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Running title: *S. pombe* Protein Kinase A

Complex Formation, Phosphorylation, and Localization of Protein Kinase A of *Schizosaccharomyces pombe* upon Glucose Starvation

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Nine *sam* mutants that undergo sexual differentiation without requiring starvation in *Schizosaccharomyces pombe* were previously isolated. In this study, we identified a nonsense mutation on the *pka1* locus in the *sam6* mutant. *pka1* encodes a catalytic subunit of protein kinase A (PKA). Replacement and overexpression of *pka1* suppressed the KCl sensitivity and hyper-mating phenotype of *sam6,* confirming that *sam6* is an allele of *pka1*. To characterize further the regulation of Pka1, we tested the physical interaction between Pka1 and Cgs1 (a regulatory subunit of PKA). Pka1 and Cgs1 physically interacted under glucose-limited conditions but not under glucose-rich conditions. In addition, the formation of a Pka1-Cgs1 complex was detected under glucose-limited conditions by Blue Native PAGE. Furthermore, the Pka1 protein was found to be phosphorylated under glucose starved conditions, and at the same time its localization shifted from the nucleus towards the cytoplasm (mainly the vacuoles), suggesting a strong relationship among phosphorylation, complex formation, and the cytoplasmic distribution of Pka1.

Key words: fission yeast; *Schizosaccharomyces pombe*; protein kinase A; sexual differentiation

53 The fission yeast *Schizosaccharomyces pombe* is an
54 excellent model organism in molecular and cellular
55 biology in the effort to understand the mitotic-meiotic
56 decision mechanism. *S. pombe* cells proliferate through
57 mitotic cell cycle under nutritionally rich conditions.
58 When nutrients (nitrogen or carbon) are limited, *S.*
59 *pombe* cells arrest at the G1 phase of the cell cycle and
60 heterothallic cells of an opposite mating type (h^+ and h^-)
61 mate to initiate sexual differentiation through a process
62 that includes conjugation, meiosis, and sporulation.

63 The sexual differentiation that precedes meiosis is
64 regulated by at least four distinct signaling pathways: the
65 cyclic AMP (cAMP) pathway, the stress-responsive
66 Sty1/Spc1 pathway, the pheromone-signaling pathway, and
67 the TOR pathways.[1-5)] Among these, the cAMP pathway is
68 the major glucose-sensing pathway in *S. pombe*. When
69 glucose is abundant, a heterotrimeric guanine nucleotide-
70 binding protein (Gpa2) becomes activated,[6)] and this then
71 activates adenylate cyclase (Cyr1) to generate cAMP from
72 ATP.[7)] Cyr1 interacts with its associated protein called
73 Cap1, which plays partly the regulatory role of adenylate
74 cyclase.[8,9)] The *cgsl* gene encodes the regulatory (R)
75 subunit of protein kinase A (PKA). Mutants defective in
76 *cgsl* are sterile due to constitutive activation of catalytic
77 (C) subunit (Pka1) of PKA. Any genetic manipulation that
78 increases the level of intracellular cAMP or the activity of
79 PKA renders *S. pombe* cells incapable of sexual
80 differentiation, whereas any manipulation that decreases

81 the cAMP level or PKA activity propels cells toward
82 sexual differentiation.[10] Sterility caused by higher cAMP
83 levels is reversed by overexpression of the *moc1-moc4*
84 genes.[11,12] Pka1 phosphorylates the Zn-finger protein Rst2,
85 which otherwise induces the expression of *ste11*, encoding
86 a key transcription factor for meiosis.[13]
87 In eukaryotic organisms, PKA is an evolutionarily
88 conserved serine/threonine kinase that contributes to the
89 regulation of diverse cell regulatory pathways.[14] It
90 modulates many cellular processes by phosphorylating
91 other proteins, and is also phosphorylated by other kinases
92 or by itself.[15] In eukaryotic cells, the major target of
93 cAMP is PKA, and the activity of PKA plays a pivotal role
94 in the regulation of adaptation to external conditions and
95 the induction of intrinsic cellular differentiation. The
96 activity of PKA is also regulated by its two R-subunits,
97 which form a dimer that binds to the two C-subunits, by
98 forming a PKA holoenzyme complex.[16] Tight interaction
99 between the R- and C-subunits requires a negatively
100 charged threonine in the activation loop region of the C-
101 subunits in the absence of cAMP.[17] The PKA holoenzyme
102 complex is primarily localized in the cytoplasm or bound
103 to specific cytoplasmic organelles *via* anchoring proteins,
104 depending on the type of R-subunit. Activation of PKA
105 occurs through the binding of cAMP to R-subunits, causing
106 dissociation of the C-subunits. The free C-subunits then
107 translocate to the nucleus and regulate cAMP-stimulated
108 transcription.[18] In *S. pombe,* the C- and the R- subunits of

109 PKA are mostly localized in the nucleus under glucose rich
110 conditions and are exported to the cytoplasm upon glucose
111 starvation.[19]
112 We have isolated nine *sam* (skips starvation for mating)
113 mutants that undergo mating and sporulation without
114 requiring nitrogen or glucose starvation, similarly to
115 Δ*pka1* and Δ*cyr1* mutants. Among the nine *sam* mutants,
116 two are dominant (s*am3* and *sam9*), and seven (*sam1*,
117 *sam2*, *sam4*, *sam5*, *sam6*, *sam7*, and *sam8*) are
118 recessive.[20] We identified *sam4* as an allele of *rad24*,
119 which encodes a 14-3-3 protein.[21] Besides this, we
120 analyzed and characterized two suppressor genes, *msa1*
121 and *msa2*,[22,23] both of which encode RNA binding
122 proteins that negatively regulate sexual differentiation.
123 Through analysis of *sam3* and *sam9,* we identified
124 *sla1*[24,25] as an inducer of sexual differentiation, and *zds1*
125 as involving both sexual differentiation and $CaCl_2$
126 tolerance.[26]
127 In this study, to identify the other *sam* mutants, we
128 sequenced the genes possibly involved in regulation of
129 sexual differentiation. We found that *sam6* is a nonsense
130 allele of *pka1*. We further found that Pka1 and Cgs1
131 interact with each other under glucose-starved conditions
132 to form a complex. Finally we found that glucose
133 starvation causes phosphorylation of the Pka1 protein and
134 a shift in its localization.
135
136 **Materials and Methods**

137

138 *Strains and media.* The *S. pombe* strains used in this
139 study are listed in Table 1. Standard yeast culture media
140 and genetic manipulations were used as described
141 previously.[27] The *S. pombe* strains were grown on YES-
142 rich medium (0.5% yeast extract, 3% glucose, and 225
143 mg/L of adenine, histidine, leucine, uracil, and lysine
144 hydrochloride) or Pombe Minimum (PM) synthetic medium
145 (0.3% potassium hydrogen phthalate, 0.22% sodium
146 phosphate, 0.5% ammonium chloride, 2% glucose,
147 vitamins, minerals, and salts), supplemented with 225
148 mg/L of adenine, leucine, and/or uracil when necessary.
149 Nitrogen-free PM medium (1% glucose without ammonium
150 chloride) was used to culture *S. pombe* when mating
151 efficiency was measured. Electroporation was used to
152 transform fission yeast cells.[28] *Escherichia coli* DH5α
153 grown in Luria-Bertani (LB) medium (1% polypeptone,
154 0.5% yeast extract, 1% sodium chloride, pH 7.2) hosted
155 all plasmid manipulations by the standard methods as
156 described previously.[29]

Table1

157

158 *Plasmid construction.* All the primers used in this
159 study are listed in Table 2. The pSLF372(L)-*pka1* plasmid
160 was constructed by amplifying the *pka1* gene with Pka1F-
161 *Bgl*II and Pka1R-*Not*I primers. The PCR product was then
162 digested with *Bgl*II and *Not*I and inserted into
163 pSLF372(L), which bears the *nmt* promoter, and three
164 copies of the HA epitope in its C-terminal region. To

