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**Intermolecular and intramolecular interactions controlling the localization and activity of the yeast kinases Kin1 and Kin2**

Keith Aaron Weise

*Yale University Graduate School of Arts and Sciences, kaweise01@gmail.com*

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Abstract

Intermolecular and Intramolecular Interactions Controlling the Localization and Activity of the Yeast Kinases Kin1 and Kin2

Keith A. Weise

2021

Kin1 and Kin2 (Kin1/2), the *S. cerevisiae* orthologs of the mammalian microtubule affinity-regulating kinases (MARKs), regulate a variety of important cellular functions, including exocytosis and the unfolded protein response (UPR). In this study, I examined the regulation of Kin1/2 kinase activity and localization, which are poorly understood. I determined the impact on Kin1/2 of interaction with Bud14, a regulatory subunit of the phosphatase Glc7, the budding yeast ortholog of PP1. Kin1/2 localization to sites of polarized growth was completely dependent on interaction with Bud14, although Kin1/2 kinase activity was not dependent on Bud14. I also examined the impact of the kinase-associated 1 (KA1) domain on both the kinase activity and localization of Kin1/2. Mutation of the KA1 domain had a partial effect on Kin1/2 localization. The KA1 domain also autoinhibits Kin1/2 catalytic activity, and mutation of the KA1 domain increases Kin1/2 activity nearly ten-fold. Kin1/2 kinase activity is also profoundly impacted by activation loop phosphorylation of a conserved threonine residue, which increases Kin1/2 kinase activity by 20-fold. I found that the protein kinases Elm1, Sak1 and Tos3, which activate related kinases, are responsible for Kin1/2 activation loop phosphorylation. The level of kinase activity, but not localization, is vital for Kin1/2 function in the unfolded protein response. Taken together, these results further elucidate the mechanisms controlling cellular Kin1/2 activity and localization.
Intermolecular and Intramolecular Interactions Controlling the Localization and Activity of the Yeast Kinases Kin1 and Kin2

A Dissertation
Presented to the Faculty of the Graduate School
Of
Yale University
In Candidacy for the Degree of
Doctor of Philosophy

By
Keith A. Weise
Dissertation Director:
Benjamin E. Turk
June 2021
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Background

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**Glossary of Terms**

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<tr>
<th>Acronym</th>
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<tr>
<td>AGC</td>
<td>Protein Kinase A, Protein Kinase G, Protein Kinase C group</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAMK</td>
<td>Ca(^{2+})/Calmodulin dependent protein kinase</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein kinase 1</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein kinase 2</td>
</tr>
<tr>
<td>CUG</td>
<td>3-carboxyumbelliferyl β-D-galactopyranoside</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorescein di-β-D-galactopyranoside</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>KA1</td>
<td>Kinase-associated 1 domain</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver kinase B1</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MAPKK</td>
<td>MAPK kinase</td>
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<td>MAPKKK</td>
<td>MAPK kinase</td>
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<td>MAPKK kinase</td>
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<tr>
<td>MARK</td>
<td>Microtubule affinity-regulating kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PH</td>
<td>Pleckstrin homology</td>
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<tr>
<td>PIP$_2$</td>
<td>Phosphatidylinositol 4,5 bisphosphate</td>
</tr>
<tr>
<td>PIP$_3$</td>
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</tr>
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<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane domain</td>
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</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SC</td>
<td>Synthetic complete</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3 domain</td>
</tr>
<tr>
<td>TAOK1</td>
<td>Thousand-and-one kinase 1</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>UBA</td>
<td>Ubiquitin-associated domain</td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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Chapter 1: Regulation of MARK/Par1/Kin1 activity in *Saccharomyces cerevisiae*

Protein kinases play necessary roles in every aspect of a living cell. From controlling metabolism, growth and division, to confronting stresses and adverse conditions, kinases are vital to cellular regulation at many levels. It is also important to understand not only how kinases regulate the cell, but how kinases themselves are regulated. Effective signal transduction in cells requires that kinases become activated at the appropriate location and time, and that they are switched off when the activating signal is no longer present. Unregulated kinase activity can lead to cellular dysfunction and can be a cause of disease.

One family of kinases that plays important roles in eukaryotic cells is the AMP-activated protein kinase related (AMPKR) family\(^1\). AMPKRs comprise a branch of the Ca\(^{2+}\)/calmodulin-dependent protein kinase-related (CAMK) group, and are conserved across all eukaryotic kingdoms\(^2\). The family is defined based on sequence similarity within an N-terminally located catalytic kinase domain, with their C-terminal regions consisting of regulatory domains that vary among family members (Fig 1.1). The best-studied member of the family is AMPK, a major regulator of cellular metabolism\(^1\). Other AMPKR subfamilies include the Brsk, Nuak, and the MARK/Par1/Kin1 groups\(^2\). My dissertation focuses on the MARK/Par1/Kin1 subfamily. Members of the subfamily in mammals are known as microtubule affinity-regulating kinases (MARKs), and orthologs other species include Par1 in *C. elegans* and *D. melanogaster*, \(^\)}
Figure 1.1: AMPKR family protein kinases in *Saccharomyces cerevisiae*. 
kin1 in *S. pombe*, and Kin1 and Kin2 (Kin1/2) in *S. cerevisiae*\(^3\)-\(^{12}\). MARK/Par1/Kin1 kinases are highly conserved across eukaryotes, with ~35-40% sequence identity between orthologs from yeast and humans. The kinases share a structural similarity, with their N-terminal kinase domains followed by a ubiquitin-associated domain UBA domain, a long, poorly conserved central region, and a C-terminal kinase-associated 1 (KA1) domain (Fig 1.2)\(^{13},\ 14\). Along with their structural similarity, the MARK/Par1/Kin1 subfamily share common functions, as many of them are implicated in the establishment of cell polarity\(^{15}\).

For example, *C. elegans* Par1 was first identified as essential for establishing a polar axis during early embryonic development, and *S. pombe* kin1 works in conjunction with several other proteins to establish polarity and maintain cellular symmetry\(^{4},\ 16\). Given the role of the MARK/Par1/Kin1 subfamily in such an important cellular process, it is not surprising that the dysfunction of these kinases can have dire implications for organisms. The human MARKs are believed to participate in the development of Alzheimer’s disease by hyperphosphorylation of their substrate Tau, which can promote the formation of neurofibrillary tangles\(^{17},\ 18\). MARKs are also implicated in various cancers, with some isoforms upregulated in brain cancers, and other isoforms downregulated or inactivated in lung cancers and stomach cancers\(^{19}-^{21}\). MARKs are also important effectors of the tumor suppressor LKB1.

Given the importance of MARK activity and the fact that dysregulation of MARKs can lead to disease, it is necessary for cells to regulate these kinases. Many kinases are activated by phosphorylation in the activation loop, a flexible region in the C-terminal lobe of the catalytic domain\(^{22}\). Canonical activation loop phosphorylation serves to stabilize the kinase in an active
Figure 1.2: Domain map of *S. cerevisiae* Kin1 (1064 amino acids) and Kin2 (1147 amino acids). Kin1/2 are representative of MARK/Par1/Kin1 kinases, with an N-terminal Ser-Thr kinase catalytic domain depicted in red (residues 120-398 in Kin1, 99-377 in Kin2) and a C-terminal kinase-associated 1 (KA1) domain, depicted in blue (residues 1015-1064 in Kin1, 1098-1147 in Kin2).
conformation by promoting substrate binding as well as proper alignment of the active site. Activation loop phosphorylation can also affect positioning of the αC helix, inducing its conserved glutamic acid residue to ion pair with the catalytic lysine in the AxK motif. AMPKR family members, including the MARK/Par1/Kin1 subfamily, are activated by phosphorylation of a conserved threonine residue in the activation loop. Activation loop phosphorylation of the MARK/Par1/Kin1 subfamily is mediated by conserved activators: liver kinase B1 (LKB1) and thousand and one kinase (TAOK1) in mammals, Par4 in C. elegans, and ssp1 in S. pombe. The S. cerevisiae orthologs, Elm1, Sak1 and Tos3, are known to activate some members of the AMPKR family, although the activating kinases of Kin1/2 remain unidentified.

MARK activity is also regulated by controlling access to the catalytic cleft of the kinase. The MARK/Par1/Kin1 subfamily contains a conserved C-terminal KA1 domain that binds to the catalytic domain and occludes substrate binding. In mammalian MARKs, the KA1 domain can reduce kinase activity toward a peptide substrate by more than 50%. Some strains of the H. pylori bacteria that cause stomach ulcers and cancers secrete the CagA protein, which binds to and occludes the catalytic cleft of MARKs through a pseudosubstrate interaction. CagA thus acts as an inhibitor of the kinase, increasing the cell polarity defects of affected cells and increasing the carcinogenic potential of the H. pylori strains that express CagA.

Substrate recognition is another important factor impacting MARK activity. In order to faithfully promulgate a signal in the proper pathway, a kinase must exhibit specificity for its appropriate substrates. Like all members of the CAMK kinase group, AMPKR family kinases are serine-threonine kinases that generally have a strong
preference for an arginine residue three residues upstream of the phosphoacceptor, known as the “-3” position. Preference for the -3 basic residue is mediated by formation of a salt bridge with a conserved glutamic acid residue in the catalytic cleft\textsuperscript{34}. AMPKRs also preferentially phosphorylate substrates with a hydrophobic residue five residues upstream (-5) and four residues downstream (+4) of the phosphoacceptor. Selectivity at the -5 position has been revealed by crystallographic and mutational analysis to be driven by four residues that form a hydrophobic pocket to accommodate the hydrophobic sidechain\textsuperscript{35}. The preference of all AMPK family kinases for a +4 hydrophobic residue is mediated by the unique presence of an alanine residue immediately upstream of the APE motif in the activation loop (\textit{AAP}E). In other serine-threonine kinases, this residue is typically Leu, Met or Arg. The small Ala residue in AMPKRs creates a hydrophobic cavity that allows binding of the +4 residue (usually Leu or Val).

Binding to lipids through non-catalytic domains is a common means of localizing kinases within the cell, with prominent examples including AKT and PKC. The KA1 domain of the MARK/Par1/Kin1 kinases, in addition to inhibiting the catalytic domain, binds anionic phospholipids and helps to localize them to cell membranes\textsuperscript{36,37}. The binding of the KA1 domain to phospholipids may also serve to release its autoinhibitory interactions with the catalytic domain, thus serving to increase kinase activity locally. Increasing the activity of the kinase while simultaneously localizing it within the cell provides a convenient means to direct the activity of the MARKs in an efficient way. This idea is reinforced by the fact that the same cluster of basic residues of the KA1 domain that mediate autoinhibition of the kinase also interact with phospholipids. Another example of regulation of kinase activity by interaction with lipids is protein kinase C (PKC). PKC is
activated by phosphorylation of its activation loop, and binds diacylglycerol (DAG) at the plasma membrane in response to cleavage of phosphatidylinositol (4,5) bisphosphate (PIP$_2$)$^{38}$. This produces DAG which promotes PKC localization to the plasma membrane, but also liberates inositol triphosphate 3 (IP$_3$), a second messenger that leads to activation of the PKC molecules now located at the plasma membrane.

