Supplementary Materials for

**Homeostatic nuclear RAGE-ATM interaction is essential for efficient DNA repair**

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This word file includes:

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Figure S1:

(A) WT and RAGE/-/- lung lysates were blotted on a 4-20% SDS PAGE gel against RAGE. No signal was observed in lungs of mice lacking RAGE.

(B) Representative immunofluorescence images of WT lungs sections stained with anti RAGE (red) and DAPI (blue), along with a secondary antibody control (upper panel) [Scale 10µm]. The dotted areas mark the zoomed portions of the respective images in the lower panel.

(C) Representative images of 30 weeks old WT and RAGE/-/- sections of kidney, intestine, liver and heart stained for senescence associated β-galactosidase [β-Gal] as described in Methods and visualized by bright field and polarized light, where the accumulated senescent areas are recognized by its blue staining (Scale 40µm).

(D) Pressure-volume loops for WT or RAGE/-/- lungs accessed via FlexiVent (for details see Methods). The curves represent group averages (n=6).
Figure S2:

Representative images of WT and RAGE^-/- lung stained with various markers such as wheat germ agglutinin (top yellow panel, to show the gross morphology), pATM (2nd panel from top in red, to show the activated ATM kinase), γH2AX (3rd panel from top in green) and 53BP1 (4th panel from top in green), showing markers associated with DNA DSB breaks. The 5th panel marks IL-6 associated persistent DNA damage signaling, as presented here in yellow and the bottom panel represents RAGE staining in red. Blue nuclear staining represents DAPI (Scale 10µm). For details see Methods.
Figure S3.

A

RAGE/EpCAM  RAGE/FSP1
A549 cells  Fibroblasts

B

Nuclear Soluble  Nuclear Soluble + RNase-A  Nuclear Soluble + DNase-I

64kDa  50kDa  62kDa  25kDa
RAGE  HDAC 1  Histone H1

C

RAGE  FSP1  DAPI  Overlay
Un-extracted  Extracted  Extracted + RNase-A  Extracted + DNase-I

D

RAGE  eIF6  RAGE/eIF6  RAGE/ DAPI  DAPI
S-phase  M-phase  G1-phase

E

RAGE-DNA complex
1  2  3  4  5
Cold competitor - - - - +
**Figure S3:**

(A) Representative immunofluorescence images of A549 (epithelial cells) and WT lung fibroblasts stained for RAGE (orange) and DAPI (blue) [Scale 40µm]. Purple staining represents RAGE nuclear localization.

(B) The nuclear soluble fraction was prepared as described in Figure 1A by a sucrose gradient. The lower RAGE band represents the less glycosylated nucleoplasmic RAGE. The upper band represents the more glycosylated RAGE. Addition of RNase-A (25µg/ml for 30 min) did not change both RAGE bands. Addition of DNase-I (10 U/µl for 30 min) increased the density of the upper band, indicating stripping of heavily glycosylated RAGE from DNA. HDAC1 and Histone 1 served as controls.

(C) Representative immunofluorescence images showing immunolocalization of RAGE in the subnuclear compartment of pulmonary fibroblasts. Cells were either stained untreated, or extracted using CSK buffer, RNase-A (25 µg/ml), or DNase-I (10 U/µl for 30 min) as described for figure S3B. The nucleus was stained by DAPI (in blue) and RAGE (red).

(D) Representative immunofluorescence images showing immunocolocalization of RAGE and the nucleolar marker eIF6 in the subnuclear compartment of the S-phase (upper panel) or M-phase (middle panel) or G1-phase synchronized and unextracted pulmonary fibroblasts. The nucleus was stained by DAPI (in blue), RAGE (red) and eIF6 (green).

(E) In a native gel shift assay, a 64mer dsDNA (approximately 75ng/reaction), labeled at the 5’ end of one of the strands, was incubated with RAGE at increasing concentrations, as indicated (12.5nM, 25nM and 50nM) (lane 2-4). Competition with a cold probe (5-fold excess) is shown in lane 5.
Figure S4.

A

Number of Cells (x10^6)

Wild-type
RAGE^−/−

Day

0 1 2 3 4 5

B

WT Zoomed RAGE^−/− Zoomed

C

WT RAGE^−/−

D

Marker WT RAGE S376A RAGE S389A RAGE S376-389A RAGE S376-398E

70kDa 55kDa mRAGE

E

Input 25% Immunoprecipitation

370 kDa 64 kDa

pATM RAGE

F

hRAGEWT hRAGE^S389A

α-S/TQ

α-FLAG

hRAGE phosphorylated hRAGE-3xFLAG

hRAGE phosphorylated

hRAGE-3xFLAG
Figure S4:

(A) Left panel: Analysis of the proliferation rate of WT (open circles) or RAGE−/− lung fibroblasts (closed circles) was determined by the MTT based micro titer plate assay. The increased cell number was plotted against time (in days). Right panel: representative cell cycle distribution of wild-type and RAGE−/− lung fibroblasts calculated from histograms. Data are represented as mean ± SEM.

