

# Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast

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**In budding yeast, ubiquitination of the cyclin-dependent kinase (Cdk) inhibitor Sic1 is catalyzed by the E2 ubiquitin conjugating enzyme Cdc34 in conjunction with an E3 ubiquitin ligase complex composed of Skp1, Cdc53 and the F-box protein, Cdc4 (the SCF<sup>Cdc4</sup> complex). Skp1 binds a motif called the F-box and in turn F-box proteins appear to recruit specific substrates for ubiquitination. We find that Skp1 interacts with Cdc53 in vivo, and that Skp1 bridges Cdc53 to three different F-box proteins, Cdc4, Met30, and Grr1. Cdc53 contains independent binding sites for Cdc34 and Skp1 suggesting it functions as a scaffold protein within an E2/E3 core complex. F-box proteins show remarkable functional specificity in vivo: Cdc4 is specific for degradation of Sic1, Grr1 is specific for degradation of the G<sub>1</sub> cyclin Cln2, and Met30 is specific for repression of methionine biosynthesis genes. In contrast, the Cdc34-Cdc53-Skp1 E2/E3 core complex is required for all three functions. Combinatorial control of SCF complexes may provide a basis for the regulation of diverse cellular processes.**

[Key Words: SCF ubiquitin ligase; glucose; methionine; cyclin; Cdk; protein-protein interaction; Met30; Grr1; Cdc4]

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Ubiquitin-dependent proteolysis is an important regulatory mechanism that controls many cellular processes (for review, see Hochstrasser 1996). In this pathway, ubiquitin is transferred from a ubiquitin activating enzyme (E1) to a ubiquitin-conjugating enzyme (E2) and, often in conjunction with a ubiquitin ligase (E3), ultimately conjugated in an isopeptide linkage to a lysine residue of a substrate protein. Reiteration of the ubiquitin transferase reaction results in formation of a poly-ubiquitin chain on the substrate, which is then recognized by the 26S proteasome, and rapidly degraded. Specificity in protein ubiquitination often derives from E3 ubiquitin ligases (Hershko et al. 1983). In some cases, an E3 facilitates recognition of the target protein by an E2, whereas in others an E3 accepts a ubiquitin thioester from an E2 and directly transfers ubiquitin to the substrate (Scheffner et al. 1995). Although substrate recognition is a key

aspect of ubiquitin mediated proteolysis, the identification of E3 enzymes has been problematic because the few known E3 families bear no sequence relationship to each other.

Ubiquitin-dependent proteolysis is essential for two major cell cycle transitions, the G<sub>1</sub>- to S-phase transition and the metaphase to anaphase transition (for review, see Hershko 1997). Key targets of the ubiquitin proteolytic pathway at these transitions include cyclins, which are positive regulators of cyclin-dependent kinases (Cdks), and Cdk inhibitors, which are negative regulators of Cdks. In budding yeast, a single Cdk, Cdc28 (a.k.a. Cdk1) is activated in G<sub>1</sub> phase by the G<sub>1</sub> cyclins Cln1-Cln3, and in S through M phase by the mitotic cyclins, Clb1-Clb6 (for review, see Nasmyth 1996). Mitotic cyclins and other proteins that regulate mitosis are targeted for degradation by a cell cycle regulated E3 ubiquitin ligase called the anaphase promoting complex (APC) or cyclosome (for review, see Hershko 1997). In contrast, G<sub>1</sub> cyclins and Cdk inhibitors are degraded via a constitutive ubiquitination pathway in which substrates are targeted by phosphorylation.

Genetic analysis in budding yeast has revealed several components required for degradation of the Cdk inhibi-

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tor Sic1: Cdc4, a WD40 repeat protein (Yochem and Byers 1987); Cdc34, an E2 ubiquitin-conjugating enzyme (Goebel et al. 1988); Cdc53, a protein that forms a complex with Cdc4 and Cdc34 (Mathias et al. 1996; Willems et al. 1996); and Skp1, a protein that binds to a motif in Cdc4 called the F-box (Bai et al. 1996). The F-box motif was originally identified in Cdc4 and two mammalian proteins, Cyclin F and Skp2, and is also found in many other eukaryotic proteins (Bai et al. 1996). Cells lacking functional Cdc4, Cdc34, Cdc53, or Skp1 arrest in G<sub>1</sub> phase because the Clb-Cdc28 inhibitor Sic1 is not degraded, and so the onset of Clb-Cdc28 activity and initiation of DNA replication cannot occur (Nugroho and Mendenhall 1994; Schwob et al. 1994; Bai et al. 1996). In late G<sub>1</sub> phase, phosphorylation of Sic1 by the Cln-Cdc28 kinases targets the inhibitor for ubiquitination and degradation (Schwob et al. 1994; Schneider et al. 1996; Tyers 1996; Verma et al. 1997a). An E3 complex has been assembled from recombinant Skp1, Cdc53, and Cdc4 (the SCF<sup>Cdc4</sup> complex), and in association with Cdc34 this complex is sufficient for ubiquitination of phosphorylated Sic1 in vitro (Feldman et al. 1997; Skowrya et al. 1997). Within the SCF<sup>Cdc4</sup> complex, Skp1 facilitates the interaction of Cdc4 with Cdc53 and, in turn, the WD40 repeats of Cdc4 confer specific recognition of phosphorylated Sic1 (Skowrya et al. 1997). Many other important regulatory proteins are degraded via the SCF<sup>Cdc4</sup> pathway, including the Cln-Cdc28 inhibitor Far1 (Henchoz et al. 1997), the replication protein Cdc6 (Drury et al. 1997), and the transcription factor Gcn4 (Kornitzer et al. 1994).

In addition to their role in Sic1 proteolysis, Cdc34, Cdc53, and Skp1 mediate Cln degradation in vivo (Deshaies et al. 1995; Yaglom et al. 1995; Bai et al. 1996; Willems et al. 1996). Cln degradation is probably triggered by autocatalytic Cdc28 dependent phosphorylation of the Cln subunit in Cln-Cdc28 complexes (Lanker et al. 1996). Consistent with this requirement, Cdc53 forms a tight complex with phosphorylated Cln2 in yeast lysates (Willems et al. 1996). Cln1 and Cln2 degradation also requires Grr1, another F-box protein that interacts with Skp1 in vivo and in vitro (Barral et al. 1995; Li and Johnston 1997; Skowrya et al. 1997). The ability of two distinct F-box proteins, Cdc4 and Grr1, to confer substrate specificity on a common ubiquitination pathway lead to the hypothesis that F-box proteins are substrate-specific recruitment factors (Bai et al. 1996). In support of this idea, Grr1 is able to recruit phosphorylated Cln1 and Cln2, but not phosphorylated Sic1, into Cdc34-Cdc53-Skp1 complexes in vitro (Skowrya et al. 1997).

Finally, Skp1 has other functions that have not yet been linked directly to proteolysis. Skp1 acts with Grr1 and Cdc53 to mediate the induction of *HXT* glucose transporter genes by glucose, although Cdc34 is apparently dispensible in this pathway (Li and Johnston 1997). Furthermore, Skp1 has a G<sub>2</sub>/M function because certain conditional alleles cause arrest in G<sub>2</sub>, and because it interacts genetically and physically with the kinetochore component Ctf13 (Connelly and Hieter 1996; Stemmann and Lechner 1996; Kaplan et al. 1997).

Through analysis of Cdc53-interacting proteins, we have determined that Cdc53 forms distinct complexes with Skp1, Cdc34, and the F-box proteins Cdc4, Grr1, and Met30 in vivo. We find that Cdc53 serves as a scaffold protein that links Skp1/F-box proteins and Cdc34, whereas the F-box proteins serve to confer functional specificity on the core Cdc34-Cdc53-Skp1 complex.

