

Degradation of Hof1 by SCF^{Grr1} is important for actomyosin contraction during cytokinesis in yeast

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SCF-type (SCF: Skp1–Cullin–F-box protein complex) E3 ligases regulate ubiquitin-dependent degradation of many cell cycle regulators, mainly at the G1/S transition. Here, we show that SCF^{Grr1} functions during cytokinesis by degrading the PCH protein Hof1. While Hof1 is required early in mitosis to assemble a functional actomyosin ring, it is specifically degraded late in mitosis and remains unstable during the entire G1 phase of the cell cycle. Degradation of Hof1 depends on its PEST motif and a functional 26S proteasome. Interestingly, degradation of Hof1 is independent of APC^{Cdh1}, but instead requires the SCF^{Grr1} E3 ligase. Grr1 is recruited to the mother–bud neck region after activation of the mitotic-exit network, and interacts with Hof1 in a PEST motif-dependent manner. Our results also show that downregulation of Hof1 at the end of mitosis is necessary to allow efficient contraction of the actomyosin ring and cell separation during cytokinesis. SCF^{Grr1}-mediated degradation of Hof1 may thus represent a novel mechanism to couple exit from mitosis with initiation of cytokinesis.

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Introduction

Ubiquitin-dependent degradation of key proteins has emerged as an important mechanism to control numerous biological responses, including signal transduction, morphogenesis and cell cycle progression. Protein destruction is triggered by the covalent attachment of ubiquitin onto lysine

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residues of substrates, which targets them for degradation by the 26S proteasome (Ciechanover and Schwartz, 2002). Three classes of enzymes are required to mediate these reactions: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzymes and the E3 ubiquitin ligases (Peters, 1998). The anaphase-promoting complex (APC) and Skp1–Cullin–F-box protein complex (SCF) are multiprotein E3 ligases that were discovered because of their essential roles in the regulation of the cell cycle (Peters, 1998). APC is thought to be involved in late cell cycle events including entry into anaphase and exit from mitosis. In budding yeast, entry into S phase requires SCF^{Cdc4}-dependent degradation of the cyclin-dependent kinase inhibitor Sic1 (Schwob *et al*, 1994; Verma *et al*, 1997). SCF^{Cdc4} is composed of the cullin Cdc53, the E2-conjugating enzyme Cdc34, the ring-finger protein Hrt1 and Skp1, which binds to the F-box motif present in a large family of proteins (Deshaies, 1999). It is thought that F-box proteins function as adaptor subunits that recruit specific substrates to a core ligase complex composed of Cdc53, Cdc34, Hrt1/Rbx1 and Skp1 (Patton *et al*, 1998; Tyers and Jorgensen, 2000). Besides Sic1, SCF^{Cdc4} is also required for the degradation of Far1, Cdc6 and Gcn4 (Drury *et al*, 1997; Henchoz *et al*, 1997; Skowyra *et al*, 1999; Meimoun *et al*, 2000). In contrast, ubiquitination of the transcription factor Met4 is dependent on SCF^{Met30} (Kaiser *et al*, 2000; Patton *et al*, 2000; Rouillon *et al*, 2000), while degradation of the G1 cyclins Cln1 and Cln2 (Barral *et al*, 1995; Seol *et al*, 1999; Skowyra *et al*, 1999) and the bud emergence protein Gic2 (Jaquenoud *et al*, 1998) is mediated by SCF^{Grr1}.

Substrate phosphorylation and the subcellular localization of F-box proteins regulate temporal and spatial aspects of protein degradation. Many SCF substrates need to be phosphorylated for subsequent ubiquitination (Deshaies, 1997), and in some cases, phosphorylation of multiple sites is required to allow substrate binding to the F-box protein, thereby setting a threshold for kinase activity before abrupt degradation is triggered (Nash *et al*, 2001). While the shared core components of the SCF complex are found in the nucleus and cytoplasm (Blondel *et al*, 2000), the localization of F-box proteins provides an important determinant for the spatial control of substrate degradation. Cdc4 is localized and active only in the nucleus, thereby ensuring that cytoplasmic Far1 is stable during mating (Blondel *et al*, 2000). Likewise, the mammalian F-box protein Skp2 is nuclear and accumulates on centrosomes in human cells (Gstaiger *et al*, 1999). These results suggest that the subcellular localization of F-box proteins may provide important clues to their function and may help to identify potential substrates. The F-box protein Grr1 is found both in the nucleus and the cytoplasm. In addition, Grr1 also accumulates at the bud neck late in mitosis (Blondel *et al*, 2000), implying that it may play a role during cytokinesis by degrading an as yet unknown substrate.

Genetic experiments revealed two redundant pathways for cytokinesis in budding yeast. The first pathway involves the type II myosin Myo1 and the formin Bni1, and is required for the assembly of a functional actomyosin-based contractile ring. The second pathway controls cell separation and septum formation and involves a number of proteins including the formin-related protein Bnr1 and Hof1/Cyk2. Hof1 is a member of the PCH family of proteins (Lippincott and Li, 2000), which contain an N-terminal FER-CIP4 homology (FCH) domain flanked by coiled-coil domains, PEST sequences and one or more SH3 domains in their C-termini. Both *Schizosaccharomyces pombe* Cdc15 and Imp2 and *Saccharomyces cerevisiae* Hof1 localize to the actin ring and are important for the completion of cytokinesis. It has been suggested that Hof1 could play a role in coupling the actomyosin system to septum formation (Vallen *et al*, 2000). Alternatively, Hof1 could act by modulating the stability of the actomyosin ring during contraction (Lippincott and Li, 1998). Interestingly, overexpression of Hof1 in *S. cerevisiae* or Cdc15 in *S. pombe* has been shown to interfere with cell separation (Fankhauser *et al*, 1995; Lippincott and Li, 1998; Vallen *et al*, 2000). Similar results were obtained when overexpressing the mouse homolog PSTPIP in *S. pombe* (Spencer *et al*, 1997).

In this study, we have investigated a role of Grr1 during cytokinesis. We found that SCF^{Grr1} is required to degrade Hof1 on the cytokinesis ring. Degradation of Hof1 is important for efficient contraction of the actomyosin ring and cell separation during cytokinesis. Because localization of Grr1 on the mother–bud neck and degradation of Hof1 require activation of the mitotic-exit network (MEN), SCF^{Grr1} may couple mitotic exit and completion of cytokinesis.

Results

Accumulation of Grr1 at the bud neck requires activation of the MEN

We have shown previously that a functional Grr1-GFP fusion protein localizes to the cytoplasm and nucleus, and also accumulates at the mother–bud neck late in mitosis (Blondel *et al*, 2000). To determine when Grr1 is recruited to the mother–bud region, we performed time-lapse microscopy in wild-type cells expressing Grr1-GFP and the spindle marker CFP-Tub1. Grr1-GFP was absent from the mother–bud region during most of the cell cycle, and accumulated on the cytokinesis ring shortly after disassembly of the mitotic spindle (Figure 1A). Quantification of more than 10 cells in at least five independent movies revealed that Grr1-GFP

transiently accumulated at the mother–bud neck shortly after disassembly of the mitotic spindle, but before cell separation as determined by DIC analysis (panel B). Consistent with these results, Grr1-GFP was not found at the mother–bud neck in *cdc16-1* cells (data not shown), which arrest at the metaphase–anaphase transition. Grr1-GFP was also absent from the mother–bud neck in *cdc14-3*

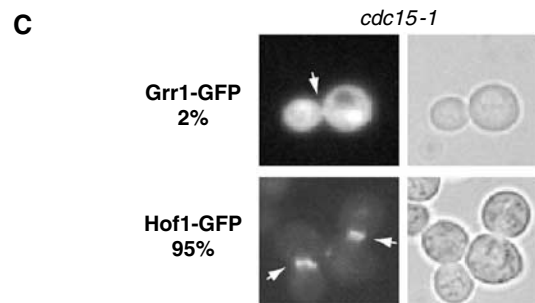
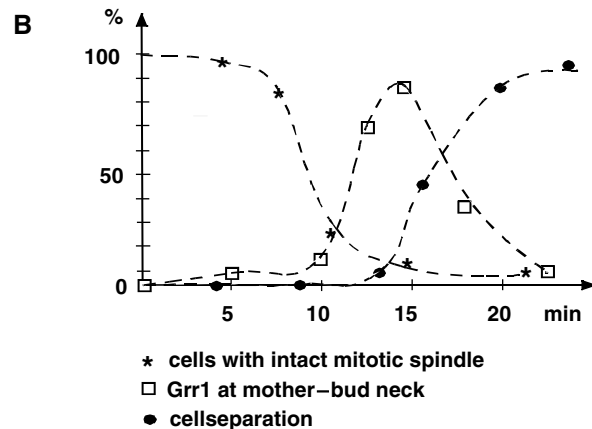
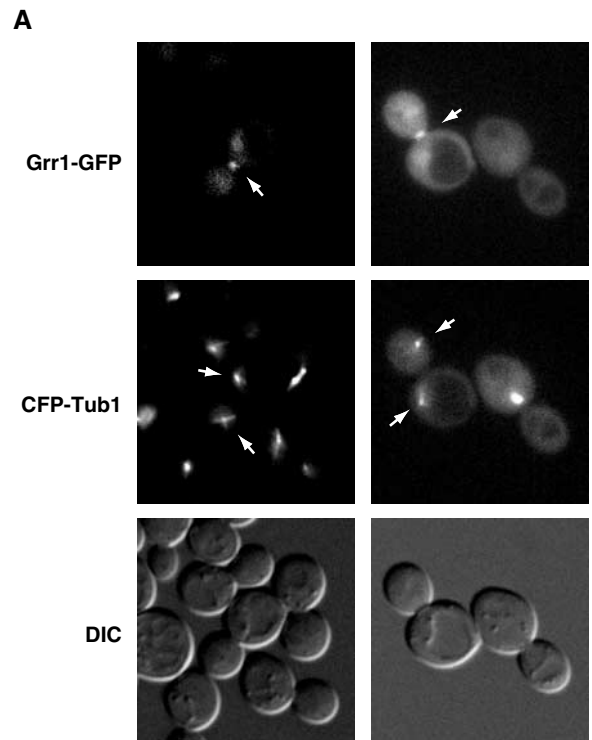


