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Functional overlap among distinct G1/S inhibitory pathways allows robust G1 arrest by yeast mating pheromones

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ABSTRACT In budding yeast, mating pheromones arrest the cell cycle in G1 phase via a pheromone-activated Cdk-inhibitor (CKI) protein, Far1. Alternate pathways must also exist, however, because deleting the cyclin CLN2 restores pheromone arrest to far1Δ cells. Here we probe whether these alternate pathways require the G1/S transcriptional repressors Whi5 and Stb1 or the CKI protein Sic1, whose metazoan analogues (Rb or p27) antagonize cell cycle entry. Removing Whi5 and Stb1 allows partial escape from G1 arrest in far1Δ cln2Δ cells, along with partial derepression of G1/S genes, which implies a repressor-independent route for inhibiting G1/S transcription. This route likely involves pheromone-induced degradation of Tec1, a transcriptional activator of the cyclin CLN1, because Tec1 stabilization also causes partial G1 escape in far1Δ cln2Δ cells, and this is additive with Whi5/Stb1 removal. Deleting SIC1 alone strongly disrupts Far1-independent G1 arrest, revealing that inhibition of B-type cyclin-Cdk activity can empower weak arrest pathways. Of interest, although far1Δ cln2Δ sic1Δ cells escaped G1 arrest, they lost viability during pheromone exposure, indicating that G1 exit is deleterious if the arrest signal remains active. Overall our findings illustrate how multiple distinct G1/S-braking mechanisms help to prevent premature cell cycle commitment and ensure a robust signal-induced G1 arrest.

INTRODUCTION Cell cycle progression in all organisms is regulated by both internal and external cues. In eukaryotes, the G1 phase of the cell cycle provides a critical period in which cells monitor whether conditions are appropriate for entry into a new division cycle (Morgan, 2007). Signals that control this decision include positive and negative growth factors, differentiation triggers, nutrient levels, and environmental stresses. These regulatory signals either promote or prevent the transition from a stable G1 state to a new round of DNA synthesis and mitosis. Often, cells become insensitive to these regulatory signals once they initiate the G1/S transition, establishing a cell cycle commitment phenomenon known as Start in yeast and the Restriction Point in animal cells (Hartwell et al., 1974; Pardee, 1974; Cross, 1995; Blagosklonny and Pardee, 2002). Accordingly, signals that influence cell cycle entry generally affect the molecular machinery that controls the G1/S transition. In all eukaryotes cell cycle transitions are promoted by cyclin-dependent kinases (Cdks), as well as by coordinate changes in gene expression, and these promoting factors are often held in check by inhibitory molecules to ensure that cell cycle progression is carefully controlled (Morgan, 2007). The overall architecture of the G1/S regulatory network is strongly conserved throughout eukaryotes, although some of the individual components may have evolved separately in yeasts versus animals (Cross et al., 2011).

In the budding yeast Saccharomyces cerevisiae, a single Cdk, Cdc28, associates with nine different cyclins that help drive distinct cell cycle events (Bloom and Cross, 2007). The transition from G1 to S phase is predominantly controlled by the G1 cyclin Cln3 along with two G1/S cyclins, Cln1 and Cln2, whereas the subsequent events of DNA synthesis and mitosis (in S and M phases) are driven...
by six B-type cyclins (Clb1–Clb6). The decision of yeast cells to enter a new cell cycle can be profoundly influenced by the presence of an external cue known as mating pheromone, which promotes fusion of two haploid mating partner cells (Hartwell, 1973). During this mating reaction, pheromone activates an intracellular signaling pathway that arrests the cell cycle in G1 phase, before Start (Dohlman and Thorner, 2001; Bardwell, 2005). An important factor in this G1 arrest pathway is the protein Far1, as far1Δ cells do not arrest in response to pheromone (Chang and Herskowitz, 1992). Far1 is believed to be a Cdk inhibitor (CKI) protein that blocks the activity of Cln-Cdc28 complexes and thereby prevents progression through Start (Peter et al., 1993; Peter and Herskowitz, 1994; Tyers and Futcher, 1993; Jeoung et al., 1998), although some findings conflict with this interpretation (Gartner et al., 1998). Of note, however, in some circumstances Far1 is dispensable for G1 arrest. For example, removing the G1/S cyclin Cln2 from far1Δ cells (i.e., far1Δ cln2Δ) restores pheromone-induced G1 arrest (Chang and Herskowitz, 1992; Cherkasova et al., 1999). Thus, even in the complete absence of Far1, pheromone signaling still can interfere with the ability of cells to pass Start and enter a new division cycle. Despite early recognition of this fact, the molecular mechanisms responsible for Far1-independent arrest remain obscure.

In this study, we probe the Far1-independent arrest mechanisms more closely in order to better understand how pheromone signaling regulates G1 arrest and provide more general insights into the multiplicity of factors that control cell cycle commitment decisions. We reasoned that pheromone arrest might involve known negative regulators of the G1/S transition that act as "brakes" to antagonize cell cycle entry in many eukaryotes (Morgan, 2007). One such negative regulator is the CKI protein Sic1 (Donovan et al., 1994; Schwob et al., 1994), which is functionally analogous to the mammalian CKI p27(Kip1) (Sherr and Roberts, 1999). During G1, these CKI proteins inhibit Cdk's bound to B-type cyclins and thereby prevent premature entry into S phase. This inhibition is eventually released in late G1, when cyclin-Cdk activity reaches levels sufficient to target the CKI for degradation (Schwob et al., 1994; Nash et al., 2001; Cross et al., 2007; Koivomagi et al., 2011). Another mode of negative regulation involves transcriptional repression of genes expressed at the G1/S boundary. In animal cells, transcription of G1/S genes is activated by the E2F family of heterodimeric transcription factors and repressed by members of the retinoblastoma protein (Rb) family (Frolov and Dyson, 2004; van den Heuvel and Dyson, 2008). Yeast cells have an analogous system (Bahler, 2005; Wittenberg and Reed, 2005), in which G1/S transcription is driven by two heterodimeric transcription factors called SBF (Swi4-Swi6) and MBF (Mbp1-Swi6) and is inhibited in early G1 by the repressors Whi5 and Sbf1 (Koch et al., 1996; Costanzo et al., 2003, 2004; de Bruin et al., 2004, 2008; Bean et al., 2005). These repressors block the action of DNA-bound SBF and MBF in part by recruiting histone deacetylases (Huang et al., 2009; Takahata et al., 2009; Wang et al., 2009); in late G1, they are dissociated via Cdk phosphorylation, allowing SBF/MBF-dependent transcription to ensue (Costanzo et al., 2004; de Bruin et al., 2004; Wagner et al., 2009; Doncic et al., 2011).

