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Research Article

cAMP-dependent protein kinase involves calcium tolerance through the regulation of Prz1 in *Schizosaccharomyces pombe*

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Running Title: Pka1 mediates calcium stress in fission yeast

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Abstract

The cAMP-dependent protein kinase Pka1 is known as a regulator of glycogenesis, meiosis, and stress responses in *Schizosaccharomyces pombe*. We demonstrated that Pka1 is responsible for calcium tolerance. Loss of functional components of the PKA pathway such as Git3, Gpa2, Cyr1, and Pka1 yields a $CaCl_2$-sensitive phenotype, while loss of Cgs1, a regulatory subunit of PKA, results in $CaCl_2$ tolerance. Cytoplasmic distribution of Cgs1 and Pka1 is increased by the addition of $CaCl_2$, suggesting that $CaCl_2$ induces dissociation of Cgs1 and Pka1. The expression of Prz1, a transcriptional regulator in calcium homeostasis, is elevated in a *pka1*Δ strain and in a wild type strain under glucose-limited conditions. Accordingly, higher expression of Prz1 in the wild type strain results in a $CaCl_2$-sensitive phenotype. These findings suggest that Pka1 is essential for tolerance to exogenous $CaCl_2$, probably because the expression level of Prz1 needs to be properly regulated by Pka1.

Key words: Pka1; Prz1; calcium tolerance

Introduction

Cyclic adenosine monophosphate (cAMP)-dependent protein kinase, also known as protein kinase A (PKA), is a serine/threonine kinase that is widely conserved among organisms from yeasts to mammals [1-4]. PKA forms a heterotetramer, in which two regulatory subunits interact with two catalytic subunits. The PKA heterotetramer is activated by disassociation after two molecules of cAMP bind to the regulatory subunits of PKA [3]. In the fission yeast *Schizosaccharomyces pombe*, the cAMP/PKA pathway consists of G-protein coupled receptor Git3, heterotrimeric G protein alpha subunit Gpa2, beta subunit Git5, gamma subunit Git11, adenylate cyclase Cyr1, regulatory subunit Cgs1, and catalytic subunit Pka1 [1, 5-7]. This pathway is known to play roles in glucose-sensing, chronological aging, regulation of transit to meiosis, and the stress response [1, 8-11]. Both Cgs1 and Pka1 are concentrated in the nucleus while being more diffusely present in the cytoplasm [12]. The nuclear localization of both these proteins is dependent on cAMP, since cytoplasmic localization of Cgs1 and Pka1 predominates following *cyr1* gene deletion [12]. The nuclear-cytoplasmic redistribution of both proteins is triggered by glucose-limitation, KCl stress, and in stationary-phase growth [12]; and Pka1 physically interacts with Cgs1 under glucose-limited conditions and not under glucose-rich conditions [13]. Pka1 is phosphorylated at threonine 356 under glucose-limited conditions [13, 14] and Pka1 phosphorylation is elevated in *cyr1*Δ mutants and is promoted by KCl [15]. In glucose-rich medium, the non-phosphorylated form of mutant Pka1 (T356A) localizes evenly between the cytoplasm and the nucleus, while the phosphomimetic mutant Pka1 (T356D) concentrates only in the nucleus. In glucose-limited medium, however, the Pka1 (T356A) and Pka1 (T356D) mutants localize to the cytoplasm [14].

Calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, consists of Ppb1 as catalytic subunit A and Cnb1 as regulatory subunit B in *S. pombe* [16, 17]. Both *chb1*Δ and *ppb1*Δ single mutants are viable and sensitive to FK506, $CaCl_2$, and $MgCl_2$ [16]. The *cnb1*Δ *ppb1*Δ double mutants exhibit phenotypes similar to those exhibited by each single deletion mutant. These observations indicate that the deletion of each single gene alone

yields the same functional defect as calcineurin deletion [16]. Calcineurin Ppb1 dephosphorylates and regulates a C2H2-type zinc finger transcription factor Prz1, which is located downstream of Ppb1 [18]. The mRNA expression of *prz1* is enhanced by the addition of $CaCl_2$ and is dependent on Ppb1 [18]. The *prz1*-deficient mutant is hypersensitive to Ca^{2+}, which results from the decreased transcription of the Pmc1 Ca^{2+} pump [18]; and overexpression of Prz1 results in defective growth in the wild type strain. Seven genes including *pka1* were isolated as multicopy suppressors of growth defect in Prz1-overexpressing cells. It was also shown by CDRE (calcineurin-dependent response element)-dependent reporter assay that the addition of cAMP represses the transcriptional activity of Prz1. Finally, an *in vitro* kinase assay demonstrated that Pka1 phosphorylates Prz1 [19]. In *Saccharomyces cerevisiae*, Crz1 (calcineurin-responsive zinc finger 1), a homologue of Prz1 [20], is phosphorylated by PKA at serines 409, 410, 423, 427, and 429 and is negatively regulated by inhibiting nuclear import [21]. These works suggest that PKA is involved in the calcineurin signaling pathway; however, it is unclear whether PKA is responsible for calcium homeostasis in *S. pombe*.

In this study, cells were found to exhibit $CaCl_2$ sensitivity following the deletion of functional PKA and $CaCl_2$ was shown to induce dramatic changes in the localization of Cgs1 and Pka1, indicating that Pka1 is activated by the addition of $CaCl_2$. Finally, we showed that the expression of *prz1* mRNA is elevated in *pka1*Δ mutants and the deletion of the *prz1* gene enhances the $CaCl_2$-sensitive phenotype of *pka1*Δ. These findings confirm that Pka1 plays a role in calcium homeostasis via Prz1 regulation.

Materials and Methods

Yeast strains, media, and genetic methods

The *S. pombe* strains used in this study are listed in Table 1. Standard yeast culture media and genetic methods were used [22, 23]. *S. pombe* cultures were grown in either YES medium (0.5% yeast extract, 3% glucose, 225 mg/L adenine, 225 mg/L uracil, 225 mg/L leucine, 225 mg/L histidine, and 225 mg/L lysine), YES glucose-limited medium (0.5% yeast extract, 0.1% glucose, 3% glycerol, 225 mg/L adenine, 225 mg/L uracil, 225 mg/L leucine, 225 mg/L histidine, and 225 mg/L lysine), or synthetic minimal medium (EMM) with appropriate auxotrophic supplements [22].

Construction of git3Δ, gpa2Δ, and prz1-GFP

The recombinant polymerase chain reaction (PCR) method described by Krawchuk et al. [24] was used to generate *git3::ura4* and *gpa2::ura4* cassettes. The pFA6a-ura4 plasmid was used as the template DNA and the oligonucleotide primer sets git3-KOW (5′-CAGTTTAATCGTGTGTCTAC-3′)/git3-KOX (5′-GGGGATCCGTCGACCTGCAGCGTACGAGAGCAACCTTGTTCCTTATTAC-3′) and git3-KOY (5′-GTTTAAACGAGCTCGAATTCATCGATAAATTTTCTCATAGCCTTTG-3′)/git3-KOZ (5′-GTATTGAGGAAATATAGTATTG-3′) were used to construct the *git3::ura4* cassette and the primer pairs gpa2-KOW (5′-CTTAATCAACCTTGGTATTCAC-3′)/gpa2-KOX (5′-GGGGATCCGTCGACCTGCAGCGTACGACACTACTGCAGAATATAAATAC-3′) and gpa2-KOY (5′-GTTTAAACGAGCTCGAATTCATCGATCTGTCTGCATATGTCTAGAGAG-3′)/gpa2-KOZ (5′-CACTCAAAGTCTCTTAGTACTC-3′) were used to construct the *gpa2::ura4* cassette. *S. pombe* strain PR109 was transformed with *git3::ura4* and *gpa2::ura4* cassettes and the resulting transformants were isolated on EMM containing leucine. Transformants in which the endogenous *git3* and *gpa2* genes were replaced by *git3::ura4* and *gpa2::ura4*, respectively, were identified by colony PCR [25].

The *prz1-GFP*-tagged strain was constructed using the recombinant PCR approach described by Krawchuk et al. [24]. The resulting DNA fragments carried the GFP(S65T)-kanMX6 cassette to the region 3′-downstream of the *prz1* gene. For this purpose, the following oligonucleotides were used: prz1-TAGW (5′-GCAATAAGCGTTTTACTAG-3′), prz1-TAGX (5′-GGGGATCCGTCGACCTGCAGCGTACGATTTTTGTTTGCTTGTCGAGG-3′), prz1-TAGY (5′-GTTTAAACGAGCTCGAATTCATCGATCACAGTTTTGCATTTAGGGT-3′), and prz1-TAGZ (5′-CTCTTGGTCGACGGGTATATG-3′). The PR109 strain was transformed with the resulting *prz1-GFP(S65T)-kanMX6* cassette and transformants were isolated on YES medium containing G418. Transformants carrying the GFP-tagged *prz1* gene were identified by colony PCR [25].

*S. pombe cyr1*Δ *cgs1*Δ*, cyr1*Δ *cgs1-GFP, cyr1*Δ *pka1-GFP, pka1*Δ *prz1*Δ*,* and *pka1*Δ *prz1-GFP* were constructed by genetic crossing using standard yeast genetic techniques [22, 23].

Plasmid construction

To construct pREP3X-Prz1, the oligonucleotide primers prz1F (5′-TATGTCGACATGGAGCGTCAAAGGTCAG-3′) and prz1R (5′-ACAGGATCCTCATTTTTGTTTGCTTGTC-3′) were used to amplify a 2.0-kb fragment of the complete *prz1* protein coding sequence from *S. pombe* genomic DNA. The amplified *prz1* gene fragment was digested with *Sal*I and *Bam*HI and ligated into the corresponding sites of pREP3X [26] to generate the plasmid pREP3X-Prz1. Wild type cells were transformed with pREP3X or pREP3X-Prz1 and selected onto EMMU with 15 μM thiamine. To test $CaCl_2$ sensitivity following *prz1* overexpression, transformants were grown on EMM containing 15 μM thiamine for 2 days at 30°C, transferred onto EMMU without thiamine, and incubated for 1 day at 30°C. The cells were then sportted onto EMMU with or without 0.3 M $CaCl_2$ and incubated for 10 days at 30°C.

