The BEG (PP2A-B55/ENSA/Greatwall) Pathway Ensures Cytokinesis follows Chromosome Separation

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Mitotic lysate (10.9mg) is loaded onto a HiTrap Q column. A NaCl gradient is used to elute the proteins, resulting in 27 x 1ml fractions.

The following proteins are detected in the fractions:

- **PP1α**
- **PP1β**
- **PP1γ**
- **PP4/PP6**
- **PP2A-B55/B56**

Proteins detected in the fractions include:

- PPP2CA/B
- PPP2R1A (Scaffold)
- PPP2R2A (B55α)
- PPP2R5C (B56γ)
- PPP2R5D (B56δ)
- PPP1CA
- PPP1CB
- PPP1CC
- PPP4C
- PPP5C
- PPP6C
- Cdk1 (Cdc2)
- Cyclin B1
- PIk1
- MASTL

The molecular weight (kD) of each protein is indicated in the left column.
Figure S1. Distribution of phosphatases in a fractionated mitotic extract, related to Figure 1.

Fractions from the ion-exchange chromatography described in Figure 1 were western blotted for the PPP subunits as shown in the figure. Peak fractions containing catalytic subunits for each PPP phosphatase are highlighted across the top. Fractions containing PP2A-B55/B56 holoenzymes that correlated with observed PRC1-pT481 and pT602 phosphatase activity in Figure 1 are boxed.
**Figure S2.** Recombinant PP2A-B55\(\alpha\) and PP2A-B56\(\gamma\) holoenzymes, related to Figure 3.

(A) Phosphatase complexes were isolated from HEK-293T cells transfected with plasmids for expression of FLAG-tagged PPP2R2A, PPP2R5C, or a FLAG vector lacking a cloned insert (FLAG-empty). Left panel, Coomassie stained gels show bands at the expected masses for PP2A catalytic (PPP2CA/B) and scaffold (PPP2R1A/B) subunits in FLAG-PPP2R2A and FLAG-PPP2R5C purifications but not those from control cells transfected with FLAG-empty. Asterisks denote proteins that bound non-specifically to beads. Isolation of complete PP2A holoenzymes was confirmed by western blot analysis (right panels). (B) Phosphatase activity against the pT481 site on PRC1 was measured by incubating recombinant PRC1-pT481 purified from insect cells with a set amount of whole cell lysate and increasing amounts of purified PP2A-B55\(\alpha\) holoenzyme. From this experiment it was estimated that 0.25x PPP2R2A gave the same specific activity as 1x lysate. (C) Purified holoenzyme fractions were diluted (37 fold) and the indicated volumes loaded per lane. Titration demonstrates PP2A-B55\(\alpha\) was 1.5x more concentrated than PP2A-B56\(\gamma\). As such, PP2A-B55\(\alpha\) was diluted 1.5 fold before use in assays described in Figure 3. (D) Following the assay described in Figure 3A, 0 min samples for each condition were blotted for PP2A catalytic and scaffold subunits. Blots confirmed the required amount of each matched holoenzyme had been added to the assay relative to lysate.
**Figure S3.** ENSA is dephosphorylated at the pS67 MASTL site, related to Figure 4.

(A) Cells were lysed at 1.0 mg/ml and incubated on ice for 1 hour in the presence or absence of okadaic acid. Mass spectrometry of ENSA from these okadaic acid treated or control cell lysates was performed as described previously (Zeng et al., 2010). (B) Blots confirming siRNA depletion for experiments described in Figure 4B. Samples from the 60 min +okadaic acid condition were blotted. Results confirm efficient siRNA depletion and demonstrate that for each condition cells start off with the same amount of pT481 phosphorylation on PRC1. (C) Samples from Figure 4B were probed for total PRC1 to confirm even protein loading. (D) ENSA phosphorylation was analysed using Phos-Tag in cells depleted of Greatwall/MASTL, ENSA, or ARPP19, lysed at 1 mg/ml and then incubated on ice for 1 hour in the presence or absence of okadaic acid. (E) Western blotting was performed using 0.5-15.0 ng recombinant His-tagged ARPP19 to determine the sensitivity of the antibody. Samples containing 15 µg protein from the 60 min +okadaic acid condition described in Figure 4B were western blotted alongside 5.0 ng His-tagged ARPP19. There was no detectable ARPP19 signal in these samples, suggesting ARPP19 is at a level <0.03 ng/µg cellular protein when it would need to be at ~1 ng/µg cell extract to be equimolar with PP2A-B55.
**Figure S4.** Detailed wiring diagram for mammalian mitotic exit, related to Figure 5.

The figure shows the components regulating anaphase progression and cytokinesis in mammalian cells, and provides a detailed wiring diagram used in the mathematical modelling. A detailed description is provided in the accompanying supplemental text.
A

CycBdbΔ=1.0

Non-degradable cyclin B

CycBdbΔ=0

A

B

Relative level

Time (min)

0 10 20 30 40 50

0 0.2 0.4 0.6 0.8 1.0

“High”

“Moderate”

“Low”

“None”

Separase

Ana I

pT481

T602

pT481Cdk-
cycB

Shifted to later time

Separase activity

Ana I

pT481Cdk-
cycB

pT602

pT481 shifted to later time

Separase

Normal Separase

SA-Separase

pT481

Cdk-
cycB

Separase

Shifted to later time
**Figure S5.** Effects of stable cyclin B and cyclin-binding mutant separase on separase and PP2A-B55 activity in mitotic exit, related to Figure 6.

(A) Simulation of separase and PP2A-B55 activity were performed using different levels of non-degradable cyclin B (CycBndT=1.0 to 0). At high levels of CycBndT =1.0 both separase and PP2A-B55 activity are inhibited. At moderate levels of CycBndT =0.5-0.125 separase becomes active, but PP2A-B55 monitored by pT481 does not. Only at low levels of CycBndT ≤ 0.05 is PP2A-B55 active, and pT481 removal efficient, leading to accumulation of the anaphase pT602 phosphorylation. Therefore the model recapitulates published work on the dose-dependent effects of stable cyclin B on mitotic exit (Wolf et al., 2006).