Figure 1 Accumulation of Grr1-GFP at the mother–bud neck occurs after spindle breakdown in a MEN-dependent manner. (A) Wild-type cells were analyzed microscopically for their localization of Grr1-GFP and the status of the mitotic spindle using CFP-Tub1. The arrows mark the position of Grr1 at the mother–bud neck and the mitotic spindle. (B) Time-lapse analysis of wild-type cells ($n = 10$) expressing CFP-Tub1 and Grr1-GFP. Time 0 represents the onset of anaphase as determined by CFP-Tub1. Cell separation was determined by DIC microscopy, as the separated G1 cells slightly tilt after separation. (C) The Localization of Grr1-GFP (upper panel) and Hof1-GFP (lower panel) was analyzed in *cdc15-1* cells at the restrictive temperature (37°C). The numbers indicate the percentage (%) of cells with Grr1-GFP (upper panel) or Hof1-GFP (lower panel) at the mother–bud neck region.

(data not shown) and *cdc15-1* arrested cells (panel C), suggesting that activation of the MEN may be necessary for its recruitment to the mother–bud neck. As a control, the localization of Hof1 at the mother–bud neck was normal, implying that the actin ring region is properly assembled under these conditions. Taken together, these results indicate that Grr1 is transiently recruited to the mother–bud neck region in a MEN-dependent manner, suggesting that Grr1 may degrade a substrate involved in cytokinesis.

The localization of Grr1-GFP at the mother–bud requires a functional leucine-rich domain but not its F-box or nuclear localization signals

To determine the domains responsible for the different subcellular localizations of Grr1-GFP, we analyzed the localization of various mutant constructs as schematically indicated in Figure 2A. The primary sequence of Grr1 can be subdivided into four domains: an N-terminal domain (1–310), the F-box (311–361), the 12 leucine-rich repeat (LRR, 413–740) and a C-terminal domain (740–1150). Interestingly, neither an intact F-box nor the C-terminal domain was required for the subcellular localization of Grr1 (panel A). These Grr1 mutant proteins efficiently accumulated in the nucleus and were specifically targeted to the mother–bud region in late mitosis. In contrast, deletion of the N-terminal domain prevented the accumulation of Grr1-GFP in the nucleus. Grr1 deletion mutants lacking 179 or 311 amino-terminal amino acids (Figure 2A and B, and data not shown) were predominantly cytoplasmic throughout the cell cycle. However, these mutants still localized to the mother–bud neck region late in mitosis, indicating that the N-terminal domain is not required for ring localization of Grr1 but rather contains sequences necessary for its nuclear import. The N-terminal domain (amino acids 1–311) contains two potential bipartite nuclear localization signals (NLS) beginning at position 152 (**RKQIHFEFKLIVGKKIQEFLVVIKRRRKE**; Figure 2A). Indeed, the N-terminal domain of Grr1 was sufficient to target GFP to the nucleus (panel A), suggesting that the amino-terminal domain is necessary and sufficient for nuclear accumulation of Grr1.

We also tested the importance of the 12 LRR for the subcellular localization of Grr1-GFP. This domain, encompassing amino acids 413 and 740, was previously described as the substrate interacting region of Grr1 and is predicted to fold into a characteristic horseshoe structure with a high density of positive charges on the concave surface (Hsiung *et al*, 2001). Based on structural predictions, deletion of a single LRR is expected to abolish its interactions with substrates (Hsiung *et al*, 2001). Interestingly, Grr1 mutant proteins lacking even small portions of the LRR domain failed to localize to the mother–bud region, while their nuclear accumulation was unaffected (Figure 2A, *grr1*^{1–661} and *grr1*^{1–480}). We conclude that the putative substrate-interactive region of Grr1 is essential for its localization to the mother–bud neck region, suggesting that Grr1 could degrade one (or more) substrate(s) at the cytokinesis ring.

In addition to the subcellular localization, we also examined the function of the various Grr1 constructs *in vivo* (Figure 2C and D). *grr1Δ* cells exhibit pronounced morphological defects and accumulate as hyperpolarized, highly elongated cells (Blacketer *et al*, 1995), most likely due to their failure to degrade the G1 cyclins Cln1 and Cln2 (Barral

and Mann, 1995). Likewise, *grr1Δ* cells fail to degrade the Cdc42 effector Gic2 after bud emergence (Jaquenoud *et al*, 1998), and as a result, overexpression of Gic2p is toxic in *grr1Δ* cells. As expected, both the Grr1 mutant lacking the F-box (*grr1-ΔF*) and the Grr1 mutant lacking LRR repeats were unable to complement these defects (Figure 2C and D, and data not shown). In contrast, cytoplasmic *grr1-ΔN* (*grr1*^{180–1150}) was able to restore both growth of *grr1Δ* cells overexpressing Gic2 and degradation of Gic2 (panels C and D). Likewise, the morphology of *grr1Δ* cells expressing cytoplasmic Grr1-ΔN was normal (data not shown). These results imply that cytoplasmic Grr1 is sufficient to restore the function of Grr1 with respect to Gic2 degradation and its morphological functions, implying that ubiquitinylation of these targets may occur outside the nucleus.

Hof1 binds Grr1 and this interaction depends on its PEST domain

To confirm that Grr1 plays a role in cytokinesis, we searched for potential substrates located at the mother–bud neck. Interestingly, a genome-wide two-hybrid screen has previously detected an interaction between Grr1 and the PCH protein Hof1 (Ito *et al*, 2001). Indeed, Hof1 accumulates at the mother–bud neck shortly after bud emergence, and abruptly disappears at the end of mitosis concomitant with the appearance of Grr1 (Figure 4A; Vallen *et al*, 2000). We confirmed the two-hybrid interaction between Grr1 and Hof1 using two different two-hybrid systems (Figure 3A). To address the functional importance of this interaction, we attempted to identify a functional Hof1 mutant that fails to interact with Grr1. Several Grr1 targets including Cln1 and Cln2 contain functional PEST domains (Lanker *et al*, 1996), which are commonly found in unstable protein. Interestingly, the PEST-Find program (Rechsteiner and Rogers, 1996) revealed a high-scoring PEST domain (score of 11.85) between amino acids 418 and 438 of Hof1 (Figure 3A). To determine the significance of this PEST motif, we constructed a Hof1 mutant lacking this domain (*hof1ΔPEST*). This mutant was found to be functional as it was able to suppress the thermo-sensitivity of *hof1Δ* cells when expressed from the endogenous *HOF1* gene promoter on a low-copy number plasmid (data not shown). Moreover, as for Hof1wt, overexpression of *hof1ΔPEST* from the inducible *GAL1,10* promoter was able to block cytokinesis (Figure 6B). Importantly, the interaction between *hof1ΔPEST* and Grr1 was greatly reduced in both two-hybrid systems (Figure 3A). Taken together, these results confirm that Grr1 binds Hof1, and suggest that a functional PEST domain of Hof1 is required for this interaction.

