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Supplementary Materials for

Extracellular matrix stiffness determines DNA repair efficiency and cellular sensitivity to genotoxic agents

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This PDF file includes:

Figure S1

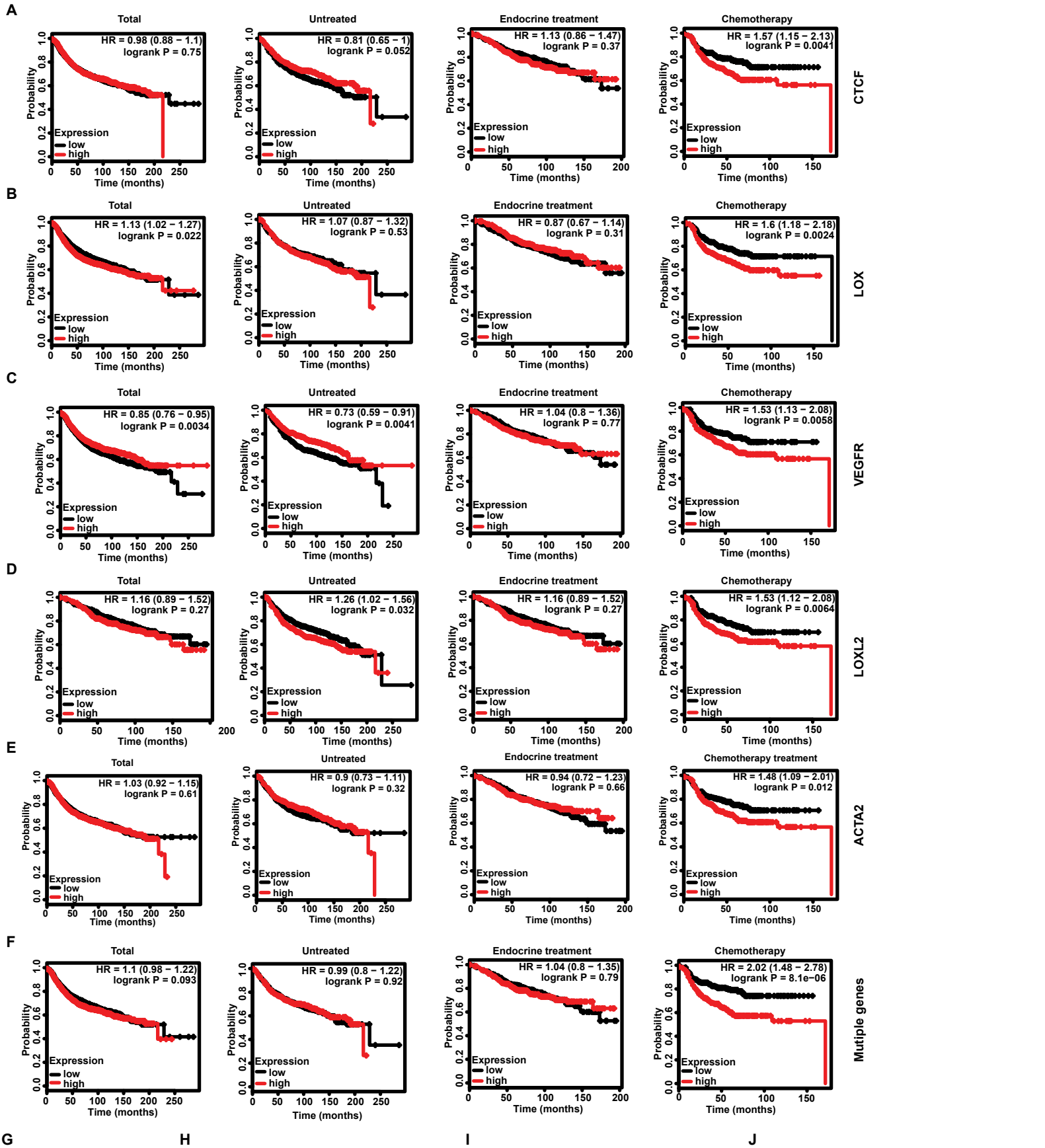

A
Total
HR = 0.98 (0.88 − 1.1)
logrank P = 0.75
Untreated
HR = 0.81 (0.65 − 1)
logrank P = 0.052
Endocrine treatment
HR = 1.13 (0.86 − 1.47)
logrank P = 0.37
Chemotherapy
HR = 1.57 (1.15 − 2.13)
logrank P = 0.0041
CTCF
B
Total
HR = 1.13 (1.02 − 1.27)
logrank P = 0.022
Untreated
HR = 1.07 (0.87 − 1.32)
logrank P = 0.53
Endocrine treatment
HR = 0.87 (0.67 − 1.14)
logrank P = 0.31
Chemotherapy
HR = 1.6 (1.18 − 2.18)
logrank P = 0.0024
LOX
C
Total
HR = 0.85 (0.76 − 0.95)
logrank P = 0.0034
Untreated
HR = 0.73 (0.59 − 0.91)
logrank P = 0.0041
Endocrine treatment
HR = 1.04 (0.8 − 1.36)
logrank P = 0.77
Chemotherapy
HR = 1.53 (1.13 − 2.08)
logrank P = 0.0058
VEGFR
D
Total
HR = 1.16 (0.89 − 1.52)
logrank P = 0.27
Untreated
HR = 1.26 (1.02 − 1.56)
logrank P = 0.032
Endocrine treatment
HR = 1.16 (0.89 − 1.52)
logrank P = 0.27
Chemotherapy
HR = 1.53 (1.12 − 2.08)
logrank P = 0.0064
LOXL2
E
Total
HR = 1.03 (0.92 − 1.15)
logrank P = 0.61
Untreated
HR = 0.9 (0.73 − 1.11)
logrank P = 0.32
Endocrine treatment
HR = 0.94 (0.72 − 1.23)
logrank P = 0.66
Chemotherapy treatment
HR = 1.48 (1.09 − 2.01)
logrank P = 0.012
ACTA2
F
Total
HR = 1.1 (0.98 − 1.22)
logrank P = 0.093
Untreated
HR = 0.99 (0.8 − 1.22)
logrank P = 0.92
Endocrine treatment
HR = 1.04 (0.8 − 1.35)
logrank P = 0.79
Chemotherapy
HR = 2.02 (1.48 − 2.78)
logrank P = 8.1e−06
Mutiple genes
Probability
Expression
low
high
Time (months)
G
Tumor weight (g)
**
Bliss Independence synergy threshold
Contorl
BAPN
Cisplatin
BAPN+Cisplatin
H
Tumor volume (mm3)
Control
Cisplatin
BAPN
BAPN+Cisplatin
**
Days post treatment
I
9kPa
9kPa+Cisplatin
0.4kPa
0.4kPa+Cisplatin
J
Control
BAPN
Cisplatin
Cisplatin +BAPN
1 2 3 1 2 3 1 2 3 1 2 3
Cleaved PARP
Cleaved caspase 3
actin
100
75
25
20
37

Fig.S1 ECM stiffness regulates tumor chemo-sensitivity.

(A to F) Survival for stiffness marker genes with different treatments in breast cancer patients. Kaplan-Meier survival plots show that higher expression of CTGF (A), LOX (B), VEGFR (C), LOXL2 (D) and ACTA2 (E) results in a worse survival after chemotherapy treatment while has subtle effect when untreated or endocrine treatment was used. Combination of these genes (F) has similar effect on chemotherapy treatment.

