PAS kinase is activated by direct SNF1-dependent phosphorylation and mediates inhibition of TORC1 through the phosphorylation and activation of Pbp1

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ABSTRACT We describe the interplay between three sensory protein kinases in yeast: AMP-regulated kinase (AMPK, or SNF1 in yeast), PAS kinase 1 (Psk1 in yeast), and the target of rapamycin complex 1 (TORC1). This signaling cascade occurs through the SNF1-dependent phosphorylation and activation of Psk1, which phosphorylates and activates poly(A) binding protein binding protein 1 (Pbp1), which then inhibits TORC1 through sequestration at stress granules. The SNF1-dependent phosphorylation of Psk1 appears to be direct, in that Snf1 is necessary and sufficient for Psk1 activation by alternate carbon sources, is required for altered Psk1 protein mobility, is able to phosphorylate Psk1 in vitro, and binds Psk1 via its substrate-targeting subunit Gal83. Evidence for the direct phosphorylation and activation of Pbp1 by Psk1 is also provided by in vitro and in vivo kinase assays, including the reduction of Pbp1 localization at distinct cytoplasmic foci and subsequent rescue of TORC1 inhibition in PAS kinase–deficient yeast. In support of this signaling cascade, Snf1-deficient cells display increased TORC1 activity, whereas cells containing hyperactive Snf1 display a PAS kinase–dependent decrease in TORC1 activity. This interplay between yeast SNF1, Psk1, and TORC1 allows for proper glucose allocation during nutrient depletion, reducing cell growth and proliferation when energy is low.

INTRODUCTION Nutrient-sensing kinases maintain metabolic homeostasis by allocating cellular resources in response to nutrient status. Their ability to control multiple central metabolic pathways has made them the target of many therapeutic approaches, including treatments for cancer and diabetes (Eglen and Reisine, 2011; Zhang and Daly, 2012; Fang et al., 2013; Lal et al., 2013; Rosilio et al., 2014). This study focuses on the interplay between three evolutionarily conserved nutrient kinases—target of rapamycin complex 1 (TORC1), AMP-regulated kinase (AMPK, known as SNF1 in yeast), and PAS kinase (PASK, or Psk1 in yeast)—which all play critical roles in maintaining cellular homeostasis. TOR forms two distinct complexes in yeast and mammalian cells, TOR complex 1 (TORC1) and 2 (TORC2), and regulates cell growth and proliferation in response to a variety of signals, including nitrogen, amino acids, insulin, growth factors, and stress factors (due to the immense literature, only recent reviews are provided: Laplante and Sabatini, 2012; Porta et al., 2014; Shimobayashi and Hall, 2014). AMPK/SNF1 regulates energy production and consumption pathways in response to the cellular AMP:ATP ratio. High AMP levels, which indicates low cellular energy, activate AMPK to stimulate energy production pathways and down-regulate energy consumption pathways (for recent reviews, see Ghillebert et al., 2011; Broach, 2012; Hardie, 2013; Liu and Jiang, 2013; Burkewitz et al., 2014; Rosilio et al., 2014; Ye et al., 2014). Mammalian PAS kinase is regulated by glucose levels and is essential for glucose homeostasis, specifically through the control of metabolic rate, insulin/glucagon secretion, and lipid/glycogen
storage (da Silva Xavier et al., 2004, 2011; Wilson et al., 2005; An et al., 2006; Hao et al., 2007; Sempli et al., 2011; Wu et al., 2014). In yeast, there are two PAS kinase homologues, Psk1 and Psk2, which play a conserved role in the regulation of respiration and glycolgen storage (Rutter et al., 2002; Grose et al., 2007, 2009; Smith and Rutter, 2007; DeMille et al., 2014). These kinases coordinate cellular energy and nutrient availability with central metabolism, making cross-talk between these pathways essential for optimal cellular health.

The interplay between AMPK/SNF1 and TORC1 is documented in several studies. In mammals, activated AMPK inhibits TOR function through direct phosphorylation of the TORC1 subunit RAPTOR (Bolster et al., 2002; Kimura et al., 2003; Cheng et al., 2004; Reiter et al., 2005), as well as the phosphorylation and stabilization of tuberous sclerosis complex 2 (TSC2), a negative regulator of TORC1 (Inoki et al., 2003a,b). This inhibition of TORC1 by AMPK appears to be conserved in yeast, in which glucose starvation has recently been shown to inhibit TORC1 in a SNF1-dependent manner (Hughes Hall et al., 2014).

There are also reported connections between PAS kinase, AMPK, and TORC1/2 in both mammalian and yeast cells. The TORC1 inhibitor TSC2 is activated by glycogen synthase kinase 3 (GSK3; Inoki et al., 2003b, 2006), and GSK3 is in turn inhibited by PAS kinase-dependent phosphorylation (Semache et al., 2013). These results suggest that PAS kinase may act as a progrowth signal in mammalian cells through the activation of TORC1. In support of this pro-growth association, PAS kinase overexpression suppresses a temperature-sensitive mutation of TOR2 in yeast (tor2ts; Cardon et al., 2012). In addition, PAS kinase was recently shown to be required for proper activation of AMPK and mTOR in the hypothalamus, where AMPK activation by nutrient depletion and TORC1 activation by refeeding are both blunted in PASK null mice (Hurtado-Carneiro et al., 2013, 2014). The converse association is found in yeast, in which AMPK/SNF1 is required for the activation of PAS kinase by non-glucose carbon sources (Grose et al., 2007). These studies solidify communication between AMPK, PAS kinase, and TORC1/2 and highlight the many overlapping complexities. Such overlap is expected to be extensive and complex, given the various mechanisms by which each kinase is activated and the importance of coordinating cellular energy and growth.

Here we provide molecular evidence for the Psk1-dependent interplay between AMPK/SNF1 and TORC1 in yeast. Both in vivo and in vitro assays support the direct phosphorylation and activation of Psk1 by Snf1. Once activated, Psk1 may phosphorylate poly(A)-binding protein binding protein 1 (Pab1 binding protein, or Pbp1), which is known to stimulate stress granule formation and inhibit TORC1 through sequestration (Takahara and Maeda, 2012). This phosphorylation is seen through direct in vitro phosphorylation assays, as well as in vivo phosphostate and phenotypic analysis. Finally, evidence is provided for the entire cascade, with SNF1 inhibiting TORC1 in a Psk1-dependent manner. Together these results support PAS kinase as a link between energy status and cell growth/proliferation, being activated by AMPK/SNF1 under nutrient/energy depletion and inhibiting cell growth/proliferation through the phosphorylation of Pbp1.

