The NHR1-1 of Prs1 and the pentameric motif $_{284}$KKCPK$_{288}$ of Prs3 permit multi-functionality of the PRPP synthetase in *Saccharomyces cerevisiae*

One sentence summary: Manipulation of the *Saccharomyces cerevisiae* Prs1/Prs3 complex identifies three different functions: PRPP synthesis, CWI maintenance and intracellular transport

Running title: Division of labour among the Prs polypeptides in *Saccharomyces cerevisiae*

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ABBREVIATIONS: aa, amino acid(s), 3-AT, 3-amino-1,2,4-triazole; CWI, cell wall integrity; 5-FOA, 5-fluoro-orotic acid; GFP, green fluorescent protein; o/n, overnight; NHR, non-homologous region; nt, nucleotide(s); ONPG, o-nitrophenyl-β-D-galactopyranoside; PRPP, 5-phosphoribosyl-1(α)-pyrophosphate; Prs, 5-phosphoribosyl-1(α)-pyrophosphate synthetase; P-Prs5, phosphorylated Prs5; P-Slt2, phosphorylated Slt2; SC, synthetic complete; WT, wild type, YEPD, yeast extract peptone dextrose; Y2H, yeast-two-hybrid analysis.

ABSTRACT

The five-membered *PRS* gene family of *Saccharomyces cerevisiae* is an example of gene duplication allowing the acquisition of novel functions. Each of the five Prs polypeptides is theoretically capable of synthesising PRPP but at least one of the following heterodimers is required for survival: Prs1/Prs3, Prs2/Prs5 and Prs4/Prs5. Prs3 contains a pentameric motif $_{284}$KKCPK$_{288}$ found only in nuclear proteins.

Deletion of $_{284}$KKCPK$_{288}$ destabilises the Prs1/Prs3 complex resulting in a cascade of events, including reduction in PRPP synthetase activity and altered CWI as measured by caffeine sensitivity and Rlm1 expression. Prs3 also interacts with the kinetochore-associated protein, Nuf2. Following the possibility of $_{284}$KKCPK$_{288}$-mediated transport of the Prs1/Prs3 complex to the nucleus it may interact with Nuf2 and phosphorylated Slt2 permitting activation of Rlm1. This scenario explains the breakdown of CWI encountered in mutants lacking *PRS3* or deleted for $_{284}$KKCPK$_{288}$. However, removal of NHR1-1 from Prs1 does not disrupt the Prs1/Prs3 interaction as shown by increased PRPP synthetase activity. This is evidence for the separation of the two metabolic functions of the PRPP-synthesising machinery: provision of PRPP and maintenance of CWI and is an example of evolutionary development when multiple copies of a gene were present in the ancestral organism.

INTRODUCTION

Yeast is an undisputed model organism for the investigation of the connection between genes and their functions in cell metabolism. As a result of the whole genome duplication that occurred approximately 100 million years ago a tetraploid yeast was created (Wolfe and Shields 1997, Dujon, Sherman, Fischer et al. 2004, Wolfe 2015). Over time this unstable tetraploid yeast lost more than 80% of the duplicated gene copies. However, at least 10% of the 6000 remaining genes which make up the genome of the current species of *Saccharomyces cerevisiae* are duplicated (Botstein and Fink 2011) and implies that these remaining gene duplications have made a positive contribution to the ancestral yeast's relative fitness.

Our research in *S. cerevisiae* has identified five copies of a gene each of which is theoretically capable of encoding the enzyme PPRPP synthetase (EC 2.7.6.1; Prs: ATP:D-ribose-5-phosphate diphosphotransferase) responsible for the production of

PRPP (5-phosphoribosyl-1(α)-pyrophosphate), a building block of nucleotides and aromatic amino acids (Hove-Jensen, Andersen, Kilstrup et al. 2017). While bacterial genomes contain only one gene encoding PRPP synthetase plants, fungi and mammals contain between two and five *PRS* genes. For instance, in the genome of the filamentous fungus *Eremothecium (Ashbya) gossypii* four genes have been found whose products contribute to cell growth and the production of riboflavin, vitamin B_2, an essential co-factor in human metabolism and often used as a food additive (Mateos Jiménez, Revuelta 2006, Jiménez, Santos and Revuelta 2008) demonstrating the ubiquity of the requirement of PRPP in cells.

Another filamentous fungus, *Aspergillus nidulans*, has three *PRS* genes, *AnprsA*, *AnprsB* and A*nprsC*, all of which are highly expressed during hyphal growth and sporulation. Genetic analysis revealed that *AnprsB* and A*nprsC* are essential or auxotrophic genes since the transformants were always heterokaryotic (Zhong, Wei, Guan et al. 2012, Jiang, Wei, Zhong et al. 2017). Further analysis revealed that the auxotrophy caused by mutation in either *AnprsB* or A*nprsC* could be at least partially compensated by including histidine, tryptophan, pyrimidine and AMP in the media, thereby confirming the multiple auxotrophy associated with these mutants. The fact that mutation of *AnprsA* does not result in auxotrophy together with the relative transcription levels of *AnprsB*>A*nprsC*>*AnprsA* is commensurate with the hypothesis that the products of these genes may contribute unequally to Prs activity. Furthermore, the identity >80% of *AnprsB* with Prs2, Prs3 and Prs4 of *Saccharomyces cerevisiae* together with the 70% identity of A*nprsA* and *AnprsC* to yeast Prs1 and Prs5, respectively, would suggest that PRPP synthesis in *A. nidulans* may, as in *S. cerevisiae*, be dependent on interactions between different Prs polypeptides.

An interesting discovery that a protein, HbPrs4, encoded in the rubber tree (*Hevea brasiliensis* Muell.Arg.) genome with 80% identity to Prs4 of *Arabidopsis thaliania* had a strong Y2H interaction with the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase regulating protein degradation through the ubiquitin/26 S proteasome pathway of the rubber tree, raising the possibility of an interaction between HbPrs4 and the cell cycle. A BlastN search of the rubber tree genome database revealed another sequence with 84% identity to *HbPRS4* gene. PRPP production is important for the nucleotide and protein biosynthesis required for latex generation. *HbPRS4* is highly expressed in the bark and responded to ethylene treatment, used in natural rubber production, to increase latex production. Ethylene treatment was found to increase both HbPrs4 expression and the ATP/ADP content of latex cells (Amalou, Bangratz and Chrestin 1992, Yu, Zhang, Zhang et al. 2017).

A recent publication has highlighted that increased expression of the five-membered *PRS* gene family of *S. cerevisiae* correlates with xylose utilization in a genetically-engineered industrial strain of *S. cerevisiae*, emphasizing the central role of PRPP synthesis in the metabolic pathway for biofuel production from lignocellulose (Feng, Liu, Weber et al. 2018).

Spinacia oleracea has four *PRS* genes. An aa sequence in the N-terminal region in spinach isozyme 2 is consistent with its localization in the chloroplast whereas isozyme 3 is postulated to be localized to the mitochondria. Isozyme 4 has no extension and is considered to be located in the cytoplasm (Krath and Hove-Jensen 1999, Krath and Hove-Jensen 2001).

The five paralogous genes, each of which is theoretically capable of encoding the enzyme PRPP synthetase that produces PRPP in *S. cerevisiae* and which, in accordance with *S. cerevisiae* nomenclature, have been designated *PRS1-5* (Carter,

Beiche, Hove-Jensen et al. 1997). Extensive genetic analysis indicates that in order to survive, the yeast genome must contain at least one of three minimal functional subunits, *viz* Prs1/Prs3, Prs2/Prs5 and Prs4/Prs5. The Prs1/Prs3 subunit is the most important since mutant strains relying on either of the other two subunits are severely impaired in their growth and PRPP-synthesizing capacity (Hernando, Carter, Parr et al. 1999, Hernando, Parr and Schweizer 1998). Furthermore, simultaneous deletion of *PRS1* and *PRS5* or *PRS3* and *PRS5* causes synthetic lethality (Hernando, Parr and Schweizer 1998). Both *PRS1* and *PRS5* genes contain non-identical in-frame insertions, not present in *PRS2*, *PRS3* or *PRS4* (Schneiter, Carter, Hernando et al. 2000, Wang, Vavassori, Schweizer et al. 2004, Vavassori, Wang, Schweizer et al. 2005a, Vavassori, Wang, Schweizer et al. 2005b, Kleineidam, Vavassori, Wang et al. 2009, Ugbogu, Wippler, Euston et al. 2013, Ugbogu, Wang, Schweizer et al. 2016). The region corresponding to NHR1-1 of Prs1 is required for bringing the Prs1/Prs3 subunit into contact with an element of the cell wall integrity (CWI) pathway as shown by Y2H and co-immunoprecipitation (Wang, Vavassori, Schweizer, et al. 2004, Ugbogu, Wang, Schweizer et al. 2016) and may explain why strains lacking *PRS1* and *PRS3* do not survive at elevated temperature (Ugbogu, Wippler, Euston, et al. 2013). *PRS5* contains two insertions, one of which is characterized by the presence of a cluster of three phosphorylatable serine residues (Ficarro, McCleland, Stukenberg et al. 2002). Mutation of these residues, either singly or multiply, influences two endpoints of the CWI pathway at both ambient and elevated temperatures (Ugbogu, Wang, Schweizer, et al. 2016). Clearly, the insertion in *PRS1* and at least one in *PRS5* are not gratuitous but fulfill essential functions in the cell. Although not ubiquitous in *PRS* genes NHR sequences are also found in eukaryotic organisms which have multiple *PRS* genes. *Aspergillus nidulans* and *Eremothecium (Ashbya) gossypii*