To construct pREP41GFP-Prz1, the oligonucleotide primers GFP-PRZ1F (5’-TATGTCGACTATGGAGCGTCAAAGGTCAG-3’) and prz1R were used to amplify a 2.0-kb

144 fragment of the complete *prz1* protein coding sequence from pREP3x-Prz1. The amplified
145 *prz1* gene fragment was digested with *Sal*I and *Bam*HI and ligated into the corresponding
146 sites of pREP41GFP [27] to generate the plasmid pREP41GFP-Prz1. Wild type cells were
147 transformed with pREP41GFP or pREP41GFP-Prz1 and selected onto EMMU containing
148 15 μM thiamine. To analyze localization and expression of Prz1, transformants were grown
149 in EMM containing 15 μM thiamine for 16 h at 30°C, washed by water, transferred into
150 EMMU without thiamine, and incubated further for 18 h at 30°C. The cells were observed
151 to check localization of proteins and proteins were analyzed by western blotting.
152
153 *Preparation of cell lysates and detection of GFP fusion protein by immunoblotting*
154 *S. pombe* cell lysates were prepared as described [28]. Lysate protein were separated
155 by SDS-PAGE, after which western blot analysis was performed using an ECL detection
156 system (GE Healthcare) according to the supplier's instructions. Mouse monoclonal anti-
157 GFP (diluted 1:1000) and rabbit polyclonal anti-PSTAIRE (Cdc2; diluted 1:1000)
158 antibodies were purchased from Roche Life Science and Santa Cruz Biotechnology,
159 respectively. Horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz
160 Biotechnology) or anti-rabbit IgG antibody (Promega) were used as secondary antibodies.
161
162 *Fluorescence microscopy of GFP fusion protein*
163 *S. pombe* cells were grown in YES liquid medium, shifted to YES medium with or
164 without 0.3 M $CaCl_2$, and incubated for 8 h at 30°C. The GFP-tagged Cgs1, Pka1, and
165 Prz1 proteins in living cells were visualized and imaged using a BX51 microscope
166 (Olympus) with a DP70 digital camera (Olympus).
167
168 *Preparation of RNA and quantitative PCR*
169 Total RNA was prepared using the RNeasy Mini Kit (QIAGEN) according to the
170 manufacturer's instruction. 500 ng of RNA of each sample was reverse transcribed by
171 GoScript Reverse Transcription System (Promega), followed by quantitative PCR analysis

using Ex-taq polymerase (Takara Bio Inc.). The oligonucleotide primers LEU1-631F (5′-GAGGAATATCCTCACCTTAC-3′) and LEU1-1072R (5′-CAGCGGTAGAAGCCTCACCTC-3′) were used to analyze the *leu1* gene as a control. The oligonucleotide primers PRZ1-740F (5′-CTGAAGTTGATTCAGAGAG-3′) and prz1R were used to analyze expression of the *prz1* gene.

Reproducibility

All experiments were conduced at least twice to confirm reproducibility of the results.

Results

Loss of functional PKA results in $CaCl_2$-sensitive phenotype

To explore the unknown functions of the cAMP/PKA pathway in *S. pombe*, the sensitivity of the wild type, *cyr1*Δ, *cgs1*Δ, *pka1*Δ, and *spc1*Δ strains to various stresses were analyzed. The wild-type and *cgs1*Δ strains grew on YES plates containing 1.5 M KCl, 0.3 M NaCl, 2 M sorbitol, and 0.3 M $CaCl_2$, the *spc1*Δ mutants, which lack stress-activated protein kinase (SAPK), did not grow well on these plates, as previously shown [14, 29-33]. The strains lacking functional components of the cAMP/PKA pathway (*cyr1*Δ and *pka1*Δ*)* were sensitive to 1.5 M KCl but not to 0.3 M NaCl and 2 M sorbitol, as previously shown [10, 13, 14, 34]. The *cyr1*Δ and *pka1*Δ strains were found to exhibit a 0.3 M $CaCl_2$-sensitive phenotype (Fig. 1). These findings indicate that the *cyr1*Δ and *pka1*Δ strains exhibit sensitivity to calcium but not to chloride and sodium.

The growth defective phenotype on KCl in strains lacking components of the cAMP/PKA pathway (Fig. 2A) [12, 34] led us to investigate how these strains grow on $CaCl_2$-containing medium. The *git3*Δ, *gpa2*Δ, *cyr1*Δ, *cgs1*Δ*,* and *pka1*Δ strains were used for this investigation, which revealed that the *git3*Δ, *gpa2*Δ, *cyr1*Δ, and *pka1*Δ strains were sensitive to 0.3 M $CaCl_2$, while the wild type and *cgs1*Δ strains grew well (Fig. 2B). The potential role of Pka1 activity in the growth defect phenotypes was then analyzed: *cyr1*Δ

*cgs1*Δ double mutants were constructed, since *cgs1* deletion results in the constitutive activation of Pka1 and Cgs1 is located downstream of Cyr1. Deletion of *cgs1* was shown to reverse the sensitive phenotype of *cyr1*Δ strains on 0.3 M $CaCl_2$, while the *cyr1*Δ strain exhibited a growth defect phenotype (Fig. 2C). The potential role of hyperactivated PKA in providing resistance to $CaCl_2$ was investigated next: the *cgs1*Δ and *cyr1*Δ *cgs1*Δ strains were found to grow on 0.4 M $CaCl_2$ while the wild type and *cyr1*Δ strains exhibited a $CaCl_2$-sensitive phenotype (Fig. 2C). These findings indicate that the loss of functional Pka1 results in growth defect on 0.3 M $CaCl_2$, and thus, the cAMP/PKA pathway is responsible for calcium tolerance.

Localization of Cgs1 and Pka1 in the presence of $CaCl_2$ in wild type and cyr1Δ *strains.*

It has been shown that Cgs1 and Pka1 localize to the nucleus and the cytoplasm under normal growth conditions [12]. Nuclear localization of Cgs1 and Pka1 depends on cAMP, and the localization of these proteins is regulated in response to physiological stresses such as KCl and glucose-limitation [12]. The effects of $CaCl_2$ on Cgs1 and Pka1 localization were thus analyzed using the *cgs1-GFP*, *cyr1*Δ *cgs1-GFP*, *pka1-GFP*, and *cyr1*Δ *pka1-GFP* strains. The cells were cultured to mid-log phase in YES, transferred into YES with or without 0.3 M $CaCl_2$, and incubated for 1, 2, and 8 h. As shown in Fig. 3A, the nuclear localizations of Cgs1-GFP and Pka1-GFP at 0 h and 8 h were dependent on Cyr1 (cAMP production) in the absence of $CaCl_2$, as previously described [12]. To determine whether Cgs1 and Pka1 localization is altered by $CaCl_2$, the *cgs1-GFP*, *cyr1*Δ *cgs1-GFP*, *pka1-GFP*, and *cyr1*Δ *pka1-GFP* strains were cultured in medium containing 0.3 M $CaCl_2$. During incubation with $CaCl_2$, nuclear-cytoplasmic redistribution of Cgs1-GFP and Pka1-GFP occurred in the wild type strain: nuclear localization (N>C; pattern 1) of Cgs1-GFP was rapidly reduced to approximately 55% of cells from 98%, while reduced nuclear localization with increased cytoplasmic localization (pattern 3) was increased to approximately 40% from 1% after incubation of 1h (Fig. 3A). Afterward, localization of Cgs1-GFP was not dramatically changed: nuclear localization (pattern 1), even localization

between nuclear and cytoplasm (N=C: pattern 2), and reduced nuclear localization with increased cytoplasmic localization (pattern 3) were observed in approximately 40%, 18%, and 45% of cells, respectively, at 8 h (Fig. 3A and B). In contrast, Pka1-GFP was gradually redistributed in the presence of 0.3 M $CaCl_2$. In most of cells, Pka1-GFP remained at nuclear at 1 h: the percentage of N>C (pattern 5) and N=C (pattern 6) were approximately 95% and 5% of cells, respectively. At the time point of 2 h, nuclear localization of Pka1-GFP was slightly reduced to approximately 77% of cells and approximately 19% of cells (pattern 6) were evenly distributed. Cytoplasm localization of Pka1-GFP was observed in approximately 70% of cells after 8 h incubation with $CaCl_2$ (pattern 7; Fig. 3A and C). High concentrations of $CaCl_2$ were thus shown to result in cytoplasmic localization of Cgs1 and Pka1, but timing of Cgs1 was earlier than Pka1. In the *cyr1*Δ strain, Cgs1-GFP and Pka1-GFP were shown to reside exclusively in the cytoplasm (more than 95% of cells), pattern 4 or 7, respectively (Fig. 3A, B, and C). We also analyzed intensities of Cgs1-GFP and Pka1-GFP from the end of one cell to another end. As shown in Supplemental Fig. 1A, cytoplasm localization of Cgs1-GFP was increased by the addition of 0.3 M $CaCl_2$. In a similar way, cytoplasmic intensity of Pka1-GFP was enhanced by $CaCl_2$ (Supplemental Fig. 1B). In the *cyr1*Δ strain, cytoplasmic intensities of Cgs1-GFP and Pka1-GFP were kept in high levels without $CaCl_2$. Thus, the localizations of these proteins are completely dependent on cAMP production. These results suggest that PKA is involved in calcium stress signaling.

Next, we analyzed intensities of Cgs1-GFP and Pka1-GFP in the presence or absence of 0.3 M $CaCl_2$ at 8h. As shown in Fig. 3D and E, both Cgs1-GFP and Pka1-GFP were increased more than two-fold in both wild type and *cyr1*Δ by incubation with 0.3 M $CaCl_2$. The expression and phosphorylation of Cgs1 and Pka1 were investigated in wild type and *cyr1*Δ strains in the presence and absence of $CaCl_2$. Pka1 is phosphorylated at threonine 356 and is hyper-phosphorylated in the *cyr1*Δ strain [13-15]. Wild type (no-tag) *cgs1-GFP*, *cyr1*Δ *cgs1-GFP*, *pka1-GFP*, and *cyr1*Δ *pka1-GFP* strains were cultured in YES liquid medium with or without 0.3 M $CaCl_2$. Cell lysates were prepared from each strain

and analyzed by western blotting, which revealed that $CaCl_2$ led to slightly increased Cgs1-GFP expression in the wild type and *cyr1*Δ strains (Supplemental Fig. 2A). The expression and phosphorylation of Cgs1-GFP and Pka1-GFP in wild type and *cyr1*Δ strains were also analyzed in the presence and absence of $CaCl_2$. Cgs1-GFP was increased by 2 and 1.6 fold by incubation of $CaCl_2$ in the wild type or *cyr1*Δ background strains, respectively, while Pka1-GFP was increased by about 1. 2 or 1.6-fold, respectively (Supplemental Fig. 2B). Incubation of $CaCl_2$ tends to increase the protein level of Cgs1-GFP and Pka1-GFP, but significance of these results is not clear. Phosphorylated Pka1 protein was detected in *cyr1*Δ background strains regardless the presence and absence of $CaCl_2$.