RESULTS

Evidence for in vivo phosphorylation and activation of Psk1 by SNF1

As mentioned, SNF1 is the master and commander of the fermentation/respiration switch in yeast and is required for the activation of Psk1 by non-glucose carbon sources (Grose et al., 2007). We recently retrieved Snf1 and two of its subunits (Gal83 and Sip2) from a large-scale screen for Psk1 binding partners (DeMille et al., 2014), suggesting that this activation is direct. To test for direct phosphorylation, in vivo Psk1 activity and phosphorylation state were monitored in response to SNF1 activation or depletion.

If this SNF1-dependent activation is due to direct phosphorylation rather than transcriptional regulation, the time required to activate Psk1 should be minimal. In support of direct in vivo phosphorylation, Psk1 was activated within 1 min of shifting cells from glucose to raffinose (Figure 1A), as determined by monitoring in vivo phosphorylation of the well-characterized substrate UDP-glucose pyrophosphorylase (Ugp1; Rutter et al., 2002; Smith and Rutter, 2007; Grose et al., 2009; Cardon et al., 2012). To provide further in vivo evidence, Psk1 was purified from wild-type (WT), Snf1-deficient (snf1Δ), and SNF1-hyperactive (reg1Δ) yeast grown in galactose (to induce expression of Psk1 from the GAL1-10 promoter due to low endogenous levels), and analyzed by SDS-PAGE electrophoretic mobility shift assay (EMSA; Figure 1B). Reg1 is an inhibitor of SNF1 that promotes dephosphorylation by protein phosphatase 1 (Ludin et al., 1998), making SNF1 constitutively active in Reg1-deficient yeast. An obvious mobility shift was observed between samples, indicating a SNF1-dependent protein modification. The shift is dramatic for such a large protein, suggesting a modification other than a single phosphorylation event. Mass spectrometry confirmed multisite phosphorylation. Samples from the WT, reg1Δ, and snf1Δ cell were submitted for mass spectrometry analysis and 17 phosphorylation sites were identified that appeared to be Snf1-dependent (S10, S101, S185, S202, S255, S307, T453, T496, T717, T919, S953, S992, S996, S1020, T1021, S1035, S1094). No other modifications were detected.

Evidence for direct phosphorylation and activation of Psk1 by Snf1

To determine whether the phosphorylation and activation of Psk1 is direct, we performed in vitro phosphorylation assays. Kinase-dead Psk1 (D1230A; DeMille et al., 2014) was purified and subjected to in vitro kinase assays with purified Snf1. PAS kinase is known to auto-phosphorylate in vitro (Rutter et al., 2001), making the kinase-dead mutant vital to ensure Snf1-dependent phosphorylation. Snf1-dependent Psk1 phosphorylation was seen in these in vitro assays (Figure 1C). These results confirm direct phosphorylation of Psk1 by Snf1.

PAS kinase contains a sensory N-terminal PAS domain and the C-terminal canonical serine/threonine kinase domain. In the model for PAS kinase regulation, the PAS domain binds and inhibits the kinase domain (Ameczua et al., 2002). Phosphorylation of the PAS or kinase domain may disrupt such binding, activating the kinase. To determine domains necessary for Snf1-dependent phosphorylation, we used a truncated Psk1 bearing the kinase-only domain (AN931PK1-KD [D1230A]; Figure 1C). Snf1 was able to phosphorylate even this kinase-only construct, suggesting that Snf1 phosphorylates Psk1 in the kinase domain. However, due to the large number of phosphorylation sites identified in the kinase domain (seven total), mapping the critical phosphorylation site(s) will require further study.

Evidence for an in vivo interaction between Psk1 and Snf1

As mentioned, Snf1 and two of its three β-subunits (Gal83 and Sip2) were retrieved as in vivo binding partners of Psk1 through copurification liquid chromatography–electrospray tandem mass spectrometry (LC–MS/MS) studies (DeMille et al., 2014). An active SNF1 complex consists of three subunits: one of the three β-subunits (Gal83, Sip2, or Sip1), the Snf1 catalytic subunit, and the regulatory Snf4 subunit. Because copurification can retrieve large protein complexes, direct interaction between Psk1 and Snf1,
Gal83, or Sip2 was examined via the yeast two-hybrid (Y2H) system. All three proteins were cloned into both the Y2H prey and bait plasmids (James et al., 1996) and tested for interaction using a Psk1 bait or prey plasmid, respectively (Figure 2A). The Y2HGold strain was used, which harbors four different reporters (ADE2, HIS3, AUR1-C, MEL1) under three different Gal4-responsive promoters (Clontech), and transformed yeast were spotted onto selective media for protein–protein interactions, as well as for plasmid maintenance (media lacking histidine and adenine, as well as tryptophan and leucine, respectively). Note that all constructs were tested with their corresponding empty vector control and were negative. A direct interaction was detected between Psk1 and Gal83 but not Sip2 or Snf1. These results are not surprising, given that the Gal83 and Sip2 β-subunits of Snf1 are each responsible for binding a subset of its targets (Vincent and Carlson, 1999; Schmidt and McCartney, 2000). Whereas a strong interaction was seen between Psk1 and Gal83 when Gal83 was used as the bait, no interaction was observed when Gal83 was used as the prey, suggesting a nonfunctional construct. The Psk1/Gal83 interaction was also strong when using either full-length Psk1 or a truncation removing the PAS domain (ΔN692Psk1). This interaction supports the in vitro phosphorylation of the kinase-only Psk1 construct observed in Figure 1C.

Previous Y2H interactions of Psk1 with its substrates were detected using the ΔN692Psk1 but not the full-length Psk1 construct (DeMille et al., 2014). The Psk1/Gal83 interaction is different, in that both full-length and truncated Psk1 bound Gal83. This finding lends additional support for Psk1 being a substrate of SNF1 rather than an upstream kinase.

Gal83 protein was then truncated to determine the regions required for interaction with Psk1 (Figure 2, B and C). Gal83 has three major regions: an N-terminal region containing a glycogen-binding domain (amino acids [aa] 1–141), which has been shown to mediate the interaction between Snf1 and Reg1 (Momcilovic et al., 2008); a “Snf1-binding domain” (aa 141–343); and a C-terminal “Snf4-interacting domain” (aa 343–417; Jiang and Carlson, 1997). The Gal83 Snf1-binding domain was clearly necessary for the Gal83 Psk1 interaction (Figure 2C). This direct in vivo interaction, combined with the foregoing in vivo and in vitro phosphorylation and activation assays, affords strong evidence for the direct phosphorylation and activation of Psk1 by Snf1.

