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Running title: Fission yeast *sam4*

Identification of *sam4* as a *rad24* Allele in *Schizosaccharomyces pombe*

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Abstract

Fission yeast requires nutritional starvation to switch mitotic cell cycle to sexual differentiation, but *sam* mutants, which we had isolated nine alleles, mate without starvation condition. Those mutants are useful for understanding the mechanism underlying how cells sense the nutritional starvation and change the cell cycle. To identify the *sam* allele, we first sought phenotypes other than the original *sam* phenotype. We found that all nine *sam* mutants were sensitive to 1M KCl, *sam2,sam3,sam4* and *sam9* were sensitive to 0.1 M $CaCl_2$ and only the *sam4* mutant was sensitive to 150 J/m^2 UV. This peculiar phenotype of *sam4* suggested us that *sam4* might be the allele of *rad24*, which encodes a 14-3-3 protein. In fact the Rad24 protein disappeared in *sam4* and the *rad24* mRNA was not transcribed in *sam4*. In addition, the mutation that changed Gln to stop codon was found in the *rad24* locus of *sam4*. Thus we concluded that *sam4* is the allele of *rad24*. We also found that over expression of *rad24* or *rad25* (a paralog of *rad24*) has a suppressive effect on *sam1*, but *sam1* was not the allele of *rad24* nor *rad25*. Thus, 14-3-3 proteins are deeply involved in switching of mitotic cell cycle to the sexual differentiation of fission yeast.

The fission yeast *Schizosaccharomyces pombe* proliferates continuously when it has abundant nutrients but arrests its cell cycle progression in the G1 phase upon depletion of glucose or nitrogen. Heterothallic cells of an opposite mating type, namely h^- and h^+, start to develop sexually through processes that include conjugation, meiosis and sporulation. Homothallic cells (h^{90}) switch frequently between the h^- and h^+ mating type.

The sexual differentiation that precedes meiosis is regulated by the cAMP pathway, the stress responsive pathway, the pheromone signaling pathway and the TOR pathway.[1-3] The cAMP pathway signals the nutrient conditions, mainly the glucose levels, to the cells. When glucose (or nitrogen) is abundant, the heterotrimeric-type guanine nucleotide-binding protein (Gpa2) becomes activated through the Git3 receptor; [4] this subsequently activates adenylyl cyclase (Cyr1) to generate cAMP from ATP.[5] Cyr1 interacts with its associated protein Cap1,[6] which plays a partly regulatory role of adenylyl cyclase and also interacts with actin and 14-3-3.[7, 8] When cAMP is abundant, it associates with the regulatory subunit Cgs1. and the catalytic protein kinase Pka1 is released.[9] Sterility caused by higher cAMP levels is reversed by over expression of the *moc1-moc4* genes.[10-14] Pka1 phosphorylates the zinc-finger protein Rst2, which otherwise induces the expression of *ste11* that encodes the key transcription factor.[15]

The pheromone signaling pathway is initiated by the binding of mating pheromone to the pheromone receptor, which

activates the receptor-coupled G protein Gpa1. The signal is then transmitted to a MAPK cascade – a process that depends on the oncoprotein homolog Ras1. The Ras1 protein recruits the MAPKK kinase, Byr2,[16] to the membrane, where it is activated. Byr2 is maintained in an inactive form by an inter-molecular interaction[17] or binding with the 14-3-3 homologs Rad24 and Rad25.[18] Activated Byr2 phosphorylates the MAPK kinase, Byr1, thereby activating it, which in turn activates the MAP kinase, Spk1, via a typical MAP kinase activation mechanism.[19, 20]

Previously, we reported the existence of *S. pombe sam* mutants, which undergo mating and sporulation without requiring nitrogen or glucose starvation. The *sam* phenotype is now re-defined as a mutation that skips the requirement of starvation for mating. Two (*sam3* and *sam9*) are dominant while seven (*sam1*, *sam2*, *sam4, sam4, sam5, sam7 and sam8*) are recessive.[21] On the basis of this characterization, we previously isolated two suppressor genes, *msa1* and *msa2*[22, 23] both of which encode RNA binding proteins that negatively regulate sexual differentiation. We also screened out *sla1*[24, 25] encoding a homolog of the mammalian La protein and *zds1* that involved in $CaCl_2$ tolerance as the genes that suppressed the *ras1* deletion phenotype through the analysis of *sam3* and *sam9*.[26]

In this study we further explored the phenotypes of nine *sam* mutants and identified the *sam4* allele for the first time among nine *sam* mutants. We found that the *sam4* mutation lies in the missense mutation of *rad24* and that abolish the production of

Rad24 and also transcription of *rad24*.

Materials and Methods

Strains and media. The *S. pombe* strains used in this study are listed in Table 1. *S. pombe* was grown in YES-rich medium (0.5% yeast extract, 3% glucose, 225 mg/liter adenine, histidine, leucine, uracil, and/or lysine hydrochloride) or Pombe Minimum (PM) synthetic medium, supplemented with 75 mg/liter adenine, leucine, and/or uracil when necessary. Nitrogen-free PM medium (1% glucose without ammonium chloride) were used to culture *S. pombe* when the mating efficiency had to be measured. *Escherichia coli* strains DH5α was used for plasmid manipulation. *E. coli* was grown in LB medium (1% polypepton, 0.5% yeast extract, 1% NaCl, pH 7.2). Table1

Plasmid. The plasmids pREP42-rad24 and pREP42-rad25 were previously described.[18] pJK148-rad24 was constructed as follows: The *rad24* DNA from wild type *S. pombe* was amplified by PCR using rad24F-SalI and rad24R-NotI primers. The PCR product was digested with *Sal*I and *Not*I and inserted into pJK148, an integration vector for the *leu1* site. pJK148-rad24(C615T) was constructed in the similar way by starting from amplifying the *rad24* locus of *sam4*.

Strain construction. The *rad24*-disrupted strain was constructed as follows: An upstream region of *rad24* from the

wild-type *S. pombe* KT34 genome was amplified by PCR using rad24d1 and rad24d2 as a primer containing the pFA6a-specific DNA sequence. Approximately a 500bp fragment from downstream of the stop codon of the *rad24* gene from KT34 was amplified by PCR using the rad24d3 primer, which contains the pFA6a-specific DNA sequence and the rad24d4 primer. In the second PCR, the *kanMX6* product amplified from pFA6a-kanMX6 by pFA6a-com5 and pFA6a-com6 was used to join the first two PCR products. Strain KT34 was transformed with the second PCR product and stable G418 resistant transformants were selected to obtain the Δ*rad24* strain. Proper integration of the *rad24* disruption was verified by colony directed PCR using Nb2 primer and rad24-P4 primer. The resulting strain was named TMS2. Homothallic *rad24* deletion strain was also derived from SP870 in a similar way, yielding TMS1.

