SYSTEMATIC ANALYSIS OF ESSENTIAL GENES REVEALS NEW REGULATORS OF G PROTEIN SIGNALING

Steven D. Cappell

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Approved by:

Advisor: Dr. Henrik Dohlman

Reader: Dr. David Siderovski

Reader: Dr. Gary Johnson

Reader: Dr. Lee Graves

Reader: Dr. Vytas Bankaitis

ABSTRACT

STEVEN D. CAPPELL: Systematic Analysis of Essential Genes Reveals New
Regulators of G Protein Signaling
(Under the direction of Dr. Henrik G. Dohlman)

Heterotrimeric G proteins are molecular switches that respond to a wide range of stimuli including light, neurotransmitters, small molecules and peptides. Due to their role in a variety of physiological responses, it is no surprise that over 50% of drugs modulate G protein signaling pathways. While many drugs function at the level of the G protein-coupled receptor, downstream signaling components are increasingly being investigated as drug targets. Therefore, discovery of new components and regulators will help identify new ways to exploit G protein-coupled signaling pathways for therapeutic utility.

Previous attempts to systematically identify new components of G protein pathways have focused on genome-wide knockout screens including gene-deletion mutants. However, these methods are inherently limited because they exclude the essential genes. In this thesis, we present studies to identify new signaling components by systematically analyzing 870 essential genes using repressible-promoter strains. Specifically, we show that the SCF^{Cdc4} E3 ubiquitin ligase complex regulates G protein turnover and catalyzes ubiquitination of the G protein α subunit, Gpa1. Also, we demonstrate that Pik1, a phosphatidylinositol (PtdIns) 4-kinase, regulates the mitogen-activated protein kinase (MAPK) cascade and helps maintain signaling fidelity. These findings reveal the essential-genome as an untapped resource for identifying new components and regulators of signal transduction pathways. Furthermore, work on this thesis has expanded our understanding of G protein signaling networks and could lead to future opportunities for drug discovery.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS AND SYMBOLS

6XHIS	Six histidine residues (used as an affinity tag)
Å	Angstrom (unit of length)
A_{600nm}	Absorbance measured at 600nm wavelength
APC	Anaphase promoting complex
BAR	Bin/Amphiphysin/Rvs
BioGRID	Biological general repository for interaction datasets
C2	Conserved region 2
CDC	Cell division cycle
CHX	Cycloheximide
CTM	C-terminal transmembrane domain
Cys or C	Cysteine
Δ	Deletion
DAG	Diacylglycerol
DHFR	Dihydrofolate reductase
DIC	Differential interference contrast
Dox	Doxycycline
DUB	De-ubiquitinating enzyme
E. coli	*Escherichia coli*
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EC50	Half maximal effective concentration
ENTH	Epsin N-terminal homology domain
FBXL	F-box with leucine-rich repeats

FBXO	F-box with unique domains
FBXW	F-box with WD repeats
FLAG	Flag epitope tag (DYKDDDDK)
FUS3	cell FUSion
FYVE	Fab1p/YOTB/Vac1p/EEA1 domain
G protein	Guanine nucleotide binding protein
G6PDH	Glucose-6-phosphate dehydrogenase
Gα	G protein alpha subunit
GAL	Galactose
GAP	GTPase accelerating protein
Gβ	G protein beta subunit
GDI	Guanosine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
Gγ	G protein gamma subunit
GO	Gene ontology
GPA1	G Protein alpha subunit
GPCR	G protein-coupled receptor
GST	Glutathione S-transferase (used as an affinity tag)
GTP	Guanosine triphosphate
HA	Human influenza hemagglutinin (used as an affinity tag)
HECT	Homologous to E6-AP carboxyl terminus
HIF	Hypoxia-inducible-factor
HOG	High osmolarity glycerol

IB	Immunoblot
Ile or I	Isoleucine
IP	Immunoprecipitate
IP	Inositol phosphate
kDa	kilo-Dalton (unit of mass)
KSS1	Kinase suppressor of Sst2 mutations
Lys or K	Lysine
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MAPKKKK	Mitogen-activated protein kinase kinase kinase kinase
MPS1	MonoPolar Spindle
MSS4	Multicopy Suppressor of Stt4 mutation
PCR	Polymerase chain reaction
PH	Pleckstrin homology domain
PIK1	Phosphatidylinositol kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PS	Phosphatidylserine
PtdIns	Phosphatidylinositol
PX	Phox homology domain
RFP	Red fluorescent protein
RGS	Regulator of G protein signaling
RING	Really interesting new gene

SCF	Skp1, cullin, F-box
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser or S	Serine
SMER	Small molecule enhancer of rapamycin
SST2	Supersensitive to pheromone
STE	STErile
STT4	Staurosporine and temperature sensitive
TAP	Tandem affinity purification (used as an affinity tag)
TCA	Trichloroacetic acid
td	Temperature-inducible degron
TetO	Tetracycline-repressible promoter
Thr or T	Threonine
ts	Temperature-sensitive
Ub	Ubiquitin
Ub^{0K}	Ubiquitin with all lysines mutated to alanine
VPS	Vacuolar protein sorting

CHAPTER I

Introduction

Organisms growing in complex environments are exposed to multiple chemical and physical stimuli. The ability to interpret and respond to such stimuli is critical for a cell to survive in changing environments. At the cellular level, external signals are transduced across the plasma membrane by a variety of cellular signaling networks. However, the most widely used networks are those coupled to heterotrimeric G proteins. Organisms as diverse as yeast and humans utilize G-protein coupled receptors (GPCRs) to respond to a wide range of stimuli including light, tastes, odors, neurotransmitters, and hormones (1-3). Given their role in critical biological processes, perturbations of G protein signaling pathways are often associated with human disease (4). Not surprisingly, G proteins and GPCRs have been extensively studied because of their potential importance as pharmacological targets. In fact, over 50% of drugs currently on the market target GPCRs, thereby modulating G protein-coupled signaling pathways (5-7). Notable examples include antidepressants such as Zoloft (sertraline hydrochloride), antipsychotics such as Zyprexa (olanzapine), and the asthma drug Singulair (montelukast). While directly targeting GPCRs has been highly successful, efforts to find new drugs have focused on downstream modulators of G protein signaling (8-10). Therefore, by studying proteins and signaling events downstream of receptor activation, it is possible to identify new drug targets in G protein signaling pathways.

This thesis will focus on new regulators of G proteins and the signaling pathways they activate. While many proteins are known to regulate G protein function, much is still unknown about other aspects of G protein signaling, such as protein turnover. In this introductory chapter, special attention will be paid to known regulators of G protein signaling, how these regulators were originally identified, and recent advancements that allow for more sophisticated methods of studying G protein signaling pathways.

Heterotrimeric G proteins

Cell surface receptors with seven-transmembrane domains couple to heterotrimeric guanine nucleotide binding proteins (G proteins), thereby linking the extracellular environment to the inside of the cell. Heterotrimeric G proteins are comprised of α, β, and γ subunits and function as molecular switches (Figure 1.1). In the absence of stimulus, the Gα subunit is bound to GDP and the obligate heterodimer G$\beta\gamma$. Activation of the receptor by an extracellular ligand results in the exchange of GDP for GTP on the Gα subunit of the heterotrimer. GTP-bound Gα undergoes conformational changes in three distinct switch regions that allow it to dissociate from the G$\beta\gamma$ dimer. Both Gα and G$\beta\gamma$ can then signal through effector proteins to elicit their downstream effects (11). Inactivation of G proteins results from the slow intrinsic GTPase activity of Gα, hydrolyzing GTP to GDP (12). After hydrolysis, GDP-bound Gα has lower affinity for effectors and a higher affinity for free G$\beta\gamma$ and thus the heterotrimeric complex reforms, terminating signaling. To accelerate inactivation, G proteins are negatively regulated by a class of proteins known as regulators of G protein signaling (RGS) (13), which stabilize the transition state of GTP hydrolysis and function as GTPase accelerating proteins (GAPs) (14).

Heterotrimeric G proteins are regulated by a number of accessory proteins. In addition to receptors and RGS proteins, which activate and inactivate G proteins respectively, there are several other classes of proteins that modulate G protein function. First, guanine nucleotide dissociation inhibitors (GDIs) function to limit activation of the Gα subunit by inhibiting the release of GDP. The most notable example is G$\beta\gamma$, which functions as a GDI to reduce basal activation of Gα. Also, the 19 amino acid GoLoco motif functions as a GDI on the G$\alpha_{i/o}$ class of G proteins (15), and is found on a number of proteins, including RGS12 (16). A crystal structure of the GoLoco motif in complex

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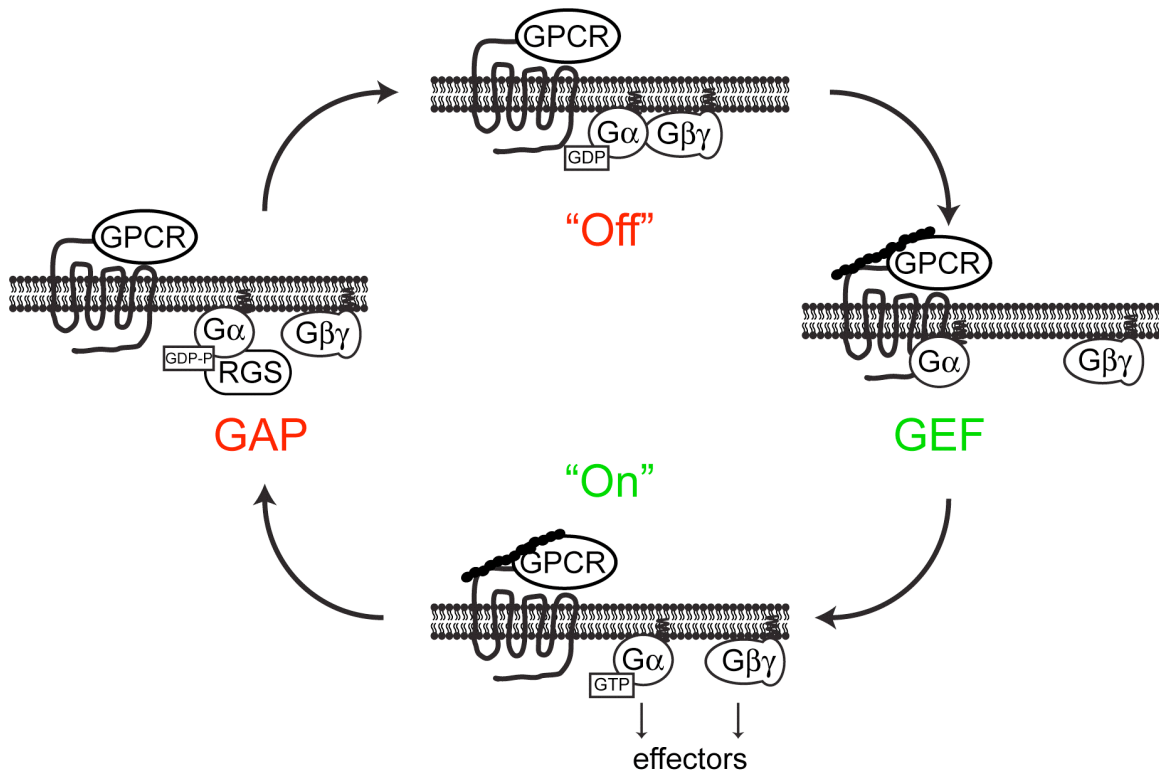
Figure 1.1

Figure 1.1 The heterotrimeric G protein activation cycle.
        G protein-coupled receptors (GPCR) respond to extracellular ligands and activate heterotrimeric G proteins inside the cell. When inactive, the Gα subunit is bound to GDP and the G$\beta\gamma$ dimer. Upon ligand binding, the receptor functions as a guanine nucleotide exchange factor (GEF) allowing Gα to exchange GDP for GTP. GTP-bound Gα undergoes a conformational change and dissociates from G$\beta\gamma$. Both subunits are then free to activate downstream effectors. Signaling is terminated by hydrolysis of GTP, a reaction that is catalyzed by GTPase accelerating proteins (GAP) such as the regulator of G protein signaling (RGS) family of proteins, which stabilize the transition state of the reaction.

with Gα_{i1}-GDP revealed a direct interaction between three conserved residues of the GoLoco motif and the α- and β-phosphates of GDP. This crystal structure indicated GDI activity is mediated by direct contact with GDP, preventing its release from Gα (17). Second, G protein activation can be modulated by non-receptor guanine nucleotide exchange factors (GEFs). To date, several non-receptor GEFs have been identified such as Ric-8A (18), Arr4 (19), and GIV (20). Similar to GPCRs, non-receptor GEFs accelerate the release of GDP on the Gα subunit, facilitating the binding of GTP, which is in excess in the cell. However, unlike GPCRs, non-receptor GEFs typically cannot accelerate nucleotide exchange on the heterotrimeric complex. They require dissociated Gα–GDP to accelerate GDP release. This observation has led to the hypothesis that non-receptor GEFs work after receptor-driven activation and function to sustain signaling by promoting faster cycling of Gα (21). Through a combination of GPCRs, RGS proteins, GDIs, and non-receptor GEFs, cells have the ability to fine-tune G protein signaling, suggesting all of these proteins could be exploited for therapeutic utility (8, 22). However, of the proteins listed, only drugs that target GPCRs have made it through clinical trials and into patients. Thus, further efforts are needed to identify new proteins that regulate G protein signaling pathways in the hopes of finding better drug targets.

Model systems for studying heterotrimeric G protein signaling

The human genome encodes more than 700 GPCRs, 20 Gα subunits, 5 Gβ subunits, and 13 Gγ subunits. Given this complexity, simpler model organisms have been extensively utilized to study fundamental principles of G protein signaling. In the yeast *S. cerevisiae*, a single canonical heterotrimeric G protein signaling pathway regulates the process of cell mating. In addition to having only one GPCR and G protein, there are several other reasons why yeast is an attractive model for studying G protein signaling. First, most of the yeast components are highly conserved in humans.

In particular, the G protein (23) and mitogen-activated protein kinase (MAPK) components (24-26) are both structurally and functionally similar to their human counterparts (Figure 1.2). Therefore, discoveries made in yeast will be applicable to human G protein signaling. Second, the signaling events downstream of the receptor and G protein are well understood. Pathway activation results in easily measured phenotypes including cell-cycle arrest, morphological changes, and new gene transcription. Third, yeast has the ability to stably grow as a haploid, making it easy to identify recessive mutations. Fourth, yeast readily undergo homologous recombination, making it easy to perform gene replacement and gene disruption at the genomic-scale. In fact, a gene-deletion strain has been made for nearly every non-essential gene in yeast (27) and a tetracycline-repressible strain has been made for nearly every essential gene (28). This combination allows for unprecedented coverage when using genome-wide screens to uncover novel signaling components.

Yeast exist as one of two haploid mating types, MAT**a** and MATα. In the proximity of a member of the opposite mating type, the yeast cell will excrete a small polypeptide pheromone: MAT**a** cells release **a** factor and MATα cells release α factor (29, 30). The pheromones activate GPCRs on the cell surface eliciting a mating response consisting of transcriptional changes, polarized growth, and cell cycle arrest at the G1 phase, which ultimately results in the fusion of two haploid cells to produce one diploid cell (31).

The pheromone response pathway has been extensively studied for over 40 years, and is arguably the most well understood signaling pathway in any eukaryotic organism. Many of the core components required to transmit signaling from the plasma membrane to the nucleus have been identified and characterized. The yeast pheromone receptor, Ste2, couples to a heterotrimeric G protein made up of Gpa1$^{G_\alpha}$ (32-

Figure 1.2

A. Humans
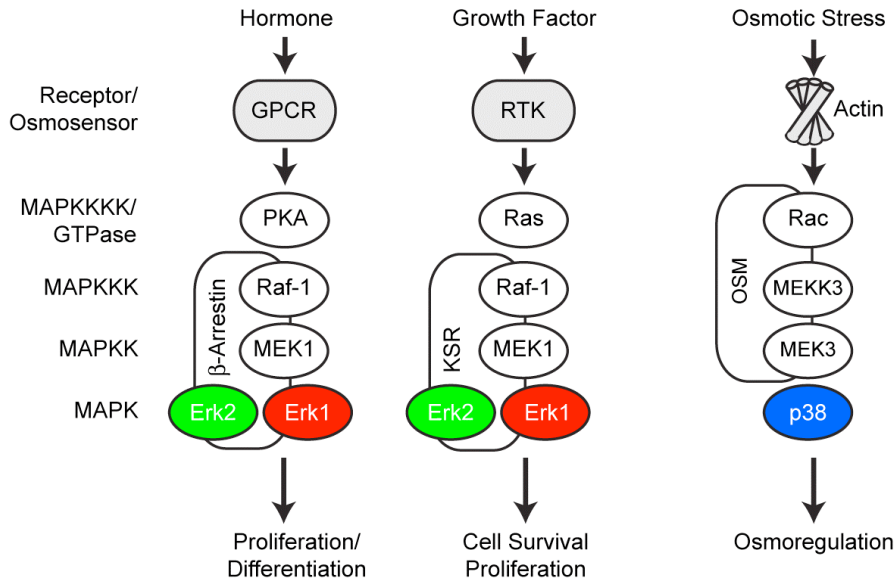
B. Yeast
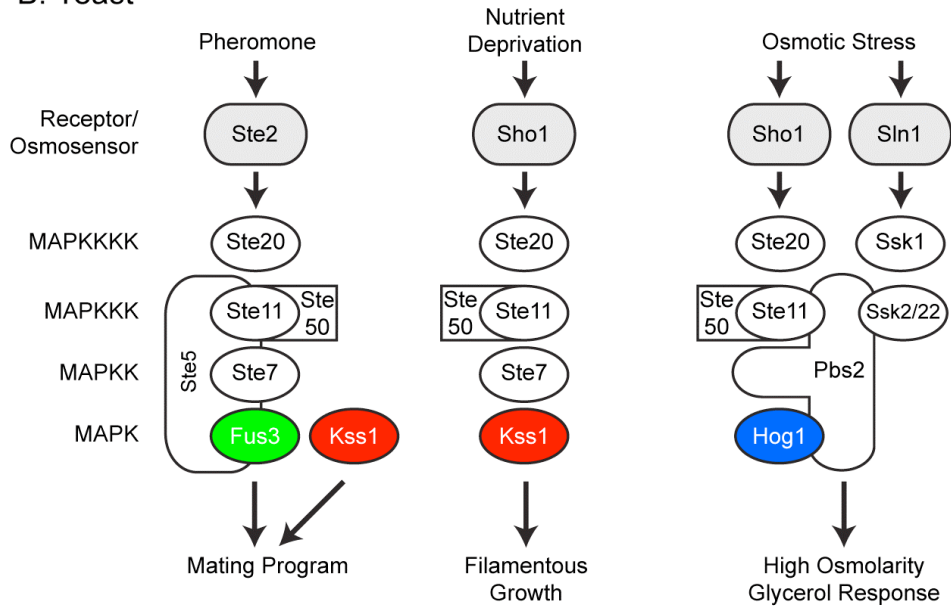
Figure 1.2 MAPK signaling pathways are conserved from yeast to humans.
(A) Human MAPK pathways. (B) Yeast MAPK pathways. Most components of yeast MAPK pathways have homologs in humans. Fus3 is homologous to Erk2 (green); Kss1 is homologous to Erk1 (Red); Hog1 is homologous to p38 (Blue). Thus, discoveries in yeast are likely to lead to discoveries in humans. G protein-coupled receptor (GPCR); receptor tyrosine kinase (RTK).

34), Ste4$^{G_\beta}$, and Ste18$^{G_\gamma}$ (35) (Figure 1.3). Pheromone binding to Ste2 promotes exchange of GDP for GTP on Gpa1. GTP-bound Gpa1 then dissociates from the Ste4/18 dimer. Dissociated Ste4/18 activates the MAPK cascade (36) comprised of Ste20, Ste11 (37, 38), Ste7 (39), the partially redundant MAPKs Fus3 and Kss1 (40), and the MAPK scaffold Ste5 (41-43). Active Fus3 then phosphorylates the transcription factor Ste12 (44, 45), which regulates the expression of genes necessary for proper mating responses (46-49).

The pheromone response pathway - Discovery of the components that comprise the pheromone pathway is the result of two key advancements in the field of biology. The first breakthrough came in the 1970's when two labs used classical genetic techniques to identify mutations that conveyed sterility to yeast. In 1974, Mackay and Manney published two papers in which they irradiated yeast cells with an ultraviolet light to induce random mutations in the yeast genome (50, 51). The mutagenized yeast were then mixed with members of the opposite mating type and spread on selective agar medium. The experiment was designed so that only unmated, sterile cells would grow on the selective medium. Genetic analysis of the sterile colonies identified 20 genes thought to be involved in pheromone signaling. Mackay and Manney designated these genes with the symbol *ste* (STErile) and numbered them in the order that they were characterized.

While Mackay and Manney were irradiating yeast to identify genes involved in mating, Lee Hartwell was using temperature-sensitive mutants to identify genes involved in the cell cycle (52-56), an endeavor that would eventually win him the Nobel Prize in Medicine in 2001 (57). Once Hartwell had identified many of the genes involved in the cell division cycle (CDC), he shifted his attention to the pheromone pathway and its ability to induce cell-cycle arrest (58). Hartwell randomly mutagenized yeast using ethylmethane sulfonate and selected for colonies that mated at 22°C but not at 34°C.

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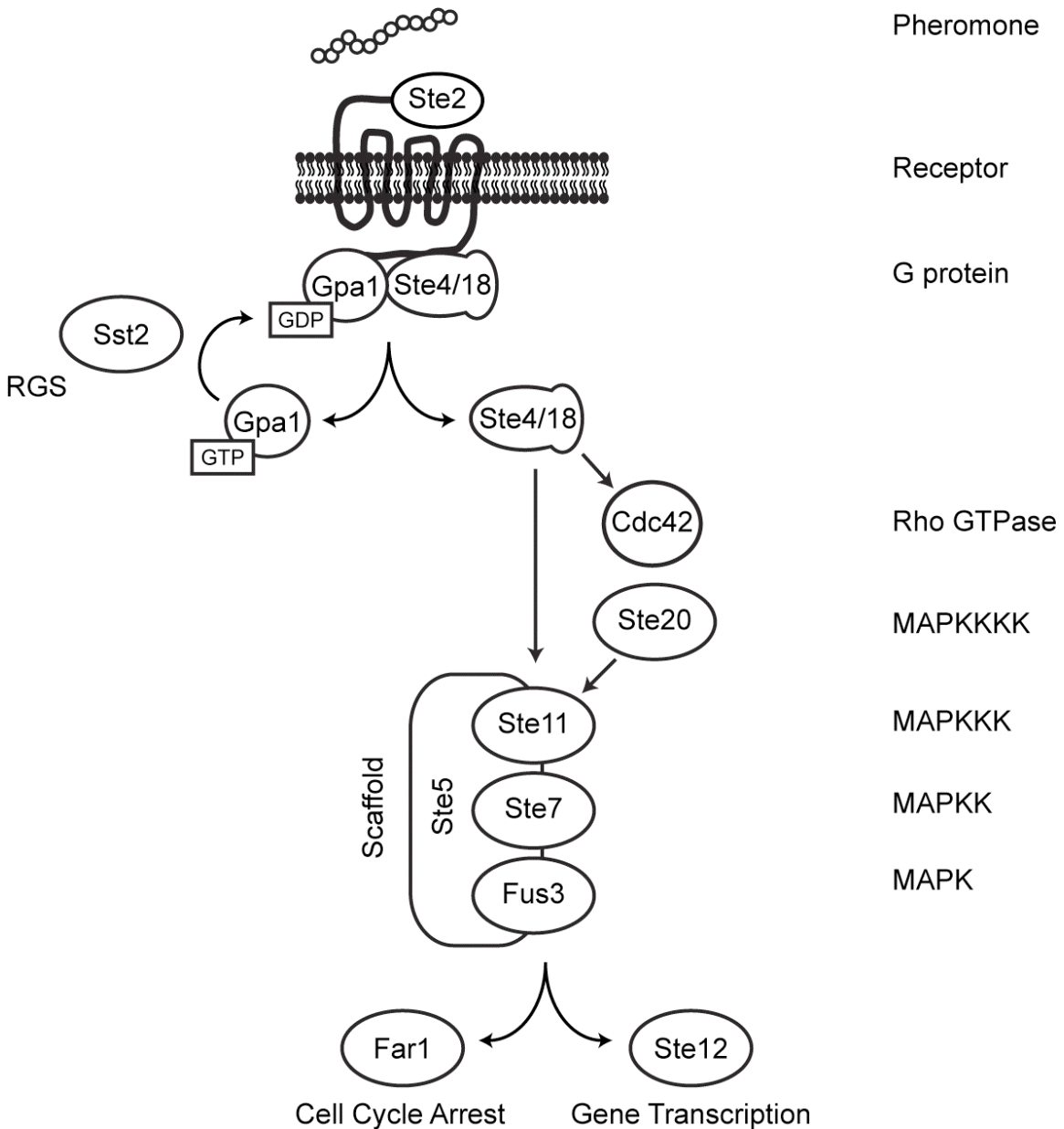
Figure 1.3

Figure 1.3 The yeast mating response pathway.
Haploid yeast respond to mating pheromones through a canonical heterotrimeric G protein-coupled signaling pathway. Pheromone causes the dissociation of GTP-bound Gpa1 and the Gβγ dimer comprised of Ste4 and Ste18. Free Ste4/18 coordinates the activation of the MAPK cascade by binding the MAPK scaffold Ste5 and activating the small G protein Cdc42. This leads to the phosphorylation of Fus3, which mainly activates two effectors: Ste12 and Far1. Ste12 is a transcription factor that regulates mating-specific genes and Far1 is a cyclin-dependent kinase inhibitor that causes cell cycle arrest in the G1 phase.

Using temperature-sensitive mutants allowed Hartwell to conduct complementation studies and thus achieve a greater understanding of the function of each *ste* gene. Hartwell was able to narrow down Mackay and Manney's list of genes in the pheromone pathway from 20 to 8, including the major pathway components Ste2, Ste4, Ste5, Ste7, Ste11, and Ste12.

Examining these early studies with the benefit of hindsight reveals the authors made some insightful conclusions about their data. For example, Hartwell was able to determine that Ste2 is specific only to MAT**a** cells, and likely encodes the pheromone receptor, without any knowledge of the sequence of the gene or the protein it encodes. Such insights, obtained only through simple genetic techniques, underscore the power of yeast genetics and illustrate why the pheromone pathway is now one of the most well-characterized signaling pathways in eukaryotes.

Early studies of the pheromone pathway identified the key genes absolutely required to transmit signaling across the plasma membrane to elicit intracellular effects. However, we now know that some genes function in the pheromone pathway but are not absolutely required for mating. These genes, such as *SST2*, *FUS3*, and *KSS1*, were missed by both Mackay and Manney's and Hartwell's screens. While these simple genetic techniques were quite successful, increasingly sophisticated technologies are required to identify genes that are not necessary for pheromone signaling but rather fine-tune signaling.

While the genes required for pheromone signaling were identified relatively quickly, discovering the proteins they encoded and their biochemical functions would take many years. From 1980-1995, many different labs worked on individual *ste* genes. For example, Whiteway *et al*. found that Ste4 encodes the Gβ subunit of the yeast heterotrimeric G protein (35). Additional research identified genes with roles in the pheromone pathway that did not yield a sterile phenotype when mutated. For example,

the MAP kinase Fus3 was identified in a screen for mutants defective in cell fusion after mating (40), the Gα subunit Gpa1 was identified as a gene homologous to mammalian Gα subunits (23, 34), and the RGS protein Sst2 was identified in a screen for mutants super-sensitive to pheromone (59). Despite the success in characterizing known components, the rapid pace of discovery of new components and regulators of the pheromone response pathway was beginning to subside in the mid-1990's.

The second major breakthrough came in 1996, when the yeast genome was completely sequenced by a worldwide collaboration (60). It was the first completely sequenced genome of a eukaryote and ushered in a new age of conducting science. Prior to this, approximately 1000 yeast genes were known (61), but the sequence incredibly identified almost 6,000 open-reading frames (ORFs). The addition of almost 5,000 new genes greatly increased the opportunity for discovering new signaling components. However, given the complexity of studying 6,000 genes, new tools and techniques would be required to study an organism at the genomic-scale.

In the years following the sequencing of the yeast genome, an international consortium was formed to systematically disrupt every ORF in the yeast genome. In 2000, the *Saccharomyces* Genome Deletion Project released the Yeast Knock-Out (YKO) deletion collection, which included almost 82% of all yeast genes (27, 62). While Manney, Hartwell, and others relied on random mutagenesis, the YKO collection allowed for the systematic analysis of almost every gene to identify new components and regulators of G protein signaling. For the first time, research could be conducted on the genomic-scale, assaying the function of every gene simultaneously, instead of relying on the variable process of generating random mutations or the time consuming process of studying one gene at a time.

