Title: Live-cell fluorescence imaging and optogenetic control of PKA kinase activity in fission yeast *Schizosaccharomyces pombe*.

Running Title: Live-cell PKA activity sensor in fission yeast cells

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Take Away

- spPKA-KTR1.0 allows visualization of PKA activity at the single-cell level
- Live-cell imaging reveals the transient decrease in PKA activity after M-phase
- Optogenetics allows simultaneous visualization and manipulation of PKA activity

Abstract

The cAMP-PKA signaling pathway plays a crucial role in sensing and responding to nutrient availability in the fission yeast *Schizosaccharomyces pombe.* This pathway monitors external glucose levels to control cell growth and sexual differentiation. However, the temporal dynamics of the cAMP-PKA pathway in response to external stimuli remains unclear mainly due to the lack of tools to quantitatively visualize the activity of the pathway. Here, we report the development of the kinase translocation reporter (KTR)-based biosensor spPKA-KTR1.0, which allows us to measure the dynamics of PKA activity in fission yeast cells. The spPKA-KTR1.0 is derived from the transcription factor Rst2, which translocates from the nucleus to the cytoplasm upon PKA activation. We found that spPKA-KTR1.0 translocates between the nucleus and cytoplasm in a cAMP-PKA pathway-dependent manner, indicating that the spPKA-KTR1.0 is a reliable indicator of the PKA activity in fission yeast cells. In addition, we implemented a system that simultaneously visualizes and manipulates the cAMP-PKA signaling dynamics by introducing bPAC, a photoactivatable adenylate cyclase, in combination with spPKA-KTR1.0. This system offers an opportunity for investigating the role of the signaling dynamics of the cAMP-PKA pathway in fission yeast cells with higher temporal resolution.

Introduction

Cells harness nutrient-sensing mechanisms to adapt to the external conditions by controlling their proliferation. In the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), the cyclic 3'-5' adenosine monophosphate (cAMP)-protein kinase A (PKA) signaling pathway is responsible for sensing glucose, which is one of the preferred carbon sources for *S. pombe* (Hoffman, 2005; Hoffman & Winston, 1990). Extracellular glucose is primarily detected by a G protein-coupled receptor, Git3, at the plasma membrane, which in turn activates adenylate cyclase, Cyr1, via heterotrimeric G proteins consisting of Gpa2 (Gα), Git5 (Gβ), and Git11 (Gγ) subunits, resulting in the cAMP production (Fig. 1A) (Hoffman & Winston, 1991; Isshiki et al., 1992; Kim et al., 1996; Landry et al., 2000; Landry & Hoffman, 2001; Maeda et al., 1990; Welton & Hoffman, 2000; Yamawaki-Kataoka et al., 1989; Young et al., 1989). The cAMP activates the catalytic subunit of PKA, Pka1, by releasing Pka1 from the inhibitory regulatory subunit, Cgs1 (DeVoti et al., 1991; Gupta et al., 2011a; Maeda et al., 1994). In contrast, a cAMP phosphodiesterase, Cgs2, negatively regulates the PKA activity by degrading cAMP (DeVoti et al., 1991; Mochizuki & Yamamoto, 1992). The cAMP-PKA pathway plays a crucial role in divergent cellular processes in fission yeast such as stress resistance (Matsuo & Kawamukai, 2017), sexual differentiation (Maeda et al., 1994), spore germination (Hatanaka & Shimoda, 2001; Sakai et al., 2023), gluconeogenesis (Hoffman & Winston, 1991), cell size control (Kelkar & Martin, 2015; Uysal Özdemir et al., 2024), heterochromatin formation (Bao et al., 2022), and autophagy (Pérez-Díaz et al., 2023). Among these, it is well known that cAMP-PKA pathway represses the expression of Ste11, a key transcription factor for sexual differentiation, by inactivating the zinc finger transcription factor Rst2 (Otsubo & Yamamoto, 2012). When the nitrogen source is depleted, Ste11 is induced in an Rst2-dependent manner (Higuchi et al., 2002), but it is not known how the regulation of PKA activity is coupled with the nitrogen starvation.

The activity of the cAMP-PKA pathway has hitherto been assessed by mating or sporulation efficiency, which is repressed by PKA in fission yeast cells (DeVoti et al., 1991; Isshiki et al., 1992; Kawamukai et al., 1991; Maeda et al., 1990, 1994; Mochizuki & Yamamoto, 1992). Alternatively, PKA activity has been measured by a transcriptional reporter using *fbp1*, a gene encoding fructose-l,6-bisphosphatase, whose transcription is enhanced when PKA is inactivated (Hoffman & Winston, 1990, 1991; Nocero et al., 1994; Welton & Hoffman, 2000). Biochemical analyses have also been used to investigate intracellular cAMP levels in fission yeast cells (DeVoti et al., 1991; Hoffman & Winston, 1991; Isshiki et al., 1992; Mochizuki & Yamamoto, 1992; Wang et al., 2005), adenylate cyclase activity *in vitro* (Kawamukai et al., 1991), and PKA activity *in vitro* and *in vivo* by using kemptide (DeVoti et al., 1991) and its endogenous target (Higuchi et al., 2002) as substrates. Although these conventional approaches have allowed the analysis of the molecular pathways and

genetic interactions involved in the cAMP-PKA pathway, it is still technically challenging to quantitatively monitor the temporal dynamics of PKA activity in a living fission yeast cell.

Genetically encoded fluorescent biosensors are promising tools for the real-time monitoring of signaling dynamics at the single-cell level (Maryu et al., 2018). Förster Resonance Energy Transfer (FRET)-based biosensors are among the most widely used for the visualization of intracellular signaling. The FRET biosensors for cAMP levels and PKA activity were originally developed for mammalian cells (Komatsu et al., 2011; Ponsioen et al., 2004; Zhang et al., 2001). Recently, the cAMP biosensors (e.g., yeast-EPAC, camp-EPAC2) (Botman et al., 2021; Colombo et al., 2017) and PKA biosensors (e.g., AKAR3, AKAR3EV, AKAR4) (Botman et al., 2023; Colombo et al., 2017, 2022; Molin et al., 2020) have been successfully adapted for the use in the budding yeast *Saccharomyces cerevisiae*. However, FRET-based biosensors for cAMP and PKA have not yet been applied to the fission yeast *S. pombe*, and therefore it is still unclear when and how PKA activation dynamics commit cell fate decisions. Here, we report new tools for the visualization and optical manipulation of PKA activity in a fission yeast cell. We first applied the FRET biosensors for cAMP and PKA activity, but these biosensors did not work in *S. pombe* for unknown reasons. Therefore, we developed a biosensor based on the principle of the kinase translocation reporter (KTR) (Maryu et al., 2016; Miura et al., 2018; Regot et al., 2014) to quantitatively monitor the signaling dynamics of the PKA in a living fission yeast cell. By using a blue light-responsive optogenetic tool in combination with the KTR-based PKA biosensor, we succeeded in visualizing and manipulating PKA activity.

Materials and Methods

Plasmids

All plasmids used in this study are summarized in Table 1, along with Benchling links including the plasmid sequences and maps. To construct pMNATZA1-spPKA-KTR1.0, the cDNA sequence of Rst2 (131–567 a.a.) was fused to the cDNA sequence of mScarlet-I by PCR and subcloned into pMNATZA1 using Gibson assembly with NEBuilder HiFi DNA Assembly (New England Biolabs, Ipswich, MA). The spPKA-KTR1.0 is stably expressed under the *Padh1* promoter from the Z locus, which is adjacent to the *zfs1* gene on chromosome 2 (Sakuno et al., 2009). To construct pMNATZA1-spPKA-KTR1.0-4A, the cDNA sequence of Rst2 (131–567 a.a.) with four mutations (S139A, T140A, T141A, T253A) was amplified, fused to the cDNA sequence of mScarlet-I by PCR and subcloned into pMNATZA1. For the construction of plasmids with truncated regions of Rst2 (pMNATZA1-spPKA-KTR1.1, pMNATZA1-spPKA-KTR1.2, pMNATZA1-spPKA-KTR1.3, and pMNATZA1-spPKA-KTR1.4), the DNA sequences of Rst2 (131–456 a.a., 131–356 a.a., 131–256 a.a., and 131–228 a.a.) were amplified, fused to the cDNA sequence of mScarlet-I by PCR and subcloned into pMNATZA1. To construct pHBCA21-bPAC_sp_opt, the cDNA sequence of bPAC optimized for fission yeast codon usage and the cDNA sequence of the *Padh21* promoter were subcloned into pHBCA1 using conventional ligation with Ligation high Ver.2 (TOYOBO, Osaka, Japan). The amino acid sequence of bPAC was obtained from pGEM-HE-h_bPAC_cmyc (Addgene plasmid #28134) (Stierl et al., 2011). The bPAC is stably expressed under the *Padh21* promoter from the C locus, which is adjacent to the *SPAC26F1.12c* gene on chromosome 1 (Sakuno et al., 2009). To construct pMNATZA1-AKAR3EV, the cDNA sequence of AKAR3EV from pCSIIbsr-AKAR3EV (Addgene plasmid #170309) (Komatsu et al., 2011) was subcloned into pMNATZA1 by conventional ligation. To construct the variant pMNATZA1-AKAR3EV-TA, a mutation (T506A) was introduced into pMNATZA1-AKAR3EV. To construct the pMNATZA1-EPACsensor-2051-ApaItoClaI, the cDNA sequence of CFP-Epac-YFP (Nakamoto et al., 2021) was subcloned into pMNATZA1 and the ApaI recognition sequence was replaced by the ClaI recognition sequence.

