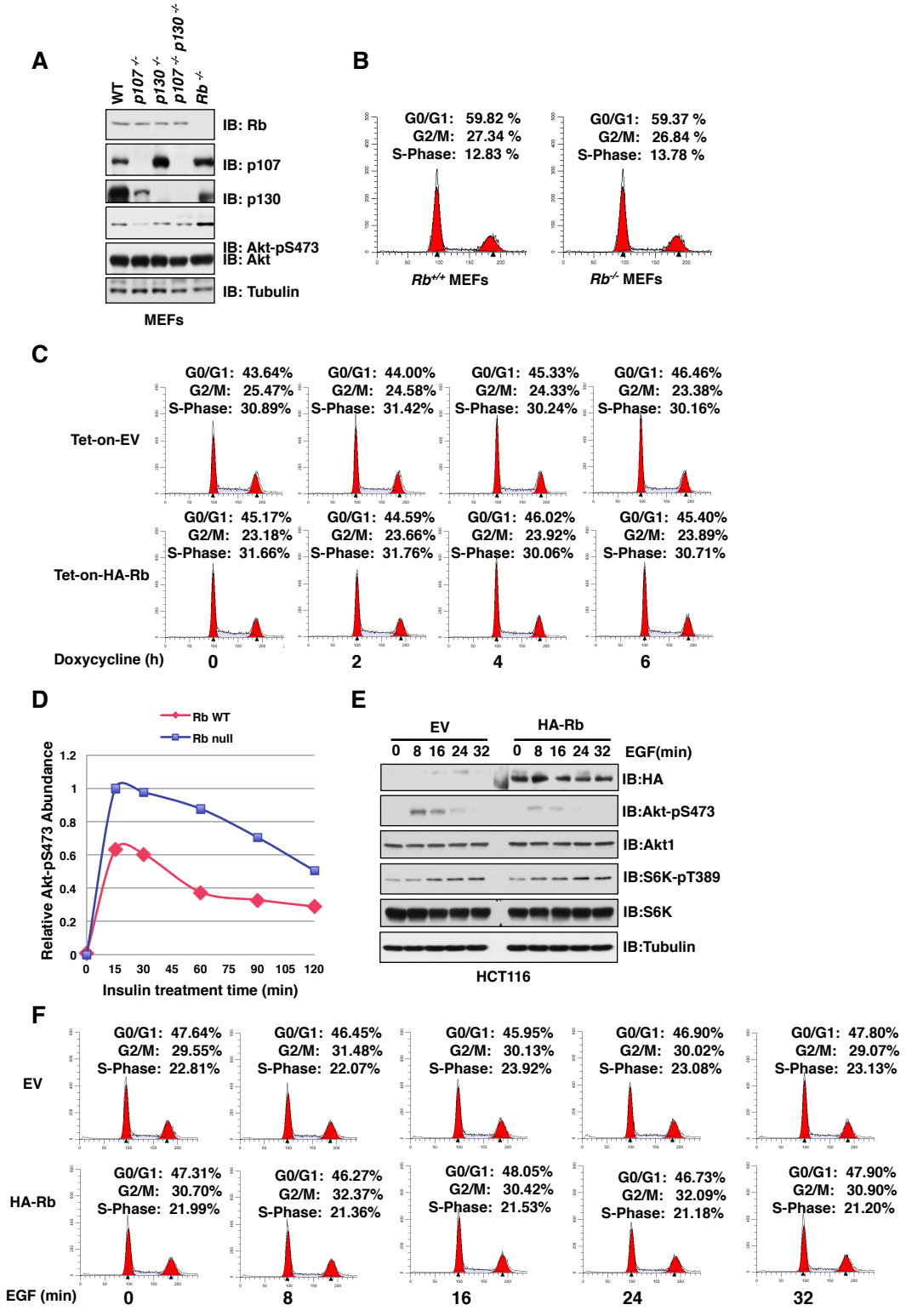

A
WT
p107 -/-
p130 -/-
p107 -/- p130 -/-
Rb -/-
IB: Rb
IB: p107
IB: p130
IB: Akt-pS473
IB: Akt
IB: Tubulin
MEFs
B
G0/G1: 59.82 %
G2/M: 27.34 %
S-Phase: 12.83 %
Rb+/+ MEFs
G0/G1: 59.37 %
G2/M: 26.84 %
S-Phase: 13.78 %
Rb-/- MEFs
C
Tet-on-EV
G0/G1: 43.64%
G2/M: 25.47%
S-Phase: 30.89%
G0/G1: 44.00%
G2/M: 24.58%
S-Phase: 31.42%
G0/G1: 45.33%
G2/M: 24.33%
S-Phase: 30.24%
G0/G1: 46.46%
G2/M: 23.38%
S-Phase: 30.16%
Tet-on-HA-Rb
G0/G1: 45.17%
G2/M: 23.18%
S-Phase: 31.66%
G0/G1: 44.59%
G2/M: 23.66%
S-Phase: 31.76%
G0/G1: 46.02%
G2/M: 23.92%
S-Phase: 30.06%
G0/G1: 45.40%
G2/M: 23.89%
S-Phase: 30.71%
Doxycycline (h)
0
2
4
6
D
Rb WT
Rb null
Relative Akt-pS473 Abundance
1.2
1
0.8
0.6
0.4
0.2
0
0 15 30 45 60 75 90 105 120
Insulin treatment time (min)
E
EV
HA-Rb
0 8 16 24 32 0 8 16 24 32 EGF(min)
IB:HA
IB:Akt-pS473
IB:Akt1
IB:S6K-pT389
IB:S6K
IB:Tubulin
HCT116
F
EV
G0/G1: 47.64%
G2/M: 29.55%
S-Phase: 22.81%
G0/G1: 46.45%
G2/M: 31.48%
S-Phase: 22.07%
G0/G1: 45.95%
G2/M: 30.13%
S-Phase: 23.92%
G0/G1: 46.90%
G2/M: 30.02%
S-Phase: 23.08%
G0/G1: 47.80%
G2/M: 29.07%
S-Phase: 23.13%
HA-Rb
G0/G1: 47.31%
G2/M: 30.70%
S-Phase: 21.99%
G0/G1: 46.27%
G2/M: 32.37%
S-Phase: 21.36%
G0/G1: 48.05%
G2/M: 30.42%
S-Phase: 21.53%
G0/G1: 46.73%
G2/M: 32.09%
S-Phase: 21.18%
G0/G1: 47.90%
G2/M: 30.90%
S-Phase: 21.20%
EGF (min)
0
8
16
24
32

Figure S1: Rb negatively regulates mTORC2, but not mTORC1 activity (related to Figure 1).

