Supplemental Materials to

ATM-dependent phosphorylation of the checkpoint clamp regulates repair pathways and maintains genomic stability

Min Hwa Shin[1], Ming Yuan[1], Hao Zhang[2], Joseph B. Margolick[2] and Mihoko Kai[1]*

SUPPLEMENTAL FIGURE LEGENDS

Figure Sl1. Knockdown of endogenous Rad9 and ATM. (a) Flag-tagged wild type Rad9 and Rad9-S272C mutant proteins were expressed exogenously, and endogenous Rad9 expression was knocked down by its shRNA on the 3'UTR resign. (b) ATM knockdown by two independent ATM specific siRNAs. Five nM siRNAs were used for knockdown.

Figure Sl2. Knockdown of ATR and DNAPKc do not affect Rad9-S272 phosphorylation. (a) ATR knockdown by two independent ATR specific siRNAs (1 & 2) (top panel), Rad9-S272 phosphorylation is not affected by ATR knockdown (bottom panel). (b) DNA-PKc knockdown by two independent DNA-PKc specific siRNAs (1 & 2) (top panel), Rad9-S272 phosphorylation is not affected by DNA-PKc knockdown (bottom panel). NC: negative control siRNA,

Figure Sl3. Nuclear localization and damage-induced foci formation of Rad9 do not require ATM. Localization and foci formation of Rad9 protein was checked by anti-Rad9 antibody in ATM knockdown cells with and without IR treatment (3 Gy). Two independent ATM specific siRNAs were used (5 nM).

Figure Sl4. Rad9^{Ser272} phosphorylation is not required for Chk1 and Chk2 phosphorylation. (a) Chk1^{Ser345} phosphorylation after damage is normal in the Rad9-S272C cells. (b) Chk2^{Thr68} phosphorylation after damage is normal in the Rad9-S272C cells.

Figure Sl5. The Rad9-S272C mutant cells show intact DNA damage response. The Rad9-S272C mutant is not sensitive to DNA damage caused by IR, CPT, UV and HU. (a) Proliferation assay. Cells were incubated with indicated amount of CPT and HU for 6 hours, or irradiated at indicated dose of IR and UV. After 1 hour, the proliferation assay was performed. (b) Colony formation assay.

Cells were treated with 300 nM CPT (6 hours) and 100 μM HU (1 hour), 2 Gy IR or 4 J/m^2 UV irradiation. After 2 weeks, numbers of colonies were counted.

Figure SI6. γ-H2AX foci are detected in Rad9-S272C and Rad9-S272A mutant cells during S phase. G1, S and G2/M phase cells were stained with anti-γ-H2AX antibody.

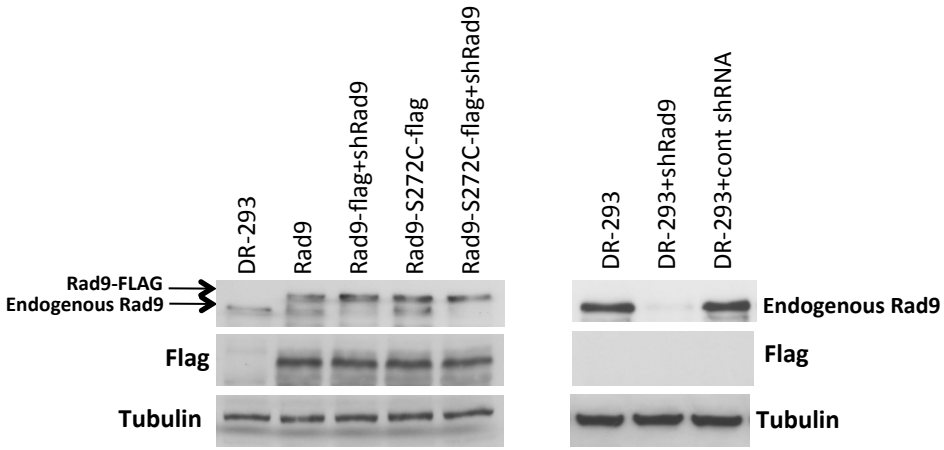
Figure SI7. Rad9^{Ser272} is phosphorylated during normal cell cycle in an ATM dependent manner. (a) Cell cycle progression and Rad9^{Ser272} phosphorylation in sorted cells with (bottom, left) and without KU55933 treatment (top, left). Cell cycle progression and Rad9^{Ser272} phosphorylation in sorted cells with siATM (bottom, right) and with control siRNA (top, right). (b) Cell cycle progression and Rad9^{S272} phosphorylation in sorted DLD1 (control) and ATR-Seckel cells.

