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Global Phosphoproteomic Mapping of Early Mitotic Exit in Human Cells Identifies Novel Substrate Dephosphorylation Motifs

Rachael A. McCloy‡, Benjamin L. Parker§, Samuel Rogers‡, Rima Chaudhuri§, Velimir Gayevskiy‡, Nolan J. Hoffman§, Naveid Ali‡, D. Neil Watkins‡¶||, Roger J. Daly**, David E. James§, Thierry Lorca‡‡, Anna Castro‡‡, and Andrew Burgess¶‡§§

Entry into mitosis is driven by the coordinated phosphorylation of thousands of proteins. For the cell to complete mitosis and divide into two identical daughter cells it must regulate dephosphorylation of these proteins in a highly ordered, temporal manner. There is currently a lack of a complete understanding of the phosphorylation changes that occur during the initial stages of mitotic exit in human cells. Therefore, we performed a large unbiased, global analysis to map the very first dephosphorylation events that occur as cells exit mitosis. We identified and quantified the modification of >16,000 phosphosites on >3300 unique proteins during early mitotic exit, providing up to eightfold greater resolution than previous studies. The data have been deposited to the ProteomeXchange with identifier PXD001559. Only a small fraction (~10%) of phosphorylation sites were dephosphorylated during early mitotic exit and these occurred on proteins involved in critical early exit events, including organization of the mitotic spindle, the spindle assembly checkpoint, and reformation of the nuclear envelope. Surprisingly this enrichment was observed across all kinase consensus motifs, indicating that it is independent of the upstream phosphorylating kinase. Therefore, dephosphorylation of these sites is likely determined by the specificity of phosphatase/s rather than the activity of kinase/s. Dephosphorylation was significantly affected by the amino acids at and surrounding the phosphorylation site, with several unique evolutionarily conserved amino acids correlating strongly with phosphorylation status. These data provide a potential mechanism for the specificity of phosphatases, and how they co-ordinate the ordered events of mitotic exit. In summary, our results provide a global overview of the phosphorylation changes that occur during the very first stages of mitotic exit, providing novel mechanistic insight into how phosphatase/s specifically regulate this critical transition. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.046938, 2194–2212, 2015.

Entry into mitosis is driven by the phosphorylation of thousands of proteins on multiple sites, triggering extensive cellular rearrangements including chromosome condensation, microtubule reorganization, and nuclear envelope breakdown (1). Many of these protein phosphorylation events are induced by cyclin dependent kinase 1 (CDK1)1, which is considered the master regulator of mitosis (2). However, several recent large-scale quantitative phosphoproteomic studies have highlighted the important role of other kinases, such as Polo like kinase 1 (PLK1), Aurora A and B, in driving many of these mitotic events (3–5). For cells to exit mitosis, the phosphorylation sites modified by these kinases must be dephosphorylated.
radation during mitotic exit (13), which although likely to be a

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supplemental Table S2.

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sodium salt (A.G. Scientifix, San Diego, CA), Nocodazole (Sigma, St.

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For many years phosphatases were thought to constantly
dephosphorylate substrates. However, recent discoveries

showed they are in fact highly regulated, and must first be

inhibited to permit mitotic entry, and then reactivated for

mitotic exit (15, 16). For the highly ordered events of mitotic

exit to occur correctly, the dephosphorylation of substrates

must occur in a rigid, timely, and ordered fashion. Failure to
do this results in a catastrophic failure of mitosis (17, 18). This

suggests that phosphatases must dictate an order of dephos-

phorylation for the correct timing of specific mitotic exit

events. This concept is supported by recent results in bud-
ding yeast where the well characterized mitotic exit phospha-
tase Cdc14 (19) drives ordered dephosphorylation during

mitotic exit (8). Evidence from higher-order mammalian, Xe-

nopus and Drosophila models indicates that both phospha-
tase PP2A (20–22) and PP1 (23, 24) are required for mitotic

exit, whereas Cdc14 appears to be dispensable (reviewed in

(25)). Yet despite many of these recent advancements, we still
do not fully understand the mechanisms controlling the global

phosphorylation changes that occur during the initial early

stages of mitotic exit. Are certain substrates preferentially
dephosphorylated, and if so how do phosphatases recognize

and specifically dephosphorylate these substrates? To an-
swer these questions we undertook a global, unbiased phos-

phoproteomic approach to characterize the full repertoire of

phosphorylation changes that occur as human cells begin

exit mitosis.

EXPERIMENTAL PROCEDURES

Chemicals, Reagents, and Antibodies—The following chemicals

were used: RO3306 (Axon MedChem, Reston, VA), Okadaic Acid

sodium salt (A.G. Scientifix, San Diego, CA), Nocodazole (Sigma, St.

Louis, MO) Thymidine and 2'-Deoxycytidine hydrate (Santa Cruz Bio-
technology, Dallas, TX). Antibodies used in this study are described in

supplemental Table S2.

Cell Synchrony—Thymidine block/release synchronizations were

performed as previously described (18). For mitotic shake off, HeLa

cells were first released from G1/S Thymidine block, and then treated

with 100 ng/ml of Nocodazole for 14 h, to capture cells in prometa-

phase (PM). Floating cells were then removed by shake-off, and treated

with 25 μM MG132 for 15 min. Mitotic cells were forced to

undergo mitotic exit by addition of 10 μM RO3306.

Western Blot Analysis—Proteins from whole-cell lysates were re-
solved under reducing conditions on 4–12% Bis-Tris polyacrylamide

gels (Life Technologies) using standard methods. Resolved proteins

were transferred to 0.45 μm Immobilon-FL PVDF membranes (Milli-

pore) and incubated with the indicated antibodies overnight at 4 °C.

Protein bands were detected by the appropriate IRDye (680/800)
fluorescently labeled secondary antibody then imaged using an Od-
yssy CLx (LI-COR). Blots were checked for equal loading by reprob-
ing with anti-β-actin.