Important targets of SBF and MBF include the G1/S cyclin genes CLN1 and CLN2, which yields increased G1/S Cdk activity, thereby creating a positive feedback loop that helps to ensure a decisive G1/S transition (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Skotheim et al., 2008). MBF/SBF-regulated genes are not expressed in pheromone-arrested cells, regardless of whether the arrest is Far1 dependent or independent (Wittenberg et al., 1990; Cherkasova et al., 1999), but it was unclear whether this inhibited transcriptional state is a cause or an effect of G1 arrest.

Here we report that the CKI Sic1 is strongly required for arrest in the absence of Far1, although not when Far1 is present. The transcriptional repressors Whi5 and Sbf1 also contribute to Far1-independent arrest, in a manner that is additive with the pheromone-induced degradation of Tec1, a transcriptional activator of CLN1. Our results reveal that multiple, functionally overlapping regulatory circuits control the sensitivity of the G1/S transition to the pheromone arrest signal. Collectively these multiple pathways provide robustness to the G1 arrest response, which may help to ensure that commitment to cell cycle entry occurs decisively rather than tentatively.

**RESULTS**

**Studying Far1-independent G1 arrest using synchronous cultures**

To test the contribution of various regulatory factors to pheromone arrest in both the presence and absence of Far1, we used synchronous cultures to measure the duration of G1 phase. The essential cell cycle gene CDC20 was placed under control of a regulated promoter (P<gal1>, allowing cells to be arrested in M phase and then released in the presence or absence of pheromone. Cell cycle progression was monitored by measuring DNA content (using flow cytometry) and/or by scoring budding. This approach offered three useful features: 1) by measuring G1 duration, we could detect even relatively subtle and temporary effects on the G1/S transition; 2) by using synchronized cells, we could readily assess the uniformity of phenotypes; and 3) we could distinguish specific effects of pheromone on G1 arrest from effects on other cell cycle stages or cell viability (which will become important later).

In this experimental context (Figure 1A), most cells finish mitosis and enter G1 (i.e., 1C DNA) by 30–60 min after release from the M-phase block and then begin a new round of DNA synthesis roughly 30 min later (at 90 min). If pheromone is added, wild-type cells complete mitosis and then arrest in G1 (for >3 h). To compare multiple different strains and replicate experiments, we plotted the percentage of cells with 2C DNA content as a function of time (Figure 1B). Generally, M-phase–arrested cultures were 80–90% 2C, and by 60 min after release they had cycled back to G1 and were predominantly 1C (i.e., only 10–15% 2C). As expected, far1Δ cells did not arrest in G1 in the presence of pheromone (Figure 1B). In addition, with or without pheromone, they showed an accelerated return to the 2C state after completing mitosis, consistent with previous findings that Far1 can alter the timing of the G1/S transition even without pheromone treatment; Alberghina et al., 2004.) In contrast, when far1Δ cln2Δ cells were released in the presence of pheromone, they remained in G1 for an extended period (Figure 1B). This arrest was not as strong as in wild-type or FAR1 cln2Δ strains, as evidenced by the gradual increase in cells with 2C DNA content beginning at 120–150 min after release. Thus G1 arrest in the far1Δ cln2Δ cells is partially leaky, but pheromone clearly imposes a durable G1 delay that affects the majority of cells in the culture.

Separately, we tested how Far1 affects the window of time in which cells commit to a new division cycle (Figure 1C). After release of cultures from the M-phase block, aliquots were removed at intervals and treated with pheromone to test whether the cells could still arrest in G1 or were already committed to division. In wild-type and cln2Δ strains, cells transitioned from fully uncommitted (>95% arrest) to substantially committed (<65% arrest) between the 45- and 60-min time points. In the far1Δ cln2Δ strain, two differences were evident (Figure 1C). First, the commitment point occurred roughly...
Role of G1/S transcriptional repressors in G1 arrest

Previous studies found that expression of G1/S transcripts is inhibited in pheromone-arrested cells (Wittenberg et al., 1990), even when the arrest is Far1 independent (Cherkasova et al., 1999). Yet it was unclear whether this inhibition is a cause or a reflection of the G1 arrest. Hence, to determine whether Far1-independent arrest relies on control of G1/S transcription, we probed the role of Whi5 and Stb1, the repressors of the G1/S transcription factors SBF and MBF. Deletion of STB1 alone produced no discernible change in either FAR1 cln2Δ or far1Δ cln2Δ strains (Figure 2, A–D). Deletion of WHI5 had no effect in FAR1 cln2Δ cells (Figure 2A), but in far1Δ cln2Δ cells (Figure 2C) it allowed a greater fraction of cells to escape G1 arrest (e.g., 38 vs. 24% 2C at 240 min). Assays using bud emergence as a marker of cell cycle progression yielded largely similar results, except that the escape phenotype caused by removing Whi5 was even more evident, and there was further enhancement when both Whi5 and Stb1 were removed (Figure 2D). Thus the transcriptional repressors contribute to Far1-independent G1 arrest. Despite these effects, the escape phenotypes were only partial, in that pheromone still imposed a significant G1 delay in the majority of cells.