genomes (www.aspgd.org, https://www.agd.unibas.ch/) (Hermida, Brachat, Voegeli et al. 2005, Jiménez, Santos and Revuelta 2008) contain three and four *PRS* genes, respectively. Two of the three *PRS* genes in *A. nidulans*, AnPrsA and AnPrsC contain NHR sequences at similar positions to those in *S. cerevisiae* Prs1 and Prs5. Two of the four genes in *Eremothecium (Ashbya) gossypii* also contain NHRs and are considered to be homologues of *S. cerevisiae* Prs1 and Prs5. *Schizosaccharomyces pombe* has three *PRS* genes (https://www.pombase.org), two of which, *S. pombe* Prs1 and Prs2 contain insertions. The insertion in *S. pombe* Prs1 is at a similar position to that of NHR5-1 of *S. cerevisiae* Prs5 and *S. pombe* Prs2 has an insertion at the same relative position as NHR5-2 of *S. cerevisiae* Prs5.

The mammalian PRPP synthetase-associated proteins, Pap-39 and Pap-41 have 76% sequence identity (Ishizuka, Kita, Sonoda et al. 1996, Katashima, Iwahana, Fujimura et al. 1998) with each other and are thought to play a negative regulatory role in PRPP synthetase activity since, following their removal by gel filtration in the presence of 1 M $MgCl_2$, Prs activity is increased (Tatibana, Kita, Taira et al. 1995, Becker 2001). Both Pap-39 and Pap-41 contain NHRs of 29 and 30 aa, respectively, occupying similar locations in the polypeptides to the locations of NHR1-1 and NHR5-2 of the corresponding *S. cerevisiae* genes. There is an interesting connection between human Prs (hPRPS1) and at least two human neuropathies, Arts syndrome and CMTX5 (Charcot-Marie-Tooth) and for which the corresponding associated mutations have been identified in hPRPS1 (de Brouwer, van Bokhoven, Nabuurs et al. 2010, Mittal, Patel, Mittal et al. 2015). We have exploited the high sequence similarity of h*PRPS1* and yeast *Prs1* to create genocopies of Arts syndrome and CMTX5 and in our current investigations have examined their influence on Prs activity.

Prs3 which interacts with Prs1 (Wang, Vavassori, Schweizer, et al. 2004) to create the minimal functional unit Prs1/Prs3 harbours a sequence five aa in length that is unique to Prs3 and close to the C-terminus of the polypeptide. When deleted, there is a negative impact on yeast physiology, consistent with Prs1/Prs3 being the most important of the three genetically-defined minimal functional subunits. Using a NADH-coupled enzyme test for PRPP synthetase in selected mutants and deletants it has proved possible to separate the production of PRPP, the metabolic function, from the maintenance of CWI, thereby emphasising the scope for evolutionary development when multiple copies of a gene are present.

MATERIALS AND METHODS

Plasmids, *in vitro* mutagenesis and strains, growth conditions

Propagation of plasmids, *Escherichia* coli DH5α and standard DNA manipulations were performed as described (Ausubel, Brent, Kingston et al. 1995). The strains and plasmids used in this study are listed in Tables S1 and S2. Expression of fusion genes cloned in pGAD$_{424}$-*LEU2* was driven by the *ADH1* promoter. The plasmid also contained the *GAL4* DNA activation domain. *In vitro* mutagenesis with the plasmid pGAD-Prs3 as the template and the mutagenic primers was carried out using the Quick-Change^{R} site-directed mutagenesis kit (Agilent^{TM}) following the manufacturer's protocol. Mutagenic primer sequences are provided under Supplementary Data (TABLE S3.). Mutations were confirmed by double-stranded sequencing of the mutagenized plasmids. Yeast genetic manipulations and media were as described in (Guthrie and Fink 1991, Kaiser, Michaelis and Mitchell 1994). Yeast cultures were grown in YEPD (1% Yeast extract, 2% peptone 2% glucose) or selective media, SC (0.67% Yeast nitrogen base without aa and $(NH_4)_2SO_4$, 2% glucose 0.5% $(NH_4)_2SO_4$) supplemented with the appropriate nutrients to select for

plasmids. Yeast transformation was performed by the 'Plate' method (Elble 1992). For Y2H analysis the high efficiency transformation protocol described in (Gietz and Woods 2002) was performed. YN96-77 was transformed with pGAD-Prs3, Tc4(pGAD-prs3Δ-KKCPK) or Tc10(pGAD-prs3-ΔKKCPK). Caffeine-mediated CWI pathway activation was determined as described previously (Ugbogu, Wippler, Euston, et al. 2013).

Y2H analysis

The host strain PJ69-4A (James, Halladay and Craig 1996) was transformed with the interacting plasmids. At least three independent transformants for each combination were tested for their ability to grow on selective media lacking adenine or histidine plus 150 mM 3-AT and assayed for β-galactosidase activity using ONPG as the substrate. The specific activity of β-galactosidase was calculated according to (Wang, Vavassori, Schweizer, et al. 2004).

Determination of Rlm1 expression

Rlm1 activation was measured in the transformed strains of YN96-77 co-transformed with the reporter plasmid pHPS100-URA (Kirchrath, Lorberg, Schmitz et al. 2000). The transformants were selected on appropriate media and three independent colonies were inoculated individually into 10 ml of selective media. Following o/n incubation the cells were diluted to $OD_{600} \approx 0.5$ in 2 x 50 ml of the same media in 250 ml Erlenmeyer flasks. One flask from each strain was incubated at 30^{o}C and the other at 37^{o}C until $OD_{660} \approx 1$ was attained. Rlm1 expression was measured with the Thermo Scientific Yeast β-galactosidase assay kit according the manufacturer's protocol (Fisher Thermo Scientific^{R}) and quantified according to (Eq. 1) where V is the volume of cells (ml) used in the assay and t is the reaction time in min.

Equation 1

$$\beta - galactosidase\ activity\ (\text{Units}) = \frac{1000 \text{ x } A_{420}}{\text{t x V x } OD_{660}}$$

Rescue of synthetic lethality

YN97-18, YN97-19 and YN97-20 which contain the synthetically lethal deletion of *PRS3* and *PRS5* and are kept alive by the presence of pVT3 (TABLE S2.) were transformed with pGAD-Prs3, Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCPK) and the transformants selected for their ability to grow in the presence of 5-FOA for the counter selection of the *URA3* plasmid pVT3.

Western blotting

Western blotting was performed essentially as described in (Ugbogu, Wippler, Euston, et al. 2013) on YN98-11 transformed with either pGAD-Prs3, Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCPK) (TABLE S1.). For each transformant crude extracts equivalent to 15 μg protein per lane were loaded and separated on 4-15% SDS-PAGE gel. Two gels were run simultaneously, one was stained with Coomassie-Blue and the other, following transfer to a PVDF membrane was probed with specific anti-GFP antibodies (Santa Cruz sc-57587) and sc-2060 as primary and secondary antibodies, respectively. Successful separation and transfer to the membrane was checked by Ponceau S. Commercially available recombinant GFP (Roche) was used as a positive control. GFP signals were detected using the ECL+Plus™ system by chemiluminescence (Bio-Rad). After processing the protein gel images were obtained with the ChemiDoc™ XRS$^+$ imager using the Image Lab software, V4.1 (Bio-Rad), for the detection of bands corresponding to the separated polypeptides. The blots were reproduced at least once in independent experiments with a representative image shown.

