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SCF ubiquitin protein ligases and phosphorylation-dependent proteolysis

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Many key activators and inhibitors of cell division are targeted for degradation by a recently described family of E3 ubiquitin protein ligases termed Skp1–Cdc53–F-box protein (SCF) complexes. SCF complexes physically link substrate proteins to the E2 ubiquitin-conjugating enzyme Cdc34, which catalyses substrate ubiquitination, leading to subsequent degradation by the 26S proteasome. SCF complexes contain a variable subunit called an F-box protein that confers substrate specificity on an invariant core complex composed of the subunits Cdc34, Skp1 and Cdc53. Here, we review the substrates and pathways regulated by the yeast F-box proteins Cdc4, Grr1 and Met30. The concepts of SCF ubiquitin ligase function are illustrated by analysis of the degradation pathway for the G1 cyclin Cln2. Through mass spectrometric analysis of Cdc53 associated proteins, we have identified three novel F-box proteins that appear to participate in SCF-like complexes. As many F-box proteins can be found in sequence databases, it appears that a host of cellular pathways will be regulated by SCF-dependent proteolysis.

Keywords: cell cycle; Skp1–Cdc53–F-box protein; Cdc34; E3 ubiquitin protein ligase; cyclin-dependent kinase

1. PROTEOLYSIS AND THE YEAST CELL CYCLE

Coordination of the transitions from one cell cycle phase to the next arises from the interplay between the cell-cycle-regulated cyclin-dependent kinases (CDKs) and highly selective proteolytic pathways that degrade cell cycle regulatory proteins. The cell cycle in the budding yeast *Saccharomyces cerevisiae* is impelled by three major cyclin families that bind and activate a single common CDK kinase subunit, Cdc28 (reviewed in Nasmyth 1996). The G1 cyclins Cln1, Cln2 and Cln3 are rate-limiting factors for commitment to cell division, an event called 'Start', which occurs in late G1 phase just prior to the onset of DNA replication. The S-phase cyclins Clb5 and Clb6 are activated as part of the Start programme and initiate the timely onset of DNA replication. Finally, the mitotic cyclins Clb1, Clb2, Clb3 and Clb4 initiate entry into mitosis. As opposed to activation by cyclin binding, CDK activity is negatively regulated by the direct binding of inhibitory proteins, generically referred to as CDK inhibitors (CKIs) (reviewed in Harper & Elledge 1996). In yeast, two CKI proteins have been identified. Far1 is a mating-pheromone-activated inhibitor of the Cln–Cdc28 kinases that arrests haploid cells at Start in response to mating pheromones (Peter & Herskowitz 1994). Sic1 is a cell-cycle-regulated inhibitor of the Clb–Cdc28 kinases that prevents premature DNA replication in G1 cells and also facilitates exit from mitosis (Schwob *et al*. 1994; Donovan *et al*. 1994). As described below, the cyclins and CKIs are among the key cell cycle targets of the ubiquitin proteolytic system.

Ubiquitin-dependent proteolysis precisely controls the abundance of many regulatory proteins (reviewed in Hershko & Ciechanover 1998; see article by Hershko, this issue). Ubiquitin is a highly conserved 76 amino-acid protein that upon covalent ligation to a substrate protein targets the substrate for rapid ATP-dependent proteolysis. Ubiquitin is conjugated to substrates via a series of trans-thioesterification reactions catalysed by the enzymes E1 (or ubiquitin-activating enzyme), E2 (or ubiquitin-conjugating enzyme) and E3 (or ubiquitin protein ligase) (figure 1). Nucleophilic attack of the E2– or E3–ubiquitin thiolester intermediate by the ε-amino group of a lysine residue on the substrate yields an isopeptide linkage between the C-terminal glycine residue of ubiquitin and the substrate. Reiteration of the ubiquitin transferase reaction on lysine residues of ubiquitin itself assembles a polyubiquitin chain on the substrate. Polyubiquitinated substrates are quickly recognized and efficiently degraded by an abundant intracellular protease particle called the 26S proteasome (reviewed in Baumeister *et al*. 1998; see article by Zwickl *et al*., this issue). The critical regulatory step in ubiquitin-dependent proteolysis is that of substrate

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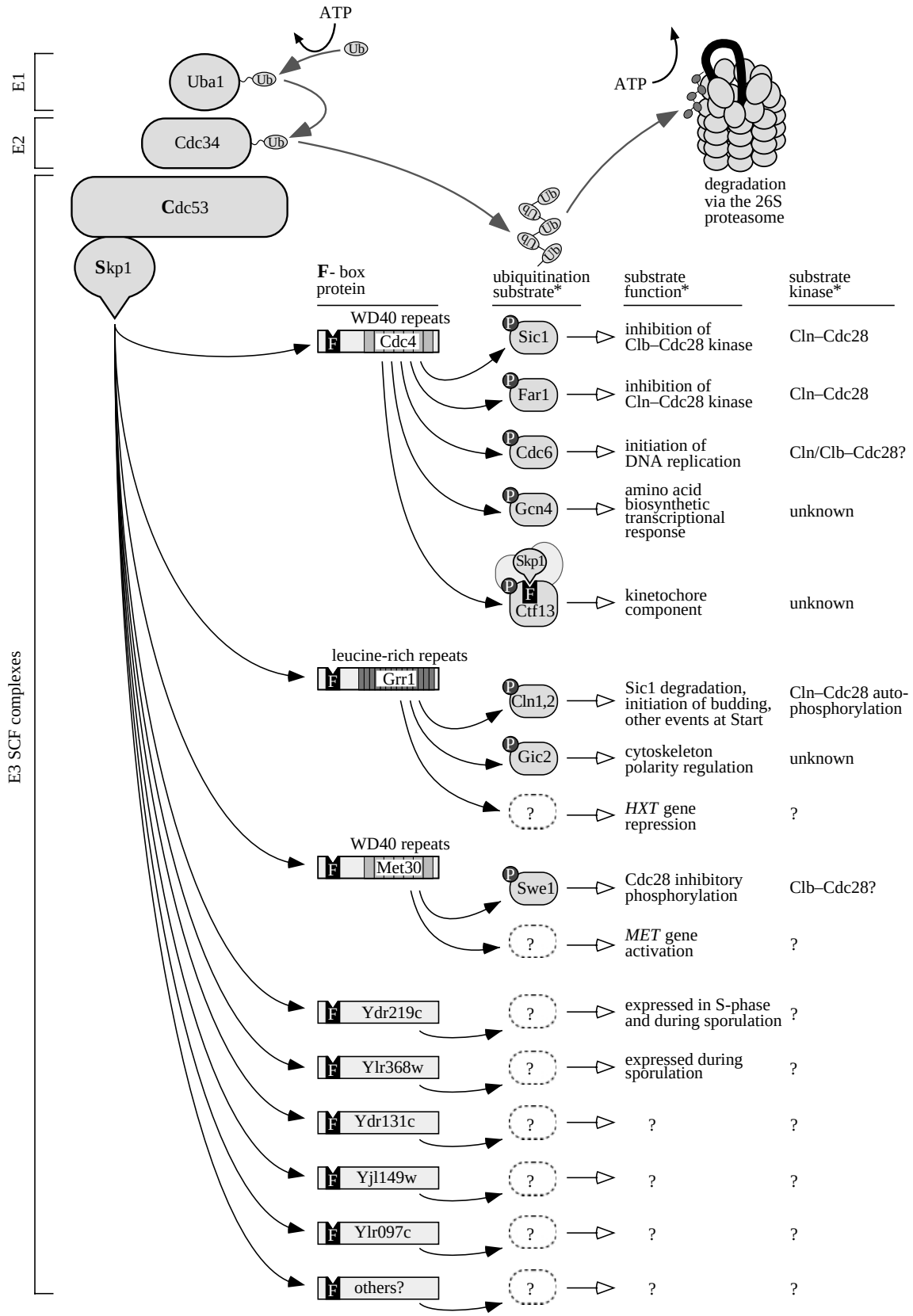

Figure 1. SCF complexes in *S. cerevisiae*. A number of proteins involved in a wide variety of physiological processes are targeted for ubiquitin (Ub)-dependent degradation by a class of E3 ubiquitin protein ligases called SCF complexes (named for their protein subunit components, Skp1, Cdc53 and an F-box protein). Skp1 and Cdc53 form a scaffold for an interchangeable F-box protein and the E2 enzyme Cdc34. F-box proteins bind to Skp1 via the F-box motif (denoted by the F; see figure 2) and recruit substrates via protein–protein interaction domains such as WD40 repeats or leucine-rich repeats. Substrate recognition is often phosphorylation dependent as indicated by the circled P. See text for details regarding substrate identities, functions and kinases (*). Note that some F-box proteins may not function with all the components represented (see text for details).

recognition, carried out by E3 ubiquitin protein ligases. An E3 enzyme is operationally defined by its ability to specifically interact with both an E2 enzyme and a substrate (Hershko *et al*. 1983). Two types of E3 enzymes, the anaphase-promoting complex (APC) and Skp1–Cdc53/cullin–F-box protein (SCF) complexes, mediate ubiquitination of many cell cycle proteins (Hershko & Ciechanover 1998). APC substrates, including the mitotic cyclins and other key proteins degraded at anaphase, contain a short sequence motif called the destruction box (see article by Peters 1998). The APC not only serves to initiate anaphase but is also required for mitotic exit and maintenance of G1 phase. The APC is thus active from anaphase until Start, a window that closely parallels the absence of CDK activity. Cell cycle regulation of the APC derives from two rate-limiting adaptor proteins, Cdc20 and Hct1/Cdh1, which are presumed to recognize the destruction box (Schwab *et al*. 1997; Visintin *et al*. 1997). In contrast to the APC, SCF complexes appear to be constitutively active throughout all phases of the cell cycle. SCF complexes specifically recognize phosphorylated substrates so that regulation is devolved to the level of substrate phosphorylation, which is often, but not always, CDK dependent (Willems *et al*. 1996; Bai *et al*. 1996; Schneider *et al*. 1998). Degradation of proteins such as the Clns, Sic1 and Far1 is controlled in this manner (reviewed in Patton *et al*. 1998*a*).

2. SCF E3 UBIQUITIN PROTEIN LIGASES

The initial insight into SCF-dependent proteolysis came from analysis of the cell division cycle (*cdc*) mutations *cdc4*, *cdc34* and *cdc53*, which cause cells to arrest with unreplicated DNA and multiple elongated buds at the non-permissive temperature. *CDC34* encodes an E2 ubiquitin-conjugating enzyme, the first hint that the ubiquitin system might eliminate an inhibitor of DNA replication (Goebl *et al*. 1988). The sequences of *CDC4* and *CDC53* provided no immediate insights into their function, although Cdc4 does contain WD40 repeats, which are implicated in protein–protein interactions in other systems (Yochem & Byers 1987; Mathias *et al*. 1996). An important clue as to the identity of the critical Cdc34 target in G1 phase was the finding that simultaneous deletion of all six *CLB* genes causes an arrest phenotype indistinguishable from the *cdc34* arrest (Schwob *et al*. 1994). This demonstrated that Clb–Cdc28 kinase activity is required for DNA replication and suggested that the Clb–Cdc28 kinase inhibitor Sic1 might be degraded in a Cdc34-dependent manner (Mendenhall 1993; Schwob *et al*. 1994). Indeed, the replication block of *cdc34* mutants is bypassed by deletion of *SIC1* (Schwob *et al*. 1994).

Phosphorylation targets Sic1 for degradation by the Cdc34 pathway. Sic1 is stable and unphosphorylated in G1 cells that lack Cln–Cdc28 kinase activity, whereas it is hyperphosphorylated and stable in *cdc34*-arrested cells, which have high levels of Cln–Cdc28 kinase activity (Schneider *et al*. 1996*b*). Surprisingly, the *cln*$^{-}$ arrest at Start is bypassed by deletion of *SIC1*, a finding that underscores the close interplay between CDK-dependent phosphorylation and degradation (Epstein & Cross 1994; Tyers 1996; Schneider *et al*. 1996*b*). Sic1 is phosphorylated on consensus CDK sites *in vivo* and a mutant version of Sic1 lacking three of these sites is stable *in vivo* (Verma *et al*. 1997). The Cln–Cdc28 kinases are not inhibited by Sic1, consistent with their role in eliminating Sic1 at Start; however, the Clb–Cdc28 kinases can also phosphorylate Sic1, which may provide a means to prevent accumulation of Sic1 until the end of mitosis (Skowyra *et al*. 1997; Visintin *et al*. 1998).

