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Supplemental Information

Transient Hysteresis in CDK4/6 Activity Underlies Passage of the Restriction Point in G1

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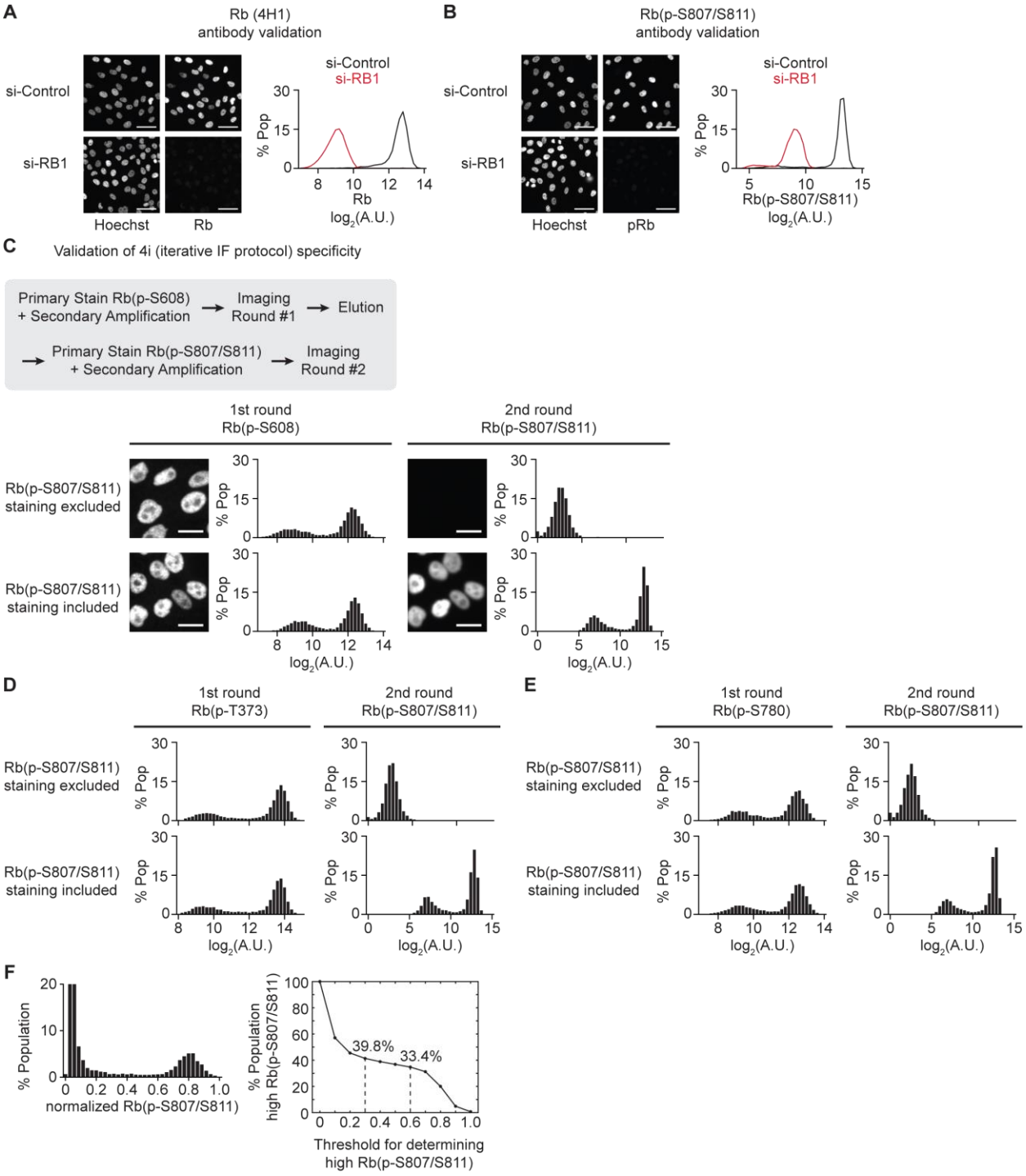
A
Rb (4H1)
antibody validation
si-Control
si-RB1
Hoechst
Rb
si-Control
si-RB1
% Pop
Rb
log2(A.U.)
B
Rb(p-S807/S811)
antibody validation
si-Control
si-RB1
Hoechst
pRb
si-Control
si-RB1
% Pop
Rb(p-S807/S811)
log2(A.U.)
C
Validation of 4i (iterative IF protocol) specificity
Primary Stain Rb(p-S608)
+ Secondary Amplification
Imaging
Round #1
Elution
Primary Stain Rb(p-S807/S811)
+ Secondary Amplification
Imaging
Round #2
1st round
Rb(p-S608)
2nd round
Rb(p-S807/S811)
Rb(p-S807/S811)
staining excluded
Rb(p-S807/S811)
staining included
% Pop
log2(A.U.)
D
1st round
Rb(p-T373)
2nd round
Rb(p-S807/S811)
Rb(p-S807/S811)
staining excluded
Rb(p-S807/S811)
staining included
% Pop
log2(A.U.)
E
1st round
Rb(p-S780)
2nd round
Rb(p-S807/S811)
Rb(p-S807/S811)
staining excluded
Rb(p-S807/S811)
staining included
% Pop
log2(A.U.)
F
% Population
normalized Rb(p-S807/S811)
% Population
high Rb(p-S807/S811)
39.8%
33.4%
Threshold for determining
high Rb(p-S807/S811)

