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Pichia pastoris 14-3-3 regulates transcriptional activity of the methanol inducible transcription factor Mxr1 by direct interaction

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Summary

The zinc-finger transcription factor, Mxr1 activates methanol utilization and peroxisome biogenesis genes in the methylotrophic yeast, *Pichia pastoris*. Expression of Mxr1-dependent genes is regulated in response to various carbon sources by an unknown mechanism. We show here that this mechanism involves the highly conserved 14-3-3 proteins. 14-3-3 proteins participate in many biological processes in different eukaryotes. We have characterized a putative 14-3-3 binding region at Mxr1 residues 212–225 and mapped the major activation domain of Mxr1 to residues 246–280, and showed that phenylalanine residues in this region are critical for its function. Furthermore, we report that a unique and previously uncharacterized 14-3-3 family protein in *P. pastoris* complements *Saccharomyces cerevisiae* 14-3-3 functions and interacts with Mxr1 through its 14-3-3 binding region via phosphorylation of Ser215 in a carbon source-dependent manner. Indeed, our *in vivo* results suggest a carbon source-dependent regulation of expression of Mxr1-activated genes by 14-3-3 in *P. pastoris*. Interestingly, we observed 14-3-3-independent binding of Mxr1 to the promoters, suggesting a post-DNA binding function of 14-3-3 in regulating transcription. We provide the first molecular explanation of carbon source-mediated regulation of Mxr1 activity, whose mechanism involves a post-DNA binding role of 14-3-3.

Introduction

The methylotrophic yeast, *Pichia pastoris*, is employed as a model system for studying peroxisome biogenesis as well as for the production of recombinant proteins (Cregg *et al.*, 1989; 2000; Cereghino and Cregg, 2000; Subramani *et al.*, 2000; Hartner and Glieder, 2006). Genes responsible for methanol catabolism (*AOX1*, *CAT* and *DHAS* encoding alcohol oxidase 1, catalase and dihydroxyacetone synthase, Aox, Cat and Dhas respectively) and peroxisome biogenesis (*PEX* genes) are induced by methanol and oleic acid (Cregg *et al.*, 1989; Hartner and Glieder, 2006). All three methanol utilizing enzymes are sequestered in peroxisomes where the initial reactions of methanol catabolism are compartmentalized, providing a rationale for the concurrent upregulation of methanol-utilizing enzymes and peroxisomes during growth in methanol (Gould *et al.*, 1992; Liu *et al.*, 1992; Johnson *et al.*, 1999; Stewart *et al.*, 2001). At the first step of methanol catabolism Aox oxidizes methanol to formaldehyde and the toxic by-product hydrogen peroxide is decomposed to oxygen and water by the action of Cat. *AOX1* expression is tightly regulated by carbon source being repressed by glucose, ethanol and glycerol and induced by methanol (Ellis *et al.*, 1985; Cregg *et al.*, 1989; Koutz *et al.*, 1989). Due to its efficient induction and tight regulation, the methanol-inducible promoter of *AOX1* (P_{AOX1}) has been employed in most heterologous recombinant protein overexpression systems in *P. pastoris* (Sakai *et al.*, 1995; Cereghino and Cregg, 2000; Cregg *et al.*, 2000; Gellissen, 2000).

Expression of the *MUT* (methanol utilizing) enzymes is regulated mainly at the transcriptional level by a zinc-finger transcription factor Mxr1 (methanol expression regulator 1) (Tschopp *et al.*, 1987; Cregg and Madden, 1988; Cregg *et al.*, 1989; Lin-Cereghino *et al.*, 2006). Functional disruption of Mxr1 causes a growth defect of *P. pastoris* in media containing methanol or oleic acid, and the expression of *AOX1* and *DHAS* was dramatically decreased. The oleic acid-induced expression of peroxisome biogenesis genes (i.e. *PEX5*, *PEX8, PEX14* and *FLD1*) was modestly affected and β-oxidation genes were affected hardly at all upon disruption of *MXR1* (Lin-Cereghino *et al.*, 2006). The binding sites for Mxr1 upstream of the *MUT* pathway and peroxisome biogenesis genes' promoters have been well characterized (Lin-Cereghino *et al.*, 2006; Hartner *et al.*, 2008; Kranthi *et al.*, 2009; 2010). A consensus sequence

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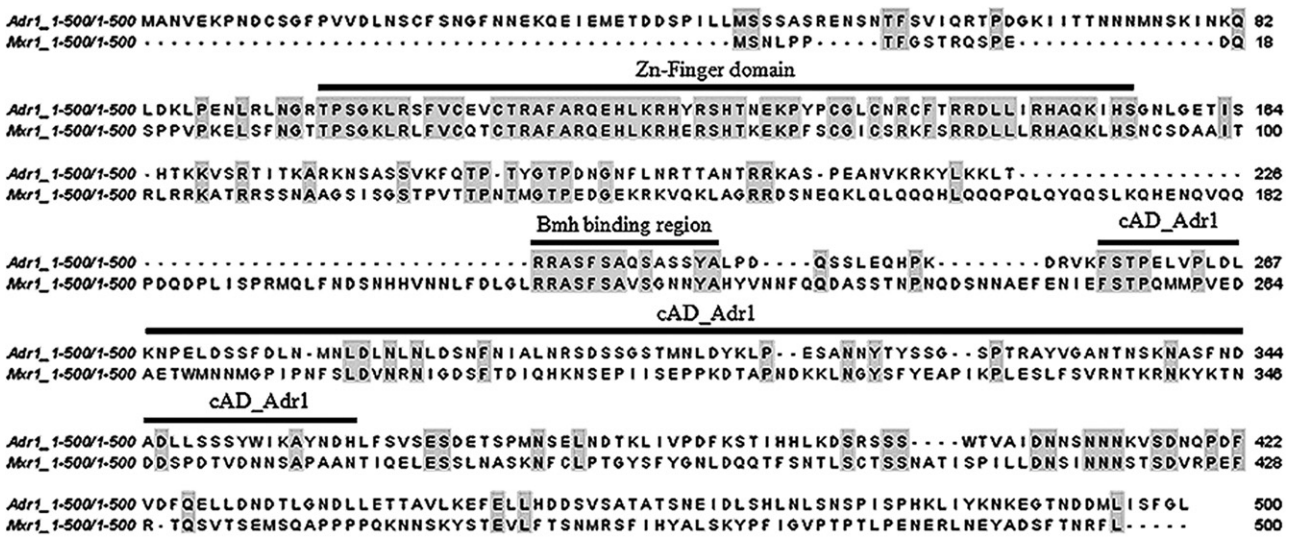

Fig. 1. Sequence alignment of Mxr1 and Adr1. The alignment was done using web server based alignment program, ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Larkin *et al*., 2007) by taking 1–500 amino acids of both the proteins. cAD, cryptic activation domain; Zn-Finger, DNA binding domain.

consisting of a core 5′-CYCC-3′ motif is recognized by Mxr1 (Kranthi *et al*., 2009). Despite the constitutive expression of Mxr1 in glucose grown cells its activity remains repressed, suggesting the existence of post-translation modification(s) that regulate its activity (Lin-Cereghino *et al*., 2006). However, any post-translation modification(s) that might regulate Mxr1 activity in response to glucose is unknown. Based on immunofluorescence studies of a Mxr1-GFP fusion protein it was proposed that decreased nuclear localization of Mxr1 in glucose grown cells might be an explanation for glucose repression of Mxr1-dependent *AOX1* and other methanol-inducible genes (Lin-Cereghino *et al*., 2006). However, it is unknown why Mxr1 remains inactive in ethanol- and glycerol-grown cells despite its nuclear localization in those growth conditions (Lin-Cereghino *et al*., 2006).

As observed by Lin-cereghino *et al*. (2006), the N-terminally located C_2H_2 zinc-finger DNA binding domain (residues 29–101) of Mxr1 shares ~ 70% sequence identity with the DNA binding domain (residues 95–156) of *Saccharomyces cerevisiae* Adr1 (alcohol dehydrogenase II synthesis regulator), a transcription factor regulating the expression of glucose-repressed genes involved in the metabolism of non-fermentable carbon sources such as glycerol, lactate, amino acids, ethanol and oleic acid (Ciriacy, 1979; Denis *et al*., 1981; Hartshorne *et al*., 1986; Simon *et al*., 1992; 1995; Young *et al*., 2003). Despite the high sequence similarities with the DNA binding region of Adr1, Mxr1 does not show any cross-reactivity for Adr1-binding *UAS* (upstream activator sequence) elements (Kranthi *et al*., 2009). Outside of their DNA binding regions, which share a similar location, Mxr1 and Adr1 share little homology, despite their similar size and analogous function in regulating the metabolism of non-fermentable substrates. The only other region of homology within Mxr1 is a short stretch of amino acids which shares approximately 70% sequence identity (Fig. 1) with the core 14-3-3 (Bmh) binding region (Conserved region 1) (Parua *et al*., 2010) located within the regulatory domain of Adr1.

14-3-3 proteins are ubiquitous, highly conserved, acidic and dimeric proteins that play important roles in controlling a wide variety of cellular processes, including cell cycle, metabolism, apoptosis and gene expression by binding to phosphoserine- or phosphothreonine-containing motifs in numerous target proteins (Jones *et al*., 1995; Xiao *et al*., 1995; Muslin *et al*., 1996; Yaffe *et al*., 1997; Fu *et al*., 2000; Ferl *et al*., 2002; Mackintosh, 2004; Wilker *et al*., 2005; Aitken, 2006; van Heusden and Steensma, 2006). The yeast *S. cerevisiae* has two genes encoding redundant 14-3-3 proteins, Bmh1 and Bmh2 (Gelperin *et al*., 1995; van Heusden *et al*., 1995; van Heusden and Steensma, 2006). Either Bmh protein can fulfil all of the essential functions in budding yeast. As in higher eukaryotes, yeast 14-3-3 proteins are involved in numerous signalling and cell differentiation pathways, including glucose repression. 14-3-3 proteins have not been reported in *P. pastoris* but their ubiquitous presence in other eukaryotes makes it likely that this yeast also contains at least one homologue which may have functional similarities to Bmh in *S. cerevisiae*.