Measurement of Prs activity

Single colonies of the strains to be tested for PRPP synthetase activity on SC-leu (YN96-77 transformants) or SC-trp (YN96-66 transformants) were grown o/n in 10 ml of the appropriate media. An aliquot of the o/n culture was added to 50 ml of the appropriate media to give an OD_{600} 0.2 and incubated at 30^0C until an $OD_{600} \approx$ 1.0-1.2 was attained. After harvesting, the cells were resuspended in sterile dist. H_2O, washed once and the wet weight determined. Cell pellets were frozen at -80^0C until cells were broken. Cell pellets were resuspended individually in 200 µl extraction buffer (50 mM KH_2PO_4/K_2PO_4 pH 7.5, 10% glycerol, 0.1% Triton X-100, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 5 mM DTT). The cell suspension was transferred into an Eppendorf™ tube containing 400 mg acid-washed glass beads (425-600 µm) (Sigma-Aldrich). The tubes were vortexed four times for 45 sec with 1 min incubation on ice between vortexing. The disrupted cells were centrifuged for 10,000 *g* for 10 min at 4^0C and the supernatants were removed by pipetting to fresh Eppendorf™ tubes. Protein content of the crude extracts was determined by the Quick Start™ Bradford protein assay (Bradford 1976) using a standard curve prepared with BSA stock solutions of known concentrations.

The specific activity of PRPP synthetase was measured in a spectrophotometric coupled-enzyme test using myokinase, pyruvate kinase and lactate dehydrogenase (Sigma-Aldrich) (Donini, Garavaglia, Ferraris et al. 2017, Jiang, Wei, Zhong, et al. 2017) as the decrease of NADH oxidised and expressed as µMol min^{-1} mg^{-1} protein using the molar extinction coefficient of 6220 M^{-1} cm^{-1} for NADH by monitoring absorption at 340 nm in a SpectraMax M series microplate reader. A conversion factor of 2 is required to calculate the specific activity since 2 moles of β-NADH are oxidised for each mole of PRPP produced. A 30 min pre-incubation of the reaction

buffer consisting of 50 mM KH_2PO_4/K_2PO_4 pH 7.5, 5 mM $MgCl_2$ x $6H_2O$, 2mM ATP, 0.2 mM NADH, 3.75 mM phosphoenol-pyruvate, 1.5 U myokinase, 3 U pyruvate kinase, 1.5 U lactate dehydrogenase without 3.75 mM ribose-5-phosphate was carried out at 37 ^{0}C. Following the addition of ribose-5-phosphate the reaction was started by adding an appropriate volume of crude extract (0.2-0.3 μg/ml) to the reaction mixture and was monitored continuously over a period of 20 min with measurement being recorded at intervals of 26 sec. Varying amounts of crude extract were used in independent experiments thus compensating for the different specific activities of PRPP synthetase of the mutants tested.

Statistical analysis

All data are calculated as the mean ± s.d. Statistical analysis was conducted using SPSS Statistics^S software, version 22. The statistics for the β-galactosidase assays were performed by two-factor ANOVA analysis in tandem with the Bonferroni test to determine the significance within the dataset. This approach was taken to test for significance within each transformant group by comparing Rlm1 expression in response to heat stress and between transformants relative to the respective culture conditions.

For the measurement of the specific PRPP synthetase activity an independent samples t-test was conducted between the crude extracts of the WT and individual mutants. Robust tests for equality of variance between samples was assessed by the Levene's test (Levene 1960). In the case of extracts with unequal variance a correction was applied to the *p*-value. In all instances a *p*-value of ≤ 0.05 was considered statistically significant.

RESULTS

Deletion of $_{284}$KKCPK$_{288}$ in Prs3

There is a high degree of sequence similarity between all five Prs gene products. We have discovered that Prs3 contains the sequence (aa $_{284}$KKCPK$_{288}$), i.e. two positively charged aa, lysine (K) flanking three residues, one of which is proline (P) located close to the C-terminus of the polypeptide and not present in the Prs1, Prs2, Prs4 or Prs5 polypeptides. Such a sequence is present in several yeast nuclear proteins but not in yeast cytoplasmic proteins (Herrero, Martinez-Campa and Moreno 1998). In a genome-wide Y2H assay it was found that Prs3 interacts with the nuclear import protein, karyopherin α homologue, importin α encoded by the *SRP1* gene (Ito, Chiba, Ozawa et al. 2001) (http://dbarchive.biosciencedbc.jp/en/yeast-y2h/download.html), implying that Prs3 has the potential to be at least temporarily a yeast nuclear protein. An obvious question is: what effect(s) does deletion of this site have on yeast physiology?

By means of specifically designed primers the fifteen nts corresponding to the motif $_{284}$KKCPK$_{288}$ were deleted from the Prs3 sequence. Fortuitously, this sequence contained a *Bae*GI restriction endonuclease site, therefore allowing a pre-screening by restriction digest of the mutagenised plasmids prior to sequencing. Sequencing of the entire length of the selected plasmids confirmed the loss of 15 nt containing a unique *Bae*GI site. This region is illustrated in Figure 1. Two plasmids Tc4(pGAD-prs3-ΔKKCPK) and Tc10(pGAD-prs3-ΔKKCPK) were selected for further experimentation.

To determine whether or not deletion of $_{284}$KKCPK$_{288}$ in Prs3 has an influence on yeast physiology we carried out plasmid shuffling using a synthetically lethal *prs3Δ*

prs5Δ double deletant (YN97-18) which is kept alive by the inclusion of a *URA3*-based plasmid, pVT3, carrying *PRS3* (Hernando, Carter, Parr, et al. 1999). Transformation of this strain with the WT Prs3 plasmid, Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCPK) followed by elimination of pVT3 on 5-FOA-containing media provided evidence that neither of the two plasmids lacking the sequence were capable of sustaining growth on 5-FOA-containing media (Figure 2). Repeating this experiment using YN97-19 and YN97-20 yielded identical results, thus providing evidence that the $_{284}$KKCPK$_{288}$ is essential for viability (data not shown).

Elimination of $_{284}$KKCPK$_{288}$ disrupts the Prs1/Prs3 complex

We have previously shown that in a strain containing an integrated GFP-labelled version of *PRS1* in combination with a deletion of *PRS3* the GFP signal is no longer visible in a Western blot. This is not the case if GFP-labelled *PRS1* is combined with deletions of *PRS2, PRS4* or *PRS5* (Ugbogu, Wippler, Euston, et al. 2013). Western blotting of transformants of YN98-11 with WT Prs3, Tc4(pGAD-prs3-ΔKKCPK)) or Tc10(pGAD-prs3-ΔKKCPK) revealed that the GFP signal was visible only when WT Prs3 was present (Figure 3). However, removal of the $_{284}$KKCPK$_{288}$ results in the loss of the strength of the GFP signal to the level observed to a strain lacking Prs3 implying that the loss of the five aa alters the Prs3 polypeptide to such an extent that it can no longer combine with Prs1 to form a stable Prs1/Prs3 heterodimer. The lower GFP-responsive band has been observed in previous Western blots and has been explained as an unknown protein which cross-hybridises with anti-GFP antibodies since it appeared in Western blots of a WT strain containing no GFP-labelled proteins (Ugbogu, Wippler, Euston, et al. 2013).

Further investigation of the transformants containing one or other of the plasmids lacking the $_{284}$KKCPK$_{288}$ sequence yielded results consistent with a breakdown of CWI maintenance. Transformants of the strain YN96-77 lacking *PRS3* with the deleted plasmids does not reverse the caffeine-sensitive phenotype of this strain. As shown in Figure 4 (A) the two transformants containing a prs3-ΔKKCPK plasmid show a similar degree of caffeine sensitivity, i.e. cessation of growth at 2-3 mM caffeine comparable with that of the negative control YN96-77 [pGAD]. However, when the WT Prs3 is present good growth is still visible at 4 mM caffeine. Growth at 37^0C is severely impaired in the presence or absence of $_{284}$KKCPK$_{288}$ but in contrast to the transformant containing WT Prs3 the addition of 1 M sorbitol to the media does not fully restore growth in YN96-77 containing either Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCPK) (Tatsiana Chyker, unpublished data).

Effect of deletion of $_{284}$KKCPK$_{288}$ on Rlm1 expression and PRPP synthetase activity

Using a reporter plasmid for Rlm1 expression following Slt2 activation there is a significant reduction in Rlm1 expression at 30^0C of over 70% in transformants of YN96-77 (*prs3Δ*) [pHPS100-URA] with Tc4(pGAD-prs3-ΔKKCPK)) or Tc10(pGAD-prs3-ΔKKCPK) in comparison to a transformant of the same strain carrying a WT version of Prs3. Incubation of the same strains at 37^0C resulted in a 1.5-4.0 fold increase in Rlm1 expression whether or not the $_{284}$KKCPK$_{288}$ sequence is present (Figure 4(B)).

