Distinct Interactions Select and Maintain a Specific Cell Fate

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SUMMARY

The ability to specify and maintain discrete cell fates is essential for development. However, the dynamics underlying selection and stability of distinct cell types remain poorly understood. Here, we provide a quantitative single-cell analysis of commitment dynamics during the mating-mitosis switch in budding yeast. Commitment to division corresponds precisely to activating the G1 cyclin positive feedback loop in competition with the cyclin inhibitor Far1. Cyclin-dependent phosphorylation and inhibition of the mating pathway scaffold Ste5 are required to ensure exclusive expression of the mitotic transcriptional program after cell cycle commitment. Failure to commit exclusively results in coexpression of both cell cycle and pheromone-induced genes, and a morphologically mixed inviable cell fate. Thus, specification and maintenance of a cellular state are performed by distinct interactions, which are likely a consequence of disparate reaction rates and may be a general feature of the interlinked regulatory networks responsible for selecting cell fates.

INTRODUCTION

The precise specification and maintenance of cell fates in response to internal and external signals are crucial for life. While unicellular organisms make vital decisions to enter different life cycle stages in response to environmental change (e.g., to sporulate in poor conditions), multicellular organisms accurately pattern diverse cell types during development. In mammals, incorrect cell fate selection can result in developmental abnormalities, while poor maintenance may play a role in oncogenesis.

Despite its importance, we lack a precise molecular understanding of cell fate selection in multicellular organisms due to the presence of multiple overlapping pathways and associated spatiotemporal complexity. In particular, commitment points are frequently invoked in the explanation of differentiation processes, yet they remain largely conceptual due to the lack of direct quantitative live-cell measurements of key regulatory proteins. This motivates the study of differentiation dynamics in unicellular organisms, which can be grown in more controlled environments and monitored with a variety of quantitative live-cell imaging techniques (Colman-Lerner et al., 2005; Yu et al., 2008). Fine temporal control of the cellular environment can be used to exogenously control signaling pathways (Charvin et al., 2008; Lee et al., 2008; Muzzey et al., 2009; Taylor et al., 2009), while time-lapse fluorescence imaging allows continual monitoring of the concentrations of key regulatory proteins, which can then be correlated with cell fates to determine causal biochemical relationships.

Here, we examine cell cycle commitment in budding yeast vis-à-vis pheromone-induced mating arrest, which exhibits all the features of terminal differentiation, including changes in gene expression, arrest of the cell cycle, and persistent alterations in morphology. Since the purpose of mating is to fuse two haploid cells, it must be restricted to the G1 phase, prior to the initiation of DNA replication. The point where a cell loses mating competence and commits to the cell cycle is called Start (Hartwell et al., 1974). Hence, upon exposure to mating pheromone, pre-Start cells arrest directly while post-Start cells complete one more round of division before arresting (Figure 1A). This physiology is reflected at the molecular level by inhibitory interactions at the interface between the cell cycle and mating pathways (see schematic in Figure 1B). Mutual inhibition ensures that the mating pathway only arrests the cell cycle pre-Start, while the cell cycle pathway only restrains mating post-Start.

The mating pathway is a mitogen-activated protein kinase (MAPK) cascade that arrests the cell cycle prior to DNA replication primarily by inhibiting G1 cyclins in complex with the cyclin-dependent kinase (Chang and Herskowitz, 1990; Jeoung et al., 1998; Peter et al., 1993; Tyers and Futcher, 1993). In haploid cells, pheromone binds a G protein-coupled receptor (Ste2 for α-factor and Ste3 for a-factor) located at the plasma membrane, which activates a heterotrimeric G protein by dissociating Gα from the Gαβγ heterotrimer (Gpa1-Ste4-Ste18) heterotrimer. Once free, the Gαβγ subunit promotes Cdc24 activation of Cdc42 (Wiget et al., 2004), which in turn activates Ste20 (Lamson et al., 2002). Then Ste20 triggers the MAPK cascade by phosphorylating and activating the MAPKKK Ste11 (Drogen et al., 2000). The scaffold protein Ste5 which interacts physically with both the kinases (Ste11, Ste7, and Fus3) and with the Gαβγ subunit is necessary for mating signaling by coupling receptor stimulation to MAPK pathway activity (Garrenton et al., 2009; Hao et al., 2008; Strickfaden et al., 2007; Takahashi and Pryciak, 2008; Whiteway et al., 1995). The downstream MAPK Fus3 activates the transcription factor Ste12 to induce the associated transcriptional program, including the CDK inhibitor Far1 (Chang and Herskowitz, 1990; Errede and Ammerer, 1989). Importantly, Far1 is activated by Fus3 phosphorylation (Chang and Herskowitz, 1992; Elion et al., 1993) to physically interact with and inhibit...
Figure 1. Start Marks the Point of Commitment to the Cell Cycle, where Cells Lose Mating Competence

(A and B) Schematics of G1 cell cycle and pheromone-induced MAPK pathway regulation (mutual inhibition indicated with red arrows).

(C) Whi5-GFP enters the nucleus right before cytokinesis and is exported from the nucleus in G1 and is a quantitative marker of cell cycle progression. A histone-mCherry fusion protein marks the nucleus.

(D and E) Composite phase and green fluorescence images showing cells growing in a microfluidic device exposed to changing α-factor concentrations shown in (E).

(F) Time course of the nuclear Whi5-GFP from the two example cells in (D) marked with red and blue outlines.
the G1 cyclins (Gartner et al., 1998; Peter and Herskowitz, 1994), suggesting a stoichiometric mechanism to CDK inhibitors (Sherr and Roberts, 1999).

Conversely, the G1 cyclins inhibit the mating pathway by promoting the phosphorylation and degradation of both Far1 (McKinney and Cross, 1995; McKinney et al., 1993; Henchoz et al., 1997; Gartner et al., 1998) and the scaffold Ste5, which is also removed from the membrane to disrupt signaling (Garrettson et al., 2009; Strickfaden et al., 2007). G1 progression is initiated by the upstream G1 cyclin Cin3, which forms a complex with the cyclin-dependent kinase Cdc28 (CDK1). Cin3-Cdc28 phosphophyrases and partially inactivates Whi5, the inhibitor of the heterodimeric transcription factor SBF (Swi4/Swi6) (Costanzo et al., 2004; de Bruin et al., 2004; Wijnen et al., 2002). Partially active SBF and the related transcription factor MBF (Mbp1/Swi6) promote the transcription of two further G1 cyclins CLN1 and CLN2 (CLN1/2), which form a positive feedback loop by completing Whi5 inactivation and SBF activation (Cross et al., 1994; Ferrezuelo et al., 2010; Flick et al., 1998; Skotheim et al., 2008; Wijnen et al., 2002). In cell cycle-synchronized cultures, an increase in cyclin expression coincides with the phosphorylation and degradation of Far1, suggesting the possibility that increases in G1 cyclin activity play an important role in determining Start (McKinney et al., 1993).

