Systematic Analysis of Essential Genes Reveals New Regulators of G protein Signaling

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SUMMARY

The yeast pheromone pathway consists of a canonical heterotrimeric G protein and MAP kinase cascade. To identify new signaling components we systematically evaluated 870 essential genes using a library of repressible-promoter strains. Quantitative transcription-reporter and MAPK activity assays were used to identify strains that exhibit altered pheromone sensitivity. Of the 92 newly identified essential genes required for proper G protein signaling, those involved with protein degradation were most highly-represented. Included in this group are members of the SCF (Skp-Cullin-F-Box) ubiquitin ligase complex. Further genetic and biochemical analysis reveals that SCF^{Cdc4} acts together with the Cdc34 ubiquitin conjugating enzyme at the level of the G protein, promotes degradation of the G protein α subunit, Gpa1, *in vivo* and catalyzes Gpa1 ubiquitination *in vitro*. These new insights to the G protein signaling network reveal the essential-genome as an untapped resource for identifying new components and regulators of signal transduction pathways.

INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is an established model for investigating fundamental biological processes including cell division, cell growth, and intracellular communication. One unique attribute of the yeast system is the availability of several thousand isogenic gene-deletion strains, which allows for unbiased genome-scale analysis of cellular functions (Giaever et al., 2002). However, of the approximately 6,000 genes in the yeast genome, nearly 1,100 are essential for viability and difficult to study using standard gene-deletion mutants. This limitation has led to a poor understanding of a substantial fraction of the yeast genome (Mnaimneh et al., 2004). Notably, these essential genes are more likely to have a human ortholog, as compared with non-essential genes (38% vs. 20%) (Hughes, 2002). Here we describe the identification of new components and new regulators

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of the G protein signaling apparatus. Our approach was to conduct a systematic analysis of the "essential genome", identify components required for efficient signal transduction, and establish their mode of action.

In yeast, a canonical heterotrimeric G protein signaling pathway regulates the process of cell mating. Yeast exists as one of two haploid cell types, **a** and α, that secrete peptide pheromones (**a** factor and α factor). These ligands bind to cell surface receptors, consequently promoting new gene transcription, morphological changes, cell cycle arrest, cell fusion, and the creation of an **a**/α diploid cell (Dohlman and Thorner, 2001).

As in other G protein pathways, agonist stimulation of the α factor receptor (Ste2) promotes exchange of GDP for GTP on the G protein α subunit (Gpa1). GTP-bound Gα undergoes conformational changes and dissociates from the Gβγ subunit dimmer (Ste4/18). Dissociated Gβγ can then signal through effector proteins including a mitogen-activated protein kinase (MAPK) cascade (Ste20, Ste11, Ste7, and Fus3). Inactivation of G protein signaling results from the slow intrinsic GTPase activity of Gα, hydrolyzing GTP to GDP, and the re-association of Gα and Gβγsubunits. GTP hydrolysis is further accelerated by the RGS (Regulator of G protein Signaling) protein Sst2. Therefore, Gpa1 functions primarily to sequester Gβγ in the absence of receptor stimulation (Dohlman and Thorner, 2001).

Many components of the yeast pheromone pathway were identified genetically, by isolating mutants that exhibit a mating-deficient (sterile) phenotype (Hartwell, 1980). Recent efforts to identify new components of G protein signaling have employed more systematic, genome-scale approaches (Slessareva et al., 2006). For example, a library of gene-deletion strains (representing almost all of the non-essential genes) was used to identify direct effectors of Gα signaling. Consequently, it was shown that Gpa1^{Gα} modulates pheromone signaling through a direct interaction with phosphatidylinositol (PtdIns) 3-kinase, resulting in elevated production of the second messenger PtdIns 3-P (Slessareva et al., 2006).

While the non-essential genes have been thoroughly studied, the essential genes are inherently less tractable and have therefore been poorly characterized. Previous approaches to investigating essential gene function have included the isolation of temperature-sensitive (ts) alleles, or fusion to a heat-inducible degron sequence (Dohmen et al., 1994; Kanemaki et al., 2003). However, the use of temperature-sensitive alleles requires growth at sub-optimal temperatures, and introduces destabilizing mutations that could alter enzyme function or protein-protein interactions. Recently a new resource for studying essential genes has been developed. Hughes and colleagues have constructed a library of repressible-promoter strains representing 870 of the yeast essential genes (Mnaimneh et al., 2004). These strains employ the tetracycline-regulatable promoter (TetO$_7$ promoter) system, allowing for precise control of gene expression, with no change in protein sequence or function. This TetO$_7$ promoter library has been used previously to identify new components of the cell division cycle (Yu et al., 2006), translation, and mitochondria import machinery (Mnaimneh et al., 2004). However, the role of essential genes in signal transduction has not been explored in any systematic manner.

While signal transduction networks, such as those mediated by heterotrimeric G proteins, are not typically thought of as essential for cell viability, they can share components with essential processes such as control of cytoskeletal rearrangements and the cell division cycle (Dohlman and Thorner, 2001). In fact, *GPA1* is an essential gene because when it is deleted, Gβγ is free to activate downstream effectors resulting in permanent cell cycle arrest (Miyajima et al., 1987).

Here, we systematically characterized 870 essential genes for participation in the yeast G protein signaling pathway. Our results show that proper G protein signaling requires the

Cdc34 E2 ubiquitin conjugating enzyme and the SCF[Cdc4] E3 ubiquitin ligase (Feldman et al., 1997; Skowyra et al., 1997). Ubiquitin ligases, such as the SCF, promote covalent modification of specific substrate proteins with ubiquitin, which can, in turn, target them for degradation by the 26S proteasome (Deshaies, 1999). Previous work has showed that Cdc34 and the SCF complex are involved in regulating the cell cycle and the mating-associated cell cycle arrest (Henchoz et al., 1997; Skowyra et al., 1997). Here we show that SCF also regulates signal initiation, through ubiquitination of the G protein α subunit. More generally, these findings reveal considerable overlap among genes required for cell viability and signal propagation.

RESULTS

Screen of essential genes for new regulators of G protein signaling

To identify new regulators of G protein signaling, we monitored the pheromone response in 870 TetO$_7$ promoter strains. This strain collection represents nearly all genes essential for viability. Pathway activation was measured initially using a pheromone-inducible promoter from the *FUS1* gene fused to the β-galactosidase (*lacZ*) gene. To validate this approach, we tested the effects of inactivation of two essential genes known to function in the pheromone response pathway: *GPA1* (Miyajima et al., 1987) and *CDC42* (Simon et al., 1995). *GPA1* encodes a negative regulator of the pheromone pathway that functions to sequester Gβγ, and thereby prevents it from activating effectors. As expected, doxycycline treatment of the TetO$_7$-GPA1 strain resulted in constitutive activation of the pathway, and a higher dose of doxycycline exacerbated this effect (Figure 1A). Conversely, *CDC42* encodes a positive regulator required for full activation of the signaling cascade. Doxycycline treatment of the TetO$_7$-CDC42 strain resulted in complete loss of the pheromone response (Figure 1B). As expected, treatment of the TetO$_7$ Wild-Type strain with doxycycline had no effect (Figure 1C). These results validate our screening method and demonstrate that the reporter assay is sufficiently sensitive to identify *bona fide* signaling components.

