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Identification of a novel Ser/Thr protein phosphatase Ppq1 as a negative regulator of mating MAP kinase pathway in *Saccharomyces cerevisiae*

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ABSTRACT

The specificity and efficiency of cell signaling is largely governed by the complex formation of signaling proteins. The precise spatio-temporal control of the complex assembly is crucial for proper signaling and cell survival. Protein phosphorylation is a key mechanism of signal processing in most of cell signaling networks. Phosphatases, along with kinases, control the phosphorylation state of many proteins and thus play a critical role in the precise regulation of signaling at each stage such as activation, propagation, and adaptation. Identification and functional analysis of pathway-specific phosphatase is, therefore, crucial for the understanding of cell signaling mechanisms. Here, we have developed a novel screening strategy to identify pathway-specific phosphatases, in which the entire repertoire of cell's phosphatases was tethered to a signaling complex and the changes in signaling response were monitored. As a model target, we have chosen the mating MAP kinase pathway in the budding yeast, which is composed of three kinases and Ste5 scaffold protein. Using this strategy, a putative Ser/Thr phosphatase, Ppq1, was identified to be mating-specific. Results show that Ppq1 down-regulates mating signaling by targeting at or upstream of the terminal MAP kinase Fus3 in the cascade. The catalytic activity of Ppq1 as a phosphatase was confirmed *in vitro* and is necessary for its function in the regulation of mating signaling. Overall, the data suggest that Ppq1 functions as a negative regulator of mating MAPK pathway by dephosphorylating target pathway protein(s) and plays a key role in the control of the background signaling noise.

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1. Introduction

When stimulated, cells go through a series of signaling processes such as activation, propagation and adaptation to generate corresponding cellular responses. The duration and magnitude of each signaling process should be precisely regulated for proper signaling. Such regulation is often mediated by phosphorylation of participating signaling proteins. Protein phosphorylation, the hallmark in the control of protein function, is a reversible and dynamic process. In many cellular signal transduction pathways, it is known that kinases are often responsible for the onset and/or activation of signaling while phosphatases are mainly involved in the adaptation and noise control of signaling [1,2]. Protein phosphatases remove covalently attached phosphate groups from Ser, Thr, and Tyr residues in target proteins. The phosphorylation state of key signaling proteins is determined by the delicate balance between these two enzyme activities, which is crucial for accurate signaling. Collapse of such balance often leads to disease states as can be seen from the progression of prostate cancer, in which adaptation step in Ras/MAP kinase pathway is abolished by constitutively active signaling proteins resulting in uncontrolled cell proliferation [3,4]. Thus, precise regulation of signal adaptation and noise control are critical for survival.

Despite the crucial role in signaling, the understanding of functional mechanisms of phosphatases, including identification and functional analysis of pathway-specific phosphatase, still remains elusive. As an enzyme, phosphatase has promiscuous characteristics such as broad substrate specificity and functional redundancy, compared to kinases [5]. These inherent properties made it difficult to identify and analyze the pathway-specific phosphatases, which is crucial for the understanding of signal adaptation mechanisms. Furthermore, the traditional approaches such as phenotypic analyses of knock-out alleles are not readily applicable to the study of phosphatases due to their functional redundancy in cellular physiology [6].

The MAP kinase signaling pathways are highly conserved in eukaryotes from yeast to human and are involved in many cellular responses from cell growth, differentiation, death and stress responses [7,8]. These pathways are composed of three kinases which are activated by sequential phosphorylation upon stimulation and are often found associated with scaffold proteins [9,10].

Abbreviations: MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; PP1, Phosphoprotein phosphatase 1; yEGFP, yeast enhanced green fluorescent protein; pNPP, p-nitrophenyl phosphate.

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One of the well-studied pathways is the mating MAP kinase pathway in the budding yeast, *Saccharomyces cerevisiae*. The pathway is composed of Ste11 (MAPKKK), Ste7 (MAPKK), Fus3 (MAPK) and Ste5 scaffold protein and is known to mediate the mating process between haploid yeast cells stimulated by mating pheromones [7]. The Ste5 scaffold protein is essential for mating signaling and is known to tether with three component kinases serving as a platform for signaling complex assembly [11,12]. Previous studies suggested that the precise spatio-temporal control of scaffold complex assembly is crucial for specific and efficient signaling, and scaffold-kinase docking interactions are mainly governed by a simple tethering mechanism [12].