Figure SI8. MRE11 is required for Rad9-S272 phosphorylation during S to G2 phase. MRE11, Rad50 and Nbs1 knockdown (5 nM) (top panels), Rad9-S272 phosphorylation in control and MRE11 specific siRNA knockdown cells (bottom panel).

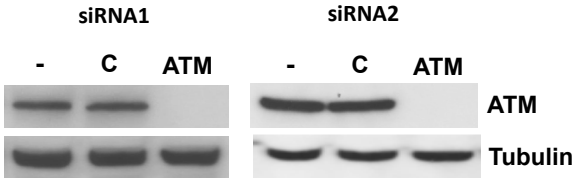
Figure SI9. Rad9^{Ser272} is phosphorylated during unperturbed cell cycle in primary cells. Cell cycle progression (left) and Rad9^{Ser272} phosphorylation (right) in sorted MRC5 cells.

Figure SI10. Phosphorylation of ATM substrates is normal in the Rad9-S272C mutant cells. Cell cycle progression and ATM substrate-phosphorylation in sorted Rad9 and Rad9-S272C cells.

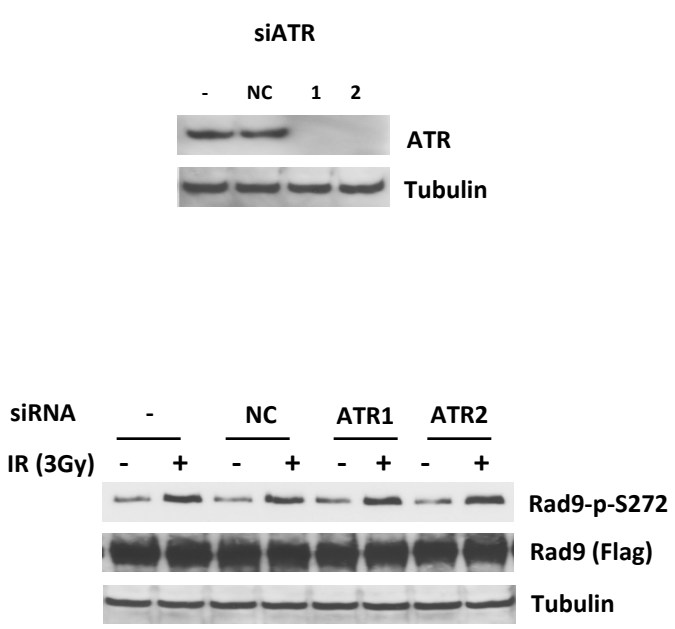
a


DR-293
Rad9
Rad9-flag+shRad9
Rad9-S272C-flag
Rad9-S272C-flag+shRad9
DR-293
DR-293+shRad9
DR-293+cont shRNA
Rad9-FLAG
Endogenous Rad9
Endogenous Rad9
Flag
Flag
Tubulin
Tubulin

