

Biochemical analyses reveal amino acid residues critical for cell cycle-dependent phosphorylation of human Cdc14A phosphatase by cyclin-dependent kinase 1

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Supplementary Information

Supplementary Figure S1. Characterization of U-2-OS cell lines stably expressing HA-Cdc14A or the phosphatase dead form HA-Cdc14A(PD). (a) HEK293T cells were transfected with Flag-tagged wild-type Cdc14A or its inactive form Cdc14A(PD). After 24 hours of transfection cellular extracts were obtained and ectopic protein expression was detected by immunoblotting with anti-Flag antibodies. (b) Comparison of Cdc14A expression levels among U-2-OS cells stably expressing retrovirally transduced HA-Cdc14A or HA-Cdc14A(PD), and transient transfection with expression vectors containing HA-Cdc14A under the control of the CMV promoter (C: U-2-OS parental cells). Full-length Cdc14A blot is shown in Supplementary Fig. S15. (c) FACS analysis of control U-2-OS cells and the U-2-OS-HA-Cdc14A cell lines. To check the mitotic index, cells were processed to detect phospho-histone H3 (Serine 10). (d) Subcellular localization of Cdc14A or Cdc14A(PD) in stable cell lines. Cells were fixed and co-stained with anti-HA tag and anti- γ -tubulin antibodies. DNA was stained with DAPI. (e) U-2-OS-HA-Cdc14A cells were synchronized at G1/S by a double thymidine treatment and then released into fresh medium to allow progression through G2 phase. After 10 hours from release, rounded mitotic cells were selected by shake off. Cellular extracts were obtained from both asynchronous and mitotic cells, resolved by Phos-tag gels and analyzed by immunoblot using anti-Cdc14A antibodies. Full-length Cdc14A blot is shown in Supplementary Fig. S15.

Supplementary Figure S2. Cdc14A interacts with PP2A. Cellular extracts were obtained from U-2-OS cell lines stably expressing HA-Cdc14A (Figure S1) and Cdc14A was immunoprecipitated with anti-HA antibodies. Interaction with the endogenous PP2A α -catalytic subunit was detected by immunoblotting with specific anti-PP2A antibodies. WCE: whole cell extracts. Unprocessed original scans of blots are shown in Supplementary Fig. S15.

Supplementary Figure S3. Alignment of Cdc14 family members. Amino acid sequence alignment of Human Cdc14A, Mouse Cdc14A, *Xenopus* Cdc14a, *Saccharomyces cerevisiae* Cdc14 and *Schizosaccharomyces pombe* Cdc14/SpFlp1. Sequence alignment was performed using Constrain-based Multiple Alignment Tool.

(COBAL program (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi)). Shadowed in gray are indicated Cdk1 phosphorylation sites described in *S. pombe* and *S. cerevisiae*. All consensus motifs for Cdk1 phosphorylation on human Cdc14A (T110, T254, S411, S453, S511, S549 and T590) are indicated. Yellow marks indicate the Cdk1-targeted phosphorylation sites of human Cdc14A.

Supplementary Figure S4. Cdc14A phosphorylation by Cdk1 does not inhibit its phosphatase activity. (a) Cdc14A was immunoprecipitated from asynchronous or mitotic nocodazole arrested U-2-OS cells stably expressing Flag-Cdc14A, and its phosphatase activity was determined on pNPP substrate. Cdc14A(PD), immunoprecipitated from asynchronous U-2-OS cells stably expressing Flag-Cdc14A(PD), was used as a negative control. Reactions were performed in triplicate. Data are representative of three independent experiments. (b) Wild-type Cdc14A, and Cdc14A-3A and -3E mutants were obtained by immunoprecipitation from U-2-OS cells stably expressing the corresponding Flag-Cdc14A version, and their phosphatase activity was determined on pNPP substrate. Reactions were performed in triplicate. Data are representative of three independent experiments. (c) HEK293T cells were co-transfected with Flag-KIBRA and EGFP-tagged wild-type Cdc14A, Cdc14A-3A, Cdc14A-5A or Cdc14A-5E mutants. After 12 hours of transfection, half of the cells were treated with nocodazole during 12 hours. Then, cellular extracts were obtained from both asynchronously growing or nocodazole treated cells, and analyzed by immunoblot with the indicated antibodies. Full-length blots are shown in Supplementary Fig. S15.

Supplementary Figure S5. Cdc14A phosphorylation by Cdk1 does not affect its association with interphase centrosomes. U-2-OS cells were transfected with EGFP-tagged Cdc14A or the phosphorylation mutants Cdc14A-3A or Cdc14A-3E. After 24 hours of transfection, cells were fixed and immunostained with antibodies against γ -Tubulin. DNA was stained with DAPI. Images were acquired using a Zeiss Axioimager Apotome microscope (objective 40X) and processed using Zeiss Zen lite software. (Scale bar: 10 μ M).

Supplementary Figure S6. Release of Cdc14A from centrosomes does not depend on Cdk1 phosphorylation. U-2-OS cells were transfected with EGFP-tagged Cdc14A or the phosphorylation mutants Cdc14A-3A or Cdc14A-3E. After 48 hours of transfection, cells were fixed and immunostained with antibodies against γ -Tubulin. DNA was stained with DAPI. Representative mitotic cells are shown. Images were acquired using a Leica DM6000B microscope (objective 40X) and processed using OpenLab 4.0.3 software. (Scale bar: 10 μ M).

Supplementary Figure S7. Phosphorylation of Cdc14A does not significantly affect its cellular stability. Derivatives of HeLa cell line, conditionally expressing low levels of Flag-tagged Cdc14A (WT), Cdc14A-3A (3A) or Cdc14A-3E (3E) in a tetracycline-inducible manner, were incubated with medium containing Doxycycline during 24 hours to induce ectopic Cdc14A expression. Cells were then treated with the inhibitor of protein synthesis cycloheximide and harvested at the indicated time points to check Cdc14A protein levels by immunoblot with anti-Cdc14A antibodies. Full-length Cdc14A blots are shown in Supplementary Fig. S16.

Supplementary Figure S8. Co-immunoprecipitation analysis of Cdc14A and Cdc14A-3A mutant.

(a) HEK293T cells were co-transfected with HA-Cdh1 and Flag-tagged wild-type Cdc14A, its inactive form Cdc14A(PD) or Cdc14A(PD)-3A mutant. After 12 hours of transfection, cells were treated with nocodazole for 12 hours. Cellular extracts were immunoprecipitated with anti-Flag antibodies and analyzed by immunoblot to detect the presence of the two proteins. Asterisks show cross-reacting bands. **(b)** HeLa cell lines conditionally expressing Flag-tagged wild-type Cdc14A or Cdc14A-3A mutant, were incubated with medium containing Doxycycline to induce protein expression and with or without nocodazole during 12 hours. Cellular extracts were obtained and Cdc14A forms were immunoprecipitated with anti-Flag antibodies. Interaction with endogenous Plk1 was analyzed by immunoblotting. Unprocessed original scans of blots are shown in Supplementary Fig. S16.

Supplementary Figure S9. Full-length immunoblots relating to Figure 1 are shown.

