Supporting Information

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SI Materials and Methods

Real-Time RT-PCR. HCT116, HeLa, and H1299 cells were either left untreated or treated with neocarzinostatin (300 ng/mL) and adriamycin (ADR; 10 μM) for different time points. RNA was extracted using TRIzol reagent (Invitrogen). Real-time RT-PCR was performed in triplicate on an MX3005P Thermal Cycler (Stratagene) using SYBR Green dye to measure amplification and ROX as a reference dye. The primer pairs used for the Ring Finger protein 144A (RNF144A) gene list are RNF144A-1F: 5′-CTG TTT GAT CCC TGT CGG ACT-3′; RNF144A-1R: 5′-GAT GGG CGC GTC ATC TTC TT-3′; RNF144A-2F: 5′-AAA CCG CAA TTA GCT GCC CA-3′; and RNF144A-2R: 5′-CGA CAG GGA TCA AAC AGC AC-3′. The primers were designed to specifically amplify RNF144A but not RNF144B. Both sets yielded similar results. The detailed PCR conditions and the primer sequences of GAPDH have been described (1). Transcript levels were normalized with GAPDH levels, which were analyzed in parallel with test genes. Results were analyzed with MxPro 4.1 Quantitative PCR software (Stratagene).

Plasmid and shRNA Construction. Mammalian expression pCMV-Tag2B vector was used for cloning of FLAG-tagged RNF144A and FLAG-tagged RNF144A truncated mutants. Briefly, human full-length RNF144A was amplified from a mixture of HeLa/H1299 cDNA pool using RT-PCR. PCR products were digested and cloned to pCMV-Tag2B in BamHI/XhoI sites directly. After sequence verification, the full-length RNF144A cDNA was used as the template for PCR cloning of the truncated mutants. The PCR primer sequences are listed below.

PCR primer names	PCR primer sequences
BamHI-RNF144A-1F	5′-ATTAGGATCCATGACCACAACAAGGTAC-3′
BamHI-RNF144A-173F	5′-ATTAGGATCCTTCAAAATGGAAGAAGAT-3′
BamHI-RNF144A-215F	5′-ATTAGGATCCCTGGAGTCTCTGGACGA-3′
XhoI-RNF144A-178R	5′-ATAACTCGAGCTAATCTTCTTCCATTTT-3′
XhoI-RNF144A-214R	5′-ATAACTCGAGGCAGTACCAGCAGAAGGCG-3′
XhoI-RNF144A-252R	5′-ATAACTCGAGTCAGCCCACAACCTGTGT-3′
XhoI-RNF144A-229R	5′-ATAACTCGAGCTACTTATCGTAGTGTAT-3′
XhoI-RNF144A-292R	5′-ATAACTCGAGCTAGGTGGGTAACGGG-3′
EcoRI-RNF144B-1F	5′-ATTAGAATTCATGGGCTCAGCTGGTAGG-3′
SalI-RNF144B-912R	5′-ATAAGTCGACTTAGGTTGTGGATGGGTCGT-3′

FLAG-tagged RNF144A point mutation mutants were directed using QuikChange II Site-Directed Mutagenesis Kits (Agilent Technologies). The primers for site-directed mutagenesis are C198A: 5′-GAG CGA GAC GAA GGC GCC GCG CAG ATG ATG TG-3′; C198A_antisense: 5′-CAC ATC ATC TGC GCG GCG CCT TCG TCT CGC TC-3′; C20A/C23A: 5′-CTC GAC CCG CTG GTG TCT GCC AAG CTC GCT CTT GGG GAG-3′; and C20A/C23A_antisense: 5′-CTC CCC AAG AGC GAG CTT GGC AGA CAC CAG CGG GTC GAG-3′. Human RNF144B was cloned into pCMV-Tag2B in EcoRI/SalI sites for expressing FLAG-tagged RNF144B. For live-image experiments, human RNF144A cDNA was subcloned into pEGFP-C1 or pmCherry-C1 in BamHI/XbaI sites. The sequences of all constructs were verified by sequencing in the BCM DNA sequencing core facility. GST-RNF144A and truncated mutants were cloned by moving the BamHI/XhoI-digested fragment of FLAG-RNF144A and corresponding mutants into pGEX-6p-1 vector. Three RNF144A hairpin shRNA constructs (V3LHS_383481, V2LHS_254611, and V2LHS_72643) were purchased from Open Biosystem. EGFP-rab7 WT plasmid was obtained from Addgene (plasmid 12605).

