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ORIGINAL PAPER

E. Asp · P. Sunnerhagen

Mkp1 and Mkp2, two MAPKAP-kinase homologues in *Schizosaccharomyces pombe*, interact with the MAP kinase Sty1

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Abstract Mkp1 (MAPKAP kinase *Schizosaccharomyces pombe* 1) and Mkp2 are two members from fission yeast of the sub-class of putative MAPK-activated protein kinases in yeasts, the other known members being Rck1 and Rck2 from *Saccharomyces cerevisiae*. The Mkp1 protein is readily co-immunoprecipitated with Sty1 from *S. pombe* extracts; Mkp2 shows a weaker interaction with Sty1. In *mkp1* mutants, conjugation and meiosis proceed more readily and rapidly than in wild-type cells, in analogy to what was previously found for *S. cerevisiae rck1* mutants. Conversely, overexpression of $mkp1^+$ delays meiosis. Mkp1 is phosphorylated in vivo in a $sty1^+$-dependent manner; this modification is removed when cells are starved for nitrogen, a condition that is conducive to entry into stationary phase and meiosis. Overexpression of $mkp1^+$, like a *sty1* mutation, also causes vegetative cells to elongate. The level of Mkp1 phosphorylation drops as cells enter mitosis. We have localised Mkp1 to the cytoplasm, excluded from the nucleus, in vegetative cells. The Mkp1 protein accumulates in zygotic asci and is concentrated within spores. The $mkp2^+$ gene has no noticeable impact on meiosis. Mkp2 is excluded from the nucleus in vegetative cells, and is concentrated at the septa of dividing cells. Mkp2 does not accumulate in meiotic cells.

Keywords Meiosis · Mitosis · Protein phosphorylation · Green fluorescent protein (GFP) · Fission yeast

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E. Asp · P. Sunnerhagen (✉)
Department of Cell and Molecular Biology,
Lundberg Laboratory, Göteborg University,
P.O. Box 462, 405 30, Göteborg, Sweden
E-mail: Per.Sunnerhagen@molbio.gu.se
Tel.: +46-31-7733830
Fax: +46-31-7733801

Introduction

In order to respond appropriately to changes in the environment or to intracellular events, the cell needs to integrate signals coming from inside and outside the cell; this is the task of the many interconnected signalling pathways. Eukaryotic cells, plant, fungal, or animal, build signalling pathways from structurally conserved modules, one of which is the mitogen-activated protein kinase (MAPK) cascade. Such a cascade is three-layered, consisting of the MAPK itself, which is activated by a tyrosine/threonine dual specificity MAPK kinase (MAPKK) that is in turn activated by a serine/threonine-specific MAPKK kinase (MAPKKK). These cascades are remarkably versatile in that they collectively respond to a wide variety of stimuli such as peptide pheromones, DNA damage, nitrogen limitation etc., to ultimately produce responses that include the activation of transcriptional programs, entry into meiosis, changes in growth pattern and so on. Typically, a given organism possesses several MAPK cascades. The specificity of signal transmission within a given cascade is guaranteed in part through physical interactions with a scaffold protein. Nevertheless communication between cascades can also occur, e.g. when one MAPKKK controls more than one MAPKK. Eukaryotic MAPK cascades have been reviewed recently (Millar 1999; Chang and Karin 2001; Kyriakis and Avruch 2001).

The Sty1 MAPK cascade in *Schizosaccharomyces pombe* is required for activation of the mating and meiotic programs. Meiosis is favoured by the same conditions as mating; newly formed zygotes normally proceed directly to meiosis, and so mating and meiosis are coupled processes in *S. pombe*. *sty1* mutants are defective in both processes (Kato et al. 1996; Shiozaki and Russell 1996; Kon et al. 1998). The requirement for Sty1 stems, at least in part, from Sty1-dependent phosphorylation of the heterodimeric transcription factor Atf1/Pcr1 (Kon et al. 1998), which is responsible for expression of the $ste11^+$ gene. The product of $ste11^+$ is a transcription

factor which in turn activates transcription of a number of genes involved in conjugation, meiosis, and sporulation. In addition, low levels of cAMP activate the expression of *ste11*$^+$ through the action of protein kinase A and the transcription factor Rst2 (Kunitomo et al. 2000). The Byr2-Byr1-Spk1 MAPK cascade, which is homologous to the Ste11-Ste7-Fus3 relay in *Saccharomyces cerevisiae*, is likewise required for mating (Neiman et al. 1993). Finally, the protein kinase Pat1 inhibits meiosis by negatively regulating Ste11 through phosphorylation (Li and McLeod 1996). Besides its involvement in regulating meiosis, Sty1 plays a central role in defence against a range of cellular stresses including osmotic shock, oxidative stress, and DNA damage (Degols and Russell 1997; Degols et al. 1996; Millar et al. 1995; Shiozaki and Russell 1995b; Stettler et al. 1996; Toone et al. 1998). It is clear that the Sty1 MAPK cascade initiates responses at the gene expression level via the transcription factors Pap1, after oxidative stress (Toone et al. 1998), and Atf1, after osmotic and a variety of other stresses (Degols and Russell1997; Shiozaki and Russell 1996; Wilkinson et al. 1996). In addition, Sty1 has recently been shown to be required for a mitotic entry checkpoint in fission yeast that monitors the integrity of the actin cytoskeleton (Gachet et al. 2001). Finally, the Sty1 pathway somehow influences mitotic cell cycle progression through G2, since *sty1* cells are elongated and have a prolonged G2 phase (Millar et al. 1995; Shiozaki and Russell 1995a; Kato et al. 1996).

In mammalian cells, many cellular responses of MAPK cascades have been shown to be mediated by MAP kinase-activated protein kinases (MAPKAPK). For instance, the MAPKAP-K1 (p90^{rsk}) family is activated by the ERK and JNK MAPK classes following exposure to UV (Zhang et al. 2001). MAPKAP kinase 2 is capable of directly phosphorylating transcription factors (Neufeld et al. 2000), and is also required for the expression of TNFα (Kotlyarov et al. 1999). The MAPK pathway in which a particular MAPKAPK functions is largely dictated by its MAPK binding domain, which is located at the C-terminus of the protein (Smith et al. 1999; 2000).

In *S. cerevisiae*, two MAPKAPK homologues have been found, Rck1 and Rck2. The *RCK1* gene was originally isolated as a suppressor of *S. pombe* checkpoint mutants, and *RCK2* was found in the genome sequence as a homologue of *RCK1* (Dahlkvist and Sunnerhagen 1994; Dahlkvist et al. 1995). The *RCK2* gene has independently been found in a search for sequences related to Ca^{2+}/calmodulin-regulated protein kinases (Melcher and Thorner 1996). A role for Rck1 in inhibition or delay of meiosis is evidenced by the finding that *rck1*/*rck1* mutants enter meiosis at an enhanced rate. Correspondingly, overexpression of *RCK1* or *RCK2* causes a decrease in the fraction of meiotic cells, but only when either of the the checkpoint genes *MEC1* and *RAD24* is mutated (Ramne et al. 2000). We have previously shown that Rck2 is a substrate for the MAP kinase Hog1, and that it is phosphorylated at Ser519 by this protein kinase following osmotic shock; this phosphorylation results in the activation of the catalytic activity of Rck2. There is also genetic evidence to link the two, in that overexpression of *RCK2* restores osmotic resistance in a *hog1* or a *pbs2* mutant, and an *rck2* deletion rescues the lethality associated with hyperactivation of the HOG pathway (Bilsland-Marchesan et al. 2000). Recently, a role for Rck2 in down-regulating translation in osmotically shocked cells via phosphorylation of EF-2 was defined. This global decrease in protein synthesis was shown to be dependent on both *RCK2* and *HOG1* (Teige et al. 2001).

Thus, Rck2 is a MAPKAPK that acts downstream of the stress-activated MAPK Hog1. We now identify two fission yeast genes that code for homologues of Rck1/Rck2, and present evidence to show that they act downstream of the Sty1, the Hog1 homologue in *S. pombe*.

Materials and methods

S. pombe genetic methods

Standard methods were used for genetic crosses (Gutz et al. 1974). *S. pombe* cells were grown on YES (0.5% yeast extract, 3% glucose, 225 mg/l each of histidine, adenine, leucine, and uracil) for vegetative growth, or Edinburgh minimal medium (EMM) (Moreno et al. 1991). Meiosis was induced on by growth on malt extract agar plates (ME), or by shifting from growth at 30°C with shaking in regular liquid EMM to growth at 25°C in nitrogen-free EMM (Kato et al.1996), as indicated.

DNA constructs for chromosomal disruptions and epitope taggings were made by PCR, and integrated by homologous recombination into the desired loci using the method outlined by Bähler et al. (1998) with minor modifications. When the *KanMX6* cassette was used as a selectable marker, transformants were grown in liquid YES medium for 16 h to allow for stable integration and expression of the kanamycin resistance gene, before plating on solid YES containing G418 (100 mg/l).