A. Immunoblot (IB) analysis of whole cell lysates (WCL) derived from wild type MEFs, $p107^{-/-}$ MEFs, $p130^{-/-}$ MEFs, $p107^{-/-}p130^{-/-}$ MEFs and $Rb^{-/-}$ MEFs.

B. Cell cycle distribution profile indicated by flow cytometry for $Rb^{+/+}$ and $Rb^{-/-}$ MEFs.

C. Cell cycle analyses for OVCAR5 cells with stable expression of Tet-on-HA-Rb or empty vector, which were treated by Doxycycline (1 μg/ml) for indicated time points to induce the ectopic expression of HA-Rb.

D. Quantification curves for the relative Akt-pSer473 intensity in the indicated time points upon insulin as presented in **Figure 1E**.

E-F. IB analysis of WCLs derived from HCT116 cells that after 36 h post-transfection with HA-Rb, cells were serum-starved for 24 h and collected after stimulation with EGF (100 ng/ml) for the indicated periods (**E**). The cell cycle distribution of both EV and HA-Rb expressing HCT116 cells from each time point were also analyzed by flow cytometry (**F**).

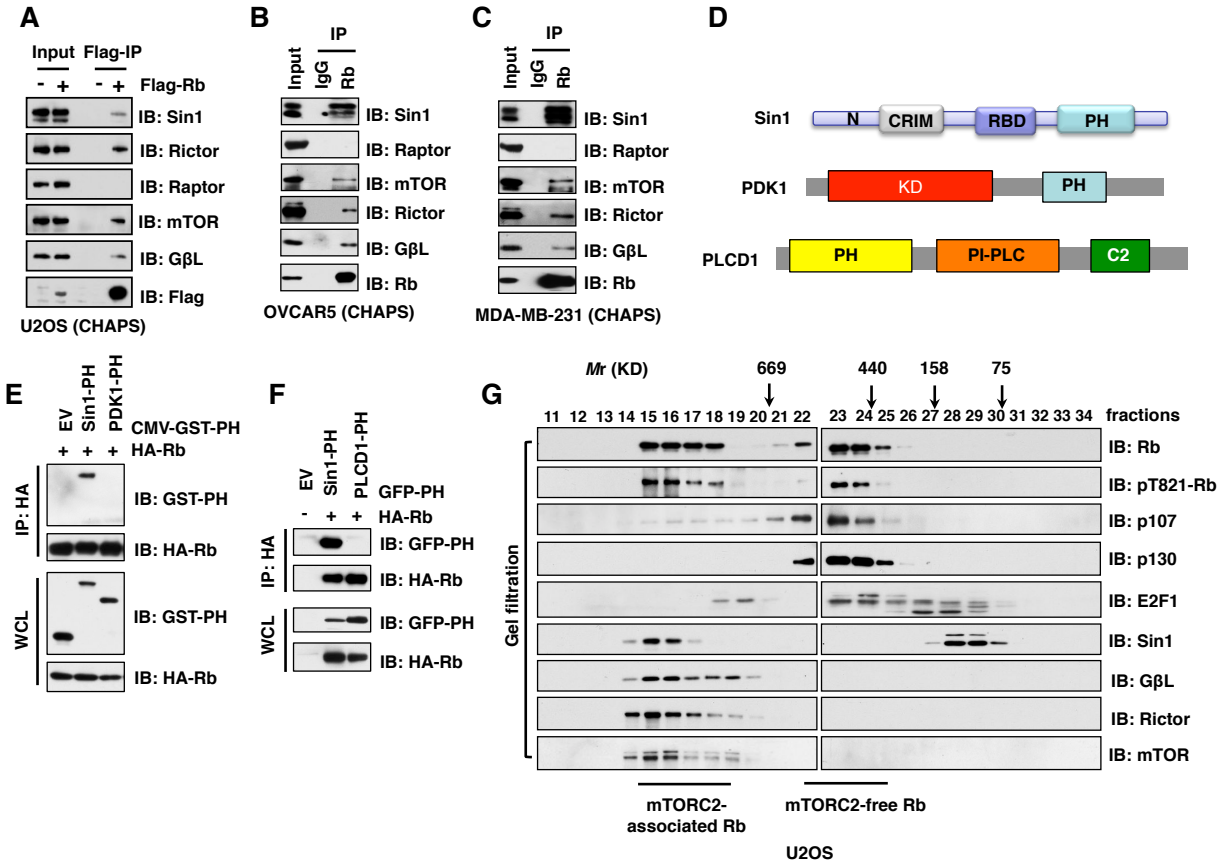

A
Input
Flag-IP
- + - +
Flag-Rb
IB: Sin1
IB: Rictor
IB: Raptor
IB: mTOR
IB: GβL
IB: Flag
U2OS (CHAPS)
B
IP
Input
IgG
Rb
IB: Sin1
IB: Raptor
IB: mTOR
IB: Rictor
IB: GβL
IB: Rb
OVCAR5 (CHAPS)
C
IP
Input
IgG
Rb
IB: Sin1
IB: Raptor
IB: mTOR
IB: Rictor
IB: GβL
IB: Rb
MDA-MB-231 (CHAPS)
D
Sin1
N
CRIM
RBD
PH
PDK1
KD
PH
PLCD1
PH
PI-PLC
C2
E
EV
Sin1-PH
PDK1-PH
CMV-GST-PH
+ + + HA-Rb
IP: HA
IB: GST-PH
IB: HA-Rb
WCL
IB: GST-PH
IB: HA-Rb
F
EV
Sin1-PH
PLCD1-PH
GFP-PH
- + + HA-Rb
IP: HA
IB: GFP-PH
IB: HA-Rb
WCL
IB: GFP-PH
IB: HA-Rb
G
Mr (KD)
669
440
158
75
11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
fractions
Gel filtration
IB: Rb
IB: pT821-Rb
IB: p107
IB: p130
IB: E2F1
IB: Sin1
IB: GβL
IB: Rictor
IB: mTOR
mTORC2-associated Rb
mTORC2-free Rb
U2OS

Figure S2: Rb interacts with the intact mTORC2 kinase complex through binding Sin1-PH domain (related to Figure 2).