Reagents and Antibodies. All reagents were purchased from Sigma-Aldrich unless indicated otherwise. ADR (doxorubicin hydrochloride) was purchased from Calbiochem. The antibody specific to RNF144A was purchased from Abcam (ab75054 and ab89260) and Novus Biological (NBP1-01049). DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) Ab-4 antibody was purchased from NeoMarkers. Phospho–DNA-PKcs (pS2056), Ku80 (ab80592), and Artemis (ab35649) antibodies were purchased from Abcam. GAPDH (sc-47724), GST (sc-138), HA (sc-805), and EEA1 (sc-6414) antibodies were purchased from Santa Cruz Biotechnology. The mAbs for p53 (Ab1) and ataxia telangiectasia mutated (ATM) (Ab-3) were purchased from Calbiochem. Antibody against phospho-p53 (pS15; 9284), phospho-AKT (pS473), and Cellular Localization IF Antibody Sampler Kit (4753) were purchased from Cell Signaling Technology. The mAbs for p53 (DO1, PAb1801, PAb240, and PAb421) were gifts from Xinbin Chen (UC Davis Cancer Center, Davis, CA). The antibody for phospho-H2AX (pS139; JBW301) was purchased from Upstate Biotech Millipore. Purified mouse anti-human poly(ADP-ribose) polymerase (556494) antibody was purchased from BD Pharmingen.

Immunoprecipitation, Western Blot Analysis, and Immunofluorescence. For coimmunoprecipitation, the transfected cells were harvested 24–72 h later with TNN buffer (50 mM Tris, 0.25 M NaCl, 5 mM EDTA, 0.5% Nonidet P-40) supplemented with 1 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate, 20 nM microcystin, and a mixture of protease inhibitors (EDTA-free protease inhibitor mixture tablets, 11873580001; Roche Diagnostics). Cells prepared for endogenous immunoprecipitation were directly lysed in TNN buffer. An aliquot of the cell lysates was saved for protein input control, and immunoprecipitation was carried out using indicated antibodies overnight and resolved by 10% or 4–10% (vol/vol) gradient SDS/PAGE (Bio-Rad). For DNase/RNase treatment, TNN-lysed cell lysates were pretreated with 10 μL RNase-free DNase (Qiagen) plus 1 μL RNase-OUT (Invitrogen) for 1 h before immunoprecipitation. The specific signals were detected with appropriate antibodies. Cells prepared for direct Western blot analysis were lysed in SDS lysis buffer (1% SDS, 60 mM Tris, pH 6.8). Equal amounts of proteins were resolved by SDS/PAGE and then probed with indicated antibodies. For immunofluorescence, the cells were stained with a mouse anti–DNA-PKcs antibody (Ab-4; NeoMarkers), a rabbit or mouse FLAG antibody (F7245 and F3165; SIGMA), and a fluorescein isothiocyanate- or Texas Red X-conjugated secondary antibody. The nuclei were stained with Hoechst 33258. Images were captured on a Zeiss fluorescence microscope equipped with ApoTome (Axio Observer Inverted Microscope).

Cell Survival Assay. Cells were seeded into six-well plates at 10,000 cells per well and treated with or without ADR, neocarzinostatin, or mitomycin C for the indicated time. Then, the DNA damaging agents were removed by washing three times with fresh medium. After incubation for an additional 24 h, the medium was aspirated, and wells were rinsed one time with PBS. Cells were then trypsinized and counted with a Beckman Coulter Counter. Alternatively, cell viability was determined by trypan blue exclusion. All assays were performed in triplicate.

Caspase 3/7 Activity Assay. Cell were plated to six wells overnight and then treated with DNA damage agents with or without 1 h of pretreatment with 1 μM ATM inhibitor (KU55933; TOCRIS Bioscience) or 0.5 μM DNA-PK inhibitors (NU7441; TOCRIS Bioscience). Cells were then harvested for caspase 3/7 activity assay using the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's instruction. All assays were performed in triplicate.

