

Electronic Supporting Information

Discovery and characterization of a novel class of cyclic peptidic compounds inhibiting the subunit interaction of the protein kinase CK2 $\alpha_2\beta_2$ holoenzyme

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1. Figures and Tables mentioned in the article

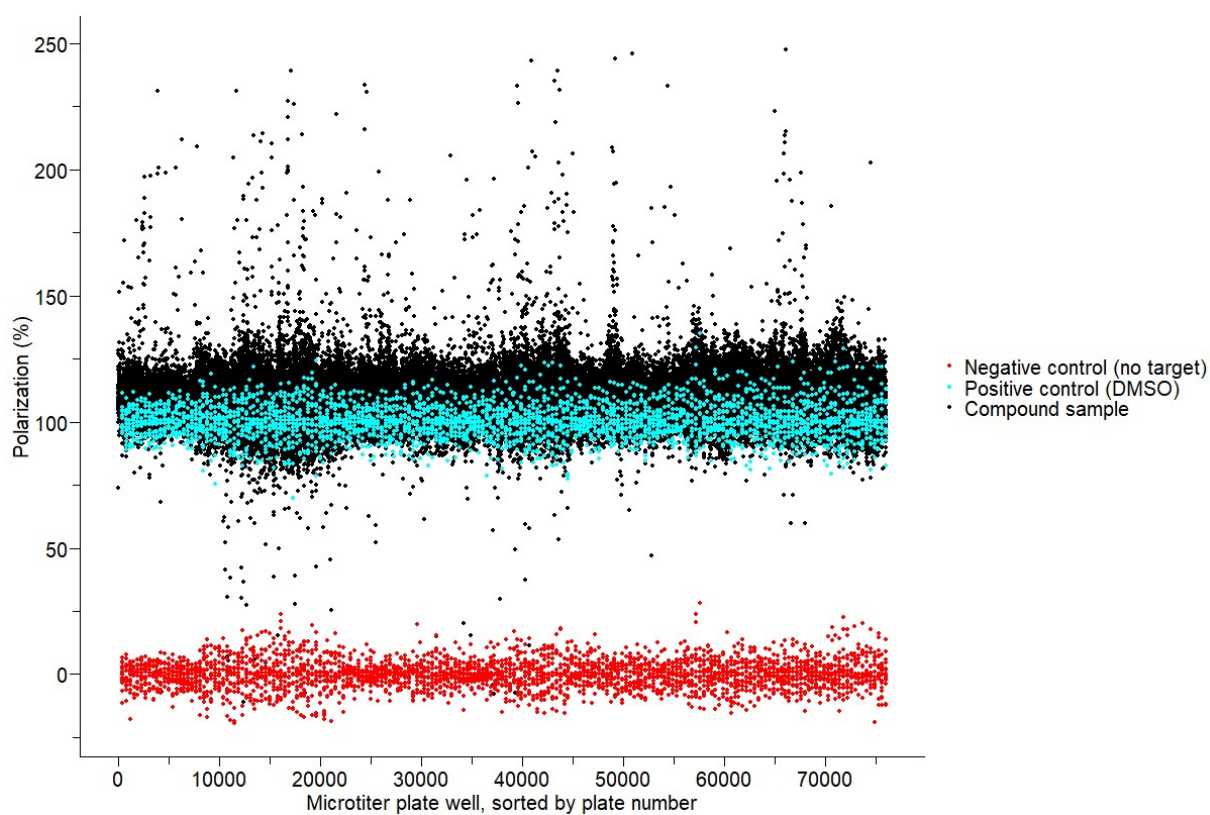
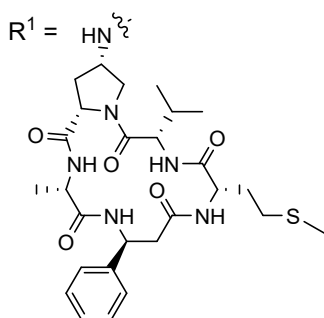
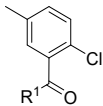
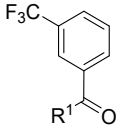
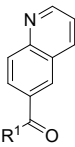
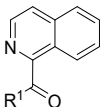
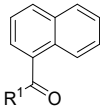
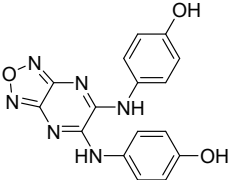


Figure S1. Scatter chart of percentage fluorescence polarization values of positive controls (CF-Ahx-Pc, CK2 α^{1-335} , and DMSO), negative controls (CF-Ahx-Pc and DMSO) and compound samples (CF-Ahx-Pc, CK2 α^{1-335} , and 20 μ M compound) from the primary screen of 67,584 compounds on 198 microplates (384-well format).

Table S1. Evaluation of hits identified in the high-throughput screening (Figure 2) by determination of the value IC_{50} for the displacement of CF-Ahx-Pc from $CK2\alpha^{1-335}$. The cyclic tridecapeptide I-Pc (Fig. 1b, $K_i = 0.158 \pm 0.051 \mu M$, mean value \pm SEM, $n = 3$)¹ served as a reference competitor to CF-Ahx-Pc. General structure of the nine cyclic pentapeptides **2**, **4**, **5**, **8**, **12**, **14**, **15**, **18** and **20** was as follows:



compd	structure	IC_{50} (μM) ^a	lower plateau ^b (<i>BOTTOM</i> , %)
I-Pc		0.885 ($K_i = 0.0669 \mu M$) ^c	2.79
FMP-109557 (5)		15.8	13.6
FMP-109594 (4)		19.1	19.3
FMP-109735 (20)		15.9	1.79×10^{-9}
FMP-109788 (2)		13.6	34.1

FMP-109957 (12)		2.67	4.28×10^{-7}
FMP-109959 (8)		9.13	7.69×10^{-6}
FMP-109969 (18)		9.88	17.4
FMP-110025 (15)		15.2	4.80×10^{-7}
FMP-110060 (14)		3.73	2.58×10^{-7}
FMP-203351		3.04	52.3 ^d

^a IC₅₀ values were obtained in a single duplicate experiment in 25 mM Tris-HCl, pH 8.5, 500 mM NaCl, 2% (v/v) DMSO, 0.05% (v/v) Tween 20 using 1 μM of CK2α¹⁻³³⁵, 0.1 μM of CF-Ahx-Pc, and 0.0391-20.0 μM (I-Pc) or 0.0488-50.0 μM (FMP compounds) of competitor. IC₅₀ values were calculated according to the four-parameter equation setting the value of *BOTTOM* to larger than zero.

^b The value of the upper plateau (*TOP*) obtained by the four-parameter equation was 94.8-105%.

^c The value of K_i was calculated according to Nikolovska-Coleska et al.² using a K_D value of 0.602 μM , obtained in a single duplicate experiment with 0.0391-20.0 μM of CK2 α^{1-335} and 0.1 μM of CF-Ahx-Pc in the absence of DMSO, and the “ K_i calculator” (http://www.umich.edu/~shaomengwanglab/software/calc_ki/index.html). When using 3 μM instead of 1 μM of CK2 α^{1-335} in the displacement assay, an IC_{50} value of 3.67 μM was obtained that gave a value of $K_i = 0.191 \mu\text{M}$. One sample t-tests comparing the reference K_i value of $0.158 \pm 0.051 \mu\text{M}$ ($n = 3$) for I-Pc with either of the two K_i values of 0.0669 and 0.191 μM yielded no significant differences with P values of 0.2160 and 0.5839, respectively.

^d FMP-203351 showed insufficient displacement of CF-Ahx-Pc from CK2 α^{1-335} (less than 50% even at high compound concentrations) and, therefore, was not further investigated herein.