Bmh in *S. cerevisiae* is involved in glucose repression at two levels. By binding to Reg1, the regulatory subunit of the Glc7 protein phosphatase, Bmh participates in the inactivation of the AMP-activated protein kinase (AMPK) Snf1

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when cells are grown in glucose (Mayordomo *et al*., 2003; Dombek *et al*., 2004; Ichimura *et al*., 2004). Snf1 is essential for activation of Adr1, and thus Bmh indirectly inhibits the expression of Adr1 target genes. Recently we described a Reg1-independent role of Bmh in glucose repression that acts directly through binding to Adr1 (Parua *et al*., 2010). Bmh binds to the Ser230-phosphorylated regulatory domain of Adr1 and inhibits the activity of a nearby cryptic activating region.

Based on this observation, we investigated whether there is 14-3-3-dependent regulation of Mxr1 activity in *P. pastoris.* We show that Mxr1 contains a highly conserved yeast 14-3-3 binding motif and *P. pastoris* has a unique and previously uncharacterized 14-3-3 family protein that interacts with Mxr1 through its 14-3-3 motif. The interaction is necessary to differentially regulate the activity of Mxr1 depending on the available carbon source. We provide evidence that 14-3-3-mediated regulation of transcription occurs at a step after DNA-binding. We also demonstrate that the first 400 amino acids of Mxr1 are sufficient for carbon source-mediated repression and activation of Mxr1-regulated genes and map the major activation domain of Mxr1 to this region of the protein. The *P. pastoris* 14-3-3 is able to complement a budding yeast strain with defective Bmh genes, indicating that it can fulfil the essential functions of Bmh in *S. cerevisiae*.

Results

S. cerevisiae *14-3-3 interacts with Mxr1*

Besides the closely related C_2H_2 Zn-finger DNA binding domains (Lin-Cereghino *et al*., 2006), the sequence alignment (Fig. 1) between residues 1–500 of both Mxr1 and Adr1 reveals the presence of only one other region of homology (~ 70% sequence identity) corresponding to the Bmh1/2 (*S. cerevisiae* 14-3-3 homologues) binding region of Adr1, residues 226–240. This putative 14-3-3 binding site (-RRASFS------YA-) is located between amino acids 212–225 of Mxr1. Based on this sequence analysis we asked whether *S. cerevisiae* 14-3-3 proteins can interact with Mxr1 through the observed putative binding motif. To answer this question, five GBD (Gal4 DNA binding domain)-Mxr1 fusion proteins, encompassing different regions of Mxr1 were generated and tested in pulldown assays with Gst (Glutathione-*S*-transferase)-Bmh1. As shown in Fig. 2A, GBD-Mxr1 proteins (encompassing amino acids 96–266, 206–246 and 206–226 of Mxr1) having the putative Bmh binding region were retained on glutathione sepharose 4B-immobilized Gst-Bmh1 resin. The fusion protein GBD-Mxr1 (206–226) showed low binding to the Gst-Bmh1 beads compared with the other two. A possible explanation is that the short 20-residue-long peptide might not be fully accessible in the fusion protein. This indicates a physical interaction between Mxr1 and *S. cerevisiae* Bmh1. Fusion proteins having amino acids 96–206 or 226–266 of Mxr1 but lacking the putative Bmh binding region, did not exhibit any interaction with Gst-Bmh1. The empty Gst-control experiments further confirmed the specificity of the interaction between Bmh1 and Mxr1. Thus, Bmh interacts with Mxr1 and does so through the putative Bmh binding region, amino acids 212–225.

14-3-3 of P. pastoris *binds to Mxr1*

Genome-wide searching and sequence alignment analyses (Fig. S1) indicate that *P. pastoris* has an uncharacterized 14-3-3 family protein, known as C4qzn3 (257 residues) located on chromosome 2. This putative 14-3-3 shares almost 90% sequence identity with 14-3-3 proteins from other eukaryotes, *S. cerevisiae*, *S. pombe*, *C. albicans*, *D. melanogaster*, plants and human (Fig. S1). Gst-pulldown experiments were performed using Gst-C4qzn3 or Gst-Bmh1 expressed in *E. coli* and yeast extracts containing GBD-Mxr1 or GBD-Adr1 fusion variants to assess their possible interaction. Comparable amounts of both GBD-Adr1 (215–260) and GBD-Mxr1 (210–226) were retained on Gst-C4qzn3-bound resin as observed for GBD-Mxr1 (210–226) on Gst-Bmh1-bound resin (Fig. 2B). GBD-Mxr1 (96–206), lacking the Bmh binding region was not pulled down with Gst-C4qzn3, suggesting that the interaction was specific for the presence of the Bmh binding region (residues 212–225) in Mxr1. These results demonstrate that the putative *Pichia pastoris* 14-3-3 protein has the ability to interact with both transcription factors, *P. pastoris* Mxr1 and *S. cerevisiae* Adr1 at their respective Bmh binding regions.

14-3-3 interacts with Mxr1 via phosphorylation of Ser215

14-3-3 proteins preferentially recognize Ser/Thr-phosphorylated targets and in our previous work we observed that Bmh interacts with Adr1 via phosphorylation of Ser230, which is located within the core Bmh binding region (Parua *et al*., 2010). The sequence alignment in Fig. 1 shows that Ser215 of Mxr1 is the residue corresponding to Ser230 of Adr1, which is known to be phosphorylated in a carbon-source regulated manner. To determine if the phosphorylation status of Ser215 plays a role in 14-3-3 binding to Mxr1, we first examined the phosphorylation status of Ser215 by performing immunoblotting experiments with anti-pSer230 antibody raised against the homologous phosphorylated Bmh1 binding motif of Adr1 (Ratnakumar *et al*., 2009). As shown in Fig. 2B, the anti-pSer230 antibody reacted with the pulled-down fraction of GBD-Mxr1 (210–226) and GBD-Adr1

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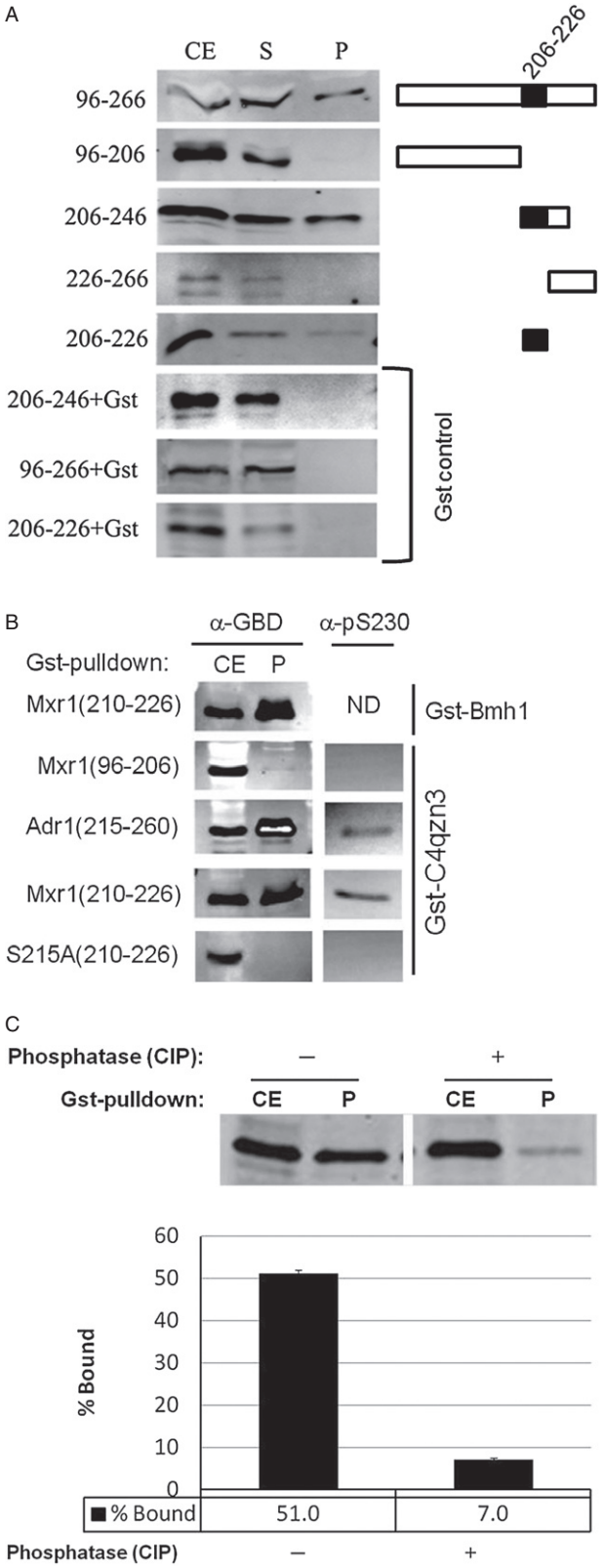

Fig. 2. A. Interaction studies between Mxr1 and Bmh1. Gst-pulldown assays were done using Gst-Bmh1 or Gst, which was expressed in *E. coli* and immobilized on glutathione sepharose-4B column, followed by application of yeast extract containing overexpressed GBD (Gal4 DNA binding domain)-Mxr1 fusion protein. All fractions were electrophoresed in 12% SDS-PAGE and visualized by Western blotting with anti-GBD antibody. Black shaded region (residues 206–226) represents the putative Bmh binding region.
B. Interaction studies of C4qzn3 with *Pichia pastoris* Mxr1 and *S. cerevisiae* Adr1. Gst-pulldown assays were done using Gst-Bmh1, Gst-C4qzn3. Yeast extracts containing either overexpressed GBD-Mxr1 or GBD-Adr1 variants was used in this study. All fractions were electrophoresed in 15% SDS-PAGE and visualized by Western blotting with anti-GBD antibody or anti-pSer230 antibody.
C. Gst-pulldown in presence and absence of phosphatase. Gst-pulldown was done following the protocol as described before using Gst-C4qzn3 and yeast extract containing GBD-Mxr1 (210–226) fusion protein in presence and absence of 10U of calf intestinal phosphatase (CIP) at 30°C. Upper panel is the Western blot profile of the pulldown fractions obtained by using anti-GBD antibody and lower panel represents the quantitative assays of the Western blot results. CE, Cell extract; S, Supernatant; P, pellet fraction.