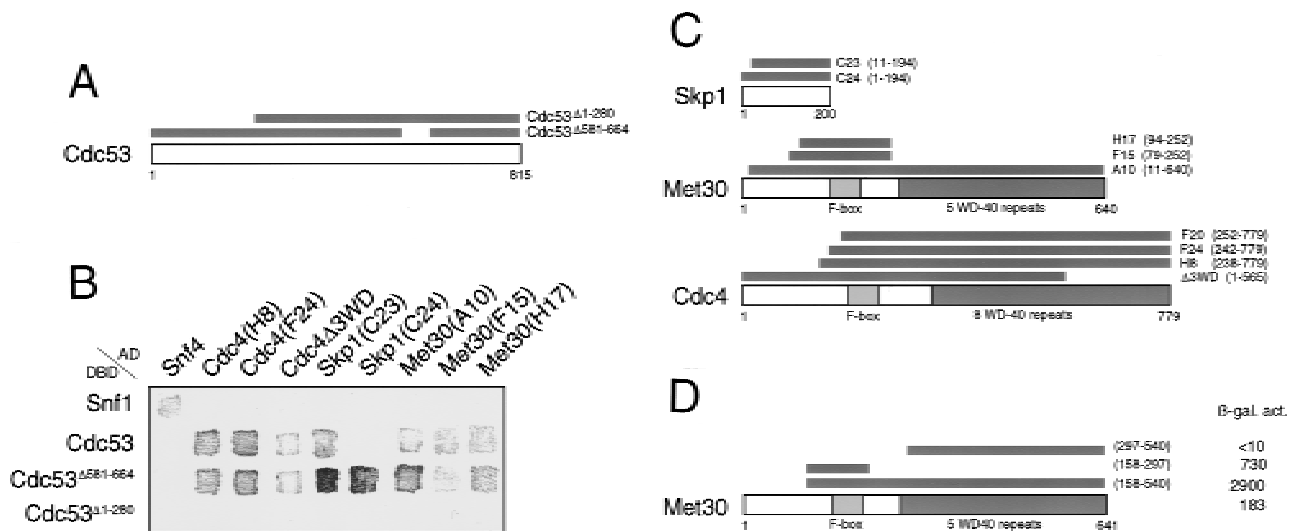
## Results

### *Interactions of Cdc53, Skp1, Cdc4, and Met30 in the two-hybrid system*

To identify proteins that interact with Cdc53, two-hybrid screens were carried out with full length Cdc53 and two Cdc53 deletion mutants (Fig. 1A). Two of the Cdc53 fusion proteins, Gal4<sup>DBD</sup>-Cdc53 and Gal4<sup>DBD</sup>-Cdc53<sup>Δ581-664</sup>, recovered multiple independent isolates of Skp1, Cdc4, and Met30 from Gal4<sup>AD</sup> genomic and cDNA libraries (Fig. 1B,C). None of the positive clones recovered interacted with Gal4<sup>DBD</sup>-Cdc53<sup>Δ1-280</sup>, suggesting that the amino-terminal region of Cdc53 was important for these interactions (see below). Met30 was originally isolated as a methionine-dependent repressor of methionine biosynthesis gene expression, and has a similar overall structure as Cdc4, with an amino-terminal F-box and carboxy-terminal WD40 repeats (Thomas et al. 1995; Bai et al. 1996). All of the Met30 and Cdc4 isolates that interacted with Cdc53 contained the F-box motif, suggesting the F-box may mediate interactions with Cdc53. In fact, two of three independent Met30 isolates contained just the F-box and a small amount of flanking region (Fig. 1C). Similarly, three independent Cdc4 isolates encompassed the F-box but lacked more amino-terminal sequences. Cdc4 and Met30 isolates, missing some or all of the WD40 repeats, did, however, interact more weakly with Cdc53 than the full-length proteins (Fig. 1B,C), which may reflect an auxiliary role for the WD40 repeats. Because Cdc4 binds Skp1 via the F-box motif (Bai et al. 1996), we directly tested for a Met30-Skp1 interaction in the two-hybrid system. The F-box of Met30 was both necessary and sufficient for interaction of Met30 with Skp1 (Fig. 1D). As for the Cdc53-Met30 interaction, the WD40 repeats of Met30 were required for maximal interaction with Skp1. Despite the known physical interaction of Cdc34 and Cdc53 (Mathias et al. 1996; Willems et al. 1996), Cdc34 was not isolated in the Cdc53 two-hybrid screens, nor did it interact in direct two-hybrid tests with Cdc53 (data not shown). In summary, two-hybrid analysis revealed a Cdc53-Skp1 interaction and suggested that Cdc53-F-box protein interactions may be bridged by Skp1.

### *Genetic interactions between CDC53 and SKP1*

To assess the in vivo relevance of the Cdc53-Skp1 two-hybrid interaction, we tested for genetic interactions between the *cdc53-1* mutation and the *skp1-11* and *skp1-12* mutations. At a semi-permissive temperature of 30°C both the *cdc53-1 skp1-11* and *cdc53-1 skp1-12* double



**Figure 1.** Cdc53 two-hybrid interactions. (A) Cdc53 two-hybrid screens were carried out with three Cdc53 fusion proteins: Gal4<sup>DBD</sup>-Cdc53, Gal4<sup>DBD</sup>-Cdc53<sup>Δ1-280</sup>, and Gal4<sup>DBD</sup>-Cdc53<sup>Δ581-664</sup>. (B) Interaction of two-hybrid Gal4<sup>AD</sup> isolates with Gal4<sup>DBD</sup> fusions in a β-galactosidase filter assay. (C) Schematic of Cdc53 interacting proteins and two-hybrid isolates. (D) Two-hybrid interactions of LexA<sup>DBD</sup>-Met30 derivatives with VP16<sup>AD</sup>-Skp1. Interactions of the indicated constructs were quantitated by liquid β-galactosidase assays in Miller units.

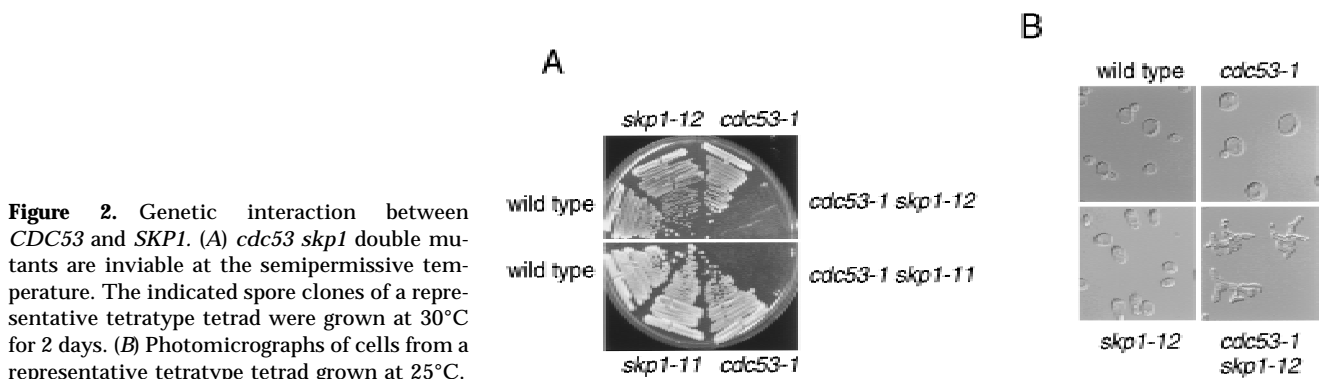
mutants were inviable, whereas either single mutant grew as well as the wild-type strain (Fig. 2A). At a permissive temperature of 25°C, *cdc53-1 skp1-12* double mutants had a severe growth defect, and accumulated multiple hyperpolarized buds (Fig. 2B), akin to the arrest phenotype of single mutants in the Cdc34 pathway (Mathias et al. 1996). Other pairwise synthetic lethal interactions between *cdc4-1*, *cdc34-2*, and *cdc53-1* have been demonstrated previously (Mathias et al. 1996). Finally, we found that overproduction of *CDC53* rescued *skp1* temperature-sensitive strains (data not shown). This genetic evidence suggested that the Cdc53-Skp1 two-hybrid interaction reflects a common function of Cdc53 and Skp1 in vivo.

*Association of Cdc53 with Cdc34, Skp1, and Cdc4 in yeast lysates*

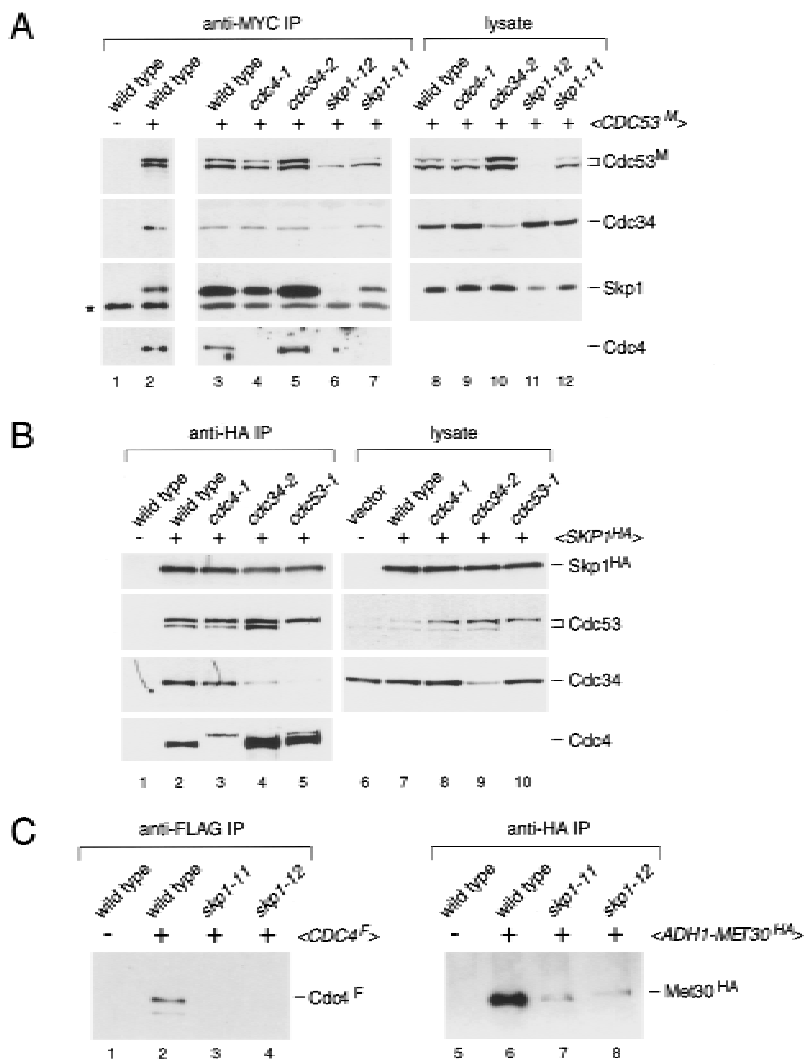
Next, we determined whether endogenous levels of Cdc53 and Skp1 form a complex in yeast lysates. To

minimize possible disruption of complexes by antibodies, we used epitope-tagged versions of Cdc53 and Skp1. Immunoprecipitation of MYC-tagged Cdc53, followed by immunoblotting with polyclonal antibodies directed against Skp1, revealed a specific association between Cdc53 and Skp1 (Fig. 3A, lane 2). Cdc4 and Cdc34 were also present in the Cdc53 complexes, consistent with the observation that Cdc4 and Cdc53 cofractionate with polyhistidine-tagged Cdc34 (Mathias et al. 1996). In the reciprocal coimmunoprecipitation experiment, Cdc53 specifically associated with HA-tagged Skp1, as did Cdc4 and Cdc34 (Fig. 3B, lane 2). Taken together, these results indicated that Cdc53 forms a multiprotein complex in vivo with Skp1, Cdc4, and Cdc34.