165 construct pSLF172(L)-*pka1*, the *pka1* gene from
166 pSLF372(L)-*pka1* was digested with *Bgl*II and *Not*I. The
167 digested fragment was cloned into the *Bgl*II and *Not*I
168 sites of pSLF172 (L) under the control of the *nmt*
169 promoter. The constructed plasmids were verified by
170 restriction digestion and sequence analysis. Plasmid
171 manipulation and bacterial transformation were done by
172 standard techniques.[29] Table2
173
174 *Strain construction.* Tag-integrated strains were
175 constructed by a PCR-based method.[30] All tagging was
176 confirmed by colony PCR and immunoblotting with
177 specific antibodies. To construct the *pka1(G1382A)-3HA*
178 mutant strain, a fragment of 500 bp from the 5' region of
179 the mutated *pka1* locus of *sam6* was amplified by PCR
180 using the Pka1-W and Pka1-X primers. Similarly, the 3'
181 region of *pka1* was amplified using the pka1-Y and Pka1-
182 Z primers. Both fragments were attached to the *kanMX6*
183 module by PCR using pFA6a–3HA–*kanMX6*. The resulting
184 tagged fragments were introduced into *S. pombe* strain
185 SP870. G418-resistant transformants were selected, and
186 proper integration was verified by PCR and sequence
187 analysis. The resulting strain was named DRG16.
188
189 *Mating and sporulation efficiency assay.* Mating and
190 sporulation efficiency was calculated using the following
191 equation:
192 $\text{Mat}\ (\%) = (2Z + 2A + 0.5S)/(H + 2Z + 2A + 0.5S)$

193 where *Z* stands for the number of zygotes, *A* for the
194 number of asci, *S* for the number of free spores, and *H* for
195 the number of cells that failed to mate.
196
197 *Western blotting*. Samples for Western blotting were
198 prepared by the simple alkali-SDS method[31)] or the boiling
199 SDS-glass bead method.[32)] Cells were harvested when they
200 reached a density of approximately 1 x 10^8 cells in an
201 appropriate medium. The harvested cells were washed
202 twice with dH_2O and dissolved in 100 μL of dH_2O, and the
203 samples were boiled at 95°C for 5 min. Subsequently, 120
204 μL of 2X Laemmli buffer (4% SDS, 20% glycerol, 0.6 M β-
205 mercaptoethanol, 8 M urea, and 0.12 M Tris-HCl, pH 6.8)
206 was added and the samples were vigorously vortexed with
207 an equal volume of acid-washed glass beads using a bead
208 homogenizer at 2,500 rpm for 3 min. The samples were
209 boiled at 95°C for 5 min and centrifuged at 10,000 x g for
210 15 min at 4°C to remove the glass beads and large debris.
211 An equal volume of cell extract was loaded onto SDS-
212 PAGE using a 10% polyacrylamide gel, and then
213 transferred to Immobilon transfer membranes (Millipore,
214 Bedford, MA) using a wet-type transfer system. To block
215 unspecific binding, the membranes were incubated in a
216 blocking buffer (PBS containing 5% non-fat dry milk)
217 supplemented with 0.1% Tween 20 at room temperature for
218 1 h. To detect Myc fusion proteins, the membrane was
219 incubated with a Myc monoclonal antibody (Santa Cruz
220 Biotechnology, Santa Cruz, CA) diluted 1:3,000 in PBS-T

221 (137 mM NaCl, 8 mM $Na_2HPO_4.12H_2O$, 2.7 mM KCl, 1.5 mM
222 $KHPO_4$, and 0.1% Tween 20) containing 5% dry milk. The
223 membrane was washed 3 times with PBS-T, and then
224 incubated with horseradish peroxidase-conjugated anti
225 mouse secondary IgG (Santa Cruz Biotechnology) diluted
226 1:3000 in PBS-T containing 5% dry milk.
227 To detect HA fusion proteins, the membrane was
228 incubated with an HA monoclonal antibody (Santa Cruz
229 Biotechnology) diluted 1:3,000 in PBS-T containing 5%
230 dry milk. The membrane was washed 3 times with PBS-T
231 every 5 min, and then incubated with an anti-mouse
232 secondary IgG, diluted 1: 3,000 in PBS-T containing 5%
233 dry milk. The membrane was again washed 3 times with
234 PBS-T every 5 min, and then secondary antibodies were
235 detected by the chemiluminescence (ECL) system, as
236 described by the manufacturer (GE Healthcare, Tokyo).
237
238 *Blue Native PAGE.* Blue Native polyacrylamide gel
239 electrophoresis (BN-PAGE) is a method of isolating intact
240 protein complexes. We followed the manufacturer's
241 instructions (Invitrogen, Tokyo). Protein complexes were
242 separated by apparent molecular mass using this standard
243 polyacrylamide gel electrophoresis system. In the first
244 dimension, separation of the complexes under native
245 conditions occurs according to molecular mass, and in the
246 second dimension, where electrophoresis is performed
247 under denaturing conditions, the individual subunits of the

248 complexes are resolved, again on the basis of their
249 molecular mass.[10,33)]
250 Cells were grown in YES medium to mid-logarithmic
251 phase, then harvested (1 to 2 x 10^8 cells) by
252 centrifugation. They were washed once with dH_2O and
253 stored at -80°C. The cell pellets were dissolved with 4X
254 Native PAGE sample buffer (25 μL) plus dH_2O (72 μL), 1
255 mM phenylmethylsulfonyl fluoride (PMSF, 1 μL) and
256 protease inhibitor (2 μL). Samples were vortexed
257 vigorously with equal volumes of acid-washed glass beads
258 using a bead homogenizer at 2,500 rpm for 3 min. After
259 centrifugation (10,000g for 15 min at 4°C), the protein
260 concentration in the supernatant was estimated.
261 Approximately 50 μg of protein was loaded per lane for
262 electrophoresis.
263 Gel strips were cut from the gel after BN-PAGE, strips
264 were transferred individually to 15 mL conical tubes, and
265 5 mL of reducing solution [0.5 mL of sample reducing
266 agent (10X), 1 mL of LDS sample buffer (4X) and 3.5 mL
267 of H_2O] was added to each tube. Samples were incubated
268 for 15 min with shaking at room temperature, and the
269 reducing solution was then decanted. Then 5 mL of
270 alkylating solution [1 mL of LDS sample buffer (4X),
271 3.72 mL of H_2O, and 28 μL of DMA] was added to each
272 tube, incubated for 30 min at room temperature with
273 shaking, and then decanted. Then 5 mL of quenching
274 solution [0.50 mL of sample reducing agent (10X), 3 mL
275 of LDS sample buffer (4X), 1 mL of EtOH, and 3.5 mL of

276 H_2O] was added to each tube and the mixture was
277 incubated at room temperature. The quenching solution
278 was decanted and the gel strips were used for 2-
279 dimensional SDS-PAGE.
280
281 *Co-immunoprecipitation. S. pombe* cells were grown in
282 YES and/or YES-glucose starved (0.1% glucose) medium
283 to mid-logarithmic phase, then harvested (2 x 10^8 cells)
284 by centrifugation, and washed once with ice-cold stop
285 buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, and 1
286 mM NaN_3 pH 8). The cells were then lysed in 100 μL of
287 ice-cold lysis buffer [50 mM Tris-HCl pH7.0, 150 mM
288 NaCl, 0.8% Nonidet-P40, 5 mM EDTA, 10% glycerol, 1
289 mM phenylmethanesulfonyl fluoride (PMSF), and protease
290 inhibitor]. The samples were vortexed vigorously with 0.5
291 mm diameter zirconia/silica beads using a bead
292 homogenizer at 2,500 rpm for 3 min. After centrifugation
293 (10,000g for 15 min at 4°C), the protein concentration in
294 the supernatant was estimated.
295 An HA monoclonal antibody and a Myc antibody were
296 used in the immunoprecipitation HA and Myc fusion
297 proteins, in which 1 mg of each cell extract was incubated
298 with 1 μg of HA antibody and 1 μg of Myc antibody for 4
299 h at 4°C. Then 40 μL of protein A sepharose beads and the
300 same volume of protein G sepharose beads were washed
301 twice with 0.5 mL of lysis buffer. The cleaned protein A
302 sepharose beads were added to the HA antibody mixture
303 and the protein G sepharose beads were added to the Myc

304 antibody mixture, followed by incubation with rotation for
305 4 h at 4°C. Sepharose beads were collected by
306 centrifugation at 10,000g over 10 min at 4°C. The
307 supernatant was discarded by aspiration and the beads
308 were washed 6 to 8 times using 0.5 mL lysis buffer
309 including protease inhibitor and PMSF. The bead pellet
310 was suspended in 30 μL lysis buffer (including protease
311 inhibitor and PMSF), and 60 μL of 2X Laemmli buffer (4%
312 SDS, 20% glycerol, 0.6 M β-mercaptoethanol, 8 M urea,
313 and 0.12 M Tris/HCl pH 6.8) was added and the mixture
314 was vortexed. The suspended beads were boiled at 95°C for
315 5 min to dissociate the immunocomplexes from the beads.
316 After centrifugation (10,000g for 10 min at 4°C), the
317 supernatant was collected in a new Eppendorf tube and
318 used for SDS-PAGE and Western blotting.
319
320 *Fluorescence microscopy.* Cells were grown in YES
321 liquid medium and then shifted to YES medium containing
322 0.1% glucose at 30°C. Pka1-GFP was observed in living
323 cells under a BX51 microscope (Olympus, Tokyo).
324 Fluorescence images were taken with a digital camera
325 DP70 (Olympus) connected to the microscope.
326
327 **Results**
328
329 *Sequencing of the* pka1 *locus of the* sam6 *mutant*
330 Because the phenotypes (such as hyper-mating and KCl
331 sensitivity) of recessive *sam* mutants are characteristics