(215–260), and not with cell extract of GBD-Mxr1 (96–206), suggesting that Ser215 within the 14-3-3 motif is phosphorylated in *S. cerevisiae*. To determine the role of pSer215 in binding of 14-3-3 to Mxr1, Ser215 was substituted with Ala in GBD-Mxr1 (210–226) and Gst-pulldown assays were performed. As shown in Fig. 2B the S215A mutant was neither pulled down nor recognized by the anti-pSer230 antibody suggesting that phosphorylation of Ser215 is essential for 14-3-3 binding. The phosphorylation-dependent interaction between 14-3-3 and Mxr1 was further confirmed by performing Gst-pulldown assays using Gst-immobilized *P. pastoris* 14-3-3 and yeast extract containing fusion protein GBD-Mxr1 (210–226) following the same protocol as described before in presence and absence of 10U of calf intestinal phosphatase (CIP; NEB) at 30°C. As shown in Fig. 2C, the phosphatase treatment significantly reduced the interaction, suggesting that the phosphorylation of Mxr1 is important for binding by 14-3-3 and most likely pSer215 is the target.

14-3-3 protein of P. pastoris *complements Bmh function(s) in* S. cerevisiae

The ability of the *P. pastoris* 14-3-3 protein to complement the loss of 14-3-3 function in *S. cerevisiae* would demonstrate that *C4QZN3* encodes a functional homologue. First, the ability of N-terminally HA-tagged *P. pastoris* 14-3-3 (from pBF-*C4QZN3*) or Bmh1 (from pBF-*BMH1*) to complement the growth defect of a *bmh2Δ bmh1-ts* mutant strain at 37°C was examined. As shown in Fig. 3A, expression of *P. pastoris* 14-3-3 rescued growth at 37°C as well as Bmh1, suggesting that *P. pastoris* 14-3-3 can perform all of

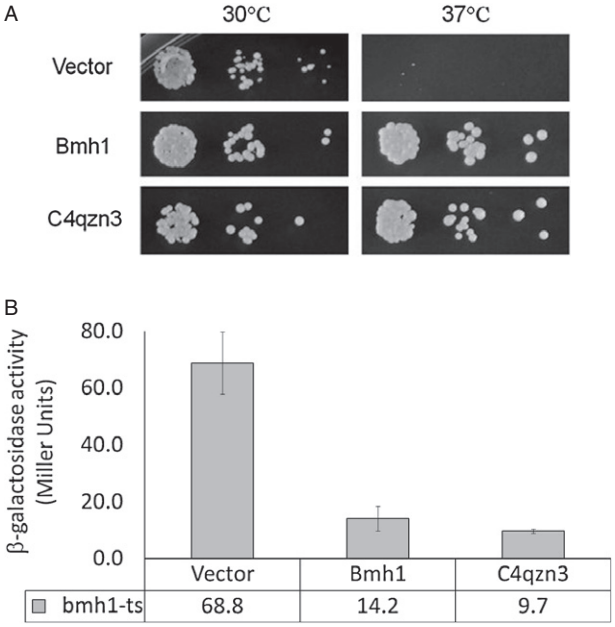

Fig. 3. A. Growth test of *bmh2Δ bmh1-ts* mutant strain expressing either HA-tagged *Pichia* 14-3-3 (C4qzn3) or Bmh1 at 30°C and 37°C on Trp⁻ plates.
B. Evaluation of the activity of endogenous Adr1 by exploiting a reporter gene (*ADH2p–lacZ*) expression assays. *S. cerevisiae bmh2Δbmh1-ts* cells harbouring a pair of plasmids, one expressing either *P. pastoris* 14-3-3 or Bmh1 or none (empty vector), another providing the reporter construct (p*LG–ADH2–lacZ*; *lacZ* under the control of *ADH2* promoter) were used as host and grown at 30°C. The error bars represent the averages of the results for three transformants ± one standard deviation.

the essential functions of the *S. cerevisiae* 14-3-3 proteins. Next, the regulatory effect of *P. pastoris* 14-3-3 protein on the transcriptional activity of Adr1 was tested. We evaluated the activity of endogenous Adr1 by assaying expression of the Adr1-dependent reporter gene, *ADH2p–lacZ* in *bmh2Δ bmh1-ts* (YLL1087) strain. The expression of the reporter is inhibited by Bmh due to its binding to the Adr1 regulatory region and consequent inhibition of Adr1 activity. Loss of Bmh activity results in a high level of reporter expression and thus high β-galactosidase activity. Restoration of Bmh function decreases this activity. *P. pastoris* 14-3-3 protein or Bmh1 expressed in *S. cerevisiae bmh2Δ bmh1-ts* cells gave rise to a fivefold lower level of β-galactosidase activity compared with the same cells without a wild-type 14-3-3 protein (Fig. 3B). This indicates that the *P. pastoris* 14-3-3 protein has the ability to inhibit Adr1 activity and suggests that it also might inhibit Mxr1 activity in *P. pastoris.*

14-3-3-dependent regulation of Mxr1 activity in response to various carbon sources

To determine whether *P. pastoris* 14-3-3 inhibits Mxr1 activity in its natural host a mutation that abrogates the *in vitro* interaction of 14-3-3 with Mxr1 (S215A) was introduced into a plasmid-borne copy of *MXR1*. The mutant protein was expressed after integration of the plasmid into the *P. pastoris* genome of a strain lacking endogenous *MXR1* (JC132, Table 1). The activity of wild-type and mutant protein was assayed by qRT-PCR analysis of mRNAs derived from Mxr1-regulated genes. Total RNA was extracted from strains lacking *MXR1* (JC132), or containing either the wild-type (JC100) or S215A mutant (PPP1) allele after growth in various repressing and inducing media. It has been reported that glucose, glycerol, and ethanol can each repress the expression of the solely Mxr1-dependent genes *AOX1* and *DHAS*, which are involved in methanol assimilation. The expression of these two genes, as well as genes involved in peroxisome biogenesis (*PEX8* and *PEX14*) and fatty acid oxidation (*POT1*, thiolase and *POX1*, oxidase) was quantified after growth in fully repressing medium containing either 0.5% glucose, or 0.5% glycerol or 0.5% ethanol; inducing medium containing 0.5% methanol; and repressing medium containing inducer, i.e. either 0.5% glucose or 0.5% glycerol or 0.5% ethanol supplemented with 0.5% methanol. As shown in Fig. 4A–C, expression of all of the genes in the wild-type Mxr1 strain was repressed in the presence of glucose as has been observed by others (Ellis *et al.*, 1985; Cregg *et al.*, 1989; Koutz *et al.*, 1989;

Table 1. Yeast strains.

Strain	Genotype	Reference
S. cerevisiae		
PJ69-4a	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7–lacZ*	James *et al.* (1996)
YLL908	W303 *MATa bmh2Δ::kanmx*	Lottersberger *et al.* (2003)
YLL1087	W303 *MATa bmh2Δ::kanmx bmh1Δ::HIS3::YIpbmh1-170(LEU2)*	Lottersberger *et al.* (2003)
P. Pastoris		
JC100	*Wild type*	Cregg *et al.* (1998)
JC132	*mxr1-1 his4*	Johnson *et al.* (1999)
PPP1	*MXR1-S215A*	This study
PPP2	*MXR1tm-S215A*	This study
PPP3	*MXR1tm*	This study