To test whether Grr1 and Hof1 interact *in vivo*, we made use of the bimolecular fluorescence complementation (BiFC) system (Hu *et al*, 2002). Grr1 was fused to the amino-terminal half of YFP (termed YFP-N), while Hof1 was fused to the carboxy-terminal half of CFP (CFP-C). The GFP-chromophore is only restored if YFP-N and CFP-C are brought together by interaction of two proteins fused to the two respective halves. As shown in Figure 3B, coexpression of Grr1p-YFP-N and Hof1-CFP-C restored GFP fluorescence specifically on the mother–bud neck late in the cell cycle. This interaction was specific as no GFP signal was detected in cells expressing either YFP-N or CFP-C alone, or CFP-C fused to another protein unable to interact with Grr1 (not shown). Moreover, no GFP signal was observed after coexpression of Grr1p-YFP-N and

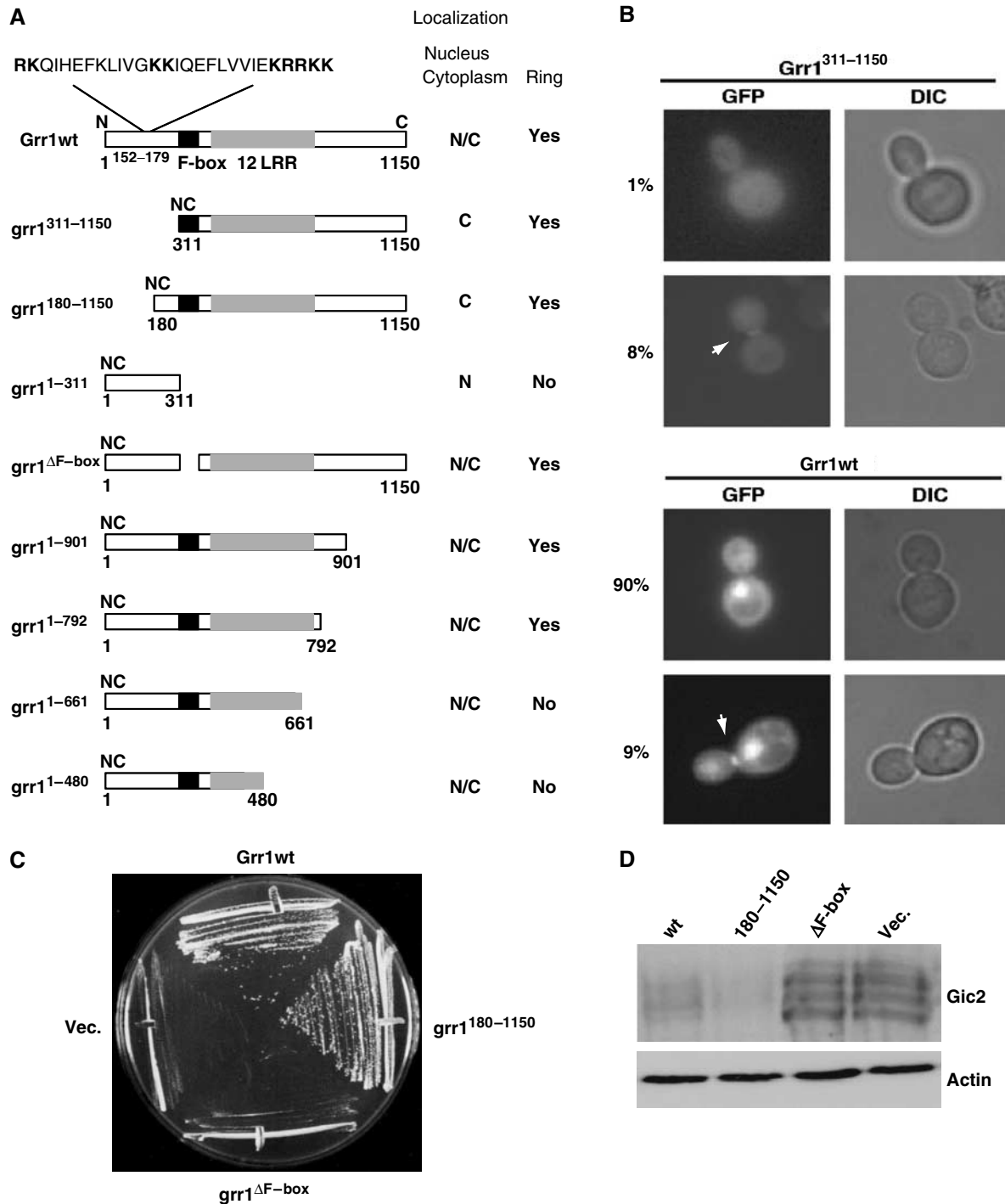


Figure 2 Grr1 domains required for function and subcellular localization. (A, B) Schematic representation of wild-type and various mutant forms of Grr1 (left panel). Black bar: F-box; gray bar: LRR. Their subcellular localization was determined microscopically using GFP fusions as indicated on the right. N: nuclear; C: cytoplasmic. The ability of wild-type and mutant GFP-Grr1 proteins to accumulate at the mother–bud neck region (ring) during cytokinesis was analyzed in at least 200 cells: ‘yes’ indicates ring localization observed in 5–10% of cells in late mitosis, ‘no’ indicates no accumulation of the GFP-Grr1 mutant protein detectable. The localization of wild-type Grr1-GFP and cytoplasmic Grr1^{311–1150}-GFP is shown in panel B. The numbers indicate the percentage (%) of cells ($n > 200$) with Grr1^{311–1150}-GFP (upper panel) or wild-type Grr1-GFP (lower panel) with nuclear accumulation (upper images), or accumulation at the mother–bud neck region of cells in late mitosis as determined by DIC (lower images). The arrows mark the position of Grr1-GFP and Grr1^{311–1150}-GFP at the mother–bud neck during cytokinesis. (C, D) Wild-type Grr1, Grr1-ΔF-box or cytoplasmic Grr1^{180–1150} expressed from the *ADH* promoter were analyzed for their ability to complement growth of *grr1Δ* cells overexpressing Gic2 (C), and degradation of Gic2 in *grr1Δ* cells by Western blot (D). An empty vector (Vec.) was included for negative control.

*hof1Δ*PEST-CFP-C, further demonstrating that the interaction of Grr1 and Hof1 requires an intact PEST domain. Importantly, no GFP signal was observed during the G1, S and G2 phases of

the cell cycle in cells coexpressing Grr1p-YFP-N and Hof1-CFP-C, demonstrating that Grr1 and Hof1 interact *in vivo* in a spatially and temporally controlled manner.

