Supplementary information, Data S1

Materials and Methods

Nuclear fractionation, Western blotting and immunoprecipitation assays

Nuclear fractionation and Western blotting were performed as described previously[6]. Briefly, after serum starvation overnight, cells were treated with or without IR, washed with phosphate buffer saline (PBS) twice, and resuspended in lysis buffer (20 mM HEPES pH7.0, 10 mM KCl, 2 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 2 μg/ml aprotinin) on ice. After homogenizing cells with 25-30 strokes in Dounce homogenizer and centrifugation at 1,500 x *g* for 5 min, supernatant was separated as cytosolic extract (CE) and nuclear pellets were wash with lysis buffer for three times and lysed in TGN buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Tween 20, 0.3% NP-40, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1X protease inhibitor mixture; Roche)[7] with sonication. Both nuclear extracts (NE) and CE were collected from supernatant after centrifugation at highest speed for 10 min at 4° C and protein concentration was determined by using BCA protein assay kit (Thermo Scientific). At least 2 mg of NE was applied to subsequent immunoprecipitation (IP) assay. Anti-EGFR (SC-03, 1:1,000; Santa Cruz), anti-ATM (1:1,000; Bethyl Lab), anti-Lamin A (1:2,000; Santa Cruz), anti-tubulin (1:10,000; Sigma), anti-Flag (1:2,000; Sigma), anti-Myc (1:10,000; Roche), anti-phospho-EGFR Y1086 (1:2,000; Cell Signaling) and anti-phosphotyrosine (4G10, 1:3,000; Millipore) antibodies were used in western blotting. Antibodies against phospho-NBS1 S343 (1:2,000; Cell Signaling), NBS1 (1:40,000; Novus), Rad50 (1:10,000; GeneTex), Mrell (1:20,000; GeneTex), phospho-ATM S1981 (1:4,000; R&D system) and ORC2 (1:2,000; Cell Signaling) were used in detection of DNA damage response signaling. For immunoprecipitation,

5 μg of anti-phosphotyrosine antibody (4G10; Millipore), 2 μg of anti-EGFR antibody (Ab-13, Thermo Scientific) or 5 μg of anti-ATM antibody (Bethyl Lab) was used in 2 mg of nuclear extracts, respectively.

Chromatin-enriched fractionation

EGFR knockdown (shRNA EGFR #1 and #2) or vector control HeLa cells were treated with or without 10 Gy IR. After IR, cells were washed by ice-cold PBS twice, resuspended with buffer A (10 mM HEPES pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, and 1X protease inhibitor cocktail mixture (Roche)) and gentle rotated at 4℃ for 30 min. After centrifugation at 6,500 x *g* for 5 min, pellets were washed with 1mL buffer A (without Triton X-100) once, resuspend with 1 mL of buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and 1X protease inhibitor mixture), and gently rotated at 4°C for 30 min. After centrifugation at 6,500 x g for 5 min, pellets were washed with 1 mL of wash buffer Ⅰ (3 mM EDTA, 0.2 mM EGTA, 10 mM Tris-HCl, pH8.0, and 150 mM NaCl) and gently rotated at 4°C for 15 min. This step was repeated with wash buffer Ⅱ (3 mM EDTA, 0.2 mM EGTA, 10 mM Tris-HCl, pH8.0, and 250 mM NaCl). Cell pellets were lysed with TGN buffer, sonicated, and applied to centrifugation at 16,000 x *g* for 10 min at 4°C. Chromatin-enriched fraction was collected from the supernatant for subsequent Western blot analysis.

Immunofluorescence staining for irradiation-induced foci formation

For detection of p-ATM S1981 irradiation-induced foci formation (IRIF), EGFR-depleted HeLa cells were exposed to 2 Gy or 10 Gy IR and subjected to staining as previously described[8]. To detect whether ATM Y370F impaired p-ATMS1981 or p-CHK2 T68 IRIF, ATM-depleted HeLa

cells were transfected with pcDNA3 vector alone, Flag-tagged ATM wild type (WT) or Y370F by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Post transfection for 24 h, cell transfectants were seeded on cover slides for subsequent IRIF staining as previous procedure. For detection of p-CHK2 IRIF, U2OS or HeLa cells were washed with PBS three times, fixed and permeabilized as previously described[9] then subjected to staining. Antibodies against phospho-ATM S1981 (1:200; Rockland Immunochemicals), phospho-CHK2 T68 (1:200; Cell Signaling), Flag (1:200; Cell Signaling), and phospho-H2AX S139 (1:200; Cell Signaling) were used in detection of IRIF.