Despite considerable study of both the cell cycle and MAPK-mating pathways, Start has remained an abstract concept without a precise biochemical definition. We show that cell cycle commitment corresponds to activating the G1 cyclin-positive feedback loop, which occurs when approximately 50% of the transcriptional inhibitor Whi5 has been exported from the nucleus. Genetic analysis of Start reveals separate functions for the Far1 and Ste5 inhibitory interactions at the interface between the cell cycle and mating pathways. While mutual inhibition between the G1 cyclins Cin1/2 and the cyclin inhibitor Far1 sets the commitment point, cyclin-dependent inhibition of the mating pathway scaffold Ste5 is required post-Start to ensure the exclusive expression of the mitotic transcriptional program. An ordinary differential equation model and in vivo kinetic measurements suggest that the observed separation of function is a consequence of the separate timescales associated with Far1 and Ste5 inhibition. Thus, selection and maintenance of a specific cellular state are performed by distinct interactions.

RESULTS

Quantitative Start Assay

To determine the molecular basis of cell cycle commitment, we developed a single-cell microfluidics assay. Cells were grown asynchronously before exposure to a step increase in pheromone (α-factor). This allowed us to classify cells based on the original operational definition of Start as the point of commitment to the cell cycle: upon exposure to α-factor, pre-Start cells arrest directly, while post-Start cells complete an additional mitotic cell cycle (Hartwell et al., 1974).

As a molecular reporter for cell cycle progression, we chose to monitor the nuclear concentration of the transcriptional inhibitor Whi5 through a C-terminal fusion protein expressed from the endogenous locus (Figure 1C). The C-terminal WHI5-GFP fusion protein is very likely to be fully functional and expressed at WT levels because it does not significantly extend G1 duration or modify cell size (Costanzo et al., 2004; de Bruin et al., 2004; Skotheim et al., 2008). Our choice of reporter was motivated by the fact that the activity of Whi5 is regulated by its subcellular localization. When Whi5 is phosphorylated and inactivated by G1 cyclin-CDK complexes, it is excluded from the nucleus (Costanzo et al., 2004; de Bruin et al., 2004; Kosugi et al., 2009; Taberner et al., 2009). The rapid nuclear entry of Whi5 at anaphase is due to dephosphorylation by Cdc14 (Taberner et al., 2009).

Throughout our assay, we use phase and fluorescence images to measure the nuclear concentration of Whi5 (see the Experimental Procedures). In the illustrative experiment shown in Figures 1D–1F, cells are first grown for 4 hr in media without pheromone. During this period, cells cycle continuously as seen by periodic Whi5-GFP nuclear entry and exit (the red and blue traces in Figure 1F correspond to the two segmented cells of the same color shown in Figure 1D). Next, mating pheromone is added, which eventually arrests all cells with a high concentration of nuclear Whi5-GFP. Both the red and blue cells are post-Start at the time of pheromone addition and complete an additional mitotic cycle before arresting. After 2 hr, the pheromone in the media is removed and cells rapidly exclude Whi5-GFP from the nucleus and re-enter the cell cycle. This experiment demonstrates our ability to rapidly add or remove pheromone from the extracellular environment.

Nuclear Whi5 Predicts Cell Fate

To identify variables that were predictive of cell fate, we recorded the amount of nuclear Whi5-GFP, cell area, cell type, and time spent in G1 prior to pheromone addition. Whi5-GFP concentration was measured relative to the maximum value after nuclear entry at anaphase (Figures 2A and 2B). Since pheromone pathway kinetics (Yu et al., 2008) and media switching (<1 min) are faster than Whi5 kinetics (~5 min) and G1 duration (~30 min), we treat pheromone pathway activity as a binary variable that instantaneously changes upon pheromone addition. We tracked cells for an additional 2 hr after pheromone addition and recorded the subsequent cell fate (arrest directly or divide once more).

To estimate the probability of each cell fate as a function of the observed variables, we used logistic regression (Hosmer and Lemeshow, 2000). We noted a very sharp correlation between nuclear Whi5-GFP and cell fate, which was weaker for the other parameters (Figures 2C–2F). Whi5-GFP predicted the cell fates of 97% ± 1% of G1 cells correctly, compared with 65% ± 2%, 65% ± 2%, and 66% ± 2% for cell type, cell size, and time spent in G1, respectively (Figure 2G). Note that a random guess predicts 50% correctly and the associated errors are estimated using crossvalidation.

To validate the accuracy of the cell fates predicted by the logistic regression and to determine whether any other parameter might improve the nuclear Whi5-GFP fit, we used L1-regularized multivariate logistic regression (Park and Hastie, 2007). This technique selects the relevant parameters (e.g., nuclear Whi5-GFP, cell size, etc.) by penalizing the L1-norm of their coefficients. Thus, a variable is not included in the model unless it is able to provide significant additional predictive value independent of the other variables. Nuclear Whi5 concentration...
accurately predicted cell fate. Indeed, no additional measured variable yielded statistically significant additional information (Figure 2G and see Figures S1A–S1D and Table S1 available online). This indicates that Start is a well-defined point that is accurately predicted by the nuclear Whi5 concentration independent of cell size, type, and G1 duration.

That nuclear Whi5 accurately predicts cell fate is reflected in the probability of cell cycle arrest decreasing from 80% to 20% as the nuclear Whi5 fraction exported prior to pheromone addition increases from 0.47 to 0.57 (inset, Figure 2C). The transition from uncommitted to committed as a function of nuclear Whi5-GFP is very sharp (Hill coefficient >14). This remarkably strong correlation between cell fate and Whi5 levels provides a rational quantitative definition of cell cycle commitment: Start is the point where half the WT cells arrest, which occurs when approximately half the Whi5-GFP (52% ± 3%) has been exported.

Sic1 is a stoichiometric inhibitor of the B-type cyclins responsible for initiating replication (Schwob et al., 1994) and has been suggested to also play a role in cell cycle commitment (Pathak et al., 2007). To investigate the potential role of Sic1 at Start, we created a strain expressing Whi5-mCherry and Sic1-GFP from their endogenous loci. Sic1 degradation follows Whi5 nuclear export, suggesting that commitment precedes Sic1 degradation (Costanzo et al., 2004) (Figures S1E and S1F).

Thus, Sic1 degradation in WT cells should be viewed as dependent on, rather than causal of, cell cycle commitment.

