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Quantitative phosphoproteomics reveals new roles for the protein phosphatase PP6 in mitotic cells

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Abstract

Protein phosphorylation is an important regulatory mechanism controlling mitotic progression. Protein phosphatase 6 (PP6) is an essential enzyme with conserved roles in chromosome segregation and spindle assembly from yeast to humans. We applied a baculovirus-mediated gene silencing approach to deplete HeLa cells of the catalytic subunit of PP6 (PP6c) and analyzed changes in the phosphoproteome and proteome in mitotic cells by quantitative mass spectrometry-based proteomics. We identified 408 phosphopeptides on 272 proteins that increased and 298 phosphopeptides on 220 proteins that decreased in phosphorylation upon PP6c depletion in mitotic cells. Motif analysis of the phosphorylated sites combined with bioinformatics pathway analysis revealed previously unknown PP6c-dependent regulatory pathways. Biochemical assays

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SUPPLEMENTARY MATERIALS

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Fig. S1. Characterization of PP6c shRNAs.

Fig. S2. Quantitative comparison of protein abundance changes upon PP6c depletion with individual PP6c shRNAs.

Fig. S3. Scheme of cell cycle synchronization.

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Fig. S8. Characterization of PP6c and NCAP-G purifications. Fig. S9. NCAP-G analysis.

Table S1. Table containing TMT protein quantification results.

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Table S3. Table containing quantification results of AURKA substrates.

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Table S6. Table containing quantification results of the PP6c in vitro phosphatase assays of NCAP-G.

Table S7. Table containing quantification results of the CK2 in vitro kinase assay of NCAP-G.

Table S8. Table containing quantification results of proteinase K digest of NCAP-G.

Author contributions: S.F.R. performed the PP6c shRNA phosphoproteomics and proteomics experiment and in vitro phosphatase assay and characterized the effects of PP6c and NCAP-G depletion on chromosome segregation and condensation. K.A.S. generated and characterized the PP6c shRNA constructs. M.E.A. managed data files and the data pipeline for all proteomics experiments and generated software for iBAQ quantification and motif analysis. A.N.K. designed the study and performed the TMT protein quantification experiment. S.F.R. and A.N.K. analyzed the data and generated figures. A.N.K. wrote the manuscript.

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demonstrated that PP6c opposed casein kinase 2–dependent phosphorylation of the condensin I subunit NCAP-G, and cellular analysis showed that depletion of PP6c resulted in defects in chromosome condensation and segregation in anaphase, consistent with dysregulation of condensin I function in the absence of PP6 activity.

INTRODUCTION

During mitosis, a cell undergoes dramatic changes in cellular structure and organization to divide its cytoplasm and organelles and equally segregate its genome to generate two viable daughter cells. Dynamic and reversible protein phosphorylation mediated by protein kinases and phosphatases is an important regulatory mechanism in this process (1, 2). Errors in cell division can lead to cells with aberrant chromosome number, a state known as aneuploidy, which is a hallmark of human cancer and the origin of many birth defects. The family of phosphoprotein phosphatases (PPPs), which includes PP1, PP2A, PP4, PP5, PP6, and PP7, is responsible for the turnover of most serine and threonine phosphorylation events in mitosis (1–3). Marine toxins, such as okadaic acid, calyculin A, and microcystin-LR, inhibit multiple members of the PPP family. Indeed, exposure of interphase cells to these compounds induces a pseudomitotic state characterized by chromosome condensation and Golgi fragmentation, and their application to mitotic cells inhibits exit from mitosis (4, 5). Thus, tight regulation of PPP activities is essential for accurate mitotic progression.

Protein phosphatase 6 (PP6) is an essential trimeric holoenzyme that consists of one catalytic (PP6c), one of three regulatory (PPP6R1, PPP6R2, and PPP6R3), and one of three scaffolding [ankyrin repeat domain-containing protein 28 (ANR28), ANR44, and ANR52, respectively] subunits (6–8). This combinatorial modularity results in nine distinct PP6 holoenzymes and is likely responsible for the diverse regulatory functions of PP6 by influencing localization, substrate targeting, and catalytic activity (9–20). Furthermore, PP6 regulatory subunits mediate interactions between the holoenzyme and protein kinases, such as DNA-dependent protein kinase (DNA-PK) (14), Aurora kinase A (AURKA) (21), transforming growth factor β -activated kinase (TAK1) (9), and kinases in the Hippo pathway (16). PP6 is highly conserved in eukaryotes from yeast to humans and has a conserved role in chromosome segregation and spindle assembly in mitosis. In fission yeast, the PP6c homolog Ppe1 promotes chromosome segregation (22), whereas in budding yeast, the PP6c homolog Sit4 is required for G₁ to S progression (23, 24). In *Caenorhabditis elegans*, PP6 regulates spindle positioning (25). In mitotic human cancer cells, PP6 inhibits the activity of the mitotic kinase AURKA by dephosphorylating Thr²⁸⁸ in the activation T-loop (21). Furthermore, PP6 opposes the mitotic kinase Polo-like kinase 1 (PLK1) by dephosphorylating a PLK1-dependent phosphorylation site on DNA-PK (20).

Genome-wide exome sequencing of malignant melanomas has identified somatic mutations in the gene encoding PP6c (26, 27). Biochemical characterization found that these mutations are heterogeneous with regards to their effect on PP6c activity, stability, and subunit binding. Consequently, the mutant enzymes can function as loss-of-function tumor suppressors or gain-of-function oncoproteins (17, 18, 28). In glioblastoma, the abundance of PP6c is increased and negatively correlated with patient survival (29). Although reduced PP6 activity

might contribute to cancer initiation or progression through activation of AURKA and promotion of the cell cycle, increased PP6 activity can promote DNA damage repair through activation of DNA-PK (12, 14, 30). Additional evidence for the importance of PP6 in cancer comes from a mouse model of skin carcinogenesis in which deletion of PP6c promotes tumor formation upon exposure of the skin to a tumor initiator (31).

The role of PP6 is complex, and PP6 integrates multiple pathways essential for cell cycle progression. To determine PP6c-dependent biological processes and pathways in mitotic cells, we analyzed phosphorylation and protein abundance changes upon PP6c depletion in Taxol-arrested HeLa cells. Because of the lack of specific PP6 inhibitors and biochemical strategies, such as substrate-trapping mutants that can be used for tyrosine-directed protein phosphatases (32, 33), we combined baculovirus-mediated RNA interference and mass spectrometry-based quantitative proteomics to identify PP6-regulated phosphorylation events. Furthermore, we characterized a role for PP6 in chromosome condensation by opposing casein kinase 2 (CK2)-mediated phosphorylation of the condensin I subunit G (NCAP-G).

RESULTS

Generation of a BacMam short hairpin RNA strategy for PP6c depletion

To determine changes in protein phosphorylation upon PP6c depletion in mitotic cells, we used a baculovirus-based short hairpin RNA (shRNA) strategy. PP6c is encoded by an essential gene, and long-term reduction of PP6c abundance results in mitotic defects (21, 31). Thus, constitutive depletion or deletion of the gene encoding PP6c in human cells is not feasible. To overcome the limitations of transient small interfering RNA (siRNA) transfection in large-scale phosphoproteomic experiments, which require $\sim 1 \times 10^7$ HeLa cells per condition and per replicate, we used an shRNA-encoding virus. We generated a recombinant baculovirus-mediated shRNA expression system because recombinant baculoviruses are safe, stable for long-term storage, amenable to amplification, and can infect a wide range of mammalian cells at high efficiency (34, 35). Recombinant baculoviruses cannot replicate in organisms other than their insect cell host. To generate a recombinant baculovirus expressing shRNAs, we combined features of baculoviral and lentiviral expression vectors (see Materials and Methods for details) into a pFastBacMam-shRNA-GFP vector (Fig. 1A), which enabled virus generation in *Spodoptera frugiperda* (Sf9) insect cells and shRNA and green fluorescent protein (GFP) expression in mammalian cells.

To identify sequences for efficient PP6c depletion, we tested five shRNA sequences targeting the PP6c coding sequence (see Materials and Methods for shRNA sequences). To determine the efficiency of infection, we infected HeLa cells with control shRNA-encoding virus or virus encoding one of the five PP6c shRNAs and monitored GFP production after 48 hours. We observed greater than 90% infection efficiency as determined by fluorescence microscopy of GFP-positive cells (Fig. 1B and fig S1A). Two baculoviruses (PP6c-sh1 and PP6c-sh4) partially reduced PP6c abundance when HeLa cells were infected with them individually (fig. S1, B and C). When used in combination, viral infection with the same titer of virus but containing 50% encoding PP6c-sh1 and 50% encoding PP6c-sh4 produced

a similar amount of GFP as infection with a 100% viral titer of either of the shRNA-encoding viruses (fig. S1, D and E). More importantly, PP6c-sh1 combined with PP6c-sh4 reduced PP6c abundance to less than 8% of that in cells infected with virus encoding a control shRNA (fig. S1, D and E).