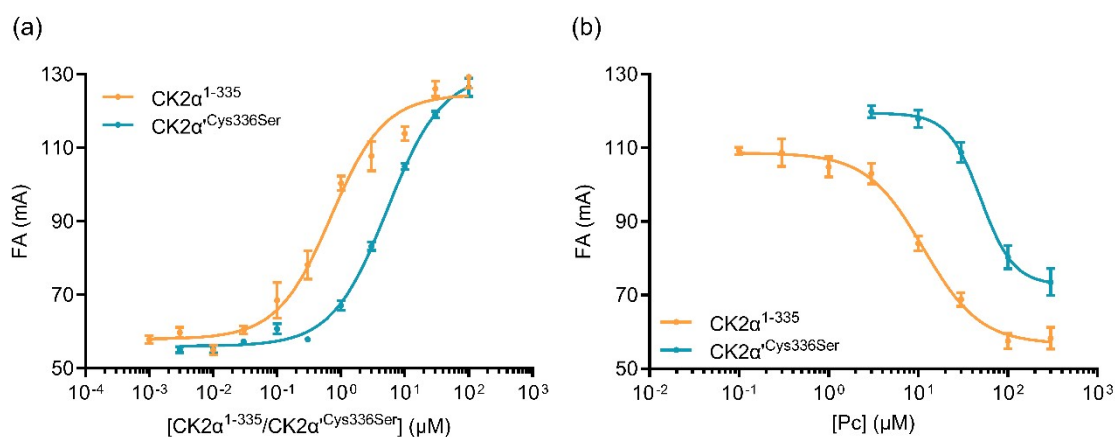


Figure S2. Binding of CF-Ahx-Pc to CK2α¹⁻³³⁵ or CK2α^{'Cys336Ser} (a) and displacement of CF-Ahx-Pc by unlabelled Pc (b) investigated on a Synergy™ 2 plate reader (BioTek, USA). All measurements were done in 25 mM Tris, 500 mM NaCl, pH = 8.5 using 0.1 μM of CF-Ahx-Pc and 0.001-100 μM CK2α¹⁻³³⁵ or 0.003-100 μM CK2α^{'Cys336Ser} for (a). (b) Experiments were done with 0.1 μM CF-Ahx-Pc and either 3 μM CK2α¹⁻³³⁵ and 0.1-300 μM Pc or 30 μM CK2α^{'Cys336Ser} and 3-300 μM Pc. CK2α¹⁻³³⁵ was investigated in a total volume of 50 μL in a 384-well plate format, whereas a total volume of 200 μL in a 96-well plate format was used for CK2α^{'Cys336Ser}. All data points shown are mean values ± SEM of three to four separate experiments, each performed in duplicate or triplicate. Analysis of data in (a) by nonlinear regression according to Hochscherf et al.³ resulted in K_D values of 0.659 ± 0.137 μM for CK2α¹⁻³³⁵ and 5.27 ± 0.60 μM for CK2α^{'Cys336Ser}, respectively. Displacement of CF-Ahx-Pc by Pc in (b) was quantified by means of the four-parameter equation, resulting in IC_{50} values of 11.6 ± 1.7 μM and 50.1 ± 3.1 μM for CK2α¹⁻³³⁵ and CK2α^{'Cys336Ser}, respectively. Coefficients of determination for all curve fits are provided in Table S4. K_i values of 1.7 ± 0.3 μM for CK2α¹⁻³³⁵ and 3.6 ± 0.5 μM for CK2α^{'Cys336Ser} were calculated according to Nikolovska-Coleska et al.² using the K_D values determined in (a) and the “ K_i calculator” (http://www.umich.edu/~shaomengwanglab/software/calc_ki/index.html).

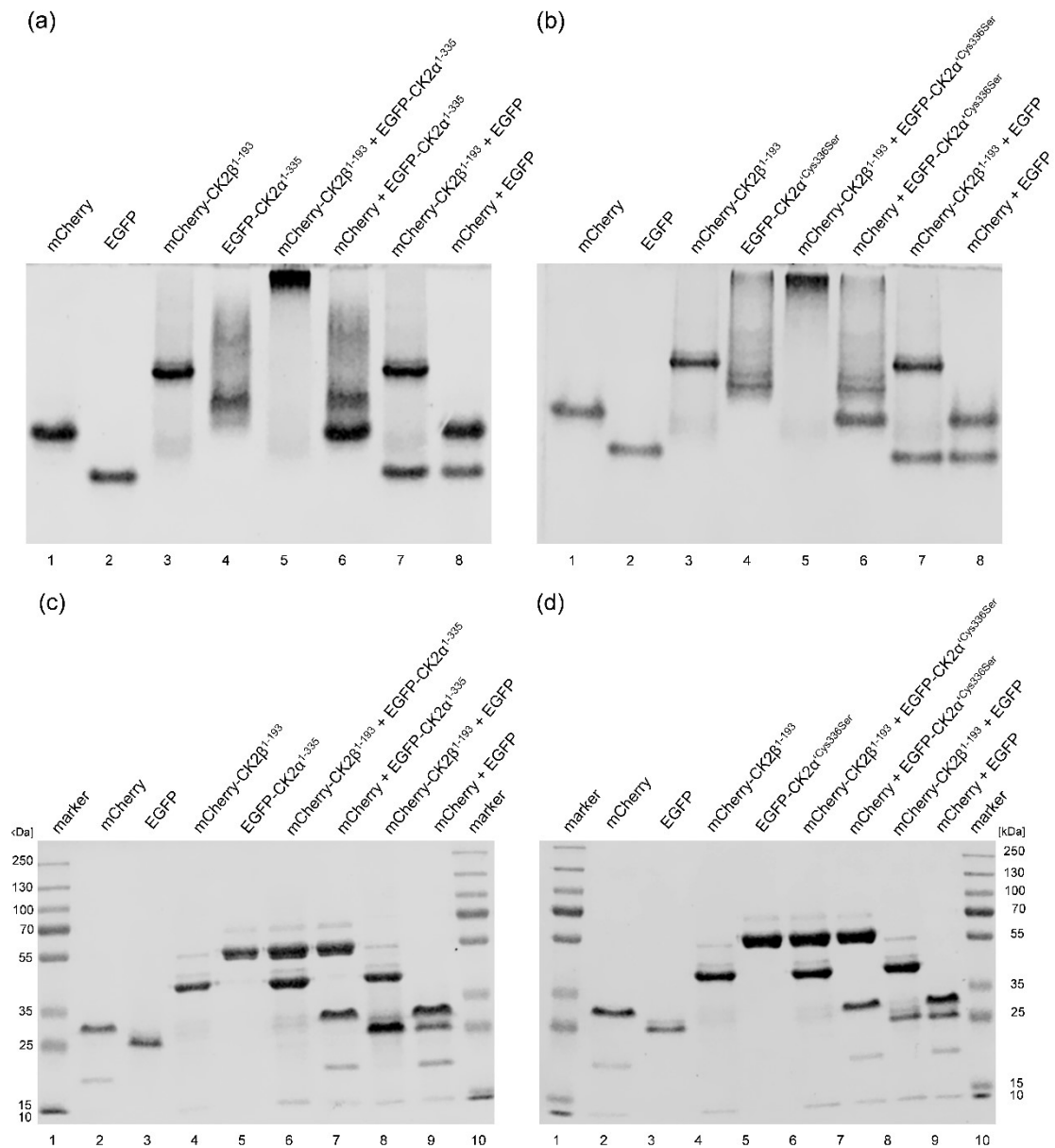


Figure S3. Protein-protein interactions visualized on Coomassie Brilliant Blue G250-stained 10% native PAGE gels (a, b) and on 10% SDS-PAGE gels (c, d). For all gels, 20 μ M of the respective protein (lane 1-4 in (a, b) and lane 2-5 in (c, d)) or protein combinations (lane 5-8 in (a, b) and lane 6-9 in (c, d)) were incubated at room temperature for 15 min in 25 mM Tris, 500 mM NaCl, pH 8.5 containing either native loading buffer (a, b) or SDS loading buffer (c, d). A volume of 5 μ L of the protein mixtures and 3 μ L of the marker PageRuler™ Plus Prestained Protein Ladder (lanes 1 and 10 in (c, d)) were loaded onto the gels and electrophoresis was performed at a constant voltage of 80 V for the native gels (a, b) or 120 V for the SDS gels (c, d). After staining, the gels were scanned on a LI-COR Odyssey Fc Imager (700 nm-channel).

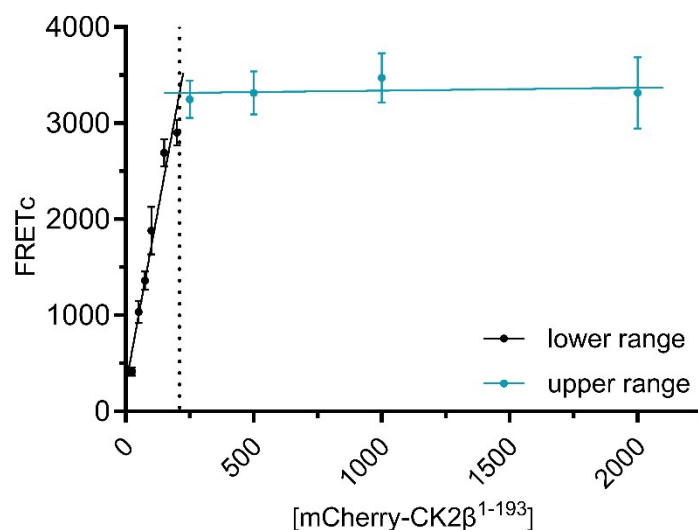


Figure S4. Titration of EGFP-CK2 α^{1-335} with mCherry-CK2 β^{1-193} . A fixed concentration of EGFP-CK2 α^{1-335} (200 nM) was incubated with mCherry-CK2 β^{1-193} (25-2000 nM) for 30 min at room temperature before FRETc was determined using an Infinite[®] M1000 PRO plate reader (Tecan Group, Switzerland). Three separate experiments were performed in duplicate in a total volume of 50 μ L on a 384-well plate. Conditions were as follows: 25 mM Tris, 500 mM NaCl, pH = 8.5, 2% (v/v) DMSO, 0.05% (v/v) Tween 20. Data points below (black) and above (cyan) the mCherry-CK2 β^{1-193} concentration of 200 nM were subjected to linear regression. The mCherry-CK2 β^{1-193} concentration able to bind to 200 nM of EGFP-CK2 α^{1-335} (x-value of the intersection point represented by a dashed line) was calculated to 210 ± 4 nM by the equation $x = (n_2 - n_1)/(m_1 - m_2)$, with m and n representing the slopes and the intercepts of the lower and upper range, respectively. Coefficients of determination for all curve fits are provided in Table S4.

