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Supplemental Information

Persistent Activation of NF-κB in BRCA1-Deficient Mammary Progenitors Drives Aberrant Proliferation and Accumulation of DNA Damage

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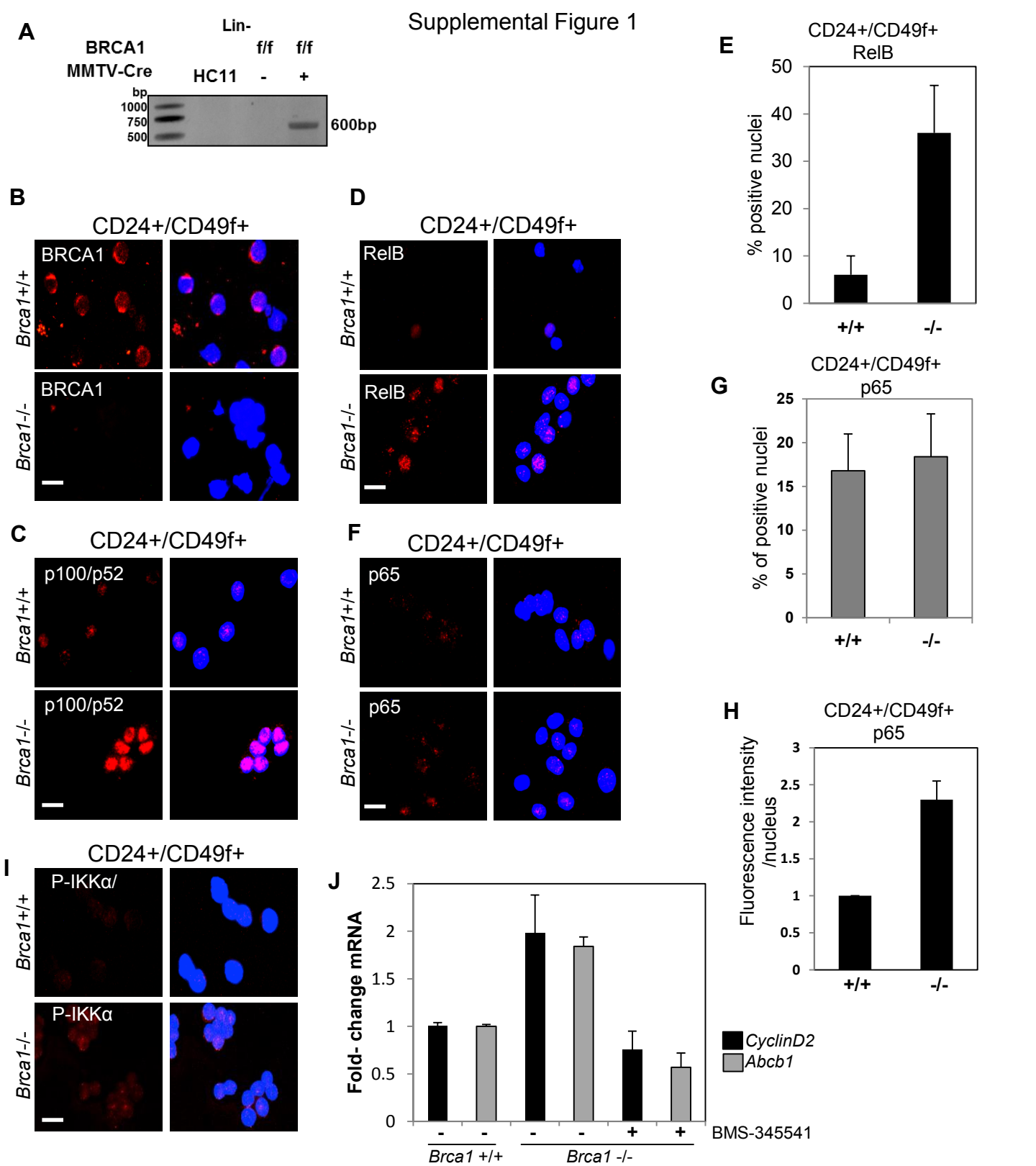
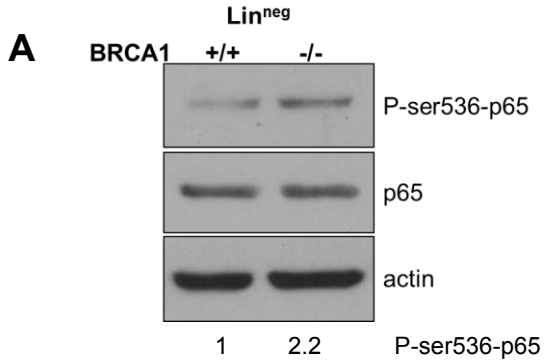
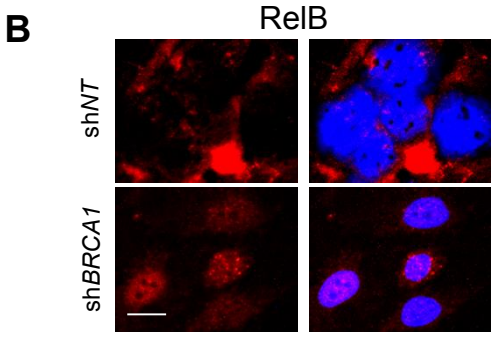
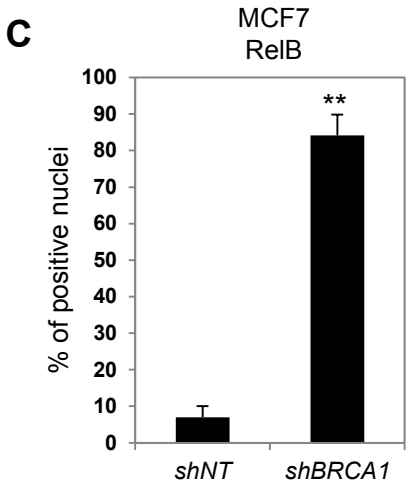
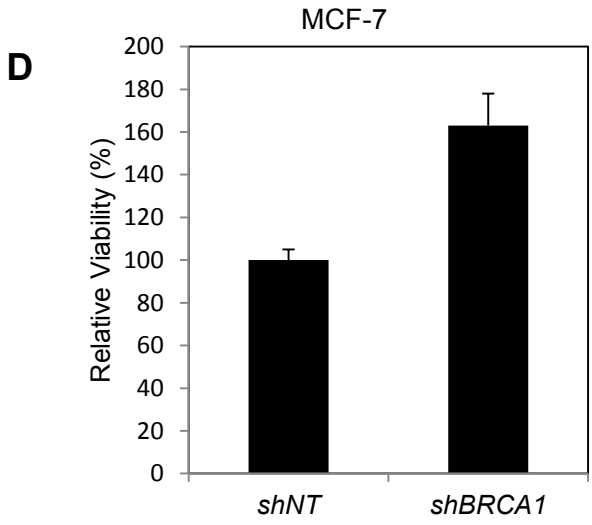