For constructs encoding Mkp1 or Mkp2 C-terminally tagged with green fluorescent protein (GFP) or $HA_{(3)}$, transformations were done with PCR fragments from pFA6a-GFP(S65T)-kanMX6 and pFA6a-3HA-kanMX6 cassettes (Bähler et al. 1998) flanked by 80 bp corresponding to the sequence immediately upstream of the stop codon and 80 bp corresponding to the sequence 80–200 bp downstream of the stop codon. To insert the wild-type or attenuated version of the *nmt1* promoter in place of the endogenous *mkp1*$^+$ or *mkp2*$^+$ promoter, pFA6a-kanMX-P3nmt1-3HA constructs flanked by 80 bp corresponding to sequences 90–200 bp upstream of the start codon of the genes and 80 bp corresponding to the N-terminal part of the gene from the start codon were used. Such constructs also carried the $HA_{(3)}$ epitope fused to the N-terminus of the protein. Gene disruption of *mkp2*$^+$ and *sty1*$^+$ was done by the same method with the KanMX6 and *ura4*$^+$ disruption cassettes flanked by 80 bp homologous to regions 5′ and 3′ to the coding sequence. Disruption of *mkp1*$^+$ was done by transforming with the PCR amplified 1.8 kb *Hind* III *ura4*$^+$ genomic fragment plus 437 bp homologous to the region 15 bp upstream of *mkp1*$^+$ and 462 bp homologous to the region 198 bp downstream of *mkp1*$^+$. In each case, correct integration at the desired chromosomal locus was verified by PCR.

Protein preparation

Cells were grown to mid-log phase and harvested in stop buffer [150 mM NaCl, 50 mM NaF, 1 mM NaN_3, 10 mM EDTA (pH 8.0)]. The cells were lysed in buffer A [50 mM TRIS-HCl (pH 8.0), 50 mM NaCl, 0.2% Triton X-100, 1% NP-40]

supplemented with Complete protease inhibitor mix (Roche), using a FastPrep 120 apparatus at a speed setting of 4.5 for 20 s. Cells to be used for co-immunoprecipitation were lysed in buffer A with 0.25% NP-40. Protein concentrations were measured using the BCA protein assay kit (Pierce) to ensure even loading.

Co-immunoprecipitation

Protein extracts (0.5 mg) were first clarified by the addition of Pansorbin (formalin-fixed *Staphylococcus aureus* cells) and centrifugation. For precipitation, 4 μg of the precipitating antibody was added to the supernatant. After 4 h of incubation on a rotary mixer at 4°C, Pansorbin was added, and incubation was continued at 4°C. Precipitates were collected by high-speed centrifugation, washed in buffer A containing 1% NP-40, and finally resolved by SDS-PAGE.

Western analysis

Fifty μg of total protein per lane was loaded on 10 % SDS-polyacrylamide gels, electrophoresed, and blotted onto a Protran nitrocellulose filter (Schleicher and Schuell) in a semi-dry blotting apparatus (Sigma Aldrich). Tagged proteins were detected with a monoclonal α-c-Myc (Santa Cruz Biotechnology) or α-HA antibody and an α-mouse Ig-POD Fab fragment as the secondary antibody (Roche) using the ECL Western blotting analysis system (Amersham Pharmacia).

Northern analysis

Total RNA was prepared using the RNeasy kit (Qiagen). Samples (10 μg) were electrophoresed under denaturing conditions on a 1 % agarose gel containing formaldehyde, blotted onto Hybond-N+ membranes (Amersham Pharmacia) and hybridised to a ^{32}P-labelled probe and washed at high stringency.

Microscopy

Cells were viewed using a Leica DM RXA microscope equipped with a Fluotar lens at 100× (fluorescence) or 63× (bright field) magnification, and photographed with a COHU cooled CCD camera. For visualisation of DNA, cells were stained for 15 min in the presence of 0.25 μg/ml of Hoechst 33258 (bisbenzimide) dissolved in growth medium.

Results

The *S. pombe* genes *mkp1*$^+$ and *mkp2*$^+$ encode homologues of the *S. cerevisiae* protein kinases Rck1 and Rck2

In a sequence similarity search for genes encoding potential homologues of *S. cerevisiae* Rck1 and Rck2 in the fission yeast (*S. pombe*) genome sequence database at the Sanger Institute (http://www.sanger.ac.uk/Projects/S_pombe/), we identified two genes encoding protein sequences closely related to those of the budding yeast kinases. The first, *mkp1*$^+$, is an open reading frame on chromosome III, which is interrupted by a short predicted intron, and has a capacity to encode a protein of 580 amino acids (cosmid SPCC1322.08; Accession No CAA22861.1). The second, *mkp2*$^+$, is located on chromosome I, has three predicted introns, and a coding capacity of 504 residues (cosmid SPAC23A1.06c; Acc. No. CAA16980.1).

In Fig. 1A, an alignment of the predicted sequences of the four members of this subfamily of protein kinases is shown, together with a mammalian MAPKAPK, and an *S. pombe* Ca^{2+}/calmodulin-regulated protein kinase, Cmk1. The four yeast protein kinases share two prominent features. First, within the catalytic domain (upper row), there is a glycine-rich sequence N-terminal to the highly conserved DFG-box, which was initially noted in Rck1 and Rck2 (Dahlkvist and Sunnerhagen 1994). This sequence is not found in any other protein kinases as far as we have been able to ascertain. We speculate that it may protrude from the otherwise conserved structure of the catalytic domain, and so we have dubbed it the "glycine loop". The second feature (bottom row), which unlike the first one is shared with mammalian MAPKAPKs, is the putative MAPK-binding site near the C-terminus of the protein, containing the conserved consensus sequence RR (Tanoue et al. 2000). Indeed, the binding of Rck2 to the MAPK Hog1 has been shown to occur through the C-terminal part of Rck2 (Bilsland-Marchesan et al. 2000). From various measures of sequence relatedness, it is not obvious if either of the two *S. pombe* proteins is more closely related to one of the two proteins in *S. cerevisiae* (Fig. 1B).

Inactivation of *mkp1*$^+$ facilitates conjugation and entry into meiosis

Since the *mkp1*$^+$/*mkp2*$^+$ homologues in *S. cerevisiae*, *RCK1* and *RCK2*, delay or depress meiosis (Ramne et al. 2000), and since Sty1, the fission yeast homologue of the Hog1 MAPK, is required for efficient meiosis (Kato et al.1996; Shiozaki and Russell 1996; Kon et al. 1998), we wanted to investigate the effects on conjugation and meiosis of disruption or up-regulation of *mkp1*$^+$ and *mkp2*$^+$. In Fig. 2A, spore staining with iodine vapour reveals that h^{90} *mkp1*Δ cells enter meiosis faster than wild-type h^{90} cells on a variety of media. After 72 h on standard meiosis-inducing medium (malt extract agar plates), markedly more staining is seen in the mutants than in the h^{90} wild-type strain (Fig. 2A); a similar difference is seen on EMM, which is semi-permissive for meiosis. On YES medium, containing yeast extract and glucose, meiosis is normally inhibited in *S. pombe* cells. However, *mkp1*Δ mutants are able to sporulate extensively on YES plates when the glucose concentration is reduced to 1%; very few wild-type cells undergo meiosis under these conditions (Fig. 2A). At 2% glucose, however, sporulation was completely suppressed in both mutant and wild-type strains (Fig. 2A).

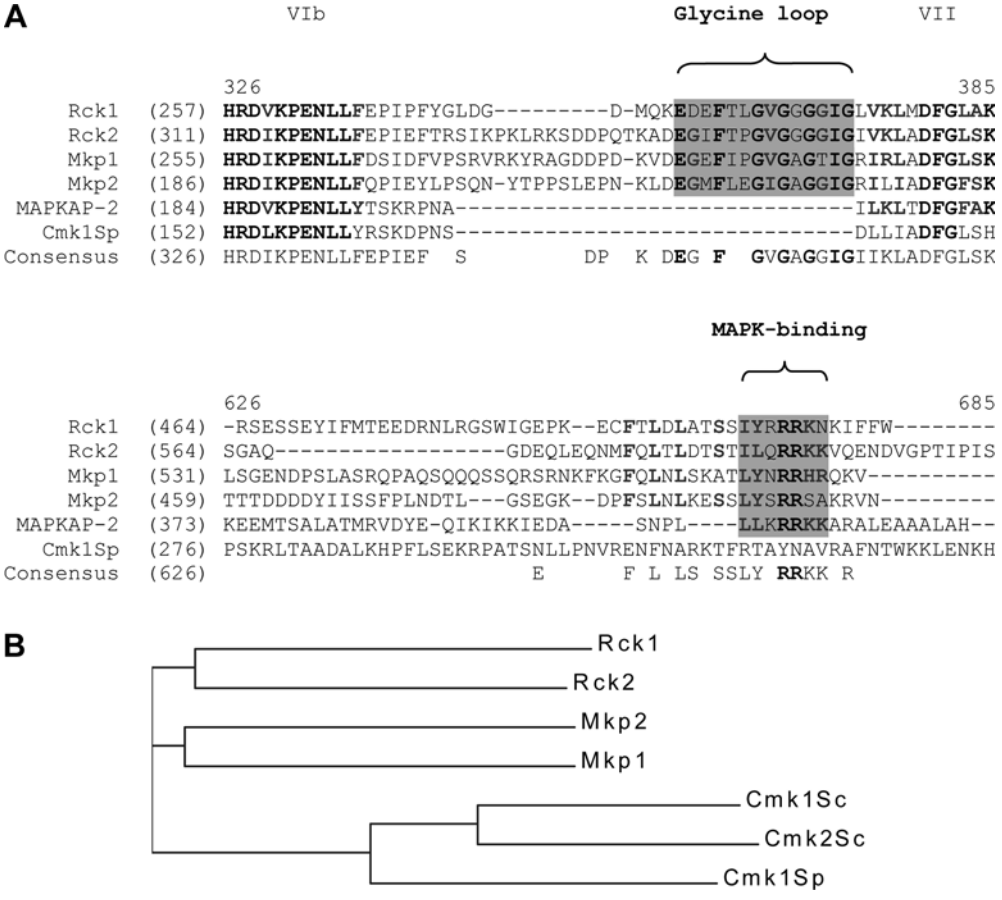
We also investigated the effect of up-regulating both genes under the control of the regulatable *nmt1*$^+$ promoter. In Fig. 2B, we see that overexpression of *mkp1*$^+$ leads to considerable depression of sporulation; this effect is abolished by lowering the expression level by the