332 of *pka1* mutants, we sequenced the *pka1* locus of the
333 recessive *sam* mutants. The genomic DNAs of the *pka1*
334 locus from the recessive *sam* mutants were amplified by
335 primer sets Pka1-F1 and Pka1-R2, and the amplified
336 fragments were sequenced directly with the primers
337 indicated in Fig. 1. In *sam6*, a mutation was found at the
338 position of the 1,382 nucleotide, which altered the TGG
339 (Trp) codon to TAG (stop codon). Sequencing of the *pka1*
340 locus of the other *sam* mutants (*sam1, sam2, sam5, sam7*,
341 and *sam8*) were done and we found that each of them
342 contained a single mutation on the *pka1* locus (data not
343 shown), but the significance of this has not been explored
344 yet.
345 Fig.1
346 *Confirmation of the* sam6 *mutant by replacement of*
347 pka1
348 To determine whether phenotypes of the *sam6* mutant
349 were reversed by exogenous expression of *pka1*, we first
350 introduced the exogenous *pka1* gene on the plasmid
351 pSLF172L-*pka1* in the *sam6* mutant. Overexpression of
352 *pka1* restored the phenotype of the *sam6* mutant with
353 respect to 1 M KCl sensitivity and the *sam* (skip starvation
354 for mating) phenotype (Fig. 2 A, B, and C).
355 Overexpression of *pka1* rendered the cells to elongate and
356 to grow as pseudohyphae, which are characteristic
357 phenotypes caused by *pka1* overexpression (Fig. 2B).[34)]
358 Furthermore, we replaced the *pka1* locus of the *sam6*
359 mutant with wild-type *pka1* by genomic integration. As

360 expected, the *sam6* mutant phenotypes were suppressed by
361 genomic replacement with wild-type *pka1* (Fig. 3 A and B).
362 To confirm further that the mutation in *sam6* of the *pka1*
363 locus is the sole mutation that leads to the *sam* phenotypes,
364 we introduced the *pka1* locus (G1382A) of *sam6* mutant
365 into the wild-type strain by genomic integration.
366 Integration was verified by colony PCR and sequencing.
367 Constructed strain DRG16 (*pka1*-G1382A) showed a
368 phenotype identical to the *sam6* mutant and the *pka1*
369 deletion mutant (Fig. 3A and B), indicating a truncated
370 version of Pka1 due to the nonsense mutation abolished its
371 function of Pka1. All these results indicate that *sam6* is
372 the allele of *pka1*.
373

Fig.2 Fig.3

374 *KCl did not kill cells but only attenuated growth*
375 It has been found that the *pka1* deletion mutant is
376 sensitive to a medium containing KCl,[35] but it is not clear
377 how much KCl affects the survival of the *pka1* mutant. We
378 compared the KCl sensitivity of the *pka1* mutant with
379 other mutants, such as *sty1*, which encodes a key
380 component of the stress-signaling pathway and is
381 responsible for growth in high salt media,[36] and of *moc1*,
382 which is also required for growth under high- salt
383 conditions.[32] The wild-type and the mutants cells were
384 pre-cultured on YES medium at mid-log phase and re-
385 cultured in YES medium containing 1 M KCl for 72 h, and
386 then spotted on a YES plate. Under this procedure, cells
387 surviving under KCl stress should grow on plates, but

388 cells dying under KCl stress should not grow after removal
389 of KCl stress. After incubation at 30°C for 72 h, the *sam6*
390 mutant as well as the Δ*pka1* mutant showed normal growth
391 on YES plates, like the wild type, but the Δ*sty1* mutant did
392 not grow, and the Δ*moc1* mutant grew only moderately (Fig.
393 4). This suggests that the *sam6* and Δ*pka1* mutants remain
394 viable during exposure to high salt (1 M KCl over 72 h of
395 exposure), but do not grow under high salt conditions (Fig.
396 2A). Under favorable conditions they continue to grow.
397 Thus, 1 M KCl confers sensitivity to *pka1* cells (Fig. 2),
398 but it does not kill them (Fig. 4), suggesting that the
399 significance and mechanism of KCl sensitivity to *pka1*
400 mutant is different from that of *sty1* and *moc1* mutants.
401

Fig.4

402 In vivo *interactions between Pka1 and Cgs1 in* S. pombe
403 In eukaryotes, generally the catalytic subunit (C) and
404 the regulatory subunit (R) of PKA associate, and the
405 binding of cAMP to the R subunit leads to dissociation of
406 the R-C complex,[15] but it is not known whether the R- and
407 C- subunits of PKA from *S. pombe* also interact at low
408 cAMP levels. To determine this, cell extracts were
409 prepared from strain DRG33 (Pka1–3HA Cgs1–13Myc). A
410 Myc antibody was used to precipitate the Cgs1–13Myc
411 protein, and the precipitates were analyzed by Western
412 blotting using the HA monoclonal antibody. Conversely,
413 the HA monoclonal antibody was used to
414 immunoprecipitate Pka1–3HA, and Cgs1–13Myc was
415 detected with a Myc antibody. The results showed that

416 under glucose limited conditions, Pka1-3HA was present in
417 the Myc immunoprecipitated sample and, reciprocally, that
418 Cgs1–13Myc was present in the HA immunoprecipitated
419 sample (Fig. 5A), indicating that Pka1 interacts with Cgs1
420 *in vivo*, but under glucose-rich conditions no interaction
421 was detected between Pka1 and Cgs1 (Fig. 5B), indicating
422 that the interaction of the R- and C- subunits of PKA
423 depends on glucose conditions.
424

Fig.5

425 *Pka1 and Cgs1 formed a complex under glucose-starved*
426 *conditions*
427 To test whether *S. pombe* PKA forms a complex of two
428 molar Pka1 and two molar Cgs1, we performed Blue
429 Native PAGE to determine the size of the PKA complex.
430 In this method, proteins remain in their native state, in
431 contrast with the cross-linked methods. Cell extracts were
432 prepared from strains DRG21 (Pka1-13Myc), DRG31
433 (*Δcgs1,* Pka1-13Myc), DRG32 (Cgs1-13Myc), and DRG34
434 (*Δpka1,* Cgs1-13Myc) after they were grown on YES (3%
435 glucose) or YES (0.1% glucose) medium. A complex of
436 about 220 kDa was detected by BN-PAGE when cells were
437 grown on low-glucose medium. The molecular mass of the
438 complex as measured by BN-PAGE was much greater than
439 the molecular mass of Pka1-13Myc (about 77kDa) and
440 Cgs1-13Myc (about 66kDa) as detected by SDS-PAGE (Fig.
441 6 A, E, and F), indicating that the Pka-Cgs1 complex
442 contains more than 1 molar of each, but it was not clear
443 whether the Pka-Cgs1 complex contains 2 molar of each.