To determine whether the partial (~25%) reduction of PP6c abundance produced by these individual shRNAs was sufficient to modulate PP6c substrate phosphorylation in mitosis, we assessed the phosphorylation status of the PP6c-regulated Thr²⁸⁸ of the AURK A T-loop (21) by Western blot analysis of infected HeLa cells that had been arrested in mitosis (fig. S1, D and E). These data indicated that the reduction in PP6 activity achieved with the individual shRNAs was insufficient to increase phosphorylation at this PP6-regulated site but showed that co-infection with both shRNA-encoding viruses produced a significant increase in phosphorylation of Thr²⁸⁸ of AURKA in mitotic cells.

Off-target effects are a potential concern when using shRNA (36, 37). We reasoned that potential off-target effects are likely unique to individual shRNAs and that the two shRNAs we used were unlikely to affect the same off-target protein. Therefore, we compared protein abundance in mitotically arrested HeLa cells that had been infected with either control shRNA-, PP6c-sh1-, or PP6c-sh4-encoding virus using multiplex quantitative proteomics in biological triplicate to validate the on-target effects of the shRNAs for PP6c (fig. S2A). We collected and digested total cellular protein, labeled the peptides using tandem mass tag (TMT) reagents, and analyzed the peptides by MS³-based quantitative liquid chromatography–tandem mass spectrometry (LC-MS/MS) (38). We identified and quantified 6700 proteins with at least two unique peptides at a 1.2% protein-level false discovery rate (FDR) in this analysis (fig. S2B and table S1).

Correlation analysis of the fold changes in protein abundance in cells infected with either of the two hairpins produced a correlation coefficient of $R^2 = 0.83$ for proteins increased or decreased by 1.4-fold ($\log_2$ ratio <0.5 or >0.5) (fig. S2B). Reassuringly, we observed the largest reduction in protein abundance in PP6c itself (fig S2B). Furthermore, we found that the abundance of the PP6c regulatory subunits PPP6R1, PPP6R2, and PPP6R3 were also reduced, which is consistent with previous reports showing that siRNA-mediated reduction in the PP2A catalytic subunit resulted in a corresponding reduction in abundance of regulatory subunits (39), indicating that PPP regulatory subunit stability depends on the ability to form holoenzyme complexes. We ascribe the differences in the amount of reduction of PPP6R1, PPP6R2, and PPP6R3 abundance to their different absolute protein abundance in HeLa cells (40), such that the relative higher amount of PPP6R3 resulted in greater destabilization upon depletion of the catalytic subunit of PP6 compared with that observed for the other PPP6R subunits. Of the 6700 quantified proteins, only 35 exhibited significantly ($P < 0.05$, two-tailed Student's t test) different amounts in cells infected with either of the two shRNA-encoding viruses (indicated by red dots in fig. S2B; table S1), with most of these differences occurring at modest fold changes [median fold change of the most reduced protein ratio between cells infected with each of the two hairpins: -0.08 ($\log_2$)], indicating that these are unlikely to be biologically important. On the basis of these analyses of the efficiency of PP6c depletion, the effect on AURKA Thr²⁸⁸ phosphorylation, and off-

target effects, we combined viruses encoding each of these two PP6c shRNAs for use in subsequent studies to ensure sufficient reduction in PP6c abundance (Fig. 1C).

Quantitative phosphoproteomics of PP6c-depleted, mitotically arrested HeLa cells

Although PP6c plays an essential role in chromosome segregation and spindle assembly from yeast to humans (21, 22, 25), only two substrates of PP6c, Thr²⁸⁸ of AURKA and Ser³²⁰⁵ of DNA-PK, have been identified in mitotic cells (20, 21, 28). To identify additional PP6c-dependent pathways important in mitosis, we infected HeLa cells with control shRNA-expressing virus or viruses expressing PP6c-sh1 and PP6c-sh4 and synchronized the cells in mitosis using a thymidine block followed by release into the microtubule-stabilizing drug Taxol (fig. S3). We collected the infected, mitotically arrested HeLa cells separately, lysed the cells, reduced and alkylated the proteins, and digested the proteins into peptides (Fig. 1D). At this point, we took aliquots of the digested peptides for quantification of changes in protein abundance between the samples (not shown in Fig. 1D). We subjected the remaining peptides to phosphopeptide enrichment, chemical labeling for quantitative comparison, and LC-MS/MS analysis. Both phosphopeptide and total peptide analyses were done in biological triplicate.

We performed Western blot analysis of PP6c abundance, as well as the phosphorylation status of the known PP6c substrate Thr²⁸⁸ on AURKA, to confirm efficient depletion of PP6c (Fig. 1E). Quantification of Western blot results normalized to the loading control lamin A/C showed that, upon infection with viruses encoding PP6c-sh1 and PP6c-sh4, but not virus encoding control shRNA, PP6c protein abundance was significantly reduced to barely detectable amounts, and that phosphorylation of Thr²⁸⁸ on AURKA was significantly increased (Fig. 1F).

Overall, we identified and quantified 29,933 phosphopeptides on 4415 proteins, of which 23,910 phosphopeptides were found in two of the three biological replicates and 16,442 were found in all three (Fig. 2A and table S2). Because we reduced PP6c abundance for a prolonged period, we corrected for changes in protein abundance as well. Using the aliquots taken after the peptide digestion, we quantified protein abundance from the three biological replicates and corrected the phosphopeptide ratios on a per-replicate basis, which reduced the total number of phosphopeptides quantified to 23,910 on 2931 proteins, of which 11,801 phosphopeptides on 2006 proteins were quantified in each of the three biological replicates (Fig. 2B).

We filtered the number of phosphopeptides and proteins to those that increased twofold or more in the PP6c-depleted cells with a *P* value of <0.1, which resulted in 658 phosphopeptides on 371 proteins (Fig. 2C). We chose this cutoff because phosphorylation site occupancy in mitotic cells is increased compared to that in interphase cells (41), and we reasoned that additional increases upon PP6c depletion might be small. Of these 658 phosphopeptides, 408 were increased because of changes in the phosphorylation abundance of the quantified phosphorylation site (fig S4A). These 408 phosphopeptides mapped to 272 proteins, of which most (203) contained only a single phosphopeptide with a significantly changed ratio (fig. S4B). Indeed, 71% of these proteins contained one or more additional phosphorylation sites that did not change in abundance. That many of the phosphorylated

sites that significantly changed were on proteins that had other sites that did not change provided further support that this subset of the data represents phosphorylation site-specific changes and not differential protein abundance. As a positive control, one of the 408 significantly increased phosphopeptides contained Ser³²⁰⁵ on DNA-PK (table S2 and fig. S5, A and B). The remaining 250 sites were due to protein abundance changes because the change in phosphopeptide abundance was less than the change in protein abundance (table S2 and fig S4A). Furthermore, we also identified 519 phosphopeptides on 319 proteins that were decreased by twofold or more with a *P* value of <0.1, of which 298 were due to changes in phosphopeptide abundance on 220 proteins and 221 in protein abundance (table S2 and fig S4A). In the subsequent analyses of PP6c-dependent signaling pathways, we concentrated on the 408 and 298 phosphopeptides that increased or decreased, respectively, because of phosphorylation changes upon PP6c depletion.

Chemical nature of PP6c-dependent phosphorylation sites

To predict which kinases PP6c might oppose, we performed motif analysis (42, 43) on the 408 or 298 phosphorylation sites that increased or decreased upon PP6c depletion, respectively. In the phosphorylation sites that increased upon depletion of PP6c, we found an enrichment of proline-directed (phospho-Ser/Thr-Pro), basophilic (Arg-X-phospho-Ser), and acidophilic (phospho-Ser-X-X-Glu) motifs (Fig. 3A), whereas the phosphorylation sites that decreased upon depletion of PP6c were enriched for proline-directed (phospho-Ser/Thr-Pro) and basophilic (Arg-X-X-phospho-Ser) but not acidophilic motifs (Fig. 3B).

