RNF168 regulates R-loop resolution and genomic stability of BRCA1/2-deficient tumors

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Figure S1

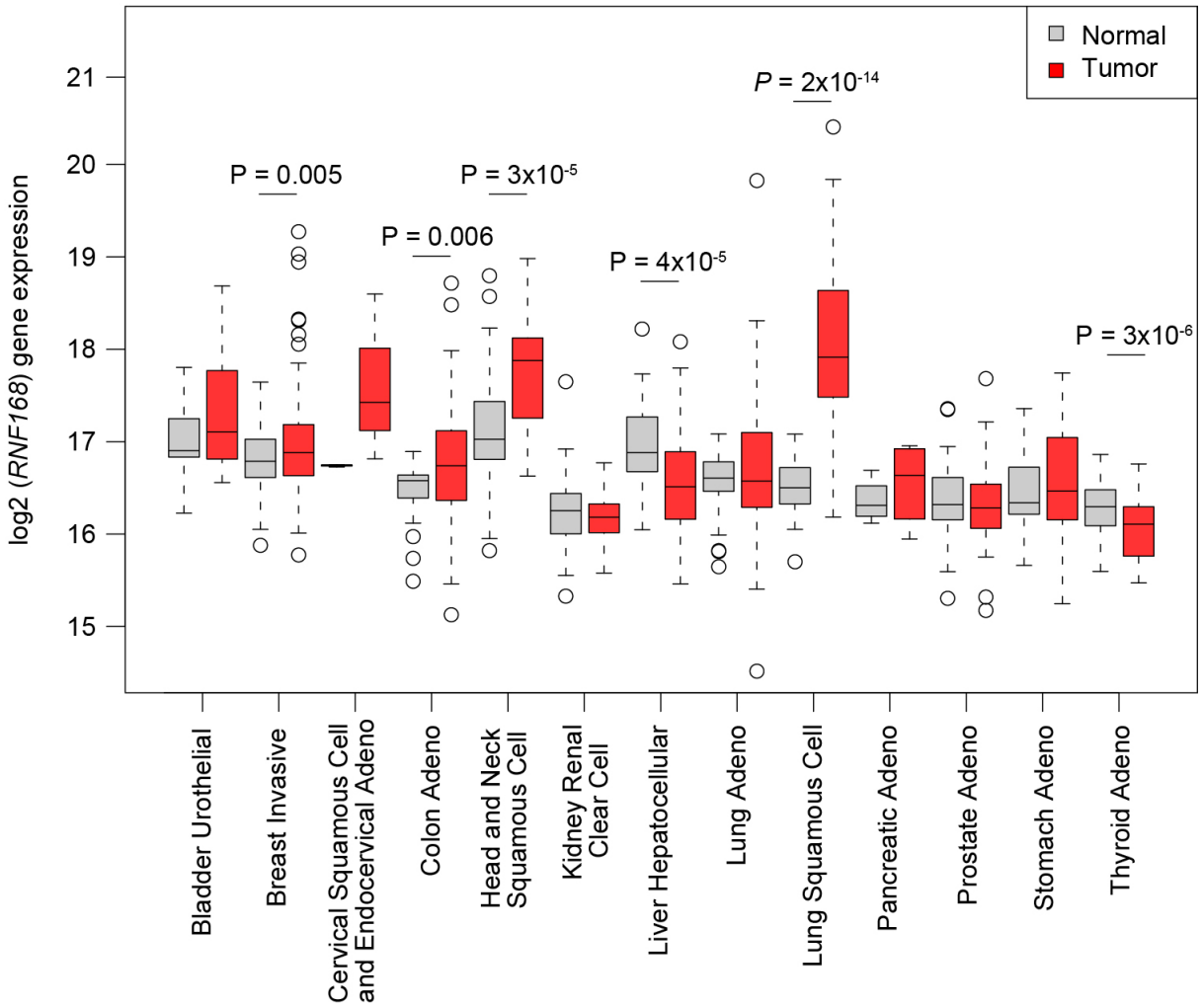


Figure S1. RNF168 is overexpressed in some primary cancers relative to its expression in normal tissues.

RNF168 mRNA expression in the indicated primary tumor types and the corresponding control normal tissues (TCGA). Significant *RNF168* overexpression was observed in breast, colon, head and neck, and lung cancers. The *P* values of paired Student's *t*-tests are indicated.

Figure S2

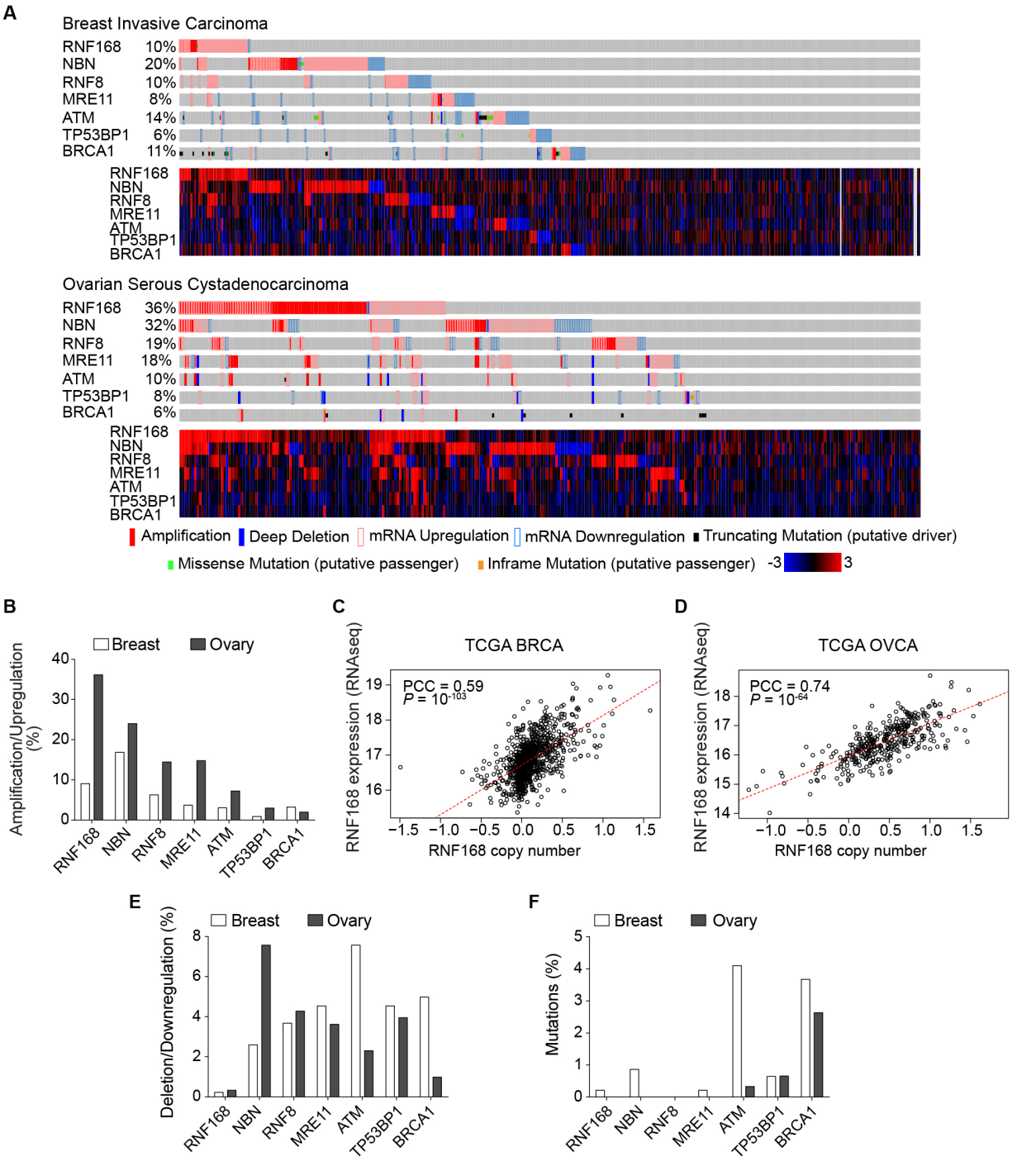


Figure S2. RNF168 is amplified and overexpressed in breast and ovarian cancers.

(A) Oncoprints generated using mRNA expression data for DSB signaling cascade genes from breast and ovarian cancer TCGA studies extracted from cBioPortal. **(B)** Quantification of the

amplification and/or upregulation of indicated genes from the Oncoprints in **A**. (**C** and **D**) Scatter plot illustrating the correlation between the *RNF168* copy number and mRNA expression in TCGA breast (n = 1,097) (**c**), and ovarian (n = 374) (**D**), datasets. The Pearson correlation coefficients (PCCs) and their corresponding *P* values are shown. (**E**) Quantification of deletion and/or downregulation of the indicated genes from the Oncoprints in **A**. (**F**) Quantification of mutations in the indicated genes from the Oncoprints in A.

