# Supporting Information Appendix

## Results

### Cdk oscillation is required for cell cycle progression

Though Cdk2 activity was reported to be nearly constant in cell cycle of ES cells (1-3) with improved synchronization protocols we clearly observed fluctuations in cyclin A protein levels (Fig. 1A). This led us to ask whether cyclin A-associated Cdk2 kinase activity also oscillates. By immunoprecipitating Cyclin A and Cdk2 at different phases of the cell cycle and performing kinase assays using recombinant histone H1 as a substrate, Cdk2 activity was found to be significantly lower in G1, as measured by either Cyclin A immunoprecipitation or Cdk2 immunoprecipitation (Fig. S3A). As expected, the levels of Cyclin A fluctuate commensurate with Cdk2 activity, while the levels of Cdk2 remain stable (Fig. S3B). Thus not only does Cdk1 (4) but also Cdk2 activity oscillates in ES cells. Since inhibition of Cdk1 and Cdk2 activity usually arrests somatic cell cycle progression, we tested whether Cdk activity is required at G1/S transition and mitosis in ES cells. When we synchronized ES cells in mitosis and subsequently released them we found that, while DMSO-treated control cells progress into S phase, cells treated with Cdk1/Cdk2 inhibitor Roscovitine arrest at the G1/S transition, as confirmed by low levels of Cyclin B and phosphorylation of Cdk1, typical of G1 phase (Fig. S3C). Though the persistence of some appreciable level of Cdk1 activity from mitosis through S phase should permit and even accelerate DNA replication, there is one problem. In somatic cells there is a need for a period in G1 where Cdk activity is absent to allow for the formation of pre-Replicative

Complexes. The persistence of some Cdk activity in G1 phase in ES cells thus raises a question of how and when the pre-RC could be assembled. To explore this apparent paradox we first investigated the order of chromatin licensing events in ES cells and compared that to somatic cells (5, 6). ES cells released from a mitotic block and harvested at different times during mitotic exit and in early G1 were lysed and the contents separated into a soluble fraction and a chromatin-containing fraction, as previously reported (7). Immunoblotting confirmed that Cdt1 loading on chromatin precedes the recruitment of Mcm proteins, as it does in somatic cells (Fig. S3D). Although the G1 progression in experiment for figure S3A-B was slightly slower than in the experiment for figure S3D (due to some heterogeneity between synchronizations), it is conceivable that Cdt1 starts to associate to chromatin before Cdk activity reaches its lowest point (which is high if compared with Cdk inhibition observed in G1 of somatic cells). Moreover, when synchronized mitotic cells were treated with Roscovitine, pre-Replicative Complexes are prematurely loaded on chromatin (Fig. S3E). These results show that, similar to somatic cells, both Cdk1 and Cdk2 fluctuate in ES cells and Cdk kinase activity is necessary for normal cell cycle progression.

## Materials and Methods

### Chemicals and siRNA

Roscovitine was from Biomol International (CC-205) and used 25 μM. Retinoic Acid was from Sigma and used 1 μM. MG132 was from Biomol International (PI-102) and used 10 μM. Nocodazole was from Biomol International (T-101) and used 50 ng/ml. Cycloheximide was from

Biomol (GR-310) and used 50 μg/ml. siRNA for mouse Cdc6 was a mixture of three siRNA from Invitrogen (CDC6MSS215567- CDC6MSS215568- CDC6MSS215569). siRNA for mouse Cdt1 was a mixture of three siRNA from Invitrogen (CDT1MSS244895-CDT1MSS244896-CDT1MSS244897). siRNA for mouse Cyclin A2 was SMARTpool L-040393-00-0005 from Thermo Scientific. siRNA for mouse Cyclin E1 was a mixture of three siRNA from Invitrogen (CCNE1MSS202709- CCNE1MSS202710- CCNE1MSS202711). siRNA for mouse Emi1 was a mixture of three siRNA from Invitrogen (FBXO5MSS244880-FBXO5MSS244881-FBXO5MSS244882). siRNA for mouse Geminin was SMARTpool L-050241-00-005 from Dharmacon RNA Technologies.

**ES cells culture and synchronizations**

Mouse ES cells (J1 cell line unless otherwise specified) were incubated at 37 C 5% $CO_2$ in DMEM medium supplemented with 15% heat inactivated serum, 0.1 mM beta-mercaptoethanol, 10% non essential amino acids and 1,000 units/ml Leukemia Inhibitory Factor (LIF) (Chemicon International). For synchronization ES cells were treated with 1.25 mM Thymidine for 14 hours, followed by treatment with 50 ng/ml Nocodazole for 7 hours; mitotic cells were collected at this time. G1 and S phase cells were collected 1 and 4 hours after release, respectively, unless differently specified. ES cells were differentiated with Retinoic Acid (RA) 1 μM and withdrawal of LIF. Cells expressing p21 were constructed from KH2 cell line. Cyclin E knockout cells were kindly provided by Sicinski laboratory (8).

**siRNA treatment**

siRNA treatments were performed by treating trypsinized ES cells with Lipofectamine 2000 (Invitrogen). Plasmids transfection was performed using FuGene 6 (Promega). Plasmids for HA-tagged versions of Geminin were from previous report (9). siRNA treatments during S-G2 were performed starting from 1 hour after thymidine release with the addition of Nocodazole for subsequent mitotic synchronization.

**Somatic cells synchronization**

U2OS cells were synchronized in mitosis with double thymidine treatment followed by Nocodazole treatment for 14 hours. Mitotic cells were collected by shake-off and released for time course; cells attached to the plate after the shake off were used as sample representative of late G2 phase (9). U2OS cells were synchronized at the G1/S transition with double thymidine treatment.

**Immunoblotting analysis and chromatin fractionation**

Immunoblots were usually performed by running proteins on 4-15% gels, transferring on nitrocellulose membranes and performing overnight primary hybridizations. Secondary hybridizations were usually performed with infrared secondary antibodies and detection of proteins was performed with Odyssey Infra-red imaging system. In some cases chemiluminescence was used. We did not detect any significant difference between the two detection methods. Chromatin fractionation was performed as previously reported (7).

**Antibodies**

The following antibodies were used in immunoblot analysis: APC2 (homemade), Cdc27 (APC3) (sc-13154), APC4 (sc21414), APC7 (sc-20987), Aurora A (Abcam 13824), Cdk1 (sc-54), p-Cdk1 (Cell Signaling #9111), Cdc20 (sc-8358), Cdh1 (Abcam 5483), Cdk2 (sc-163), Cdt1 (P26A6) (9), Cyclin A2 (sc-596), Cyclin B1 (sc-245), Cyclin E (Abcam 7959), Emi1 (Zymed 38-5000), Geminin (sc-13015), p-Histone H3 (Upstate #06-570), Oct4 (sc-8628 and sc-5279), Nanog (Bethyl A300-397A), Mcm2 (sc-9839), Mcm6 (sc-9843), p21 (sc-397), Plk1 (Upstate #06-813), Securin (MBL K0090-3), Vinculin (sc-25336), Tubulin (sc-6217).