Fission yeast strains and culture conditions

All fission yeast strains used in this study are summarized in Table 2 along with their origins. Unless otherwise noted, the standard methods and media selection followed those previously described (Moreno et al., 1991). Fission yeast cells were cultured in YEA medium supplemented with high (2%, 111 mM), low (0.2%, 11 mM), or no (0%) glucose. To adjust the osmolyte concentration in the low-glucose medium (glucose concentration of 0 and 0.2%), 2% and 1.8%

glycerol were added, respectively. The fission yeast transformation protocol was modified from a previously reported one (Suga & Hatakeyama, 2005).

Live-cell fluorescence imaging of fission yeast cells

Fission yeast cells were imaged using an IX83 inverted microscope (Olympus) equipped with an sCMOS camera (ORCA-Fusion BT; Hamamatsu Photonics), a spinning disk confocal unit (CSU-W1; Yokogawa Electric Corporation), an oil immersion objective lenses (UPLXAPO 60X, NA = 1.42, WD = 0.15 mm; Olympus), and diode lasers at wavelengths of 445 nm, 488 nm, 561 nm, and 640 nm. The excitation laser and fluorescence filter settings were as follows: Excitation laser, 445 nm for Turquoise2-GL and mTFP, 488 nm for mNeonGreen, 561 nm for mScarlet-I, and 640 nm for iRFP; excitation dichroic mirrors, DM405/488/561/640 for mNeonGreen, mScarlet-I, and iRFP and DM445/514/640 for Turquoise2-GL, mTFP, YPet, and mVenus; emission filters, 482/35 for Turquoise2-GL and mTFP, 525/50 for mNeonGreen, YPet, and mVenus, 617/73 for mScarlet-I, and 685/40 for iRFP (Yokogawa Electric Corporation). The microscope was controlled by MetaMorph software (ver. 7.10.3). To illuminate cells expressing bPAC with the blue light, blue LED light (450 nm) (LED-41VIS450; OptoCode Corp.) was used from the top of the stage.

For snapshot imaging, fission yeast cells were concentrated by centrifugation at 860 g, mounted on a slide glass (thickness, 0.9–1.2 mm; Matsunami), and sealed with a glass coverslip (thickness, 0.13–0.17 mm; Matsunami). For time-lapse imaging, fission yeast cells were observed by using the CellASIC ONIX2 Microfluidic platform (Merck). The cell suspension was loaded into a trapping chamber (Y04T-04 or Y04C-02) by applying a pressure of 8 psi for 15 seconds. During the time-lapse imaging, the medium was perfused by applying a pressure of 1 psi and the cells were incubated at 32°C. During the medium exchange, the new medium was perfused at 8 psi pressure for 15 seconds for washout and then perfused at 1 psi pressure.

For the glucose dose-dependent response of spPKA-KTR1.0 in Figure 3, cells were cultured in YEA medium containing 3% glucose overnight, transferred to low-glucose medium (YEA medium containing 0.1% glucose and 3% glycerol), and then cultured at 32°C for 6 hours. Cells were collected by centrifugation, suspended in YEA medium containing various concentrations of glucose (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 0.9, 1.5, or 3.0%), and cultured at 32°C for 5 minutes. Cells were then fixed with ice-cold methanol and stored at -30°C overnight. After removal of methanol, cells were resuspended in DDW and imaged as described above.

Images and data analysis

All imaging data were analyzed and quantified using Fiji/ImageJ (https://fiji.sc/) (Schindelin et al., 2012) and Python. For all images, the background was subtracted using the rolling ball method

(rolling ball radius, 50.0 pixels) adopted in Fiji. For the quantification of snapshot images, two regions of interest (ROIs) appropriate for nucleus and cytoplasm were manually selected, the mean fluorescence signal intensities in these ROIs were measured, and the cytoplasm/nucleus (C/N) ratio values for spPKA-KTR1.0 or spPKA-KTR1.0-4A were calculated by dividing the mean intensity in the cytoplasm by that in the nucleus. For quantification of AKAR3EV and CFP-Epac-YFP, ROIs in the cytoplasm were manually selected, the mean fluorescence signal intensities in these ROIs were measured, and the FRET/CFP ratios and CFP/FRET ratios were calculated for AKAR3EV and CFP-Epac-YFP, respectively. For the segmentation, tracking, and quantification of time-lapse images, we used CellTK (Kudo et al., 2018) to quantify the PKA activity from translocation of spPKA-KTR1.0. Briefly, pre-processing, segmentation, tracking, and post-processing were performed using the CellTK functions as we adapted this method in mammalian cells (Tany et al., 2022). In pre-processing, nuclear images, Hta1-mNG or NLS-iRFP-NLS, were processed with a Gaussian blur filter. In the segmentation process, an adaptive thresholding algorithm was used. Segmented nuclear labels were tracked using the Linear Assignment Problem (LAP) algorithm (Jaqaman et al., 2008) and then processed using a track_neck_cut function in CellTK. In post-processing, short tracking data were excluded, and the cytoplasmic region was defined as an expanding ring around the nuclear segmentation. Quantified feature values (i.e., mean intensity, nuclear area, etc.) of each tracked cell were exported and cleaned in Python. To visualize the heatmaps in Figure 4D and 5E, PKA activities (C/N ratio) of each representative 26 cells were shown from nuclear division to the next. The timing of nuclear division was determined from the acute change in nuclear morphology based on Hta1-mNG images. For the visualization of Figure 2B, 2D, and 2F, each dot represents the C/N ratio of a single cell. In the boxplots, lines represent the median values, boxes represent the interquartile range and whiskers represent the minimum and maximum values, except for outliers, which are defined as 1.5 times the interquartile range. Data visualization and graph generation were performed using Python 3.10 with the modules Numpy 1.21.3, Pandas 1.3.4, Matplotlib 3.4.3, and Seaborn 0.11.2. In Figure 3B, the glucose dose-dependent response of spPKA-KTR1.0 was fitted by the Hill function ($y = Min + (Max - Min)\frac{x^n}{k^n + x^n}$), where n is the Hill coefficient and k is the EC_{50}.

Results and Discussion

FRET-based biosensors for PKA activity and cAMP levels did not work in the *S. pombe*

To monitor PKA activity and cAMP levels in the fission yeast *S. pombe*, we first tried the previously reported FRET-based biosensors. Unfortunately, neither the FRET-based the PKA biosensor AKAR3EV (Komatsu et al., 2011)nor the cAMP biosensor CFP-Epac-YFP (Ponsioen et al., 2004) could be used in fission yeast cells (Fig. S1). AKAR3EV is a FRET-based PKA biosensor that was originally developed to measure PKA activity in mammalian cells (Komatsu et al., 2011; Zhang et al., 2005). When AKAR3EV is phosphorylated by PKA, it undergoes conformational changes and exhibits a higher FRET/CFP ratio than in the non-phosphorylated state. As expected, AKAR3EV-TA, in which the threonine residue of PKA phosphorylation is substituted with an alanine residue, showed lower FRET/CFP ratio values (Fig. S1A and S1B). However, deletion of either the catalytic subunit *pka1* or the regulatory subunit *cgs1* did not alter the FRET/CFP ratio values of AKAR3EV (Fig. S1A and S1B), suggesting that AKAR3EV is phosphorylated by other kinases.