We also tested the role of Mbp1, which is the DNA-binding component of the MBF heterodimer (Bahler, 2005). Although Mbp1 is required for transcriptional activation by MBF, it is also required for full repression of MBF-bound genes (Koch et al., 1993; Bean et al., 2005; de Bruin et al., 2006). We found that removing Mbp1 from far1Δ cln2Δ cells caused a more complete arrest rather than increased escape (Figure 2E), suggesting that either its role as an
activator outweighs its role as a repressor in this setting or its removal allows stronger gene repression during pheromone treatment, possibly due to increased binding of SBF to MBF target genes (de Bruin et al., 2006). Deleting WHI5 from these far1Δ cln2Δ mbp1Δ cells allowed increased escape from G1 arrest (Figure 2E), with an especially strong difference (i.e., with vs. without Whi5), although the effect remained partial such that the majority of cells still arrested.

Whi5 and Stb1 repress transcription in part through the recruitment of the histone deacetylase Rpd3 (Huang et al., 2009; Takahata et al., 2009; Wang et al., 2009). To determine whether the reduced arrest proficiency of the far1Δ cln2Δ whi5Δ strain was due to the loss of Rpd3 recruitment, we tested far1Δ cln2Δ rpd3Δ strains (Figure 2F). In fact, deletion of Rpd3 did not increase escape from G1 arrest and instead seemed to make G1 exit even slower, although this may reflect a slightly decreased growth rate of rpd3Δ mutants. Furthermore, Rpd3 is not required for the G1 arrest role of Whi5, because removing Whi5 from the far1Δ cln2Δ rpd3Δ strain still led to increased escape, indicating that the whi5Δ phenotype cannot be attributed solely to a defect in Rpd3 recruitment.

FIGURE 2: Partial role for transcriptional repressors in Far1-independent arrest. (A) Removal of Whi5 and/or Stb1 does not affect G1 arrest when Far1 is present. P_GAL1-CDC20 strains in the FAR1 cln2Δ background were arrested in M phase and released in the presence or absence of pheromone. Cell cycle progression and G1 arrest were measured by the flow cytometry assay of DNA content. Graphs show mean ± SEM (n = 4–8). (B) The strains in A were tested for G1 arrest by the budding assay. Budding was scored 120 min after release from M-phase arrest in the presence of α-factor. Bars, mean ± SEM (n = 3–4). (C) Removal of Whi5 partially compromises Far1-independent arrest. Cell cycle progression and G1 arrest were measured in far1Δ cln2Δ strains by the DNA assay. Graphs show mean ± SEM (n = 3–4). (D) Strains from C were tested for G1 arrest by the budding assay as in B. Bars show mean ± SEM (n = 6). (E) Mbp1 is not required for Far1-independent arrest or for the role of Whi5. G1 arrest was measured by the DNA (left) and budding (right) assays. Data points, mean ± SEM (n = 3). (F) Rpd3 is not required for the role of Whi5. Graphs show mean ± SEM (n = 4) for far1Δ cln2Δ and far1Δ cln2Δ rpd3Δ or mean ± range (n = 2) for far1Δ cln2Δ rpd3Δ whi5Δ.

P. A. Pope and P. M. Pryciak

Molecular Biology of the Cell

3678
Collectively these results show that Whi5 and Stb1 are not required for the strong G1 arrest seen when Far1 is present, but they contribute to the weaker G1 arrest observed in cells that lack Far1. Even so, their removal from far1Δ cln2Δ cells causes only a partial defect in G1 arrest, indicating that pheromone signaling can still inhibit the G1/S transition in the absence of both Far1 and these transcriptional repressors.

Loss of repressors only partially derepresses transcription

We considered two possible explanations for why removing the transcriptional repressors did not fully eliminate G1 arrest in far1Δ cln2Δ cells: 1) pheromone signaling might still be able to inhibit G1/S transcription even without the repressors; and 2) the G1/S transcripts could be fully derepressed, but pheromone signaling might exert nontranscriptional effects that inhibit exit from G1. To test these possibilities, we analyzed G1/S transcript levels via real-time quantitative PCR (RT-qPCR). We chose five representative genes (CLN1, YOX1, POL1, RNR1, and CDC21) that are induced by SBF and/or MBF at the G1/S transition (Bean et al., 2005; de Bruin et al., 2006; Eser et al., 2011). For comparison, we also monitored a gene expressed at the earlier M/G1 boundary (SIC1). We first conducted single-time-course experiments for eight different strains, in which we measured transcript levels at numerous time points in synchronous cultures (Figure 3). Then we analyzed the most informative time points in multiple independent trials (Figure 4).

In cells released from mitosis without pheromone, the M/G1 and G1/S transcripts peaked at 30 and 60 min, respectively (Figure 3). This agrees with the timing of DNA synthesis and budding (which begin at 60–90 min). Adding pheromone prevented the G1/S peak in FAR1 cln2Δ cells, and instead these transcripts declined to a minimum at 60 min and remained low for up to 4 h (Figure 3). In the absence of Far1 (far1Δ cln2Δ cells), pheromone still inhibited the G1/S peak, but after 60 min these transcripts gradually increased, reaching levels that were higher than the corresponding FAR1 cln2Δ cells but below the peak levels in untreated cells. This gradual increase is consistent with the leaky arrest phenotype of far1Δ cln2Δ.
cells. As expected for transcriptional repressors, removal of Whi5 and/or Stb1 allowed G1/S transcripts to start increasing earlier and/or to reach elevated levels in pheromone-treated cells. More specifically, the stb1Δ mutation seemed to reduce the pheromone-mediated decrease in all transcripts upon release from the mitotic block, whereas the whi5Δ mutation was more selective for specific transcripts. Nevertheless, in the absence of either repressor, or both simultaneously, pheromone still interfered with peak gene expression. These patterns of partial derepression were seen in both FAR1 cln2Δ and far1Δ cln2Δ backgrounds, although inhibition by pheromone was generally more potent and durable when Far1 was present. Clearly, pheromone signaling can prevent peak G1/S transcription even without Whi5 and Stb1.

