

Supplementary Information

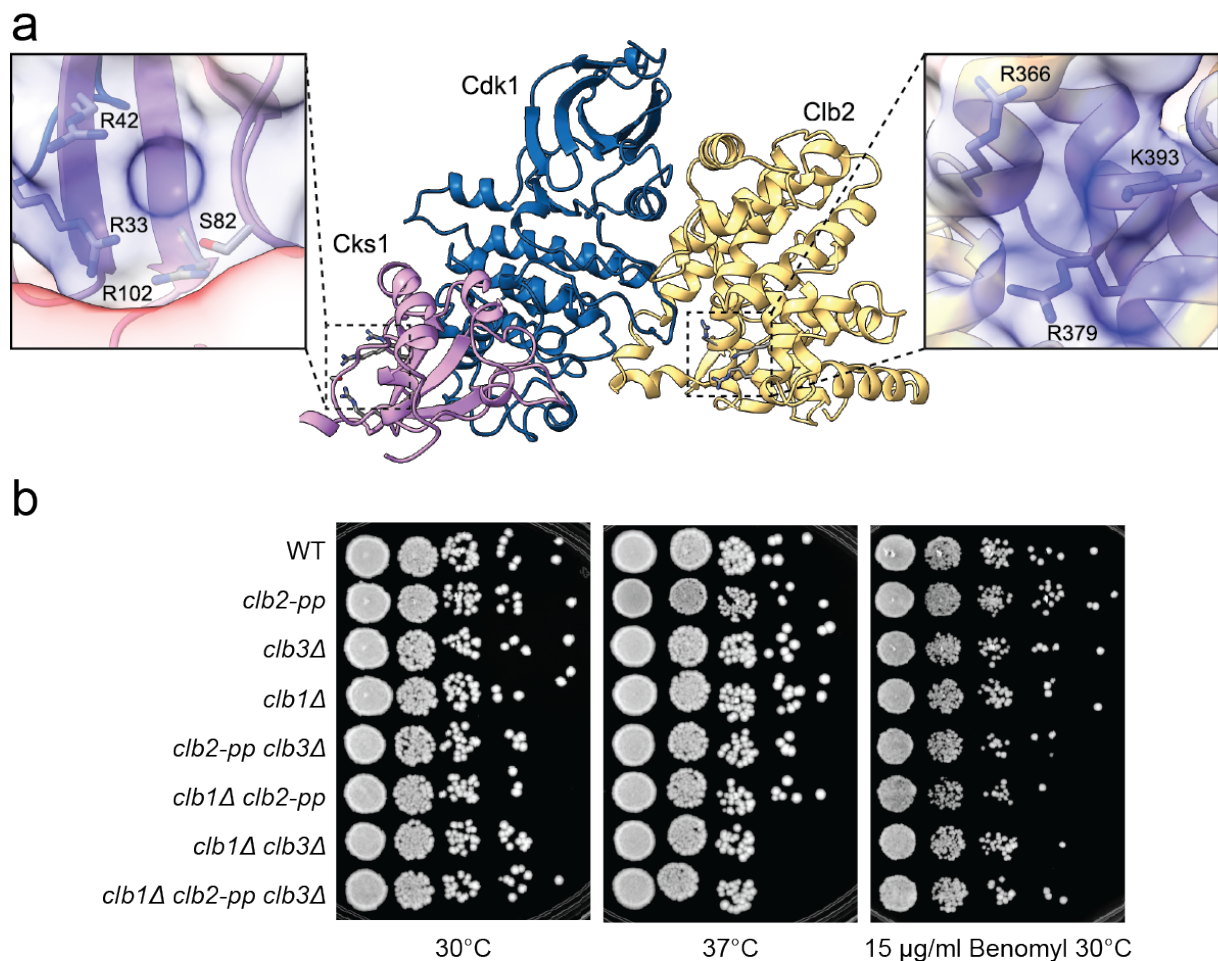
Phosphate-binding pocket on cyclin B governs CDK substrate phosphorylation and mitotic timing

Henry Y. Ng, Devon H. Whelpley, Armin N. Adly, Robert A. Maxwell, and David O. Morgan