(B) Representative images showing morphological differences among passage matched WT or affected RAGE−/− lung fibroblasts growing under similar culture conditions, as visualized by bright field and polarized light (Scale 100µm). The dotted areas mark the zoomed portions of the main image.

(C) Representative images of passage matched WT or RAGE−/− lung fibroblasts stained for the cellular senescence associated marker, β-galactosidase [β-Gal] (upper panel, for details see Methods) as visualized by bright field and polarized light. Cytoplasmic β-Gal activity is shown in blue (scale 40µm). The dotted areas mark the zoomed portions of the main image (lower panel).

(D) Purification profile of purified WT RAGE or its indicated point mutants after expressing it in an E.coli expression host (for details see Methods) on a 4-20% SDS-PAGE. The purified RAGE, (WT or mutants as indicated) were used in in vitro pATM kinase assay.

(E) Cultured lung adenocarcinoma (A549) cells were pre-sensitized using BrdU (10µM for 24 hr). Cells were either left untreated or stimulated using bleomycin (30µg/ml for 60 minutes). The cell extract was immunoprecipitated using antibodies against pATM (lane 5, 7 and 9), RAGE (lane 4, 6 and 8), or a non-specific species control antibody (lane 10). The input loading controls were shown in lanes 1-3.

(F) Detection of human RAGE phosphorylation in lung adenocarcinoma cells (A549) with (α-pS/TQ-ab) 1 h after exposure to either Camptothecin (1µM), Bleomycin (30µg/ml), or Camptothecin with the ATM kinase inhibitor (Ku55933; 15µM, 60 minutes before CPT treatment) or with the DNA-PK inhibitor (Nu7026; 10µM, 60 minutes before CPT treatment), added 36 hours after transfection with the hRAGEWT FLAG or hRAGES389A construct. The phosphorylated RAGE was collected from the cell lysates on M2 magnetic beads followed by analyzing them by western blotting. For specificity of detection in immunoblotting a λ-phosphatase treated sample was used as a negative control.
Figure S5.

A

| Repair Factor | RAGE | DAPI | Overlay |
|---------------|------|------|---------|
| γH2AX         |      |      |         |
| MRE11         |      |      |         |
| Rad50         |      |      |         |
| Nbs1          |      |      |         |

B

| Repair Factor | RAGE | DAPI | Overlay |
|---------------|------|------|---------|
| γH2AX         |      |      |         |
| MRE11         |      |      |         |
| Rad50         |      |      |         |
| Nbs1          |      |      |         |

C

Nbs1         | RAGE | Overlay |

D

Input (10%) | GFP-RAGE | GFP cyto RAGE | Co-IP |
|------------|----------|--------------|-------|
| Marker     |           |              |       |
| 150 kDa    | Rad50    |              |       |
| 81 kDa     | MRE11    |              |       |
| 95/72 kDa  |          |              |       |
| 30/26 kDa  |          |              |       |

E

| Conditions | MRE11 | RAGE\textsuperscript{\textsc{wt}} | RAGE\textsuperscript{S76A-D299A} |
|------------|-------|--------------------------------|-------------------------------|
| NaCl       | +     | +                              | +                             |
| NaCl       | -     | -                              | -                             |

F

| Conditions | 53BP1 | Overlay | Zoom |
|------------|-------|---------|------|
| Input      |       |         |      |
| RAGE       |       |         |      |
| 53BP1      |       |         |      |
Figure S5:
(A) Immunocolocalization of recruitment of endogenous RAGE to the site of CPT (1µM, for 60 minutes) induced DNA damage in pre-sensitized (BrdU; 10µm for 24 hours in phenol red free DMEM-medium) WT lung fibroblasts, analyzed by immunofluorescence after 60 minutes of camptothecin treatment. Cells were detergent extracted before fixing in 4% paraformaldehyde.

(B) Immunocolocalization of recruitment of endogenous RAGE to the site of bleomycin (30µg/ml, for 60 minutes) induced DNA damage in pre-sensitized (BrdU; 10µm for 24 hours in phenol red free DMEM-medium) WT lung fibroblasts, analyzed by immunofluorescence as described for Figure 3A and S4A.

(C) Human lung adenocarcinoma (A549) cells co-expressing RFP-Nbs1 along with either GFP-RAGEWT or GFP-RAGES376A-S389A mutant were pre-sensitized with BrdU (10µM for 24 hours), were either left untreated or treated with camptothecin (CPT; 1µM for 60 minutes). The recruitment kinetics of RFP-Nbs1 and GFP-RAGE (WT and mutant) was analyzed by fluorescence microscopy for DSB associated foci formation after fixing with 4% paraformaldehyde in PBS for 15 minutes at 4°C. The BrdU treatment was started 6 hours after transfection, thus the CPT treatment began 30 hours after transfection.

(D) Co-immunoprecipitation analysis of camptothecin (CPT) treated (1µM for 60 minutes) WT lung fibroblasts ectopically expressing GFP-RAGE (lane 1 and 4), GFP-Cyto RAGE (lane 2 and 5), or GFP alone (lane 3 and 6). Cells were transfected with the respective constructs 36 hours prior to drug treatment and were sensitized with BrdU (10µm for 24hrs). Shown are the controls before GFP co-immunoprecipitation and after immunoprecipitation using an anti-GFP antibody. Rad50 is shown on top, MRE11 in the middle and GFP in the bottom. The lower band represents GFP alone or in lane 5 the cyto-RAGE (MW 30 kDa) fused to GFP (26 kDa).