The 870 TetO$_7$ strains were next arrayed in a 96-well format and transformed with the *FUS1-lacZ* reporter. Since gene product depletion varies depending on mRNA and protein half-life, we treated each strain with two doses of doxycycline (10ng/mL and 100ng/mL) for 15hrs. Each strain was then exposed to a range of six pheromone concentrations and β-galactosidase activity was measured using a spectrofluorometer. During the screening process, 61 essential genes repeatedly failed to either transform with the reporter plasmid or grow to a suitable cell density (A$_{600nm}$ = 0.8) required to conduct the reporter assay, and were not tested.

After the initial rounds of screening, we identified 92 genes required for normal pheromone response as measured by our transcriptional reporter (Figure 2). By using a highly specific reporter assay, we were assured that components of the mating pathway would be identified. However we excluded an additional 97 genes likely to have global effects on transcription and translation. The list of excluded genes is comprised of those involved in mRNA production, protein synthesis, DNA replication, RNA processing, or ribosome biogenesis (Table S1).

We classified our hits by functional category using Gene Ontology (GO) annotations generated by Osprey, whereby each gene was classified according to one of 30 GO processes. When compared with all essential genes, our pheromone pathway hits were enriched for the following GO processes: protein degradation, cell cycle, protein transport, and phosphorylation (compare Figures 1D and 1E). Interestingly, genes involved in protein degradation were enriched 3-fold, by far the most highly represented group of functionally related genes (Figure 1F).

In order to further prioritize our investigations, we generated an interaction map using the Osprey Network Visualization System (Breitkreutz et al., 2002), which incorporates known physical and genetic interactions from the Biological General Repository for Interaction Datasets (BioGRID) (Stark et al., 2006) (Figure S1). We found several previously known interactions between pheromone pathway components and genes identified in our screen. In particular, genes involved in cell organization and biogenesis (dark blue), protein transport (green), and protein degradation (orange) were highly connected to known pheromone pathway components. We also used hierarchical clustering to organize genes into phenotypically similar groups based on changes in the maximal response, EC50, and basal activity (Figure 2). We found that genes involved with protein degradation tended to cluster more closely than genes involved in other cellular processes such as metabolism, cell organization and biogenesis. Given that genes involved in protein degradation were (*i*) over-represented in our screen, (*ii*) highly connected with known pathway components, and (*iii*) clustered more closely than any other functional group, we reasoned they must play a particularly important role in regulating G protein signaling.

Screen validation

Based on the above analysis, we selected six genes deemed likely to participate in cell signaling (Table 1). *MPS1* is the yeast ortholog of mammalian TKK (Liu et al., 2003), and is known to encode a dual-specificity kinase (Lauze et al., 1995). According to our hierarchical analysis it was the only gene that failed to cluster with any of the other 91 hits. We speculate that Mps1 phosphorylates some component of the pheromone pathway. We also selected for further consideration two PtdIns 4-kinases, *STT4* and *PIK1* (Flanagan et al., 1993;Yoshida et al., 1994). Recently, components of the PtdIns 3-kinase, Vps15 and Vps34, were shown to bind directly to Gα and to be required for full activation of the pheromone signaling pathway (Slessareva et al., 2006). Given this precedent, we postulated a broad role for mono-phosphorylated inositides in G protein signaling. Validation of *STT4* and *PIK1* involvement in G protein signaling would strengthen the proposed model. Among genes involved in protein degradation, we were particularly interested in *CDC4, CDC34*, and *CDC53* because they encode proteins that either form (*CDC53, CDC4*) or function with (*CDC34*) the SCF^{Cdc4} ubiquitin ligase complex (Feldman et al., 1997;Skowyra et al., 1997). The Cdc34/SCF family of ubiquitin ligases regulates a variety of proteins, in most cases promoting their degradation by the proteasome. Among SCF^{Cdc4} targets are proteins that play key roles in the regulation of cell growth and division, and the mating-associated cell cycle arrest. However, we were interested in determining a role of SCF^{Cdc4} in regulating components of the G protein-coupled signaling cascade.

The six TetO$_7$ strains described above were re-transformed with the *FUS1-lacZ* reporter and re-tested individually using a broader range of pheromone concentrations, as well as a higher dose of doxycycline (10μg/mL). Testing individual strains in this manner confirmed results obtained by the high-throughput screening method. For further validation, we transformed each TetO$_7$ strain with a single-copy plasmid containing the absent wild-type gene, and showed this restored proper signaling (Table 1).

Transcriptional reporter assays are susceptible to false positives by proteins affecting overall gene expression. To determine if any of the six components have global effects on transcription or translation, we used a dual-reporter assay containing red fluorescent protein (RFP) under the control of a constitutive promoter (*ADH1*-RFP) and green fluorescent protein (GFP) under the control of a pheromone-responsive promoter (*FUS1*-GFP). None of the six genes exhibited any change in RFP abundance, despite clear differences in GFP expression. Investigation of five other randomly-selected genes revealed three that alter RFP as well as GFP expression. Thus each of the six genes of interest regulates signaling in a pathway-specific manner (Table 1 and Figure S2).

Analysis of pathway regulation by the newly identified essential genes

Information from dose-response curves can be used to infer function. For example, a gene mutation leading to an increase in maximal response typically indicates a negative role in signaling. Knockdown of five genes, *CDC4, CDC34, CDC53, STT4* and *PIK1*, dampened the pheromone response (Figures 3 B–F), indicating a positive role in signaling. Conversely, knockdown of *MPS1* resulted in an increase in maximal activation (Figure 3G), indicating a negative role in signaling. Knockdown of *PIK1* resulted in substantial pathway activity even in the absence of pheromone addition (Figure 3F). These findings indicate that *PIK1* (or its catalytic product PtdIns 4-P) serves to suppress basal signaling.

To further define the function of each candidate gene, we measured pathway activity upstream of transcriptional regulation. Phosphorylation of the MAPK is a prerequisite for transcription of pheromone-responsive genes. Therefore we measured MAPK phosphorylation using an antibody that recognizes the dually-phosphorylated, fully-activated form of Fus3 and the partially redundant MAPK Kss1. Compared to the $TetO_7$ wild-type control strain (Figure 3A), knockdown of *CDC4, CDC34, CDC53, STT4*, or *PIK1* resulted in a decrease in Fus3 phosphorylation (Figures 3B–F) in relation to total Fus3 levels (Figure S3). Conversely, knockdown of *MPS1* resulted in an increase in Fus3 activation (Figure 3G). Thus the changes in Fus3 phosphorylation mirror the changes in β-galactosidase activity reported above. These results confirm a role for each gene in pheromone pathway regulation, and indicate they all function at a point upstream of the MAPK.

Most genes involved in the pheromone response pathway regulate phosphorylation of both Fus3 and Kss1 in tandem. Interestingly, doxycycline treatment of both $TetO_7$-PIK1 and $TetO_7$-STT4 strains resulted in a reduction of Fus3 but not Kss1 activity (Figures 3E and 3F). Selective regulation of Fus3 has only been observed for a small number of gene deletion mutants, but this list includes the Fus3-binding protein Ste5 (Choi et al., 1994; Marcus et al., 1994) as well as the PtdIns 3-kinase Vps34 and its binding partner Vps15 (Slessareva et al., 2006). Pik1 and Stt4 are both PtdIns 4-kinases, but are present at different subcellular locations: Stt4 at the plasma membrane (Audhya and Emr, 2002) and Pik1 at the Golgi and the nucleus (Strahl et al., 2005). Taken together, these findings reveal a possible role for mono-phosphorylated inositides in Fus3 signaling.