**Evidence for direct phosphorylation of Pbp1 by Psk1**

In addition to Snf1 and its subunits, Pbp1 was recently identified as a Psk1 binding partner (DeMille et al., 2014). Although Pbp1 was retrieved from both yeast two-hybrid and copurification screens, we did not observe phosphorylation of full-length Pbp1 when purified from bacteria (DeMille et al., 2014) or yeast (unpublished data). However, the Y2H retrieved two truncations of Pbp1 (ΔN196Pbp1 and ΔN355Pbp1), and when a further truncated (ΔN419Pbp1) Pbp1 was used in the in vitro kinase assays, a strong phosphorylation was observed (Figure 3A). The ability of Psk1 to phosphorylate the truncated but not full-length Pbp1 suggests that Pbp1 may adopt different conformations regulated by its N-terminal end.

Pbp1 contains three regions: the Sm domain, the LSmAD domain, and the self-interaction region (Figure 3B). The Sm domain (Sm-ATX) is required for interaction with Psk1 (Figure 2, B and C). Gal83 has three main regions: an N-terminal region containing a glycogen-binding domain (amino acids [aa] 1–141), which has been shown to mediate the interaction between Snf1 and Reg1 (Momcilovic et al., 2008); a “Snf1-binding domain” (aa 141–343); and a C-terminal “Snf4-interacting domain” (aa 343–417; Jiang and Carlson, 1997). The Gal83 Snf1-binding domain was clearly necessary for the Gal83 Psk1 interaction (Figure 2C). This direct in vivo interaction, combined with the foregoing in vivo and in vitro phosphorylation and activation assays, affords strong evidence for the direct phosphorylation and activation of Psk1 by Snf1.

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Pbp1 contains three regions: the Sm domain, the LSmAD domain, and the self-interaction region (Figure 3B). The Sm domain (Sm-ATX or LSm) is required for interaction with other proteins, namely Lsm12, Pbp4, and Rpl12a. The LSmAD domain is usually associated with Sm domains and enhances protein binding, and the self-interaction region is required for binding Pab1 and itself (Mangus et al., 2013). To map the Pbp1 regions necessary for Psk1 interaction, we tested several Pbp1 truncations via the Y2H assay (Figures 3, B and C; note that all constructs were tested with their corresponding empty vector control and were negative). The results mirrored the in vitro kinase assays, in that Psk1 was not able to bind the full-length Pbp1 protein but bound truncated constructs in which the N-terminal 196 amino acids were removed. Larger N-terminal truncations were made to further characterize the minimal region necessary for interaction.
Evidence for in vivo phosphorylation and activation of Pbp1 by Psk1

The combined evidence of in vivo protein–protein interaction with in vitro kinase assays makes Pbp1 a high-confidence cellular Psk1 substrate. To determine whether Pbp1 is phosphorylated by Psk1 in vivo, we monitored the Psk1-dependent phosphorylation of Pbp1 through nonspecific PhosphoThreonine (Cell Signaling Technology) or PhosphoSerine (Qiagen) antibodies (Figure 4A). HIS-HA epitope-tagged Pbp1 was purified from yeast grown on galactose, and a Western blot revealed Psk1-dependent phosphorylation of at least one threonine residue, whereas phosphoserine presence was independent of Psk1. Phosphorylation state was assessed in cells deficient in both Psk1 and Psk2 (PSK1PSK2 deficient) as compared with

FIGURE 2: Yeast two-hybrid assays reveal an interaction between the Gal83 Snf1-binding domain and the Psk1 kinase domain. (A) The SNF1 complex subunit Gal83, but not Sip2 or Snf1, interacts with full-length Psk1. Y2HGold cells (Clontech) containing the Gal83 (pJG1236 or pJG1237), Sip2 (pJG1238 or pJG1239), and Snf1 (pJG1240 or pJG1241) bait (BD) and prey (AD) plasmids, respectively, were cotransformed with bait and prey plasmids harboring full-length Psk1 (pJG441 or pJG442) or empty vector (pJG424 or pJG421). Only representative interactions are shown (all other combinations of bait and prey were negative). (B) Psk1 truncations used to screen for interactions with Gal83 are diagrammed. (C) Gal83 interacts with truncated versions of Psk1 that harbor the kinase domain. Y2HGold cells were cotransformed with bait plasmids harboring either Gal83 (pJG1236) or the empty vector (pJG424), and prey plasmids harboring truncations of Psk1 (ΔN692Psk1 [pJG709] or ΔN931Psk1 [pJG1276]). (D) The Snf1-binding domain is necessary for interaction with Psk1. Truncations used to screen for the Gal83 domains necessary for Psk1 binding are diagrammed. Constructs are marked with an asterisk if any binding to Psk1 was detected in the Y2H assays shown in E. (E) Y2HGold cells containing the Psk1 prey plasmid (pJG442) were cotransformed with bait plasmids harboring full-length Gal83 (pJG1236), truncated Gal83 (aa 1–342 [pJG1258], 141–417 [pJG1260], 1–140 [pJG1262], 141–342 [pJG1264]), or empty vector (pJG424). Overnight samples were grown in SD-Leu-Trp for plasmid maintenance, diluted fivefold serially, and plated on Y2H selective medium (SD-Leu-Trp-His-Ade), as well as on a control plate (SD-Leu-Trp). Plates were incubated at 30°C for 3–4 d.

Removal of the N-terminal 419 amino acids still allowed for a Psk1 interaction, whereas removal of the N-terminal 469 amino acids did not. Further attempts to narrow in on the essential interaction region of Pbp1 by creating C-terminal truncations in the self-interaction domain (removing aa 420–722, 566–722, 581–722, or 661–722) did not result in constructs that interact, even when the N-terminus was also removed. Thus the Pbp1 self-interaction region (aa 420–722) is necessary for in vivo association with Psk1, whereas the N-terminal Sm region (aa 1–197) of Pbp1 appears to inhibit interaction. Because this C-terminal region is involved in enhancing protein binding (Mangus et al., 2004; Kimura et al., 2013), the phosphorylation of Pbp1 by Psk1 may either enhance or inhibit its activity.
whether Psk1 also localizes to the same distinct cytoplasmic foci as Pbp1, red fluorescent protein (RFP)–tagged Psk1 was transformed into yeast containing Pbp1–green fluorescent protein (GFP). Cytoplasmic foci were induced by glucose deprivation, a condition known to induce both P-body and stress granule formation. Psk1 colocalized to distinct cytoplasmic foci with Pbp1 in ∼74% of cells in which Pbp1-GFP foci were visible (Figure 4C).