Figure S3

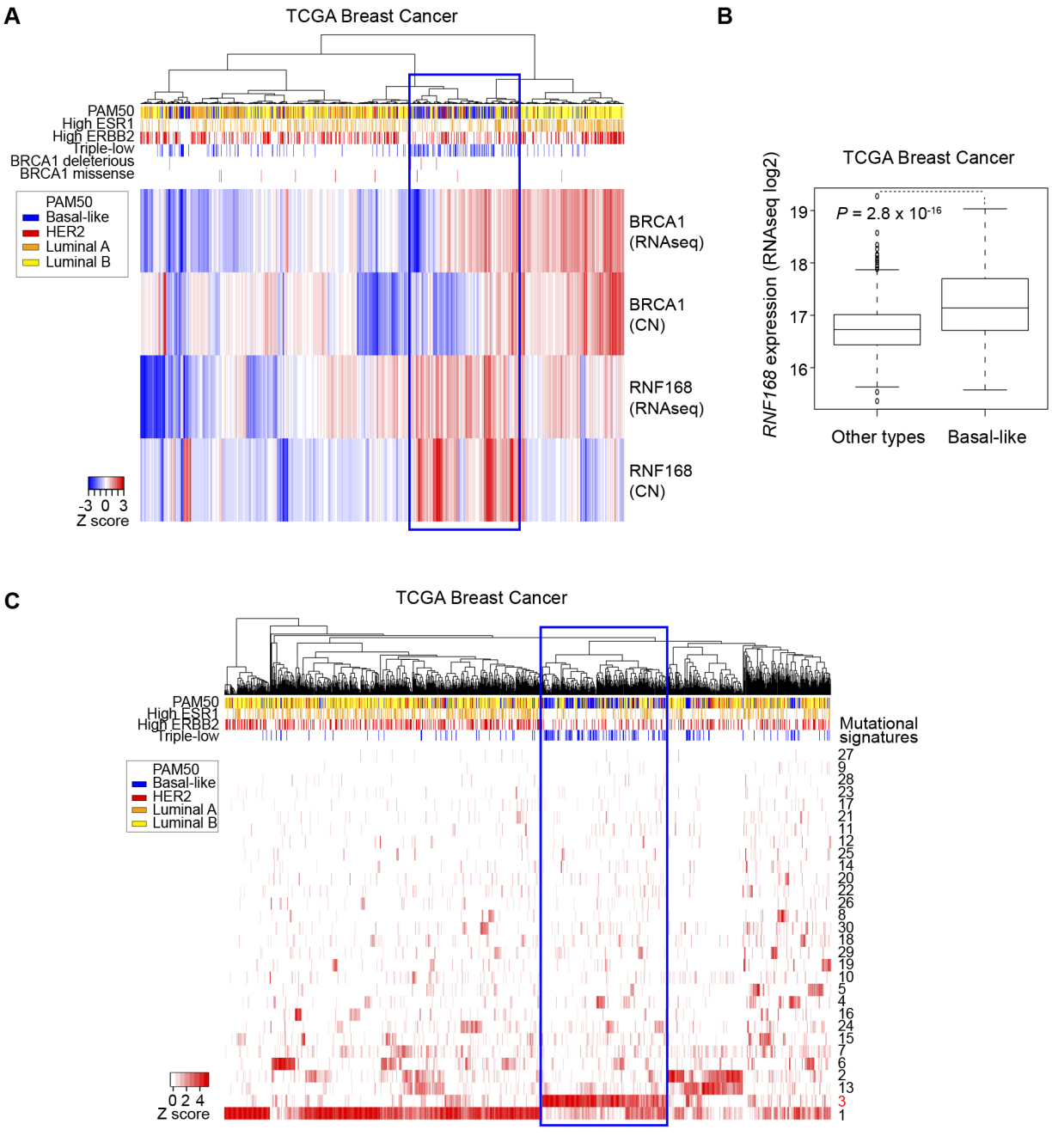


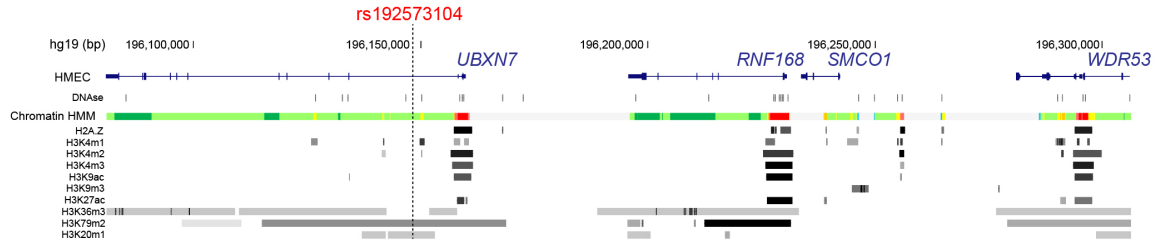
Figure S3. The *BRCA1* copy number is negatively correlated with *RNF168* expression.

(A) Heatmap generated from the TCGA breast invasive carcinoma dataset (n = 1,097), indicating the negative correlation between the *BRCA1* copy number and *RNF168* expression in patients with

the low *ESR1-PGR-ERBB2* signature. The data were scaled using the z-score and clustered using Ward's method for Euclidean distances. **(B)** Box plot indicating *RNF168* expression in TCGA BRCA patients in the indicated tumor subsets. Basal tumors (n = 211), other tumors (n = 874). The *P* value of an unpaired Student's *t*-test is indicated. **(C)** Somatic signatures were applied to the TCGA breast cancer dataset. Signature 3 corresponds to tumors whose molecular profile indicates an HR deficiency signature. The data were scaled using z-scores, and hierarchical clustering was performed using the complete linkage method with Euclidean distance.

Figure S4

A



B

GTEx rs192573104

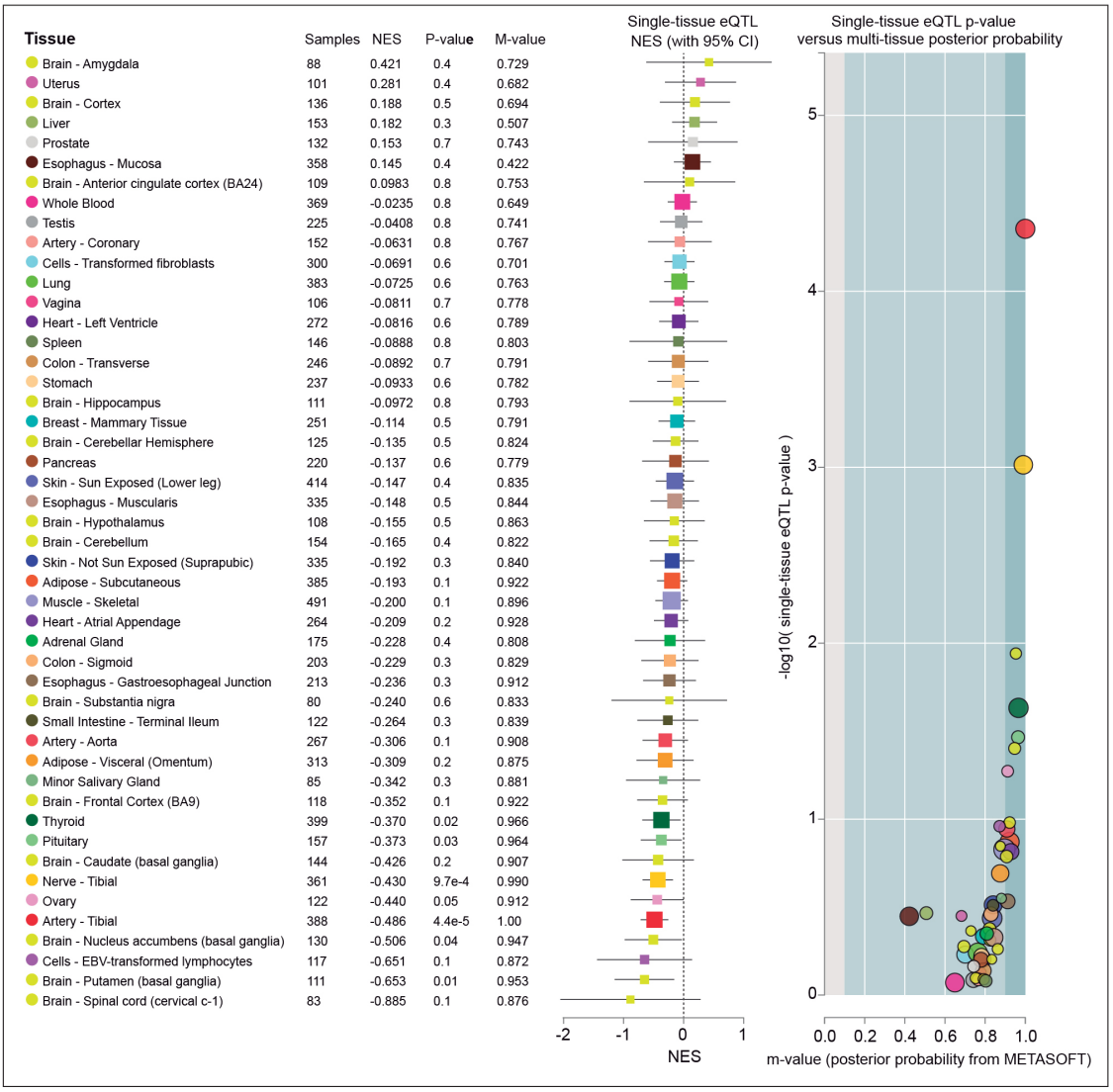
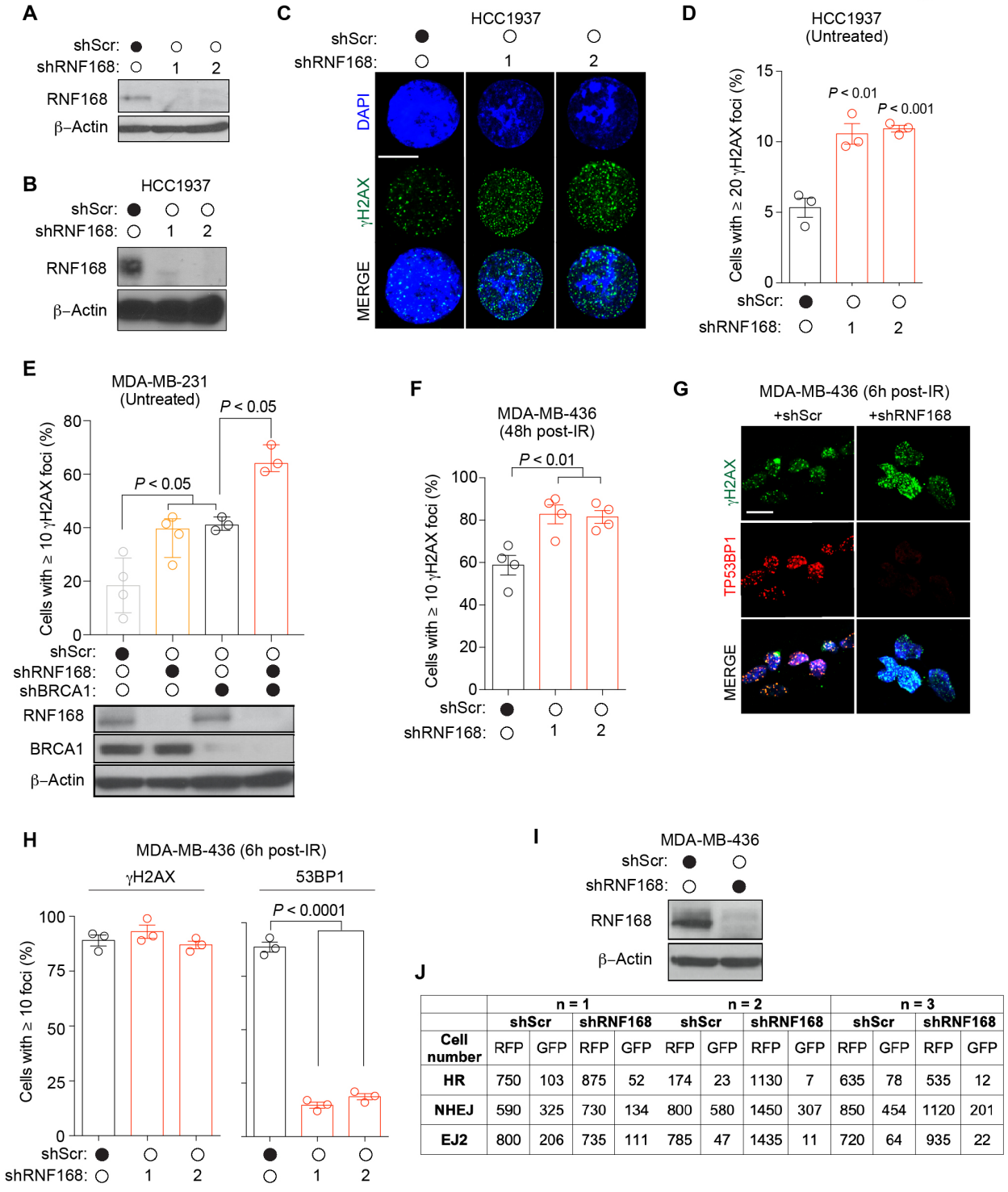


Figure S4. SNP rs192573104 is associated with decreased *RNF168* expression. **(A)** Schematic indicating the genomic location of rs192573104. **(B)** Forest plot of rs192573104

effect estimates (normalized effect size (NES)) across normal human tissues in the GTEx project. The size of the rectangles is proportional to the precision of the corresponding study. The horizontal lines indicate 95% confidence intervals. Most tissues show a tendency towards negative NES values. Right panel, plot showing the -log10 *P* values (Y-axis) and posterior probability values (X-axis) for all tissues studied.

Figure S5



J

	n = 1				n = 2				n = 3			
	shScr		shRNF168		shScr		shRNF168		shScr		shRNF168	
Cell number	RFP	GFP	RFP	GFP	RFP	GFP	RFP	GFP	RFP	GFP	RFP	GFP
HR	750	103	875	52	174	23	1130	7	635	78	535	12
NHEJ	590	325	730	134	800	580	1450	307	850	454	1120	201
EJ2	800	206	735	111	785	47	1435	11	720	64	935	22

Figure S5. Loss of RNF168 impairs DNA DSB repair in BRCA1-deficient cells.