In this study, a novel, yet simple approach is developed to identify pathway-specific phosphatases using the yeast mating MAP kinase pathway as a model target. The entire repertoire of phosphatases in yeast cells was collected and individually enforced in proximity to Ste5 complex via covalent tethering, and a decrease or loss of signaling output was monitored for identification of mating pathway-specific phosphatases. The limitations of traditional phosphatase researches were overcome by taking advantage of the fact that simple tethering mechanism of scaffold assembly is crucial for signaling. Using the screening, we have identified Ppq1, a putative Ser/Thr phosphatase, as a novel negative regulator of the mating MAP kinase pathway along with two previously known phosphatases, Msg5 and Ptp3 [13]. The results show that Ppq1 negatively controls the mating signaling and the catalytic activity of Ppq1 is required for its function, which is presumably targeted at or upstream of Fus3 kinase. These findings suggest that Ppq1 may play a key role in control of signaling noise by minimizing the basal level response.

2. Materials and methods

2.1. Strains, constructs and growth conditions

All yeast strains used in this study (Supplementary Table S1) were derived from the strain RB200, which is the wild-type W303a strain carrying the Fus1-LacZ reporter gene, using a PCR-based tagging technique [14]. The ORF's of yeast phosphatase genes were individually amplified by PCR (Supplementary Table S3) using the yeast genomic DNA as a template and were cloned into pRS-based pSH95 vector at BamHI and NotI sites to generate Ste5-phosphatase fusions. Plasmid constructs used in this study are listed in the Supplementary Table S2. Cells were grown at 30 °C in a YEPD or synthetic complete (SC) medium using glucose or galactose (2%, w/v) as a carbon source.

2.2. Cell lysis, immunoprecipitation and immunoblotting

Yeast cells at the mid-log phase (OD_{600} = 0.5 ~ 0.7) were treated with α-factor (1 ~ 10 μM) to trigger mating signaling response and were grown for various time periods prior to harvest. Cell harvest, lysis, immunoprecipitation and immunoblotting were performed as described [15]. The antibodies used in this study are as follows: anti-Fus3 (Santa Cruz Biotechnology), anti-phospho p44/42 (Cell Signaling Technology), anti-myc (Millipore), anti-HA (Santa Cruz Biotechnology), and anti-polyhistidine (Sigma–Aldrich) antibodies. Proteins were detected by a LAS-3000 (Fuji) imaging system using the West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer's instructions.

2.3. Mating analyses

Quantitative mating assay was performed by mixing RB201 cells harboring appropriate plasmids with IH1793 tester strain as described before [16]. For Fus1-lacZ transcription assay, cells at the mid-log phase were treated with α-factor (1 μM) for 2 h before harvest. Harvested cells were frozen in liquid nitrogen for β-galactosidase assay. The β-galactosidase activity was quantified by a standard protocol [16]. A peptide corresponding to α-factor was chemically synthesized using F-moc chemistry and was purified by HPLC. The halo assay for the yeast cells carrying appropriate plasmids was also performed as described previously [16]. The FACS analysis of mating response was performed with SH180 yeast cells carrying a yEGFP reporter gene whose expression was under control of the Fus1 promoter. Aliquots of SH180 cells treated with α-factor (1 μM) for 90 min followed by washing with a medium lacking α-factor. Stimulated cells were harvested at every 30 min up to 300 min followed by sonication for 3 s to separate cell clumps. The fluorescence intensity from approximately 10,000 cells was quantitatively measured using a FACSCalibur flow cytometer (BD Biosciences) equipped with a 488 nm blue laser source. The mating analyses described here were performed in triplicate.

2.4. Protein expression and purification from Escherichia coli

The recombinant His6-tagged proteins were expressed in Rosetta (DE3) pLysS cells with the treatment of IPTG (0.5 mM) for 30 min at 37 °C. Harvested cells were lysed by sonication on ice in PBS buffer containing PMSF (1 mM). The cleared cell lysate was applied to a Ni-Sepharose 6 resin (GE Healthcare) and His6-tagged proteins were purified according to the manufacturer's instructions and standard procedures.

