Title

Multistep regulation of protein kinase A in its localization, phosphorylation and binding with a regulatory subunit in fission yeast

Author(s)

Dipali Rani Gupta, Swapan Kumar Paul, Yasuo Oowatari, Yasuhiro Matsuo, Makoto Kawamukai

Journal

Current genetics, 57, Article number: 353 (2011)

Published

31 August 2011

URL

https://doi.org/10.1007/s00294-011-0354-2

Multistep regulation of protein kinase A in its localization, phosphorylation and binding with a regulatory subunit in fission yeast

Dipali Rani Gupta, Swapan Kumar Paul, Yasuo Oowatari, Yasuhiro Matsuo, Makoto Kawamukai

Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Japan

Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu, Matsue 690-8504, Japan.

Running title

Pka1 in *S. pombe*

+To whom correspondence should be addressed.

Tel: +81-852-32-6587; Fax: +81-852-32-6092; E-mail: kawamuka@life.shimane-u.ac.jp

Abstract

The cAMP-PKA pathway is the major glucose-sensing pathway that controls sexual differentiation in *Schizosaccharomyces pombe*. Sequencing from the *pka1* locus of recessive *sam* mutants, in which cells are highly inclined to sexual differentiation, led to the identification of mutations in the *pka1* locus in *sam5* (*pka1*-G441E) and *sam7* (*pka1*-G441R). Rst2 and Ste11 proteins were induced and localized to the nucleus of *sam5* and *sam7* mutants even under rich glucose conditions, indicating that the function of Pka1 was completely abolished by mutations. Pka1-G441E and Pka1-G441R mutant proteins reside in the cytoplasm, even under glucose-rich conditions, while wild-type Pka1 resides in the nucleus, indicating that the functionality of Pka1 is important for its nuclear localization. This is supported by the observation that the Pka1-T356A mutant, which partially lacks Pka1 function, was localized to both the cytoplasm and the nucleus, but an active phosphomimetic Pka1-T356D mutant prtotein was localized to the nucleus under glucose-rich conditions. In addition to the basal phosphorylation of Pka1 at T356, hyperphosphorylation of Pka1 was observed under glucose-starved conditions, and such hyperphosphorylation was not observed in *pka1*-G441E, *pka1*-G441R, *pka1*-T356A or *pka1*-T356D mutant. As these mutant proteins failed to interact with a regulatory subunit Cgs1, hyperphosphorylation of Pka1 mutant proteins was considered to be dependent on Cgs1 interaction. Consistent with a role for Cgs1 in Pka1 phosphorylation, we detected the formation of a Cgs1-Pka1 complex prior to Pka1 hyperphosphorylation. Together, these results indicate that nuclear localization of Pka1 depends on its activity and hyperphosphorylation of Pka1 depends on Cgs1 interaction.

48 **Introduction**

49 The fission yeast *Schizosaccharomyces pombe* undergoes sexual differentiation upon nutritional
50 starvation because such conditions induce the expression of genes that result in the enhanced
51 production of mating pheromones and their receptors. Cells recognize the pheromones secreted by
52 cells of the opposite mating type (h^+ or h^-) and elongate conjugation tubes toward each other. After
53 the fusion of mated cells, they proceed to karyogamy, pre-meiotic DNA synthesis, meiosis I,
54 meiosis II, and sporulation (Yamamoto 2003). Switching the cell cycle from mitosis to meiosis is
55 regulated by many factors with complex mechanisms. The cAMP-dependent protein kinase (PKA)
56 pathway is the major glucose-sensing pathway that regulates sexual differentiation through the
57 activation of PKA. The glucose signal is transferred from its receptor Git3 to a heterotrimeric
58 guanine nucleotide-binding protein (Gpa2, Git5 and Git11) (Hoffman 2005; Welton and Hoffman
59 2000) by releasing α subunit Gpa2, which then activates adenylate cyclase (Cyr1) to generate
60 cAMP from ATP (Kawamukai et al. 1991). Cyr1 interacts with at least two associated proteins, Git1
61 (Kao et al. 2006), which is required for glucose activation of Cyr1, and Cap1, which assists Cyr1
62 activity and also interacts with actin and 14-3-3 (Kawamukai et al. 1992; Zhou et al. 2000). The
63 *cgsl* gene of *S. pombe* encodes the regulatory subunit of PKA. Mutants defective in *cgsl* are
64 partially sterile because of the constitutive activation of PKA. Intracellular cAMP levels positively
65 regulate Pka1 activity (DeVoti et al. 1991), and cells with high Pka1 activity are incapable of sexual
66 differentiation, whereas any manipulation that decreases the cAMP level or the PKA activity
67 propels cells toward sexual differentiation (Maeda et al. 1994). Sterility caused by higher cAMP
68 levels is reversed by the over-expression of *moc1-moc4* genes, whose products are thought to
69 control Ste11, a key transcription factor for meiosis, at the translational level (Goldar et al. 2005;
70 Kawamukai 1999; Paul et al. 2009; Yakura et al. 2006a). Under glucose-starved conditions, Pka1
71 resides in the cytoplasm, but it enters into the nucleus under nutrient-rich conditions (Matsuo et al.
72 2008) and phosphorylates the Zn-finger protein Rst2 that induces the expression of *ste11* (Higuchi

et al. 2002; Kunitomo et al. 2000). In addition to the regulation of meiosis, PKA is involved in other cellular processes including cell cycle control, cytoskeletal dynamics, metabolism, and physiological stress responses (McInnis et al.). The cAMP-PKA pathway is known to be required for the growth of cells in medium with high KCl (Yang et al. 2003).

PKA is a conserved serine or threonine kinase found among many organisms, including yeasts and mammals. PKA is a heterotetramer formed by two regulatory (R) subunits and two catalytic (C) subunits (Taylor et al. 1990). Binding of cAMP to the R subunit results in the release of C subunits. When high glucose conditions allow the cAMP level to increase in fission yeast, the C subunit and the R subunit both reside in the nucleus and are only diffusely present in the cytoplasm. When cells are grown in a non-fermentable carbon source and the cAMP level is low, both subunits are largely localized to the cytoplasm (Tudisca et al. 2010). In cells defective in *cgs1*, Pka1 is concentrated in the nucleus regardless of culture conditions, whereas in stationary phase, Pka1 is distributed in the cytoplasm (Matsuo et al. 2008).

PKA is synthesized as an inactive form that is activated by phosphorylation at amino acid residues on an activation loop in mammals (Moore et al. 2002). The C subunit of mammalian PKA is phosphorylated at two conserved amino acid residues, Thr-197 and Ser-338 (Yonemoto et al. 1997). The Thr-197 residue is conserved in all known mammalian C-subunits and is analogous to the fission yeast Thr-356 residue. However, the Ser-338 residue of the mammalian C-subunits is not conserved in any of the yeast isozymes. Considering its homology with the mammalian protein, the Thr-356 residue of the C-subunit from fission yeast was expected to be phosphorylated and is likely phosphorylated by Ksg1 (Tang and McLeod 2004). Substitution of alanine for Thr-356 resulted in an unregulated Pka1 phenotype (Tang and McLeod 2004), but the resulting substrate specificities or catalytic activities have not been measured. It was recently shown that Pka1 is hyperphosphorylated, presumably at residue(s) other than Thr-356, when cells are either depleted of cAMP or grown under glucose-limited conditions. Although Cgs1 was shown to be required for this

hyperphosphorylation (McInnis et al. 2010), the significance of Pka1 hyperphosphorylation was not clear.

Nine *sam* (skips starvation for mating) mutants that entered sexual differentiation in the absence of nutritional starvation were previously reported (Katayama et al. 1996). Among these, *sam4* was identified as carrying a nonsense allele of *rad24* (Oowatari et al. 2009). Two RNA binding proteins, Msa1 and Msa2, which negatively regulate sexual differentiation, were isolated as suppressors of *sam1* (Jeong et al. 2004a; Jeong et al. 2004b). Through analysis of *sam3* and *sam9, sla1* was identified as an inducer of sexual differentiation and *zds1* as shown to be involved in both sexual differentiation and $CaCl_2$ tolerance (Tanabe et al. 2003; Tanabe et al. 2004).

In this paper, both *sam5* and *sam7* mutants are demonstrated to carry missense alleles of *pka1*. A point mutation in the conserved amino acid residues of the *pka1* locus of *sam5* and *sam7* mutants caused the downregulation of Pka1 biological activities and defects in both Pka1 hyperphosphorylation and its interaction with a regulatory subunit, Cgs1. In addition, it was found that the Thr-356 residue of the activation loop of Pka1 is important for its nuclear localization, binding to Cgs1 and that the binding of Cgs1 is required for the hyperphosphorylation of Pka1. We demonstrate here that the activity of Pka1 is important for nuclear localization of Pka1 and Pka1 is hyperphosphorylated after binding with Cgs1.

Materials and methods

Media and genetic manipulations

Standard yeast culture media and genetic manipulations were used as described previously (Alfa et al. 1993). The *S. pombe* strains were grown in YES medium (0.5% yeast extract, 3% glucose, and 225 mg/liter adenine, histidine, leucine, uracil, and/or lysine hydrochloride), YES glucose-poor medium (only glucose concentration (0.1%) is different from YES) or Pombe Minimum (PM)

123 medium (0.3% potassium hydrogen phthalate, 0.22% sodium phosphate, 0.5% ammonium chloride,
124 and 2% glucose, vitamins, minerals, and salts), supplemented with 0.0075% adenine, leucine,
125 and/or uracil when necessary. PMAU contains adenine and uracil in PM. Nitrogen-free PM
126 medium (1% glucose without ammonium chloride) was used to culture *S. pombe* when the mating
127 efficiency was measured. Electroporation was used to transform yeast cells (Prentice 1992).
128 *Escherichia coli* DH5α was grown in Luria-Bertani (LB) medium (1% polypeptone, 0.5% yeast
129 extract, 1% sodium chloride, pH 7.2) and was used as a host for all plasmid manipulations using
130 standard methods as described (Sambrook et al. 1989).
131
132 Plasmid construction
133 The pSLF372(L)-*pka1* plasmid was constructed by amplifying the *pka1* gene with the Pka1F-*Bgl*II
134 and Pka1R-*Not*I primers listed in supplementary Table 1. The PCR product was then digested with
135 *Bgl*II and *Not*I and inserted into pSLF372(L) plasmid, which has an *nmt81* promoter and three
136 copies of the HA epitope in its C-terminal region. To construct pSLF172 (L)-*pka1* plasmid, the
137 *pka1* gene from the plasmid pSLF372(L)-*pka1* was digested with the restriction enzymes *Bgl*II and
138 *Not*I. The digested fragment was cloned into the *Bgl*II and *Not*I sites of pSLF172 (L) that are
139 located in the downstream of *nmt1* promoter region. The nucleotide sequences of the constructed
140 plasmids were verified by restriction digestion and DNA sequence analysis.
141
142 Strain construction
143 The *S. pombe* strains used in the study are listed in Table 1. Tag-integrated versions of *pka1-13Myc*,
144 *cgs1-3HA*, *rst2-3HA*, *gpd1-3HA* and *ste11-GFP* genes were generated by a PCR-based method
145 (Krawchuk and Wahls 1999). To construct the *sam5-pka1*$^{+}$-13Myc and *sam7-pka1*$^{+}$-13Myc strains,
146 the *pka1* locus of wild-type strain SP870 covering the mutation site was amplified using the primers
147 Pka1-W/Pka1-X. Similarly, the *pka1(G1322A)*-13Myc and *pka1(G1321A)*-13Myc strains were

148 constructed by amplifying the *pka1* locus from *sam5(G1322A)* and *sam7(G1321A)* mutants,
149 including the mutation site, using the primers Pka1-W/Pka1-X. The amplified fragments were
150 attached to the end of the *kanMX6* module by PCR using pFA6a-13Myc-kanMX6. The wild-type
151 strain SP870 was transformed with the tagged DNA fragments from each of the second PCR
152 products and G418-resistant transformants were selected. Proper integration was verified by
153 western blotting and DNA sequencing. The resulting strains were named DRG15 and DRG17. The
154 Pka1-T356A and Pka1-T356D mutants were constructed by amplifying the 500 bp of *pka1* gene
155 from the 3' region of pSLF172L-$pka1^{T356A}$ and pSLF172L-$pka1^{T356D}$, which carries the T356A and
156 T356D mutations, using the respective primers. The downstream fragment of the *pka1* gene was
157 amplified as for DRG15 or DRG17 mutants. Both fragments were attached to the kanMX6 module
158 by PCR using pFA6a-13Myc-kanMX6.
159
160 Mating and sporulation efficiency assay
161 The mating and sporulation efficiency was calculated using the following equation: Mat (%) = (2*Z*
162 + 2*A* + 0.5*S*)/(*H* + 2*Z* + 2*A* + 0.5*S*), where *Z* stands for the number of Zygotes, *A* for the number of
163 asci, *S* for the number of free spores, and *H* for the number of cells that failed to mate.
164
165 Western Blotting
166 Protein extraction for western blotting was performed by the alkali-SDS method (Matsuo et al.
167 2006) and the boiling SDS-glass bead method (Yakura et al. 2006b). Mouse monoclonal anti-HA,
168 and anti-Myc, and rabbit polyclonal anti-PSTAIRE (Cdc2) antibodies were purchased from Santa
169 Cruz Biotechnology (Santa Cruz, CA).
170
171 Co-immunoprecipitation
172 *S. pombe* cells were grown in YES and/or YES glucose-starved (0.1% glucose) medium to the mid-

logarithmic phase, and then harvested ($2x10^8$ cells) by centrifugation and washed once with ice-cold stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, and 1 mM NaN_3, pH 8). The cells were then lysed in 100 µL ice-cold lysis buffer [50 mM Tris, 150 mM NaCl, 0.8% Nonidet-P40, 5 mM EDTA, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride (PMSF), and protease inhibitor]. The samples were vortexed vigorously with 0.5 mm diameter zirconia/silica beads using a bead homogenizer at 2,500 rpm for 3 min. After centrifugation (10,000-x g for 15 min at 4° C), the protein concentration in the supernatant was estimated. Co-immunoprecipitation was carried out as previously described (Paul et al. 2009).