Supplementary Figure S10. Full-length gels and immunoblots relating to Figure 2 are shown.

Supplementary Figure S11. Full-length gels and immunoblots relating to Figure 3 are shown.

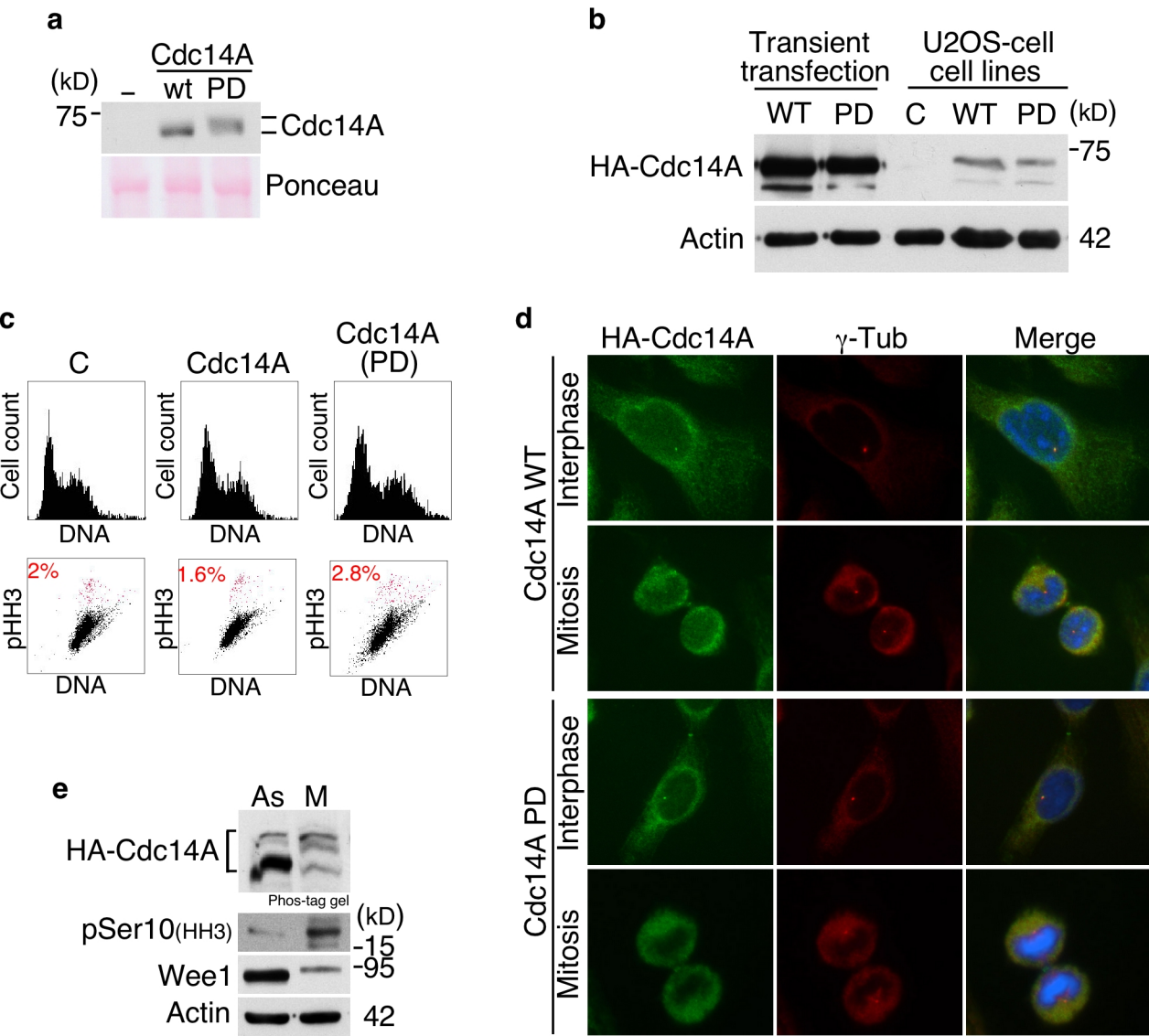
Supplementary Figure S12. Full-length gels and immunoblots relating to Figure 4 are shown.

Supplementary Figure S13. Full-length gels and immunoblots relating to Figure 5 are shown.

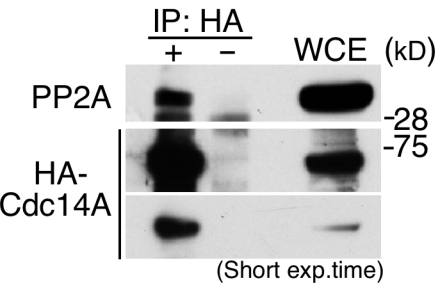
Supplementary Figure S14. Full-length immunoblots relating to Figure 6 are shown.

Supplementary Figure S15. Full-length immunoblots relating to Supplementary Figures S1, S2 and S4 are shown.

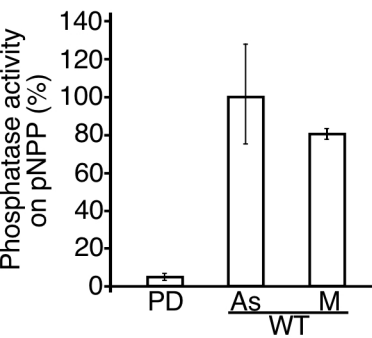
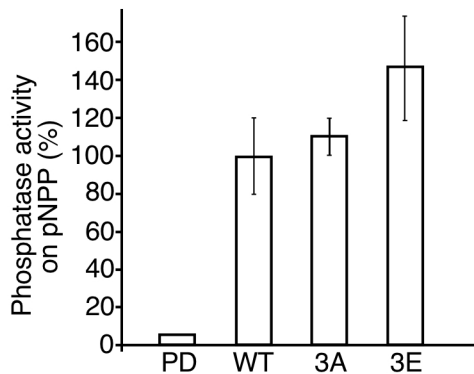
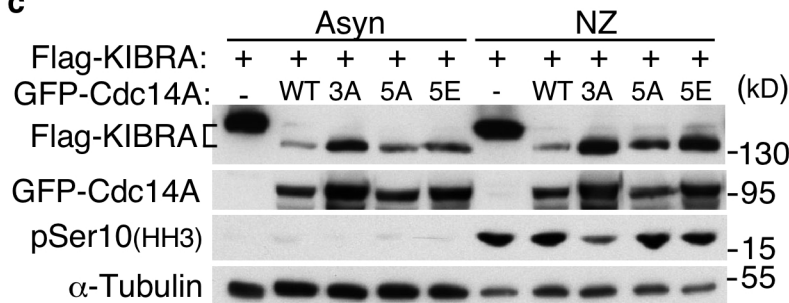
Supplementary Figure S16. Full-length immunoblots relating to Supplementary Figures S7 and S8 are shown.