Table 2

Mating efficiency. The mating and sporulation efficiency was calculated using following equation:

Mat (%) = $(2Z + 2A + 0.5S)/(H + 2Z + 2A + 0.5S)$ where Z stands for the number of Zygotes, *A* for the number of asci, *S* for the number of free spores, and *H* for the number of cells that failed to mate.[23)]

Western blotting. Approximately $1x10^8$ cells were harvested after growth in the appropriate medium, washed twice with dH_2O, dissolved in 100 µl of dH_2O, and samples were boiled at 95˚C for 5 min. Subsequently, 120 µl of 2x Laemmli buffer (4% SDS, 20% glycerol, 0.6M ß-mercaptoethanol, 8M urea, 0.12M

Tris-HCl [pH 6.8]) was added and the samples were vigorously vortexed with acid-washed glass beads for 3min. The samples were heated at 95˚C for 5min. The glass beads and large debris were removed by centrifugation at 16,000 x g for 10min (Fig. 4). Each sample was analyzed on SDS-PAGE with a 7.5% or 10% polyacrylamide gel and then transferred to Immobilon Transfer Membranes (Millipore) by using a wet-type transfer system or semidry transfer system. To detect the Rad24 protein, the membrane was incubated with a Rad24 antibody diluted 1:2,000 in 5% skim milk in PBS-T (137mM NaCl, 8mM $Na_2HPO_4 \cdot 12H_2O$, 2.7mM KCl, 1.5mM KH_2PO_4, 0.1% Tween20). The membrane was washed with PBS-T for 15min and 5min twice per wash and then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Bio-Rad Laboratories, Inc.) diluted 1:3,000 in 5% skim milk in PBS-T. After the membrane was washed, the secondary antibodies were detected with the ECL system as described by the manufacturer (Amersham). To detect tubulin, the membrane was incubated with an anti-tubulin monoclonal antibody (Sigma T-5168) diluted 1:5000 in 5% skim milk in PBS-T, washed and then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody diluted 1:1000 in 5% skim milk in PBS-T. After the membrane was washed, the secondary antibodies were detected with the ECL system (Amersham)

Preparation of RNA and RT-PCR.

Total RNA was prepared for RT-PCR and it was performed as

described previously.[23] *S. pombe* cells were grown in PM medium at 30°C to a density of 2 x 10^8 cells/ml. The cells were pelleted by centrifugation, washed with DEPC treated H_2O. The cells were suspended in 1 ml of ISOGEN (RNA isolation reagent; Nippon Gene) and vigorously vortexed 6 minutes with glass beads. After centrifugation (10,000 x g for 15 minutes at 4°C), the supernatant was precipitated with isopropanol. Then, RNA samples were treated with RQ1 RNase free DNase (Promega) for 30 min at 37°C. Each 500ng RNA was reverse transcribed by TAKARA PrimerScript followed by semi quantitative PCR using Ex taq polymerase.

Results

Identification of sam alleles

We have previously isolated nine *sam* mutants that mate and sporulate without any starvation condition in *S. pombe*.[21] Wild type *S. pombe* strain dose not form spores under nutrient rich condition such as on YES rich medium, but all *sam* mutants formed the spores under the same condition. Thus, the *sam* phenotype is now re-defined as a mutation that skips the requirement of starvation for mating. The degree of mating efficiency were variable among nine *sam* mutants but which are clearly higher than wild type (Fig.1). Rounder cell shaped cells were often observed in *sam3, sam4* and *sam9* strains (Fig. 1)

Fig.1

To identify the corresponding *sam* mutations, we first looked for the phenotypes in which we can use for the screening to

identify the *sam* gene. We checked the sensitivity of nine *sam* mutants to 0.1M $CaCl_2$, 1M KCl, 0.18M NaCl, 0.2M $MgCl_2$, 0.2M $MgSO_4$, 0.25mM $MnCl_2$, and 1.2M sorbitol. Although no sensitivity was found to 0.2M $MgCl_2$, 0.2M $MgSO_4$, 0.25mM $MnCl_2$, and 1.2M sorbitol (data not shown), we found that *sam2*, *sam3*, *sam4*, and *sam9* are sensitive to 0.1 M $CaCl_2$, all nine *sam* mutants are sensitive to 1M KCl but with different degree (Fig. 2) and *sam4* was a little sensitive to 0.18M NaCl (data not shown). Because KCl sensitivity is a characteristic phenotype of *cyr1* and *pka1* mutants, [27, 28] which are also categorized as *sam* mutants, those results suggest the tight linkage of *sam* phenotype and KCl sensitivity. Fig.2

We next tested UV sensitivity of all *sam* mutants and found only *sam4* is clearly sensitive to the UV in the dose higher than 120 J/m^2 (Fig. 3). The observation of UV sensitivity of *sam4* lead us to compare its sensitivity in the *rad24* mutant, because of which is known to be sensitive to UV[29] and also displayed the *sam* phenotype.[18] As the result, the UV sensitivity of *sam4* was found to be in a similar level to a *rad24* mutant. Fig.3

Detection of Rad24 *protein level in sam mutants*

Above result prompted us to check the protein level of Rad24 in *sam4*, because *rad24* is known to mate without any starvation condition like all *sam* mutants.[18] To know the existence of the Rad24 protein in the *sam4* mutant, we used the antibody against Rad24. This antibody was raised against the *S. pombe* Rad24 protein and recognized Rad24 specifically.[30] When we tested the presence of Rad24 in nine *sam* mutants by

western blotting, Rad24 was detected in all *sam* mutants except for *sam4*. Thus, Rad24 was completely lost in the *sam4* mutant. This result indicated that *sam4* is the allele of *rad24* or the mutation that affect the Rad24 protein. Fig.4

Then, we tested the expression level of *rad24* by Reverse Transcription (RT) coupled PCR. The total RNA was extracted from *sam4* and wild type SP870, and reverse transcribed with Reverse Transcription System (Promega). RT-PCR was conducted by the primer sets of rad24ORF-F and rad24ORF-R. The actin mRNA was amplified by primers set of act1-F and act1-R as a control. As the result, there was no band of *rad24* appeared in the RNA sample taken from *sam4*, while *rad24* was detected in the wild type strain (Fig. 5). Thus, *rad24* was not expressed or very low in the *sam4* mutant. This result suggested that the *sam4* mutation lies within the *rad24* gene or the other gene that affected the transcription of *rad24*. Fig.5

We then sequenced the *rad24* locus of the *sam4* mutant by amplifying the genomic DNA of *rad24* from the *sam4* mutant. The primers of rad24 P-1 and rad24 P-4 were used for amplification of the *rad24* locus and surrounding region. The amplified fragments were directly sequenced by primers indicated in Fig. 6. As the result, only one mutation was found at the position of 615 nucleotide from the start codon of *rad24*. By this mutation, the CAA (Gln) condon was changed to TAA (stop) codon, that lead to generate truncated or non functional Rad24 protein (Fig. 6). There was no other mutation including the promoter region and the terminator region. The result that only the nonsense mutation was found in *rad24* was a little

surprising since we did not detect the transcription of *rad24* in the *sam4* mutant, which will be discussed later.

Fig.6

Suppression of sam4 mutant by rad24

We next introduced the exogenous *rad24* by expression on the plasmid in the *sam4* mutant to test whether all phenotypes of *sam4* was reversed. We confirmed that the plasmid restored its phenotypes of *sam4* in sensitivity to 0.1M $CaCl_2$, 1M KCl and 150 J/m^2 UV (Fig. 7A). The *sam* phenotype, namely, mate without any starvation, was also reversed by expression of *rad24* (Fig. 7B). Further more, we amplified the *rad24* allele from *sam4* and introduced into the *leu1* locus of an authentic *rad24* mutant. As expected, there was no suppression of *rad24* by *rad24* allele of *sam4* in the derived strain named TM4. TM4 was phenotypically identical with the *rad24* deletion mutant (data not shown). All these results consistently indicate that the *sam4* is the allele of *rad24* and also there is no phenotypically difference between the *sam4* mutant (HS418) and *rad24* null allele. From all those results, we concluded that the *sam4* strain contains only the nonsense mutation in *rad24*.

Fig.7

Suppression of the sam1 mutant by rad24 or rad25.