Indirect immunofluorescence

Indirect immunofluorescence was performed by fixing the cells in 3.7% formaldehyde, as described previously (Matsuo et al. 2011). Cell walls were digested with 0.3 mg/ml zymolyase 100T (Seikagaku) at 37°C for 30~40 minutes. Cell pellets were permeabilized using 1.0% Triton X-100 and then incubated with the first antibody (anti-HA or anti-Myc) overnight at 30°C and, subsequently, with the secondary antibody (Alexafluor 488 goat anti-mouse IgG). To visualize the nucleus, cells were stained with 0.1% DAPI.

Fluorescence microscopy

Fluorescence microscopy was performed using a BX51 microscope (Olympus, Tokyo, Japan). Images were captured using a digital camera DP70 (Olympus).

Results

Identification of *sam5* and *sam7* mutants carry mutations of *pka1* alleles

198 All nine *sam* mutants were growth inhibited on medium containing 1 M KCl and underwent sexual
199 differentiation despite the presence of a high glucose concentration in the medium (Oowatari et al.
200 2009). Because the cAMP-PKA pathway is required for the survival of *S. pombe* cells under high-
201 salt conditions (Yang et al. 2003), and a *pka1* deletion mutant showed a hyper mating tendency in
202 the nutrient-rich medium (Maeda et al. 1994), it is possible that one or more of the *sam* mutants
203 carry a mutant allele of *pka1*. Genomic DNAs were isolated from the recessive *sam* mutants and
204 the *pka1* locus was amplified using a set of primers used for sequencing *pka1*. Both *sam5* and *sam7*
205 mutants contained a single nucleotide change at the *pka1* locus that changed the codon for amino
206 acid glycine 441. A G to A transition at base pair 1322 was found in the *pka1* gene of the *sam5*
207 mutant that changed a glycine to glutamic acid (G441E), and a G to A transition at base pair 1321
208 nucleotide mutation was found in the *pka1* gene of the *sam7* mutant that changed a glycine to
209 arginine (G441R).
210
211 Suppression of *sam5* and *sam7* mutants by *pka1*
212
213 To test whether the *pka1* mutations found in *sam5* and *sam7* mutants were the cause of the
214 phenotypes observed, *pka1* was expressed from a plasmid to determine if it suppressed the
215 phenotypes of *sam5* and *sam7* mutants. The *sam5* and *sam7* mutants that expressed *pka1* grew well
216 on PMAU containing 1.0 M KCl, whereas the same cells harboring a control plasmid did not (Fig.
217 1A). Expression of *pka1* lowered the mating efficiency of *sam5* and *sam7* mutants and cells
218 became elongated upon expression of *pka1* (Fig. 1 B and C). The phenotypes were typical for high-
219 levels of expression of *pka1*. Next, the *pka1* loci of *sam5* and *sam7* mutants were replaced with
220 wild-type $pka1^{+}$. Consequently, the KCl sensitivity and hyper mating phenotypes were reversed in
221 strains DRG25 ($pka1^{+}$) and DRG27 ($pka1^{+}$) (Fig. 2 A and data not shown), indicating that the *sam5*
222 and *sam7* phenotypes were caused by mutant *pka1* alleles.

223

224 Pka1 protein was synthesized in *sam5* and *sam7* mutants

225

226 To determine whether the Pka1 protein was synthesized in *sam5* and *sam7* mutants, the *pka1* loci of
227 *sam5* and *sam7* mutants were introduced into the wild-type background using a PCR-mediated
228 method and, concomitantly, the Pka1 C-termini were tagged with 13Myc. Wild-type *S. pombe* cells
229 containing Pka1-G441E or Pka1-G441R mutations were growth inhibited on medium with 1 M
230 KCl (Fig. 2 A). The mating efficiencies of the DRG15 (*pka1-G441E*) or DRG17 (*pka1-G441R*)
231 strains growing on YES rich medium were measured by counting the cells using a microscope.
232 Unlike the wild-type cells, *pka1-G441E* or *pka1-G441R* mutant cells conjugated and sporulated at a
233 high frequency like other *sam* mutants (Fig. 2 B). To examine the Pka1 protein expression of the
234 newly constructed mutants, crude protein was extracted from the wild-type, DRG15 (*pka1-G441E*)
235 and DRG17 (*pka1-G441R*) strains and analyzed using a 10% SDS-polyacrylamide gel. An anti-
236 Myc antibody was used to detect the Pka1 protein, which was found to be present in crude extracts
237 from both mutants as well as the wild type (Fig. 2 C). Thus, the *pka1* mutations *sam5* and *sam7* did
238 not affect Pka1 protein synthesis but somehow altered protein function.

239

240 Ste11 is constitutively expressed in *pka1-G441E* and *pka1-G441R* mutants

241

242 Ste11 is the key transcriptional factor for the onset of meiosis in fission yeast. Ste11 shuttles
243 between the cytoplasm and the nucleus, and it is present in the cytoplasm under nutritionally-rich
244 conditions (Higuchi et al. 2002; Kunitomo et al. 2000). The mating pheromone and nutrient
245 starvation induce the expression of Ste11 and trigger its accumulation in the nucleus (Qin et al.
246 2003). To determine Ste11 expression and localization in *pka1* mutants, Ste11 was tagged with
247 GFP at its C-terminus in wild-type, *sam5*, and *sam7* mutants. In wild-type cells growing on rich

248 medium, Ste11 was found in the cytoplasm and the GFP signal was weak. In *sam5* and *sam7*
249 mutants, Ste11 accumulated in the nucleus, even in nutrient-rich medium, and showed a strong GFP
250 signal. However, under starved conditions, all 3 strains showed a nuclear GFP signal
251 (supplementary Fig. 1 A). In *sam5* and *sam7* mutants GFP signals were stronger than in wild type,
252 which led us to test Ste11 protein levels. Cells were grown on nitrogen-free medium to induce
253 mating and samples were collected at different times to examine the expression of Ste11 protein in
254 wild type, *sam5*, and *sam7*. Ste11 expression was induced at a higher level in *sam5* and *sam7*
255 mutants, even at 0 hour of nitrogen starvation, than in wild-type cells (supplementary Fig. 1 B).
256 Thus, in *sam5* and *sam7* mutants, Pka1 inactivation resulted in a higher expression of Ste11 even
257 under nutrient-rich conditions, providing additional evidence that Pka1 is non-functional in *sam5*
258 and *sam7* mutants.
259
260 Pka1-G441E and Pka1-G441R mutations affect the phosphorylation and localization of Rst2 under
261 nutrient-rich conditions
262
263 Rst2 is a transcription factor that is required for the expression of *ste11* under glucose-starved
264 conditions. Pka1 negatively regulates Rst2 by causing it to be excluded from the nucleus, thereby
265 inhibiting the expression of *ste11*. The Rst2 protein becomes hyperphosphorylated under de-
266 repressed conditions and Pka1 inhibits this hyperphosphorylation (Higuchi et al. 2002). The Rst2
267 phosphorylation state of *pka1-G441E* and *pka1-G441R* mutants under glucose starvation was
268 analyzed. Rst2 from Δ*pka1* cells, as well as *pka1-G441E* and *pka1-G441R* mutants grown on
269 glucose-rich medium, was phosphorylated, whereas Rst2 from wild-type cells was not
270 (supplementary Fig. 2A). Under glucose-starved conditions, Rst2 was hyperphosphorylated in all
271 strains, including the wild type. Indirect immunofluorescence was used to determine the sub-
272 cellular localization of Rst2 under nutrient-rich and nutrient-poor conditions. Under nutrient-rich

conditions, Rst2 was localized to the cytoplasm in wild-type cells; however, in *pka1-G441E* and *pka1-G441R* mutants, Rst2 was localized to the nucleus, as found for the Δ*pka1* strain (supplementary Fig. 2B). Rst2-3HA from all strains was localized to the nucleus under nutrient-starved conditions (data not shown). Thus, Rst2 expression and localization in the *pka1-G441E* and *pka1-G441R* mutants displayed a pattern similar to that in Δ*pka1* cells, which again suggests that *pka1* is non-functional in *pka1-G441E* and *pka1-G441R* mutants and that the changed amino acids are critical for Pka1 function.

Pka1 protein remains in the cytoplasm in *pka1-G441E* and *pka1-G441R* mutants under nutrient-rich conditions

Cells growing in high glucose medium generate cAMP, which causes nuclear import of Pka1 (Matsuo et al. 2008). The localization of Pka1-G441E and Pka1-G441R proteins was analyzed using indirect immunofluorescence microscopy. The cells were fixed with formaldehyde and stained with DAPI and anti-Myc antibodies directed against the wild type, *pka1-13Myc*, *pka1-G441E-13Myc*, and *pka1 -G441R-13Myc* strains. The fluorescence of Pka1-13Myc from the wild-type cells was concentrated in the nucleus under nutrient-rich conditions. However, the Pka1-13Myc fluorescence was detected throughout the cytoplasm in *pka1-G441E-13Myc* and *pka1-G441R-13Myc* strains under the same conditions (Fig. 3A) When the wild-type cells were starved for glucose, Pka1 protein showed a cytoplasmic distribution. The results demonstrated that Pka1-G441E and Pka1-G441R mutations affect the nuclear localization of Pka1 protein even under glucose-rich conditions and suggested that the activity of Pka1 is important for its nuclear localization.

Pka1-G441E and Pka1-G441R mutations affect the phosphorylation of Pka1

298

299 Pka1 is hyperphosphorylated under conditions of glucose starvation (data not shown and McInnis
300 et al. 2010). To learn whether these mutations affect the hyperphosphorylation of Pka1-G441E and
301 Pka1-G441R, the phosphorylation states of Pka1 in *pka1-G441E* and *pka1-G441R* mutants were
302 examined. Cells were cultured on YES medium containing 0.1% glucose and protein extracts were
303 prepared at different times and analyzed by SDS-PAGE. The Pka1-G441E and Pka1-G441R
304 proteins were not hyperphosphorylated under glucose starvation even after a long incubation time,
305 whereas the wild-type Pka1 became phosphorylated under the same conditions (Fig. 3B). Thus, the
306 inactivation of Pka1 by G441 mutation abolished the phosphorylation of Pka1.
307

308 Involvement of the Pka1-T356 residue in Pka1 function and phosphorylation
309

310 Mammalian C subunits of PKAs are regulated by the phosphorylation of T197 residue on the
311 activation loop. In fission yeast, the phosphorylation of Pka1-T356 corresponding to mammalian
312 T197 was identified by wide-range mass spectrometric analysis (Seidler et al. 2009). Substitution of
313 Thr-356 with alanine was previously shown to inactivate Pka1 function (Tang and McLeod 2004).
314 The role of Thr-356 in Pka1 function was reexamined by creating *pka1-T356A* and *pka1-T356D*
315 mutants. The *pka1-T356A* mutant conjugated on rich medium with a higher mating efficiency than
316 that found for wild-type cells (9.34% versus 1.03% on glucose-rich medium). Conversely, the
317 *pka1-T356D* mutant (to mimic a constitutively phosphorylated site) showed vegetative growth in
318 glucose-rich medium and had a slightly lower mating efficiency than the wild type on glucose-rich
319 medium (Fig. 4A and B). The growth of the *pka1-T356A* mutant was inhibited on medium
320 containing 1.2 M KCl, whereas the *pka1-T356D* mutant was resistant to KCl in medium containing
321 1.6 M KCl (Fig. 4C), which confirmed the importance of the phosphorylation state of T356 in the
322 control of Pka1 function.