2.5. In vitro phosphatase assay

Ppq1 protein purified from *E. coli* was incubated with pNPP substrate (2.5 mM) in the assay buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 2 mM $MnCl_2$ and 5 mM DTT) in the volume of 0.2 mL. After incubation at 37 °C for 30 min, phosphatase activity was quantified by measuring the absorbance at 405 nm followed by standard protocols. Lambda phosphatase (λPPase, Millipore) was used as a positive control in the assay.

3. Results

3.1. Screening of mating pathway-specific phosphatases

Identification of pathway-specific phosphatases is critical for understanding of functional mechanisms of cell signaling. Promiscuity is an inherent property of phosphatases due to broad substrate specificity and functional redundancy. Therefore, it has been challenging to screen or identify pathway-specific phosphatases using traditional approaches such as phenotypic analyses of gene deletion [6] or over-expression. Based on the fact that proper signaling is governed by the formation of signaling complex upon stimulation [12], we have developed a novel screening strategy to identify the pathway-specific phosphatases. In the screening, the entire repertoire of phosphatases in cell – phosphatome – was individually enforced in proximity to the signaling complex via a covalent tether to the pathway scaffold and a loss of or decrease in the signaling response was quantitatively monitored for each phosphatase. This strategy was applied to the mating MAP kinase pathway of the budding yeast, in which Ste5 scaffold protein is known to function as a platform for signaling complex assembly (Fig. 1A). All thirty-two phosphatases in yeast cells [6] were individually cloned and expressed as a fusion to Ste5 scaffold with a GS_{10} linker in a Δ*ste5* deletion strain (Fig. 1B). Quantitative mating assays were performed to monitor the change in mating signaling response for cells carrying each Ste5-phosphatases fusion protein. Among thirty-two screened, three phosphatases – Msg5, Ptp3 and