MARK/Par1/Kin1 localization may also be regulated by phosphorylation at sites outside of the catalytic domain and by interaction with protein binding partners. For example, phosphorylation of human MARKs by PKC impacts their localization. PKC phosphorylation creates a binding site for 14-3-3 proteins, which sequester the MARKs in the cytoplasm, away from peripherally localized substrates, and serve to inhibit kinase activity$^{39,40}$. MARKs are also known to interact with Gab1, a scaffold protein that increases kinase activity of the MARKs by preventing the KA1 domain from binding to the active site of the kinase$^{41}$. In S. pombe, kin1 is localized to the sites of polarized growth by a combination of the KA1 domain, which assists with membrane localization in general, and by its association with pal1, a protein binding partner. pal1 is also a substrate of kin1, and co-localizes with it to the tips of growing cells. kin1 and pal1 are mutually dependent for localization to these polar sites. In addition to pal1, kin1 localization to sites of polarized growth is also aided by tea4, a regulatory subunit of dis2, the fission yeast ortholog of protein phosphatase-1 (PP1). It is unknown whether the KA1 domain of S. cerevisiae Kin1/2 plays a role in membrane localization, or if Kin1/2 interact with protein binding partners that determine their distribution within the cell, questions that I will address in this dissertation.
The subsequent research in this study is concerned with the budding yeast *Saccharomyces cerevisiae* AMPKR family, in particular with the members of the MARK/Par1/Kin1 subfamily, Kin1 and Kin2. The yeast AMPKR family has six members. Snf1, the best characterized member of the family, is orthologous to mammalian AMPK, and as such fulfills the same role in the cell of acting as a master regulator of metabolism.

Three other members of the family, Gin4, Kcc4, and Hsl1, have roles in septin organization during cell division, consistent with the AMPKR family role in establishment of cell polarity. Hsl1 in particular participates in the bud morphogenesis checkpoint, coupling proper septin formation to cell cycle progression by mediating degradation of the Cdc28 kinase Swe1. Each of these family members is activated by phosphorylation of a conserved threonine by some combination of Elm1, Sak1, or Tos3, all of which are orthologs of LKB1/Par4/Ssp1. The final two family members and the closest budding yeast homologs of animal MARK/Par1 kinases are Kin1 and Kin2.

While Kin1 and Kin2 (Kin1/2) were first cloned more than 30 years ago, comparatively little is known about their function or regulation. An issue that limits understanding the function of Kin1/2 has been the lack of a phenotype under normal growth conditions when both kinases are deleted. One role for Kin1/2 was discovered through a high-copy suppressor screen for secretory pathway mutants. Overexpression of Kin1 or Kin2 rescued the temperature-sensitive growth defect of late secretory pathway mutant strains, suggesting a role for Kin1/2 in exocytosis. This research also revealed that Kin1/2 directly interact with and phosphorylate Sec9, a t-SNARE protein involved in vesicle fusion. Kin1/2 phosphorylation of Sec9 leads to its dissociation from the plasma membrane, and an increase in the cytosolic pool of Sec9, which presumably aids in Sec9
incorporation into vesicles ready for fusion during exocytosis. There is also evidence of an interaction between Kin2 and Rho3, a GTPase that plays a critical role in exocytosis as well as cell polarity\textsuperscript{46}. It is unclear if the function of Kin1/2 in exocytosis is related to a broader function in establishing cell polarity, and no role for endogenous Kin1/2 in regulating exocytosis has been described.

Kin2 was also reported to localize to sites of polarized growth, an interesting observation given the role of orthologous proteins in establishing cell polarity in other organisms. In budded cells, GFP-tagged Kin2 was found to localize to the bud neck and bud tip, and also displayed more general localization to the cell cortex\textsuperscript{46, 47}. Structure-function analysis determined that two regions within Kin2 were important for localization. The N-terminal portion of the protein (residues 1-510) including the catalytic domain was necessary for localization to sites of polarized growth, although the Kin2 signal detected at the bud tip was reduced for this construct. The KA1 domain was found to be necessary for general localization to the plasma membrane but dispensable for localization to sites of polarized growth. It was also found that the ability of Kin2 to localize to sites of polarized growth was necessary for proper function in exocytosis. However, mechanisms controlling this localization have not been uncovered.

Kin1/2 also play a role in proper cell wall organization. \textit{KIN2} overexpression caused a minor defect in cell morphology and budding\textsuperscript{46}. Overexpression of Kin2 also sensitized budding yeast to cell wall disrupting agents such as SDS and calcofluor white. This phenomenon was also observed upon overexpression of a Kin2 mutant lacking an intact KA1 domain, a notable result given the role of the KA1 domain in localizing MARKs to cell membranes. Chitin staining of the cell wall suggests that overexpression of Kin2
leads to a defect in cell wall organization. The same N-terminal region of Kin2 (residues 1-510) that is necessary for localization to sites of polarized growth suppressed growth when overexpressed. The kinase activity of Kin2 was required for this growth suppression. Likewise, Kin2 lacking a KA1 domain failed to support growth when overexpressed. Kin2 was also found to interact with Tos1, a protein known to bind the cell wall, perhaps explaining the cell wall disorganization results. The N-termin of Kin2 interacted with Bmh1, a 14-3-3 protein in budding yeast. Given the important regulatory nature of the interaction between the human MARKs and 14-3-3 proteins, this interaction suggests that 14-3-3 binding may be a more general means of regulating the MARK/Par1/Kin1 family. All of these results imply that there may be deeper role for Kin1/2 in cell polarity, despite the lack of an observable phenotype in *kin1Δ kin2Δ* strains.

Several interactions between Kin2 and other proteins have also been discovered that provide insight into Kin2 function in proper septin formation and regulation of the cell wall. These interactions also hint at an overall function for Kin2 in establishing cell polarity. As mentioned, *KIN2* overexpression causes a minor defect in cell morphology and budding, an effect that was exacerbated in a strain lacking *GIN4*. This is noteworthy given the role of Gin4 in proper septin organization during budding. Kin2 was found to interact with Cdc11, a septin subunit in budding yeast. Kin2 also interacted with Pea2, a protein that is a component of the polarisome and also believed to function in septin formation. Both of these interactions occurred in the same region of Kin2 (residues 780-1147). Overexpression of this region alone lead to septation defects in yeast, suggesting that the interaction with one or both of these proteins could be responsible for the septation
defects observed. A role for Kin2 in septin regulation would also indicate a role in cytoskeletal regulation congruous with MARK/Par1/Kin1 family members across species.

More recently, it has come to light that combined deletion of KIN1 and KIN2 strongly sensitizes cells to agents such as tunicamycin and DTT that cause ER stress and stimulate the unfolded protein response (UPR)\textsuperscript{48,49}. The UPR, the only ER stress pathway in yeast, is activated by the dual kinase/RNAse Ire1. When Ire1 detects misfolded protein in the lumen of the ER, it forms foci and cleaves introns from HAC1 mRNA (orthologous to XBP1 mRNA in humans). HAC1 mRNA is actively targeted to Ire1 to begin this process. This spliced HAC1 is translated into Hac1 protein, which is translocated into the nucleus to act as a transcription factor that leads to the production of a number of genes that assist with resolving ER stress and protein folding. Kin1/2 kinase function is necessary for function in the UPR. Overexpression of Kin1/2 was able to overcome targeting defects associated with a mutant variant of HAC1 mRNA. Deletion of both KIN1 and KIN2 disrupts multiple processes in the UPR, from Ire1 foci formation to the targeting of HAC1 mRNA to Ire1. It remains unclear exactly how Kin1/2 promote the UPR, or what substrate(s) they phosphorylate to mediate this response.

One interesting feature of Kin1/2 is that they possess a unique phosphorylation motif preference relative to the other yeast AMPKRs\textsuperscript{50,51}. The other four members of the family recognize a motif similar to human AMPK, with a preference for an arginine residue at the -3 position, as well as a preference for a hydrophobic amino acid at the -5 and +4 position relative to the phosphoacceptor (Fig 1.3). Among these features, Kin1/2 only share a preference for a hydrophobic residue at the +4 position, in keeping with the presence of an alanine residue upstream of the APE motif shared with all other AMPKR
Figure 1.3: Canonical phosphorylation motifs of AMPK family kinases (top row) and \textit{S. cerevisiae} Kin1 and Kin2 (bottom). The phosphoacceptor is indicated in red.

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<th>-4</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
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<th>+3</th>
<th>+4</th>
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<td>AMPKR family motif</td>
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<td>x</td>
<td>R</td>
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<td>Kin1/2 motif</td>
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family members. However, in contrast to essentially all members of the larger CAMK group, Kin1/2 lacks a basophilic preference at the -3 position. In other kinases, the preference for a -3 Arg is mediated by a key Glu residue (Glu100 in human AMPK), which forms a salt bridge with the charged headgroup of the basic residue. In Kin1/2, this Glu residue is substituted with Gln (Gln230 in Kin1, Gln209 in Kin2), neutralizing the charge and rendering Kin1/2 non-selective at the -3 position. It is not immediately apparent why Kin1/2 lack a preference for a -5 hydrophobic residue in their substrates. Residues that make contact with the -5 residue in other AMPKR family members are largely conserved in Kin1/2. However, unlike other members of the family, two of these residues are isoleucine in Kin1/2. It is possible that these larger branched hydrophobic residues occlude the -5 interaction pocket, preventing access by larger residues. In addition, Kin1/2 have a lysine residue in the vicinity of the -5 interaction site, and the positive charge may preclude binding of a hydrophobic -5 residue. Kin1/2 also show a unique preference for an asparagine residue two positions upstream (-2) and a phosphorylated serine/threonine residue two residues downstream (+2) of the phosphoacceptor.

Kin1/2 activity against a peptide substrate is increased by ~25 fold when the substrate is phosphorylated at the +2 position. This phenomenon whereby a kinase recognizes a previously phosphorylated substrate is known as substrate priming or hierarchical phosphorylation. Substrate priming is an established method of regulating kinase activity on a substrate, as it requires an extra input for phosphorylation to occur. Some kinases, such as glycogen synthase kinase 3 (GSK3) and Cdc7, recognize primed substrates through structural determinants in their kinase catalytic domain. The Turk laboratory previously found that a patch of basic residues in the αC helix and the αC helix-β3 loop of Kin1/2 are positioned to interact with
the +2 residue on the substrate. Mutational analysis of these residues revealed that Lys183 and Arg187 in Kin1 (corresponding to Lys162 and Arg166 in Kin2) were required for Kin1/2 recognition of primed substrates. Mutation of both of these residues to alanine reduced the activity of Kin1/2 on primed substrates to the same levels they exhibited on unprimed substrates. It is interesting to note that the basic residues needed for Kin1/2 recognition of primed substrates are conserved through most yeast species, although they are not conserved in the human MARKs, which lack a preference for primed substrates\textsuperscript{56}. It was found that the ability to recognize primed substrates is necessary for optimal Kin1/2 function in the UPR, particularly at high concentrations of tunicamycin, although it is dispensable for Kin1/2 function in exocytosis.