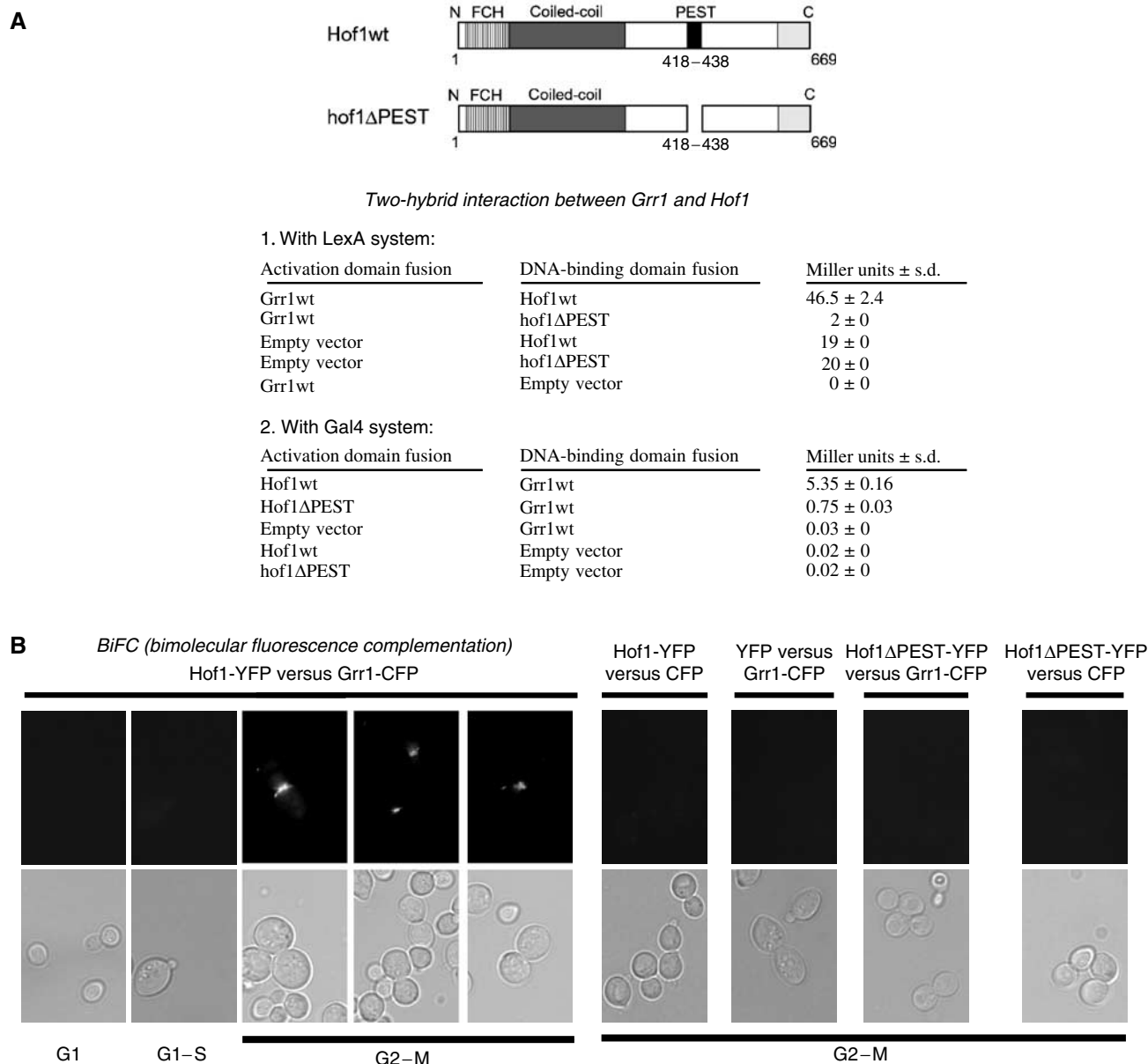


Figure 3 Interaction of Hof1 with Grr1 in a PEST-dependent manner. (A) Schematic representation of wild-type Hof1 and hof1ΔPEST. The various functional domains (FCH, coiled-coil, PEST and SH3) are indicated. Two-hybrid analysis between Grr1 and wild type and hof1ΔPEST using either the LexA (upper panel) or Gal4 system (lower panel). The numbers indicate Miller units (MU) with standard deviations (s.d.) of liquid β -galactosidase reporter assays determined from at least four independent experiments. (B) BiFC analysis of Grr1 and Hof1. Wild-type cells (K699) expressing Grr1 fused to CFP-C (Grr1-CFP) and Hof1 fused to YFP-N (Hof1-YFP) were analyzed by GFP microscopy. Cells expressing Grr1-CFP and YFP-N alone, and Hof1-YFP or hof1ΔPEST-YFP and CFP-C alone were included for control.

Cell cycle-dependent degradation of Hof1 but not hof1ΔPEST

Like other members of the *CLB2* cluster (Spellman *et al*, 1998), *HOF1* transcription gradually increases from S phase to mitosis and peaks around anaphase. Indeed, Hof1-GFP expressed from its endogenous promoter was absent during the G1 phase of the cell cycle, and assembled at the mother-bud neck only after S phase (Figure 4A; Vallen *et al*, 2000). Hof1-GFP expressed from the constitutive *CYC1* or *ADH* promoters was also absent in unbudded G1 cells, while it assembled at the mother-bud region shortly after bud emergence (Figure 4A and B). The mother-bud neck signal increased in large budded cells and then abruptly disap-

peared during cytokinesis. The premature accumulation of Hof1-GFP expressed from the *CYC1* promoter but not the endogenous promoter confirms that the transcriptional regulation of the *HOF1* promoter contributes to its G2/M-specific expression *in vivo*. Importantly however, these data imply that the absence of Hof1 in late mitosis and the G1 phase of the cell cycle must be predominantly regulated at the post-transcriptional level. While wild-type Hof1-GFP was undetectable in most G1 cells, hof1ΔPEST-GFP expressed from the constitutive *CYC1* or *ADH* promoters was present at all stages of the cell cycle and accumulated in a ring-like structure at the cell cortex of G1 cells (Figure 4A and B). This difference was also evident in α -factor-arrested G1 cells. Taken together,

these data strongly suggest that Hof1, but not hof1ΔPEST-GFP, is degraded during the G1 phase of the cell cycle.

To measure directly the half-life ($t_{1/2}$) of Hof1, we expressed HA- and GFP-tagged Hof1 and hof1ΔPEST from the *GAL1,10* promoter, which allows a transcriptional shutoff by the addition of glucose. In asynchronous cells ($-\alpha F$), HA- and GFP-Hof1 were degraded with a half-life of about 55 min (Figure 4C, and data not shown). Similar results were obtained when the half-life of HA-Hof1 expressed in the

normal chromosomal context was determined after blocking protein translation by the addition of cycloheximide (CX, data not shown). When cells were synchronized in G1 by α -factor treatment ($+\alpha F$), HA- and GFP-Hof1 were degraded with a half-life of about 30 min (Figure 4C, and data not shown). These results are consistent with the microscopy analysis presented above, and suggest that Hof1 is degraded during the end of mitosis and bud emergence. In contrast, GFP- and HA-hof1ΔPEST were stabilized at all cell cycle stages (Figure 4A–C), with a half-life of more than 120 min in α -factor-arrested cells. In asynchronous cells, slower migrating forms of HA-Hof1 corresponding to phosphorylated species were detectable (Figure 4C and D; Vallen *et al*, 2000). Such phosphorylated species were not observed with HA-hof1ΔPEST, suggesting that the PEST domain of Hof1 may be important for phosphorylation. Taken together, these results demonstrate that Hof1 is rapidly degraded during the G1 phase of the cell cycle by a PEST- and perhaps phosphorylation-dependent mechanism.

Degradation of Hof1 during G1 requires the 26S proteasome and SCF^{Grr1}

Because Grr1 interacted with Hof1 in a PEST-dependent manner, we next examined the expression of wild-type Hof1-GFP in cells defective for components of the SCF^{Grr1} complex. Interestingly, we were unable to obtain *grr1Δ* cells expressing Hof1-GFP from the constitutive *ADH* promoter, probably because it is toxic without efficient degradation. However, although the cells grew very poorly, we were able to analyze *grr1Δ* cells expressing Hof1-GFP from the weaker *CYC* promoter. Interestingly, strong Hof1-GFP staining was observed in *grr1Δ* cells at all cell cycle stages including G1 (Figure 5A). Similarly, Hof1-GFP was present in G1 in *cdc34-2* and *cdc53-1* cells grown at the semipermissive temperature of 30°C (panel B, and data not shown). In contrast, Hof1-GFP was absent in G1 cells in *cdc4-1* cells (panel C), which like *cdc34-2* and *cdc53-1* cells fail to degrade the Cdk inhibitor Sic1 at the G1/S-phase transition (Schwob *et al*, 1994). Half-life measurements confirmed that HA-Hof1 was stable in *grr1Δ rgt1* and *cdc34-2* cells (Figure 5D), while it was rapidly degraded in *cdh1Δ* cells, which are deficient for APC activity during the G1 phase of the cell cycle (Peters, 1999). Stabilization of HA-Hof1 expressed from its endogenous promoter was also observed in *grr1Δ* cells using CX chase (data not shown). HA-Hof1 but not HA-hof1ΔPEST