(G) 1×10^6 mouse mammary tumor 4T1 cells were injected into the mammary gland of Balb/c mice. After the tumor size reached 100 mm^3, mice were treated with cisplatin, BAPN or combination. Tumor weight of mice with subcutaneous injection at day 28 after treatment. The Bliss Independence model of additivity indicates an additive effect of the drug combination for a synergistic effect for BAPN and cisplatin. **: $p < 0.01$.

(H) Tumor growth was measured at the indicated times after treatment. n = 5 for each group. (**: $p < 0.01$, by two-way ANOVA.)

(I) Tumor images from (G).

(J) Protein extraction of tumor from (G) were run on SDS-PAGE gel and probed with indicated antibodies.

Figure S2

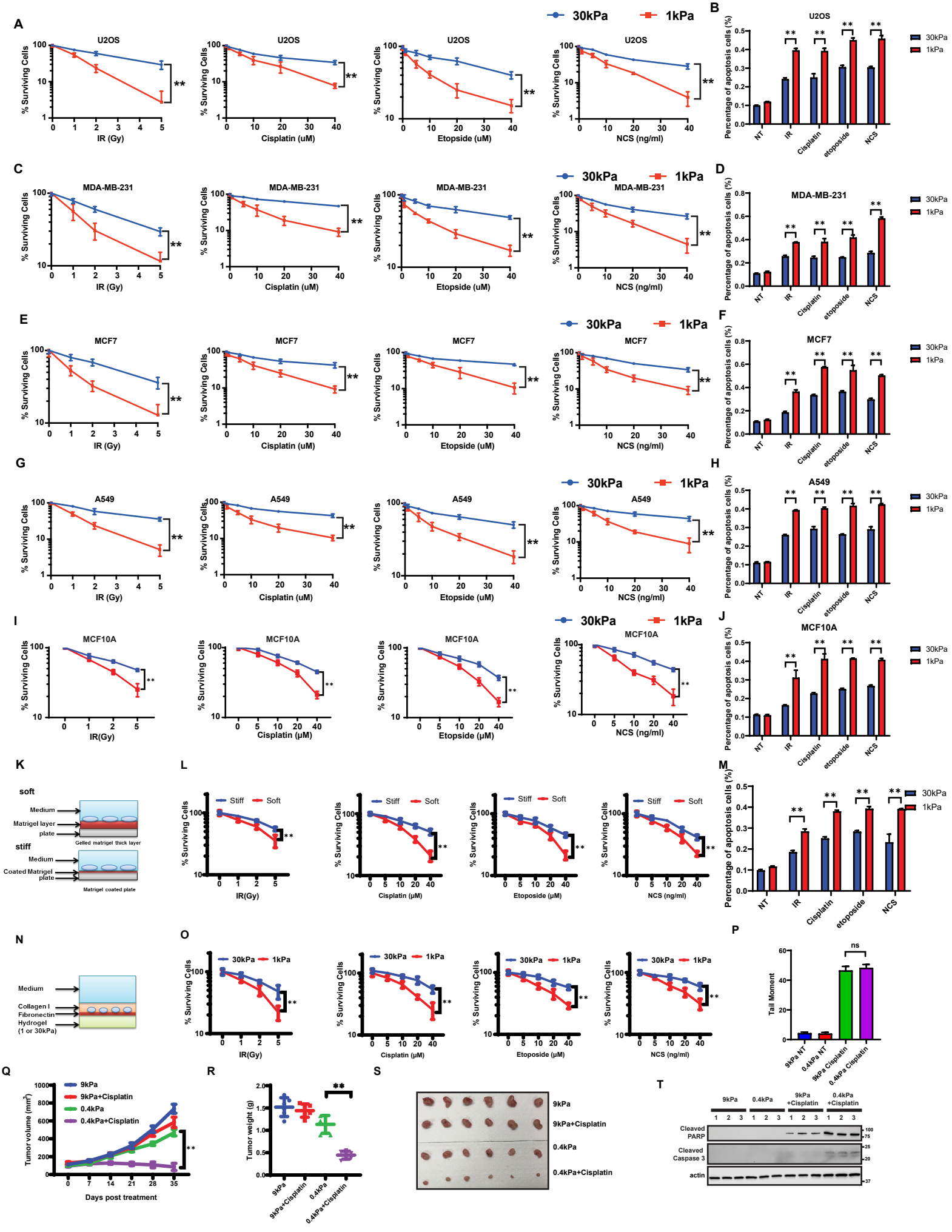

A
U2OS
% Surviving Cells
IR (Gy)
Cisplatin (uM)
Etopside (uM)
NCS (ng/ml)
30kPa
1kPa
B
Percentage of apoptosis cells (%)
NT
IR
Cisplatin
etoposide
NCS
C
MDA-MB-231
D
E
MCF7
F
G
A549
H
I
MCF10A
IR(Gy)
Etopside (μM)
J
K
soft
Medium
Matrigel layer
plate
Gelled matrigel thick layer
stiff
Coated Matrigel
Matrigel coated plate
L
Stiff
Soft
M
N
Collagen I
Fibronectin
Hydrogel
(1 or 30kPa)
O
P
Tail Moment
ns
9kPa NT
0.4kPa NT
9kPa Cisplatin
0.4kPa Cisplatin
Q
Tumor volume (mm³)
9kPa
9kPa+Cisplatin
0.4kPa
0.4kPa+Cisplatin
Days post treatment
R
Tumor weight (g)
S
T
Cleaved PARP
Cleaved Caspase 3
actin

Fig.S2 Low stiffness impairs DSB repair and increases cellular sensitivity to genotoxic agents.

(A).U2OS cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents. Colonogenic assays were performed to detect cell survival on different ECM.

(B).U2OS cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents (10 Gy IR, 10 μM cisplatin, 10 μM etoposide, 10 ng/ml NCS). Cells were trypsinized after 48h and cellular apoptosis was detected by Annexin V and PI staining. Error bars represent SD of the mean for 3 replicate samples analyzed in one experiment.

(C). MDA-MB-231 cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents. Colonogenic assays were performed to detect cell survival on different ECM.

(D). MDA-MB-231 cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents (10 Gy IR, 10 μM cisplatin, 10 μM etoposide, 10 ng/ml NCS). Cells were trypsinized after 48h and cellular apoptosis was detected by Annexin V and PI staining. Error bars represent SD of the mean for 3 replicate samples analyzed in one experiment.

(E). MCF7 cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents. Colonogenic assays were performed to detect cell survival on different ECM.

(F). MCF7 cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents (10 Gy IR, 10 μM cisplatin, 10 μM etoposide, 10 ng/ml NCS). Cells were trypsinized after 48h and cellular apoptosis was detected by Annexin V and PI staining. Error bars represent SD of the mean for 3 replicate samples analyzed in one experiment.

(G). A549 cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents. Colonogenic assays were performed to detect cell survival on different ECM.

(H). A549 cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents (10 Gy IR, 10 μM cisplatin, 10 μM etoposide, 10 ng/ml NCS). Cells were trypsinized after 48h and cellular apoptosis was detected by Annexin V and PI staining. Error bars represent SD of the mean for 3 replicate samples analyzed in one experiment.

(I).MCF10A cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents. Colonogenic assays were performed to detect cell survival on different ECM.

(J).MCF10A cells were plated on low (1 kPa) and high (30 kPa) stiff hydrogel coated with fibronectin. Cells were treated with indicated genotoxic agents (10 Gy IR, 10 μM cisplatin, 10 μM etoposide, 10 ng/ml NCS). Cells were trypsinized after 48h and cellular apoptosis was detected by Annexin V and PI staining. Error bars represent SD of the mean for 3 replicate samples analyzed in one experiment.

