Supplemental Data

Supplemental Information

Numerical analysis of the phosphorylation sites

From 226 LC-MS/MS runs for proteome and phosphoproteome quantification, we have identified 3395 unique proteins at a false discovery rate (FDR) of 2%. About 60% of these (1996 proteins) were phosphorylated and more than 90% (3108 proteins) had quantification profile (could be quantified with SILAC). The raw phosphopeptide output of the MaxQuant software assigned 9544 phosphopeptides at a FDR of 2.2% (Table S1A). This included, however, phosphopeptides with low identification Mascot scores and multiple entries in the cases of different phosphopeptide-isoforms, for which the exact site of phosphorylation within the peptide sequences could not be determined. After applying a Mascot score cut-off \( \geq 12 \) (Mascot score from the best identification scans, see below) to all identified phosphopeptides with localization probability \( \geq 0.75 \) (class I site) (1), 4420 unique phosphorylation sites with high confidence were retained at a FDR of 0.89% from 4 different experiments (3526 from two inhibitor experiments and 3212 from two shRNA experiments) (Figure 1H and Table S1B, C). In MaxQuant, the best identification scan of a peptide is the MS/MS scan which results in the highest identification confidence of the phosphopeptide sequence. MaxQuant assigns this Mascot score from the best identification scan also to each identified phosphopeptide-isoform. To control for fluctuations in the levels of individual proteins between the different experimental conditions, all measured phosphopeptide ratios were normalized to protein levels. Collectively, these analyses led to a final number of 3894 unique phosphorylation sites at a FDR of 0.38% with SILAC and corresponding protein ratios, including 3092
and 2808 phosphorylation sites from experiments performed with small molecule inhibitors and the shRNA cell lines, respectively (Figure 1H). In total, more than 85% of the high confidence phosphorylation sites could be quantified (had SILAC pairs and could be normalized against protein ratios). However, in a small number of cases, only the protein levels and/or phosphopeptides from one cell population were identified impeding quantitation. This is likely due to significant delocalization of a protein from Plk1-inhibited spindles or downregulation of a phosphorylation site to levels below the detection limit.

Distributions of phosphoserines (79.8%), phosphothreonines (19.6%) and phosphotyrosines (0.6%) sites (localization probability > 0.75 (1)), resembled the ones reported in an independent quantitative analysis of the human spindle phosphoproteome at distinct mitotic stages (2).
Supplemental Experimental Procedures

Materials (Mass Spectrometry): Glycine, trifluoroacetic acid (25%), acetic acid, formic acid (98-100%), acetonitrile, methanol and ammonia solution (25%) were obtained from Merck. 2-Propanol was from Carl Roth. Lactic acid (~90%) was ordered from Fluka. NuPAGE Bis-Tris gels (1.0mm thick, 12 lanes per gel) and NuPAGE lithium dodecyl sulfate (LDS, 4 ×) sample buffer were from Invitrogen. DL-Dithiothreitol (DTT, >99%) and iodoacetamide were obtained from Sigma. Proteomics grade trypsin was purchased from Roche (Mannheim, Germany). Titansphere (10 µm) was from GL Sciences Inc. (Japan) and GELoader tips were purchased from Eppendorf. The 3M Empore C8 and C18 disks were obtained from 3M Bioanalytical Technologies (St. Paul, MN).

Label-free quantification: Label-free quantitation was employed for a control experiment to confirm that downregulated phosphorylation sites found upon Plk1 inhibition were not affected by MA treatment (see Results and Discussion). The twelve LC-MS runs acquired (four for each treatment, i.e. MA, TAL and MA+TAL were quantified using the feature extraction and alignment software tool Progenesis LC-MS (v2.5, Non-linear dynamics). After assigning the database search results to the feature table, the extracted ion intensities of the individual runs were normalized (by mean), the median intensities were determined for each of the three perturbations and the ratios (in log10) to the control (MA treatment) were calculated. Additionally, the significances of the abundance changes were determined (p-value, ANOVA-test) for each identified phosphopeptide based on the normalized peak intensities of the individual LC-MS runs. Only phosphopeptides with a p-value of 0.05 or lower were considered significantly regulated.
Overall, 4430 phosphopeptide ions could be identified and quantified by this approach. Of these 128 (192) were significantly down and 313 (372) were significantly up regulated after TAL (MAL+TAL) treatment.