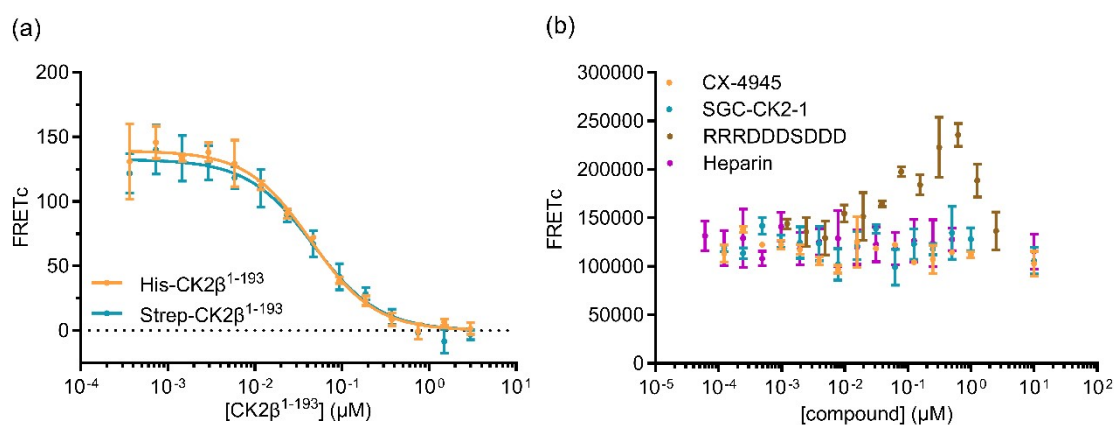
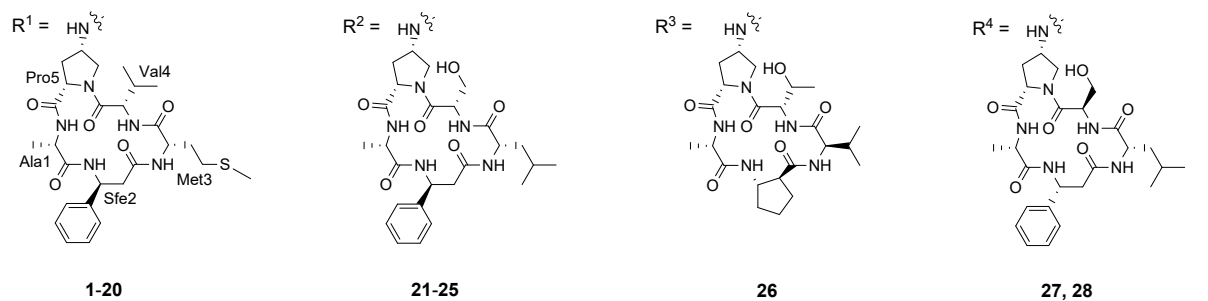











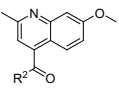



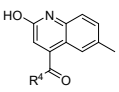



Figure S5. Investigations on the inhibition of binding of mCherry-CK2β¹⁻¹⁹³ to EGFP-CK2α¹⁻³³⁵ by tagged CK2β¹⁻¹⁹³ (a) and ATP or substrate site-directed compounds (b), respectively. Conditions were as follows: 25 mM Tris, 500 mM NaCl, pH = 8.5, 2% (v/v) DMSO, 0.05% (v/v) Tween 20. Three separate experiments in duplicate were conducted in a total volume of 50 μL in the 384-well plate format using 30 nM of EGFP-CK2α¹⁻³³⁵ and 50 nM of mCherry-CK2β¹⁻¹⁹³. Displacement of mCherry-CK2β¹⁻¹⁹³ was investigated in the presence of 3.66×10⁻⁴-3 μM His-CK2β¹⁻¹⁹³ or Strep-CK2β¹⁻¹⁹³ using an Infinite® M1000 PRO plate reader (Tecan Group, Switzerland) (a) or with 1.2×10⁻⁴-10 μM of CX-4945 or SGC-CK2-1, 0.0012-10 μM of RRRDDDSDDD and 6×10⁻⁵-10 μM Heparin, respectively, on a Synergy™ 2 plate reader (BioTek, USA) (b). IC₅₀ values in (a) were calculated according to the four-parameter equation, yielding 0.0444 ± 0.0088 μM and 0.0578 ± 0.0095 μM for His-CK2β¹⁻¹⁹³ and Strep-CK2β¹⁻¹⁹³, respectively. Coefficients of determination for all curve fits are provided in Table S4. K_i values of 0.0022 ± 0.0007 μM for His-CK2β¹⁻¹⁹³ and 0.0040 ± 0.0009 μM for Strep-CK2β¹⁻¹⁹³ were obtained by applying K_D values of 3.41 nM (SEM: 0.41 nM) and 4.43 nM (SEM: 1.17 nM), respectively, obtained in two independent sets of three triplicate experiments for the binding of mCherry-CK2β¹⁻¹⁹³ to EGFP-CK2α¹⁻³³⁵, in the “K_i calculator” (http://www.umich.edu/~shaomengwanglab/software/calc_ki/index.html).

Table S2. Characterization of cyclic pentapeptides with regard to binding to CK2 α^{1-335} and disturbing the interaction of mCherry-CK2 β^{1-193} with EGFP-CK2 α^{1-335} and EGFP-CK2 α^{1-335} Cys336Ser, respectively.



compd	structure	CK2 α^{1-335}		EGFP-CK2 α^{1-335}		EGFP-CK2 α^{1-335} Cys336Ser	
		IC ₅₀ (μM)	K _i (μM) ^a	IC ₅₀ (μM)	K _i (μM) ^a	IC ₅₀ (μM)	K _i (μM) ^a
1		> 300 ^b	> 70	~ 200 ^c	~ 15	> 200 ^b	> 30
2		35.7 \pm 3.3	8.0 \pm 0.8	33.6 \pm 1.4	2.5 \pm 0.1	> 100 ^b	> 15
3		> 100 ^b	> 23	~ 100 ^c	~ 7.5	> 100 ^b	> 15
4		89.4 \pm 12.8	21 \pm 3	42.6 \pm 12.4	3.2 \pm 0.9	> 20 ^b	> 3.0
5		50.0 \pm 7.4	11 \pm 2	16.0 \pm 4.2	1.6 \pm 0.4	96.8 \pm 10.9	14 \pm 2
6		43.3 \pm 0.3	9.8 \pm 0.1	32.7 \pm 10.2	2.5 \pm 0.8	110 \pm 20	16 \pm 3
7		62.1 \pm 8.8	14 \pm 2	103 \pm 9	7.7 \pm 0.7	> 20 ^b	> 3.0
8		22.4 \pm 2.6	4.9 \pm 0.6	15.8 \pm 1.9	1.2 \pm 0.1	58.6 \pm 9.1	8.7 \pm 1.4
9		10.9 \pm 1.6	2.2 \pm 0.4	10.6 \pm 2.8	0.80 \pm 0.21	41.3 \pm 9.5	6.1 \pm 1.4
10		12.4 \pm 0.6	2.5 \pm 0.1	7.83 \pm 2.42	0.59 \pm 0.18	> 20 ^b	> 3.0
11		11.0 \pm 0.6	2.2 \pm 0.2	6.56 \pm 0.46	0.49 \pm 0.03	34.5 \pm 7.1	5.1 \pm 1.1

