Supplemental Data

Temporal Profiling of the Chromatin Proteome Reveals System-wide Responses to Replication Inhibition

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Supplemental Experimental Procedures

Materials and Equipment

Trypsin Gold, Mass Spectrometry Grade, was purchased from Promega. Expression and purification of non-degradable geminin^{DEL} were described in [S1]. Mcm2, Mcm3, Smc2 ,and Nup153 antibodies were as described previously [S2]. Rabbit polyclonal antiserum was raised against a synthetic peptide corresponding to the alpha-peptide of *Xenopus* pol δ (GTKQA SIMGFFQKK) as described [S3].

Xenopus Chromatin Manipulations

Mitotically arrested *Xenopus* egg extract preparation, chromatin reconstitution, and isolation was carried out as previously described [S4, S5], with small modifications. Extracts were characterized before being used for proteomic data collection and were rejected if they did not support efficient nuclear assembly and DNA replication, with a sharp entry and exit into S phase. Defrosted extracts were precleared by centrifugation at 20,000 g for 15 min at 4°C. The assembly reactions including 6000 sperm nuclei/μl were combined on ice and released into interphase by addition of 0.3 mM $CaCl_2$ and transfer to 23°C.

Samples of reconstituted chromatin were isolated at 5 min and then at 10 min intervals between 10 and 90 min. One hundred and fifty microliter aliquots of extract were diluted with 300 μl nuclear isolation buffer (NIB: 50 mM KCl, 50 mM Hepes [pH 7.6], 5 mM $MgCl_2$, 2 mM dithiothreitol [DTT], 0.5 mM spermidine, 0.15 mM spermine, and 1 μg/ml each leupeptin, pepstatin, and aprotinin), supplemented with 2.5 mM Mg-ATP and 0.1% Triton X-100, and chromatin was pelleted through a 30% sucrose cushion at 6000 g for 15 min at 4°C. Chromatin proteins were eluted in 90 μl XBE2 (10 mM K-Hepes [pH 7.7], 0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM ethylene glycol tetraacetic acid [EGTA], and 50 mM sucrose), supplemented with 0.1% Triton X-100 and 0.5 M NaCl, and were then chloroform/methanol precipitated. Protein pellets were dissolved and propagated for trypsin digestion as previously described [S6]. So that technical variations could be minimized and enough protein could be accumulated for analysis, experiments were repeated with the same extract at least twice for all conditions, and samples from corresponding time points were pooled.

Mcm2- and Mcm3-associated chromatin proteins were isolated by immunoprecipitation as described [S2]. In brief, replicating chromatin was isolated and treated with micrococcal nuclease to digest DNA to < 160 bp fragments. After centrifugation, soluble material was immunoprecipitated with antibodies against Mcm2 or Mcm3 or with nonimmune antibodies coupled to protein G Sepharose beads. After the washing, bound protein was eluted with 500 mM NaCl and was analyzed by LC-MS/MS.

Mass Spectrometry and Quantification

LC-MS analysis was performed with a quadruple time-of-flight instrument (QSTAR-XL, ABI-MDS-Sciex, Toronto, Canada) or a linear ion trap Fourier-transform ion-cyclotron resonance mass spectrometer (LTQ-FT-ICR, Thermo-Finnigan, Bremen). Samples were loaded onto a fused silica capillary with a 75 μm ID and an 8 μm tip opening (New Objective, Woburn, Massachusetts) filled with Reprosil 3 μm reverse phase material (Dr. Maisch, Ammerbuch, Germany). Peptides were eluted with a 140 min linear gradient of 92% buffer A (0.5% acetic acid in H_2O) to 50% buffer B (80% acetonitrile, 0.5% acetic acid in H_2O).

Mass spectra were collected automatically with information depended acquisition. The QSTAR-XL was operated in the pulsing mode to enhance the signal in both MS and MS/MS. Precursor ion spectra (from m/z 350–1500) were collected for 1 s. The four most intense ions were isolated and product ion spectra (m/z 70–1500) were collected for 1.5 s. The LTQ-FT-ICR instrument was also operated in the data-dependent mode to acquire high-resolution precursor ion spectra (from m/z 300–1500, R = 25,000, and ion accumulation to a target value of 5,000,000) in the ICR cell. The five most intense ions were sequentially isolated for accurate mass measurements by SIM scans (10 Da mass window, R = 50,000, and a target accumulation value of 50,000). The ions were simultaneously fragmented in the linear ion trap with a normalized collision energy setting of 27% and a target value of 2,000.

