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Supplemental Information

PLK1 Activation in Late G2 Sets Up Commitment to Mitosis

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Supplemental information

Supplemental figures

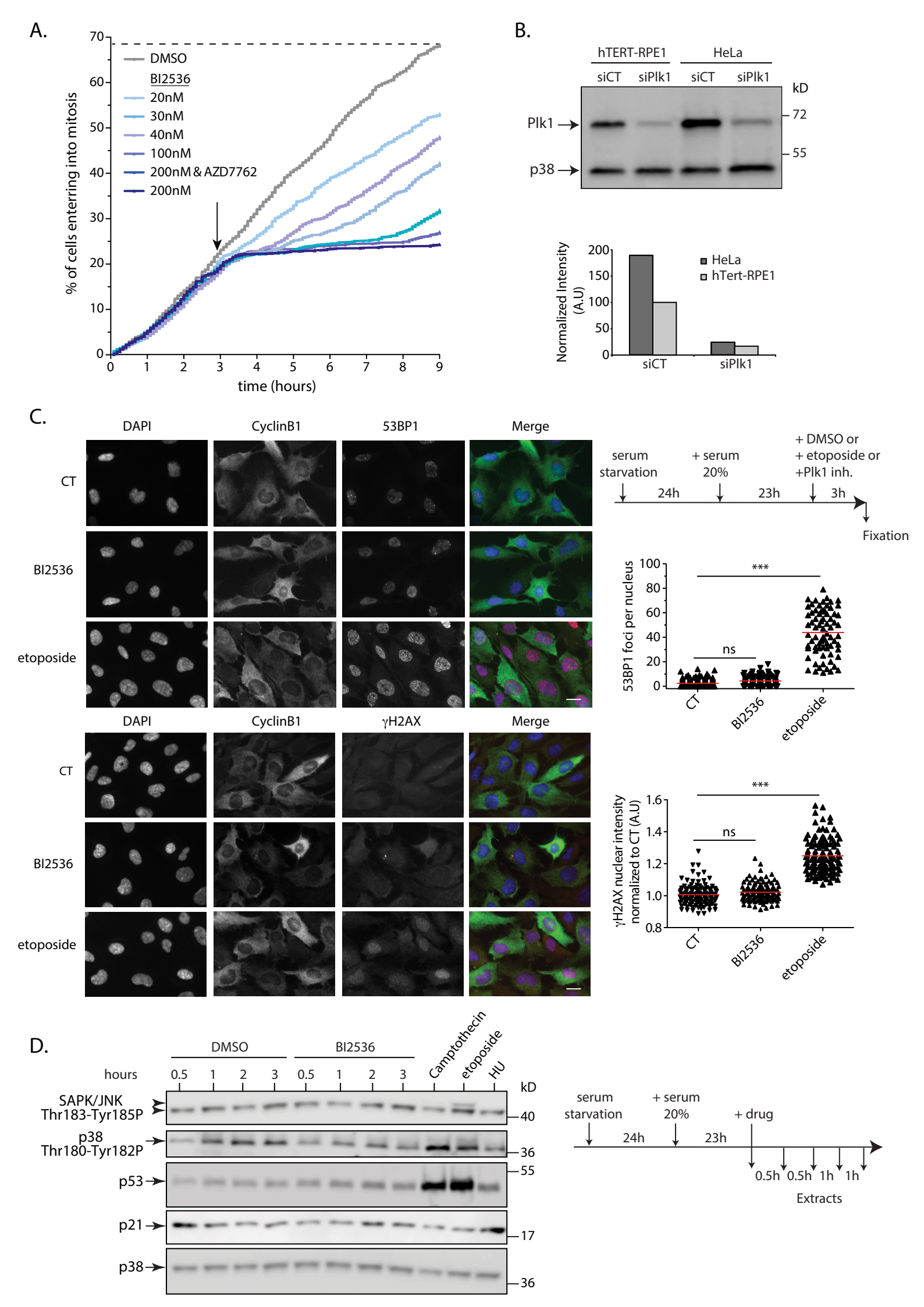

A.
% of cells entering into mitosis
time (hours)
DMSO
BI2536
20nM
30nM
40nM
100nM
200nM & AZD7762
200nM
B.
hTERT-RPE1
HeLa
siCT
siPlk1
siCT
siPlk1
kD
Plk1
p38
72
55
Normalized Intensity (A.U)
HeLa
hTert-RPE1
siCT
siPlk1
C.
DAPI
CyclinB1
53BP1
Merge
CT
BI2536
etoposide
serum starvation
+ serum 20%
+ DMSO or + etoposide or +Plk1 inh.
24h
23h
3h
Fixation
53BP1 foci per nucleus
***
ns
CT
BI2536
etoposide
DAPI
CyclinB1
γH2AX
Merge
γH2AX nuclear intensity normalized to CT (A.U)
***
ns
D.
hours
DMSO
BI2536
Camptothecin
etoposide
HU
kD
SAPK/JNK Thr183-Tyr185P
p38 Thr180-Tyr182P
p53
p21
p38
40
36
55
17
36
serum starvation
+ serum 20%
+ drug
24h
23h
0.5h
0.5h
1h
1h
Extracts

Figure S1 (related to Figure 1)
DNA damage or stress pathways are not induced following Plk1 inhibition during G2
(A) Entry into mitosis of asynchronous hTERT-RPE1 cells was recorded by phase contrast video-microscopy at 1 image/5 min following the addition of Plk1 inhibitor BI2536 (arrow) at increasing concentrations. When indicated, Plk1 (BI2536 200nM) and Chk1&Chk2 (AZD7762 100nM) inhibitors were added simultaneously. n=900-1100 cells analyzed for each condition; two independent experiments. Graphs represent the percentage of cells that have entered into mitosis over time, normalized to cell density. (B) Immunoblot displays Plk1 protein levels in asynchronous HeLa and hTERT-RPE1 cells (siCT) or 24 hours after RNAi-mediated Plk1 depletion (siPlk1). Bottom: Quantification of Plk1 protein levels in the different conditions, normalized to p38. (C) G2-enriched hTERT-RPE1 cells (see schematic scheme) were treated or not (CT: DMSO) with Plk1 inhibitor BI2536 (200nM) or etoposide (25 μM) for three hours. Immunostaining of CyclinB1 and 53BP1 or γH2AX are displayed in the different conditions. Right: Quantification in CyclinB1 positive cells of 53BP1 foci number per nucleus. n=70-170 cells per condition; two independent experiments. ANOVA test was used for statistical analyses; ns: no significant variation; p value <0.0001 *** between etoposide versus CT. For γH2AX signal in CyclinB1 positive cells, whole nuclear signal is normalized to average CT value. n=110-120 cells per condition; two independent experiments. ANOVA test; ns: no significant variation; p value <0.0001 *** between etoposide versus CT. (D) as in (C). G2-enriched hTERT-RPE1 cells were treated or not (DMSO) with Plk1 inhibitor (BI2536 200nM) for 0.5, 1, 2 or 3 hours. Treatments with camptothecin (1μM), etoposide (25μM) or hydroxyurea (HU 1mM) for 3 hours were used as controls. Immunoblots showing phosphorylation profiles of SAPK/JNK (Thr 183 and Tyr 185P) and p38 MAPK (Thr 180 and Tyr 182P) as well as p53 and p21 protein levels. Bar, 10 μm.