In the absence of $_{284}$KKCPK$_{288}$ the response of Rlm1 expression with respect to time is also affected (Figure 4(C)). Transformants of YN96-77 (*prs3Δ*) [pHPS100-URA] were grown at 30^0C before shifting to 37^0C. Aliquots of each culture were removed

at 60 and 120 min for measurement of Rlm1 expression at 37^0C. Under these culture conditions both the vector control and the WT Prs3 showed a steady increase in Rlm1 expression whereas removal of $_{284}$KKCPK$_{288}$ disrupts this pattern. Removal of $_{284}$KKCPK$_{288}$ has apparently a 2-fold effect: a reduction in the β-galactosidase activity at time zero and no steady increase over time (Figure 4(C)).

Prs activity in YN96-77 (Figure 5) was examined with the transformants harbouring WT Prs3, the vector and Tc4(pGAD-prs3-ΔKKCPK)) or Tc10(pGAD-prs3-ΔKKCPK). When Prs3 was present Prs activity is equivalent to the WT. However, in the absence of $_{284}$KKCPK$_{288}$ the enzyme activity was significantly reduced in comparison to that of the WT and YN96-77 [pGAD-Prs3]. Specifically, with Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCPK) the enzyme activity was only 25% higher than that of the vector control which measures Prs activity produced by complexes of Prs2/Prs5 and Prs4/Prs5. This is further evidence that the deletion of $_{284}$KKCPK$_{288}$ has a deleterious effect on the Prs1/Prs3 complex. A result which is not unexpected since it has been shown in Western blotting that neither of these two plasmids is capable of restoring the GFP signal as evidence of a Prs1/Prs3 heterodimer (Figure 3).

NHR1-1 is not required for binding of Prs1 with Prs2 and Prs3

In contrast to the impact of the deletion of $_{284}$KKCPK$_{288}$ on the integrity of the Prs1/Prs3 complex, removal of NHR1-1 of Prs1 does not alter this complex. Y2H analysis of prs1-ΔNHR1-1 with Prs3 or Prs2 was carried out and the results are shown in Figure 6. The transformants with both pairwise combinations were tested for β-galactosidase activity (Figure 6) and their ability to grow on media lacking either adenine or histidine (data not shown). It is clear that NHR1-1 does not play a part in

the interactions between Prs1 and Prs2 or Prs3 emphasising that the interaction of Prs1 with Slt2 is solely dependent on the presence of NHR1-1 (Wang, Vavassori, Schweizer, et al. 2004).

Interaction of Prs3 with the kinetochore-associated protein Nuf2

A connection between PRPP-synthesising machinery and the cell cycle is suggested by the altered morphology of *prs3Δ* and *prs5Δ* strains, the former appear as highly vacuolated, large spherical cells (Schneiter, Carter, Hernando, et al. 2000) that suggested a possible defect in polarised growth or cell division. It was observed that a *prs3Δ* strain failed to arrest the cell cycle in the G1 upon nutrient deprivation (Binley, Radcliffe, Trevethick et al. 1999). These observations gained more importance in light of the fact that the kinetochore-associated protein Nuf2 interacted with Prs2 in a genome-wide Y2H assay (Uetz, Giot, Cagney et al. 2000). On the basis of this result we undertook a Y2H analysis of Nuf2 with Prs1-Prs5 which was carried out in both orientations. The data for the combination Nuf2Gal4BD and Prs1-5Gal4AD is summarised in Table 1. Only the interaction between Nuf2 and Prs4 could not be confirmed by Histidine or Adenine prototrophy and had a lower β-galactosidase activity than the negative control with the empty vectors. Interestingly, the interaction between Nuf2 and each of the five Prs polypeptides as measured by β-galactosidase activity can be summarised as Prs3/Nuf2>Prs2/Nuf2>Prs5/Nuf2>Prs1/Nuf2. These results are compatible with each of the Prs polypeptides being at least temporarily in the nucleus by virtue of interaction with the nuclear resident protein Nuf2 (Suzuki, Badger, Haase et al. 2016).

NHR1-1 of Prs1 is essential for maintenance of CWI but its loss does not impair PRPP synthetase activity

We have shown that NHR1-1 of Prs1 is essential for rescuing the synthetic lethality of a *prs1Δ pr5Δ* strain, most likely by virtue of its interaction with Slt2 since when NHR1-1 is removed from Prs1 there is no longer any interaction with Slt2 (Wang, Vavassori, Schweizer, et al. 2004). As shown above (Figure 6), loss of NHR1-1 does not interfere with the interaction of Prs1 with Prs3. However, in contrast removal of NHR1-1 caused a significant increase in PRPP synthetase activity in comparison to the Prs1 plasmid in YN96-66. (Figure 7(B)). Furthermore, deletion of the NHR1-1 results in bringing the divalent-cation-binding site and the PRPP-binding site (ribose-5-phosphate loop) closer together resembling the situation in Prs3, Prs2 and Prs4 which could in fact give rise to increased enzyme activity. Nevertheless, this result is particularly interesting since for the first time it has been shown that the removal of NHR1-1 disrupts the maintenance of CWI (Ugbogu, Wippler, Euston, et al. 2013) but does not have a negative effect on PRPP synthetase activity.

Figure 7(B) also displays the values for Prs activity in strains representing genocopies of human neuropathies and sites essential for enzyme activity. The mutation Q133P is the yeast genocopy of the mutation associated with Arts syndrome (de Brouwer, van Bokhoven, Nabuurs, et al. 2010, de Brouwer, Williams, Duley et al. 2007). This mutation Q133P could only relieve the synthetic lethality of a *prs1Δ pr5Δ* strain to a limited extent (Ugbogu, Wippler, Euston, et al. 2013) and reduces PRPP synthetase activity by over 50% of that measured in YN96-66 [pGBT9-Prs1]. CMTX5, the human neuropathy, is associated with the mutation M115T in hPRPS1 (Kim, Sohn, Shy et al. 2007, Synofzik, Müller vom Hagen, Haack et al. 2014) and the yeast genocopy (L115T) still retains 80% Prs activity in comparison to YN96-66 [pGBT9-

Prs1]. The Prs activity was also measured in two further mutations, one affecting the ribose-5-phosphate binding site at position 326 (D326A) and the double mutation (H130A/D326A) affecting both the divalent-cation and the PRPP-binding (ribose-5-phosphate) sites. There was no measurable effect on PRPP synthetase activity when the ribose-5-phosphate site was mutated whereas the double mutation H130A/D326A had an effect on enzyme activity similar to that of the Arts syndrome (Q133P) genocopy. The importance of the H130A divalent cation-binding site in contrast to the D326A PRPP-binding site (ribose-5-phosphate) has been observed previously in another scenario since H130A was incapable of rescuing a *prs1Δ pr5Δ* strain whereas D326A was more efficient than the WT (Ugbogu, Wippler, Euston, et al. 2013).

Discussion

PRPP is an extremely important metabolite due to its crucial role in the synthesis of purine and pyrimidine nucleotides and the fact that it links carbon and nitrogen metabolism. Our collection of mutants and deletions in the five *PRS*-encoding genes existing in the *S.cerevisiae* genome which affect both PRPP synthesis and CWI has provided the opportunity to investigate their relative contributions to these two vital aspects of yeast physiology. We have shown that the integrity of the minimal functional unit Prs1/Prs3 is dependent on the presence of Prs3 (Ugbogu, Wippler, Euston, et al. 2013). The results presented here demonstrate that the $_{284}$KKCPK$_{288}$ sequence close to the C-terminus of Prs3 and not found in any of the other four Prs polypeptides of *S.cerevisiae*, is essential for the maintenance of the Prs1/Prs3 subcomplex. Removal of these five aa, as is the case with a *PRS3* deletion, also results in the loss of the GFP-Prs1 signal (Figure 3). Furthermore, the failure of a Prs3 plasmid lacking the $_{284}$KKCPK$_{288}$ sequence to rescue the synthetic lethality of *prs3Δ prs5Δ* strain (Figure 2) is further evidence for the sequence $_{284}$KKCPK$_{288}$ being