Combined peak lists were searched in the NCBI database with the Mascot program (Matrix Science, London, United Kingdom) and specifying *Xenopus laevis* species. QSTAR-XL data were searched with initial mass tolerance of 0.2 Da for precursors and product ions. Iterative calibration algorithms on the basis of identified peptides were used to achieve an average absolute mass accuracy of better than 20 ppm in both precursors and product ions. Proteins identified with a combined peptide score of higher than 60 were considered significant, and lower-scoring proteins were manually verified or rejected. LTQ-FT-ICR data were searched with a peptide mass tolerance of 5 ppm and a fragment mass tolerance of 0.8 Da. Iterative calibration algorithms on the basis of identified peptides resulted in an average absolute peptide mass accuracy of better than 1 ppm. Stringent criteria were used for protein identification based on the LTQ-FT-ICR data: at least two matching peptides per protein, a mass accuracy within 3 ppm, a Mascot score for individual ions of > 20, and a delta score of > 5. In those cases where protein identification was the only target, the public version of Phenyx software (http://www.phenyx-ms.com) was also employed.

MSQuant software [S7] was used to extract information from the Mascot HTML database search files and to manually validate the certainty in peptide identification, and in peptide abundance, on the basis of extracted ion chromatograms. A few proteins with a single high-scored peptide identified in at least seven time points were included in the analysis. To avoid rejection of polypeptides transiently associating with chromatin, we also analyzed proteins identified by only two or more highly scoring peptides at a single time point.

Mass-spectrometric analysis was repeated three times for the replicating chromatin and once for chromatin reconstituted in the presence of replication inhibitors. Temporal profiles from technical repeats were averaged at the protein level.

Clustering of Temporal Profiles

For each protein identified, the extracted ion current of up to the 12 most intense peptides were linearly transformed to the same mean value and averaged at each time point. The resulting temporal profiles were subjected to two rounds of smoothing (Figure 1B) and normalized so that over the time series the maximum abundance of each protein was set to 1. Proteins were combined into a single entry if the homology between related isoforms was > 93%. The resulting temporal profiles from replicating chromatin were clustered with fuzzy c mean (FCM) soft clustering procedure available as Mfuzz toolbox for R [S8]. The software package Cluster 3.0 [S9] was used for hierarchical cluster analysis.

DAVID [S10] was used to carry out annotation term enrichment analysis and functional clustering of FCM groups. The annotation categories are considered to be strongly enriched if p values (Figure 2) associated with them are equal or smaller than 0.05. The overall group enrichment score (Figure 2: GES column) is the negative log of the geometric mean of the statistical significance (p value) for all members of the cluster. For statistical calculation, all genes that were identified in our experiments were used as a background.

To estimate the reproducibility of temporal profiles, we used 276 proteins identified in three LCMS replicates of untreated chromatin and separated them into 12 FCM clusters. We defined the level of reproducibility (Rp) for cluster as a percentage of cases where at least two individual profiles we present in a cluster together with corresponding averaged one.

References

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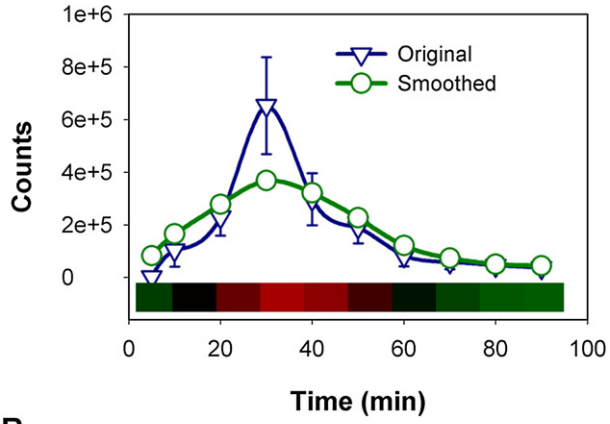
S2. Gillespie, P.J., Khoudoli, G.A., Stewart, G., Swedlow, J.R., and Blow, J.J. (2007). ELYS/MEL-28 chromatin association coordinates nuclear

