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YIL113w encodes a functional dual-specificity protein phosphatase which specifically interacts with and inactivates the Slt2/Mpk1p MAP kinase in *S. cerevisiae*

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Abstract We show here that the *YIL113w* gene of *Saccharomyces cerevisiae* encodes a functional protein phosphatase. Yil113p shows no activity in vitro towards either phosphorylated casein or myelin basic protein. However, Yil113p dephosphorylates activated extracellular signal-regulated kinase 2 MAP kinase indicating that it is a dual-specificity MAP kinase phosphatase. In support of this we find that Yil113p specifically interacts with the stress-activated Slt2/Mpk1p MAP kinase of *S. cerevisiae*. Furthermore, expression of Yil113p causes the dephosphorylation of Slt2/Mpk1p in vivo, while expression of an inactive mutant of Yil113p causes the accumulation of phosphorylated Slt2/Mpk1p. We conclude that the physiological target of YIL113p is Slt2/Mpk1p.

Key words: Signalling; Dephosphorylation; Cell integrity; Stress response; *SDP1*

1. Introduction

The mitogen-activated protein kinases (MAPKs) are key components of cellular signal transduction pathways which are activated in response to a wide variety of extracellular stimuli and act to relay, amplify and integrate complex signals in order to elicit appropriate physiological responses [1]. The budding yeast *Saccharomyces cerevisiae* has five distinct MAPK signalling cascades which are involved in mediating diverse physiological functions [2]. Fus3p and Kss1p are involved in the mating response to pheromone and the regulation of pseudohyphal/invasive growth respectively. Slt2/Mpk1p and Hog1p are stress-activated MAPKs which mediate cell integrity and the response to osmotic stress respectively while Smk1/Sps1p is required for spore wall morphogenesis [2,3].

The magnitude and duration of MAPK signalling are known to be critical determinants of biological outcome [4,5] and it is now clear that the MAPK itself is a major target for regulation through the action of specific protein phosphatases. MAPK activation is uniquely dependent on the phosphorylation of both threonine and tyrosine residues within the 'activation loop' of the kinase and dephosphorylation of either residue is sufficient for inactivation. This can be achieved by protein-tyrosine phosphatases (PTPs), serine/threonine-specific phosphatases or by dual-specificity (threonine/tyrosine) protein phosphatases. Work in a variety of model systems, including both budding and fission yeast, has demonstrated that all three classes of enzyme are involved [6].

Thus far it has been demonstrated that two tyrosine-specific phosphatases, Ptp2p and Ptp3p, are involved in regulating MAPK signalling in *S. cerevisiae*. These enzymes were first implicated in the regulation of the Hog1p pathway in response to osmotic stress [7,8]. Subsequently it was shown that both Ptp2p and Ptp3p also regulate Fus3p [9]. Interestingly, Fus3p had already been identified as a target for negative feedback regulation by the pheromone inducible dual-specificity phosphatase Msg5p [10] and was therefore the first example of a MAPK which is regulated by both a dual-specificity and one or more tyrosine-specific phosphatases [9]. More recently both Msg5p and Ptp2p/Ptp3p have been implicated in the dephosphorylation of Slt2/Mpk1p indicating that the participation of multiple classes of protein phosphatase in the regulation of a single MAPK is more widespread [11,12].

The completion of the genomic sequence of *S. cerevisiae* has facilitated the complete annotation of the yeast proteome and allowed targeted surveys of the extent of particular protein families, revealing many previously unidentified proteins [13]. A database search using the nucleotide sequence of the human dual-specificity MAPK phosphatase Pyst1/MAPK phosphatase (MKP)-3 [14] as a probe revealed significant amino acid sequence homology with an open reading frame (ORF) designated *YIL113w* located on chromosome 9. The *YIL113w* ORF encodes a putative protein of 209 amino acids which contains a single copy of the consensus sequence for the PTP active site. This coupled with the fact that the closest relative of *YIL113w* within the *S. cerevisiae* genome is *MSG5* (56% identity), indicates that this protein might play a role in regulating MAPK signalling in yeast. Here we demonstrate that *YIL113w* encodes a functional protein phosphatase which specifically interacts with and inactivates the Slt2/

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Abbreviations: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MKP, MAPK phosphatase; ORF, open reading frame; PP2A, protein phosphatase type-2A; PTP, protein-tyrosine phosphatase

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Mpk1p MAPK in vivo. Our data strongly suggest that the physiological target of Yil113p is the stress-activated Slt2/Mpk1p MAPK.

2. Materials and methods

2.1. Yeast strains

AYS927 (*MAT***a**/*MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100 ssd1-d2/ssd1/d2* Gal$^+$); PJ69-4A (*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*); PJ69-4α (*MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) (kindly provided by Dr P. James, University of Wisconsin, USA).