12		10.2 ± 2.3	2.0 ± 0.5	7.60 ± 2.06	0.57 ± 0.16	30.0 ± 6.1	4.5 ± 0.9
13		5.50 ± 0.52	0.92 ± 0.12	1.99 ± 0.18	0.15 ± 0.01	16.3 ± 2.0	2.4 ± 0.3
14		5.79 ± 0.99	0.99 ± 0.23	4.33 ± 0.97	0.32 ± 0.07	57.3 ± 5.7	8.5 ± 0.8
15		13.3 ± 1.8	2.7 ± 0.4	9.88 ± 1.17	0.74 ± 0.09	$> 200^b$	> 30
16		11.2 ± 1.6	2.4 ± 0.4	13.4 ± 3.4	1.0 ± 0.3	74.6 ± 19.7	11 ± 3
17		39.0 ± 9.2	9.3 ± 2.3	20.3 ± 8.2	1.5 ± 0.6	$> 200^b$	> 30
18		$\sim 100^c$	~ 23	21.0 ± 3.1	2.1 ± 0.3	$> 100^b$	> 15
19		22.0 ± 0.3	5.1 ± 0.1	22.5 ± 2.0	1.7 ± 0.2	$> 100^b$	> 15
20		32.9 ± 11.4	7.3 ± 2.7	30.5 ± 2.7	2.3 ± 0.2	117 ± 28	17 ± 4
21		$> 100^b$	> 23	$> 100^b$	> 7.5	$> 100^b$	> 15
22		$> 100^b$	> 23	$> 100^b$	> 7.5	$> 100^b$	> 15
23		$> 100^b$	> 23	$> 100^b$	> 7.5	$> 100^b$	> 15
24		$> 300^b$	> 70	$> 200^b$	> 15	$> 100^b$	> 15
25		$> 300^b$	> 70	$> 200^b$	> 15	$> 100^b$	> 15
26		$> 300^b$	> 70	$> 200^b$	> 15	$> 100^b$	> 15
27		$> 300^b$	> 70	$> 200^b$	> 15	$> 100^b$	> 15
28		$> 300^b$	> 70	$> 200^b$	> 15	$> 100^b$	> 15

^a Dissociation constants K_i are the same as those in Table 3 and were calculated from the respective IC_{50} values (mean values \pm SEM, $n = 3-4$) obtained on a Synergy™ 2 plate reader (BioTek, USA) by means of the “ K_i calculator” (http://www.umich.edu/~shaomengwanglab/software/calc_ki/index.html) using K_D values of protein preparations of 0.609 μ M (CK2 α^{1-335}), 3.28 nM (EGFP-CK2 α^{1-335}) and 7.90 nM (EGFP-CK2 $\alpha^{Cys336Ser}$), respectively. ^b Less than 50% inhibition was observed at the highest compound concentration investigated. ^c About 50% inhibition was observed at the highest compound concentration investigated.

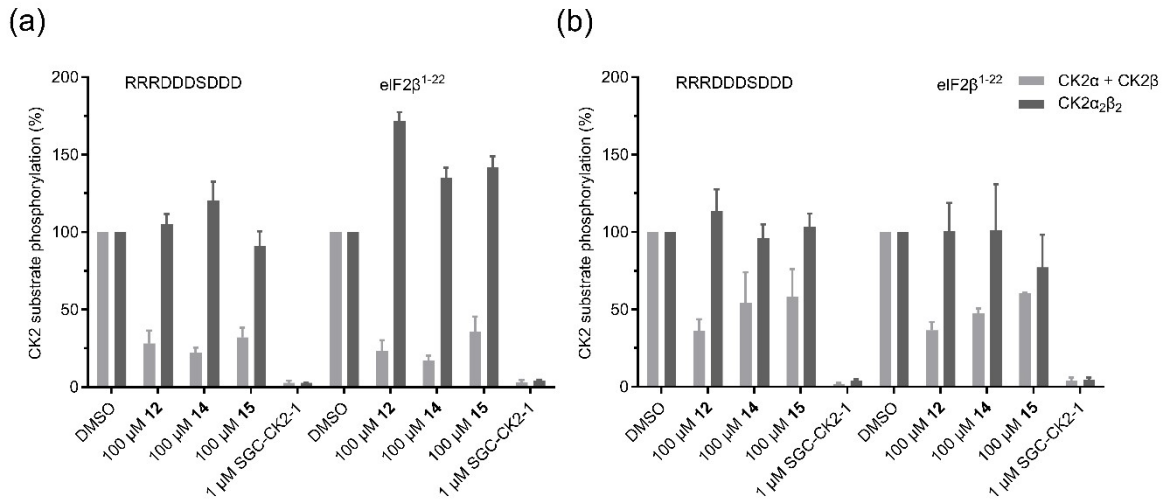


Figure S6. Determination of the kinase activity of full-length CK2α in presence of full-length CK2β (light grey bars) and CK2α₂β₂ holoenzyme (dark grey bars) by analysing the phosphorylation of the peptides RRRDDDSDDD (class I substrate, left) and eIF2β¹⁻²² (class III substrate, right) by γ -³²P-ATP starting either directly (a) or after 10 min of incubation of inhibitors with protein at room temperature (b). Measurements were done using the following conditions: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT. For experiments using the individual subunits (light grey bars), a volume of 1 μL of 5 mM cyclic pentapeptide or 0.05 mM SGC-CK2-1 in DMSO was added to CK2α (final concentration of 90 nM). The same concentration of CK2β was added to the mixture after 1-2 min of incubation at room temperature. For experiments with the CK2 holoenzyme, a final concentration of 10 nM CK2α₂β₂ was used. The reaction was started either immediately or after 10 min of incubation at room temperature by addition of a mixture of substrate peptide (0.19 mM RRRDDDSDDD or eIF2β¹⁻²²) and ATP (20 μCi mL⁻¹ γ -³²P-ATP and 50 μM ATP). Measurements in the presence of DMSO and the reported ATP site-directed CK2 inhibitor SGC-CK2-1⁴ served as negative and positive controls, respectively. Depicted are mean values \pm SEM of five (a) or two (b) experiments, each performed in duplicate.

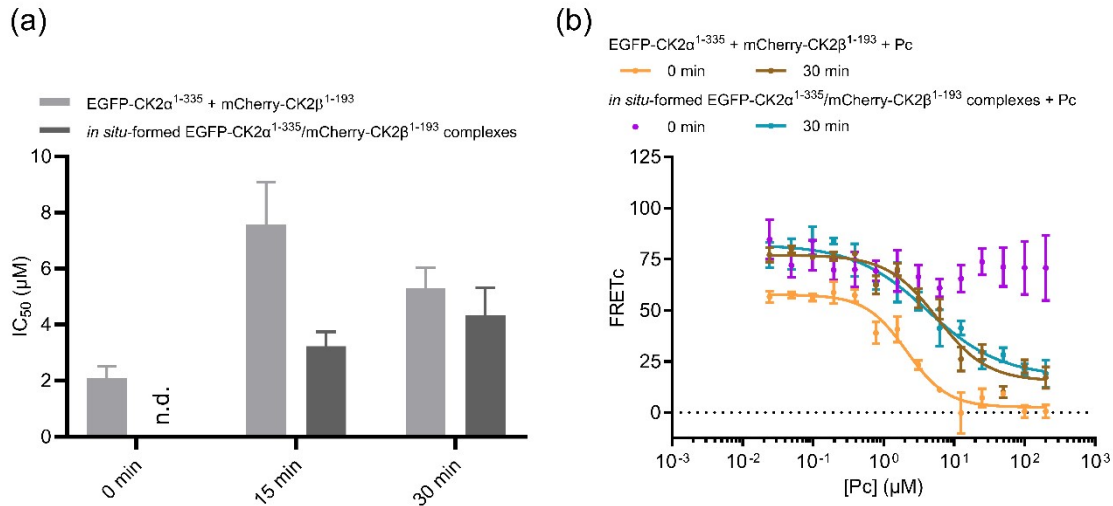


Figure S7. Inhibition of binding of mCherry-CK2 β^{1-193} to EGFP-CK2 α^{1-335} and dissociation of pre-formed EGFP-CK2 α^{1-335} /mCherry-CK2 β^{1-193} complexes by Pc. Results shown in Figure S7a have also been provided in Figure 3e. Three separate experiments were performed in duplicate using the following conditions: 25 mM Tris, 500 mM NaCl, pH = 8.5, 2% (v/v) DMSO, 0.05% (v/v) Tween 20. Experiments were done in a total volume of 50 μ L using a 384-well-plate. Either the individual subunits (75 nM each) or a mixture of both subunits (37.5 nM each) were incubated for 30 min at 30 °C. After incubation, the subunits or the mixture were added to Pc to obtain a final concentration of the individual subunits or the complex of 30 nM each and displacement of mCherry-CK2 β^{1-193} from EGFP-CK2 α^{1-335} was investigated in the presence of 0.024-200 μ M Pc using an Infinite® M1000 PRO plate reader (Tecan Group, Switzerland). FRET was investigated either directly after addition of the subunits and the mixture, respectively, or after an incubation period of 15 or 30 min. IC₅₀ values in (a) were calculated according to the four-parameter equation, yielding 2.08 ± 0.43 μ M (0 min), 7.56 ± 1.53 μ M (15 min) and 5.30 ± 0.73 μ M (30 min) for the individual subunits (light grey bars). Disruption of *in situ*-formed EGFP-CK2 α^{1-335} /mCherry-CK2 β^{1-193} complexes by Pc (dark grey bars in (a)) was quantified by the following IC₅₀ values: 3.23 ± 0.52 μ M (15 min), 4.33 ± 0.99 μ M (30 min). Dose-response curves of the displacement of mCherry-CK2 β^{1-193} by Pc after 0 and 30 min are shown in (b). No disruption of the EGFP-CK2 α^{1-335} /mCherry-CK2 β^{1-193} complex was observed when the pre-incubated mixture was added to Pc and FRET was immediately investigated

after addition (purple curve, (b)). Coefficients of determination for all curve fits are provided in Table S4.