Our lab utilized the YKO collection to identify genes required for proper pheromone signaling and answer a long-standing question about the role of the yeast

Gα subunit. In the yeast mating pathway pathway, the signal generated by pheromone stimulation is mostly propagated by G$\beta\gamma$ effectors (63, 64). For many years, it was thought that Gpa1 functions solely to sequester G$\beta\gamma$, and prevent it from activating downstream effectors. Therefore, Gpa1 was considered a negative regulator of the pathway. In support of this model, deletion of Gpa1 results in constitutive activation of the pheromone pathway and permanent cell-cycle arrest. However, given the role of human Gα subunits in activating downstream effectors, it was speculated that the yeast Gα subunit may also activate downstream effecters. In 2006, Slessareva *et al*. sought to identify novel effectors of Gpa1 by screening the YKO collection for deletion strains that block signaling by a constitutively active Gpa1^{QL} mutant. Using this systematic approach, they found that GTP-bound Gpa1 activates the phosphatidylinositol (PtdIns) 3-Kinase Vps34 at the endosome, resulting in increased PtdIns 3-P production, translocation of signaling components to the endosome, and enhanced MAPK activation (65). Thus, Gpa1 has a positive role in signaling and can, in fact, activate effectors. Given that Vps34 or its human homolog had never been implicated in G protein signaling, it is unlikely that a more directed, hypothesis-driven approach would have identified it as a Gα effector. Therefore, the systematic approach utilized by Slessareva *et al*. was instrumental in discovering the first new component of the pheromone pathway in almost 8 years (66). Despite the yeast pheromone pathway being mapped out in incredible detail over the previous 35 years, this result demonstrated that more signaling components are still waiting to be discovered using systematic screening methods.

Essential genes

One of the outcomes of the yeast Genome Deletion Project was the realization that 18% of yeast genes are essential for growth. Genes involved in cellular processes

required for cell viability such as cell division, DNA replication, and cytoskeletal rearrangements tend to be essential (27, 62). Interestingly, essential genes are more highly conserved than the non-essential genes; 38% of essential genes have a human ortholog compared to only 20% of non-essential genes (67). Thus, any discoveries made while studying yeast essential genes are more likely to translate to humans. Additionally, their role in cell viability makes them attractive drug targets for diseases such as cancer.

Despite the benefits of studying essential genes, they have remained poorly characterized. This is due primarily to the fact that essential genes are incredibly difficult to study. They cannot be permanently deleted in haploid cells and mutations that alter their function or enzymatic properties tend to be lethal. Classical genetic techniques, such as random mutagenesis employed by Mackay and Manney, are not suited for identifying essential genes. For example, the yeast Gα subunit *GPA1* is essential, because when it is deleted, the G$\beta\gamma$ dimer is free to activate signaling, and induces permanent cell-cycle arrest, terminating growth. *GPA1* was not originally identified in screens conducted by Mackay and Manney or Hartwell, and its role in the pheromone pathway escaped detection until 1987, when it was identified due to its similarity to human Gα subunits (23, 34). The fact that the G protein alpha subunit, arguably the most important component of the pheromone pathway, could not be identified by random mutagenesis screens underscores the limitation of previous techniques to identify novel signaling components, and highlights the need for more sophisticated screening methods in order to study the role of essential genes.

Temperature-sensitive mutants - The best way to identify the function of a given gene is to delete it and look for altered cellular behavior or morphology. Since an essential gene cannot be stably deleted, conditional mutants are required. The most common method of generating a conditional mutant is to create a temperature-sensitive

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(*ts*) mutant (68). A plasmid containing the wild-type gene is randomly mutagenized and transformed into a strain lacking the same gene. For essential genes, the mutagenized plasmid must be transformed at the same time the genomic allele is deleted. Once colonies form, they are patched onto two different plates, one placed at 25°C and the other placed at 37°C. The *ts* mutants are those that grow at 25°C, the permissive temperature, but not at 37°C, the restrictive temperature. Mutations that convey temperature-sensitivity usually alter protein stability and result in unfolded protein at the restrictive temperature. Thus, the protein can be rapidly knocked-down by growing the cells at elevated temperatures. The main drawback to this technique however, is that the exact function of the randomly generated mutation is not known (69). For example, the mutation could only cause local instability of a particular domain within the protein, disrupting some functions but retaining others. This could cause unforeseen effects such as making a dominant-negative. Alternatively, the mutations could be overly effective, resulting in low activity at the permissive temperature, and rapid loss of protein function at the restrictive temperature. This could alter cell viability or induce cell stress pathways that could alter the experimental outcome. Thus, experimental conditions cannot be properly controlled if the function of the mutation is not fully understood. Furthermore, this method is time consuming, results can vary from gene to gene, and it is not suited for high-throughput systematic screens.

Alternative methods have been developed to generate *ts* alleles in a more systematic manner and remove the variability and uncertainty that comes with generating random mutants. In 1986, Alexander Varshavsky discovered that the half-life of a given protein is determined by the amino-acid composition of the N-terminus. Proteins with amino-terminal residues such as arginine or lysine are degraded more quickly than proteins with amino-terminal residues such as glycine or alanine, a phenomenon called the "N-end rule" (70). Subsequently, it was found that proteins

degraded by the N-end rule are ubiquitinated on a lysine close to the N-terminus and degraded by the proteasome (71, 72).

Varshavsky wanted to use the N-end rule as a tool to generate a *ts* N-degron sequence. To this end, he found a mutant form of the mouse protein dihydrofolate reductase (*DHFR*) that contains a destabilizing arginine residue at its N-terminus, yet is long-lived in yeast. Using the standard methods described above, Dohmen *et al.* created a *ts* mutant of *DHFR* (*DHFR^{ts}*) that is stable at the permissive temperature but is rapidly degraded by the N-end rule at the restrictive temperature. They reasoned that fusion of *DHFR^{ts}* to the N-terminus of other proteins should convey temperature-sensitivity to that protein. To test this hypothesis, they fused *DHFR^{ts}* to the essential gene *CDC28* and created a protein (Cdc28^{td}) that was also stable at 25°C but rapidly degraded at 37°C. Thus, *DHFR^{ts}* can serve as a temperature-inducible degron (*td*) sequence when fused to another gene (73). This strategy offers several improvements over the traditional method of generating *ts* mutants. First, the *td* method does not typically alter the function of the protein of interest because many proteins can tolerate modification of the N-terminus. In contrast, random destabilizing mutations are likely to affect protein function. Second, degradation of the *td* sequence is rapid and predictable, whereas *ts* alleles can cause only partial disruption of protein stability, resulting in slow or incomplete degradation of the protein of interest. Thus, the *td* method normalizes the time required for degradation between two proteins. Finally, by simply fusing the *td* sequence to the protein of interest, one can bypass the need for an exhaustive search for *ts* alleles generated by random mutagenesis. This makes the *td* method preferable when conducting large-scale systematic screens. For example, Kanemaki *et al*. fused the *td* sequence to half of the essential genes with an unknown function to determine if any have a role in regulating the cell cycle (74). This experiment would not be possible if *ts* alleles had to be independently generated, one at a time.

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Despite having many benefits for studying essential genes, *ts* and *td* strains have several major drawbacks. The most obvious limitation to using both methods is the requirement for growth at sub-optimal temperatures that could have unintended effects. At 37°C, enzymes will exhibit altered kinetics, cells will divide more quickly, off-target genes will likely be de-stabilized, and cellular stress pathways will be activated, all of which could affect the outcome of the experiment. For example, growth at 37°C causes a 50% reduction in MAPK activation in response to pheromone in wild-type cells (75). Therefore, any phenotype caused by a temperature-sensitive allele may be masked by the effect of non-physiological temperatures on normal cell growth. A second limitation is that both techniques require altering the open-reading frame of the protein, which could affect normal protein function. As mentioned above, random mutations can affect protein function in a number of unknown and undesired ways. Fusion of the *td* sequence to proteins helps eliminate most of this uncertainty, but protein function can still be affected. The heat-inducible N-degron sequence is actually a 21 kDa protein, which could interfere with normal protein folding, protein-protein interactions, and localization. Furthermore, some proteins cannot tolerate alterations to their N-terminus. For example, Gpa1 is mysristoylated and palmitoylated at its N-terminus and both modifications are required for proper localization to the plasma membrane (76-79). Disruption of either modification by adding additional residues to the N-terminus results in mislocalized Gpa1, constitutive activation of the pheromone pathway, and permanent cell cycle arrest. Likewise, transmembrane proteins do not tolerate alteration of their N-termini. Therefore, *ts* and *td* mutants are not suitable for studying a significant portion of essential genes.

Regulatable-promoter strains - Conditional mutants can also be generated by using a regulatable-promoter. Such an approach is an attractive alternative to temperature-sensitive mutants because it allows for tight control of gene expression

without changing the open-reading frame, protein function, or the temperature at which cells are grown. In yeast, there are several regulatable-promoters that can be used, and selecting the correct promoter requires a detailed understanding of the advantages and disadvantages of each.

The most widely used regulatable-promoter is the *GAL1-10* promoter, which can be induced up to 1000-fold by galactose (80, 81). This makes the *GAL* promoter useful when overexpression is required. In addition, gene repression can be achieved by the removal of galactose from the growth medium. The main drawback of the *GAL* promoter is that it requires changing the carbon source from glucose to galactose, which alters cell metabolism. The presence of glucose in the growth medium actively represses the *GAL1-10* promoter (82). Thus, transcriptional induction can be delayed as much as 1 hr while the cells metabolize any glucose still remaining in the medium. To counterbalance this delay, cells can be grown in medium containing the alternative carbon source raffinose, which does not repress the *GAL1-10* promoter. However, cellular growth is not optimal in raffinose-containing medium (83). Regardless, the required changes in carbon source affect cell metabolism and growth-rate, complicating the interpretation of any phenotypes that may arise upon overexpression of the gene of interest. Therefore, the *GAL1-10* promoter should be used with caution.

An alternative solution is the *MET3* promoter, which is repressed by methionine (84). Removal of exogenous methionine results in low-level induction of genes under the control of the *MET3* promoter. This method is useful when overexpression is not desired. However, low gene induction and changes in the nutritional composition of the growth medium limit its usefulness in many applications (85). The copper-inducible promoter *CUP1* has slightly higher induction ratios than *MET3*, but gene expression in the absence of copper can be quite high (86-88). Thus, the *CUP1* promoter cannot be as tightly regulated as the *MET3* and *GAL1-10* promoters. Furthermore, at high enough

concentrations, copper can be lethal, and the introduction of copper into the growth medium at concentrations sufficient for *CUP1* induction could have pleiotropic effects on the cellular stress response (89). All three of these regulatable-promoters, *GAL1-10*, *MET3*, and *CUP1*, were designed from endogenous promoter systems and thus all suffer from the same disadvantage; they require nutritional changes to the growth medium. A better solution would incorporate a promoter not induced by nutrients, but rather a substance that is relatively inert in yeast.

The tetracycline-resistance operon of *Escherichia coli* provides a system of regulating gene transcription in yeast that relies on a non-endogenous control element (90). In *E. coli*, the transcription of resistance-mediating genes is inhibited by the tetracycline repressor (TetR) protein (91). In the presence of the antibiotics tetracycline, TetR can no longer bind to the tet operon promoter sequence (*tet*O) and transcriptional repression is alleviated (91). Thus, tetracycline induces gene expression. In 1992, Gossen and Bujard adapted this system so that it could be used in other organisms (90) (Figure 1.4). They fused TetR to the viral transcription factor virion protein 16 (VP16) (92), to create a tetracycline-controlled transactivator (tTA). They also adapted the *tet*O sequence by fusing it to the minimal promoter sequence for VP16 (TetO). In the absence of tetracycline, the tTA binds with high affinity to TetO, and transcription is induced by VP16 up to 1000-fold. In the presence of tetracycline, the tTA falls off the TetO and transcription is terminated. Thus, tetracycline inhibits gene expression. This system is often referred to as "Tet-Off."

Of the regulatable-promoters outlined above, the tetracycline-repressible promoter system is the most attractive for several reasons. First, the affinity of tetracycline or the more stable analog doxycycline for TetR is very high (~1nM) (93). Therefore only small doses are required to achieve sufficient gene repression. Second, tetracycline and doxycycline have limited physiological effects on organisms other than

Figure 1.4
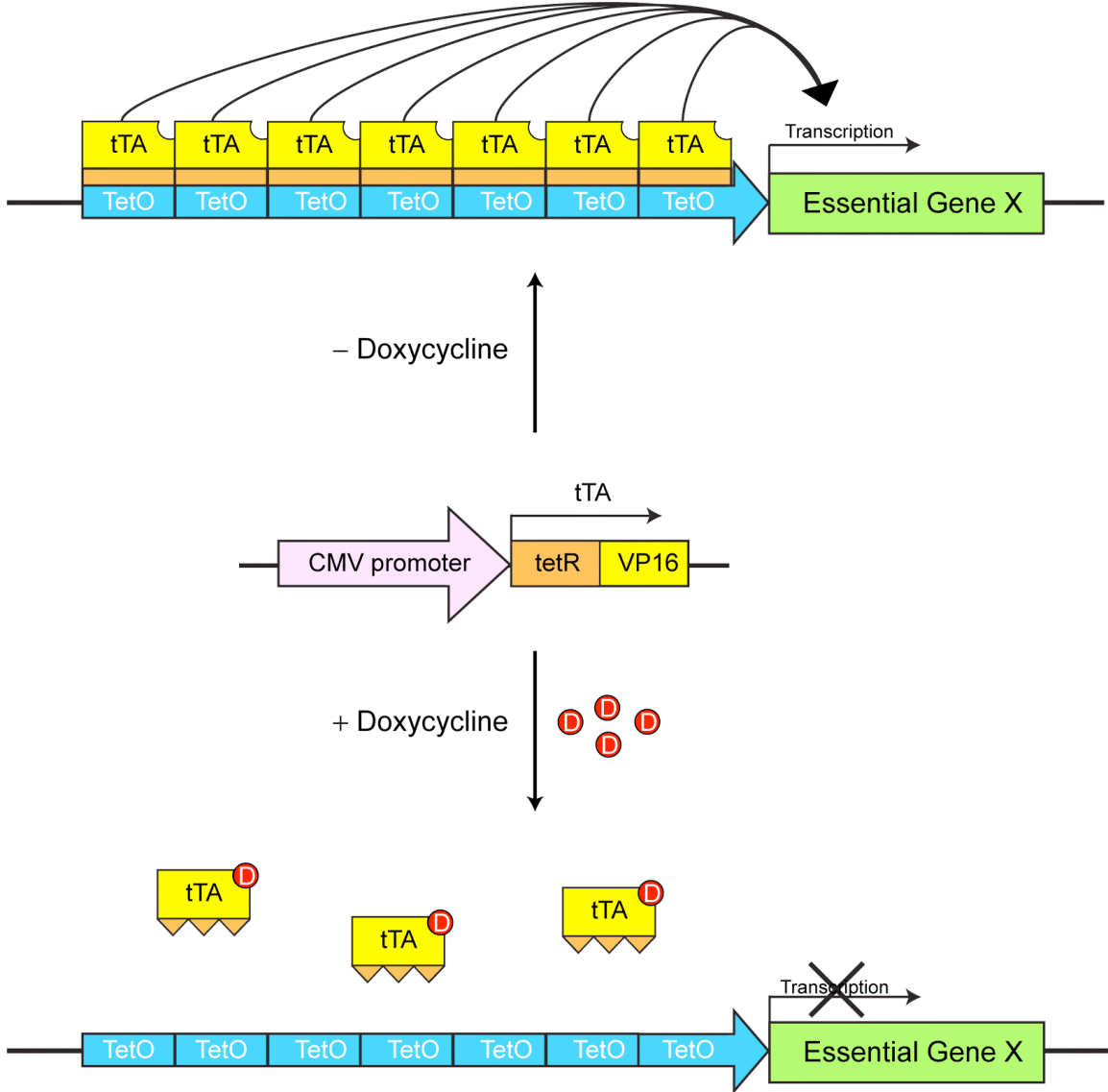
Figure 1.4 The tetracycline-repressible promoter system.
      The tetracycline-repressible promoter system requires two components. First, a fusion of the tetracycline repressor (TetR) protein and virion protein 16 (VP16) is expressed under the control of a constitutive CMV promoter. This fusion protein is called the tetracycline transactivator (tTA). Second, the promoter of the gene of interest is replaced with seven repeats of the tetracycline operon (TetO) promoter sequence. In the absence of doxycycline, the tTA binds the TetO and drives expression of the gene of interest. When added to the growth medium, doxycycline binds the tTA and causes it to fall off the TetO and transcription is terminated.

bacteria, including yeast and humans, and no changes in the nutritional content of the growth medium are required. Thus, unlike the other regulatable promoters *GAL1-10*, *MET3*, *CUP1*, the Tet-Off promoter system is unlikely to produce pleiotropic effects on cell physiology. Third, the binding interaction between tTA and TetO is highly specific so off-target gene induction is unlikely (90). Fourth, the Tet-Off system has a very high induction ratio (91). In the absence of doxycycline gene expression is very high, while in the presence of even low concentrations of doxycycline gene expression is extremely low.

The major drawback of the Tet-Off system, and indeed all regulatable promoter systems, is that mRNA production can be rapidly inhibited while protein levels decrease much more slowly. Depending on the natural rate of decay for a given protein, the emergence of phenotypes due to gene depletion can be significantly, and sometimes unpredictably, delayed. This caveat must be taken into account when characterizing a large number of proteins whose half-lives may vary considerably. Regardless, the Tet-Off system remains the most attractive approach for characterizing large numbers of genes, because TetO strains can be easily generated and only minimal changes to growth conditions are required.

Since the sequencing of the yeast genome in 1996, all the non-essential genes have been systematically deleted, but the essential genes have eluded systematic analysis. In a recent effort to overcome this deficit, Hughes and colleagues generated a library of tetracycline-repressible promoter strains for 602 of the 1,100 yeast essential genes (28). They incorporated the tTA originally developed by Gossen and Bujard (90) into the yeast genome and then systematically replaced the promoter of each essential gene with seven repeats of the TetO cassette (TetO$_7$). In the years since, the library has expanded to include over 900 essential genes. This powerful new tool allows for the systematic analysis of almost all (~80%) the yeast essential genes. Indeed, Mnaimneh

et al. analyzed the original 602 TetO$_7$-strains using four phenotypic assays including cellular morphology, cell size, growth rates under 15 different conditions, and transcriptional profiles using microarrays (28). This approach helped assign functions to several previously uncharacterized genes and identify new genes involved in ribosome biogenesis and mitochondrial import. The Hughes TetO$_7$ library has also been used to identify essential genes involved in the cell division cycle (94), mitochondrial morphogenesis (95), and tombusvirus RNA recombination (96). In Chapter II, I describe a systematic screen of the essential genes using the TetO$_7$ library to identify new regulators of the yeast pheromone response pathway.

Ubiquitin-proteasome system

Efficient protein degradation is essential for many cellular processes such as progression through the cell cycle (97), the DNA damage response (98), and regulation of metabolic pathways (99). Some proteins undergo normal turnover to maintain steady-state levels while others must be rapidly degraded to switch cellular mechanisms on or off. In either case, the 26S proteasome functions to degrade the majority of proteins in the cytoplasm and the nucleus (100, 101). Proteins destined for proteolysis by the 26S proteasome are marked by the covalent attachment of multi-ubiquitin chains, allowing the 26S proteasome to recognize which proteins should be degraded. Tight control of the ubiquitination process is required to target the correct proteins for degradation and prevent the unnecessary degradation of the incorrect proteins. Not surprisingly, deregulation of the ubiquitin-proteasome system is often associated with human diseases such as cancer (102) and Parkinson's disease (103).

Ubiquitin is an 8 kDa protein (104, 105) that is attached to substrates through the processive action of E1, E2, and E3 enzymes (106). Ubiquitin is activated for attachment to substrates by the E1 ubiquitin-activating enzyme (107). In an ATP-

dependent reaction, a thioester bond is formed between the active site cysteine of the E1 and the C-terminal carboxyl group of ubiquitin (108). Then, ubiquitin is transferred to the active site cysteine of an E2 ubiquitin-conjugating enzyme via a trans(thio)esterification reaction (106). Finally, an E3 ligase is required to transfer ubiquitin either directly or indirectly from the E2 to an internal lysine residue on the substrate, resulting in an isopeptide bond between the lysine residue and the C-terminal glycine of ubiquitin. E3 ligases directly bind substrates and therefore confer substrate specificity to the ubiquitination machinery (109). Given that ubiquitin contains seven internal lysines, it also can serve as a substrate for ubiquitination, resulting in multi-ubiquitin chains (72).

Substrate proteins can either be mono- or poly-ubiquitinated and each modification results in a different fate. Monoubiquitination is defined as the attachment of three or less ubiquitins to a substrate (110). Typically, transmembrane proteins or proteins localized to membranes are monoubiquitinated, which results in their internalization by endocytosis and subsequent degradation by the lysosome or vacuole (111). Polyubiquitination is defined as the attachment of a multi-ubiquitin chain consisting of four or more ubiquitins (112). Chain elongation can occur through any of the seven lysines on ubiquitin, and linkages through different lysines can have different effects. Chains made of K48-linked ubiquitin are the most common, and target proteins for degradation by the 26S proteasome (112). Linkages by other lysines can have proteolysis-independent functions (113). Most notably, K63-linked polyubiquitin chains have a role in signal transduction and can activate kinases. For example, upon stimulation of cells with proinflammatory cytokines, the protein TRAF6 is modified with K63-linked ubiquitin chains. Proteins containing ubiquitin-binding domains specific for K63-linked chains are recruited to TRAF6, which results in the activation of IkappaB kinase (IKK) (114). Distinct E3 ligases are required to generate ubiquitin chains through

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different lysines. Likewise, distinct E3 ligases are required for mono- versus poly-ubiquitination (115).

Typically, organisms such as yeast and humans have only one E1 enzyme that is responsible for activating the entire cellular pool of ubiquitin (116, 117). There are slightly more E2 enzymes. Yeast have 13 E2 enzymes, but each is capable of coupling with multiple E3 ligases. E3 ligases are by far the most diverse; humans have ~500 and yeast have ~40. Across all species, more than 1000 E3 ligases have been identified (115). Considering E3 ligases provide substrate specificity to the ubiquitination reaction, it is no surprise they are by far the most diverse component of the ubiquitin machinery. There are three main subfamilies of E3 ubiquitin ligases: homologous to E6-AP carboxyl terminus (HECT) domain-containing E3s, single-subunit really interesting new gene (RING) domain-containing E3s, and multi-subunit RING domain-containing E3s.

HECT domain E3s – The HECT domain is a conserved 350 amino acid sequence that was originally discovered in the E6-associated protein (E6-AP) (118). Five yeast genes are classified as HECT domain-containing E3 ligases due to their similarity to E6-AP. Aside from the conserved sequence, the ubiquitination reaction catalyzed by HECT domain-containing E3 ligases further distinguishes them from RING domain-containing E3s. The HECT domain first binds an E2 conjugating enzyme and ubiquitin is transferred from the E2 to the active site cysteine of the HECT domain (115). Through an additional protein-protein interaction domain, HECT E3s bind the substrate and directly catalyze the transfer of ubiquitin to an internal lysine residue on the substrate. HECT E3s contain a number of different protein-protein interaction domains but the most common is the WW domain, which is capable of binding phosphoserine and phosphothreonine residues (119) . The most well characterized HECT E3 ligase in yeast is Rsp5 (118). Rsp5 contains a C2 domain for binding phospholipids at membranes and a WW domain for binding substrates that contain the consensus

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sequence Proline-Proline-X-Tyrosine (120).

Ubiquitination of substrates by HECT E3s can be regulated by several different mechanisms. Many HECT E3s are constitutively active and are regulated by an adapter protein that recruits a ubiquitin-charged E2 activating enzyme to the E3-substrate complex (121). Such is the case with the human E3 ligase Smurf1 and its substrate the TGF-β receptor (122). The adapter protein Smad7 binds both Smurf1 and the TGF-β receptor and then recruits ubiquitin-charged UbcH7[E2] to initiate ubiquitination. Alternatively, constitutively active HECT E3s can be associated with a deubiquitinating enzyme (DUB) which functions to remove ubiquitin from substrates. Rsp5 associates with Ubp2, a ubiquitin-specific protease, that functions to "edit" substrate ubiquitination by Rsp5. Rsp5 has been shown to polyubiquitinate some substrates and monoubiquitinate others (123). It is hypothesized that Ubp2 functions to partially degrade multi-ubiquitin chains to produce monoubiquitinated substrates. However, it is not known how Ubp2 recognizes which proteins should be monoubiquitinated. Other HECT E3 ligases are basally inactive and can be activated by phosphorylation (121). The human E3 Itch is inactive due to intra-molecular interactions between its WW domain and HECT domain (124). Phosphorylation by the MAP kinase JNK1 disrupts this intra-molecular interaction and activates Itch (125). These examples demonstrate that HECT E3s have acquired a variety of different mechanisms, some quite elegant, to tightly regulate protein ubiquitination. As will be discussed below, RING domain-containing E3 ligases are typically regulated via only one mechanism.

RING domain E3s – The RING domain was originally identified as a conserved zinc-binding motif in the human protein Really Interesting New Gene (RING) 1 due to its similarity to other known zinc fingers. Since their discovery, over 200 proteins have been found to contain RING domains. RING domains are comprised of repeating histidine and cysteine residues that allow binding of two zinc molecules (121). While the

exact primary sequence is not highly conserved, the spacing of the two zinc molecules is invariant in almost all RING domains, indicating they play a particularly important role in regulating function. For years, the exact function of the RING domain was unknown and it was thought that they were DNA binding motifs, like other zinc fingers. However, in 1999 three independent groups found proteins with RING domains bound E2 ubiquitin-conjugating enzymes, suggesting RING domains play an important role in ubiquitination (126-128). Subsequent discoveries revealed these RING domain-containing proteins were actually E3 ubiquitin ligases. This revelation had a huge impact in the ubiquitination field since it meant that the more than 200 proteins with RING domains were all E3 ligases.

RING E3s are catalytically distinct from HECT E3s. Whereas HECT E3s have an active site cysteine and catalyze ubiquitination, RING E3s have no enzymatic activity and function solely as scaffolds. They function to bring the substrate and the ubiquitin-conjugated E2 sufficiently close to each other for efficient transfer of ubiquitin to the substrate (115). This lack of enzymatic activity is most likely why the RING E3s escaped detection for almost a decade, and their initial characterization actually triggered a fierce debate in the ubiquitination field as to whether they should in fact be called E3 "ligases." As such, a protein with a RING domain is often referred to as simply an "E3".

There are two different types of RING E3s: single-subunit and multi-subunit. Single-subunit RING E3s, such as the human proteins c-Cbl (129) and Mdm2 (130), contain both a RING domain and a substrate-binding domain. Multi-subunit RING E3s are comprised of multiple proteins that, together, form a functional E3. Typically, one protein in the complex contains a RING domain and binds the E2 while another protein contains a substrate-binding domain (131). Other proteins in the complex function as scaffolds, adapters, or regulatory subunits. The two major types of multi-subunit RING E3s are the Skp1-cullin-F-box (SCF) complex and the anaphase promoting complex

(APC) (reviewed in (131-133)). For this introduction, the main focus will be the SCF complex since it is discussed further in Chapter II of this thesis.

SCF complex – The SCF complex is a heterotetrameric complex that functions to bind both a substrate and an E2 conjugating-enzyme to facilitate ubiquitination. The SCF complex was first identified in yeast and was named after three of its key components: **S**kp1, **C**ullin, and **F**-box protein (134, 135). The F-box protein conveys substrate specificity to the complex through a substrate-binding domain, and is denoted in superscript to differentiate SCF complexes (e.g SCF^{Cdc4} where Cdc4 is the F-box). Typically, several different F-box proteins can couple to the same SCF complex, greatly increasing the variety and number of different proteins that can be ubiquitinated by the SCF.   Regulation of SCF-mediated ubiquitination is generally through the phosphorylation of target proteins (131). Almost all the substrates identified so far must be phosphorylated before they can bind the F-box protein (135). This provides the cell with a simple mechanism to regulate which proteins should be ubiquitinated by the SCF complex.

In yeast, the most well-studied SCF complex is SCF^{Cdc4} and it is comprised of Cdc4, Skp1, Cdc53, and Roc1 and couples with the E2 enzyme Cdc34. These four essential components form a scaffold that holds the substrate and E2 enzyme close enough to allow the transfer of ubiquitin. The F-box protein, Cdc4, binds substrates, Cdc53 and Roc1 bind the E2 conjugating enzyme, and Skp1 is an adapter protein that binds both Cdc4 and Cdc53, linking the entire complex (Figure 1.5).

