



Cdc14 activation requires coordinated Cdk1-dependent phosphorylation of Net1 and PP2A–Cdc55 at anaphase onset

Soraya Játiva¹ · Ines Calabria^{1,2} · Yolanda Moyano-Rodriguez¹ · Patricia Garcia¹ · Ethel Queralt¹

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Abstract

Exit from mitosis and completion of cytokinesis require the inactivation of mitotic cyclin-dependent kinase (Cdk) activity. In budding yeast, Cdc14 phosphatase is a key mitotic regulator that is activated in anaphase to counteract Cdk activity. In metaphase, Cdc14 is kept inactive in the nucleolus, where it is sequestered by its inhibitor, Net1. At anaphase onset, downregulation of PP2A^{Cdc55} phosphatase by separase and Zds1 protein promotes Net1 phosphorylation and, consequently, Cdc14 release from the nucleolus. The mechanism by which PP2A^{Cdc55} activity is downregulated during anaphase remains to be elucidated. Here, we demonstrate that Cdc55 regulatory subunit is phosphorylated in anaphase in a Cdk1–Clb2-dependent manner. Interestingly, *cdc55-ED* phosphomimetic mutant inactivates PP2A^{Cdc55} phosphatase activity towards Net1 and promotes Cdc14 activation. Separase and Zds1 facilitate Cdk-dependent Net1 phosphorylation and Cdc14 release from the nucleolus by modulating PP2A^{Cdc55} activity via Cdc55 phosphorylation. In addition, human Cdk1–CyclinB1 phosphorylates human B55, indicating that the mechanism is conserved in higher eukaryotes.

Keywords Cell cycle · Phosphatases · Mitosis · Mitotic exit · PP2A–Cdc55 · Zds1 · Separase · FEAR

Introduction

Mitotic exit encompasses an ordered series of events that occur between chromosome segregation during anaphase and the completion of cell division by cytokinesis (reviewed in Refs. [1, 2]). The budding yeast phosphatase, Cdc14, is a major regulator of these events. Cdc14 is the major Cdk-counteracting phosphatase, which promotes cyclin destruction, inducing the accumulation of the Cdk1 inhibitor Sic1 and dephosphorylating Cdk1 targets [3, 4]. Cdc14 is kept inactive in the nucleolus by binding to its nucleolar inhibitor, Net1 (also called Cfi1), for most of the cell cycle [5,

6]. Release of Cdc14 from the nucleolus depends on two regulatory networks: the Cdc-Fourteen Early Anaphase Release (FEAR) network, which initiates Cdc14 release, and the mitotic exit network (MEN, also known as the Hippo pathway in higher eukaryotes) that maintains Cdc14 activity following release. Numerous proteins, including separase, Cdk1–Clb2, PP2A^{Cdc55}, Zds1, Slk19, polo-like kinase Cdc5, Spo12, Fob1 and Bns1, have been implicated in FEAR-Cdc14 release (reviewed in Refs. [7–9]). The MEN is a G-protein signaling cascade (reviewed in Ref. [10, 11]) that is associated with the spindle pole body (SPBs, yeast centrosomes) and this localization is crucial for their function in mitotic exit [12–16].

In metaphase, upon activation of the anaphase-promoting complex (APC) by its co-activator Cdc20, APC^{Cdc20}, securin is ubiquitinated, thereby activating separase. The release of separase from its inhibitor securin is crucial to Cdc14 activation [17–19]. FEAR-dependent Cdc14 release requires Net1 phosphorylation at Cdk consensus sites [20]. Net1 is maintained in an under-phosphorylated state in metaphase by the phosphatase PP2A^{Cdc55} [17]. Separase and Zds1/2-dependent PP2A^{Cdc55} downregulation specifically initiates Cdk1-dependent phosphorylation of Net1 in anaphase [21, 22]. Cdc14 release from the nucleolus is also

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✉ Ethel Queralt
equeralt@idibell.cat

¹ Cell Cycle Group, Cancer Epigenetics and Biology Program (PEBC), Institut d'Investigacions Biomèdiques de Bellvitge (IDIBELL), Av. Gran Via de L'Hospitalet 199-203, 08908 L'Hospitalet de Llobregat, Barcelona, Spain

² Present Address: Genomics Unit, Medical Research Institute La Fe, Valencia, Spain

regulated by Cdc5-dependent Net1 phosphorylation [23, 24]. The first wave of Cdc14 release induced by FEAR eventually activates MEN by dephosphorylating the PAK kinase Cdc15, which further activates the downstream effectors Dbf2–Mob1 and Cdc14 [18, 25–28]. Downregulation of PP2A^{Cdc55} phosphatase also regulates MEN activity through its action on Bfa1 and Mob1 [29]. However, the mechanism by which PP2A^{Cdc55} activity is inactivated during anaphase has yet to be elucidated.

It has been proposed that Zds1 acts as a PP2A^{Cdc55} regulator by recruiting Cdc55 to the nucleolus [22] via their physical interaction through the Zds1-C motif [22, 30]. Nevertheless, PP2A^{Cdc55} cannot be modulated solely as a function of its localization; an additional regulatory step may be required. Here, we demonstrate that Cdc55 phosphorylation is increased upon PP2A^{Cdc55} down-regulation at anaphase onset. Remarkably, Cdc55 phosphorylation depends on Cdk1–Clb2, regulates Cdc14 activation and directly controls PP2A^{Cdc55} phosphatase activity towards Net1. Moreover, Cdc55 phosphorylation requires active separase and functional Zds1 protein. Excitingly, down-regulation of PP2A^{Cdc55} is achieved by increasing the PP2A^{Cdc55} and Net1 physical interaction acting as a substrate trap and could serve as a new paradigm for how PP2A may regulate its substrates. Strikingly, human PP2A-B55 is also phosphorylated by Cdk1–CyclinB1, suggesting that the mechanism is conserved in mammals.

Methods

Yeast strains, plasmids and cell-cycle synchronization procedures

All yeast strains used in this study were derivatives of W303 and are summarized in Table 1. Epitope tagging of endogenous genes and gene deletions was performed by gene targeting using polymerase chain reaction (PCR) products. Cell synchronization using α -factor and metaphase arrest by Cdc20 depletion and entry into synchronous anaphase by Cdc20 re-introduction were also performed as previously described [31]. The plasmids containing Cdc55 mutations were obtained by site-directed mutagenesis using the plasmids E362 (*promCDC55-HA3-CDC55-3'utrCDC55*) and E427 (*promCDC55-Pk3-CDC55-3'utrCDC55*); E367 (*promCDC55-HA3-CDC55-T174E*), E368 (*promCDC55-HA3-CDC55-S301D*), E377 (*promCDC55-HA3-CDC55-T174D-S301D*), E378 (*promCDC55-HA3-CDC55-T174A*), E379 (*promCDC55-HA3-CDC55-S301A*), E380 (*promCDC55-HA3-CDC55-T174A-S301A*), E430 (*promCDC55-Pk3-CDC55-T174A-S301A*) and E463 (*promCDC55-Pk3-CDC55-T174E-S301D*). All experiments were done at least three times.

Immunoprecipitation, phosphatase assay, and kinase assays

The co-immunoprecipitation assays were performed as described [22, 24]. Protein extracts were prepared by mechanical lysis using glass beads. The clarified extracts were incubated with antibody, and the immunocomplexes were adsorbed onto magnetic protein-A Dynabeads® (Life Technologies). The beads were washed in extraction buffer and the protein-bound fraction eluted with SDS-PAGE loading buffer. The antibody used for immunoprecipitation was the α -Pk clone SV5-Pk1 (Serotec), α -Clb2 (Santa Cruz Biotechnologies) and α -myc 9E10 (Covance). The images were obtained using the Amersham Imager 600 (GE Healthcare) and quantified using ImageQuant software (GE Healthcare). For the quantification of the western blots, all data points from a replicate were first normalized to the control and later mean and SEM were calculated.

The radioactive Cdk1–Clb2 kinase assays were done as described [17]. The non-radioactive kinase assays were done using Pro-Q Diamond to detect phosphorylation [24]. Pk epitope-tagged Clb2 or Clb2 protein were immunopurified on protein-A Dynabeads, as above. The beads were washed two times more with kinase buffer (50 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 5 mM β -glycerolphosphate, 150 μ M ATP) and incubated with 20 μ L kinase reaction mix containing bacteria-purified GST-Cdc55(1–193) or GST-Cdc55(1–193)-T174A for 30 min at 20 °C. Reactions were terminated by adding 2 \times SDS-PAGE loading buffer. Phosphorylation was detected by Pro-Q Diamond staining (Life Technologies) following the manufacturer's instructions, and images were acquired using Typhoon FLA 9500 (GE Healthcare). Clb2 recovered from the beads and the GST-Cdc55 were visualized using the Amersham Imager 600 (GE Healthcare) and quantified using ImageQuant software (GE Healthcare). For the quantification of the specific kinase activity, the radioactive signal was first normalized by the Clb2 kinase and GST-Cdc55 levels for each point. Then, all data points from a replicate were normalized to the control, mean and SEM were calculated and the analysis of the unpaired two-tailed *t* test was performed to assess statistical significance.

For the PP2A–Cdc55 phosphatase assays, bacteria-purified Net1(1–600) was previously phosphorylated with Cdk1–Clb2, as before, but after the kinase reaction the phosphorylated-Net1 was recovered from the supernatant and reserved for the phosphatase assays. Pk epitope-tagged Cdc55 was immunopurified on protein-A Dynabeads, as before, and the beads were washed two times more with phosphatase buffer (50 mM Tris–HCl, pH 7.5, 1 mM EGTA, 0.1% β -mercaptoethanol). Reaction