(E) The Ni-NTA pulldown assay was performed by using FLAG tagged MRE11 as Prey and either His tagged WT RAGE or RAGE S376A-S389A as bait. The beads were washed with 100mM, 200mM and 300mM NaCl in wash buffer and then beads were boiled in Lamelli buffer and analyzed for the interaction with MRE11 (α-FLAG). The RAGE was probed by using anti (His)_6 antibody.

(F) In G1-phase cultured WT pulmonary fibroblasts, using serum starvation as described in Methods, were stained for endogenous RAGE (red) or the Oct-1-PTF transcription domain marker 53BP1 (green). Scale 40µm.
Figure. S6:

(A) Representative immunofluorescence images of WT or RAGE-/- pulmonary fibroblasts treated with either bleomycin (30μg/ml) or camptothecin (1μM) for 60 minutes and analyzed for the indicated markers (Scale 40μm).

(B) Synchronized cultured fibroblasts from WT and RAGE-/- lungs were treated with camptothecin (1μM for the indicated times). The expression and phosphorylation status of the indicated factors was analyzed by western-blotting of total extracts at the indicated times. Shown are phosphorylated CHK1 (Ser 345) and total CHK1. The cyclin-A was probed as an S-phase control, and γH2AX as a DNA DSB control.

(C) Synchronized pulmonary wild type or RAGE-/- fibroblasts were either untreated (lane 1, 2), or treated with 1μM CPT (lane 3, 4), 30μg/ml bleomycin (lane 5, 6), 5μM etoposide (7, 8), or 5mM hydroxyurea (9, 10) for 60 minutes. RAGE, total RPA, RPA2S4&S8, was probed along with cyclin-A as an S-phase control, γH2AX and total H2AX.

(D) Representative immunofluorescence images of pre-sensitized (BrdU, 10μM for 24 hr) WT or RAGE-/- pulmonary fibroblasts treated with camptothecin (1μM) for 60 minutes, analyzed for γH2AX, BrdU (under native and non-denaturing conditions). These cells were detergent extracted before fixing in 4% paraformaldehyde.
Figure S7.

Representative immunofluorescence images of the indicated markers in WT or RAGE−/− lungs and in WT irradiated lungs (12Gys irradiated and after 4 hours of radiation recovery lungs were collected) as positive control (Scale 10µm).
Figure S8.

(A) Schematic representation of the modified DR-GFP based HR assay, where the 3XFLAG tag was incorporated into the c-terminus of SceGFP (EGFP-A).

(B) Endonuclease activity assay using a 1% agarose gel, along with the markers in lane 1. The nucleolytic processing of circular single stranded DNA derived from the viral plasmid PhiX174 was studied as described in Methods. Lane 2,3,4,5 show the presence of MRN, RAGE<sup>WT</sup>, RAGE<sup>S376E-S389E</sup>, RAGE<sup>S376A-S389A</sup> alone respectively, lane 5 MRN with RAGE<sup>WT</sup>, lane 6 MRN with RAGE<sup>S376E-S389E</sup>, lane 7 MRN with RAGE<sup>S376A-S389A</sup>, lane 8 buffer with DNA alone as negative control. The reaction buffer contained MRN (50nM) or RAGE (2.0μM) in 5mM MnCl<sub>2</sub>.
Figure S9:

(A) Lungs of RAGE−/− mice were studied 4 weeks after AAV 2/8 transduction. RFP was used to proof transduction efficiency, since the transduced proteins were fused to RFP (upper panel). γH2AX was used to determine DNA damage repair (3 panel). The two bottom panels show the ECM deposition, evidenced by Masson’s trichrome staining). Mice were transduced either with vector control, full length WT RAGE (RAGE1−402), or non-phosphorylatable RAGE (RAGE S376A-S389A). Shown is the mean ± SD of 6 animals per group **: P<0.01. The zoomed portion represents the dotted windows (scale 10µm for fluorescent images) and 40µm for TM staining).

(B) Quantitative analysis of 53BP1 positive nuclei in lungs of RAGE−/− mice transduced with AAV2/8 as described in Methods. 6 months old mice were harvested 4 weeks after viral transduction. The empty vector served as control. Mean ± SD of 6 animals per group is shown. ***: P<0.01.
Figure S10

A

Contralateral

Ipsilateral

Contralateral

Ipsilateral

Brain

Kidney

B

RAGE

γH2AX

Overlay

Untreated

Camptothecin
Figure S10:

(A) Representative immunofluorescence images showing nuclear accumulation (blue, DAPI) and co-localization of RAGE (red) with the DSB marker γH2AX (green), post ischemia-reperfusion induced oxidative damage in the brain and kidney (Scale 40µm). The contralateral side represents the unclamped control and the ipsilateral side represents reperfusion injury.