*Generation of stable, inducible shRNA cell lines:* Short hairpin RNAs (shRNA) was designed to target the mRNA of Plk1 and Eg5. The shRNA sequences were synthesized as two complementary DNA oligonucleotides:

**Eg5** 5′-GATCCCCCTAGATGGCTTTCTCAGTATTCAAGAGATACTGAGAAAGCCATCTA GTTTTTGGAAA- 3′ and 5′-GCTTTTCCAAAAACTAGATGGCTTTTCTCAGTATCTCTTTGA ATACTGAGAAAGCCATCTAGGG- 3′ and **Plk1** 5′-GATCCCCGAGCTGCTTAATGACGAGTTCAAGAGACTCGTCATTAAGCAGCTCCTTTGGAAA- 3′ and 5′-AGCTTTTCCAAAAACGAGCTGCTTAATGACGAGTCTCTTGAACTCGTCATTAAGCAGCTCCTGGG - 3′

The oligonucleotides were mixed in equimolar amounts, heated for 5 min at 95°C, and then gradually cooled to room temperature in annealing buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM MgCl₂). The resultant duplex was ethanol-precipitated and ligated into a modified pTER+ vector (containing a TetO element under the control of an H1 promoter, puromycin resistant) by BglII-HindIII. To generate Tet inducible cell lines for the expression of shEg5 and shPlk1, the corresponding plasmids were transfected in HeLa S3 cells expressing a TetR upon Tet induction (Tet-ON system).

*Immunofluorescence (IF) microscopy:* Cells were grown on coverslips, washed in PBS and fixed and permeabilized with PTEMF solution (4% formaldehyde; 0.2% Triton X-100; 10 mM EGTA; and 1 mM MgCl₂ in 20 mM PIPES pH 6.8) for 15 min at room temperature. For centrosomal staining, cells were fixed in MeOH at -20°C for 5 min.
Cells were blocked for 30 min with 1% BSA in PBS and the primary and secondary antibodies were prepared in the same solution. Immunofluorescence microscopy was performed using a Zeiss Axioplan II microscope (Carl Zeiss) equipped with an Apochromat 63x oil immersion objective, and images were acquired using a Micromax charge coupled device (CCD) camera and Metamorph software (Visitron Systems). Alternatively, a Deltavision microscope on a Nikon Eclipse TE200 base (Applied Precision) equipped with an Apo 60x/1.4 oil immersion objective and a CoolSnap HQ camera (Photometrics) was used for collecting 0.15-μm distanced optical sections in the z-axis. Images at single focal planes were processed with a deconvolution algorithm dependent on the objective used. Settings were conservative, with noise filtering set to low and 2 deconvolution cycles. The number of z-stacks collected was variable (between 5 and 20), depending on the thickness of the individual cell. Images were projected into one picture using the Softworx software (Applied Precision). Exposure times and settings for image processing (deconvolution) were constant for all samples to be compared within any given experiment. Images were processed with Adobe Photoshop CS (AdobeSystems).

Cell extracts, immunoprecipitation, Western blotting analysis: Preparation of lysates and Western blotting analysis were performed as described previously (3). For Plk1 and Aurora A immunoprecipitations, 1 μg of antibody was couple to 1 μl Sepharose-G beads (20 μl beads in total) (Pierce). After protein capture, beads were washed 4x with HEPES buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM DTT, 30 μg/ml RNase A, 30 μg/ml DNase, protease inhibitors and phosphatase inhibitors) and 1x with the
corresponding kinase buffer before kinase assays were performed. Part of the samples
was resuspended in sample buffer and proteins were analyzed by SDS-PAGE and
Western blotting.