Table 1 List of strains used in this study

Strain	Genotype	Origin
Y610	<i>MATα pph22::HA-PPH21 TRP1::GAL1-FLAG3-ZDS1 CDC14-MYC18 cdc20::MET-CDC20</i>	This study
Y888	<i>MATα cdc55::HA3-CDC55 TRP1::GAL1-FLAG3-ZDS1 CDC14-Pk9 cdc20::MET-CDC20</i>	This study
Y769	<i>Mata cdc20::MET-CDC20 TRP1::GAL1-FLAG3-ZDS1 cdc55::HA3-CDC55</i>	This study
Y770	<i>Mata cdc20::MET-CDC20 cdc55::HA3-CDC55 TRP1::GAL1-FLAG3-zds1Δ803-916</i>	This study
Y1015	<i>Mata cdc20::MET-CDC20 TRP1::GAL1-FLAG3-ZDS1 cdc55::HA3-CDC55 CDC14-Pk9 NET1-GFP</i>	This study
Y2541	<i>Mata cdc20::GAL1-CDC20 cdc55::HA3-CDC55</i>	This study
Y492	<i>Mata cdc20::GAL1-CDC20 cdc55::HA3-CDC55 cdc28::cdc28-as1</i>	This study
Y537	<i>MATα HA3-CDC55 TRP1::GAL1-FLAG3-ZDS1 cdc55::HA3-CDC55 CDC14-Pk9 cdc20::MET-CDC20 cdc28::cdc28-as1</i>	This study
Y1125	<i>Mata cdc20::MET-CDC20 clb2Δ TRP1::GAL1-FLAG3-ZDS1 cdc55::HA3-CDC55 CDC14-Pk9</i>	This study
Y569	<i>MATα cdc20::MET-CDC20 cdc55::HA3-CDC55</i>	This study
Y480	<i>MATα cdc20::MET-CDC20 cdc55::HA3-CDC55 cdc28::cdc28-as1</i>	This study
Y1281	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55 NET1-MYC18</i>	This study
Y1284	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-2A NET1-MYC18</i>	This study
Y1081	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55 GAL1-FLAG3-ZDS1</i>	This study
Y1087	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-2A GAL1-FLAG3-ZDS1</i>	This study
Y1211	<i>Mata cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55 CDC14-Pk9</i>	This study
Y1215	<i>Mata cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-T174A CDC14-Pk9</i>	This study
Y1216	<i>Mata cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55 -S301A CDC14-Pk9</i>	This study
Y1217	<i>Mata cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55 -2A CDC14-Pk9</i>	This study
Y3034	<i>MATα cdc20::MET-CDC20 zds1Δ CDC14-Pk9</i>	Our lab
Y564	<i>MATα cdc20::MET-CDC20 cdc55::HA3-CDC55</i>	This study
Y879	<i>Mata cdc20::MET-CDC20 cdc55Δ CDC14-Pk9</i>	Our lab
Y1212	<i>Mata cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-T174E CDC14-Pk9</i>	This study
Y1213	<i>Mata cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-S301D CDC14-Pk9</i>	This study
Y1214	<i>Mata cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-ED CDC14-Pk9</i>	This study
Y500	<i>Mata cdc20::GAL1-CDC20 cdc55::Pk3-CDC55</i>	This study
Y2312	<i>MATα cdc20::GAL1-CDC20 CDC14-HA6 CLB2-Pk3</i>	Our lab
Y1049	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55</i>	This study
Y1077	<i>MATα cdc20::MET-CDC20 cdc55Δ cdc55::HA3-CDC55</i>	This study
Y1080	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-2A</i>	This study
Y824	<i>MATα cdc20::MET-CDC20</i>	Our lab
Y1294	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-ED NET1-MYC18</i>	This study
Y1285	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55 ZDS1-PK6</i>	This study
Y639	<i>MATα cdc20::MET-CDC20 NET1-MYC18 CDC14-Pk9</i>	Our lab
Y1118	<i>MATα cdc20::MET-CDC20 zds1Δzds2Δ cdc55::HA3-CDC55 YCplac22</i>	This study
Y1192	<i>MATα cdc20::MET-CDC20 zds1Δzds2Δ cdc55::HA3-CDC55 YCplac22-Zds1Promoter-FLAG3-ZDS1_full length</i>	This study
Y1119	<i>MATα cdc20::MET-CDC20 zds1Δzds2Δ cdc55::HA3-CDC55 YCplac22-Zds1Promoter-FLAG3-zds1Δ461-916</i>	This study
Y1122	<i>MATα cdc20::MET-CDC20 zds1Δzds2Δ cdc55::HA3-CDC55 YCplac22-Zds1Promoter-FLAG3-zds1Δ1-802</i>	This study
Y1117	<i>MATα cdc20::MET-CDC20 zds1Δzds2Δ cdc55::HA3-CDC55 YCplac22-Zds1Promoter-FLAG3-zds1Δ74-802</i>	This study
Y1120	<i>MATα cdc20::MET-CDC20 zds1Δzds2Δ cdc55::HA3-CDC55 YCplac22-Zds1Promoter-FLAG3-zds1Δ461-802</i>	This study
Y1288	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-ED ZDS1-PK6</i>	This study
Y1291	<i>MATα cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-2A ZDS1-PK6</i>	This study
Y1597	<i>MATα cdc20::MET-CDC20 zds1Δ zds2Δ cdc28::cdc28Y19F cdc55Δ CDC14-HA6 pRS316</i>	This study
Y1598	<i>MATα cdc20::MET-CDC20 zds1Δ zds2Δ cdc28::cdc28Y19F cdc55Δ CDC14-HA6 pRS316-Pk3-CDC55</i>	This study
Y1600	<i>MATα cdc20::MET-CDC20 zds1Δ zds2Δ cdc28::cdc28Y19F cdc55Δ CDC14-HA6 pRS316-Pk3-CDC55-ED</i>	This study
Y536	<i>MATα cdc55::HA3-CDC55</i>	This study
Y567	<i>MATα cdc55::HA3-CDC55 esp1::esp1-2</i>	This study
Y568	<i>MATα cdc55::HA3-CDC55 esp1::esp1-2</i>	This study
Y565	<i>MATα cdc20::MET-CDC20 cdc55::HA3-CDC55 esp1::esp1-2</i>	This study
Y888	<i>MATα cdc20::MET-CDC20 TRP1::GAL1-FLAG3-ZDS1 cdc55::HA3-CDC55 CDC14-Pk9</i>	This study

Table 1 (continued)

Strain	Genotype	Origin
Y524	<i>MATa cdc20::MET-CDC20 esp1::esp1-2 TRP1::GAL1-FLAG3-ZDS1 cdc55::HA3-CDC55 CDC14-Pk9</i>	This study
Y2782	<i>MATa cdc20::MET-CDC20 esp1::esp1-2 cdc55Δ CDC14-HA6 pRS316</i>	This study
Y1561	<i>MATa cdc20::MET-CDC20 esp1::esp1-2 cdc55Δ CDC14-HA6 pRS316-Pk3-CDC55</i>	This study
Y1481	<i>MATa cdc20::MET-CDC20 esp1::esp1-2 cdc55Δ CDC14-HA6 pRS316-Pk3-CDC55-ED</i>	This study
Y755	<i>MATa cdc55::HA3-CDC55 ZDS1-FLAG3</i>	This study
Y986	<i>MATa cdc20::MET-CDC20 cdc55Δ</i>	Our lab
Y616	<i>Mata cdc20::MET-CDC20 pph22::PPH21-HA</i>	This study
Y1649	<i>MATa cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55</i>	This study
Y1651	<i>MATa cdc20::MET-CDC20 cdc55Δ URA3::HA3-CDC55-2A</i>	This study
Y3227	<i>MATa cdc20::GAL1-CDC20 CDC14-Pk9</i>	Our lab
Y3034	<i>Mata cdc20::MET-CDC20 zds1Δ CDC14-Pk9</i>	Our lab

mix (phosphatase buffer containing P-Net1) was added to the beads and incubated at 30 °C for 15 min. Reactions were terminated by adding 2 × SDS-PAGE loading buffer. Remaining Net1 phosphorylation was detected by Pro-Q Diamond staining and images were acquired using Typhoon FLA 9500 (GE Healthcare). Cdc55 recovered on the beads was quantified using the Amersham Imager 600 (GE Healthcare) and ImageQuant software (GE Healthcare). For the quantification of the remaining Net1 phosphorylation, the Pro-Q diamond signal was first normalized by the Cdc55 and Net1(1–600)-His levels for each point. Then, all data points from a replicate were first normalized to the control and, mean values and SEM were calculated.

For the kinase assays of human proteins (Fig. 7a), commercially available purified recombinant Cdk1 and Cyclin B1 co-expressed in baculovirus Sf9 insect cells (SRP5009, Sigma-Aldrich) and recombinant human PPP2R2A (B55 alpha subunit) purified from wheat germ (ab159172, Abcam) were purchased and the kinase assays were performed following the manufacturer's instructions. Briefly, a mix of 10 μL Kinase solution (0.05 μg/μL), 3 μL of substrate solution (0.05 μg/μL), 5 μL of ATP (at final concentrations of 20, 80, and 160 μM) and 5 μL of cold water per sample were prepared, incubated at 30 °C for 15 min and reaction was terminated by adding 2 × SDS-PAGE loading buffer. Phosphorylation was detected by Pro-Q Diamond staining (Life Technologies) following the manufacturer's instructions, and images were acquired using Typhoon FLA 9500 (GE Healthcare).

Identification of Cdc55 phosphorylation by mass-spectrometry

The immunoprecipitation of Cdc55 was performed as before but using 2 × 10⁹ cells (1 L cultures). Purified Cdc55 were resolved in 4–15% SDS-PAGE gels (Bio-rad) and stained

using EZ Blue Coomassie (Sigma). The bands were then excised and in-gel digested with trypsin for LC–MS/MS analysis. Before the LC–MS/MS analysis the peptides were purified with a C18 reverse-phase column to remove any contamination and traces of acrylamide. Peptides were analyzed by LC–MS/MS using an LTQ-Orbitrap Lumos mass spectrometer (Thermo Scientific). Eluted peptides were subjected to electrospray ionization in an emitter needle (PicoTip™, New Objective, Scientific Instrument Services, Ringoes, NJ, USA) with an applied voltage of 2000 V. Peptides were fragmented using CID (collision-induced dissociation) in the linear ion trap using helium as collision gas with 38% normalized collision energy. Multistage activation was enabled to favor the detection of phosphopeptides. Peptides were identified using Thermo Xcalibur software (v.2.2) (Termo Fisher Scientific). The data analysis was performed in the MaxQuant software (v.1.6.2.6a) using the Andromeda Search Engine with the *Saccharomyces cerevisiae* SwissProt database (July 2018). The searches were filtered to get a 1% FDR at both protein and peptide level.

rDNA chromatin immunoprecipitation assays

The ChIP-qPCR experiments were performed as previously described [32]. The antibody used for immunoprecipitation was the α-Pk clone SV5-Pk1 (Serotec). Oligonucleotide sequences for quantitative real-time PCR were described in Ref. [33].

Molecular interactions by surface plasmon resonance experiments

The surface plasmon resonance experiments were performed using a BIACORE T200 (GE Healthcare) equipped with a research-grade CM5 sensor chip. The Net1(1–600)-His ligand was immobilized using amine-coupling chemistry. The ligand at a concentration of 0.5 μM in 10 mM sodium acetate, pH 5.0,

was immobilized at a density of 500 RU on flow cell 2 and 4; flow cell 1 and 3 were left blank to serve as a reference surface. To collect kinetic binding data, the GST(1–193)-Cdc55 and GST(1–193)-Cdc55-P analytes in 10 mM HEPES, 500 mM NaCl, 0.005% P-20, pH 7.4, were injected at concentrations of 0.35–5 μ M at a flow rate of 30 μ L/min and at 25 °C. The complex was allowed to associate and dissociate for 120 and 300 s, respectively. The surfaces were regenerated with a 30 s injection of 50 mM NaOH. Duplicate injections of each analyte were flowed over the surfaces. The data were fit to a simple 1:1 kinetics interaction model using the global data analysis option available or to a steady-state affinity curve within BiaEvaluation 3.1 software.

Other techniques

Protein extracts for western blots were obtained by NaOH or TCA protein extraction. For the phostag gels, 20 μ M Phostag (Wako) 6% gels containing 100 μ M MnCl₂ were used. Protein gels were washed with 1 mM EDTA before protein transfer to remove the manganese. For the quantification of the Cdc55 phosphorylation, the ratio of the quantified upper and lower bands for each point was calculated. Then, all data points from a replicate were normalized to the control and, mean and SEM were calculated.

Antibodies used for western blots and immunofluorescence were α -HA clone 12CA5 (Roche), α -phosphoserine/threonine antibody clone 22A (BD Biosciences), α -Cdc14 (yE-17) sc-12045 (Santa Cruz Biotechnology), α -myc 9E10 (Covance), α -FLAG clone M2 (Sigma), α -Pk clone SV5-Pk1 (Serotec), α -Clb2 (y-180) sc-907 (Santa Cruz Biotechnology), α -tubulin clone YOL1/34 (Serotec), α -GST (GE Healthcare), α -his ab9108 (Abcam), α -phosphoCdc28Y19 (phosphoCdc2-Tyr15) clone 9111 (Cell Signaling Technology), α -Cdc28 sc-53 (Santa Cruz Biotechnology) α -Tpd3 (Innovagen) and α -phosphoglycerate kinase (Life Technologies). The secondary antibodies were Cy3-labeled α -mouse (GE Healthcare), fluorescein-conjugated α -rat (Millipore) and Cy3-labeled α -goat (Jackson ImmunoResearch).

Statistical analysis

Statistical significance was tested by analysis of the unpaired two-tailed Student's *t* test (GraphPad Prism). Values of $p < 0.05$ were considered statistically significant, $p < 0.001$ ***, $p > 0.001$ **; $p > 0.01$ *.