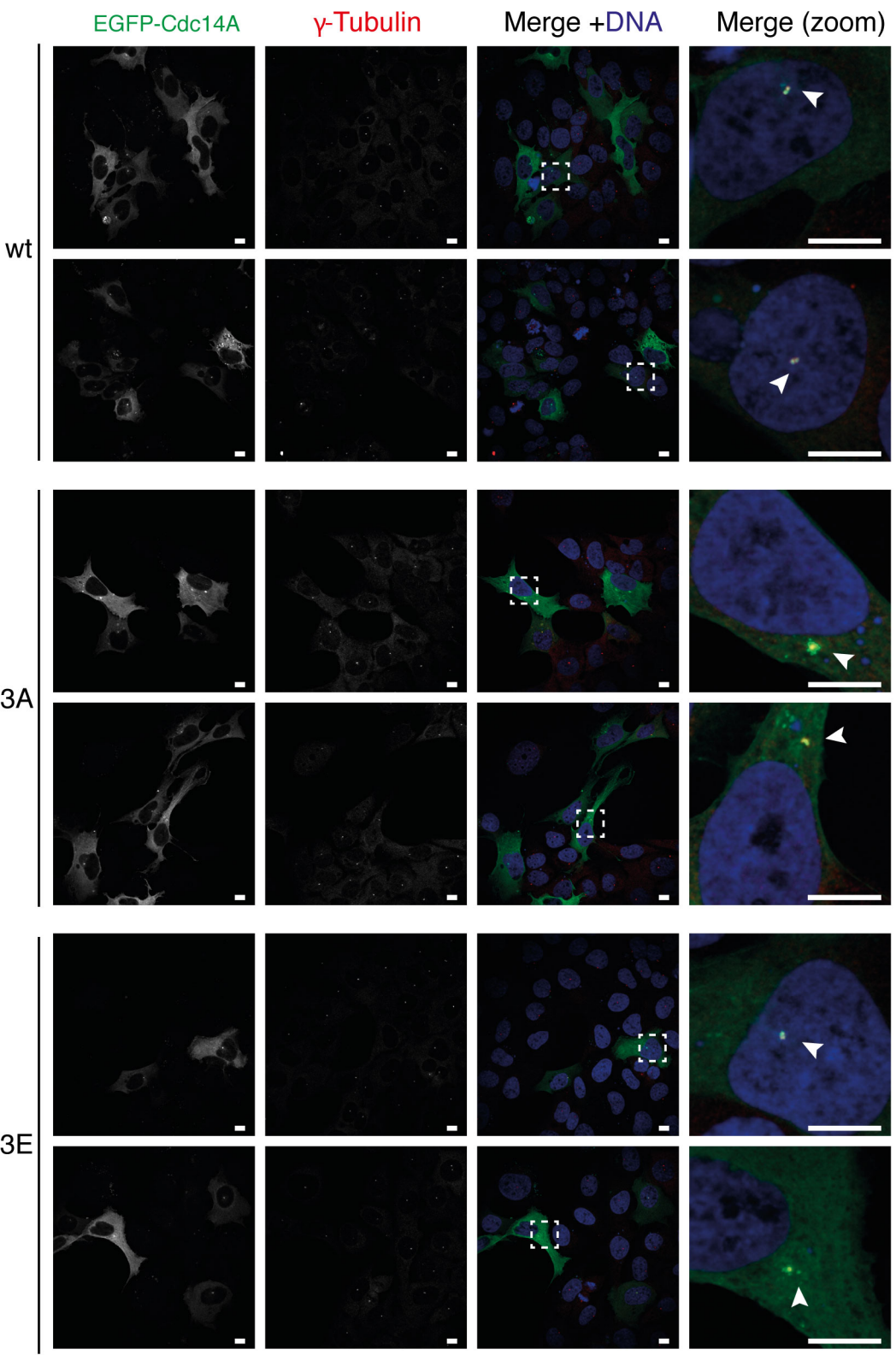


Supplementary Figure S2



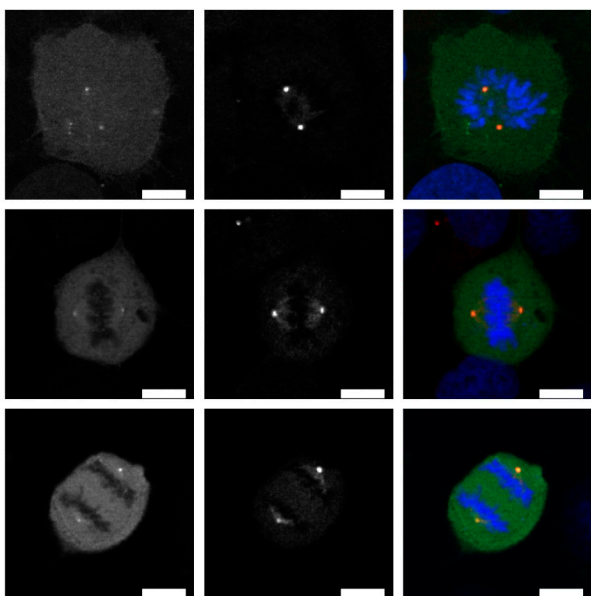
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XlCDC14A	1	M--DN---QELISASEVIKDRLYFAILRNKPK-ST-LNTHYFCTDEEFVYENFYADFGPLNLAQLYRYCCKLNKKLKSFS	73
SpFlp1	1	MDYQDdglGEMT---EFLEDKLYYTSLSQPPKaElyPHMHFFTIDDELIYNPFYHDFGPLNVSHLIRFAVIVHGMGKHR	77
ScCDC14	1	MRRSVy-lDNTI---EFLRGRVYLGAYDYTPe-DT-DELVFFTVEDAIFYNSFHLDGPMNIGHLYRFAVIFHEILNDPE	74
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MmCDC14A	76	LSR--KKIVHYTSFDQRKRANAFLIGAYAVIYLKKTPEEAYRALLSGSNPPYLPRDASFGNCTYNLTIVLDCLQGIRKG	153
XlCDC14A	74	LSR--KKIVHYTSFEQRKRNSAGFLISAYAVIYLKKSPEEVYRALLSGSNAQYLPFRDASFGSCTYNLTILDCLQIRKA	151
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SpFlp1	422	SKQSIQ ^{EN} ENKASYSSYEDSEIQNDDETRTV ^{CT} PTETISV ^{VR} LRSSSSQSNIEP ^{NG} VR ^{SP} TSSPTGSPIRR ^T SGNRWSSGS	501
ScCDC14	436	GQNGS-----NTIEDINNRNPTSHANR ^{KV} VIESNNSDDESMQDTNGTNSH----YPKVSRKKNDISS	494
		S(549)	..T(590) ..
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XlCDC14A	534	-----NQNPSTP-----KDMYGSFRPSYTG--LASSPRFLRSRIP SLQSEYAQI	576
SpFlp1	502	SHSKKSA -QRSVMS-SLNN ^T SNGRVAKPKSK-----SRLIS -----	537
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a**b****c**

