Supplementary Materials and Methods

Chemicals, antibodies, and reagents

MG115, MG262, and cycloheximide were obtained from Sigma (St. Louis, MO). Antibodies specific for rpS3 (sc-376098), Tubulin (sc-5546), Lamin A/C (sc-6215), Ubiqutin (sc-9133), FLAG (sc-807), RNF138 (sc-133967), DDIT3 (sc-166682), and GADD34 (sc-8327), which were purchased from Santa Cruz Biotechology (Santa Cruz, CA), and HA (3724), which was purchased from Cell Signaling Technology (Beverly, MA), were all used for Western blotting, immunoprecipitation (IP), or immunofluorescence. Cell culture medium (RPMI-1640), FBS, glutamine, penicillin, and streptomycin were acquired from Gibco (Grand Island, NY). Scrambled siRNA, rpS3 siRNA1, and RNF138 siRNA1 were obtained from Dharmacon (Chicago, IL). RpS3 siRNA2 and RNF138 siRNA2 were obtained from Bioneer Co. (Daejeon, Republic of Korea).

Western blotting and Immunoprecipitation (IP)

Following the experimental treatments, Western blotting and immunoprecipitation studies were performed as previously described.[1] Whole cell lysate (WCL) was prepared using RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 25 mM NaF, 1 mM DTT, 20 mM EGTA, 1 mM Na_3VO_4, 0.3 mM PMSF, and 5 U/ml aprotinin) and protein concentration in the lysates was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). To prepare cytoplasmic extract (CE) and nuclear extract (NE), the cells were suspended in buffer A (10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 1 μg/ml Aprotinin, 5 μg/ml Leupeptin, and 1 μg/ml Pepstatin A) for 20 min on ice. Then, an equal volume of buffer B (buffer A + 0.1% NP-40) was added and the suspension was allowed to sit for 20 min on ice. Following centrifugation, the

supernatant CE was collected and subjected to centrifugation at 5000 *g* for 2 min to remove cellular debris. Nuclear pellet was washed two times with buffer A and resuspended using buffer C (10 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA). The NE was cleared of debris by centrifugation at 13,000 rpm for 15 min at 4°C. The protein samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, followed by blocking 5% skim milk in TBST (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. The membranes were then probed with specific primary antibodies and peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Proteins probing antibodies were visualized using an ECL detection system (Roche Applied Science, Indianapolis, IN). Densitometric analysis was performed by using Scion Image software (Scion Corporation, Frederick, MD). For IP studies, we prepared lysates for protein samples obtained from treatment of non-denaturing buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% NP-40, 50 mM NaF, 100 μM Na_3VO_4, 50 μM PMSF, 2 μg/ml Aprotinin, 1 μg/ml Leupeptin) for 30 min at 4°C, or treatment of denaturing buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 100 μM Na_3VO_4, 50 μM PMSF, 2 μg/ml Aprotinin, 1 μg/ml Leupeptin) for 5 min at 95°C (after heating, the suspension of denaturing lysates was diluted with non-denaturing buffer).[2] The lysates for protein samples were immunoprecipitated overnight with a specific primary antibody and protein A/G-agarose beads (Santa Cruz Biotechnology). After washing three times with a lysis buffer, immunoprecipitates were then boiled in 2×SDS sample buffer for 10 min, followed by centrifugation. They were detected by Western blot analysis using specific antibodies.

Cell viability assay

The effects of rpS3 knockdown on cell growth was measured by a cell viability assay using

thiazolyl blue tetrazolium bromide solution (Sigma, St. Louis, MO) as previously described.[3] The cells were cultured in 96-well plates (1,000 cells per well) and transfected with scrambled or rpS3-specific siRNAs for 24 h. After being exposed to radiation for 6 h, the cells were subjected to a cell viability assay. Media were removed and 0.05% thiazolyl blue tetrazolium bromide solution (Sigma) was added before the cells were incubated at 37°C for 2 h. The thiazolyl blue tetrazolium bromide solution was subsequently replaced with dimethyl sulfoxide and the plates were incubated for 10 min. After incubation, the solution was aliquoted into 96-well plates in duplicate, and absorbance was measured at 570 nm.