444 No complex band was detected in the cells growing on
445 glucose-rich medium, which is consistent with the results
446 shown in Fig. 5. Cells lacking *cgs1* or *pka1* did not form
447 a complex on the glucose-limited medium (Fig. 6). The
448 smaller molecular weight bands observed in the lane of
449 Cgs1-Myc (0.1% G) may indicate that most Cgs1 exists in
450 free form. The proteins, separated by BN-PAGE in the
451 first dimension, were further separated by SDS-PAGE in
452 the second dimension, and subsequently detected with a
453 Myc antibody. During electrophoresis in the second
454 dimension, the complex was separated according to the
455 molecular masses of the individual subunits, and the
456 proteins were detected by Western blotting (Fig. 6B, C,
457 and D). The proteins from strains DRG21 (Pka1-13Myc)
458 and DRG32 (Cgs1-13Myc) that grew under glucose-
459 starved conditions had strong signals and separated in a
460 pattern similar to the cells grown under glucose-rich
461 conditions (3% glucose). These results indicate that the
462 C- and R- subunits of PKA exist as a complex in glucose-
463 starved cells and are released under glucose-rich
464 conditions.
465

Fig.6

466 *The Pka1 protein was phosphorylated under glucose-*
467 *starved conditions*
468 Protein kinases are often regulated by phosphorylation,
469 either in an autocatalytic way or by other kinases.[37] We
470 observed that the Pka1 protein was in a higher molecular
471 form when samples were collected from cells growing on a

472 glucose-limited medium, but not when samples were
473 collected from the cells growing on a glucose-rich medium
474 (Fig. 5A and B). This lead us to investigate whether
475 glucose starvation induces a band shift in the Pka1 protein.
476 We tested the effects of different levels of glucose on
477 band shift in the Pka1 protein in the YMSM101 (Pka1-
478 GFP) strain by Western blotting. The medium containing
479 0.1% glucose had a dramatic effect on Pka1 band shift,
480 whereas higher concentrations of glucose failed to induce
481 any clear band shift in Pka1 (Fig. 7A). To test further the
482 conditions of band shift in Pka1, the strain YMSM101
483 (Pka1-GFP) was cultured in YES (3% glucose) to mid-log
484 phase (5 x 10^6/mL). Then the cells were harvested, washed
485 with glucose-free YES medium, and further incubated in
486 the same medium. They were collected at the indicated
487 times and Western blot analysis was performed. As Fig. 7B
488 indicates, the Pka1-GFP band shifted to the upper position
489 under glucose-starved conditions. This band shift started
490 at 1 h after glucose starvation, and reached a steady level
491 after 6 h. Phosphatase treatment using λPPase downshifted
492 the Pka1 band, and a concomitant addition of phosphatase
493 inhibitor inhibited band shift in Pka1 (Fig. 7C). These
494 results clearly indicate that the Pka1 protein is
495 phosphorylated under glucose-starvation conditions. We
496 further examined the relevance of the R-subunit of PKA to
497 the phosphorylation of Pka1. The results showed that Cgs1
498 is required for the phosphorylation of Pka1 (Fig. 7D).
499

Fig.7

500 *Cellular dynamics of Pka1-GFP under glucose*
501 *starvation*
502 It has been found that the Pka1 protein resides in the
503 nucleus under glucose-rich conditions and is exported to
504 the cytoplasm under glucose-starved conditions.[19] The
505 Pka1 protein was in a phosphorylated form under the
506 glucose-starved conditions (Fig. 7A). We tested to
507 determine whether there is any relationship among Pka1
508 phosphorylation, complex formation, and localization. To
509 examine the time point of localization of the Pka1 protein
510 under glucose starvation, we used a strain that contains
511 the Pka1 protein with GFP tagging. The Pka1-GFP strain
512 was first cultured on YES medium containing 3% glucose
513 at early log phase, and then shifted to growth on YES
514 medium containing 0.1% glucose. Then localization of the
515 Pka1-GFP protein was observed by direct fluorescence. At
516 0 h, the Pka1-GFP protein was found in the nucleus. At 6
517 h, Pka1-GFP was detected mostly at the periphery of the
518 nucleus, and at 8 h it started to form dot structures. At
519 24 h, the Pka1-GFP protein was distributed throughout
520 the cytoplasm (Fig. 8). This observation suggests that
521 phosphorylation of Pka1 protein occurs in the nucleus,
522 because the Pka1 protein was fully phosphorylated before
523 6 h of glucose starvation, at which time the Pka1 protein
524 remained in the nucleus. We then tested whether the dot
525 structure observed at 8-10 h co-localized with vacuoles or
526 stress granules. Vacuoles were stained with FM4-64, and
527 stress granules were observed by co-localization with

528 Pabp-RFP.[38] The results indicated that Pka1 co-localized
529 with the vacuoles, but not with Pabp-RFP.
530 Fig.8
531 **Discussion**
532
533 In this study we found that *sam6* was a nonsense allele
534 of *pka1*, which encodes a C- subunit of PKA. Among nine
535 *sam* mutants, this is the second example of identification
536 of its mutant locus next to the identification of *sam4* as a
537 *rad24* allele.[21] It has been found that the *S. pombe* Δ*pka1*
538 cells are de-repressed for sexual differentiation even in
539 the presence of rich nutrition, and that Δ*pka1* spores are
540 apparently impaired in germination and often fail to
541 resume vegetative growth.[10,39] These *pka1* deletion
542 mutant phenotypes were similarly observed in *sam6*,
543 which was selected to undergo sexual differentiation
544 under nutrient-rich conditions. We found that the
545 phenotypes of the *sam6* and *pka1* deletion mutants were
546 indistinguishable, indicating that a nonsense mutation at
547 position 461 aa of Pka1 abolished its function. Because
548 this mutation site is located in the kinase domain of Pka1,
549 Pka1 is not functional even if the truncated Pka1 is
550 synthesized.
551 It has been found that the cAMP pathway is required
552 for survival of salt stress in *S. pombe*,[35] but the
553 mechanism is not yet clearly understood. Both the *pka1*
554 deletion mutant and a *sam6* mutant were sensitive to KCl.
555 But we found that a condition (exposure to 1 M KCl for 72

556 h) that kills the *sty1* mutant did not kill the *pka1* deletion
557 mutant. Apparently, sensitivity to KCl is different in the
558 *pka1* deletion mutant than in the *sty1* mutant. A
559 comparison of mutants on a plate containing 1 M KCl did
560 not identify this difference (Fig. 2A *versus* Fig. 4), since
561 gain-of-function in the cAMP pathway rescued the salt-
562 sensitive growth defect of the deletion mutant of *plb1,*
563 which encodes the phospholipase B homolog, the cAMP
564 pathway was proposed to be a potential target of Plb1 in
565 *S. pombe*.[35] Further study of the connection with *plb1* is
566 necessary to understand the KCl-sensitive phenotype of
567 the *pka1* mutants.

568 Cells containing high intracellular cAMP activate PKA,
569 whereas lower cAMP has an opposite effect on cells. In
570 both fission yeast and budding yeast, the levels of
571 intracellular cAMP are high during mitotic growth.[7]
572 Conditions unfavorable to growth in these two yeasts lead
573 to downregulation of PKA, which triggers adaptation to
574 the adverse conditions. Disruption of *pka1* slows cell
575 growth but is not lethal in *S. pombe*. On the other hand, at
576 least one of the three C-subunits of PKA is required for
577 survival in *S. cerevisiae*.[40] This difference makes *S.*
578 *pombe* much more tractable to functional analysis of PKA.
579 Here, for the first time in *S. pombe*, we found interaction
580 between Pka1 and Cgs1. This interaction occurred under
581 glucose-limited conditions, but not under glucose-rich
582 conditions. By BN-PAGE, we found that Pka1 and Cgs1
583 formed a complex under glucose-limited conditions but no

584 complex formation was detected under glucose-rich
585 conditions. The molecular size of the Pka1-Cgs1 complex
586 confirms that the complex is multimeric. Our results
587 clearly indicate that the formation of a complex between
588 Pka1 and Cgs1 is dependent on the glucose concentration,
589 which has only been assumed as a model in *S. pombe*.
590 We found that the Pka1 protein is phosphorylated under
591 glucose-starved conditions. Very recently, it was reported
592 that cAMP deficiency and other stresses result in the
593 phosphorylation of Pka1 in *S. pombe*.[14] This is consistent
594 with our results, although our experimental conditions of
595 glucose starvation were more physiological than the
596 experiments done in a deletion mutant of *cyr1*. The cAMP-
597 PKA pathway has been found to be a glucose-sensing
598 pathway in *S. pombe*, and cAMP is maintained in cells by
599 uptake of glucose from the environment.[6] It was clearly
600 observed in our study and in others that low glucose in the
601 medium is a cause of low levels of basal cAMP, which
602 induces the phosphorylation of Pka1. Cgs1 is required for
603 the phosphorylation of Pka1, suggesting that R- and C-
604 complex formation is important for Pka1 phosphorylation.
605 We also observed that the Pka1 protein was in the
606 nucleus until 6 h and then moved toward the cytoplasm.
607 Phosphorylation of the Pka1 protein occurred at the same
608 time point, and no obvious change in band shifting
609 occurred after 6 h. Glucose starvation after 6 h might be
610 the transition point for Pka1 to move from the nucleus to
611 the cytoplasm, because at that time point Pka1 was

612 localized in the nuclear peripheral of most cells (Fig. 8).
613 We found that the Pka1 protein localized in the vacuoles
614 when cells were starved for 6-10 h. Pka1 might be
615 transported to the vacuoles, because it is not required to
616 be functional under glucose starvation. In fact, some lower
617 molecular weight Pka1 proteins were observed on BN-
618 PAGE under glucose-starved conditions (Fig. 6), which
619 might reflect partial degradation of Pka1 in the vacuoles.
620 Under glucose-limited conditions, after 24 h both PKA
621 subunits were distributed in both cytoplasm and nucleus.
622 It was found that in cells lacking Cgs1, Pka1 became
623 concentrated in the nucleus and was more diffusely present
624 in the cytoplasm. Similarly, under glucose-rich conditions
625 both subunits were concentrated in the nucleus, and were
626 more diffusely present in the cytoplasm. The nuclear
627 localization of Pka1 was independent of Cgs1.[19]
628 Combining these results and ours, the Pka1 and Cgs1
629 proteins remain in the cytoplasm as a complex under
630 glucose-starved conditions, but under rich conditions, the
631 binding of cAMP with its R-subunit release the C-subunit
632 to be imported into the nucleus. In mammalian cells, it has
633 been found that the C- and R-subunits reside in the
634 cytoplasm, forming an inactive holoenzyme complex, and
635 that the Pka1 protein is fully phosphorylated when it
636 assembles into an inactive complex at a lower cAMP
637 level.[15,18] A similar mechanism in *S. pombe* is envisaged
638 based on our results.