Because of the known role of PP6 in reducing AURKA activity (21), a kinase with substrate preference for argininerich motifs (44), we expected the enrichment of a basophilic motif in phosphopeptides that exhibited an increase in PP6-depleted cells. Furthermore, we considered that we could assess the regulation of AURKA substrates by comparing this PP6-dependent data set with our previously published data set of AURKA substrates in Taxol-arrested HeLa cells that were identified using the AURKA-specific small molecule inhibitor MLN8054 (45) and quantitative phosphoproteomics (44). Of the 36 AURKA substrates present in the protein-corrected data set from the PP6c-depleted cells (table S3), only 12 were significantly increased in phosphorylation occupancy upon PP6c depletion (Fig. 3C). We also assessed what fraction of PP6c-dependent phosphorylation sites that contain the Arg-X-phospho-Ser motif were identified as AURKA substrates in the MLN8054 study (table S4). Of these 42 phosphopeptides with the basophilic motif that increased in PP6c-depleted cells, 8 were reported as sensitive to AURKA inhibition, 18 were not significantly affected by AURKA inhibition, and 15 were not identified or quantified in the MLN8054 study (Fig. 3D). Together, these results suggested that increased AURKA activity as indicated by increased phosphorylation of the AURKA activation T-loop upon PP6c depletion results in increased phosphorylation site occupancy of a subset of AURKA substrates.

In addition, the observed enrichment of proline-directed motifs was also not surprising because these are canonical kinase motifs for cyclin-dependent kinases (CDKs), including Cdk1, which functions in chromosome condensation, nuclear envelope breakdown, organelle fragmentation, spindle assembly, and chromosome segregation (46–49). Proline-directed

motifs are also recognized by mitogen-activated kinases (MAPKs) (50) and glycogen synthase kinase 3 (GSK-3) (50). The acidophilic motif could be ascribed to CK2, which requires an acidic residue at the third position downstream of the phosphorylatable amino acid (50–52).

Protein network analysis

To elucidate the biological processes regulated by PP6c, we performed network analysis on the 272 proteins that contain phosphorylation sites that increased upon PP6c depletion. We gathered protein-protein interaction data among the 272 proteins from the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (53) and analyzed them in Cytoscape (54, 55) (fig. S6A). We determined densely connected clusters of protein interactions within the network to identify nodes predicted as involved in specific regulatory processes, which identified four clusters with a *P* value of <0.01 (Fig. 4A). Gene ontology (GO) analysis of each of the four clusters indicated their enrichment in biological processes and cellular components (56). The cluster shown in black contained proteins implicated in chromosome segregation and condensation, with spindle and chromosomes as primary cellular components, which is consistent with the conserved role of PP6 in chromosome segregation and spindle formation through the direct regulation of spindle and kinetochore proteins, including AURKA (21, 22, 25). However, the identification of proteins involved in chromosome condensation, including NCAP-G, SMC4, TOP2A, and KIF4A, as potentially regulated by PP6c is new, representing a previously unknown function of PP6c. The cluster shown in blue contained proteins involved in RNA processing that belong to nucleolar and preribosomal complexes. During mitosis, the nucleolus is not present as a discrete structure, nucleolar proteins localize around chromosomes, and ribosomal DNA (rDNA) transcription is suppressed (57). Suppression of rDNA transcription and ribosomal RNA (rRNA) processing is mediated by protein phosphorylation in prophase by Cdk1 and, later in the cell cycle, is relieved by dephosphorylation (58). We also identified proteins involved in RNA splicing and processing that are components of the spliceosome (yellow cluster). PP6c coimmunoprecipitates with small nuclear ribonucleoproteins (snRNPs) that are part of the spliceosome (59) and associates with the spliceosome throughout the splicing reaction by binding to U1 snRNP (60). Protein phosphorylation is required for the formation of the spliceosome, and dephosphorylation is necessary for progression of the catalytic splicing reaction (61). Phosphatases might function in the exchange of snRNPs, regulate protein-protein interactions during splicing, or control splicing-independent functions of U1 snRNP (60). The fourth cluster of densely connected proteins contained proteins involved in protein translation on cytoplasmic ribosomes (magenta cluster). Like rDNA transcription and rRNA processing, most protein translation is suppressed in mitosis by protein phosphorylation (62). PP6c activity might be involved in the reactivation of these processes in telophase when net phosphorylation by mitotic kinases decreases, and phosphatase activity on these substrates can lead to a decrease in the phosphorylation of inhibitory sites in the protein translational machinery.

Performing the same type of analysis on proteins that contain phosphorylation sites that decreased upon PP6c depletion resulted in a network with an overall less well connected structure (compare the number of nodes that were not significantly densely clustered in fig.

S6, A and B). We identified three clusters of moderate connectivity (Fig. 4B), all of which had fewer nodes than the clusters found in the interaction network of proteins with increased phosphorylation. We identified four proteins involved in the regulation of Rab guanosine triphosphatase activity (yellow cluster), as well as a cluster (magenta) containing components of the nuclear envelope and nuclear core complex that regulate RNA transport. The largest cluster (shown in black) contains microtubule-associated proteins, such as the kinesins KIF4A, KIF20, and KIF18B, which are proteins that regulate microtubule-based processes at the spindle (63, 64).

Forty proteins identified in our analyses contained phosphorylation sites that increased upon PP6c depletion, whereas other sites decreased in abundance (table S2). Some of these differentially occupied sites are related. For instance, we observed an increase in phosphorylation of the doubly phosphorylated peptide Ser⁴³⁸⁴.Ser⁴³⁹⁶ on Plectin, whereas the single phosphorylated peptide Ser⁴³⁸⁴ decreased, which is suggestive of stepwise enzyme activity. However, most anticorrelated abundance changes of phosphorylation sites on one protein appeared unrelated, suggesting more complex regulatory mechanisms by PP6 holoenzymes and opposing kinases governing these proteins in mitotic cells.

Investigation of PP6c- and CK2-regulated phosphorylation site occupancy in NCAP-G by in vitro assays

By depleting PP6c for an extended period, we cannot distinguish the direct effect of the lack of PP6c phosphatase activity (a substrate of PP6) from an indirect effect of PP6c depletion and reduced activity. Therefore, we conducted in vitro phosphatase assays with purified PP6 holoenzyme complex to determine whether PP6 directly dephosphorylated select phosphorylation sites from our data set. Because of the unknown role for PP6 in chromosome condensation and the importance of proper chromosome condensation in chromosome stability, we focused on the phosphorylation site with increased abundance in PP6c-depleted mitotically arrested HeLa cells identified on NCAP-G, a member of the condensin I complex.

We found that the abundance of a singly phosphorylated peptide on NCAP-G (identified as either pSer⁹⁷³ or pSer⁹⁷⁵; hereafter referred to as pSer^{973/5}) was significantly increased upon PP6c depletion (table S2 and fig. S7, A and B). We could not establish the specific site of phosphorylation because the MS/MS spectra lacked the site-determining ions required to distinguish between them (fig. S7B). Thus, PP6c depletion may affect either site or both of them when singly phosphorylated. Additionally, we also observed the corresponding doubly phosphorylated (both pSer⁹⁷³ and pSer⁹⁷⁵; hereafter referred to as pSer^{973:975}) peptide, which did not significantly change upon PP6c depletion (table S2). To validate that PP6c can directly dephosphorylate NCAP-G, we purified the condensin I complex from 293T cells stably expressing 3×Flag-NCAP-H and purified the PP6 holoenzyme from 293T cells stably expressing 3×Flag-PP6c for an in vitro phosphatase assay (fig. S8A). We confirmed the composition of the purified condensin I complex and PP6 holoenzymes by LC-MS/MS (table S5). We detected the endogenous inhibitors a4 and TIPRL (5, 65, 66) and the PPP6R and ANR subunits with PP6c (fig. S8B). This is consistent with the observation that free

catalytic PPP subunits are unstable and rapidly degraded in cells and require stabilization through binding to an endogenous inhibitor or regulatory and scaffolding subunits (67).