RNF138 gene knockout (KO)

U87MG cells were co-transfected with pooled CRISPR/Cas9 KO plasmids for knockout of *RNF138* gene and homology-directed DNA repair plasmids (Santa Cruz Biotechnology, Santa Cruz, CA), corresponding to the cut sites generated by the CRISPR/Cas9 KO plasmids that coded for a puromycin resistance cassette and red fluorescent protein. Two days after transfection, the stable knockout cells were selected using a previously optimized dose of puromycin dihydrochloride (Santa Cruz Biotechnology), and cultured for 2 weeks. The stable *RNF138* gene knockout was confirmed by real-time qRT-PCR and Western blotting.

Interactome analysis

For interactome analysis, the normal or Δ*RNF138* U87MG cells were transfected with HA-FLAG-rpS3 and harvested after irradiation. NE was then used for double immunoaffinity purification of rpS3-containing complexes, as previously described.[4] NE was incubated with anti-HA antibody-conjugated agarose beads (Sigma). After washing the beads three times with a wash buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 1mM EDTA, 1% NP-40, 1 mM PMSF, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml

pepstatin A), rpS3 and associated proteins were eluted in an elution buffer (Thermo Scientific, Rockford, IL) containing HA peptide (Sigma). The elution sample was incubated with anti-FLAG antibody-conjugated agarose beads (Sigma). After washing the beads three times with a wash buffer, rpS3 and associated proteins were eluted in an elution buffer (Thermo Scientific) containing FLAG peptide (Sigma). The final elution samples were subjected to SDS-PAGE and silver-stained using Pierce® Silver Stain for Mass Spectrometry (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions. Subsequently, the stained gel was analyzed by peptide mass fingerprint (PMF) including MALDI-TOF at Genomine Inc (Pohang, Republic of Korea).

Peptide mass fingerprint

For protein identification by PMF, protein spots were excised, digested with trypsin (Promega, Madison, WI), mixed with α cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA, and subjected to MALDI-TOF analysis (Microflex LRF 20, Bruker Daltonics, Breman, Germany), as described by Fernandez *et al*.[5] Spectra were collected from 300 shots per spectrum over an m/z range of 600–3000, which were calibrated by two point internal calibration using the trypsin auto-digestion peaks (m/z 842.5099, 2211.1046). A peak list was generated using Flex Analysis 3.0. The threshold used for the selection of peaks was 500 for minimum resolution of monoisotopic mass and 5 for S/N. The MASCOT search program developed by Matrixscience (http://www.matrixscience.com/) was used to identify proteins by PMF. The following parameters were used to search the database: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. The PMF acceptance criteria were based on probability scoring.

Luciferase reporter gene assay

A luciferase assay was performed as previously described.[6] Following the co-transfection with DDIT3 Luciferase reporter gene (DDIT3-Luc) and specific combination of plasmids as indicated, the normal or Δ*RNF138* U87MG cells were washed twice with cold PBS and lysed in a reporter lysis buffer (Promega, Madison, WI). After vortexing the lysates and centrifugation at 12,000 *g* for 1 min at 4°C, the cell extract and luciferase assay reagent (Promega) were mixed at room temperature, and placed in a luminometer (AutoLumat LB 953, EG & G Berthold, Bad Widbad, Germany) to measure the luciferase activity.

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed as previously described.[1] Following the overnight transfection with specific constructs and IR irradiation as indicated, the normal or Δ*RNF138* U87MG cells (5×10^8 cells) were cross-linked in 1% formaldehyde and quenched in 125 mM glycine followed by washing two times in chilled PBS. The cells were then lysed (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, and protease inhibitors) and sonicated with 30-s pulses and 30-s idle time. The sonicated extract was centrifuged for 10 min at maximum speed and diluted 5-fold in a dilution buffer (0.01% SDS, 1.1% Triton X-100, 1 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 200 mM NaCl). To reduce unspecific binding in the IP, the diluted lysates were precleared using protein A/G Plus-agarose and calf thymus DNA at 4°C for 1 h. IP was performed with anti-DDIT3 or anti-IgG. Immune-complexes were collected and washed in chilled low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 150 mM NaCl), high salt buffer (as before, containing 500 mM NaCl), and LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1), which were then washed two times in 10 mM Tris/5 mM EDTA. DNA was extracted from the beads twice using a 100 μL of elution buffer (1% SDS and 0.1 M NaHCO)

and supplemented with 0.25 M NaCl. Following an overnight incubation at 65°C for reversal of cross-links, the samples were incubated for an additional hour at 65°C with 10 μM EDTA, 40 μM Tris, pH 6.8, and 2 μg of proteinase K. DNA was purified using the QIAquick PCR purification kit for PCR. PCRs were performed with primers that encompass the *GADD34* promoter (Table S2).

References

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