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Regulating the Yeast Kinetochore by Ubiquitin-Dependent Degradation and Skp1p-Mediated Phosphorylation

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Summary

In S. cerevisiae, the four-protein Cbf3 complex binds to the essential *CDEIII* region of centromeric DNA to initiate kinetochore assembly. We report the reconstitution of Cbf3p from recombinant proteins and an analysis of its p58^{Ctf13} and p23^{Skp1} subunits. p23^{Skp1} has both G1- and G2-specific functions in yeast and binds to p58^{Ctf13} and to the essential Cdc4p component of the ubiquitin conjugating complex Scul^{Cdc4}. We show that the function of p23^{Skp1} in Cbf3p is to activate p58^{Ctf13} by phosphorylation. p58^{Ctf13} is an unstable protein that is targeted to the proteosome, probably by Scul^{Cdc4}-mediated ubiquitination. Thus, p58 appears to be activated by phosphorylation in a p23^{Skp1}-dependent step and degraded by the proteosome in a ubiquitin-dependent step. We propose that coupled activation and destruction link the assembly of Cbf3p to the duplication of centromeres in S phase.

Introduction

The attachment of chromosomes to the microtubules of the mitotic spindle is mediated by kinetochores, DNA–protein complexes that assemble on centromeric DNA. Failure to form a functional kinetochore or the formation of two kinetochores per chromosome results in missegregation. To understand how the accuracy of centromere assembly is maintained, we are studying the assembly of the relatively simple kinetochore of Saccharomyces cerevisiae. Centromeres in S. cerevisiae are composed of three DNA elements: *CDEI*, *CDEII*, and *CDEIII* (Clarke and Carbon, 1980; Fitzgerald-Hayes et al., 1982; Hegemann and Fleig, 1993). The deletion of *CDEI* or the *CDEI*-binding protein, Cbf1p, increases chromosome loss 10-fold (Baker and Masison, 1990; Cai and Davis, 1990). Deletions in the A/T-rich *CDEII* element inactivate the centromere, although no *CDEII*-binding proteins have been identified (Gaudet and Fitzgerald-Hayes, 1987). Deletions and point mutations in highly conserved *CDEIII* bases completely abolish centromere function (McGrew et al., 1986; Ng and Carbon, 1987).

Because it is absolutely required for kinetochore function, we have focused our attention on Cbf3, a protein complex that binds to *CDEIII*. Initial purification of Cbf3p from yeast has shown it to consist of three protein subunits with molecular masses of 110, 64, and 58 kDa (Lechner and Carbon, 1991). p110 is encoded by the *NDC10/CTF14/CBF2* genes, p64 is encoded by the *CEP3/CBF3b* genes, and p58 is encoded by the *CTF13* gene (Doheny et al., 1993; Goh and Kilmartin, 1993; Jiang and Carbon, 1993; Lechner, 1994; Strunnikov et al., 1995). For simplicity, *CBF3* gene subunits will be referred to by their molecular weights (i.e., p58, p64, and p110). All three genes are essential for cell viability, and conditional mutations abolish the *CDEIII* binding activity of Cbf3p (Sorger et al., 1994). This shows that all three proteins are required to assemble a Cbf3p complex active for DNA binding (Lechner, 1994; Sorger et al., 1995).

Recent analysis has shown that the product of the *SKP1* gene (p23^{Skp1}) is a fourth component of Cbf3p and that p23^{Skp1} copurifies with p58 from yeast extracts (Stemmann and Lechner, 1996). *SKP1* was also found as a high copy suppressor of a mutation in p58 (*ctf13-30*; Connelly and Hieter, 1996). Analysis of *SKP1* has shown that it is an essential gene that appears to be required for both the G1–S and G2–M transitions. Yeast carrying the *skp1-3* allele arrest before the initiation of DNA synthesis, while yeast carrying the *skp1-4* allele arrest in G2. At semipermissive temperatures, *skp1-4* cells exhibit an increased rate of chromosome loss (Connelly and Hieter, 1996).

In addition to the role of *SKP1* in kinetochore assembly, *SKP1* was also isolated as a suppressor of a mutation in the cell division control gene *CDC4* (Bai et al., 1996). *CDC4* is required for the degradation of Sic1p, an inhibitor of the cell cycle kinase p34^{Cdc28}. Mutants in *CDC4* arrest cells at the G1–S transition, presumably because they cannot degrade Sic1p and thus fail to activate p34^{Cdc28} (Schwob et al., 1994). Cdc4p has recently been shown to be a component of the multiprotein Scul^{Cdc4} (also termed SCF^{Cdc4p}) complex, which also contains the ubiquitin ligase Cdc34p, the cullin Cdc53p, and p23^{Skp1} (Feldman et al., 1997; Skowyra et al., 1997). p23^{Skp1} binds directly to Cdc4p via a protein sequence called the F box (Bai et al., 1996). Scul^{Cdc4} complexes transfer ubiquitin to phosphorylated Sic1p, and substrate recognition is thought to involve the Cdc4p and p23^{Skp1} subunits (Feldman et al., 1997; Skowyra et al., 1997). Following the Scul^{Cdc4}-mediated monoubiquitination of Sic1p, a polyubiquitin chain is added, and ubiquitinated Sic1p is then degraded by the 26S proteosome (Hochstrasser, 1995).

The discovery that p23^{Skp1} is required for both kinetochore function and ubiquitin-mediated proteolysis is intriguing. In this paper, we investigate this connection by determining the function of p23^{Skp1} in the formation of active Cbf3p. We report that the *CDEIII* binding activity of Cbf3p can be reconstituted in insect cells by expressing four proteins: p110, p64, p58, and p23^{Skp1}. We demonstrate that p23^{Skp1} mediates the phosphorylation-dependent activation of p58 and that p58 is an unstable protein, targeted for degradation in a Cdc34p-dependent manner. These findings link the role of p23^{Skp1} at the kinetochore with its role in targeting proteins for ubiquitin conjugation. We propose that by tightly coupling p58 activation and p58 destruction, the cell regulates the amount of Cbf3p available for assembly on

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DNA. This may be one of the mechanisms that ensures that only one kinetochore forms per chromosome.

Results

Production of Active Recombinant Cbf3p

The components of Cbf3p are present in very small amounts. In nuclear and whole-cell extracts we typically recover 5–10 active Cbf3p complexes per cell (as assayed by *CDEIII* binding activity). We therefore set out to generate recombinant Cbf3p. First, we determined which yeast proteins are required for the formation of active Cbf3p. We had previously shown that mutations in genes encoding p58, p64, and p110 abolish the *CDEIII* binding activity of Cbf3p (Sorger et al., 1995). To determine if this is also true for p23^{Skp1}, we assayed congenic *SKP1*, *skp1-3*, and *skp1-4* strains for activity. Extracts from *skp1-3* mutant cells, which arrest in G1, had essentially the same *CDEIII* binding activity as extracts from *SKP1* cells (Figure 1A, lanes 1 and 5), but extracts from *skp1-4* cells, which arrest in G2–M, were 8-fold less active (lane 4). Thus, mutations in any one of the four *CBF3* genes eliminate *CDEIII* binding in yeast extracts.

These results suggested that to reconstitute Cbf3p in vitro, we would need to express all four Cbf3 proteins. We therefore infected insect cells with recombinant baculoviruses expressing wild-type p23^{Skp1}, p58, p64, and p110 (Figure 1B). First, we expressed the four Cbf3p subunits separately, made extracts from infected cells, mixed the extracts, and assayed *CDEIII* binding on bandshift gels (Figure 1C, lane 12). No DNA binding activity could be detected in mixtures of extracts from individually infected cells. We therefore attempted to generate Cbf3p by coinfecting insect cells with four viruses, each of which expressed one Cbf3p subunit. In a quadruple coinfection, active Cbf3p was formed (Figure 1C, lane 13) and shown to be a specific for *CDEIII* using a competition assay (lanes 14 and 15).

