Modular bioengineered kinase activity sensors via scaffold protein-mediated split-luciferase complementation

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Contents

1. Materials and methods

1.1 Sensor design details

To generate sensors for protein kinases including PKA, PKB, CHK1, the optimal peptides (Table S1) were respectively selected. These peptides have been reported to bind to 14-3-3 upon phosphorylation. The monovalent peptide for the **Mono-PKA** sensor (mPKA) was derived from the C-terminus of the plant plasma membrane H^+-ATPase PMA2[1,2]. Effectiveness of this 14-3-3/CT32 interaction has already been shown in a synthetic biology approach.[3] The kinase that naturally phosphorylates the wild-type CT32 remains unknown, however, introduction of the well-defined PKA recognition motif (R-R-X-S-Φ)[4] was hypothesized to result in a CT32 that requires the phosphorylation by PKA to induce binding to 14-3-3. Since CT32 possesses a C-terminal mode III 14-3-3 binding motif, e.g. requires a free C-terminus, this peptide does not allow for the generation of a bivalent 14-3-3 binding motif. Therefore, the well-studied natural PKA substrate motif derived from C-RAF was selected to generate the **Bi-PKA** sensor, since it contains two PKA phosphorylation sites and has been shown to interact with 14-3-3 (K_D=346 nM)[5]. The monovalent peptide for **Mono-CHK1** sensor (mCHK1) is derived from Cdc25C, which is a known substrate of Checkpoint Kinase 1.[6] CHK1 phosphorylation of this peptide on serine-216 has shown to induce the binding to 14-3-3.[7,8] The length of the peptide was based on the crystal structure (Figure S1), as observed electron density for residues is indicative for rigidity, which might be the result of interaction with 14-3-3 and are therefore important residues for binding. For the **Bi-CHK1** sensor, the bivalent peptide was obtained from covalent attachment of two monovalent peptides via a flexible $(Gly\text{-}Gly\text{-}Ser)_3$ – linker, the length of this linker is chosen to be long enough to span the distance between two peptides binding in separate binding grooves. The monovalent peptide for **Mono-PKB** sensor (mPKB) was derived from the protein glycogen synthase kinase (GSK3α), which is a known substrate for protein kinase B[9]. Since no crystal structure of the interaction between 14-3-3 and CDC25C is available, the length of the peptide was chosen to include the known PKB recognition motif (RXRXXS)[10] on the N-terminal side and to include residues that might play a role in interaction of CDC25C with 14-3-3 on the C-terminal side, more specifically, the glycine residues flanking the chosen peptide sequence are not incorporated. The hypothesized 14-3-3 binding of mPKB was evaluated via online software http://www.compbio.dundee.ac.uk/1433pred/ (cut-

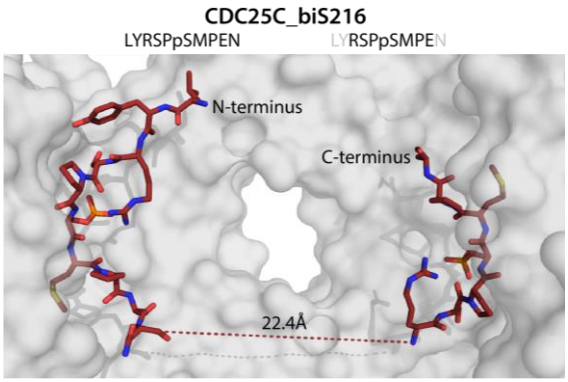

Figure S1 Crystal structure for the CDC25C/14-3-3 interaction (PDB 5M35).

off = 0.50). For the **Bi-PKB** sensor, the bivalent peptide was obtained from covalent attachment of two monovalent peptides via a flexible $(Gly\text{-}Gly\text{-}Ser)_3$ – linker, the length of this linker is chosen to be long enough to span the distance between two peptides binding in separate binding grooves. The Gblocks were synthesized by IDT (USA).

Table S1 Details of monovalent and bivalent peptides.

Name	Source	Motif	Kinase	Strategy
mPKA	CT32_RRYSI	RQRELHTLKGHVESVVKLKGLDIETIQ**RRYSI**	PKA	Monovalent
mPKB	GSK3α_S21	**RARTSSFAEP**	PKB	Monovalent
mCHK1	Cdc25C_S216	**LYRSPSMPEN**	CHK1	Monovalent
biPKA	C-RAF_S233_S259	**QHRYSTPHAF**TFNTSSPSSEGSLSQ**RQRSTSTPNVH**	PKA	Bivalent
biPKB	GSK3A_biS21	**RARTSSFAEP**GGSGGSGGS**RARTSSFAEP**	PKB	Bivalent
biCHK1	Cdc25C_biS216	**LYRSPSMPEN**GGSGGSGGS**LYRSPSMPEN**	CHK1	Bivalent

Note: The amino acids in bold fonts show the recognitions motifs for kinases, and the putative phosphorylation sites are highlighted with underline.

1.2 Experimental section

1.2.1 Plasmid preparation and protein purification

C-terminal fusion of the monovalent peptides to the large NanoBiT (LgNB) and the small NanoBiT (SmNB) fragments of split-NanoLuc[11] were obtained via restriction and ligation of the synthesized Gblocks encoding the monovalent peptides. In the case of bivalent systems, only LgNB was fused to the bivalent peptides via restriction and ligation of the Gblocks encoding the bivalent peptides. SmNB was fused to the C-terminus of the covalent dimeric scaffold protein 14-3-3[12] (dT14-3-3-SmNB), also via restriction and ligation. All the LgNB/SmNB fusion proteins were encoded into a pET28a vector and expressed in *E.coli* BL21(DE3) (Novagen) in sterile medium supplemented with 30 μg/mL kanamycin. For the induction, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultures when the cell density OD_{600} reached 1. Subsequently, the cultures were incubated for 15 hours at 18 °C, shaking at 160 rpm. Additionally, the plasmid pOPINF encoding T14-3-3cΔC[13,14] was used to express the tobacco monomer scaffold protein 14-3-3 (T14-3-3cΔC) in *E.coli* BL21-CodonPlus(DE3)-RIL (Novagen) with ampicillin (100 μg/mL) as the selective agent. Furthermore, pET28a SUMO-PKA was expressed in *E. coli* BL21(DE3), after induction with 0.5 mM IPTG at 37 °C, 160 rpm for 4 hours. The empty vector (EV) was obtained by excluding SUMO-PKA fragment from the pET28a SUMO-PKA vector. All the sequence information is shown in Figure S3.

Cells were harvested by centrifugation at 8,000 rpm at 4 °C for 10 min. The pellets were resuspended in buffer (50 mM Tris, 300 mM NaCl, 5 mM $MgCl_2$, 50 mM imidazole, pH 8.0) with Benzonase® Nuclease (25 U per 10 mL buffer, Novagen). After cell lysis with EmulsiFlexC3 High Pressure homogenizer (Avestin), the cell lysate was centrifuged at 20,000 rpm, 4 °C for 20 minutes.

To purify the proteins, the supernatants containing Lg/SmNB fused peptides and SUMO-PKA were individually applied to a Ni-loaded column (His-Bind® Resin, Novagen) and washed with wash buffer (50 mM Tris, 300 mM NaCl, 50 mM imidazole, pH 8.0) in presence and subsequent absence of 0.1% Triton-X-100. The protein was eluted from the column with elution buffer (50 mM Tris, 300 mM NaCl, 250 mM Imidazole, pH 8.0). For SmNB fused monovalent peptides, samples were cleaved with SUMO protease dtUD1 (17.2 mg/mL)[13,15] during overnight dialysis (MWCO 3.5 kDa, Thermo Scientific). The cleaved and dialyzed sample was further purified with Ni-loaded column and the flow-through containing SmNB fused proteins were collected.

Amicon Ultra Centrifugal Filters (Millipore) were used to concentrate the fractions. Finally, the buffer was exchanged into assay buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) using a PD-10 desalting column (GE Healthcare). After determining the concentration by ND-1000 spectrophotometer (Thermo Scientific) at 280 nm (the extinction coefficients (ε) in water under reducing conditions have been listed in SI 1.3), the protein aliquots were snap-freezed with liquid nitrogen and stored at -80 °C. The purification was analyzed on precast SDS-PAGE gels (4-20% Mini-PROTEAN® TGX™, Bio-Rad), using Coomassie Brilliant Blue (R-250, Bio-Rad) to stain the proteins. Gel pictures were taken with an ImageQuant 350 (GE Healthcare).

1.2.2 *In vitro* phosphorylation assay

To obtain the phosphorylated peptides, the purified fusion proteins, including LgNB/SmNB-mPKA, LgNB/SmNB-mPKB, LgNB/SmNB-mCHK1, LgNB-biPKA, LgNB-biPKB, LgNB-biCHK1 (20 μM, total volume 150 μL) were incubated separately with their relevant commercial kinases, i.e. PKA (10 μL, P6000S, 2500 U/μL, New England Biolabs), PKB (10 μL, SRP5001, 10.3 U/μL, Sigma), CHK1 (15 μL, C0870, 16.9 U/μL, Sigma) in the absence or presence of adenosine triphosphate (ATP, 200 μM) in the phosphorylation buffer (10 mM HEPES, 150 mM NaCl, 20 mM MgAcetate, pH7.4) at 30 °C, 300 rpm for 3 hours. The phosphorylated protein aliquots were stored at -80 °C prior to use. The reaction buffer was changed to 0.1% formic acid before confirming the phosphorylation via Q-ToF-Mass Spectrometry.

