Supplemental Figures

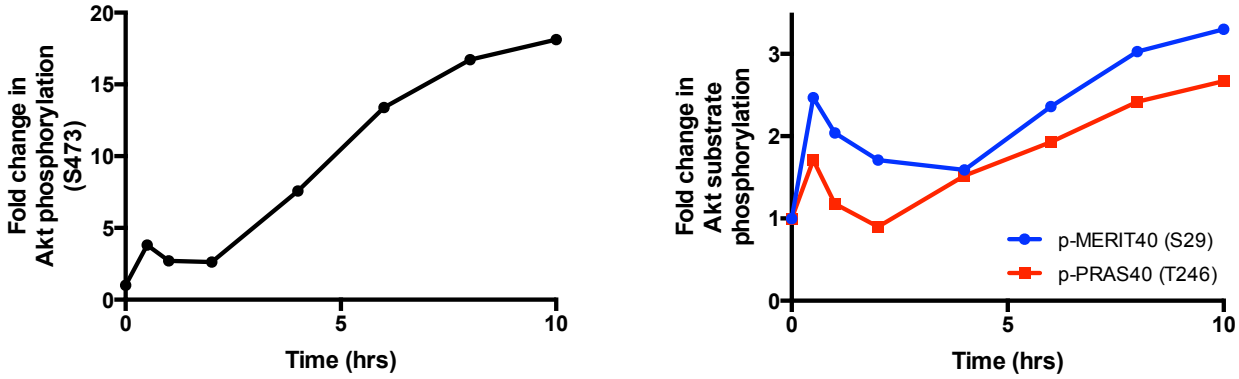

Fig. S1, related to Fig. 2. Effect of doxorubicin on Akt activity and Akt substrate phosphorylation.
MCF10A cells were serum-starved and exposed to 2 μM doxorubicin over a 10 hour time course. Levels of **(A)** Akt Ser473 phosphorylation and **(B)** MERIT40 Ser29 or PRAS40 Thr246 phosphorylation were quantified using ImageJ software (NIH).

Supplemental Figures

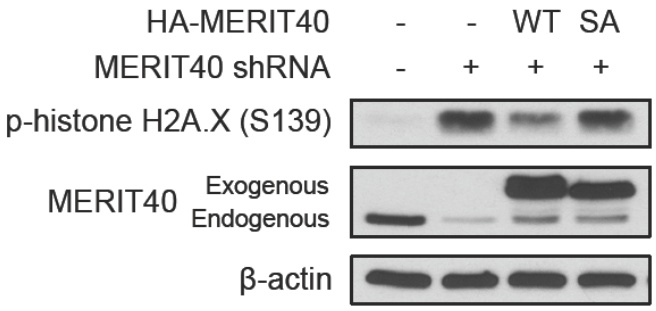

Fig. S2, related to Fig. 4. Contribution of MERIT40 phosphorylation toward the resolution of spontaneous DNA damage in MCF10A cells.
MCF10A cells were infected with empty vector or a MERIT40 shRNA construct and empty vector, wild-type MERIT40 or MERIT40 S29A mutant expression constructs. Histone H2A.X Ser139 phosphorylation was monitored under full serum conditions.

Supplemental Experimental Procedures

Cell culture

MCF7 and HEK293T cells were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Cellgro) supplemented with 10% FBS (Gibco). T47D cells obtained from ATCC were grown in RPMI-1640 medium (Cellgro) supplemented with 10% FBS and 10 µg/mL insulin (Gibco). SUM-159-PT cells were cultured in Ham's F12 medium (Cellgro) containing 5% FBS, 1 µg/mL hydrocortisone (Sigma-Aldrich) and 5 µg/mL insulin. MCF10A cells were obtained from ATCC and maintained in DMEM/Ham's F12 medium supplemented with 5% equine serum (Cellgro), 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, 20 ng/mL EGF (R&D Systems), and 100 ng/mL cholera toxin (List Biological Laboratories). All cell lines were authenticated using short tandem repeat (STR) profiling. Cells were maintained in culture for no longer than 4 months and were routinely assayed for mycoplasma contamination.

Doxorubicin, growth factors and inhibitors

Doxorubicin (Cell Signaling Technology), BKM120 (Active Biochem), MK2206 (Active Biochem), Nu7441 (Selleck Chemicals), gefitinib (Selleck Chemicals), rapamycin (Cayman Chemicals) and Torin1 (Selleck Chemicals) were reconstituted in DMSO (Sigma-Aldrich). Cells were stimulated with recombinant human IGF-1 (R&D Systems) at a final concentration of 100 ng/mL.