Protein Purification. *Escherichia coli* strain DH5α transformed with pGEX-6P vector, pGEX-6P-RNF144A (amino acids 1–229), or pGEX-6P-RNF144A (amino acids 1–292) was cultured in LB medium containing Ampicillin at 37 °C to an A_{600} value of 0.6. GST fusion proteins were induced by 0.5 mM isopropyl-D-thiogalactopyranoside at 37 °C for 3 h. Cells were then lysed in sodium chloride-Tris·EDTA buffer (100 mM NaCl, 1 mM EDTA) supplemented with lysozyme and protease inhibitors. After 15 min of treatment, the lysate was sonicated for 30 s and then purified using Glutathione Sepharose 4B (GE Healthcare). Sepharose beads were washed four times with sodium chloride-Tris-EDTA buffer. The GST proteins were eluted out by reduced glutathione if needed. For purification of DNA-PKcs protein, HEK293T cellular extracts were prepared by lysing the cells with TNN buffer supplemented with 1 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate, 20 nM microcystin, and a mixture of protease inhibitors, including leupeptin (10 μg/mL), aprotinin (10 μg/mL), pepstatin (10 μg/mL), PMSF (1 mM), antipain (2 μg/mL), and chymostatin (1 μg/mL). DNA-PKcs was isolated on anti–DNA-PKcs antibody (Ab-4; NeoMarkers) and protein G agarose (Pierce). The beads were washed by TNN buffer five times.

Subcellular Fractionation Assay. HEK293T cells transiently expressed FLAG-tagged RNF144A for 24 h and then were harvested for subcellular fractionation in hypotonic solution (20 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 mM DTT, 0.1% Nonidet P-40). The nuclei were spun down in a microfuge at 0.5 × *g* for 5 min two times. Supernatant includes cytosol, subcellular organelles, and plasma membrane. The enriched nuclei were dissolved in TNN lysis buffer. The total cell lysates, nuclear extract, and supernatant were subjected to immunoprecipitation using FLAG M2 agarose beads overnight followed by immunoblotting. The blot was then probed with an anti-GAPDH antibody and an anti-Histone 3 antibody as cytosolic and nuclear markers, respectively.

Colony Formation Assay. To determine long-term viability, 500–1,000 cells from each cell line were plated in each 10-cm diameter dish overnight in triplicate experiments. The cells were then either mock-treated or treated with indicated concentrations of ADR for 3 h; then, they were released by three times by 1× PBS washing. Cells were grown for 10–14 d to form colonies and then stained with 5% crystal violet (0.5 g crystal violet, 25 mL methanol, and 75 mL H_2O). Cell number was counted.

Statistical Analysis. All experiments were performed at least three times. Data are presented as mean ± SD and represent three independent experiments. Student *t* test was used for comparing group means, and *P* values < 0.05 were considered significant.

1. Liu K, et al. (2009) Regulation of p53 by TopBP1: A potential mechanism for p53 inactivation in cancer. *Mol Cell Biol* 29(10):2673–2693.

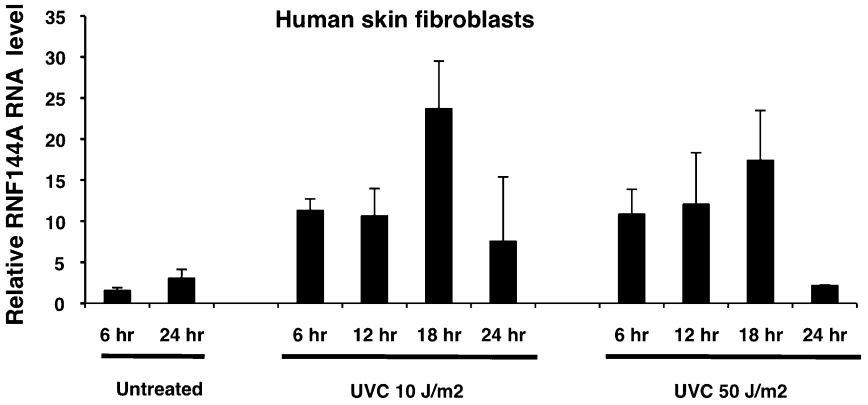

Fig. S1. DNA damage induces RNF144A expression. UV radiation induced RNF144A mRNA in normal human skin fibroblasts (WS1, CRL-1502; ATCC). Data were accessed from Gene Expression Omnibus accession no. GDS400 (1). UVC, ultraviolet electromagnetic radiation subtype C.

1. Gentile M, Latonen L, Laiho M (2003) Cell cycle arrest and apoptosis provoked by UV radiation-induced DNA damage are transcriptionally highly divergent responses. *Nucleic Acids Res* 31(16):4779–4790.