Supplementary Figures 1-9

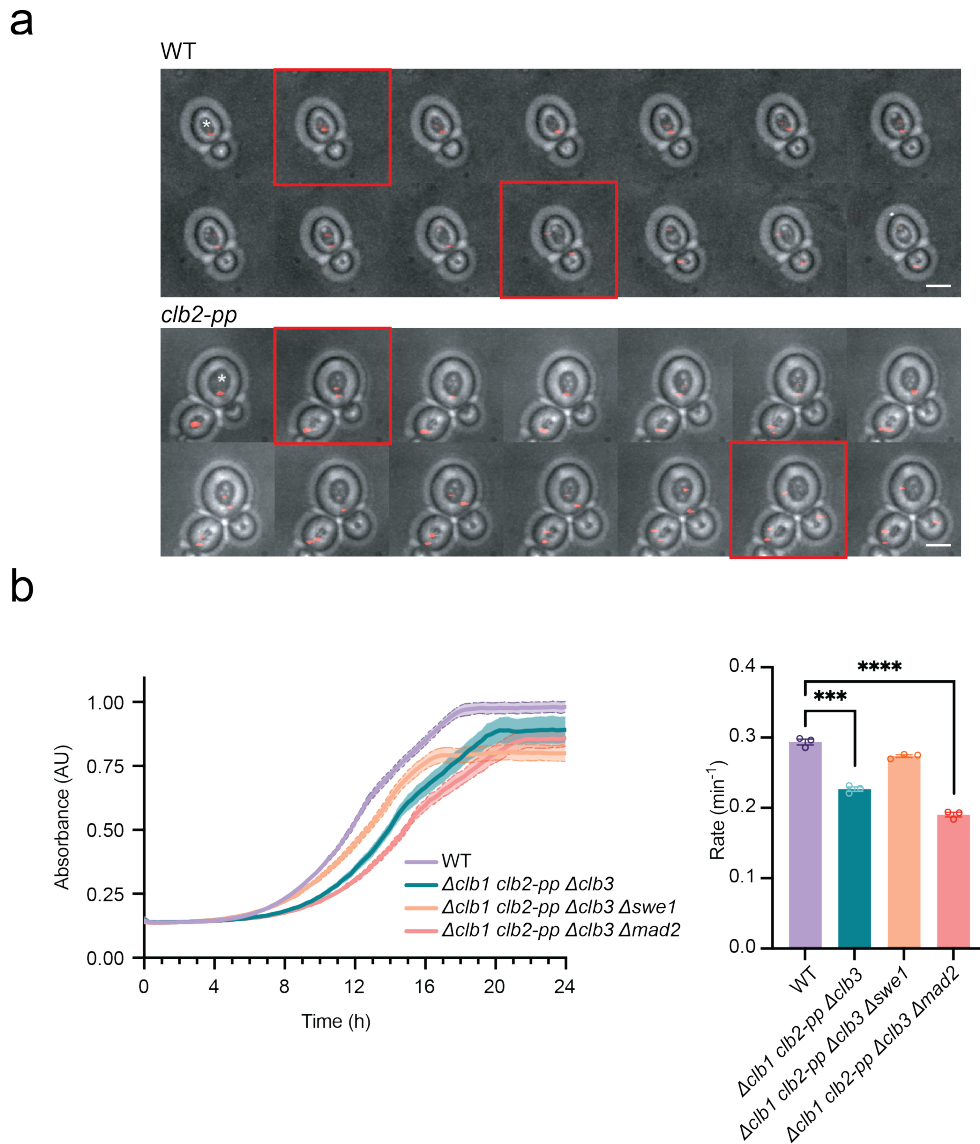
Supplementary Tables 1, 2

Supplementary References



Supplementary Fig. 1: Mutation of the conserved phosphate-binding pocket in Clb2 has little effect on yeast colony growth on plates.

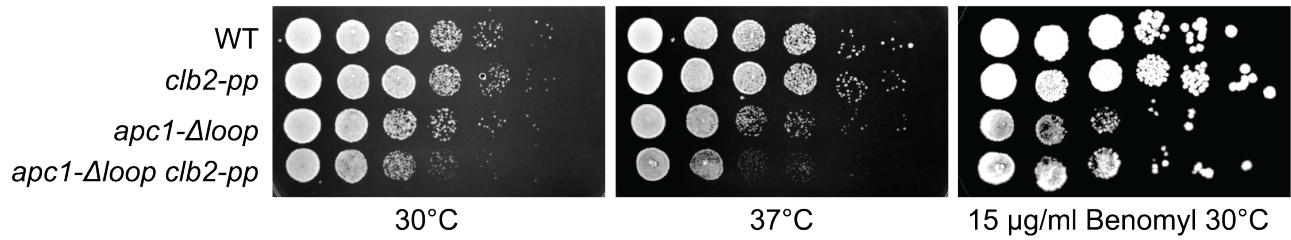
a, AlphaFold structure model of yeast Cdk1-Clb2-Cks1 complex. Insets show electrostatic surfaces of phosphate-binding pockets in Cks1 (left) and Clb2 (right), colored blue to indicate positive charge. **b**, The indicated yeast strains (with endogenous Myc-tagged securin) were spotted on YPD plates and incubated for 48 h at the indicated temperatures. Right panel shows growth on plates containing benomyl (15 µg/ml). Each spot represents 5 µl of a yeast culture at $OD_{600} = 0.1$ followed by four 1:10 serial dilutions. Source data are provided as a Source Data file.



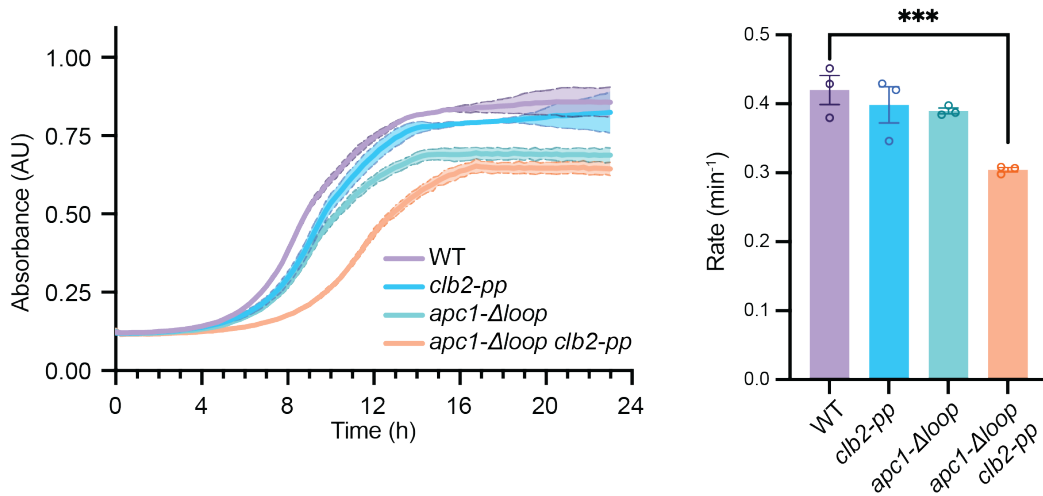
Supplementary Fig. 2: The *clb2-pp* mutation causes a mitotic delay.