1.2.3 Cell culture and Pharmacology

Human embryonic kidney cells HEK293T were maintained and proliferated in DMEM (Dulbecco's Modified Eagle Medium) solutions supplemented with 10% fetal bovine serum (FBS, Gibco®), and 1%

penicillin-streptomycin (Gibco®) at 37 °C in an atmosphere of 5% CO_2 and 70% humidity.[16] For PKA stimulation, $5x10^6$ cells per well were incubated in 6-well plate for 48 hours prior to cell treatment with forskolin (FSK, 100 µM, Sigma) for 1 hour. Control cells were treated with an equal amount of dimethyl sulfoxide (DMSO, vehicle). After detaching the cells from the bottom of the plate via pipetting with PBS, the culture was transferred into Eppendorf cups and centrifuged at 4 °C, 20,000 rpm for 10 min. The cell pellet was lysed by 100 µL pre-cooled lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Tween 20) with 1 % (v/v) protease inhibitor (P8340, Sigma) and 1% (v/v) phosphatase inhibitor (P2850, Sigma). After spinning down at 20,000 rpm for 30 min, the supernatant was kept at -80 °C before use.

1.2.4 Activity assays

In the monovalent system, 50 nM of the phosphorylated LgNB and SmNB fusion protein were incubated simultaneously with the 14-3-3 monomer in the complementation buffer (10 mM HEPES, 150 mM NaCl, 1 mg/mL BSA (Sigma), pH 7.4). In the bivalent system, LgNB fusion protein was incubated with 14-3-3 dimer for each sensor. Furimazine (substrate for Nano-Glo® Luciferase assay, Promega) was added into the mixture (total volume 100 µL, LumiNunc 96 wells-plate) before recording the bioluminescent spectra from 398 nm to 608 nm on a Tecan Spark 10M platereader (integration time: 1000 ms).

In order to get insight into the efficiency of the bivalent sensors, unphosphorylated LgNB-bivalent peptides (10 nM) with the equal amount of 14-3-3–SmNB were incubated directly with ATP (200 µM ATP) and their corresponding kinase in the reaction buffer (10 mM HEPES, 150 mM NaCl, 20 mM MgAcetate, 1 mg/mL BSA, pH 7.4). Furimazine was added at the start of recording the bioluminescent intensity (at 458 nm) at 30 °C for 1 h (total volume 100 µL, LumiNunc 96 wells-plate, EASYfilm cover). The selectivity of the kinase sensors was investigated in the same way while incubating with different kinases.

1.2.5 AssayQuant PKA PhosphoSens assay

Considerations

The kinase concentrations recommended by the supplier are within the range of 0.02-20 nM. Using the following constants; specific activity: ~$5*10^6$ U/mg and molecular weight: 38 kDa (derived from the NEB product summary sheet), a PKA activity concentration range of 0.02-1.28 U/µL translates to a PKA concentration range of ~ 0.1-7 nM. These concentrations are within the recommended concentration range.

Protocol

The commercial AssayQuant PKA PhosphoSens assay was performed following the instructions

manual. First, a mastermix was prepared, to which serial dilutions of PKA (0.02-1.28 U/µL, New England Biolabs) prepared in Enzyme dilution buffer were added. The final mixture contains HEPES (54 mM, pH 7.5), ATP (1 mM), Brij-35 (0.012%), $MgCl_2$ (10 mM), AQT0458 (10 µM). Fluorescence intensity (ex: 460(20) nm, em: 492(20) nm, gain: 85, Z-position: 22848 µm) was measured on a Tecan Spark 10M platereader for 1h at 30°C (total volume 25 µL, white 384 wells-plate (Corning, small volume, NBS treated), ClearVue cover (MolecularDimensions)).

1.2.6 Application of Bi-PKA sensor in *E.coli* lysate

To validate the feasibility of the kinase sensors in *E.coli* cell lysate, LgNB-biPKA (20 µM) was incubated with cell lysate (5 µL) containing expressed PKA in the absence or presence of ATP (200 µM) in a total volume of 150 µL, at 30 °C, 300 rpm for 3 hours in the phosphorylation buffer (see above, pH7.4). *E.coli* lysate containing *ev* plasmid was used as control. After that, the pre-incubated LgNB-biPKA was diluted in the complementation buffer (see above, pH 7.4) to arrive at a final concentration of 25 nM, with equal concentration of 14-3-3–SmNB (25 nM). The bioluminescent intensity was recorded immediately after adding the substrate furimazine.

1.2.7 Application of Bi-PKA sensor in human cell lysate

To validate the feasibility of the Bi-PKA sensor in human cell lysate, LgNB-biPKA (25 nM) with/without 14-3-3–SmNB (25 nM) was incubated with 293T cell lysate (5 µL) in the reaction buffer (see above, pH7.4) in the presence or absence of ATP (200 µM) at 37 °C. Cyclic adenosine monophosphate (cAMP, 2 mM, A9501, Sigma) was added to stimulate the PKA activity *in vitro*. On the contrast, the inhibitor H89 (10 µM, B1427, Sigma) was used to inhibit the PKA activity in cell lysate. After 1 h, luminescent intensity (at 458 nm) was recorded immediately after adding substrate furimazine.

1.3 Sequences of constructs

T14-3-3 cΔC (ε = 27,390 M^{-1} cm^{-1})

1 MAHHHHHHSS GLEVLFQGMA VAPTAREENV YMAKLAEQAE RYEEMVEFME KVSNSLGSEE LTVEERNLLS

71 VAYKNVIGAR RASWRIISSI EQKEESRGNE EHVNSIREYR SKIENELSKI CDGILKLLDA KLIPSAASGD

141 SKVFYLKMKG DYHRYLAEFK TGAERKEAAE STLTAYKAAQ DIATTELAPT HPIRLGLALN FSVFYYEILN

211 SPDRACNLAK QAFDEAIAEL DTLGEESYKD STLIMQLLRD NLTLWTSD

LgNB-CT32_RRYSI (LgNB-mPKA; ε = 21,430 M^{-1} cm^{-1})

1 MGSSHHHHHH SSGVFTLEDF VGDWEQTAAY NLDQVLEQGG VSSLLQNLAV SVTPIQRIVR SGENALKIDI

71 HVIIPYEGLS ADQMAQIEEV FKVVYPVDDH HFKVILPYGT LVIDGVTPNM LNYFGRPYEG IAVFDGKKIT

141 VTGTLWNGNK IIDERLITPD GSMLFRVTIN SGGSGGSGGS GGSGGSGGSG GSGGSGGSGG SRQRELHTLK

211 GHVESVVKLK GLDIETIQQS YDI

SmNB-CT32_RRYSI (SmNB-mPKA; ε = 2,980 M^{-1} cm^{-1})

1 VTGYRLFEEI LGGSGGSGGS GGSGGSGGSG GSGGSGGSGG SRQRELHTLK GHVESVVKLK GLDIETIQQS

71 YDI

LgNB-GSK3A_S21 (LgNB-mPKB; ε = 25,440 M^{-1} cm^{-1})

1 MGSSHHHHHH SSGVFTLEDF VGDWEQTAAY NLDQVLEQGG VSSLLQNLAV SVTPIQRIVR SGENALKIDI

71 HVIIPYEGLS ADQMAQIEEV FKVVYPVDDH HFKVILPYGT LVIDGVTPNM LNYFGRPYEG IAVFDGKKIT

141 VTGTLWNGNK IIDERLITPD GSMLFRVTIN SGGSGGSGGS GGSGGSGGSG GSGGSGGSGG GTRARTSSFA

211 EPGGSGGSGG SWSHPQFEK

SmNB-GSK3A_S21 (SmNB-mPKB; ε = 6,990 M^{-1} cm^{-1})

1 VTGYRLFEEI LGGSGGSGGS GGSGGSGGSG GSGGSGGSGG SGTRARTSSF AEPGGSGGSG GSWSHPQFEK

LgNB-CDC25C_S216 (LgNB-mCHK1; ε = 26,930 M^{-1} cm^{-1})

1 MGSSHHHHHH SSGVFTLEDF VGDWEQTAAY NLDQVLEQGG VSSLLQNLAV SVTPIQRIVR SGENALKIDI

71 HVIIPYEGLS ADQMAQIEEV FKVVYPVDDH HFKVILPYGT LVIDGVTPNM LNYFGRPYEG IAVFDGKKIT

141 VTGTLWNGNK IIDERLITPD GSMLFRVTIN SGGSGGSGGS GGSGGSGGSG GSGGSGGSGG GTLYRSPSME

211 NGGSGGSGGS WSHPQFEK

SmNB-CDC25C_S216 (SmNB-mCHK1; ε = 8,480 M^{-1} cm^{-1})

1 VTGYRLFEEI LGGSGGSGGS GGSGGSGGSG GSGGSGGSGG GTLYRSPSME NGGSGGSGGS WSHPQFEK

dT14-3-3-SmNB (ε = 61,770 M^{-1} cm^{-1})