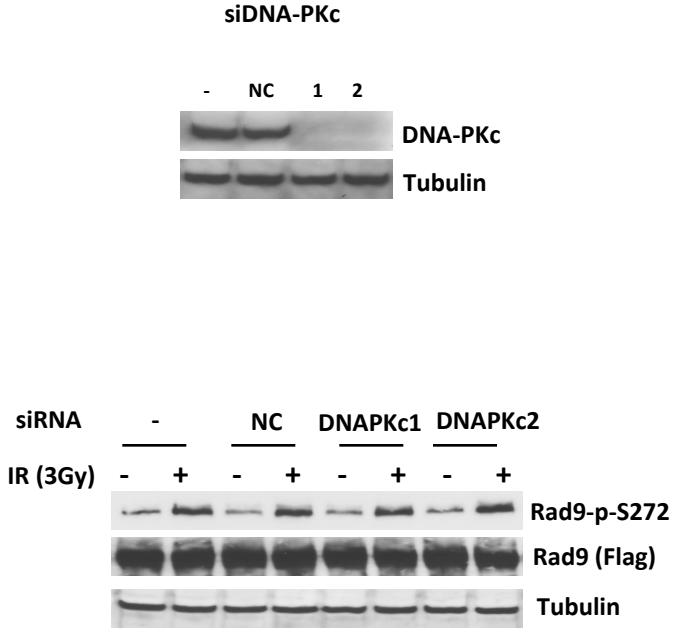
b

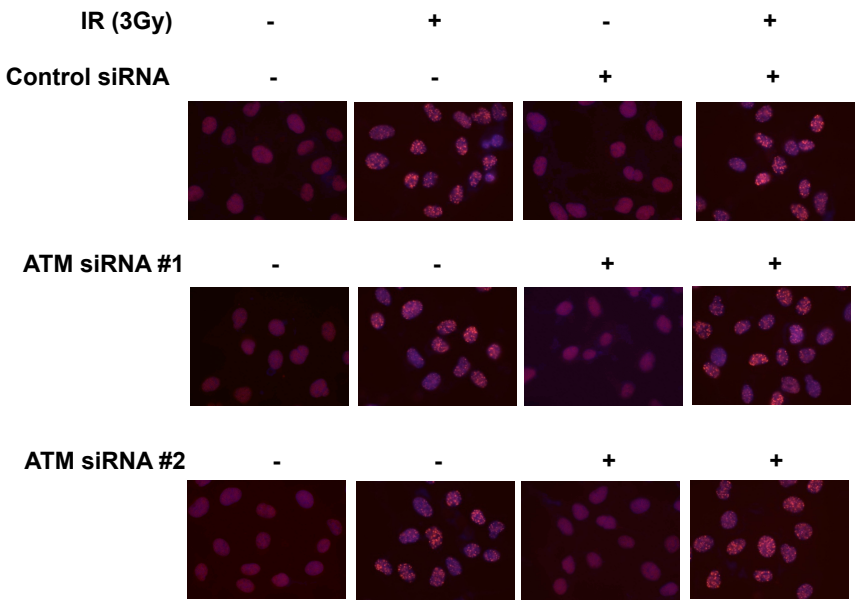

siRNA1
siRNA2
-
C
ATM
-
C
ATM
ATM
Tubulin

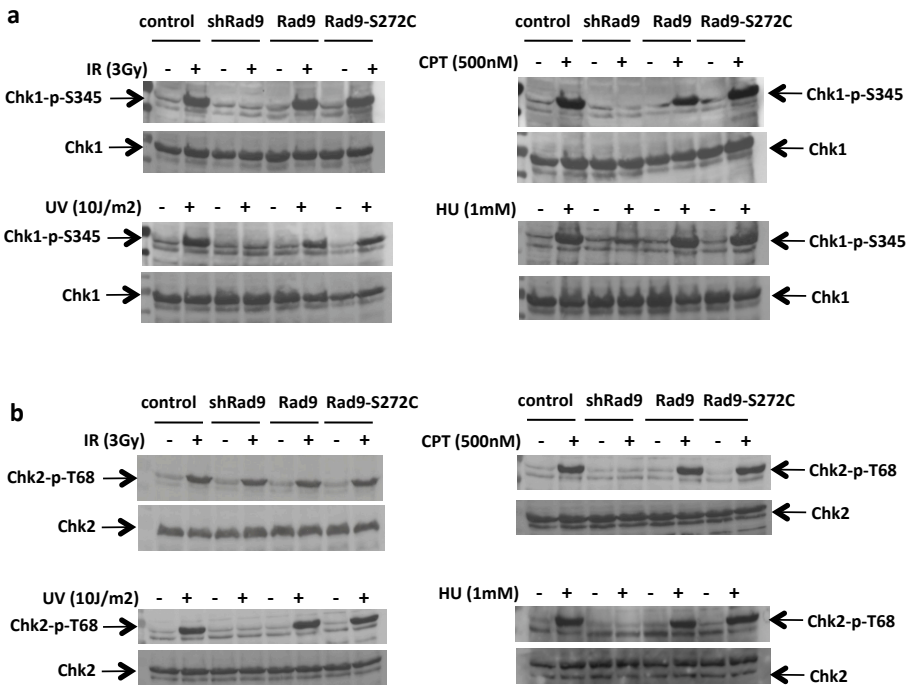
a


siATR
- NC 1 2
ATR
Tubulin
siRNA - NC ATR1 ATR2
IR (3Gy) - + - + - + - +
Rad9-p-S272
Rad9 (Flag)
Tubulin

