Supplementary experimental procedures

Cell culture and cell harvesting

HeLa cells were grown as described in (1). Mitotic cells for the regulatory cut-off definition were arrested for 18h using 330 ng/µl nocodazole (Sigma-Aldrich, USA), harvested by scraping, washed twice with PBS with the same nocodazole concentration as in the cell culture buffer, frozen in liquid nitrogen and stored at -80°C until analysis.

Immunofluorescence microscopy and image processing

Cells cytopun onto microscopy slides were washed with 0.5% Triton-X100 in PBS for 2 min and fixed with 4% formaldehyde in PBS (2). After fixation samples were permeabilized with 0.5% Triton-X100 in PBS for 15 min and thereafter blocked with 10% FCS in PBS containing 0.01% Triton-X100. Slides were incubated for 1 h each at RT with primary (Lamin A, see antibody details) and secondary antibodies (at 2 µg/ml in 3% BSA in PBS containing 0.01% Triton-X100), DNA was counterstained with 4'-6-Diamidino-2-phenylindole (DAPI, Molecular Probes, Invitrogen, UK). Coverslips were mounted with either ProLong Gold (Molecular Probes, Invitrogen, UK) or Vectashield Mounting Medium (H1000, Vector Laboratories, USA) onto slides. Images were aquired according to (3) on a Zeiss Axioplan 2 microscope using 63x Plan-Apochromat objective lens (Carl Zeiss, Jena, Germany) equipped with CoolSnapHQ CCD camera (Photometrics, USA). Images shown in the same figure have been scaled identically.

Protein extraction and precipitation

The lysis buffers were prepared as described (4). Equal volumes of cell pellet and lysis buffer were used to resuspend the pellet. For the preparation of the cell suspension the protocol described in Poser et al, 2008 was utilized. Crude cell extracts were centrifuged at 13200 rpm in a table top centrifuge at 4°C for 15 minutes and the supernatant kept. The protein content was determined using the Bradford method. 5 sample volumes of ice-cold 100% acetone (Sigma-Aldrich, Germany) were added and the sample transferred to -20°C over night. Precipitated proteins were collected by centrifugation at 13200 rpm for 30 min. Then the supernatant was discarded and the pellet was resuspended in 150 µl of cold 80% acetone (-20°C). To resuspend the pellet it was forced through the tip of a 200 µl pipette several times. The sample was centrifuged at 13200 for 5 min. Finally the supernatant was removed again and the pellet was air-dried for 5 to 10 min. Then it was dissolved with 8M urea (Amresco, USA) in 0.5 M ammonium bicarbonate (ABC, Fluka, Switzerland). The protein concentration was determined using Bradford (Bio-Rad, USA) and BCA Protein Assay protocols (Thermo Fisher Scientific, USA).

Strong cation exchange fractionation

The separation system consisted of:
1. BioLC inert chromatography system (Dionex, USA) with
   a. AS50 Autosampler with column oven and 1 ml sample loop
   b. GS50 HPLC Pump
   c. UVD340U UV detector
2. Probot, µ-fraction collection unit (Dionex, USA)
3. PolySULFOETHYL A (aspartate SCX) separation columns (Poly LC, USA) of the
dimension: 4.6 mm ID x 15 cm length (operated at 1 ml/min)

Mobile phases for SCX separation:
A. 5 mM phosphate buffer + 15% acetonitrile (ACN), pH = 2.7
B. 5 mM phosphate buffer + 15% acetonitrile (ACN), pH = 2.7 + 0.5M NaCl
C. 5 mM phosphate buffer + 15% acetonitrile (ACN), pH = 6.0

Preparation of mobile phases:

Chemicals:
- ortho-phosphoric acid 85% p.a.; Merck, P/N: 1.00573
- NaH2PO4.H2O ACS reagent; Sigma-Aldrich, P/N: S9638
- Na2HPO4.2H2O p.a.; Merck, P/N: 1.06580
- NaCl p.a.; Merck, P/N: 1.06404
- Acetonitrile "LiChrosolv"; Merck, P/N: 1.00030
- 18.2 Milli-Q H2O or ddH2O
- Media-Plus filter Units MF75 Series, 0.22µm Nylon Membrane; Nalgene, P/N: 164-0020