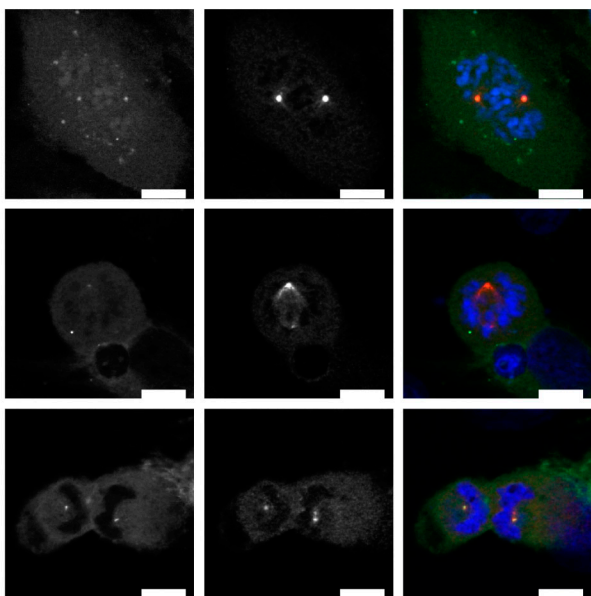


EGFP-Cdc14A γ -Tubulin Merge +DNA

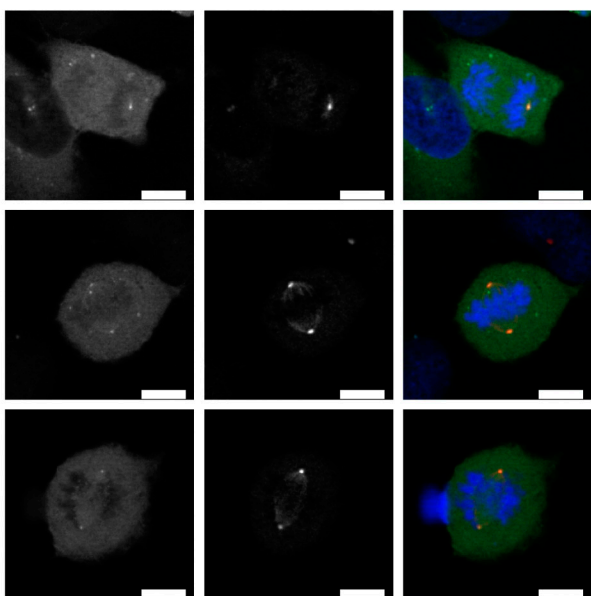
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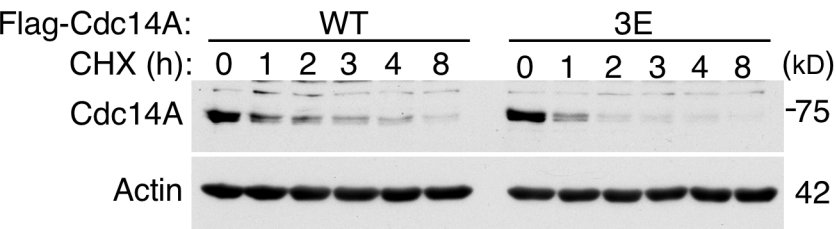
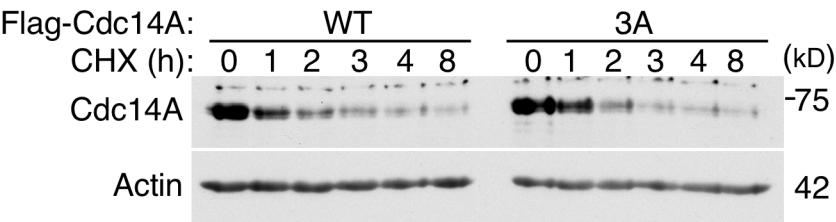


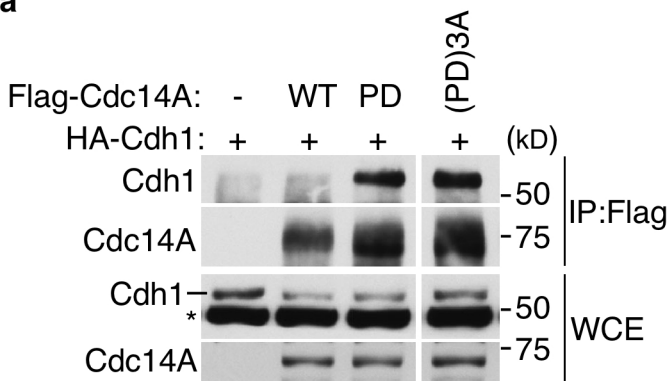
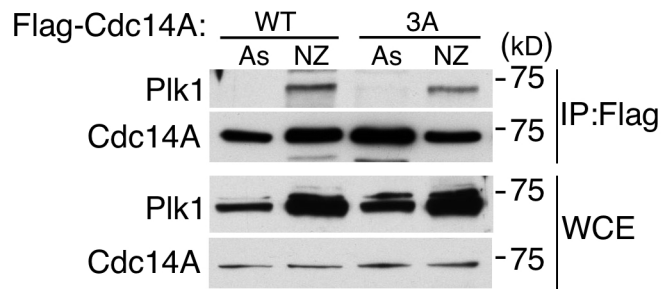
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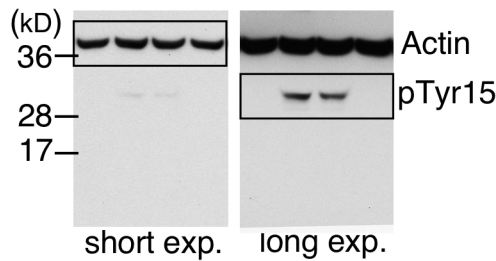
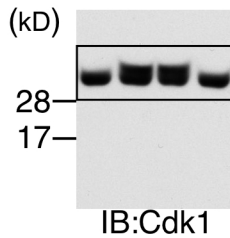
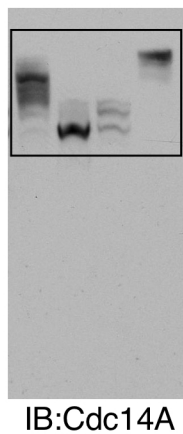
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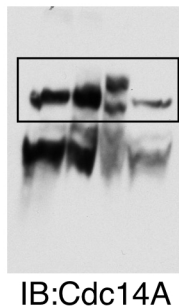