In HeLa cells, condensin I subunit and PP6c protein abundance are comparable (40). We incubated the purified condensin I complex with or without the purified PP6 holoenzyme at a 1:20 enzyme/substrate ratio (Fig. 5A). We used this high enzyme/substrate ratio because we expected that much of the purified PP6c was inactive due to binding to endogenous inhibitors (fig. S8B). After quenching the *in vitro* reactions, we resolved the proteins by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), excised the bands corresponding to NCAP-G from the gel (Fig. 5B), and digested the protein into peptides. After labeling the peptides using reductive dimethyl labeling chemistry, we mixed the labeled peptides and performed LC-MS/MS (Fig. 5A). We observed a strong decrease in NCAP-G phosphorylation on Ser^{973/5} upon PP6c addition and an increase in the corresponding unphosphorylated peptide (Fig. 5, C and D, and table S6), whereas the doubly phosphorylated peptide was unchanged (table S6). Furthermore, another phosphorylation site on NCAP-G, Ser⁶⁷⁴, which also contains an acidophilic motif but was unchanged in PP6c–depleted cells (table S2), was also not dephosphorylated *in vitro* with purified PP6 (table S6). Thus, we concluded that PP6 holoenzymes specifically dephosphorylate pSer⁹⁷³ or pSer⁹⁷⁵ on NCAP-G but only when singly phosphorylated in mitotic HeLa cells and *in vitro*.

The two candidate PP6 substrate sites on NCAP-G, Ser⁹⁷³ and Ser⁹⁷⁵, are surrounded by acidic amino acids (Fig. 5C), which is similar to the substrate motif preference of CK2 (51). To establish that Ser^{973/5} is a CK2 phosphorylation site, we performed an *in vitro* kinase assay with CK2 and purified condensin I subunits previously dephosphorylated with nonspecific λ -phosphatases, and identified phosphorylated peptides by LC-MS/MS (Fig. 5E and table S7). We observed an increase in Ser^{973/5} phosphorylation and a decrease in the corresponding unphosphorylated peptide in the samples exposed to CK2 (Fig. 5, F and G). We did not observe the generation of pSer^{973:975} in the CK2 reaction. Thus, these results indicated that CK2 is likely responsible for the single phosphorylation of either Ser⁹⁷³ or Ser⁹⁷⁵ on NCAP-G and that PP6c dephosphorylates this site in mitotic cells.

Defects in chromosome condensation and segregation upon PP6c depletion

Cells begin to condense their chromosomes when they enter mitosis; this process continues throughout prometaphase. If mitotic progression is halted and cells are arrested in prometaphase by spindle poisons, condensin complex activity continues in the arrested cells, which results in gradual hypercondensation of chromosomes (Fig. 6A). Disruption of the condensin I complex by depletion of NCAP-D2 reduces this chromosome hypercondensation effect (70). To determine whether depletion of NCAP-G had the same effect on chromosome condensation as depletion of NCAP-D2 and whether PP6c depletion mimics those effects, we depleted HeLa cells of PP6c by infection with the viruses encoding PP6c–sh1 and PP6c–sh4, treated the cells with nocodazole (which arrests cells in prophase), performed chromosome spread assays, and analyzed the DNA for hypercondensation by microscopy (Fig. 6A). Indeed, we observed a similar decrease (~30%) in chromosome hypercondensation and a corresponding increase in normally condensed chromosomes in

chromosome spreads from cells depleted of NCAP-G or PP6c compared to cells infected with the control virus (Fig. 6B).

Additionally, reduced condensin I function can result in chromosome segregation errors, including lagging chromosomes and chromatin bridges (Fig. 6C) (71). We hypothesized that PP6-dependent dephosphorylation of condensin I may prevent such chromosome segregation errors in mitosis. We depleted HeLa cells of PP6c or NCAP-G and imaged anaphase cells by fluorescence microscopy. Whereas NCAP-G knockdown resulted in a significant increase in both chromatin bridges and lagging chromosomes, PP6c depletion only produced a significant increase in chromatin bridges (Fig. 6D). Thus, as previously observed for NCAP-D2 (70, 71), NCAP-G is necessary for chromosome condensation upon prolonged prometaphase arrest as well as accurate chromosome segregation. Furthermore, depletion of PP6c results in similar defects in hypercondensation, suggesting that the CK2-dependent phosphorylation sites on NCAP-G and potentially other members of the condensin I complex reduces the activity of the complex in mitotic PP6c-depleted cells.

DISCUSSION

Here, we developed a baculovirus-mediated shRNA approach and combined it with quantitative phosphoproteomics to determine changes in phosphorylation and protein abundance upon depletion of PP6c. We chose a baculovirus-mediated gene silencing approach because recombinant baculoviruses are safe, stable for long-term storage, amenable to amplification, and can infect a wide range of mammalian cells at high efficiency (34, 35). Because of these characteristics, baculoviruses are ideal for efficient and reproducible gene depletion in large numbers necessary for large-scale phosphoproteomics experiments. We identified two shRNA sequences that in combination efficiently reduced PP6c abundance and increased PP6c substrate phosphorylation. However, as with other gene silencing approaches, potential off-target effects are a concern. Thus, we globally compared protein abundance in mitotic HeLa cells upon infection with each of these shRNAs individually. We found that the abundance of only 35 proteins was statistically different between infections with the different shRNAs, and the median fold change of this difference was only modest. Although statistically significant because of highly reproducible fold changes in each triplicate analysis, we predicted that the minor absolute differences in the protein abundance of the 35 proteins were not likely biologically important in the context of the large increases in phosphorylation occupancy we observed in our combined hairpin shPP6 data set. Although we cannot rule out that these hairpins may exhibit off-target effects on proteins not among the 6700 in this experiment, the lack of any compelling off-target candidates among this relatively deep proteomics data set is supportive of their selectivity for PP6c.

Using this strategy, we identified 408 phosphopeptides on 272 proteins that increased and 298 phosphopeptides on 220 proteins that decreased upon PP6c depletion in mitotically arrested HeLa cells. Characterization of the motifs surrounding the phosphorylation sites that increased upon PP6c depletion identified an arginine-containing basophilic motif, which is reminiscent of the substrate motif of the known PP6c substrate AURKA (21). Comparison with previously identified AURKA substrates (44) revealed that phosphorylation of only a

subset of AURKA substrates increased upon PP6c depletion and that not all basophilic PP6c-dependent phosphorylation sites are increased because of higher AURKA activity. One explanation for this involves the relatively high phosphorylation site occupancy of mitotic phosphoproteins that may preclude some AURKA substrates from increasing in site occupancy any further upon PP6c depletion (40, 41). This may also reflect the complex structural and regulatory requirements for kinase activation that extend beyond activation T-loop phosphorylation status, in which AURKA may exhibit increased activity toward select substrates. In addition, other phosphatases that directly target AURKA substrates may exhibit differential activity on these substrates to tightly regulate their occupancy. Furthermore, although some Arg-X-phospho-Ser-containing phosphopeptides might be increased upon PP6c depletion due to increased AURKA activity, it is possible that others might be directly affected by PP6c depletion through opposition of AURKA or other basophilic kinases. Alternatively, the analysis of AURKA inhibitor-sensitive phosphorylation sites may have not identified changes in phosphorylation site occupancy of some of these PP6c-specific phosphopeptides because of experimental conditions, including short-term inhibition of kinase activity (30 min) that might not be sufficient to allow for phosphorylation site turnover by opposing protein phosphatases. Finally, there are many other basophilic protein kinases that could play a role in regulating these sites, such as CHK2, calcium and calmodulin-dependent kinase II (CaMKII), protein kinase A (PKA), protein kinase B (PKB), and serum-and glucocorticoid-regulated kinase (SGK) (50). These differences in responses of certain phosphorylation sites to selective kinase and opposing phosphatase inhibition further serve to highlight the difficulty in studying the posttranslational mechanisms that regulate systems as complex as cell division. We only observed an enrichment for acidophilic sites in the portion of phosphorylation sites that were increased in abundance upon PP6c depletion. This motif is similar to the substrate preference of CK2 (50–52), which has a wide array of substrates and is an important regulator in many signaling pathways (72); however, its role in mitosis is just emerging (68, 73–76).