**Antibodies:** Anti-pS1324 CENP-F polyclonal antibody was generated by immunizing
rabbits with KLH-conjugated phosphopeptide (H-CVTELNDpSRSE-OH) and the serum
was used for immunofluorescence experiments (1:1000 dilution). Other primary
antibodies used in this study are monoclonal anti-Plk1 (4), rabbit anti-Eg5 (5), mouse
anti-α-tubulin (clone DM1A) (Sigma), mouse anti-Aurora-A (Signal Transduction),
rabbit anti-pT288 Aurora A (Cell Signaling), rabbit anti-Pericentrin (Abcam), rabbit anti-
Cep192 (6), rabbit anti-Cep215 (7), rabbit anti-Cep135 (8), mouse anti-CENP-F
(Transduction Laboratories), mouse anti-BubR1 (9), mouse anti-cyclin B1 (Upstate),
CREST human autoimmune serum was purchased from Immunovision. Primary
antibodies used for immunofluorescence were detected with cy2/cy3/cy5-conjugated
donkey antibodies (Dianova). DNA was stained with DAPI (2 mg/ml). For all Western
blotting, signals were detected using HRP-conjugated anti-mouse or anti-rabbit
antibodies (Pierce).

**In vitro kinase assays:** Plk1 and Aurora A were immunoprecipitated from cells and their
corresponding activity was assayed in kinase buffer (50mM Tris-HCl pH7.5, 10 mM
MgCl2, 1 mM DTT, 100 uM NaF, 10uM Sodium Vanadate) and (20 mM Hepes pH 7.4,
150 mM KCl, 5 mM MnCl2, 5 mM NaF and 1mM DTT), respectively. Casein and MBP
were used as substrates, respectively. Reactions were mixed, incubated for 30 min at 30
°C and stopped by the addition of sample buffer. Proteins were resolved by SDS-PAGE and gels were dried before being visualized by autoradiography.

**Statistical analysis:** We compared the significance of the presence of D or E (classical motif), N, Q, S or L at -2 position of the *in vitro* validated peptides. The resulted *p-values* after applying a Fisher's test were 1.19 e-6, 1.06 e-5, 0.000727, 0.098034 and 0.103435, respectively, indicating that only the presence of a D or E, an N, or a Q was statistically significant, considering a p-value cut-off of 0.05.
Supplemental Figures

Figure S1. Control experiments. (A) Technical replicates: Reproducibility of phosphorylation sites identification in SILAC samples. Enriched spindle proteins from 20 gel slices of one shRNA experiment (shPlk1 light-labeled/shEg5 heavy-labeled) were analyzed separately twice in LC-MS/MS, datasets named shRNA-2b1 and shRNA-2b2. For phosphorylation sites identified in both datasets, the log transformed abundance ratios (H/L) from shRNA-2b1 were plotted against that from shRNAi-2b2 (1236 unique phosphorylation sites). (B) Phosphopeptide abundance distributions. Log2-transformed heavy/light (TAL:TAL) ratios for all quantified phosphopeptides. Discontinuous vertical lines show selected cutoffs corresponding to signal changes of more than ±50%. (C) Correlation of phosphopeptide abundance changes between TAL and MA+TAL treated cells. Scatterplot of phosphopeptide abundance ratios (in log10) calculated for TAL and MA+TAL-treated cells, respectively, using cells treated with MA alone as base. Phosphopeptides that show a significant change in abundance in TAL-treated cells (p-value <0.05, ANOVA-test, N=441) were considered. The respective squared Pearson correlation R² is indicated.