Figure S1 (Related to Figure 1): ***Verification of mammary Brca1f/f recombination and NF-kB activation in mouse mammary progenitor cells.*** **(A)** DNA was isolated from Lin- cells and PCR performed to confirm BRCA1 knock-out. Cre-mediated excision of exons 5-13 was detected using primers for *Brca1* INT4 fwd and *Brca1* INT13 rev to yield a 600 bp product. HC11 cells were used as negative control. CD24+/CD49f+ *Brca1*+/+ and -/- mouse MECs were FACS-sorted, and immunostained with antibodies against **(B)** BRCA1 (n=2), **(C)** p100/p52 (n=10), **(D)** RelB (n=10) or **(F)** p65 (n=3). Graph of % of **(E)** RelB-positive and **(G)** p65-positive nuclei from FACS-sorted MECs. Average fluorescence intensities/nucleus for p65 are shown in **(H)**, ± S.E.M values derived from 10-20X microscopic fields. ** $p<0.01$, unpaired t-test. **(I)** P-IKKα IF on 2 day monolayer cultures of *Brca1*-/- and +/+ CD24+/CD49f+ cells. **(J)** mRNA levels for NF-κB-responsive genes *Abcb1* (Zhou and Kuo,1997) and *CyclinD2* (Huang et al., 2001) were analyzed by qRT-PCR. mRNA was collected from Lin- cells cultured for 4 days in the presence of vehicle or 10 μM BMS-345541. Images are of representative IF-positive cells. Scale bars= 10 μm.









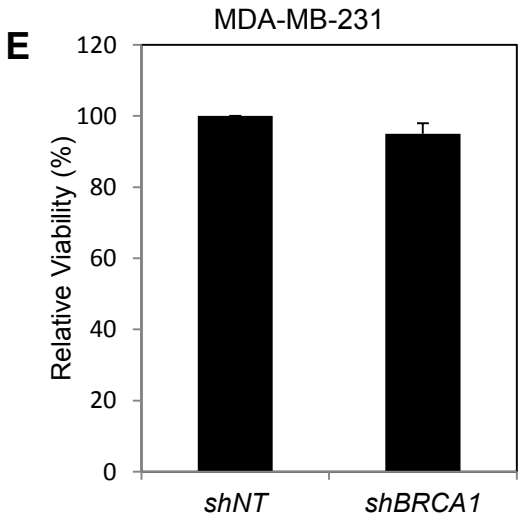


Figure S2 (Related to Figure 2): **P-ser536 p65 in *Brca1*-/- mice and effects of BRCA1 depletion on RelB and proliferation of MCF-7 and MDA-MB-231 cells. (A**) Lysates from 4 day cultures of Lin- cells from *Brca1*+/+ and -/- mice immunoblotted with anti-P-ser536-p65 and anti-p65. **(B)** MCF-7 cells cultured on coverslips were infected with NTshRNA or shBRCA1 lentivirus and immunofluorescence was performed 72 hrs later for RelB. Bar=10μm. **(C)** Average fluorescence intensity/nucleus of RelB IF based on 10 microscopic fields (n=3). **(D)** MCF-7 and **(E)** MDA-MB-231 cells were infected with sh*NT* or sh*BRCA1* lentiviruses and cell numbers determined by trypan blue exclusion after 72hrs of culture. ** $p<0.01$, unpaired t-test. Bars are S.E.M.