necessary for the integrity of the heterodimer, Prs1/Prs3, the most important of the three minimal functional units. Furthermore, it supports the hypothesis that a *prs3Δ prs5Δ* strain is, in fact, a triple deletant, *prs1Δ prs3Δ prs5Δ*, as postulated previously (Ugbogu, Wippler, Euston, et al. 2013). The existence of such a triple deletant is incompatible with the requirement for the presence of at least one of the minimal functional units, Prs1/Prs3, Prs2/Prs5 or Prs4/Prs5 since any one of these subunits must be capable of synthesising PRPP at a level necessary for viability and to maintain CWI. The most important connection between PRPP synthesis and CWI is dependent on the NHR1-1 of Prs1 that directly interacts with the MAPK Slt2 when this is dually phosphorylated (Ugbogu, Wang, Schweizer, et al. 2016). However, the two minimal functional units, Prs2/Prs5 and Prs4/Prs5 can, by virtue of NHR5-2 of Prs5, play at least a supporting role in the maintenance of CWI. In addition, Y2H analysis has revealed that each of the four remaining polypeptides interact with Slt2. Although it has been shown that Prs1 is co-immunoprecipitated with phosphorylated Slt2 (Ugbogu, Wang, Schweizer, et al. 2016), the Y2H interaction between Slt2 and either Prs3 or Prs1 was stronger than between Slt2 and Prs2 or Prs4 or Prs5 (Stefano Vavassori, unpublished data) emphasising the importance of the Prs1/Prs3 complex and the necessity for cooperation of the five Prs polypeptides in the maintenance of cell viability. We have evidence that the phosphorylation status of Slt2 is different from the pattern found in the WT when one or more of the three phosphorylation sites in NHR5-2 is mutated. In a premature truncation of Prs5 that removed most of NHR5-2, impaired phosphorylation of Slt2 following mild heat shock was shown by Western blotting (Ugbogu, Wang, Schweizer, et al. 2016). Mutation of three specific serine residues, $S_{364}A$, $S_{367}A$ and $S_{369}A$, within NHR5-2 results in hyperphosphorylation of Slt2 and this was reflected in the expression of Rlm1, an endpoint of the CWI

pathway and Fks2, a component of the 1,3-β-glucan synthase, which is expressed only under conditions of stress (Levin 2011). A connection between Fks2 and Prs polypeptides is feasible *via* the Paf1 transcription complex. Elimination of *PAF1* results in a 50% reduction of the transcription of Prs1 (Chang, French-Cornay, Fan et al. 1999) and there is an interaction at the *FKS2* promoter between Paf1 and Slt2/Swi4/Swi6 when *FKS2* is transcribed (Kim and Levin 2011).

Alignment of the sequence of PRPP synthetase of *Bacillus subtilis* (Eriksen, Kadziola, Bentsen et al. 2000, Hove-Jensen, Andersen, Kilstrup et al. 2017) and the sequences of the five Prs polypeptides from yeast shows that NHR1-1 and NHR5-2 have locations external to the hexameric complex of *B.subtilis* Prs, implying that the interactions of these two non-homologous regions with other proteins are likely to be favoured. In a strain in which the sequence of NHR1-1 has been deleted from Prs1 a 50% increase in Prs activity in comparison to the WT grown in YEPD, is achieved (Figure 7(B)). A lower, albeit possibly a more accurate factor, 15%, for the increase in Prs activity in the absence of NHR1-1 is obtained by comparing the activities of YN96-66 [pGBT9-Prs1] and YN96-66 [pGBT9-prs1-ΔNHR1-1] since in both instances the constructs are plasmid-borne. Taking into consideration the alignment of Prs3 with *B.subtilis* Prs it is feasible that the presence of NHR1-1 could interfere with binding of Prs1 with Prs3. However, the presence of NHR1-1 and its ability to interact with phosphorylated Slt2 ensures two important properties of cellular homeostasis, PRPP synthesis and maintenance of CWI, are upheld, even though NHR1-1 is located C-terminally within the catalytic flexible loop of Prs1 (Figure 8). We know that the presence of NHR1-1 is essential for rescuing synthetic lethality of a *prs1Δ prs5Δ* strain and have hypothesised that the cause of synthetic lethality for such

a strain when *PRS1* and *PRS5* are simultaneously deleted is a breakdown of CWI (Ugbogu, Wippler, Euston, et al. 2013). In addition, we have shown that removal of NHR1-1 does not compromise the Prs1/Prs3 interaction as demonstrated by Y2H (Figure 6) and strengthens the argument that the synthetic lethality associated with simultaneous deletion of *PRS1* and *PRS5* is due to a breakdown of the CWI pathway. This is the first time it has been demonstrated that it is possible to separate Prs activity and maintenance of CWI as shown by the increase of Prs activity when NHR1-1 is no longer present. This is further substantiated by the inability of a strain lacking NHR1-1 to increase Rlm1 expression in response to elevated temperature (Ugbogu, Wippler, Euston, et al. 2013).

While it cannot be determined if the reduction of Rlm1 expression in *prs3Δ* strains containing either Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCP) is due to the lack of the five aa or the concomitant loss of Prs1, there is evidence that Prs1, Prs3 and Prs5 all interact with Rlm1 (Ugbogu, Wang, Schweizer, et al. 2016), a transcription factor always resident in the nucleus (Levin 2005, Levin 2011, Engelberg, Perlman and Levitzki 2014). It is tempting to hypothesise that Prs3 on account of its $_{284}$KKCPK$_{288}$ sequence is responsible for the transport of itself, Prs1 and Prs5 into the nucleus, thereby activating expression of Rlm1 and Fks2 and ensuring the maintenance of CWI. The interaction of Prs3 with Nuf2, a component of the kinetochore Ndc80 complex which is localised in the nucleus, provides further support to our hypothesis that Prs1, Prs3 and Prs5 must be transported to the nucleus. In our Y2H-based analysis of Nuf2 with each of the five Prs polypeptides the strongest interaction was between Nuf2 and Prs3 followed by Prs2>Prs5/Prs1 (Table 1). The interaction between Prs4 and Nuf2 was below the level of the negative control. These results support our working hypothesis that Prs3 can function as a

transport protein in times of stress bringing a Prs1/Prs3/Prs5 complex into the nucleus, thereby ensuring Prs1 *via* NHR1-1 comes into contact with activated Slt2. In an earlier publication (Schneiter, Carter, Hernando, et al. 2000) Western blotting with Prs1-specific antibodies revealed the presence of Prs1 in the nuclear fraction.

Furthermore, it would appear that the loss of $_{284}$KKCPK$_{288}$ causes such a conformational change in Prs3 that it prevents stabilisation of the Prs1/Prs3 dimer. The Prs3 polypeptide, although incapable *per se*, as are the four other Prs polypeptides, of PRPP synthesis nevertheless makes a major contribution to yeast survival by stabilising the most important minimal functional subunit, Prs1/Prs3 and ensuring its transport, with or without Prs5, to the nucleus in order to fulfill the requirement for the maintenance of CWI. Some support for this interpretation is provided by the measurement of Prs activity in transformants of YN96-77. Introduction of Prs3 into the *prs3Δ* strain YN96-77 restores Prs activity to that of the WT but introduction of either Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCPK) into YN96-77 does not bring Prs activity up to the level measured in YN96-77 [pGAD-Prs3] (Figure 5). Taken together the average Prs activity of the transformants carrying the deleted plasmids display a reduced Prs activity. In comparison to the activity of YN96-77 [pGAD] in which Prs activity is supplied by the Prs2/Prs5 and/or Prs4/Prs5 minimal subunits, a mean activity of 70% is achieved with Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCPK) relative to YN96-77 [pGAD] (Figure 5). This is in agreement with the inability of either of these two constructs to support the existence of a Prs1/Prs3 complex.

Alterations in the specific activity of Prs have been shown to be associated with the human neuropathies, Arts syndrome and CMTX5 (de Brouwer, van Bokhoven, Nabuurs, et al. 2010, Mittal, Patel, Mittal, et al. 2015). Genocopies in yeast of the

human mutations associated with both of these neuropathies had a negative influence on Prs activity; the mutation Q133P associated with Arts syndrome resulted in halving the Prs activity measured (Figure 7(B)). It is known that proline is a helix breaker and the substitution of glutamine (Q) by proline (P) prevents helix stabilisation through intermolecular H-bonding (de Brouwer, Williams, Duley, et al. 2007, de Brouwer, van Bokhoven, Nabuurs, et al. 2010). The CMTX5 genocopy in yeast, L115T, has a less severe effect on Prs activity, reducing it by approximately 35%. In keeping with its influence on Prs activity in humans the L115T mutation is found to be more adept than Q133P in rescuing the synthetic lethality of a *prs1Δ prs5Δ* strain. The double mutation H130A/D326A affecting the divalent cation- and PRPP-binding (ribose-5-phosphate) sites, respectively, was unable to rescue the synthetic lethality (Ugbogu, Wippler, Euston, et al. 2013) and like, Q133P, caused >50% reduction in Prs activity as measured in YN96-66 [pGB T9-Prs1] and would suggest that the integrity of the divalent cation-binding site is vital for Prs activity whereas D326A alone does not affect Prs activity.