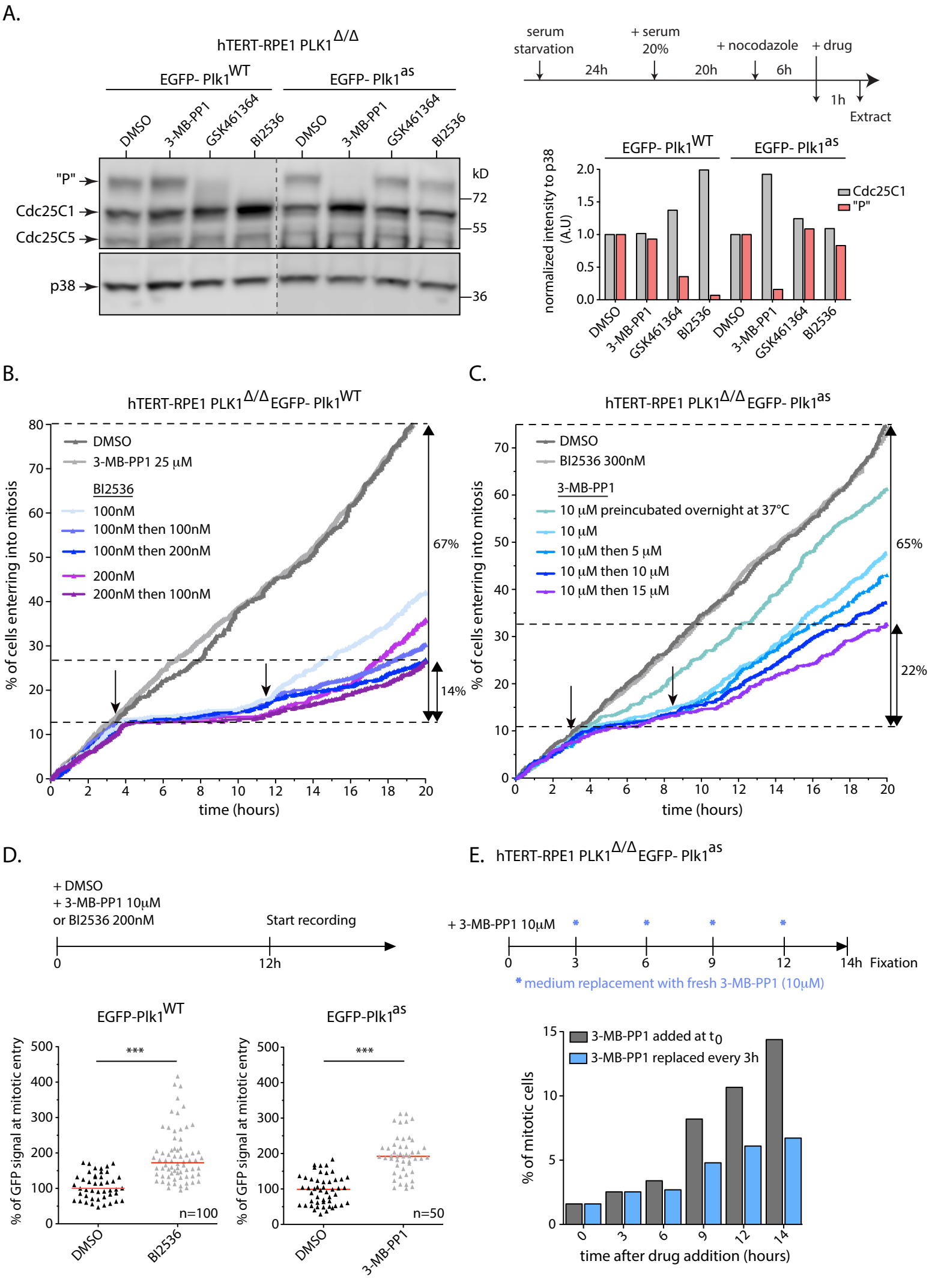

A.
hTERT-RPE1 PLK1Δ/Δ
EGFP- Plk1WT
EGFP- Plk1as
DMSO
3-MB-PP1
GSK461364
BI2536
"P"
Cdc25C1
Cdc25C5
p38
kD
72
55
36
serum starvation
24h
+ serum 20%
20h
+ nocodazole
6h
+ drug
1h
Extract
normalized intensity to p38 (A.U)
Cdc25C1
"P"
B.
hTERT-RPE1 PLK1Δ/Δ EGFP- Plk1WT
% of cells entering into mitosis
time (hours)
DMSO
3-MB-PP1 25 μM
BI2536
100nM
100nM then 100nM
100nM then 200nM
200nM
200nM then 100nM
67%
14%
C.
hTERT-RPE1 PLK1Δ/Δ EGFP- Plk1as
DMSO
BI2536 300nM
3-MB-PP1
10 μM preincubated overnight at 37°C
10 μM
10 μM then 5 μM
10 μM then 10 μM
10 μM then 15 μM
65%
22%
D.
+ DMSO
+ 3-MB-PP1 10μM
or BI2536 200nM
Start recording
0
12h
EGFP-Plk1WT
EGFP-Plk1as
% of GFP signal at mitotic entry
***
n=100
n=50
E.
hTERT-RPE1 PLK1Δ/Δ EGFP- Plk1as
+ 3-MB-PP1 10μM
0
3
6
9
12
14h Fixation
* medium replacement with fresh 3-MB-PP1 (10μM)
3-MB-PP1 added at t0
3-MB-PP1 replaced every 3h
% of mitotic cells
time after drug addition (hours)

Figure S2 (related to Figure 1)
Long term effects of Plk1 inhibition using BI2536 or 3-MB-PP1 ATP analog on mitotic entry
(A) M-phase arrested hTERT-RPE1 *PLK1*$^{\Delta/\Delta}$-EGFP-Plk1^{wt} or -EGFP-Plk1^{as} cells were treated with a bulky purine analog (3-MB-PP1, 10 μM) or a Plk1 inhibitor (GSK461364 250nM or BI2536 200nM) for one hour. Immunoblot showing phosphorylation levels ("P") of Cdc25C1 in each condition. Right: Normalized quantification to p38 level is displayed. (B, C) Entry into mitosis of asynchronous hTERT-RPE1 *PLK1*$^{\Delta/\Delta}$-EGFP-Plk1^{wt} (B) or hTERT-RPE1 *PLK1*$^{\Delta/\Delta}$-EGFP-Plk1^{as} (C) cells over a 20-hour period were recorded by phase contrast video-microscopy at 1 image/5 min. (B) A first treatment was performed at $t_{3.5h}$ using DMSO, BI2536 (100 or 200nM) or 3-MB-PP1 (25 μM). When indicated, a second treatment was performed at $t_{11.5h}$ by adding BI2536 at 100 or 200nM. Note that hTERT-RPE1 *PLK1*$^{\Delta/\Delta}$-EGFP-Plk1^{wt} cells are insensitive to 3-MB-PP1 (25 μM). (C) A first treatment was performed at t_{3h} using DMSO, BI2536 (300nM), 3-MB-PP1 (10 μM) or 3-MB-PP1 (10 μM) pre-incubated in fresh culture medium at 37°C for 12 hours. When indicated, a second treatment was performed at $t_{8.5h}$ by adding 3-MB-PP1 at 5, 10 or 15 μM. Note that hTERT-RPE1 *PLK1*$^{\Delta/\Delta}$-EGFP-Plk1^{as} cells are insensitive to BI2536 (300 nM) and that 3-MB-PP1 pre-incubated at 37°C for 12h lose its potency to inhibit entry into mitosis. (B, C) n=800-1000 cells for each condition; three independent experiments. Graphs represent the percentage of cells that have entered into mitosis over time, normalized to cell density. (D) Quantification of whole EGFP signal, expressed as percent of average CT value, in hTERT-RPE1 *PLK1*$^{\Delta/\Delta}$-EGFP-Plk1^{wt} (n=100 per condition) or EGFP-Plk1^{as} cells (n=50 per condition) that entered into mitosis from 12 hours after DMSO, 3-MB-PP1 (10 μM) or BI2536 (200nM) treatment. (E) Percentage of cells that have entered into mitosis over time (using H3S10P and DAPI staining) after 3-MB-PP1 (10μM) addition at t_0 or following the replacement of the culture medium containing fresh 3-MB-PP1 (10μM) each 3 hours. n=500 cells analyzed per time point; two independent experiments.