The effect of PAS kinase on Pbp1 localization to distinct foci was then assessed. Pbp1-GFP foci were significantly reduced in the psk1psk2 yeast when compared with WT (Figure 4D), suggesting that PAS kinase activates Pbp1 by increasing localization to stress granules or P-bodies.

Snf1 inhibits TORC1 phosphorylation of Sch9

We provided evidence for the Snf1-dependent phosphorylation and activation of Psk1, which then leads to the phosphorylation and activation of Pbp1, inhibiting TORC1. To further support this model, we monitored TORC1 activity in response to both Snf1 and PAS kinase. TORC1 activity was assessed through the in vivo phosphostate of the S6 kinase, Sch9, commonly used as a readout of TORC1 activity (Kingsbury et al., 2014; Urban et al., 2007). Sch9 is phosphorylated when both Psk1 and Psk2 phosphorylate the well-characterized PAS kinase substrate Ugp1. Psk2 is unlikely to phosphorylate Pbp1 in this study, however, because Psk2 is not expressed on carbon sources other than glucose, and is thus not activated by Snf1 (Grose et al., 2007). Therefore phosphorylation of Pbp1 grown in galactose is attributed to Psk1 activity.

To determine the effects of this phosphorylation on Pbp1 function, Pbp1-dependent caffeine sensitivity was assessed in WT and PSK1PSK2-deficient yeast. Pbp1 has been shown to inhibit TORC1 through its sequestration to stress granules (Takahara and Maeda, 2012), suggesting a role for PAS kinase in TORC1 regulation. This sequestration and inactivation of TORC1 produces a caffeine sensitive phenotype. PSK1PSK2 deficiency alleviates the caffeine sensitivity of cells overexpressing PBP1, consistent with Psk1-dependent activation of Pbp1 (Figure 4B). Combined these results suggest that Psk1 phosphorylates Pbp1 at a threonine residue, which activates Pbp1, inducing stress granule formation and subsequent TORC1 sequestration.

**Psk1 increases Pbp1 localization at cytoplasmic foci**

The Pbp1-dependent localization of TORC1 to stress granules was previously shown by Takahara and Maeda (2012). To determine whether Psk1 also localizes to the same distinct cytoplasmic foci as Pbp1, red fluorescent protein (RFP)–tagged Psk1 was transformed into yeast containing Pbp1–green fluorescent protein (GFP). Cytoplasmic foci were induced by glucose deprivation, a condition known to induce both P-body and stress granule formation. Psk1 colocalized to distinct cytoplasmic foci with Pbp1 in ∼74% of cells in which Pbp1-GFP foci were visible (Figure 4C).

The effect of PAS kinase on Pbp1 localization to distinct foci was then assessed. Pbp1-GFP foci were significantly reduced in the psk1psk2 yeast when compared with WT (Figure 4D), suggesting that PAS kinase activates Pbp1 by increasing localization to stress granules or P-bodies.
Caffeine sensitivity is associated with inhibition of TORC1, and Psk1Psk2 deficiency can rescue the caffeine sensitivity of cells overexpressing Pbp1 (Figure 4B), suggesting that phospho-rylation of Pbp1 by Psk1 is activating. In support of this finding, Psk1Psk2 deficiency reduces the amount of Pbp1 localized to distant cytoplasmic foci (Figure 4D).

This model for the PAS kinase–dependent interplay between SNF1 and TORC1 was further supported by monitoring the in vivo phosphorylation state of Sch9, a TORC1 substrate. Sch9 was fully phosphorylated in WT yeast grown on glucose. This phosphorylation was increased in snf1 yeast and inhibited in reg1 yeast, in which Snf1 is constitutively active (Figure 5). In addition, PAS kinase was necessary for this inhibition. During the revision of the manuscript, an article was published validating the Snf1-dependent inhibition of TORC1 signaling on alternate carbon sources (Hughes Hallett et al., 2014). Our results describe the molecular pathways underlying this observation.

The present article describes the second recent connection between yeast PAS kinase and TOR. In yeast, two TOR paralogues exist, Tor1 (which is a subunit of TORC1) and Tor2 (which can function in both TORC1 and TORC2 complexes). TORC1 primarily controls growth in response to nutrients through the regulation of transcription, translation, ribosome biogenesis, nutrient transport, and autophagy; TORC2 controls cell cycle–dependent polarization of the actin cytoskeleton (for a review, see Loewith and Hall, 2011).
Overexpression of yeast PSK1 or PSK2 suppresses a tor2ts mutation (Cardon et al., 2012; Cardon and Rutter, 2012), suggesting rescue of the TORC2 complex, since TORC1 is still functional in this background. In contrast, Psk1 appears to be a negative regulator of TORC1 activity (Figures 4 and 5). These findings are consistent with the cellular selection for two differentially controlled TOR complexes that regulate separate pathways involved in cell growth and proliferation.

In a cell’s adaptation to its environment, there cannot simply be one “progrowth” pathway, but instead alternate pathways, such as those allowing for growth with or without division. Not only are there separate TOR complexes, but these complexes regulate different pathways in response to different stimuli. This complexity is necessary due to the multiple metabolic inputs and outputs the cell must sense (e.g., energy, amino acids, nitrogen, carbon source, etc.). It remains to be determined whether PAS kinase inhibits only the phosphorylation of Sch9, a progrowth kinase stimulating many pathways including protein synthesis, or whether other TORC1 pathways are also affected. The PAS kinase–dependent interplay between SNF1 and TORC1 would allow for an additional regulatory input, rather than just direct SNF1-dependent phosphorylation of TORC1.

The complexity of these pathways is further demonstrated by comparing different cell types. Although the direct inhibition of TORC1 by AMPK reported in mammalian cells (Bolster et al., 2002; Kimura et al., 2003; Cheng et al., 2004; Reiter et al., 2005) is consistent with the overall pathway mediated by PAS kinase described here, PAS kinase is predicted to activate TORC1 in mammalian cells through the inhibition of GSK3 (Inoki et al., 2003b, 2006; Semache et al., 2013). This alternate role for PAS kinase may be due to the differential metabolic responses of different cell types. For example, yeast ferment when glucose is high and respire on poor carbon sources, whereas mammalian cells activate respiration in response to nutrient-rich conditions (high glucose). These differences are reflected in AMPK/SNF1 activity, which is stimulated by alternate carbon sources in yeast and high glucose in mammalian cells (Ghillebert et al., 2011; Broach, 2012; Hardie, 2013; Liu and Jiang, 2013; Burkewitz et al., 2014; Ye et al., 2014). Whether PAS kinase also mediates inhibition of TORC1 through the mammalian Pbp1 homologue, ataxin-2, remains to be seen.