a, Representative fluorescence image montages of yeast strains carrying mCherry-labeled Spc42, captured over 48 min at 2 min intervals. Images depict representative yeast cells with distinct mCherry foci (red). Frames indicated with red outlines depict (1) SPB separation and (2) initiation of spindle elongation. White asterisks mark the cells entering mitosis used for quantification. The scale bar represents 3 μm . See Fig. 2a for data from multiple cells. **b**, (left) Growth curves of indicated yeast strains, with OD₆₀₀ recorded every 15 min. Data represent mean $\pm$ SD of three independent biological replicates. (right) Growth rates calculated by fitting OD₆₀₀/min to a logistic growth model. Data represent mean $\pm$ SD of three independent biological replicates. Statistical significance was determined using ANOVA (**p < 0.001; ****p < 0.0001). Source data are provided as a Source Data file.

a



b



Supplementary Fig. 3: The *clb2-pp* defect is not rescued by deletion of the putative regulatory loop in Apc1.

a, The indicated yeast strains were spotted on YPD plates and incubated for 48 h at the indicated temperatures. Right panel shows growth on plates containing benomyl (15 µg/ml). Each spot represents 5 µl of a yeast culture at OD₆₀₀=0.1 followed by four 1:10 serial dilutions. **b**, (left) Growth curves of indicated yeast strains, with OD₆₀₀ recorded every 15 min. Data represent mean ± SD of three independent biological replicates. (right) Growth rates calculated by fitting OD₆₀₀/min to a logistic growth model. Data represent mean ± SD of three independent biological replicates. Statistical significance was determined using ANOVA (***p<0.001). Source data are provided as a Source Data file.

Cdc16

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1 MKFCLYCCHC YIVICGKATH YYKSSKATS N LKSSNRVLMR NPMSPSEQHS QHNSTLAASP FVSNVSAART QQSLPTDAQN
81 DRLQQPWNRT NTATSPYQSL ANSPLIQKLQ ANIMTPHQPS ANSNSNSNSI TGNVVNDNNL LASMSKNSMF GSTIPSTLRK
161 VSLQREYKDS VDGVRDEEDN DEDVHNNGDA AANANNDRS KLGHNGPLTT TLTITTTTAT QLDVSELSAI ERLRLWRFDA
241 LMQHMYRTAE YIADKVYNIS NDPDDAFWLQ QVYNNNNQYV RAVELITRNN LDGVNLCRY LLGLSFVKLQ RFDDALDVIG
321 EYNPFSEDPG TTAANTMSNN GNNNTSQPV TDGGIKMESS LCFLRGKIYF AQNNFNKARD AFREAILVDI KNFEAFEMLL
401 SKNLLTPQEE WDLFDSLDFK EFGEDKEIMK NLYKINLSKY INTEDITKSN EILAKDYKLA DNDVWVRSKV DICYTQCKFN
481 ECLELCETVL ENDEFNTNIL PAYIGCLYEL SNKNKLFLLS HRLAETFPKS AITWFSVATY YMSLDRISEA QKYYSKSSIL
561 DPSFAAAWLQ FAHTYALEGE QDQALTAYST ASRFFPGMHL PKLFLGMQFM AMNSLNLAES YFVLAYDICP NDPLVLNEMG
641 VMYFKKNEFV KAKKYLKAL EVVKDLPSS RTTISIQNLN GHTYRKLNN EIAIKCFRCV LEKNDKNSEI HCSLGVLK
721 TKKLQKAIDH LHSKLYLKP NNSATALLKN ALELNVLTSL DASHPLIDKS NLMSQASKDK ASLNKKRSSL TYDPVNMMAKR
801 LRTQKEIFDQ NNKALRKGGH DSKTGSNNAD DDFDADMELE *
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Cdc27

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1 MAVNPELAPF TLRGIPSPFD DQALSTIIQL QDCIQQAIQQ LNSTAEFLA ELLYAECSIL DKSSVWSDA VVLYALSFL
81 NKSYPHTAFQI SKEFKEYHLG IAYIFGRCL QLSQGVNEAI LTLISIINF SSNSNTRIN MVLNSNLVHI PDLATLNCLL
161 GNLPMKLDHS KEFAGYHSEA LAINPYLWES YEAIKMRAT VDLKRVFFDI AGKKSNSHNN NAASSFPSTS LSHFEPRSQP
241 SLYSKTNKNG NNNINNNVNT LQSSNSPPS TSASSFSSIQ HFSRSQQQQA NTSIRTCQNK NTQTTPKNPAI NSKTSSALPN
321 NISMNLVSPS SKOPTISSLA KVYNNRKLTT TTPSKLLNND RNHQNNNNNNN NNNNNNNNNN NNNNNNNNII NKTTFKTPRN
401 LYSSTGRITL SKKNPRSLII SNSILTSYD ITPLEIMYNF ALILRSSSQY NSFKAIRLFE SQIPSHIKDT MPWCLVQLGK
481 LHFEIINYDM SLKYFNRLKD LQPARVKDME IFSTLLWLHL DKVKSSNLAN GLMDTMPNKP ETWCCIGNLL SLQKDHDAI
561 KAFKATQQLD PNFAYAYTLQ GHEHSSNDSS DSAKTCYRKA LACDPQHYN YGLGTSAMK LGQYEEALLY FEKARSINPV
641 NVVLICCCGG SLEKLGKKEK ALQYYELACH LQPTSSLSKY KMGQLYSMT RYNVALQTFE ELVKLVPPDA TAHYLLGQTY
721 RIVGRKKDAI KELTVAMNLD PKGNQVIIDE LQKCHMQE *
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Bud6

