Supplementary Materials for

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

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This word file includes:

Supplementary figure S1-S11

Supplementary methods

Supplementary tables S1 to S4

Supplementary references 1 to 8

Figure S1.

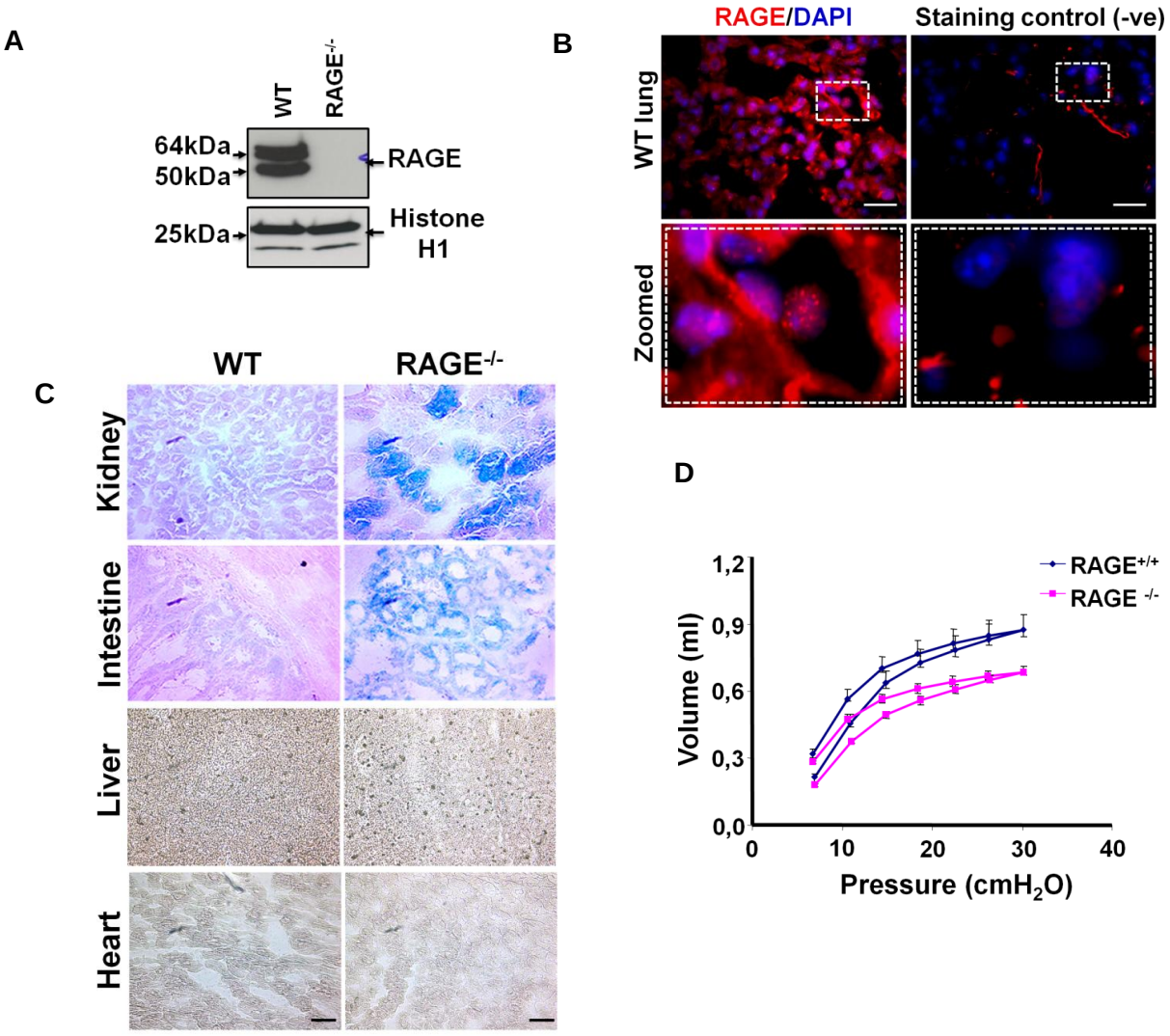


Figure S1:

(**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.