While these recent insights have increased our understanding of Kin1/2, there is still much we do not understand about these kinases. While it is known that Kin1/2 are activated by phosphorylation at a conserved threonine in the activation loop, it is not known what kinase(s) is responsible for activation at this site. It is unclear what role the KA1 domain plays in Kin1/2 regulation. Does the KA1 domain autoinhibit the kinase domain of Kin1/2 as it does for the human MARKs? Does it assist with localization of the kinase to sites of polarized growth? Further, are there other factors that assist with Kin1/2 localization to sites of polarized growth, such as interaction with Bud14? High-throughput analyses have hinted at an interaction between Kin1/2 and Bud14, the budding yeast ortholog of tea4. However, an interaction between Kin1/2 and Bud14 has never been independently verified, and it is unknown what impact Bud14 has on Kin1/2 localization or kinase activity. Furthermore, how does localization and kinase activity impact the
function of Kin1/2 in the unfolded protein response. The goal of this study is to answer these questions relating to the regulation of Kin1/2 by kinase activity and localization.
Chapter 2: Intermolecular and intramolecular interactions controlling the localization and activity of the yeast kinases Kin1 and Kin2

BACKGROUND

Protein kinases play a vital role in controlling a wide variety of cellular processes within all living organisms. In order to function in normal physiology, the activity of kinases must be tightly regulated, as evidenced by the pathological impact of kinase hyperactivation in cancer and other human diseases. Numerous mechanisms exist within cells to regulate kinases. Perhaps the most common form of regulation is through phosphorylation by upstream kinases or through autophosphorylation, which typically increases the intrinsic catalytic activity of a kinase. About two-thirds of human kinases, for example, are established or predicted to be activated by phosphorylation at conserved residues within the activation loop, a conformationally flexible region in the catalytic domain. Many kinases are regulated by intra- and/or intermolecular interactions between the catalytic domain and other regions of the protein. Finally, localization of kinase to specific areas or compartments of the cell can target kinases to spatially restricted pools of their substrates. These mechanisms are not mutually exclusive, and often act in cooperation.

Microtubule affinity-regulating kinases (MARKs, also called Par1) are a group of serine-threonine kinases conserved throughout eukaryotes. Based on similarity within
their catalytic domains, MARK/Par1 kinases belong to the AMP-activated kinase related (AMPKR) family\(^2\). MARK/Par1 orthologs across species include the MARKs in mammals, Par1 in \textit{C. elegans} and \textit{D. melanogaster}, kin1 in \textit{S. pombe} and Kin1 and Kin2 (Kin1/2) in \textit{S. cerevisiae}\(^4\)\(^-\)\(^12\). The MARK/Par1 kinases contain an N-terminal catalytic kinase domain, a kinase-associated 1 (KA1) domain at their C terminus, and a large, poorly conserved central region of unknown structure. All isoforms are activated by phosphorylation of a key activation loop threonine residue. Activation loop phosphorylation of mammalian MARKs is catalyzed by the tumor suppressor liver kinase B1 (LKB1) or thousand-and-one kinase (TAOK1)\(^23\), \(^24\). Additionally, the KA1 domain regulates kinase activity by autoinhibiting the kinase domain\(^30\), \(^31\), \(^57\). The KA1 domain also binds to anionic phospholipids, and as such may play a role in localization of MARKs to the plasma membrane\(^36\). It has been suggested that binding of the KA1 domain to the plasma membrane could couple localization to activation of the kinase. In multicellular organisms, MARKs are also regulated by interactions with binding partners such as 14-3-3 proteins, which serve to disrupt membrane localization of the MARKs in response to aPKC phosphorylation\(^39\).

In multicellular organisms, MARKs have an important role in establishing and maintaining epithelial cell polarity\(^3\). This study focuses on the budding yeast MARK/Par1 orthologs Kin1/2\(^9\). Kin1/2 peripherally associate with the plasma membrane, with Kin2 reportedly localizing specifically to sites of polarized growth\(^46\), \(^47\). While Kin1/2 are members of the AMPKR family based on similarity within their catalytic domain, Kin1/2 phosphorylate a divergent motif compared to other members of the family\(^50\). Typical AMPKRs are basophilic and have a strong preference for substrates with an Arg residue.
three positions upstream (-3) of the phosphoacceptor, and for hydrophobic amino acids five residues upstream (-5) and four residues downstream (+4) of the phosphoacceptor. While Kin1/2 share the preference for the +4 hydrophobic residue with other family members, analysis of peptide substrates revealed that they prefer to phosphorylate sites with an Asn two residues upstream of the phosphoacceptor (-2), and a phosphorylated Ser or Thr residue two positions downstream (+2). This preference for phosphorylated, or “primed” substrates is a method of regulation that has been reported in other kinases such as glycogen synthase kinase 3 (GSK3), Cdc7, and the casein kinases. Structural analysis using human MARK2 bound to a pseudosubstrate as a model determined that a cluster of basic residues in the αC helix and the β3-αC helix loop interact with the primed residue on the substrate. Mutation of these basic residues (Lys183/Arg187 in Kin1 or Lys162/Arg166 in Kin2) created mutants that abolished the preference for phosphorylated substrates while maintaining activity towards unphosphorylated substrates.

Kin1/2 have ascribed roles in several cellular processes. A high-copy suppressor screen of secretory pathway mutants revealed that Kin1/2 play a role in exocytosis, where they promote fusion of exocytic vesicles with the plasma membrane through phosphorylation of the t-SNARE protein Sec9. The role of Kin1/2 in exocytosis appears to be dependent on localization to the plasma membrane. Overexpression of Kin2 also leads to defects in septin organization, particularly in conjunction with deletion of the septin-regulating kinase Gin4. Kin2 overexpression furthermore impairs chitin deposition at the cell wall and sensitizes yeast to cell wall disrupting agents such as SDS and calcofluor white. Recently it was reported that Kin1/2 are important for promoting the ER stress-mediated unfolded protein response (UPR). Kin1/2 are believed to promote
processing of Hac1 mRNA by the ER resident transmembrane kinase/RNase Ire1 to produce the mature Hac1 transcription factor. Further, deletion of both Kin1 and Kin2 sensitizes budding yeast to ER stress agents such as tunicamycin. The kinase activity of Kin1/2 is necessary for function in the UPR, and the ability of Kin1/2 to recognize primed substrates is required for optimal function, especially at high concentrations of tunicamycin. It is unclear if Kin1/2 participation in the UPR is related to other cellular functions of Kin1/2, such as exocytosis.

In this study I investigate how Kin1/2 localization and activity are influenced by interaction with the protein Bud14. No fewer than four large scale protein interaction screens have detected an interaction between Bud14 and/or Kin1 and Kin2, although the interaction has never been independently confirmed\textsuperscript{60-63}. Bud14 is a regulatory subunit of the phosphatase Glc7, the budding yeast ortholog of PP1\textsuperscript{64}. Bud14 is a 79 kDa protein containing a central SH3 domain important in the establishment of polarized growth. Bud14 localizes to sites of polarized growth through an interaction with the Kelch proteins Kel1 and Kel2, where it participates in cell polarization in at least two ways\textsuperscript{65, 66}. First, it acts as an inhibitor of the formin Bnr1 to locally regulate actin dynamics\textsuperscript{67, 68}. By acting as an adapter subunit for Glc7, Bud14 promotes local activation of the dynein/dynactin complex to impact microtubule dynamics\textsuperscript{69}. Bud14 also functions in other pathways that share a role in regulating polarity, such as MAPK pathways controlling mating and filamentous growth. Bud14 deletion confers sensitivity to mating pheromone, aberrant formation of mating projections, hyperpolarized growth of projections during filamentous growth. The connection between Bud14 and Kin1/2 is supported by observations that in \textit{S. pombe}, deletion of the single Kin1/2 ortholog \textit{kinl} exacerbates the aberrant growth
phenotype associated with mutants of the Bud14 ortholog tea4. Furthermore, a phosphoproteomics study demonstrated kin1-dependent phosphorylation of tea4 in vivo, though whether this is functionally important is unknown. Given the limited knowledge of both the function and regulation of Kin1/2 in budding yeast, I investigated regulation of the activity and localization of budding yeast Kin1/2. Of particular interest was the impact of interaction with the putative protein binding partner Bud14 on Kin1/2 localization to sites of polarized growth, and whether Kin1/2 localization was also mediated by the KA1 domain, as is the case with the mammalian MARKs. I also investigated the impact of both activation loop phosphorylation and KA1-mediated autoinhibition of Kin1/2 kinase activity. I established that Bud14 interacts with two discrete regions of Kin1/2 to localize the kinases to sites of polarized growth. I also found that the KA1 domain promotes proper localization of Kin1/2, and also regulates their kinase activity. I also examined the extent to which activation loop phosphorylation increases Kin1/2 kinase activity and identified the responsible upstream kinases. Disruption of proper Kin1/2 localization did not impact its ability to function in the UPR. However, regulation of Kin1/2 kinase activity by either the KA1 domain or activation loop phosphorylation influenced its capacity to support growth under conditions of ER stress. Thus, Kin1/2 kinase activity, but not localization, correlates with Kin1/2 function in the UPR.

RESULTS

*Kin1 and Kin2 localize to sites of polarized growth in a manner dependent on Bud14*
It has been reported previously that Kin2 localizes partly to sites of polarized growth in budding yeast\(^4^6\). To compare the localization patterns of Kin1 and Kin2, I expressed them as GFP fusion proteins from their native promoters on low-copy plasmids and visualized them by fluorescence microscopy. As previously reported, I found that in a subset of cells, GFP-Kin2 localized to sites in a diffuse manner at the bud neck or bud tip (Fig 2.1). GFP-Kin1 localized to the same regions, but in some cells formed bright puncta at the cell periphery (Fig 2.2). Bud14 reportedly interacts with Kin1/2 and has a similar pattern of localization\(^6^0-6^3,6^5\). We therefore examined whether localization of Kin1 or Kin2 was dependent on Bud14. We found that in a \(bud14\Delta\) strain, neither Kin1 nor Kin2 localized to sites of polarized growth (Fig 2.3). Re-expression of mCherry-tagged Bud14 rescued Kin1-GFP localization in the \(bud14\Delta\) strain (Fig 2.4). Furthermore, we observed complete co-localization of mCherry-Bud14 with Kin1-GFP (Fig 2.5). These observations are consistent with Bud14 having a role in controlling the subcellular localization of Kin1.

**Proper localization of Kin1/2 requires a direct physical interaction with Bud14**

While large scale screens have implicated Kin1/2 and Bud14 as interaction partners, they have also uncovered a number of common interactors for the two proteins, including Kel1/2, Swe1, Dhh1, and Glc7\(^6^0-6^3,6^6\). It is plausible that Bud14 influences Kin1/2 localization indirectly through an intermediary protein. To examine the importance of a direct Bud14-Kin1/2 interaction, we sought to map their region(s) of interaction using a yeast two-hybrid strategy. We observed a robust interaction between full-length (FL) Kin2
Figure 2.1: Localization of GFP-Kin2 to bud tip (left) and bud neck (right) in dividing yeast cells. GFP-Kin2 was expressed from a low-copy plasmid. Data was collected at 100x magnification.
Figure 2.2: Localization of Kin1-GFP in budding yeast cells. Images are representative of localization to puncta (left), bud tip (center images), and bud neck (right). Kin1-GFP was expressed from a low-copy plasmid. Data was collected at 100x magnification.
Figure 2.3: Quantification of localization of Kin1-GFP in *bud14Δ* cells. Kin1-GFP was expressed from a low-copy plasmid. Every budding cell for each strain was assessed in approximately 20 fields of view at 100x magnification. The percentage of these budding cells displaying the indicated phenotype (P = puncta formation; BT = bud tip localization; BN = bud neck localization) was determined. Five replicates were averaged in the above graph. Data was collected at 100x magnification. Localization of Kin1-GFP WT in *kin1Δ kin2Δ* strain is representative of Kin1-GFP WT localization in subsequent microscopy quantifications when indicated.
Figure 2.4: Expression of mCherry-Bud14 from a low-copy plasmid rescues the localization phenotype of Kin1-GFP. Quantification was performed as described in the legend for Figure 2.3.
Figure 2.5: mCherry-Bud14 and Kin1-GFP colocalize to sites of polarized growth in budding yeast cells. Images are representative of results observed over three replicates.
and Bud14 using a yeast two-hybrid assay (Fig 2.6). In order to map the interacting region, we examined a series of Kin2 fragments in which the protein was truncated from either the N-terminus or C-terminus (Fig 2.7). We found that fragments harboring only the N-terminal 459 residues (including the kinase catalytic domain) (Fig 2.8) or the C-terminal 863 residues (including the KA1 domain) provided only background signals in the assay (Fig 2.9). All larger N- and C-terminal fragments of Kin2, to varying degrees, interacted with Bud14. Because some N- and C-terminal fragments that provided positive signals did not overlap, our results implied that Bud14 interacts with multiple sites within the region comprising residues 459 – 863. To more finely map the sites of interaction, we took advantage of the conserved nature of the Kin1/2-Bud14 interaction. Alignment of the sequences of Kin1 and Kin2 in *S. cerevisiae* with orthologs from yeast species as divergent as *S. pombe* revealed six conserved regions located in the central interacting portion of Kin1 and Kin2 (Fig 2.10). To determine the importance of these regions, we made mutants that deleted each of them in the context of FL Kin2. Two of these mutants, deleting amino acids 749-756 and 801-833, greatly reduced the interaction between Kin2 and Bud14 (Figs 2.11, 2.12). When these two deletions were combined (to generate Kin2-Δ749-756,Δ801-833), the two-hybrid signal was reduced to near background levels (Fig 2.12).