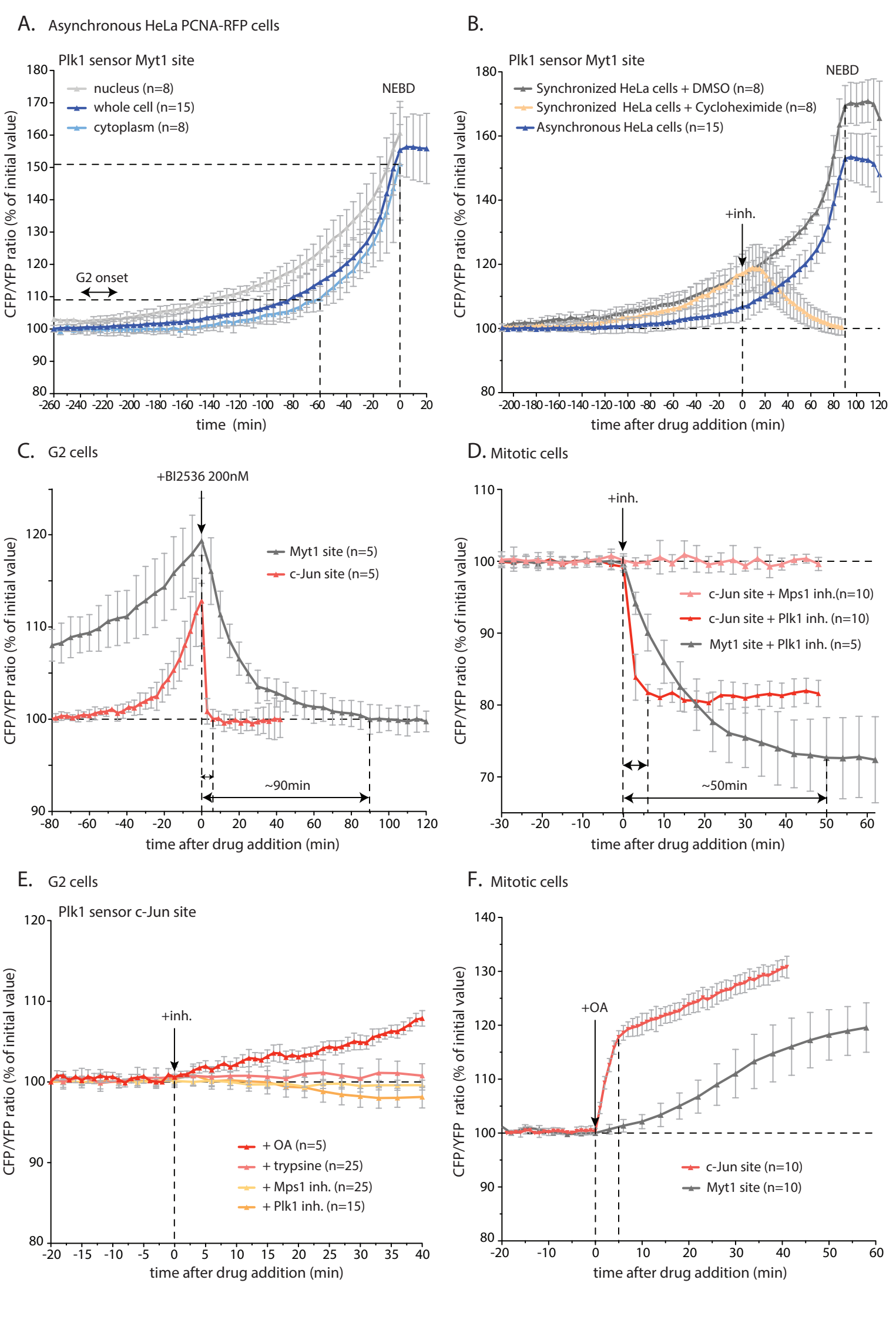

A. Asynchronous HeLa PCNA-RFP cells
Plk1 sensor Myt1 site
nucleus (n=8)
whole cell (n=15)
cytoplasm (n=8)
NEBD
G2 onset
CFP/YFP ratio (% of initial value)
time (min)
B.
Plk1 sensor Myt1 site
Synchronized HeLa cells + DMSO (n=8)
Synchronized HeLa cells + Cycloheximide (n=8)
Asynchronous HeLa cells (n=15)
NEBD
+inh.
CFP/YFP ratio (% of initial value)
time after drug addition (min)
C. G2 cells
+BI2536 200nM
Myt1 site (n=5)
c-Jun site (n=5)
~90min
CFP/YFP ratio (% of initial value)
time after drug addition (min)
D. Mitotic cells
+inh.
c-Jun site + Mps1 inh.(n=10)
c-Jun site + Plk1 inh. (n=10)
Myt1 site + Plk1 inh. (n=5)
~50min
CFP/YFP ratio (% of initial value)
time after drug addition (min)
E. G2 cells
Plk1 sensor c-Jun site
+inh.
+ OA (n=5)
+ trypsine (n=25)
+ Mps1 inh. (n=25)
+ Plk1 inh. (n=15)
CFP/YFP ratio (% of initial value)
time after drug addition (min)
F. Mitotic cells
+OA
c-Jun site (n=10)
Myt1 site (n=10)
CFP/YFP ratio (% of initial value)
time after drug addition (min)

Figure S3 (related to Figure 2)
Characterization of c-Jun-based Plk1 phosphorylation sensor
(A) Asynchronous PCNA-RFP HeLa cell line transiently expressing the Myt1-based Plk1 sensor. Recording was performed at 1 image/5 min. Displayed are whole cell (n=15) or cytoplasmic and nuclear (n=8) CFP/YFP emission ratio changes over time. G2 onset is defined from the disappearance of PCNA foci. Curves were aligned on NEBD. Data are means ± SD; two independent experiments. (B) Comparison of whole cell CFP/YFP ratio changes in asynchronous versus synchronized (thymidine-release regime) Myt1-based sensor-expressing HeLa cells, following or not (CT: DMSO) protein synthesis inhibition (cycloheximide 10μM) at ~7 hours post-thymidine release. 1 image/5 min. Data are means ± SD; two independent experiments. Note that protein synthesis inhibition rapidly blocks CFP/YFP ratio increase. (C) Whole CFP/YFP emission ratio changes following Plk1 inhibition (BI2536 200nM) at 7 hours post-release in synchronized Myt1-based sensor-expressing HeLa cells. Comparison with Plk1 inhibition (BI2536 200nM) in late G2 (8 hours post-release) in synchronized c-Jun-based sensor-expressing HeLa cells (see also Figure 2B). Curves were aligned on the time of BI2536 addition. Data are means ± SD; two independent experiments. (D) Mitotic arrested HeLa cells (thymidine-release + nocodazole) expressing either Myt1-based or c-Jun-based Plk1 sensor were treated with Mps1 (reversine 5μM + MG132) or Plk1 (BI2536 200nM) inhibitor. Mean quantification curves (± SD) of emission ratio changes are displayed. (C, D) Note the differential dephosphorylation kinetics of both sensors following Plk1 inhibition during G2 and mitosis. (E) C-Jun-based sensor-expressing HeLa cells. G2-enriched (7 hours post-thymidine release) cells were treated with either Mps1 (reversine 5μM), Plk1 (BI2536 200nM), PP2A&PP1 (okadaic acid 250nM) inhibitor or the serine protease trypsin. Data are means ± SD.
(F) as in (D). Mitotic arrested HeLa cells expressing either Myt1-based or c-Jun-based Plk1 sensor were treated with okadaic acid (OA 250nM). Data are means ± SD, n=10.