Coexpression of p23^{Skp1} and p58 Is Required For Cbf3p Activity

Why is the coexpression of four Cbf3p subunits required to generate an active Cbf3p complex? Differences in the steady-state levels of Cbf3 proteins did not appear to be responsible for the differences in activity between individually expressed and coexpressed proteins (Figure 1B and data not shown). To assay the activities of recombinant Cbf3 proteins individually, we added them to extracts from yeast cells carrying mutations in Cbf3p-encoding genes. When recombinant p110 was added to extracts prepared from *ndc10-42* cells, *CDEIII* binding was restored to wild-type levels (Figure 1D, lanes 16 and 17). Similarly, recombinant p64 reconstituted *CDEIII* binding activity in *cep3-2* extracts (lane 18 and 19). However, neither p23^{Skp1} nor p58 was active in reconstituting

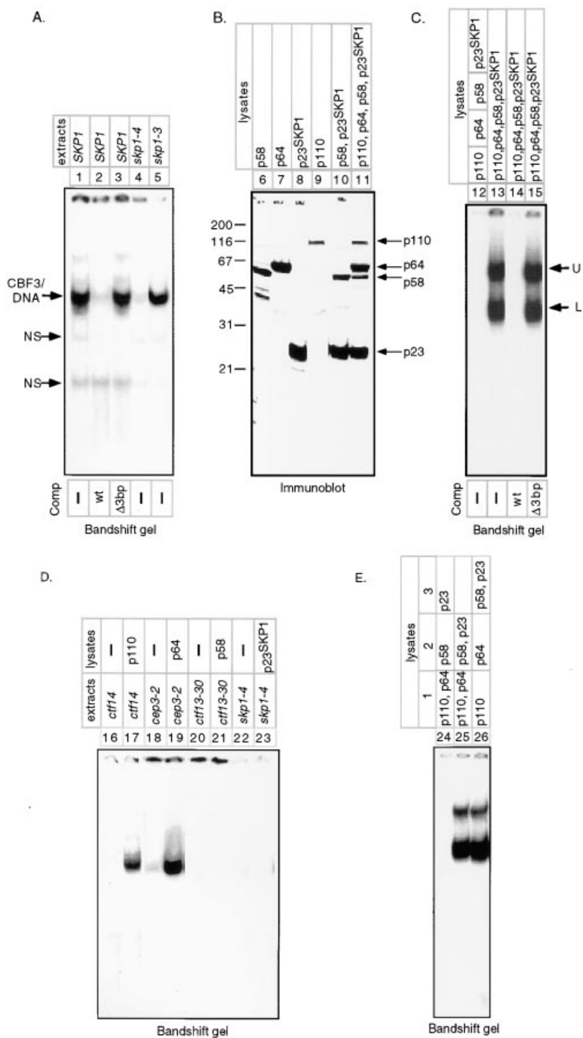

Figure 1. p23^{Skp1} Is Required for the Formation of Active Cbf3p

(A) *CDEIII* binding activity requires *SKP1*. Yeast extracts (60 μg) were prepared from congenic wild-type (lanes 1–3), *skp1-4* (lane 4), and *skp1-3* yeast strains (lane 5) grown at 37°C for 3 hr and analyzed on bandshift gels using an 88 bp *CEN* DNA probe. *CDEIII*-Cbf3p complexes were specifically inhibited by a 64-fold molar excess of wild-type (lane 2) but not mutant unlabeled *CEN*-DNA competitor (lane 3). Nonspecific proteins bound to radiolabeled probe are labeled NS.

(B) Recombinant Cbf3p expression. Insect cells were infected with individual or combinations of recombinant baculoviruses. Lysates from cells expressing p58 (lane 6), p64 (lane 7), p23^{Skp1} (lane 8), p110 (lane 9), both p58 and p23^{Skp1} (lane 10), or all four subunits (lane 11) were analyzed by immunoblotting using four polyclonal sera raised against each Cbf3p subunit followed by incubation with ^{125}I-protein-A.

(C) Active recombinant Cbf3p. Equal amounts of recombinant p110, p64, p58, and p23^{Skp1} produced in four independent infections were mixed together (lane 12) or were produced in a quadruple coinfection (lane 13) and analyzed on bandshift gels. Two bandshift products were observed with the 88 bp *CEN* DNA probe (arrows) and both products (slower migrating, U; faster migrating, L) were shown to be specific by competition analysis (lanes 14–15).

(D) Complementing mutant yeast extracts with recombinant proteins. Extract (60 μg) from *ctf14* (lanes 16 and 17), *cep3-2* (lanes 18 and 19), *ctf13-30* (lanes 20 and 21), or *skp1-4* (lanes 22 and 23) cells were supplemented with buffer alone (lanes 16, 18, 20, and 22) or 200 pmol of recombinant p110 (lane 17), p64 (lane 19), p58 (lane 21), or p23^{Skp1} (lane 23) and analyzed on bandshift gels.

(E) Activity requires p58 and p23^{Skp1} coexpression. A p110/p64-lysate was mixed with a p58 and a p23^{Skp1}-lysate (lane 24) or with a p58/p23^{Skp1}-lysate (lane 25). Alternatively, a p110-lysate was mixed with a p64-lysate and then added to a p58/p23^{Skp1}-lysate (lane 26). All reactions analyzed on bandshift gels.

skp1-4 or *ctf13-30* extracts (Figure 1D, lanes 20–23). We conclude that our inability to reconstitute Cbf3p from separately expressed proteins is probably a consequence of the inactivity of either p23^{Skp1} or p58.

To confirm this, we asked which proteins required coexpression for activity. Lysates from insect cells infected with different combinations of viruses were mixed so that all four Cbf3p subunits were present in the final mix. *CDEIII* binding activity was then measured on bandshift gels. We found that only p23^{Skp1} and p58 had to be coexpressed to form active Cbf3p (Figure 1E, lanes 24 and 25). When p64 and p110 were expressed individually and added to coexpressed p23^{Skp1} and p58, *CDEIII* binding activity was as high as when all four Cbf3p subunits were expressed in one cell (Figure 1E, lane 26).

p23^{Skp1}-Mediated Activation of p58

To investigate the mechanism by which the coexpression of p23^{Skp1} and p58 leads to Cbf3p assembly, HA-p23^{Skp1} and His$_6$HA-p58 were coexpressed in insect cells and then separated on an NTA-Ni resin (which binds to hexa-histidine sequences). Because both HA-p23^{Skp1} and His$_6$HA-p58 carry the same influenza-derived epitope tag, their concentrations could be compared directly on immunoblots using anti-HA antibody and ^{125}I-protein-A. The sample loaded onto the NTA-Ni column contained p23^{Skp1} and p58 in approximately equimolar amounts (Figure 2A, lane 1). When the column was washed in 300 mM NaCl, essentially all of the p23^{Skp1} eluted along with about 1% of the p58 (lane 3). p58 could be recovered free of detectable p23^{Skp1} by eluting the column with imidazole (lanes 4–6). Samples from the column were then added to *ctf13-30* or *skp1-4* yeast extracts, and *CDEIII* binding activity was measured on bandshift gels. When NTA-Ni–purified p58 (fraction 2) was added to *ctf13-30* extracts, *CDEIII* binding activity was reconstituted, suggesting that the recombinant p58 free of p23^{Skp1} is active (but only if it had previously been exposed to p23^{Skp1}). More remarkable was the ability of purified p58 to reconstitute activity when added to *skp1-4* extracts (Figure 2B, lanes 10 and 11). This suggests that the biochemical defect preventing the formation of active Cbf3p in *skp1-4* yeast cells is the inactivity of p58.