2.2. Plasmids

All manipulations were performed using standard techniques and plasmid structures were verified by DNA sequencing [15]. The *YIL113w* ORF was amplified by PCR using yeast genomic DNA (strain AYS927) as template and subcloned into the bacterial expression vector pET15b (Novagen, Madison, WI, USA) as an *Xho*I–*Bam*HI fragment. The codon specifying cysteine 140 within *YIL113w* was mutated to serine by overlap extension PCR [16] using yeast genomic DNA as template and the mutant ORF was subcloned into pET15b as above. The Slt2/Mpk1 ORF was amplified by PCR using yeast genomic DNA (strain AYS927) as template and subcloned into pET15b as an *Nde*I–*Xho*I fragment. For expression of myc-tagged Slt2/Mpk1p and Hog1p in bacteria, the ORFs were amplified by PCR as above using primers which deleted the stop codon and introduced a single copy of the myc epitope tag followed by a stop codon and *Xho*I site and subcloned into pET15b as *Nde*I–*Xho*I fragments.

For yeast two-hybrid assays the MAPKs *FUS3,KSS1,SLT2/MPK1* and *SMK1/SPS1* were amplified by PCR using yeast genomic DNA (strain AYS927) as template and subcloned into pGBKT7 (Clontech, Palo Alto, CA, USA) as either an *Eco*RI–*Bam*HI (*FUS3,KSS1* and *SLT2MPK1*) or an *Xma*I–*Bam*HI (*SMK1/SPS1*) fragment. A 'kinase dead' mutant of *HOG1* in which the codon specifying an essential aspartic acid (residue 162) was mutated to asparagine was created by overlap extension PCR [16] using yeast genomic DNA (strain AYS927) as template and subcloned into pGBKT7 as an *Nde*I–*Bam*HI fragment. Wild-type *YIL113w* and its corresponding cysteine 140 to serine mutant were subcloned into pGADT7 (Clontech, Palo Alto, CA, USA) as *Bam*HI–*Xho*I fragments. Plasmids pGBD-C1 encoding *SNF1* fused to the *GAL4* DNA-binding domain and pGAD-C1 encoding *SNF4* fused to the *GAL4* activation domain were kindly provided by S. Fields (University of Washington, Seattle, WA, USA).

Wild-type *YIL113w* and its corresponding cysteine 140 to serine mutant were subcloned as *Hin*dIII–*Bam*HI fragments into a modified yeast expression vector, pYES2 (Invitrogen, Carlsbad, CA, USA) encoding a C-terminal myc tag (pYES2.8myc). The *MSG5* ORF was amplified by PCR using yeast genomic DNA (strain AYS927) as template and subcloned into pYES2.8myc as a *Sac*I–*Bam*HI fragment. The codon specifying cysteine 319 within *MSG5* was mutated to serine by overlap extension PCR [16], using yeast genomic DNA as template and the mutant ORF was subcloned into pYES2.8myc as above.

2.3. Expression and purification of recombinant Yil113p, Yil113p^{C140S}, Slt2p/Mpk1p and Hog1p

pET15b expression plasmids encoding His-tagged Yil113p, Yil113p^{C140S}, Slt2/Mpk1p, Slt2/Mpk1p-myc and Hog1p-myc were transformed into the host bacterial strain BL21 (DE3) (Novagen, Madison, WI, USA) using standard techniques. Following expression, proteins were purified using nickel–agarose resin (Ni^{2+}-nitriloacetate; Qiagen Inc., Valencia, CA, USA) under either native (Yil113p) or denaturing (Slt2/Mpk1p and Hog1p) conditions in accordance with the manufacturers instructions. Recombinant proteins were analysed by SDS–PAGE followed by staining with Coomassie blue.

2.4. Co-immunoprecipitations

Anti-myc monoclonal antibody (9B11, Cell Signalling, Beverly, MA, USA) was coupled to protein-G Sepharose overnight. Coupled antibody was then incubated for 1 h at 4°C either alone or with 1 μg of either recombinant Slt2/Mpk1p-myc or Hog1p-myc. Recombinant Yil113p (1 μg) was then added and incubated for a further 1 h at 4°C before centrifugation at 16 000×*g* for 1 min. Finally, immunoprecipitates were washed three times with CB100 buffer (20 mM HEPES, pH 8.0, 0.2 mM EDTA, 100 mM KCl, 1 mM DTT, 20% v/v glycerol and 0.1% v/v NP-40) and analysed by SDS–PAGE and Western blotting.