(B) Nuclear accumulation as well as colocalization of endogenous RAGE and γH2AX to the site of CPT (1µM, for 60 minutes) induced DNA damage in pre-sensitized (BrdU; 10µm for 24 hours in phenol red free DMEM-medium) lung adenocarcinoma cells (A549), analyzed by immunofluorescence after 60 minutes of camptothecin treatment. Cells were fixed in 4% paraformaldehyde. The nucleus was stained by DAPI (in blue), RAGE (red) and γH2AX (green).
Figure S11:

Representative images showing immunocolocalization of nuclear DAPI (blue), RAGE (red), with the DNA DSB marker γH2AX (green) in heart, kidney, liver, intestine and brain before and after irradiation performed as described in Methods. (Scale 10µm).
Supplementary methods:

Primary fibroblast culture:

The isolation of primary murine lungs fibroblasts from Wild type (WT) and RAGE-/- murine lungs was performed as described earlier (1). Respectively genotyped mice were sacrificed and perfused with 0.9% saline. Lavaged lungs were then injected with 600µl of dispase (Sigma-aldrich,St Louis, USA), excised and transferred to a tissue culture plate containing additional volume of Dispase (sufficient to cover the area of lungs) and incubated at 37ºC for 30-45 minutes. After incubation the tissue was disaggregated using a scalpel. The recovered tissue was then supplemented with 10 ml of Dulbecco’s modified Eagle’s media (DMEM)–DNase-I mix and incubated on a rotatory shaker at RT for 15 minutes. After incubation the undigested tissue was separated using a 100 µm cell strainer (BD biosciences; Erembodegen, Belgium) and cells were collected by centrifugation at 1000 rpm for 15 minutes at 4ºC. The pellet was then resuspended and seeded in DMEM supplemented with fetal calf serum (15%), penicillin-streptomycin (1%), non-essential amino acids (1%) and L-glutamine (1%). This preparation was numbered as passage zero and subsequent passages were numbered as P1, P2 etc. The characterization of these isolated fibroblasts was performed using anti-vimentin antibody (Sigma-Aldrich, St Louis; USA, anti-vimentin, V5255; clone VIM-13.2, 1:200). We preferred using PAA FCS for murine lung fibroblast growth.

Cell culture and transfection:

Murine pulmonary fibroblasts, human A549 and HEK293 were grown in DMEM medium supplemented with fetal calf serum (10-15%), penicillin-streptomycin (1%), non-essential amino acids (1%) and L-glutamine (1%). The plasmid DNA transfections in primary cells and A549 were performed using the Neon (Invitrogen, GmbH) transfection system. HEK 293 cells were transfected using standard CaCl₂ method of virus production. Transfected cells were analyzed after 24-48 hours of transfection.
Cell lysis and immunoblotting:

Total cell extracts or tissue extracts (liquid N\textsubscript{2} grinded powder of respective tissue) were obtained by resuspending them in 20mM Tris-Cl pH 7.5, 40mM NaCl, 2mM MgCl\textsubscript{2}, 0.5% NP40, 50U/ml benzonase, supplemented with protease and phosphatase inhibitors and after 15 minutes of incubation on ice, the NaCl concentration was adjusted to 150/450 mM and then it was further incubated for 15 more minutes. The lysate was then centrifuged at 14K for 15 minutes at 4°C. The supernatant was collected in labeled tubes and the total protein was quantified using Bradford’s reagent. About 10 or 20\(\mu\)g of total protein was used for loading in Laemmli buffer (0.8% SDS, 4% glycerol, 100 mM dithiothreitol, 25 mM Tris-HCl pH 6.8, 0.005% bromophenol blue). Proteins were resolved by 4-20 % SDS-PAGE (Mini-PROTEAN TGX Biorad), transferred onto nitrocellulose (Protran) and probed using the appropriate primary (Supplementary table S3) and secondary antibodies coupled to horseradish peroxidase (Cell Signaling or Santa Cruz). Protein detection was done with ECL reagents (GE Healthcare).

Immunoprecipitation:

Immuno-precipitation of the various complexes was performed from WT pulmonary fibroblasts or lungs (collected tissues were first grinded in liquid nitrogen to a powder and then processed in a similar way as for cells) after respective treatments and then lysing in 20mM Tris pH 7.5, 40 mM NaCl, 2mM MgCl\textsubscript{2}, 0.5% NP40, 50U/ml benzonase, supplemented with protease and phosphatase inhibitors and adjusted to 150 mM salt concentration. Lysates were clarified by centrifugation (13200 rpm, 20 min, 4°C) and at least 0.5 to 1.0 mg proteins were used per immune-precipitation in IP buffer (25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1.5 mM DTT, 10% glycerol, 0.5% NP40) supplemented with protease and phosphatase inhibitors. Where indicated the samples were pre-treated with either ethidium bromide (25 \(\mu\)g/ml). Endogenous proteins were captured onto protein A/G- agarose beads (Santa cruz) coupled to the indicated antibody,
while GFP-(His)$_6$ tagged proteins were directly captured onto Ni NTA beads (Thermo scientific). Complexes were extensively washed in IP buffer. Immuno-precipitation with non-specific IgG or from cells expressing GFP only was used as negative control.

