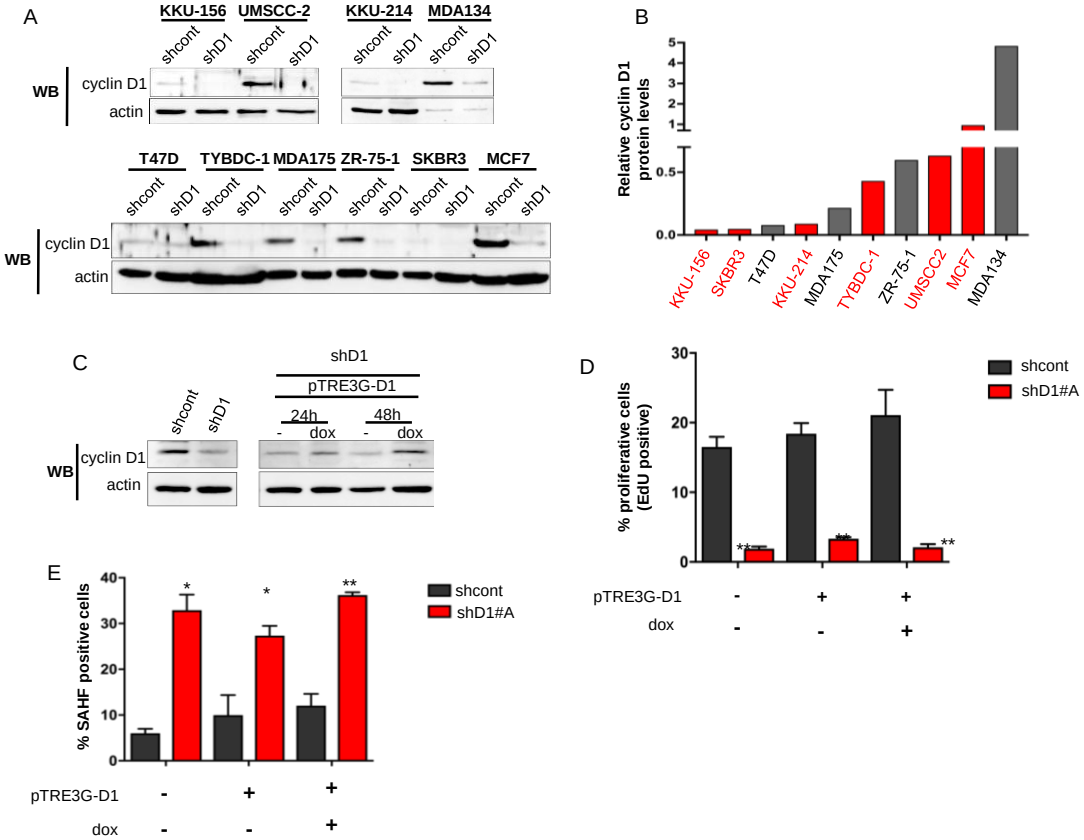

Fig. S1.

A. Western blotting (WB) analysis of cyclin D1-knockdown efficiency in several types of cancer cell lines. Protein lysates were harvested 4 days after transduced with non-targeting shRNA (shcont) or cyclin D1-specific shRNA (shD1#A). Actin was used as a loading control.

B. Relative protein levels of cyclin D1 in the cell lines from (a). Bars in grey are cell lines that did not undergo senescence and red bars are cell lines that underwent senescence upon cyclin D1 depletion.

C. Western blotting (WB) analysis of re-expressing cyclin D1-HA in the senescent cells. The inducible system (Takara Bio Inc., Mountain view, CA, USA), composed of pEF1α-Tet3G and pTRE3G-cyclin D1-HA plasmids was co-transfected into MCF7 cells, followed by cyclin D1 depletion using cyclin D1-specific shRNA (shD1#A). Expression of cyclin D1-HA was achieved by addition of 1 μM doxycycline. Re-expressions of cyclin D1-HA were examined at 24 and 48 hours after the induction. Actin was used as a loading control.