Network analysis of proteins containing the regulated phosphorylation sites revealed known and new biological processes and pathways that are potentially directly or indirectly regulated by PP6. The identification of PP6c-regulated phosphorylation sites on proteins linked to chromosome condensation is intriguing. Protein phosphorylation is an important regulatory mechanism in chromosome condensation, and the integration of specific kinase activities in both interphase and mitosis is important for the fidelity of chromosome condensation (69, 77–82). Furthermore, the role of protein phosphatases in chromosome condensation and decondensation is just emerging (83–85). We identified several phosphorylation sites on members of the condensin I complex that increased upon PP6c depletion. Condensin I is a pentameric protein complex, containing NCAP-G, NCAP-H, NCAP-D2, SMC2, and SMC4, that associates with chromosomes in prometaphase after nuclear envelope breakdown (82). Condensin I is required for longitudinal chromosome compaction in prolonged prometaphase, complete dissociation of cohesin from chromosome arms, and normal timing of mitotic progression (70). Furthermore, condensin I is important for mechanical rigidity and assembly of mitotic chromosomes and faithful chromosome segregation. Depletion of condensin I subunits leads to defects in anaphase, including the

formation of chromatin bridges and lagging chromosomes (71). In vitro, condensin I introduces positive supercoils into closed circular DNA in an adenosine triphosphate (ATP)-dependent manner (86). CK2 phosphorylates and thereby negatively regulates condensin I function in interphase by decreasing the supercoiling activity of condensin I (81); this is thought to prevent premature or inappropriate chromosome condensation outside of mitosis. As cells enter mitosis, condensin I is phosphorylated by Cdk1, which targets condensin I to chromosomes and partially activates it (81). To achieve full condensin I activation as cells proceed through mitosis, the CK2-phosphorylated sites on condensin I must be dephosphorylated relative to their status in interphase (81). This reduced phosphorylation could either result from a change in CK2 activity or substrate accessibility, or from increased activity of a protein phosphatase, or both. On the basis of the CK2 consensus motif, previous research suggested that CK2 phosphorylates Thr⁹⁵¹ on SMC4, Ser¹³⁷¹ on NCAP-D2, Ser⁹⁷⁵ on NCAP-G, and Ser⁵⁷⁰ on NCAP-H (81). However, this was only confirmed experimentally for Ser⁵⁷⁰ on NCAP-H (81). Of the four potential CK2 phosphorylation sites in proteins in the condensin I complex [Thr⁹⁵¹ on SMC4, Ser¹³⁷¹ on NCAP-D2, Ser⁹⁷⁵ on NCAP-G, and Ser⁵⁷⁰ on NCAP-H (81)], we only detected peptides covering Ser^{973/5} on NCAP-G; in silico sequence analysis revealed that many of these potential CK2 phosphorylation sites are in highly acidic, lysine- and arginine-free regions of protein sequence that are not accessible by trypsin digestion. We confirmed that NCAP-G Ser^{973/5} is a direct substrate of PP6c by in vitro phosphatase assays, and data from in vitro kinase assays showed that PP6c opposes CK2 phosphorylation on the NCAP-G, indicating that PP6c could contribute to condensin I activation through dephosphorylation of CK2 phosphorylation sites (Fig. 7).

Inhibition of condensin I function by subunit depletion results in chromosome condensation defects in cells experiencing prolonged mitotic arrest and chromosome segregation defects in cells in anaphase (70, 71). We found that depletion of PP6c resulted in chromosome condensation and segregation defects similar to those observed with depletion of NCAP-G. Given that we were unable to profile other known CK2-dependent sites on condensin complex subunits in our large-scale PP6c screen, possibly because of their inaccessibility by trypsin-mediated proteolysis, we recognize that not all of the inhibitory function of condensin I is likely to be regulated through dynamic phosphorylation of Ser^{973/5} on NCAP-G. However, we established that CK2 can mediate NCAP-G phosphorylation at Ser^{973/5} and that PP6 dephosphorylates Ser^{973/5}, thereby opposing this activity of CK2. In vitro, CK2 did not generate pSer^{973:975}, suggesting that a different protein kinase may mediate the second phosphorylation event or that priming phosphorylation events (87) that were likely removed by previous λ -phosphatase treatment are required for CK2 processivity at this locus. PP6 neither in cells nor in vitro dephosphorylated this locus when both sites were phosphorylated, suggesting that the second phosphorylation site may function to inhibit PP6 activity here. Clearly, there are additional layers of complexity between PP6, CK2, and condensin I function that remain to be explored.

Broadly speaking, the interaction and counteraction between protein kinases and phosphatases are essential for cellular function and organismal survival; these delicate balances are often deregulated in human cancer. As cells progress through mitosis, they undergo marked changes in cellular structure and organization to successfully divide. These

changes are regulated and controlled by dynamic protein phosphorylation to ensure faithful chromosome segregation and distribution of cellular content (1, 2). Although the importance of protein phosphatases is well recognized, the identification of candidate protein phosphatase substrates is hindered by the lack of small molecule inhibitors that specifically target the catalytic subunit of a single PPP family member. Thus, whereas the approach that we present cannot distinguish direct substrates of the phosphatase from indirect effects of phosphatase depletion, it provides valuable insights into phosphatase biology and pathways and provides another strategy for revealing previously unknown protein kinase—phosphatase interactions in the regulation of phosphorylation site occupancy of common substrates.

MATERIALS AND METHODS

Cells

HeLa cells were grown as adherent cultures in Dulbecco's modified Eagle's medium (Cellgro Mediatech Inc.) with 10% heat-inactivated fetal bovine serum (FBS) (HyClone) and penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively; Cellgro Mediatech Inc.) at 37°C in a humidified incubator with 5% CO₂. Sf9 cells were grown in Grace's supplemented insect cell medium (Life Technologies) with 10% heat-inactivated FBS (HyClone), gentamicin (10 µg/ml; Sigma), and amphotericin B (0.25 µg/ml; Sigma). Sf9 cells were maintained at 27°C in a nonhumidified incubator.

Recombinant baculovirus-based shRNA generation

To generate recombinant baculovirus expressing shRNAs, the polyhedrin promoter was deleted from pFastBac1 (Life Technologies). A fragment of the pLL3.7 lentivirus vector (88) containing the U6 promoter, shRNA, CMV promoter, and EGFP complementary DNA was subcloned into pFastBac1 to generate pFastBacMam-shRNA-GFP. shRNA sequences targeting PP6c were chosen with the siDESIGN Center (Dharmacon) and from Zeng *et al.* (21): PP6c-1 (GTTTGGAGACCTTCACTT), PP6c-2 (CGCTA-GACCTGGACAAGT), PP6c-3 (GTAAATACAAGAGAACCA), PP6c-4 (CTAAATGGCCTGATCGTA), and PP6c-5 (GTAGACAGAGGTTACTAT). Baculoviruses of PP6c-1 and PP6c-4 were efficient in reducing PP6c expression. Specificity for PP6c was confirmed using BLAST search against the human genome. shRNA sequences were cloned into pLL3.7, subcloned into the modified pFastBac1, and confirmed by DNA sequencing. Sequence-confirmed vectors were transformed in DH10Bac (Life Technologies) to generate bacmids. Recombinant baculoviruses were generated according to the manufacturer's instructions. Viruses were further amplified in Sf9 insect cells.

shRNA testing

HeLa cells were infected with P0 baculovirus. Infection efficiency was determined by monitoring GFP expression 24 hours after infection. Depletion of PP6c was monitored at 24, 48, and 72 hours after infection by Western blotting with a PP6c-specific antibody (Abcam). For Western blot analysis, cells were collected, washed with phosphate-buffered saline (PBS) (pH 7.4), and lysed in lysis buffer [50 mM tris-HCl (pH 8.1), 150 mM NaCl, 15% glycerol, 1% SDS, phosphatase inhibitors, protease inhibitors, 1 mM dithiothreitol (DTT)].

TMT quantitative proteomics

HeLa cells treated with control, shPP6c-1, or shPP6c-4 baculovirus were synchronized and arrested in mitosis in biological triplicate. Mitotic cells were collected by shake-off, washed twice with PBS, pelleted, and lysed in 9 M urea, 50 mM tris (pH 8.2), 100 mM NaCl buffer in the presence of protease inhibitors by sonication. A 50- μ l aliquot of the lysate was removed for quantification by bicinchoninic acid assay (BCA) (Pierce); the remainder of the lysate was reduced with 5 mM DTT at 50°C for 25 min, followed by alkylation with 13 mM iodoacetamide at room temperature for 1 hour. The lysates were then diluted fivefold with 50 mM tris (pH 8.2) and 75 mM NaCl and digested with trypsin (1:100 w/w) for 16 hours at 37°C. The digests were desalted using C₁₈ solid-phase extraction cartridges (Grace-Vydac); an aliquot of each of the desalted eluates corresponding to 50 μ g of peptide digest was dried by vacuum centrifugation in separate tubes.

