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Pkh1 interacts with and phosphorylates components of the yeast Gcn2/eIF2α system

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ABSTRACT

The yeast *Saccharomyces cerevisiae* responds to amino acid deprivation by increasing translation of the transcription factor Gcn4, which enhances expression of amino acid biosynthetic genes. Accumulation of uncharged tRNAs activates the Gcn2 protein kinase, which phosphorylates the alpha subunit of the eukaryotic initiation factor 2 (eIF2α). The resulting downregulation of eIF2 activity causes reduction of general translation and stimulation of *GCN4* translation. *S. cerevisiae* contains three PDK1 orthologs (encoded by *PKH1*, *PKH2* and *PKH3*) that have been implicated in nutrient signaling. Using heterologously expressed proteins, we demonstrate physical interaction between Pkh1 and all three subunits of eIF2 as well as Gcn2. We confirm the interaction between Pkh1 and Gcn2 by co-immunoprecipitation in yeast cell extracts and show that Pkh1 can phosphorylate Gcn2 *in vitro*. However, Pkh1 inactivation did not affect eIF2α-S51 phosphorylation *in vivo* or *GCN4* translation in response to amino acid deprivation. Hence, the physiological importance of the close interactions between Pkh1 and Gcn2 or eIF2 could depend on other conditions and/or other targets of the Gcn2/eIF2 system.

1. Introduction

Nutrient signaling allows yeast cells to adapt rapidly to changing environmental conditions. When starved for amino acids, *Saccharomyces cerevisiae* accumulates uncharged tRNAs to activate the eukaryotic initiation factor 2α (eIF2α) kinase Gcn2 [1,2]. The heterotrimeric initiation factor eIF2 consists of an alpha/Sui2, beta/Sui3 and gamma/Gcd11 subunit, of which the latter binds GTP [3–5]. Phosphorylation of the α-subunit on Ser51 by Gcn2 impedes general translation, but derepresses translation of the transcription factor *GCN4* which mediates general amino acid control (GAAC) response [6].

Gcn2 consists of a protein kinase domain (res. 530–910) and a juxtaposed C-terminal histidyl-tRNA synthetase-like domain (HisRSs) (res. 920–1450), which is involved in binding and activation of Gcn2 by uncharged tRNAs [7,8]. In addition, Gcn2 activity requires autophosphorylation on Thr-882 and Thr-887 whereas phosphorylation on Ser-577 has been reported to inhibit Gcn2 function [9,10].

Mammalian PDK1 is an important regulatory kinase, involved in several signaling events downstream of receptors that stimulate PI3kinases (PI3K). Binding of growth factors or insulin to their corresponding receptors triggers activation of Class I PI3K which phosphorylate inositol phospholipids, generating phosphoinositides (PtdIns) [11]. These lipid species recruit PDK1 to the membrane, where PDK1 can phosphorylate some of its substrates. Most PDK1 substrates interact with PDK1 through a hydrophobic motif (HM) located carboxy-terminal to their catalytic domain, with consensus sequence F–X–X–F–(**S**/**T**)–(F/Y) (**S**/**T** = phosphorylated residues, X = any amino acid). This HM can bind to a hydrophobic pocket in PDK1, called PIF-pocket (PDK1-interacting fragment) and functions as a docking site for PDK1 [12–15]. PDK1 then phosphorylates the substrate activation loop on the so-called PDK1-site with consensus sequence **T**–F–C–G–T–X–E–Y (**T** = phosphorylated residue) [16,17].

The three *S. cerevisiae* PDK1 orthologs (encoded by *PKH1*, *PKH2* and *PKH3*) are involved in cell wall integrity signaling, endocytosis and nutrient signaling [18–20]. Several substrates of the Pkh protein kinases have been identified over the past few years, including the yeast SGK (serum- and glucocorticoid regulated protein kinases) homologs (Ypk1 and Ypk2) [21,22], the yeast PKC1 homolog

Abbreviations: GAAC, general amino acid control; HM, hydrophobic motif; PIF, PDK1-interacting fragment.

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Pkc1, the PKB and/or S6K homolog Sch9 [20,23,24] and Tpk1, a catalytic subunit of yeast PKA [20,25].

Previous observations have suggested a possible connection between PI3K and Gcn2/eIF2 signaling. In mammalian cells, eIF2α kinases (mainly PKR and PERK) have been suggested as activators of the PI3K signaling pathway [26]. In addition, Hao et al. [27] showed that sensing of essential amino acid deficiency by Gcn2 in mammalian brain cells is sensitive to the PI3K inhibitor wortmannin. How these pathways are connected remains unclear.

In this work, we show that Pkh1 physically interacts with all three eIF2 subunits *in vitro*, whereas two other kinases of the protein kinase A, G and C (AGC) family, Sch9 and Tpk1, do not. In addition, Pkh1 interacts *in vitro* and *in vivo* with the eIF2α kinase Gcn2. This interaction is not dependent on the hydrophobic pocket in the kinase domain of Pkh1, nor on the hydrophobic motif in Gcn2. Using *in vitro* kinase assays, we show that Pkh1 is able to phosphorylate full-length Gcn2. In addition, Pkh1 co-precipitates with a kinase other than Gcn2 that is able to phosphorylate eIF2α *in vitro*, indicating possible additional functions of Pkh protein kinases in regulation of eIF2α phosphorylation. However, no significant differences were observed in eIF2α-S51 phosphorylation or *GCN4* translation *in vivo* in response to amino acid starvation upon inactivation of Pkh1. Together, these results indicate a possible role for the yeast PDK1 orthologs in regulation of Gcn2, although the conditions and/or mechanisms involved remain unclear. The identification of Gcn2 as a potential Pkh1 substrate may have implications for the identification of novel PDK1 substrates.