D. Re-expression of cyclin D1-HA did not increase cell proliferation in cyclin D1 depletion-induced senescent cells. Percentages of EdU-positive cells were analyzed at 48 hours after the induction. Bars represent averages of 3 experiments± s.d. (**$p \leq 0.01$)

E. Re-expression of cyclin D1-HA in the senescent cells did not reduced number of SAHF-positive cell. Analyses of SAHF-positive cell numbers were performed at the same time point as in (d). Doxycycline treatment; dox. Bars represent averages of 3 experiments ± s.d. (*$p \leq 0.05$ and **$p \leq 0.01$)

Statistical significance was determined by Student's t-test

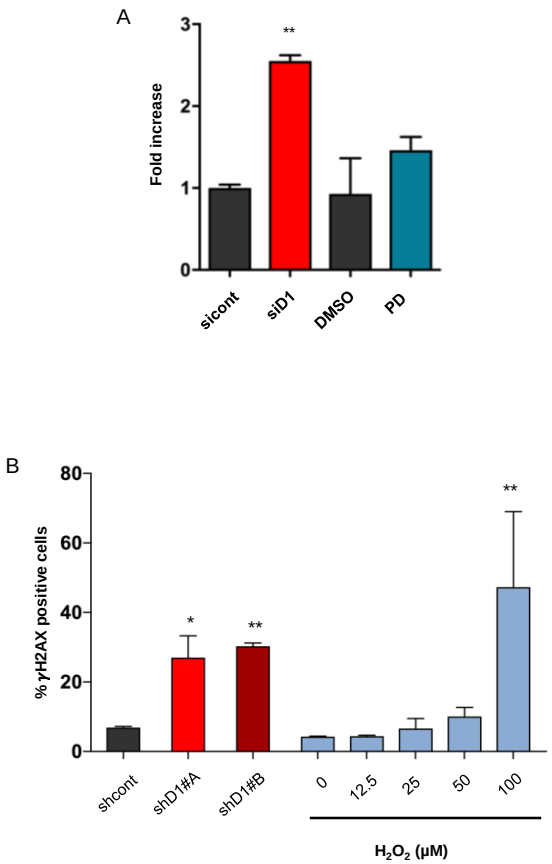

Fig. S2.

A. Apoptosis detection of cyclin D1-specific siRNA (siD1) and CDK4/6 inhibitor treatment (PD), compared to control cells (sicont) and vehicle control (DMSO). Bars represent averages from 3 independent experiments ± s.d. Statistical significance was determined by Student's t-test (***p*≤*0.01*).

B. Cyclin D1-depleted cells (shD1#A, shD1#B) showed significant elevation of γH2AX foci-positive cells, compared to control cells (shcont). Percentages of γH2AX foci-positive MCF7 cells treated with increasing concentrations of H_2O_2 are shown as a reference (grey bars). At least 5,000 γH2AX-positive cells were analyzed. Bars represent averages from 3 independent experiments ± s.d. Statistical significance was determined by Student's t-test (**p*≤*0.05 and* ***p*≤*0.01*).