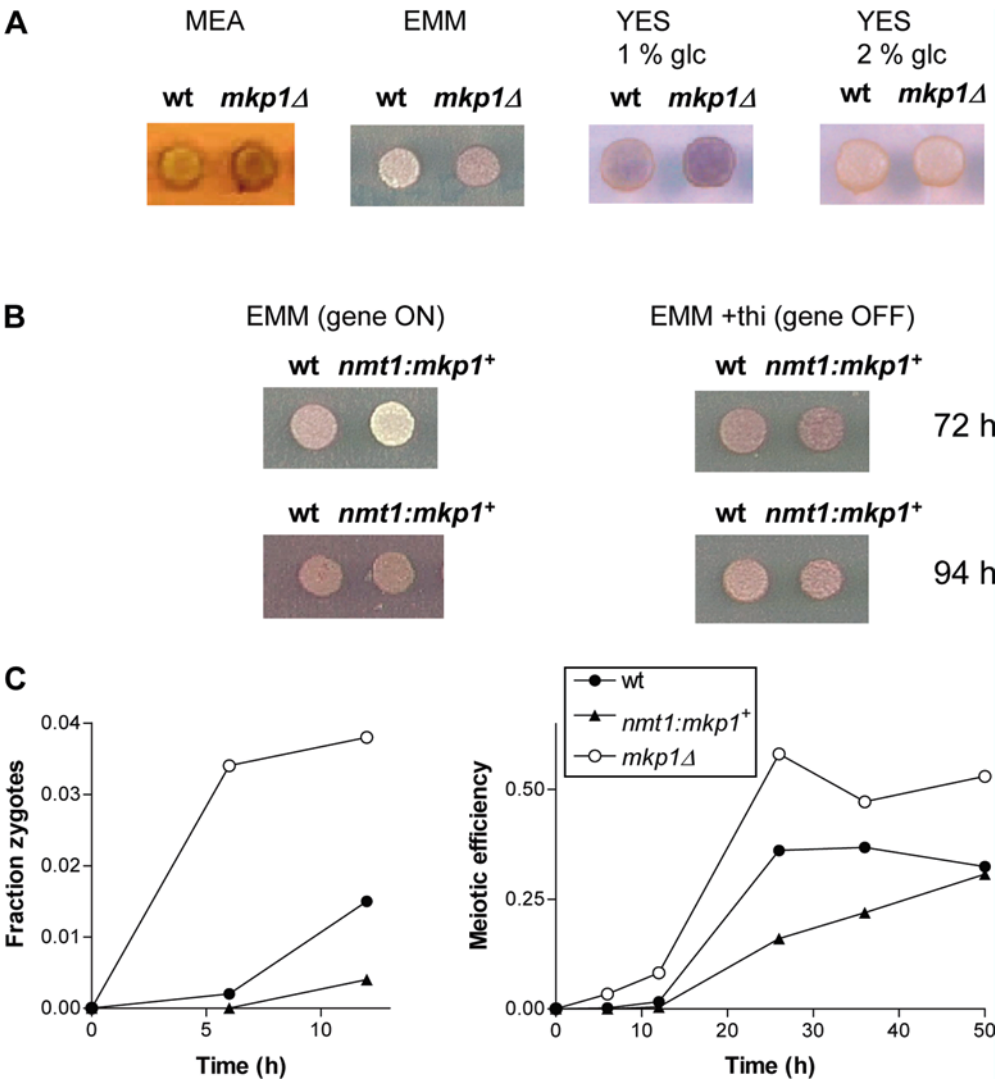
addition of thiamine to the medium. It should be noted that the *nmt1-P3* promoter variant used permits sufficient transcription even in the presence of thiamine that no phenotypic effects of down-regulation are seen here. In contrast, neither up-regulation nor disruption of the *mkp2*$^+$ gene had any obvious impact on the kinetics of spore formation (not shown).

Because of the obvious impact by *mkp1*$^+$, but not by *mkp2*$^+$, on the timing of spore production, we wanted to investigate the kinetics of appearance of zygotes (products of conjugation), as well as asci and spores (products of meiosis), in strains in which *mkp1*$^+$ was disrupted or ectopically overexpressed. Zygotes appear in the *mkp1*

Fig. 1A, B The Rck family of protein kinases: Rck1, *S. cerevisiae* (Acc. No. S47900); Rck2, *S. cerevisiae* (Acc. No. S59394); Mkp1, *S. pombe* (Acc. No. O94547); Mkp2, *S. pombe* (Acc. No. O42844). For comparison, the following protein kinase sequences are shown: MAPKAP-2, human MAPK-activated protein kinase 2 (Acc. No. P49137); Cmk1Sp, *S. pombe* calmodulin-activated protein kinase 1 (Acc. No. O74235); Cmk1Sc, *S. cerevisiae* calmodulin-activated protein kinase 1 (Acc. No. A40896); Cmk2Sc, *S. cerevisiae* calmodulin-activated protein kinase 2 (Acc. No. B40896). **A** Defining sequence features. The subdomains VIb and VII of the protein kinase catalytic domain (Hunter 1991) are marked *above* the sequence. The "glycine loop", which is not present in other protein kinases, upstream of the highly conserved DFG box in the kinase catalytic domain, and the MAP kinase binding domain near the C-terminus are *boxed in grey*. Conserved positions within these domains are marked in *bold* in the consensus sequence. **B** Phylogenetic comparison of the yeast MAPKAP kinases and their relationship to Ca^{2+}/CaM-regulated protein kinases in yeast. Estimated relative phylogenetic distances are indicated by the tree, which was calculated using Kimura's correction (Kimura and Takahata 1983)

Fig. 2A–C Conjugation and meiosis in strains with altered expression of *mkp1*$^+$ or *mkp2*$^+$. All strains are in the meiosis-proficient h^{90} genetic background. Cells were grown at 30°C to mid log-phase in liquid EMM with the required supplements prior to spotting on solid media as indicated. In panels **A** and **B** sporulation was detected by staining with iodine vapour. **A** Sporulation in wild type (h^{90}) and *mkp1Δ* mutants (EA31) on different solid media. Pictures were taken at 72 h after plating and incubation at 25°C. **B** Sporulation in wild type (h^{90}) and in a strain with ectopic overexpression of *mkp1*$^+$ (EA64) on solid EMM. Expression from the *nmt1* promoter (*nmt1-P3*) was induced at maximal levels by cultivation in the absence of thiamine, or repressed by the addition of thiamine (10 μM) to the medium. Strains were initially cultured in liquid medium of the same composition as that on which they were subsequently plated. Pictures were taken at 72 or 96 h after plating and incubation at 25°C. **C** Time course of conjugation and meiosis in the wild-type strain, and in strains lacking or overexpressing *mkp1*$^+$. Cells were first cultivated in liquid standard EMM (without added thiamine) at 30°C. The meiotic pathway was induced by transfer to nitrogen-free EMM at 25°C (time 0), and cultivation with shaking was continued. Ectopic expression from the *nmt1-P3:mkp1*$^+$ construct was maximised by cultivation in medium without thiamine throughout the experiment. Samples were taken at the indicated times after shift and examined by microscopy for the presence of zygotes, four-spored asci and free spores. Meiotic efficiency was defined as $(2Z+2A+0.5S)/(2Z+2A+0.5S+V)$, where Z is the number of zygotes; A the number of asci; S the number of free spores; and V the number of vegetative cells (Kunitomo et al. 1995)





cultures (Fig. 2C, left panel) within 6 h after transfer to nitrogen-free liquid medium and downshift to 25°C, whereas no zygotes are present in wild-type cultures. Again, the opposite effect is observed when *mkp1*$^+$ is overexpressed from the *nmt1* promoter; the number of zygotes is much lower. The same effects are observed when the products of meiosis are considered (right panel). In the *mkp1*Δ mutant, asci appear at 12 h; at this stage no asci are visible in the wild-type. Initially, in the population overexpressing *mkp1*$^+$, the efficiency of meiosis is clearly depressed compared to the wild-type. At later time points, however, it reaches similar levels. The converse is seen in the *mkp1*Δ mutant; here the number of meiotic products is considerably higher than in the wild-type.

Mkp1 and Mkp2 associate with the Sty1 MAPK in vivo

Because of the apparent similarities between Mkp1 and Mkp2, on one hand, and the *S. cerevisiae* Hog1-binding MAPKAPK Rck2 (Bilsland-Marchesan et al. 2000) on the other, we wanted to investigate whether these fission yeast kinases physically associate with the Hog1 homologue Sty1. In Fig. 3 (upper panel, lane 5), myc-Sty1 is seen to be clearly co-precipitated by a monoclonal antibody against Mkp1-HA; a faint band representing Sty1-myc co-precipitated with Mkp2-HA is also visible (lane 2). The co-precipitation of this protein species is dependent on both Sty1-myc and Mkp1-HA or Mkp2-HA, respectively (lanes 3, 4, and 6). In the converse experiment (lower panel), two bands representing Mkp1-HA co-precipitated with Sty1-myc are also seen (lane 5); however, we could not detect co-precipitated Mkp2-HA under these conditions. Again, recovery of these bands is dependent on the presence of both protein partners (lanes 3, 4 and 6).