Prz1 is regulated by Pka1 in the presence of $CaCl_2$

The C2H2-type zinc finger transcription factor Prz1 has been shown to regulate genes involved in calcium homeostasis in *S. pombe* [18]. The overexpression of Prz1 results in extremely slow growth and seven genes including *pka1* were identified as suppressors of the slow growth phenotype induced by Prz1 overexpression [19]. An *in vitro* kinase assay was used to demonstrate that Prz1 is phosphorylated by Pka1 [19], which, together with the identification of Pka1 as a suppressor of the slow growth phenotype induced by Prz1 overexpression reported here, led us to hypothesize that Pka1 may regulate Prz1 in the presence of $CaCl_2$. To test this hypothesis, a *S. pombe* strain that expresses *prz1-GFP*, in which the GFP is fused to the C-terminal coding end of Prz1, was constructed. The *prz1-GFP* strain was considered phenotypically indistinguishable from wild type cells by growth on YES containing 0.3 M $CaCl_2$ (data not shown).

Because it was previously shown that GFP-Prz1 localizes predominantly to cytoplasm [18, 19], we examined the localization of GFP-Prz1 by expressing it under the *nmt41* promoter. We confirmed that GFP-Prz1 localized predominantly to cytoplasm (Supplemental Fig. 3A) as described previously [18, 19]. We also analyzed localization of GFP-Prz1 in the *pka1*Δ strain in the presence or absence of 0.3 M $CaCl_2$. Nuclear

284 localization of GFP-Prz1 was observed in both wild type and *pka1*Δ strains, indicating
285 Pka1 does not regulate the localization of Prz1 via calcium (Supplemental Fig. 3B). To
286 investigate the expression of Prz1, we constructed C-terminal GFP tagging strain in wild
287 type and *pka1*Δ background strains, yielding Prz1-GFP and *pka1*Δ Prz1-GFP strains,
288 respectively. When we checked the size of GFP-Prz1 protein together with Prz1-GFP by
289 western blotting, we detected a protein around 130 kDa in both GFP-Prz1 and Prz1-GFP
290 proteins (Supplemental Fig. 3C), therefore it appears to be a full length of GFP and Prz1
291 fusion, as Prz1 is 74 kDa and GFP is 27 kDa. We also detected cleaved bands around 40
292 kDa in GFP-Prz1 and a band around 80 kDa in Prz1-GFP (Supplemental Fig. 3C and Fig.
293 4A). The size difference of cleaved proteins in two fusion forms may suggest that Prz1 is
294 cleaved at the N-terminal proximal region and this may create the difference of protein
295 localization in these two forms, although it needs further clarification. We hereafter tested
296 in the expression of Prz1-GFP fusion protein. The expression of Prz1-GFP was analyzed
297 by western blotting. Because localizations of Cgs1 and Pka1 were most clearly changed at
298 8h after the addition of 0.3 M $CaCl_2$ (Fig. 3), cell lysates were prepared from the *prz1-GFP*
299 and *pka1*Δ *prz1-GFP* strains cultured for 8 h in YES liquid medium in the presence or
300 absence of 0.3 M $CaCl_2$. As shown in Fig. 4A, Prz1-GFP protein expression was markedly
301 increased in the *pka1*Δ strain compared with the wild type.
302 To determine whether Prz1 is regulated at transcriptional level, we analyzed
303 expression of mRNA in the presence or absence of 0.3 M $CaCl_2$. Cells were cultured
304 overnight to log-phase and were then incubated for 8 h at 30°C in the presence or
305 absence of 0.3 M $CaCl_2$. Total mRNAs were prepared, subjected to RT-PCR and
306 quantitative PCR was performed. As shown in Fig. 4B and C, the *prz1* mRNA was
307 significantly increased in the *pka1*Δ strain compared with the wild type under normal
308 growth condition. This result clearly indicates that *prz1* is regulated by Pka1 at the
309 transcriptional level. Expression of *prz1* mRNA was increased in wild type by the addition
310 of 0.3 M $CaCl_2$ as previously observed [18], but was decreased in the *pka1*Δ strain (Fig. 4B
311 and C).

Next, Prz1 overexpression was analyzed as a potential inducer of $CaCl_2$ sensitivity. The wild type strain was transformed with pREP3x or pREP3x-prz1, which encode *prz1* under the control of a thiamine-repressible *nmt1* promoter. Transformed cells were spotted onto EMMU in presence or absence of 0.3 M $CaCl_2$. The overexpression of Prz1 was found to slow the growth on EMMU lacking thiamine, as previously described (Fig. 4D and [19]). Overexpression of Prz1 clearly induced the $CaCl_2$-sensitive phenotype (Fig. 4D), which is in agreement with the finding that the *pka1*Δ strain is sensitive to $CaCl_2$ and expresses a higher level of Prz1.

Prz1 is a target of the cAMP/PKA pathway

Taken together with previous reports [19], the findings described above suggest that Pka1 regulates Prz1 downstream of the cAMP/PKA pathway. To confirm this, *pka1*Δ *prz1*Δ double mutants were used to analyze whether calcium sensitivity induced by the loss of functional Pka1 is completely dependent on Prz1. As shown in Fig. 5, *prz1*Δ and *pka1*Δ single mutants exhibited growth defects on YES plates containing 0.05 M or 0.2 M $CaCl_2$, respectively. However, *pka1*Δ *prz1*Δ double mutants exhibited sensitivity to as little as 0.025 M $CaCl_2$, indicating that the deletion of *prz1* enhanced the $CaCl_2$ sensitivity of the *pka1*Δ strain, which in turn suggests that Pka1 has at least another target in addition to Prz1 in the context of calcium stress signaling.

Expression of Prz1 is induced by glucose-limitation

The cAMP/PKA pathway is known to regulate glucose signaling [7, 8] and thus the role of Prz1 was analyzed under glucose-limited conditions using the wild type, *pka1*Δ, *prz1*Δ, and *pka1*Δ *prz1*Δ strains. As shown in Fig. 6A, the wild type and *pka1*Δ strains were not sensitive to 0.025 M or 0.05 M $CaCl_2$ on glucose rich (3 % glucose) and glucose-limited (0.1 % glucose + 3 % glycerol) media, but the *pka1*Δ *prz1*Δ double mutant exhibited $CaCl_2$ sensitivity on 0.025 M $CaCl_2$. The $CaCl_2$ sensitivities of the *prz1*Δ single and *pka1*Δ *prz1*Δ double mutants, however, were enhanced by glucose-limitation.

Next, we analyzed whether the expression of Prz1 is induced by glucose-limitation by incubating the *prz1-GFP* and *pka1*Δ *prz1-GFP* strains under glucose rich or glucose-limited conditions for 8 h. As shown in Fig. 6B, the expression of Prz1-GFP was significantly increased by glucose-limitation. The expression of Prz1-GFP was, moreover, shown to be further increased by glucose-limitation in the *pka1*Δ strain. These findings suggest that glucose signaling regulates the calcium sensing transcription factor Prz1 via expression level alterations.

Finally, we analyzed whether expression of the *prz1* mRNA is induced by glucose-limitation. We incubated the wild type and *pka1*Δ strains under glucose rich or glucose-limited condition for 8 h and quantified mRNA by PCR. As shown in Fig. 6C, expression of *prz1* mRNA was remarkably increased by glucose-limitation in wild type strain. These results suggest that *prz1* is regulated at mRNA expression level of upon glucose-limitation.

Discussion

In this study, the cAMP/PKA pathway was newly identified as being functionally important for calcium tolerance in *S. pombe*, which represents the discovery of a further role for the cAMP/PKA pathway in addition to the many roles it is known to play, such as regulating various processes including chronological aging, transition into meiosis, glycogenesis, and osmotic stress (KCl) response [1, 7, 10, 12-14, 35]. Loss of functional Git3, Gpa2, Cyr1, and Pka1 resulted in growth defect in the presence of 0.2 M $CaCl_2$, while loss of functional Cgs1 resulted in resistance to 0.4 M $CaCl_2$. Four components (Git3, Gpa2, Cyr1, and Pka1) of the cAMP/PKA pathway function to enhance the pathway, while Cgs1 downregulates the pathway. Our observations regarding the $CaCl_2$ sensitivity (or resistance) of these mutants are therefore consistent with the roles of the individual proteins. The $CaCl_2$ sensitivity in the *pka1*Δ strain is explainable by increased expression of *prz1*, but the reason of resistance to high concentration of $CaCl_2$ in the *cgs1*Δ strain is not simply explained by the regulation of *prz1*. Based on our genetic evidence, we predict