Three pieces of data argue that the biochemical complementation of *skp1-4* extracts by p58 was not due to the presence of a small amount of contaminating p23^{Skp1}. First, the amount of p23^{Skp1} present in a sample had no effect on its ability to reconstitute *CDEIII* binding (compare lanes 12 and 14 in Figure 2). Second, no p23^{Skp1} could be detected in Cbf3p-*CDEIII* complexes formed with purified p58. When lysates from cells coexpressing HA-p23^{Skp1}, p58, p64, and p110 were assayed on bandshift gels, the incorporation of HA-p23^{Skp1} into *CDEIII* binding complexes could be detected by adding anti-HA antiserum and then looking for an antibody-dependent supershift (Connelly and Hieter, 1996). Following the purification of p58 away from HA-p23 by ion exchange chromatography, no supershift was detected (data not shown). Third, no *CDEIII* binding activity could be detected when purified His$_6$-p23^{Skp1} was added to *skp1-4* yeast extracts, showing that even at high levels, recombinant p23^{Skp1} was not active (data not shown). From

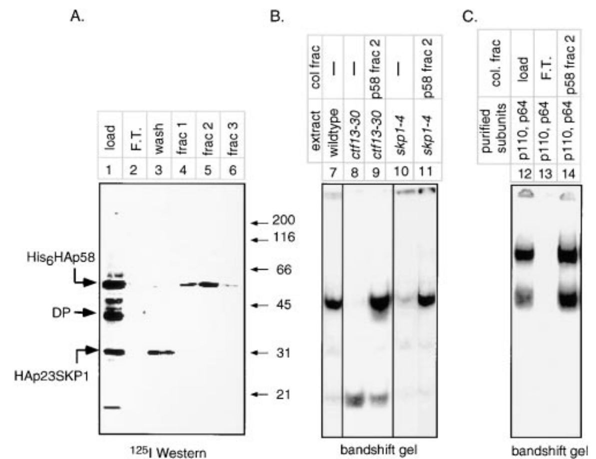

Figure 2. p58 Is Activated by Transient Association with p23^{Skp1}

(A and B) Purification of p58 (A). His$_6$HA-p58 from an insect cell lysate containing both His$_6$HA-p58 and HA-p23^{Skp1} was purified by binding to NTA-Ni resin and elution with imidazole. An equivalent percentage of the load (load, lane 1), flow-through (FT, lane 2), wash (W, lane 3), and the first three eluted fractions (frac, lanes 4–6) were analyzed by immunoblotting using a monoclonal antibody against the HA epitope (MS101R; Babco). Degraded p58 products that appeared during the purification are labeled "DP". Immunoblotting with ^{125}I-protein-A was linear over a 10-fold range of His$_6$HA-p58 (data not shown). (B) Activity of purified p58. Extracts (60 μg) from congenic wild-type (lane 7), *ctf13-30* (lane 8), and *skp1-4* (lanes 10 and 11) yeast strains were analyzed on bandshift gels. *ctf13-30* (lane 9) and *skp1-4* extracts (lane 11) were supplemented with equal amounts of purified His$_6$HA-p58 from fraction 2 (A) and analyzed as in Figure 1D.

(C) Forming active Cbf3p with purified p58, p64, p110. Purified p110 and p64 were mixed with insect cell lysate containing His$_6$HA-p58 and HA-p23^{Skp1} (load, lane 12), column flow-through (FT, lane 13), or purified p58 (fraction 2, lane 14), and analyzed on bandshift gels. Equivalent amounts of His$_6$HA-p58 were analyzed in lanes 12 and 14. Control experiments show that the amounts of p58 added to p64 and p110 were within the linear range of the assay (data not shown).

these data, we conclude that the biochemical defect preventing the formation of active Cbf3p in *skp1-4* yeast cells is the inactivity of p58. This strongly suggests that the function of p23^{Skp1} in Cbf3p assembly is to promote p58 activation. We presume that, in insect cells, this activation can take place only when p58 and p23^{Skp1} are coexpressed.

An implication of these findings is that active Cbf3p can be formed from purified p64, p110, and activated p58. To test this, we added the NTA-Ni–purified p58 described above to purified p110 and p64 and assayed *CDEIII* binding activity on bandshift gels (Figure 2C). The NTA-Ni–purified p58 was as active per mole of p58 in this assay as unfractionated lysates from cells coexpressing p58 and p23^{Skp1} (lanes 12 and 14). We therefore conclude that p58, p64, and p110 can assemble into an active DNA binding complex in the absence of p23^{Skp1}. Thus, p23^{Skp1} appears to be required only transiently for the *CDEIII* binding activity of Cbf3p.

p23^{Skp1} Binds to p58

To examine the mechanism by which p23^{Skp1} activates p58 in insect cells, we asked whether p58 and p23^{Skp1}

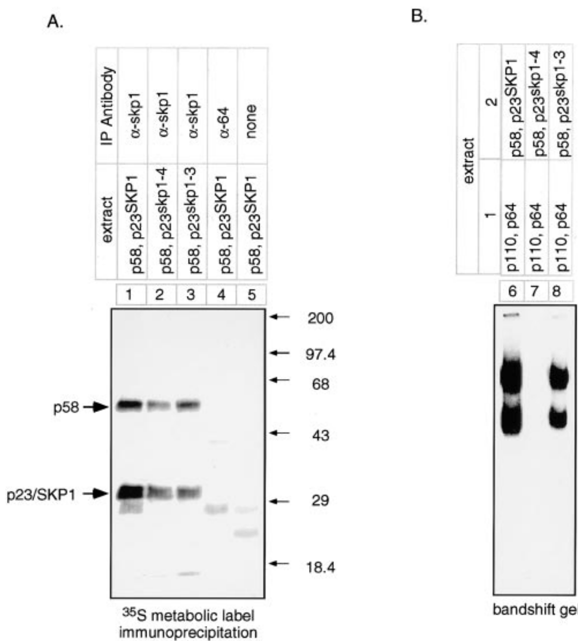

Figure 3. $p23^{Skp1}$ Association Is Not Sufficient for p58 Activity

(A) Binding of p23 to p58. Insect cells coexpressing p58 and $p23^{Skp1}$, $p23^{skp1\text{-}4}$, or $p23^{skp1\text{-}3}$ were metabolically labeled with ^{35}S-methionine and cysteine. Cells were lysed in Triton-X 100 buffer and 250 μg of total protein immunoprecipitated with α-$p23^{Skp1}$ antibodies and protein A-Sepharose. Immune complexes containing p58 and $p23^{Skp1}$(lane 1), p58 and $p23^{skp1\text{-}4}$ (lane 2), or p58 and $p23^{skp1\text{-}3}$ (lane 3) were analyzed on SDS-PAGE and then by autoradiography. As a negative control, lysate containing p58 and $p23^{Skp1}$ were immunoprecipitated with α-p64 antibody and protein A-Sepharose (lane 4) or with protein A-Sepharose alone (lane 5).

(B) Activity of p58 coexpressed with wild-type and mutant $p23^{Skp1}$. Equal amounts p58 from lysates containing p58 and $p23^{Skp1}$ (lane 6), p58 and $p23^{skp1\text{-}4}$ (lane 7), or p58 and $p23^{skp1\text{-}3}$ (lane 8) were mixed with recombinant p110 and p64 and assayed on bandshift gels.

bind to each other in the absence of other Cbf3 proteins. Lysates were prepared from ^{35}S-labeled cells coexpressing p58 and $p23^{Skp1}$, incubated with anti-$p23^{Skp1}$ or control antibodies absorbed to protein A-Sepharose, and the immunoprecipitates then analyzed on SDS-containing gels. Both $p23^{Skp1}$ and p58 were present in anti-$p23^{Skp1}$ immunoprecipitates (Figure 3A, lanes 1), but neither was present in control precipitates using an unrelated antibody or protein A-Sepharose alone (lane 4 and 5). We conclude that $p23^{Skp1}$ and p58 bind to each other in insect cells.