The nine samples were prepared for TMT-based quantitative proteomics as one sixplex (biological replicates 1 and 2) and one triplex (biological replicate 3). Previously dried, individual TMT reagent ali-quots corresponding to 133 μ g of reagent were resuspended in 40 μ l of 150 mM Hepes (pH 8.5)/20% acetonitrile (ACN), followed by transfer of the resuspended reagent aliquots to tubes containing 50 μ g of peptide digest and vortexing to mix reagent and peptides. After 1 hour at room temperature, each digest was quenched with 5 μ l of 500 mM ammonium bicarbonate solution for 10 min, mixed into the two sets of multiplexes, diluted threefold with 0.1% trifluoroacetic acid (TFA) in water, and desalted using C₁₈ solid-phase extraction cartridges as above. The desalted multiplexes were dried by vacuum centrifugation, resuspended in 110 μ l of 3% ACN/0.1% TFA in water, and separated on a pentafluorophenyl (PFP) analytical column [XSelect HSS PFP XP column, Waters; 100 A, 2.5 mm inner diameter (ID), 150 mm in length] into 48 fractions by gradient elution [buffer A (3% ACN, 0.1% TFA); buffer B (95% ACN, 0.1% TFA)] with 5% B, 0 to 1 min; 5 to 11% B, 1 to 2 min; 11 to 47% B, 2 to 60 min; 47 to 100% B, 60 to 61 min; 100% B, 61 to 69 min; 100 to 5% B, 69 to 70 min; 5% B, 70 to 110 min, all at a flow rate of 0.15 ml/min. The 48 fractions were reduced to 24 by mixing fraction 1 with fraction 13, fraction 2 with fraction 14, etc., and dried by vacuum centrifugation. Basic pH reverse-phase system is as described below. The equivalent of 1.5 μ g of peptide from each fraction was analyzed using an Easy LC-1000 (Proxeon) and Orbitrap Fusion (89) (Thermo Fisher Scientific) LC-MS/MS platform across a 2-hour gradient from 9% ACN/0.1% formic acid to 37% ACN/0.1% formic acid. The Orbitrap Fusion was operated in data-dependent, SPS-MS³ quantification mode (38, 90) wherein an Orbitrap MS¹ scan was taken [scan range, 350 to 1500 mass-to-charge ratio (m/z); resolution (R), 120K; automatic gain control (AGC) target, 2.5×10^5 ; max ion injection time, 100 ms], followed by ion trap MS² scans on the most abundant precursors for 4 s [max speed mode; quadrupole isolation, 0.6 m/z ; AGC target, 4×10^3 ; scan rate, rapid; max ion injection time, 60 ms; minimum MS¹ scan signal, 5×10^5 normalized units; charge states 2, 3, and 4 included; collision-induced dissociation (CID) energy, 33%] and Orbitrap MS³ scans for quantification [R, 15K; AGC target, 2×10^4 ; max ion injection time, 125 ms; higher-energy collisional dissociation (HCD) energy, 48%; scan range, 120 to 140 m/z ; synchronous precursors selected, 10). The resulting data files were searched using Comet with a static mass of 229.162932 daltons on peptide N-termini and lysines and 57.02146 daltons on cysteines and a variable mass of 15.99491 daltons on

methionines against the target-decoy version of the human proteome FASTA (UniProt) and filtered to a ~1% FDR at the peptide level. TMT reporterions were filtered to require a minimum signal of 3000 U, and at least two unique peptide sequences were required to contain quantification results per protein. These requirements resulted in 6700 protein quantifications across the two multiplexes (1.2% protein FDR by target-decoy). To assess differences in protein abundance between the two shRNAs, we required protein quantifications in both hairpins to be statistically significant ($P < 0.05$, two-tailed Student's t test), and assessed differences between the two hairpins from those protein abundances ($P < 0.05$, two-tailed Student's t test).

Proteome-wide analysis of PP6c depletion

HeLa cells were seeded at 15% confluency and infected with PP6c-sh1- and PPc-sh4-expressing virus or control shRNA-expressing virus the next day. Twenty-four hours after infection, HeLa cells were synchronized at the G₁/S transition by the addition of thymidine (final concentration, 2 mM; Sigma) for 16 hours followed by a washout for 3 hours and addition of Taxol (100 nM, Sigma) for 16 hours. For thymidine washout, cells were washed three times with 15 ml of PBS (Cellgro Mediatech Inc.). Mitotic HeLa cells were collected by mitotic shake-off, washed with PBS, snap-frozen in liquid nitrogen, and stored at -80°C . Efficiency of PP6c depletion and increased phosphorylation of Thr²⁸⁸ of AURKA were determined by Western blotting with specific antibodies against PP6c (Abcam) and AURK A Thr²⁸⁸ (Cell Signaling Technologies). Western blot intensities were quantified using ImageJ (91). HeLa cell pellets were thawed on ice, lysed in ice-cold lysis buffer [8 M urea, 25 mM tris-HCl (pH 8.6), 150 mM NaCl, phosphatase inhibitors (2.5 mM β -glycerophosphate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium molybdate), and protease inhibitors (1 mini-Complete EDTA-free tablet per 10 ml of lysis buffer; Roche Life Sciences)], and sonicated three times for 15 s each with intermittent cooling on ice. Lysates were centrifuged at 15,000g for 30 min at 4°C . Supernatants were transferred to a new tube, and the protein concentration was determined using a BCA assay (Pierce/Thermo Fisher Scientific). For reduction, DTT was added to the lysates to a final concentration of 5 mM and incubated for 30 min at 55°C . Afterward lysates were cooled to room temperature and alkylated with 15 mM iodoacetamide at room temperature for 45 min. The alkylation was then quenched by the addition of an additional 5 mM DTT. After sixfold dilution with 25 mM tris-HCl (pH 8), the samples were digested overnight at 37°C with 2.5% (w/w) trypsin. The next day, the digest was stopped by the addition of 0.25% TFA (final, v/v) and centrifuged at 3500g for 30 min at room temperature to pellet precipitated lipids, and peptides were desalted on a 500-mg (sorbent weight) solid-phase extraction C₁₈ cartridge (Grace Davison). Aliquots of peptide samples (250 μg each) were taken for protein abundance analysis. Peptides were lyophilized and stored at -80°C until further use.

Phosphopeptide enrichment

Phosphopeptide purification was performed as previously described (92). Briefly peptides were resuspended in 2 M lactic acid in 50% ACN ("binding solution"). Titanium dioxide microspheres were added and vortexed by affixing to the top of a vortex mixer on the highest speed setting at room temperature for 1 hour. Afterward microspheres were washed twice with binding solution and three times with 50% ACN/0.1% TFA. Peptides were eluted twice

with 50 mM KH_2PO_4 (adjusted to pH 10 with ammonium hydroxide). Peptide elutions were combined quenched with 50% ACN/5% formic acid dried and desalted on a μHLB OASIS C_{18} desalting plate (Waters).

Dimethyl labeling

Dimethyl labeling was performed essentially as described (93). Peptides and phosphopeptides were resuspended in 100 mM triethyl ammonium bicarbonate (TEAB) by vortexing. For “light” labeling, formaldehyde (final concentration of 0.25%) and cyanoborohydride (final concentration of 25 mM) were added to the samples followed by vortexing. For “heavy” labeling, formaldehyde- ^{13}C d_2 (final concentration of 0.25%) and cyanoborodeuteride (final concentration of 25 mM) were added to the sample followed by vortexing. Samples were incubated at room temperature for 1 hour. Afterward, reactions were quenched by the addition of NH_3 solution (final concentration of 0.2%), followed by vortexing and incubation for 5 min at room temperature. Reactions were further quenched by the addition of formic acid (final concentration of 0.5%), followed by vortexing and incubation for 5 min at room temperature. Heavy and light samples were mixed, acidified with TFA (final concentration of 0.1%), and desalted.

SCX chromatography

Phosphopeptides were resuspended in SCX buffer A [7 mM KH_2PO_4 (pH 2.65)/30% ACN] and separated per injection on an SCX column (Phenomenex Luna SCX; 2.1-mm ID $\times$ 200-mm length) as previously described (92) and under the following conditions: using a gradient of 0 to 10% SCX buffer B [350 mM KCl /7 mM KH_2PO_4 (pH 2.65)/30% ACN] for 10 min, 10 to 17% SCX buffer B for 17 min, 17 to 32% SCX buffer B for 13 min, 32 to 60% SCX buffer B for 10 min, 60 to 100% SCX buffer B for 2 min, holding at 100% SCX buffer B for 5 min, from 100 to 0% SCX buffer B for 2 min, and equilibration at 0% SCX buffer B for 65 min, all at a flow rate of 0.2 ml/min, after a full blank injection of the same program was run to equilibrate the column. Sixteen fractions were collected, dried, and desalted using a μHLB OASIS C_{18} 96-well desalting plate and manifold (Waters).