The SCF^{Cdc4} was discovered through a series of papers that slowly identified the function of all the components of the complex. *CDC4*, *CDC53*, and *CDC34* were identified in screens for mutants with defects in the cell division cycle (136, 137). Subsequently, all three were found to be required for the degradation of the G1/S phase checkpoint protein Sic1 (136, 137), indicating they may play a role in regulating Sic1

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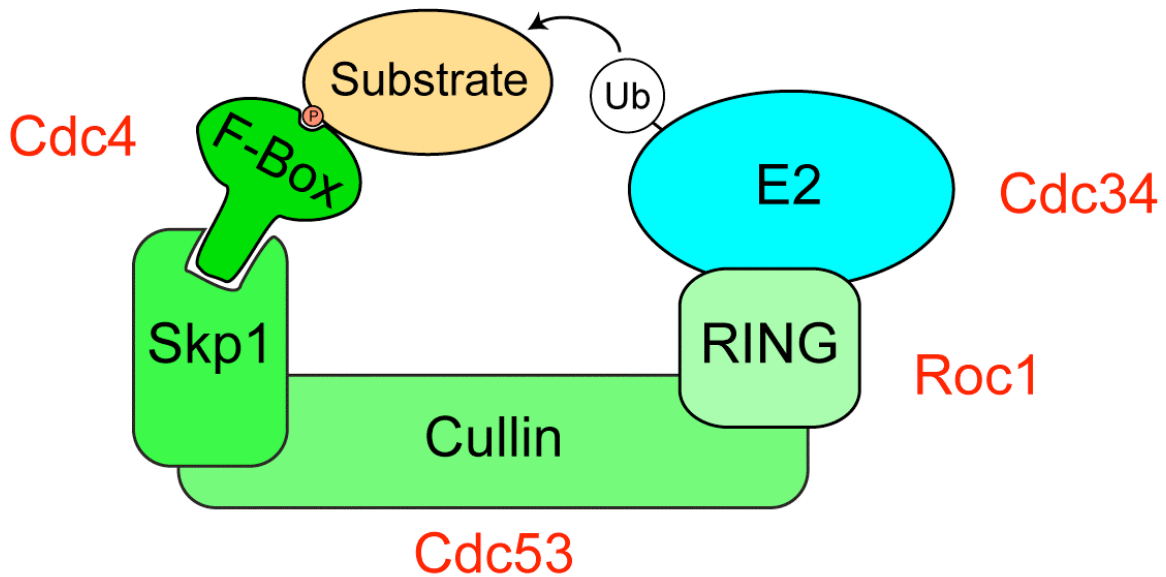
Figure 1.5

Figure 1.5 The SCF E3 ubiquitin ligase complex.
The SCF complex is an E3 ubiquitin ligase and is comprised of four essential components. A protein that contains a RING domain binds the E2 ubiquitin-conjugating enzyme. The F-box protein functions as the substrate receptor and typically binds phosphorylated substrates. Skp1 is an adapter that links the F-box protein to the rest of the complex. The cullin functions as a scaffold and organizes the complex by binding multiple components. In yeast, the SCF is comprised of the yeast proteins Cdc4, Skp1, Cdc53, and Roc1 and couples to the E2 enzyme Cdc34.

turnover. At the time, it was known that Cdc34 was an E2 ubiquitin-conjugating enzyme, further supporting this hypothesis (138). Cdc53 was also found to be part of a recently identified protein family called Cullins which were involved in protein turnover (139). However, the exact role of Cdc4 and Cdc53 in Sic1 ubiquitination and degradation was unclear. Further complicating the model, *SKP1* was identified in a screen for genes that suppress *cdc4^{ts}* mutants when overexpressed. It was then shown that *skp1^{ts}* mutants were also defective in Sic1 degradation and that Skp1 binds Cdc4 at a conserved motif called an F-box (140). Thus, Cdc4, Cdc53, and Skp1 were thought to form a multi-protein complex that along with Cdc34 is required for ubiquitination of Sic1. To definitively test this hypothesis, Skowyra *et al*. and Feldman *et al.* expressed Cdc4, Cdc53, and Skp1 from insect cells and showed they formed a functional E3 ubiquitin ligase, that along with Cdc34 purified from bacteria, was capable of ubiquitinating phosphorylated Sic1 *in vitro* (134, 135). Interestingly, Skowyra *et al*. also showed that another protein containing an F-box domain, Grr1, was capable of forming an SCF complex with Skp1 and Cdc53. SCF^{Grr1} was unable to bind Sic1, but was able to bind a different SCF substrate, Cln1 (135). These data indicate F-box proteins are interchangeable substrate receptors and, by coupling to different F-box proteins, the SCF can target a wide range of proteins for ubiquitination (141).

As mentioned above, SCF complexes contain four different proteins, yet Skowyra *et al*. and Feldman *et al.* expressed only three proteins in insect cells to form a functional SCF ligase (134, 135). The fourth protein in the SCF complex was eventually discovered a few years after their papers were published. Three independent groups identified Roc1 as a protein that co-purified with Cdc53, was required for Sic1 degradation, and contained a RING domain (142-144). Coincidentally, this discovery was made around the same time that RING domains were found to be E3 ligases that bind E2 enzymes (126-128). This led to the obvious hypothesis that Roc1 must be a

missing link between the core SCF components Cdc4, Cdc53, and Skp1 and the E2 enzyme Cdc34. Indeed, when Roc1 was added to Cdc4, Cdc53, and Skp1 purified from insect cells, Sic1 ubiquitination was greatly enhanced (144). Later, it was determined that by expressing Cdc4, Cdc53, and Skp1 in insect cells, Skowyra *et al.* and Feldman *et al.* inadvertently co-purified the insect homolog of Roc1, thus forming a functional SCF complex (131). This highlights the fact that SCF components are highly conserved across species, and discoveries made in model organisms such as yeast are likely to yield similar results in humans.

Typically, the SCF is regulated through its interaction with substrates. Given the F-box protein is the substrate receptor for the complex, it is the most functionally relevant subunit. All F-box proteins contain a short conserved ~50 amino acid domain called an F-box that mediates binding to Skp1. The F-box was first identified as a shared motif between Cyclin F and Cdc4 (140). Since then, over 400 F-box proteins (18 in yeast) have been identified (145, 146). F-box proteins also contain a substrate-binding domain and a short linker that connects the F-box to the substrate-binding domain, which has been implicated in regulating protein turnover (147) and dimerization (148). F-box proteins are divided up into three main families based on their substrate binding domains which can be seven WD40 repeats (FBXW), multiple leucine-rich repeats (FBXL), or a unique binding domain (FBXO) (146). Cdc4, the prototype F-box protein, contains seven WD repeats (crystal structures reveal a possible eighth cryptic WD40 repeat) and is highly conserved (149). The human homolog of Cdc4 is Fbw7 (150, 151). While F-box proteins contain a diverse array of substrate-binding domains, they all seem to selectively bind phosphorylated substrates. Thus, despite evolutionary divergence of the F-box protein structure, they have maintained a conserved regulatory mechanism, suggesting that control of protein ubiquitination by phosphorylation conveys an evolutionary advantage.

Since the discovery of the SCF^{Cdc4} over 13 years ago, much effort has gone into identifying all of its substrates. Only six have been identified, including Sic1 (152). This lack in progress is mostly due to the difficulty in identifying protein substrates of E3 ligases. Typically, one must demonstrate both a functional and a physical interaction with the E3 before a protein can be considered a substrate. However, demonstrating a physical interaction can be very difficult since interactions are often very weak, substrate levels in the cell are usually low, and when interactions occur they lead to rapid ubiquitination and degradation of the substrate (152, 153). Furthermore, most substrates must first be phosphorylated before they can bind the SCF. Unless the cellular conditions that promote substrate phosphorylation are known, no physical interactions will be detected. Thus, the identification of SCF substrates remains a high priority.

Gα ubiquitination

Gα proteins undergo a variety of different post-translational modifications including i) myristoylation, ii) palmitoylation, iii) ADP-ribosylation, iv) phosphorylation, and v) ubiquitination. These diverse modifications regulate cellular localization, the ability to interact with other proteins, GTP hydrolysis, and GDP release, and play a critical role in the regulation of G protein signaling. Of all these post-translational modifications however, G protein ubiquitination is the least studied.

The first evidence of G protein ubiquitination came in 1994 when it was shown that the yeast G protein, Gpa1, was degraded by the N-end rule (154). In 1996, Obin *et al*. purified a ubiquitinated protein out of retinal rod outer segments that had the same molecular weight as G_t (155). Human $Gα_{i3}$ was shown to be degraded in HEK293 cells in a proteasomal-dependent manner, and it was shown that overexpression of a putative E3 ubiquitin ligase enhanced degradation. However, direct evidence of Gα

ubiquitination was lacking in this report (156). Recently, more convincing evidence of G protein ubiquitination in humans has emerged. Nagai *et al.* was able to detect ubiquitinated $G\alpha_s$ in HEK293 cells by using the proteasomal inhibitor MG132. Furthermore, they showed that the protein Ric-8B (a homolog of Ric-8A which functions as a non-receptor GEF) binds $G\alpha_s$ and prevents its ubiquitination and subsequent degradation (157). While the details of human G protein ubiquitination are still emerging, it is clear that ubiquitination does play a role in regulating $G\alpha$ abundance in several different organisms. Ubiquitination of the yeast $G\alpha$ subunit, Gpa1, is by far the most studied (154, 158-161). As further evidence of human G protein ubiquitination is gathered, Gpa1 is likely to serve as a model for studying both the regulation of G protein ubiquitination and its role in G protein-mediated signaling pathways.

Gpa1 is one of only a few proteins that is known to be both mono- and poly-ubiquitinated. Consistent with its localization at the plasma membrane, Gpa1 is monoubiquitinated, endocytosed, and delivered to the vacuole for degradation (159). Rsp5, a HECT E3, is necessary and sufficient for Gpa1 monoubiquitination both *in vivo* and *in vitro* (160). Interestingly, Rsp5 also monoubiquitinates the α factor receptor Ste2 and mediates receptor internalization after pheromone stimulation (162, 163). The fact that Gpa1 and Ste2 are targeted for internalization by the same enzyme indicates Gpa1 and Ste2 monoubiquitination may both be required for desensitization. However, it is not currently known how Gpa1 monoubiquitination by Rsp5 is regulated. While Ste2 ubiquitination is stimulated by pheromone treatment, Gpa1 monoubiquitination is not (159, 162). Thus, the exact role of Gpa1 monoubiquitination is unknown and is an active area of research.

Gpa1 is also polyubiquitinated and degraded by the 26S proteasome (159). Mass spectrometry analysis revealed that Gpa1 is modified with ubiquitin on K165 (158).

Polyubiquitination of Gpa1 can be blocked by deletion of the ubiquitinated subdomain (residues 128-236) and results in a dampened pheromone response (159). Such a phenotype is consistent with overexpression of Gpa1 which functions to sequester $G\beta\gamma$ and inhibit signaling (164). However, contrary to this phenotype, blocking poly-ubiquitination of Gpa1 did not alter total cellular abundance of Gpa1 as measured by western blotting (159). These data indicate that polyubiquitination may regulate specific subpopulations of Gpa1. Thus, while total cellular Gpa1 levels are unchanged, specific subpopulations of Gpa1 could be elevated, resulting in dampened pheromone signaling. Further study of Gpa1 polyubiquitination is needed to fully understand its role in regulating G protein signaling.

The E3 ligase that mediates Gpa1 polyubiquitination is unknown, but it is distinct from Rsp5. Why yeast require two different modes of ubiquitinating Gpa1 is not well understood, but identifying the E3 ligase involved in polyubiquitination could reveal important differences in the regulation of mono- vs poly-ubiquitination. Furthermore, E3 ligases involved in yeast G protein ubiquitination are likely to be conserved in humans and could be useful drug targets.

Phosphatidylinositol kinases

Phosphatidylinositol (PtdIns) is a component of cell membranes and has a role in regulating many cell signaling pathways. PtdIns has both polar and non-polar groups allowing it associate with both the plasma membrane and cytosolic proteins (165). It is comprised of a glycerol backbone, two fatty acids, and a phosphate group attached to an inositol head group (Figure 1.6). The inositol head group has five hydroxyls and each are capable of being phosphorylated by specific PtdIns kinases to produce phosphoinositides (166). Yeast have six such kinases and are capable of producing five distinct derivatives of PtdIns, each with its own cellular function (165). Typically,

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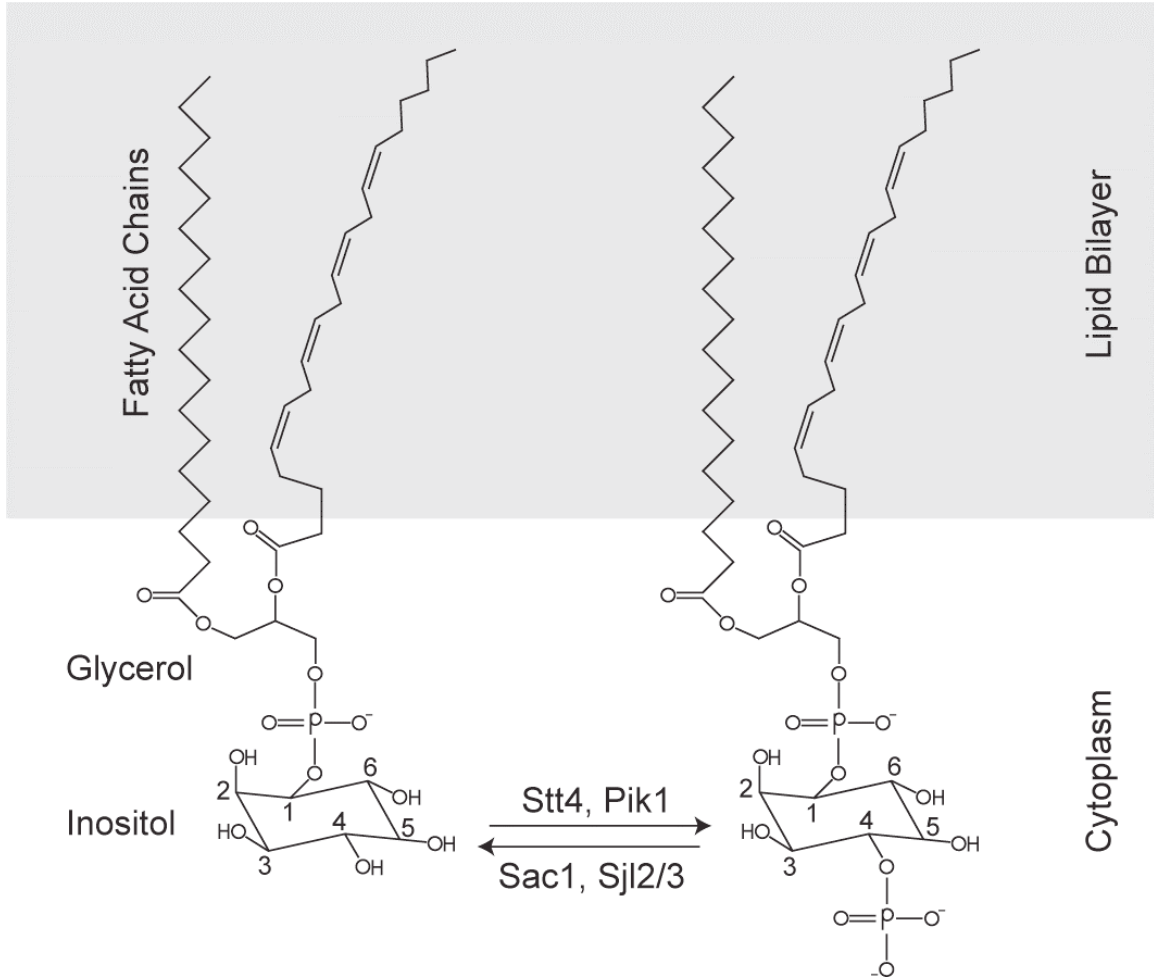
Figure 1.6

Figure 1.6 Phosphatidylinositol is phosphorylated by lipid kinases
    Phosphatidylinositol (PtdIns) is an amphiphatic lipid that associates with both non-polar lipid membranes and the cytosol. Phosphorylated derivatives of PtdIns are called phosphoinositides and function as membrane anchors for many proteins. They are comprised of two non-polar fatty acid chains, a glycerol backbone, and a polar inositol headgroup. Inositol can be phosphorylated by a number of lipid kinases to produce different PtdIns derivatives. For example, Stt4 and Pik1 phosphorylate PtdIns at the 4 position to make PtdIns 4-P. Phosphatases such as Sac1 and Sjl2 catalyze the reverse reaction and remove the phosphorylation.

phosphoinositides can regulate cell signaling pathways in one of two ways. First, they can serve as a substrate for phospholipases, which cleave PtdIns and produce the second messengers inositol phosphate (IP) and diacyglyercol (DAG) (167, 168). Second, phosphoinositides can bind cytosolic proteins altering either their localization or activity. A variety of phospholipid-binding domains have been identified so far including the pleckstrin homology (PH) domain, phox homology (PX) domain, epsin N-terminal homology (ENTH) domain, and Fab1p/YOTB/Vac1p/EEA1 (FYVE) domain (169). Each domain can have a different specificity for phosphorylated subspecies. For example, the PX domain of Bem3 binds only to PtdIns 3-P while the PX domain from Bem1 binds PtdIns 3-P and PtdIns 4-P (170). Such differences allow for the regulation of only a subset of lipid-binding domains by a given PtdIns kinase.

PtdIns have long been known to play an important role in G protein signaling (171-173). In mammals, G_q activates phospholipase C (PLC) which cleaves PtdIns $3,4,5-P_3$ to produce IP_3 and DAG (174). Both of these second messengers then trigger the release of intracellular calcium stores from the endoplasmic reticulum. More recently however, a new role for PtdIns in G protein signaling was identified in yeast. Slessareva *et al.* found that GTP-bound Gpa1 activates the PtdIns 3-kinase Vps34 and stimulates the production of PtdIns 3-P at the endosome (65). PtdIns 3-P then recruits the PX domain-containing protein Bem1 to the endosome and is required for full activation of the MAPK Fus3. This new discovery indicates PtdIns may have a larger role in pheromone signaling than was previously recognized.

Thesis summary

The pheromone pathway has served as a model system for studying heterotrimeric G proteins for many years. It all started with Mackay, Manney, and Hartwell who discovered the first sterile genes in yeast (50, 51, 58). Since then, the

yeast pheromone pathway has been one of the most intensely studied signaling pathways in all eukaryotes. Insights made in yeast have often led to discoveries in humans. Relevant examples include the RGS protein Sst2 (13, 59, 175), the MAPK scaffold Ste5 (176), and the F-box protein Cdc4 (150). At the time research began on this thesis, systematic screens to identify new components of the pheromone pathway had attained impressive coverage, including deletion strains representing almost 5,000 genes (177). Thus after more than 30 years of research, almost 80% of genes had been assayed for activity in the pheromone pathway. The last 20% however, has proven the most difficult to study since they are essential for cell growth and survival. The essential genes represent one of the last frontiers of yeast genetics and are a virtually untapped resource for identifying new components of signaling pathways coupled to heterotrimeric G proteins. For my thesis research, I intended to identify new components of the yeast pheromone pathway by systematically screening the essential genes, and to finally achieve near 100% coverage of the yeast genome. I undertook this challenge in the hopes that discovering new regulators of G protein signaling would lead to a better understanding of human G protein signaling and perhaps to the development of new therapies to treat G protein-related diseases.

The remainder of this thesis is divided into 3 chapters. In Chapter II, "Systematic screen of essential genes reveals important regulators of G protein signaling," I present a genome-scale screen of the $TetO_7$ promoter library, including the design and validation of the screening method. Also in this chapter, I characterize several essential genes that were identified in the screen, with special attention paid to the SCF^{Cdc4} complex and its role in regulating G protein ubiquitination. In Chapter III, "Selective regulation of MAP kinase signaling by an endomembrane phosphatidylinositol 4-kinase," I compare and contrast the role of two PtdIns 4-kinases, Pik1 and Stt4, in regulating the pheromone pathway and reveal a unique role for Pik1 in regulating parallel MAP kinase pathways.

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Finally, in Chapter IV, "Conclusions and general discussion," I discuss the broad impact

of these findings and speculate on the future direction of the field.

CHAPTER II

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

Figures contributed by:

Steven D. Cappell: 2.1; 2.2; 2.3; 2.4; 2.5; 2.6; 2.7; 2.8; 2.9B and C; 2.10; Table 2.1
Rachael Baker: 2.9A

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 (27). 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 (28). Notably, these essential genes are more likely to have a human ortholog, as compared with non-essential genes (38% vs. 20%) (67). Here we describe the identification of new components and new regulators 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 (178).

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 G$\beta\gamma$ subunit dimer (Ste4/18). Dissociated G$\beta\gamma$ 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 G$\beta\gamma$ subunits. GTP hydrolysis is further accelerated by the RGS (Regulator of G protein Signaling) protein Sst2. Therefore, Gpa1 functions primarily to sequester G$\beta\gamma$ in the absence of receptor stimulation (178).

Many components of the yeast pheromone pathway were identified genetically, by isolating mutants that exhibit a mating-deficient (sterile) phenotype (58). Recent efforts to identify new components of G protein signaling have employed more systematic, genome-scale approaches (65). 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$^{G\alpha}$ modulates pheromone signaling through a direct interaction with phosphatidylinositol (PtdIns) 3-kinase, resulting in elevated production of the second messenger PtdIns 3-P (65).

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 (73, 74). 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 (28). 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 (94), translation, and mitochondria import machinery (28). 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 (178). 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 (34).

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 (134, 135). 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 (131). Previous work has showed that Cdc34 and the SCF complex are involved in regulating the cell cycle and the mating-associated cell cycle arrest (135, 179). 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* (34) and *CDC42*

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(180). *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 2.1 A). 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 2.1 B). As expected, treatment of the TetO$_7$ Wild-Type strain with doxycycline had no effect (Figure 2.1 C). 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 (10 ng/mL and 100 ng/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 2.2). 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.

We classified our hits by functional category using Gene Ontology (GO)

Figure 2.1
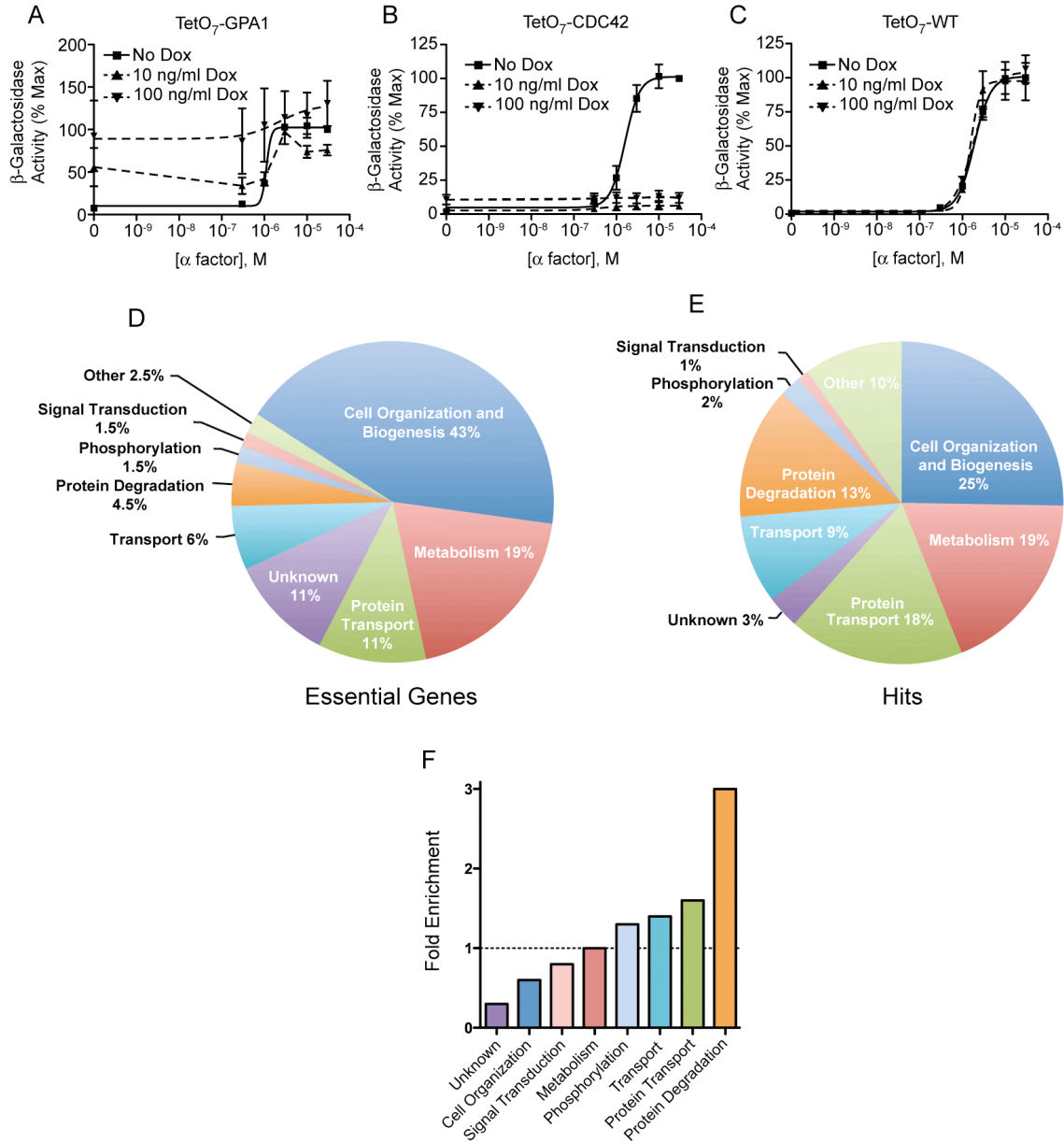
Figure 2.1 Validation of the TetO$_7$ promoter essential gene screen.
(A) Transcriptional activation (β-galactosidase activity) in response to α factor treatment was measured spectrofluorometrically in TetO$_7$-GPA1 cells treated with doxycycline (Dox, 10 ng/mL and 100 ng/mL) or untreated control. Cells 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) TetO$_7$-CDC42 cells treated as in (A). (C) TetO$_7$-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 2.2
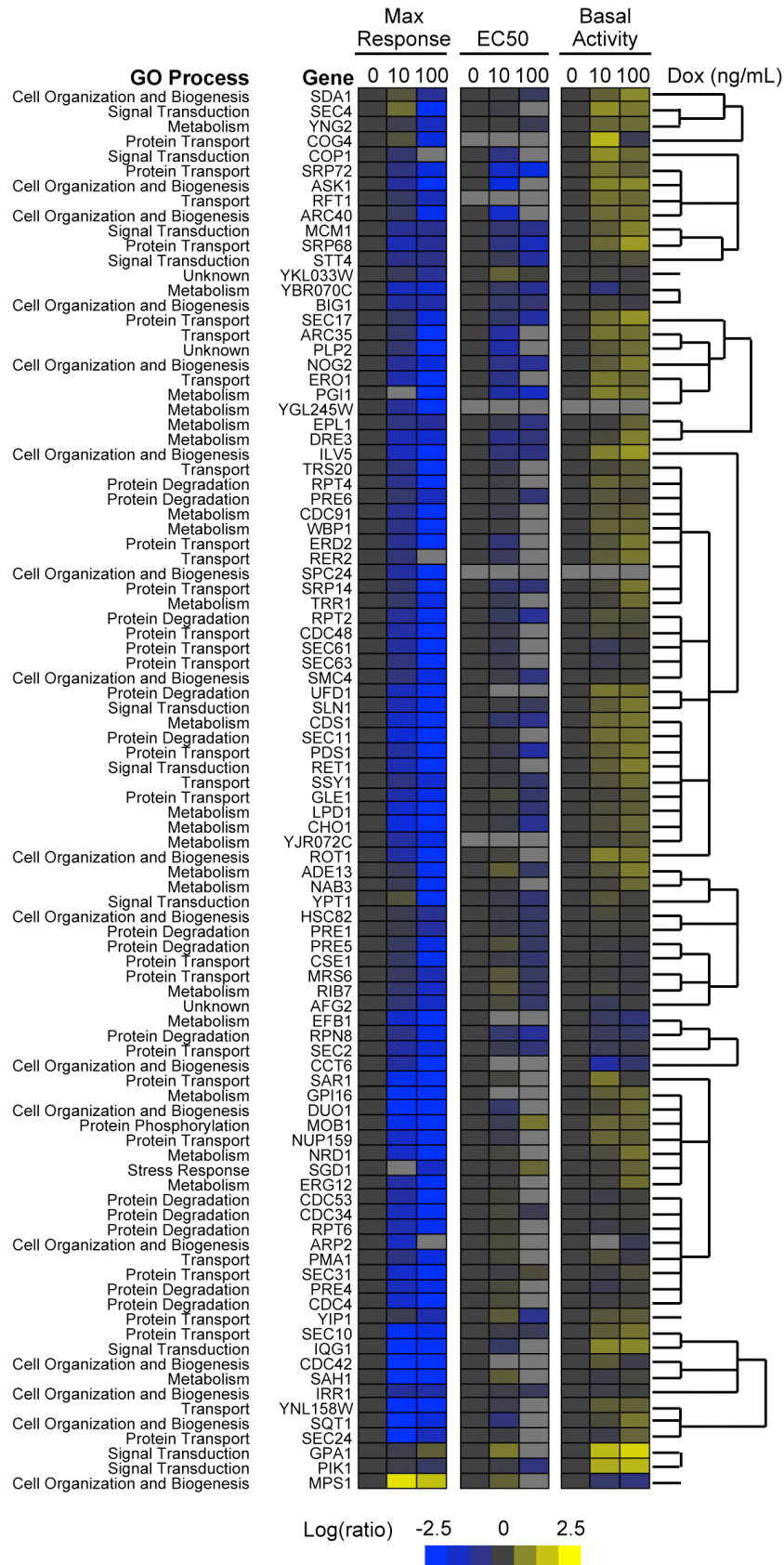
Figure 2.2 Phenotype clustering analysis.

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.

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 2.1 D and E). Interestingly, genes involved in protein degradation were enriched 3-fold, by far the most highly represented group of functionally degradation were related genes (Figure 2.1F).