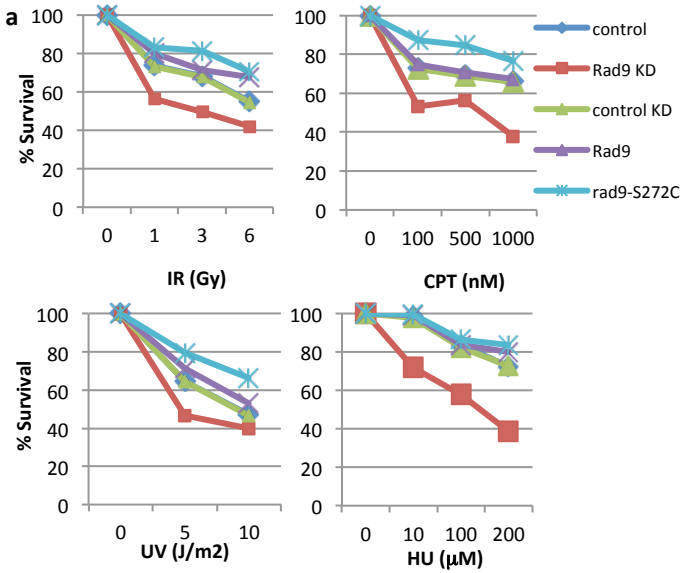
b

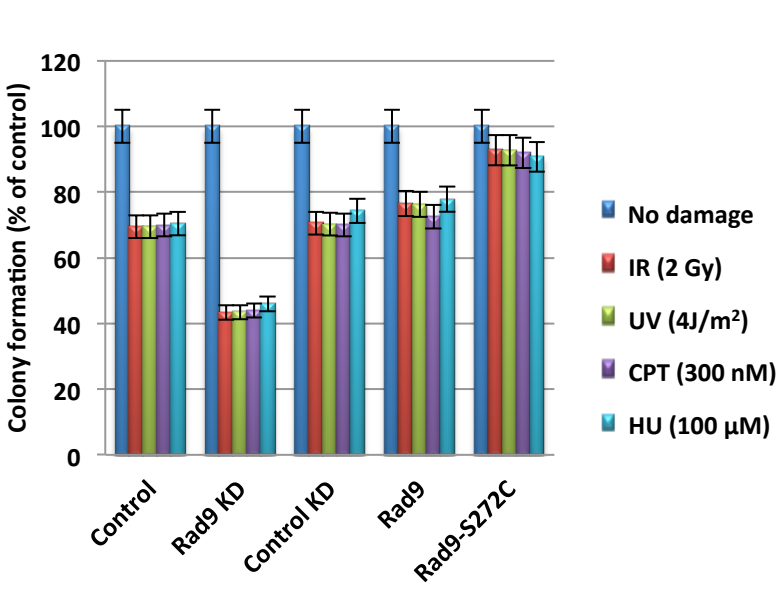

siDNA-PKc
- NC 1 2
DNA-PKc
Tubulin
siRNA - NC DNAPKc1 DNAPKc2
IR (3Gy) - + - + - + - +
Rad9-p-S272
Rad9 (Flag)
Tubulin