1 MGGSHHHHHH GTGASSSGMA VAPTAREENV YMAKLAEQAE RYEEMVEFME KVSNSLGSEE LTVEERNLLS

71 VAYKNVIGAR RASWRIISSI EQKEESRGNE EHVNSIREYR SKIENELSKI CDGILKLLDA KLIPSAASGD

141 SKVFYLKMKG DYHRYLAEFK TGAERKEAAE STLTAYKAAQ DIATTELAPT HPIRLGLALN FSVFYYEILN

211 SPDRACNLAK QAFDEAIAEL DTLGEESYKD STLIMQLLRD NLTLWTSDMQ GGSGGSGGSG GSGGGSGGSG

281 GSGSGGSGGS MAVAPTAREE NVYMAKLAEQ AERYEEMVEF MEKVSNSLGS EELTVEERNL LSVAYKNVIG

351 ARRASWRIIS SIEQKEESRG NEEHVNSIRE YRSKIENELS KICDGILKLL DAKLIPSAAS GDSKVFYLKM

421 KGDYHRYLAE FKTGAERKEA AESTLTAYKA AQDIATTELA PTHPIRLGLA LNFSVFYYEI LNSPDRACNL

491 AKQAFDEAIA ELDTLGEESY KDSTLIMQLL RDNLTLWTSD MQGTGGNGSS GGVTGYRLFE EILGGSGGSW

561 SHPQFEKGGS

LgNB-CRAF_biS233-S259 (LgNB-biPKA; ε = 26,930 M^{-1} cm^{-1})

1 MGSSHHHHHH SSGVFTLEDF VGDWEQTAAY NLDQVLEQGG VSSLLQNLAV SVTPIQRIVR SGENALKIDI

71 HVIIPYEGLS ADQMAQIEEV FKVVYPVDDH HFKVILPYGT LVIDGVTPNM LNYFGRPYEG IAVFDGKKIT

141 VTGTLWNGNK IIDERLITPD GSMLFRVTIN SGGSGGSGGS GGSGGSGGSG GSGGSGGSGG TQHRYSTPHA

211 FTFNTSSPSS EGSLSQRQRS TSTPNVHGGS GGSGGSWSHP QFEK

LgNB-GSK3A_biS21 (LgNB-biPKB; ε = 25,440 M^{-1} cm^{-1})

1 MGSSHHHHHH SSGVFTLEDF VGDWEQTAAY NLDQVLEQGG VSSLLQNLAV SVTPIQRIVR SGENALKIDI

71 HVIIPYEGLS ADQMAQIEEV FKVVYPVDDH HFKVILPYGT LVIDGVTPNM LNYFGRPYEG IAVFDGKKIT

141 VTGTLWNGNK IIDERLITPD GSMLFRVTIN SGGSGGSGGS GGSGGSGGSG GSGGSGGSGG TRARTSSFAE

211 PGGSGGSGGS GSRARTSSFA EPGGSGGSGG SWSHPQFEK

LgNB-CDC25C_biS216 (LgNB-biCHK1; ε = 28,420 M^{-1} cm^{-1})

1 MGSSHHHHHH SSGVFTLEDF VGDWEQTAAY NLDQVLEQGG VSSLLQNLAV SVTPIQRIVR SGENALKIDI

71 HVIIPYEGLS ADQMAQIEEV FKVVYPVDDH HFKVILPYGT LVIDGVTPNM LNYFGRPYEG IAVFDGKKIT

141 VTGTLWNGNK IIDERLITPD GSMLFRVTIN SGGSGGSGGS GGSGGSGGSG GSGGSGGSGG TLYRSPSMPE

211 NGGSGGSGGS GSLYRSPSMP ENGGSGGSGG SWSHPQFEK

SUMO-PKA (ε = 55,350 M^{-1} cm^{-1})

1 MGSSHHHHHH SSGLVPRGSH MSDSEVNQEA KPEVKPEVKP ETHINLKVSD GSSEIFFKIK KTTPLRRLME

71 AFAKRQGKEM DSLRFLYDGI RIQADQTPED LDMEDNDIIE AHREQIGGMG NAAAAKKGSE QESVKEFLAK

141 AKEDFLKKWE TPSQNTAQLD QFDRIKTLGT GSFGRVMLVK HKESGNHYAM KILDKQKVVK LKQIEHTLNE

211 KRILQAVNFP FLVKLEFSFK DNSNLYMVME YVAGGEMFSH LRRIGRFSEP HARFYAAQIV LTFEYLHSLD

281 LIYRDLKPEN LLIDQQGYIQ VTDFGFAKRV KGRTWTLCGT PEYLAPEIIL SKGYNKAVDW WALGVLIYEM

351 AAGYPPFFAD QPIQIYEKIV SGKVRFPSHF SSDLKDLLRN LLQVDLTKRF GNLKNGVNDI KNHKWFATTD

421 WIAIYQRKVE APFIPKFKGP GDTSNFDDYE EEEIRVSINE KCGKEFTEF

2. Supporting figures

2.1 SDS-PAGE and Q-ToF LC/MS analysis

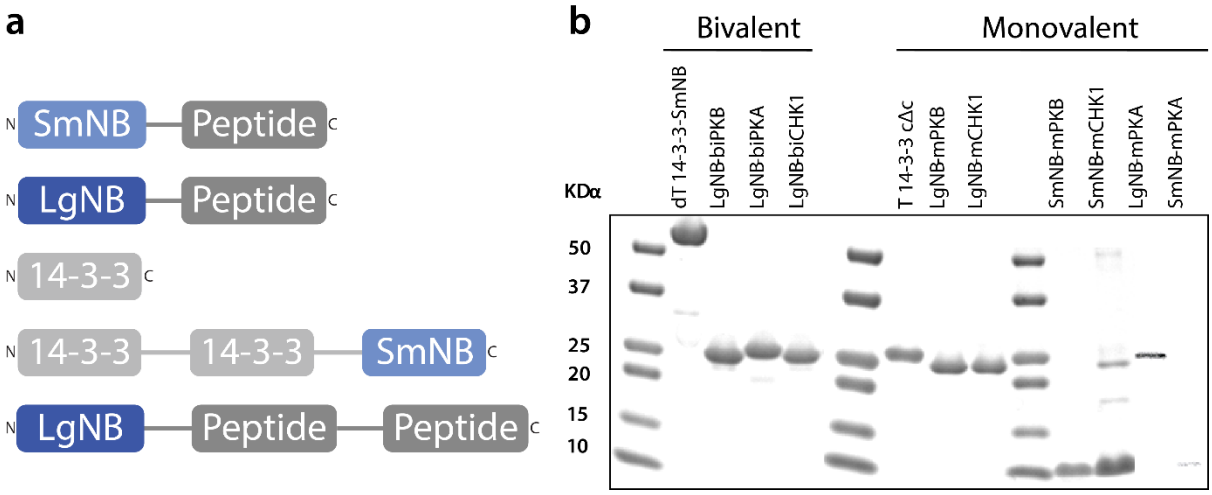

Figure S2 (a) Schematic representation of the constructs. (b) SDS-PAGE gel analysis of Ni-NTA purified proteins.