To determine if any of these protein-protein interactions correlated with function in vivo, we examined the composition of the complex in strains bearing various temperature-sensitive alleles. In one set of experiments, Cdc53 immune complexes were immunoblotted with antibodies against Cdc4, Cdc34, and Skp1 (Fig. 3A). In



**Figure 2.** Genetic interaction between *CDC53* and *SKP1*. (A) *cdc53 skp1* double mutants are inviable at the semipermissive temperature. The indicated spore clones of a representative tetrad type were grown at 30°C for 2 days. (B) Photomicrographs of cells from a representative tetrad type grown at 25°C.



**Figure 3.** Characterization of Cdc53 complexes in yeast lysates. (A) Effects of temperature-sensitive mutations on the composition of Cdc53 immune complexes. The indicated strains containing  $\langle CDC53 TRP1 CEN \rangle$  or  $\langle CDC53^M TRP1 CEN \rangle$  plasmids were arrested at 37°C for 2 hr. Anti-MYC immunoprecipitates from each strain were immunoblotted and sequentially probed with antibodies against Cdc4, Cdc34, Skp1, and the MYC epitope. The antibody against Cdc4 did not reliably detect Cdc4 in lysates and, therefore, these panels were omitted (see part C, below). (B) Effects of temperature-sensitive mutations on the composition of Skp1 immune complexes. Analysis was as above except that strains contained either empty vector or  $\langle SKP1^{HA} LEU2 CEN \rangle$  plasmids. Anti-HA immunoprecipitates were probed as in A. The cause of the lower mobility form of Cdc4 in lanes 3 and 5 has not been determined. (C) Abundance of Cdc4 and Met30 in *skp1* mutants. Wild-type, *skp1-11*, *skp1-12* strains containing either empty vector,  $\langle CDC4^F TRP1 CEN \rangle$  or  $\langle pADH1-MET30^{HA} TRP1 2\mu \rangle$  plasmids, were analyzed as above. Anti-FLAG and anti-HA immunoprecipitates were probed with antibodies against Cdc4 and the HA epitope, respectively.

*cdc4-1* and *skp1-11* mutants, Cdc4 was not detected in Cdc53 immune complexes; however, the absence of Cdc4 from the complexes was attributable, at least in part, to decreased Cdc4 abundance in the mutants (see Fig. 3C). The *skp1-12* mutation severely decreased the abundance of Cdc4, Cdc53, and Skp1 itself, and so the absence of associated proteins in Cdc53 complexes from *skp1-12* cells was not informative.

In another set of experiments, Skp1 immune complexes from temperature-sensitive strains were immunoblotted with antibodies against Cdc4, Cdc34, and Cdc53 (Fig. 3B). In this configuration, the amount of Cdc4 in the complex was also reduced by the *cdc4-1* mutation. In contrast, the amount of Cdc4 in the complex was increased by both the *cdc34-2* and *cdc53-1* mutations. Interestingly, Cdc4 mobility was altered in *cdc4-1* and *cdc53-1* strains, but the cause of this mobility shift has not been determined. Relative to the abundance of Cdc34 in lysates, the amount of Cdc34 in Skp1 complexes was severely compromised by the *cdc53-1* mutation, suggesting that Cdc53 may bridge the Cdc34-Skp1 interaction (see below).

As the anti-Cdc4 antibodies we used could not reliably detect Cdc4 in yeast lysates, we were unable to determine directly whether the *skp1* mutations reduced the abundance of Cdc4. Immunoprecipitation of a FLAG-tagged version of Cdc4, followed by immunoblotting with anti-Cdc4 polyclonal antibody, however, revealed that Cdc4 abundance is greatly diminished in *skp1-11* and *skp1-12* strains (Fig. 3C). The abundance of another F-box protein, Met30, was similarly reduced by the *skp1-11* and *skp1-12* mutations (Fig. 3C). As noted above, the abundance of Cdc53 is also decreased by the *skp1-12* mutation. Thus, Skp1 may function, at least in part, to stabilize both Cdc53 and F-box proteins. Overall, each of the temperature-sensitive mutations perturbed the mutual interactions by altering the abundance of a given component in lysates and/or the immune complexes.

#### Interaction of Cdc53 with multiple F-box proteins

To corroborate the Cdc53-Met30 and Skp1-Met30 two-hybrid interactions, we determined whether Met30 formed complexes with Cdc53 and Skp1 in yeast lysates.

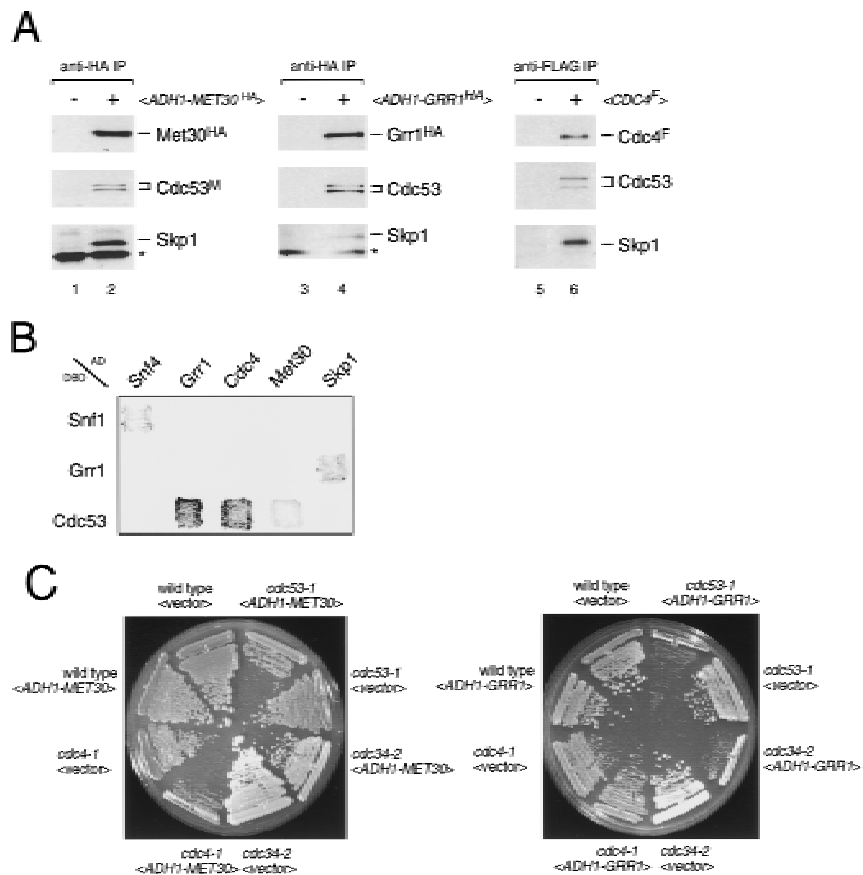
For this purpose, we used an HA-tagged version of Met30 expressed from the constitutive *ADH1* promoter. Immunoprecipitation of Met30, followed by immunoblotting against Cdc53 and Skp1, revealed the presence of both Cdc53 and Skp1 in Met30 immune complexes (Fig. 4A).

Because Grr1 functions with Skp1 and Cdc53 to mediate Cln1/2 degradation and glucose regulation (Barral et al. 1995; Bai et al. 1996; Willems et al. 1996; Li and Johnston 1997), we asked if Grr1 interacted with Cdc53 in yeast lysates. We found that Cdc53 specifically coimmunoprecipitated with an HA-tagged version of Grr1 (Fig. 4A). Skp1 was also present in the Grr1 immune complexes, as shown previously (Li and Johnston 1997). In a control experiment, FLAG-tagged Cdc4 immune complexes also contained Cdc53 and Skp1, thereby completing the set of all possible pairwise coimmunoprecipitations between Cdc4, Cdc34, Cdc53, and Skp1 (Fig. 3A,B; Mathias et al. 1996). We were unable to reproducibly detect Cdc34 in the various F-box protein immune complexes, perhaps because each of these complexes necessarily contains only a fraction of the total Cdc34, Cdc53, and Skp1.

To determine whether Cdc53 forms independent complexes with the various F-box proteins, we tested for interactions between Cdc4, Met30, and Grr1 in the two-hybrid system. A Gal4<sup>DBD</sup>-Grr1 fusion known to interact with Gal4<sup>AD</sup>-Skp1 (Li and Johnston 1997) failed to

interact with either Gal4<sup>AD</sup>-Cdc4 or VP16<sup>AD</sup>-Met30 fusions, or with a Gal4<sup>AD</sup>-Grr1 fusion (Fig. 4B). Each of the F-box protein fusions interacted with a Gal4<sup>DBD</sup>-Cdc53 fusion as expected. We have also been unable to detect Cdc4 in either Met30 or Grr1 immune complexes in direct coprecipitation experiments (data not shown). Thus, the available evidence suggests that Skp1 and Cdc53 form mutually exclusive complexes with at least three different F-box proteins in vivo.