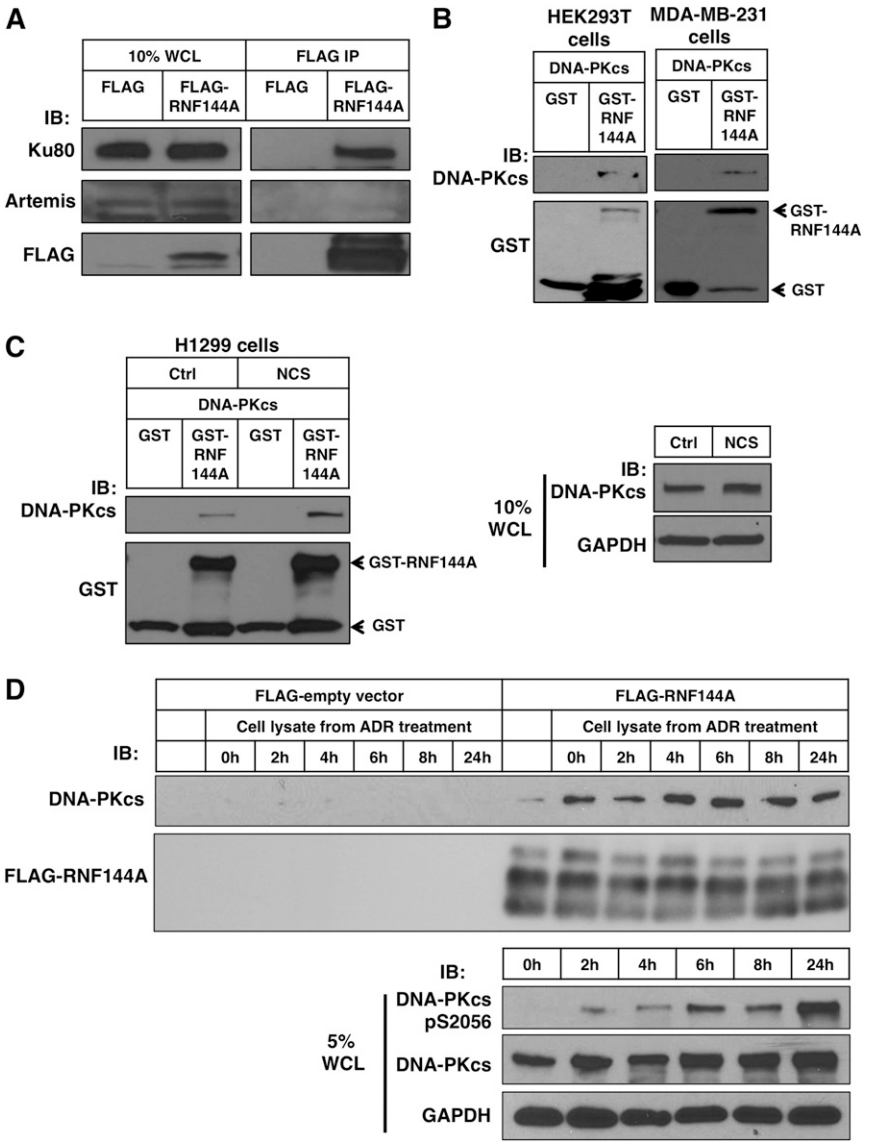

Fig. S2. RNF144A interacts with DNA-PKcs. (*A*) Western blot shows that FLAG-RNF144A interacted with Ku80 under MG132 treatment. (*B* and *C*) GST pull-down experiment shows that RNF144A directly bound to DNA-PKcs. Cell lysates from (*B*, *Left*) ADR-treated HEK293T and (*B*, *Right*) MDA-MB-231 cells and (*C*) neocarzinostatin (NCS) -treated or untreated H1299 cells were incubated with purified GST or GST-RNF144A protein followed by Glutathione Sepharose pull-down and SDS/PAGE and Western blot analyses. (*D*) HEK293T cells were treated with ADR for 2–24 h, and the cellular lysates were incubated with the same amount of FLAG-RNF144A (which was immunoprecipitated by anti-FLAG beads from other HEK293T cells transfected with an empty vector control plasmid or FLAG-RNF144A). The weak DNA-PKcs signal in FLAG-RNF144A alone immunoprecipitation is coming from coimmunoprecipitated DNA-PKcs in FLAG-RNF144A–transfected cells. On FLAG-RNF144A immunoblot, the middle major bands represent FLAG-RNF144A. The bands above and below the major bands are occasionally seen in FLAG-RNF144A immunoprecipitation/FLAG IB and FLAG-RNF144A immunoprecipitation/anti-RNF144A IB, and represent modified species of RNF144A. Anti-pS2056–DNA-PKcs immunoblot is performed to ensure the DNA damage response to ADR treatment in this experiment. Ctrl, control; IB, immunoblot; WCL, whole cell lysates.