The Cdc34 pathway was further elaborated through analysis of Cln degradation. The extreme instability of Cln1 and Cln2 is essential for coupling Cln–Cdc28 activity to changes in *CLN* gene expression at Start (Tyers *et al*. 1992, 1993; Lanker *et al*. 1996). This coupling is essential for proper cell cycle regulation because stable mutants such as Cln3-1 and Cln2-1 cause pheromone and nutrient insensitivity (Cross 1988; Nash *et al*. 1988; Hadwiger *et al*. 1989; Tyers *et al*. 1993; Lanker *et al*. 1996). The first indication that phosphorylation might be important in Cdc34-dependent degradation was the finding that hyperphosphorylated forms of Cln3 and Far1 accumulate in *cdc34*-arrested cells (Tyers *et al*. 1992; McKinney *et al*. 1993). Indeed, Cdc34 is required for ubiquitination of Cln2 in an extract system and for degradation of Cln2 *in vivo*, both of which are phosphorylation dependent (Deshaies *et al*. 1995; Lanker *et al*. 1996). Cln2 appears to be targeted for degradation by its Cdc28-dependent autophosphorylation on several CDK consensus sites because Cln2 mutants that lack these sites are stable *in vivo* (Lanker *et al*. 1996). As might be predicted by this model, the Clns are unstable in pre-Start G1 cells, which also implies that the Cln degradation machinery is active throughout the cell cycle (Schneider *et al*. 1998). Cln1 and Cln2 instability *in vivo* also depends on a leucine-rich repeat containing protein called Grr1 (Flick & Johnston 1991), which was revealed in a genetic screen for mutants defective in Cln1 degradation (Barral *et al*. 1995).

Another component of the Cdc34 pathway, Cdc53, was directly identified by sequencing proteins that bind to Cln2 (Willems *et al*. 1996). The interaction between Cln2 and Cdc53 depends on Cln2 phosphorylation because a stable multiple CDK phosphorylation site mutant of Cln2 called Cln2^{4T3S} does not bind Cdc53. Cln2 instability *in vivo* depends on Cdc53, and furthermore, Cln2 is ubiquitinated *in vivo* in a phosphorylation and Cdc34/Cdc53-dependent manner. Cdc53 also interacts with Cln1 and Cln3, suggesting that all three Clns are targeted by a Cdc53-dependent pathway. Finally, Cdc53 physically interacts with Cdc34 *in vivo* and *in vitro* (Willems *et al*. 1996; Mathias *et al*. 1996). Thus, by the standard criteria of substrate and E2 interaction, Cdc53 is an E3 ubiquitin protein ligase. It is now apparent that Cdc53 fulfils this function as part of a multi-protein E3 complex.

Another component of the Cdc34 pathway, Skp1, was first identified in a complex with cyclin A–CDK2 (hence its designation S-phase kinase-associated protein (SKP)) along with a leucine-rich repeat containing protein, SKP2 (Zhang *et al*. 1995). Yeast Skp1 was subsequently isolated as a high copy suppressor of the *cdc4-1* mutation, which allowed a subtle reinterpretation of the gene name as a suppressor of a CDK inhibitor proteolysis defect (Bai *et al*. 1996). Skp1 was also isolated in a two-hybrid screen with human cyclin F, an interaction that is still of

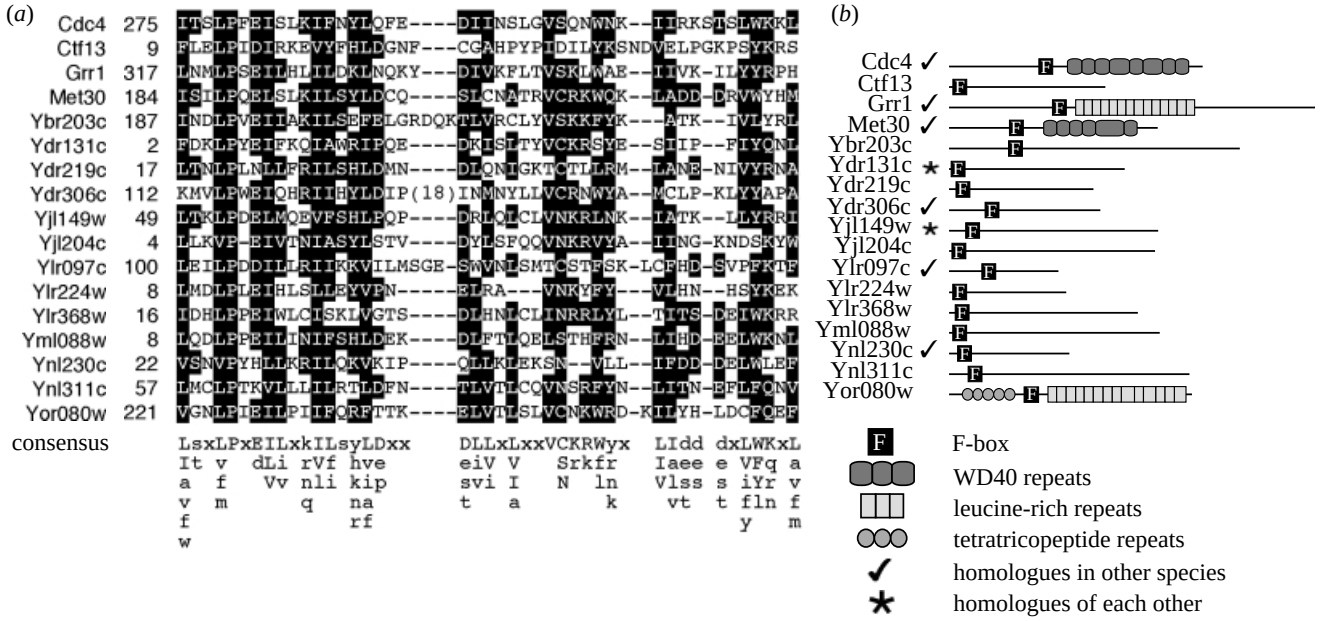

Figure 2. F-box proteins in *S. cerevisiae*. (*a*) Database searches of the *S. cerevisiae* genome reveal at least 17 putative F-box-containing proteins. Conserved amino acids are highlighted. A consensus F-box motif is indicated below the alignment. Upper-case letters indicate highly conserved residues, lower-case letters indicate partially conserved residues and x indicates non-conserved positions. The amino-acid position of the first F-box motif residue in each protein is indicated to the left of each sequence. Ydr306c has an 18 amino-acid insertion in the central portion of the F-box. (*b*) Schematic representation of known and putative *S. cerevisiae* F-box proteins.

unknown significance (Bai *et al*. 1996). Skp1 binds directly to Cdc4 and cyclin F *in vitro* (Bai *et al*. 1996). Finally, Skp1 was identified as a component of the CBF3 kinetochore complex, which binds the CDEIII element of yeast centromeres (Stemmann & Lechner 1996) and as a high copy suppressor of a defect in Ctf13, another component of the CBF3 complex (Connelly & Hieter 1996). Amusingly, this G2 function of Skp1 was also accommodated by its original designation, as a suppressor of a kinetochore protein. Some alleles of *SKP1* cause arrest with a *cdc34*-like phenotype, and indeed Sic1 is stabilized in such *skp1* mutants, as are Cln2 and Clb5 (Bai *et al*. 1996). Other alleles of *SKP1* cause a G2 arrest, which presumably reflects defective kinetochore function (Connelly & Hieter 1996; Bai *et al*. 1996).

3. THE F-BOX HYPOTHESIS

Alignment of the three Skp1 interacting proteins, cyclin F, Skp2 and Cdc4, revealed a shared motif referred to as the F-box, for cyclin F (Bai *et al*. 1996). The F-box motif is a highly degenerate, hydrophobic sequence that is approximately 40 amino acids in length (figure 2). Subsequent inspection also revealed F-box motifs in Ctf13 and in Grr1. The F-box appears to be a binding site for Skp1 because conserved residues within the F-box are necessary for the interaction of Skp1 and Cdc4 *in vitro* (Bai *et al*. 1996). Importantly, the Sic1 and Cln2 degradation pathways appeared to rely on both common (i.e. Cdc34, Cdc53 and Skp1) and divergent (i.e. Cdc4 versus Grr1) components. The observations were drawn together by an idea now referred to as the 'F-box hypothesis' (Bai *et al*. 1996). This hypothesis has two main tenets:

(i) Skp1 directly binds F-box proteins via the F-box motif. In this way, Skp1 links various F-box proteins to the Cdc34/Cdc53 ubiquitination machinery.
(ii) F-box proteins in turn recruit substrates for ubiquitination via specific protein–protein interaction domains present in the C-terminal portion of the F-box protein, such as the WD40 repeats in Cdc4 or the leucine-rich repeats in Grr1.

The physical interactions detected between the various components of the Cdc34 pathway consolidated the idea that Skp1, Cdc53 and various F-box proteins might form E3 complexes that recruit various phosphorylated substrates for ubiquitination by Cdc34 (Willems *et al*. 1996; Bai *et al*. 1996; Mathias *et al*. 1996; see figure 1). The basic configuration of these E3 complexes was established with the finding that Cdc53 independently binds both Skp1 and Cdc34 (Skowyra *et al*. 1997; Feldman *et al*. 1997). A short region at the extreme N-terminus of Cdc53 binds Skp1, while a conserved central region of Cdc53, sometimes referred to as the cullin homology domain, is required for binding Cdc34 (Patton *et al*. 1998*b*). The Skp1–Cdc53 subcomplex thus acts as a scaffold that links F-box proteins to Cdc34 (see figure 1). The F-box seems to behave as a modular unit as F-box sequences can function in the context of heterologous F-box proteins (Kumar & Paietta 1998). Unlike the HECT domain class of E3 enzymes, which form catalytically essential ubiquitin thiolester intermediates (Scheffner *et al*. 1995), genetic analysis in yeast suggests that none of the SCF components bear essential cysteine residues (Patton *et al*. 1998*b*).

An important prediction of the F-box hypothesis is that Cdc53 and Skp1 should be necessary for all F-box

protein-mediated degradation pathways, whereas the various F-box proteins should exhibit specificity in degradation pathways. The primary example of such specificity is that of Cdc4 for Sic1 degradation and of Grr1 for Cln1/2 degradation (Schwob *et al*. 1994; Barral *et al*. 1995; Skowyra *et al*. 1997; Patton *et al*. 1998*b*). A second example of specificity is the requirement of another F-box protein called Met30, but not Cdc4 or Grr1, for repression of methionine biosynthesis genes (Patton *et al*. 1998*b*). In contrast to the specificity of F-box protein function, Skp1, Cdc53 and Cdc34 are required in each of these pathways (Patton *et al*. 1998*b*). In order to accommodate the numerous permutations that derive from interactions of the Skp1–Cdc53 subcomplex with many different F-box proteins, the acronym SCF was coined for Skp1–Cdc53–F-box protein complexes (Skowyra *et al*. 1997; Feldman *et al*. 1997). For example, an SCF complex consisting of Skp1, Cdc53 and the F-box protein Cdc4 is called SCF^{Cdc4}.