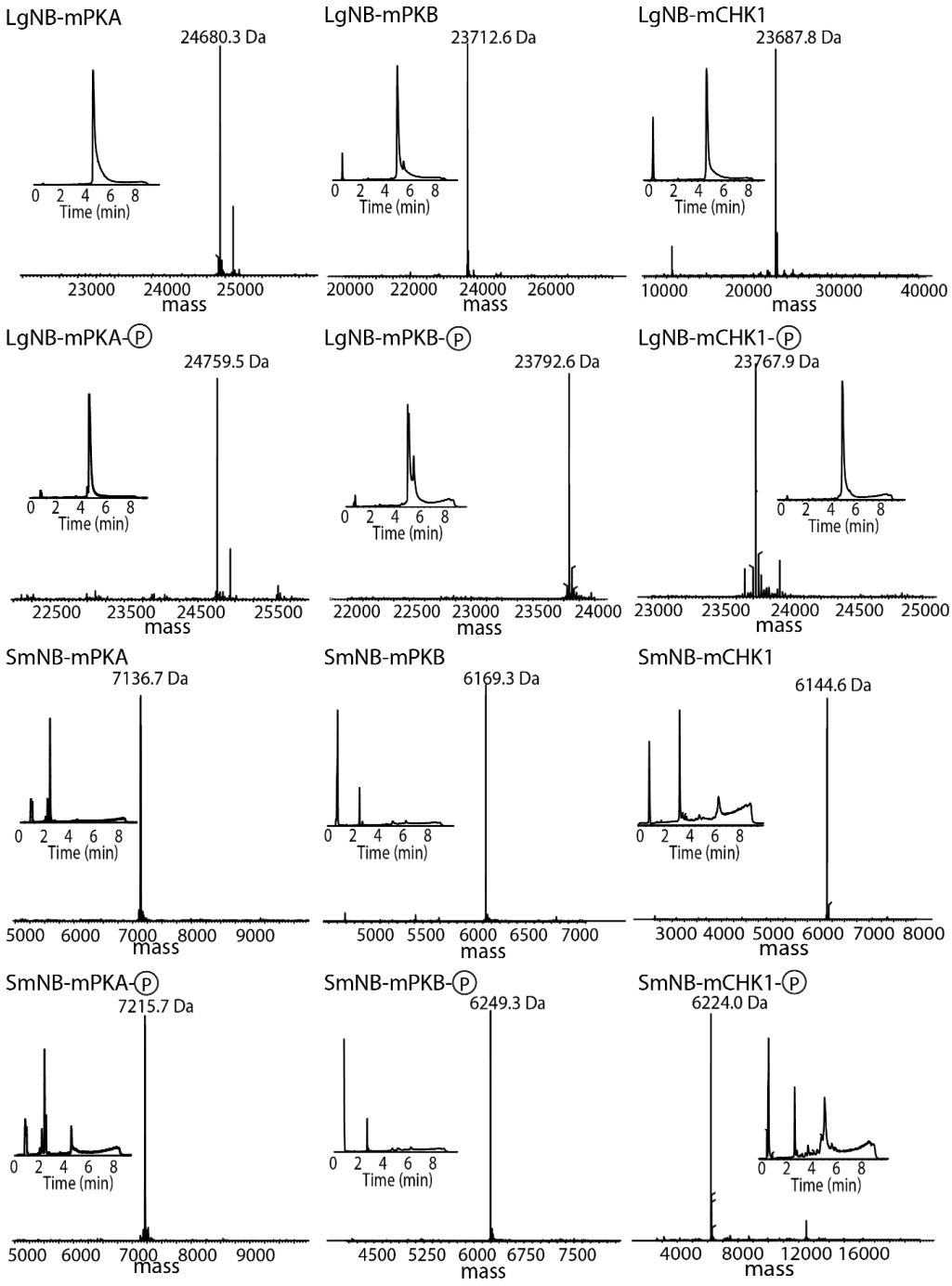

Figure S3 Qtof-LC/MS analysis of unphosphorylated and phosphorylated monovalent Lg/SmNB-peptides. These proteins containing specific phosphorylation sites have been incubated with relative kinases in the presence or absence of ATP. The graphs represent the mass spectrums and the chimeric ones show the total ion count chromatograms. The left three exhibits the successfully phosphorylated LgNB-mPKA (24759 Da), SmNB-mPKA (7215 Da) with expected masses which show an 80 Da increase compared to the unphosphorylated LgNB-mPKA (24679 Da) and SmNB-mPKA (7136 Da). The middle and right columns show the successfully phosphorylated Lg/SmNB-mPKB and Lg/SmNB-mCHK1.

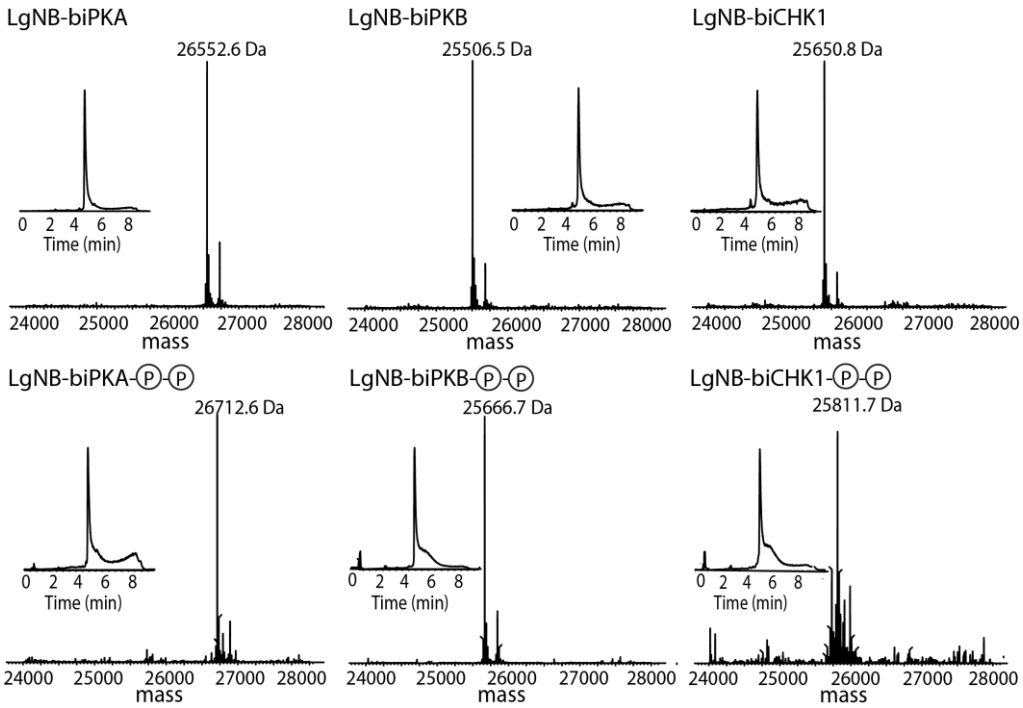

Figure S4 Qtof-LC/MS analysis of unphosphorylated and phosphorylated bivalent LgNB-peptides. These proteins containing double specific phosphorylation sites have been incubated with relative kinases in the presence or absence of ATP. The graphs represent the mass spectrums and the chimeric ones show the total ion count chromatograms. The top three exhibits the successfully phosphorylated LgNB-biPKA (25712 Da), LgNB-biPKB (25667 Da) and LgNB-biCHK1 (25811 Da) with expected masses which show a 160 Da increase compared to the unphosphorylated ones in the bottom row.

2.2 Full bioluminescence spectra

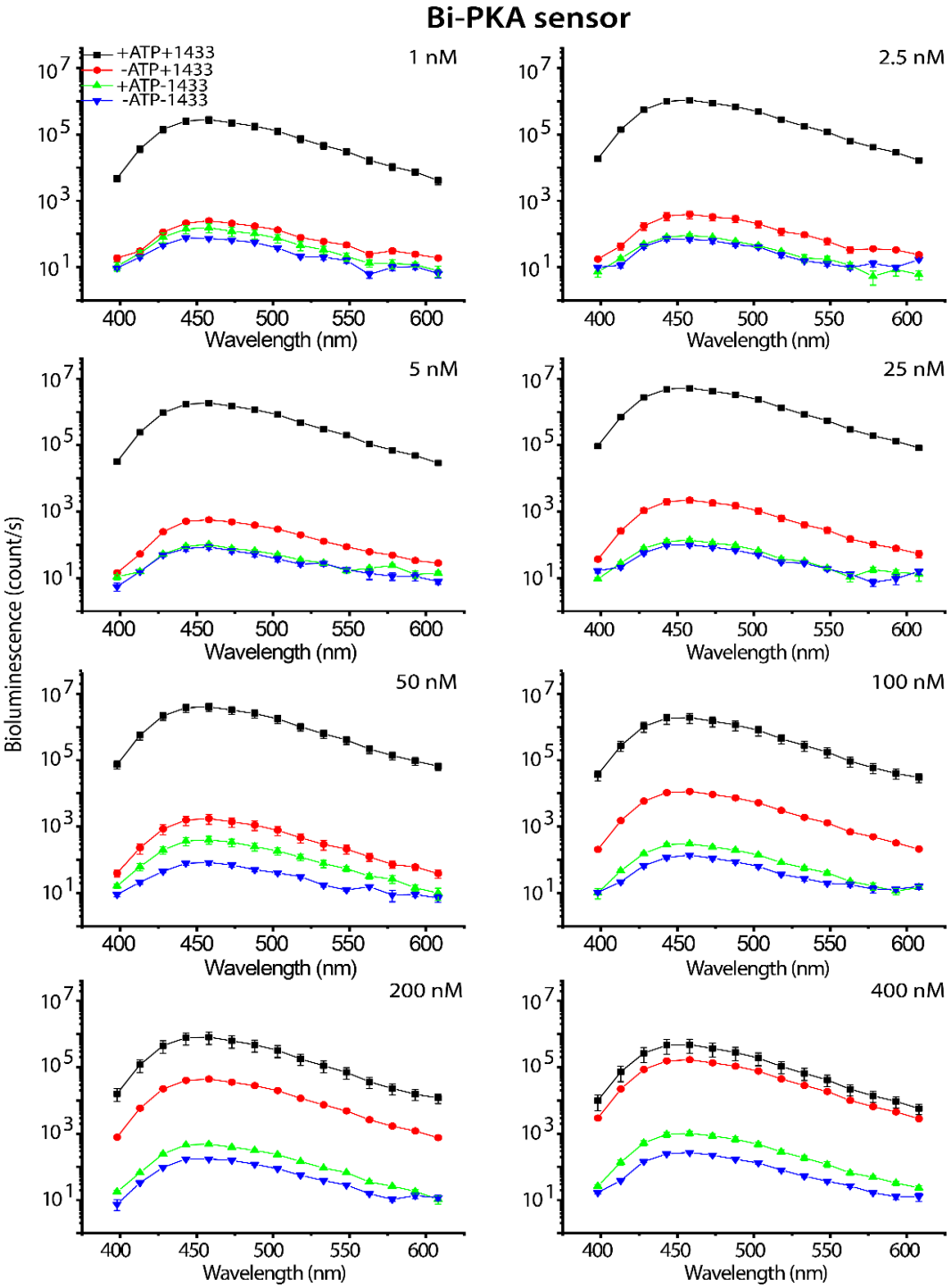

Figure S5 Activation assay of the reconstituted luciferase based on the **Bi-PKA** sensor showing the bioluminescence intensity for different wavelengths. Phosphorylated (+ATP) or unphosphorylated (-ATP) LgNB-biPKA (1–400 nM) was incubated with/without the same concentration of 14-3-3–SmNB. Furimazine was used at a final dilution of 1:8000. Error bars represent the standard error of triplicates.