Figure S2. Comparative assessment of phosphoproteomics data. (A) Venn diagrams illustrating overlap of the phosphorylation sites identified in this study. Overlap between the two different strategies (upper diagram) and the two biological replicates from shRNA or inhibitor experiments (middle and lower diagram, respectively) are shown. (B) Overlap of downregulated phosphorylation sites identified in this study between the
different Plk1 inactivation strategies. 93 sites were found downregulated with both strategies.

**Figure S3. SILAC KiF4A phosphopeptides.** Four independent experiments were carried out with different SILAC conditions. Due to the SILAC strategy for metabolic labeling, arginine- and/or lysine-containing peptides coeluted in LC-MS as doublets with a defined mass difference. The ion signals allowed accurate measurement of relative peptide abundance. (A) Representative MS spectra of a KIF4A phosphopeptides following the classical Plk1 consensus motif quantified on spindles isolated from MA/TAL-treated cells and (B) shEg5/shPlk1-induced cells. (C-E) Representative MS spectra of a KIF4A phosphopeptide containing a N at -2 position, quantified on spindles isolated from MA/TAL-treated cells (C and D) or shEg5/shPlk1-induced cells (E). Reciprocal SILAC labeling in experiments shown in C and D resulted in inversed peptide ratios in the MS spectra (crossover experiment). MaxQuant assigned the same phosphorylation site within the peptide with high confidence (localization probability score 1). Peptide, protein and corrected (peptide/protein) ratios are shown.

**Figure S4. Comparative analysis with other studies.** (A) Percentage of proteins in IPI human (version 3.59) containing at least one potential PBD conforming to the motif S-S/T-P, were plotted relative to the protein length. The results followed the exponential trend-line shown. (B) Spindle proteins previously identified as PBD-Plk1 interaction partners by (Lowery et al., 2007), containing downregulated or unchanged phosphorylation sites upon Plk1 depletion/inhibition, or that were not found in our study.
**Figure S5. Plk1 interaction networks.** Networks were generated using the STRING database (Jensen et al., 2009). A medium confidence setting (0.400) was used and only experimental information (pink) or information from databases (light blue) was used. (A) Complete interaction network of all proteins containing downregulated phosphorylation sites (see Table S2A-B). (B) Sub-network of centrosomal proteins that are linked to Plk4 (left panel) or Plk1 (right panel), respectively. (C) Sub-network of NUPs (Nup98, Nup188, Nup93, Nup107, Nup153 and TPR) clearly linked to kinetochore proteins (via CENP-F).

**Figure S6. In vitro Plk1 kinase assays on peptide arrays.** (A) Example of membranes illustrating the *in vitro* peptide kinase assays. Membranes incubated in the presence of recombinant Plk1 are shown on the left and membranes incubated with ATP in the absence of Plk1 are shown on the right, as control. Red squares indicate spots shown in Figure 4C. Blue square shows the spot of the peptide used in Figure 4G. (B) Membrane control corresponding to Figure 4G incubated with ATP in the absence of Plk1.

**Figure S7.** Permutational analysis for Plk1 phosphorylation on 6 different peptides carrying a N at the -2 position. Phosphoacceptor residues are marked in red, -2 positions in green. Red arrows indicate tolerance for E/D or Q respectively.

**Figure S8. Characterization of the pS1324-CenpF antibody.** (A) HeLa S3 cells were fixed and stained with CENP-F pS1324 (red), CENP-F (green) and CREST serum (far
red, depicted in blue). DNA was visualized using DAPI (blue). (B) Hela S3 cells treated with GL2 (control) or CENP-F siRNA for 48 h were fixed and stained with CENP-F pS1324 (red), CENP-F (green) and CREST serum (far red, depicted in blue). DNA was visualized using DAPI (blue). (C) HeLa S3 cells treated with MA were fixed and stained with pS1324 (red) and CREST serum (green). Antibody dilutions were preincubated on ice for 30 min with 1mg/ml untreated (competition) or alkaline phosphatase-treated (CIP, control) pS1324 peptide to block specific antibody reactivity. DNA was visualized using DAPI (blue). Scale bars, 10 μm.