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1 MKMAVDDPTY GTPKIKRTAS SSSSIETTVT KLLMSTKHL QVLTQWSKGT TSGRLVSDAY VQLGNDKFVV SKFFMHAKVD
81 MSVDGVDPMA LRRVLEVTLR EPPSDETLN HLPKIREIIV TLLDKLKVQK AILKNMQQEH RISVKSHHQ NPSFTSNLSL
161 GSEGTREGTP LSRKSSIVR DQRQSDSVEN SYGEKVNSTG TGPASQSAE ATLTKPRNI KQNLKSNAP NASDDDDALS
241 QLKKGTNLQR RASKRYSAYH MAKLTNQSTT EAAAAAGLMT TSPSPMLHLE ETVRKSKLYG NNNNDDDRNI NSAENKGS
321 DDVSKASPLA KTPLPIENVR ASPRRLSSV TTPSPDKAMNG TCPVFLRIGD KTKKCHVQLP TTKNALRLIF IERFAYSPGA
401 NSFPIIYIMD PQYGVFYELE ELNLLDIKEG FVIELKLEEN PNNTIKEFID TVKMEISNSQ NDIIRHLKEM SFGSAISGKQ
481 TEVLPQPGLE ANKHDLVGQN KKDDDKTIK IQYELGKIQ VHNINRSNIN ETIFNIRKV DNFKSLFSFA KNSSNRMYME
561 KSQTELGDLS DTLISKVDDL QDVEIMRKD VAERRSQPAK KKLTVSKDL ENAQADVLKL QEFIDTEKPH WKTWEAELD
641 KVCEEQQLT LQEELILDLE EDLGKALETF DLIKLCCEEQ EKNPSRSKSN PILPIMRPGT FNQVREQVMV AVQSLNPDDH
721 SRVEAIDKAE KMWEMERKLK ASNEFDDELE NFGVNSNLK SGGFEEVERI RKQKDEANLR AYFGPGFT *
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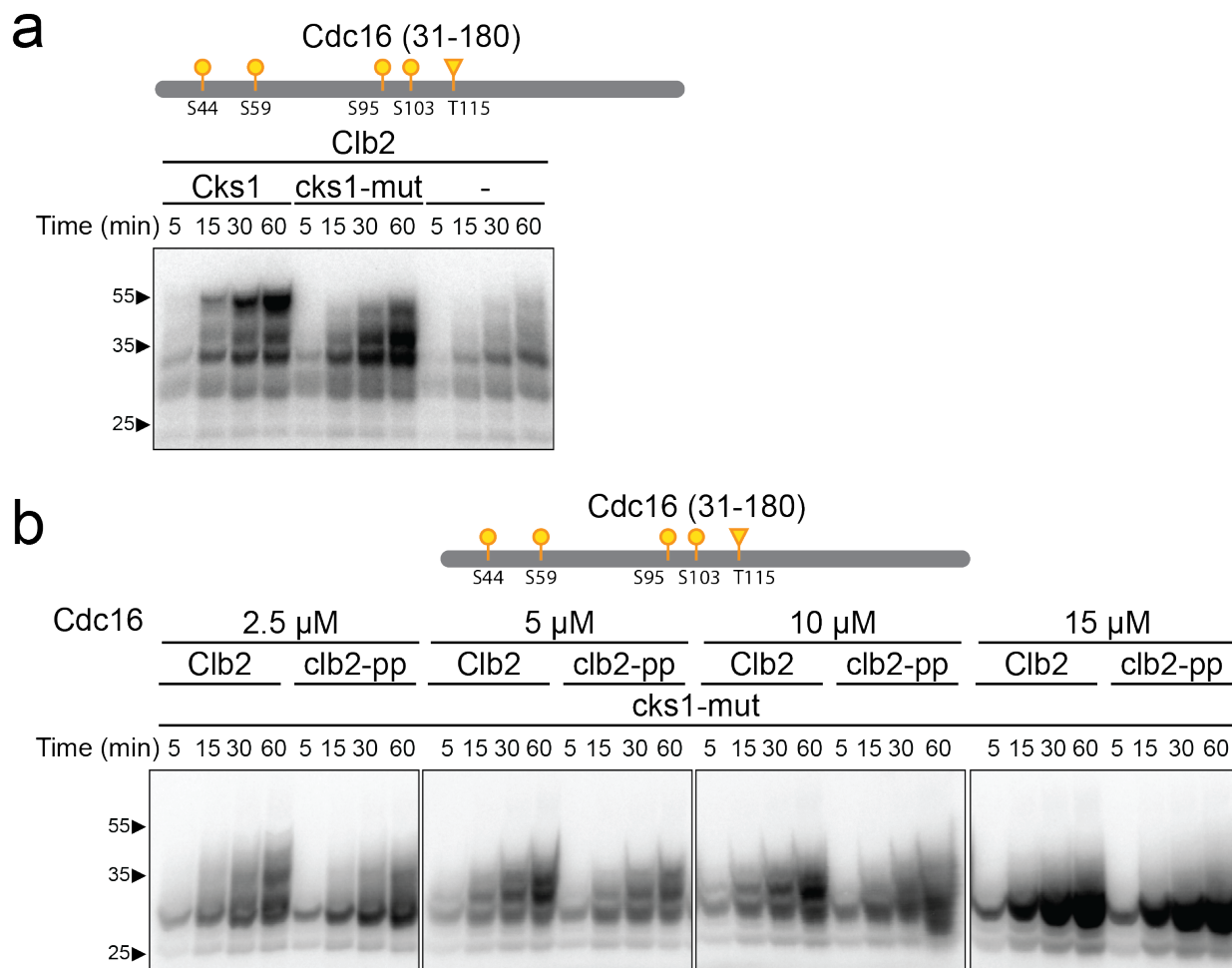
Spa2