By comparing the relative mobilities of the species of Mkp1-HA which co-immunoprecipitated with Sty1-myc in Fig. 3 with those of the λ phosphatase-sensitive Mkp1 protein species (Fig. 4A), we conclude that the upper of the two major Mkp1 species associated with Sty1 is phosphorylated. Thus, both unphosphorylated and phosphorylated forms of Mkp1 associate with Sty1 under these conditions.

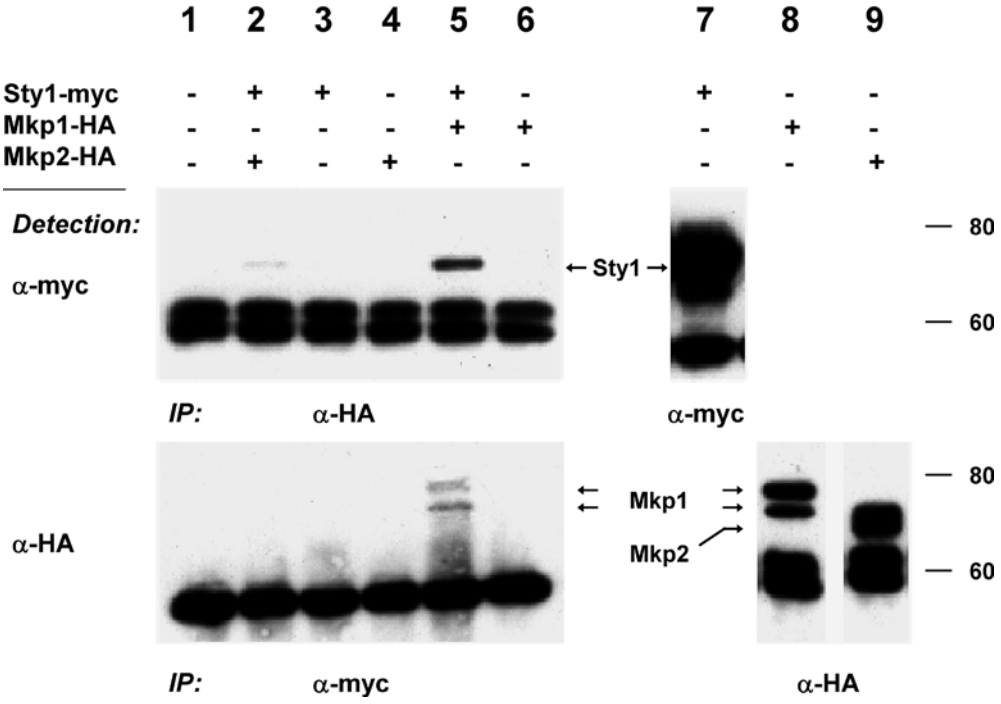


Fig. 3 Co-immunoprecipitation of Sty1 with Mkp1 and Mkp2. Cells expressing Sty1-myc$_{(9)}$, Mkp1-HA$_{(3)}$, and/or Mkp2-HA$_{(3)}$ from their respective chromosomal promoters were grown in liquid EMM at 30°C with shaking until the A_{595nm} had reached 0.7–0.8. Native extracts were prepared as detailed in Materials and methods, and immunoprecipitated with the indicated monoclonal antibodies. The immunoprecipitates were electrophoresed on 8% SDS-PAGE gels and transferred to PVDF membranes, which were subsequently probed with the indicated antibodies. The *bars on the right* indicate positions of molecular weight markers (sizes are given in kDa). Bands representing immunoglobulins are present in all lanes in the lower part of each panel. Strains used: 1, 972 h^- (wild-type, no tags); 2, CM1; 3, EA56; 4, EA60; 5, CM3; 6, EA61; 7, EA56; 8, EA61; 9, EA60. The presence or absence of the respective protein tags is indicated above each lane; strains are listed in Table 1

Mkp1 is phosphorylated in a *sty1*$^+$-dependent fashion, and this modification disappears upon nitrogen starvation

In *S. cerevisiae*, the level of a highly phosphorylated species of Rck2 increases upon osmotic shock, leading to activation of the HOG pathway; this phosphorylation is dependent on the MAPK-encoding *HOG1* gene (Bilsland-Marchesan et al. 2000). Since Mkp1 and Mkp2 physically associate with Sty1, it is reasonable to expect that they too will be phosphorylated by Sty1 when this MAPK cascade is activated. Indeed, it has recently been shown that Mkp2 is phosphorylated by Sty1 under oxidative stress conditions (Sanchez-Piris et al. 2002). As seen in Fig. 4A (lane 1), in cells growing under normal vegetative conditions, two major bands of Mkp1 appear, with about equal intensities. Phosphatase treatment identifies the upper band as phosphorylated, as it disappears after treatment with λ phosphatase, leaving only the lower band (lane 3). In *sty1*Δ cells, the upper, phosphorylated, band is absent (lane 2), demonstrating that it is dependent on Sty1. The total amount of Mkp1 also appears reduced in *sty1*Δ cells. Overexpression of Mkp1, on the other hand, results not only in a higher total amount of Mkp1 protein, but, notably, in a predominance of the phosphorylated species (lane 6).

Nitrogen limitation is a potent inducer of the meiotic programme in *S. pombe*, causing cells to accumulate in G1 and initiate conjugation and meiosis. Because of the phenotypes related to meiosis in cells lacking or overexpressing Mkp1, we wanted to investigate the phosphorylation status of Mkp1 under nitrogen starvation. In Fig. 4B, it can be seen that phosphorylated Mkp1 rapidly disappears after transfer to nitrogen-free medium. At 1 h after transfer, all of the phosphorylated Mkp1 still remains. By 1.5 h, dephosphorylation has clearly begun, and at 2 h, almost all of the phosphorylated species has disappeared, and only unphosphorylated Mkp1 remains throughout the rest of the period tested. A similar ratio of phosphorylated to unphosphorylated Mkp1 in logarithmically growing cells was seen in h^{90} cells expressing Mkp1 tagged with GFP, and the kinetics of disappearance of phosphorylated Mkp1 under conditions of nitrogen limitation were the same (data not shown).

Overexpression of *mkp1*$^+$ does not abolish *ste11*$^+$ expression upon nitrogen starvation

The transcription factor Ste11 is responsible for the activation of a number of genes that are essential for mating and meiosis, including *mei2*$^+$ and the genes *mat1-Pc* and *mat1-Mc* (Sugimoto et al. 1991). The *ste11*$^+$ gene is induced by nitrogen starvation, but this induction is absent in *sty1* mutants, which are also

Table 1 *S. pombe* strains

Strain	Genotype	Source/reference
EA34	h^{90}	Wild type
EA31	h^{90} *mkp1::ura4*$^+$ *ura4-D18*	This work
EA99	h^{90} *mkp2::KanMX6*	This work
EA64	h^{90} *KanMX6:nmt1-P3:HA*$_{(3)}$*:mkp1*$^+$	This work
EA45	h^{90} *KanMX6:nmt1-P3:HA*$_{(3)}$*:mkp2*$^+$	This work
EA69	h^{90} *mkp1*$^+$*:GFP:KanMX6*	This work
EA66	h^{90} *mkp2*$^+$*:GFP:KanMX6*	This work
972	h^-	Wild type
EA1	h^-*his3 leu1–32 ura4-D18*	P. Russell
EA18	h^-*mkp1::ura4*$^+$ *his3 leu1-32 ura4-D18*	This work
EA98	h^-*mkp2::KanMX6*	This work
EA56	h^-*sty1*$^+$*:myc*$_{(9)}$*:ura4*$^+$ *leu132 ura4D-18 ade6-M210*	J. Millar (JM1698)
CM1	h^-*sty1* $^+$*:myc*$_{(9)}$*:ura4*$^+$*mkp2* $^+$*:HA*$_{(3)}$*:KanMX6 leu132 ura4D-18 ade6-M210*	This work
CM3	h^-*sty1*$^+$*:myc*$_{(9)}$*:ura4*$^+$ *mkp1*$^+$*:HA*$_{(3)}$*:KanMX6 leu1-32 ura4D-18 ade6-M210*	This work
EA61	h^-*mkp1* $^+$*:HA*$_{(3)}$*:KanMX6 leu1-32*	This work
EA60	h^-*mkp2*$^+$*:HA*$_{(3)}$*:KanMX6 leu1-32*	This work
CM9	h^-*KanMX6:nmt1-P3:HA*$_{(3)}$*:mkp1*$^+$	This work
CM10	h^-*KanMX6:nmt1-P3:HA*$_{(3)}$*:mkp2*$^+$	This work
EA79	h^-*mkp2*$^+$*:GFP:KanMX6*	This work
EA82	h^-*mkp1*$^+$*:GFP:KanMX6*	This work
EA36	h$^-$cdc25-*22 leu1-32*	S. Moreno
EA87	h$^-$mkp1$^+$*:HA*$_{(3)}$*:KanMX6 cdc25-22 leu1-32*	This work
EA95	h^-*mkp1*$^+$*:HA*$_{(3)}$*:KanMX6 leu1-32 ura4-D18*	This work
EA100	h$^-$mkp1$^+$*:HA*$_{(3)}$*:KanMX6sty1::ura4*$^+$ *ura4D-18 leu1-32*	This work

defective in meiosis (Shiozaki and Russell 1996). The requirement for Sty1 for *ste11*$^+$ expression is mediated through the transcription factor Atf1/Pcr1 (Kon et al. 1998). Since overexpression of *mkp1*$^+$ causes a delay in meiosis, and since Sty1 is required for entry into meiosis, we wanted to see if the delay could be ascribed to a lack of expression of *ste11*$^+$. In Fig. 5, it is seen that *ste11*$^+$ induction is somewhat delayed in cells overexpressing *mkp1*$^+$, but that the transcript is clearly induced to essentially wild-type levels following nitrogen starvation, in contrast to the case in *sty1* mutants.