In order to further prioritize our investigations, we generated an interaction map using the Osprey Network Visualization System (181), which incorporates known physical and genetic interactions from the Biological General Repository for Interaction Datasets (BioGRID) (182) (Figure 2.3). 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 2.2). 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 2.1). *MPS1* is the yeast ortholog of mammalian TKK (183), and

Figure 2.3
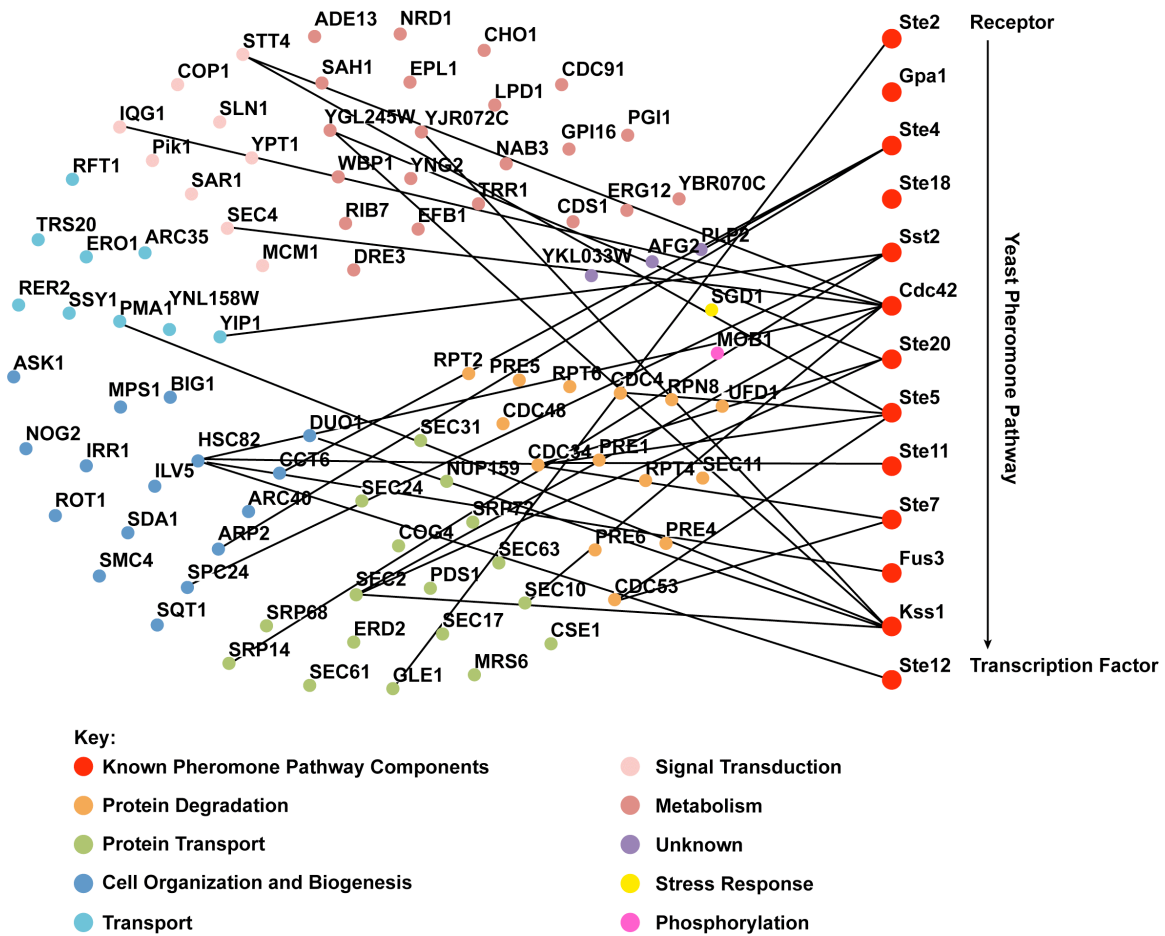
Figure 2.3   Many of the hits from the essential-gene screen have known interactions with pheromone pathway components.

The Osprey Network Visualization System software was used to generate an interaction network based on known genetic and physical interactions from BioGRID.

Table 2.1 Selected essential genes required for proper pheromone signaling

Systematic Name[a]	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 indicated TetO$_7$ 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]LogEC$_{50}$ for TetO$_7$ Wild-Type strain is -5.60 ± 0.01 M
[f]Showed 4x increase in basal activity when treated with doxycycline.

is known to encode a dual-specificity kinase (184). 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* (185, 186). 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 (65). 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 (134, 135). 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 (10 μg/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 2.1).

Transcriptional reporter assays are susceptible to false positives by proteins affecting overall gene expression. To determine if any of the six components have

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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 (Figure 2.4). Thus each of the six genes of interest regulates pheromone signaling specifically and not general gene transcription (Table 2.1).

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 2.5 B-F), indicating a positive role in signaling. Conversely, knockdown of *MPS1* resulted in an increase in maximal activation (Figure 2.5 G), indicating a negative role in signaling. Knockdown of *PIK1* resulted in substantial pathway activity even in the absence of pheromone addition (Figure 2.5 F). 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 2.5 A), knockdown of *CDC4*, *CDC34*, *CDC53*, *STT4*, or *PIK1* resulted in a decrease in Fus3 phosphorylation (Figures 2.5 B-F) in

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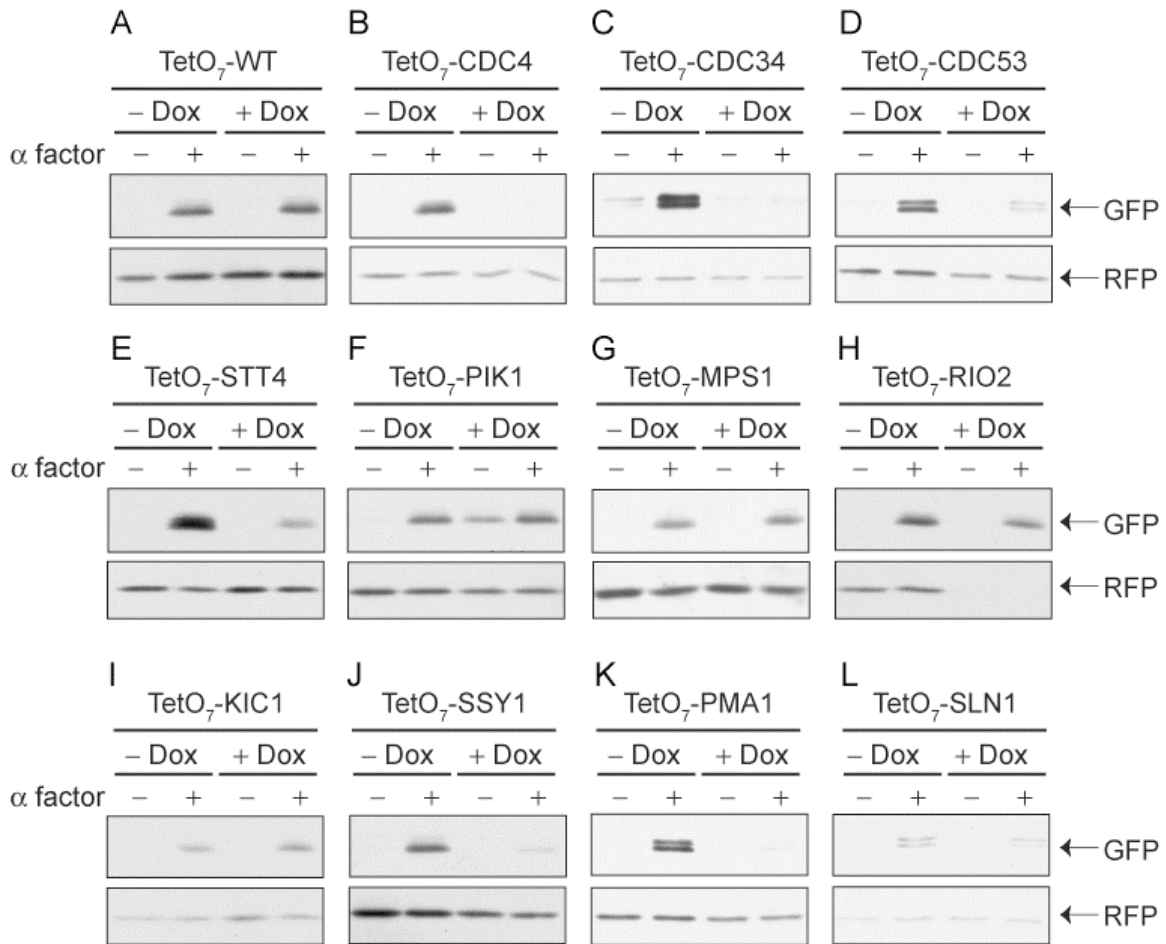
Figure 2.4

Figure 2.4   Hits from the essential-gene screen regulate G protein signaling upstream of translation.

(A-L) The indicated TetO$_7$ strains expressing the pRS315 AR/FG dual reporter plasmid were treated with 10 μg/mL doxycycline for 15 hrs and 3 μM α factor for 30min. Cell lysates were probed with GFP and RFP antibodies. Five additional strains, TetO$_7$-RIO2, TetO$_7$-KIC1, TetO$_7$-SSY1, TetO$_7$-PMA1 and TetO$_7$-SLN1, were also tested. Strains representing *RIO2*, *KIC1*, and *SSY1* exhibited changes in RFP expression. Strains representing *PMA1* and *SLN1* showed no changes in RFP expression but were not considered further.

Figure 2.5
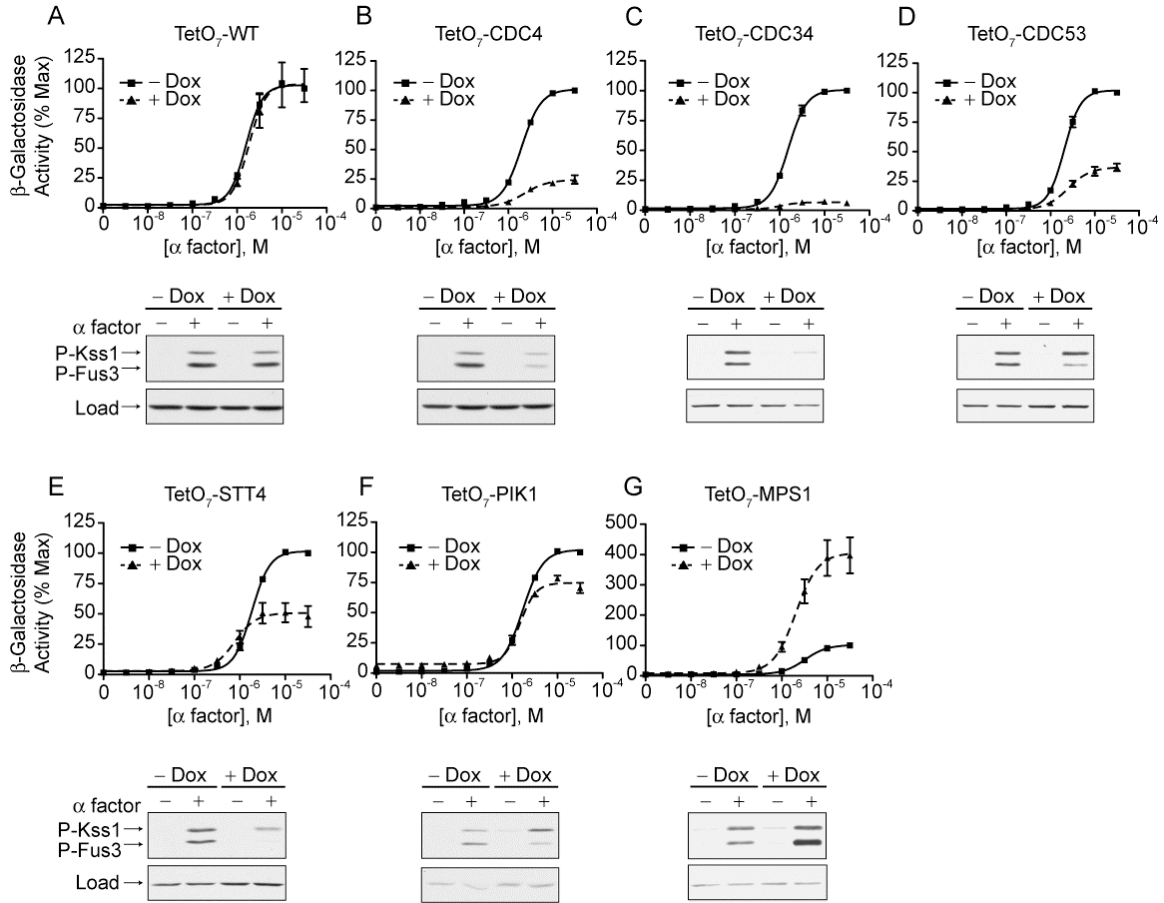
Figure 2.5   Verification of the roles of selected essential genes in pheromone signaling.

(A-G) The indicated essential genes were chosen for further validation and analysis. TetO$_7$ strains expressing *FUS1-lacZ* were treated with 10 μg/mL doxycycline for 15 hrs and exposed to the indicated concentrations of α factor for 90 min. 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 10 μg/mL doxycycline for 15 hrs and then 3 μM α factor for 30min. Results are the mean ± S.E. (n=5).

relation to total Fus3 levels (Figure 2.6). Conversely, knockdown of *MPS1* resulted in an increase in Fus3 activation (Figure 2.5 G). 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 2.5 E and F). 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 (176, 187) as well as the PtdIns 3-kinase Vps34 and its binding partner Vps15 (65). Pik1 and Stt4 are both PtdIns 4-kinases, but are present at different subcellular locations: Stt4 at the plasma membrane (188) and Pik1 at the Golgi and the nucleus (189). Taken together, these findings reveal a possible role for mono-phosphorylated inositides in Fus3 signaling.

SCF^{Cdc4} regulates the pheromone pathway upstream of $Ste4^{G_\beta}$

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 (134, 135). 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 2.5 B-D). The similarity of

Figure 2.6
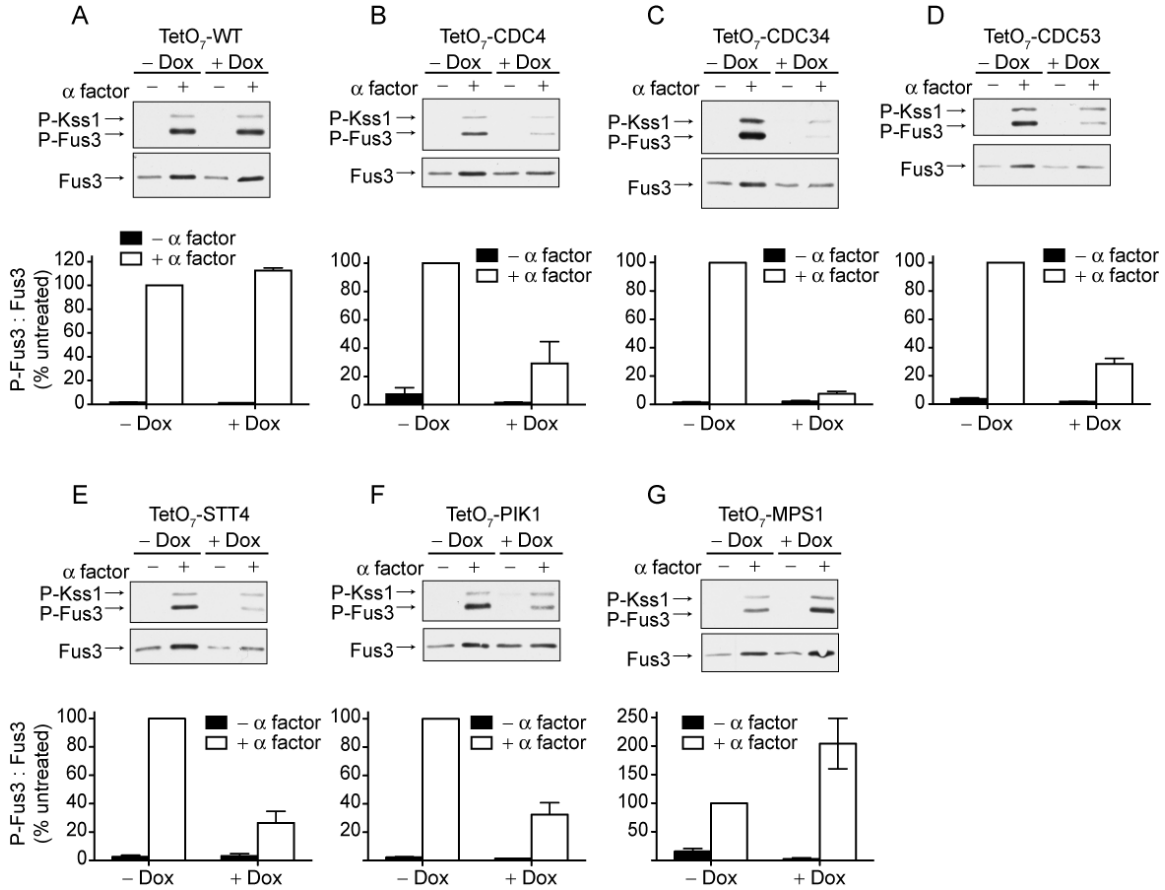
Figure 2.6 Knock-down of essential genes affects Fus3 activation.
(A-G) The indicated TetO$_7$ strains were treated with 10μg/mL doxycycline for 15 hrs and 3 μM α factor for 30 min. Cell lysates were probed with phospho-p42/44 (P-Fus3, P-Kss1) and Fus3 antibodies. Bands were quantified by densitometry and the ratio of P-Fus3:Fus3 is shown below each immunoblot. Results are the mean ± S.E. (n=3).

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 (190). *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 2.7 A). These results indicate that SCF^{Cdc4} acts on a protein component that is upstream of the MAPKKK.

Next, we over-expressed *STE4*$^{G_\beta}$ using a galactose-inducible promoter. Since Gpa1 cannot sequester excess Ste4$^{G_\beta}$ (34), the overproduced Ste4$^{G_\beta}$ 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 2.7 B). Thus knock-down of Cdc4 attenuates signaling by pheromone but not the G protein β 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

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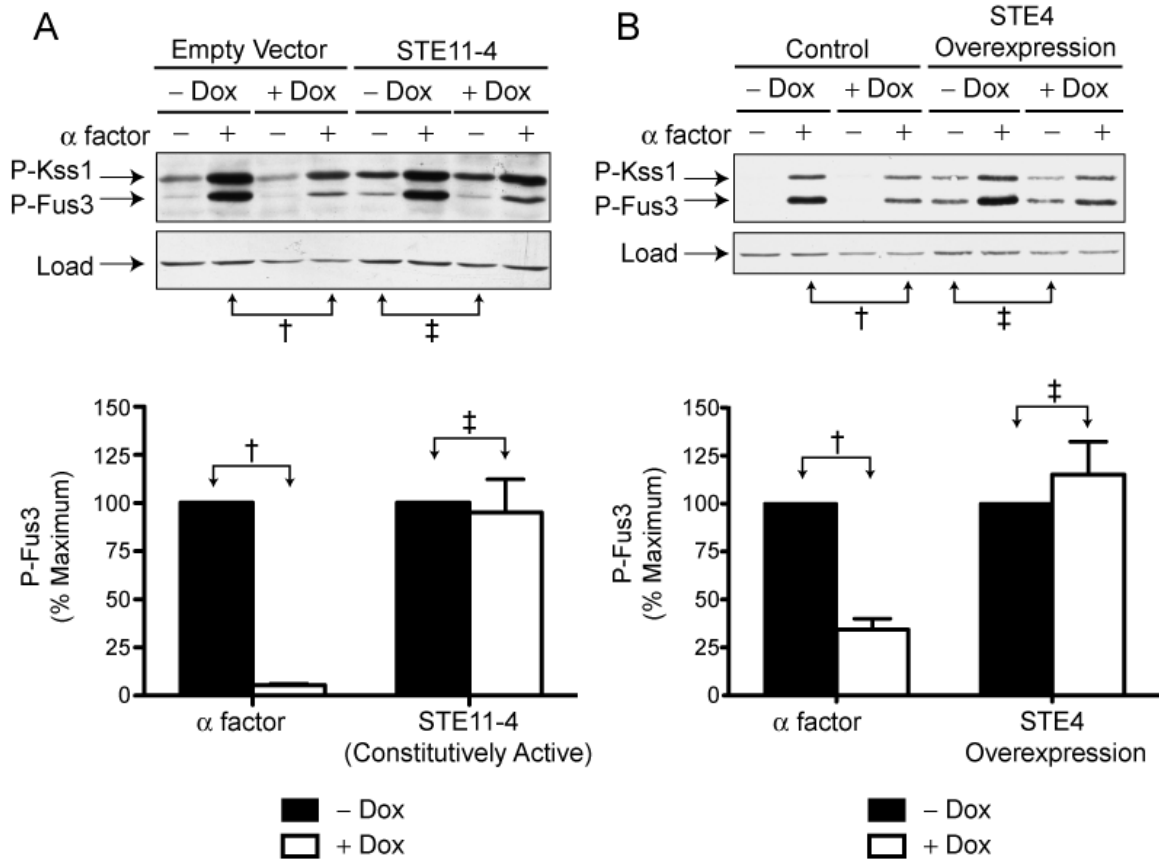
Figure 2.7

Figure 2.7 The Cdc34 E2 and SCF^{Cdc4} regulate signaling upstream of Ste4$^{G_\beta}$.
(A) TetO$_7$-CDC4 cells were transformed with a plasmid containing either *STE11-4* (constitutively active mutant) or no insert. Cells were treated with 10 μg/mL doxycycline for 15 hrs and then 3 μM α factor for 30 min. 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 10 μg/mL doxycycline for 12 hrs in medium containing either dextrose or switched to galactose (2% w/v final concentration) for 3 hrs prior to α factor treatment (3 μM for 30 min). 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).

of Cdc4 resulted in a decrease in signaling. There are only two well-characterized negative regulators that function upstream of Ste4$^{G_\beta}$, Gpa1 and the RGS protein Sst2, and both are known to be ubiquitinated (154, 191). 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 (152). The rate of Sst2 degradation was similar in both the wild-type and *cdc4-1* cells (Figures 2.8 B and D). 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 344 min (Figures 2.8 A and C). 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 (159), comparable to that seen in the SCF mutant strains (Table 2.1). 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 2.9 A). These data corroborate reports that the Cdc34/SCF pathway promotes the degradation of Ste7 and Ste5 (192, 193), 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) (192, 193). Strikingly, despite stabilized expression of multiple components that *propagate* the signal, loss of Cdc34 or SCF^{Cdc4}

Figure 2.8
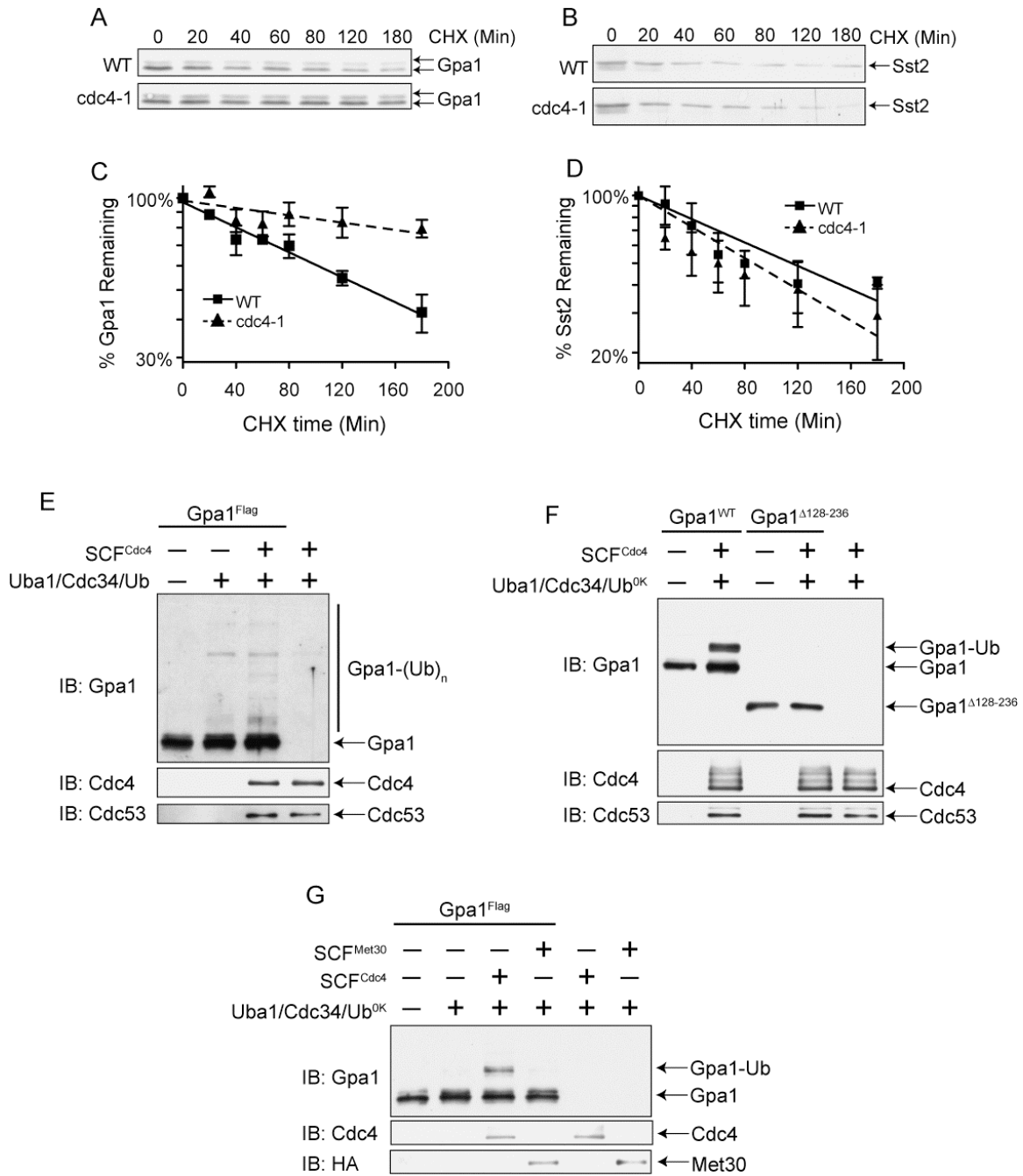
Figure 2.8 SCF^{Cdc4} ubiquitinates Gpa1 *in vitro* and facilitates its turnover *in vivo*.

(A) Gpa1 stability in wild-type vs. temperature-sensitive *cdc4-1* mutant cells. Cultures were grown at 25°C, shifted to 37°C for 1 hr, 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$_{\Delta128-236}$, 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 2.9 B).

Figure 2.9
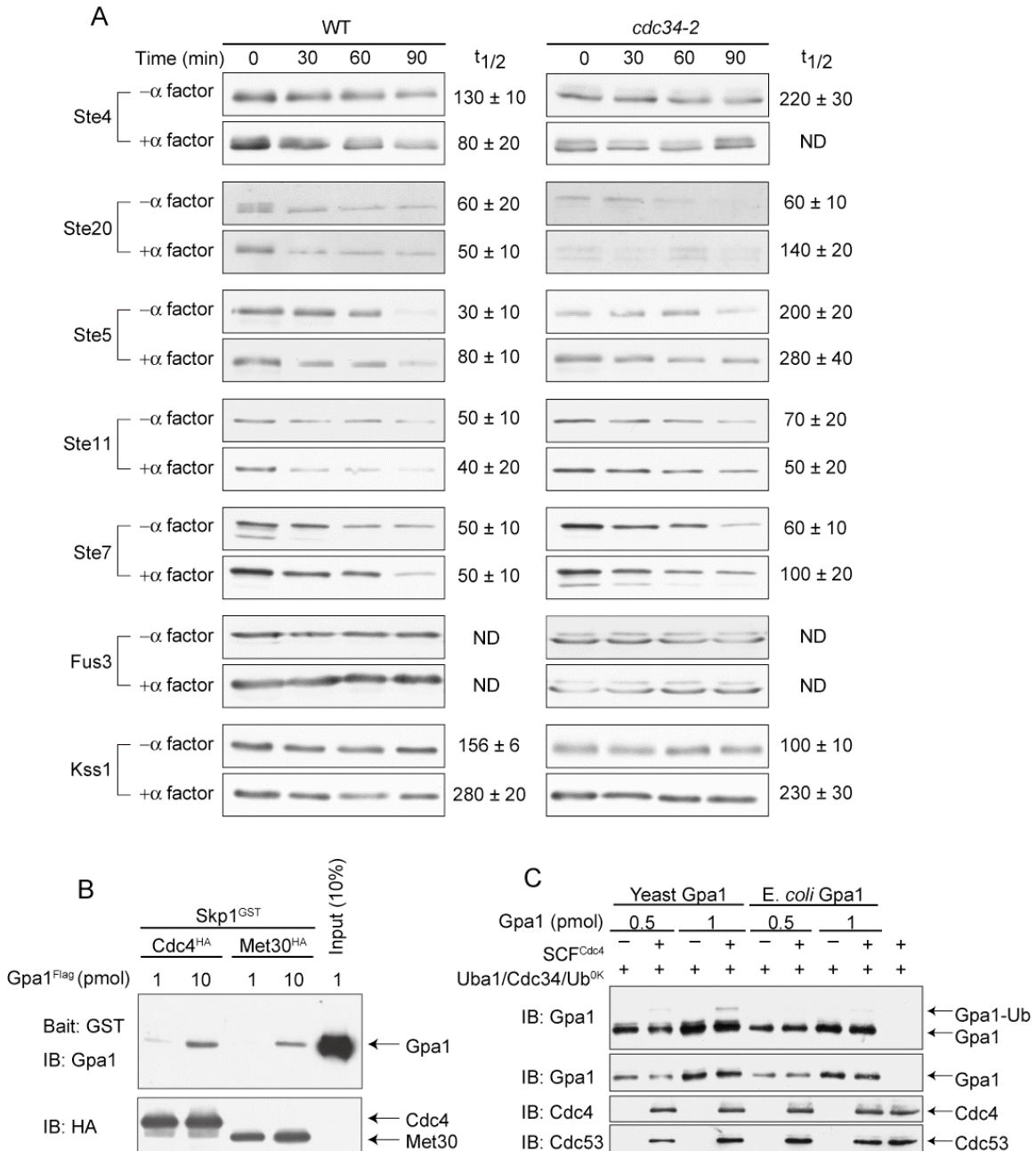
Figure 2.9 The SCF does not destabilize any positive regulators of G protein signaling and primarily functions to ubiquitinate Gpa1.