(A and **B)** Western blot showing RNF168 knockdown in BRCA1-deficient MDA-MB-436 (A) and HCC1937 (B) cells. **(C)** Representative images of endogenous γH2AX foci in the indicated

HCC1937 cells (bar = 25 μm). **(D)** Quantification of HCC1937 cells (from **C**) with ≥ 20 endogenous γH2AX foci (n = 3). **(E)** Quantification of MDA-MB-231 cells with ≥ 10 endogenous γH2AX foci and representative western blot showing RNF168 and BRCA1 expression (n = 3-4). **(F)** Quantification of the indicated MDA-MB-436 cells with ≥ 10 γH2AX foci 48 h post IR exposure (5 Gy) (n = 4). **(G** and **H)** Representative images (**G**, bar = 25 μm) and quantification (**H**), of the indicated MDA-MB-436 cells with ≥ 10 γH2AX or TP53BP1 foci 6 h post IR exposure (5 Gy) (n = 3). **(I)** A representative western blot showing RNF168 knockdown in BRCA1-deficient MDA-MB-436 (corresponds to Figure 2E). **(J)** Cell counts for repair assays. RFP represents transfection efficiency and GFP represents repair events (corresponds to Figure 2E; n = 3). Data were analyzed using one-way ANOVA with Dunnett's multiple comparison test (D, F, H) and one-way ANOVA with Tukey's multiple comparison test (E). The data in the scatter plots are shown as the means ± SEMs of 3-4 biological replicates.

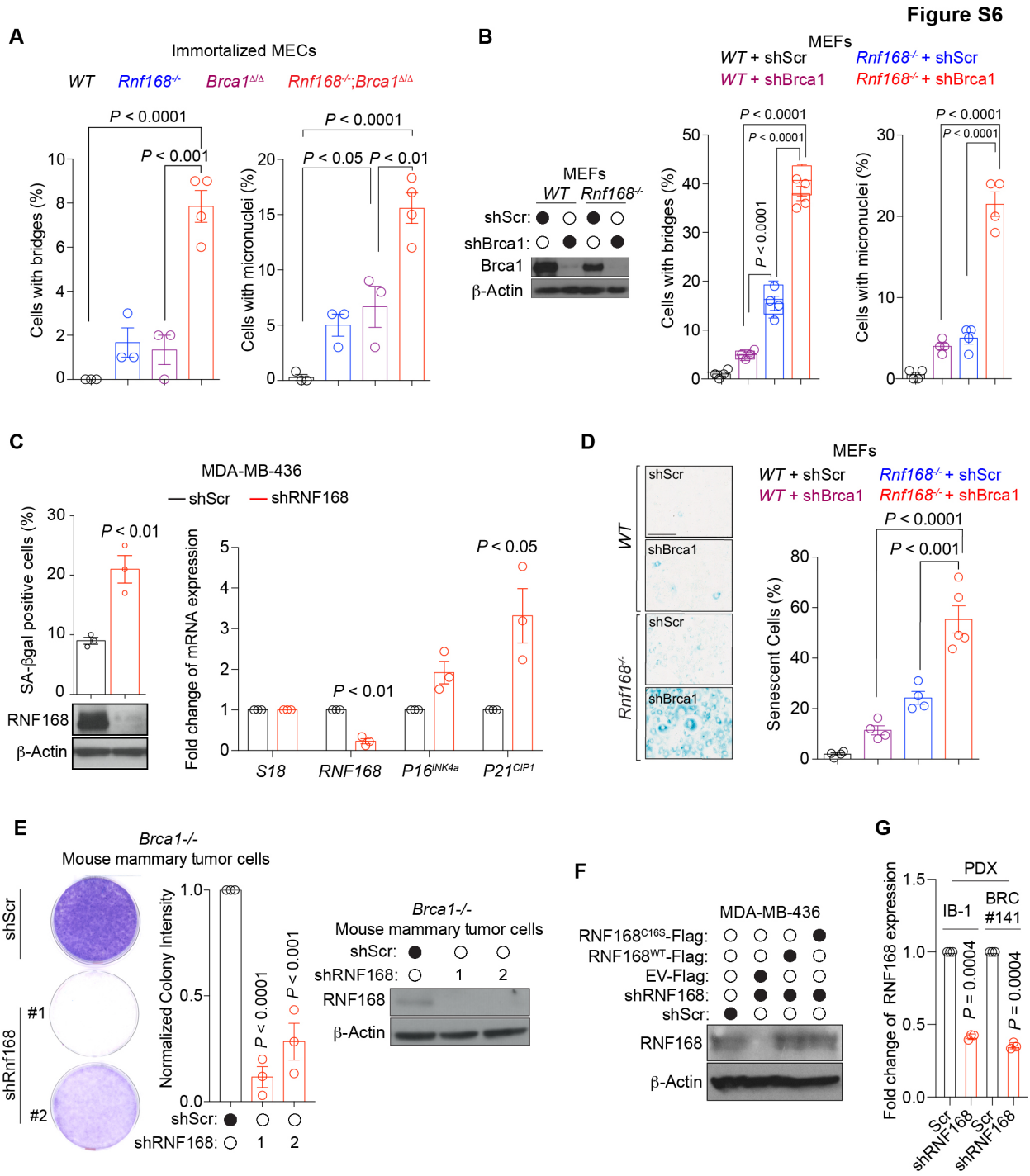


Figure S6. Loss of RNF168 causes genomic instability, senescence, and impaired growth in *Brca1/BRCA1* mutant mouse and human cells.

(A) Quantification of chromosomal bridges and micronuclei in the indicated MECs (n = 3-4). **(B)** Western blot indicating BRCA1 expression in the indicated MEFs and quantification of

chromosomal bridges and micronuclei in *WT* and *Rnf168*$^{-/-}$ MEFs transduced with shScr or sh*Brca1* (n = 4). **(C)** Quantification of SA-β-gal-positivity and RT-qPCR determination of the relative mRNA expression of *RNF168*, *p16*^{INK4a}, and *p21*^{CIP1} in the indicated MDA-MB-436 cells (n = 3). **(D)** Representative images and quantification of SA-β-gal-positive MEFs of the indicated genotypes (n = 4-5). **(E)** Long-term colony-forming capacity of *Brca1* knockout mouse mammary tumor cells transduced with the indicated shRNA (n = 3). **(F)** Representative western blot showing the expression of WT and mutant forms of mouse RNF168 in the indicated cells. **(G)** RT-qPCR determination of the relative RNF168 mRNA expression in cells from the IB-1 (*BRCA1* mutant) and BRC#141 (*BRCA1* wild-type) PDXs (n = 3). A minimum of three independent experiments were scored, and a minimum of 100 cells from at least five fields of view were counted (A-E). Data were analyzed using one-way ANOVA with Tukey's multiple comparison test (A, B, D), unpaired Students *t*-test (C), one-way ANOVA with Dunnett's multiple comparison test (E), Welch's *t*-test (F). The data in the scatter plots are presented as the means ± SEMs of 3-4 biological replicates.

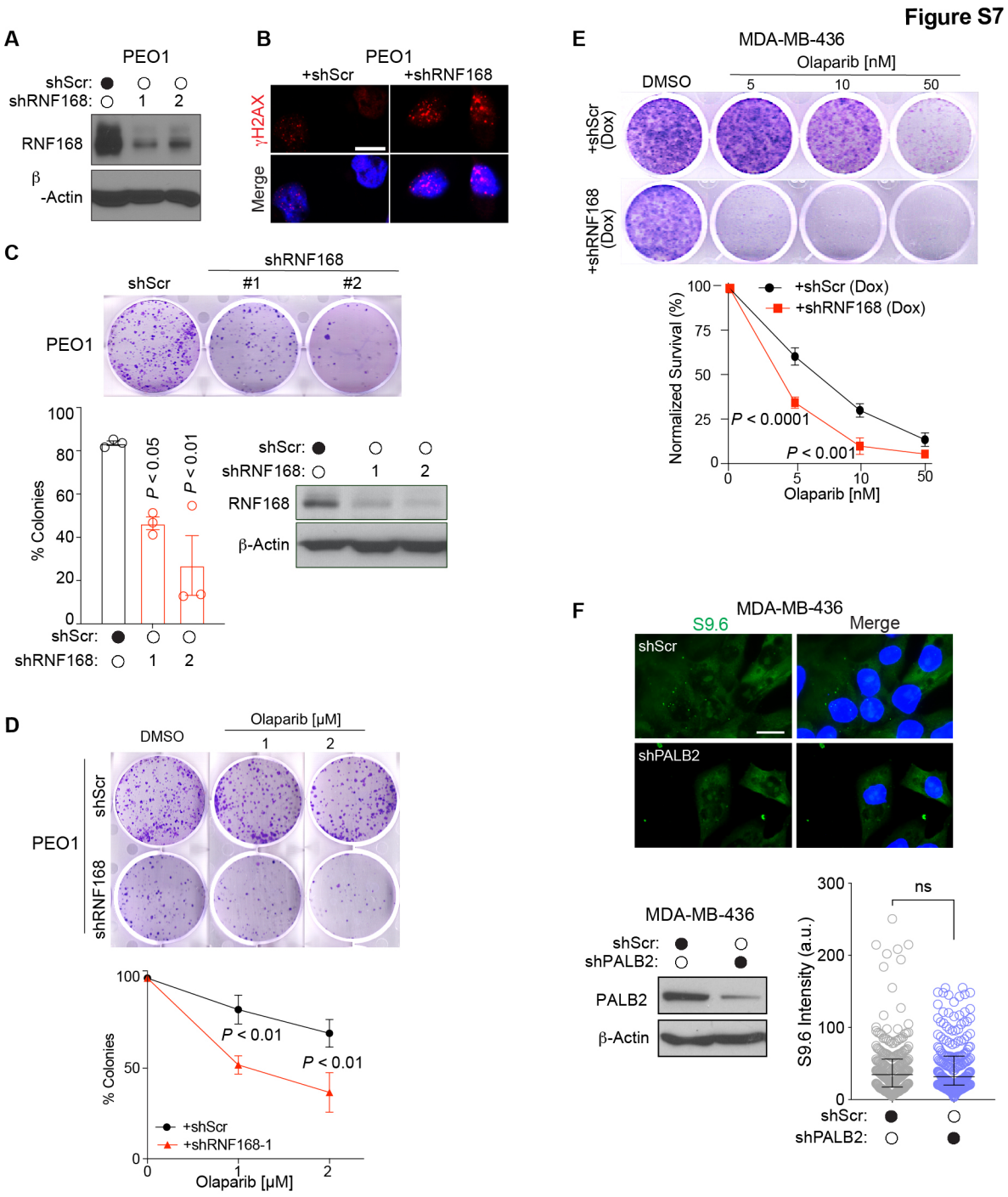


Figure S7. RNF168 depletion impairs growth and enhances sensitivity to PARPi in *BRCA*-mutant breast and ovarian cancer cells and leads to R-loop accumulation.

(A) Western blot displaying the RNF168 levels in the indicated PEO1 cells. **(B)** Representative images showing endogenous γH2AX foci in PEO1 cells proficient and deficient in RNF168 as

indicated (bar = 50 μm). A minimum of three independent experiments were performed. **(C)** Representative images and quantification of dishes showing colony-forming ability of the indicated PEO1 cells under untreated conditions (n = 3). **(D)** Representative images of dishes showing colony-forming ability of the indicated PEO1 cells either untreated (DMSO) or treated with the PARP inhibitor olaparib and colony quantification (n = 4). **(E)** Representative images of dishes showing colony-forming ability of the indicated MDA-MB-436 cells either untreated (DMSO) or treated with the PARP inhibitor Olaparib (n = 4). **(F)** S9.6 immunofluorescences and nuclear intensity in indicated MDA-MB-436 cell (n = 3). At least 200 cells were quantified (bar = 50 μm). Western blot for PALB2 expression is shown. Data were analyzed using one-way ANOVA with Dunnett's multiple comparison test (C), two-way ANOVA with Sidak's multiple comparision test (D, E), and two-tailed, unpaired Student's *t*-test (F). Data are shown as the mean ± SEM of three biological replicates (C-E). Median and interquartile range are shown for F.