For further analysis we performed multiple repetitions of each time course experiment and then measured mRNA levels at 60 min after release from M phase (Figure 4), as this was when expression peaked in the absence of pheromone. Without pheromone, G1/S transcripts reached roughly the same peak level in all strains (Figure 4), consistent with previous findings that Whi5 and Stb1 only slightly affect peak expression (Costanzo et al., 2003, 2004; de Bruin et al., 2004, 2008; Takahata et al., 2009). However, derepression was clearly evident in the pheromone-treated samples, as G1/S transcripts were no longer fully repressed when Whi5 and/or Stb1 were absent. Of note, however, even in the absence of both Whi5 and Stb1, G1/S transcript levels in pheromone-treated cells did not reach the maximum seen in untreated samples. Therefore inactivation of these repressors is not sufficient for full expression of G1/S transcripts, as pheromone signaling can still prevent their full expression. For several mRNAs the highest levels were seen in the far1Δ cln2Δ whi5Δ and far1Δ cln2Δ stb1Δ whi5Δ strains, which agrees with the finding that these strains have the strongest G1 escape phenotype, yet the ability of pheromone to prevent peak expression in these strains also agrees with the finding that even the strongest G1 escape phenotypes were partial.

The mRNA analyses revealed striking synergy between Far1 and Whi5 (Figure 4). That is, for several genes there was an additive effect of removing both proteins (i.e., far1Δ cln2Δ whi5Δ), whereas pheromone treatment could still exert a strong repressive effect if either one was present (i.e., FAR1 cln2Δ whi5Δ or far1Δ cln2Δ WHI5). Again, this correlates with the arrest behavior, in that removing Whi5 caused a notable escape phenotype only when Far1 was absent. By contrast, the derepression caused by removing Stb1 was not further enhanced when Far1 was also removed (compare FAR1 cln2Δ stb1Δ with far1Δ cln2Δ stb1Δ), and the additive relationship between Far1 and Whi5 did not require Stb1 (compare FAR1 cln2Δ stb1Δ whi5Δ with far1Δ cln2Δ stb1Δ whi5Δ). Together these results suggest that Far1 and Whi5 contribute additively to transcriptional repression, with a corresponding additive effect on G1 arrest. Because Far1 is believed to inhibit Cln-Cdk activity (Peter et al., 1993; Peter and Herskowitz, 1994; Tyers and Futcher, 1993; Jeoung et al., 1998), the finding that it contributes to transcriptional repression even in the absence of Whi5 and Stb1 suggests that Cdk activity promotes G1/S transcription by additional mechanisms distinct from repressor displacement (see Discussion).

The CLN1 transcription factor Tec1 antagonizes G1 arrest

One route by which pheromone might inhibit G1/S transcription could involve the ability of the pheromone-activated mitogen-activated protein kinase (MAPK) Fus3 to trigger degradation of the transcription factor Tec1 (Bao et al., 2004; Bruckner et al., 2004; Chou et al., 2004). Because Tec1 positively regulates CLN1 expression

FIGURE 4: Loss of repressors only partially derepresses transcription. G1/S transcripts were assayed at a fixed time corresponding to the transition from G1 to S phase. The PGAL1-CDC20 arrest/release experiments shown in Figure 3 were repeated three times, and mRNA levels were measured before and 60 min after release in either the presence or absence of α factor. Bars, mRNA levels (mean ± SD; n = 3) at the 60-min time points, expressed relative to the levels in the M-phase–arrested cells. The effects of Whi5 and Stb1 were compared in FAR1 cln2Δ (left) and far1Δ cln2Δ (right) backgrounds. Statistical analysis of all 12 pairwise comparisons, using the Benjamini–Hochberg false discovery rate method, is provided in Supplemental Table S1. The most pertinent points are 1) the inhibition by α factor is statistically significant (q < 0.05) for each of the eight individual genotypes and 2) the derepression due to removal of Whi5/Stb1 is significant in most cases except for comparisons involving the far1Δ cln2Δ whi5Δ strain, for which greater variability prevents a firm conclusion, but the effect of Whi5 is separately supported by comparing far1Δ cln2Δ stb1Δ with far1Δ cln2Δ stb1Δ whi5Δ (e.g., q = 0.007 for CLN1 mRNA).
DNA content was monitored at the indicated times. Graphs show mean (pPP680, pPP4051). Cells were released from the mitotic block into medium containing α-factor, range; the two strains at right (±n containing two-tailed unpaired TEC1 (wild type and T273M) sets were ranked significant by a together in a set of experiments separate from the four strains at left (C) PGAL1-CDC20 selective media (SC –Ura), overlaid with filter disks containing 20 μl of TEC1 plasmids (pPP681, pPP4042, pPP4043) were introduced into the (A) Empty vector or pPP4050, pPP4051) were introduced into the indicated PGAL1-CDC20 strains (PPY2014, PPY2063, YPAP165, YPAP171). Cells were released from the mitotic block into medium containing α-factor, and the percentage of cells that escaped G1 arrest was measured by scoring budding after 120 min. Bars, mean ± range; the two strains at right (n = 3) were assayed together in a set of experiments separate from the four strains at left (n = 2–5). In each strain, differences between vector and TEC1 (wild type and T273M) sets were ranked significant by a two-tailed unpaired t test (from left to right, p = 0.008, 0.009, 0.014, 0.002, 0.005, 0.0002). (C) PGAL1-CDC20 strains (PPY2014, YPAP171) contained empty vector or a TEC1-T273M plasmid (pPP680, pPP4051). Cells were released from the mitotic block into medium containing α-factor, and DNA content was monitored at the indicated times. Graphs show mean ± range of two separate experiments (some error bars are smaller than symbols).

(Stanley and Sprague, 2012). Nonetheless, the similar effects of both extra and stabilized Tec1 agree with the notion that pheromone arrest depends in part on blocking Tec1 accumulation. Taken together, our results indicate that Far1-independent arrest is promoted by two parallel pathways that act additively to reduce G1/S gene expression: Whi5/Stb1-mediated repression of SBF/MBF genes, and pheromone-triggered degradation of the CLN1 transcription factor Tec1.

Finally, to assess how strongly Far1-independent pathways can contribute to arrest in an otherwise wild-type cell, we tested the same TEC1 plasmids in a strain with intact copies of both FAR1 and CLN2. Remarkably, the TEC1-T273M mutant caused much stronger pheromone resistance in FAR1 CLN2 cells than in FAR1 cln2Δ cells (Figure 5, A, leftmost vs. rightmost columns, and B, right). Thus the same pathway can promote arrest in both far1Δ cln2Δ cells and wild-type cells. In addition, these results reveal an interesting parallel between Far1-dependent and Far1-independent pathways, as the pheromone resistance phenotype of either far1Δ or TEC1-T273M is suppressed by removing Cln2.