Supplemental Figure 3

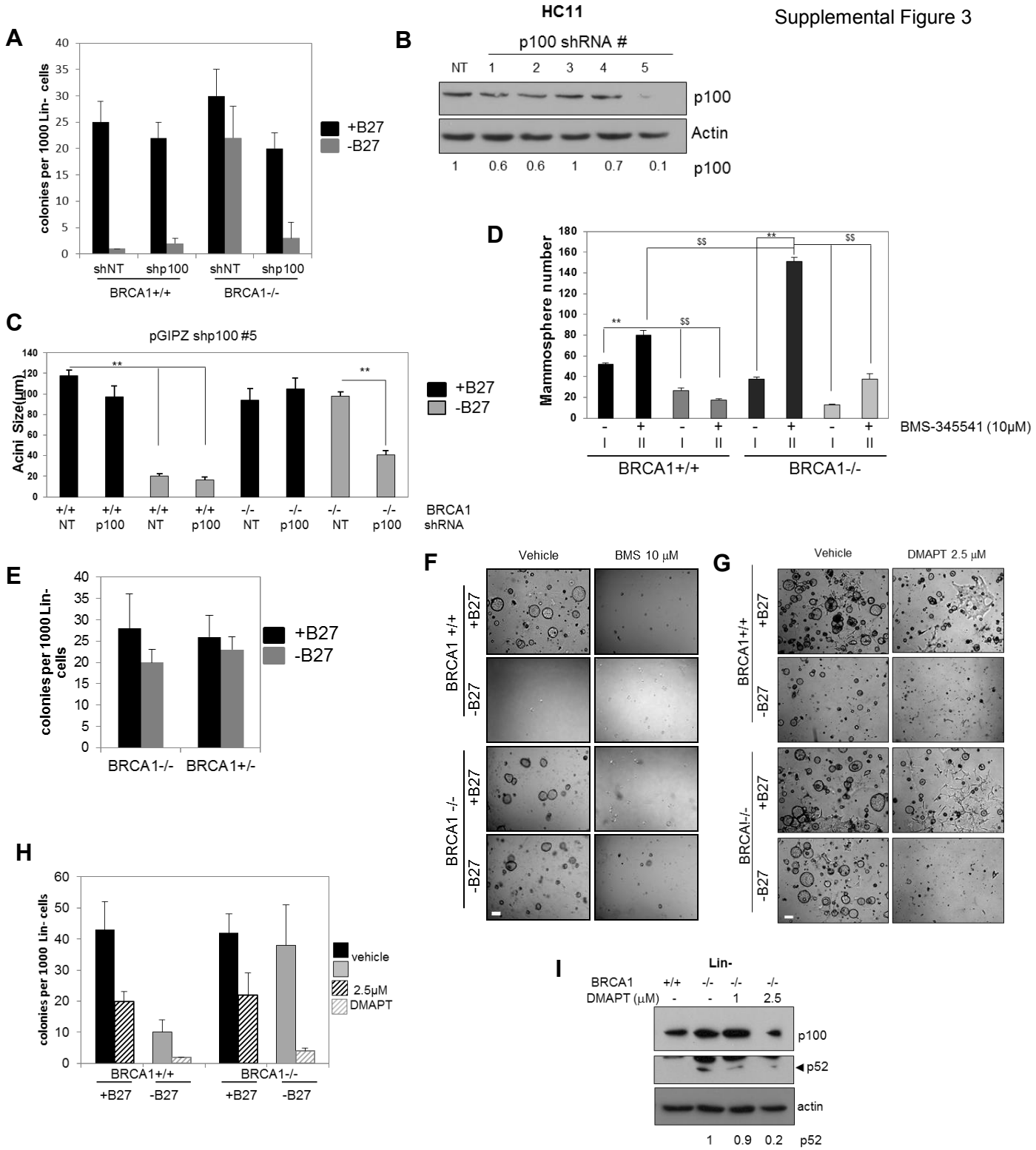


Figure S3 (Related to Figure 3): **Alternative NF-kB activation promotes B27-independent proliferation in BRCA1-deficient cells**. **(A)** Bar chart of CFCs by *Brca1*+/+ (n=3) and -/- (n=3) Lin- cells following infection with lenti-shNT or lenti-shp100.Bars are S.E.M. of 3 experiments in triplicate. **(B)** Five p100/p52 lenti-shRNAs or non-targeting shRNA (NT) were used to infect HC11 cells to test knockdown efficiencies. After 72h cells were harvested and lysates immunoblotted to detect p100. p100 quantification was analyzed using ImageJ and normalized to actin. **(C)** CFC assay of Lin- cells infected with lenti-shp100 clone #5. **(D)** Lin- cells were plated in non-adherent conditions and sphere formation assessed after 7 days (I). Spheres were then dissociated and replated then secondary sphere formation assessed after another 7 days (II). Cells were cultured in the presence of 10 μM of BMS-345541 or vehicle. ($$ - **, p<0.01, Two-way ANOVA followed by Tukey test). **(E)** Results of CFC assay of *Brca1*-/- (n=6) or +/- (n=3) Lin- cells cultured in triplicate in the presence or absence of B27+/- SEM **(F)** CFC assay of Lin- cells from *Brca1*+/+ and -/- mice in the presence or absence of BMS-345541 or **(G)** DMAPT at the indicated concentrations. Representative images are shown for both (n=4). **(H)** Bar graph showing colony formation by *Brca1*+/+ (n=4) and -/- (n=4) Lin- cells in the presence or absence of B27 or vehicle or 2.5μM DMAPT +/- SEM. **(I)** Lysates from Lin- cells cultured in the presence or absence of DMAPT for 4 days immunoblotted with anti-p100/p52.