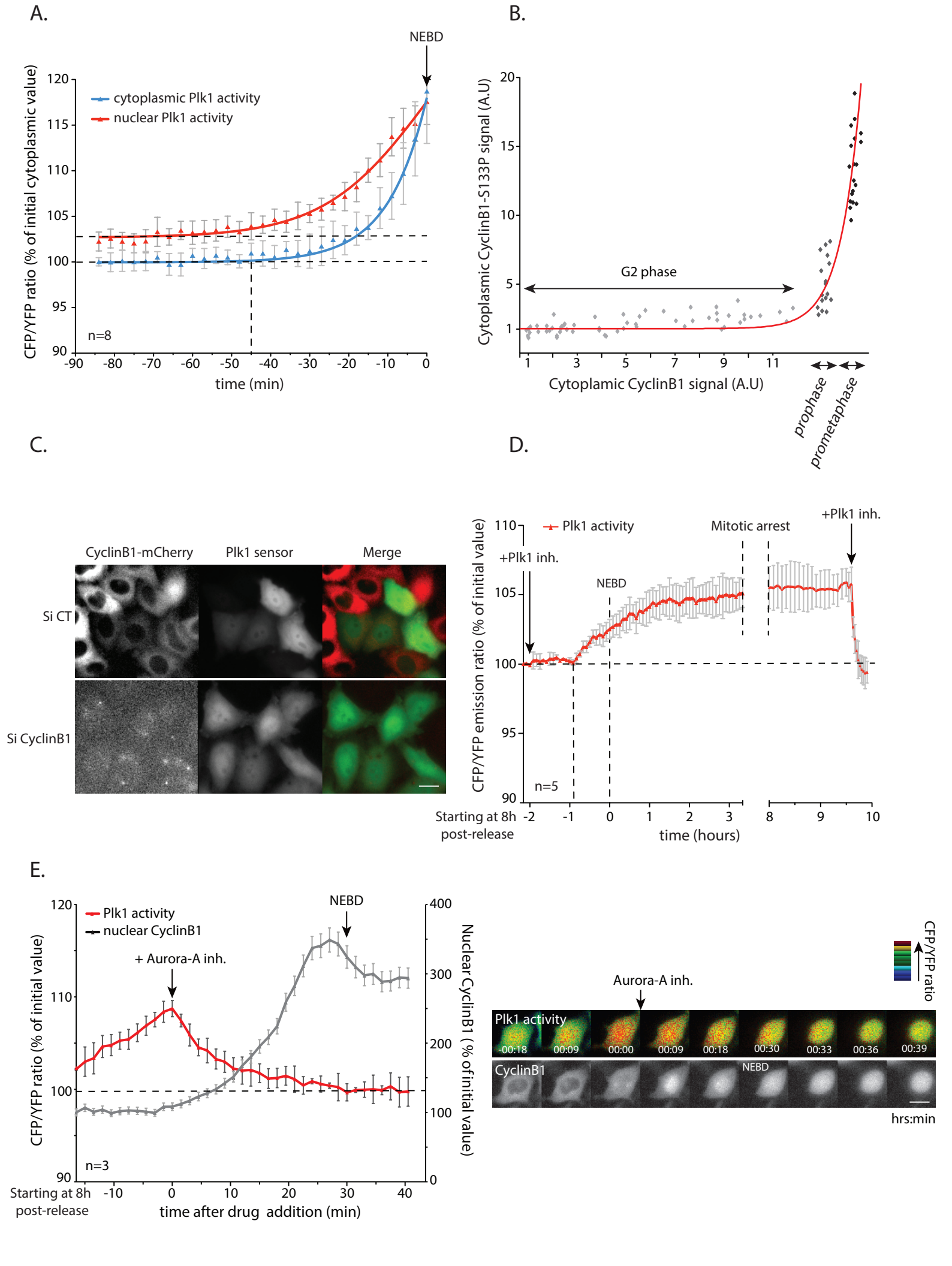

A.
NEBD
cytoplasmic Plk1 activity
nuclear Plk1 activity
CFP/YFP ratio (% of initial cytoplasmic value)
n=8
time (min)
B.
Cytoplasmic CyclinB1-S133P signal (A.U)
G2 phase
Cytoplamic CyclinB1 signal (A.U)
prophase
prometaphase
C.
CyclinB1-mCherry
Plk1 sensor
Merge
Si CT
Si CyclinB1
D.
Plk1 activity
+Plk1 inh.
Mitotic arrest
+Plk1 inh.
NEBD
CFP/YFP emission ratio (% of initial value)
n=5
Starting at 8h post-release
time (hours)
E.
Plk1 activity
nuclear CyclinB1
+ Aurora-A inh.
NEBD
CFP/YFP ratio (% of initial value)
Nuclear CyclinB1 (% of initial value)
n=3
Starting at 8h post-release
time after drug addition (min)
Aurora-A inh.
CFP/YFP ratio
Plk1 activity
CyclinB1
NEBD
hrs:min

Figure S4 (related to Figure 2 and Figure 3)
Regulation of Plk1 activity at G2/M transition
(A) Comparison of cytoplasmic versus nuclear CFP/YFP emission ratio changes in asynchronous HeLa cells transiently expressing (c-Jun based) Plk1 sensor. Recording was performed at 1 image/3 min. Curves were aligned on NEBD. Data are means ± SD. n=8; two independent experiments.
(B) Quantification of cytoplasmic CyclinB1-S133P and CyclinB1 immunofluorescent signals from asynchronous hTERT-RPE1 cells. n>100.
(C) Representative images of CyclinB1 depletion efficiency in CyclinB1-mCherry - Plk1 sensor HeLa stable cell line. Images were acquired on G2 synchronized cells, ~30h after RNAi treatment.
(D) Plk1 sensor-expressing HeLa cells that still achieved mitotic entry following Plk1 inhibition (BI2536 200nM at t_{8h} post-release) were recorded at 1 image/5 min and 12 hours later, Plk1 inhibitor concentration was increased by two-fold (BI2536 400nM) (1 image/min). Data are mean ± SD. n=5; two independent experiments.
(E) Synchronized CyclinB1-mcherry HeLa cells transiently expressing the Plk1 sensor were treated with Aurora-A inhibitor MLN8054 at mitosis onset and recorded at 1 image/1.5 min. Mean quantification curves (± SD) of CFP/YFP emission ratio changes are displayed. n=3; three independent experiments. Right: IMD representation of CFP/YFP ratio changes and nuclear CyclinB1 translocation before and after treatment. Bars, 10 μm.