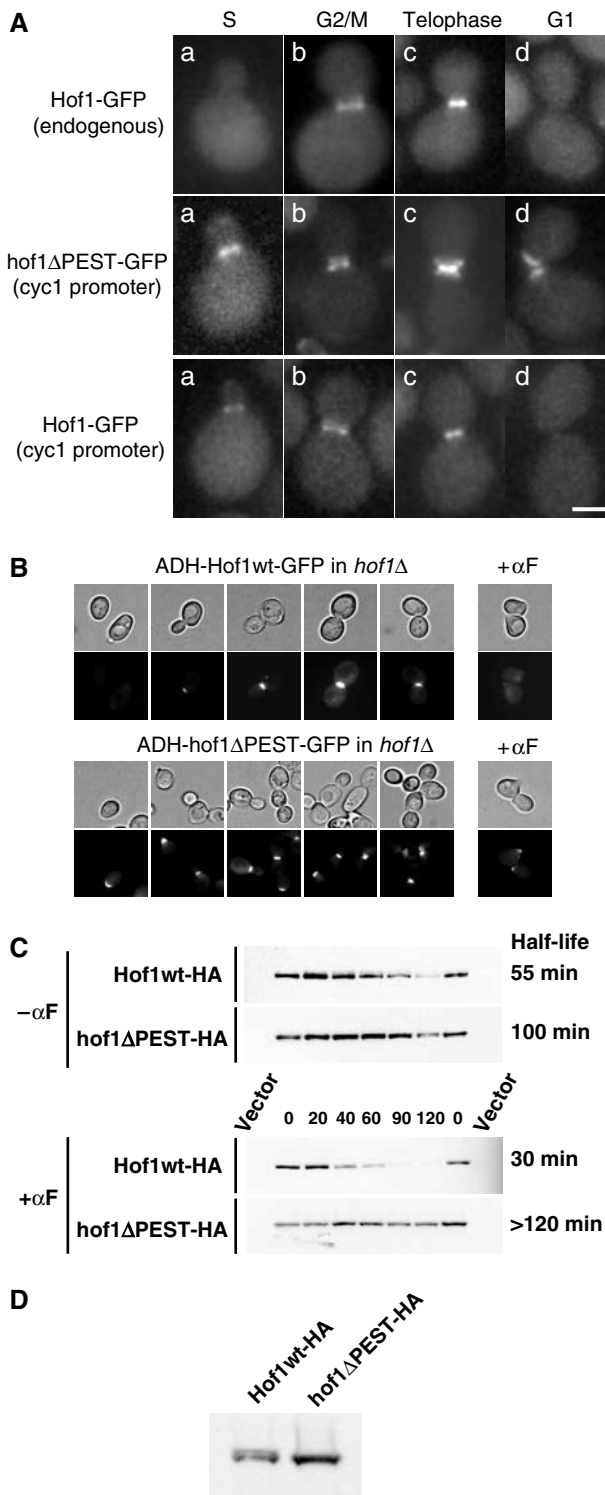


Figure 4 Cell cycle-dependent degradation of Hof1. (A, B) The localization of Hof1-GFP and hof1ΔPEST-GFP expressed from either the endogenous promoter or the constitutive *CYC1* (A) or *ADH* promoters (B) was analyzed in *hof1Δ* cells (YSB100) by GFP microscopy at different cell cycle stages. The scale bar in panel A represents 2 μm. Where indicated, the cells were arrested with α -factor ($+\alpha F$) for 90 min. (C) The half-life of Hof1-HA and hof1ΔPEST-HA in wild-type cells (K699) was determined by *GAL* shutoff experiments as described in Materials and methods. Times are listed in minutes after the addition of glucose (time 0) to turn off expression of Hof1-HA and hof1ΔPEST-HA. Where indicated, the cells have been arrested in G1 phase by α -factor ($+\alpha F$). An empty vector was used to control for the specificity of the Western blot. The half-life (in minutes) on the right was averaged from at least three independent experiments. (D) The phosphorylation status of Hof1-HA and hof1ΔPEST-HA expressed from the *ADH* promoter was determined by Western blot analysis of extracts prepared from asynchronous *hof1Δ* cultures.

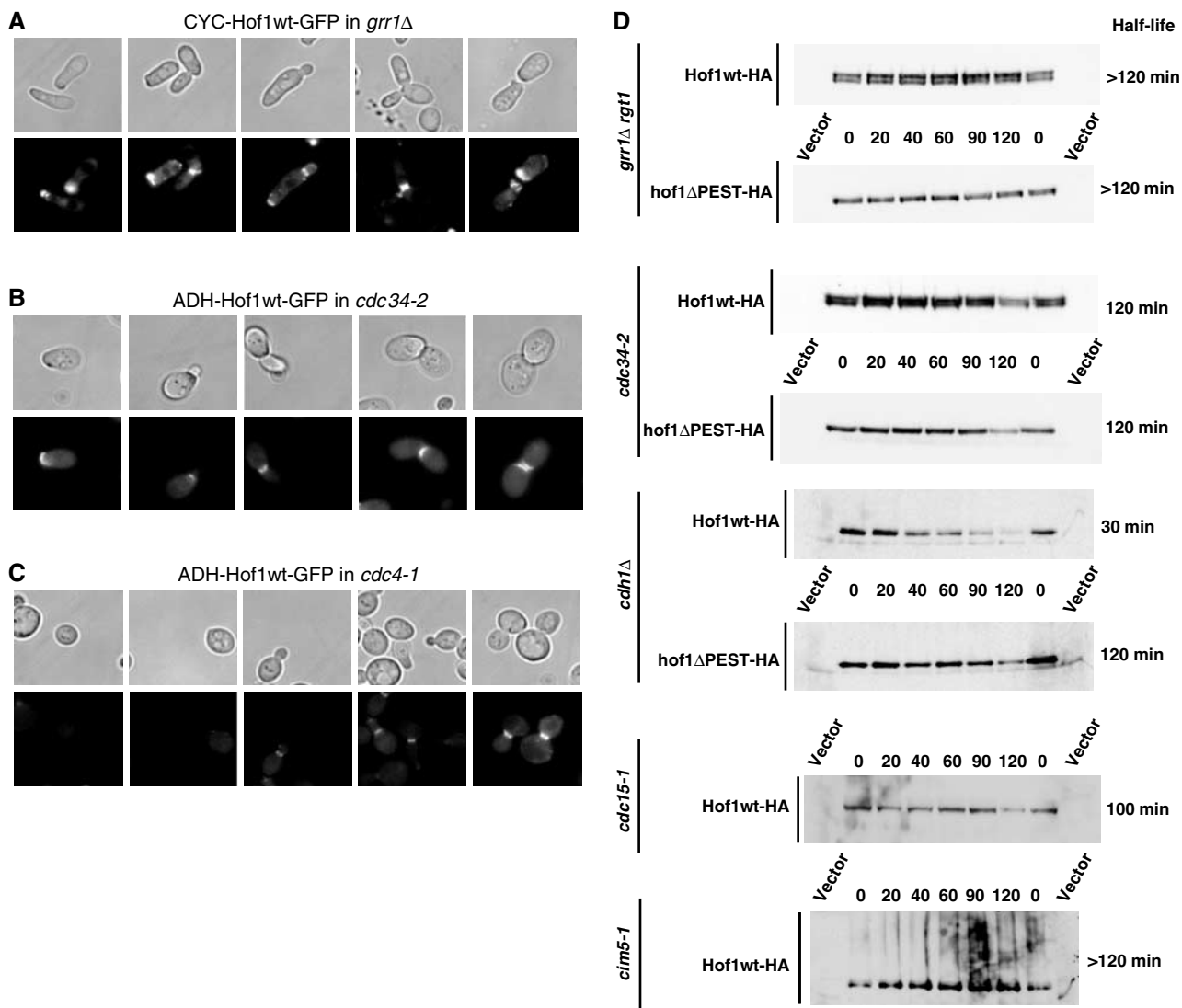


Figure 5 Hof1 degradation depends on SCF^{Grr1} and a functional MEN pathway. (A–C) *grr1Δ rgt1* (YMJ166 (A)), *cdc34-2* (YMT670 (B)) or *cdc4-1* (YMT668 (C)) cells expressing Hof1-GFP from the constitutive *CYC1* or *ADH* promoter were grown to early log phase in selective media at 30°C (semipermissive temperature), and analyzed by phase (upper panels) and GFP microscopy (lower panels). (D) The half-lives of HA-tagged Hof1 or hof1ΔPEST were determined by *GAL* shutoff experiments and the half-lives (in minutes) are shown on the right. The following strains were analyzed at 30°C (except where indicated): *grr1Δ rgt1* (YMJ166), *cdc34-2* (YMT670, 35°C), *cdh1Δ* (MJ1075), *cdc15-1* (YMP690) and *cim5-1* (YMP241, 35°C). Cells were treated with pheromones for 2 h (+ α F).

accumulated in its phosphorylated form in *grr1Δ rgt1* and *cdc34-2* cells, suggesting that the failure to degrade Hof1 is not caused by a defect in its phosphorylation. Hof1 was also stabilized in *cim5-1* cells analyzed at the restrictive temperature (35°C) (half-life >120 min), implying that Hof1 is degraded by the 26S proteasome. Interestingly, a smear of high molecular mass HA-Hof1 species accumulated in these cells, which may represent ubiquitinated species of Hof1. Consistent with this notion, such higher molecular weight Hof1 species were absent in *grr1Δ rgt1* or *cdc34-2* cells. Taken together, these results strongly suggest that Hof1 is ubiquitinated by SCF^{Grr1}, and specifically degraded by the 26S proteasome *in vivo*.

To test whether activation of the MEN is necessary for Hof1 degradation, we determined the half-life of HA-Hof1 in *cdc15-1* cells at semipermissive temperature (30°C). Interestingly, HA-Hof1 was stabilized (half-life approximately 100 min) and

as expected accumulated in its unphosphorylated form (Vallen *et al*, 2000), suggesting that phosphorylation of Hof1 at the end of mitosis may trigger its degradation. Cdc15 and/or Dbf2 may directly phosphorylate Hof1 or, alternatively, another MEN-dependent kinase(s) may be responsible for these modifications.

Degradation of Hof1 is required for efficient actin ring contraction and cell separation during cytokinesis

To determine whether downregulation of Hof1 at the end of mitosis is important for cell viability, we expressed wild-type Hof1 or stabilized hof1ΔPEST from its endogenous promoter or under the control of the constitutive *CYC*, *ADH* or *TEF* promoters (Funk *et al*, 2002) in *hof1Δ* cells. Expression of Hof1 from the *ADH* promoter resulted in protein levels comparable to levels obtained from the endogenous promo-

ter, while Hof1 is approximately three-fold overexpressed from the *TEF* promoter (data not shown). While constitutive expression of Hof1 in wild-type cells has no effect (Figure 6A), expression of *hof1ΔPEST* from the *ADH* or *TEF* promoters significantly diminished the growth rate. Likewise, *grr1Δ* cells expressing wild-type Hof1 from the constitutive *ADH* promoter were not viable (data not shown). Taken together, these results suggest that efficient inactivation of Hof1 at the end of mitosis is functionally important and is achieved both by transcriptional downregulation and ubiquitin-dependent proteolysis.