**Sic1 plays a strong role in Far1-independent arrest**

Another regulator of the G1/S transition is the protein Sic1, a CKI that inhibits S-phase cyclin-Cdks during early G1 and is inactivated in late G1 via phosphorylation by G1/S cyclin-Cdks. Because of its role as an antagonist of the G1/S transition, we tested whether Sic1 is involved in the Far1-independent arrest by pheromone (Figure 6). (These experiments exclusively used the budding assay because the sic1Δ mutation caused extremely broad DNA flow cytometry profiles in PGAL1-CDC20 strains, which obscured the analysis.) Indeed, although deleting SIC1 had no effect on G1 arrest when Far1 was present (Figure 6A), it had a substantial effect on Far1-independent arrest (Figure 6B). That is, in far1Δ cln2Δ sic1Δ cells the ability of...
Strickfaden et al., 1997), and a Cdk-resistant form of the signaling antagonist of S- and M-phase cyclins, namely, the APC component Cdh1, which inhibits accumulation of B-type cyclins during G1 (Visintin et al., 1997; Morgan, 2007). The presence of Cdh1 in the far1Δ cln2Δ background caused a negligible change in G1 escape (Figure 6B), although for unknown reasons we did observe an increased escape phenotype in rare isolates (legend to Figure 6). Therefore these findings suggest that Far1-independent arrest depends more strongly on the inhibitor of Clb protein accumulation (Cdh1). Note, however, that we cannot rule out the possibility that the absence of Cdh1 was suppressed in our experiments by ectopic expression of its functional relative, Cdc20, which enabled the cell synchronization protocol; for example, Pgal1-Cdc20 expression may limit accumulation of the B-type cyclin Clb2, which was shown previously to allow premature escape from pheromone arrest in a fraction of cdh1Δ (hct1-3) mutant cells (Schwab et al., 1997).

**G1 arrest failure can compromise cell viability**

Although removing Sic1 allowed far1Δ cln2Δ cells to escape pheromone-induced G1 arrest more readily, it appeared to cause enhanced sensitivity to pheromone when growth arrest was measured by a long-term halo assay (Figure 7A). This paradox led us to consider the possibility that the failure of these cells to arrest in G1 caused reduced viability during continuous incubation with pheromone. Therefore we assayed cell viability in asynchronous cultures treated with pheromone for various times (Figure 7B). Wild-type strains maintained viability for several hours, but the far1Δ cln2Δ sic1Δ strain showed a clear loss of viability after only a short period (2–4 h). This reduced viability was not seen in the absence of pheromone (legend to Figure 7) or when either Far1 or Sic1 was present (i.e., far1Δ cln2Δ sic1Δ or far1Δ cln2Δ Sic1 strains), suggesting that it is not the presence or absence of either protein per se but rather the rapid escape from G1 that ultimately causes reduced viability in the far1Δ cln2Δ sic1Δ strain. Furthermore, simultaneous absence of both proteins was tolerated if strains retained Cln2 (i.e., far1Δ sic1Δ strains; Figure 7C). Because Cln2 plays a prominent role in blocking pheromone response as cells pass Start (Oehlen and Cross, 1994; Strickfaden et al., 2007), the combined results suggest that the observed inviability is due to cells exiting G1 without down-regulating pheromone signaling.

These implications were further corroborated by other experiments that did not involve Far1, Sic1, or Cln2. In particular, overproduction of the S-phase cyclin Clb5 can override G1 arrest by pheromone (Oehlen et al., 1998), and a Cdk-resistant form of the signaling protein Ste5 (Ste5-8A) prevents pheromone response from being shut down in post-Start cells (Strickfaden et al., 2007). The presence

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**FIGURE 6:** Sic1 plays a strong role in Far1-independent arrest. Pgal1-Cdc20 strains of the indicated genotypes were synchronized and then released into the presence or absence of α factor (αF). Cell cycle progression and G1 arrest was assayed by budding. (A, B) Results in the Far1 cln2Δ and Far1 cln2Δ backgrounds, respectively. All graphs show the mean ± SD (n = 4–6). Note that far1Δ cln2Δ cdh1Δ strains showed phenotypic heterogeneity that was isolate dependent. Specifically, we tested 12 isolates: six Pgal1-Cdc20 derivatives from each of two independent far1Δ cln2Δ cdh1Δ strains. The results shown are an average of three strains (YPAP242, 244, and 245) that displayed the majority phenotype seen in 10 of 12 isolates. In two of 12 isolates, both derived from the same initial far1Δ cln2Δ cdh1Δ parent strain, we observed a notable escape phenotype (e.g., for YPAP243, ~40% budded cells after 120–180 min in αF). The reason for this heterogeneity is unknown, but the observation of the escape phenotype in only a minority of derivatives (2/6) of one parent strain and in no derivatives (0/6) of the other suggests that a rare enhancer mutation may be responsible.
of both features together (i.e., STE5–8A P<sub>GDY</sub>-CLB5 double mutants) caused cells to lose viability during pheromone exposure, whereas the single mutants did not (Figure 7C), arguing that inviability is a specific consequence of allowing pheromone signaling to continue in cells that escape the G1 arrest. Taken together, these findings illustrate the physiological importance of maintaining a robust G1 arrest in cells that are still transmitting the arrest signal. They also reveal that, under some circumstances, mutations that compromise G1 arrest would not be identified using only a growth arrest assay (see Discussion).