SilAC Labeling and Mass Spectrometry—HeLa cells were SILAC-
labeled by culturing in DMEM in which the natural “light” Lysine and

Arginine were replaced by “heavy” isotope-labeled amino acids

13C615N4-L-Arginine (Arg 10) and 13C615N2-L-Lysine (Lys 8) (Silan-
tes GmbH, Goliester, Germany) which was supplemented with 10% dialyzed FBS and 4 mM glutamine. Cells were cultured for approxi-
mately six doublings to reach complete labeling of (25)). Yet despite many of these recent advancements, we still

match between runs option. Peptides were assigned incorporating

processed with version 1.2.7.4 of the MaxQuant software package

aLTQ of up to 20 most abundant precursor ions. Mass spectra were

alyzed on an LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific). A

precursor MS scan (350 - 1650 m/z) was acquired in the Orbitrap at

acetonitrile containing 0.5% acetic acid. Phosphopeptides were an-

alyzed on an Easy nLC-II (ThermoFisher Scientific) and eluted with a linear gradient of 0 - 30%

acetoni trile containing 0.5% acetic acid. Phosphopeptides were an-

alyzed on an LTO-Orbitrap Velos Pro (Thermo Fisher Scientific). A

precursor MS scan (350 - 1650 m/z) was acquired in the Orbitrap at

resolution of 60,000 followed by data-dependent CID MS/MS in the

LTQ of up to 20 most abundant precursor ions. Mass spectra were

processed with version 1.2.7.4 of the MaxQuant software package

(http://www.maxquant.org) using default settings with the inclusion of

match between runs option. Peptides were assigned incorporating

modified arginine-10 and lysine-6, with a maximum of two missed

cleavages, using the fixed modification carboxyamidomethyl, and

modified arginine-10 and lysine-6, with a maximum of two missed

cleavages, using the fixed modification carboxyamidomethyl, and

variable methionine oxidation and STY phosphorylation. Database

searching was performed using the Andromeda search engine inte-
grated into the MaxQuant environment (28) against the complete

human proteome containing 88,820 sequence entries (UniProt re-

lease-2013_06, ftp://ftp.uniprot.org). Precursor mass tolerance was

set at 20 ppm for initial search, fragmentation peptide to 0.6 Da. To

ensure high quality protein identifications, MS spectra were also

searched against a reverse database of a similar size with the false
discovery rate limited to < 1%. Known contaminants identified by

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MaxQuant were filtered out of the initial data set. Phosphosite identifications were filtered for greater than 0.75 localization probability and a minimum MaxQuant score of 30 and a maximum posterior error probability of 1% to ensure that only highly confident protein identifications were reported. A fold change cutoff of $2 \leq \log_2$ ratio $> 2$ or $\leq -2$ was used to identify increased and dephosphorylated phosphopeptides, respectively. A moderated $t$ test was used to identify phosphopeptides that are significantly up or down-regulated using Linear Models for Microarray and RNA-Seq Data (LIMMA) package in R (29). LIMMA allows for global variance shrinkage using an empirical Bayes model. Identified sites were then corrected for multiple hypothesis testing using Benjamini and Hochberg method (controlling for 5% false discovery rate). Phosphopeptides were considered to be stable if they were nonsignificant proteins (adj. $P$ value $> 0.05$), and had log$_2$ ratios between $-0.25$ and $+0.25$ with a standard deviation $<0.5$. All raw mass spectrometry data files and MaxQuant output files have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (30) with the identifier PXD001559. Annotated spectra can be viewed using the free MS-viewer (31) (http://prospector2.ucsf.edu) with the search key gsmtp1s5q7.supplemental Table S1 contains a summary of all the phosphosites identified, along with moderated $t$-statistics, $p$ values and adjusted $p$ values for all phosphosites. In addition, each phosphosite is annotated for all potential upstream kinases based on motif, PhosphoSitePlus, and KinomeXplorer prediction methods.

Data Analysis—Data analysis was performed using Microsoft Excel and the bioinformatics platform Perseus (Max Planck Institute of Biochemistry, Munich), and enrichment analysis was performed using Venny (bioinfogp.cnb.csic.es/tools/venny/). Clustering and protein interactions were performed using STRING 9.1 (http://string-db.org/) (32), whereas Gene Ontology (GO) enrichment was performed with Enrichr (http://amp.pharm.mssm.edu/Enrichr/) (33). Motif enrichment analysis of phosphopeptides was performed with MMFP (34), IceLogo (35) and WebLogo 3 (36). Novel motifs were scanned for potential upstream kinases using the Phospho Motif Finder software incorporated into the HPRD database (http://www.hprd.org/PhosphoMotif_finder), and were created using KinomeXplorer (http://kinomeexplorer.info) (37). Heat maps were created using Matrix2png (38). Geneous version R.7.1 was used for sequence alignments of Myc and PRC1 (39). Statistical analysis was performed using GraphPad Prism 6 software using an unpaired nonparametric Kolmogorov-Smirnov $t$ test to determine population distribution differences.

Short Peptide Motif Modeling—The short peptides H-Thr-Pro-Pro-OH and H-Thr-Pro-Gly-OH were modeled in ChemBio3D Ultra 14.0.0.117 (CambridgeSoft Dist. By PerkinElmer, Waltham, MA). Each peptide underwent molecular dynamics simulation using MM2 force field calculations (40) in 2.0 fs steps over 10,000 intervals for a temperature of 300 K. The structures were then MM2 energy minimized using a RMS (root-mean square derivative) gradient of 0.01 producing each energy term.

Immunofluorescence Staining—Cells were grown on Histogrip (Life Technologies) coated glass coverslips and fixed using ice-cold 100% methanol (Lamin A/C) or with 3.7% formaldehyde diluted in PBS with 0.5% Triton X-100 for 10 min (Aurora B, Kif23). All cells were washed and then blocked (3% BSA, 0.1% TWEEN 20 in PBS) for 30 min. Cells were incubated with primary antibodies for 2 h at room temperature in blocking solution. DNA was stained with DAPI. For Lamin A/C staining, a Leica DM6000 SP8 confocal with a 63 X lens was used. All other images were captured using Leica DM5500 microscope coupled with a CoolSpin HQ2 camera, using a Leica 100X or 40X APO 1.4 lens, powered by Leica LAS AF v3 software. In all cases 0.3 μm Z-sections were taken, deconvolved, and displayed as 2D maximum projections using Adobe Photoshop CC 2014 software.

Live Cell Imaging—Hela cells co-expressing pH2B_mCherry, IRES_neo3 (Addgene plasmid # 21044) and pmEGFP_a_tubulin_IRES_puro2b (Addgene plasmid # 21042), gifts from Daniel Gerlich (41), were filmed with using a Leica TCS SP8 MP confocal microscope coupled with Leica HyD nondescanned detectors and a HC PL APO 63x/1.20 W motCORR CS2 lens powered by LAS AF v3 software. Images, both Tubulin-GFP and H2B-CherryFP fluorescence, were taken every 2.5 min. The resulting images were processed, analyzed and false colored using Image J (v1.49) and Adobe Photoshop CC 2014 software.