SCF[Cdc4] regulates the pheromone pathway upstream of Ste4[Gβ]

Of the essential genes found to regulate pheromone signaling, we were particularly interested in those that promote protein degradation. As noted above, genes involved in degradation were enriched almost 3-fold compared to all the essential genes. Moreover they were clustered more closely than any other functional group in the hierarchical analysis. Among the genes identified in our screen, *CDC4, CDC34*, and *CDC53* encode components of the Skp1, Cullin, F-box protein (SCF)-type ubiquitin ligase (Feldman et al., 1997; Skowyra et al., 1997). When treated with doxycycline, the $TetO_7$-CDC4, $TetO_7$-CDC34, and $TetO_7$-CDC53 strains all exhibited a significant decrease in the maximal response to pheromone as well as diminished MAPK activation (Figures 3B, 3C, and 3D). The similarity of the responses of these strains, and the fact that the affected proteins exist as a complex in cells, suggests the SCF[Cdc4] has a particularly important role in signal regulation.

While each component of the SCF is necessary for ubiquitin-ligase function, the F-box protein, Cdc4, binds directly to the substrate and therefore defines substrate specificity of the SCF complex. For this reason we focused further efforts on characterizing the role of Cdc4. To narrow our search for Cdc4 substrates we activated the pathway at several points downstream of the pheromone receptor. First, we over-expressed the constitutively active *STE11-4* mutant (Stevenson et al., 1992). *STE11* encodes the MAPK kinase kinase

(MAPKKK) that phosphorylates Ste7, which in turn phosphorylates and activates Fus3 and Kss1. TetO$_7$-CDC4 cells expressing *STE11-4* triggered MAPK phosphorylation in the absence of pheromone. However, doxycycline treatment of these cells had no effect on Fus3 activity, in contrast to the reduction seen in pheromone-stimulated cells (Figure 4A). These results indicate that SCF^{Cdc4} acts on a protein component that is upstream of the MAPKKK.

Next, we over-expressed *STE4*^{Gβ} using a galactose-inducible promoter. Since Gpa1 cannot sequester excess Ste4^{Gβ} (Miyajima et al., 1987), the overproduced Ste4^{Gβ} is free to activate effectors even in the absence of any stimulus. TetO$_7$-CDC4 cells containing *GAL-STE4* were grown in dextrose- or galactose-containing medium to induce protein expression. Overexpression of *STE4* resulted in MAPK activation in the absence of pheromone. Once again, doxycycline treatment failed to dampen this signal (Figure 4B). Thus knock-down of Cdc4 attenuates signaling by pheromone but not the G protein subunit. These data indicate that SCF acts at the level of the G protein or the receptor.

Loss of Cdc4 stabilizes Gpa1 protein levels

Our genetic epistasis analysis indicates that Cdc4 acts early in the pathway. We hypothesized further that Cdc4 must ubiquitinate a negative regulator, since knockdown of Cdc4 resulted in a decrease in signaling. There are only two well-characterized negative regulators that function upstream of Ste4^{Gβ}, Gpa1 and the RGS protein Sst2, and both are known to be ubiquitinated (Hao et al., 2003; Madura and Varshavsky, 1994). To determine if Cdc4 affects Gpa1 or Sst2 stability, we tracked their rates of degradation in a temperature-sensitive *cdc4-1* strain. This mutant strain has been used previously in similar experiments to identify Cdc4 substrates (Tang et al., 2005). The rate of Sst2 degradation was similar in both the wild-type and *cdc4-1* cells (Figures 5B and 5D). In contrast, Gpa1 was considerably more stable in the absence of Cdc4 function. Whereas Gpa1 in wild-type cells exhibited an approximate half-life of 141min, in *cdc4-1* cells the half-life was extended to 344min (Figures 5A and 5C). These data demonstrate that Cdc4 is required for proper turnover of Gpa1 *in vivo*. Interestingly, Wang *et al.* showed that a mutant Gpa1 that cannot be ubiquitinated *in vivo* produces a dampened pheromone response (Wang et al., 2005), comparable to that seen in the SCF mutant strains (Table 1). Taken together, these data indicate that SCF^{Cdc4} regulates the function of Gpa1.

We also considered whether the Cdc34/SCF complex regulates other components involved in G protein signaling. To this end we measured the abundance and stability of proteins downstream of the receptor but upstream of the transcription factor (Ste4, Ste20, Ste5, Ste11, Ste7, Fus3, and Kss1). To identify Cdc34/SCF substrates, including those that could be recruited by F-box proteins other than Cdc4, we used a temperature sensitive *cdc34-2* mutant strain. Of the proteins tested, four (Ste4, Ste20, Ste7, and Ste5) were significantly stabilized in the *cdc34-2* mutant as compared with wild-type cells (Figure S4A). These data corroborate reports that the Cdc34/SCF pathway promotes the degradation of Ste7 and Ste5 (Garrenton et al., 2009; Wang and Dohlman, 2002), and suggest that it may likewise act on Ste20 and Ste4. Of these proteins at least one binds to Cdc4 (Ste5) and another is clearly ubiquitinated (Ste7) (Garrenton et al., 2009; Wang and Dohlman, 2002). Strikingly, despite stabilized expression of multiple components that *propagate* the signal, loss of Cdc34 or SCF^{Cdc4} function leads to a *reduction* in pheromone signaling. Furthermore, loss of *CDC4* gene expression has no effect when the pathway is activated at the level of Ste4^{Gβ} (Figure 4B). Taken together, these findings highlight the importance of a negative regulator as the most functionally significant target for Cdc34/SCF^{Cdc4}-dependent degradation, and demonstrate that the dampened pheromone signaling in SCF-depleted cells is due primarily to the stabilization of Gpa1.

Cdc34 poly-ubiquitinates purified Gpa1 *in vitro* in an SCF^{Cdc4}-dependent manner

Protein turnover is often dependent upon polyubiquitination. Gpa1 was shown previously to undergo ubiquitination *in vivo* (Madura and Varshavsky, 1994). To test the possibility that SCF^{Cdc4} is directly responsible for the modification, we sought to establish whether SCF^{Cdc4} could interact with Gpa1. We affinity purified Skp1-GST/Cdc4-HA from insect cell lysates and added purified Gpa1-Flag. As shown in Figure S4B, Gpa1 copurified with Cdc4. Additionally, we tested a closely related F-box protein, Met30. Both Met30 and Cdc4 contain a substrate-binding domain comprised of WD repeats (Chandrasekaran et al., 2006). Gpa1 bound to Met30 (Figure S4B), but with a lower affinity than Cdc4. These results suggest that the Cdc4 component of the SCF^{Cdc4} ubiquitin ligase complex binds Gpa1, and as a consequence may promote its ubiquitination by Cdc34.

To establish whether Gpa1 is ubiquitinated by Cdc34/SCF^{Cdc4} we sought to reconstitute Gpa1 ubiquitination *in vitro*. We purified Gpa1-Flag from yeast (to maintain post-translational modifications) and purified the SCF^{Cdc4} complex (comprised of yeast Flag-Skp1, Cdc53, Rbx1, and Cdc4) from insect cells. As shown in Figure 5E, Gpa1 is polyubiquitinated in reaction mixtures composed of purified SCF^{Cdc4}, Cdc34, Uba1, Ubiquitin and ATP, but not in the absence of SCF^{Cdc4} or Cdc34. To determine if the SCF polyubiquitinates Gpa1 at a single site, or instead monoubiquitinates Gpa1 at multiple sites, we performed the *in vitro* ubiquitination reactions with a mutant form of ubiquitin (Ub^{0K}) that, due to the replacement of all the lysines with arginine, cannot be incorporated into poly-ubiquitin chains. Under such conditions, we observed only a single 8kD shift, suggesting Gpa1 is modified at a single lysine residue (Figure 5F).