**Immunoprecipitation of APC/C**

APC/C complexes were isolated by immunoprecipitation of Cdc20 protein from cells lysates prepared using RIPA buffer. 5 micrograms of Cdc20 antibody (sc-1906) or Oct4 antibody (sc-8628)(control) were added to 2 mg of total protein lysates and incubated for 30' on ice, before addition of protein G-Agarose (Roche) and rocking at 4 C for other 1h30'.

**Degradation assay**

Cells were treated with swelling buffer (20 mM Hepes pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, protease inhibitors) plus 2% total volume of energy mix solution (375 mM Phosphocreatinine, 50 mM ATP, 5 mM EGTA, 50 mM $MgCl_2$). After 30' on ice the samples were frozen in liquid nitrogen and thawed at 30 C for two rapid cycles. Cells were then lysed with a 20G1/2 needle and the soluble extracts were separated from cell pellet by centrifugation for 45' at 4 C. Degradation reactions were then assembled in this way: 50% cell extracts, 10% degradation cocktail (3 mg/ml Ubiquitin, 30 % energy mix solution), 0.1 mg/ml UbcH10 from Boston Biochem, 10% reticulocytes-extracts $^{35}$S-labeled in vitro-translated substrate. N-terminal

truncated recombinant Emi1 was used to inhibit degradation of APC/C substrates. Reactions were performed at 30 C for the indicated times and blocked with the addition of Laemmli buffer before loading on gel and detection by autoradiography or immunoblotting analysis.

**Ubiquitination assay**

The purification of the anaphase promoting complex and the following ubiquitination assays were described in detail previously (10). Briefly, APC/C was purified from mitotic arrested ES cells (either incubated in presence of LIF or previously incubated for 48 hours in presence of retinoic acid) followed by incubation of the cell extract with 3 μM UBCH10 at room temperature for 1 hr. In the ubiquitination assay, 100 nM recombinant human Securin was incubated with 50 nM E1, 1 μM UBCH10, 1 mg/mL ubiquitin and 30 nM purified APC/C for 30 min and 60 min at 30 ºC prior to treatment with sample buffer and immunoblotting analysis. 100 nM recombinant human N-terminal deleted Emi1 was added as a control to inhibit the reaction.

**Kinase assay**

Cell extracts were prepared by separation of the soluble fraction from the chromatin-containing fraction after lysis with CSK-Triton buffer, as previously described (7). Cyclin A2 (sc-596), Cdk2 (sc-163) or Cdk1 (sc-54) antibodies together with protein A-agarose beads were used to immunoprecipitate Cdk complexes from the soluble fraction. After washing three times with CSK-Triton buffer, the agarose beads were used to assemble kinsase reactions with 0.1 mg/ml of recombinant histone H1, 0.37 mM ATP, 0.75 μCi $^{32}$P-ATP. Reactions were incubated at 25 C for 20’ under constant shaking. Kinase reactions were finally stopped with addition of Laemmli

buffer and loaded on gel before transfer on nitrocellulose membrane and detection of radioactive signal by phosphoimaging detection (Personal Molecular Imager FX – Bio-Rad).

**RNA isolation, cDNA synthesis and Quantitative RT-PCR**

Three independent biological samples were collected at different stage of cell cycle (late S phase, S-G2 transition and G2-M transition). Total RNA was isolated from by RNeasy mini kit according to the manufacture (Qiagen). Five μg of total RNA were treated with RNase-free DNase (Ambion, Inc., TX) for 30 min at 37 $^{o}$C to remove residue DNA contamination. cDNA were synthesized by SuperScript III First Stand Synthesis System according to the manufacture (Invitrogen, CA). Quantitative PCR reaction was performed using the 2x Brilliant II SYBR QPCR low ROX master mix (Agilent Technologies, CA) on Mx3005p QPCR system (Agilent Technologies, CA). All samples were normalized to β-actin.

**FACS analysis**

FACS analysis was performed according to standard protocols after Propidium Iodide, BrdU or SSEA1 stainings. Analysis of the cells was performed with Flow Cytometry Analysis Software FlowJo.

**Generation of ES cells expressing p21$^{CIP1}$**

Open reading frame of p21$^{CIP1}$ was cloned into the pBS31 plasmid and was electroporated into the KH2 mouse ES cells to target the Col1A1 locus under the control of a minimal CMV promoter with doxycycline response element, as described previously. The KH2 cells also express the M2-rtTA from the ROSA26 locus. 10 days after electroporation, ES cell clones with

proper pBS31-p21$^{CIP1}$ integration that are resistant to 140 μg/ml of hygromycin B were individually picked and expanded. Doxycycline induction of p21 of each clone was confirmed by immunoblotting analysis.

**Synchronization of human ES and iPS cell lines**

BGO1 human ES cells and dH1f-iPS human iPS cells were used for synchronization. Cells were cultured in F12/DMEM medium (Stemcell technologies), supplemented with 20% knockout serum replacement (Invitrogen, CA), 0.1 mM non-essential amino acid, 1 mM L-glutamine, 50 U/ml penicillin/streptomycin and 10 ng/ml basic FGF (Invitrogen, CA) on irradiated mouse embryonic fibroblasts. Cells were synchronized at M-phase by treating with 2 mM thymidine (Sigma, MO) for 15 hours followed by 50 ng/ml of Nocodazole (Sigma, MO) for another 12 hours.

References


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2. Fujii-Yamamoto H, Kim JM, Arai K, & Masai H (2005) Cell cycle and developmental regulations of replication factors in mouse embryonic stem cells. *J Biol Chem* 280(13):12976-12987.
3. Yang VS (2011) Geminin escapes degradation in G1 of mouse pluripotent cells and mediates the expression of Oct4, Sox2, and Nanog. *Curr Biol* 21(8):8.
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5. Dutta A & Bell SP (1997) Initiation of DNA replication in eukaryotic cells. *Annu Rev Cell Dev Biol* 13:293-332.
6. Lutzmann M & Mechali M (2009) How to load a replicative helicase onto chromatin: a more and more complex matter during evolution. *Cell Cycle* 8(9):1309-1313 .

