The Cdc42p effector Gic2p is targeted for ubiquitin-dependent degradation by the SCF^{Grr1} complex

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Cdc42p, a Rho-related GTP-binding protein, regulates cytoskeletal polarization and rearrangements in eukaryotic cells. In yeast, Gic1p and Gic2p are effectors of Cdc42p involved in actin polarization at bud emergence. Gic2p is expressed in a cell cycle-dependent manner and rapidly disappears shortly after bud emergence concomitant with the activation of the G₁ cyclin-dependent kinase Cdc28p–Clnp. Here we have shown that the rapid disappearance of Gic2p results from ubiquitin-dependent proteolysis. Biochemical and genetic evidence demonstrates that degradation of Gic2p required the Skp1–cullin–F-box protein complex (SCF) components Cdc34p, Cdc53p, Skp1p and Grr1p, but not Cdc4p. Phosphorylation of several C-terminal sites of Gic2p served as part of the recognition signal for ubiquitination. In addition, binding of Gic2p to Cdc42p was a prerequisite for degradation, suggesting that specifically the active form of Gic2p is targeted for destruction. Finally, our data indicate that degradation of Gic2p may be part of a mechanism which restricts cytoskeletal polarization in the G₁ phase of the cell cycle.

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Introduction

The actin cytoskeleton mediates many biological functions in all eukaryotic cells, such as maintenance of cell shape and polarity, cell motility, cytokinesis and phagocytosis (Hall, 1998). Members of the Rho family of small GTPases including Rho, Rac and Cdc42 have emerged as key regulators that control the organization and dynamics of the actin cytoskeleton and the assembly of focal adhesion contacts (Burridge *et al.*, 1997; Hall, 1998; Keely *et al.*, 1998). In addition, these GTPases play important roles in the establishment of cell polarity (Drubin and Nelson, 1996) and may be directly involved in axon guidance (Gallo and Letourneau, 1998). The biological effects of GTPases are mediated through their interaction with multiple target proteins, which ensure coordinated control of the actin cytoskeleton with other cellular activities such as gene transcription and adhesion.

In yeast, activation of Cdc42p by its exchange factor Cdc24p is required to organize the actin cytoskeleton towards the incipient bud site during vegetative growth and towards the pheromone-secreting partner during mating (Chant, 1996). Cytoskeletal polarization also requires Bem1p, a protein with two SH3 domains which is thought to function as an adaptor for Cdc42p and Cdc24p (Chenevert *et al.*, 1990; Leeuw *et al.*, 1995). In the absence of Cdc42p function, cells fail to grow in a polarized manner and instead increase in size isotropically (Adams *et al.*, 1990; Johnson and Pringle, 1990). Several effectors of Cdc42p recently have been identified, including the PAK kinases Ste20p, Cla4p and Smk1p (Cvrckova *et al.*, 1995; Peter *et al.*, 1996; Leberer *et al.*, 1997a; Martin *et al.*, 1997; Ebi *et al.*, 1998), the formins Bni1p and Bnr1p (Evangelista *et al.*, 1997; Imamura *et al.*, 1997), and Gic1p and Gic2p (Brown *et al.*, 1997; Chen *et al.*, 1997). None of these proteins alone can account for the effect of Cdc42p on polarized growth, suggesting that Cdc42p might affect the cytoskeleton by interacting with multiple targets.

The actin cytoskeleton is highly dynamic during all stages of the yeast life cycle and is regulated by both internal and external signals (Lew and Reed, 1995; Leberer *et al.*, 1997b). During mating, pheromones activate a signal transduction cascade leading to polarization and projection formation. In contrast, activation of the cyclin-dependent kinase (CDK) Cdc28p–Clnp during the G₁ phase of the cell cycle triggers polarization of the cytoskeleton resulting in bud emergence. In G₂, activation of the Cdc28p–Clbp kinase inactivates polarized growth, leading to uniform expansion of the bud. Finally, in mitosis, actin is redistributed to form a cleavage ring in the middle of the two separating cells. How these different cytoskeletal rearrangements are regulated in response to extracellular signals or by the cell cycle machinery is poorly understood. Regulation may alter the activity of the Cdc42p GTPase module and/or may be mediated through one or several downstream effectors.

Ubiquitin-dependent degradation of key components has emerged as an important mechanism controlling multiple cell cycle transitions. For example, anaphase is initiated by the destruction of Pds1p, an inhibitor of sister chromosome separation (Cohen-Fix *et al.*, 1996; Michaelis *et al.*, 1997). Degradation of the cyclin-dependent kinase inhibitor (CKI) Sic1p activates Cdc28p–Clbp kinase which in turn initiates DNA replication (Schwob *et al.*, 1994). At least two distinct ubiquitination systems trigger these events: Pds1p and mitotic cyclins are degraded by the anaphase-promoting complex (APC; King *et al.*, 1996), whereas Sic1p is ubiquitinated by a complex termed SCF (Skp1–cullin–F-box protein complex; Deshaies, 1997; Feldman *et al.*, 1997; Skowyra *et al.*, 1997; Patton *et al.*, 1998a). Homologues of several APC and SCF components

have been identified in a variety of systems and, like in yeast, they have been shown to function in ubiquitin-dependent degradation of proteins involved in cell cycle control and other biological processes (Krek, 1998). SCF complexes are ubiquitin ligases which interact with both a ubiquitin-conjugating enzyme and substrates (Hershko, 1997; Patton *et al.*, 1998a). Several substrates need to be phosphorylated for subsequent ubiquitination, and it is thought that phosphorylation of the substrate constitutes at least part of the recognition signal which mediates binding to the SCF complex (Willems *et al.*, 1996; Feldman *et al.*, 1997; Henchoz *et al.*, 1997; Skowrya *et al.*, 1997; Verma *et al.*, 1997).

SCF^{Cdc4} is composed of Cdc53p, Cdc34p, Skp1p and Cdc4p, and is necessary and sufficient for the ubiquitination of Sic1p (Feldman *et al.*, 1997; Skowrya *et al.*, 1997). It is also involved in the degradation of Far1p (Henchoz *et al.*, 1997), Cdc6p (Drury *et al.*, 1997), Ctf13p (Kaplan *et al.*, 1997) and possibly Gcn4p (Kornitzer *et al.*, 1994). *CDC53* encodes a cullin (Willems *et al.*, 1996; Mathias *et al.*, 1996) whereas Cdc34p shows homology to ubiquitin-conjugating enzymes (Goebel *et al.*, 1988). Cdc4p is a member of the growing family of proteins containing an F-box, a small sequence motif which has been shown to mediate the interaction with Skp1p (Bai *et al.*, 1996; Lisztwan *et al.*, 1998).

In addition to Cdc4p, yeast cells express several other F-box proteins including Grr1p and Met30p (Bai *et al.*, 1996; Patton *et al.*, 1998a). Cells lacking Grr1p exhibit severe morphological abnormalities and defects in glucose repression (Flick and Johnston, 1991), whereas cells deleted for *MET30* are unable to grow in the absence of methionine (Thomas *et al.*, 1995). Both Grr1p and Met30p are able to form complexes with Skp1p, Cdc34p and Cdc53p (Li and Johnston, 1997; Skowrya *et al.*, 1997; Patton *et al.*, 1998b). However, whereas Grr1p is required for the degradation of the G₁ cyclins Cln1p and Cln2p, it is dispensable for the degradation of Sic1p (Barral *et al.*, 1995; Patton *et al.*, 1998b), suggesting that the F-box proteins provide substrate specificity to the SCF complex.

To understand the function of individual F-box proteins, it is crucial to identify the substrates which they target for destruction. Here we show that Gic2p degradation at bud emergence is dependent on the SCF^{Grr1} complex, and is regulated by specific phosphorylation which requires active, GTP-bound Cdc42p. Cells overexpressing an undegradable Gic2 protein exhibit severe morphological defects in the G₁ phase of the cell cycle, suggesting that after the Gic proteins have polarized the actin cytoskeleton their subsequent destruction is needed for efficient bud emergence. Taken together, our results suggest that ubiquitin-dependent degradation of the Gic proteins coordinates polarization of the actin cytoskeleton in the G₁ phase of the yeast cell cycle.

Results

Cell cycle-dependent expression of Gic2p requires ubiquitin-dependent degradation by the SCF^{Grr1} complex

Gic2p is expressed in a cell cycle-dependent manner and accumulates throughout G₁, reaching maximal levels just before bud emergence (Brown *et al.*, 1997). Gic2p abruptly

disappears shortly after cells have polarized their cytoskeleton and is virtually undetectable during S and G₂ phases (Figure 1A; Brown *et al.*, 1997). The expression pattern of Gic2p is regulated in part at the level of transcription: expression of *GIC2* mRNA is maximal at bud emergence but is absent at later stages of the cell cycle (data not shown; R.Tabtiang, unpublished results). To test whether regulated transcription is responsible for the abrupt disappearance of Gic2p, we placed Gic2p under the control of the constitutive *CYC1* promoter. No obvious growth or morphological differences were apparent between Δ *gic2* cells expressing Gic2p from the constitutive *CYC1* promoter or from the endogenous promoter, suggesting that transcriptional regulation of *GIC2* was not essential for cell cycle progression and morphogenesis (data not shown). Moreover, both strains initiated bud formation ~30 min after release from an α -factor block (Figure 1B). However, even when *GIC2* was expressed constitutively, Gic2 protein levels still decreased shortly after bud emergence (Figure 1A, lower panel), demonstrating that post-transcriptional mechanism(s) downregulate Gic2 protein levels. In contrast, Gic2p was present later in the cell cycle only when expressed from constitutive promoters, suggesting that a transcriptional mechanism may regulate the levels of Gic2p during S and G₂ (Figure 1A).