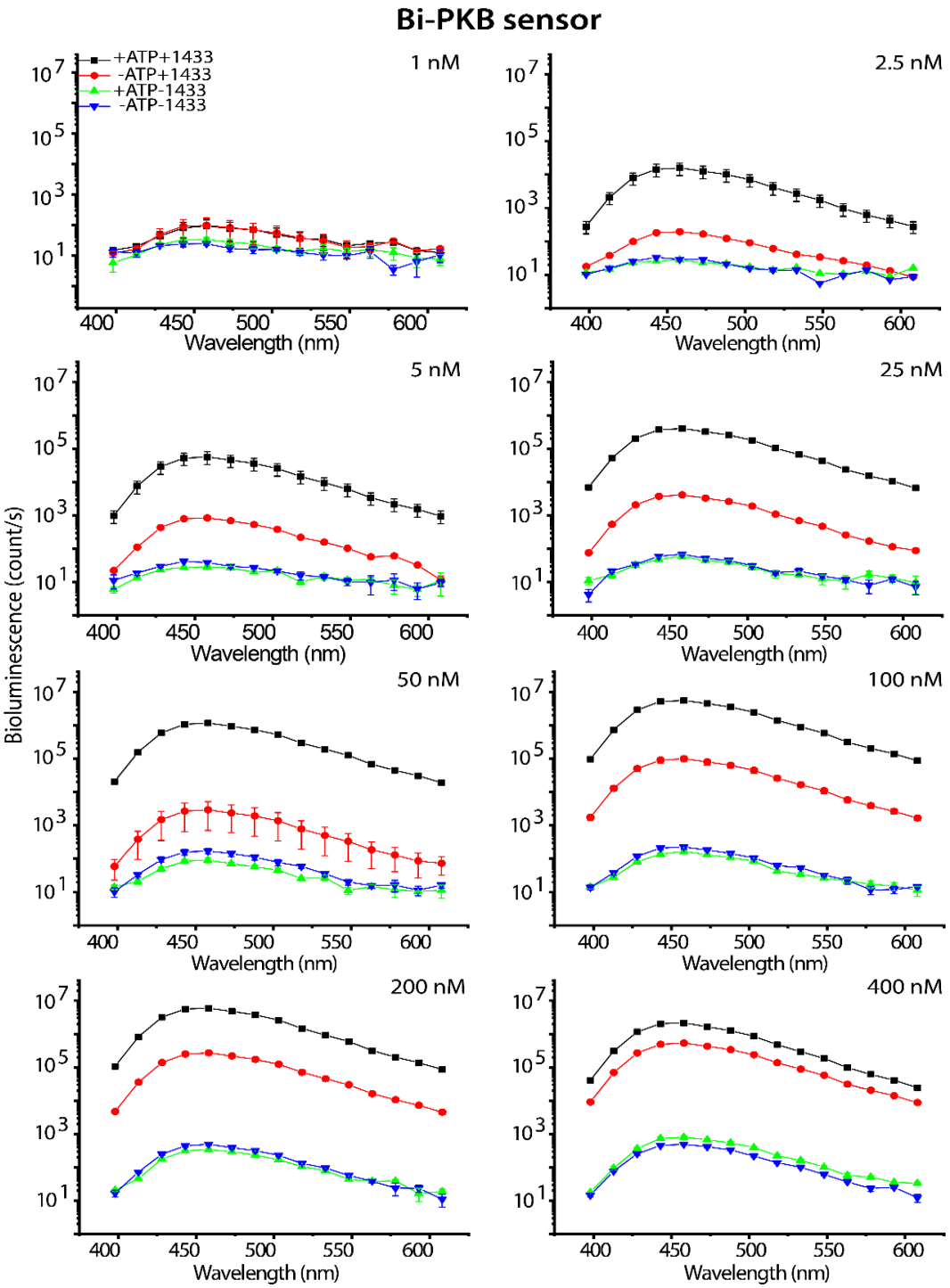

Figure S6 Activation assay of the reconstituted luciferase based on the **Bi-PKB** sensor showing the bioluminescence intensity for different wavelengths. Phosphorylated (+ATP) or unphosphorylated (-ATP) LgNB-biPKB (1–400 nM) was incubated with/without the same concentration of 14-3-3–SmNB. Furimazine was used at a final dilution of 1:8000. Error bars represent the standard error of triplicates.

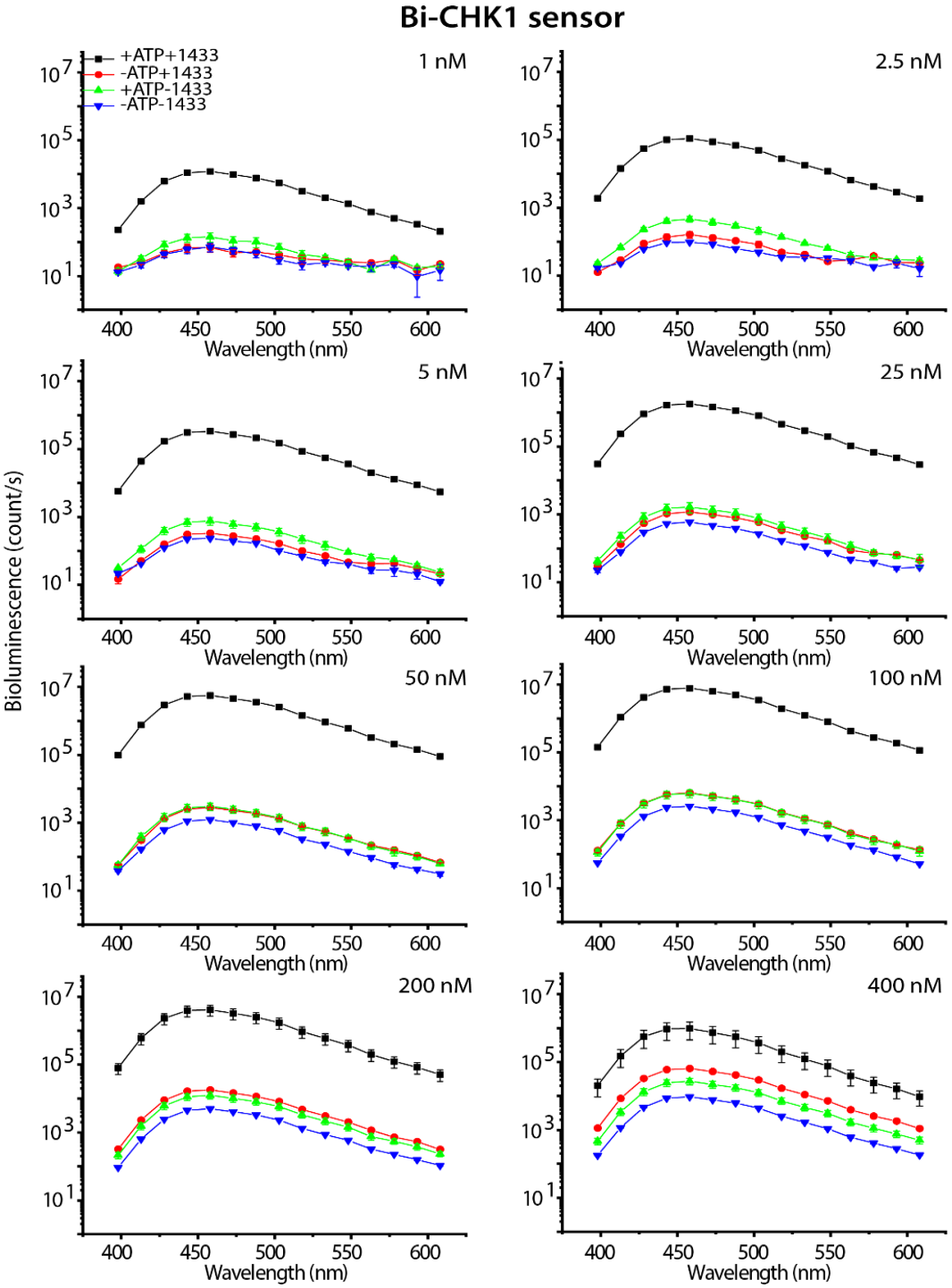

Figure S7 Activation assay of the reconstituted luciferase based on the **Bi-CHK1** sensor showing the bioluminescence intensity for different wavelengths. Phosphorylated (+ATP) or unphosphorylated (-ATP) LgNB-biCHK1 (1–400 nM) was incubated with/without the same concentration of 14-3-3–SmNB. Furimazine was used at a final dilution of 1:8000. Error bars represent the standard error of triplicates.