2. Materials and methods

2.1. Strains and growth media

The following yeast strains were used in this work: BY4742 wild type, BY4742 *gcn2Δ*, BY4741 *gcd11::GCD11-HA* (MK22, this work), BJ2168, S288C ura3–52 (RH2520, provided by G. Braus, Gottingen). S288C ura3–52 *gcn2::KanMX6* (MK227, this work) and *pkh1^{ts}pkh2Δ pkh3Δ* [20]. Standard rich (YP) and minimal media (SC) containing either 2% glucose, raffinose or galactose, supplemented with appropriate nutrients to maintain selection for plasmids were used for yeast cultivation. Cells were routinely grown at 30 °C, with the exception of temperature sensitive strains, which were grown at 24 °C.

2.2. Plasmids

Constructs containing HA- or GST-tagged Tpk1, Sch9 and Pkh1 and Pkh1^{L199E} were described previously [20]. YEP351pGAL-Pkh1-HA, YEP351pGAL-Pkh1^{K154R}-HA, and P180 (p*GCN4*::LacZ) were provided by J. Thorner (Berkeley) and A. Hinnebusch (Bethesda) respectively. The Ycplac22-Pkh1 was obtained from [19]. The His_6-tagged Pkh1 was expressed in a pBevyU vector (Haesendonckx S., unpublished results). The pRS313-*HIS2-ADE1* plasmid and pGEX-4T-1 constructs containing GST-tagged eIF2α/Sui2, eIF2β/Sui3, eIF2γ/Gcd11 and Gcn2 were created in this work. Site-directed mutagenesis on pGEX-4T-1-Gcn2 was performed using the Quick Change site-directed mutagenesis kit (Stratagene). All constructs were sequenced to verify presence of the desired mutation(s) as well as absence of any erroneous mutations.

2.3. Expression and purification of GST-tagged proteins from Escherichia coli and GST pull-down assay

Expression and purification of GST-tagged proteins from *E. coli* and GST pull-down assay were performed as previously described [20]. After washing, proteins were eluted in SDS-loading dye, equal amounts of eluate were separated by SDS–PAGE. The gels were stained with Coomassie Brilliant Blue or subjected to western blotting using rat monoclonal anti-HA peroxidase high affinity antibody (Roche) or antibodies against yeast eIF2α/Sui2, eIF2β/Sui3 (provided by T. Dever, Bethesda) or Gcn2 (provided by A. Hinnebusch, Bethesda).

2.4. Expression and purification of proteins from S. cerevisiae and co-immunoprecipitation

Expression and purification of proteins from *S. cerevisiae* were done as previously described [20], with slight modification for the RNaseA treatment. The immunoprecipitates were incubated in lysis buffer at 26 °C for 30 min in the presence or absence of RNaseA (250 μg, Roche). Following RNase treatment, immunocomplexes were collected by centrifugation and washed three times with lysis buffer lacking protease inhibitors and supplemented with 0.015% SDS (w/v). Finally, proteins were eluted in SDS-loading dye and equal amounts of eluate were separated by SDS–PAGE. The gels were subjected to western blotting using anti-Gcn2 (provided by A. Hinnebusch, Bethesda) or anti-His (GE Healthcare) antibodies.

2.5. In vitro kinase assay

In vitro kinase assays were performed as previously described [20]. Proteins were resolved by SDS–PAGE. Gels were stained with Coomassie Brilliant Blue and analyzed by autoradiography using a PhosphorImager and by western blotting using anti-HA (Roche) antibodies.

2.6. β-Galactosidase assay (GCN4 expression)

Beta-galactosidase activity in permeabilized cells was measured according to Dever [28]. Specific β-galactosidase activity was normalized to the total protein in each extract. The relative amounts of *GCN4* expression have been calculated in β-galactosidase Units: 1 Unit = $(OD_{420} \times 1.7)/(0.0045 \times$ reaction time in min $\times$ volume extract in mL $\times$ protein concentration in μg/μL). Assays were performed for at least three independent transformants, and the mean value is presented. The standard errors of the means were below 15%.

2.7. Determining eIF2α phosphorylation

Amino acid starvation with 5-MT (5-methyltryptophan) and sample taking was performed according to Dever [28]. Cell pellets were resuspended in 500 μL 20% TCA, proteins were further extracted by TCA precipitation. Finally, proteins were eluted in SDS-loading dye (supplemented with 1 M Tris) and equal amounts of eluate were separated by SDS–PAGE. The gels were subjected to western blotting using anti-eIF2α(phospho S51) (Abcam-ab26197) and anti-eIF2α (T. Dever, Bethesda) antibodies for detection.