IR (3Gy) - + - +
Control siRNA - - + +
ATM siRNA #1 - - + +
ATM siRNA #2 - - + +

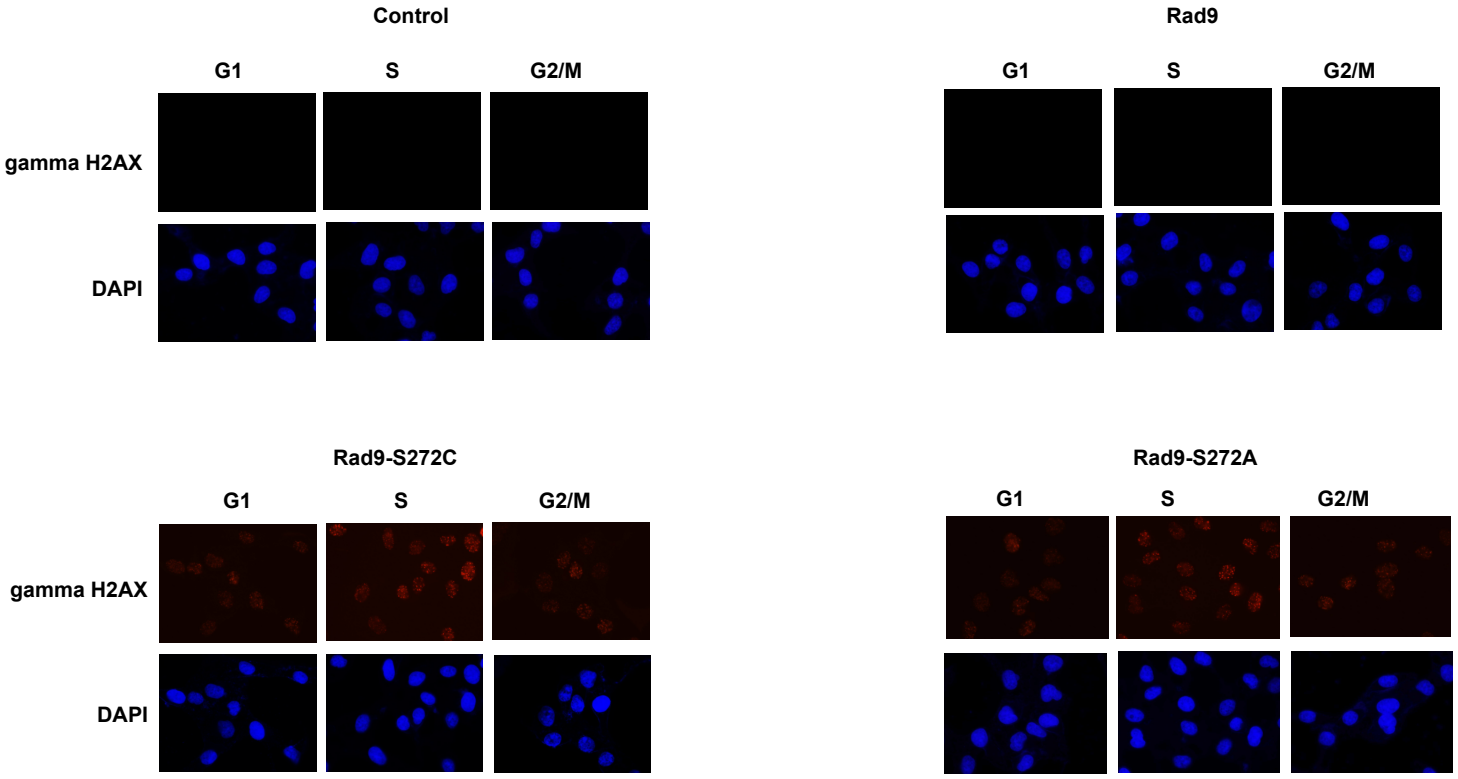

a
control
shRad9
Rad9
Rad9-S272C
IR (3Gy)
Chk1-p-S345
Chk1
UV (10J/m2)
CPT (500nM)
HU (1mM)
b
Chk2-p-T68
Chk2