Results

The PP2A regulatory subunit Cdc55 is phosphorylated at anaphase onset

Ectopic Zds1 expression induces the downregulation of PP2A^{Cdc55} and promotes Net1 phosphorylation [21], the *Zds_C motif* being sufficient to achieve this [22]. As a first approach to studying the detailed mechanism underlying PP2A^{Cdc55} downregulation at anaphase onset, we visualized the three PP2A^{Cdc55} subunits (Pph21, Tpd3 and Cdc55) by western blot after ectopic Zds1 expression. Two isoforms were detected for Cdc55 and Pph21 (Fig. 1a and Fig. S1). We observed that the overexpression of Zds1 in metaphase-arrested cells by Cdc20 depletion promotes the accumulation of the slower migrating form of Cdc55 simultaneously with the nucleolar release of Cdc14 (Fig. 1a). This slowly migrating Cdc55 form is not accumulated when a truncated Zds1 protein lacking the *Zds1_C motif* is induced. This protein cannot promote the nucleolar release of Cdc14, and is therefore incapable of downregulating PP2A^{Cdc55} [22] (Fig. 1b). These results suggest that Cdc55 suffers a post-translational modification upon Zds1 ectopic expression.

To ascertain the nature of the Cdc55 post-translational modification we purified Cdc55 after Zds1 ectopic expression in metaphase-arrested cells and treated it with alkaline phosphatase (AP) (Fig. 1c). In the presence of alkaline phosphatase, the slower migrating form of Cdc55 was greatly reduced but was partially recovered when the alkaline phosphatase was combined with phosphatase inhibitors (PI). Therefore, these results suggest that Cdc55 is phosphorylated upon Zds1 induction.

Next, we wondered whether the post-translational modification corresponds to a Ser/Thr phosphorylation. First, we repeat the alkaline phosphatase treatment in immunopurified-Cdc55 as in (c) and incubate the immunoblots with an anti-phosphoSer/Thr antibody (Fig. 1d). In the presence of AP the Ser/Thr phosphorylation signal was greatly reduced, suggesting that Cdc55 phosphorylation is almost abrogated upon AP treatment. On the contrary, upon inhibition of the AP by PI addition, the Ser/Thr phosphorylation signal was recovered. This suggests that the anti-phosphoSer/Thr antibody does not cross-react with the non-phosphorylated Cdc55 isoforms. Interestingly, these data indicate that the two Cdc55 migrating isoforms contain a mix of phosphorylated and non-phosphorylated Cdc55. Second, we purified Cdc55 in synchronized cells at the metaphase-to-anaphase transition by Cdc20 depletion and release into anaphase by Cdc20 re-addition (Fig. 1e). Purified-Cdc55 was resolved in Phostag gels to improve the visualization of the phosphorylated forms. Two Cdc55

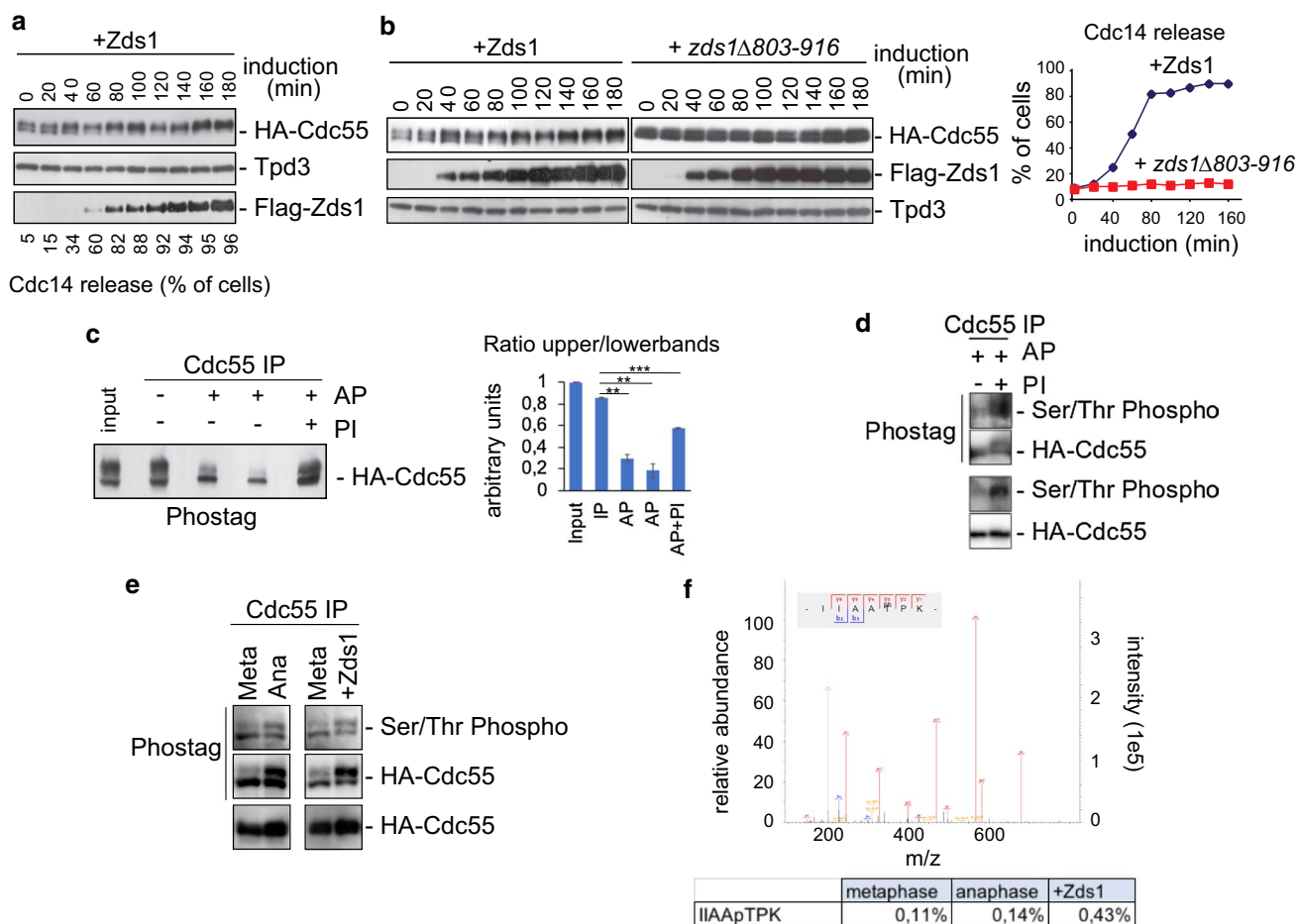


Fig. 1 PP2A regulatory subunit Cdc55 is phosphorylated upon PP2A^{Cdc55} downregulation. **a** PP2A^{Cdc55} downregulation induces the accumulation of a Cdc55 post-translational modification. Strain Y888 was arrested in metaphase by Cdc20 depletion, and Zds1 ectopic expression was induced by galactose addition. Cdc55, Tpd3 and Zds1 proteins were analyzed by western blot. Cdc14 release from the nucleolus was visualized by immunofluorescence. **b** Ectopic expression of a non-functional version of Zds1 cannot promote the Cdc55 post-translational modification. Strains Y769 and Y770 were arrested in metaphase by Cdc20 depletion, and Zds1 ectopic expression was induced. Cdc55, Zds1 and Tpd3 proteins were analyzed by western blot (left). Tpd3 was used as a loading control. Cdc14 release from the nucleolus was visualized by immunofluorescence (right). **c** Cdc55 experiences phosphorylation modifications. Strain Y1015 was arrested in metaphase by Cdc20 depletion, and Zds1 ectopic expression was induced. After 180 min of Zds1 induction, protein extracts were prepared, Cdc55 was immunoprecipitated, and alkaline phosphatase (AP) treatment was performed. 2x PhosS-TOP (Roche) was used as an alkaline phosphatase inhibitor (PI).

isoforms were resolved in the Phostag gels. The anti-Ser/Thr antibody recognized the lower and faster migration Cdc55 isoforms, suggesting that both bands contained Cdc55-phosphorylated isoforms. Interestingly, the signal of the slower migration band increased in anaphase and upon Zds1 induction. These results indicate that Cdc55 is phosphorylated at Ser/Thr residues and that the

A representative experiment of one of the assays is shown (left). A quantification of the slower and faster Cdc55 migration isoforms is depicted (right). Mean values and SEM are shown. **d** Cdc55 is phosphorylated in Ser/Thr residues. Cdc55 immunopurified as in **c** were detected using an anti-phosphoserine/threonine antibody and anti-HA as a control. One independent experiment is shown. **e** Phosphorylation of Cdc55 in Ser/Thr residues is enhanced in anaphase and upon Zds1 induction. Protein extracts from strain Y2541 arrested in metaphase (Meta) by Cdc20 depletion, and 20 min (Ana) after released into synchronous anaphase were prepared. Cell extract from Y1015 after 180 min of Zds1 induction was also prepared. Cdc55 was immunopurified, resolved in phostag gels and Ser/Thr phosphorylation was detected using anti-phosphoserine/threonine antibody. Purified HA-Cdc55 protein was also detected by western blot in phostag and normal gels as controls. **f** In vivo phosphorylation of Cdc55 at T174. Cdc55 immunopurified as in **e** were submitted to mass-spectrometry. The best MS/MS spectrum corresponding to the anaphase sample is shown. The ratios of modified/unmodified peptide are depicted

phosphorylation intensifies during anaphase and upon Zds1 induction. Finally, to unequivocally identify the Cdc55 phosphorylation we submitted the purified-Cdc55 to mass-spectrometry analysis (Fig. 1f). The phosphopeptide IIAApTPK corresponding to the Thr174 of Cdc55 was identified in metaphase, anaphase and upon Zds1 induction. More interestingly, the relative amount of the

phosphopeptide increased 27% at anaphase compare to metaphase cells. This demonstrates that the Cdc55 phosphorylation at Thr174 enhances in anaphase and upon Zds1 induction as suggested by the western blot.

We next wanted to establish whether Cdc55 phosphorylation is cell cycle-dependent. This prompted us to analyze the Cdc55 phospho-isoforms in cell-cycle progression after synchronous release from G1 (Fig. S2). To improve the visualization of the phosphorylated forms we used Phostag gels. Two slower migrating Cdc55 isoforms appeared by 60 min that was coincident with the peak of Clb2 levels, suggesting that Cdc55 was hyperphosphorylated during mitosis. To investigate the Cdc55 phosphorylation further we synchronized cells at the metaphase-to-anaphase transition by Cdc20 depletion and release into anaphase by Cdc20 re-addition (Fig. 2a, WT panel). An accumulation of the slower migration form of Cdc55 was detected during anaphase (after 15–30 min). These results suggest that Cdc55 is hyperphosphorylated during mitosis, specifically during anaphase, coinciding with the PP2A^{Cdc55} downregulation required for Cdc14 activation. All these findings lead us to propose that Cdc55 phosphorylation at anaphase might be the mechanism controlling PP2A^{Cdc55} activity.

Cdc28–Clb2 participates in Cdc55 phosphorylation

Cdc14 activation in early anaphase depends on Cdk1-dependent Net1 phosphorylation [17, 20]. In order to test whether Cdc55 phosphorylation is also regulated by Cdk1, we used Phostag gels to check the Cdc55 isoforms in cells carrying the ATP analog-sensitive allele *cdc28-as1* [34]. Cells were arrested in metaphase by Cdc20 depletion, then *cdc28-as1* was inhibited with 1NM-PP1 and Cdc20 was re-induced. Control cells containing wild-type Cdc28 showed two Cdc55 isoforms at metaphase and the slower migrating isoform accumulated during anaphase (Fig. 2a). In contrast, the Cdc55 slower migrating isoform was greatly reduced in the *cdc28-as1* cells in metaphase and anaphase cells. This result suggests that Cdk1 is responsible for Cdc55 phosphorylation. Nevertheless, since the Cdc55 slower migrating band in the *cdc28-as1* cells is not completely abolished, we cannot rule out the possibility that another kinase also contributes to Cdc55 phosphorylation.