RESULTS

Establishment of a Synchronized, Phosphatase Dependent Mitotic Exit Model—In order to study the phosphorylation changes that occur during initial early stages of mitotic exit (EME), we created highly enriched populations of mitotic cells using a two-step synchronization process, which were then treated with the proteasome inhibitor MG132, to minimize the loss of phosphorylation from protein degradation. Normal mitotic exit is triggered by the loss of CDK1 activity (42), therefore, we took advantage of the specific CDK1 inhibitor RO3306 (43) to induce a highly synchronized mitotic exit (Fig. 1A). A similar method has been used by several other groups to analyze various aspects of mitotic exit (44–46). This model allowed us to perform highly reproducible biochemical analysis of EME, with cells beginning to rapidly exit mitosis within 5 min of CDK1 inhibition. At 10 min, clear invagination of the plasma membrane could be observed at the center of the cell, along with elongation of the mitotic spindle (Fig. 1B, supplemental Video S1). Chromatin remained clearly condensed until 30–45 min, at which point the mitotic spindle had largely regressed, and a clear cytokinesis furrow had formed. Furthermore, dephosphorylation of phospho-Histone H3 (Ser10) reached a plateau between 45–60 min, which corresponded with the decondensation of chromatin by time-lapse microscopy (Fig. 1C). Inhibition of the proteasome by MG132 was confirmed by the presence of stable cyclin B1 and securin throughout the time course (Fig. 1C), with stable securin explaining the failure to separate chromosomes (47). Therefore, our EME model followed the general pattern of a normal mitotic exit, with cells reorganizing the mitotic spindle and forming the midspindle/cytokinesis furrow prior to decondensation of the DNA.

This exit was driven by the activity of phosphatases, as it could be inhibited by cotreatment with the phosphatase inhibitor Okadaic Acid (Fig. 1D). Given these results, the time point of 15 min was chosen for subsequent mass spectrometry (MS) analysis, as cells showed clear evidence of EME events including changes in the mitotic spindle, formation of a cytokinesis furrow, yet still contained significant levels of pH3 and condensed chromosomes.

Phosphoproteomic Analysis of the EME—To accurately quantify the global phosphorylation changes during EME, we metabolically labeled cells using stable isotope labeling by amino acids in cell culture (SILAC). SILAC labeled cells were...
treated either with (MG+RO) or without (MG) the CDK1 inhibitor RO3306 for 15 min and then harvested. Following SILAC cell harvesting, protein contents of each isotopically labeled heavy and light cell population were equally mixed 1:1, and precipitated proteins were trypsinized. The resulting peptides were desalted using C18 columns, fractionated by strong-cation exchange, and then phosphopeptides were enriched using titanium dioxide (TiO2) chromatography. Enriched phosphopeptides were analyzed by tandem MS (LC-MS/MS) (Fig. 2A).

A combined total of 40,982 phosphosites were identified across three biological replicates. The majority of phosphopeptides contained two or less phosphorylated residues (Fig. 2B). Therefore, only high quality phosphopeptides with a PEP score <0.01 and phosphorylation localization score >0.75 were included. The data set contained 141 contaminants and reverse database hits (FDR of 0.84%), which reduced the data set to 16,716 unique phosphorylation sites (supplemental Table S1). Approximately 84% of phosphopeptides were identified on Serine (S) residues, with ~15% on Threonine (T) and <1% on Tyrosine (Y) residues (Fig. 2B). Of the 16,716 phosphorylation sites identified, 12,711 are currently annotated in the PhosphoSitePlus database (PSP, phosphosite.org) (48). Therefore, this study has identified an additional
4005 novel mitotic phosphorylation sites that have not previously been annotated in the PSP database (Fig. 2C and supplemental Table S1).

Although there have been several other large-scale mitotic phosphoproteomic screens (3, 49), to date only one other study has specifically analyzed the phosphorylation changes...
During mitotic exit (50). However, the analysis performed by Malik et al. was limited to proteins bound to the mitotic spindle (50). In contrast, in the current study we analyzed whole cell phosphorylation, resulting in a six- to eightfold increase in protein and phosphorylation information respectively (Supplemental Fig. S1A). Consequently, this study provides both a significant increase in resolution and depth, and a unique focus on the global phosphorylation changes that occur specifically during EME.

Reproducibility of Experimental System—To ensure accuracy of the experimental system three independent biological replicates, including a SILAC label switch (Run B), were performed on SILAC labeled cells. Normalized log$_2$ transformed heavy to light (H/L) ratios were present for more than 98% of the 16,716 phosphorylation sites identified, and each experiment showed a similar spread of ratios (Supplemental Fig. S1B). An investigation of the variation between the 3 biological replicates found the correlation between samples was 67% on average, and the missing value counts were acceptable (Fig. 1D and Supplemental Fig. S1C). The small difference seen between experiments is likely because of contamination with small, variable amounts of interphase cells during the mitotic shake off enrichment procedure. In summary, the final data set contained quantitative phosphorylation data in at least two experiments for 12,315 sites on 2355 unique proteins (Fig. 2E).

Prediction of Upstream Kinase—We began the analysis of this large data set by separating all of the phosphopeptides based on the predicted upstream kinase responsible for each phosphorylation event. To do this we first utilized all of the known kinase-substrate relationships annotated in the PSP database, however; only 654 of the 12,315 sites identified contained a known upstream kinase. Therefore, to include as much of the data as possible we segregated all sites into either Proline (S/T-P) or non-Proline (Non-P) directed kinase families. These were then further separated into the major mammalian kinases (CDK1, GSK3, MAPK, PLK1, Aurora, CKII, PKA, and ATM) using the minimal reported consensus phosphorylation motif for each kinase (Supplemental Fig. S2).