3. Results and discussion

3.1. Pkh1 interacts with eIF2 and Gcn2

Interaction of all three eIF2 subunits and the protein kinase Gcn2 with yeast Pkh1 was demonstrated using a GST pull-down assay. N-terminal GST-fusions of the eIF2 subunits and Gcn2 were expressed in *E. coli* and used as bait in pull-down experiments with full-length HA_3-Tpk1, HA_3-Sch9 or Pkh1-HA, expressed in yeast. These experiments showed that Pkh1 interacted with all three eIF2 subunits and Gcn2 *in vitro* (Fig. 1(A)). These results were

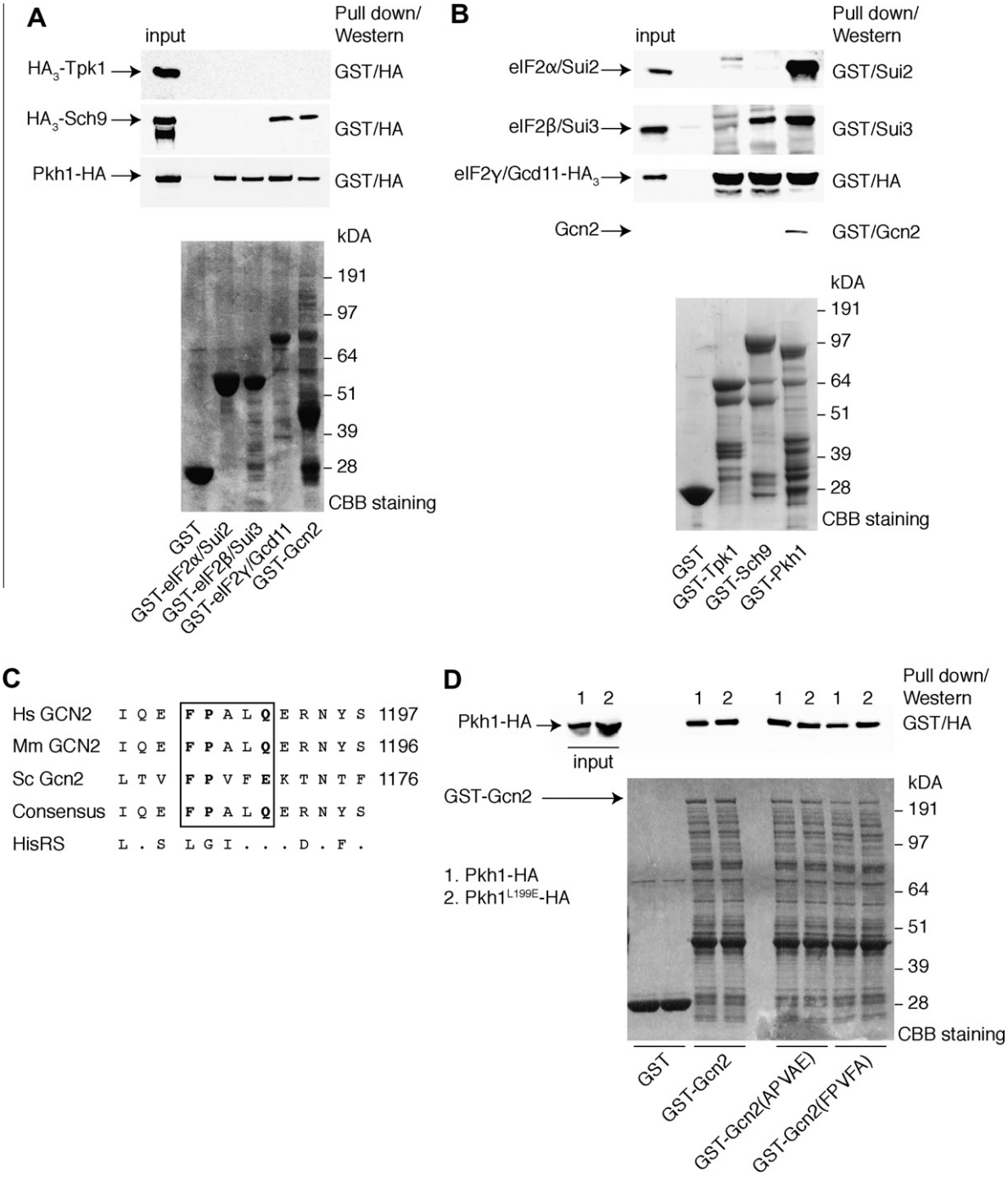

Fig. 1. Pkh1 interacts *in vitro* with all eIF2 subunits and Gcn2 produced in *E. coli*. (A) GST-fusion proteins were purified from *E. coli* onto glutathione-Sepharose beads and incubated with cell extracts from strain BJ2168 expressing either HA_3-Tpk1, HA_3-Sch9 or Pkh1-HA. A GST pull-down demonstrates interaction between Pkh1 and GST-tagged eIF2α/Sui2 (~60 kDa), eIF2β/Sui3 (~57 kDa), eIF2γ/Gcd11 (~84 kDa) and Gcn2 (~200 kDa). (B) GST-fusion proteins were purified from *E. coli* onto glutathione-Sepharose beads and incubated with cell extracts from strain MK22 expressing Gcd11-HA_3. A GST pull-down demonstrates interaction between all three eIF2 subunits and Gcn2 with Pkh1. (C) Sequence alignment of mouse and human GCN2 with the region surrounding the HM in ScGcn2. The HM is partly conserved in human (Hs) and mouse (Mm) GCN2 and differs from the conserved HisRS domain. (D) Mutagenesis of the HM (1167-FPVFE-1171) in Gcn2 or mutagenesis of the PIF pocket in Pkh1 (L199E) does not affect the interaction. CBB staining: Coomassie Brilliant Blue staining.

confirmed using N-terminal GST-fusions of Tpk1, Sch9 and Pkh1 (Fig. 1(B)). Tpk1 and Sch9 showed limited interaction with some of the eIF2 subunits and/or or Gcn2 (Fig. 1(B)).