Figure S8

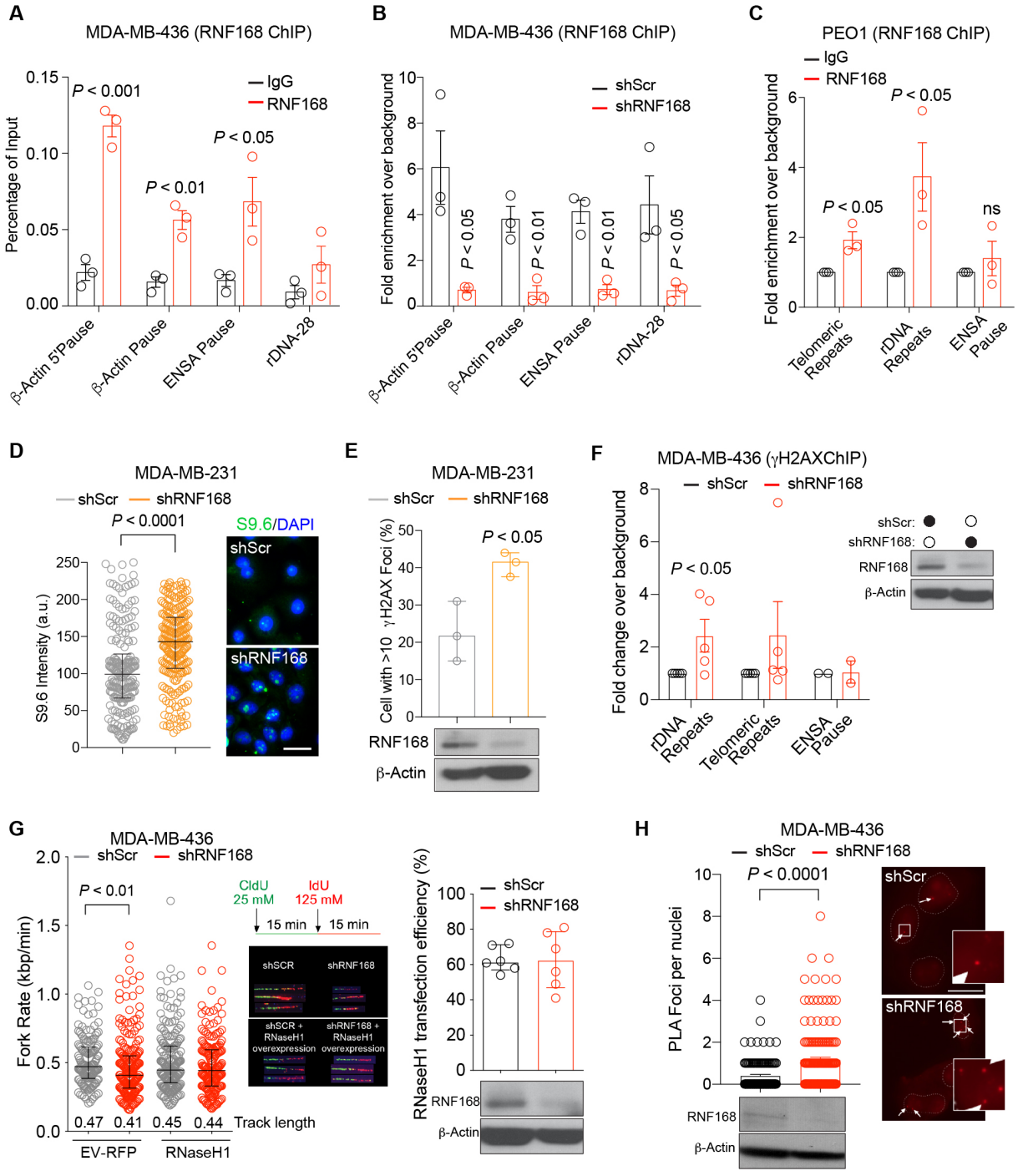


Figure S8. RNF168 is recruited to R-loop-enriched loci and its loss results in decreased replication fork rate and increased transcription-replication collision.

(A) ChIP-qPCR analysis of RNF168 in MDA-MB-436 cells compared to mock IgG (n = 3). **(B)** ChIP-qPCR analysis of RNF168 in indicated MDA-MB-436 cells (n = 3). **(C)** ChIP-qPCR analysis

of RNF168 recruitment to R-loop-prone loci in PEO1 cells (n = 3). **(D)** S9.6 signal intensity and representative images in control (shScr) and RNF168-depleted MDA-MB-231 cells (n = 3; bar = 100 μm). **(E)** Quantification of indicated MDA-MB-231 cells (n = 3) with >10 γH2AX foci. A representative western blot for RNF168 expression is shown. **(F)** ChIP-qPCR analysis of γH2AX enrichment to R-loop-prone loci in MDA-MB-436 cells (n = 2-5) A representative western blot for RNF168 expression is shown. **(G)** DNA fibre assay in indicated control and shRNF168 cells. Representative images of track length are shown. RNAseH1 or empty vector (EV) transfection efficiency, measured through immunofluorescence from 6 different fields of view is shown. A representative western blot for RNF168 expression is shown. **(H)** Quantification and representative images of PLA foci indicative of interaction between phosphorylated Serine 2- PolII and PCNA is shown (n = 3; bar = 50 μm). A representative western blot for RNF168 expression is shown. Data were analyzed using two-tailed unpaired Student's *t*-test (A-C, E, F, and right panel of G), Kruskal-Wallis test with Dunn's multiple comparison test (G), and Mann-Whitney test (D, H). The data in A-C, E, F, G (right panel) and H scatter plots are summarized as the means ± SEMs of three biological replicates. The data in D and G (left panel) are summarized as median with interquartile range.

Figure S9

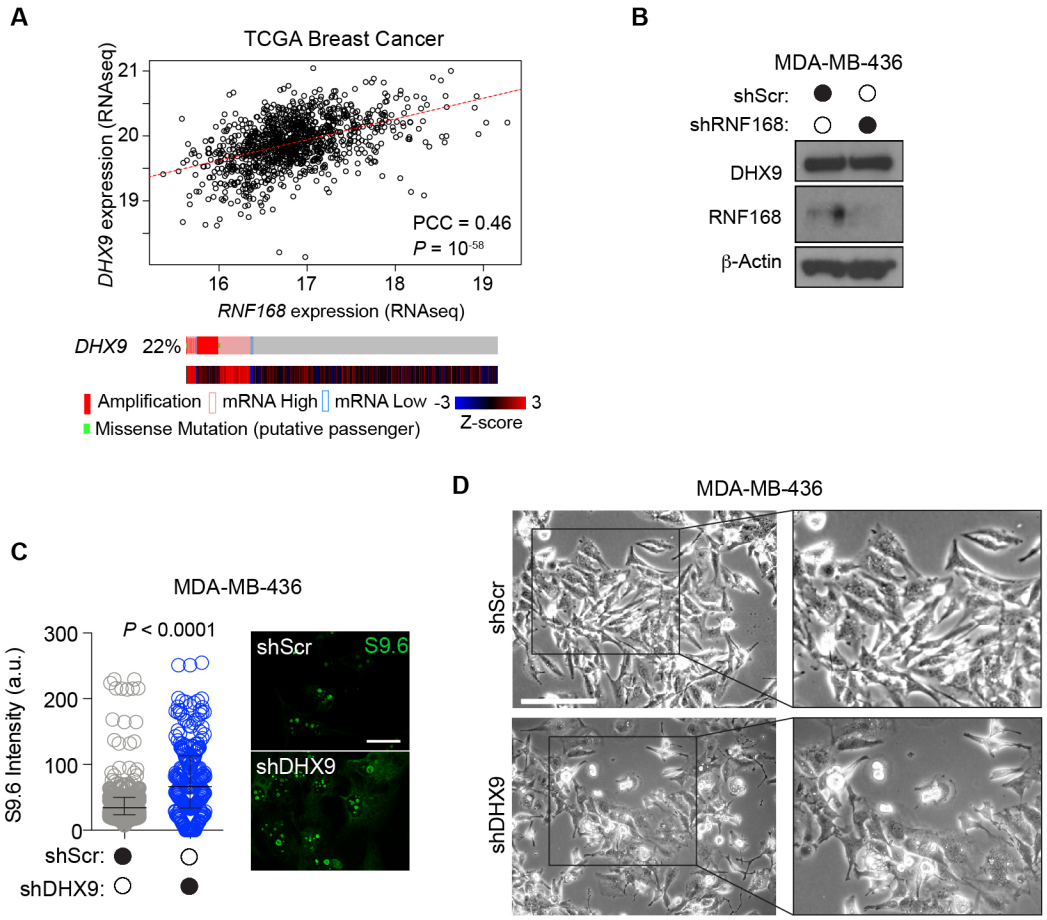


Figure S9. RNF168 expression is positively correlated with the expression level of its interactor and R-loop-suppressing helicase DHX9.

(A) Scatter plot indicating the positive correlation of *RNF168* with *DHX9*. RNA sequencing data were derived from the TCGA breast cancer dataset (n = 1,097) on cBioPortal (the PCC is shown). **(B)** Western blot showing the expression levels of DHX9 and RNF168 in the indicated cells. **(C)** Quantification and representative images of S9.6 signal intensity in the indicated MDA-MB-436 cells. **(D)** Representative images of cellular phenotypes of indicated control and shDHX9 cells (bar = 50 μm). Data were analyzes using one-way ANOVA with Mann-Whitney test (C). The data in the scatter plots are summarized as the means ± SEMs of three biological replicates. Median and interquartile range is shown for C.

Supplemental Table 1. Association results at the *RNF168* locus (± 60 kb) for the modification of breast and/or ovarian cancer risk in *BRCA1/2* mutation carriers. File uploaded online

The table shows the genetic variants included in the region (± 60 kb at the *RNF168* gene locus), their position on chromosome 3, their frequencies, their effect sizes (β), the standard error (S.E.), the associated *P* value, and the effect direction in the iCOGS and OncoArray genome-wide association studies (GWASs) for *BRCA1* and *BRCA2* mutation carriers and for breast cancer (BC) and ovarian (OC) cancer risk.

Supplemental Table 2. Mass spectrometry analysis of RNF168 interacting partners.

File uploaded online

List of protein spectral counts identified with a TPP probability of 1.00 in mock or FLAG-RNF168 expressing HEK293T cells. Gene names, NCBI gene, and protein ID along with observed spectral counts in each gel chunk are given for each protein.

Supplemental Table 3. Analysis of distribution of putative quadruplex-forming G-rich sequences in displaced single-strand DNA in the R-loop.

Locus	Quadruplex forming G-Rich Sequences (QGRS)	G score
rDNA Repeats	GGTTTCACCACGTTGGCCGGGCTGG	26
Telomeric Repeat	GGCGAGGGGCTGCATTAAAGG	26
ENSA Pause	GGGTCTTGAGGGAGATTAAAAAGAAAGATCTCTTCC ATCCTGGGG	7
β-actin Pause	GGTGAAGGCAAAGGCGTGG	35
	GGAAATGCAGGTGCCAACCAGCCCCAGTGAGGCATGG	20
β-actin 5' Pause	GGCTGCTCAATGTCAAGGCAGGAGGG	24

Supplemental Table 4. Table of mass spectrometry results from DHX9 in vitro ubiquitylation.