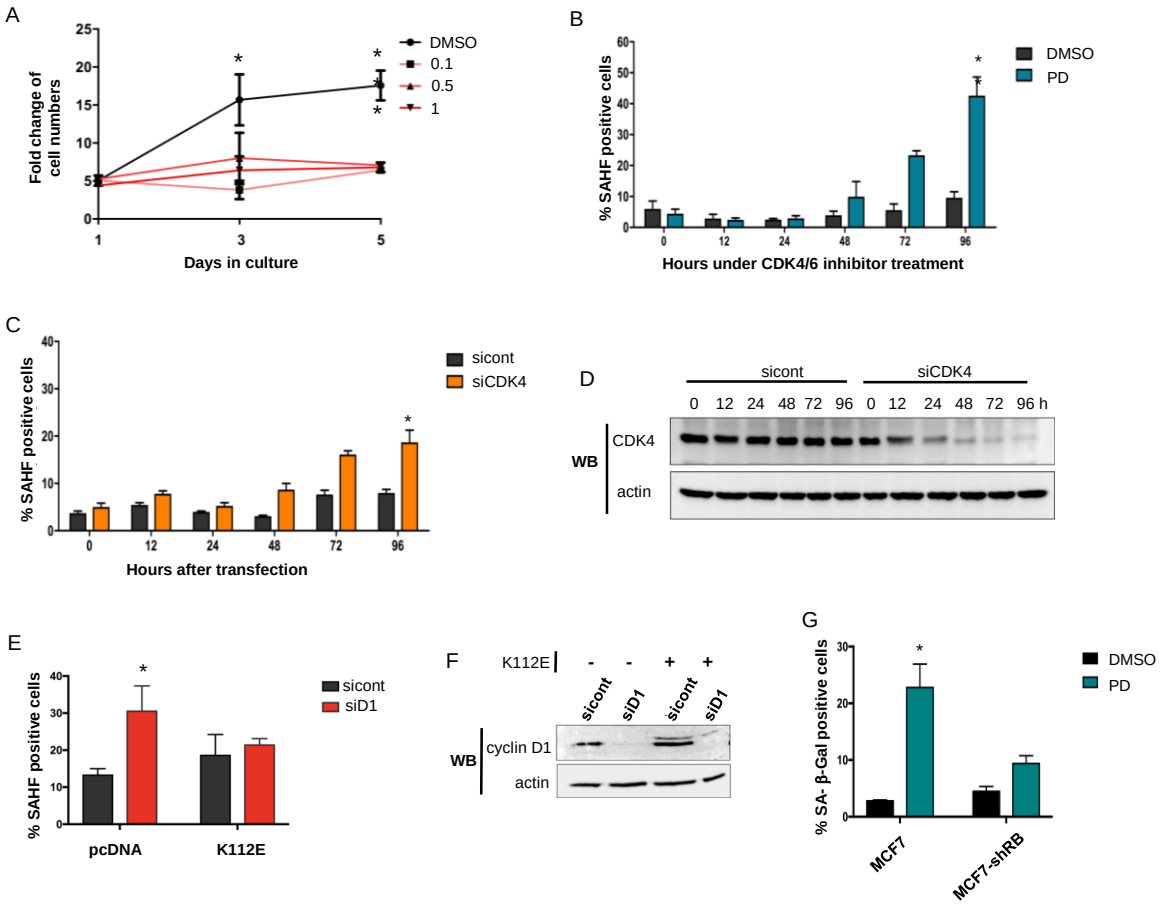

Fig. S3.

A. Growth curves of MCF7 cells treated with CDK4/6 inhibitor (PD) at indicated concentration (0, 0.1, 0.5, and 1 μM).

B. Percentages of SAHF-positive MCF7 cells were analyzed at 0, 12, 24, 48, 72, and 96 hours after the cells were treated with 0.5 μM PD 0332991 (PD). DMSO was use as a vehicle control. Bars represent averages from 3 independent experiments ± s.d. *(**p≤0.01*).

C. Percentages of SAHF-positive MCF7 cells were analyzed at 0, 12, 24, 48, 72, and 96 hours after the cells were transfected with CDK4-specific siRNA (siCDK4), or non-target control siRNA (sicont). Bars represent averages from 3 independent experiments ± s.d. *(*p≤0.05*).

D. WB analysis of CDK4 depletion by the CDK4-specific siRNA at indicated time points as in (c). Actin was used as a loading control.

E. Cells were co-transfected control pladmid pcDNA or kinase dead cyclin D1 (K112E), with GFP expressing plasmid peGFP, followed by cyclin D1 depletion using cyclin D1-specific siRNA targeting 5'UTR of cyclin D1 mRNA. Percentages of SAHF-positive MCF7 cells were analyzed in GFP-positive cells after 72 hours of cyclin D1 depletion (**p≤0.05*).

F. WB analysis of MCF7 cells expressing kinase-dead cyclin D1 (K112E). Lysates were collected after 72 hours of siRNA transfection.

G. Percentages of SA-β-Gal-positive cells after 5 days of PD0332991 (PD) or vehicle (DMSO) treatment . Bars represent averages from 3 independent experiments ± s.d. (**p≤0.05*).