Gcn2 contains a possible hydrophobic motif at position 1167-FPVFE-1171, with Glu possibly acting as a phosphomimic residue. Interestingly, the HM is partially conserved in mammalian and mouse GCN2 (Fig. 1(C)), the sequence in this region differs from that seen in the conserved HisRS domain (res. 920–1450) [29]. This prompted us to investigate the importance of the HM in Gcn2, and the PIF pocket in Pkh1, for the interaction between Gcn2 and Pkh1. Mutagenesis of the PIF-pocket in Pkh1 ($Pkh1^{L199E}$) or the HM in Gcn2 ($Gcn2^{\underline{A}PV\underline{A}E}$ and $Gcn2^{FPVF\underline{A}}$) did not abolish the interaction (Fig. 1(D)). These results indicate that neither the PIF pocket in Pkh1, nor the conserved HM in Gcn2 are required for their *in vitro* interaction. Unlike other Pkh1 substrates, Gcn2 is not an AGC kinase, which could indicate a different way of interaction

of PDK1 with AGC and non-AGC substrates. Since none of the eIF2 subunits contain a hydrophobic motif, these interactions were not further investigated.

These results show that the yeast PDK1 ortholog Pkh1 interacts *in vitro* with eIF2 and the eIF2α kinase Gcn2. Although Gcn2 contains a HM, mutagenesis of the PIF pocket of Pkh1 or the HM in Gcn2 does not abolish the observed interaction.

3.2. Interaction of Pkh1 with Gcn2 in yeast cell extracts

We then used co-immunoprecipitation experiments in yeast cell extracts to confirm the interactions shown by the GST pull-down assays. His_6-tagged Pkh1 was expressed and purified from yeast, and immunocomplexes were analyzed by SDS–PAGE and western blotting using antibodies against the eIF2 subunits and Gcn2. As a control, extracts from a strain containing the empty pBevyU plasmid were used. Although expression of Gcn2 was weak in both input samples, Gcn2 clearly co-precipitates with the immunopurified His_6-tagged Pkh1. Gcn2 was not precipitated with the anti-His antibody in the control sample. Since Gcn2 is known to bind to ribosomes, tRNAs and potentially also mRNA, part of the interaction might be mediated by interactions with ribonucleic acids. To investigate if the interaction between Pkh1 and Gcn2 requires the presence of RNA, the immunocomplexes were treated with RNaseA. RNaseA treatment did not abolish the observed interaction (Fig. 2). Similar co-immunoprecipitation experiments did not confirm the interaction of Pkh1 with the eIF2 subunits (results not shown). Also the interactions between Tpk1 or Sch9 and the different eIF2 subunits could not be confirmed by means of co-immunoprecipitation (results not shown). These results indicate that yeast Pkh1 interacts with Gcn2 in yeast cell extracts and that this interaction is not mediated by interactions with ribonucleic acids. The observed interactions indicate a possible connection between Gcn2/eIF2 and Pkh1 signaling in yeast.

3.3. Pkh1 phosphorylates Gcn2 in vitro and co-precipitates an eIF2α-kinase other than Gcn2

Following these results, we investigated whether the observed interactions of Pkh1 with Gcn2 or the different eIF2 subunits indicated a possible kinase-substrate interaction. *In vitro* kinase assays were performed with eIF2 and Gcn2 as substrates using Pkh1 and a kinase dead version of Pkh1. A kinase dead variant of Gcn2 was used as a substrate, to avoid autophosphorylation *in vitro*. The results indicated that Pkh1 is able to phosphorylate Gcn2 *in vitro*, whereas a kinase dead variant of Pkh1 could not (Fig. 3(A)).

A strong phosphorylation of eIF2α/Sui2 was observed when incubated with Pkh1 purified from yeast (Fig. 3(A)). This phosphorylation

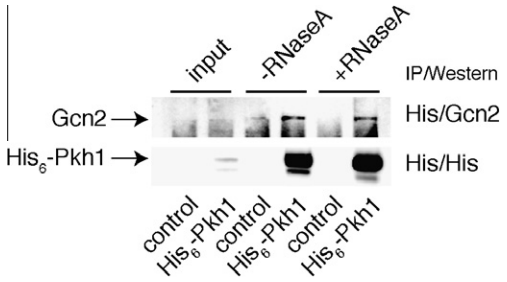

Fig. 2. Pkh protein kinases interact with Gcn2 in yeast cell extracts. His_6-Pkh1 was expressed and purified from yeast extracts. As a control, samples of a wild type strain harbouring an empty pRS316 vector were treated in the same way as the samples containing His_6-tagged Pkh1. The interaction between Pkh1 and Gcn2 is independent of the presence of ribonucleic acids, since treatment with RNaseA does not abolish the interaction *in vivo*.

was still present when incubated with the kinase dead variant of Pkh1, indicating that it is not performed by Pkh1 but by a co-precipitating protein kinase. Since the kinase assays for Pkh1 were performed with either Pkh1 or a kinase dead variant of Pkh1 expressed as a HA-fusion protein, in a *pkh1^{ts} pkh2Δ pkh3Δ* strain, it is unlikely that Pkh2 or Pkh3 are the kinases responsible for the observed phosphorylation of eIF2α/Sui2.