**DISCUSSION**

In this study we sought to increase our understanding of the mechanisms that yeast cells use to activate G1 arrest in response to extracellular mating pheromones. Previous findings indicate that this arrest does not absolutely require the pheromone-activated CKI Far1, because removal of Cln2 restores pheromone arrest to far1Δ cells. Thus here we compared Far1-dependent and Far1-independent G1 arrest in terms of their molecular phenotypes and their dependence on other inhibitory factors. We found that Far1 is not absolutely required for establishing a commitment point at Start, but it lengthens the time window in which pheromone can block this commitment step. Far1 also makes the G1 arrest process less dependent on other antagonists of the G1/S transition, such as repressors of G1/S transcription and inhibitors of S- and M-phase Cdk activity. Conversely, these repressors and inhibitors are not absolutely required for G1 arrest, but they reduce the dependence on Far1. Thus G1 arrest is made robust by functional overlap among multiple distinct G1/S inhibitory pathways (Figure 8).

In many eukaryotes it is believed that inhibition of G1/S transcription by Rb-like repressors and inhibition of S- and M-phase Cdk activity by Cip/Kip-like CKIs are key factors that restrain cell cycle entry and thereby impose a G1 waiting period (Morgan, 2007). In this study we found that pheromone-induced G1 arrest in budding yeast can still be imposed in the absence of the transcriptional repressors.

![Diagram](image-url)

**FIGURE 7:** Failure to arrest in G1 causes loss of viability during pheromone exposure. (A) Removing Sic1 from far1Δ cln2Δ cells causes enhanced pheromone sensitivity when measured by a chronic growth arrest (halo) assay. Cells were spread on solid growth medium, overlaid with filter disks containing 20 μl of α factor (20 or 100 μM), and then incubated at 30°C for 2 d. (B) Pheromone treatment causes loss of viability in far1Δ cln2Δ sic1Δ cells. Asynchronous liquid cultures were incubated with pheromone for 4–1 h, and then cell viability was measured by plating on medium lacking pheromone and counting colony formation (see Materials and Methods). Viable cells at each time point were expressed relative to the number present before treatment (t = 0). Graphs show mean ± range (n = 2). In parallel cultures incubated without pheromone, no differences in viability were observed among these strains (unpublished data). (C) Loss of cell viability is a consequence of escaping G1 arrest without inhibiting pheromone signaling. Asynchronous cultures were incubated with pheromone for 4 h, and viable cells were measured and expressed relative to the pretreated cultures (t = 0) as in B. Bars, mean ± SD (n = 4). Strains that continue dividing in the presence of pheromone (e.g., far1Δ) show an increased number of viable cells at 4 h compared with pretreated culture. See the text for further explanation.

![Diagram](image-url)

**FIGURE 8:** Simple illustration of multiple pathways contributing to pheromone arrest. Regulatory effects that inhibit or promote the G1/S transition are indicated in red or green, respectively. Dashed arrows with question marks emphasize that, although we found roles for Whi5/Stb1 and Sic1 in Far1-independent arrest, it is not known whether pheromone signaling enhances their inhibitory activity or simply depends on their constitutive effects. This simplified view is elaborated in greater detail in Supplemental Figure S1, which also includes a comparison of expected differences in wild-type, far1Δ, and far1Δ cln2Δ cells. See the text for further discussion.
Whi5/Stb1 or the CKI Sic1, or even both Whi5 and Sic1 simultaneously, but these negative factors become important for pheromone arrest in far1Δ cells. The existence of multiple braking mechanisms may help to prevent premature cell cycle entry when antiproliferative signals are present and expand the commitment decision period, perhaps by imposing a requirement that G1/S Cdk activity exceeds a sufficiently high threshold to counteract multiple antagonists. Such functional overlap, while in principle not required for a basic cell cycle, may increase the opportunities for regulatory control. Indeed, in animals there is evidence that Rb and CKIs can have additive effects in restraining proliferation of undifferentiated cells or redundant effects in terminally differentiated cells (Bragurolas et al., 1998; Buttitta et al., 2010; Wirt et al., 2010).

It is noteworthy that removal of Whi5 and Stb1 was not sufficient for full expression of G1/S transcripts, despite evident derepression. Instead, we found that pheromone signaling inhibits expression of G1/S genes even in the absence of either repressors, similar to previous results in whi5Δ or stb1Δ single mutants (Costanzo et al., 2004; de Bruin et al., 2008). Because this inhibition was maximal when Far1 was present, it suggests that Cdk activity promotes full G1/S transcription by an additional route separate from its role in releasing repression by Whi5/Stb1, perhaps via direct phosphorylation of SBF and/or MBF. Indeed, Cdk sites have been identified in both Swi6 and Whi5, but direct evidence that Cdk phosphorylation of SBF/MBF enhances their activity is lacking (Sidorova et al., 1995; Wijnen et al., 2002); instead, mutational analyses suggest that Cdk phosphorylation of Swi6 or Whi5 acts redundantly to block repression by Whi5/Stb1, perhaps via direct phosphorylation of SBF/MBF expression and hence Cln1-Cdk activity, was able to increase escape from G1 arrest even when Whi5/Stb1 were already absent.

Although Whi5/Stb1 and Sic1 contribute to pheromone-induced arrest when Far1 is absent, it is not clear whether they are regulated by pheromone or function instead as constitutive inhibitors (see question marks in Figure 8). Because Sic1 can be phosphorylated and stabilized by another MAPK, Hog1 (Escote et al., 2004), a similar effect could be exerted by the pheromone-regulated MAPK, Fus3. It is unlikely that the identical mechanism is used because the Hog1-phosphorylated form of Sic1 was absent in pheromone-arrested cells (Escote et al., 2004), but another study did observe partial phosphorylation of the Sic1 N-terminus in pheromone-arrested cells (Koivomagi et al., 2011), although the sites and responsible kinases are unknown. The effects of pheromone on G1/S transcription may be caused primarily by Far1-mediated inhibition of Cdk activity and MAPK-mediated degradation of Tec1, although it is conceivable that MAPK phosphorylation of SBF/MBF or Whi5/Stb1 provides another route for inhibition. Other pertinent mechanisms may include pheromone-induced reduction in Cln protein levels (Valdivieso et al., 1993) or reductions in total protein synthesis rates (Goranov et al., 2009; see question marks in Supplemental Figure S1), which may cause a rapid drop in the levels of short-lived cyclins and hence reduce cyclin-Cdk activity.