To investigate further the role of Cdk1–Clb2 in regulating the Cdc55 phosphorylation, we checked Cdc55 phosphorylation upon Zds1 ectopic expression in cells lacking Cdk1 activity. Cells carrying the *cdc28-as1* allele or a *clb2* deletion were arrested in metaphase by Cdc20 depletion and Zds1 expression was induced (Fig. 2b). 1NM-PP1 and galactose to induce Zds1 were added simultaneously in *cdc28-as1* cells. Cells containing wild-type Cdc28 accumulated the slower migrating form of Cdc55, as expected. But inhibition of Cdc28-as1 by 1NM-PP1 avoided further phosphorylation

of Cdc55 upon Zds1 induction. Accumulation of the Cdc55 phosphorylated form was delayed in *clb2Δ* cells concomitantly with a delay in Cdc14 release from the nucleolus. These data suggest that Cdk1–Clb2 participates in Cdc55 phosphorylation.

To explicitly test Cdk1-dependent Cdc55 phosphorylation we measured Cdk1–Clb2 in vitro activity against Cdc55 after Clb2 purification from metaphase-arrested cells, when Cdk1–Clb2 is more active (Fig. 2c). The substrate was a recombinant fragment of Cdc55 spanning amino acids 1–193, containing the phosphorylated residue detected by mass spectrometry: Thr174. Cdc55 phosphorylation was observed in the presence of Cdk1–Clb2: wild-type cells (lane 6) and *cdc28-as1* strain without drug (lanes 2, 4). Remarkably, the phosphorylation was impaired in the *cdc28-as1* allele inhibited by 1NM-PP1 (lanes 3, 5). Therefore, Clb2-associated Cdk1 activity against GST-Cdc55 was determined, indicating that Cdc55 is an in vitro substrate of Cdk1–Clb2.

Cdc55 phosphorylation regulates nucleolar release of Cdc14

The results reported before indicate that Cdk1–Clb2 participates in Cdc55 phosphorylation during anaphase. Within Cdc55 we identified one residue, Thr174, with a full consensus for Cdk1 (S/TxP-x-K/R), and a second residue, Ser301, containing the Cdk1 minimal consensus site (S/TxP). We prepared strains containing these residues mutated to alanine (non-phosphorylatable mutants) and to aspartic/glutamic (phosphorylated mimetic mutants) in a *cdc55Δ* background. The growth and the mitotic kinetics of the control HA–Cdc55 *cdc55Δ* were equivalent to the control strain with endogenous Cdc55 epitope tagging (data not shown). The control HA–Cdc55 and the mutant alleles *cdc55-T174A-S301A* (hereafter, *cdc55-2A*), *cdc55-T174E-S301D* (hereafter, *cdc55-ED*) showed equivalent growth rates, rescued the cold-sensitivity of the *cdc55Δ* mutant (Fig. S3a), suppressed the elongated bud phenotypes of the *cdc55Δ* mutant (Fig. S3b), physically interact with the PP2A catalytic subunit Pph21 (Fig. S3c) and presented similar amounts of inhibitory Cdk1 phosphorylation at Y19 (Fig. S3d). All these results indicate that the mutant Cdc55 alleles behave similar to the control Cdc55 outside mitosis.

To determine whether either of these residues is required for Cdc55 phosphorylation in vivo, first, we analyzed Cdc55 phosphorylation in the non-phosphorylatable mutants (Fig. 3a) in metaphase cells. Reduced Cdc55 phosphorylation was observed in the *cdc55-2A* mutant allele compared with levels in control Cdc55 and in the single mutants *cdc55-T174A* and *cdc55-S301A*. These data indicate that the slower migrating form of Cdc55 corresponds to the phosphorylation in both residues (T174

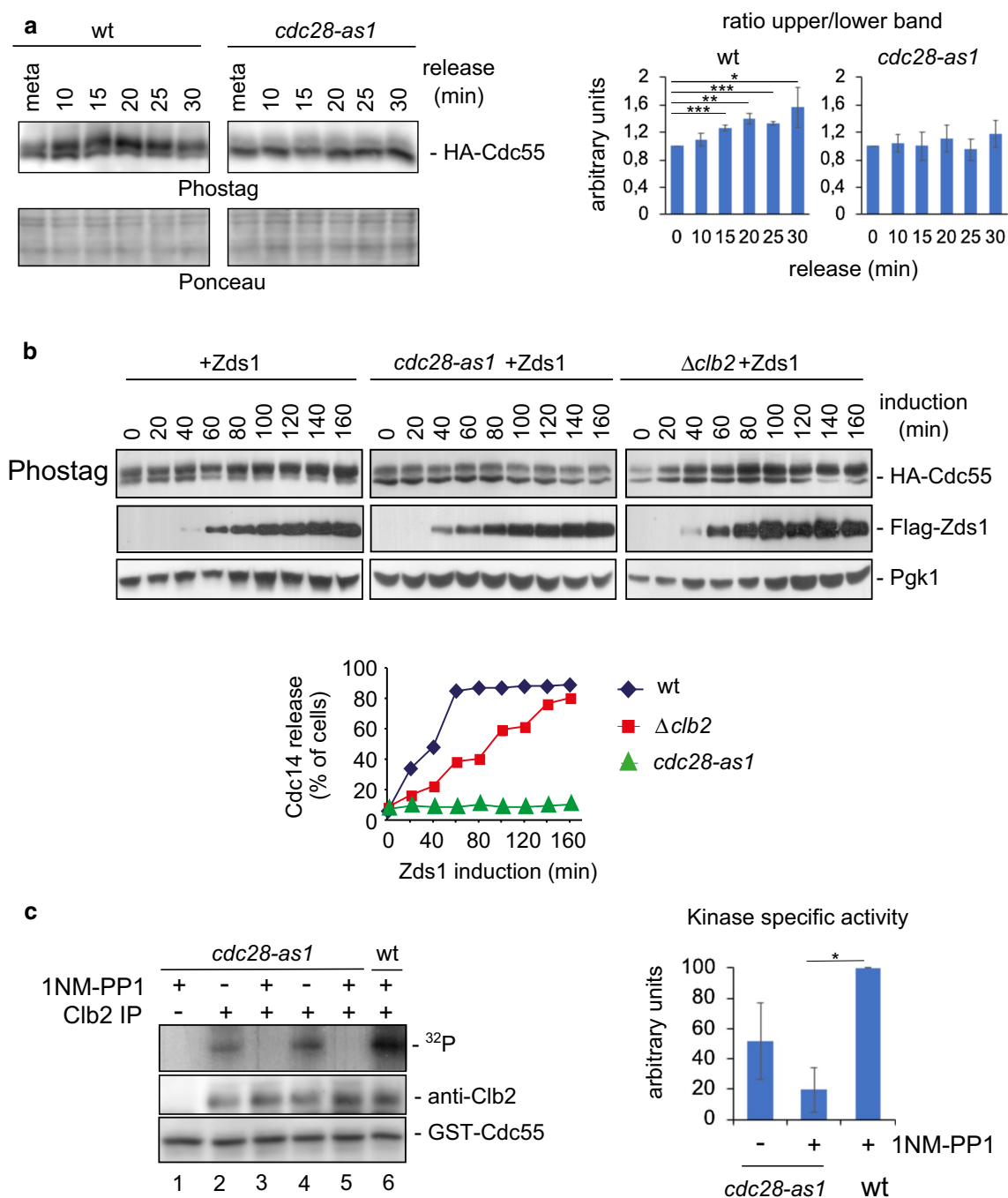


Fig. 2 Cdk1–Clb2 complex phosphorylates Cdc55. **a** Cdk1 is required for Cdc55 phosphorylation. Strains Y2541 and Y492 were arrested in metaphase by depletion of Cdc20, the *cdc28-as1* allele was inactivated by addition of 1 μ M 1NM-PP1 20 min before release into anaphase by Cdc20 re-addition. Cdc55 protein phosphorylation was analyzed by western blot. Ponceau staining is presented as loading control. A representative experiment of one of the assays is shown (left). Quantifications of the slower and faster Cdc55 migration isoforms are depicted (right). Mean values and SEM are illustrated. **b** The Cdk1–Clb2 complex is necessary for proper Zds1-induced Cdc55 phosphorylation. Strains Y1015, Y537, and Y1125 were arrested in metaphase, the *cdc28-as1* allele was inhibited by the addition of 1NM-PP1 during 20 min, and Zds1 expression was induced. Cdc14 release from the nucleolus was monitored

by in situ immunofluorescence (bottom) and the Cdc55 phosphorylation and Zds1 levels were analyzed by western blot. Pgk1 was used as loading control. **c** The Cdk1–Clb2 complex phosphorylates Cdc55 in vitro. Strains Y564 and Y480 were arrested in metaphase by Cdc20 depletion and the *cdc28-as1* allele was inactivated using 1 μ M 1NM-PP1. Cib2 was purified by immunoprecipitation, and the Cdc28–Clb2 kinase assay was performed using bacteria-purified GST-Cdc55(1–193) as the substrate. An immunoprecipitation using rabbit IgG, instead of the Cib2 antibody, was used as a negative control for the kinase assay. A representative image of one of the experiments is depicted (left). A quantification of four independent immunoprecipitations was performed (right). Mean values and SEM are shown

and S301) and suggests that the two residues are phosphorylated *in vivo*. Second, we investigated the mitotic progression and Cdc14 release from the nucleolus in anaphase-synchronized cells by Cdc20 depletion in the non-phosphorylatable mutants (Fig. 3b, c). Control cells released Cdc14 from the nucleolus at anaphase onset, as expected. In contrast, in *cdc55-T174A*, *cdc55-S301A* and *cdc55-2A*, Cdc14 release from the nucleolus was retarded by 10 min relative to the onset of spindle elongation, a delay similar to that observed in FEAR mutants [21]. These results suggest that Cdc55 phosphorylation is required for proper Cdc14 release from the nucleolus in anaphase.

To study the delay in Cdc14 activation relative to an internal marker for mitotic progression further, we measured Cdc14 release from the nucleolus with respect to the spindle length (Fig. 3d). Control cells started to release Cdc14 at spindle lengths of $> 2 \mu\text{m}$. In contrast, the non-phosphorylatable mutants released Cdc14 when the spindle length was 4–6 μm , similar to what occurs in *zds1Δ* cells [21]. Similar results were obtained synchronizing cells from G1 (Fig. S3). Therefore, Cdc55 phosphorylation is required for the proper release of Cdc14 from the nucleolus in early anaphase. Thus, phosphorylation of both residues, T174A and S301A, contributes similarly to timely Cdc14 activation in early anaphase.

Next, we examined whether these Cdc55 phosphorylation sites are responsible for the Cdk1-Clb2 phosphorylation of Cdc55 observed before *in vitro*. We were not able to purify the Cdc55 full-length recombinant protein, so we could only test the T174 residue by changing the residue to alanine, GST-Cdc55-T174A. Cdk1-Clb2 activity against control GST-Cdc55 was again detected as before (Figs. 2c, 3e); in contrast, GST-Cdc55-T174A phosphorylation was greatly reduced, indicating that T174 is responsible for the Cdk1-Clb2-dependent phosphorylation of Cdc55 *in vitro*.