Gene Symbol		In vitro DHX9 ubiquitylation	
DHX9		**(-) HA-Ub**	**(+) HA-Ub**
GG-K Peptide	**GG-K Site**	2488	2037
K.NFLYAWCGK.R	14	0	1
R.KMTPSYEIR.A	16	0	3
K.KLAAQSCALSLVR.Q	236	0	3
R.QLYHLGVVEAYSGLTK.K	264	3	4
K.EGETVEPYK.V	275	0	6
R.KLEAGIR.G	497	0	5
R.KVFDPVPVGVTK.V	697	8	15
K.VFDPVPVGVTK.V	708	0	6
R.LGGIGQFLAK.A	819	7	12
R.KILTTEGR.N	1011	0	2
K.KVQSDGQIVLVDDWIK.L	1074	0	3
R.YGDGPRPPK.M	1163	2	5
Total		20	65

Supplemental Table 5. Sequences of PCR primers

qPCR primers		
Gene	**Forward Primer**	**Reverse Primer**
β-Actin 5' pause	TTACCCAGAGTGCAGGTGTG	CCCCAATAAGCAGGAACAGA
β-Actin Pause	GGGACTATTTGGGGGTGTCT	TCCCATAGGTGAAGGCAAAG
rDNA-28	CCTTCCACGAGAGTGAGAAG	GACCTCCCGAAATCGTACAC
rDNA repeats	CAGTGGCTCACGTCTGTCAT	CGCCTGACTCCATTTCGTAT
Telomeric Repeats	CAGCGAGATTCTCCCAAGCTAAG	AACCCTAACCACATGAGCAATG
ENSA Pause	TCTGCCTGTATTTGTGTGCTG	GCAGCCCTCGTCTGTATAATG
RNF168	GGATCTGCATGGAAATCCTCG	ACTGGAAGCACGGTTTACACA
S18	CAGCCACCCGAGATTGAGCA	TAGTAGCGACGGGCGGTGTG
$p16^{INK4a}$	CCCAACGCACCGAATAGTTA	ACCAGCGTGTCCAGGAAG
$p21^{CIP1}$	CGAAGTCAGTTCCTTGTGGA	CATGGGTTCTGACGGACAT
PCR primers used for cloning DHX9 WT and its deletion mutants		
Gene	**Forward Primer**	**Reverse Primer**
DHX9	AAACGCGTCGACATGGGTGACGTTA AAAATTTTCTGTATGCC	AAATCCGGATCCTTAATAGCCG CCACCTCCTC
DHX9 ΔN	AAACGCGTCGACATGTCAGTTGTCA TTATTAGAGGGGCTACTGG	AAATCCGGATCCTTAATAGCCG CCACCTCCTC
DHX9 ΔC	AAACGCGGATCCATGGGTGACGTTA AAAATTTTCTGTATGCCTG	AAATCCCCGCGGATTTTGGCTG ATTGCTTCCAGAA
DHX9 ΔCN	AAACGCGTCGACATGTACCATCTTG GAGTGGTTGAAGC	AAATCCGGATCCTTAATATCCG CTTCCATTGTCGTATC

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Supplementary Data

Supplemental Methods

Chromatin immunoprecipitation

For each condition, cells were fixed in 1% formaldehyde for 10 min at room temperature, followed by glycine neutralization for 5 min. Medium was removed, cells were washed twice with ice-cold PBS, and lysed with 10 ml lysis buffer, supplemented with protease inhibitor. Lysed cells were scraped and collected in 15-ml tubes and incubated on ice for 10 min. Nuclei were pelleted by centrifugation for 10 min at 4ºC and then lysed in 500 µl of nuclear lysis buffer. Chromatin was sonicated to produce an average fragment length of 250-400 nucleotides, then centrifuged for 10 min at 4ºC. A volume corresponding to a 20% input was recovered and stored overnight at 4ºC. For each IP, 50 µl of chromatin was diluted 10 times in IP dilution buffer and incubated with 2.5 µg anti-RNF168 antibody (#AF7217; R&D Systems), 5 µg anti-Flag (#F1804; Sigma-Aldrich), 4 µg anti-DHX9 (#A300-855A; Bethyl), or 10 µg anti-γH2AX (#05-636 JBW301, Millipore) overnight with rotation at 4ºC. The next day, 25 µl pre-washed magnetic beads (Dynabeads, Thermo Fisher Scientific) or G-protein agarose beads (BioShop) were added to each sample and incubated for 2 h at 4ºC with rotation. Beads were pelleted, resuspended, and washed with 1 ml of a series of cold buffers in the following order: low salt wash, high salt wash, LiCl wash, and TE buffer (twice). Each wash was performed for 5 min with rotation at room temperature. To elute the chromatin, beads were subjected to two rounds of 15 min incubation with 100 µl of elution buffer at room temperature. All tubes (including inputs) were supplemented with 8 µl of 5 M NaCl

and subjected to reverse crosslinking overnight in a 65ºC hybridization oven with rotation. On ChIP day 3, samples were incubated with 3 µl of Rnase A (10 mg/ml) for 30 min at room temperature followed by a second incubation (2 h, 45ºC) after addition of 4 µl EDTA (5 M), 8 µl of Tris-HCl (1 M, pH 8.0), and 1 µl of proteinase K (10 mg/ml, Wisent) to each sample. DNA was then purified using a GeneAid PCR purification kit (GeneAid Biotech, DFH100, DFH300) and associated protocols, and eluted in a total volume of approximately 200 µl. qPCR was run in triplicate using an ABI PRISM 7900HT Sequence Detection System or Bio-Rad CF96.

DNA-RNA immunoprecipitation

For each DRIP experiment, one 10-cm plate of 70% confluent shScr and sh*RNF168* MDA-MB-436 cells were harvested. On DRIP day 1, old medium was removed, and cells were washed twice with ice-cold PBS. Cells were detached in cold PBS by scraping, transferred to 15 ml tubes, and then pelleted by centrifugation. Pelleted cells were resuspended, lysed in 1.6 ml TE containing 41.5 µl of 20% SDS and 5 µl of Proteinase K (Roche), and incubated at 37ºC overnight with rotation. On DRIP day 2, lysed cells were subjected to two rounds of phenol:chloroform:isoamyl alcohol (25:24:1) extraction. The viscous aqueous phase was carefully transferred to new tubes and precipitated by the addition of two volumes of 100% ethanol. Precipitated DNA was carefully spooled onto cut pipette tips, transferred to 1.5-ml Eppendorf tubes, and washed five times with 70% ethanol. The ethanol was carefully removed, and genomic DNA (gDNA) pellets were dried for 1 h, followed by gentle resuspension in TE buffer. Half of the gDNA was mixed with a cocktail of restriction enzymes (HindIII, Bsrg1, Xba1, and Ssp1 from NEB and EcoRI; Thermo Fisher Scientific), spermidine (BioShop), and buffer 2.1 (NEB) and digested overnight at 37ºC with gentle rotation. On DRIP day 3, digested DNA was extracted by phenol:chloroform:isoamyl alcohol extraction. For each condition, 4.4 µg of digested DNA was incubated with either

100U RNASEH1 (NEB) as a control, or RNASE H buffer without the enzyme at 37ºC overnight. On DRIP day 4, samples were diluted with 10X binding buffer and TE buffer. 50 µl of each sample was recovered for input, and the remaining nucleic acid mixture was incubated with 5 µg of S9.6 antibody (Kerafast, ENH001) at 4ºC overnight with rotation. On DRIP day 5, 50 µl of Dynabeads (Thermo Fisher Scientific) were prewashed three times with 1x binding buffer. Antibody-DNA mixture was then transferred into tubes containing washed beads and incubated at 4ºC for 2 h. Beads were pelleted and washed three times with 1x binding buffer. Ips and inputs were then resuspended in 250 µl of elution buffer (final) containing 7 µl of proteinase K (Roche) and incubated at 55ºC in a hybridization oven for 45 min. Beads were pelleted and eluates transferred to new tubes. DNA was then purified using a GeneAid PCR purification kit (GeneAid Biotech, DFH100, DFH300). qPCR was run in triplicate.

Proximity ligation assay

Cells seeded on coverslips were pre-extracted using cold 0.5% NP-40 (I8896, Sigma-Aldrich) for 4 min on ice and fixed with 4% paraformaldehyde for 15 min. Coverslips were then washed 3 times and blocked with 2% BSA for 1 h at room temperature. Cells were incubated with 1:500 anti-RNAP II CTD (phospho S2) antibody (ab5095; Abcam) and 1:500 anti-PCNA antibody (FL-261, #7907; SCBT) overnight at 4°C. On the following day, cells were washed 3 times with PBS and incubated in a pre-mixed solution of PLA probe anti-mouse minus (DUO92004; Sigma-Aldrich) and PLA probe anti-rabbit plus (DUO92002; Sigma-Aldrich) for 1 h at 37°C. The Duolink In Situ Detection Reagents Red ((DUO92008; Sigma-Aldrich) were used to perform the PLA reaction according to manufacturer's instructions. The coverslips were stained with DAPI (Invitrogen) for 5 min and then mounted using Mowiol (Sigma-Aldrich).

Reconstitution of RNF168-depleted tumor cells with WT and E3 ligase dead forms of RNF168

To complement the RNF168-depleted tumor cell lines with WT and E3 ligase dead mutant RNF168, cDNA for mouse *Rnf168* (WT and C16S) was cloned into MSCV-Flag. *Rnf168* retroviral constructs and empty MSCV-Flag were co-transfected into HEK293T cells with pCL-10A packaging plasmid. Virus supernatants were collected 48 h and 72 h post-transfection. Tumor cell lines were transduced with the harvested lentiviral or retroviral supernatants in the presence of polybrene (8 μg/mL; Sigma-Aldrich) and selected for puromycin or blasticidin resistance (BioShop).

DHX9 constructs, mutagenesis, and transfection

Full-length DHX9 and DHX9 deletion mutants were amplified by PCR using complementary DNA from pGFP-DHX9 (Gift from R. Santoro) and cloned into MSCV-Flag using primers indicated in Supplemental Table 5. WT and lysine to arginine DHX9 mutant expression constructs were generated using fusion PCR and ligated into MSCV-Flag. These constructs were transfected in HEK293T and MDA-MB-436 cells using a standard calcium phosphate transfection protocol or using GeneJet (#SL100489; SignaGen) as per manufacturer's protocol, respectively.

Generation of viable PDX-derived tumor cells, lentiviral transduction, and cell viability assay

Freshly collected PDX-derived xenograft tissues were minced using sterile scalpels and dissociated for a maximum of 90 min in DMEM/F12/HEPES (GIBCO), 1 mg/ml collagenase (Roche), 100 U/ml hyaluronidase (Sigma), 25% BSA fraction V (GIBCO), 5 μg/ml insulin, and 50 μg/ml gentamycin (GIBCO). This was followed by further dissociation using trypsin (GIBCO), dispase (STEMCELL Technologies Inc.) and DNase (Sigma). Red blood cell lysis was performed

by washing the cell pellet in a 1:4 solution of HF media (GIBCO): ammonium chloride (STEMCELL Technologies Inc.). Cells were resuspended in HuMEC medium (GIBCO), divided into three equal aliquots, each of which was then transduced with lentivirus (carrying shScr or sh*RNF168*) plus polybrene (8 μg/ml). The next day, cells were washed twice with PBS, and single-cell suspensions were plated in triplicate in 96-well plates. Cell viability reading intensities were obtained using CellTiter-Glo (Promega) on day 0 and subsequent days, the counts being normalized with respect to that of day 0.

Xenotransplantation

All xenotransplantation studies were approved by and performed in compliance with PMCC Animal Care Committee guidelines. Equal parts of cells (1.5 million) and Matrigel matrix were injected into inguinal mammary fat pads of 6-7-week-old NSG mice (Jackson Laboratory). Tumor volume was calculated weekly using caliper measurements (length x width^2)/2. Mice were euthanized by CO_2 inhalation when the tumor size reached the ethical endpoint of 2 cm, in accordance with the rules of PMCC's Animal Resources Centre.

Bioluminescence

Human tumor cells transduced with pLenti-CMV-Luciferase (Addgene# 17477) were xenotransplanted as previously described. XenoLight-D-Luciferin potassium salt (PerkinElmer, #122799) was prepared in accordance with the manufacturer's instructions and injected intraperitoneally at 150 mg/kg of the mouse's body weight. Bioluminescent intensities were measured weekly using Xenogen IVIS Spectrum (Xenogen, MA, USA) and data were analyzed using LivingImage 4.0 Software (Caliper Life Sciences, MA, USA).

Assessment of nuclear abnormalities

Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min at 4°C, stained with DAPI (Invitrogen) for 5 min and then mounted using Mowiol (Sigma-Aldrich). A minimum of 300 cells were assessed per cell line using a Leica DM4000B fluorescence microscope.