Which molecular targets can explain the escape phenotypes we obtained by inactivating inhibitory circuits in far1Δ cln2Δ cells? Ultimately, the key factor dictating whether cells can pass the G1/S transition despite the presence of pheromone may be acquisition of B-type Cdk activity, as pheromone cannot arrest cells in G1 if the S-phase cyclin Clb5 is expressed from a strong foreign promoter (Oehlen et al., 1998; Strickfaden et al., 2007). This view can explain the effects of removing Sic1, which directly inhibits Clb5-Cdk activity (Schwob et al., 1994). Furthermore, CLB5 is an SBF-regulated gene, as are CLN1 and CLN2, which engage in a positive feedback loop that enhances their own expression plus other SBF/MBF-dependent genes (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Skotheim et al., 2008). Thus, in far1Δ cln2Δ cells, levels of CLN1 and CLB5 expression may determine whether sufficient Clb5-Cdk activity accumulates to pass Start, and hence their increased expression upon removal of Whi5/Stb1 and/or addition of stabilized Tec1 may cause the escape phenotype. In far1Δ single mutants, the presence of Cln2 may tip the balance in favor of Start by a mix of several effects: 1) it might simply increase the total cyclin dosage, equal to an extra copy of CLN1; 2) Cln2 might be a more effective promoter of Start than Cln1 (Tyers and Futcher, 1993), perhaps by providing greater Cdk activity toward key substrates like Whi5 and Sic1; and 3) Cln2 counteracts pheromone arrest by directly inhibiting pheromone signaling, and it does so more potently than does Cln1 (Oehlen and Cross, 1994; Strickfaden et al., 2007). Thus, when Far1 is absent, the weaker Far1-independent arrest mechanisms can be overridden by Cln2 or genetic changes that increase Clb5-Cdk activity. When Far1 is present, the stronger inhibition of G1/S transcription may dampen Clb5 accumulation to a degree such that Sic1 is not needed for pheromone arrest. A comparison of these scenarios is illustrated in Supplemental Figure S1.

Finally, our findings reveal that robust G1 arrest is physiologically important, because if cell cycle entry occurs when cells are responding to pheromone, it can lead to irreversible, lethal effects. In particular, this was observed when far1Δ cln2Δ sic1Δ cells were exposed to pheromone; they were unable to arrest in G1, but they were nevertheless unable to divide and grow. Similar inviability was observed during pheromone treatment of STE5-8A PGR11:-CLB5 cells, in which G1 arrest is bypassed but signaling cannot be shut down. The cause of this inviability is not known, but ongoing studies suggest that pheromone signaling disrupts the function of the microtubule cytoskeleton during nuclear division (unpublished observations). Our findings clarify earlier results that implied that Sic1 was not required for Far1-independent arrest: namely, pheromone was able to arrest growth of cln1Δ cln2Δ cln3Δ far1Δ sic1Δ cells (Tyers, 1996). In retrospect, those cells may not have arrested in G1 but instead became inviable due to failed G1 arrest. These notions also illuminate an important consideration when analyzing other mutants for defects in pheromone-mediated arrest: if they allow slippage past the G1/S transition but do not prevent pheromone signaling, then the G1 arrest defect will not be noticeable by growth arrest assays. Hence it is conceivable that a distinct class of pheromone arrest mutants exists that would have gone undetected in prior genetic screens.

**MATERIALS AND METHODS**

**Yeast strains and plasmids**

Standard procedures were used for growth and genetic manipulation of yeast (Rothstein, 1991; Sherman, 2002). Yeast cultures were grown at 30°C. Strains and plasmids are listed in Tables 1 and 2, respectively; all yeast strains were derived from the W303 background (Thomas and Rothstein, 1989) and harbored the bar1Δ mutation to block α factor degradation. PCR-mediated gene targeting used methods described previously (Longtine et al., 1998); selectable markers included antibiotic resistance genes (kanMX6, natMX6) and orthologues of biosynthesis genes from other yeasts (Saccharomyces kluyveri HIS3, Candida glabrata TRP1, Kluveromyces lactis URA3). To ensure that genetic effects were reproducible, independently
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**TABLE 1:** Yeast strains used in this study.
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*All strains are in the W303 background (ade2-1 his3-11,15 leu2-3112 trp1-1 ura3-1 can1). In the \(PGAL1\)-CDC20 strains, a cassette containing the URA3\(^k^\) marker and GAL1 promoter is inserted in place of the CDC20 promoter at the native CDC20 locus.

Synchronous culture assays

To synchronize cell cultures, we placed the CDC20 gene under control of the GAL1 promoter (Cosma et al., 2001; Bean et al., 2005; Takahata et al., 2009). These \(PGAL1\)-CDC20 strains were grown asynchronously in liquid YP gal medium (containing 2% galactose), and then cultures were arrested in M phase by pelleting and resuspending in YPD medium (2% glucose), followed by incubation for 3 h. Cultures were released from the M-phase block by two rounds of pelleting and washing in YPGal, resuspension in YPGal either with or without α factor (0.2 μM), and incubation (with shaking) for 0–240 min. In experiments using TEC1 plasmids, before the arrest in YPD and release in YPGal, cultures were grown overnight in synthetic media (SC –Leu, with 2% raffinose and 2% galactose) to maintain selection for LEU2 plasmids.

Flow cytometry and budding assays

DNA content was measured by flow cytometry using previous methods (Haase and Reed, 2002; Strickfaden et al., 2007). Briefly, cell aliquots (0.5 ml) were harvested by centrifugation, resuspended in 0.3 ml water, fixed by addition of 0.7 ml of 100% ethanol, mixed by inversion, and incubated overnight at 4°C. Fixed cells were pelleted, washed once with water, resuspended in 0.5 ml of freshly prepared RNase solution (2 mg/ml RNase A in 50 mM Tris-HCl, pH 8.0, 15 mM NaCl), and incubated for 2 h at 36°C. They were then pelleted and resuspended in 0.2 ml of 0.1 M proteinase K solution (1 mg/ml proteinase K in 50 mM Tris-HCl, pH 8.0, 15 mM NaCl), incubated for 1 h at 36°C, then pelleted and resuspended in 0.5 ml of buffer (50 mM Tris-HCl, pH 7.5), and stored at 4°C. Before analysis, suspensions were sonicated (10 pulses with a microtip probe), and then 50 μl was mixed with 1 ml of fresh Sytox Green solution (1 μM in 50 mM Tris-HCl, pH 7.5), gently vortexed, and analyzed with a FACScan flow cytometer (BD, Franklin Lakes, NJ). For experiments that were directly compared with each other, a uniform range of fluorescence values was defined for both the 2C peak (generally 100–150 U) and the total (including 1C, 2C, and intermediate; generally 40–200 U), and then percentage 2C was calculated as 100% x 2C/total.