In order to determine if Gpa1 is ubiquitinated by the SCF^{Cdc4} at the previously identified site, we conducted an *in vitro* ubiquitination assay using a mutant form of Gpa1 that lacks the ubiquitinated subdomain (Gpa1$^{\Delta128-236}$). As shown in Figure 5F, Gpa1$^{\Delta128-236}$ is not ubiquitinated *in vitro*, indicating the SCF^{Cdc4} ubiquitinates Gpa1 at the known site of ubiquitination. Interestingly, Gpa1$^{\Delta128-236}$ is not ubiquitinated, and the response to pheromone is partially abrogated. This gain-of-function phenotype is likely due to stabilized expression of the protein. However we cannot rule out the possibility that there are other functional differences between the mutant and wild-type protein. As an additional control, we conducted an *in vitro* reaction with the F-box protein Met30. We show above that Met30 was able to bind Gpa1. However, SCF^{Met30} complexes were not able to ubiquitinate Gpa1 (Figure 5G).

Finally, the SCF complex is typically recruited to substrates in response to substrate phosphorylation (Ardley and Robinson, 2005). To determine if Gpa1 ubiquitination by the SCF^{Cdc4} is regulated by phosphorylation we conducted *in vitro* ubiquitination assays with Gpa1 from *E. coli*, which should lack all post-translational modifications. As shown in Figure S4C, Gpa1 expressed from *E. coli* is ubiquitinated poorly, indicating that Gpa1 ubiquitination by SCF^{Cdc4} may be regulated by a post-translational modification.

Notably, the Gpa1$^{\Delta128-236}$ mutant, which is not ubiquitinated *in vivo* or *in vitro* (Wang et al., 2005) (Figure 5F), exhibits a dampened pheromone response comparable to that seen following knock-down of SCF^{Cdc4} expression (Table 1). The ability of SCF^{Cdc4} to ubiquitinate Gpa1 *in vitro*, and to accelerate Gpa1 turnover *in vivo*, reveals Gpa1 as a critical target of SCF regulation. To date, only a handful of SCF^{Cdc4} substrates have been verified by direct ubiquitination using purified components. Indeed, of the mating pathway components only the cell cycle regulatory protein Far1 has been characterized in this manner (Figure 6) (Blondel et al., 2000). The identification of Gpa1 as an SCF^{Cdc4} substrate could explain the critical role of SCF^{Cdc4} in pheromone signaling, and expands the repertoire of regulatory SCF^{cdc4} functions in the pheromone pathway (Figure 6).

DISCUSSION

The yeast pheromone response system is perhaps the most thoroughly characterized of any signaling pathway. The contributions of non-essential gene products have been exhaustively and systematically characterized over much of the past three decades. Much less is known about genes that are essential to cell viability, but which nevertheless contribute to effective signal transduction. Until now, only two major components of the pheromone signaling cascade, Gpa1 and Cdc42, were known to be encoded by essential genes. To address this deficit, we conducted a systematic analysis of the essential yeast genome. Our results implicate 92 essential genes in the regulation of G protein signaling, verify the involvement of 6 selected genes, and define their mode of action. These findings suggest a level of complexity in G protein signaling that has not been fully appreciated.

Protein Degradation

Among the genes required for proper pheromone responses, those involved in protein degradation (13 of 92 genes) were over-represented in our screen, indicating that they may have a particularly important role in signal regulation. Included in this group are four components of the 20S proteasome, four components of the 19S regulatory particle (together forms the 26S proteasome complex), as well as the Cdc34 E2 ubiquitin conjugating enzyme and the SCF^{Cdc4} E3 ubiquitin ligase. These data are consistent with the observation that many of the core components of the pheromone pathway are ubiquitinated, including the receptor, G protein, RGS protein, and components of the MAP kinase cascade (Esch and Errede, 2002; Garrenton et al., 2009; Hao et al., 2003; Henchoz et al., 1997; Hicke and Riezman, 1996; Madura and Varshavsky, 1994; Wang and Dohlman, 2002). Further, there is *indirect* evidence that SCF complexes promote ubiquitination of several proteins that propagate the signal, including Ste5 (Garrenton et al., 2009), Ste7 (Wang and Dohlman, 2002), Ste4 and Ste20 (Figure S3). Strikingly, despite stabilized expression of these positive regulators, loss of SCF^{Cdc4} function results in a *reduction* in pheromone signaling. A negative regulator is thus the most functionally significant target of the SCF^{Cdc4} in signal transduction. Our analysis indicates that Gpa1 is this critical target of the SCF^{Cdc4}.

These findings expand the classic roles of SCF complexes in orchestrating the events leading to mating (Figure 6). Arrest of cell division in the G1 phase is needed to ensure cell cycle synchrony prior to cell fusion. Essential to G1 cell cycle arrest is inactivation of the cyclin-dependent kinase (CDK) Cdc28. Several mechanisms contribute to the G1-specific functions of Cdc28; these include degradation of the G1 cyclins Cln1 and Cln2, and direct inhibition of Cln/Cdc28 activity by Far1 (Peter and Herskowitz, 1994), a protein that is upregulated in a Ste12-dependent manner during mating (Chang and Herskowitz, 1990). Additionally, these changes prevent proteolysis of the Sic1 S-phase CDK inhibitor, which normally depends on G1 CDK activity, thereby ensuring that the S-phase Cdc28 functions are not activated in mating cells. Each of these processes is regulated by SCF complexes. Whereas SCF^{Cdc4} promotes the ubiquitination and degradation of Far1 (Henchoz et al., 1997) and Sic1 (Feldman et al., 1997;Skowyra et al., 1997), SCF^{Grr1} promotes the degradation of Cln1 and Cln2 (Barral et al., 1995;Schweitzer et al., 2005).

As noted above, loss of Gpa1 leads to G1 cell cycle arrest. Thus an additional role for SCF^{Cdc4} in ubiquitination and degradation of Gpa1 is likely to contribute to cell cycle regulation. Inhibition of SCF^{Cdc4} could promote a timely recovery following pheromone stimulation, and also prevent pheromone signaling in non-G1 phases of the cell cycle when improper activation of cell mating could result in aneuploidy. The observation that many of the pheromone pathway components are not expressed in diploid cells suggests that SCF could also promote degradation of unnecessary (haploid-specific) signaling proteins and, as such, prevent aberrant pathway activation after mating.

Gpa1 as a target for proteasomal and vacuolar proteolysis

Gpa1 is a rare example of a protein that can be either mono- or poly-ubiquitinated (Madura and Varshavsky, 1994). Whereas polyubiquitinated Gpa1 is targeted to the proteasome, monoubiquitinated Gpa1 is internalized and degraded within the vacuole (Wang et al., 2005). Left unresolved was whether mono- and poly-ubiquitination of Gpa1 requires two distinct sets of E2 and E3 enzymes. Recently, we showed that Rsp5 monoubiquitinates Gpa1 (Torres et al., 2009). Here we show that the SCF complex polyubiquitinates Gpa1. Taken together these findings reveal that Gpa1 mono- and poly-ubiquitination occur by distinct pathways and/or mechanisms.