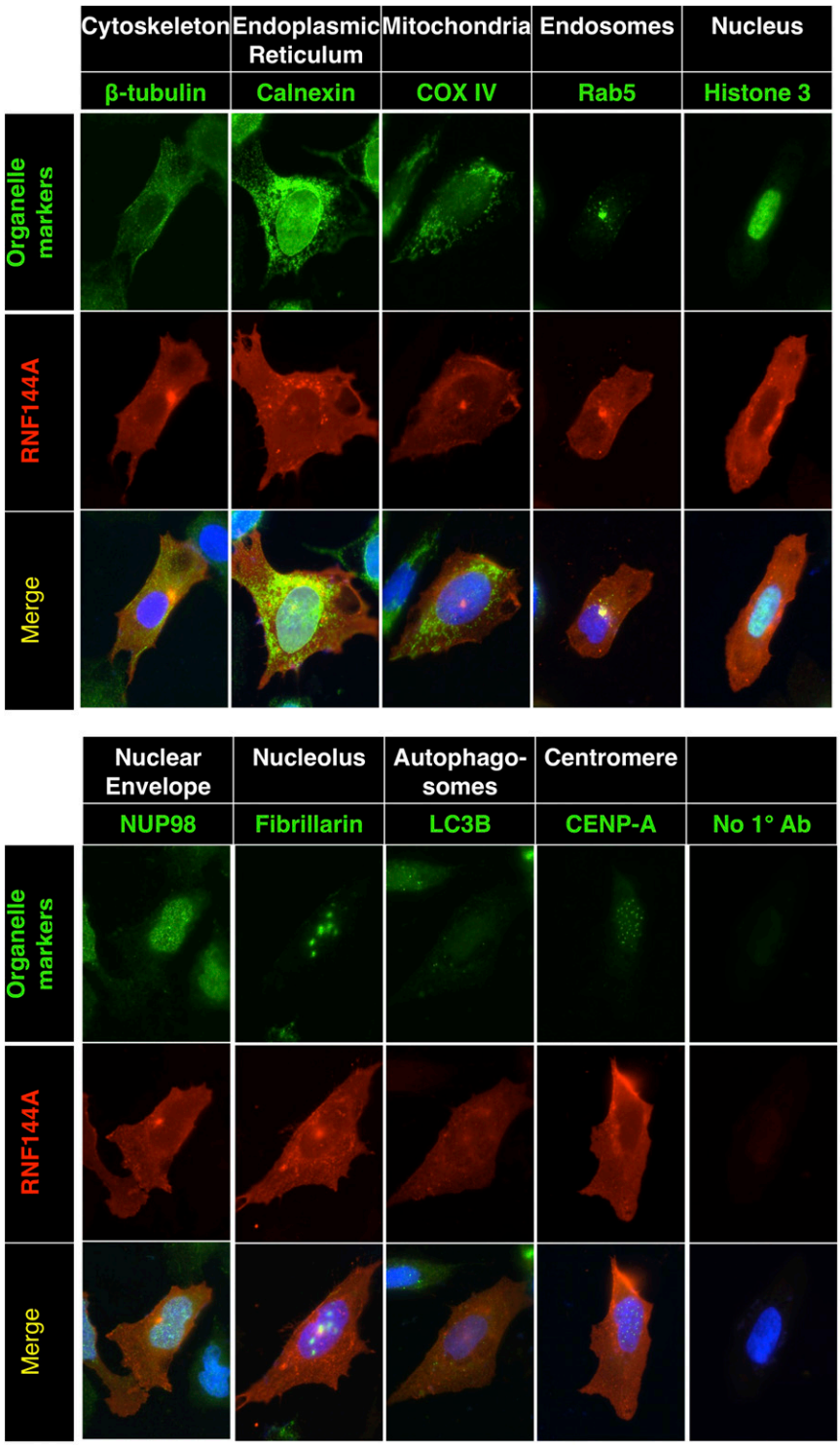

Fig. S3. RNF144A mainly localizes in the plasma membrane and endosomes. FLAG-tagged RNF144A was transfected into U2OS cells. On the next day, cells were fixed and stained with anti-FLAG antibody (red) and the indicated subcellular organelle markers (green). The immunofluorescence images (100×) show that RNF144A located in the cytoplasmic vesicles (endoplasmic reticulum and endosome) and plasma membrane.