Since Gcn2 was shown to interact with Pkh1, the observed phosphorylation of eIF2α/Sui2 could be caused by co-precipitation of Gcn2. Repetition of the kinase assay with Pkh1 extracted from a *gcn2Δ* strain, however, still resulted in high levels of phosphorylated eIF2α/Sui2 (Fig. 3(B)).

Together, these results indicate that Pkh1 is able to phosphorylate Gcn2 *in vitro*. Although most well-characterized substrates of Pkh1 contain a PDK1 site, sequence analyses failed to identify a putative PDK1 phosphorylation site in Gcn2. The identification of the residue(s) in Gcn2 phosphorylated by Pkh1, will be relevant towards the full understanding of Gcn2 kinase activity and its regulation by nutrients or stress conditions. In addition, eIF2α/Sui2 is phosphorylated by a kinase co-precipitating with Pkh1 but other than Gcn2. Besides Gcn2, yeast casein kinase II (CKII) is capable of phosphorylating eIF2α/Sui2 in yeast [30]. Recently, the alpha subunit of CKII, Cka2, was identified as a potential interaction partner of Pkh1 [31]. It remains to be investigated if CKII is the co-precipitated kinase responsible for the observed eIF2α/Sui2 phosphorylation *in vitro*.

3.4. Yeast Pkh protein kinases do not affect Ser51 phosphorylation of eIF2α/Sui2 or GCN4 translation during amino acid starvation

The observed interactions of Pkh1 with eIF2 and the eIF2α kinase Gcn2 could indicate a possible involvement of Pkh1 in the known Gcn2-dependent phosphorylation of eIF2α on Ser51 and/or the Gcn2-dependent translational derepression of *GCN4* under conditions of amino acid starvation. We compared phosphorylation of eIF2α on Ser51 and the level of *GCN4* translation in a (prototrophic) *pkh1^{ts} pkh2Δ pkh3Δ* mutant strain, harboring the p180 plasmid (containing *GCN4::lacZ*) and either an empty YCplac22 plasmid ('Pkh1^{ts}') or a YCplac22 plasmid containing wild type Pkh1 ('Pkh1 WT'). The strains were grown on 24 °C and shifted to the restrictive temperature of 35 °C in the presence of 1.2 M sorbitol. Since the commonly used 3-amino-1,2,4-triazole (3-AT, a competitive inhibitor of histidine biosynthesis) did not elicit a proper amino acid starvation response in the 15 Dau background (results not shown), the structural tryptophan analog 5-MT (5-methyl tryptophan) was used. As a control, the responses in a wild type and a *gcn2Δ* S288C strain are shown. Inactivation of Pkh1 did not result in significant differences in the relative phosphorylation state of eIF2α on Ser51 or *GCN4* translation levels during amino acid starvation conditions (Fig. 4).

Together these results indicate that although yeast Pkh1 physically interacts with and phosphorylates Gcn2, Pkh1 is not a regulator of Gcn2 kinase activity under conditions of amino acid starvation. Nonetheless, Pkh1 is still present under the tested conditions although catalytically inactive, and could therefore still mediate a physical interaction between Gcn2, eIF2 and/or an unknown kinase. Besides amino acid starvation, Gcn2 kinase activity is also stimulated by other stress conditions, including glucose starvation, purine deprivation, accumulation of unfolded proteins and heat shock [32,33]. Interestingly, reduced function of the Pkh protein kinases results in a translation initiation defect during heat stress [34].

It would be interesting to investigate the direct interaction between mammalian PDK1 and GCN2, which could be responsible for the observed (indirect) regulation of the PI3K pathway by eIF2α kinases. These findings therefore hint towards new modes of