a**b**

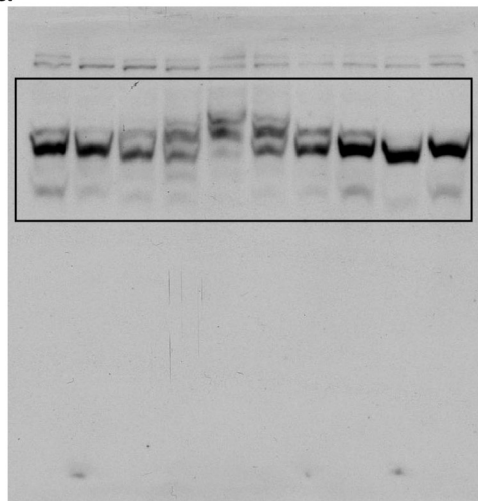
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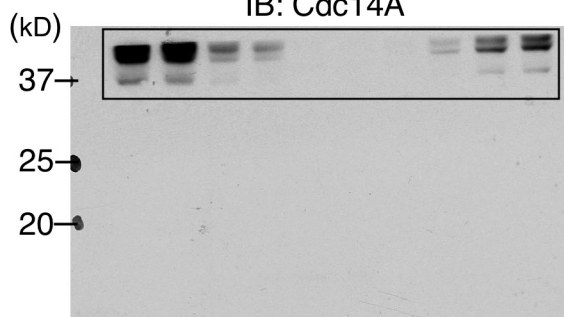
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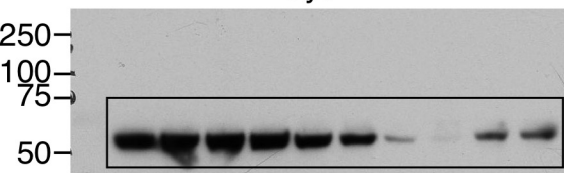
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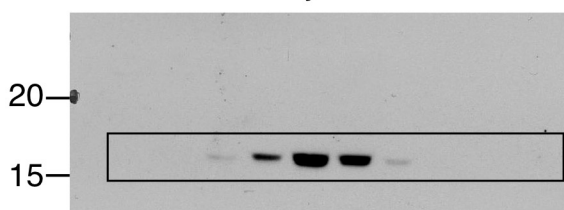
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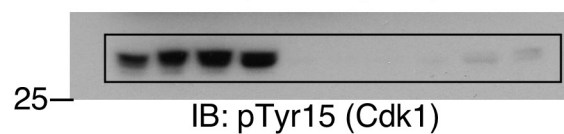
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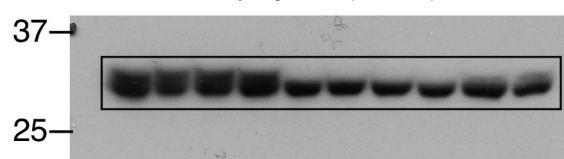
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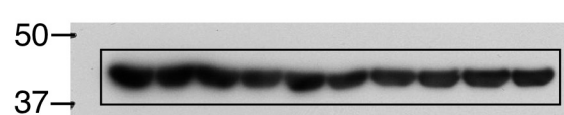
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IB: pTyr15 (Cdk1)

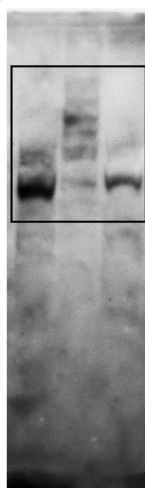


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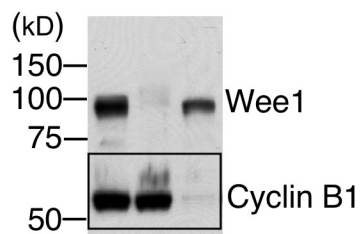


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2b



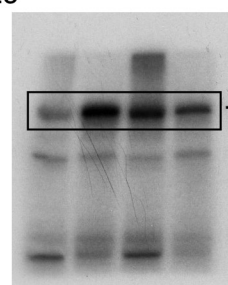
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Wee1

Cyclin B1

2c

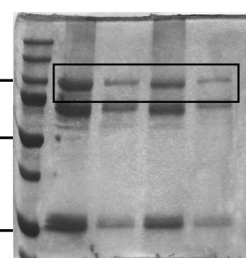
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(kD)

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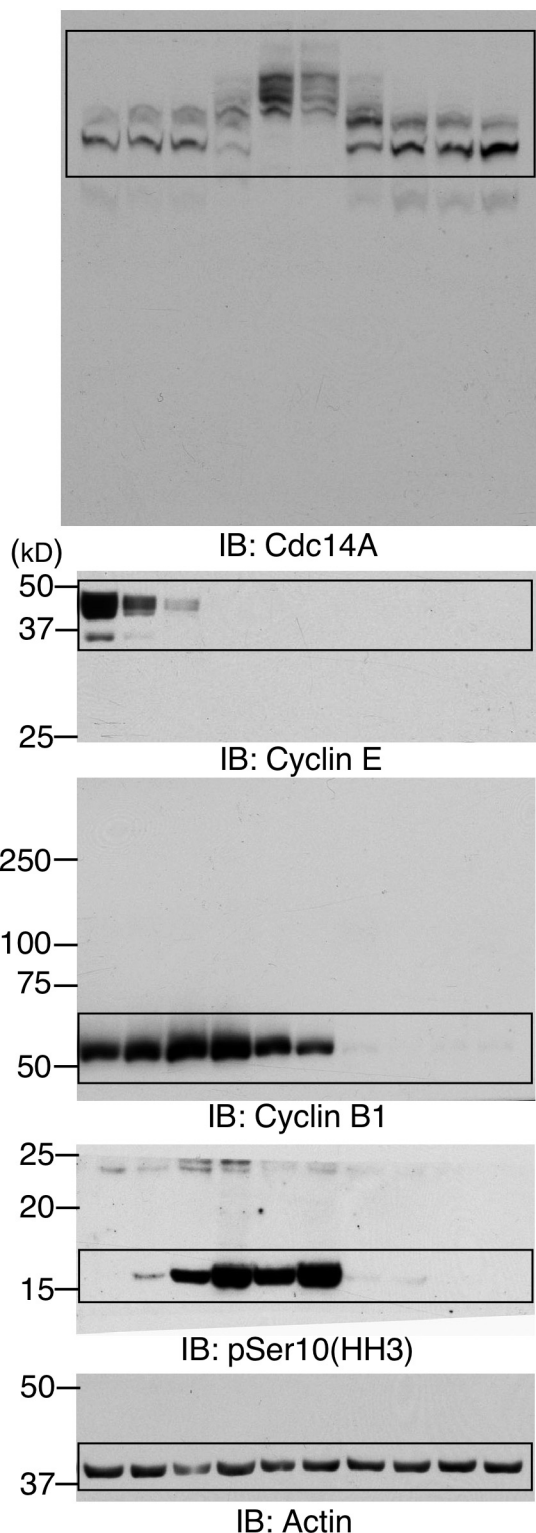
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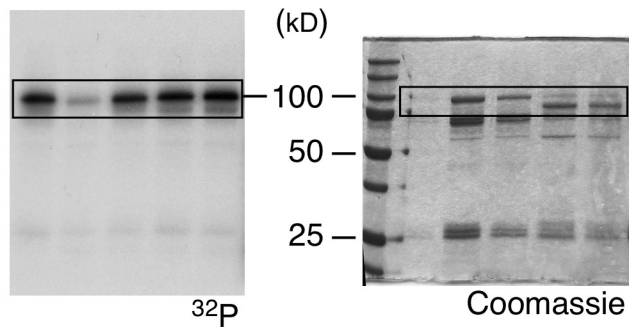