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**Supplementary figure legend**

**Figure S1**

List of all tested synchronization protocols. Protocols selection was based on FACS analysis (not shown for clarity). 14 hours with 1.25 mM Thymidine treatment followed by 7 hours 50 ng/ml Nocodazole treatment was chosen as standard synchronization protocol. "G1-like state" symbolizes a protocol that synchronizes cells with 4N content but with features typical of G1 phases, as similarly observed in somatic cells treated with Nocodazole for extended times. "No mitosis arrest" symbolizes protocols that do not arrest cells in mitosis.

**Figure S2**

FACS profiles of standard protocol. ES cells synchronized in mitosis (i.e. Nocodazole arrest) and released for the indicated times are shown. "As" represents asynchronized cells. The inset shows DAPI staining of Nocodazole-synchronized cells.

**Figure S3**

Cdk activity oscillates in ES cells. (A) Kinase assay is performed with Cdk complexes obtained by immunoprecipitation of Cyclin A or Cdk2. Immunoprecipitations are performed with cells

synchronized in mitosis or different times after Nocodazole release. Histone H1 is used as substrate. Autoradiography for $^{32}$P-H1 is shown. (B) Cdk2 protein levels are constant along the cell cycle of ES cells. Total lysates, Cyclin A and Cdk2 immunoprecipitates are used for evaluation of the indicated proteins by immunoblotting analysis. Samples correspond to lysates and immunoprecipitations from kinase assay shown in panel (A). (C) Cells treated with Roscovitine during G1 phase display impaired G1/S progression. ES cells are released for 6 hours from mitotic block in presence or absence of Cdk inhibitor Roscovitine. FACS profiles are shown on the top and immunoblotting analysis for the indicated proteins is shown at the bottom. (D) Pre-Replicative Complexes (pre-RC) are formed at mitotic exit in ES cells. Cdt1 association to chromatin precedes Mcm proteins loading. ES cells released from mitosis are fractioned as previously described (7) and the chromatin-containing fraction is used to measure the loading of pre-RC components on chromatin. Immunoblotting analysis for the indicated proteins is shown. Mcm2 and Mcm6 hybridizations were performed on the same membrane. * represents proteins of chromatin-containing fraction. (E) Inhibition of Cdk activity during mitosis leads to premature formation of pre-RC. Mitotic ES cells are treated for 3 hours with Roscovitine and immunoblotting analysis for the indicated proteins is shown. * chromatin-containing fraction. Note that cells in panels A+B and D are from distinct experiments (though with same synchronization protocols) and for this reason there are small differences in speed of G1 progression.

**Figure S4**

Somatic oscillations of Cyclin A and Cyclin B levels. U2OS cells are synchronized in G2 phase, mitosis or S phase as described in the methods section. Cells are released from mitotic or S phase

block for the indicated times and immunoblotting analysis for the indicated proteins is shown. Images were cut to exclude the 11 hours time point due to a technical problem in that lane. Short and long exposure times are shown for Cyclin A and B.

**Figure S5**

Intrinsic APC/C activity is not changed upon differentiation. (A) Degradation assay from mitotic undifferentiated ES cells or ES cells induced to differentiation for 48 hours with retinoic acid is shown. In vitro translated $^{35}$S-Geminin is used. Autoradiography for $^{35}$S-Geminin is shown. The panel on the bottom shows the quantification of the degradation rate; levels of $^{35}$S-Geminin are expressed in arbitrary units (dashed line for undifferentiated cells and solid line for differentiated cells). (B) Degradation assay from mitotic undifferentiated ES cells or ES cells induced to differentiation for 48 hours with retinoic acid is shown. Endogenous levels of Cdt1 protein are detected by immunoblotting analysis. (C) Intrinsic APC/C activity is not changed upon differentiation. Ubiquitination assay is shown for recombinant Securin after immunoprecipitation of endogenous APC/C from mitotic undifferentiated ES cells or ES cells induced to differentiation for 48 hours with retinoic acid. The reaction is performed for the indicated times at 30 C and immunoblotting for Securin is shown. Recombinant N-terminal deleted Emi1 is used as a control to inhibit the reaction. (D) APC/C subunits levels are constant along the cell cycle and not affected by differentiation. ES cells are treated with retinoic acid for the indicated times. Cells are synchronized in S phase with thymidine and then released for 3 hours (i.e. S/G2 phase) or synchronized in mitosis. Immunoblotting analysis for the indicated proteins is shown. (E) Emi1 depletion induces S-G2 accumulation and re-replication, similarly to Cyclin A and Geminin depletion, respectively. FACS profiles are shown for cells treated for only 24 hours

with the indicated siRNA and stained with Propidium Iodide. S-G2 accumulation and re-replication are greater at later time points (not shown for clarity).

**Figure S6**

Cyclin E fluctuates along the cell cycle of ES cells, with lowest levels during S phase. (A) Specificity test for Cyclin E antibodies. Cyclin E wild type (WT) or knockout (KO) mouse ES cells (kindly provided by Peter Sicinski laboratory) are used for testing different commercial Cyclin E antibodies for immunoblotting analysis. Whole cell extracts are used after lysis with Laemmli buffer and sonication. Block arrow represents 47.5 kDa marker (New England BioLabs #P7708S). Total protein levels differ slightly between the two lanes, as shown by different intensities of background bands. sc-198 and sc-481 antibodies do not detect specific bands even at increased exposures or using chemiluminescence detection (not shown for clarity). (B) ES cells are synchronized as previously described in mitosis before release for the indicated times. Abcam antibody (Ab 7959) is used for immunoblotting analysis. Uniformity of loading is evaluated by Ponceau staining of the membrane used for immunoblotting analysis.

**Figure S7**

p21 expression delays cell cycle progression and inhibits Cdk2 activity. (A) FACS profiles of ES cells expressing or not-expressing p21, synchronized in mitosis and released for the indicated times. (B) Kinase assay with cells synchronized as in (A). Cdk complexes are obtained by immunoprecipitation of Cdk1 or Cdk2. $^{32}$P-Histone H1 is used as a substrate. Immunoblotting analysis for Cdk1 and Cdk2 is shown. Autoradiography for $^{32}$P-H1 is shown.

**Figure S8**

Cyclin A depletion decreases Cdt1 levels and pre-RC formation. (A) ES cells are treated with siRNA for Cyclin A and incubated for 24 hours before thymidine treatment for standard synchronization protocol. Cells are therefore released in presence of Nocodazole for the indicated times. Time points are taken at different times after thymidine release and other samples are used for subsequent Nocodazole release. Immunoblotting analysis for the indicated proteins is shown. * represents the chromatin-containing fraction. (B) Related FACS analysis profiles are shown.