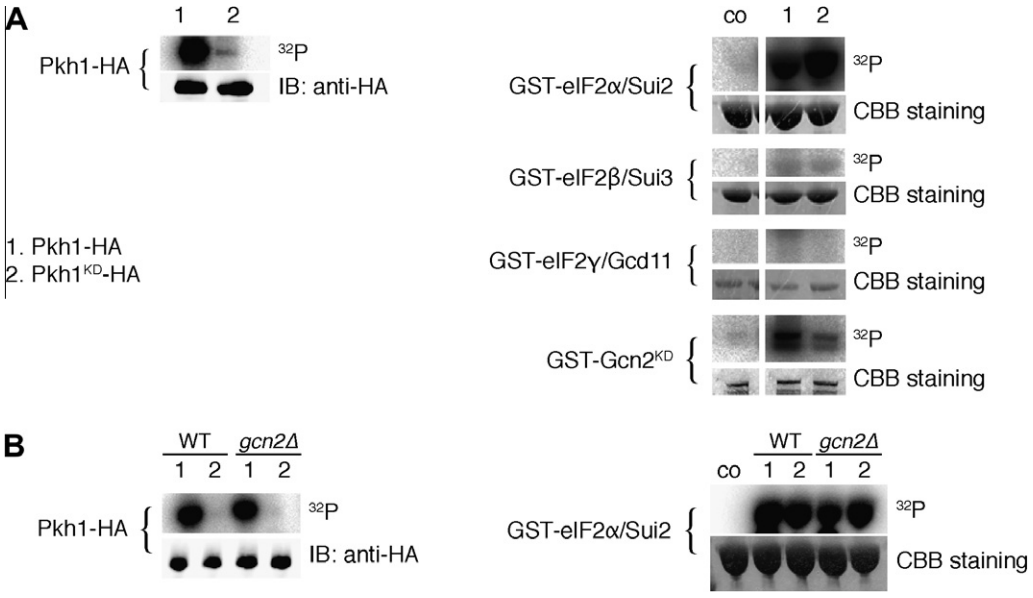

Fig. 3. Pkh1 phosphorylates Gcn2 *in vitro*. (A) Pkh1 (1) or a kinase dead variant of Pkh1 (2) were expressed as HA-fusion proteins in a *pkh1^{ts} pkh2Δ pkh3Δ* strain (using YEP351pGAL-Pkh1-HA and YEP351pGAL-Pkh1^{K154R}-HA, respectively). A control experiment showing the kinase activities and expression levels of the respective constructs is shown on the left. The isolated proteins were incubated with purified GST-tagged eIF2α/Sui2, eIF2β/Sui3, eIF2γ/Gcd11 or a kinase dead variant of Gcn2 (K628R) expressed in *E. coli*) (right). (B) Pkh1 (1) and a kinase dead variant of Pkh1 (2) were expressed and purified from a wild type and a *gcn2Δ* BY4742 strain. A control experiment showing the kinase activities and expression levels of the respective constructs is shown on the left. Incubation of GST-eIF2α/Sui2 with Pkh1 extracted from the *gcn2Δ* strain still results in strong phosphorylation of eIF2α/Sui2. Phosphorylation was not reduced when a KD mutant of Pkh1 was used. IB: immunoblot, CBB staining: Coomassie Brilliant Blue staining, ^{32}P: autoradiogram.