Whi5 Export Correlates with Cln1/2 Feedback Induction

The correlation between cell fates and nuclear level of Whi5 prompted us to investigate its functional role in cell cycle commitment. Whi5 is bound to SBF on the CLN2 promoter, which likely inhibits CLN2 expression in early G1 (Cross et al., 1994; de Bruin et al., 2004). We examined GFP expression from a CLN2 promoter integrated into the genome of a cell already expressing WHI5-GFP and the nuclear marker HTB2-mCherry (Mateus and Avery, 2000). After a simple subtraction, the Whi5-GFP signal does not interfere with the CLN2-pr-GFP signal, and vice versa (Skotheim et al., 2008). In WT cells, the CLN2-pr is measured to be induced when 76% ± 2% Whi5-GFP has been exported (Figure S2). The rapid feedback-driven Whi5-GFP nuclear exit kinetics likely results in an overestimation of the amount of Whi5 exported by the time of CLN2-pr induction, because our measurement of the transcription induction depends on the maturation of a fluorescent reporter. We therefore decided to examine the correlation in cln1Δ cln2Δ cells in which the Whi5-GFP kinetics is substantially slower (Figure 3A). We recorded the amount of Whi5 exported at the time of CLN2-pr induction. On average, 49% ± 1% (±SEM) Whi5-GFP has been exported (Figure 3B), which is very similar to the commitment point that occurs at 52% ± 3%. Taken together, our analysis confirms the link between the Whi5 nuclear concentration at Start and CLN2-pr induction and strongly suggests that commitment to cell division, i.e., Start, closely follows activation of the Cln1/2-positive feedback loop.
mutants containing Cln3 may affect the timing, but not the constitution, of concentration, indicating that upstream signals acting through than in WT cells (consistent with previous results (Oehlen and Cross, 1994). We found that Far1 phosphorylation and degradation contributes to the ability to arrest the cell cycle and to stop mating (Ydenberg and Rose, 2009). Indeed, cells exposed to α-factor just past Start had a high likelihood (~75%) of a defective cell cycle (Figures 5C and 5D). Further beyond Start, the aberrant arrest fraction likely decreases due to the activation of the mitotic cyclins that inhibit mating via a different mechanism (Ydenberg and Rose, 2009).

To test our prediction that aberrant cell fate selection correlates with mixed gene expression, we constructed STE5-8A and wild-type strains containing mating pathway (FUS1pr-GFP) and cell cycle (CLN2pr-mCherry) gene expression reporters (Figures 5E and 5F). We found that the aberrant STE5-8A cells showed significant coexpression of mating and cell cycle genes relative to both normally arrested STE5-8A and wild-type cells (Figure 5G). Furthermore, STE5-8A cells exhibiting a significant mitotic delay (>2 hr) were found to have an intermediate level of coexpression. Thus, specific phosphorylation of Ste5 by G1 cyclins is required for cell fate exclusivity.

Our observations give some insight into previously observed aberrant cell cycle arrests. Since far1Δ STE5-8A cells lack both the ability to arrest the cell cycle and to stop mating pathway activity when exposed to α-factor, each cell is expected to suffer a mixed cell fate every cell cycle it remains in α-factor. Indeed, ~80% of all far1Δ STE5-8A cells arrest aberrantly after exposure to α-factor (Figure 5D). This is consistent with previous results showing far1Δ STE5-8A sensitivity to α-factor and increased 2C arrest (Strickfaden et al., 2007). Furthermore, constitutive expression of Clb5 in a ctn1Δ ctn2Δ ctn3Δ cell exposed to mating pheromone results in a similar coexpression of mating and cell cycle genes and 2C arrest (Oehlen et al., 1998).
Figure 4. Genetic Analysis of Start Reveals the Primacy of the Cln1/2-Far1 Interaction in Determining Commitment to Cell Division

(A–F) For each genotype, we performed the Whi5-based quantitative Start assay as shown in Figure 2C for WT cells. Cells were grown for 2 hr prior to exposure to a step increase in pheromone concentration. Subsequent cell fate (arrest directly or commit to an additional division) and nuclear Whi5-GFP concentration were measured. Each panel shows a histogram of the nuclear Whi5-GFP concentration ($\delta/\gamma$) at the time of pheromone addition for each cell fate (red, arrest; blue, commit). For each mutant, we used logistic regression to estimate the probability of arrest as a function of nuclear Whi5-GFP, which is always compared to the corresponding curve for WT cells shown in black. The shaded regions indicate 95% confidence intervals estimated from 10,000 bootstrapping iterations. Indicated $p$ values are computed relative to WT using a $\chi^2$ test. The number of cells used for each mutant is $N_{\text{WT}} = 315$, $N_{\text{STE5-8A}} = 218$, $N_{\text{FAR1-S87A}} = 273$, $N_{\text{cln1D,cln2D}} = 477$, $N_{\text{cln3D}} = 266$, $N_{\text{FAR1-S87A STE5-8A}} = 216$, $N_{\text{3XFAR1}} = 193$.

(G) Summary of the genetic analysis reveals two clusters (WT, STE5-8A, cln3D) and (FAR1-S87A, cln1D,cln2D, FAR1-S87A STE5-8A, 3XFAR1). Each mutant is not significantly different from the other mutants in its cluster ($p > 0.07$ for all comparisons) but significantly different from all mutants in the other cluster ($p < 0.002$ for all comparisons; see Table S2).
Differential Rate Constants May Explain Separation of Function at Start

Our observation that Cln1/2-CDK phosphorylation of Far1, but not Ste5, impacts Start is consistent with cyclins phosphorylating and inhibiting Far1 before Ste5. In this model, the Cln1/2-Far1 inhibition is rapidly resolved to either a high or low Far1 state corresponding to mating arrest or the mitotic cell cycle, respectively. Thus, if the cell cycle is selected, Far1 is rapidly phosphorylated and degraded while Ste5 phosphorylation occurs more slowly.

To address whether differences in inhibition rate could explain the observed separation of function, we constructed...
a mathematical model based on ordinary differential equations (Figure 6A; Figures S3A and S3B). The goal of our modeling effort is not to fit data, but rather to gain a qualitative understanding for how differential rate constants may lead to a separation of function at the interface of competing signaling pathways. In our simplified model, cell cycle pathway activity is represented by the Cln1/2 level, while mating pathway activity is represented by the level of Ste5. Far1 is activated by Ste5 and inhibits Cln1/2, whereas Cln1/2 inhibits both Ste5 and Far1. To model the dynamics, Cln1/2 synthesis ($k_7$) representing Cln3 activity is initiated at time = 0. The mating pathway is then activated at a rate ($k_8$) at time $t$. Intuitively, if $t$ is large enough, Cln1/2 accumulates sufficiently to activate positive feedback which commits the cell to division (high Cln1/2, low Ste5 and Far1; Figure S3C). However, if $t$ is small, the mating pathway is activated before Cln1/2 feedback, and then Far1 inhibits Cln1/2 and the cell arrests (low Cln1/2, high Ste5 and Far1; Figure S3D). For each set of parameters there is a critical time, $t_{crit}$, above which the cell is committed to the cell cycle and below which the mating pathway is engaged. $t_{crit}$ corresponds to a critical amount of Cln1/2 which can be accumulated before commitment.