The FRET-based cAMP biosensor CFP-Epac-YFP (Ponsioen et al., 2004), leading to a decrease in FRET efficiency. Therefore, we quantified the CFP/FRET ratio of CFP-Epac-YFP as a proxy for cAMP binding. As expected, deletion of the phosphodiesterase gene *cgs2* increased the CFP/FRET values (Fig. S1C and S1D). However, the adenylate cyclase-deficient strain, *cyr1*Δ cells, did not show a significant decrease in the CFP/FRET ratio in fission yeast grown in rich medium (Fig. S1C and S1D). These results suggest that CFP-Epac-YFP is unable to detect the physiological cAMP levels in fission yeast cells due to its lower sensitivity to cAMP than the physiological cAMP levels.

Development of the KTR-based PKA biosensor, spPKA-KTR1.0, for *S. pombe*

The principle of KTR relies on the concept of converting a phosphorylation event into a nucleocytoplasmic shuttling event. The reporter is exported from the nucleus to the cytoplasm when it is phosphorylated, and imported back into the nucleus when it is dephosphorylated (Fig. 1B). Thus, this type of reporter allows us to estimate kinase activity based on the ratio of cytoplasmic to nuclear fluorescence intensity (C/N ratio). To construct a KTR-based PKA biosensor for fission yeast, we focused on the transcription factor Rst2, which is directly phosphorylated by PKA and exhibits nucleocytoplasmic shuttling in a PKA activity-dependent manner (Fig. 1C) (Gupta et al., 2011b; Higuchi et al., 2002; Jiang et al., 2021; Koike et al., 2012). Since overexpression of full-length Rst2 induces growth defects in fission yeast cells (Koike et al., 2012; Takenaka et al., 2018), we deleted the N-terminus of Rst2 up to 130 amino acids (a.a.), which contains the two Cys_2His_2-

type zinc finger motifs (Kunitomo et al., 2000). The truncated Rst2 fused to mScarlet-I is referred to as spPKA-KTR1.0 (Fig. 1C). We established a fission yeast strain that stably expresses the spPKA-KTR1.0 under the constitutive *adh1* promoter (*Padh1*). The spPKA-KTR1.0 accumulated in the nucleus of fission yeast cells cultured in medium without glucose (Fig. 1D). The reporter rapidly translocated from the nucleus to the cytoplasm when glucose was supplied into the medium (Fig. 1D, Movie S1). The cytoplasmic translocation of the reporter upon glucose stimulation was not observed in adenylate cyclase-deficient cells (*cyr1*Δ) (Fig. 1D). We quantified the C/N ratios of spPKA-KTR1.0, and found that the values started to increase 5 minutes after glucose stimulation and reached a plateau within a minute (Fig. 1E). After glucose washout, the C/N ratios returned to basal levels within 10 minutes (Fig. 1E). In contrast, the C/N ratios of spPKA-KTR1.0 in *cyr1*Δ cells did not change under the same conditions (Fig. 1E). These results support the idea that the nucleocytoplasmic translocation of spPKA-KTR1.0 depends on Cry1-induced cAMP production and subsequent PKA activation. The spPKA-KTR1.0 was excluded from the nucleolus (Fig. 1B and 1D), suggesting that this reporter may still have some ability to associate with chromatin in the nucleus despite the absence of the zinc finger motifs. A recent study has reported that transcription factors lacking DNA binding domains often localize to chromatin by using their intrinsically disordered regions (IDRs) (Kumar et al., 2023). Rst2 also has IDRs throughout its amino acid sequence, suggesting that spPKA-KTR1.0 preferentially localizes to the chromatin region through IDRs.

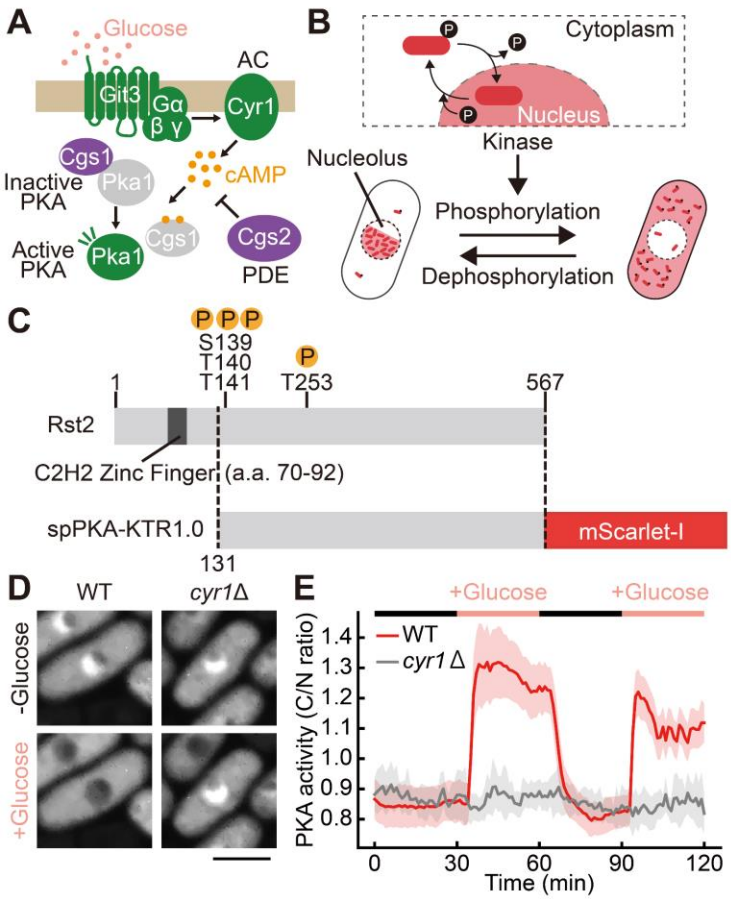

Fig. 1. Development of the PKA biosensor spPKA-KTR1.0 for fission yeast cells.
(A) Schematic representation of the cAMP-PKA signaling pathway in fission yeast cells. AC, adenylate cyclase; PDE, cAMP phosphodiesterase. (B) Schematic illustration of the phosphorylation-dependent translocation of the kinase translocation reporters (KTRs). Unphosphorylated KTR-based biosensors are primarily localized to the nucleus, whereas upon the kinase activation, the biosensors are phosphorylated and translocated to the cytoplasm. The PKA biosensor, spPKA-KTR1.0, was excluded from the nucleolus. (C) Structure of the KTR-based PKA biosensor spPKA-KTR1.0 (bottom), which consists of the protein region of Rst2 (131–567 a.a., top) and mScarlet-I. To avoid affecting the gene expression, this biosensor does not contain the two Cys_2His_2-type zinc finger motifs in the N-terminal region of Rst2 (Kunitomo et al., 2000). The amino acids (S139, T140, T141, T253) in Rst2 are the putative phosphorylation target sites of PKA (Higuchi et al., 2002). (D) Representative confocal fluorescence images of wild-type (WT, left) and *cyr1*Δ (right) strains expressing the spPKA-KTR1.0 before (top) and after (bottom) glucose stimulation. Scale bar, 5 μm. (E) Glucose-stimulated PKA activation in wild-type (red, n = 67 cells) and *cyr1*Δ (gray, n = 10 cells) strains expressing the spPKA-KTR1.0. Cells were cultured in 2% glycerol supplemented YEA medium for 16 hours and then stimulated with 2% glucose supplemented YEA medium (+ Glucose). The cytoplasmic-to-nuclear (C/N) ratio of spPKA-KTR1.0 was calculated and the mean values of the C/N ratio are shown with error bars (SD).