Although complex, there may be specific benefits to having two ubiquitinating pathways that can target the same protein for distinct proteolytic machineries. One such benefit would be that degradation could be triggered in response to different signals and/or functional states of the protein. In support of this model, we showed that the Rsp5 E3 ligase ubiquitinates only the fully myristoylated (fully mature) form of the G protein. Myristoylated Gpa1 would localize to the plasma membrane and its degradation in vacuoles could therefore be linked to endocytosis. Conversely, there may be another modification that directs Gpa1 to SCF^{Cdc4}. Indeed, many E3 ubiquitin ligases including the SCF, are recruited to substrates in response to substrate phosphorylation (Ardley and Robinson, 2005). Recently, two independent phospho-proteomic screens revealed that Gpa1 is phosphorylated at Thr-189 and Ser-200 (Li et al., 2007; Smolka et al., 2007), and it is noteworthy that both sites are located near a known Gpa1 ubiquitination site, Lys-165, established by mass spectrometry (Marotti et al., 2002). In this study, we show that Gpa1 purified from *E. coli* and lacking any post-translational modifications is not ubiquitinated *in vitro*. Thus phosphorylation of Gpa1 may serve to recruit the SCF ubiquitination machinery. It is also possible, however, that phosphorylation of a Gpa1-binding partner, rather than Gpa1 itself, is sufficient to direct the protein to the SCF^{Cdc4} complex. Knowing when Gpa1 is phosphorylated, whether there are additional sites of phosphorylation, and the identity of the protein kinase(s) could further establish the role of Gpa1 ubiquitination in regulating the pheromone pathway.

Interestingly, depletion of Cdc4 does not result in increased steady-state levels of Gpa1 (data not shown). While any differences in Gpa1 abundance are small, even small differences in abundance could account for the 54% loss of signal observed in the TetO$_7$-CDC4 strain. The SCF^{Cdc4} likely targets a small pool of Gpa1 that is functionally important for signaling as has been shown for other proteins (Chandrasekaran et al., 2006; Tworkowski et al., 2002). In this case, small changes in protein levels, in a specific functional context, can result in large changes in signaling. We further expect that slower Gpa1 turnover in the TetO$_7$-CDC4 strain would increase the proportion of protein that has had time to be correctly folded, fully modified, properly localized, and assembled into the heterotrimeric complex. Unfortunately, it is not currently feasible to distinguish Gpa1 that is folded and functional from protein that is non-functional but nevertheless expressed in the cell.

G protein ubiquitination may also be regulated by external stimuli. F-box proteins appear to serve as receptors for the plant hormones auxin and jasmonates; in each case binding to these hormones enhances the interaction between SCF and its substrates (Dharmasiri et al., 2005; Katsir et al., 2008; Kepinski and Leyser, 2005). Thus F-box proteins and ubiquitin ligases can function as hormone receptors. By extension, F-box proteins might also serve as targets for drugs that enhance or diminish signaling by G proteins and G protein-coupled receptors.

Non-proteolytic new essential regulators of G protein signaling

We also characterized three other essential genes required for proper G protein signaling. Of particular interest are the PtdIns 4-kinases Pik1 and Stt4. Whereas pheromone stimulation leads to activation of both Fus3 and Kss1, we found that knockdown of *PIK1* or *STT4* leads to a selective diminution of Fus3 activity. These observations suggest a positive role for PtdIns 4-P in signal transduction, and in particular for Fus3. Similarly, there is a selective loss of Fus3 activation in cells that lack the PtdIns 3-kinase components Vps34 and Vps15, or in cells treated with the PtdIns 3-kinase inhibitor Wortmannin (Slessareva et al., 2006). Taken together, these findings indicate a potential role for mono-phosphorylated phosphoinositides in Fus3 activation. Notably, Fus3 activation requires the MAPK scaffolding protein Ste5, while Kss1 activation does not. Moreover, Ste5 was shown to bind to PtdIns 4-P and PtdIns 4,5-P_2 *in vitro* (Garrenton et al., 2006). We hypothesize that PtdIns 4-P interaction is required for Ste5 activity or proper localization. Additionally, PtdIns 3-P and 4-P are produced in different sub-cellular locations, and these differences could contribute further to signaling specificity and activity. In any event, these discoveries suggest an important and expanded role for phosphoinositides in the pheromone-response pathway.

Conclusions

We have systematically characterized 870 essential genes for participation in the yeast G protein signaling pathway, identified up to 92 new regulators of the pathway, and characterized six in detail. Our findings reveal considerable overlap among genes required for cell viability and signal propagation. More significantly, our work reveals that there are still many new pathway components to be found. While we focused on components for which there was a specific and rigorously-testable hypothesis, there are still dozens of others that will be pursued in the future. Based on these results, we regard the essential-genome as an under-utilized resource for the identification of new signal transduction factors. Further efforts to screen the essential genes in yeast and other model systems will undoubtedly lead to a more complete understanding of signal transduction networks. Given the conservation of G protein function across species, newly described functions in yeast are likely to extend as well to humans. Due to the established importance of G proteins in physiology and pharmacology, our findings may also reveal future opportunities for drug discovery.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

See Supplemental Methods.

Cell-Extract Preparation and Immuno-Blot Analysis

See Supplemental Methods

Preparation and Purification of Recombinant Proteins

See Supplemental Methods.

Screen of Essential Genes

852 TetO$_7$ promoter strains (Open Biosystems, yTHC) were transformed with pRS423 *FUS1*-lacZ, and β-galactosidase activity was measured in a 96-well plate format as described previously (Chasse and Dohlman, 2004). Prior to pheromone stimulation, cells were grown for ~15hrs in either untreated medium or medium containing 10ng/mL, or 100ng/mL doxycycline hyclate (Sigma-Aldrich). β-galactosidase activity was measured in triplicate for each condition. Strains were considered for further analysis if doxycycline

treatment resulted in a >50% increase or decrease in maximal response, at least a two standard deviation shift in the EC50, or at least a four-fold increase in the basal activity as determined by non-linear regression analysis (Graphpad Prism). Twenty-six candidate strains were re-tested individually using twelve concentrations of α factor pheromone and a higher dose of doxycycline (10μg/mL). Eleven confirmed strains were transformed with the pRS316-AR/FG dual reporter and tested for GFP and RFP expression by immunoblotting. While all eleven strains exhibited changes in GFP expression, three TetO$_7$-strains representing the essential genes *RIO2, KIC1*, and *SSY1* also exhibited detectable changes in RFP expression and were not considered further. The remaining eight TetO$_7$-strains representing the essential genes *MPS1, STT4, PIK1, CDC4, CDC53, CDC34, PMA1*, and *SLN1* showed no change in RFP expression. Of the remaining eight strains, six were selected for transformation with a single-copy plasmid containing the corresponding wild-type gene, and these were re-tested for restoration of normal β-galactosidase activity (Table 1).

Protein Turnover Measurements

Cells were grown in 100mL of selective medium at room temperature to A$_{600nm}$ ~0.6, and then shifted to 37°C for 1hr. Cells were then treated with cycloheximide (final concentrations, 10μg/mL in 0.1% ethanol) for up to 3hrs. Growth was stopped by the addition of TCA (5% final concentration) and samples were normalized to the same A$_{600nm}$. Cell pellets were washed and brought up directly in boiling SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.0005% bromophenol blue) and lysed using glass beads as previously described (Wang et al., 2003). Protein extracts were resolved by 10% SDS-PAGE and immunoblotting with Gpa1 antibodies at 1:1000 (Dohlman et al., 1993) and Sst2 antibodies at 1:2000 (Dohlman et al., 1996).