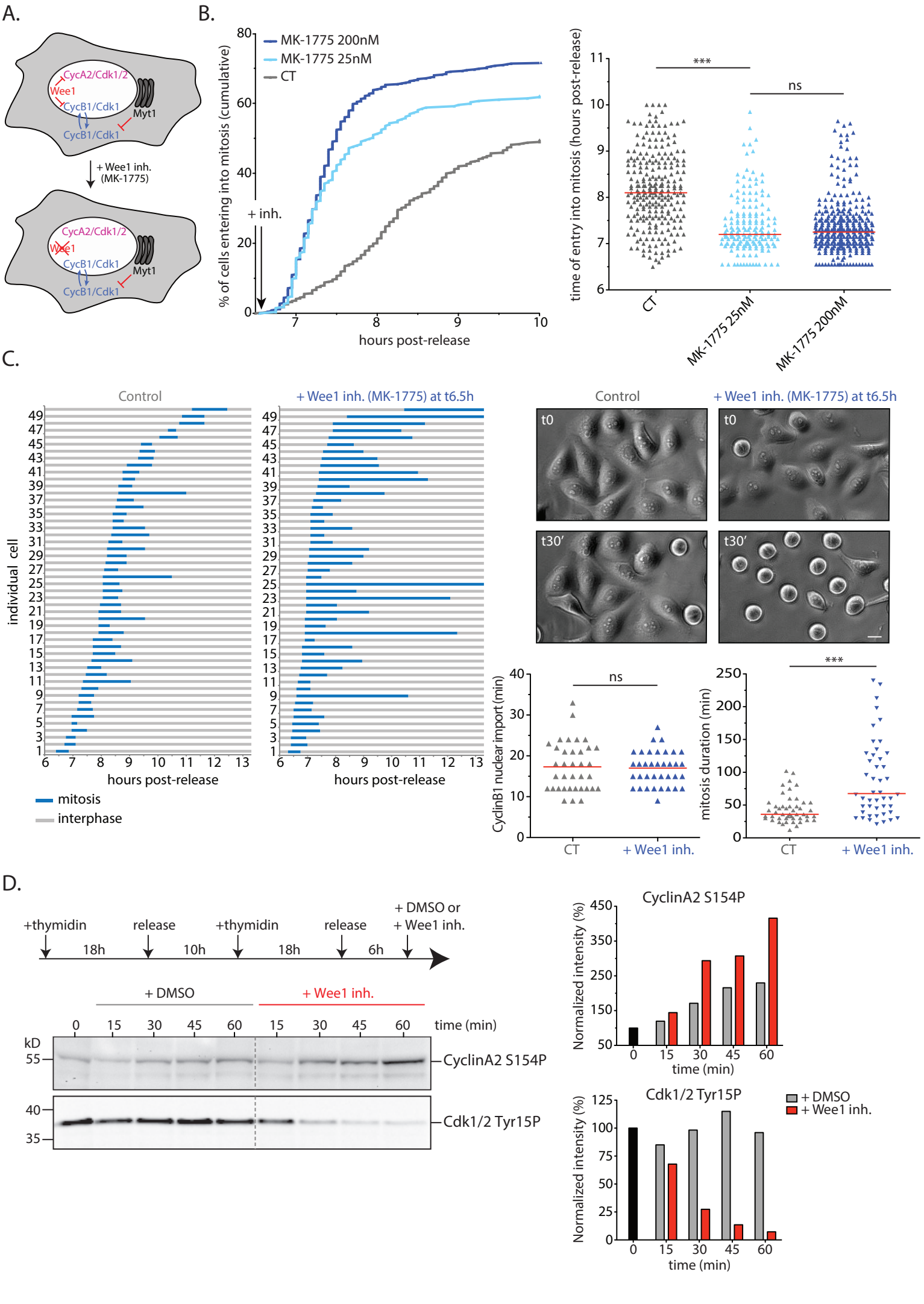
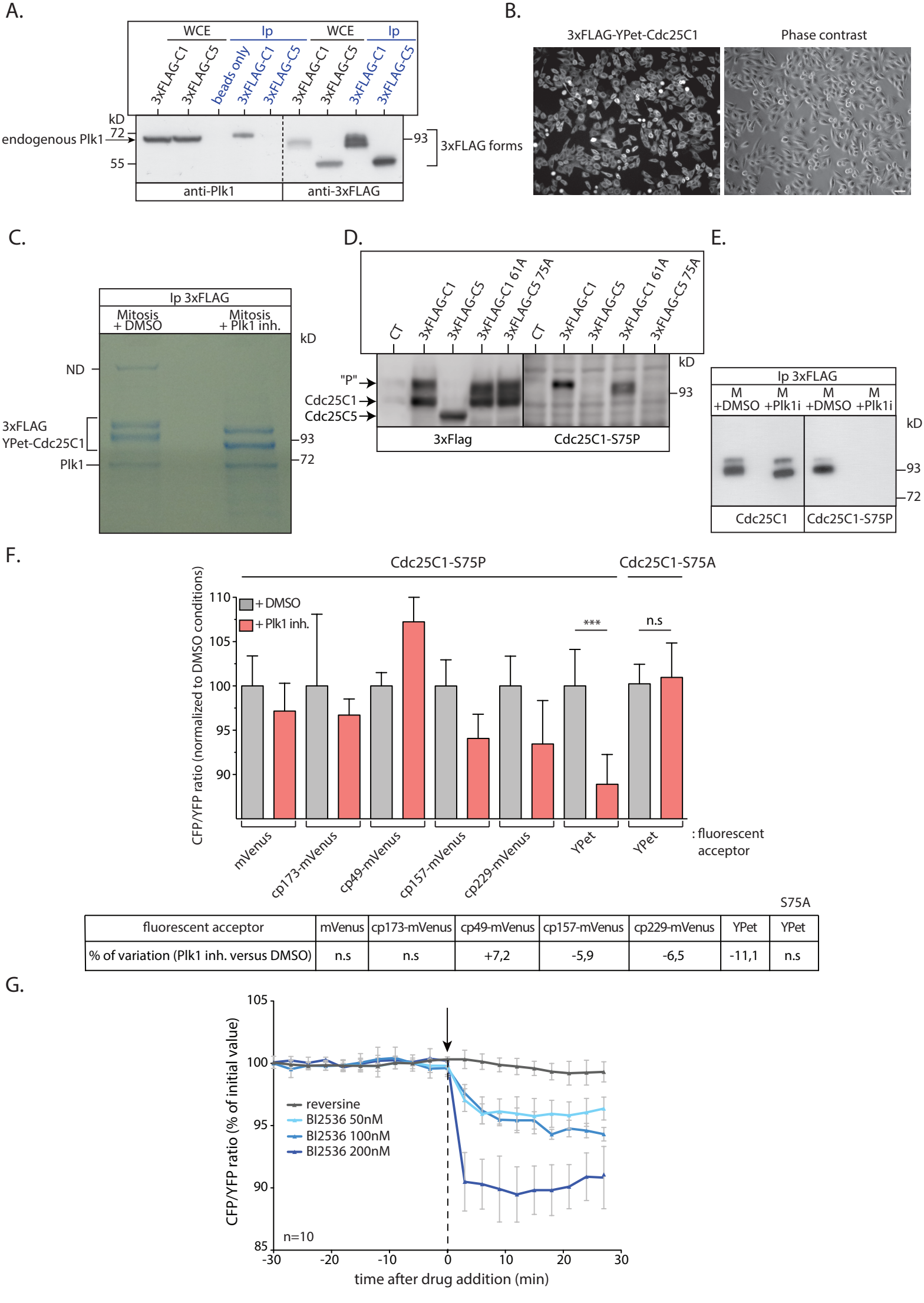

A.
CycA2/Cdk1/2
Wee1
CycB1/Cdk1
Myt1
CycB1/Cdk1
+ Wee1 inh.
(MK-1775)
CycA2/Cdk1/2
Wee1
CycB1/Cdk1
Myt1
CycB1/Cdk1
B.
MK-1775 200nM
MK-1775 25nM
CT
% of cells entering into mitosis (cumulative)
+ inh.
hours post-release
time of entry into mitosis (hours post-release)
***
ns
CT
MK-1775 25nM
MK-1775 200nM
C.
Control
+ Wee1 inh. (MK-1775) at t6.5h
individual cell
hours post-release
mitosis
interphase
t0
t30'
CyclinB1 nuclear import (min)
ns
mitosis duration (min)
***
CT
+ Wee1 inh.
D.
+thymidin
18h
release
10h
+thymidin
18h
release
6h
+ DMSO or
+ Wee1 inh.
+ DMSO
+ Wee1 inh.
time (min)
kD
55
CyclinA2 S154P
40
35
Cdk1/2 Tyr15P
CyclinA2 S154P
Normalized intensity (%)
time (min)
Cdk1/2 Tyr15P
+ DMSO
+ Wee1 inh.

Figure S5 (related to Figure 4)
Cell cycle progression following Wee1 inhibition
(A) Schematic representation of Wee1 inhibition strategy. Intracellular localization of CyclinA2-Cdk, CyclinB1-Cdk1, Wee1 and Myt1 proteins are displayed.
(B) Early G2 synchronized HeLa cells were treated, except in control (CT, + DMSO), by Wee1 inhibitor MK-1775 at $t_{6.5h}$, using indicated concentrations. Cumulative curves of cells entering into mitosis over time, normalized to cell density, are displayed (n=200-500 cells per condition). Right: Comparison of times of entry into mitosis following or not Wee1 inhibition. Statistical analyses (t-test), ns: no significant difference, p value <0.001 ***.
(C) Display of cell cycle progression of 50 individual HeLa cells stably expressing CyclinB1-mCherry recorded in the absence (CT, DMSO) or presence of Wee1 inhibitor MK-1775 added at $t_{6.5h}$ post-release. Right: representative phase contrast images. Bottom: quantification of the mean duration of CyclinB1 nuclear translocation in prophase (n= 40) and mitosis duration (from NEDB to anaphase onset) (n=50), following or not Wee1 inhibition. Statistical analyses (t-test), ns: no significant difference, p value <0.001 ***.
(D) Immunoblot showing phosphorylation profiles of CyclinA2-S154P and Cdk1/2 Tyr15P over a one-hour period, following or not Wee1 inhibition (MK-1775) at t_{6h} post-release. Right: normalized quantitation of the corresponding immunoblot.
Bar, 10 μm.



fluorescent acceptor	mVenus	cp173-mVenus	cp49-mVenus	cp157-mVenus	cp229-mVenus	YPet	YPet
% of variation (Plk1 inh. versus DMSO)	n.s	n.s	+7,2	-5,9	-6,5	-11,1	n.s

Figure S6 (related to Figure 6)
Plk1 targets the Cdc25C1 phosphatase
(A) HeLa cells transiently expressing 3xFLAG-YPet-Cdc25C1 or 3xFLAG-YPet-Cdc25C5 construct were synchronized into mitosis (thymidine-release + nocodazole). Following mitotic check off, immunoprecipitations were performed using anti-FLAG (M2) antibody except in control (beads only). Immunoblot showing the level of indicated proteins. WCE: whole cell extract.
(B, C, E) 3xFLAG-YPet-Cdc25C1 HeLa cell line used for mass spectrometry assays.
(B) Representative YFP emission and phase contrast images of HeLa cells stably expressing 3xFLAG-YPet-Cdc25C1 construct. Note the cytoplasmic distribution of Cdc25C1 in interphase cells.
(C) Colloidal blue staining gel of immunoprecipitated 3xFLAG-YPet-Cdc25C1 from mitotic arrested HeLa cells (+ nocodazole) incubated or not with Plk1 inhibitor (BI2536 200nM) for 1 hour. Note that the interaction between Plk1 and 3xFLAG-YPet-Cdc25C1 is independent of Plk1 activity. ND: Not Determined.
(D) HeLa cells transiently expressing 3xFLAG-YPet-Cdc25C1, -Cdc25C5, -Cdc25C1 S61A or -Cdc25C1 S75A construct were synchronized into mitosis (thymidine-release + nocodazole). Immunoblot showing the level of indicated proteins (left) and Ser75 phosphorylation state (right).
(E) Immunoblot showing immunoprecipitated 3xFLAG-YPet-Cdc25C1 from mitotic arrested HeLa cells, incubated or not with Plk1 inhibitor BI2536 (200nM). Right: Cdc25C1 Ser75 phosphorylation state was analyzed using the corresponding phospho-specific antibody.
(F, G) Specificity of Cdc25C1-S75P FRET sensor.
(F) CFP/YFP emission ratio of mitotic arrested HeLa cells expressing different constructs used to generate a Cdc25C1-S75P phosphorylation sensor, following or not Plk1 inhibition (BI2536 200nM) for 45min. Data are means ± SD. n=10 cells per condition; two independent experiments. Statistical analyses (t-test), ns: no significant difference, p value <0.001 ***. Table: Percentages of variation of CFP/YFP ratio between Plk1 inhibition versus DMSO conditions.
(G) Mitotic arrested Cdc25C1-S75P sensor-expressing HeLa cells were treated with either a Plk1 (BI2536 at indicated concentrations) or Mps1 (reversine + MG132) inhibitor. Displayed are means ± SD of the CFP/YFP emission ratio. n=10 cells per condition. Recordings 1 image/3 min.
Bar, 20 μm.