Figure S1, Related to Figure 2. (A,B) MCF-10A reverse-transfected with siRNA against RB1, fixed 24hrs later, and stained for (A) Rb and (B) Rb(p-S807/S811). Scale bar 50μm. n=8000 cells per condition. (C-E) Negative controls for 4i protocol to demonstrate complete stripping of first round primary stain. Top and bottom are identical, except second round primary antibody excluded in top. Controls for stripping of antibodies against (C) Rb(p-S608), (D) Rb(p-T373), and (E) Rb(p-S780). n=5000 cells per condition. (F) Same data as normalized Rb(p-S807/S811) from Figure 2F analyzed for percentage of cells above thresholds spanning entire range of normalized signal. Results highlight relative insensitivity of taking an arbitrary threshold of 0.5 to report the signal as high Rb(p-S807/S811). n=4629 cells.

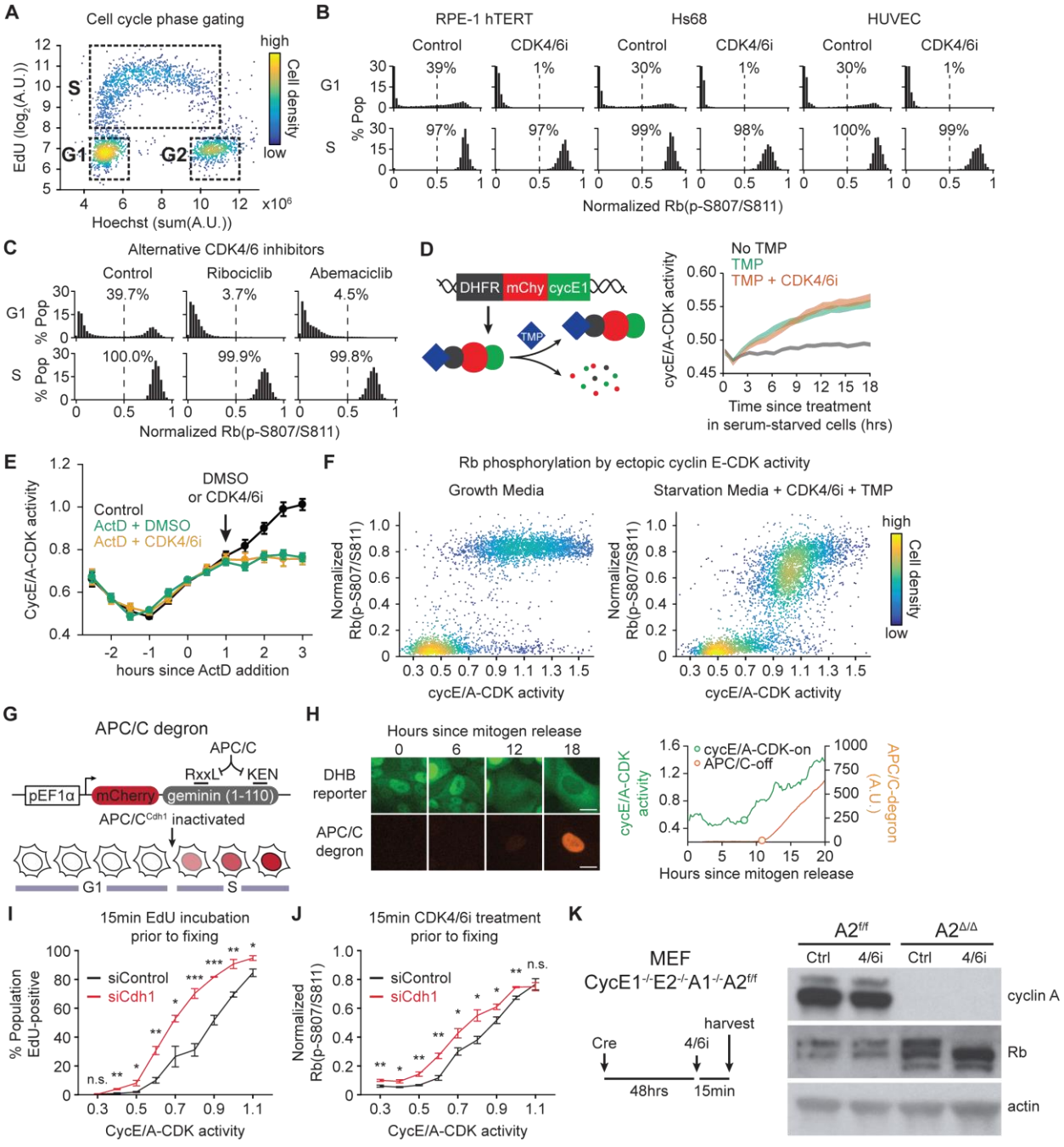
A
Cell cycle phase gating
EdU (log2(A.U.))
Hoechst (sum(A.U.))
S
G1
G2
high
low
Cell density
B
RPE-1 hTERT
Hs68
HUVEC
Control
CDK4/6i
G1
S
% Pop
39%
1%
30%
97%
99%
98%
100%
Normalized Rb(p-S807/S811)
C