To determine whether Cdc55 phosphorylation is sufficient to bring about PP2A^{Cdc55} downregulation we prepared phospho-mimetic mutants by changing the threonine into a glutamic residue and the serine to an aspartic residue. If Cdc55 phosphorylation induces PP2A^{Cdc55} inactivation, the phospho-mimetic mutants should lead to a premature Cdc14 release from the nucleolus, reminiscent of the *cdc55Δ* deletion mutant [17]. We constructed the single and double phospho-mimetic mutants, *cdc55-T174E*, *cdc55-S301D* and *cdc55-ED* and measured the Cdc14 release in metaphase-arrested cells by Cdc20 depletion (Fig. 3f, g). In controls cells, Cdc14 was sequestered in the nucleoli of metaphase cells, as expected. Conversely, Cdc14 was prematurely released from the nucleolus in all the phospho-mimetic mutants, similar to the phenotype of the *cdc55Δ* mutant. All these results suggest that PP2A^{Cdc55} inactivation by Cdc55 phosphorylation regulates timely Cdc14 activation.

Cdc55 phosphorylation modulates PP2A^{Cdc55} phosphatase activity

It has been previously reported that Cdc55 and its substrate, Net1, are located at the nucleolus and that this location does not change throughout mitosis [17, 22]. By contrast, a reduction of Cdc55 signal during mitosis was also proposed [35]. To find out whether nucleolar Cdc55 localization is regulated during anaphase, we used a non-subjective and quantitative technique: ChIP-qPCR. This assay allows nucleolar localization to be evaluated by quantifying the enrichment of a given protein at the ribosomal DNA (rDNA) region [32], and it has often been used to detect Net1 and Cdc14 nucleolar localization. To test whether we can detect Cdc14 redistribution throughout the entire cell during anaphase using this assay, we performed Cdc14-Pk ChIP-qPCR in metaphase- and anaphase-synchronized cells by Cdc20 depletion and reintroduction (Fig. S5). Cdc14 was enriched at the NTS1 and NTS2 region at metaphase, and this enrichment was greatly reduced during anaphase, concomitantly with the Cdc14 release. Therefore, we can use this technique to detect nucleolar localization of Cdc55.

Next, we repeated the ChIP-qPCR assays for Pk-Cdc55 at metaphase and anaphase cells (Fig. 4a). Cdc55 was enriched at the NTS1 and NTS2 region in metaphase cells, indicating that Cdc55 is bound to the nucleolus (rDNA). In anaphase cells, Cdc55 enrichment at the NTS1 region and most of the NTS2 was similar to that in metaphase cells; nevertheless, enrichment at the end of the NTS2 region and downstream towards the 18S region was reduced in anaphase cells. This differential Cdc55 enrichment along the rDNA during anaphase might explain the controversial results previously reported using other localization approaches. Therefore, nucleolar Cdc55 localization might be mildly altered during anaphase and could contribute to the regulation of PP2A^{Cdc55} activity. We do not fully understand what the reduction at the NTS2-18S region means, but overall, our experiments suggest that Cdc55 is still bound to the rDNA in anaphase cells (at least at the NTS1 region and partially at the NTS2 region) and changes in Cdc55 localization alone cannot explain the PP2A^{Cdc55} inactivation during anaphase.

Downregulation of PP2A^{Cdc55} phosphatase-specific activity occurs at anaphase onset [17] and upon Zds1 ectopic expression [21]. To establish whether Cdc55 phosphorylation modulates PP2A^{Cdc55} phosphatase activity, we measured the *in vitro* phosphatase activity of the non-phosphorylatable and the phospho-mimetic mutants of Cdc55. As a substrate we prepared *in vitro*-phosphorylated Net1(1–600)-his by Cdk1-Clb2 (Fig. 4b). Cdc55 immunoprecipitates from metaphase-arrested cells which were incubated with this substrate and the remaining Net1 phosphorylation was quantified using Pro-Q Diamond-stained gels (Fig. 4c). Cells containing wild-type Cdc55 dephosphorylated Net1, as expected.

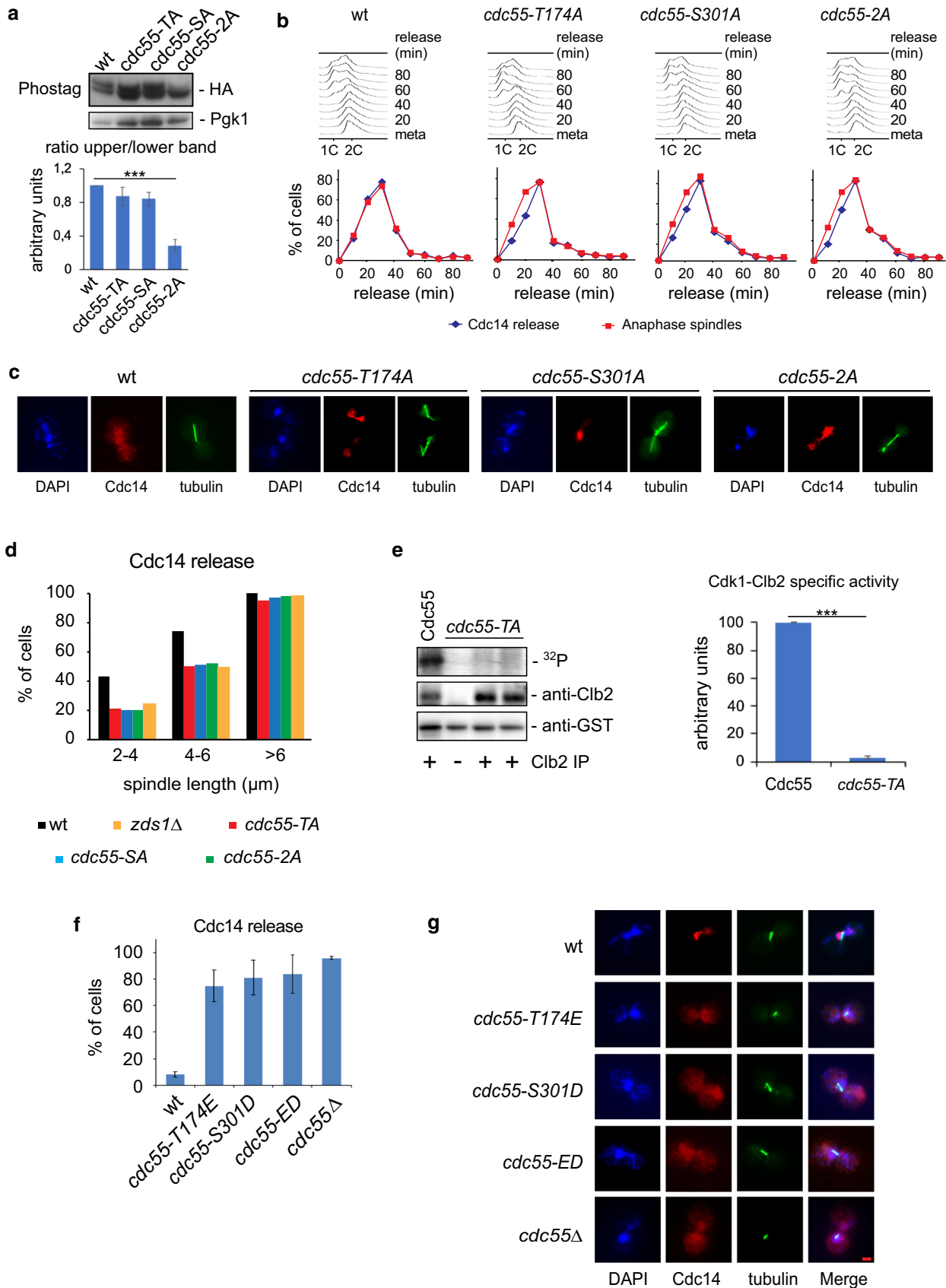


Fig. 3 Cdc55 phosphorylation alters Cdc14 release from the nucleolus. **a** Cdc55 phosphorylation is impaired in the non-phosphorylatable *cdc55-2A* mutant. Protein extracts from strains Y1211, Y1215, Y1216 and Y1217 arrested in metaphase by Cdc20 depletion were prepared. Cdc55 phosphorylation was analyzed by western blot. Pgk1 protein levels are shown as loading control. A representative experiment of one of the assays is presented (top). Quantifications of the slower and faster Cdc55 migration isoforms are depicted (bottom). Mean values and SEM are shown. **b** Cdc14 release is impaired during early anaphase in non-phosphorylatable Cdc55 mutants. Strains Y1211, Y1215, Y1216, and Y1217 were arrested in metaphase by Cdc20 depletion and released into synchronous anaphase by Cdc20 re-introduction. Mitosis progression was monitored by FACS analysis of DNA content (up). Cdc14 release from the nucleolus and anaphase spindles were monitored by immunofluorescence (middle). Representative images of cells in early anaphase with a spindle length of 3–5 μm are depicted in **c**. Scale bar, 2 μm . **d** Cdc14 nucleolar release from **b** was analyzed as a function of spindle length. The strain Y3034 was used as a FEAR mutant control. At least 50 cells were scored for each spindle length. **e** Mutation of the T174A within Cdc55 impaired Cdk1-Clb2 phosphorylation in vitro. Strain Y564 was arrested in metaphase by Cdc20 depletion, Clb2 was purified by immunoprecipitation, and the Cdc28-Clb2 kinase assay was performed using bacteria-purified GST-Cdc55(1–193) and GST-Cdc55(1–193)-T174A as substrates. A representative experiment is depicted (left). Mean values and SEM of four independent immunoprecipitations are shown (right). **f** Cdc14 is prematurely released during metaphase in Cdc55 phospho-mimetic mutants. Strains Y879, Y1211, Y1212, Y1213, and Y1214 expressing Cdc55 phospho-mimetic mutants of the Cdk1 consensus sites within Cdc55 were arrested in metaphase by Cdc20 depletion. Cdc14 release was checked by immunofluorescence. At least 100 cells were scored for each strain. Mean values and SD of three independent experiments are shown. Photographs are of cells in early anaphase with a spindle length of 3–5 μm (**g**). Scale bar, 2 μm

The non-phosphorylatable mutant *cdc55-2A* presented a mild increase in phosphatase activity compared to control Cdc55 cells. The quantification of the Net1 remaining phosphorylation (Fig. 4c right panel) showed a 30% reduction in the Net1 phosphorylation, suggesting that the *cdc55-2A* allele is hyperactive. In the phospho-mimetic mutant *cdc55-ED* the Net1 phosphorylation detected was similar to the levels in negative control cells in which Cdc55 was not tagged. Quantification of the phosphatase-specific activity was consistent with PP2A^{Cdc55} activity being inactivated in the *cdc55-ED* mutant (Fig. 4c right panel). We conclude that the phosphorylation of Cdc55 regulates PP2A^{Cdc55} phosphatase activity.

PP2A regulatory subunits provide substrate specificity. Co-immunoprecipitation experiments showed that Cdc55 interacts with Net1 in metaphase-arrested cells (Fig. 4d). To establish whether Cdc55 phosphorylation affects substrate recognition we examined whether Cdc55 phospho-isoforms physically interact with Net1 substrate.