368 an alternative target apart from Prz1 in $CaCl_2$ response mediated by Pka1 (Fig. 5). There
369 must be yet an unidentified mechanism how *cgs1*Δ tolerates to high concentrated calcium
370 stress. Cgs1 and Pka1 normally exhibit nuclear and cytoplasmic localization, where this
371 localization depends on Cyr1 [12]. When cells are exposed in KCl, Cgs1 and Pka1
372 translocate to the cytoplasm [12]. Similar to the response to KCl, Cgs1 and Pka1 were
373 shown to predominantly localize to the cytoplasm in the presence of $CaCl_2$. The
374 cytoplasmic localization of Pka1 induced by $CaCl_2$ suggests that Pka1 likely dissociates
375 from Cgs1 to be activated in the presence of $CaCl_2$.
376 The C2H2-type zinc finger transcription factor Prz1 plays a key role in the response
377 to calcium perturbation. Prz1 is dephosphorylated by Ppb1, a catalytic subunit of
378 calcineurin [16]. In the presence of calcium, the phosphorylated Prz1 is then translocated to
379 the nucleus, where it upregulates the transcription of Pmc1, which encodes a vacuolar
380 Ca^{2+} pump [16, 18]. Prz1 was previously shown to localize in the cytoplasm under normal
381 growth conditions [18, 19]. Pka1 is not responsible for localization of Prz1 even in the
382 presence of $CaCl_2$. However, we newly identified that Prz1 is cleaved at the N-terminal
383 proximal region in this study. Localization of Prz1 was shown to change from cytoplasm to
384 nucleus when $CaCl_2$, DTT, NaCl, or micafungin was added, or by deletion of *msn5* [18, 19, 36].
385 Msn5 is though to be responsible for export of Prz1 from nucleus [19], but it is not yet
386 cleared how localization of Prz1 is precisely controlled. The *prz1* mRNA is increased after
387 the addition of $CaCl_2$ but is not enhanced in *ppb1*Δ [18]. The possible cooperation of Ppb1
388 and Pka1 in the regulation of *prz1* was not investigated in this work, but it is another
389 interesting subject. Further studies regarding localization, modification, and cleavage of
390 Prz1 should be conducted.
391 Under glucose-limited condition, Prz1 was shown to accumulate in the *pka1*Δ and the
392 wild type strains. Since Pka1 is inactivated by binding with Cgs1 under glucose-limited
393 conditions, this observation indicates that the glucose condition is relevant to $CaCl_2$
394 sensitivity. In fact, $CaCl_2$ sensitivity of *prz1*Δ and *pka1*Δ *prz1*Δ were found to be increased
395 in glucose-limited medium (Fig. 6). The accumulation of Prz1 in the *pka1*Δ strain was

shown to result from increased expression of the *prz1* mRNA. It was also noted that Prz1 (~130 kDa) were present on western blot membranes for wild type and *pka1*Δ strains (Fig. 4B), but cleaved Prz1-GFP was increased in *pka1*Δ or by glucose-limitation. It is not clear which transcription factor regulates expression of *prz1* and how post-translational modification of Prz1 is regulated, but would be interesting subjects for further investigation.

In this study, we not only demonstrated the regulation of Prz1 transcriptional levels by Pka1, but also showed that the regulation of Prz1 by Pka1 is not the sole cause of the calcium-sensitive phenotype of the *pka1*Δ strain, since *pka1*Δ *prz1*Δ double mutants exhibited hypersensitivity to 0.025 M $CaCl_2$. Prz1 thus seems not to be the sole target of Pka1 in the response to exogenous calcium. The sensitivity to different concentrations of $CaCl_2$ in various mutants likely reflects the contribution of the different genes to calcium homeostasis. Since the central regulator consisting of the calcineurin-Prz1 pathway is dominantly responsible for calcium homeostasis, a lack of genes in this pathway generally results in sensitivity to 0.1 M $CaCl_2$. The lack of *pka1* alone results in sensitivity to 0.2 M $CaCl_2$; however, this sensitivity is extended to 0.025 M $CaCl_2$ by deletion of *prz1*, indicating that more factors are involved in calcium homeostasis. In fact, there are more mutants that show sensitivity to $CaCl_2$, including *rad24*Δ, *ncs1*Δ, and *cap1*Δ mutants exhibiting sensitivity to 0.1 M $CaCl_2$ [37-39]; and the *gsf1*Δ mutant exhibiting sensitivity to 0.25 M $CaCl_2$ [40]. The relevance of each of these genes in calcium homeostasis is not always clear, but the list of such mutants is increasing [41]. More works is thus required to understand how calcium homeostasis is controlled in *S. pombe*.

From the study findings reported here, we conclude that Pka1 is responsible for calcium homeostasis in *S. pombe*. Pka1 negatively regulates the expression of Prz1 and furthermore plays a role in protein modification of Prz1. Our findings contribute insight into how Prz1 is regulated by calcium through Pka1 in *S. pombe*.

Author contributions

Y.M. and M.K. designed the experiments, analyzed the data, and wrote the manuscript;

Y.M. performed the experiments; M.K. carried out discussion.

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562 **Figure legends**

563 **Fig. 1 The cAMP/PKA and SAPK pathways play a role in tolerance to $CaCl_2$.** Wild
564 type, *cyr1*Δ, *cgs1*Δ*, pka1*Δ*,* and *spc1*Δ *S. pombe* strains were streaked onto YES, YES+1.5
565 M KCl, YES+0.3 M NaCl, YES+0.3 M $CaCl_2$, and YES+2 M sorbitol plates and incubated
566 at 30°C for 2–5 days (YES and YES+2 M sorbitol for 2 days; YES+1.5 M KCl, YES+0.3 M
567 NaCl, and YES+0.3 M $CaCl_2$ for 5 days).
568
569 **Fig. 2 Loss of functional components in the cAMP/PKA pathway cause $CaCl_2$-**
570 **induced growth defect.** (A) Schematic diagram of the cAMP-dependent protein kinase
571 pathway in *S. pombe*. (B) Wild type, *git3*Δ*, gpa2*Δ*, cyr1*Δ*, cgs1*Δ, and *pka1*Δ strains were
572 grown on YES, harvested, and resuspended in water at 10^7 cells/mL. The cell suspensions
573 were serially diluted (1:10) and each dilution was spotted onto YES and YEAU+0.3 M
574 $CaCl_2$ as indicated and incubated for 3 or 10 days at 30°C (YES for 3 days and YES+0.3
575 M $CaCl_2$ for 10 days). (C) Wild type, *cyr1*Δ*, cgs1*Δ*,* and *cyr1*Δ *cgs1*Δ strains were grown on
576 YES, harvested, and resuspended in water at 10^7 cells/ml. Culture dilutions, spotted onto
577 plates, which were then incubated for the same number of days as in (B). Spotted plates
578 were incubated for 3 or 6 days (YES for 3 days and YES+0.3 and 0.4 M $CaCl_2$ for 6 days).
579
580 **Fig. 3 Subcellular localization of Cgs1 and Pka1 in the presence of $CaCl_2$.** (A) *cgs1-*
581 *GFP*, *cyr1*Δ *cgs1-GFP, pka1-GFP,* and *cyr1*Δ *pka1-GFP* strains were cultured in YES
582 liquid medium to mid-log phase (~4 × 10^6 cells/mL), harvested by centrifugation, and
583 resuspended in YES with or without 0.3 M $CaCl_2$. After 1, 2, and 8 h, the cultures were
584 examined by fluorescence microscopy. Scale bar: 10 μm. Arrows indicate representative
585 cells categorized in (B) and (C). Cgs1-GFP localizations are categorized in nuclear
586 localization (pattern1), even localization between nuclear and cytoplasm (pattern 2),
587 reduced nuclear localization with increased cytoplasmic localization (pattern 3), and
588 cytoplasmic localization (pattern 4), respectively. Pka1-GFP localizations are categorized
589 in nuclear localization (pattern 5), even localization between nuclear and cytoplasm

(pattern 6), and cytoplasmic localization (pattern 7), respectively. (B) Distribution of Cgs1-GFP in wild type and *cyr1*Δ cells after 8 h incubation of 0.3 M $CaCl_2$. (C) Distribution of Pka1-GFP in wild type and *cyr1*Δ cells after 8 h incubation of 0.3 M $CaCl_2$. About 200 cells were analyzed in individual strains and experiments were performed twice: averages with S.D. are shown (B and C). Box and whisker plots of Cgs1-GFP (D) and Pka1-GFP (E) are shown. Fluorescence intensities in one entire cell in both wild type and *cyr1*Δ background cells grown in YES in the presence or absence of 0.3 M $CaCl_2$ for 8 h were quantified. Fifty cells were analyzed in individual strains and experiments were performed twice: representative one is shown (D and E).

Fig. 4 Prz1-GFP protein accumulates in *pka1*Δ. (A) Immunoblot analysis of Prz1-GFP fusion protein in cell lysates prepared from wild type (no-tag), *prz1-GFP*, and *pka1*Δ *prz1-GFP* strains. Cells were cultured overnight to log phase (~5 × 10^6 cells/mL) in YES liquid medium. Cells were then harvested by centrifugation and resuspended in YES with or without 0.3 M $CaCl_2$. Prz1-GFP protein was detected using an anti-GFP antibody. Anti-PSTAIRE was used as an internal loading control. (B) Wild type, *pka1*Δ, and *prz1*Δ cells were cultured overnight to log phase (~5 × 10^6 cells/mL) in YES liquid medium. Cells were then harvested by centrifugation and resuspended in YES in the presence or absence of 0.3 M $CaCl_2$. Total mRNAs were prepared from each sample. cDNAs were synthesized by reverse transcriptase. Expression level of mRNA was analyzed by quantitative PCR. The *leu1* gene was used as control. (C) Expression levels of *prz1* mRNA were quantified and normalized to wild type. Experiments were performed three times: averages with S.D. are shown. (D) Wild-type transformants were grown on EMMU containing 15 μM thiamine at 30°C for 2 days. Cells were transferred onto EMMU without thiamine and incubated for 1 day at 30°C. To analyze $CaCl_2$ sensitivity, cells were harvested and resuspended in water to 1 × 10^7 cells/mL. Culture dilutions and spotted plates were as described for Fig. 2B. The plates were incubated for 10 days at 30°C.

Fig. 5 *pka1*Δ *prz1*Δ exhibits higher $CaCl_2$ sensitivity than *pka1*Δ. Wild type, *pka1Δ*, *prz1*Δ*,* and *pka1*Δ *prz1*Δ *S. pombe* strains were grown on YES, harvested by centrifugation, and resuspended in water at 1×10^7 cells/mL. Culture dilutions and spotted plates were as described for Fig. 2B. The plates were incubated for 3 to 5 days at 30°C (YES, YES+0.025M $CaCl_2$, and YES+0.05M $CaCl_2$ for 3 days; YES+0.1M $CaCl_2$, and YES+0.2M $CaCl_2$ for 5 days).