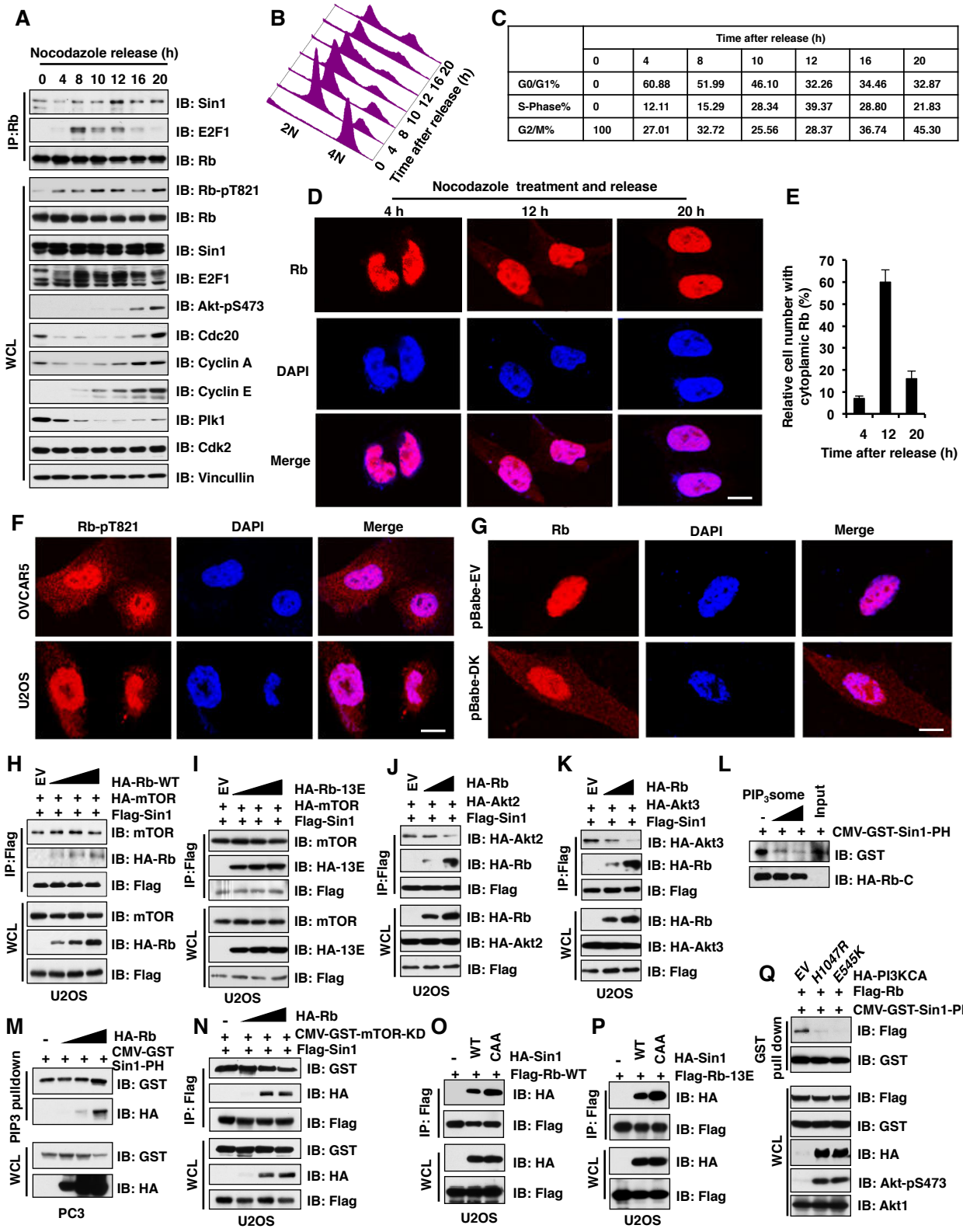
A. Immunoblot (IB) analysis of whole cell lysates (WCL) and Flag-IP derived from U2OS cells lysed in 0.3% CHAPS buffer 36 h after transfected with empty vector and Flag-Rb.

B-C. IB analysis of WCL and endogenous Rb-IP derived from OVCAR5 (**B**) and MDA-MB-231 (**C**) cells lysed in 0.3% CHAPS buffer.

D. A schematic representation of the indicated domains of Sin1, PDK1 and PLCD1.

E-F. IB analysis of WCL and HA-IP derived from U2OS cells lysed in 1% Triton buffer 36 h after transfected with indicated constructs.

G. Gel filtration experiments to illustrate that endogenous Rb, associates with the intact mTORC2 complex in U2OS cells that were arrested at late-G1/S phase by double thymidine treatment. Before running cell lysates, the molecular weight resolution of the Superdex 200 was estimated by running native molecular weight markers (thyroglobulin, relative molecular mass of 669,000 (Mr ~669K); ferritin, Mr ~440K; aldolase, Mr ~158K; conalbumin, Mr ~75K and ovalbumin, Mr ~44K) to determine their retention times on Coomassie Blue-stained SDS-PAGE protein gels.



	Time after release (h)						
	0	4	8	10	12	16	20
G0/G1%	0	60.88	51.99	46.10	32.26	34.46	32.87
S-Phase%	0	12.11	15.29	28.34	39.37	28.80	21.83
G2/M%	100	27.01	32.72	25.56	28.37	36.74	45.30

Figure S3: Rb interacts with Sin1 in a phosphorylation-dependent manner (related to Figure 3).

A. Immunoblot (IB) analysis of whole cell lysates (WCL) and anti-Rb IP derived from U2OS cells synchronized in M phase with 330 nM nocodazole for 16 h and released back to cell cycle with indicated time points and lysed in 1% Triton buffer.

B-C. Fluorescence activated cell sorting (FACS) analysis was performed to monitor the corresponding cell-cycle changes for U2OS cells in (**A**).

D. Immunofluorescence (IF) assays by confocal microscopy to show the subcellular localization of Rb for the indicated time points from U2OS cells in (**A**). Scale bar: 5 μm.