The molecular mechanisms of the spPKA-KTR1.0 translocation in fission yeast cells

We further investigated the mechanisms of the spPKA-KTR1.0 translocation using three approaches. First, we measured the C/N ratio in mutant strains lacking components of the cAMP-PKA pathway (*cyr1*, *pka1*, *cgs1*, or *cgs2*) in the medium containing 2% glucose. The spPKA-KTR1.0 was localized in the cytoplasm in wild-type, *cgs1*Δ, and *cgs2*Δ strains, whereas *cyr1*Δ and

*pka1*Δ strains showed nuclear accumulation of spPKA-KTR1.0 (Fig. 2A). The C/N ratios of the *cyr1*Δ and *pka1*Δ strains were reduced compared to that of the wild-type strain, whereas those of the *cgs1*Δ and *cgs2*Δ strains were comparable or higher than that of the wild-type strain (Fig. 2B). These results are consistent with previous findings that Cyr1 and Pka1 are required for PKA activation (Maeda et al., 1990, 1994) and that PKA is constitutively activated in *cgs1*Δ and *cgs2*Δ strains (DeVoti et al., 1991).

Second, we examined whether PKA-mediated phosphorylation directly controls spPKA-KTR1.0 translocation. Since spPKA-KTR1.0 has two putative PKA target sites in its sequence (Fig. 1C) (Higuchi et al., 2002), we substituted the putative clustered phosphorylation sites (S139, T140, T141) and T253 with alanine residues to generate a mutant of spPKA-KTR1.0 (spPKA-KTR1.0-4A). As expected, spPKA-KTR1.0-4A accumulated in the nucleus even in wild-type and *cgs1*Δ strains as well as in the *pka1*Δ strain (Fig. 2C). The cells expressing spPKA-KTR1.0-4A exhibited a lower C/N ratio at the same level as the *pka1*Δ strain expressing spPKA-KTR1.0 (Fig. 2D). These results indicate that the spPKA-KTR1.0 shuttles between the nucleus and cytoplasm in a PKA phosphorylation-dependent manner.

Finally, we examined which regions of spPKA-KTR1.0 are required for its translocation. There seemed to be no nuclear localization signals (NLS) or nuclear export signals (NES) in the amino acid sequence of Rst2. Therefore, for further investigation, we generated a series of truncated spPKA-KTRs, each containing the Rst2 regions 131–456 a.a., 131–356 a.a., 131–256 a.a., and 131–228 a.a., respectively (Fig. 2E). We quantified the C/N ratio of the truncated spPKA-KTRs in the wild-type, *cgs1*Δ, and *pka1*Δ strains, and found that spPKA-KTR1.1-1.4 did not change their localization in the *cgs1*Δ and *pka1*Δ strains (Fig. 2F). Among the series of spPKA-KTRs, the original spPKA-KTR1.0 showed the highest contrast in C/N ratio between *cgs1*Δ and *pka1*Δ (Fig. 2G), suggesting that multiple domains within the spPKA-KTR1.0 are required for efficient translocation in response to the PKA activity, although we could not find any putative motifs. One possible mechanism for spPKA-KTR1.0 translocation is nuclear export via the 14-3-3 proteins. The 14-3-3 proteins bind to phosphorylated proteins (Muslin et al., 1996) and regulate various cellular functions including nuclear export (Fu et al., 2003). Although there are no reports demonstrating a direct interaction between Rst2 and 14-3-3 proteins, growth inhibition in Rst2-overexpressing cells is weakly suppressed by overexpression of 14-3-3 proteins, Rad24 or Rad25, suggesting a genetic interaction between Rst2 and 14-3-3 proteins (Koike et al., 2012). Therefore, nuclear export of phosphorylated Rst2 may be regulated by the Rad24 and Rad25 in a manner similar to the well-studied nuclear export of Cdc25 phosphatase, a cell cycle regulator protein (Dalal et al., 1999; Kumagai & Dunphy, 1999; Lopez-Girona et al., 1999).

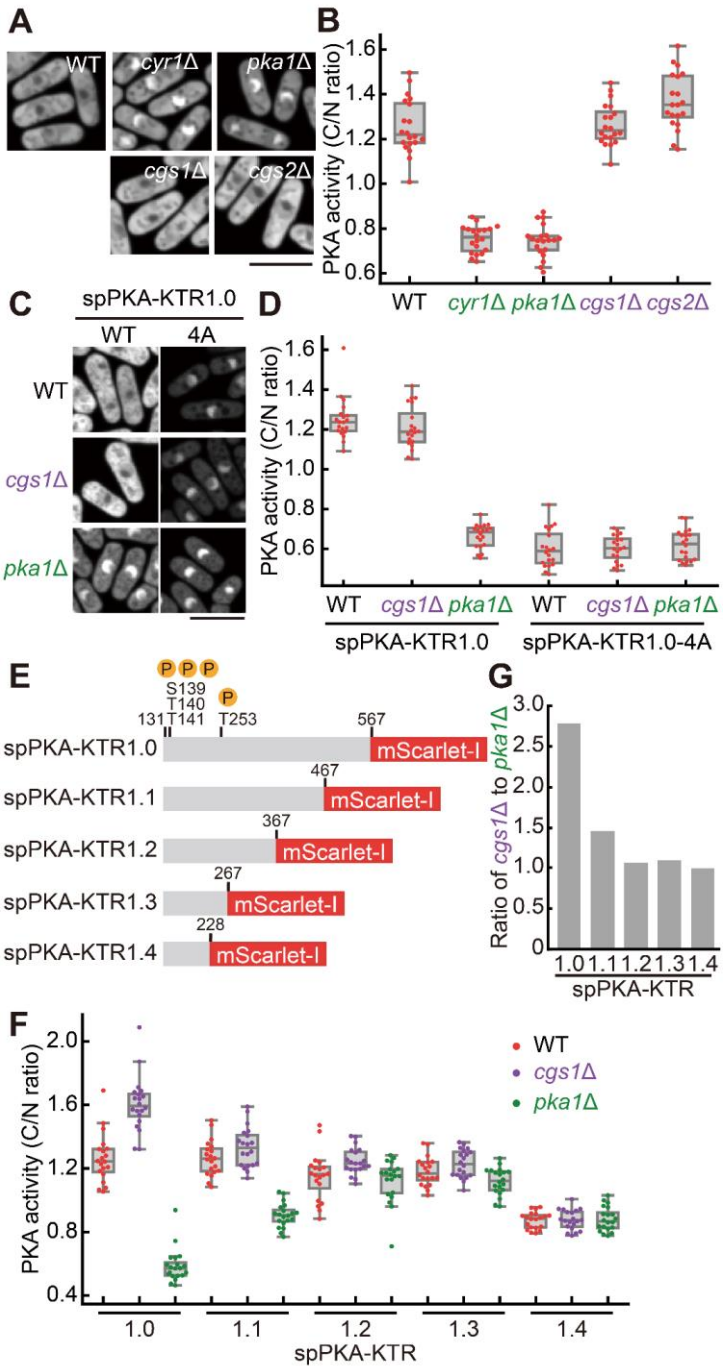

Fig. 2. Characterization of the translocation mechanisms of spPKA-KTR1.0.
(A) Representative confocal fluorescence images of wild-type (WT), *cyr1*Δ, *pka1*Δ, *cgs1*Δ, and *cgs2*Δ strains expressing the spPKA-KTR1.0 in the 2% glucose-supplemented YEA medium. Scale bar, 10 μm. (B) The PKA activity (C/N ratio) in wild-type (WT), *cyr1*Δ, *pka1*Δ, *cgs1*Δ, and *cgs2*Δ strains expressing the spPKA-KTR1.0 (n = 20 cells). Cells were cultured in YEA medium supplemented with 2% glucose. (C) Representative confocal fluorescence images of wild-type (WT), *cgs1*Δ, and *pka1*Δ strains expressing the spPKA-KTR1.0 or its variant, spPKA-KTR1.0-4A. Scale bar, 10 μm. (D) The PKA activity (C/N ratio) in wild-type (WT), c*gs1*Δ, and *pka1*Δ strains expressing the spPKA-KTR1.0 or its variant, spPKA-KTR1.0-4A (n = 20 cells). Cells were cultured in YEA medium supplemented with 2% glucose. (E) Structure of truncated versions of spPKA-KTR1.0, namely spPKA-KTR1.1–1.4. Each of them has the protein region of Rst2 from 131–456 a.a., 131–356 a.a., 131–256 a.a., and 131–228 a.a., respectively. (F) The C/N ratio of spPKA-KTR1.0–1.4 in wild-type (WT), c*gs1*Δ, and *pka1*Δ strains (n = 20 cells). Cells were grown in YEA

medium supplemented with 2% glucose. (G) The ratio of C/N ratio in *cgs1*Δ to that in *pka1*Δ strain expressing the spPKA-KTR1.0–1.4. The ratios were calculated from the data in panel F.