We further examined whether expression of *rad24* or *rad25* affect the phenotypes on other *sam* mutants. We introduced *rad24* or *rad25* in all *sam* mutants to see any phenotypes are reversed by expression of those genes. As the result, the KCl sensitive phenotype and high mating ratio of *sam1* was found

to be reversed by expression of *rad24* or *rad25* (Fig. 8,A & B). To seek out any possibility of *sam1* is the allele of *rad24* or *rad25,* we sequenced the *rad25* and *rad24* loci of the *sam1* mutant (HS412) in a similar way as tested in the *sam4* mutant (HS418), but did not find any alteration in those two genes. We concluded that *sam1* is not a allele of *rad24* nor *rad25*. On the contrary to the *rad24* deletion strain, the *rad25* deletion strain did not show the *sam* phenotype, nor sensitive to KCl, UV, and $CaCl_2$ (Fig. 8,C ,D and data not shown). It is unlikely that any other *sam* mutants are either the allele of *rad25* or *rad24*.

Fig.8

Discussion

Despite a long on-going analysis of *sam* mutants in our laboratory, we had not succeeded to identify any *sam* alleles and only isolated suppressors genes such as *msa1* and *msa2*. [22, 23] We also isolated and characterized *zds1*[26] and *sla1* [24, 25] as inducers of sexual differentiation through the analysis of *sam3* and *sam9* dominant mutants. But here we clearly showed that *sam4* is the allele of *rad24* that encodes one of 14-3-3 proteins. 14-3-3 is a protein widely found in eukaryotes and modulate many cellular process by interacting primarily phospholyrated protein.[31] Fission yeast has one paralogous gene of *rad24* for 14-3-3 called *rad25*. It can be dispensable for either one of *rad24* and *rad25* genes but not for both.[29] A *rad24* deletion mutant is highly inclined to sexual differentiation, but a *rad25* deletion strain is not (Fig. 8). This can be explained by the different expression level of *rad24* and *rad25* in *S. pombe*. Rad24 is much abundant than Rad25 in fission yeast. When

rad25 was expressed under the *nmt1* promoter, it behaves like *rad24* so that the function of Rad24 and Rad25 is not indistinguishable.

It is reasonable that *rad24* allele was included in one of nine *sam* mutants, as it has been known that deletion of *rad24* lead to the *sam* phenotype.[18] The phenotype of *sam4* and *rad24* deletion mutants were identical and no evidence was obtained that argue against the existence of other mutations in *sam4* as far as we examined in this study.

The *sam* phenotype, namely skip starvation for mating, were currently considered in mainly defective at two different points. One defective point resides in the signal transduction pathway such as the cAMP pathway. Typically, *cyr1* that encodes adenylyl cyclase or *pka1* that encodes protein kinase A deletion mutants showed *sam* phenotype.[5, 9] The other is the mutation defective in cell cycle controller like *cig2/cyc17* encoding one of cyclin.[32] If the cell cycle progression was not inhibited at G1 phase, cells easily stop at the G1 phase, so that, cells skip the requirement of nutritional starvation for mating. Since Rad24 interacted with proteins related meiosis such as Ste11, Mei2, and Byr2, and negatively regulate those proteins, the deletion mutant of *rad24* skip requirement of starvation for mating.

Because it has been shown that Rad24 (or Rad25 in some cases) bound with many proteins like Cdc25,[33] Clp1,[34] Plc1,[30] Chk1,[35] Cid13 and CAP[8] in addition to Byr2,[18] Ste11,[36] and Mei2[36] in fission yeast, Rad24 (or Rad25) involves in many cellular events other than sexual

differentiation. In addition to the phenotypes of UV sensitivity and *sam* phenotype, we found *rad24* deletion mutant is sensitive to $CaCl_2$, which was not shown before. Sensitivity to UV can be explainable from its property of Rad24 to interact with Chk1, a check point involved kinase or Plc1, Phospholipase C, and *sam* phenotype can be explainable of its property of Rad24 to interact with Byr2, Ste11 and Mei2. But, $CaCl_2$ and KCl sensitive phenotype can not be clearly explainable at this point. One possibility of $CaCl_2$ sensitivity of the *rad24* deletion mutant might be relevant to the role of Zds1 because deletion of *zds1* causes $CaCl_2$ sensitivity [26] and it interacted with Rad24 (our unpublished observation). But, as it was shown that more than 200 proteins interact with 14-3-3 in *S. cerevisiae*, [37] it is not an easy task to dissect the each function of Rad24 to be responsible for each different phenotype in *S. pombe* by current information.

There was a point mutation in *rad24,* so that we generally expect the transcription is normal. But no or very little transcription of *rad24* was observed in *sam4*. This result was a little surprising. But, we think this observation probably reflect the response of Nonsense mediated mRNA decay (NMD). NMD is the mechanism that detects and eliminates aberrant mRNAs whose expression would result in truncated proteins by premature termination codon.[38] This NMD mechanism is known to be highly conserved in eukaryotes including budding yeast, worms, mammals and plants, although no clear evidence was shown experimentally in fission yeast yet.[38] Our result might be one of the example that fission yeast uses the NMD

mechanism as well.

Among other *sam* mutants *sam1* was suppressed by expression of *rad24* or *rad25*. But, no mutation was found in *rad24* nor *rad25* in *sam1*. Rad24 was detected by western blot so that it is unlikely that *sam1* is the allele of *rad24* or that affect the expression of *rad24*. It is also considered that all nine *sam* are independent allele. We do not know what gene is mutated in *sam1*. But, all our results suggested that 14-3-3 proteins are deeply involved in the sexual differentiation of fission yeast.

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Fig. 1. Phenotypes and mating efficiency of nine *sam* mutants. Cells were grown on YES medium for 3 days and pictures were taken. Mating efficiency was calculated by counting 300 cells.

Fig. 2. Sensitivity of *sam* mutants by KCl and $CaCl_2$.
SP870 (wild-type), and *sam1-sam9* cells were cultured at 30˚C in liquid medium until they reached log phase. They were concentrated to 1 x 10^7 cells/ml and then diluted sequentially 5-fold (in the right-hand direction). The cells were spotted on YES plates and incubated at 30˚C for 3 days with 1 M KCl and 0.1 M $CaCl_2$.

Fig. 3. Sensitivity of *sam* mutants to UV.
Indicated cells were grown and diluted as in Fig. 2. Cells were exposed to 120, 150 J/m^2 UV or none. The cells were spotted on YES plates and incubated at 30˚C for 3 days.

Fig. 4. Western blot of *sam* mutants by Rad24 antibody.
Indicated cells were grown in YES at 30˚C and crude proteins were extracted. The samples were analyzed by SDS-PAGE with a 10% polyacrylamide gel. Rad24 was detected with a Rad24 specific antibody. Tublin was detected with a tubulin specific antibody as a loading control.

Fig. 5. mRNA level of *rad24*.
The mRNA level was monitored by RT-PCR. Samples were taken from a *sam4* mutant and wild type as indicated. Experiments were done with reverse transcriptase (Left panel)

and without reverse transcriptase (Right panel). The *act1* gene encoding actin was used as a control. PCR was done with two different cycles.

Fig. 6. A mutation site of *rad24* in the *sam4* mutant. The *rad24* gene was amplified by rad24 P1 and P-6 primers. The sequence of the *rad24* region was determined by the primers indicated by arrows. A mutation was found at the 615 position from the start codon. By this mutation Gln codon was changed to stop codon.

Fig. 7. Suppression of *sam4* by *rad24*.
A *sam4* mutant that expressed *rad24* or not was grown for 3 days on PM minimum medium under indicated condition(A). Cells were taken by photography (B). The phenotypes of *sam4* were reversed by expression of *rad24*.