639 In this study we monitored the four events involving the

640 Pka1 protein: interaction with Cgs1, complex formation of
641 Pka1 and Cgs1, the phosphorylation of Pka1, and the
642 distribution of Pka1. All these events are controlled by
643 glucose starvation. Phosphorylation of Pka1 occurred at
644 the time of the nuclear localization of Pka1, and the PKA
645 complex resided in the cytoplasm, suggesting the
646 possibility that phosphorylation occurs in cells before
647 complex formation, but, it is unclear whether the C- and
648 R- subunits interact in the nucleus or out-side of the
649 nucleus in *S. pombe* cells. However, under the nutrient-
650 rich conditions, both subunits remain in the nucleus and
651 are distributed in the cytoplasm upon starvation.[19] It is
652 not clear yet which events, of phosphorylation, complex
653 formation, and the distribution of Pka1 occur first, or how
654 these events are correlated with each other. Further
655 analysis is necessary to answer these questions.
656
657 **Acknowledgments**
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660 We thank Dr. T. Kaino, Dr. T. Nakagawa, and Dr. Y.
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662
663

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756

757 **Figure legends.**

758 **Fig. 1.** The Mutation Site of *pka1* in the *sam6* Mutant.

759 The *pka1* gene was amplified with pka1 F1 and R2
760 primers. The DNA sequence of the amplified *pka1* region
761 was determined by the primers indicated by arrows. A
762 mutation was found at the 1,382 position from the start
763 codon in the *sam6* mutant. By this mutation, the Trp
764 codon was changed to a stop codon in the *sam6* mutant.

765

766 **Fig. 2.** Suppression of *sam6* by *pka1*.

767 A, The HS422 (*sam6*) mutant, which expressed *pka1*, was
768 grown on PMAU medium. To check KCl sensitivity, the
769 indicated cells were spotted on PMAU or PMAU
770 containing 1 M KCl with a serial dilution, and were grown
771 for 5 d at 30°C. B, The morphology of the cells was
772 observed in the HS422 (*sam6*) mutant, expressed *pka1* or
773 not. C. Mating efficiency was measured by culturing the
774 indicated strains for 24 h in PMAU medium. The cells
775 were fixed with glutaraldehyde, and mating efficiency
776 was calculated as described in "Materials and Methods".

777

778 **Fig. 3.** Phenotypes of the *pka1* and *pka1(G1382A)*
779 Integrated Strains.

780 A, The SP870 (wild type), HS422 (*sam6*), JZ633 (*Δpka1*),
781 DRG10 (*pka1^{+}-3HA*), DRG6 (*sam6-pka1^{+}-3HA*), and
782 DRG16 (*pka1-G1382A-3HA*) strains were grown on YES
783 and YES containing 1 M KCl plates and incubated for 3 d
784 at 30°C. B, The hyper mating phenotype of DRG16 cells

785 was observed microscopically after incubation for 3 d at
786 30°C on YES medium.
787
788 **Fig. 4.** Viability of *sam6* Mutants under KCl Stress.
789 SP870 (wild type), HS422 (*sam6*), TK105 (Δ*sty1*), MYM1
790 (Δ*moc1*), and JZ633 (Δ*pka1*) cells were cultured at 30°C in
791 YES liquid medium until they reached to log phase, and
792 then were re-cultured for 72 h on YES medium containing
793 1 M KCl or not. They were concentrated to 2 x 10^7
794 cells/mL, and then diluted sequentially 5-fold. These cells
795 were spotted on YES plates and incubated at 30°C for 3 d.
796
797 **Fig. 5.** Interaction between Pka1 and Cgs1 *in Vivo*.
798 Cell extract was prepared from *S. pombe* cells carrying
799 Cgs1-13Myc, Pka1–3HA, Pka1–3HA, and Cgs1–13Myc, or
800 the un-tagged strain (the wild type). The individual cell
801 extracts were incubated with an HA antibody and a Myc
802 antibody. Protein A Sepharose beads were added to the
803 mixtures to co-immunoprecipitate Pka1, and protein G
804 Sepharose beads were added to co-immunoprecipitate Cgs1.
805 The co-immunoprecipitates were analyzed by Western
806 blotting using HA and Myc antibodies. A, Interaction of
807 Pka1 and Cgs1 in glucose-limited (YES+0.1%G) medium. B,
808 Interaction between Pka1 and Cgs1 in glucose-rich (YES+
809 3%G) medium. The same immunoprecipitated samples were
810 loaded in panels 1 and 2, and the same samples in panels 3
811 and 4.
812

813 **Fig. 6.** Western Blot Analysis of Pka1 Followed by Blue
814 Native PAGE and Two-dimensional SDS-PAGE.
815 A, Cells extracts from *S. pombe* DRG21 (Pka1-13Myc),
816 DRG32 (Cgs1-13Myc), DRG31 (*Δcgs1*; Pka1-13Myc), and
817 DRG34 (*Δpka1*; Cgs1-13Myc) growing on glucose-rich
818 (lanes 1 and 2) or glucose-limited medium (lanes 3, 4, 5,
819 and 6) were separated on 4% to 16% gels by BN-PAGE.
820 Western blotting was performed using a Myc antibody
821 (1/3,000), followed by anti-mouse IgG (1/3,000). Arrows
822 and arrowheads indicate the positions of the PKA complex
823 and the Pka1 protein itself respectively. B, Gel strips were
824 excised from the first- dimensional gel, and incubated
825 with dissociation buffers, and placed horizontally on top
826 of the second dimensional gel. Arrowhead indicates the
827 Pka1 protein. B, 10% SDS-PAGE was then performed in the
828 second dimension. When the gel strip was treated with
829 dissociation buffer, the protein complexes dissociated into
830 their constituent polypeptides, and the subunits of the
831 protein complexes separated during two-dimensional
832 electrophoresis. After the two-dimensional SDS-PAGE,
833 Western blotting of the strain DRG21 (Pka1-Myc) growing
834 on glucose rich-medium (YES +3%G) was performed using
835 a Myc antibody (1/3,000) and subsequent anti-mouse IgG
836 (1/3,000). C and D, Two-dimensional electrophoresis was
837 performed using the DRG21 (Pka1-Myc) and DRG32 (Cgs1-
838 Myc) strains growing under glucose-limited conditions.
839 Arrowheads indicate the Pka1 protein (C) and the Cgs1
840 protein (D). E and F, Pka1-13Myc and Cgs1-13Myc were

841 detected by Western blotting with a Myc antibody
842 (1/3,000) and subsequent anti-mouse IgG (1/3,000) on
843 SDS-PAGE alone. Numbers to the left indicate size
844 markers (kD) in all figures.
845
846 **Fig. 7.** Pka1 Phosphorylation under Glucose-Starved
847 Conditions.
848 A, Cells were cultured on YES medium containing 3%
849 glucose at mid-log phase, and then shifted to YES medium
850 containing 2%, 1%, 0.5%, 0.2%, 0.1%, and PMAU medium
851 containing 2% glucose. They were incubated for 6 h, and
852 samples were collected and loaded onto SDS-PAGE. B.
853 Cell extract was prepared from cells containing Pka1-GFP
854 at the time of glucose starvation (YES containing 0.1%
855 glucose). They were grown at 30°C. Samples were loaded
856 onto SDS-PAGE, and epitope-tagged Pka1 proteins were
857 visualized by Western blot developed with anti-GFP
858 antibodies. Tubulin was detected with a tubulin specific
859 antibody (Sigma, St. Louis, MO) as loading control. C, For
860 phosphatase treatments, immunoprecipitated samples at 6 h
861 of glucose starvation were treated with λPPase (New
862 England Bio Labs, Beverly, MA) and phosphatase inhibitor
863 for 1 h of incubation at 30°C in a water bath prior to SDS-
864 PAGE. D, YMSM101 (Pka1-GFP) and YMSM103 (Δ*cgs1,*
865 Pka1-GFP) strains were cultured on YES medium to mid-
866 log phase, harvested by centrifugation, and washed with
867 YES medium containing 0.1% glucose. The cells were then
868 re-suspended in YES medium containing 0.1% glucose and

869 incubated at 30°C for the indicated times.