Antibodies

Anti-phospho-Akt S473, anti-phospho-Akt T308, anti-Akt, anti-phospho-PRAS40 T246, anti-PRAS40, anti-phospho-H2A.X S139, anti-PARP, anti-actin, anti-phospho-MERIT40 S29, anti-MERIT40, anti-phospho S6 S235/236, anti-S6, anti-p110α, anti-HA, anti-Akt1, anti-Akt2, anti-Akt3 and anti-myc antibodies were obtained from Cell Signaling Technology. Anti-TNKS 1/2 and anti-BRCA1 antibody was from Santa Cruz Biotechnology. Anti-Rap80, anti-Abraxas, anti-BRE and anti-BRCC36 antibodies were purchased from Bethyl Laboratories. The Alexa Fluor® 647-conjugated anti-phospho H2A.X S139 antibody used for immunofluorescence was purchased from BD Biosciences. The anti-Rap80 antibody used for immunofluorescence was purchased from Abcam. The Alexa Fluor® 647-conjugated anti-rabbit antibody was purchased from Jackson ImmunoResearch Laboratories. Horseradish peroxidase conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Chemicon.

Plasmids

An MSCV-HA-Flag-MERIT40 construct was obtained from Stephen Elledge (Wang et al., 2009). A phosphorylation site Ser29Ala mutant was generated by site-directed mutagenesis using the primer 5´-GCC CCG CAC TCG CGC CAA TCC TGA AGG-3´. JP1520-GFP, JP1520-PIK3CA-WT-HA, JP1520-PIK3CA-H1047R-HA and JP1520-PIK3CA-E545K-HA plasmids were from Joan Brugge (Addgene plasmids 14570, 14571, 15572). To produce retroviral supernatants, 293T cells were cotransfected with MSCV or JP1520 vectors, R-VSVG and pUMVC for 48 hours. A pLP-dmyc-TNKS2-WT construct was obtained from Frank Sicheri (Guettler et al., 2011). pcDNA3-myr-HA-

Akt1, pcDNA3-myr-HA-Akt2 and pcDNA3-myr-HA-Akt3 constructs were from William Sellers (Addgene plasmids 9008, 9016, 9017). Cells were transfected with myr-Akt constructs using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's protocol.

RNA interference

For shRNA silencing of MERIT40 a single-stranded oligonucleotide encoding MERIT40 target shRNA, and its complement, were synthesized: MERIT40 sense, 5´-CCG GAG GAA GTC CTT GGC CTA AAG CCT CGA GGC TTT AGG CCA AGG ACT TCC TTT TTT G-3´; MERIT40 antisense, 5´-AAT TCA AAA AAG GAA GTC CTT GGC CTA AAG CCT CGA GGC TTT AGG CCA AGG ACT TCC T-3´. For shRNA silencing of Akt isoforms single-stranded oligonucleotides encoding Akt1/2/3 target shRNAs, and their complements, were synthesized: Akt1 sense, 5´-CCG GGA GTT TGA GTA CCT GAA GCT GCT CGA GCA GCT TCA GGT ACT CAA ACT CTT TTT G-3´; Akt 1 antisense, 5´-AAT TCA AAA AGA GTT TGA GTA CCT GAA GCT GCT CGA GCA GCT TCA GGT ACT CAA ACT C-3´; Akt2 sense, 5´-CCG GGC GTG GTG AAT ACA TCA AGA CCT CGA GGT CTT GAT GTA TTC ACC ACG CTT TTT G-3´; Akt2 antisense, 5´-AAT TCA AAA AGC GTG GTG AAT ACA TCA AGA CCT CGA GGT CTT GAT GTA TTC ACC ACG C-3´; Akt3 sense, 5´-CCG GCT GCC TTG GAC TAT CTA CAT TCT CGA GAA TGT AGA TAG TCC AAG GCA GTT TTT G-3´; Akt3 antisense, 5´-AAT TCA AAA ACT GCC TTG GAC TAT CTA CAT TCT CGA GAA TGT AGA TAG TCC AAG GCA G-3´. The oligonucleotide sense and antisense pair was annealed and inserted into the pLKO.1 backbone. To produce lentiviral supernatants, 293T cells were cotransfected

with control or shRNA-containing pLKO.1 vectors, VSVG and psPAX2 for 48 hours. For siRNA-mediated knockdown of MERIT40, siGENOME BABAM1 siRNA oligos (D-020702-17) were purchased from Dharmacon. Cells were transfected with BABAM1 or control siRNA (Dharmacon) using DharmaFECT 1 transfection reagent (Dharmacon) according to the manufacturer's protocol.

In vitro kinase assay

MCF10A cells expressing MSCV-HA-Flag-MERIT40 or MSCV-HA-Flag-MERIT40 Ser29Ala were serum starved for 16 hours. MERIT40 was immunoprecipitated from cell extracts and incubated with 500 ng of recombinant Akt1, Akt2 or Akt3 (Sigma-Aldrich) in the presence of 250 μmol/L cold ATP in a kinase buffer for 1 hour at 30°C. The kinase reaction was terminated by addition of SDS-PAGE sample buffer.