To examine whether its interaction with Bud14 is required for proper localization of Kin1, we made the corresponding mutant (Kin1-Δ709-716,Δ775-806) in the context of the yeast GFP fusion construct. We indeed found that mutating the Bud14 binding sites substantially reduced Kin1 localization to puncta, the bud tip and bud neck (Fig 2.13). The partial localization of Kin1 to these sites may reflect residual weak interaction of Bud14 with this mutant, or some contribution from indirect association with other proteins.
Figure 2.6: Full-length Kin2 and Bud14 interact in yeast two-hybrid analysis. The indicated fusion proteins were expressed from high-copy plasmids. Cultures were grown to mid-exponential phase, and 5-fold dilutions were prepared starting at an OD$_{600}$ of 0.5. 2 μL were spotted onto plates with histidine (+His) or without histidine but containing 3-aminotriazole, and plates were photographed after incubation at 30˚C for 48-72 hours.
Figure 2.7: Truncation constructs used for yeast two-hybrid analysis. Full-length Kin2 (residues 1-1147) is depicted at the top for reference. Red sections denote the catalytic kinase domain of Kin2, while blue sections correspond to the KA1 domain.
**Figure 2.8:** Interaction of Gal4 AD-Kin2 N-terminal fragments with full-length Bud14. Samples were prepared as described in Figure 2.6. Only Gal4 AD-Kin2 constructs that included the N-terminal 863 residues were able to recapitulate the interaction between full-length Kin2 and Bud14. Results are representative of three replicates.
**Figure 2.9:** Interaction of Gal4 AD-Kin2 C-terminal fragments with full-length Bud14. Samples were prepared as described in Figure 2.6. Gal4 AD-Kin2 C-terminal fragments required the region between residues 784 and 863 to recapitulate the interaction between full-length Kin2 and Bud14. Results are representative of three replicates.
Figure 2.10: Alignment of *S. cerevisiae* Kin1 (top row), *S. cerevisiae* Kin2 (middle), and *S. pombe* kin1 (bottom). The N-terminal kinase domain and C-terminal KA1 domain are highlighted in yellow. The six regions highlighted in red are conserved from *S. cerevisiae* to *S. pombe* and are located within the section of *S. cerevisiae* Kin1/2 determined to mediate the interaction between Kin2 and Bud14.
Figure 2.11: Deletion constructs used for yeast two-hybrid analysis. Full-length Kin2 (residues 1-1147) is depicted at the top for reference. Red sections denote the catalytic kinase domain of Kin2, while blue sections correspond to the KA1 domain.
Figure 2.12: Interaction of Gal4 AD-Kin2 deletion constructs with full-length Bud14. Samples were prepared as described in Figure 2.6. Deletion of two regions (corresponding to residues 749-756 and 801-833 in Kin2) resulted in a decreased interaction between Kin2 and Bud14. Combining these two deletions reduced the interaction to near background levels. Results are representative of three replicates.
Figure 2.13: Kin1-GFP lacking the two regions that interact with Bud14 are unable to localize properly to sites of polarized growth. All three localization patterns (puncta formation, bud tip, and bud neck) were greatly reduced compared to Kin1-GFP WT (see Figure 2.3 for comparison). Results are the average of three replicates.
**Kin1 localization is partly dependent on its KA1 domain**

The kinase-associated 1 (KA1) domain of MARKs has been shown to both autoinhibit the kinase domain and to bind anionic phospholipids in a way that may promote localization to cellular membranes. To determine the extent to which the KA1 domain in Kin1 and Kin2 may have similar roles, we first sought to identify likely functional residues within the domain. Alignment of budding yeast Kin1 and Kin2 with the human MARK2 sequence revealed a cluster of conserved basic residues in the Kin1/Kin2 KA1 domains analogous to residues in MARK2 implicated in both kinase autoinhibition and in membrane association (H1040/K1042/K1043 of Kin1 and H1123/K1125/K1126 of Kin2). To test for a role for the KA1 domain in controlling Kin1 localization, we mutated each of these residues to neutral polar amino acids (H1040Q/K1042S/K1043S, hereafter referred to as Kin1-KA1mut) in the context of the Kin1-GFP expression construct. Strikingly, mutation of the KA1 domain entirely eliminated formation of discrete puncta at the periphery (Fig 2.14). Localization to the bud tip was reduced about 50%, while bud neck localization did not change significantly. We do note that the intensities of the signal at the bud neck and bud tip were reduced when compared to that of wild-type (WT) Kin1-GFP (Fig 2.15).

**Kin1 kinase activity is regulated by its KA1 domain independent of its localization or activation loop phosphorylation**

Kin1/2, like other kinases in the AMPKR family have a conserved Thr phosphorylation site in the activation loop that reportedly promotes kinase activity. To examine the impact of activation loop phosphorylation, we mutated the corresponding
Figure 2.14: Kin1-GFP KA1mut is unable to localize properly to sites of polarized growth. Puncta formation was completely abolished in the KA1 mutant (see Figure 2.3 for comparison). Results are the average of three replicates.
Figure 2.15: Kin1-GFP KA1mut bud tip and bud neck localization were reduced compared to WT. Maximum intensity of individual cells was determined using ImageJ. Approximately 100 cells of both WT and the KA1 mutant were analyzed for each replicate. Results are the average of three replicates.
residue in Kin1 (Thr302) or Kin2 (Thr281) and compared their activities to the WT kinases in vitro. For these assays we purified GFP-tagged Kin1 and Kin2 expressed in yeast from their own promoters. Unlike previous investigations of activation loop mutants that used generic substrates such as casein, we used a “primed” peptide substrate derived from the authentic Kin1/2 substrate Sec9. For both Kin1 and Kin2, we observed an approximately 95% reduction in the kinase activity of the activation loop mutants relative to the WT kinases (Fig 2.16). These results indicate that substrate priming does not bypass a requirement for activation loop phosphorylation, as is the case for other kinases such as glycogen synthase kinase 3β. We also observed no change in the localization of the GFP-Kin1 T302A or GFP-Kin2 T281A mutants by fluorescence microscopy (Fig 2.17).

While activation loop phosphorylation has been previously shown to enhance Kin1 and Kin2 activity, the upstream kinase(s) responsible for phosphorylating Kin1/Kin2 have not been identified. Budding yeast have three orthologs of the human MARK kinase LKB1: Elm1, Sak1, and Tos3. All three kinases play a role in activating other members of the yeast AMPK family, either individually or in combination. To determine if these kinases are responsible for phosphorylation of the Kin2 activation loop, we purified GFP-Kin2 from an elm1∆ sak1∆ tos3∆ strain lacking all three of the putative upstream activating kinases. Similar to mutation of Thr281, we observed a more than 95% reduction in the activity of WT GFP-Kin2 purified from the elm1∆ sak1∆ tos3∆ strain relative to an otherwise isogenic WT strain (Fig 2.18). We assessed Kin2 Thr281 phosphorylation by immunoblotting the purified protein with an antibody broadly reactive against the phosphorylated activation loops of AGC and CAMK group kinases. When isolated from WT cells, we detected robust phosphorylation of WT Kin2 at Thr281, but the signal was
Figure 2.16: Activation loop phosphorylation is required for optimal Kin1/2 kinase activity. Point mutants of the putative activation loop threonine were generated for both Kin1 (T302A) and Kin2 (T281A). Activity against a phosphopeptide substrate was determined as described in methods. Results are the average of four replicates. KD = kinase dead.
Figure 2.17: Mutation of the activation loop threonine does not impact localization of Kin1-GFP (see Figure 2.3 for comparison). Results are the average of three replicates.
Figure 2.18: Elm1/Sak1/Tos3 are responsible for activation of Kin1/2 in budding yeast. Wild-type and T281A GFP-Kin2 was purified from both wild-type yeast and a strain lacking Elm1, Sak1 and Tos3. Samples were subjected to a kinase assay as described in methods. Results are the average of three replicates.
greatly reduced in the T281A mutant. Likewise, Kin2 isolated from the elm1Δ sak1Δ tos3Δ strain did not react with the activation loop antibody (Fig 2.19). These results provide strong evidence that some combination of Elm1, Sak1 and Tos3 is responsible for the activation loop phosphorylation of Kin1 and Kin2 in vivo.

Given previous reports that human MARKs are autoinhibited by their KA1 domains, we examined whether the KA1 domain of Kin1/2 had the same function. WT and KA1 domain mutant GFP-Kin1 were purified from yeast and subjected to an in vitro kinase assay against the same phosphopeptide substrate used above. We found a more than eightfold increase in the kinase activity of the Kin1-GFP KA1 mutant relative to WT Kin1-GFP (Fig 2.20). By contrast, we found no difference between WT Kin1 and Kin-KA1mut in the level of activation loop phosphorylation, suggesting that the KA1 domain restrains the kinase activity through a direct autoinhibitory interaction rather than by restricting activating phosphorylation.

To assess any impact that association with Bud14 may have on the kinase activity of Kin1 and Kin2, we performed in vitro kinase assays on the Kin1-GFP-Δ709-716,Δ775-806 construct. We observed a statistically significant, albeit minor, decrease in the kinase activity of the mutant in comparison to WT Kin1-GFP (Fig 2.21). We did not observe any change in the activation loop phosphorylation state of the Kin1-GFP Δ709-716,Δ775-806 mutant relative to wild type Kin1-GFP. We also performed a kinase assay on WT Kin1-GFP that had been purified from both wild type and bud14Δ yeast cells. In this case, we found no difference in either the kinase activity or activation loop phosphorylation of Kin1-GFP regardless of the host strain (Fig 2.22). Collectively, these results suggest that neither
**Figure 2.19:** Elm1/Sak1/Tos3 are required for phosphorylation of the activation loop threonine of GFP-Kin2. Wild-type and T281A GFP-Kin2 was immunoprecipitated from both wild-type yeast and a strain lacking Elm1, Sak1 and Tos3. Phosphorylation was assessed using an antibody reactive to activation loop phosphorylation in AGC family kinases. The result is representative of three replicates.
Figure 2.20: The KA1 domain of Kin1/2 autoinhibits the activity of the kinase. Kin1-GFP WT and KA1 mut were purified from *kin1Δ kin2Δ* yeast. Kinase assays were performed using a phosphopeptide substrate as described in methods. The data is an average of three replicates. The corresponding IP/Immunoblot is representative of three replicates.
Figure 2.21: The Kin1-GFP Δ709-716,Δ775-806 mutant has a minor significant impact on Kin1 kinase activity. Kin1-GFP WT and Δ709-716,Δ775-806 were purified from *kin1Δ kin2Δ* yeast. Kinase assays were performed using a phosphopeptide substrate as described in methods. The data is an average of three replicates. The corresponding IP/immunoblot is representative of three replicates.
Figure 2.22: Bud14 has no impact on Kin1 kinase activity. Kin1-GFP WT was purified from both \textit{kin1}\Delta \textit{kin2}\Delta and \textit{kin1}\Delta \textit{kin2}\Delta \textit{bud14}\Delta yeast. Kinase assays were performed using a phosphopeptide substrate as described in methods. The data is an average of three replicates. The corresponding IP/Immunoblot is representative of three replicates.
interaction with Bud14 nor localization to the cell periphery promote the kinase activity of Kin1.