Figure S9. Plk1 inactivation abolishes Pericentrin, Cep192 and Cep215 recruitment to mitotic centrosomes. HeLa S3 cells were treated with DMSO MA, TAL or MA+TAL, fixed and immunostained for pericentrin (A), Cep192 (B), Cep215 (C) and Cep135 (D) (red) and α-Tubulin (green). DNA was visualized using DAPI (blue). Scale bars, 10 μm.
Supplemental Tables

Table S1. **(A) MaxQuant phosphopeptide output** (FDR 2.2%, without any additional qualitative control cut-offs, comprehensive information on all identified phosphopeptides is presented) **(B,C) Lists of phosphorylation sites identified in Inhibitor (B) and shRNA (C) experiments, respectively, with Mascot score ≥12.** Class I sites are indicated in column F. Only class I sites from these lists were counted and shown in Figures 1H and S3A). The SILAC H/L ratios correspond to TAL/MA and shPlk1/shEg5. Mascot scores for the best identification scans and SILAC H/L ratios corresponding to each experiment are shown in yellow and green, respectively. (The best localization scan is defined as the MS/MS scan which results in the highest confidence of the phosphorylation site assignment within the identified peptide sequence).

Table S2. **Downregulated phosphorylation sites identified in Inhibitor (A) and shRNA experiments (B) with Mascot score ≥12.** Phosphopeptide SILAC ratios are shown for each independent experiment. H/L ratios correspond to TAL/MA (A) or shPlk1/shEg5 (B). Phosphorylation sites with ratios < 2/3 are considered downregulated (see Experimental Procedures for detailed description of cut-off values). Contradictory phosphorylation sites between biological replicates (upregulated in one replicate but downregulated in the other) have been excluded. Class I sites are indicated in column F and only class I sites from this list were counted and shown in Figures 1H and S2B). Mascot scores for the best identification scans and SILAC H/L ratios are shown in yellow and green, respectively.
Table S3. (A) Downregulated phosphorylation sites identified on spindle proteins upon Plk1 inactivation. SILAC ratios corresponding from each of the four independent experiments are shown. H/L ratios correspond to TAL/MA or shPlk1/shEg5. Sites with ratios < 2/3 are considered downregulated. Phosphorylation sites following a different trend between the different Plk1 inactivation strategies (downregulated upon TAL treatment but upregulated upon shPlk1 depletion and vice-versa) were kept and included in the subsequent verification step. Note that SILAC ratios of phosphorylation sites that were contradictory between the two biological replicates of one strategy (and therefore excluded from Table S2A or B) will appear in this list if they were downregulated in the other strategy (depicted in blue). Results from in vitro Plk1 kinase assay on peptides are shown in column J. NC signifies non-conclusive results. Information about conservation in different species (column P) and presence of potential PBD recognition motif (S-S/T-P) (column O) are included in the list. Columns showing the Mascot scores for the best identification scans are shown in yellow, SILAC H/L ratios in green. Class I sites are indicated in column H and only class I sites from this list were counted and shown in Figure 3B. (B) Summary of phosphorylation sites identified on spindle proteins. Asterisk indicates sites with localization probability <0.75. (C) List of phosphorylation site candidates for Aurora A. Phosphorylation sites downregulated upon Plk1 inactivation that were synthesized and assayed for Aurora A phosphorylation in vitro. Results of the in vitro Aurora A kinase assay are shown (column J). NC signifies non-conclusive results. Mascot scores for the best identification scans are shown in yellow and the SILAC H/L ratios are shown in green.
Table S4. Spindle protein levels upon Plk1 inhibition. (A) The protein ratios from the two experiments performed with isolated spindles from MA/TAL-treated cells were normalized against internal α/β-Tubulin levels and averaged. Proteins with ratios above and below 1±0.2 between biological replicates were not considered. Only proteins with at least three quantified non-modified peptides were considered. H/L spindle ratios were then normalized against H/L ratios from total cell lysates of inhibitor-treated cells. Lysate levels were also corrected against α/β-Tubulin amounts. Spindle to total lysate ratios are shown. (B, C) MaxQuant protein identification output from spindle (B) or lysate (C) experiments, respectively. In Tables S4B and C comprehensive information on identified protein groups without additional qualitative control cut-offs is presented.