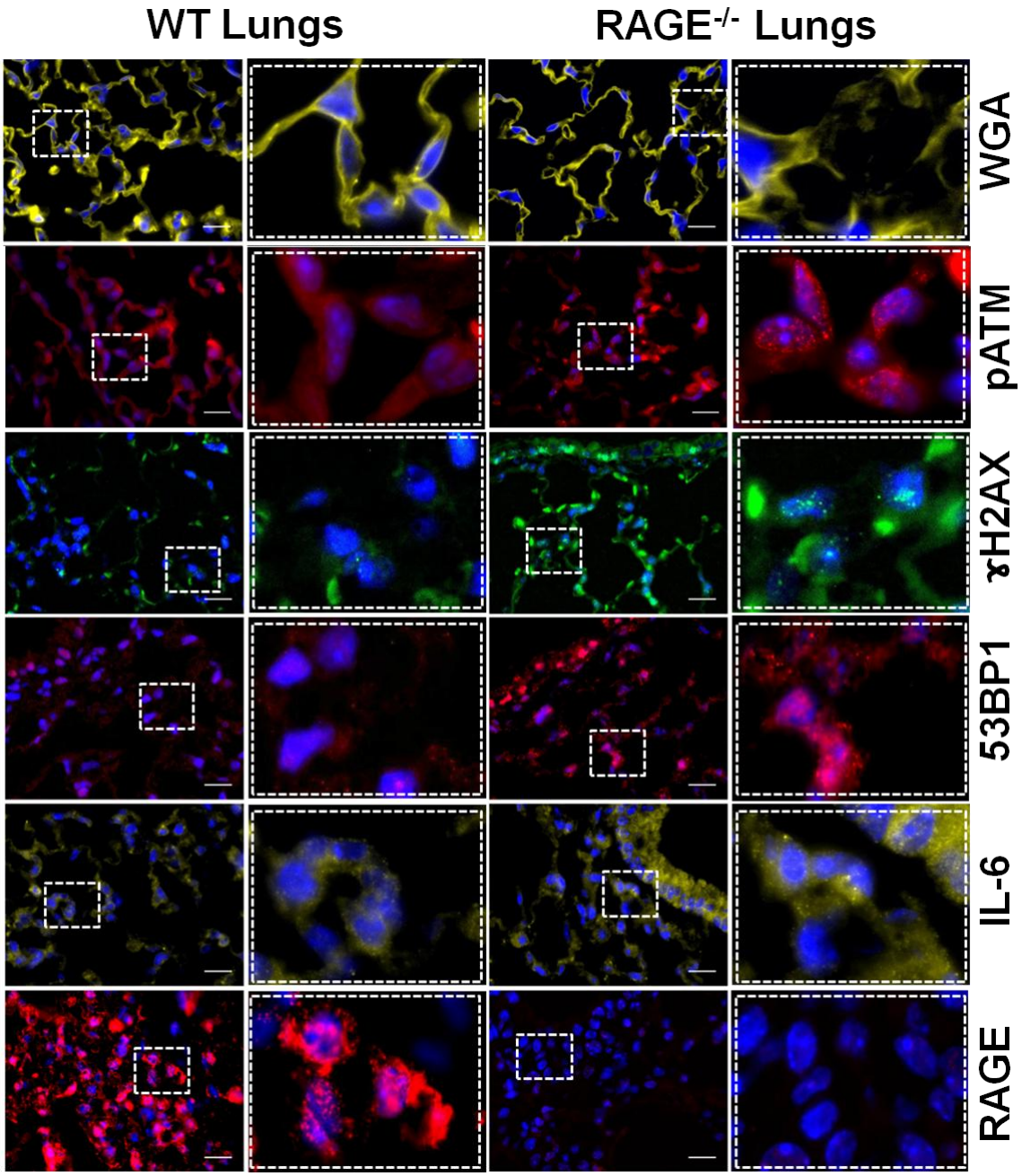


Figure S2:

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.

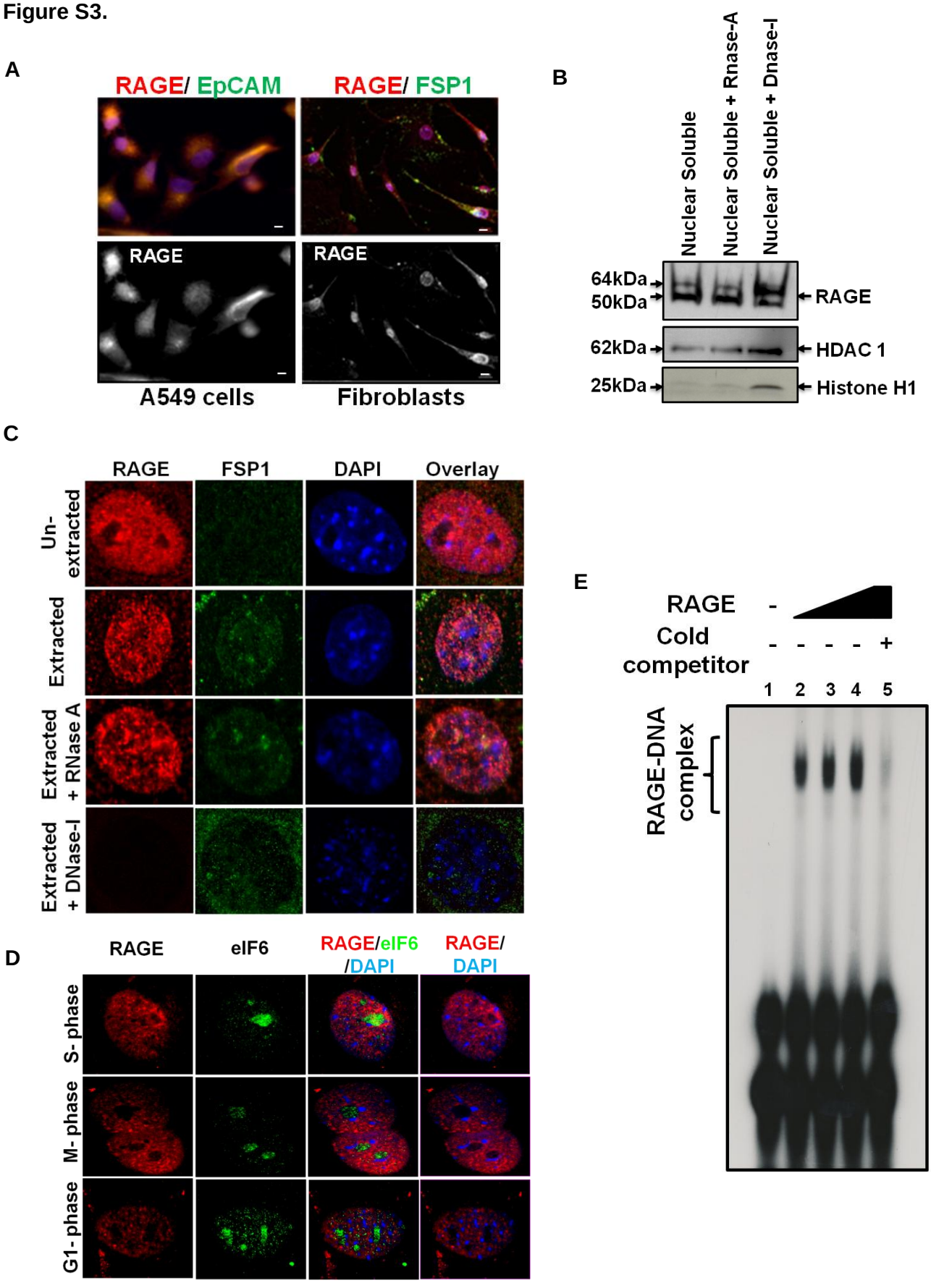

A
RAGE/ EpCAM
RAGE/ FSP1
RAGE
RAGE
A549 cells
Fibroblasts
B
Nuclear Soluble
Nuclear Soluble + Rnase-A
Nuclear Soluble + Dnase-I
64kDa
50kDa
RAGE
62kDa
HDAC 1
25kDa
Histone H1
C
RAGE
FSP1
DAPI
Overlay
Un-extracted
Extracted
Extracted + RNase A
Extracted + DNase-I
D
RAGE
eIF6
RAGE/eIF6 /DAPI
RAGE/ DAPI
S- phase
M- phase
G1- phase
E
RAGE
Cold competitor
1 2 3 4 5
RAGE-DNA complex

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.