*Kin1 activation loop phosphorylation, but not its localization to sites of polarized growth, promotes its function in the UPR*

We assessed the function of the various Kin1 mutants in the unfolded protein response (UPR) by performing growth assays in the presence of tunicamycin. We found that yeast expressing Kin1/2 with mutated activation loops were more sensitive to tunicamycin, suggesting that reducing their kinase activity decreases their capacity to promote the UPR (Fig 2.23). We further found that yeast expressing the KA1 domain mutant had increased resistance to tunicamycin. Because this mutation both hyperactivates Kin1 and causes its mislocalization, we further examined tunicamycin sensitivity of yeast expressing the Kin1-Δ709-716,Δ775-806 mutant, which mislocalizes but has similar kinase activity to WT Kin1. This mutant was not impaired in its ability to support growth in the presence of tunicamycin. Collectively, these results provide evidence that the localization of Kin1 and Kin2 has little to no impact on the ability of yeast to maintain a robust UPR. They further suggest that the kinase activity of Kin1 and Kin2 correlates with their ability to act in the UPR.

**DISCUSSION**

Here we have shown that the localization of Kin1/2 is dependent on direct association with Bud14, which interacts with Kin1/2 through two discrete regions in the C-terminal region of Kin1/2. We note that one of the Bud14 binding sites on Kin1/2 contains a P-x-x-P
Figure 2.23: (Top) Kin1 function in the unfolded protein response correlates with its protein kinase activity. UPR function was assessed by growth assay. Mid-exponential phase cultures of Kin1-GFP were spotted on plates in 5-fold dilutions starting at an OD$_{600}$ of 0.5. 2 μL aliquots were deposited onto plates with the indicated concentration of tunicamycin. Results are representative of three replicates. (Bottom) Representative immunoblot showing protein levels. Homocitrate synthase was used as a loading control.
sequence, a common recognition motif for SH3 domains. We were intrigued by the possibility that the interaction was SH3-mediated, although attempts to analyze this using Bud14 with a mutated SH3 domain resulted in a high level of autoactivation in the yeast two-hybrid system (data not shown). In light of previous observations that kin1 phosphorylates tea4 in fission yeast, it is interesting to note that Bud14 contains several sites that nominally fit the preferred phosphorylation motif of Kin1/2, although none of these sites are conserved in fission yeast. While I observed no change in Kin1/2 activation loop phosphorylation in a bud14Δ strain, it is possible that Bud14 facilitates dephosphorylation of other sites on Kin1/2 through recruitment of the Glc7 phosphatase. It is still unknown whether Bud14 is mutually dependent on Kin1/2 for localization to sites of polarized growth. It is also unclear what role localization of Kin1/2 to sites of polarized growth plays in yeast cells generally. It is possible that Kin1/2 regulate Bud14 (and by extension, Glc7), or perhaps Kin1/2 and the Bud14/Glc7 phosphatase complex act upon common substrates at sites of polarized growth.

As for a variety of orthologous kinases from human MARKs to C. elegans Par1 and fission yeast kin1, we have shown that the KA1 domain in Kin1 is necessary to properly localize the kinase to sites of polarized growth, in particular being necessary for puncta formation. Since I also found that the KA1 domain autoinhibits the kinase domain of Kin1/2, my results support a model by which KA1-domain binding to anionic phospholipids serves to both localize and activate the kinase. Previous research on human MARKs also suggests that protein binding partners such as Gab1 serve to activate the kinase by disrupting the interaction between the KA1 domain and the kinase domain. Given the lack of impact on kinase activity even in the absence of Bud14, the data suggest
that Bud14 does not play a similar role in yeast. My research found that the autoinhibition of Kin1/2 by the KA1 domain was more dramatic than that found in the MARKs, where an approximately 2-fold impact on kinase activity has been reported.

The activation loop phosphorylation of Kin1/2 by Elm1/Sak1/Tos3 confirms that this trio of kinases activates all six members of the AMPK family in budding yeast\textsuperscript{25-29}. Elm1/Sak1/Tos3 are partially redundant in their ability to activate other members of the AMPK family, although some members of the family exhibit a preference for one upstream activating kinase or another. Sak1, for example, is the primary activating kinase of Snf1, while Elm1 is responsible for activation of Gin4. Further research will be needed to show the degree to which Elm1, Sak1, and Tos3 are redundant in activation of Kin1/2.

We are interested in how Kin1/2 may participate in regulation of cell polarity in budding yeast cells. Cell wall biosynthesis kinase (Cbk1) is an important regulator of cell growth, division, and establishment of cell polarity. Cbk1 and Kin1/2 have overlapping patterns of localization to the bud neck and cortex and Cbk1 participates in cellular processes in common with other MARK/Par1/Kin orthologs, and notably Cbk1 was reported to interact with both Kin1 and Kin2 in two high throughput studies\textsuperscript{61,63}. The more C-terminal region of Bud14 interaction on Kin1/2 (775-806 in Kin1, 801-833 in Kin2) contains sequence that perfectly matches the phosphorylation motif of Cbk1 (H-x-R-x-x-pS at Ser799 on Kin1, Ser826 on Kin2) it is possible that phosphorylation at this site regulates the interaction between Kin1/2 and Bud14\textsuperscript{73}. In a preliminary study, I examined localization of Kin1/2 in a strain deficient for Cbk1 (\textit{cbk1Δ ssd1Δ}), but no significant defect in Kin1/2 localization was observed (not shown). Mutational analysis of the putative Cbk1
site on Kin1/2 would be required to definitively rule out a regulatory interaction between Cbk1 and Kin1/2.

While we did not observe localization of Kin1/2 to the ER, it may be that they relocalize under conditions of ER stress. Alternatively, it is possible that a small pool of Kin1/2 undetectable by fluorescence microscopy is sufficient to promote the UPR. It is also possible that Kin1/2 function in the UPR and exocytosis are connected to an overall role in cell polarization in ways that are not fully understood.

**EXPERIMENTAL PROCEDURES**

*Antibodies* – Antibodies used in this study and their sources were: anti-GFP (600-101-215; Rockland), anti-phospho-Kin1/2 (AGC kinase motif antibody, clone A1K4K; Cell Signaling Technology), anti-Gal4 activation domain (135398 14-7E10G101; Abcam). All other antibodies were from Santa Cruz Biotechnology: anti-Gal4 DNA binding domain (RKC51 sc-510), anti-Fus3 (yC-19 sc-6773), and Anti-HCS (31F5).

*Plasmids* - Low-copy plasmids expressing C-terminally GFP-tagged Kin1 and Kin2 (pRS316-Kin1-GFP, pRS316-Kin2-GFP) were generous gifts from Peter Pryciak (University of Massachusetts). The low copy plasmid expressing N-terminally GFP-tagged Kin2 from its own promoter was previously described\(^5\). All point mutants made on these plasmid backgrounds were constructed by site-directed mutagenesis using the QuikChange protocol. The pGBT9 bait and the pGAD GH prey yeast two-hybrid plasmids were
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generously supplied by Chris Burd’s laboratory (Yale University). pGBT9-Bud14 was produced by PCR amplification of the Bud14 coding sequence from the *S. cerevisiae* genomic tiling collection and inserted into pGBT9 using the EcoRI site. Kin2 coding sequences for construction of the two-hybrid prey vectors were PCR amplified using pRS416-GFP-Kin2 as a template. N-terminal Kin2 fragments were cloned into pGAD GH using the BamHI and XhoI sites; fragments of Kin2 C-terminus were cloned using an inserted 5’ EcoRI site (generated from pRS416: GFP-Kin2 by PCR amplification) and SalI. Full-length pGAD GH-Kin2 was created by inserting the Kin2-N457 fragment into pGAD GH-Kin2-C457. All loop-out mutants were created by the QuikChange protocol using pGAD GH-Kin2 as a template. pRS315-mCherry-Bud14 was produced by Gibson assembly using the pBG1442 vector generously provided by Bruce Goode as a template for the mCherry-Bud14 sequence.

**Yeast strains**- Single deletion strains replacing the relevant gene with the kanMX cassette in the BY4741 background were from the yeast deletion collection (Stanford University) and purchased from Open Biosystems. The *kin1Δ kin2Δ* strain was described previously. To construct the *kin1Δ kin2Δ bud14Δ* strain, we first disrupted *KIN2* by insertion of a *LEU2* expression cassette in the *bud14Δ* single deletion strain, and then inserted the *HIS3* gene at the *KIN1* locus.

In vitro *kinase assays*- Yeast strains expressing GFP-tagged Kin1 or Kin2 and their respective mutants were grown in 500 mL of selective media to mid-exponential phase
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growth (~0.8 OD\textsubscript{600}). Approximately 500 OD\textsubscript{600} units of cells were centrifuged at 4°C, 22,000 x g for 15 min, washed with dH\textsubscript{2}O, centrifuged again at 4°C for 10 min at 6,000 x g, washed with dH\textsubscript{2}O, flash-frozen with liquid nitrogen, and stored at -80°C for later use in kinase assays or immunoblots. Cell pellets were thawed on ice and lysed in buffer containing 50 mM Tris, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 10% glycerol, 2 mM EGTA, 2 mM EDTA, 10 mM β-glycerophosphate, 2 mM NaF, 800 μM Na\textsubscript{3}VO\textsubscript{4}, 1 μM aprotinin, 10 μM leupeptin, 1 μM pepstatin A, and 2 mM DTT. Cells were disrupted by beating with 400 mg of glass beads per 1 mL of lysate (10 x 1 min rounds of vortexing at 4°C), and the lysate was clarified by centrifugation at 4°C for 10 min at 16,000 x g, transferred to a new Eppendorf tube, and protein concentration determined by Bradford assay. Equal amounts of protein were incubated with GFP-nanotrap beads (10 μL of packed beads per 1 mL of cell lysate, pre-equilibrated with lysis buffer) with rotation at 4°C for 1-4 hr. Beads were pelleted by centrifugation at 2,000 x g for 2 min at 4°C, washed twice in lysis buffer, then washed twice in kinase assay buffer (50 mM HEPES pH 7.4, 10 mM MgCl\textsubscript{2}, 0.1% Tween-20, 1 mM DTT).