(K) Model of cell culture on Matrigel coated plates (stiff) and Matrigel thick layer (soft).

(L) HEK293 cells were plated on Matrigel coated plates (stiff) and Matrigel thick layer (soft). Cells were treated with indicated doses of genotoxic agents. Colony formation assays were performed to detect cell survival on different ECM. Data are presented as mean ± S.D., n = 3 biologically independent samples. (**: $p < 0.01$)

(M) HEK293 cells were plated on Matrigel coated plates (stiff) and Matrigel thick layer (soft). Cells were treated with indicated genotoxic agents (10 Gy IR, 10 μM cisplatin, 10 μM etoposide, 10 ng/ml NCS). Cells were trypsinized after 48h and cellular apoptosis was detected by Annexin V and PI staining. Error bars represent SD of the mean for 3 replicate samples analyzed in one experiment.

(N) Model of 3D cell culture on stiff (30 kPa) and soft (1 kPa) hydrogels.

(O) HEK293 cells were grown on stiff (30 kPa) and soft (1 kPa) hydrogels and overlaid with Collagen I. Cells were treated with indicated doses of genotoxic agents. Colony formation assays were performed to detect cell survival on different ECM. Data are presented as mean ± S.D., n = 3 biologically independent samples. (**: $p < 0.01$)

(P) 5×10^6 MDA-MB-231 cells were embedded in 200 μl of 0.4 or 9.0 kPa hyaluronan-based gel and were subcutaneously injected into nude mice. Mice were treated with or without cisplatin at 1 mg/kg for 6h. Cells from tumor were trypsinized and harvested for neutral comet assay.

(Q) 5×10^6 MDA-MB-231 cells were embedded in 200 μl of 0.4 or 9.0 kPa hyaluronan-based gel and were subcutaneously injected into nude mice. Mice were treated with or without cisplatin at 1 mg/kg/day. Tumor growth was measured at the indicated times after treatment. n = 6 for each group. (**: $p < 0.01$, by two-way ANOVA.)

(R) Tumor weight of mice with subcutaneous injection as described in (l) at day 35 after treatment. **: $p < 0.01$, by two-way ANOVA.

(S) Images of tumors from (R).

(T) Protein extraction of tumor from (R) were run on SDS-PAGE gel and probed with indicated antibodies.

Figure S3

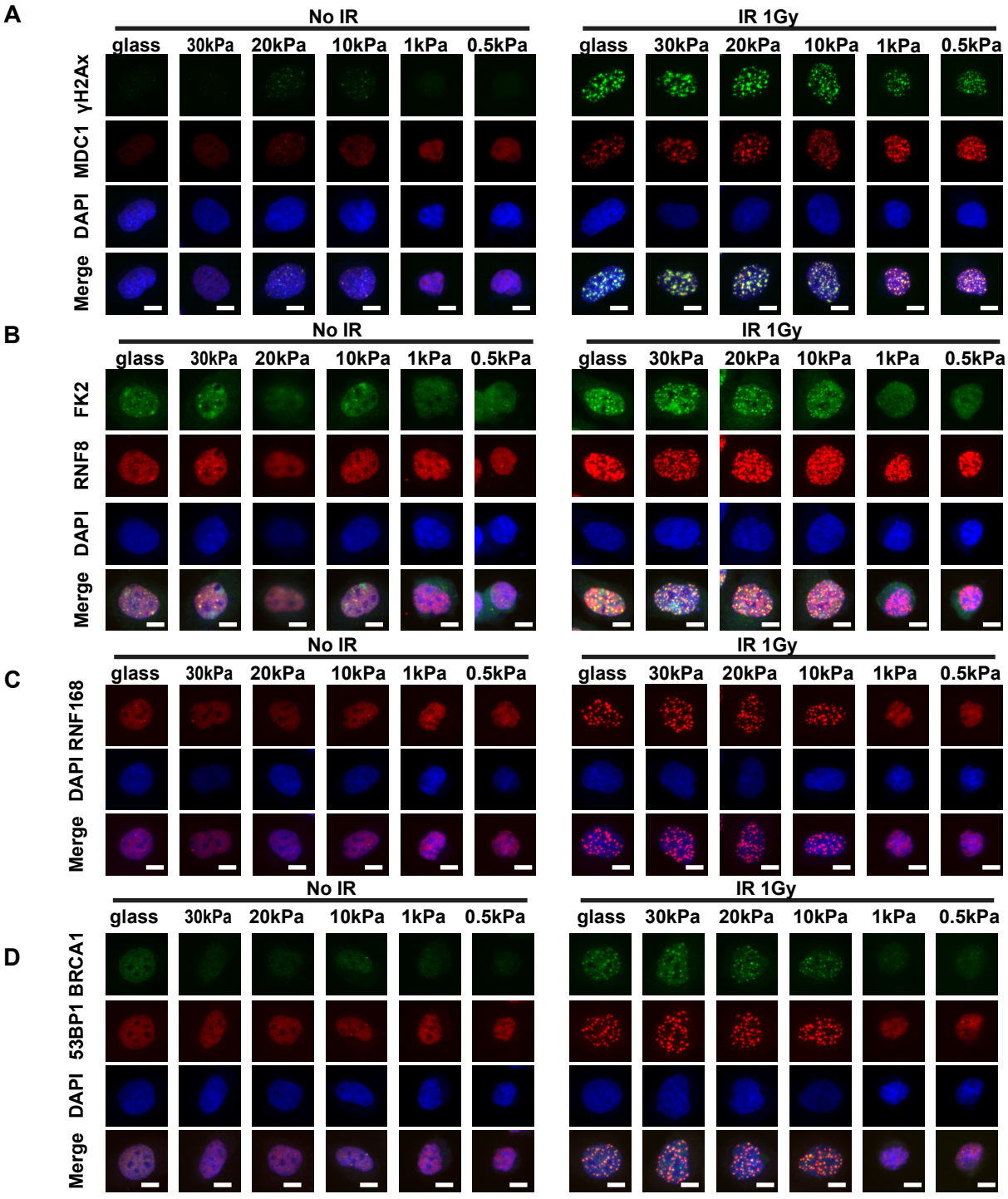

A
No IR
IR 1Gy
glass
30kPa
20kPa
10kPa
1kPa
0.5kPa
γH2Ax
MDC1
DAPI
Merge
B
FK2
RNF8
C
RNF168
D
BRCA1
53BP1

Fig.S3 Low stiffness inhibits DSB repair at the level of RNF8 in the DSB repair pathway.