Fig. 8. Suppression of the *sam1* mutant by *rad24* or *rad25*.
rad24 or *rad25* was expressed in the *sam1* mutant. They were tested for the phenotypes of KCl sensitivity (A) and mating efficiency (B). Phenotypic comparison of homothallic and heterothallic *rad24* and *rad25* deletion mutants on KCl sensitivity was done (C). Cells of the *rad24* deletion mutant made spores on YES rich medium but cells of the *rad25* deletion did not (D).

References

1) Kawamukai M, *Nippon Nogeikagaku Kaish (in Japanese)*, **73**, 1147-1153 (1999).

2) Yamamoto M, Initiation of meiosis. *In* "The Molecular Biology of *Schizosaccharomyces pombe*." Springer-Verlag, Berlin, pp. 297-309 (2003).

3) Matsuo T, Otsubo Y, Urano J, Tamanoi F and Yamamoto M, *Mol Cell Biol*, **27**, 3154-64 (2007).

4) Hoffman CS, *Biochem Soc Trans*, **33**, 257-260 (2005).

5) Kawamukai M, Ferguson K, Wigler M and Young D, *Cell Regul.*, **2**, 155-64 (1991).

6) Kawamukai M, *Seikagaku*, **68**, 31-5 (1996).

7) Kawamukai M, Gerst J, Field J, Riggs M, Rodgers L, Wigler M and Young D, *Mol. Biol. Cell*, **3**, 167-80 (1992).

8) Zhou GL, Yamamoto T, Ozoe F, Yano D, Tanaka K, Matsuda H and Kawamukai M, *Biosci. Biotechnol. Biochem.*, **64**, 149-59 (2000).

9) Maeda T, Watanabe Y, Kunitomo H and Yamamoto M, *J. Biol. Chem.*, **269**, 9632-9637 (1994).

10) Kawamukai M, *Biochim. Biophys. Acta*, **1446**, 93-101 (1999).

11) Goldar MM, Nishie T, Ishikura Y, Fukuda T, Takegawa K and Kawamukai M, *Biosci. Biotechnol. Biochem.*, **69**, 1422-6 (2005).

12) Goldar MM, Jeong HT, Tanaka K, Matsuda H and Kawamukai M, *Curr. Genet.*, **48**, 345-55 (2005).

13) Yakura M, Ishikura Y, Adachi Y and Kawamukai M, *Biosci Biotechnol Biochem*, **70**, 1740-9 (2006).

14) Paul SK, Goldar MM, Yakura M, OOWATARI Y and Kawamukai M, *Biosci Biotechnol Biochem*, **in press**.
15) Higuchi T, Watanabe Y and Yamamoto M, *Mol Cell Biol*, **22**, 1-11 (2002).
16) Wang Y, Xu HP, Riggs M, Rodgers L and Wigler M, *Mol. Cell. Biol.*, **11**, 3554-3563 (1991).
17) Tu H, Barr M, Dong DL and Wigler M, *Mol. Cell. Biol.*, **17**, 5876-87 (1997).
18) Ozoe F, Kurokawa R, Kobayashi Y, Jeong HT, Tanaka K, Sen K, Nakagawa T, Matsuda H and Kawamukai M, *Mol. Cell. Biol.*, **22**, 7105-19 (2002).
19) Toda T, Shimanuki M and Yanagida M, *Genes Dev.*, **5**, 60-73 (1991).
20) Yamamoto TG, Chikashige Y, Ozoe F, Kawamukai M and Hiraoka Y, *J. Cell Sci.*, **117**, 3875-86 (2004).
21) Katayama S, Ozoe F, Kurokawa R, Tanaka K, Nakagawa T, Matsuda H and Kawamukai M, *Biosci. Biotechnol. Biochem.*, **60**, 994-999 (1996).
22) Jeong HT, Oowatari Y, Abe M, Tanaka K, Matsuda H and Kawamukai M, *Biosci. Biotechnol. Biochem.*, **68**, 1621-6 (2004).
23) Jeong HT, Ozoe F, Tanaka K, Nakagawa T, Matsuda H and Kawamukai M, *Genetics*, **167**, 77-91 (2004).
24) Tanabe K, Ito N, Wakuri T, Ozoe F, Umeda M, Katayama S, Tanaka K, Matsuda H and Kawamukai M, *Eukaryot. Cell*, **2**, 1274-1287 (2003).
25) Tanabe K, Tanaka K, Matsuda H and Kawamukai M, *Biosci. Biotechnol. Biochem.*, **68**, 266-270 (2004).

26) Yakura M, Ozoe F, Ishida H, Nakagawa T, Tanaka K, Matsuda H and Kawamukai M, *Genetics*, **172**, 811-25 (2006).
27) Yang P, Du H, Hoffman CS and Marcus S, *Mol Genet Genomics*, **269**, 116-125 (2003).
28) Matsuo Y, McInnis B and Marcus S, *Eukaryot Cell*, **7**, 1450-1459 (2008).
29) Ford JC, al-Khodairy F, Fotou E, Sheldrick KS, Griffiths DJ and Carr AM, *Science*, **265**, 533-5 (1994).
30) Andoh T, Kato T, Jr., Matsui Y and Toh-e A, *Mol. Gen. Genet.*, **258**, 139-47 (1998).
31) van Heusden GP and Steensma HY, *Yeast*, **23**, 159-71 (2006).
32) Obara-Ishihara T and Okayama H, *Embo J*, **13**, 1863-72 (1994).
33) Lopez-Girona A, Furnari B, Mondesert O and Russell P, *Nature*, **397**, 172-5 (1999).
34) Mishra M, Karagiannis J, Sevugan M, Singh P and Balasubramanian MK, *Curr. Biol.*, **15**, 1376-83 (2005).
35) Dunaway S, Liu HY and Walworth NC, *J. Cell Sci.*, **118**, 39-50 (2005).
36) Kitamura K, Katayama S, Dhut S, Sato M, Watanabe Y, Yamamoto M and Toda T, *Dev. Cell*, **1**, 389-399 (2001).
37) Kakiuchi K, Yamauchi Y, Taoka M, Iwago M, Fujita T, Ito T, Song SY, Sakai A, Isobe T and Ichimura T, *Biochemistry*, **46**, 7781-92 (2007).
38) Shyu AB, Wilkinson MF and van Hoof A, *EMBO J*, **27**, 471-81 (2008).

Table 1

Strain	Genotype	Source
L972	*h-*	Lab. stock
SP66	*h90 ade6-M210 leu1-32*	Lab. stock
SP870	*h90 ade6-M210 leu1-32 ura4-D18*	5)
KT34	*h- ade6-M210 leu1-32 ura4-D18*	21)
HS412	*h90 ade6-M216 leu1-32 ura4-D18, sam1*	21)
HS414	*h90 ade6-M216 ura4-D18, sam2*	21)
HS416	*h90 ade6-M216 ura4-D18, sam3*	21)
HS418	*h90 ade6-M216 leu1-32 ura4-D18, sam4*	21)
HS420	*h90 ade6-M216 leu1-32 ura4-D18, sam5*	21)
HS422	*h90 ade6-M216 leu1-32 ura4-D18 sam6*	21)
HS424	*h90 ade6-M216 leu1-32 ura4-D18 sam7*	21)
HS428	*h90 ade6-M216 ura4-D18 sam8*	21)
HS430	*h90 ade6-M216 ura4-D18 sam9*	21)
r24.d	*h- ade6-M704 leu1-32 ura4-D18 rad24::ura4*	29)
r25.d	*h- ade6-M704 leu1-32 ura4-D18 rad25::ura4*	29)
SP24U1	*h90 ade6-M210 leu1-32 ura4-D18 rad24::ura4*	18)
SP25U1	*h90 ade6-M210 leu1-32 ura4-D18 rad25::ura4*	18)
TMS1	*h^{90} ade6-M210 leu1-32 ura4-D18 rad24::kanMX6*	this study
TMS2	*h^{-} ade6-M210 leu1-32 ura4-D18 rad24::kanMX6*	this study
TMS3	*h^{90} ade6-M210 ura4-D18 rad24::kanMX6 leu1-32::leu1:rad24*	this study
TMS4	*h^{90} ade6-M210 ura4-D18 rad24::kanMX6 leu1-32::leu1:rad24(C615T)*	this study