A



B

Correlation	P interval	Percentage
Large	0.5 - 1.0	72.7
Medium	0.3 - 0.5	6.0
Small	0.1 - 0.3	4.0

C

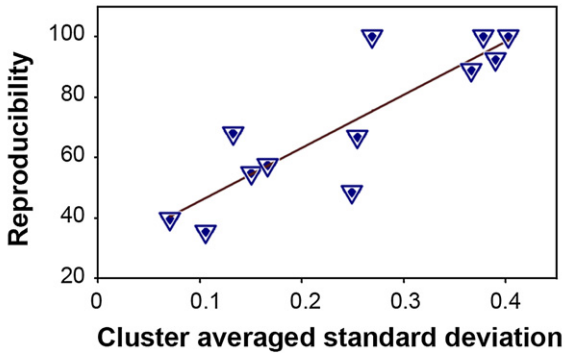


Figure S1. Data Presentation and Reproducibility Analysis

(A) Comparison of raw ion current values (blue) and smoothed values for Mcm2. Below, a heat map of the smoothed values is shown (red, highest; green, lowest; black, intermediate). Error bars indicate the SD.

(B) Reproducibility of protein behavior observed in three LCMS runs of untreated chromatin estimated by Pearson coefficient of correlation and graded according to Cohen [S11]. Coefficients of correlation were calculated pair wise for 276 proteins identified in all three LCMS runs of untreated chromatin.

(C) Regression analysis of correlation between reproducibility of temporal profiles assignment to cluster and amplitude of profiles variation presented by averaged cluster standard deviation. Only proteins identified in all three repeated LCMS analysis of untreated chromatin (276) were used for comparison.