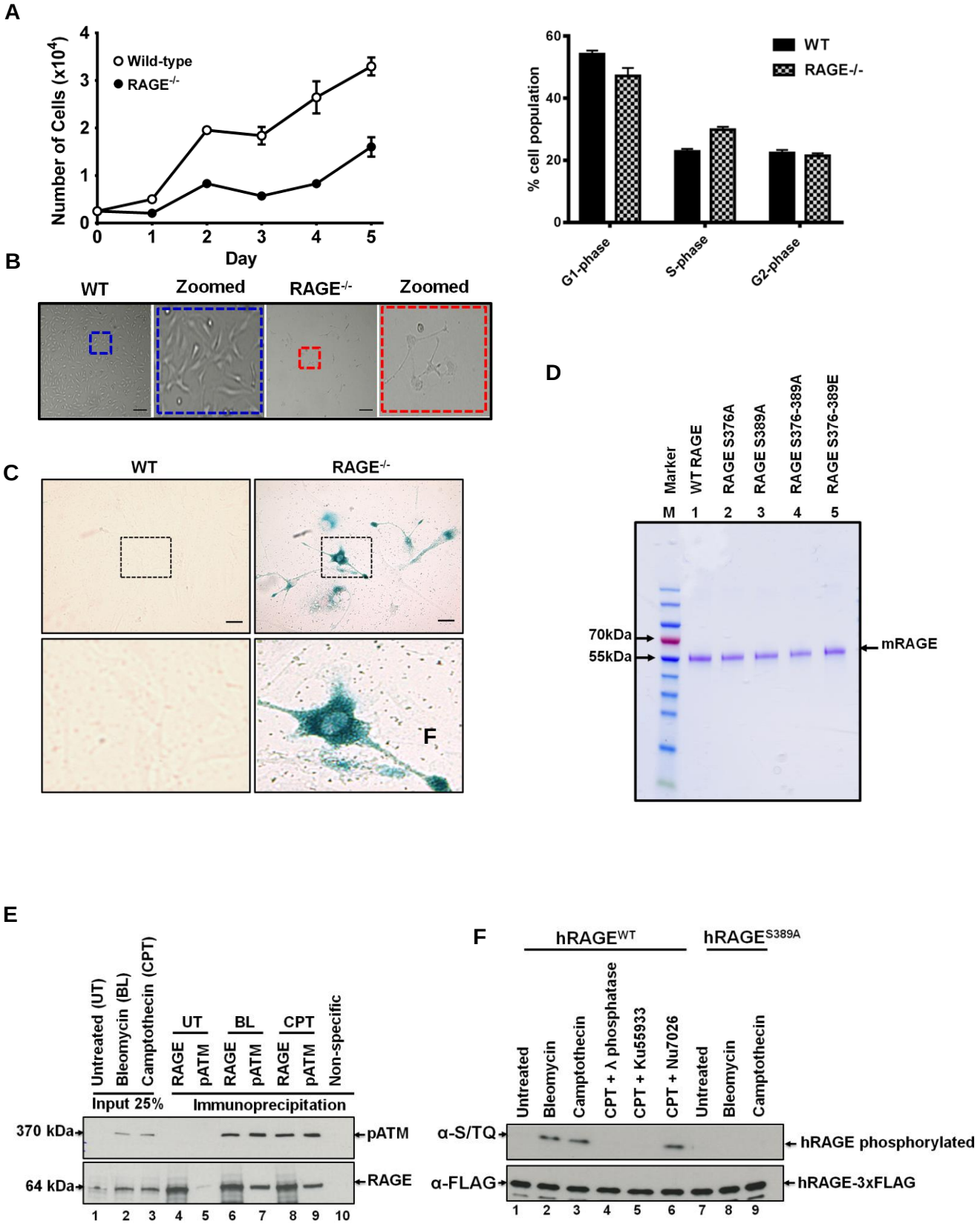

A
Number of Cells (x10^4)
Wild-type
RAGE-/-
Day
% cell population
WT
RAGE-/-
G1-phase
S-phase
G2-phase
B
WT
Zoomed
RAGE-/-
Zoomed
C
WT
RAGE-/-
F
D
Marker
WT RAGE
RAGE S376A
RAGE S389A
RAGE S376-389A
RAGE S376-389E
M 1 2 3 4 5
70kDa
55kDa
mRAGE
E
Untreated (UT)
Bleomycin (BL)
Camptothecin (CPT)
UT
BL
CPT
RAGE
pATM
Non-specific
Input 25%
Immunoprecipitation
370 kDa
64 kDa
pATM
RAGE
1 2 3 4 5 6 7 8 9 10
F
hRAGE^WT
hRAGE^S389A
Untreated
Bleomycin
Camptothecin
CPT + λ phosphatase
CPT + Ku55933
CPT + Nu7026
α-S/TQ
α-FLAG
hRAGE phosphorylated
hRAGE-3xFLAG
1 2 3 4 5 6 7 8 9

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 ß-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 hRAGE^{WT} FLAG or hRAGE^{S389A} construct. The phosphorylated RAGE was collected from the cell lysates on M_2 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.