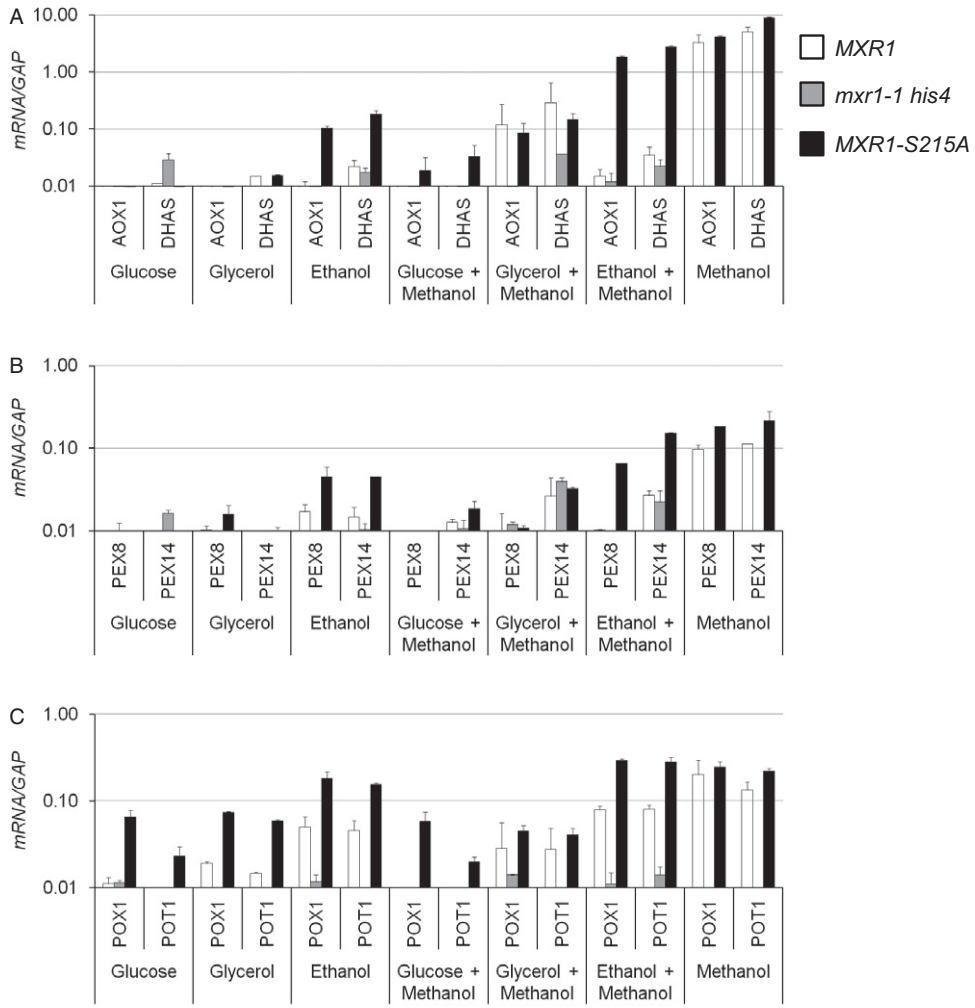

Fig. 4. Studies on Mxr1-dependent expression of genes involved in methanol utilization (*AOX1* and *DHAS*), peroxisome biogenesis (*PEX8* and *PEX14*) and fatty acid oxidation (*POT1 and POX1*) in *P. pastoris* in response to different carbon sources. mRNA was extracted from strains JC100 (*MXR1WT*), JC132 (*mxr1-1 his4*), PPP1 (*MXR1-S215A*) both in repressed (0.5% glucose, 0.5% glycerol and 0.5% ethanol) and induced (0.5% methanol) as well as repressed plus methanol growth conditions. Quantitative RT-PCR (qRT-PCR) was performed to determine the levels of mRNA. The mRNA levels were normalized to the levels of *GAP* mRNA in each sample. The error bars represent the mean of three biological replicates assayed in duplicate.

Lin-Cereghino *et al*., 2006). Moreover, glycerol-mediated repression of all of these genes was also observed in the wild-type Mxr1 strain. This was surprising because the *PEX* and fatty acid oxidation genes of *S. cerevisiae* are derepressed when glycerol is the sole carbon source. *MUT* pathway genes and genes involved in peroxisome biogenesis were repressed significantly in wild-type ethanol grown cells (Fig. 4A and B). However, fatty acid oxidation genes were not repressed in ethanol (Fig. 4C), which is analogous to Adr1-dependent derepression of the β-oxidation pathway genes in ethanol media in *S. cerevisiae*. Furthermore, their complete repression in the *mxr1-1* null mutant strain suggests that expression of β-oxidation pathway genes is highly Mxr1-dependent.

Interestingly, there was derepression of the expression of fatty acid oxidation genes (~ 7- to 8-fold in glucose media and ~ 2- to 3-fold in glycerol media), methanol assimilation pathway genes (more than 10-fold in ethanol growth condition) and peroxisome biogenesis genes (~ 2- to 3-fold in ethanol media) in the strain carrying the S215A mutant allele, suggesting that disruption of the interaction with 14-3-3 reduces carbon source-inhibited transcriptional activation by Mxr1 (Fig. 4). Addition of methanol into the ethanol growth media further increased the expression of the *AOX1*, *DHAS* and *PEX14* in the *S215A* mutant strain to a level comparable to that observed in inducing condition (Fig. 4A and B). This suggests the existence of at least two layers of control over the expression of *MUT* and *PEX* genes. All of the genes tested remained repressed after addition of methanol to the glucose media (Fig. 4A–C). However modest induction was observed for *MUT* pathway and β-oxidation genes in the wild-type strain upon

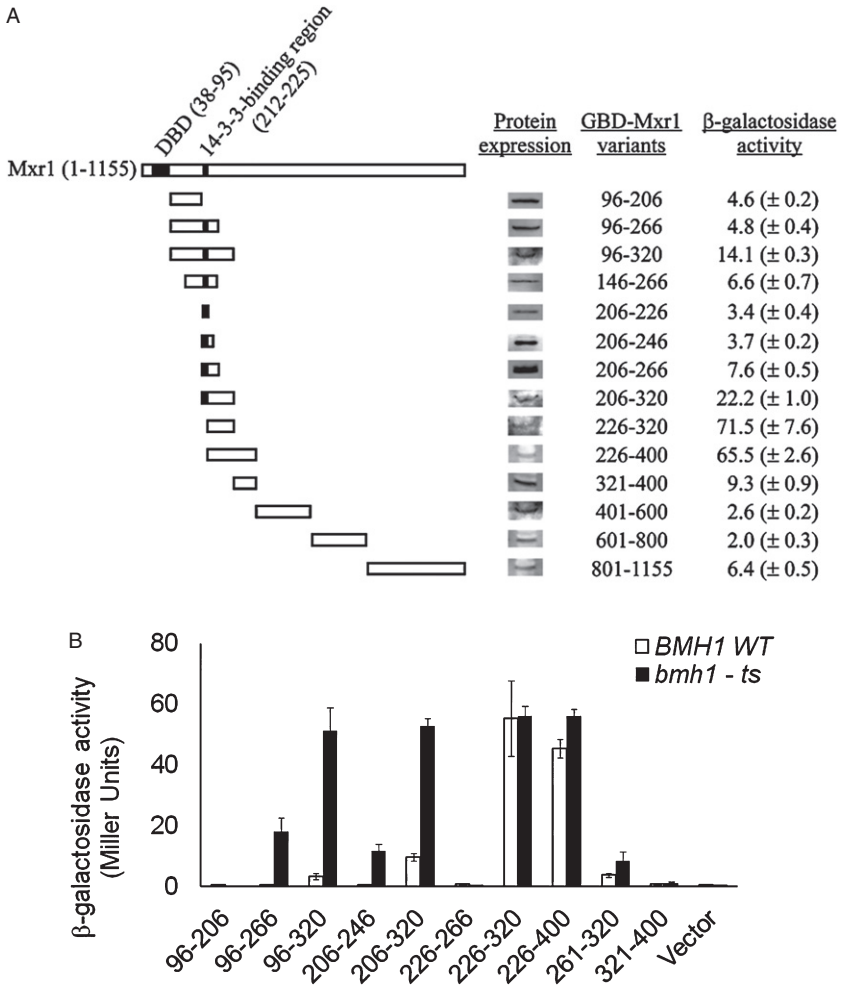

Fig. 5. A. Activity assays of GBD-Mxr1 variants. Diagram represents the various Mxr1 fragments, which were generated as GBD (Gal4 DNA binding domain) fusion protein. DBD, DNA binding region and the conserved 14-3-3 binding region (residues 212–225) are highlighted by black shade. Activity was measured by exploiting the reporter gene (*lacZ*) expression assays, where *S. cerevisiae* PJ69-4a strain harbouring plasmid, which expressed Mxr1 variants as GBD fusion proteins. The strain was also harbouring a chromosomal copy of a *GAL7p–lacZ* reporter cassette. The values are the averages of the results for three transformants ± one standard deviation. Expression level of all GBD-fused Mxr1 peptides was also shown in the figure obtained by Western blotting using anti-GBD antibody.
B. Activity assays of GBD-Mxr1 proteins in *BMH1* wild type (YLL908; *bmh2Δ BMH1*) and *bmh1-ts* (YLL1087: *bmh2Δ bmh1-170*) strain. Activity was assayed by measuring the activity of expressed β-galactosidase from *lacZ* gene, where cells harbouring a pair of plasmids, one having *GAL10p–CYC1–lacZ* reporter cassette (pHZ18′) and another expressing Mxr1 variants as GBD fusion proteins were grown at 30°C. The error bars represent the averages of the results for three transformants ± one standard deviation.

addition of methanol to glycerol-containing medium (Fig. 4A and C), suggesting that glucose and glycerol repress Mxr1 activity through distinct pathways. In the *mxr1-1* null mutant none of the genes were expressed in any growth condition and all of them were significantly induced in *MXR1* wild-type and *S215A* mutant cells when methanol was the sole carbon source. In conclusion, a mutation that disrupts the interaction of 14-3-3 with Mxr1 allows significant methanol-independent expression of Mxr1-dependent genes.

Mapping of activation domain(s) of Mxr1

Although Mxr1 is an important transcription factor for the expression of genes involved in methanol utilization, oleic acid metabolism and peroxisome biogenesis, the existence and location of activation region(s) of the protein have not been identified. Sequence comparison to Adr1 did not identify a region with significant homology to any of its activation domains. To identify and map potential activation domains of Mxr1 we employed yeast one-hybrid assays where GBD-fused variants of Mxr1 (Fig. 5A) were expressed in *S. cerevisiae* strain PJ69-4a (Table 1) and their activity was assayed by evaluating the expression of three Gal4-dependent reporter genes, *GAL2p–ADE2*, *GAL1p–HIS3* and *GAL7p–lacZ*. As shown in Fig. 5A, the highest β-galactosidase activity was observed for the GBD-Mxr1 fusion proteins encompassing residues 226–320 and 226–400, suggesting that a major activation domain is located between residues 226–320. Despite the presence of residues 226–320 the fusion proteins with amino acids 96–320 and 206–320 showed approximately 3–5 times lower activity. Interestingly, both of the Mxr1 fusions contain the 14-3-3 binding region (residues 212–225), while fragments, 226–320 and 226–400 do not, suggesting that the 14-3-3 binding region acts to inhibit the function of this activation domain. Furthermore, as shown in Fig. 5A, all of the GBD-Mxr1 fusion proteins were expressed significantly, suggesting that the observed activity was not a reflection of the level of expression and the stability of a peptide. The results of the β-galactosidase assays were confirmed by measuring the activity of the

GAL1p–HIS3 and *GAL2p–ADE2* reporter genes by observing the growth on Trp⁻, Trp⁻Ade⁻ and Trp⁻His⁻ plates (Fig. S2). Only cells expressing GBD-Mxr1 variants encompassing regions 226–320 or 226–400 showed substantial growth on both Trp⁻Ade⁻ and Trp⁻His⁻ plates, confirming the location of a strong activation domain at amino acids 226–320. Consistent with the observed low level of β-galactosidase activity, the fusion proteins encompassing residues, 96–320 and 96–400 showed reduced growth on Trp⁻His⁻ and no growth on Trp⁻Ade⁻ plates. As both of the above two fusion proteins contain the observed Bmh binding region (212–225 amino acids), supporting an inhibitory role of the 14-3-3 binding region on activation domain function. Moreover our results indicate the presence of a strong activation region(s) located in the vicinity of the N-terminus just downstream of the zinc-finger DNA binding domain.