In Vitro Ubiquitination Assay

Ubiquitination reactions were prepared with [Flag]SCF complexes containing Flag-Skp1/Cdc53/Myc-Rbx1/Cdc4 or Met30 purified from insect cells as described in Supplemental Methods, and in (Skowyra et al., 1997). 9μL of purified and eluted SCF complex (~2 pmol) was combined with 13.5 pmol [His]Cdc34, 1.0 pmol [His]Uba1, 120 pmol Ub or 0K Ub (Boston Biochem), 1.5 pmol Gpa1-Flag and supplemented with 1mM ATP, 5mM MgCl$_2$, and 20μM GDP in a volume of 15μL. Reactions were allowed to proceed at 30°C and stopped after 90min with the addition of boiling SDS-PAGE sample buffer, followed by SDS-PAGE and immunoblotting with Gpa1 antibodies. Membranes were stripped and re-probed with Cdc4 antibodies at 1:1000 and Cdc53 antibodies at 1:1000 (provided by Mark Goebl, University of Indiana Medical School).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Yu L, Pena Castillo L, Mnaimneh S, Hughes TR, Brown GW. A survey of essential gene function in the yeast cell division cycle. Mol Biol Cell. 2006; 17:4736–4747. [PubMed: 16943325]

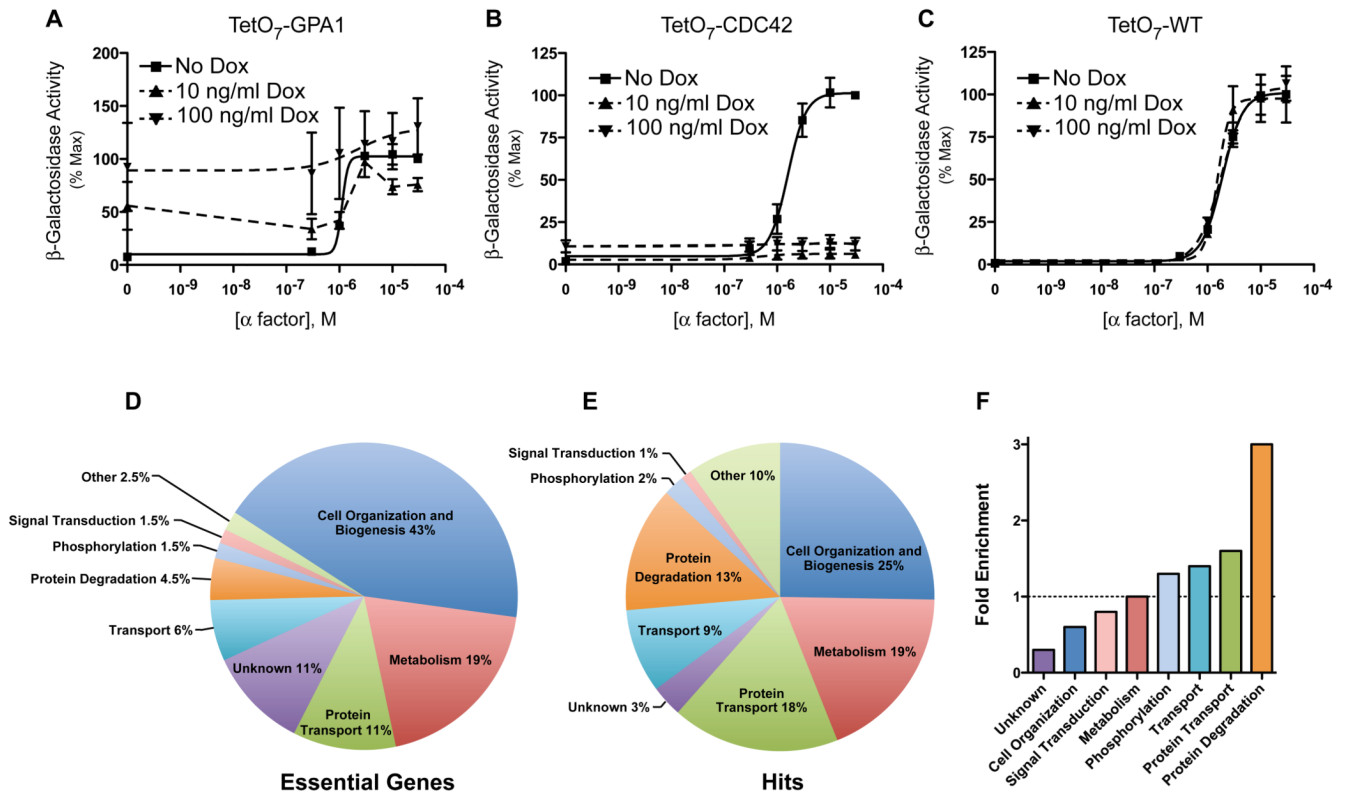**Figure 1. Validation of the TetO₇ Promoter Essential Gene Screen**

(A) Transcriptional activation (β-galactosidase activity) in response to α factor treatment was measured spectrofluorometrically in TetO₇-GPA1 cells treated with doxycycline (Dox, 10ng/mL and 100ng/mL) or untreated control. Cells were transformed with a plasmid containing the pheromone-inducible reporter *FUS1-lacZ*. Data were analyzed by non-linear regression (sigmoidal-dose response, variable slope) using GraphPad Prism software. Results are the mean ± S.E. for three individual experiments each performed in triplicate.

(B) TetO₇-CDC42 cells treated as in (A).

(C) TetO₇-WT cells treated as in (A).

(D) Percentage of essential genes associated with the indicated GO Process.

(E) Percentage of essential gene hits associated with the indicated GO Process.

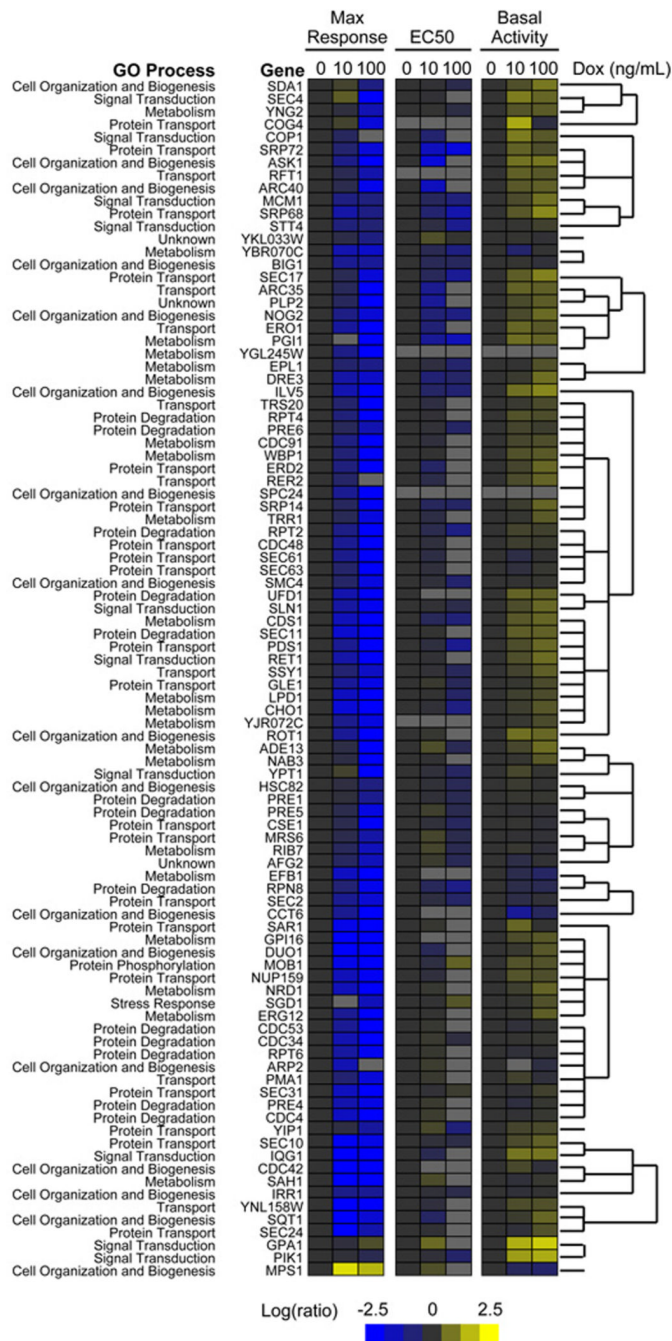(F) Fold-enrichment of hits compared to all essential genes for each GO Process.