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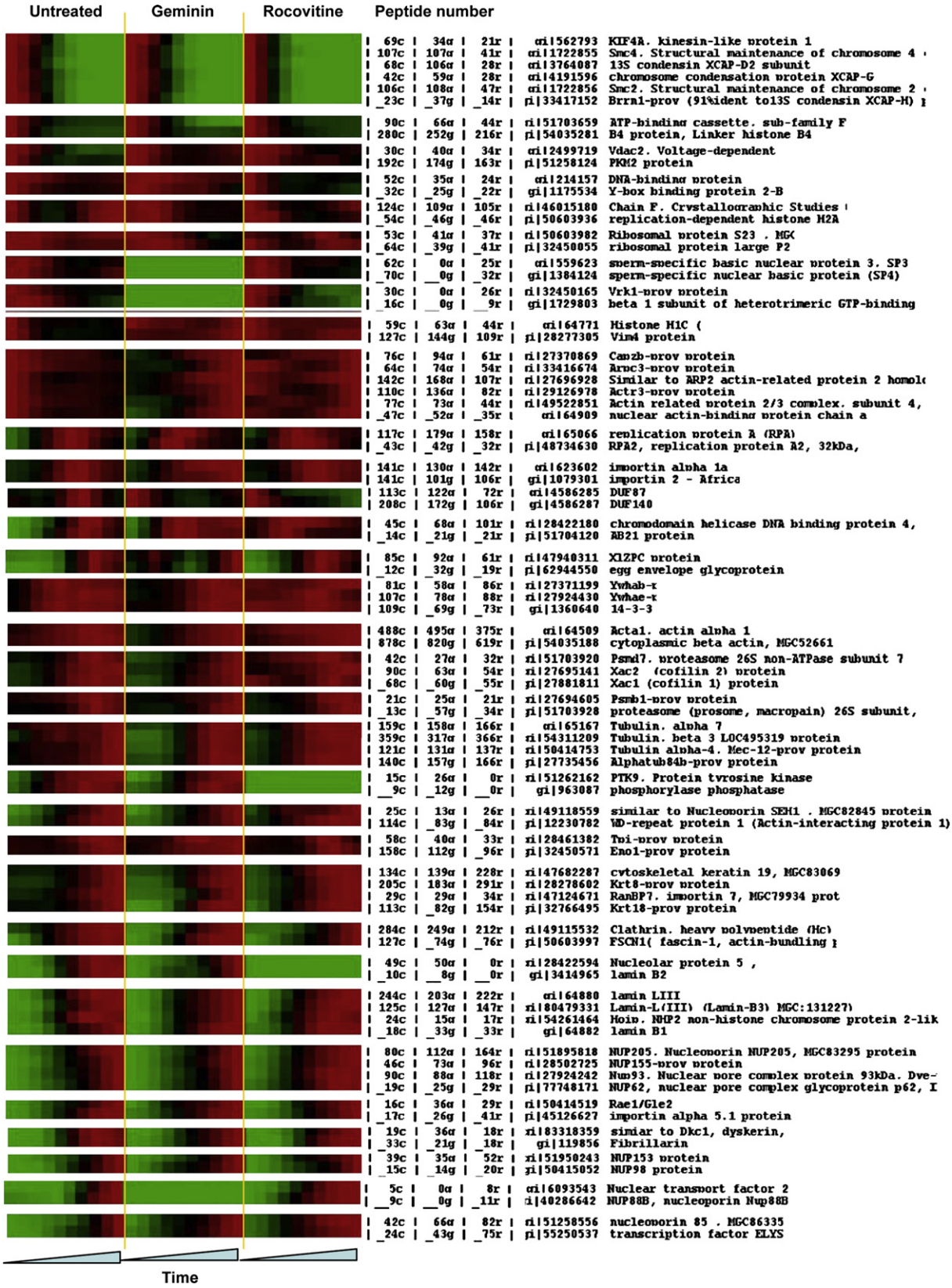
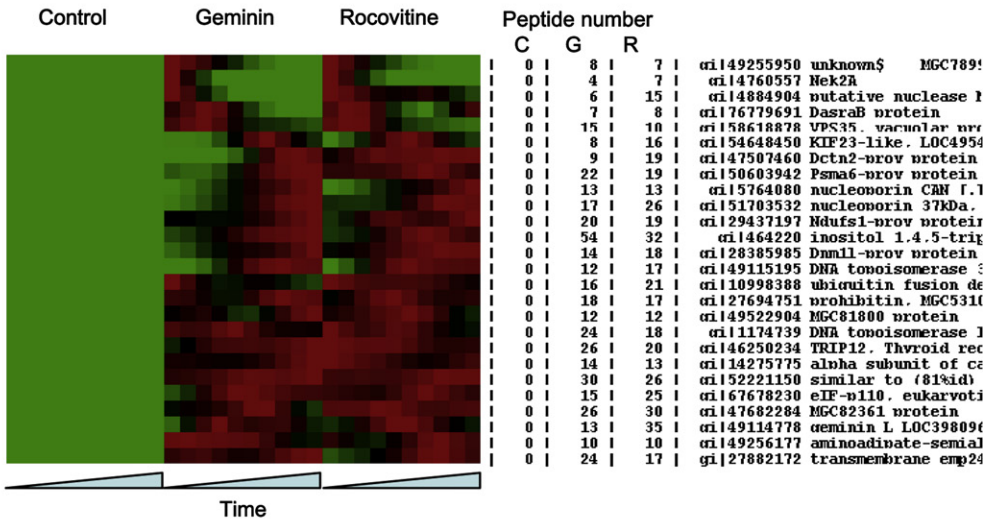


Figure S2. Protein Groups with Highly Correlated Temporal Behavior on Interphase Chromatin across All Experimental Treatments

The combined dataset was generated by integration of profiles from inhibitor-free, geminin, and roscovitine experiments for each protein. Hierarchical clustering was performed on complete combined dataset. Branches of hierarchical trees with the correlation level above the threshold defined by the MCM licensing complex are presented. The color coding is as follows: 0.0, green; 0.5, black; and 1.0, red. Number of peptides identified for proteins in each treatment is also indicated.