(B) Simulation of separase and PP2A-B55 activity were performed using either wild-type separase or a cyclin B binding mutant (SA-separase). SA-Separase behaves essentially like the wild-type enzyme due to the securin component in the anaphase inhibitor term (Ana I) in agreement with published findings (Shindo et al., 2012). Separase inhibition of Cdk1-cyclin B is also lost, and results in an ~10 min shift in PP2A-B55 activity to later times, thus delaying activation of the anaphase spindle elongation protein PRC1. Simulation of mitotic exit with SA-separase was performed with a model in which all anaphase inhibitor (Ana I) containing dynamic variables were separated into two ODE’s in order to distinguish the temporal behaviour of cyclin B and securin.
**Figure S6.** Cell cycle progression and checkpoint silencing in Greatwall depleted cells, related to Figure 7.

(A) Hela S3 cells were transfected with siRNA oligonucleotides targeting control, MASTL/Greatwall, PP2A B55 or B56 subunits, incubated for 60h and then arrested in interphase for 20h by addition of 2 mM thymidine. Cells were released from the arrest in 4 ml of fresh media following washing with 4 ml of pre-warmed PBS three times, then once in growth medium. At the indicated time points after removal of thymidine, cells were processed for microscopy and stained for PRC1, PLK1 and DNA. Cell-cycle distribution was analysed for each time point and siRNA condition (n=300). (B) A double stable eGFP-Mad2 mCherry-securin cell line was treated with control, or (C) MASTL/Greatwall siRNA for 36 hours, arrested in interphase (2 mM Thymidine) and then released from arrest for 8 hrs into fresh medium before imaging. Scale bar is 10µm. Securin and Mad2 levels were measured and plotted in the lines graphs (n=5).
Supplemental experimental procedures

Reagents and antibodies

Antibodies used are listed in the table:

| Target            | Species | Supplier (Catalogue/Lot)                                                                 |
|-------------------|---------|-----------------------------------------------------------------------------------------|
| PRC1 (Total)      | Rb      | Published antibody (Neef et al., 2007)                                                  |
| PRC1 pT481        | Rb      | Epitomics (cat 2189-1 / lot GR66072-5)                                                   |
| PRC1 pT602        | Rb      | Published antibody (Neef et al., 2007)                                                  |
| Aurora A pT288    | Rb      | Cell Signalling (cat 3079S / lot 5)                                                      |
| Aurora A (Total)  | Rb      | Cell Signalling (cat 4718S / lot 3)                                                      |
| PRC1 pT602        | Rb      | Published antibody (Neef et al., 2007)                                                  |
| PPP1CA            | Rb      | Bethyl Laboratories (cat A300-904A / lot A300-904A-1)                                    |
| PPP1CB            | Rb      | Bethyl Laboratories (cat A300-905A / lot A300-905A-1)                                    |
| PPP1CC            | Go      | Santa Cruz (cat sc-6108 / lot L2206)                                                     |
| PPP2CA/B          | Mo      | BD Transduction Laboratories (cat 610555 / lot 2146900)                                  |
| PPP4C             | Rb      | Bethyl Laboratories (cat A300-835A / lot A300-835A-1)                                    |
| PPP5C             | Rb      | Bethyl Laboratories (cat A300-909A / lot A300-909A-1)                                    |
| PPP6C             | Rb      | Published antibody (Zeng et al., 2010)                                                   |
| Cyclin B1         | Mo      | Millipore (cat 05-373 / lot DAM1776410)                                                  |
| Tubulin           | Mo      | Sigma (cat T6199-200UL / lot 110M4849)                                                   |
| PPP2R1A/B         | Go      | Santa Cruz (cat sc-6112 / lot G0106)                                                     |
| PPP2R2A           | Mo      | Cell Signalling (cat 5689S / lot 1)                                                      |
| PPP2R2B           | Rb      | Abcam (cat ab157461 / lot GR117938-1)                                                    |
| PPP2R2D           | Rb      | GeneTex (cat GTX116609 / lot 40268)                                                      |
| PPP2R5A           | Rb      | Bethyl Laboratories (cat A300-967A / lot A300-967A-1)                                    |
| PPP2R5C           | Rb      | Santa Cruz (cat sc-374380 / lot I2711)                                                   |
| PPP2R5D           | Rb      | Bethyl Laboratories (cat A301-100A / lot A301-100A-1)                                    |
| ENSA              | Sh      | This study; full-length recombinant antigen, affinity purified                            |
| ARPP19            | Sh      | This study; full-length recombinant antigen, affinity purified                            |
| PPP1CA pT320      | Rb      | Epitomics (cat 2167-1 / lot GR27139-4)                                                   |
| PPP1CA            | Rb      | Bethyl Laboratories (cat A300-904A / lot A300-904A-1)                                    |
| Pan Cdk pT        | Mo      | Cell Signalling (cat 9391S / lot 6)                                                      |
| Securin           | Rb      | Epitomics (cat 2603-1 / lot YF-11-12-06C)                                                |
| Separase          | Mo      | Abcam (cat ab16170 / lot 946095)                                                        |
| Sds22             | Go      | Santa Cruz (cat sc-162164 / lot C1510)                                                   |
| MYPT1             | Rb      | Epitomics (cat 1519-1 / lot YH011803C)                                                    |
| MASTL             | Rb      | Bethyl Laboratories (cat A302-190A / lot A302-190A-2)                                    |
| Plk1              | Mo      | Abcam (cat ab135637 / lot GR99892-1)                                                     |
| Cdc2 (Cdk1)       | Rb      | Epitomics (cat 1053-1 / C31801)                                                          |
| FLAG              | Rb      | Sigma (cat F7425)                                                                        |

Key: Rb = Rabbit; Sh = Sheep; Mo = Mouse; Go = Goat.

