xenopus* egg extracts or budding yeast extracts contain at least 8–12 subunits, including Cdc16 (Apc6), Cdc23 (Apc8) and Cdc27 (Apc3) (Peters *et al.*, 1996; Zachariae *et al.*, 1996, 1998; Yu *et al.*, 1998), although none of them have yet been reported to associate with the D-box. Identification of the D-box receptor in the anti-Cdc27 immunoprecipitate apparently is not a straightforward matter. We conducted yeast two-hybrid screens to identify binding partners for the D-box without success. There is a problem with this approach, however, in that for much of the cell cycle, the D-box may not be recognized with high affinity, and when it is recognized, the ‘bait’ construct becomes highly unstable. We have also made unsuccessful attempts to isolate multicopy suppressors of the toxicity due to strong expression of N70. If the binding site for D-box-containing substrates is formed from more than one polypeptide, such approaches would probably not succeed. We continue to explore other ways of identifying the elusive D-box receptor, and to understand how the recognition is regulated.

Materials and methods

Fission yeast strains and methods

Schizosaccharomyces pombe haploid wild type h⁻ strain was used. Strains with mutations in 20S APC/C subunits were *cut9-665* and *nuc2-663* (Hirano *et al.*, 1988; Samejima and Yanagida, 1994). All media and growth conditions are as described by Moreno *et al.* (1991). Temperature-sensitive mutants used were normally grown at the permissive temperature of 25°C, whereas 29 and 36°C were used as semi-permissive and restrictive temperatures, respectively. Wild-type cells were cultured at 32°C. Transformation of *S.pombe* was performed using the high-efficiency lithium method (Okazaki *et al.*, 1990).

To induce expression from the *mtl1* promoter, cells were grown in minimal medium containing 2 µM thiamine to mid-exponential phase, spun down and washed three times with minimal medium and resuspended in fresh medium lacking thiamine at a density calculated to produce 3 × 10⁶ cells/ml after 14 h of induction.

Plasmids and DNA

The plasmids pREP1(N70) and pREP1(N70/dm) have been described in Yamano *et al.* (1996). The N-terminal 70 residues of Cdc13 (N70) have five lysine residues (K32, K46, K47, K57 and K58) and the D-box spans residues 59–67. Using PCR-based site-directed mutagenesis (Horton and Pease, 1991), lysine-less (K0-N70) and single-lysine (K1-N70) mutants were created by replacing all five lysines (K32, K46, K47, K57 and K58) and four lysines (K46, K47, K57 and K58) to arginine, respectively, except that K58 was changed to proline because the residue just before the D-box is proline in vertebrate cyclin B1. The final PCR products of K0-N70 and K1-N70 were digested with *NdeI* and *BamHI* and ligated with *NdeI* and *BamHI*-cut pREP1 vector. These N70, N70/dm, K0-N70 and K0-N70/dm *NdeI*–*BamHI* fragments were subcloned into pET16b for bacterial expression. To construct ubiquitin fused to N70 (Ub-N70) and N70/dm (Ub-N70/dm), the *Xenopus* ubiquitin gene was prepared by PCR as an *NdeI*–*BfaI* fragment, then ligated with *NdeI*-cut pREP1(N70) and pREP1(N70/dm), respectively. pREP1(Ub) was constructed by

subcloning *Xenopus* ubiquitin gene into the pREP1 vector. All constructs were sequenced to confirm the mutation sites.

Plasmids pHY22(Cdc13) and pHY22(Cdc13 NΔ67) used for *in vitro* translation were described in Yamano *et al.* (1996). Cdc13 NΔ32, NΔ50, NΔ58 and a series of N70 deletions fused to Gal4bd (Figure 6) were amplified by PCR with the T7 promoter sequence (TAATACGACTCATATAGGGGAGACCGGAAG) at the 5' end, which enabled the preparation of substrates for the destruction assay as described below.