E. Quantification of cell number with cytoplasmic Rb. Data are shown as mean ± s.d. from three independent experiments.

F. IF assays by confocal microscopy to show the localization of phosphorylated Rb (Rb-pT821) in OVCAR5 and U2OS cells arrested at the late G1/S boundary by double thymidine treatment. Scale bar: 5 μm.

G. IF assays by confocal microscopy to show the subcellular localization of Rb for human primary fibroblast cells stably expressing pBabe-cyclin D/Cdk4-R24C (termed pBabe-DK) (with empty vector as a negative control). Scale bar: 5 μm.

H-I. Ectopic expression of Rb-WT (**H**) or Rb-13E (**I**) does not lead to the dissociation of the mTORC2 complex. IB analysis of anti-Flag IP and WCL derived from U2OS cells lysed in 0.3% CHAPS buffer 36 h after transfection with indicated constructs.

J-K. Rb competed with Akt2 and Akt3 in binding to Sin1. IB analysis of WCL and anti-Flag IP derived from U2OS cells lysed in 1% Triton buffer 36 h after transfected with the indicated constructs.

L. PIP3 competes with Rb-C terminal domain binding with Sin1-PH domain. HA-Rb-C was transfected into U2OS cells and immunoprecipitated by HA-beads and thoroughly washed to eliminate non-specific bindings. Subsequently, HA-Rb-C beads were used as the bait to pull down WCLs with CMV-GST-Sin1-PH from U2OS cells, in the presence of increasing doses of $PI(3,4,5)P_3$ polysomes.

M. IB analysis of WCL and $PI(3,4,5)P_3$ pull-down derived from U2OS cells lysed in 1% Triton buffer 36 h after transfected with indicated constructs.

N-P. IB analysis of WCL and anti-Flag immunoprecipitates (IP) derived from U2OS cells lysed in 1% Triton buffer 36 h after transfected with indicated constructs.

Q. IB analysis of WCL and GST pull-down derived from 293 cells lysed in 1% Triton buffer 36 h after being transfected with the indicated constructs.

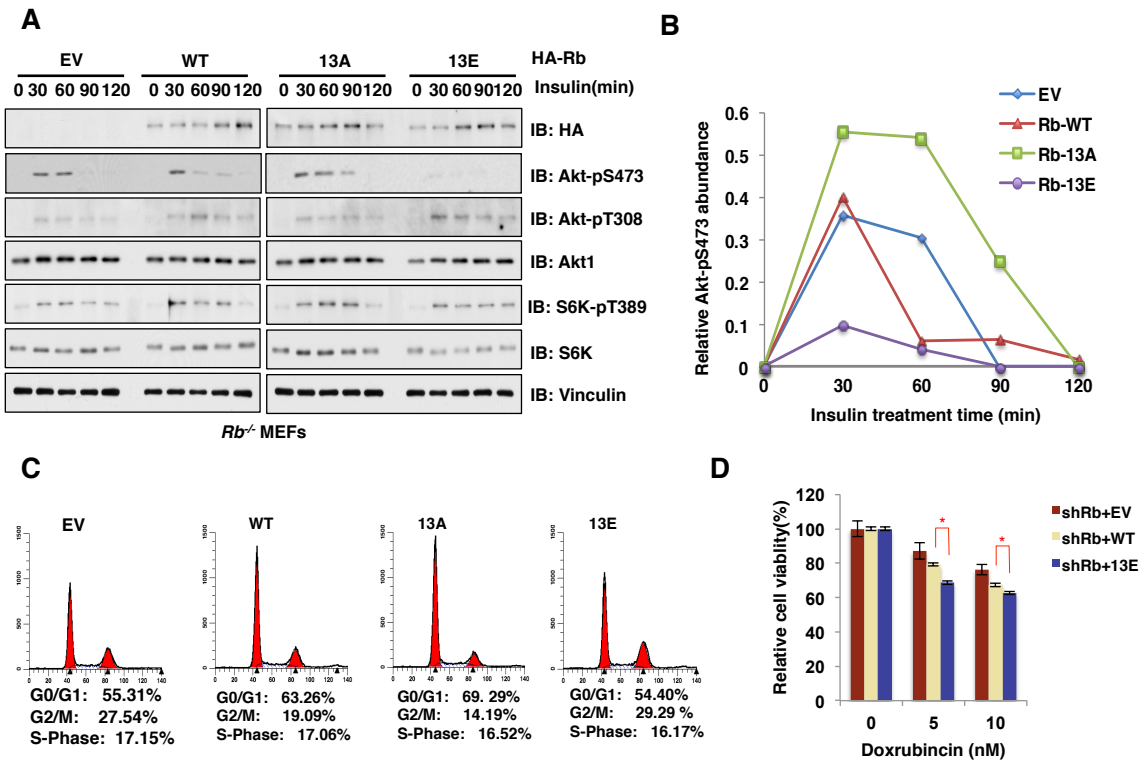


Figure S4: Hyper-phosphorylated Rb inhibits mTORC2 kinase activity to suppress cell proliferation and sensitize cells to chemotherapeutic treatment (related to Figure 4).

- **A.** Ectopic expression of the hyper-phosphorylated mutant form of Rb-13E led to attenuated Akt Ser473 phosphorylation response to insulin stimulation. $Rb^{-/-}$ MEFs were transfected with the indicated HA-Rb constructs (with empty vector as a negative control). 36 h post-transfection, the resulting cells were serum-starved for 24 h and collected after refed with 10% FBS DMEM for the indicated periods.
- **B.** Quantification of Figure **S4A** for the band intensity of Akt-pS473.
- **C.** Cell cycle analysis of $Rb^{-/-}$ MEFs stably expressing EV, HA-Rb-WT, HA-Rb-13A and HA-Rb-13E. DNA contents were measured by propidium iodide staining followed by FACS analysis.
- **D.** *Rb*-depleted MDA-MB-231 cells stably expressing the indicated Rb constructs were treated with indicated concentration of doxorubincin for 48 h before performing cell viability assay. Data was shown as mean ± s.d. for 3 independent experiments. * indicates $p<0.05$ by student's *t*-test.