Table S3. X-ray diffraction data and refinement statistics of CK2 α^{1-335} and CK2 $\alpha^{\text{Cys336Ser}}$ complex crystals

Crystallized complex	CK2 $\alpha^{\text{Cys336Ser}}$ with cpd. 12	CK2 α^{1-335} with cpd. 12	CK2 α^{1-335} with cpd. 15
PDB code	9FBI	9FBM	9FBL
DOI access of raw X-ray diffraction data	10.15151/ESRF-ES-1007017045	10.15151/ESRF-ES-1317816410	10.15151/ESRF-ES-1118696478
<i>X-ray diffraction data quality</i>			
Wavelength	0.8856 Å	0.9677 Å	0.8856 Å
Synchrotron (beamline)	ESRF (ID30B) ⁵	ESRF (ID30A-3) ⁶	ESRF (ID23-1) ⁷
Space group	P2 ₁	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2
Cell dimensions			
a, b, c [Å]	46.452, 71.068, 102.878	78.016, 208.360, 76.718	77.952, 206.56, 76.772
α , β , γ [°]	90, 91.967, 90	90, 90, 90	90, 90, 90
Resolution [Å] (highest resolution shell) [#]	58.462 - 1.161 (1.329-1.161)	76.718 - 2.054 (2.182-2.054)	72.931 - 2.176 (2.403-2.176)
R_{sym} [%] [#]	8.1(127.5)	56.2 (664.1)	47.9 (839.7)
CC1/2 [#]	0.906 (0.535)	0.994 (0.563)	0.990 (0.502)
Signal-to-noise ratio (I/σ) [#]	7.2 (1.8)	9.6 (1.5)	8.1 (1.6)
No. of unique reflections [#]	119426 (5971)	68956 (3448)	39662 (1983)
Completeness (spherical) [%] [#]	52.0 (7.8)	87.5 (26.8)	60.2 (11.9)
Completeness (ellips.) [%] ^{§, #}	86.0 (62.0)	94.7 (48.3)	93.6 (68.1)
Multiplicity [#]	4.9 (6.3)	35.3 (37.4)	17.6 (17.7)
Wilson-plot B-factor [Å ²]	13.04	21.56	38.94
<i>Refinement and structure quality</i>			
No. of refl. for $R_{\text{work}}/R_{\text{free}}$	118163/1205	67912/1023	38434/1186
$R_{\text{work}}/R_{\text{free}}$ [%]	0.1452/0.1847	0.1847/0.2388	0.2068/0.2644
Protomers per asymmetric unit	2	3	3
No. of non-H-atoms	6236	9450	8840
Protein	5634	8435	8397
Ligand/ion	117	226	246
Water	485	789	197
Average B-Factors [Å ²]	20.49	32.82	51.90
Protein	19.44	32.67	51.81
Ligand/ion	29.13	39.03	66.25
Water	30.67	32.65	37.87
RMS deviations			
Bond lengths [Å]	0.013	0.007	0.002
Bond angles [°]	1.16	0.90	0.48
Ramachandran plot			
Favored [%]	96.93	96.76	96.14
Allowed [%]	2.91	3.14	3.66
Outliers [%]	0.15	0.10	0.20

[#]Values in brackets refer to the highest resolution shell.

[§]According to anisotropic analysis with STARANISO⁸

Table S4. Coefficients of determination (R^2) for data analyses depicted in the main text and the Electronic Supporting Information. Mean values of $R^2 \pm$ SEM from three to four separate experiments, each performed in duplicate to triplicate, are listed.

Figure	Condition or compound	R^2
3a	w/ DMSO & Tween 20 (EGFP-CK2 α^{1-335})	0.935 \pm 0.012
	w/ DMSO (EGFP-CK2 α^{1-335})	0.884 \pm 0.051
	w/o DMSO & Tween (EGFP-CK2 α^{1-335})	0.903 \pm 0.011
	w/ DMSO & Tween 20 (EGFP-CK2 α^{C336S})	0.931 \pm 0.013
	w/ DMSO (EGFP-CK2 α^{C336S})	0.955 \pm 0.010
	w/ Tween (EGFP-CK2 α^{C336S})	0.979 \pm 0.004
	w/o DMSO & Tween (EGFP-CK2 α^{C336S})	0.949 \pm 0.013
4	CK2 β^{1-193} (EGFP-CK2 α^{1-335})	0.884 \pm 0.009
	I-Pc (EGFP-CK2 α^{1-335})	0.877 \pm 0.023
	Pc (EGFP-CK2 α^{1-335})	0.961 \pm 0.017
	Pc (EGFP-CK2 α^{C336S})	0.858 \pm 0.011
5a	12	0.896 \pm 0.011
	13	0.853 \pm 0.029
	14	0.819 \pm 0.011
	15	0.798 \pm 0.009
5b	12	0.815 \pm 0.038
	13	0.799 \pm 0.017
	14	0.664 \pm 0.074
	15	/
5c	12	0.966 \pm 0.006
	13	0.976 \pm 0.0002
	14	0.963 \pm 0.003
	15	0.926 \pm 0.019
5f	EGFP-CK2 α^{1-335} + mCherry-CK2 β^{1-193} + 12 (0 min)	0.862 \pm 0.024
	EGFP-CK2 α^{1-335} + mCherry-CK2 β^{1-193} + 12 (30 min)	0.848 \pm 0.031
	<i>in situ</i> -formed EGFP-CK2 α^{1-335} /mCherry-CK2 β^{1-193} complexes + 12 (0 min)	/
	<i>in situ</i> -formed EGFP-CK2 α^{1-335} /mCherry-CK2 β^{1-193} complexes + 12 (30 min)	0.884 \pm 0.031
S2a	CK2 α^{1-335}	0.959 \pm 0.007
	CK2 α^{C336S}	0.988 \pm 0.006
S2b	CK2 α^{1-335}	0.954 \pm 0.004
	CK2 α^{C336S}	0.983 \pm 0.007
S4	Lower range	0.938 \pm 0.012
	Upper range	0.236 \pm 0.082
S5a	His-CK2 β^{1-193}	0.929 \pm 0.027
	Strep-CK2 β^{1-193}	0.955 \pm 0.008
S7b	EGFP-CK2 α^{1-335} + mCherry-CK2 β^{1-193} + Pc (0 min)	0.843 \pm 0.020
	EGFP-CK2 α^{1-335} + mCherry-CK2 β^{1-193} + Pc (30 min)	0.867 \pm 0.011
	<i>in situ</i> -formed EGFP-CK2 α^{1-335} /mCherry-CK2 β^{1-193} complexes + Pc (0 min)	/
	<i>in situ</i> -formed EGFP-CK2 α^{1-335} /mCherry-CK2 β^{1-193} complexes + Pc (30 min)	0.857 \pm 0.010

2. Experimental Section

2.1 Plasmid construction

Genetic information of EGFP and mCherry embedded in the pIRES plasmid and lentiCRISPR v2 plasmid, respectively, was cloned into the vector pPSG IBA 105 (IBA-Lifesciences, Göttingen, Germany) using the Esp3I restriction sites. To generate the respective CK2 construct, genetic information of CK2 α^{1-335} (pET22b(+)), CK2 $\alpha^{\text{Cys336Ser}}$ (pET-28a(+), Gene Universal, Newark, DE, USA) or CK2 β^{1-193} (pT7-7) was cloned into the respective plasmid using BamHI and SacI for orientation. Successful cloning was verified by sequencing. The three CK2 constructs contained an N-terminal Twin-Strep-Tag followed by EGFP or mCherry, which is connected to the respective CK2 subunit via a GGDGGGGG and a GGGGGGGGEL linker, respectively. The fusion proteins are referred to as EGFP-CK2 α^{1-335} , EGFP-CK2 $\alpha^{\text{Cys336Ser}}$ and mCherry-CK2 β^{1-193} , respectively.

2.2 Protein expression and purification

For crystallization experiments, CK2 α^{1-335} and CK2 $\alpha^{\text{Cys336Ser}}$ – both provided with an N-terminal (His)₆-tag – were prepared as described by Werner et al.^{9, 10}. N-terminal (His)₆-tagged CK2 α^{1-335} , which was used for compound library screening and all FA experiments with that CK2 subunit, was expressed and purified as reported by Lindenblatt et al.¹.