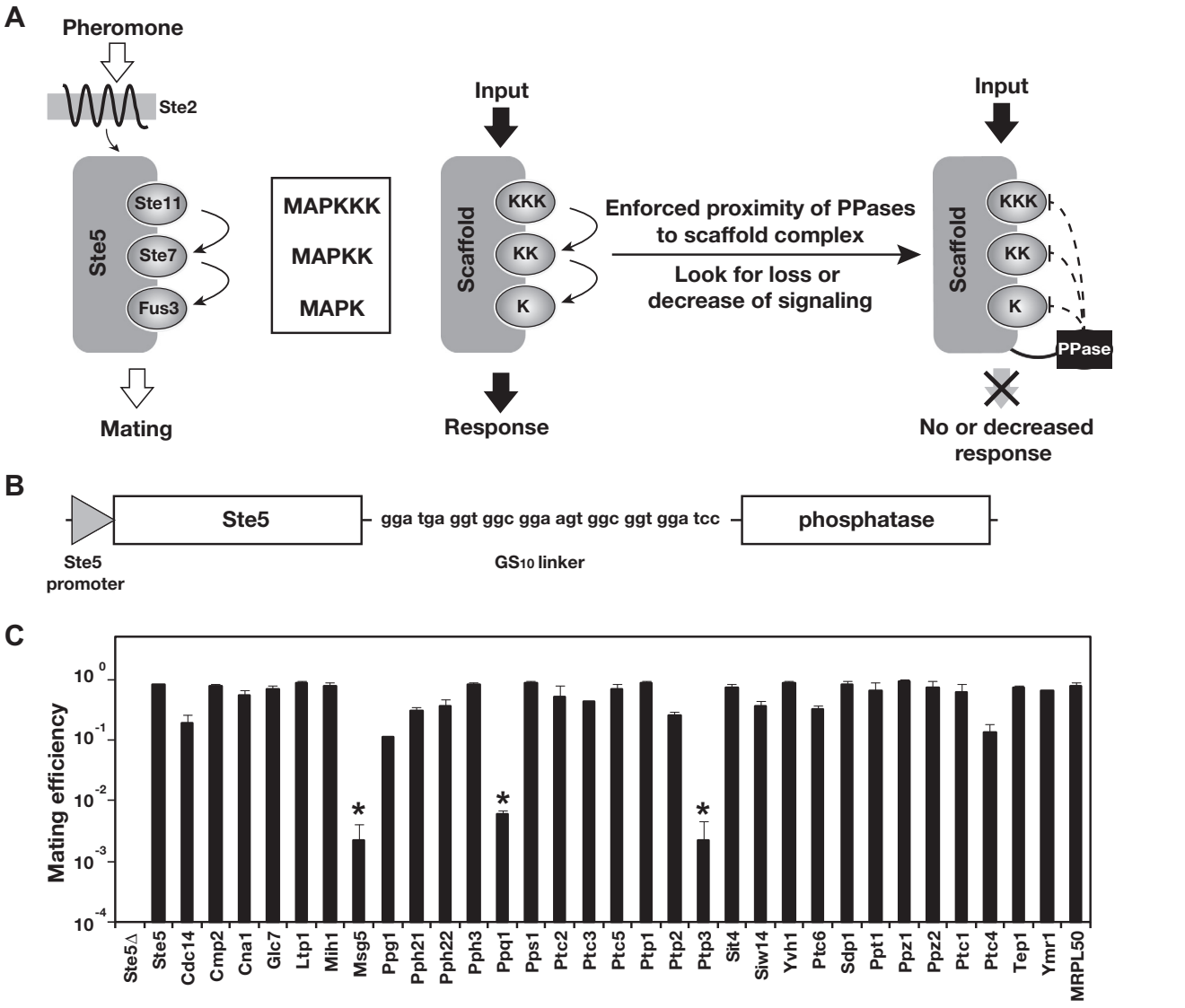

Fig. 1. Screening of phosphatases specific for the mating MAP kinase pathway in *S. cerevisiae*. (A) A novel approach to identify pathway-specific phosphatases was developed. The loss of or decrease in signaling output by a phosphatase (in black) tethered to scaffold complex can be monitored using mating functional assays. The tethered phosphatase may target (dashed lines) pathway members in the complex. (B) The ORF's of yeast phosphatase genes were tethered to Ste5 scaffold using GS_{10} linker. Each Ste5-phosphatase fusion was expressed in a Δ*ste5* strain (RB201) under Ste5 promoter. (C) Three phosphatases (Msg5, Ptp3 and Ppq1) were identified using quantitative mating assay and are indicated with asterisks.

Ppq1 – showed a dramatic decrease in the quantitative mating response by more than 100-fold compared to the wild-type response (Fig. 1C). Msg5 and Ptp3 phosphatases are previously known as mating-specific negative regulators by dephosphorylating active Fus3 MAP kinase [13,17,18]. However, the screening resulted in the identification of a Ser/Thr phosphatase, Ppq1, whose function is not clearly understood [19–21] with no known relevance to the mating signaling. When artificially recruited to Ste5 scaffold complex, Ppq1 showed a strong negative effect on mating response along with known phosphatases suggesting that Ppq1 might be a novel, putative mating-specific phosphatase.

3.2. Ppq1 functions as a negative regulator of mating signaling

To investigate the function of Ppq1 as a negative regulator of mating signaling, we have attempted a series of mating analyses under various conditions. Mating signaling is known to induce an arrest of cell cycle at G1, which allows for a simple halo assay [22,23]. Halo assays were performed for wild-type, Δ*ste5*, and Δ*ppq1* cells with or without over-expressed Ppq1 (Fig. 2A). When challenged by α-factor, wild-type and Δ*ste5* cells showed a clear and no halo, respectively. However, Δ*ppq1* cells showed more pronounced halo compared to the wild-type cells and over-expression of Ppq1 protein in Δ*ppq1* cells dramatically reduced the halo effect to the level of Δ*ste5* cells. As a recapitulation of the screening condition, Δ*ste5* cells carrying Ste5-Ppq1 fusion protein also showed little halo effect when tested. For more quantitative analysis of Ppq1 function on the mating signaling, the Fus1-LacZ reporter assay was performed for the same set of cells used in the halo assay (Fig. 2B). The deletion of Ppq1 resulted in ~20% increase in the LacZ activity and the over-expression of Ppq1 resulted in ~50% decrease in the LacZ activity compared to the wild-type response. To determine the effect of Ppq1 function on immediate pathway output, the dual phosphorylation of Fus3 MAP kinase upon α-factor stimulation was monitored (Fig. 2C). The deletion and over-expression of Ppq1 caused similar effects on the dual phosphorylation of Fus3 MAP kinase like the Fus1-LacZ or halo assay described above. Interestingly, the expression level of Ppq1