To examine the idea that differences in timescales can lead to separation of function in the network, we decided to fix all the rates except the Cln1/2 inhibition of Ste5 ($k_1$) and Far1 ($k_3$) whose ratio we varied from 500:1 to 1:500. For each ratio we determined the critical level of Cln1/2 beyond which the cell is committed to division: $Cln1/2_{crit}(k_1, k_3)$. Next we examined the effects of the $STE5-8A$ and $FAR1-S87A$ mutations in our model by setting $k_1$ or $k_3$ to zero, respectively, and then repeating our calculation. The critical Cln1/2 levels for the $STE5-8A$ and $FAR1-S87A$ mutants are $Cln1/2_{crit}(0, k_3)$ and $Cln1/2_{crit}(k_1, 0)$, respectively. We calculated how commitment was delayed, as represented by a decreased ability to activate the Cln1/2 feedback in each mutant relative to WT. The relative increase in the critical Cln1/2 level was denoted as $I_{FAR1-S87A}$ and $I_{STE5-8A}$ so that

$$I_{FAR1-S87A} = \frac{Cln1/2_{crit}(0, k_3)}{Cln1/2_{crit}(k_1, k_3)}$$

and

$$I_{STE5-8A} = \frac{Cln1/2_{crit}(k_1, 0)}{Cln1/2_{crit}(k_1, k_3)}.$$
Next, we compared the impact of $\text{STE5-8A}$ mutation on increasing the commitment threshold relative to the impact of the $\text{FAR1-S87A}$ mutation: impact of $\text{STE5-8A}$ relative to $\text{FAR1-S87A} = \frac{1}{(1/\text{STE5-8A}) + (1/\text{FAR1-S87A})}$, which is plotted in Figure 6B.

When comparing the relative impact of the $\text{STE5-8A}$ and $\text{FAR1-S87A}$ mutations while varying the inhibition rates, we found that the relative impact from the mutations is determined by the ratio between the $\text{Cln1/2-Far1}$ and $\text{Cln1/2-Ste5}$ inhibition rates (Figure 6B, Table S3). In a counterfactual analysis, we find that $\text{Ste5}$ inhibition would determine the commitment point if it were significantly faster than $\text{Far1}$ inhibition ($k_1 > k_2$). Thus, commitment is determined by the fastest rate, suggesting that a separation of timescales of $\text{Far1}$ relative to $\text{Ste5}$ inhibition may underlie the observed separation of function.

Exogenously Controlled $\text{CLN2}$ Expression Inhibits $\text{Far1}$ before $\text{Ste5}$

Our model predicts that separation of function at the cell cycle-MAPK interface arises due to more rapid inhibition of $\text{Far1}$ than $\text{Ste5}$ by G1 cyclins. To test this hypothesis, we examined a yeast strain expressing a $\text{FAR1-Venus}$ fusion from the endogenous locus in addition to an integrated $\text{STE5-YFP}$ allele. This strain also contains an integrated $\text{CLN2}$ allele driven by the $\text{MET3}$ promoter, which expresses $\text{CLN2}$ at physiological levels in media lacking methionine (Chavin et al., 2008). Upon pheromone arrest, $\text{Far1-Venus}$ accumulates primarily in the nucleus, while $\text{Ste5-YFP}$ accumulates at the shmoo tip. Control experiments on two strains containing only one of the two yellow markers indicates negligible contributions of $\text{Far1}$ and $\text{Ste5}$ to the shmoo tip and nuclear signals, respectively. Pheromone-arrested cells, showing significant nuclear and shmoo yellow fluorescence, were exposed to a new media containing pheromone (see the Experimental Procedures; Figures 6C and 6D, Figures S3E and S3F). This result supports our argument that separation of function at network interfaces may arise from a separation of timescales.

We note that the effect of $\text{Far1}$ and $\text{Ste5}$ mutations was not a priori obvious because the number of links in a pathway does not necessarily predict the speed of signal propagation. Kinetic rate constants may significantly differ, perhaps due to spatial considerations, and should ideally be determined in vivo. Here, $\text{Far1}$ inactivation via $\text{Ste5}$ inactivation must come after we observe $\text{Ste5}$ dissociation from the membrane. Our observations and analysis suggest that $\text{Ste5}$-mediated $\text{Far1}$ inhibition is slower than direct inhibition of $\text{Far1}$ by G1 cyclins, which likely underlies the separation of function shown in Figures 4 and 5. Thus, the separation of function of inhibitory interactions may be a general feature emerging from the interfaces between competing signaling pathways. Our data are consistent with theoretical analysis suggesting that linking fast ($\text{Cln1/2-Far1}$) and slow ($\text{Cln1/2-Ste5}$) feedback loops can produce rapidly inducible yet noise-resistant switches (Brandman et al., 2005).

DISCUSSION

Here, we showed how the interaction between the cell cycle and the mating pathway determines $\text{Start}$. The point of commitment to the mitotic cell cycle corresponds to activating the G1 cyclin-positive feedback loop controlled by the transcription factors $\text{SBF}$ and $\text{MBF}$, which have $>200$ additional targets. In a related paper appearing in this issue, we show that increased expression of the G1 cyclins $\text{CLN1}$ and $\text{CLN2}$ precedes activation of the bulk of the $>200$ coregulated genes, implying that genome-wide changes in transcription depend on cell cycle commitment (Eser et al., 2011).

Our work emphasizes the importance of the G1 cyclin-dependent inhibition of the MAPK scaffold $\text{Ste5}$ (Strickfaden et al., 2007). Once $\text{Cln1/2}$ has inhibited $\text{Far1}$ to pass $\text{Start}$, there must be an additional negative regulation due to $\text{Far1}$’s terminal location in the pheromone pathway. Without the ability of $\text{Cln1/2}$ to inactivate $\text{Ste5}$, the post-$\text{Start}$ cell cycle cannot short circuit pheromone signaling and downstream gene expression with potentially fatal consequences.

We show that commitment at $\text{Start}$ corresponds precisely to the induction of $\text{Cln1/2}$ feedback and is controlled by mutual inhibition of $\text{Cln1/2}$ and $\text{Far1}$, thereby providing a precise biochemical definition for $\text{Start}$ (Figure 7). That the commitment point is determined by the relative strengths of a direct mutual inhibition suggests a single axis through which evolution can rapidly tune the fundamental mating-mitotic switch. The complete separation of function of CDK phosphorylation of $\text{Ste5}$ and $\text{Far1}$ allows tuning the commitment point without risking cell cycle and mating pathway coexpression and reduced fitness.