To determine why degradation of Hof1 is important for mitotic exit, we first analyzed the morphological phenotype of wild-type cells overexpressing *hof1ΔPEST* from the indu-

cible *GAL* promoter. As shown in Figure 6B, these cells arrested as large groups of cells that remained attached to each other, characteristic for a defect during cytokinesis. Interestingly, after cell wall digestion by zymolyase, *hof1Δ* and *hof1Δ* cells expressing stabilized *hof1ΔPEST* from the *ADH* promoter exhibited an increased number of cells arrested in cytokinesis (Figure 6C), implying that cytokinesis could not be efficiently completed and that the cells continue to share the same cytoplasm. To determine more precisely what process during cytokinesis may require degradation of Hof1, we studied wild-type cells expressing stabilized *hof1ΔPEST* from the *ADH* promoter by time-lapse microscopy (Figure 6D and E). As expected, the cells had no significant defects at bud emergence,

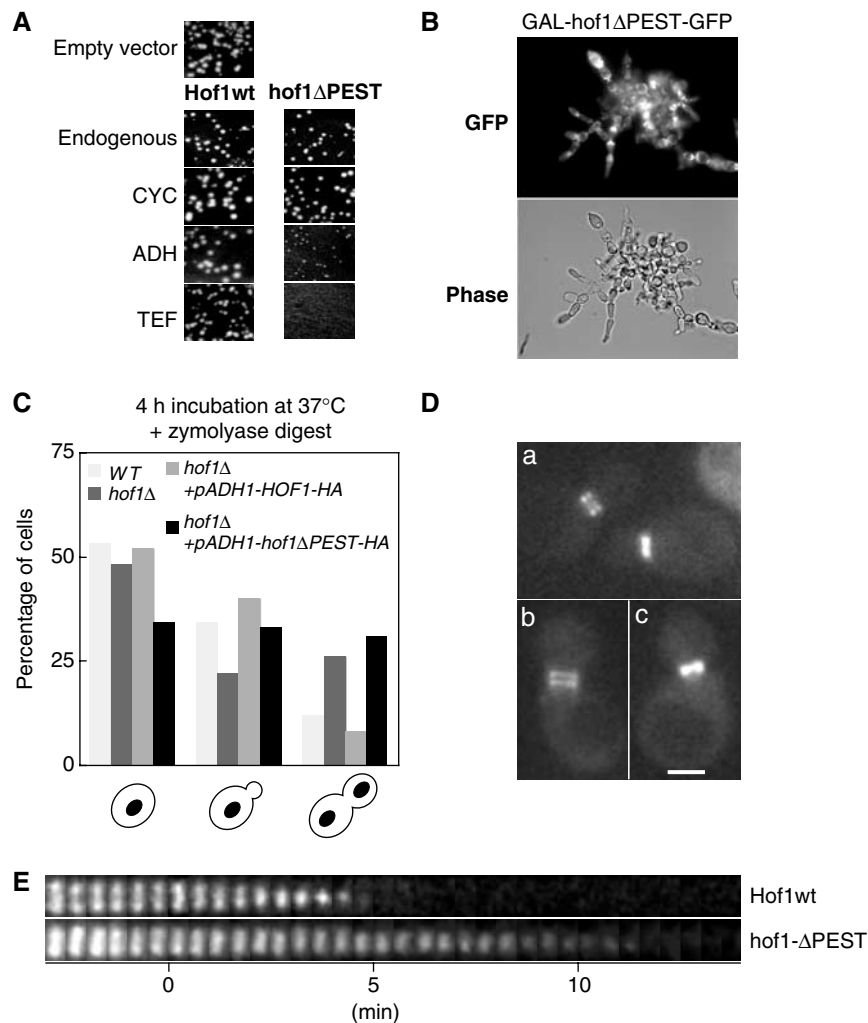


Figure 6 Stabilization of Hof1 inhibits efficient actomyosin contraction during cytokinesis. (A) Wild-type cells (K699) were transformed with the same quantity of an empty control vector (empty vector), or plasmids expressing either Hof1-GFP or *hof1ΔPEST*-GFP from the constitutive *CYC* (pBM360 and 365), *ADH* (pBM361 and 366) or *TEF* (pBM362 and 367) promoters. Sectors of the transformation plates were photographed after 3 days at 30°C. (B) Wild-type cells (K699) expressing *hof1ΔPEST*-GFP from the inducible *GAL* promoter were grown to early log phase in 2% raffinose, and photographed 24 h after addition of 2% galactose to induce expression of *hof1ΔPEST*-GFP. (C) Wild-type (S288C) or isogenic *hof1Δ* cells (DJ640), and *hof1Δ* cells with integrated plasmids expressing as indicated either Hof1-HA (DJ1600) or *hof1ΔPEST*-HA (DJ1608) from the *ADH* promoter were shifted to 37°C for 4 h, fixed, treated with zymolyase for 30 min and vortexed. Cell cycle stages were counted as shown in the graph. Note that expression of stabilized *hof1ΔPEST*-HA increases the number of cells arrested in cytokinesis. (D) The splitting of the septin ring was monitored using GFP-Cdc3 in cells expressing *pADH1-HOF1-HA* (a, DJ1600) cells and cells expressing *pADH1-hof1ΔPEST-HA* (b and c, DJ1608). The scale bar represents 2 μm. (E) Actomyosin ring (Myo1-CFP) assembly and contraction were analyzed in cells expressing *pADH1-HOF1-HA* (DJ1619, upper panel) and cells expressing *pADH1-hof1ΔPEST-HA* (DJ1618, lower panel). Cell cycle progression was monitored using a GFP-tagged septin (GFP-CDC3). Splitting of the septin ring marks the start point (0 min) and pictures were taken every 30 s.

and initiated anaphase with normal kinetics (data not shown). The splitting of the septin ring as assayed by Cdc3-GFP occurred efficiently (panel D; Dobbelaere and Barral, 2004). Wild-type Hof1 was degraded at this stage of cytokinesis, while stabilized hof1 Δ PEST abnormally accumulated between the two septin rings (data not shown). Interestingly, degradation of Hof1 was not required to assemble the actomyosin ring at the mother–bud neck (Figure 6E). However, time-lapse analysis of Myo1-CFP in cells expressing hof1 Δ PEST revealed that initiation of actomyosin ring contraction was significantly delayed compared to cell expressing wild-type Hof1. Moreover, subsequent disassembly of the actomyosin ring was slower compared to wild-type cells. Taken together, these results strongly suggest that degradation of Hof1 occurs after splitting of the septin ring, and is required for efficient contraction of the actomyosin ring and proper cell separation during cytokinesis.

Discussion

We show here that Grr1 functions during cytokinesis by promoting degradation of the PCH protein Hof1, demonstrating that SCF-ubiquitin ligases also function during mitosis in budding yeast. Because degradation of Hof1 requires prior phosphorylation in a MEN-dependent manner, SCF^{Grr1} may be part of a mechanism to ensure that cytokinesis cannot occur until chromosome segregation has been completed.

Specific subcellular localization of F-box proteins provides information on their function

SCF-type E3 ligases target a large variety of substrates for degradation by the 26S proteasome. Not surprisingly therefore, the common core subunits of yeast SCF complexes including Cdc53, Skp1, Cdc34 and Hrt1 are distributed in the nucleus and cytoplasm (Blondel *et al*, 2000). In contrast, several F-box proteins localize to specific cellular sites. Cdc4 and Met30 are nuclear proteins (Choi *et al*, 1990; Blondel *et al*, 2000; Rouillon *et al*, 2000) and it has been shown that SCF^{Cdc4} is active only in the nucleus, at least when tested for its substrates Far1, Sic1, Gcn4 and Cdc6 (Blondel *et al*, 2000; Pries *et al*, 2002; Luo *et al*, 2003). Interestingly, Grr1 has several substrates that localize to distinct cellular locations. The accumulation of Grr1 at the mother–bud neck region late in mitosis triggers degradation of Hof1, while the Cdc42 effector Gic2 is likely degraded at bud tips shortly after bud emergence (Jaquenoud *et al*, 1998). Because cytoplasmic Grr1 is able to complement the morphology defects of *grr1 Δ cells, it is likely that the G1 cyclins Cln1 and Cln2 are degraded in the cytoplasm (Miller and Cross, 2000). However, Grr1 is also found in the nucleus of cells throughout the cell cycle, and we have identified a specific region in the amino-terminus that contains functional NLS. Although these NLS sequences are not conserved in Grr1 orthologs from other species, we suspect that Grr1 in budding yeast may target an unknown nuclear protein for degradation. Grr1 is required for the repression of genes involved in glucose fermentation (Flick and Johnston, 1991), and it is possible that Grr1 may regulate a nuclear target involved in the regulation of gene expression.*