Figure 2. Phenotype Clustering Analysis
Gene hits were analyzed by Cluster 3.0 software based on maximum response, EC50, and basal activity normalized to the untreated control and converted to Log_2. Gene similarity was calculated using Pearson correlation (uncentered correlation) and clusters were generated using centroid linkage. Clustering data was visualized by Java TreeView (v 1.1.3). Genes were labeled by their involvement in the indicated GO Process. See also Figure S1 and Table S1.
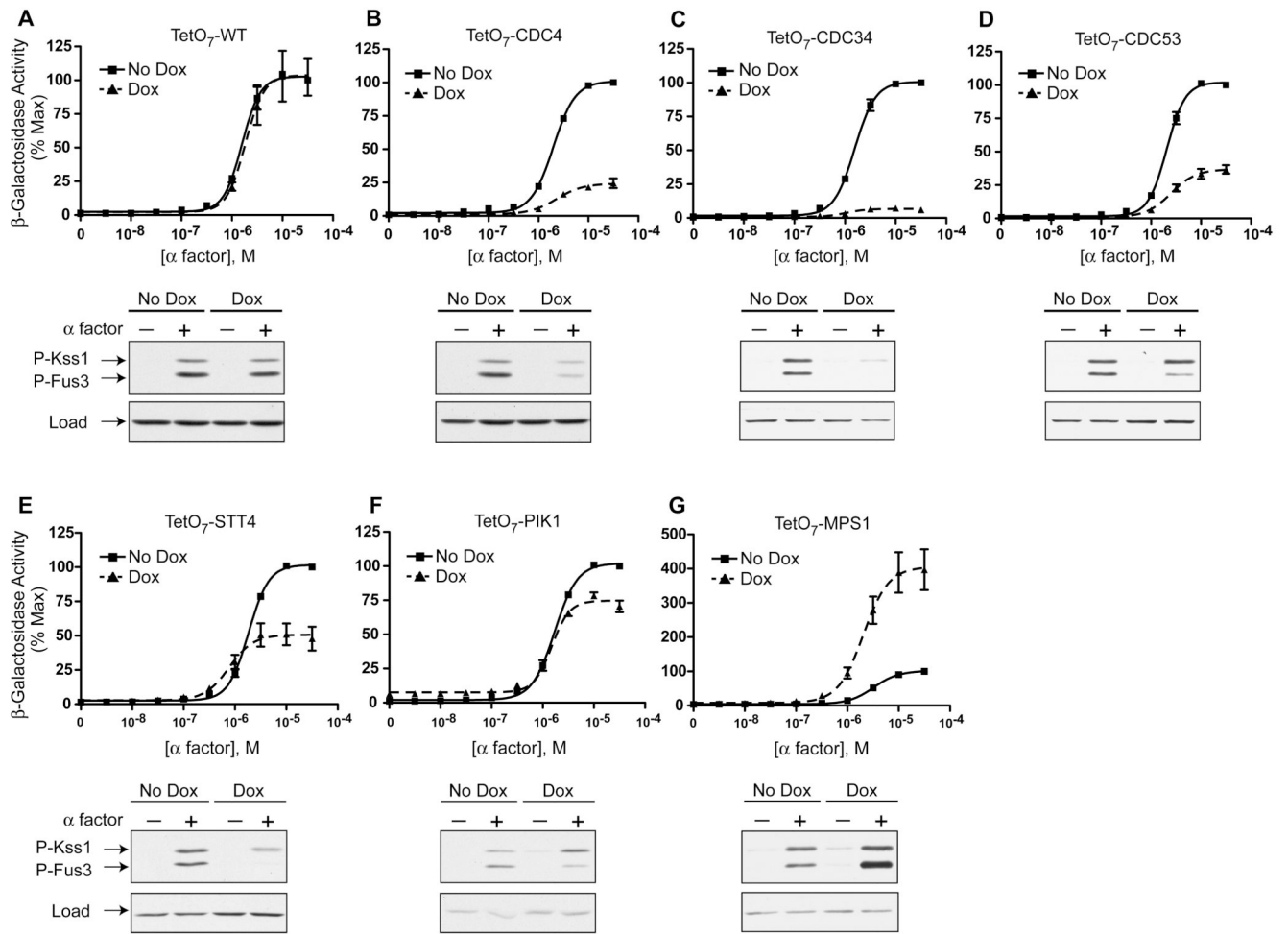
Figure 3. Verification of the Roles of Selected Essential Genes in Pheromone Signaling
(A–G) The indicated essential genes were chosen for further validation and analysis. $TetO_7$ strains expressing *FUS1-lacZ* were treated with 10μg/mL doxycycline for 15hrs and exposed to the indicated concentrations of α factor for 90min. Below each pheromone dose-response curve is a corresponding immunoblot probed using phospho-p42/44 (P-Fus3, P-Kss1) or G6PDH (load control) antibodies. $TetO_7$ strains were treated with 10μg/mL doxycycline for 15hrs and then 3μM α factor for 30min. Results are the mean ± S.E. (n=5). See also Figure S3.
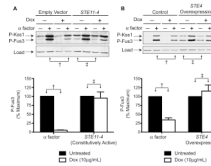
Figure 4. The Cdc34 E2 and SCF^Cdc4 Regulate Signaling Upstream of Ste4^Gβ

(A) TetO$_7$-CDC4 cells were transformed with a plasmid containing either *STE11-4* (constitutively active mutant) or no insert. Cells were treated with 10µg/mL doxycycline for 15hrs and then 3µM α factor for 30min. Samples were analyzed by immunoblotting using phospho-p42/44 or G6PDH antibodies. Bar graphs represent quantification of the indicated bands. Results are the mean ± S.E. (n=3).

(B) TetO$_7$-CDC4 cells were transformed with a plasmid containing *STE4* under the control of a galactose-inducible promoter. Cells were treated with 10µg/mL doxycycline for 12hrs in medium containing either dextrose or switched to galactose (2% w/v final concentration) for 3hrs prior to α factor treatment (3µM for 30min). Samples were analyzed by immunoblotting using phospho-p42/44 or G6PDH antibodies. Bar graphs represent quantification of the indicated bands. Results are the mean ± S.E. (n=3).
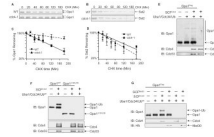
Figure 5. SCF^{Cdc4} Ubiquitinates Gpa1 *in vitro* and Facilitates its Turnover *in vivo*

(A) Gpa1 stability in wild-type vs. temperature-sensitive *cdc4-1* mutant cells. Cultures were grown at 25°C, shifted to 37°C for 1hr, and then treated with cycloheximide (CHX) for the indicated times. Myristoylated (bottom band) and unmyristoylated (top band) Gpa1 detected by immunoblotting with Gpa1 antibodies.