Cause and Consequences of a Predictive Measurement

The accuracy with which we determine $\text{Start}$ stands in sharp contrast to the molecular noise often observed in single cells and associated with cellular decisions (Balázsi et al., 2011). Indeed, that we are able to predict the cell fate of 97% of G1 cells exposed to mating pheromone strongly suggests that nuclear $\text{Whi5-GFP}$ is a direct measurement of a well-defined continuous...
cell cycle phase in G1. Had we chosen different reporters, that less directly measure the cell cycle phase, it is unlikely that we could achieve such accuracy and we may have concluded that the process is fundamentally “noisy.” Instead, we infer that the precise nature of cell cycle commitment likely reflects its fundamental importance in the yeast life cycle.

That nuclear Whi5 corresponds to cell cycle phase arises from molecular considerations. Whi5 export during G1 depends on the karyopherin Msn5 and is due to G1 cyclin phosphorylation disrupting nuclear localization sequences, while Whi5 import is constitutive throughout the cell cycle and depends on the classical nuclear import pathway (Kosugi et al., 2009; Taberner et al., 2009). Since acute removal of CDK activity leads to substantial nuclear re-entry within 10 min (Charvin et al., 2010), the nuclear Whi5-GFP concentration likely reflects a balance of import and export rates that is shifted by increasing CDK activity through G1. Thus, the monotonically decreasing Whi5 nuclear concentration is likely to be a direct measurement of G1 cyclin-CDK activity, whose dynamic range is precisely tuned to monitor progression through Start.

From a dynamical systems point of view, Start is a bistable switch (Charvin et al., 2010; Novak et al., 2007; Skotheim et al., 2008). At a critical level of CDK activity the CLN1/2 positive feedback loop is irreversibly engaged as the dynamical system passes through a saddle-node bifurcation. The result that we can accurately predict cell fate, i.e., the location of the bifurcation point, is remarkable considering the numerous factors (some essential) that have been identified through genetic analysis to play a role in G1 kinetics (Jorgensen et al., 2002). Our present results do not exclude a role at Start for changes in the concentration of these factors because they may exist at different levels in different environmental conditions leading to a qualitatively different, but qualitatively similar, Start. However, for a given growth condition, the yeast cell cycle appears robust to concentration variations of other factors and the relevant dynamics collapse to a single axis, CDK activity, with a well-defined threshold.

**General Aspects of Commitment**

The existence of an alternate cell fate is essential for the definition of a commitment point, which emerges naturally from the interactions at the interface between any two pathways inducing competing cell fates. The fact that multiple signaling pathways induced by stress (Barbet et al., 1996; Gray et al., 2004; Trotter et al., 2001) can arrest cells in a low-CDK activity G1 state suggests the potential for a multiplicity of G1 commitment points. In these other arrests, commitment may be determined through alternate interfaces of the core G1/S cell cycle regulatory network with stress-activated kinase pathways. Thus, cell cycle commitment defined vis-à-vis stress or nutrient deprivation rather than mating arrest may well occur at a different CDK activity level. However, if the commitment point is defined by G1 cyclin inhibition, it is likely to coincide with the rapid increase in G1 cyclin synthesis occurring when half of the nuclear Whi5 has been removed. Thus, the possibility remains of a universal point of commitment.

Here, we defined cell cycle commitment in budding yeast to reveal a surprising degree of modularity in a regulatory interface essential for all living cells. Indeed, cell fate selection, as a result of interactions at the interface between the core cell cycle and extrinsic signaling pathways, is fundamental for successful proliferation and development. The loss of restriction point control regulating cell division in mammals is associated with cancer and developmental defects (Chen et al., 2009; Zetterberg et al., 1995). We expect our methods and conceptual framework to be extended to the study of human cells, thereby giving insight into both development and disease.

**EXPERIMENTAL PROCEDURES**

**Microscopy, Flowcell, and Image Analysis**

Experiments were performed with a Cellasic microfluidic device (http://www.cellasic.com/) using Y16 and Y4 plates with a flow rate of 5 psi, which exchanged the media in the chamber in about 60 s. Images were taken for up to 16 positions every 3 min with a Zeiss Observer Z1 microscope with an automated stage using a plan-apo 63×1.4NA oil immersion objective. Automatic focusing was performed using Definite Focus hardware. Whi5-GFP and FUS1pr-GFP strains were exposed for 100 ms and 25 ms, respectively, using the Colibri LED 470 module at 25% power. HTB2-mCherry and CLN2pr-mCherry strains were exposed for 10 ms and 300 ms, respectively, using the Colibri 540-80 LED module at 25% power. All images were subsequently analyzed using custom MATLAB software that segments, tracks, and calculates the mean nuclear fluorescence relative to the cytoplasm (Skotheim et al., 2008). See supplemental information in Skotheim et al. (2008) for a detailed justification of the use of Whi5-GFP and CLN2pr-GFP dual-labeled cells.

**Yeast Strains and Growth Conditions**

Strains are congenic with W303 (see the Supplemental Experimental Procedures). Prior to an experiment, cells were grown in log phase (OD 0–0.1) in synthetic complete medium with 2% glucose (SCD) and then sonicated (~5 s 3W). All media used were mixed with 20 μg/ml cascin (Sigma) to inhibit x-factor surface adhesion (Yu et al., 2008). cln1Δ cln2Δ cells were treated slightly differently and were grown in log phase in SCD lacking methionine (SCD – met) to express Cln2 from an integrated MET3 promoter. cln1Δ cln2Δ cells were switched to SCD after 1 hr in the flowcell and then treated as the other strains.

**Measurement of Start**

Cells were grown in the flowcell for at least 1.5 hr before being exposed to 240 nM x-factor and tracked for at least 2 additional hours. For each G1 cell, we compare the amount of Whi5-GFP exported to the largest value of nuclear Whi5-GFP within 30 min prior to pheromone exposure. The Whi5-GFP baseline is calculated semiautomatically for each cell. Cells with segmentation errors or nuclei outside of the plane of focus were discarded.

**Mixed Cell Fate Experiment**

Cells were grown in the flow cell for 2 hr before being exposed to 240 nM x-factor for 270 min. Cells that first bud then shmoo without an intervening cytokinesis were scored as aberrant. Cells exhibiting prolonged mitosis until the movie limit, likely reflecting a bimodal population of cells either ultimately managing to go through cytokinesis or failing, were scored separately as “mitotic delay.” FUS1-CLN2 coexpression was measured using the product of the baseline-subtracted and peak-normalized CLN2pr-mCherry and FUS1pr-GFP signals.