To examine how Gic2p is down-regulated at bud emergence, we first determined Gic2p levels in cells lacking components involved in ubiquitin-dependent degradation (Figure 1C). Interestingly, we found that Gic2p levels were dramatically increased in *cdc34-2*, *cdc53-1* cells and cells deleted for *GRR1*. Moreover, a characteristic slower migrating Gic2p species became apparent in all of these mutants. Gic2p accumulated in *skp1* cells in an allele-dependent manner: Gic2p levels were high in *skp1-12* cells which arrest in G₂, but were low in cells harbouring a G₁-specific allele of *SKP1* (*skp1-11*). In contrast, low levels and no slower migrating forms of Gic2p were present in *cdc4-1* mutants which arrest at the same stage of the cell cycle as *cdc34-2* and *cdc53-1* cells (Figure 1C). Thus, we conclude that decreasing the levels of Gic2p at the time of bud emergence requires the SCF^{Grr1} but not the SCF^{Cdc4} complex. To exclude further the possibility that cell cycle arrest rather than a direct involvement of these proteins accounted for the accumulation of Gic2p, we compared Gic2p levels in *cdc53-1* and *cdc34-2* cells, which arrest in the G₁-phase of the cell cycle, and *cdc53-1* Δ *sic1* and *cdc34-2* Δ *sic1* strains which replicate their DNA and arrest in G₂ (Figure 1F; data not shown; Schwob *et al.*, 1994). Clearly, Gic2p accumulated in both strains (Figure 1E), demonstrating that Cdc53p and Cdc34p are directly involved in controlling Gic2p levels around bud emergence.

Finally, elevated levels of Gic2p were also apparent in *cim3*, *cim5* and *sen3* cells which arrest at the G₂-M boundary because of a defect in the 26S proteasome (Figure 1D; Ghislain *et al.*, 1993; Hilt and Wolf, 1995). We conclude from these results that Gic2p degradation requires a functional proteasome. Importantly, Gic2p accumulated in these proteasome mutants as a ladder of high molecular weight species distinct from the characteristic slower migrating form (Figure 1D). We believe that these high molecular species represent ubiquitinated forms

Fig. 1. Gic2p protein levels are regulated by ubiquitin-dependent degradation which requires components of the SCF^{Grr1} complex. (**A** and **B**) Δ *gic2* cells expressing Gic2p either from the endogenous (upper panel) or the constitutive *CYC1* promoter (lower panel) were released from α -factor arrest and Gic2p levels were determined by immunoblotting at the times indicated (minutes after washing out α -factor). The asterisk marks the position of a protein which cross-reacts with the Gic2p antiserum. Cell cycle entry was monitored microscopically by analysing the percentage of unbudded cells (**B**; $\circ$, endogenous promoter; $\blacksquare$, constitutive *CYC1* promoter). (**C**) Gic2p protein levels (upper panel) were determined by immunoblotting in strains harbouring defects in ubiquitin-dependent degradation. Lane 1, *skp1-11*; lane 2, *skp1-12*; lane 3, *cdc4-1*; lane 4, *cdc34-2*; lane 5, *cdc53-1*; lane 6, *cim3-1*; lane 7, Δ *grr1*; lane 8, wild-type. Immunoblotting against actin was used as a loading control (lower panel). The arrow marks the position of Gic2p; the arrowhead points to the slower migrating form of Gic2p which accumulates in some mutants. (**D**) Immunoblot demonstrating that Gic2p accumulates as a ladder of high molecular forms (bracket) in mutants defective in proteasome function. The following strains were analysed: lane 1, *cim3-1*; lane 2, *cim5-1*; lane 3, *sen3-1*; lane 4, wild-type; lane 5, Δ *gic2*. (**E** and **F**) Accumulation of Gic2p is independent of the cell cycle arrest point. Gic2p levels in *cdc4-1*, *cdc34-2* and *cdc53-1* mutants (lanes 3, 5 and 7) were compared with accumulation of Gic2p in *cdc4-1*, *cdc34-2* and *cdc53-1* mutants lacking *SIC1* (lanes 4, 6 and 8). FACS analysis confirming that *cdc53-1* mutant cells arrest with a G₁ DNA content (left panel) whereas *cdc53-1 sic1Δ* cells replicate their DNA and arrest in G₂ (right panel).

of Gic2p, although at present we are unable to demonstrate this directly.

To determine whether the SCF^{Grr1} complex was required to degrade Gic2p, we compared the stability of Gic2p in *cdc4-1*, Δ *grr1 rgt1* and *cdc34-2* mutants (Figure 2A). We used the Δ *grr1 rgt1* strain because the *rgt1* mutation restores efficient glucose repression in cells lacking Grr1p (Barral *et al.*, 1995). Gic2p was expressed from the *GAL1* promoter, at which time synthesis was turned off by addition of glucose. Interestingly, Gic2p was stable throughout the time course and accumulated in the slower migrating form in Δ *grr1 rgt1* and *cdc34-2* cells (half-life >60 min). In contrast, rapid degradation of Gic2p was observed in *cdc4-1* cells (half-life ~10 min), demonstrating that Grr1p and Cdc34p but not Cdc4p are required for Gic2p degradation. Consistent with these results, we found that overexpression of Gic2p is lethal in Δ *grr1 rgt1* or *cdc34-2* cells grown at the semi-permissive temperature, whereas *cdc4-1* cells overexpressing Gic2p grew normally (Figure 2B). Moreover, Gic2p tagged with the green fluorescent protein (GFP) was detected readily as a crescent at bud tips of *cdc34-2* cells arrested at 37°C (Figure 2C, bottom panel), whereas Gic2p-GFP was degraded rapidly and was virtually undetectable in *cdc4-1* cells (top panel). Taken together, these results strongly suggest that the stability of Gic2p is regulated by the SCF^{Grr1} complex.

Phosphorylation of Gic2p is required for ubiquitin-mediated degradation

Ubiquitin-dependent degradation of Sic1p, Far1p and the G₁ cyclins requires phosphorylation of the substrate (Lanker *et al.*, 1996; Schneider *et al.*, 1996; Feldman

et al., 1997; Henchoz *et al.*, 1997; Skowrya *et al.*, 1997; Verma *et al.*, 1997). As noted earlier, Gic2p accumulated as slower migrating forms in cells lacking any component of the SCF^{Grr1} complex. To determine whether these slower migrating forms represented phosphorylated Gic2p, we incubated Gic2p isolated from *cdc34-2* cells with alkaline phosphatase (calf intestine phosphatase; CIP). As shown in Figure 3A, the slower migrating forms of Gic2p were converted quantitatively to the faster migrating form (lane 4), demonstrating that Gic2p was indeed phosphorylated. No downshift was observed if CIP was inactivated by boiling (lane 5) or by the addition of phosphatase inhibitors (data not shown).

Interestingly, Gic2p accumulated in an unphosphorylated form in cells lacking a functional Cdc28p-Clnp kinase, suggesting that either the Cdc28p-Clnp kinase itself or a kinase dependent on Cdc28p-Clnp was responsible for Gic2p phosphorylation (Figure 3A, lane 1, and B). No accumulation of Gic2p was observed in strains lacking G₂ cyclins (Figure 3B, lanes 7 and 8) or in strains harbouring the *cdc28-1N* allele (which arrests cells in the G₂ phase of the cell cycle; Figure 3B, lane 5), indicating that Gic2p was phosphorylated efficiently. To corroborate these results further, we used a *cdc34* strain deleted for *CLN1*, *CLN2* and *CLN3* harbouring *CLN3* under the control of the inducible *GAL* promoter (Schneider *et al.*, 1996). As expected, Gic2p accumulated in the unphosphorylated form when these cells were arrested by the addition of glucose to turn off expression of Cln3p (Figure 3C, lane 2), even when Cdc34p was inactivated simultaneously by shifting the culture to the restrictive temperature. Induction of Cln3p by the addition of

Fig. 2. Gic2p is stabilized in *Δgrr1 rgt1* and *cdc34-2*, but not in *cdc4-1* mutant cells. (A) Cells carrying a plasmid coding for Gic2p from the inducible *GAL* promoter were grown in raffinose and expression was induced by the addition of galactose for 3 h (see Materials and methods). Glucose was then added to shut off the *GAL* promoter, and samples were taken every 10 min as indicated and immunoblotted for the presence of Gic2p. Equal loading was confirmed by immunoblotting the samples with antibodies against actin (lower panels). The following strains were analysed: top panel, *cdc4-1 Δgic2*; middle panel, *Δgrr1 rgt1 Δgic2*; bottom panel, *cdc34-2 Δgic2*. The *rgt1* mutation suppresses the glucose repression defect of *Δgrr1* strains. (B) Overexpression of wild-type Gic2p from the *GAL* promoter is lethal in *Δgrr1 rgt1* and *cdc34-2* cells at the semipermissive temperature (30°C), whereas *cdc4-1* cells tolerate overexpression of Gic2p. All strains grew normally when plated on medium containing glucose where expression of Gic2p was not induced (data not shown). (C) Overexpressed wild-type Gic2p fused to GFP is visualized readily at bud tips in arrested *cdc34-2* cells (bottom panel), but is rapidly degraded and virtually undetectable in *cdc4-1* cells (top panel). Photographs show GFP fluorescence superimposed with phase contrast.

galactose resulted in rapid phosphorylation of Gic2p (Figure 3C, lanes 3–8), consistent with the observation that the *G₁* cyclins are required for Gic2p phosphorylation. Subsequent repression of Cln3p by addition of glucose for 3 h led to dephosphorylation of Gic2p and its accumulation in the unphosphorylated form (Figure 3C, lanes 9–15), demonstrating that the continuous presence of the *G₁* cyclins was needed to keep Gic2p in the phosphorylated state. These findings enabled us to ask whether phosphorylation was required for degradation of Gic2p *in vivo* (Figure 3D). Cells grown in galactose medium at 25°C were arrested at the *cdc34* arrest point by shifting the culture to the restrictive temperature. The culture was then divided and glucose was added to one half to repress Cln3p expression. After 2 h, Cdc34p was activated by shifting the temperature to 25°C and degradation of Gic2p was monitored by immunoblotting. Interestingly, Gic2p was degraded rapidly when Cln3p was present, but Gic2p was stable when Clnp activity was depleted. Thus, these results suggest that Clnp-dependent phosphorylation of Gic2p is required for subsequent ubiquitin-dependent degradation.