Table 2 primers

Primer name	Sequence (5' to 3')
Rad25F-NotI	TAGCGGCCGCATGAGTAATTCTCGTG
Rad25R-NotI	TAGCGGCCGCAAGCTTTAACAGTGTCA
rad24ORF-F	ACCTTTCCAGTGCCAACCAC
rad24ORF-R	GGCATCAATCATGAGCAACG
rad24F-SalI	TATGTCGACACACCTGGTGGCATAGCAGAC
rad24R-NotI	CTGGCGGCCGCACAGCAGCTACCAAATACACAC
rad24-d1	TTTGCACGTGTTCAGGCACC
rad24-d2	TCGACCTGCAGCGTACGATTGGCACTGGAAAGGTTGCG
rad24-d3	TAAACGAGCTCGAATTCATCGATAGCTCGTTGCTCATGATTGATGCC
rad24-d4	AGTGGTACATCGGTCGTATGC
rad24 P-1	GGAACGTGTCGCAAAATTG
rad24 P-2	CTTCCGCCGATAGAACCTTG
rad24 P-3	TCTCCGAGTTGGACAGCCTTTC
rad24 P-4	AGGGTCAACCGACTTTCTCG
rad24 P-5	TACATCATGCTGCGCATCTC
rad24 P-6	AGAAAACCTTGGATTCGGCAG
rad25 P-1	TGGGAAAATCGTATTGACCAG
rad25 P-2	GGCCATTTCAGAGCTCGATAGTC
rad25 P-3	TCCCACATACAGTAGCAAACTGC
rad25 P-4	CAATCAAGTTGCCCACAACG
rad25 P-5	CTTGTCGAGTGTTGCCCCTAC
rad25 P-6	TCAGGAGTAACACGAAACACCG
pFA6a-com5	TCGTACGCTGCAGGTCGA
pFA6a-com6	ATCGATGAATTCGAGCTCGTTTA
act1-F	GGCATCACACTTTCTACAACG
act1-R	GAGTCCAAGACGATACCAGTG
nmt1-F	CCGGATAATGGACCTGTTAA
nmt1-R	GAATGGGCTTCCATAGTTTG
Nb2	GTTTAAACGAGCTCGAATTCATCGAT

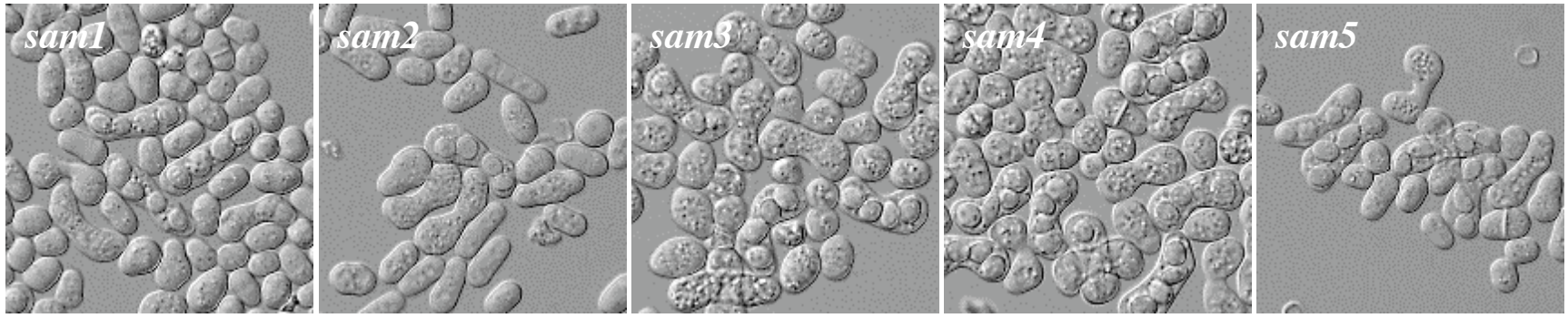

sam1
sam2
sam3
sam4
sam5
24.6%
11.5%
27.6%
29.2%
33.8%
sam6
sam7
sam8
sam9
Wild Type
51.2%
15.8%
33.1%
24.3%
0.7%

Fig.1

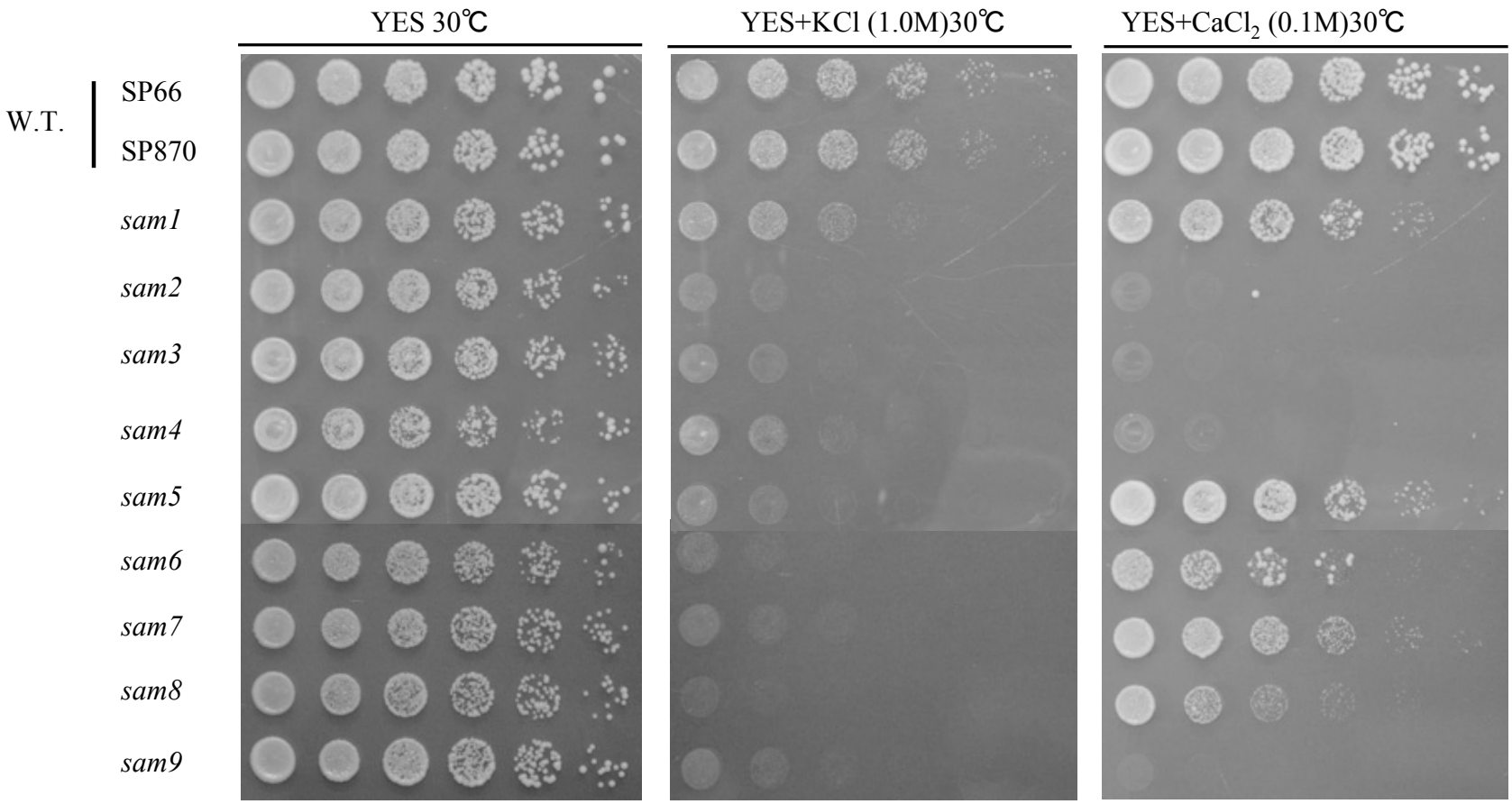

YES 30℃
YES+KCl (1.0M)30℃
YES+CaCl2 (0.1M)30℃
W.T.
SP66
SP870
sam1
sam2
sam3
sam4
sam5
sam6
sam7
sam8
sam9