A.

(3xFLAG-YPet)-Cdc25C1 sequence coverage (%)				HsCdc25C1 sequence coverage (%)		
trypsin	trypsin + GluC	AspN	Total	trypsin	AspN	Total
62	77.8	29.4	87.1	36.2	8	44.2

B.

Phosphorylated residue	(3xFLAG-YPet)-cdc25C1						HsCdc25C1 (endogenous)				upstream kinase	Phospho proteome analyses
	trypsin	Phospho RS (%)	trypsin + GluC	Phospho RS (%)	AspN	Phospho RS (%)	trypsin	Phospho RS (%)	AspN	Phospho RS (%)		
S7			X	94.27								
S14			X	99.14								X
S15	X	77.76	X	99.14								X
S17	X	87.72	X	88.93								X
S20	X	99.87	X	99.94							Plk1	
S38	X	99.63	X	49.73 for S38 orT37							Plk1	X
T48	X	100	X	100	X	100					Cdk1	X
S57			X	100								
S61			X	99.96							Plk1	X
S64	X	96.64	X	99.41			X	99.84				X
T67	X	99.99	X	99.8							Cdk1	X
S75			X	100							Plk1	
S78 or S79					X	?						
S90					X	96.35			X	83.90		
T87, T88 or S90			X	33.33 for each					X	49.78		
S96					X	100						
S101					X	100						
S102					X	100					Plk1	
S122	X	100	X	100			X	100			Cdk1	X
S129 or T130	X	50 for each	X	50 for each								X
T130	X	99.56									Cdk1	
S146, 147 or 148	X	33.33 for each			X	33.33 for each	X	33.33 for each				X
S148	X	99.4										X
S161					X	84.91						
S168	X	99.93	X	99.97	X	99.48	X	91.51			Cdk1	X
T172			X	89.80								
S191			X	100	X	100					Plk1/3	X
S198			X	100	X	100					Plk1	X
S207			X	99.83								
S209			X	99.61								
S214	X	98.35	X	100							Cdk1	X
S216	X	93.28	X	99.59							Chk1/2	
T236	X	100	X	100								
S247	X	99.99										
T268					X	99.96					Plk1	
S472			X	99.68	X	100						

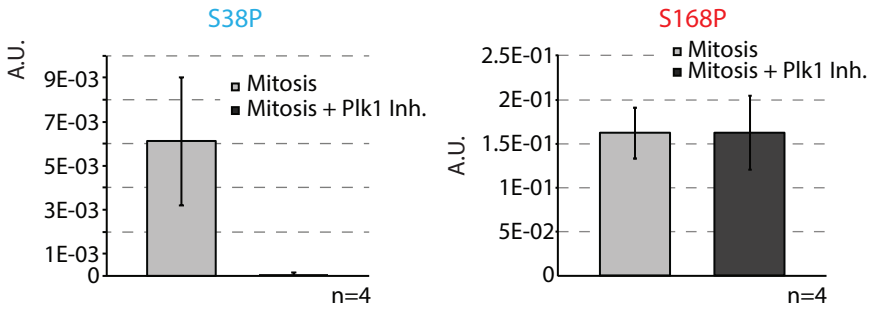
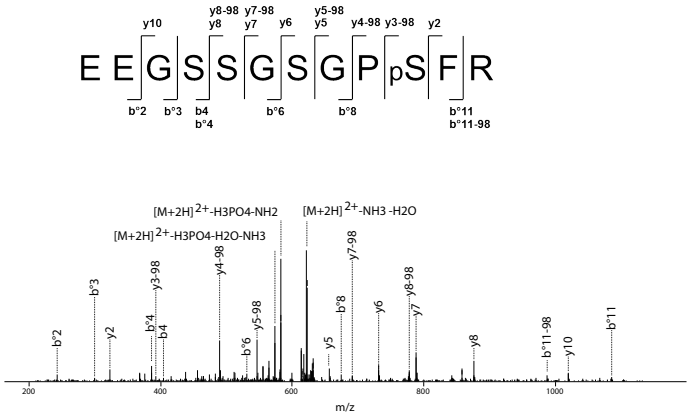
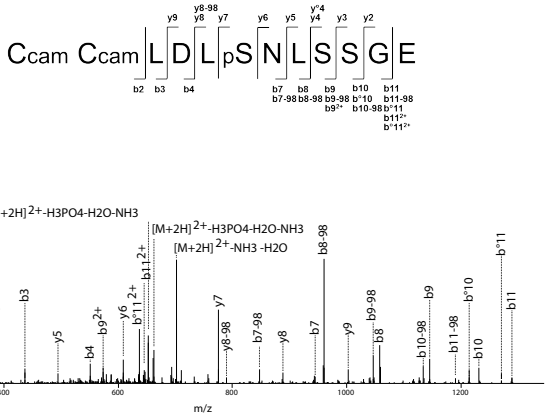


Table S1 (related to Figure 6)
Identification of Plk1-dependent Cdc25C1 phosphorylation sites
(A) Percentage coverage of exogenous (3xFLAG-YPet)-Cdc25C1 and endogenous HsCdc25C1 protein sequence by mass spectrometry analyzes following indicated protease digestions.
(B) Mitotic Cdc25C1 phosphorylation sites identified in the present work. Indicated are percentages of confidence. Sites phosphorylated in a Plk1-dependent manner (± Plk1 inhibitor BI2536 for 1h) are in blue. Cdk1/2-dependent phosphorylation sites are displayed in red. Phosphorylation sites identified by previous phosphoproteome analyzes are indicated in the right column. Bottom panels: Mean phosphorylation levels (± SD) of Plk1 and Cdk1-dependent Ser38 and Ser168 sites, respectively, in the presence or absence of Plk1 inhibitor. n=4.