The essential features of the F-box hypothesis have been proven by *in vitro* reconstitution of SCF^{Cdc4}-dependent ubiquitination of Sic1 with purified recombinant proteins (Skowyra *et al*. 1997; Feldman *et al*. 1997). As anticipated from *in vivo* studies, Sic1 ubiquitination in the *in vitro* system depends absolutely on its prior phosphorylation by Cdc28 kinases. A key prediction of the F-box hypothesis was fulfilled with the finding that Cdc4 directly binds phospho-Sic1 and that the WD40 repeat domain of Cdc4 is essential for this interaction (Skowyra *et al*. 1997). Furthermore, F-box proteins confer specificity on reconstituted SCF complexes as SCF^{Cdc4} binds phospho-Sic1 but not phospho-Cln2, whereas SCF^{Grr1} preferentially binds phospho-Cln2 (Skowyra *et al*. 1997). Thus, SCF complexes are bona fide E3 ubiquitin protein ligases because they interact with substrates and an E2 enzyme (Hershko *et al*. 1983).

Many putative F-box proteins can be identified in sequence databases, suggesting that an enormous number of cellular pathways may be regulated by SCF-dependent proteolysis (Bai *et al*. 1996; Patton *et al*. 1998*a*). For instance yeast contains at least 17 F-box proteins (see figure 2). However, despite the predictive successes of the F-box hypothesis, one should not assume that every SCF complex will be wired in an identical manner, or even that every F-box protein will function in a degradation pathway. Indeed, Skp1 and the F-box protein Ctf13 interact as structural components of the CBF3 kinetochore complex but there is no evidence that Ctf13 functions in a degradation pathway (Kaplan *et al*. 1997). Furthermore, while the F-box is necessary for interaction with Skp1, other regions of F-box proteins may contribute to the interaction, as for example in the case of Grr1 (Li & Johnston 1997). Finally, despite all efforts, only SCF^{Cdc4} activity has been reconstituted from recombinant components which may indicate that other SCF complexes require additional modifications or co-factors for activity (Skowyra *et al*. 1997).

SCF pathways appear conserved through evolution, as homologues of each SCF component can be identified in most eukaryotic species. The first metazoan homologue of Cdc53, called CUL-1 (for 'cullin'), was identified in worms, where loss of function mutations cause hyperproliferation in all post-embryonic tissues, consistent with a possible cell cycle function for SCF pathways in development (Kipreos *et al*. 1996). Mammals contain at least ten cullin homologues (Kipreos *et al*. 1996; Mathias *et al*. 1996; Zachariae *et al*. 1998; A. R. Willems and M. Tyers, unpublished data). Of these, CUL-1 assembles into an SCF complex with human Skp1 and the F-box protein Skp2 (Lisztwan *et al*. 1998; Lyapina *et al*. 1998; Michel & Xiong 1998; Yu *et al*. 1998). The existence of numerous F-box-containing proteins in sequence databases suggests that as in yeast, many cellular processes may be regulated by SCF complexes in mammals (Bai *et al*. 1996).

4. SUBSTRATES FOR SCF DEGRADATION PATHWAYS IN YEAST

Each SCF complex identified to date targets more than one substrate for degradation (figure 1). In addition to Sic1, SCF^{Cdc4} is implicated either directly or indirectly in the degradation of Far1, the replication protein Cdc6, the transcription factor Gcn4 and the kinetochore protein Ctf13. Like Sic1, Far1 appears to be targeted by Cln–Cdc28-dependent phosphorylation (McKinney *et al*. 1993). Because Far1 is an inhibitor of Cln–Cdc28 kinases, it appears that these two mutually antagonistic activities generate a bistable state in the pheromone response—either Cln–Cdc28 kinases are inhibited by Far1, leading to G1 arrest, or Far1 is targeted for degradation by Cln2–Cdc28 kinase-dependent phosphorylation, leading to recovery from G1 arrest. The N-terminus of Far1 mediates its instability (McKinney & Cross 1995) and mutation of a single phosphorylation site within this region, S87, results in Far1 stabilization and an increased sensitivity to pheromone (Henchoz *et al*. 1997; Gartner *et al*. 1998).

Cdc6 is essential for the assembly of pre-replicative complexes at chromosomal origins of replication (reviewed in Newlon 1997). Cdc6 instability is mediated by SCF^{Cdc4} *in vivo* and Cdc6 may be targeted for degradation by the Cln–Cdc28 kinases (Drury *et al*. 1997). The physiological significance of Cdc6 instability in *S. cerevisiae* is not clear because stabilization of Cdc6 by deletion of residues 2–47, which encompasses several Cdc28 consensus phosphorylation sites, does not cause any obvious replication defect (Drury *et al*. 1997). However, stabilization or overexpression of the *Schizosaccharomyces pombe* Cdc6 homologue, Cdc18, does cause re-replication, suggesting that additional redundant controls may prevent re-replication in *S. cerevisiae* (Jallepalli *et al*. 1997). The degradation pathway for Cdc18 and a CDK inhibitor called Rum1 in *S. pombe* is highly analogous to SCF^{Cdc4}-mediated degradation in *S. cerevisiae* (Kominami & Toda 1997; Jallepalli *et al*. 1998; see article by Toda *et al*., this issue). The pathways that control SCF^{Cdc4}-mediated degradation of Gcn4 and Ctf13 are not well understood, in part because the relevant kinases have not been identified (Kornitzer *et al*. 1994; Kaplan *et al*. 1997).

In addition to Cln1 and Cln2, SCF^{Grr1} also targets Gic2, an effector of the Rho-related GTP-binding protein Cdc42 that initiates polarization of the actin cytoskeleton during bud emergence (Brown *et al*. 1997; Chen *et al*. 1997). Gic2 degradation *in vivo* depends on Grr1 but not Cdc4, and is phosphorylation dependent because phosphorylation site mutants of Gic2 are stable and cause

hyperpolarized growth. Although Gic2 degradation requires Cln–Cdc28 activity *in vivo*, this requirement appears to be indirect because hyperactivation of Cdc42 alleviates the Cln–Cdc28 dependence but not the phosphorylation dependence (Jaquenoud *et al*. 1998). Activation of Gic2 by Cdc42 presumably allows an unknown kinase to phosphorylate Gic2, which results in its subsequent recognition by SCF^{Grr1}. The coupling of Gic2 activation and proteolysis might help limit Gic2 activity to a narrow window in G1 phase (Jaquenoud *et al*. 1998).

Grr1 was originally identified because of its role in glucose repression (Flick & Johnston 1991). In the presence of glucose, Grr1 inhibits Rgt1, a transcriptional repressor of *HXT* genes, such that *grr1* mutants are unable to express the Hxt transporters and therefore grow poorly on glucose medium. Although SCF^{Grr1} may target Rgt1 for degradation, there is no evidence that Rgt1 abundance is regulated by glucose (Li & Johnston 1997). Interestingly, the interaction between Grr1 and Skp1 may be stimulated by glucose, the first indication that SCF activity may be regulated at the level of complex assembly (Li & Johnston 1997). Additional targets of SCF^{Grr1} can also be inferred from the cation and amino-acid transport defects of *grr1* mutants (Li & Johnston 1997).

The third F-box protein of known function in *S. cerevisiae* is Met30, a WD40-repeat-containing protein that was originally identified as a repressor of methionine biosynthesis gene transcription (Thomas *et al*. 1995). Met30 binds the transcriptional activator Met4 and, in response to increased levels of intracellular S-adenosylmethionine, represses transcriptional activation by the Met4-based transcription factor complex. Repression also requires Skp1, Cdc34 and Cdc53, suggesting it is mediated by an SCF^{Met30} complex, perhaps via degradation of a component of the Met4 complex (Patton *et al*. 1998*b*). The Cdc28 inhibitory kinase Swe1 has recently been identified as a substrate of SCF^{Met30} (Kaiser *et al*. 1998). Swe1 may be targeted for degradation by the Clb–Cdc28 kinases because Swe1 abundance is increased in cells with low Clb activity. Again, this mutual antagonism may serve to establish a bistable state between a kinase and its inhibitor, in this case to facilitate exit from the Swe1-dependent cell morphology checkpoint arrest and possibly also to sharpen the transition from G2 into mitosis (Kaiser *et al*. 1998).

5. PHOSPHORYLATION-DEPENDENT RECOGNITION OF SUBSTRATES BY SCF COMPLEXES

A common feature of all SCF degradation pathways identified to date is phosphorylation-dependent substrate recognition. In addition to phosphorylation, most SCF substrates contain so-called 'PEST' regions, which are simply defined by a preponderance of proline (P), glutamate (E), serine (S) and threonine (T) residues. Many unstable proteins contain PEST-rich regions and it has been suggested that PEST sequences represent instability determinants, an idea referred to as the 'PEST hypothesis' (Rogers *et al*. 1986). In support of the PEST hypothesis, truncation of the PEST-rich C-terminal regions of Cln2 and Cln3 causes their stabilization (Nash *et al*. 1988; Cross 1988; Hadwiger *et al*. 1989), and a PEST region derived from Cln3 acts as a portable degradation motif (Yaglom *et al*. 1995). However, some key phospho-acceptor residues in Cln2 and Sic1 do not lie within PEST sequences *per se* (Lanker *et al*. 1996; Verma *et al*. 1997). Thus PEST sequences serve as common but not obligatory 'hosts' for proline-directed kinase phosphorylation sites. At least one unstable PEST-containing protein, ornithine decarboxylase, is degraded by the proteasome in a ubiquitin-independent manner (reviewed in Coffino 1998). It remains to be determined whether or not the majority of the many unstable PEST-containing proteins will be degraded by SCF pathways.

Unlike other phosphorylation-dependent protein–protein interactions that are mediated by short continuous phosphopeptide sequences, such as the recognition of phosphotyrosine motifs by SH2 domains and phosphoserine motifs by 14-3-3 proteins (reviewed in Pawson 1995), the mechanism by which phosphorylated substrates are recognized by F-box proteins is not well understood. For Sic1 and Cln2, phosphorylation at multiple redundant sites appears necessary for interaction with their cognate F-box proteins (Lanker *et al*. 1996; Verma *et al*. 1997). In other cases, such as for Far1, a single site may mediate recognition (Henchoz *et al*. 1997). Alignment of the many known phosphorylation sites that target substrates to SCF^{Cdc4} does not reveal any obvious consensus sequence. Thus, at present, it is not known whether phosphorylated residues participate directly in binding to F-box proteins through ionic interactions or only indirectly by causing conformational changes that expose a binding epitope. It is quite conceivable that different SCF substrates will be recognized via different mechanisms. It is puzzling that ostensibly similar phosphorylated sequences in Cln2 and Sic1 are recognized by completely different F-box proteins, Grr1 and Cdc4, respectively. In spite of the confusion for cell-cycle substrates, the mechanism of phosphorylation-dependent recognition by an E3 complex has recently been determined for IκB, a mammalian protein which prevents nuclear localization of the transcription factor NFκB (see article by Hay *et al.*, this issue). Phosphorylation of IκB on two closely spaced serine residues results in IκB degradation by the ubiquitin pathway and nuclear entry of NFκB. A simple di-phosphorylated peptide corresponding to the IκB sites outcompetes binding to the IκB E3 complex and stabilizes IκB *in vivo*, suggesting that a simple ionic interaction underlies recognition of phospho-IκB (Yaron *et al*. 1997). Recent exciting results suggest that the IκB E3 complex contains a WD40-repeat-containing F-box protein called βTrCP which appears to directly bind IκB (Yaron *et al*. 1998).