Statistical significance was determined by Student's t-test

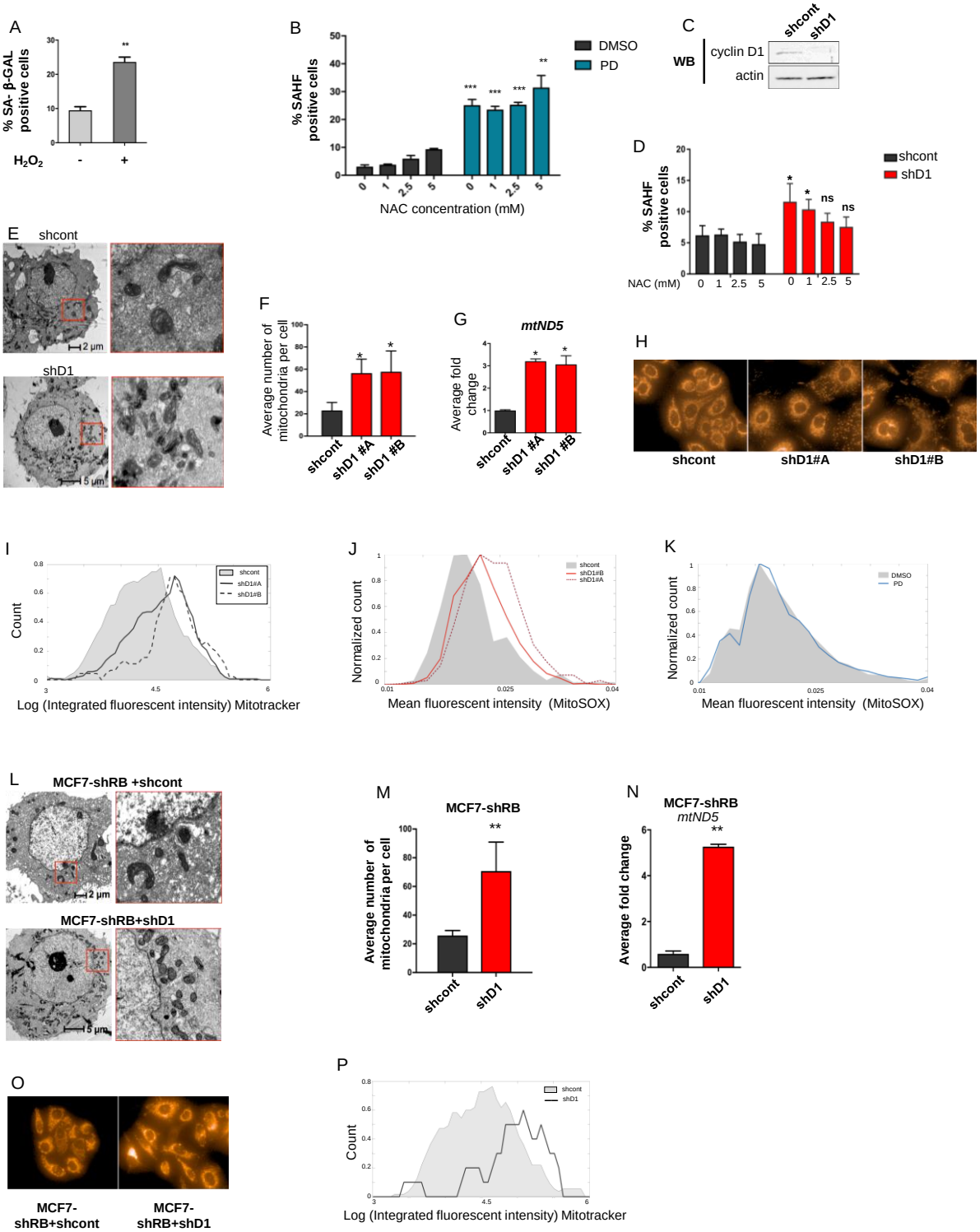
A
% SA- β-GAL positive cells
H2O2
B
% SAHF positive cells
DMSO
PD
NAC concentration (mM)
C
shcont
shD1
WB
cyclin D1
actin
D
% SAHF positive cells
shcont
shD1
NAC (mM)
E
shcont
shD1
2 µm
5 µm
F
Average number of mitochondria per cell
shcont
shD1 #A
shD1 #B
G
mtND5
Average fold change
H
shcont
shD1#A
shD1#B
I
Count
Log (Integrated fluorescent intensity) Mitotracker
J
Normalized count
Mean fluorescent intensity (MitoSOX)
K
Normalized count
DMSO
PD
Mean fluorescent intensity (MitoSOX)
L
MCF7-shRB +shcont
MCF7-shRB+shD1
M
MCF7-shRB
Average number of mitochondria per cell
N
MCF7-shRB
mtND5
Average fold change
O
MCF7-shRB+shcont
MCF7-shRB+shD1
P
Count
Log (Integrated fluorescent intensity) Mitotracker

Fig. S4.