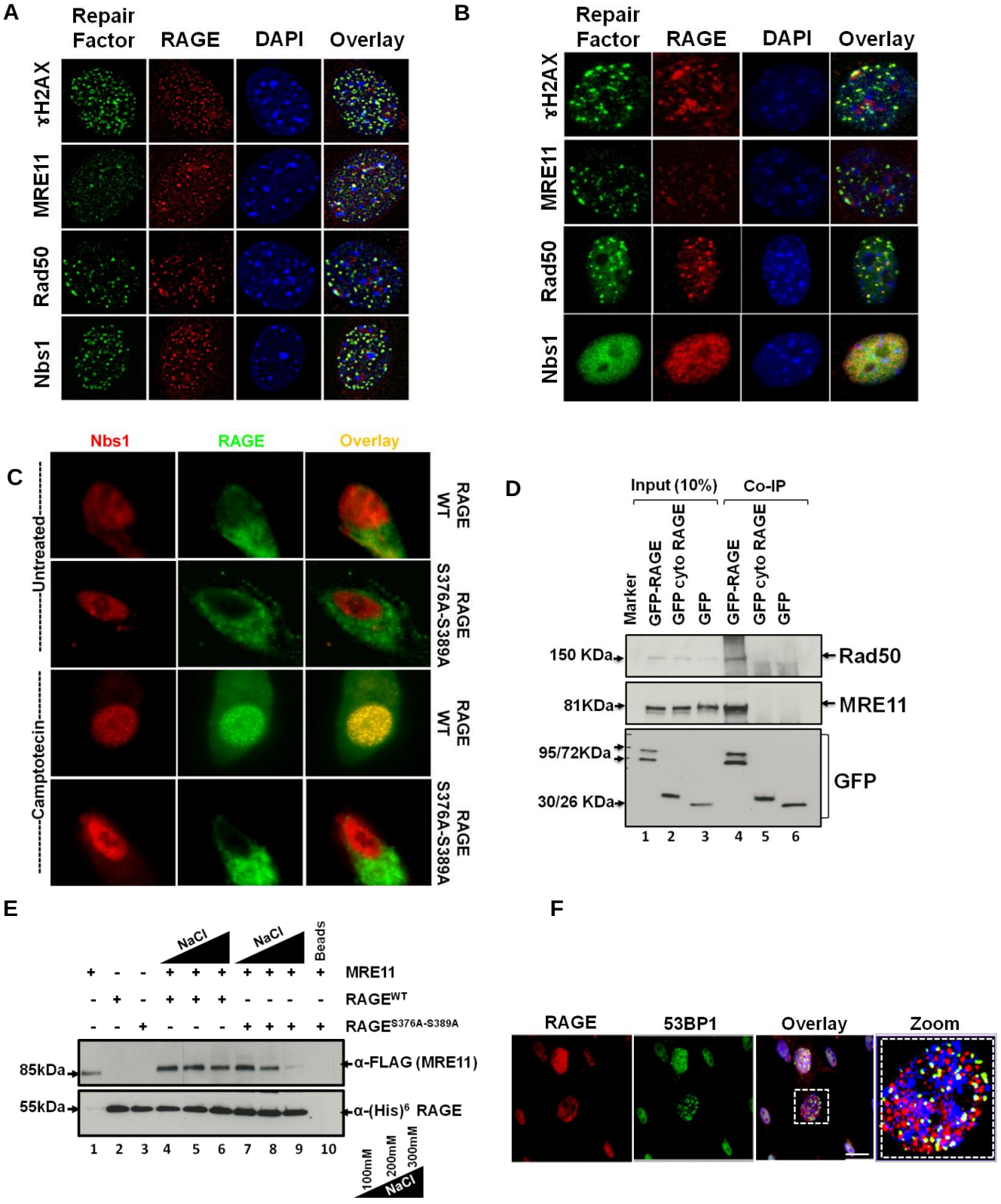

A
Repair Factor
RAGE
DAPI
Overlay
γH2AX
MRE11
Rad50
Nbs1
B
Repair Factor
RAGE
DAPI
Overlay
γH2AX
MRE11
Rad50
Nbs1
C
Nbs1
RAGE
Overlay
Untreated
Camptotecin
RAGE WT
RAGE S376A-S389A
RAGE WT
RAGE S376A-S389A
D
Input (10%)
Co-IP
Marker
GFP-RAGE
GFP cyto RAGE
GFP
GFP-RAGE
GFP cyto RAGE
GFP
150 KDa
81KDa
95/72KDa
30/26 KDa
Rad50
MRE11
GFP
1 2 3 4 5 6
E
NaCl
NaCl
Beads
MRE11
RAGE^WT
RAGE^S376A-S389A
85kDa
55kDa
α-FLAG (MRE11)
α-(His)6 RAGE
1 2 3 4 5 6 7 8 9 10
100mM
200mM
300mM
NaCl
F
RAGE
53BP1
Overlay
Zoom

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-RAGE^{WT} or GFP-RAGE$^{S376A\text{-}S389A}$ 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 $^{S376A\text{-}S389A}$ 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.