KillerRed system

KillerRed (KR; Supplementary information, Figure S2B) is a light-stimulated ROS-inducer fused to a tet-repressor (tetR-KR), whichbinds to a TRE cassette (~ 90 kb) integrated at a defined genomic locus in U2OS cells (U2OS TRE cell line)[1]. KR facilitates the formation of oxygen radicals and superoxide through the excited chromophore[3, 4] to induce DNA damage. By targeting the expression of KR to one specific genome site, we can visualize the recruitment of proteins at genetic loci. To activate KR, tetR-KR was exposed to 559 nm laser light for 50 scans (over a total of 10 s) at a power rate of 1 mW/scan (equal to 50 mW). At "the KR-TRE array" induced localized damage, we have detected γ-H2AX at the site of tetR-KR but not tetR-monomer cherry (tetR-mcherry) after laser light exposure[2] For bleaching KR, a 559 nm laser (1 mW/scan) in a selected area was used (FV1000 SIM Scanner set with 405 nm laser diode, Cat. F10OSIM405, Olympus). The dose that was delivered to the KillerRed spot was calculated based on the pixel size, the pixel size for irradiation is (0.138 um/pixel) and the dwell time per pixel is (8 us/pixel). The irradiation is at 1.0 mW (1.0 mJ/s). With a dwell time of 8 us/pixel, this

irradiates each pixel with 8.0 nJ/pixel/scan. Multiplying by the number of scans gives the total energy per pixel.

Neutral comet assay

Neutral comet assay was carried out to determine the levels of DSB in EGFR-depleted and control HeLa cells according to the manufacturer's instruction of Trevigen's Comet Assay Kit (4250-050-K, R&D systems). The comet tail movement was measured by using CometScore software (www.autocomet.com) among more than 100 randomly selected cells in each experiment.

Cell culture, transient transfection, and viral infection

HeLa cell, U2OS cell, and HEK 293T cell were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum. All cell lines are characterized as mycoplasma negative. For transient transfection, cells were transfected with indicated plasmids by either SN liposome[10] or Lipofectamine 2000 (Invitrogen). For lentiviral short hairpin RNA (shRNA) infection, HEK 293T cells were contransfected with vector control, two various EGFR-targeting or two different ATM-targeting shRNA with packing plasmids including deltaVPR8.2 and envelope plasmid VSVG using SN liposome or Lipofectamine 2000 according to the manufacturer's instructions. Virus-particles containing media was harvested to infect HeLa cell or U2OS cell after 48 h cotransfection. All the infected cells were maintained in media containing 0.5 μg/mL puromycin.

In vitro kinase assay

Equal amounts of purified GST, GST-tagged ATM2 or ATM2/Y370F protein were incubated with purified recombinant human EGFR kinase (#7706, Cell Signaling), 10 μCi [γ-^{32}P] ATP, 10 μM ATP in kinase assay buffer (5 mM $MgCl_2$, 5 mM $MnCl_2$, 50 μM Na_3VO_4, 50 mM HEPES, pH7.4, and 5 mM DTT) at 30°C for 30 min. The kinase reaction was stopped by addition of SDS samples buffer and boiling. The samples were analyzed by SDS-PAGE, transferred to PVDF membrane, and detected by autoradiography. To detect EGFR directly phosphorylated full-length Flag-tagged ATM, purified Flag-tagged ATM protein was incubated with or without immunoprecipitated Myc-tagged EGFR wild type (WT) or kinase dead (KD) mutant in the kinase assay buffer as previously mentioned without [γ-^{32}P] ATP. The resulting signal was detected by Western blotting with the indicated antibody.

Chromatin immunoprecipitation assay

To detect EGFR or ATM localized at DSB sites, DR-GFP integrated U2OS cells were transiently transfected with vector alone or I-*SceI* plasmid as described previously[8]. After transfection for 24 h, transfectants were harvested for nuclear fractionation and intact nuclear pellets were collected for crosslink with formaldehyde and subsequent ChIP assays by using EZ-ChIP kit (Upstate) in accordance with the manufacturer's instructions. Anti-EGFR (Thermo Scientific, Ab-13) and anti-pATM S1981 (Rockland Immunochemicals) antibodies were used for immunoprecipitation. PCR was carried out with primers flanking the I-*SceI* restriction enzyme cutting site shown in Supplementary information, Figure S2A.

Colony formation assay

HeLa cells were re-plated and exposed to various doses of ionizing radiation (IR) the next day. After 10-day culture, cells were fixed with Acetic acid:methanol (1:3) solution and colonies were stained with 0.5% Crystal violet. The number of colonies was quantitated and results were normalized to plating efficiency.

Supplementary References

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