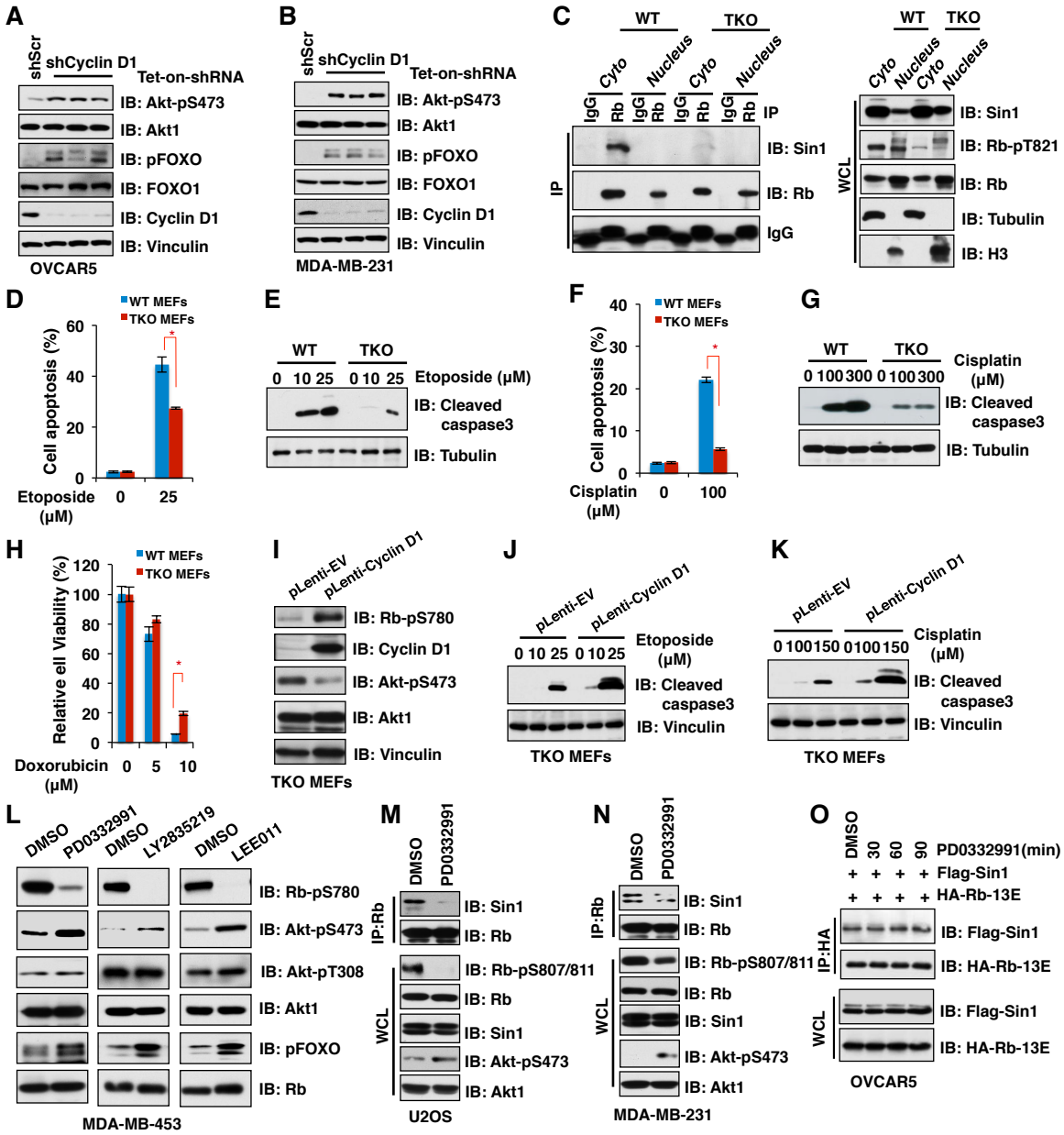

A
shScr
shCyclin D1
Tet-on-shRNA
IB: Akt-pS473
IB: Akt1
IB: pFOXO
IB: FOXO1
IB: Cyclin D1
IB: Vinculin
OVCAR5
B
shScr
shCyclin D1
Tet-on-shRNA
IB: Akt-pS473
IB: Akt1
IB: pFOXO
IB: FOXO1
IB: Cyclin D1
IB: Vinculin
MDA-MB-231
C
WT
TKO
Cyto
Nucleus
IgG
Rb
IP
IB: Sin1
IB: Rb
IgG
WCL
IB: Sin1
IB: Rb-pT821
IB: Rb
IB: Tubulin
IB: H3
D
WT MEFs
TKO MEFs
Cell apoptosis (%)
Etoposide (μM)
0
25
E
WT
TKO
0 10 25 0 10 25
Etoposide (μM)
IB: Cleaved caspase3
IB: Tubulin
F
WT MEFs
TKO MEFs
Cell apoptosis (%)
Cisplatin (μM)
0
100
G
WT
TKO
0 100 300 0 100 300
Cisplatin (μM)
IB: Cleaved caspase3
IB: Tubulin
H
WT MEFs
TKO MEFs
Relative ell Viability (%)
Doxorubicin (μM)
0
5
10
I
pLenti-EV
pLenti-Cyclin D1
IB: Rb-pS780
IB: Cyclin D1
IB: Akt-pS473
IB: Akt1
IB: Vinculin
TKO MEFs
J
pLenti-EV
pLenti-Cyclin D1
0 10 25 0 10 25
Etoposide (μM)
IB: Cleaved caspase3
IB: Vinculin
TKO MEFs
K
pLenti-EV
pLenti-Cyclin D1
0 100 150 0 100 150
Cisplatin (μM)
IB: Cleaved caspase3
IB: Vinculin
TKO MEFs
L
DMSO
PD0332991
DMSO
LY2835219
DMSO
LEE011
IB: Rb-pS780
IB: Akt-pS473
IB: Akt-pT308
IB: Akt1
IB: pFOXO
IB: Rb
MDA-MB-453
M
DMSO
PD0332991
IP:Rb
IB: Sin1
IB: Rb
WCL
IB: Rb-pS807/811
IB: Rb
IB: Sin1
IB: Akt-pS473
IB: Akt1
U2OS
N
DMSO
PD0332991
IP:Rb
IB: Sin1
IB: Rb
WCL
IB: Rb-pS807/811
IB: Rb
IB: Sin1
IB: Akt-pS473
IB: Akt1
MDA-MB-231
O
DMSO
30
60
90
PD0332991(min)
Flag-Sin1
HA-Rb-13E
IP:HA
IB: Flag-Sin1
IB: HA-Rb-13E
WCL
IB: Flag-Sin1
IB: HA-Rb-13E
OVCAR5

Figure S5: Inhibition of CDK4/cyclin D-mediated Rb phosphorylation by either genetic deletion of *cyclin D* or CDK4/6 inhibitor treatment leads to activation of mTORC2 to confer resistance to chemotherapeutic drugs (related to Figure 5).