A. MCF7 cells were treated with 1000 μM of H_2O_2 for 72 hours. Bars represent average percentages ± s.d. of SA-β-Gal-positive cells from 3 experiments *(**p≤0.01*)

B. PD0332991-induced senescence could not be prevented by N-acetyl cysteine (NAC). Percentages of SAHF-positive MCF7 cells were analyzed after 96 hours of 0.5 μM PD0332991 (PD) treatment. Indicated concentrations of NAC were added to the culture medium at the same time as PD treatment. DMSO was used as vehicle control. Bar represent average ± s.d *(**p≤0.01, ***p≤0.001*)

C. WB analysis of cyclin D1 in Capan-1 cells after transduced with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1) for 4 days.

D. Percentages of SAHF-positive Capan-1 cells were analyzed after transfected with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1) for 4 days . Indicated concentrations of NAC were added to the culture medium at the same time as cyclin D1 depletion. Bar represent average ± s.d *(*p≤0.05, ns; statistically not significant)*

E. MCF7 cells were transduced with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1) for 4 days before analyzed by transmission electron microscope (TEM). Red boxes are enhanced to the right figures.

F. Average numbers of mitochondria per cell analyzed by TEM from (c) (shcont; n = 4 cells) (shD1 #A, and #B; n = 5 cells). Bars represent averages of 3 experiments ± s.d. (**p≤0.05*)

G. Mitochondrial DNA contents of *mtND5* gene in cyclin D1-depleted, and control cells. Bars represent averages of 3 experiments ± s.d. (**p≤0.05*)

H. Mitotracker staining of MCF7 cells expressing non-targeting control shRNA (shcont), or cyclin D1-specific shRNAs (shD1#A, and shD1#B).

I. Histograms show Log integrated fluorescent intensity of Mitotracker stained mitochondria from MCF7 cells expressing either non-targeting shRNA (shcont), or cyclin D1-specific shRNAs (shD1#A, and shD1#B), from (f).

J. Histograms show mean fluorescent intensities of MitoSox Red stained mitochondria in MCF7 cells expressing either non-targeting shRNA (shcont), or cyclin D1-specific shRNAs (shD1#A, and shD1#B).

K. Histograms show mean fluorescent intensities of MitoSox Red stained mitochondria in MCF7 cells treated either with vehicle (DMSO), or 0.5μM PD0332991 (PD)

L. TEM analyses of pRB-deficient MCF7 cells (upper panel) and pRB-deficient MCF7 cells expressing cyclin D1-specific shRNA (shD1# C) (lower panel). Red boxes are enhanced to the right figures.

M. Average numbers of mitochondria per cell from (L). Bars represent averages ± s.d. (shcont, n = 5 cells; shD1, n = 5 cells) (**p≤0.01*)

N. Mitochondrial DNA content of *mtND5* gene in MCF7-shRB cells. Bars represent averages ± s.d. from 3 experiments (**p≤0.01*)

O. Mitotracker staining of MCF7-shRB cells expressing non-targeting shRNA (shcont), or a cyclin D1-specific shRNA (shD1#C).

P. Histograms show log integrated fluorescence intensity of Mitotracker stained mitochondria from MCF7-shRB cells expressing either control shcont (shcont), or cyclin D1-specific shRNA (shD1), from (m).