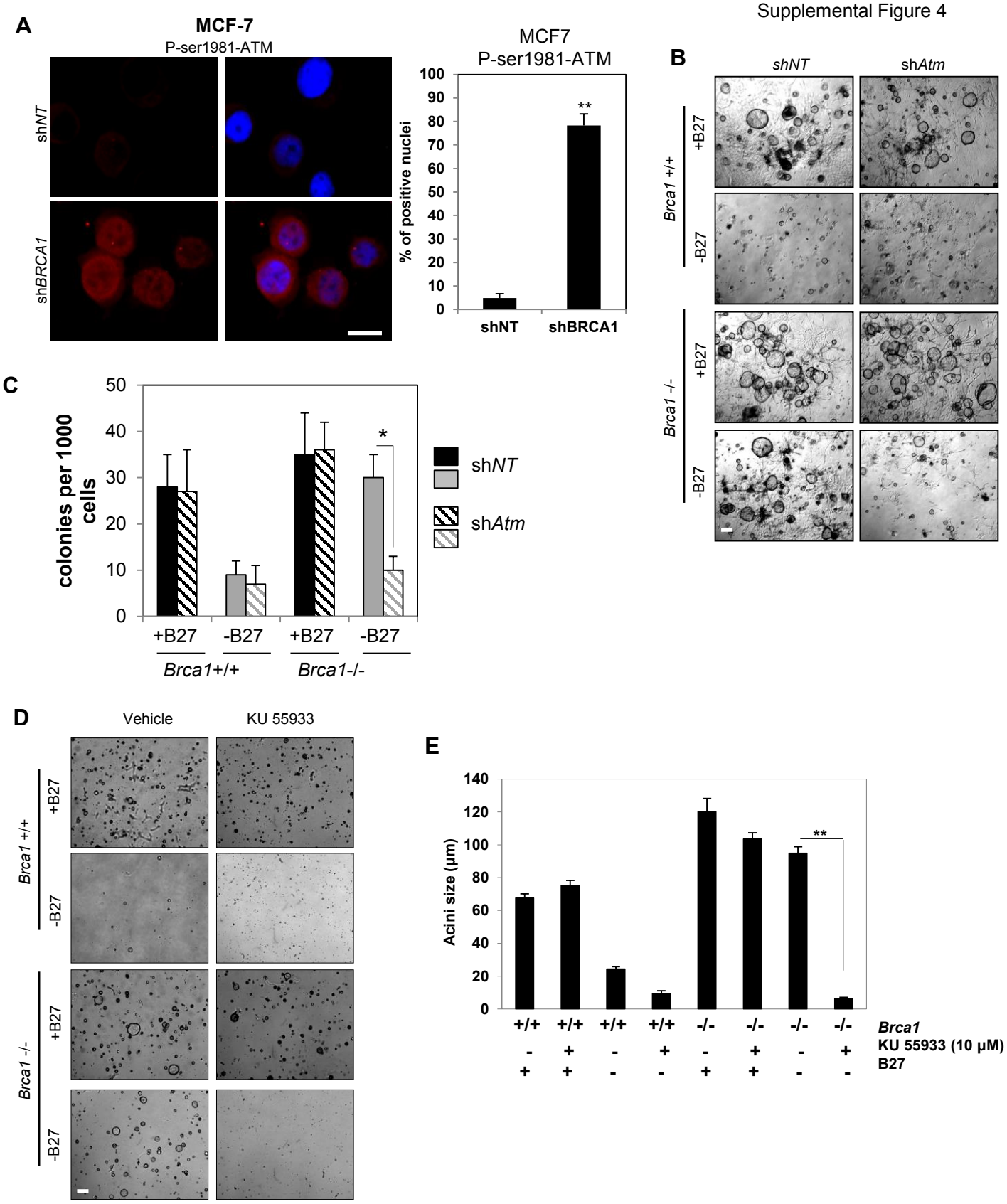
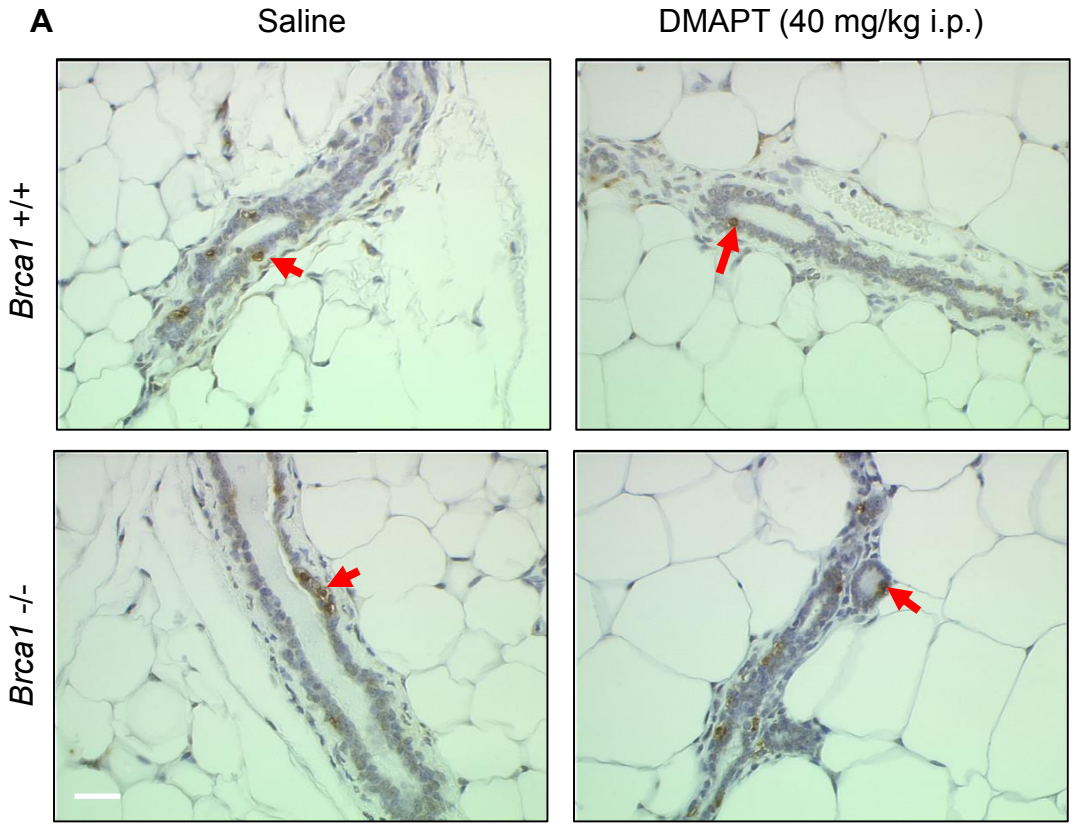


Figure S4 (Related to Figure 4): **BRCA1 knockdown activates ATM, which is required for B27-independent colony formation: (A)** MCF-7 were infected with lenti-sh*BRCA1* and after 72h cells were immunostained with anti-phospho-ser1981-ATM. Relative fluorescence intensities are shown on the graph on right. **p<.01, t-test. Scale bar= 10 μm **(B)** Representative acini assays of Lin- cells infected with lenti-sh*Atm* or lenti-sh*NT* and cultured with or without B27 from *Brca1*-/- (n=3) or +/+ (n=3) mice. **(C)** Bar graph depicting colony formation +/- S.E.M. from triplicate values for each mouse in (B)(* p<.05, t-test). **(D)** Acini assays of Lin- cells from *Brca1*-/- (n=3) or +/+ (n=3) mice cultured with 10μM of ATM inhibitor KU 55933 or vehicle in the presence or absence of B27. Scale bar= 200 μm **(E)** Acini size was assessed after 15 days in culture and measured using Northern Eclipse software. Each column in the graph represents the diameter of the acini ± S.E.M values derived from the 30 largest acini. (** p<0.01, Two-way ANOVA followed by Tukey test).