Our hypothesis that the 14-3-3 binding region inhibits the major activation domain of Mxr1 was confirmed by evaluating the activity of selected GBD-fused Mxr1 variants in both *BMH1* wild type (YLL908; *bmh2Δ BMH1*) and *bmh1-ts* mutant (YLL1087; *bmh2Δ bmh1-170*) strains using the *GAL10p–CYC1–lacZ* reporter gene at 30°C, a temperature sufficiently high to cause a *bmh⁻* phenotype (Parua *et al*., 2010). In both *bmh2Δ BMH1* and *bmh2Δ bmh1-ts* mutant strains we observed comparable amounts of activity for the GBD-Mxr1 (226–320) and GBD-Mxr1 (226–400) fusion proteins, which lack the Bmh binding region (Fig. 5B). Fusion proteins containing both the Bmh binding region and all or part of the activation region (96–266, 96–320, 206–246 and 206–320), were approximately 5- to 15-fold more active in the *bmh2Δ bmh1-ts* mutant than in the wild-type strain (Fig. 5B). All other fusion proteins tested had little or no activity in both strains. The absence of activity for the fusion protein encompassing residues 226–266 and the low activity of the 261–320 fragment suggests the critical role of the entire 226–320 region for the activity of Mxr1. Inactivation of Bmh or removal of the 14-3-3 binding region suppressed the inhibition of activation. This suggests that the binding of 14-3-3 to amino acids 212–225 of Mxr1 inhibits the activity of the major activation domain located at residues 226–320.

Characterization of the major activation domain of Mxr1

To map the activation domain more precisely we generated additional GBD-Mxr1 fusion proteins spanning residues 226–320 of Mxr1 and measured their activity in strain PJ69-4a. The results of the β-galactosidase (Fig. 6A) and growth assays (Fig. 6B) indicate that the shortest region capable of conferring activating function is located between amino acids 246 and 280.

Interestingly, the shortest functional region of Mxr1 (residues 246–280) is enriched in hydrophobic and acidic residues (see Fig. 1), characteristics of canonical activation domains in which bulky hydrophobic residues are frequently important for activation domain function (Cress and Triezenberg, 1991; Regier *et al*., 1993; Blair *et al*., 1994; Gill *et al*., 1994; Lin *et al*., 1994; Drysdale *et al*., 1995; Folkers *et al*., 1995; Almlöf *et al*., 1997; Young *et al*., 1998). The importance of the three phenylalanine residues located within this region of Mxr1 at positions 249, 254 and 278 was tested by substituting them by Ala either individually or in combination. All of the mutants were expressed as GBD-fusion proteins in *S. cerevisiae* strain PJ69-4a and their activity was measured by means of β-galactosidase activity and growth assays as described above. A threefold decrease in *GAL7p–lacZ* expression was observed when individual Phe residues were changed to Ala. Substitution of all three to Ala caused an approximately fivefold decrease in expression compared with the wild-type fusion protein (Fig. 6C). Furthermore, the cells expressing either a single, double, or triple mutant protein were inviable on Trp⁻Ade⁻ medium (Fig. 6D). Western blot analysis of the GBD fusion proteins indicated that all the mutant proteins were expressed at a significant level (Fig. 6C). These observations corroborate the importance of the hydrophobic Phe residues for the activity of the GBD-Mxr1 fusion protein and show that this region of Mxr1 functions as a canonical activation domain.

The N-terminal 400 amino acids of Mxr1 are sufficient for full transcriptional activity in P. pastoris

We have shown that the functions of Mxr1 required for activating transcription in *S. cerevisiae* are located within the N-terminal 400 amino acids of the protein. Because this region of Mxr1 also contains its DNA binding domain we could determine whether it is sufficient for activation of Mxr1 target genes in its natural host, *P. pastoris*. A plasmid carrying the *MXR1* promoter followed by either the *S215A* mutant or wild-type coding sequence truncated to amino acid 400 was introduced into the *his4* gene of *P. pastoris* JC132 to create strain PPP2 (*MXR1tm-S215A*) and PPP3 (*MXR1tm*) respectively. These strains as well as a strain expressing the full-length wild-type protein, JC100, a *mxr1* null mutant strain, JC132, and a strain expressing the full-length Mxr1-S215A mutant protein, PPP1 were grown in repressing medium, 0.5% ethanol, inducing medium, 0.5% methanol, and repressing medium containing inducer, 0.5% ethanol and 0.5% methanol. Total RNA was isolated from each culture and *AOX1* mRNA, as a measure of Mxr1 activity, was quantified using qRT-PCR. As shown in Fig. 7, the truncated Mxr1 wild type (Mxr1tm-WT) and mutant (Mxr1tm-S215A) displayed a level of *AOX1* expression comparable to that found in the full-length wild type and Mxr1-S215A mutant in inducing condition. Furthermore, both S215A mutants exhibited comparable levels of

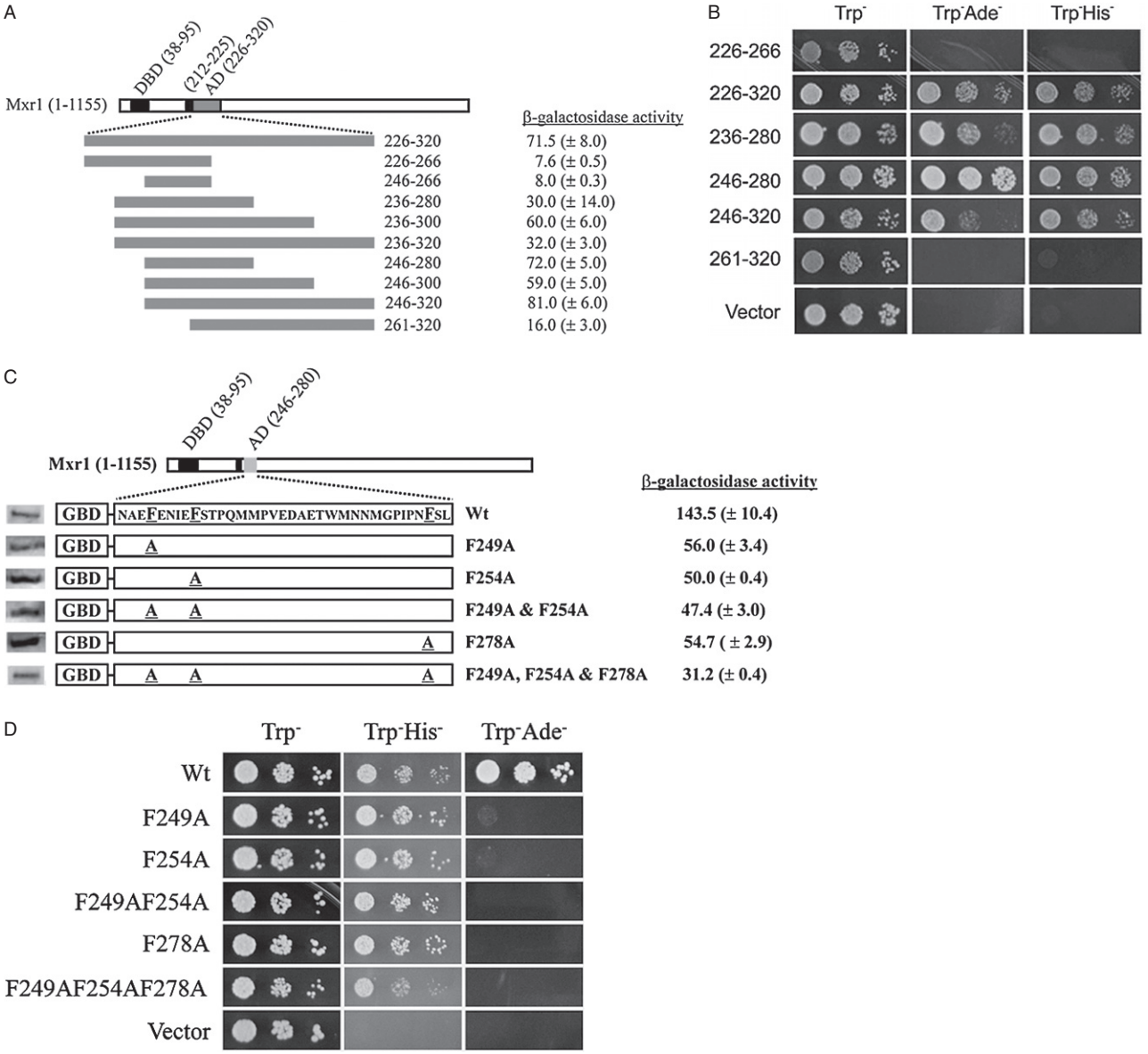

Fig. 6. A. Indicated different Mxr1 fragments encompassing the 226–320 amino acids region, were expressed as GBD (Gal4 DNA binding domain) fusion protein in yeast two hybrid strain PJ69-4a. The activity was measured by monitoring *lac*Z expression from *GAL7p* promoter. The values are the averages of the results for three transformants ± one standard deviation.
B. Growth test of PJ69-4a harbouring different GBD-Mxr1 constructs at 30°C on selective amino acid (Trp^- and Trp^-His^-) and/or amino acid-nucleotide (Trp^-Ade^-) deficient plates.
C. Indicated Mxr1 fragments encompassing the 246–280 amino acids region having specific Phe to Ala mutation(s) were expressed as GBD (Gal4 DNA binding domain) fusion proteins and activity was measured as described before. Expression level of all of the fusions was also shown in the figure. The values are the averages of the results for three transformants ± one standard deviation.
D. Growth test of PJ69-4a was performed at 30°C as described before.

derepression of *AOX1* expression in ethanol media upon disruption of the interaction with 14-3-3. These observations suggest that the N-terminal 400 amino acids of Mxr1 are sufficient to activate carbon source-dependent expression of Mxr1-dependent genes and provide *in vivo* evidence that the major activation domain identified in *S. cerevisiae* is functional in *P. pastoris*.