(A-D) HEK293 cells were grown on fibronectin coated hydrogels of different stiffness for 24 hours. Cells were fixed 1 hour after irradiation (1 Gy) and stained with anti- γ-H2AX and MDC1 (A), RNF8 and FK2 (B), 53BP1 and BRCA1 (D) antibodies. For RNF168 (C), HEK293 cells expressing mCherry-RNF168 were plated on fibronectin coated hydrogels of different stiffness. Cells were fixed 1 hour after irradiation (1 Gy) and RNF168 foci were visualized with mCherry. Representative images showing IR induced foci after 1Gy irradiation. Scale bar, 10 μM

Figure S4

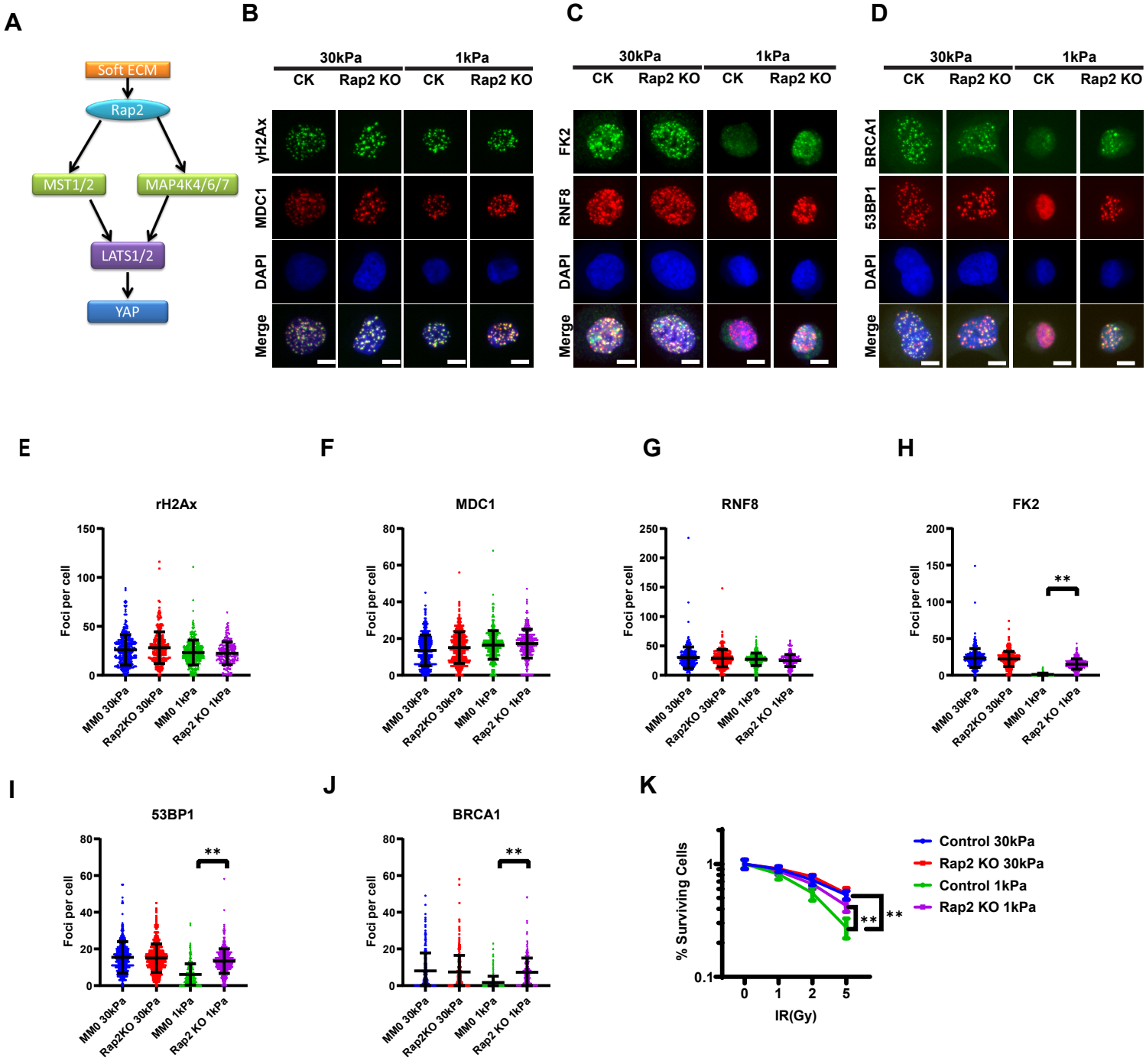

A
Soft ECM
Rap2
MST1/2
MAP4K4/6/7
LATS1/2
YAP
B
30kPa
1kPa
CK
Rap2 KO
CK
Rap2 KO
γH2Ax
MDC1
DAPI
Merge
C
30kPa
1kPa
CK
Rap2 KO
CK
Rap2 KO
FK2
RNF8
DAPI
Merge
D
30kPa
1kPa
CK
Rap2 KO
CK
Rap2 KO
BRCA1
53BP1
DAPI
Merge
E
rH2Ax
Foci per cell
MM0 30kPa
Rap2KO 30kPa
MM0 1kPa
Rap2 KO 1kPa
F
MDC1
Foci per cell
MM0 30kPa
Rap2KO 30kPa
MM0 1kPa
Rap2 KO 1kPa
G
RNF8
Foci per cell
MM0 30kPa
Rap2KO 30kPa
MM0 1kPa
Rap2 KO 1kPa
H
FK2
Foci per cell
**
MM0 30kPa
Rap2KO 30kPa
MM0 1kPa
Rap2 KO 1kPa
I
53BP1
Foci per cell
**
MM0 30kPa
Rap2KO 30kPa
MM0 1kPa
Rap2 KO 1kPa
J
BRCA1
Foci per cell
**
MM0 30kPa
Rap2KO 30kPa
MM0 1kPa
Rap2 KO 1kPa
K
% Surviving Cells
IR(Gy)
Control 30kPa
Rap2 KO 30kPa
Control 1kPa
Rap2 KO 1kPa
**
**

Fig.S4 Inhibition of DSB repair by low stiffness is dependent on Rap2.

(A) A model showing the role of Rap2 in mechanotransduction.

(B to D) Control (CK) and Rap2 knockout (Rap2KO) HEK293 cells were grown on soft (1 kPa) and stiff (30 kPa) fibronectin coated hydrogels. Cells were fixed 1 hour after irradiation (1Gy) and stained with anti- γ-H2AX and MDC1 (B), RNF8 and FK2 (C), 53BP1 and BRCA1 (D) antibodies. Scale bar, 10 μM.

(E to J) Quantification of (B to D) is described in Methods. Data are presented as mean ± S.D., n = 3 biologically independent samples. (**: $p < 0.01$)

(K) Rap2 is required for regulation of stiffness-induced radiation sensitivity. Colony formation assays were performed to examine radio-sensitivity of wild type (Control) and Rap2 knockout (Rap2KO) HEK293 cells on soft (1 kPa) and stiff (30 kPa) fibronectin coated hydrogels. Data are presented as mean ± S.D., n = 3 biologically independent samples. (**: $p < 0.01$)