The ability of Cdc53 to interact with multiple F-box proteins in independent complexes raised the possibility that different F-box proteins may compete for binding to a Cdc34-Cdc53-Skp1 core complex. We tested this possibility by overexpressing *MET30* or *GRR1* in *cdc4-1*, *cdc34-2*, and *cdc53-1* temperature-sensitive strains. Overexpression of *MET30* dramatically impaired growth of a *cdc4-1* strain at 30°C, and caused a mild growth defect in *cdc34-2* and *cdc53-1* strains (Fig. 4C), but had no effect on either *skp1-11* or *skp1-12* strains (data not shown). Although overexpression of *GRR1* did not affect growth of a *cdc4-1* strain, the growth of *cdc34-2* and *cdc53-1* strains was retarded at 30°C (Fig. 4C). It has been noted previously that high-level expression of *GRR1* is lethal in *skp1-12* strains at 30°C (Li and Johnston 1997), and high-level expression of Cdc4 causes inviability of *cdc34* and *cdc53* strains at 23°C (Mathias et al. 1996). Taken together, the above results suggest various F-box



**Figure 4.** Interactions of different F-box proteins. (A) Interaction of Met30, Grr1, and Cdc4 with Cdc53 and Skp1. The indicated immunoprecipitates from wild-type cells containing either vector, <pADH1-MET30<sup>HA</sup> TRP1 2μ>, <pADH1-GRR1<sup>HA</sup> TRP1 2μ> and <CDC4<sup>F</sup> TRP1 CEN > plasmids were probed as indicated with antibodies against Cdc53, Skp1, Cdc4, and the HA epitope. IgG light chain is indicated by an asterisk. (B) Different F-box proteins do not interact with each other. Gal4<sup>AD</sup>-Met30 (A10), Gal4<sup>AD</sup>-Grr1 and Gal4<sup>AD</sup>-Cdc4 were tested against Gal4<sup>DBD</sup>-Grr1 and Gal4<sup>DBD</sup>-Cdc53 in the two-hybrid system by a β-galactosidase filter assay. (C) Effects of *MET30* or *GRR1* overexpression. Strains of the indicated genotype containing an empty vector plasmid <pADH1-MET30<sup>HA</sup> TRP1 2μ> (left) or <pADH1-GRR1<sup>HA</sup> TRP1 2μ> (right) plasmids were grown at 30°C for 3 days and photographed.

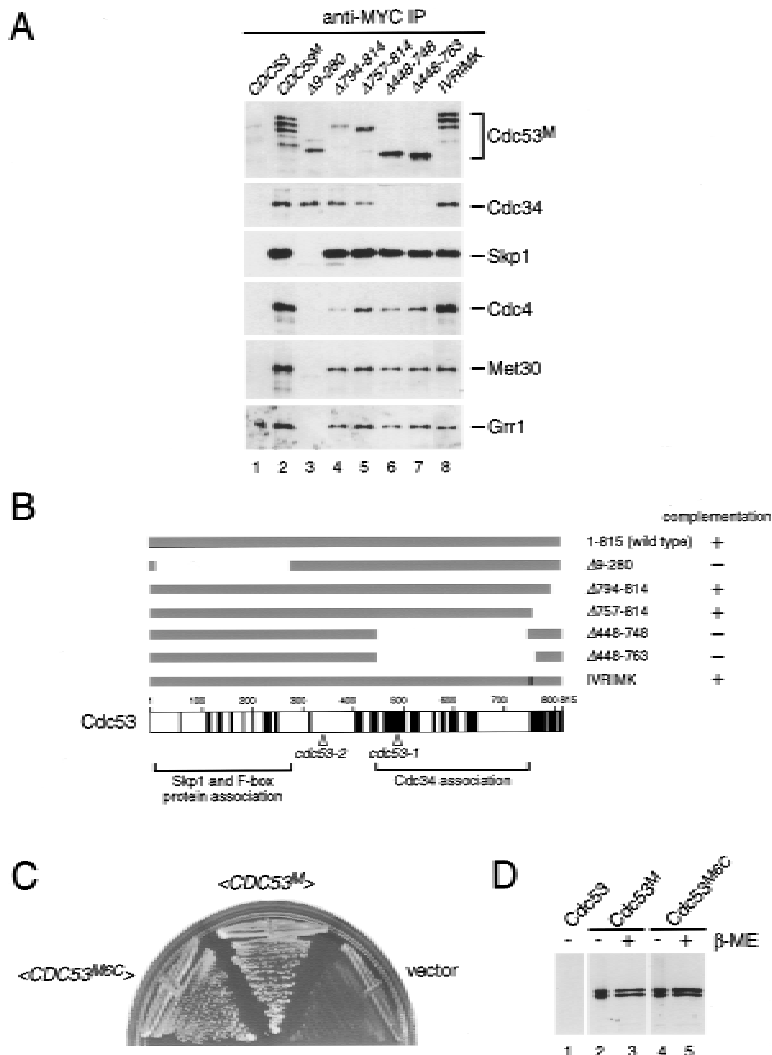
proteins may compete for binding to a Cdc34-Cdc53-Skp1 core complex in vivo, and that the relative stoichiometry of the various complexes may be critical for viability.

#### *Cdc53 is scaffold for Cdc34 and Skp1/F-box proteins*

To identify potential protein-protein interaction domains of Cdc53, we constructed a series of Cdc53 deletion mutants using natural and engineered restriction sites (see Materials and Methods). Each of the mutant proteins was expressed in yeast to similar levels as wild-type Cdc53 (Fig. 5A). The ability of each Cdc53 mutant protein to interact with Cdc34, Skp1, and the three F-box proteins Cdc4, Grr1, and Met30 was assessed by immunoblot analysis of MYC-tagged Cdc53 immune complexes with specific polyclonal antibodies (Fig. 5A). In this experiment, each of the interactions detected involved approximately wild-type levels of Cdc53 (which was expressed from a low copy plasmid) and endogenous levels of each of the associated proteins. Deletion of an

amino-terminal region of Cdc53 (residues 9–280) completely disrupted Skp1 binding. In parallel, the binding of all three F-box proteins was specifically disrupted. Importantly, Cdc34 still interacted with Cdc53<sup>Δ9–280</sup>, eliminating the possibility that the truncated protein was simply misfolded and entirely nonfunctional. Conversely, deletion of an internal region of Cdc53 (residues 448–748) abrogated Cdc34 binding but did not affect binding of Skp1 or any of the F-box proteins. The correlation between the Cdc53-Skp1 interaction and Cdc53-F-box protein interactions is most easily explained by a bridging role for Skp1. Furthermore, the independent nonoverlapping binding regions in Cdc53 indicate that the protein-protein interactions within Cdc53 complexes occur in a modular fashion.

Importantly, Cdc53 mutants that were unable to bind either Skp1/F-box proteins or Cdc34 could not complement a *cdc53* deletion strain, whereas mutants unaffected in protein-protein interactions could complement (Fig. 5B). To determine if the Skp1/F-box protein and Cdc34 binding domains of Cdc53 corresponded to con-



**Figure 5.** Mutational analysis of Cdc53. (A) Deletion analysis of Cdc53 protein-protein interaction domains. Cells were transformed with untagged Cdc53 (lane 1), MYC-tagged Cdc53 (lane 2) or MYC-tagged versions of the indicated Cdc53 mutants (lanes 3–8). All of the proteins were expressed from the wild-type *CDC53* promoter on a *CEN* plasmid. Lysates from each strain were immunoprecipitated with anti-MYC antibody, immunoblotted, and probed with antibodies specific to each of the indicated proteins. (B) Schematic representation of Cdc53 mutant proteins and their ability to complement a *cdc53* deletion strain. Regions of amino acid sequence conservation in the Cdc53 family are indicated in black (see Materials and Methods). The positions of the *cdc53-1* (R488C) and *cdc53-2* (G340D) point mutations, and the regions required for binding to Skp1/F-box proteins and Cdc34 are also indicated. (C) Cdc53 does not contain essential cysteine residues. A *cdc53::ADE2* deletion strain containing a <*CDC53<sup>HA</sup> URA3 CEN*> plasmid was transformed with <*CDC53<sup>M6C</sup> TRP1 CEN*>, <*CDC53<sup>M</sup> TRP1 CEN*>, or an empty vector plasmid, plated on 5-FOA medium to select for Ura<sup>-</sup> cells, and photographed after 2 days. (D) Cysteine residues in Cdc53 are not required for posttranslational modification of Cdc53. Lysates from strains containing a <*CDC53<sup>M</sup> TRP1 CEN*> or <*CDC53<sup>M6C</sup> TRP1 CEN*> plasmid were separated by SDS-PAGE in the absence or presence of reducing agent and immunoblotted with anti-MYC antibody.

served regions of Cdc53, we aligned 15 different members of the Cdc53 family (Fig. 5B; see Materials and Methods for details of the sequence alignment). Sequence similarity within the Cdc53 family is highest over a broad internal region and a narrow region at the extreme carboxyl terminus. Surprisingly, the latter region is not required for binding to Skp1/F-box proteins or Cdc34, nor for viability (Fig. 5A,B). The internal conserved region, however, overlaps with the Cdc34 binding site. There is relatively poor conservation in the amino terminus of Cdc53, despite the fact that this region contains the Skp1 binding site, suggesting that only a subset of the Cdc53 family may interact with Skp1.