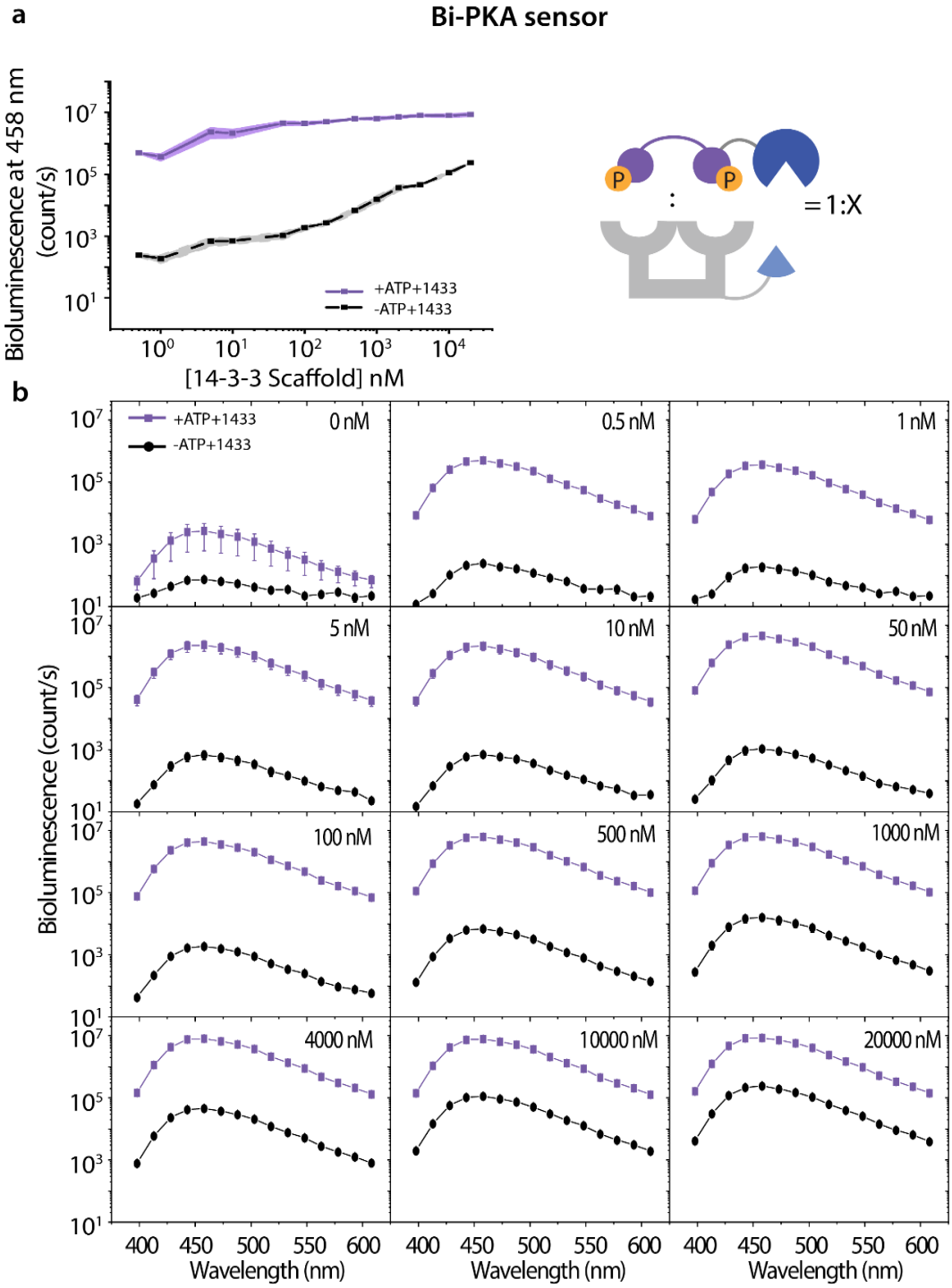

Figure S8 Titration of the 14-3-3–SmNB scaffold to constant concentrations of LgNB-biPKA. (a) LgNB-biPKA (+ATP, 10 nM) was incubated with various concentrations of 14-3-3–SmNB (0 nM – 20 µM). The unphosphorylated peptides (-ATP) were used as control. The furimazine was used at a final dilution of 1:8000. Error bars represent the standard error of triplicates. (b) Bioluminescence spectra from 375 to 625 nm.

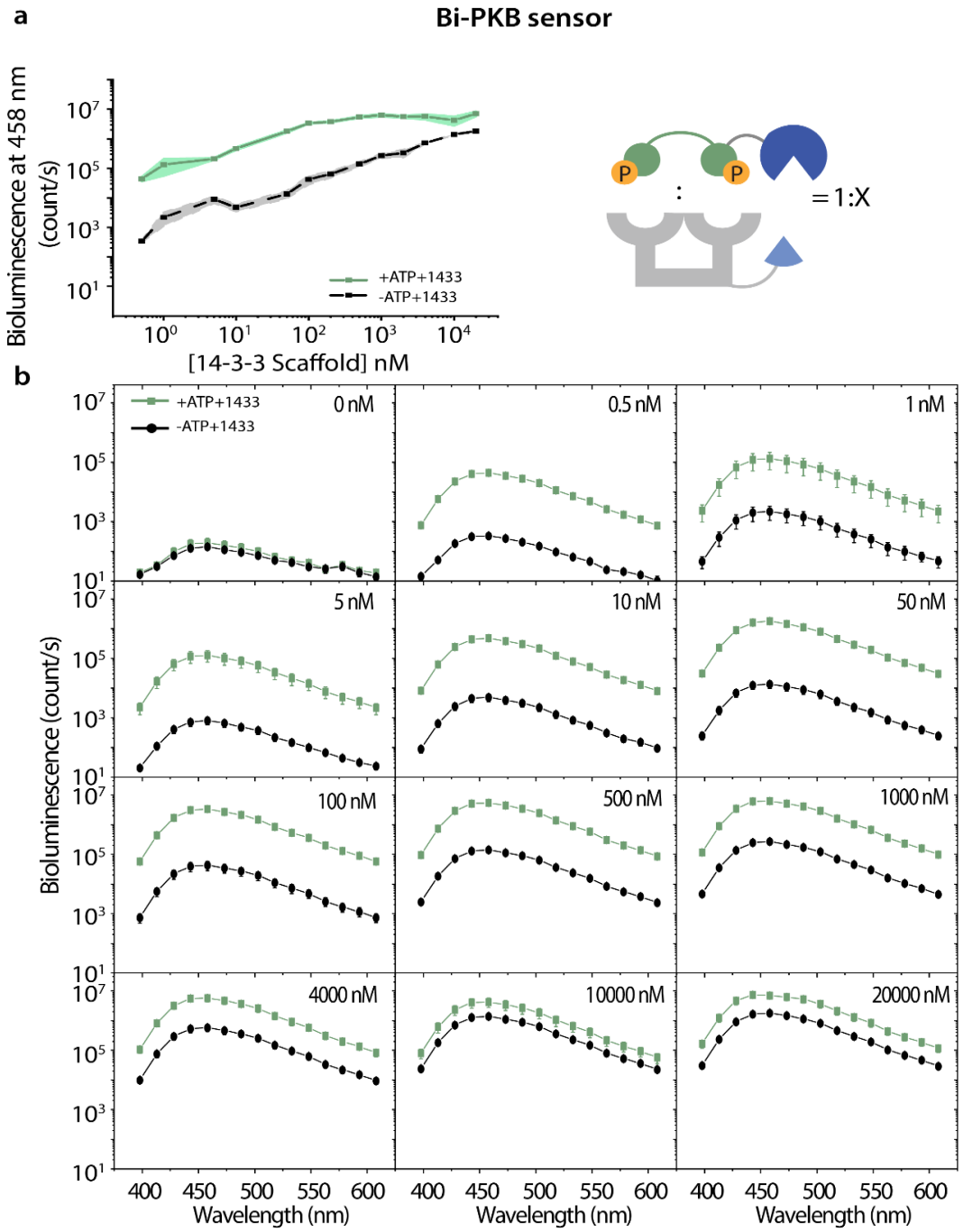

Figure S9 Titration of the 14-3-3–SmNB scaffold to constant concentrations of LgNB-biPKB. (a) LgNB-biPKB (+ATP, 50 nM) was incubated with various concentrations of 14-3-3–SmNB (0 nM – 20 µM). The unphosphorylated peptides (-ATP) were used as control. The furimazine was used at a final dilution of 1:8000. Error bars represent the standard error of triplicates. (b) Bioluminescence spectra from 375 to 625 nm.

