Supplementary Materials for

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

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Figs. S1 to S9
Figure S1

A

B

C

D

E

F

G

H

I

J

- **Control**
- **Cisplatin**
- **BAPN**
- **BAPN+Cisplatin**

**Control BAPN** Cisplatin Cisplatin +BAPN

- **Cleaved PARP**
- **Cleaved caspase 3**
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) 1×10⁶ mouse mammary tumor 4T1 cells were injected into the mammary gland of Balb/c mice. After the tumor size reached 100 mm³, 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.
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) 5×10⁶ 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) 5×10⁶ 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 | 0.5kPa |
| 30kPa | 1kPa   |
| 20kPa | 10kPa  |
| 10kPa | 0.5kPa |

B

| No IR | IR 1Gy |
|-------|--------|
| glass | 0.5kPa |
| 30kPa | 1kPa   |
| 20kPa | 10kPa  |
| 10kPa | 0.5kPa |

C

| No IR | IR 1Gy |
|-------|--------|
| glass | 0.5kPa |
| 30kPa | 1kPa   |
| 20kPa | 10kPa  |
| 10kPa | 0.5kPa |

D

| No IR | IR 1Gy |
|-------|--------|
| glass | 0.5kPa |
| 30kPa | 1kPa   |
| 20kPa | 10kPa  |
| 10kPa | 0.5kPa |
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

B

C

D

E

F

G

H

I

J

K

mm0

30kPa

Rap2 KO

1kPa

MDC1

RNF8

FK2

53BP1

BRCA1

Control 30kPa

Rap2 KO 30kPa

Control 1kPa

Rap2 KO 1kPa

% Surviving Cells

IR(Gy)
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)
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

B

"Ube1/Ubc13/Uve1/RNF8 MAP4K4 MAP4K6 MAP4K7"

Time (min) 0 20 40 0 20 40 0 20 40 0 20 40

WB: Ub

C

"Ube1 MAP4K4 MAP4K6 MAP4K7"

non-phostag gel phostag gel

D

"UbcH5c MAP4K4 MAP4K6 MAP4K7"

non-phostag gel phostag gel

E

"Ubc13/Uve1 MAP4K4 MAP4K6 MAP4K7"

non-phostag gel phostag gel

F

"RNF8 MAP4K4 MAP4K6 MAP4K7"

non-phostag gel phostag gel
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 1 hour 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).
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. Cells were fixed and stained with anti-pT66 antibody. Scale bar, 10 µM.

(D) Lysates from HEK293 cells grown on soft hydrogel (1 kPa) were trypsinized and subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. One peptide (ESP TLHLVLR) corresponding to ubiquitin Thr66 phosphorylation was identified.
Figure S8

A

Ub + + + + + + + + - - - - -
pUb - - - - - + + + + + + +
Time (min) 0 5 10 20 40 5 0 10 20 40
E2-ub E2 ub

UbCh5c charging assay

B

Ub + + + + + - - - - - - -
pUb - - - - - + + + + + + +
Time (min) 0 20 40 0 10 20 40
E2-ub E2 ub

No E3

RNF8

C

Ube1/UbcH5c/MDM2/p53
E1/E3/E3 + + + + + + + + + + +
Ub + + + + + - - - - - - -
pUb - - - - - + + + + + + +
Time (min) 0 10 20 40 0 10 20 40

D

Ube1/Ube2D2/XIAP
E1/E3/E3 + + + + + + + + + + +
Ub + + + + + - - - - - - -
pUb - - - - - + + + + + + +
Time (min) 0 10 20 40 0 10 20 40

MW (kd)
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-His₆ or phosUb-His₆ 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

Ub-WT
Ub-T66A
Ub-T66E

Tet On

Ub-xiRNA

UBA52
RPS27
UBB
UBC

Ub WT
Ub T66A
Ub T66E

L40
S27a

HA

Tet On

Ub shRNA

Control

Ub-WT
Ub-T66A
Ub-T66E

WB: Ub

HA

actin

WB: pT66

actin

30kPa
1kPa

F

30kPa
1kPa

DAPI

Merge

DAPI

Merge

37
25
20
15
10
250
150
100
75
50
37
25
20
15
10
37
25
20
15
10

Tet On

D

30kPa
1kPa

WT
T66A
T66E

WT
T66A
T66E

WT
T66A
T66E

WT
T66A
T66E

WT
T66A
T66E

WT
T66A
T66E

WT
T66A
T66E

G

High Stiffness

Low Stiffness

Rap2
MAP4K4/6/7

MAP4K4/6/7

Rap2

BRCA1/53BP1

BRCA1/53BP1

BRCA1/53BP1

BRCA1/53BP1

BRCA1/53BP1

BRCA1/53BP1
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.