Table S5. (A, B) Downregulated phosphorylation sites identified from Inhibitor (A) and shRNA experiments (B) with Mascot score 7 ≤ X < 12. H/L ratios correspond to TAL/MA or shPlk1/shEg5. Sites with ratios < 2/3 are considered downregulated. Mascot scores for the best identification scans are shown in yellow, and the SILAC H/L ratios are shown in green. Class I sites are indicated in column F.
Supplemental References


Santamaria et al., Figure S1

**A**

\[
y = 0.9551x - 0.0299 \\
R^2 = 0.843 \\
N = 1236
\]

**B**

CTL Experiment: TAL:TAL (1:1)

No. of phosphopeptides

**C**

\[
y = 0.9166x + 0.0433 \\
R^2 = 0.83727 \\
N = 441
\]
Santamaria et al., Figure S2

A

Quantified phosphorylation sites

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shRNA - 1

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Heavy TAL/Light MA

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Heavy MA/Light TAL

B

Downregulated phosphorylation sites

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<td>432</td>
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Santamaria et al., Figure S2
A. R. GQVSESED(ph)ITK
Chromosome-associated kinesin KIF4A

Peptide ratio (H/L): 0.279
Protein ratio (H/L): 0.663
Corrected ratio (peptide/protein): 0.42
Downregulation: 58%

B. Peptide ratio (H/L): 1.781
Protein ratio (H/L): 0.879
Corrected ratio (peptide/protein): 2.03
Downregulation: 51%

C. K. NQS(ph)LVEENEK
Chromosome-associated kinesin KIF4A

Peptide ratio (H/L): 0.039
Protein ratio (H/L): 0.663
Corrected ratio (peptide/protein): 0.06
Downregulation: 94%

D. Peptide ratio (H/L): 6.654
Protein ratio (H/L): 0.510
Corrected ratio (peptide/protein): 13.05
Downregulation: 92%

E. Peptide ratio (H/L): 2.112
Protein ratio (H/L): 0.879
Corrected ratio (peptide/protein): 2.40
Downregulation: 58%

Santamaria et al., Figure S3
A

Spindle proteins with downregulated sites upon Plk1 depletion/inhibition

- NUMA
- TPX2
- RACGAP1
- DLG7 (HURP)
- VIMENTIN
- CIT
- EML3

Spindle proteins with unchanged sites upon Plk1 depletion/inactivation

- ANLN
- Septin-9
- CENPF
- TPR

B

Known PBD-Plk1 interacting proteins containing putative Plk1 phosphorylation sites (Lowery et al., 2007)

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Spindle proteins not found in our study

- Tao1
- Cdc2
- Kif2
- PKB
Santamaria et al., Figure S6

A

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CENP-F S1324 VTELNDSRSECI

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Santamaria et al., Figure S8

A

Prophase Prometaphase Metaphase Anaphase Telophase

pS1324

CENP-F

pS1324 CENP-F

CREST

pS1324 CENP-F

DAPI

Merge+CREST

Merge+DAPI

Merge+CREST

B

GL2 siRNA Cenp-F siRNA

pS324

CENP-F

pS1324 CENP-F

Merge+CREST

Merge+DAPI

C

- +p-peptide p-peptide

pS1324

CREST

pS1324 CREST

Merge+DAPI
Santamaria et al., Figure S9

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