Antibodies to ENSA and ARPP19 were produced against full-length recombinant protein antigen. Specific antibodies were purified using antigen conjugated to Affigel-15, eluted with 0.2M glycine pH 2.8, then dialyzed against PBS before storage at -80°C. Antibodies to PRC1, PRC1 pT602, and
Plk1 were described previously (Bastos and Barr, 2010; Neef et al., 2007; Neef et al., 2003). Affinity purified primary and HRP-coupled secondary antibodies (Jackson ImmunoResearch Laboratories, Inc) were used at 1µg/ml final concentration. All western blots were revealed using ECL (GE Healthcare).

General laboratory chemicals and reagents were obtained from Sigma-Aldrich and Thermo Fisher Scientific. Inhibitors were obtained from Sigma-Aldrich (flavopiridol 5mM 1000x stock, and MG132 20mM 2000x stock), Tocris Bioscience (AZ3146 20mM 1000x stock), and Axon Medchem (BI2536 1mM 1000x stock). A benchtop microfuge (5417R; Eppendorf) was used for all centrifugations unless otherwise indicated. For western blotting, proteins were separated by SDS-PAGE and transferred to nitrocellulose using a Trans-blot Turbo system (Bio-Rad). Protein concentrations were measured by Bradford assay using Protein Assay Dye Reagent Concentrate (Bio-Rad).

**Preparation of cells for phosphatase siRNA library screening assay**

Hela S3 cells were seeded at a density of 125,000 cells per 10cm dish in 8ml growth medium and left to adhere for 24h prior to transfection with specific siRNA oligonucleotides. Cells were then incubated for 54h followed by 18h in 100ng/ml nocodazole. Mitotic cells were isolated by shake off, washed three times in 5ml ice cold PBS, pelleted at 200g for 3mins at 4°C and then lysed for 15min on ice at 1,000 cells/µl (0.75-1.0mg/ml final cleared lysate) ice cold mitotic lysis buffer (50mM Tris-HCl pH7.35, 150mM NaCl, 1% [vol/vol] IGEPAL, 1mM DTT, protease inhibitor cocktail (Sigma-Aldrich, P8340)) supplemented with 100nM okadaic acid as required.
siRNA library target sequences:

| Gene   | Accession | Company | Catalog # | Sequence                        |
|--------|-----------|---------|-----------|---------------------------------|
| PPP1CA | NM_206873 | Thermo  | J-008927-05 | GCAAGAGACGCUACAACAU            |
| PPP1CA | NM_206873 | Thermo  | J-008927-06 | GAGCAGAUUCGCGGGAUCA            |
| PPP1CA | NM_206873 | Thermo  | J-008927-07 | CAUCUUGGGUUUCUACGUA            |
| PPP1CA | NM_206873 | Thermo  | J-008927-08 | GAAGCAGCGUGCCGUCUUC           |
| PPP1CB | NM_206877 | Thermo  | J-008685-05 | GCAAGAUUUGGUCAGUA             |
| PPP1CB | NM_206877 | Thermo  | J-008685-06 | GCGUGAGAUUUCUCAACGUA          |
| PPP1CB | NM_206877 | Thermo  | J-008685-07 | GUGCUUACUGUCUAAU             |
| PPP1CB | NM_206877 | Thermo  | J-008685-08 | GAGAGAGACUAAUCCCA           |
| PPP1CC | NM_002710 | Thermo  | J-006827-05 | GCGGAGAGUUGUACAGU             |
| PPP1CC | NM_002710 | Thermo  | J-006827-06 | CGAUUUGCUAGGCAACUG            |
| PPP1CC | NM_002710 | Thermo  | J-006827-07 | UAGAUAACACGAACACGUA          |
| PPP1CC | NM_002710 | Thermo  | J-006827-08 | UGACUACGAUUGGACAGAAA         |
| PPP2CA | NM_002715 | Thermo  | J-003598-05 | GACUUUGACGUAUACUUA            |
| PPP2CA | NM_002715 | Thermo  | J-003598-06 | GCUGUAGUCUCUUAAGGU           |
| PPP2CA | NM_002715 | Thermo  | J-003598-07 | GCGAAGAUUUCUGAGAC            |
| PPP2CA | NM_002715 | Thermo  | J-003598-08 | GCAAAUCAACGACAGUACAA         |
| PPP2CB | NM_004156 | Thermo  | J-003599-05 | CAGAAGCGCCUAAUAA              |
| PPP2CB | NM_004156 | Thermo  | J-003599-06 | CACGAGGCUAGUCAAC           |
| PPP2CB | NM_004156 | Thermo  | J-003599-07 | CAGAAGCGCCUAAUAA             |
| PPP2CB | NM_004156 | Thermo  | J-003599-08 | CAGAAGCGCCUAAUAA             |
| PPP2CB | NM_004156 | Thermo  | J-003599-09 | CAGAAGCGCCUAAUAA             |
| PPP2CB | NM_004156 | Thermo  | J-003599-10 | CAGAAGCGCCUAAUAA             |
| PPP4C  | NM_002720 | Thermo  | J-008486-05 | GCAUAAUGCUUUGCAUC           |
| PPP4C  | NM_002720 | Thermo  | J-008486-06 | GCAUAAUGCUUUGCAUC           |
| PPP4C  | NM_002720 | Thermo  | J-008486-07 | GCAUAAUGCUUUGCAUC           |
| PPP4C  | NM_002720 | Thermo  | J-008486-08 | GCAUAAUGCUUUGCAUC           |
| PPP4C  | NM_002720 | Thermo  | J-008486-09 | GCAUAAUGCUUUGCAUC           |
| PPP5C  | NM_006247 | Thermo  | J-009259-05 | GGCUCUGAGCUGACUGU             |
| PPP5C  | NM_006247 | Thermo  | J-009259-06 | GCAAAUCAACGACAGUA             |
| PPP5C  | NM_006247 | Thermo  | J-009259-07 | GCAAAUCAACGACAGUA             |
| PPP5C  | NM_006247 | Thermo  | J-009259-08 | GCAAAUCAACGACAGUA             |
| PPP6C  | NM_002721 | Thermo  | J-009935-05 | CUAACGGAACAGAAGGUA            |
| PPP6C  | NM_002721 | Thermo  | J-009935-06 | CUAACGGAACAGAAGGUA            |
| PPP6C  | NM_002721 | Thermo  | J-009935-07 | CUAACGGAACAGAAGGUA            |
| PPP6C  | NM_002721 | Thermo  | J-009935-08 | CUAACGGAACAGAAGGUA            |
| PPP2R1A| NM_014225 | Thermo  | J-010259-05 | AGCGGAACUUUGCAGA             |
| PPP2R1A| NM_014225 | Thermo  | J-010259-06 | AGCGGAACUUUGCAGA             |
| PPP2R1A| NM_014225 | Thermo  | J-010259-07 | AGCGGAACUUUGCAGA             |
| PPP2R1B| NM_181699 | Thermo  | J-017592-05 | GCAAAUUCGAGUAA             |
| PPP2R1B| NM_181699 | Thermo  | J-017592-06 | GCAAAUUCGAGUAA             |
| PPP2R1B| NM_181699 | Thermo  | J-017592-07 | GCAAAUUCGAGUAA             |
| PPP2R1B| NM_181699 | Thermo  | J-017592-08 | GCAAAUUCGAGUAA             |
| PPP2R1B| NM_181699 | Thermo  | J-017592-09 | GCAAAUUCGAGUAA             |
| PPP2R2A| NM_002717 | Thermo  | J-004824-05 | CAUACGGAACAGAAGA             |
| PPP2R2A| NM_002717 | Thermo  | J-004824-06 | CAUACGGAACAGAAGA             |
| PPP2R2A| NM_002717 | Thermo  | J-004824-07 | CAUACGGAACAGAAGA             |
| PPP2R2A| NM_002717 | Thermo  | J-004824-08 | CAUACGGAACAGAAGA             |
| PPP2R2B| NM_181676 | Thermo  | J-003022-05 | UGCAUUACUGAAGU             |
| PPP2R2B| NM_181676 | Thermo  | J-003022-06 | UGCAUUACUGAAGU             |
| PPP2R2B| NM_181676 | Thermo  | J-003022-07 | UGCAUUACUGAAGU             |
| PPP2R2B| NM_181676 | Thermo  | J-003022-08 | UGCAUUACUGAAGU             |
| PPP2R2C| NM_181876 | Thermo  | J-019167-05 | CGAGGAGUUCUUGGCAU             |
| PPP2R2C| NM_181876 | Thermo  | J-019167-06 | GAUAAACCCUGAGGUA             |
| PPP2R2C| NM_181876 | Thermo  | J-019167-07 | GAUAAACCCUGAGGUA             |
| PPP2R2C| NM_181876 | Thermo  | J-019167-08 | GAUAAACCCUGAGGUA             |
| Gene      | Accession | Vendor | ID          | Description |
|-----------|-----------|--------|-------------|-------------|
| PPP2R2D   | NM_001003656 | Thermo | J-032298-05 | GUAGGUUCCUCUUCUCAGA |
| PPP2R2D   | NM_001003656 | Thermo | J-032298-06 | UCGGAUAGCACCACUAUAGA |
| PPP2R2D   | NM_001003656 | Thermo | J-032298-07 | GAGACUACCUCUGGUGGAA |
| PPP2R2D   | NM_001003656 | Thermo | J-032298-08 | GAGACGACUGCAUCUUUG |
| PPP2R3A   | NM_181897   | Thermo | J-017376-05 | GGAAGGCUUAGAAGAUAU |
| PPP2R3A   | NM_181897   | Thermo | J-017376-06 | CAGAAUUGCUCAACAUUG |
| PPP2R3A   | NM_181897   | Thermo | J-017376-07 | CAAUAACAUUCCACGGG |
| PPP2R3A   | NM_181897   | Thermo | J-017376-08 | GGAAGAAGUGUGCAUAA |
| PPP2R3B   | NM_199326   | Thermo | J-019459-05 | CGCCGAGGAGAUGCAU |
| PPP2R3B   | NM_199326   | Thermo | J-019459-06 | GGUAAACGCGAGACUAG |
| PPP2R3B   | NM_199326   | Thermo | J-019459-07 | GGAAGAUCACGUGCAUG |
| PPP2R3B   | NM_199326   | Thermo | J-019459-08 | GACAGCAGGCAUCAGAG |
| PPP2R3C   | NM_017917   | Thermo | J-018203-09 | GAAGAAGCUCAACUGCAU |
| PPP2R3C   | NM_017917   | Thermo | J-018203-10 | GGAAGAUGCAUAAUAGAA |
| PPP2R3C   | NM_017917   | Thermo | J-018203-11 | CACCAUUAUACUAGAUU |
| PPP2R3C   | NM_017917   | Thermo | J-018203-12 | AAACAUAGGCAUCAGUUA |
| PPP2R4    | NM_178003   | Thermo | J-005214-05 | GCAGUUUGCAGCUAGA |
| PPP2R4    | NM_178003   | Thermo | J-005214-06 | UGGGAUGUAUCUGUAGUUA |
| PPP2R4    | NM_178003   | Thermo | J-005214-07 | GAAUAGACUGGCAUAA |
| PPP2R4    | NM_178003   | Thermo | J-005214-08 | CCAACAGCGUGGAGCAU |
| PPP2R5A   | NM_006243   | Thermo | J-009352-07 | GUCUAAAGAGGCAUUA |
| PPP2R5A   | NM_006243   | Thermo | J-009352-08 | CAUAACAGAUGGCAUA |
| PPP2R5A   | NM_006243   | Thermo | J-009352-09 | UGAAGAAGACGUGGAG |
| PPP2R5A   | NM_006243   | Thermo | J-009352-10 | GGAAGAUGCAUAGGCAU |
| PPP2R5B   | NM_006244   | Thermo | J-009366-05 | CGCAUAGCUGCAUGA |
| PPP2R5B   | NM_006244   | Thermo | J-009366-06 | UCAACGUGCUGCAUGU |
| PPP2R5B   | NM_006244   | Thermo | J-009366-07 | CAAACAGCUCAUCAG |
| PPP2R5B   | NM_006244   | Thermo | J-009366-08 | AAACAUAGGAGCAUUA |
| PPP2R5C   | NM_178588   | Thermo | J-009433-05 | GGAUUUCUGCUACCA |
| PPP2R5C   | NM_178588   | Thermo | J-009433-06 | GGAAGAUGAAGCAUGA |
| PPP2R5C   | NM_178588   | Thermo | J-009433-07 | CAUCAGAAUUGAGAGA |
| PPP2R5C   | NM_178588   | Thermo | J-009433-08 | CAGAGAUGUCUAAGUUA |
| PPP2R5D   | NM_180977   | Thermo | J-009799-06 | GUCAACUGAACCACAG |
| PPP2R5D   | NM_180977   | Thermo | J-009799-07 | UCCAAGGAGCAUAA |
| PPP2R5D   | NM_180977   | Thermo | J-009799-08 | UGAUCAGGCUGGAGU |
| PPP2R5D   | NM_180977   | Thermo | J-009799-09 | GUAGGCAUAGCACCAC |
| PPP2R5E   | NM_006246   | Thermo | J-008531-06 | UUAAGAAGUGGGAGCA |
| PPP2R5E   | NM_006246   | Thermo | J-008531-07 | GACACGGUGCAUAGG |
| PPP2R5E   | NM_006246   | Thermo | J-008531-08 | GACACGGUGCAUAGG |
| PPP2R5E   | NM_006246   | Thermo | J-008531-09 | GGAUAAAGGAGCAUGA |
| ARPP19    | NM_006628   | Thermo | J-015338-05 | CAAGCGUGCGUGCGUAG |
| ARPP19    | NM_006628   | Thermo | J-015338-06 | CAACAUGCCUAAGCAAA |
| ARPP19    | NM_006628   | Thermo | J-015338-07 | GUCAGAAUUCUAAAGAG |
| ARPP19    | NM_006628   | Thermo | J-015338-08 | GCAACAGGUUCUACAC |
| MASTL     | NM_001172303| Thermo | J-004020-09 | GGAACACUGUAACUGUA |
| MASTL     | NM_001172303| Thermo | J-004020-10 | ACUGGAGCGCGUGAGU |
| MASTL     | NM_001172303| Thermo | J-004020-11 | GGAUAAAGUGAGAGAG |
| MASTL     | NM_001172303| Thermo | J-004020-12 | CCAUAGGAGAGAAGG |
| ENSA      | NM_207042   | Thermo | J-011852-05 | GCJAUAGCAGCAGAA |
| ENSA      | NM_207042   | Thermo | J-011852-06 | AGAGAGCAGGAGGCA |
| ENSA      | NM_207042   | Thermo | J-011852-07 | UGAAGAUGGCACAGAA |
| ENSA      | NM_207042   | Thermo | J-011852-08 | AGAGACCAAGGAGGAA |
| ENSA      | NM_207042   | QiAgen | SI03042326 | AGAGGCAAGCUAAAGG |
| ENSA      | NM_207042   | QiAgen | SI03093356 | CUGAGAGACGUGAGAGG |
**Protein expression and purification**