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1 MGTSSSEVSLA HHRDIFHYV SLKTFEFTV ENRDRSNSTR AQKARAKLLK LSSSQFYELS TDVSDQLRR IGEDANQPDY
81 LLPKANFHMK RNQARQKLAN LSQTRFNDLL DDILFEIKRR GFDKDLAPR PPLPQPMQK VSKDSDDTAR TSTNSSSVTQ
161 VAPNVSVQPS LVIPKMASID WSSEEEEEEQ VKEKNEPEG KQTSMDKEKE AKPALNPIVT DSDLPDSQVL ARDITSMART
241 PTTTHKNYWD VNDSPIIKVD KDIDNEKGPE QLKSPVEQRA ENNNPNSEME DKVKELTDLN SDLHLQIEDL NAKLASLTSE
321 KEKEKKEEKE EKEKENLKI NYTIDESFQK ELLSLNSQIG ELSIENENLK QKISEFELHQ KKNNDNNDLK ITDGFISKYS
401 SADGLIPAQY ILNANNLIQ FTRLASVPI GDSTAISHQI GEELFQILSQ LSNLISQLLL SADLLQYKQD VILLKASLSH
481 AITSIRYFSV YGPVLIPKIT VQAAVSEVCF AMCNLIDSAK IKSDSNGEST TSNEGNRQVL EYSPTATT TPPTFPSTSG
561 INMKKGFINP RKPASFLNDV EEEE SPVKPL KITQKAINSP IIRPSSSNGV PTTSRKPSGT GLFSLMIDSS IAKNSSHKED
641 NDKYVSPIKA VTSASNSASS NISEIPKLTL PPQAKIGTVI PPSENQVPI KIENTEEDNK RSDITNEISV KPTSSIADKL
721 KQFEQSSSEK SSPKENPIAK EEMDSKPKLS NKFITSMDV STDDSSSDGN ENDDADDDDD FTYMALKQTM KREGSKIEKN
801 NDKSLPANIV ELDLHESPEK VKIESPEK EITSSEMSE MPSSSLPKRL VEDVEPSEMP EKGASVESVR KKNFQEPGLN
881 VESPDMTQKV KSLGMTGKAV GPESDSRVS PGMTGQIKSL NMAGKVVGPE ADSRVESPGM KEQIKSLGMT GKITAQESIK
961 SPEAARKLAS SGEVDKIESP RMVRESSELE AVGNTIPSNM TVKME SPNLK GNTVSEPQEI RRDIASSEPI ENVDPPKVLK
1041 KIVFPKAVNR TGSPKSVEKT PSSATLKSG LPEPNSQIVS PELAKNSPLA PIKKNVELRE TNKPTTETIT SVEPTNKDAN
1121 TSWRDADLNR TIKREEEDED FDRVNHNIQI TGAYTKTGKI DYHKIPVDRK AKSEAEVHTS EEDIDESNNV NGKRADAQIH
1201 ITERKHAFVN PTENSQVKT SHSPFLNSKP VQYENSESNG GINNHIKIKN TGETTAHDEK HYSDDDDSSY QFVPMKHEEQ
1281 EQEQNRSEEE ESEDDDEEEE DSDFDVDTFD IENPDNTLSE LLLYLEHQTM DVISTIQSL TSIKKPQVTK GNLRGESNAI
1361 NQVIGQMADA TSISMEQSRN ANLKKHGDWV VQSLRDCSRR MTILCQLTGD GILAKEKSDQ DYADKNFKQR LAGIAFDVAK
1441 CTKELVKTVE EASLKDEINY LNSKLK *
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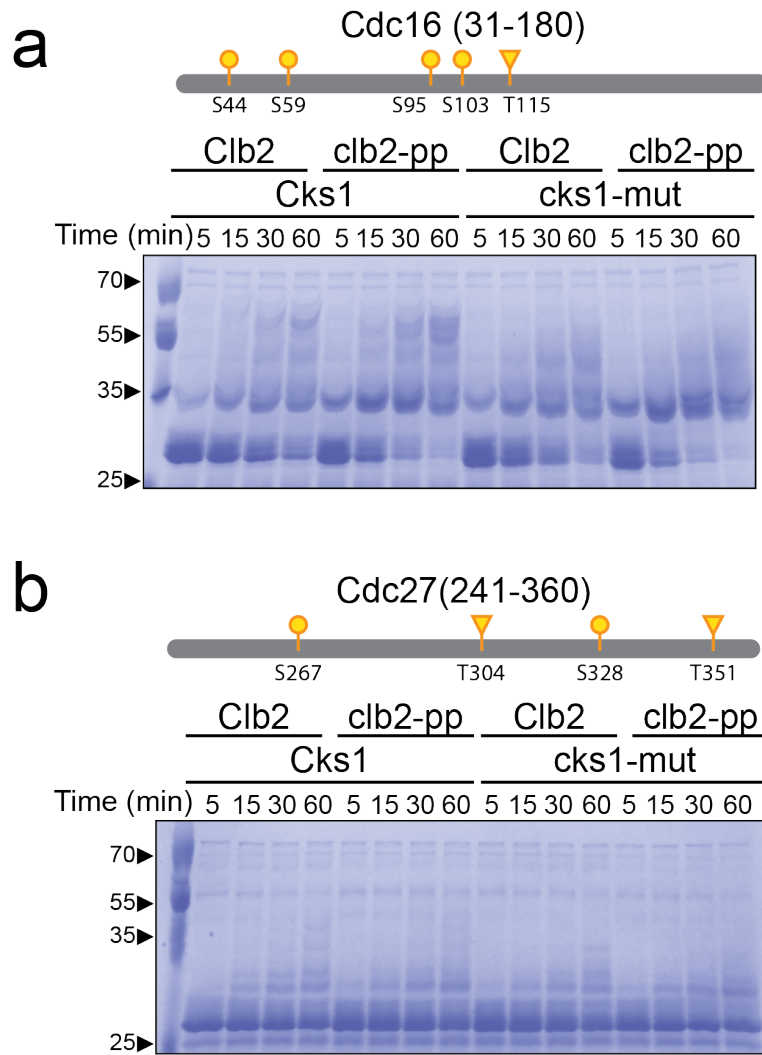
Supplementary Fig. 4: Amino acid sequences of the CDK substrates analyzed in this study.