Dose-dependent response of spPKA-KTR1.0 to different glucose concentrations in fission yeast cells

To further evaluate whether spPKA-KTR1.0 acts as a reliable biosensor for PKA activity, we measured the dose-dependent response of spPKA-KTR1.0 to a range of glucose concentrations. Cells expressing spPKA-KTR1.0 were cultured in low-glucose medium (YEA medium containing 0.1% glucose and 3% glycerol) and then stimulated with medium containing different glucose concentrations (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 0.9, 1.5, or 3.0%) (Fig. 3A).The C/N ratio was quantitatively correlated with the glucose concentration, and the EC_{50} and Hill coefficient were 6.7×10^{-2}% (3.7 mM) and 1.9, respectively (Fig. 3B). Compared with WT cells, the *cgs1*Δ cells expressing spPKA-KTR1.0 exhibited higher C/N ratios in a glucose concentration-independent manner, whereas the *pka1*Δ cells showed constant low C/N ratios independent of glucose concentration (Fig. 3B). A wide range of C/N ratios within these upper and lower limits were observed in WT cells (Fig. 3B), indicating that the C/N ratios of the *cgs1*Δ cells and *pka1*Δ cells correspond to the upper and lower limits of PKA activity measured by spPKA-KTR1.0. The EC_{50} value (6.7×10^{-2}%) is consistent with reports that cells can proliferate normally at glucose concentrations greater than 0.08% (Masuda et al., 2016; Pluskal et al., 2011; Takeda et al., 2015). Moreover, consistent with previous reports on the glucose concentration that determines the transition between proliferation and quiescence (Pluskal et al., 2011), the C/N ratio was close to the basal level upon 0.03% glucose stimulation (Fig. 3B). These results indicate that spPKA-KTR1.0 allows the monitoring of PKA activity under the physiological conditions in fission yeast.

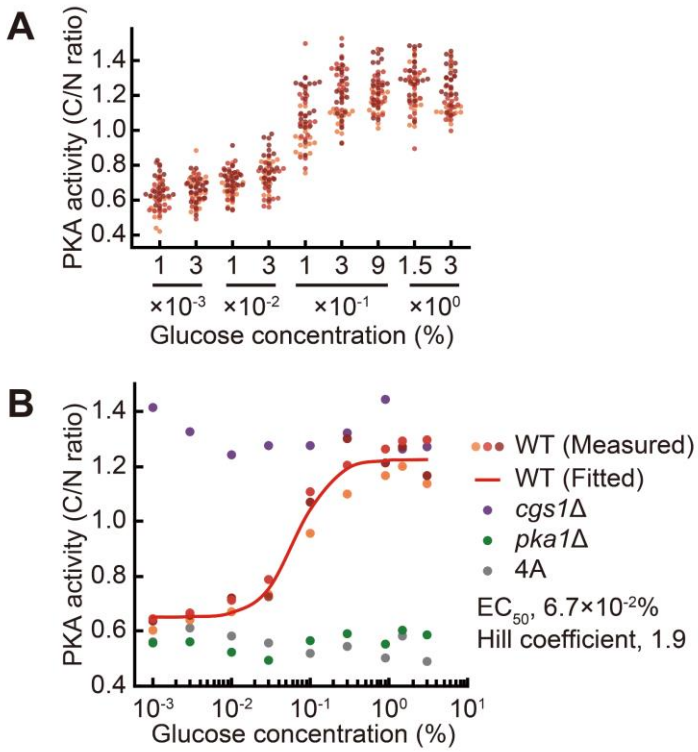

Fig. 3. Dose-dependent response of spPKA-KTR1.0 to glucose concentration.
(A) PKA activity (C/N ratio) in fission yeast cells expressing spPKA-KTR1.0 in response to various glucose concentrations. Cells were pre-cultured in low-glucose medium (YEA medium supplemented with 0.1% glucose and 3% glycerol), stimulated with YEA medium supplemented with different glucose concentrations (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 0.9, 1.5, or 3.0%), and then the localization of spPKA-KTR1.0 was observed 5 min after stimulation. The C/N ratios of 20 cells were calculated from each of three independent experiments and plotted with different colored dots. (B) PKA activity (C/N ratio) in wild-type (WT), *cgs1*Δ, and *pka1*Δ strains expressing the spPKA-KTR1.0 or spPKA-KTR1.0-4A (4A) in response to various glucose concentrations (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 0.9, 1.5, or 3.0%). Each dot represents the median C/N ratio of 20 cells. The solid red line represents the Hill function-based fitted curve for the data from the WT strain expressing spPKA-KTR1.0 (EC_{50}, 6.7×10^{-2}%; Hill coefficient, 1.9).

Visualization of PKA activation dynamics in fission yeast cells

Using the KTR-based PKA biosensor spPKA-KTR1.0, we visualized the dynamics of PKA activity in fission yeast cells under two different conditions. First, the dynamics of PKA activity was measured when cells were cultured in a medium with high glucose concentration (2% glucose), where fission yeast cells grow well. To track the nucleus and cytoplasm over time, endogenous Hta1 (histone H2A) was fused to mNeonGreen (Hta1-mNG) as a nuclear marker. By single-cell tracking of PKA activity, we found that PKA activity was transiently decreased after nuclear division (Fig. 4A and 4B), and gradually recovered within 40 minutes, coinciding with the cell separation (Fig. 4B and 4C). The transient decrease in PKA activity was observed in most cells (Fig. 4D). The spPKA-KTR1.0-4A showed a constant low level of the C/N ratio (Fig. 4B and 4D), indicating that the change in the C/N ratio of spPKA-KTR1.0 is regulated by the phosphorylation.