**Far1 and Ste5 Inhibition Kinetics Experiment**

Cells were initially grown in SCD for 90 min and then exposed to x-factor for 120 min. Next we induced exogenous CLN2 by switching to media containing pheromone but lacking methionine. Nuclear Far1-Venus was quantified using the same algorithm as Whi5-GFP, whereas we used custom matlab software to detect the brightest spot on the shmoo (manually selected) for the Ste5-YFP signal (Figures S3E and S3F).
SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2011.06.025.

ACKNOWLEDGMENTS

In particular, we thank Fred Cross and Peter Pryciak for extensive discussions, insightful suggestions, and generous sharing of reagents. We also thank Richard Yu and Gustavo Pesce for generous sharing of reagents. We thank Chris Aakre, Amanda Amodeo, Nick Buchler, Stefano DiTalia, Carlos Gomez Uribe, Danny Lew, and Eric Siggia for comments on the manuscript. We thank Gilles Chavirin for advice on microfluidics. Research was funded by the Burroughs Welcome Fund, the National Institutes of Health (GM092925), the National Science Foundation (CAREER award #1054025), and the Hellman Foundation.

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REFERENCES


Supplemental Information

Distinct Interactions Select and Maintain a Specific Cell Fate

Andreas Dončić, Melody Falleur-Fettig, and Jan M. Skotheim

Supplemental Information Inventory

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Figure S2: Correlation of CLN2pr-GFP and Whi5-GFP in WT cells ............... p5
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Supplemental Information

Figure S1, related to Figure 2: A,B. L₁-regression reveals that nuclear Whi5-GFP is the only variable required to predict cell fate. Analysis was performed using the R package from [1]. Briefly, the L₁-regression package performs a multivariate logistic regression in which the L₁-norm of the variables is penalized. As this penalty is
reduced, more variables are included in the model and the L1-norm of the coefficients increases (x-axis on plots). The coefficient path of the L1-regression (A) shows that the only significant variable in predicting cell fate (arrest or divide) is the nuclear Whi5 concentration at the time of pheromone addition. At an L1-norm > 4, cell type is included in the model, but this yields no additional predictive value as the cross validated error shown in (B) does not significantly decrease. The regression was performed using the following variables: nuclear Whi5-GFP concentration at the time of pheromone addition, maximum nuclear Whi5-GFP concentration, cell type (mother or daughter), cell size at the time of pheromone addition, G1 duration by the time of pheromone addition, and the dummy variable cell number (cells included in the data set were labeled 1 to 315). This analysis reveals that once nuclear Whi5-GFP is measured, there is no additional information from the rest of the variables. C. Comparison of Start for mother and daughter cells shows no significant difference. D. Comparison of Start using a pheromone step from 0 to 12nM yields the same result as for the 0 to 240nM step used in all the data presented in Figure 2. E,F. Start precedes Sic1 degradation. Sic1 is an inhibitor of B-type cyclins (Cln1-6) which is active in late mitosis and stays active until post-Start G1 where its degradation is instrumental for the promotion of S-phase [2]. Sic1 is nuclear when it is active so its concentration can be measured using the same techniques we used for Whi5-GFP. To measure Sic1 degradation relative to cell cycle commitment, we constructed a strain containing SIC1-GFP and WHI5-mCherry and observed their nuclear concentration dynamics in 49 cells. E. An example trace of Whi5-mCherry and Sic1-GFP concentrations. F. Whi5 exit always precedes Sic1 degradation, which suggests Sic1 is not directly involved in Start. Note that due to the short G1 in most mothers (~15min) the Sic1-GFP does not have enough time to mature and is difficult to observe so that the majority of our cells are daughters (39/49). Shown here are mothers and daughters together. Separately the time difference between Whi5 exit and Sic1 degradation for mothers and daughters was on average 6.3±2.6 min and 7.8±3 min respectively (p=0.15).
<table>
<thead>
<tr>
<th>Model</th>
<th>p-value comparison with the model based only on nuclear Whi5-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear Whi5-GFP &amp; Max nuclear Whi5-GFP</td>
<td>0.41</td>
</tr>
<tr>
<td>Nuclear Whi5-GFP &amp; Cell type (mother or daughter cell)</td>
<td>0.06</td>
</tr>
<tr>
<td>Nuclear Whi5-GFP &amp; time in G1 prior to pheromone addition</td>
<td>0.20</td>
</tr>
<tr>
<td>Nuclear Whi5-GFP &amp; Cell size at the time of pheromone addition</td>
<td>0.61</td>
</tr>
<tr>
<td>Nuclear Whi5-GFP &amp; Pheromone concentration (12 or 240nM)</td>
<td>0.78</td>
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<tr>
<td>Nuclear Whi5-GFP &amp; Cell number (dummy variable)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table S1, related to Figure 2: For WT G1 cells we recorded the maximum nuclear intensity, the relative nuclear intensity at the time of pheromone addition, cell type, time in G1 prior to pheromone addition, cell size at the time of pheromone addition, the pheromone concentration used to block cells and the cell number. A model based solely on the relative nuclear concentration of Whi5-GFP predicts the cell fate of 97% of G1 cells. Here, we compare this model with models based on nuclear Whi5-GFP and an additional factor. None of these two component models are significantly different from the nuclear Whi5-GFP model (p>0.05 for all comparisons). This comparison was based on a chi-squared test comparing generalized linear models implemented in R using the `add1` function.
Figure S2, related to Figure 3: The amount of Whi5-GFP exported at the time of CLN2 promoter induction in a WT-cell (same method as for the cln1Δcln2Δ cells shown in Figure 3B).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>cln3Δ</th>
<th>STE5-8A</th>
<th>cln1Δcln2Δ</th>
<th>FAR1-S87A</th>
<th>STE5-8A FAR1-S87A</th>
<th>3xFAR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1</td>
<td>0.40</td>
<td>0.19</td>
<td>2.8e-4</td>
<td>1.6e-5</td>
<td>8.3e-7</td>
<td>1.7e-3</td>
</tr>
<tr>
<td>cln3Δ</td>
<td>1</td>
<td>0.64</td>
<td>8.2e-4</td>
<td>2.3e-5</td>
<td>2.5e-6</td>
<td></td>
<td>4.0e-4</td>
</tr>
<tr>
<td>STE5-8A</td>
<td>1</td>
<td>9.3e-6</td>
<td>5.0e-7</td>
<td>4.6e-8</td>
<td>7.9e-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cln1Δcln2Δ</td>
<td>1</td>
<td>1</td>
<td>0.30</td>
<td>0.066</td>
<td>0.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAR1-S87A</td>
<td></td>
<td></td>
<td>1</td>
<td>0.29</td>
<td></td>
<td></td>
<td>0.71</td>
</tr>
<tr>
<td>STE5-8A</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAR1-S87A</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3xFAR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Table S2, related to Figure 4: P-values for all pairs of Start mutants as described in Figure 4. For a pairwise comparison, commitment data for both datasets was pooled and a model based solely on nuclear Whi5-GFP was constructed. A second variable, valued 0 for one genotype and 1 for the other, was then added. P-values for genotype influence were then calculated using a χ² test implemented with the add1 function in R.
A production-term(s) degradation-term(s)
\[
\frac{d\text{Cln1/2}}{dt} = k_p + \frac{k_s \text{Cln1/2}}{k_p + \text{Cln1/2}^n} - k_d \text{Cln1/2} - k_c \text{Cln1/2Far1}
\]
\[
\frac{d\text{Ste5}}{dt} = k_q(t) \text{Ste5}^* - k_c \text{Ste5Cln1/2}
\]
\[
\frac{d\text{Far1}}{dt} = k_r \text{Ste5} - k_c \text{Cln1/2Far1} - k_s \text{Far1}
\]
\[
\frac{d\text{Ste5}^*}{dt} = k_r \text{Ste5Cln1/2} - k_q(t) \text{Ste5}^*
\]
\[
\text{Ste5}_{\text{tot}} = \text{Ste5} + \text{Ste5}^*
\]