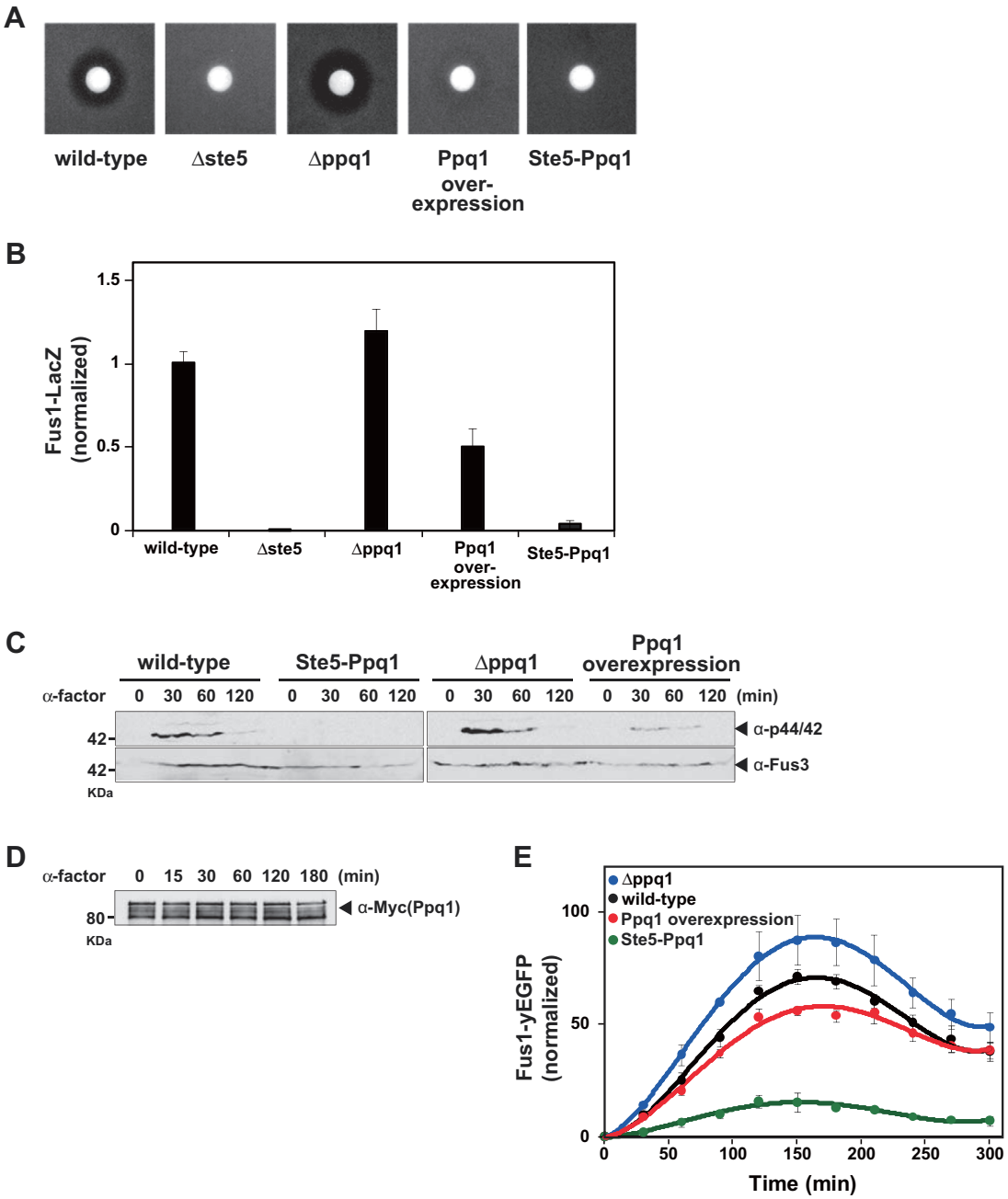

Fig. 2. Ppq1 functions as a negative regulator of mating signaling. Down-regulation of the mating signaling by Ppq1 was confirmed using various mating assays. Ppq1 was over-expressed using the TEF2 promoter in a Δ*ppq1* strain, and Ste5-Ppq1 was expressed in a Δ*ste5*Δ*ppq1* strain (A) Halo assay. (B) Fus1-LacZ reporter assay. (C) The dual phosphorylation of MAP kinase Fus3. The total Fus3 and dually phosphorylated Fus3 were detected using anti-Fus3 and anti-phospho p44/42 antibodies, respectively. (D) Expression level of cellular Ppq1 during the mating signaling was monitored. Ppq1-myc protein was detected using anti-myc antibody. (E) FACS analysis was performed to monitor signaling kinetics of mating responses using Fus1-yEGFP reporter. Perturbations in Ppq1 expression level did not alter the signaling kinetics or shape.

protein remained constant upon α-factor stimulation indicating that Ppq1 may not participate in a feedback control of signaling (Fig. 2D).