CDK consensus sequences (S/T*-P and S/T*-PxK/R) are highlighted in yellow. Highlighted in green is the disordered fragment used for kinase reactions *in vitro*. Amino acids colored in red were identified as phosphorylation sites in our mass spectrometry analysis (Supplementary Data 2, Fig. 6).



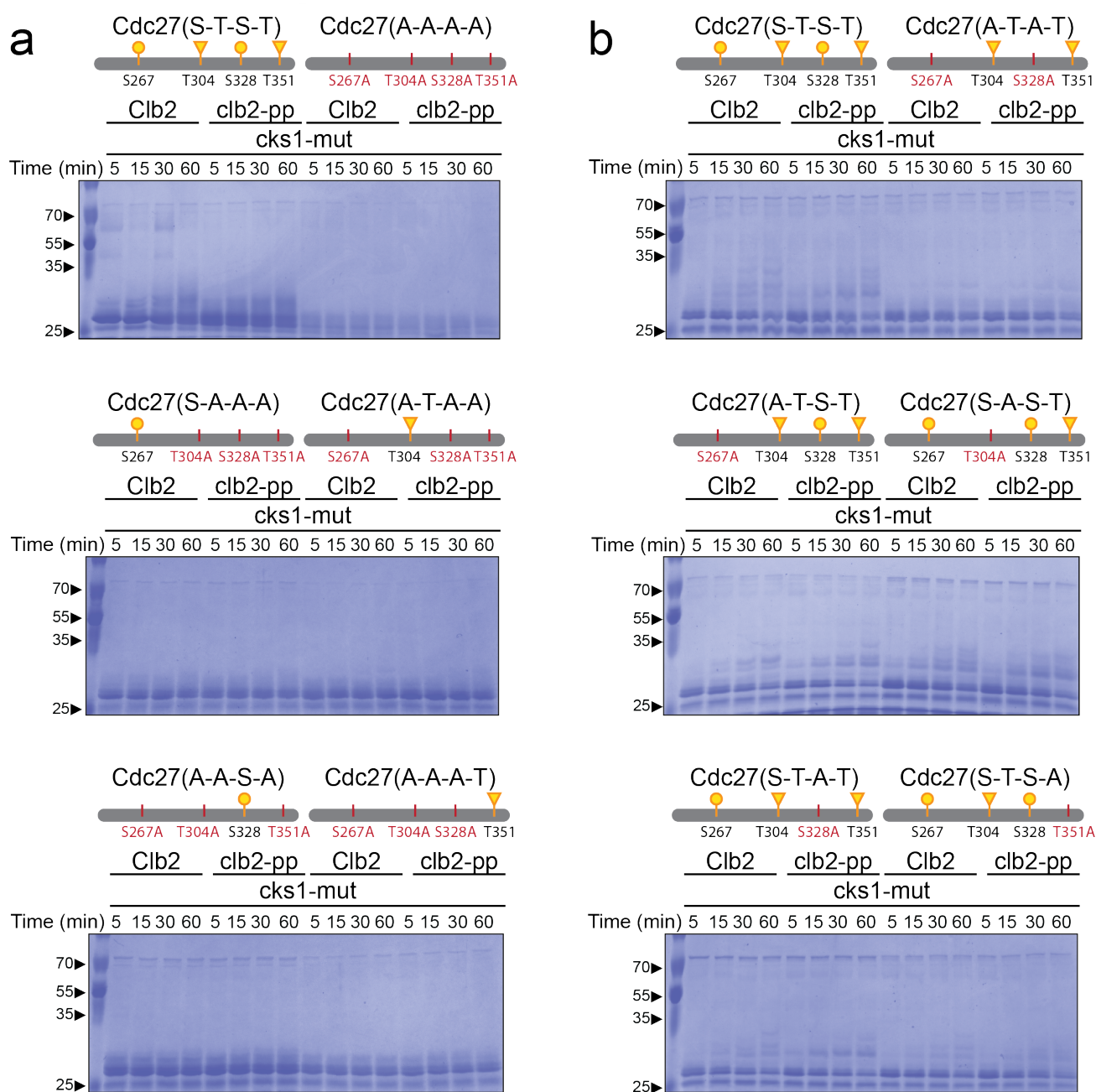
Supplementary Fig. 5: Multi-site phosphorylation of CDK substrates *in vitro* is dependent on the presence of Cks1 and substrate concentration.

a, 2.5 μ M purified Cdc16 fragment (aa 31-180) was incubated with 150 nM wild-type or *clb2-pp* Clb2-Cdk1 plus wild-type, mutant or no Cks1 and radiolabeled ATP. **b**, The indicated concentrations of purified Cdc16 fragment (aa 31-180) were incubated with 150 nM wild-type or *clb2-pp* Clb2-Cdk1 plus mutant Cks1 and radiolabeled ATP. Diagrams at top indicate suboptimal (S/T*-P; yellow) CDK consensus sites (S: circle; T: triangle) in the tested fragment (see Supplementary Fig. 4 for complete sequences). Source data are provided as a Source Data file.