Is it the inability of p58 to bind to mutant $p23^{skp1\text{-}4}$ that is responsible for the inactivity of p58 in *skp1-4* mutants? To answer this, p58 activity and binding to $p23^{Skp1}$ were compared following p58 coexpression with wild-type or mutant $p23^{Skp1}$. p58 activity was measured by mixing lysates from cells coexpressing p58 and $p23^{Skp1}$ with recombinant p64 and p110 and then assaying *CDEIII* binding activity (p58 that functions in this assay will be referred to as "active" p58). p58 coexpressed with either $p23^{Skp1}$ or $p23^{skp1\text{-}3}$ was active (Figure 3B, lanes 6 and 8) but p58 coexpressed with $p23^{skp1\text{-}4}$ was inactive (lane 7). This is the same relationship between p58 activity and *skp1* mutant alleles that is observed in yeast. The binding of 58 to $p23^{Skp1}$ was assayed by immunoprecipitation as described above. Gel analysis of anti-p23 immune complexes isolated from each of the three lysates showed that the amounts of p58 bound to p23 were indistinguishable (Figure 3A, lanes 1–5). We conclude that p58 and $p23^{skp1\text{-}4}$ can bind to each other as efficiently as p58 and wild-type $p23^{Skp1}$ and, thus, that binding is not sufficient to activate p58.

Phosphorylation of p58 Is Required for Activity

To look for a $p23^{Skp1}$-mediated modification of p58 that might be responsible for activation, we asked whether p58 is phosphorylated. Insect cells expressing a His_6HA-tagged version of p58 alone or coexpressing His_6HA-p58 and either wild-type or mutant $p23^{Skp1}$ were labeled with ^{32}P-orthophosphate, and p58 was isolated by immunoprecipitation. A small amount of ^{32}P was present when His_6HA-p58 was expressed alone, but it rose 10-fold when p58 was coexpressed with $p23^{Skp1}$ (Figure 4 legend and 4A, lanes 1 and 2). Moreover, His_6HA-p58 phosphorylation was high following coexpression with $p23^{skp1\text{-}3}$ but low after coexpression with $p23^{skp1\text{-}4}$ (Figure 4A, lanes 2–4; $p23^{Skp1}$ was also phosphorylated at an approximately constant level). We conclude that the coexpression of p58 and $p23^{Skp1}$ causes p58 to become phosphorylated, and this has the same dependence on *SKP1* alleles as p58 activity. Because $p23^{Skp1}$ bears no resemblance to known protein kinases, we presume that it promotes the phosphorylation of p58 by an as-yet-unidentified kinase present in insect cells (and presumably also yeast; see below).

The expression of $p23^{Skp1}$ stimulates p58 phosphorylation, but is phosphorylation required for activity? To answer this, we treated purified His_6HA-p58 with calf intestinal phosphatase (CIP) coupled to beads, removed the beads by centrifugation, and measured p58 activity by mixing it with recombinant p64 and p110. The treatment of p58 with CIP reduced *CDEIII* binding activity 20-fold, but a control treatment with CIP in the presence of sodium vanadate, a phosphatase inhibitor, had no effect (Figure 4C, lanes 22–24). We conclude that p58 phosphorylation is required for the formation of a Cbf3p complex active in binding to *CDEIII* DNA.

It had previously been shown that $p23^{Skp1}$ binds to Cdc4p via a short sequence known as the F box (Bai et al., 1996). p58 contains a match to the F-box consensus near its amino terminus. We have recently completed a mutagenic analysis of this sequence showing that it is required for p58 function in yeast cells and for the binding of p58 to $p23^{Skp1}$ in rabbit reticulocyte lysates (I. Russell and P. K. S., unpublished data). To determine the role that the F box plays in p58 phosphorylation and activation, we coexpressed $p23^{Skp1}$ with a p58 variant that carries two point mutations in the F-box consensus sequence (p58-L12A/P13A). p58-L12A/P13A is expressed as well as wild-type p58 (Figure 4B, lanes 16–18), but its level of phosphorylation is 5-fold lower (Figure 4B, lanes 13–15), and it is unable to reconstitute *CDEIII* binding when added to recombinant p64 and p110 (Figure 4B, lanes 19–21). This shows that the F box in p58 is required for efficient p58 phosphorylation and for activity. Because F-box sequences are involved in $p23^{Skp1}$ interaction, the implication of this data is that $p23^{Skp1}$ physically links p58 to an activating kinase.

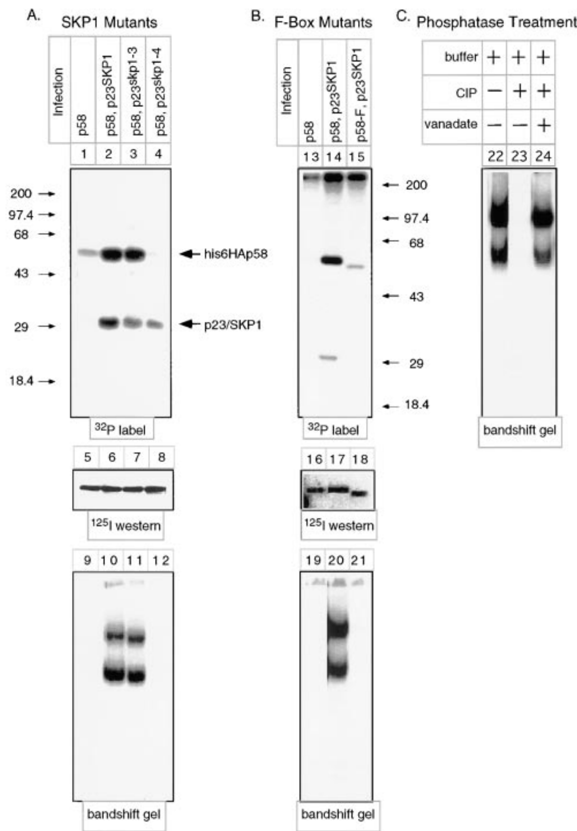

Figure 4. A $p23^{Skp1}$-Dependent Phosphorylation of p58 Is Required for Activity

(A and B) Phosphorylation of p58 mediated by $p23^{skp1-3}$ but not $p23^{skp1-4}$ (A). Insect cells expressing His_6HA-p58 alone (lane 1), or coexpressing His_6HA-p58 and $p23^{Skp1}$(lane 2), p58 and $p23^{skp1-3}$ (lane 3), p58 and $p23^{skp1-4}$ (lane 4) were labeled in vivo with ^{32}P-orthophosphoric acid. Lysate (500 μg) was immunoprecipitated with anti-HA antibody (MS101R; Babco). Immunoprecipitates were divided into two equal fractions and analyzed on SDS-PAGE. One-half of the immunoprecipitate was subjected to autoradiography to quantitate ^{32}P incorporation (lanes 1–4), and one-half was immunoblotted with ^{125}I-protein-A to determine protein levels (lanes 5–8). Cell lysates were also analyzed for *CDEIII* binding activity (lanes 9–12). ^{32}P and ^{125}I signals were quantified using a Phosphorimager, and the amount of phosphate incorporated per unit protein was calculated. (B) Phosphorylation of p58 but not F-box mutant p58. Extracts from ^{32}P-labeled insect cells expressing His_6HA-p58 alone (lanes 13, 16, and 19), coexpressing His_6HA-p58 and $p23^{Skp1}$ (lanes 14, 17, and 20) or coexpressing F-box mutated His_6-p58 (L12A/P13A, labeled p58-F) and $p23^{Skp1}$ (lanes 15, 18, and 21) were analyzed by immunoprecipitation (lanes 13–15), Western blotting (lanes 16–18), or bandshift gels (lanes 19–21) as in (A).

(C) p58 phosphorylation and activity. His_6HA-p58 coexpressed with $p23^{Skp1}$ was isolated from insect cell lysates using NTA-Ni chromatography, and equal amounts were treated with buffer alone (lane 22), 15 units of immobilized-CIP on agarose beads (lane 23), or immobilized-CIP and 100 mM sodium orthovanadate (lane 24) for 15 min at 30°C. Equal volumes of the reaction were mixed with recombinant p64 and p110 and assayed on bandshift gels. A fraction of each reaction was analyzed by immunoblotting to show that the incubations did not affect the levels of p58 (data not shown).