Shin_Fig Sl5

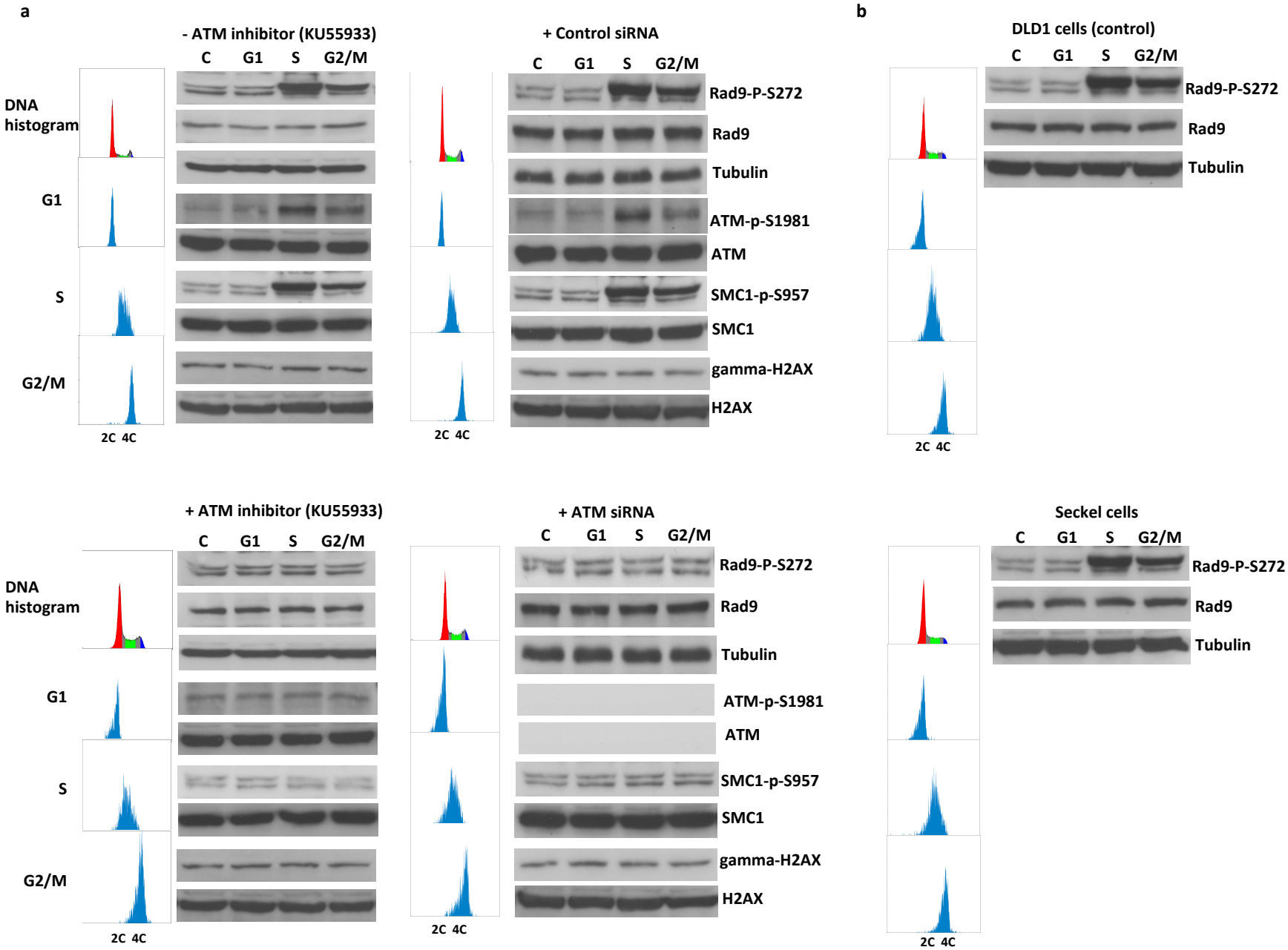

a
% Survival
IR (Gy)
CPT (nM)
UV (J/m2)
HU (μM)
control
Rad9 KD
control KD
Rad9
rad9-S272C


b
Colony formation (% of control)
Control
Rad9 KD
Control KD
Rad9
Rad9-S272C
No damage
IR (2 Gy)
UV (4J/m²)
CPT (300 nM)
HU (100 μM)