DNA content analysis

Cells were fixed and stored in 70% ethanol overnight. Subsequently, fixed cells were washed twice with PBS and then treated with 50 µl of 100 µg/ml Rnase A (Thermo Scientific) for 20 min before adding 5 µg/ml propidium iodide (Sigma). The samples were then washed once in PBS before flow cytometric analysis using BD FACSCanto and FlowJo.

Senescence-associated β-galactosidase assay

MECs, MEFs, and MDA-MB-436 cells were fixed and stained for senescence-associated β-galactosidase using the senescence β-galactosidase staining kit (#9860; Cell Signaling), following the manufacturer's instructions. The cells were visualized using a brightfield setting on a Leica DM4000B fluorescence microscope under 40X or 63X magnifications.

RNA extraction, cDNA synthesis and qPCR

Total RNA was isolated using TRIZOL (Invitrogen). To quantify RNA expression levels, equal amounts of cDNA were synthesized using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). cDNA was mixed with SYBR Green PCR master mix (Applied Biosystems) and the indicated forward and reverse primers for each gene. Samples were amplified and analyzed using default settings in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Primers used for RT-PCR are indicated in Supplemental Table 4.

Clonogenic assay

MDA-MB-436 (10,000 cells, 5000 cells, or 500 cells), *Brca1*$^{-/-}$ mouse mammary tumor (10,000 cells), and PEO1 (500 cells) were seeded on six-well plates, treated with DMSO or the indicated

drug, and cultured for 14-21 days. Colonies were stained with crystal violet and scored. Cells were seeded in triplicate and three independent experiments were performed.

Growth curve

100,000 MDA-MB-436 cells transduced with shScr or sh*RNF168*, and MDA-MB-436+sh*RNF168* cells transduced with MSCV-Rnf168^{WT}-Flag or MSCV-Rnf168^{C16S}-Flag were seeded in six-well plates. Every 3 days the cells were trypsinized, counted, and 100,000 cells were re-seeded. Cells were seeded in triplicate and three independent experiments were performed for each cell line.

Luciferase viability assay

10,000 MDA-MB-436 cells transduced with shScr and shRNF168 were further transduced with pLenti-CMV-luciferase and seeded on a 96-well plate. Cells were treated with various concentrations of pyridostatin (Sigma-Aldrich) 1 day after seeding. On day 10, viability was measured using the Luciferase Assay System (Promega, E1500) according to the manufacturer's protocol using a CLARIOStar Monochromator Microplate Reader (BMG Labtech). Cells were seeded in triplicate and three independent experiments were performed for each cell line.

Immunohistochemical staining

Formalin-fixed paraffin-embedded sections of harvested xenografts of MDA-MB-436 cells transduced with shScr or shRNF168 were stained using Ki-67 monoclonal antibody (Clone SolA15; eBioscience). All slides were counterstained with DAPI (Invitrogen). Four xenograft sections and positive cells in at least five 40X fields were visualized and scored using a Leica DM4000B fluorescence microscope.

Replication fork progression

Nucleoside labeling and plug preparation

Cells were seeded 36 h before labeling. Cells were pulse-labeled with 25 μM CldU for 30 min, and washed with pre-warmed PBS before pulse-labeling with 125 μM IdU for 30 min. Next, cells were trypsinized, resuspended in PBS and counted. An equal volume of 1% low melting point agarose was added to cells ($8x10^6$ cells/ml) and poured into a plug castor (Bio-Rad #170-3713) and incubated at 4ºC for 45 min to solidify.

Agarose plug digestion

Plugs were washed three times with 1 ml TE_{50} buffer and incubated with proteinase K solution (1 mg/ml proteinase K, 1% w/v sarkosyl, 10mM Tris-Cl pH 7.5, 50 mM EDTA, heated to 50°C for 30 min before use) for 3 days, renewing with fresh proteinase K solution each day. Plugs were removed from the proteinase K solution and washed five times in TE_{50} for 10 min at room temperature. The plugs were stored in 1 ml TE_{50} at 4°C, protected from light.

Plug melting, molecular combing, and immunodetection

Digested agarose plugs were incubated in MES buffer (7:3 v/v MES hydrate:MES sodium salt, 50 mM, pH 5.7) for 5 min at room temperature. These plugs were then melted at 72°C for 10 min, gently agitated, and incubated for another 10 min. DNA was combed onto silanized coverslips at a constant speed of 710 μm/s and fixed at 60°C for 90 min. Once the coverslips had been mounted on glass slides, they were dehydrated sequentially in 70%, 90%, and anhydrous ethanol for 5 min each. DNA was denatured in 1 M NaOH solution for 25 min at room temperature. Coverslips were blocked in 21 μl of blocking buffer (10% w/v albumin in PBS-T, 0.05% Tween-20, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) for 30 min, and stained with anti-CldU and anti-IdU solution (AbD Serotec MCA2060 1:40 dilution in blocking buffer, Becton Dickinson 347580 1:10 dilution), anti-DNA solution (Millipore MAB3034 1:50 dilution in blocking buffer), and anti-secondary solution (Alexa Fluor 546 anti-mouse IgG1, Molecular Probes A21123, 1:50 dilution,

Alexa Fluor 648 anti-mouse IgG2, Molecular Probes A21241, 1:50 dilution in blocking buffer) for 1 h at 37°C in a humid chamber, washing three times with PBS-T for 5 min between each staining. ProLong Gold was added to the coverslip and imaged.

Immunoprecipitation

Nuclei of MDA-MB-436 cells were harvested and lysed using Pierce IP buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol) or NETN (100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5% (v/v) NP-40) buffer supplemented with complete protease inhibitor cocktail tablet (Sigma) on ice for 30 min. Debris was removed by 20,000 x g centrifugation for 10 min at 4°C. 2 μg of anti-DHX9 (A300-855A, Bethyl) and anti-IgG (#197767, Abcam) were added to lysates and the mixture was rotated overnight at 4°C. Interaction domain mapping was conducted by transiently transfecting full-length and DHX9 deletion mutants into HEK293T cells. Cell lysates were prepared 48 h post-transfection using aforementioned conditions. 2 μg of anti-Flag (M2, Sigma-Aldrich) was used for immunoprecipitation overnight at 4°C. The next day, G protein agarose beads or Dynabeads were used to elute interacting proteins, which were then subjected to standard SDS-PAGE and immunoblotting using standard protocols.

Expression of recombinant DHX9 in Sf9 cells

The recombinant donor vector DHX9-pFastBac plasmid was transformed into DH10Bac E. coli cells (Invitrogen) to generate recombinant viral DNA. Sf9 cells (Invitrogen) were transfected with Bacmid DNA using jetPRIME® transfection reagent (PolyPlus Transfection), and recombinant baculovirus particles were recovered. The recombinant virus was sequentially amplified from P1 to P3 viral stocks.

Sf9 cells grown in I-Max Insect Serum Free Medium (Wisent) to a density of 4×106 cells/mL and with viability not less than 97% were infected with 12 mL of P3 viral stock for each 1 L of cell

culture. Cell culture medium was collected after 4 days of incubation on a shaker at 150 RPM and 27°C when culture viability dropped to 75-80%.

Purification of E1-His and DHX9-His proteins using insect cell pellets

Insect cell pellet from 400ml culture was resuspended in 40ml of lysis buffer containing 50mMTris, 500mM NaCl, 10% glycerol, 5mm Imidazole, 1mM PMSF, 2mM Benzamidine, 2mM TCEP, 0.5% NP-40, pH 7.5, and complete EDTA-free protease inhibitor (Roche), then sonicated at 20% output for 1 minute for 3 times. The lysate was clarified using centrifugation at 15000rpm for 30 minutes and the supernatant was incubated with pre-washed 2.5 ml of Talon beads (Clontech) at 4°C for 1 hour with rotation. The beads were washed with 25 ml of washing buffer, which is the lysis buffer without NP-40 but with 10mM Imidazole, twice. The His-tagged proteins were eluated with the same buffer containing 500mM Imidazole. E2E2-His and Ub-His were expressed in bacteria BL21(DE3)-CodonPlus-RIL strain and were purified the same way as E1-His and DHX9-His proteins using Talon beads.

Purification of RNF168-GST protein

RNF168-GST protein was expressed in bacteria BL21(DE3)-CodonPlus-RIL strain and was purified using Glutathione-sepharose 4B beads in a buffer of 25mM Tris, 500mM NaCl, 10%glycerol, 5mM beta-mercaptoethanol, 5mM DTT, 2mM TCEP, 1mM Benzamidine, 1mM PMSF, 1mM EDTA, pH 7.2, and protease inhibitors. The GST-fusion protein was eluated with 20mM reduced glutathione, 25mM Tris, 500mM NaCl, 2mM TCEP, pH 7.2, and was further purified using gel-filtration chromatography on a column of Superdex 200. The fractions containing the full length RNF168-GST protein was used for ubiquitination assay.

LC-MS Sample Preparation

SDS-PAGE bands were cut into a slurry and subjected to dehydration/rehydration cycles by sequential CH3CN/NH4HCO3 incubation in order to reduce (DTT, 5mM, 60°C, 20 min.), alkylate (iodoacetamide, 10mM, 20°C, 15 min.) and digest (TPCK-treated, sequencing-grade trypsin (Promega), 1μg/sample, 37°C, 16 hrs.) proteins contained in the gel bands. The released peptides were collected, samples were acidified with TFA (1%), desalted using C18 chromatography columns and lyophilized.

LC-MS Sample Analysis

Samples were reconstituted in HCOOH (0.1%), loaded on a pre-column (C18 Acclaim PepMapTM 100, 75 μm x 2 cm, 3 μm, 100Å, Thermo Scientific) and separated on an analytical column (C18 Acclaim PepMapTM RSLC, 75 μm x 50 cm, 3mm, 100Å, Thermo Scientific) via high performance liquid chromatography (LC) over a 120-minute, reversed-phase gradient (5-30% CH3CN in 0.1% HCOOH) running at 250 nl/min on an EASY-nLC1200 pump in-line with a Q-Exactive HF mass spectrometer (Thermo Scientific) operated in positive ESI mode. An MS1 ion scan was performed at 60,000 FWHM followed by MS/MS scans (HCD, 15,000 FWHM) of the twenty most intense parent ions (minimum ion count of 1000 for activation). Dynamic exclusion (within 10 ppm) was set for 5 seconds.

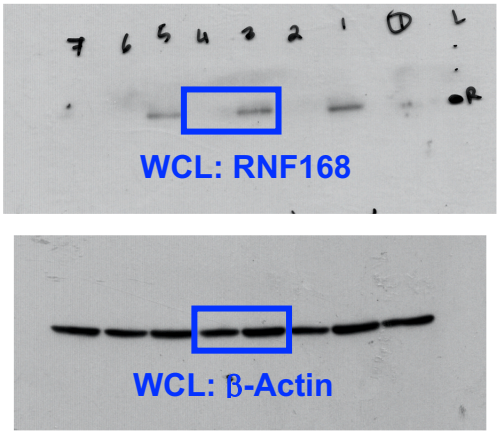
LC-MS Data Processing

Raw files (.raw) were converted to .mzML format using Proteowizard (v3.0.19311), then searched using X!Tandem (v2013.06.15.1) and Comet (2014.02 rev. 2) against Human RefSeqV104 (containing 36,113 entries). Search parameters specified a parent MS tolerance of 15 ppm and an MS/MS fragment ion tolerance of 0.4 Da, with up to two missed cleavages allowed for trypsin. Carbamidomethyl c, was set as a fixed modification, while deamidation (NQ), oxidation (M), acetylation (protein N-term) and diglycine (K) were set as variable modifications. Search results

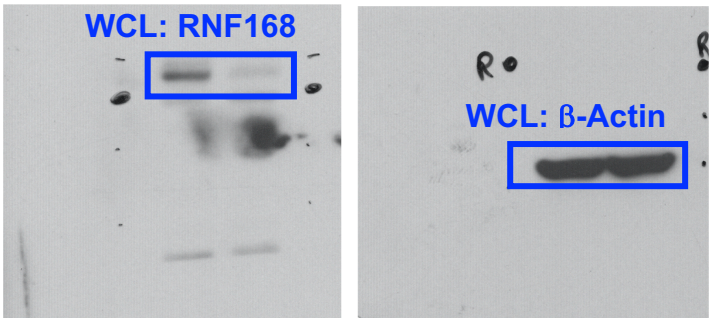
were processed through the trans-proteomic pipeline (TPP v4.7) and proteins to which ≥2 unique peptides were assigned and showing an iProphet probability ≥0.9 were considered confident identifications. Manual spectral inspection was performed to confirm proper assignment of peptide matches and modifications. All raw mass spectrometry data have been deposited in the MassIVE repository (massive.ucsd.edu) with accession ID MSV000084981.