To investigate the signaling dynamics in detail, FACS analysis was performed for same set of cells carrying a Fus1-yEGFP reporter gene (Fig. 2E). As in the Fus1-LacZ assays, the Δ*ppq1* cells showed a clear increase in signaling magnitude whereas cells with over-expressed Ppq1 resulted in decreased response compared to the wild-type cells. In all cases, a similar signaling kinetics was observed, in which the maximal response was reached around 160 min after α-factor stimulation followed by signaling adaptation. Unlike Msg5 or Ptp3 that are known to mainly function in the adaptation stage via negative feedback regulation [17,18], Ppq1 seems to be working to lower overall magnitude of signaling. The fact that perturbations in Ppq1 level did not alter the signaling kinetics or shape suggests that Ppq1 negatively regulates the mating pathway by controlling the noise or basal level of signaling.

3.3. Catalytic activity of Ppq1 phosphatase is essential for the regulation of mating signaling

Although the alignment of amino acid sequences suggests Ppq1 to be a putative Ser/Thr phosphatase [19,20], its enzymatic activity has never been confirmed. To verify the catalytic activity of Ppq1 as

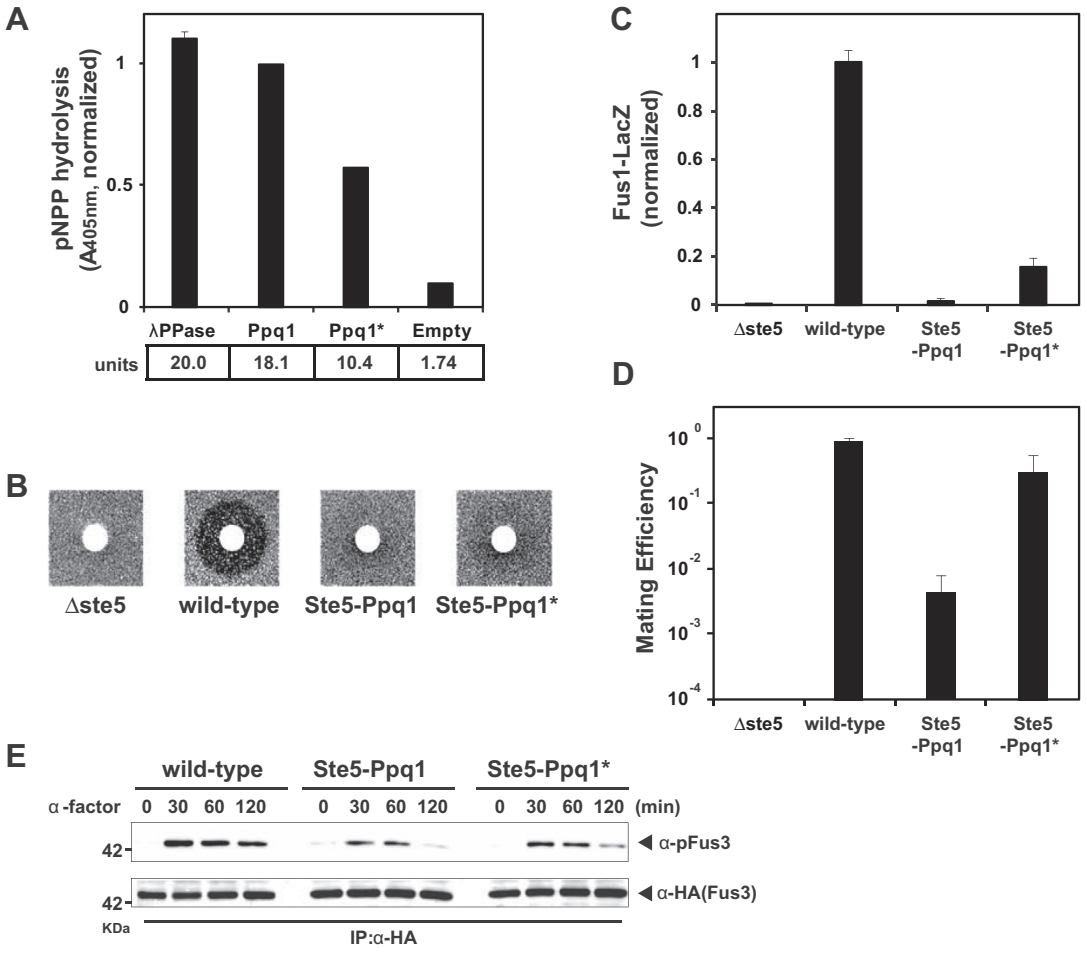

Fig. 3. Catalytic activity of Ppq1 phosphatase is essential for the regulation of mating signaling. (A) Catalytic activity of Ppq1 and Ppq1* purified from *E. coli* was verified. Ppq1* exhibited attenuated catalytic activity, which is about 57% of the wild-type activity. The units of enzymes used in the assay were calculated based on the activity of λPPase as described in datasheets. The empty sample was prepared from *E. coli* cells expressing Ppq1 without IPTG induction. (B) Cell cycle arrest at G1 by Ppq1 was released by Ppq1*. The suppression of mating response by Ppq1 was relieved by Ppq1*, which was monitored by (C) Fus1-LacZ reporter assay, (D) quantitative mating assay, and (E) immunoblotting of dually phosphorylated Fus3 MAP kinase.