Preparation of yeast extract and immunoblotting

Yeast extracts were prepared essentially as described by Stone *et al.* (1993). Cultures were grown to 3–5 × 10⁶ cells/ml, harvested by centrifugation and washed in 1 ml of lysis buffer (50 mM Tris–HCl pH 8.0, 0.4 M NaCl, 10 mM EDTA, 5 mM EGTA, 80 mM sodium β-glycerophosphate, 1 mM Na₃VO₄, 1 mM 2-mercaptoethanol, 1 mM PMSF). Cells were disrupted in the lysis buffer by vortexing vigorously with glass beads. Cell debris was removed by a 1 min spin in a microcentrifuge and the protein concentration determined by Bradford assay. Equal amounts of protein (20–50 µg) were analysed by SDS–PAGE, transferred to nitrocellulose and immunoblotted with anti-Cdc13 antiserum, affinity-purified anti-Cut2 (a gift of Dr M. Yanagida), anti-Rum1 antiserum (a gift of Dr P. Nurse), anti-Cdc18 antiserum (a gift of Dr H. Nishitani), the monoclonal anti-Cdc2 antibody Y100 (Yamano *et al.*, 1996) or an anti-ubiquitin monoclonal antibody (mAb1510, Chemicon Inc.). The immunoblots were developed using the enhanced chemiluminescent (ECL) system (Amersham).

Cyclin destruction assay in *Xenopus* egg extract

Cyclin destruction assays were carried out essentially as described previously (Yamano *et al.*, 1996). The intensities of the labelled cyclin bands were quantified using a Molecular Dynamics PhosphorImager in conjunction with ImageQuant software. Destruction of cyclin in the cell-free system was triggered by the addition of 0.4 mM CaCl₂. Substrates for destruction were labelled with [³⁵S]methionine + cysteine (Promix, Amersham) in a coupled *in vitro* transcription–translation system (Craig *et al.*, 1992) except for N70 and its variants, which were labelled by an *E. coli* T7 S30 extract system (Promega). As inhibitors of the ubiquitin–proteasome pathway, 5 mM NEM, 100 µM MG132 (Peptide Institute, Inc., Japan) or Z-L₃VM (carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone; a gift of Dr M. Bogoy) were added to reactions.

The sequence of the D-box peptide was CSTNVPKRRHALDDVSNFHN (D-box underlined), and the mutant Dm-box peptide CSTNVPKKAHAADDVSNFHN (mutated residues underlined). Bacterially expressed and purified N70 protein was also tested as a competitor of proteolysis of Cdc13 in the destruction assays.

Preparation of mouse monoclonal antibody against human Cdc27 (mAb AF3)

A peptide corresponding to the C-terminal 10 residues of human Cdc27 (CQLHAAESDEF) was coupled to KLH and injected into Balb/c mice in Freund's complete adjuvant. Hybridoma cells produced from the spleen cells from a hyperimmune mouse were fused with Sp/0 myeloma cells using standard procedures (Harlow and Lane, 1988). Positive clones were identified using immunoadsorbent assays and immunoblotting of HeLa cell extracts. mAb AF3 gave the strongest signal in these assays. Immunoprecipitates of *Xenopus* extracts using AF3 contained a polypeptide that was recognized by other anti-Cdc27 antibodies (Transduction labs C40920), and showed APC/C activity *in vitro*.

Ubiquitination assays

His-tagged mouse E1, *Xenopus* UBCx and substrates (N70, N70/dm, K0-N70, K0-N70/dm and *Xenopus* cyclin B1 N100-GST) were expressed in *E. coli* and purified essentially as previously described (Hoffmann and Roeder, 1991; Poon *et al.*, 1993). The 20S APC/C was immunoprecipitated from okadaic-acid-treated *Xenopus* CSF extracts using mAb AF3. To perform *in vitro* ubiquitination of substrates, a 5 µl reaction mix (50 mM Tris–HCl pH 9.0, 10 mM MgCl₂, 1 mM DTT, 2 mM ATP) containing 30 ng mouse E1, 75 ng *Xenopus* UBCx, immunoprecipitates of 20S APC/C by AF3 coupled-Affi-Prep protein A beads (Bio-Rad) from 10 µl *Xenopus* extracts, ¹²⁵I-labelled bovine ubiquitin and 0.1 µg of substrate were incubated at 23°C for 60 min. Substrates were immunoprecipitated by anti-Cdc13 antibody in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.1% SDS and 1 mM EDTA, and analysed by SDS–PAGE and Molecular Dynamics PhosphorImager using ImageQuant software. For competition experiments (Figure 8), 0.1 µg of *Xenopus* cyclin B1 N-terminal 100 residues fused to GST was used as a substrate, and 8 µM K0-N70 or K0-N70/dm proteins, or 400 µM,

40 μ M and 4 μ M of either K0/D-box peptide CSTNVLRPRHALD-DVSNFHN, Dm-box peptide CSTNVPKKAHAADDVSNFHN or D-box peptide CSTNVPKKRHALDDVSNFHN were used as competitors.

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