Recombinant baculoviruses encoding hexahistidine-tagged PRC1 or Plk1 were used to infect 4x10^7 insect cells in 20x15cm dishes with a multiplicity of infection of 10. To infect cells, the medium was removed, and the virus added in 3ml of insect growth medium per dish. Dishes were gently rocked for 1h; every 10min the dishes were rotated by 90 degrees. After 1h, 17ml insect cell growth medium were added and the cells left for 48h prior to harvesting by centrifugation at 200g. Dually phosphorylated PRC1-pT481/pT602 substrate was generated by treatment for 4h with 100nM okadaic acid before harvesting. Cell pellets were washed once in ice cold PBS, and then lysed in 10ml of insect cell lysis buffer (20mM Tris-HCl pH 8.0, 300mM NaCl, 20mM imidazole, 0.2% [vol/vol] Triton-X 100, and protease inhibitor cocktail (Sigma-Aldrich)) for 20min on ice. Clarified cell lysate was prepared by centrifugation at 100,000g in a TLA100.3 rotor (Beckman), and then loaded on a 1ml HisTrap FF column (GE Healthcare) at 0.5ml/min. The column was then washed with 30ml of 20mM Tris-HCl pH 8.0, 300mM NaCl, 20mM imidazole, and eluted with a 20ml linear gradient from 20-200mM imidazole collecting 1ml fractions. Peak fractions judged by SDS-PAGE and absorbance at 280nm were concentrated using Ultrace -10K centrifugal filters (Millipore) to a final volume of 1ml. Samples were buffer exchanged into 20mM Tris-HCl pH 8.0, 300mM NaCl and 1mM DTT using 5ml Zeba desalt spin columns (Perbio). Purified protein was snap frozen in 15µl aliquots and stored at -80°C. To generate the PRC1-pT602 substrate lacking pT481 phosphorylation, 158µg PRC1 purified from insect cells that had not been treated with okadaic acid or nocodazole was phosphorylated *in vitro* by incubating with 300µg Plk1 in a
final volume of 500µl 20mM Tris-HCl pH 8.0, 300mM NaCl, 1mM DTT, 3mM Mg-ATP for 3h at 21°C. This sample was further purified by gel filtration on a Superose 6 10/300 column at 0.2ml/min and the fractions containing PRC1-pT602, confirmed by western blotting, snap frozen and stored at -80°C.