**Figure S9**

APC/C substrate Geminin protein levels oscillate in other mouse and human pluripotent cells. (A) CCE mouse ES cell line displays fluctuation of APC/C substrate Geminin protein during cell cycle progression, with lowest levels in G1 phase. CCE cell line is synchronized using the same protocol used for synchronization of J1 cells. Immunoblotting analysis for the indicated proteins is shown on the left and FACS profiles of the same time points along the cell cycle are shown on the right. (B) Human ES cells display fluctuation of Geminin protein during cell cycle progression, with lowest levels in G1 phase. BGO1 human ES cells are used for synchronization. Cells are synchronized in mitosis by treating with 2 mM thymidine for 15 hours followed by Nocodazole treatment for another 12 hours. Immunoblotting analysis is shown on the left and corresponding FACS profiles are shown on the right. Loading is evaluated by Ponceau staining of the membrane used for immunoblotting analysis. (C) Human iPS cells display fluctuation of Geminin protein during cell cycle progression, with lowest levels in G1 phase. dH1f-iPS human iPS cells are used for synchronization. Cells are synchronized in mitosis by treating with 2 mM

thymidine for 15 hours followed by Nocodazole treatment for another 12 hours. Immunoblotting analysis is shown on the left and corresponding FACS profiles are shown on the right. Loading is evaluated by Ponceau staining of the membrane used for immunoblotting analysis.

| S phase (Pre-Synchronization) | Mitosis | | | | |
|---|---|---|---|---|---|
| ---------no---------- | Nocodazole | 12h | 50 ng/ml | | G1-like state |
| Thymidine 14h 2.50 mM | Nocodazole | 5h | 50 ng/ml | | |
| Thymidine 14h 2.50 mM | Nocodazole | 6h | 50 ng/ml | | |
| Thymidine 14h 2.50 mM | Nocodazole | 7h | 50 ng/ml | | |
| Thymidine 14h 2.50 mM | Nocodazole | 8h | 50 ng/ml | | |
| Thymidine 14h 2.50 mM | Nocodazole | 9h | 50 ng/ml | | |
| Thymidine 14h 2.50 mM | Nocodazole | 10h | 50 ng/ml | | |
| Thymidine 14h 2.50 mM | Nocodazole | 12h | 50 ng/ml | | |
| Thymidine 14h 2.50 mM | Nocodazole | 16h | 50 ng/ml | | G1-like state |
| Thymidine 14h 2.50 mM | Nocodazole | 8h | | 17 ng/ml | No Mitosis Arrest |
| Thymidine 14h 2.50 mM | Nocodazole | 8h | | 25 ng/ml | |
| Thymidine 14h 2.50 mM | Nocodazole | 8h | | 37 ng/ml | |
| Thymidine 14h 2.50 mM | Nocodazole | 8h | 50 ng/ml | + Deoxycytidine | |
| Thymidine 14h 2.50 mM | TN16 | 8h | 50 ng/ml | | |
| Thymidine 14h 2.50 mM | TN16 | 8h | 50 ng/ml | | |
| Thymidine 14h 1.25 mM | Nocodazole | 7h | 50 ng/ml | | |
| Thymidine 14h 1.25 mM | Nocodazole | 10h | 50 ng/ml | | |
| Thymidine 14h 1.25 mM | Nocodazole | 12h | 50 ng/ml | | |