To substantiate further the link between phosphorylation and degradation, we attempted to identify the phosphorylation sites on Gic2p. We found that a truncated Gic2p

lacking the C-terminal domain (Gic2p-N^{1–208}) was not fully phosphorylated in *cdc34-2* mutant cells (Figure 4A, lane 4). Importantly, half-life measurements of Gic2p-N^{1–208} in *cdc4-1* mutant cells confirmed that the protein was stable (half-life ~60 min; Figure 4B), suggesting that the critical phosphorylation sites involved in Gic2p degradation are located in the C-terminal domain of the protein. Sequence alignment of Gic1p and Gic2p revealed two closely spaced, conserved serine residues in this domain (serines 254 and 258 of Gic2p); both residues are followed by a proline, and hence conform to the minimal consensus site for both CDKs and MAP kinases (Nigg, 1995). We therefore mutated the two putative phosphorylation sites to non-phosphorylatable alanine residues and examined the phosphorylation state of the Gic2-S254A/S258A protein in *cdc34-2* mutant cells. As shown in Figure 4A, the fully phosphorylated form of Gic2p was absent, suggesting that S254 and/or S258 are phosphorylated *in vivo*. However, S254 and S258 are not the only phosphorylation sites, because mutant Gic2p still migrated more slowly than unphosphorylated Gic2p. Nevertheless, Gic2p-S254A/S258A was significantly stabilized in *cdc4-1* mutants with a half-life of 25 min compared with <10 min for wild-type Gic2p (Figure 4B). Consistent with these

Fig. 3. Cdc28p–Clnp-dependent phosphorylation of Gic2p is required for degradation. (A) Gic2p accumulates in a phosphorylated form in *cdc34-2* cells. Total cell extracts (T.E.) prepared from *cdc34-2* cells (lanes 2–5) were incubated with purified calf intestine phosphatase (CIP; lane 4), buffer (lane 3), or CIP which was inactivated prior to addition by boiling for 5 min (lane 5). Note that CIP quantitatively converts the slower migrating forms of Gic2p (arrowhead) to the unphosphorylated form present in *cdc28-4* cells (lane 1; arrow). (B) Phosphorylation and accumulation of Gic2p depends on the presence of Cdc28p–Clnp kinase. Gic2p levels were analysed by immunoblotting in cells lacking Cdc28p function (lanes 3–5) or various cyclins (lanes 6–8). The following strains were analysed: lane 1, wild-type (K699); lane 2, *cdc34-2* (YMT670); lane 3, *cdc28-1* (MJ254); lane 4, *cdc28-4* (YMP599); lane 5, *cdc28-1N* (YML50), lane 6, $\Delta\Delta\Delta\text{cln1},2,3$ (EY569); lane 7, $\Delta\Delta\text{clb5},6$ (MJ202), lane 8, $\Delta\Delta\Delta\Delta\Delta\text{clb1},2,3,4,5,6$ (MJ207). The arrowhead marks the position of phosphorylated Gic2p; the arrow points towards the position of unphosphorylated Gic2p. Equal loading of the lanes was confirmed by immunoblotting actin (lower panel). (C) MJ270 were arrested at 25°C by depletion of the G₁ cyclins *CLN1*, 2 and 3, and released by addition of galactose to induce expression of Cln3p. The culture simultaneously was shifted to 37°C to inactivate Cdc34p. Samples were collected at the times indicated and immunoblotted with antibodies against Gic2p (lanes 2–8). After 90 min at 37°C, glucose was added to half of the culture to repress Cln3p expression. Aliquots were removed at the times indicated (0' represents addition of glucose) and analysed by immunoblotting with Gic2p antibodies (lanes 9–15). Note that maintenance of Gic2p phosphorylation depends on the presence of Cln3p. (D) Degradation of Gic2p depends on the presence of Clnp. Cells were arrested as in (C) and released from the *cdc34-2* block by shifting the temperature to 25°C either in the presence (Cln3p expressed, upper panel) or absence (Cln3p repressed, lower panel) of galactose. Samples were removed at the times indicated and immunoblotted with Gic2p antibodies. Time 0 indicates release of the culture from the *cdc34-2* block. The immunoblots were quantified by phosphoimager and plotted against time in minutes after release. ■, +galactose (Cln3p expressed); ♦, –galactose (Cln3p repressed).

tin-conjugated forms of Gic2p (Figure 1D). Interestingly, no slower migrating forms of Gic2p-N¹⁻²⁰⁸ could be detected after expression in *cim3* cells (Figure 4D, lane 4) and, similarly, ubiquitination of Gic2p-S254A/S258A was significantly reduced (lane 2). Thus, the defects of these Gic2 mutant proteins correlated with their defects in phosphorylation and degradation. We conclude from these results that phosphorylation of Gic2p is required for ubiquitination *in vivo*.

Phosphorylation and degradation of Gic2p depends on its ability to bind Cdc42p

Previous studies have suggested that the Gic proteins function in a complex with Cdc42p in its active, GTP-bound form (Brown *et al.*, 1997; Chen *et al.*, 1997). To determine whether Cdc42p is involved in the degradation of Gic2p, we measured Gic2p levels in mutants lacking Cdc42p. Interestingly, we found that Gic2p accumulated

Fig. 4. Phosphorylation of C-terminal phosphorylation sites triggers ubiquitination of Gic2p *in vivo*. (A) Phosphorylation of wild-type and mutant forms of Gic2p expressed in *cdc34-2* (lanes 1–4) or for control *cdc4-1* cells (lane 5) was analysed by Western blotting with Gic2-specific antibodies. The following Gic2p mutants were analysed: lane 1, wild-type; lane 2, Gic2p-S254A/S258A; lane 3, Gic2p-crib⁻; lanes 4 and 5, Gic2p-N¹⁻²⁰⁸. (B) The half-life of wild-type and Gic2p mutant proteins was analysed in *cdc4-1* cells as described in the legend to Figure 2 and quantified by phosphorimager. ■, Gic2p-N¹⁻²⁰⁸; ▲, Gic2p-S254A/S258A; ◆, wild-type. (C) Overexpression of either phosphorylation mutant of Gic2p (S254A/S258A or N¹⁻²⁰⁸) from the *GAL* promoter is lethal in *cdc4-1* cells incubated at 30°C. In contrast, cells harbouring a control plasmid (vect.) or overexpressing wild-type Gic2p (WT) grew normally. All strains grew normally when plated on medium containing glucose (data not shown). (D) Phosphorylation of Gic2p is required for ubiquitination. *Cim3-1 Δgic2* cells (MJ416) were transformed with plasmids expressing various Gic2p mutant proteins and grown at 37°C for 3 h. Extracts were prepared after 3 h and immunoblotted with polyclonal Gic2p antibodies. The following Gic2p proteins were analysed: lane 1, wild-type; lane 2, Gic2p-S254A/S258A; lane 3, Gic2p-crib⁻; lane 4, Gic2p-N¹⁻²⁰⁸; lane 5, vector. Note that only wild-type Gic2p accumulates as a characteristic ladder of high molecular forms indicative of ubiquitination (bracket).

in *cdc42-1* cells (Figure 5A, lane 3), indicating that Cdc42p is necessary for degradation. In contrast, low levels of Gic2p were detected in cells lacking Bem1p (Figure 5A, lane 1) or the Rho1p GTPase (Figure 5A, lane 4). Increased levels of Gic2p were also observed in *cdc24-1* mutant cells (Figure 5A, lane 2), suggesting that specifically the GTP-bound form of Cdc42p is involved in the degradation of Gic2p. Gic2p accumulated in an unphosphorylated form in both *cdc42* and *cdc24* mutants, indicating that Cdc42p-GTP is required for phosphorylation of Gic2p. Taken together, these results indicate that the GTP-bound form of Cdc42p is required for phosphorylation and degradation of Gic2p.

The Gic proteins interact with Cdc42p-GTP through their N-terminal CRIB domain (Cdc42/Rac interactive binding domain; Brown *et al.*, 1997; Chen *et al.*, 1997). Strikingly, we found that a mutant Gic2p which is not able to interact with Cdc42p due to mutations in the CRIB domain (Gic2p-crib⁻, Brown *et al.*, 1997) was not phosphorylated in *cdc34-2* cells (Figure 4A, lane 3), demonstrating that phosphorylation of Gic2p requires the interaction with Cdc42p. Furthermore, Gic2p-crib⁻ was no longer ubiquitinated *in vivo* (Figure 4D, lane 3) and, as a consequence, Gic2p-crib⁻ protein was stable in *cdc4-1* mutants with a half-life of ~50 min (Figure 5B). However,

in contrast to other stabilized forms of Gic2p (see above), overexpression of Gic2p-crib⁻ did not cause lethality when expressed in *cdc4-1* cells (Figure 5C), consistent with the previous observations that binding of Cdc42p is required for the function of the Gic proteins (Brown *et al.*, 1997; Chen *et al.*, 1997). Taken together, we conclude that binding of Cdc42p is required for phosphorylation and subsequent destruction of Gic2p and, importantly, these results suggest that only active Gic2p complexed with Cdc42p-GTP is targeted for ubiquitin-dependent degradation.