Fig.2

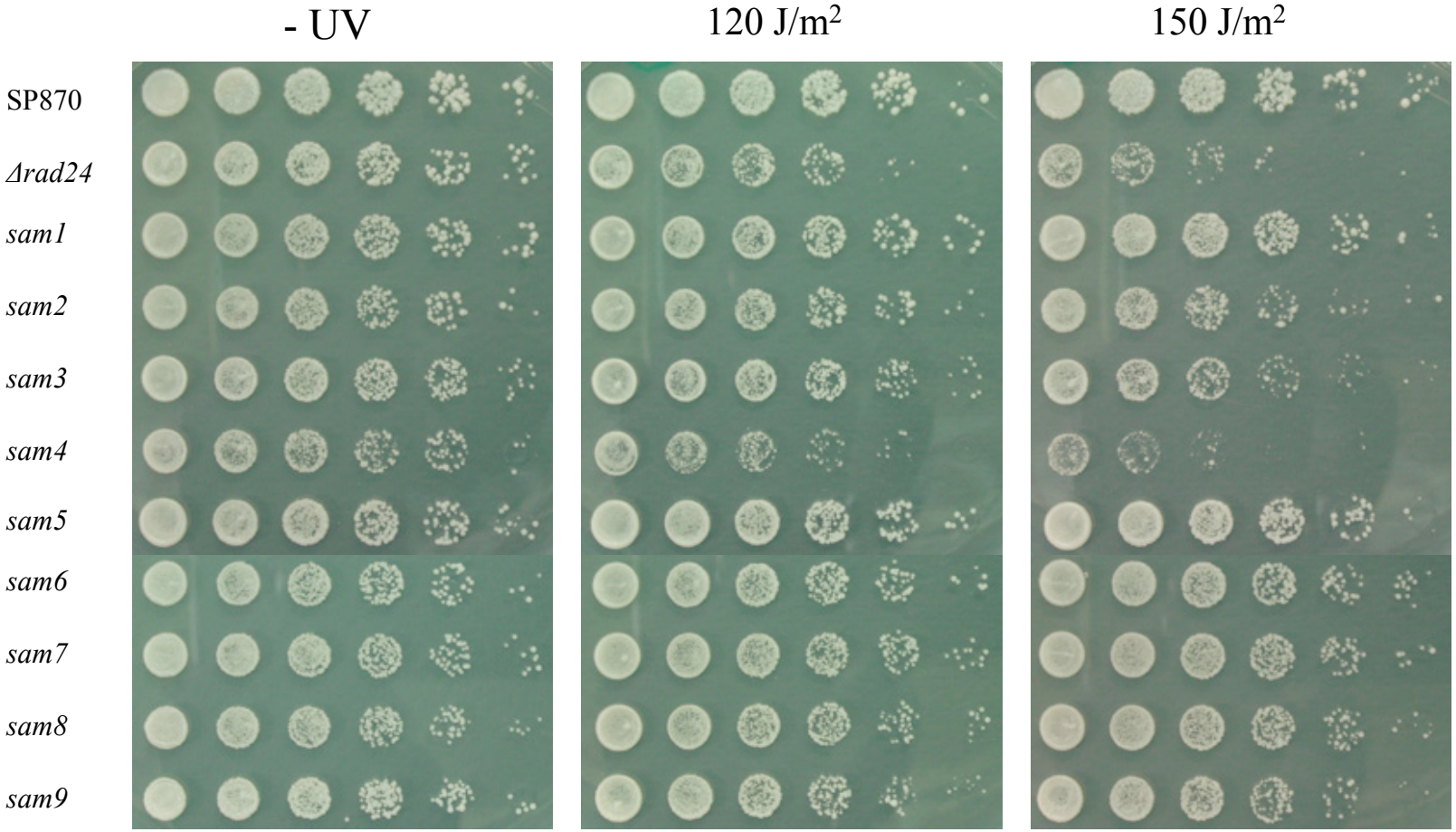

- UV
120 J/m2
150 J/m2
SP870
Δrad24
sam1
sam2
sam3
sam4
sam5
sam6
sam7
sam8
sam9

Fig.3

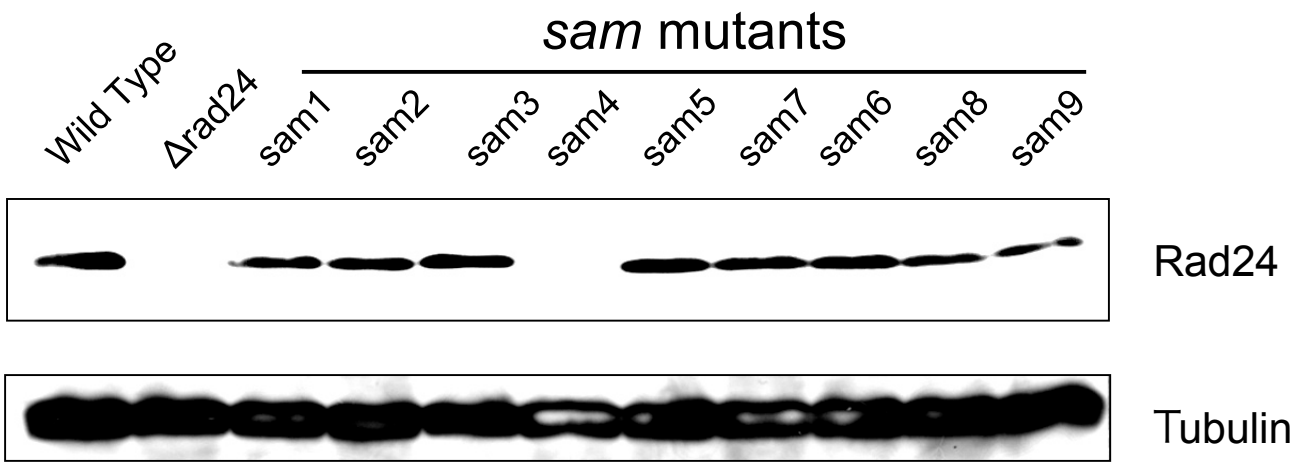

sam mutants
Wild Type
Δrad24
sam1
sam2
sam3
sam4
sam5
sam7
sam6
sam8
sam9
Rad24
Tubulin