Carbon source-dependent phosphorylation of Ser215 and binding of 14-3-3 to Mxr1

The data just presented suggest that phosphorylation-dependent binding of 14-3-3 differentially regulates Mxr1 activity in response to various carbon sources. To examine whether phosphorylation of Ser215 and 14-3-3 binding are

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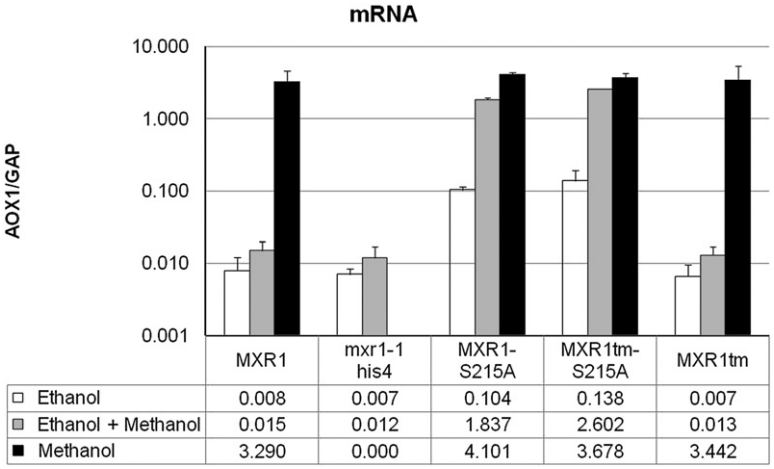

Fig. 7. Studies on expression of major Mxr1-dependent gene, *AOX1* in *P. pastoris* in response to different carbon sources. mRNA was extracted from strains JC100 (*MXR1WT*), JC132 (*mxr1-1 his4*), PPP1 (*MXR1-S215A*), PPP2 (*MXR1tm-S215A*) and PPP3 (*MXR1tm*) both in repressed (0.5% ethanol) and induced (0.5% methanol) as well as ethanol plus methanol growth conditions. Quantitative RT-PCR (qRT-PCR) was performed to determine the levels of *AOX1* mRNA. The mRNA levels were normalized to the levels of *GAP* mRNA in each sample. The error bars represent the mean of three biological replicates assayed in duplicate.

carbon source-regulated we performed Ni^{2+}-NTA and Gst-pulldown assays using *P. pastoris* cell extracts containing C-terminal Myc-$(His)_6$-tagged truncated Mxr1 (Mxr1tm, 1–400 amino acids) and *E. coli* expressed Gst-C4qzn3. We were unable to detect Mxr1 in the cell extracts either due to its very low level of expression or due to its highly unstable nature. To enrich the protein we performed Ni^{2+}-pulldown using protein extracts from *P. pastoris* strain PPP3 (expressing Mxr1tm-Myc-His_6) after growth in fully repressing medium containing either 0.5% glucose, or 0.5% ethanol; and inducing medium containing 0.5% methanol, PPP2 (expressing Mxr1tm-S215A-Myc-His_6) after growth in 0.5% ethanol containing medium, and JC132, a *mxr1-1* null mutant strain after growth in 0.5% glucose media. Pulldown fractions were immunoblotted with anti-cMyc and anti-pSer230 antibody, as described before. As shown in Fig. 8A Ser215-phosphorylation was diminished significantly in both glucose and methanol growth conditions compared with that observed in ethanol growth condition for wild-type protein. Phosphorylation of the Ser to Ala mutant was also significantly diminished. No protein band was observed corresponding to Mxr1tm for the *mxr1-1* null mutant strain, confirming the specificity of the pulldown assay. A Gst-pulldown was done to test 14-3-3 binding to Mxr1 in different growth conditions using the same cell extracts (except the *mxr1-1* null mutant extract). As shown in Fig. 8B, 14-3-3 binding was also significantly impaired in glucose and methanol grown cells, and completely abolished due to the substitution of Ser to Ala even in ethanol growth media. These results are consistent with the phosphorylation data and together they suggest that Ser215-phosphorylation is regulated by the carbon source available for growth and this in turn regulates 14-3-3 binding to Mxr1. The result in glucose media was quite surprising and interesting. Despite the absence of 14-3-3 binding Mxr1-activated genes are completely repressed, suggesting the presence of a 14-3-3-independent pathway for the regulation of expression of Mxr1-dependent genes.

14-3-3 acts at a post DNA binding step

The ability of Mxr1 to activate transcription might be regulated at different steps in response to carbon source availability. These include nuclear localization, DNA-binding and post-DNA binding. 14-3-3 could regulate any of these steps. It has been reported that Mxr1 was mostly localized in the cytoplasm in glucose-grown cells and its nuclear localization was increased when cells were grown in ethanol, glycerol, and methanol containing media (Lin-Cereghino *et al.*, 2006). Despite its localization in the nucleus Mxr1 activity remains repressed in glycerol and ethanol media and introduction of a mutation that disrupts its interaction with 14-3-3 partially relieves the repression. These observations prompted us to test whether 14-3-3 inhibits DNA binding by Mxr1 *in vivo*. We performed chromatin immunoprecipitation (ChIP) using cross-linked protein extract containing either C-terminal Myc-tagged truncated wild-type (Mxr1tm) or S215A mutant variant (Mxr1tm-S215A). *P. pastoris* strains PPP2 (expressing Mxr1tm-S215A-Myc) and PPP3 (expressing Mxr1tm-Myc) were grown in 0.5% glucose, 0.5% ethanol and 0.5% methanol-containing media. We tested carbon source-dependent Mxr1-binding to three Mxr1-activated gene promoters, *AOX1prm*, *PEX8prm* and *POX1prm*. As shown in Fig. 9, binding of Mxr1 to these promoters was significantly increased both in ethanol- and methanol-grown cells compared with that observed in glucose grown cells, The binding of Mxr1 to these promoters was consistent with the expression level of the respective genes (Fig. 4A–C). The low level of Mxr1 binding in the presence of glucose could be due to its reduced level of nuclear localization. Interestingly, there was a comparable level of binding of wild-type and mutant protein in both repressing (ethanol) and induc-

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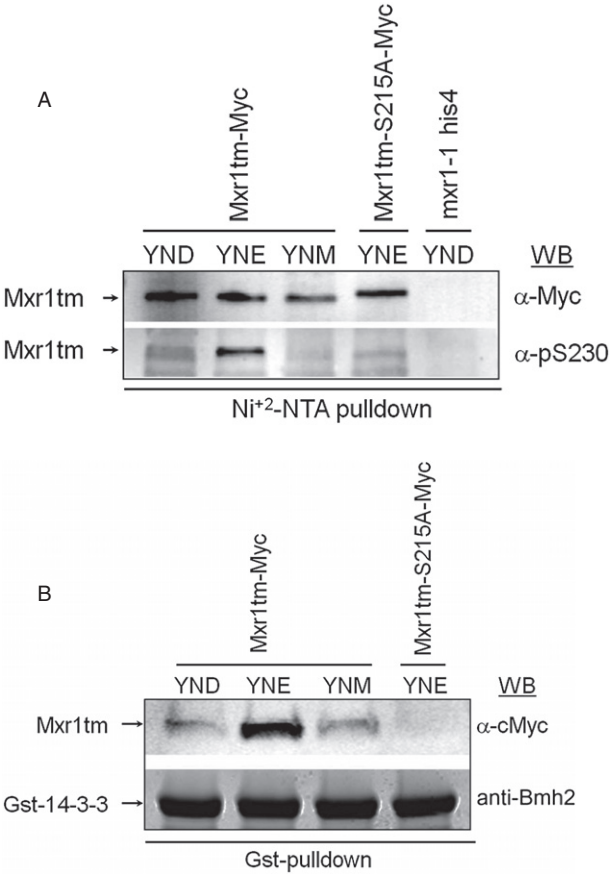

Fig. 8. A. Determination of phosphorylation of Mxr1-Ser215 in response to various carbon sources. Ni^{2+}-pulldown was done using *P. pastoris* protein extract containing either truncated wild-type Mxr1(Mxr1tm-Myc-His_6) prepared from cells grown in 0.5% glucose (YND), 0.5% ethanol (YNE) and 0.5% methanol (YNM) containing media, or truncated mutant Mxr1 (Mxr1tm-S215A-Myc-His_6) prepared from cells grown in 0.5% ethanol (YNE) containing media, or extract prepared from *mxr1-1* null mutant strain grown in 0.5% glucose containing media (YND). Pulldown fractions were electrophoresed in 4–20% SDS-PAGE and visualized by immunoblotting with anti-cMyc and anti-pSer230 antibody.
B. Interaction studies between Mxr1 and 14-3-3 in response to various carbon sources. Gst-pulldown was performed using the *P. pastoris* cell extracts and *E. coli* expressed Gst-C4qzn3 as described above. Pulldown fractions were electrophoresed in 4–20% SDS-PAGE and visualized by Western blotting with anti-cMyc and anti-Bmh2 antibody.

ing (methanol) media, suggests that 14-3-3 does not act to regulate the DNA binding activity of Mxr1, indicating a post-DNA binding role of 14-3-3 in carbon source-dependent regulation of Mxr1 activity. Moreover our results also indicate that besides 14-3-3 other regulatory pathways, are also acting at a post-DNA binding step to regulate Mxr1-dependent gene expression in ethanol.