**Cellular immunofluorescence (IF):**

Cells grown on poly L-lysine coated coverslips (Thermo) or glass bottom dishes(Ibidi) were fixed with 4% paraformaldehyde for 15mins at 4°C and permeabilized with 0.3% Triton X-100 in PBS for 5 mins at room temperature. Cells undergoing drug treatment were processed differently such that after drug treatment or laser induced DNA damage, cells were pre-extracted with CSK buffer (10 mM PIPES (pH 7.0), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl$_2$, and 0.01% Triton X-100) for 1 min at room temperature. Samples were then blocked in 5% bovine serum albumin and immune-stained using indicated primary antibodies (listed in Supplementary table S4) and secondary antibodies. Species adsorbed AlexaFluor 488/555/647 secondary antibodies were purchased from Abcam. Fluorescent images of infected and control cells were captured with a CCD camera connected to an inverted fluorescence microscope (Cell Observer, Carl Zeiss, GmbH, Göttingen, Germany). Samples were scanned using an x63 oil objective. Images were further processed using ImageJ (Fiji) and Photoshop CS5 (Adobe).

**Tissue sections immunofluorescence:**

Paraffin embedded tissue sections were deparaffinized using a series of washes with xylol (10 mins x 2 times), isopropanol (5 mins x 1 times), 96% ethanol (5 mins x 1 times), 85% (5 mins x 1 times), 70% (5 mins x 1 times) and then in aquadest (5 mins x 1 times). Antigen retrieval was performed by incubating sections in Retrieval buffer A (10 mM Tris-Cl pH-9.0, 1mM EDTA.0.05% Tween 20) for 20 minutes at RT. Sections were then extensively washed with water and permeabilized using 0.1% aqueous solution of saponin for 30 minutes at room temperature. These permeabilized sections were then washed extensively with Tris buffered saline/
0.2% Triton X100 (TBS-T) for 5 mins x 3 times. These prepared sections were then blocked with 10% goat serum in TBS-0.2% Triton X100 for 45 minutes at rt. After incubation, the respective antibodies were diluted in the blocking buffer and incubated at 4°C for 8/10 hrs. The sections were then washed with TBS-T (10 minutes x 3 times). Fluorochrome conjugated and species adsorbed respected secondary antibodies were then used to detect the signal. The imaging of the section was performed as described for the cellular immunofluorescence staining.

**Cellular DNA damage induction:**

WT or RAGE−/− lung fibroblasts were isolated and grown to 40% confluency, after which the medium was changed to Syn-DMEM (DMEM + 10% FCS, 1% pen-strep, 1% glutamine) + 2mM thymidine for 24 h (first block). After first thymidine block, cells were washed twice with PBS (1X) and grown in normal fibroblast growth medium such as DMEM (10% FCS, 1% pen-strep, 1% glutamine) for 3 hours to release cells, after which the medium was changed to DMEM (10% FCS, 1% pen-strep, 1% glutamine), 100ng/ml nocodazole was added to the cells for 12 h (second block). After the second block, thymidine was removed by washing with PBS and cells were released by adding fresh Phenol red free DMEM (10% FCS, 1% pen-strep, 1% glutamine) along with 10μM BrdU to pre-sensitized for 24 hours and then these S-phase cells were used in DNA damage treatment. The drug treatment was performed as described in the supplementary table 1.

The G-phase synchronization was induced by serum starvation of cells by growing them (for 60 to 72 hours) in above described growth medium, but supplemented with 0.1% serum.

**Whole body irradiation of mice:**

WT C57BL/6 male mice were purchased from Janvier Labs and housed in individually ventilated cages in the animal facility. Total body irradiation (TBI) of mice was performed with gamma irradiation using 137Cs using BioBeam 8000 (Gamma Service Medical GmbH, Germany) at a dose rate of 12Gy. Mice were
anesthetized and held in ventilated radiation chamber for TBI. Post radiation induced DNA damage mice were housed for another 4 hours and then sacrificed and organs were harvested and preserved as earlier described in immunohistochemistry section. Tissues from control mice were processed the same way, but were not exposed to radiation.

**Bleomycin induced DNA damage in lungs:**

The mice were anesthesized with specified doses of Avertin (using 2.5% avertin/gm body weight). After that 2mg/kg of bleomycin implanted through nostrils in a minimum volume of 15μl and then mice were allowed to rest under normal housing conditions. 24 hours after first induction, mice were administered afresh dose of bleomycin, as described. 1 hour after this 2nd administration, the mice were sacrificed and their lungs were collected and processed for respective studies.

**Comet assay:**

Comet assay was carried out under alkaline conditions as described earlier (2). In brief frosted slides were coated with 1% normal melting point (NMP) agarose (Sigma) and left to dry overnight at room temperature. About 10000 cells were applied to these dried slides in 0.5% low melting point (LMP) agarose (Sigma) and the solidified cell layer was then stabilized using 1% LMP agarose. These immobilized cells were then lysed in ice-cold lysis solution [2.5M NaCl, 100mM EDTA, 10mM Tris–HCl, 1% Na-sarcosinate (pH-10) with 1% Triton X-100 (Sigma) and 10% dimethyl sulfoxide]. Electrophoresis of denaturated DNA was then carried out using a horizontal electrophoresis unit in electrophoresis buffer (300mM NaOH, 1mM EDTA, pH 13.0) for 20 minutes at 25 V (300 mA). After electrophoresis the slides were rinsed gently three times with a neutralization buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20μg/ml) and covered with a cover slip. Slides were stored at 4°C in sealed boxes until analysis. A total of 100-200 comets were
scored using the image analysis system (Comet Assay analysis Trevigen; Cat. no. 4260-000-CS). Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail.