323 To test whether this mutation also affected the hyperphosphorylation of Pka1 that is induced by
324 glucose starvation, wild-type, *pka1-T356A*, and *pka1-T356D* strains were grown on YES medium
325 to mid-log phase and then shifted to YES medium containing 0.1% glucose to induce glucose
326 starvation. Samples were collected after six hours and the phosphorylation of proteins was
327 determined by SDS-PAGE and western blotting. The Pka1 protein was not shifted at 0 hr of
328 starvation (Fig. 4D) but after 6 hr it was shifted to a higher molecular weight, while Pka1-T356A
329 and Pka1-T356D were not. This finding suggested a role for T356 in the starvation-induced
330 hyperphosphorylation of Pka1.
331 As shown in Fig. 3A, inactive Pka1 was found largely in the cytoplasm. The localization of Pka1-
332 T356A and Pka1-T356D proteins was then assessed using indirect immunofluorescence
333 microscopy with an anti-Myc antibody and DAPI staining. Pka1-13Myc fluorescence was detected
334 throughout the cytoplasm and in the nucleus in the *pka1-T356A* mutant when cells were grown
335 under glucose-rich conditions. Interestingly, the fluorescence of Pka1-13Myc in the *pka1-T356D*
336 mutant was concentrated in the nucleus when cells were grown under glucose-rich conditions and it
337 showed a cytoplasmic distribution under glucose-starved conditions. The localization of Pka1-
338 T356D resembled that of wild-type Pka1 (Fig. 4E). The results showed that T356 is a critical amino
339 acid residue for the regulation of Pka1 and that the activity of Pka1 is important for its nuclear
340 import but not for export upon starvation.
341
342 Wild-type Pka1 cannot restore the function of mutant forms of Pka1
343 The findings that inactive Pka1-G441E/G441R mutant proteins were localized to the cytoplasm
344 and the active Pka1-T356D mutant protein was localized to the nucleus led us to test the possibility
345 of whether wild-type Pka1 could reverse the nuclear localization of G441E/G441R. Wild-type Pka1
346 was exogenously expressed in the DRG15 (*pka1-G441E-13Myc*) and DRG17 (*pka1-G441R-*
347 *13Myc*) strains that endogenously expressed 13Myc-tagged mutant Pka1 proteins. Indirect

348 immunofluorescence revealed that exogenously supplied wild-type Pka1 was unable to change the
349 nuclear localization of Pka1 mutant proteins under glucose-rich conditions. Mutant Pka1 proteins
350 were distributed throughout the cytoplasm (Fig. 5A). The same transformants were cultured on
351 PMAU medium, shifted to PMAU medium containing 0.1% glucose, and samples were collected.
352 As shown in Fig. 5B, the mutant Pka1 proteins were not hyperphosphorylated even after expressing
353 wild-type Pka1. Note that the expression of wild-type Pka1 in these mutants restored their
354 phenotypes, as in Fig. 1. These observations indicated that G441/G441R were inactive forms
355 regardless of the presence of active Pka1 and also suggested that Pka1 is not the kinase that
356 hyperphosphorylates Pka1.
357
358 Interaction of the regulatory subunit with the catalytic subunit in mutant strains
359 The catalytic subunit of PKA binds with its regulatory subunit when cAMP is absent or under
360 glucose-starved conditions. Pka1 and Cgs1 should interact with each other in cells grown on
361 glucose-limited medium. To observe the effect of Pka1 mutations on Pka1 interaction with the
362 regulatory subunit, a co-immunoprecipitation experiment was performed. Cell extracts were
363 prepared from DRG21C (*pka1-13Myc, cgs1-3HA*), DRG356DC (*pka1-T356D-13Myc, cgs1-3HA*),
364 DRG356AC (*pka1-T356A-13Myc, cgs1-3HA*), DRG15C (*pka1-G441E-13Myc, cgs1-3HA*), and
365 DRG17C (*pka1-G441R-13Myc, cgs1-3HA*) strains, which were grown on YES medium until mid-
366 log phase and then shifted to YES medium containing 0.1% glucose. The wild-type Pka1-13Myc
367 protein co-immunoprecipitated with Cgs1-3HA in wild-type cells but not in any of the *pka1*
368 mutants (Fig. 6A). In a reverse experiment, the Cgs1-3HA protein was coimmunoprecipitated with
369 the wild-type Pka1-13Myc protein but not with any of the Pka1 mutant proteins, which shows that
370 the hyperphosphorylation of Pka1 and its interaction with Cgs1 is interrelated.
371 To further confirm the requirement of Cgs1 binding for the hyperphosphorylation of Pka1, we
372 performed the coimmunprecipitation experiment in Pka1-13Myc, Cgs1-3HA doubled tagged strain

373 at different time points after glucose starvation. As shown in Fig. 6B the hyperphosphorylated Pka1
374 protein was found after 3 hr of glucose starvation and the coimmunprecipitation with Cgs1 was
375 observed after 1 hr of glucose starvation. In a reverse experiment, the coimmunprecipitation of
376 Pka1 with Cgs1 was observed after 1 hr of glucose starvation (Fig. 6B), prior to Pka1
377 hyperphosphorylation. Thus, these studies clearly demonstrate that the formation of a Pka1-Cgs1
378 complex precedes the hyperphosphorylation of Pka1. All results consistently indicate that the
379 hyperphosphorylation of Pka1 occurs after Pka1- Cgs1 complex formation.
380
381 **Discussion**
382 Identification of *pka1* mutants.
383 The cAMP-PKA pathway plays a central role in the transmission of glucose signals to the
384 expression of genes under the control of transcription factor Ste11 in *S. pombe* Pka1 (Hoffman
385 2005; Maeda et al. 1994), the catalytic subunit of cAMP-dependent protein kinase, is a tightly
386 regulated enzyme that has multiple roles in growth regulation, transcription and metabolism
387 (Gibson and Taylor 1997). Disruption of *pka1* slows cell growth but is not lethal in *S. pombe*;
388 whereas, at least one of three C subunits of PKA variants in *S. cerevisiae* is required for survival
389 (Toda et al. 1987). This difference makes *S. pombe* more tractable for the functional analysis of
390 PKA. From the analysis of *sam* mutants, which do not need to undergo starvation for mating
391 (Oowatari et al.; Oowatari et al. 2009), non-functional *pka1* mutations in *sam5* and *sam7* mutants
392 were identified (Fig. 1). Despite the importance of the role of Pka1 in fission yeast, mutational
393 analysis of *pka1* has not been extensively pursued. The finding that *sam5* and *sam7* mutants possess
394 a missense allele (G441E and G441R) of *pka1* has provided a way to study the regulation of Pka1.
395 Since the same amino acid is altered in *sam5* and *sam7*, glycine 441 must be important for Pka1
396 function. When the *pka1* locus was replaced with either a *pka1-G441E* or a *pka1-G441R* mutant
397 gene in wild-type cells, the phenotypes were indistinguishable from those of *sam* mutants,

398 confirming that *sam5* and *sam7* phenotypes are caused by mutations in *pka*1 (Fig. 2). In *sam5* and
399 *sam7* mutants, Ste11 and Rst2 were localized to the nucleus, even under glucose-rich conditions,
400 and Rst2 was hyperphosphorylated. These results were consistent with the results obtained with a
401 *pka1* deletion mutant (Higuchi et al. 2002) and indicated that Pka1 activity should be very low in
402 *pka1-G441E* and *pka1-G441R* mutants.
403 Distribution of Pka1
404 Activation of PKA is mediated by increases in the concentrations of cAMP that result in the release
405 of the catalytic subunit. PKA is then imported into the nucleus where it regulates the expression of
406 its substrates. It has been shown that, under glucose-limited conditions, both Pka1 and Cgs1 are
407 distributed in both the cytoplasm and the nucleus (Matsuo et al. 2008). In cells lacking Cgs1, Pka1
408 is primarily concentrated in the nucleus and displays only a diffuse staining pattern in the cytoplasm.
409 Similarly, under glucose-rich conditions, both subunits are concentrated in the nucleus, with a
410 diffuse distribution in the cytoplasm; however, the nuclear localization of Pka1 is independent of
411 Cgs1. We found that the inactive Pka1-T356A protein resides in both the cytoplasm and the nucleus,
412 even under glucose-rich conditions (Fig. 4). Pka1-T356A retained some activity, as judged by the
413 phenotypes of its mutants, and its nuclear exclusion was incomplete. Conversely, the active Pka1-
414 T356D protein was completely localized to the nucleus. These observations suggest that Pka1
415 protein activity is important for its nuclear import and are consistent with the observation that the
416 G441E and G441R Pka1 mutations block the nuclear localization of Pka1. However, how this is
417 controlled is not clear. Autoregulatory mechanisms of Pka1 are unlikely because the expression of
418 wild-type Pka1 did affect the localization of Pka1- G441R (Fig. 5).
419 Interaction of Pka1 and Cgs1
420 Pka1 interacted with Cgs1 in wild-type cells grown under glucose-starved conditions. However,
421 Pka1-T356A, which lacks Pka1 activity, did not interact with Cgs1. This defect could not be
422 ascribed to a defect in the interaction between Pka1 and Cgs1, as Pka1-T356D, an active form, also

failed to interact with Cgs1 under conditions of glucose starvation. These results indicated that phosphorylation at T356 is important for the interaction of Pka1 with Cgs1. In *S. cerevisiae*, interactions between catalytic and regulatory subunits of PKA depend on a conserved phospho-Thr-241 in Tpk1 when cAMP levels are low. A substitution of Thr-241 with alanine reduced binding between catalytic and regulatory subunits, but substitution of Thr-241 with aspartate did not (Levin and Zoller 1990). Although the fission yeast Pka1-T356D mutant is active as a kinase, it may not completely mimic Pka1 in its ability to bind to the R-subunit. The Thr-197 residue of the mammalian C-subunit of PKA is located in region that makes contact with the R- subunit, and this region is important for both the activity of the R-subunit and its interaction with the C-subunit. Our results suggest that Thr-356 of Pka1 is important for both its activity and interaction with Cgs1 in fission yeast; however, the loss of the interaction between the C and R subunits observed in the Pka1-G441E and G441R mutant might require another explanation. The Pka1-T356D G441R double mutant integrated at the chromosomal locus displayed a phenotype indistinguishable from that of the Pka1-G441R mutant (data not shown), indicating that the G441R mutation at least did not block the T356 site. Gly-441 in Pka1 is part of the catalytic sub-domain XI, which is known as a COOH terminal boundary region according to the classification of protein kinase superfamily (Hanks and Hunter 1995). However, the function of this boundary domain is poorly defined. We found that this region was important for both Pka1 activity and its interaction with the R-subunit. The corresponding residue (Gly-282) is conserved in mammalian C-subunits and the three dimensional structure of mammalian PKA complex shows that sub-domain XI is located at the interaction surface with the R-subunit (Kim et al. 2007). Sub-domain XI of the G441R mutant might be too disordered to serve as a scaffold for the R-subunit.