All buffers were prepared and stored in plastic bottles to minimize buffer aging.
The solvents were prepared as followed:
A Buffer: 5mM Na-PO4 pH 2.7, 15% ACN
- 337.1 µl ortho-phosphoric acid was dissolved in 800 ml H2O
- the pH was set to 2.7 with 2M NaOH (ca. 870µl)
- the phosphoric acid solution was filtered through a 0.22µm nylon filter into a bottle containing
  150 ml ACN
- the bottle was filled up to 1000ml with water
B Buffer: 5mM Na-PO4, 0.5M NaCl pH 2.7, 15% ACN
- 337.1 µl ortho-phosphoric acid and 29.22g NaCl were dissolved in 800 ml H2O
- the pH of phosphoric acid was set to 2.7 with 2M NaOH (ca 2000µl)
- the phosphoric acid solution was filtered through a 0.22µm nylon filter into a bottle containing
  150 ml ACN
- the bottle was filled up to 1000ml with water
C Buffer: 5mM Na-PO4 pH 6.0, 15% ACN
- 690 mg NaH2PO4.H2O was dissolved in 800 ml H2O
- the pH of phosphoric acid was set to pH6 with 2M NaOH (ca 150µl)
- the solution was filtered through a 0.22µm nylon filter into the bottle containing 150 ml ACN
- the bottle was filled up to 1000ml with water
Table 1 Triple SCX-gradient

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (ml/min)</th>
<th>% Buffer B</th>
<th>% Buffer C</th>
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<tbody>
<tr>
<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
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<td>0</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

96 fractions eluting from the SCX column were collected through the PROBOT fraction collector and then pooled according to their signal intensity in 25 pools.

Antibody Details

To detect proteins on western blots (WB) or in immunofluorescence microscopy (IF), the following antibodies were used (antibodies are from rabbit, if not stated otherwise): mouse α-Lamin A antibody [133A2] (Abcam, ab8980), α-STAG2 (5), α-phospho-STAG2 (6) mouse α-phospho-S10-H3 (05-499, Upstate), goat α-H3 (sc8654, Santa Cruz), α-CENPF (Abcam, ab5), α-EIF4B (Aviva Systems Biology, ARP40359_T100), α-WAPAL (6) α-phospho-CDK1 (Santa Cruz, sc-28435-R), mouse α -CDK1 (Santa Cruz, sc-54), α -phospho-RAD21 (Hegemann, in revision), mouse α-RAD21 (Millipore, 05-908).

Secondary antibodies used for western blotting were α-mouse, α-rabbit or α-goat coupled to horse radish peroxidase (HRP); for immunofluorescence Alexa 488, Alexa 568 and Alexa 633 labeled secondary antibodies were used (Molecular Probes, Invitrogen, USA). DAPI was used to stain chromatin.

Table 3: Peptide information of generated phospho-specific antibodies

<table>
<thead>
<tr>
<th>name of protein</th>
<th>peptide sequence</th>
<th>host animal</th>
<th>purification taken for W.B. (Magnesium elution (M))</th>
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</thead>
<tbody>
<tr>
<td>CENPF</td>
<td>CLQGLDLS(p04)S(p04)RSLLGI</td>
<td>rabbit</td>
<td>M</td>
</tr>
<tr>
<td>EIF4B</td>
<td>CPRRGDDS(p04)FGDKYR</td>
<td>rabbit</td>
<td>M</td>
</tr>
</tbody>
</table>

The phospho-specific antibodies CENPF, EIF4B and WAPAL (Hegemann et al., in preparation) were used at a final concentration of 2µg/ml for immunoblotting.

Motif searching

Preparation of phosphopeptide quantification data for motif enrichment analysis

4061 unique sites of phosphorylation were identified in this study. Foregrounds of 134 and 518 phospho-sites up- and downregulated, respectively, in the context of PLK1 inhibition were
created by selecting those sites with at least a 2-fold change in phosphorylation in the presence of inhibitor in the context of at least one peptide observed by MS, and removing duplicate instances of any 15-mer sequence found in more than one tryptic fragment.

**Determination of empirical statistical significance cut-off (see Supplementary Figure S2)**
In order to control for the rate of false positive motif discovery, 1000 foregrounds were generated by randomly selecting phospho-sites from the full background of 4061 sites for both the Plk1 inhibitor upregulated and downregulated foregrounds. Each random foreground contained the same number of phospho-sites as its associated biological foreground. For each random foreground, the motif search procedure was repeated. For each true foreground an empirical statistical significance cut-off was determined as the most significant p-value that could be found in more than 5% of random backgrounds of the same size. These values were $7.24 \times 10^{-7}$ for the PLK1 inhibitor upregulated sites and $3.16 \times 10^{-6}$ for the PLK1 inhibitor downregulated sites (data not shown). Only motifs with statistical significances of enrichment more significant than these values are accepted as significant and reported here.

**Supplementary references:**