Parameters used:
k_1 = \text{varies from 0 (deleted) to 100, } k_2 = 1, k_3 = \text{as 'k1'},
k_4 = 5, k_5 = 10, k_6 = 1, k_7 = 0.01, k_8 = \begin{cases} 1 & \text{if } t > \tau_1 \; \text{if } t < \tau_1 \end{cases},
k_9 = 0.5, k_{10} = 5, n = 2, \text{Ste5}_{\text{tot}} = 1

B mating pathway activity as a function of time

C relative activity (a.u.)
\[
\tau < \tau_{\text{crit.}} \quad \text{Cln1/2 feedback not yet activated}
\]

D relative activity (a.u.)
\[
\tau > \tau_{\text{crit.}} \quad \text{Cln1/2 feedback activated}
\]

E relative activity (a.u.)

F time between start of FAR1 and STE5 inhibition [min]
Figure S3, related to Figure 6: Mathematical model with experimental validation of the cell cycle-mating pathway interface. A. Ordinary differential equations for the model in Figure 6A-B. For simplicity we assume that the total amount of Ste5 remains constant and exists in either an active (Ste5) or inactive (Ste5*) form. All parameters were chosen for simplicity as a formal exploration of the 10-dimensional parameter space is not possible. Apart from $k_1$ and $k_3$, we did not vary any other parameters during our analysis. We model a cell entering G1 as having a low but fixed activity of Cln3 (represented by $k_f$). After some time, Cln1/2 accumulates to a high enough level to trigger the feedback loop and the cells commit to the cell cycle. B. We activate the mating pathway at a time $\tau$ after the initiation of Cln1/2 accumulation by increasing $k_8$ from 0. C,D. show pre- and post-Start cells respectively. Start is defined as the point where the system loses its mating competence. In our model this corresponds to the largest time, $\tau_{crit}$ at which the mating pathway can initiate arrest. For each set of parameters the critical amount of Cln1/2 was taken to be the amount present at $\tau_{crit}$. E. Example traces of Far1-venus (nuclear, black) and Ste5-YFP (shmoo, blue). Cells were grown in $\alpha$-factor for 120min and then exposed to media without methionine to induce Cln2 (at time=0). Far1 localizes both to the nucleus and to the cytoplasm but is degraded in the nucleus [3]. Hence, we measure the relative nuclear Far1-Venus intensity (black) and note the initiation of Far1 disappearance (dashed black line) as well as the relative Ste5-YFP fluorescence in the Shmoo tip (blue) noting the initiation of its dissociation from the Shmoo tip (dashed blue line). We note that sometimes the nuclear Far1-Venus was only partially removed and sometimes appeared to reappear. In these cases, we score the initial depletion of Far1-Venus from the nucleus. The intensity of Ste5-YFP localized at the membrane was measured as follows: First we manually selected the shmoo in each cell that has a visible Ste5-YFP signal at the onset of Methionine removal. Next we identify the 5 brightest pixels along the membrane and their surroundings (a region of 3X3 pixels was selected). Pixels in this region which belonged to the interior of the cell were excluded leaving a 2X3 pixel region which was scored for total intensity. The result of this process was monitored frame by frame to prevent misregistrations. F. Histogram of time differences between the initiation of Far1 and Ste5 inhibition reveals that most cells initiate the Far1 degradation before Ste5 dissociates. N=43; P = 0.016. Mean disappearance initiation times were 8±1min (s.e.m.) for Far1 and 11±1min (s.e.m.) for Ste5.
Calculating the relative impact of the STE5-8A and FAR1-S87A mutants

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Choose parameter values for $k_1$ and $k_3$ (all other parameters are fixed)</td>
<td>$k_1 = 5, k_3 = 100$</td>
</tr>
<tr>
<td>2. Calculate the maximal amount of Cln1/2 this set of parameters can inhibit before committing to the cell cycle (at time $\tau_{\text{crit}}$).</td>
<td><img src="image1" alt="Graph" /></td>
</tr>
<tr>
<td>3. Redo step 2 for $k_1 = 0$ and then for $k_3 = 0$.</td>
<td><img src="image2" alt="Graph" /></td>
</tr>
</tbody>
</table>
| 4. Calculate the relative impact on the ability to inhibit Cln1/2 for each of the mutants. | \[
I_{\text{STE5-8A}} = \frac{Cln1/2^{\text{STE5-8A}}(k_1 = 0, k_3 = 100)}{Cln1/2^{\text{WT}}(k_1 = 5, k_3 = 100)} \quad \text{here} = 1.13
\]
\[
I_{\text{FAR1-S87A}} = \frac{Cln1/2^{\text{FAR1-S87A}}(k_1 = 5, k_3 = 0)}{Cln1/2^{\text{WT}}(k_1 = 5, k_3 = 100)} \quad \text{here} = 1.59
\]
| 5. Compare the relative impacts of the mutants with each other. | ![Graph](image3) |
| 6. Redo step 1-5 for a wide range of rates. | ![Graph](image4) |

Table S3, related to Figure 6: Algorithm to calculate the relative impact of removing Cln1/2 inhibition of Ste5 or Far1 for a series of inhibition rates of Ste5 ($k_1$) and Far1 ($k_3$).
Supplemental Experimental Procedures

Yeast strains and growth conditions

The strains used in this study are congenic with W303 (see Tables S1-2). Before the experiments, cells were grown in log-phase (OD ~ 0.1) in synthetic complete medium with 2% glucose (SCD) and then sonicated (~5s 3W). All media used were mixed with 20µg/ml casein (Sigma) to inhibit α-factor surface adhesion [4]. cln1Δ cln2Δ cells were treated slightly differently and were grown in log-phase in SCD lacking methionine (SCD - met) to express Cln2 from an integrated MET3 promoter [5]. cln1Δ cln2Δ cells were switched to SCD after one hour in the flowcell and then treated as the other strains.