6. Cln2 IS MODIFIED *IN VIVO* IN A Grr1-DEPENDENT MANNER

A considerable body of evidence suggests that Cln2 ubiquitination *in vivo* is dependent on phosphorylation as well as on Cdc53 and Cdc34 function (Willems *et al*. 1996; Lanker *et al*. 1996). By their very nature substrate–ubiquitin conjugates are transient and difficult to detect. The initial detection of Cln2–ubiquitin conjugates required overexpression of *CLN2* and overexpression of an epitope-tagged ubiquitin mutant that is unable to form polyubiquitin chains and is resistant to de-ubiquitination

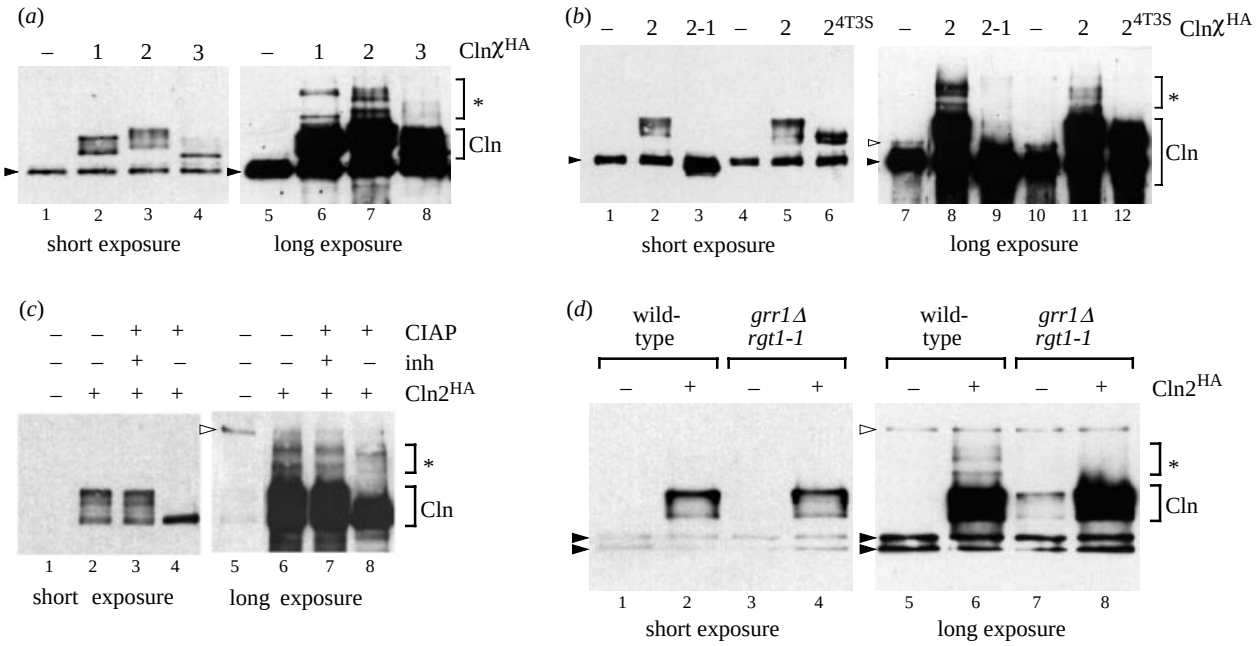

Figure 3. Cln2 modification *in vivo* is dependent on phosphorylation and on the F-box protein Grr1. (*a*) A small fraction of Cln1, Cln2 and Cln3 exhibits lower mobility by SDS–PAGE analysis. Proteins from lysates of wild-type cells (K699) containing an empty plasmid (pRS316) or plasmids expressing $CLN1^{HA}$ (pMT485), $CLN2^{HA}$ (pMT634) or $CLN3^{HA}$ (pMT41) from the *GAL1* promoter were immunoprecipitated with the 12CA5 anti-HA monoclonal antibody, and detected by immunoblotting with the same antibody. A short exposure reveals the bulk of the 12CA5 reactive protein (labelled 'Cln'), while a long exposure reveals low abundance low mobility isoforms (labelled with an asterisk). Solid arrowheads indicate secondary antibody cross-reactivity with immunoglobulin heavy chain. Open arrowheads indicate other non-specific cross-reacting bands. All experiments in this figure were performed in a similar manner except as noted. (*b*) Cln2 modification is phosphorylation dependent. Lysates from the following strains were analysed as in part (*a*): no tag (K699, lanes 1 and 7), $CLN2^{HA}$ (MTY738, lanes 2 and 8), $CLN2\text{-}1^{HA}$ (MTY251, lanes 3 and 9), no tag (K699 with pRS316, lanes 4 and 10), $pGAL1\text{-}CLN2^{HA}$ (K699 with pSL46, lanes 5 and 11) or $pGAL1\text{-}CLN2^{4T3S\text{-}HA}$ (K699 with pSL122, lanes 6 and 12). (*c*) Cln2 modification is not due to phosphorylation alone. Immunoprecipitations were performed on lysates from cells harbouring an empty plasmid (pRS316, lanes 1 and 5) or expressing $CLN2^{HA}$ (pSL46, lanes 2–4 and 6–8). Prior to immunoblot analysis, the immunoprecipitates were either mock treated (lanes 1, 2, 5 and 6) or treated with calf intestinal alkaline phosphatase in the presence (lanes 3 and 7) or absence (lanes 4 and 8) of phosphatase inhibitors. (*d*) Cln2 modification is dependent on Grr1 function. Lysates from wild-type cells (MTY623, lanes 1, 2, 5 and 6) or *grr1 rgt1-1* cells (MTY608, lanes 3, 4, 7 and 8) containing either no plasmid or a plasmid expressing $CLN2^{HA}$ (pSL46) were analysed as in (*a*).

(Willems *et al*. 1996). Here we demonstrate a modification of Cln proteins that is consistent with the existence of Cln–ubiquitin conjugates in wild-type cells. Upon immunoprecipitation from yeast lysates, low-mobility forms of all three G1 cyclins, Cln1, Cln2 and Cln3, could be detected by long exposures of immunoblots visualized by sensitive chemiluminescent detection (figure 3*a*). A specific and unique pattern of low-mobility species was associated with each Cln (lanes 5–8). These high molecular weight species were not an artefact of Cln overexpression, as the pattern of Cln2 isoforms was identical regardless of whether Cln2 was expressed from its own promoter or from the *GAL1* promoter (figure 3*b*, compare lanes 8 and 11).

We next correlated the modified forms of Cln2 with Cln2 stability *in vivo*. First, elimination of CDK phosphorylation sites in Cln2 resulted in a loss of these isoforms (figure 3*b*). The modified species were not apparent in cells expressing Cln2-1, a truncated protein which lacks the C-terminal region that contains many Cdc28 phosphorylation sites (lanes 8 and 9) (Hadwiger *et al*. 1989). Similarly, the modified forms were not associated with $Cln2^{4T3S}$ in which seven Cdc28 phosphorylation sites in the C-terminal region are mutated to non-phosphorylatable residues (lanes 11 and 12) (Lanker *et al*. 1996). Phosphorylation itself does not account for the modified forms, as dephosphorylation of wild-type Cln2 *in vitro* did not collapse the low-mobility forms (figure 3*c*, lane 8) despite removal of the phosphates that cause a smear of Cln2 phosphoisoforms (lane 4). In fact, the mobility of the modified species was slightly advanced after dephosphorylation, consistent with the idea that it is the phosphorylated versions of Cln2 that are modified by ubiquitin *in vivo* (Willems *et al*. 1996). Finally, the low-mobility Cln2 isoforms depended on Grr1 function *in vivo* (figure 3*d*, lanes 6 and 8). The Grr1 requirement was not an indirect effect of defective glucose repression, because the *grr1* strain also contained an *rgt1-1* mutation, which suppresses the glucose repression defects of *grr1* cells but has no effect on Cln2 stability (Ozcan & Johnston 1995; Barral *et al*. 1995). Consistent with the Grr1 dependence, the modified forms of Cln2 also depended on Cdc34 function (data not shown).

While the above data are consistent with the presence of Cln–Ub conjugates, we stress that we have not directly proven that the modified forms of each Cln are

ubiquitinated intermediates in the Cln degradation pathway. It is possible that anti-HA monoclonal cross-reactive species specifically co-immunoprecipitate with phospho-Cln2. However, such proteins would have to cross-react only in immunoblots but not in immunoprecipitations by the same antibody from yeast extracts in the negative controls, and different sets of such cross-reacting proteins would have to be present in immunoprecipitations of each G1 cyclin. Also, the modified forms of Cln2 were evident in denatured Cln2 immunoprecipitations in which the Cln2–Cdc28 interaction is disrupted (data not shown). It thus seems unlikely that the cross-reactive species are non-covalently associated with Cln2. Finally, the pattern of Cln2 modification is reminiscent of rare isoforms of other proteins degraded by SCF pathways, such as Cdc18 in *S. pombe* (Jallepalli *et al*. 1998), again suggesting that these isoforms indeed represent substrate–ubiquitin conjugates.

7. Grr1 MEDIATES THE INTERACTION BETWEEN Cln2 AND Cdc53

In our original analysis of the Cln2 degradation pathway, we showed that Cdc53 specifically binds phospho-Cln2 (Willems *et al*. 1996). However, we did not determine if the Cln2–Cdc53 interaction was direct or whether it was bridged by another protein in yeast lysates. The F-box protein Grr1 is an obvious candidate, as it is required for Cln2 degradation *in vivo* (Barral *et al*. 1995; Patton *et al*. 1998*b*), binds phospho-Cln2 in an *in vitro* reconstituted system (Skowyra *et al*. 1997) and is required for Cln2 modification *in vivo* (figure 3*d*). As predicted, we were able to detect a Cln2–Grr1 interaction in yeast lysates, as shown by specific co-immunoprecipitation of Grr1 and Cln2 (figure 4*a*). We next examined whether the interaction between Cln2 and Cdc53 in yeast lysates depends on Grr1. Indeed, while Cln2 was associated with Cdc53 in wild-type cells (figure 4*b*, lane 2), this interaction was lost in a *grr1* deletion strain (lane 4). As a control, the Cln2–Cdc28 interaction was not affected in the *grr1* strain. Abrogation of the Cln2–Cdc53 interaction in *grr1* cells was not simply due to inactivation of the ubiquitination pathway, because the interaction is unaffected by the *cdc34-2* mutation (Willems *et al*. 1996). Furthermore, loss of the Cln2–Cdc53 interaction was not an indirect effect of growth defects of the *grr1* strain because the strain we used contained an *rgt1* mutation which bypasses this growth defect. A similar recent analysis of Cln2–Grr1 interactions indicated that Skp1 interacts with Cln2 in a Grr1-dependent manner, and that the interaction of Cln2 with Grr1 depends on the leucine-rich repeats of Grr1 (Kishi & Yamao 1998). Taken together, these observations fully support the model that SCF^{Grr1} targets Cln2 for ubiquitin-mediated degradation *in vivo*.

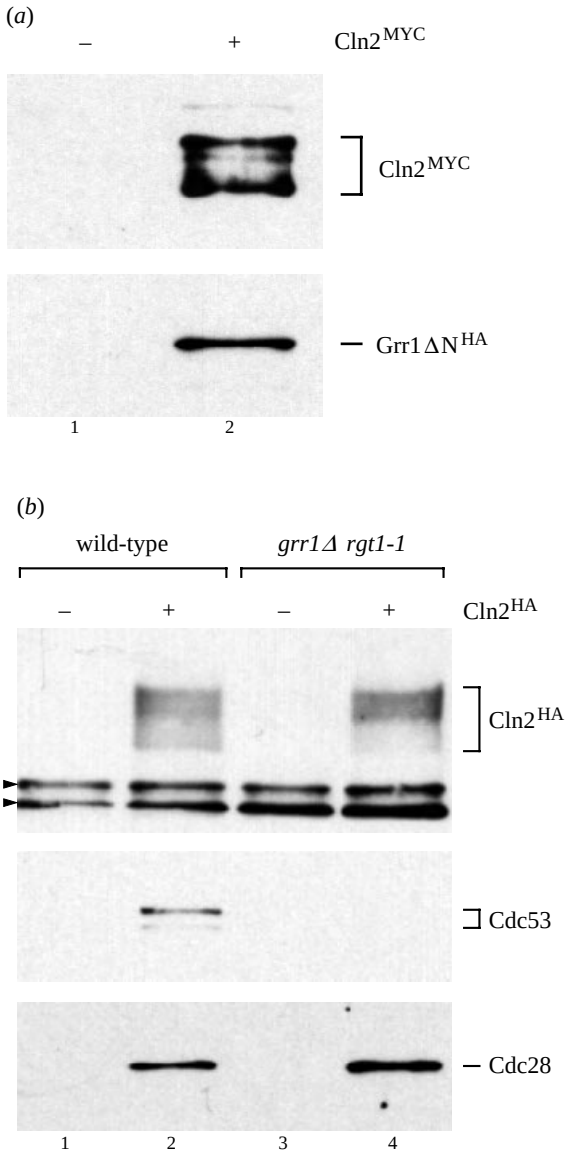

Figure 4. Grr1 binds Cln2 and mediates the Cln2–Cdc53 interaction. (*a*) Cln2 binds Grr1. Lysates from wild-type (K699) or *pGAL1-CLN2^{MYC}* cells (MTY1386) that contained a *pADH1-GRR1ΔN^{HA}* plasmid (pBF494) were immunoprecipitated with the 9E10 anti-MYC monoclonal antibody and analysed by immunoblotting with 9E10 anti-MYC antibody and 12CA5 anti-HA antibody. (*b*) The Cln2–Cdc53 interaction is dependent on Grr1. Lysates from wild-type (S288C) or *grr1 rgt1-1* (YM3378) cells that either contained no plasmid or contained a *pGAL1-CLN2^{HA}* plasmid (pSL46) were immunoprecipitated with the 12CA5 anti-HA antibody and then analysed by immunoblotting with anti-HA antibody, polyclonal anti-Cdc53 antibody and polyclonal anti-Cdc28 antibody.