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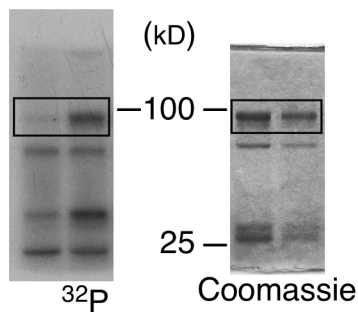
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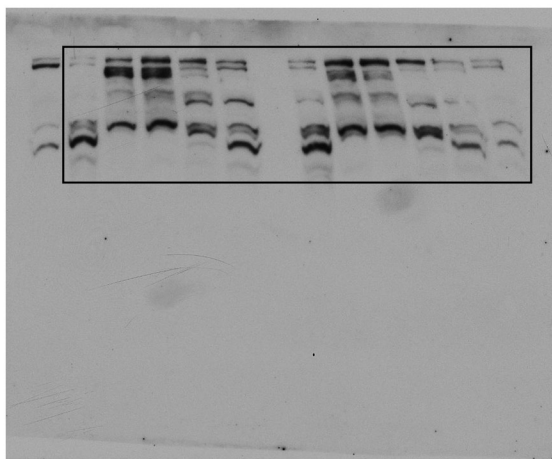
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3c

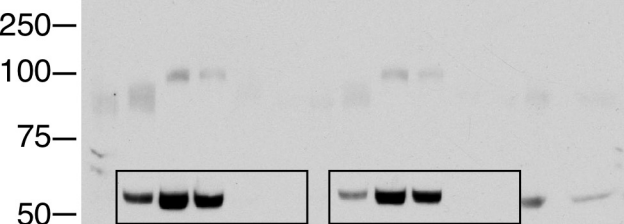


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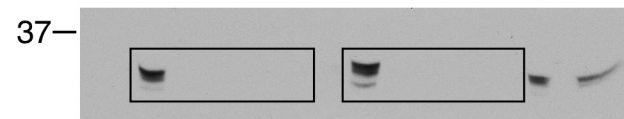


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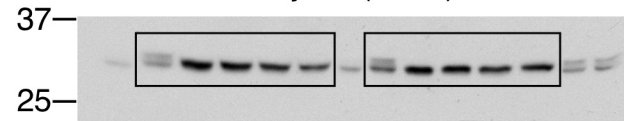
(kD) * * * * unrelated samples



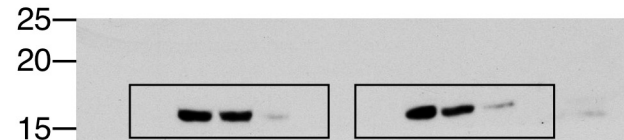
IB: Cyclin B1



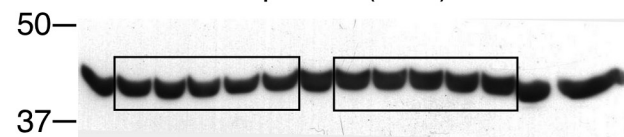
IB: Tyr15 (Cdk1)



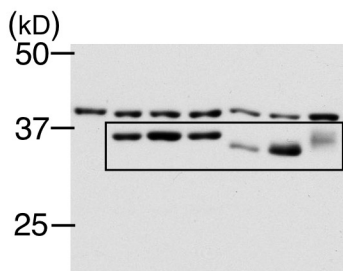
IB: Cdk1



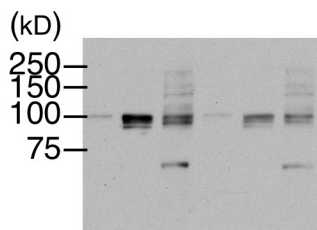
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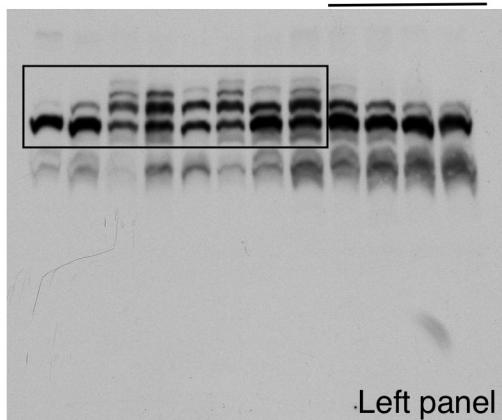
IB: Actin

4b

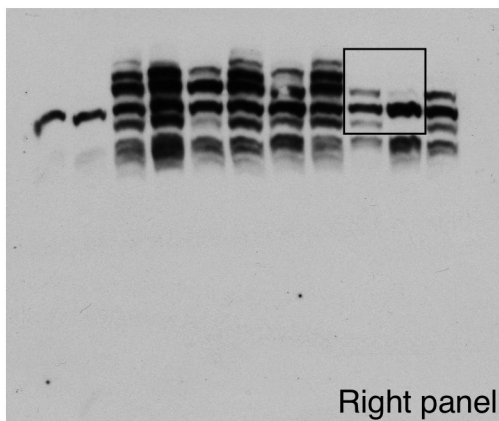
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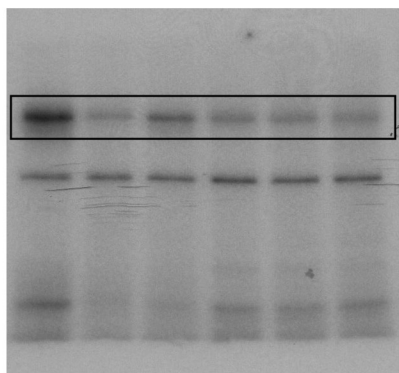
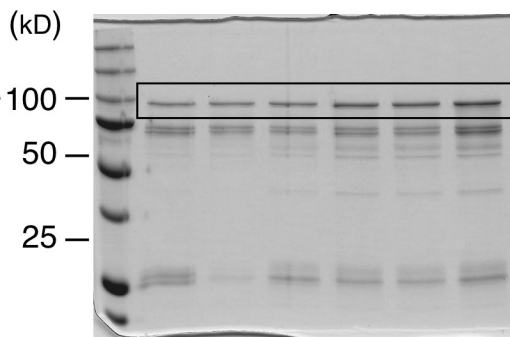
IB:pSer(CDKs)

4dother
mutants

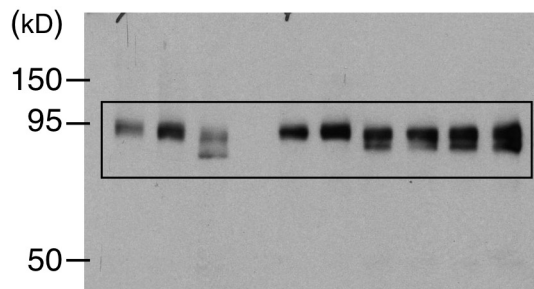
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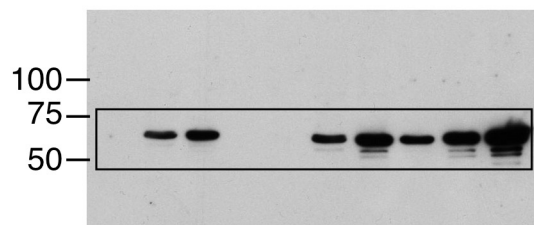
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4e³²P