A



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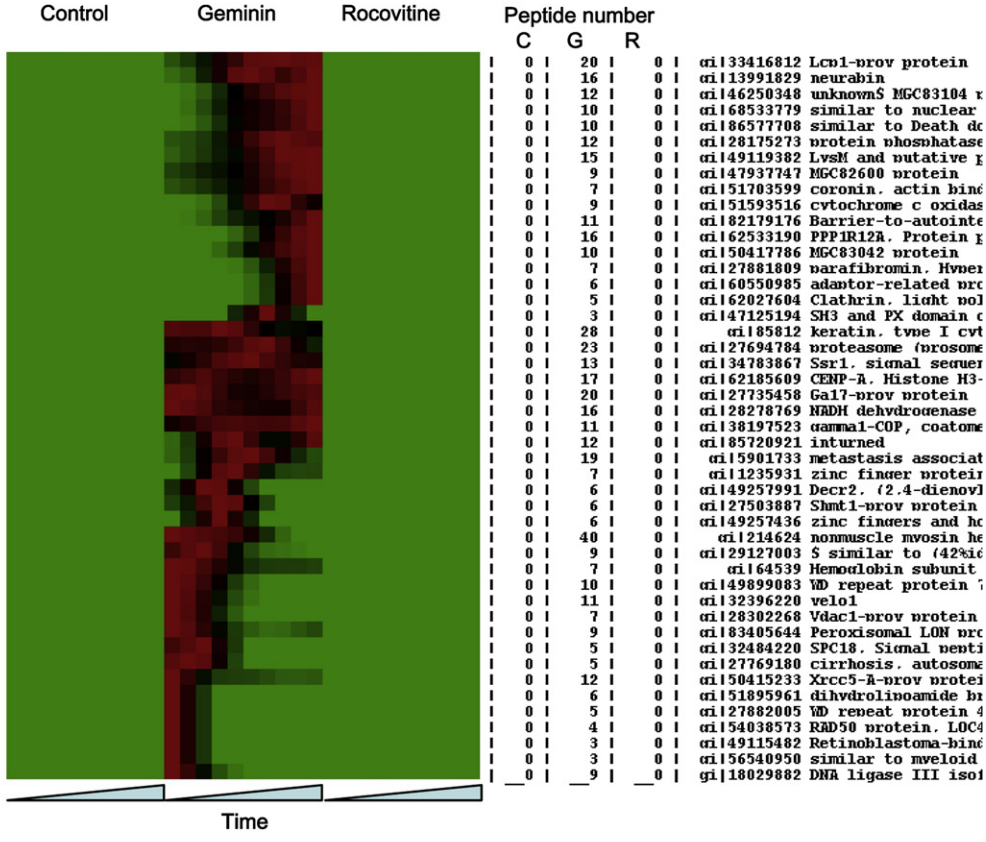

(Figure S3. Continued)

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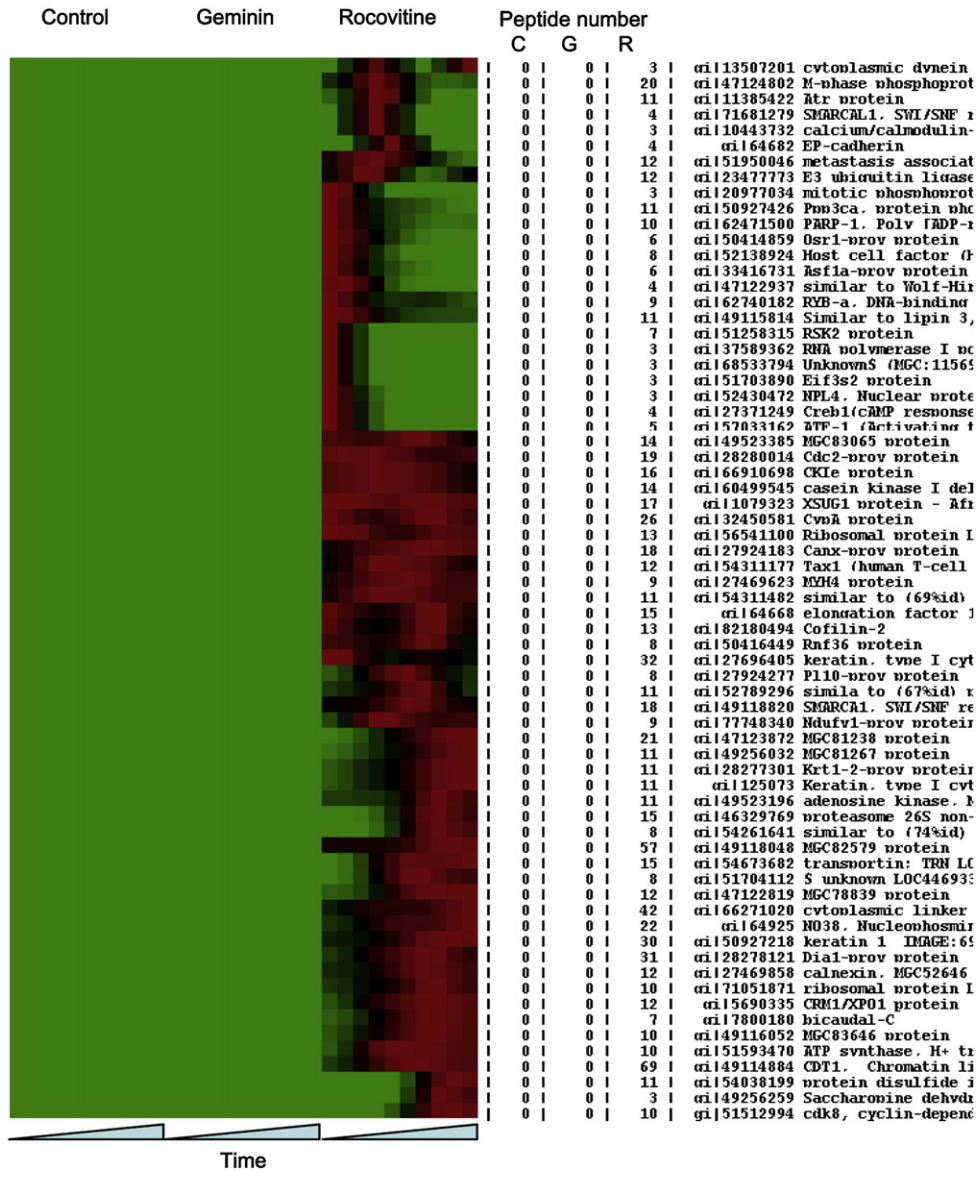