**Proliferation assay:**

The change in cell number and cellular growth activity was measured by a colorimetric assay using the MTT reagent, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (Sigma). In brief, about 500, WT or RAGE-/- lung fibroblasts were grown in 96 well plates for the indicated time points. Cells were incubated with 0.6 mg/ml MTT reagent for 4 h. The reagent was reduced by living cells to form insoluble blue formazan product. The cells were washed, solubilized with DMSO and the absorbance at 570 nm was quantified on Fluostar microplate reader. The data was analyzed using Microsoft Excel.

**Fluorescence activated cell sorting (FACS):**

Fluorescent activated cell sorter (FACS) was used to sort cells expressing only GFP or GFP and RFP from the transfected cells. The gating was done using untransfected and RFP alone transfected cells.

**Expression and purification of recombinant proteins:**

The pET28a-RAGE (full-length, mutant or deletion) constructs was transformed into *E. coli* BL21 (DE3) codon plus (Novagen) competent cells. The bacteria were cultured in LB or TB medium with vigorous shaking (200 rpm) at 37 °C to a density of OD_{600} = 0.8 and then transferred to 25°C and induced with IPTG (0.4 mM in water). Cultures were further incubated at the same conditions for 8 hours. After induction, the cell culture was centrifuged at 6000 rpm for 15 min at 4 °C. The cell pellet was frozen in -80°C for later use.

The purification of the recombinant proteins was done using Ni NTA beads using QIA expressionist method and the eluted protein was analyzed on SDS PAGE. The ultrapure fractions were further subjected to 2 step polishing steps on Heparin
agarose column and Sephacryl S200 column. The pure fractions collected were
diazyed in 1x PBS or indicated reaction buffer at 4°C overnight. The ATM,
MRE11, RAD50, Nbs1 and MRN complex was purified as described by Prof.
Tanya Paull (3).

Ni-NTA pull-down assay:

Recombinant RAGE-(His)_6 fragments were purified from soluble extracts. Ni-
NTA pull-down assays were performed using 2μg of the His_(6)-tagged RAGE, mixed
with either 5μg of MRE11, RAD50 or Nbs1 in the pull down buffer (2X buffer: 20
mM Tris-HCl, pH 7.9, 20% Glycerol, 10mM MgCl_2, 300mM KCl, 0.2% NP40,
30mM imidazole, 1mM PMSF and Phosphatase inhibitors) along with the Ni-
nitritriacetic acid (NTA) His bind resin (Novagen). The mixture was incubated
for 3 h at 4°C on a rotary shaker. Where indicated the samples were pre-treated
with either ethidium bromide (25 μg/ml) or DNase-I (10U/μl, 30 min at 30°C @
10mM MgCl_2). After which, the beads were extensively washed in the pull down
buffer (while comparing the stability and specificity of the RAGE-MRE11
interaction column was washed with either 100mM, 200mM or 300mM
NaCl/KCl), the proteins were extracted from the beads into the sodium dodecyl
sulfate (SDS) sample buffer, separated on an SDS-12% polyacrylamide gel
electrophoresis (PAGE) gel, and either visualized by Coomassie Brilliant Blue
(CBB) staining or blotted and probed with appropriate antibodies.

Virus production:

The production of recombinant AAV virions in HEK293 cells was performed as
described earlier (4). In brief cells were seeded at 5 x 10^6 cells/flask for each vector
type. Once cells reached 80% confluency, cells were transfected with three plasmids
for each AAV virus type to be packaged (Supplementary table: 2). The triple-
transfection of HEK 293T cells was set up as follow: for each confluent T150 flask,
12.5μg of AAV backbone plasmid, 25μg pDp2rs helper plasmid and 12μg pDP8
plasmid were added to 2.4 ml of sterile water in a 15 ml Falcon tube, then 330μl of
2.5 M CaCl₂ was added to the mixture. The transfection mixture was filtered through a 0.2 μm syringe filter into another 15 ml Falcon tube and whilst vortexing the solution vigorously, 2.5 mL of 2x HBSS buffer (280 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM HEPES, PH 7.5) was added quickly. The mixture was incubated 5 mins at room temperature and 5 ml was applied to the corresponding T150 flask. Transfected cells were incubated at 37°C/5% CO₂. 16 h post-transfection, media was removed and replaced with fresh complete DMDM. After 96 hours of transfection, packaging cells were lysed in packing lysis buffer (50 mM Tris, 150 mM NaCl at pH 8.4) with vigorous pipetting to release the AAV virions. Lysate and culture medium samples were incubated at 37°C for 30 min in water bath, and the cell debris was removed by centrifugation at 3000 g for 15 min at 4°C. Virions were purified and concentrated using an Iodixanol gradient and concentrated using the Vivaspin centrifugal concentrator (50KDa cutoff).