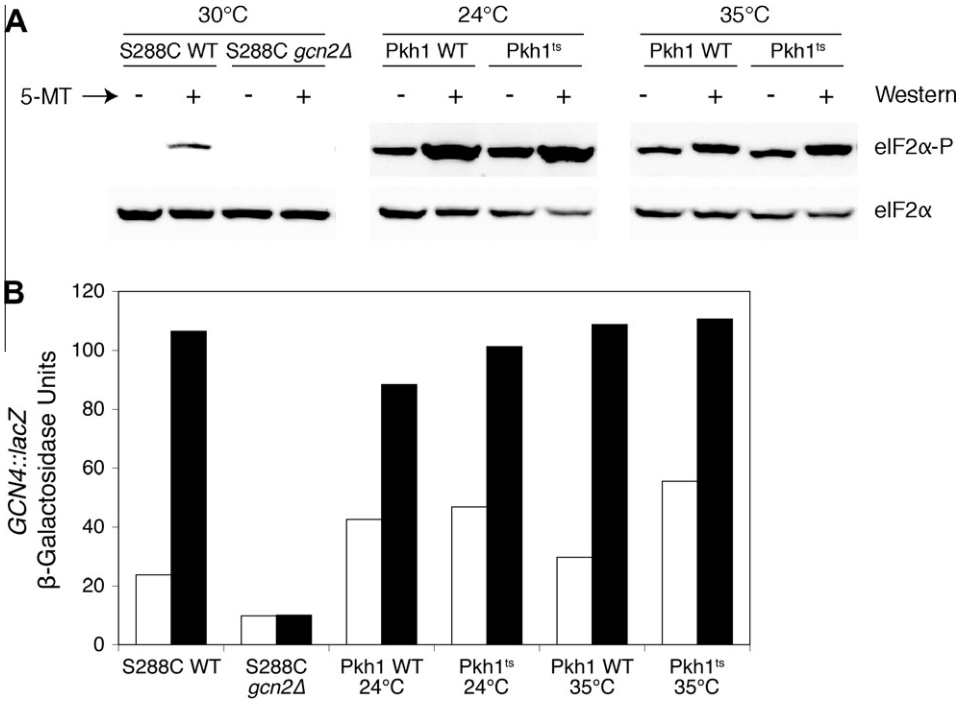

Fig. 4. Inactivation of Pkh1 does not abolish amino acid starvation induced eIF2α phosphorylation and induction of *GCN4* translation. (A) TCA extracts were prepared from yeast S288C wild type or *gcn2Δ* strains or (prototrophic) *pkh1^{ts} pkh2Δ pkh3Δ* strains harbouring, pRS313-*HIS2-ADE1*, the p180 plasmid and either YCplac22 containing wild type Pkh1 ('Pkh1 WT') or an empty YCplac22 plasmid ('Pkh1^{ts}'). Cells were grown under non-starvation conditions or treated for 1 h with 2 mM 5-MT. The amount of eIF2α-P and total eIF2 present in isolated protein extracts were analyzed by Western blot using a phosphorylation-specific polyclonal anti-eFI2α (Ser51-P) antibody or polyclonal anti-eIF2α antibodies respectively. (B) Crude protein extracts were prepared from yeast S288C wild type or *gcn2Δ* strains or (prototrophic) *pkh1^{ts} pkh2Δ pkh3Δ* strains harbouring, pRS313-*HIS2-ADE1*, the p180 plasmid and either YCplac22 containing wild type Pkh1 ('Pkh1 WT') or an empty YCplac22 plasmid ('Pkh1^{ts}'). Cells from an overnight culture were diluted and incubated for 5 h under non-starvation conditions (log phase, white bars) or amino acid starvation conditions by adding 2 mM 5-MT (black bars). The relative amounts of *GCN4* expression were calculated in β-galactosidase Units [2]. Bars depict means of at least three independent measurements with a standard deviation not exceeding 15%.

regulation of Gcn2/eIF2 signaling as well as the identification of novel PDK1 substrates.