Ubiquitin-Mediated Degradation of p58

We have shown that an essential role of $p23^{Skp1}$ at the yeast kinetochore is to activate DNA binding. However, it has recently been reported that $p23^{Skp1}$ is an essential component of a ubiquitin-conjugating complex (Feldman et al., 1997; Skowyra et al., 1997). To explore the connection between these two roles for $p23^{Skp1}$, we asked whether any Cbf3p components are unstable. We monitored protein levels following the transcriptional shutoff of fusions to the galactose-inducible *GAL1* promoter, a method previously used to monitor destruction of cyclins (Amon and Nasmyth, 1994) and *PDS1* (Cohen-Fix et al., 1996). Yeast cells carrying integrated *GAL1-CTF13*, *GAL1-CEP3*, or *GAL1-NDC10* were grown in the presence of galactose for 1–3 hr to induce transcription and to express proteins. The cells were then switched into glucose-containing media to repress transcription and protein levels were monitored by immunoblotting (Figure 5A). This analysis showed that p58 was very unstable (half-life of 15 min) but that both p64 and p110 were stable (half-life of >300 min). Thus, alone among known Cbf3p subunits, p58 is rapidly degraded.

Is p58 degraded by a ubiquitin-dependent pathway? With a low-abundance protein, it can be difficult to detect ubiquitin conjugates in vivo, so we measured p58 half-life in yeast cells carrying the *pre1-1,4*, *cdc34-2*, or *cdc4-1* mutations. Pre1p is a component of the 26S proteosome (Heinemeyer et al., 1991), a multisubunit complex that degrades ubiquitin-conjugated proteins. In *pre1-1,4* cells, p58 half-life was 5-fold greater than in wild-type cells (90 min versus 15 min), showing that proteosome activity is required for p58 degradation (Figure 5B). Cdc34p and Cdc4p are components of a multiprotein ubiquitin-conjugating complex (the Scul complex) that includes $p23^{Skp1}$. In *cdc4-1* cells, p58 half-life was 4-fold greater than in wild-type cells, and a putative p58-ubiquitin conjugate could be detected (Figure 5D; 30 min time point). In *cdc34-2* cells, p58 was dramatically stabilized, and little degradation was apparent 90 min after *GAL1-CTF13* transcriptional shutoff (Figure 5C) (as a control, we determined that, in both wild-type and mutant cells, RNA stability was identical, Figure 5F). In *cdc34-2* cells, we also observed that the overexpression of p58 at permissive temperatures resulted in a rapid loss of viability (data not shown). In control congenic cells, p58 overexpression increased chromosome loss but viability remained high. A similar synthetic lethal interaction between *cdc34-2* and substrates has previously been described for *CLN2* and *CLN3* (Deshaies et al., 1995). The synthetic lethal interaction between *GAL-CTF13* and *cdc34-2* is additional evidence that p58 is a Cdc34p substrate in vivo.

Both *cdc34-2* and *cdc4-1* cause cells to arrest in G1, and it was necessary to demonstrate that p58 stabilization was not a secondary consequence of cell cycle arrest. We therefore examined p58 degradation in *cdc7-1* cells, which, like *cdc4-1* and *cdc34-2* cells, arrest early in G1–S (Hartwell et al., 1970). The half-life of p58 in congenic wild-type and *cdc7-1* cells was not significantly different, showing that p58 is degraded in G1-arrested cells (Figure 5D). As a further control, we arrested wild-type and *cdc34-2* cells in S phase with hydroxyurea and then shifted them to 37°C. Cells arrested in G1 exhibited the same *cdc34*-dependent degradation of p58, suggesting that it is the defect in *CDC34* and not the cell cycle arrest that alters the stability of p58 in *cdc34-2* cells. Taken together, these data show that p58 degradation requires components of the ubiquitin-mediated

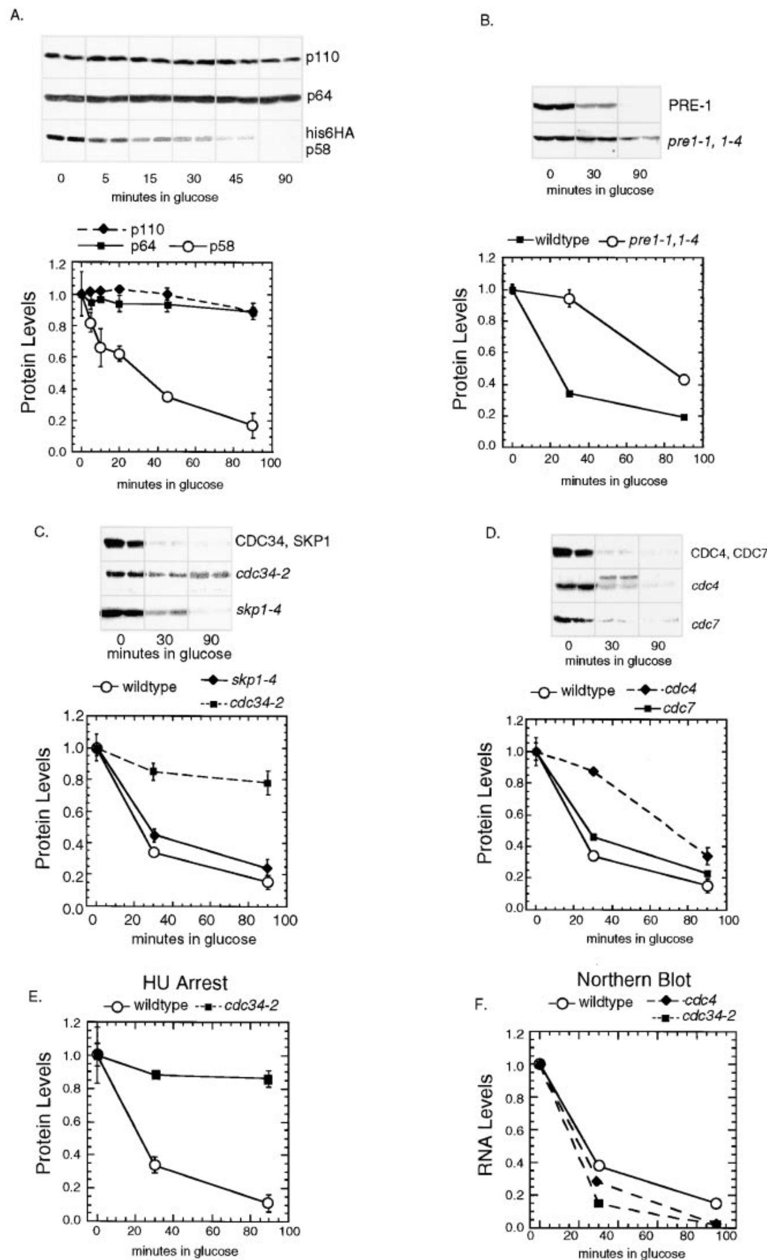

Figure 5. Cdc34p-Mediated Ubiquitin-Dependent Proteolysis Is Involved in Degrading p58 but Not Other Cbf3 Proteins

(A–E) Stability of p58, p64, and p110 in wild-type yeast cells (A). Yeast cells containing either *GAL1-NDC10* , *GAL1-CEP3*, or *GAL1-His$_6$HA-CTF13* were grown to log phase at 30°C in media containing 2% galactose and 2% raffinose for 4–6 hr. A sample was chilled as the 0 time point and the remaining cells were washed into media containing 2% glucose, grown at 30°C, and aliquots placed on ice 5, 15, 30, 45, and 90 min after the addition of glucose. Extracts were prepared and 100 μg of total protein loaded in duplicate lanes on 10% SDS-PAGE gels. Cbf3p subunits were detected by immunoblotting with polyclonal α-p110 and α-p64 antisera or with a anti-HA antibody against His$_6$HA-p58. Antibody binding was quantified using ^{125}I-protein-A and a Phosphorimager. (B) Stability of p58 in mutant yeast cells. The *pre1-1,1-4*, (C) *cdc34-2* or *skp1-4*, (D) *cdc4-1* or *cdc7* mutant strains and their congenic wild-type controls carrying plasmid-borne *GAL1-His$_6$*HA-*CTF13* were grown in selective media and then switched to 37°C for 2 hr; one-third of the cells were placed on ice, and the remaining cells were washed into prewarmed, selective media containing 2% glucose and 10 μg/ml cyclohexamide. Cells were harvested at 30 and 90 min after addition of glucose and analyzed as above (A). (E) p58 stability in S phase. A *cdc34-2* mutant and congenic wild-type (as in B–D) were grown in the presence of 15 mg/ml hydroxyurea for 2 hr to synchronize the cells in S phase and then analyzed as above (B–D). Cells arrested in hydroxyurea were more than 90% arrested based on their large-budded morphology at the end of the time course.