To analyze cell cycle position by budding, cells aliquots (0.5 ml) were fixed by the addition of formaldehyde to 3.7% final concentration, incubated on a nutator (room temperature, 10 min), washed three times with phosphate-buffered saline (PBS), and resuspended in 500 μl of PBS. Fixed cells were spotted on glass slides and dried.
RNA was prepared as described previously (de Bruin et al., 2008) using an RNeasy Plus Mini Kit (74134; Qiagen, Valencia, CA). Cells (~5 × 10^7) were harvested by centrifugation and frozen in liquid nitrogen. Cell pellets were resuspended in 400 μl of Qiagen RLT-plus buffer freshly supplemented with β-mercaptooethanol (10 μl/ml of buffer) and transferred to a microcentrifuge tube. Approximately 400 μl of acid-washed glass beads was added, and cells were lysed by vortexing (four cycles of 1 min, interspersed with rest periods of 3 min on ice). The tube was punctured at the bottom with a needle, placed in another tube, and centrifuged briefly (10 s) to transfer the lysate to the fresh tube. Then cell debris was removed by centrifugation (2 min, full speed). The supernatant was loaded onto Qiagen gDNA Eliminator columns, and then mRNA was prepared according to the manufacturer's instructions, diluted in sterile PBS, and stored at −20°C.

**mRNA preparation and RT-qPCR analysis**

Quantitative real-time PCRs were performed using Power SYBR Green PCR Master Mix (4367659; Applied Biosystems, Foster City, CA). Reaction mixtures (15 μl) contained 7.5 μl of SYBR Green reaction mix, 2 μl of primer mix (3 μM each primer), 1 μl of cDNA (2.5 ng/μl), and 4.5 μl of water. Reactions were performed in 96-well plates, in duplicate, using an Applied Biosystems StepOnePlus instrument. Preliminary trials using multiple primers for each gene were performed to identify primer sets with optimal properties (linearity of amplification) for use in all subsequent experiments. Primers are listed in Table 3. The ΔΔCt method was used to convert real-time amplification kinetics into relative mRNA levels; ACT1 mRNA served as the internal control.

### Cell viability assays

Asynchronous cultures were treated with pheromone (0.2 μM) for 1–4 h. Aliquots were collected before and after treatment, sonicated (seven pulses with a micropipette probe), diluted in sterile PBS, and spread on solid synthetic medium, and incubated for 2 d. Viable colonies were counted and expressed as a percentage of the number obtained from equivalent aliquots of the initial, untreated cultures.

**REFERENCES**


Cross FR, Schroeder L, Bean JM (2007). Phosphorylation of the S1 inhibitor of B-type cyclins in Saccharomyces cerevisiae is not essential but contributes to cell cycle robustness. Genetics 176, 1541–1555.


**TABLE 3: Oligonucleotide primers used for RT-qPCR analysis.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (′5′ to 3′)</th>
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<tbody>
<tr>
<td>CLN1-fw1</td>
<td>CTTGTTGTACGCGCAGAAC</td>
</tr>
<tr>
<td>CLN1-rev1</td>
<td>AGAAAGGCCTGGAATACGAG</td>
</tr>
<tr>
<td>YOX1-up1</td>
<td>AAATAGGGCCTATCCACAC</td>
</tr>
<tr>
<td>YOX1-dn1</td>
<td>ACGTTCCTACGGGAGTCAC</td>
</tr>
<tr>
<td>RNR1-up1</td>
<td>TCGAGGCTTCTTGAAGAC</td>
</tr>
<tr>
<td>RNR1-dn1</td>
<td>GGCACAACAGAAAAACAGAG</td>
</tr>
<tr>
<td>POL1-fw1</td>
<td>TGACATTGGCTCTGTTAGGC</td>
</tr>
<tr>
<td>POL1-rev1</td>
<td>CGGCTTATGTCCTTTTAC</td>
</tr>
<tr>
<td>CDC21-fw1</td>
<td>GGAACCCAGCTGATTGAC</td>
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<td>ACT1-fw1</td>
<td>TTCCAGCCTTACGTTTTC</td>
</tr>
<tr>
<td>ACT1-rev1</td>
<td>CCAGCGTAAATTTGAAGAC</td>
</tr>
</tbody>
</table>

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Figure S1. Detailed illustration of regulatory pathways contributing to either pheromone arrest or cell cycle entry. As in Figure 8, regulatory effects that inhibit or promote the G1/S transition are indicated in red or green, respectively. In addition, inhibitory effects of Cln-Cdk activity on the pheromone pathway are indicated in blue. The question marks indicate that, in addition to regulating Far1 and Tec1, the pheromone-activated MAPK may cause post-translational effects that interfere with the synthesis and/or stability of cyclin proteins (see Discussion).

(A) The pheromone pathway and the cell cycle are mutually antagonistic. In wild-type cells, the ability of pheromone to cause G1 arrest is likely dependent on whether the pheromone signal is received prior to the accumulation of Cln-Cdk activity. 

(B) In far1Δ CLN2 cells, uninhibited Cln-Cdk can more potently drive events that promote the G1/S transition (green arrows) and that inhibit pheromone signaling (blue inhibitory arrow), resulting in resistance to pheromone arrest.

(C) In far1Δ cln2Δ cells, the loss of Cln2-Cdk activity can allow other pheromone-induced effects to effectively antagonize the G1/S transition, in a manner dependent on Tec1 destruction and the activities of Whi5/Stb1 and Sic1.