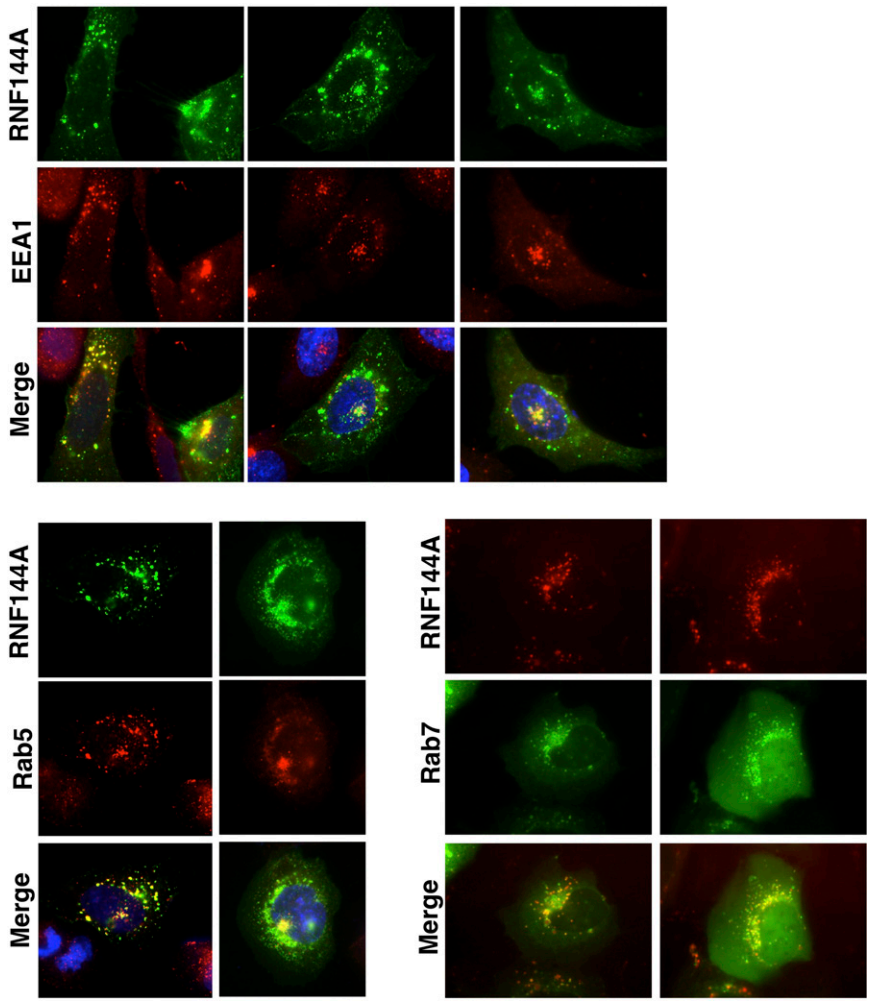

Fig. S4. Expanded image data for Fig. 5*B*. RNF144A colocalization with different endosomal markers: EEA1 and Rab5 (early endosome) and Rab7 (late endosome). Shown are several representative images from each experiment performed in U2OS as described in Fig. 5*B*.

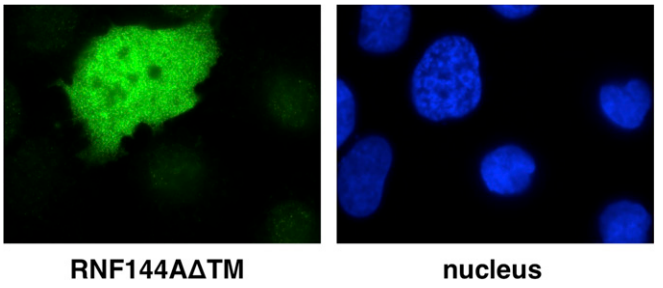

Fig. S5. Homogeneous distribution of RNF144AΔ transmembrane (TM) in the cells. Deletion of TM domain (amino acids 250–270) caused a redistribution of RNF144A to a homogenous pattern throughout the cells. FLAG-tagged RNF144AΔTM was transfected into U2OS cells. On the next day, cells were fixed and stained with anti-FLAG antibody (green).