These findings may provide valuable insight into the function of PAS kinase in mammalian cells, in that all of the proteins in this pathway have conserved homologues. The human homologue of Pbp1, ataxin-2, is associated with neurodegenerative disease. This study provides both in vitro and in vivo evidence for the direct, Psk1-dependent phosphorylation of Pbp1 and the inhibition of phosphorylation by the Pbp1 N-terminal domain (Figure 3). This N-terminal truncation may trap Pbp1 into a conformation that is naturally obtained under certain in vivo conditions because WT Pbp1 copurifies with Psk1 (DeMille et al., 2014) and is phosphorylated in a Psk1-dependent manner within cells (Figure 4A). The N-terminus of Pbp1, which inhibits Psk1-dependent phosphorylation, also harbors the ataxin-2 domain, a conserved domain required for localization to

**FIGURE 5:** SNF1 inhibits TORC1 phosphorylation of Sch9 through PAS kinase. (A) Western blots of phospho-Sch9 and total Sch9. (B) Quantification of band intensities in A. A plasmid expressing Sch9 under the yeast ADH promoter was transformed into yeast [WT [JGY1], reg1 [JGY95], snf1 [JGY91], reg1psk1psk2 [JGY283], reg1snf1psk1psk2 [JGY280]]. Yeast were grown in YPAD until OD<sub>600</sub> ~ 1.0. Preparation of Sch9 protein extract was performed as described by Miller-Fleming et al. (2014). Samples were normalized and loaded on 8% SDS–PAGE, transferred to nitrocellulose membrane, and incubated overnight with anti–phospho-Thr737-Sch9 and anti–Thr737-Sch9 antibodies (Kingsbury et al., 2014). Intensity signals were quantified using ImageJ. Phosphorylation was determined in duplicate. Error bars represent SEM.
P-bodies and stress granules and for promoting their formation (Mangus et al., 1998; Nonhoff et al., 2007; Swisher and Parker, 2010; Kaehler et al., 2012). The conservation of this N-terminal domain suggests that this regulatory role may also be conserved. Because mutations in ataxin-2 are associated with cerebral ataxia (SCA2), amyotrophic lateral sclerosis (ALS), and parkinsonism (for recent reviews, see Magana et al., 2013; Borza, 2014), understanding the regulation of this protein may provide key therapeutic targets for disease treatment.

The cross-talk between AMPK/SNF1, PAS kinase, and TOR seems vital for appropriate metabolic regulation, linking energy production to growth in response to a wide variation in available nutrients. These pathways are central to understanding a cell’s basic metabolism. In addition, both AMPK and TOR are key therapeutic targets for the treatment of a variety of diseases, including obesity, diabetes, and cancer (for recent reviews, see Khan et al., 2013; Quinn et al., 2013). As a nonessential protein, PAS kinase may prove to be a safer target for therapeutic treatment of metabolic disease.

MATERIALS AND METHODS

Growth assays

Lists of strains, plasmids, and primers used in this study are provided in Table 1. For plasmid construction, standard PCR-based cloning methods were used. All restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Yeast two-hybrid bait and prey plasmids were made by PCR amplification and subsequent cloning into the EcoRI/SalI sites of pJG424 (pJG1236 [JG3199/3200], pJG1198) or the EcoRI/SalI sites of pJG421 (pJG1003 [JG3136/3138], pJG1004 [JG3137/3138], pJG1005 [JG2916/2917], pJG1196 [JG2917/3461], pJG1197 [JG2917/3462]).

FIGURE 6: A model for the cross-talk between the nutrient-sensing kinases SNF1, TORC1, and PsK1 proposed in this study. SNF1 becomes activated when cellular energy levels are low (such as in conditions of glucose deprivation) and phosphorylates PAS kinase (PsK1). This phosphorylation leads to a disruption of the inhibitory binding of the PAS domain, leading to activation of the kinase. Once active, PsK1 phosphorylates Pbp1, which inhibits TORC1 by sequestering it to stress granules. The inhibition of TORC1 inactivates the S6 kinase, Sch9, and possibly other pathways that control cellular growth and proliferation.

Histidine- and Myc-tagged protein purification

Yeast harboring plasmids for histidine (His)- or Myc-tagged protein expression were grown in selective SD-Leu-Trp media overnight, diluted 1:100-fold into 100 or 500 ml of SD-Ura, and grown for 10–12 h, pelleted, and resuspended in SGal-Ura for 36 h to induce expression under the GAL1-10 promoter. Yeast were pelleted and resuspended in lysis buffer for His purification (50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid [HEPES], 300 mM NaCl, 20 mM imidazole, 10 mM KCl, 1 mM β-mercaptoethanol, and 1:300 mammalian protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO] or Complete Protease Inhibitor Cocktail Tablet [Roche], pH 7.8, with phosphatase inhibitors, 50 mM NaF, and glycerophosphate when necessary) or for Myc purification (20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid [EGTA], 50 mM NaCl, 10% glycerol, 0.5 M N-ε-acetyl-L-lysine, 1 M β-mercaptoethanol, 0.1 M phenylmethylsulfonyl fluoride, and 0.01% Tween 20). Yeast cells were then lysed and cleared by centrifugation, and the supernatants were collected and applied to appropriate affinity matrices for purification.
| Strain   | Background | Genotype                                                                 | Abbreviation | a/α | Reference or source                  |
|---------|------------|---------------------------------------------------------------------------|--------------|-----|---------------------------------------|
| JGY1    | W303       | ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1                         | WT           | a   | David Stillman (University of Utah, Salt Lake City, UT) |
| JGY3    | W303       | psk2::kan-MX4 ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1          | PSK1psk2     | a   | Grose et al. (2007)                   |
| JGY4    | W303       | psk1::his3 psk2::kan-MX4 ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 | psk1psk2     | a   | Grose et al. (2007)                   |
| JGY91   | W303       | snf1::hphMX4 ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1           | snf1         | a   | Grose et al. (2007)                   |
| JGY95   | W303       | reg1::hphMX4 ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1           | reg1         | a   | Grose et al. (2007)                   |
| JGY299  | S288C      | PSK2-TAPtag::kanMX ura3-0 trp1-0 SUC2 mal mel gal2 CUP1 flo1 flo8-1       | WT           | α   | Jared Rutter (University of Utah, Salt Lake City, UT) |
| JGY280  | W303       | psk1::His3 psk2::kanMX4 reg1::hphMX4 snf1::URA3 ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 | snf1reg1psk1psk2 | a | Jared Rutter |
| JGY283  | W303       | psk1::His3 psk2::kanMX4 reg1::hphMX4 ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1 | reg1psk1p-sk2 | a | Jared Rutter |
| Y2H Gold | (JGY1031) | LYS2::GAL1UAS-GAL1TATA-His3, GAL2UAS-Gal2TATA-Ade2 URA3::MELUAS-MELITATA, AUR1-CMEL1, ura3-52 his3-1-200 ade2-101 trp1-1-901 leu2-3, 112 gal4del gal80del met- | Y2H Gold   | α | Clontech |
| JGY1144 | BY4741     | Chromosomally-tagged Pbp1-GFP::His3 from Invitrogen his3-1 leu2-0 met15-0 ura3-0 | WT           | a   | Invitrogen                            |
| JGY1160 | BY4741     | Pbp1-GFP::His3, psk1::hygro, psk2::NAT his3-1 leu2-0 met15-0 ura3-0        | psk1psk2     | a   | This study                            |
| JGY1161 | S288C      | psk1::hygro psk2::NAT ura3-0 trp1-0 SUC2 mal mel gal2 CUP1 flo1 flo8-1   | psk1psk2     | α   | This study                            |