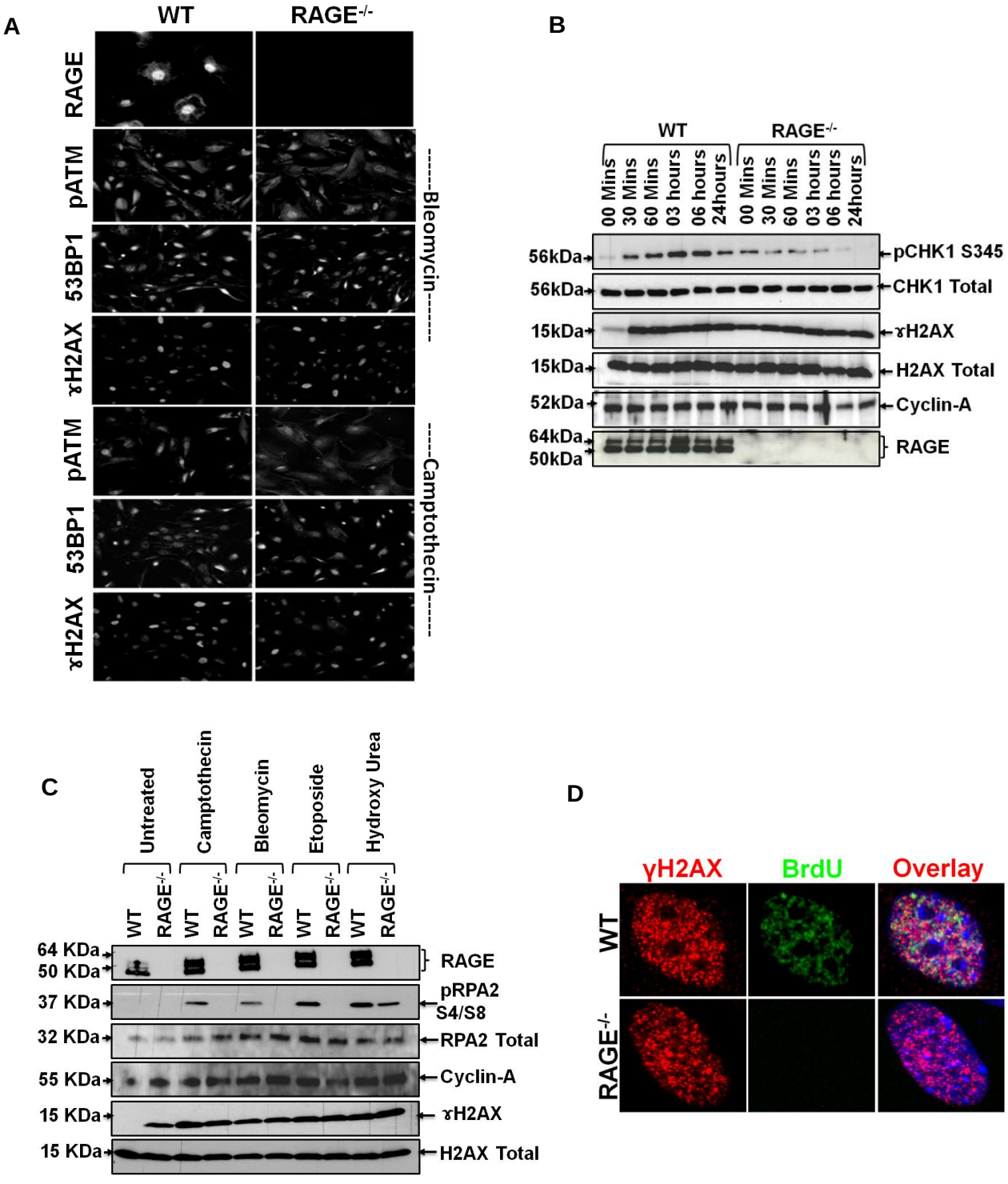

A
WT
RAGE-/-
RAGE
pATM
53BP1
γH2AX
pATM
53BP1
γH2AX
-----Bleomycin-----
-----Camptothecin-----
B
WT
RAGE-/-
00 Mins
30 Mins
60 Mins
03 hours
06 hours
24hours
00 Mins
30 Mins
60 Mins
03 hours
06 hours
24hours
56kDa
pCHK1 S345
56kDa
CHK1 Total
15kDa
γH2AX
15kDa
H2AX Total
52kDa
Cyclin-A
64kDa
50kDa
RAGE
C
Untreated
Camptothecin
Bleomycin
Etoposide
Hydroxy Urea
WT
RAGE-/-
64 KDa
50 KDa
RAGE
37 KDa
pRPA2 S4/S8
32 KDa
RPA2 Total
55 KDa
Cyclin-A
15 KDa
γH2AX
15 KDa
H2AX Total
D
γH2AX
BrdU
Overlay
WT
RAGE-/-

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, RPA2$^{S4\ \&\ S8}$, 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.

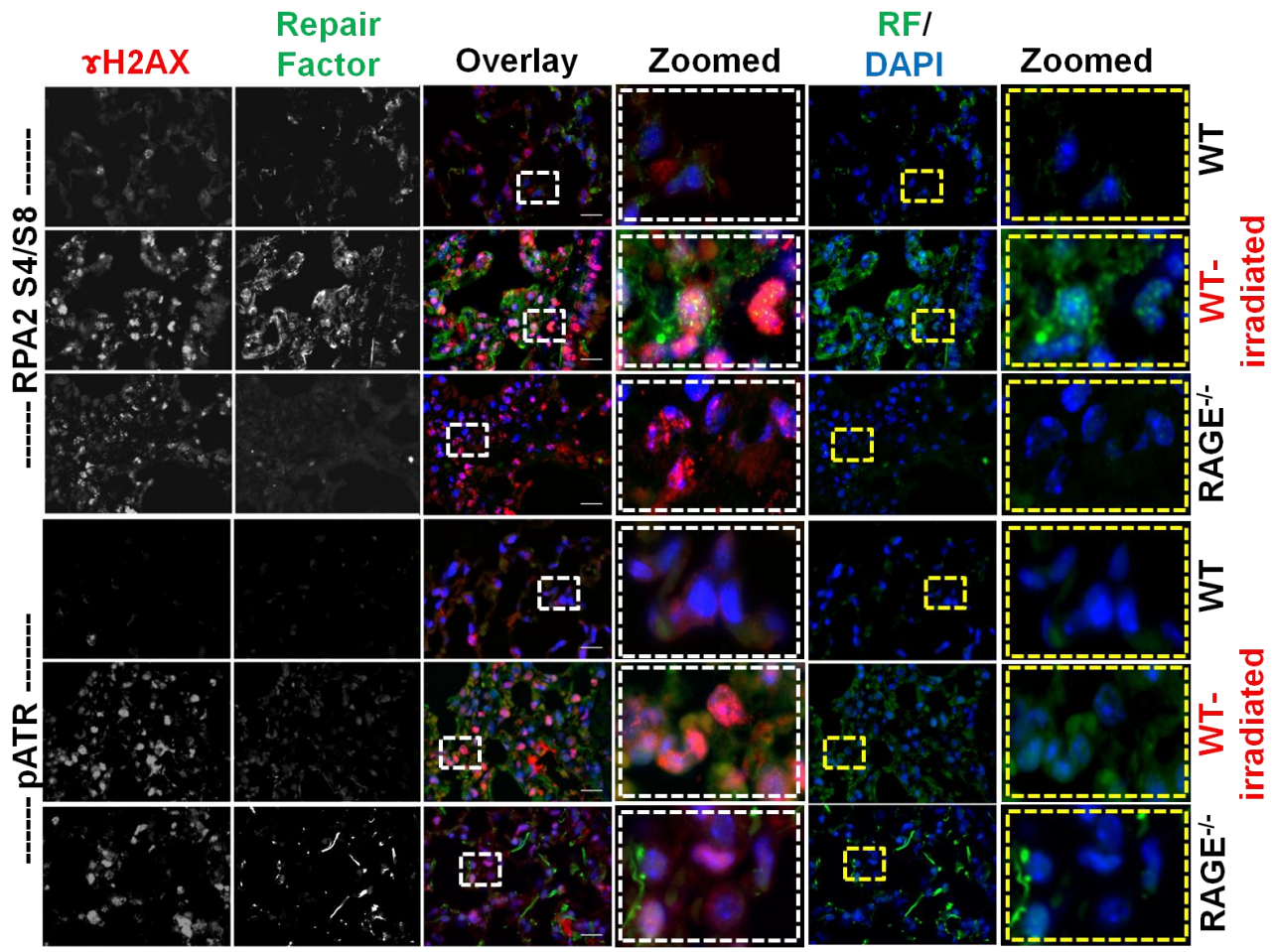


Figure S7.

Representative **i**mmunofluorescence 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.