Figure S1

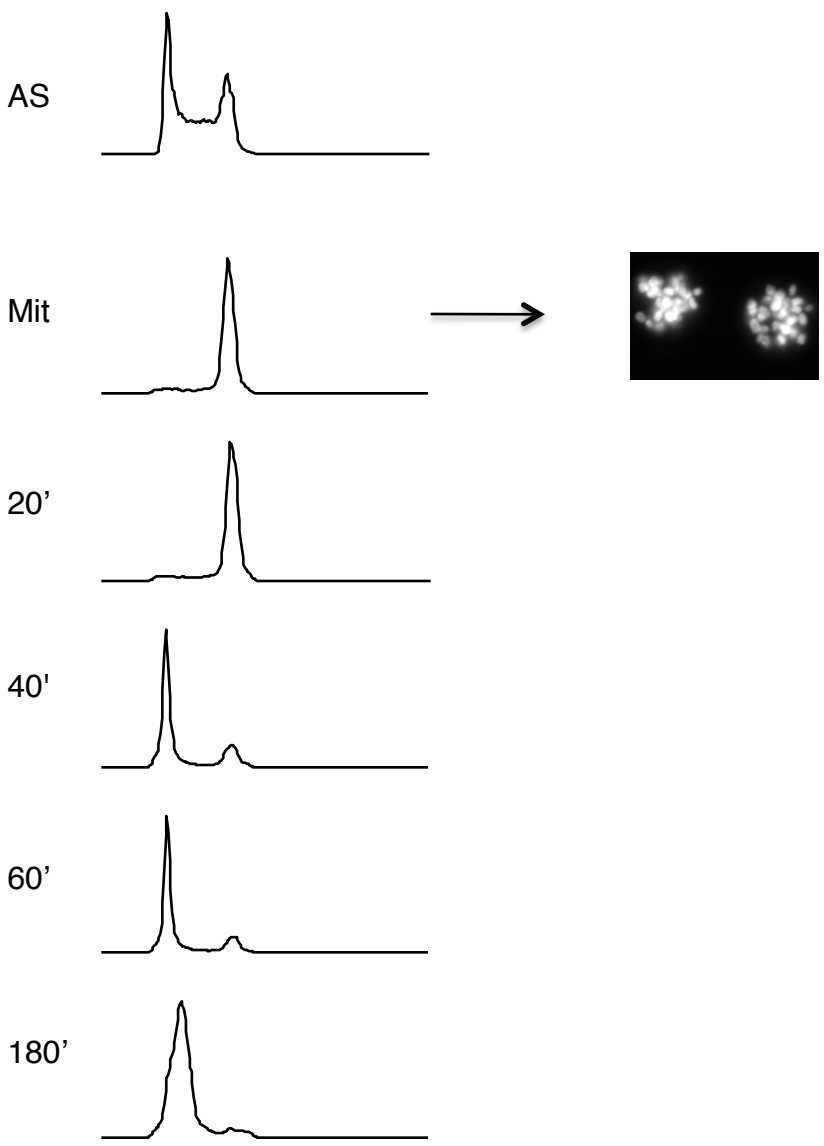
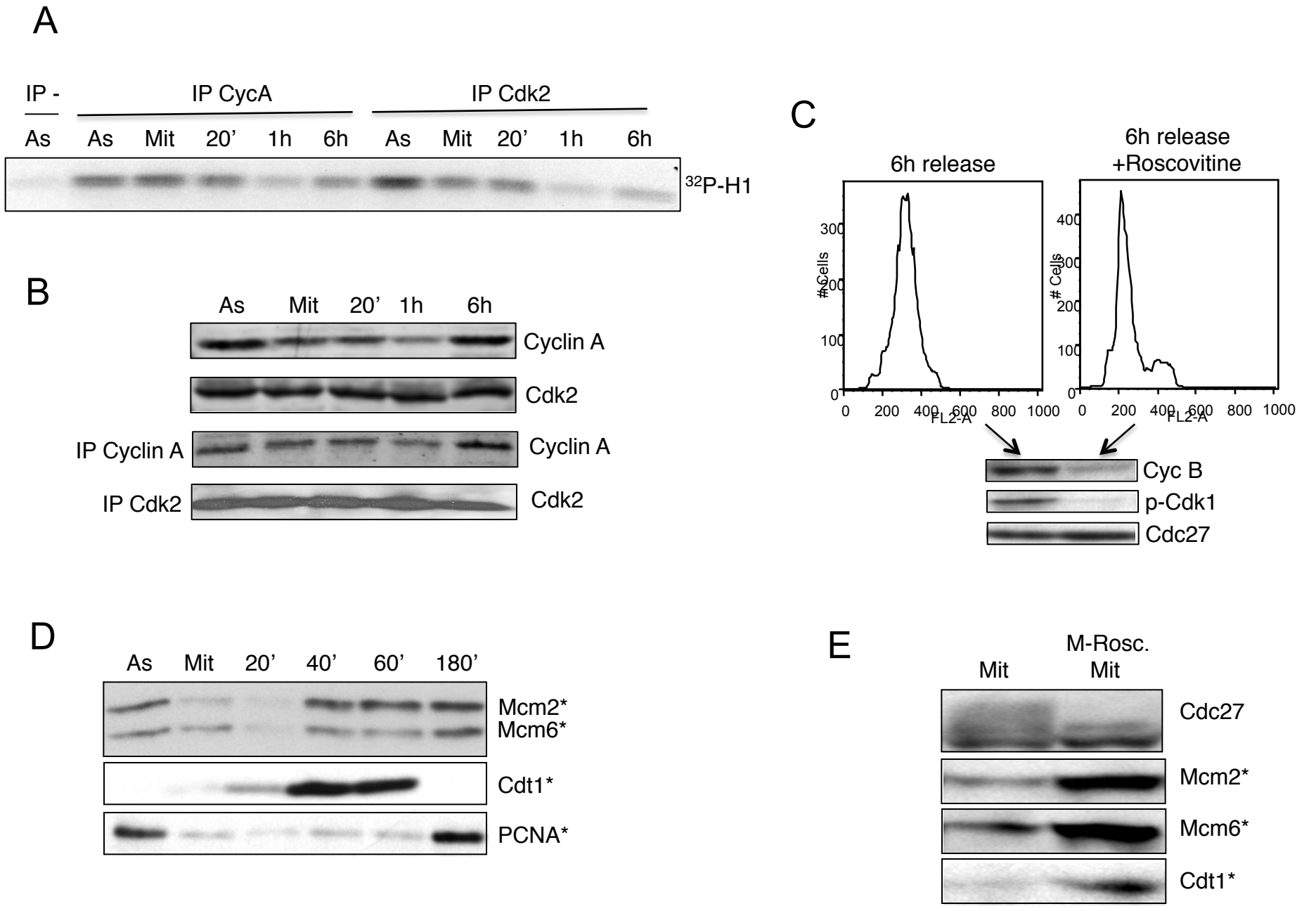



Figure S2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B
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20'
1h
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Cyclin A
Cdk2
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Cyclin A
IP Cdk2
Cdk2
C
6h release
6h release
+Roscovitine
# Cells
FL2-A
Cyc B
p-Cdk1
Cdc27
D
As
Mit
20'
40'
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Figure S3