We were able to assign ~53% of all the identified phosphopeptides as potential substrates for one of these kinases (Fig. 3A and Supplemental Table S1). To identify potential remaining kinases in the unassigned pools, motif analysis was performed using MMFPPh software on all of the phosphopeptides from our data set that did not fit the minimal consensus motif of CDK1, MAPK, GSK3, PLK1, CK2, Aurora, or PKA. MMFPPh identifies over-represented amino acid/position pairs in order to identify all statistically significant, sufficiently frequent motifs present in a large phosphoproteomic data set (34). Proline directed sites contained an additional 16 (SP) and 12 (TP) motifs (Supplemental Fig. S3A); however, all of these additional motifs were likely phosphorylated by the one of the major Proline direct kinases GSK3, MAPK or CDK1 (HPRD) (S1). In contrast, only four of the 15 Serine, and one of the five Threonine based Non-P motifs were associated with a potential kinase (Supplemental Fig. S3B). Searching the PhosphoSitePlus database helped identify several validated kinase and substrates relationships for the majority of these novel motifs. Kinases from all families were random distributed across the motifs suggesting that hierarchical clustering of motifs does not provide any additional enrichment (Supplemental Fig. S3B). Interestingly, several of the motifs (e.g. S-x-x-K/R and S/T-x-P) were associated with CDK1, suggesting that these sites are residual CDK1 sites. In support, a recent paper identified these atypical motifs can be phosphorylated by CDK1 (52). Finally, to further validate our predictions we utilized the KinomeXplorer platform to predict potential cellular kinase-substrate relationships based on motif and cellular context (37). Using this method we were able to identify 127 potential upstream kinases that belong to 50 different kinase families for ~60% of the phosphosites in our data set. The majority of sites were assigned to the MAPK, CDK, PKA, and CK1/2 family of kinases, yet surprisingly only 32 Aurora sites and no PLK1 sites were identified despite their well-known roles in mitosis (Fig. 3B and Supplemental Table S1). Comparison between the different methods showed varying degrees of agreement, with KinomeXplorer enriching for similar motifs to the minimal reported kinase motifs (Supplemental Fig. S2A and S2B). In addition, both methods were able to identify validated kinase-substrate relationships annotated in the PSP database (Fig. 3C). Taken together, these results suggest that both basic motif and more advanced prediction models are able to successfully enrich for validated substrates of the upstream kinase, albeit with varying levels of success.

Impact of Upstream Kinase on Dephosphorylation—The above data demonstrate that multiple upstream kinases are responsible for phosphorylating >12,000 sites during mitosis. To determine if phosphorylation status of specific phosphosites correlated with any specific upstream kinase, we first limited the data set to phosphosites that showed consistency (<2 standard deviations from the mean) between replicates. The final data of 11,954 phosphorylation sites on 2823 proteins was then analyzed using the kinase predictions (both motif and KinomeXplorer based) made above. A statistically significant shift in the cumulative distribution toward dephosphorylation was observed for S/T-P compared with Non-P directed sites, indicating that S/T-P sites were preferentially dephosphorylated during EME (Fig. 4A). Separation of phosphosites into each upstream kinase motif also revealed several significant differences. Surprisingly, potential substrates of Aurora kinase (from both prediction methods) along with all unassigned peptides showed a significant shift toward dephosphorylation compared with total Non-P directed sites during EME. Potential PKA and PKC sites were also shifted toward dephosphorylation, but only the KinomeXplorer based prediction method was statistically significant. Finally, TTK (Mps1) sites (KinomeXplorer) were also significantly shifted.
toward dephosphorylation. In contrast, phosphorylation sites targeted by both PLK1 and CK1 and CK2 were significantly shifted to the right and therefore more stable compared with global Non-P sites (Fig. 4A). PLK1 has a well-established role during mitotic exit, and its activity is required for correct cytokinesis furrow formation (53). Therefore, it is possible that high PLK1 activity may counterbalance phosphatase activity resulting in stabilization of phosphorylation status. In support, phosphorylation of PLK1 on T210, a marker of PLK1 activity, was stable by both MS analysis (mean log2 ratio: 0.653) and Western blot analysis, indicating that the majority of PLK1 was still active during EME (Fig. 4B). Notably, potential CK2 phosphosites showed an even greater distribution shift than PLK1, suggesting that CK2 activity is also high during mitotic exit.

For Proline directed motifs both GSK3 and all unassigned sites showed no significant difference to total S/T-P sites; however, both CDK1 and to a lesser extent MAPK sites were significantly more stable during EME (Fig. 4A). Phosphorylation of the inhibitory Y15 residue on CDK1 was weakly detected at 15 min and noticeably increased after 45 min by Western blot, which correlated with the slight increase in log2 score observed by MS (Fig. 4B). Therefore, it is possible that the stability of the majority of CDK1 phosphosites is because of continued activity of CDK1 kinase. However, the dose of RO3306 used in our system is sufficient to completely block mitotic entry and specifically inhibit 90% of CDK1 kinase activity (18, 43). Given that the addition of RO3306 rapidly triggered mitotic exit, it is likely that most of CDK1 is inhibited in our samples. In summary, these data indicate that during EME the majority of phosphosites from all potential upstream kinases remain stable. The activity of Non-P directed kinases do appear to play a more significant role in dictating the phosphorylation status during EME. In particular, continued PLK1 and potentially CK2 activity, likely explains the stability of these sites. In contrast, the phosphorylation status of S/T-P
Fig. 4. Only a small percentage of phosphosites are dephosphorylated during EME. A, Cumulative frequency distribution graphs for all phosphopeptides separated according to potential upstream phosphorylating kinase. Significant differences in the population distributions are shown. p values * = <0.01, ** = <0.001, *** = <0.0001, **** = <0.00001, NS = not significant. B, Log2 ratios from each replicate and their mean are shown for phosphorylation at PLK1 (T210), and CDK1 (Y15). HeLa cells were treated as per Fig. 1A, then harvested at the indicated times postrelease, lysed, and analyzed by Western blot with the indicated antibodies. Densitometry of three independent experiments was
Motifs Regulate Dephosphorylation During Early Mitotic Exit

directed sites, especially CDK1 sites, appears to be independent of the upstream kinase indicating that additional factors determine the specificity of dephosphorylation during EME.

Dephosphorylation of Specific Substrates Drives EME—The above results suggest that changes in the activity of the upstream kinase, especially S/T-P directed sites, are not sufficient to fully account for the specificity of dephosphorylation during EME. To explore this further, we identified phosphosites that were significantly altered during EME. A fold change cut off of a ≥four-fold decrease (log2 ratio < -2) was used to classify dephosphorylated phosphopeptides, whereas phosphopeptides were considered to be stable if they had consistent (s.d. <0.5) log2 ratios between −0.25 and +0.25, and increased phosphorylation was limited to phosphopeptides with a log2 ratio ≤ +2 (Fig. 4C). A one sample moderated t test (with Benjamini/Hochberg correction) was then performed to identify high confidence (adj. p value <0.05) differentially expressed phosphosites. Approximately 9.5% of all phosphosites (>4 fold and 5% FDR) were found to be significantly dephosphorylated during EME (Fig. 4D and supplemental Table S1).