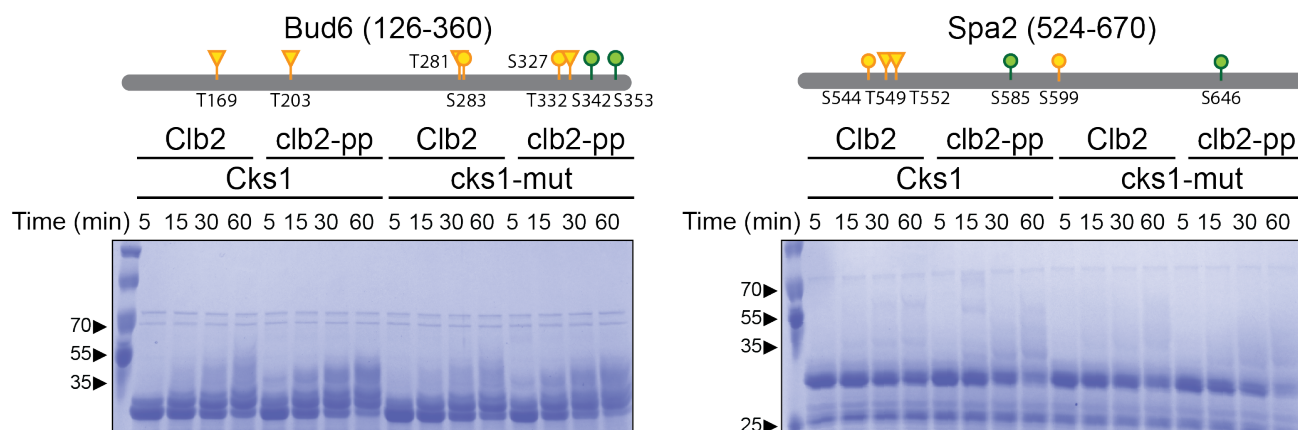
Supplementary Fig. 6: Phospho-pocket mutations reduce phosphorylation of APC/C subunits *in vitro*.

Phos-tag polyacrylamide gels for the kinase assays in Fig. 3b and c were stained with Coomassie Brilliant Blue to demonstrate extensive PP-dependent multisite phosphorylation of some substrates. Source data are provided as a Source Data file.



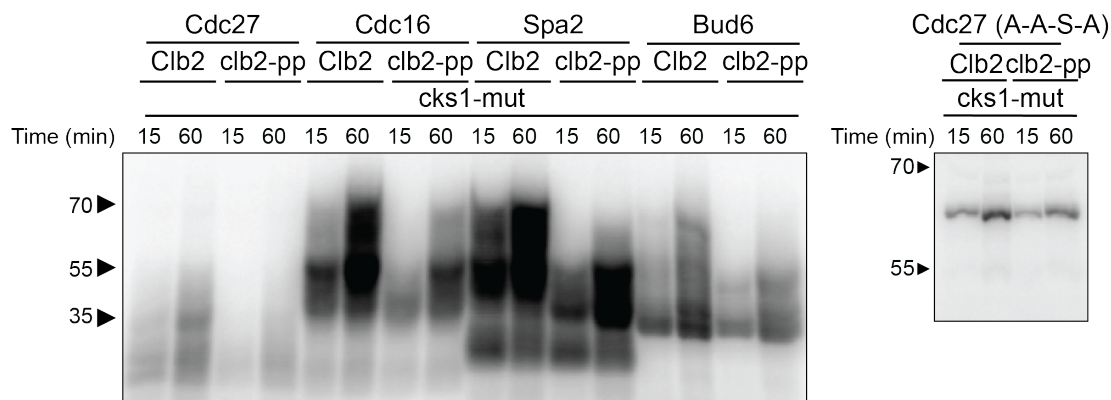
Supplementary Fig. 7: Phospho-pocket mutations reduce phosphorylation of CDK consensus sites on Cdc27.

Phos-tag polyacrylamide gels for the kinase assays in Fig. 4 were stained with Coomassie Brilliant Blue to demonstrate extensive PP-dependent multisite phosphorylation of some substrates. Source data are provided as a Source Data file.



Supplementary Fig. 8: Phospho-pocket mutations reduce phosphorylation of polarisome subunits

Phos-tag polyacrylamide gels for the kinase assays in Fig. 5 were stained with Coomassie Brilliant Blue to demonstrate extensive PP-dependent multisite phosphorylation of some substrates. Source data are provided as a Source Data file.



Supplementary Fig. 9: Kinase reaction products for samples analyzed by mass spectrometry.

Radiolabeled kinase reactions with the five indicated protein fragments were prepared in parallel to the unlabeled samples used for mass spectrometry analysis. Kinase concentrations were 200 nM (+ 200 nM mutant Cks1); substrate concentrations were 10 μ M. Reaction products were analyzed by Phos-tag SDS-PAGE (7.5% for gel on left; 12.5% polyacrylamide for gel on right). Source data are provided as a Source Data file.

Supplementary Table 1: Yeast Strains used in this work.