(F) RNA control. Total cellular RNA was isolated from *cdc34-2*, *cdc4-1*, and a congenic wild-type strain during the course of GAL induction and repression, and the levels of *His$_6$*HA-*CTF13* message determined by Northern blotting.

degradation pathway that lie both upstream, such as the Cdc34p ubiquitin ligase, and downstream, such as the Pre1p subunit of the 26S proteosome. p58 stability does not appear to be affected by the *skp1-3* and *skp1-4* mutations (Figure 5C). This does not exclude a role for p23^{Skp1} in p58 degradation but does show that the *skp1-3* and *skp1-4* mutations do not exert their biological effects by stabilizing p58 (see discussion).

Homeostatic Control of p58 Levels

The data described above suggest that the amount of active p58 in a cell is regulated positively by p58 phosphorylation and negatively by p58 degradation. To examine the interplay between positive and negative regulation, we compared the levels of total p58 protein with the levels of active p58 following induction and repression of a *GAL-CTF13* construct. Total p58 levels were determined by immunoblotting. The levels of active p58 were assayed by adding excess recombinant p64 and p110 to yeast extract and measuring the levels of Cbf3p complex by bandshift. Unlike bandshift assays with yeast extract alone, the addition of recombinant p64 and p110 allowed the activity of overexpressed p58 to be measured linearly over at least a 5-fold range (Figure 6A). When total p58 protein levels were measured after *GAL-CTF13* repression, they fell steadily and were down 10–15-fold at 90 min (Figure 6C). In contrast, the total amount of active p58 had fallen only 1.3-fold by 90 min. (Figure 6B). These data show that the level of active p58 remained nearly constant despite a large change in total

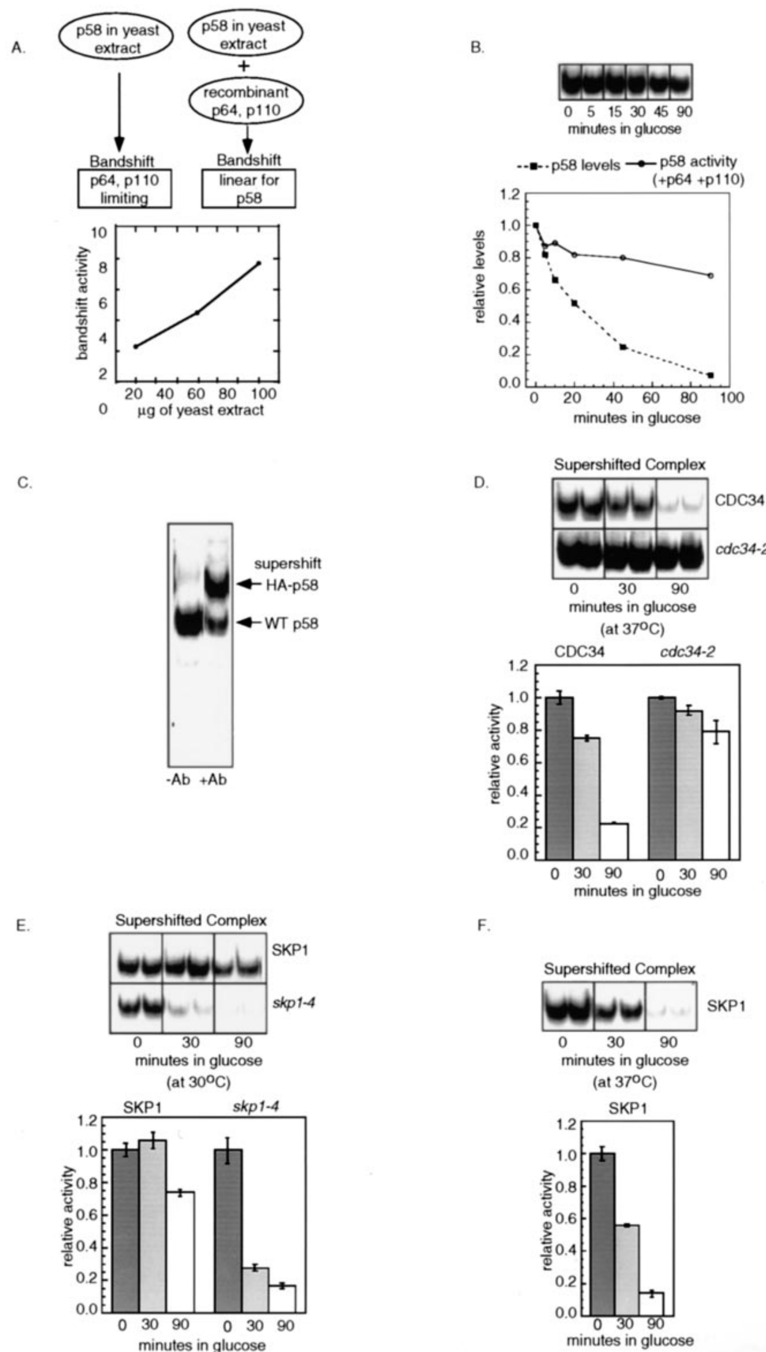

Figure 6. Relationship between the Levels of Total and Active p58 Protein

(A and B) Establishing an assay for active p58 (A). Yeast carrying the integrated *GAL1-His$_6$HA-CTF13* were grown as in Figure 5A. Extracts were made and analyzed for the levels of active p58. To establish a linear assay for active p58, yeast extracts containing His$_6$HA-p58 were added to excess recombinant p64 and p110 (ca. 800 fmol) in a bandshift reaction with 56 bp radiolabeled *CEN* DNA probe. (B). Active and total p58 compared. Yeast extracts (60 μg) from 0, 5, 15, 30, 45, and 90 min after addition of glucose were analyzed as in (A) and the amount of *CDEIII* binding determined on bandshift gels.
(C) Distinguishing endogenous and GALinduced p58. To determine the amount of active His$_6$HA-p58, a monoclonal antibody against the HA epitope was used to supershift Cbf3p complexes containing His$_6$HA-p58.
(D–F). Stability of activated Cbf3p. The mutant strains *cdc34-2* (D), *skp1-4* (E), and their congenic wild-type controls containing the *GAL1-His$_6$HA-CTF13* were analyzed as in Figure 5 and the levels of active His$_6$HA-p58 measured by anti-HA supershift of the Cbf3p complex (only supershifted complex shown; D, E, and F). At 30°C (E, top panel), His$_6$HA-p58 protein was more stable than at 37°C (F).

p58 levels, implying that active p58 is maintained by a homeostatic mechanism that balances phosphorylation and degradation.