On the basis of the sequence alignment, we mutated many conserved charged residues in Cdc53 to alanines, but none of the mutants had any overt phenotype (data not shown). For instance, mutation of the most conserved stretch in the entire protein, IVRIMK (residues 755–760), to polyalanine did not cause an obvious defect in Cdc53 function or in binding to Skp1/F-box proteins or Cdc34 (Fig. 5A,B). To further explore the structure/function relationship of Cdc53, we determined the sequence of two temperature-sensitive alleles of *CDC53* (Mathias et al. 1996). The *cdc53-1* mutation causes an R488C substitution whereas the *cdc53-2* mutation causes a G340D substitution. Both mutations alter highly conserved residues, even though G340 does not lie within a window of conserved residues. Interestingly, the *cdc53-1* mutation occurs within the Cdc34 binding region. In conjunction with the defective Skp1–Cdc34 interaction in *cdc53-1* strains (Fig. 3B), this result strongly suggests that the *cdc53-1* mutation specifically perturbs the Cdc34 binding site.

#### *Cdc53 does not form essential ubiquitin thioesters in vivo*

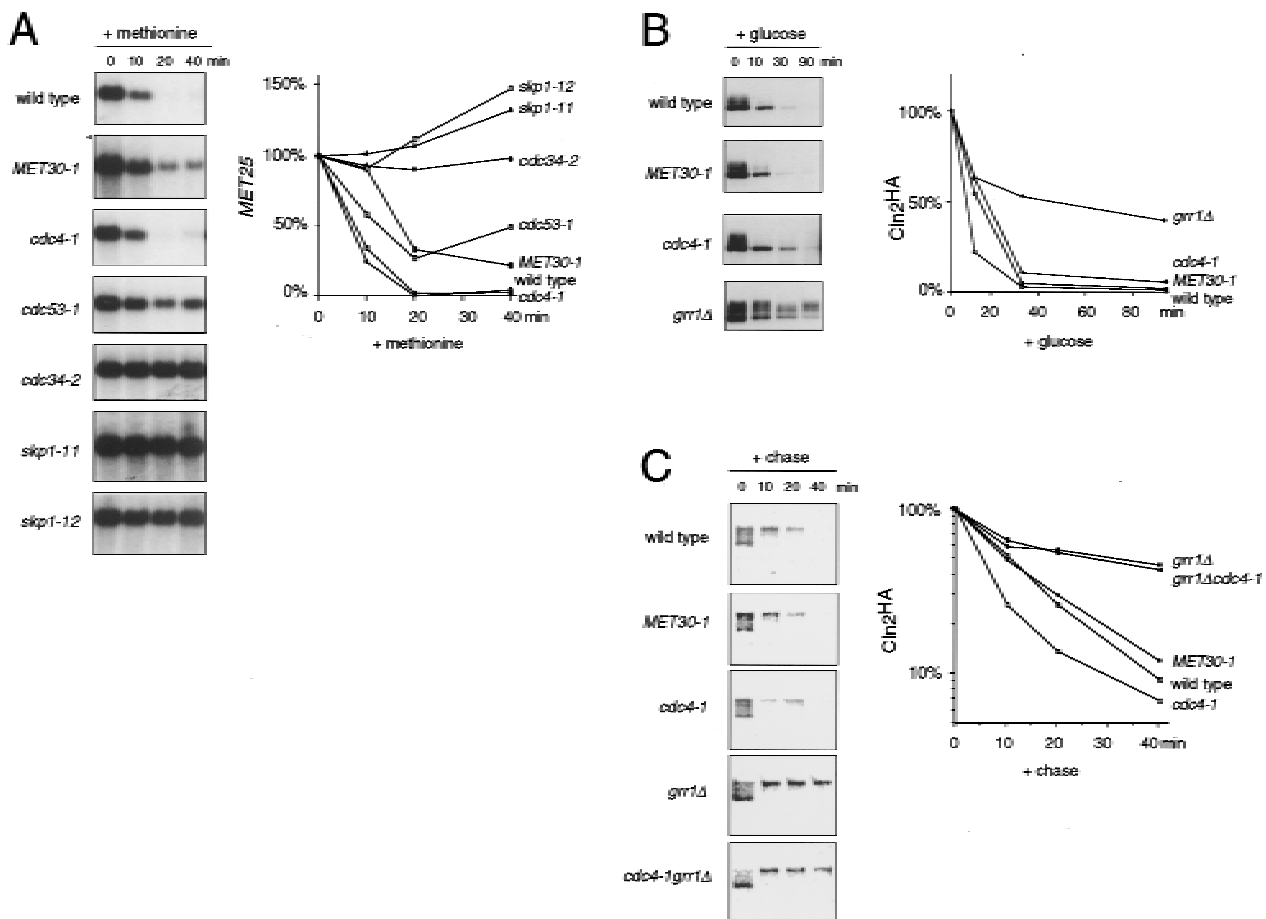
In addition to target protein recognition, some E3 ubiquitin ligases participate in the catalytic transfer of ubiquitin onto substrates through formation of ubiquitin thioesters on cysteine residues (Scheffner et al. 1995). Therefore, we determined whether any of the cysteine residues in Cdc53 were required for function in vivo. Simultaneous mutation of all six cysteine residues in Cdc53 to alanines (Cdc53<sup>M6C</sup>) did not impair complementation of a *cdc53* deletion strain (Fig. 5C). Cdc53 is covalently conjugated to a ubiquitin-cross reactive species in vivo (Willems et al. 1996), which is not ubiquitin itself, but is Rub1, a ubiquitin-related protein (M. Estelle, pers. comm.). Therefore, we compared the extent of modification of the cysteineless Cdc53 to wild-type Cdc53. The mutant protein was modified by Rub1 to the same extent as wild type, and the modification was not sensitive to reducing agents, demonstrating that this modification does not occur on cysteine residues (Fig. 5D). Although this mutational analysis does not rule out thioester formation on Cdc53 in vivo, such intermediates clearly cannot be essential for viability. Cdc53 thus appears to act as a noncatalytic scaffold protein for Cdc34 and Skp1/F-box proteins.

#### *Cdc34, Cdc53, and Skp1 function in methionine repression*

To assess the biological significance of the Cdc53–Met30 and Skp1–Met30 interactions, we determined whether Cdc34, Cdc53, or Skp1 were required for proper regulation of methionine biosynthesis genes. We examined the regulation of *MET25*, which encodes homocysteine synthase and is one of several methionine regulated genes. *MET25* is activated by the Cbf1–Met4–Met28 transcriptional complex and repressed by Met30 (Thomas et al. 1995; Kuras et al. 1996). As expected, methionine repressed *MET25* expression in wild-type cells (Fig. 6A). As *MET30* is an essential gene, we used an antimorphic allele called *MET30-1* as a positive control for derepression in methionine medium (Thomas et al. 1995). As shown previously, *MET25* was incompletely repressed by methionine in *MET30-1* cells. Strikingly, repression of *MET25* by methionine was severely compromised in *cdc53-1* cells and completely defective in *cdc34-2*, *skp1-11*, and *skp1-12* cells (Fig. 6A). The derepression of *MET25* in methionine medium did not depend on G<sub>1</sub> phase cell cycle arrest because derepression did not occur in *cdc4-1* cells that arrest at the identical point in G<sub>1</sub>, and yet did occur in *skp1-12* mutants that arrest in G<sub>2</sub> phase. Because *MET25* was effectively repressed with wild-type kinetics in *cdc4-1* cells, methionine biosynthesis gene regulation specifically requires the F-box protein Met30. We were unable to assess the role of Grr1 in methionine repression because *grr1Δ* strains were inviable when grown on standard B medium used for analysis of methionine repression (data not shown).

#### *F-box protein function in Cln2 degradation*

It has been shown previously that Cln2 is stabilized in *grr1Δ*, *cdc34-2*, *cdc53-1*, and *skp1-12* strains (Barral et al. 1995; Deshaies et al. 1995; Bai et al. 1996; Willems et al. 1996). To directly assess the specificity of F-box protein function in Cln2 degradation, we compared the half-life of Cln2 in *cdc4-1*, *MET30-1*, and *grr1Δ* strains. Because Grr1 and Met30 are required for proper responses to glucose and methionine, respectively, we carried out half-life experiments in each F-box mutant strain by both glucose repression of a *pGAL1-CLN2<sup>HA</sup>* construct and [<sup>35</sup>S]methionine/cysteine pulse-chase analysis of endogenous Cln2. In promoter shut-off experiments, Cln2 half-life was greatly increased in a *grr1Δ* strain as expected (Barral et al. 1995), but was not altered in either *cdc4-1* or *MET30-1* strains (Fig. 6B). Virtually identical results were obtained in pulse-chase experiments, as Cln2 was stabilized only in the *grr1Δ* strain (Fig. 6C). To determine whether Cdc4 might have any ancillary role in Cln2 degradation, we also examined the half-life of Cln2 in a *grr1Δ cdc4-1* double mutant strain by pulse-chase analysis. Cln2 was no more stable in the *cdc4-1 grr1Δ* double mutant than in the *grr1Δ* single mutant (Fig. 6C). Thus, Grr1 is the primary mediator of Cln2 degradation in vivo. Finally, consistent with previous results (Schwob et al. 1994; Bai et al. 1996), we found that