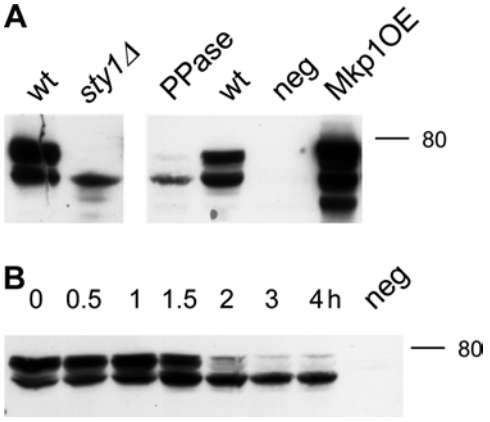


Fig. 4A, B Phosphorylation of Mkp1 in vivo. Cells expressing HA-tagged Mkp1 were grown in liquid medium as detailed below. Protein samples were processed for analysis by Western blotting, and probed with α-HA antibodies. **A** Phosphorylated and unphosphorylated species of Mkp1. From left to right: logarithmically growing wild-type cells (EA61; *mkp1*$^+$*:HA*$_{(3)}$) in normal EMM medium; *sty1*Δ (EA100; *mkp1*$^+$*:HA*$_{(3)}$ *sty1::ura4*$^+$); as in lane 1 but treated with 400 U of λ phosphatase (New England Biolabs) at 37°C for 15 min.; same as in lane 1; negative control without HA tag (972 h^-); Mkp1 overexpression (CM9; *nmt1-P3:HA*$_{(3)}$*:mkp1*$^+$), cells grown in the absence of thiamine to ensure maximal induction of the *nmt1* promoter. **B** Mkp1 phosphorylation status during nitrogen starvation. Wild-type h^- cells (EA61; *mkp1*$^+$*:HA*$_{(3)}$) were grown to mid-logarithmic phase in normal EMM and then transferred to nitrogen-free EMM medium as described in the legend to Fig. 2C. Samples were taken at the indicated times thereafter. The last lane shows a negative control without the HA tag (972 h^-)

Overexpression of Mkp1 causes elongation of mitotic cells

It has previously been shown that *sty1* cells are elongated and the G2 phase of the cell cycle is prolonged (Millar et al. 1995; Shiozaki and Russell 1995a; Kato et al. 1996). When Mkp1 is ectopically expressed from the fully induced *nmt1-P3* promoter for an extended period, the strain displays a marked increase in average cell length, as well as in length at septation (Fig. 6). The converse situation, an *mkp1*Δ background, does not have such an obvious effect on cell length, nor does up-regulation or deletion of *mkp2*$^+$ have a noticeable influence in this regard (not shown).

Mkp1 phosphorylation decreases as cells enter mitosis

The G2/M transition in the mitotic cell cycle is regulated through multiple phosphorylation events, including phosphorylation of Cdc2 on Tyr 15. Our observation that overexpression of Mkp1, like a *sty1* mutation, causes cell elongation, and the fact that phosphorylation of Mkp1 is Sty1-dependent, led us to examine the level

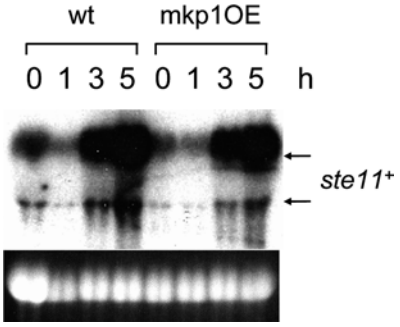


Fig. 5 Expression of *ste11*$^+$ after nitrogen starvation in wild-type cells and cells overexpressing *mkp1*$^+$. Cells growing logarithmically in liquid EMM at 30°C were washed and transferred to nitrogen-free EMM at 25°C; cultivation was continued under these conditions for the indicated times. Ectopic overexpression of *mkp1*$^+$ from the *nmt1-P3* promoter was maximised before and after nitrogen starvation by omitting thiamine from the culture medium. RNA was prepared from samples taken at the indicated times after transfer. A probe for *ste11*$^+$ was made by PCR using the primers Ste11-F (5′-AGCTTTATTAGCTCCCGCTC-3′) and Ste11-R (5′-ACTTTGCTCACTCACGGTTC-3′). Lanes 1–4, wild-type cells (972 *h*$^-$); lanes 5–8, cells overexpressing *mkp1*$^+$ (CM9, *h*$^-$ *nmt1-P3:HA*$_{(3)}$*:mkp1*$^+$). *Lower panel* Loading controls (28S ribosomal RNA) stained with ethidium bromide

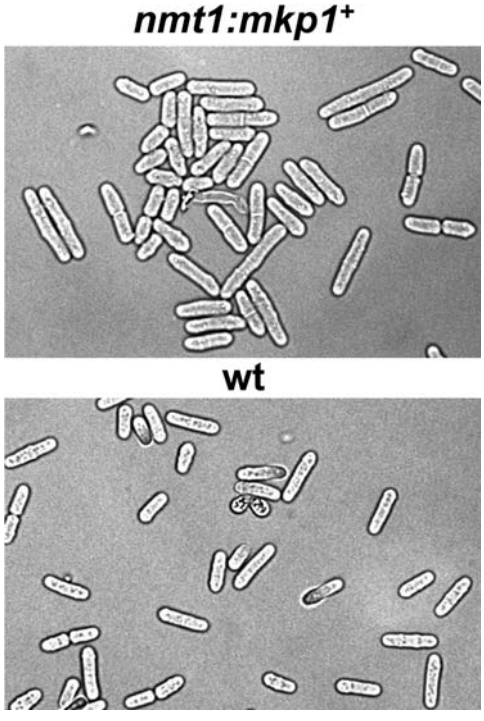


Fig. 6 Elongation of cells overexpressing Mkp1. Cells were grown in liquid EMM in the absence of thiamine (maximal induction of the *nmt1-P3* promoter) at 30°C for 48 h, and bright-field micrographs were taken. *Upper panel* CM9 (expressing Mkp1 from the strong *nmt1-P3* promoter). *Lower panel* Wild-type (972 *h*$^-$)

and phosphorylation status of Mkp1 during the mitotic cell cycle. In Fig. 7, the result of a *cdc25* block and release experiment is shown. The total amount of Mkp1 does not change appreciably during the mitotic cell cycle. However, at time points 30 and 180 min after shift-

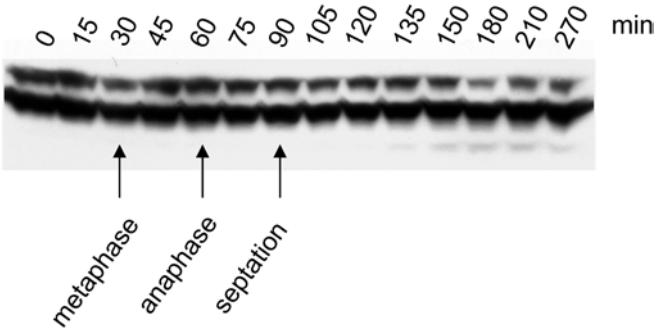


Fig. 7 Expression and phosphorylation status of Mkp1 during the mitotic cell cycle. *mkp1-HA cdc25-22* cells (EA87) grown in liquid EMM were synchronised in G2 by incubation for 3 h at 35°C, and then released from the block by lowering the temperature to 25°C. Samples were taken at the indicated times thereafter and immediately placed on ice. From these, denatured protein extracts were prepared and subjected to Western analysis with α-HA antibodies. An aliquot of each sample was stained with DAPI and analysed by fluorescence microscopy to determine the the cell cycle stage (indicated in the *lower* panel)

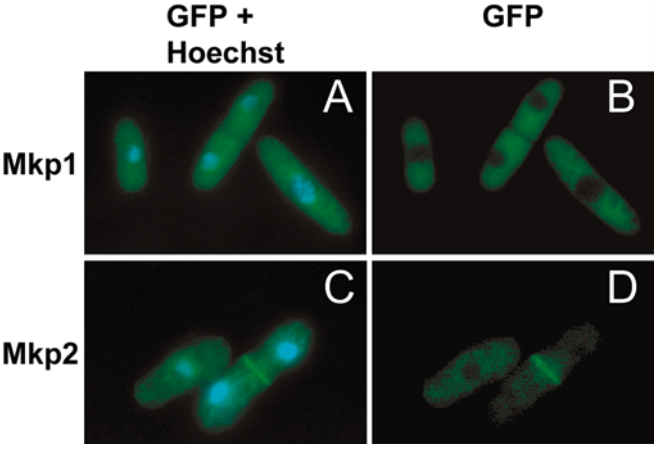


Fig. 8 Localisation of Mkp1-GFP and Mkp2-GFP in vegetative cells. Cells carrying constructs expressing *mkp1*$^+$*:GFP*(EA82) or *mkp2*$^+$*:GFP*(EA79) from their respective endogenous chromosomal loci in a *h*$^-$ background were grown to mid-logarithmic phase in normal EMM at 30°C and examined by fluorescence microscopy

down, there is a clear decrease in the upper band, representing phosphorylated Mkp1. These timepoints are coincident with the two metaphases of the cycles represented in this experiment. The decrease is only transient, as normal phosphorylation levels reappear in the following samples, corresponding to anaphase.