Fig.4

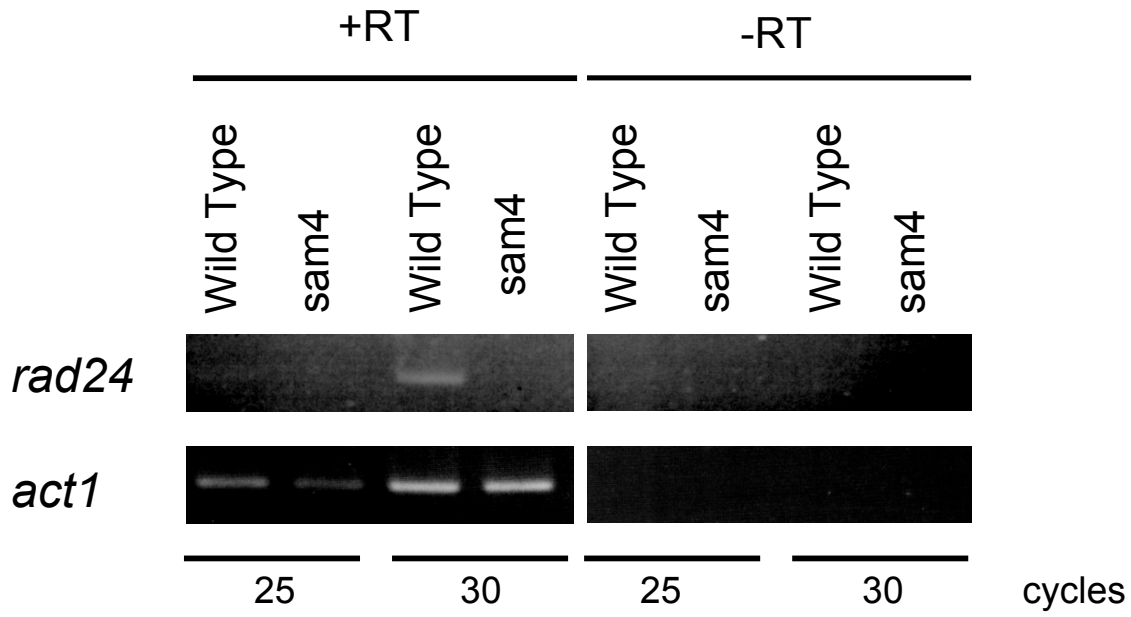

+RT
-RT
Wild Type
sam4
Wild Type
sam4
Wild Type
sam4
Wild Type
sam4
rad24
act1
25
30
25
30
cycles

Fig.5

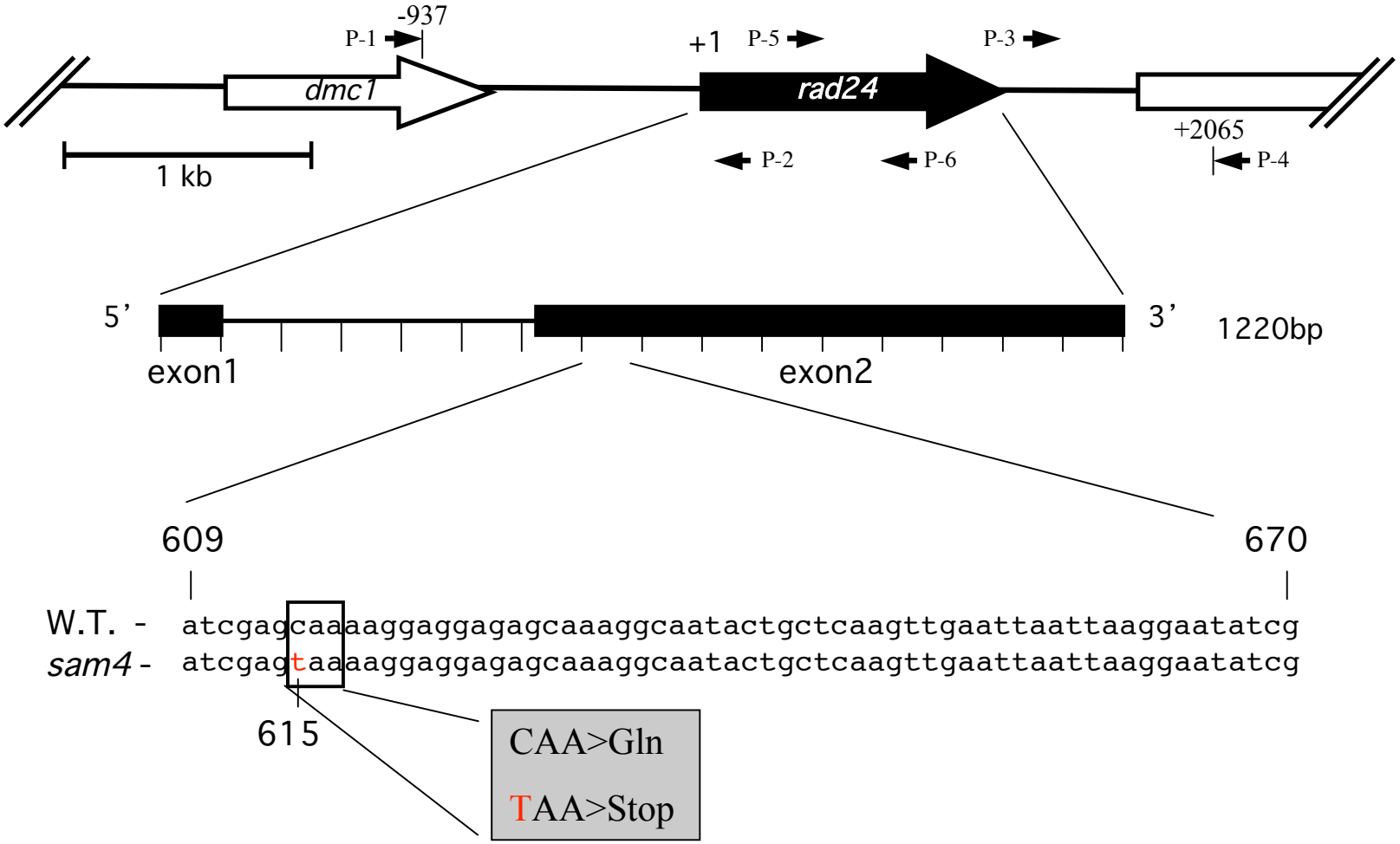
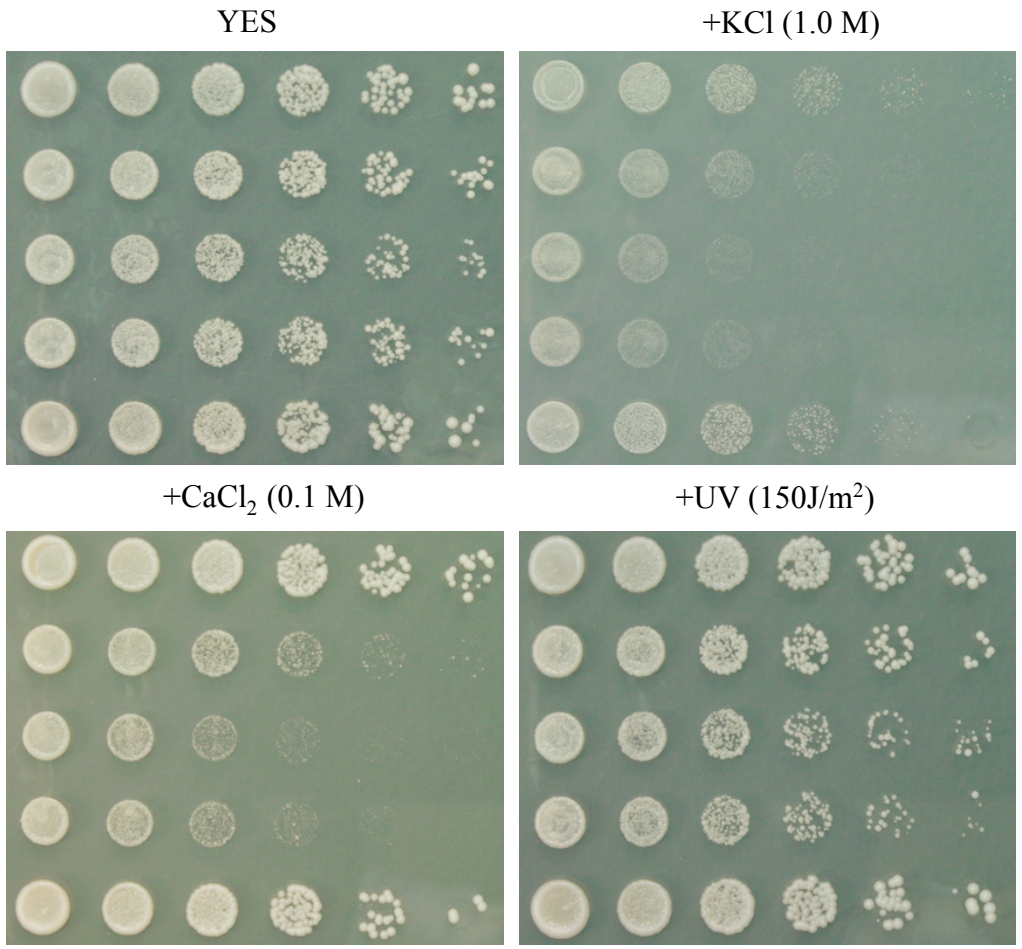