**Mitotic extract preparation and ion-exchange chromatography**

Twenty 15cm dishes (500,000 Hela S3 cells per plate) were grown for 72h prior to 18h treatment with 100ng/ml nocodazole. Mitotic cells isolated by shake off were washed in ice cold PBS (3x50ml) and then lysed on ice for 15min in 50mM Tris-HCl pH 7.35, 1mM DTT, 1mM MgCl₂, 0.1mM MnCl₂, 50mM NaCl, 1% [vol/vol] IGEPAL supplemented with protease inhibitor cocktail (Sigma-Aldrich, P8340). Extracts were clarified at 20,817g for 10min at 4°C. Soluble extract proteins, 10.9mg, were injected onto a 1ml HiTrap-Q HP anion exchange column (GE Healthcare) pre-equilibrated in Q50 buffer (50mM Tris-HCl pH 7.35, 1mM DTT, 1mM MgCl₂, 0.1mM MnCl₂, 50mM NaCl, 0.1% [vol/vol] IGEPAL). The column was washed with 5ml Q50 buffer. Proteins were eluted with a 20ml linear gradient from 0-50% [vol/vol] Q1000 (50mM Tris-HCl pH 7.35, 1mM DTT, 1mM MgCl₂, 0.1mM MnCl₂, 1 M NaCl, 0.1% [vol/vol] IGEPAL) followed by 2ml 100% Q1000 and then 5ml 100% Q50. Flow rate was 1ml/min and 1ml fractions were collected then aliquoted prior to snap freezing with liquid nitrogen. Fractions were screened for phosphatase activity using Cdk and Plk1-phosphorylated PRC1 substrates. In all instances 10µl of each fraction was incubated on ice for 35min with either 95ng PRC1-Cdk substrate or 20ng PRC1-Plk substrate in a total volume of 100µl 20mM Tris-HCl pH 7.35 supplemented with 5µM flavopiridol (Cdk
inhibitor), 1µM BI2536 (Plk1 inhibitor) and 0.2mg/ml bovine serum albumin carrier protein.

**Purification of PP2A holoenzymes**

HEK293T cells were seeded onto 15-cm dishes (7.325x10⁶ cells per dish) and grown for 24h. Six dishes per condition were transiently transfected with 8 µg of pCDNA5-Flag (empty vector control), pCDNA5-FLAG-PPP2R2A or pCDNA5-FLAG-PPP2R5C. After harvesting, cells were washed 3x in 50ml ice cold PBS, centrifuged at 400g, 3min, 4°C, and then pellets lysed in 2ml ice-cold T-lysis buffer (20mM Tris-HCl pH 7.35, 150mM NaCl, 1% [vol/vol] Triton-X 100, protease inhibitor cocktail (Sigma-Aldrich)) on ice for 15min. Extracts were clarified at 20,817g for 15min at 4°C, supernatants isolated and then 200µl of FLAG M2-agarose beads (Sigma-Aldrich) pre-washed in T-lysis buffer were added per condition. After 3h at 4°C beads were washed 2x in T-lysis buffer, 4x in TBS (20mM Tris-HCl pH 7.35, 150mM NaCl) containing 0.1% [vol/vol] Triton-X 100, 1x in TBS and 1x in elution buffer (20mM Tris-HCl pH 7.35, 150mM NaCl, 1mM MnCl₂). Bound proteins were eluted from beads in 110µl elution buffer containing 200µg/ml FLAG peptide (Sigma-Aldrich, F3290) at 22°C for 30min. Glycerol ([25%]final) and DTT ([1mM]final) were added to the eluate before aliquoting and snap freezing.

**Activity assays with purified PP2A holoenzymes**

PPP2R2A holoenzymes purified as described in the online supplemental material were matched to lysate generated in the chromatography experiment by titration to yield the same relative activity against PRC1 pT481. Reactions
were setup on ice and performed in tubes treated with a blocking buffer (50mM Tris pH7.35, 0.1mM MnCl₂, 1mM MgCl₂, 1mM DTT, 0.1% [vol/vol] IGEPAL, 300mM NaCl, 2mg/ml BSA) to limit non-specific loss of protein components on tube walls. Standard assays used 375ng PRC1 pT481/pT602 substrate in 185µl of reaction buffer (50mM Tris pH7.35, 0.1mM MnCl₂, 1mM MgCl₂, 1mM DTT, 0.1% [vol/vol] IGEPAL, 300mM NaCl, 0.2mg/ml BSA, 5µM flavopiridol and 1µM BI2536). Activity in 110µg of mitotic lysate was tested alongside increasing amounts of PPP2R2A holoenzyme: 540ng (0.16x), 970ng (0.3x) and 1300ng (0.4x)). From this titration it was estimated that 810ng (0.25x) gave a relative activity that matched the lysate condition. Western blotting of catalytic and scaffold subunits revealed that the concentration of isolated PPP2R5C holoenzymes was 1.5x less than PPP2R2A; as such, PPP2R2A was diluted 1.5 fold in elution buffer containing 25% [vol/vol] glycerol and 1mM DTT to bring holoenzymes to the same concentration. Assays were then performed using 100µg lysate or 770ng of each holoenzyme against either 380ng PRC1-pT481/pT602 or 90ng PRC1-pT602 (equivalent to a matched amount of pT602) in 175µl reaction buffer supplemented where appropriate with 100nM okadaic acid. Recombinant PRC1 and mitotic cell extract were prepared as described in the supplemental online material. An equal volume of undiluted eluate from the pCDNA5-Flag (empty vector) control IP was used in mock reactions to ensure that non-specific phosphatase activity was not associated with the beads. For both titration and assay experiments samples were removed at indicated time points, added to SDS-sample buffer and boiled immediately for 5min to stop reactions.
**Live and fixed cell microscopy**