(A) The indicated TAP-fusion genes were integrated into wild-type and cdc34-2 ts strains. Cycloheximide was administered at time zero and protein levels measured by immunoblotting with protein A antibodies. (B) GST-Skp1/Cdc4-HA or Met30-HA complexes were immobilized on Glutathione SepharoseTM resin and incubated with purified Gpa1-Flag, followed by washing and analysis of the bound proteins with Gpa1-antibodies to detect co-purification of Gpa1. (C) In vitro ubiquitination of Gpa1 expressed in yeast and E coli, which lacks most post-translational modifications.

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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$^{G_\beta}$ (Figure 2.7). 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* (154). 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 2.9 B, 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 (99). Gpa1 bound to Met30 (Figure 2.9 B), 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 2.8 E, 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

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(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 2.8 F).

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 ($Gpa1_{\Delta}^{128-236}$). As shown in Figure 2.8 F, $Gpa1_{\Delta}^{128-236}$ is not ubiquitinated *in vitro*, indicating the SCF^{Cdc4} ubiquitinates Gpa1 at the known site of ubiquitination. Interestingly, $Gpa1_{\Delta}^{128-236}$ 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 2.8 G).

Finally, the SCF complex is typically recruited to substrates in response to substrate phosphorylation (194). 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 2.9 C, 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 $Gpa1_{\Delta}^{128-236}$ mutant, which is not ubiquitinated *in vivo* or *in vitro* (159) (Figure 2.8 F), exhibits a dampened pheromone response comparable to that seen following knock-down of SCF^{Cdc4} expression (Table 2.1). 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 2.10) (195). 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 2.10).

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

Figure 2.10
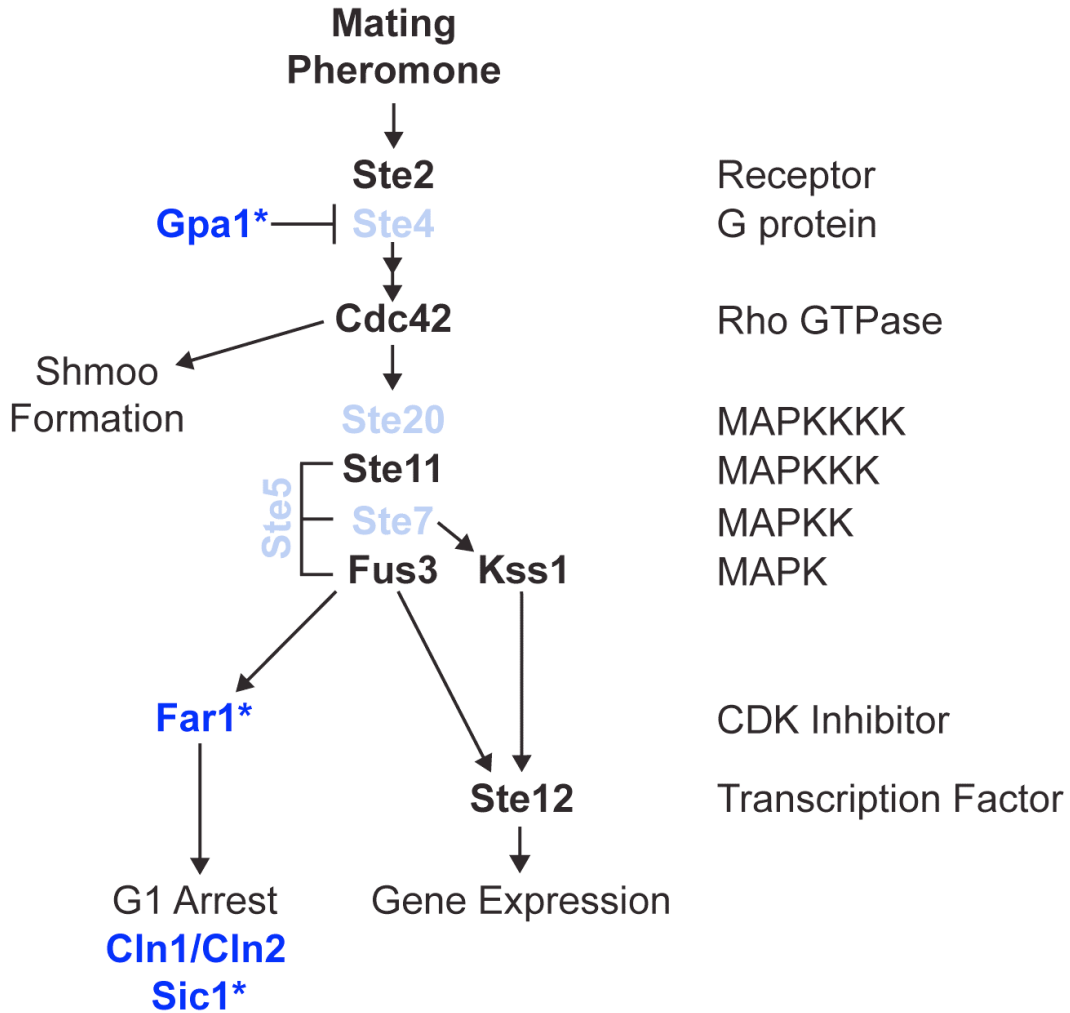
Figure 2.10 The pheromone response pathway.
The Cdc34/SCF complex targets several known substrates in the pheromone response pathway. Known substrates of Cdc34/SCF are shown in dark blue. Likely substrates of the SCF are shown in light blue. SCF^{Cdc4} substrates that have been verified using in vitro ubiquitination assays are designated with an asterisk (*).

pathway are ubiquitinated, including the receptor, G protein, RGS protein, and components of the MAP kinase cascade (154, 162, 179, 191-193, 196). Further, there is *indirect* evidence that SCF complexes promote ubiquitination of several proteins that propagate the signal, including Ste5 (192), Ste7 (193), Ste4 and Ste20 (Figure 2.9 A). 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 2.10). 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 (197), a protein that is upregulated in a Ste12-dependent manner during mating (198). 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 (179) and Sic1 (134, 135), SCF^{Grr1} promotes the degradation of Cln1 and Cln2 (199, 200).

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 (154). Whereas polyubiquitinated Gpa1 is targeted to the proteasome, monoubiquitinated Gpa1 is internalized and degraded within the vacuole (159). 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 (160). 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 (194). Recently, two independent phospho-proteomic screens revealed that Gpa1 is phosphorylated at Thr-189 and Ser-200 (201, 202), and it is noteworthy that both sites are located near a known Gpa1 ubiquitination site, Lys-165, established by mass spectrometry (158). In this study, we show that Gpa1 purified from *E. coli* and lacking

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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 (99, 203). 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 (204-206). 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 (65). 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* (207). 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 - Standard procedures for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout. Yeast *Saccharomyces cerevisiae* strains used in this study were BY4741 (*MAT**a** leu2Δ met15Δ his3-1 ura3Δ*), MTY235 (*MAT**a** ade2*-1 *his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*), MTY670 (MTY235 *cdc34-2*), and MTY668 (MTY235 *cdc4-1*) (provided by Mike Tyers, Samuel Lunenfield Research Institute) (152), BY4741-derived strains containing a C-terminal tandem affinity purification (TAP)-tag (Yeast TAP-Fusion Library, Open Biosystems), and the BY4741-derived strain R1158 (*MAT**a** URA3::CMV-tTA leu2Δ met15Δ his3-1 ura3Δ*) (208).   The tetracycline-repressible strains were purchased as the yeast Tet-promoter Hughes Collection (yTHC, Open Biosystems) (28).

      Yeast shuttle plasmids used were pRS315 (CEN, amp^R, *LEU2*), and pRS316 (CEN, amp^R, *URA3*). Expression plasmids described previously were pRS423-FUS1-lacZ (209), pRS316-ADH1, pRS316-ADH1-GPA1 (78), pFAGa-mRFP1-KanMX6 (210), pUG35 (provided by Johannes Hegemann, Heinrich-Heine-Universität), YCp50-STE11-4 (from George Sprague, University of Oregon) (190), and pRS315-GAL-STE4 (175). Plasmid pRS316-ADH-GPA1-Flag was constructed by PCR amplification of a 384 bp fragment of *GPA1* from pRS316-ADH-GPA1 using primers 1 and 2 (see Appendix I). Plasmid pRS316-ADH-GPA1$_Δ^{128-236}$-Flag was constructed by QuikChange (Stratagene)

using primers 3 and 4 to remove the 324 bp fragment corresponding to amino acids 128-236. Plasmid pRS315-STE11 was constructed by PCR amplification of *STE11* from BY4741 genomic DNA, followed by SacI and XmaI digestion and ligation into the corresponding sites of pRS315.   Plasmid pRS315-STE11-4 was constructed by engineering the single point mutation Thr-596-Ile (190) into pRS315-STE11 using QuikChange and primer 5 and its complement. Rescue plasmids for *CDC4*, *CDC34*, and *STT4* were constructed by PCR amplification of each gene from BY4741 genomic DNA, followed by SacI and XmaI digestion and ligation into the corresponding sites of pRS315. Rescue plasmids for *PIK1* and *MPS1* were made in a similar manner except that SacII was used in place of SacI.

The pRS316-ADH1-RFP/FUS1-GFP (AR/FG) dual reporter was constructed using the steps outlined below. The plasmid pRS316-ADH1-GFP was constructed by PCR amplification of *GFP* from the plasmid pUG35 using primers 6 and 7 including SacI sites, followed by SacI digestion and insertion into the corresponding site in pRS316-ADH1.   The *ADH1* terminator sequence ($ADH1_t$) (from the stop codon to 600bp downstream) was PCR amplified from genomic DNA with primers 8 and 9 including XmaI and SalI sites. The resulting PCR product was digested with XmaI and SalI and inserted into the corresponding sites in pRS316-ADH1-GFP, resulting in pRS316-$ADH1_p$-$ADH1_t$-GFP.   RFP was PCR amplified from pFA6a-mRFP1-KanMX6 using primers 10 and 11 with XmaI sites. This fragment was ligated into the corresponding XmaI sites of pRS316-$ADH1_p$-$ADH1_t$-GFP, resulting in the plasmid pRS316-$ADH1_p$-RFP-$ADH1_t$-GFP. The *FUS1* promoter (*FUS1p*) (600bp upstream of the start codon of *FUS1*) was PCR amplified from genomic DNA using primers 12 and 13 containing SalI sites. The resulting fragment was digested with SalI and inserted into the corresponding sites of pRS316-$ADH1_p$-RFP-$ADH1_t$-GFP resulting in the plasmid pRS316-$ADH1_p$-RFP-$ADH1_t$-FUS1-GFP (designated pRS316-AR/FG). pRS315-AR/FG was constructed by

digestion of pRS316-AR/FG with PvuI and ligation into the corresponding sites in pRS315.

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 (177). Prior to pheromone stimulation, cells were grown for ~15 hrs in either untreated medium or medium containing 10 ng/mL, or 100 ng/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 (10 μg/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 2.1).

Bioinformatics - Physical and genetic interactions among the genes identified in the screen were analyzed using Osprey Network Visualization System (181) which

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incorporates published data from the Biological General Repository for Interaction Datasets (BioGRID) (182). Functional categories were assigned based on Gene Ontology annotations using Functional Clustering in Osprey. Genes involved in multiple GO processes were assigned a single GO term based on Osprey's hierarchical GO process order. Hierarchical clustering of $TetO_7$-Strain phenotypes was conducted with the open source software Cluster 3.0 (211) using uncentered correlation and centroid linkage. The generated clustering data was visualized with the open source software Java TreeView (v1.1.3) (212).

Cell-extract preparation and immuno-blot analysis - The yeast $TetO_7$ strains were grown in selective medium to A_{600nm}~0.8 and re-inoculated at 1:80 into medium containing doxycycline at a final concentration of 10μg/ml and grown to A_{600nm}~0.8. Cell cultures were then divided in half, and either treated with 3 μM α factor pheromone or left untreated at 30°C for 30 min. Protein extracts were produced by glass bead lysis in trichloroacetic acid (TCA) as previously described (19). Protein extracts were resolved by 12% SDS-PAGE and immunoblotting with Phospho-p44/42 MAPK antibodies (9101L, Cell Signaling Technology) at 1:500, Fus3 antibodies (sc-6773, Santa Cruz Biotechnology, inc.) at 1:500, GFP antibodies (632375, BD Biosciences) at 1:500, dSRed antibodies (632496, Clontech) at 1:1000, and G6PDH antibodies (A9521, Sigma-Aldrich) at 1:100,000. Immunoreactive species were visualized by chemiluminescent detection (PerkinElmer Life Sciences LAS) of horseradish peroxidase-conjugated antibodies (170-5047 and 170-5046, Bio-Rad). Protein concentration was determined by Dc protein assay (Bio-Rad Laboratories). Where indicated, $TetO_7$ cells were transformed with pRS315-GAL-STE4, pRS315-STE11-4, pRS315-ADH-RFP/FUS1-GFP, or empty vector, and grown in selective medium containing 2% (w/v) dextrose or galactose to induce *STE4* expression.

Protein turnover measurements - Cells were grown in 100 mL 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, 10 μg/mL in 0.1% ethanol) for up to 3 hrs. 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 (213). Protein extracts were resolved by 10% SDS-PAGE and immunoblotting with Gpa1 antibodies at 1:1000 (76) and Sst2 antibodies at 1:2000 (214).

TAP-fusion protein turnover screen - TAP-fusion genes were PCR amplified and integrated into MTY235 and *cdc34-2* cells. Cells were grown at room temperature to A_{600nm} ~0.25, shifted to 37°C for 3h rs treated with 3 μM α factor for 1 hr, and treated with cycloheximide for up to 90 min. Protein extracts were resolved by 7.5% SDS-PAGE and immunoblotting with protein A (P3775, Sigma-Aldrich) antibodies at 1:50,000. Experiments were performed in triplicate, and bands were quantified by densitometry.

Preparation and purification of recombinant proteins - BY4741 yeast cells were transformed with pRS316-ADH1-GPA1-Flag and pRS316-ADH1-GPA1$^{\Delta128-236}$-Flag and grown to early log phase (A_{600nm} ~1.0) before harvesting by centrifugation. The cell pellet was frozen in liquid nitrogen and lysed by grinding cells blast-frozen in a 1:0.7 ratio of lysis buffer (50 mM Tris-HCl, pH 7.5, 400 mM KCl, 0.1% Triton, 0.2 mM dithiothreitol) supplemented with 20 μM GDP, 10 mM NaF, 10 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 proteinase inhibitor tablet per 50mL (11873580001, Roche Applied Science). The cell lysate was thawed on ice, and centrifuged at 15,000xg for 30 min at 4°C. The supernatant was transferred and incubated with EZview anti-Flag M2 beads (Sigma-Aldrich) for 2 hrs rotating at 4°C.

Beads were harvested by centrifugation and washed 3 times with 100x bead-bed volume of ubiquitination buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.2 mM dithiothreitol, 2 mM $MgCl_2$, 20 μM GDP) supplemented with 5% glycerol, followed by elution with 2x bead-bed volume of the supplemented ubiquitination buffer containing 0.25 mg/mL 3XFlag peptide (Sigma-Aldrich). Protein aliquots were frozen and stored at -80°C.

Yeast ^{His}Cdc4 E2 was purified from *E. coli*, yeast ^{His}Uba1 E1 was purified from yeast, and yeast SCF E3 Complexes were purified from insect cells infected with the baculoviruses expressing yeast Flag-Skp1, Cdc53, Myc-Rbx1, and HA-Cdc4 or HA-Met30 for 40 hrs as described previously (135). Cells were disrupted in NETN buffer (50 mM Tris-HCl, pH7.5, 150 mM KCl, 0.5% Nonidet P-40, 0.2 mM dithiothreitol, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride) supplemented with proteinase inhibitor tablets and cleared by centrifugation at 15,000xg for 30 min at 4°C. Typically 3 mL of NETN buffer was used per 0.5 x 10^8 cells. For immunopurification 300 μL of cell lysate was incubated with 10 μL EZview anti-Flag M2 beads with rotating for 1 hr at 4°C. Beads were washed 3 times with 500 μL of NETN buffer for 5 min each with rocking and 3x quick washes with 500 μL of ubiquitination buffer. Bound protein was eluted from the beads with 2x 10 μL of ubiquitination buffer supplemented 0.25 mg/mL 3XFlag peptide for 10 min each. Eluted protein was added directly to ubiquitination reactions.

6xHIS-Gpa1 expression plasmid was described previously (215) and transformed into BL21 (DE3) *E. coli*. Cells were grown from a single colony overnight at 37°C in Luria Broth (LB) supplemented with 100 μg/mL carbenicillin and then diluted 1:100 into fresh media. Once cells grew to A_{600nm} ~0.7, 6xHIS-Gpa1 expression was induced by addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside and incubation at room temperature for 5 hrs with shaking. Cells were harvested by centrifugation, resuspended

in Buffer A (20 mM Tris pH 8.0, 200 mM NaCl, 5% glycerol, 20 μM GDP, 2 mM MgCl$_2$, 1 mM DTT) supplemented with protease inhibitor tablets (Roche), and homogenized with an Emulsiflex-C5 Homogenizer (Avestin). Lysates were clarified by centrifugation at 12,000xg for 30 min, and the resulting supernatent was mixed with Buffer A-equilibrated Ni-Sepharose^{TM}6 Fast Flow resin (GE Healthcare) for 2 hrs rotating at 4°C. Resin was collected by centrifugation at 500xg for 5 min and washed 3 times with Buffer A followed by elution with Buffer A supplemented with 250 mM imidazole. The elution was mixed with His-tagged tobacco etch virus protease (to remove the N-terminal 6xHIS from Gpa1) and dializyed in 1 L of Buffer B (20 mM Tris, pH 8.0, 100 mM NaCl, 5% glycerol, 20 μM GDP, 2 mM MgCl$_2$, 1 mM DTT) overnight. Sample was incubated with Ni-Sepharose resin for 1hr to remove tobacco etch virus protease and cleavage products. Flow-through from the Ni-Sepharose was concentrated using Vivaspin concentrators (Vivascience AG).

Co-immunoprecipitation assay - Insect cell lysates containing GST-Skp1 and either Cdc4-HA or Met30-HA where mixed with Glutathione Sepharose^{TM} 4 Fast Flow resin (GE Healthcare) for 1hr rotating at 4°C. The beads were then washed 3x with 50x bead-bed volume of binding buffer (50 mM Tris-HCl, pH 7.5, 15 0mM KCl, 0.5% NP-40, 0.2 mM dithiothreitol, 2 mM MgCl$_2$, 20 μM GDP, 10 mM NaF, 10 mM β-glycerol phosphate, 1 mM sodium orthovanadate, and proteinase inhibitor tablets). Gpa1-Flag purified from yeast was added to the beads and incubated for 2 hrs at 4°C with rotating. The beads were washed 3x with 50x bead-bed volume of binding buffer for 5min each with rocking and 3x quick washes with 50x bead-volume of binding buffer. Bound protein was then eluted in 2x bead-bed volume of binding buffer supplemented with 20 mM glutathione. Protein samples were resolved by 10% SDS-Page and immunoblotting with Gpa1 antibodies at 1:1000 and HA antibodies (3F10, Roche Applied Sciences) at 1:2000.

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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 (135). 9 μL 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 15 μL. 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).

CHAPTER III

Selective Regulation of Mitogen-Activated Protein Kinase Signaling By an Endomembrane Phosphatidylinositol 4-Kinase

All figures contributed by Steven D. Cappell

Summary

Multiple mitogen-activated protein (MAP) kinase pathways share components yet initiate distinct biological processes. Signaling fidelity can be maintained by scaffold proteins and restriction of signaling complexes to discreet subcellular locations. For example, the yeast MAP kinase scaffold Ste5 binds to phospholipids produced at the plasma membrane and promotes selective MAP kinase activation. Here we show that Pik1, a phosphatidylinositol (PtdIns) 4-kinase that localizes primarily to the Golgi, also regulates MAP kinase specificity, but does so independently of Ste5. Pik1 is required for full activation of the MAP kinases Fus3 and Hog1 and represses activation of Kss1. Further, we show by genetic epistasis analysis that Pik1 likely regulates Ste11 and Ste50, components shared by all three MAP kinase pathways. These findings reveal a new regulator of signaling specificity functioning at endomembranes rather than at the plasma membrane.

Introduction

Cells growing in complex environments are exposed to multiple chemical and physical stimuli. Many external stimuli activate mitogen-activated protein (MAP) kinase pathways to elicit intracellular biological processes. In some cases, a single stimulus will activate multiple MAP kinases, yet signaling specificity is maintained (216). How cells regulate the activation of different MAP kinase pathways to invoke the appropriate biological response is not well understood.

The yeast *Saccharomyces cerevisiae* provides a versatile model for understanding the coordinated regulation of multiple MAP kinases. In yeast, three well-characterized MAP kinase pathways respond to different external stimuli to initiate distinct, and sometimes mutually exclusive, biological processes (Figure 3.1 A) (217). First, mating pheromones activate a pathway that induces cell cycle arrest, polarized cell expansion, and the fusion of haploid **a**- and α-type cells to form an **a**/α diploid. This process is mediated by a heterotrimeric G protein and a protein kinase cascade comprised of Ste20, Ste11, Ste7, and the MAP kinase Fus3 (178). Second, nutrient deprivation results in the activation of the same kinase components, with the exception of the MAP kinase, Kss1 (218, 219), and induces filamentous growth as well as increased adherence and invasion into the substratum (220). Third, osmotic stress activates the MAP kinase Hog1 in the high osmolarity glycerol (HOG) response pathway (221), and induces glycerol production to counterbalance osmotic pressure and enable cell survival (222, 223).

Despite profound differences in stimulus and response, different MAP kinase pathways will often share signaling components. For example, the MAP kinase kinase kinase Ste11 and its adapter protein Ste50 are shared by the mating, filamentous growth, and HOG pathways (224-226). Ste7 is shared by the mating and filamentous

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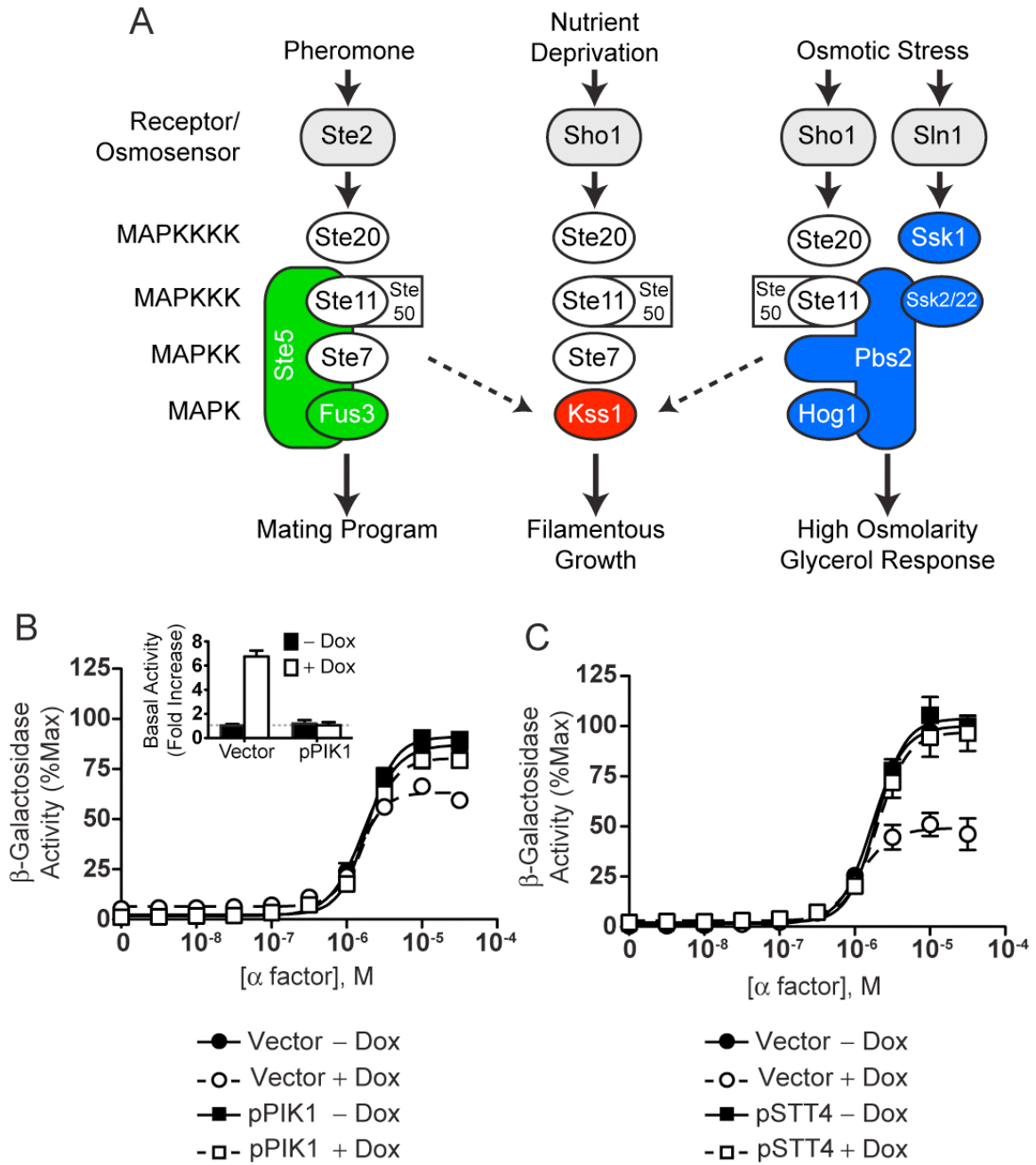
Figure 3.1

Figure 3.1 Pik1 is required for proper pheromone signaling.

(A) Three yeast MAP kinase pathways respond to different stimuli and regulate distinct biological processes, yet all three pathways share signaling components. The MAP kinase kinase kinase Ste11 and the adapter Ste50 regulate all three pathways. (B) $TetO_7$-PIK1 cells were transformed with the *FUS1-lacZ* transcription reporter and pRS315-PIK1 (pPIK1) or pRS315 (Vector) and treated with a factor pheromone at the indicated concentration for 90 min. β-galactosidase activity was measured spectrofluorometrically. Inset is activity in cells not stimulated with a factor. (C) $TetO_7$-STT4 cells were transformed with the *FUS1-lacZ* reporter and pRS315-STT4 (pSTT4) or empty vector (Vector) and treated with a factor pheromone. Results are the mean ± S.E. for three individual experiments each performed in triplicate.

pathways (217). Yet remarkably little pathway cross-talk is observed. In the pheromone response pathway, MAP kinase specificity is maintained by the scaffold protein Ste5, which binds Ste11, Ste7, and Fus3 (227). Upon pheromone stimulation, Ste5 translocates to the plasma membrane by binding to G protein $\beta\gamma$ subunits (228) and facilitates signal propagation through Fus3, but not Kss1 or Hog1 (229). In the HOG pathway, specificity is maintained by another scaffold protein Pbs2, which also functions as the kinase that activates Hog1. However, despite the existence of these scaffolds, Kss1 is still partially activated in response to pheromone (230, 231) and osmotic stress (232). Therefore, scaffolds that associate with Fus3 or Hog1 are not sufficient to prevent activation of Kss1. Additional mechanisms are likely to be required to maintain proper balance between Fus3, Hog1, and Kss1 activation.

Previous reports demonstrated a role for phospholipids in maintaining signaling fidelity. For example, deletion of the PtdIns 3-kinase Vps34, alters Fus3 activation but not Kss1 (65), indicating that phosphorylated phosphoinositides may play an important role in maintaining MAP kinase specificity. Ste5 is likewise required for Fus3 activation; Ste5 binds PtdIns 4-P and PtdIns 4,5-P$_2$ *in vitro* (207, 233) and Ste5 translocation to the plasma membrane *in vivo* requires the PtdIns 4-kinase Stt4 and the PtdIns 4-P 5-kinase Mss4 (75, 233). While both Stt4 and Mss4 localize primarily to the plasma membrane (188, 234), Vps34 localizes to endosomes (235, 236). Together these findings show that phospholipids play an important role in maintaining MAP kinase specificity, and these phospholipids do not necessarily function at the plasma membrane.

In a recent screen of essential genes we identified regulators of pheromone signaling including Stt4, as well as a second PtdIns 4-kinase Pik1 (161). As noted above Stt4 promotes the activation of Fus3. Here we show that Pik1 regulates the activity of three different MAP kinases in yeast. Whereas Pik1 enhances Fus3 and Hog1 activation, it inhibits Kss1 activation. We demonstrate further that Pik1 regulates MAP

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kinase signaling through a mechanism distinct from that of Stt4. Whereas Stt4 acts by promoting Ste5 translocation to the plasma membrane (75), Pik1 exerts its effects through Ste11 and the adapter protein Ste50. These findings reveal a novel role for PtdIns 4-P at endomembranes in maintaining specificity across multiple MAP kinase pathways.