The substrate peptide was previously reported and had the sequence RNSLNHSNpSTLNKKK, where pS indicates phosphoserine\textsuperscript{51}. Peptide kinase assays were performed by suspending approximately 10 μL of packed GFP nanotrap beads loaded with GFP-tagged Kin1 or Kin2 (prepared as described above) in 20 μL of kinase assay buffer containing 100 μM peptide. Reactions were initiated by adding ATP to 50 μM (with 0.05 μCi/ml [γ-\textsuperscript{32}P]-ATP) and transferring to a 30°C heat block. Samples were agitated by hand every 2-3 min to prevent settling of beads. Aliquots (5 μL) were removed at 15 min intervals and spotted onto P81 phosphocellulose filters, which were immediately quenched.
in 75 mM phosphoric acid. Filters were washed three times in the same solution, air dried, and analyzed by scintillation counting. Phosphate incorporation into peptides was calculated from standards consisting of varying amounts of reaction mixtures spotted onto filters that were left unwashed.

*Fluorescence microscopy*- Cells were grown overnight in selective medium, and the density was adjusted by dilution into fresh medium to allow for ~4 doublings before harvest at mid-exponential phase growth (OD$_{600}$ = 0.5-1.0). To image cells, 1 mL of culture was centrifuged (2 min at 4K rpm, room temperature), the supernatant was aspirated, and the cells were resuspended in 10-20 μL of fresh selective medium. The suspension (1-2 μL) was then deposited on a glass slide and visualized using a 100x magnification oil-immersion objective on a Nikon Eclipse Ti microscope using μ-Manager software. Subsequent analysis was performed using ImageJ.

*Yeast two-hybrid analysis and growth assays*- For two-hybrid analysis, yeast (strain PJ69-4a) transformed with the indicated plasmids were grown to mid-exponential phase in 50 mL of selective medium (SC-Leu-Trp). Aliquots (2 μl) of a 5-fold dilution series (highest OD$_{600}$ = 0.5) were spotted onto control agar plates (SC-Leu-Trp) and agar plates (SC-Leu-Trp-His) containing the indicated concentrations of 3-aminotriazole (3AT), which were grown at 30 °C for 72–96 h. The remainder of the cultures (~50 mL) was pelleted by centrifugation (5 min at 6,000 x g, 4°C), washed with ddH$_2$O, repelleted, snap frozen in liquid N$_2$, and stored at -80°C and subsequently used for immunoblot analysis.
For growth assays, the indicated yeast strains were grown to mid-exponential phase in 5 mL of selective medium. Dilutions were prepared as above and spotted onto SC-Ura agar plates containing the indicated concentrations of tunicamycin, which were grown at 30 °C for 48–72h. For experiments examining tunicamycin sensitivity of Kin2 mutants, a portion of the culture was retained to prepare cell lysates for immunoblotting. The cells (~10 OD\textsubscript{600} units) were collected by centrifugation, washed with ddH\textsubscript{2}O, repelleted, flash frozen in liquid N\textsubscript{2}, and stored at -80 °C.

*Immunoblotting*- Cell lysates for immunoblots were prepared by mechanical disruption and TCA precipitation as described using 1 mL of 10% TCA extraction buffer (10 mM Tris, pH 8.0; 10% trichloroacetic acid; 25 mM NH\textsubscript{4}OAc; 1 mM Na\textsubscript{2}EDTA). For samples harvested from yeast two-hybrid experiments, the protein pellet was then resuspended in 300 μL of 500 mM Tris-HCl, pH 11.0 and 3% SDS, heated at 95°C for 5 min, and centrifuged for 1 min at 16,000 x g at 4°C. 240 μL of the lysate was combined with 80 μL of 4x SDS-PAGE loading buffer and boiled for 5 min. The remainder was used to determine protein concentration by BCA assay. Equal amounts of protein were separated on a 7.5% acrylamide gel, transferred to a PVDF membrane overnight (18V for 15hr), and blocked in 5% milk for 1 hr at room temperature. The samples were visualized on a LiCor Odyssey CLx using fluorophore-conjugated secondary antibodies. Samples prepared from microscopy experiments were prepared in the same way as above with the exception of being resuspended in 150 μl of 100 mM Tris HCl, pH 11.0, and 3% SDS prior to boiling.

To immunoblot samples in pulldown experiments, approximately 10 μL of packed beads were suspended in 25 μL of 4x SDS-PAGE loading buffer, heated at 95°C for 5 min,
separated on a 7.5% acrylamide Laemmli gel, and transferred to PVDF membranes. Membranes were blocked in 5% milk for 1 hr at room temperature and incubated overnight at 4°C with primary antibodies diluted in 5% BSA. Membranes were treated with fluorophore-conjugated secondary antibodies and visualized on a LiCor Odyssey CLx system.
Appendix: Phosphorylation-dependent regulation of Ste5 in the *S. cerevisiae* mating pheromone pathway

**ABSTRACT**

Scaffold proteins can perform important functions in the regulation of signal transduction cascades, such as providing specificity and promoting signal amplification. Post-translational modification of scaffold proteins provides a mechanism for crosstalk regulation between signaling pathways. In this study I have shown that the budding yeast MAPK scaffold Ste5 is regulated by phosphorylation. Ste5 is phosphorylated at Ser898 *in vivo* to stabilize the protein, thus promoting signal promulgation within the cell. Although it is unclear what condition(s) are responsible for regulating phosphorylation at Ser898, my results suggest that Ser898 phosphorylation is an important facet in the control of Ste5 protein levels.

**BACKGROUND**

Signal transduction cascades are utilized by living cells to adapt to changes in their extracellular environment. Scaffold proteins can have important roles in these processes by arranging components of signaling pathways to optimize relay of the signal\textsuperscript{75, 76}. In addition, scaffold proteins can contribute to dynamic regulation of signaling flux. A key example of the essential role played by scaffold proteins in signal transduction involves mitogen-activated protein kinase (MAPK) cascades in the budding yeast *Saccharomyces cerevisiae*\textsuperscript{77, 78}. The four yeast MAPK pathways control various functions including mating, filamentous growth, response to osmotic stress, and cell wall integrity. The mating
and filamentous growth pathways in particular mediate distinct morphological changes under disparate conditions. Mating pathway activation prepares yeast for fusion with another cell and is optimal under nutrient rich conditions\textsuperscript{79, 80}. By contrast filamentous growth leads to morphological changes that enable foraging for nutrients and is activated during starvation\textsuperscript{81}. Notably, while these two pathways lead to different cellular responses, they share key signaling intermediates\textsuperscript{82}. The mating pathway is activated by the binding of pheromone to the G protein-coupled receptors (GPCR) Ste2 and Ste3, while the filamentous growth pathway is activated by the cell surface mucin Msb2, which detects nutrient deficiency by an unknown mechanism\textsuperscript{77}. These signals are promulgated inside the cell by both heterotrimeric G-proteins (Gpa1/Ste4/Ste18) and small G-proteins (Cdc42) in the case of the mating pathway, while Cdc42 also plays an important role in activation of the filamentous growth pathway\textsuperscript{77, 81}. The two pathways share common MAPKKKKs (Ste20), MAPKKKs (Ste11), and MAPKKs (Ste7). One key factor in mediating the differential cellular outputs is activation of the respective MAPKs, Fus3 in the case of the mating pathway, and Kss1 in the case of filamentous growth. Activation of Fus3 versus Kss1 involves the scaffold protein Ste5, which diverts signaling preferentially to Fus3 and promotes transcription of mating pheromone-responsive genes\textsuperscript{83, 84}.

Ste5 is a large protein that contains binding sites for Ste11, Ste7, and Fus3, orienting the constituents of the MAPK cascade for activation by phosphorylation\textsuperscript{77, 85-87}. Ste5 also induces conformational changes in Fus3 to promote its phosphorylation by Ste7\textsuperscript{88}. Ste5 undergoes shuttling between the nucleus and cytoplasm under vegetative growth conditions\textsuperscript{89-91}. Once a cell receives a pheromone signal, Ste5 is localized to the plasma membrane via a unique plasma membrane targeting domain, a pleckstrin homology (PH)
domain, and a binding site for the heterotrimeric G-protein Gβ subunit Ste4\textsuperscript{92}. Adaptor proteins such as Ste50 also help to localize Ste5 at the cell membrane and facilitate Ste11 activation by Ste20, thus promoting proper and efficient interplay of the crosstalk between the mating and filamentous growth pathways.

Given the nutrient-regulated nature of both mating and filamentous growth, it stands to reason that signaling between the two pathways may be regulated by crosstalk with other nutrient-sensitive pathways. Indeed, Elm1, one of the three upstream activating kinases of the budding yeast AMP-activated protein kinase (AMPK) ortholog Snf1, may negatively regulate the mating pathway through phosphorylation of Gpa1, the Gα subunit associated with the Ste2/3 pheromone receptors\textsuperscript{93}. Likewise, deletion of Reg1, the phosphatase that dephosphorylates and inactivates Snf1 in the presence of glucose, leads to downregulation of the mating pathway.

In this study, I investigate the possibility that the scaffold protein Ste5 serves to regulate nutrient-sensitive crosstalk between the mating pathway and filamentous growth pathway. In particular, I explore phosphorylation of Ste5 at Ser898 as a potential means of regulating this crosstalk. I found that Ste5 Ser898 is indeed phosphorylated \textit{in vivo}, and that phosphorylation at this site stabilizes the protein. While phosphorylation of Ste5 at Ser898 does not appear to be glucose sensitive, under some conditions it appears to promote signaling through the mating pathway. Collectively, my studies reveal a new mode of crosstalk regulation of Ste5 by an as yet unidentified kinase.
RESULTS

In vivo phosphorylation of Ste5 on Ser898

I first assessed whether Ste5 Ser898 was phosphorylated in vivo. Prior phosphoproteomic studies have detected phosphorylation of Ste5 at Ser898, and the residues surrounding Ser898 conform to the preferred phosphorylation motifs of both Snf1 and Tpk1/2/3, major nutrient-sensitive kinases (Fig A.1). Attempts to generate a Ste5 phospho-Ser898 antibody from a commercial source were unsuccessful (not shown). As an alternative, I used an anti-phospho PKA substrate antibody that recognizes the epitope R-R-x-pS/pT (hereafter called anti-phosphoSer898) on protein immunoprecipitated from cell lysates via a Myc epitope tag. I was able to use this system to detect phosphorylation of Ste5 Ser898, and the reactivity of the antibody was greatly reduced when Ser898 was mutated to alanine (Fig A.2).