A and **B.** Immunoblot (IB) analysis of whole cell lysates (WCL) derived from OVCAR5 (**A**) and MDA-MB-231 (**B**) cells acutely depleted of *cyclin D1* by three independent Tet-inducible shRNAs (shScramble as a negative control). Acute *Cyclin D1* depletion was achieved by addition of 1 μg/ml Doxycline in cell culture medium for 48 hours before harvesting.

C. IB analysis of anti-Rb IP and WCL derived from equal amounts of cytoplasmic and nuclear lysates of wild type (WT) and *cyclin D1*$^{-/-}$/*cyclin D2*$^{-/-}$/*cyclin D3*$^{-/-}$ triple knock out (TKO) MEFs, respectively.

D and **F.** Wild type and TKO MEFs were treated with the indicated concentration of etoposide (**D**) or cisplatin (**F**) for 24 h before performing cell apoptosis assay. Data are shown as mean ± s.d. from three independent experiments. * $p<0.05$ (*t*-test).

E and **G.** IB analysis of WCL derived from wild type and TKO MEFs treated with indicated concentration of etoposide (**E**) or cisplatin (**G**) for 24 h.

H. Wild type and TKO MEFs were treated with the indicated concentration doxorubicin for 24 h before performing cell viability assay. Data are shown as mean ± s.d. from three independent experiments. * $p<0.05$ (*t*-test).

I. IB analysis of WCL derived from TKO MEFs stably expressing cyclin D1 via lentiviral infection (with pLenti-EV as a negative control).

J and **K.** TKO MEFs stably expressing cyclin D1 via lentiviral infection (with pLenti-EV as a negative control) were treated with the indicated concentration of etoposide (**J**) and cisplatin (**K**) before harvesting for IB analysis.

L. MDA-MB-453 cells were treated with 500 nM CDK4/6 inhibitors PD0332991, LY2835219 or LEE011, respectively, for 48 h before harvesting for IB analysis.

M and **N.** The CDK4/6 inhibitor PD0332991 decreased the endogenous interaction of Rb with Sin1. IB analysis of WCL and anti-Rb IP derived from U2OS (**M**) and MDA-MB-231 (**N**) cells treated with 1 μM PD0332992 for 90 minutes.