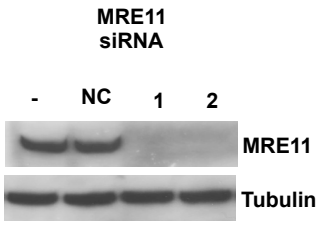
Shin_Fig Sl6

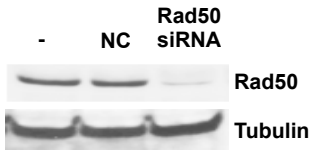

Control
G1
S
G2/M
gamma H2AX
DAPI
Rad9
G1
S
G2/M
Rad9-S272C
G1
S
G2/M
gamma H2AX
DAPI
Rad9-S272A
G1
S
G2/M

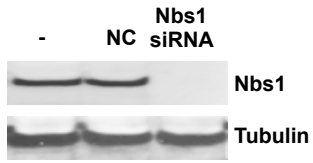
Shin_Fig Sl7


a
- ATM inhibitor (KU55933)
+ Control siRNA
C G1 S G2/M
DNA histogram
G1
S
G2/M
2C 4C
Rad9-P-S272
Rad9
Tubulin
ATM-p-S1981
ATM
SMC1-p-S957
SMC1
gamma-H2AX
H2AX
b
DLD1 cells (control)
+ ATM inhibitor (KU55933)
+ ATM siRNA
Seckel cells