Analysis of upstream kinases for the significantly dephosphorylated sites, found that there was approximately equal distribution of potential kinases for both motif and KinomeXplorer based prediction methods, with the exception of CK1/2, which was notably reduced in both (Fig. 5A). This data again supports the idea that dephosphorylation during EME is not solely dictated by the activity of the upstream kinase. Therefore, we next determined if these dephosphorylated sites were enriched for any specific functional groups of proteins independent of the upstream kinase. This was done by segregating dephosphorylated phosphosites into S/T-P and Non-P directed sites and then comparing any commonly dephosphorylated proteins. This produced a list of 156 proteins that are commonly dephosphorylated independent of the upstream kinase (Fig. 5B). STRING analysis (32) of these proteins resulted in strong clustering into several interconnected groups known to regulate EME events (Fig. 5B). These included proteins critical for the regulation of the spindle assembly checkpoint (SAC), nuclear pore organization, and microtubule dynamics (Fig. 5B and 5C). We performed a similar enrichment on common proteins that remained stable during EME and found that they were strongly enriched for proteins involved in regulating RNA processing, nucleic acid metabolism, gene expression, and DNA replication (Fig. 5C and supplemental Fig. S4). Furthermore, there was a clear separation between dephosphorylated and stable proteins according to specific cellular compartments, with proteins localized at the central spindle and nuclear pore basket all significantly enriched in the dephosphorylated pool. In comparison, stable phosphorylation sites were highly enriched for ribonucleoproteins, nuclear specks and the nucleolus (Fig. 5D). Taken together, these results suggest proteins that regulate and are localized near the mitotic spindle, kinetochores and nuclear envelope are preferentially dephosphorylated during EME.

Dephosphorylation of EME Proteins Correlates with Specific Mitotic Exit Events—The above results suggest that specific proteins are preferentially dephosphorylated during EME; however, these data were based on a single phosphorylation site. The majority of proteins are phosphorylated on multiple sites, and there were 50 proteins that appeared in both dephosphorylated and stable groups (supplemental Figs. S4 and S5A). Therefore, we examined all of the phosphorylation sites across a selection of proteins that have known critical roles in regulating the SAC, nuclear envelope and central spindle formation. Although the majority of proteins contained at least one site that was dephosphorylated, there was a large amount of variability in the phosphorylation states of neighboring sites within each individual protein. Therefore, we ordered proteins by their median log2 phosphorylation ratio to identify and rank proteins that were strongly dephosphorylated (Fig. 6A). Using this method, the top ranked dephosphorylated protein was the nuclear pore component NUP107. Interestingly, NUP107 is one of the first proteins recruited to chromatin and is required for the initiation of nuclear pore complex reassembly during mitotic exit (54). Therefore, we analyzed the other major components of nuclear pore reassembly (POM121C, NUP53, NUP93, and NUP188) and found that the amount of dephosphorylation at 15 min tightly corresponds with the specific order of recruitment of these proteins to the reforming nuclear pore during mitotic exit (Fig. 6B). In support, partial reformation of the nuclear envelope was observed in our model during EME (Fig. 6C).

We further validated this ordering of proteins, based on the level of dephosphorylation, using band shift as a marker of phosphorylation by Western blot. Both TPX2 and CDC27 were rapidly dephosphorylated within 15 min, with significant loss of the slower migrating phosphorylated band, whereas INCENP showed a gradual stepwise decrease in band shift, matching the heterogeneous dephosphorylation observed at 15 min by MS. BUBR1 remained in its slowly migrating phosphorylated state, with partial dephosphorylation only observed after ~90 min (supplemental Fig. S5B). The stability of

performed, normalized to actin and expressed as a percentage of 0 min time point for PLK1 (T210) and against the 120 min time point for CDK1 (Y15). All error bars are S.E. C, Phosphopeptides with log2 fold change scores of <-2 were considered to be significantly dephosphorylated (blue). Those with log2 scores between −0.25 and +0.25 were classified as stable (yellow), and those >+2 were classified as having increased phosphorylation (red). Mean (μ), standard deviation (σ) and z-score cutoffs are shown. D, Volcano plot showing phosphosites that are significantly up or down-regulated, based on results from a moderated t test with correction for multiple hypothesis testing (Benjamini & Hochberg method), controlling for 5% FDR.
BUBR1 phosphorylation was surprising as phosphorylated BUBR1 has been reported to inhibit the APC, thereby blocking anaphase onset (55). However, phosphorylation of BUBR1 at S670 was recently shown to be required for targeting the phosphatase PP2A-B56 to kinetochores (56), where PP2A is required for opposing MPS1 (TTK) activity and silencing of the SAC (57). In support, we found that BUBR1 remained localized at the kinetochore during EME (supplemental Fig. S5C). Interestingly, we found that MPS1 was significantly dephosphorylated on S281 (adj. p value 0.01, Fig. 6A), which would allow the binding of CDC27 and subsequent APC mediated degradation of MPS1 during later stages of mitotic exit (58). Notably, our MS results show that CDC20 is significantly dephosphorylated (adj. p value 0.019) on S51 (Fig. 6A and supplemental Table S1). This was confirmed using a phosphospecific antibody against the S51 site in CDC20, which showed rapid dephosphorylation within 5 min of triggering mitotic exit with RO3306 (Fig. 6D). This dephosphorylation of CDC20 likely ensures prompt and efficient activation of APC and subsequent degradation of MPS1 (58). Subsequent loss of MPS1 activity (through dephosphorylation and degradation) then shifts the balance toward dephosphorylation of downstream SAC proteins at the kinetochore. In support, significant dephosphorylation of several sites on BUB1 and the PP2A-B56 substrate CASC5 (knl1) (57), were observed in our MS results (Fig. 6A and supplemental Table S1). Furthermore, this rapid loss of MPS1 activity likely explains the global shift toward dephosphorylation of potential MPS1 (TTK) substrates identified by KinomeXplorer (Fig. 4A). Thus, efficient silencing of the SAC during EME appears to be dependent on the preferential dephosphorylation of MPS1 and CDC20. Finally, to examine the formation of the central-spindle, immunofluorescence analysis was performed on KIF23 (Mklp1) and Aurora B. Both proteins underwent rapid relocalization to the central-spindle indicating that, as implicated by our MS results, KIF23 is rapidly dephosphorylated during EME (supplemental Fig. S5D). Taken together, these results provide strong evidence that dephosphorylation plays a critical role in determining the order of events during mitotic exit by precisely dephosphorylating specific sites on a subset of substrates involved in regulating EME. However, strong dephosphorylation of a specific site within a protein does not guarantee that the remaining sites within that protein will also be dephosphorylated.