870

871 **Fig. 8.** Cellular Localization of the Pka1-GFP Protein.

872 YM101 (Pka1-GFP) and DRG101P (Pka1-GFP, Pabp-RFP)

873 cells were cultured in YES medium at 30°C to early-log

874 phase and harvested by centrifugation. The cells were

875 washed twice with YES containing 0.1% glucose medium

876 and re-suspended in YES medium containing 0.1% glucose.

877 YM101 cells were incubated in vacuole-staining dye FM4-

878 64 (Invitrogen, Carlsbad, CA) for 40-45 min (left-hand

879 panel). Pka1-FGP and Pabp-RFP localization was

880 monitored with a fluorescence microscope (right-hand

881 panel). Bar, 10μM.

Table 1. *S. pombe* Strains Used in This Study

Strain	Genotype	Source
SP870	h^{90} *ade6.210 leu1.32 ura4-D18*	8)
HS422	h^{90} *ade6.216 leu1.32 ura4-D18, sam6*	20)
JZ633	h^{90} *ade6.216 leu1.32 ura4-D18 pka1::ura4*	13)
JZ858	h^{90} *ade6.216 leu1.32 ura4-D18 cgs1::ura4*	13)
MYM1	h^{90} *ade6.210 leu1.32 ura4-D18moc1:: kanMX6*	32)
TK105	h^{90} *leu1.32 ura4-D18 sty1::ura4*	22)
YMSM101	h^{90} *ade6.210 leu1.32 ura4-D18 pka1-GFP(S65T)-kanMX6*	19)
DRG6	h^{90} *ade6.216 leu1.32 ura4-D18 pka1:: pka1(WT)-3HA-kanMX6*, *sam6*	This study
DRG10	h^{90} *ade6.210 leu1.32 ura4-D18 pka1-3HA-kanMX6*	This study
DRG16	h^{90} *ade6.210 leu1.32 ura4-D18 pka1::pka1 (G1382A)-3HA- kanMX6*	This study
DRG21	h^{90} *ade6.210 leu1.32 ura4-D18 pka1-13Myc-kanMX6*	This study
DRG31	h^{90} *ade6.216 leu1.32 ura4-D18 cgs1::ura4 pka1-13Myc-kanMX6*	This study
DRG32	h^{90} *ade6.210 leu1.32 ura4-D18 cgs1-13Myc-kanMX6*	This study
DRG33	h^{90} *ade6.210 leu1.32 ura4-D18 pka1-3HA-kanMX6 cgs1-13Myc-hphMX6*	This study
DRG34	h^{90} *ade6.216 leu1.32 ura4-D18 pka1::ura4 cgs1-13Myc-kanMX6*	This study
DRG101P	h^{90} *ade6.210 leu1.32 ura4-D18 pka1-GFP(S65T)-kanMX6 pabp-mRFP-hphMX6*	This study

Table 2. Oligonucleotide Primers Used in This Study

Primer name	Sequence
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'
Pka1-F-*Bgl*II	5'-ACATTAGATCT (*Bgl*II) CATGGATACGACTGC-3'
Pka1-R-*Not*I	5'-CACGCGGCCGC (*Not*I) AAAAGTCCTTAAAGATAG-3'
Pka1-W	5'- TTGCCAAACGCGTCTCTAC-3'
Pka1-X	5'-GGGGATCCGTCGACCTGCAGCGTACGAAAAGTCCTTAAAGATAGAAG-3'
Pka1-Y	5'- GTTTAAACGAGCTCGAATTCATCGATGCGTTGAGCAACGAATGCC-3'
Pka1-Z	5'- TTTGGGAGCCTGTGCTTAG-3'
Cgs1-W	5'- GATCGTACAAGTTTCCGTC-3'
Cgs1-X	5'- GGGGATCCGTCGACCTGCAGCGTACGATGCTTTAGTTGATGGAGGTG-3'
Cgs1-Y	5'- GTTTAAACGAGCTCGAATTCATCGATTGGTCATGCATGCATGTG-3'
Cgs1-Z	5'- TGCGTTACTCCAATGCCAAG-3'

Restriction enzyme sites are underlined.

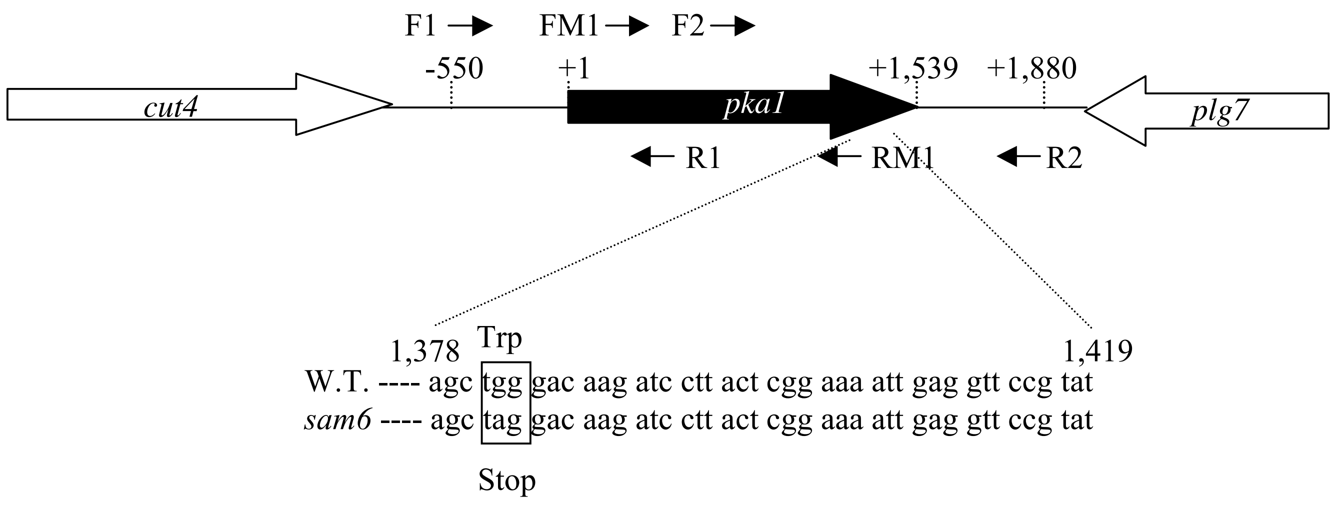
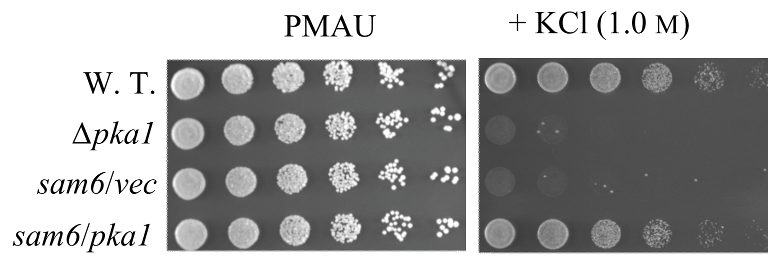


Figure 1, GUPTA et. al.