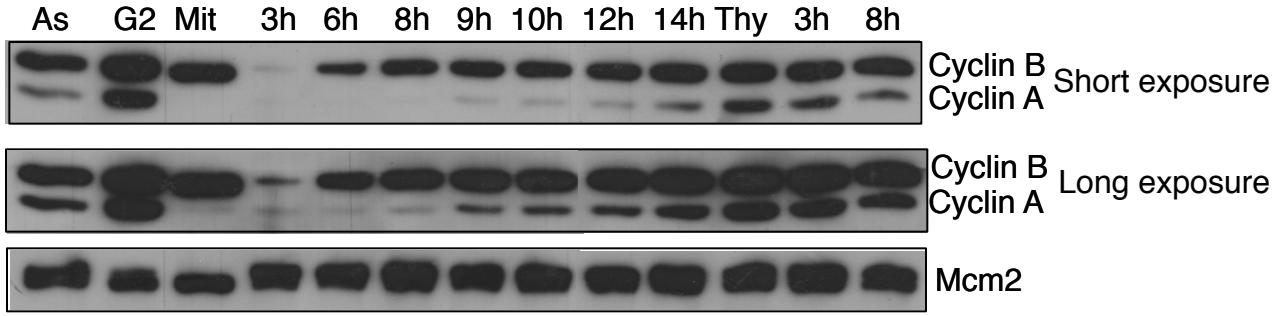
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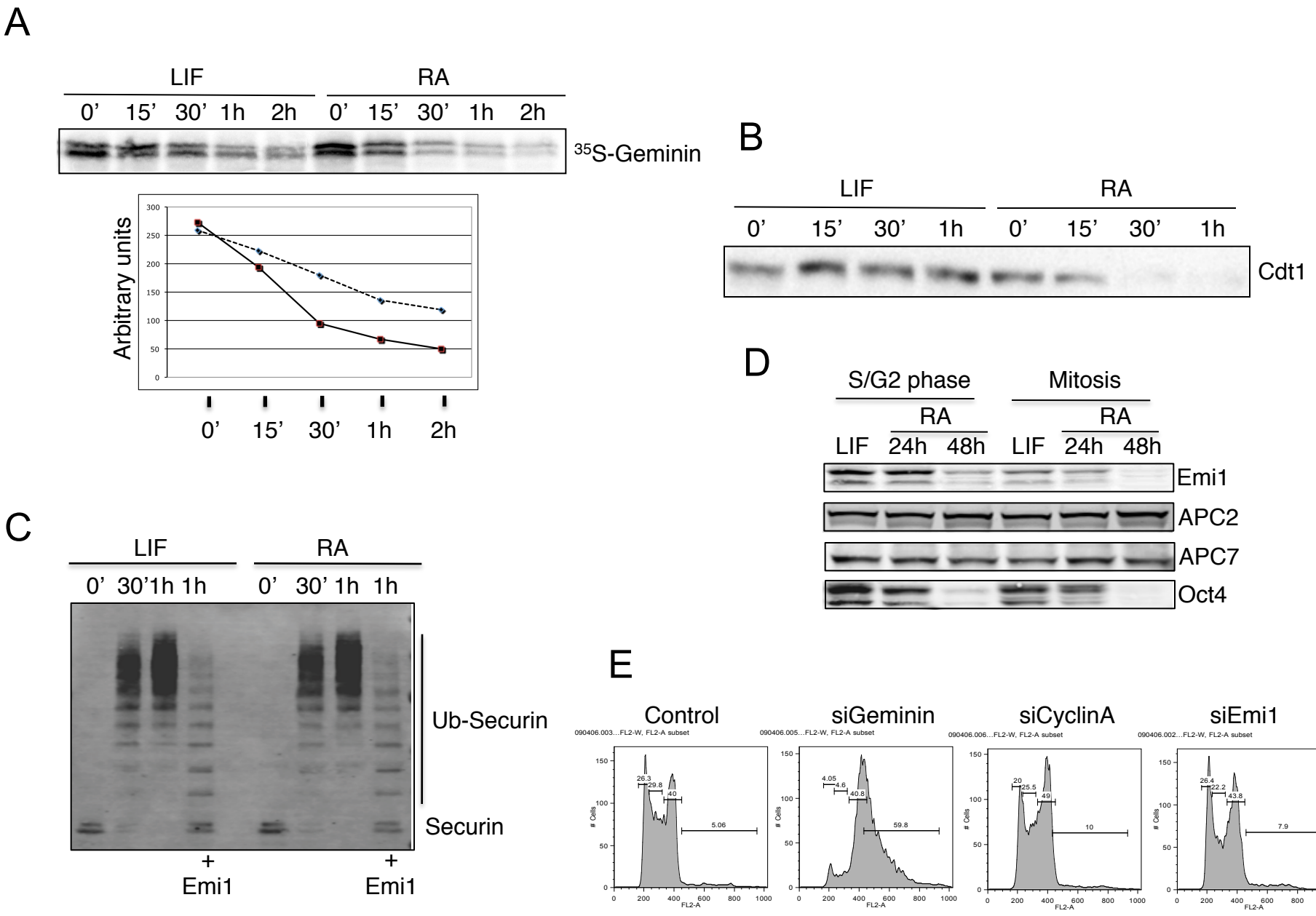
Figure S4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D
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Mitosis
RA
RA
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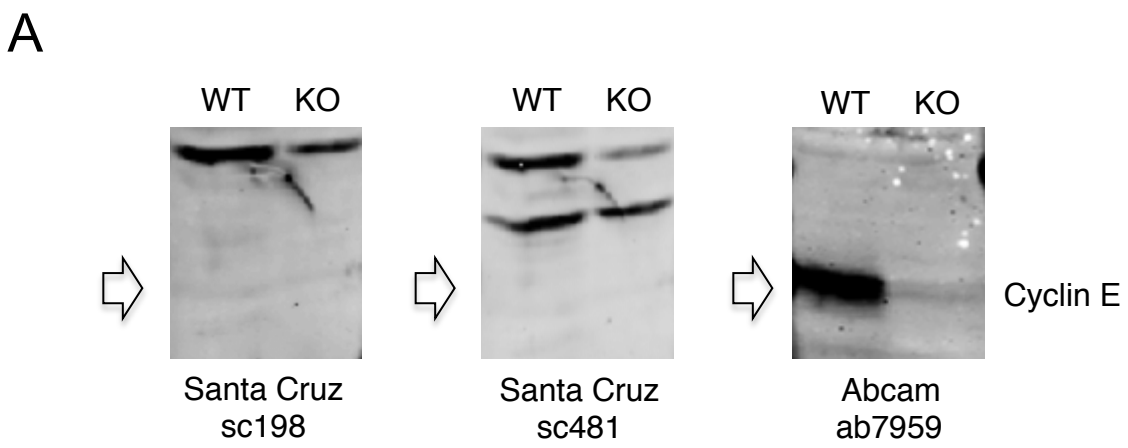
Figure S5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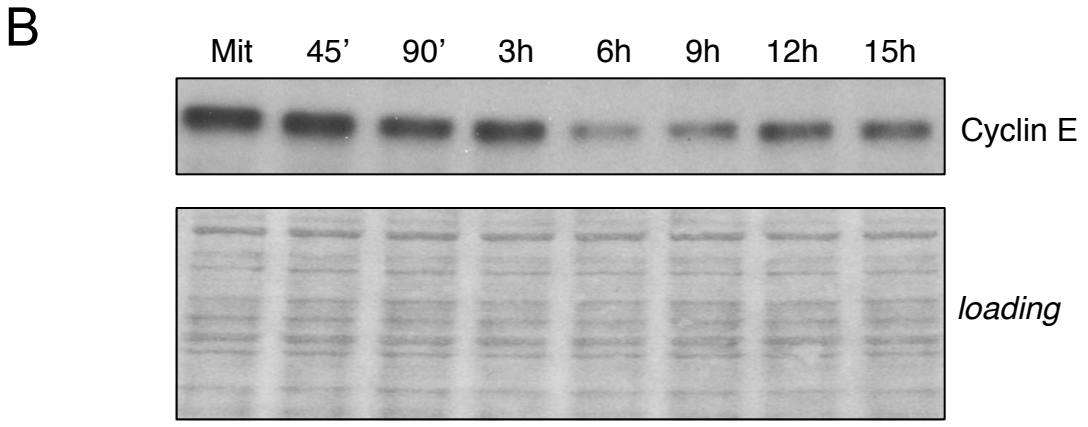
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Cyclin E
loading