Figure S5

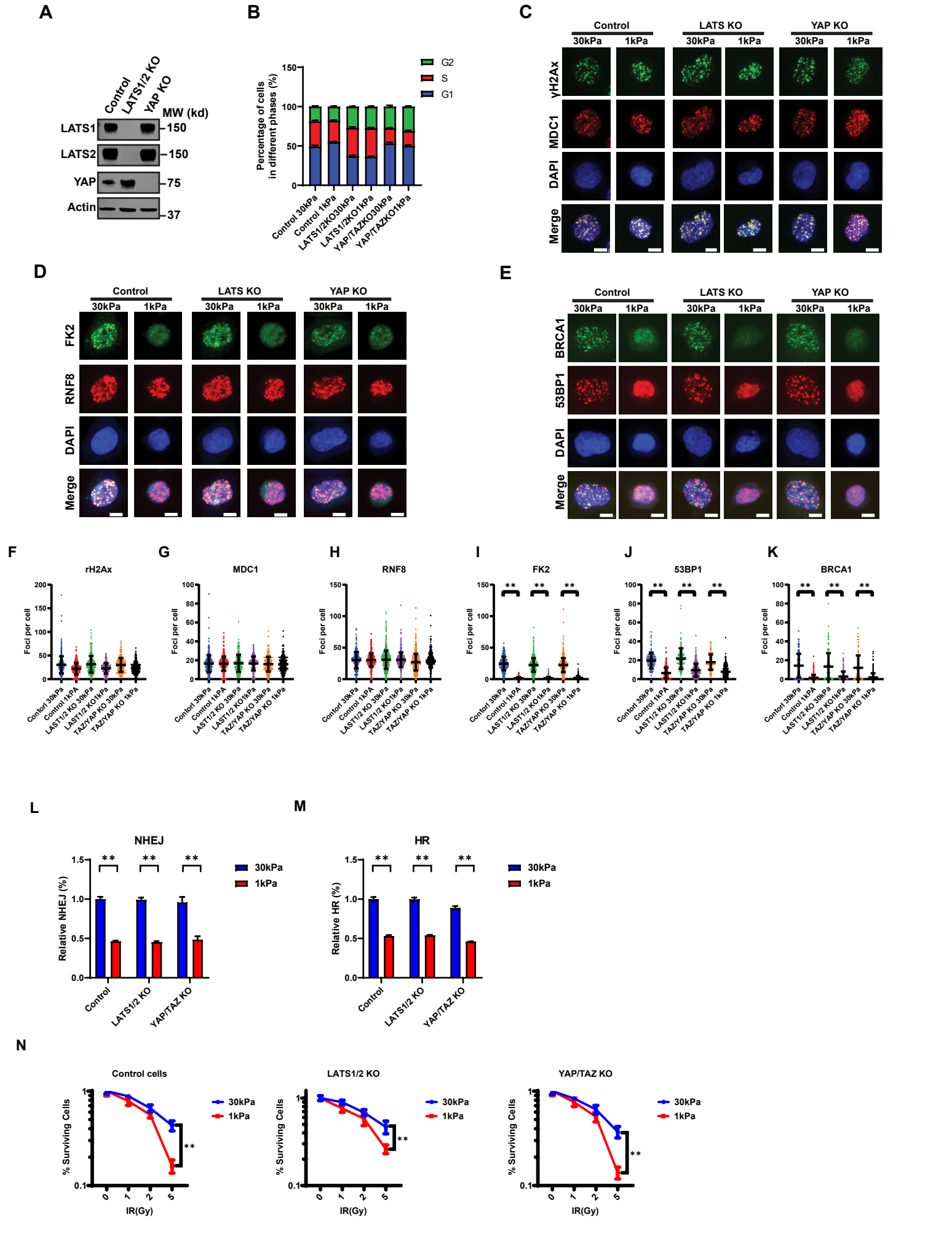

A
Control
LATS1/2 KO
YAP KO
MW (kd)
LATS1
LATS2
YAP
Actin
150
150
75
37
B
Percentage of cells in different phases (%)
G2
S
G1
Control 30kPa
Control 1kPa
LATS1/2KO30kPa
LATS1/2KO1kPa
YAP/TAZKO30kPa
YAP/TAZKO1kPa
C
Control
LATS KO
YAP KO
30kPa
1kPa
γH2Ax
MDC1
DAPI
Merge
D
FK2
RNF8
E
BRCA1
53BP1
F
rH2Ax
G
MDC1
H
RNF8
I
FK2
J
53BP1
K
BRCA1
Foci per cell
L
NHEJ
Relative NHEJ (%)
M
HR
Relative HR (%)
Control
LATS1/2 KO
YAP/TAZ KO
N
Control cells
LATS1/2 KO
YAP/TAZ KO
% Surviving Cells
IR(Gy)

Fig.S5 Inhibition of DSB repair by low stiffness is independent LATS/YAP.

(A) Western blots showing the expression levels of LATS1/2 and YAP in control, LATS1/2 knockout (LATS1/2 KO) and YAP/TAZ knockout (YAP/TAZ KO) HEK293 cells.

(B) Control (MM0), LATS1/2 KO (LATS KO) and YAP/TAZ KO (YAP KO) cells were grown on soft (1 kPa) and stiff (30 kPa) fibronectin coated hydrogels for 24 hours. Cell cycle distribution was detected by flow cytometry.

(C to E) Control (MM0), LATS1/2 KO (LATS KO) and YAP/TAZ KO (YAP/TAZ KO) cells were grown on soft (1 kPa) and stiff (30 kPa) fibronectin coated hydrogels for 24 hours. Cells were fixed 1 hour after irradiation and stained with indicated antibodies. Scale bar, 10μM.

(F to K) Quantification of (C to E) is described in Methods.

(L and M) Control, LATS1/2 KO and YAP/TAZ KO cells were grown on soft (1 kPa) and stiff (30 kPa) fibronectin coated hydrogels. Effect of ECM stiffness on the efficiency of NHEJ (L) and HR (M) in indicated cells was analyzed by flow cytometry.

(N) LATS1/2 kinases and YAP/TAZ are not required for stiffness-induced regulation of radiation sensitivity. Colony formation assays were performed to examine survival of Control, LATS1/2 KO and YAP/TAZ KO cells on soft (1 kPa) and stiff (30 kPa) fibronectin coated hydrogels.