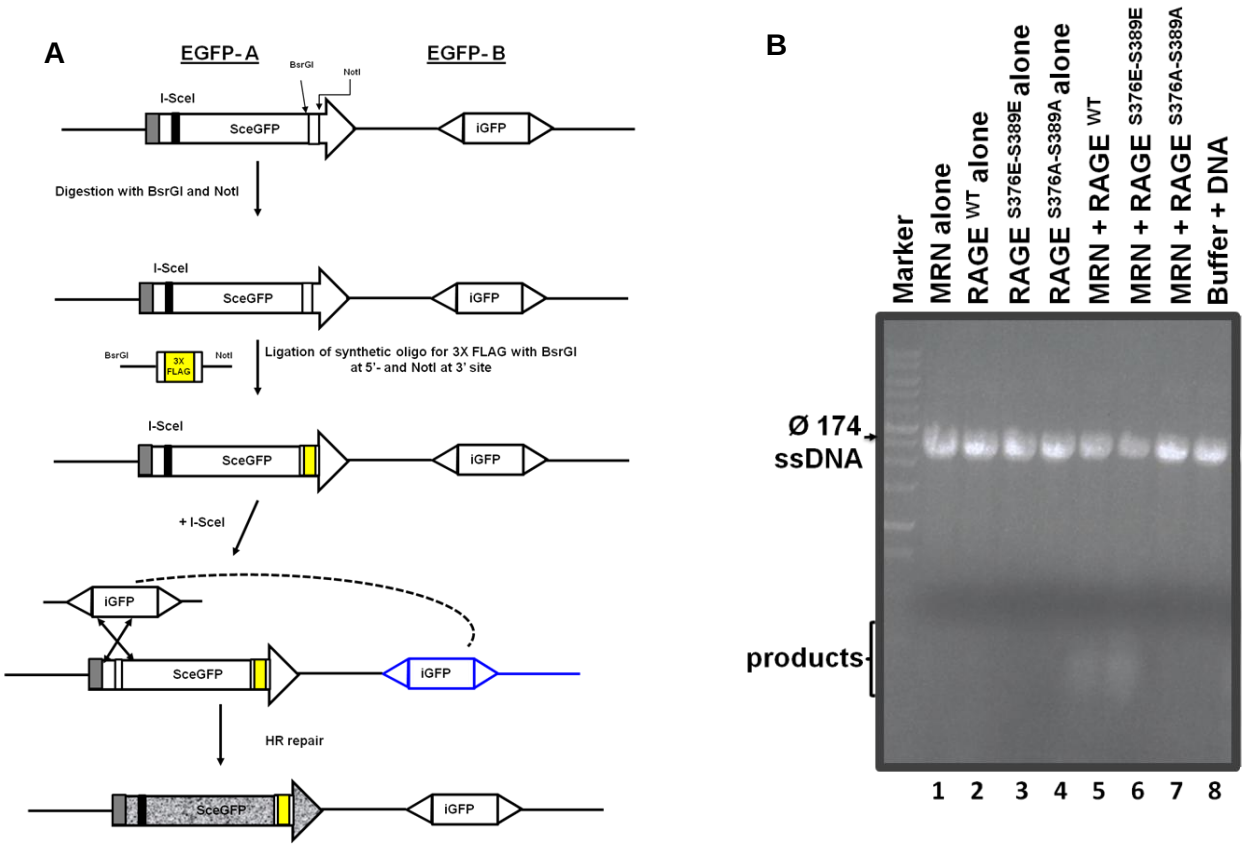


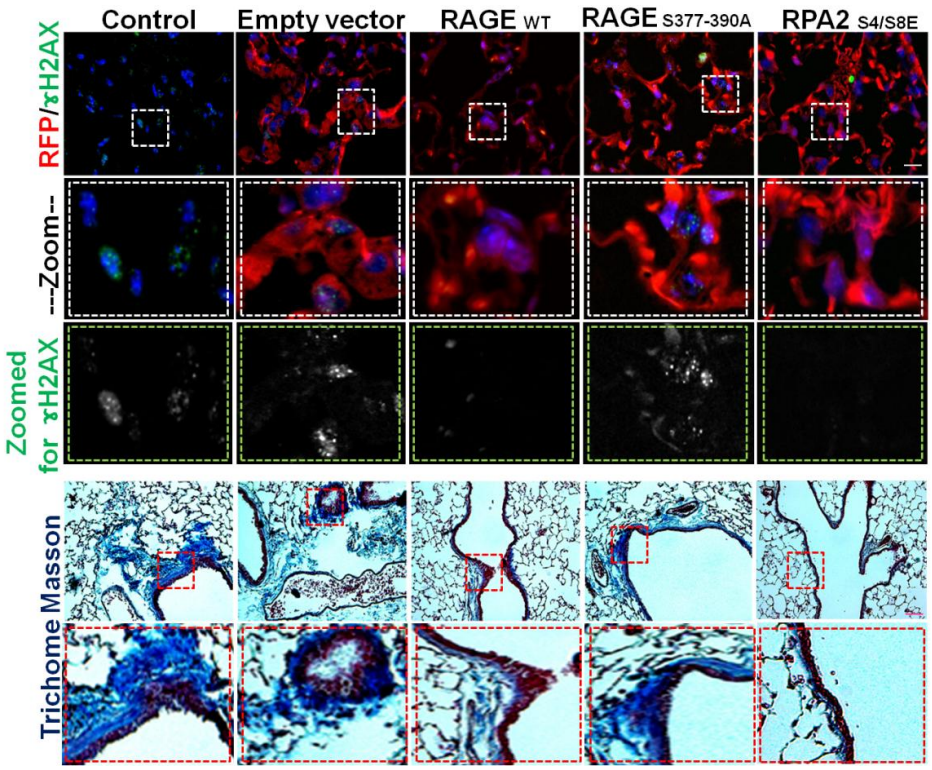
Figure. S8:

(**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, $RAGE^{WT}$, $RAGE^{S376E-S389E}$, $RAGE^{S376A-S389A}$ alone respectively, lane 5 MRN with $RAGE^{WT}$, lane 6 MRN with $RAGE^{S376E-S389E}$, lane 7 MRN with $RAGE^{S376A-S389A}$, lane 8 buffer with DNA alone as negative control. The reaction buffer contained MRN (50nM) or RAGE (2.0μM) in 5mM $MnCl_2$.

Figure S9.