Degradation of Hof1 by SCF^{Grr1} is required for efficient actomyosin contraction and cell separation during cytokinesis

Several lines of evidence strongly suggest that SCF^{Grr1} plays a role in cytokinesis. First, Grr1 is recruited to the mother–bud neck region just after completion of anaphase but before cell separation initiates. Second, this ring localization depends on its substrate interacting domain (LRR). Third, *grr1 Δ cells accumulate with a G2 DNA content and exhibit clear cell separation defects (Barral *et al*, 1995). Finally, we have identified the PCH protein Hof1 as a likely substrate of SCF^{Grr1} and the 26S proteasome during cytokinesis. Hof1 is degraded at the end of mitosis, and remains unstable during the entire G1 phase of the cell cycle. Hof1 degradation depends on its PEST motif, which is required for its interaction with the F-box protein Grr1. Cells expressing the stabilized mutant version hof1 Δ PEST exhibit severe defects during cytokinesis. While assembly of the actomyosin structure and splitting of the septin ring occurred normally, contraction and subsequent ingression of the actomyosin ring were delayed and the cells were unable to separate their cytoplasm. This phenotype is reminiscent of the function of Imp2 in *S. pombe* (Demeter and Sazer, 1998), which is dispensable for actomyosin ring assembly but is required for proper contraction during cytokinesis. However, our data further suggest that SCF^{Grr1} may have substrates other than Hof1 during cytokinesis. For example, deletion of *HOF1* in *grr1 Δ cells did not suppress the cell separation defect, but instead exacerbated the cytokinesis defects (data not shown). Moreover, although the substrate-binding domain of Grr1 was required for its localization to the mother–bud neck, Grr1 still accumulated on the cytokinetic ring in *hof1 Δ cells (data not shown). Additional SCF^{Grr1} substrates may include Bni1, which contains PEST domains, and the PCH protein Bzz1, which was also found to interact with Grr1 by comprehensive two-hybrid analysis (Ito *et al*, 2001). Finally, SCF^{Grr1} may also be involved in the degradation of Gic1 (M Blondel, unpublished observation), which localizes to the mother–bud neck during cytokinesis (Chen *et al*, 1997).***

Dual regulation of Hof1 expression: cell cycle-dependent transcription and degradation

HOF1 transcription peaks in mitosis and is downregulated as cells exit from mitosis (Spellman *et al*, 1998). Our results show that this transcriptional regulation is not essential for cell viability as cells expressing the *HOF1* gene under the control of the constitutive *ADH* promoter are viable and grow with wild-type rates. Likewise, decreasing the rate of Hof1 degradation alone does not have deleterious effects, as expression of hof1 Δ PEST under the control of its normal promoter does not severely interfere with cell division. However, affecting both transcriptional regulation and proteolysis is toxic and blocks cytokinesis. These results indicate that yeast cells use two distinct and redundant pathways to downregulate Hof1 levels at the end of mitosis.

Hof1 degradation: a mechanism to couple cytokinesis to mitotic exit?

Binding of substrates to their F-box protein is often dependent on site-specific phosphorylation (Deshaies,

1997), providing the possibility to control protein degradation by regulating the kinase activity. Hof1 is heavily phosphorylated at the end of mitosis (Vallen *et al*, 2000), and this hyperphosphorylation depends on its PEST motif. PEST motifs are rich in proline, serine and threonine residues (Rechsteiner and Rogers, 1996), and are often directly phosphorylated. For example, the PEST motif of the G1 cyclin Cln2 is phosphorylated and regulates its interaction with SCF^{Grr1} (Lanker *et al*, 1996). Likewise, hof1ΔPEST was stable, most likely because the mutant protein fails to interact with Grr1. These results suggest that phosphorylation of Hof1 may regulate its binding to SCF^{Grr1}. Available results suggest that Hof1 is phosphorylated by a kinase dependent on the MEN pathway. Hyperphosphorylation of Hof1 correlates with the activation of the MEN pathway, and Hof1 is strongly stabilized and accumulated in an unphosphorylated form in MEN mutants including *cdc15-1* and *dbf2-2* cells. Interestingly, Dbf2 also requires MEN activity for its accumulation at the mother–bud neck (Frenz *et al*, 2000), raising the possibility that Hof1 may be a direct substrate of Mob1/Dbf2. However, our results indicate that Hof1 is unstable in α-factor-arrested cells, and Mob1/Dbf2 is not thought to be active during the G1 phase of the cell cycle (Visintin and Amon, 2001). Nevertheless, it is possible that Mob1/Dbf2 may initiate Hof1 degradation as cells exit from mitosis, and that another kinase phosphorylates Hof1 during G1. Taken together, these results suggest that activation of the MEN pathway after completion of anaphase triggers phosphorylation of Hof1 (and maybe other substrates of SCF^{Grr1}) at the mother–bud neck region. This allows its recognition by SCF^{Grr1} and subsequent degradation by 26S proteasomes. Because Hof1 inhibits actomyosin ring ingression, its degradation may trigger completion of cytokinesis. This mechanism may thus ensure that cell separation cannot be initiated as long as mitosis is not completed.

Degradation of PCH family members: a conserved mechanism to regulate cytokinesis?

PCH proteins constitute a conserved protein family including *S. pombe* Cdc15, Imp2 and YB65, *S. cerevisiae* Hof1 and Bzz1, *Mus musculus* PSTPIP, PSTPIP2, PACSIN and PACSIN 2 and *Homo sapiens* CIP4. Although these proteins share only around 20% sequence identity, they have the same domain structure. Most PCH family proteins contain an N-terminal FCH domain, coiled-coil domains followed by PEST sequences and finally one or more SH3 domains in their C-termini. Recent studies suggest that PCH proteins regulate actin-based processes and perhaps polarized secretion of membrane material during cytokinesis (Lippincott and Li, 2000). Several lines of evidence suggest that degradation of PCH proteins may be a widely conserved mechanism. First, all PCH members contain a conserved PEST domain. Second, transcriptional regulation of the *S. pombe* Hof1 ortholog Cdc15 is not essential (Utzig *et al*, 2000). However, overexpression of this protein is toxic and blocks cytokinesis, suggesting that an alternative pathway involving degradation of Cdc15 may also exist in *S. pombe*. Indeed, *S. pombe* Cdc15 is phosphorylated around the time of cytokinesis (Fankhauser *et al*, 1995) and these phosphorylations depend on its PEST domain (V Simanis, personal communication). Finally, overexpression of the *M. musculus* homolog PSTPIP2 is toxic in *S. pombe* cells (Spencer *et al*, 1997). Because Grr1 itself is a conserved protein among eukaryotes, it is possible that degradation of PCH proteins by SCF^{Grr1} or other SCF complexes may generally regulate cytokinesis in many organisms. However, although expression of the putative *S. pombe* Grr1 homolog Pof2 was able to at least partially complement the cryosensitivity of budding yeast *grr1Δ* cells, its inactivation in *S. pombe* did not result in any obvious cytokinesis phenotype (S Bamps and J Vandenhaute, unpublished observation).

Table I Strain list

Strain name	Relevant genotype	Background	Source
K699	Mat a	W303	Kim Nasmyth
YMP190	Mat a <i>cdc16-1</i>	W303	Kim Nasmyth
YMP809	Mat a <i>cdc14-3</i>	W303	Toni Hyman
YMP690	Mat a <i>cdc15-1</i>	W303	Ray Deshaies
YMP1118	Mat a <i>dbf2-2</i>	W303	Ray Deshaies
YSB100	Mat a <i>hof1::KANMX</i>	W303	This study
YMP2957	Mat a <i>grr1::LEU2</i>	W303	Jaquenoud <i>et al</i> (1998)
YMJ166	Mat a <i>grr1::LEU2 rgt1-101</i>	S288C	Carl Mann
YMT670	Mat a <i>cdc34-2</i>	W303	Mike Tyers
YMT740	Mat a <i>cdc53-1</i>	W303	Mike Tyers
YMT668	Mat a <i>cdc4-1</i>	W303	Mike Tyers
MJ1075	Mat a <i>cdh1::LEU2</i>	W303	Angelika Amon
YMP241	Mat a <i>cim5-1</i>	S288C	Ghislain <i>et al</i> (1993)
YSB101	Mat a <i>bni1::KANMX</i>	W303	This study
DJ640	Mat α <i>hof1::HIS3</i>	S288C	Dobbelaere and Barral (2004)
DJ641	Mat a <i>HOF1-GFP::His3MX6</i>	S288C	Dobbelaere and Barral (2004)
DJ1316	Mat a <i>MYO1-CFP::Kan ura3-52</i>	S288C	Dobbelaere and Barral (2004)
DJ1601	<i>pCYC1-hof1-PEST-GFP::URA3 hof1::HIS3</i>	S288C	This study
DJ1602	<i>pCYC1-HOF1-GFP::URA3 hof1::HIS3</i>	S288C	This study
DJ1600	<i>pADH1-HOF1-HA::URA hof1::HIS3</i>	S288C	This study
DJ1608	<i>pADH1-hof1-PEST-HA::URA hof1::HIS3</i>	S288C	This study
DJ1618	<i>pADH1-HOF1-PEST-HA::URA hof1::HIS3 Myo1-CFP::Kan</i>	S288C	This study
DJ1619	<i>pADH1-HOF1-HA::URA3hof1::HIS3 Myo1-CFP::Kan</i>	S288C	This study
DJ1620	<i>hof1::HIS3 Myo1-CFP::Kan</i>	S288C	This study

At present, the molecular understanding of how PCH proteins first promote and then inhibit efficient actomyosin contraction and cell separation during cytokinesis is still unclear and will be an important goal for future experiments.