Figure S6

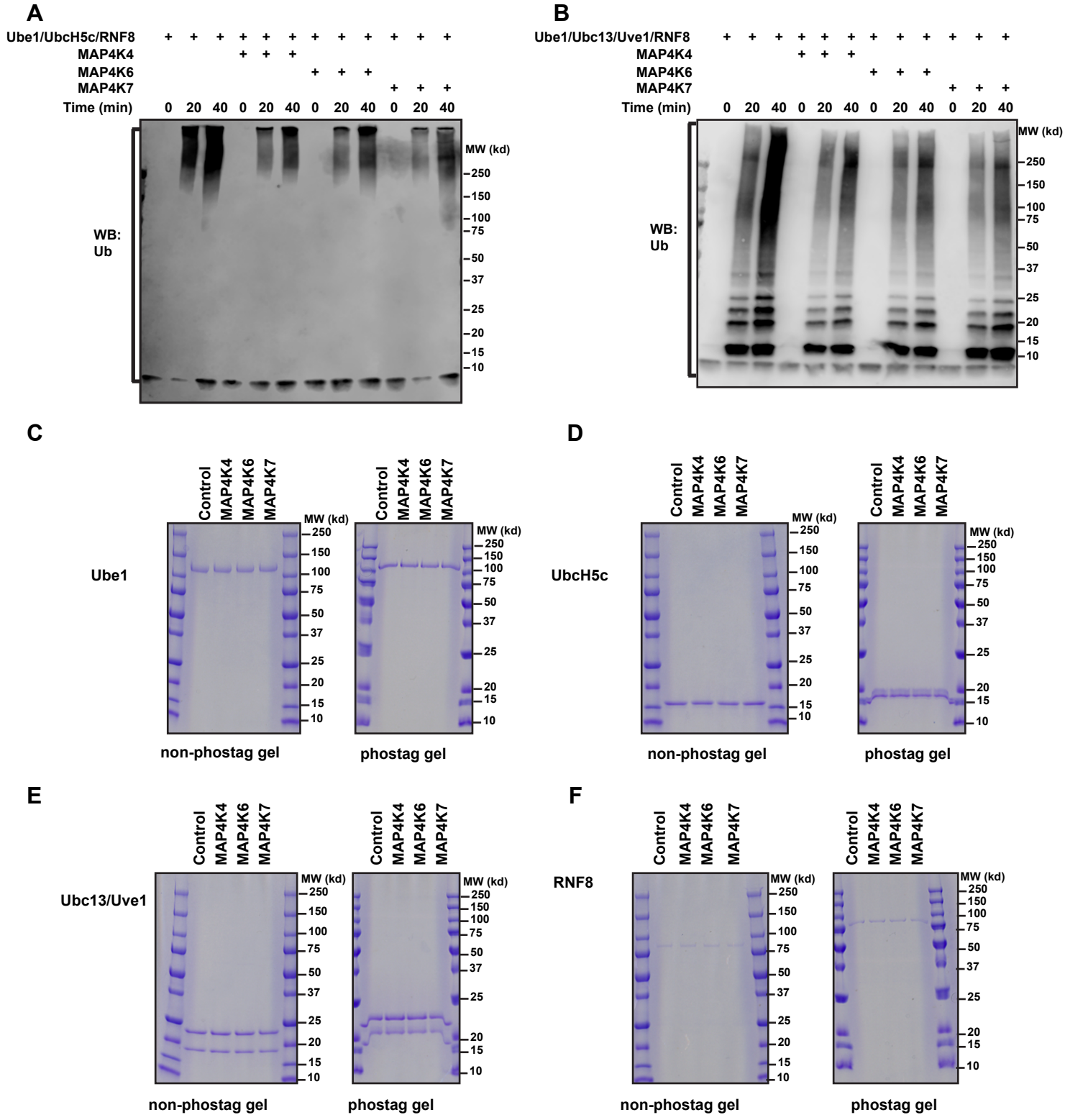
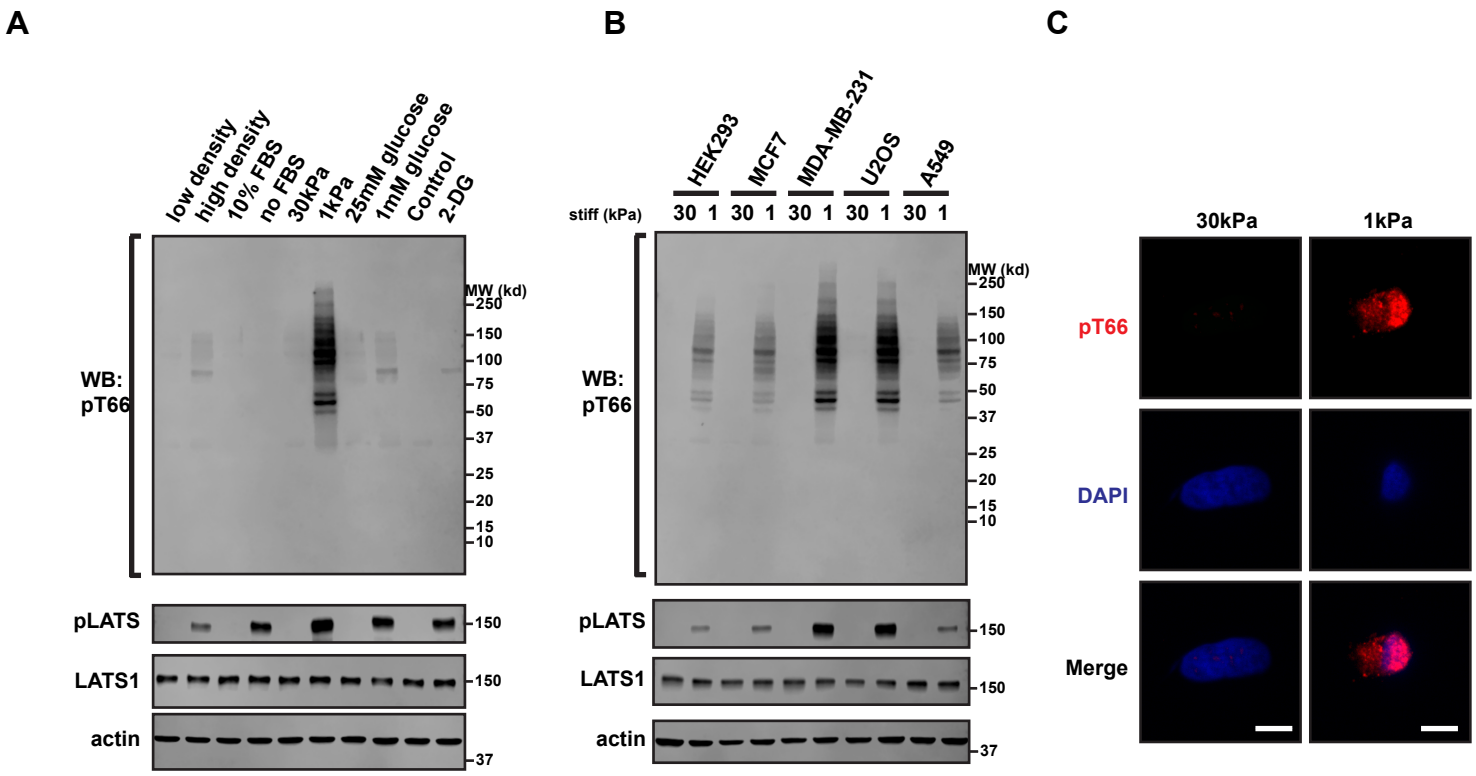

A
Ube1/UbcH5c/RNF8
MAP4K4
MAP4K6
MAP4K7
Time (min)
0 20 40 0 20 40 0 20 40 0 20 40
WB: Ub
MW (kd)
250
150
100
75
50
37
25
20
15
10
B
Ube1/Ubc13/Uve1/RNF8
MAP4K4
MAP4K6
MAP4K7
Time (min)
0 20 40 0 20 40 0 20 40 0 20 40
WB: Ub
MW (kd)
C
Control
MAP4K4
MAP4K6
MAP4K7
Ube1
non-phostag gel
phostag gel
D
UbcH5c
E
Ubc13/Uve1
F
RNF8

Fig.S6 MAP4K4/6/7 kinases directly inhibit RNF8 mediated ubiquitin chain formation *in vitro*.

(A and B) MAP4K4/6/7 kinases impair RNF8 mediated ubiquitin chain formation *in vitro*. The assembly of ubiquitin chains was determined at 30°C in the presence of Ube1, UbcH5c (A) or Ubc13/Uve1 (B) and RNF8 with indicated kinases. Samples were taken at the indicated time points, and polyubiquitin chains were detected by immunoblotting with an anti-Ub antibody.

(C to F) MAP4K4/6/7 kinases do not phosphorylate Ube1, UbcH5c, Ubc13/Uve1 or RNF8 *in vitro*. An *in vitro* kinase assay was performed at 30°C for 1hour in the presence of Ube1(C), UbcH5c (D), Ubc13/Uve1 (E) or RNF8 (F) and indicated kinases. Samples were run on non-phos-tag or phos-tag PAGE and the gels were stained with Coomassie Brilliant Blue (CBB).