Basic pH reversed-phase high-performance liquid chromatography

Peptides were resuspended in buffer A [5% ACN, 10 mM ammonium bicarbonate (pH 8.0)] and separated per injection on an Agilent 300 Extend C_{18} column (5- μm particles, 4.6-mm ID, and 200-mm length) using a 45-min linear gradient from 8 to 35% ACN (for dimethyl-labeled peptides) and from 11 to 39% ACN (for TMT-labeled peptides) in 10 mM ammonium bicarbonate (pH 8) on an Agilent 1100 liquid chromatography system. Peptides were separated into 96 fractions. The 96 fractions were combined into 12 samples (for dimethyl-labeled peptides) and 24 samples (for TMT-labeled peptides), dried, and desalted using a μHLB OASIS C_{18} 96-well desalting plate and manifold (Waters).

LC-MS/MS Analysis

LC-MS/MS analysis for peptides and phosphopeptides was performed on a Fusion Orbitrap Tribrid mass spectrometer (Thermo Fisher Scientific) equipped with an Easy-nLC 1000 (Thermo Fisher Scientific) and nanospray source (Thermo Fisher Scientific).

Phosphopeptides were redissolved in 5% ACN/1% formic acid and loaded onto a trap column at 2500 nL/min [1.5-cm length, 100- μ m ID; ReproSil, C₁₈ AQ 5- μ m 200-Å pore (Dr. Maisch)] vented to waste through a micro-tee and eluted across a fritless analytical resolving column (35-cm length, 100- μ m ID; ReproSil, C₁₈ AQ 3- μ m 200-Å pore) pulled in-house (Sutter P-2000, Sutter Instruments) with a 60-min gradient of 5 to 30% LC-MS buffer B (LC-MS buffer A: 0.0625% formic acid, 3% ACN; LC-MS buffer B: (0.0625% formic acid, 95% ACN). The Orbitrap Fusion was set to perform an Orbitrap MS¹ scan (*R*, 120K; AGC target, 2.5×10^5) from 350 to 1500 Thomson, followed by HCD MS² spectra on the most abundant precursor ions detected by Orbitrap scanning (*R*, 15K; AGC target, 40,000; max ion time, 50 ms) for 2.5 s before repeating the cycle. Precursor ions were isolated for HCD with a quadrupole isolation width of 0.6 Thomson and HCD fragmentation at 30% collision energy. Charge state 2, 3, and 4 ions were selected for MS².

Peptide spectral matching and bioinformatics

Raw data were searched using SEQUEST (94, 95) (Thermo Fisher Scientific) against a target-decoy (reversed) (96) version of the human proteome sequence database (UniProt; downloaded February 2013) with a precursor mass tolerance of ± 1 dalton and requiring fully tryptic peptides with up to three miscleavages. Carbamidomethylcysteine, dimethylation at peptide N-termini, and lysines were enabled as fixed modifications. Oxidized methionine; phosphorylated serine, threonine, and tyrosine; and isotopically heavy label (+8.04437) at peptide N-termini and lysines were enabled as variable modifications. The resulting peptide spectral matches were filtered to <1% FDR for peptides and 2% FDR for phosphopeptides, on the basis of reverse-hit counting [mass measurement accuracy cutoffs within ± 2.5 ppm, a δ -XCorr (dCn) of greater than 0.08, and appropriate XCorr values). Probability of phosphorylation site localization was determined by phosphoRS (97). Peptide ratio quantification was performed using MassChroQ (98). Log₂ H/L ratios were median-adjusted for mixing errors. Phosphopeptide fold changes were adjusted for changes in protein abundance on a per-sample basis. Significance of log₂ protein-corrected phosphopeptide fold change was determined by two-tailed Student's *t* test assuming unequal variance. For further analysis, we required an at least twofold increase at *P* < 0.1. Peptide motif analysis was performed on phosphopeptides increased by twofold or more and a *P* value of <0.1 based on (42). Protein-protein interactions of proteins belonging to phosphopeptides with significant increase in phosphorylation occupancy were determined using the STRING database and analyzed in Cytoscape (54, 55). Edges present protein-protein interactions based on the STRING database. Densely connected clusters with the networks were identified using ClusterONE (99) in Cytoscape (minimum size, 4; node penalty, 2.4; overlap threshold, 0.5). GO annotations were performed in Cytoscape using BiNGO to test for ontology enrichment. To determine significance of enrichment of terms, a Bonferroni-corrected *P* value cutoff of 0.05 was used.

PP6 in vitro phosphatase assay

293T cells were transfected with p3×Flag-CMV10-PP6c or p3×Flag-CMV10-NCAP-H, and stable cells lines were selected using G418. PP6c holoenzymes and condensin I complex were purified separately using anti-Flag M2 affinity gel (Sigma) (15 μ l of resin for each 15-cm tissue culture dish lysed) and eluted with 3×Flag peptide (final concentration, 150 ng/ μ l).

For dephosphorylation reactions, condensin I was incubated in the presence or absence of PP6c holoenzymes in phosphatase buffer [50 mM Hepes (pH 7.5), 10 mM NaCl, 2 mM DTT, 1 mM MnCl₂, 0.01% Brij 35] and incubated for 2 hours or overnight at room temperature. We found that NCAP-G was efficiently dephosphorylated after 2 hours, and we did not observe any further dephosphorylation of other phosphorylation sites on NCAP-G in the overnight reactions. Reactions were quenched by the addition of SDS-PAGE sample buffer, reduced, and alkylated (as described above), and then separated by SDS-PAGE gel electrophoresis. To better access the region of NCAP-G corresponding to Ser⁹⁷³ and Ser⁹⁷⁵, bands corresponding to NCAP-G were excised and digested with proteinase K in 50 mM TEAB for 1 hour at 37°C. Peptides were extracted using 5% formic acid/50% ACN and dried. Peptides were labeled heavy and light by reductive dimethylation (as described above), mixed, and desalted. Peptides were analyzed on a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) or Orbitrap Fusion mass spectrometer equipped with an Easy-nLC 1000 (Thermo Fisher Scientific). Raw data were searched using COMET in high-resolution mode (100) against a NCAP-G sequence database with a precursor mass tolerance of ± 20 ppm, no enzyme specificity, and carbamidomethylcysteine, and dimethylation at peptide N-termini, and lysines enabled as fixed modifications. Oxidized methionine; phosphorylated serine, threonine, and tyrosine; isotopically heavy label (+8.04437) at peptide N-termini and lysines were searched as variable modifications. Probability of phosphorylation site localization was determined by phosphoRS (97). Quantification of LC-MS/MS spectra was performed using MassChroQ (98). Reproducibility of proteinase K digest is depicted in fig. S9, and results are shown in table S8.

CK2 in vitro kinase assay

Condensin I was purified from 293T cells expressing p3×Flag-CMV10-NCAP-H as described above and dephosphorylated with 200 U of λ -phosphatase (New England Biolabs). λ -Phosphatase was inhibited by the addition of 5 mM sodium vanadate, and condensin I was incubated with 125 U of CK2 (New England Biolabs) in protein kinase buffer (New England Biolabs) supplemented with 200 mM ATP (Sigma) at 30°C for 1 hour. Reactions were quenched by the addition of SDS-PAGE sample buffer, reduced, and alkylated (as described above), and then separated by SDS-PAGE gel electrophoresis. Bands corresponding to NCAP-G were excised and analyzed by proteinase K digest and reductive dimethyl labeling as described above, as well as by LC-MS/MS.

Analysis of mitotic defects

HeLa cells were seeded on glass coverslips and infected with viruses expressing PP6c-sh1 and PP6c-sh4 or viruses expressing control shRNA for 48 hours. Cells were fixed in 3.7% formaldehyde for 7 min at room temperature, permeabilized with PBS containing 0.1% Triton X-100 (PBST), and blocked with 3% bovine serum albumin (BSA) in PBST for 30 min at room temperature. Afterward, cells were incubated with primary antibodies against CRESTautoimmune serum (ImmunoVision) and α -tubulin (Sigma) diluted in 3% BSA in PBST for 30 min at room temperature. Cells were washed with PBST and incubated with anti-human Alexa 568 and anti-mouse Alexa 488 (Molecular Probes) secondary antibodies diluted in 3% BSA in PBST for 30 min at room temperature. DNA was labeled with

NucBlue Fixed Cell ReadyProbes reagent (Life Technologies). Images were collected on an Axioplan2 Zeiss fluorescence microscope. Experiments were done in biological triplicates. At least 100 HeLa cells in anaphase were counted per condition per experiment.