**Figure 6.** Specificity of F-box protein function. (A) Methionine repression is mediated by Met30, Cdc34, Cdc53, and Skp1 but not Cdc4. The indicated strains were grown in methionine-free medium and then repressed with 1.0 mM methionine for the indicated times. *MET25* expression was determined by Northern analysis and normalized to *ACT1* expression. Values are expressed as percent of the signal at  $t = 0$ . (B) *Grr1* specifically mediates *Cln2* degradation. *Cln2* stability was examined in the indicated strains carrying a  $\langle pGAL1-CLN2^{HA} URA3 LEU2 CEN \rangle$  plasmid by repression of the *GAL1* promoter. *Cln2*<sup>HA</sup> was detected by immunoblotting with anti-HA antibody. Exposures were adjusted to give approximately equal *Cln2*<sup>HA</sup> signals at  $t = 0$ . *Cln2*<sup>HA</sup> was quantitated by densitometry and normalized to *Cdc28* signals from the same blot probed with anti-*Cdc28* antibody. Values are expressed as percent of the signal at  $t = 0$ . (C) *Cdc4* does not overlap with *Grr1* in *Cln2* degradation. *Cln2* stability was determined by [<sup>35</sup>S]methionine/cysteine pulse-chase analysis in the indicated strains. The <sup>35</sup>S-labeled *Cln2*<sup>HA</sup> signal was quantitated by PhosphorImager and normalized to the <sup>35</sup>S-labeled total lysate signal for each sample. Values are expressed as percent of the signal at  $t = 0$ .

*Sic1* degradation in vivo required *Cdc4*, but not *Grr1* or *Met30* (data not shown).

## Discussion

### Architecture of *Skp1-Cdc53-F-box protein (SCF) complexes*

*Cdc53* forms multiprotein complexes in vivo with *Cdc34*, *Skp1*, and three independent F-box proteins, *Cdc4*, *Met30*, and *Grr1*. To simplify description of the various F-box containing complexes, we have adopted the term SCF, for *Skp1-Cdc53-F-box protein complex* (Feldman et al. 1997; Skowrya et al. 1997). The specific F-box complexes described here are thus designated SCF<sup>Cdc4</sup>, SCF<sup>Met30</sup>, and SCF<sup>Grr1</sup>. Formally, SCF complexes are E3 ubiquitin ligases, as they interact with both

substrates and an E2 enzyme, *Cdc34* (Hershko 1997). The following observations bear on the general architecture of SCF complexes in vivo: (1) the *Cdc34-Cdc53* interaction is independent of *Skp1* binding; (2) the *Skp1/F-box protein-Cdc53* interaction is independent of *Cdc34* binding; (3) the *Skp1-Cdc34* interaction is abrogated by the *cdc53-1* mutation, which lies in the *Cdc34* binding site; (4) the F-box of *Met30* is sufficient for interaction with *Skp1* and *Cdc53* in the two-hybrid system; (5) the binding region for *Skp1* on *Cdc53* precisely overlaps that of three different F-box proteins, *Cdc4*, *Grr1*, and *Met30*. These in vivo observations are consistent with the assembly of recombinant SCF complexes in vitro (Bai et al. 1996; Feldman et al. 1997; Skowrya et al. 1997). We envisage that *Cdc53* is a scaffold protein that bridges *Cdc34* to *Skp1*, which in turn binds to vari-



ous F-box proteins, which in turn target various substrates (Fig. 7). Although the various protein-protein interactions within SCF complexes are largely modular in nature, the interaction of Skp1 with Grr1 in the two-hybrid system requires both the F-box and the leucine rich repeats of Grr1 (Li and Johnston 1997). We also observe that the WD40 repeats of Cdc4 and Met30 are required for maximal interaction with Skp1. Thus, Skp1 may recruit F-box proteins to Cdc53 complexes in conjunction with other domains within F-box proteins.

Unlike some other E3 ligases, Cdc53 does not form essential ubiquitin thioester intermediates. We have also mutated several conserved cysteine residues in Cdc4 (residues 454, 455, 468, and 562) and found that such mutants provide full Cdc4 function in vivo, even when combined with a cysteineless Cdc53 mutant (data not shown). In addition, the only conserved cysteine residue in Skp1 is nonessential (S. Elledge, pers. comm.). Thus, SCF-mediated ubiquitin transfer may rely solely on Cdc34 for catalytic activity. Genetic evidence suggests that Cdc34 is the only E2 able to provide function in Sic1 degradation, Cln degradation, and methionine repression, indicating that Cdc34 may be specific for SCF complexes. Glucose induction of *HXT1* expression, however, occurs normally in *cdc34-2* strains, suggesting that either another E2 is required or that the *cdc34-2* allele is able to function in *HXT1* induction (Li and Johnston 1997). It will be of interest to determine whether the conserved central region in other Cdc53 homologs mediates interactions with E2 enzymes.

Finally, although three distinct SCF complexes conform to the generic SCF architecture, the Skp1-Ctf13

interaction occurs in the context of the CBF3 kinetochore complex, which is composed of p23<sup>Skp1</sup>, p58<sup>Ctf13</sup>, p64<sup>Cep3</sup>, and p110<sup>Ndc10</sup> (Connelly and Hieter 1996; Stemmann and Lechner 1996; Kaplan et al. 1997). Surprisingly, Skp1 is not an obligate structural component of this complex, but rather appears to facilitate phosphorylation dependent conversion of Ctf13 into an active form (Kaplan et al. 1997). It is possible that the Skp1-F-box interaction may be a widely utilized protein motif that functions in contexts other than ubiquitin dependent proteolysis.

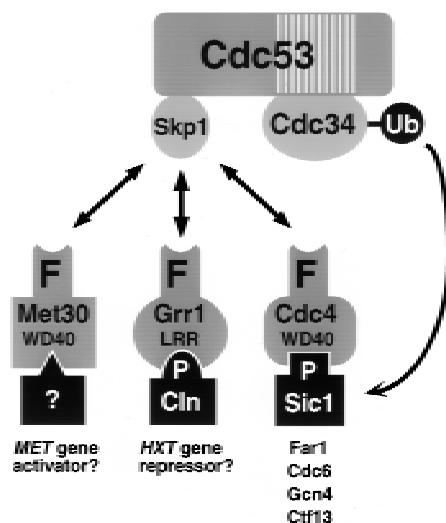
#### F-box proteins confer specificity on SCF function

The dramatic cell cycle arrest phenotype caused by the inability to degrade Sic1 has, to some extent, obscured the pleiotropic functions of the Cdc34-Cdc53-Skp1 E2/E3 core complex. Specific functions are conferred on the E2/E3 core complex by its association with different F-box proteins. SCF<sup>Cdc4</sup> is required for specific degradation of Sic1 and other cell-cycle regulators. SCF<sup>Grr1</sup> mediates specific degradation of Cln1 and Cln2, and is also required for transcriptional induction of glucose transporter genes (Barral et al. 1995; Li and Johnston 1997). As *grr1* mutants have many other defects, it seems likely that SCF<sup>Grr1</sup> will regulate several other pathways as well (Li and Johnston 1997). Here, we have described a third SCF complex, SCF<sup>Met30</sup>, and demonstrated that in addition to Met30, Cdc34, Cdc53, and Skp1 are required for appropriate repression of methionine biosynthesis genes.

The specificity of each SCF complex for different cellular processes is demonstrated by a remarkable absence of cross talk. The *cdc4-1* mutation does not affect *MET25* repression and conversely, the *MET30-1* mutation does not affect either Sic1 or Cln2 degradation. Similarly, Sic1 degradation requires only Cdc4, whereas Cln2 degradation requires only Grr1. It should be pointed out that this latter result is at odds with a model in which Clb-Cdc28 activity instigates Cln degradation, an issue that has yet to be resolved (Blondel and Mann 1996).

The growth defects caused by high level expression of *CDC4*, *GRR1*, or *MET30* in various SCF mutants suggests that different F-box proteins may be in equilibrium with a limiting amount of the E2/E3 core complex. There are other indications that F-box proteins may be subject to stringent regulation. The Skp1-Grr1 interaction is stimulated by glucose (Li and Johnston 1997), and Ctf13 levels appear to be precisely controlled by the SCF<sup>Cdc4</sup> pathway (Kaplan et al. 1997). Furthermore, Ctf13 is activated by phosphorylation in a Skp1 dependent manner (Kaplan et al. 1997). It will be of interest to determine whether the Skp1-Met30 interaction is modulated by methionine, and to determine whether other F-box proteins are regulated by phosphorylation.

It seems likely that other SCF complexes will regulate many other processes because yeast contain at least nine additional F-box proteins of unknown function, at least two of which associate with Cdc53 (A. Willems and A. Shevchenko, unpubl.). Yeast also contain three identi-



**Figure 7.** Multiple F-box proteins recruit different substrates to an E2/E3 core ubiquitination complex. The hatched box in Cdc53 represents a conserved region found in all Cdc53 homologs that overlaps with the Cdc34 binding site in Cdc53. The F-box protein-substrate interactions are most firmly established for Cln1, Cln2, and Sic1. P indicates a phosphorylation dependent interaction, WD40 indicates WD40 repeats, and LRR indicates leucine rich repeats. See text for details.

able Cdc53 homologs and a Skp1 homolog, all of unknown function. The use of specific adaptor proteins to recruit multiple substrates to core ubiquitination complexes may be a common theme, as two putative adaptor proteins for the APC, Cdc20, and Hct1/Cdh1, have recently been identified (Schwab et al. 1997; Visintin et al. 1997) and shown to confer substrate specificity on APC function (Visintin et al. 1997).