Figure S7


A
low density
high density
10% FBS
no FBS
30kPa
1kPa
25mM glucose
1mM glucose
Control
2-DG
WB: pT66
MW (kd)
250
150
100
75
50
37
25
20
15
10
pLATS
LATS1
actin
B
HEK293
MCF7
MDA-MB-231
U2OS
A549
stiff (kPa)
30 1 30 1 30 1 30 1 30 1
C
30kPa
1kPa
pT66
DAPI
Merge

D

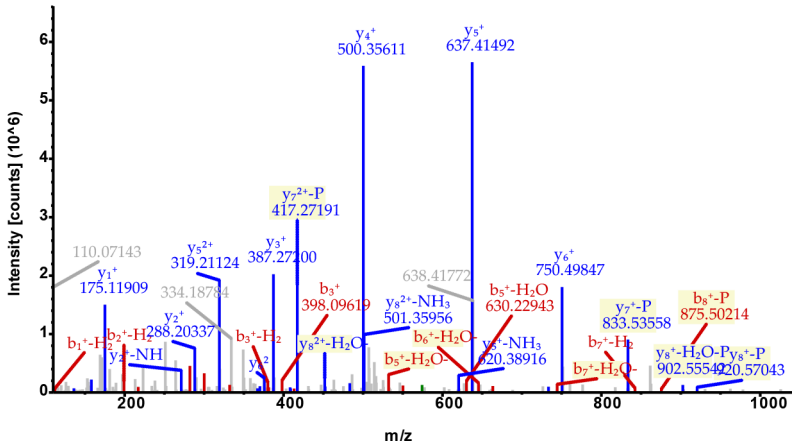

Intensity [counts] (10^6)
m/z
y4+ 500.35611
y5+ 637.41492
y7 2+-P 417.27191
y3+ 387.27200
y5 2+ 319.21124
y1+ 175.11909
110.07143
334.18784
y2+ 288.20337
b3+ 398.09619
y6+ 750.49847
b5+-H2O 630.22943
y7+-P 833.53558
b8+-P 875.50214
y8+-H2O-P 902.55542
y8+-P 920.57043

Fig.S7 Phosphorylation of Ub is regulated by stiffness in cells.

(A) HEK293 cells were treated with indicated conditions and cell lysates were blotted with indicated antibodies.

(B) Different cells were grown on soft (1 kPa) and stiff (30 kPa) fibronectin coated hydrogels. Cell lysates were blotted with indicated antibodies.

(C) HEK293 cells were grown on soft (1 kPa) and stiff (30 kPa) fibronectin coated hydrogels for 24 hours. Cell were fixed and stained with anti-pT66 antibody. Scale bar, 10 μM.

(D) Lysates from HEK293 cells grown on soft hydrogel (1 kPa) was trypsinized and subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. One peptide (ESpTLHLVLR) corresponding to ubiquitin Thr66 phosphorylation was identified.

Figure S8

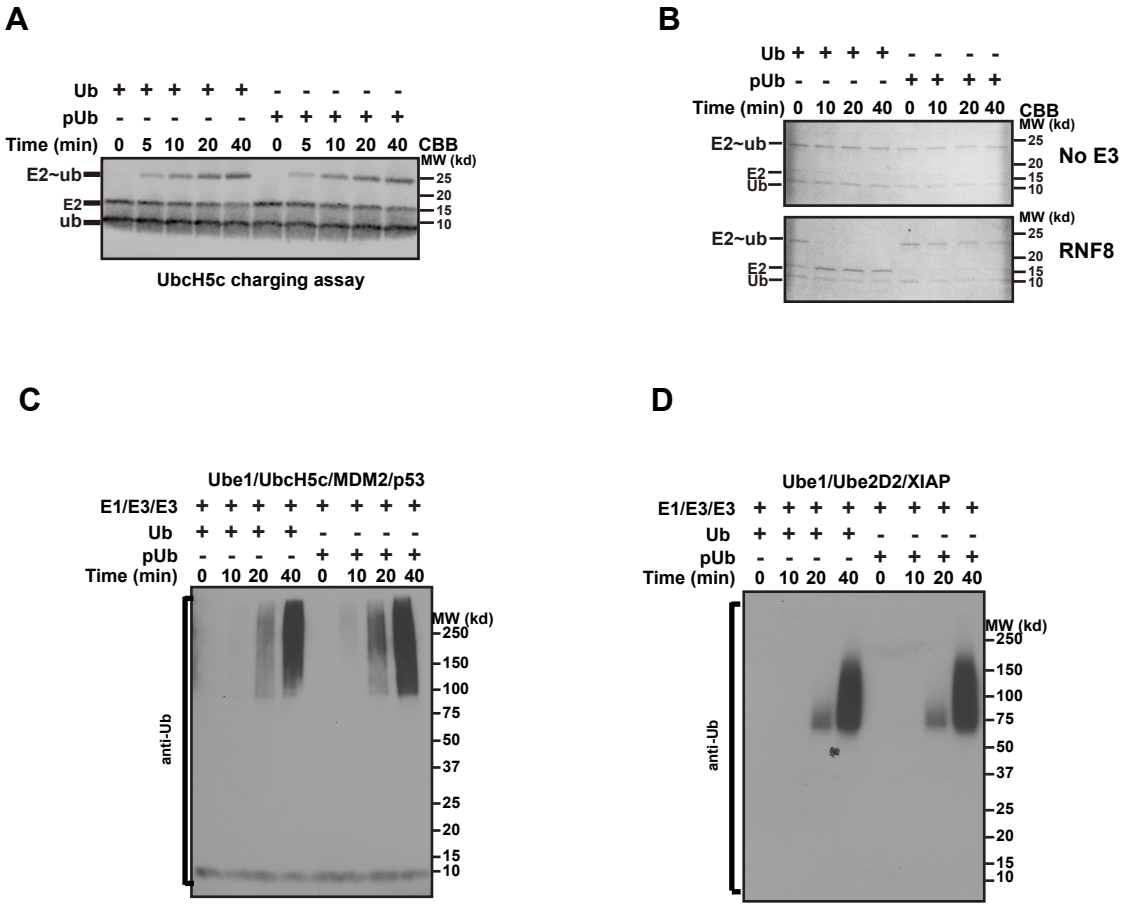

A
Ub + + + + + - - - - -
pUb - - - - - + + + + +
Time (min) 0 5 10 20 40 0 5 10 20 40 CBB
E2~ub
E2
ub
MW (kd)
25
20
15
10
UbcH5c charging assay
B
Ub + + + + - - - -
pUb - - - - + + + +
Time (min) 0 10 20 40 0 10 20 40 CBB
E2~ub
E2
Ub
MW (kd)
25
20
15
10
No E3
E2~ub
E2
Ub
MW (kd)
25
20
15
10
RNF8
C
Ube1/UbcH5c/MDM2/p53
E1/E3/E3 + + + + + + + +
Ub + + + + - - - -
pUb - - - - + + + +
Time (min) 0 10 20 40 0 10 20 40
anti-Ub
MW (kd)
250
150
100
75
50
37
25
20
15
10
D
Ube1/Ube2D2/XIAP
E1/E3/E3 + + + + + + + +
Ub + + + + - - - -
pUb - - - - + + + +
Time (min) 0 10 20 40 0 10 20 40
anti-Ub
MW (kd)
250
150
100
75
50
37
25
20
15
10

Fig.S8 Phosphorylation of ubiquitin blocks RNF8 mediated ubiquitin conjugation *in vitro*.

(A) E1-mediated UbcH5c charging is not affected by ubiquitin T66 phosphorylation. E1-mediated charging of E2 enzyme by Ub and phos-Ub was examined in a time-course analysis. Reactions were stopped with 2 ×non-reducing buffer and processed on non-reducing SDS–PAGE. "~Ub~" refers to generation of a thioester with E2 enzyme.