CONCLUSIONS

The non-homologous regions of Prs1 and Prs5 are responsible for interaction with components of the CWI pathway (Ugbogu, Wippler, Euston, et al. 2013) (Ugbogu 2016) and the $_{284}$KKCPK$_{288}$ sequence of Prs3, which should perhaps be renamed NHR3-1, is essential for the stability of Prs1 and possibly necessary for transport of Prs1 and Prs5 to the nucleus. These findings provide a further explanation for the requirement of at least one heterodimeric complex for the survival of the yeast cell, since in addition to synthesising PRPP, there has to be a link to the mainteance of CWI. Support for such a link is provided by interaction of Prs polypeptides with Nuf2, a component of the kinetochore complex and Srp1 (importin α). Slt2 is found

both in the nucleus and cytoplasm (Kamada, Jung, Piotrowski et al. 1995, Hahn and Thiele 2002). Given the interaction of Prs1 with Slt2, Prs3 and Rlm1 it could well be that Prs3 facilitates the entry of Slt2 into the nucleus under conditions of stress. Therefore, Prs3 may be considered to both stabilise the Prs1/Prs3 minimal functional unit and ensure, under conditions of stress, together with Prs5 entry into the nucleus where the transient four-component complex, Prs1/Prs3/P-Prs5/P-Slt2 comes into contact with Rlm1 to initiate the stress response. It is also likely that there is an interaction with the Paf1 transcription complex that has responsibility for the transcriptional elongation of Fks2 under conditions of stress. The interaction of Prs1 and Prs3 is not compromised upon removal of NHR1-1. Our finding that removal of NHR1-1 from Prs1 results in a significant increase in Prs activity is a first time demonstration that the functions, CWI maintenance and PRPP production, associated with the Prs gene family can be separated.

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AUTHORS' CONTRIBUTION

Maëlle S., AR, RC, FH, SV, KW, GB, CGN, TC, EE conducted the experiments, LMS and MS conceived the idea of the project. LMS and MS interpreted the data and wrote the paper.

Conflict of interest. None declared.

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Figure 1

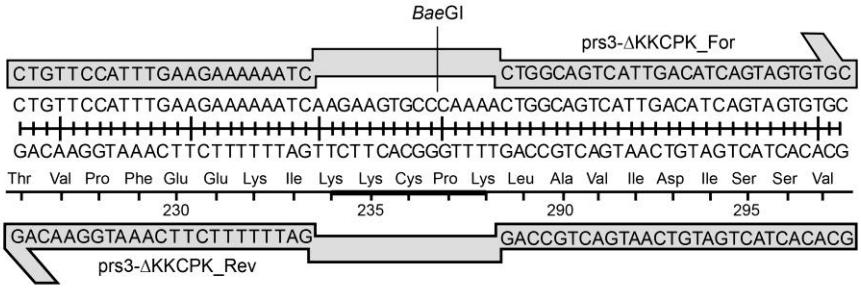

FIGURE 1. Schematic representation of the C-terminal region of Prs3 containing the $_{284}$KKCPK$_{288}$ motif and indicating the position of the *Bae*GI site used for the initial screening of mutagenized plasmids. The mutagenic primers prs3-ΔKKCPK_For and Prs3--ΔKKCPK_Rev (Table S3.) responsible for the deletion of the 15 nt corresponding to $_{284}$KKCPK$_{288}$ (highlighted as a black bar) are indicated above and below the nucleotide and protein sequences of $_{284}$KKCPK$_{288}$ and its flanking regions.

Figure 2

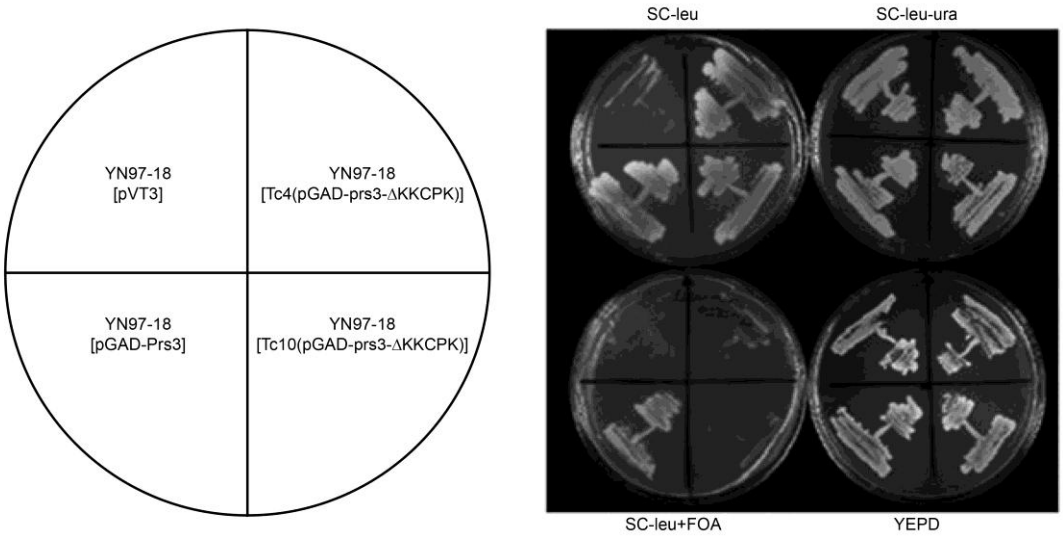

FIGURE 2. Rescue of synthetic lethality of YN97-18 (*prs3Δ prs5Δ*) [pVT3]. Plasmid shuffling was performed using FOA-counterselection. The strains were streaked onto appropriate media as indicated above and below the photographed plates and incubated at 30^0C for five days. The cartoon on the left indicates the plasmids used for testing the rescue of synthetic lethality.

Figure 3

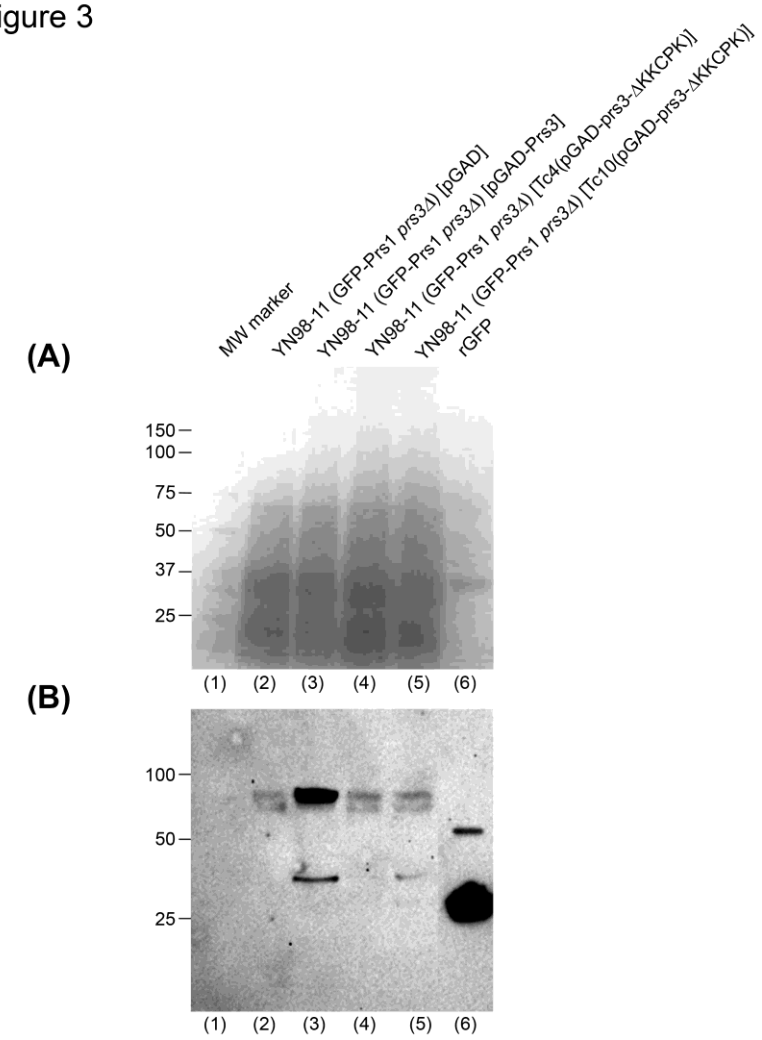

FIGURE 3. Western blotting reveals that the deletion of $_{284}$KKCPK$_{288}$ from Prs3 results in the loss of the GFP-signal associated with Prs1 in YN98-11. Crude extracts equivalent to 15 µg protein per lane were prepared from YN98-11 transformed with either pGAD (lane 2), pGAD-Prs3 (lane 3), Tc4(pGAD-prs3-ΔKKCPK) (lane 4) or Tc10(pGAD-prs3-ΔKKCPK) (lane 5) and separated on two simultaneously run 4-15% SDS-PAGE gels. (A) Coomassie-Blue stained gel as loading control and (B) following transfer of the duplicated gel to a PVDF membrane Western blotting was performed with specific anti-GFP antibodies (Santa Cruz sc-57587) and sc-2060 as primary and secondary antibodies, respectively. Successful electrophoresis and transfer to a PVDF membrane were checked by Ponceau S staining. The GFP-signal

was detected by chemiluminescence with anti-GFP antibodies. (A) Lane (1) molecular weight marker from Coomassie-stained gel. Lane (6) 10 μg rGFP standard.