Fig. 6 Expression of Prz1 is increased by glucose-limitation. (A) Wild type, *pka1Δ*, *prz1*Δ*,* and *pka1*Δ *prz1*Δ *S. pombe* strains were grown on YES, harvested by centrifugation, and resuspended in water at 1×10^7 cells/mL. Culture dilutions and spotted plates were as described for Fig. 2B. The plates were incubated for 3 or 4 days at 30°C (YES for 3 days and YES glucose-limited medium for 4 days). (B) Cells were cultured overnight to log phase (~5×10^6 cells/mL) in YES liquid medium. Cells were harvested by centrifugation and resuspended in YES (3% glucose) or YES glucose-limited medium (0.1% glucose + 3% glycerol) as indicated. Prz1-GFP protein was detected using an anti-GFP antibody. Anti-PSTAIRE was used as an internal loading control. (C) Wild type, *pka1*Δ, and *prz1*Δ cells were cultured overnight to log phase (~5×10^6 cells/mL) in YES liquid medium. Cells were then harvested by centrifugation and resuspended in YES (3% glucose) or YES glucose-limited medium (0.1% glucose + 3% glycerol) as indicated. Total mRNAs were prepared from each sample. cDNAs were synthesized by reverse transcriptase. Expression level of mRNA was analyzed by quantitative PCR. The *leu1* gene was used as control.

Supplemental Fig. 1 GFP fluorescence intensities of Cgs1-GFP and Pka1-GFP in the presence of $CaCl_2$. Intensities of Cgs1-GFP (A) and Pka1-GFP (B) were measured alongside of the line indicated in cells by ImageJ software. Twenty cells of Fig. 3A were analyzed in individual strains and representative one is shown. Intensities of Cgs1-GFP and Pka1-GFP are shown as arbitrary units.

Supplemental Fig. 2 Immunoblot analysis of Cgs1-GFP and Pka1-GFP in the presence of $CaCl_2$. *cgs1-GFP*, *cyr1*Δ *cgs1-GFP, pka1-GFP,* and *cyr1*Δ *pka1-GFP* strains were cultured in YES liquid medium to mid-log phase (~4 × 10^6 cells/mL), harvested by centrifugation, and resuspended in YES with or without 0.3 M $CaCl_2$. Cells were harvested by centrifugation after 8 h to prepare cell lysates. Cgs1-GFP (A) and Pka1-GFP (B) proteins were detected using an anti-GFP antibody. Anti-PSTAIRE was used as an internal loading control.

Supplemental Fig. 3 Microscope and iImmunoblot analysis of GFP-Prz1. (A) Wild-type (PR109) transformants were cultured into EMMU containing 15 μM thiamine at 30°C. Cells were washed, transferred into EMMU without thiamine, and incubated for 18h at 30°C. The cells were examined by fluorescence microscopy at the indicated time points. Scale bar: 10 μm. (B) Wild-type (PR109) and *pka1*Δ (YMP36) transformants were culture into EMMU containing 15 μM thiamine at 30˚C. Cells were washed, transferred into EMMU without thiamine and incubated for 18 h at 30˚C. Cells were centrifuged, resuspended into YES in the presence or absence of 0.3 M $CaCl_2$, and incubated for 8 h at 30˚C. The cells were examined by fluorescence microscopy at the indicated time points. Scale bar: 10 μm. (C) Immunoblot analysis of GFP-Prz1 and Prz1-GFP fusion proteins in cell lysate prepared from wild-type harboring pREP41GFP, or pREP41GFP-Prz1, and *prz1-GFP* strains. Wild-type harboring pREP41GFP or pREP41GFP-Prz1 cells were cultured overnight to log phase (~5 × 10^6 cells/mL) in EMMU. The prz1-GFP strain was cultured in YES liquid medium, transferred into YES containing 0.3 M $CaCl_2$, and incubated for 8 h. Cells were then harvested by centrifugation and cell lysate was prepared. GFP-Prz1 and Prz1-GFP proteins were detected using an anti-GFP antibody. Anti-PSTAIRE was used as an internal loading control.

Supplemental Fig. 4 Full scans of uncropped immunoblot result.

674 The data of Fig. 4A, 6B, supplemental Fig. 2, and 3C are shown.

675

676 Table 1 *S. pombe* strains used in this study

Strain	Genotype	Source
PR109	*h⁻ leu1-32 ura4-D18*	P. Russell
DY114	*h^{90} ade6-216 leu1-32 ura4-D18 cyr1::ura4*	[6]
MK7	*h^{90} ade6-216 leu1-32 ura4-D18 cyr1::LEU2*	[6]
JZ858	*h^{90} ade6-216 leu1-32 ura4-D18 cgs1::ura4*	[42]
JZ633	*h^{90} ade6-216 leu1-32 ura4-D18 pka1::ura4*	[42]
TK105	*h^{90} leu1-32 ura4-D18 spc1::ura4*	Lab stock
YMSM101	*h^{90} ade6-210 leu1-32 ura4-D18 pka1-GFP(S65T)-kanMX6*	[12]
YMSM105	*h^{90} ade6-210 leu1-32 ura4-D18 cgs1-GFP(S65T)-kanMX6*	[12]
NHP083 (FY17838)	*h⁺ ade6-216 leu1-32 ura4-D18 his3-D1 prz1::LEU2*	[43]
YMP43	*h⁻ leu1-32 ura4-D18 git3::ura4*	This study
YMP39	*h⁻ leu1-32 ura4-D18 gpa2::ura4*	This study
YMP28	*h⁻ leu1-32 ura4-D18 cyr1::ura4*	This study
YMP40	*h⁻ leu1-32 ura4-D18 cgs1::ura4*	This study
YMP36	*h⁻ leu1-32 ura4-D18 pka1::ura4*	This study
YMP58	*h⁻ leu1-32 ura4-D18 cyr1::LEU2 cgs1::ura4*	This study
YMP44	*h⁻ leu1-32 ura4-D18 spc1::ura4*	This study
YMP26	*h⁻ leu1-32 ura4-D18 cgs1-GFP(S65T)-kanMX6*	This study
YMP45	*h⁻ leu1-32 ura4-D18 cyr1::ura4 cgs1-GFP(S65T)-kanMX6*	This study
YMP19	*h⁻ leu1-32 ura4-D18 pka1-GFP(S65T)-kanMX6*	This study
YMP48	*h⁻ leu1-32 ura4-D18 cyr1::ura4 pka1-GFP(S65T)-kanMX6*	This study
YMP529	*h⁻ leu1-32 ura4-D18 prz1::LEU2*	This study
YMP531	*h⁻ leu1-32 ura4-D18 pka1::ura4 prz1::LEU2*	This study
YMP543	*h⁻ leu1-32 ura4-D18 prz1-GFP(S65T)-kanMX6*	This study
YMP544	*h⁻ leu1-32 ura4-D18 pka1::ura4 prz1-GFP(S65T)-kanMX6*	This study