| Plasmid | Gene | Description | Backbone | Yeast origin | Selection | Reference or source                  |
|---------|------|-------------|----------|--------------|-----------|---------------------------------------|
| pJG9    | PSK1 | Psk1 in pRS424 | pRS424   | 2u           | Trp       | Jared Rutter                         |
| pJG124  | EV   | EV          | pRS424   | 2u           | Trp       | Bruce Horazdovsky (Mayo Clinic, Minneapolis, MN) |
| pJG232  | PSK1 | C-terminal HIS/HA-tagged Psk1 | pRS426   | 2u           | Ura       | Jared Rutter                         |
| pJG410  | PSK1 | Psk1-D1230A in pJG232 | pRS426   | 2u           | Ura       | DeMille et al. (2014)                |
| pJG421  | EV   | pGAD-C1 empty Y2H prey vector | YEp-GAD | 2u           | Leu       | James et al. (1996)                  |
| pJG422  | EV   | pGAD-C2 empty Y2H prey vector | YEp-GAD | 2u           | Leu       | James et al. (1996)                  |
| pJG423  | EV   | pGAD-C3 empty Y2H prey vector | YEp-GAD | 2u           | Leu       | James et al. (1996)                  |
| pJG424  | EV   | pGBD-C1 empty Y2H bait vector | YEp-GBD  | 2u           | Trp       | James et al. (1996)                  |
| pJG425  | EV   | pGBD-C2 empty Y2H bait vector | YEp-GBD  | 2u           | Trp       | James et al. (1996)                  |
| pJG441  | PSK1 | Full-length Psk1 in pJG425 | YEp-GBD  | 2u           | Trp       | DeMille et al. (2014)                |
| pJG442  | PSK1 | Full-length Psk1 in pJG422 | YEp-GBD  | 2u           | Trp       | DeMille et al. (2014)                |
| pJG598  | PSK1 | ΔN692Psk1 in pJG425 | YEp-GBD  | 2u           | Trp       | DeMille et al. (2014)                |

**TABLE 1:** Strains, plasmids, and primers used in this study.

Continues
| Plasmid  | Gene          | Description                                           | Backbone     | Yeast origin | Selection | Reference or source                              |
|---------|---------------|-------------------------------------------------------|--------------|--------------|-----------|------------------------------------------------|
| pJG709  | PSK1          | ΔN692Psk1 in pJG422                                    | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG858  | PSK1          | pGAL1-10, Psk1-His/HA                                  | pRS426       | 2u           | Ura       | DeMille et al. (2014)                          |
| pJG859  | EV            | pGAL1-10, His/HA                                       | pRS426       | 2u           | Ura       | DeMille et al. (2014)                          |
| pJG925  | PBP1          | Full-length Pbp1 in pJG859                             | pRS426       | 2u           | Ura       | This study                                      |
| pJG960  | PSK1          | ΔN692Psk1-His/HA in pJG858                            | pRS426       | 2u           | Ura       | DeMille et al. (2014)                          |
| pJG998  | EV            | pET15b with pGADT7 MCS                                 | pET15b       |             | Amp       | This study                                      |
| pJG1000 | PSK1          | ΔN931Psk1-His/HA in pJG858                            | pRS426       | 2u           | Ura       | DeMille et al. (2014)                          |
| pJG1001 | PBP1          | aa356-722Pbp1                                         | YEp-GAD      | 2u           | Leu       | DeMille et al. (2014)                          |
| pJG1002 | PBP1          | aa197-722Pbp1                                         | YEp-GAD      | 2u           | Leu       | DeMille et al. (2014)                          |
| pJG1003 | PBP1          | aa420-722Pbp1                                         | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1004 | PBP1          | aa565-722Pbp1                                         | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1008 | PBP1          | Full-length Pbp1                                      | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1046 | GAL83         | pGAL1-10, Gal83-His/HA                                 | pRS426       | 2u           | Ura       | This study                                      |
| pJG1047 | SIP2          | pGAL1-10, Sip2-His/HA                                  | pRS426       | 2u           | Ura       | This study                                      |
| pJG1119 | PSK1          | pGAL1-10, Psk1-RFP                                     | pRS426       | 2u           | Ura       | This study                                      |
| pJG1168 | PBP1          | aa1-565Pbp1                                           | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1169 | PBP1          | aa420-565Pbp1                                         | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1170 | PSK1          | pGAL1-10-Psk1-D1230A-His/HA                            | pRS426       | 2u           | Ura       | DeMille et al. (2014)                          |
| pJG1181 | SK1           | pGAL1-10-Psk1-Myc                                     | pRS426       | 2u           | Ura       | DeMille et al. (2014)                          |
| pJG1183 | EV            | pGAL1-10, EV-Myc                                       | pRS426       | 2u           | Ura       | DeMille et al. (2014)                          |
| pJG1193 | SNF1          | Snf1-8xmyc                                            | pRS313       | 2u           | Ura       | Mark Johnston (University of Colorado School of Medicine, Aurora, CO) |
| pJG1195 | PBP1          | aa470-722Pbp1                                         | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1196 | PBP1          | aa1-420Pbp1                                           | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1197 | PBP1          | aa1-580Pbp1                                           | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1198 | PBP1          | aa1-660Pbp1                                           | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1215 | PSK1          | pGAL1-10-Psk1-D1230A-Myc                               | pRS426       | 2u           | Ura       | This study                                      |
| pJG1216 | PSK1          | pGAL1-10-ΔN931Psk1-Myc                                 | pRS426       | 2u           | Ura       | This study                                      |
| pJG1230 | PBP1          | aa420-580Pbp1                                         | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1231 | PBP1          | aa420-660Pbp1                                         | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1236 | GAL83         | Gal83 in pJG424                                       | YEp-GBD      | 2u           | Trp       | This study                                      |
| pJG1237 | GAL83         | Gal83 in pJG421                                       | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1238 | SIP2          | Sip2 in pJG424                                        | YEp-GBD      | 2u           | Trp       | This study                                      |
| pJG1239 | SIP2          | Sip2 in pJG421                                        | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1240 | SNF1          | Snf1 in pJG424                                        | YEp-GBD      | 2u           | Trp       | This study                                      |
| pJG1241 | SNF1          | Snf1 in pJG421                                        | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1250 | PBP1          | pGAL1-10, ΔN419Pbp1-Myc                                | pRS426       | 2u           | Ura       | This study                                      |
| pJG1251 | PBP1          | pGAL1-10, Pbp1-Myc                                     | pRS426       | 2u           | Ura       | This study                                      |
| pJG1258 | GAL83         | aa1-342Gal83 in pJG424                                 | YEp-GBD      | 2u           | Trp       | This study                                      |
| pJG1259 | GAL83         | aa1-342Gal83 in pJG424                                 | YEp-GAD      | 2u           | Leu       | This study                                      |
| pJG1260 | GAL83         | aa141-417Gal83 in pJG424                               | YEp-GBD      | 2u           | Trp       | This study                                      |
| pJG1261 | GAL83         | aa141-417Gal83 in pJG424                               | YEp-GAD      | 2u           | Leu       | This study                                      |