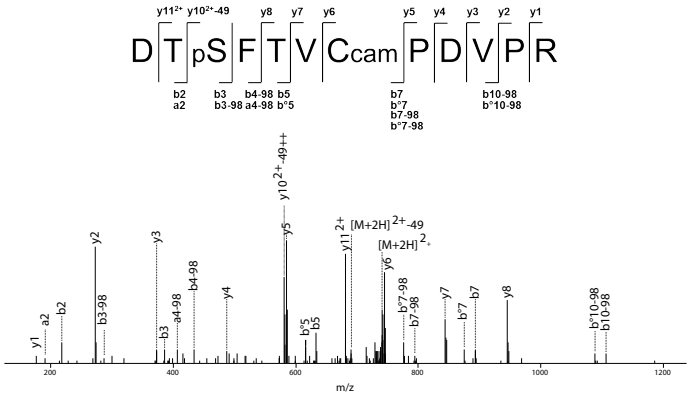
S20



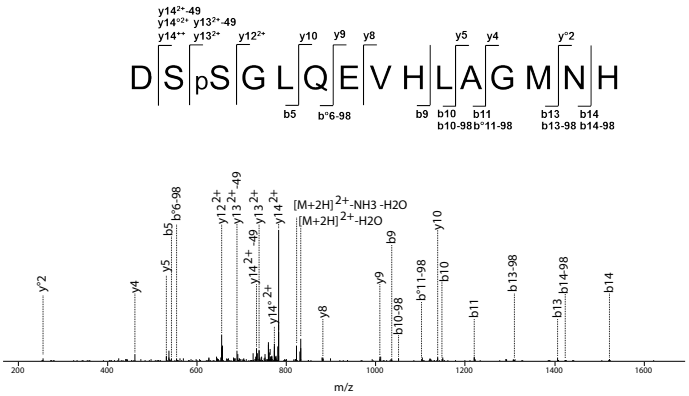
S75



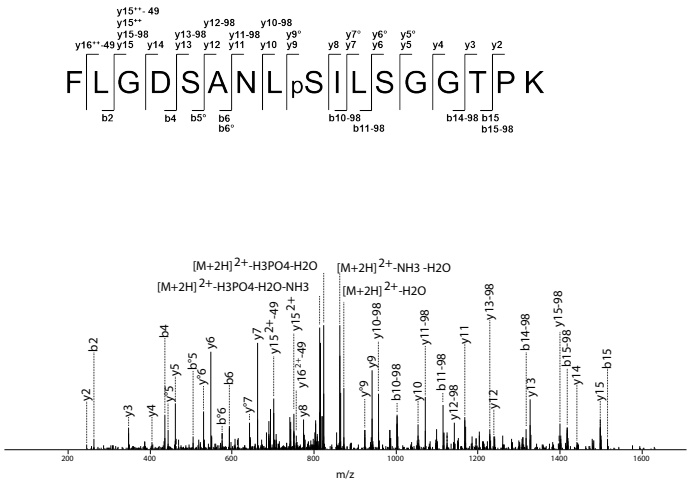
S38



S102



S61



T268

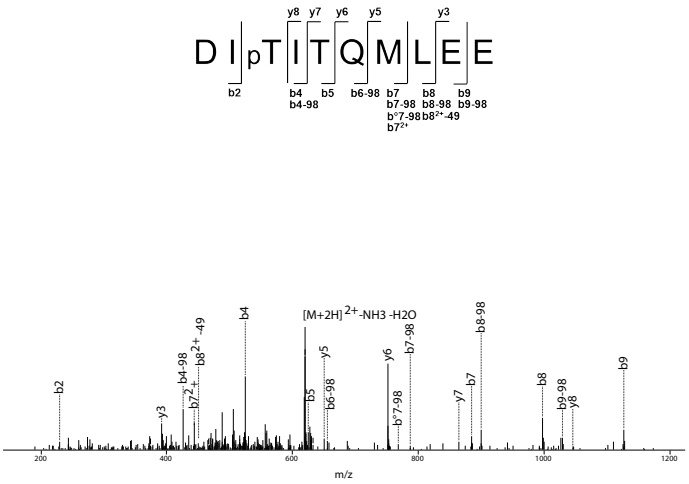


Figure S7 (related to Figure 6)
Phosphopeptide sequence of Plk1-dependent Cdc25C1 phosphorylation sites
Liquid chromatography-MS/MS spectra of Cdc25C1 phosphopeptides containing Plk1-dependent phosphorylation sites; Ser20 (EEGSSGSGP^{20}SFR: 638.75$^{(2+)}$*m/z*, trypsin), Ser38 (DT^{38}SFTVCPDVPR: 679.36 $^{(2+)}$*m/z*, Trypsin/GluC), Ser61 (FLGDSANL^{61}SILSGGTPK: 878.93$^{(2+)}$*m/z*, Trypsin/GluC), Ser75 ($C_{cam}C_{cam}$LDL^{75}SNLSSGE: 717.77$^{(2+)}$*m/z*, Trypsin/GluC), Ser102 (DS^{102}SGLQEVHLAGMNH: 837.85$^{(2+)}$*m/z*, AspN) and Thr268 (DI^{268}TITQMLEE: 636.78 ($^{2+}$)*m/z*, AspN). The fragmentation spectra are trypsin, Trypsin/GluC or AspN derived peptides and show the peptide sequence based on the interpretation of the observed ions obtained. Tandem mass spectrums were labeled to show singly and doubly charged *b* and *y* ions, as well as ions corresponding to neutral losses of water (*circles* °), NH3 (*asterisks* *) and H3PO4 (*98 Da and 49$^{(2+)}$ Da*) groups; *M*: parent ion mass; *Cam*, carbamidomethyl cysteines are shown.

Supplemental experimental procedures

Reagents
Chemical compounds dissolved in DMSO were used at the following concentrations unless stated otherwise: BI2536 (Axon Medchem) 200nM, BI6727 (Axon Medchem) 200nM, reversine (Axon Medchem) 5 μM, AZD7762 (Axon Medchem) 100nM, MLN8054 (Selleckchem) 2.5 μM, MK-1775 (Selleckchem) 200nM, okadaic acid (Santa Cruz Biotechnology) 250nM, MG132 (Tocris Bioscience) 1 μM, PD166285 (Tocris Bioscience) 50 nM, GSK461364 (MedChem Express) 250nM, caffeine (Sigma Aldrich) 5 μM, Nocodazole (Sigma Aldrich) 100nM and 3-MB-PP1 (Calbiochem) 10 μM. Oligonucleotides targeting Plk1 (sense sequence 5'-C.G.A.G.C.U.G.C.U.U.A.A.U.G.A.C.G.A.G.U.U-3') (Liu et al., 2012) or CyclinB1 (ON-target plus SMART pool: 5'-C.A.A.C.A.U.U.A.C.C.U.G.U.C.A.U.A.U.A.U.U-3', 5'-U.G.C.A.C.U.A.G.U.U.C.A.A.G.A.U.U.U.A.U.U-3', 5'-G.A.A.U.G.U.A.G.U.C.A.U.G.G.U.A.A.A.U.U.U-3', 5'-C.U.A.A.U.U.G.A.C.U.G.G.C.U.A.G.U.A.C.U.U-3') were purchased form Thermo Scientific.

Antibodies
Antibodies used were anti-Cdc25C (C20, sc-327), Cdc25B (C20, sc-326), Cdc25A (DCS122, sc-65503), anti-CyclinA2 (C-19, sc-596), anti-p53 (DO-1, sc-126), anti-p21 (F5, sc-6246), and anti-CyclinB1 (GNS1, sc-245) from Santa Cruz Biotechnology, anti-phospho Thr210 of Plk1 (K50-483) from BD Pharmigen, anti-phospho Ser10 of Histone H3 (06-570) and anti-PLK1 (clone 35-206, 05-844) from Millipore, anti-p38 MAPK (D13E1), anti-phospho p38 MAPK (Thr180/Tyr182)(D3F9), anti-phospho SAPK/JNK (Thr183/Tyr185)(81E11), anti-phospho histone H2AX (Ser139)(20E3) and anti-phospho Tyr15 of Cdk1 (10A11) from Cell signaling. Note that due to the conservation of surrounding amino-acids, the latter may not discriminate between phospho-Tyr15 of Cdk1 versus Cdk2 as described in Sakurikar et al. 2015. Specificity of each Cdc25 antibody used was confirmed using transfected cells with different Cdc25 tagged forms (our unpublished results). Anti-phospho Ser154 of CyclinA2 and anti-phospho Ser126 and Ser133 of CyclinB1 were a kind gift of Dr Jon Pines. Anti-phospho S75 of Cdc25C1 was produced and affinity purified by Eurogentec using the following NH_2-K-R-C-L-D-L-**S**-N-L-S-S-G-E-COOH peptide.

Cell transfection
For transient expression assays, transfection were performed using jet PRIME reagent (Polyplus) with a 200 000 cells/1μg/4μl ratio for HeLa cells and a 50 000 cells/1μg/2μl ratio for HEK-293 cells. Following a 3-4 hours period, cells were incubated with fresh medium. For cell cycle progression analyzes of HEK-293 cells, a short synchronization schedule was used to reach maximum expression levels of the transfected forms around G2 phase and to record the first divisions (thymidine regime for 18 hours, 4 hours post-transfection). For RNAi assays, 200 000 cells were transfected using 4μl Darmafect-1 reagent (Thermo Scientific) and 100nM of siRNA Plk1 or 20nM of siRNA CT or CyclinB1 into serum-free-medium. Cells were incubated for 7 h and supplemented with fresh medium (10% serum final).