Results

Pik1 is required for proper pheromone signaling

In a recent screen to identify essential genes required for proper pheromone signaling, we identified two PtdIns 4-kinases, Stt4 and Pik1. While Stt4 and Pik1 have the same enzymatic activity, they are both essential, suggesting they have non-redundant functions *in vivo* (237). Furthermore, Stt4 and Pik1 localize to different parts of the cell: Stt4 at the plasma membrane (188), and Pik1 at the Golgi and nucleus (189). Given that depleting the cell of *either STT4* or *PIK1* diminishs pheromone signaling (161), we reasoned they might likewise have non-redundant functions in the pheromone response pathway. Stt4 has a known role promoting Ste5 translocation to the plasma membrane (75). Here we investigate the signaling properties of Pik1.

Previous research on Pik1 and Stt4 was conducted using temperature-sensitive (*ts*) alleles. The use of *ts* alleles requires growth at suboptimal temperatures and introduces destabilizing mutations that could alter enzyme function or protein-protein interactions. Growth at high temperatures can impair MAP kinase activity independent of any gene mutations. For example Garrenton *et al*. reported a ~50% reduction in Fus3 activation in wild-type cells grown at 37°C vs. 26°C, (see Figure 5 in (75)). Thus the use of higher growth temperatures could obscure small differences resulting from partial loss of function *ts* alleles.

To best determine the contribution of Pik1 in pheromone signaling, and to verify a

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role for Stt4, we used strains where the native promoter was replaced with a doxycycline-repressible (TetO$_7$) promoter. Cells were grown in the presence or absence of doxycycline to repress gene expression. Pathway activation was measured using a highly specific pheromone-inducible promoter (from *FUS1*) fused to the β-galactosidase gene. As shown in Figure 3.1, knockdown expression of TetO$_7$-PIK1 or TetO$_7$-STT4 results in dampened transcriptional output upon pheromone stimulation. Furthermore, knockdown of *PIK1* results in constitutive activation in the absence of pheromone (161). Thus, knockdown of *PIK1* paradoxically yields both a dampened maximum response and increased basal activity. To confirm the integrity of these strains, we expressed single-copy plasmids containing the wild-type gene in the corresponding TetO$_7$ strain. For both *PIK1* (Figure 3.1 B) and *STT4* (Figure 3.1 C), introduction of the absent gene restored normal pheromone responses.

To determine if depletion of *PIK1* results in a subsequent loss of intracellular PtdIns 4-P, we visualized PtdIns 4-P *in vivo* using three well-characterized GFP-tagged biosensors. First, the pleckstrin homology (PH) domain from phospholipase Cδ (PH$^{PLC_\delta}$-GFP) binds specifically to PtdIns 4,5-P$_2$ at the plasma membrane and has been used to monitor plasma membrane pools of both PtdIns 4-P and PtdIns 4,5-P$_2$ (238). Second, the PH domain from FAPP1 (PH^{FAPP1}-GFP) binds specifically to PtdIns 4-P at the Golgi (238). Third, the C2 (conserved region-2) domain from bovine lactadherin (C2^{lact}-GFP) binds phosphatidylserine (PS), an abundant component of all membranes (239) and serves as a reference control. Knockdown of *PIK1* resulted in a partial loss of Golgi staining of PH^{FAPP1}-GFP but no change in localization of PH$^{PLC_\delta}$-GFP or C2^{lact}-GFP (Figure 3.2 A). In contrast, knockdown of *STT4* resulted in a partial loss of plasma membrane staining of PH$^{PLC_\delta}$-GFP but no change in localization of PH^{FAPP1}-GFP or C2^{lact}-GFP (Figure 3.2 B) (188). Thus, partial knockdown of either *PIK1* or *STT4* is sufficient to

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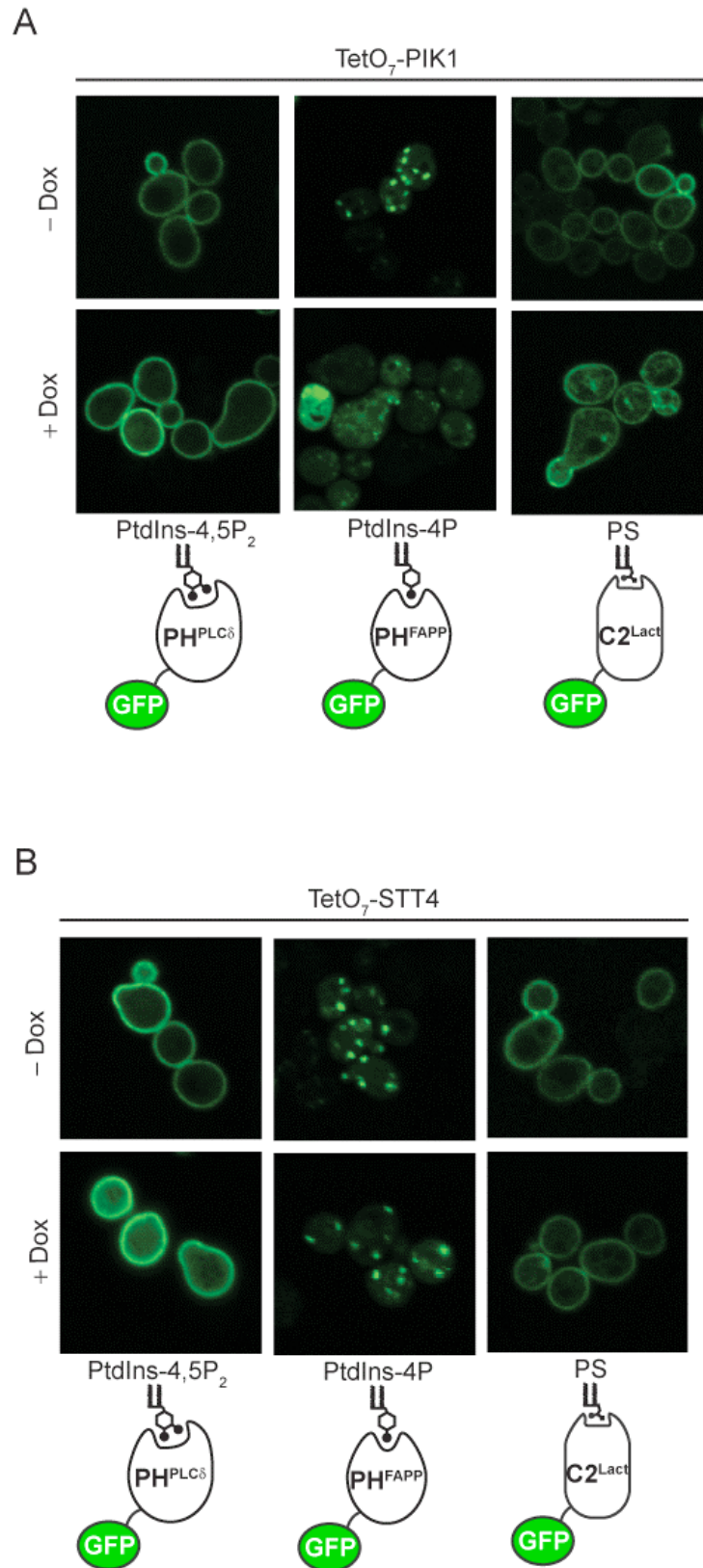
Figure 3.2 Knockdown of *PIK1* or *STT4* results in loss of PtdIns 4-P *in vivo*.
GFP fluorescence of cells expressing a single copy plasmid pRS316 containing GFP fusion proteins of PH$^{PLC_\delta}$, PH^{FAPP1}, or C2^{Lact} and treated with 10 μg/mL doxycycline (Dox) for 15 hr, as indicated. (A) TetO$_7$-PIK1. (B) TetO$_7$-STT4.

observe a dampened pheromone response, underscoring the importance of PtdIns 4-P in maintaining proper pheromone signaling.

Loss of Pik1 induces elongated growth

Cells exposed to pheromone undergo cell cycle arrest and form mating projections in preparation for mating ("shmoo" morphology). At low doses of pheromone, cells continue to divide in a bipolar fashion and elongate along pheromone gradients in the direction of a potential mating partner (chemotropic growth) (240-243). When elongated cells encounter a sufficiently high level of pheromone, they undergo cell cycle arrest and form shmoos. Since knockdown of *PIK1* results in constitutive induction of pheromone-responsive genes (see Figure 3.1 B and inset), we considered whether these cells also exhibit an altered morphology. DIC microscopy revealed that *PIK1* knockdown results in large and elongated cells, even in the absence of pheromone (Figure 3.3 A). The observed cellular elongation was most similar to that of cells exposed to low doses of pheromone (240-243) and is consistent with our observation that *PIK1* knockdown results in a small, but significant, increase in basal activation of the pheromone pathway. Conversely, knockdown of *STT4* has no effect on cell morphology, suggesting further that Pik1 and Stt4 regulate signaling in fundamentally different ways.

Pik1 regulates pheromone signaling independently of Ste5

Prior to pheromone stimulation, the MAP kinase scaffold Ste5 is localized diffusely in the nucleus and cytoplasm. After pheromone stimulation, Ste5 translocates to the plasma membrane. In an *stt4^{ts}* strain, however, Ste5 is no longer localized to the plasma membrane, most likely due to diminished synthesis of PtdIns 4-P or PtdIns 4,5-P_2 (75, 228, 233). Accordingly, Stt4 (like Ste5) is required for full activation of Fus3. Pik1 also generates PtdIns 4-P and is required for Fus3 activity, yet Pik1 is absent from the plasma membrane. Thus we investigated whether Pik1 affects Ste5 localization in

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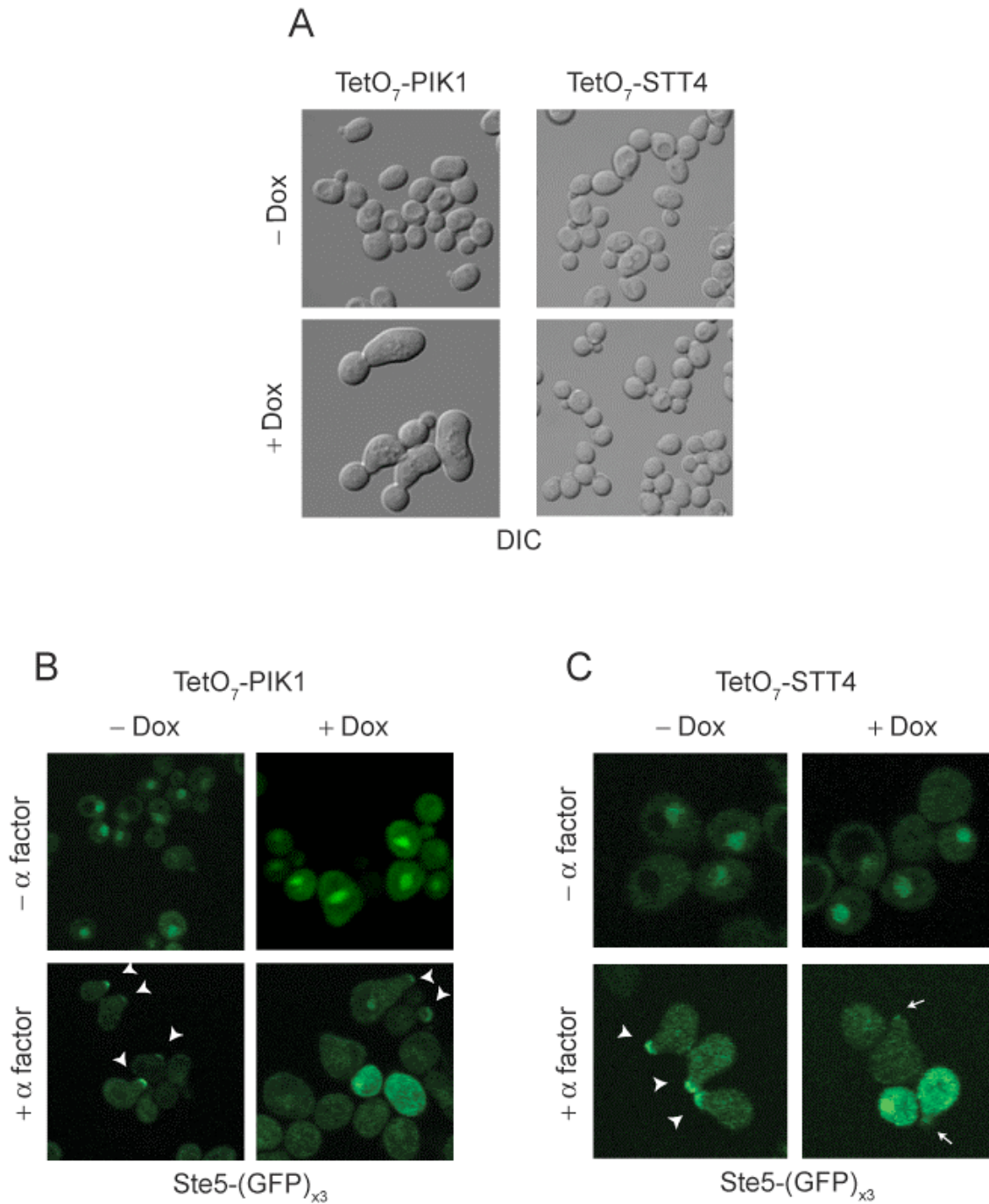
Figure 3.3 Pik1 does not alter Ste5 localization.

(A) DIC image of TetO$_7$-PIK1 and TetO$_7$-STT4 cells treated with 10 μg/mL doxycycline for 90 min where indicated (+ Dox). (B) GFP fluorescence of TetO$_7$-PIK1 cells expressing pRS316-Ste5-(GFP)$_{x3}$. Cells were treated with doxycycline for 90 min and 3 μM α factor pheromone for 90 min. Arrow heads indicate Ste5-GFP localized to shmoo tips. Arrows indicate absence of Ste5-GFP at shmoo tips. (C) TetO$_7$-STT4 cells treated as in (B).

some other way. To this end we expressed *STE5-(GFP)*$_{x3}$ in a TetO$_7$-PIK1 strain and treated cells with and without α factor pheromone. Consistent with previous data from *ts* strains, knock down of *PIK1* had no effect on Ste5-GFP localization (Figure 3.3 B) while knock down of *STT4* resulted in a marked loss of Ste5-GFP from the shmoo tip (Figure 3.3 C). These results indicate that Ste5 translocation to the plasma membrane requires Stt4 but is unaffected by Pik1.

In addition to binding phospholipids, Ste5 binds several signaling components including G$\beta\gamma$ (228) and all three kinases in the MAP kinase cascade: Ste11, Ste7, and Fus3 (176, 187). While available evidence indicates that Stt4 helps to recruit this scaffolded complex to the plasma membrane, the differences between Stt4 and Pik1 suggest that Pik1 might activate the complex without contributing to Ste5-membrane association. To test this model, we tethered Ste5 to the plasma membrane using a C-terminal transmembrane (CTM) fusion protein and expressed it under the control of a galactose-inducible promoter. Ste5-CTM results in constitutive association of the MAP kinase cascade with upstream activators, and therefore results in constitutive activation of Fus3. Ste5-CTM is localized only at the plasma membrane and therefore should not interact with Pik1 or PtdIns 4-P at the Golgi or nucleus (228). We then monitored Fus3 activation, using an antibody that recognizes the dually phosphorylated and fully activated form of the kinase. As shown in Figure 3.4, Ste5-CTM strongly activates Fus3 (228). As expected, Fus3 activity was largely unaffected by the loss of *STT4* (Figure 3.4 A). In contrast, this response was substantially diminished by the loss of *PIK1* (Figure 3.4 B). Thus Stt4 and Pik1 regulate MAP kinase signaling by distinct mechanisms. Whereas expression of Ste5-CTM bypasses a need for PtdIns 4-P at the plasma membrane, these cells remain sensitive to changes in PtdIns 4-P by Pik1 within the cell.

Figure 3.4
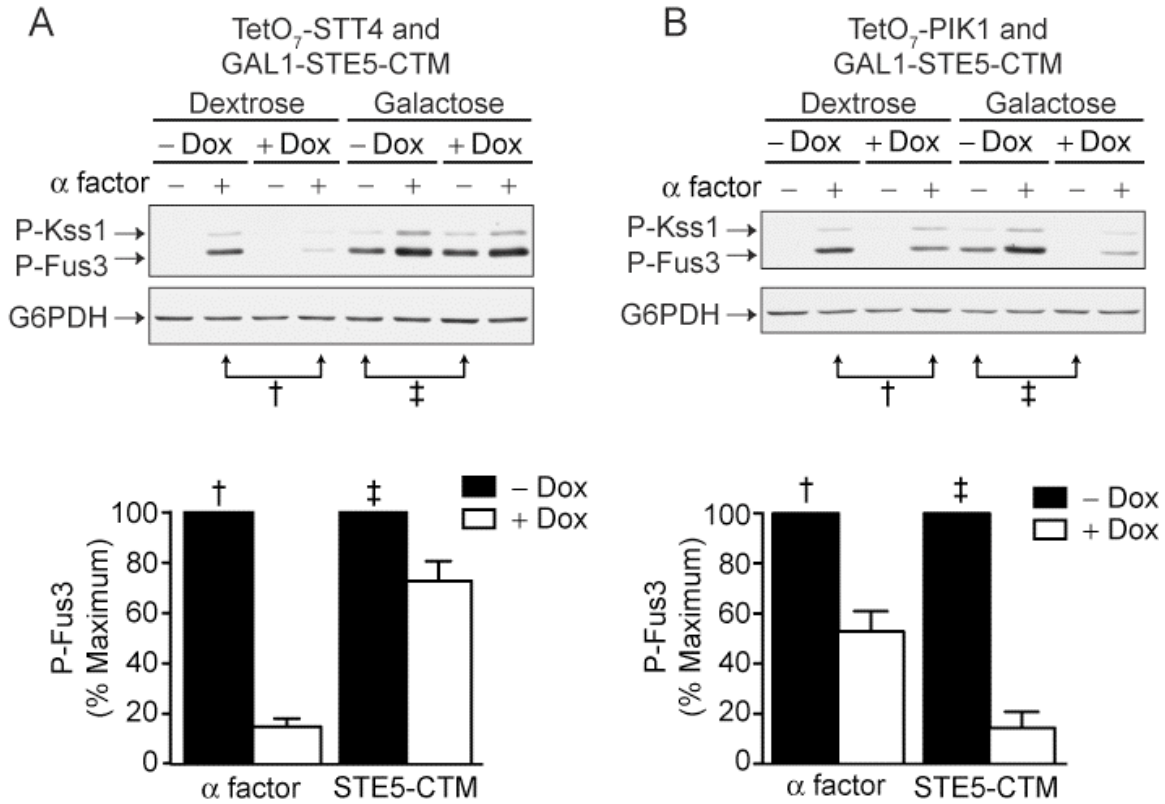
Figure 3.4 Pik1 regulates pheromone signaling independently of Ste5.
(A) TetO$_7$-STT4 cells were transformed with pRS313-GAL1-STE5-CTM and grown in selective medium containing dextrose or galactose to induce STE5-CTM expression. Cells were treated with 10 μg/mL doxycycline for 90 min and 3 μM α factor pheromone for 30 min, as indicated. Cell lysates were resolved by 12.5% SDS-PAGE and immunoblotting with phospho-p44/42 antibodies (P-Kss1, P-Fus3) and G6PDH antibodies as a loading control. Phosphorylated and activated Fus3 (P-Fus3) was quantified by scanning densitometry and analyzed with ImageJ software. Results were normalized to P-Fus3 levels of untreated samples. Bar graphs represent quantification of the indicated bands. Results are the mean ± S.E. (n=3). (B)TetO$_7$-PIK1 cells treated as in (A). Results are the mean ± S.E. (n=3).

Pik1 is required for full Fus3 activation and inhibits Kss1 activation

Although necessary for Fus3 signaling, Ste5 actually slows the rate of Fus3 activation (244). A second MAP kinase Kss1 is also activated by pheromone, but is not scaffolded and is activated comparatively quickly (240). To determine if *STT4* or *PIK1* affects the kinetics of activation, we monitored Fus3 and Kss1 at multiple time points following pheromone treatment. While knockdown in either case reduced the magnitude of Fus3 phosphorylation, the dynamics of activation were largely unchanged: Fus3 activation remained slow while Kss1 activation remained fast (Figure 3.5 A and 3.5 B). There were however notable differences in the behavior of Kss1. In contrast to *STT4*, loss of *PIK1* resulted in marked elevation of Kss1 activity, particularly in the absence of pheromone stimulation (Figure 3.5 A). The 22-51% increase in Kss1 activation is particularly striking when compared to the 28-61% reduction in Fus3 activation. Considering that Kss1 induces chemotropic growth at low doses of pheromone, these data are consistent with the observed elongated growth upon *PIK1* knockdown (Figure 3.3 A). Treatment of cells harboring the $TetO_7$ promoter attached to a non-expressible genetic element ($TetO_7$-WT) with doxycycline had no effect on activation of Fus3 or Kss1, indicating that doxycycline alone has no effect on pathway activation (Figure 3.6 A).

As an additional control we monitored MAP kinase activity in cells lacking Vps34, a PtdIns 3-kinase required for full activation of Fus3 (65) (Figure 3.5 C). Again the dynamics of activation were largely unaltered (Figure 3.5 C), even as overall Fus3 activity was diminished by 29-66%. The reduction we observed here is comparable to that reported by Slessareva *et al*. (65) but somewhat greater than the ~20% difference reported by Garrenton *et al.* (75). These data reveal that Pik1 differentially regulates MAP kinase activation and is required to maintain MAP kinase specificity. Whereas Pik1

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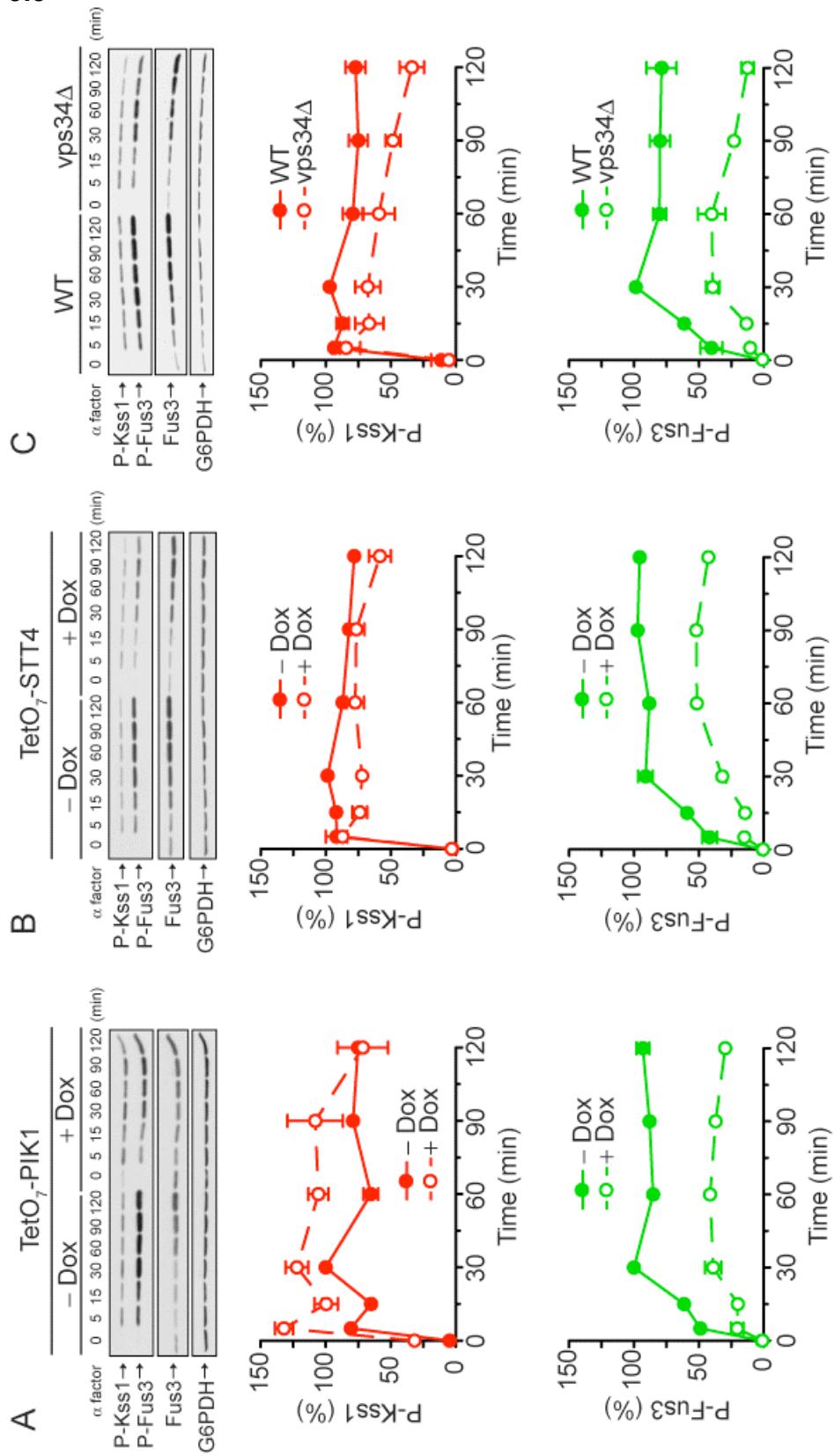
Figure 3.5   Pik1 is required for full Fus3 activation and represses basal Kss1 activation.

(A) TetO$_7$-PIK1 cells were treated with 10 μg/mL doxycycline for 90 min and 3 μM α factor pheromone for the times indicated. Cell lysates were resolved by 12.5% SDS-PAGE and immunoblotting with phospho-p44/42 antibodies (P-Fus3 and P-Kss1), Fus3 antibodies, or G6PDH antibodies as a loading control. Note that pheromone stimulation induces *FUS3* but not *KSS1* expression. Bands were quantified by scanning densitometry and analyzed with ImageJ software. Results are the mean ± S.E. (n=3). (B) TetO$_7$-STT4 cells treated as in (A).   (C) BY4741 (WT) and isogenic *vps34Δ* cells treated as in (A) except that no doxycycline was added to the cultures.

Figure 3.6
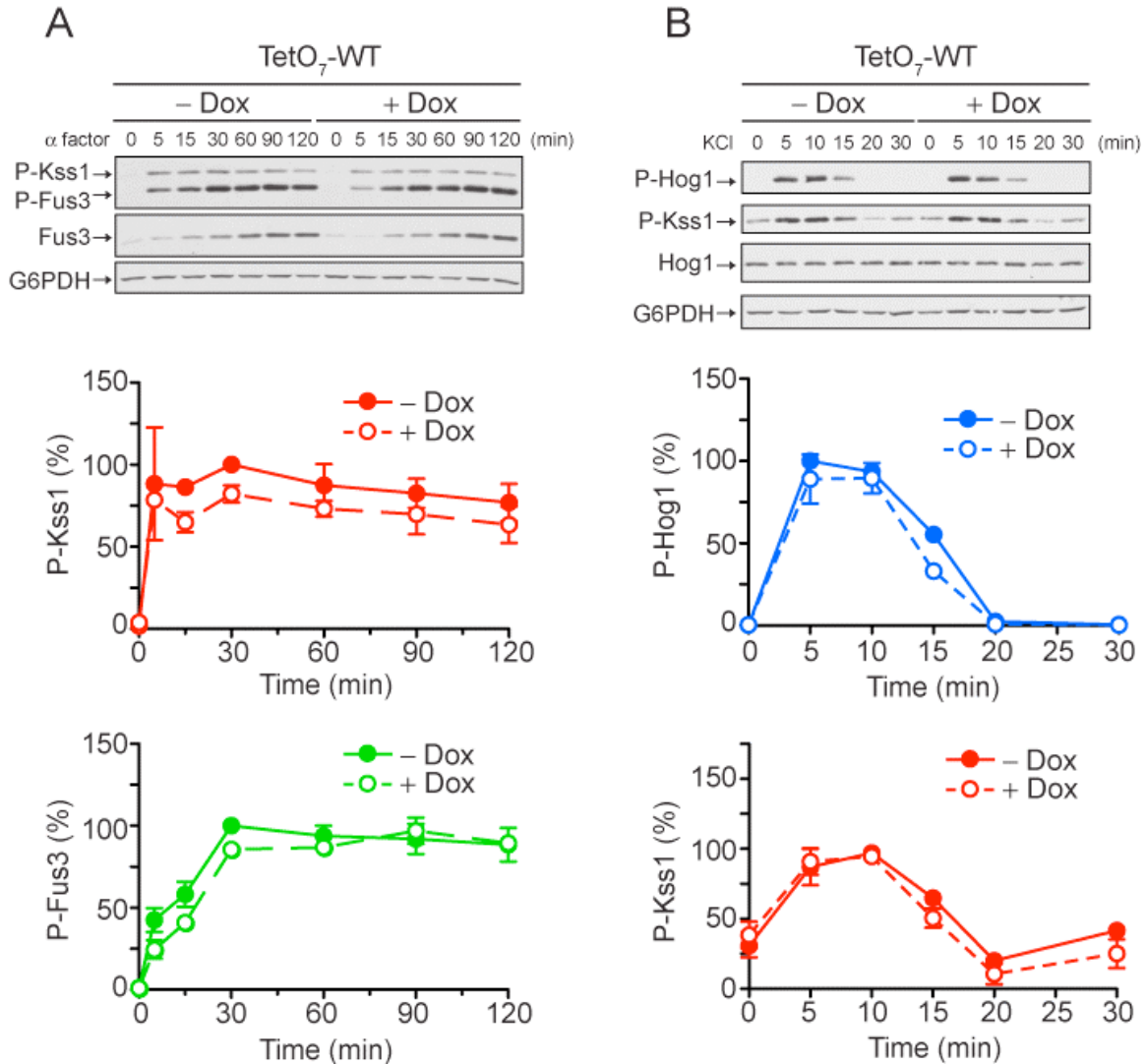
Figure 3.6 Doxycycline treatment does not affect Fus3, Kss1, or Hog1 activation.
(A) Cells harboring a doxycycline-repressible promoter attached to a non-expressible genetic element (TetO$_7$-WT) were treated with 10 μg/ml doxycycline (Dox) for 15 hr and 3 μM α factor pheromone for the times indicated. Cell lysates were resolved by 12.5% SDS-PAGE and immunoblotting with phospho-p44/42 antibodies, which recognize the dually phosphorylated and activated form of Fus3 (P-Fus3) and Kss1 (P-Kss1), Fus3 antibodies, or glucose-6-phosphate dehydrogenase (G6PDH) antibodies as a loading control. Note that pheromone stimulation induces *FUS3* but not *KSS1* expression. Bands were quantified by scanning densitometry and analyzed with ImageJ software. Results are the mean ± S.E. (n=3). (B), as in (A), but cells were treated plus and minus 0.5 M KCl for the times indicated instead of pheromone. Immunoblots were analyzed with phospho-p38 (P-Hog1) antibodies, phospho-p44/42 (P-Kss1) antibodies, Hog1 antibodies, or G6PDH antibodies as a loading control.

is required for stimulus-dependent activation of Fus3, it is also required to limit the activation of Kss1.