(B) Samples from panel (A) analyzed with Sst2 antibodies.

(C and D) The intensity of bands from (A) and (B), analyzed by densitometry. Results are the mean ± S.E. (n=3).

(E) *In vitro* ubiquitination of Gpa1. Purified Gpa1-Flag was incubated with purified SCF^{Cdc4} complex (Flag-Skp1/Cdc53/Myc-Rbx1/Cdc4), His6-Uba1, His6-Cdc34, and ubiquitin as indicated, followed by SDS-PAGE and immunoblotting. Unmodified (Gpa1) and ubiquitinated (Gpa1-$(Ub)_n$) Gpa1 protein was visualized with Gpa1 antibodies. Membranes were also probed with Cdc4 antibodies and Cdc53 antibodies.

(F) *In vitro* ubiquitination using Lys-less ubiquitin (Ub^{0K}). Reactions contain either Gpa1 or Gpa1$^{\Delta 128-236}$, a mutant form of Gpa1 lacking the ubiquitinated subdomain.

(G) *In vitro* ubiquitination of Gpa1 using Ub^{0K} and SCF complexes containing either Cdc4 or Met30 as indicated. Note that Met30 appears to bind weakly to Gpa1 but does not sustain Gpa1 ubiquitination (Figure S4B).
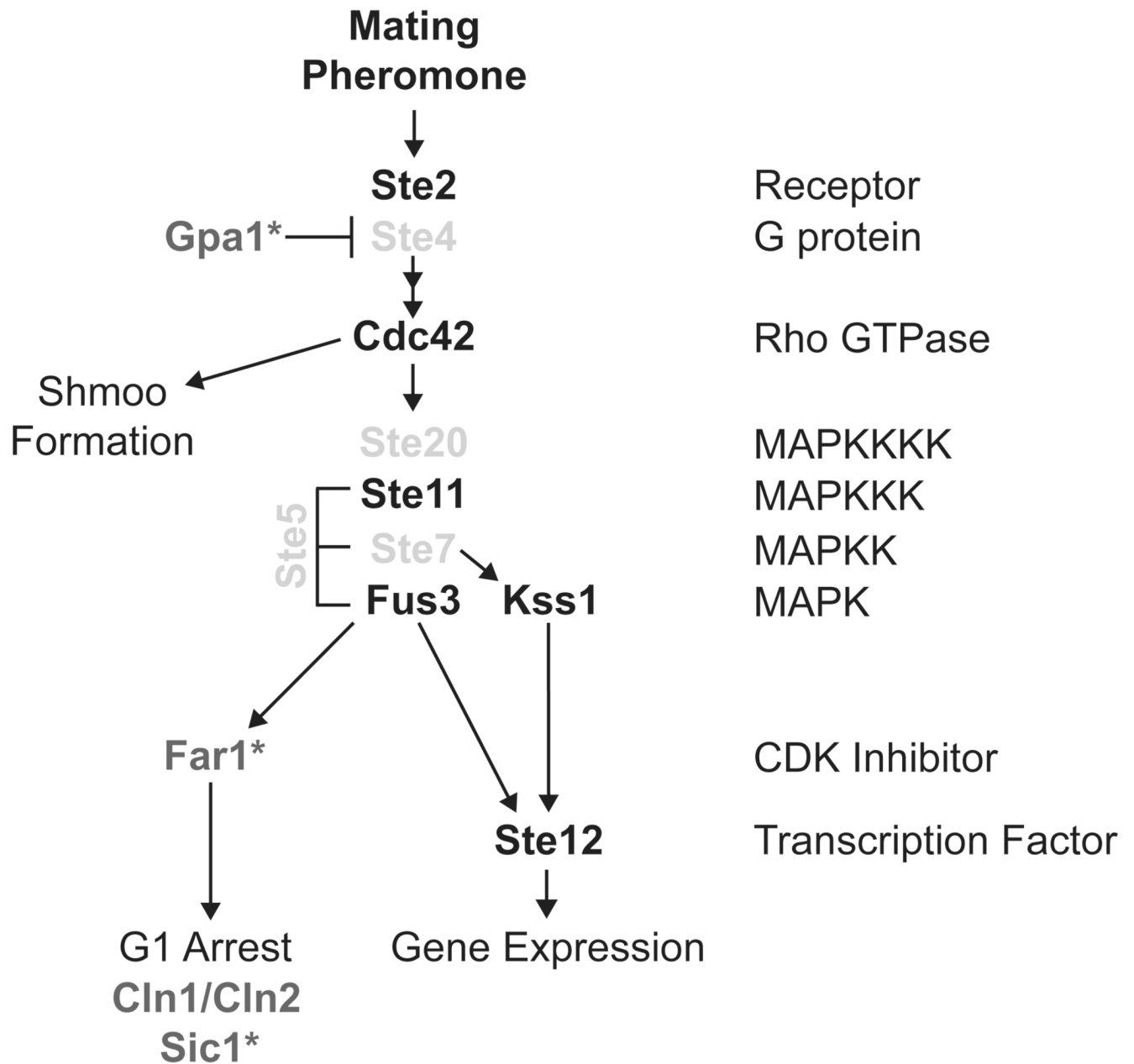
See also Figure S4.

Figure 6. The Pheromone Response Pathway
The Cdc34/SCF complex targets several known substrates in the pheromone response
pathway. Known substrates of Cdc34/SCF are shown in dark grey. Likely substrates of the
SCF are shown in light grey. SCF[Cdc4] substrates that have been verified using *in vitro*
ubiquitination assays are designated with an asterisk (*).

Table 1

Selected Essential Genes Required for Proper Pheromone Signaling[a] (See also Figure S2)

Systematic Name	Standard Name	Function[b]	Plasmid Rescue	α factor Max Response[c,d]	α factor LogEC$_{50}$[c,d,e]
YDL028C	MPS1	Dual-specific kinase; required for spindle checkpoint function	Yes	178.7% ± 5.6%	−5.71 ± 0.04
YLR305C	STT4	Phosphatidylinositol-4-kinase; involved in the Pkc1 pathway	Yes	44.8% ± 0.7%	−6.10 ± 0.04
YNL267W	PIK1[f]	Phosphatidylinositol-4-kinase; may control cytokinesis through actin cytoskeleton	Yes	88.3% ± 4.9%	−5.82 ± 0.05
YFL009W	CDC4	F-Box protein; component of the SCF ubiquitin ligase	Yes	46.1% ± 3.1%	−5.40 ± 0.03
YDR054C	CDC34	E2 ubiquitin conjugating enzyme; component of the SCF ubiquitin ligase	Yes	6.2% ± 0.2%	−5.98 ± 0.05
YDL132W	CDC53	Cullin; component of the SCF ubiquitin ligase	Not Done	36.1% ± 1.9%	−5.59 ± 0.06

[a] The essential genes *PMA1* and *SLN1* were confirmed but were not characterized further.

[b] *Saccharomyces* Genome Database (www.yeastgenome.org).

[c] *FUS1* reporter transcription was measured in the indicted TetO7 strains treated with doxycycline or untreated control (average ± SEM).

[d] Transcriptional response with α factor pheromone; α factor Max Response and LogEC$_{50}$ data derived from nonlinear regression analysis (sigmoidal dose-response).

[e] LogEC50 for TetO7 Wild-Type strain is −5.60 ± 0.01

[f] Showed 4× increase in basal activity when treated with doxycycline.