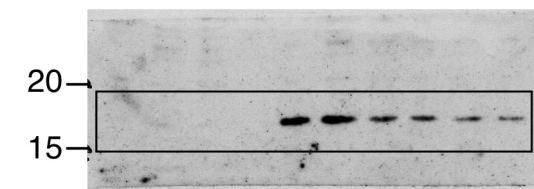
Coomassie

5a

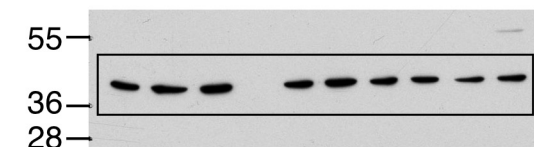
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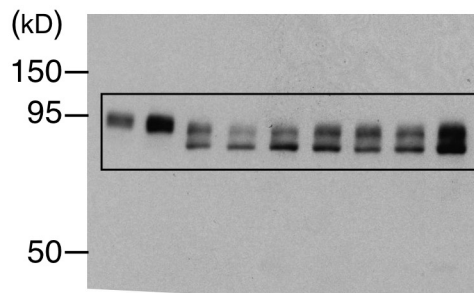
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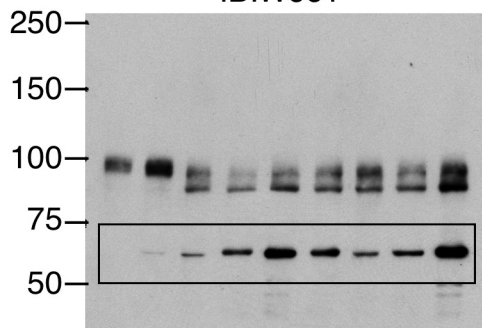
IB: pSer10 (HH3)



IB: Actin

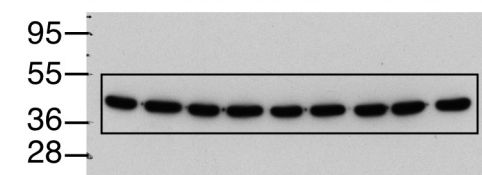
5b

IB: Wee1

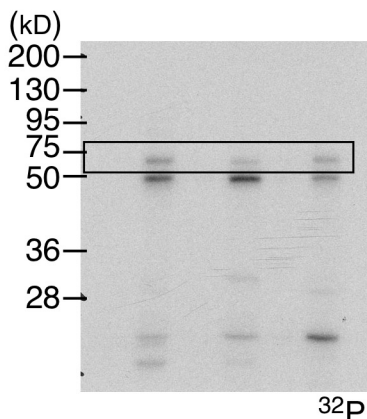
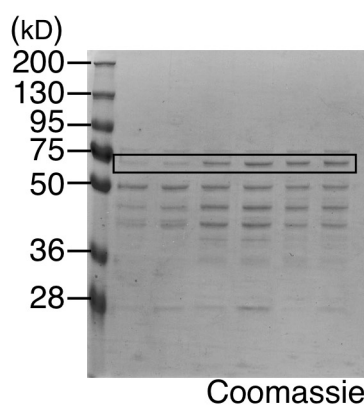


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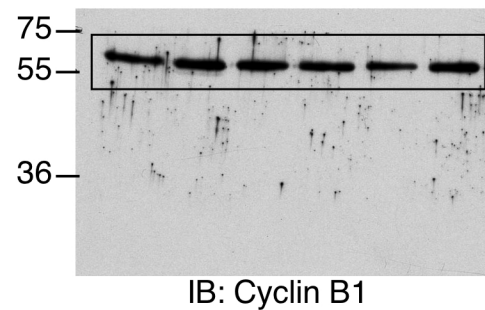
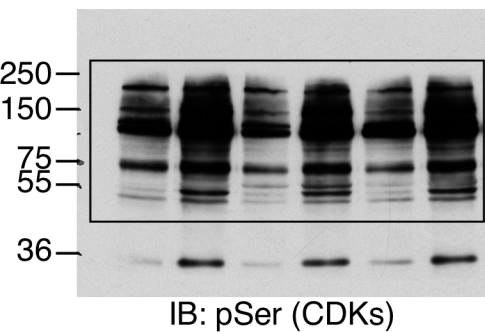
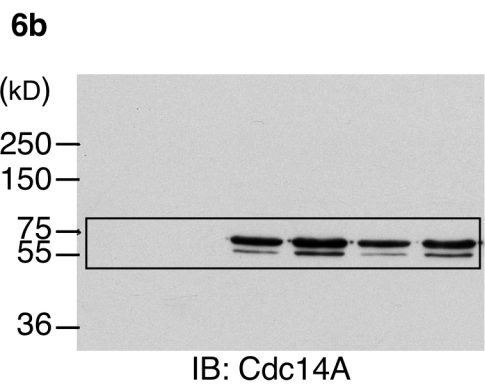
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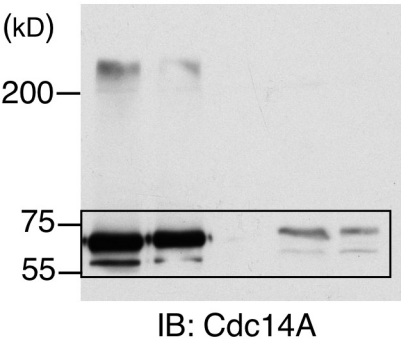
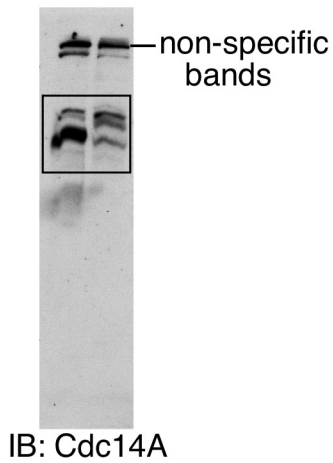
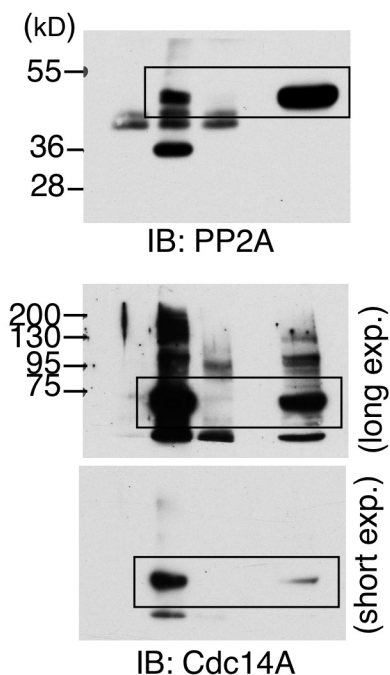
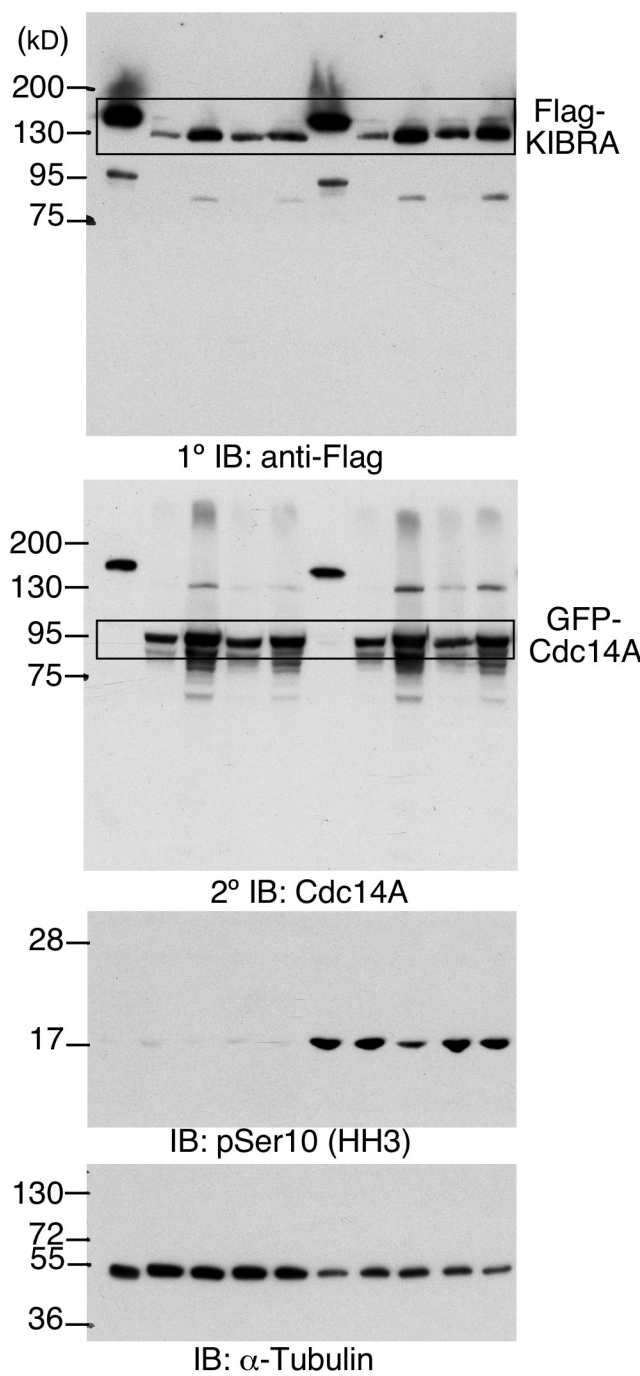