(His)₆-tagged CK2 $\alpha^{\text{Cys336Ser}}$ was prepared following Werner et al.¹⁰ but with some alterations as detailed below. Cells expressing CK2 $\alpha^{\text{Cys336Ser}}$ were pre-cultured at 37 °C overnight in 50 mL of LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) containing 100 $\mu\text{g}/\text{mL}$ ampicillin. Main culture (600 mL) was treated as described but cells were grown at 37 °C for 3h instead at 18 °C for 18 h. Cells were then centrifuged (4 °C, 7000 \times g) and washed with ice-cold 0.9% NaCl containing 0.2 mM PMSF and the pellet was stored at -80 °C. Cells were lysed as described but in the presence of 300 mM instead of 500 mM NaCl. Purification of CK2 $\alpha^{\text{Cys336Ser}}$ was done using 50 mM HEPES, pH 7.5 with 300 mM NaCl, 10 mM imidazole, 5% (v/v) glycerol 0.5 mM TCEP for equilibrating and washing the column (5 mL HisTrap FF

column (Cytiva, Marlborough, MA, USA)), whereas the same buffer but with a gradient from 10 to 250 mM imidazole was used for elution of CK2 α '^{Cys336Ser}.

Expression of untagged CK2 β ¹⁻¹⁹³, (His)₆-tagged CK2 β ¹⁻¹⁹³, CK2 β ¹⁻¹⁹³ tagged with Strep-tag[®] II and the Twin-Strep-tagged[®] proteins EGFP, mCherry, EGFP-CK2 α ¹⁻³³⁵, EGFP-CK2 α '^{Cys336Ser} and mCherry-CK2 β ¹⁻¹⁹³ was as follows: The plasmid containing the genetic information for the respective protein was transformed into *E. coli* BL21(DE3) bacteria using the heat shock method. For expression, single cell colonies were grown at 37 °C overnight in 200 mL LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) containing either 100 μ g/mL ampicillin (for untagged CK2 β ¹⁻¹⁹³, CK2 β ¹⁻¹⁹³ tagged with Strep-tag[®] II, EGFP, mCherry, EGFP-CK2 α ¹⁻³³⁵, EGFP-CK2 α '^{Cys336Ser}, and mCherry-CK2 β ¹⁻¹⁹³) or 75 μ g/mL kanamycin (His-CK2 β ¹⁻¹⁹³). The pre-culture was diluted 1:50 into 4x 1 L of fresh LB-Miller G medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 4 g/L glucose, 0.5 g/L MgSO₄, 5.4 g/L KH₂PO₄, 10.5 g/L K₂HPO₄, pH 7.0) containing 100 μ g/mL ampicillin and 75 μ g/mL kanamycin, respectively, and grown at 37 °C until an optical density (OD₆₀₀) of 0.4-0.8 was reached. Protein expression was induced by addition of isopropyl thio-D-galactopyranoside (IPTG) (final concentration of 0.5 mM) and cells were grown at 20 °C overnight. Cells were harvested by centrifugation (4 °C, 7000 -10,000 \times g) and the cell pellet was stored at -80 °C until further use. For lysis, the cell pellet was thawed and suspended in the respective buffer (25 mM Tris, 300 mM NaCl, pH 8.5 for untagged CK2 β ¹⁻¹⁹³; 20 mM Tris, 300 mM NaCl, pH 7.8 for His-tagged CK2 β ¹⁻¹⁹³ and 100 mM Tris, 150 mM NaCl, pH 8.0 for all the other proteins). Cells expressing His-CK2 β ¹⁻¹⁹³ or CK2 β ¹⁻¹⁹³ tagged with Strep-tag[®] II were lysed for 1 h at 4 °C using 2 mg/mL lysozyme, 0.35 mg/mL PMSF, 0.2 mg/mL DNaseI and 50 mg/mL MgSO₄. Those cells expressing the fluorescently labelled proteins were lysed under the same conditions but with additional 50 μ L of protease inhibitor cocktail (product no. ab201111, abcam, Cambridge, UK). Lysis of cells producing the untagged CK2 β ¹⁻¹⁹³ was performed with 1 mg/mL lysozyme, 0.17 mg/mL PMSF, 0.1 mg/mL DNaseI and 25 mg/mL MgSO₄. The lysates were stored at -80 °C until further use.

Purification of untagged CK2 β ¹⁻¹⁹³ was conducted as described by Raaf et al.¹¹. Purification of proteins carrying the Strep-tag[®] II or the Twin-Strep-tag[®] was done by affinity chromatography on a 5 mL Strep-tactin XT superflow column (IBA-Lifesciences, Göttingen, Germany). After loading the supernatant onto the column and washing unbound material away with 100 mM Tris, 150 mM NaCl, pH 8.0, the protein was eluted using BXT-buffer (100 mM Tris, 150 mM NaCl, 1 mM EDTA and 50 mM biotin). For EGFP-CK2 α ¹⁻¹³³⁵ and EGFP-CK2 α ^{Cys336Ser}, a second purification step was added using a 1 mL HiTrap Heparin column (GE Healthcare, Düsseldorf, Germany). Equilibration of the column and loading of pre-purified proteins was done with a low salt buffer (25 mM Tris-HCl, 150 mM NaCl, pH 8.5). After washing the column with the same buffer, the purified proteins were eluted by a gradient containing an increasing proportion of high salt buffer (25 mM Tris-HCl, 1 M NaCl, pH 8.5). His-tagged CK2 β ¹⁻¹⁹³ was purified using a 1 mL HiTrap Talon crude column (Cytiva, Marlborough, MA, USA). After equilibration and loading of the protein onto the column, unbound material was washed away using 20 mM Tris, 300 mM NaCl, 5 mM imidazole, pH 7.8. A gradient elution was done using the same buffer, which had a high imidazole content (300 mM).

All purified proteins in this work were concentrated and re-buffered in 25 mM Tris, 500 mM NaCl, pH 8.5 by ultracentrifugation using Merck Amicon[™] Ultra-15 filters and stored at -80 °C.

2.3 Synthesis of Pc peptides

The cyclic peptides G(CRLYGXKIHGC)G (X = Phe, Pc, and X = 3-I-Phe, I-Pc) and the fluorescent FA probe CF-Ahx-Pc consisting of 5,6-carboxyfluorescein (CF) coupled via a 6-aminohexanoic acid linker (Ahx) to the N-terminus of Pc were synthesized by the group of Ines Neundorf (University of Cologne, Department of Chemistry and Biochemistry, Institute of Biochemistry, Germany) according to the procedure described by Lindenblatt et al.¹ Analytical data for the three peptides have previously been provided^{1, 3, 12}. Stock solutions of Pc (10 mM),

I-Pc (10 mM) and CF-Ahx-Pc (200 μ M) was prepared in distilled water and stored at -80 °C in the dark.

2.4 High-throughput screening of compound library

Screening of a library of 67,584 compounds and initial hit validation was performed in assay buffer, i.e. 25 mM Tris, 500 mM NaCl, pH 8.5, 0.05% (v/v) Tween 20, with additional 2%(v/v) DMSO. For library screening, binding of 0.1 μ M CF-Ahx-Pc to 1 μ M CK2 α^{1-335} was followed in the presence of 20 μ M of each compound. Positive and negative controls, i.e. 100% and 0% of signal, respectively, were determined with CF-Ahx-Pc in the presence and absence of CK2 α^{1-335} . Experiments were conducted in Corning 384-well low flange black flat bottom polystyrene plates (product no. 3573); liquid handling was done with a BioTek MultiFlo Fx Multimode Dispenser and a Tecan Freedom EVO Workstation. All assays were performed in a total volume of 20.4 μ L, with 0.4 μ L of compound in DMSO being added to 10 μ L of assay buffer followed by addition of 5 μ L of 4 \times CF-Ahx-Pc and 5 μ L of 4 \times CK2 α^{1-335} both dissolved in assay buffer. The assay mixture was incubated for 30 min at room temperature and the parallel and perpendicular fluorescence intensities were measured on a Perkin Elmer EnVision plate reader (excitation at 480/30 nm, emission at 535/40 nm). Fluorescence polarization (G factor = 0.95) was calculated according to the equation Polarization (mP) = 1000 * (S-G*P)/(S+G*P) with S and P being the parallel and the perpendicular fluorescence intensity, respectively.

Initial validation of primary hits was done by determination of their IC₅₀ values as detailed in legend to Table S1. IC₅₀ values of compounds, I, were calculated according to the four-parameter equation $Y = \text{Bottom} + ((\text{TOP} - \text{BOTTOM}) / (1 + ([I] / \text{IC}_{50})^{nH}))$. In this equation, TOP and BOTTOM correspond to the upper and lower plateau of the dose-response curve, respectively, whereas nH represents the Hill slope, i.e. the steepness of the curve. All calculations described in chapters 2.4, 2.5, and 2.7, were done using Microsoft Excel as part of Microsoft Office Professional Plus 2016 (Microsoft Cooperation, Redmond, WA, USA) and GrapPad Prism v10.2.3 for Windows (GraphPad Software, Boston, MA, USA). Cyclic pentapeptides identified in the initial library screening and selected for further

characterisation were commercially obtained from ChemBridge Corporation (San Diego, CA, USA).