An interaction was also detected between *cdc55-2A* and Net1, similar to what is observed in control cells. Strikingly, the *cdc55-ED* and Net1 interaction was stronger (Fig. 4d right panel). To further confirm the Net1-Cdc55-binding dynamics we studied the molecular interactions using

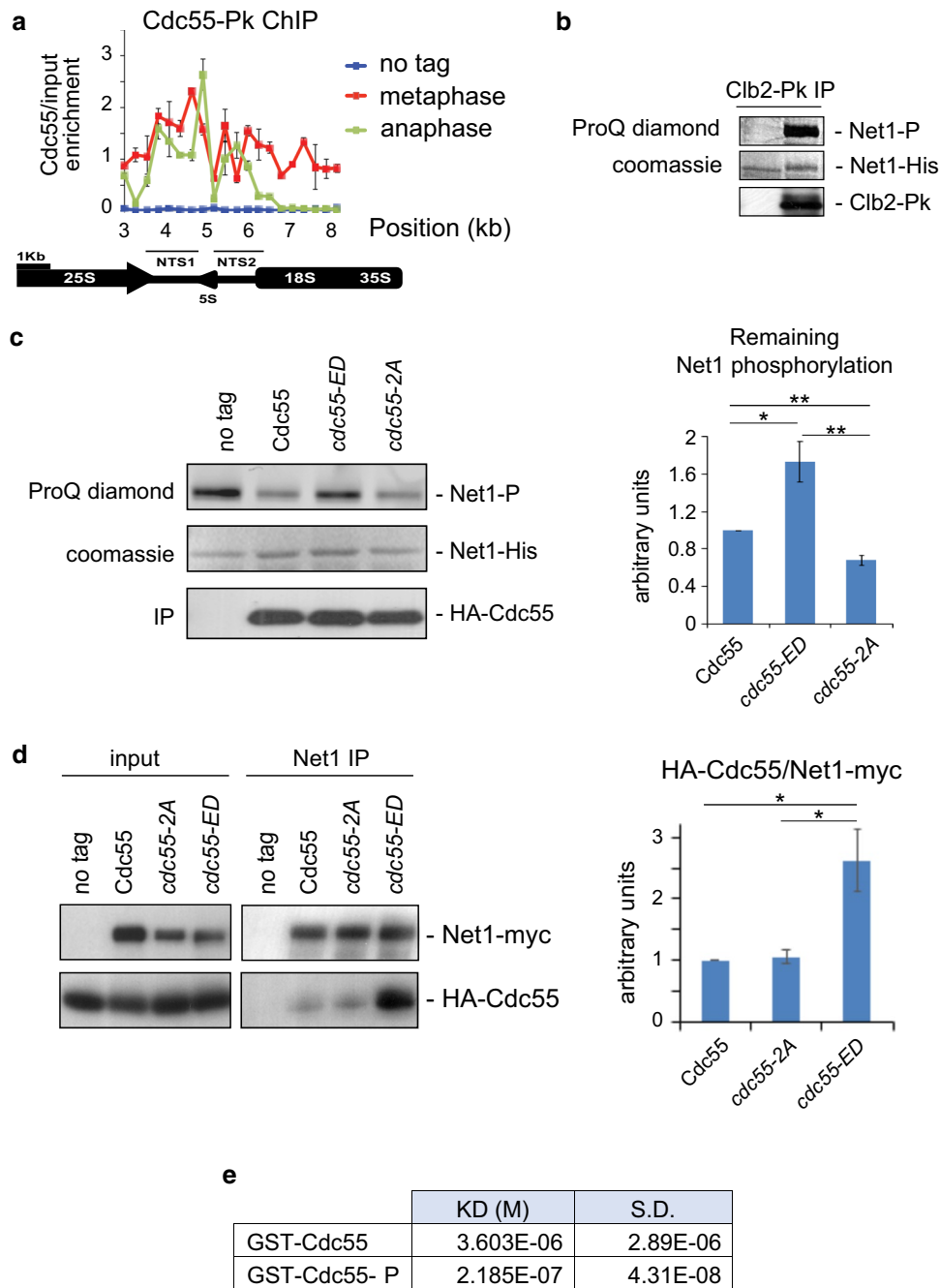
the surface plasmon resonance Biacore system (Fig. 4e). Net1(1–600)-his was purified from *E. coli* and immobilized in CM5 chips. Bacteria-purified GST-Cdc55 was phosphorylated in vitro by Cdk1-Clb2 and phosphorylated and non-phosphorylated GST-Cdc55 were used as analytes. The phosphorylated GST-Cdc55-P presented a lower KD 2.18E–07 compared to the non-phosphorylated GST-Cdc55 (KD 3.6E–06) one, indicating a higher affinity towards Net1. These results indicate that phosphorylation of Cdc55 modulates PP2A^{Cdc55} activity by altering enzyme–substrate-binding dynamics. In this case, the *cdc55-ED* mutant acts as a “substrate trapping” mutant [36] that retains a high binding affinity for substrates but blocks catalysis, making the interaction between the enzyme and its substrate less dynamic.

Functional Zds1 is required for proper Cdc55 phosphorylation

As reported above, Zds1 ectopic expression promotes the accumulation of the Cdc55 phosphorylation. Conversely, Cdc55 phosphorylation should be altered in absence of Zds1. Cdc55 phosphorylation was checked in the double mutant *zds1Δzds2Δ* in cycling cells (Fig. 5a, lane 1). Phosphorylation levels of Cdc55 were lower in the absence of Zds1/2 (compare lane 1 and 2), indicating that the presence of Zds1/2 is required to properly maintain Cdc55 phosphorylation. To test which domain of the Zds1 protein is required for the Cdc55 phosphorylation, we introduced truncated versions of Zds1 in the *zds1Δ zds2Δ* double mutant (lanes 3–6). We previously described that the truncated *zds1Δ461-802* protein (lane 6) is the only functional to release Cdc14 from the nucleolus [22]. Only the truncated version containing the functional protein *zds1Δ461-802* (lane 6) was able to restore the Cdc55 phosphorylation to comparable levels of the full length Zds1 (lane 2). Thus, not only Zds1’s presence, but its functional form is also required to promote Cdc14 activation for proper Cdc55 phosphorylation.

Zds1 physically interacts with PP2A^{Cdc55} via its regulatory subunit Cdc55 [22, 30]. Therefore, we examined whether the Zds1 and Cdc55 physical interaction is affected by Cdc55 phosphorylation. To test this possibility, co-immunoprecipitation experiments were performed using the Cdc55 phospho-mutants. In Zds1 immunoprecipitates from metaphase-arrested cells, we detected similar levels of co-purified Cdc55, *cdc55-ED* and *cdc55-2A* by western blot (Fig. 5b). These results indicate that Zds1 and Cdc55 interact independent of Cdc55 phosphorylation status.

If the defects in the Cdc14 release from the nucleolus observed in *zds* mutants are due to the impaired Cdc55 phosphorylation, the introduction of the *cdc55-ED* phospho-mimetic allele should rescue the Cdc14 release. To study in greater depth whether Zds1 promotes Cdc14 activation by regulating Cdc55 phosphorylation,



we introduced the *cdc55-ED* phospho-mimetic mutant into a *zds1Δzds2Δ cdc55Δ CDC28^{Y19F}* background. The *CDC28^{Y19F}* is refractory to inhibition by phosphorylation at Cdc28-Y19 and rescues the slow-growing phenotype and the elongated morphology of the *zds1Δzds2Δ* double mutant, allowing us to synchronize cells. We previously showed that the defective-Cdc14 release was equivalent in *zds1Δzds2Δ* and *zds1Δzds2Δ CDC28^{Y19F}* mutants [22]. We arrested cells at the metaphase-to-anaphase transition by Cdc20 depletion and analyzed the Cdc14 localization. When we introduced the wild-type Cdc55, the Cdc14

release was delayed as expected for *zds* mutants (Fig. 5c). On the other hand, in the absence of Cdc55 (empty vector), Cdc14 was released from the nucleolus prematurely in most of the metaphase-arrested cells (Fig. 5c, and [21]). The inactive *cdc55-ED* allele also promoted the premature release of Cdc14 from the nucleolus in the *zds1Δzds2Δ* background. Therefore, inactivation of Cdc55 by phosphorylation also promotes Cdc14 activation in the absence of Zds1. This is consistent with Zds1 being involved in the regulation of Cdc55 phosphorylation.

Fig. 4 Cdc55 phosphorylation regulates PP2A^{Cdc55} phosphatase activity. **a** Cdc55 nucleoli localization downstream from the NTS2 is reduced in anaphase. Strain Y500 was synchronized at the metaphase-to-anaphase transition. Binding to the NTS1 and NTS2 regions of the rDNA was determined by Pk-Cdc55 chromatin immunoprecipitation experiments. Mean values and SEM of three independent experiments are shown. **b** Net1 phosphorylation by Cdk1-Clb2. Strain Y2312 was arrested in metaphase by Cdc20 depletion, Clb2-Pk was purified by immunoprecipitation and Cdc28-Clb2 kinase assay was performed using bacteria-purified Net1(1–600)-his as substrate. Strain Y2541 without the Clb2-Pk epitope was used as negative control for the kinase assay. **c** PP2A^{Cdc55} phosphatase activity in the Cdc55 phosphorylation mutants. Strains Y1049, Y1077 and Y1080 were arrested in metaphase by Cdc20 depletion. Phosphatase activity of Cdc55-immunopurified complexes was measured as described in the “Methods”. Strain Y824 without HA-Cdc55 epitope was used as a negative control for the phosphatase assay. A representative image of one of the experiments is depicted (left). Mean values and SEM of remaining Net1 phosphorylation in three independent experiments are presented (right). **d** The Cdc55 and Net1 interaction is less dynamic in the Cdc55 phospho-mimetic mutants. Cell extracts were prepared from strains Y1281, Y1284 and Y1294 arrested in metaphase and co-immunoprecipitation of Cdc55 with Net1 was determined. Strain Y1285 without Net1-myc epitope was used as a negative control. Immunoblots of one representative experiment are illustrated (left). Mean values and SEM of three independent experiments are shown (right). **e** Cdc55 phosphorylation increases Net1-Cdc55 interaction. Bacteria-purified Net1(1–600)-his was immobilized in CM5 chips and GST-Cdc55(1–193) and GST-Cdc55(1–193)-P (phosphorylated by Cdk1-Clb2) were used as analytes in surface plasmon resonance experiments. The mean KD and the SD are depicted

Separase is needed to maintain Cdc55 phosphorylation

Separase activation is essential for Cdc14 nucleolar release, and downregulation of PP2A^{Cdc55} at anaphase onset depends on separase [17]. To discover how separase regulates PP2A^{Cdc55} we studied Cdc55 phosphorylation in separase thermo-sensitive mutants. In cycling cells, after 6–8 h at the restrictive temperature when the separase alleles *esp1-1* and *esp1-2* are inactivated, the slower migrating form of Cdc55 was reduced (Fig. 6a), suggesting that separase inactivation impaired Cdc55 phosphorylation. Later on, we synchronized cells at the metaphase-to-anaphase transition by Cdc20 depletion in wild-type and *esp1-2* mutant cells (Fig. 6b). Upon inactivation of the separase allele, *esp1-2*, the slower migrating form of Cdc55 was reduced at the restrictive temperature already at metaphase cells (time 0). In addition, the accumulation of the slower migration form of Cdc55 was not detected during anaphase in *esp1-2* cells, contrary to control cells. This indicates that separase activation is required to maintain Cdc55 phosphorylation.

Zds1 is required for separase-induced Cdc14 activation while Zds1 can release Cdc14 in absence of separase activity, suggesting that Zds1 acts in concert with separase, or downstream of it [21]. To analyze the relationship of these proteins, we examined Cdc55 phosphorylation upon Zds1

ectopic expression in the *esp1-2* cells in metaphase-arrested cells by Cdc20 depletion at the restrictive temperature (Fig. 6c). Zds1 expression in metaphase-arrested wild-type or separase mutant *esp1-2* cells led to Cdc55 phosphorylation with similar kinetics. These results are consistent with Zds1 acting in parallel or downstream of separase regulating PP2A^{Cdc55} activity, as previously described.