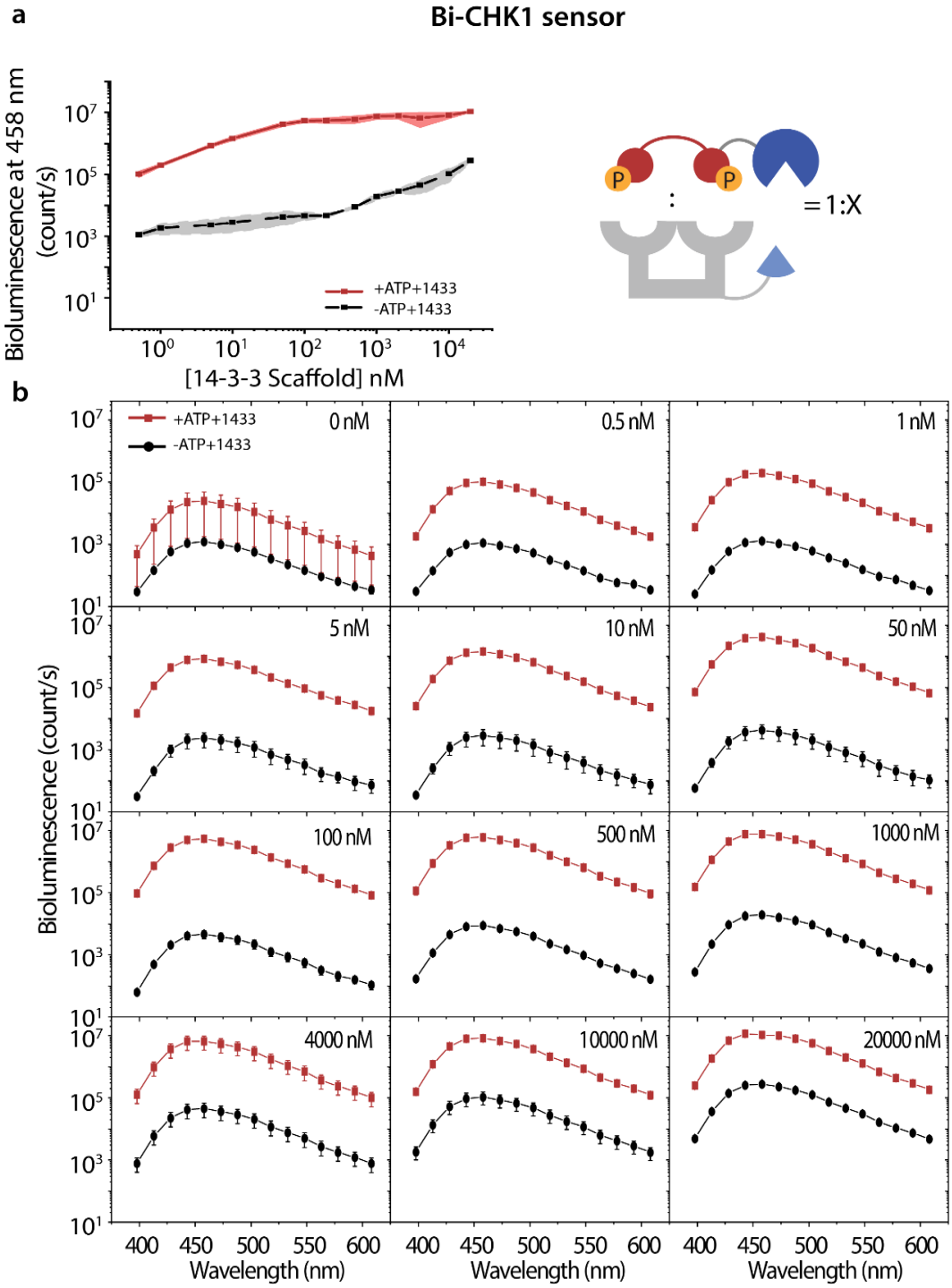

Figure S10 Titration of the 14-3-3–SmNB scaffold to constant concentrations of LgNB-biCHK1. (a) LgNB-biCHK1 (+ATP, 50 nM) was incubated with various concentrations of 14-3-3–SmNB (0 nM – 20 µM). The unphosphorylated peptides (-ATP) were used as control. The furimazine was used at a final dilution of 1:8000. Error bars represent the standard error of triplicates. (b) Bioluminescence spectra from 375 to 625 nm.

2.3 Time-responsiveness of bivalent sensors

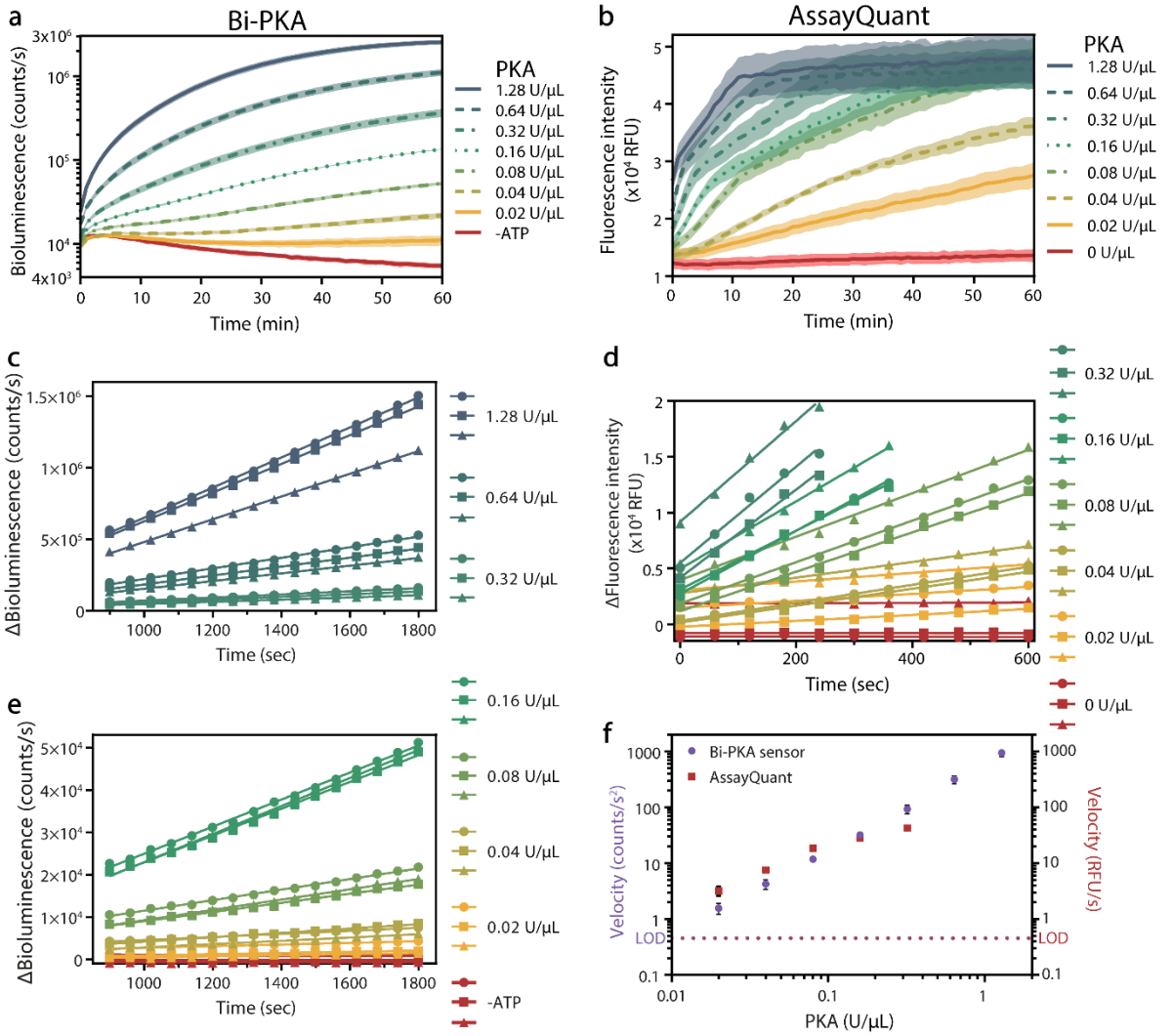

Figure S11 Time-responsiveness of bivalent sensor Bi-PKA and commercial kit AssayQuant PhosphoSens. (a) Bioluminescence intensity at 458 nm of Bi-PKA (10 nM) incubated with PKA (0.02 – 1.28 U/µL). Furimazine was used at a final dilution of 1:2000. Error clouds represent the standard error of triplicates. (b) Fluorescence intensity at 492(20) nm of AQT0458 (10 µM, AssayQuant) incubated with PKA (0.02 – 1.28 U/µL). Error clouds represent the standard error of triplicates. (c,e) Linear regression of background (-ATP) subtracted Bi-PKA traces. (d) Linear regression of background (0 U/µL) subtracted AssayQuant PhosphoSens traces. Note: the traces for 1.28 and 0.64 U/µL PKA are not taken along, due to limited datapoints in the initial linear part of the traces. (f) Velocity upon phosphorylation varying PKA concentrations (0.02-1.28 and 0.02-0.32 U/µL, respectively). Same as Figure 4b, smaller symbols show error bars, which represent the standard deviation of triplicates. The limit of detection (LOD) is defined as three times the standard deviation of the background.