Phosphorylation of Pka1

Tang and McLeod showed that the upper band shift of Pka1 on an SDS-PAGE gel was abolished in a *ksg1* mutant and also by the T356A mutation (Tang and McLeod 2004), demonstrating that

448 Thr-356 of Pka1 is phosphorylated by Ksg1 (a phosphoinositide-dependent protein kinase).
449 McInnis et al. reported that Pka1 was hyperphosphorylated in a *cyr1* deletion mutant and that the
450 hyperphosphorylated form was even more abundant in cells grown under glucose-starved
451 conditions or in stationary phase (McInnis et al. 2010). They also found no band shift of Pka1 in
452 cells grown on glucose rich medium. The results presented in the present study are consistent with
453 those of McInnis et al. A clear band shift of Pka1 was observed in cells grown under glucose-
454 starved conditions, but not in cells grown under glucose rich conditions. We found that substitution
455 of Thr-356 with alanine abolished the band shift of Pka1 in cells grown under conditions of glucose
456 starvation, and also we observed that the upper band shift of Pka1 found after glucose stravations
457 was abolished in a *ksg1* mutant (data not show), which confirms the results of Tang and McLeod
458 with the notable exception that they used glucose rich medium. If stationary phase cells had been
459 used, a Pka1 band shift would have been observed in their experiments, but the interpretations of a
460 Pka1 band shift would have been different. Our results indicate that the idea describing
461 phosphorylation of Thr-356 of Pka1 by Ksg1 was prematurely concluded by Tang and McLeod.
462 Since wide-range proteomic analysis identified the phosphopeptide containing Thr-356 (Wilson-
463 Grady et al. 2008) as the sole phosphopeptide in Pka1, Thr-356 is apparently the major
464 phosphorylation site of Pka1 in fission yeast. However, our results and the results of McInnis et al.
465 suggested that the upper band shift of Pka1 cannot be ascribed to phosphorylation of Thr-356 for
466 the reasons listed below. Under glucose-starved conditions, Pka1 and Cgs1 form a holoenzyme
467 complex in which Pka1 is hyperphosphorylated, as indicated by the band shift and also the
468 hyperphosphorylation of Pka1 occurs only after Pka1- Cgs1 complex formation (Fig. 6). Deletion
469 of Cgs1 results in a loss of its interaction with Pka1, and Pka1 protein is not hyperphosphorylated
470 under this condition; however, it remains sufficiently active for the execution of its function as a
471 protein kinase. If the detected band shift is caused by the phosphorylation of Thr-356 and the lower
472 band is the dephosphorylated form, it would contradict the observed state of Pka1 activity. The

three dimensional structure of the PKA holoenzyme also predicts that Thr-356 is located on the surface that makes contact with the R subunit (Kim et al. 2007). Furthermore, the Pka1 mutant proteins, such as G441R, G441E, T356A, and T356D that failed to interact with Cgs1 were not hyperphosphorylated under glucose-starved conditions. In mammals, Thr197 is constitutively phosphorylated, but is not detected as an upper band shift during SDS-PAGE. Those obsevations strongly suggest that the hyperphosphorylation of Pka1 detected as an upper band shift after binding with Cgs1 is an inhibitory phosphorylation of Pka1.

Conclusions

This study demonstrates that Thr-356 phosphorylation in the activation loop of Pka1 is important for Pka1 activity and that its activity is required for its nuclear localization. Pka1-Thr-356 is also critical for binding of Pka1 to its regulatory subunit Cgs1. The Gly-441 residue plays an important role for both Pka1 activity and its binding to Cgs1. Hyperphosphorylation of Pka1 occurs after complex formation with Cgs1. Fission yeast Pka1 is regulated spatially and physically by glucose signaling and phosphorylation.

Acknowledgments

This study was partly supported by the Sasakawa Scientific Research Grant from The Japan Science Society. We thank Dr. T. Kaino, Dr. T Nakagawa, K. Tanae, and Y. Matsuo for helpful discussion and for experimental support.

References

Alfa C, Fantes P, Hyams J, McLeod M, Warbrick E (1993) Experiments with fission yeast. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

DeVoti J, Seydoux G, Beach D, McLeod M (1991) Interaction between ran1+ protein kinase and cAMP dependent protein kinase as negative regulators of fission

yeast meiosis. Embo J 10: 3759-3768
Gibson RM, Taylor SS (1997) Dissecting the cooperative reassociation of the regulatory and catalytic subunits of cAMP-dependent protein kinase. Role of Trp-196 in the catalytic subunit. J Biol Chem 272: 31998-32005
Goldar MM, Jeong HT, Tanaka K, Matsuda H, Kawamukai M (2005) Moc3, a novel Zn finger type protein involved in sexual development, ascus formation, and stress response of *Schizosaccharomyces pombe.* Curr Genet 48: 345-355
Hanks SK, Hunter T (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. Faseb J 9: 576-596
Higuchi T, Watanabe Y, Yamamoto M (2002) Protein kinase A regulates sexual development and gluconeogenesis through phosphorylation of the Zn finger transcriptional activator Rst2p in fission yeast. Mol Cell Biol 22: 1-11
Hoffman CS (2005) Glucose sensing via the protein kinase A pathway in *Schizosaccharomyces pombe*. Biochem Soc Trans 33: 257-260
Jeong HT, Oowatari Y, Abe M, Tanaka K, Matsuda H, Kawamukai M (2004a) Interaction between a negative regulator (Msa2/Nrd1) and a positive regulator (Cpc2) of sexual differentiation in *Schizosaccharomyces pombe.* Biosci Biotechnol Biochem 68: 1621-1626
Jeong HT, Ozoe F, Tanaka K, Nakagawa T, Matsuda H, Kawamukai M (2004b) A novel gene, *msa1*, inhibits sexual differentiation in *Schizosaccharomyces pombe.* Genetics 167: 77-91
Kao RS, Morreale E, Wang L, Ivey FD, Hoffman CS (2006) *Schizosaccharomyces pombe* Git1 is a C2-domain protein required for glucose activation of adenylate cyclase. Genetics 173: 49-61
Katayama S, Ozoe F, Kurokawa R, Tanaka K, Nakagawa T, Matsuda H, Kawamukai M (1996) Genetic analysis of the *sam* mutations, which induce sexual development with no requirement for nutritional starvation in fission yeast. Biosci. Biotechnol. Biochem. 60: 994-999
Kawamukai M (1999) Isolation of a novel gene, *moc2*, encoding a putative RNA helicase as a suppressor of sterile strains in *Schizosaccharomyces pombe.* Biochim Biophys Acta 1446: 93-101
Kawamukai M, Ferguson K, Wigler M, Young D (1991) Genetic and biochemical analysis of the adenylyl cyclase of *Schizosaccharomyces pombe.* Cell Regul 2: 155-164
Kawamukai M, Gerst J, Field J, Riggs M, Rodgers L, Wigler M, Young D (1992) Genetic and biochemical analysis of the adenylyl cyclase-associated protein, cap, in *Schizosaccharomyces pombe*. Mol Biol Cell 3: 167-180

539 Kim C, Cheng CY, Saldanha SA, Taylor SS (2007) PKA-I holoenzyme structure
540 reveals a mechanism for cAMP-dependent activation. Cell 130: 1032-1043
541 Krawchuk MD, Wahls WP (1999) High-efficiency gene targeting in
542 *Schizosaccharomyces pombe* using a modular, PCR-based approach with long
543 tracts of flanking homology. Yeast 15: 1419-1427
544 Kunitomo H, Higuchi T, Iino Y, Yamamoto M (2000) A zinc-finger protein, Rst2p,
545 regulates transcription of the fission yeast *ste11*(+) gene, which encodes a
546 pivotal transcription factor for sexual development. Mol Biol Cell 11: 3205-
547 3217
548 Levin LR, Kuret J, Johnson KE, Powers S, Cameron S, Michaeli T, Wigler M, Zoller
549 MJ (1988) A mutation in the catalytic subunit of cAMP-dependent protein
550 kinase that disrupts regulation. Science 240: 68-70
551 Levin LR, Zoller MJ (1990) Association of catalytic and regulatory subunits of cyclic
552 AMP-dependent protein kinase requires a negatively charged side group at a
553 conserved threonine. Mol Cell Biol 10: 1066-1075
554 Maeda T, Watanabe Y, Kunitomo H, Yamamoto M (1994) Cloning of the *pka1* gene
555 encoding the catalytic subunit of the cAMP-dependent protein kinase in
556 *Schizosaccharomyces pombe*. J Biol Chem 269: 9632-9637
557 Matsuo Y, Asakawa K, Toda T, Katayama S (2006) A rapid method for protein
558 extraction from fission yeast. Biosci Biotechnol Biochem 70: 1992-1994
559 Matsuo Y, Kishimoto H, Tanae K, Kitamura K, Katayama S, Kawamukai M (2011)
560 Nuclear protein quality is regulated by the ubiquitin-proteasome system
561 through the activity of Ubc4 and San1 in fission yeast. J Biol Chem 286:
562 13775-13790
563 Matsuo Y, McInnis B, Marcus S (2008) Regulation of the subcellular localization of
564 cyclic AMP-dependent protein kinase in response to physiological stresses and
565 sexual differentiation in the fission yeast *Schizosaccharomyces pombe*.
566 Eukaryot Cell 7: 1450-1459
567 McInnis B, Mitchell J, Marcus S (2010) Phosphorylation of the protein kinase A
568 catalytic subunit is induced by cyclic AMP deficiency and physiological
569 stresses in the fission yeast, *Schizosaccharomyces pombe*. Biochem Biophys
570 Res Commun 399: 665-669
571 Moore MJ, Kanter JR, Jones KC, Taylor SS (2002) Phosphorylation of the catalytic
572 subunit of protein kinase A. Autophosphorylation versus phosphorylation by
573 phosphoinositide-dependent kinase-1. J Biol Chem 277: 47878-47884
574 Oowatari Y, Jeong HT, Tanae K, Nakagawa T, Kawamukai M (2011) Regulation and
575 role of an RNA-binding protein Msa2 in controlling the sexual differentiation
576 of fission yeast. Current Genetics 57: 191-200

577 Oowatari Y, Toma K, Ozoe F, Kawamukai M (2009) Identification of *sam4* as a *rad24*
578 allele in *Schizosaccharomyces pombe*. Biosci Biotechnol Biochem 73: 1591-
579 1598
580 Paul SK, Oowatari Y, Kawamukai M (2009) A large complex mediated by Moc1, Moc2
581 and Cpc2 regulates sexual differentiation in fission yeast. FEBS J 276: 5076-
582 5093
583 Prentice HL (1992) High efficiency transformation of *Schizosaccharomyces pombe* by
584 electroporation. Nucleic Acids Res 20: 621
585 Qin J, Kang W, Leung B, McLeod M (2003) Ste11p, a high-mobility-group box DNA-
586 binding protein, undergoes pheromone- and nutrient-regulated nuclear-
587 cytoplasmic shuttling. Mol. Cell Biol. 23: 3253-3264
588 Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a lablatory manual,
589 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
590 Seidler J, Adal M, Kubler D, Bossemeyer D, Lehmann WD (2009) Analysis of
591 autophosphorylation sites in the recombinant catalytic subunit alpha of
592 cAMP-dependent kinase by nano-UPLC-ESI-MS/MS. Anal Bioanal Chem
593 395: 1713-1720
594 Tanabe K, Ito N, Wakuri T, Ozoe F, Umeda M, Katayama S, Tanaka K, Matsuda H,
595 Kawamukai M (2003) Sla1, a *Schizosaccharomyces pombe* homolog of the
596 human La protein, induces ectopic meiosis when its C terminus is truncated.
597 Eukaryot Cell 2: 1274-1287
598 Tanabe K, Tanaka K, Matsuda H, Kawamukai M (2004) Truncated Sla1 induces
599 haploid meiosis through the Pat1-Mei2 system in fission yeast. Biosci
600 Biotechnol Biochem 68: 266-270
601 Tang Y, McLeod M (2004) In vivo activation of protein kinase A in
602 *Schizosaccharomyces pombe* requires threonine phosphorylation at its
603 activation loop and is dependent on PDK1. Genetics 168: 1843-1853
604 Taylor SS, Buechler JA, Yonemoto W (1990) cAMP-dependent protein kinase:
605 framework for a diverse family of regulatory enzymes. Annu Rev Biochem 59:
606 971-1005
607 Toda T, Cameron S, Sass P, Zoller M, Wigler M (1987) Three different genes in *S.*
608 *cerevisiae* encode the catalytic subunits of the cAMP-dependent protein
609 kinase. Cell 50: 277-287
610 Tudisca V, Recouvreux V, Moreno S, Boy-Marcotte E, Jacquet M, Portela P (2010)
611 Differential localization to cytoplasm, nucleus or P-bodies of yeast PKA
612 subunits under different growth conditions. Eur J Cell Biol 89: 339-348
613 Welton RM, Hoffman CS (2000) Glucose monitoring in fission yeast via the Gpa2
614 galpha, the git5 Gbeta and the git3 putative glucose receptor. Genetics 156:

513-521
Wilson-Grady JT, Villen J, Gygi SP (2008) Phosphoproteome analysis of fission yeast. J Proteome Res 7: 1088-1097
Yakura M, Ishikura Y, Adachi Y, Kawamukai M (2006a) Involvement of Moc1 in sexual development and survival of *Schizosaccharomyces pombe.* Biosci Biotechnol Biochem 70: 1740-1749
Yakura M, Ozoe F, Ishida H, Nakagawa T, Tanaka K, Matsuda H, Kawamukai M (2006b) *zds1*, a novel gene encoding an ortholog of Zds1 and Zds2, controls sexual differentiation, cell wall integrity and cell morphology in fission yeast. Genetics 172: 811-825
Yamamoto M, 2003 *Initiation of meiosis.* Springer-Verlag, Berlin.
Yang P, Du H, Hoffman CS, Marcus S (2003) The phospholipase B homolog Plb1 is a mediator of osmotic stress response and of nutrient-dependent repression of sexual differentiation in the fission yeast *Schizosaccharomyces pombe*. Mol Genet Genomics 269: 116-125
Yonemoto W, McGlone ML, Grant B, Taylor SS (1997) Autophosphorylation of the catalytic subunit of cAMP-dependent protein kinase in Escherichia coli. Protein Eng 10: 915-925
Zhou GL, Yamamoto T, Ozoe F, Yano D, Tanaka K, Matsuda H, Kawamukai M (2000) Identification of a 14-3-3 protein from *Lentinus edodes* that interacts with CAP (adenylyl cyclase-associated protein), and conservation of this interaction in fission yeast. Biosci. Biotechnol. Biochem. 64: 149-159

639 **Figure legends**

640 **Fig. 1. Suppression of *sam5* and *sam7* by the expression of *pka1*.**

641 (A) The *pka1* gene was expressed in HS422 (*sam5*) and HS426 (*sam7*) mutants. Each strain
642 harboring pSLF172L (vector) or pSLF172L-*pka1* was grown under repressed and de-repressed
643 conditions and spotted on PMAU and PMAU containing 1 M KCl to assess KCl sensitivity. (B)
644 Transformants were observed under a microscope. (C) Mating efficiencies (%) of the same strains
645 were measured.

646

647 **Fig. 2. Phenotypes of the *pka1* single mutant.**

648 (A) KCl sensitivity of wild type and different *pka1* mutants was tested. Cells were streaked on YES
649 and YES containing 1 M KCl and incubated at 30°C for 5 days. (B) DRG21 (*pka1-13Myc*), DRG15
650 (*pka1-G441E-13Myc*) and DRG17 (*pka1-G441R-13Myc*) strains grown under glucose-rich
651 medium were observed by microscopy and their mating efficiencies were measured. (C) The Pka1
652 protein in DRG21, DRG15, and DRG17 strains was detected by western blotting. Cell extracts
653 prepared from indicated strains were analyzed on a 10% polyacrylamide gel and the Pka1-13Myc
654 protein was detected using an anti-Myc antibody.

655

656 **Fig. 3. Localization and phosphorylation of Pka1 in wild type, *sam5,* and *sam7* mutants.**

657 (A) DRG21 (*pka1-13Myc*), DRG15 (*pka1-G441E-13Myc*) and DRG17 (*pka1-G441R-13Myc*)
658 strains were grown on YES or glucose-depleted YES medium and indirect immunofluorescence
659 was performed with anti-Myc antibody as described in Fig. 4B. (B) Cell extracts were prepared
660 from DRG21 (WT *pka1-13Myc*), DRG15 (*pka1-G441E-13Myc*), and DRG17 (*pka1-G441R-*
661 *13Myc*) strains at indicated time of glucose starvation and subjected to SDS-PAGE as described in
662 Fig. 4A. Pka1-13Myc protein was detected using an anti-Myc antibody. Anti-tubulin was used as
663 an internal loading control.

664

665 **Fig. 4. Phenotype, phosphorylation, and localization of Thr-356 residue of Pka1 under**
666 **glucose starvation.**

667 (A) Phenotype of Pka1-T356D and Pka1-T356D mutants. DRG21 (WT; *pka1-13Myc*), DRG356D
668 (*pka1-T356D-13Myc*) and DRG356A (*pka1-T356A-13Myc*) strains were grown on YES medium
669 plates and photographs were taken after two days of incubation. (B) Mating efficiency of the *pka1*
670 mutants. Cells were cultured on PMALU medium and then shifted to PMALU medium containing
671 1% glucose without nitrogen and incubated for 24 hours. Cells were fixed with formaldehyde and
672 mating cells were counted using a microscope. (C) KCl sensitivity. Cells were grown in YES
673 medium to mid-log phase, synchronized at 1 x 10^7 /ml, spotted on YES plates containing different
674 concentrations of KCl, and then incubated for 5 days. A *sty1* mutant was included as a negative
675 control. (D) Phosphorylation of T356 under glucose starvation. Cell extracts were prepared from
676 DRG21 (WT; *pka1-13Myc*), DRG356D (*pka1-T356D-13Myc*) and DRG356A (*pka1-T356A-*
677 *13Myc*) strains and loaded on SDS-PAGE. Proteins were detected using an anti-Myc or anti-tubulin
678 antibody. (E) DRG356D (*pka1-T356D*) and DRG356A (*pka1-T356A*) mutant cells were grown on
679 YES or YES containing 0.1% glucose medium and indirect immunoflourescence was performed
680 with an anti-Myc antibody.

681

682 **Fig. 5. Effect of expressing wild type Pka1 in *pka1* mutants.**

683 (A) Localization of mutant Pka1 proteins in RDG15 (Pka1-G441E) and DRG17 (Pka1-G441R)
684 strains expressing the wild type Pka1 on a plasmid (pSLF172L-*pka1*). Localization of Pka1
685 proteins was observed by indirect immunofluorescence micrsoscopy using an anti-Myc antibody as
686 in Fig. 4B. (B) Phosphorylation of mutant Pka1 proteins in cells expressing the wild type Pka1. Cell
687 extracts were prepared as described in Materials and methods and subjected to SDS-PAGE for
688 Western blot analysis.

Fig. 6. Phosphorylation at T356 is important for interactions between catalytic and regulatory subunits.

Cell extracts were prepared from DRG21C (*pka1-13Myc, cgs1-3HA*), DRG356DC (*pka1-T356D-13Myc, cgs1-3HA*), DRG356AC (*pka1-T356A-13Myc, cgs1-3HA*), DRG15C (*pka1-G441E-13Myc, cgs1-3HA*), DRG17C (*pka1-G441R-13Myc, cgs1-3HA*), and SP870 (wild-type). All strains were grown in YES medium to mid log phase and then shifted to YES medium containing 0.1% glucose. Cell extracts were incubated with an HA antibody and a Myc antibody. Protein G Sepharose beads were added to the mixtures to co-immunoprecipitate Pka1, and protein A Sepharose beads were added to coimmunoprecipitate Cgs1. The co-immunoprecipitated proteins were analyzed by western blotting using an anti-Myc or an anti-HA antibody. (B) Cell extracts were prepared from DRG21 strain at the indicated times and coimmunoprecipitation was performed as described above. Proteins were detected by an anti-Myc or an anti-HA antibody. * indicates a nonspecific background protein.

Table 1. *S. pombe* strains used in the study.

SP870	*h^{90} ade6-210 leu1-32 ura4-D18*	M. Kawamukai
HS422	*h^{90} ade6-216 leu1-32 ura4-D18, sam5*	M. Kawamukai
HS426	*h^{90} ade6-216 leu1-32 ura4-D18, sam7*	M. Kawamukai
JZ633	*h^{90} ade6-216 leu1-32 ura4-D18 pka1::ura4*	M. Yamamoto
TK105	*h^{90} leu1-32 ura4-D18 sty1::ura4*	T. Kato
DRG15	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-G441E-13Myc- kanMX6*	This study
DRG17	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-G441R-13Myc- kanMX6*	This study
DRG21	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-13Myc-kanMX6*	This study
DRG21C	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-13Myc-kanMX6 cgs1-3HA-hphMX6*	This study
DRG25	*h^{90} ade6-216 leu1-32 ura4-D18 sam5-pka1^{+}-13Myc- kanMX6*	This study
DRG27	*h^{90} ade6-216 leu1-32 ura4-D18 sam7-pka1^{+}-13Myc- kanMX6*	This study
DRG70	*h^{90} ade6-210 leu1-32 ura4-D18 rst2-3HA-hphMX6*	This study
DRG15R	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-G441E-13Myc-kanMX6 rst2-3HA-hphMX6*	This study
DRG17R	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-G441R-13Myc-kanMX6 rst2-3HA-hphMX6*	This study
DRG74	*h^{90} ade6-216 leu1-32 ura4-D18 pka1::ura4 rst2-3HA-hphMX6*	This study
DRG50	*h^{90} ade6-210 leu1-32 ura4-D18 ste11-GFP-kanMX6*	This study
DRG55	*h^{90} ade6-216 leu1-32 ura4-D18 ste11-GFP-kanMX6 sam5*	This study
DRG57	*h^{90} ade6-216 leu1-32 ura4-D18 ste11-GFP-kanMX6 sam7*	This study
DRG15C	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-G441E-13Myc-kanMX6 cgs1-3HA-hphMX6*	This study
DRG17C	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-G441R-13Myc-kanMX6 cgs1-3HA-hphMX6*	This study
DRG356A	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-T356A-13Myc-kanMX6*	This study
DRG356D	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-T356D-13Myc-kanMX6*	This study
DRG356AC	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-T356A-13Myc-kanMX6 cgs1-3HA-hphMX6*	This study
DRG356DC	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-T356D-13Myc-kanMX6 cgs1-3HA-hphMX6*	This study

Supplementary Table 1. List of primers

Sequencing primer		
Pka1-F1	5'-CTTTGAAGGACTCAGAGTCG -3'	
Pka1-F2	5'-TAGTAGCCAAAGCAGCCATC -3'	
Pka1-FM1	5'-GACCTTTTTGCCTCGACC-3'	
Pka1-R1	5'-ACGAGCCAGTGCCCAATG-3'	
Pka1-R2	5'-CATCAGAGCAGGCTAATTGC-3'	
Pka1-RM1	5'-AGGAACATACGGAACCTC-3'	
Primers for plasmid construction		
Pka1-F-*Bgl*II	5'-ACATTAGATCT (*Bgl*II) CATGGATACGACTGC-3'	
Pka1-R-*Not*I	5'-CACGCGGCCGC (*Not*I) AAAAGTCCTTAAAGATAG-3'	
Mutagenesis primers		
Pka1T356A-F	5'- CTACTAGCAACTGTTGTGCTCTTTGTGGTACCCCC-3'	
Pka1T356A-R	5'-GGGGGTACCACAAAGAGCACAACAGTTGCTAGTAG-3'	
Pka1T356D-F	5'- CTACTAGCAACTGTTGTGATCTTTGTGGTACCCCC-3'	
Pka1T356D-R	5'-GGGGGTACCACAAAGATCACAACAGTTGCTAGTAG-3'	
Pka1 tagging primers		
Pka1-W	5'- TTGCCAAACGCGTCTCTAC-3'	
Pka1-X	5'-GGGGATCCGTCGACCTGCAGCGTACGAAAAGTCCTTAAAGATAGAAG-3'	
Pka1-Y	5'- GTTTAAACGAGCTCGAATTCATCGATGCGTTGAGCAACGAATGCC-3'	
Pka1-Z	5'- TTTGGGAGCCTGTGCTTAG-3'	
Rst2 tagging primers		
Rst2-W	5' TTCGATCCCTTCTGGATTGC-3'	
Rst2-X	5' GGGGATCCGTCGACCTGCAGCGTACGAAAATGAGGGCGGTTGATTC-3'	
Rst2-Y	5' GTTTAAACGAGCTCGAATTCATCGATAGGATTGATCTGAAGTTTTG-3'	
Rst2-Z	5' AGATTCAGGG CAGTAAAC-3'	

Restriction enzyme sites are underlined.