-937
P-1
+1
P-5
P-3
dmc1
rad24
+2065
1 kb
P-2
P-6
P-4
5'
3'
1220bp
exon1
exon2
609
670
W.T. - atcgagcaaaaggaggagagcaaaggcaatactgctcaagttgaattaattaaggaatatcg
sam4 - atcgagtaaaaggaggagagcaaaggcaatactgctcaagttgaattaattaaggaatatcg
615
CAA>Gln
TAA>Stop

Fig.6

A


YES
+KCl (1.0 M)
W.T. (SP870)
Δrad24
sam4
sam4/vector
sam4/rad24
+CaCl2 (0.1 M)
+UV (150J/m2)
W.T. (SP870)
Δrad24
sam4
sam4/vector
sam4/rad24

B

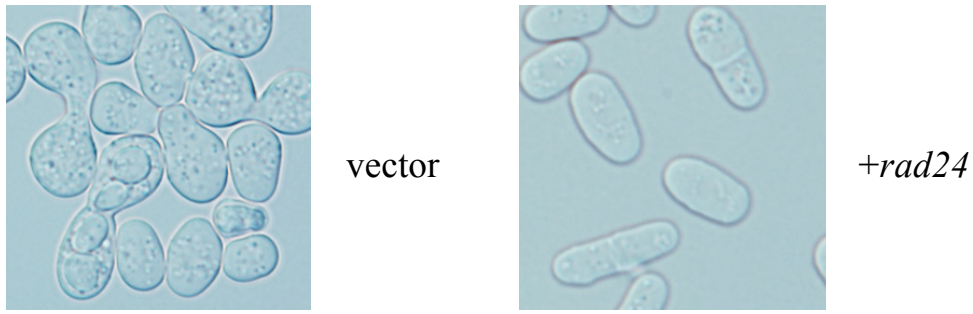
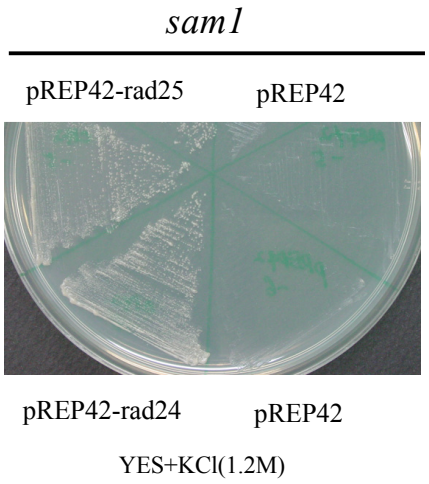
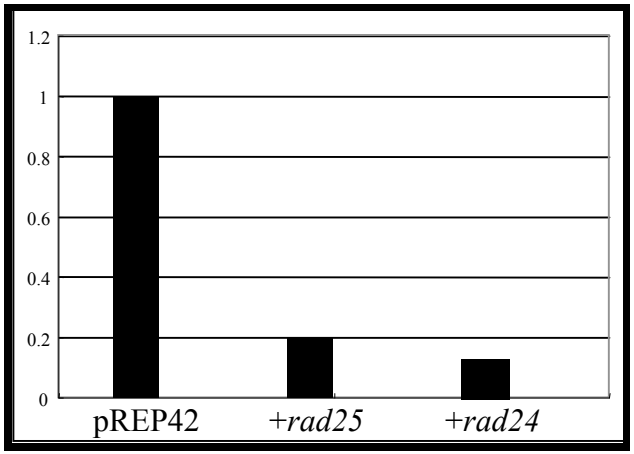

vector
+rad24

Fig.7

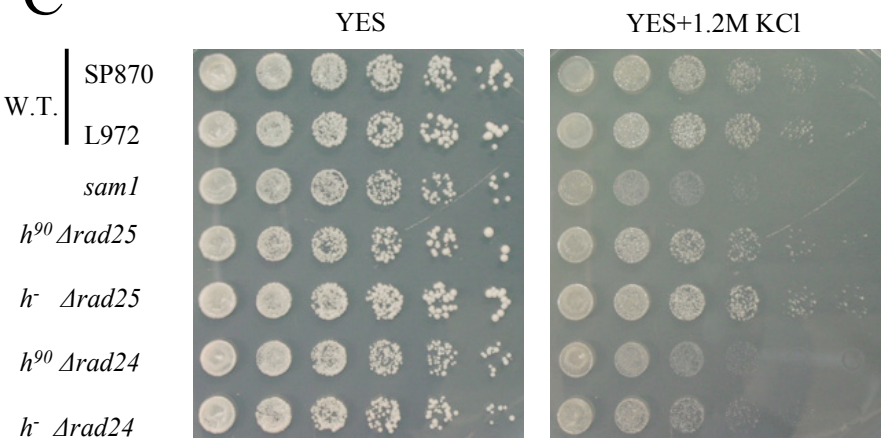
A


sam1
pREP42-rad25
pREP42
pREP42-rad24
pREP42
YES+KCl(1.2M)

B


Relative spore formation
1.2
1
0.8
0.6
0.4
0.2
0
pREP42
+rad25
+rad24

C


YES
YES+1.2M KCl
W.T.
SP870
L972
sam1
h90 Δrad25
h- Δrad25
h90 Δrad24
h- Δrad24

D

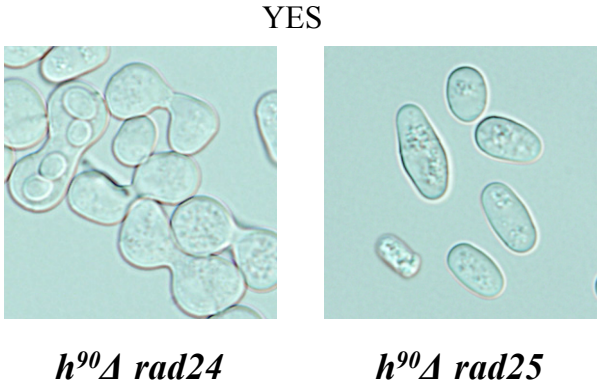

YES
h90Δ rad24
h90Δ rad25

Fig.8