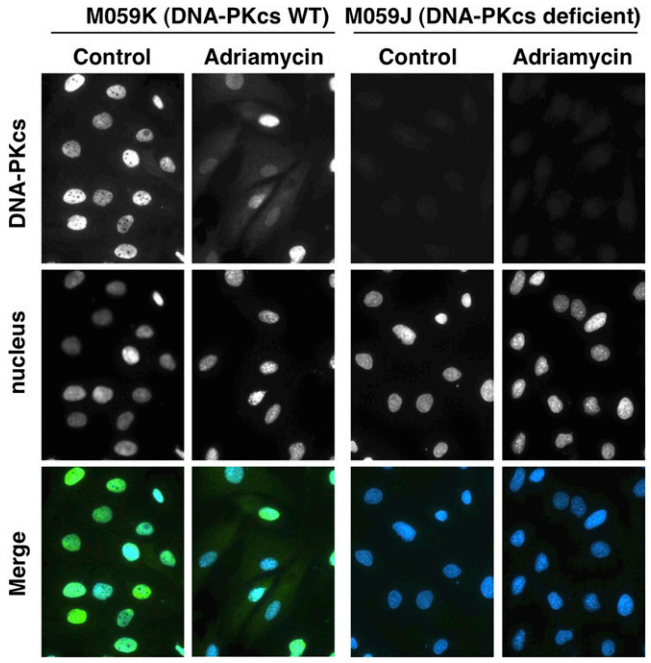

Fig. S6. ADR-induced cytosolic localization of DNA-PKcs. M059K (DNA-PKcs–proficient) and M059J (DNA-PKcs–deficient; as a control for antibody specificity) cells were treated with ADR (5 μM) for 9 h and then fixed and stained with anti–DNA-PKcs (green) and Hoechst 33258 (blue). Black and white images are shown for better resolution.

Table S1. MS identified 66 peptide sequences matched to DNA-PKcs from RNF144A interacting proteins (related to Fig. 3*A*)

DNA-dependent protein kinase catalytic subunit (*Homo sapiens*)