To confirm the influence of separase on Cdc55 phosphorylation, we introduced the *cdc55-ED* phospho-mimetic mutant into the *esp1-2* mutant allele. We arrested cells in metaphase by Cdc20 depletion and incubated the cells at the restrictive temperature to inactivate the separase. Upon introduction of the control Cdc55 into the *esp1-2 cdc55Δ* mutant (Fig. 6d), Cdc14 was sequestered in the nucleolus, as previously described [17]. By contrast, the inactive *cdc55-ED* allele promoted the release of Cdc14 from the nucleolus, similar to what occurs in the absence of Cdc55. This is consistent with separase-facilitating Cdk-dependent Net1 phosphorylation and Cdc14 release from the nucleolus by modulating PP2A^{Cdc55} activity via Cdc55 phosphorylation.

Cdk1-CyclinB1 phosphorylates PP2A-B55 in higher eukaryotes

PP2A phosphatases regulate almost every stage of the cell cycle by forming diverse heterotrimeric holoenzymes (reviewed in Refs. [37, 38]). Many aspects and components of the mitotic exit are conserved in higher eukaryotes. Human PP2A-B55 has several roles in mitosis. Before mitosis initiation, PP2A-B55 dephosphorylates Wee1 and Greatwall kinase, thereby inactivating them [39–41]. CDC25 dephosphorylation by PP2A-B55 subsequently promotes mitotic exit [42, 43]. On the other hand, Cdk1 phosphorylates Greatwall and in turn Greatwall-ENSA inhibits PP2A-B55 [41, 44–47]. Therefore, we considered it worthwhile checking whether PP2A^{Cdc55} regulation by Cdk1 phosphorylation is conserved. For this purpose, we investigated whether the human PP2A-B55 was phosphorylated by Cdk1-CyclinB1 in vitro. Recombinant human PP2A-B55 was incubated with a greater amount of human recombinant Cdk1-CyclinB1 at 30 °C for 30 min. PP2A-B55 phosphorylation levels were visualized by Pro-Q Diamond staining (Fig. 7a). PP2A-B55 was phosphorylated in vitro in the presence of Cdk1-CyclinB1. This result suggests that Cdk1-dependent phosphorylation of the B regulatory subunit is conserved in human cells. Strikingly, the yeast Thr174 is a conserved residue corresponding to human Ser167, which is a minimal Cdk1 consensus site SP (Fig. 7b). The yeast Ser301 is also conserved in human B55, which corresponds to Ser294, although in this case it does not match with any Cdk1 consensus site. Therefore, we propose that phosphorylation at T174 (or S167 in human) might be involved in the conserved mechanism of Cdk1-dependent phosphorylation

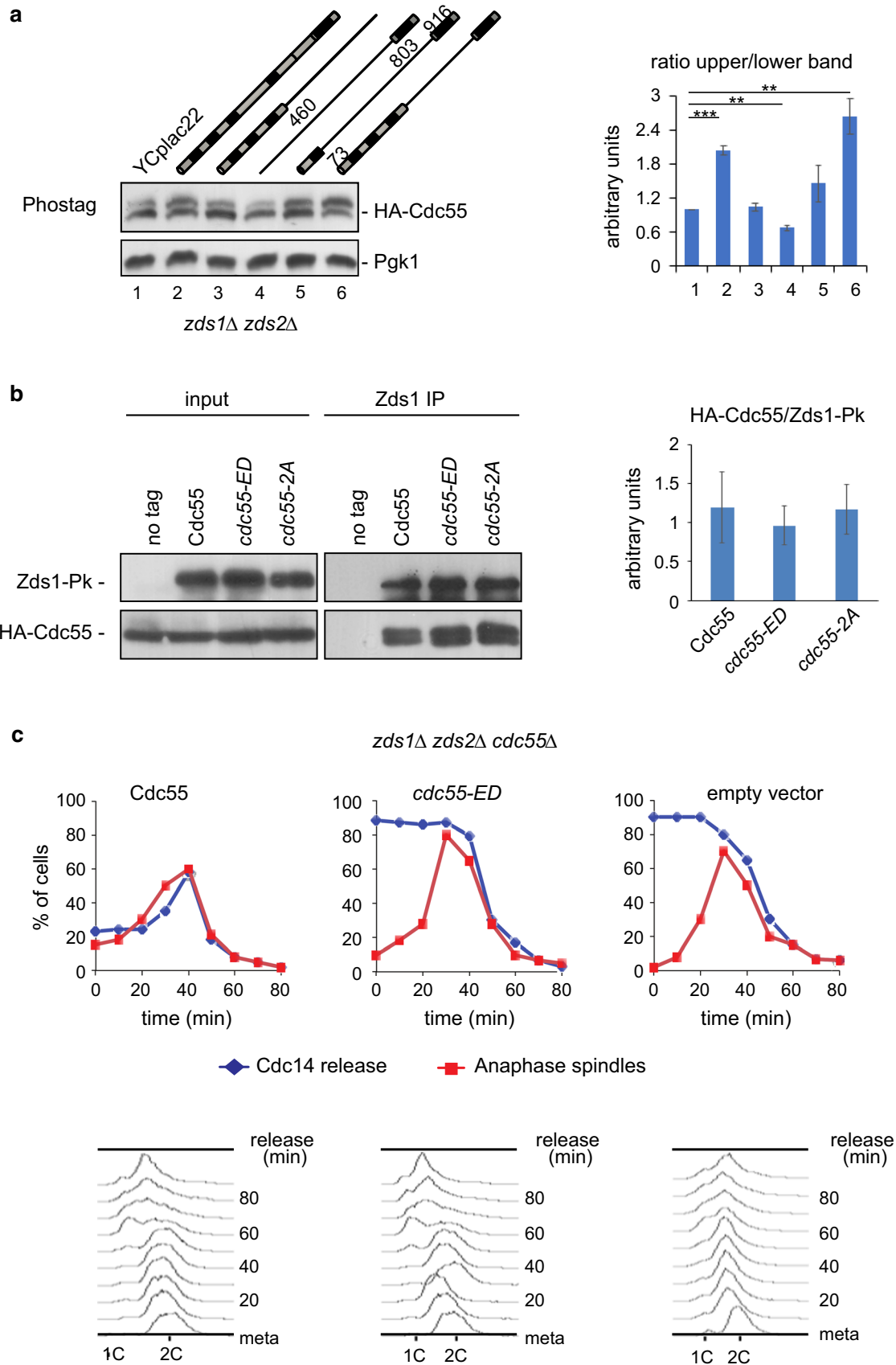


Fig. 5 Zds1 is required for proper Cdc55 phosphorylation. **a** Cdc55 phosphorylation is impaired in the absence of Zds proteins. Protein cell extracts were prepared from strains Y1118, Y1192, Y1119, Y1122, Y1117 and Y1120 in cycling cells. Cdc55 phosphorylation was analyzed by western blot. Pgk1 protein levels are shown as loading control. A representative image of one of the experiments is depicted (left). Mean values and SEM of three independent experiments are presented (right). **b** Cdc55 and Zds1's physical interaction is not affected by Cdc55 phosphorylation. Protein cell extracts of strains Y1285, Y1288 and Y1291 arrested in metaphase were prepared, and co-immunoprecipitation of Cdc55 with Zds1 was determined (left). The strain Y1281 without the Zds1-Pk epitope was used as a negative control. Mean values and SEM of two independent experiments are shown (right). **c** The inactive *cdc55-ED* phosphomimetic mutant promotes premature Cdc14 release from the nucleolus in the *zds1Δ zds2Δ* mutant. Strains Y1597, Y1598 and Y1600 were arrested in metaphase by Cdc20 depletion and release into anaphase by Cdc20 re-introduction. Cdc14 release from the nucleolus and anaphase spindles was determined by immunofluorescence. Mitosis progression was monitored by FACS analysis of DNA content

of Cdc55 (human B55). Although some of the effectors may differ in mammalian cells, studying the molecular mechanism governing yeast mitotic exit may yield insights into mitotic progression in other contexts and organisms.

Discussion

Cdk1-dependent Cdc55 phosphorylation regulates timely Cdc14 activation

In *S. cerevisiae* Cdc14 phosphatase is a key mitotic component whose essential function is to counteract Cdk1-Clb2 activity during anaphase, allowing cells to exit from mitosis [4]. Initial FEAR-Cdc14 activation at anaphase onset depends on downregulation of PP2A^{Cdc55}, promoting Net1 phosphorylation and Cdc14 release from the nucleolus [17]. Anaphase-specific inactivation of PP2A^{Cdc55} phosphatase activity arises from the phosphorylation of its regulatory B subunit, Cdc55, by the Cdk1-Clb2 kinase. Cdk1-dependent Cdc55 phosphorylation does not provoke a change in substrate recognition; instead, Cdc55 phosphorylation affects the dynamic interaction between Net1 and Cdc55. Notably, active separase and functional Zds1 are required to sustain Cdc55 phosphorylation and a constitutive inactive version of Cdc55, *cdc55-ED*, can compensate for the defects of Cdc14 release from the nucleolus in the absence of Zds1 and separase activities. Therefore, separase and Zds1 control Cdc14 activation by modulating Cdc55 phosphorylation or PP2A^{Cdc55} phosphatase activity.

Phosphorylation of Cdc55 at the Cdk1 consensus sites within T174 and Ser301 is equally required for Cdc14 activation since no additive effect was observed in the double mutants *cdc55-2A* and *cdc55-ED* compared to the single phospho-mutants. It has recently been reported that PP2A^{Cdc55}

imposes ordered cell-cycle phosphorylation by opposing threonine phosphorylation [48] and PP2A-B55 preference for threonine residues is conserved in mammals [49]. Therefore, Cdk1-Clb2 can phosphorylate both residues during mitosis, but the fine-tuning of Cdc55 phosphorylation might be regulated by preferential (auto)-dephosphorylation of T174 by PP2A^{Cdc55}.

The different subunits of PP2A^{Cdc55} are known to suffer post-translational modifications, such as phosphorylation and carboxy-methylations [50–55]. The Leu309 carboxy-methylation of Pph21 is involved in the assembly of the holoenzymes [55] and the Pph21/22 phosphorylation at Tyr307 destabilized the heterotrimeric PP2A^{Cdc55} complex. Although we cannot rule out the possibility that some other post-translational modification might also affect PP2A^{Cdc55} phosphatase activity, we did not detect any anaphase-specific modification in Pph21 and Tpd3 by western blot or mass-spectrometry. In addition, in global yeast phosphoproteome studies, several phosphorylations for Cdc55 were described in the S124, S155 and S440 residues in asynchronous cells [56–59]. In our mass-spectrometry analysis of Cdc55 phosphorylation we identified the T174 phosphorylation. We were not able to detect any peptide (modified or unmodified) containing the S301. Although, we detected phosphorylation at (non-Cdk1) residues T133 and S403 during mitosis. Therefore, we cannot discount the possibility that Cdc55 undergoes other non-Cdk1-dependent phosphorylation or even other post-translational modifications, since in our experiments in the absence of Cdk1 and with the alkaline phosphatase treatment, a residual slower migrating form of Cdc55 is still detectable.