Immunoblotting

Cells were washed with PBS and lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, protease inhibitor cocktail, 50 nmol/L calyculin A, 1 mmol/L sodium pyrophosphate, and 20 mmol/L sodium fluoride). Lysates were resolved by SDS-PAGE and transferred electrophorectically to nitrocellulose membrane (Bio-Rad) followed by immunoblotting. Membranes were developed using enhanced chemiluminescence substrate (EMD Millipore).

Immunoprecipitation

To prepare nuclear extracts, cells were collected by scraping and rinsed by centrifugation with cold PBS. The pellet was resuspended in hypotonic buffer (10mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, protease inhibitor cocktail, 50 nmol/L calyculin A, 1 mmol/L sodium pyrophosphate, and 20 mmol/L sodium fluoride) for 10 min at 4°C. Nuclei were collected by centrifugation (10 min at 4,000 rpm) and resuspended in hypertonic buffer (20mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.6% NP-40, protease inhibitor cocktail, 50 nmol/L calyculin A, 1 mmol/L sodium pyrophosphate, and 20 mmol/L sodium fluoride). After rotation for 15 min at 4°C the supernatant was collected by centrifugation (5 min at 10,000 rpm).

For whole cell lysate preparation, cells were washed with PBS and lysed in EBC lysis buffer (0.5% NP-40, 50 mmol/L Tris, pH 7.4, 120 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, protease inhibitor cocktail, 50 nmol/L calyculin A, 1 mmol/L sodium pyrophosphate, and 20 mmol/L sodium fluoride). Cell extracts were passed 5 times through a 23G needle prior to centrifugation.

Nuclear lysates or whole cell lysates were incubated with 1-2 µg of antibody overnight at 4°C followed by incubation with protein A/G Sepharose beads (Amersham Biosciences). Immune complexes were washed with NETN buffer (0.5% NP-40, 20 mmol/L Tris, pH 8, 100 mmol/L NaCl, 1 mmol/L EDTA) and eluted by incubation for 5 minutes at 95°C in SDS-PAGE sample buffer (62.5 mmol/L Tris, pH 6.8, 10% glycerol, 2% SDS, 5% beta-mercaptoethanol and bromophenol blue). Eluates were resolved by SDS-PAGE.

Sulforhodamine B assay

Cell viability was monitored using the Sulforhodamine B (SRB) assay. Adherent cells were fixed by addition of 12.5% (w/v) trichloroacetic acid and incubation at 4°C for 1 hour. Wells were washed with water and cells were stained by addition of SRB solution (0.5% (w/v) SRB, 1% acetic acid). Wells were washed twice with 1% acetic acid and allowed to dry at room temperature. SRB was solubilized with 10 mmol/L Tris, pH 10.5 and absorbance at 510 nm was measured.

Immunofluorescence

Cells plated on coverslips were fixed with 2% paraformaldehyde for 10 minutes, permeabilized with 0.5% Triton X-100, and blocked with 1% BSA in 20 mmol/L Tris-HCl, pH 7.5, for 20 minutes. Coverslips were then incubated with Alexa Fluor® 647-conjugated anti-phospho H2A.X S139 antibody (1:100) for 3 hours or anti-Rap80 antibody (1:100) for 2 hours followed by Alexa Fluor® 647-conjugated anti-rabbit antibody (1:50) for 1 hour. After washing twice with PBS, coverslips were mounted with Prolong Gold antifade reagent containing DAPI (Life Technologies). Images of cells were acquired using a fluorescence microscope (Nikon Eclipse Ti) and digital image analysis software (NIS-Elements, Nikon).

Tissue microarrays and image analysis of immunohistochemistry

To construct a tissue microarray, slides and formalin fixed paraffin embedded blocks were selected from breast tissue specimens from the archives of the Department of Pathology at the Beth Israel Deaconess Medical Center, as previously described (Elloul

et al., 2014). Briefly, two tissue microarrays were constructed using a computer controlled machine (TMA-Master, 3D-Histech LTD) from 50 cases and then four core samples (core size=1 mm) were obtained per each case consisting of invasive tumor in center of tissue sample, invasive tumor in periphery of tissue sample, normal terminal duct lobular units and stroma. Two 5-μm paraffin sections were cut from each of the TMA blocks and immunohistochemistry staining was done for total MERIT40 and p-MERIT40 Ser29. Computational image analysis of protein expression was performed using Definiens TissueStudio 3.6.1 (Munich, Germany) to yield the intensity of nuclear expression of p-MERIT40 Ser 29 and MERIT 40 in the cancer epithelium. The proportion of positively staining epithelial nuclei was recorded for each core and reported as the Nuclear Positive Index.