Chromosome spreads

HeLa cells were infected with PP6c or control shRNA for 48 hours and treated for 4 hours with nocodazole (100 ng/ml). Cells were collected by mitotic shake-off and pelleted. Cells were resuspended gently in 75 mM KCl, incubated at room temperature for 5 min and at 4°C for 1 min, and pelleted by centrifugation. Cells were fixed with methanol/acetic acid (3:1) and pelleted. Cells were washed twice with methanol/acetic acid (3:1), resuspended, and dropped onto glass slides. Slides were allowed to dry, and DNA was stained with NucBlue Fixed Cell ReadyProbes reagent (Life Technologies) diluted in PBS for 30 min and sealed with coverslips using ProLong Gold (Life Technologies). Experiments were done in biological triplicates. At least 100 chromosome spreads were counted per condition per experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Strategy to determine PP6c-dependent phosphorylation changes in cells by quantitative phosphoproteomics

(A) Vector map of pFastBacMam-shRNA-GFP. SV40 PA term, simian virus 40 polyadenylation terminator; EGFP, enhanced GFP; CMV, cytomegalovirus promoter; Amp, ampicillin resistance gene. (B) Representative immunofluorescence micrographs of HeLa cells infected with dual PP6c shRNA- and GFP-expressing baculoviruses. Scale bar, 50 μ m. GFP protein abundance was comparable for all tested PP6c shRNA and control viruses (fig. S1A). GFP protein abundance was performed in all experiments to determine infection

efficiency. DAPI, 4',6-diamidino-2-phenylindole. (C) Western blot analysis of PP6c and GFP abundance in HeLa cells infected with viruses encoding control or both PP6c-1 and PP6c-4 shRNAs (PP6c shRNA). Lamin A/C is the loading control. (D) Scheme depicting experimental strategy for determining PP6c-dependent changes in phosphorylation site occupancy upon PP6c depletion. Mitotically arrested HeLa cells infected with viruses encoding control or PP6c shRNAs were separately lysed, reduced, alkylated, and trypsin digested. Phosphopeptides (p-peptides) were enriched using titanium dioxide microspheres, heavy or light labeled by reductive dimethylation, and mixed. Phosphopeptides were separated by strong cation exchange (SCX) chromatography and analyzed by LC-MS/MS ($n = 3$ independent experiments). (E) Western blot analysis of PP6c abundance and AURKA pThr²⁸⁸ in HeLa cell lysates as described in (D). Lamin A/C is the loading control. (F) Quantification of PP6c, AURKA pThr²⁸⁸, and lamin A/C abundance. PP6c and AURKA pThr²⁸⁸ abundances were normalized to Lamin A/C. * $P < 0.05$, *** $P < 0.001$ ($n = 3$ independent experiments).

Fig. 2. Candidate phosphorylation site substrates of PP6 in mitosis

(A) Venn diagrams depicting overlap of identified and quantified phospho-peptides and proteins in biological triplicate experiments. (B) Venn diagrams depicting overlap of identified and quantified phosphopeptides and proteins after protein correction in these experiments. In (A) and (B), each experiment is represented by a color, and gray is the overlap in all three experiments. (C) Volcano plot of $\log_2$ ratios of protein-corrected phosphopeptides from (B) plotted against the negative $\log_{10}$ of the P value of their fold

change. We used the 298 phosphopeptides that decreased and the 408 phosphopeptides that increased because of phosphorylation abundance changes for subsequent analysis.

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Fig. 3. Motif analysis and comparison of candidate PP6c substrates with known AURKA phosphorylation sites

(A) Enriched phosphorylation site motifs of phosphopeptides significantly increased by twofold or more upon PP6c depletion. (B) Enriched phosphorylation site motifs of phosphopeptides significantly decreased by twofold or more upon PP6c depletion. (C) Scatter plot of phosphopeptides that were previously ascribed to AURKA activity versus their fold change upon PP6c depletion. (D) Scatter plot of phosphopeptides that contain a basophilic [RXp(S/T)] motif and increase by twofold or more upon PP6c depletion versus

their fold change upon inhibition with AURKA inhibitor. In (C) and (D), red circles represent phosphorylation sites regulated upon both AURKA inhibition and PP6c depletion, and black circles represent sites regulated upon either AURKA inhibition (C) or PP6c depletion (D). Dotted black lines indicate zero coordinates.

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Fig. 4. Processes regulated by PP6c in mitotic cells
(A) Subnetwork depicting the connectivity of highly connected clusters in the protein-protein interaction network from phosphopeptides significantly increased in phosphorylation upon PP6c depletion in mitotically arrested cells. Proteins in the different colored clusters and their enrichment in biological processes and cellular components are shown. (B) Subnetwork depicting the connectivity of highly connected clusters in the protein-protein interaction network from phosphopeptides significantly decreased in phosphorylation upon

PP6c depletion in mitotically arrested cells. Proteins in the different colored clusters and their enrichment in biological processes and cellular components are shown.

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Fig. 5. In vitro PP6 dephosphorylation analysis of NCAP-G

(A) Scheme depicting the experimental strategy for PP6 in vitro phosphatase assay. Purified condensin I was incubated with or without purified PP6 holoenzyme and resolved by SDS-PAGE. NCAP-G was excised and digested, and peptides were differentially labeled by reductive dimethylation, mixed, and analyzed by LC-MS/MS ($n = 3$ independent experiments). (B) SDS-PAGE gel of condensin I purification. (C) Relative intensities of LC-MS/MS traces of heavy- and light-labeled phosphopeptides corresponding to the singly phosphorylated Ser^{973/5} phosphorylation site in NCAP-G, extracted to ± 2 parts per million

(ppm). Phosphopeptide sequence, charge state, and ion mass-to-charge ratios are indicated. Acidic amino acids are highlighted in red. **(D)** Relative intensities of LC-MS/MS traces of the heavy- and light-labeled unphosphorylated peptide spanning Ser^{973/5}, extracted to ± 2 ppm. Peptide sequence, charge state, and ion mass-to-charge ratios are indicated. Acidic amino acids are highlighted in red. Blue lines in (C) and (D) represent the samples incubated with buffer, and gray lines represent the samples incubated with purified PP6c. **(E)** Scheme depicting experimental strategy for CK2 in vitro kinase assay. Purified and λ -phosphatase–dephosphorylated condensin I was incubated with or without purified CK2 and resolved by SDS-PAGE. NCAP-G was excised and digested, and peptides were labeled by reductive dimethylation, mixed, and analyzed by LC-MS/MS ($n = 3$ independent experiments). **(F)** Relative intensities of LC-MS/MS traces of heavy- and light-labeled phosphopeptides covering the Ser^{973/5} phosphorylation site in NCAP-G, extracted to ± 2 ppm. Phosphopeptide sequence, charge state, and peptide ion mass-to-charge values are indicated. Acidic amino acids are highlighted in red. **(G)** Relative intensities of LC-MS/MS traces of the heavy- and light-labeled corresponding unphosphorylated peptide, extracted to ± 2 ppm. Peptide sequence, charge state, and peptide ion mass-to-charge values are indicated. Acidic amino acids are highlighted in red. Blue lines in (F) and (G) represent the samples incubated with buffer and gray lines represent the samples incubated with purified CK2.

Fig. 6. PP6-dependent defects in chromosome condensation and segregation in mitosis

(A) Effect of NCAP-G or PP6c depletion on chromosome hypercondensation.

Immunofluorescence micrographs depicting representative hypercondensed or normally condensed chromosomes in chromosome spreads of HeLa cells. Scale bar, 10 μ m. (B)

Quantification of differences between normally condensed and hypercondensed chromosomes upon NCAP-G depletion (left) and PP6c depletion (right). (C) Effect of NCAP-G or PP6c depletion on the formation of lagging chromosomes and chromosome bridges. Immunofluorescence micrographs depicting representative images of chromosome segregation defects in anaphase in PP6c shRNA-infected HeLa cells. Scale bar, 5 μ m. (D) Quantification of the number of lagging chromosomes and chromatin bridges in NCAP-G-depleted (left) or PP6c-depleted (right) HeLa cells. Quantified data are shown as means with SD. ** $P < 0.005$; * $P < 0.05$, paired Student's t test ($n = 3$ independent experiments).

Fig. 7. Model of regulation of condensin I activity

Scheme depicting mechanism of condensin I regulation adapted from Takemoto *et al.* (81). In G₁/S, condensin I is phosphorylated by CK2, maintaining condensin I in an inactive state. In G₂, Cdk1 phosphorylates condensin I, which partially activates the DNA supercoiling activity of the complex. As cells enter mitosis and during mitosis, CK2-dependent phosphorylation sites become dephosphorylated, maximally activating condensin I. On the basis of our results, PP6c contributes to this process by dephosphorylating CK2 sites on NCAP-G.