Name	Genotype	Source
DOM123	<i>W303, MATa, bar1-HisG</i>	ATCC
JBA53	<i>trp1::pGAL-NDD1-9xMYC-TRP1</i>	¹
HN40	<i>clb2Δ::HYGR, trp1::pGAL-NDD1-9xMYC-TRP1</i>	This Study
JBA71	<i>clb2::CLB2-R336A,R379A,K383A-HYGR, trp1::pGAL-NDD1-9xMYC-TRP1</i>	¹
HN01	<i>clb3Δ::pAgTEF-KANMX-tAgTEF, trp1::pGAL-NDD1-9xMYC-TRP1</i>	This Study
HN02	<i>clb1Δ::pAgTEF-NATNT-tADH1, trp1::pGAL-NDD1-9xMYC-TRP1</i>	This Study
HN03	<i>clb3Δ::pAgTEF-KANMX-tAgTEF, clb2::CLB2-R336A,R379A,K383A-HYGR, trp1::pGAL-NDD1-9xMYC-TRP1</i>	This Study
HN04	<i>clb1Δ::pAgTEF-NATNT-tADH1, clb2::CLB2-R336A,R379A,K383A-HYGR, trp1::pGAL-NDD1-9xMYC-TRP1</i>	This Study
HN05	<i>clb3Δ::pAgTEF-KANMX-tAgTEF, clb1Δ::pAgTEF-NATNT-tADH1, trp1::pGAL-NDD1-9xMYC-TRP1</i>	This Study
HN06	<i>clb3Δ::pAgTEF-KANMX-tAgTEF, clb1Δ::pAgTEF-NATNT-tADH1, clb2::CLB2-R336A,R379A,K383A-HYGR, trp1::pGAL-NDD1-9xMYC-TRP1</i>	This Study
DOM1324	<i>SPC42-mCherry-HIS3</i>	²
HN24	<i>SPC42-mCherry-HIS3, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
DOM1228	<i>pds1::PDS1-myc13-TRP1</i>	³
HN10	<i>pds1::PDS1-myc13-TRP1, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
HN18	<i>pds1::PDS1-myc13-TRP1, clb3Δ::pAgTEF-KANMX-tAgTEF</i>	This Study
HN19	<i>pds1::PDS1-myc13-TRP1, clb1Δ::pAgTEF-NATNT-tADH1</i>	This Study
HN13	<i>pds1::PDS1-myc13-TRP1, clb3Δ::pAgTEF-KANMX-tAgTEF, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
HN16	<i>pds1::PDS1-myc13-TRP1, clb1Δ::pAgTEF-NATNT-tADH1, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
HN22	<i>pds1::PDS1-myc13-TRP1, clb3Δ::pAgTEF-KANMX-tAgTEF, clb1Δ::pAgTEF-NATNT-tADH1</i>	This Study
HN21	<i>pds1::PDS1-myc13-TRP1, clb3Δ::pAgTEF-KANMX-tAgTEF, clb1Δ::pAgTEF-NATNT-tADH1, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
HN43	<i>pds1::PDS1-myc13-TRP1, swe1Δ::pAgTEF-NATNT-tADH1</i>	This Study
HN44	<i>pds1::PDS1-myc13-TRP1, swe1Δ::pAgTEF-NATNT-tADH1, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
DW03	<i>pds1::PDS1-myc13-TRP1, swe1Δ::pAgTEF-NATNT-tADH1, clb3Δ::pAgTEF-KANMX-tAgTEF, clb1Δ::pAgTEF-NATNT-tADH1</i>	This Study
DW04	<i>pds1::PDS1-myc13-TRP1, swe1Δ::pAgTEF-NATNT-tADH1, clb3Δ::pAgTEF-KANMX-tAgTEF, clb1Δ::pAgTEF-NATNT-tADH1, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
DOM1231	<i>pds1::PDS1-MYC-TRP1, mad2Δ::KAN</i>	³
HN39	<i>pds1::PDS1-MYC-TRP1, mad2Δ::KAN, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
DW01	<i>pds1::PDS1-MYC-TRP1, mad2Δ::KAN, clb3Δ::pAgTEF-KANMX-tAgTEF, clb1Δ::pAgTEF-NATNT-tADH1</i>	This Study
DW02	<i>pds1::PDS1-MYC-TRP1, mad2Δ::KAN, clb3Δ::pAgTEF-KANMX-tAgTEF, clb1Δ::pAgTEF-NATNT-tADH1, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
HN41	<i>pds1::PDS1-myc13-TRP1, apc1::APC1Δ225-365-NATNT</i>	This Study
HN42	<i>pds1::PDS1-myc13-TRP1, apc1::APC1Δ225-365-NATNT, clb2::CLB2-R336A,R379A,K383A-HYGR</i>	This Study
DOM900	<i>cdc16::CDC16-TAP-HIS3</i>	⁴

Supplementary Table 2: Plasmids used in this work.

Name	Description	Source
pHN56	pET28a-6xHis-SUMO-Cib2(187-491)	This Study
pHN57	pET28a-6xHis-SUMO-Cib2(187-491, R336A, R379A, K383A)	This Study
pHN58	pLIB-GST-TEV-Cdc28	This Study
pEV652	pET11a-Cks1 (untagged)	⁵
pEV653	pET11a-Cks1 mutant (R33A, S82E, R102A)	⁶
pDW5	pET28a-6xHis-SUMO-Cdc16 (31-180)	This Study
pHN44	pET28a-6xHis-SUMO-Cdc27 (241-360)	This Study
pDW10	pET28a-6xHis-SUMO-Cdc27 (241-360, S267A, T304A, S328A, T351A)	This Study
pHN61	pET28a-6xHis-SUMO-Cdc27 (241-360, T304A, S328A, T351A)	This Study
pHN62	pET28a-6xHis-SUMO-Cdc27 (241-360, S267A, S328A, T351A)	This Study
pHN63	pET28a-6xHis-SUMO-Cdc27 (241-360, S267A, T304A, T351A)	This Study
pHN64	pET28a-6xHis-SUMO-Cdc27 (241-360, S267A, T304A, S328A)	This Study
pAA29	pET28a-6xHis-SUMO-Cdc27 (241-360, S267A, S328A)	This Study
pAA25	pET28a-6xHis-SUMO-Cdc27 (241-360, S267A)	This Study
pAA26	pET28a-6xHis-SUMO-Cdc27 (241-360, T304A)	This Study
pAA27	pET28a-6xHis-SUMO-Cdc27 (241-360, S328A)	This Study
pAA28	pET28a-6xHis-SUMO-Cdc27 (241-360, S351A)	This Study
pHN47	pET28a-6xHis-SUMO-Bud6 (126-360)	This Study
pHN46	pET28a-6xHis-SUMO-Spa2 (524-670)	This Study

Supplementary References

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