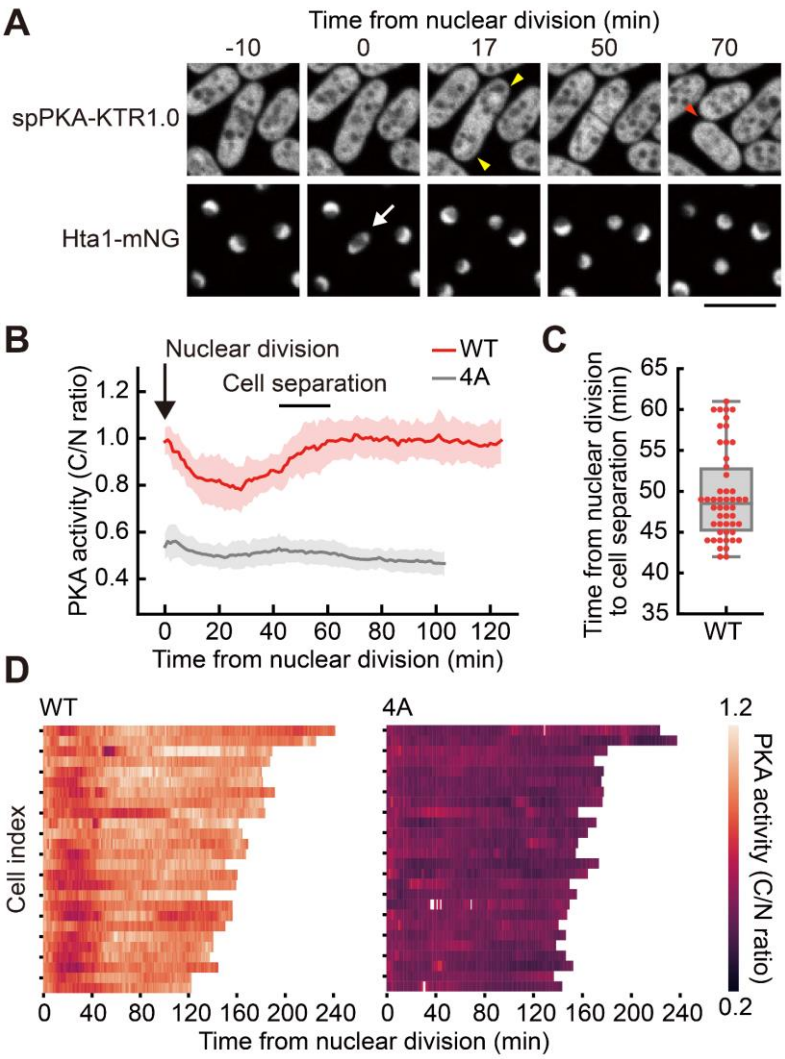

Fig. 4. Decreased PKA activity during the cell cycle under high glucose conditions.
(A) Representative confocal fluorescence images of the spPKA-KTR1.0 (top) and Hta1 (histone H2A) fused to mNeonGreen (Hta1-mNG, bottom) during nuclear division in wild-type cells. Cells were cultured in YEA medium containing 2% glucose. spPKA-KTR1.0 transiently accumulated in the nucleus during nuclear division in some cells. A white arrow indicates the onset of the nuclear division. Yellow arrows indicate the accumulation of spPKA-KTR1.0 in the nucleus. A red arrow indicates the cell separation. Scale bar, 10 μm. (B) Decreased PKA activity after nuclear division of fission yeast cells cultured in YEA medium containing 2% glucose. The C/N ratios of spPKA-KTR1.0 (WT, n = 26 cells) or spPKA-KTR1.0-4A (4A, n = 64 cells) were calculated, and the mean values of C/N ratios are shown with error bars (SD). Cell separation was indicated by the range of maximum and minimum values quantified in panel C. (C) Quantification of the time from nuclear division to cell separation in cells expressing spPKA-KTR1.0 (n = 50 cells). (D) Heatmap of time courses for PKA activity after nuclear division of fission yeast cells cultured in YEA medium containing 2% glucose. Each row represents a single cell trace of the C/N ratio of spPKA-KTR1.0 (WT, left, n = 26 cells) or spPKA-KTR1.0-4A (4A, right, n = 26 cells). Cells were aligned with the peak of the nuclear area as time 0.

Second, we measured PKA activity in response to acute glucose limitation. In budding yeast, a PKA-regulated transcription factor, Msn2, is known to undergo oscillatory nucleocytoplasmic shuttling in response to the reduction of glucose concentration in the culture medium (Hao et al., 2013). To investigate the dynamics of PKA activity in glucose-limited fission yeast cells, cells expressing spPKA-KTR1.0 were cultured in a high-glucose medium (2% glucose) and then transferred to a low-glucose medium (0.2% glucose). Cell growth was halted soon after the glucose limitation, and spPKA-KTR1.0 was accumulated in the nucleus within 10 min after glucose limitation (Fig. 5A), indicating the reduced PKA activity. Unlike the dynamics of Msn2 in response to glucose in budding yeast cells (Hao et al., 2013), we did not observe the oscillatory nucleocytoplasmic shuttling of spPKA-KTR1.0 in fission yeast cells, suggesting that the dynamics of PKA activity in fission yeast are different from those in budding yeast cells. PKA activity was gradually restored to a lower level than before glucose limitation for approximately 3 hr after glucose limitation (Fig. 5B). Next, we examined whether PKA activity decreased after nuclear division under the glucose-limited condition (0.2% glucose) as well as the high-glucose condition (2% glucose) (Fig. 4B and 4D). We focused on PKA activity after 280 minutes from 0.2% glucose stimulation (400 min in Fig. 5B), when PKA activity adapted to glucose-limited conditions (Fig. 5B). As in the high-glucose condition, PKA activity was also decreased in the glucose-limited conditions although the transient decrease in the glucose-limited condition was less significant than that in the rich medium due to the low basal PKA level (Fig. 5D and 5E). The similar activation dynamics of PKA reported in mammalian and budding yeast cells using a FRET biosensor and the transcription factor localization, respectively (Guerra et al., 2022; Vandame et al., 2014) suggested a conserved function of PKA in the timely M-phase events.

Although it is known that reduced PKA activity is necessary for sexual differentiation induced by nitrogen depletion (Higuchi et al., 2002; Otsubo & Yamamoto, 2012), it is not well understood how PKA activity is reduced under these conditions even in the presence of glucose. In this study, we found that during vegetative growth, PKA activity is transiently decreased after nuclear division even in the presence of glucose. If such a transient decrease in PKA activity also occurs during sexual differentiation by arresting cell cycle in G1 phase, the low PKA activity in G1 phase may contribute to the efficient induction of sexual differentiation. The molecular mechanism underlying the reduction of PKA activity from late M to G1 phase is still an open question.

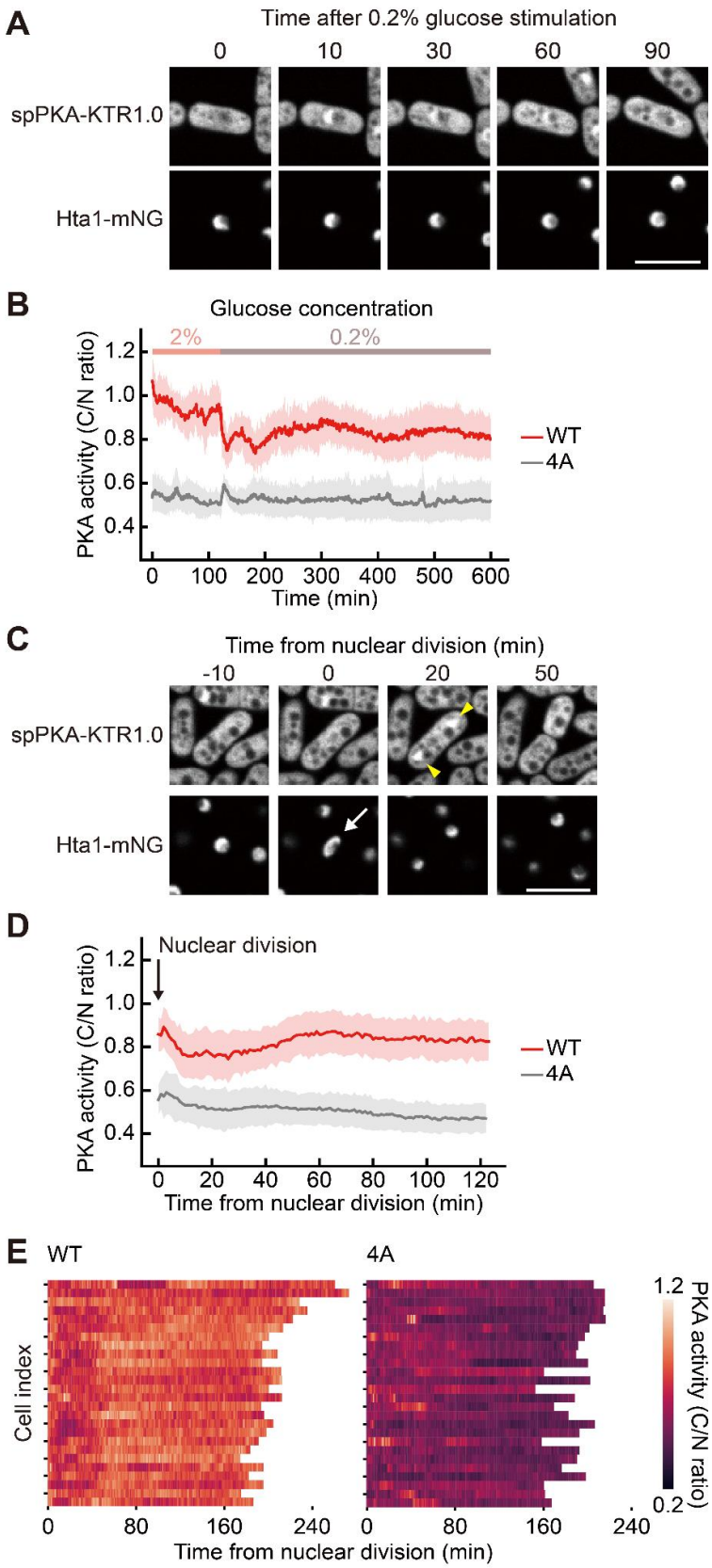

Fig. 5. Decreased PKA activity during the cell cycle under glucose-limited conditions. (A) Representative confocal fluorescence images of the spPKA-KTR1.0 (top) and Hta1-mNG (bottom) in wild-type cells after changing the glucose concentration in the medium from 2% to 0.2%. Scale bar, 10 μm. (B) Decreased PKA activity after changing the glucose concentration in the medium from 2% to 0.2% in fission yeast cells. The C/N ratios of spPKA-KTR1.0 (WT, n = 171 cells) or spPKA-KTR1.0-4A (4A, n = 379 cells) were calculated, and the mean values of C/N ratios

are shown with error bars (SD). (C) Representative confocal fluorescence images of the spPKA-KTR1.0 (top) and Hta1-mNG (bottom) during nuclear division in wild-type cells. Cells were grown in YEA medium supplemented with 0.2% glucose. A white arrow indicates the onset of the nuclear division. Yellow arrows indicate the accumulation of spPKA-KTR1.0 in the nucleus. Scale bar, 10 μm. (D) Decreased PKA activity after nuclear division of fission yeast cells cultured in YEA medium containing 0.2% glucose. The C/N ratios of spPKA-KTR1.0 (WT, n = 77 cells) or spPKA-KTR1.0-4A (4A, n = 121 cells) were calculated and the mean values of C/N ratios are shown with error bars (SD). (E) Heatmap of the time course of PKA activity after nuclear division of fission yeast cells cultured in YEA medium containing 0.2% glucose. Each row represents a single cell trace of the C/N ratio of spPKA-KTR1.0 (WT, left, n = 26 cells) or spPKA-KTR1.0-4A (4A, right, n = 26 cells). Cells were aligned with the peak of the nuclear area as time 0.