A


Control
Empty vector
RAGE WT
RAGE S377-390A
RPA2 S4/S8E
RFP/γH2AX
---Zoom--
Zoomed for γH2AX
Trichome Masson

B

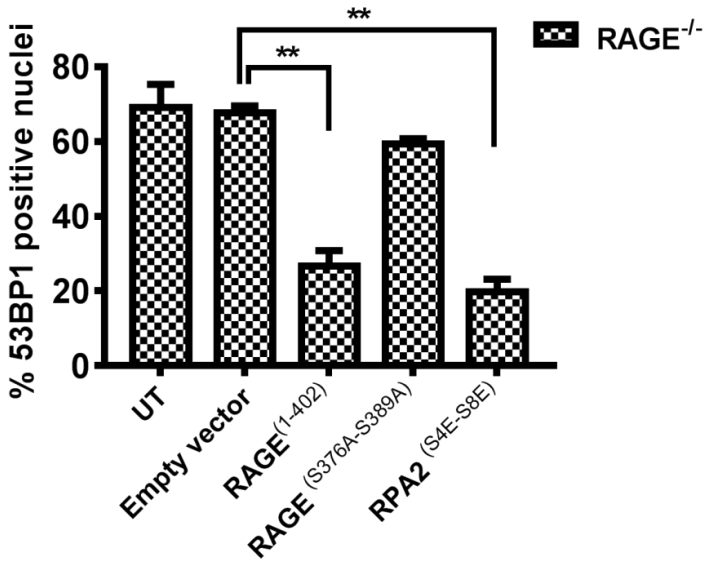

% 53BP1 positive nuclei
80
60
40
20
0
**
**
RAGE-/-
UT
Empty vector
RAGE(1-402)
RAGE (S376A-S389A)
RPA2 (S4E-S8E)

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 (RAGE$^{1-402}$), 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

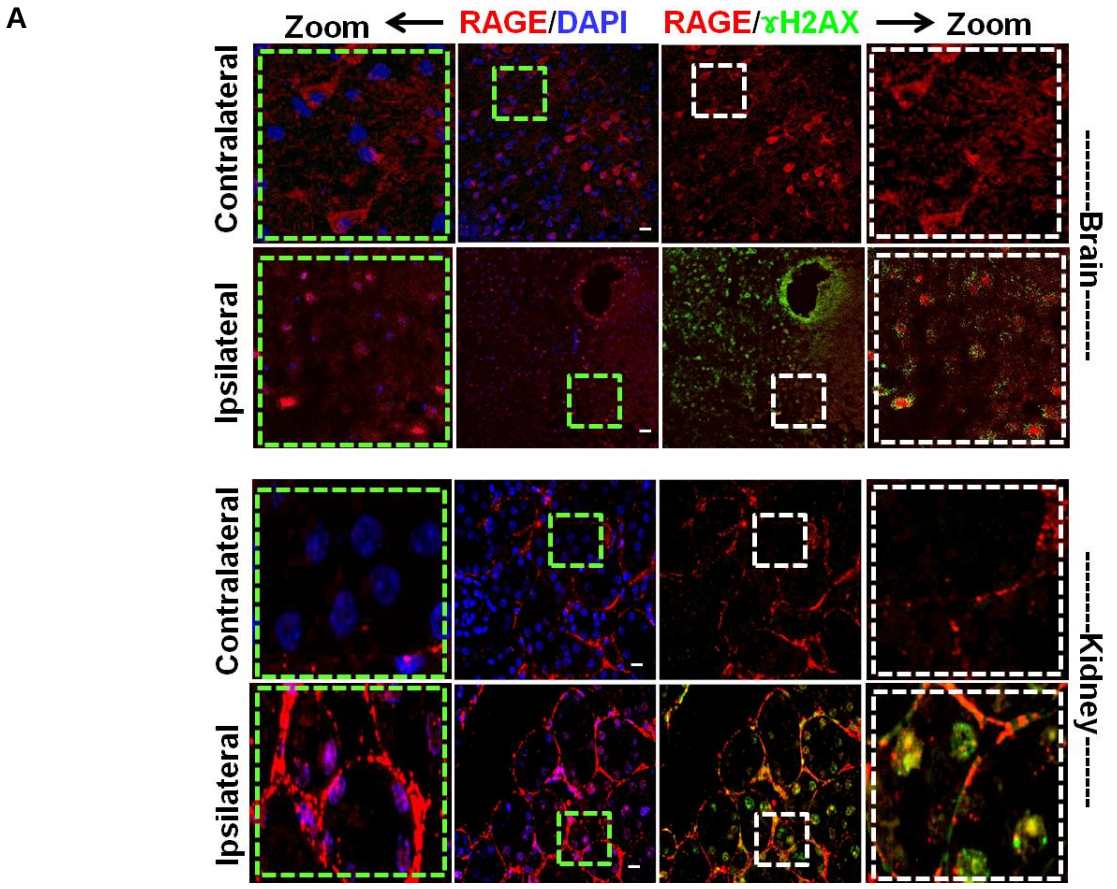

A
Zoom
RAGE/DAPI
RAGE/γH2AX
Zoom
Contralateral
Ipsilateral
Brain
Contralateral
Ipsilateral
Kidney
B
RAGE
γH2AX
Overlay
Untreated
Camptothecin

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:

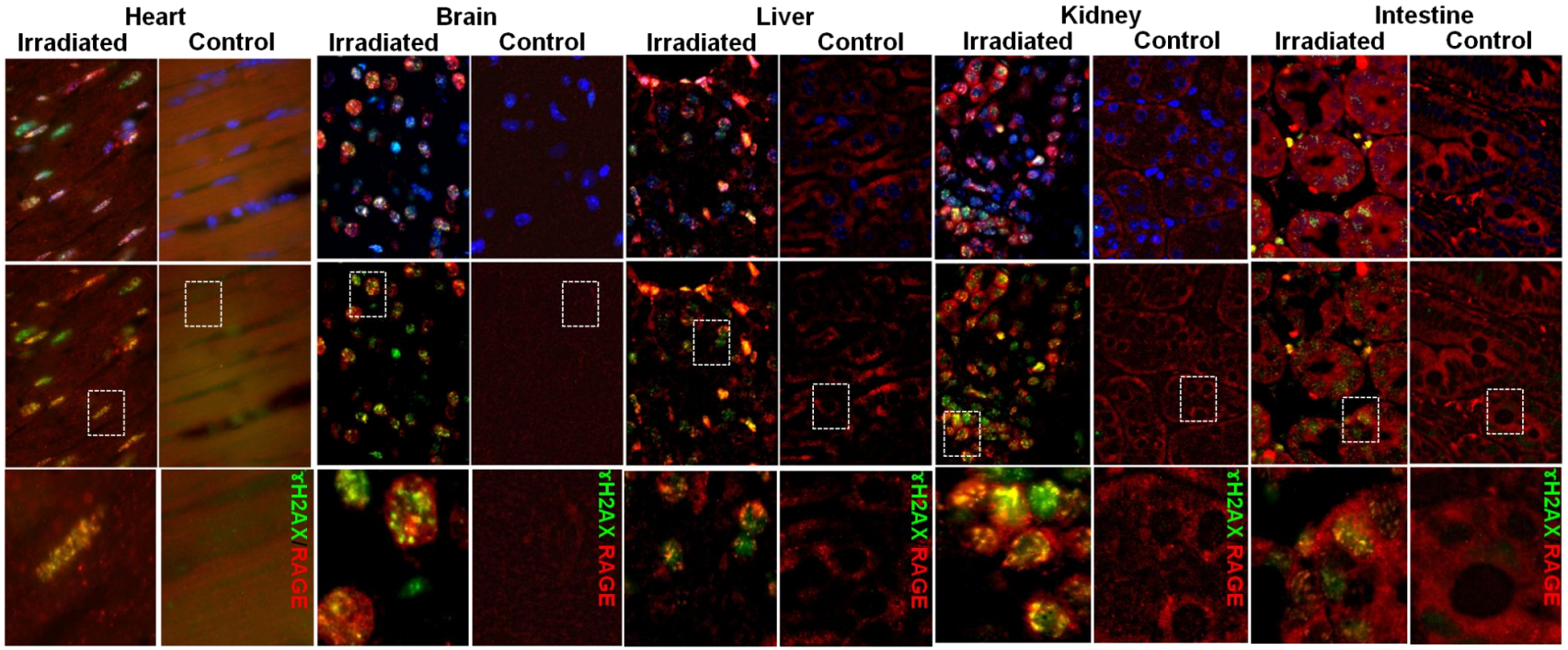


Figure S11:

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 $CaCl_2$ 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_2 grinded powder of respective tissue) were obtained by resuspending them in 20mM Tris-Cl pH 7.5, 40mM NaCl, 2mM $MgCl_2$, 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 20µg 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 $MgCl_2$, 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 µg/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 Retrival 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°Cfor 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-dimethythiazol-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 formazon 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 dialyzed 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-nitrilotriacetic 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 30ºC @ 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 $CaCl_2$ was added to the mixture. The transfection mixture was filtered through a 0.2um 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 Na_2HPO_4, 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 cels 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 18hours. After first block cells were washed with PBS and supplemented with normal growth medium for 9 hours. The 2^{nd} Thymidine block was initiated after 9 hours by incubating with Thymidine containing medium for another 17hours. After five hours of 2^{nd} 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 $Alexa^{647}$ 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-*Sce*I plasmid backbone along with the indicated RAGE constructs; and as 100% control WT cells were just transfected with modified reporter and I-*Sce*I plasmid (+ve control; 100%). WT untransfected (no plasmid) and $RAGE^{-/-}$ cells transfected with modified reporter and I-*Sce*I 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 $GFP\text{-}FLAG^{+}$ 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

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
ɣH2AX	Rabbit	9718(Cell Signaling)	IB,IF
ɣH2AX	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	TTACGGTCCCCCGGCACCATTCTCT
4	RAGE FL FP F	ATGCCAGCGGGGACAGCAGCTAGAGCCTG
5	RAGE FL FP R	CGGTCCCCCGGCACCATTCTCTG
6	RAGE Tr FP F	ATGCTGGCCTTGGGGATCCTGGGAGGC
7	shRAGE	GATCCCCAAGCCAGAAATTGTGGATCCTTTCAAGAGAAGG ATCCACAATTTCTGGCTTTTTTTA
8	RAGE EM F	GGACTCCAGGTCCAGGACCGCCGTTTTTCGCGCGCACGGC GCGGGAGGTCCAGCTGGTCCACCTCC
9	RAGE EM R	GGAGGTGGACCAGCTGGACCTCCCGCGCCGTGCGCGCGA AAAACGGCGGTCCTGGACCTGGAGTCC
10	RPA2 F	ATGTGGAATAGCGGATTCGAA
11	RPA2 R	TCACTCTGCATCTGTAGACTTAAAGTGAT
12	RPA2 S8F	AATAGCGGATTCGAAGAGTTCAGCAGCTCCA
13	RPA2 S4 S8F	GAGGGATTCGAAGAGTTCAGCAGCTCCA

14	RPA S4 F	GCGAGATCTATGTGGAATGAGGGATTCGAA
15	RPA S33 F	CCACACCGGAGCAGGCGGAGAAGAA
16	RPA 2 S33 R	TCCGCCTGCTCCGGTGTGGGCGA
17	RAGE S376A F	GAAGGCCCCGGAAGCCCAGGAGGATGAG
18	RAGE S376A R	ATCCTCCTGGGCTTCCGGGGCCTTCCTC
19	RAGE S390A F	GTGCAGAGCTGAATCAGGCGGAGGAAGCGGAGATG
20	RAGE S390A R	CATCTCCGCTTCCTCCGCCTGATTCAGCTCTGCAC
21	hRAGE F	ATGGCAGCCGGAACAGCAGTTGGAGC
22	hRAGE R	AGGCCCTCCAGTACTACTCTCGCCT
23	RAGE S376E F	GAAGGCCCCGGAAGAACAGGAGGATGAG
24	RAGE S376E R	CTCATCCTCCTGTTCTTCCGGGGCCTTC
25	RAGE S390E F	GTGCAGAGCTGAATCAGGAAGAGGAAGCGGAGATG
26	RAGE S390E R	CATCTCCGCTTCCTCTTCCTGATTCAGCTCTGCAC
27	HR-F modification	GTACAAGGACTACAAGGACCACGACGGCGACTACAAGGAC CACGACATCGACTACAAGGACGATGATGACAAGTAAAGC
28	HR-R modification	GGCCGCTTTACTTGTCATCATCGTCCTTGTAGTCGATGTCG TGGTCCTTGTAGTCGCCGTCGTGGTCCTTGTAGTCCTT

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