O. IB analysis of WCL and anti-HA IP derived from OVCAR5 treated with 1 μM PD0332992 for the indicated time points.

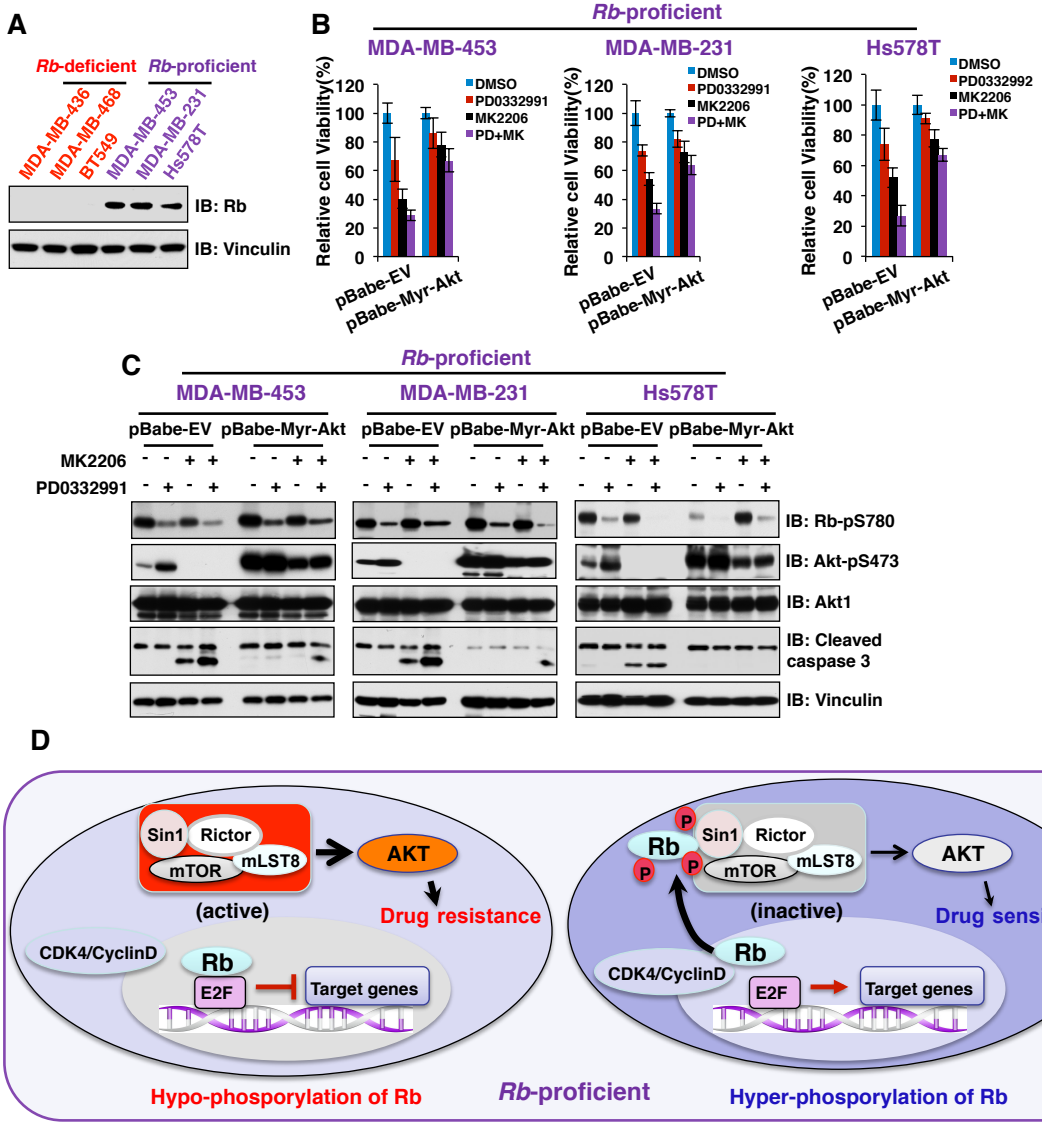

A
Rb-deficient
Rb-proficient
MDA-MB-436
MDA-MB-468
BT549
MDA-MB-453
MDA-MB-231
Hs578T
IB: Rb
IB: Vinculin
B
Rb-proficient
MDA-MB-453
MDA-MB-231
Hs578T
Relative cell Viability(%)
DMSO
PD0332991
MK2206
PD+MK
PD0332992
pBabe-EV
pBabe-Myr-Akt
C
Rb-proficient
MDA-MB-453
MDA-MB-231
Hs578T
pBabe-EV
pBabe-Myr-Akt
MK2206
PD0332991
IB: Rb-pS780
IB: Akt-pS473
IB: Akt1
IB: Cleaved caspase 3
IB: Vinculin
D
Sin1
Rictor
mTOR
mLST8
AKT
(active)
Drug resistance
CDK4/CyclinD
Rb
E2F
Target genes
Hypo-phosporylation of Rb
Rb-proficient
P
(inactive)
Drug sensitivity
Hyper-phosporylation of Rb

Figure S6: The CDK4/6 inhibitor synergizes with the Akt inhibitor in treating *Rb*-proficient breast cancer cell lines (related to Figure 6).

- **A.** Immunoblot (IB) analysis of whole cell lysates (WCL) derived from human breast cancer cell lines with Rb-negative (MDA-MB-436, MDA-MB-468, and BT549) and positive status (MDA-MB-453, MDA-MB-231, and Hs578T).
- **B.** *Rb*-proficient breast cancer cells stably expressing pBabe-EV or Myr-Akt were treated with CDK4/6 inhibitor PD0332991 (500 nM), or Akt inhibitor MD2206 (1 μM), either alone or in combination for 48 h before performing cell viability assays.
- **C.** IB analysis of WCL derived from *Rb*-proficient breast cancer cells stably expressing pBabe-EV or Myr-Akt treated with CDK4/6 inhibitor PD0332991 (500 nM), or Akt inhibitor MD2206 (1 μM), either alone or in combination for 48 h.
- **D.** A proposed model illustrating an, E2F-independent, physiological role for Rb by directly binding the Sin1-PH domain to suppress the mTORC2 kinase activity. Hypo-phosphorylated form of Rb mainly regulates the cell cycle progression through inhibiting E2F family of transcription factors (left panel). On the other hand, hyper-phosphorylation of Rb by CDK4/cyclin D releases Rb binding with E2F family transcription factors, while on the other hand, favors the interaction between the hyper-phosphorylated form of Rb with the Sin1-PH domain to suppress the kinase activity of mTORC2 (right panel).

Extended Experimental Procedures

Cell culture

Rb$^{loxp/loxp}$ MEFs (a gift of Dr. Julien Sage, Department of Pediatrics and Genetics, Stanford University), *Rb*$^{-/-}$ MEFs, WT MEFs, *cyclin D1*$^{-/-}$ MEFs, *cyclin D2*$^{-/-}$ MEFs, *cyclin D3*$^{-/-}$ MEFs, *cyclin D1*$^{-/-}$ *D2*$^{-/-}$*D3*$^{-/-}$ MEFs, *p107*$^{-/-}$ MEFs, *p130*$^{-/-}$ MEFs, *p107*$^{-/-}$ *p130*$^{-/-}$ MEFs (a gift of Dr. Nicolas Dyson in Department of Medicine, Massachusetts General Hospital Cancer Center), OVCAR5, HeLa, U2OS, HEK293T, HCT116, MDA-MB-231, Hs578T cells were cultured in DMEM medium (Life Technologies, CA) supplemented with 10% FBS, 100 units of penicillin and 100 μg/ml streptomycin. MDA-MB436, MDA-MB468, MDA-MB453 cells were cultured in McCoy's5A (Corning, NY) medium supplemented with 10% FBS. BT549 cell was cultured in RPMI medium (Corning, NY) with 10% FBS. The cells were maintained in a 5% CO_2-humidified atmosphere at 37°C, as previously described. Cell transfection and cell fraction was performed as described previously (Gao et al., 2009). Packaging of lentiviral short hairpin RNA (shRNA), pTRIPZ as well as pLenti viruses was preformed as described previously (Liu et al., 2015; Liu et al., 2013). Kinase inhibitors, PD0332991 (Selleckchem, S1116), LY2835219 (Selleckchem, S7158), LEE011 (Selleckchem, S7440), and MK-2206 (Selleckchem, S1078), were used as the dose indicated.

Cell transfection

Cells were transfected using Lipofectamine 2000 (Life Technologies) in Opti-MEM medium (Life Technologies) according to the manufacturer's instructions. 36 hours post-transfection, transfected cells were further subjected to immunoblot analysis to detect the efficacy of transfection.

Plasmids

HA-Rb, HA-Sin1, HA-mTOR, HA-Raptor, HA-Rictor and HA-GβL were constructed by cloning the indicated cDNAs into pcDNA3-HA vector. Flag-Rb, Flag-p107, Flag-p130, Flag-Rb-N, Flag-Rb-M and Flag-Rb-C were constructed by cloning the corresponding cDNAs into pcDNA3-Flag vector. pTripz-HA-Rb indicated constructs and pLenti-HA-Rb indicated constructs were constructed by subcloning the indicated

HA-Rb constructs or Rb constructs into pTripz-puro and pLenti-hygro-HA vector, respectively. Rb-13A and 13E mutants were gifts from David Goodrich (Roswell Park Cancer Institute). Plasmids pGEX-Sin1, pGEX-Sin1-N, pGEX-Sin1-CRIM, pGEX-Sin1-RBD and pGEX-Sin1-PH, HA-Sin1, HA-Sin1-N, HA-Sin1-CRIM, HA-Sin1-RBD and HA-Sin1-PH were described previously (Liu et al., 2013). HA-Akt and HA-SGK1 constructs were described previously (Gao et al., 2009).