Pik1 functions at the level of Ste11

Given that Pik1 activates Fus3 while inhibiting Kss1, we hypothesized that Pik1 must regulate a pathway component upstream of both kinases. In order to better define which component is targeted by Pik1, we took a genetic epistasis approach. Using constitutively active mutants we stimulated the pathway at multiple points, bypassing the need for pheromone and the pheromone receptor. First we overexpressed the G protein βγ subunits (*STE4*$^{G_\beta}$ expressed using a galactose-inducible promoter). Since Gpa1 cannot sequester excess Gβγ (63, 64, 245), the overproduced Ste4$^{G_\beta}$ is free to activate effectors even in the absence of any stimulus. As shown in Figure 3.7, knockdown of *PIK1* dampened Gβγ-mediated activation of Fus3 (Figure 3.7 A and 3.7 C). Next, we overexpressed the constitutively active *STE11-4* mutant (190). *STE11* encodes the kinase that phosphorylates Ste7, which in turn phosphorylates and activates Fus3 and Kss1. Once again, knockdown of *PIK1* dampened *STE11-4*-mediated activation of Fus3 (Figure 3.7 B and 3.7 C). Knockdown of *STT4* likewise dampened *STE11-4*-mediated activation of Fus3 (Figure 3.7 E and 3.7 F). Thus Pik1 promotes signaling by pheromone, the G protein, the kinase scaffold, and the protein kinase Ste11.

Pik1 regulates HOG pathway

The data presented above indicate that Pik1 regulates the pheromone pathway, and that Pik1 acts on, or downstream of, Ste11. We have largely excluded Ste5 as a target for Pik1 regulation, leaving three likely targets: Ste11, its binding-partner Ste50 or its direct substrate Ste7 (Figure 3.1 A). To further distinguish between these candidate targets, we examined Pik1 regulation of the HOG pathway (224-226). The pheromone and HOG pathways share the use of Ste11 and Ste50, but not Ste7. We measured

Figure 3.7
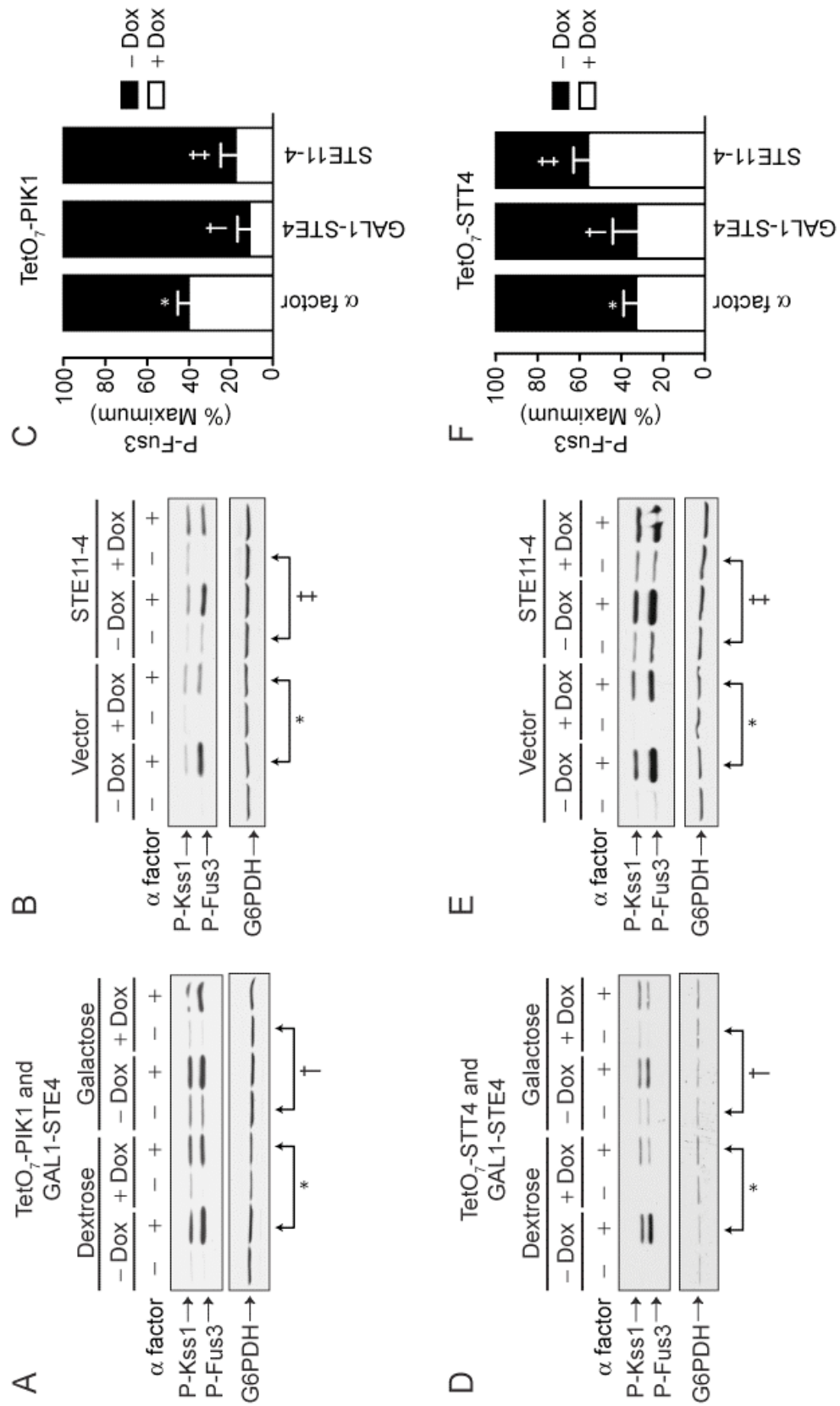
Figure 3.7 Pik1 acts on Ste11 or a pathway component downstream of Ste11.
(A) TetO$_7$-PIK1 cells were transformed with pRS315-GAL1-STE4 and grown in selective medium containing dextrose or galactose to induce Ste4 (Gβ) protein expression. Cells were treated with 10 μg/mL doxycycline for 90 min and 3 μM α factor pheromone for 30 min, as indicated. Cell lysates were resolved by 12.5% SDS-PAGE and immunoblotting with phospho-p44/42 antibodies (P-Kss1, P-Fus3) and G6PDH antibodies as a loading control. (B) TetO$_7$-PIK1 cells were transformed with pRS425 (Vector) or pRS425-STE11-4. (C) P-Fus3 levels from (A) and (B) were quantified by scanning densitometry and analyzed with ImageJ software. Results are the mean ± S.E. (n=3). (D) TetO$_7$-STT4 cells treated as in (A). (E) TetO$_7$-STT4 cells treated as in (B). (F) P-Fus3 levels from (D) and (E) were quantified as in (C).

activation of Hog1 and Kss1 in response to the addition of 0.5 M KCl. As shown in Figure 3.8, knockdown of *PIK1* resulted in diminished Hog1 activity (Figure 3.8 A). Furthermore, we again observed constitutive activation of Kss1 as well as an overall increase in Kss1 activation in response to salt stress.

Recent reports indicate that Kss1 activates a Hog1-specific phosphatase Ptp2 (246). To determine whether high basal activation of Kss1 was in any way responsible for the diminished Hog1 response, we deleted *KSS1* from the TetO$_7$-PIK1 strain. As shown in Figure 3.8, the loss of *KSS1* did not affect the ability of Pik1 to regulate activation of either Fus3 (Figure 3.8 D) or Hog1 (Figure 3.8 C). Finally, the available data indicate that Pik1 and Stt4 act in different ways to promote Fus3 signaling; Pik1 acts via Ste11 while Stt4 acts via Ste5. However Pik1 also acts to limit Kss1 signaling. As a further test of our model, we monitored Kss1 activity in the absence of Ste11 and Pik1 (TetO$_7$-PIK1 *ste11Δ* strain). In accordance with the model, we found that Ste11 is necessary for the constitutive activation of Kss1 (Figure 3.8 D) while deletion of *STE5* had no effect. These data further confirm that Pik1 regulates the function of Ste11 but not Ste5.

Discussion

Signal transduction systems will often share core signaling components yet maintain specificity and avoid pathway cross-talk. In yeast, three proteins have been found to preferentially regulate Fus3 and not Kss1. First, the scaffold Ste5 binds Fus3 and is required for Fus3 catalytic activity. Ste5 is not required by other MAP kinases and thus helps to differentiate pheromone signaling from other signaling systems. Second, the PtdIns 4-kinse Stt4 promotes activation of Fus3 (but not Kss1), and does so by helping to recruit Ste5 to the plasma membrane. Third, the PtdIns 3-kinase Vps34 promotes activation of Fus3 in preference to Kss1. While functionally similar to Stt4 (see

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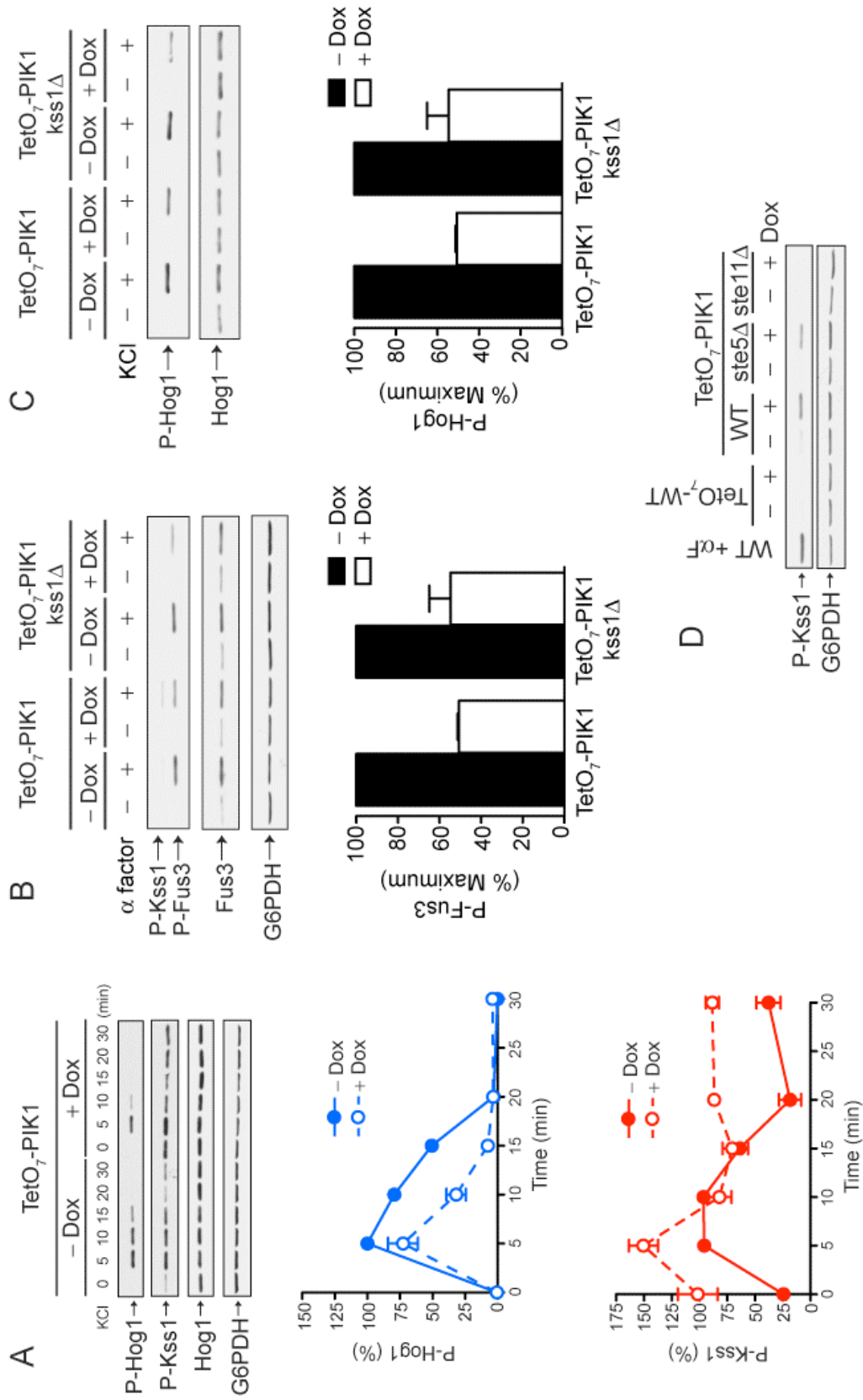
Figure 3.8 Pik1 acts via Ste11.

(A) TetO$_7$-PIK1 cells were treated with doxycycline for 90 min and 0.5 M KCl for the times indicated and analyzed by immunoblotting with phospho-p38 (P-Hog1) antibodies, phospho-p44/42 (P-Kss1) antibodies, Hog1 antibodies, or G6PDH antibodies as a loading control. (B) TetO$_7$-PIK1 and TetO$_7$-PIK1 *kss1Δ* cells were treated with doxycycline and 3 μM α factor pheromone for 30 min, as indicated. Immunoblots were analyzed with phospho-p44/42, Fus3 and G6PDH antibodies. (C) TetO$_7$-PIK1 and TetO$_7$-PIK1 *kss1Δ* cells were treated with doxycycline and 0.5 M KCl for 10 min. Immunoblots were analyzed with phospho-p38 and G6PDH antibodies. (D) Wild-type, TetO$_7$-PIK1 and isogenic cells carrying *ste5Δ* or *ste11Δ* mutations were treated with doxycycline and α factor (αF) for 30 min and analyzed with phospho-p44/42 antibodies (P-Kss1). All bands were quantified by scanning densitometry and analyzed with ImageJ software. Results are the mean ± S.E. (n=3).

Figure 3.5), Vps34 is expressed at endosomes rather than at the plasma membrane. Here we have investigated the function of another PtdIns 4-kinase Pik1. Like Stt4, Pik1 generates PtdIns 4-P and selectively regulates MAP kinase activity. Like Vps34, Pik1 is an endomembrane protein. Thus Pik1 joins a small but growing number of factors that promote MAP kinase signaling specificity. Unlike any of the previously characterized regulators however, Pik1 activates two MAP kinases (Fus3 and Hog1) while simultaneously inhibiting a third, competing MAP kinase (Kss1).

While much has been learned, important questions remain for the future. For instance, is the location of Pik1 at endomembranes related to its unique function in signaling? Activation of intracellular pathways usually requires the assembly of signaling components at the plasma membrane. In pheromone signaling, several mechanisms are required to recruit components to activated transmembrane receptors. The heterotrimeric G-proteins subunits Gα and Gγ (79, 247) and the small G-protein Cdc42 (248) are covalently modified with lipid moieties that anchor them to the plasma membrane. The scaffold Ste5 translocates from the cytoplasm to the plasma membrane by binding to the G$\beta\gamma$ dimer (228) as well as to Stt4-derived PtdIns 4-P and PtdIns 4,5-P$_2$ (207, 233). Additionally, the PAK-family kinase Ste20, as well as the closely related kinase Cla4, translocates to the plasma membrane by binding both PtdIns 4,5-P$_2$ and Cdc42 (249, 250). Therefore, spatial restriction of signaling components to areas near activated receptors helps prevent aberrant activation of parallel pathways.

Another question is whether Pik1 acts by altering the distribution of some signaling protein within the cell. Our epistasis analysis reveals that the likely target of Pik1 is Ste11 or its binding partner Ste50. Although required for Ste11 catalytic activity, the role of Ste50 in yeast MAP kinase signaling is not well understood (225, 251-255). Both proteins are shared among three different MAP kinase pathways, and have previously been shown to regulate cross-talk between Hog1 and Kss1 (232, 256). This

makes them ideal candidates for regulation of cross-talk between all three pathways. Furthermore, Ste11 and Ste50 translocate from the cytoplasm to puncta after exposure to osmotic stress (224). While the puncta were not identified by co-localization with known organelle markers, it might be useful to determine if they coincide with the distribution of Pik1 or PtdIns 4-P. While localization studies can be informative, a more pressing (and difficult) question is the direct target of Pik1 and PtdIns 4-P. We consider it unlikely that either Ste11 or Ste50 bind to PtdIns 4-P. Neither protein contains a typical phospholipid-binding domain such as a PH or PX domain (169). However, the BLAST CDD database does predict a low confidence BAR (Bin/Amphiphysin/Rvs) domain in Ste50 (E-value=0.19). BAR domains are dimerization, lipid binding, and curvature sensing modules found on many proteins involved in protein trafficking (257). It is possible this putative BAR domain is responsible for osmotic stress-induced localization of Ste11 and Ste50 at puncta, thereby regulating MAP kinase specificity. Alternatively, Ste11 and Ste50 could interact with PtdIns 4-P indirectly through interaction with a protein containing a known lipid-binding domain. This model is particularly attractive since Ste50 binds the transmembrane protein Opy2, and this interaction is required for Ste11 and Ste50 localization at the plasma membrane (255). Perhaps an interaction with some auxiliary protein is likewise required for Ste11 and Ste50 localization to endomembranes.

Fus3 is known to down-regulate Kss1 (231). Thus, a decrease in Fus3 activation could lead to elevated Kss1 activation, similar to that observed after *PIK1* knockdown. While we propose that Pik1 affects a shared upstream component that results in the differential regulation of both Fus3 and Kss1, it is possible that Pik1 regulates Fus3 directly, but Kss1 indirectly. We consider this unlikely however, since loss of *STT4* or *VPS34* dampens Fus3 activation without a concomitant increase in Kss1 activation. Thus, simply dampening Fus3 activation does not result in constitutively-active Kss1.

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Another question is the mechanism of Pik1 regulation. Currently, there is little evidence to suggest the enzymatic activity of Pik1 is dynamically regulated by pheromone. Garrenton *et al*. showed that pheromone treatment does not change total cellular PtdIns 3-P or PtdIns 4-P levels (75). Therefore, it is likely that direct regulation of an unidentified binding-partner is required. Interestingly, Hog1 feedback-phosphorylates Ste50 in response to osmotic-stress and thereby accelerates both Kss1 and Hog1 inactivation (232, 256). It is possible that Ste50 phosphorylation affects its subcellular localization or its ability to activate Ste11. We also considered a previous suggestion that Pik1 is needed for efficient mRNA export and protein synthesis (75). Under conditions where MAP kinase activity is severely affected, however, we observed no changes in the expression of control proteins including alcohol dehydrogenase (*ADH1-RFP*), glucose 6-phosphate dehydrogenase, or Hog1 *(161)*. Furthermore, selective knockdown of Pik1 at the Golgi, and not the nucleus, is responsible for dampened pheromone signaling (data not shown). Therefore, Pik1 regulation of MAP kinase signaling is likely due to the direct regulation of signaling components present at the Golgi membrane.

In conclusion, we show that Pik1 and PtdIns 4-P promote the activation of Fus3 and Hog1 while repressing activation of Kss1. By acting on all three MAP kinases, Pik1 appears well-positioned to coordinate cellular responses in the face of competing signals. Together with previous demonstrations of signal regulation by a PtdIns 3-kinase at the endosome, there is growing evidence for signal coordination by endomembrane-associated second messengers (65, 258-261). Finally, Ste11 is homologous to human MEKK3 and Ste50 is highly similar to human OSM (Osmosensing Scaffold for MEKK3) (262). Given the conservation of MAP kinase signaling across species, our findings are likely to translate to human MAP kinase pathways as well.

Experimental Procedures

Strains, plasmids, and growth conditions - Standard procedures for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout. Cells were grown in selective medium containing 2% (w/v) dextrose or galactose to induce gene expression. Yeast strains used are listed in Table 3.1. Plasmids used are listed in Table 3.2. Plasmid pRS313 GAL-STE5-CTM was created by SacI and ApaI digestion of pGS5-CTM (228) and ligation into the corresponding sites of pRS313.

The yeast $TetO_7$ strains (28) were grown in selective medium to $A_{600nm}\sim0.8$, re-inoculated at 1:80 into medium containing doxycycline at a final concentration of 10 μg/mL and grown to $A_{600nm}\sim0.8$. To activate the pheromone pathway, α factor pheromone was added at a final concentration of 3 μM for 30 min unless otherwise noted. To induce osmotic stress, KCl was added to a final concentration of 0.5 M for 10 min unless otherwise noted. Time courses were halted by the addition of trichloroacetic acid (TCA) at a final concentration of 5%.

Cell extracts and immunoblotting - Protein extracts were produced by glass bead lysis in TCA as previously described (19). Protein extracts were resolved by 12.5% SDS-PAGE and immunoblotting with phospho-p44/42 MAPK antibodies (9101L, Cell Signaling Technology) at 1:500, Fus3 antibodies (sc-6773, Santa Cruz Biotechnology, Inc.) at 1:500, phospho-p38 MAPK antibodies (9211L, Cell Signaling Technology) at 1:500, Hog1 antibodies (sc-6815, Santa Cruz Biotechnology) at 1:500, and glucose-6-phosphate dehydrogenase (G6PDH) antibodies (A9521, Sigma-Aldrich) at 1:50,000. Immunoreactive species were visualized by chemiluminescent detection (PerkinElmer Life Sciences LAS) of horseradish peroxidase-conjugated antibodies (170-5047 and 170-5046, Bio-Rad Laboratories). Protein concentration was determined by Dc protein assay (500-0112, Bio-Rad Laboratories). Band intensity was quantified by

Table 3.1 Strains Used

Strain Name	Genotype	Source
BY4741*	*MATa leu2D met15D his3-1 ura3D*	(263)
TetO$_7$-STT4	*MATa URA3::CMV-tTA kan^R-TetO$_7$-TATA$_{CYC1}$-STT4*	(28)
TetO$_7$-PIK1	*MATa URA3::CMV-tTA kan^R-TetO$_7$-TATA$_{CYC1}$-PIK1*	(28)
TetO$_7$-PIK1 *kss1Δ*	*MATa URA3::CMV-tTA kan^R-TetO$_7$-TATA$_{CYC1}$-PIK1 kss1::LEU2*	This study
TetO$_7$-PIK1 *ste11Δ*	*MATa URA3::CMV-tTA kan^R-TetO$_7$-TATA$_{CYC1}$-PIK1 ste11::LEU2*	This study
TetO$_7$-PIK1 *ste5Δ*	*MATa URA3::CMV-tTA kan^R-TetO$_7$-TATA$_{CYC1}$-PIK1 ste5::LEU2*	This study
vps34Δ	*MATa vps34::kan^R*	Invitrogen

*All strains derived from BY4741

Table 3.2 Plasmids Used

Fig	Plasmid Name	Alias	Description	Source
3.1	pRS315	SC-2-013	CEN LEU2 vector	(264)
3.7	pRS425	SC-2-006	2mM LEU2 vector	(265)
3.1	pRS423 FUS1-LacZ	SC-2-001	2mM HIS3 P_{FUS1}-lacZ	(222)
3.1	pRS315-STT4	SC-2-075	CEN LEU2 STT4	(161)
3.1	pRS315-PIK1	SC-2-076	CEN LEU2 PIK1	(161)
3.7	pRS425-STE11-4	SC-2-025	2mM LEU2 STE11-4	(161)
3.7	pRS315 GAL1-STE4	SC-2-024	CEN LEU2 P_{GAL1}-STE4	(175)
3.4	pRS313 GAL1-STE5-CTM	SC-2-035	CEN HIS3 P_{GAL1}-STE5-CTM	This Study
	pGS5-CTM	SC-2-027	CEN TRP1 P_{GAL1}-STE5-CTM	(228)
3.3	pRS316 STE5-GFPx3	SC-1-055	CEN URA3 GFP-STE5x3-T_{CYC1}	(233)
3.2	pRS426 GFP-2xPH-PLCδ	SC-3-008	2mM URA3 GFP-2xPH-PLCδ	(238)
3.2	pRS426 GFP-2xPH-FAPP	SC-3-009	2mM URA3 GFP-2xPH-FAPP	(238)
3.2	pRS416 GFP-Lact-C2	SC-3-010	CEN URA3 GFP-Lact-C2	(239)

scanning densitometry using Image J (National Institutes of Health). Phospho-Fus3 and phospho-Kss1 values were normalized to G6PDH loading control and phospho-Hog1 values were normalized to total Hog1.

Transcriptional reporter assay - *FUS1-LacZ* levels were measured 90 min after treatment with α factor pheromone using a β-galactosidase assay and fluorescein di-β-D-galactopyranoside as described previously (209).

Microscopy - Cells were visualized with differential interference contrast (DIC) and fluorescence microscopy using an Olympus Fluoview 1000 confocal microscope equipped with a 488 nm laser (blue argon, for GFP). Images were analyzed using ImageJ (National Institutes of Health).

CHAPTER IV

Conclusions and General Discussion

All figures contributed by Steven D. Cappell

Summary

        G protein-coupled signaling pathways have been extensively studied because of their role in human disease and their importance as pharmacological targets. Most current drugs target the G protein-coupled receptor, but alternative therapies are being developed that modulate G protein signaling downstream of the receptor (5-7). Thus, a complete understanding of the proteins that comprise G protein signaling networks will aid in the development of new pharmacological therapies.

        In this thesis, we report the identification of 92 new proteins required for proper G protein signaling. We utilized a repressible-promoter library and quantitative transcriptional reporter assays to systematically analyze the essential genes in yeast, a group of genes that accounts for ~20% of the yeast genome and has not been extensively characterized (62). Three components of the SCF ubiquitin ligase complex, Cdc4, Cdc53, and Cdc34, were among the genes identified in our screen. We showed that the SCF^{Cdc4} polyubiquitinates and regulates turnover of the Gα subunit, Gpa1. This discovery reveals a new mode of regulating G protein signaling via ubiquitination and indicates the SCF^{Cdc4} could be a viable drug target. However, how ubiquitination of Gpa1 by the SCF^{Cdc4} is regulated and what is its role in the pheromone pathway remains to be determined.

        Also in our screen, we identified two PtdIns 4-kinases, Stt4 and Pik1. We show that both Stt4 and Pik1 are required to maintain MAPK specificity in the pheromone pathway, but Pik1, unlike Stt4, also regulates MAPK specificity in the high-osmolarity glycerol (HOG) response pathway. Thus, we defined a novel role for PtdIns 4-P in maintaining signaling fidelity in multiple MAPK signaling pathways. This work reveals two new mechanisms that regulate signal transduction, both at the level of the heterotrimeric G protein and downstream effectors. In this chapter, I relate the work in this thesis to the existing literature, discuss its implications, and speculate on the future

direction of yeast G protein research.

Role of essential genes in pheromone signaling

Genes that are involved in critical cellular processes such as transcription, translation, and metabolism tend to be essential, and genes involved in dispensable cellular processes such as mating tend to be non-essential (27, 62). Our systematic analysis unexpectedly revealed a great deal of overlap between essential genes and genes required for proper mating response. In particular, genes that are involved in protein degradation, protein transport, and the cell cycle were over represented in our screen, indicating these cellular processes are especially important in both G protein signal propagation and cell mating.

As discussed in Chapter II, genes involved in protein degradation were enriched 3-fold in our screen. We demonstrated a role for three such genes, *CDC4*, *CDC53*, and *CDC34*, in directly regulating Gpa1 turnover. However, we also identified 9 other genes involved in protein degradation, including 8 components of the 26S proteasome (266, 267). This indicates protein degradation must be playing a much larger role in regulating the pheromone pathway than just simply regulating Gpa1 turnover. Indeed, many of the components of the pheromone pathway are known to be ubiquitinated, including Ste2, Sst2, Gpa1, Ste7, and Ste5 (154, 162, 191, 192). Still others may await identification.

Why are so many components of this pathway regulated by ubiquitination? There are several potential answers to this question. First, in the absence of pheromone, pathway components are distributed uniformly in the cell. When cells are exposed to pheromone they become polarized and many localize to the tip of the mating projection, or shmoo (268, 269). How these proteins become so polarized is not completely understood, but it is possible that ubiquitination may play a role. In support of this hypothesis, pheromone triggers the ubiquitination and internalization of the

pheromone receptor Ste2 (162). Subsequently, newly transcribed Ste2 is delivered to the plasma membrane in a polarized fashion to what will eventually become the shmoo tip (62, 270). If ubiquitination of Ste2 is blocked by mutation of its C-terminus, cells are unable to polarize and mating is blocked. This example demonstrates how ubiquitination may regulate cell polarization in response to a graded signal, and indicates that other proteins in the pheromone pathway may be regulated in a similar fashion.