Cdc28p–Clnp kinase may be required indirectly for Gic2p phosphorylation by promoting formation of Cdc42p-GTP

The results presented so far demonstrate that phosphorylation and degradation of Gic2p are dependent on the Cdc28p–Clnp kinase and binding to Cdc42p-GTP. It is possible, therefore, that the Cdc28p–Clnp kinase may not phosphorylate Gic2p directly but instead be involved in the formation of Cdc42p-GTP. Supporting the latter view, we were unable to phosphorylate Gic2p *in vitro* using Cdc28p–Clnp kinase purified from baculovirus-infected cells (data not shown). To distinguish further between the two possibilities, we tested whether Cdc42p-GTP is

Fig. 5. Binding of Cdc42p is required for phosphorylation and degradation of Gic2p. (A) Gic2p accumulates in an unphosphorylated form in *cdc42-1* and *cdc24-1* mutants. Levels and phosphorylation state of Gic2p were determined by immunoblotting of total cell extracts prepared from cells grown at 37°C for 3 h. The following strains were analysed: lane 1, *Δbem1* (YML295); lane 2, *cdc24-1* (MJ393); lane 3, *cdc42-1* (MJ391); lane 4, *rho1-104* (YMP276); lane 5, wild-type (YMP277). (B) The half-life of a Gic2p mutant unable to interact with Cdc42p (Gic2p-crib⁻; upper panel) and wild-type Gic2p (lower panel) was determined in *cdc4-1 Δgic2* cells (MJ261) as described in the legend to Figure 2. Western blots against actin were used as a loading control (bottom panels). (C) In contrast to Gic2p-N¹⁻²⁰⁸, *cdc4-1* cells overexpressing Gic2p-crib⁻ from the *GAL* promoter at 30°C were viable, confirming that binding of Cdc42p to Gic2p is required for its function (Brown *et al.*, 1997).

sufficient to trigger degradation of Gic2p in the absence of the G₁ cyclins (Figure 6A) or functional Cdc28p kinase (Figure 6B). We therefore arrested a strain deleted for the three G₁ cyclins *CLN1*, *CLN2* and *CLN3* expressing Cln2p from the repressible *MET25* promoter at START by the addition of methionine (Figure 6A; Rönicke *et al.*, 1997). The strain also harbours Cdc42p under the control of the inducible *GAL* promoter; thus expression of Cdc42p was induced by the addition of galactose, and subsequent phosphorylation and degradation of Gic2p was monitored by immunoblotting. Strikingly, Gic2p was phosphorylated rapidly and degraded if Cdc42p was expressed in its GTP-bound active form (Figure 6A, lane 1) whereas no phosphorylation and degradation of Gic2p was observed after expression of the GDP-bound form of Cdc42p (Figure 6A, lane 3). Similar results were obtained when *cdc28-13* cells were arrested at the non-permissive temperature for 3 h (Figure 6B). Thus, these results suggest that Cdc28p–Clnp kinase is not directly phosphorylating Gic2p *in vivo*, but instead that Cdc28p–Clnp kinase may be involved in the formation of active Cdc42p-GTP.

Degradation of Gic2p contributes to morphogenesis and correct bud emergence

To determine the role of degradation of Gic2p, we first tested whether degradation of Gic2p is required for viability. Cells lacking *GIC1* and *GIC2* were transformed with plasmids expressing stabilized forms of Gic2p from the endogenous promoter. As shown in Figure 7C, both Gic2p-S254A/S258A and Gic2p-N¹⁻²⁰⁸ were able to support growth at the restrictive temperature (37°C), demonstrating that degradation of Gic2p is not essential for cell cycle progression. In contrast, expression of stabilized forms of Gic2p from the *GAL* promoter in wild-type cells induced lethality (Figure 7A and B; see also Figure 4C), suggesting that transcriptional control of *GIC2* may contribute to the regulation of Gic2p levels. Importantly, cells expressing stable forms of Gic2p exhibited several morphological abnormalities. First, more than half of the cells appeared

Fig. 6. Expression of Cdc42p-GTP is sufficient to induce phosphorylation and degradation of Gic2p in the absence of Cdc28p–Clnp function. Cells were arrested at START either by depletion of the G₁ cyclins [EY569; (A)] or by use of the *cdc28-13* allele [MJ255; (B)], at which time expression of either Cdc42p-GTP [(A and B), lanes 1 and 2] or Cdc42p-GDP [(A), lanes 3 and 4] was induced by the addition of galactose ('+' sign) for 3 h. For control, cells were either transformed with empty vectors [(A), lanes 5 and 6; (B), lanes 3 and 4] or expression of the Cdc42p forms was not induced (no addition of galactose; '-' signs). Gic2p levels were determined by immunoblotting (upper panel); immunoblots against actin were used as an internal loading control (lower panel). The arrowhead marks the position of phosphorylated Gic2p; the arrow points towards unphosphorylated Gic2p. Note that Cdc42p-GTP induces phosphorylation and degradation of Gic2p.

large and round, with an actin cytoskeleton either unpolarized or polarized towards multiple sites, indicating that these cells have a problem during bud emergence (Figure 7A and B). Secondly, some cells (2%) accumulated with multiple buds; these buds are similar in size and originate from the mother cell, suggesting that they were formed simultaneously (Figure 7A, insert). Elutriation of cells expressing a stable form of Gic2p confirmed that morphological abnormalities appeared at bud emergence in G₁, whereas DNA replication occurred on schedule (data not shown). Taken together, these results indicate that while degradation of Gic2p is not essential for viability, it is involved in proper morphogenesis during bud emergence

Fig. 7. Degradation of Gic2p is not essential for viability but contributes to proper morphology and organization of the actin cytoskeleton. (A and B) Wild-type cells harbouring a plasmid expressing non-degradable Gic2p-N¹⁻²⁰⁸ (A, upper panels) from the inducible *GAL* promoter or an empty plasmid (A, lower panels) were grown at 30°C in raffinose medium to log phase, at which time expression was induced by the addition of galactose to 2% final concentration. After 4 h, cells were sonicated, fixed with 3.7% formaldehyde and analysed microscopically. The actin cytoskeleton was visualized after staining with rhodamine-phalloidin (left panels). Representative examples of accumulated large unpolarized and multibudded cells (insert) are shown in (A). Enlarged cells with an unpolarized actin cytoskeleton were defined as abnormal. At least 300 cells were counted for the quantitation shown in (B). Similar results were also obtained with cells overexpressing Gic2p-S254A/S258A (data not shown). (C) MJ398 cells transformed with either an empty plasmid (vector) or plasmids expressing wild-type Gic2p (GIC2 WT), Gic2p-S254A/S258A (S254A S258A) or Gic2p-N¹⁻²⁰⁸ (N 1-208) from the endogenous promoter were grown for 3 days at 37°C. Note that stable Gic2p mutant proteins were able to complement the growth defect of Δ *gic1* Δ *gic2* cells when expressed from the endogenous promoter, suggesting that degradation is not essential for viability.

and may prevent polarization of the actin cytoskeleton towards multiple sites.

Discussion

Gic2p is targeted specifically for degradation by the SCF^{Grr1} complex

Our results identify Gic2p, a protein involved in cytoskeletal organization and morphogenesis, as a novel target of the SCF^{Grr1} complex. Degradation of Gic2p was mediated by the proteasome and required the components Sen3p, Cim3p and Cim5p (Hilt and Wolf, 1995). Interestingly, Gic2p accumulated as a ladder of higher molecular

weight forms in proteasome mutants, which most likely represent ubiquitinated forms of Gic2p.

Several lines of evidence suggest that phosphorylation of Gic2p is required for ubiquitin-dependent degradation. First, Gic2p accumulated in a phosphorylated form in mutants defective in components of the SCF^{Grr1}. Secondly, phosphorylation and degradation of Gic2p required active Cdc28p-Clnp kinase and, finally, mutations which abolished phosphorylation of Gic2p not only stabilized Gic2p but also prevented ubiquitination of Gic2p. Taken together, these results suggest that phosphorylation of Gic2p is necessary for ubiquitination *in vivo*, further supporting the view that phosphorylation of SCF substrates

Fig. 8. Model for the degradation of Gic2p and its role in actin polarization. **(A)** Model for the degradation of Gic2p. Gic2p is first phosphorylated in a Cdc42p-dependent manner; phosphorylated Gic2p is then recognized by the SCF^{Grr1} complex containing Grr1p, Cdc34p, Cdc53p and Skp1p, which in turn ubiquitinates Gic2p. Ubiquitinated Gic2p is degraded by the proteasome. **(B)** Cdc42p plays a dual role during bud emergence. Activation of the Cdc28p–Clnp kinase triggers polarization of the actin cytoskeleton at START, possibly by activating the exchange factor Cdc24p. Cdc42p–GTP then binds the Gic proteins which in turn organize the actin cytoskeleton (Brown *et al.*, 1997; Chen *et al.*, 1997). Cdc42p–GTP is also required for phosphorylation of Gic2p, which triggers its subsequent inactivation by ubiquitin-dependent degradation. Destruction of Gic2p after polarization of the actin cytoskeleton may restrict cytoskeletal polarization and thereby contribute to efficient bud emergence. Arrows indicate activation; blunt arrows indicate inhibition.

signals their destruction (Figure 8A). Because binding of Sic1p and Cln2p to the SCF complex is regulated by phosphorylation (Feldman *et al.*, 1997; Skowyra *et al.*, 1997) it is likely that phosphorylation of Gic2p similarly promotes the interaction between Gic2p and SCF^{Grr1} complexes. However, we have not yet demonstrated direct binding of Gic2p to the SCF^{Grr1} complex and, therefore, cannot exclude that loss of Grr1p may cause stabilization of Gic2p indirectly *in vivo*. Phosphorylated Sic1p not only binds to reconstituted SCF^{Cdc4} complexes *in vitro*, but is also ubiquitinated efficiently (Feldman *et al.*, 1997; Skowyra *et al.*, 1997). In contrast, reconstituted SCF^{Grr1} containing Cdc53p, Cdc4p, Cdc34p, Skp1p and Grr1p is unable to ubiquitinate Cln2p, although Cln2p binds in a phosphorylation-dependent manner (Skowyra *et al.*, 1997). These results suggest that the SCF^{Grr1} complex may require post-translational modifications or that the complex lacks an additional component required for ubiquitination of the bound substrate. Taken together, our results suggest the following model for the degradation of Gic2p (Figure 8A): first, Gic2p is phosphorylated; phosphorylated Gic2p then binds to the SCF^{Grr1} complex, which in turn ubiquitinates Gic2p. Finally, ubiquitinated Gic2p is recognized and degraded by the proteasome (Peters, 1994).