Antibodies

All antibodies were used in 1:1000 dilutions in 5% non-fat milk for western blot. Anti-Akt1 antibody (2938), anti-phospho-Ser473-Akt antibody (4060), anti-phospho-Thr308-Akt antibody (13038), anti-phospho-Thr389-S6K antibody (9234), anti-S6K antibody (9202), anti-Rb antibody (9309), anti-phospho-Ser807/811-Rb antibody (8516), anti-phospho-Ser780-Rb antibody (8180), anti-phospho-Thr346-NDRG1 (5482), anti-phospho-Thr246-PRAS40 (13175), anti-phospho-Ser227-RSK2 (3556), anti-phospho-Thr32-FOXO1 antibody (9464), anti-FOXO1 antibody (9454), anti-Rictor antibody (2114), anti-Raptor antibody (2280), anti-GβL antibody (3274) and anti-mTOR antibody (2983) were purchased from Cell Signaling Technology. Anti-Skp2 antibody (sc-7164), anti-p27 antibody (sc-527), anti-cyclin A antibody (sc-751), anti-E2F1 antibody (sc-193), anti-cyclin D1 antibody (sc-450), and anti-cyclin D3 antibody (sc-182), polyclonal anti-HA antibody (sc-805), anti-Rb conjugated beads (sc-102) and anti-E2F1 conjugated beads (sc-251) were purchased from Santa Cruz. Anti-phospho-Thr821-Rb (R4153), anti-vinculin antibody (V-4505), anti-tubulin antibody (T5168), polyclonal anti-Flag antibody (F-2425), monoclonal anti-Flag antibody (F-3165, clone M2), anti-HA agarose beads (A-2095), anti-Flag agarose beads (A-2220), peroxidase-conjugated anti-mouse secondary antibody (A-4416) and peroxidase-conjugated anti-rabbit secondary antibody (A-4914) were purchased from Sigma. Anti-cyclin D2 (554201) was purchased from BD Pharmingen. Monoclonal anti-HA antibody (MMS-101P) was purchased from Covance. Anti-phospho-Thr821-Rb (AT-6026) was purchased from MBL International Corporation. Lipofectamine2000, Lipofectamine and Plus reagents were purchased from Life Technologies.

shRNA

Rb shRNA1 was purchased from Addgene (Plasmid #25641). Other two Rb shRNAs (shRNA2 and shRNA3) were generated using the following sequences to deplete endogenous Rb (denoted 2 with the targeting sequence 5'-TCGCTTGTATTACCGAGTAAT-3', denoted 3 with the targeting sequence 5'-GTGCGCTCTTGAGGTTGTAAT-3').

Gel filtration chromatography analysis

U2OS cells treated with thymidine for 16 hours were washed with PBS for twices and then added fresh normal medium. After 9 hours, the cells were treated with thymidine for another 16 hours to arrest cells at G1/S phase. Cells were washed with PBS, lysed in 0.5 ml of CHAPS lysis buffer (50 mM Tris pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS) containing protease inhibitors and phosphatase inhibitors, and filtered through a 0.22 μm syringe filter. 3 mg total protein lysed with CHAPS buffer was loaded onto a Superdex 200 10/300 GL column (GE Lifesciences, Cat. No. 17-5175-01). Chromatography was performed on the AKTA-FPLC (GE Lifesciences, Cat. No. 18-1900-26) with CHAPS buffer as described previously (Liu et al., 2013). One column volume of eluates were fractionated with 500 μl in each fraction, at the elution speed of 0.5 ml/min. 30 μl aliquots of each fraction were loaded onto SDS-PAGE gels and detected with indicated antibodies.

Cell cycle distribution analysis by flow cytometry

Cells treated with doxycycline, EGF or synchronized with nocodazole/thymidine-arrest and release were collected at the indicated time points. Briefly, the cells were fixed by 70% ethanol at -20°C overnight and washed 3 times using cold PBS. The samples were digested with RNase for 30 minutes at 37°C and stained with propidium iodide (Roche) according to the manufacturer's instructions. Stained cells were sorted with BD FACSCanto™ II Flow Cytometer. The results were analyzed by ModFit LT 4.1 and FSC express 5 softwares.

Cytoplasm/nuclear fractionation

Cytoplasm/nuclear fractionation procedures were performed as described previously (Gao et al., 2009; Inuzuka et al., 2012). Briefly, cells with 80% confluence were harvested and washed once using cold PBS. The cells were resuspended in 8 volumes of cell pellets with hypotonic buffer (10 mM HEPES pH7.9, 0.2 mM EDTA, 1 mM DTT, protease inhibitors and phosphatase inhibitors). After incubating on ice for 10 minutes, cells were then added 10% Triton X-100 to the final concentration of 0.1% and vortexed for 15 seconds to make sure that clear nuclei were observed under a microscope. The samples were centrifuged at 7,500 rpm for 5 minutes to get the supernatant as the cytoplasmic part. The remaining pellets should be washed twice and resuspended in 3 volumes of pellets with hypotonic buffer. The samples were sonicated and centrifuged at 14,000 rpm for 10 minutes to get the supernatant as the nuclear part. Equivalent lysates derived from cytoplasmic and nuclear part (add 5 M NaCl to the final concentration of 150 mM) were used for immunoprecipitation assays.

Immunofluorescence assays by confocal microscopy

Cells were grown on glass coverslips and fixed with 4% formaldehyde in PBS for 15 minutes at room temperature. The cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes on ice. Coverslips were washed 3 times in PBS for 5 minutes each time and were then blocked for 30 minutes with 5% control goat serum. The samples were incubated with primary antibodies for 2 hours at room tempreture and then rinsed 3 times using PBST containing 0.1% Tween-20. After the coverslips incubated with Alexa-594-conjugated goat anti-mouse secondary antibody (Invitrogen) for 1 hour and washed 3 times with PBST, the nucleus was stained with 4, 6-diamidino-2-phenylindole (DAPI) for 10 minutes. Coverslips were rinsed 2 times with PBS and mounted onto slides. Fluorescence images were captured on a ZEISS LSM 880 confocal microscope.

Supplemental References:

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