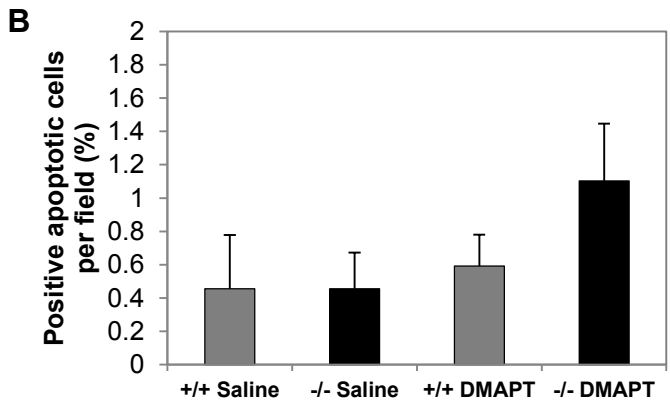


***Figure S5 (Related to Figure 6*): DMAPT injections do not induce apoptosis in mouse mammary glands: (A)** *Brca1*+/+ and *Brca1*-/- mice were treated as in mammary glands were paraffin-embedded and sectioned. Apoptag (EMD Millipore) assay was performed. Red arrows indicate apoptotic nuclei. Scale bar= 30 μm **(B)** Apoptotic nuclei were counted per field (15 different fields) and values calculated as percentage. Bars are ± S.E.M.

Table 1. List of qRT-PCR primers (*related to Experimental Procedures*)

	Forward 5' to 3'	Reverse 5' to 3'
Mm *Abcb1*	AGTGGACCCAACAGTACTCTGAT	GCACCAATCCCGGTGTAATA
Mm *Atm*	AGGATCTCCCTGGAAACGAG	CGGTGCAGAGAACACACAAG
Mm *β-actin*	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
Mm *Brca1*	TGACAGTGCCAAAGAACTCG	GATACGCTGGTGCTCTCCTC
Mm *CyclinD2*	AAGCCTGCCAGGAGCAAA	ATCCGGCGTTATGCTGCTCT

Table 2. List of lentiviral constructs (*related to Experimental Procedures*)

Hs/Mm pGIPZ sh*BRCA1* (Thermo Scientific)	Clone ID: V2LHS_254648
Hs/Mm pLKO3.G sh*p100* (TRC#0000006512)	F: GCTGCTAAATGCTGCTCAGAA R: TTCTGAGCAGCATTTAGCAGC
Mm pGIPZ sh*p100* #5 (Thermo Scientific)	Clone ID: V3LMM_485472
Mm pGIPZ sh*Ikk*α	Clone ID: V2LMM_32
Mm pGIPZ sh*Ikk*α	Clone ID: V3LMM_494894
Mm pFLRU-sh*Atm* (Gapud et al., 2011)	F: GTGGAAAGGACGAAACACCGCTTGAGGCTGATCCATATCTTCAAGAGAGAATAT R: GTGGAAAGGACGAAACACCGCTTGAGGCTGATCCATATTCTTCAAGAGAGAATAT
Mm pFLRU sh*NT* (Gapud et al., 2011)	F: GTGGAAAGGACGAAACACCGGTTCGATGTCCCAATTCTGTTCAAGAGACAGAAT R: TCCAGCTCGAGAAAAAGGTTCGATGTCCCAATTCTGTCTCTTGAACAGAAT
Hs pLKO.1 sh*p105/p50* (Battaglia et al, 2008)	TRCN0000006520
pGIPZ sh*NT* (Thermo Scientific)	F: ATCTCGCTTGGGCGAGAGTAAG R: CTTACTCTCGCCCAAGCGAGAG
pLKO.1 sh*NT* (Sigma)	F: CAACAAGATGAAGAGCACCAA R: TTGGTGCTCTTCATCTTGTTG
Lenti-κB-dEGFP (Chen et al, 2005)	

Supplemental Experimental Procedures

Reagents

KU 55933 was purchased from Tocris Bioscience, while all other chemicals including BMS-345541, RU486 (mifepristone) and progesterone were purchased from Sigma, unless otherwise specified. The synthesis of dimethylaminoparthenolide (DMAPT) has been previously described (Neelakantan et al., 2009).

Quantitative RT PCR

Cells were seeded in triplicate on 35 mm dishes. Total RNA extraction was performed using Trizol (Invitrogen) according to the manufacturer's instructions. Reverse transcription-quantitative PCR (RT-qPCR) reactions were performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) with 100 ng of RNA and 0.5 µM of the respective primers, according to manufacturer's protocol. Forward and reverse primers for *Brca1*, *Atm*, β-*actin* were purchased from Invitrogen. Primer sequences are shown in supplemental table 1. Reverse transcription was performed at 50°C for 30 min followed by initial PCR activation at 95°C for 15 min. Forty cycles consisting of a denaturing step at 94°C for 15 sec, an annealing step at 55°C for 30 sec, then an extension step at 72°C for 30 sec were performed.