Discussion

Expression of the methanol utilizing genes among methylotrophic yeasts (*P. pastoris*, *H. polymorpha*, *C. boidinii* and *P. methanolica*) is regulated differently in response to various carbon sources (Hartner and Glieder, 2006). Independent of the yeast species all of the *MUT* genes are strongly repressed either in the presence of glucose or ethanol and highly induced by methanol. Species-dependent regulation of their expression has been observed in growth media containing either glycerol or glycerol and methanol. The alcohol oxidase genes, *AOX1 and AOX2* in *P. pastoris* are repressed in growth media containing either glycerol or glycerol and methanol whereas those genes in other methylotrophs are significantly derepressed by glycerol and induced to different levels upon addition of methanol. The differential response to mixed carbon sources suggests the existence of unknown species-specific regulatory machinery(s) for modulating their expression. The zinc-finger transcription factor, Mxr1 is essential for methanol utilization and peroxisome biogenesis in *P. pastoris* (Lin-Cereghino *et al.*, 2006). The molecular mechanism(s) of carbon source-dependent inhibition and methanol-induced activation of Mxr1 is also unknown. Here we report that a *P. pastoris* 14-3-3 protein is involved in this carbon source-dependent regulation.

In the present study we have identified a highly conserved region of Mxr1, residues 212–225, which contains the core 14-3-3 binding region RRASFSA (Parua *et al.*, 2010). As for Adr1, this putative 14-3-3 binding region of Mxr1 differs significantly from mammalian 14-3-3 motifs because it lacks the well-conserved Pro at the +2 position. We report here a previously uncharacterized 14-3-3 family protein in *P. pastoris* that has the ability to complement

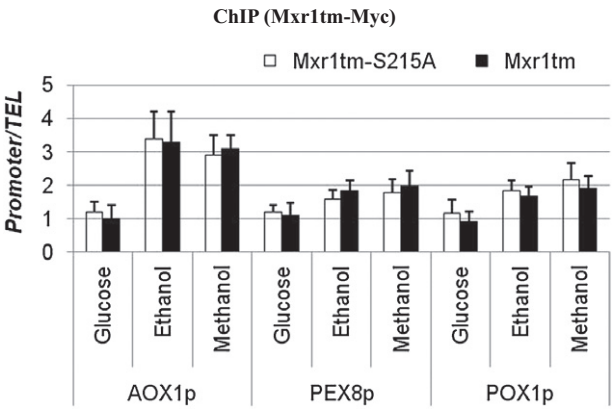

Fig. 9. Chromatin immunoprecipitation. Chromatin immunoprecipitation was done using cross-linked protein extract prepared either from strain PPP2 (*MXR1tm-S215A*) or PPP3 (*MXR1tm*) after growth in 0.5% glucose, 0.5% ethanol and 0.5% methanol containing media. Immunoprecipitaion was done using anti-cMyc antibody against C-terminal Myc-tagged truncated Mxr1 wild-type and mutant protein, Mxr1tm-Myc and Mxr1tm-S215A-Myc respectively. The data are expressed as binding (ChIP/input) for *AOX1p*, *PEX8p* and *POX1p* relative to ChIP/input at the TEL region used as a reference.

S. cerevisiae 14-3-3 functions and to interact with Mxr1 through the 14-3-3-binding motif at residues 212–225 via phosphorylation of Ser215, suggesting that 14-3-3 is a biologically relevant regulator of Mxr1 in *P. pastoris*. Consistent with this interpretation we have observed gene- and carbon source-specific regulation of Mxr1 activity in *P. pastoris* that was dependent on an intact, phosphorylatable 14-3-3 binding motif. Indeed, we observed carbon source-dependent regulation of phosphorylation of Ser215 and consequent carbon source-dependent regulation of 14-3-3 binding to Mxr1, confirming the presence of 14-3-3-mediated regulation of Mxr1 activity in a carbon source-dependent way. Although we observed 14-3-3-mediated inhibition of expression of Mxr1-dependent genes involved in methanol assimilation pathways (*AOX1* and *DHAS*) and peroxisome biogenesis (*PEX8* and *PEX14*) in response to ethanol, there was no 14-3-3-dependent inhibition observed for the genes in glucose- and glycerol-containing media in *P. pastoris*. Ethanol and glucose have been shown to repress the *MUT* pathway genes via two distinct mechanisms (Sakai *et al*., 1987; Alamae and Liiv, 1998; Parpinello *et al*., 1998). Indeed, our experimental observations suggest that there is a distinct 14-3-3-independent pathway in glucose-dependent regulation of Mxr1 activity, where nuclear localization could be the predominant pathway that might be regulated by a different mechanism. Based on our present observations we propose that 14-3-3 is a key factor in the mechanism of repression utilized by ethanol, but not by glucose. ChIP results indicate that 14-3-3 might be functioning at a post-DNA binding step, where it could regulate either RNA polymerase II recruitment or pre-initiation complex formation, either by recruiting some repressor or by inhibiting the recruitment of some activating factors. Surprisingly, a different scenario was observed for β-oxidation pathway genes. They were expressed constitutively in ethanol medium essentially through an Mxr1-dependent pathway, although at lower levels than that observed in methanol. 14-3-3-mediated regulation of their expression was also observed in glucose and glycerol. This suggests that the Mxr1-dependent activation mechanism for expression of β-oxidation pathway genes is distinct from that of the *MUT* pathway genes. We propose that Mxr1 might be acting through and/or in conjunction with the Pip2/Oaf1 homologues of *P. pastoris* to regulate the expression of β-oxidation pathway genes as has been observed for Adr1 in *S. cerevisiae* (Gurvitz *et al*., 2000; 2001; Biddick *et al*., 2008; Ratnakumar and Young, 2010).

We have mapped the location of the major activation domain of Mxr1 to residues 246–280. We have confirmed this mapping in *P. pastoris* by showing that full activity resides within the first 400 amino acids of Mxr1, which includes the N-terminally located DNA binding domain at residues 29–101. The amino acid sequence alignment in Fig. 1 shows that the C-terminal end of the Mxr1 activation domain is in a position analogous to the location of the cryptic activation domain of Adr1 at residues 260–360 (Cook *et al*., 1994; Parua *et al*., 2010). Although the two amino acid sequences show no significant overall homology, they are both enriched in hydrophobic and acidic residues, a characteristic of canonical activation domains. Indeed, our mutational analyses indicate that the Phe residues at positions 249, 254 and 278 are important for Mxr1 activity. This is consistent with reports that hydrophobic residues are important for the activity of a number of other activation domains (Cress and Triezenberg, 1991; Regier *et al*., 1993; Blair *et al*., 1994; Gill *et al*., 1994; Lin *et al*., 1994; Drysdale *et al*., 1995; Folkers *et al*., 1995; Almlöf *et al*., 1997), including the major activation domain of Adr1 (Young *et al*., 1998) located at amino acids 420–462 (Cook *et al*., 1994; Young *et al*., 2002). The presence of the major activation domain of Mxr1 at a location analogous to the cryptic activation domain of Adr1 suggests that either Mxr1 has lost a second activation domain analogous to the Adr1 major activation domain or Adr1 has gained this domain.

Despite the presence of remarkable N-terminal sequence similarities between *S. cerevisiae* Adr1 and *P. pastoris* Mxr1, these proteins share no other regions of significant sequence similarity. Both proteins have similar but not identical regulatory features. Adr1 activates genes that are important for growth on non-fermentable carbon sources, for peroxisome biogenesis, and for β-oxidation of fatty acids (Ciriacy, 1979; Simon *et al*., 1992; 1995; Sloan *et al*., 1999; Young *et al*., 2003). Mxr1 activates genes responsible for methanol utilization, peroxisome biogenesis (Lin-Cereghino *et al*., 2006) and our present study suggests its involvement in the activation of β-oxidation pathway genes. A major difference in the gene regulation in which these factors participate is that distinct methanol-mediated induction and derepression mechanisms exist for Mxr1-regulated genes, while only derepression in the absence of induction has been reported for Adr1-dependent genes. The canonical Mxr1-regulated genes, *AOX1* and *DHAS*, are repressed tightly by glucose, glycerol and ethanol, and highly induced by methanol in the absence of another carbon source (Ellis *et al*., 1985; Cregg *et al*., 1989; Koutz *et al*., 1989; Lin-Cereghino *et al*., 2006), while repression of the canonical Adr1-dependent gene, *ADH2*, is lifted by shifting the cells to growth on glycerol, ethanol or other non-fermentable carbon sources or upon glucose starvation (Ciriacy, 1975; Denis, 1987; Young *et al*., 2003). Our studies suggest that phosphorylation-dependent 14-3-3 binding to a domain located between the DNA-binding domain and the major activation domain of Mxr1 and Adr1 plays an important role in regulating the activity of both transcription factors. This suggests that carbon source- and 14-3-3-dependent regulation of tran-

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scription factor activation domain function is conserved in fermentative yeast like *S. cerevisiae* and *P. pastoris* and may be present in other species containing analogous transcription factors. It will be interesting to determine whether 14-3-3 in the divergent yeasts regulates the activities of these transcription factors in a similar manner despite their lack of overall sequence conservation.

Experimental procedures

Yeast strains and growth of cultures

The *S. cerevisiae* strains used in this study are listed in Table 1. *S. cerevisiae* cultures were grown in either yeast extract peptone medium or in synthetic medium containing 2–5% glucose and lacking the appropriate amino acid or nucleotide for plasmid selection. To maintain selection for plasmids containing *TRP1* and/or *URA3* the synthetic selective medium contained 0.2% casamino acids rather than the standard dropout solution. All *S. cerevisiae* strains were grown at 30°C unless otherwise mentioned.