Figure S6

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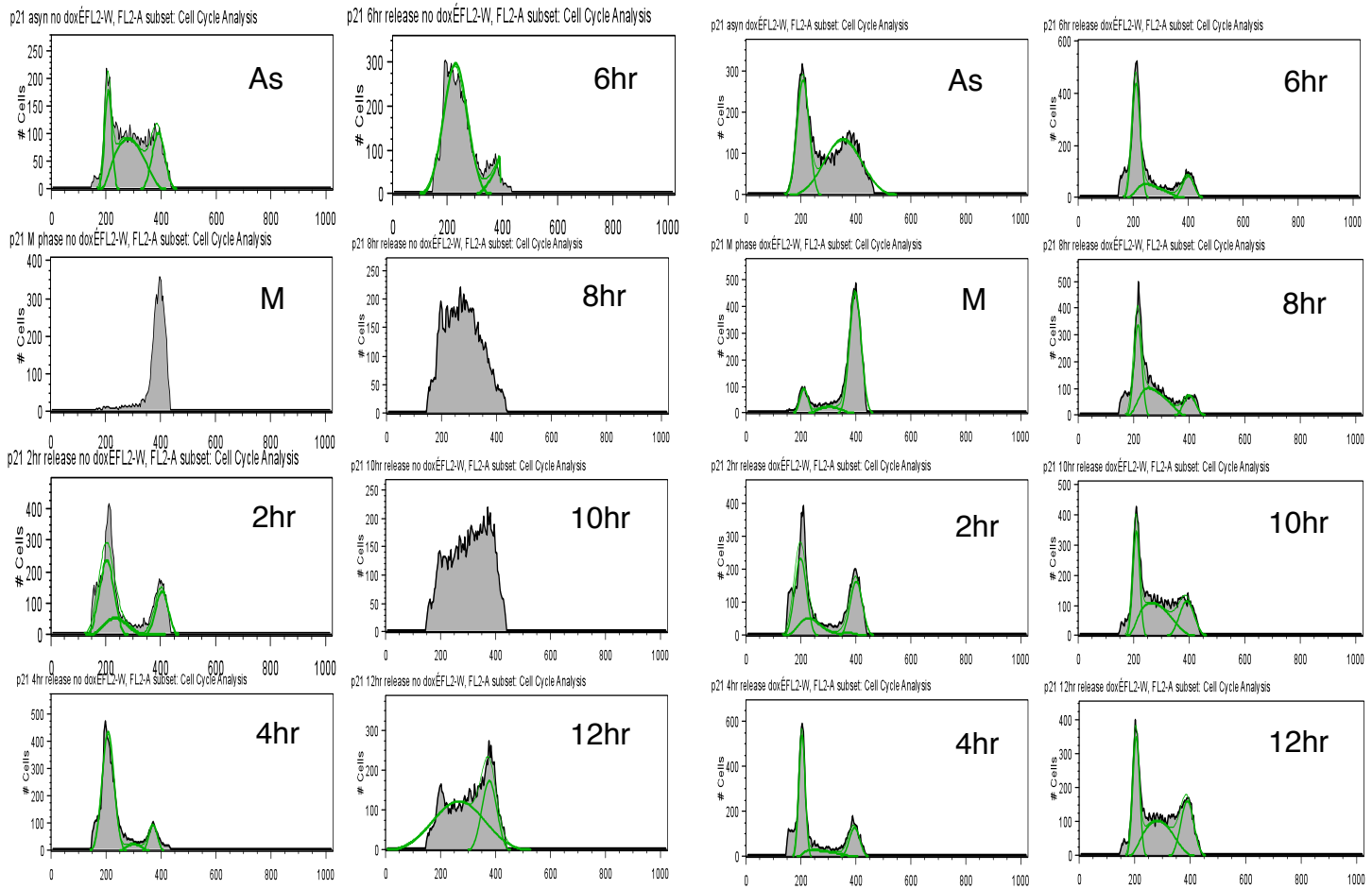
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2hr
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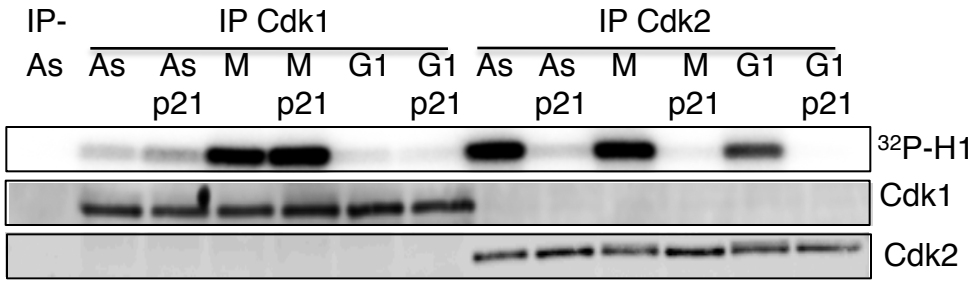
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IP Cdk2
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As p21
M
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G1 p21
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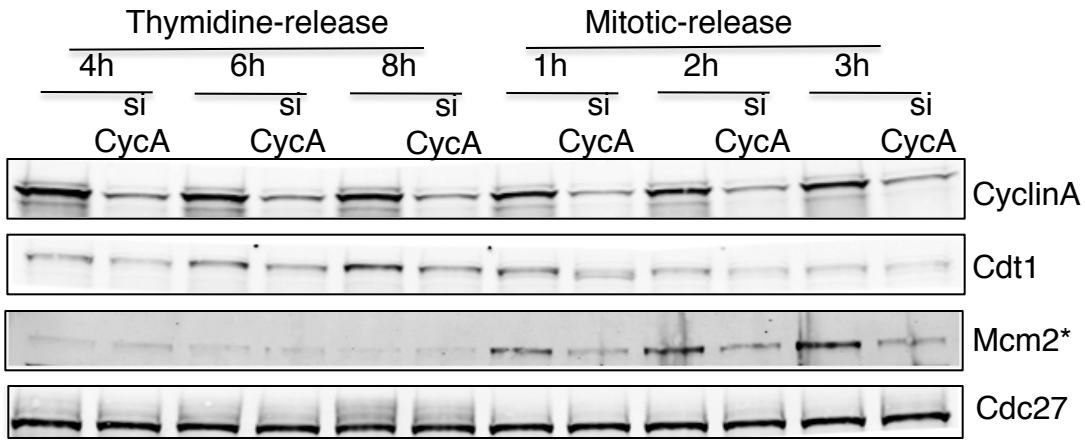
Cdk1
Cdk2

Figure S7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Mitotic-release
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6h
8h
1h
2h
3h
si CycA
CyclinA
Cdt1
Mcm2*
Cdc27