Ser898 controls Ste5 protein levels

Phosphorylation can impact protein stability and play a role in regulating cellular levels of a given protein. I noted that mutation of Ser898 led to an approximately 50% decrease in the levels of Ste5 protein in the cell (see Fig A.2, Fig A.3). Phosphorylation of Ser898 stabilizes Ste5 and increases its half-life (Fig A.4). Exposure to pheromone has been shown to increase the half-life of Ste5. I found that, even after pheromone treatment, phosphorylation of Ser898 increased cellular levels of Ste5 protein by 50% (Fig A.5). I also observed that pheromone treatment did not have an impact on Ser898 phosphorylation (see Fig A.5). These results indicate that phosphorylation of Ste5 Ser898 controls Ste5 protein levels independently of mating pathway activation.
Figure A.1: (Above) Schematic of Ste5 scaffold protein, to scale. Amino acids important for interaction with proteins in the budding yeast MAPK cascade are depicted as black boxes. Regions of the protein that interact with lipids or participate in structural rearrangement are depicted underneath the protein. Ser898 is denoted by a red dash. (Below) The Ste5 Ser898 site matches the consensus phosphorylation sequence of Snf1 and Tpk1/2/3.
**Figure A.2:** Ste5 Ser898 is phosphorylated *in vivo*. Myc-Ste5 (WT and S898A) was expressed in ste5Δ cells on a low-copy plasmid and purified using a Myc antibody conjugated to agarose beads. Phosphorylation was determined using a phospho-PKA substrate antibody. Three-fold increases in total protein was loaded into lanes left to right.
Figure A.3: Ser898 controls Ste5 protein levels. Mutation of Ser898 to alanine leads to a ~50% decrease in Ste5 protein level. Myc-Ste5 was immunoprecipitated as described in methods. Results are averaged from five experiments. Error bars represent +/- S.E.M.
Figure A.4: Ser898 controls Ste5 protein stability. The half-life of Ste5 was assessed by a cycloheximide pulse experiment. Cultures of Myc-Ste5 WT and S898A mutant were grown to mid-exponential phase, treated with 1 μM cycloheximide, and aliquots were removed at the indicated times. Homocitrate synthase was used as a loading control. Results are the average of three replicates.
Figure A.5: Ste5 Ser898 phosphorylation is not pheromone-responsive. Myc-Ste5 was purified from ste5Δ cells and assessed for levels of Ste5 protein and for in vivo phosphorylation of Ser898. Results are representative of three replicates.
Ste5 Ser898 is required for an optimal mating pheromone response

As a measure of flux through the mating pathway, I examined phosphorylation of the MAPK Fus3 within its activation loop. For the experiments described in this section, I expressed Ste5-Myc on a low-copy plasmid under the control of its native promoter in a ste5Δ strain. I performed a time course monitoring Fus3 activation loop phosphorylation after stimulation with α-factor mating pheromone in yeast expressing either wild-type (WT) Ste5 or the Ste5-S898A mutant. I found that activation loop phosphorylation of Fus3 was significantly reduced in the strain expressing the S898A mutant (Fig A.6).

Activation of the budding yeast mating pathway ultimately leads to transcription of genes necessary for the mating process. I used a transcriptional reporter consisting of the FUS1 promoter placed upstream of the lacZ gene to measure the output of the mating pathway. I found that mutation of Ser898 to Ala lead to an approximately 50% reduction in transcriptional activation of the mating pathway as indicated by β-galactosidase activity measured in cell lysates (Fig A.7) or intact cells (Fig A.8). I observed a similar reduction in mating pathway activation when mutating Ser898 to asparagine (Fig A.9). Mutation of Ser898 to a phosphomimetic Glu residue restored much of the WT transcriptional activation phenotype, suggesting that phosphorylation of Ser898 is vital to its role in promoting mating pathway signaling. To determine whether impaired pheromone signaling was due to reduced Ste5 protein levels, I examined the impact of increasing levels of Ste5-S898A by expressing it from two separate plasmids. I found that adding a second Ste5-S898A plasmid restored WT levels of transcriptional activity (Fig A.10, Fig A.11) without impacting the half-life of Ste5 (data not shown).
Figure A.6: Phosphorylation of the Fus3 activation loop. *ste5Δ* cells in exponential-phase growth were treated with 1 μM α-factor and aliquots were removed at the indicated time points. Activated Fus3 (pFus3) was quantified against both total Fus3 levels and against a loading control (homocitrate synthase) by immunoblotting. Results are the average of five replicates.
Ser898 controls transcriptional activation of the yeast mating pathway. β-galactosidase activity was quantified in whole cell lysates prepared from WT and ste5Δ cells. Cells were exposed to 1 μM α-factor for one hour prior to harvest. Results were normalized to ste5Δ:Ste5-WT +/- S.E.M. Results are the average of five replicates.

Figure A.7: Ser898 controls transcriptional activation of the yeast mating pathway. β-galactosidase activity was quantified in whole cell lysates prepared from WT and ste5Δ cells. Cells were exposed to 1 μM α-factor for one hour prior to harvest. Results were normalized to ste5Δ:Ste5-WT +/- S.E.M. Results are the average of five replicates.
Figure A.8: β-galactosidase activity was quantified in vivo in \( ste5^\Delta \) cells as described in methods. Results were normalized to \( ste5^\Delta:Ste5\,WT +/- \) S.E.M. Results are the average of five replicates.
Figure A.9: β-galactosidase activity of the S898N mutant was quantified in vivo in ste5Δ cells. WT and S898A results are included for comparison. Results are the average of three replicates.
**Figure A.10:** Expressing Ste5 from two plasmids restored levels of Ste5 protein in cells to an intermediate level. Levels of total Ste5 were determined using homocitrate synthase as a loading control. Results are the average of three replicates.
Figure A.11: Expressing Ste5 from two plasmids restored the transcriptional activation of the mating pathway to WT levels. β-galactosidase activity of the S898A mutant expressed from two plasmids was quantified in vivo in ste5Δ cells. Results are the average of three replicates.
There are three additional phosphorylatable residues in the immediate vicinity of Ser898 (Ser897, Ser900, and Ser901). I examined the impact of mutation of each of these three residues to alanine individually and found that none of them had a profound impact on transcriptional activity in the mating pathway. However, combined mutation of all four Ser residues to Ala (Ste5-AAAA) modestly reduced the level of transcriptional activation in comparison to the single S898A mutation (Fig A.12). Previous research had demonstrated that mutation of Arg895 to Gly impaired the ability of Ste5 to bind Ste794. I mutated Arg895 to Gly under the assumption that this residue may additionally play a key role in recognition by the kinase that phosphorylates Ser898. I found that the R895G mutant showed a significant decrease in transcriptional activation of the mating pathway, similar to the decrease I observed in the Ser898Ala mutant (Fig A.13).

As another measure of signaling output from the mating pathway, I examined pheromone-induced cell cycle arrest using a halo assay. I noted a marked reduction in cell cycle arrest as evident from a reduction in both the diameter and intensity of the halo for the S898A mutant (Fig A.14). I found that mutation of Ser898 to the phosphomimetic Glu residue restored the cell cycle arrest phenotype of WT Ste5. Taken together, these results suggest that phosphorylation at Ser898 is required for optimal activation of the mating pathway and for maximal downstream responses.

*Mutation of Ste5 Ser898 at its endogenous locus impacts protein levels but not signaling output from the mating pathway*

The results above had been obtained by expressing Ste5 from a low-copy plasmid in a ste5Δ strain. I noted that in both transcriptional reporter assays and in halo assays, re-
Figure A.12: Mutation of three serines adjacent to Ser898 lead to a minor additional decrease in transcriptional activation of the mating pathway relative to the S898A mutant. β-galactosidase activity of the AAAA mutant was quantified in vivo in ste5Δ cells. Results are the average of four replicates.
Figure A.13: Mutation of a key Arg residue adjacent to Ser898 leads to a significant decrease in transcriptional activation of the mating pathway equivalent to S898A mutation. β-galactosidase activity of the R895G mutant was quantified in vivo in ste5Δ cells. Results are the average of three replicates.
Figure A.14: Ste5 Ser898 phosphorylation is required for optimal mating response. Wild-type and ste5Δ yeast were transformed with the indicated plasmid and a halo assay was performed as described in methods. The size and intensity of the halo is markedly decreased in the S898A mutant, while the halo produced by the S898E mutant is similar to or stronger than ste5Δ: Myc-Ste5 WT. The results are representative of three replicates.
expression of WT Ste5 from a plasmid did not result in full activation of the pathway as seen in \textit{STE5} cells. This suggested that the low copy plasmid may underexpress Ste5 relative to expression from its endogenous locus. To examine the requirement for phosphorylation of endogenously expressed Ste5, strains were engineered to incorporate a C-terminal Myc epitope tag at the endogenous \textit{STE5} locus, in conjunction with either the WT sequence, the S898A mutation, or the AAAA mutation. I found that the S898A mutation reduced total Ste5 protein levels by roughly 50\%, consistent with what I had observed when expressing Ste5 from a plasmid, confirming that Ser898 controls Ste5 protein levels (Fig A.15). However, I observed no change in pheromone-induced cell-cycle arrest or activation of the transcriptional reporter between cells harboring WT Ste5 and either Ste5-S898A or Ste5-AAAA. These results suggest that while Ser898 indeed controls Ste5 protein levels, the endogenous levels of Ste5 protein are sufficient to support an optimal mating response even when its phosphorylation is prevented (Fig A.16).

\textit{Ste5 Ser898 is phosphorylated by an as yet unidentified kinase}

I attempted to determine the kinase(s) responsible for phosphorylating Ste5 Ser898 \textit{in vivo} by observing the phosphorylation of Ser898 in yeast strains harboring deletions of various kinases of interest. These kinases were chosen based on having a phosphorylation site motif matching the sequence surrounding Ser898 and for their roles in nutrient sensing. I found that Ser898 phosphorylation was unchanged in the absence of Snf1, under both high and low glucose conditions, suggesting that Snf1 plays no role in regulating this site. I also examined other members of the AMPK family: Hsl1, Kcc4, and Gin4, and found that deletion of any of these kinases had no effect on Ste5 Ser898 phosphorylation. I examined
Figure A.15: Levels of endogenously expressed Ste5 are controlled by Ser898 phosphorylation. The S898A mutation reduces Ste5 protein levels by 50% when expressed under the control of the endogenous STE5 promoter. Results are the average of three replicates.
Figure A.16: When Ste5 is expressed under its endogenous promoter, Ser898 phosphorylation does not impact transcriptional activation of the mating pathway. Results of three replicates are depicted for each clone of Ste5 WT (n=8), Ste5 S898A mutant (n=7), and Ste5 AAAA mutant (n=5). The x-axis represents clones, each of which was assayed four times.
a strain lacking all three upstream activating kinases for the AMPK family members, Elm1/Sak1/Tos3, and found no effect on Ser898 phosphorylation, ruling out redundant phosphorylation of Ser898 by multiple members of the Snf1 family. Since the sequence surrounding Ser898 also matched the phosphorylation motif of Tpk1/2/3, the yeast homolog of PKA and another important glucose-sensitive kinase, I examined strains for each of these kinases and found no change in Ser898 phosphorylation. Likewise, Ser898 is not phosphorylated by Sch9, the budding yeast ortholog of mammalian S6 kinase and a major target of TOR phosphorylation, or Ste20, the MAPKKKK in the yeast mating pathway. These results indicate that the kinase responsible for Ser898 phosphorylation may not be directed by the motif surrounding the site.

Ste5 Ser898 is constitutively phosphorylated under a variety of culture conditions

Given that Ser898 phosphorylation leads to a reduction in Ste5 protein levels, I wanted to find a condition that regulated phosphorylation at that site, as this may lead to a reduction in Ste5 that could impact mating efficiency. I made use of our integrated S898A construct to examine the impact of glucose withdrawal, growth in alternative carbon sources, high salt conditions (0.4 M NaCl, 1 M NaCl), osmotic stress induced by sorbitol (0.5 M and 1 M), and inhibition of protein synthesis using the mTORC1 inhibitor rapamycin (50 nM and 200 nM). As judged by both halo assays and transcriptional reporter assays, I was unable to discern any impact in the mating response under these various conditions.

Ste5 is known to shuttle between the nucleus and cytoplasm by means of a nuclear localization sequence (NLS). Previous studies have shown a decrease in transcriptional activation of the mating pathway in yeast expressing Ste5 with a non-functional NLS. I
mutated the NLS in both WT and S898A Ste5 to produce the Ste5-NLSmut and determined the impact of a functional NLS on transcriptional activation of the mating pathway. I discerned no Ser898-specific impact on transcriptional activation from the NLSmut (data not shown). This suggests that Ser898 does not influence nuclear shuttling via the NLS.