Alternative CDK4/6 inhibitors
Control
Ribociclib
Abemaciclib
39.7%
3.7%
4.5%
100.0%
99.9%
99.8%
Normalized Rb(p-S807/S811)
D
DHFR
mChy
cycE1
TMP
No TMP
TMP
TMP + CDK4/6i
cycE/A-CDK activity
Time since treatment
in serum-starved cells (hrs)
E
CycE/A-CDK activity
DMSO
or CDK4/6i
Control
ActD + DMSO
ActD + CDK4/6i
hours since ActD addition
F
Rb phosphorylation by ectopic cyclin E-CDK activity
Growth Media
Starvation Media + CDK4/6i + TMP
Normalized
Rb(p-S807/S811)
cycE/A-CDK activity
high
low
Cell density
G
APC/C degron
APC/C
RxxL
KEN
pEF1α
mCherry
geminin (1-110)
APC/C^Cdh1 inactivated
G1
S
H
Hours since mitogen release
DHB
reporter
APC/C
degron
cycE/A-CDK-on
APC/C-off
cycE/A-CDK
activity
APC/C-degron
(A.U.)
Hours since mitogen release
I
15min EdU incubation
prior to fixing
% Population
EdU-positive
siControl
siCdh1
n.s.
CycE/A-CDK activity
J
15min CDK4/6i treatment
prior to fixing
Normalized
Rb(p-S807/S811)
siControl
siCdh1
n.s.
CycE/A-CDK activity
K
MEF
CycE1-/-E2-/-A1-/-A2f/f
Cre
4/6i
harvest
48hrs
15min
A2f/f
A2Δ/Δ
Ctrl
4/6i
Ctrl
4/6i
cyclin A
Rb
actin