Localisation of Mkp1 and Mkp2 in vegetative cells

We constructed gene fusions expressing green fluorescent protein (GFP) C-terminally linked to Mkp1 or Mkp2, integrated at their respective chromosomal locus and under control of their endogenous promoters. The sub-cellular localisation of Mkp1-GFP and Mkp2-GFP was examined by fluorescence microscopy (Fig. 8).

For Mkp1, the overall fluorescent signal is somewhat stronger than for Mkp2. Mkp1-GFP is seen in the cytoplasm, and is clearly excluded from the nucleus (Fig. 8A, B).

In non-septated cells, a weak signal from Mkp2-GFP is seen in the cytoplasm, excluding the nucleus (Fig. 8C, D). This exclusion is less pronounced than for Mkp1, however. In post-mitotic cells, we observe a distinct signal from Mkp2 at the newly formed septa.

Localisation of Mkp1 and Mkp2 in meiotic cells

Because of the effects of the *mkp1*$^+$ gene on meiosis, we investigated the status of the Mkp1 and Mkp2 proteins under conditions conducive to meiosis. The Mkp1-GFP and Mkp2-GFP fusion proteins were also expressed in meiotic cells by integrating the same DNA constructs as above into the corresponding loci in the genome of wild-type h^{90} cells. Cells were induced to enter meiosis by cultivation in liquid nitrogen-free medium, and samples were taken at various time-points.

In Fig. 9A and B, a bright fluorescent signal is seen in vegetative and early meiotic cells expressing Mkp1-GFP. This represents a considerable increase over that seen in cells grown in rich medium. No equivalent accumulation was seen for Mkp2-GFP (Fig. 9D, E). The localisation of Mkp1 in conjugating and early meiotic cells resembles that in vegetative cells, the protein is found in the cytoplasm surrounding, but not in, the nucleus. This includes cells undergoing karyogamy (marked with an arrow in Fig. 9A). For Mkp2, although there was no clear accumulation during conjugation and meiosis, we did observe a translocation of the protein. Whereas Mkp2 is diffusely distributed in the cytoplasm in vegetative cells, it concentrates near the nuclear rim in cells during the late stages of meiosis (marked an arrow in Fig. 9E). This suggests an association with the structure known as the forespore membrane (Nakamura et al. 2001).

After completion of meiosis, when spores have formed, a marked difference in distribution between the two proteins is again seen. A very bright signal from Mkp1-GFP is seen within spores in tetrads (Fig. 9C). Mkp2, by contrast, does not accumulate to similar levels in asci. Instead, a dim signal from Mkp2-GFP is seen surrounding spores (Fig. 9F).

Discussion

The role of Mkp1 in the meiotic and mitotic cell cycles

In Fig. 10, we present a formal model for the action of Mkp1 on meiosis. Its key feature is that the phosphorylated form of Mkp1 (Mkp1-P) has a negative or delaying effect on conjugation and meiosis. The model is consistent with the following observations: (1) Mkp1-P disappears completely between 1 and 2 h of nitrogen starvation, leaving only unphosphorylated Mkp1 (this study); (2) Sty1 is phosphorylated on Tyr within 1 h of nitrogen starvation (Shiozaki and Russell 1996); (3) in an *mkp1*Δ mutant, Mkp1-P is absent, so the meiotic pathway is free to proceed faster and under conditions where it is normally restrained (this study); (4) in cells that overexpress Mkp1, there is an overabundance of Mkp1-P, leading to a delay in the meiotic programme (this study); (5) in a *sty1*Δ mutant, mating and meiosis are blocked even though Mkp1-P is absent, because Sty1 is also required for activation of *ste11*$^+$ through the transcription factor Atf1 (Kon et al. 1998).

Another piece of evidence that supports this view is that *ste11*$^+$ induction still occurs after nitrogen

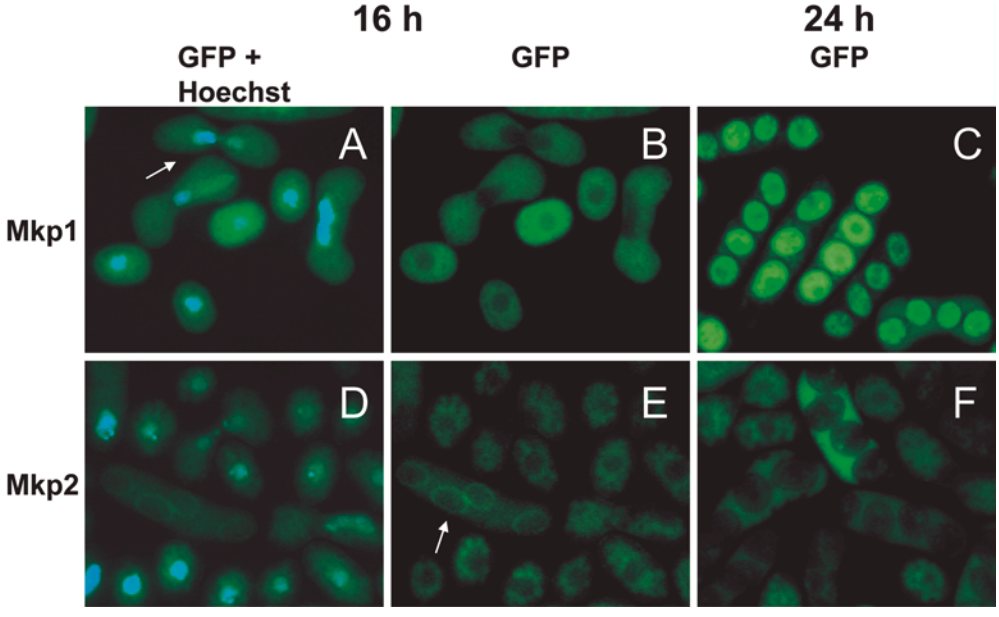


Fig. 9 Localisation of Mkp1-GFP and Mkp2-GFP in meiotic cells. Cells carrying constructs expressing *mkp1*$^+$*:GFP* (EA69) or *mkp2*$^+$*:GFP* (EA66) from their respective endogenous chromosomal loci in a h^{90} (meiosis-proficient) background were grown to mid-logarithmic phase in normal EMM at 30°C, then transferred to nitrogen-free EMM at 25°C and examined by fluorescence microscopy at the indicated times after transfer. Since the DNA stain Hoechst 33258 does not enter spores under these conditions, only GFP was visualised in the images made 24 h post-transfer

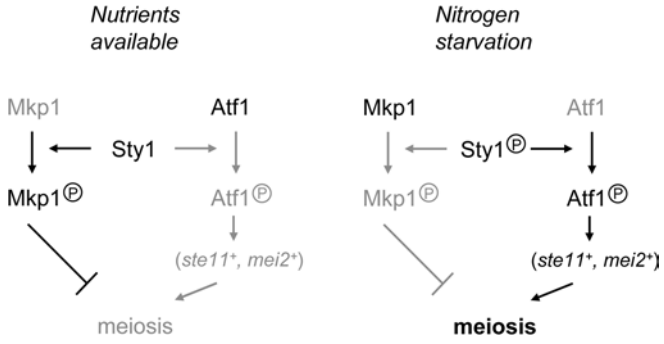


Fig. 10 Model for the action of Sty1 on Mkp1 and the Atf1 pathway. In this scheme, "meiosis" refers to the entire process from G1 arrest through mating, meiosis and sporulation. Components that are present or active under a particular condition are marked in *black*, inactive or absent components are shaded *grey*. →, Activating; ⊣, deactivating

starvation in cells overexpressing Mkp1. In wild-type cells this induction requires Sty1, and is known to occur through the action of Atf1. The Mkp1 and Atf1 branches of Sty1 action would then be relatively independent of each other. The difference with respect to meiosis between the situation in a *sty1*Δ mutant and that in cells overexpressing *mkp1*$^+$ is further underscored by the fact that meiosis is completely inhibited in the former. In the latter case, however, it does proceed, although with a delay, and the level of meiotic products ultimately reaches that seen in the wild-type strain (Fig. 2B, C). This is directly paralleled by the behaviour of *S. cerevisiae rck1*Δ/*rck1*Δ mutants, where meiosis proceeds more rapidly than in wild-type cells but reaches a plateau at the same level (Ramne et al. 2000).

A prediction of the model is that under conditions of nitrogen starvation, Sty1 shifts its phosphorylating activity from Mkp1 to Atf1 (and to other targets required for G1 arrest and commitment to the meiotic pathway). It should be noted that Sty1 is activated by Wis1-dependent Tyr phosphorylation upon nitrogen starvation (Shiozaki and Russell 1996); apparently this event leads not to increased, but instead to decreased, phosphorylation of Mkp1.

This could occur by a variety of mechanisms. Given the substantial fraction of Sty1 apparently associated with Mkp1, this switch could occur if Mkp1 were dephosphorylated (by the increased action of a phosphatase or by reduced Sty1 activity towards Mkp1), and this caused the dissociation of Sty1 from Mkp1, freeing it to interact with Atf1 instead. There is no support for this idea from our co-immunoprecipitation experiments, however. Sty1 seems to bind at least as strongly to unphosphorylated Mkp1 as to Mkp1-P (Fig. 3, lower panel, lane 5).