While it is clear that inactive p58 can be degraded (Figure 5C, *skp1-4*), a more interesting question is whether activated p58 is also subject to ubiquitin-dependent proteolysis. We examined the stability of active p58 by measuring its levels in extracts prepared from cells carrying *GAL1His$_6$HA-CTF13* in which transcription was briefly induced and then repressed for 0, 30, and 90 min. To distinguish Cbf3p complexes that contained endogenous p58 (expressed from the chromosomal *CTF13* gene) and His$_6$HA-p58, we added anti-HA antibodies to extracts prior to analyzing *CDEIII* DNA binding activity (Figure 6C). We had previously shown that anti-HA antibodies quantitatively "supershift" DNA-protein complexes containing HA-p58 (Doheny et al., 1993).

In wild-type cells grown at 25°C and then shifted to 37°C, the levels of active His$_6$HA-p58 fell rapidly following the transcriptional shutoff of the *GAL-His$_6$HA-CTF13* construct. In *cdc34-2* cells, however, active His$_6$HA-p58 was dramatically stabilized (Figure 6D). This shows that active His$_6$HA-p58 is indeed unstable and that this instability is dependent on the Cdc34p ubiquitin ligase. Next,

we analyzed *skp1-4* cells. In these cells, the steady-state levels of active His_6HA-p58 were very low when cells were shifted to 37°C prior to *GAL* repression (data not shown). This precluded our determining the half-life of the active pool at 37°C. We therefore grew cells under semipermissive conditions (30°C) prior to shifting them into glucose (by comparison, growing wild-type cells at 30°C instead of 37°C resulted in a 2-fold increase in p58 stability; Figures 6E and 6F). Strikingly, active His_6HA-p58 was significantly less stable in *skp1-4* cells than in the congenic wild type cells grown at 30°C. This can be explained if we postulate that $p23^{Skp1}$ is required to activate p58 (as shown above, Figure 1A) but not to degrade it. In *skp1-4* cells, the conversion of a large pool of inactive p58 into activated phospho-p58 is blocked and it therefore disappears rapidly. Taken together, these data show that activated p58 is rapidly degraded in a *CDC34*-dependent and *SKP1*-independent manner.

Discussion

The Cbf3p complex contains four protein subunits: $p23^{Skp1}$, p58, p64, and p110 (Hyman and Sorger, 1995). All four proteins are required for the binding of Cbf3p to centromeric DNA (see introduction). In this paper, we show that $p23^{Skp1}$ is essential for Cbf3p function because it is required for the activation of p58. This is demonstrated most dramatically by the finding that p58 purified from p58-$p23^{Skp1}$–coexpressing cells is able to complement *skp1-4* yeast extracts for *CDEIII* binding activity, but purified $p23^{Skp1}$ is not. Thus, a genetic lesion in $p23^{Skp1}$ has as a biochemical consequence the inactivity of p58. Presumably, it is the failure to activate p58 and thus to assemble Cbf3p that is responsible for the high rate of chromosome loss in *skp1-4* cells under semipermissive conditions and for cell cycle arrest in G2 at high temperatures (Connelly and Hieter, 1996). It had previously been shown that $p23^{Skp1}$ can associate with a DNA-bound Cbf3p complex (Connelly and Hieter, 1996). We have shown, however, that Cbf3p active in DNA binding can assemble in the absence of $p23^{Skp1}$. This suggests that, while $p23^{Skp1}$ can associate with DNA-bound p58, p64, and p110, it does not play an essential structural role. We propose that $p23^{Skp1}$ is a p58 activator and that p58, p64, and p110 are structural components of Cbf3p in direct contact with DNA (Espelin et al., submitted).

p58 is subject not only to positive regulation by phosphorylation, but also to negative regulation by ubiquitin-mediated protein degradation. The degradation of p58 requires the activity of the Cdc34p ubiquitin ligase and also of Cdc4p. Cdc34p and Cdc4p are components of a multiprotein ubiquitin-conjugating complex that also includes $p23^{Skp1}$ as an essential component (Feldman et al., 1997; Skowyra et al., 1997). This finding links the role of $p23^{Skp1}$ at the kinetochore to its previously discovered role in targeting proteins for ubiquitination. We propose that the phosphorylation-dependent activation and ubiquitin-mediated destruction of p58 are coupled to maintain tight control over the level of active Cbf3p.

Activation of p58 by a $p23^{Skp1}$-Associated Kinase

In the baculovirus expression system used in this paper, p58 phosphorylation occurs only when p58 and $p23^{Skp1}$ are coexpressed. This implies that the p58-activating kinase is present in insect cells as well as in yeast cells. Because human p23 binds to a cyclin dependent kinase (via the F box–containing protein Skp2p; Zhang et al., 1995) $p34^{Cdc28}$ is a possible candidate for the p58-activating kinase in yeast. We have, however, examined extracts from cells carrying the *cdc28-4* and *cdc28-13* mutations and found that p58 is as active in these extracts as in extracts from congenic wild-type cells (unpublished data). Thus, either a kinase other than $p34^{Cdc28}$ is involved in phosphorylating p58, or that in the absence of $p34^{Cdc28}$ activity, another kinase can substitute.

We propose that $p23^{Skp1}$ forms a complex with an as-yet-unknown kinase and then links the kinase to p58 via its F box. The F box sequence was first discovered in Cdc4p and shown to be involved in its binding to $p23^{Skp1}$ (Bai et al., 1996). Sequence comparisons reveal a good match to an F box in p58, and *CTF13* genes carrying mutations in the F box are unable to complement a chromosomal deletion of *CTF13*. In addition, F-box mutant p58 proteins cannot bind to $p23^{Skp1}$ in rabbit reticulocyte lysates (I. Russell and P. K. S., unpublished data). Thus, the F box in p58, like the F box in Cdc4p, plays an essential role in $p23^{Skp1}$ binding. We show here that a functional F box is required for the efficient phosphorylation and activation of p58, suggesting that the formation of a p58, $p23^{Skp1}$, and p58-activating kinase complex is F box–dependent.

However, the binding of $p23^{Skp1}$ to p58 is not sufficient for p58 activation. When coexpressed with p58, mutant $p23^{skp1-4}$ protein binds efficiently to p58, but the $p23^{skp1-4}$ mutant does not mediate phosphorylation and activation of p58. This suggests that $p23^{skp1-4}$, while competent to bind p58 through its F box, is unable to bind productively to the p58 activating kinase. Thus, the F-box mutation appears to disrupt one part of the $p23^{Skp1}$-mediated link, and the $p23^{skp1-4}$ mutation appears to disrupt the other. This view of $p23^{Skp1}$ as a linker protein strongly argues for the existence in cells of a complex containing p58, $p23^{Skp1}$, and a kinase.

Degradation of p58 by Ubiquitin-Mediated Proteolysis

Ubiquitin-mediated proteolysis proceeds via a series of steps that starts with the E1-dependent conjugation of ubiquitin to an E2 ligase, followed by transfer of a single ubiquitin to the substrate, and elaboration of the monoubiquitin adduct to create a polyubiquitin chain. Polyubiquitinated substrates are then degraded by the 26S proteosome (Hochstrasser, 1995). We have demonstrated that p58 is degraded in a ubiquitin-dependent manner by showing that p58 is stabilized in cells carrying mutations in an E2 enzyme (*CDC34*), a putative substrate recognition factor (*CDC4*), and a proteosome subunit (*PRE1*). Cdc34p and Cdc4p are both components of the multiprotein $Scul^{Cdc4}$ complex that also contains the Cdc53p cullin and $p23^{Skp1}$ (Feldman et al., 1997; Skowyra et al., 1997). Because $Scul^{Cdc4}$ appears to be required for the degradation of both the Sic1p cell cycle regulator and p58, p58 degradation may be coupled via $Scul^{Cdc4}$ to cell cycle progression. p58 appears to be the only unstable component of Cbf3p, but because all four subunits of Cbf3p are required for DNA binding, the degradation of a single subunit is sufficient to prevent Cbf3p assembly.