Specific Motifs Correlate with Intra-Protein Phosphosite Status—One possible explanation for the heterogeneity in intraprotein dephosphorylation is that some sites may be more easily accessible compared with others and are therefore more readily dephosphorylated. We examined this by mapping the log2 ratios on to the 3D crystal structure of PKMYT1, a protein involved in regulating the auto-amplification loop of CDK1, which we identified as containing a high level of heterogeneously dephosphorylated sites (supplemental Fig. S6A). However, there was no obvious correlation with the level of dephosphorylation and the 3D location of the phosphosite, indicating that accessibility does not necessarily correlate with the dephosphorylation of specific sites within a protein.

Another possibility for this heterogeneity is that certain local amino acid sequences at and around the phosphorylation sites may be favored by phosphatases and are therefore preferentially dephosphorylated. To test this we performed comparative motif analysis between dephosphorylated and stable motifs for both S/T-P and Non-P sequences. Several distinct enrichments were correlated with dephosphorylation or with stable phosphosites (Fig. 7A). For Non-P sites, the presence of acidic amino acids (D/E) upstream (right), and to a lesser extent downstream (left) of the phosphorylation site strongly correlated with stable phosphosites, suggesting that these were impeding dephosphorylation (supplemental Fig. S6B). However, it should be noted that PLK1 and CK2 both favor acidic residues surrounding the phosphorylation site (supplemental Fig. S2), so this could simply reflect the continued activity of these kinases. In contrast, basic amino acids upstream (especially at +3 position) were highly enriched in dephosphorylated motifs, suggesting that these residues may promote dephosphorylation (supplemental Fig. S6B). For S/T-P sites, T sites were strongly and significantly dephosphorylated compared with total S/T-P site, whereas S sites were significantly more stable (Fig. 7A). This prominent preference for T over S was dependent on a P in the +1 position, as the preference for T was weakly, but not significantly seen in Non-P sites (supplemental Fig. S6B). Western blot analysis of synchronized mitotic exit samples using antibodies that specifically recognize SP or TP phosphorylated sites confirmed this observation, with global TP sites rapidly lost within 5 min of RO addition, whereas SP sites were slowly removed over the 120 min time-course (Fig. 7B).

Dephosphorylation of TP Sites is Regulated by Local Amino Acids—The above results suggest that TP sites are preferentially dephosphorylated compared with SP sites. However, only ~26% of all TP phosphosites were significantly dephospho-
Motifs Regulate Dephosphorylation During Early Mitotic Exit

A

| Proteins | Phosphorylation Sites |
|----------|-----------------------|
| NUP107  | S11, T45, S58, T64, S69, T82, S86 |
| CDC20   | S41, S51, T59 |
| RACGAP1 | S203, S206, T277, T579, T580, S591, T601, T606, S628 |
| CDC5    | S21, S33, S75, S79, S107, T111, T113, T115, S154, T159, S181 |
| TPX2    | T59, S125, T369, S486, S738 |
| KIF23   | S682, S684, S686, T738, S744, S902, S906, S911, S912 |
| PRC1    | T481, S571 |
| TTK     | S281, S321, S393, S436, S621 |
| ECT2    | S15, T359, S367, T373, S376, S391, S397, S716, T846, S861 |
| CDCA8   | S108, S110, S180, T185, T189, T199, S219, S260 |
| CDC27   | S219, S220, S334, S336, S339, S369, S384 |
| CASC5   | S32, S1076, S1088, S1773 |
| CENPF   | S1748, S1750, S1770, S1822, S1988, S2993, S2996, S3007, S3013, T3042, T3045 |
| S3048   | T3090, T3093, S3094, S3119, S3122, S3150, S3154, T3163, S3175, S3179 |
| CENPA   | S17, S19, T23, S32, S36, S37 |
| NDC80   | S82, S69, S76 |
| INCENP  | S72, S93, S94, S119, S148, S197, T199, S214, T219, S234, S235, T239 |
| POM121C | S269, S291, T292, S296, S300, S306, S311, S312, S314, S420, S421, T424 |
| BUB1    | S446, S476, S481, T507, T509, S510, S798, T907, S931, T932, S983, S984 |
| LMNA    | S2, T182, S165, T180, S322, S328, S348, S412, S416, S674 |
| ANAPC1  | S307, S445, T452, S593, S596, S608, T609, S655, S661 |
| LMNB2   | S12, S17, S18, T19, S22, S390, S392, T394, S395, S403, S404, S405 |
| FZR1    | S407, S414, S458, S612, S613, S616, S619, S628, S632, S636, S652 |
| NUP53   | T291, S298, S299, S300, S313, S317, S341, S355, S362 |
| CENPE   | S364, S372, S373, S377, T530, T537, S586, S688 |
| MAD2L1  | S34, S37, S391, S393, S419, S420, T421, T422, S424, S426 |
| MAD2L1BP| S66, S73, S99, S100, T106, T109, S121, S138, S259, T273, T270, T280, T308 |
| NUP53   | S2605, S2608, S2639, S2651, S2654 |
| DS11    | S28, S30, S57, S58, S77, S81, S86, S109, S331 |
| S3A3    | S119, S155, T190, S267, S318 |
| MAD21BP | S102 |
| BUB1B   | S343, S670 |
| NUP188  | S170, S177 |
| NUP93   | S52, S72, S75, S757 |

B

Nuclear Pore Basket Assembly

C

MG132  +RO3306

Lamin a/c

0 min  15 min  30 min

D

Synchronised Mitotic exit

CDC20-S51

CDC20

Actin

Time Post RO3306 (min)
phorylated during EME, indicating that additional factors beyond a TP were affecting dephosphorylation. Therefore, we analyzed TP sites in greater detail to determine if any additional amino acids surrounding the TP site correlated with dephosphorylation. Comparative motif alignments of dephosphorylated and stable TP sites showed that the $\text{H}_{11001}2$ position played the strongest role in determining phosphorylation status (Fig. 8A). Specifically, small nonpolar amino acids (G, A, L, or V) were significantly enriched in dephosphorylated peptides (TPG motif). In contrast, a Proline at $\text{H}_{11001}2$ (TPP motif) was significantly enriched in stable phosphopeptides, indicating that this residue inhibited dephosphorylation (Fig. 8A and 8B). Notably, similar enrichments were also found in SP sites (supplemental Fig. S6C). Comparing the TPG motif (dephos-

![Image](image-url)

**Fig. 7.** **Local amino-acids predict phosphorylation status.** A, Dephosphorylated ($\log_2 < -2$) and stable ($0.25$ to $0.25$) S/T-P phosphopeptides were compared using IceLogo motif analysis software, to identify differentially enriched amino acids. Increasing letter size, and distance away from center, corresponds to stronger enrichment of amino acids. B, Validation of preferential dephosphorylation of TP compared with SP sites. HeLa cells were synchronized as per Fig. 1A, then harvested at the indicated times post-release, lysed, and analyzed by Western blot with antibodies that recognize proline directed phosphorylations at serine ($\text{pSer-P}$) and threonine ($\text{pThr-P}$) sites. Densitometry of 3 independent experiments was performed, normalized to actin and expressed as a percentage of 0 min time point. $p$ value $*** = <0.0001$. All error bars are S.E.