The *P. pastoris* strains used in this study are listed in Table 1. *P. pastoris* cells were cultured in either YPD medium (1.0% yeast extract, 2.0% peptone, 2.0% glucose) or minimal YNB medium (0.17% yeast nitrogen base without amino acids and 0.5% ammonium sulphate) supplemented with either 0.5% glucose (YND), 0.5% methanol (YNM), 0.5% glycerol (YNG) or 0.5% ethanol (YNE). Amino acids were added to 50 μg ml^{-1} when required. *P. pastoris* strains were grown at 30°C and transformations were done by using electro-transformation (Cregg and Russell, 1998).

Escherichia coli cells, mainly DH10β and BL21 (DE3), were cultured in Luria–Bertani medium at 37°C for recombinant DNA techniques and protein overexpression.

Gene expression studies on different carbon sources

For gene expression studies in response to different carbon sources, cells were first grown in YND medium to an optical density at 600 nm (λ_{600}) of approximately 0.5. These glucose-grown cells were centrifuged, washed with water and resuspended either in pre-warmed YNM, YNE, YNG or YND supplemented with any other necessary ingredients. The cells were grown with vigorous shaking at 30°C for 8–10 h, and harvested by centrifugation and processed for RNA extraction.

Plasmid constructs

All *MXR1* constructs were made by cloning the corresponding coding sequence into the *TRP1-CEN4* vector, pOBD2 using gap repair methods(Orr-Weaver and Szostak, 1983; Szostak *et al.*, 1983), where proteins were expressed as N-terminal GBD (Gal4 DNA binding domain)-fusion as described in Parua *et al.* (2010). PCR fragments representing various regions of *MXR1* were generated using forward and reverse primers that contained homology to the vector sequences flanking the poly-linker region of pOBD2 as well as homology to *MXR1*. The NcoI and PvuII-digested pOBD2 plasmid (Uetz *et al.*, 2000) and a PCR fragment were used to transform PJ69-4a to Trp^+ prototrophy. Plasmid DNA from two or three Trp^+ transformants was rescued and sequenced to confirm that recombination had produced the correct in-frame gene fusion using primers, OBDsF and OBDsR (see Table S1). Western analysis with an anti-Gal4 DBD monoclonal antibody (RK5C1, Santa Cruz Biotechnology) was used to confirm the synthesis of a fusion protein of the correct size. The primers used are listed in Table S1.

pGEX-3X-*C4QZN3* and pBF-*C4QZN3* were made by PCR amplifying C4qzn3 (*Pichia pastoris* 14-3-3) coding sequence from *P. pastoris* genomic DNA using C4QZN3_BamHI_F and C4QZN3_BamHI_R primers (see Table S1). BamHI-digested PCR fragment was then cloned into the BamHI site of pGEX-3X (GE Healthcare Life Sciences) to produce pGEX-3X-*C4QZN3*. pBF-*C4QZN3* was generated by replacing *BMH1* from pBF-*BMH1* [a gift from S. Zheng; (Bertram *et al.*, 1998)] with the above mentioned BamHI digested PCR product. Positive clones were checked by restriction digestion with BamHI and EcoRI and the sequence and directionality of the insert was tested by DNA sequencing analysis using sequencing primer GEX_sF for pGEX-3X-*C4QZN3* and BF339_sF for pBF-*C4QZN3* (listed in Table S1).

All the plasmids used in this study are listed in Table S2.

Western blot

Western blot analyses were performed according to the manufacturer's instructions for the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE), using 1:500 to 1:1000 diluted monoclonal anti-GBD (Santa Cruz Biotechnology, RK5C1) or anti-cMyc (Santa Cruz Biotechnology, 9E10) or polyclonal anti-Bmh2 (a gift from S. Lemmon) or anti-pSer230 (custom raised against Ser230-phosphorylated Adr1 sequence from BETHYL Laboratories) antibody as primary antibodies and Licor λ_{800} secondary antibodies.

Preparation of protein extracts from yeast cells

Protein extracts from yeast cells were prepared following the procedure as described in Parua *et al.* (2010). Typically 50–100 ml of yeast cell culture grown to an A_{600} of ~ 1 was used for protein extraction. Cells were collected by centrifugation at 1600 *g* for 5 min at 4°C in a Sorvall RC3B-plus centrifuge, washed once with 15% glycerol containing 1 mM phenylmethylsulphonyl fluoride (PMSF), and resuspended in an equal volume of ChIP lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) containing protease (Sigma) and phosphatase inhibitors (Roche Applied Science). The cells were broken with glass beads in a Savant FP120 FastPrep machine with two disruption cycles of 45 sec at a speed setting of 4.0. The unbroken cells and debris were pelleted by centrifugation in a microcentrifuge at 13 600 *g* for 10 min. The clarified extract was collected in a fresh microcentrifuge tube containing 1 mM PMSF and 1× phosphatase inhibitor and used in subsequent experiments.

GST-pulldown assays

The Gst-pulldown assays were done following the same protocol essentially as described in Parua *et al.* (2010). GST-

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Bmh1 and *P. pastoris* Gst-14-3-3 fusion proteins were expressed from pGEX-3X-*BMH1* and pGEX-3X-*C4QZN3*, respectively in *E. coli* BL21 (DE3). These fusion proteins were then immobilized on glutathione-sepharose 4B beads as described by the manufacturer (GE Healthcare Life Sciences). Pulldown assays were performed using 30–40 μg of sepharose 4B coupled Gst-fused proteins and yeast extract containing ~ 2 mg of total proteins in 1× PBST (Phosphate buffered saline containing 0.1 % Tween 40), 1× protease inhibitor and 1× phosphatase inhibitor. The suspension was incubated at 4°C for one hour with continuous nutation. The beads were then pelleted by centrifugation at 320 *g* for 1 min, washed three times with 1× PBST and re-suspended in 60 μl of 2× LDS-NuPAGE sample buffer followed by boiling at 95°C for 5 min. Fractions collected at different steps of the pulldown assays were then analysed by SDS-PAGE and Western blotting.

Ni^{2+}-NTA pulldown assays

Ni^{2+}-NTA pulldown assays were done using *P. pastoris* cell extract expressing C-terminal His_6-tagged truncated wild-type or S215A Mxr1 (encodes 1–400 amino acids) mutant. Yeast extract containing approximately 10 mg total protein was incubated with Ni^{2+}-NTA beads at 4°C for one hour with continuous nutation in binding buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5% glycerol and 10 mM imidazole). The beads were then pelleted by centrifugation at 320 *g* for 1 min, washed three times with wash buffer (same as binding buffer except 25 mM imidazole) and re-suspended in 2× LDS-NuPAGE sample buffer followed by boiling at 95°C for 5 min. Pulldown fractions collected were then analysed by SDS-PAGE and Western blotting.

mRNA isolation and qRT-PCR

mRNA was isolated from yeast strains grown in either repressing or inducing medium using the acid phenol method described in Collart and Oliviero (2001). Residual DNA in the RNA preparation was reduced by treatment with DNase I (Ambion) following the manufacturer's recommendations. cDNA was synthesized using SuperScript III (Invitrogen) following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed for measuring mRNA levels using diluted cDNA. A standard curve was generated with *GAP* primers and used to quantify the mRNA levels. Samples were prepared from biological triplicates and analysed in duplicate.

β-Galactosidase assays

β-Galactosidase assays were performed as described by Guarente (1983) after growing the cells at 30°C in selective medium containing 2–5% glucose to an A_{600} of approximately 1. The reported values in Miller units are the averages of three transformants.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed as described in Biddick *et al.* (2008). In brief, cells from a 50 ml culture at an A_{600} of ~1 were pelleted at 1600 *g* at room temperature in a Sorvall RC3B-plus centrifuge and resuspended in 8.75 ml of phosphate-buffered saline (PBS). Ethylene glycol-bis (succinimidylsuccinate) (EGS; Thermo Scientific; 21565) cross-linker dissolved in DMSO was added to a final concentration of 3 mM and the sample was swirled at room temperature for 45 min. The EGS cross-linked cells were pelleted at 1600 *g* at room temperature and resuspended in 25 ml of 1× PBS supplemented with 1% formaldehyde. After shaking gently for 15 min at room temperature, 1.5 ml of 2.5 M glycine was added and the cells were pelleted. The cell pellet was washed once with 10 ml of tris-buffered saline (TBS) containing 125 mM glycine and once with 10 ml of TBS only. Protein extract was prepared as described above after resuspending the cells into ChIP lysis buffer containing protease and phosphatase inhibitor cocktail and the clarified extract was then used in subsequent immunopreciptation (IP) experiments. IP of ~ 6 mg of protein extract (Tachibana *et al.*, 2007) was performed overnight with constant nutation at 4°C with anti-cMyc (9E10; Santa Cruz Sc40). After IP, 50 μl of Protein A-coated Mag Sepharose™ Xtra beads from GE Healthcare (Cat. No. 28-9670-62) were added and nutation was continued for 1–2 h at 4°C. After separating the beads, 10 μl of the supernatant was aliquot out and mixed with 140 μl of ChIP elution buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM EDTA) for measuring the amount of input DNA. The beads were processed by washing twice with ChIP lysis buffer, twice with high salt ChIP lysis buffer (ChIP lysis buffer with 0.5 M NaCl), twice with ChIP wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% Noniodet-P40, 0.5% sodium deoxycholate), and twice with ChIP TE (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). The immunoprecipitated DNA bound to the beads was eluted at 65°C for 10 min with 150 μl elution buffer. The eluted and input DNA were incubated for 12–16 h at 65°C and then purified using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's protocol. The eluted and input DNA were diluted 20- and 100-fold, respectively, and quantification of specific sequences was performed by qPCR using Power SYBR Green master mix (Applied Biosystems) in a PTC-200 Thermocycler coupled to a Chromo 4 continuous fluorescence detector (MJ-Research). Opticon 3 software (MJ-Research) was used for the data analysis. Occupancy of a protein is expressed as fold-increase of the IP to input ratio of the amount of the specific amplicon for the gene sequence over the IP to input ratio corresponding to the amplicon for the telomeric sequence. Primers used for ChIP analysis are listed in Table S1.

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