Uncropped/unedited versions of all blot images that appear in the main and supplemental figures.

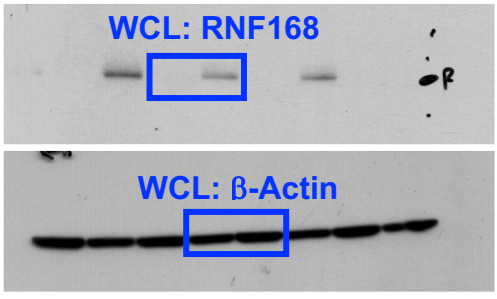
Full unedited gel for Figure 2G



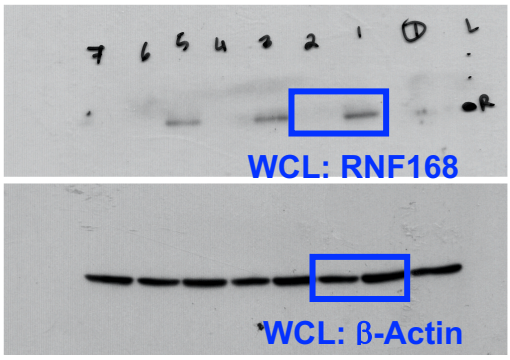
Full unedited gel for Figure 3B



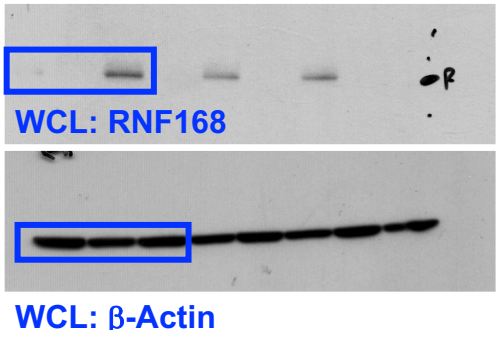
Full unedited gel for Figure 4A



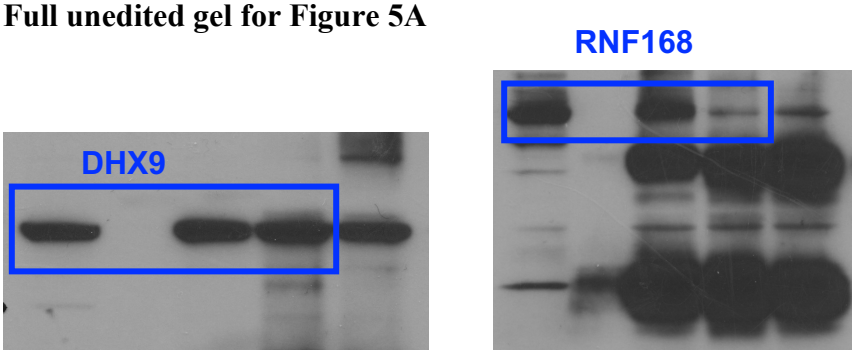
Full unedited gel for Figure 4B



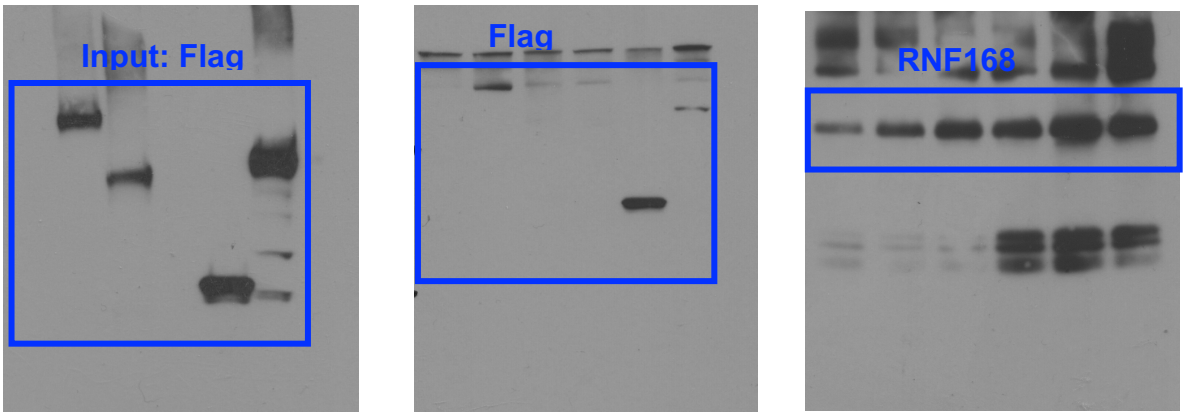
Full unedited gel for Figure 4H



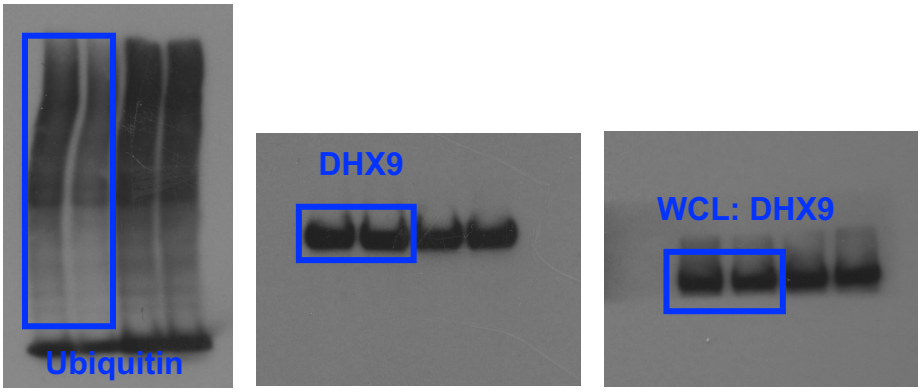
Full unedited gel for Figure 5A

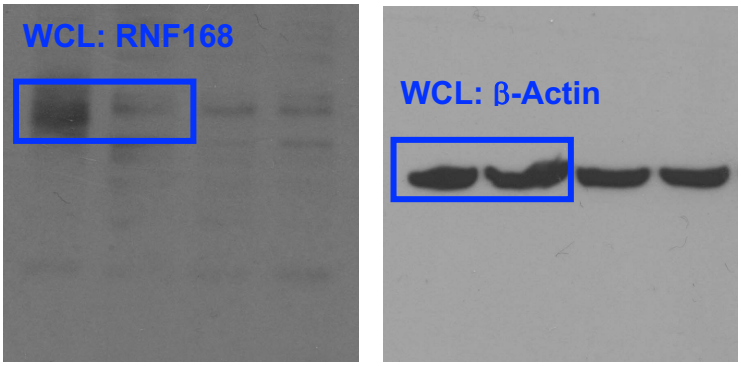


Full unedited gel for Figure 5B

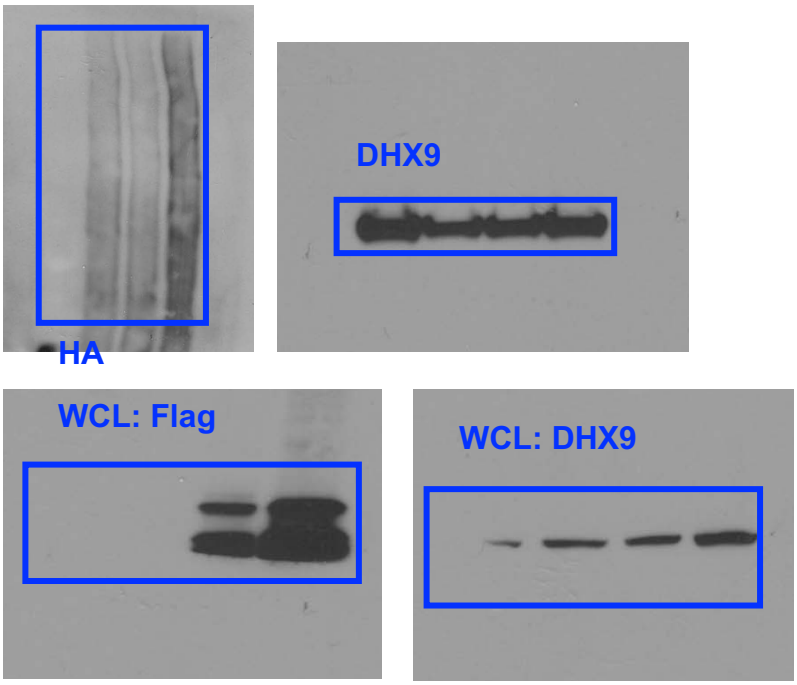


Full unedited gel for Figure 5E

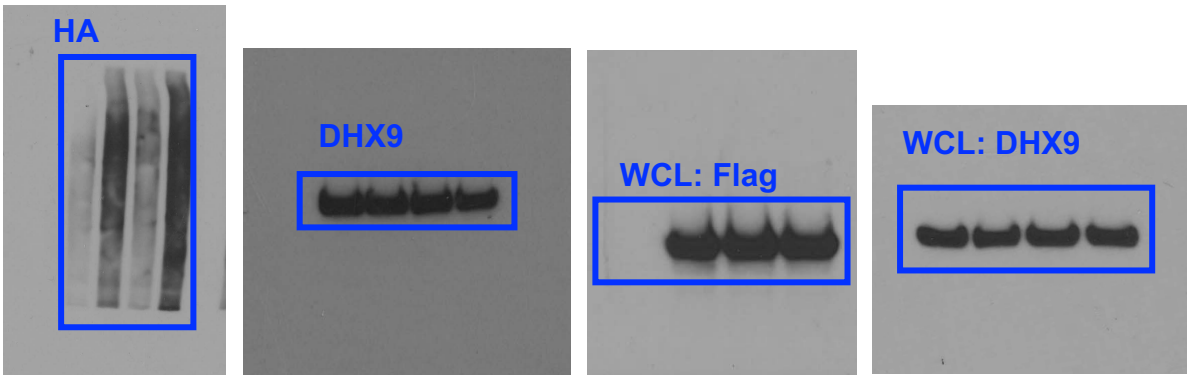




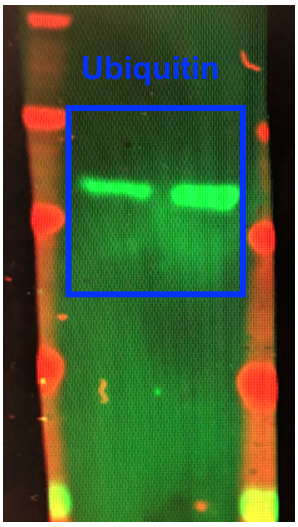
Full unedited gel for Figure 5F



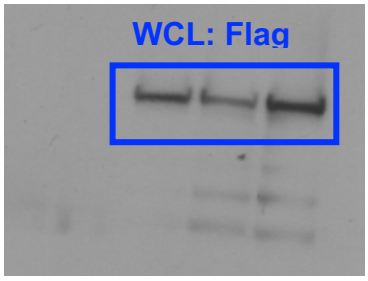
Full unedited gel for Figure 5G

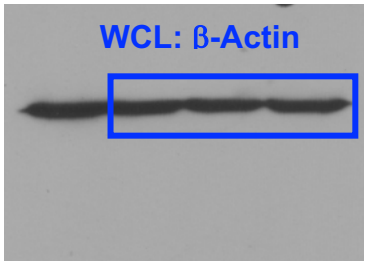


Full unedited gel for Figure 5H

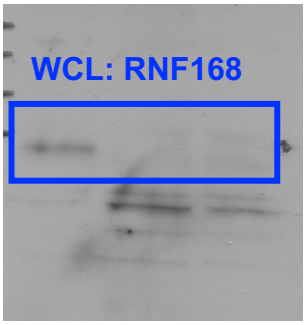


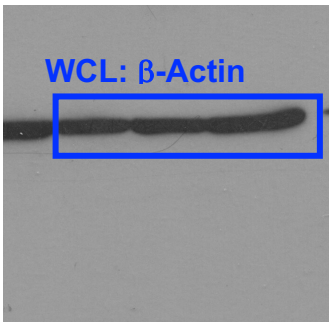
Full unedited gel for Figure 6D



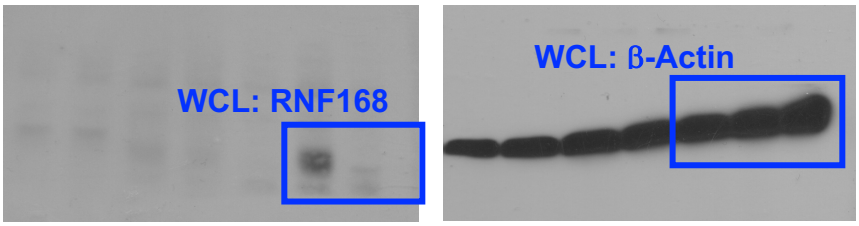


Full unedited gel for Supplementary Figure 5A

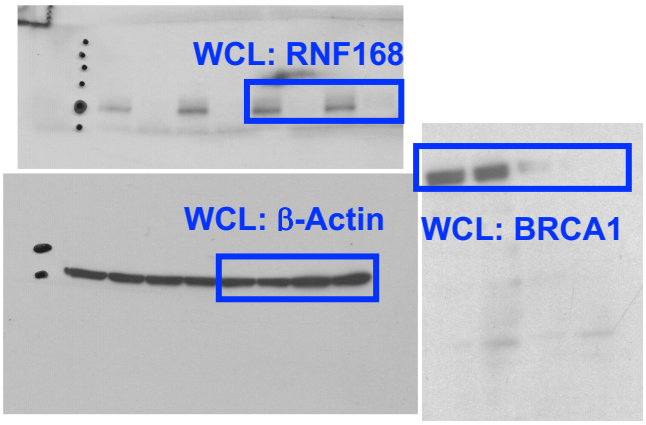