**Fig. 6.** **Intra-protein variations in neighboring phosphorylation sites.** A, Intraprotein phosphorylation changes of common proteins involved in regulation of EME. Proteins were ranked by the average fold change across all sites. Bold text indicates phosphorylation sites with a known role in regulating the function of the protein. B, Proteins from A involved in nuclear envelope reformation were ordered according to their mean log$_2$ ratio, which corresponded to the order of recruitment during nuclear envelope reassembly. C, HeLa cells were synchronized as per 1A treated with MG132 and then 15 min later RO3306, fixed at the indicated time-points, and stained with lamin a/c antibody. Partial reassembly of the nuclear envelope was observed at 15 min. All scale bars = 5 $\mu$m. D) HeLa cells were synchronized as per Fig. 1A, then harvested at the indicated times post-release, lysed, and analyzed by Western blot with the indicated antibodies. Shown are representative blots from three independent experiments.
Motifs Regulate Dephosphorylation During Early Mitotic Exit

A

B

C

D

| Gene     | Site | Log2 | Kinase       | Phosphosite Function                                                                 |
|----------|------|------|--------------|---------------------------------------------------------------------------------------|
| CDC25B   | T355 | nd   | p90RSK      | Activation during G2/M                                                                   |
| EIF4EBP1 | T70  | -0.2640 | mTOR, Cdk1 | Inhibits activity                                                                       |
| FOXM1    | T627 | 0.0688 | Cdk4        | Protein stabilization                                                                   |
| JUNB     | T255 | 0.2355 | GSK3B       | FBXW7 mediated degradation                                                              |
| MCL1     | T163 | nd   | ERK1/2, JNK | Protein stabilization, Pin1 association                                                 |
| MED1     | T1032| nd   | ERK1/2      | Activity induced, protein stabilization                                                |
| MYC      | T58  | 0.0056 | GSK3        | Pin1 and degradation                                                                    |
| PKMYT1   | T412 | nd   | ?           | Induces binding with Pin1                                                               |
| WDR77    | T5   | 0.1605 | Cdk4        | Carcinogenesis                                                                         |
| WEE1     | T173 | 0.2711 | ?           | ? Degradation                                                                          |
| ANLN     | T364 | -3.4772 | ?           | ?                                                                                     |
| CDC20    | T59  | -2.7717 | ?           | ?                                                                                     |
| CDC5L    | T438 | -3.2910 | ? Cdk      | RNA splicing, altered enzymatic activity, induced                                       |
| CDC7     | T376 | nd   | Cdk1        | Phosphorylation induces interaction with Pik1                                          |
| CDC46    | T159 | -2.2860 | Cdk1        | ?                                                                                     |
| CDC68    | T185 | -2.4589 | ?           | ?                                                                                     |
| MASTL    | T207 | -2.4129 | Cdk1?       | Activation of Kinase                                                                    |
| MEK1     | T292 | nd   | Cdk1, Erk1/2| Inhibits activity                                                                       |
| NUP133   | T10  | -2.0605 | ?           | ? Phosphorylated in mitosis                                                              |
| PRC1     | T481 | -2.1865 | Cdk1        | Prevents mid-zone localisation                                                         |

E

Synchronised Mitotic exit

% Change

Time Post RO3306 (min)
Motifs Regulate Dephosphorylation During Early Mitotic Exit

...phorylated) to the stable TPP motif using MM2 molecular dynamics simulation and energy minimization illustrated that TPP has far greater energy associated with torsional strain (Fig. 8C). This indicates that the TPP is far more conformationally restricted than the TPG motif. The lower energy barrier to rotation could allow the TPP motif to switch to a geometry that is more favorable for dephosphorylation by a phosphatase.

Interestingly, phosphorylation on TPG and TPP motifs are currently annotated on 831 and 1109 proteins in PhosphoSitePlus, respectively, and our analysis identified an additional 24 (TPG) and 36 (TPP) sites that have not previously been reported (Fig. 8D). These two motifs are evolutionarily conserved in numerous proteins such as FOXM1, JUNB, MYC, CDCA5 (Sororin) and PRC1, where they have known critical roles in regulating protein function (Fig. 8D, supplemental Figs. S7A and S7B). To validate these results we analyzed PRC1, a key regulator of cytokinesis and mid-spindle formation, and the transcription factor and oncogene MYC, as examples of a TPG and TPP motifs respectively. Phosphorylation at T481 prevents PRC1 from localizing to the central spindle; therefore, rapid dephosphorylation at this site would be beneficial to ensure timely relocation of PRC1 during EME. Western blot analysis confirmed that this site is rapidly dephosphorylated during mitotic exit (Fig. 8E), which results in PRC1 relocating to the mid-spindle (supplemental Fig. S7C).

In contrast, phosphorylation of the TPP motif in MYC was highly stable during mitotic exit with significant levels of phosphorylation on T58 still present 120 min after triggering of mitotic exit (Fig. 8E). Interestingly, the residues from T58 to S60 are a hot spot for missense mutation in cancer (supplemental Fig. S7D), with the annotated mutation of P60A observed in cancer cell lines creating at TPA motif, which our modeling predicts to be rapidly dephosphorylated (Fig. 8A). In summary, these results suggest that phosphorylation on the physically restrictive TPP motif is highly stable, whereas the more flexible TPG motif is more readily dephosphorylated.

**DISCUSSION**

This study shows that during EME in human cells, only a small fraction of protein phosphorylation modifications are removed and responsible for the events that occur during anaphase. Not surprisingly, these dephosphorylation events occur on proteins that are involved in regulating nuclear pore assembly, central-spindle formation, and SAC function, which are all critical for these EME events. Consequently, dephosphorylation is critical for ensuring that mitotic exit occurs in the correct order. This raises an important question of how specific dephosphorylation of these critical EME proteins is determined. One possibility is that as cells exit mitosis, kinase activity decreases, shifting the balance toward dephosphorylation. Many kinases have well established preferences for certain substrates, and this preference could determine the rate as which specific sites and proteins are dephosphorylated. However, our results suggest that there are additional factors beyond kinase activity that determine specificity. First, our use of a specific CDK1 inhibitor did not result in a preferential dephosphorylation of CDK1 substrates, suggesting that the activity of CDK1 does not necessarily determine the specific dephosphorylation of EME substrates. This is supported by work in yeast, which showed during early mitotic exit, the specificity of CDK toward specific substrates does not correlate with substrate dephosphorylation. Furthermore, forced persistent CDK activity during exit only prevented the dephosphorylation of late mitotic exit substrates, with EME substrates continuing to be dephosphorylated independently of CDK activity (8). We also found that there was a similar enrichment of EME proteins dephosphorylated across all the kinases we analyzed, reducing the likelihood that kinase activity and/or specificity is driving the order of dephosphorylation during EME.