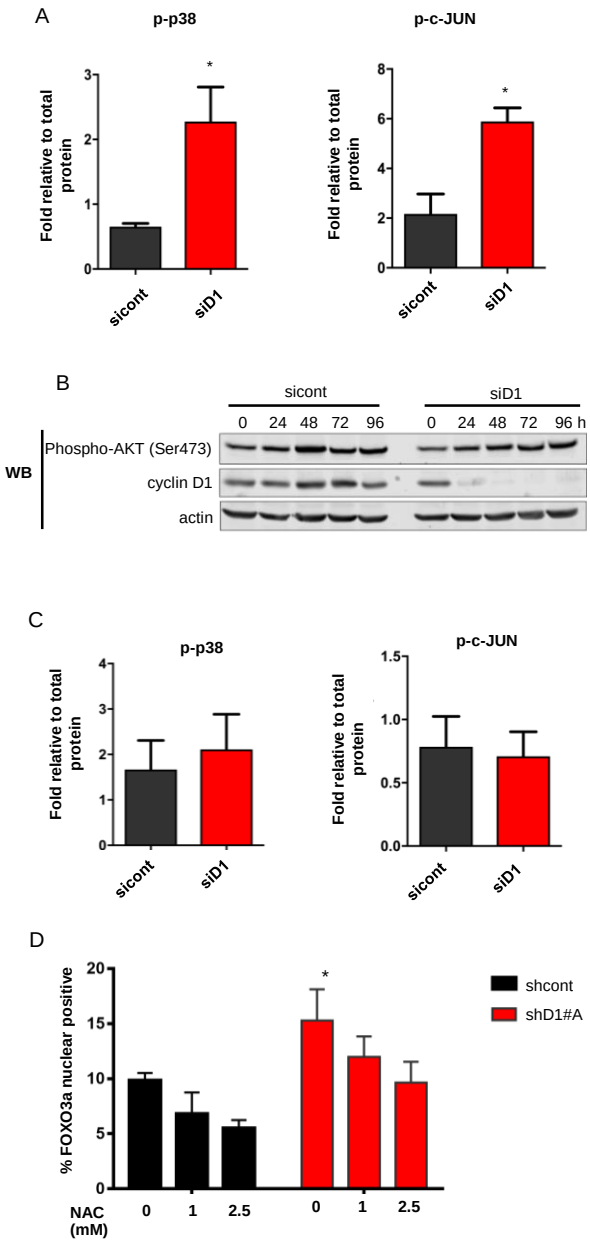

Fig. S5.

A. Cells were transfected with non-targeted siRNA (sicont) or cyclin D1 siRNA (siD1) transfection. Bars represent quantified signals of phospho-p38, and phospho-c-JUN normalized by total proteins at the 96-hour time point. (**p≤0.05*)
B. WB analysis of phospho-AKT Ser473, and cyclin D1 after transfected with with non-targeted siRNA (sicont) or cyclin D1 siRNA (siD1) at indicated time points. Actin was used as a loading control.
C. WB analysis was performed similarly as in **(A)**, except that freshly prepared NAC was added to the cells every other day from the 0 hour until the cells were harvested. Bars represent quantified signals of phospho-p38, and phospho-c-JUN normalized by total proteins at the 96-hour time point.
D. MCF7 cells were transduced with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1) for 4 days under treatment of NAC at indicated concentration. At least 5,000 FOXO3a nuclear-positive cells were analyzed. Bars represent percentage of cell that have nuclear FOXO3a localization. *(*p≤0.05*).