8. ISOLATION OF NOVEL Cdc53-ASSOCIATED PROTEINS

As described above, three distinct SCF complexes have been identified in budding yeast: SCF^{Cdc4}, SCF^{Grr1} and SCF^{Met30} (Patton *et al*. 1998*b*). Thus, Skp1, Cdc34 and a variety of F-box proteins can be detected in Cdc53 immune complexes, as illustrated in figure 5*a*. Yet database searches reveal at least 12 other F-box proteins (Bai *et al*. 1996; Patton *et al*. 1998*a*; see figure 2). To test the hypothesis that Cdc53 may interact with other uncharacterized F-box proteins, we tagged Cdc53 with six copies of the MYC epitope and immunoprecipitated Cdc53 complexes with an anti-MYC antibody. Proteins associated with Cdc53MYC were separated by one-dimensional SDS-PAGE and visualized by silver staining (figure 5*b*). We performed this experiment in a *cdc34-2* temperature-sensitive strain, as the association between Cdc53 or Skp1

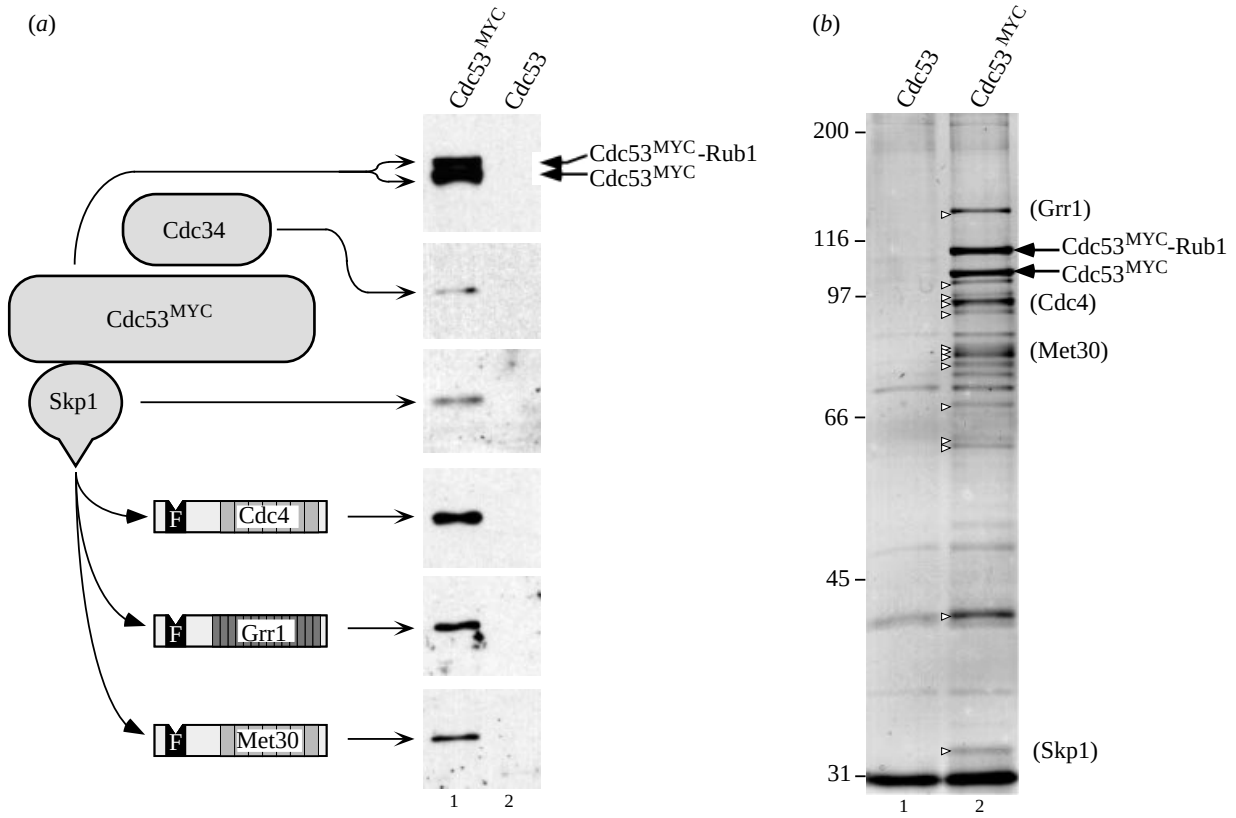

Figure 5. Profiles of proteins that interact with Cdc53 in yeast lysates. (*a*) Cdc53 physically associates with Cdc34, Skp1 and the F-box proteins Cdc4, Grr1 and Met30. Lysates from wild-type cells (K699) expressing either untagged *CDC53* (pMT817) or *CDC53*^{MYC} (pMT843) were immunoprecipitated with 9E10 anti-MYC antibody. Cdc53^{MYC}-associated proteins were visualized by immunoblotting with antibodies specific for the indicated proteins. Cdc53 migrates as a doublet because it is partially modified by Rub1, a ubiquitin-related protein. (*b*) Numerous other proteins associate with Cdc53 in yeast lysates. *cdc34-2* cells (MTY670) harbouring a low copy *CEN* plasmid expressing either untagged *CDC53* (pMT817) or *CDC53*^{MYC} (pMT843) from its own promoter were grown at the permissive temperature (25 °C), shifted to the restrictive temperature (37 °C) for 1 h and harvested. Immunoprecipitations were performed with 9E10 anti-MYC antibody that was cross-linked to a protein A-sepharose matrix. After separation by SDS–PAGE, proteins were detected with an alkaline silver staining procedure (Wray *et al*. 1981). The apparent molecular weights in kiloDaltons of protein standards are indicated on the left. Open arrowheads indicate proteins that specifically associate with Cdc53. The approximate positions of the known Cdc53-associated proteins Grr1, Cdc4, Met30 and Skp1 are shown.

with at least one other F-box protein, Cdc4, is enhanced by the *cdc34-2* mutation (Patton *et al*. 1998*b*). Cdc53 was the predominant protein in the immune complexes, a substantial fraction of which was covalently modified by the ubiquitin-like protein Rub1. The function of the Rub1 modification on Cdc53 is unknown, but it is not essential for SCF activity in wild-type cells (Lammer *et al*. 1998; Liakopoulos *et al*. 1998; A. R. Willems and M. Tyers, unpublished results). As expected, Skp1 was present in the Cdc53 complex and, although it is not visualized well with silver stain, Coomassie blue staining revealed a stoichiometric association of Skp1 with Cdc53 in wild-type cells (data not shown). Cdc34 appears to associate with Cdc53 rather weakly and was not identifiable by direct staining, but Cdc34 can be detected in Cdc53 immunoprecipitations by immunoblotting (Willems *et al*. 1996; Mathias *et al*. 1996; Patton *et al*. 1998*b*; see figure 5*a*). In contrast to Skp1, many proteins were associated with Cdc53 at substoichiometric levels (figure 5*b*). Cdc53 complexes isolated from wild-type cells contained a similar spectrum of associated proteins and furthermore the association of most of these proteins depends on a functional Skp1–Cdc53 interaction, a characteristic expected of F-box proteins (data not shown). It is also conceivable that some of the Cdc53-associated proteins are substrates for particular SCF complexes or are other, as yet unidentified, components of one or more SCF complexes.

9. CHARACTERIZATION OF THREE NOVEL F-BOX PROTEINS

We identified two of the Cdc53-associated proteins by mass spectrometry. Two bands, having apparent molecular weights of approximately 61 kDa (p61) and 41 kDa (p41) and which contained approximately 500 fmol of protein material each (as was estimated by comparison of their staining intensity to the staining intensities of molecular weight standards) were excised from the gel and in-gel-digested with trypsin. The recovered unseparated pools of tryptic peptides were sequenced by nanoelectrospray tandem mass spectrometry (figure 6*a*,*b*). Three peptide ions observed in the spectrum of the p61 digest and one peptide ion observed in the spectrum of the p41 digest were fragmented. It is not unusual to recover only a few peptides after in-gel-digestion of proteins which are