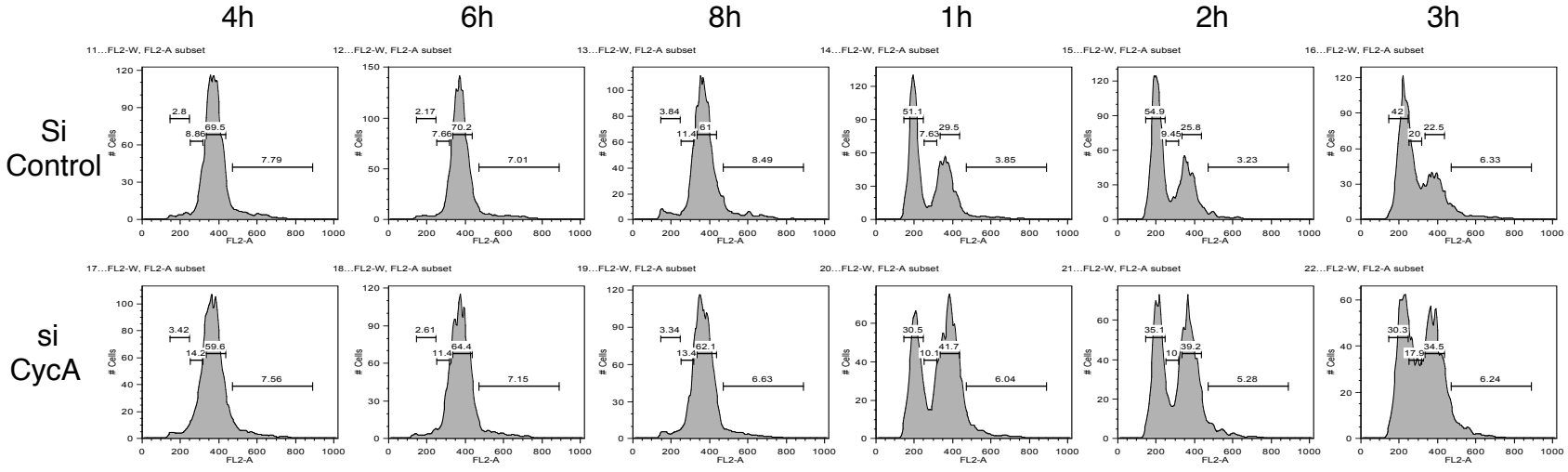
4h
6h
8h
1h
2h
3h
Si Control
si CycA
# Cells
FL2-A

Figure S8

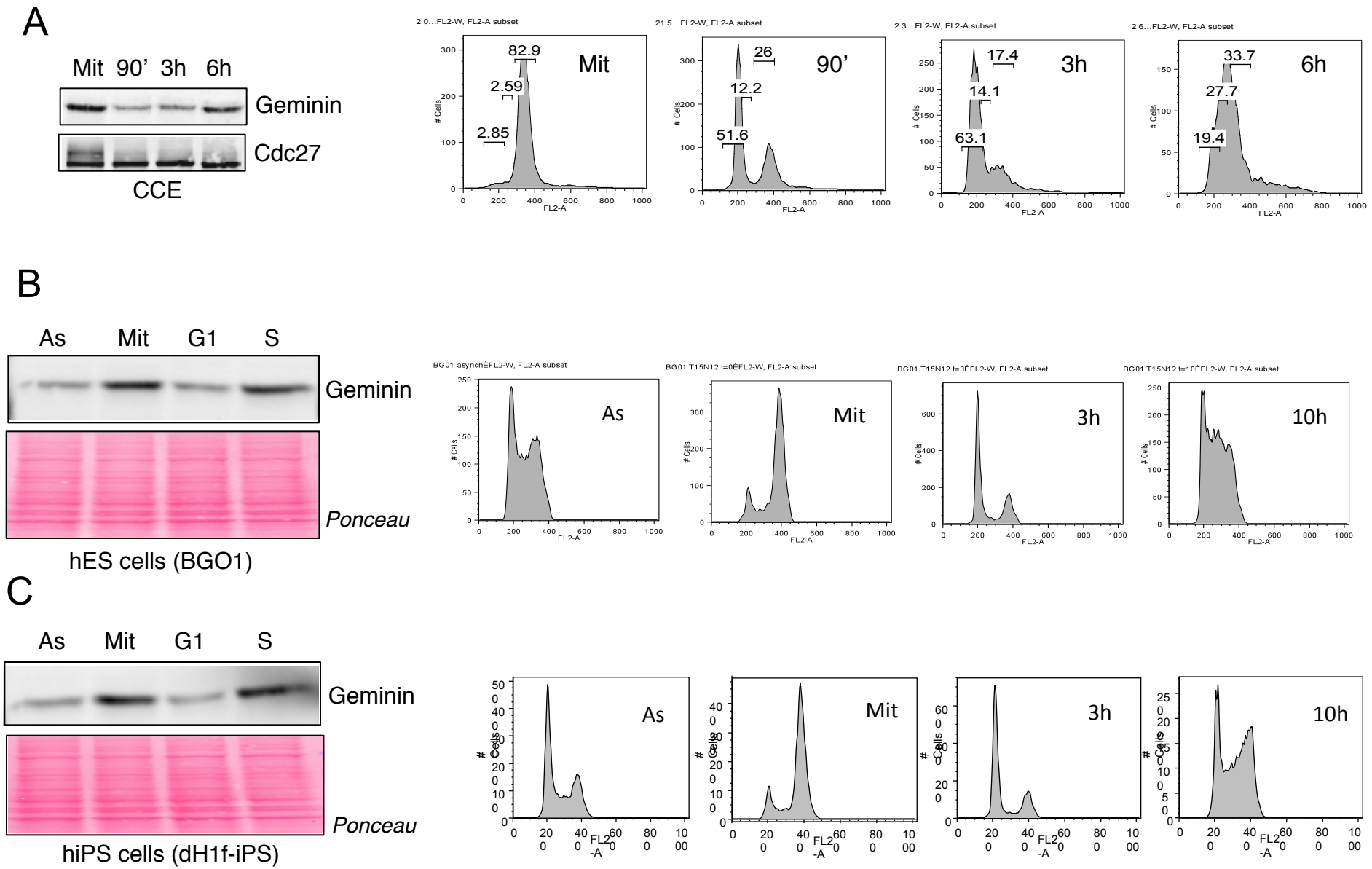
A
Mit 90' 3h 6h
Geminin
Cdc27
CCE
2 0…FL2-W, FL2-A subset
82.9
2.59
2.85
Mit
21.5…FL2-W, FL2-A subset
26
12.2
51.6
90'
2 3…FL2-W, FL2-A subset
17.4
14.1
63.1
3h
2 6…FL2-W, FL2-A subset
33.7
27.7
19.4
6h
# Cells
FL2-A
B
As Mit G1 S
Geminin
Ponceau
hES cells (BGO1)
BG01 asynchÉFL2-W, FL2-A subset
As
BG01 T15N12 t=0ÉFL2-W, FL2-A subset
Mit
BG01 T15N12 t=3ÉFL2-W, FL2-A subset
3h
BG01 T15N12 t=10ÉFL2-W, FL2-A subset
10h
C
As Mit G1 S
Geminin
Ponceau
hiPS cells (dH1f-iPS)
As
Mit
3h
10h
# Cells
FL2-A

Figure S9