2.5. Phosphatase assays

Phosphatase activity of either recombinant Yil113p or Yil113p^{C140S} was determined using *p*-NPP hydrolysis exactly as described previously [14]. For inhibitor studies, reactions were supplemented with either 2 mM sodium orthovanadate (Sigma Aldrich St. Louis, MO, USA), 1 μM okadaic acid (Calbiochem, La Jolla, CA, USA) or 1 μM microcystin (Calbiochem, La Jolla, CA, USA). Catalytic activation was assessed by adding the indicated amounts of either recombinant Slt2/Mpk1p or extracellular signal-regulated kinase 2 (ERK2) MAPKs. Preparation of ^{32}P-labelled myelin basic protein (MBP) and casein and protein phosphatase assays using these substrates have been described previously [17]. Dephosphorylation of ERK2 by Yil113p in vitro was assayed using using rabbit polyclonal antibodies specific for either pThr^{183}-ERK2 (Promega Corp., Madison, WI, USA) or pTyr^{185}-ERK2 (Calbiochem, La Jolla, CA, USA) exactly as described previously [18].

2.6. Yeast two-hybrid analysis

Plasmids encoding *GAL4* DNA-binding domain and *GAL4* activation domain fusion proteins were transformed into the *S. cerevisiae* strains PJ69-4A and PJ69-4α respectively, exactly as described previously [19]. Following mating of these strains two-hybrid interactions were quantified in the resulting diploids by activation of the *lacZ* reporter using a cell permeable β-galactosidase assay, according to the method described by Guarente et al. [20]. At least three independent two-hybrid assays were performed for each interaction studied and, following correction for background activities (*GAL4* DNA-binding domain fusion and *GAL4* activation domain alone), mean values with associated standard errors are presented. Expression of *GAL4* DNA-binding domain and *GAL4* activation domain fusion proteins was verified by Western blotting of whole cell extracts prepared from yeast diploids using monoclonal antibodies which recognise either the Gal4p DNA-binding domain or the Gal4p activation domain respectively (Clontech, Palo Alto, CA, USA).

2.7. Monitoring the activation of endogenous Slt2/Mpk1p using anti-phospho p44/42 MAPK antibodies

Yeast strain AYS927 was transformed with either empty pYES2 or pYES2.8myc containing each of the following ORFs: *YIL113w*, *yil113w*^{C140S}, *MSG5* and *msg5*^{C319S}. Cells were then grown at 25°C in SC plus raffinose (minus uracil) to an absorbance at 600 nm of 0.3–0.4. Galactose was then added to a final concentration of 2% w/v and cells were incubated at 25°C for a further 2 h to induce protein expression. Yeasts were either left untreated or exposed to heat shock at 39°C for 30 min before lysis and clarification of protein extracts as described by Kamada et al. [21]. Equal amounts of total yeast protein (10–15 μg) for each sample were analysed by SDS–PAGE. Activated Slt2/Mpk1p was detected by Western blotting using an antibody against phosphorylated ERK1/2 MAPK (NEB, Beverly, MA, USA) as described previously [12]. Expression of myc-tagged Yil113p, Yil113p^{C140S}, Msg5p and Msg5p^{C319S} was detected by Western blotting using the anti-myc (9E10) monoclonal antibody. Levels of total Slt2/Mpk1p in yeast cell extracts were analysed by Western blotting using an antibody against Slt2/Mpk1p (sc-6802, Santa Cruz Biotech, CA USA).

3. Results

3.1. YIL113w encodes a functional protein phosphatase

Yil113p was expressed as a His-tagged fusion protein and purified using nickel–agarose resin. A mutant protein Yil113p^{C140S}, in which the predicted PTP active site cysteine was substituted by serine (Cys140 to Ser) was also produced under identical conditions (Fig. 1A). Yil113p is catalytically active as it readily hydrolyses *p*-NPP. Furthermore, this activity is absolutely dependent on the cysteine residue within the PTP active site as Yil113p^{C140S} is inactive (Fig. 1B). Finally,