a protein phosphatase, an *in vitro* phosphatase assay was performed for the wild-type and a mutant Ppq1 protein purified from *E. coli*. To obtain the mutant Ppq1 (Ppq1*) with reduced catalytic activity, known conserved amino acids in the PP1 phosphatase family were substituted as follows: D301N, H303A, D329N, N361A, H410A, and H485A. In the phosphatase assay using pNPP as a substrate, the wild-type Ppq1 exhibited a clear catalytic activity comparable to that of lambda protein phosphatase used as a positive control and the mutant Ppq1* exhibited decreased activity by 57% (Fig. 3A).

To determine whether the catalytic activity of Ppq1 is necessary for its function on mating regulation, the Δ*ste5* cells carrying Ste5-Ppq1 or Ste5-Ppq1* fusion were tested for mating responses. According to the halo assay, cells carrying Ste5-Ppq1* fusion showed a slight increase in halo formation compared to the cells carrying Ste5-Ppq1 fusion (Fig. 3B). The magnitude of mating signaling was significantly repressed by covalent tethering of the active Ppq1 to Ste5 scaffold. However, the repression of mating signaling caused by Ste5-Ppq1 was dramatically released by the fusion with catalytically attenuated Ppq1* (Ste5-Ppq1*) as monitored by the Fus1-LacZ reporter assays (Fig. 3C), the quantitative mating assays (Fig. 3D) and the phospho Fus3 immunoblotting (Fig. 3E). The release of signaling by Ste5-Ppq1* is not due to a likely instability of Ppq1* mutant because the protein stability of Ppq1* seemed to be comparable to that of wild-type Ppq1 (data not shown). It is noteworthy that Ste5-Ppq1* did not fully restore mating repression caused by Ste5-Ppq1 because of residual activity of Ppq1*. In addition, the facts that near full mating responses exhibited by most of phosphatases larger than Ppq1 (Fig. 1C) and Ste5 assembly is highly modular [12], and the use of a flexible GS_{10} linker [24] ensure that the partial recovery of mating responses by Ste5-Ppq1* (Fig. 3C) was not due to physical interferences possibly caused by tethering of bulky proteins near Ste5 scaffold. Together, these data suggest that Ppq1 manifests its regulatory functions in mating signaling via its catalytic activity, not ruling out the possibility of functioning via alternative mechanisms such as binding or assembly with other proteins.