677
678

Fig. 1, Matsuo & Kawamukai

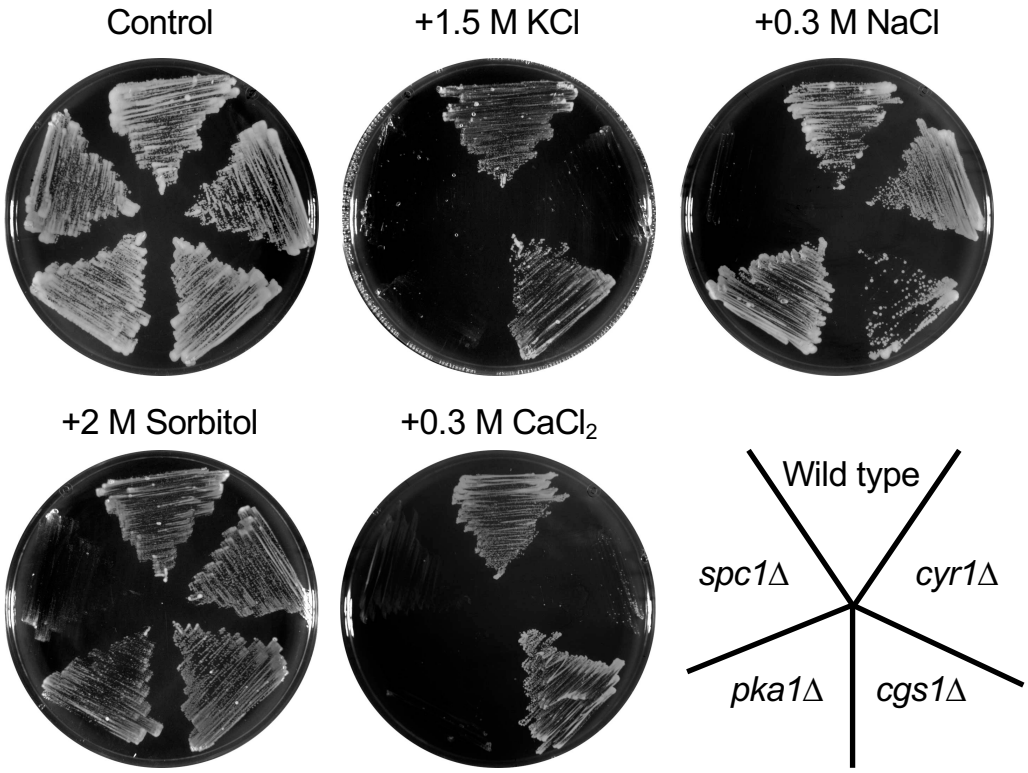


Fig. 2, Matsuo & Kawamukai

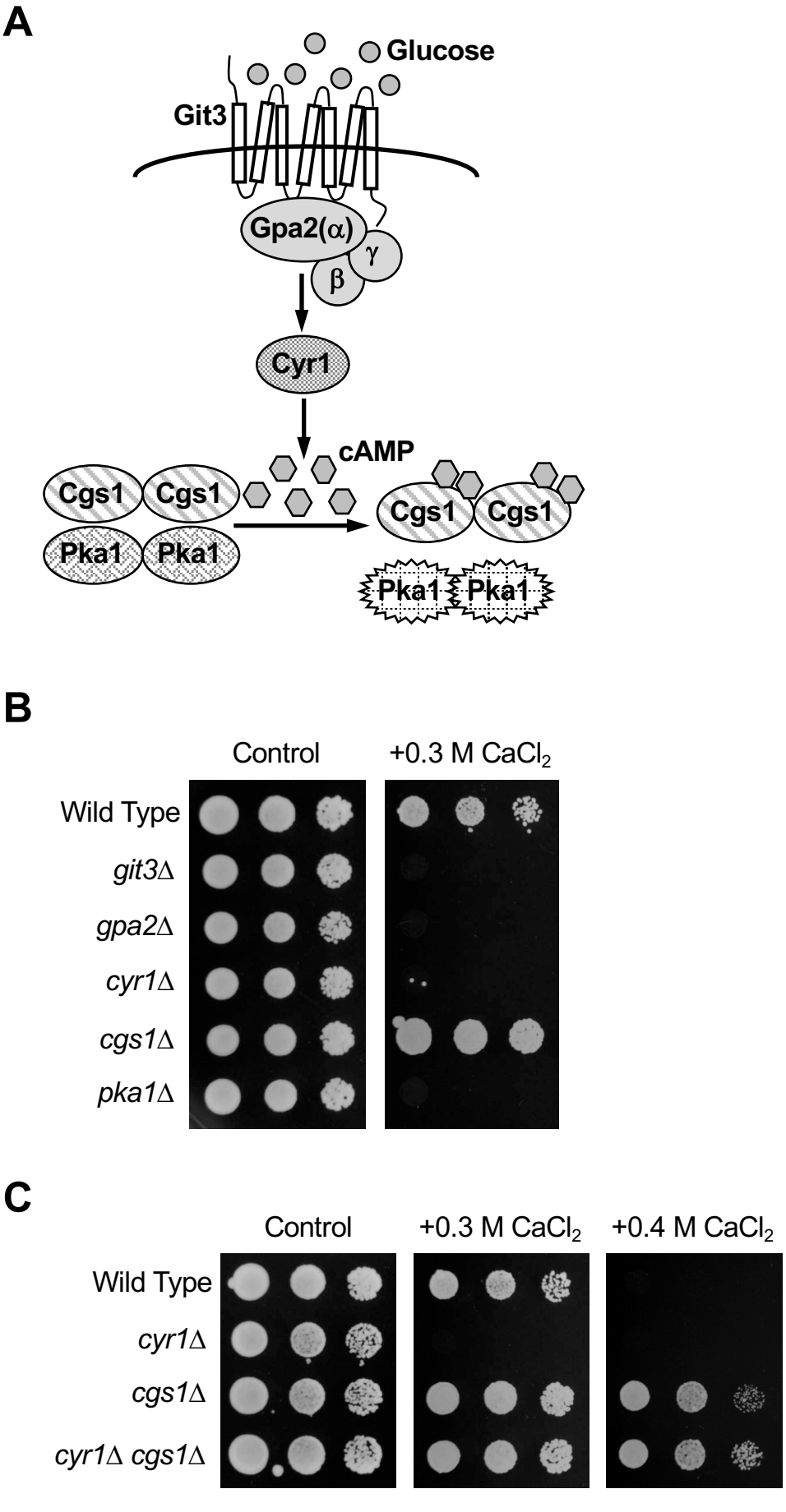


Fig. 3, Matsuo & Kawamukai

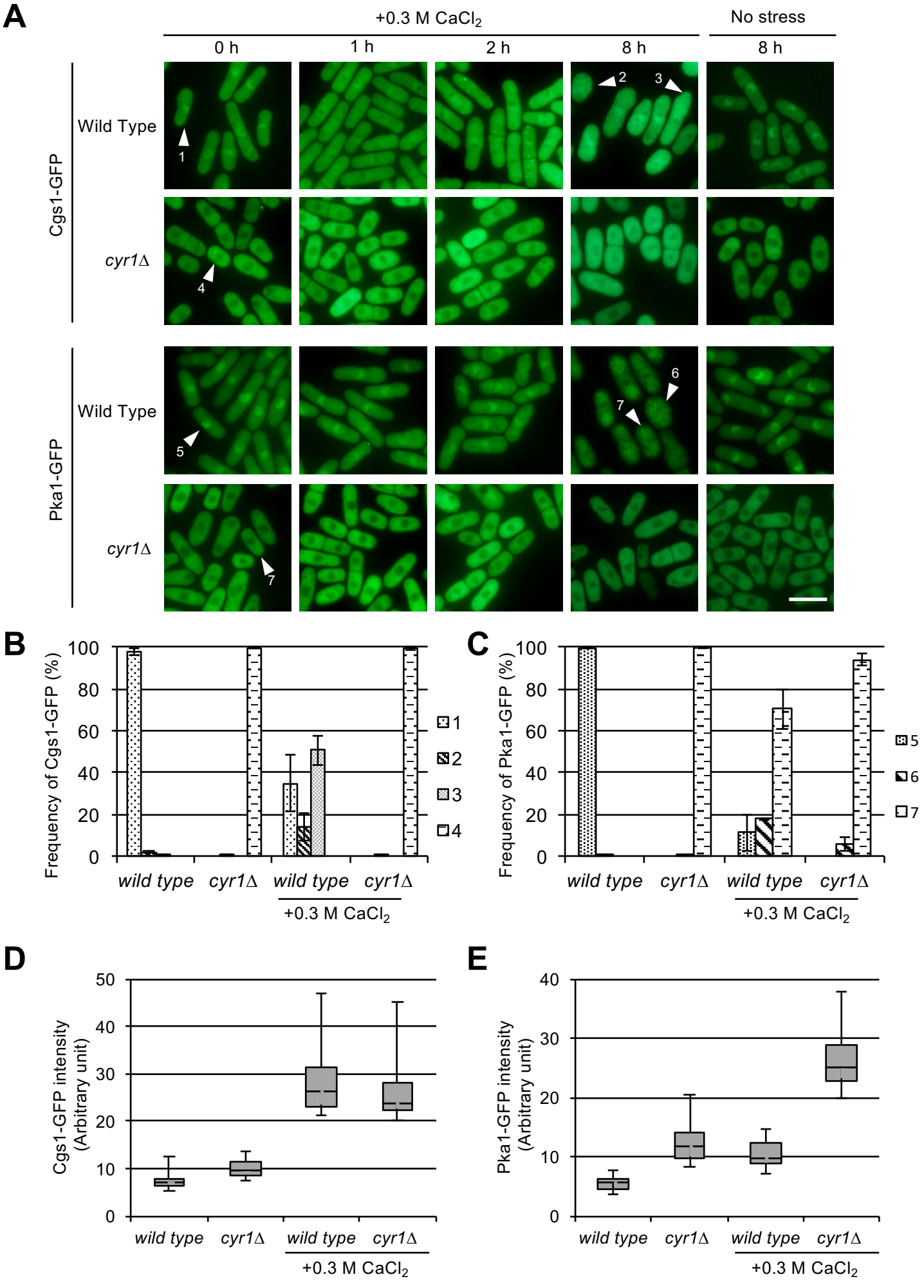