### Substrates of SCF complexes

To date, only SCF<sup>Cdc4</sup> has been unequivocally shown to directly mediate ubiquitination of target proteins. Ubiquitination of phosphorylated Sic1 has been reconstituted in vitro, in both a yeast extract system and in a purified system with recombinant proteins (Feldman et al. 1997; Skowrya et al. 1997; Verma et al. 1997b). Recently, phosphorylation-dependent ubiquitination of Far1 by SCF<sup>Cdc4</sup> has been achieved in yeast extracts (Henchoz et al. 1997). Substantial evidence also suggests that SCF<sup>Cdc4</sup> also targets Cdc6, Ctf13 and Gcn4 for degradation in a phosphorylation-dependent manner (Kornitzer et al. 1994; Drury et al. 1997; Kaplan et al. 1997).

Although ubiquitination of Cln1/2 has not yet been reconstituted in vitro, SCF<sup>Grr1</sup> specifically binds to phosphorylated Cln1/2, consistent with Grr1-dependent degradation of Cln1/2 in vivo (Barral et al. 1995; Skowrya et al. 1997). Somewhat surprisingly, Cln3 is not stabilized in *grr1* strains (Barral et al. 1995), but is stabilized in *cdc34-2* strains (Yaglom et al. 1995), perhaps indicating that another F-box protein may target Cln3 for degradation. A negative regulator of *HXT* expression called Rgt1 is a possible target of the SCF<sup>Grr1</sup> complex; however, it is not yet known if Rgt1 is regulated by ubiquitin-dependent proteolysis (Li and Johnston 1997). The requirement for SCF<sup>Met30</sup> function in methionine repression also implicates ubiquitin-dependent proteolysis, but, again, this remains to be proven. Because Met30 forms a complex with the transactivator Met4, it is possible that Met30 targets Met4 for degradation, although other components of the Met4 transcriptional complex are also candidate targets (Kuras et al. 1996).

### Diverse functions of SCF complexes

In instances where function is known, F-box protein complexes have emerged as key regulators of cell division in other organisms. In *Schizosaccharomyces pombe*, a Cdc4 homolog called Pop1 regulates DNA replication by targeting a Cdk inhibitor, Rum1, and a Cdc6 homolog, Cdc18, for ubiquitin dependent proteolysis (Kominami and Toda 1997). In *Caenorhabditis elegans*, a Cdc53 homolog called CUL-1 appears to negatively regulate activators of division as *cul-1* mutants exhibit hyperplasia in all tissues (Kipreos et al. 1996). As in yeast, degradation of mammalian G<sub>1</sub> cyclins and Cdk inhibitors is phosphorylation dependent (Clurman et al. 1996; Won and Reed 1996; Diehl et al. 1997; Sheaff et al. 1997) and, therefore, it will be of prime importance to determine the role of SCF complexes in these pathways. It is

likely that SCF architecture will be conserved, as human Skp1 forms a specific complex with human Cul-1 and binds to cyclin A-Cdk2 through its associated F-box protein, Skp2 (Zhang et al. 1995; Lisztwan et al. 1998; Y. Xiong, pers. comm.).

The regulation of various transcription factors by SCF<sup>Cdc4</sup>, SCF<sup>Grr1</sup>, and SCF<sup>Met30</sup> suggests that SCF pathways may be a common means of transcriptional control, particularly as gene expression is often regulated by proteolysis (Pahl and Baeuerle 1996). Transcriptional regulation of methionine biosynthesis by SCF complexes is conserved in fungi as the Met30 homologs Scon2 and SconB, and the Skp1 homolog SconC regulate sulfur metabolism in *Neurospora* and *Aspergillus*, respectively (Natorff et al. 1993; Kumar and Paietta 1995). Another human Cdc53 homolog, Cul-2, may also regulate transcription as it physically associates with the VHL tumour suppressor protein in a complex with elongin B and elongin C, two transcriptional elongation factors that have similarity to ubiquitin and Skp1, respectively (Pause et al. 1997). Intriguingly, another F-box protein, elongin A, participates in a complex with elongin B and elongin C (Aso et al. 1996).

Finally, F-box proteins are implicated in developmental decisions, often in the context of transcriptional control. A Cdc4 homolog in *C. elegans*, SEL-10, influences a lateral cell fate decision through negative regulation of the notch homolog LIN-12, possibly via ubiquitin-dependent degradation (Hubbard et al. 1997). In *Arabidopsis*, the F-box protein UFO controls floral development through regulation of a floral organ identity gene *APETALA3* (Lee et al. 1997). The analogous F-box protein in *Antirrhinum*, FIMBRIATA, functions in a similar manner, and moreover, physically interacts with three Skp1 homologs (Ingram et al. 1997).

SCF complexes represent a versatile regulatory system that has been exploited in many cellular processes. At least seven metazoan homologs of Cdc53 have been identified and are generically referred to as cullins, after the *C. elegans* Cdc53 homolog, CUL-1 (Kipreos et al. 1996). Similarly, database searches reveal several Skp1 homologs and a large number of metazoan proteins containing putative F-box motifs. If the combinatorial control of SCF complexes defined to date applies to these homologs, then many other SCF pathways remain to be elucidated.

## Materials and methods

### Plasmids

Plasmids were constructed by use of standard molecular cloning techniques (Table 1). For two-hybrid screens, the *CDC53* open reading frame was cloned into the *Bam*HI site of pAS2 (provided by S. Elledge, Baylor College of Medicine, Houston, TX) to create a Gal4<sup>DBD</sup>-Cdc53 fusion. Versions that lacked either the amino-terminal 280 residues (Gal4<sup>DBD</sup>-Cdc53<sup>Δ1-280</sup>) or internal residues 581-664 (Gal4<sup>DBD</sup>-Cdc53<sup>Δ581-664</sup>) were created by digestion with *Nco*I or *Kpn*I, respectively and religating. Gal4<sup>AD</sup>-Cdc4<sup>Δ566-779</sup> is a truncated *CDC4* PCR product cloned into the *Bam*HI site of pGAD424 (Skowrya et al. 1997). To test the Skp1-Met30 interaction in the two-hybrid system, a *SKP1*

**Table 1.** *Plasmids used in this study*

Plasmid	Relevant characteristics	Source
pBF339	<i>pADH1<sup>HA</sup> TRP1 2μ</i>	Li and Johnston (1997)
pBF494	<i>pADH1<sup>HA</sup>-GRR1<sup>ΔN</sup> TRP1 2μ</i>	Li and Johnston (1997)
pBM2576	<i>pADH1-GAL4<sup>DBD</sup>-GRR1<sup>ΔN</sup> TRP1</i>	Li and Johnston (1997)
pBM2868	<i>pADH1-GAL4<sup>AD</sup>-GRR1<sup>ΔN</sup> TRP1</i>	Li and Johnston (1997)
pMT634	<i>pGAL1-CLN2-HA LEU2 URA3 CEN</i>	Willems et al. (1996)
pMT817	<i>CDC53-C-NotI TRP1 CEN</i>	Willems et al. (1996)
pMT843	<i>CDC53<sup>M</sup> TRP1 CEN</i>	Willems et al. (1996)
pMT915	<i>pADH1-GAL4<sup>AD</sup>-CDC4<sup>Δ3WD</sup> LEU2 2μ</i>	this study
pMT918	<i>pADH1-GAL4<sup>DBD</sup>-CDC53 TRP1 2μ</i>	this study
pMT951	<i>CDC53<sup>HA</sup> URA3 CEN</i>	this study
pMT954	<i>pADH1-GAL4<sup>DBD</sup>-CDC53<sup>Δ1-280</sup> TRP1 2μ</i>	this study
pMT955	<i>pADH1-GAL4<sup>DBD</sup>-CDC53<sup>Δ581-664</sup> TRP1 2μ</i>	this study
pMT1511	<i>SKP1<sup>HA</sup> LEU2 CEN</i>	P. Heiter (University of British Columbia Vancouver, Canada)
pMT1514	<i>cdc53::ADE2</i> in pGEM3	this study
pMT1567	<i>CDC4<sup>F</sup> TRP1 CEN</i>	this study
pMT1707	<i>pADH1<sup>HA</sup>-MET30 TRP1 2μ</i>	this study
pMT1850	<i>CDC53<sup>MΔ9-280</sup> TRP1 CEN</i>	this study
pMT1854	<i>CDC53<sup>MΔ448-763,H767A</sup> TRP1 CEN</i>	this study
pMT1856	<i>CDC53<sup>MΔ448-748</sup> TRP1 CEN</i>	this study
pMT1857	<i>CDC53<sup>MΔ757-815</sup> TRP1 CEN</i>	this study
pMT1858	<i>CDC53<sup>MΔ794-815</sup> TRP1 CEN</i>	this study
pMT1859	<i>CDC53<sup>MIVRIMK</sup> TRP1 CEN</i>	this study
pMT1861	<i>CDC53<sup>M6C</sup> TRP1 CEN</i>	this study
pMT1589	<i>LEXA<sup>DBD</sup>-MET30 TRP1 2μ</i>	this study
pLexM30-4(297-540)	<i>LEXA<sup>DBD</sup>-MET30<sup>Δ297-540</sup> TRP1 2μ</i>	this study
pLexM30-4(158-297)	<i>LEXA<sup>DBD</sup>-MET30<sup>Δ158-297</sup> TRP1 2μ</i>	this study
pLexM30-4(158-540)	<i>LEXA<sup>DBD</sup>-MET30<sup>Δ158-540</sup> TRP1 2μ</i>	this study
pVAD1-SKP1	<i>VAD-SKP1 LEU2 2μ</i>	this study
pSE1111	<i>pADH1-GAL4<sup>AD</sup>-SNF1 LEU2 2μ</i>	S. Elledge
pSE1112	<i>pADH1-GAL4<sup>DBD</sup>-SNF4 TRP1 2μ</i>	S. Elledge