Materials and methods

Yeast strains and genetic manipulations

Yeast strains are described in Table I. Strains are derived from K699: *Mat a*, *ade2-1*, *trp1-1*, *can1-100*, *leu2-3,112*, *his3-11,15*, *ura3*, *GAL+*, *psi+*, *ssd1-d2* (W303 background) or S288c: *ade2-101*, *ura3-52*, *lys2-801*, *trp1-Δ1*, *his3Δ200*, *leu2-Δ*. Standard yeast growth conditions and genetic manipulations were as described previously (Guthrie and Fink, 1991; Wach *et al*, 1994). The plasmids pDJ228, pDJ230, pDJ233 and pDJ234 were digested with *StuI* for integration at the *URA3* locus of the *hof1Δ* strain DJ640. To analyze actomyosin ring behavior, the strains harboring an HA-tagged Hof1 construct were crossed with a strain containing Myo1-CFP (DJ1316).

DNA manipulations, Western blots and antibodies

Standard molecular biology techniques were used for plasmid constructions (Table II) and details will be provided upon request. PCR reactions were performed with the Expand polymerase kit as recommended by the manufacturer (Roche), and confirmed by sequencing. Standard procedures were used for yeast cell extract preparation and immunoblotting. Antibodies against GFP (Roche) or HA (Babco) were used as recommended by the manufacturers.

Microscopy

GFP-tagged proteins were visualized using a Chroma GFP4 filter (excitation 455–495 nm) on an Olympus BX61 microscope, photographed with a Spot RT cooled CCD camera and analyzed with Photoshop 5.0 software (Adobe). Cells expressing GFP fusion proteins from the inducible *GAL1*, *10* promoter were grown to early log phase at 25°C in selective media containing raffinose (2% final concentration), at which time galactose was added (2% final concentration) for 5 h as previously described (Blondel *et al*, 1999). The ability of wild-type and mutant Grr1-GFP proteins to accumulate at the mother–bud neck region (ring) was quantified by analyzing for each construct at least 200 cells in late mitosis (as

Table II Plasmid list

pBM200	GAL GRR1wt-GFP URA3 CEN	Blondel <i>et al</i> (2000)
pJMG48	GAL GRR1wt-GFP TRP1 CEN	Blondel <i>et al</i> (2000)
pMJ200	GAL GFP TRP1 CEN	Jaquenoud <i>et al</i> (1998)
pBM210	GAL grr1(1–901)-GFP TRP1 CEN	This study
pBM211	GAL grr1(1–661)-GFP TRP1 CEN	This study
pBM213	GAL grr1(1–311)-GFP TRP1 CEN	This study
pBM223	GAL grr1(311–1150)-GFP TRP1 CEN	This study
pBM241	GAL grr1(180–1150)-GFP TRP1 CEN	This study
pLC45	GAL grr1(1–180)-GFP TRP1 CEN	This study
pLC50	ADH GRR1wt LEU2 CEN	This study
pLC51	ADH grr1(180–1150) LEU2 CEN	This study
pLC52	ADH grr1ΔFbox LEU2 CEN	This study
pLC53	ADH GRR1wt-GFP LEU2 CEN	This study
pLC13	HOF1wt-GFP TRP1 CEN	This study
pBM320	hof1Δ418–438-GFP TRP1 CEN	This study
pLC12	GAL HOF1wt -GFP TRP1 CEN	This study
pBM321	GAL hof1Δ418–438-GFP TRP1 CEN	This study
pBM325	HOF1wt-GFP URA3 CEN	This study
pBM326	hof1Δ418–438-GFP URA3 CEN	This study
pBM327	GAL HOF1wt-GFP URA3 CEN	This study
pBM328	GAL hof1Δ418–438-GFP URA3 CEN	This study
pBM351	GAL grr1ΔFbox-GFP TRP1 CEN	This study
pBM352	GAL grr1(1–792)-GFP TRP1 CEN	This study
pBM360	CYC1 HOF1wt-GFP URA3 CEN	This study
pBM361	ADH HOF1wt-GFP URA3 CEN	This study
pBM362	TEF HOF1wt-GFP URA3 CEN	This study
pBM365	CYC1 hof1Δ418–438-GFP URA3 CEN	This study
pBM366	ADH hof1Δ418–438-GFP URA3 CEN	This study
pBM367	TEF hof1Δ418–438-GFP URA3 CEN	This study
pBM370	pEG203 HOF1 wt (from plasmid DNA)	This study
pBM371	pEG203 hof1Δ418–438	This study
pBM375	pEG203 HOF1 wt (from genomic DNA)	This study
pBM385	GAL HA ³ TRP1 CEN	This study
pBM390	GAL HOF1wt-HA ³ TRP1 CEN	This study
pBM391	GAL hof1Δ418–438-HA ³ TRP1 CEN	This study
pBM400	pACT2 HOF1wt	This study
pBM401	pACT2 hof1Δ418–438	This study
pBM402	pGBT9 GRR1wt	This study
pBM405	GAL HOF1wt-HA ³ URA3 CEN	This study
pBM406	GAL hof1Δ418–438-HA ³ URA3 CEN	This study
pSOB1	GAL linker-CFP(155–238) TRP1 CEN	This study
pSOB2	GAL linker-YFP(1–173) URA3 CEN	This study
pSOB4	GAL HOF1wt-linker-YFP(1–173) URA3 CEN	This study
pSOB11	GAL GRR1wt-linker-CFP(155–238) TRP1 CEN	This study
pSOB15	GAL hof1Δ418–438-linker-YFP(1–173) URA3 CEN	This study
pDJ63	GFP-CDC3wt LEU2 CEN	Dobbelaere <i>et al</i> (2003)
pDJ228	CYC1-HOF1wt-GFP URA3	This study
pDJ230	CYC1-hof1Δ418–438-GFP URA3	This study
pDJ233	ADH-HOF1wt-HA ³ URA3	This study
pDJ234	ADH-hof1Δ418–438-HA ³ URA3	This study

judged by DIC). For the BiFC experiments, wild-type cells expressing either wild-type Hof1 or hof1ΔPEST fused to the N-terminal part of YFP (1–173) together with Grr1 fused to the C-terminal part of CFP (155–238) were analyzed using a Chroma GFP4 filter (Hu and Kerppola, 2003).

Time-lapse movies were acquired at room temperature unless noted otherwise, with a BX51 Olympus microscope connected to a Till Vision monochromator. All pictures/movies were the result of the projection of five different stacks. The examined strains were grown overnight on YPD plates, and then put on an agarose pad containing nonfluorescent medium (Dobbelaere *et al*, 2003). Analysis of septin ring behavior in both wild-type (S288C) and hof1ΔPEST (DJ1608) strains was performed after shifting the cells for 1 h to 35°C and then mounting them under a preheated microscope. These strains were transformed with a plasmid containing GFP-CDC3 (pDJ63). Actomyosin ring contraction was analyzed at 30°C in at least five different movies for each strain.

Cytokinetic assays

Cytokinetic assays were performed essentially as described previously (Dobbelaere *et al*, 2003). Briefly, S288C, DJ640, DJ1608 and DJ1600 were grown overnight at room temperature in YPD. Cells were diluted to OD = 0.1 in fresh YPD and grown for 1 h to reach ±OD = 0.2. Cells were shifted to 37°C and samples were taken every hour, fixed with ethanol and stained with DAPI. To analyze if cells were connected to each other, ethanol-fixed cells were treated

with zymolyase (2 mg/ml) for 30 min at 30°C. Cells were vortexed extensively and then stained with DAPI. Cell cycle stages were determined microscopically and compared before and after zymolyase treatment.

Protein half-life determination

Half-lives were measured as described previously (Blondel *et al*, 2000) in strains that carry plasmids expressing HA-tagged Hof1 or hof1ΔPEST from the inducible *GAL1,10* promoter. Half-life measurements by CX chase were performed as described (Galan and Peter, 1999).

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