Strains and plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>JS94-2b</td>
<td>MATa WHI5-GFP::KanMX HTB2-mCherry-spHIS5 ADE2 CLN2pr-GFPPEST-HIS3::cln2 MET3pr-CLN2::TRP1</td>
<td>[6]</td>
</tr>
<tr>
<td>JS95-11d</td>
<td>MATa WHI5-GFP::KanMX HTB2-mCherry-spHIS5 ADE2 CLN2pr-GFPPEST-HIS3::cln2::LEU2 cln1-del MET3pr-CLN2::TRP1</td>
<td>[6]</td>
</tr>
<tr>
<td>JS136-3c</td>
<td>MATa bar1::URA3 trp1::TRP1-MET3pr-CLN2 WHI5-GFP-kanMX HTB2-mCherry-spHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>JS146-8c</td>
<td>MATa bar1::URA3 cln1::HIS3 cln2Δ trp1::TRP1-MET3pr-CLN2 WHI5-GFP-kanMX HTB2-mCherry-spHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>JS150-5b</td>
<td>MATa bar1Δ STE5-8A trp1::TRP1-MET3pr-CLN2 WHI5-GFP-kanMX HTB2-mCherry-spHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>JS183-13</td>
<td>MATa bar1Δ STE5-8A 3XCLN2pr-mCherry-PEST-NLS WHI5-GFP-kanMX HTB2-mCherry-spHIS5</td>
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</tr>
<tr>
<td>JS187-1</td>
<td>MATa bar1::URA3 FAR1-S87A STE5-8A trp1::TRP1-MET3pr-CLN2 WHI5-GFP-kanMX HTB2-mCherry-spHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>JS193-5c</td>
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<td>JS196-4</td>
<td>MATa bar1Δ CLN2::3xCLN2-URA3 trp1::TRP1-MET3pr-CLN2 WHI5-GFP-kanMX HTB2-mCherry-spHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>JS197-29b</td>
<td>MATa bar1Δ ura3::URA3 trp1::TRP1-MET3pr-CLN2 WHI5-GFP-kanMX HTB2-mCherry-spHIS5</td>
<td>This study</td>
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<tr>
<td>JS198-2</td>
<td>MATa bar1Δ STE5-8A far1::CglaEU2 trp1::TRP1-MET3pr-CLN2 WHI5-GFP-kanMX HTB2-mCherry-spHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>JS211-5b</td>
<td>MATa bar1::URA3 FAR1-S87A trp1::TRP1-MET3pr-CLN2 WHI5-GFP-kanMX HTB2-mCherry-spHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>JS212-11b</td>
<td>MATa bar1Δ SIC1-GFP-HIS3 WHI5-mCherry-spHIS5 MYO1-GFP-kanMX ADE2 trp1::TRP1-MET3pr-CLN2</td>
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</tr>
<tr>
<td>JS221-4</td>
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<tr>
<td>AD6-29a</td>
<td>MATa bar1Δ trp1::TRP1-MET3pr-CLN2 HTB2-mCherry-spHIS5 FAR1-venus-kanMX ste5::STE5-1FP</td>
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<td>TCY3057</td>
<td>MATa bar1Δ ste5::STE5-1FP trp1::STE5-1FP-STE5-TRP1</td>
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</tr>
<tr>
<td>PPy1748</td>
<td>MATa bar1Δ STE5-8A</td>
<td>[7]</td>
</tr>
</tbody>
</table>

List of strains. All strains were obtained by standard methods, are congenic with W303 (leu2-3,112 his3-11,15 ura3-1 trp1-1 can1-1), and were constructed from stocks originating in the Cross laboratory at the Rockefeller University.

To create JS183-13, pJS20 was integrated at the CLN2 locus after EcoNI digestion and pPP1552 was integrated at the URA3 locus after SstI digestion. To create JS196-4, pJS23 was integrated at the CLN2 locus after EcoNI digestion. To create JS208-1,
pJS24 was digested with XcmI and integrated at the URA3 locus. The SIC1-GFP-HIS3 allele was a kind gift of the C. Tang lab at UCSF. Number and location of plasmid integrations was checked by qPCR. PCR based gene deletion of FAR1 was performed using pPP3129, which contains LEU2 gene from C. glabrata.

To construct JS187-1, we replaced the endogenous FAR1 allele with the FAR1-S87A phosphorylation site mutant allele. We synthesized (Epoch Biolabs) a fragment containing 200 bp of the FAR1 promoter just upstream of the start codon followed by the first 500 bp of FAR1-S87A allele. This fragment was flanked by SalI and EcoRI restriction sites and subcloned into the pRS406 plasmid to create pJS21, which was integrated into the yeast genome at the FAR1 locus after HindIII digestion. Next, we selected on FOA for clones that had lost URA3 resistance by popping out a WT gene fragment to leave only the complete FAR1-S87A allele at the endogenous locus. The perfect gene replacement of the STE5 allele with the STE5-8A mutant was also done using FOA selection by P. Pryciak[7].

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
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<tr>
<td>pJS20</td>
<td>pRS404-CLN2pr-mCherry-PEST-NLS</td>
<td>This study</td>
</tr>
<tr>
<td>pJS21</td>
<td>pRS406-200bpFAR1pr-500bpFAR1-S87A</td>
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<td>pRS406-CLN2</td>
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</tr>
<tr>
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<tr>
<td>pPP1552</td>
<td>pRS306-FUS1pr-GFP-NLS</td>
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<tr>
<td>pPP3129</td>
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<td>P. Pryciak</td>
</tr>
<tr>
<td>pSD03</td>
<td>pRS403-CLN2</td>
<td>[9]</td>
</tr>
</tbody>
</table>

Plasmids used in this study.

To construct pJS23, the CLN2 insert from pSD03 was ligated to the pRS406 vector following digestion with BglII. The insert contains 1410 bp of the CLN2 promoter, the CLN2 ORF, and 1120 bp of the downstream region. Dual active NLS-A from [10] was used in both pJS20 and pPP1552. The destabilizing PEST sequence is from CLN2 [11]. Unless, specified otherwise, promoters are the 1000bp upstream of the start codon. pJS24 was created by inserting a WHI5-GFP [12] fragment obtained by PCR of genomic DNA between the HindIII and BamHI sites of a pRS406 plasmid. pPP3129 was a kind gift of Prof. P. Pryciak (UMass).