Figure 4

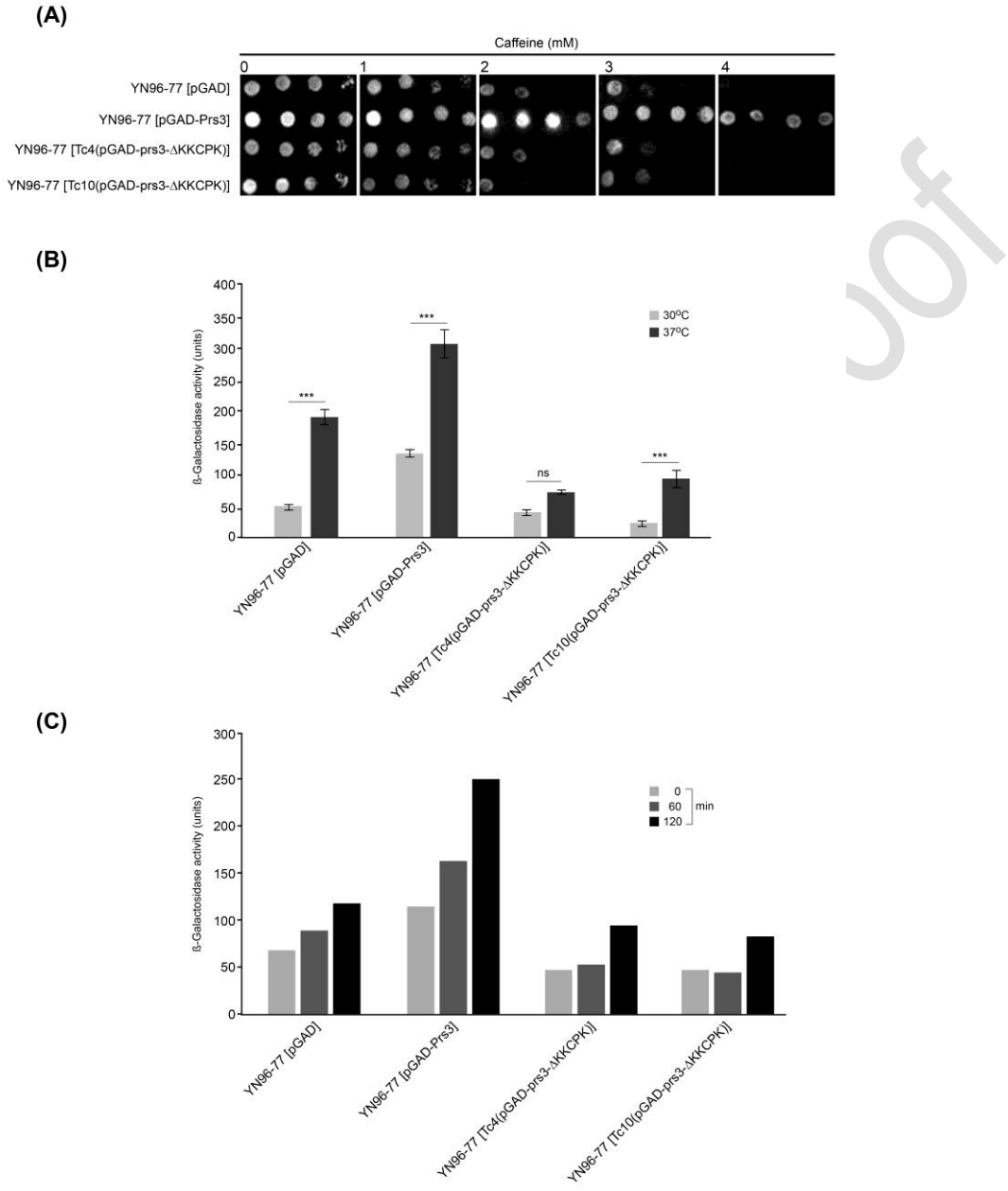

Figure 4. (A) The sequence $_{284}$KKCPK$_{288}$ is required for growth on caffeine. Cultures of YN96-77 (*prs3Δ*) transformed with pGAD, pGAD-Prs3, Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCPK) were adjusted to OD_{600} 0.5 and 3 μl of 10-fold serial dilutions thereof were spotted onto SC-leu caffeine-containing media at

the caffeine concentrations indicated. Plates were incubated at 30^0C for three days prior to documentation in a ChemiDoc^{TM} XRS+imager. The experiment was repeated four times and a representative image shown. (B) The effect of deletion of $_{284}$KKCPK$_{288}$ on Rlm1 expression. The four strains described in (A) were transformed with the reporter plasmid pHPS100-URA that expresses β-galactosidase as a measure of Rlm1 expression. The cultures were grown at either 30^0C or 37^0C to an OD_{600} 1.0 and Rlm1 expression was measured as β-galactosidase activity using the Thermo Scientific^{TM} yeast β-galactosidase kit. Nine independent repeat experiments were performed. The data are presented as the mean ± s.e.m. Asterisks represent significant temperature-dependent Rlm1 expression within transformants (p-value <0.01 = ***), ns = not significant. (C) Kinetics of Rlm1 expression in response to heat stress. The strains described in the legend of Figure 4(B) were grown at 30^0C to an OD_{600} 1.0 and an aliquot taken for β-galactosidase measurement prior to shifting the cultures to 37^0C. Aliquots for β-galactosidase measurements were removed after 60 and 120 min at 37^0C.

Figure 5

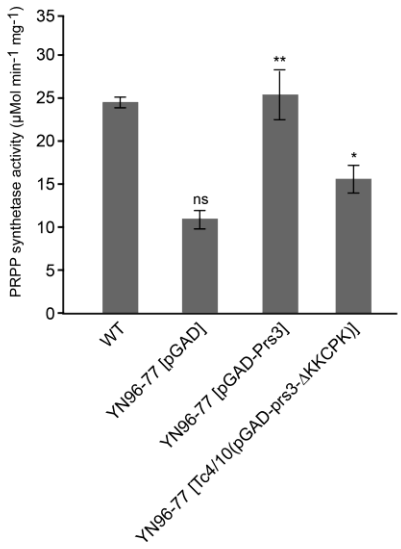

Figure 5. Deletion of $_{284}$KKCPK$_{288}$ from Prs3 reduces PRPP synthetase activity. YN94-1 (WT) and YN96-77 (*prs3Δ*) transformed with pGAD, pGAD-Prs3, Tc4(pGAD-prs3-ΔKKCPK) or Tc10(pGAD-prs3-ΔKKCPK) were grown o/n from a single colony at 30^{0}C in 10 ml SC-leu or YEPD for WT. Cell extracts were prepared as described in Materials and Methods. Following protein determination and pre-incubation of the reaction buffer at 37^{0}C for 30 min the reaction was started by the addition of ribose-5-phosphate and appropriate amounts of extracts dispensed into a 96-well plate. The results are expressed as µMol min^{-1} mg^{-1}. The significance level of YN96-77 [pGAD-Prs3] and YN96-77 [Tc4/10(pGAD-prs3-ΔKKCPK)] are calculated with respect to the specific activity of YN96-77 [pGAD]. Data is shown as mean ± s.d. Number of measurements for each transformant: WT, pGAD-Prs3, Tc4(pGAD-prs3-ΔKKCPK) (n=3) and for pGAD, Tc10(pGAD-prs3-ΔKKCPK) (n=5). For illustration purposes the data for Tc4(pGAD-prs3-ΔKKCPK) and Tc10(pGAD-prs3-

ΔKKCPK) have been combined (Tc4/Tc10(pGAD-prs3-ΔKKCPK). *P*-values: * = p≤0.05, ** = p≤0.01, ns = not significant.