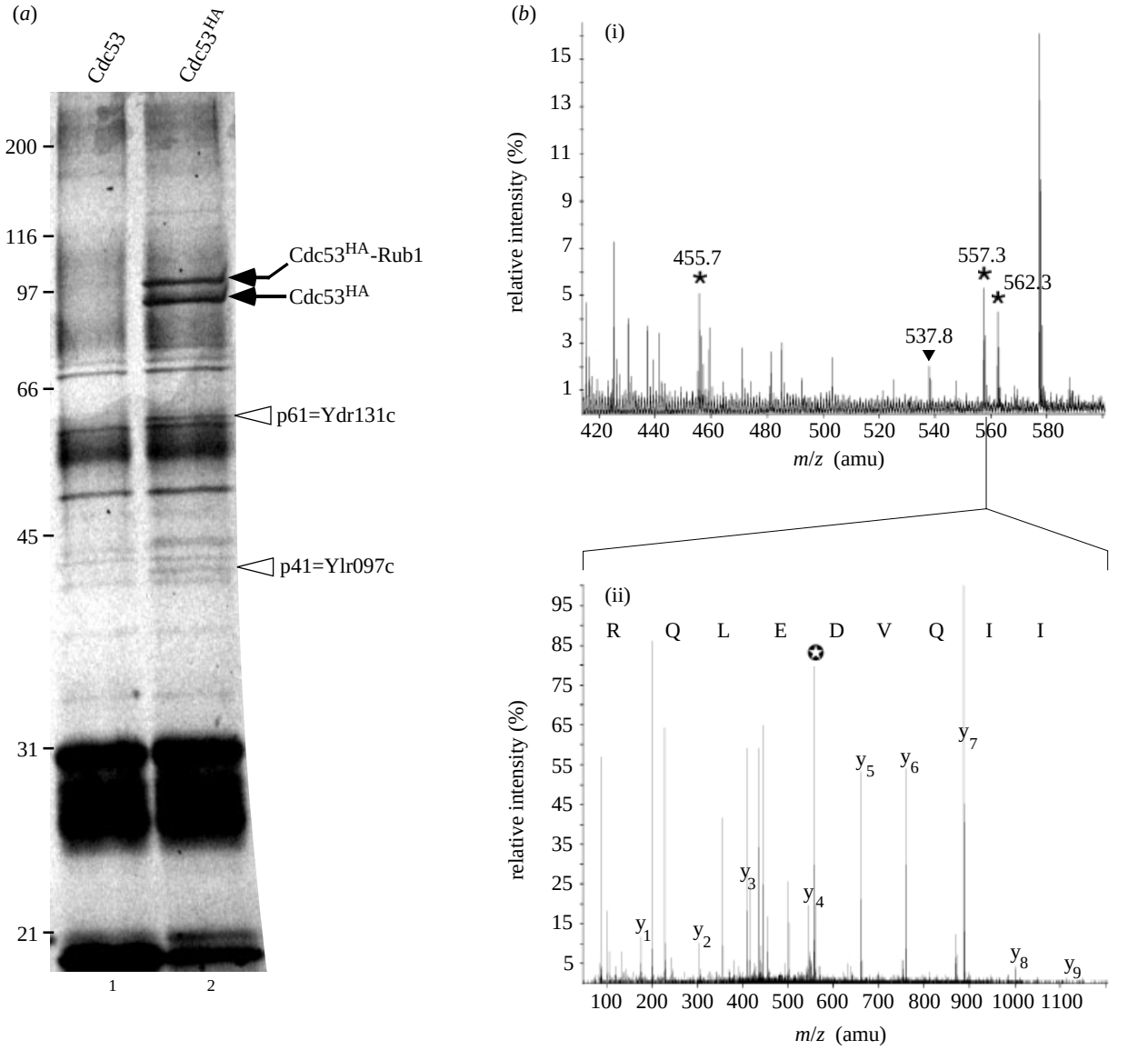

Figure 6. Identification of novel F-box proteins in Cdc53 complexes. (*a*) Lysates from wild-type cells (K699) that contained low copy plasmids expressing untagged *CDC53* (pMT817) or *CDC53^{HA}* (pMT839) from its own promoter were immunoprecipitated with the 12CA5 anti-HA antibody that was cross-linked to protein A-sepharose matrix. Proteins eluted from the matrix were separated by one-dimensional SDS-PAGE and detected by silver staining (Shevchenko *et al*. 1996). Bands corresponding to two proteins, p61 and p41, were excised from the gel for mass spectrometric sequencing and subsequently identified as Ydr131c and Ylr097c, respectively. (*b*) Sequencing of p61 by mass spectrometry; (i) shows a part of the mass spectrum of the in-gel-tryptic digest of p61. Peptide ions labelled in (*b*)(i) were sequenced by tandem mass spectrometry. Sequencing of the doubly charged ion with $m/z = 557.3$ is discussed here as an example. The ion was isolated by a quadrupole mass filter and subsequently fragmented in a collision cell. A spectrum of the daughter ions (*b*)(ii) was acquired using a TOF mass analyser which allowed >7000 mass resolution (FWHM) without compromising the sensitivity. Upon collisional fragmentation, the peptide gave rise to a continuous series of fragment ions containing the C-terminus of the peptide (y-ion series) (Biemann 1988). Short stretches of the peptide sequence were then determined by considering the precise mass differences between the adjacent ions in the series. A piece of peptide sequence deduced from the mass spectrum was combined with both the mass of the intact peptide and the masses of the corresponding fragment ions into a peptide sequence tag (Mann & Wilm 1994), which was used for searching a database with PeptideSearch v. 3.0 software. A matching sequence was retrieved from the database, and the masses of fragment ions from other series (a-series, b-series etc.) were used to confirm the hit. By this approach the peptide ions designated by asterisks in (*b*)(ii) were matched to corresponding tryptic peptides originating from Ydr131c. The arrowhead indicates a peptide originating from an immunoglobulin contaminant. The white star indicates the intact parent ion. Peaks not labelled in the spectrum are ions of known trypsin autolysis products and singly charged ions originating from chemical noise.

present on a gel in femtomole amounts. However, the very high mass accuracy of a hybrid quadrupole-time-of-flight (QqTOF) instrument dramatically increases specificity of database searching and thus allows unambiguous protein identification even when the tandem mass spectrum from only a single peptide ion is acquired (Shevchenko *et al*. 1997). Using tandem mass spectrometric data, p61 was identified as the product of the previously uncharacterized gene *YDR131c*. Similarly the single sequenced peptide unambiguously identified p41 as a product of another uncharacterized gene *YLR097w*. Close inspection of the sequences of both *YDR131c* and *YLR097w* showed that

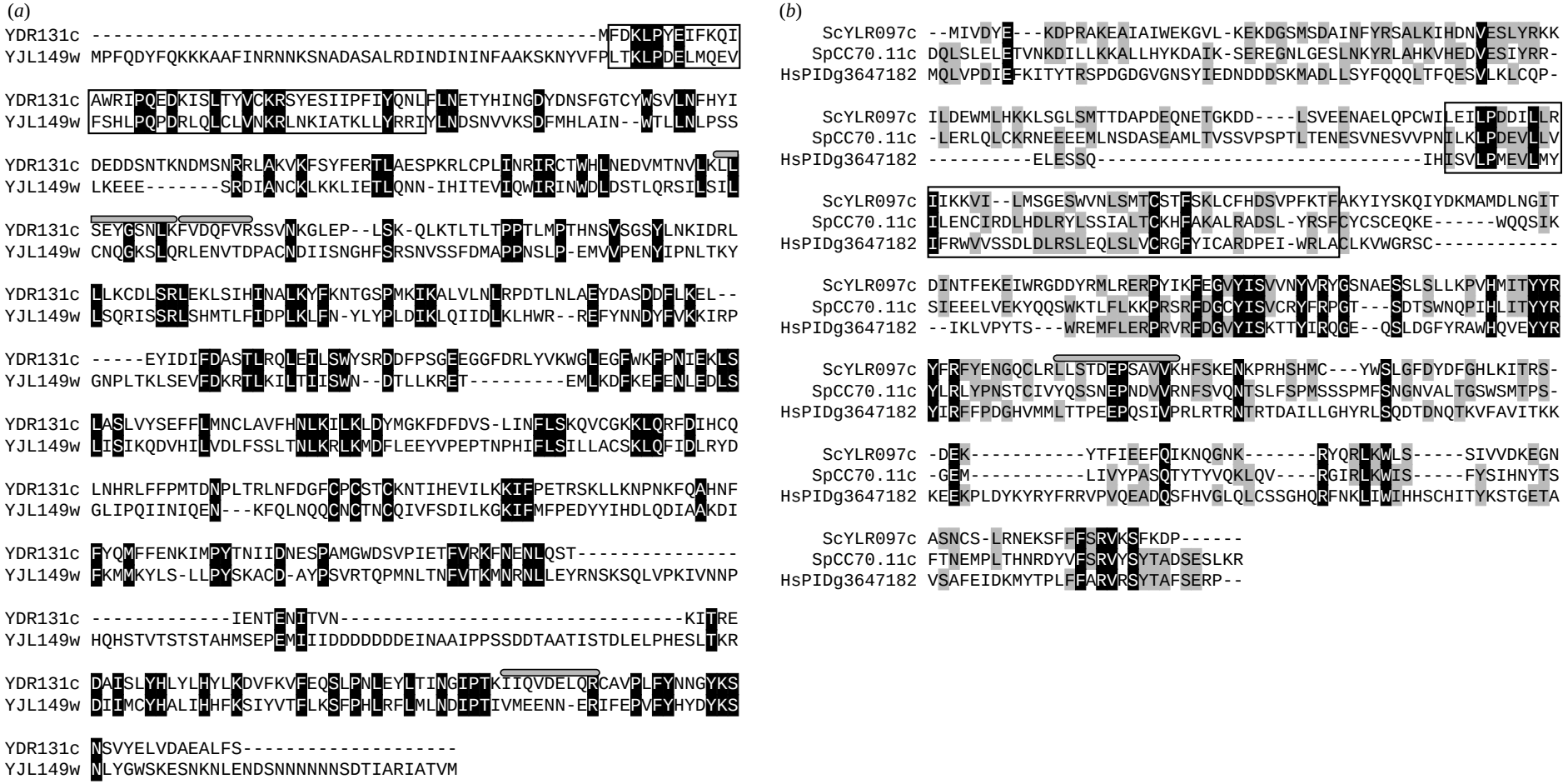

Figure 7. Homologues of Ydr131c and Ylr097c. (*a*) p61 is Ydr131c. Three peptides from p61 were sequenced by mass spectrometry and found to match the yeast hypothetical protein Ydr131c (grey bars). Ydr131c is 24% identical to another hypothetical yeast protein Yjl149w. Identical residues are shown in black. The F-box motif, present in both homologues, is indicated by the box. Outside the F-box, no similarity to other proteins in the databases was found. (*b*) p41 is Ylr097c. One peptide from p41 was sequenced by mass spectrometry and found to match the yeast hypothetical protein Ylr097c (grey bar). Ylr097c has homologues in *Schizosaccharomyces pombe* (CC70.11c) and in humans (PIDg3647182). A partial mouse EST consensus sequence (TC38577) is almost identical to the human homologue (data not shown). Identical residues are shown in black where they occur in all three homologues, grey where they occur in only two. The F-box motif is indicated by a box. Organism abbreviations: Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Hs, *Homo sapiens*.

each predicted open reading frame (ORF) contains an F-box motif (figure 7*a*,*b*). Thus, by the criterion of association with Cdc53, both of these F-box-containing proteins might participate in SCF complexes. Aside from the F-box, neither Ydrl3lc nor Ylr097c has any obvious sequence motifs. However, Ydrl3lc is similar to another candidate F-box protein from *S. cerevisiae*, Yjll49w (figure 7*a*). In addition, Ylr097c is a conserved protein, as it has sequence homologues in *S. pombe*, mice and humans (figure 7*b*).

To further characterize the new F-box proteins, epitope-tagged versions of Yjll49w and Ylr097c were immunoprecipitated from yeast lysates and the immune complexes probed with anti-Skpl and anti-Cdc53 antibodies. Skpl and Cdc53 associated with both Yjll49w and Ylr097c, suggesting that both new F-box proteins form bona fide SCF complexes (figure 8*a*). As controls, a functional N-terminally truncated form of Grrl (Li & Johnston 1997) and full-length Met30 interacted with Cdc53 and Skpl to a similar extent. We also determined whether the new F-box proteins interact genetically with other SCF pathway components. *CDC34* has been implicated genetically and/or biochemically in the function of SCF^{Cdc4}, SCF^{Grrl} and SCF^{Met30}. For example, overexpression of *GRR1* interferes with growth of a *cdc34-2* strain (Patton *et al*. 1998*b*). We found a similar genetic interaction between overexpression of either *YLR097c* or *YJL149w* and the *cdc34-2* mutation (figure 8*b* and data not shown). The morphology of *cdc34-2* cells overexpressing *YLR097c* is similar to that caused by numerous other mutations that stabilize Sic1 (figure 8*b*). The simplest interpretation of this phenotype is that overexpression of any given F-box protein may titrate away Skpl–Cdc53 from SCF^{Cdc4}, which in combination with the *cdc34-2* mutation leads to synthetic growth defects. Synthetic lethal interactions are known to occur between *cdc4*, *cdc34* and *cdc53* mutations (Mathias *et al*. 1996). Based on their physical interaction with Cdc53 and Skpl and their genetic interaction with *cdc34-2*, we anticipate that $SCF^{Yjl149w}$ and $SCF^{Ylr097c}$ may target one or more unknown substrates for proteolysis.