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References

[1] R.C. Wek, B.M. Jackson, A.G. Hinnebusch, Juxtaposition of domains homologous to protein kinases and histidyl-tRNA synthetases in GCN2 protein suggests a mechanism for coupling GCN4 expression to amino acid availability, Proc. Nat. Acad. Sci. USA 86 (1989) 4579–4583.
[2] T.E. Dever, L. Feng, R.C. Wek, A.M. Cigan, T.F. Donahue, A.G. Hinnebusch, Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast, Cell 68 (1992) 585–596.
[3] E.M. Hannig, A.M. Cigan, B.A. Freeman, T.G. Kinzy, GCD11, a negative regulator of GCN4 expression, encodes the gamma subunit of eIF-2 in *Saccharomyces cerevisiae*, Mol. Cell Biol. 13 (1993) 506–520.
[4] F.L. Erickson, L.D. Harding, D.R. Dorris, E.M. Hannig, Functional analysis of homologs of translation initiation factor 2gamma in yeast, Mol. Gen. Genet. 253 (1997) 711–719.
[5] F.L. Erickson, J. Nika, S. Rippel, E.M. Hannig, Minimum requirements for the function of eukaryotic translation initiation factor 2, Genetics 158 (2001) 123–132.
[6] A.G. Hinnebusch, Translational regulation of GCN4 and the general amino acid control of yeast, Annu. Rev. Microbiol. 59 (2005) 407–450.
[7] R.C. Wek, M. Ramirez, B.M. Jackson, A.G. Hinnebusch, Identification of positive-acting domains in GCN2 protein kinase required for translational activation of GCN4 expression, Mol. Cell. Biol. 10 (1990) 2820–2831.
[8] S. Zhu, A.Y. Sobolev, R.C. Wek, Histidyl-tRNA synthetase-related sequences in GCN2 protein kinase regulate in vitro phosphorylation of eIF-2, J. Biol. Chem. 271 (1996) 24989–24994.
[9] P.R. Romano, M.T. Garcia-Barrio, X. Zhang, Q. Wang, D.R. Taylor, F. Zhang, C. Herring, M.B. Mathews, J. Qin, A.G. Hinnebusch, Autophosphorylation in the activation loop is required for full kinase activity in vivo of human and yeast eukaryotic initiation factor 2alpha kinases PKR and GCN2, Mol. Cell. Biol. 18 (1998) 2282–2297.
[10] M. Garcia-Barrio, J. Dong, V.A. Cherkasova, X. Zhang, F. Zhang, S. Ufano, R. Lai, J. Qin, A.G. Hinnebusch, Serine 577 is phosphorylated and negatively affects the tRNA binding and eIF2alpha kinase activities of GCN2, J. Biol. Chem. 277 (2002) 30675–30683.
[11] B. Vanhaesebroeck, S.J. Leevers, G. Panayotou, M.D. Waterfield, Phosphoinositide 3-kinases: a conserved family of signal transducers, Trends Biochem. Sci. 22 (1997) 267–272.
[12] M. Frodin, T.L. Antal, B.A. Dummler, C.J. Jensen, M. Deak, S. Gammeltoft, R.M. Biondi, A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation, EMBO J. 21 (2002) 5396–5407.
[13] M. Frodin, C.J. Jensen, K. Merienne, S. Gammeltoft, A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1, EMBO J. 19 (2000) 2924–2934.
[14] R.M. Biondi, A. Kieloch, R.A. Currie, M. Deak, D.R. Alessi, The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB, EMBO J. 20 (2001) 4380–4390.
[15] R.M. Biondi, D. Komander, C.C. Thomas, J.M. Lizcano, M. Deak, D.R. Alessi, D.M. van Aalten, High resolution crystal structure of the human PDK1 catalytic domain defines the regulatory phosphopeptide docking site, EMBO J. 21 (2002) 4219–4228.
[16] R.M. Biondi, Phosphoinositide-dependent protein kinase 1, a sensor of protein conformation, Trends Biochem. Sci. 29 (2004) 136–142.
[17] L.R. Pearce, D. Komander, D.R. Alessi, The nuts and bolts of AGC protein kinases, Nat. Rev. Mol. Cell Biol. 11 (2010) 9–22.
[18] A. Casamayor, P.D. Torrance, T. Kobayashi, J. Thorner, D.R. Alessi, Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast, Curr. Biol. 9 (1999) 186–197.
[19] M. Inagaki, T. Schmelzle, K. Yamaguchi, K. Irie, M.N. Hall, K. Matsumoto, PDK1 homologs activate the Pkc1-mitogen-activated protein kinase pathway in yeast, Mol. Cell Biol. 19 (1999) 8344–8352.
[20] K. Voordeckers, M. Kimpe, S. Haesendonckx, W. Louwet, M. Versele, J.M. Thevelein, Yeast 3-phosphoinositide-dependent protein kinase-1 (PDK1) orthologs Pkh1-3 differentially regulate phosphorylation of protein kinase A (PKA) and the protein kinase B (PKB)/S6K ortholog Sch9, J. Biol. Chem. 286 (2011) 22017–22027.
[21] A.K. deHart, J.D. Schnell, D.A. Allen, L. Hicke, The conserved Pkh-Ypk kinase cascade is required for endocytosis in yeast, J. Cell Biol. 156 (2002) 241–248.
[22] F.M. Roelants, P.D. Torrance, N. Bezman, J. Thorner, Pkh1 and Pkh2 differentially phosphorylate and activate Ypk1 and Ykr2 and define protein kinase modules required for maintenance of cell wall integrity, Mol. Biol. Cell 13 (2002) 3005–3028.
[23] J. Urban, A. Soulard, A. Huber, S. Lippman, D. Mukhopadhyay, O. Deloche, V. Wanke, D. Anrather, G. Ammerer, H. Riezman, J.R. Broach, C. De Virgilio, M.N. Hall, R. Loewith, Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae*, Mol. Cell 26 (2007) 663–674.
[24] K. Liu, X. Zhang, R.L. Lester, R.C. Dickson, The sphingoid long chain base phytosphingosine activates AGC-type protein kinases in *Saccharomyces cerevisiae* including Ypk1, Ypk2, and Sch9, J. Biol. Chem. 280 (2005) 22679–22687.
[25] L.R. Levin, J. Kuret, K.E. Johnson, S. Powers, S. Cameron, T. Michaeli, M. Wigler, M.J. Zoller, A mutation in the catalytic subunit of cAMP-dependent protein kinase that disrupts regulation, Science 240 (1988) 68–70.
[26] S. Kazemi, Z. Mounir, D. Baltzis, J.F. Raven, S. Wang, J.L. Krishnamoorthy, O. Pluquet, J. Pelletier, A.E. Koromilas, A novel function of eIF2alpha kinases as inducers of the phosphoinositide-3 kinase signaling pathway, Mol. Biol. Cell 18 (2007) 3635–3644.
[27] S. Hao, C.M. Ross-Inta, D.W. Gietzen, The sensing of essential amino acid deficiency in the anterior piriform cortex, that requires the uncharged tRNA/GCN2 pathway, is sensitive to wortmannin but not rapamycin, Pharmacol. Biochem. Behav. 94 (2010) 333–340.
[28] T.E. Dever, Using GCN4 as a reporter of eIF2 alpha phosphorylation and translational regulation in yeast, Methods 11 (1997) 403–417.
[29] M. Ramirez, R.C. Wek, C.R. Vazquez de Aldana, B.M. Jackson, B. Freeman, A.G. Hinnebusch, Mutations activating the yeast eIF-2 alpha kinase GCN2: isolation of alleles altering the domain related to histidyl-tRNA synthetases, Mol. Cell Biol. 12 (1992) 5801–5815.
[30] L. Feng, H. Yoon, T.F. Donahue, Casein kinase II mediates multiple phosphorylation of Saccharomyces cerevisiae eIF-2 alpha (encoded by SUI2), which is required for optimal eIF-2 function in *S. cerevisiae*, Mol. Cell Biol. 14 (1994) 5139–5153.
[31] A. Breitkreutz, H. Choi, J.R. Sharom, L. Boucher, V. Neduva, B. Larsen, Z.Y. Lin, B.J. Breitkreutz, C. Stark, G. Liu, J. Ahn, D. Dewar-Darch, T. Reguly, X. Tang, R. Almeida, Z.S. Qin, T. Pawson, A.C. Gingras, A.I. Nesvizhskii, M. Tyers, A global protein kinase and phosphatase interaction network in yeast, Science 328 (2010) 1043–1046.
[32] M.J. Clemens, Initiation factor eIF2 alpha phosphorylation in stress responses and apoptosis, Prog. Mol. Subcell Biol. 27 (2001) 57–89.
[33] T. Grousl, P. Ivanov, I. Frydlova, P. Vasicova, F. Janda, J. Vojtova, K. Malinska, I. Malcova, L. Novakova, D. Janoskova, L. Valasek, J. Hasek, Robust heat shock induces eIF2alpha-phosphorylation-independent assembly of stress granules containing eIF3 and 40S ribosomal subunits in budding yeast, *Saccharomyces cerevisiae*, J. Cell Sci. 122 (2009) 2078–2088.
[34] K.D. Meier, O. Deloche, K. Kajiwara, K. Funato, H. Riezman, Sphingoid base is required for translation initiation during heat stress in *Saccharomyces cerevisiae*, Mol. Biol. Cell 17 (2006) 1164–1175.