Figure 6

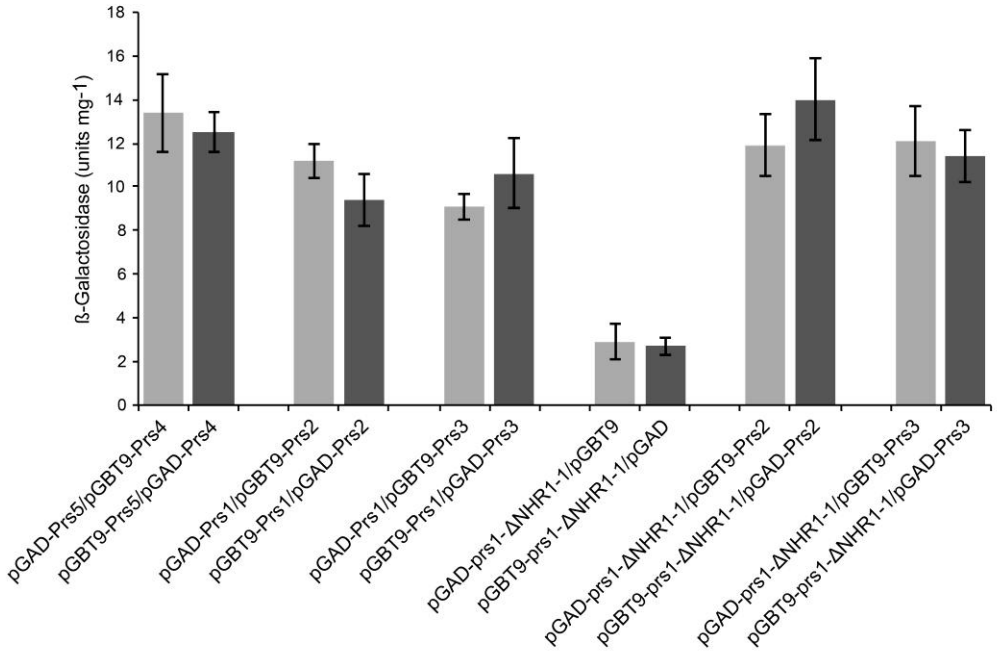

Figure 6. β-galactosidase activity of strains testing for interactions between NHR1-1 less Prs1 with Prs2 and Prs3. Cell extracts from strains assessing the indicated interactions were analysed using the ONPG β-galactosidase assay. The columns represent the mean of values obtained in three independent experiments and the error bars are representative of the s.d. between them.

Figure 7

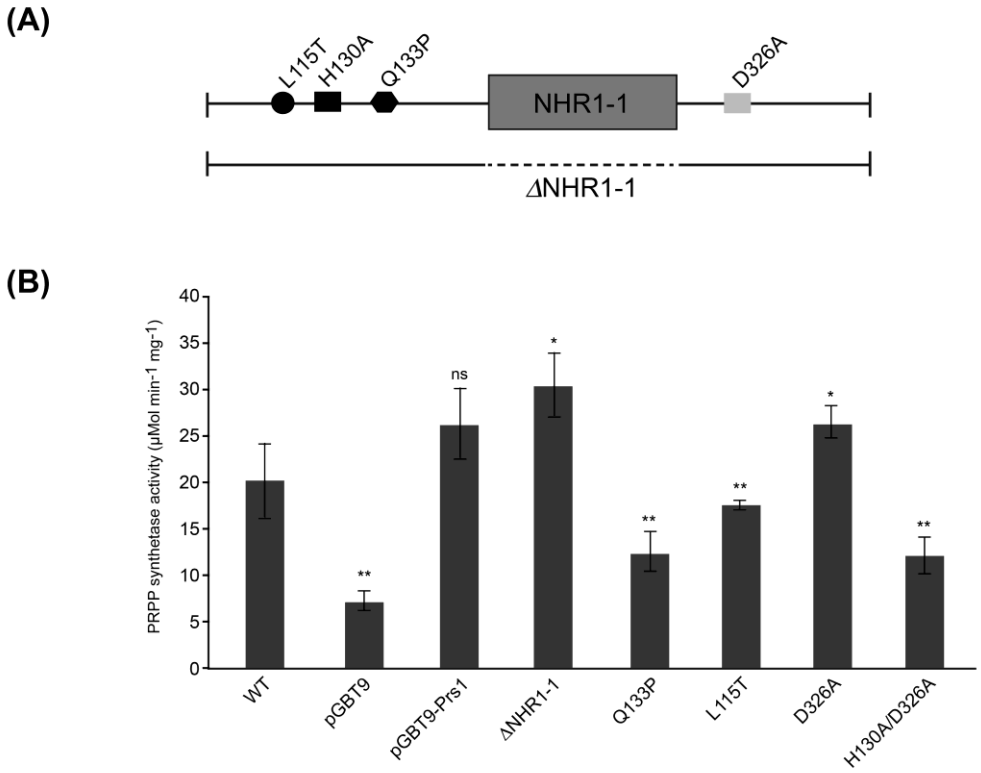

Figure 7. (A) Schematic representation of Prs1 showing the positions of the genocopies corresponding to CMTX5 (L115T), Arts syndrome (Q133P), the divalent cation-binding site (H130A), the PRPP-binding site (ribose-5-phosphate) (D326A) of PRPP synthetase, the location of NHR1-1 (gray box) and the extent of its deletion as a broken line. (B) PRPP synthetase of the constructs illustrated in (A) was measured as described in Figure 5. Transformants of YN96-66 with indicated plasmids were grown in SC-trp or YEPD for WT. Data is shown as mean ± s.d. Number of measurements for each transformant: WT and pGBT9 (n=5); pGBT9-Prs1 (n=6); ΔNHR1-1 and Q133P (n=4); L115T (n=3); D326A (n=8) and H130A/D326A (n=10). *P*-values: * = p≤0.05; ** = p≤0.01, ns = not significant with respect to pGBT9-Prs1.

Figure 8

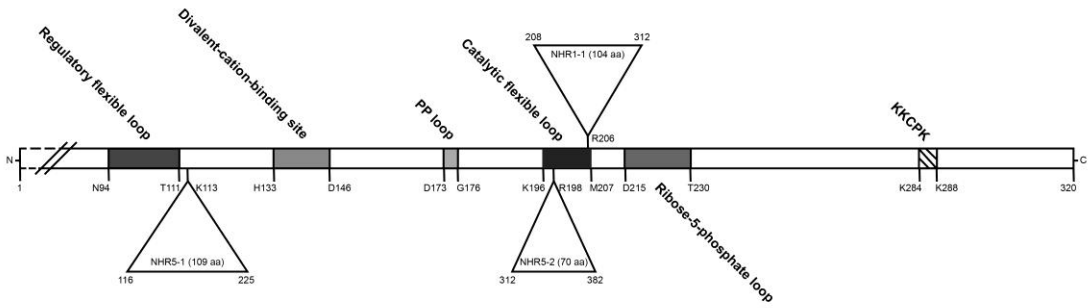

Figure 8. Schematic distribution of characteristic motifs of Prs polypeptides. The Prs3 polypeptide consists of 320 aa and the position of $_{284}$KKCPK$_{288}$ is indicated by a hatched box. The positions of the diagnostic features of a Prs polypeptide are indicated on the open bar, their coordinates below the open bar were obtained by comparing the sequence of *B.subtilis* Prs lacking its initial methionine with that of *S.cerevisiae* Prs3. The insertion of Prs1, NHR1-1, is indicated by a triangle above the open bar. The two insertions in Prs5, NHR5-1 and NHR5-2, are indicated by triangles below the open bar. The coordinates of the insertions refer to their positions in the sequences of Prs1 and Prs5, respectively. The numbering of their insertion points corresponds to the sequence of the Prs3 polypeptide. All sequence comparisons were performed using the CLUSTALW multiple alignment tool (weight matrix = Gonnet) available in Expasy (NPS@: Network Protein Sequence Analysis (Combet, Blanchet, Geourjon et al. 2000)

Table 1. Y2H interactions of Prs polypeptides with Nuf2 in PJ69-4A. The β-galactosidase activity (nmole ONPG min^{-1} mg^{-1}) represented the mean ± s.d. from at least three independent experiments.

Gal4 BD	Gal4 AD	β-galactosidase filter assay	Histidine prototrophy	Adenine prototrophy	β-galactosidase activity
-	-	-	-	-	2.8±0.3
Snf4	Snf1	+	+	+	11.4±0.7
Prs5	Prs4	+	+	+	11.1±0.1
Nuf2	Prs1	+	+	+	25.7±1.3
Nuf2	Prs2	+	+	+	39.9±0.5
Nuf2	Prs3	+	+	+	56.3±0.6
Nuf2	Prs4	+	-	-	1.7±0.1
Nuf2	Prs5	+	+	+	26±0.3