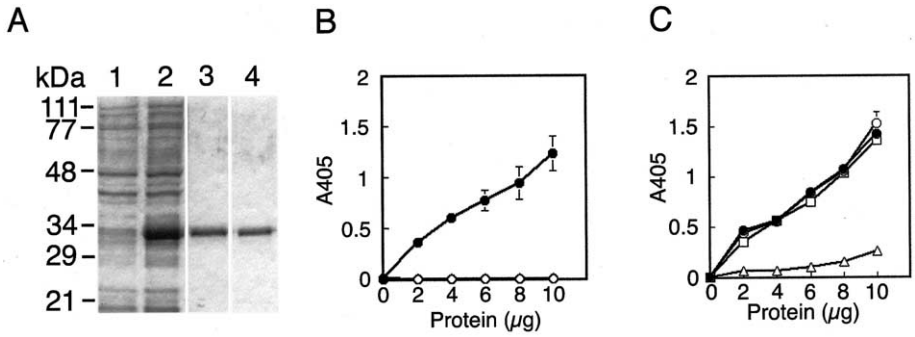

Fig. 1. Expression and characterisation of recombinant Yil113p protein in vitro. A: Expression of His-tagged Yil113p in *Escherichia coli*. Coomassie stained SDS–PAGE gel of soluble protein fractions from uninduced (lane 1) and induced (lane 2) bacteria expressing wild-type Yil113p. Yil113p (lane 3) and Yil113p^{C140S} proteins (lane 4) after purification using nickel–agarose resin. B: The Yil113p protein possesses intrinsic phosphatase activity. The indicated amount of either purified Yil113p (●) or Yil113p^{C140S} (○) was assayed for its ability to hydrolyse *p*-NPP. C: The phosphatase activity of Yil113p is inhibited by sodium vanadate. The indicated amount of purified Yil113p was assayed for its ability to hydrolyse *p*-NPP either in the absence (●) or presence of 1 μM okadaic acid (○), 1 μM microcystin (□) or 2 mM sodium vanadate (△).

Yil113p is inhibited by the PTP inhibitor sodium orthovanadate but its activity is unaffected by addition of either microcystin or okadaic acid, which are specific inhibitors of type 1/2 serine/threonine phosphatases (Fig. 1C).

The prototypic dual-specificity phosphatase of vaccinia virus (VH1) was first characterised by its ability to dephosphorylate protein substrates modified on either tyrosine or serine residues [22]. To determine if Yil113p has a similar activity we examined its ability to dephosphorylate casein (modified on serine/threonine residues) and MBP (modified on tyrosine residues). As positive controls for these reactions we used PP2A, a type-2 serine/threonine phosphatase, and PTP1B, a tyrosine-specific phosphatase respectively. We could detect no measurable liberation of [^{32}P] from either of these two model substrates on incubation with recombinant Yil113p under conditions where either PP2A (casein) or PTP1B (MBP) readily dephosphorylated these proteins (Fig. 2A,B). This strongly suggests that YIL113p is highly specific for its physiological substrate and is reminiscent of the activity of the mammalian dual-specificity MKP, CL100/MKP-1, which was also without activity towards either casein or MBP but readily dephosphorylated ERK2 MAPK in vitro [17].

To determine whether Yil113p has activity towards MAPKs, activated ERK2 was incubated with Yil113p and

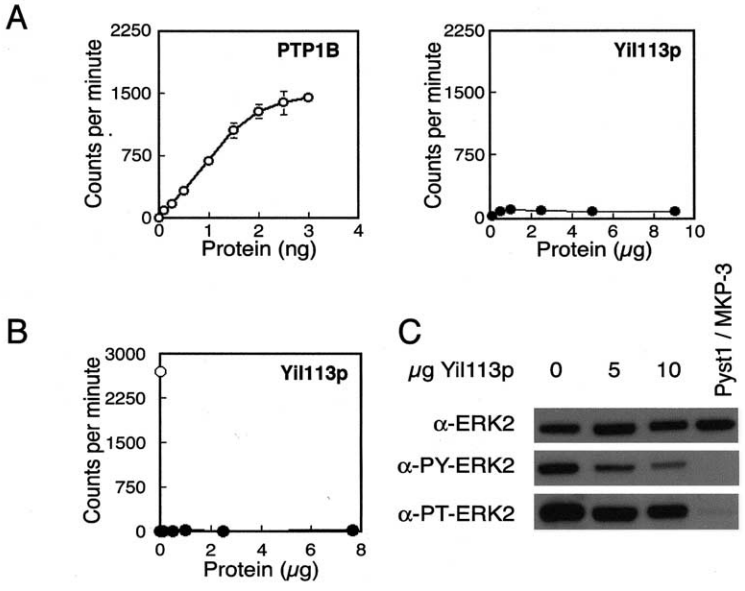

Fig. 2. Yil113p is a functional dual-specificity MAPK phosphatase in vitro. A: Yil113p is unable to dephosphorylate tyrosine residues on MBP. The indicated amount of either PTP1B (left panel) or Yil113p (right panel) was incubated with ^{32}P-labelled MBP and hydrolysis of phosphotyrosine was determined by measuring the release of acid-soluble counts (cpm) using scintillation counting. B: Yil113p is unable to dephosphorylate serine/threonine residues on casein. The indicated amount of Yil113p was incubated with ^{32}P-labelled casein and hydrolysis of phosphoserine/threonine (cpm) was determined by measuring the release of acid-soluble counts (cpm) using scintillation counting. The point on the *X*-axis (○) shows the number of counts released on a single incubation of this substrate with the catalytic subunit of PP2A (120 μU) over the time course of this assay. C: Dephosphorylation of recombinant activated ERK2 by Yil113p in vitro. Immunoblot of activated ERK2 protein following incubation with the indicated amount of recombinant Yil113p. The blot was probed with an anti-ERK2 antibody as a loading control (upper panel) and then with antibodies which specifically recognise either the phosphorylated tyrosine (middle panel) or threonine (lower panel) residues within the conserved T-E-Y motif of ERK2. As a positive control ERK2 was also incubated with 12.5 μg of recombinant Pyst1/MKP-3 protein (lane 6).