To investigate the role of the new F-box proteins *in vivo*, we deleted the genes for *YDR131c*, *YJL149w* and *YLR097c*. Because *YDR131c* and *YJL149w* are homologues we suspected that they might have overlapping functions, so we also created a *ydr131c yjl149w* double mutant. Each single F-box protein deletion mutant and the double deletion mutant were viable. We assayed each of the deletion strains for growth defects under a variety of conditions. The deletion strains had no growth defects on either glucose or glycerol medium at either 16 °C or 37 °C, nor was there any growth defect on medium containing 0.4 M NaCl or on low pH medium. The *ydr131c yjl149w* double mutant was slightly more sensitive to mating pheromone than wild-type, but this effect was only apparent at 37 °C. Several checkpoint pathways also appeared to be intact in the F-box protein mutants. The deletion strains exhibited wild-type sensitivity to the ribonucleotide reductase inhibitor hydroxyurea and the DNA-damaging agent methyl methane sulphonate, suggesting that the F-box proteins are not required for the DNA replication and DNA damage checkpoints. The *ydr131c yjl149w* double mutant did exhibit a slight sensitivity to nocodazole relative to wild-type cells at 37 °C, which may indicate a subtle defect in the mitotic spindle checkpoint pathway. Finally, the cell cycle distribution of an asynchronously growing population of each deletion strain appeared normal by FACS analysis of DNA content. Further

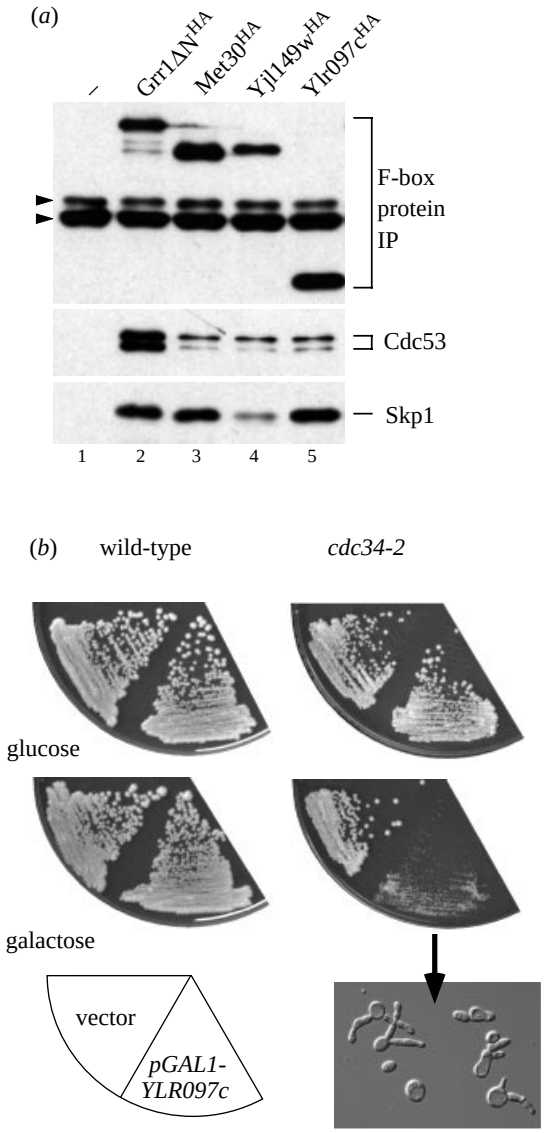

Figure 8. Yjl149w and Ylr097c interact biochemically and genetically with SCF core components. (*a*) Yjl149w and Ylr097c form SCF complexes *in vivo*. Lysates from wild-type cells (K699) harbouring either an empty plasmid (pBF339) or a plasmid expressing ^{HA}*GRR1ΔN* (pBF494), ^{HA}*MET30* (pMT1707), ^{HA}*YJL149w* (pMT1925) or ^{HA}*YLR097c* (pMT1930) from the *ADH1* promoter were immunoprecipitated with 12CA5 anti-HA antibody and analysed by immunoblotting with anti-HA, anti-Cdc53 and anti-Skp1 antibodies. Immunoglobulin heavy chain is indicated with the solid arrowheads. (*b*) Overexpression of *YLR097c* interferes with *CDC34* function. Wild-type (K699) or *cdc34-2* strains (MTY670) harbouring an empty plasmid (pMT1985) or a high copy 2 μ plasmid expressing *YLR097c* (pMT2001) from the *GAL1* promoter were grown on rich medium at 30 °C, a semi-permissive temperature for the *cdc34-2* strain. *YLR097c* expression was either repressed by growth on glucose medium or induced by growth on galactose medium. Cellular morphology of the *cdc34-2* cells expressing *pGAL1-YLR097c* on galactose medium is shown.

analysis will be required to determine the functions of these new F-box proteins.

10. PERSPECTIVE: CONNECTING SCF PATHWAYS AND THEIR SUBSTRATES

Many SCF pathways await the identification of physiological substrates, relevant F-box proteins and/or relevant kinases. One important unresolved issue is the putative substrate for SCF^{Met30} that presumably activates *MET* gene transcription (Patton *et al*. 1998*b*). As Met30 binds the *MET* gene transcription factor Met4, it is a logical candidate, as are other components of the *MET* transcription factor complex (Thomas & Surdin-Kerjan 1997). The means by which methionine limitation regulates Met30 activity is also a mystery. Similarly, the target of SCF^{Grr1} in glucose regulation of *HXT* genes has yet to be identified. Additional substrates for SCF^{Cdc4} can also be inferred from the G2 terminal arrest phenotype of *cdc34 sic1* double mutants (Schwob *et al*. 1994). Intriguingly, a *cdc34 sic1 grr1* triple mutant is viable at 35 °C, yet the *cdc34 sic1* defect is not bypassed by Cln overexpression or by defects in glucose signalling (Kishi *et al*. 1998). The F-box proteins that target Cln3 and Clb5 for SCF-dependent degradation have so far eluded genetic and biochemical characterization. Cln3 instability *in vivo* clearly depends on Cdc34 and Cdc53, but apparently not Grr1 (Barral *et al*. 1995), so it seems probable that Cdc4, Met30 or one of the novel F-box proteins may target Cln3 for degradation (Tyers *et al*. 1992; Yaglom *et al*. 1995; Willems *et al*. 1996). Clb5 degradation also depends on Skp1 and Cdc28, despite the fact that Clb5 contains a destruction box, which might also target it to the APC (Bai *et al*. 1996; Germain *et al*. 1997). Finally, several of the kinases that target substrates for SCF-mediated ubiquitination have not been found, most notably for the Gcn4 and Gic2 pathways (Kornitzer *et al*. 1994; Jaquenoud *et al*. 1998).

As our initial genetic analysis did not reveal a function for any of the three novel F-box proteins, *YDR131c*, *YJL149w* and *YLR097c*, it may not be trivial to identify the function and presumed substrates for the many putative F-box proteins in yeast. To complicate matters further, deletions of most of the other 11 putative F-box proteins (see figure 2) also do not affect viability or responses to various stresses (T. Goh and M. Tyers, unpublished data). The absence of an obvious phenotype may simply reflect the fact that stabilization of the putative substrates for these pathways does not cause lethality under laboratory conditions. Such is the case for the Clns, Far1 and Cdc6. Alternatively, some F-box proteins may carry out redundant functions with other pathways. However, the available approaches to identify F-box substrates and/or functions are by no means exhausted. First and foremost, the physical association between substrates and their cognate F-box proteins may allow substrates to be identified directly. As precedent, the SCF substrates Cdc6, Swe1, Cln1, Cln2 and Cln3 all physically associate with SCF complexes *in vivo* (Willems *et al*. 1996; Kaiser *et al*. 1998; Drury *et al*. 1997). We have recently detected a protein that binds Ylr097c, which may be a substrate for this F-box protein (A. R. Willems and M. Tyers, unpublished data). In other cases a stable interaction between an F-box protein and its substrate may not occur in cell extracts. For example, while Cdc4 binds phospho-Sic1 with high affinity in a reconstituted *in vitro* system (Skowyra *et al*. 1997; Feldman *et al*. 1997), a phospho-Sic1–Cdc4 complex has not been detected in yeast extracts. In fact, it is extremely difficult to even detect phosphorylated isoforms of Sic1, even in cells arrested by the *cdc34-2* mutation (Schneider *et al*. 1996*b*). This may be because a phosphatase actively removes critical phosphates from Sic1 *in vivo*. The Cdc14 phosphatase, which is required for mitotic exit, may function in part as a Sic1-specific phosphatase that stabilizes Sic1 *in vivo* (Visintin *et al*. 1998). In contrast to Sic1, Cln phosphoisoforms are stable in yeast extracts (Tyers *et al*. 1993), which undoubtedly facilitates detection of the Cln2–SCF^{Grr1} interaction. Inactivation of phosphatases by either chemical or genetic means might aid in identification of phosphorylation-dependent F-box protein substrates. Likewise, genetic inactivation of SCF pathways might trap an F-box protein–substrate interaction. Genetic interactions between F-box proteins and their substrates may also be exploited to identify potential substrates. For instance, *CLN* overexpression is detrimental to *grr1* and *cdc53* mutants, *SIC1* overexpression is lethal in *cdc4* and *skp1* mutants, and *FAR1* overexpression is lethal in *cdc4*, *cdc34*, *cdc53* and *skp1* mutants (Barral *et al*. 1995; Willems *et al*. 1996; Bai *et al*. 1996; Henchoz *et al*. 1997).

Expression patterns may also yield valuable clues regarding the function of novel F-box genes. Genome-wide transcriptional analysis of cell-cycle-regulated and sporulation-specific gene expression in yeast has recently revealed that the F-box genes *YDR219c* and *YLR368w* are induced during sporulation, and that *YDR219c* is also up-regulated during mitotic S phase (Chu *et al*. 1998; Cho *et al*. 1998). Some F-box genes and *SKP1* homologues are also developmentally regulated in plants (Lee *et al*. 1997; Ingram *et al*. 1997). Interestingly, all known F-box proteins appear to directly regulate the expression of one or more genes, as for example, *MET* genes by Met30, *HXT* genes by Grr1 and amino-acid biosynthesis genes by Cdc4. Genome-wide transcriptional profiles of F-box protein deletion strains may thus provide clues as to their function.

Finally, in addition to substrate specificity, different F-box proteins may also direct specific interactions with E2 enzymes other than Cdc34. For example, Cdc34 does not appear to participate with SCF^{Grr1} in the regulation of *HXT* gene expression (Li & Johnston 1997), even though it is clearly required for Cln1/2 and Gic2 degradation (Deshaies *et al*. 1995; Willems *et al*. 1996; Jaquenoud *et al*. 1998). In support of the idea that F-box proteins may select particular E2 enzymes, we have found that Cdc4 interacts specifically with Cdc34 in the absence of other yeast proteins (X. Tang and M. Tyers, unpublished data).

The assumption that all F-box proteins function in SCF-dependent proteolytic pathways is certainly an over-simplification. Skp1 appears to play a largely structural role in the CBF3 complex and there is no reason not to expect that Skp1 will do so in other contexts. It is also evident that not all F-box proteins will interact with Skp1. The mammalian transcriptional elongation factor Elongin A contains an F-box-like sequence, but instead of interacting with Skp1 it forms a complex with Elongin B and Elongin C, which are distant homologues of ubiquitin and Skp1, respectively (Aso *et al*. 1996). Similarly, the

Table 1. *Plasmids used in this study*

plasmid	relevant characteristics	source
pBF339	*pADH1-^{HA} TRP1* 2 μ	M. Johnston
pBF494	*pADH1-^{HA}GRR1ΔN TRP1* 2 μ	M. Johnston
pMPY-ZAP	*hisG-URA3-hisG* in pBluescript	(Schneider *et al*. 1996*a*)
pMT41	*pGAL1-CLN3$^{HA}_{3}$ URA3* 2 μ	G. Tokiwa
pMT485	*pGAL1-CLN1$^{HA}_{3}$ URA3 CEN*	G. Tokiwa
pMT634	*pGAL1-CLN2$^{HA}_{3}$ LEU2 URA3 CEN*	(Willems *et al*. 1996)
pMT817	*CDC53 TRP1 CEN*	(Willems *et al*. 1996)
pMT839	*CDC53$^{HA}_{3}$ TRP1 CEN*	this study
pMT843	*CDC53$^{MYC}_{6}$ TRP1 CEN*	(Willems *et al*. 1996)
pMT1111	*cln2::pGAL1-CLN2^{MYC3}-LEU2* in pUC119	B. Schneider
pMT1707	*pADH1-^{HA}MET30 TRP1* 2 μ	(Patton *et al*. 1998*b*)
pMT1925	*pADH1-^{HA}YJL149w TRP1* 2 μ	this study
pMT1930	*pADH1-^{HA}YLR097c TRP1* 2 μ	this study
pMT1985	*pGAL1-$^{HA\text{-}HIS}_{10}$ TRP1* 2 μ	this study
pMT2001	*pGAL1-$^{HA\text{-}HIS}_{10}$YLR097c TRP1* 2 μ	this study
pRS316	*URA3 CEN*	(Sikorski & Hieter 1989)
pSL46	*pGAL1-CLN2^{HA} URA3 CEN*	(Willems *et al*. 1996)
pSL122	*pGAL1-CLN2$^{4T3S\text{-}HA}$ URA3 CEN*	(Willems *et al*. 1996)

cullin family participates in diverse complexes. The mammalian Cdc53 homologues CUL2 through CUL5 do not interact with SKP1 (Michel & Xiong 1998), although CUL2 does form an SCF-like complex with Elongin B, Elongin C and the tumour suppressor protein VHL. However, there is as yet no evidence that this complex functions as an E3 ligase (reviewed in Kaelin & Maher 1998). Budding yeast contains three other Cdc53 homologues and, intriguingly, the most distantly related homologue, Apc2, is a component of the APC (Zachariae *et al*. 1998). While the APC does not contain any F-box proteins, it does use substrate-specificity factors presumed to function as adaptors in a manner analogous to SCF complexes (Visintin *et al*. 1997; Schwab *et al*. 1997). By analogy to Cdc53, the cullin homology domain of Apc2 might be a binding site for an E2 enzyme.