Therefore, specific dephosphorylation of EME proteins must be driven by the activity of phosphatases. In support, inhibition of phosphatases with okadaic acid prevented mitotic exit and dephosphorylation, indicating that the PP1/2A/4/6 families of phosphatases are responsible for dephosphorylating substrates during EME. This is supported by previous studies which have shown that both PP1 (23, 59, 60) and PP2A (45, 57, 61) are responsible for dephosphorylating critical substrates during EME in mammals. However, this still does not answer the question of how these phosphatases specifically dephosphorylate only a discrete subset of phosphorylation sites during EME. The answer in part is likely because of the nature of these families of phosphatases, which are multimeric protein complexes that can be regulated by a variety of mechanisms. These include the binding of

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**Fig. 8.** Evolutionary conserved TPP and TPG motifs dictate phosphorylation status. A, Similar to 7B, dephosphorylated (dephos.) and stable (stable) sites from TP phosphorylated phosphopeptides were compared using IceLogo software. B, The distribution of all phosphopeptides corresponding to the top dephosphorylated and stable motifs were compared, with significant differences (p values) shown, p values * = <0.01, ** = <0.001, *** = <0.0001, **** = <0.00001, NS = not significant. C, Comparison of stretch, bend, torsion and total energy requirements of the TPP and TPG short peptide motifs using MM2 dynamics simulation. D, Venn diagrams comparing the number of proteins identified with a phosphorylated TPP or TPG motif in our MS data compared with PhosphoSitePlus. A table of 10 notable proteins from PhosphoSitePlus and our data set that contain the TPP and TPG motifs, the upstream phosphorylating kinase, and the function this phosphorylation has on protein function. E, Dephosphorylation of PRC1-T481 TPG motif and stability of MYC-T58 TPP motif. Briefly, HeLa cells were synchronized as per Fig. 1A, then harvested at the indicated times post-release, lysed, and analyzed by Western blot with the indicated antibodies. Densitometry of three independent experiments was performed, normalized to actin and expressed as a percentage of 0 min time point. * t tests were performed to compare pPRC1 (blue) or pMYC (red) to total pThr-P sites (green). p value *** = <0.0001. All error bars are S.E.
regulatory subunits and inhibitory proteins, which can target these phosphatases to specific subcellular localizations (reviewed in (62, 63)). For example, during mitosis PP2A bound with the B56 regulatory subunit (PP2A-B56) is targeted to the kinetochore region (57) and PP2A-B55 to the mitotic spindle (64), where they plays critical roles in silencing the SAC and activating the APC respectively. Likewise, PP1-RepoMan (cdca2) is also targeted to the kinetochore region, where it regulates the microtubule-kinetochore interface during anaphase (65). PP1 in combination with Sds22 is targeted to the cell cortex (66), chromatin via PNUTs (59), and binding with AKAP149 targets to the nuclear envelope (67). Targeting of PP1 and PP2A to these regions corresponds with the proteins that we found to be dephosphorylated during EME, and therefore sub-cellular localization clearly plays a key role in determining the specificity of the phosphatases (Fig. 9). However, it does not explain why some proteins at these locations are not dephosphorylated, or the large variation in dephosphorylation of neighboring sites seen in many individual proteins observed at these subcellular locations.

Our results suggest that local amino acids at and around the phosphorylation site could provide a mechanism for the specificity of the phosphatases. This was most clearly observed with Proline directed sites, which showed a strong preference for dephosphorylation of Throneine compared with Serine residues. Malik et al. also observed a similar preference for the dephosphorylation of TP sites during mitotic exit (50), whereas in vitro dephosphorylation assays using synthetic peptides also found TP sites were heavily favored over SP sites (68). Our results also identified that residues surrounding the phosphorylation site have an impact on dephosphorylation. For Non-P directed motifs, acidic amino acids with negatively charged side chains (D, E) upstream from the phosphorylation site appear to protect and prevent dephosphorylation, whereas basic amino acids (K, R) generally promote dephosphorylation (supplementary Fig. S6B). In support, arginine (R) residues proximal to the (S31/S40) phosphorylation sites in tyrosine hydroxylase have been shown to promote dephosphorylation by PP2A (69). For Proline directed motifs, the amino acid at the +2 position heavily

**Fig. 9. Proposed model of mechanisms controlling specific dephosphorylation during EME.** Proteins that are involved in regulating midspindle formation, kinetochore function and nuclear pore assembly are preferentially dephosphorylated (blue) during EME. In addition, specific amino acids at and surrounding the phosphorylation site on individual proteins, helps dictate the dephosphorylation. Together these two mechanisms help provide the specific dephosphorylation pattern and ordering of events during mitotic exit.
impacts dephosphorylation. Specifically, small non-polar residues (e.g. G, A, V, and L) promote dephosphorylation. This was best exemplified by the evolutionary conserved TPG motif found in PRC1, which is rapidly dephosphorylated during EME prompting its relocation to the central-spindle. We also identified several inhibitory motifs that prevented dephosphorylation, with a Proline or glutamic acid at the +2 position (S/T-P-P/E motif) strongly correlating with stable phosphorylation sites. Interestingly, there are several well-known TPP motifs in proteins such as MYC, JunB, and FoxM1, which have all been found to play critical roles in regulating the degradation and function of these proteins. For example, phosphorylation of the TPP site in MYC (T58) is required for efficient degradation of MYC. Phosphorylation at T58 and the upstream S62, provides a docking site for the isomerase Pin1, which then promotes the cis-conformational change between the S62-P63 bond. This allows PP2A to bind and dephosphorylate the S63 site, which in turn creates a motif for targeting MYC for degradation (70). This could also be an explanation for why the T58-P60 site is commonly mutated in a range of cancers (71, 72), as rapid dephosphorylation impacts dephosphorylation. Specifically, small non-polar residues (G, A, V, and L) promote dephosphorylation. This motif found in PRC1, which is rapidly dephosphorylated during mitotic exit.

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S This article contains supplemental Tables S1 and S2, Figs. S1 to S7 and Video S1.

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