Degradation of Gic2p required Cdc34p, Cdc53p and

Skp1p, components which have been shown to interact with several F-box proteins including Grr1p, Cdc4p and Met30p and are therefore thought to be common components functioning in multiple SCF complexes (Patton *et al.*, 1998b). Interestingly, degradation of Gic2p was dependent on Grr1p but not Cdc4p, whereas, conversely, degradation of Sic1p, Far1p and Cdc6p requires Cdc4p but not Grr1p, supporting the view that F-box proteins provide substrate specificity to the SCF complexes (Patton *et al.*, 1998a). SCF^{Grr1} complexes are also involved in degradation of the G₁ cyclins Cln1p and Cln2p (Bai *et al.*, 1996) and an unknown substrate necessary for glucose repression (Li and Johnston, 1997). Specific alleles of Skp1p are also able to distinguish between different substrates: *skp1-12* cells were unable to degrade Gic2p, but efficiently degrade Sic1p and Far1p (Bai *et al.*, 1996; Henchoz *et al.*, 1997). Conversely, *skp1-11* cells fail to degrade Sic1p and Far1p, while degradation of Gic2p was unaffected. It is possible, therefore, that the Skp1-11 protein is able to assemble SCF^{Grr1} complexes, but is unable to form SCF^{Cdc4} complexes.

Although we have not tested whether Gic1p is degraded by a similar mechanism, we consider it likely given the high degree of sequence conservation and functional overlap between Gic1p and Gic2p (Brown *et al.*, 1997; Chen *et al.*, 1997).

Degradation of Gic2p requires Cdc42p-GTP

Several lines of evidence demonstrate that degradation of Gic2p is dependent on the presence of active, GTP-bound Cdc42p. First, analysis of synchronized cultures showed that Gic2p was degraded shortly after bud emergence, concomitant with the activation of Cdc28p–Clnp kinase and the formation of Cdc42p–GTP (Figure 1A and B; Brown *et al.*, 1997). Secondly, degradation of Gic2p was abolished in strains lacking Cdc42p or Cdc24p function, and thirdly, a mutant Gic2p unable to bind Cdc42p was stabilized *in vivo*. What is the role of Cdc42p in degradation of Gic2p? Our results demonstrate that Cdc42p is required for the first step in the pathway leading to ubiquitin-dependent degradation (Figure 8A): Gic2p isolated from cells lacking functional Cdc42p–GTP or a mutant Gic2p protein unable to bind Cdc42p failed to be phosphorylated *in vivo*. Consistent with the notion that phosphorylation targets Gic2p to the SCF^{Grr1} complex, Gic2p was no longer ubiquitinated and remained stable. Although phosphorylation of Gic2p was dependent on active Cdc28p–Clnp kinase *in vivo*, phosphorylation and degradation of Gic2p could be triggered in the absence of G₁ cyclins following expression of Cdc42p–GTP. These results suggest that the Gic2p kinase may not be Cdc28p–Clnp itself, but rather that Cdc28p–Clnp kinase activity may be required to produce Cdc42p–GTP. Consistent with these results, we found recently that expression of Cdc42p in its GTP form is able to bypass the requirement for the Cdc28p–Clnp kinase with respect to cytoskeletal polarization (M.-P.Gulli and M.Peter, unpublished results). Because Cdc42p is necessary to localize Gic2p efficiently to the presumptive bud site (Brown *et al.*, 1997; M.Jaquenoud and M.Peter, unpublished results), phosphorylation of Gic2p may be dependent on its correct localization. Thus, the Gic2p protein kinase may be localized at the presumptive bud site. Alternatively, the

Gic2p kinase might only recognize Gic2p as a substrate when it is bound to Cdc42p. The phosphorylation site mutant of Gic2p suggests that the Gic2p kinase may be a member of the proline-directed kinase family, which includes the CDK Pho85p-Pclp and the MAP kinase Mpk1p. Mpk1p relocates from the nucleus to the cytoplasm and has been found at the presumptive bud site (Kamada *et al.*, 1995); however, in cells lacking either Mpk1p or Pho85p, Gic2p was still phosphorylated and degraded efficiently (M.Jaquenoud and M.Peter, unpublished results). It is also unlikely that members of the PAK-like family are responsible for phosphorylation of Gic2p. First, the half-life of Gic2p is not altered in *ste20Δ*, *cla4Δ* or *smk1Δ* mutants and Gic2p is only slightly stabilized in a temperature-sensitive *cla4-104 ste20Δ* strain (data not shown). Secondly, Gic2p was still phosphorylated in *cdc34-2* cells even when Ste20p and Cla4p were depleted (M.Jaquenoud and M.Peter, unpublished results). Thus, further experiments will be required to determine the identity of the kinase responsible for targeting Gic2p for degradation.

A dual role for Cdc42p at bud emergence

Our results suggest that Cdc42p may play a dual role in the regulation of bud emergence (Figure 8B). On one hand, Cdc42p binds and activates the Gic proteins, which in turn polarize the actin cytoskeleton towards the incipient bud site. On the other hand, Gic2p bound to Cdc42p is phosphorylated rapidly and targeted for degradation by the SCF^{Grr1} ubiquitin system. Thus, activation of Gic2p by Cdc42p is intimately coupled to its destruction. As a consequence, the function of Gic2p appears to be limited to a short window during G₁ when the actin cytoskeleton needs to be polarized. This coordinated mechanism ensures that Gic2p cannot be degraded before Cdc42p has been converted to the GTP-bound form. Because Cdc42p is required for both function and degradation of Gic2p, these findings strongly suggest that specifically the active pool of Gic2p is targeted for degradation. It is thus unlikely that ubiquitin-dependent degradation eliminates predominantly unassembled Gic2p which may be produced in excess to ensure that activated Cdc42p is quantitatively bound to Gic2p. Such a model has been proposed to safeguard the assembly of kinetochore complexes thereby ensuring that every chromosome is attached properly to microtubules during cell division (Kaplan *et al.*, 1997).

Because phosphorylation and degradation of Gic2p are dependent on the presence of Cdc42p-GTP, phosphorylation and degradation of Gic2p may provide a useful *in vivo* marker to detect Cdc42p-GTP. For example, Bem1p plays an important role in polarizing the actin cytoskeleton in G₁ and directly interacts with Cdc42p and Cdc24p, but it is unclear whether Bem1p is required to produce Cdc42p-GTP *in vivo*. However, our finding that Gic2p was phosphorylated efficiently and degraded in *bem1Δ* cells now indicates that Bem1p may not function to produce Cdc42p-GTP, thus favouring a model where Bem1p may be involved in properly localizing the polarity establishment proteins.

Degradation of Gic2p may prevent multiple rounds of actin polarization

Why do cells degrade Gic2p shortly after it has performed its function? Our data indicate that cells expressing stable

Gic2p from their endogenous promoter were viable but exhibit morphological abnormalities which resulted from a failure to polarize the actin cytoskeleton properly. Cells overexpressing stable Gic2p were inviable and arrested predominantly as large unbudded cells or with two or more buds. The actin cytoskeleton of these arrested cells was either unpolarized, polarized towards a broad area or polarized towards multiple sites. Thus, cells unable to efficiently degrade the Gic proteins exhibit morphological abnormalities most likely due to defects in actin polarization. It is important to note that loss of Gic function and overproduction of a stable form of Gic2p produce a superficially similar phenotype. However, this terminal phenotype may be achieved in a very different manner: loss of the Gic proteins appears to result in a failure to polarize (Brown *et al.*, 1997; Chen *et al.*, 1997), whereas cells unable to degrade Gic2p appear to polarize their cytoskeleton towards multiple sites thereby leading to uniform surface growth. It is difficult at present to distinguish between these two opposing possibilities, but the appearance of irregularly shaped and multibudded cells supports our interpretation.

Elimination of the Gic proteins after polarization may ensure alternation of different Cdc42p complexes: in addition to the Gic proteins, Cdc42p also interacts with the three PAK-like kinases Ste20p, Cla4p and Smk1p (Peter *et al.*, 1996; Leberer *et al.*, 1997a; Martin *et al.*, 1997), and loss of these kinases causes defects in actin polarization (Ebi *et al.*, 1998). These Cdc42p effectors share a common binding motif termed the CRIB domain (Burbelo *et al.*, 1995), which is required for proper localization and function of these proteins *in vivo* (Peter *et al.*, 1996; Brown *et al.*, 1997; Chen *et al.*, 1997; Leberer *et al.*, 1997a). It is not known whether Cdc42p simultaneously binds the different effectors or whether distinct complexes are assembled sequentially. In the latter case, degradation of Gic2p might liberate Cdc42p to interact with other effectors such as the PAK-like kinases, which in turn prepare the polarized cell for later events such as cytokinesis.