Figure S2, Related to Figure 3. (A) Cycling MCF-10A treated with EdU for 15min prior to fixing and staining with Hoechst. Scatter plot of Hoechst versus EdU. n=20000 cells. (B) Cycling indicated cell types treated with EdU + CDK4/6i and fixed and stained for normalized Rb(p-S807/S811) and DNA content. n>900 per condition. (C) Cycling MCF-10A treated with EdU + vehicle (DMSO), Ribociclib 3μM, or Abemaciclib 300nM for 15min prior to fixing and staining as in (B). n>4671 cells per condition. (D) MCF-10A expressing cyclin E/A-CDK activity reporter and DHFR-mCherry-cyclin E1 mitogen-starved for 48hrs, then treated with vehicle, TMP 5μM, or TMP 5μM + CDK4/6i in starvation media. Shading represents 95% C-I; n>750 cells per condition. (E) Asynchronously cycling MCF-10A expressing cyclin E/A-CDK activity reporter treated with Actinomycin D (1μg/mL) for 1hr prior to addition of vehicle or CDK4/6i. Cells computationally gated for increasing E/A activity in G1 at time of Actinomycin D treatment. n>2973 cells per condition; error bars are std dev. (F) Same cells as in (D) treated for 18hrs with continued growth media or starvation media + CDK4/6i + TMP 10μM. n=5000 cells per scatter plot. (G) Schematic of APC/C degron reporter. (H) MCF-10A expressing both cyclin E/A-CDK activity reporter and APC/C degron reporter. Images and quantification of sample cell. Scale bar 20μm. (I) Cycling cells as in (H) transfected with siRNA for 28hrs, then treated with EdU for 15min prior to fixing and staining. n>1432 cells per condition. n=3 replicates; SEM; 2-sample t-test (left to right): p=9.13E-1, 4.27E-3, 1.79E-2, 1.76E-3, 1.25E-2, 9.67E-4, 3.72E-4, 1.97E-3, 1.54E-2. (J) Same experiment as (I), but cells incubated with CDK4/6i for 15min prior to fixing and staining for normalized Rb(p-S807/S811). n>1980 cells per condition. n=3 replicates; SEM; 2-sample t-test: p=8.05E-3, 1.32E-2, 3.55E-3, 1.64E-3, 2.00E-2, 1.13E-2, 1.78E-2, 1.47E-3, 6.93E-1. (K) Indicated MEFs treated as shown and harvested for western blot. Representative of 2 replicates. Control/vehicle (DMSO), CDK4/6i (Palbociclib) 1μM, and EdU 10μM wherever indicated.