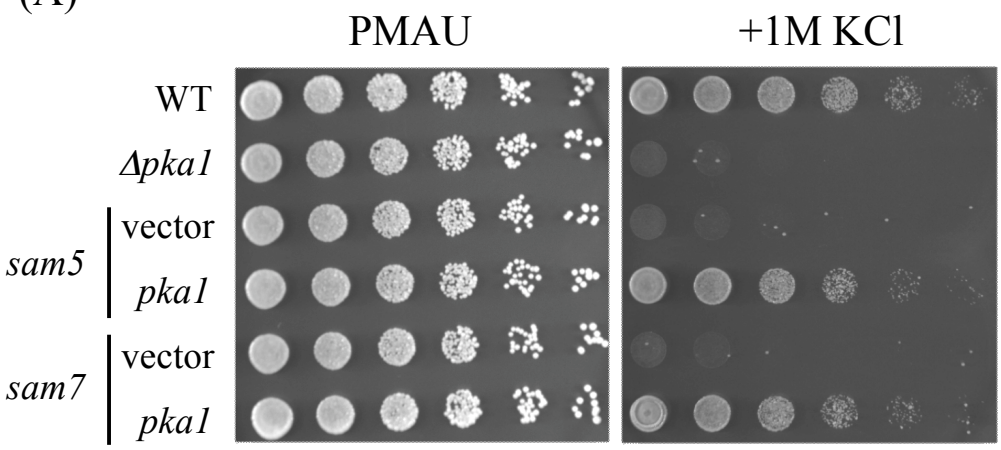
Supplementary Fig. 1 Ste11 expression and localization in *sam5* and *sam7* mutants under nutrient-rich conditions.

(A) Localization of Ste11-GFP protein in DRG50 (WT; *ste11-GFP*), DRG55 (*sam5*, *ste11-GFP*) and DRG57 (*sam7*, *ste11-GFP*) mutants. Cells were grown on PMALU medium with or without nitrogen to early log-phase and GFP fluorescence images were observed using a fluorescence microscope. (B) Ste11 expression in *sam5* and *sam7* mutants. DRG50, DRG55, and DRG57 strains were grown on PMALU medium to mid-log phase and then shifted to PMALU-nitrogen containing 1% glucose. Samples were collected and subjected to SDS-PAGE. An anti-GFP antibody was used to detect the Ste11-GFP protein. An anti-PSTAIRE (Cdc2) was used as a loading control.

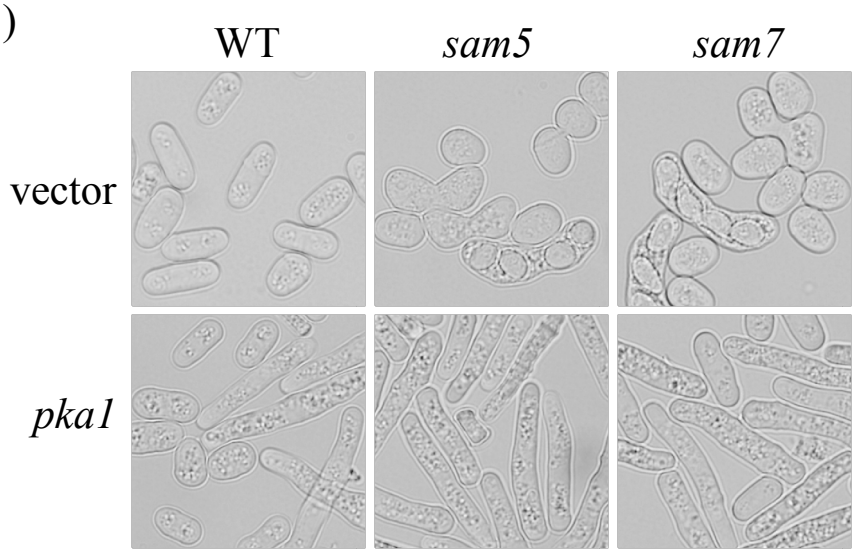
Supplementary Fig. 2. Phosphorylation and localization of Rst2 in *sam5* and *sam7* mutants under nutrient-rich conditions.

(A) To determine the phosphorylation status of Rst2, extracts were prepared from cells grown in YES containing 3% glucose and then half of the culture was shifted to YES medium containing 0.1% glucose and incubated for 2 hours. Proteins were analyzed using SDS-PAGE and western blot analysis. (B) Indirect immunofluorescence of Rst2-3HA. DRG70 (WT; *pka1*$^{+}$, *rst2-3HA*), DRG15R (*pka1-G441E, rst2-3HA*), DRG17R (*pka1-G441R*, *rst2-3HA*), and DRG74 (Δ*pka1*, *rst2-3HA*) cells were grown on YES medium containing 3% glucose to early-log phase and fixed with formaldehyde. Cells were stained with an anti-HA antibody and counterstained with DAPI to visualize the nucleus.

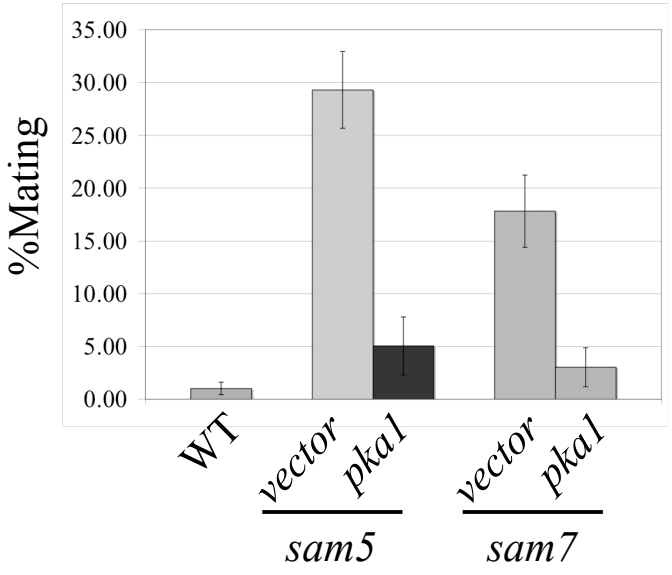
(A)



(B)

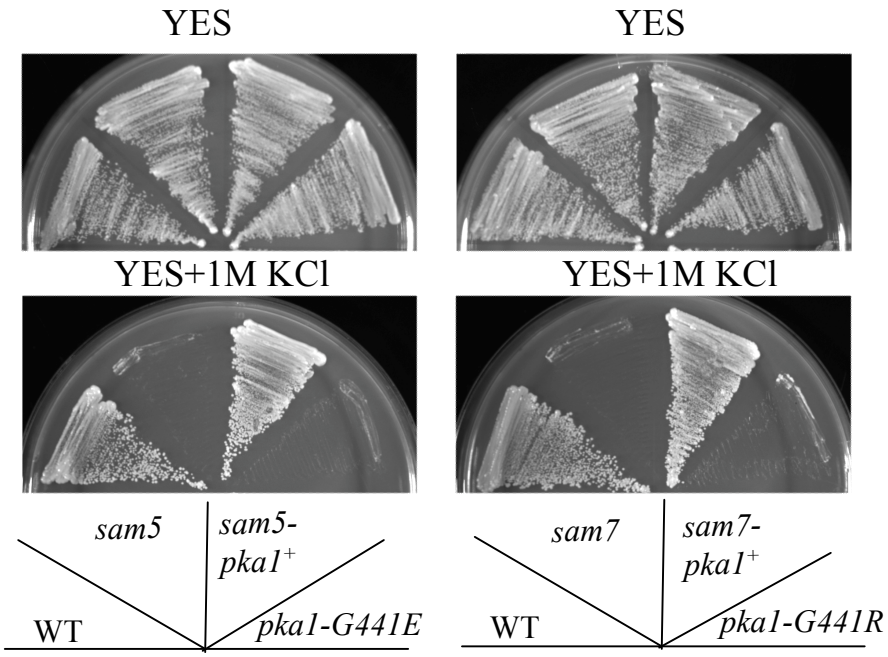


(C)

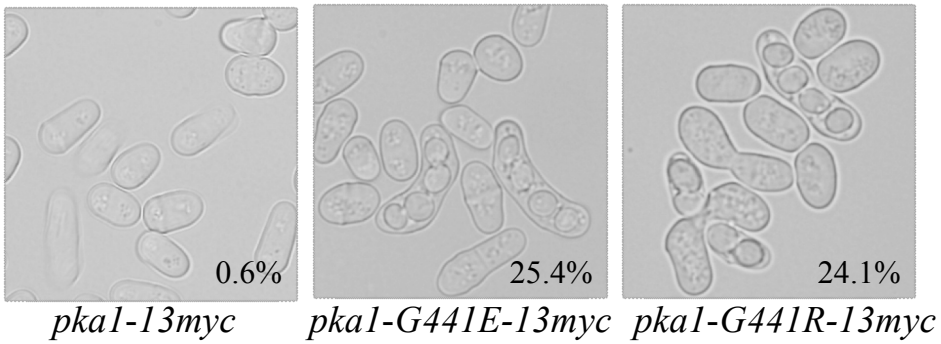


Gupta et al., Fig. 1

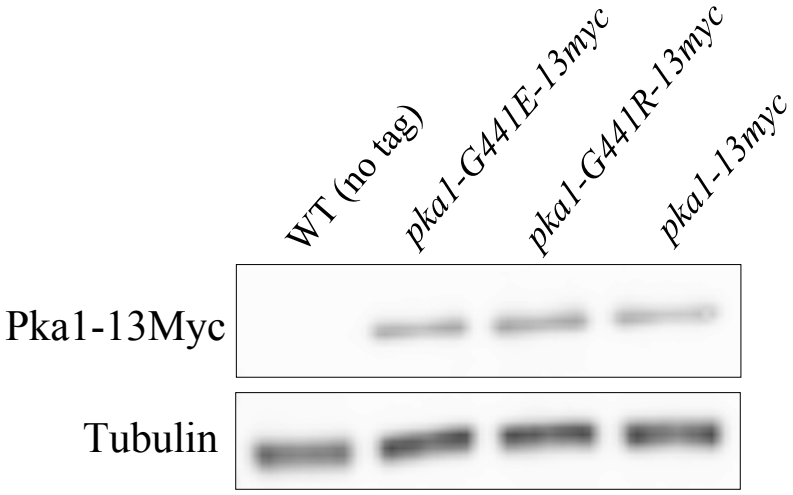
(A)



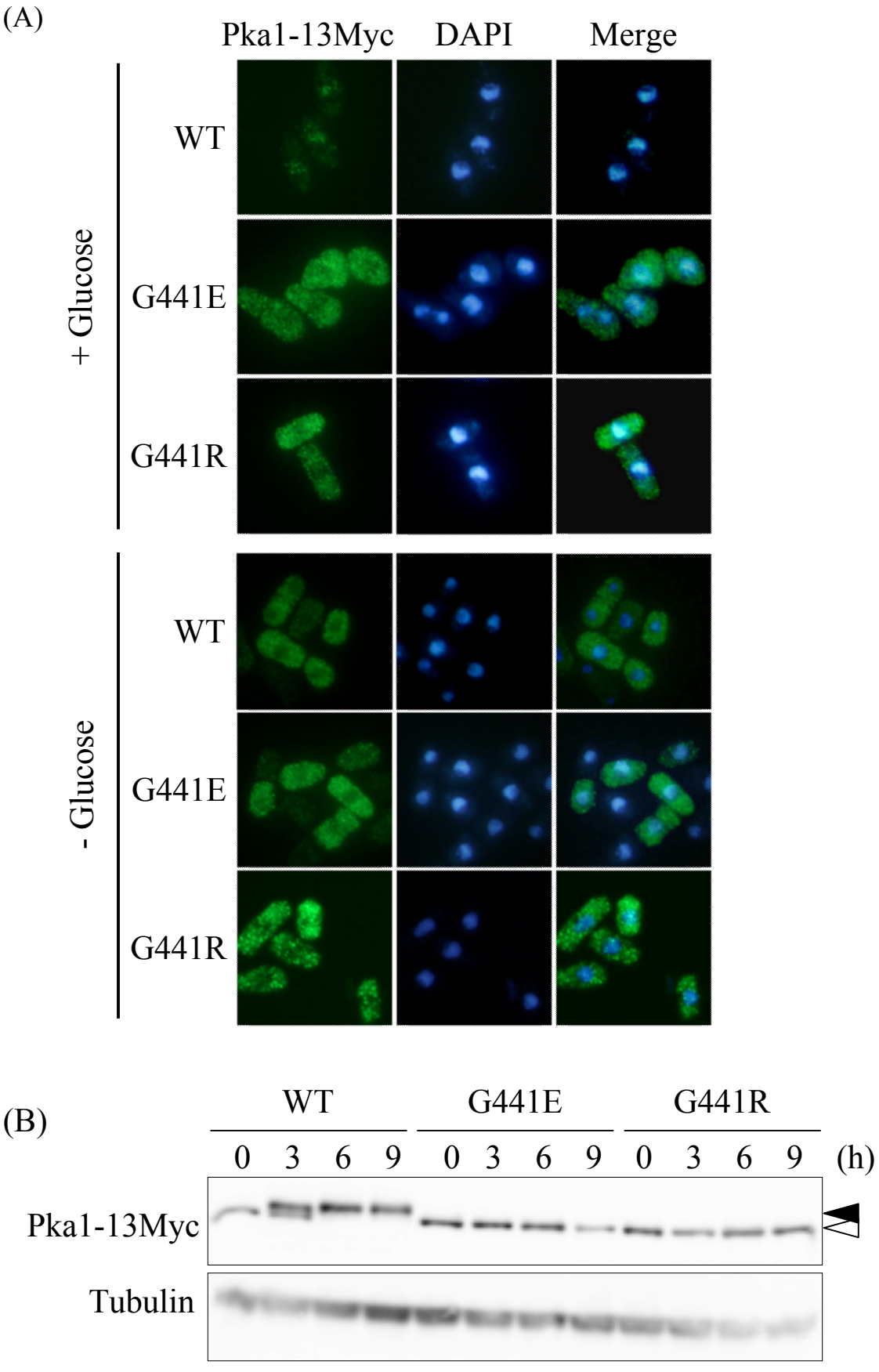
(B)