the phosphorylation state of the ERK2 was analysed using antibodies which specifically recognise either the phosphothreonine or phosphotyrosine residues within the Thr-Xaa-Tyr motif. Our results clearly show that recombinant Yil113p acts directly on ERK2 causing significant dephosphorylation of both phosphotyrosine and phosphothreonine (Fig. 2C). This activity is significantly lower than is seen on incubation with the ERK-specific human dual-specificity MKP Pyst1/MKP-3 (see Fig. 2C, lane 4) and Yil113p preferentially dephosphorylates the tyrosine residue of ERK2. This probably reflects the fact that mammalian ERK2 is a poor model for the *S. cerevisiae* MAPK which is the physiological target of Yil113p. However, we can conclude that Yil113p is able to act as a bona-fide dual-specificity MAPK phosphatase in vitro.

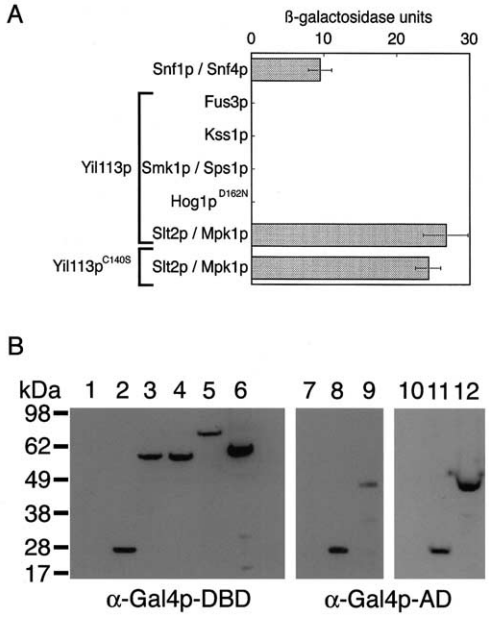

Fig. 3. Analysis of two-hybrid interactions between Yil113p and the MAPKs of *S. cerevisiae*. A: Yil113p selectively interacts with the Slt2/Mpk1p MAPK in vivo. Plasmids encoding *GAL4* DNA-binding domain ('bait') fusions of each of the MAPKs Fus3p, Kss1p, Slt2/Mpk1p, Smk1/Sps1p and hog1p^{D162N} were transformed into yeast strain PJ69-4A while plasmids encoding either the Yil113p or Yil113p^{C140S} (Slt2/Mpk1p only) *GAL4* activation domain fusion proteins were transformed into the *S. cerevisiae* strain PJ69-4α. Following mating, two-hybrid interactions were quantified in the resulting diploids by activation of the *lacZ* reporter using a cell permeable β-galactosidase assay. At least three independent two-hybrid assays were performed for each interaction studied and following correction for background activities, mean values with associated standard errors are presented. As a positive control in these assays the well characterised interaction between the yeast proteins Snf4p and Snf1p was used. B: Western blot analysis showing expression of *GAL4* DNA-binding domain ('bait') fusions of each of the MAPKs in yeast diploids using an antibody against the Gal4p DNA-binding domain (α-Gal4p-DBD, left panel). PJ69-4A/α control (lane 1), Gal4pDBD control (lane 2), Fus3p (lane 3) Kss1p (lane 4), Slt2/Mpk1p (lane 5) and Smk1/Sps1 (lane 6). Expression of Yil113p *GAL4* activation domain ('prey') fusion protein using an antibody against the Gal4p activation domain (α-Gal4p-AD, middle panel). PJ69-4A/α control (lane 7), Gal4pAD control (lane 8), Yil113p (lane 9). Expression of Yil113p^{C140S} *GAL4* activation domain ('prey') fusion protein (α-Gal4p-AD, right panel). PJ69-4A/α control (lane 10), Gal4pAD control (lane 11), Yil113p^{C140S} (lane 12).