Second, ubiquitination may help the cell eliminate signaling components after mating has occurred. When two haploid yeast cells mate, they form a diploid cell. Interestingly, many of the components of the pheromone response pathway are not expressed in a diploid cell (271). This likely prevents two diploid cells from mating and creating a tetraploid cell. Thus, immediately following a successful mating event, cells must quickly degrade the components of the mating pathway, and the most efficient way to accomplish this is through the ubiquitin/proteasome system. This may explain why many *ste* genes are ubiquitinated.

In our screen, we also identified many genes involved in protein transport and the secretory pathway. We hypothesize that efficient protein transport is needed to deliver newly transcribed signaling molecules to the plasma membrane. This is certainly true for Ste2 and the G protein components, Gpa1, Ste4, and Ste18, which must be available at the plasma membrane in sufficient quantities to respond to any pheromone in the local environment. But even after pheromone stimulation, proteins must be delivered to the plasma membrane to continue signaling and respond to changing gradients of pheromone (272, 273). For example, in response to pheromone the small G protein Cdc42 localizes to the shmoo tip and regulates actin polymerization (274, 275). Actin fibers function to deliver vesicles containing extra membrane material to "build" the mating projection and other proteins required for cell-cell fusion. Cdc42 and actin are also important if a cell needs to alter the direction of its mating projection. Disruption of

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the protein transport machinery would most likely halt delivery of these key mating response proteins and prevent mating. Thus, our screen of essential genes highlights the broad importance of protein transport in maintaining proper signaling.

Regulation of Gpa1 ubiquitination

Here we showed that Gpa1 is polyubiquitinated by the SCF^{Cdc4} complex. However, it is still not known how Gpa1 ubiquitination is regulated. Typically, substrates of the SCF complex must be phosphorylated in order to bind to the F-box protein. In fact, almost all substrates of F-box proteins identified to date are phosphorylated, suggesting that Gpa1 must also be phosphorylated before it can be ubiquitinated (152). While there is no substantial evidence so far that Gpa1 is phosphorylated, there is some information in the literature that indicates it is. Two proteome-wide screens of phosphorylated proteins in yeast found Gpa1 is phosphorylated on two residues, threonine 189 (202) and serine 200 (201). These data were not confirmed and phospho-proteomic screens are prone to false positives. However, these data do indicate that Gpa1 may be phosphorylated. Interestingly, the two proposed phosphorylation sites, T189 and S200, are close to the previously identified site of ubiquitination, K169 (158). In fact, all three residues are located on the same structurally distinct subdomain, specifically a 110 amino acid unstructured region that extends from the all-helical domain of Gpa1 (276).

As discussed in Chapter II, and shown in Figure 2.8F, deletion of the ubiquitinated subdomain blocks ubiquitination of Gpa1 by purified SCF^{Cdc4}. This could result because the primary site of ubiquitination was deleted. However, E3 ligases are notoriously promiscuous when it comes to choosing a lysine for ubiquitin attachment. An E3 ligase will favor one particular lysine when given a choice, but if that lysine is mutated to arginine, the E3 ligase will almost always find a nearby lysine to ubiquitinate (115).

Thus, one might expect that despite the deletion of the primary lysine site in the ubiquitinated subdomain, the SCF^{Cdc4} would find another site to ubiquitinate, but we observed a complete loss of ubiquitination of Gpa1 that lacks the ubiquitinated subdomain (Gpa1$_\Delta^{128-236}$). Alternatively, we hypothesize that the 110 amino acid ubiquitinated subdomain also functions as the SCF^{Cdc4} binding site when T189 and/or S200 are phosphorylated. In support of this hypothesis, we showed that purified Gpa1 from *E. coli*, which should not contain post-translational modifications such as phosphorylation, was not ubiquitinated by the SCF^{Cdc4}. Further experiments are required in order to make firm conclusions. Specifically, pull-down experiments should be performed between Cdc4 and Gpa1$^{T189A/S200A}$ mutants, Gpa1$^{110}_\Delta$ mutants, and wild-type Gpa1 that has been treated with λ phosphatase. No interaction between Cdc4 and Gpa1 should be detected in any of these three conditions. However, given that phosphorylation of T189 and S200 were identified in phospho-proteomic screens, it is possible that there are additional phosphorylation sites on Gpa1, and mutation of T189 and S200 is insufficient to block binding to Cdc4. Mass spectroscopy of Gpa1 may be required to identify all sites of phosphorylation in order to better understand how Gpa1 ubiquitination by the SCF^{Cdc4} may be regulated.

Cell-cycle regulation of Gpa1 ubiquitination – Gpa1 ubiquitination does not seem to be regulated by pheromone. In early studies of Gpa1 ubiquitination, it was thought that pheromone signaling may trigger degradation of Gpa1, providing a mechanism whereby signaling could be enhanced or prolonged. However, experiments have shown pheromone does not induce Gpa1 polyubiquitination (159). Since then, the conditions that promote Gpa1 ubiquitination have remained elusive, and it is simply thought that a basal level of polyubiquitinated Gpa1 is present in the cell at all times. This has prompted the hypothesis that polyubiquitination may be regulating and degrading misfolded Gpa1 (159, 160). However, several pieces of evidence refute this

hypothesis. Most notably, polyubiquitination of Gpa1 has been shown to require localization to the plasma membrane. When the sites of lipid modification of Gpa1 are mutated, C2A and C3A, Gpa1 can no longer bind the plasma membrane, and surprisingly polyubiquitination is no longer detected in the cell (160). Replacement of the lipid-attachment residues with a polybasic region, Gpa1$^{C2A/4K}$, restores both plasma membrane binding and polyubiquitination. Only properly folded protein would make it to the plasma membrane, suggesting fully formed and properly folded Gpa1 is polyubiquitinated.

We suggest an alternative hypothesis, where Gpa1 polyubiquitination is regulated by progression through the cell cycle. Given that several proteins known to regulate the cell cycle, Cdc4, Cdc34, and Cdc53, are directly involved in Gpa1 ubiquitination, it is likely the cell cycle is involved in regulating Gpa1 ubiquitination. All of the other known substrates of Cdc4, such as Sic1 and Far1, are ubiquitinated in specific stages of the cell cycle (152). To test the hypothesis that Gpa1 ubiquitination is regulated by the cell cycle, levels of Gpa1 ubiquitination should be measured in different cell cycle stages. Preliminary experiments conducted by members of our lab using cell cycle inhibitors do in fact show that polyubiquitinated Gpa1 is present in G1 but not S or G2/M phases (data not shown). This indicates that Gpa1 is polyubiquitinated and degraded in G1 phase, and stabilized in S and G2/M phase. This correlates well with other known Cdc4 substrates, which are also ubiquitinated in G1 (135).

Regulation of Gpa1 ubiquitination in different cell cycle stages is an attractive model because mating is prevented in non-G1 phases of the cell cycle in order to prevent aneuploidy. One mechanism by which this can be achieved is regulating the abundance of key signaling components in different stages of the cell cycle (Figure 4.1). Since Gpa1 is a negative regulator of pheromone signaling, it is desirable for the cell to

Figure 4.1
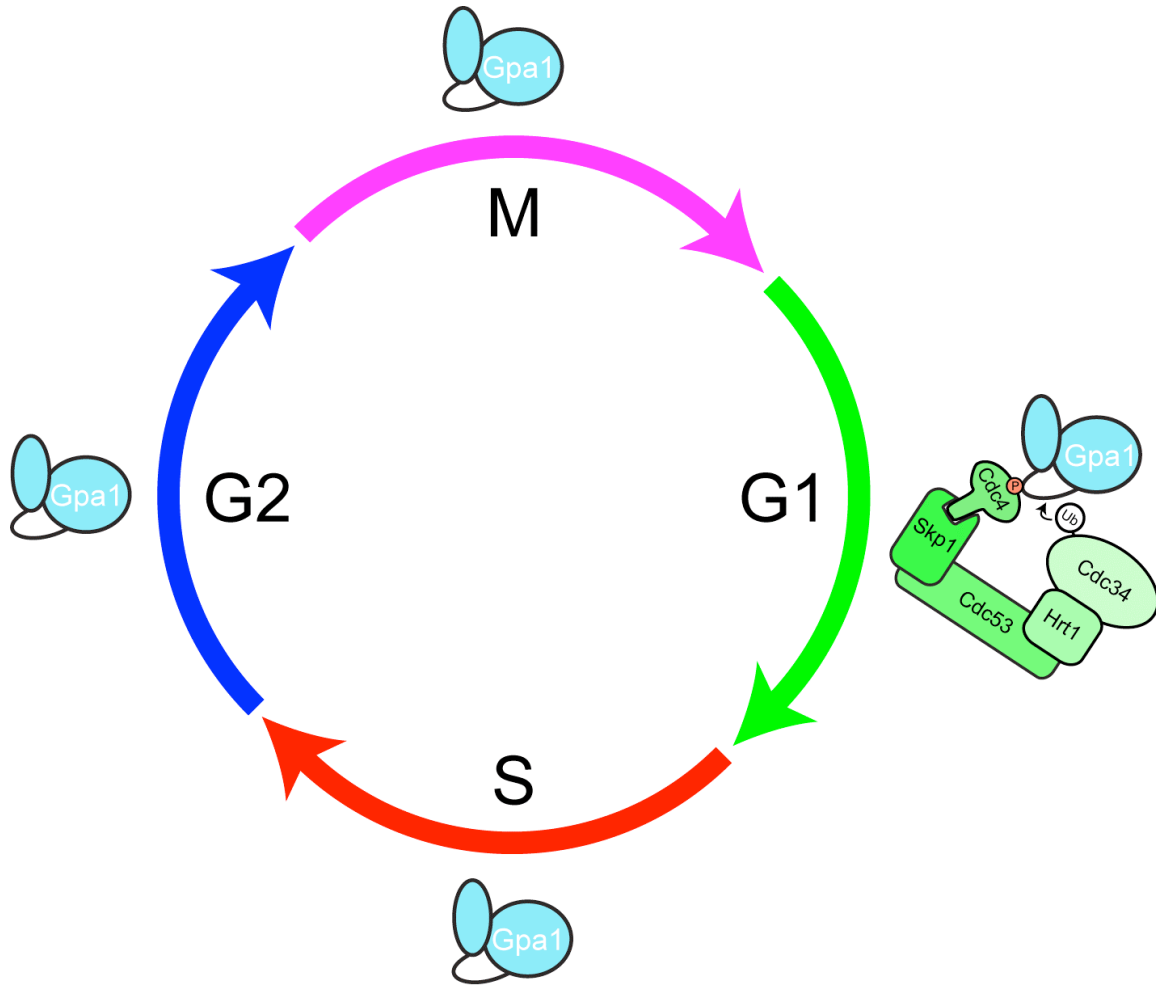
Figure 4.1 G protein ubiquitination cycle.

Model showing G protein ubiquitination is regulated by the cell cycle. In S, G2 and M phase, Gpa1 is stabilized and prevents activation of the pheromone response pathway. Fusion of two cells in any of these phases would result in aneuploidy. In G1 phase, excess Gpa1 is ubiquitinated by the SCF^{Cdc4} complex and degraded by the 26S proteasome. This restores a more favorable stoichiometry between Gpa1 and Ste4, allowing efficient signaling to occur.

accumulate Gpa1 in S, G2, and M phases to prevent aberrant signaling. Then, in G1

phase, the cell would want to degrade excess Gpa1 to allow greater sensitivity to

pheromone. Thus, by regulating Gpa1 levels in different cell cycle stages, the cell can

fine-tune its ability to respond to mating pheromones. To test this hypothesis, cells

arrested in different stages of the cell cycle could be assayed for total cellular Gpa1,

levels of ubiquitinated Gpa1, and mating efficiency.   Gpa1 could also be purified from

yeast arrested at different stages of the cell cycle and subjected to *in vitro* ubiquitination

assays with SCF^{Cdc4}. Gpa1 purified from cells arrested in G1 should be a suitable

substrate for the SCF^{Cdc4} while Gpa1 purified from cells arrested in G2/M should not.

Regulation of total Gpa1 levels – We showed that the SCF^{Cdc4} complex

polyubiquitinates Gpa1 and targets it for degradation by the 26S proteasome. Based on

this knowledge, one would assume that deletion of the SCF complex would result in an

accumulation of total cellular Gpa1 levels. However, when we knocked down Cdc4 we

observed only a slight change in total cellular Gpa1 levels.   There are several

explanations as to why we did not observe Gpa1 accumulation. First, we measured

Gpa1 levels using western blotting, which is a notoriously poor method for protein

quantification.   The linear range for ECL is rather narrow, which can mask small

differences in protein concentration. To better quantify protein levels, we could use a

secondary antibody fused to a fluorescent probe which has a wider linear range, rather

then horseradish peroxidase which has a narrow linear range. Second, small changes

in Gpa1 are known to have large effects on pheromone signaling. Gpa1 functions to

sequester Ste4$^{G_\beta}$, and small increases in Gpa1 levels function to buffer free Ste4 and

prevent normal pheromone signaling (164). Thus, the small changes we observed may

be sufficient to yield a large decrease in pheromone signaling. Third, we measured total

cellular protein levels in a population of cells. This cannot account for variability in the

population. Gpa1 levels could be higher in a proportion of cells and unchanged in the

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rest, masking an accumulation phenotype. Fourth, western blotting measures total protein levels and does not provide any information about subcellular localization. Given that the mating response involves cell polarization, it is reasonable to assume that a subpopulation of Gpa1 is targeted for degradation by polyubiquitination. For example, Gpa1 is known to localize to endosomes in small quantities. It is possible that this small pool of endosomal Gpa1 is targeted for ubiquitination and would accumulate in SCF-deficient cells. Unfortunately, at this time, we have not had the opportunity to accurately quantify Gpa1 levels. However, the fact remains that depletion of the SCF^{Cdc4} increases the half-life of Gpa1 and decreases pheromone signaling, which is rescued by overexpressing Ste4$^{G_\beta}$. Furthermore, Gpa1 is ubiquitinated by the SCF^{Cdc4} *in vitro*. These data provide overwhelming evidence that the SCF^{Cdc4} ubiquitinates Gpa1.

SCF and disease

Like other E3s, deregulation of F-box proteins and their ability to recognize substrates is often associated with human diseases including cancer and Von Hippel-Lindau (VHL) disease. The human homolog of Cdc4, Fbw7, is involved in cancer (277). SCF^{Fbw7} degrades several different proto-oncogenes including c-MYC (278), cyclin E (151), and c-JUN (279). Fbw7 is often inactivated by a truncation mutation in breast and pancreatic cancer that prevents it from recognizing substrates (280). Accumulation of SCF^{Fbw7} substrates leads to genomic instability and cancer. Other diseases also involve mutated F-box proteins. VHL is a hereditary disease that results in a predisposition for malignant tumors in a number of different organs including the brain, kidney, and pancreas. VHL is caused by a germline mutation in an F-box protein that prevents it from recognizing substrates, including hypoxia-inducible-factor (HIF) (281). HIF controls the expression of genes that induce angiogenesis and when overexpressed, supports tumor growth by supplying the tumor with a constant supply of glucose and growth

factors. Thus, patients who carry mutations in VHL have elevated levels of HIF, and are highly prone to tumor formation (282). Currently it is unclear how to therapeutically re-activate mutant F-box proteins that cannot bind substrates such as those found in VHL and breast cancer, but this is an active area of research (283).

SCF^{Skp2} also involved in human cancer due to its role in the degradation of the cyclin-dependent kinase inhibitor p27 (284). p27 inhibits cell division and promotes quiescence. Loss of p27 results in unchecked progression through the cell cycle (285). Thus, control of p27 levels is required for proper regulation of the cell cycle, and the prevention of tumor formation. When the F-box protein Skp2 is overexpressed in mice, p27 levels decrease and tumors start to form (286). Indeed, Skp2 is found overexpressed in many different types of human cancers, and is always correlated with low levels of p27 (287, 288). Drugs that inhibit Skp2 and prevent degradation of p27 could prove to be a highly successful chemotherapeutics (283, 289).

Recently, two labs discovered small molecule inhibitors of F-box proteins (290, 291). Aghajan *et al.* found an inhibitor of the yeast F-box protein Met30, which contains seven WD40 repeats and is structurally very similar to Cdc4 (290). The inhibitor, small-molecule enhancers of rapamycin (SMER) 3, was found to disrupt binding between Met30 and Skp1. It is hypothesized that SMER3 binds at or near the F-box protein of Met30 and prevents its interaction with Skp1. Interestingly, the authors demonstrated that SMER3 was only active with Met30, and not Cdc4. Thus, despite binding a common domain, SMER3 shows remarkable specificity. Given the role of many F-box proteins in human disease (283), these results are encouraging and indicate a common mechanism whereby diverse F-box proteins could be inhibited by small molecules.

Tyers and colleagues found a small molecule inhibitor of Cdc4 that functions via a different mechanism than SMER3. Using yeast as a model, Orlicky *et al.* identified a small molecule called SCF-I2 that disrupts the Cdc4 substrate-binding domain (291). A

crystal structure of Cdc4 in complex with SCF-I2 shows the small molecule binds between two β-propellers formed by WD40 repeats number 5 and 6, and is 25 Å from the substrate binding pocket. By wedging between these two propellers, SCF-I2 induces a large conformational shift of the main chain, which disrupts the substrate-binding pocket. Given the high degree of homology between Cdc4 and the human F-box protein Fbw7, it is likely that human Gα proteins will also be ubiquitinated by the SCF[Fbw7]. It is our hope that a small molecule similar to SCF-I2 will be found to inhibit Fbw7. If such a drug were developed, it could be used to enhance G protein signaling by stabilizing the Gα subunit. This is an exciting proposition and further reinforces the theory that new drugs can be derived from studies of G protein signaling in yeast.

The crystal structure of SCF-I2 and Cdc4 also indicates a general mechanism where all WD40 repeat proteins can be therapeutically targeted. The list of proteins containing WD40 repeats is not restricted to F-box proteins (292-294). Most notably, the Gβ subunit of the heterotrimeric G protein is comprised of 7 WD40 repeats (295, 296) and forms the same β-propeller structure as Cdc4 (149, 297). Perhaps the discovery of SCF-I2 could lead to a new class of drugs that directly regulates heterotrimeric G protein signaling by disrupting the interaction between Gβ and Gα (298). Nevertheless, SCF-I2 and SMER3 prove that small molecule inhibitors of F-box proteins are indeed feasible, and future research is likely to focus on F-box proteins involved in human disease, such as Skp2 and Fbw7 (289).

Phosphoinositides in MAPK signaling

Recently, several reports have detailed new roles for phosphoinositides in regulating MAPK signaling pathways. Specifically, the PtdIns kinases Stt4, Vps34, and Pik1 have all been implicated in regulating the pheromone response pathway (65, 75, 207, 233). Despite the fact that PtdIns kinases all produce phosphorylated

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phospholipids, they all seem to regulate signaling via a distinct mechanism. Stt4 produces PtdIns 4-P at the plasma membrane and is necessary to recruit the scaffold Ste5 to the cell periphery where it can interact with upstream activators such as Ste20 (207, 233). This process is required for Fus3 activation but not Kss1 activation. Vps34 is localized to early endosomes and produces PtdIns 3-P when stimulated by GTP-bound Gpa1. Deletion of *VPS34* diminishes Fus3 activation but does not affect Kss1 activation (65). The exact mechanism of action of Vps34-derived PtdIns 3-P is not currently known. Pik1 produces PtdIns 4-P at the Golgi and in the nucleus. Like Vps34, knockdown of Pik1 causes a reduction in Fus3 activation. However, it also causes constitutive Kss1 activation. Thus, in yeast, three separate PtdIns kinases regulate MAPK specificity by selectively affecting Fus3 and not Kss1.

Although Stt4, Vps34, and Pik1 produce different catalytic products at different cellular locations, they all differentially regulate Fus3 and Kss1. This suggests a general requirement for phosphoinositides in maintaining signaling fidelity between multiple MAPK signaling pathways that share components. The most logical hypothesis is that phosphoinositides insulate signaling pathways by physically tethering components to different subcellular locations. For example, Stt4 helps tether Ste5, and by association Ste11, Ste7, and Fus3, to the plasma membrane in locations of pheromone-bound receptors (233). This most likely helps prevent aberrant activation of Kss1 and a third MAPK, Hog1. Similar mechanisms are likely to exist for Vps34 and Pik1 at endosomes and the Golgi respectively. Examining the localization of proteins that contain lipid-binding domains could reveal the function of Vps34 and Pik1.

Proteins can contain a number of different lipid-binding domains including PH, PX, ENTH, and FYVE domains (169). Of these examples, ENTH domain-containing proteins are intriguing candidates for proteins that may directly bind phosphoinositides at endomembranes and regulate pheromone signaling. Proteins that contain ENTH

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domains typically localize to endosomes or the Golgi due to their ability to bind phosphoinositides, clathrin coated pits, and possibly actin (299, 300). Yeast have five proteins that contain an ENTH domain, designated Ent1-5 (301, 302). Ent5 is a particularly interesting protein because it is involved in trafficking between the Golgi and endosomes (301).

Ent5 has been shown to physically interact with Fus3 (303). This indicates Pik1 and/or Vps34 could be regulating Fus3 activation by physically sequestering Fus3 to sites of intracellular compartments through Ent5. Preliminary data show that Fus3-GFP localizes to intracellular puncta in addition to the nucleus (Figure 4.2). While the identity of these spots has not yet been determined, this observation is consistent with the hypothesis that PtdIns kinases regulate Fus3 localization to endomembrane compartments.

Genetic studies have also shown a negative interaction between Ent5 and Ste50, which is an adapter protein for the MAPKKK Ste11 (304). Ste50 regulates cross-talk between the HOG pathway and the filamentous growth pathway (305). The genetic interaction between Ent5 and Ste50 indicates they function in the same pathway. Furthermore, Ste50 localizes to intracellular puncta upon exposure to osmotic stress, indicating a physical interaction between Ent5 and Ste50 is possible (176). It has not been determined whether Ste50 localization to intracellular spots is dependent on either PtdIns 3-P or PtdIns 4-P. Nevertheless, the several pieces of evidence outlined above suggest Ent5 may be the target of phosphoinositides regulation of MAPK signaling.

Future experiments should aim at identifying proteins that link phosphoinositides to pheromone signaling. GFP fusions to proteins with known lipid binding domains can be monitored in the presence and absence of specific PtdIns kinases. Candidate genes can then be subjected to co-Immunoprecipitation experiments with components of the MAPK cascade, specifically Ste50, Ste11, and possibly Fus3. Ent1-5 will be a high

Figure 4.2
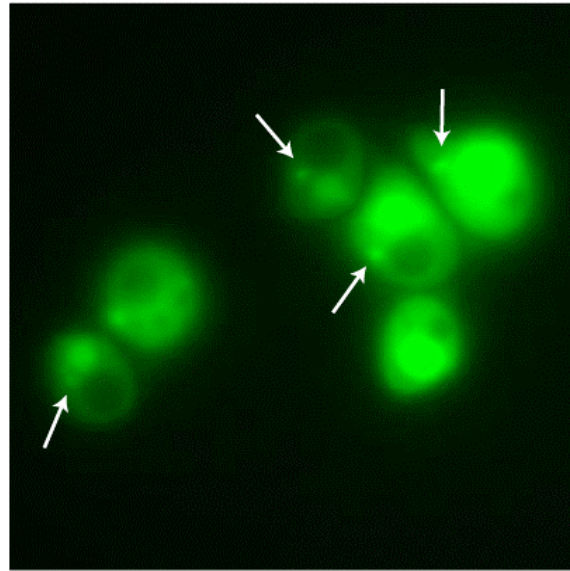
Fus3-GFP

Figure 4.2 Fus3-GFP localizes to intracellular puncta.
     FUS3-GFP was integrated into the genome of wild-type BY4741 cells. Cells were treated with 3 μM α factor for 2 hrs. Cells were visualized and fluorescence microscopy using an Olympus Fluoview 1000 confocal microscope equipped with a 488 nm laser (blue argon, for GFP). Images were analyzed using ImageJ (National Institutes of Health).

priority for analysis, as will other proteins that have physical and genetic interactions with known pheromone pathway components.

Conclusions

We identified 92 essential genes required for proper pheromone signaling in yeast. Of these, we thoroughly characterized five genes to reveal how they regulate G protein signaling. This work revealed a link between genes that regulate the cell cycle via protein ubiquitination and G protein turnover, indicating the cell may control its ability to respond to external stimuli by altering the abundance of key signaling components. Furthermore, we uncovered new roles for phosphoinositides in regulating fidelity in signal transduction networks. Together, these findings reveal the essential genes are a virtually untapped resource for identifying new signaling components. Given the high degree of conservation between yeast and human G protein pathways, discoveries made during the course of this research are likely to translate to humans, leading to new avenues for drug discovery.

APPENDIX I

PCR Primers (Used In Chapter II)

Name	Primer Sequence
1	5'–TAG GAT CCG TAG GAA ATA ATG GGG TGT AC–3'
2[a]	5'–TAA AGC TTT **GAT TAC AAG GAT GAC GAC GAT AAG** AGC CTT TAG CAG TAT TCT C–3'
3	5'–GAA GGA TGA CGA CGA TAA GAA ACT TAT TCA CGA AGA CAT
4	5'–GCA ATG TCT TCG TGA ATA AGT TTC TTA TCG TCG TCA TCC
5	5'–GGT TGC GTA AAA ATT ATT GAT TTT GGT ATT TC–3'
6	5'–GAG CTC GCC ATG TCT AAA GGT GAA GAA TTA TTC ACT GGT G–3'
7	5'–GAG CTC GCC ATG TCT AAA GGT GAA GAA TTA TTC ACT GGT G–3'
8	5'–CTC GAG CCC GGG GCG AAT TCT TAT GAT TTT ATG ATT TTT ATT ATT AAA TAA G–3'
9	5'–GAA TTC GGT ACC CAA TAG CAA TGG GGT TTT TTT CAG–3'
10	5'–CCC GGG AAC ATG GCC TCC TCC GAG GAC–3'
11	5'–CCC GGG GCC TTA GGC GCC GGT GG–3'
12	5'–GTC GAC CCA TGC AGA AGC TGT TGC GAA G–3'
13	5'–GTC GAC TTT GAT TTT CAG AAA CTT GAT GGC TTA TAT CCT GC–3'
Cdc4 F	5'–CAG TTA GAG CTC TGG TAT TCA TCA TCA AAA ACA GCC TTC CAG–3'
Cdc4 R	5'–CAG TTA CCC GGG AAA AAT CAG CAG AAT CAA CTT CTT AAC G–3'
Cdc34 F	5'–TAG AGC TCG GCA ATT ACT AAT GCG GGT TC–3'
Cdc34 R	5'–TAC CCG GGT CGC AAA GAT CGA CCT AAG AC–3'
Stt4 F	5'–GAG CTC AAA TTA GGC TGA ATA GAA CTG C–3'
Stt4 R	5'–CCC GGG AGA TTT TCC TTG TCC TCC CTT T–3'
Pik1 F	5'–CCG CGG CTA GAA GAT ATT GAC ATC GAT TCC G–3'
Pik1 R	5'–CCC GGG CGA TGT GCC ATA TAG TAA GCT GG–3'
Mps1 F	5'–CCG CGG ATC ACA ACA AAT GGT GAT TCT GG–3'
Mps1 R	5'–CCC GGG TCT AAG CAA GAA TGG CAA GAA AG–3'
Ste4 F	5'–CAG GTA CAC ATT ACG ATG GAA TTC CAT CAG ATG GAC TCG ATA ACG–
Ste4 R	5'–CGT TAT CGA GTC CAT CTG ATG GAA TTC CAT CGT AAT GTG TAC CTG–
Ste20 F	5'–AAG GAA AAA AGC GGC CGC CTC GCT TAT GGA TTG TGG GAT CTC ACC–
Ste20 R	5'–CCG CTC GAG CCT TTC CGT CAC TGT TCC ATG–3'
Ste5 F	5'–TCC CCG CGG GAC ATC AGC TGA TTT CTC ATA GAG–3'
Ste5 R	5'–AAG GAA AAA AGC GGC CGC GCT CAT TAT GTA ACC ATT CCG ATT GAC–
Ste11 F	5'–AAG GAA AAA AGC GGC CGC CTG CGC AGC TTT ATA CAA GTT AGC–3'
Ste11 R	5'–CCG CTC GAG ACG CTT CTA GAG GTA CAG GCG–3'
Ste7 F	5'–CGC GGA TCC TGG TAT CCT TCT TGG GAA TAT TCA ATG C–3'
Ste7 R	5'–CCG CTC GAG AGC GAG TAC AGT CAT TGT GTG CCA CC–3'
Fus3 F	5'–CGC GGA TCC GCA GCG ACT GCA CTA AAC TAG AGG–3'
Fus3 R	5'–CCG GAA TTC TCT AGT AAG ACG GTC TCT TGC TGC–3'
Kss1 F	5'–AAG GAA AAA AGC GGC CGC GAC AGA TTA CGA GAG TCC AGC C–3'
Kss1 R	5'–CCG CTC GAG CTT GGC TGG GTA TTA GTT GTG–3'
Ste12 F	5'–CGC GGA TCC TCT TCC CTT CCC AGA GAG AAA AAA GG–3'
Ste12 R	5'–CCG CTC GAG CTG CTT TTA TTA TTT GTT AGT GCC–3'

[a]FLAG sequence highlighted in bold

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