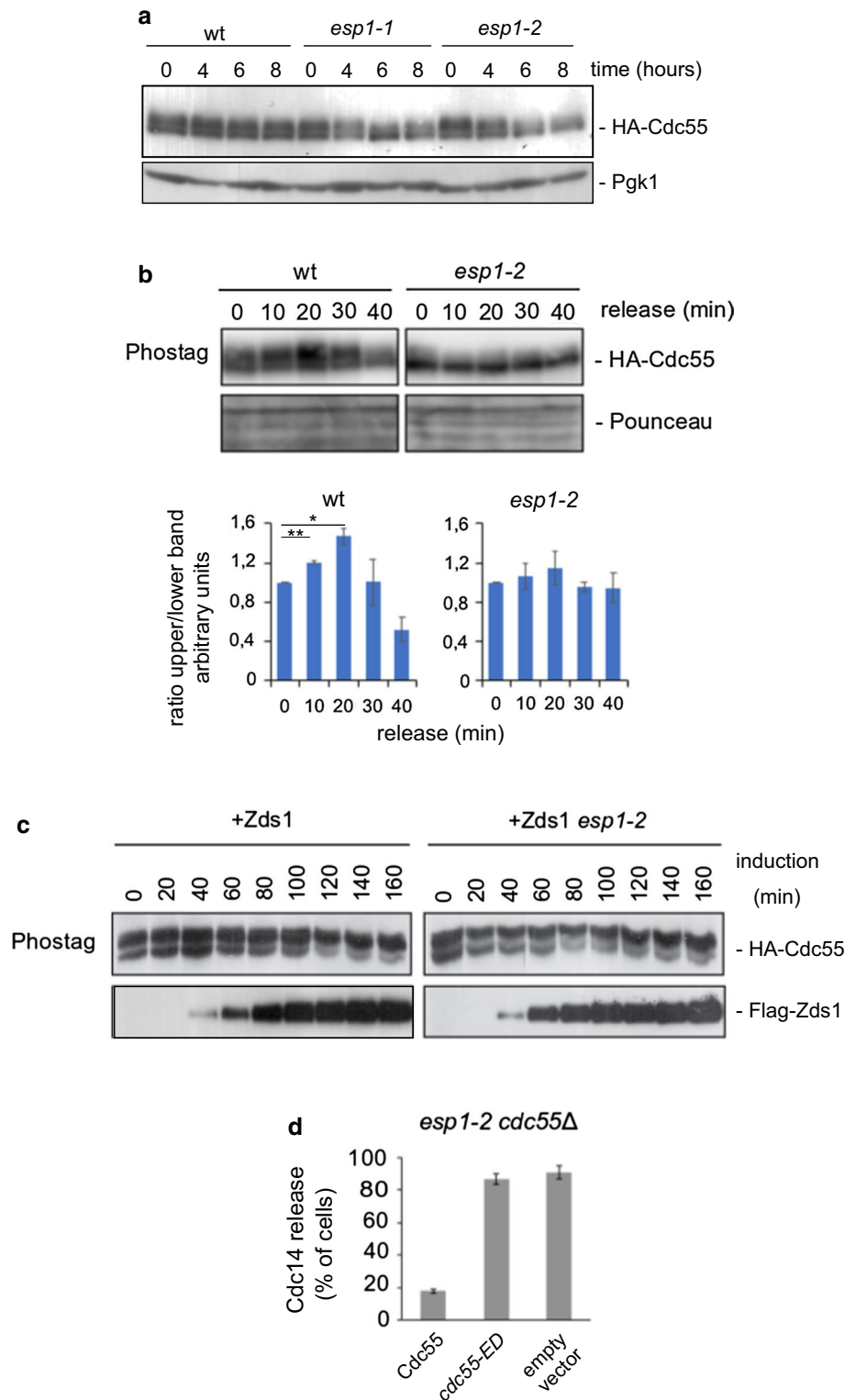
Zds1 and separase are involved in maintaining proper Cdc55 phosphorylation

Our results demonstrate that functional Zds1 is required for proper Cdc55 phosphorylation, and that the same functional domains of Zds1 regulating Cdc14 activation [22] are also involved in maintaining proper Cdc55 phosphorylation (Fig. 5a). In addition, introduction of the inactive *cdc55-ED* allele promotes the premature Cdc14 release from the nucleolus in the absence of Zds1 (Fig. 5c). In the same way, the need for active separase to maintain proper Cdc55 phosphorylation and Cdc14 activation (Fig. 6a–c) is compensated by a constitutive Cdc55 phosphorylation (*cdc55-ED*, Fig. 6d). All these findings prompted us to propose a model in which separase and Zds1 control the activity of the proteins regulating Cdc55 phosphorylation (Fig. 7c).

Proposed model of the regulation of PP2A^{Cdc55} and Cdc14 during mitosis

Protein phosphorylation and dephosphorylation are regulated by the interplay of protein kinases and phosphatases.

Fig. 6 Separase is necessary for proper Cdc55 phosphorylation. **a** Cdc55 phosphorylation is defective in separase mutants. Protein cell extracts were prepared in cycling cells from strains Y536, Y568 and Y567 incubated at 32 °C for the indicated times. Cdc55 phosphorylation was analyzed by western blot. Pgk1 is shown as loading control. **b** Impaired Cdc55 phosphorylation in anaphase in separase mutants. Strains Y564 and Y565 were arrested in metaphase, incubated at 32 °C for 2 h to inactivate the *esp1-2* allele and release into anaphase. Cdc55 phosphorylation was analyzed by western blot. Ponceau staining is shown as loading control. A representative image of one of the experiments is depicted (top). Mean values and SEM of three independent experiments are presented (bottom). **c** Separase is not required for Zds1-induced Cdc55 phosphorylation. Strains Y888 and Y524 were arrested in metaphase by Cdc20 depletion, incubated at 32 °C for 2 h, and Zds1 ectopic expression was induced. **d** Premature Cdc14 release in separase mutants upon expression of the Cdc55 phospho-mimetic mutant, *cdc55-ED*. Strains Y2782, Y1561 and Y1481 were arrested in metaphase. Cdc14 release was analyzed by immunofluorescence. Mean values and SEM of three independent experiments are shown



Reversible phosphorylation of protein substrates commonly involved futile cycles correlated with an exponential signal-response curve [17, 60]. Nevertheless, coherent feed-forward loops reduce futile cycling, ensuring that the kinase and phosphatase are not fully active at the same time and

generate a sigmoidal signal–response curve helping to make more definitive decisions. In this sense, our proposed model (Fig. 7c) fits perfectly with a coherent feed-forward loop in which the kinase, Cdk1–Clb2, phosphorylates the Net1 substrate and helps inactivate the phosphatase, PP2A^{Cdc55},

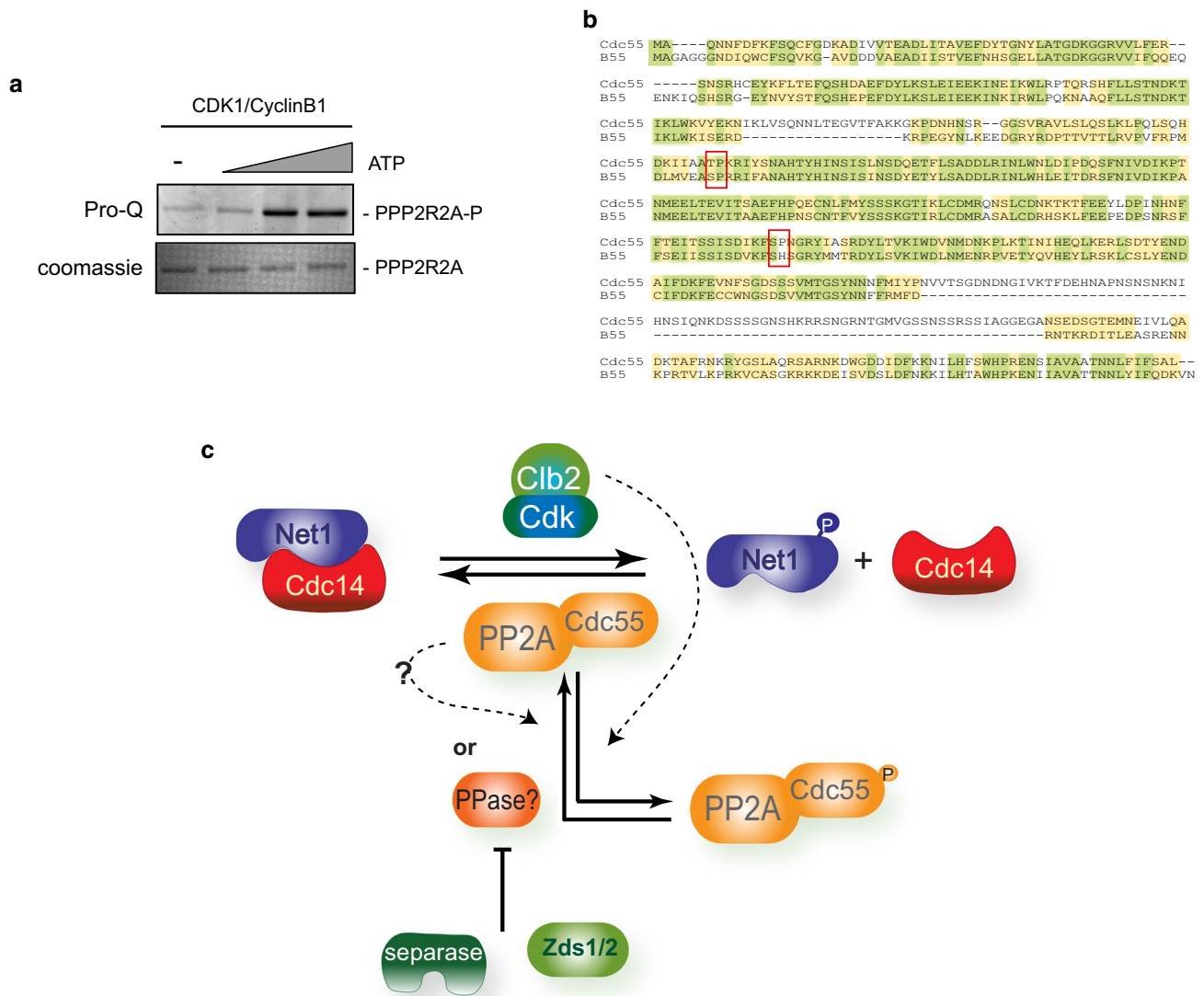


Fig. 7 Phosphorylation of B55 by Cdk1 is conserved in humans. **a** Cdk1–CyclinB1 phosphorylates human B55. Kinase assays using recombinant human Cdk1–Cyclin B1 and B55 were performed. A representative image of one of the experiments is depicted. **b** Alignment of the budding yeast Cdc55 and human B55. Red boxes mark the conserved T174 and S301 in Cdc55. **c** Model of the Net1 phosphorylation and Cdc14 release regulated by downregulation of the

PP2A^{Cdc55} via phosphorylation of the regulatory subunit Cdc55. During metaphase, futile cycles of Cdk1–Clb2 phosphorylation and PP2A^{Cdc55} dephosphorylation maintain low levels of phosphorylated-Net1. During anaphase, Cdc55 phosphorylation promotes the inactivation of the phosphatase activity of the PP2A^{Cdc55} (indicated as black dashed lines) and, as a consequence, Net1 phosphorylation is increased and Cdc14 is released from the nucleolus

by phosphorylating it (the new mechanism described in this work, indicated as black dashed lines). In this model, the active form of the phosphatase PP2A^{Cdc55} can promote its own activation by auto-dephosphorylation or a second phosphatase (marked as PPase) might contribute to PP2A^{Cdc55} dephosphorylation (marked with a question mark since this step is not proven).

In the proposed model, some protein effectors of the system might regulate the kinase, Cdk1–Clb2 or the phosphatase, PP2A^{Cdc55}. Zds1 and separate could therefore be involved in regulating Cdk1–Clb2 or PP2A^{Cdc55}. Our results

favor a model in which separate and Zds1 act to regulate PP2A^{Cdc55} and/or the second phosphatase (PPase). Further work is required to elucidate the mechanism by which Zds1 and separate control the activity of the phosphatases. PP1 (Glc7 in budding yeast) is known to have different roles in yeast during mitosis [61–66], *Xenopus* [67–69] and human cells [70–72]. Therefore, PP1 is a candidate phosphatase to regulate PP2A^{Cdc55}. In addition, PP1 and PP2A physically interact and are mutually regulated [66], suggesting that both phosphatases may be closely regulated in different organisms.

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Author contributions SJ, IC, YMR, PG and EQ performed the experiments. SJ, IC and EQ designed the experiments and interpreted the data. EQ wrote the manuscript. All authors read and discussed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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