2.5 Fluorescence anisotropy assay

Fluorescence anisotropy (FA) assays following binding of CF-Ahx-Pc to both CK2 α^{1-335} and CK2 $\alpha^{Cys336Ser}$ and displacement of the probe from the proteins by competitors were conducted according to the procedure by Hochscherf et al.³ and Lindenblatt et al.¹ with some alterations. FA was determined without any pre-incubation of probe and protein.

CK2 α^{1-335} was investigated in Corning 384-well low flange black flat bottom polystyrene plates (product no. 3573) in a total volume of 50 μ L containing 0.1 μ M CF-Ahx-Pc and 2-3 μ M of protein. Displacement of probe by 0.1-300 μ M Pc was followed using 25 mM Tris, 500 mM NaCl, pH 8.5, whereas 2% (v/v) of DMSO was added to the buffer for analysis of the cyclic peptides (0.1-100/300 μ M). For CK2 $\alpha^{Cys336Ser}$, black BRANDplates pureGrade 96-well plates with F-bottom wells (Brand, Wertheim, Germany) and a total volume of 200 μ L were used. Displacement of 0.1 μ M CF-Ahx-Pc from 30 μ M of protein by 3-300 μ M Pc was investigated in 25 mM Tris, 500 mM NaCl, pH 8.5. The dissociation constant of CF-Ahx-Pc (0.1 μ M) on the two proteins was determined with 0.001-100 μ M CK2 α^{1-335} and 0.003-100 μ M CK2 $\alpha^{Cys336Ser}$, respectively.

2.6 Native PAGE for analysis of protein-protein interactions

For native polyacrylamide gel electrophoresis (native PAGE) and sodium dodecyl sulphate-PAGE (SDS-PAGE) a 10% separating gel and a 4% stacking gel were used. For native PAGE, the stacking gel consisted of 0.125 M Tris, pH 6.8, and 10% (v/v) acrylamide-bisacrylamide, whereas the separating gel contained 0.375 M Tris, pH 8.8, and 10% (v/v) acrylamide-bisacrylamide. For SDS-PAGE, both gels had the same composition but contained additional 0.1% (m/v) SDS. Loading of the samples onto the gel was conducted using three parts of protein mixed with one part of loading buffer (native PAGE: 240 mM Tris-HCl pH 6.8, 40% (v/v) glycerine and 0.08% bromophenol blue; SDS-PAGE: the same buffer with addition of 8% (m/v)

SDS and 2% (v/v) 2-mercaptoethanol). To analyse protein-protein interaction, proteins were prepared either separately or in combination to give a final concentration of 20 μ M and incubated for 15 min at room temperature. Electrophoretic runs were conducted at 80 V, 300 mA, 30 W (native PAGE) or 120 V, 300 mA, 30 W (SDS-PAGE) in running buffer (native PAGE: 25 mM Tris, pH 8.3, 0.2 M glycine; SDS-PAGE: the same buffer with additional 0.5% (m/v) SDS). Fluorescence of native PAGE gels was visualized on an Invitrogen™ iBright™ FL1500 imaging system using both the green ($\lambda_{\text{ex}} = 455\text{-}485$ nm, $\lambda_{\text{em}} = 508\text{-}557$ nm) and red ($\lambda_{\text{ex}} = 515\text{-}545$ nm, $\lambda_{\text{em}} = 568\text{-}617$ nm) channels. In addition, gels were stained with colloidal Coomassie Brilliant Blue G250 and destained several times with 10% (v/v) aqueous ethanol containing 2% (v/v) phosphoric acid¹³. Afterwards, gels were scanned using a LI-COR Odyssey Fc Imager (700 nm-channel) and analysed with Image Studio software version 5.2.5.

2.7 Analysis of protein-protein interactions by FRET

2.7.1 General information

FRET-based assays were conducted at 30 °C in either Corning 384-well low flange black flat bottom polystyrene plates (product no. 3573, total assay volume: 50 μ L) or black BRANDplates pureGrade 96-well plates with F-bottom wells (total assay volume: 200 μ L). Experiments were performed on a Synergy™ 2 plate reader (BioTek, USA) using filters for excitation and emission in combination with a 50% mirror or an Infinite® M1000 Pro plate reader (Tecan Group, Switzerland) equipped with a monochromator. The following excitation and emission wavelengths were used on a Synergy™ 2 plate reader: donor channel (D) 485/25 nm, 528/20 nm; FRET channel (F) 485/25 nm, 620/40 nm; acceptor channel (A) 540/25 nm, 620/40 nm. The gain factor was automatically determined. Excitation and emission wavelengths on the Infinite® M1000 Pro plate reader were the same, except using a bandwidth of 10 nm and a gain factor of 100 or 110. If not stated otherwise, experiments were conducted in 25 mM Tris-HCl, 500 mM NaCl, pH 8.5 (assay buffer) containing both 2% (v/v) DMSO and 0.05% (v/v) Tween 20.

Fluorescence intensity was measured for the following assay compositions: either EGFP/EGFP-CK2 α^{1-335} /EGFP-CK2 $\alpha^{Cys336Ser}$ (donor) or mCherry/mCherry-CK2 β^{1-193} (acceptor) alone (sample controls) or both in combination or blank wells containing assay buffer (with or without co-solvents). Values of fluorescence intensity obtained for blank wells were subtracted from those of containing fluorescent protein(s). Due to spectral bleed through, corrected sample controls were needed to calculate the corrected FRET signal (FRET_c) according to Youvan et al.¹⁴ and Hochreiter et al.¹⁵. In this process, corrected fluorescence intensities with negative values were set to zero.

2.7.2 Dissociation constants (K_D) for binding of mCherry-CK2 β^{1-193} to EGFP-CK2 α^{1-335} or EGFP-CK2 $\alpha^{Cys336Ser}$

Protein stocks kept on ice were freshly diluted with assay buffer containing either no additive or 2% (v/v) DMSO or 0.05% (v/v) Tween 20 or both co-solvents. A dilution series of mCherry-CK2 β^{1-193} or mCherry (final acceptor concentrations of 0.6 – 300 nM) was mixed with a constant amount of EGFP-CK2 α^{1-335} , EGFP-CK2 $\alpha^{Cys336Ser}$ or EGFP (final donor concentration of 30 nM). In addition, sample controls containing the same concentrations of acceptor or donor were prepared to calculate FRET_c. Three independent experiments were conducted in triplicate. Total assay volume was 200 μ L for the following donor/acceptor combinations: EGFP-CK2 α^{1-335} /mCherry-CK2 β^{1-193} (w/ DMSO, w/o DMSO and Tween) and EGFP-CK2 $\alpha^{Cys336Ser}$ /mCherry-CK2 β^{1-193} (w/ DMSO & Tween, w/ DMSO, w/ Tween, w/o DMSO and Tween). All the other donor/acceptor combinations shown in Figure 1a were investigated in a total volume of 50 μ L. The dissociation constant, K_D , was calculated according to the equation

$$FRET_c = FRET_{c_{free}} + \frac{(FRET_{c_{bound}} - FRET_{c_{free}})([A] + [D] + K_D - \sqrt{([A] + [D] + K_D)^2 - 4[A][D]})}{2[D]}$$

with FRET_{c_{free}} and FRET_{c_{bound}} representing the FRET_c values for unbound donor and donor completely bound to acceptor, respectively, while [A] and [D] being the concentrations of the acceptor and the donor, respectively.

2.7.3 Dissociation constants (K_i) of CK2 inhibitors

A dilution series of different concentrations of the inhibitor was pre-mixed with mCherry-CK2 β^{1-193} in assay buffer containing 2% (v/v) DMSO and 0.05% (v/v) Tween 20 to obtain 2 \times inhibitor and 2 \times acceptor. A volume of 25 μ L of 2 \times EGFP-CK2 α^{1-335} or 2 \times EGFP-CK2 $\alpha^{\text{Cys336Ser}}$ in assay buffer with 2% (v/v) DMSO and 0.05% (v/v) Tween 20 was added to 25 μ L of the pre-mix on a 384-well plate to obtain final acceptor and donor concentrations of 50 and 30 nM (1 \times), respectively. Sample controls were prepared by addition of 50 μ L of either 1 \times donor or 1 \times acceptor to the well.