Manipulation of PKA activity by using photoactivatable adenylate cyclase bPAC

To analyze the causal relationship between intracellular signaling and cellular function, rapid and reversible perturbation of signaling molecules is required. Optogenetics is a promising technique to manipulate signaling molecules and has been widely used to perturb signaling pathways (Goto et al., 2021). A photoactivatable adenylate cyclase, bPAC, produces cAMP upon blue light illumination (Iseki et al., 2002; Stierl et al., 2011), which leads to the activation of PKA. Therefore, we implemented bPAC into fission yeast cells to manipulate the signaling dynamics of PKA. We constructed the fission yeast strain expressing bPAC with spPKA-KTR1.0 and NLS-iRFP-NLS (Sakai et al., 2021) to visualize and measure PKA activity in real time (Fig. 6A). The adenylate cyclase gene, *cyr1*, was deleted to prevent bPAC-independent cAMP production. Under dark conditions, spPKA-KTR1.0 accumulated in the nucleus and was exported from the nucleus to the cytoplasm upon blue light stimulation (Fig. 6B). PKA activity increased within 2 minutes after the blue light illumination, whereas it decreased within 5 minutes after the illumination was turned off (Fig. 6C, Movie S2). Importantly, the level of PKA activity could be fine-tuned by changing the intensity of blue light (Fig. 6D). These results suggest that bPAC-induced PKA activation (~2 min) is faster than glucose-induced PKA activation (~5 min) (Fig. 1E). This difference in PKA activation by bPAC and glucose stimulation could be due to three possibilities. One possibility is that glucose stimulation requires time for the glucose signal to be transmitted from the G protein-coupled receptor Git3 to the adenylate cyclase Cyr1, whereas bPAC directly produces cAMP in response to blue light simulation. Second possibility is that the media exchange of glucose stimulation in the microfluidic chamber takes longer than blue light stimulation. The third possibility is that the physiological state is different between glucose-stimulated cells and bPAC-expressing cells because the former have been cultured in the glycerol medium for a long time. Although further investigation is required to determine which possibility is feasible, we found that spPKA-KTR1.0 detects both fast (~2 min) and slow (~5 min) activation of PKA. Together, we have confirmed that bPAC can be used to precisely manipulate PKA activity in fission yeast cells with high temporal resolution.

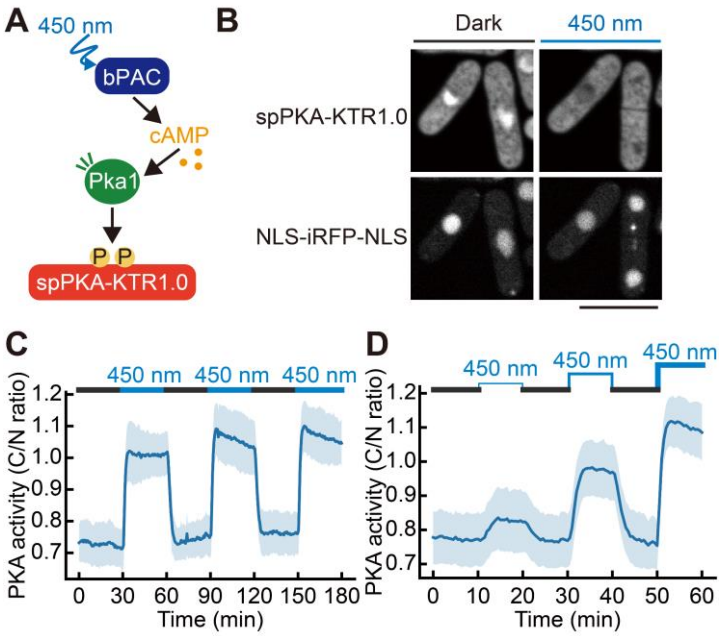

Fig. 6. Optogenetic manipulation and visualization of PKA activity by using photoactivatable adenylate cyclase bPAC and spPKA-KTR1.0.
(A) Schematic illustration of visualization and manipulation of PKA activity by using spPKA-KTR1.0 and photoactivatable adenylate cyclase bPAC. Upon illumination with blue light (450 nm), bPAC catalyzes the production of cAMP from ATP, resulting in the activation of PKA, which is monitored by spPKA-KTR1.0. (B) Representative confocal fluorescence images of the *cyr1*Δ strain expressing the spPKA-KTR1.0 (top), NLS-iRFP-NLS (bottom), and bPAC. Cells were cultured under dark conditions (left) in 2% glucose-supplemented YEA medium and then stimulated with blue light (450 nm) for 8 min (right). Scale bar, 10 μm. (C and D) Optogenetic control of PKA signaling dynamics in the *cyr1*Δ strain expressing the spPKA-KTR1.0 and bPAC. Due to the deletion of the *cyr1* gene, cAMP production in this strain is dependent on the bPAC. The intensity of blue light illumination was set to constant (C) or stepwise (D). The C/N ratios of spPKA-KTR1.0 were calculated and the mean values of the C/N ratios are shown with error bars (SD).

Conclusions

In conclusion, we have developed and characterized the KTR-based PKA biosensor, spPKA-KTR1.0, which is a reliable indicator for the PKA activity in fission yeast cells. Taking advantage of KTR, we have also implemented the photoactivatable adenylate cyclase, bPAC, in fission yeast to simultaneously visualize and manipulate PKA activity with a high temporal resolution. The spPKA-KTR1.0 and bPAC would allow us to investigate the dynamics of PKA activity under different environmental conditions. For example, PKA activity is still required to regulate cell size via microtubule destabilization even under the glucose-limited conditions, where PKA activity is thought to be low (Kelkar & Martin, 2015)In future studies, measuring and manipulating the dynamics of PKA activity under such conditions will reveal the physiological relevance of the dynamics of the cAMP-PKA pathway throughout the fission yeast life cycle.

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Conflict of Interest Statement

The authors declare no conflict of interests.

Tables

Table 1. Plasmid list

Plasmid name	Description	Source	Benchling Link
pSKI-BSD-1L-A1-M-SynPCB2.1	SynPCB2.1	K. Sakai et al., 2021	https://benchling.com/s/seq-AElby3f7vUr2vmtWEdpE?m=slm-jsJ3UgDKiMpJigQfl4kr
pMNATZA1-AKAR3EV	AKAR3EV	this study	https://benchling.com/s/seq-Npd47bLBHJC9UUzLHkRa?m=slm-v3avoZM5Hogb5QunTnPa
pMNATZA1-AKAR3EV-TA	AKAR3EV-TA	this study	https://benchling.com/s/seq-kX3yozQJGEkOREZfy3Tt
pMNATZA1-EPACsensor-2051-ApaItoClaI	CFP-Epac-YFP	this study	https://benchling.com/s/seq-IovqJHiSekcK5ze27MdA?m=slm-zLjqBTyZK44aK8h7V7Wo
pMNATZA1-spPKA-KTR1.0	spPKA-KTR1.0	this study	https://benchling.com/s/seq-HEVuRywPSzKJefrQTHoL?m=slm-1H8qZuWadi2KPOo612uN
pHBCA21-bPAC_sp_opt	bPAC	this study	https://benchling.com/s/seq-c7hwKyXTcOxHfc7NncUy
pSKI-BSD-2L-A1-M-NLS-iRFP-NLS	NLS-iRFP-NLS	K. Sakai et al., 2021	https://benchling.com/s/seq-obPOLIX96PllBreCKNXY?m=slm-Vxfn8ljcb4QTIhfAiAr1
pMNATZA1-spPKA-KTR1.0-4A	spPKA-KTR1.0-4A	this study	https://benchling.com/s/seq-sIk8riZ2Gud8dprQcioU?m=slm-7JL8BJgORdEeJ7RHt75m
pMNATZA1-spPKA-KTR1.1	spPKA-KTR1.1	this study	https://benchling.com/s/seq-iutW6vFAfpzasWXVLB2Q
pMNATZA1-spPKA-KTR1.2	spPKA-KTR1.2	this study	https://benchling.com/s/seq-MOsdmXgKlfohwAKbUB3H
pMNATZA1-spPKA-KTR1.3	spPKA-KTR1.3	this study	https://benchling.com/s/seq-bMxY3fIjbkKU4C3gIyof
pMNATZA1-spPKA-KTR1.4	spPKA-KTR1.4	this study	https://benchling.com/s/seq-PPv6DRwc23WIlmIuqjJq