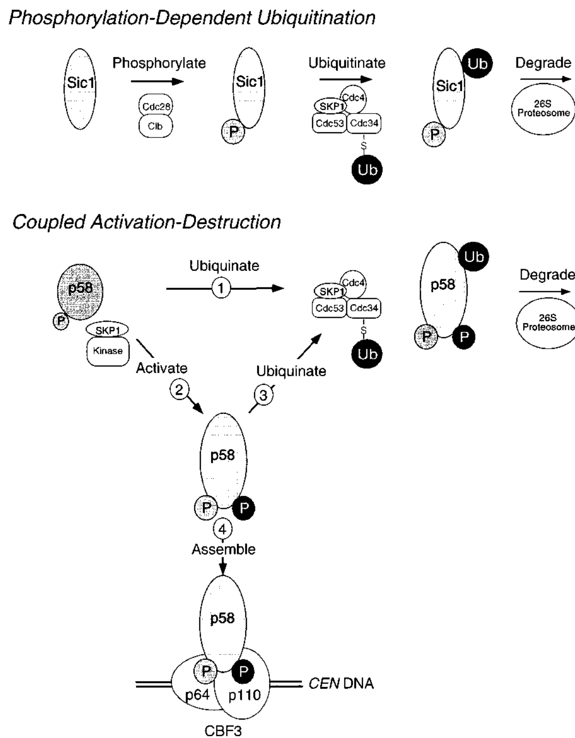

Figure 7. Speculative Model for Coupled Activation and Destruction

A speculative model comparing the phosphorylation-dependent ubiquitination of Sic1p (Feldman et al., 1997; Skowyra et al., 1997) with the coupled activation and destruction of p58 (see text for details).

p23^{Skp1} Roles in Activation and Destruction

We have shown that both total p58 protein and the subset of p58 that has been activated for DNA binding are degraded in a Cdc34p and presumably ubiquitin-dependent fashion. We propose that inside cells, p58 has several fates: ubiquitination without activation, activation followed by ubiquitination, and activation followed by assembly into functional Cbf3p. The interplay among these fates is diagrammed in Figure 7 and contrasted with the phosphorylation-dependent ubiquitination of the cell cycle regulator Sic1p. In pathway one, inactive p58 is ubiquitinated and targeted for degradation. p23^{Skp1}-dependent activation is not a prerequisite for destruction of p58 because p58 is degraded as rapidly in *skp1-4* cells (in which activation is blocked) as in wild-type cells. Nevertheless, by analogy with Sic1p, we speculate that p58 must be phosphorylated to be ubiquitinated. Consistent with this, we have determined that there is p23^{Skp1}-independent phosphorylation of p58 (data not shown). p58 that follows pathway two and is activated by p23^{Skp1}-dependent phosphorylation has two possible fates. One is assembly with p64 and p110 into a Cbf3p complex that can bind to *CEN* DNA and is resistant to degradation (pathway 4; see below) and the other is ubiquitination and degradation (pathway 3). Although we have not yet determined the fluxes through pathways 1–3, the effect of the *skp1-4* mutation on the levels of activated p58 at semipermissive temperatures is striking. In *skp1-4* cells, activated p58 levels fall much more rapidly than in wild-type cells. We can explain this by noting that the *skp1-4* mutation blocks phosphorylation via pathway 2, apparently without affecting ubiquitination via pathways 1 and 3. Thus, following the shift of *skp1-4* cells to semipermissive temperatures, activated p58 is degraded while the generation of new activated p58 is blocked.

The initial discovery of *SKP1* as a suppressor of the *ctf13-30* and *cdc4* mutations implicated p23^{Skp1} in two apparently distinct aspects of cellular physiology: kinetochore regulation and protein ubiquitination. We have shown that these are linked, at least in a general sense, since p58 is both activated in a p23^{Skp1}-dependent manner and targeted for destruction by a protein complex, Scul^{Cdc4}, that probably contains p23^{Skp1}as an essential component. It is therefore surprising that the *skp1-3* and *skp1-4* mutations have no apparent effect on p58 half-life. One possible explanation is that these *SKP1* alleles are specific for other functions and that we need to isolate further *SKP1* mutants to demonstrate an effect on p58 half-life. The second possibility suggested by our data is that p23^{Skp1} is dispensable for p58 ubiquitination and that the role of p23^{Skp1} is restricted to p58 activation. In this light, it will be interesting to examine more closely the role of p23^{Skp1} in the Scul^{Cdc4} complex. It is not impossible that a critical function of p23^{Skp1} is to activate Scul^{Cdc4}, perhaps by mediating the phosphorylation of its Cdc4p component.

Editing Kinetochore Assembly

Although both active and inactive p58 are rapidly degraded, it is the fate of the active p58 that is the most important for cellular physiology. We propose that p58 is subject to degradation when part of an incomplete or incorrectly assembled kinetochore, but resistant to degradation when part of a functional kinetochore. A prediction of this model is that the association of p58 with other Cbf3p subunits should increase its half-life. Consistent with this prediction, we have observed that p58 associates with p64 to form a binary complex and that the overexpression of p64 in yeast makes p58 more stable (unpublished data). Its seems likely that coupled activation and destruction of p58 is an important part of the process that regulates Cbf3p assembly. We speculate that p23^{Skp1}-mediated phosphorylation and Scul^{Cdc4}-mediated degradation of p58 links kinetochore formation to the replication of centromeric DNA in S phase. It will be interesting to look for other examples in which coupled activation and destruction may be involved in linking the assembly of critical mitotic structures to cell cycle progression.

Experimental Procedures

Plasmids, Yeast Strains, and Extracts

The *ctf13-30*, *ndc10-42* (Doheny et al., 1993), *skp1-3*, and *skp1-4* (Connelly and Hieter, 1996) mutant strains and congenic wild-type controls were provided by Dr. P. Hieter; *cdc4-1*, *cdc34-2*, and *cdc7* by Dr. R. Deshaies (Hartwell et al., 1970); and the *pre1-1,1-4* double mutation by Dr. D. Finley. Mutant strains were transformed with a CEN/ARS plasmids containing *URA3* and *GAL1-His$_6$HA-CTF13*. Wild-type strains overexpressing Cbf3p proteins carried *CBF3* genes linked to the *GAL1/10* promoter and integrated at the *ura3-52* locus (Sorger et al., 1995). Yeast extracts were prepared as described (Kaplan and Sorger, 1997).

Production and Purification of Recombinant Cbf3p

The production of recombinant proteins in insects cells will be described elsewhere (K. B. K. et al., unpublished data). Individual, six-His–tagged Cbf3 proteins were purified from insect cell lysates using metal-chelate chromatography. Following precipitation with a 70% saturated ammonium sulfate, proteins were resuspended and dialyzed into Ni binding buffer (10mM HEPES [pH 8.0], 300mM NaCl, 5mM imidazole, 10% glycerol, 10mM β-mercaptoethanol, and 10 μg/ml leupeptin, pepstatin, chymotrypsin, and 100mM phenylmethylsulfonyl fluoride) and then incubated in batch with 250 μl of NTA-Ni resin (Qiagen) for 8–12 hr at 4°C. Protein-bound resin was washed in binding buffer supplemented with 20 mM imidazole and eluted in binding buffer containing 500 mM imidazole.

Cbf3p Bandshifts

Bandshift assays were carried out as described in Sorger et al. (1995). Between 0.1 and 2.0 μl of insect cell lysate and between 30 and 90 μg of yeast extract was added to a standard 30 μl bandshift reaction containing 40 fmol of 88 bp *CDEIII*-radiolabeled probe as described (Sorger et al., 1995). Alternatively, a 56 bp *CDEIII* probe was used as indicated (Espelin et al., submitted).

Acknowledgments

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Note Added in Proof

The reconstitution of the $SCUL^{Cdc4}$ complex has been published recently by Feldman et al. (1997) and Skowyra et al. (1997) and has been named SCF^{Cdc4p}.