A



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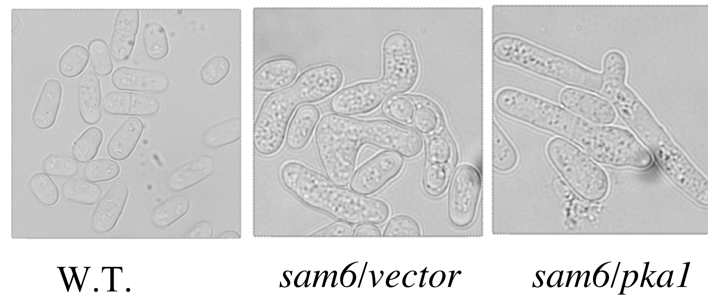

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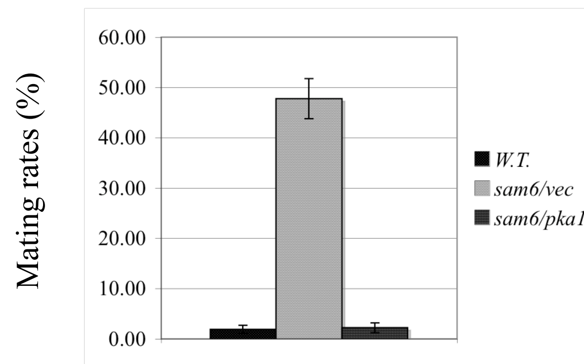


Figure 2, GUPTA et. al.

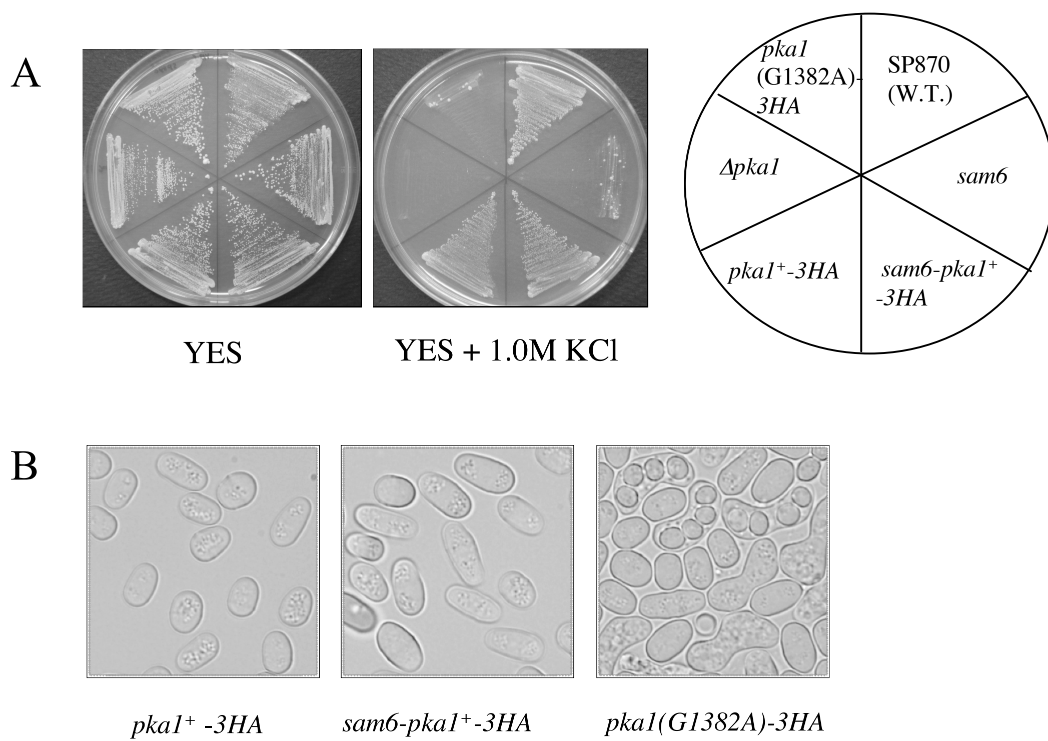


Figure 3, GUPTA et. al.

YES

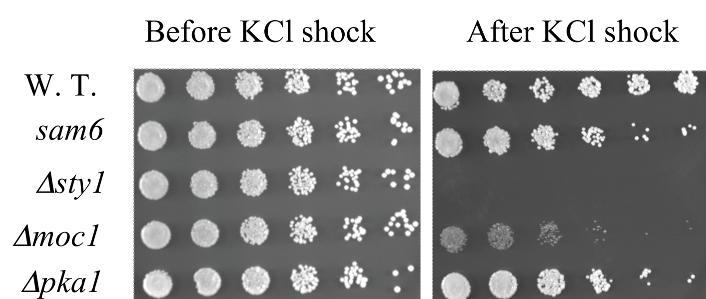
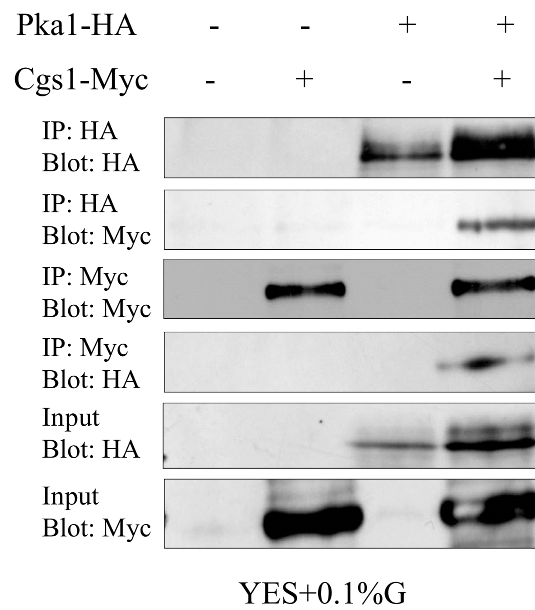


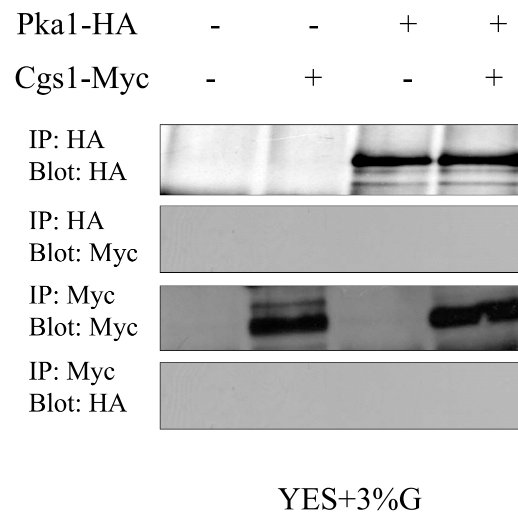
Figure 4, GUPTA et. al.

A



YES+0.1%G

B



YES+3%G

Figure 5, GUPTA et. al.