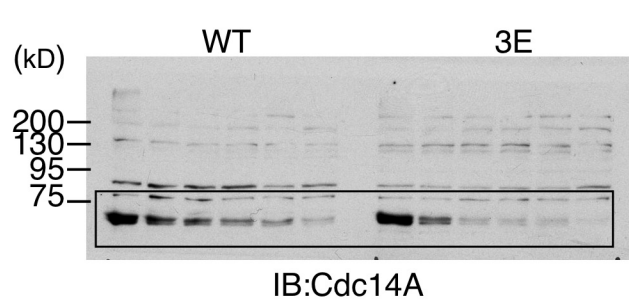
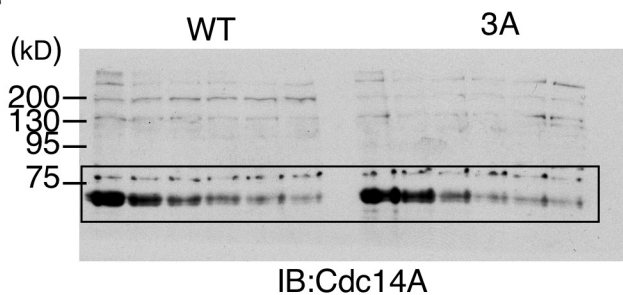
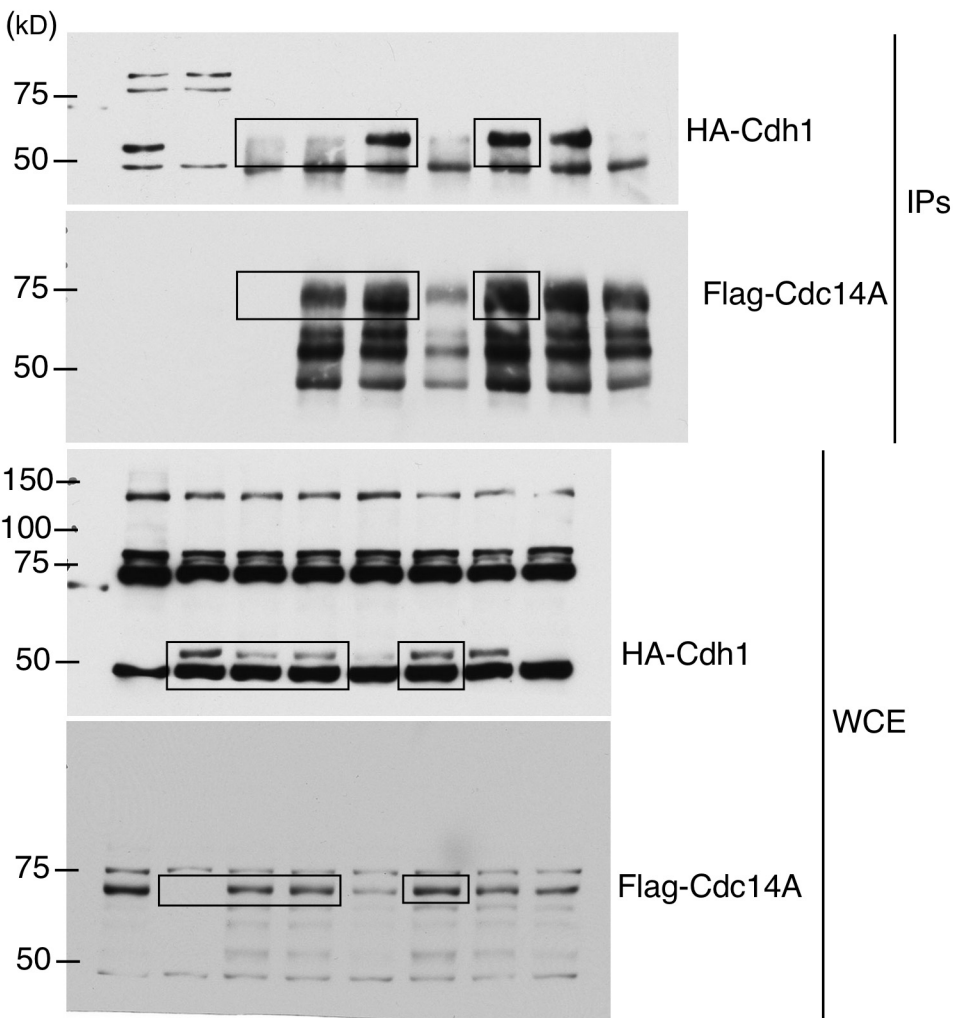
IB: Actin

5c³²P

Coomassie



S1b**S1e****S2****S4**

S7**S8a****S8b**