Ubiquitin-dependent degradation of regulators may be a common strategy to ensure the correct order of events and to prevent cell cycle transitions being repeated multiple times. For example, degradation of Sic1p allows activation of the Clb kinases, thereby ensuring correct alternation between S- and M-phases (Schwob *et al.*, 1994; Dahmann *et al.*, 1995). Likewise, Cdc20p, an essential activator of the APC, is itself a substrate of the APC, thereby preventing inappropriate reactivation of the APC later in the cell cycle (Prinz *et al.*, 1998; Shirayama *et al.*, 1998). Thus, the identification of the Gic proteins as substrates of the SCF^{Grr1} complex suggests that ubiquitin-dependent degradation may similarly regulate morphological changes in a spatially and temporally restricted manner.

Grr1p and morphogenesis

Cells lacking Grr1p exhibit severe morphological defects and accumulate as large, elongated cells with a G₂ DNA content (Flick and Johnston, 1991; Barral *et al.*, 1995). Similar to cells expressing stable Gic2p, *Δgrr1* cells fail to restrict actin polarization towards a defined site and instead polarize towards a broad area, suggesting that the Gic proteins may be important targets of Grr1p involved

Table I. Strains list

Strain name	Relevant genotype	Background	Source
YMT670	<i>cdc34-2</i>	W303	C.Mann
ES464	<i>cdc34-2 sic1::HIS3</i>	W303	E.Schwob
YMT668	<i>cdc4-1</i>	W303	M.Tyers
ES205	<i>cdc4-1 sic1::HIS3</i>	W303	E.Schwob
YMT740	<i>cdc53-1</i>	W303	M.Tyers
MJ225	<i>cdc53-1 sic1::URA3</i>	W303	this study
Y552	<i>skp1-11</i>	W303	S.Ellege
Y554	<i>skp1-12</i>	W303	S.Ellege
YMP273	<i>cim3-1</i>	S288C	C.Mann
MJ416	<i>cim3-1 gic2::LEU2</i>	S288C	this study
YMP241	<i>cim5-1</i>	S288C	C.Mann
YM2957	<i>grr1::LEU2</i>	S288C	C.Mann
MJ263	<i>grr1::LEU2 gic2::hisG URA3</i>	S288C	this study
MJ511	<i>grr1::LEU2 rgt1-101 gic2::hisG URA3</i>	S288C	this study
MJ217	<i>sen3::TRP1</i>	S288C	M.Hochstrasser
YMP599	<i>cdc28-4</i>	A649a	J.Li
YML50	<i>cdc28-1N</i>	A649a	J.Li
MJ255	<i>cdc28-13</i>	A649a	J.Li
EY569	<i>cln1::HisG cln2Δ cln3::LEU2 METCLN2</i>	W303	E.Schwob
MJ202	<i>clb5::URA3 clb6::LEU2</i>	W303	E.Schwob
MJ207	<i>clb1 clb2ts clb3::TRP1 clb4::HIS3 clb5:: GALCLB5 clb6::LEU2</i>	W303	E.Schwob
MJ270	<i>cdc34-2 cln1::HIS3 cln2::TRP1 cln3::GALCLN3</i>	W303	A.B.Futcher
MJ398	<i>gic1::URA3 gic2::HisGURA3</i>	W303	this study
MJ83	<i>gic2::LEU2</i>	W303	this study
MJ393	<i>cdc24-1</i>	W303	I.Herskowitz
MJ391	<i>cdc42-1</i>	W303	I.Herskowitz
YMP276	<i>bem1::LEU2</i>	W303	I.Herskowitz
YMP276	<i>rho1-104</i>	S288C	Y.Takai

in morphogenesis. Grr1p is also required for the degradation of the G₁ cyclins Cln1p and Cln2p (Barral *et al.*, 1995; Patton *et al.*, 1998b), and overproduction of either Cln1p or Cln2p results in hyperpolarized growth (Lew and Reed, 1993), possibly by triggering constitutive production of Cdc42p-GTP (M.-P.Gulli and M.Peter, unpublished results). Therefore, loss of Grr1p may lead to morphological abnormalities by both increasing production of Cdc42p-GTP and stabilization of its effectors Gic1p and Gic2p. The finding that Gic2p is stabilized in *grr1Δ* mutants may also explain why loss of *GRR1* was found as an extragenic suppressor of cells lacking Bem2p (Kim *et al.*, 1994). *BEM2* encodes a protein with a GTPase-activating domain and is involved in bud emergence (Bender and Pringle, 1991). Overexpression of the Gic proteins is able to restore viability of *bem2Δ* cells (Brown *et al.*, 1997; Chen *et al.*, 1997). As shown here, loss of Grr1p function dramatically increases Gic2 protein levels which, as a result, might restore viability of cells lacking Bem2p.

Homologues of F-box proteins have been identified in a wide variety of systems including human (Bai *et al.*, 1996; Patton *et al.*, 1998a). As in yeast, these proteins appear to assemble into multiprotein complexes (Lisztwan *et al.*, 1998), which function in ubiquitin-dependent degradation during diverse biological processes. Potential homologues of Grr1p include human Skp2 (Zhang *et al.*, 1995), an open reading frame from *Caenorhabditis elegans* (CO2F5.7), and a recently identified protein from *Arabidopsis*, which functions in the response to auxin (Ruegger *et al.*, 1998). It will be interesting to determine whether these Grr1p homologues are also involved in morphogenesis, possibly by degrading substrates such as the Gic proteins.

Materials and methods

Yeast strains and genetic experiments

Yeast strains are described in Table I. The genotypes of the yeast strains are: W303, *ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL+, psi+, ssd1-d2*; S288C, *ade2-101, ura3-52, lys2-801, trp1-Δ1, his3Δ200, leu2-Δ*; and A364a, *trp1-289, leu2-3,112, his3-11,15, ura3-52, GAL+*, unless noted otherwise. Standard yeast growth conditions and genetic manipulations were used as described (Guthrie and Fink, 1991). Yeast transformations were performed by the lithium acetate procedure (Ito *et al.*, 1983). Strains deleted for *GIC2* marked with *URA3* were constructed using plasmid pMJ43 digested with *HindIII* and *NotI*. Pheromone response and mating assays were performed as previously described (Valtz and Peter, 1997).

DNA manipulations

Plasmids and oligonucleotides are described in Table II. Standard procedures were used for recombinant DNA manipulations (Sambrook *et al.*, 1989; Ausubel *et al.*, 1991). PCRs were performed with the Expand polymerase kit as recommended by the manufacturer (Boehringer Mannheim). Oligonucleotides were synthesized by Genset (France). Site-directed mutagenesis was performed with the oligonucleotide oTP440 using the method developed by Kunkel *et al.* (1987). The correct sequence of the mutants was confirmed by sequencing. To express Gic2p under the control of the inducible *GAL* promoter, the full-length *GIC2* coding sequence was amplified by PCR to introduce an *EcoRI* site immediately upstream of the start codon using the oligos oTP409 and oTP357 and plasmid pMJ410 as a template. The 1431 bp *EcoRI* fragment was then ligated into yeast expression vectors as indicated in Table II (Mumberg *et al.*, 1995). *GIC2* fused to GFP was constructed as follows. Plasmid pTP43 containing the GFP-coding sequence with the S35T mutation (Heim *et al.*, 1995) was digested with *BamHI*, the ends filled with T4 DNA polymerase and further digested with *EcoRI*. The fragment was isolated and ligated into pRS314 (Sikorski and Hieter, 1989) which was digested with *XhoI*, the ends filled in with T4 DNA polymerase and further digested with *EcoRI*. The resulting plasmid was then linearized with *EcoRI* and ligated with the *EcoRI*-digested PCR fragment of *GIC2* generated by oTP409 and oTP491 to yield plasmid pMJ383.

Antibodies and Western blots

Standard procedures were used for yeast cell extract preparation and immunoblotting (Harlow and Lane, 1988; Brown *et al.*, 1997). Polyclonal

Table II. Plasmids and oligonucleotides

Plasmids	Relevant characteristics	Source
MJ172	GIC2 TRP1 CEN	this study
KP10	CYC GIC2 HIS3 CEN	this study
MJ62	GAL GIC2 TRP1 CEN	this study
MJ77	GAL GIC2 ¹⁻²⁰⁸ TRP1 CEN	this study
MJ341	GIC2 ¹⁻²⁰⁸ TRP1 CEN	this study
MJ280	GAL GIC2 ^{S254A S258A} TRP1 CEN	this study
MJ409	GIC2 ^{S254A S258A} TRP1 CEN	this study
MJ198	GAL GIC2 ^{crib-} TRP1 CEN	this study
MJ173	GIC2 ^{crib-} TRP1 CEN	this study
TP520	GAL CDC42 ^{G12V} URA3 CEN	D.Kellogg
TP521	GAL CDC42 ^{D118A} URA3 CEN	D.Kellogg
MJ383	GAL Gic2 GFP TRP1 CEN	this study
Oligonucleotides Sequences 5' to 3'		
OTP356	GCATTGCGGCCGCTTATAATTTTGGCGTTCAGCAA-GCGCGCGG	
OTP357	GTTAATTCGAAGATATAATAACGAATGTATGGGA-TAACGCC	
OTP409	ACTGGAATTCAATATGACTAGTGCAAGTATTACC	
OTP423	ACGCTCGAGTCATAGTCTTGATGCTTATTTCGT-GCG	
OTP440	TTTGAGTACGTACCAAAACGCAGCTCCAAAAC	
OTP491	GGTTTGCAGGAATTCGAGCTGG	

anti-Gic2p antibodies have been described previously (Brown *et al.*, 1997). Antibodies specific for actin were purchased from Boehringer Mannheim and used as recommended by the manufacturer.