Fig. 4, Matsuo & Kawamukai

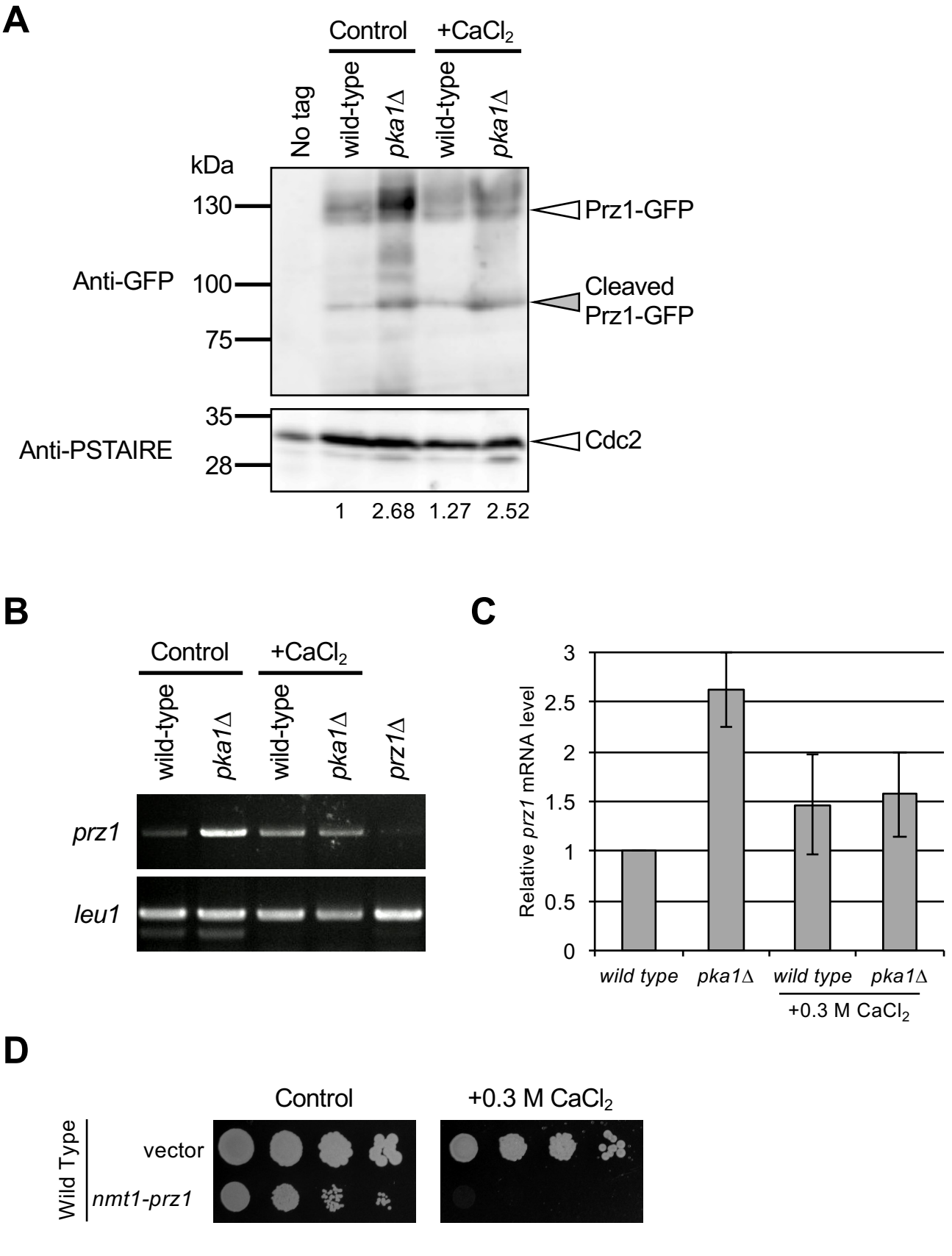


Fig. 5, Matsuo & Kawamukai

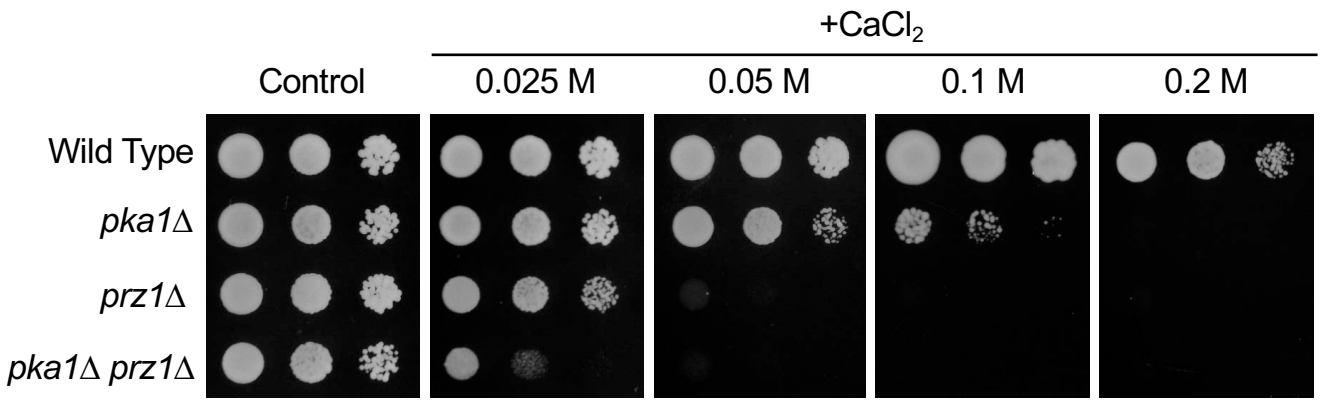
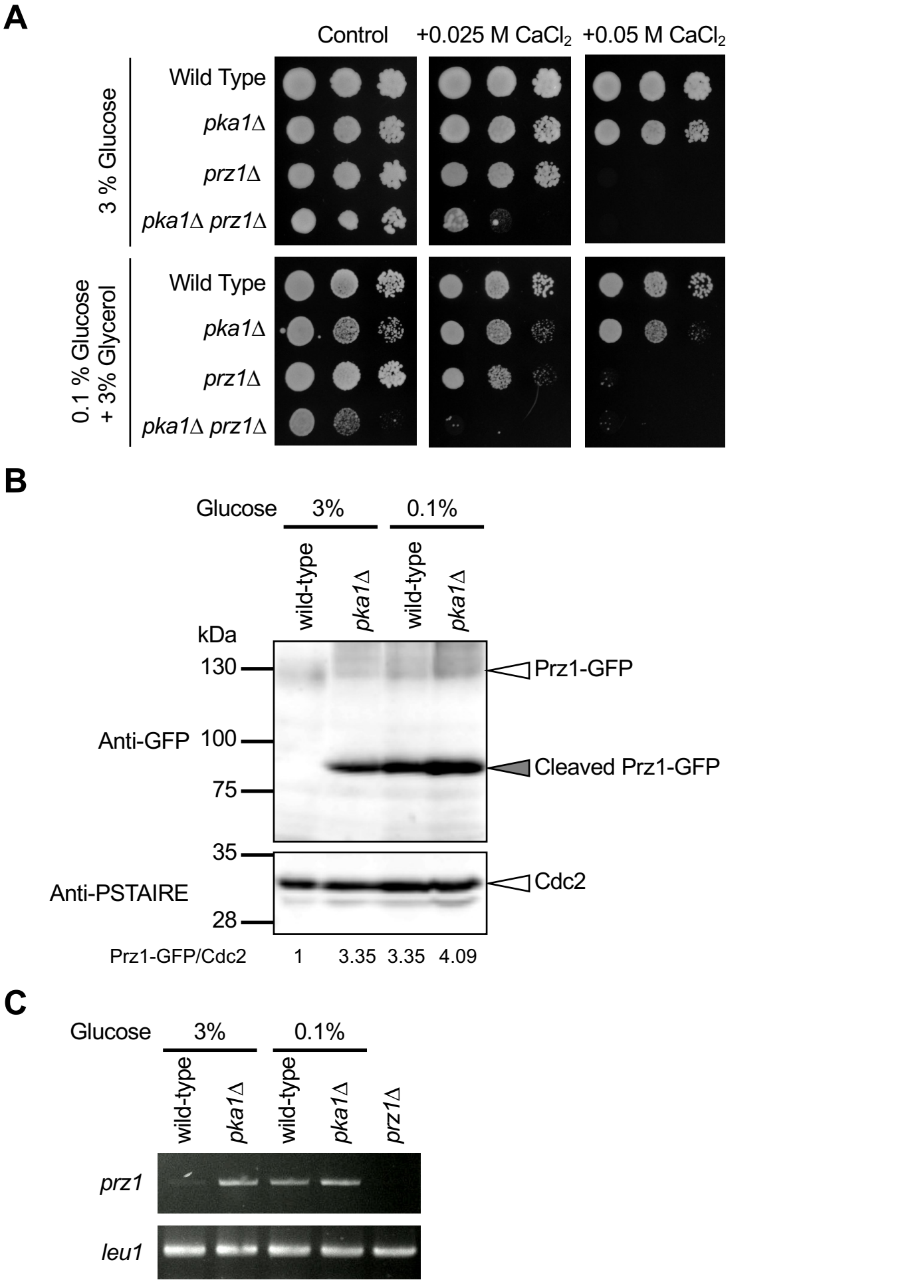
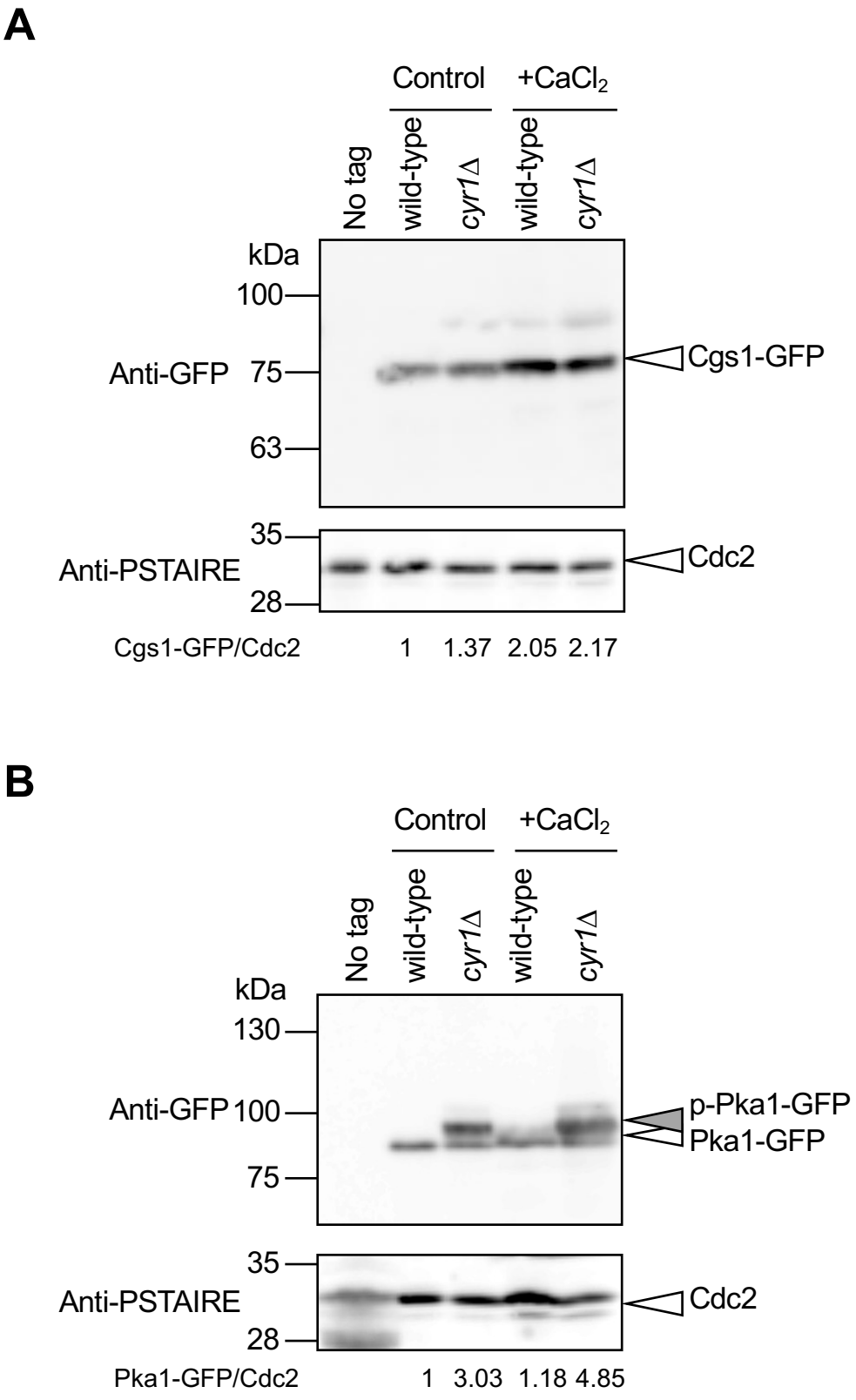