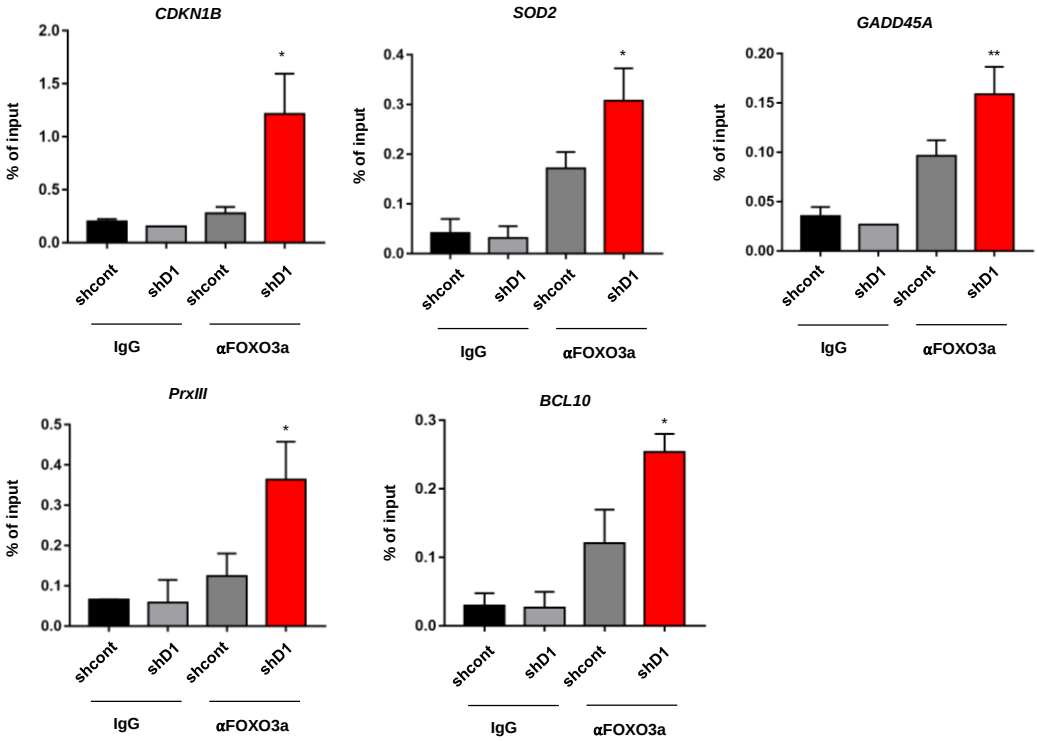

Fig. S6.

FOXO3a chromatin immunoprecipitation (ChIP) in cells expressing non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1). ChIP products were analyzed by semi-quantitative PCRs. IgG was used as a negative control for chromatin immunoprecipitation. Bars represent percent of input ± s.d. *(*p≤0.05, **p≤0.01*).

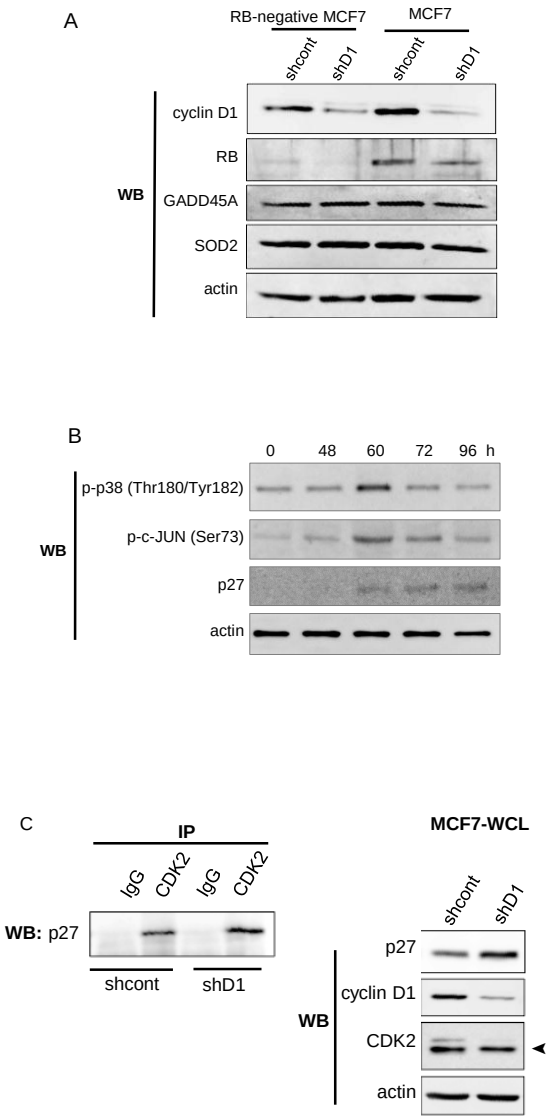

Fig. S7.

A. MCF7 cells and MCF7-expressing shRB were transduced with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1#C). Four days after transduction the cell lysates were collected and indicated proteins were analyzed by WB analyses. Actin was used as a loading control.

B. The upregulation of p27 coincided with p38 and c-JUN phosphorylations. WB analysis of p27, and p38/c-JUN specific phosphorylations. Protein lysates were prepared at 0, 48, 60, 72, and 96 hours after cyclin D1 siRNA (siD1) transfection.

C. (Left) WB analysis of p27 from immunoprecipitation by using IgG or CDK2 antibody in MCF7 cells expressing non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1). (Right) WB analyses of whole cell lysate (WCL) from MCF7 cell with non-targeting shRNA (shcont), or cyclin D1 shRNA (shD1). Arrow indicated the CDK2 band.