TABLE 1: Strains, plasmids, and primers used in this study.
TABLE 1: Strains, plasmids, and primers used in this study. Continued
1 mM β-mercaptoethanol, cComplete Protease Inhibitor Cocktail Tablet, pH 7.4, with phosphatase inhibitors when necessary. Resuspended cultures were lysed using the Microfludics M-110P homogenizer (Microfludics, Westwood, MA [for 500-ml cultures]) or bead blasted for 1 min, followed by 1 min on ice repeated three times using 0.2-mm glass beads (for 100-ml cultures). Cell debris was then pelleted at 12,000 rpm for 20–30 min. Supernatants were transferred to new tubes and incubated with 200 μl of nickel-nitritroltriacetic acid (Ni-NTA) agarose beads (Qiagen, Valencia, CA [for 500-ml HIS purification]) or 5–10 μl of Myc-conjugated magnetic beads (Cell Signaling, Danvers, MA [for 100-ml Myc purification]) for 2–3 h at 4°C. For His-epitope purification, beads were washed twice with 15 ml of lysis buffer and then transferred to a polypropylene column and washed with 30–50 ml of lysis buffer. For Myc-protein purification, beads were separated using magnetic force and washed four times with 500 μl of lysis buffer lacking the protease inhibitor. HIS-tagged proteins were eluted three times with 0.3 ml of lysis buffer containing 250 mM imidazole and 100 mM NaCl but lacking protease and phosphatase inhibitors. Beads containing Myc-tagged proteins were used directly for in vitro kinase assays without eluting.

Quantification of in vivo phosphorylation of Ugp1
As a measure of Psk1 activity, the amount of Ugp1 phosphorylated in vivo was assessed. Briefly, an overnight sample of Psk2-deficient cells (PSK1psk2, JGY3) was diluted 1:100 in yeast extract/peptone/dextrose/adenine (YPAD) and grown for 3–4 h at 30°C to an OD600 of 0.5. Medium was then changed by filtering with a 0.2-μm filter and resuspending in YPA-raffinose (2%). Cells were harvested at various time points by filtering and flash freezing at –80°C. Percentage Ugp1 phosphorylation was assayed as previously described (Smith and Rutter, 2007). Briefly, cells were lysed by bead blast, and the supernatant was fractionated on a monoQ column, which separates the phosphorylated and unphosphorylated forms of Ugp1. The activity of Ugp1 in each fraction was assessed, and the percentage of phosphorylated Ugp1 was calculated by fitting the sum of two Gaussians to the curves to determine the area under the peak using KaleidaGraph (Synergy Software, Reading, PA).

In vivo Psk1 EMSA
HIS-HA epitope-tagged Psk1 (pJG858) was purified from WT (JGY1), snf1 (JGY91), and reg1 (JGY95) yeast grown in galactose, run on 8% SDS–PAGE, and silver stained to visualize any electrophoretic mobility shift.

In vitro kinase assays
Constructs of Psk1-Myc and Pbp1-Myc epitope-tagged plasmids were made as follows: full-length Pbp1 (pJG1251) was constructed by PCR amplification using primers JG2916/2917 and cloning into the EcoRI/Xhol sites of pJG1183. ΔN419Pbp1 (pJG1250) was constructed by PCR amplification using primers JG3384/2916 and cloning into the EcoRI/Xhol sites of pJG1183. ΔN931Psk1 (pJG1216) was constructed by PCR amplification using primers JG3140/1158 and cloning into the EcoRI/Xhol sites of pJG1181. Full-length Psk1-D1230A (kinase dead) (pJG1215) was constructed by digesting pJG410 and subcloning into BglII/Xhol sites of pJG1181. ΔN931Psk1-D1230A (pJG1281) was constructed by PCR amplification of pJG1170 using primers JG3140/1158 and cloning into the EcoRI/Xhol sites of pJG1181. Full-length Psk1 (pJG1181) was made previously (DeMille et al., 2014).