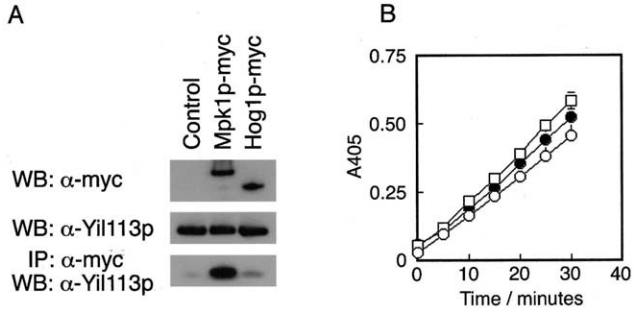

Fig. 4. The interaction between Yil113p and Slt2/Mpk1p is specific and direct but the phosphatase activity of Yil113p is not increased upon incubation with recombinant MAPK. A: Recombinant Yil113p was incubated alone or with either myc-tagged Mkp1p or myc-tagged Hog1p before immunoprecipitation using an anti-myc monoclonal antibody. Immunoprecipitates were then analysed by SDS–PAGE and Western blotting using a polyclonal antibody against Yil113p (lower panel). B: The indicated amount of purified Yil113p was assayed for its ability to hydrolyse *p*-NPP either in the absence (●) or presence of either 20 μg of recombinant ERK2 (○) or 10 μg of recombinant Slt2/Mpk1 (□).

3.2. *Yil113p interacts specifically with the Slt2/Mpk1p MAPK in S. cerevisiae*

The inactivation of mammalian MAPKs by dual-specificity MKPs is accompanied by the formation of a physical complex between phosphatase and substrate [6]. To determine whether Yil113p is capable of specific protein–protein interactions with the five MAPKs of *S. cerevisiae* in vivo we have used a yeast two-hybrid assay.

We were unable to demonstrate any interactions between Yil113p and either Fus3p, Kss1p, Hog1p^{D162N} or Smk1/Sps1p irrespective of whether Yil113p or each of the individual MAPKs were used as the 'bait' in these assays. However, the Slt2/Mpk1p MAPK showed a strong interaction with Yil113p when used as a 'bait' in the two-hybrid system as revealed by semi-quantitative β-galactosidase assays (Fig. 3A). Slt2/Mpk1 also interacted strongly with the inactive Cys140 to Ser mutant Yil113p^{C140S} under these conditions (Fig. 3A). Our failure to detect any interaction between Yil113p and the other yeast MAPK isoforms is not due to a lack of expression during these assays as verified by Western blotting (Fig. 3B). The interaction between Yil113p and Slt2/Mpk1p is both direct and specific as recombinant Yil113p co-immunoprecipitates with recombinant myc-tagged Slt2/Mpk1p but not myc-tagged Hog1p in vitro (Fig. 4A).

3.3. *Yil113p does not undergo catalytic activation on incubation with MAPKs*

A significant breakthrough in our understanding of MKP function came with the discovery that the catalytic activity of the mammalian dual-specificity MKP Pyst1/MKP-3 could be specifically increased on binding to ERK2 MAPK in vitro [23]. Catalytic activation has subsequently been demonstrated to be a general property of these enzymes and is thought to be an important mechanism which underpins substrate selectivity [24,25]. To determine whether Yil113p is activated in this way, *p*-NPP hydrolysis was monitored either in the absence or presence of either ERK2 MAPK or Slt2/Mpk1p, the specific binding partner for Yil113p in *S. cerevisiae* (Fig. 4B). Our results show that the catalytic activity of recombinant Yil113p is unchanged upon incubation with either of these