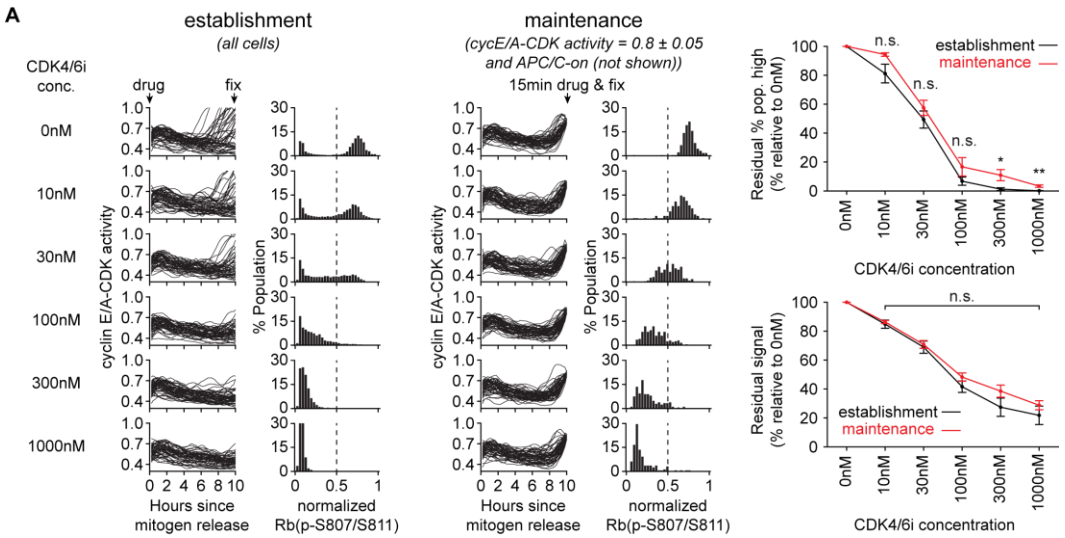

Figure S3, Related to Figure 4. (A) MCF-10A expressing cyclin E/A-CDK activity reporter and APC/C degron reporter mitogen-released and treated with CDK4/6i either at the time of mitogen release (establishment) or for 15min prior to fixing (maintenance). For establishment test, no gating performed. For maintenance test, cells computationally gated for E/A=0.8±0.05 and APC/C deg not rising (not shown) at the time of drug treatment. 50 sample traces per condition shown. (right top) Percentage of cells with normalized Rb(p-S807/S811) > 0.5 calculated for each concentration of CDK4/6i and normalized to the 0nM condition. Error bars are SEM; n>57; n=3 replicates; 2-sample t-test between each dose (10nM-1000nM) and 0nM: p=0.07, 0.28, 0.16, 0.042, 0.011. (right bottom) Alternate analysis: mean of normalized Rb(p-S807/S811) signal calculated for each concentration of CDK4/6i and normalized to the 0nM condition. Error bars are SEM; n>57 cells per condition; n=3 replicates; 2-sample t-test: p=0.59, 0.67, 0.17, 0.15, 0.30.

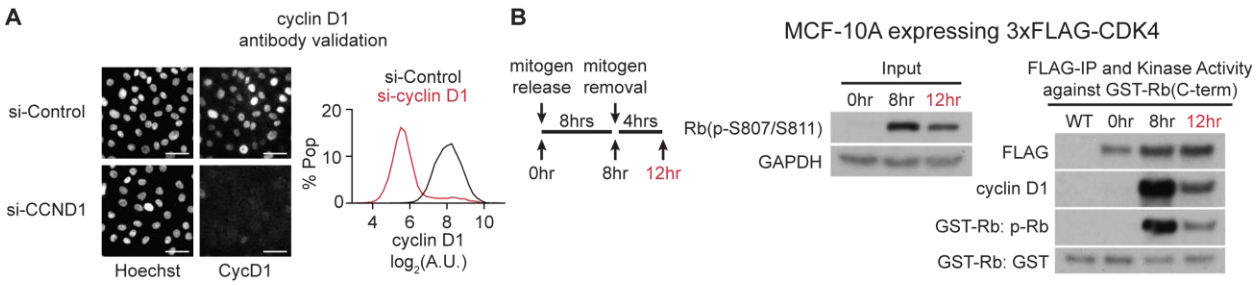

Figure S4, Related to Figure 5. (A) MCF-10A mitogen-starved, transfected with siRNA against CCND1, and released for 8hrs prior to fixing and staining for cyclin D1. Scale bar 50µm. n=8000 cells per condition. (B) MCF-10A expressing 3xFLAG-CDK4 treated as indicated and harvested at the indicated times for IP and IP-kinase measurement of cyclin D1 binding and phosphorylation of a GST-Rb fragment (See Methods). Representative of 2 replicates.