**Cell-cycle analysis:**

To evaluate the cell-cycle distribution, the DNA content of the cells was accessed by flow cytometry. Briefly, the cells were fixed with ice-cold 70% ethanol and stored on ice for 45 minutes, and then treated stained with PI/RNaseA (PI: 50μg/ml/ and RNaseA: 0.5μg/ml) mix. Cells were analyzed on an LSRII flow cytometer (BD Bioscience): we calculated the proportion in G0/G1-, S- and G2-phase using the FlowJo Ver.10 software.

**Cell synchronization method:**

The early S-phase synchronization was performed by using double Thymidine block method. In brief 50-60% confluent cells were incubated with growth medium supplemented with 2mM Thymidine for 18 hours. After first block cells were washed with PBS and supplemented with normal growth medium for 9 hours. The 2nd Thymidine block was initiated after 9 hours by incubating with Thymidine containing medium for another 17 hours. After five hours of 2nd release, most of cells propagate through S-Phase.
G1-phase synchronization: The G1-phase cell synchronization was performed by incubating 30-40% confluent cells in 0.1% serum containing medium for 72 hours.

The G2/M-phase synchronization was induced by Thymidine-Nocadazole treatment: In brief cells were incubated with Thymidine containing medium for 24 hours and released for 3 hours by incubating them in Thymidine free medium. After 3 hours, cells were further incubated in Nocadazole (100ng/ml) containing medium for 12 hours.

HR-DGFP Assay

To measure HR-mediated repair of DNA DSBs, we used the DR-GFP/SceI assay as described previously, The DR-GFP plasmid (originally generated by Dr Maria Jasin (5), Memorial Sloan-Kettering Hospital, New York, NY, we obtained this plasmid from Addgene) was further modified to attach 3X Flag at the 3'- of the EGFP-A (refer Figure S8A) in BsrGI and NotI site. After repair this insertion results in the expression of modified GFP that has 3X-FLAG fused at the C-terminus of the expressed protein. This GFP-3X-FLAG can be detected with Alexa647 conjugated α-FLAG antibody and can be analyzed by either flow cytometry or by microscopy. In this assay, WT or RAGE/− lung fibroblasts were co-transfected with the modified DR-GFP HR reporter plasmid and I-SceI plasmid backbone along with the indicated RAGE constructs; and as 100% control WT cells were just transfected with modified reporter and I-SceI plasmid (+ve control; 100%). WT untransfected (no plasmid) and RAGE/− cells transfected with modified reporter and I-SceI plasmid served as negative control (-ve control) of the assay. Proficient HR repair system then repairs these DSBs, resulting in a functional GFP-FLAG gene. The percentage of GFP-FLAG+ cells was measured 72 hours later by flow cytometry and/or observed under microscope.
**Supplementary tables:**

**Supplementary table. 1: Substances used for DNA damage induction**

| Drug          | Purpose                  | Concentration          | References |
|---------------|--------------------------|------------------------|------------|
| Camptothecin  | DNA DSB induction        | 1μM (for 60mins)       | (6)        |
| Bleomycin     | DNA DSB induction        | 30μg/ml (for 60mins)   | (7)        |
| Etoposide     | DNA DSB induction        | 5μM (for 60mins)       | (8)        |
| Hydroxy Urea  | Replication stress induction | 2mM(for 60mins)     | (6)        |

**Supplementary table. 2: Details of plasmids used for AAV production**

| Packaged AAVs     | Plasmid                                                                 |
|-------------------|-------------------------------------------------------------------------|
| RAGE RFP ^WT      | pAM CBA RAGERFP WPRE bGH & pDP2rs + pDP8.                               |
| RAGERFP ^S376A-S389A | pAM CBA RAGE mut RFP WPRE bGH & pDP2rs + pDP8.                         |
| RPA2 RFP ^S4E-S8E  | pAM CBA RPARFP WPRE bGH & pDP2rs + pDP8.                               |
| RFP alone         | pAM CBA RFP WPRE bGH & pDP2rs + pDP8.                                  |