Co-immunoprecipitation (co-IP)

MCF7 cells infected with lenti-sh*BRCA1* and lenti-sh*NT* for 72 hours and HCC1937 cells were harvested in co-IP buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 0.5 mM EDTA pH8, 0.5% Triton-X, 5 mM β-glycerophosphate, 5% glycerol, 1 mM DTT, 1 mM PMSF, and 1 mM Na_3VO_4). Cells were then left rotating for 30 minutes at 4 °C, and the spun for 15 minutes at 14 000 x g at 4 °C. The supernatant was collected and protein quantified as in the immunoblotting section. 200 to 300 µg of protein were used for incubation with antibody overnight. Two different ATM antibodies were used at a 50:50 ratio at a final concentration of 2 µg:1 µg of mouse monoclonal ATM (Clone MAT3-4G10/8 Sigma #A1106) and 1 µg of rabbit polyclonal ATM (Clone Ab-3 819-844 Calbiochem #PC-116). Anti-mouse and rabbit IgG (Jackson) were used a negative control. The following day 20 µL of protein A/G PLUS-Agarose (Santa Cruz #2003) were added and incubated in rotation for 3 hours at 4 °C. Protein A/G bound with immunoreactive complexes were washed 4 times with co-IP buffer, each time rotating for 5 minutes at 4 °C followed by centrifugation at 200 x g for 2 minutes. After the final wash, immunoprecipitated complexes were eluted in 50 µL of 2X sample buffer and boiled for 5 minutes. 40 µL of each sample was analyzed using SDS-PAGE as described above with 20 µg of input protein.

Antibodies

The following primary antibodies were used for immunoblot analysis: anti-actin #A-2066 (Sigma); anti-NF-κB p52 #06-413 (Millipore); anti-BRCA1 (I-20) #sc-646, anti-phospho-ATM (10H11.E12) (Ser1981) #sc-47739, anti-RelB (C-19) #sc-226, anti-Ki67 (H-300) #sc-15402, anti-NF-κB p50 (E-10) #sc-8414, (all Santa Cruz); anti-p65 #ab16502; anti-vinculin #ab129002 (both Abcam), anti-E-cadherin #3195, anti-IKKα #2682, phospho-IKKα/β (Ser176/180) #2697, anti-IKBα #9242, anti-phospho-Histone-H2AX (Ser139) #2577, anti-phospho-ser536-p65 #3031 (all Cell Signaling).

Immunohistochemistry analysis

Mouse and human paraffin-embedded sections were prepared by the University of Ottawa Department of Pathology and Laboratory Medicine. Briefly, slides were deparaffinized in

toluene, hydrated (100%, 95%, 70% ethanol and water, 10 min each) and processed for antigen retrieval in the presence of 10 mM sodium citrate,pH 6. Slides were then blocked for 1 hour at room temperature in blocking solution containing 2% normal goat serum (NGS), 1% BSA, 0.3% Triton X-100. After incubating with the primary rabbit antibody overnight at 4 °C, slides were incubated 1 h at room temperature with DAKO Envision + Peroxidase (DAKO K4002), then developed for 5 to 30 min using 1 drop of DAB Chromogen per mL of substrate buffer (DAKO K3467). Slides were then rinsed in distilled water. Counterstaining was performed by dipping the slides for 10 seconds in filtered hematoxylin (Fisher), followed by 2 minutes in distilled water. Dehydration was performed in 70, 95, and 100% ethanol followed by toluene. Slides were mounted with coverslips using permanent mounting media (Permount, Fisher).

Immunofluorescence analysis

Cells were plated on coverslips and fixed with 4% paraformaldehyde. After permeabilizing with 0.5% TritonX-100, cells were blocked with 1% Bovine Serum Albumin (BSA). Human and mouse mammary gland paraffin-embedded sections were deparaffinized as described above and antigen retrieval performed in the presence of 10mM sodium citrate, pH 6 and blocked in 1% BSA. Primary and secondary antibodies were diluted in antibody dilution buffer (DAKO #S0809). Cy3-conjugated AffiniPure donkey anti-rabbit antibodies (Jackson Immunoresearch # 711-165-152) were incubated for 1 hour at room temperature, protected from light. Cells or tissue section were covered with VECTASHIELD® Mounting Media with DAPI (Vector H-1200) and images were taken using a Zeiss Axioskop 2 *mot plus* microscope. For some slides the corrected total nuclear fluorescence (CTNF) was calculated using ImageJ where CTNF = Integrated Density – (Area of selected nucleus X Mean fluorescence of background readings). Values presented are average fluorescence/nucleus based on 50 random cells.