Phosphatase assays

Fifty millilitres of *cdc34-2* cells (YMT670) were grown at 25°C to mid-log phase before the temperature was shifted to 37°C for 3 h. Cells were lysed as described (Brown *et al.*, 1997), and the proteins precipitated with trichloroacetic acid (TCA) and resuspended in RIPA buffer (50 mM Tris pH 8.0, 50 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin and pepstatin. The extract was incubated for 30 min with 20 U of CIP (Boehringer Mannheim) at 30°C and the reaction was stopped by the addition of gel sample buffer. Control incubations were performed in the presence of phosphatase inhibitors (80 mM β -glycerophosphate, 10 mM NaF, 2 mM Na vanadate), or with CIP which had been boiled for 5 min at 95°C prior to the addition. Proteins were analysed after SDS-PAGE and immunoblotted with Gic2p antibodies.

Determination of levels and half-life of Gic2p

To follow the accumulation of Gic2p in various temperature-sensitive strains, cells were grown in rich medium at 25°C to mid-log phase and then shifted to 37°C for 3 h. Cell extracts were prepared as described and immunoblotted with Gic2p antibodies.

The half-lives of either wild-type or mutant forms of Gic2p were determined essentially as described previously (Henchoz *et al.*, 1997). Briefly, various strains deleted for *GIC2* but harbouring plasmids encoding *GIC2* under the control of the inducible *GAL* promoter were grown at 30°C (25°C for temperature-sensitive mutants) to early log phase in raffinose medium (2% raffinose), at which time expression of Gic2p was induced by the addition of galactose for 3 h. Temperature-sensitive strains were shifted to 37°C 3 h after the addition of galactose. A first aliquot was removed before expression was turned off by addition of glucose to a final concentration of 2% (time 0). Aliquots were collected every 10 min, and Gic2p levels were analysed by immunoblotting. Western blots were quantified with the Image QuantTM software (Molecular Dynamics, Inc.).

Degradation of Gic2p in the strain MJ270 was determined as follows (Schneider *et al.*, 1996): cells were grown in galactose medium (2% galactose) at 25°C to early log phase, at which time they were washed and further incubated at 37°C to inactivate Cdc34p and in rich medium (YPD) to shut off expression of Cln3p. After 3 h, cells were washed with pre-warmed medium containing 2% galactose (to induce Cln3p) and released from the START arrest while maintaining the temperature at 37°C. Aliquots were taken every 30 min, and the phosphorylation of

Gic2p was analysed by immunoblotting with Gic2p antibodies. After 2 h, half the culture was washed with pre-warmed YPD medium (2% glucose), the other half with pre-warmed YP-galactose medium (2% galactose), and cells were incubated for an additional 3 h at 37°C in the same medium. The cells were then released from the *cdc34* block by shifting the temperature to 25°C (time 0). Aliquots were taken every 30 min, and Gic2p levels were analysed by immunoblotting.

The strain EY569 harbouring either an empty plasmid (pRS316), or a plasmid encoding CDC42-G12V (pTP520) or CDC42-D118A (pTP521), respectively, was grown to early log phase at 30°C in raffinose medium lacking methionine (2% raffinose). Cells were arrested for 3 h by the addition of methionine (final concentration of 2 mM) to shut off expression of Cln2p (Rönnicke *et al.*, 1997), at which time galactose was added (2% final concentration) to induce the expression of Cdc42p. Extracts were prepared 3 h later and Gic2p levels were analysed by immunoblotting.

Strain MJ255 harbouring a plasmid encoding either CDC42-G12V (pTP520) or CDC42-D118A (pTP521) was grown at 25°C to early log phase in raffinose medium (2% final). Galactose was added (2% final) for 30 min before the temperature was shifted to 37°C to arrest the cells at START. After 3 h, extracts were prepared and analysed for Gic2p levels as described above.

Cell synchronization

Cell synchronization experiments by release from α -factor arrest were performed as described (Breedon, 1997); synchrony was monitored by fluorescence-activated cell sorting (FACS) analysis and microscopic determination of the budding index.

Cells for elutriation were grown in selective medium containing raffinose (2%) to early log phase before galactose was added to 2% final concentration. Cells were elutriated using a Beckman JS 5.0 system essentially as described (Johnston and Johnson, 1997). Small unbudded cells were inoculated into pre-warmed selective medium containing 2% galactose and grown at 30°C. Samples were collected as indicated and cell cycle progression was monitored by FACS analysis. Cells were examined microscopically and the actin cytoskeleton was visualized after staining with rhodamine-phalloidin. Multibudded and unusually large cells with an unpolarized actin cytoskeleton were counted as abnormal. At least 300 cells were counted for each time point.

Microscopy and flow cytometry

Yeast actin was visualized with rhodamine-phalloidin (Molecular Probes, Inc., Leiden, The Netherlands). Briefly, cells were fixed with formaldehyde (3.7% final concentration) for 60 min, washed and stained for 20 min on ice with rhodamine-phalloidin (diluted 1:5 in methanol), washed three times with PBS and viewed on a Zeiss axiophot fluorescence microscope. At least 300 cells were counted for the morphological analysis. Proteins tagged with GFP were visualized on a Zeiss axiophot fluorescence microscope using a Chroma GFP filter (excitation 440–470 nm), photographed with a Photometrics CCD camera and analysed with Photoshop 4.0 software (Microsoft). Photographs shown are overlays of phase contrast and fluorescence images.

Flow cytometry was carried out as described in Epstein and Cross (1992). Briefly, cells were fixed in 70% ethanol, washed and digested with RNase A for 5 h. The DNA was then stained with propidium iodide and analysed on a FACScan (Becton Dickinson).

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References

Adams, A.E., Johnson, D.I., Longnecker, R.M., Sloat, B.F. and Pringle, J.R. (1990) *CDC42* and *CDC43*, two additional genes involved in budding