The targets of Mkp1 responsible for its inhibitory effect on meiosis are not known. However, it is very likely that at least one of them is involved in the very first steps of commitment to the meiotic pathway, since dephosphorylation of Mkp1 occurs at an early stage in this process, at 2 h after the onset of nitrogen starvation. This is when the decision is taken to arrest in G1 and enter the meiotic pathway. Importantly, this dephosphorylation (as well as induction of *ste11*$^+$) occurs also in a pure *h*$^-$ population, which is incompetent for mating and meiosis. It is possible that the effects of Mkp1 observed here all occur within the mitotic cell cycle and in stages preceding commitment to meiosis. Of course, this does not exclude the possibility that there may be additional, later, targets in the meiotic pathway yet to be identified. The effect of Mkp1 on meiosis is not restricted to conditions of nitrogen limitation. The balance point for entry into the meiotic programme with respect to glucose availability is also shifted in *mkp1*Δ cells, as they are able to produce spores at higher glucose concentrations than wild-type cells. The picture one gets from these observations is that Mkp1, or its upstream regulator Sty1, integrates signals from different sources in the decision to proceed into meiosis.

Another scenario in which the model presented in Fig. 10, with separate actions of Mkp1 and Atf1, may have some relevance, is the G2 to M transition in the mitotic cell cycle. Disruption of *sty1*$^+$, like overexpression of *mkp1*$^+$, leads to elongation of cells. For *sty1*Δ cells, this has been coupled to a delay in G2 (Millar et al. 1995; Shiozaki and Russell 1995a; Kato et al. 1996). It should be noted that mutation or overexpression of neither Atf1 nor Pap1, the transcription factors known to be directly or indirectly regulated by Sty1, affects cell elongation (Shiozaki and Russell 1996; Takeda et al. 1995; Wilkinson et al. 1996). Thus, this effect is likely to be mediated by factors other than Atf1 or Pap1. We show here that *mkp1*$^+$ overexpression results in cell elongation. This again is paralleled by the effects of the *S. cerevisiae RCK1* and *RCK2* genes, homologues of *mkp1*$^+$ and *mkp2*$^+$. Ectopic expression of these genes in *S. pombe* causes considerable elongation of cells (Dahlkvist et al. 1995).

We have demonstrated that a transient drop in Mkp1 phosphorylation coincides with mitotic metaphase. At this stage in the cell cycle, MPF activity is high, whereas in anaphase, when Mkp1 phosphorylation reappears, MPF activity is low. A speculative model for the mechanism underlying the impact of Mkp1 on the efficiency of meiosis would be that Mkp1-P acts as a brake on mitotic cell cycle progression in G2. In a population of cells overexpressing Mkp1, there would be an accumulation of cells in G2, and so fewer cells would be available for immediate entry into meiosis. Conversely, in *mkp1*Δ cells, the diminished fraction of cells in G2 leads to a higher proportion of G1 cells, ready to embark on the meiotic pathway. It should be emphasised, however, that our data are compatible with models in Mkp1 acts also at G1 or further down the meiotic pathway. The increased intensity and distinctive localisation of Mkp1-GFP at later time points in meiosis are certainly consistent with this possibility.

The nature of the interaction between the MAPK Sty1 and Mkp1/2

There appears to be quite a tight association between Sty1 and Mkp1, since a considerable fraction of Sty1 is co-precipitated with Mkp1 and vice versa, when both proteins are expressed from their endogenous promoters. This view is reinforced by the finding that Mkp1 is constitutively phosphorylated; in our hands about half of total Mkp1 is in the form of Mkp1-P. By contrast, we find only a weak interaction between Sty1 and Mkp2. Recently, Sanchez-Piris et al. (2002) demonstrated an interaction between these two proteins by overexpressing both of them in the same cells. We can detect Sty1 co-immunoprecipitated with Mkp2 when both proteins are expressed at normal levels from their endogenous promoters at their normal chromosomal loci; however, the signal is faint. Since Mkp1 and Mkp2 are expressed at similar levels in wild-type cells under standard conditions, it follows that under normal, unstressed, conditions, Sty1 binds to Mkp2 with only a small fraction of the affinity that it has for Mkp1.

We have shown here that the *S. pombe* proteins Mkp1 and Mkp2, homologues of Rck1 and Rck2, bind to the MAPK Sty1. Of course, this does not exclude potential additional regulatory mechanisms for Mkp1 and Mkp2. Grouping of eukaryotic protein kinase sequences places the Rck/Mkp subfamily close to Ca^{2+}/calmodulin (CaM)-regulated kinases (Dahlkvist and Sunnerhagen 1994; Melcher and Thorner 1996; Teige et al. 2001), yet experimental evidence supporting regulation of Rck1 or Rck2 from *S. cerevisiae*, or of *S. pombe* Mkp2 by Ca^{2+}/CaM is lacking (Melcher and Thorner 1996; Ramne et al. 2000; Sanchez-Piris et al.2002). It should be noted that a bona fide Ca^{2+}/CaM protein kinase from *S. pombe*, Cmk1, has already been described. There is clear evidence that this protein both binds to and is activated by Ca^{2+}/CaM, in contrast to the Rck/Mkp subfamily (Rasmussen 2000). Mkp2 has both sequence features typical of the Rck subfamily, whereas Cmk1 lacks both (Fig. 1A). The contention that Mkp2 is more closely related to the Rck/Mkp subfamily than to CaM-regulated kinases, on the one hand, and that *S. pombe* Cmk1 is more related to the *S. cerevisiae* CaM-regulated kinases (Cmk1 and Cmk2) on the other, is also supported by the clustering based on sequence similarity (Fig. 1B).

How many MAPKAPKs are there in yeast?

Although a relatively large number of protein kinases with putative MAPK-binding domains are found in plant, animal and fungal species, so far we have only identified the "glycine loop" in the four members of the Rck subfamily. Do these four then make up the whole set of MAPKAPKs in *S. pombe* and *S. cerevisiae*? Using less stringent homology criteria, excluding the "glycine loop" but including a protein kinase domain and a sequence similar to a MAPK binding box at the C-terminus, one can find between five and eight candidate genes in each of the yeast genomes. Only experiment can tell if any of these other candidates correspond to actual MAPKAPKs. Until we know the number of MAPKAPKs in yeast, we cannot tell if all MAPKAPKs are specific to one MAPK cascade, or if one or a few MAPKAPKs integrate the responses of several MAPK cascades.

We have found both Mkp1, and, to a lesser degree, Mkp2, to be physically associated with Sty1. It is not unreasonable to suppose that both these putative MAPKAPKs are linked to the same MAPK, given the following considerations regarding the situation in *S. cerevisiae*. Rck2 has been shown to interact with Hog1 by co-immunoprecipitation as well as two-hybrid analysis (Bilsland-Marchesan et al. 2000). In global screens for protein-protein interactions, Hog1 has been found to bind to Rck1, using both two-hybrid analysis (Uetz et al. 2000) and affinity tag purification/mass spectrometry (Ho et al. 2002). In addition, an interaction between Rck1 and Fus3 has been reported (Ho et al. 2002). Thus, Rck1 potentially binds two MAPKs, Hog1 and Fus3. The *RCK1* transcript is strongly upregulated in *fus3* mutants and after treatment with α pheromone (Roberts et al. 2000), hinting at a role for Rck1 in the mating pathway. By analogy, one may speculate that Mkp1 could interact with Spk1, the Fus3 homologue. In fission yeast, the Sty1 cascade, as well as Spk1, has a major impact on mating and meiosis. The same is not true for its budding yeast homologue, the Hog1 MAPK cascade. It is conceivable that Mkp2, besides its weak interaction with Sty1, has another MAPK interaction partner; at this stage we cannot speculate about likely candidates.

Additional functions of Mkp1 and Mkp2

Besides its effect on mating and meiosis, Sty1 is also required for the activation of responses to osmotic and oxidative stress conditions. Mutation of the *sty1*$^+$ gene causes sensitivity to a wide range of oxidative and DNA-damaging agents, as well as to osmotic shock (Millar et al. 1995; Shiozaki and Russell 1995a; Kato et al. 1996; Degols and Russell 1997). Its homologue in budding yeast, *HOG1*, is required for survival under osmotic stress (Brewster et al. 1993). Recently, *mkp2* mutants were shown to be moderately sensitive to arsenite (Sanchez-Piris et al.2002). From the similarity of the meiotic phenotypes, one gets the impression that Mkp1 is more similar to Rck1 than to Rck2; however, as mentioned above, sequence similarities do not yield a clear-cut confirmation of this. In a limited search for phenotypes related to osmotic and oxidative stress in mkp1 mutants, none were found (not shown). In *S. cerevisiae*, no phenotypes coupled to osmotic stress survival were detected in *rck1*, *rck2*, or *rck1 rck2* disruption mutants (Dahlkvist and Sunnerhagen 1994;

Bilsland-Marchesan et al. 2000). Only the expression of a dominant-negative kinase-dead *RCK2* mutant allele confers sensitivity to osmotic stress (Bilsland-Marchesan et al. 2000; E. Bilsland-Marchesan, S. Swaminathan and Z. Åkerblom, unpublished data). Thus, such phenotypes may well be revealed by further investigations of *mkp1* mutants.