Table 2. *Schizosaccharomyces pombe* strain list

Strain name	Genotype	Fig.	Source
L972	h-		NBRP
L975	h+		NBRP
YG038	h- hta1-mNeonGreen<<kan		this study, L972
YG509	h- 1L::Padh1-SynPCB2.1<<bsd		this study, L972
SK014	h- Z::Padh1-AKAR3EV<<nat	Fig. S1A, S1B	this study, L972
SK023	h- pka1::kan		this study, L972
SK029	h+ cyr1::kan		this study, L975
SK034	h+ cyr1::kan Z::Padh1-EPACsensor-2051<<nat	Fig. S1C, S1D	this study,

			SK029
SK045	h+ cgs2::bsd		this study, L975
SK076	h- cgs1::kan		this study, L972
SK077	h+ cgs1::kan		this study, L975
SK094	h- pka1::kan z::Padh1-AKAR3EV<<nat	Fig. S1A, S1B	this study, SK023
SK095	h- cgs1::kan z::Padh1-AKAR3EV<<nat	Fig. S1A, S1B	this study, SK076
SK096	h- z::Padh1-AKAR3EV-TA<<nat	Fig. S1A, S1B	this study, L972
SK097	h- cgs1::kan z::Padh1-AKAR3EV-TA<<nat	Fig. S1A, S1B	this study, SK076
SK104	h- pka1::kan z::Padh1-AKAR3EV-TA<<nat	Fig. S1A, S1B	this study, SK023
SK112	h+ z::Padh1-EPACsensor-2051<<nat	Fig. S1C, S1D	this study, L975
SK113	h+ cgs2::bsd z::Padh1-EPACsensor-2051<<nat	Fig. S1C, S1D	this study, SK045
SK186	h- hta1-mNeonGreen<<kan z::Padh1-spPKA-KTR1.0(mScarlet-I)<<nat	Fig. 1D, 1E, Fig. 2A, 2B, 2C, 2D, 2F, Fig. 3A, 3B, Fig. 4A, 4B, 4C, 4D, Fig. 5A, 5B, 5C, 5D, 5E, Movie S1	this study, YG038
SK188	h+ cyr1::kan z::Padh1-spPKA-KTR1.0(mScarlet-I)<<nat	Fig. 1D, 1E, Fig. 2A, 2B	this study, SK029
SK192	h+ cgs2::bsd z::Padh1-spPKA-KTR1.0(mScarlet-I)<<nat	Fig. 2A, 2B	this study, SK045
SK193	h+ cgs1::kan z::Padh1-spPKA-KTR1.0(mScarlet-I)<<nat	Fig. 2A, 2B, 2C, 2D, 2F, 2G, Fig. 3B	this study, SK077
SK194	h- pka1::kan z::Padh1-spPKA-KTR1.0(mScarlet-I)<<nat	Fig. 2A, 2B, 2C, 2D, 2F, 2G, Fig. 3B	this study, SK023
SK210	h- 1L::Padh1-SynPCB2.1<<bsd c::Padh21-bPAC_sp_opt<<hyg		this study, YG509
SK211	h+ cyr1::kan z::Padh1-spPKA-KTR1.0(mScarlet-I)<<nat 2L::Padh1-NLS-iRFP-NLS<<bsd		this study, SK188
SK230	h+ 1L::Padh1-SynPCB2.1<<bsd c::Padh21-bPAC_sp_opt<<hyg cyr1::kan z::Padh1-spPKA-KTR1.0(mScarlet-I)<<nat 2L::Padh1-NLS-iRFP-NLS<<bsd	Fig. 6B, 6C, 6D, Movie S2	this study, SK210×211
SK261	h+ cgs1::kan z::Padh1-spPKA-KTR1.0-4A<<nat	Fig. 2C, 2D	this study, SK077
SK264	h- pka1::kan z::Padh1-spPKA-KTR1.0-4A<<nat	Fig. 2C, 2D	this study, SK023
SK270	h- hta1-mNeonGreen-kan z::Padh1-spPKA-KTR1.0-4A<<nat	Fig. 2C, 2D, Fig. 3B, Fig. 4B, 4D, Fig. 5B, 5D, 5E	this study, YG038
SK306	h- hta1-mNeonGreen<<kan z::Padh1-spPKA-KTR1.1<<nat	Fig. 2F	this study, YG038

SK307	h- hta1-mNeonGreen<<kan z::Padh1-spPKA-KTR1.2<<nat	Fig. 2F	this study, YG038
SK308	h- hta1-mNeonGreen<<kan z::Padh1-spPKA-KTR1.3<<nat	Fig. 2F	this study, YG038
SK309	h- hta1-mNeonGreen<<kan z::Padh1-spPKA-KTR1.4<<nat	Fig. 2F	this study, YG038
SK310	h+ cgs1::kan z::Padh1-spPKA-KTR1.1<<nat	Fig. 2F, 2G	this study, SK077
SK311	h+ cgs1::kan z::Padh1-spPKA-KTR1.2<<nat	Fig. 2F, 2G	this study, SK077
SK312	h+ cgs1::kan z::Padh1-spPKA-KTR1.3<<nat	Fig. 2F, 2G	this study, SK077
SK313	h+ cgs1::kan z::Padh1-spPKA-KTR1.4<<nat	Fig. 2F, 2G	this study, SK077
SK314	h- pka1::kan z::Padh1-spPKA-KTR1.1<<nat	Fig. 2F, 2G	this study, SK023
SK315	h- pka1::kan z::Padh1-spPKA-KTR1.2<<nat	Fig. 2F, 2G	this study, SK023
SK316	h- pka1::kan z::Padh1-spPKA-KTR1.3<<nat	Fig. 2F, 2G	this study, SK023
SK317	h- pka1::kan z::Padh1-spPKA-KTR1.4<<nat	Fig. 2F, 2G	this study, SK023

Supplementary Information

Movie S1. Translocation of the KTR-based PKA biosensor, spPKA-KTR1.0, in response to glucose stimulation in fission yeast cells.

Representative confocal fluorescence images of spPKA-KTR1.0 (left) and Hta1 (histone H2A) fused to mNeonGreen (Hta1-mNG, right) in fission yeast cells. Cells were cultured in YEA supplemented with 2% glycerol, and then stimulated with YEA containing 2% glucose from 30 minutes to 60 minutes (marked with "+ Glucose" in the movie). Fluorescence images were taken every one minute, and the playback speed was set to 40 frames per second. The time stamp indicates the time in hours: minutes. Scale bar, 10 μm.

Movie S2. Optogenetic control of PKA kinase activity by using a photoactivatable adenylate cyclase bPAC in fission yeast cells.

Representative confocal fluorescence images of the *cyr1*Δ strain expressing the spPKA-KTR1.0 (left), NLS-iRFP-NLS (right), and bPAC. Cells were cultured under dark conditions in 2% glucose-supplemented YEA medium and then stimulated with blue light (labeled "Blue light" in the movie). Fluorescence images were taken every 30 seconds, and the playback speed is set to 40 frames per second. The time stamp indicates the time in hours: minutes. Scale bar, 10 μm.

Supplementary Figure 1. Characterization of the FRET-based PKA biosensor AKAR3EV and the cAMP biosensor CFP-Epac-YFP in *S. pombe*.

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