Isolation and FACS analysis of mouse mammary epithelial cells

Briefly, the 4th inguinal mammary glands were resected from 8 to 12 week-old *Brca1*f/f; MMTV-cre or *Brca1*f/f mice and subjected to overnight enzymatic digestion using gentle collagenase/hyaluronidase (Stem Cell Technologies #07919). A single cell suspension was generated using 0.05% trypsin (GIBCO) and 5mg/ml dispase (Stem Cell Technologies #07913). For the mammosphere and acini colony forming assay, stem/progenitor cells were isolated using EasySep™ kit (Stem Cell Technologies #19757). For FACS analysis MECs were incubated with PE-conjugated CD24 antibody, clone M1/69, (Stem Cell Technologies #19757) and Alexa647-conjugated CD49f antibody, clone GoH3, (AbD Serotec). Luminal progenitor cells (Ma-CFC) were identified as CD49flo/CD24hi and MRU (stem cell enriched) were considered as the CD49fhi/CD24lo population. CD24+/CD49f+ progenitor cells were sorted using a Beckman-Coulter MoFlo XDP instrument and then cytospun onto coverslips for immuno-staining. ML and LP enrichment was done using Pacific Blue-conjugated anti-CD24, FITC-conjugated anti-CD29, and PE-conjugated anti-CD61 (BioLegend #101819, #102205, #104307 respectively). CD24+/CD29+/CD61+/- cells were sorted on a MoFlo Astrios instrument.

Mammospheres and acinar colony forming cell (CFC) assays

Mammosphere assay: Single cell suspensions of EasySep™ -derived lineage- (Lin-) cells were plated in 96 well low attachment plate (Corning #3474) and mammosphere formation was scored after 7 days. Colony forming assay: Lin- cells (2000) were seeded in 20 μl Matrigel (BD Biosciences) and acini formation was scored after 15 days. For mammospheres and CFC assays DMEM:F12 (1:1) media containing 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 20 ng/ml EGF, 20 ng/ml cholera toxin and B27 supplement (GIBCO)

was added to the culture unless otherwise stated. The diameter of at least 30 of the largest acini in each well was measured using Northern Eclipse software and mean and standard error calculated.

Human tissue procurement
We obtained normal breast tissue from reduction mammoplasties and prophylactic mastectomies of known *BRCA1* mutation carriers through kConFab and the Royal Melbourne Hospital Tissue Bank. Tissue was obtained from consenting individuals between the ages of 33 and 45 with approval from the Human Research Ethics Committees of The Walter and Eliza Hall Institute of Medical Research and Melbourne Health. Patient paraffin blocks from prophylactic mastectomies and mammoplasties were obtained from the Queensway-Carleton Hospital and the Ottawa Hospital, Ottawa, Ontario in compliance with the University of Ottawa Research Ethics Board.

Human mammary epithelial cell isolation (organoids)
Breast tissue was minced and digested for 6 – 10 h at 37 °C with 150 U/ml collagenase (Sigma), 50 U/ml hyaluronidase (Sigma) and 100 U/ml DNase (Worthington Biochemical) in DMEM with nutrient mixture F-12 Ham (DME-HAM) supplemented with 5% fetal calf serum, 5g/ml insulin, 2 mM glutamine, 10 ng/ml epidermal growth factor and 500 ng/ml hydrocortisone.

Cell line cultures
MCF-10A human mammary epithelial cells were maintained in Ham's F12:DMEM (1:1) (GIBCO), 20 ng/mL epidermal growth factor (EGF) (Sigma), 10 µg/mL insulin (Sigma), 20ng/ml cholera toxin (Sigma), 500 ng/mL hydrocortisone (Sigma) and 5% horse serum (GIBCO). MCF-7 cells were maintained in DMEM high glucose (HyClone) supplemented with 5% fetal bovine serum (FBS) (GIBCO). MDA-MB-231 cells were maintained in DMEM low glucose supplemented with 5% FBS. HCC1937 cells were maintained in RPMI (HyClone) supplemented with 10% FBS. HC11 mouse mammary epithelial cells were maintained in RPMI supplemented with 5 µg/mL insulin, 10 ng/ml EGF and 10% FBS. 293T and PT67 were grown in DMEM high glucose (HyClone) supplemented with 10% FBS (GIBCO).

Lentivirus and retrovirus infection
Lentivirus was prepared in 293T cells transfected using polyethyleneimine (4 µg/ml) with lentiviral vector and packaging plasmids psPAX2 and pMD2.G. After 48 and 72 hours media containing virus was collected and filtered through 0.45 µm filter and the virus concentrated using Lenti-X Concentrator (Clontech #631231). For reconstitution experiments, pBABE-puro HA-BRCA1 (Addgene #14999, Cortez et al) or pBABE-puro (empty vector) were transfected into PT67 cells and media containing retrovirus collected after 72 hours. A complete list of lentiviral constructs used is available in supplemental table 2.

Statistics
Experimental results represent a minimum of three independent trials unless stated otherwise. Values presented are means ±S.E.M. A Student's t-test was used for statistical analysis and $p<0.05$ was considered significant. Comparisons between multiple groups were performed by two-way analysis of variance (ANOVA). Tukey tests were used to determine the differences between groups. A p value < 0.05 was considered significant.

Supplementary References

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