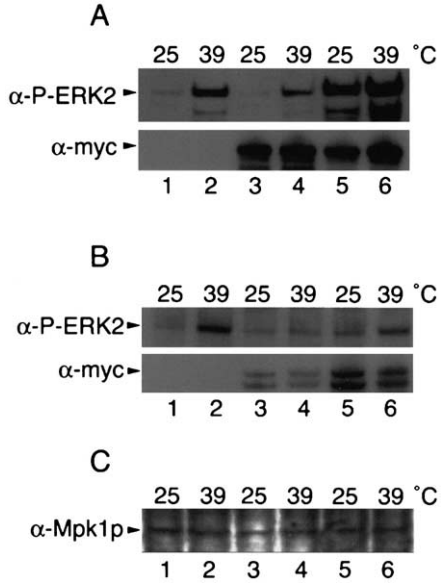

Fig. 5. Yil113p dephosphorylates Slt2/Mpk1p in vivo. A: Western blot analysis of phosphorylated Slt2/Mpk1p in yeast cells at the indicated temperature using an antibody against phosphorylated ERK2 MAPK (α-P-ERK2, upper panel). Cells contained either empty pYES2 vector (lanes 1 and 2) or pYES2 expressing either myc-tagged wild-type Yil113p (lanes 3 and 4) or Yil113p^{C140S} (lanes 5 and 6). Expression of phosphatases was verified using the anti-myc (9E10) monoclonal antibody (α-myc, lower panel). B: Western blot analysis of phosphorylated Slt2/Mpk1p in yeast cells at the indicated temperature using an antibody against phosphorylated ERK2 MAPK (α-P-ERK2, upper panel). Cells contained either empty pYES2 vector (lanes 1 and 2) or pYES2 expressing either myc-tagged wild-type Msg5 (lanes 3 and 4) or Msg5p^{C319S} (lanes 5 and 6). Expression of phosphatases was verified using the anti-myc (9E10) monoclonal antibody (α-myc, lower panel). C: Western blot of endogenous Slt2/Mpk1p protein in yeast cells grown at the indicated temperature using a polyclonal antibody against Slt2/Mpk1p (α-Mkp1p).

proteins suggesting that specific interaction between Yil113p and Slt2/Mpk1p may be sufficient for the substrate selectivity of this phosphatase.

3.4. *Yil113p dephosphorylates Slt2/Mpk1p in vivo*

An antibody which specifically recognises the phosphorylated (activated) form of ERK2 MAPK also recognises phosphorylated Slt2/Mpk1p in *S. cerevisiae* [12]. To determine if Yil113p was able to dephosphorylate Slt2/Mpk1p in vivo both myc-tagged wild-type and Yil113p^{C140S} were expressed in *S. cerevisiae* using the galactose-inducible yeast expression vector pYES2. pYES2 containing either myc-tagged wild-type or a cysteine to serine mutant of Msg5p were used as positive controls for these experiments. These expression plasmids along with empty pYES2 vector were then transformed into yeast, grown at 25°C in medium containing galactose to induce the expression of either Yil113p or Msg5p and either left untreated or exposed to a 39°C heat shock for 30 min. The phosphorylation state of endogenous Slt2/Mpk1p and the expression of the myc-tagged phosphatases was then monitored by Western blotting. In agreement with previously published data, our results show that the activation of endogenous Slt2/Mpk1p in response to heat shock can be monitored using the anti-phospho ERK antibody (Fig. 5A,B, lanes 1 and 2). Furthermore, expression of either wild-type Yil113p or Msg5p significantly reduces the activation of this MAPK (Fig. 5A,B, lanes 3 and 4). Mutation of the active site cysteine within all PTPs abolishes catalytic activity (see Fig. 2B). In addition, PTPs carrying this inactivating mutation act as 'substrate traps' forming a stable complex with their physiological targets when expressed in mammalian cells [26,27]. Our results show clearly that expression of mutant Yil113p causes the accumulation of phosphorylated Slt2/Mpk1p even at the normal growth temperature (compare lanes 1 and 5 in Fig. 5A). Furthermore, expression of Yil113p^{C140S} significantly increased the levels of phosphorylated Slt2/Mpk1p following heat shock (compare lanes 2 and 6 in Fig. 5A). Msg5^{C319S}p while unable to dephosphorylate Slt2/Mpk1p (compare lanes 4 and 6 Fig. 5B) did not appear to cause significant accumulation of phosphorylated MAPK. None of the changes in the levels of phosphorylated Slt2/Mpk1p which we observe are due to heat shock-induced changes in the levels of the endogenous protein as verified by Western blotting using an antibody against Slt2/Mpk1p (Fig. 5C). We conclude that Yil113p can dephosphorylate Slt2/Mpk1p in vivo and that the mutant form of Yil113p is able to bind specifically to phosphorylated Slt2/Mpk1p, blocking its subsequent dephosphorylation by an endogenous phosphatase.

4. Discussion

The *YIL113w* gene of *S. cerevisiae* encodes a putative protein phosphatase which is highly related to both mammalian MKPs and the yeast dual-specificity MAPK phosphatase Msg5p. In the present study we have demonstrated that *YIL113w* encodes a catalytically active phosphatase which shows specific activity towards the ERK2 MAPK in vitro, where it acts as a bona-fide dual-specificity MAPK phosphatase. Furthermore, using the yeast two-hybrid assay to detect protein–protein interactions we have shown that Yil113p interacts specifically with the Slt2/Mpk1p MAPK in *S. cerevisiae*. Very recently, two systematic global surveys of protein–protein complexes within the yeast proteome using mass spectrometry were published. In one of these, Yil113p was found to be complexed with Slt2/Mpk1p [28] confirming both our results and those of a preliminary global two-hybrid analysis in which Yil113p also interacted with Slt2/Mpk1p [29]. Neither in our two-hybrid assays nor in any of these global surveys was Yil113p able to interact with any of the other four MAPKs of *S. cerevisiae*. This latter finding coupled with our observation that expression of wild-type Yil113p causes dephosphorylation of Slt2/Mpk1p in vivo while a substrate trapping mutant of Yil113p enhances the phosphorylation of Slt2/Mpk1p strongly suggests that the Yil113p phosphatase is a specific regulator of signalling through the Slt2/Mpk1p MAPK pathway. This conclusion is supported by our own findings in which a *YIL113w* disruptant strain exhibited a normal response to mating pheromone, indicating that Yil113p is not involved in regulating the Fus3p MAPK (M.C., unpublished observations) and by the results of a study in which disruption of *YIL113w* alone or in combination with *PTP2* and/or *MSG5* was found to have no effect on phosphorylation of the Hog1p MAPK, indicating that Yil113p is not involved in regulating the response to osmotic stress in *S. cerevisiae* [7]. Finally, during preparation of this manuscript an independent study by Hahn and Thiele which also strongly supports a role for *YIL113w* in regulating the