- and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.*, **111**, 131–142.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1991) *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York.
- Bai, C., Sen, P., Hofmann, K., Ma, L., Goebel, M., Harper, J.W. and Elledge, S.J. (1996) *SKP1* connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell*, **86**, 263–274.
- Barral, Y., Jentsch, S. and Mann, C. (1995) G1 cyclin turnover and nutrient uptake are controlled by a common pathway in yeast. *Genes Dev.*, **9**, 399–409.
- Bender, A. and Pringle, J.R. (1991) Use of a screen for synthetic lethal and multicopy suppressor mutants to identify two new genes involved in morphogenesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **11**, 1295–1305.
- Breeden, L.L. (1997) α -Factor synchronization of budding yeast. *Methods Enzymol.*, **283**, 332–341.
- Brown, J.L., Jaquenoud, M., Gulli, M.P., Chant, J. and Peter, M. (1997) Novel Cdc42-binding proteins Gic1 and Gic2 control cell polarity in yeast. *Genes Dev.*, **11**, 2972–2982.
- Burbelo, P.D., Drechsel, D. and Hall, A. (1995) A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J. Biol. Chem.*, **270**, 29071–29074.
- Burridge, K., Chrzanowskawodnicka, M. and Zhong, C.L. (1997) Focal adhesion assembly. *Trends Cell Biol.*, **7**, 342–347.
- Chant, J. (1996) Generation of cell polarity in yeast. *Curr. Opin. Cell Biol.*, **8**, 557–565.
- Chen, G.C., Kim, Y.J. and Chan, C.S. (1997) The Cdc42 GTPase-associated proteins Gic1 and Gic2 are required for polarized cell growth in *Saccharomyces cerevisiae*. *Genes Dev.*, **11**, 2958–2971.
- Chenevert, J., Corrado, K., Bender, A., Pringle, J. and Herskowitz, I. (1990) A yeast gene (*BEM1*) necessary for cell polarization whose product contains two SH3 domains. *Nature*, **356**, 77–79.
- Cohen-Fix, O., Peters, J.M., Kirschner, M.W. and Koshland, D. (1996) Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.*, **10**, 3081–3093.
- Cvrckova, F., De Virgilio, C., Manser, E., Pringle, J.R. and Nasmyth, K. (1995) Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. *Genes Dev.*, **9**, 1817–1830.
- Dahmann, C., Diffley, J.F. and Nasmyth, K.A. (1995) S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Curr. Biol.*, **5**, 1257–1269.
- Deshaies, R.J. (1997) Phosphorylation and proteolysis: partners in the regulation of cell division in budding yeast. *Curr. Opin. Genet. Dev.*, **7**, 7–16.
- Drubin, D.G. and Nelson, W.J. (1996) Origins of cell polarity. *Cell*, **84**, 335–344.
- Drury, L.S., Perkins, G. and Diffley, J.F. (1997) The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. *EMBO J.*, **16**, 5966–5976.
- Ebi, J.J., Holly, S.P., van Drogen, F., Grishin, A.V., Peter, M., Drubin, D.G. and Blumer, K.J. (1998) Actin cytoskeleton organization regulated by PAK kinases. *Curr. Biol.*, in press.
- Epstein, C.B. and Cross, F.R. (1992) *CLB5*, a novel B-type cyclin from budding yeast with a role in S-phase. *Genes Dev.*, **6**, 1695–1703.
- Evangelista, M., Blundell, K., Longtine, M.S., Chow, C.J., Adames, N., Pringle, J.R., Peter, M. and Boone, C. (1997) Bni1p, a yeast formin linking Cdc42p and the actin cytoskeleton during polarized morphogenesis. *Science*, **276**, 118–122.
- Feldman, R.M., Correll, C.C., Kaplan, K.B. and Deshaies, R.J. (1997) A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell*, **91**, 221–230.
- Flick, J.S. and Johnston, M. (1991) *GRR1* of *Saccharomyces cerevisiae* is required for glucose repression and encodes a protein with leucine-rich repeats. *Mol. Cell. Biol.*, **11**, 5101–5112.
- Gallo, G. and Letourneau, P.C. (1998) Axon guidance—GTPases help axons reach their targets. *Curr. Biol.*, **8**, R80–R82.
- Ghislain, M., Udvardy, A. and Mann, C. (1993) *S.cerevisiae* 26S protease mutants arrest cell division in G2/metaphase. *Nature*, **366**, 358–362.
- Goebel, M.G., Yochem, J., Jentsch, S., McGrath, J.P., Varshavsky, A. and Byers, B. (1988) The yeast cell cycle gene *CDC34* encodes a ubiquitin-conjugating enzyme. *Science*, **241**, 1331–1335.
- Guthrie, C. and Fink, G.R. (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press Inc., San Diego, CA.
- Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science*, **279**, 509–514.
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Heim, R., Cubitt, A.B. and Tsien, R.Y. (1995) Improved green fluorescence. *Nature*, **373**, 663–664.
- Henchoz, S., Chi, Y., Catarin, B., Herskowitz, I., Deshaies, R.J. and Peter, M. (1997) Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast. *Genes Dev.*, **11**, 3046–3060.
- Hershko, A. (1997) Roles of ubiquitin-mediated proteolysis in cell cycle control. *Curr. Opin. Cell Biol.*, **9**, 788–799.
- Hilt, W. and Wolf, D.H. (1995) Proteasomes of the yeast *S.cerevisiae*: genes, structure and functions. *Mol. Biol. Rep.*, **21**, 3–10.
- Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163–168.
- Imamura, H., Tanaka, K., Hihara, T., Umikawa, M., Kamei, T., Takahashi, K., Sasaki, T. and Takai, Y. (1997) Bni1p and Bnr1p: downstream targets of the Rho family of small G-proteins which interact with profilin and regulate actin cytoskeleton in *Saccharomyces cerevisiae*. *EMBO J.*, **16**, 2745–2755.
- Johnson, D.I. and Pringle, J.R. (1990) Molecular characterization of *CDC42*, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. *J. Cell Biol.*, **111**, 143–152.
- Johnston, L.H. and Johnson, A.L. (1997) Elutriation of budding yeast. *Methods Enzymol.*, **283**, 342–350.
- Kamada, Y., Jung, U.S., Pietrowski, J. and Levin, D.E. (1995) The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. *Genes Dev.*, **9**, 1559–1571.
- Kaplan, K.B., Hyman, A.A. and Sorger, P.K. (1997) Regulating the yeast kinetochore by ubiquitin-dependent degradation and Skp1p-mediated phosphorylation. *Cell*, **91**, 491–500.
- Keely, P., Parise, L. and Juliano, R. (1998) Integrins and GTPases in tumour cell growth, motility and invasion. *Trends Cell Biol.*, **8**, 101–106.
- Kim, Y.J., Francisco, L., Chen, G.C., Marcotte, E. and Chan, C.S. (1994) Control of cellular morphogenesis by the Ip12/Bem2 GTPase-activating protein: possible role of protein phosphorylation. *J. Cell Biol.*, **127**, 1381–1394.
- King, R.W., Deshaies, R.J., Peters, J.M. and Kirschner, M.W. (1996) How proteolysis drives the cell cycle. *Science*, **274**, 1652–1659.
- Kornitzer, D., Raboy, B., Kulka, R.G. and Fink, G.R. (1994) Regulated degradation of the transcription factor Gcn4. *EMBO J.*, **13**, 6021–6030.
- Krek, W. (1998) Proteolysis and the G₁–S transition: the SCF connection. *Curr. Opin. Genet. Dev.*, **8**, 36–42.
- Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.
- Lanker, S., Valdivieso, M.H. and Wittenberg, C. (1996) Rapid degradation of the G1 cyclin Cln2 induced by Cdk-dependent phosphorylation. *Science*, **271**, 1597–1601.
- Leberer, E., Wu, C.L., Leeuw, T., Fourest-Lieuvain, A., Segall, J.E. and Thomas, D.Y. (1997a) Functional characterization of the Cdc42p binding domain of yeast Ste20p protein kinase. *EMBO J.*, **16**, 83–97.
- Leberer, E., Thomas, D.Y. and Whiteway, M. (1997b) Pheromone signalling and polarized morphogenesis in yeast. *Curr. Opin. Genet. Dev.*, **7**, 59–66.
- Leeuw, T., Fourest-Lieuvain, A., Wu, C., Chenevert, J., Clark, K., Whiteway, M., Thomas, D.Y. and Leberer, E. (1995) Pheromone response in yeast: association of Bem1p with proteins of the MAP kinase cascade and actin. *Science*, **270**, 1210–1213.
- Lew, D.J. and Reed, S.I. (1993) Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *J. Cell Biol.*, **120**, 1305–1320.
- Lew, D.J. and Reed, S.I. (1995) Cell cycle control of morphogenesis in budding yeast. *Curr. Opin. Genet. Dev.*, **5**, 17–23.
- Li, F.N. and Johnston, M. (1997) Grr1 of *Saccharomyces cerevisiae* is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and the cell cycle. *EMBO J.*, **16**, 5629–5638.
- Lisztwan, J., Marti, A., Sutterluty, H., Gstaiger, M., Wirbelauer, C. and Krek, W. (1998) Association of human CUL-1 and ubiquitin-conjugating enzyme CDC34 with the F-box protein p45(SKP2): evidence for evolutionary conservation in the subunit composition of the CDC34–SCF pathway. *EMBO J.*, **17**, 368–383.

- Martin,H., Mendoza,A., Rodriguez-Pachon,J.M., Molina,M. and Nombela,C. (1997) Characterization of *SKM1*, a *Saccharomyces cerevisiae* gene encoding a novel Ste20/PAK-like protein kinase. *Mol. Microbiol.*, **23**, 431–444.
- Mathias,N., Johnson,S.L., Winey,M., Adams,A.E.M., Goetsch,L., Pringle,J.R., Byers,B. and Goehl,M.G. (1996) Cdc53p acts in concert with Cdc4p and Cdc34p to control the G1-to-S-phase transition and identifies a conserved family of proteins. *Mol. Cell. Biol.*, **16**, 6634–6643.
- Michaelis,C., Ciosk,R. and Nasmyth,K. (1997) Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell*, **91**, 35–45.
- Mumberg,D., Müller,R. and Funk,M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*, **156**, 119–122.
- Nigg,E.A. (1995) Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *BioEssays*, **17**, 471–480.
- Patton,E.E., Willems,A.R. and Tyers,M. (1998a) Combinatorial control of ubiquitin dependent proteolysis: Skp1, Cdc53/cullin and F-box protein complexes. *Trends Genet.*, **14**, 236–243.
- Patton,E.E., Willems,A.R., Sa,D., Kuras,L., Thomas,D., Craig,K.L. and Tyers,M. (1998b) Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. *Genes Dev.*, **12**, 692–705.
- Peter,M., Neiman,A.M., Park,H.O., Van Lohuizen,M. and Herskowitz,I. (1996) Functional analysis of the interaction between the small GTP-binding protein Cdc42 and the Ste20 protein kinase in yeast. *EMBO J.*, **15**, 7046–7059.
- Peters,J.M. (1994) Proteasomes: protein degradation machines of the cell. *Trends Biochem. Sci.*, **19**, 377–382.
- Prinz,S., Hwang,E.S., Visintin,R. and Amon,A. (1998) The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis. *Curr. Biol.*, **8**, 750–760.
- Rönicke,V., Graulich,W., Mumberg,D., Müller,R. and Funk,M. (1997) Use of conditional promoters for expression of heterologous proteins in *Saccharomyces cerevisiae*. *Methods Enzymol.*, **283**, 313–322.
- Ruegger,M., Dewey,E., Gray,W.M., Hobbie,L., Turner,J. and Estelle,M. (1998) The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast Grr1p. *Genes Dev.*, **12**, 198–207.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schneider,B.L., Yang,Q.H. and Futcher,A.B. (1996) Linkage of replication to start by the Cdk inhibitor Sic1. *Science*, **272**, 560–562.
- Schwob,E., Böhm,T., Mendenhall,M.D. and Nasmyth,K. (1994) The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S.cerevisiae*. *Cell*, **79**, 233–244.
- Shirayama,M., Zachariae,W., Ciosk,R. and Nasmyth,K. (1998) The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO J.*, **17**, 1336–1349.
- Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
- Skowyra,D., Craig,K.L., Tyers,M., Elledge,S.J. and Harper,J.W. (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell*, **91**, 209–219.
- Thomas,D., Kuras,L., Barbey,R., Cherest,H., Blaiseau,P.L. and Surdin-Kerjan,Y. (1995) Met30p, a yeast transcriptional inhibitor that responds to S-adenosylmethionine, is an essential protein with WD40 repeats. *Mol. Cell. Biol.*, **15**, 6526–6534.
- Valtz,N. and Peter,M. (1997) Functional analysis of *FAR1* in yeast. *Methods Enzymol.*, **283**, 350–365.
- Verma,R., Annan,R.S., Huddleston,M.J., Carr,S.A., Reynard,G. and Deshaies,R.J. (1997) Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science*, **278**, 455–460.
- Willems,A.R., Lanker,S., Patton,E.E., Craig,K.L., Nason,T.F., Mathias,N., Kobayashi,R., Wittenberg,C. and Tyers,M. (1996) Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. *Cell*, **86**, 453–463.
- Zhang,H., Kobayashi,R., Galaktionov,K. and Beach,D. (1995) p19Skp1 and p45Skp2 are essential elements of the cyclin A-CDK2 S phase kinase. *Cell*, **82**, 915–925.

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