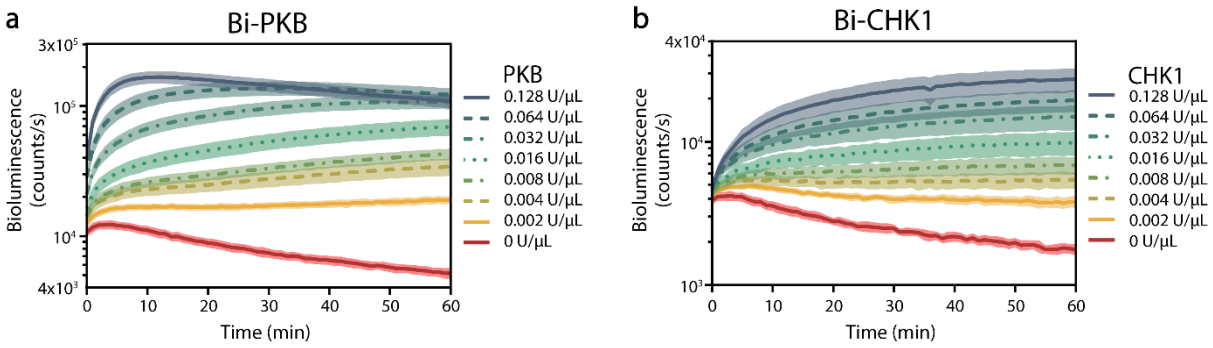

Figure S12 Time-responsiveness of bivalent sensors Bi-PKB and Bi-CHK1. Bioluminescence intensity at 458 nm of Bi-PKB (a, 10 nM) and Bi-CHK1 (b, 10 nM) incubated with their respective kinase (0 – 0.128 U/µL). Furimazine was used at a final dilution of 1:2000. Error clouds represent the standard error of triplicates.

2.4 Bi-PKA application in *E. coli* lysate

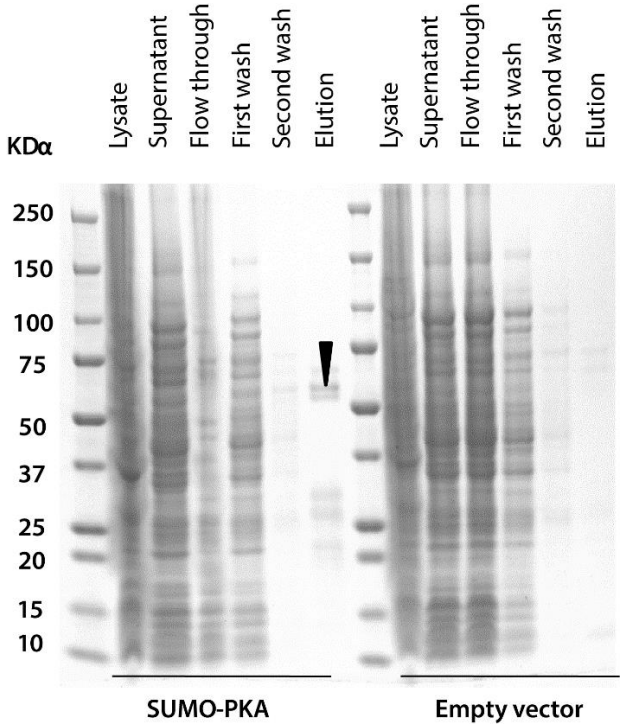

Figure S13 SDS-PAGE gel showing the expression and purification of SUMO-PKA (left). As a control, the process using the same pET28a vector without insert (right) is shown. The arrow indicates the expressed PKA with an expected mass at 54 kDa.

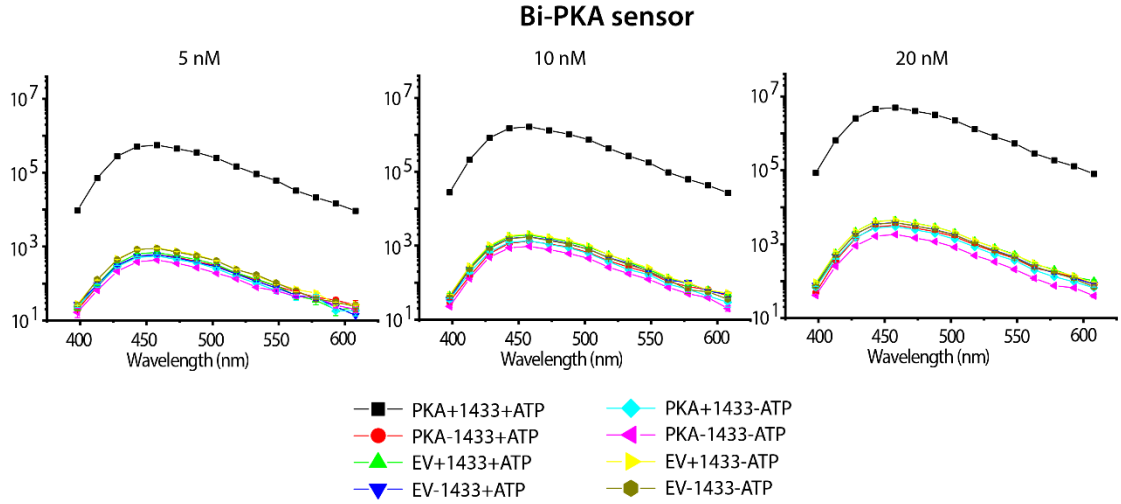

Figure S14 Bioluminescence spectra of the **Bi-PKA** sensor applied in *E. coli* cell lysate. LgNB-biPKA (5 nM, 10 nM or 20 nM) was pre-incubated with 5 µL of cell lysate containing overexpressed PKA in the presence (+ATP) or absence (-ATP) for 3 hours. Then the pre-incubated sample was incubated with or without the same amount of 14-3-3 dimer. Cell lysate containing empty vector (EV) was used as control. The substrate furimazine was used at a final dilution of 1:4000. Error bars represent the standard error of triplicates.

2.5 Bi-PKA application in HEK293T lysate

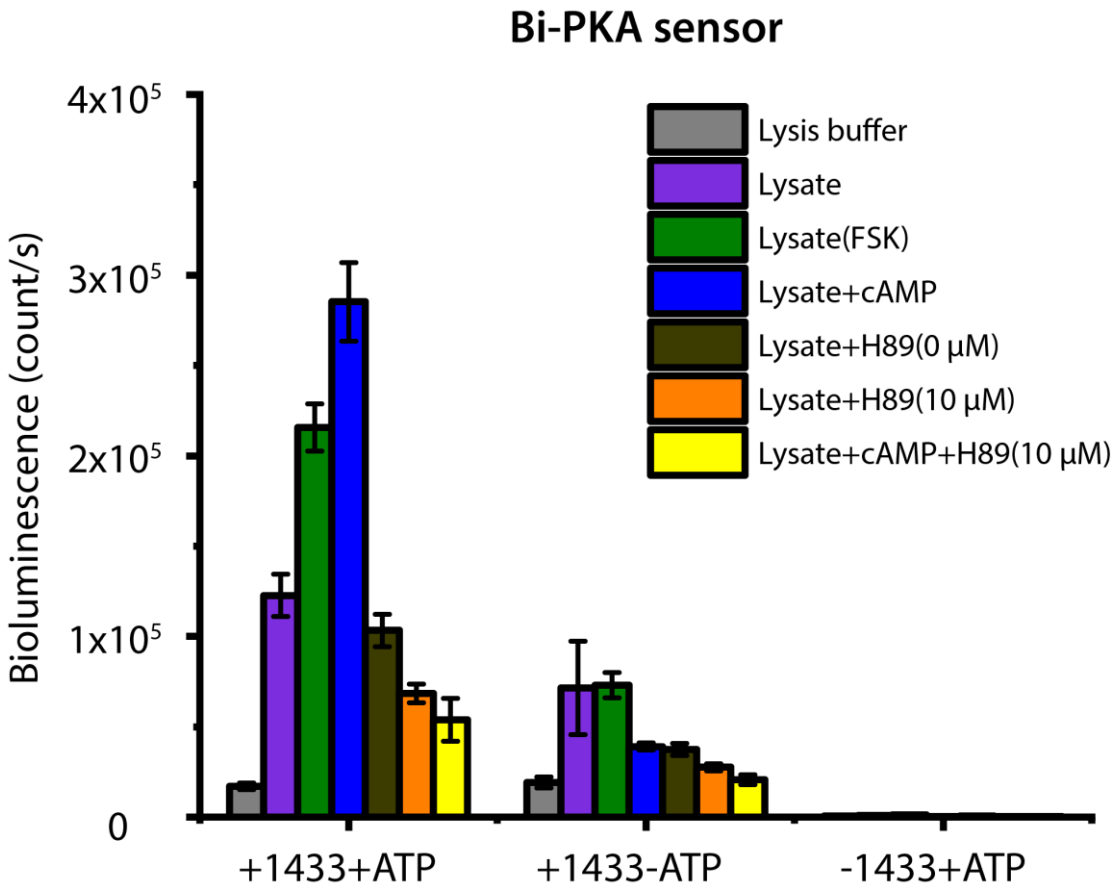

Figure S15 Bioluminescent intensity at 458 nm of the **Bi-PKA** sensor was applied in human cell lysate. 293T cells were treated with Forskolin (FSK, 100 μM) or vehicle (1% DMSO) for 1 h before cell lysis. LgNB-biPKA (25 nM) was incubated with cell lysate in the presence/absence of 14-3-3–SmNB (25 nM) and ATP (200 μM). Additionally, the PKA inhibitor H89 (10 μM), the PKA activator cAMP (2 mM) or vehicle (0 μM, 1% DMSO) were added. Lysis buffer was used to detect the background. The substrate furimazine was used at a final dilution of 1:2000. Error bars represent the standard error of triplicates.

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