Fixed cell microscopy was carried out exactly as described previously (Dunsch et al., 2012). Secondary antibodies conjugated to Alexa Fluor 488, 555, and 647 were obtained from Invitrogen. DNA was stained with DAPI (Sigma-Aldrich). For live cell imaging, cells were plated in 35mm dishes with a 14mm No. 1.5 thickness coverglass window (MatTek Corporation) then treated as described in the figure legends. The dishes were placed in a 37°C and 5% CO$_2$ environment chamber (Tokai Hit) on an Olympus IX81 inverted microscope with a 60x 1.42NA oil immersion objective coupled to an Ultraview Vox spinning disk confocal system (Perkin Elmer). Images were captured with a C9100-13 EM-CCD camera (Hamamatsu Corporation). Exposure times were 30msec for all markers using 4% laser power for both 488 and 561nm lasers. Image stacks of 24 planes spaced 0.6µm apart were taken at 1-4 stage positions every min as cells passed through mitosis. A brightfield reference image was taken to visualise cell shape. Maximum intensity projection of the fluorescent channels was performed in Volocity to create 24-bit RGB or 8-bit grayscale TIFF files. Images were then placed into Adobe Illustrator CS3 to produce the figures.

**Mathematical model for mammalian mitotic exit**

Figure 5A illustrates our working hypothesis in the form of an influence diagram for the components regulating anaphase progression and cytokinesis in mammalian cells. This is explored in a more detailed wiring diagram that provided the mechanistic basis of mathematical modelling (Figure S4). In the model, the time dependent change of each network component is described.
by a nonlinear ordinary differential equation (ODE). Each term on the right side of the ODE corresponds to a biochemical reaction. All the reactions are described by the law of mass action following zero (e.g. protein synthesis), first (degradation and complex dissociation) or second (association of proteins) order kinetics.

Equations for the mitotic exit control model:

\[
\begin{align*}
\frac{d[MCC]}{dt} &= -k_d[MCC] \times [MCC : APC] \\
\frac{d[MCC : APC]}{dt} &= k_{d,MA} \times (MCC : APC) \times [APC] \\
&\quad - (k_{d,MA} + k_{d,MCC}) \times [MCC : APC] \\
\frac{d[AnaT]}{dt} &= k_{s,AnaT} - k_d[AnaT] \times [AnaT] + k_{d,AnaT} \times (AnaT : APC) + [AnaT : APC : SEP] \\
\frac{d[Ana : APC]}{dt} &= k_{s,AA} \times [Ana] \times [APC] - (k_{d,AA} + k_{d,AnaT} + k_{d,Ap}) \times [Ana : APC] \\
&\quad \times (k_{d,AA} \times [Ana] \times [SEP] + k_{d,AS} \times [Ana] : APC : SEP] \\
\frac{d[Ana : SEP]}{dt} &= k_{s,AS} \times [Ana] \times [SEP] - (k_{d,AS} + k_{d,AnaT}) \times [Ana : SEP] \\
&\quad \times (k_{d,AS} \times [Ana] \times [SEP] + k_{d,AA} \times [Ana] : APC : SEP] \\
\frac{d[Ana : APC : SEP]}{dt} &= k_{s,AP} \times [Ana] \times [APC] \times [SEP] + k_{s,AA} \times [Ana] \times [SEP] \times [APC] \\
&\quad - (k_{d,AS} + k_{d,AA} + k_{d,Ap}) \times [Ana] : APC : SEP] \\
\frac{d[CycBd : SEP]}{dt} &= k_{s,AS} \times (CycBd : SEP) - (CycBd : SEP) \times [SEP] \\
&\quad - k_{d,AS} \times [CycBd : SEP] \\
\frac{d[GWP]}{dt} &= k_{p,GW} \times [CycBA] \times (GWT - GWP) - (k_{dp,GW} \times [B55] + [GWP]) \times [GWP] \\
\frac{d[ENSAPT]}{dt} &= k_{p,E} \times [GWP] \times (ENSAPT - [ENSAPT]) - k_{dp,E} \times [ENSAPT] \\
\frac{d[PEcom]}{dt} &= k_{p,E} \times [GWP] \times (ENSAPT - [ENSAPT]) - k_{dp,E} \times [ENSAPT] \\
\frac{d[T481]}{dt} &= k_{p,T481} \times [CycBA] \times ([PRCI] + [T602]) - (k_{dp,T481} \times [B55] \times [T481] \\
\frac{d[T602]}{dt} &= k_{p,GW} \times [PRCI] - k_{dp,GW} \times [T602] - k_{p,T481} \times [CycBA] \times [T602] \\
[APC] &= [APCT] - [MCC : APC] - [Ana : APC] - [Ana : APC : SEP] \\
[Ana] &= [AnaT] - [Ana : APC] - [Ana : SEP] - [Ana : APC : SEP] \\
[SEP] &= [SEP] - [Ana : SEP] - [Ana : APC : SEP] - [CycBd : SEP] \\
[B55] &= [B551] \times [PEcom] \\
[PRCI] &= [PRCT] - [T602] - [T481] \\
[CycBA] &= \frac{\epsilon \times [Ana]}{[CycBd : SEP] + FLA}
\end{align*}
\]
The dynamic variables of the model, which represent relative protein concentrations, are dimensionless. The rate constants \( k \) have a dimension of \( \text{min}^{-1} \) and the form of \( k_{a,b}^c \). The subscript \('a'\) indicates the type of reaction: s, synthesis; as, association; d, degradation; ds, dissociation; p, phosphorylation; dp, dephosphorylation. The subscript \('b'\) indicates the proteins participating in the reaction. The superscript \('c'\) indicates whether it is a non-regulated spontaneous reaction ('') or a catalysed reaction (labelled by the catalysing enzyme).