fragment was cloned into the *Bam*HI site of pVAD1, to create a VP16-Skp1 fusion. LexA-Met30 derivatives were based on pLEXM30-4 (Thomas et al. 1995). Met30 was tagged at the amino terminus with an HA epitope by insertion of a *MET30* fragment encoding amino acids 7–640 from pLEXM30-4 into a *pADH1-HA* expression plasmid (Li and Johnston 1997). Cdc4 was tagged at the amino terminus with a FLAG epitope by site directed mutagenesis. A *CDC53* deletion construct was made by replacing an internal *Bgl*III fragment of pGEM53-8 (Mathias et al. 1996) with an *ADE2* fragment. To allow for negative selection of wild-type *CDC53* in the *cdc53Δ* shuffle strain a 3.6-kb *Eco*RI fragment of *CDC53* was cloned into a *<URA3 CEN>* plasmid. Mutagenesis of Cdc53 was carried out in pMT843, as described previously (Willems et al. 1996) and restriction sites incorporated during mutagenesis were used to construct the deletions shown in Figure 5. The version of Cdc53 in which all six cysteine residues are replaced by alanines (C59A, C157A, C412A, C606A, C754A, and C774A) was created in a single site-directed mutagenesis reaction.

#### Yeast strains and culture

Yeast strains are listed in Table 2. All strains were isogenic with the W303 background except for Y187, Y190, Y533, Y555, and WX131-2C. Standard methods were used for yeast culture and transformation (Kaiser et al. 1994). A *cdc53Δ* shuffle strain was constructed by deleting one copy of *CDC53* with pMT1514 in a K699 a/α diploid, transforming with pMT951, sporulating and isolating a Ura<sup>+</sup> Ade<sup>+</sup> segregant. Complementation of the

shuffle strain by various *<CDC53<sup>M</sup> TRP1 CEN >* plasmids was tested by plating on 0.1% FOA medium. *cdc53 skp1* double mutants were generated in crosses of MTY871 with Y553 and Y555 (Bai et al. 1996). The *MET30-1* strain (CC786-1A) was created by crossing W303-1B with CM100-1A (Thomas et al. 1995).

#### Two-hybrid analysis

Strain Y187 expressing Gal4<sup>DBD</sup> fusions was transformed with a yeast Gal4<sup>AD</sup>-cDNA library (provided by S. Elledge) or a Gal4<sup>AD</sup> genomic DNA library (James et al. 1996) and screened as described (Durfee et al. 1993). With the cDNA library, Gal4<sup>DBD</sup>-Cdc53 recovered one positive clone (Met30-A10) from 140,000 transformants, and Gal4<sup>DBD</sup>-Cdc53<sup>Δ581-664</sup> recovered two positive clones (Skp1-C23, Skp1-C24) from 225,000 transformants. Gal4<sup>DBD</sup>-Cdc53<sup>Δ1-280</sup> recovered no positive clones from 427,000 transformants. With the genomic DNA library, Gal4<sup>DBD</sup>-Cdc53 recovered five positive clones (Cdc4-F20, Cdc4-F23, Cdc4-F24; Met30-F15, Met30-F19) from 1,000,000 transformants and Gal4<sup>DBD</sup>-Cdc53<sup>Δ581-664</sup> recovered seven positive clones (Cdc4-H1, Cdc4-H8; Met30-H6, Met30-H9, Met30-H17; Skp1-H11, Skp1-H13) from 500,000 transformants. Some clones were isolated several independent times, but all unique clones are represented in Figure 1.

#### RNA analysis

Preparation of yeast lysates and analysis of total RNA were

**Table 2.** Yeast strains used in this study

Strain	Relevant genotype	Source
K699	<i>MATa ade2-1 can1-100 his3-1,15 leu2-3,112 trp1-1 ura3</i>	K. Nasmyth (Research Institute of Molecular Pathology, Vienna, Austria)
MTY668	<i>MATa cdc4-1</i>	this study
MTY670	<i>MATa cdc34-2</i>	Willems et al. (1996)
MTY871	<i>MATa cdc53-1</i>	Willems et al. (1996)
MTY1243	<i>cdc53::ADE2 &lt;CDC53<sup>HA</sup> URA3 CEN&gt;</i>	this study
MTY1293	<i>cdc53-1 skp1-11</i>	this study
MTY1294	<i>cdc53-1 skp1-12</i>	this study
MTY1280	<i>grr1::LEU2</i>	
MTY1310	<i>cdc4-1 grr1::LEU2</i>	
Y187	<i>MATa ade2-101 his3-Δ200 leu2-3,112 trp1-901 ura3-52 gal4Δ gal80Δ URA3::GAL-lacZ LYS2::GAL-HIS3</i>	S. Elledge
Y190	as for Y187 but <i>MATα</i>	S. Elledge
Y553	<i>MATα skp1-11</i>	Bai et al. (1996)
Y555	<i>MATα skp1-12</i>	Bai et al. (1996)
WX131-2c	<i>MATα cdc53-2 trp1-7 ura3-52 ade2</i>	M. Goebel (Indiana University School of Medicine, Indianapolis)
CC786-1A	<i>ade2 his3 leu2 ura3 trp1 MET30-1</i>	this study
C170	<i>ade2 his3 leu2 trp1 met4::TRP1 ura3::lexA op-lacZ::URA3</i>	Kuras et al. (1996)

carried out as described previously (Willems et al. 1996). *MET25* mRNA expression was assayed in cultures grown in B media supplemented with 0.1 mM methionine (Thomas et al. 1995). At a density of  $0.5 \times 10^7$  cells/ml cultures were shifted to 37°C for 2 hr, repressed with 1.0 mM methionine, and time points taken for RNA extraction. Northern blots were probed with a 1.3-kb *MET25* fragment and a 0.6-kb *ACT1* fragment. mRNA abundance was quantitated on a Molecular Dynamics Storm PhosphorImager.

#### Protein analysis

Immunoblots were processed for ECL detection as described (Willems et al. 1996) and where indicated, immunoreactivity was quantitated by densitometry and NIH Image software. Antibodies against Cdc4, Cdc34, and Cdc53 (Mathias et al. 1996), Cdc28 (Tyers et al. 1992) and Skp1 (Bai et al. 1996) were used as described. Anti-Grr1 antiserum was adsorbed against polyacrylamide to eliminate background binding and used at 1:100 (Flick and Johnston 1991). Anti-Met30 antiserum was raised against

recombinant Gst-Met30 (residues 297–640, which encompass the WD40 repeats), affinity purified and used at a dilution of 1:100. 9E10 anti-MYC and 12CA5 anti-HA monoclonal antibodies were produced as ascites fluid and used at a dilution of 1:10,000. Anti-FLAG M2 antibody conjugated to Sepharose beads (Kodak) and HRP-conjugated secondary antibodies (Amersham) were used according to manufacturer's instructions.

For promoter shut-off of *pGAL1-CLN2<sup>HA</sup>* constructs, cultures grown in galactose for 2 hr at 37°C were rapidly pelleted, rinsed, and resuspended in glucose medium at 37°C for the indicated timecourse (Willems et al. 1996). Northern analysis of the *CLN2* mRNA confirmed that the *GAL1* promoter was rapidly repressed in all strains. Determination of Cln2 stability by pulse-chase analysis was carried out as described (Amon et al. 1994). Briefly, 40 ml of culture was grown to  $1 \times 10^7$  cells/ml in selective medium that contained 0.1 mM methionine. Cells were washed by filtration, resuspended in 1 ml of medium lacking methionine, incubated for 5 min, pulse-labeled with [<sup>35</sup>S]-methionine/cysteine (ICN) for 5 min, and chased for various times with cold 1 mM methionine/cysteine and 1 μg/ml of cycloheximide. Cells were killed by addition of 10% TCA, and disrupted by glass bead lysis followed by boiling in 1% SDS. Extracts were diluted to RIPA conditions, incubated with 1 μl of 12CA5 anti-HA antibody for 2 hr on ice, and immune complexes collected on protein A-Sepharose for 2 hr. For each pulse-chase series, equivalent amounts of radioactivity were immunoprecipitated for each sample. Immunoprecipitated proteins were separated by 10% SDS-PAGE and the dried gel exposed to a PhosphorImager screen. <sup>35</sup>S-labeled Cln2<sup>HA</sup> signal was quantitated with a Molecular Dynamics Storm PhosphorImager and normalized to total radioactivity in the lysate.

#### Sequence analysis

Regions of sequence conservation between Cdc53 homologs identified in database searches were determined by amino acid alignment with ClustalW (Thompson 1994). Conserved residues with a weight of 10 or higher were identified by analysis of 15 full-length homologs with the Wisconsin Package program Pretty. Black lines in Figure 5B indicate the central residue of an 11 residue window containing four or more such conserved residues.

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