Figure S3. Hierarchical Clustering of Temporal Profiles for Proteins Identified on Chromatin Only in the Presence of Inhibitors

(A) Proteins whose binding to chromatin was induced by both inhibitors.

(B) Proteins identified on chromatin only in the presence of geminin.

(C) Proteins identified on chromatin only in the presence of roscovitine.

Table S1. Functional Annotation Clustering of Proteins Induced by Replication Inhibitors

Drug	FG	GES	Overrepresented Annotation Terms with Highest p Value	p Value
Gem (72)				
	1	1.84	WD-40repeat (IN)	1.00E-02
	3	0.87	Ion transporter activity (GM)	1.80E-02
	6	0.61	DNA repair (GB)	1.20E-02
	7	0.42	Phosphorylation (GB)	3.60E-01
Ros (94)				
	1	3.41	Phosphorylation (GB)	4.10E-08
	2	1.05	transporter activity (GM)	1.90E-02
	5	0.66	cellular physiological process (GB)	5.10E-02
	8	0.38	Armadillo-like helical (IN)	2.60E-02

Number of genes unique for each treatment is indicated in parentheses. "FG" indicates functional groups identified by DAVID; "GES" indicates group enrichment score. The annotation source is indicated next to a term: GB, GOTERM_BP_ALL; GM, GOTERM_MF_ALL; GC, GOTERM_CC_ALL; IN, INTERPRO_NAME; SN, SMART_NAME; SP, SP_PIR_KEYWORDS; KP, KEGG_PATHWAY; and HC, Hierarchical Clustering.

Table S2. Proteins Identified in Mcm2 and Mcm3 ChIPs

Gene Name	Number of Identified Peptide	
	Mcm2 IP	Mcm3 IP
MCM2	45	44
MCM3	32	9
MCM4	57	51
MCM5	35	3
MCM6	30	31
MCM7	37	37
ORC1	–	7
ORC2	–	4
ORC3	–	2
ORC4	–	2
ORC5	–	1
UBF1	2	–
HP1	2	3
Cdc6	–	1
DNA polymerase epsilon	1	–
RuvBL2	2	2
Duf87	1	–
AND-1	–	1
RECQL4	–	1
MCM10	–	1
Actr3	13	7
ARP2	7	4
Arp2/3 complex, subunit 2	4	4
Arp2/3 complex, subunit 4	2	3
Arpc1a	1	4
Capzb	6	4
Cortactin	4	11
Aurora B	–	13
XL-INCENP	–	5
Survivin	–	5
DasraA protein	–	1
Plk1	1	2
ELYS/Mel-28	2	1
Nup160	–	1
Nup107	3	2
Nup 85	3	1
Nup53	1	–
Nup43	1	–
SEH1	1	–