Query	Observed	M_r(expt)	M_r(calc)	Δ	Miss	Score	Expect	Peptide
2,534	469.22	936.42	936.11	0.31	0	44	0.096	R.LSFAVPFR.E
2,546	473.79	945.56	945.22	0.34	0	34	1.1	K. MAVLALLAK.I
2,556	479.15	956.29	956.14	0.16	0	55	0.0075	R. LLEEALLR.L
2,623	506.17	1,010.32	1,009.20	1.12	0	30	2	R.EIFNFVLK.A
2,678	527.19	1,052.36	1,051.15	1.21	0	40	0.24	R.SIGEYDVLR.G
2,694	534.19	1,066.36	1,068.27	1.90	1	39	0.32	K. FYGELALKK.K
2,749	552.41	1,102.80	1,103.18	−0.38	0	87	4.8e-006	R.LQETLSAADR.C (Query 2748)
2,774	563.38	1,124.75	1,125.30	0.55	1	62	0.0014	K. SKDFVQVMR.H (Query 2775)
2,779	568.62	1,135.22	1,135.27	0.05	0	55	0.0085	R.HGDLPDIQIK.H
2,783	575.62	1,149.22	1,149.34	0.11	0	79	2.8e-005	K. AALSALESFLK.Q
2,908	627.86	1,253.71	1,254.50	0.78	0	42	0.14	R.AYVPALQMAFK.L
2,996	651.26	1,300.51	1,300.46	0.05	0	63	0.0012	K.QITQSALLAEAR.S
3,096	664.03	1,326.05	1,326.50	−0.45	1	45	0.084	K. QGNLSSQVPLKR.L
3,098	664.25	1,326.48	1,325.47	1.02	1	43	0.14	R.FNNYVDCMKK. F
3,212	696.85	1,391.69	1,390.45	1.24	0	62	0.0014	R.NELEIPGQYDGR.G
3,218	699.14	1,396.27	1,396.50	−0.23	0	63	0.0012	K. LNESTFDTQITK.K
3,293	722.63	1,443.25	1,444.71	1.46	0	74	0.0001	K.NLLIFENLIDLK.R
3,327	731.05	1,460.09	1,459.71	0.38	0	65	0.00081	R.VVQMLGSLGGQINK.N
3,348	734.75	1,467.49	1,467.54	0.04	0	49	0.029	R.SLGPPQGEEDSVPR.D
3,364	738.17	1,474.32	1,473.69	0.63	0	56	0.0059	R.VEGMTELYFQLK.S
3,492	508.21	1,521.61	1,520.73	0.88	0	49	0.029	K. HSSLITPLQAVAQR.D
3,539	773.06	1,544.11	1,543.74	0.37	0	70	0.00023	K. DVDFMYVELIQR.C
3,571	787.35	1,572.68	1,572.72	0.04	1	83	1.1e-005	R.NVDSNNKELSIAIR.G
3,692	812.62	1,623.22	1,624.81	1.59	0	45	0.08	R.YNFPVEVEVPMER.K
3,716	817.18	1,632.34	1,631.69	0.65	0	80	2.4e-005	R.AQEPESGLSEETQVK.C
3,748	828.18	1,654.34	1,652.91	1.44	0	66	0.00061	K. LSDFNDITNMLLLK.M
3,783	840.87	1,679.72	1,678.93	0.80	0	70	0.00023	K. LVINTEEVFRPYAK.H
3,949	884.09	1,766.16	1,765.96	0.20	0	53	0.012	K. MDPMNIWDDIITNR.C
3,950	885.67	1,769.32	1,768.92	0.41	0	77	4.9e-005	K. TVSLLDENNVSSYLSK.N
4,006	908.33	1,814.64	1,815.07	0.43	0	54	0.0092	R.TVGALQVLGTEAQSSLLK. A (Query 4005)
4,099	953.20	1,904.38	1,904.16	0.22	0	87	4.2e-006	K. LLLQGEADQSLLTFIDK. A (Query 4092)
4,115	959.75	1,917.49	1,917.99	−0.49	0	68	0.00037	R.ATQQQHDFTLTQTADGR.S (Query 4116)
4,203	656.61	1,966.80	1,966.26	0.54	1	37	0.43	K.NKLQYFMEQFYGIIR.N
4,240	993.21	1,984.40	1,984.23	0.17	0	92	1.5e-006	R.IMEFTTTLLNTSPEGWK.L
4,282	1,003.33	2,004.65	2,006.24	1.58	0	95	7.5e-007	K. NLDLAVLELMQSSVDNTK.M (Query 4301)
4,415	703.74	2,108.21	2,108.22	0.01	1	49	0.026	K. TSALSDETKNNWEVSALSR.A
4,424	1,060.49	2,118.97	2,119.46	0.49	0	127	4.7e-010	K. DVLIQGLIDENPGLQLIIR.N (Query 4423, 4425)
4,459	720.77	2,159.30	2,158.50	0.80	1	42	0.14	K. HSSLITPLQAVAQRDPIIAK.Q
4,478	1,091.70	2,181.39	2,181.48	0.09	0	81	1.5e-005	K. LQSVQALTEIQEFISFISK.Q
4,502	731.92	2,192.74	2,193.56	0.82	1	85	7.5e-006	R. LLQIIERYPEETLSLMTK.E 4511
4,719	1,156.26	2,310.50	2,311.57	1.06	0	91	1.7e-006	R.STVLTPMFVETQASQGTLQTR.T
4,783	1,191.79	2,381.56	2,381.59	0.03	1	110	2e-008	R. ILELSGSSSEDSEKVIAGLYQR.A (Query 4782, 4784)
4,886	835.72	2,504.15	2,504.70	0.55	0	33	1.1	K. EEENASVIDSAELQAYPALVVEK.M
4,901	848.64	2,542.89	2,542.81	0.08	1	78	3.2e-005	R.SDPGLLTNTMDVFVKEPSFDWK.N
4,994	878.43	2,632.26	2,632.87	0.61	1	37	0.42	R.KEEENASVIDSAELQAYPALVVEK.M
5,040	916.39	2,746.14	2,747.06	0.92	0	77	3.7e-005	K. AILELHYSQELSLLYLLQDDVDR.A
5,066	939.46	2,815.37	2,815.20	0.17	1	70	0.00019	K. LLLQGEADQSLLTFIDKAMHGELQK. A
5,082	959.19	2,874.54	2,874.23	0.31	0	100	2.1e-007	K. VYELLGLLGEVHPSEMINNAENLFR. A
5,146	1,008.24	3,021.69	3,021.32	0.37	0	98	2.8e-007	K. YYIQNGIQSFMQNYSSIDVLLHQSR. L (Query 5147)
5,160	1,049.26	3,144.75	3,143.55	1.20	0	92	1.1e-006	R.AGLLHNILPSQSTDLHHSVGTELLSLVYK.G (Query 5159)
5,165	1,060.49	3,178.45	3,177.57	0.88	1	72	0.0001	K.DVLIOGLIDENPGLQLIIRNFWSHETR. L (Query 5164)
5,171	1,079.49	3,235.45	3,235.42	0.03	0	89	2.2e-006	R.SSFDWLTGSSTDPLVDHTSPSSDSLLFAHK. R

Mass spectrometry data were processed using Mascot Daemon software. All 66 peptides identified were ranked one and matched to human DNA-PKcs (gi: 13606056, mass: 465559) with score of 3156 and emPAI of 0.54. emPAI, the Exponentially Modified Protein Abundance Index represents relative quantitation of the proteins in a mixture based on protein coverage by the peptide matches in a database search result.