(C)

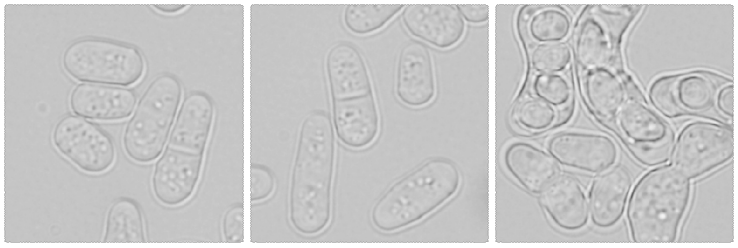


Gupta et al., Fig. 2



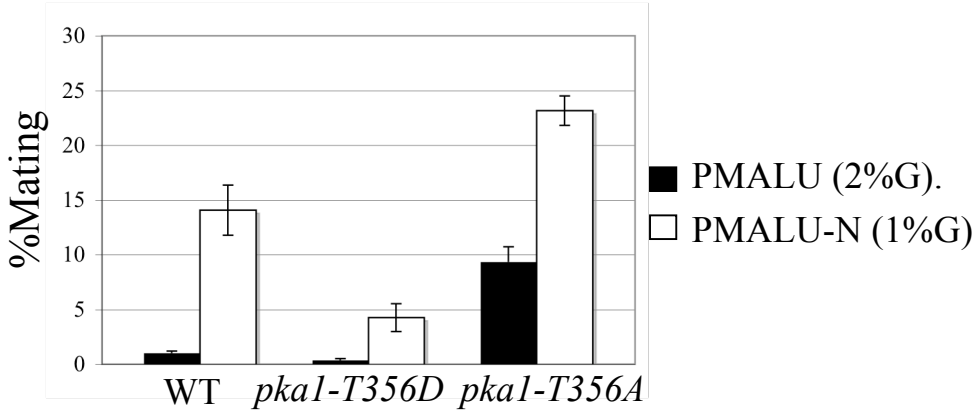
Gupta et al., Fig. 3

(A)

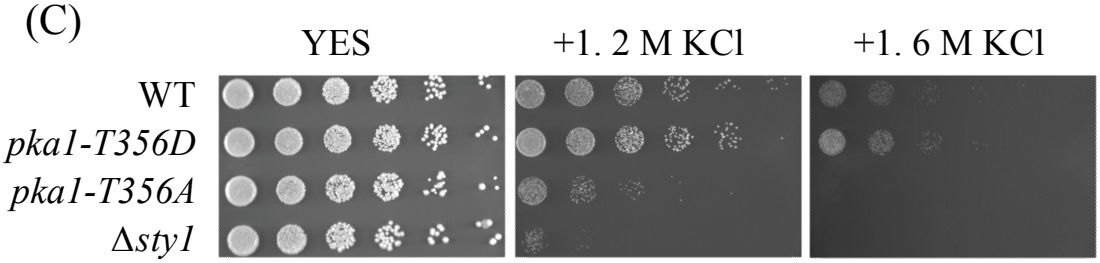


WT *pka1-T356D* *pka1-T356A*

(B)

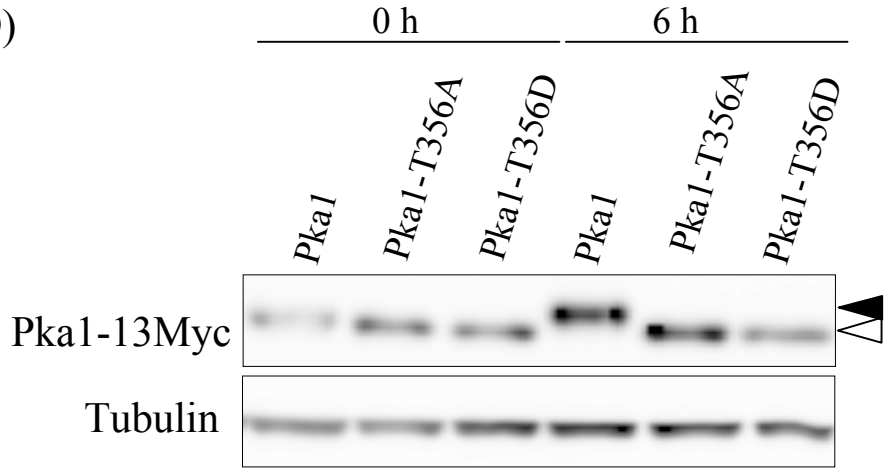


(C)

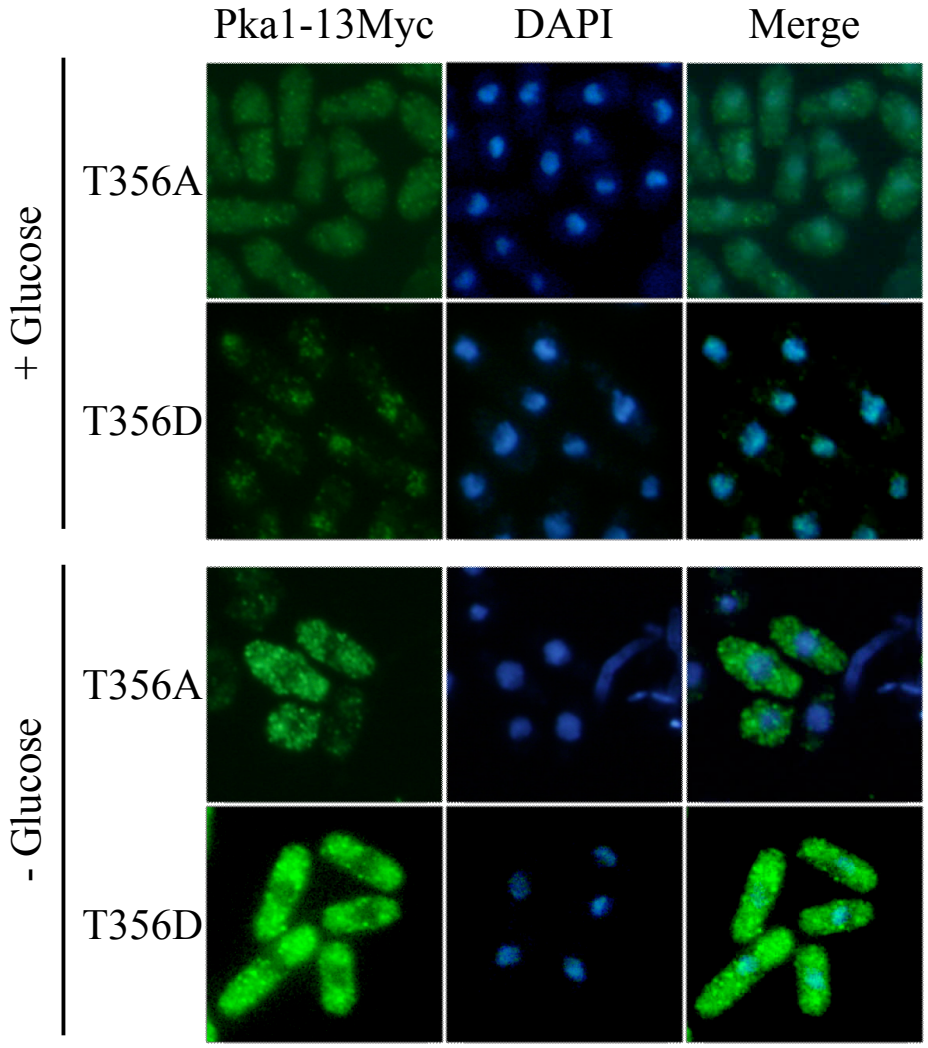


Gupta et al., Fig. 4A, B, C

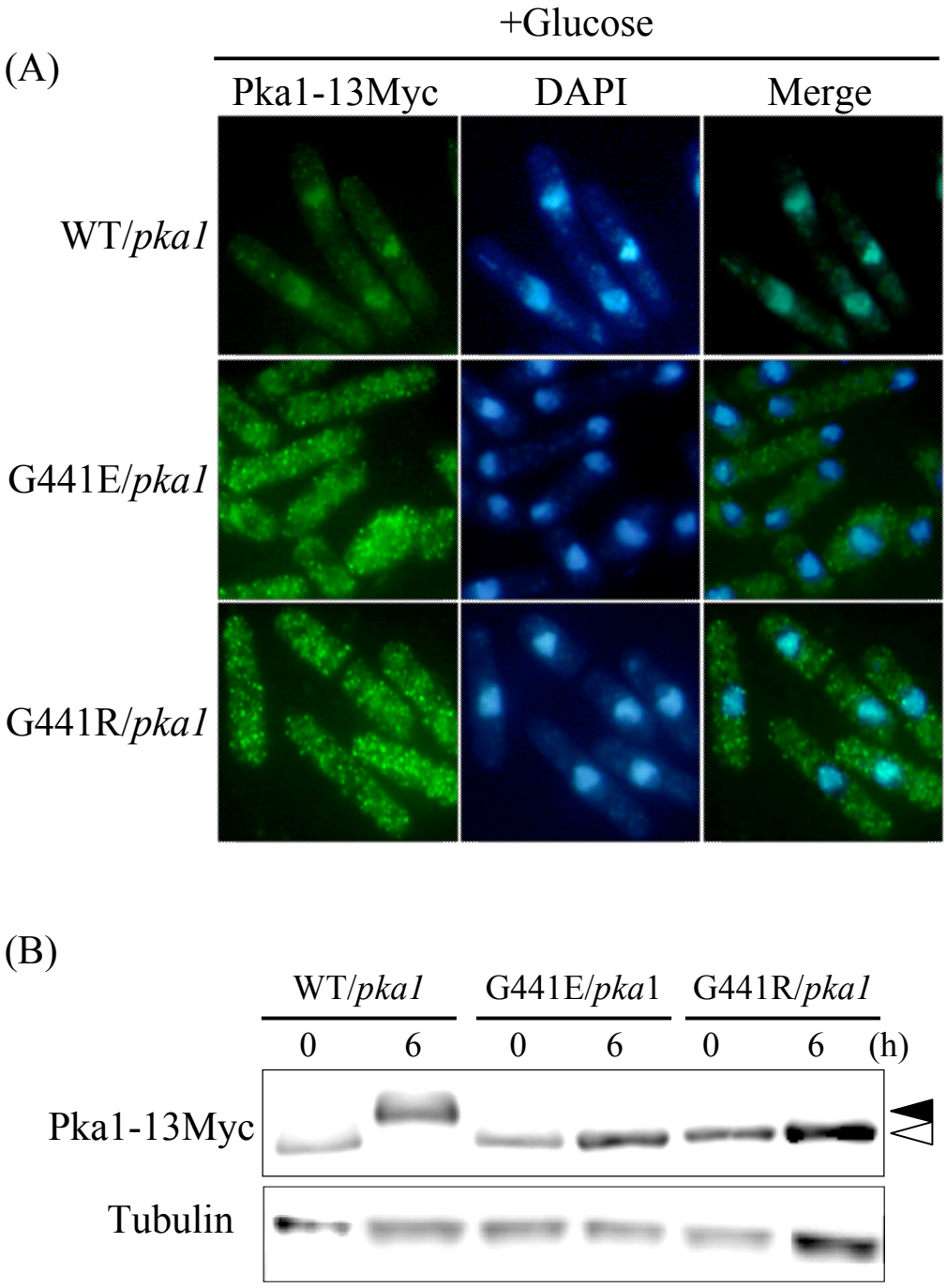
(D)