(B) RNF8 mediated E2 discharging is blocked by phosphorylation of Ub. UbcH5c charged with ubiquitin (UbcH5c~Ub) was mixed with Ub-His_6 or phosUb-His_6 in E2 discharging buffer. RNF8 or MDM2 were included as indicated and reaction were performed at 37°C for the indicated times. Reactions were stopped with 2 ×non-reducing buffer and processed on non-reducing SDS–PAGE.

(C) Ubiquitin T66 phosphorylation does not affect MDM2 mediated ubiquitin chain formation. Phospho-Ub was prepared and purified as described in Methods. The assembly of ubiquitin chains was determined at 30°C in the presence of Ube1, UbcH5c, MDM2, p53 and indicated Ub variants. Samples were taken at the indicated time points, and polyubiquitin chains were detected by immunoblotting with an anti-Ub antibody.

(D) Ubiquitin T66 phosphorylation does not affect XIAP mediated ubiquitin chain formation. The assembly of ubiquitin chains was determined at 30°C in the presence of Ube1, Ube2D2, XIAP and indicated Ub variants. Samples were taken at the indicated time points, and polyubiquitin chains were detected by immunoblotting with an anti-Ub antibody.

Figure S9

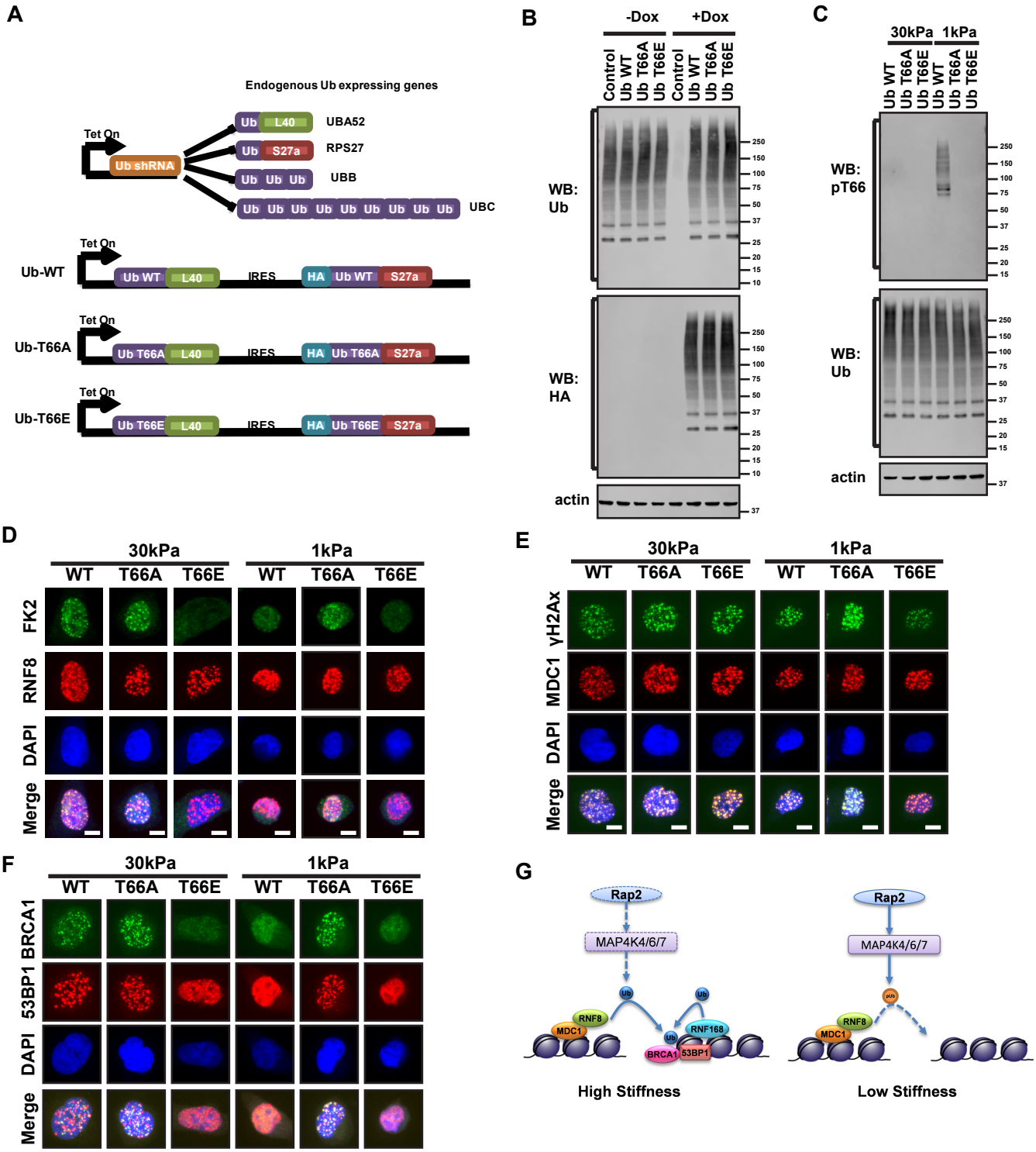

A
Endogenous Ub expressing genes
Tet On
Ub shRNA
Ub L40 UBA52
Ub S27a RPS27
Ub Ub Ub UBB
Ub Ub Ub Ub Ub Ub Ub Ub Ub UBC
Ub-WT
Ub WT L40 IRES HA Ub WT S27a
Ub-T66A
Ub T66A L40 IRES HA Ub T66A S27a
Ub-T66E
Ub T66E L40 IRES HA Ub T66E S27a
B
-Dox
+Dox
Control
Ub WT
Ub T66A
Ub T66E
WB: Ub
WB: HA
actin
C
30kPa
1kPa
WB: pT66
WB: Ub
actin
D
30kPa
1kPa
WT
T66A
T66E
FK2
RNF8
DAPI
Merge
E
γH2Ax
MDC1
F
BRCA1
53BP1
G
Rap2
MAP4K4/6/7
RNF8
MDC1
RNF168
BRCA1
53BP1
High Stiffness
Low Stiffness

Fig.S9 Phosphorylation of ubiquitin mediates DNA repair blockage in cells at low stiffness.
(A) Schematic of the ubiquitin-replacement system used here to replace endogenous Ub with WT Ub, Ub T66A and Ub T66E mutants.
(b) Validation of ubiquitin-replacement HEK293 cells. The indicated cells induced with 2 μM Dox for 72 hours. The levels of HA-Ub, Ub T66A and Ub T66E were measured by immunoblotting. Actin was used as a loading control.
(C) Replacement of ubiquitin with phospho-deficient ubiquitin mutant T66A or T66E blocks ubiquitin phosphorylation at low stiffness. Replacement of ubiquitin was induced by 2 μM Dox for 48h. Cells were then plated on low (1 kPa) or high (30 kPa) stiff hydrogel coated with fibronectin and cultured for 24h. The level of ubiquitin phosphorylation was detected with indicated antibodies.
(D to F) Replacement of ubiquitin with phospho-deficient ubiquitin mutant T66A restores FK2, 53BP1 and BRCA1 foci at low stiffness. Ubiquitin-replacement HEK293 cells expressing wild-type (WT), T66A and T66E ubiquitin were grown on soft (1 kPa) and stiff (30 kPa) fibronectin coated hydrogels. Cells were fixed 1 hour after irradiation (1 Gy) and stained with anti- γ-H2AX and MDC1 (D), RNF8 and FK2 (E), 53BP1 and BRCA1 (F) antibodies. Representative images showing IR induced foci after 1 Gy irradiation. Scale bar, 10μM.
(G) A working model for this study.