**Supplementary table. 3: Details of primary and secondary antibodies**

| Antibody       | Species | Supplier                  | Application |
|----------------|---------|---------------------------|-------------|
| Calreticulin A | Rabbit  | (Cell Signaling)          | IB          |
| Cyclin A       | Goat    | Sc-751(Santa Cruz)        | IB          |
| Cytochrome C   | Mouse   | NB100-56503(Novus Biologicals) | IB          |
| CHK1           | Mouse   | 2360(Cell Signaling)      | IB          |
| CHK 2          | Rabbit  | 2662(Cell Signaling)      | IB          |
| eIF6           | Rabbit  | 3263(Cell Signaling)      | IF          |
| Antibody | Species | Catalog Number | Company | Format |
|----------|---------|----------------|---------|--------|
| FLAG     | Rat     | MA1-142-A647   | Thermo  | IF/FC  |
| FSP1     | Rabbit  | Arb (13415)    |         | IF     |
| GFP      | Goat    | Sc-8334        | Santa Cruz | IP     |
| GFP      | Rabbit  | Ab6556         | Abcam   | IB     |
| HDAC1    | Rabbit  | 2062           | Cell Signaling | IB     |
| Histone H1 | Mouse | (Abcam)        |         | IB     |
| H2AX     | Rabbit  | IHC-0008       | Bethyl  | IB     |
| γH2AX    | Rabbit  | 9718           | Cell Signaling | IB,IF  |
| γH2AX    | Mouse   | 05-636         | Millipore | IF     |
| IL-6     | Rabbit  | Ab6672         | Abcam   | IF     |
| MRE11    | Rabbit  | 4895           | Cell Signaling | IB     |
| MRE11    | Rabbit  | Nb100-142      | Novus Biologicals | IF     |
| NBS1     | Rabbit  | A301-284A      | Bethyl  | IP     |
| NBS1     | Rabbit  | NB100-143      | Novus Biologicals | IF,IP  |
| pATM     | Mouse   | NB100-306      | Novus Biologicals | IF     |
| pATM     | Mouse   | 201-300-400    | Rockland | IF, IB |
| pATR     | Rabbit  | 2853           | Cell Signaling | IF     |
| pCHK1 S345 | Rabbit | AF2475         | R&D     | IF,IB  |
| pCHK2 T68 | Rabbit | NB100-92502    | Novus Biologicals | IB     |
| pSQE      | Rabbit | 9607 (Cell Signaling) | IB |
|----------|--------|-----------------------|----|
| RAD50    | Rabbit | A300-184A (Bethyl)    | IF,IP |
| RAGE     | Goat   | Sc-8230 (Santa Cruz)  | IF,IP,IB |
| RAGE     | Rat    | MAB1179 (R&D)         | IF |
| RPA2     | Rabbit | AP9115a (Abgent)      | IB |
| RPA2S4/S8| Rabbit | A300-245A (Bethyl)    | IF,IB |
| Tom20    | Rabbit | 13929 (Cell signaling)| IB |
| Transketolase | Rabbit | Sc-67120 (Santa Cruz) | IB |
| WGA 647  | XXXX   | (Life Technologies)   | IF |
| 53BP1    | Rabbit | NB100-304 (Novus Biologicals) | IF |

**Secondary antibodies**

| Anti-Goat-HRP | Donkey | sc-2033 (Santa Cruz) | IB |
|---------------|--------|----------------------|----|
| Anti-Rabbit IgG-HRP | Goat | sc-2030 (Santa cruz) | IB |
| Anti-Mice IgG-HRP | Goat | sc-2062 (Santa Cruz) | IB |
| Anti-Rabbit IgG-HRP | Goat | 7074 (Cell Signaling) | IB |
| Anti-mouse   | Goat   | 7076 (Cell Signaling) | IB |
| IgG-HRP          | Anti Goat-Alexa-647 | Donkey | Ab150139 (Abcam) | IF |
|------------------|--------------------|--------|-----------------|----|
|                  | Anti mice-Alexa-647| Donkey | Ab150111 (Abcam) | IF |
|                  | Anti Rabbit-Alexa-555| Donkey | Ab150074 (Abcam) | IF |

**Supplementary table.4: Cloning primers used**

| Name       | Sequence                                                                 |
|------------|----------------------------------------------------------------------------|
| 1 RAGE F   | ATGCCAGCGGGGACAGCAGCTAGAGCCT                                              |
| 2 RAGE R   | TTACAGGGCTAGCGTACCCAGCCCAGACT                                              |
| 3 RAGE R ST| TTACGGTCCCCCGGCAACCATTTCTCT                                               |
| 4 RAGE FL FP F | ATGCCAGCGGGGACAGCAGCTAGAGCCT                                               |
| 5 RAGE FL FP R | CCGTCCCCCGGCAACCATTTCTCT                                               |
| 6 RAGE Tr FP F | ATGCTGGCCTTTGGGGGATCCTTGGGGAGGC                                           |
| 7 shRAGE   | GATCCCCAAGCCAGAAATTGTGGGATCCTTTTCAAGAGAAGGG ATCCACAATTCTGCTTTTTTTTTTA    |
| 8 RAGE EM F | GGAACCGAAGCTGGGACGCTGGGGCTCCCGGCGCAGGCGGCCAGGCTGGGACGCTGGGACGCTGGGACGCTGG |
| 9 RAGE EM R | GGAGGTGGGACAGCTGGGACGCTGGGGCTCCCGGCGCAGGCGGCCAGGCTGGGACGCTGGGACGCTGGGACGCTGGG |
| 10 RPA2 F  | ATGTGGAATAGCGGATTCGAA                                                     |
| 11 RPA2 R  | TCACCTCTGCTAGGAGACTTTAAAGTGAT                                              |
| 12 RPA2 S8F | AATAGCGGATTCGAGAGAGTTACGAGCTCC                                       |
| 13 RPA2 S4 S8F | GAGGGATTCGAAGAGGTTACGAGCTCC                                            |
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