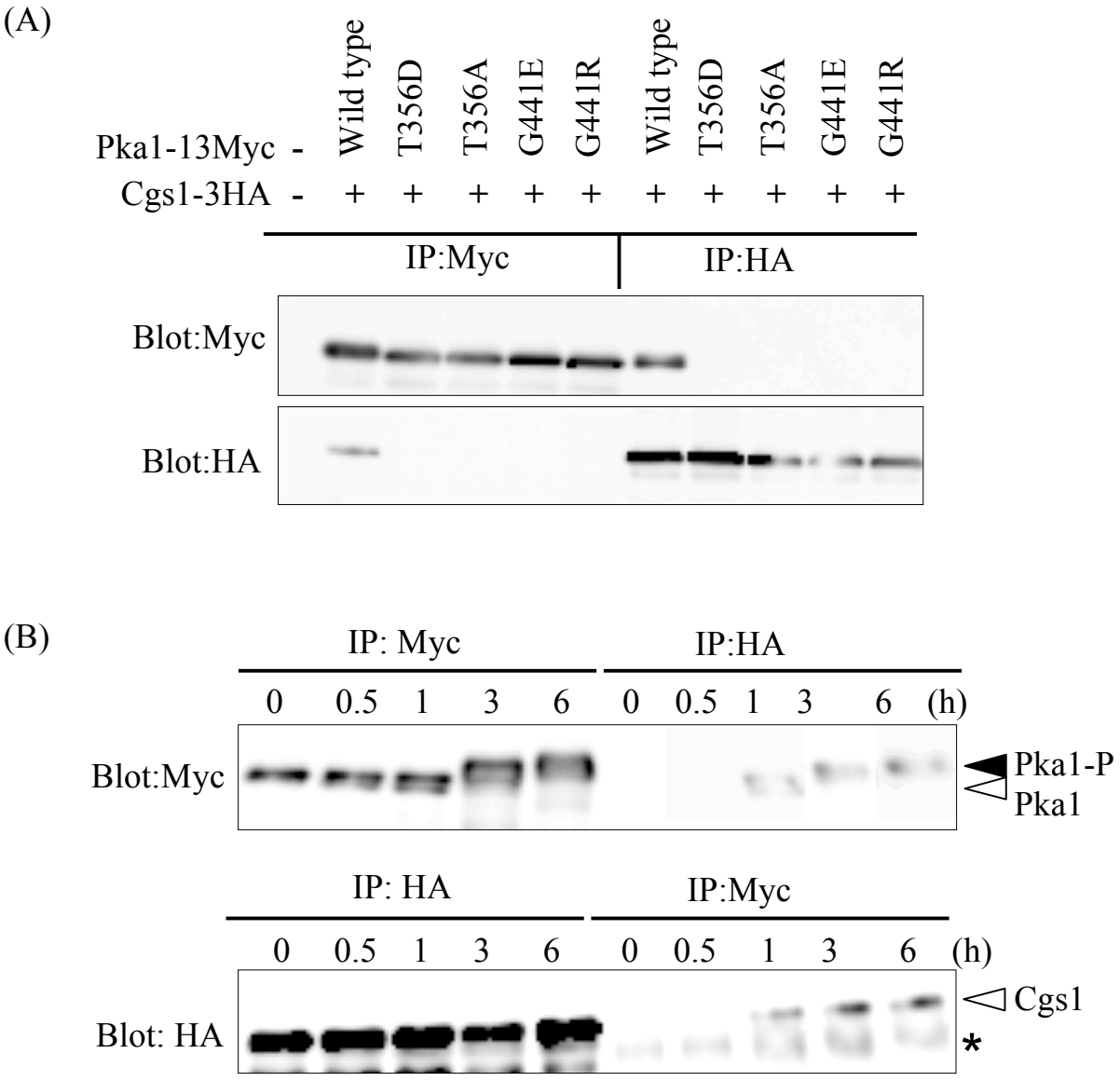
(E)



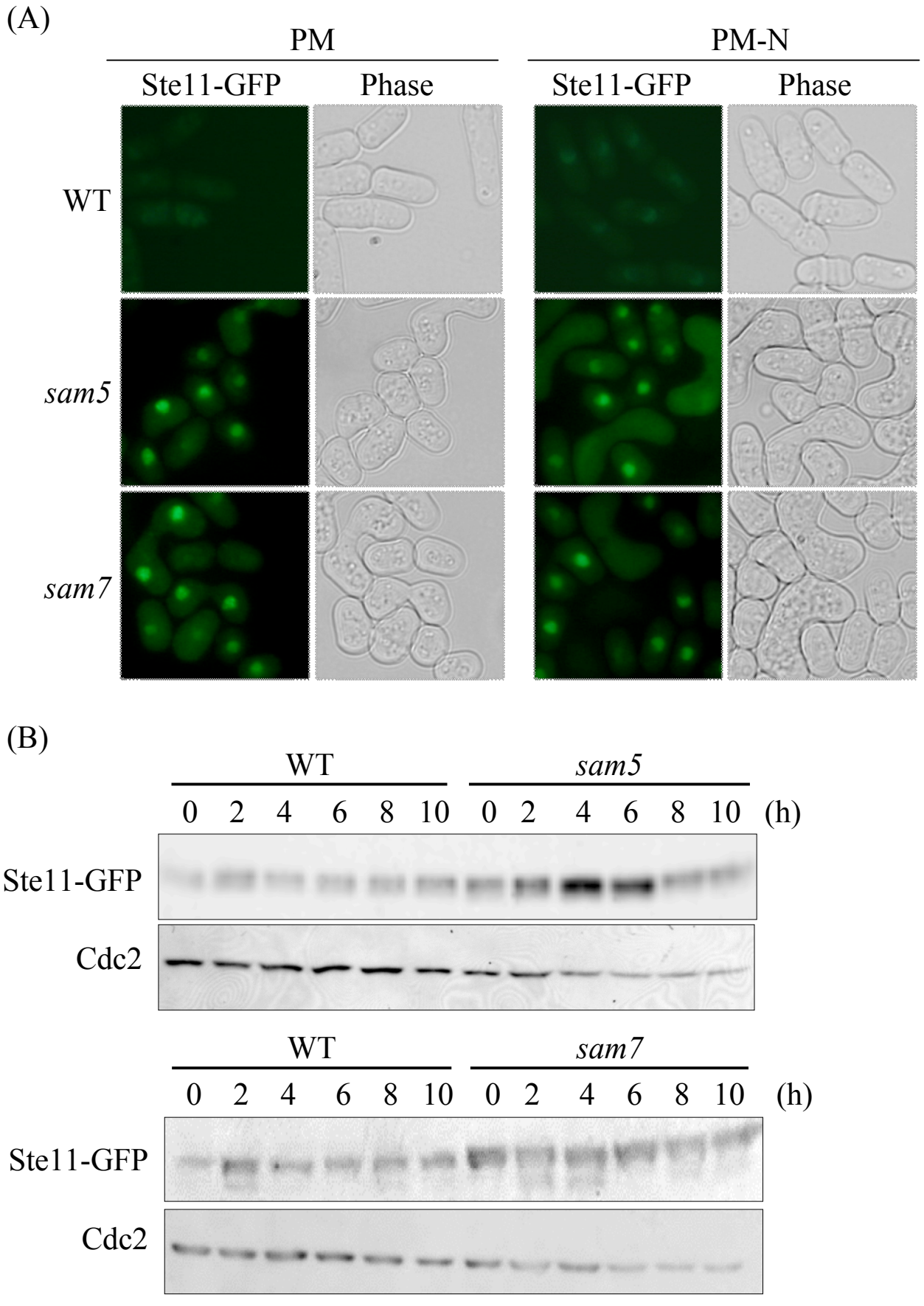
Gupta et al., Fig. 4D and E



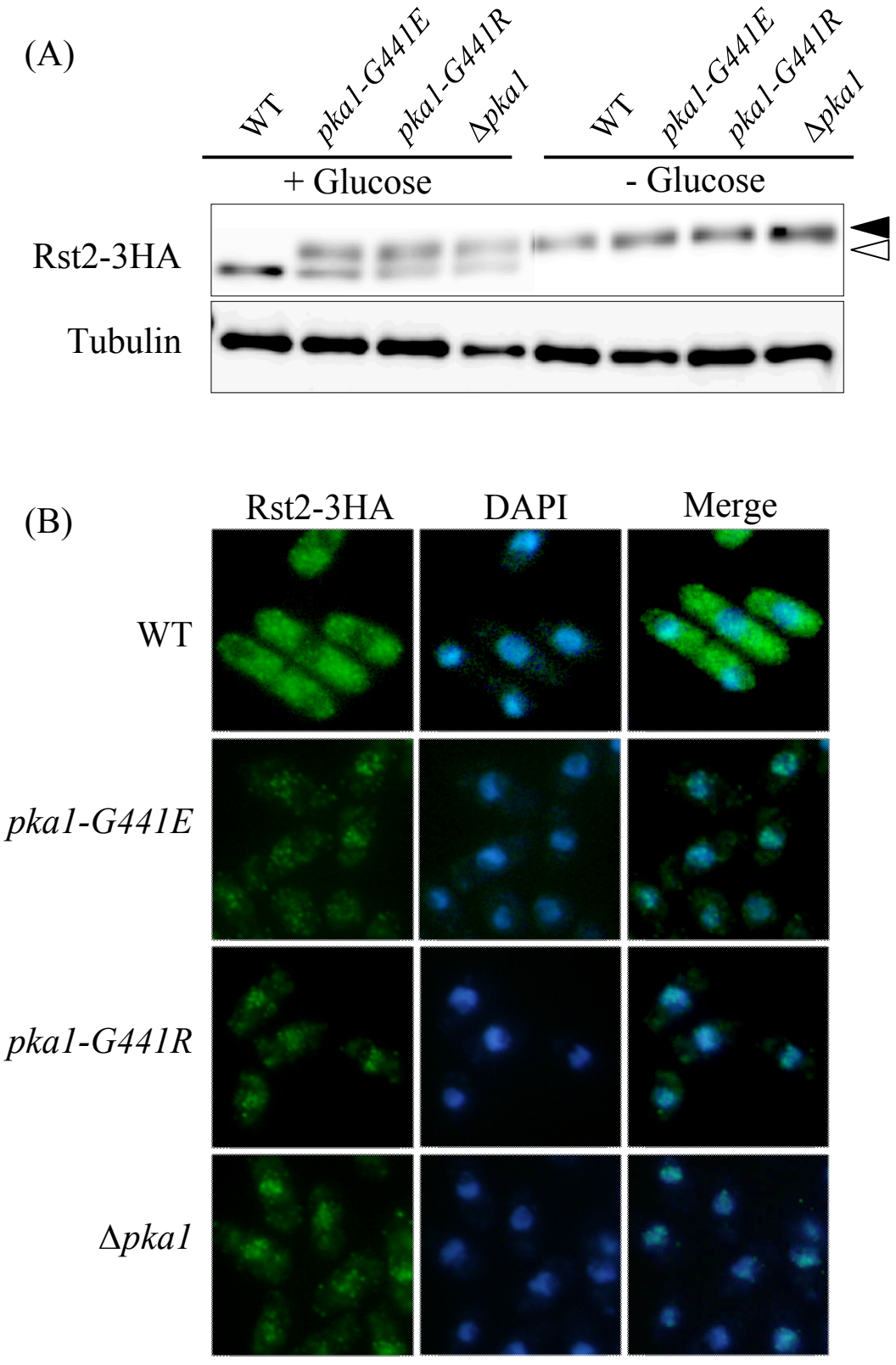
Gupta et al., Fig. 5



Gupta et al., Fig. 6



Gupta et al., suppl. Fig. 1



Gupta et al., suppl Fig. 2