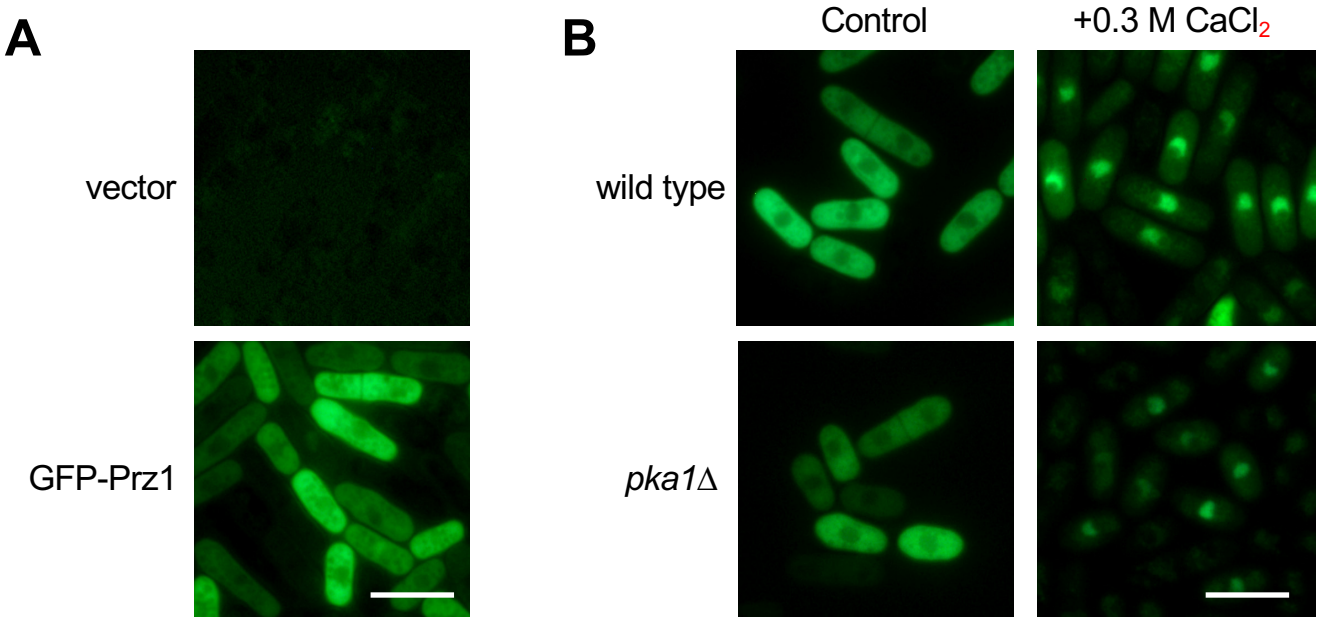
Fig. 6, Matsuo & Kawamukai

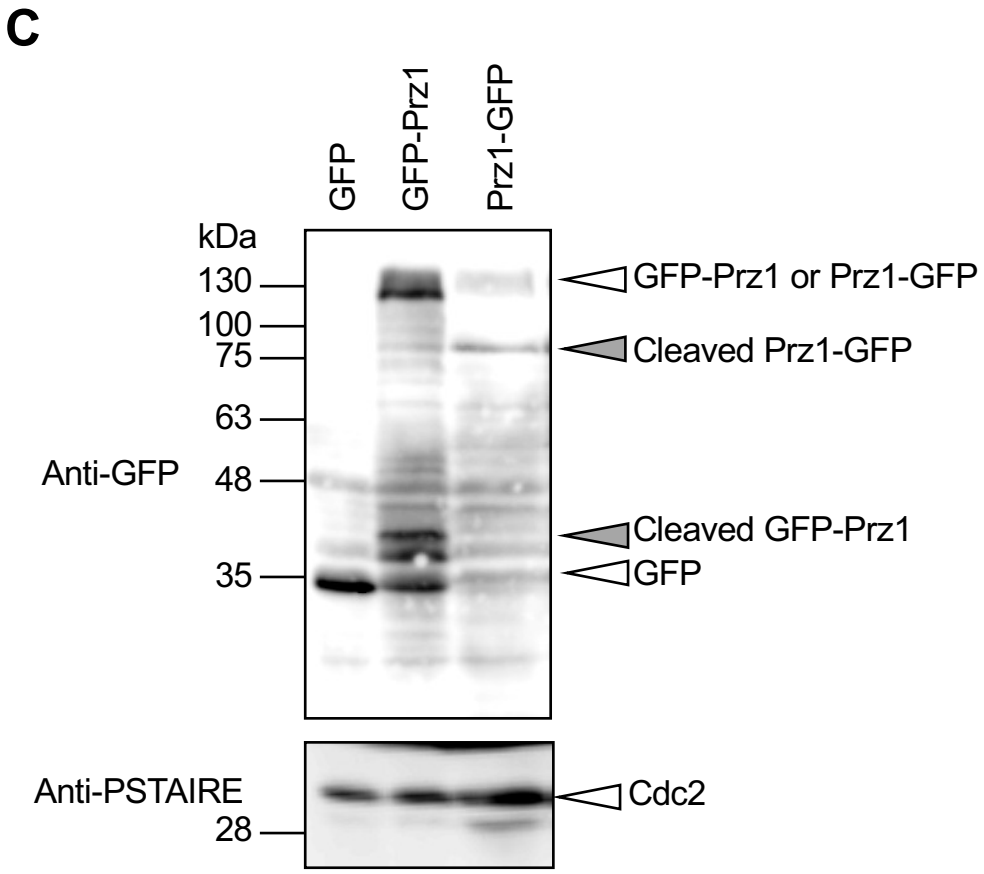


Supplemental Fig. 1, Matsuo & Kawamukai



Supplemental Fig. 2, Matsuo & Kawamukai





Supplemental Fig. 3, Matsuo & Kawamukai

Fig. 4A

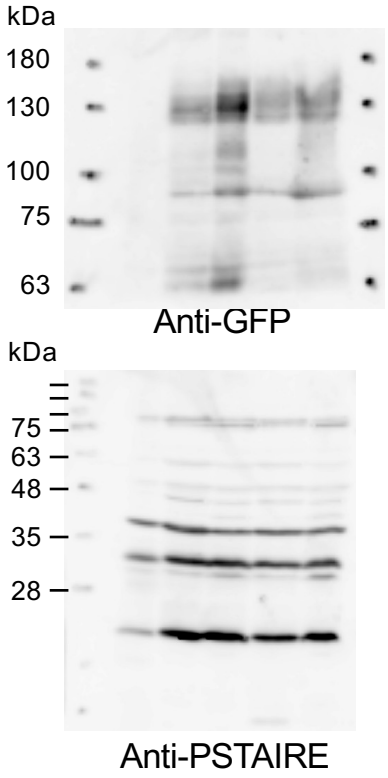
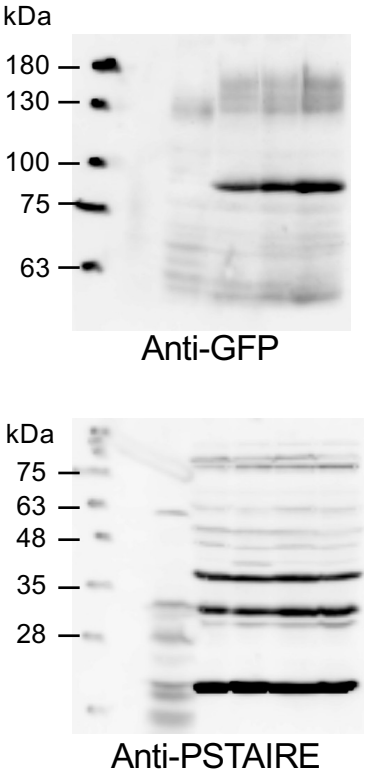
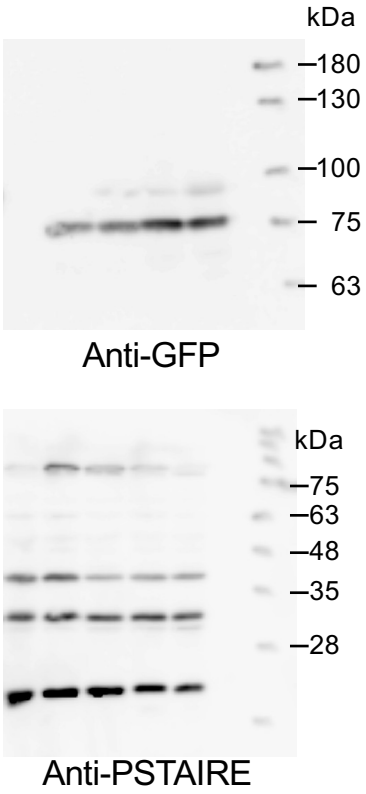


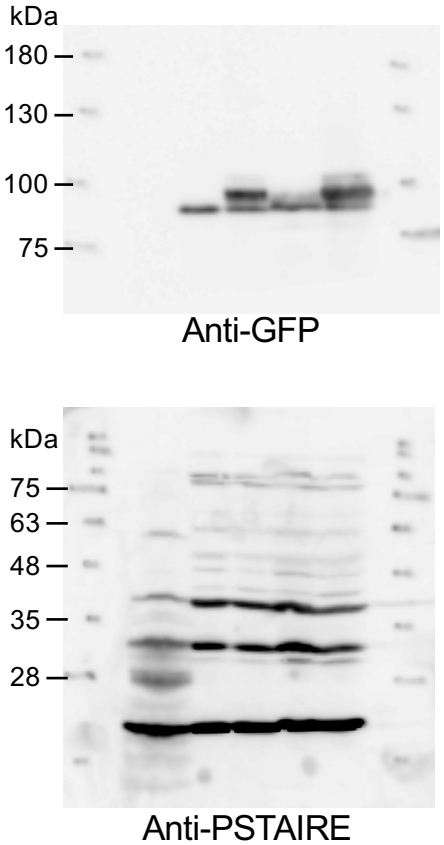
Fig. 6B



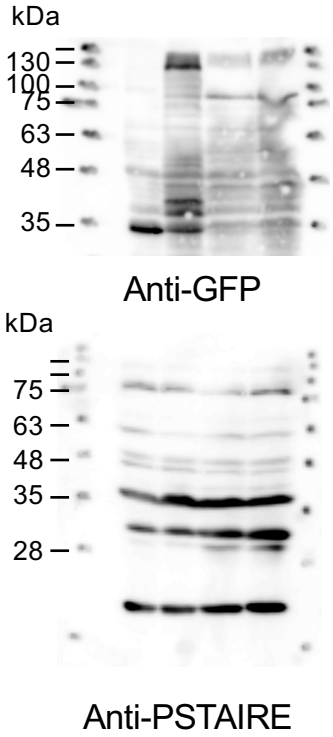
Supplemental Fig. 1A



Supplemental Fig. 1B



Supplemental Fig. 2C



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