4. Discussion

Reversible protein phosphorylation plays a pivotal role in cell signaling and reciprocal interplay of kinases and phosphatases is a key mechanism for the control of phosphorylation status of target proteins. Phosphatases are thought to function in cell signaling in two ways: to suppress signaling magnitude in adaptation step via negative feedback controls and to minimize background signaling for noise control [1,2]. However, in general, the detailed mechanisms of the phosphatase function are not well understood compared to kinase function, mainly because of the promiscuous

characteristics of phosphatases. Especially, the physiological redundancy and broad substrate specificity of phosphatases severely impeded the utility of traditional phenotypic screens to identify pathway-specific phosphatases. It was reported that single- or double-knockouts of phosphatase genes in yeast rarely generated detectable phenotypes mainly due to the physiological redundancy [6,25]. Therefore, there was a pressing demand for alternative approaches to identify and analyze phosphatase functions to better understand the mechanisms of cell signaling.

In this study, we have developed a novel strategy to screen for pathway-specific phosphatases of the mating MAP kinase signaling in yeast as a model system. All thirty-two phosphatase genes in yeast cells were cloned and were individually expressed as a fusion to the Ste5 scaffold protein in Δ*ste5* cells. A mating-specific phosphatase, when enforced in proximity to the putative substrates via tethering to scaffold complex, can be easily identified by monitoring the decrease in mating responses. Using the strategy, we have identified three phosphatases, of which Ppq1 was novel with no previous implication in mating signaling whereas Msg5 and Ptp3 phosphatases were already known to be mating-specific [13,17,19]. It seems that the identification of Msg5 and Ptp3 validates the integrity of the screening. The screen appears to be quite selective from the fact that the rest of phosphatases in the screen caused only a slight decrease in mating responses, which was probably due to the non-specific catalytic activities enforced in close proximity to the active signaling complex. We think the strategy developed in this study provides for a useful and efficient research tool for the study of phosphatase functions in signaling and should be applicable to other scaffold-dependent signaling pathways in higher eukaryotes, such as JIP1 or KSR1-medicated MAP kinase pathways in mammals [26].

Ppq1 is a putative Ser/Thr phosphatase in the PP1 family and is previously known to be involved in the control of translational suppression and the maintenance of cell wall integrity under selective nutritional conditions [19–21]. To investigate the functional mechanism of Ppq1 as a negative regulator of the mating signaling, the null mutation and over-expression of Ppq1 were tested for effects on mating responses. The Δ*ppq1* mutation caused a clear increase in mating response compared to the wild-type and the over-expression of Ppq1 suppressed the mating outputs in several mating readouts (Fig. 2A–C). This result strongly suggests that Ppq1 is directly involved in the down-regulation of mating signaling. Interestingly, the cellular expression level of Ppq1 remained relatively constant regardless of α-factor stimulation and the knockout or over-expression Ppq1 did not perturb the signaling kinetics (Fig. 2D and E). This finding seems to rule out the possibility of negative feedback regulation by Ppq1 and rather suggests its implication in the noise control via minimizing the background signaling [27]. The experiments using Ste5 scaffold tethered with a catalytically-attenuated mutant (Ste5-Ppq1*) demonstrated that the phosphatase activity was required for its function in the control of mating signaling (Fig. 3B–D). When a purified, autophosphorylated Fus3 [17] was treated with active Ppq1 *in vitro*, no decrease in phosphorylation of Thr or Tyr residues in Fus3 was observed (data not shown). It is known that Ste7 and Ste11 are phosphorylated at Ser or Thr residues. Taken together, it seems that Ste7 and/or Ste11 are more likely to be substrate(s) of Ser/Thr-specific phosphatase Ppq1 than Fus3 [28,29]. But for now, the substrate specificity of Ppq1 is yet to be identified.

In nature, cells are continuously bombarded by many extracellular stimuli and the corresponding signaling cascades should be turned on and off, reversibly. This scenario requires the active or phosphorylated signaling proteins to be readily recycled to an inactive or dephosphorylated form in the absence of stimulation. The mating-specific phosphatases identified so far, Msg5 and Ptp3, are known to target the terminal MAP kinase Fus3 [13,17]. However, phosphatases directed to the upstream kinases such as Ste7 and Ste11 are yet to be discovered. In conclusion, the present study demonstrated that Ppq1 functions as a negative regulator in mating pathway by targeting at Fus3 or upstream kinases. Furthermore, the screening strategy, based on the artificial enforcement of phosphatases to signaling complex, can be used for identification of novel pathway-specific phosphatases in other signaling pathways in higher organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.110.

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