Plasmid constructs
Plk1 c-Jun-based FRET sensor was obtained from addgene (ref: 45203).
HsCdc25C1 coding sequence was amplified by PCR and inserted using KpnI and BamHI restriction sites in a pEYFP-C1 vector (Clontech) in which EYFP has been replaced by mVenus. Mutagenesis of phosphorylation sites was performed using QuickChange II site-directed mutagenesis kit (Agilent) according to manufacturer's recommendations.
Complete sequences are available upon request.

Immunofluorescence
Cells were grown on glass coverslips and fixed for 10 min at RT with 4% formaldehyde in PBS or for 20 min at 4°C in 10% trichloroacetic acid (TCA)/DMEM. Coverslips were incubated at RT for 30 min in 3% BSA/PBS; 1 hour each with primary and fluorescently conjugated Alexa Fluor 488 or 568 secondary antibodies (Life technologies) and mounted in Prolong gold mounting medium with DAPI (Molecular Probes). Images were taken using an Axio imager Z1 microscope (Zeiss) equipped with an ORCA-ER digital camera (Hamamatsu) and 63x, 1.40 numerical aperture; oil immersion lens. For γH2A.X signal quantification, cell nuclei were segmented using the DNA staining and nuclear γH2A.X-integrated fluorescence intensity in CyclinB1 positive cells was measured using imageJ software. For 53BP1 foci quantification in CyclinB1 positive cells, all foci ≥ 2-fold above background fluorescence intensity were scored.

Immunoprecipitation and immunoblot
Monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) and anti-Cdc25C (C20) were incubated with protein G-coated magnetic beads (Dynabeads, Invitrogen) for 1 h at room temperature in PBS+0.02% Tween-20. Cross-linking was performed with 5.4 mg/ml of dimethyl pimelimidate (DMP) in 0.2M triethanolamine pH 8.5 for 2x30min. Quenching was performed in 50mM tris-HCl pH 7.5 for 15min. Unbounded antibodies were eluted with 2x1min 0.1M Glycine pH 3 treatments. Cell lysis was performed in 20mM tris-HCl pH 7.5, 150mM NaCl, 10% Glycerol, 0.5% NP-40, complete EDTA-free protease inhibitor cocktail (Roche) and anti-phosphatase inhibitor cocktail 3 (Sigma-Aldrich) at 4°C. After centrifugation (12 000 rpm, 20min), supernatants were incubated with antibody coupled beads for 2 h at 4°C. Following 4 washes in the same lysis buffer, elution was performed using either 3xFLAG peptide (F4799, Sigma-Aldrich) for 20min at 4°C or 1x NuPAGE LDS sample buffer (Life Technologies) for 10min at 65°C (for HsCdc25C immunoprecipitation assays). Immunoprecipitation of mVenus-tagged CyclinA2 were performed using GFP-trap beads (chromotek) using manufacturer's recommendations.
Protein quantification of cell extracts was performed in triplicate using BCA Protein Assay kit - reducing agent compatible (Thermo Scientific). All Western blot (16-bit) images were acquired using an Amersham imager 600 (GE healthcare) and quantified using imageJ software (National Institutes of Health).

Two-dimensional gel analyses
Mitotic cells were detached by shake off and washed in 10mM Tris pH7.5, 250mM Saccharose. Protein extraction was performed in 7M Urea, 2M thiourea, 4% CHAPS, 20mM DTT, 2% ampholytes (3-10, Pharmacia) extraction buffer according to Weiss and al. recommendations (Methods in Mol. Biology, 2009). After incubation at RT for 1 hour under gentle shaking, ultracentrifugation at 30 000 rpm was performed for 30'. Supernatants (100µg) were incubated with 7 cm pH range 3-10, non-linear (NL) or 5-7 strips (Immobiline DryStrip, GE Healthcare) followed by active rehydratation overnight. Electrofocalisation was performed using linear voltage increase up to 8000V (BioRad) followed by SDS-PAGE and immunoblotting.

Cdc25C1-S75P sensor
Cdc25C1-S75P sensor was generated using a FRET-dedicated plasmid library, a kind gift from C. Schultz (Piljic et al., 2011). Briefly, this library encodes for mTurquoise variant as a CFP fluorescent donor and mVenus or various cpVenus as YFP fluorescent acceptors, linked to short linkers of different sizes (2, 4 or 8 amino-acids) to modulate FRET performance. YFP fluorescent variant YPet was also evaluated as an alternative acceptor. Nucleotide sequences coding for FHA2 phosphorylation binding domain from scRad51 and the following peptide NH_2-G-G-A-P-K-R-C-L-L-**T**-N-L-I-COOH were inserted into the plasmid library. The latter sequence is from HsCdc25C1, residues 66–78, with Ser75 replaced with Thr and an Ile amino-acid inserted at +3 position to promote FHA2 binding. Plasmid constructs containing different fluorescent acceptors were first evaluated in ± Plk1 inhibitor conditions on mitotic arrested cells (see Figure S6E) to select a candidate exhibiting the highest FRET dynamic range. A second round of optimization was performed using plasmid constructs encoding amino-acid linkers of different sizes (not shown) (Piljic et al., 2011). The final construct selected contains mTurquoise as donor, 8 amino acids linker, S75P phosphorylation site, FHA2 domain, 2 amino acid linker, and YPet as fluorescent acceptor. Full nucleotide sequence of FRET-based Cdc25C1-S75 phosphorylation sensor is available upon request.

Mass Spectrometry Analyses
After immunoprecipitation, proteins were separated on SDS–PAGE gels and stained with colloidal blue staining (LabSafe Gel Blue GBiosciences). Gel slices were excised and proteins were reduced with 10mM DTT prior to alkylation with 55 mM iodoacetamide. After washing and shrinking the gel pieces with 100% MeCN, in-gel digestion was performed using trypsin, AspN, trypsin/GluC (Promega) or Elastase (Sigma-Aldrich) overnight in 25 mM NH_4HCO_3 at 30 °C. Peptides were extracted and analyzed by nano-LC-MS/MS using an Ultimate 3000 system (Dionex S.A.) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Samples were loaded on a C18 precolumn (300 μm inner diameter x 5 mm; Dionex) at 20 μl/min in 5% MeCN, 0.1% TFA. After a desalting for 3 min, the precolumn was switched for an analytical C18 column (75 µm i.d. x 15 cm or 50 cm, packed with C18 PepMap™, 3 µm, 100 Å; LC Packings) equilibrated in solvent A (2% MeCN, 0.1% HCO_2H). Bound peptides were eluted using a 57 min linear gradient (from 5 to 52%) of solvent B (80% MeCN, 0.085% HCO_2H) at a 200 nl/min flow rate for the short column and with a 100 min linear gradient (0 to 30% (v/v)) of solvent B at a 150 nl/min flow rate and an oven temperature of 40°C for the long column. A LTQ-Orbitrap mass spectrometer in the positive ion mode was used. We acquired Survey MS scans in the Orbitrap on the 400-1200 m/z range with the resolution set to a value of 100,000. Each scan was recalibrated in real time by co-injecting an internal standard from ambient air into the C-trap. The five most intense ions per survey scan were selected for CID fragmentation and the resulting fragments were analyzed in the linear trap (LTQ). Target ions already selected for MS/MS were dynamically excluded for 20 s. Data were acquired using the Xcalibur

software (v 2.0) and the resulting spectra were interrogated by the Mascot™ Software through Proteome Discoverer (v 1.4, Thermo Scientific) with the in house database containing the Cdc25 (Cdc25C4, C2 and C3, 3xFLAG-YPet-Cdc25C, HsCdc25C1 and C2) sequences. Phosphorylation of Ser and Thr was used as a variable modification for all searches. We set specificity of trypsin or AspN or trypsin/GluC digestion and allowed 4 missed cleavage sites whereas mass tolerances in MS and MS/MS was set to 2 ppm and 0.8 Da, respectively. Phosphorylated peptides were validated by combining the phosphoRS information and by manually inspecting the peak assignment. In case of phosphorylation ambiguity, where the spectra did not permit assignment, all potential phosphorylation residues are displayed.

Label-free Quantification

To quantify the phophorylated peptides, we manually extracted from the MS survey of nano-LC-MS/MS raw files the extracted ion chromatogram (XIC) signal of the well-characterized tryptic peptide ions using Xcalibur. We integrated XIC areas in Xcalibur under the QualBrowser interface using the ICIS algorithm. Mean values and S.D. were calculated from four independent experiences.