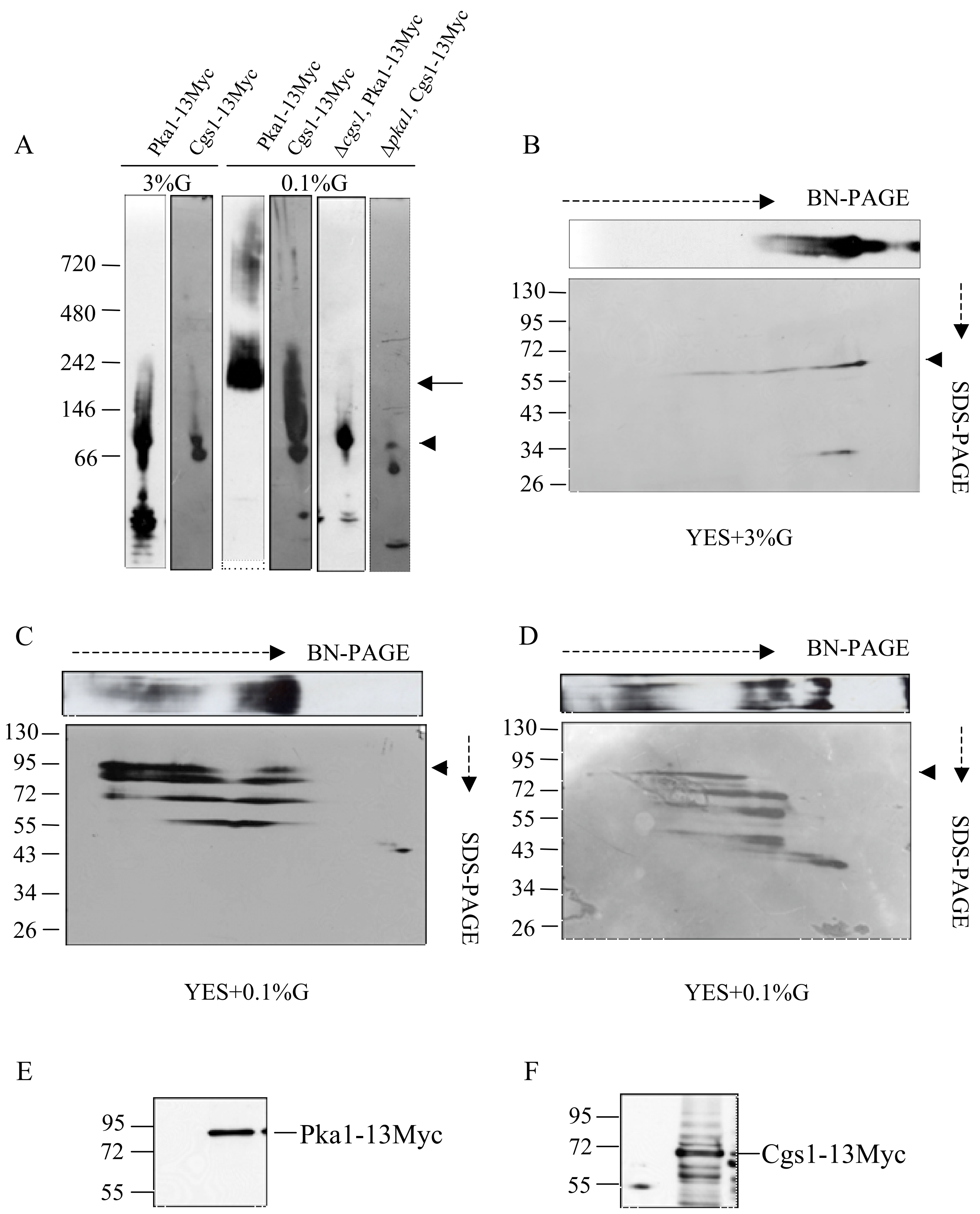


Figure 6, GUPTA et. al.

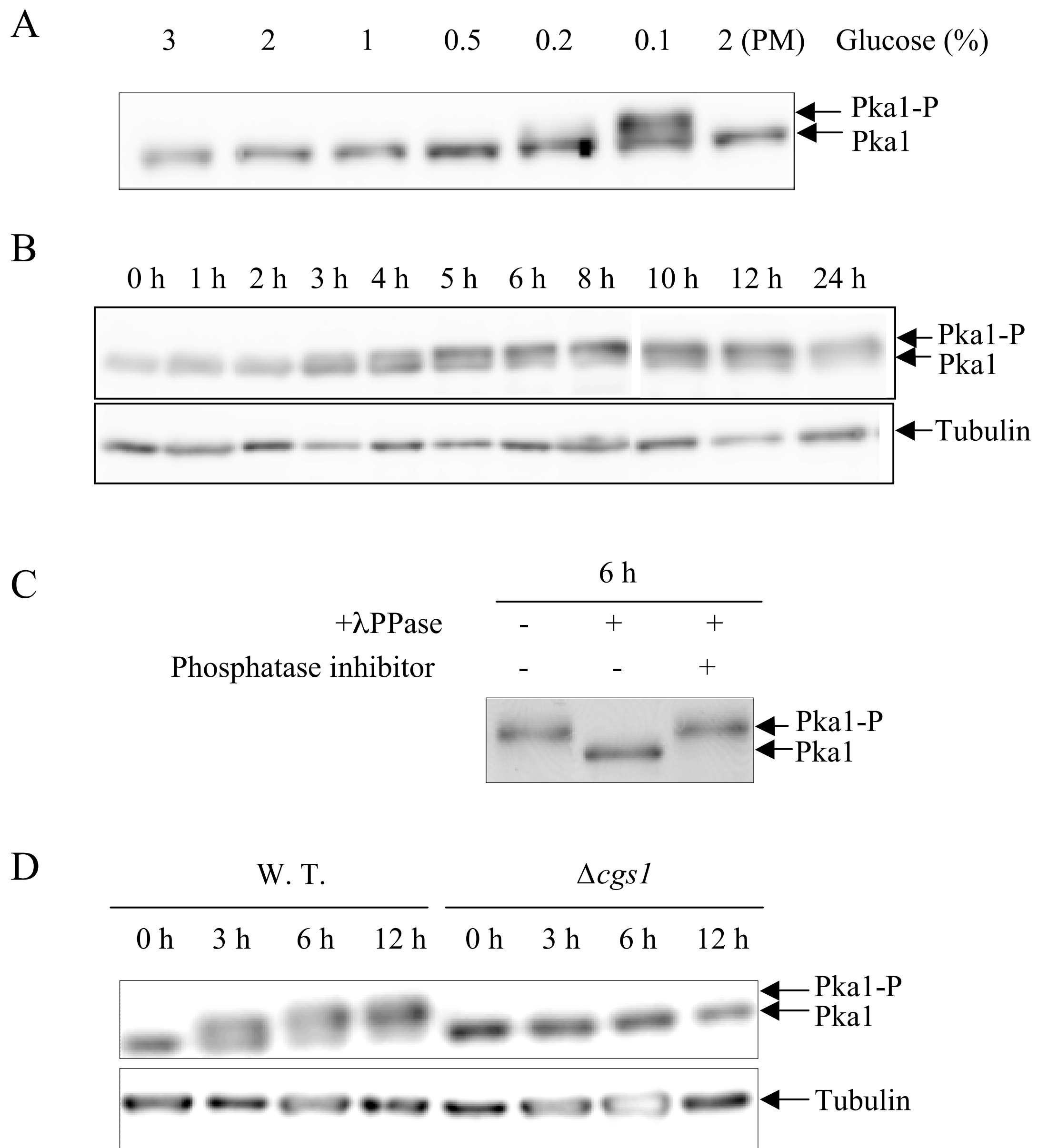


Figure 7, GUPTA et. al.

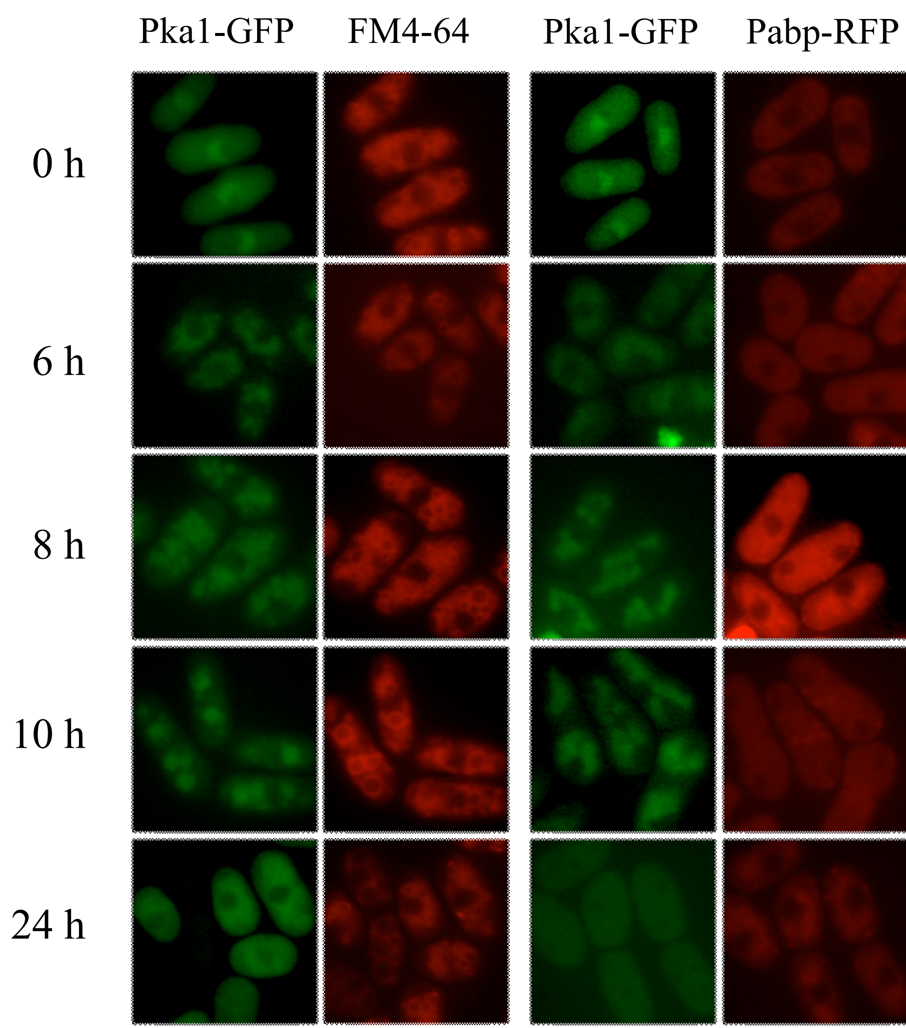


Figure 8, GUPTA et. al.