Slt2/Mpk1p MAPK has appeared [30]. This shows that deletion of *YIL113w* exacerbates growth defects due to overexpression of a constitutively active mutant of Slt2/Mpk1p, that the heat shock-induced phosphorylation level of Slt2/Mpk1p is elevated in this deletant when compared to a wild-type strain and that heat shock-activated phospho-Slt2/Mpk1p is dephosphorylated by recombinant Yil113p in vitro.

Mammalian dual-specificity MKPs contain two distinct domains, a catalytic domain which contains the PTP active site and an amino-terminal non-catalytic domain which carries two diagnostic regions of sequence similarity with the cdc25 cell cycle regulatory phosphatase [31]. It is this latter domain which mediates specific interactions with MAPKs leading to catalytic activation of these enzymes, thus determining substrate selectivity [32]. Yil113p is a small protein when compared to the mammalian MKPs and has sequence homology only with the catalytic domain of these enzymes. We have demonstrated that Yil113p interacts directly with the Slt2/Mpk1p MAPK but, unlike the prototypic mammalian MKP CL100/MKP-1, it does not undergo catalytic activation when incubated with recombinant MAPKs. Interestingly, the mammalian dual-specificity phosphatase VHR, which also lacks an amino-terminal non-catalytic domain, has also been shown to interact with and dephosphorylate the ERK2 MAPK [33]. Furthermore, a *Drosophila* phosphatase (DMKP) of 203 amino acids which is similar in sequence to VHR and also lacks an amino-terminal non-catalytic domain has recently been demonstrated to inactivate the *Drosophila* ERK and c-Jun-N-terminal kinase MAPKs and also to inactivate Fus3p when expressed in *S. cerevisiae* [34]. Yil113p may thus be a member of an emerging subfamily of dual-specificity MKPs in which substrate recognition is mediated by sequences which reside within the catalytic domain and which do not require a conformational change within the active site in order to dephosphorylate MAPKs.

The Slt2/Mpk1p MAPK cascade is composed of Bck1p (MAPK kinase kinase), Mkk1p/Mkk2p (MAPK kinase) and Slt2/Mpk1p (MAPK) and lies downstream of protein kinase-C signalling [2]. The pathway is involved in the maintenance of cell wall integrity and is activated in response to a variety of stress conditions including heat shock [21] and hypotonic shock [35]. Slt2/Mpk1p is also activated in response to mating pheromone. However, this latter effect is indirect and results from re-organisation of the cell wall induced by the mating pheromone pathway [36]. With respect to the regulation of Slt2/Mpk1p by protein phosphatases it has been demonstrated that the tyrosine-specific phosphatases Ptp2p and Ptp3p negatively regulate this kinase with Ptp2p playing the major role [11]. Furthermore, the expression of Ptp2p, but not Ptp3p, is increased in response to heat shock in a Slt2/Mpk1p dependent manner suggesting that Ptp2p acts in a negative feedback loop to regulate Slt2/Mpk1p. Slt2/Mpk1p is also targeted by the dual-specificity phosphatase Msg5p [12]. Msg5p is inducible by mating pheromone in a Fus3p dependent manner [10] and thus may represent a point of crosstalk between these two MAPK pathways.

In conclusion, we have demonstrated that the product of the *YIL113w* gene of *S. cerevisiae* encodes a functional dual-specificity MAPK phosphatase which interacts selectively with and inactivates the Slt2/Mpk1p MAPK in vivo. Our results add to the degree of complexity involved in the negative regulation of this pathway by adding Yil113p to the previously characterised activities of Ptp2p,Ptp3p and Msg5p in regulating Slt2/Mpk1p. The challenge of future work will be to understand how these different enzymes act in concert to determine both the basal and induced levels of signalling through this critical MAPK pathway.

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