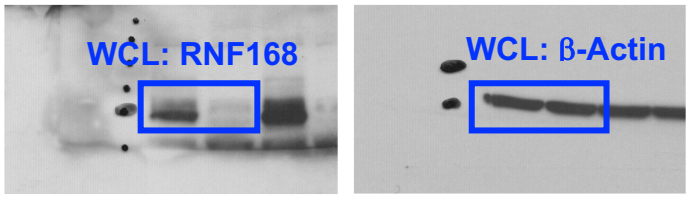
Full unedited gel for Supplementary Figure 5B



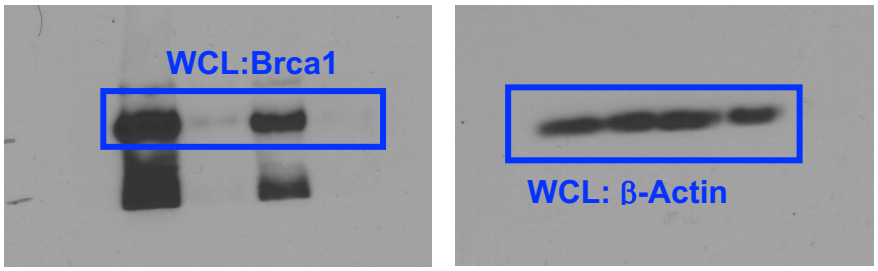
Full unedited gel for Supplementary Figure 5E



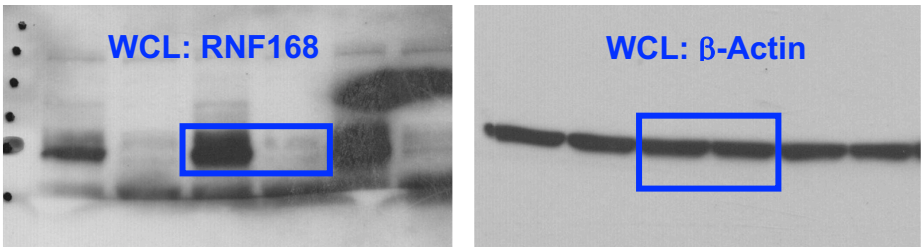
Full unedited gel for Supplementary Figure 5I



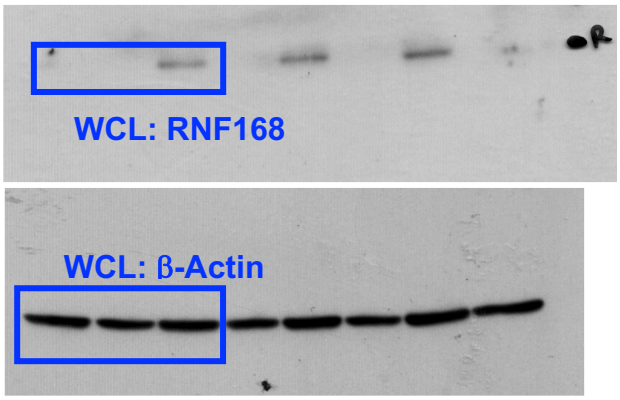
Full unedited gel for Supplementary Figure 6B



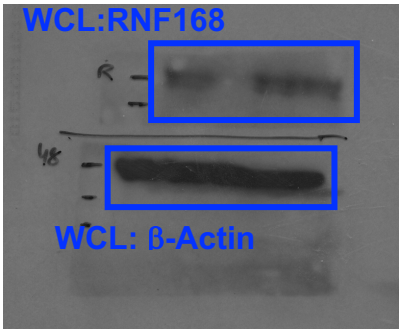
Full unedited gel for Supplementary Figure 6C



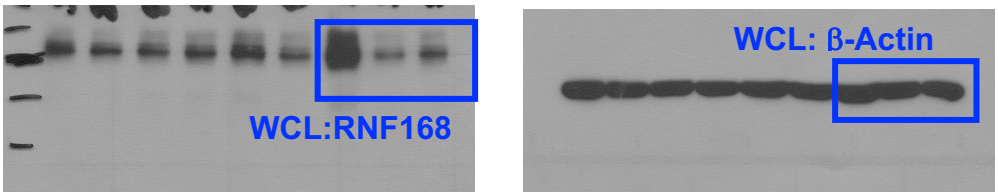
Full unedited gel for Supplementary Figure 6E



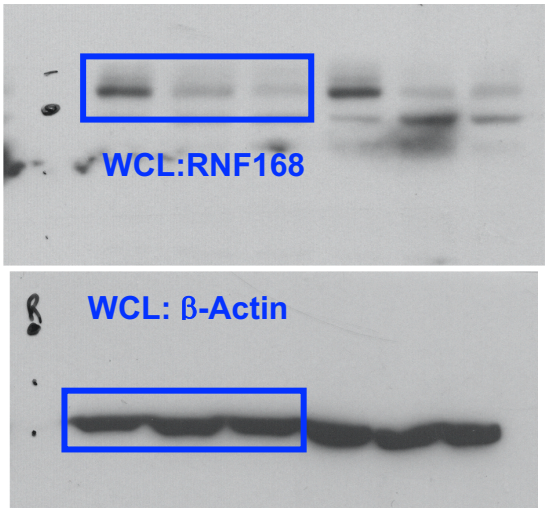
Full unedited gel for Supplementary Figure 6F



Full unedited gel for Supplementary Figure 7A



Full unedited gel for Supplementary Figure 7C



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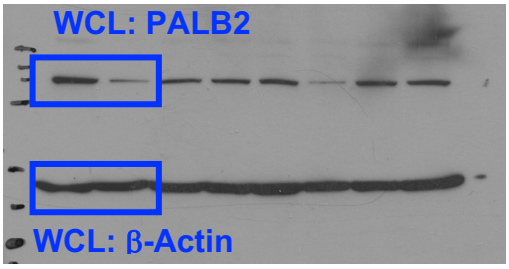

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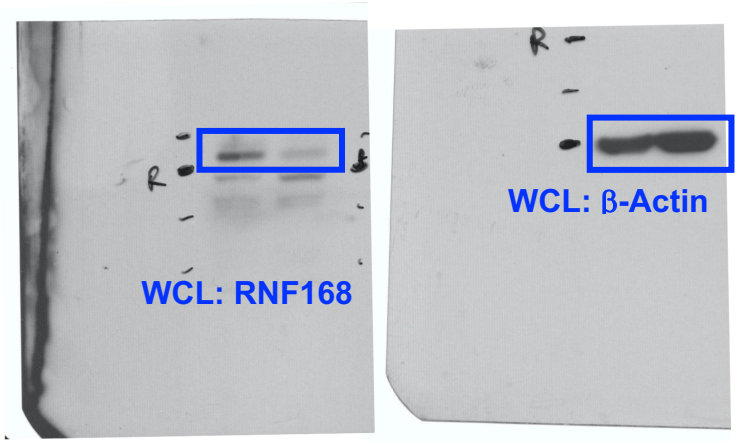

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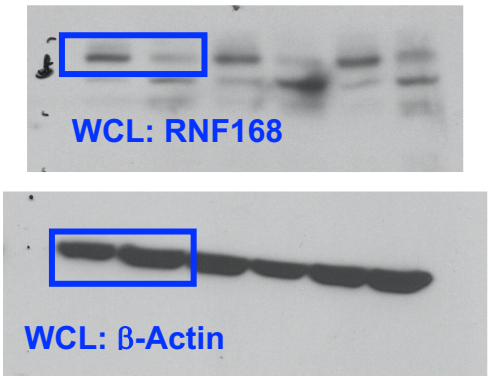
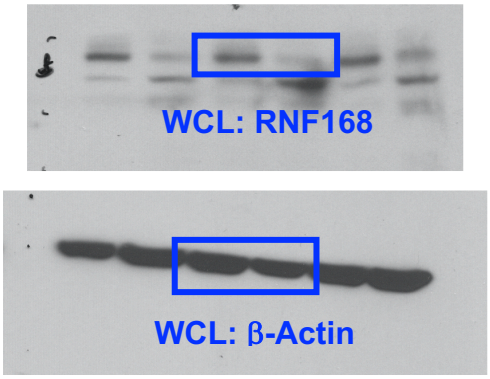
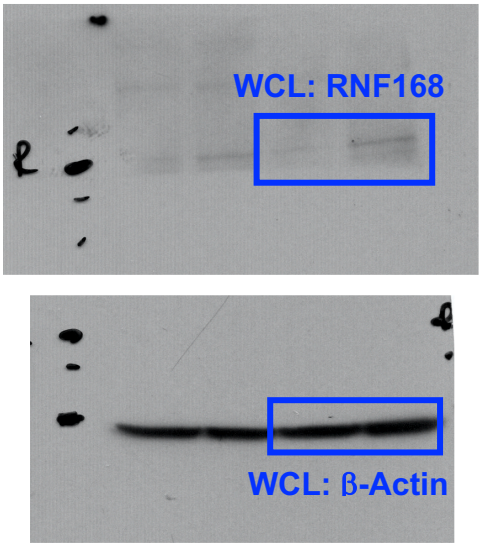


Full unedited gel for Supplementary Figure 8G



Full unedited gel for Supplementary Figure 8H



Full unedited gel for Supplementary Figure 9B