Many putative F-box proteins can be found in sequence databases. For example, the complete *Caenorhabditis elegans* genome encodes approximately 100 potential F-box proteins. Recent genetic analysis in many model organisms has revealed a plethora of F-box proteins that function in diverse signalling pathways, including the wingless pathway, the hedgehog pathway, the Notch pathway, the NFκB pathway and the auxin response (reviewed in Patton *et al*. 1998*a*). Biochemical analysis suggests that key cell cycle regulators in mammalian cells are also targeted for degradation by phosphorylation, including cyclin D, cyclin E, the CDK inhibitor $p27^{KIP1}$ and the transcription factor E2F-1 (reviewed in Krek 1998). Although hard evidence is not yet in place, it is certainly highly anticipated that SCF-like complexes will target these cell cycle proteins for degradation and that SCF pathways may be perturbed in human cancer cells.

11. MATERIAL AND METHODS

(a) *Plasmid construction*

Plasmids and oligonucleotides used in this study are listed in tables 1 and 2. Novel F-box proteins encoded by *YJL149w* and *YLR097c* were amplified by PCR from yeast genomic DNA with the primer pairs MTO497/498 and MTO499/500, respectively. For expression of HA-tagged F-box proteins in yeast, each PCR product was digested with NcoI and XhoI and ligated into an NcoI/SalI-digested *pADH1* expression vector (pBF339) to create pMT1925 and pMT1930. Another yeast expression vector, pMT1985, was constructed by replacing the SacI/SalI *ADH1* promoter/N-terminal epitope tag region of pBF339 with a SacI/EcoRI *GAL1* promoter fragment and an EcoRI/SalI fragment encoding an HA/HIS_{10} double epitope tag. pMT2001 was constructed by inserting the NcoI/SacI *GAL1* promoter/$HA\text{-}HIS_{10}$ tag region of pMT1985 into the *YLR097c* vector pMT1930.

(b) *Yeast strains and culture*

Yeast strains are listed in table 3. Standard methods were used for yeast culture and transformation (Adams *et al*. 1998). The ORFs encoding new F-box proteins were replaced with the *URA3* gene by PCR-mediated gene disruption using pMPY-ZAP (Schneider *et al*. 1996*a*). Deletion constructs for *YDR131c*, *YJL149w* and *YLR097c* were generated by PCR with the primer pairs MTO486/488, 489/491 and 492/494. PCR fragments were used to transform diploid strain K699 a/α, correct integrants identified by PCR were sporulated and Ura^+ segregants, which segregated 2:2 with Ura^- segregants, were selected for analysis. Phenotypes of F-box gene deletion strains were determined by serial dilution on to rich medium (Adams *et al*. 1998) containing the following additions: 3% glycerol; 0.4 M NaCl; 6.7% v/v phosphate-citrate buffer (0.21 g ml^{-1} citric acid, 0.28 g ml^{-1} K_2HPO_4, adjusted to pH 4.5 with citric acid or KH_2PO_4); 1.67 μg ml^{-1} alpha factor; 150 mM hydroxyurea; 0.01% v/v methyl methane sulphonate; and 7.5 μg ml^{-1} nocodazole. FACS analysis of DNA content was performed as described (Tyers *et al*. 1993).

(c) *Protein and sequence analysis*

Immunoprecipitations and immunoblots were performed as described (Willems *et al*. 1996). Proteins eluted from protein A-IgG beads were separated by one-dimensional gel electrophoresis and visualized by silver staining. Protein bands, which were observed only in an immunoprecipitate from the tagged

Table 2. *Oligonucleotides used in this study*

oligo	name	sequence
MTO486	*YDR131c*-Z-N-fwd	GTCTTAAAATAGATAAAAATAACCCTACTGCC AATTTGAAAGGGCCCGAAAAATGAGGGAACA AAAGCTGG
MTO488	*YDR131c*-Z-C-rev	AGTATTTAGAGTATATATCATTATGTACGTGT TATAACCGCCATGTCTCACAGTACTATAGGGC GAATTGG
MTO489	*YJL149w*-Z-N-fwd	AATATCCAGGACGTCTATACACAGTGTTTACA ACTCAGCTTATATTCATATCATGAGGGAACAA AAGCTGG
MTO491	*YJL149w*-Z-C-rev	CTAGCTTAAAAAATGCGTTGAATATATATTAT TAAATATATATATTTGAAGGGGAGTTGACTAT AGGGCGAATTGG
MTO492	*YLR097c*-Z-N-fwd	CATCATCTTTGGCGACGACACATGTGTTCCAT AAGCTAAACTCAAGGAGCAAATGAGGGAACA AAAGCTGG
MTO494	*YLR097c*-Z-C-rev	GTTCTACATAAATCTATAAGAATAATAAATAA AGTAAAACAACTGCAAAAAACATCGATGCTAT AGGGCGAATTGG
MTO497	*YJL149w*-ORF-fwd	GGCGTCGACCCATGGCGCCATTTCAAGATTAT TTTCAG
MTO498	*YJL149w*-ORF-rev	CCGCTCGAGTCAGCGGCCGCTCATAACAGTGG CTATTC
MTO499	*YLR097c*-ORF-fwd	GGCGTCGACCCATGGCGATAGTAGATTATGAA AAGG
MTO500	*YLR097c*-ORF-rev	CCGCTCGAGTCAGCGGCCGCTTCCAGGATCCTT GAAAG

Table 3. *Yeast strains used in this study*

strain	background	relevant genotype[a]	source
K699	W303	*ade2-1 can1-100 his3-1,15 leu2-3,112 trp1-1 ura3*	K. Nasmyth
K699 **a**/α	W303	*MAT***a**/*MAT*α *ade2-1/ade2-1 can1-100/can1-100 his3-1,15/his3-1,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3/ura3*	K. Nasmyth
MTY251	W303	*cln2::CLN2-1*$^{HA}{}_{3}$*-LEU2*	(Willems *et al*. 1996)
MTY738	W303	*cln2::CLN2*$^{HA}{}_{3}$	(Willems *et al*. 1996)
MTY670	W303	*cdc34-2*	(Willems *et al*. 1996)
MTY1355	W303	*yjl149w::URA3*	this study
MTY1363	W303	*ydr131c::URA3*	this study
MTY1364	W303	*ydr131c::URA3 yjl149w::URA3*	this study
MTY1380	W303	*ylr097c::URA3*	this study
MTY1386	W303	*cln2::pGAL1-CLN2*$^{MYC}{}_{3}$*-LEU2*	this study
S288C	S288C	*ade2-101 his3-200 leu2-1 lys2-801 ura3-52 GAL +*	B. Andrews
YM3378	S288C	*ade2-101 his3-200 lys2-801 ura3-52 gal80 grr1-1829 rgt1-101*	M. Johnston

[a] All strains are *MAT***a** except as noted.

strain but not in the control, were excised from the gel and in-gel-digested with trypsin (Boehringer Mannheim, unmodified, sequencing grade) as described (Shevchenko *et al*. 1996). Tryptic peptides were extracted from the gel matrix by 5% formic acid and acetonitrile and combined extracts were dried down in a vacuum centrifuge. Unseparated pools of tryptic peptides were sequenced by nanoelectrospray tandem mass spectrometry as described (Wilm *et al*. 1996). Mass spectrometry was performed on a prototype hybrid quadrupole-time-of-flight mass spectrometer (MDS Sciex, Concord, Ontario) equipped with a nanoelectrospray ion source (Wilm & Mann 1996) developed at Protana (Odense, Denmark). Database searches were performed in a comprehensive sequence database using PeptideSearch v. 3.0 software developed in EMBL. No restrictions on species of origin or protein molecular weight were applied. Identification and alignment of putative yeast F-box proteins by database searches were as described (Patton *et al*. 1998*a*).

We thank numerous colleagues for many stimulating discussions and communication of their unpublished results. M.T. is supported by grants from the National Cancer Institute of Canada, the Medical Research Council of Canada and the Protein Engineering Network Centre of Excellence, and is a Research Scientist of the National Cancer Institute of Canada. A.R.W. was supported in part by an Ontario Graduate Studentship. We thank Dr B. Thomson (MDS Sciex) for his assistance in experiments performed on the QqTOF mass spectrometer.

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Note added in proof. A fourth SCF subunit, a RING finger protein of 16 kDa variously called Rbx1, Roc1 and Hrt1, has recently been identified by several laboratories (Kamura *et al.* 1999; Skowyra *et al.* 1999; Ohta *et al.* 1999; Tan *et al.* 1999; Seol *et al.* 1999).

Discussion

M. Hochstrasser (*University of Chicago, Illinois, USA*). What is the E2 enzyme for Grr1? I thought Mark Johnston's lab showed that with his substrate, Cdc34 was not the E2.

M. Tyers. I have simplified the story here considerably. Grr1 was originally identified in a genetic screen by Mark Johnston as being defective for growth on glucose. It turns out that Grr1 is needed to induce glucose transporters. Cdc34 is not required to induce these *HXT* genes. This led Mark to conclude there might be some other E2 involved. Of course, one needs to remember that all of the alleles in the SCF pathway were selected for being defective in Sic1 degradation. So, for example, the Cdc34-3 allele has no defect in Cln degradation but a very severe defect in Sic1 degradation.

P. D. Andrews (*University of Dundee, Scotland*). In *S. cerevisiae* there are four proteins with cullin homology. Do you know anything about the roles of the other two? Also, do you have any evidence that the leucine-rich repeat (LRR) of Grr1 contributes to substrate selection?

M. Tyers. Deletion of the other two cullin homologues, which is work from Mark Goebl's lab, has no obvious phenotype. The evidence that Grr1 participates in substrate recognition is that if you make a Grr1 complex in insect cells, it will recruit Clns specifically; there are no mutations in the LRR as yet.

K. A. Nasmyth (*Research Institute of Molecular Pathology, Vienna, Austria*). What is the phenotype of *met30* knockouts? Do they arrest in G1?

M. Tyers. Our Gal shut-off strains arrest at Start as unbudded cells with a 1C DNA content. Interestingly, this arrest cannot be bypassed by Cln overexpression.

A. Hershko (*Technion—Israel Institute for Technology, Haifa, Israel*). What is the common feature of the proteins that are recognized by Cdc4?

M. Tyers. It is difficult to pick out a consensus sequence. With the G1 cyclins, many cdk phosphorylation sites need to be knocked out to stabilize the protein. There also appear to be multiple phosphorylations involved in targetting Sic1 for degradation. However, even millimolar concentrations of a synthetic phosphopeptide from Sic1 will not outcompete the binding, so recognition may be more complex than just recognition of phosphorylation sites.