Our mathematical model describes the temporal dynamics of mitotic exit in mammalian cells from the beginning of metaphase, when spindles are already properly aligned and the production of the Mitotic Checkpoint Complexes (MCC) is already terminated. During the early stage of mitotic exit, the MCC that has accumulated in prometaphase is inactivated by Anaphase Promoting Complex (APC) dependent ubiquitylation (Eq. 1 & 2). After MCC is degraded, APC is released to promote the ubiquitin dependent degradation of two other anaphase substrates, securin and cyclin B (Eq. 3 & 4). These two APC substrates are combined in the model under the name of Anaphase Inhibitors (Ana I), because both of them bind and inhibit separase (Eq. 5 & 6), which is the protease responsible for cohesin cleavage and anaphase initiation. We assume that cyclin B is a constant fraction of Ana I (the fraction is represented with the parameter \( \epsilon \)) and that cyclinB is in complex with Cdk1. For some experiments the model was supplemented with a constant level (zero during normal mitotic exit) of non-degradable cyclin B that can bind to and inhibit separase (Eq. 7). When Cdk1-cyclin B is not associated with APC or separase, it is able to phosphorylate its substrates Greatwall/MASTL and
PRC1. Both of these substrates are dephosphorylated by PP2A-B55. Cdk1-cyclin B phosphorylates Greatwall/MASTL (Eq. 8), and then this active Gwl in turn phosphorylates ENSA to convert it to a PP2A-B55 inhibitor (Eq. 9). The phosphorylated ENSA (ENSA-P) binds reversibly to PP2A-B55 (Eq. 10) and thereby blocks Gwl and PRC1 T481 dephosphorylation by PP2A-B55. Dephosphorylation of ENSA-P might be catalysed by PP2A-B55 or by another phosphatase, and we assume PP2A-B55 is not essential for ENSA dephosphorylation.

PRC1 is phosphorylated at two sites (T481 and T602), which could define four species with different phosphorylation states. Cdk1-cyclin B and Polo-kinase are responsible for T481 and T602 phosphorylation, respectively (Eq. 11 & 12). Dephosphorylation of T481 is catalysed by PP2A-B55 while phosphorylated T602 is targeted by PP2A-B56. According to our experimental data, T481 phosphorylation promotes T602 dephosphorylation. This allows us to neglect the double-phosphorylated form in the model (see Suppl. Fig. S4).

The conservation relationship, that the total level of a protein is the sum of all its forms, allows us to calculate the free (not in complex) forms of APC, Ana I, separase and PP2A-B55 (Eq. 13-16). The same conservation relationship also allows us to calculate the unphosphorylated form of PRC1 by subtracting the phosphorylated ones from the constant total level (Eq. 17). Cdk1-cyclin B inhibition by flavopiridol (FLA) is described by Eq. 18.

Mitotic exit which is a short window of the cell cycle, and we assume that APC, separase, PP2A-B55 and PRC1 levels are constant during this time window. Therefore the total levels of these proteins are described with constant parameters.
Model parameters

Values of the parameters (rate constants and relative protein levels) are largely unknown and have to be estimated within reasonable bounds. For example, stoichiometric inhibitors should be present in excess in order to provide significant inhibition. Therefore we assume that [MCCT] > [APCT] and [Ana IT] > [SEPT] at the beginning of metaphase. We also assume that [ENSAT] > [B55T], which is consistent with our observation that ENSA is in about 5-fold excess with respect of PP2A-B55. Association constants are large but less than 200 min\(^{-1}\), therefore not faster than diffusion-limited rate of second order reactions at typical cellular protein concentrations (Verdugo et al., 2013). The choice of rate constants is also constrained by known cellular physiology. For example, we require that separase activation precedes PRC1 T481 dephosphorylation during normal mitotic exit.

Numerical simulation of the model starts with initial condition characteristic for the beginning of metaphase in mitosis (high level of MCC and Ana I, inactive APC and PRC1 phosphorylated on T481 etc.). These initial conditions correspond to a checkpoint arrested mitotic state, which is achieved in the model by setting by \(k_{d,MCC} = 0\). Simulated mitotic exit starts at time zero by increasing \(k_{d,MCC}\) to 0.2.

\[
\begin{align*}
  k_{as,MA} &= 50, \quad k_{ds,MA} = 0.05, \quad k_{as,aa} = 1, \quad k_{dsaa} = 0.5, \quad k_{asas} = 50, \quad k_{dsas} = 0.5, \\
  k_{as,EP} &= 200, \quad k_{ds,EP} = 0.1, \\
  k_{sAna\;I} &= 0.005, \quad k_{dA\_bk} = 0.005, \quad k_{dA\_AC} = 0.75, \quad k_{dmcc} = 0.2 (\text{=0 for proteasome inhibition by MG132}). \\
  k_{pGW} &= 100, \quad k_{dpGW\_bk} = 0, \quad k_{dpGW\_B55} = 50, \quad k_{pE} = 8, \quad k_{dpE} = 5, \quad k_{p481} = 10, \\
  k_{dp481} &= 0.5, \quad k_{p602} = 2, \quad k_{dp602} = 1.
\end{align*}
\]
APCT=1, SEPT=0.25, GWT=1 (=0 for Gwl depleted cells), ENSAT=6, B55T=1, PRC1T=1, CycBndT=0 (>0 if non-degradable cyclin B is added).

ε=0.5, FLA = 0 (=100 for flavopiridol inhibition)

**Initial conditions**

[MCC:APC]=0.998, [MCCT]=2, [ANA IT]=0.91, [ANA I:APC]=0, [ANA I:SEP]=0.246, [ANA I:APC:SEP]=0, [GWP]=0.978, [ENSAPT]=3.661, [PEcom]=0.991, [T481]=0.998, [T602]=0.001

**Model simulation and analysis**

Time series simulation and bifurcation analysis of the model were carried out with the freely available software XPPAUT (http://www.math.pitt.edu/~bard/xpp/xpp.html).
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