**DISCUSSION**

This study establishes a role for Ser898 phosphorylation in controlling the stability of the Ste5 scaffold protein in budding yeast. The functions of scaffold proteins are critically dependent on their level of expression\textsuperscript{95}. Insufficient concentrations of a scaffold protein will fail to bring signaling components into proximity, while too many copies can titrate components away from each other. Interestingly, prior studies have suggested that under standard growth conditions, cellular Ste5 levels do not provide maximal output from the mating pathway\textsuperscript{96}. Expression of Ste5 from promoters of varying strength indicated that endogenous levels of Ste5 are about 90% less than necessary to maximize signal output. Systems-level modeling suggested that there is a trade-off between the level of signal output and the dynamic range of the system. The levels of Ste5 protein appear to be a key determinant in maintaining this balance, illustrating the importance of Ste5 scaffold levels in cellular decision-making. Further indication of the importance of Ste5 levels is suggested by the regulation of Ste5 stability in response to pheromone stimulation. In cells that have not been exposed to pheromone, Ste5 is localized primarily in the nucleus, and the half-life of the protein is \(~30\text{ min}\) due to ubiquitin-proteasome mediated degradation. Ste5 is shuttled between the nucleus and the cytoplasm, providing a pool of the scaffold available when a pheromone signal is received. Upon pheromone stimulation, Ste5 translocates to
the plasma membrane, and is stabilized such that its half-life is in excess of two hours. Stabilization of Ste5 in response to pheromone treatment is likely important in propagation of signaling through the mating pathway as well as in preventing aberrant activation of the mating response in the absence of pheromone.

The notion that phosphorylation is vital to stabilization of Ste5 is not without precedent. For example, degradation of Ste5 in the nucleus in the absence of pheromone is mediated by Cdc28 phosphorylation\textsuperscript{97-99}. Cdc28 phosphorylation also prevents Ste5 association with the plasma membrane, thus promoting the return of Ste5 to the nucleus for degradation\textsuperscript{98}. Cdc28 involvement also ties Ste5 stability to the cell cycle, helping to enforce pheromone-induced cell-cycle arrest in G\textsubscript{1} phase. Upon pheromone stimulation and ensuing relocation to the plasma membrane, Ste5 is phosphorylated by various members of the budding yeast mating pathway, especially Fus3 but also Kss1\textsuperscript{97, 100, 101}. This constitutes a positive feedback loop that assists with Ste5 localization to the plasma membrane, thus preventing nuclear re-entry and further stabilizing Ste5 during mating pathway activation.

While I have confirmed that phosphorylation of Ste5 Ser898 controls the stability of the scaffold protein, it remains unclear what impact this phosphorylation has on mating pathway signaling with endogenous levels of Ste5. It is possible that under certain conditions phosphorylation at Ser898 has a discernable impact on mating pathway activation. For example, growth conditions in which Ste5 protein levels are reduced could require phosphorylation at Ser898 to promote mating pathway signaling. While we were unable to identify such conditions in this study, one can envision untested stimuli that could impact the ability of a yeast cell to mate, such as exposure to extremes of temperature or
pH. Determination of such a condition would also facilitate the discovery of the kinase(s) responsible for phosphorylation of Ste5 Ser898, and yield insight into dynamic regulation of the budding yeast mating pathway.

**EXPERIMENTAL PROCEDURES**

*Analysis of Ste5 phosphorylation*- Yeast strains expressing Myc-tagged WT Ste5 and respective mutants were grown in 50 mL of selective medium to mid-exponential phase growth (OD$_{600}$ = ~0.8). Approximately 50 OD$_{600}$ units of cells were centrifuged at 4°C, 22,000 x g for 15 min, washed with ddH$_2$O, and re-centrifuged twice, flash-frozen with liquid nitrogen, and stored at -80°C. Cell pellets were thawed on ice and lysed in buffer containing 50 mM Tris, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 10% glycerol, 2 mM EGTA, 2 mM EDTA, 10 mM β-glycerophosphate, 2 mM NaF, 800 μM Na$_3$VO$_4$, 1 μM aprotinin, 10 μM leupeptin, 1 μM pepstatin A, and 2 mM DTT. Cells were disrupted with 400 mg of glass beads per 1 mL of lysate with 10 x 1 min rounds of vortexing at 4°C. The lysate was clarified by centrifugation at 4°C for 10 min at 16,000 x g, and protein concentration determined by Bradford assay. Equal amounts of protein were incubated with 20 μL of packed agarose beads bound to anti-Myc antibody (ThermoFisher, #20168) per 1 mL of cell lysate, pre-equilibrated to lysis buffer and mixed by rotation at 4°C for 4 hr. Beads were pelleted by centrifugation at 2,000 x g for 2 min at 4°C, and washed twice in lysis buffer in preparation for immunoblotting. Packed beads were suspended in 25 μL of 4x SDS-PAGE loading buffer for 20 μL of packed beads, heated at 95°C for 5 min, and separated on a 7.5% polyacrylamide Laemmli gel, and transferred to PVDF membranes. Membranes were blocked in 5% milk for 1 hr at room temperature (RT) and incubated
overnight at 4°C with primary antibodies diluted in 5% BSA. Membranes were treated with fluorophore-conjugated secondary antibodies and visualized on a LiCor Odyssey CLx system.

For samples used in experiments to investigate putative kinases that phosphorylate Ste5 Ser898, cultures were grown in 400 mL of selective media to mid-exponential phase growth and harvested by quenching in TCA (final concentration 20%) for 20 min at 4°C. Cells were centrifuged at 4°C, 22,000 x g for 15 min, and washed twice with ddH2O. Samples were prepared for immunoprecipitation and immunoblotting as above.

*Cell growth, lysis and immunoblotting*- Yeast cultures were grown to mid-exponential phase in the appropriate selective media and treated with the indicated concentration of cycloheximide (for analysis of Ste5 stability) or α-factor (for analysis of Fus3 phosphorylation). At the indicated time points, the necessary amount of OD600 units of cells (50 OD units for analysis of Ste5 levels or 5 OD units for analysis of Fus3 phosphorylation) were removed, centrifuged at 4°C for 10 min at 6,000 x g, washed with ddH2O, flash-frozen with liquid nitrogen, and stored at -80°C. Cell lysates were prepared by mechanical disruption and TCA precipitation in 1 mL of 10% TCA extraction buffer (10% TCA, 10 mM Tris HCl pH 8.0, 25 mM NH4OAc, and 1 mM Na2EDTA)[102]. Proteins were precipitated from the lysates by centrifugation at 4°C, 16,000 x g, for 10 min. The supernatant was aspirated, and the pellet was resuspended in 0.1 M Tris HCl, pH 11.0 and 3% SDS, heated at 95°C for 5 min, and centrifuged for 1 min at 4°C, 16,000 x g. The supernatant was removed for analysis, and a portion was used to determine protein concentration by BCA assay. Equal amounts of protein were separated on polyacrylamide
gels (7.5% for Ste5 levels analysis or 10% for analysis of Fus3 phosphorylation) and analyzed by immunoblotting as described above.

**Strains**- To epitope tag Ste5 at its endogenous locus, the tag coding sequence and HIS3 cassette were PCR amplified from the plasmid pFA6a-6Gly-Myc-His3 with primers incorporating 48 – 49 nucleotide homology arms flanking the 3’ end of the STE5 coding sequence. To introduce point mutations, the PCR product was used as a template for a second PCR reaction using mutagenic primers incorporating an additional 60 nucleotides of sequence upstream of the mutation site. Yeast (strain BY4741) were transformed with the PCR products and selected on SC-His media. Transformants were genotyped by colony PCR using primers flanking the 5’ and 3’ insertion sites. Introduction of the desired point mutations was confirmed by Sanger sequencing of the relevant PCR product.

**Plasmids**- FUS1-lacZ fusion reporter plasmids and the His6-Myc-Ste5 from plasmid pCJ93 were obtained from the laboratory of Jeremy Thorner. To produce pRS315-His6-Myc-Ste5, I subcloned the entire ORF into the BamHI site of pRS315. Subsequent mutations in Ste5 were generated by site-directed mutagenesis using an Agilent QuikChange kit.

**Halo Assays**- overnight yeast cultures were diluted to an OD600 of 1.0, and 1 mL was spread on SC-Leu-Ura plates. After 1 hr, excess moisture was removed by pipette and 10 μL of ddH2O with the indicated concentration of α-factor pheromone was applied to 1 cm filter paper disks (Difco) and plates were incubated for 24-48 hr before imaging.
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<tr>
<th>Plasmid</th>
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<td>YEplU-FUS1Z</td>
<td>2µM URA3 pFUS1-lacZ</td>
<td>Jeremy Thorner</td>
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**Antibodies**- antibodies used in this study and their sources were: anti-homocitrate synthase (Santa Cruz Biotechnology; 31F5), anti-Fus3 yC-19 (Santa Cruz Biotechnology; 6773), anti-GFP (600-101-215; Rockland). All other antibodies used were from Cell Signaling Technologies: anti-myc (9B11) (for immunoblotting), anti-myc (71D10) (for immunoprecipitation), anti-phospho PKA substrate 100G7E (9624), anti-phospho p44/42 (4370).

**Reporter Assays**- For *in vitro* reporter assays, the indicated strains were grown overnight in SC-Leu-Ura media. Samples were diluted to an OD<sub>600</sub> of 0.1 and grown for 2-3 population doublings, and α-factor was added to the indicated concentration for 1 hr. Subsequently, 5 OD<sub>600</sub> units of cells were collected by centrifugation, washed with 500 μL of ddH<sub>2</sub>O, re-centrifuged and resuspended in 250 μL of lysis buffer (100 mM Tris pH 8.0, 10% glycerol, 1 mM DTT, 2 mM PMSF). The suspension was flash frozen in liquid nitrogen and stored at -80 °C. Cell lysates were prepared by mechanical disruption and clarified as described above. A portion of the lysate was used to determine protein concentration by Bradford assay. Assays were performed using the FluoReporter® lacZ/Galactosidase Quantitation Kit (ThermoFisher, #F-2905). Equal amounts of protein were loaded in quadruplicate for each sample, and total volume was brought to 10 μL using reaction buffer (100 mM phosphate buffer, 1mM MgCl₂, and 45 mM β-mercaptoethanol), and 100 μL of 3-carboxyumbelliferyl β-D-galactopyranoside (CUG) working solution (final concentration of CUG = 1.1 mM), shaken gently to mix, and incubated for 30 min in the dark at RT. The reaction was quenched with 50 μL of 200 mM sodium carbonate and read in a plate reader (390 nm excitation, 460 nm emission).
In vivo analysis of transcriptional reporter activity was performed as described\textsuperscript{104}. Cultures were grown to early exponential phase (OD\textsubscript{600} = ~0.1-0.2) in quadruplicate, and 90 μL of cells were pipetted into each well of a 96-well plate containing either vehicle or α-factor at final concentrations ranging from 64 nM to 64 μM. Plates were incubated in a 30°C shaker at 205 rpm for 90 min, and 20 μL of fluorescein di-β-D-galactopyranoside (FDG) reagent (0.5 mM FDG in 130 mM PIPES, 0.25% Triton X-100) was added to each well. Plates incubated at 30°C for ~60 min, reactions were quenched by addition of 20 μL of 1 M sodium carbonate and read in a plate reader (485 nm excitation, 530 nm emission).
REFERENCES