Untagged CK2 β^{1-193} and I-Pc were investigated with the same final acceptor and donor concentrations but in a total volume of 200 μ L by adding 20 μ L 10 \times inhibitor and 20 μ L 10 \times mCherry-CK2 β^{1-193} to 140 μ L of assay buffer. Fluorescence intensities were measured after addition of 20 μ L 10 \times EGFP-CK2 α^{1-335} and corrected by those obtained for 200 μ L 1 \times sample controls. Three to four individual experiments were conducted in duplicate to triplicate. To test for self-fluorescence of the inhibitors, a concentration of 100 μ M of inhibitor was investigated in the absence of the fluorescent proteins. In case of self-fluorescence as found for tagged and untagged CK2 β^{1-193} as well as for Pc and I-Pc, the fluorescence intensities were determined as described above without addition of either donor (tagged CK2 β^{1-193} and Pc) or donor and acceptor (untagged CK2 β^{1-193} and I-Pc) and used to correct fluorescence intensities obtained in the presence of both donor and acceptor. As the compounds from the library screen and their derivatives did not show any self-fluorescence at 100 μ M, measured fluorescence intensities were not further corrected in this regard. IC₅₀ values were determined by means of the four-parameter equation and used to calculate K_i values according to Nikolovska-Coleska et al.². Herein, K_D values determined for several preparations of acceptor and donor proteins were applied.

2.7.4 Disruption of *in situ*-formed EGFP-CK2 α^{1-335} /mCherry-CK2 β^{1-193} complexes

A mixture of EGFP-CK2 α^{1-335} and mCherry-CK2 β^{1-193} in assay buffer containing 2% (v/v) DMSO and 0.05% (v/v) Tween 20 (37.5 nM each in a total volume of 640 μ L (for measurements shown in Figure 3d) or 1440 μ L for IC₅₀ determinations (Figures 3e,f, S6a,b)) was incubated for 30 min at 30 °C to form complexes. As a control, either EGFP-CK2 α^{1-335} or mCherry-CK2 β^{1-193} alone in the same buffer (75 nM each in a total volume of 320 μ L (for measurements shown in Figure 3d) or 800 μ L for IC₅₀ determinations (Figures 3e,f, S6a,b)) was incubated for 30 min at 30 °C. A volume of 10 μ L of either 5 \times inhibitor in assay buffer with 2% (v/v) DMSO and 0.05% (v/v) Tween 20 (final concentration of 100 μ M or 0.024-200 μ M for IC₅₀ determination) or the same buffer without inhibitor was pipetted into a 384 well plate. A volume of 40 μ L of the pre-formed CK2 complexes or 20 μ L of each of the two CK2 subunits (final concentration of 30 nM each) was added and fluorescence intensities were measured as described above immediately after addition of the proteins or after 15 min and 30 min, respectively. To correct for self-fluorescence, as it is the case for Pc, fluorescence intensities were determined without addition of either donor or acceptor and used to correct fluorescence intensities obtained in the presence of both donor and acceptor. To correct for spectral bleed-through, sample controls with 50 μ L of 1 \times EGFP-CK2 α^{1-335} (30 nM) or 1 \times mCherry-CK2 β^{1-193} (30 nM) were analysed. Three to four separate experiments were performed in duplicate. Relative subunit binding for measurements shown in Figure 3d was calculated using the FRET_c values obtained without compound as 100%. IC₅₀ values were determined by means of the four-parameter equation.

2.8 CK2-catalyzed substrate phosphorylation

Kinase activity of CK2 α and CK2 $\alpha_2\beta_2$ holoenzyme was quantified by analysing phosphorylation of the peptides RRRDDDSDDD and eIF2 β^{1-22} by γ -³²P-ATP as previously described by Lindenblatt et al.¹ with some alterations.

For experiments with the individual CK2 subunits, full-length CK2 α and full-length CK2 β (90 nM each) were investigated in a total volume of 50 μ L. To a volume of 18 μ L of 4.4 pmol CK2 α in kinase buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT), a

volume of 1 μL of 5 mM inhibitor in DMSO or 1 μL of 0.05 mM SGC-CK2-1 in DMSO (final concentrations: 100 μM for compounds **12**, **14** and **15**, 1 μM for SGC-CK2-1) was added. After 1-2 min of incubation at room temperature, a volume of 1 μL of 4.4 pmol CK2 β was added. The reaction was started by addition of 30 μL of a mixture of the substrate peptides (0.19 mM RRRDDDSDDD or eIF2 β ¹⁻²²) and ATP (20 $\mu\text{Ci mL}^{-1}$ γ -³²P-ATP and 50 μM ATP) in assay buffer (25 mM Tris-HCl, pH 8.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT) either immediately or after 10 min of pre-incubation at room temperature. For experiments with CK2 holoenzyme, a final concentration of 10 nM CK2 $\alpha_2\beta_2$ was used.

2.9 Crystallization and crystal structure determination

All protein crystallization experiments were performed using the sitting drop variant of the vapour diffusion method. For CK2 $\alpha^{\text{Cys336Ser}}$, crystallization conditions to provide atomic-resolution structures are established¹⁶; in the underlying triclinic crystal packing, however, the CK2 β interface is not accessible for small molecule ligands. Therefore, we adapted a CK2 $\alpha^{\text{Cys336Ser}}$ crystallization strategy recently described by Werner et al.¹⁰ and based on a slight reduction of the LiCl concentration. This procedure provided an alternative monoclinic crystal packing in which one of two CK2 $\alpha^{\text{Cys336Ser}}$ protomers is no longer blocked at the CK2 β interface. In this way, we could successfully co-crystallize CK2 $\alpha^{\text{Cys336Ser}}$ with compound **12** (but not with compound **15**).

The optimal reservoir composition for the crystallization of the CK2 $\alpha^{\text{Cys336Ser}}$ /**12** complex was 28 % (w/v) PEG 6000, 810 mM LiCl, 100 mM Tris-HCl, pH 8.5. CK2 $\alpha^{\text{Cys336Ser}}$ with a concentration of 5 mg/mL was mixed in a 1:10 ratio with 10 mM compound **12** in DMSO. This mixture was incubated on ice for 30 min. Afterwards, any precipitate was removed by centrifugation (20000 \times g, room temperature, 2 min). Using sitting-drop crystallization plates, the CK2 $\alpha^{\text{Cys336Ser}}$ /**12** solution was mixed in a 2:1 ratio with reservoir solution and pre-incubated at 20 °C for one week. To induce crystal formation, micro seeds from another set of monoclinic CK2 $\alpha^{\text{Cys336Ser}}$ crystals were added. This led to crystals that were subsequently used as macro seeds. The resulting crystals were cryo-protected by transferring them to a mixture of 70%

reservoir solution and 30% ethylene glycol and afterwards flash-frozen in liquid nitrogen. X-ray diffraction data were collected at beamline ID30B⁵ of the ESRF.

For co-crystallization of CK2 α ¹⁻³³⁵ with compounds **12** and **15**, no known conditions were adapted; rather, we tested several commercially available crystallization screens, different protein-to-ligand ratios and temperatures as well as micro seeding. The final hit used for any subsequent crystallization experiment with CK2 α ¹⁻³³⁵ and either compound **12** or **15** was reagent 16 (1.5 M lithium sulphate, 0.1 M Na-HEPES, pH 7.5) of the Crystal screen from Hampton Research. After optimization, the best procedure was as follows: 7 mg/ml CK2 α ¹⁻³³⁵ was mixed in a 1:40 ratio with a 20 mM solution of compound **12** or **15** in DMSO. The CK2 α ¹⁻³³⁵/ligand mixture was incubated at 20 °C for 30 min. After removing precipitate by centrifugation (20000 × g, 2 min, 20 °C), the CK2 α ¹⁻³³⁵/ligand solution was mixed in a 2:1 ratio with the reservoir solution (1.5 M lithium sulphate, 0.1 M Na-HEPES, pH 7.5) in a 24-well sitting drop plate. The drop was pre-equilibrated against 700 μ L reservoir solution. CK2 α ¹⁻³³⁵/**15** crystals formed spontaneously. In the case of CK2 α ¹⁻³³⁵/**12**, crystals growth had to be induced by micro seeding using a seed suspension prepared as 1:1000 dilution of one CK2 α ¹⁻³³⁵/**15** crystal crushed in 100 μ l reservoir solution. Crystals were harvested after 3 weeks (with compound **12**) or 2 months (with compound **15**) by flash-freezing in liquid nitrogen without any additional cryo-protection. X-ray diffraction data of the CK2 α ¹⁻³³⁵ crystals were collected at beamlines ID30A-3 (MASSIF-3)⁶ or ID23-1⁷ of the ESRF.

All X-ray diffraction data were processed using the autoPROC pipeline¹⁷. This includes indexing and integration by XDS¹⁸, symmetry analysis by POINTLESS¹⁹ and scaling by AIMLESS²⁰ from the CCP4 software suite²¹. Finally, STARANISO⁸ was applied for anisotropy analysis. It provided ellipsoidal diffraction data sets that were applied for structure determination by molecular replacement using PHASER²² within Phenix²³. Structure optimization were performed by manual modelling with Coot²⁴ alternating with computational refinement with Phenix²⁵. The novel ligands **12** and **15** were parametrized with eLBOW²⁶.

3. References

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