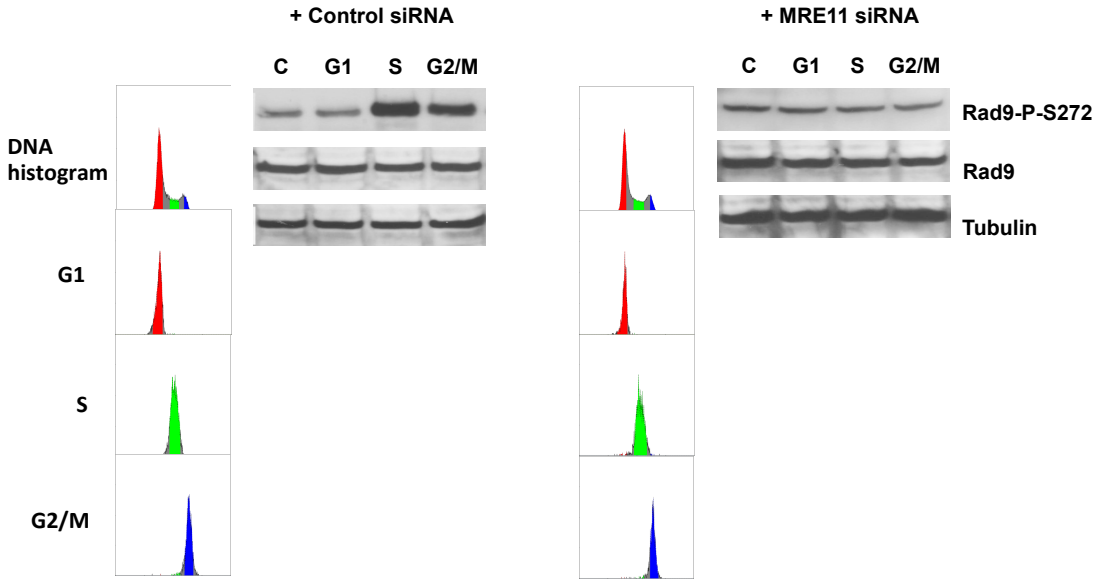
Shin_ Fig Sl8



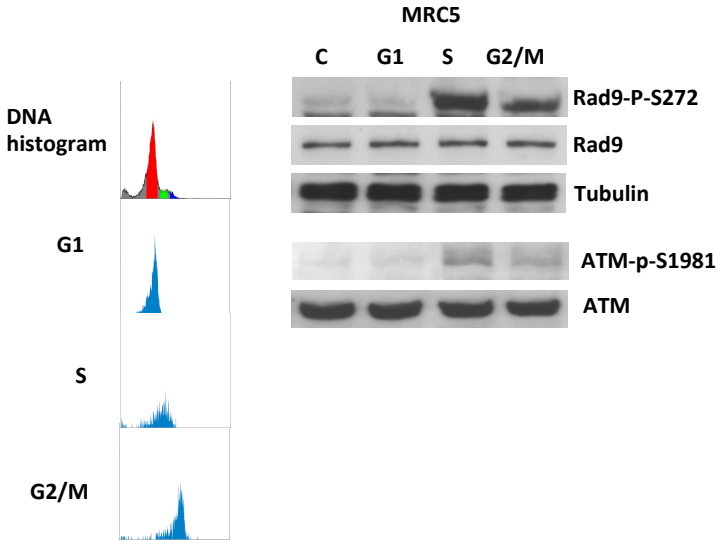


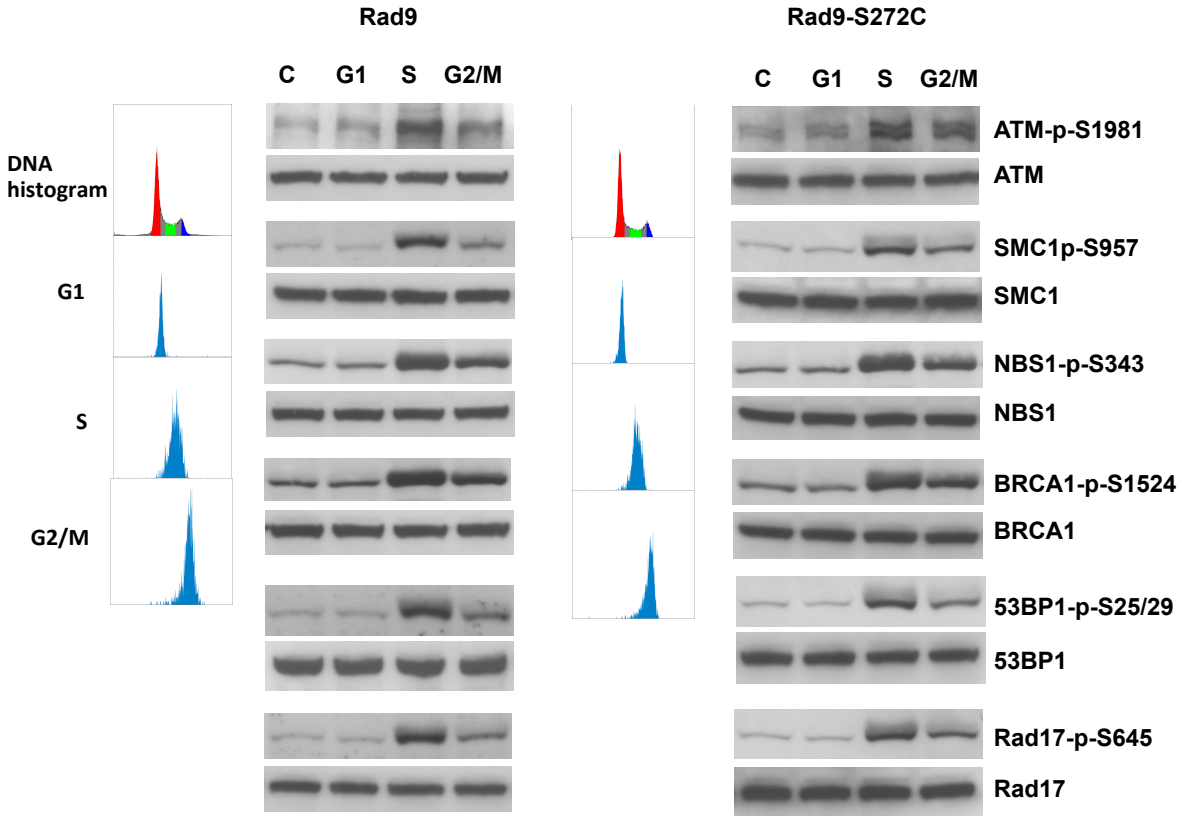


NC: Negative Control



Shin_ Fig Sl9


MRC5
C
G1
S
G2/M
DNA histogram
G1
S
G2/M
Rad9-P-S272
Rad9
Tubulin
ATM-p-S1981
ATM


Rad9
Rad9-S272C
C
G1
S
G2/M
C
G1
S
G2/M
DNA histogram
G1
S
G2/M
ATM-p-S1981
ATM
SMC1p-S957
SMC1
NBS1-p-S343
NBS1
BRCA1-p-S1524
BRCA1
53BP1-p-S25/29
53BP1
Rad17-p-S645
Rad17