Full-length Psk1 and ΔN931Psk1 were purified from WT yeast (JGY1), and kinase-dead Psk1 and both Pbp1 constructs were purified from psk1psk2 yeast (JGY4). Purified full-length and ΔN419Pbp1-Myc–tagged proteins were assayed for PAS kinase–dependent phosphorylation by incubating purified protein in 30 μl of reaction buffer containing 1× Psk1 kinase buffer (0.4 M HEPES, 0.1 M KCl, 5 mM MgCl₂, pH 7.0), 0.2 mM ATP, 32P-ATP (5 μCi; MP Biomedicals, Santa Ana, CA) in the presence or absence of purified full-length Psk1, truncated ΔN931Psk1, or kinase-dead Psk1-D1230A. Kinase assays were started with the addition of Psk1 and stopped with SDS–PAGE sample buffer. Reactions were incubated for 12 min at 30°C. For in vitro kinase assays of Snf1 (pJG1193) and Psk1-D1230A (pJG1215) or ΔN931Psk1-D1230A (pJG1281), reaction conditions were similar, except the following changes: 1× Snf1 kinase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol [DTT], pH 7.5), 10 μM ATP, and 7.5 μCi of 32P-ATP, and reactions were incubated for 35 min.

In vivo Pbp1 phosphostate analysis
Pbp1-HIS expressed under the GAL1-10 promoter (pJG925) was made by PCR amplifying Pbp1 with primers JG2916/2917 and cloning into the EcoRI/Xhol sites of pJG859. HIS-tagged Pbp1 (pJG925) was expressed in JGY1 (WT) overexpressing full-length Psk1 on a plasmid (pJG9) or in JGY4 (psk1psk2) containing an empty vector (pJG124). Proteins were grown in duplicate in SD-Ura overnight, induced with Sgal-Ura for 36 h, and purified on Ni-NTA as described. Eluates were run on 8% SDS–PAGE, transferred to nitrocellulose membrane, and incubated overnight with nonspecific Phosphothreonine (Cell Signaling Technology) antibody. Blots were imaged, stripped, and then incubated overnight with PhosphoSerine (Q5; Qiagen) antibody. Total Pbp1 was assessed by stripping the membrane once more, incubating overnight with hemagglutinin (HA) antibody (Roche), and imaging.

Microscopy
Pbp1-GFP fusion yeast (JGY1144) was obtained from the Invitrogen yeast-GFP clone collection (Invitrogen, Grand Island, NY) and transformed with Psk1-RFP (pJG1119) for colocalization analysis. pJG1119 was made by PCR amplification of dsRED using JG3334/3352 and Bba J04450 (iGEM registry plasmid) as DNA template and cloned into the Xhol/SphI sites of pJG858 to replace the HISHA tag with the RFP fusion. Overnight samples were grown in SD-Ura medium, and then yeast were pelleted and resuspended in Sgal-Ura medium to induce expression of Psk1-RFP. Cultures were grown in Sgal-Ura for 3–5 d, cells were pelleted, washed once with synthetic complete (SC) medium lacking a carbon source, and resuspended in fresh SC medium. Cultures were incubated at 30°C for an additional 1 h to allow them to grow under glucose deprivation conditions. Images were then captured using a Fluoview FV1000 confocal laser-scanning microscope (Olympus, Tokyo, Japan) with 60× magnification lens and the appropriate filter sets (GFP, dsRED). For percentage of Pbp1-GFP foci in WT (JGY1144) and psk1psk2 (JGY1160) yeast, strains were grown in SD complete medium for 4 d and then washed, resuspended in SC lacking a carbon source, incubated, and imaged as described. For percentage of Psk1 and Pbp1 colocalization, 50 cells (96 distinct cytoplasmic foci) were counted. For percentage of Pbp1-GFP foci, 1199 cells for WT and 782 for psk1psk2 were counted. SEM was used for error bars, and Student’s t test was used for statistical significance calculations.

Sch9 phosphorylation assays
A plasmid expressing Sch9 under the ADH1 promoter (pJG1288) was made by amplifying Sch9-HIS using primers JG3330 and JG3331
and cloning into pJG1285. This Sch9 plasmid was transformed into yeast (WT [JGY1], reg1 [JGY95], snf1 [JGY91], reg1psk1psk2 [JGY283], reg1snf1psk1psk2 [JGY280]), grown in SD-Trp overnight, and then diluted 1:100 to YPAD and grown until OD600 ~1.0. For rapamycin controls, 100 nM rapamycin (Research Products International, Mount Prospect, IL) was added 1 h before harvesting cells. Preparation of Sch9 protein extract was performed as described by Miller-Fleming et al. (2014). Samples were normalized and loaded on 8% SDS–PAGE, transferred to nitrocellulose membrane, and incubated overnight with anti–phospho-Thr737-Sch9 and anti–Thr737-Sch9 antibodies (Kingsbury et al., 2014). Intensity signals were quantified using ImageJ (National Institutes of Health, Bethesda, MD).

**Mass spectrometry**

PsK1 samples were prepared using a modified version of filter-aided sample preparation (Wisniewski et al., 2009). Briefly, samples were placed on filters, denatured by adding 8 M urea (Molecular Biology Grade; EMD Chemicals, Gibbstown, NJ), reduced with dithiothreitol (DTT, Sigma-Aldrich), alkylated with iodoacetamide (BioUltra Grade; Sigma-Aldrich), and digested with trypsin (Sequencing Grade Modified; Promega, Madison, WI). Digested protein was analyzed by LC-MS using the Eksigent NanoLC Ultra coupled with the LTQ Orbitrap XL mass spectrometer. Peptides were separated on a Waters Pepsitide Separation Technology c18 column with a water/acetonitrile gradient containing 0.1% formic acid (Optima LC-MS Grade, Fisher Scientific, Pittsburgh, PA). MS scans were collected in the Orbitrap. Fragmentation scans were collected in the LTQ, excluding charge state 1 and unassigned charge states using collision-induced dissociation (CID). MS3 multistage activation or MS3 CID (Kall et al., 2007) was used, with neutral losses corresponding to phosphate groups (32.66, 48.99, and 97.97 m/z) in some cases (Schroeder et al., 2004).

Data were analyzed using Mascot, Sequest, and/or Sequest HT with or without Percolator (Perkins et al., 1999; Tabb et al., 2001; Kall et al., 2007). The Proteome Discoverer Package was also used on some samples. Search settings included dynamic modifications: phosphorylation (STY) and oxidation (M). Carbamidomethyl (C) was set as a dynamic or static modification. Searches were performed against the uni_yeast database or a modified uni_yeast database set as a dynamic or static modification. Searches were performed with or without Percolator (Perkins et al., 2007) was used, with neutral losses corresponding to phosphate groups.

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