The different localisation patterns of Mkp1 and Mkp2 suggest that they have divergent roles in the cell, despite their sequence similarity. The localisation of Mkp1 and Mkp2 in vegetative cells, in the cytoplasm and excluded from the nucleus, is consistent with that of *S. cerevisiae* Rck2 (Melcher and Thorner 1996; Ramne et al. 2000). In meiotic cells, their patterns are clearly dissimilar. Mkp1 increases in overall intensity at later stages of meiosis, and localises to the interior of forming spores. One may speculate that Mkp1 is necessary for spore survival or germination. It is interesting to note that the proteins Psy1, a fission yeast syntaxin homolog, and Spo3 both localise near septa during vegetative growth, but relocate to forespore membranes during meiosis (Nakamura et al. 2001). This is the same localisation pattern as we have observed for Mkp2 in mitotic and meiotic cells, and may reflect a role for this MAPKAP kinase homologue in the organisation of these structures.

Future work with Mkp1 and Mkp2, and their homologues in budding yeast, will aim at identifying critical cellular targets of these protein kinases in the context of their roles in the meiotic and mitotic cell cycles, as well as in stress responses.

Note added in proof. Independently of our work, the gene mkp1+ has recently been described as srk1+ (Smith DA, Toone WM, Chen D, Bähler J, Jones N et al. (2002) The Srk1 protein kinase is a target for the Sty1 stress-activated MAPK in fission yeast. J Biol Chem 277:33411–33421).

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References

Bilsland-Marchesan E, Ariño J, Saito H, Sunnerhagen P, Posas F (2000) Rck2 kinase is a substrate for the osmotic-stress activated MAP kinase Hog1. Mol Cell Biol 20:3887–3895

Bähler J, Wu JQ, Longtine MS, Shah NG, McKenzie A, 3rd et al. (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast 14:943–951

Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC (1993) An osmosensing signal transduction pathway in yeast. Science 259:1760–1763

Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. Nature 410:37–40

Dahlkvist A, Sunnerhagen P (1994) Two novel deduced serine/threonine protein kinases from *Saccharomyces cerevisiae*. Gene 139:27–33

Dahlkvist A, Kanter-Smoler G, Sunnerhagen P (1995) The *RCK1* and *RCK2* protein kinase genes from *Saccharomyces cerevisiae* suppress cell cycle checkpoint mutations in *Schizosaccharomyces pombe*. Mol Gen Genet 246:316–326

Degols G, Russell P (1997) Discrete roles of the Spc1 kinase and the Atf1 transcription factor in the UV response of *Schizosaccharomyces pombe*. Mol Cell Biol 17:3356–63

Degols G, Shiozaki K, Russell P (1996) Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. Mol Cell Biol 16:2870–2877

Gachet Y, Tournier S, Millar JB, Hyams JS (2001) A MAP kinase-dependent actin checkpoint ensures proper spindle orientation in fission yeast. Nature 412:352–355

Gutz H, Heslot H, Leupold U, Loprieno N. 1974. *Schizosaccharomyces pombe*. *In* King RC (ed) Handbook of genetics, Vol. 1: Bacteria, bacteriophages, and fungi. Plenum, New York, pp. 395–446.

Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L et al. (2002) Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature 415:180–183

Hunter T (1991) Protein kinase classification. Meth Enzymol 200:3–37

Kato T Jr, Okazaki K, Murakami H, Stettler S, Fantes PA, Okayama H (1996) Stress signal, mediated by a Hog1-like MAP kinase, controls sexual development in fission yeast. FEBS Lett 378:207–12

Kimura M, Takahata N (1983) Selective constraint in protein polymorphism: study of the effectively neutral mutation model by using an improved pseudosampling method. Proc Natl Acad Sci U S A 80:1048–52

Kon N, Schroeder SC, Krawchuk MD, Wahls WP (1998) Regulation of the Mts1-Mts2-dependent *ade6-M26* meiotic recombination hot spot and developmental decisions by the Spc1 mitogen-activated protein kinase of fission yeast. Mol Cell Biol 18:7575–7583

Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C et al. (1999) MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. Nat Cell Biol 1:94–97

Kunitomo H, Sugimoto A, Wilkinson CR, Yamamoto M (1995) *Schizosaccharomyces pombe pac2*$^+$ controls the onset of sexual development via a pathway independent of the cAMP cascade. Curr Genet 28:32–38

Kunitomo H, Higuchi T, Iino Y, Yamamoto M (2000) A zinc-finger protein, Rst2p, regulates transcription of the fission yeast *ste11*$^+$ gene, which encodes a pivotal transcription factor for sexual development. Mol Biol Cell 11:3205–3217

Kyriakis JM, Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81:807–869

Li P, McLeod M (1996) Molecular mimicry in development: identification of ste11+ as a substrate and mei3+ as a pseudosubstrate inhibitor of ran1+ kinase. Cell 87:869–880

Melcher ML, Thorner J (1996) Identification and characterization of the *CLK1* gene product, a novel CaM kinase-like protein kinase from the yeast *Saccharomyces cerevisiae*. J Biol Chem 271:29958–29968

Millar JB (1999) Stress-activated MAP kinase (mitogen-activated protein kinase) pathways of budding and fission yeasts. Biochem Soc Symp 64:49–62

Millar JB, Buck V, Wilkinson MG (1995) Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. Genes Dev 9:2117–2130

Moreno S, Klar A, Nurse P (1991) Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. Meth Enzymol 194:795–823

Nakamura T, Nakamura-Kubo M, Hirata A, Shimoda C (2001) The *Schizosaccharomyces pombe spo3*$^+$ gene is required for assembly of the forespore membrane and genetically interacts with *psy1*$^+$ encoding syntaxin-like protein. Mol Biol Cell 12:3955–72

Neiman AM, Stevenson BJ, Xu HP, Sprague GF, Jr., Herskowitz I et al. (1993) Functional homology of protein kinases required for sexual differentiation in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* suggests a conserved signal transduction module in eukaryotic organisms. Mol Biol Cell 4:107–120

Neufeld B, Grosse-Wilde A, Hoffmeyer A, Jordan BW, Chen P, Dinev D, Ludwig S, Rapp UR (2000) Serine/Threonine kinases 3pK and MAPK-activated protein kinase 2 interact with the basic helix-loop-helix transcription factor E47 and repress its transcriptional activity. J Biol Chem 275:20239–20242

Ramne A, Bilsland-Marchesan E, Erickson S, Sunnerhagen P (2000) The protein kinases Rck1 and Rck2 inhibit meiosis in budding yeast. Mol Gen Genet 263:253–261

Rasmussen CD (2000) Cloning of a calmodulin kinase I homologue from *Schizosaccharomyces pombe*. J Biol Chem 275:685–690

Roberts CJ, Nelson B, Marton MJ, Stoughton R, Meyer MR et al. (2000) Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. Science 287:873–880

Sanchez-Piris M, Posas F, Alemany V, Winge I, Hidalgo E et al. (2002) The serine/threonine kinase Cmk2 is required for oxidative stress response in fission yeast. J Biol Chem 277:17722–17727

Shiozaki K, Russell P (1995a) Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature 378:739–743

Shiozaki K, Russell P (1995b) Counteractive roles of protein phosphatase 2C (PP2C) and a MAP kinase kinase homolog in the osmoregulation of fission yeast. EMBO J 14:492–502

Shiozaki K, Russell P (1996) Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. Genes Dev 10:2276–2288

Smith JA, Poteet-Smith CE, Malarkey K, Sturgill TW (1999) Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK in vivo. J Biol Chem 274:2893–2898

Smith JA, Poteet-Smith CE, Lannigan DA, Freed TA, Zoltoski AJ, Sturgill TW (2000) Creation of a stress-activated p90 ribosomal S6 kinase. The carboxyl-terminal tail of the MAPK-activated protein kinases dictates the signal transduction pathway in which they function. J Biol Chem 275:31588–31593

Stettler S, Warbrick E, Prochnik S, Mackie S, Fantes P (1996) The wis1 signal transduction pathway is required for expression of cAMP- repressed genes in fission yeast. J Cell Sci 109:1927–35

Sugimoto A, Iino Y, Maeda T, Watanabe Y, Yamamoto M (1991) *Schizosaccharomyces pombe ste11*$^{+}$ encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. Genes Dev 5:1990–1999

Takeda T, Toda T, Kominami K, Kohnosu A, Yanagida M, Jones N (1995) *Schizosaccharomyces pombe atf1*$^{+}$ encodes a transcription factor required for sexual development and entry into stationary phase. EMBO J 14:6193–6208

Tanoue T, Adachi M, Moriguchi T, Nishida E (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nat Cell Biol 2:110–116

Teige M, Scheikl E, Reiser V, Ruis H, Ammerer G (2001) Rck2, a member of the calmodulin-protein kinase family, links protein synthesis to high osmolarity MAP kinase signaling in budding yeast. Proc Natl Acad Sci USA 98:5625–5630

Toone WM, Kuge S, Samuels M, Morgan BA, Toda T, Jones N (1998) Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. Genes Dev 12:1453-63

Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS et al. (2000) A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature 403:623–627

Wilkinson MG, Samuels M, Takeda T, Toone WM, Shieh JC et al. (1996) The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. Genes Dev 10:2289–2301

Zhang Y, Zhong S, Dong Z, Chen N, Bode AM, Ma W (2001) UVA induces Ser381 phosphorylation of $p90^{RSK}$/MAPKAP-K1 via ERK and JNK pathways. J Biol Chem 276:14572–14580